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ORIGINAL RESEARCH

Hot-water extract of the branches of *Hovenia dulcis* Thunb. (Rhamnaceae) ameliorates low-fiber diet-induced constipation in rats

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Abstract: *Hovenia dulcis* Thunb. (Rhamnaceae), also known as oriental raisin tree, is used in traditional herbal medicine. Its extracts have been reported to show various pharmacological effects such as hepatoprotection, antitumor, antiatopic dermatitis, antilipid peroxidation, anti-steatotic, anti-inflammatory, and antiallergic activities. However, there have been no reports on the effect of *H. dulcis* extracts in relieving constipation so far. The aim of this study was to investigate the effects of a hot-water extract of the branches of *H. dulcis* (WEHD) on low-fiber diet-induced constipation in Sprague Dawley rats. The in vivo laxative activity of WEHD was assessed by measuring the intestinal transit of charcoal meal and stool parameters. Furthermore, the in vitro spasmogenic activity of WEHD was evaluated by monitoring the temporal profiles of contraction of rat colon in the absence or presence of WEHD. In addition, constituent profiling was conducted using high-performance liquid chromatography analysis. Pretreatment with WEHD significantly enhanced the intestinal transit of charcoal meal and increased the frequency and weight of stools in rats. In addition, the frequency and amplitude of contractile responses of isolated rat colon were markedly enhanced by WEHD. Two organic phenolic acids, ferulic and vanillic acids, were identified in WEHD, of which vanillic acid exhibited spasmogenic activity. To the best of our knowledge, this is the first study to report the laxative and spasmogenic activities of *H. dulcis* and its constituents, suggesting that WEHD can serve as a complementary and/or alternative laxative in alleviating chronic constipation.

Keywords: *Hovenia dulcis*, constipation, low-fiber diet, charcoal meal, vanillic acid, laxative

Introduction

Functional gastrointestinal disorders are regarded as a major public health issue, and their prevalence is estimated to be 70% in general population.[1] In particular, constipation is one of the most common gastrointestinal disorders affecting an estimated 12%–19% of Americans and 14% of Asians.[2] It is characterized by infrequent bowel movements, usually three or fewer stools per week.[3,4] Other symptoms and complications associated with constipation may include fecal impaction, gut obstruction, abdominal distension, bloating, anal fissure, and hemorrhoids.[5,6] In the USA, the mean annual direct health care cost for constipation in each patient was reported to be ~US$7,900, accounting for 6.5% of the total lower gastrointestinal costs.[7] Moreover, previous estimates indicated that more than US$800 million is annually spent on purchasing laxatives and 20%–30% of the population aged 60 years and older take laxatives at least once a week.[2,6]

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Oral or rectal preparations containing bisacodyl, sennosides, and/or magnesium sulfate have been typically prescribed for the treatment of constipation owing to their potent and rapid laxative activity.[6] However, these laxatives have side effects, such as severe diarrhea, dehydration, hypotension, and postural dizziness. In addition, the prolonged use of stimulant laxatives can potentially lead to rhabdomyolysis, steatorrhea, renal failure, pancreatitis, and melanosis coli.[8–11] Over the past decade, botanical extracts have attracted increasing interest as complementary and alternative medicines for managing functional gastrointestinal disorders owing to their constituents, which can improve primary treatment with reduced adverse effects.[12]

Hovenia dulcis Thunb., also known as oriental raisin tree, is a deciduous tree belonging to the family Rhamnaceae.[13] In Asian countries, *H. dulcis* has been used in traditional herbal medicine for quenching thirst, alleviating alcohol intoxication, promoting digestion, and relieving fever.[14] Moreover, recent studies have shown that extracts of the fruits, seeds, and branches of *H. dulcis* attenuate acute liver toxicity and atopic dermatitis-like skin lesions and exert antitumor, antilipid peroxidation, antisteatotic, anti-inflammatory, and antiallergic activities.[14–17] However, there have been no studies reporting the effect of *H. dulcis* extracts in alleviating constipation.

In this study, we investigated the effects of a hot-water extract of the branches of *H. dulcis* (WEHD) on low-fiber diet-induced constipation in Sprague Dawley rats. This rat model of constipation is known to naturally mimic humans who have constipation owing to poor dietary habits.[6] The in vivo laxative activity of WEHD was assessed by measuring the intestinal transit of charcoal meal and stool parameters. Furthermore, the in vitro spasmogenic activity of WEHD was evaluated by monitoring the temporal profiles of contraction of rat colon in the absence or presence of WEHD. In addition, constituent profiling was conducted using high-performance liquid chromatography (HPLC) analysis.

Materials and methods

Plant materials

The branches of *H. dulcis* were kindly provided by the Jeonnam Institute of Natural Resources Research (Jeonnam, South Korea), and their identification was confirmed by Dr C-YC (the first author). A voucher specimen was deposited at the Jeonnam Institute of Natural Resources Research. Air-dried branches (1 kg) were chopped into small pieces and extracted thrice with 20 L of distilled water (DW) at 100°C for 3 h. After filtration, the resultant aqueous solution was evaporated and freeze-dried at −40°C for 48 h. Subsequently, 47 g crude extract of the branches of *H. dulcis* (WEHD) was obtained in the form of powder. To prepare solvent fractions of WEHD, 40 g WEHD was dissolved in 1 L of DW and sequentially partitioned with hexane, chloroform, ethyl acetate, and butanol. The resultant solvent fractions were filtered, evaporated, and freeze-dried at −40°C for 48 h and stored at −80°C in the form of powder for further studies.

Purification and constituent profiling of WEHD by HPLC analysis

WEHD was purified in the following order: solvent extraction, liquid chromatography, and preparative thin-layer chromatography. Briefly, 40 g of WEHD was suspended in 200 mL of DW for 3 h. Then, the aqueous layer was partitioned twice with ethyl acetate (1:1, v/v). After evaporation, the resultant residue (2.0 g) was subjected to flash RP-C18 silica gel chromatography (3×20 cm, flow rate: 5 mL/min), using 10% acetonitrile to afford the major constituents HD1 and HD2. HD1 and HD2 were further purified by preparative thin-layer column chromatography to an acceptable analytical purity. The final amounts of HD1 and HD2 were measured to be 20 and 15 mg, respectively. A nuclear magnetic resonance (NMR) analysis indicated that HD1 and HD2 contained vanillic acid and ferulic acid, respectively (data not shown). Then, constituent profiling of WEHD was performed using an Alliance 2695 HPLC System (Waters Co., Milford, MA, USA) equipped with a photodiode array detector. The analytical column used was Waters SunFire C18 (C18, 4.6×250 mm, 5 μm), with a mobile phase consisting of solvent A (methanol) and B (0.1% formic acid). Gradient elution (from 35/65 to 100/0, v/v) was used at a flow rate of 1.0 mL/min. The column temperature was maintained at 25°C, and the detection wavelength was set at 264 nm for vanillic acid and ferulic acid (Table 1).

Animals

Male Sprague Dawley rats (7–9 weeks old; 230–300 g) were purchased from Orient Bio Inc. (Seongnam, South Korea). The rats were retained in a clean room (Jeonnam Institute of Natural Resources Research) at a temperature of 20°C–23°C with 12 h light (07:00–19:00) and dark (19:00–07:00) cycles and a relative humidity of 50%±5%. They were housed in metabolic cages (Tecniplast USA Inc., West Chester, PA, USA) under filtered and pathogen-free air, with free access to water. The rats were fed a normal or low-fiber diet. The normal diet (LabDiet 5053) was purchased from Orient Bio Inc. The low-fiber diet containing corn starch (41.5%),

Table 1 Analytical conditions of the HPLC methods used in this study

Parameter	Condition		
Column	Waters SunFire C18 (C18, 4.6×250 mm, 5 μm)		
Flow rate	1 mL/min		
Injection volume	10 μL		
UV detection	264 nm		
Run time	37 min		
	Time (min)	**MeOH (v/v%)**	**0.1% formic acid (v/v%)**
Gradient flow	0	35	65
	18	35	65
	25	100	0
	31	100	0
	33	35	65
	37	35	65

Abbreviation: HPLC, high-performance liquid chromatography.

sucrose (10.0%), dextrin (10.0%), milk casein (24.5%), corn oil (6.0%), a mineral mixture (7.0%), and a vitamin mixture (1.0%) was purchased from Samtako Bio Korea (Osan, South Korea). The composition of normal diet and low-fiber diet is listed in Table 2. To prepare the rat model of constipation, the rats were fed a low-fiber diet for 5 weeks to induce constipation prior to the experiments (Figure 1). Induction of constipation was evaluated on the basis of stool parameters (frequency, weight, and water content). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jeonnam Bioindustry Foundation (date of approval: June 01, 2015; approval number: JINR1516) and conducted in full compliance with the IACUC guidelines.

In vivo study on intestinal transit of charcoal meal

Intestinal transit of a charcoal meal was evaluated as reported previously.[18,19] WEHD, its solvent fractions, and Dulcolax® (bisacodyl; kindly donated by Kolon Pharma, Inc., Gwacheon, South Korea) were suspended in saline. Then, saline, WEHD at a dose of 200 mg/kg, Dulcolax at a dose of 200 mg/kg (containing 5 mg/kg of bisacodyl and 16.75 mg/kg of docusate sodium), and solvent fractions of WEHD at a dose of

Table 2 Composition of normal and low-fiber diet

Ingredient	Contents (%)	
	Normal diet	**Low-fiber diet**
Moisture	9.2	9.0
Fiber	4.7	0.1
Protein	20.0	21.9
Fat	9.9	6.1
Ash	6.0	5.9
Nitrogen-free extract	50.2	57.0

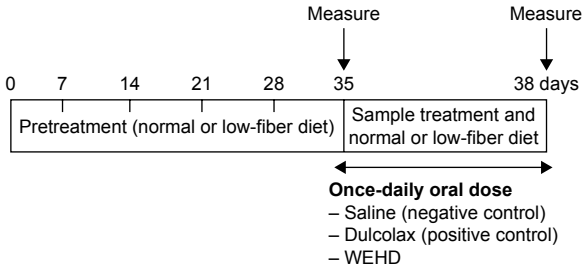

Figure 1 Schematic diagram illustrating the schedule for the preparation of low-fiber diet-induced constipation rat model and sample treatment.
Abbreviation: WEHD, hot-water extract of the branches of *Hovenia dulcis*.

100 mg/kg were orally administered to normal rats. After 15 min, the rats received 0.2 mL of charcoal meal containing 10% gum arabic (Sigma–Aldrich Co., St Louis, MO, USA) and 5% active charcoal (Sigma–Aldrich) suspended in DW. After 30 min, the rats were sacrificed by cervical dislocation and the whole small intestine was excised. Then, the total length of the small intestine was measured by laying out the small intestine on a measuring tape and measuring the distance from the pyloric sphincter to the ileocecal valve. The distance traveled by the charcoal meal was measured from the pyloric sphincter to the most caudal edge of the charcoal. The percentage of movement of charcoal meal was calculated by comparing the charcoal transit distance and total small intestine length.

In vivo stool test

The rats were divided into four groups as follows: normal diet-fed rats receiving saline (normal group); rats with low-fiber diet-induced constipation receiving saline (negative control); rats with low-fiber diet-induced constipation receiving Dulcolax at a dose of 100 mg/kg (positive control; containing 2.5 mg/kg of bisacodyl and 8.375 mg/kg of docusate sodium); and rats with low-fiber diet-induced constipation receiving WEHD at a dose of 200 mg/kg. Saline, Dulcolax, and WEHD were administered orally once daily for 3 days. Meanwhile, the frequency and weight of stools from each rat were measured on the third day. The water content of stools was determined as described previously with slight modifications.[20,21] The stools were collected and dried at 70°C for 24 h, and their water contents were determined by measuring the difference between wet weight and dry weight of stool sample.

In vitro study on isolated rat colon contractions

The in vitro spasmogenic activity of WEHD in the rat colon segments was determined as described previously.[22] The whole colon of rats was excised, transferred to a dish

containing Krebs–Henseleit solution continuously aerated with carbogen (a mixture of 5% carbon dioxide and 95% oxygen) at 37°C, and cut into segments (2 cm in length). Then, the colon segments were mounted in 10 mL of tissue baths containing Krebs–Henseleit solution maintained at 37°C and aerated with carbogen. A preload of 0.5 g was applied to each colon segment, and the contractile responses were recorded using PowerLab 4/30 (ADInstruments, Sydney, NSW, Australia) and a PowerLab data-acquisition system. The colon segments were allowed to equilibrate for a period of 30 min and then stabilized with a submaximal concentration of acetylcholine (0.3 μM). The tissue segments were assumed stable only after the reproducibility of the said responses. Then, WEHD at a concentration of 0.2 mg/mL or Dulcolax at a concentration of 1 mg/mL (containing 0.025 mg/mL of bisacodyl and 0.08375 mg/mL of docusate sodium) were applied to examine its spasmogenic activity on the rat colon segments. In addition, the in vitro spasmogenic activity of ferulic and vanillic acids in the rat colon segments was evaluated in the same manner as WEHD.

Statistical analysis

Unless indicated otherwise, all data were expressed as mean ± standard deviation and they were rounded to three significant figures. We conducted statistical analysis using one-way analysis of variance with Tukey's multiple range test (SAS Version 9.4 statistical software, SAS Institute Inc., Cary, NC, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Effect of WEHD and its solvent fractions on in vivo intestinal transit of charcoal meal in rats

To investigate the laxative activity of WEHD and its various solvent fractions, the intestinal transit of orally administered charcoal meal was measured in normal rats. As shown in Figure 2, the percentage of movement of charcoal meal through the small intestine was significantly higher in normal rats treated with 200 mg/kg WEHD (by 27.1%; $P=0.0293$) or Dulcolax (by 24.8%; $P=0.0404$) than in normal rats treated with saline (control rats). As shown in Figure 3, the percentage of movement of charcoal meal through the small intestine was slightly but significantly higher (by 14.5%; $P=0.0325$) in rats treated with 100 mg/kg of water fraction of WEHD than in control rats. However, no significant increases in intestinal transit of charcoal meal were observed in rat groups treated with the other solvent fractions at a dose of 100 mg/kg.

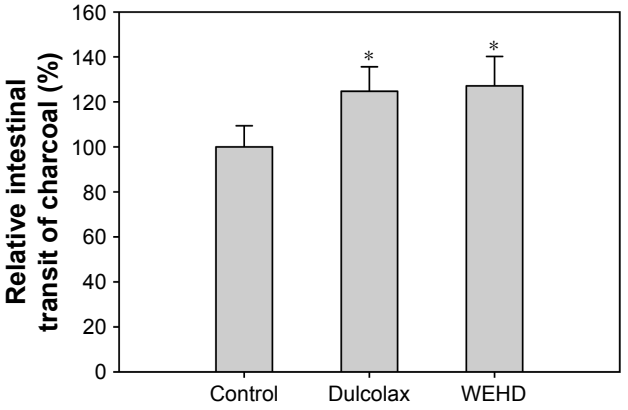

Figure 2 Intestinal transit of orally administered charcoal meal without or with oral administration of WEHD or Dulcolax in normal rats.
Notes: The rectangular bars and their error bars represent the mean and standard deviation, respectively (n=3–4). *Significantly different from the control (without WEHD or Dulcolax) group ($P<0.05$; ANOVA followed by Tukey's test).
Abbreviations: ANOVA, analysis of variance; WEHD, hot-water extract of the branches of *Hovenia dulcis*.

Effect of WEHD on low-fiber diet-induced constipation in rats

To investigate the laxative activity of WEHD, stool parameters were measured in the rat model of low-fiber diet-induced constipation. The stool frequencies, weights, and water content after oral administration of saline in normal rats and oral administration of saline, 100 mg/kg of Dulcolax, or 200 mg/kg of WEHD in rats with low-fiber diet-induced constipation (constipated rats) are shown in Figure 4. The stool frequency was significantly lower in constipated rats treated with saline than in normal rats; however, the

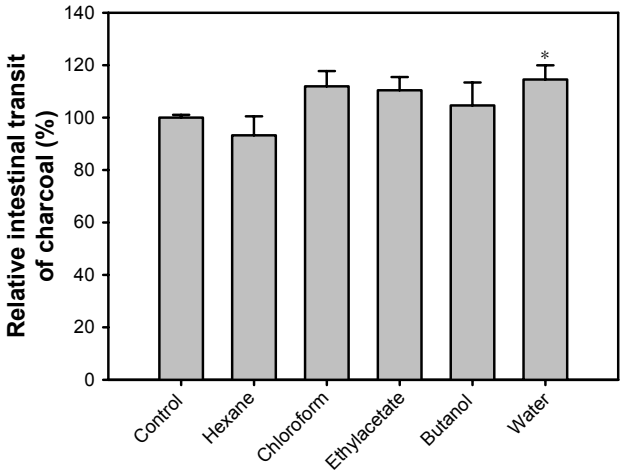

Figure 3 Intestinal transit of orally administered charcoal meal with or without oral administration of various solvent fractions of WEHD in normal rats.
Notes: The rectangular bars and their error bars represent the mean and standard deviation, respectively (n=3–4). *Significantly different from the control (without solvent fractions) group ($P<0.05$; ANOVA followed by Tukey's test).
Abbreviations: ANOVA, analysis of variance; WEHD, hot-water extract of the branches of *Hovenia dulcis*.

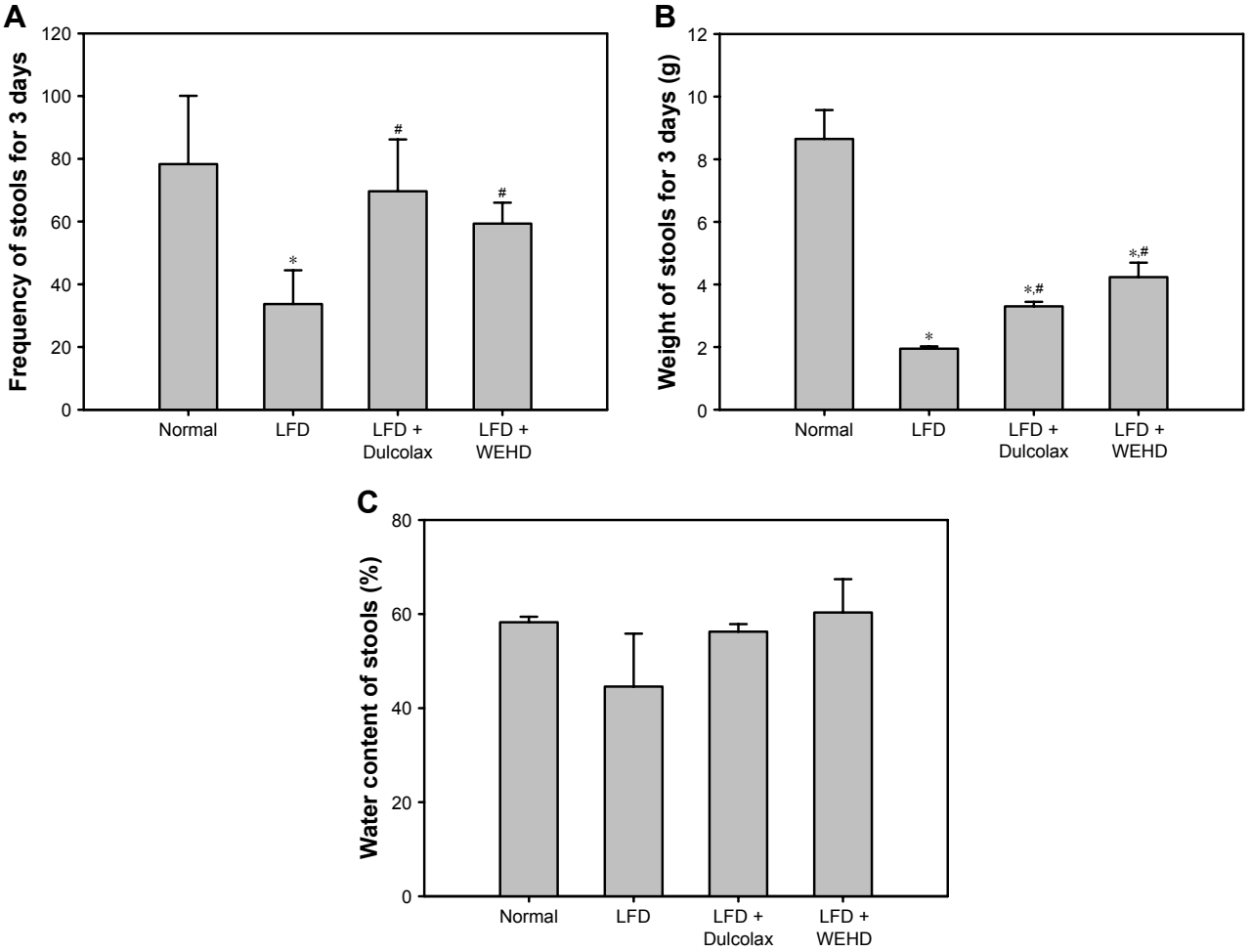

Figure 4 Frequencies (**A**), weights (**B**), and water contents (**C**) of stools after oral administration of saline in normal rats (normal) and after oral administration of saline (LFD), Dulcolax (LFD + Dulcolax), or WEHD (LFD + WEHD) in rats with LFD-induced constipation. The rectangular bars and their error bars represent the mean and standard deviation, respectively (n=3–4). *Significantly different from the normal (negative control) group. #Significantly different from the LFD (positive control) group ($P<0.05$; ANOVA followed by Tukey's test).

Abbreviations: ANOVA, analysis of variance; LFD, low-fiber diet; WEHD, hot-water extract of the branches of *Hovenia dulcis*.

frequencies were comparable among normal rats and constipated rats treated with Dulcolax or WEHD (Figure 4A). The weights of stools were significantly lower in negative control rats than in normal rats, while they were significantly higher in constipated rats treated with Dulcolax or WEHD than in negative control rats (Figure 4B). The water contents of stools were considerably lower (by 23.5%; $P=0.053$) in constipated rats treated with saline than in normal rats, while they were comparable among normal rats and constipated rats treated with Dulcolax or WEHD (Figure 4C).

Effect of WEHD on in vitro contraction of isolated rat colon

To examine the spasmogenic activity of WEHD, its effect on the contraction of isolated rat colon was assessed. The temporal profiles of contractile activity of isolated rat colon in the absence or presence of WEHD or Dulcolax (positive control) are shown in Figure 5. At predosing, stable and constant contractile responses were confirmed (Figure 5A). Notably, the frequency and amplitude of contractile responses were markedly enhanced by the addition of WEHD (Figure 5B) or Dulcolax (Figure 5C).

Constituent profiling of WEHD by HPLC analysis

To determine the constituent profiles of WEHD, WEHD purification, marker compound identification, and constituent profiling were performed using HPLC and NMR. Two organic compounds, ferulic acid and vanillic acid, were identified in WEHD (Figure 6). The contents of the phenolic acids were determined as 2.5% for ferulic acid and 6.2% for vanillic acid.

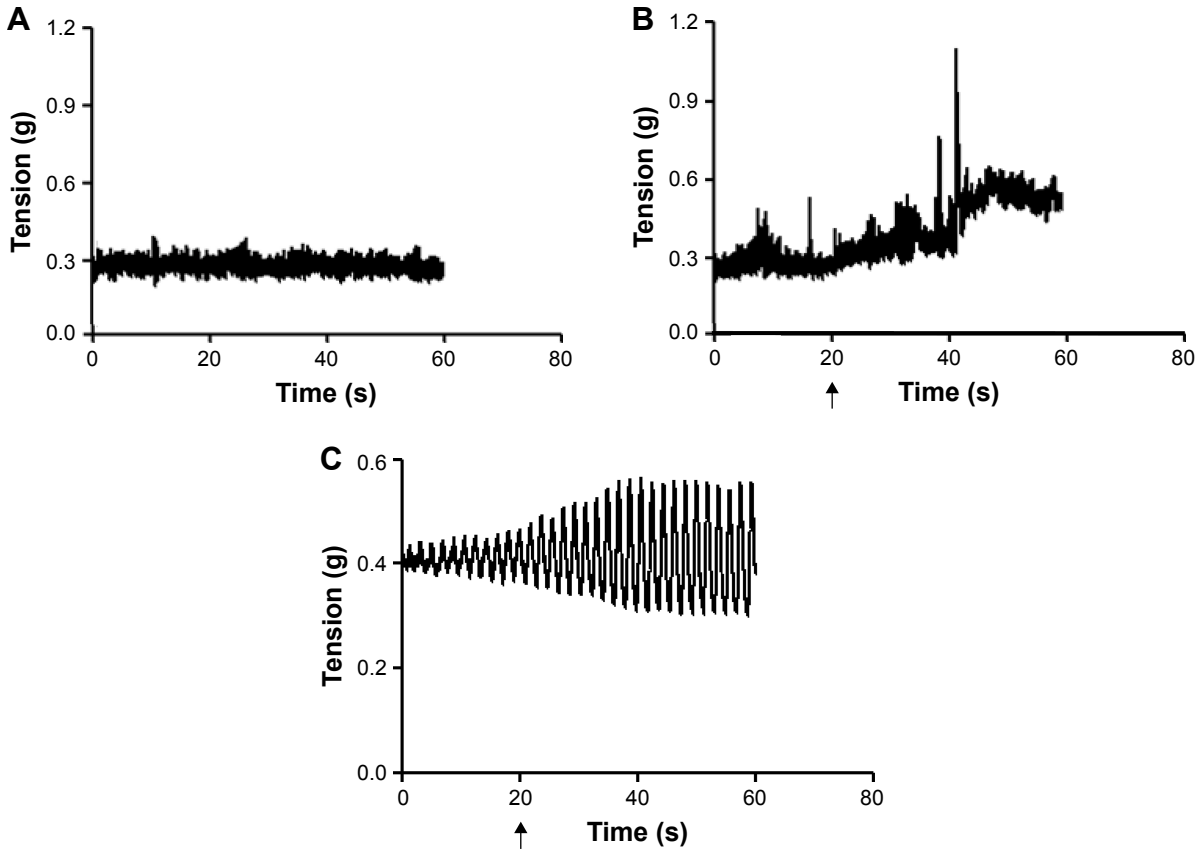

Figure 5 Representative temporal profiles of contractile activity of isolated rat colon in the absence (**A**) and presence of WEHD (**B**) or Dulcolax (**C**).
Abbreviation: WEHD, hot-water extract of the branches of *Hovenia dulcis*.

Effect of ferulic and vanillic acids on in vitro contraction of isolated rat colon

To examine the spasmogenic activity of ferulic and vanillic acids, its effect on the contraction of isolated rat colon was assessed. The temporal profiles of contractile activity of isolated rat colon in the absence or presence of vanillic acid at the concentrations of 0.1 and 0.2 mg/mL are shown in Figure 7. Notably, the frequency and amplitude of contractile responses were markedly enhanced by the addition of vanillic acid. However, no discernible change was observed following the addition of ferulic acid.

Discussion

This study provides novel data on the effects of WEHD on the intestinal transit of charcoal meal, low-fiber diet-induced constipation, and colon contractile activity in Sprague

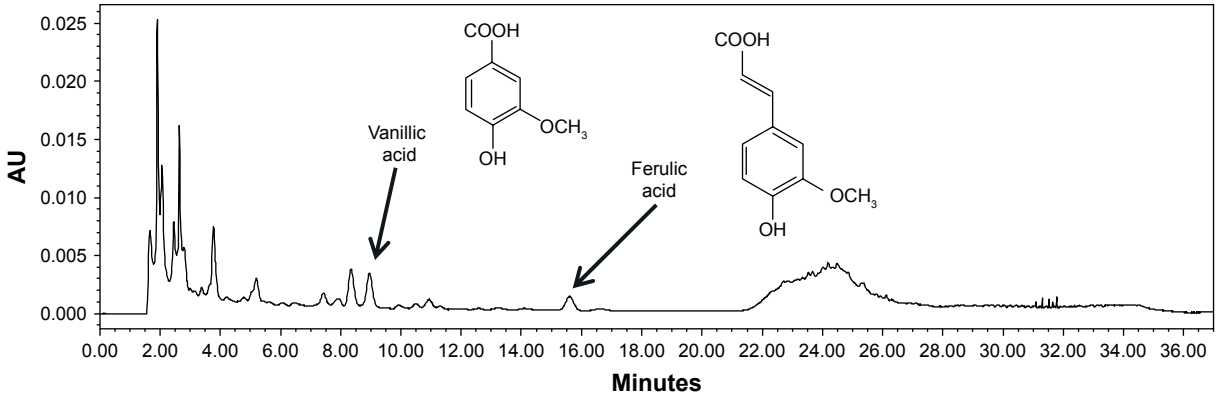

Figure 6 Representative HPLC chromatogram of WEHD.
Abbreviations: HPLC, high-performance liquid chromatography; WEHD, hot-water extract of the branches of *Hovenia dulcis*.

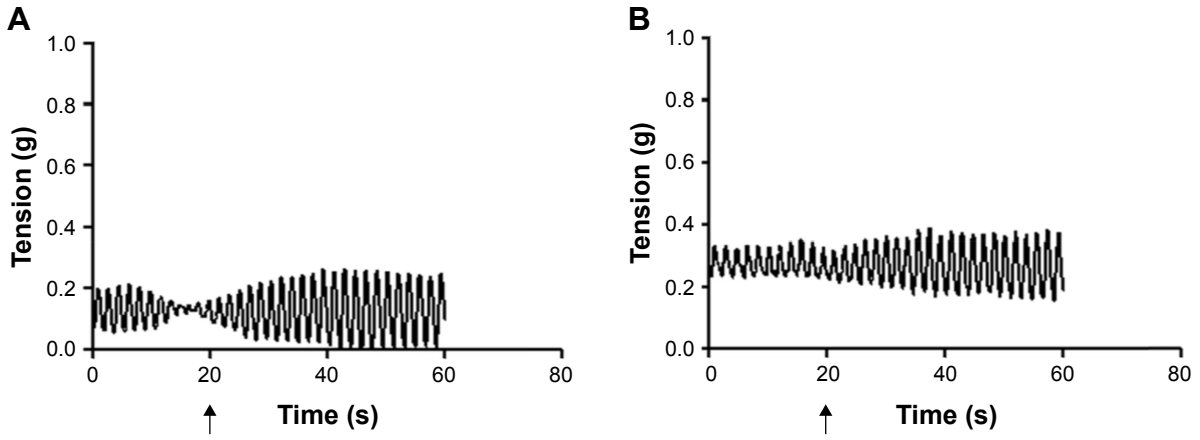

Figure 7 Representative temporal profiles of contractile activity of isolated rat colon in the presence of vanillic acid at 0.2 mg/mL (**A**) or 0.1 mg/mL (**B**).

Dawley rats. The intestinal transit time has most often been evaluated by administering an aqueous suspension of active charcoal to rodents, and the distance traveled by charcoal is reflective of intestinal motility.[19] The results shown in Figure 2 clearly indicate that WEHD at a dose of 200 mg/kg significantly increased the distance traveled by charcoal meal, suggesting that WEHD could have stimulated intestinal peristaltic motility, consequently leading to the increase in propulsive movement of charcoal meal. Moreover, among the various solvent fractions of WEHD, only the water fraction significantly enhanced the intestinal transit of charcoal meal. This suggests that the water fraction may contain higher levels of active constituents that show laxative activity. Notably, the extent of enhancement of charcoal meal intestinal transit by WEHD tended to be higher than that by its solvent fractions. This observation could be attributed to the higher dose of WEHD and possibly higher levels of synergistic interactions among the active constituents in WEHD when compared with that of the solvent fractions. Further investigation on dose dependency and chemical profiling is warranted to clarify this finding.

Various factors are responsible for constipation, including psychological stress, dietary habits, and drugs, such as morphine, an opioid-receptor agonist. Loperamide, an opioid-receptor agonist, has been used to induce constipation in murine, which mimics morphine-induced constipation in human patients.[23] In this study, a low-fiber diet was used to induce constipation in rats, which corresponds to constipation caused by poor dietary habits in humans.[6] The in vivo study using the rat model of low-fiber diet-induced constipation shows that WEHD can ameliorate the frequency and weight of stools in constipated rats (Figure 4A and B), suggesting the laxative activity of WEHD in low-fiber diet-induced constipation. However, the water contents of stools did not differ significantly after WEHD treatment between the normal and constipated rats (Figure 4C). This result suggests that WEHD may exert laxative activity via a mechanism that is distinct from that of hyperosmotic laxatives, and saline laxatives (eg, glycerin, polyethylene glycol, and magnesium sulfate).[24,25] Moreover, the in vitro study using rat colon preparations clearly shows that WEHD can stimulate the spontaneous contraction of isolated rat colon (Figure 5). Based on the in vitro and in vivo results, it can be speculated that WEHD shows stimulatory effect on intestinal contractile responses, potentially acting as a stimulant laxative.

Ferulic acid and vanillic acid were identified in WEHD (Figure 6). Consistent with our current results, these two phenolic acids were isolated from a methanol extract of the branches of *H. dulcis* in another study.[17] Ferulic acid is widely found in various fruits, leaves, vegetables, and flowers.[26] In humans, it is rapidly absorbed after oral administration but undergoes extensive first-pass metabolism in the liver, resulting in a low oral bioavailability of $<$20%.[27] Ferulic acid exhibits a wide variety of biomedical effects, including anti-inflammatory, antioxidant, hepatoprotective, antiallergic, and anticancer activities.[28] Vanillic acid is an oxidized form of vanillin, which is used as a flavoring agent.[29] A few studies have shown that vanillic acid could modulate inflammation or immune responses.[30,31] In a previous study, vanillic acid exhibited hepatoprotective activity via suppression of immune-mediated hepatitis induced by concanavalin A.[31] Another study reported that vanillic acid showed a beneficial effect on dextran sodium sulfate-induced ulcerative colitis.[30] However, to date, no studies on the intestinal contractility-stimulant or laxative effects of ferulic acid or vanillic acid have been reported.

In this study, vanillic acid exhibited spasmogenic activity (Figure 7), while ferulic acid did not. Some organic acids have been shown to stimulate contractile response in rat intestine. For instance, Na et al[32] reported that malic and citric acids, the

major components in crude water extracts of *Prunus mume* Siebold & Zucc fruits, enhanced the contractile amplitude and frequency of rat colon in a concentration-dependent manner. In a previous study, it was reported that organic acids present in red wine and white wine stimulated the contraction of rat duodenum in a decreasing order of contractile potency as follows: citric acid > tartaric acid ≥ malic acid > lactic acid.[33] These findings can inspire us to extend the scope of investigation further to other organic acids and their related compounds. Thus, further research is warranted to investigate the stimulant effects of other organic compounds with acidic properties on intestinal motility and evaluate their feasibility as potential laxative agents.

Conclusion

This study investigated the pharmacological effects of WEHD on constipation. The results of our in vivo and in vitro studies demonstrated that WEHD could ameliorate low-fiber diet-induced constipation in rats and enhance the spontaneous contraction of rat intestine. In addition, two phenolic acids, ferulic and vanillic acids, were identified in WEHD by HPLC analysis of which vanillic acid exhibited spasmogenic activity. To the best of our knowledge, this is the first study to report the laxative and spasmogenic activities of *H. dulcis* and its constituents. Our results suggest that WEHD can serve as a complementary and/or alternative laxative in alleviating chronic constipation.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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