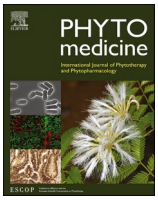
Phytomedicine 115 (2023) 154826

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed



Original Article

Tibetan medicine Liuwei Muxiang pills (LWMX pills) effectively protects mice from chronic non-atrophic gastritis☆

Rinchen Dhondrup [a,*], Tawni Tidwell [b], XiaoKang Zhang [c], Xuemei Feng [d], Dhondrup Lobsang [a], Qincuo Hua [d], Duojie Geri [a], Duojie Caidan Suonan [a], Gang Fan [e], Gyal Samdrup [a]

[a] Tibetan Medical College of Qinghai University, Xining 810016, Qinghai, People's Republic of China
[b] University of Wisconsin-Madison, Center for Healthy Minds, Madison, WI, 53703 United States
[c] Jingjie PTM Bio (Hangzhou) Co., Ltd., Hangzhou 310018, Zhejiang, People's Republic of China
[d] Qinghai Provincial Tibetan Hospital, Xining 810007, Qinghai, People's Republic of China
[e] School of Ethnic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, Sichuan, People's Republic of China

ARTICLE INFO

Keywords:
Chronic non-atrophic gastritis (CNG)
Liuwei Muxiang pill (lwmx pills)
Mice
Anti-inflammation
Cancer development

ABSTRACT

Background: Chronic non-atrophic gastritis (CNG) is the most common type of chronic gastritis. If not actively treated, it may induce gastric cancer (GC). Western medicine is effective in CNG, but there are more adverse reactions after long-term medication, and it is easy to relapse after treatment, which affects patients' health and life. Tibetan medicine Liuwei Muxiang Pills (LWMX pills) is a traditional Tibetan medicine compound, which has a unique curative effect in the treatment of gastric inflammation, especially chronic non-atrophic gastritis. However, the mechanisms of LWMX pills for treatment CNG still remain poor known.

Purpose: The aim of this study was to evaluate the therapeutic intervention potential of Tibetan medicine LWMX pills on CNG and explore its potential mechanisms in mice models.

Methods: The mice models was established to evaluate the therapeutic effect of LWMX pills on CNG. The main components of LWMX pills were analyzed by GC-MS. HE staining, immunohistochemistry, proteomics and Western Blot were used to analyze the potential mechanism of LWMX pills for CNG treatment.

Results: In the present study, LWMX pills containing costunolide, dehydrocostuslactone and antioxidants were found. IF results showed that the expression of ALDH1B1 in the control group was significantly lower than that in the model group in the gastric mucosa tissue, and the expression of ALDH1B1 was significantly lower in the 25 mg/ml LWMX Pills group (one month) and 25 mg/ml LWMX Pills group (two months) than in the model group. IHC revealed that model group samples expressed higher levels of Furin than 25 mg/ml LWMX Pills group samples, as evidenced by very strong staining of Furin in gastric mucosal cells. However, AMY2 staining in gastric mucosal cells did not differ significantly between the treated and control groups. the protein expression levels of these proteins were decreased in 25 mg/mL LWMX pills. Meanwhile, we found that the CAM1 protein expression in the in 25 mg/ml LWMX pills group (two mouths) was increased compared to the in 25 mg/ml LWMX pills group (one mouths).Western blotting showed that the protein expression levels of Furin, AMY2A, CPA3, ALDH1B1, Cam1, COXII, IL-6, IL-1β were decreased in 25 mg/mL LWMX pills. Meanwhile, that the CAM1 protein expression in the in 25 mg/ml LWMX pills group (two mouths) was increased compared to the in 25 mg/ml LWMX pills group (one mouths).

Conclusion: 25mg/ml LWMX pill treatment for one month had better therapeutic effect on mice CNG. Further proteomic results showed that LWMX pills maintain gastric function by inhibiting inflammation and oxidative

Abbreviations: LWMX, pills Liuwei Muxiang Pills; CNG, Chronic non-atrophic gastritis; GC, gastric cancer; HP, Helicobacter pylori; TTM, Traditional Tibetan medicine; IF, Immunofluorescence; IHC, Immunohistochemical; HE staining, hematoxylin-eosin staining; KEGG, Kyoto Encyclopedia of Genes and Genomes; GC-MS, Gas Chromatography-Mass Spectrometer..

☆ **Contributions:** (I) Conception and design: R Dhondrup; (II) Administrative support: G Samdrup; (III) Provision of study materials: D Lobsang, Q Hua; (IV) Collection and assembly of data: D Geri, D C Suonan, X Feng; (V) Data analysis and interpretation: X Zhang, T Tawni, G Fan; (VI) Manuscript writing: all authors; (VII) Final approval of manuscript: all authors.

* Corresponding author.
E-mail address: 2731435914@qq.com (R. Dhondrup).

https://doi.org/10.1016/j.phymed.2023.154826
Received 16 November 2022; Received in revised form 5 April 2023; Accepted 16 April 2023
Available online 17 April 2023

stress, and we also found that LWMX pills regulate the expression of proteins associated with cancer development (Amy2, Furin).

Introduction

Chronic gastritis (CG) is a very common digestive system disease in clinical. It can be divided into three types: nonatrophic gastritis (CNG), chronic atrophic gastritis (CAG), and other special types (Rugge and Genta, 2005). CNG is characterized by chronic inflammatory cellular infiltration of the gastric mucosa, mainly plasma cells and lymphocytes, without atrophic changes of the gastric mucosa (Du et al., 2014). Further evidence suggested that without effective treatment, CNG may develop into CAG, which is an important precursor lesion in the development of gastric cancer (Ohata et al., 2004). CNG is mainly caused by Helicobacter pylori (HP) infection, and the main treatment for CNG is HP eradication (Yue et al., 2021). Western medicine mainly aims to eradicate H pylori by antibiotics, but due to the widespread use of antibiotics, many adverse effects such as antibiotic resistance and gastrointestinal side effects have emerged (Wang et al., 2022). HP eradication rate did not achieve the desired results. CNG patients urgently need drugs with ideal efficacy. Tibetan medicine has significant advantages in the treatment of avoiding the side effects of Western medicine as much as possible (Yue et al., 2021).

Traditional Tibetan medicine (TTM) has a long history of developed medical knowledge regarding the etiology, diagnostics and treatment for chronic gastritis, originating as early as the eighth century, and explicated extensively in the Tibetan medical classic known as the *Four Medical Tantras* (*Rgyud bzhi*) where it is a type of disease within the greater category of Béken (phlegm) diseases (Fu et al., 2020) . As one of the great Asian medical traditions, it has a unique theory-praxis system with demonstrated clinical efficacy, particularly in treating digestive diseases. The practice of TTM is also extensively guided by cumulative empirical experience and expertise developed over decades of experience among its practitioners. Populations across China and other Asian countries frequently seek TTM for treatment of gastrointestinal diseases (Chen et al., 2022). Studies have shown that TTM can significantly improve the clinical symptoms of patients with CNG, and with fewer adverse drug reactions (Tong et al., 2021), so it is widely used in clinical practice. LWXM Pills is a widely used classical Tibetan medicine, which was recorded in the Tibetan Medicine Standard" by the Chinese Ministry of Health in the 1995s (WS3-BC-0283–95). LWMX pills are composed of 6 Tibetan medicinal plants (Aucklandiae radix, Pomegranate, Cardamom, Piper longum, Paxiaga, and Phyllanthus emblica), which can be used for diseases such as gastrointestinal diseases. It has the functions of enhancing stomach fire, slowing blood heat, relieving stomach bloating and stomach pain caused by blood-wind disorder. According to the theory of Tibetan medicine, it has a certain potential value of anti-cancer effect through the smooth flow of blood, removing blood stasis and increasing fire, slowing down blood heat and preventing the formation of gastric mucosal tumors. Clinical reports show that LWMX pills are effective in acute and chronic gastroenteritis, chronic atrophic gastritis and gastroduodenal ulcers. However, the mechanisms of LWMX pills for treatment CNG still remain poor known.

In the present study, we analyzed the main components of LWMX pills and treated mice suffering from CNG with 25 mg/ml LWMX pills. Further, we analyzed the molecular biological mechanism of LWMX pills for CNG treatment with proteomics.

Materials and methods

Animals

SPF grade Wistar 120 male mice (10 weeks old), purchased from Zhejiang Viton Lihua Laboratory Animal Technology Co (SCXK (Zhejiang) 2018–0001). All experiments in the current study were approved by the Institutional Animal Care and Use Committee of the Qinghai University, Xining 810,016, Qinghai (Approval No.:20,201,018).

Chromatographic conditions

In this study, LWMX pills were obtained from Tibetan GanLu traditional medicine Co., Ltd. (Lhasa, China, Z54020033). 1 g LWMX pills powder was placed in a 50 ml conical flask, followed by the addition 20 ml methanol and sonication for 30 min. The samples were then centrifuged (11,000 r/min, 5 min) and the supernatant was passed through a 2 μm microporous membrane to obtain the solution to be measured. The chromatographic and mass spectrometric conditions are described as follows chromatographic column WondaSil C18 (4.6 × 250 mm, 5 μm); mobile phase: acetonitrile (A)−0.2% phosphoric acid aqueous solution (B), gradient elution program is 0∼12 min, 20%∼28%A; 12∼15 min , 28%A; 15∼20 min, 28%∼55%A; 20 min∼35 min, 55%∼60%A; 35 min∼45 min, 60%∼65%A; column temperature: 30 °C; detection Wavelength: 210 nm; Flow rate: 1.0 ml/min; Injection volume: 10 μl.

CNG mouse model

Wistar mice were randomly divided into control group, model group, low treatment group, medium treatment group and high treatment group, 10 mice in each group. The mice in the control group drank water freely, and the mice in the other groups dosed with 1 g/l of MNNG mother liquor in double-distilled water every week for mice to drink freely and gavaged with a dose of 2.25 g/l ranitidine salt solution, and the test mice were taken to be full for 2 days and starved for 1 day without water fasting, and the model was observed from six months onwards Two mice were taken under pathology for HE staining every two weeks to determine the success of CNG mouse model construction. The control group and CNG mice were treated with 0.2 ml of saline by gavage, while the treatment group was given 12.5 mg/ ml, 25 mg/ml and 50 mg/ ml of LWMX Pills by gavage in the low, medium and high doses, respectively, once a day in the morning and once a day in the afternoon.

HE staining of mouse gastric tissues

At seven month, three mice in the modeling group, three mice in the treatment group and three mice in the control group were randomly taken, and the whole stomachs were washed with pre-cooled PBS at 4 °C to clean the blood stains, loaded into lyophilization tubes, snap-frozen in liquid nitrogen for 10 min, removed and stored at −80 °C. Finally, 1 mm^3 of gastric mucosal tissue at the junction of the body-sinus of the gastric lesser curvature was cut along the gastric greater curvature, and the lesions were taken at 5 mm intervals, fixed with 4% formaldehyde solution, dehydrated, paraffin-embedded, and sectioned at a thickness of 4 μm. The pathological changes of gastric mucosal tissue in mice with heterogeneous hyperplastic lesions were observed by HE and Alisin blue-periodic acid Schiff's (AB-PAS) staining. Suitable samples were selected for proteomic analysis using the results based on gastric mucosal histopathology.

Proteomics analysis

The sample was grinded with liquid nitrogen into cell powder and then transferred to a 5-ml centrifuge tube. After that, four volumes of lysis buffer (8 M urea, 1% protease inhibitor cocktail) was added to the

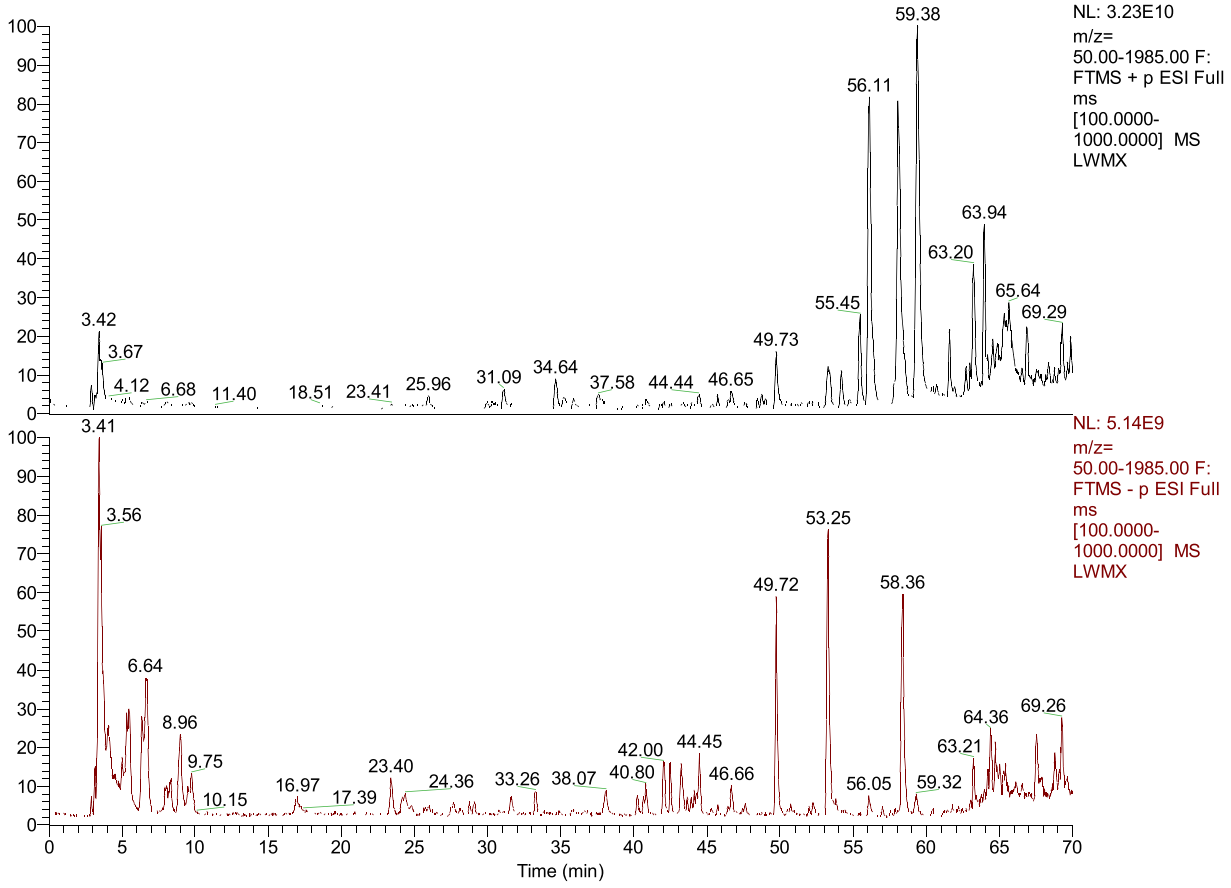


Fig. 1. Analysis of the chemical components of the extract of Liuwei Muxiang pills.

cell powder, followed by sonication three times on ice using a high intensity ultrasonic processor (Scientz). (Note: For PTM experiments, inhibitors were also added to the lysis buffer, e.g. 3 μM TSA and 50 mM NAM for acetylation, 1% phosphatase inhibitor for phosphorylation). The remaining debris was removed by centrifugation at 12,000 g at 4 °C for 10 min. Finally, the supernatant was collected and the protein concentration was determined with BCA kit according to the manufacturer's instructions.

For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 °C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion. Finally, the peptides were desalted by C18 SPE column.

Tryptic peptides were firstly dissolved in 0.5 M TEAB. Each channel of peptide was labeled with their respective TMT reagent (based on manufacturer's protocol, ThermoFisher Scientific), and incubated for 2 h at room temperature. Five microliters of each sample were pooled, desalted and analyzed by MS to check labeling efficiency. After labeling efficiency check, samples were quenched by adding 5% hydroxylamine. The pooled samples were then desalted with Strata X C18 SPE column (Phenomenex) and dried by vacuum centrifugation.

LC-MS/MS analysis

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for TMT kit. Briefly, one unit of TMT reagent was thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation. The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). The tryptic peptides were treated and subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™Plus (Thermo Scientific, Waltham, MA, USA) coupled online to the UPLC. A data-dependent procedure alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Raw data were analyzed by MaxQuant software version 1.5.3.8. The FDR (False discovery rate) was set to 1% for all peptides and proteins. Protein expression more than 1-log fold changes were found to be associated with liver cancer.

Functional enrichment

Proteins were classified by GO annotation into three categories: biological process, cellular compartment and molecular function. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. The GO with a corrected P-value < 0.05 is considered significant. Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways by a two-tailed Fisher's exact test to test the enrichment of the differentially expressed protein against all identified proteins. The pathway with a corrected P-value < 0.05 was considered significant. These pathways were classified into hierarchical categories according to the KEGG website.

Western blot analyses

All samples were lysed with RIPA buffer to obtain protein extracts.

Table 1
Identification of chemical components of Liuwei Muxiang pills by UPLC-Q-exactive orbitrap MS.

Peak No	tR (min)	Chemical formula	Ionic mode	Measured value (*m/z*)	Theoretical value (*m/z*)	Error (ppm)	Secondary fragment ion information	Identification results
1	36.60	$C_{15}H_{18}O_2$	+	231.1379	231.1380	−0.43	231.1379(90); 213.1271(16); 203.1429(10);185.1324 (100);175.0752(16); 157.1011(25); 143.0855(39); 131.0856(27); 128.0624(9)	Dehydrocostuslactone
2	42.52	$C_{14}H_6O_8$	–	300.9992	300.9979	4.32	300.9992(100);283.9962(7);257.0103(3);229.0147 (6);201.0189(4); 185.0237(4)	Ellagic acid
3	46.79	$C_{15}H_{10}O_7$	–	301.0351	301.0343	2.66	301.0351(100);273.0407(5);151.0030(94);121.0286 (31);107.0130(27)	Quercetin
4	47.51	$C_{16}H_{20}NO_3$	+	274.1438	274.1438	0.00	274.1438(100);189.0547(73);171.0440(11);143.0493 (8);115.0545(16)	Piperlonguminine
5	47.78	$C_{15}H_{10}O_6$	–	285.0405	285.0394	3.86	285.0405(100);241.0501(1);199.0397(5);151.0032 (10)	Luteolin
6	49.22	$C_{15}H_{10}O_6$	–	285.0409	285.0394	5.26	285.0409(100);239.0354(2);229.0506(2);211.0398 (2);185.0604 (3);169.0653(1);109.0285(1);93.0337(4)	Kaempferol
7	56.09	$C_{17}H_{19}NO_3$	+	286.1438	286.1438	0.00	286.1438(70);201.0547(100);171.0441(16);143.0492 (21);115.0545(29)	Piperine
8	58.07	$C_{15}H_{20}O_2$	+	233.1536	233.1536	0.00	233.1536(63); 215.1431(29); 187.1481(100); 159.1168(16); 145.1012(33); 131.0856(34); 119.0858 (17); 105.0703(33); 91.0547(16); 81.0705(31)	Costunolide

Protein samples were separated on 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, proteins were transferred from PAGE onto a nitrocellulose membrane (Millipore Corporation, Billerica, MA). The membranes were blocked for 2 h at room temperature with 5% powdered skim milk and then were incubated with primary antibodies (1:1000; Cell Signalling Technology, Boston, MA, USA) at 4 °C overnight. Subsequently, the membranes were washed with TBST three times (ten minutes each time) and incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Bioss, Beijing, China) for 1 h at 37 °C. The membrane was washed with TBST three times (ten minutes each time), and then the reactive proteins on the membrane was detected by chemilumescent detection kit (Beyotime, Shanghai, China). Bands on the blots were quantified by Image J software (Media Cybernetics, Rockville, MD). The intensity of the β-actin bands was used for normalization.

Immunofluorescence (IF) of gastric tissues

Gastric tissues were collected for the preparation of tissue sections in 4% (w/v) paraformaldehyde solution. Tissue sections were cut out and paraffin-embedded. Subsequently, the sections were dewaxed with xylene and then hydrated with a gradient of alcohol. Next, the slices were incubated with 3% H_2O_2 in the dark at room temperature (18–22 min). Tissue sections were incubated with the primary anti-ALDH1B1 (1:2000; bs-6601; Bioss Antibodies, Woburn, MA, USA), anti-cpa3 (1:2000; bs-6601; Bioss Antibodies, Woburn, MA, USA), anti-cma1 (1:2000; ab180610; Abcam, Cambridge, MA, USA) monoclonal antibodies at 4 °C for 12 h. Next, the tissue sections were washed with Phosphate-Buffered Saline (PBS) and then incubated with Cy3-conjugated goat anti-rabbit IgG (1:2000). Fluorescein peanut agglutinin isothiocyanate (FITC-PNA, 100 μg/ml in PBS; a marker of acrosome in round spermatid and sperm) and DAPI (cell nuclear staining) were used to visualize antigens by an epifluorescence microscope (Nikon Eclipse C1; Nikon, Tokyo, Japan). The sample was observed by a phase-contrast microscope (Nikon Eclipse C1).

Immunohistochemical (IHC) staining

Paraffin-embedded rat stomach tissues were deparaffinized and subjected to antigen retrieval. Then, the samples were incubated with a primary rabbit monoclonal anti-amy2 antibody (Cat No.: ab191434, Abcam, 1: 2000), rabbit polyclonal anti-Furin antibody (Cat No.: 12,375–1-AP, Proteintech, 1:100), rabbit polyclonal anti-Zurin antibody at 4 °C overnight. Antibody diluent (Cat No.: G2025–100, Servicebio)

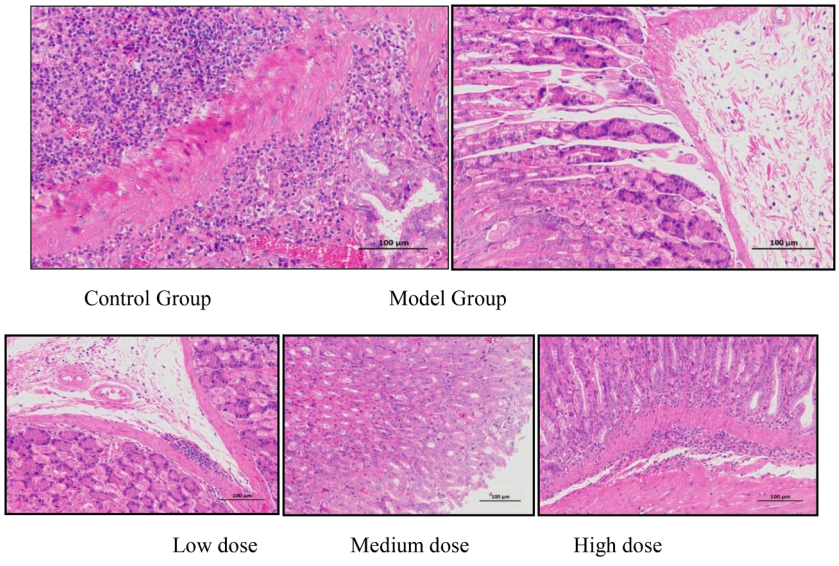


Fig. 2. HE staining to observe the pathological changes of mice gastric mucosa in each group (X200).

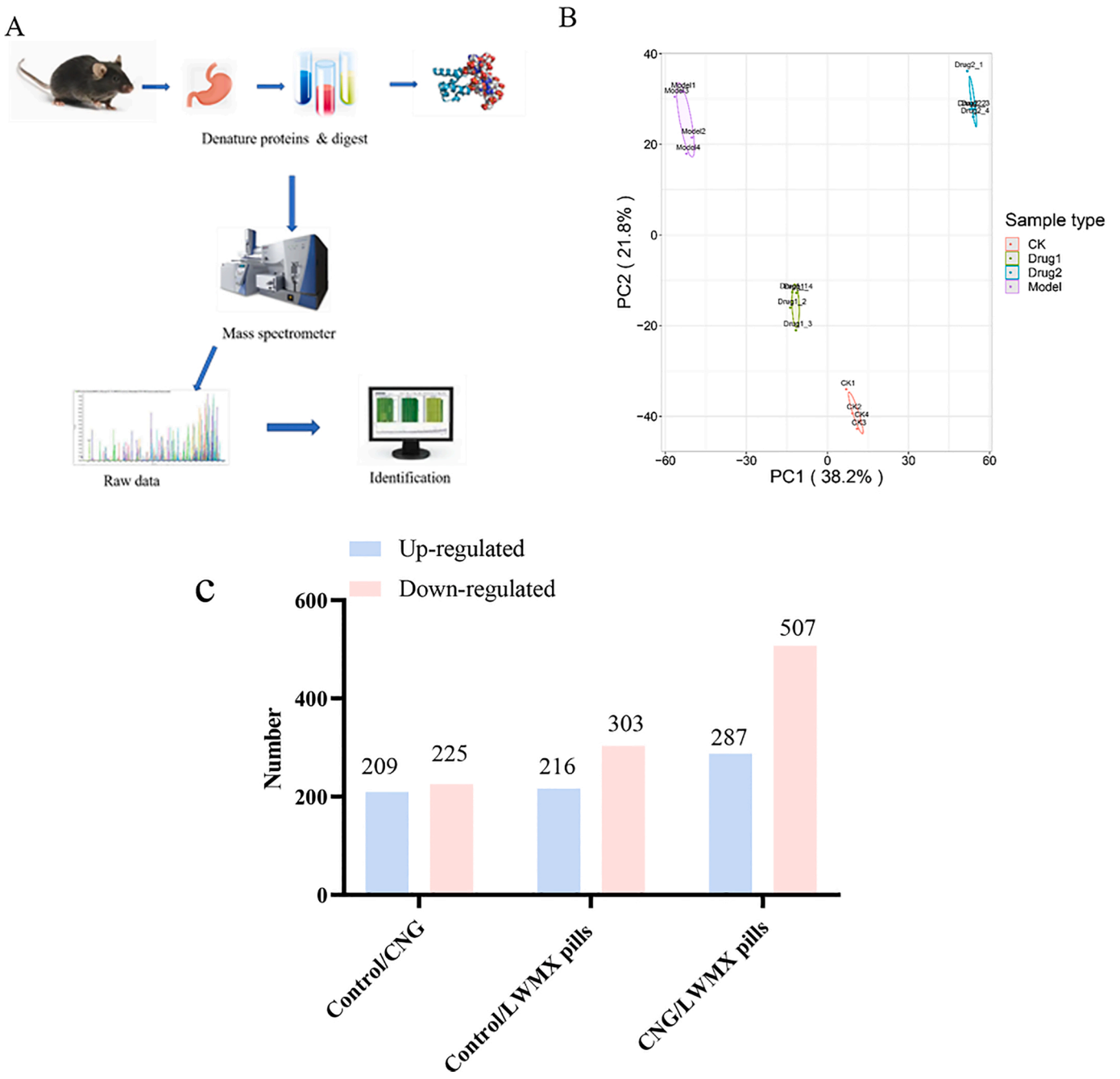


Fig. 3. Proteomic analysis in mice gastric mucosa. (A) General workflow of MS-based quantitative proteomics and bioinformatics analyses. (B) Two-dimensional scatter plot of PCA (principal component analysis) distribution of all samples using quantified proteins.(C) Differentially expressed proteins were analyzed in different groups.

was purchased from Wuhan Servicebio Biotechnology Co., Ltd. The sections were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cat No.: GB23303, Servicebio, 1:5000) and counterstained with hematoxylin. The sections were imaged by light microscopy (Olympus, Japan) at 200 × and 400 × magnification was applied to photograph images.

Data analysis

In this study, protein quantification were presented as mean ± SEM. Data were compared using Student's *t*-test by SPSS version 20 for Windows. $p < 0.05$ between groups were considered statistically significant.

Results

Analysis of the chemical components of the extract of Liuwei Muxiang pills

The extract of LWMX pills was analyzed by UPLC-Q/TOF-MS. As shown in the Fig. 1 and Table 1, it mainly contains dehydrocostuslactone, ellagic acid, quercetin, piperlonguminine, luteolin, kaempferol, piperine, costunolide and other ingredients (Fig. 1, Table 1).

Histopathological analysis of gastric mucosa in mice

HE staining showed that the gastric mucosa of control mice had a

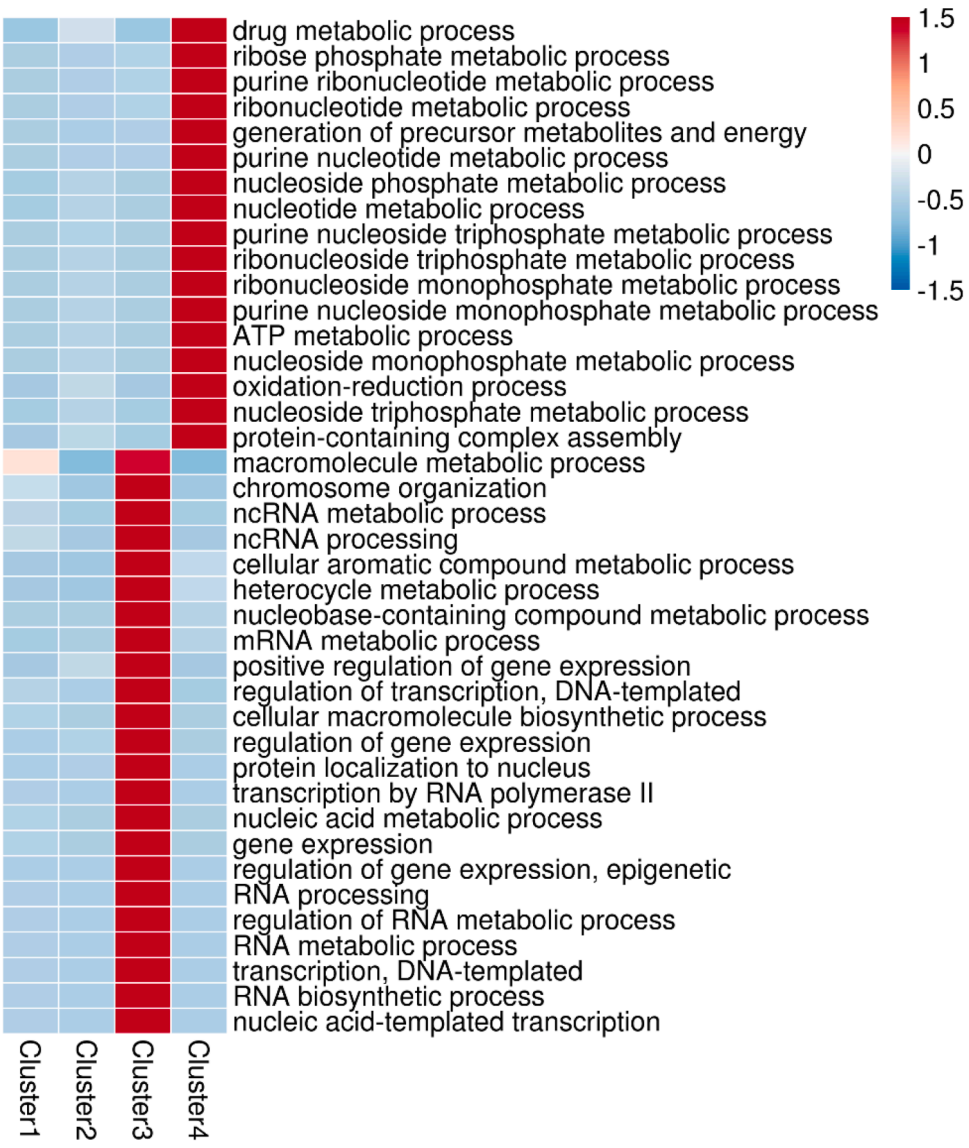


Fig. 4. Biological process analyses of the differentially expressed proteins by GO database in CK group, Model group, Drug1 groups and Drug2 groups.

small area of heavy edema, loose arrangement of connective tissue, increased number of blood vessels, and a small amount of lymphocyte infiltration, no obvious intestinal hyperplasia, and no obvious atrophy of gastric glands. The submucosal layer was edematous, with loose arrangement of connective tissue and lymphocyte infiltration, no obvious intestinal hyperplasia and mucosal gland atrophy were observed. In the low dose group, the submucosa was moderately edematous with loose connective tissue arrangement and increased number of blood vessels, accompanied by lymphocyte infiltration (Fig. 2). In the middle dose group, the epithelial cells in the mucosal layer were normal in shape, and the gastric glands were abundant, evenly distributed and closely arranged, with no obvious intestinalization or glandular atrophy. The gastric mucosa in the high-dose group did not show any obvious abnormalities, and a small amount of inflammatory cell infiltration was seen in the mucosal muscle layer, mucosal layer and submucosal layer.

Proteomic analysis

To further understand the protective mechanism of gastric mucosa protection by LWMX pills, we performed proteomic analysis on gastric mucosa tissues of mice in groups (control group, model group, 25 mg/ml LWMX pills group). The results of differential proteins showed that 209 proteins were up-regulated and 225 proteins were down-regulated in control group and CNG group (Fig. 3). Such as, the expression of CELA1, Muc6, Tgfbi, Pnliprp1, Lamb2 proteins were down-regulated. The expression of Obp2a, Aldh1b1, H2-Ea, Mgst1, Nolc1 proteins was upregulated. The up-regulated proteins were mainly involved in biological Processes including metabolic process, positive regulation of gene expression, regulation of RNA metabolic process, etc. The down-regulated proteins were mainly involved in biological Processes including extracellular matrix organization, negative regulation of cell motility, leukocyte migration (Fig. 4). KEGG analysis revealed that up-regulated proteins were mainly including spliceosome, cell adhesion molecules. The down-regulated proteins were mainly involved in signaling pathways such as protein digestion and absorption, pancreatic secretion, focal adhesion (Fig. 5). There were 519 differential proteins in the control and 25 mg/ml LWMX pills groups, of which 216 proteins were up-regulated and 303 proteins were down-regulated (Fig. 3). For instance, the expression of Chil4, Bpifb1, Cma1 Mcpt2, and Bcas1 proteins was upregulated, and the expression of Ca3, Amy2, Maoa, and Furin Ywhae proteins was downregulated. The results of protein enrichment showed that the up-regulated proteins were mainly involved in biological processes including regulation of defense response, positive

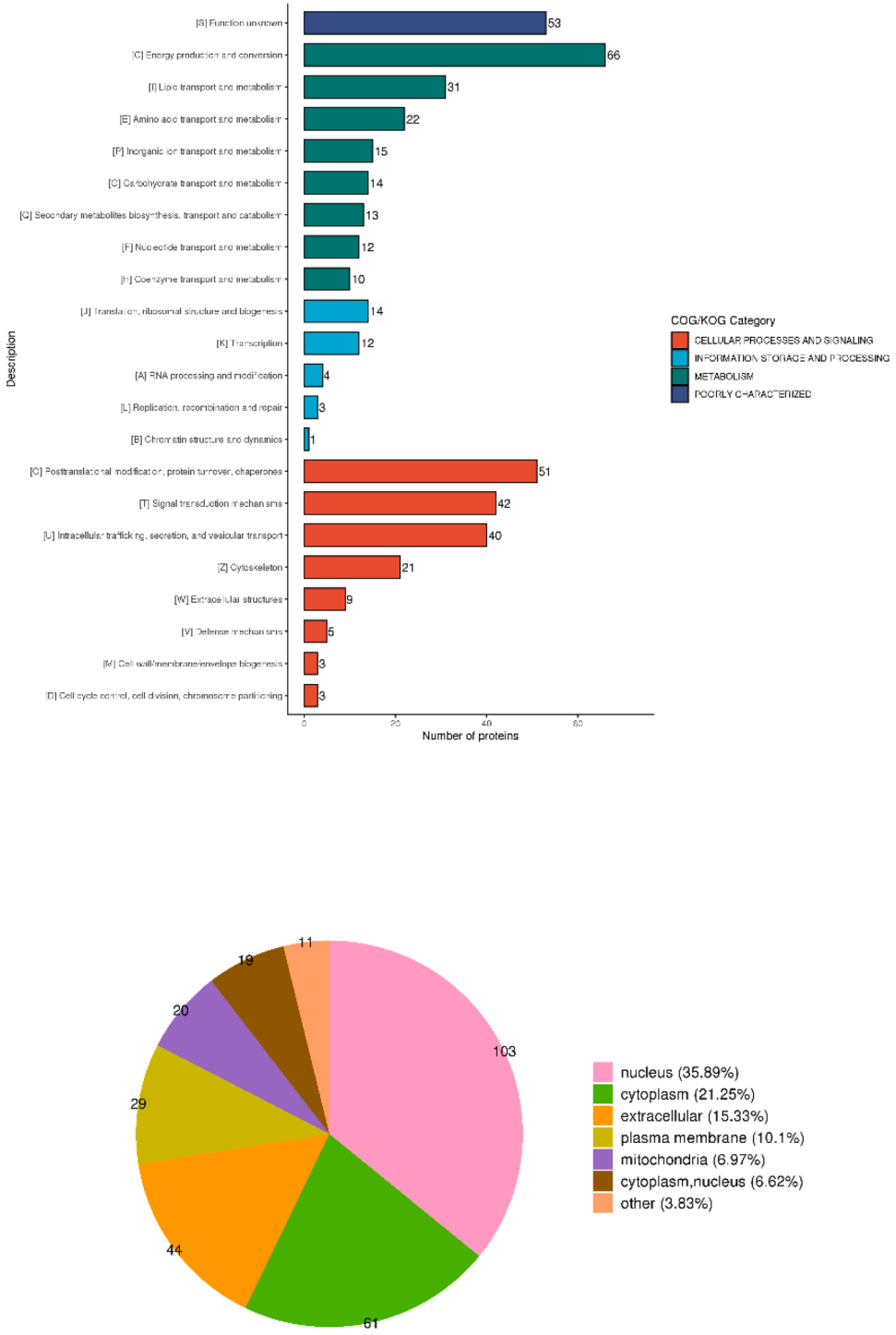


Fig. 5. Functional classification of differential proteins by GOG database in CK group, Model group, Drug1 groups and Drug2 groups.

regulation of immune response, response to bacterium, animal organ development, etc. The down-regulated proteins were mainly involved in biological Processes including protein-containing complex assembly, nucleoside phosphate metabolic process, ribose phosphate (Fig. 4). KEGG analysis revealed that the up-regulated proteins were mainly involved in signaling pathways including phagosome, complement and coagulation cascades, natural killer cell mediated cytotoxicity. The signaling pathways of the down-regulated proteins were mainly in Oxidative phosphorylation. chemical carcinogenesis - reactive oxygen species, thermogenesis (Fig. 5). There were 794 differential proteins in the CNG group and 25 mg/ml LWMX pills group, of which 287 proteins were upregulated and 507 proteins were down-regulated (Fig. 3). For example, the expression of Chil4, Mybbp1a, Bpifb1, and Cpa3 proteins was upregulated, and the expression of Muc6, Amy2, Furin, and Atp5mf proteins was downregulated. The enrichment of differential proteins showed up-regulated proteins involved in biological Processes including RNA metabolic process, nucleic acid metabolic process. The down-regulated proteins were mainly involved in biological Processes including mitochondrion organization, nucleoside phosphate metabolic process, carboxylic acid metabolic process (Figs. 4 and 6). KEGG analysis revealed that the up-regulated proteins were mainly involved in signaling pathways including Ribosome biogenesis in eukaryotes. The

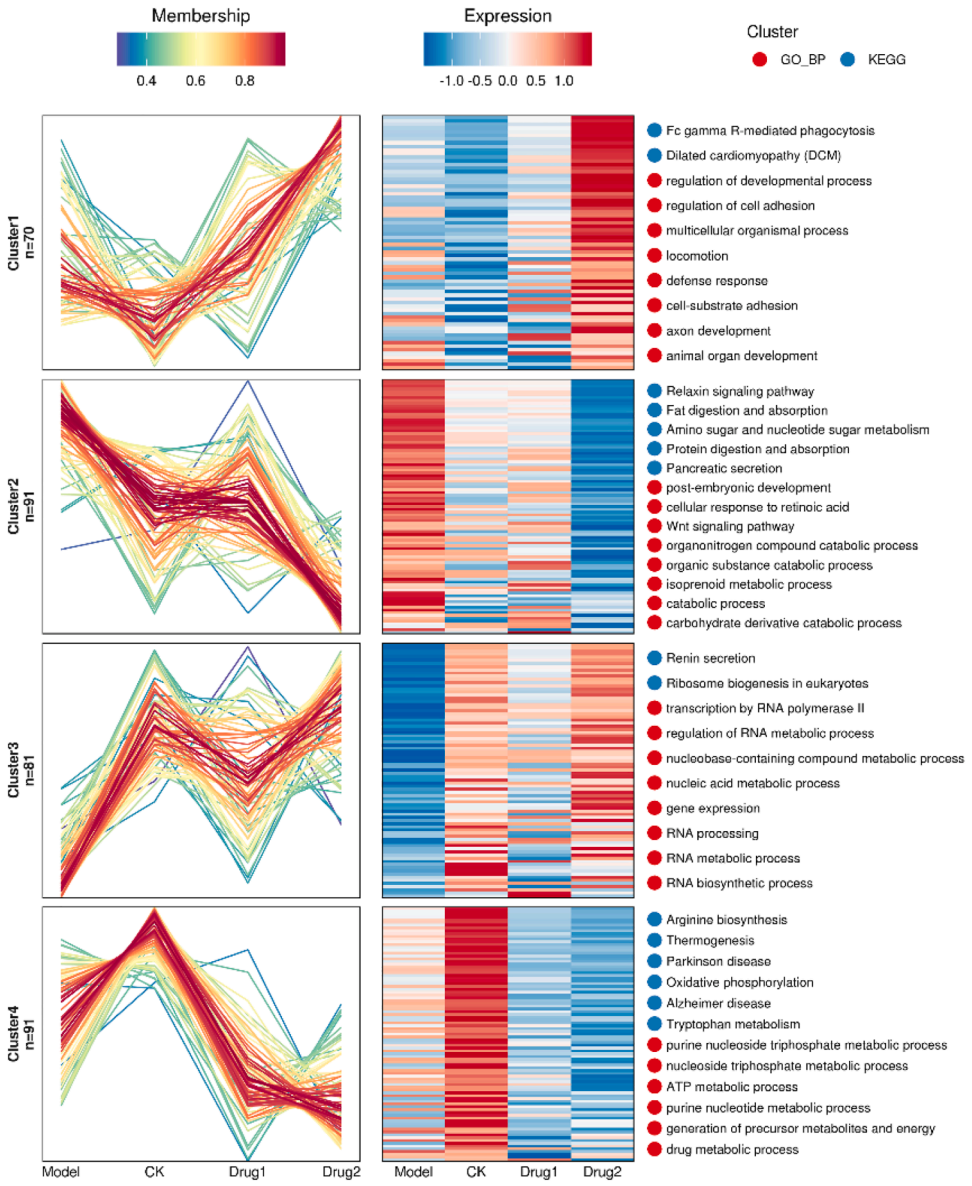


Fig. 6. Proteins clustered and analyzed in Model groups, CK groups, LWMX groups according to biological processes with the GO and KEGG databases. Four clusters were determined by co-expression analysis. Left: co-expression patterns of the proteins in the five clusters. Right: representative GO terms for each cluster.

signaling pathways of the down-regulated proteins mainly included In Chemical carcinogenesis - reactive oxygen species, Oxidative phosphorylation (Fig. 5).

IF analysis of gastric mucosa in mice

IF analysis was presented in Fig. 7. The results showed that the expression of ALDH1B1 in the control group was significantly lower than that in the model group in the gastric mucosa tissue, and the expression of ALDH1B1 was significantly lower in the 25 mg/ml LWMX Pills group (one month) and 25 mg/ml LWMX Pills group (two months) than in the model group, which indicated that the treatment with LWMX pills could reduce the expression of ALDH1B1. The expression of Cma1 in the control group was higher than that in the LWMX pills and model groups. The expression of Cma1 in the model group was higher than that in the LWMX pills groups, while we found that the staining of Cma1 was weakest in 25 mg/ml LWMX Pills. The expression of Cpa3 was the highest among all groups, while the expression of Cpa3 in the 25 mg/ml LWMX Pills group (one mouth) was higher than that in the 25 mg/ml LWMX Pills (two mouths)group and the model group, however interestingly the staining of Cpa3 in the 25 mg/ml LWMX Pills group(two months) was weaker than in the model group, these suggest that 25 mg/ml LWMX pills treated for 1 month have a therapeutic effect on chronic gastritis, but the same concentration treated for 2 months may accelerate the inflammation of gastric mucosal tissue.

IHC analysis of gastric mucosa in mice

IHC revealed that model group samples expressed higher levels of Furin than 25 mg/ml LWMX Pills group samples, as evidenced by very strong staining of Furin in gastric mucosal cells. However, AMY2 staining in gastric mucosal cells did not differ significantly between the treated and control groups (Fig. 8).

The relative expression of proteins in gastric mucosa

Western blotting was used to explore the effect of LWMX pills intervention on the chronic gastritis (Fig. 9). Compared with the control

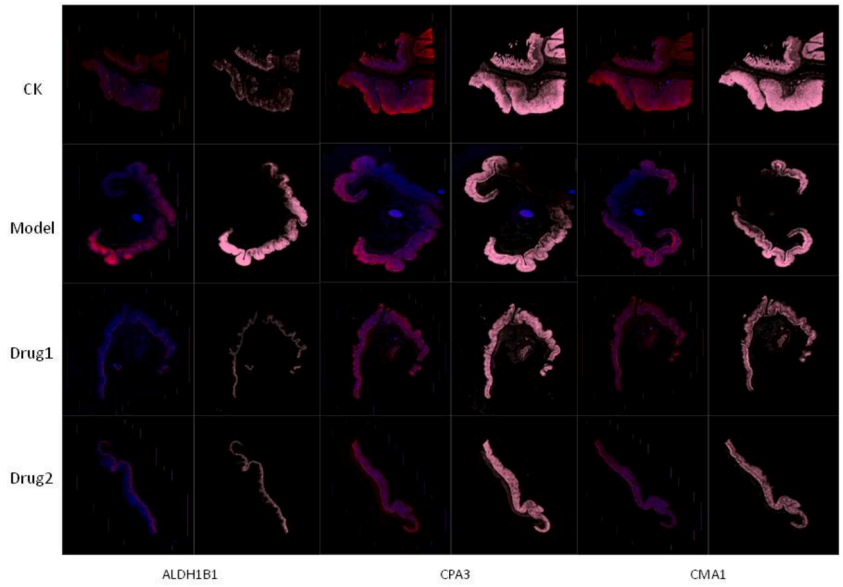


Fig. 7. The protein expression of ALDH1B1, Cpa3 and Cma1 was analyzed by IF in the gastric mucosa.

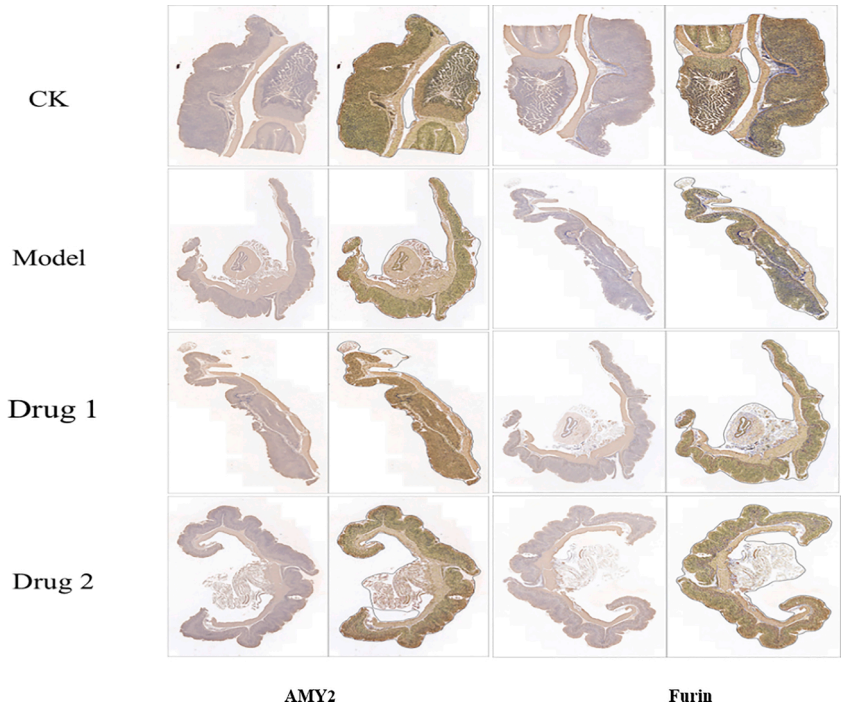


Fig. 8. The expression of AMY2, Furin was detected using IHC.

group, chronic gastritis could increase protein expression of Furin, AMY2A, CPA3, ALDH1B1, Cam1, COXII, IL-6, IL-1β. However, the protein expression levels of these proteins were decreased in 25 mg/ml LWMX pills. Meanwhile, we found that the CAM1 protein expression in the in 25 mg/ml LWMX pills group (two mouths) was increased compared to the in 25 mg/ml LWMX pills group (one mouths).

Discussion

CNG was the most common type of CG. Without effective treatment, patients may suffer from upper gastrointestinal symptoms, influencing their normal life and work, and it may develop into CAG, a precursor lesion of gastric carcinoma (GC) (Ohata et al., 2004) There is no ideal treatment option for CNG, and previous studies have found that herbal medicine has a good effect in treating CNG (Hwang et al., 2018).LWMX pills, a classic prescription of TTM, has been widely used for centuries in the treatment of gastrointestinal disorders. At present, the underlying mechanisms of these results were unclear. In this study, we found that LWMX pills have a good therapeutic effect on CNG, and further we found that LWXM pills can be used mainly by increasing resistance to inflammation and downregulating genes associated with cancer development through proteomic analysis. To the best of our knowledge, this is the first proteomic analysis of the possible molecular mechanisms of LWMX pills in the treatment of CNG, and our study provides a scientific reference for the promotion of TTM.

The results of liquid chromatography analysis showed that

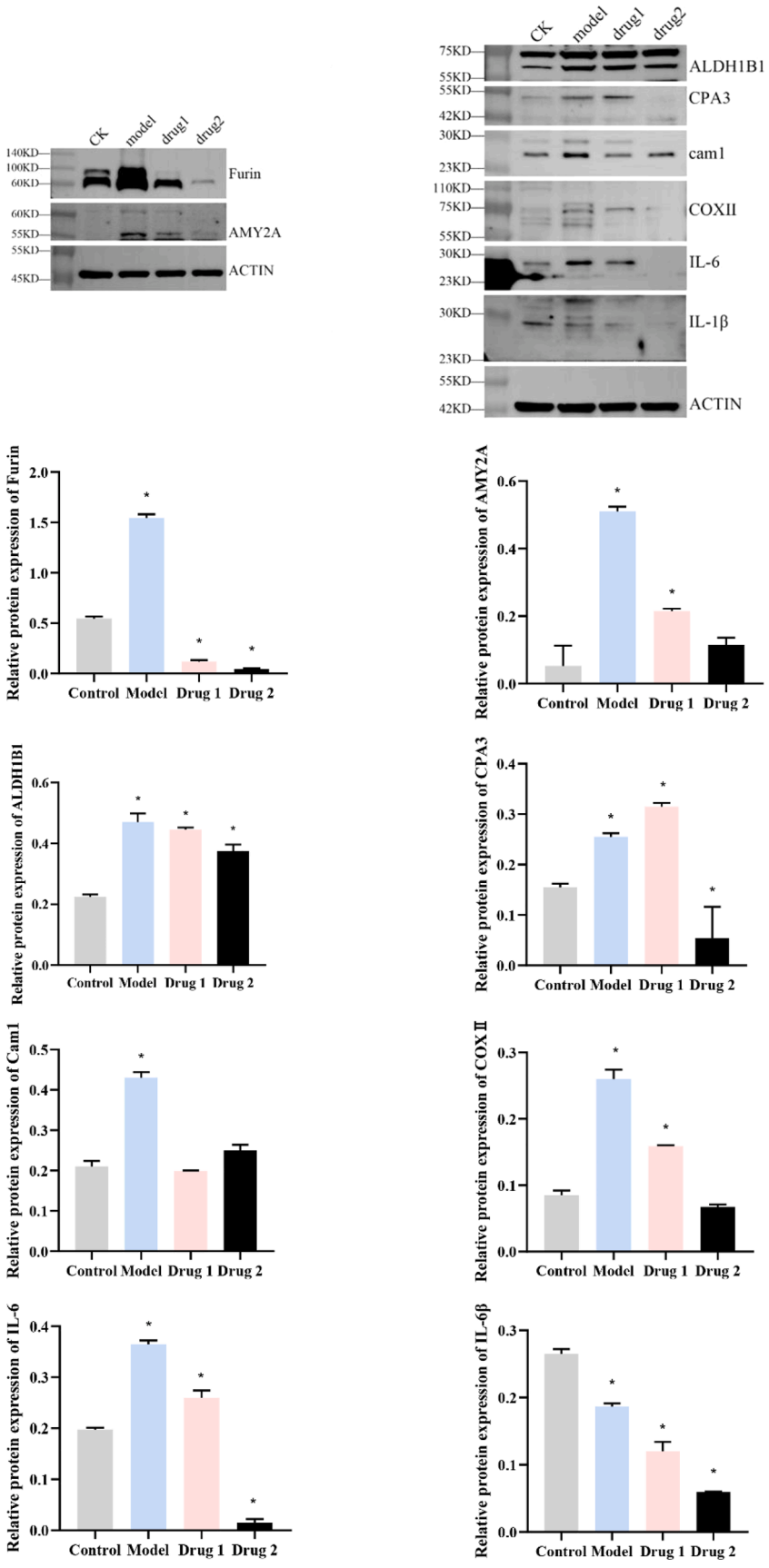


Fig. 9. The expression of ALDH1B1, CPA3, CAM1, COXII, IL-6, IL-1β was analyzed by Western blotting.

costunolide and dehydrocostuslactone were found in the extract of LWXM pills, a large number of antioxidants (Piperine glycosides, quercetin, luteolin, catechin, β-sitosterol, kaempferol). Previous studies have shown that costunolide and dehydrocostuslactone have significant protective effects against gastric ulcers (Zheng et al., 2016) and that H. pylori causes excessive oxidative stress in the gastrointestinal tract inducing chronic gastritis and even gastric cancer. Antioxidants are able to maintain gastric function by inhibiting the inflammatory response and oxidative stress(Fu et al., 2018). HE staining showed better results with 25 mg/ml LWMX pills (one mouths, two mouths) for CNG. The efficacy of drugs is mostly dose-related, so in this trial we chose the optimal concentration of 25 mg/ml for LWMX pills treatment for subsequent proteomic analysis.

CELA1 can act as a protein marker of tumor progression in colorectal cancer (Xie et al., 2016). Muc6 is associated with cancer migration and invasion (Giraldi et al., 2018), and previous studies found that MUC6 expression was significantly lower in gastric cancers with lymph node metastasis and poor pathological staging than in gastric cancers without lymph node metastasis and good pathological staging, respectively (Lee et al., 2001). TGFBI protein has an important role in tumor growth, metastasis and immunity. TGFBI knockdown inhibits tumor growth and metastasis in vivo (Poulsen et al., 2018; Wang et al., 2019). These lipase genes Pnliprp1 expression was significantly reduced in pancreatic tumors compared to non-tumor tissues (Zhu et al., 2021). LAMB2 could be a potential biomarker for colorectal cancer (Long et al., 2016) In our study, the expression of CELA1, Muc6, TGFBI, Pnliprp1, LAMB2 was decreased in the control group compared to the model group, affirming that CAG is a potential for further cancer development, and we also found that the expression of ALDH1B1 (Yano et al., 2017), H2-Ea (Du et al., 2014), Mgst1 (Kuang et al., 2021), and Nolc1(Kong et al., 2021) were upregulated in relation to tumor development, indicating that our model mice met the requirements of the experiment and confirming the reliability of our sequencing data. At the same time, we found that the downregulated proteins were mainly in the signaling pathways of protein digestion and absorption, pancreatic secretion, and focal adhesion, which are closely related to cell invasion and metastasis, and gastrointestinal digestion and absorption. Dietary proteins are usually absorbed in an efficient manner by gastrointestinal proteases, and many pathological conditions alter this process (Lee et al., 2021). CAG as precancerous state of gastric cancer is accompanied by alterations in proteins associated with cell invasion and metastasis (Abbas et al., 2017), which is similar to our findings. Interestingly, Cytoplasmic and mitochondrial protein expression is down-regulated and nucleus is up-regulated after LWMX pills treatment (Fig. 5).

HE staining of mouse gastric tissues showed improvement of the tissue phenomenon of chronic gastritis after 25 mg/ml LWMX pills treatment. The results of proteomic analysis showed that the inflammation-related factor Chil4 (Wang et al., 2021), the cancer suppressor-associated protein Rnf213 (Wang et al., 2020), Mybbp1a (Felipe-Abrio and Carnero, 2020), Bpifb1 (Wei et al., 2018), Cpa3 (Hamalainen et al., 2022) are up-regulated. Muc6 (Lee et al., 2001), Amy2 (Carpenter et al., 2015), Furin (Luo et al., 2022), and Atp5mf (Ianiro et al., 2016) are down-regulated. Amy2 and Atp5mf digestive enzyme, amylase-related genes, FURIN are highly expressed in lung adenocarcinoma and significantly associated with poorer overall survival (Luo et al., 2022). These results suggest that LWXM Pills significantly improved the pathological status of CNG and increased anti-inflammatory factors, however, the expression of genes related to amylase and digestive enzymes was downregulated in this result. Amylase, digestive enzymes are involved in maintaining normal gastrointestinal tract function (Giraldi et al., 2018). Therefore, we speculate that although LWMX pills improve the pathological state of CNG, the effect of inflammation on amylase and digestive enzymes in gastric tissues is irreversible, but the exact molecular mechanism needs to be shown in further studies. Further, KEGG enrichment revealed that the downregulated proteins were mainly focused on oxidative stress, which causes inflammation of the gastric mucosa (Yisireyili et al., 2020). The control and LWMX pills groups showed that LWMX enhanced the anti-inflammatory capacity of the body and down-regulated the expression of proteins associated with cancer development, and very interestingly, the treatment with LWMX pills down-regulated the expression of Ywhae (Helicobacter pylori interacting protein) (Zhang et al., 2018). Notably, after LWMX pills treatment, the protein expression of Chil4 and Mybbp1a were upregulated in gastric tissues, and the expression of Amy2 and Furin protein were downregulated in comparison with the control and model groups, respectively. This result suggests that LWMX pills maintain gastric function by suppressing inflammation and oxidative stress, and we also found that LWMX pills regulate the expression of protein related to cancer development (Amy2, Furin).

Conclusion

In this study, we found that LWMX pills contain costunolide, dehydrocostuslactone and antioxidants. Further study found that 25 mg/ml LWMX pill treatment for one month had better therapeutic effect on CNG. Further proteomic results showed that LWMX pills maintain gastric function by inhibiting inflammation and oxidative stress, and we also found that LWMX pills regulate the expression of proteins associated with cancer development (Amy2, Furin). Our study is the first to explain the possible mechanism of LWMX pills through proteomics, providing a theoretical basis for promoting TTM.

Note

The authors have no competing financial interests to declare.

CRediT authorship contribution statement

Rinchen Dhondrup: Writing – original draft, Methodology, Data curation, Investigation, Validation, Project administration, Funding acquisition. **Tawni Tidwell:** Methodology, Writing – review & editing. **XiaoKang Zhang:** Methodology, Data curation, Investigation. **Xuemei Feng:** Conceptualization, Resources. **Dhondrup Lobsang:** Data curation, Investigation. **Qincuo Hua:** Investigation. **Duojie Geri:** Investigation. **Duojie Caidan Suonan:** Data curation, Investigation. **Gang Fan:** Conceptualization, Methodology, Writing – review & editing. **Gyal Samdrup:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest in this article.

Acknowledgments

This study was supported by Qinghai Provincial Science and Technology Project – Applied Basic Research Program (Grant No. 2018-ZJ-732) and the National Key R&D Program of China (Grant No. 2017YFC1703902).

References

Abbas, M., Habib, M., Naveed, M., Karthik, K., Dhama, K., Shi, M.Q., Chen, D.D., 2017. The relevance of gastric cancer biomarkers in prognosis and pre- and post-chemotherapy in clinical practice. Biomed. Pharmacother. 95, 1082–1090.

Carpenter, D., Dhar, S., Mitchell, L.M., Fu, B.Y., Tyson, J., Shwan, N.A.A., Yang, F.T., Thomas, M.G., Armour, J.A.L., 2015. Obesity, starch digestion and amylase: association between copy number variants at human salivary (AMY1) and pancreatic (AMY2) amylase genes. Hum. Mol. Genet. 24, 3472–3480.

Chen, X., Shen, K., Deng, Y., Mo, J., Ni, J., Hendi, M., Chen, S., Wang, L., Si, J., 2022. A randomized double-blind clinical trial of weierkang pills for the treatment of chronic atrophic gastritis. J. Clin. Gastroenterol. 57, 165–171.

Du, Y.Q., Bai, Y., Xie, P., Fang, J.Y., Wang, X.Z., Hou, X.H., Tian, D., Wang, C.D., Liu, Y. D., Sha, W.H., Wang, B.M., Li, Y.Q., Zhang, G.L., Li, Y., Shi, R.H., Xu, J.M., Li, Y.M., Huang, M.H., Han, S.X., Liu, J., Ren, X., Xie, P.Y., Wang, Z.L., Cui, L.H., Sheng, J.Q., Luo, H.S., Wang, Z.H., Zhao, X.Y., Dai, N., Nie, Y.Q., Zou, Y.Y., Xia, B., Fan, Z.N., Chen, Z.T., Lin, S.R., Li, Z.S., 2014. Chronic gastritis in China: a national multi-center survey. BMC Gastroenterol. 14, 24502423.

Felipe-Abrio, B., Carnero, A., 2020. The tumor suppressor roles of MYBBP1A, a major contributor to metabolism plasticity and stemness. Cancers (Basel) 12, 254.

Fu, K., Xu, M., Zhou, Y., Li, X., Wang, Z., Liu, X., Meng, X., Zeng, Y., Zhang, H., 2020. The Status quo and way forwards on the development of Tibetan medicine and the pharmacological research of tibetan materia Medica. Pharmacol. Res. 155, 104688.

Fu, Y., Wu, H.Q., Cui, H.L., Li, Y.Y., Li, C.Z., 2018. Gastroprotective and anti-ulcer effects of oxymatrine against several gastric ulcer models in rats: possible roles of antioxidant, antiinflammatory, and prosurvival mechanisms. Phytother. Res. 32, 2047–2058.

Giraldi, L., Michelazzo, M.B., Arzani, D., Persiani, R., Pastorino, R., Boccia, S., 2018. MUC1, MUC5AC, and MUC6 polymorphisms, Helicobacter pylori infection, and gastric cancer: a systematic review and meta-analysis. Eur. J. Cancer Prev. 27, 323–330.

Hamalainen, S., Kareinen, L., Sukura, A., Kareinen, I., 2022. Carboxypeptidase A3 expression in canine mast cell tumors and tissue-resident mast cells. Vet. Pathol. 59, 236–243.

Hwang, Y.J., Kim, N., Lee, H.S., Lee, J.B., Choi, Y.J., Yoon, H., Shin, C.M., Park, Y.S., Lee, D.H., 2018. Reversibility of atrophic gastritis and intestinal metaplasia after Helicobacter pylori eradication - a prospective study for up to 10 years. Aliment. Pharmacol. Ther. 47, 380–390.

Ianiro, G., Pecere, S., Giorgio, V., Gasbarrini, A., Cammarota, G., 2016. Digestive enzyme supplementation in gastrointestinal diseases. Curr. Drug Metab. 17, 187–193.

Kong, F.G., Shang, Y.S., Diao, X.Y., Huang, J.G., Liu, H., 2021. Knockdown of NOLC1 Inhibits PI3K-AKT pathway to improve the poor prognosis of esophageal carcinoma. J. Oncol. 2021, 9944132.

Kuang, F.M., Liu, J., Xie, Y.C., Tang, D.L., Kang, R., 2021. MGST1 is a redox-sensitive repressor of ferroptosis in pancreatic cancer cells. Cell Chem. Biol. 28, 765–775.

Lee, H.S., Lee, H.K., Kim, H.S., Yang, H.K., Kim, Y.I., Kim, W.H., 2001. MUC1, MUC2, MUC5AC, and MUC6 expressions in gastric carcinomas: their roles as prognostic indicators. Cancer 92, 1427–1434.

Lee, S., Choi, Y.S., Jo, K., Yong, H.I., Jeong, H.G., Jung, S., 2021. Improvement of meat protein digestibility in infants and the elderly. Food Chem. 356, 129707.

Long, N.P., Lee, W.J., Huy, N.T., Lee, S.J., Park, J.H., Kwon, S.W., 2016. Novel biomarker candidates for colorectal cancer metastasis: a meta-analysis of in vitro studies. Cancer Inform. 15, 11–17.

Luo, L.X., Li, M.S., Su, J.T., Yao, X.Y., Luo, H., 2022. FURIN correlated with immune infiltration serves as a potential biomarker in SARS-CoV-2 infection-related lung adenocarcinoma. Clin. Exp. Med. 22, 371–384.

Ohata, H., Kitauchi, S., Yoshimura, N., Mugitani, K., Iwane, M., Nakamura, H., Yoshikawa, A., Yanaoka, K., Arii, K., Tamai, H., Shimizu, Y., Takeshita, T., Mohara, O., Ichinose, M., 2004. Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer. Int. J. Cancer. 109, 138–143.

Poulsen, E.T., Runager, K., Nielsen, N.S., Lukassen, M.V., Thomsen, K., Snider, P., Simmons, O., Vorum, H., Conway, S.J., Enghild, J.J., 2018. Proteomic profiling of TGFBI-null mouse corneas reveals only minor changes in matrix composition supportive of TGFBI knockdown as therapy against TGFBI-linked corneal dystrophies. FEBS J. 285, 101–114.

Rugge, M., Genta, R.M., 2005. Staging and grading of chronic gastritis. Hum. Pathol. 36, 228–233.

Tong, Y.L., Wang, R.L., Liu, X., Tian, M., Wang, Y.L., Cui, Y.F., Zou, W.J., Zhao, Y.L., 2021. Zuojin Pill ameliorates chronic atrophic gastritis induced by MNNG through TGF-beta 1/PI3K/Akt axis. J. Ethnopharmacol. 271, 113893.

Wang, B.J., Chi, K.P., Shen, R.L., Zheng, S.W., Guo, Y., Li, J.F., Fei, J., He, Y., 2019. TGFBI promotes tumor growth and is associated with poor prognosis in oral squamous cell carcinoma. J. Cancer 10, 4902–4912.

Wang, L., Ren, W.W., Li, X.W., Zhang, X.J., Tian, H.K., Bhattarai, J.P., Challis, R.C., Lee, A.C., Zhao, S.H., Yu, H.M., Ma, M.H., Yu, Y.Q., 2021. Chitinase-like protein ym2 (chil4) regulates regeneration of the olfactory epithelium via interaction with inflammation. J. Neurosci. 41, 5620–5637.

Wang, R.L., Wang, Y.L., Lu, Z., Jing, J., Wang, Z.X., He, T.T., Tian, M., Yuan, Z.Y., Cui, Y. F., Rong, W.Y., Ma, X., Zhao, Y.L., 2022. Efficacy and safety of zuojin pill for the treatment of chronic nonatrophic gastritis: a randomized active-controlled clinical trial. Evid. Based Complement. Alternat. Med. 2022, 2266023.

Wang, X., Ye, M., Wu, M., Fang, H., Xiao, B., Xie, L., Zhu, X., 2020. RNF213 suppresses carcinogenesis in glioblastoma by affecting MAPK/JNK signaling pathway. Clin. Transl. Oncol. 22, 1506–1516.

Wei, F., Tang, L., He, Y., Wu, Y.F., Shi, L., Xiong, F., Gong, Z.J., Guo, C., Li, X.Y., Liao, Q. J., Zhang, W.L., Ni, Q.X., Luo, J., Li, X.L., Li, Y., Peng, C., Chen, X., Li, G.Y., Xiong, W., Zeng, Z.Y., 2018. BPIFB1 (LPLUNC1) inhibits radioresistance in nasopharyngeal carcinoma by inhibiting VTN expression. Cell Death Dis. 9, 432.

Xie, Y.Y., Chen, L.H., Lv, X.L., Hou, G.X., Wang, Y., Jiang, C.C., Zhu, H.X., Xu, N.Z., Wu, L., Lou, X.M., Liu, S.Q., 2016. The levels of serine proteases in colon tissue interstitial fluid and serum serve as an indicator of colorectal cancer progression. Oncotarget 7, 32592–32606.

Yano, S., Takehara, K., Tazawa, H., Kishimoto, H., Urata, Y., Kagawa, S., Fujiwara, T., Hoffman, R.M., 2017. Therapeutic cell-cycle-decoy efficacy of a telomerase-dependent adenovirus in an orthotopic model of chemotherapy-resistant human stomach carcinomatosis peritonitis visualized with FUCCI imaging. J. Cell. Biochem. 118, 3635–3642.

Yisireyili, M., Alimujiang, A., Aili, A., Li, Y.L., Yisireyili, S., Abudureyimu, K., 2020. Chronic restraint stress induces gastric mucosal inflammation with enhanced oxidative stress in a murine model. Psychol. Res. Behav. Ma 13, 383–393.

Yue, P., Zhong, J., Huang, J.J., Lan, Z.X., Zhong, S., 2021. The efficacy and safety of Xiangsha Liujunzi decoction in the treatment of chronic non-atrophic gastritis A protocol for a systematic review and meta-analysis. Medicine (Baltimore) 100, e24504.

Zhang, X.Y., Zeng, B.W., Wen, C.Y., Zheng, S.R., Chen, H., She, F.F., 2018. YWHAE is a novel interaction partner of Helicobacter pylori CagA. FEMS Microbiol. Lett. 365 (10), 1093.

Zheng, H., Chen, Y.L., Zhang, J.Z., Wang, L., Jin, Z.X., Huang, H.H., Man, S.L., Gao, W.H., 2016. Evaluation of protective effects of costunolide and dehydrocostuslactone on ethanol-induced gastric ulcer in mice based on multi-pathway regulation. Chem.-Biol. Interact. 250, 68–77.

Zhu, G., Fang, Q., Zhu, F., Huang, D., Yang, C., 2021. Structure and function of pancreatic lipase-related protein 2 and its relationship with pathological states. Front. Genet. 12, 693538.