

RESEARCH ARTICLE



WDJ-S4, a standardized herbal formula, promotes gastrointestinal motility via modulation of the acetylcholine pathway in a loperamide-induced functional dyspepsia mice

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ABSTRACT

Context: WDJ-S4, a standardized herbal formula, has been prescribed for refractory functional dyspepsia (FD) in Korea, but the detailed mechanisms are lacking.

Objective: The present study investigates the acceleration of gastrointestinal (GI) motility by WDJ-S4 and its potential mechanisms.

Materials and methods: For five days, WDJ-S4 (50, 100 and 200 mg/kg) or mosapride (3 mg/kg) was orally given to BALB/c mice. After 20 h of fasting, loperamide (10 mg/kg, i.p.) was given to the mice except for normal group. To assess gastric emptying or intestinal propulsion, 500 μ L of 0.05% phenol red or 200 μ L of 5% charcoal diet was given once orally.

Results: Loperamide delayed gastric emptying and intestinal propulsion, while WDJ-S4 ameliorated peristaltic dysfunction, evidenced by reductions of remaining phenol red in the stomach and a marked increase of charcoal propulsion in the intestine. WDJ-S4 also normalized levels of acetylcholine and acetylcholine-related enzymes, including choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), in the gastric antrum and jejunum. C-kit level and smooth muscle contraction-related genes were elevated by WDJ-S4 in both the gastric antrum and jejunum.

Conclusion: Overall, WDJ-S4 can effectively promote GI motility. The efficacy is associated with modulation of acetylcholine pathway and the interstitial cells of Cajal (ICCs) activation. All the results provide scientific evidence supporting the clinical usage of WDJ-S4 for FD.

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Introduction

Dyspepsia is highly prevalent in the general population, yet underlying organic or structural problems are only found in a minority of patients (Ford et al. 2015). Approximately, 80% of these cases cannot be attributed to any structural or organic causes and are termed functional dyspepsia (FD) (Ford et al. 2020). The global prevalence of FD is estimated to be around 11.5–29.2% (Mahadeva and Goh 2006). While it does not significantly affect overall life expectancy, FD has a substantial impact on the quality of life and results in a significant economic burden, estimated at about \$18.4 billion annually in the United States (Lacy et al. 2013).

FD is classified into three distinct subtypes: postprandial distress syndrome (PDS), epigastric pain syndrome (EPS) and a combination of both PDS and EPS symptoms, as defined by the ROME IV consensus (Stanghellini et al. 2016). PDS primarily includes symptoms like early satiation and postprandial fullness, while epigastric discomfort or burning is commonly associated

with EPS (Miwa et al. 2022). Although the precise etiology of FD remains unclear, several factors have been proposed to be linked to the pathophysiology. These factors include abnormal gastrointestinal (GI) motility, heightened visceral sensitivity, excessive gastric acid output and infection with *Helicobacter pylori* (Enck et al. 2017). Given these etiological factors, various therapeutic strategies have been employed to treat FD, including the use of prokinetics, proton pump inhibitors and *Helicobacter pylori* eradication (Masuy et al. 2019).

Targeting the normalization of delayed GI motility holds promise in alleviating overall FD symptoms, due to 84% of total FD patients having PDS symptoms (Carbone et al. 2015). Therefore, as a primary option, prokinetic agents such as cisapride, mosapride and domperidone are commonly used for FD (Goelen et al. 2023). Although these drugs have been approved to enhance GI motility in patients with FD, their limitations still exist regarding low efficacy and adverse effects, including myocardial infarction and ventricular arrhythmias occasionally (Black et al. 2022). So, new drug development for FD is still required.

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Herbal medicine has emerged as a treatment option for patients with FD. A study reported that 36.7% of surveyed patients with functional GI disorders utilized herbal medicines (Lahner et al. 2013). Many patients with FD have turned to herbal products likely traditional Korean medicine (TKM) and traditional Chinese medicine (TCM). Numerous studies have presented the therapeutic effects of multi-herbal decoctions on FD in clinical trials and animal studies (Hwang et al. 2021; Kim et al. 2021). Notably, the pharmacological theories of TKM and TCM emphasize the synergistic actions of multi-herbal combinations in treating multifactorial disorders, such as FD (Yoon et al. 2022). WDJ-S4, a standardized herbal formula, is comprised of 13 herbs employed to alleviate dyspeptic symptoms. Since 2016, it has been prescribed for patients suffering with refractory functional dyspepsia (RFD). However, information regarding the mechanism of action of this formula is still lacking.

Therefore, the main purpose of the present study is to investigate the efficacy of WDJ-S4 on FD and explore the potential mechanisms through an animal model.

Materials and methods

Chemicals and reagents

The following reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO): loperamide hydrochloride (Cat. No. L4762), mosapride citrate salt dihydrate (Cat. No. M2946), phenol red (Cat. No. P3532), sodium carboxymethyl cellulose (Na-CMC, Cat. No. 419303), sodium hydroxide (Cat. No. 655103), hydrochloride (Cat. No. 258148), trichloroacetic acid (TCA, Cat. No. T6399), tris base (Cat. No. 93362), sodium chloride (Cat. No. S9888), triton X-100 (Cat. No. T8787), 10% neutral formalin (Cat. No. HT501128), calcium carbonate (Cat. No. 239216), calcium sulfate (Cat. No. 237132), aqueous mounting buffer (Cat. No. HC08) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Cat. No. D9542).

Other reagents and chemicals were purchased from the following manufacturers: activated charcoal power (Cat. No. 07909, YAKURI, Tokyo, Japan), Arabic gum (Cat. No. 12505-1201, JUNSEI, Tokyo, Japan), tween 20 (Cat. No. GD9856, Glentham Life Sciences, Corsham, UK), bovine serum albumin (Cat. No. A0100, GenDEPOT, Houston, TX) and skim milk (Cat. No. SKI500, LPS Solution, Daejeon, South Korea). Hydroperoxide (Cat. No. 000H1408), methanol (Cat. No. 502M3032) and ethanol (Cat. No. 000E1166) were purchased from SAMCHUN Pure Chemical (Seoul, South Korea). Ten marker analytes selected for high-performance liquid chromatography (HPLC) fingerprinting analysis of WDJ-S4 were purchased from natural product manufacturers: hydroxymethylfurfural ($\geq 99.0\%$, Cat. No. W501808) and chlorogenic acid (99.7%, Cat. No. 00500590) from Merck KGaA (Darmstadt, Germany), neochlorogenic acid (98.6%, Cat. No. DR11568), cryptochlorogenic acid (99.1%, Cat. No. DR12124), liquiritin apioside (99.6%, Cat. No. DR10690) and decursin (98.7%, Cat. No. DR11193) from Shanghai Sunny Biotech (Shanghai, China), liquiritin (99.6%, Cat. No. BP0874), glycyrrhizin (99.1%, Cat. No. BP0682) and decursinol angelate (98.3%, Cat. No. BP1812) from Biopurify Phytochemicals (Chengdu, China), and nodakenin (99.5%, Cat. No. CFN90232) from ChemFaces Biochemical (Wuhan, China). Solvents including methanol (Cat. No. 9093-88), acetonitrile (Cat. No. 9017-88) and distilled water (Cat. No. 4218-88) were all HPLC grade and purchased from J. T. Baker (Avantor, Radnor, PA). Formic acid (Cat. No. 695076) was purchased from Merck KGaA (Darmstadt, Germany).

Preparation and fingerprinting analyses of WDJ-S4

WDJ-S4 was obtained by mixing 13 types of herbs in the ratio of 7:7:7:4:3:3:3:3:2:2:2:1.5:1.5 and extracting them with hot water. The composition of herbs and preparation process are as follows: dried *Glycyrrhiza uralensis* Fisch. (Leguminosae) (70g, Voucher specimen No. DJU-2023-1), *Angelica gigas* Nakai (Apiaceae) (70g, No. DJU-2023-2), *Prunus mume* (Siebold) Siebold & Zucc. (Rosaceae) (70g, No. DJU-2023-3), *Ostrea gigas* Thunb. (Ostreidae) (40g, No. DJU-2023-4), *Laminaria japonica* Aresch. (Laminariaceae) (30g, No. DJU-2023-5), *Raphanus raphanistrum* subsp. *sativus* (L.) Domin (Brassicaceae) (30g, No. DJU-2023-6), *Crataegus pinnatifida* Bunge (Rosaceae) (30g, No. DJU-2023-7), *Pterocarpus santalinus* L.f. (Leguminosae) (30g, No. DJU-2023-8), *Phyllostachys nigra* (Lodd. ex Lindl.) Munro (Poaceae) (20g, No. DJU-2023-9), *Zingiber officinale* Roscoe (Zingiberaceae) (20g, No. DJU-2023-10), *Gallus gallus domesticus* Briss. (Phasianidae) (20g, No. DJU-2023-11), *Aucklandia lappa* DC. (Compositae) (15g, No. DJU-2023-12) and *Aconitum kusnezoffii* Rchb. (Ranunculaceae) (15g, No. DJU-2023-13) were pulverized and extracted with hot distilled water (46L) for 4h. All herbs were obtained from Korean Institute of Oriental Medicine (KIOM), a Korean-governed national institute for herbal and traditional medicine (<https://kiom.re.kr/>). As these particular herbs are not subject to special regulations, no additional permissions or approvals were required. Herb pictures are presented in [Supplementary Figure 2](#). In addition, all plant samples were retained and stored in the laboratory with assigned batch numbers to ensure traceability and reproducibility. The extract solution was filtered through a 53 μ m mesh filter, concentrated in a rotary evaporator, and then freeze-dried to obtain the extract powder (WDJ-S4, 76g, 16.5% yield, Deposit No. DJU-2023-14).

HPLC fingerprinting analysis of WDJ-S4 was performed with a Shimadzu Prominence LC-20A series system (Shimadzu Co., Kyoto, Japan) coupled with a photodiode array detector, controlled by LabSolutions software (version 5.117). Chromatographic separation of 10 marker analytes was carried out using a Waters SunFire C₁₈ analytical column (250 $\times$ 4.6mm, 5 μ m, Milford, MA) maintained at 40°C. The mobile phases consisted of distilled water (A) and acetonitrile (B), both of which contained 0.1% (v/v) formic acid. Gradient elution using two mobile phases is as follows: 5% B (0 min), 60% B (40 min), 95% B (50 min; hold for 10 min) and 5% B (70 min). The re-equilibration time was 10 min. The flow rate was 1.0 mL/min and the injection volume was 10.0 μ L. For HPLC fingerprinting analysis, the PDA detector was scanned simultaneously in the range of 190–800 nm.

Animal ethics and ARRIVE compliance statement

All animals in this study were reviewed and approved by the Daejeon University Institutional Animal Care and Use Committee (IACUC; approval number DJUAR2023-017, granted on 7 August 2023) and conducted in accordance with ARRIVE guidelines. We used 90 male BALB/C mice (6 weeks old, 19–21g) obtained from KOATECH (Seoul, South Korea), specifically selected to investigate the prokinetic effects of WDJ-S4 on GI motility and to explore its underlying mechanisms. The mice were housed under room temperature (22 $\pm$ 2°C) and relative humidity of 60 $\pm$ 5% with a 12:12-hour light–dark cycle and were provided free access to standard feed (Daehanbio-link, Eumseong-gun, South Korea) and water. Humane endpoints were determined based on clinical criteria, and all mice were euthanized in a CO₂ chamber, in compliance with established ethical standards.

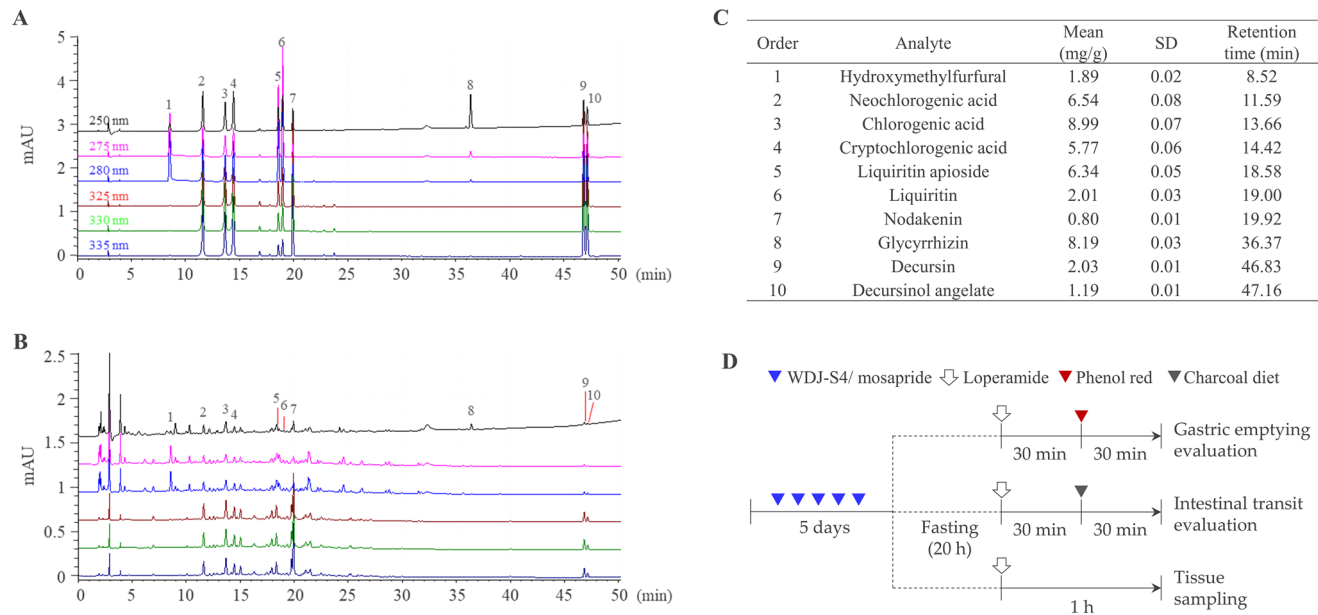


Figure 1. HPLC fingerprinting analysis of WDJ-S4 and experimental schedule. High-performance liquid chromatography (HPLC) analysis of 10 reference standards (A). HPLC analysis of WDJ-S4 (B). Quantitative analysis of WDJ-S4 (C). Experimental schedule followed during the study (D). The 10 reference standards analyzed are as follows: hydroxymethylfurfural (1), neochlorogenic acid (2), chlorogenic acid (3), cryptochlorogenic acid (4), liquiritin apioside (5), liquiritin (6), nodakenin (7), glycyrrhizin (8), decursin (9) and decursinol angelate (10).

Experimental design

After a week of acclimation, mice were randomly divided into three experimental sets: (1) evaluation of gastric emptying ($n = 30$), (2) assessment of intestinal propulsion ($n = 30$) and (3) collection of stomach and small intestine ($n = 30$). In each set, mice were separated into six groups ($n = 5/\text{group}$), including normal, control, three WDJ-S4 dosages (50, 100 and 200 mg/kg), and mosapride (3 mg/kg). WDJ-S4, mosapride and distilled water were administered orally to each group once daily for five days. The dose of WDJ-S4 is determined by the human adult dosage prescribed in a clinic (1.5 g/day/70 kg adult), which is equivalent to 315 mg/kg of a mouse by animal equivalent dose calculation based on body surface area (Nair and Jacob 2016). After a pilot study to select the optimal concentration of WDJ-S4, the maximal dose was determined to be 200 mg/kg. On the last day of the investigation, the mice fasted for 20 h. Then, mice were examined according to the protocol for each experimental set: (1) gastric emptying test, (2) intestinal propulsion test and (3) biomolecular analysis. For biomolecular analysis, the antrum of the stomach and jejunum of the small intestine were utilized. The experimental scheme is presented in Figure 1(D).

To analyze molecular changes, the gastric antrum was chosen due to its crucial role in regulating gastric motility and emptying, while the jejunum was selected for its importance in nutrient absorption and motility. These sites were specifically selected based on their anatomical and functional significance: the gastric antrum is located just before the junction with the duodenum, playing a key role in controlling gastric emptying, whereas the jejunum represents the middle section of the small intestine, essential for digestion and nutrient absorption.

Determination of gastric emptying using phenol red and stomach weight

The mice underwent a 20-hour fasting period and were provided unrestricted access to tap water. All mice, excluding those in the

normal group, received intraperitoneal injections of loperamide hydrochloride (10 mg/kg, dissolved in normal saline). After 30 min, all the mice were orally administered with a phenol red solution (500 μL each mouse). Phenol red was solubilized in a 1.5% Na-CMC solution (prepared with distilled water) at a concentration of 0.05%. Thirty minutes post-phenol red treatment, the mice were euthanized in a CO_2 chamber (Jeungdo Bio & Plant, Seoul, South Korea), after which the stomachs were promptly excised and weighed. To observe the stomach tissues, each tissue was photographed individually. The amount of the phenol red solution and the time point at which roughly 60% delayed stomach emptying was seen were determined based on our prior study (Hwang et al. 2021) and additional protocols (Asano et al. 2016; Lee et al. 2016).

To determine the absorbance of phenol red retained in the stomach, gastric samples were homogenized in 5 mL of 0.1 N sodium hydroxide solution and 0.5 mL of 20% TCA. The homogenates were subjected to centrifugation at 3000 rpm for 20 min, after which 1 mL of supernatant was combined with 4 mL of 0.5 N sodium hydroxide. The absorbance of the pink solutions was measured at 560 nm using a spectrophotometer.

The stomach emptying rates were determined using the formula: gastric emptying (%) = $(1 - X/Y) \times 100$. X: absorbance of phenol red retained in the stomach; Y: absorbance of normal phenol red combined with sodium hydroxide.

Determination of intestinal propulsion rate using charcoal diet

To evaluate the intestinal propulsion rate, the mice (except those in the normal group) were intraperitoneally injected with loperamide hydrochloride (10 mg/kg). After 30 min, they were orally administered 5% charcoal diets in 10% Arabic gum (200 μL /mouse). Thirty minutes later, the mice were sacrificed in CO_2 chamber (Jeungdo Bio & Plant, Seoul, South Korea). Intestine tissues were immediately dissected and photographed by digital camera (Product No. TG-6BLK, Olympus, Hachioji, Japan), and intestinal propulsion was measured by determining the distance

the charcoal traveled from the pylorus to the cecum using ImageJ (NIH, 1.46r Version, Bethesda, MD).

Determination of acetylcholine levels in gastric antrum and jejunum by ELISA

Gastric antrum and jejunum were immediately collected after sacrifice. Isolated tissues were homogenized with RIPA buffer. Then, to determine the level of acetylcholine, homogenates were measured by using a commercial acetylcholine ELISA kit (ab287812, Abcam, Cambridge, UK). Once all reagents, standards and samples are at room temperature, 40 µL of sample dilution buffer and 10 µL of sample are added to the well of 96-well plate, followed by 100 µL of HRP-conjugate reagent. Then, the plate is sealed and incubated at 37°C for 60 min. After removing the sealer, each well is washed five times with wash solution, gently pat-dried, and then, 50 µL of chromogen solution A and 50 µL of chromogen solution B are added and incubated at 37°C in the dark for 15 min. The reaction is stopped with 50 µL of stop solution, and the absorbance is read at 450 nm. Using the standard curve, the acetylcholine concentration of sample is calculated by comparing its OD value with the standard curve.

Protein expression analysis by Western blotting in gastric antrum and jejunum

To determine the expression of C-kit, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and actin in the gastric antrum and jejunum, tissues were lysed in RIPA lysis buffer. To separate the proteins, gastric antrum and jejunum lysates were dissolved in Laemmli sample buffer (L1100-001, GenDEPOT, Houston, TX) and denaturalized under 100°C for 5 min. Then, the samples were loaded at 20 µg per well, with a total volume of 20 µL. The proteins were separated by 7.5% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk for 1 h at room temperature, the membranes were incubated with primary antibodies, such as C-kit (1:400, AF1356, polyclonal goat IgG, RRID: AB_354750, R&D Systems, Minneapolis, MN), nNOS (1:1000, ab76067, monoclonal rabbit IgG, RRID: AB_2152469, Abcam, Cambridge, UK), ChAT (1:1000, ab178850, monoclonal rabbit IgG, RRID: AB_2721842, Abcam, Cambridge, UK), AChE (1:1000, ab183591, monoclonal rabbit IgG, RRID: AB_2857345, Abcam, Cambridge, UK) or actin (1:1000, MA5-11869, monoclonal mouse IgG1, RRID: AB_11004139, Invitrogen, Waltham, MA) antibodies, overnight at 4°C. After washing with 0.1% TBS-T, the membranes were incubated with HRP-conjugated donkey anti-goat (against C-kit, 1:2500, GTX232040-01, RRID: AB_2887583, GeneTex, Irvine, CA), goat anti-rabbit (against nNOS, ChAT and AChE, 1:5000, GTX213110-01, RRID: AB_10618573, GeneTex, Irvine, CA) or goat anti-mouse (against actin, 1:5000, GTX213111-01, RRID: AB_10618076, GeneTex, Irvine, CA) antibodies. These proteins were visualized using an enhanced chemiluminescence (ECL) advanced kit (Thermo Fisher Scientific, Waltham, MA) and imaged using a FUSION Solo System (Vilber Lourmat, Collégien, France). Protein expression was semiquantified using ImageJ (NIH, Bethesda, MD).

Stomach and small intestine gene expression analysis by quantitative real-time PCR

To investigate smooth muscle contraction, quantitative real-time PCR analysis was performed to assess the expression of four

Table 1. Primer sequence for real-time quantitative PCR.

Gene	Sense (5' → 3')	Anti-sense (5' → 3')
Ano1	GGT GTC GGG TTT GTG AAG AT	TGC ACG TTG TTC TCT TCA GG
Ryr3	GGC CAA GAA CAT CAG AGT	TCA CTT CTG CCC TGT CAG TTT C
	GAC TAA	
5-HT ₄ R	ATG GTC AAC AAG CCC TAT GC	AGG AAG GCA CGT CTG AAA GA
smMLCK	AGA AGT CAA GGA GGT AAA	CGG GTC GCT TTT CAT TGC
	GAA TGA TGT	

ANO1: anoctamin-1; Ryr3: ryanodine receptor 3; 5-HT₄R: 5-hydroxytryptamine receptor type 4; smMLCK: smooth muscle myosin light chain kinase.

contraction-related genes including 5-hydroxytryptamine receptor 4 (5-HT₄R), anoctamin-1 (ANO1), ryanodine receptor 3 (Ryr3) and smooth muscle cell myosin light chain kinase (smMLCK). Details of the primer sequences used for quantitative real-time PCR are provided in Table 1.

Total RNA was isolated from gastric antrum and jejunum tissues using QIAzol reagent (QIAGEN, Hilden, Germany). From each sample, 2 µg of total RNA was used for cDNA synthesis with a High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814, Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR was then carried out using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA), and gene expression was analyzed on an IQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA).

Immunofluorescence staining analysis in gastric antrum and jejunum

Paraffin-embedded sections of gastric antrum and jejunum tissues (8 µm in thickness) were first dried at 60°C for 15 min. Next, the sections underwent deparaffinization in xylene followed by rehydration in a graded ethanol series (100%, 95%, 85%, 70% and 50%) for 3 min each, with a final rinse in tap water. Antigen retrieval was carried out by incubating the sections with 10 mM sodium citrate buffer for 10 min. After three washes, non-specific binding sites were blocked for 1 h with normal chicken serum, and the sections were then incubated overnight at 4°C with an anti-C-kit (1:200, AF1356, polyclonal goat IgG, RRID: AB_354750, R&D Systems, Minneapolis, MN). Following another wash, the slides were incubated for 1 h at room temperature with a donkey anti-goat Alexa Fluor 488-conjugated secondary antibody (1:500, ab150129, polyclonal donkey IgG, RRID: AB_2687506, Abcam, Cambridge, UK). After three 10-minute washing, the sections were stained with DAPI (1 µg/mL) for 3 min at room temperature in the dark condition. Finally, C-kit signals were visualized using an Axio-phot microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The data are presented as mean ± standard deviation (SD) or as fold changes in means. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test. A *p* value less than 0.05 was considered statistically significant.

Results

Fingerprinting analysis of WDJ-S4

Ten analytes, hydroxymethylfurfural, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, liquiritin apioside, liquiritin, nodakenin, glycyrrhizin, decursin and decursinol angelate, were selected as markers for HPLC fingerprinting analysis of WDJ-S4.

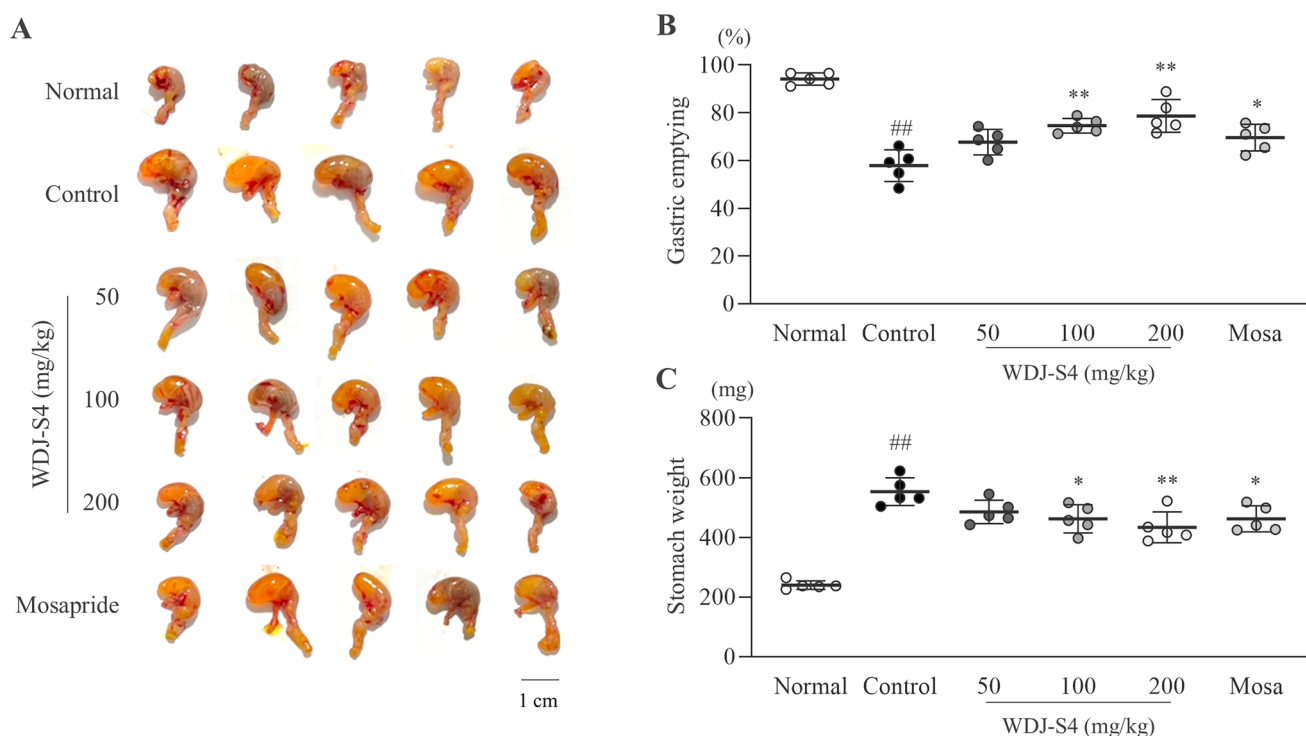


Figure 2. WDJ-S4 reversed loperamide-induced delay in gastric emptying. Mice were orally administered with WDJ-S4 (50, 100 and 200 mg/kg) or mosapride (3 mg/kg) for five days, followed by intraperitoneal loperamide (10 mg/kg) injection. Subsequently, phenol red was administered. After 30 min, the following assessments were conducted. Visual observation of stomach (A), gastric emptying rate (B) and stomach weight (C). To observe the stomach tissues, each tissue was photographed individually. Data are presented as the mean $\pm$ SD ($n = 5$ /group). Statistical significance is indicated as follows: ^{##} $p < 0.01$ compared with the normal group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared with the loperamide-treated control group.

The analytical method was appropriately applied to the fingerprinting analysis of WDJ-S4, and all analytes were eluted within 50 min (Figure 1(A,B)). The retention times of these marker analytes were 8.52, 11.59, 13.66, 14.42, 18.58, 19.00, 19.92, 36.37, 46.83 and 47.16 min, respectively. For simultaneous determination of these analytes in the WDJ-S4 sample, the wavelengths were set to 250 nm (glycyrrhizin), 275 nm (liquiritin apioside and liquiritin), 280 nm (hydroxymethylfurfural), 325 nm (neochlorogenic acid, chlorogenic acid and cryptochlorogenic acid), 330 nm (decursin and decursinol angelate) and 335 nm (nodakenin), respectively. In established assay, 10 marker analytes were detected at concentrations of 0.80–8.99 mg/lyophilized g (Figure 1(C)). Structures used as reference compounds are presented in Supplementary Figure 1.

WDJ-S4 reversed loperamide-induced delay in gastric emptying and intestinal transit

The administration of loperamide resulted in a significant inhibition of phenol red passage, leading to gastric fullness. However, this effect was markedly mitigated by pretreatment with WDJ-S4 (Figure 2). This observation was supported by visual assessment (Figure 2(A)), quantification of the remaining phenol red in the stomach (Figure 2(B)), and measurement of stomach weight (Figure 2(C)), all of which demonstrated statistical significance ($p < 0.01$ for 100 and 200 mg/kg of WDJ-S4). The efficacy of mosapride was found to be similar to that of 100 mg/kg of WDJ-S4.

As expected, loperamide injection also notably decreased intestinal propulsion. WDJ-S4 treatment accelerated the passage of the charcoal diet ($p < 0.05$ or 0.01 for 100 and 200 mg/kg of WDJ-S4, Figure 3(A,B)). Additionally, it was evident that

mosapride had a comparable prokinetic effect to 100 mg/kg of WDJ-S4 in terms of intestinal propulsion.

WDJ-S4 increased acetylcholine release through modulation of acetylcholine metabolism

The injection of loperamide led to a reduction in acetylcholine concentration in both the gastric antrum and jejunum. However, WDJ-S4 treatment significantly mitigated these alterations in both the gastric antrum and jejunum ($p < 0.05$ or 0.01 for 100 and 200 mg/kg of WDJ-S4, Figure 4(A,D)).

Moreover, loperamide had a pronounced effect on the protein expressions of ChAT and AChE, causing a notable downregulation and upregulation, respectively, in both the gastric antrum and jejunum. Nonetheless, WDJ-S4 treatment, particularly at a dose of 200 mg/kg, effectively normalized these protein expression changes ($p < 0.01$ or 0.05) in both the gastric antrum and jejunum (Figure 4(B,C,E,F)). Additionally, it is worth noting that mosapride exhibited a normalizing effect only on ChAT expression in the gastric antrum.

WDJ-S4 enhanced C-kit expression in the gastric antrum and jejunum

The expression of C-kit was significantly reduced by loperamide in the gastric antrum and jejunum. However, pretreatment with WDJ-S4 effectively reversed these alterations, as evidenced by immunohistochemistry (Figure 5(A,D)) and western blot assays ($p < 0.05$ or 0.01 for 100 or 200 mg/kg of WDJ-S4, Figure 5(B,C,E,F)). The administration of mosapride resulted in the upregulation of C-kit expression only in the gastric antrum, while no such effect was observed in the jejunum.

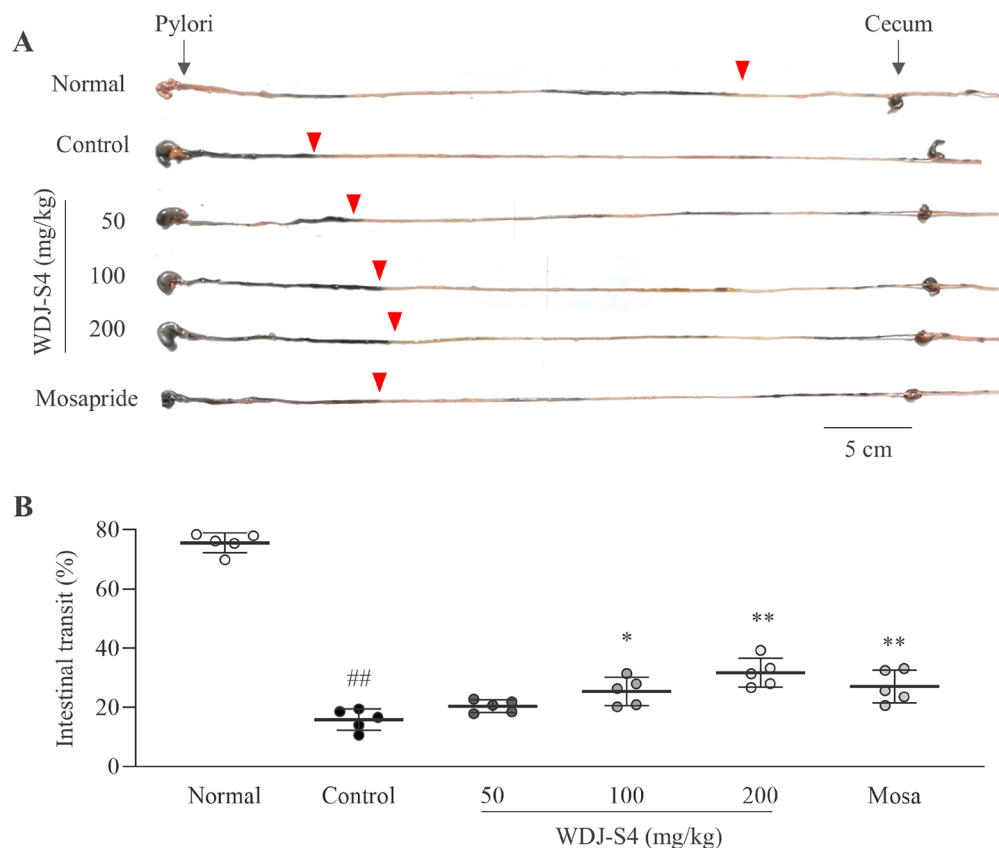


Figure 3. WDJ-S4 reversed loperamide-induced delay in intestinal propulsion. Mice were orally administered with WDJ-S4 (50, 100 and 200 mg/kg) or mosapride (3 mg/kg) for five days, followed by intraperitoneal loperamide (10 mg/kg) injection. Thirty minutes after the administration of the charcoal diet, the traveled distance of the charcoal diet was pictured (A) and quantified (B). The red arrowhead indicates how much charcoal diet moved into the cecum. Data are presented as the mean $\pm$ SD ($n = 5$ /group). Statistical significance is indicated as follows: ## $p < 0.01$ compared with the normal group; * $p < 0.05$ and ** $p < 0.01$ compared with the loperamide-treated control group.

WDJ-S4 upregulated the expression of protein and genes associated with GI motility

WDJ-S4 pretreatment significantly mitigated the loperamide-induced downregulation of nNOS expression in both gastric antrum and jejunum ($p < 0.01$ for 100 and 200 mg/kg of WDJ-S4, Figure 6(A,B,D,E)). In addition, loperamide downregulated the expression of smooth muscle contraction-related genes, such as *Ano1*, *Ryr3*, *5-HT₄R* and *smMLCK*, in the gastric antrum and jejunum. These alterations were notably normalized by treatment with 200 mg/kg of WDJ-S4 in the gastric antrum and jejunum ($p < 0.05$ or 0.01 for 200 mg/kg of WDJ-S4, Figure 6(C,F)), and effects of WDJ-S4 increased in a concentration-dependent manner (Supplementary Figure 3).

Discussion

To investigate the pharmacological potential of WDJ-S4 in treating FD, we utilized a loperamide-induced mouse model of FD. Loperamide, a μ_2 -opioid receptor agonist, inhibits the function of myenteric plexus by reducing acetylcholine secretion, leading to decreased contractile activity of circular and longitudinal smooth muscles in the GI tract (Chen W et al. 2012). Clinically, loperamide is commonly used to manage diarrhea but is associated with adverse effects such as abdominal discomfort, nausea, dyspepsia and constipation (Hanauer 2008). Therefore, several previous preclinical studies have employed high doses of loperamide to establish animal models of FD and constipation (Hwang et al. 2021; Li et al. 2021).

As expected, loperamide administration significantly delayed gastric emptying and intestinal propulsion (Figures 2 and 3). However, pretreatment with WDJ-S4 substantially enhanced the propulsion of gastric and intestinal contents, as indicated by stomach weight and the amount of phenol red retained in the stomach (Figure 2). These results demonstrate that WDJ-S4 exhibits a superior potential to promote GI motility compared to mosapride, the positive control agent in this study. Furthermore, it was observed that both WDJ-S4 and mosapride exhibited a considerable restorative effect on the downregulated *5-HT₄R* gene expression by loperamide in the gastric antrum (Figure 6(C)). Mosapride and cisapride, both *5-HT₄R* agonists, are typically the first-choice options for treating PDS-type FD because *5-HT* and its receptors play a role in regulating smooth muscle contraction (Yang et al. 2017). However, these treatments have clinical limitations such as a high recurrence rate after cessation and adverse effects (Shinozaki et al. 2020). Thus, WDJ-S4 can be an alternative therapeutic option to care for patients, especially for non-responders to prokinetic agents. In addition, given the multi-factorial pathophysiological features of FD, WDJ-S4 would be further effective for at least certain patients owing to the multiple herbal components with multi-action modes.

Delayed GI motility affects 30–70% of FD patients, causing symptoms like postprandial fullness and early satiety (Thumshirn 2002). GI motility relies on rhythmic segmental and peristaltic contractions contributed by interstitial cells of Cajal (ICCs), which act as pacemakers generating electrical slow waves transmitted to smooth muscle cells (Sanders et al. 2014). Dysfunctional

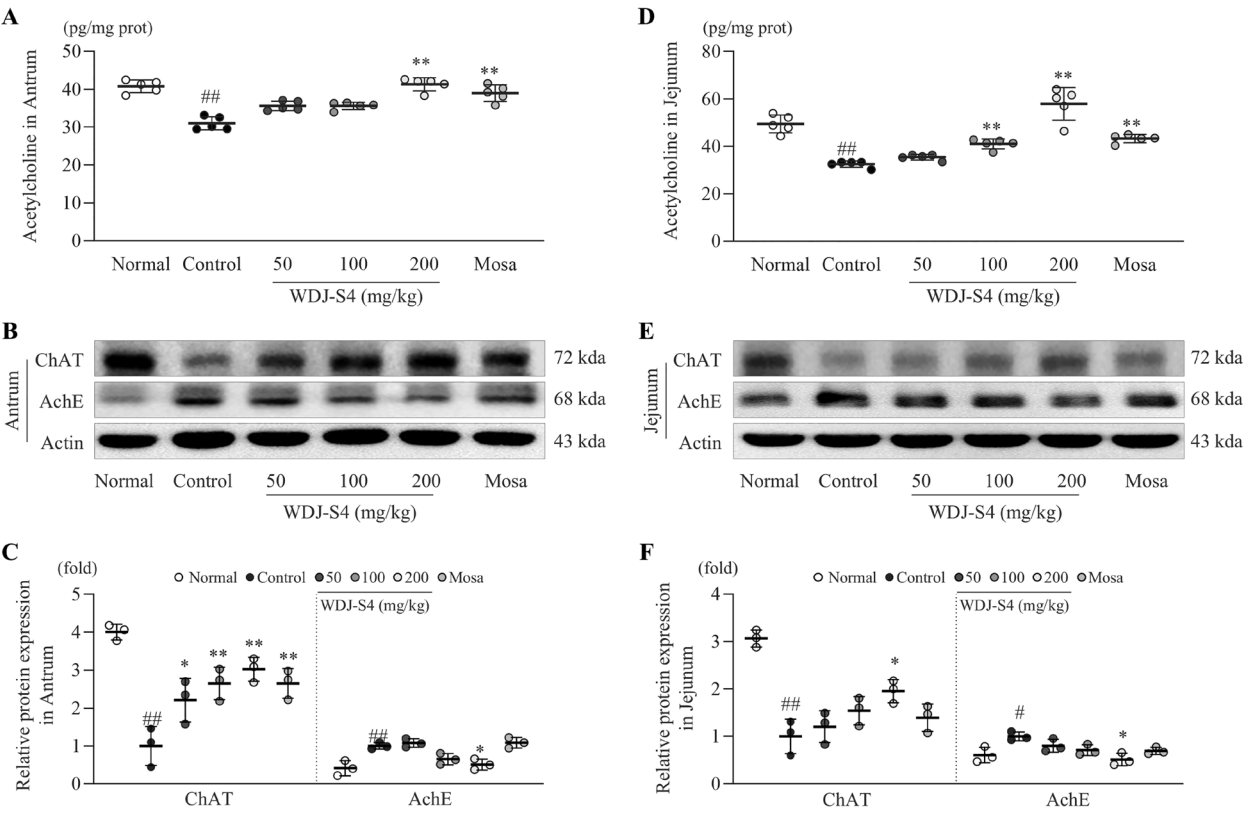


Figure 4. WDJ-S4 increased acetylcholine release through modulation of acetylcholine metabolism in the gastric antrum and jejunum. The level of acetylcholine was detected in the gastric antrum (A) and jejunum (D) using the ELISA analysis method. Protein expressions of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) were detected by using Western blot assay and intensity was semi-quantified in the gastric antrum (B, C) and jejunum (E, F) by using ImageJ software program (Bethesda, MD). Western blot was performed three times. Data are presented as the mean $\pm$ SD ($n = 5$ /group for acetylcholine ELISA and triplication for Western blot). Statistical significance is indicated as follows: [#] $p < 0.05$ and ^{##} $p < 0.01$ compared with the normal group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared with the loperamide-treated control group.

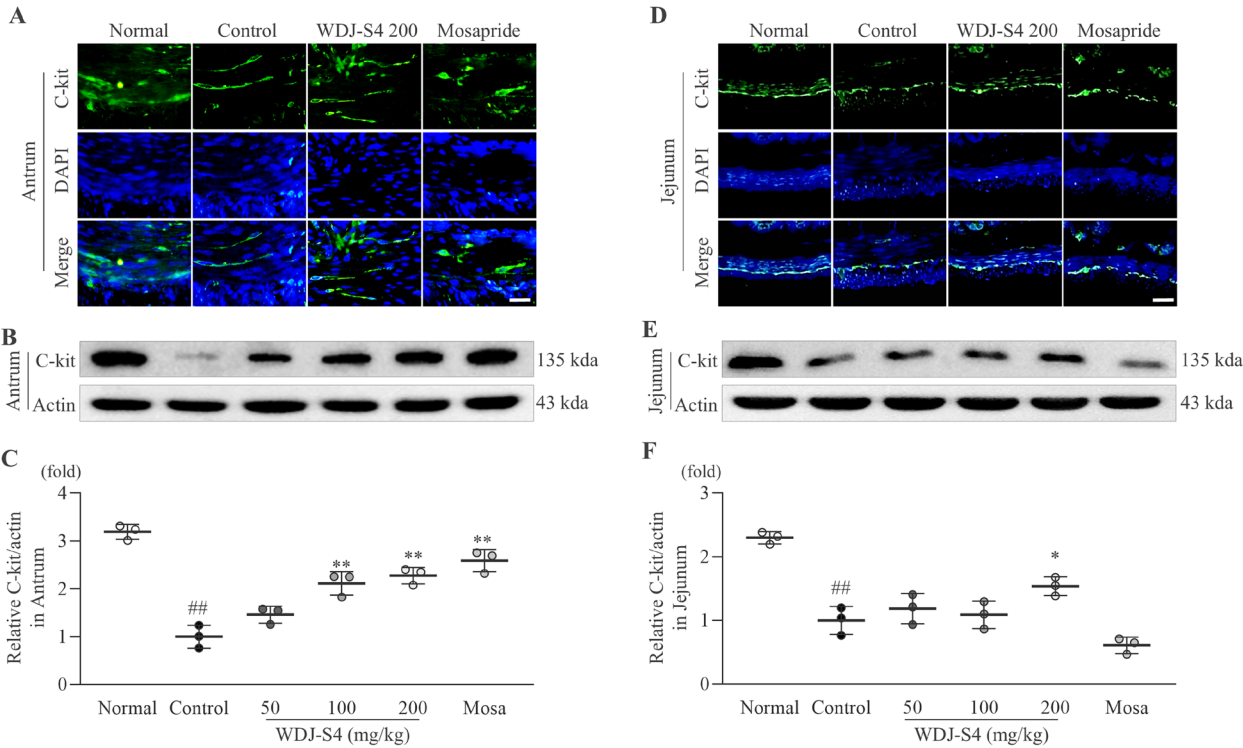


Figure 5. WDJ-S4 upregulated C-kit expression in the gastric antrum and jejunum. C-kit protein expression was visualized by immunofluorescence (A, D) and Western blotting (B, E) in the gastric antrum and jejunum. Western blot signals were semiquantified (C, F) by the ImageJ software program (Bethesda, MD). Immunofluorescence was observed under a microscope ($\times 200$ magnification), and the scale bar indicates 100 μ m. Data are presented as the mean $\pm$ SD. Statistical significance is indicated as follows: ^{##} $p < 0.01$ compared with the normal group; ^{*} $p < 0.05$ and ^{**} $p < 0.01$ compared with the loperamide-treated control group.

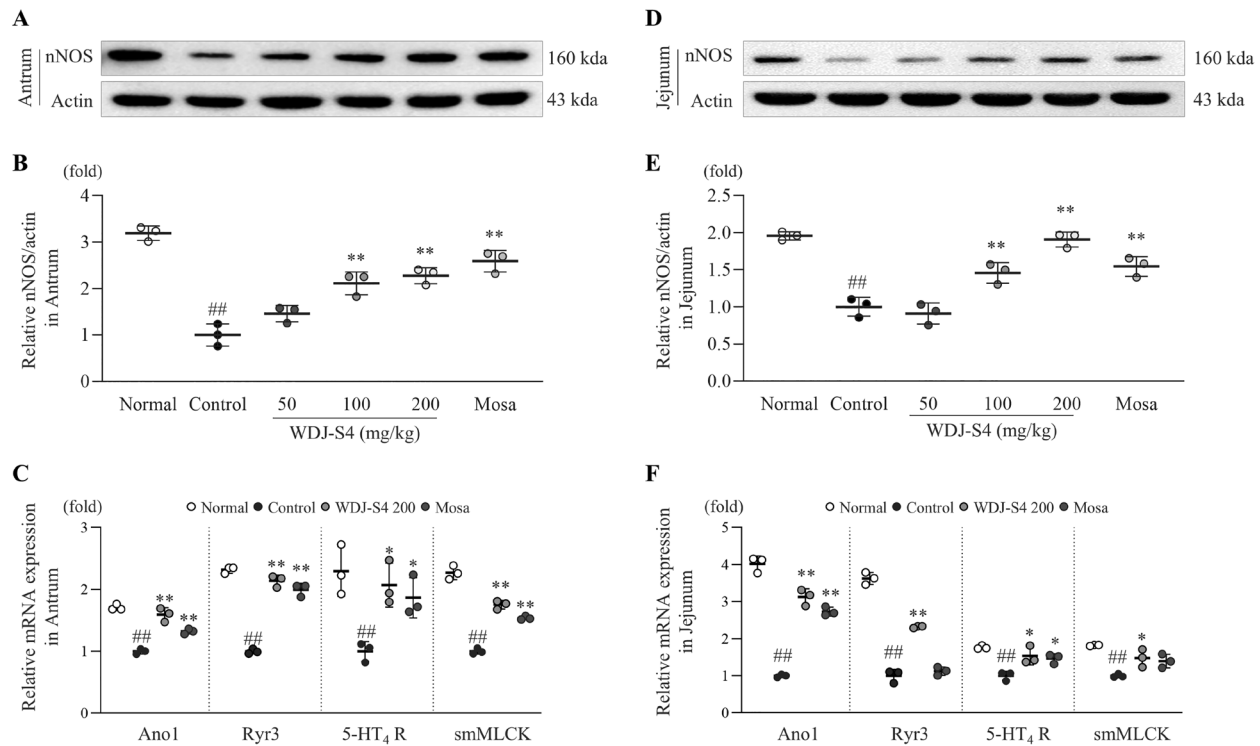


Figure 6. WDJ-S4 stimulated the expression of the nNOS protein and contraction-related genes in the gastric antrum and jejunum. Western blot analysis of nNOS and semi-quantification were performed in the gastric antrum (A, B) and jejunum (D, E). mRNA expression analyses for four gastrointestinal motility-related genes were conducted in the gastric antrum (C) and jejunum (F) tissues. Data are presented as the mean $\pm$ SD. Statistical significance is indicated as follows: ## p < 0.01 compared with the normal group; * p < 0.05 and ** p < 0.01 compared with the loperamide-treated control group. nNOS: neuronal nitric oxide synthase; Ano1: anoctamin-1; Ryr3: ryanodine receptor 3; 5-HT₄R: 5-hydroxytryptamine receptor 4; smMLCK: smooth muscle myosin light chain kinase.

or lost ICCs are linked to GI motility-related disorders, including functional GI disorders (Forster et al. 2005). To evaluate the involvement with ICCs of WDJ-S4, we detected C-kit, a key ICCs marker. Some animal studies have shown that mice lacking C-kit have disrupted electrical networks of ICCs and impaired GI motility (Torihashi et al. 1995; Chen X et al. 2020). WDJ-S4 administration effectively countered the loss of C-kit by loperamide in the gastric antrum (Figure 5). ICCs generate the slow waves via molecules like Ano1 and Ryr3 (Sanders et al. 2006, 2012). Furthermore, voltage-dependent L-type calcium channel (Cav_L)-positive cell also help facilitate calcium influx into ICCs, which is a key step in generating and propagating the slow waves necessary for GI motility (Cobine et al. 2017). The slow waves transmit electrical signals to neighboring smooth muscle cells via gap junctions and then result in smMLCK activation leading to smooth muscle contraction (Surks 2007). WDJ-S4 pretreatment normalized the low expression of the above-mentioned genes such as Ano1, Ryr3 and smMLCK (Figure 6(C)). Furthermore, WDJ-S4 significantly restored the nNOS protein level in the gastric antrum (Figure 6(A,B)). Smooth muscle relaxation in the GI tract depends on nitric oxide (NO) production mediated by nNOS, a retrograde neurotransmitter in vagal nerve synapses (Terauchi et al. 2005). In clinical and animal studies, the administration of the NOS inhibitor, N^G-nitro-L-arginine methyl ester, delayed gastric emptying and nNOS^{-/-} mice also exhibited delayed gastric emptying (Tack et al. 2002; Micci et al. 2005). These findings suggest that WDJ-S4 primarily accelerating GI motility through modulating expression of ICCs and related-genes.

Acetylcholine, synthesized by ChAT and degraded by AChE in enteric nerves of the GI tract, plays a pivotal role in regulating GI muscle peristalsis (Yajima et al. 2011). A preclinical study showed that acetylcholine binds to muscarinic acetylcholine receptors on smooth muscle and ICCs, leading to contraction of

smooth muscle (Iino and Horiguchi 2006). Therefore, upregulation of the acetylcholine level via modulating activities of ChAT and AChE is a promising therapeutic target to promote the GI motility associated with ICCs. In our results, WDJ-S4 exerted the effect of increasing the acetylcholine levels and C-kit expression in the gastric antrum (Figures 4(A) and 5(A-C)). Additionally, the alterations of ChAT and AChE expression are also normalized in gastric antrum (Figure 4(B,C,E,F)). Therefore, acetylcholine release causing ICCs activation might be a potential mechanism of GI motility promotion by WDJ-S4. Acotiamide, a pharmacological compound known for its selective antagonistic effects on AChE, has demonstrated the ability to improve gastric emptying and accommodation (Altan et al. 2012; Vandenberghe et al. 2020). In the European phase 3 trial, acotiamide showed a significantly positive effect on the elimination of individual PDS symptoms (Tack et al. 2018). However, acotiamide is also not a complete FD treatment because dyspeptic symptoms recur about one year after cessation of acotiamide therapy with a recurrence rate of 51% (Shinozaki et al. 2020). As far as we know, there are currently no reports suggesting that acotiamide regulates the function of ICCs. Therefore, the combined regulation of acetylcholine and ICC function will make WDJ-S4 as a potentially useful treatment option for FD.

Irritable bowel syndrome (IBS) is a common condition characterized by abdominal discomfort associated with abnormal bowel movements and not a uniform condition but includes an array of particular forms called subtypes such as constipation-dominant (IBS-C) or diarrhea-dominant (IBS-D) (Kibune Nagasako et al. 2016). While FD and IBS are typically considered distinct conditions, they often co-occur, with 32% of FD cases also having IBS (Suzuki and Hibi 2011). Herein, we found that WDJ-S4 treatment promoted the not only gastric emptying but also intestinal propulsion (Figure 3). Their action

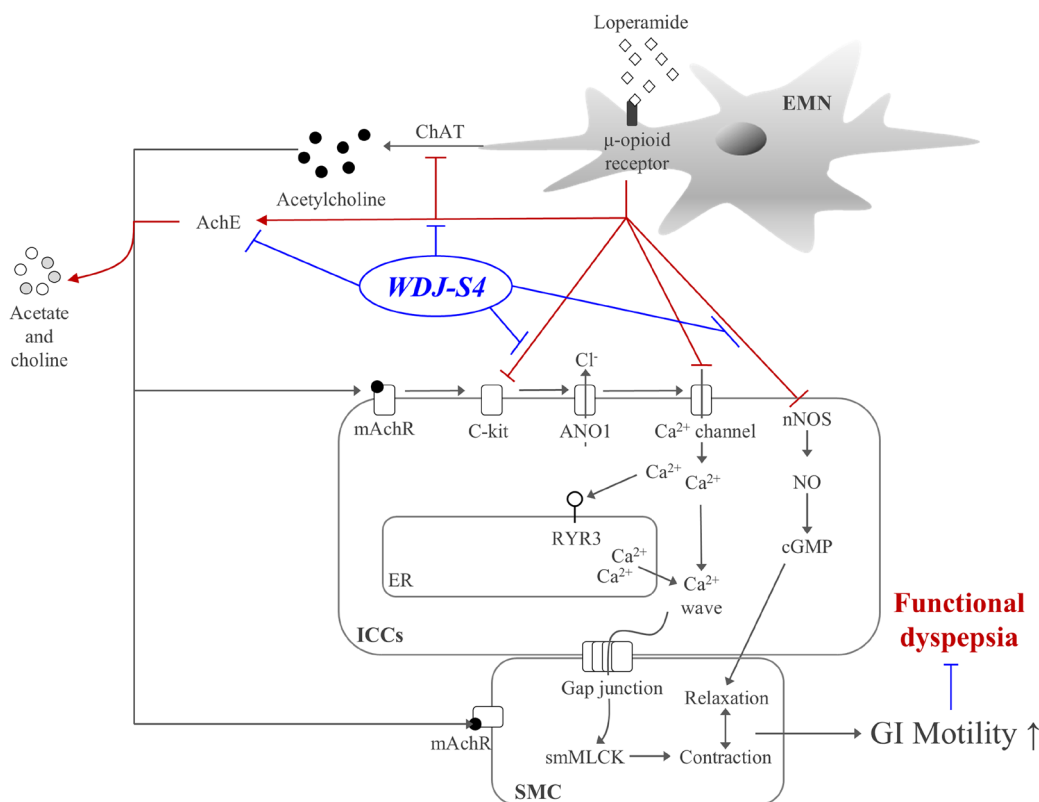


Figure 7. Summary of WDJ-S4 action mechanism. AChE: acetylcholine esterase; ANO1: anoctamin 1; cGMP: cyclic guanosine monophosphate; ChAT: choline acetyltransferase; EMN: enteric motor neuron; ER: endoplasmic reticulum; GI: gastrointestinal; ICCs: interstitial cells of Cajal; mAChR: muscarinic acetylcholine receptor; nNOS: neuronal nitric oxide synthase; RYR3: ryanodine receptor 3; SMC: smooth muscle cell; smMLCK: smooth muscle myosin light chain kinase. Red line indicated the effect by loperamide. Blue line is the effect of WDJ-S4.

mechanism is similar to it in gastric antrum evidenced by normalization of acetylcholine level (Figure 4(D)), acetylcholine metabolism-related proteins (ChAT and AChE, Figure 4(E,F)), GI motility-related proteins (C-kit and nNOS, Figures 5(D–F) and 6(D,E)) and genes (Ano1, Ryr3, 5-HT₄R and smMLCK, Figure 6(F)) expression in jejunum. Therefore, WDJ-S4 might be more beneficial for curing FD patients with IBS-C.

Given the clinical limitations of currently available drugs for FD, such as the relatively high recurrence rate following cessation and low efficacy (Shinozaki et al. 2020), there has been a growing interest in exploring herb-derived natural compounds as a potential approach for drug development (Tan et al. 2020). In the current study, we aimed to assess the prokinetic effects of WDJ-S4, an herbal formula consisting of 13 specific herbs. Some component herbs of WDJ-S4 including *Glycyrrhiza uralensis* Fisch. and *Angelica gigas* Nakai, showed the anti-inflammatory effect of inflammatory GI disease (Oh et al. 2017; Shi et al. 2022). Furthermore, *Crataegus pinnatifida* Bunge and *Zingiber officinale* Roscoe showed prokinetic effects of GI tract (Haniadka et al. 2013; Wu J et al. 2014). WDJ-S4 formula has undergone modification and enhancement in alignment with TKM theory and clinical experience at WeeDahm Hospital in Korea, with a specific focus on addressing RFD. WeeDahm Hospital has been a specialized center for managing RFD since 1992 (<http://weedahm.com/eng/main/main.php>).

RFD represents a distinct category of FD characterized by the persistence of symptoms despite having undergone at least two medical interventions, and it is estimated to account for approximately 10–20% of all FD cases (Jiang et al. 2015). Since 2016, WDJ-S4 has been employed as a standardized herbal formula, and its composition has been verified to include 10 specific compounds, including chlorogenic acid and glycyrrhizin (Figure 1(A–C)). Chlorogenic acid and glycyrrhizin, which are abundant in

WDJ-S4, have demonstrated antidepressant effects in various rodent models of depression (Dhingra and Sharma 2005; Zhao et al. 2022). Furthermore, the combination of glycyrrhizin with escitalopram, a selective serotonin reuptake inhibitor, has demonstrated a synergistic antidepressant effect in a clinical study (Cao et al. 2020). The existing body of literature supports the notion that depressive mood or serotonin dysregulation can suppress the peristaltic movement in the GI tract (Haj Kheder et al. 2018) and it is also worth noting that patients with FD frequently had a high comorbidity of depression (Esterita et al. 2021). In addition, there have been reports indicating that chlorogenic acid has the potential to elevate serotonin levels in the GI tract of various animal species, such as mice and pigs (Wu Y et al. 2018; Chen XD et al. 2021). In current study, WDJ-S4 increases the expression of 5-HT₄R gene in both gastric antrum and jejunum (Figure 6(C,F)). These previous findings and our result collectively provide support for the potential positive effects of WDJ-S4 in the GI tract.

Consequently, we anticipate that the synergistic action of WDJ-S4 may substantiate its clinical utility in the management of patients with FD. In fact, loperamide-induced mouse model does not fully explain the full range of symptoms outline in ROME IV diagnostic criteria, evaluate particularly the subjective symptoms like epigastric pain and early satiety due to this model is effective for studying certain motility-related aspects of FD. Considering the characteristics of this animal model, WDJ-S4 is likely more suitable for the treatment of the PDS type of FD. While the intricate mechanistic details remain elusive, we propose that the effect of WDJ-S4 on peristaltic movement is achieved through the modulation of ICCs activity with the acetylcholine pathway (Figure 7). However, our studies have some limitations like requirements ensuring the toxicity profile of WDJ-S4, although no toxicity-related event has been noticed

from clinical and animal observation. It is also essential to acknowledge that a constraint of the present study lies in the absence of specific compound identification. As such, further investigation is warranted to address this shortcoming. Furthermore, it is imperative to explore the optimal formulation of these multi-herbal combinations to maximize their prokinetic effects and elucidate the underlying mechanisms.

Conclusions

In conclusion, our present data showed the prokinetic effect of WDJ-S4 on FD in a loperamide-induced FD mouse model. The main mechanisms corresponding to these effects may involve the modulation of the acetylcholine pathway, including activation of ICCs in GI tract.

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Author contributions

CRedit: **Seung-Ju Hwang**: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing; **Chang-Seob Seo**: Formal analysis, Resources, Visualization, Writing – review & editing; **Dong-Cheol Baek**: Formal analysis, Investigation, Validation; **Tae-Wook Woo**: Formal analysis, Investigation, Validation; **Jing-Hua Wang**: Visualization, Writing – review & editing; **Jin-Seok Lee**: Visualization, Writing – review & editing; **Yu-Jin Choi**: Formal analysis, Investigation, Validation; **Ji-Yeon Gu**: Formal analysis, Investigation, Validation; **Dong-Seon Kim**: Formal analysis, Resources; **Chang-Gue Son**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files), further inquiries can be directed to the Chang-Gue Son (corresponding author).

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