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Exploring the material basis and mechanism of *Moringa oleifera* in alleviating slow transit constipation based on network pharmacology and animal models

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ABSTRACT: *Moringa oleifera*(*M. oleifera)* have laxative effects, but their active compositions and mechanisms are not very clear thus far. To this end, we systematically explored the active components and mechanism of *M. oleifera* leaves in relieving constipation by using the slow transit constipation (STC) mouse model and network pharmacology. The results of animal experiments showed that *M. oleifera* aqueous extract (MOA) had good laxative activity, and its 70% alcohol soluble part (ASP) also showed significant laxative activity($P < 0.01$). Network pharmacological prediction results suggested that L-phenylalanine (Phe) was the key compound of ASP, and it might relieve constipation through tachykinin receptor 1 (TACR1) and three kinds of adrenergic receptors, including alpha-1a (ADRA1A), alpha-2a (ADRA2A), and alpha-2b (ADRA2B). Further animal experiment results showed that Phe significantly promoted gastrointestinal motility. Phe may relieve STC by enhancing the release of substance P (SP) and upregulating the mRNA expression of *TACR1* in the ileum. Importantly, Phe may also promote intestinal movement by downregulating *ADRA2A* and *ADRA2B* and upregulating *calmodulin* and the mRNA and protein expression of myosin light chain 9 (MYL9) in the ileum, thereby activating the GPCR-MLC signaling pathway. These results lay a foundation for the application of *M. oleifera* and Phe in constipation.

Keywords: *Moringa oleifera*; Network pharmacology; L-phenylalanine; Gastrointestinal motility; Laxative

1. Introduction

Constipation is a common digestive system disease. Constipation affects approximately 11%-20% of the adult population each year [1]. According to the etiology, constipation is divided into organic constipation and slow transit constipation (STC) [2]. STC refers to chronic constipation without organic causes, structural abnormalities or metabolic disorders except irritable bowel syndrome [3]. Its main clinical symptoms include reduced defecation, dry stool, defecation difficulty and incomplete defecation [4-5]. Although constipation is

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not life-threatening, long-term constipation can cause complications of anorectal diseases such as hemorrhoids and anal fissure and increase the mortality of hypertension, cardio-cerebrovascular diseases and the prevalence of colon cancer [5]. Intestinal dyskinesia is the most common cause of STC. At present, the exact pathogenesis of STC is not clear, and there is no effective radical cure in the clinic. Previous studies have shown that many factors are involved in the occurrence and development of STC, such as alteration of aquaporin [6-7], structural abnormality of smooth muscle cells [8], disturbance of gastrointestinal hormone secretion [9], and activation of signaling pathways [10].

Network pharmacology provides new ideas and methods for analyzing massive biomedical data and building a bridge from data to knowledge [11]. As a means of systematic analysis, network pharmacology can provide the potential relevance of studying genes, targets, and signaling pathways at multiple levels. Research on traditional Chinese medicine and even Western medicine based on network pharmacology is systematic, relevant and predictive. These studies can provide support for clinical drugs to optimize and explain the mechanism of action of traditional drugs and have broad application [10]. Network pharmacology can also provide a new perspective for the study of constipation. For example, the potential relationship between constipation and depression has been found through network pharmacological analysis [12], and the potential signaling pathway of paclitaxel in improving functional constipation was identified by network pharmacology [13].

At present, the treatment of constipation is mainly focused on permeability, irritation and other Western medicines and traditional Chinese medicines, such as the proprietary Chinese medicine Senna and rhubarb [14]. However, these drugs are always associated with the risk of adverse reactions such as arterial contraction, coronary artery spasm and myocardial infarction [15]. Therefore, the treatment of constipation with natural plant-based diets with few side effects is preferable.

M. oleifera leaves have a variety of health benefits, including reducing blood lipids and blood sugar, antioxidant activity, antibacterial activity, anticancer activity, and anti-inflammatory activity [6,16]. *M. oleifera* leaves can regulate the number of stools, wet weight of feces and water content of feces to varying degrees, achieve the effect of defecation, and restore the thickness and mucus of the colonic muscle layer [17]. *M. oleifera*, as a traditional medicinal plant, has been used in folk medicine to relieve constipation [18]. Further study found that three flavonoids in *M. oleifera* leaves showed good inhibitory effects on constipation [19]. Although these previous studies have reported the laxative function and some active compositions of *M. oleifera* leaves, the material basis and mechanism of its laxative activity need to be further investigated along with its rich material compositions and biological activities. To this end, we systematically explored the effective compositions of *M. oleifera* leaves and their mechanism of relieving functional constipation using network pharmacology and animal experiments in this study.

2. Materials and methods

2.1. Preparation of M. oleifera leaf extracts

M. oleifera leaf aqueous extract (MOA): A total of 5,000 g powder of *M. oleifera* leaves was accurately weighed, added to boiled deionized water according to the ratio of material to liquid (1:9) (g/mL), mixed well and boiled for 1 min, and then 4 layers of medical gauze were used to filter immediately. After the rough filtration was completed, the filtrate was centrifuged (4,500 rpm for 5 min), and the supernatant was collected. Then, the filter residue was poured into boiled deionized water (as before) and boiled for 1 min again. The above operation was repeated twice, the supernatant was pooled, vacuum freeze-dried (48 h), and the MOA dry powder was obtained. The yield was 20.0%.

Alcohol-soluble part (ASP) and alcohol-precipitated part (APP) of MOA: 500 g of MOA dry powder was accurately weighed, sterilized water was added according to the ratio of material to liquid (1:10) (g/mL), anhydrous ethanol was slowly added, and the mixture was stirred quickly so that the ethanol concentration reached 70% (v/w). After standing at 4 ℃ for 24 h, the extract was obviously stratified, and the supernatant (ASP) was separated from the precipitate (APP) by centrifugation (4,500 rpm, 5 min). ASP was concentrated, ethanol was removed by rotary evaporation, and a light green extract was obtained. Both ASP and APP were dried using a vacuum freeze-drying method (48 h). The yields of ASP and APP were 58.8% and 28.6%, respectively, with a ratio of approximately 2:1.

2.2. Animal experimental design

Healthy male Kunming(KM) mice (4 weeks old, 20-25 g) were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. The animals were placed in an SPF environment (24 ± 1 °C, 12 h light cycle, lights off at 20:00) with 3 mice per cage, and the mice had free access to food and water. All animals were acclimatized for 1 week and then randomly divided into 4 or 6 groups of 12 animals each (n = 12), ensuring similar body weight in different groups. Then, 8 mg/kg·bw loperamide (loperamide, mg/kg·body weight, Sigma) was used for 8 days to construct the STC mouse model, and the mice received daily oral dosages of 300 μL of solutions that were assigned to the appropriate experimental group. The Maren pill (positive medicine, Beijing Tongrentang Pharmaceutical Co., Ltd.), MOA, ASP, APP, and L-phenylalanine (Phe) were dissolved in 300 μL of saline solution. Four animal experiments were performed in this study, and the group settings are described subsequently.

Animal Experiment 1: The mice were separated into 6 groups of 12 mice each after 7 days of acclimatization. The dose of MOA was set with reference to the dose of the traditional constipation treatment drug, MaRen pills (the positive drug). The 6 groups included the CON group (control group, received saline solution as vehicle), LOP group (model group, received loperamide), POS group (positive control group, received 900 mg/kg·bw Maren pill from Beijing Tongrentang Pharmaceutical Co., Ltd.), LMOA group (received loperamide and 300 mg/kg·bw MOA, low dosage), MMOA group (received loperamide and 600 mg/kg·bw MOA, medium dosage) and HMOA group (received loperamide and 900 mg/kg·bw MOA, high dosage).

Animal Experiment 2: The mice were separated into 6 groups of 12 mice each after 7 days of acclimatization. Based on the ASP yield (58.8%) and MOA dosage, six dose groups were established. The 6

groups included the CON group, LOP group, POS group, LASP group (received loperamide and 300 mg/kg·bw ASP, low dosage), MASP group (received loperamide and 450 mg/kg·bw ASP, medium dosage) and HASP group (received loperamide and 600 mg/kg·bw ASP, high dosage).

Animal Experiment 3: The mice were separated into 6 groups of 12 mice each after 7 days of acclimatization. Based on the APP yield (28.6%) and MOA dosage, six dose groups were established. The 6 groups included the CON group, LOP group, POS group, LAPP group (received loperamide and 150 mg/kg·bw APP, low dosage), MAPP group (received loperamide and 225 mg/kg·bw APP, medium dosage) and HAPP group (received loperamide and 300 mg/kg·bw APP, high dosage).

Animal Experiment 4: The mice were separated into 4 groups of 12 mice each after 7 days of acclimatization. Based on the body weight and feed intake of the mice, as well as the amount of Phe in the mice's feed, the daily intake of Phe from the feed can be converted to approximately 500-700 mg/kg. Therefore, in order to investigate whether supplemental Phe has laxative activity, two doses of Phe equivalent to the dose obtained from the feed were set, which were comparable to the doses of MOA and ASP.

The 4 groups included the CON group, LOP group, LPhe group (received loperamide and 500 mg/kg·bw Phe, low dosage) and HPhe group (received loperamide and 750 mg/kg·bw Phe, high dosage).

All mice were deprived of food but not water overnight before the defecation test and gastrointestinal transit test. The protocols of animal experiments used in this study were previously approved by the Animal Ethics Committee of Yunnan Agriculture University (Approval No.: 202201018).

2.3. Defecation test, gastrointestinal transit test and tissue collection

Defecation test: On the evening of the 6th day after intervention, the mice in each group were withdrawn from food. After 10 hours, each group was intragastrically administered the corresponding treatments. After 30 min, mice in each group were given intragastric administration of ink and began to be timed. After intragastric administration, the mice were placed in independent cages and given sufficient water. The first black stool time (FBST) of each mouse was recorded. The feces of each mouse were collected within 6 hours, and fecal wet weight (FW), fecal water content, and fecal number (FN) were all examined to assess the laxative effect [20].

Gastrointestinal transit test: On the 7th night after intragastric administration of the corresponding subjects, the mice in each group were starved. Ten hours later, each group was intragastrically administered the corresponding treatments. The ink (300 μL) was administered to each mouse 30 min later. The mice were euthanized in a box filled with CO_2 after 20 min, and the abdominal cavity was opened to collect blood from the abdominal aorta. The length of the small intestine was measured as the total length of the small intestine, and the ink advancing distance from the pylorus to the ink front was used to determine the gastrointestinal transmission rate. The collected blood was incubated at 37 ℃ for 30 min and centrifuged at 4 ℃ at 3,500 rpm for 10 min, and serum was collected. The ileum of each mouse was precisely dissected simultaneously. The contents of the ileum were rinsed extensively with cold PBS. Then, the ileum was snap-frozen in liquid nitrogen and stored at -80 ℃.

2.4. Phytochemical composition determination by widely targeted metabolomics

A total of 10 mg freeze-dried ASP powder was combined in a centrifuge tube with 2 small steel balls and 500 μL extract (methanol: water, volume ratio 1: 2). The mixture was precooled at 40 ℃, including the internal standard. The mixture was mixed for 30 s, homogenized at 35 Hz for 4 min, and placed in an ice water bath for ultrasonic homogenization for 5 min. The ultrasonic homogenization was repeated 3 times. Then the mixture was incubated overnight at 4 ℃ on the homogenizer. Next, the mixture was centrifuged for 15 min at 12,000 rpm (centrifugal force 13,800 g, radius 8.6 cm) at 4 ℃. The supernatant was carefully filtered through a 0.22 μm microporous membrane. One hundred microliters of each sample was mixed into QC samples and stored at -80 ℃ until computer analysis.

The target compositions were separated by EXIONLCSystem (SCIEX) performance liquid chromatography (EXIONLCSystem) and a Waters UPLC liquid chromatography column. The liquid chromatography phase was an aqueous solution containing 0.1% formic acid, and phase B was acetonitrile. The temperature of the column incubator was 40 °C, the temperature of the automatic injector was 4 °C, and the injection volume was 2 μL. A Sciex QTrap 6500+ (Sciex Technologies) was used for assay development. Typical ion source parameters were as follows: ion spray voltage: +5500/-4500 V, curtain gas: 35 psi, temperature: 400 ℃, ion source gas 1:60 psi, ion source gas 2: 60 psi, DP: ± 100 V.

SCIEX Analyst Work Station Software (Version 1.6.3) was used for MRM data acquisition and processing. MS raw data (wiff) files were converted to the TXT format using MS conventer. An in-house R program and database were used for peak detection and annotation.

2.5. Network pharmacology analysis

Screening the targets based on the identified compositions of ASP: Based on UHPLC–MS technology, the chemical compositions of ASP were analyzed. Their CAS number was put into the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) for standardization, the corresponding 2D structure diagram was downloaded, and the 2D structure was imported into the "SwissTargetPrediction" website (http://www.swisstargetprediction.ch/). The compositions were determined by gastrointestinal absorption rate (GI absorption) and drug likeness in the screening condition of "Swiss ADME", and the target with probability > 0.1 was selected for follow-up analysis.

Screening the targets related to constipation: The "GeneCards" database (https://www.genecards.org/), "OMIM" database (https://omim.org/), and "Drugbank" database (https://www.drugbank.org/) were searched with "constipation, intestinal peristalsis, interstitial cells of Cajal, intestinal inflammation" to collect and sort out the related targets of constipation. The collected constipation targets are shown in Table S1.

A composition-target interaction network was constructed using Cytoscape v3.9.1. Then, the ASP composition target was associated with the collected constipation target, the target intersection network was established by the Venn method, and the overlapping target was selected as the key analysis object.

Approaches and networks for predicting the role of major aspartic acid compositions: the intersection targets were selected from the above analysis and uploaded to the Kyoto Encyclopedia of Genes and Genomes

(KEGG, http://www.kegg.jp/) and Metascape bioinformatics resource databases to obtain information about the pathways. The FDR error control method was used to test the P value, and the threshold was set to $P < 0.05$.

2.6. RNA extraction and real-time quantitative PCR (RT–qPCR)

Total RNA was extracted from mouse tissues by a FastPure® Cell/Tissue Total RNA Extraction Kit (Nanjing, China, Vazyme), and the complement gene was reverse transcribed by a HiScript8 III RT SuperMix for qPCR (+ gDNA wiper) kit (Beijing, China, Summer Bio). RT–qPCR amplification was performed using SYBR quantitative polymerase chain reaction (Nanjing, China, Vazyme) on a LightCycler 480 real-time fluorescence quantitative system. The primer sequences used to evaluate the expression of various genes are shown in Supplementary Table S2, including tachykinin receptor (*TACR1*), adrenoceptor alpha 1A (*ADRA1A*), adrenoceptor alpha 2A (*ADRA2A*), adrenoceptor alpha 2B (*ADRA2B*), calmodulin (*Calm*), and myosin light chain 9 (*MYL9*). The relative expression of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.7. Enzyme-linked immunosorbent assay

Substance P (SP) and induced noradrenaline/norepinephrine (NA/NE) in serum were determined using the substance P (SP) ELISA Kit Instruction (Sangon Biotech, China) and Mouse Noradrenaline/Norepinephrine (NA/NE) ELISA Kit Instruction (Sangon Biotech, China), respectively.

2.8. Western blot analysis

Approximately 30 mg of tissue from the ileum was lysed in 300 μl of radioimmunoprecipitation assay (RIPA) buffer (Strong, catalog number E121-01; Genstar, China) containing phenylmethylsulfonyl fluoride (PMSF) (1 mM) and then homogenized. Protein samples were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for 2 h and then incubated with anti-MYL9 (Wuhan, China, Proteintech) and anti-β-actin antibodies (Wuhan, China, Proteintech) overnight at 4 ℃. The membrane was washed with PBST 3 times for 10 min each time, incubated with HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Wuhan, China, Proteintech) at room temperature for 1 h, and washed with PBST 3 times for 10 min each time. Then, the protein was visualized by ECL developer, and the gray level of the protein was analyzed. The relative expression of protein was expressed as target protein/β-actin.

2.9. Statistical analysis

The data are expressed as the means ± standard errors of the means (SEMs). The unpaired two-tailed Student's t test was performed to analyze two independent groups. Unless otherwise specified in the figure legends, the results were considered statistically significant at a P value of < 0.05.

3. Results

3.1. MOA alleviates the symptoms of loperamide-induced STC in mice

At present, the relief effect of constipation is mainly evaluated by defecation tests and gastrointestinal transit tests (Fig. 1A). The therapeutic effect of MOA was evaluated by the mouse model of STC induced by loperamide. Compared with the LOP group, the FBST of mice in the MMOA (600 mg/kg·BW) and HMOA groups (900 mg/kg·BW) decreased by 40.3% and 46.3%, respectively (Fig. 1B, $P < 0.01$). Although MOA treatment did not significantly increase the number and weight of feces in STC KM mice within 6 h (Fig. 1C, 1D and 1F), the middle and high doses of MOA significantly increased the fecal water content of feces (Fig. 1E and 1F). In the LOP group, the high doses of MOA significantly increased the gastrointestinal transit rate (GTR) by 32.7% (Fig. 1G and 1H, $P < 0.001$). The tendency of increased defecation and enhanced gastrointestinal motility in the MOA group was consistent with that in the POS group, and it is worth noting that we did not observe any negative effects of MOA treatment on food intake, drinking or basic physical indicators (Fig. S1).

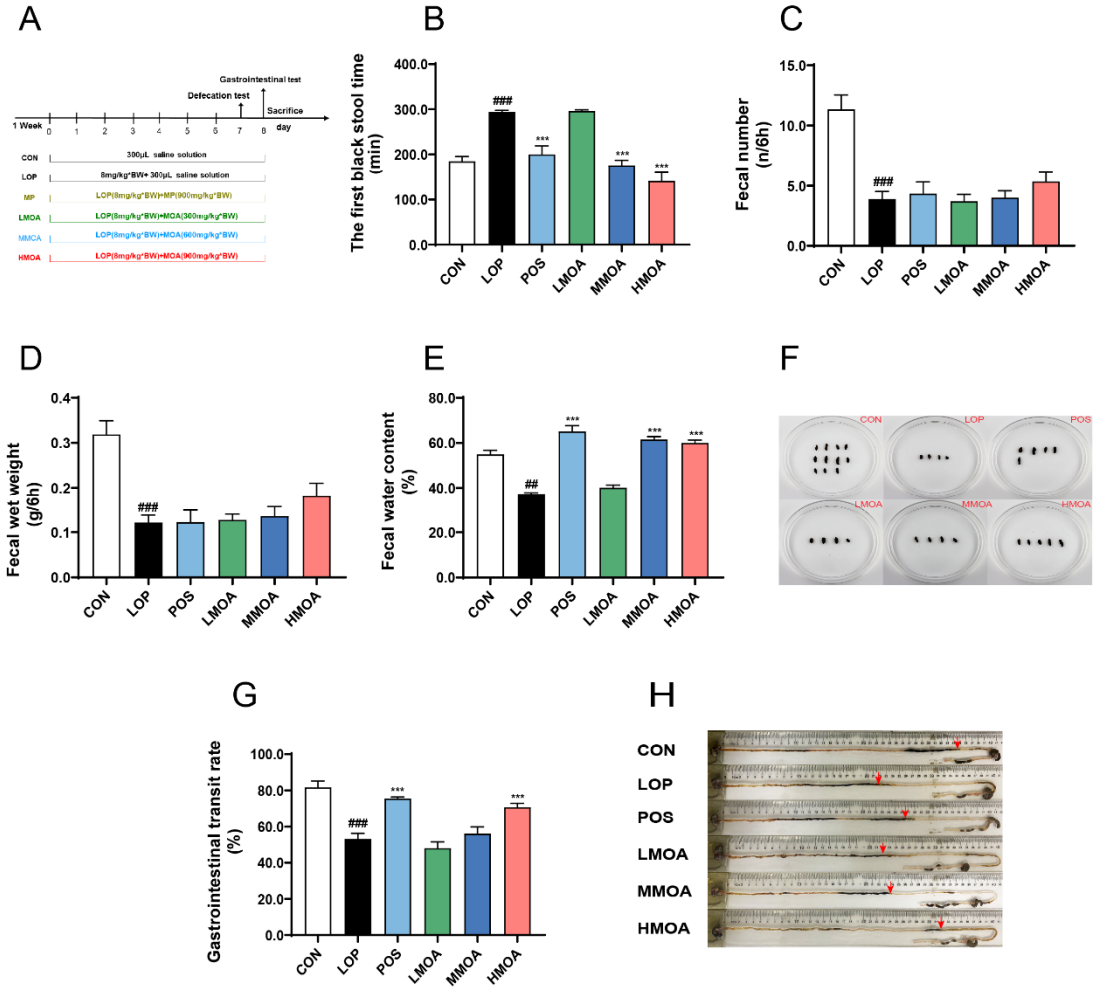

Fig. 1 Influence of MOA on loperamide-induced constipation symptoms in mice. (A) Animal experimental procedure; (B) The first black stool time; (C) Fecal number; (D) Fecal wet weight ; (E) Fecal water content; (F) Representative fecal morphology of each group; (G) Gastrointestinal transit rate and (H) Representative pictures of ink propulsion distance. The data are expressed as the means ± SEMss ($n = 12$). #, compared with the CON group; *, compared with the LOP group. ##, $P < 0.01$; ###, $P < 0.001$. ***, $P < 0.001$.

3.2. ASP is the active fraction involved in MOA laxative activity

Small molecular active compositions often play an important role in the process of constipation [21]. As a complex containing a large number of flavonoids, polyphenols and amino acids, ASP may have potential value in improving STC. Through animal experiments, we found that ASP could significantly shorten the FBST (Fig. 2A) and increase the GTR in loperamide-induced STC mice (Fig. 2E and 2I), although FN and FW failed to show significant changes (Fig. 2B-2D). In addition, we found that APP did not show a significant relieving effect on STC in either the defecation test or gastrointestinal transit test (Fig. 2F and 2G, Fig. S2 and S3). It is worth noting that, similar to MOA treatment, no side effects of ASP on STC mice were observed during the whole process (Fig. S2).

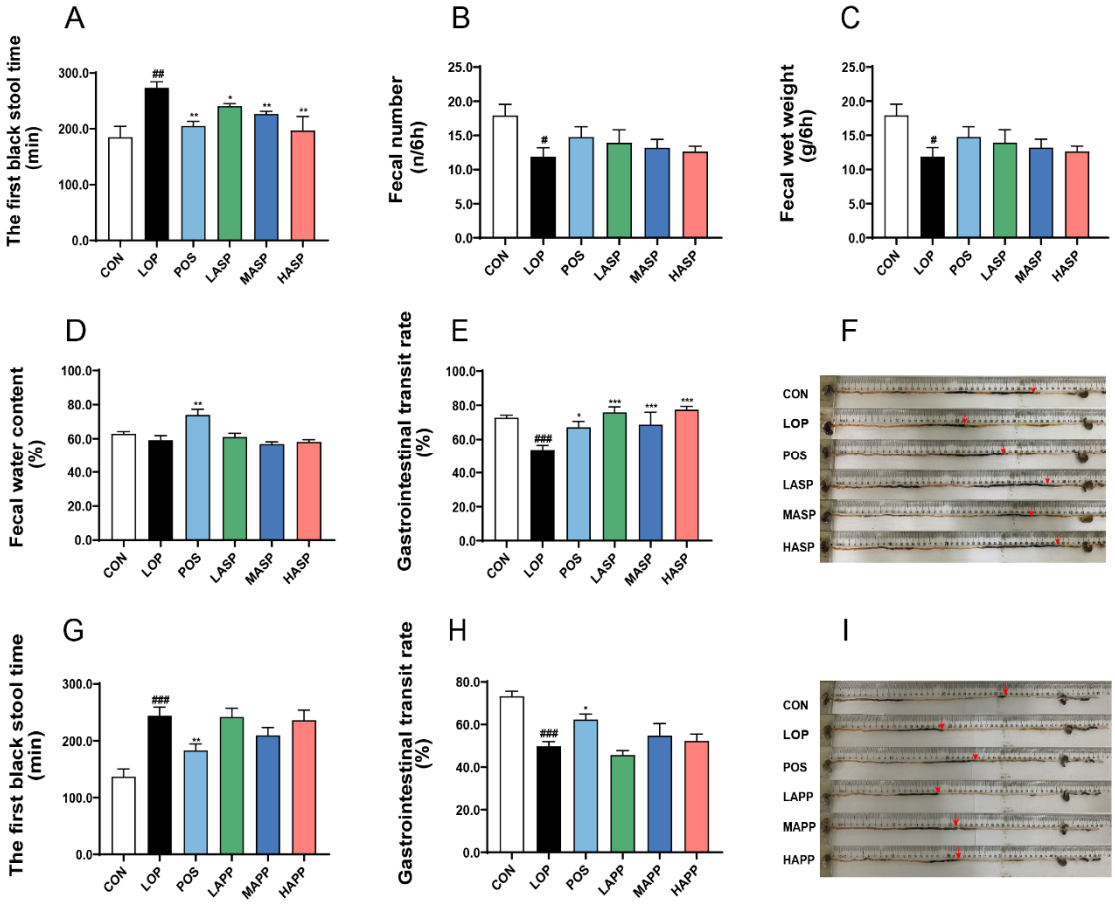

Fig. 2 Influences of fraction ASP and APP on loperamide-induced constipation symptoms in mice. Animal Experiments 2 and 3 were used to assess the laxative effect of ASP and APP on loperamide-induced STC. (A and G) The first black stool time; (B) Fecal number; (C) Fecal wet weight; (D) Fecal water content; (E and H) Gastrointestinal transit rate; (F and I) Representative pictures of ink propulsion distance. The data are expressed as the means ± SEMss (n = 12). #, compared with the CON group; *, compared with the LOP group. #, $P < 0.05$, ##, $P < 0.01$; ###, $P < 0.001$. *, $P < 0.05$, **, $P < 0.01$ ***, $P < 0.001$.

3.3. Analysis of ASP composition based on UHPLC–MS

Based on the results of UHPLC–MS, we classified the identifiable compounds (Fig. 3C), and the top 10 classifications were flavonoids, amino acids, nucleotides, lipids and aromatics, alkaloids, phenols, vitamins and organic acids, phenylpropanoids, tryptamine, indole and pyridines. As a single compound, L-phenylalanine (Phe) had the highest relative abundance (Fig. 3D). The relative abundances of the identified compounds in ASP were ranked. The top 62 compounds in terms of relative abundance were further analyzed

by network pharmacology because the sum of the relative abundance of these 62 compounds was just greater than 95%. The specific compositions are shown in Table S3.

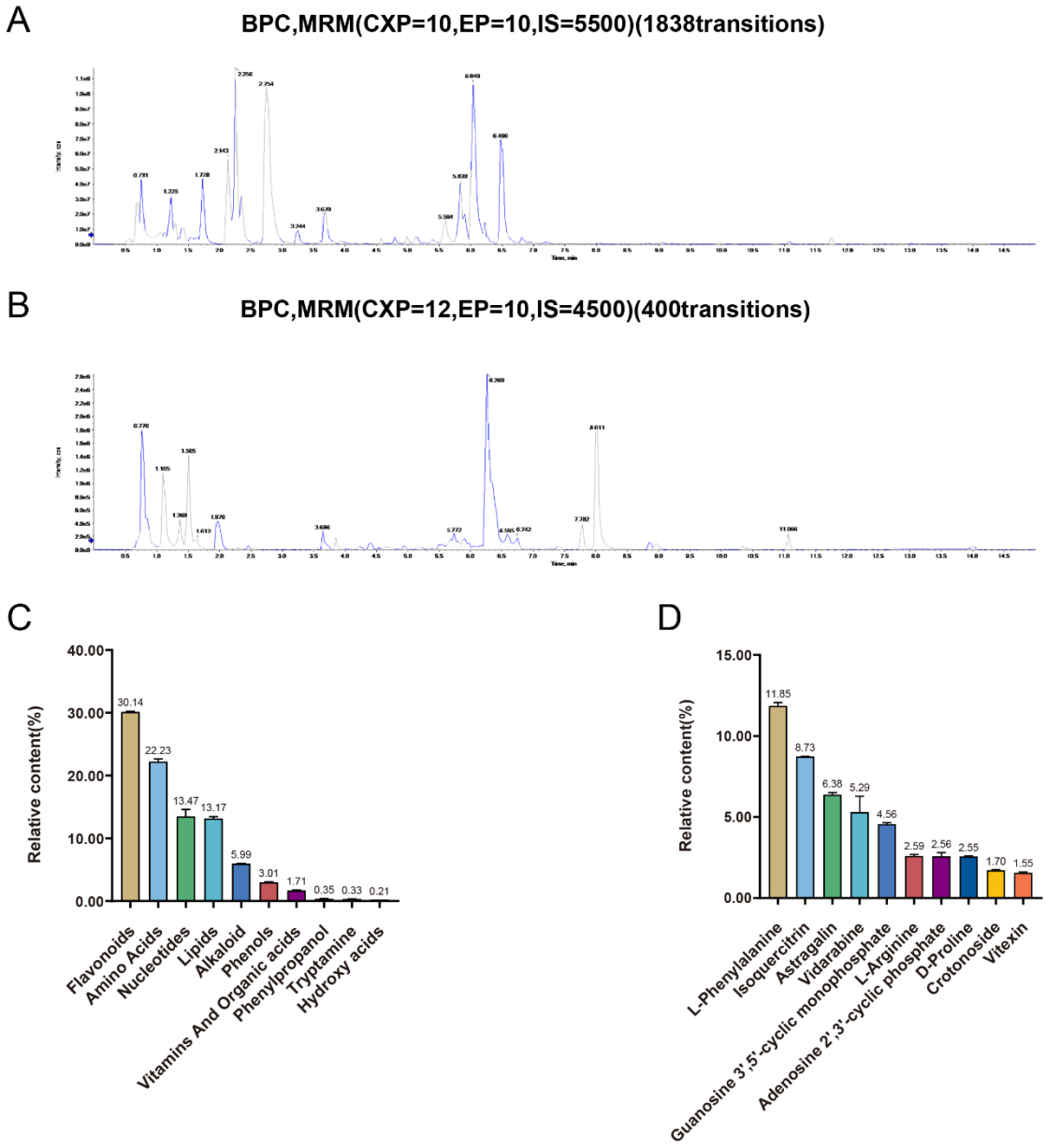

Fig. 3 The main chemical components of ASP based on widely targeted metabolomics

Total ion flow map: positive ions (A), negative ions (B); (C) the top 10 classifications of ASP; the relative abundance of major categories of ASP components; (D) the relative abundance of top 10 single compounds in ASP. The data are expressed as the means ± SEMs (n = 3).

The 62 compounds were screened by ADME on the "SwissTargetPrediction" platform, and 29 potentially active compounds were obtained (Table S4). The composition-target network is shown in Fig. S4. Taking the parameter probability > 0.1 as the screening criterion, 296 possible targets were obtained. The potential 296 targets of 29 compounds intersected with the collected constipation targets and 146 potential targets related to constipation were obtained (Fig. 4A and Table S4 and Table S5).

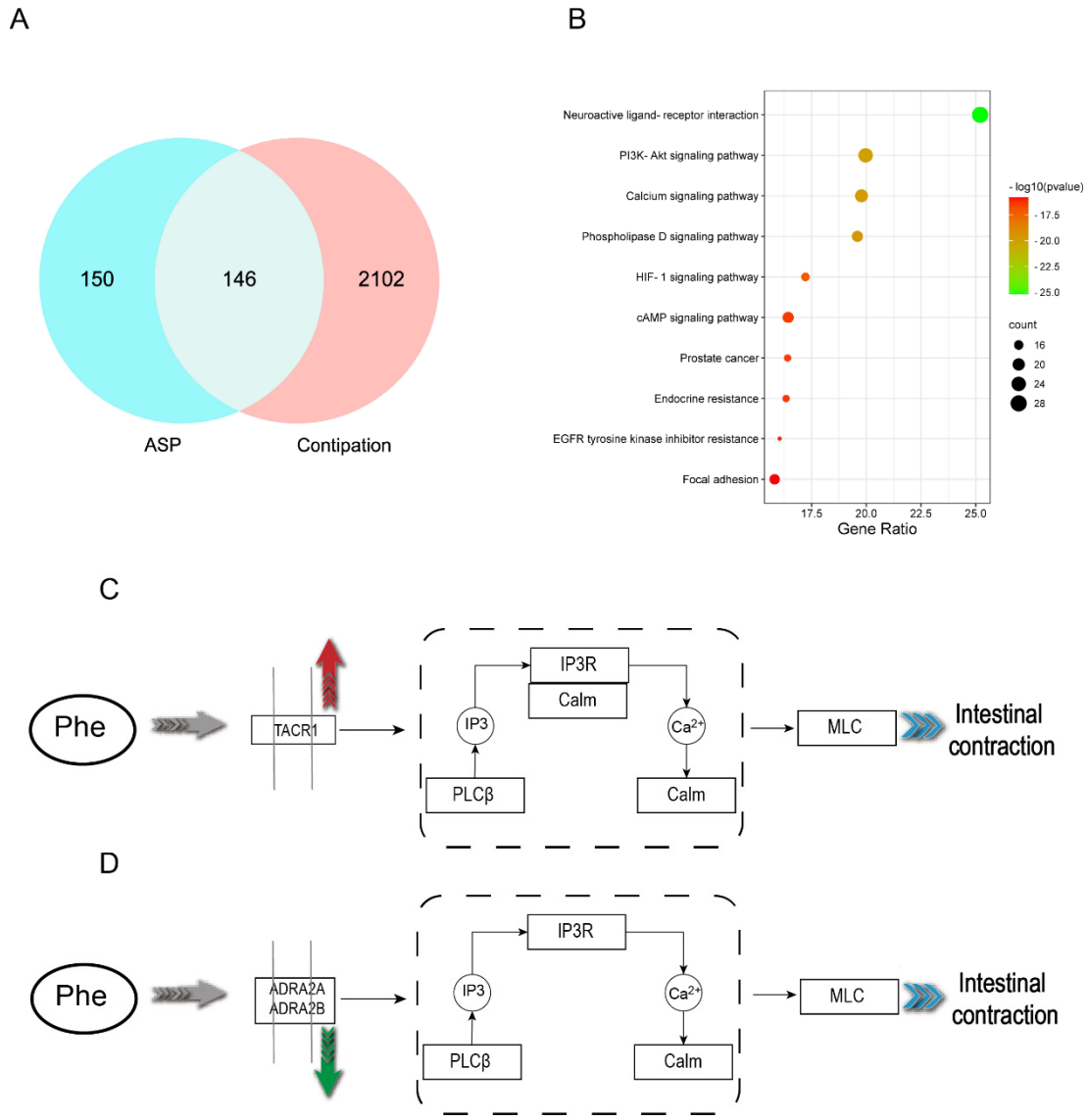

Fig. 4 Prediction and analysis of ASP target and signaling pathways. (A) The intersection target of ASP and constipation; (B) KEGG enrichment analysis of intersection target (top 10 pathways); (C) TACR1, a predicted target of Phe, was located in the GPCR-MLC signaling pathway; (D) ADRA2A and ADRA2B, predicted targets of Phe, were located in the GPCR-MLC signaling pathway.

3.4. Analysis of ASP composition based on network pharmacology

The 146 targets were imported into the "Metascape" database for KEGG signaling pathway enrichment analysis. The enrichment results showed that a total of 156 signal pathways were obtained by KEGG enrichment analysis. Taking the *P* value as the screening condition and combined with the research status of constipation-related mechanisms, the first 10 enrichment pathways were selected, the results were visualized by the “bioinformatics” platform (http://www.bioinformatics.com.cn), and the results are displayed in a bubble chart (Fig. 4B). Neuroactive ligand–receptor interaction, PI3K-Akt, calcium signaling and other signaling pathways may be involved in the process by which ASP relieves constipation.

To explore the key components of ASP in improving constipation, we conducted a comprehensive analysis of the predicted signaling pathways. We focused on the most enriched neuroactive ligand–receptor interaction signaling pathway, and Table 1 shows the correspondence of the relevant compound-relative

abundance targets for this signaling pathway. Based on the highest relative abundance and more targets enriched in neuroactive ligand–receptor, we hypothesized that L-phenylalanine (Phe) may be an important component of ASP and MOA in relieving constipation. Notably, although turanose has more targets than Phe in this pathway, its low abundance (0.16%) makes it unlikely to be a candidate compound to explain the relief effect of ASP on STC.

Table 1 The correspondence of the relevant compound-relative abundance targets with the neuroactive ligand–receptor interaction signaling pathway.

Compounds	Relative abundance (%)	Target
L-Phenylalanine	11.84	TACR1, ADRA1A, ADRA2A, ADRA2B
Crotonoside	1.70	ADORA1, ADORA2A, F2
L-Lysine	0.49	GRIA1, GRIA2, GRIA4
L-Glutamic acid	0.24	GRIA1, GRIA2, GRIA4, GRM2, GRM5
p-Octopamine	0.22	ADRA1A, ADRA2A, ADRA2B, DRD2
Turanose	0.16	ADRA1A, ADRA2A, ADRA2B, DRD1, DRD2, F2, HTR2A, HTR2C, TRPV1
Tricetin	0.15	ADORA1, ADORA2A, F2
Pinocembrin	0.11	ADORA1, GRM2, GRM5

Therefore, we hypothesized that ADRA1A, ADRA2A, ADRA2B and TACR1 may be the core targets of Phe. We next analyzed these targets using the KEGG database. Interestingly, these four potential targets belong to GPCR, and all targets are related to the GPCR-MLC signaling pathway. This suggests that Phe may alleviate STC through the GPCR-MLC signaling pathway (Fig. 4C).

3.5 Phe can also alleviate the symptoms of loperamide-induced STC

Loperamide-induced STC mice were used to observe the therapeutic effects of Phe. Compared with the LOP group, the FBST in the HPhe group (high dose of Phe) was the shortest (Fig. 5A). Similar to ASP, Phe had no significant effect on FN or FW (Fig. 5B and 5C). However, Phe treatment significantly increased the FWC in STC mice (Fig. 5D and 5E). The gastrointestinal transit test shows the speed at which food spreads throughout the gastrointestinal system, especially the peristaltic frequency. The GTR of mice in the Phe groups increased rapidly with increasing doses of concentrations and was significantly enhanced compared with the LOP group (Fig. 5F-5H). At the same time, Phe did not significantly change the basic physical indices of mice (Fig. S5).

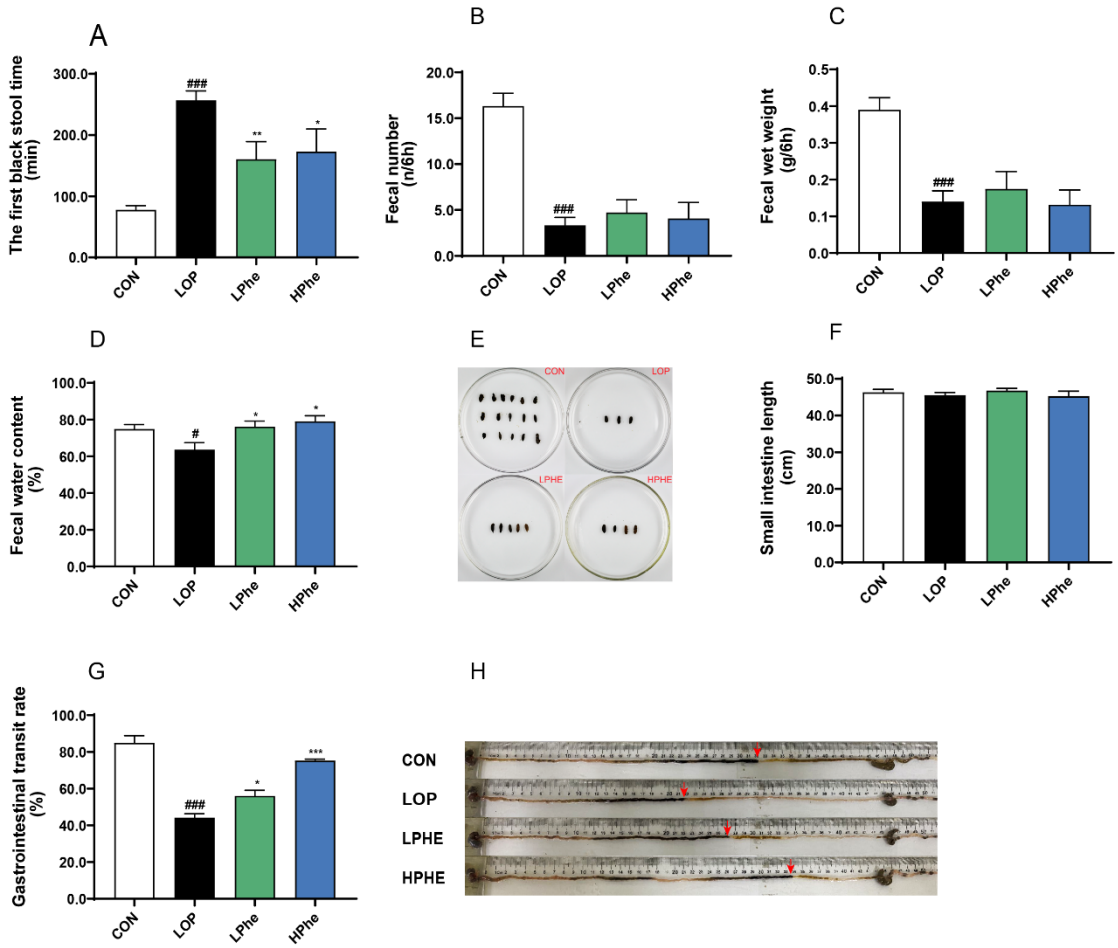

Fig. 5 Effects of Phe on defecation and intestinal motility in STC mice (A) FBST; (B) FN; (C) FW; (D) Fecal WC; (E) Representative fecal morphology of each group; (F) Small intestine length; (G) Gastrointestinal transit rate; (H) Representative pictures of ink propulsion distance. The data are expressed as the means ± SEMs (n = 12). #, compared with the CON group; *, compared with the LOP group. #, $P < 0.01$, ##, $P < 0.01$; ###, $P < 0.001$. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

3.6 Phe alleviates STC by regulating the GPCR-MLC signaling pathway

Currently, the mechanism by which Phe relieves constipation is not completely clear. Here, we investigated the mechanism by which Phe relieves constipation. First, we obtained the potential target of Phe defecation through network pharmacological analysis and noted that these targets are located in the GPCR-MLC signaling pathway. Subsequently, through animal experiments, we found that Phe may downregulate the mRNA expression of *TACR1* (Fig. 6A), downregulate *ADRA2A* and *ADRA2B* in the ileum (Fig. 6C and 6D), and then downregulate the mRNA expression of *Calm* (Fig. 6E) and *MYL9* (Fig. 6F), thus promoting intestinal contraction.

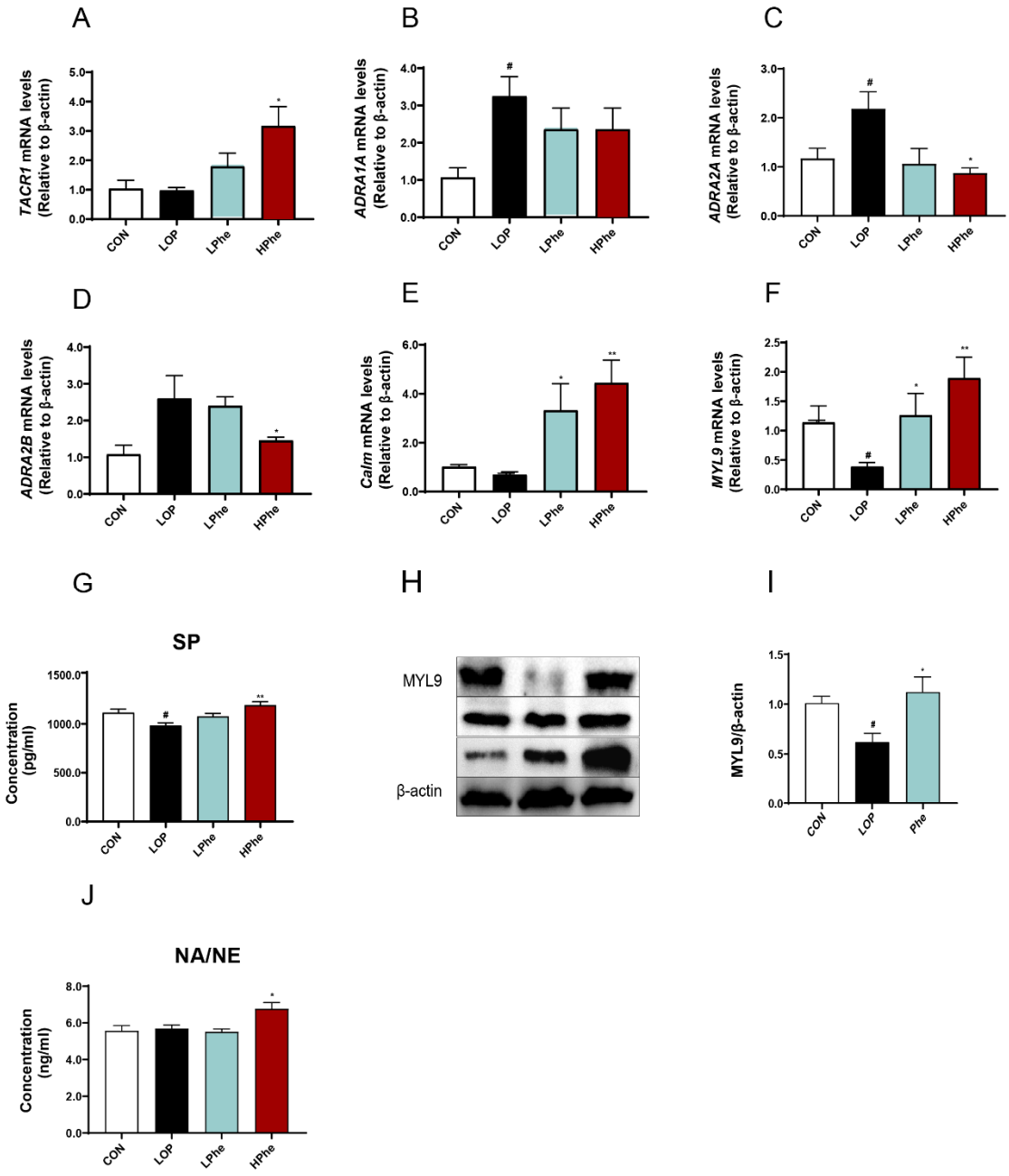

Fig. 6 Phe improves constipation by regulating the GPCR-MLC signaling pathway.

F) Expression of the ileal genes *TACR1*, *ADRA1A*, *ADRA2A*, *ADRA2B*, Calm and *MYL9* mRNA. (G) SP content in the serum of mice. (H-I) Effects of SCB on the expression of MYL9. (J) Content of NA/NE in the serum of mice. Compared with the control, #, $P < 0.05$, Compared with the model group,*, $P < 0.05$, **, $P < 0.01$.

As the receptor of substance P (SP), TACR1 plays an important role in promoting intestinal motility [22]. By detecting the serum content of SP, we found that the serum level of SP in STC mice decreased significantly ($P < 0.05$), and the levels of SP in the HPhe group were significantly higher than those in the LOP group ($P < 0.05$, Fig. 6G).

The regulatory MLC of smooth muscle is encoded by MYL9, also named MLC2 [23]. MYL9 plays an important role in the contraction of gastrointestinal smooth muscle [24]. As shown in Fig. 6H-6I, loperamide treatment significantly inhibited the expression of MYL9 in the ileum of STC mice, and high-dose Phe treatment reversed this change, thereby promoting intestinal motility.

As an important neurotransmitter, noradrenaline/norepinephrine (NA/NE) is also a metabolite of Phe in vivo. NA/NE can increase the concentration of intracellular calcium by acting on its receptor, thus promoting intestinal contraction [25]. The concentration of NA/NE can be regulated by Phe [26], and its content in serum increases with the dose of Phe (Fig. 6J). Our data suggest that Phe may promote intestinal motility and relieve constipation symptoms by promoting the release of gastrointestinal neurotransmitters, activating motility factors related to intestinal motility, and then activating the GPCR-MLC signaling pathway.

4. Discussion

STC is mainly characterized by infrequent defecation, difficulty in defecation and dry stools. With the tense pace of modern life, increasing social pressure and unhealthy eating habits, constipation has become an important human health problem [27]. *M. oleifera*, the tree of life, has a variety of physiological functions. It plays an active role in anti-inflammatory, antioxidant, and anticancer activities [28]. *M. oleifera* leaves have been shown to be effective in relieving symptoms of constipation, but the key components behind its laxative activity and the mechanism of action are not yet clear [17, 29]. It has been found that not only macromolecular substances but also small molecular compositions in edible Chinese herbal medicine, such as steroidal saponins, quercetin and naringin, have laxative effects [30-32]. Therefore, in this study, we used ethanol to enrich the small molecular substances in the aqueous extract of *M. oleifera* leaves and obtained ASP. Next, we found that ASP could relieve constipation symptoms in STC mice by promoting intestinal motility and then promoting defecation. However, the material basis and mechanism of its laxative effect are still unclear.

In view of the complexity of the chemical composition of ASP, we used plant MRM technology to carry out extensive targeted metabolomics analysis of ASP. Combined with a literature search, we preliminarily identified 62 high-abundance chemical components in ASP. However, it is important to note that ASP still contains small amounts of macromolecular substances, such as polysaccharides and proteins.

Traditional medicine often exerts its curative effect through multiple targets and multiple components. Network pharmacology is used to analyze the biological basis of traditional medicine and its components through a combination of calculations and experiments and to identify the components with therapeutic activity against various diseases from many traditional medicines. In addition, the specificity of key driving factors and targets of the disease need to be identified, and the weight in network pharmacological analysis should be properly considered [33-35]. Network pharmacology has great potential to systematically clarify the mechanism of drug action and guide drug research and development into the frontier research field of clinical diagnosis and treatment [36]. Network pharmacology has a variety of applications, including plant composition target/disease gene prediction, network balance regulation, cooperative component pairs and active component group elucidation [37-38].

In this study, on the basis of the main chemical constituents of ASP, a total of 29 potentially active components, 296 targets and 2,248 constipation-related targets were obtained using network pharmacology. Through network pharmacology prediction, we found that Phe and other active components in ASP may

improve constipation through multiple signaling pathways, such as the neuroactive ligand–receptor, calcium and PI3K-AKT signaling pathways.

Disorders in the neurologic and calcium signaling pathways have been widely reported as classic and important pathways in the study of constipation [39-40]. Bauhinia root alleviates constipation by antagonizing the binding of acetylcholine to the muscarinic receptor, inhibiting calcium influx and anti-inflammation [41]. In this study, we found that Phe had a significant advantage in the relative abundance and the number of corresponding targets through the analysis of the corresponding components of the enriched signaling pathway, suggesting that Phe may be the key component of MOA and ASP to alleviate STC. Phe may play an important role in the pathophysiological process of chronic constipation, but its molecular mechanism is not clear [42].

Phe is an essential aromatic amino acid for the human body, and it is the precursor of the monoamine neurotransmitter 5-hydroxytryptamine, catecholamine, dopamine, noradrenaline/norepinephrine (NA/NE) and epinephrine [43]. Phe is also an effective glucagon-like peptide-1 secretion trigger and causes an increase in intracellular calcium concentration [44]. Phe metabolism is closely related to gastrointestinal function [45-46]. Recent studies have also noted that Phe may regulate the intestinal microenvironment through extracellular calcium-sensitive receptors and help restore intestinal homeostasis [47]. These findings support the association of Phe with constipation. Our animal experiment results show that Phe can significantly shorten the time of the first black stool, stimulate the peristalsis of the small intestine and then increase the propulsive rate of small intestinal ink. Its laxative effect is similar to that of other reports [42].

GPCRs are the largest family of membrane protein receptors that transmit signals from the extracellular space to the intracellular space by coupling to downstream heterotrimeric G proteins. Generally, GPCRs are considered to have the functions of regulating smell, taste, light perception and pheromone signal transmission. GPCRs regulate signaling through ligands that range from small molecules to peptides and proteins. They are the target of most clinical drugs, although only a small fraction of these receptors are used therapeutically. GPCRs can be divided into five categories: glutamate family, rhodopsin family, adhesion family, Frizzled family, and secretin family [48].

GPCRs have been reported to regulate intestinal health [49], interfere with intestinal movement and improve constipation [50-51]. Many studies have proven that the GPCR-MLC signaling pathway plays an important role in regulating the onset of gastrointestinal symptoms [52-54]. Interestingly, TACR1, ADRA1A, ADRA2A, and ADRA2B belong to the GPCR family and are potential targets of Phe to improve constipation. Our results indicate that Phe can significantly affect the mRNA expression of *TACR1*, *ADRA2A*, and *ADRA2B* in the ileum of STC mice.

As an important member of the GPCR family, TACR1 plays an important regulatory role in improving intestinal motility in constipated mice [55]. After Phe intervention, the expression of TACR1 increased significantly. This may indicate a potential change in the content of substance P [56]. Through ELISA analysis, we found that the serum content of SP in STC mice with a high dose of Phe was significantly

increased. The increase in SP content can promote intestinal peristalsis by changing the intracellular Ca^{2+} concentration, which may be one of the important ways for Phe to improve STC symptoms. In addition, the increase in NA/NE can also further increase the content of SP [57], which is consistent with the results observed in this study. Through the study of the GPCR signaling pathway and the above receptors, we found that TACR1, ADRA2A, and ADRA2B may regulate intestinal movement and relieve constipation symptoms through MLC, which may be the potential mechanism by which Phe improves constipation.

Different II-like myosin complexes, also known as conventional myosin, are present in muscle cells and all cells. In muscle cells, they are responsible for muscle contraction, and in all cells they are necessary for contractile bundles involved in cellular structure, movement, adhesion and cytokinesis. The myosin II complex consists of two myosin heavy chains (MHC) and four myosin light chains (MLCs). MHC is responsible for oligomerization and interaction with actin, while MLCs are necessary to maintain the stability of the holographic complex [58]. One MLC, MYL9 plays an important role in regulating the contraction of smooth muscle [59]. The *MYL9* gene encodes a regulatory myosin MLC, which is the key to the contraction of smooth muscle cells. The absence of *MYL9* can cause diseases characterized by gastrointestinal functional obstruction [60]. MYL9 deficiency can also lead to smooth muscle dysfunction in many parts of the intestine [61]. Importantly, smooth muscle dysfunction is closely related to constipation [62]. Our results showed that Phe significantly enhanced the expression of MYL9 in the ileum of STC mice. This suggests that Phe may alleviate STC through the GPCR-MLC pathway. Taken together, this evidence suggests that Phe may increase the SP content and NA/NE release, upregulate TACR1 expression, activate the GPCR-MLC signaling pathway, and promote intestinal peristalsis, thus alleviating loperamide-induced STC. In addition, microbial metabolism of aromatic amino acids in the diet promotes the biosynthesis of serotonin, which stimulates gastrointestinal transport through a TACR1-dependent mechanism [63]. This may be another potential mechanism by which Phe improves constipation symptoms. Although we have clarified the key active components and the laxative mechanism of MOA to a certain extent through network pharmacology and STC mouse models and found the possible pathway of Phe laxative, we have not fully revealed the laxative material basis and mechanism of *M. oleifera* leaves. For example, further over-expression and down-regulation studies of TACR1 and ADRA2A may reveal the laxative mechanism of Phe more clearly, but this will require many in-depth research efforts. It is worth noting that the composition of most foods and herbs is very complex. In MOA, Phe is not the only compound with laxative activity, and there may be synergistic or antagonistic effects among various substances, which cannot be fully revealed in this study for the time being. Whether other Phe-rich foods also have laxative activity depends on their specific chemical composition.

5. Conclusion

In conclusion, we systematically investigated the effects of MOA on loperamide-induced STC in mice. We found that ASP significantly improved loperamide-induced constipation. We hypothesized that Phe is a key component of ASP laxative activity using network pharmacology. The results of the animal experiments showed that Phe can improve constipation by increasing the release of gastrointestinal hormones, regulating

intestinal peristalsis-related factors, and activating the GPCR-MLC signaling pathway. These results provide a theoretical basis for the application of *M. oleifera* leaf extract and Phe in relieving constipation.

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