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Protective effects of fresh *Dendrobium officinale* against gastric ulcer by regulating Keap1-Nrf2 signaling pathway

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ABSTRACT

Objective: This study aimed to systematically investigate the gastroprotective effects and underlying mechanisms of fresh *Dendrobium officinale* granules (FDG), a clinically convenient formulation of fresh *Dendrobium officinale* (*D. officinale*).

Methods: For the convenience of clinical use, fresh *D. officinale* was prepared into granules. The active ingredients of FDG were detected by HPLC. The gastroprotective efficacy of FDG was investigated in mice with alcoholic, indometacin, and reserpine gastric ulcer models, and in rats with acetic acid chronic gastric ulcers. The ulcer index, histopathology of gastric mucosa, and the levels of nitric oxide (NO), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) were measured. Network pharmacology method was used to predict the potential mechanism of FDG. Lastly, the expression levels of Keap1, Nrf2, and HO-1 were assessed by qRT-PCR and Western blot methods.

Results: HPLC result showed that the four active ingredients of FDG were vicenin-2, isoschaftoside, schaftoside, and 6-C-xylosyl-8-C-glucosylapigenin. FDG exerted significant effects against acute and chronic gastric ulcers in a dose-dependent manner. The network pharmacology analysis predicted the potential mechanism on oxidative stress and its related pathways. FDG remarkably increased SOD, NO, and CAT levels and lowered MDA levels in various gastric ulcer models, indicating it could enhance the antioxidant capacity to prevent gastric ulcers. FDG obviously down-regulated Keap1 expression and up-regulated Nrf2 and HO-1 expression.

Conclusion: This study proved the gastroprotective efficacy of fresh *D. officinale*, and its mechanism may be related to the antioxidation activity and the regulation of Keap1-Nrf2 signaling pathway.

1. Introduction

Peptic ulcer is a globally prevalent gastrointestinal disease, which is a general term for gastric ulcer and duodenal ulcer, which penetrates beyond the gastric or duodenal mucosa, respectively.[1] Gastric ulcer is prevalent and multiple, with the main clinical manifestations of epigastric pain, loss of appetite, gastric acid reflux, and hemorrhage. The main clinical manifestations are epigastric pain, loss of appetite, acid reflux, and blood in stool, etc. In China, the prevalence of gastric ulcer is as high as 6.07% in the general population, and 22.5% of patients with gastrointestinal diseases suffer from gastric ulcer.[2] Approximately 200, 000 people are hospitalised each year with a diagnosis of this disease, with treatment costs of up to 4 billion dollars. Various factors such as gastric acid and pepsin, *Helicobacter pylori* (*H. pylori*), non-steroidal anti-inflammatory drugs (NSAIDs), imbalances in the gastric mucosa's own mechanisms and psychological and environmental factors can lead to the formation of ulcers in the gastrointestinal system.[3] Gastric ulcers

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Abbreviations

CAT	Catalase
D. officinale	*Dendrobium officinale* Kimura & Migo
FDG	Fresh *Dendrobium officinale* granules
H. pylori	Helicobacter pylori
MDA	Malondialdehyde
NO	Nitric oxide
SOD	Superoxide dismutase
NSAIDs	Non-steroidal anti-inflammatory drugs

are usually treated with histamine-2 receptor blockers or proton pump inhibitors (omeprazole). These drugs are effective but produce some side effects with long-term use. Clinical and experimental studies have shown that Chinese herbal medicine has therapeutic benefits for gastric ulcers with fewer side effects, and the cost of treating gastric ulcers is only about one-sixth of that of Western medicine.[4]

Studies on human and animal models have shown that herbal medicines exert beneficial effects on gastric ulcers through a variety of mechanisms, including antioxidant activity, inhibition of acid production and secretion, and inhibition of inflammation.[5–7] Fresh medicine has a long history of clinical application in traditional Chinese medicine. *Dendrobium officinale* Kimura & Migo (*Tiě pí shí hú, D. officinale*), is a kind of traditional Chinese medicine for both medicine and food, which is known as "life-saving fairy grass" in the folk. It has a medicinal history of more than 2000 years in China and the Asia-Pacific region, and has high medicinal value.[8,9] *D. officinale* was first recorded in one of the traditional Chinese medicine scriptures, "*the Divine Husbandman's Classic of the Materia Medica* (*Shén Nóng bèn cǎo jīng*)", the book pointed out that it could strengthen the spleen and stomach. "*Comprehensive Outline of the Materia Medica* (*Běn cǎo gāng mù*)" also recorded its effect of tonifying the stomach. It can be seen that *D. officinale* has been used to treat gastrointestinal diseases since ancient times.[10,11] In the ancient books such as "Discussion of Seasonal Diseases (*Shí bìng lùn*)" and "*The Refined in Medicine Remembered* (*Yī chún shèng yì*)", fresh *D. officinale* and other fresh herbs were used to treat diseases, "*Qufan Yangwei decoction*", "*Ye Shi Yangwei decoction*" and other prescriptions were all used fresh *D. officinale* for stomach disorders. It can be seen that fresh *D. officinale* is a traditional method to treat stomach disease.[10] Studies have shown that *D. officinale* has many pharmacological effects, such as protecting gastric mucosa and regulating intestinal movement, antioxidation, lowering blood sugar, lowering blood pressure, enhancing immunity, anti-tumor and so on.[12–14] But so far the depth and breadth of literature on the treatment of digestive system diseases is still inferior to that of others. There are also very few reports of fresh *D. officinale*. Therefore, it is important to deeply study the pharmacological effects of fresh *D. officinale* and clarify its mechanisms. In the folk and some traditional Chinese medicine hospitals in Guangdong Province, fresh *D. officinale* has proved to be effective in treating gastric ulcers. However, fresh medicine is difficult to preserve and inconvenient to use. So the purpose of this study was to observe the effect of fresh *D. officinale* on gastric ulcers and to explore the mechanism of gastric protection, also to explore the development and utilization of fresh Chinese herbal medicine.

2. Material and methods

2.1. Chemicals and antibodies

Absolute ethanol, xylene, neutral gum, and glacial acetic acid were purchased from National Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China). Malondialdehyde (MDA), Nitric oxide (NO), Superoxide dismutase (SOD) and Catalase (CAT) kits were bought from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China). Methanol (HPLC grade) was supplied by Merck Chemicals (Darmstadt, Germany). Formic acid was from Sigma-Aldrich (St Louis, MO, USA). vicenin-2, isoschaftoside, schaftoside, and 6-C-xylosyl-8-C-glucosylapigenin were supplied by Chemfaces Co., Ltd. (Wuhan, Hubei, China). RIPA lysate (strong), SDS-PAGE protein up-sample buffer, BCA protein concentration determination, and SDS-PAGE gel rapid preparation kits were obtained from Biyuntian biotechnology Co., Ltd. (Shanghai, China). Ranitidine hydrochloride capsule, indomethacin enteric coated tablets were purchased from Guangdong South China Pharmaceutical Group Co., Ltd. (Guangzhou, China). Hypersensitive ECL chemiluminescence reagent, β-actin antibody, HRP labeled goat antirabbit IgG (hogl) and rabbit anti-mouse IgG labeled with HRP were purchased from Wanlei biotechnology Co., Ltd. (Shenyang, China). Reserpine powder ($\geq$98%, Shanghai Yuanye Biotechnology Co., Ltd. batch No. X02M7Y1025). Hematoxylin and eosin dyesolution (servicebio, batch No. G1005). Differentiation solution (servicebio, batch No. G10053). Reverse blue solution (servicebio, batch No. G10054).

2.2. Plant material

The fresh *D. officinale* was collected from Mangshi of Yunnan province, China, batch No. 20161219. The specimen was identified by Associate Professor Xuanxuan Cheng, Guangdong Pharmaceutical University. A voucher specimen has been deposited at the lab 218 of new drug research and Development Center, Guangdong Pharmaceutical University (Accession number: TCM007).

2.3. Dendrobium officinale granules (FDG) preparation

Fresh *D. officinale* was broken into 1 cm fragments, then added to water to crush and homogenize at low temperature for 40 min. The juice was concentrated to a thick paste with a relative density of 1.35–1.40 (60 °C), and a proper amount of dextrin was added to make granules. Granule specification: 7 g/bag (containing 6.67 g fresh herbs, equivalent to 1 g dry products). Each bag contained mannose ($C_6H_{12}O_6$) 0.13–0.38 g.[15]

2.4. Animals and environmental conditions

Kunming mice of either sex (18–22 g, experimental animal license number: SYXK 2018-0002) and Sprague-Dawley rats of either sex (180–200 g, experimental animal license number: SYXK 2013-0002) were provided by the Guangdong medical experimental animal center. Animals were housed in the laboratory animal center of Guangdong Pharmaceutical University, under a 12h light/dark cycle at a temperature of 22 $\pm$ 2 °C and relative humidity ranging from 65% to 75%. The animals had free access to a standard diet and water throughout the experiments. This research was approved by the Animal Feeding and Use Ethics Committee of Guangdong Pharmaceutical University (No. gdpulacspf2017271). All of the animals involved in this protocol were bred and managed in strict accordance with the Chinese Regulations on the Use and Breeding of Experimental Animals.

2.5. Analysis of the potential components of FDG by HPLC

The sample solution was prepared according to the following steps. 10 g of FDG was extracted with 50 mL of 70% methanol for 30 min by sonication, and then centrifuged at 3000 rpm for 20 min. Subsequently, 20 mL of the supernatant was concentrated and the final volume was fixed to 2 mL. Finally, 0.2 mL of the concentrated solution was filtrated with a 0.45 μm membrane and then subjected to HPLC analysis.

HPLC determination was performed on a DIONEX 3000 UHPLC system (Thermo Fisher, Dreieich, Germany), equipped with a diode array detector and a Chromelon™ Chromatography Data System (Thermo Fisher, Dreieich, Germany). Chromatographic separation was performed on a Kromasil C_{18} column (Akzonobel, Sweden, 250 $\times$ 4.6

mm, particle size 5 μm) at a flow rate of 1.0 mL/min, and monitored at 280 nm. In this case, methanol (A) and water with 0.2% formic acid (B) were used as the mobile phases. The gradient profile was as follows: 0–10 min, 73% → 68% of B; 10–26 min, 68% → 64% of B; 26–30 min, 64% → 55% of B; 30–32 min, 55% → 5% of B.

2.6. Studies of network pharmacology analysis

2.6.1. Metabolites and targets of D. officinale

D. Officinale was identified using ETCM (tcmip.cn), meeting the criteria of Oral Bioavailability (OB) ≥ 30% and Drug-Likeness (DL) ≥ 0.18. PubChem (https://Pubchem.ncbi.nlm.nih.gov/) provided chemical structures; Swiss Target Prediction (http://www.swisstargetprediction.ch/) predicted targets.

2.6.2. Collecting therapeutic targets for gastric ulcer

The GeneCards website (https://www.Genecards.org/) was used to seek for gastric ulcers and download disease targets. Subsequently, a Venn diagram illustrating the interaction between the therapeutic targets of *D. officinale* and the disease targets was generated using the Microbioinformatics platform (https://www.bioinformatics.com.cn/), resulting in the identification of intersecting targets.

2.6.3. Analysis of PPI network

The intersection targets of *D. officinale* and gastric ulcer were input into the STRING database (http://string.embl.de/), and data with a confidence score greater than 0.4 were selected to obtain the PPI network. The results were input into Cytoscape software to visualize the PPI network diagram of key targets.

2.6.4. GO and KEGG analysis

Target genes were analyzed for KEGG pathway enrichment using David database (https://david.ncifcrf.gov/home.jsp; min overlap 3, $P < 0.05$, enrichment score > 1.5). Enrichment analysis results were visualized on the online charting platform, MicBio.

2.6.5. Establish the network of D. officinale-target-pathway-gastric ulcer

D. officinale, putative gastric ulcer targets, and enriched KEGG pathways (top 20) were integrated into a Cytoscape 3.9.1 network.

2.7. Effect of FDG on different gastric ulcer models

2.7.1. Ethanol-induced gastric ulcer test

60 SPF-grade Kunming mice were used in this experiment and randomly divided into 6 groups, with 10 mice per group: normal control group (abbreviated as N group), model group (M group), ranitidine group (R group, ranitidine 0.06 g/kg), FDG high-dose group (FH group, 8 g/kg), FDG medium-dose group (FM group, 4 g/kg), and FDG low-dose group (FL group, 2 g/kg). The FH, FM, and FL groups were treated orally with 8, 4 and 2 g/kg FDG, the N and M groups received orally only vehicle (0.5% CMC-Na), and the R group received orally 0.06 g/kg ranitidine, respectively. All treatments were administrated once daily. After continuous administration for 6 days, mice were fasted for 24 h, then all groups were orally treated with the drug/vehicle for the last day. 1 h later, each mouse was administered intragastrically with absolute ethanol. The mice were sacrificed after intragastric administration of ethanol for 1 h, and the ulcer index was calculated for each mouse, and the gastric tissues were taken for pathological examination. The ulcer inhibition percentage was calculated through the following formula: (the average ulcer index of model group-the average ulcer index of drug group)/the average ulcer index of model group × 100%.

2.7.2. Indometacin-induced gastric ulcer test

This method referred to the research of other scholars.[16] After 3 days of adaptive feeding, 50 SPF-grade Kunming mice were used in this experiment and randomly divided into 5 groups, with 10 mice per group: model group (M group), ranitidine group (R group, 0.06 g/kg), FDG high-dose group (FH group, 8 g/kg), FDG medium-dose group (FM group, 4 g/kg), and FDG low-dose group (FL group, 2 g/kg). The FH, FM, and FL groups were treated orally with 8, 4 and 2 g/kg FDG, the M groups received orally only vehicle (0.5% CMC-Na), and the R group received orally 0.06 g/kg ranitidine, respectively. All treatments were administrated once daily, and were continuously administrated for 6 days. Mice were fasted for 24 h, then all groups were treated orally for the last time. One h later, each mouse was given a subcutaneous injection of indomethacin (80 mg/kg), after 7 h, mice were sacrificed and the ulcer index of each mouse was calculated, the gastric tissues were taken for pathological examination. The calculation method for ulcer inhibition percentage is the same as above 2.7.1.

2.7.3. Reserpine-induced gastric ulcer test

This method referred to the research of other scholars.[17] After 3 days of adaptive feeding, the mice were randomly divided into groups. Animal specifications, grouping method, number of mice per group and treatment in this experiment were consistent with those in section 2.7.1. The mice were subjected to continuous administration for 6 days (once daily). After fasting for 24 h, mice of N, M, R, FH, FM, and FL groups were treated orally with vehicle, vehicle, ranitidine (0.06 g/kg), high, middle, and low doses of FDG (8, 4 and 2 g/kg) respectively. 1 h later, each mouse was injected with reserpine solution (10 mg/kg), then 6 h later the mice were sacrificed. The ulcer index was calculated for each mouse, and the gastric tissues were taken to determine the levels of SOD, NO, and MDA. The calculation method for ulcer inhibition percentage is the same as above 2.7.1.

2.8. Mechanism of FDG treatment for gastric ulcer caused by acetic acid

2.8.1. Acetic acid-induced ulcer test

60 SPF-grade Sprague-Dawley rats were used in this experiment and randomly divided into 6 groups, with 10 rats per group: the sham operation group (abbreviated as S group), the model group (M group), the ranitidine group (R group) and high, middle, low doses of FDG groups (FH group, FM group, FL group). After fasting for 24 h, the rats were anesthetized to cut open the abdomen and find the gastric body. The round filter paper soaked in glacial acetic acid (diameter 6 mm) was affixed to the junction of the gastric body and pyloric antrum for 30 s. The S group was only applied with circular filter paper soaked in normal saline. Iodine tincture was applied daily on the sutures. On the second day after the operation, the drug groups were given FDG (4, 2 and 1 g/kg) or ranitidine (0.03 g/kg), the sham operation group was given the same volume of distilled water, and the M group was given 0.5% CMC-Na. After fasting for 12 h, rats were given a final administration, and 1 h later rats were anesthetized, blood was collected from abdominal cavity, centrifuged supernatant was obtained, and the levels of SOD, CAT, and MDA in serum were detected according to the instructions of the biochemical kit. Rats were finally sacrificed and the ulcer index was calculated for each rat. The gastric tissues were taken for pathological examination and other indicators were tested. The calculation method for ulcer inhibition percentage is the same as above 2.7.1.

2.8.2. Detection of Keap1, Nrf2 and HO-1 mRNA by RT-PCR

The gastric tissue was taken from −80 °C refrigerator, the total RNA was extracted strictly according to the instructions of the kit, the content and purity of RNA were determined by a nucleic acid analyzer. Then the RNA was reverse transcribed to cDNA, and the cDNA was used as a template for DNA amplification. The expression of Keap1, Nrf2, and HO-1 was detected and analyzed by real-time quantitative PCR, each sample was repeated 3 times, and the result was shown as Ct value. The primer sequence is shown in Table 1.

2.8.3. Detection of Keap1, Nrf2 and HO-1 protein by Western blot

Frozen stomach tissue was retrieved for protein extraction, followed

Table 1
Primer sequence.

Gene name	Upstream primers (5′ to 3′)	Downstream primers (5′ to 3′)
Keap1	TGCTCAACCGCTTGCTGTATGC	TCATCCGCCACTCATTCCTTCC
Nrf2	GCTGCCATTAGTCAGTCGCTCTC	ACCGTGCCTTCAGTGTGCTTC
HO-1	TCTGGTATGGGCCTCACTGG	AATGTTGAGCAGGAAGGCGG
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

by the preparation of SDS-PAGE gels. Following sample application, proteins were separated using electrophoresis and then transferred onto membranes. After a blocking step to reduce non-specific binding, the membranes were probed with primary antibodies specific to Keap1 (1:1000), Nrf2 (1:500), HO-1 (1:5000), and β-Actin (1:8000). Secondary antibodies were applied for room temperature incubation, followed by development. Keap1, Nrf2 and HO-1 expression levels were quantified relative to the gray values of the internal control protein, β-Actin.

2.9. HE of gastric mucosa

Gastric samples underwent a fixation process using 4% paraformaldehyde, then were embedded in paraffin, sliced, stained with hematoxylin and eosin, and finally sealed with coverslips. Gastric tissue structure and morphology were assessed via microscopy, and images were acquired for histopathological scoring purposes.

The images were collected and analyzed by TIGER tImaPro software. The pathological scoring criteria were based on the previously described method.[18] The degree of surface epithelial exfoliation, glandular cell degeneration, submucosal edema, and inflammatory cell infiltration was assessed using 0–3 points respectively. Then the cumulative score of each section was calculated.

Gastric injury was assessed semiquantitatively using the previously described criteria: Desquamation of surface epithelium (0–3, where 0, None; l, Mild; 2. Moderate; 3, Severe), Hemorrhage, focal necrosis andmucosal congestion (0–3), Degeneration of glandularcells (0–3), Infammatory cell infltration (0–3), with a maximum score of 12.

2.10. Statistical analysis

All the results were presented as the means ± standard deviation (SD) and were analyzed by SPSS 24.0 software. One-way analysis of variance (ANOVA) and Dunnett's *t*-test were used to evaluate the significant differences among the groups, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Component analysis of FDG

FDG was derived from fresh *D. officinale* via low-temperature homogenization and dextrin granulation. The HPLC analysis results of FDG solution are showed in Fig. 1. The FDG solution (B) was confirmed to contain 4 kinds of flavone C-glycosides by the retention time verified in the chromatographic analysis of the mixed reference substances solution (A). In addition, it can be inferred that vicenin-2, isoschaftoside, schaftoside, and 6-C-xylosyl-8-C-glucosylapigenin are the ingredients of FDG, which is related to improving the antioxidant capacity of gastric tissue of mice and the protective effect on gastric mucosa.

3.2. Results of network pharmacology analysis

3.2.1. Acquisition of potential D. officinale action targets for the therapy of gastric ulcer

Following the screening, 4 potential metabolites of *D. officinale* (Table 2) and 204 potential action targets were predicted. To identify and integrate the targets associated with gastric ulcer, GeneCards

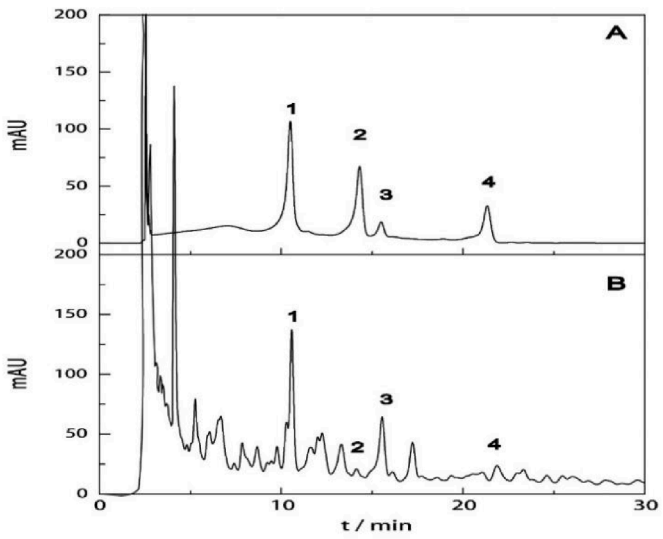

Fig. 1. Representative HPLC chromatograph of mixed reference substances solution (A) and FDG solution (B). Vicenin-2 (1), isoschaftoside (2), schaftoside (3), 6-C-xylosyl-8-C-glucosylapigenin (4).

Table 2
Basic information on the metabolites of *D. officinale*.

No.	Chemical compound	MolID	OB (%)	DL
1	5,7-Dimethoxyphenanthrene-2,6-diol	MOL010154	80.73	0.24
2	Evodiamine	MOL003958	86.02	0.64
3	Nodakenetin	MOL004793	84.77	0.18
4	Rutaecarpine	MOL002662	40.30	0.60

databases were utilized. Duplicate target proteins were removed, resulting in a total of 6077 targets. A total of 202 target genes were obtained by cross-referencing of the *D. officinale* target and the disease target (Fig. 2A).

3.2.2. Establishing the PPI network

202 Obtained intersecting targets were imported into the STRING database, where one isolated target was removed. The data was then visualized using Cytoscape version 3.9.1, resulting in the PPI network displayed in Fig. 2B. The PPI network comprises 199 nodes and 1898 edges. Notably, larger nodes exhibit a color that approaches purple and a higher degree value. Core targets ($n = 50$) were selected based on degree, betweenness, and closeness centrality above median values.

3.2.3. GO and KEGG analysis

Metascape was used for GO and KEGG enrichment of core target genes, as illustrated in Fig. 2C. GO analysis mainly focused on protein phosphorylation, response to oxidative stress, and inflammatory response. To refine the analysis based on the current state of research, unrelated pathways were excluded, and the Microbioinformatics platform was used to visualize the 20 most highly enriched pathways, as shown in Fig. 2D. Major signaling routes involved encompass the MAPK signaling pathway, cAMP signaling pathway, and pathways in cancer.

3.2.4. Construction of the drug-target-pathway network

Using Cytoscape 3.9.1, we constructed a network of drug-targets-pathways by integrating *D. officinale* and its components with 23 core targets and 20 key pathways, as shown in Fig. 2E. The network comprised 106 nodes and 195 edges: diamonds represent *D. officinale*, circles denote the four active ingredients, 80 rectangular nodes indicate overlapping targets, 20 inverted triangular nodes symbolize pathway intersections, and triangular nodes stand for gastric ulcer-related targets.

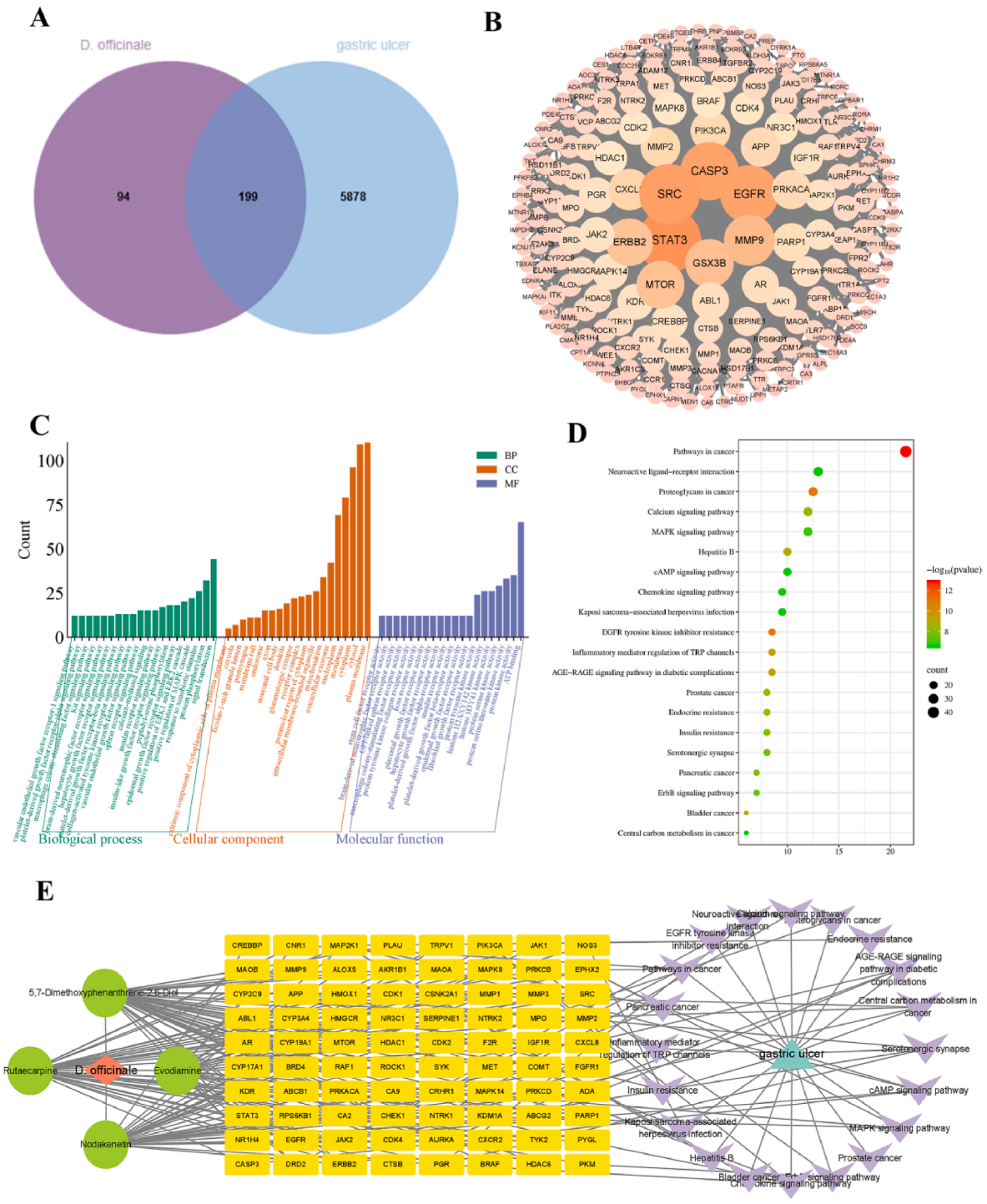

Fig. 2. Network pharmacology of *D. officinale* for the therapy of gastric ulcer. A: Venn diagram of component targets and disease targets. B: PPI network visualization. C: GO enrichment analysis. D: KEGG enrichment analysis. E: *D. officinale*-target- pathway network diagram.

3.3. Effect of FDG on different gastric ulcer models

3.3.1. *Ethanol-induced gastric ulcer*

In the model group, intragastric administration of ethanol produced extensive bleeding and erosive ulcers in the gastric mucosa (Fig. 3). However, FDG and ranitidine both showed obvious anti-ulcer effects ($P < 0.01$ or $P < 0.05$). The dose of 8 g/kg FDG group and ranitidine group significantly inhibited the appearance of lesions, the ulcer inhibition percentages of the two groups were 66.51% and 63.04% respectively. FDG at doses of 4 g/kg, 2 g/kg inhibited the lesions by 49.87% and 38.73% respectively.

As shown in Fig. 4, the gastric mucosal injury in the model group was prominently manifested in submucosal edema, mucosal structure disorder, atrophy, and accumulation of inflammatory cells. In ranitidine group, the structure of gastric mucosa was intact, some glands were loosely arranged and damaged. The gastric mucosa of high-dose FDG was intact, the glands were arranged closely, and there was no obvious inflammatory reaction, while in the middle-dose group, the gastric mucosa became thinner, the glands were damaged, and no obvious inflammatory reaction was observed. Compared with the model group, the

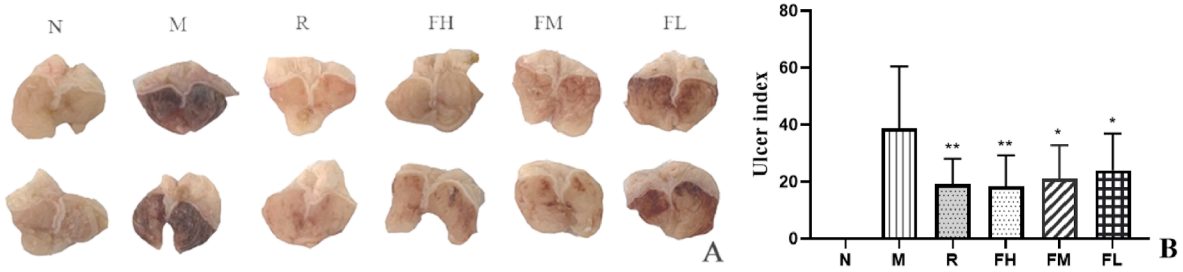

Fig. 3. Effect of FDG on ethanol induced gastric ulcer in mice. A: Pictures of gastric ulcer; B: Ulcer index in each group ($\bar{x} \pm s$, $n = 10$); vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

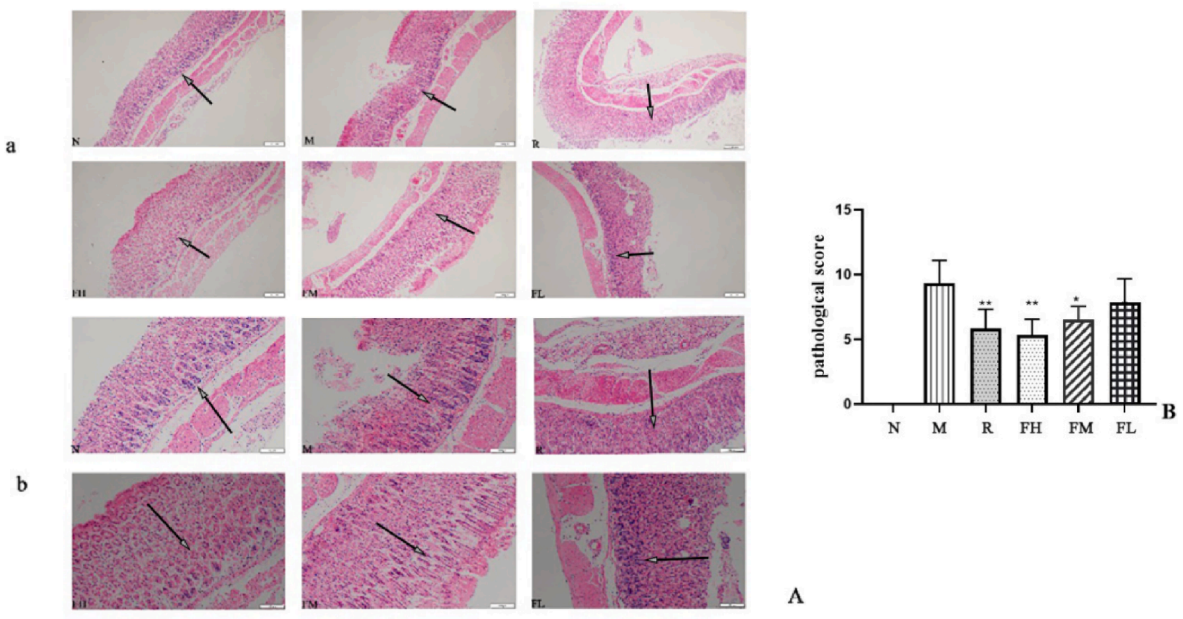

Fig. 4. Pathomorphology of FDG's effect on ethanol induced gastric ulcer in mice. A: Pathological chart; B: Pathological score ($\bar{x} \pm s$, $n = 10$); Black arrow indicates inflammatory cell infiltration in HE staining; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

gastric mucosal injury in the ranitidine group and the high dose group was significantly improved, and the pathological score was lower than that of model group ($P < 0.01$), and the gastric mucosal injury in the middle dose FDG group was also improved ($P < 0.05$).

3.3.2. Indometacin-induced gastric ulcer

Traditional NSAIDs can cause obvious gastrointestinal adverse reactions, such as gastric ulcers. From this test we found a single injection of indometacin in mice could induce an obvious strip or punctate lesion of gastric mucosa, the ulcer index of model group was 32.86 ± 12.23, as depicted in Fig. 5. FDG demonstrated significant antiulcer activity at doses of 8 and 4 g/kg, the ulcer index was 4.56 ± 1.99, 12.92 ± 2.63, respectively. By calculating, the ulcer inhibition percentage of the two groups was 86.12% and 60.68%. Ranitidine also showed an obvious anti-ulcer effect. The ulcer index was 7.18 ± 3.40, and the ulcer inhibition percentage was 78.15%. However, FDG at 2 g/kg had no significant effect.

As shown in Fig. 6, the gastric mucosa of model group became thinner, the structure was incomplete, the arrangement of glands was loose, and inflammatory cells gathered, while the structure of gastric mucosa in ranitidine group was intact, and some glands were loosely arranged and damaged. The gastric mucosal structure of the high dose

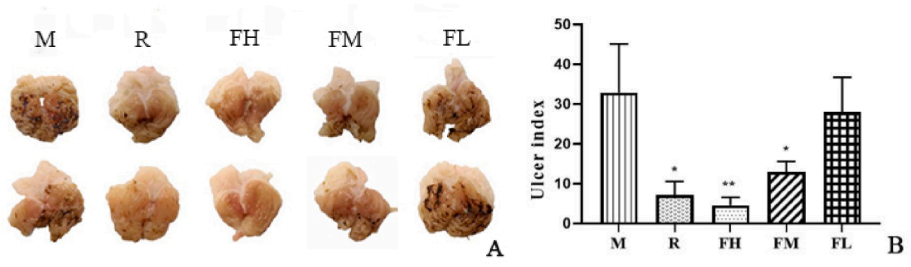

Fig. 5. Effect of FDG on indometacin induced gastric ulcer in mice. A: Pictures of gastric ulcer; B: Ulcer index in each group ($\bar{x} \pm s$, $n = 10$); vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

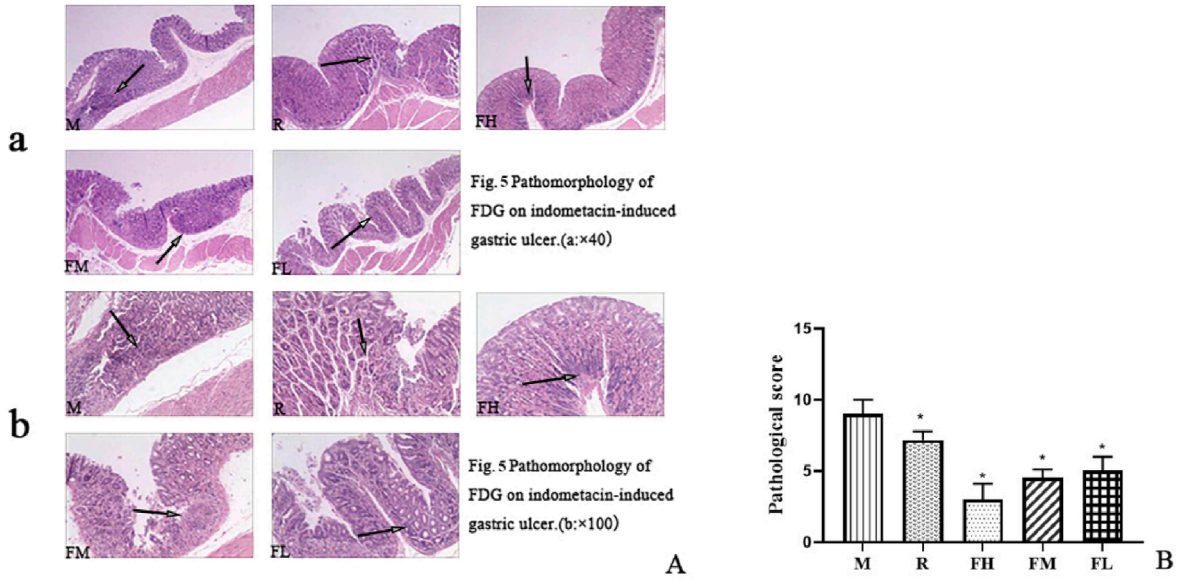

Fig. 6. Pathomorphology of FDG's effect on indometacin-induced gastric ulcer in mice. A: Pathological chart; B: Pathological score ($\bar{x}$ ±s, $n = 10$); Black arrow indicates inflammatory cell infiltration in HE staining; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

group of FDG was intact, the glands were arranged closely, and there was no obvious inflammatory reaction, while in the middle and low dose groups, the gastric mucosa became thinner, the glands were damaged, and no obvious inflammatory reaction was observed. Compared with the model group, both the ranitidine group and the FDG groups showed significant improvement in gastric mucosal damage, and the pathological scores were lower than that of model group ($P < 0.05$).

3.3.3. Reserpine-induced gastric ulcer

Reserpine depletes catecholamine neurotransmitters in adrenergic nerve endings, resulting in a relative increase in vagal excitability, then stimulates the release of 5-HT or histamine, which leads to excessive secretion of gastric juice and changes in vascular motion of gastric wall, resulting in ulceration. The present study proved that intraperitoneal injection of reserpine at a dose of 10 mg/kg successfully induced gastric ulcers in mice, as demonstrated by many extensively elongated thick, blackish red bands of hemorrhagic lesions on the glandular part of the stomach. Compared with the model group, FDG at doses of 8 g/kg and 4 g/kg significantly inhibited the appearance of lesions, the ulcer inhibition percentages of the two groups were 61.50% and 40.50%, respectively (Fig. 7).

The formation of gastric ulcers is closely related to the cell damage caused by oxygen free radical, which is also one of the damage mechanisms of reserpine gastric ulcer model. Furthermore, several pieces of evidence showed that NO can be used as a protective agent for gastric ulcers by promoting mucosal blood flow, stimulating gastric mucus secretion and reducing leukocyte infiltration. Therefore, we also detected the activities of SOD, NO, and MDA in gastric mucosal tissue. Compared with the normal control group, the activity of total SOD in the gastric tissue of the model group decreased significantly, while the contents of MDA increased significantly ($P < 0.01$). Compared with the model group, the levels of SOD and NO in the high and middle dose groups of FDG were significantly increased ($P < 0.01$ or $P < 0.05$), and the levels of MDA were decreased significantly ($P < 0.01$ or $P < 0.05$). The results showed that FDG could improve the antioxidant capacity of gastric tissue of mice with acute gastric ulcer induced by reserpine, which caused a protective effect on gastric mucosa (Fig. 8).

3.4. The mechanism of FDG treatment for acetic acid induced gastric ulcer

3.4.1. Ulcer index

Chronic gastric ulcer is a very common clinical disease. Chronic acetic acid gastric ulcer is caused by acetic acid corroding gastric mucosa, and is a commonly used and standardized chronic gastric ulcer model both domestically and internationally. Its pathological form and repair process are similar to human gastric ulcers, the ulcer is usually deep and big, around mucous membrane hyperemia and edema, serious rats appear perforation. As shown in Fig. 9, the ulcer index of model group was 6.06 ± 0.38, however, the ulcer index of 8 and 4 g/kg FDG groups was 1.92 ± 1.12 and 4.01 ± 0.72, the ulcer inhibition percentages were 68.32% and 33.83%, respectively. The ulcer index of

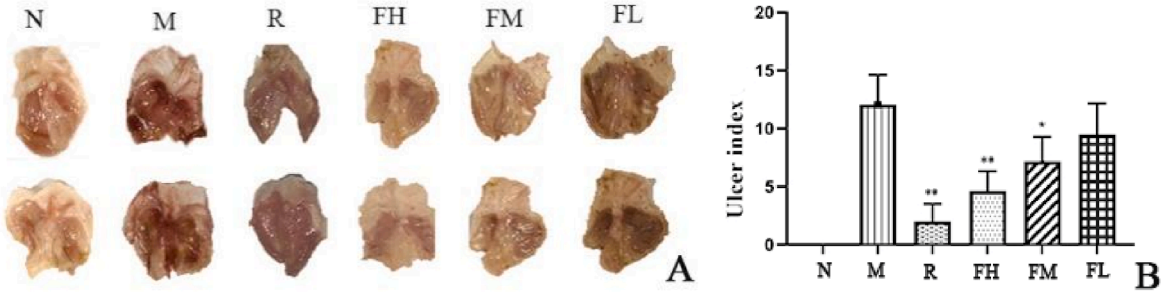

Fig. 7. Effect of FDG on reserpine induced gastric ulcer in mice. A: Pictures of gastric ulcer; B: Ulcer index in each group ($\bar{x}$ ±s, $n = 10$); vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

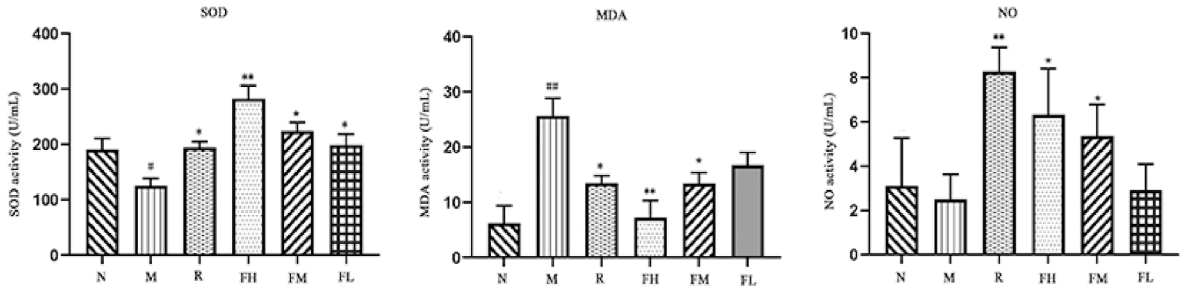

Fig. 8. Effect of FDG on the levels of SOD, MDA and NO in the reserpine-induced gastric ulcer mice ($\bar{x} \pm s$, $n = 10$). vs normal group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

ranitidine group was 3.56 ± 0.23, and the ulcer inhibition percentage was 41.25%. Compared with the model group, the three groups all significantly inhibited the appearance of lesions ($P < 0.05$).

3.4.2. *Histopathology examination*

As shown in Fig. 10, the structure of each layer of gastric mucosa in the sham operation group was intact, the glands were arranged tightly and without injury, and no inflammatory cells were found. However, in the model group, the gastric mucosal injury was serious and the area was large, the structure of gastric mucosa almost disappeared, the arrangement of glands was disordered and bleeding, and a large number of inflammatory cells were found. The gastric mucosal glands of rats in ranitidine group were missing and interrupted with a small amount of inflammatory cell infiltration; the gastric mucosal glands of rats in high-dose FDG group were slightly damaged and loosely arranged; in the middle-dose group, the glands of gastric mucosa were missing and a small number of inflammatory cells infiltrated; in the low-dose group, the mucosal layer was interrupted and the structure of glands was disordered, inflammatory cells infiltrated. Compared with the sham operation group, the gastric mucosal injury in the model group was more serious and the pathological score was significantly higher ($P < 0.05$). Compared with the model group, the gastric mucosal injury was significantly improved and the pathological score significantly decreased in ranitidine group and all FDG groups ($P < 0.05$).

3.4.3. *Levels of SOD, CAT and MDA*

As shown in Fig. 11, compared with the sham operation group, the activities of SOD and CAT in the model group decreased significantly, while the MDA content increased significantly; compared with the model group, the activities of SOD and CAT in the high and middle dose groups of FDG increased significantly, and the MDA content decreased significantly in a dose-dependent manner, and the high dose group was slightly better than the ranitidine group, and the middle concentration group was similar to the ranitidine group.

3.4.4. *The mRNA expression of Keap1, Nrf2 and HO-1*

As shown in Fig. 12, compared with the sham operation group, the mRNA expression of Keap1 and HO-1 in the model group increased significantly ($P < 0.05$), but there was no significant difference on the expression of Nrf2. Compared with the model group, the expression of Keap1 in all treatment groups decreased, especially the high dose group of FDG had obvious differences ($P < 0.05$); the mRNA expression of Nrf2 in ranitidine group, high and middle dose groups of FDG increased significantly ($P < 0.05$); the expression of HO-1 in ranitidine group and high dose group increased significantly ($P < 0.05$). Compared with the sham operation group, the expression of Keap1 increased significantly in low dose group, decreased in high dose group; the expression of Nrf2 increased significantly in ranitidine group, high and middle dose groups of FDG; and HO-1 mRNA expression increased significantly in ranitidine group and all FDG groups.

3.4.5. *The protein expression of Keap1, Nrf2 and HO-1*

As shown in Fig. 13, the protein expression of Keap1 was significantly higher in the model group than in the sham operation group, and the expression of Nrf2 and HO-1 was also higher in the model group than in the sham operation group, but without significant difference. Compared with the model group, the expression of Keap1 protein was significantly reduced in the high-dose group of FDG, and decreased in the middle-dose group of FDG and ranitidine group, but not significantly; however, the expression of Nrf2 and HO-1 protein increased significantly in ranitidine group, high and middle dose of FDG groups ($P < 0.05$). Compared with the sham operation group, the expression of Keap1 protein in the low-dose group was significantly increased, and the high-dose group was slightly lower than the sham operation group. The expression of Nrf2 protein in ranitidine group, high-dose group and middle-dose group was significantly higher than that in sham-operation

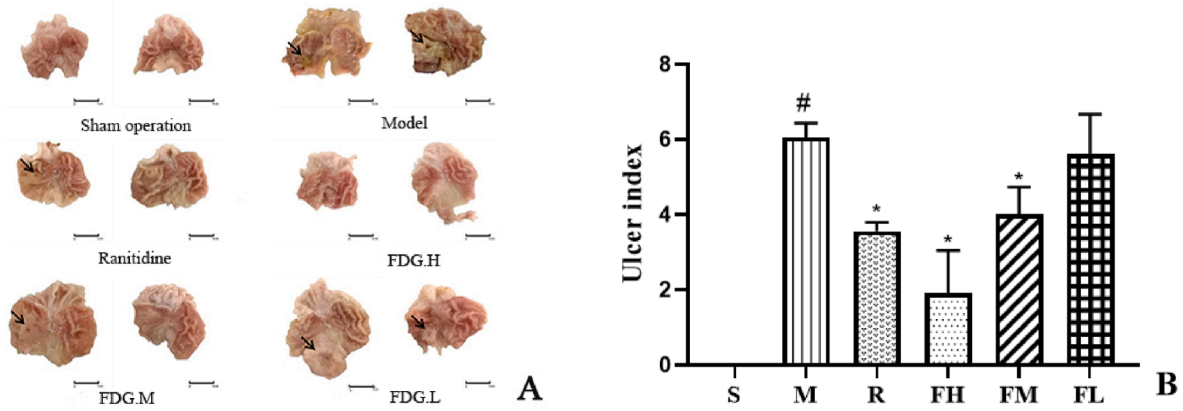

Fig. 9. Effect of FDG on acetic acid induced gastric ulcer in rats. A: Pictures of gastric ulcer; B: Ulcer index in each group ($\bar{x} \pm s$, $n = 10$); vs sham operation group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

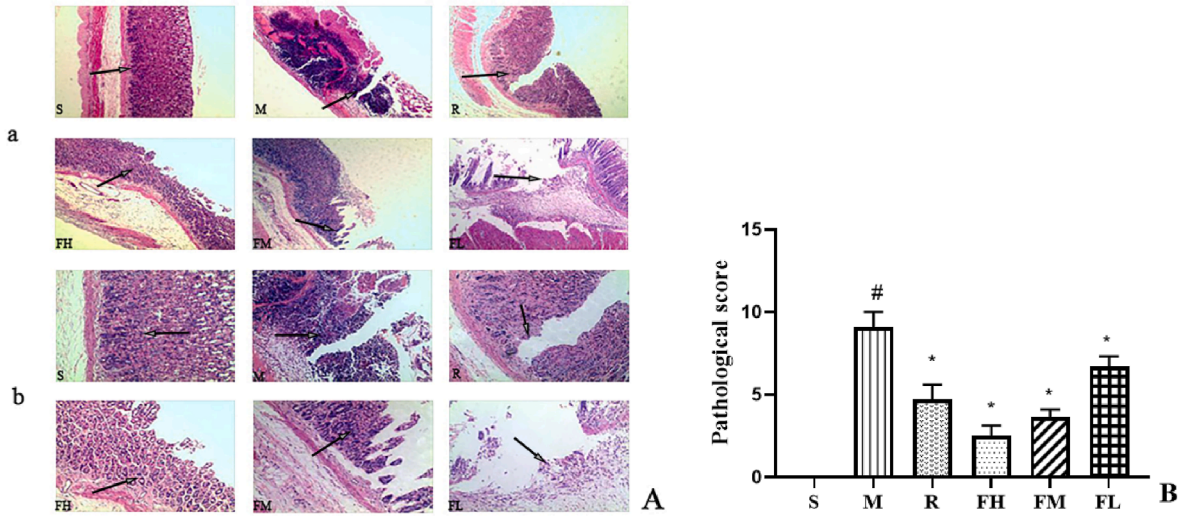

Fig. 10. Pathomorphology of FDG's effect on acetic acid induced gastric ulcer rats. A: Pathological chart; B: Pathological score ($\bar{x} \pm s$, $n = 10$); Black arrow indicates inflammatory cell infiltration in HE staining; vs sham operation group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

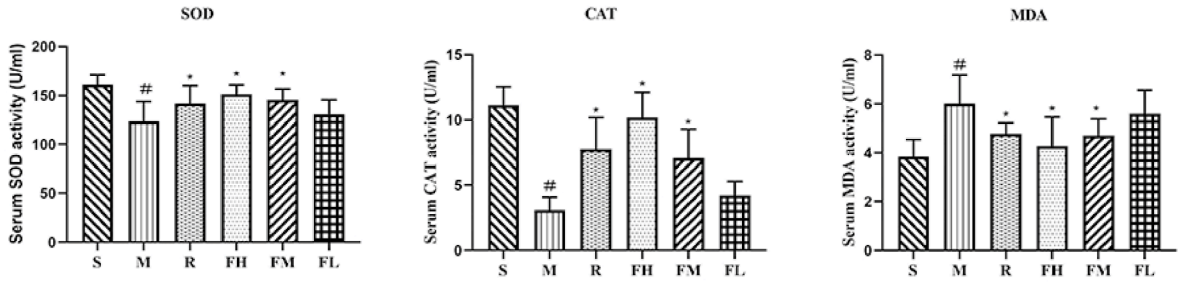

Fig. 11. Effect of FDG on the activities of SOD, CAT and MDA in the acetic acid induced gastric ulcer rats ($\bar{x} \pm s$, $n = 3$). vs sham operation group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

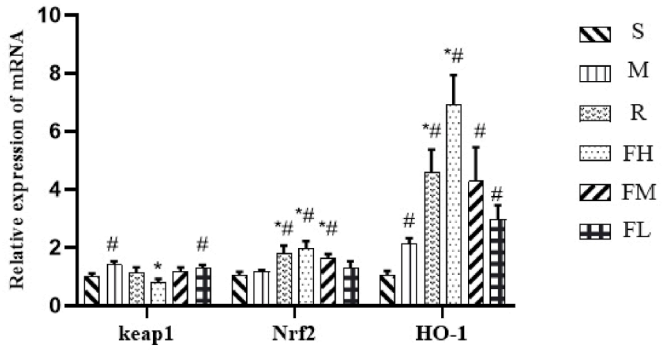

Fig. 12. The mRNA expression of Keap1, Nrf2 and HO-1 in the acetic acid induced gastric ulcer rats. vs sham operation group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

group ($P < 0.05$), and the expression of HO-1 protein in ranitidine group, high-dose group, middle-dose group and low-dose group was significantly higher than that in sham-operation group ($P < 0.05$).

4. Discussion

Fresh medicine has a special effect in the clinical treatment of traditional Chinese medicine, especially in the treatment of acute syndrome, heat syndrome, and surgical diseases. In the ancient book *the Divine Husbandman's Classic of the Materia Medica (shén nóng běn cǎo jīng),* there are some records of fresh use of traditional Chinese medicine, such as Ginger and Dandelion. The discovery of artemisinin also benefited from the use of the juice of *Artemisia carvifolia* in the treatment of malaria. Therefore, the use of fresh traditional Chinese medicine can make it play a special role in treating disease. Fresh *D. officinale* has been proved to be effective in treating gastric ulcers in folk, also also applied in Qingyuan hospital of Chinese medicine for many years.

D. officinale contains a variety of chemical components, including polysaccharides, flavonoids, alkaloids, amino acids, volatile components and trace elements, etc. Among them, polysaccharides are the main components for their pharmacological activity and quality control. It has been proved that polysaccharide components of *D. officinale* can increase the activity of SOD and PGE_2 in gastric mucosa, reduce the content of MDA, and exert its protective effect on gastric mucosa, thereby accelerating ulcer healing.[19–21] Since the clinical use of fresh *D. officinale* in the treatment of stomach diseases has achieved good efficacy, this study also proved that the use of fresh products is beneficial in improving the dissolution rate and amount of polysaccharides. However, due to the storage difficulties of fresh herbs, the clinical application is limited, so it is urgent to find a better way to use them. Furthermore, there is limited scientific research on the gastroprotective efficacy of fresh *D. officinale.* Therefore, we developed a modern fresh medicine formulation, namely FDG, which could facilitate clinical use, and explored its protective effect and mechanism on gastric ulcers. Firstly, we conducted preliminary research on the quality standards of FDG. Each bag (7 g) contained 6.67 g fresh herbs, which was equivalent to 1 g dry products and contained mannose ($C_6H_{12}O_6$) 0.13–0.38 g. HPLC analysis revealed that FDG contained four flavonoid compounds:

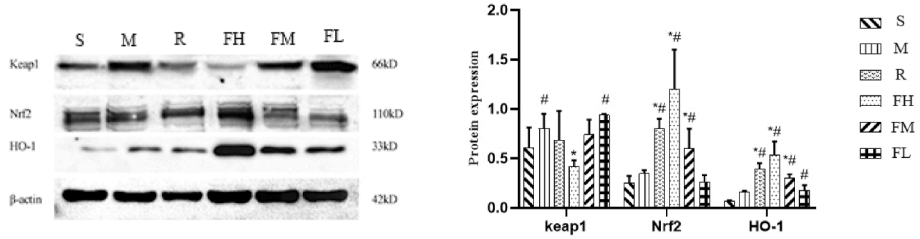

Fig. 13. The protein expression of Keap1, Nrf2 and HO-1 in the acetic acid induced gastric ulcer rats. vs sham operation group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; vs model group: $^{*}P < 0.05$, $^{**}P < 0.01$.

vicenin-2, isoschaftoside, schaftoside, and 6-C-xylosyl-8-C-glucosylapigenin. Flavonoids have been found in multiple studies to be associated with antioxidant and gastrointestinal mucosal protective effects.[22,23] In this study, FDG demonstrated a clear gastric protective effect, suggesting that the aforementioned components may serve as the potential molecular basis for its mechanism of action.

Gastric ulcer is a common and frequently occurring disease in clinics. Furthermore, it has the potential crisis of transforming into gastric cancer and has been classified as a precancerous lesion by the World Health Organization.[24] The intervention of ethanol, reserpine, indomethacin, and acetic acid in animals damages the gastric mucosa, and destroys the adipose protein and microcirculation at the tight junctions between gastric epithelial cells. The results of this study proved that FDG markedly inhibited the occurrence of various acute and chronic gastric ulcers. Based on preliminary experimental results, we employed network pharmacology to predict the multifaceted mechanisms of *D. officinale* in treating gastric ulcers. The identified pathways primarily included the MAPK signaling pathway, cAMP signaling pathway, and other pathways. *D. officinale* may primarily achieve therapeutic effects through mechanisms such as antioxidant stress and anti-inflammatory actions to improve gastric ulcer conditions. The Keap1-Nrf2-ARE pathway is one of the most important antioxidant stress response pathways in the body. Located downstream of the MAPK pathway, it is involved in various aspects such as inflammatory cancer transformation, cell apoptosis, inflammatory response, and mucosal repair, which is widely distributed in mammalian tissues.[25,26] Under normal conditions, Nrf2 remains at a low level under the regulation of its inhibitor protein Keap1 and the enzymatic hydrolysis of proteasome. When the body is attacked by oxidant factors, the conformation of Keap1 changes. Nrf2 can accumulate and enter the nucleus through decoupling with Keap1 and reducing the degradation of proteasome, and start the transcription of downstream antioxidant factors (such as HO-1, SOD, and CAT, GSH, etc.), then regulate the body's antioxidant capacity and reduce oxidative damage.[27] The loss of Nrf2 function leads to the activation of MAPK signal and the increase of inflammation. The abnormal signaling transduction of Nrf2 and MAPK promotes the occurrence and development of diseases by regulating oxidative stress and inflammation.[28]

The results of this study also suggested that Keap1-Nrf2 signaling pathway played an important role in the pathogenesis of gastric ulcer, and FDG significantly increased the levels of Nrf2 and HO-1, SOD, CAT, and down-regulated the expression of Keap1, indicating that the pharmacodynamic mechanism of FDG in treating gastric ulcer may be related to its antioxidation activity and the activation of Keap1-Nrf2 signaling pathway. Studies have shown that HO-1 plays an important role in maintaining the integrity of the gastric mucosa.[29] The expression of HO-1 in gastric ulcer rats induced by cold restraint stress and acetic acid both increased after modeling, further increasing the expression of HO-1 can promote the self-repair ability of the body to the injury. SOD is an important enzyme in organisms that scavenges superoxide anion free radicals, and it is effective in making organisms resistant to the toxicity of oxygen free radicals. CAT is also one of the key enzymes in biological defense system, which can eliminate hydrogen peroxide in the body and protect cells from the toxicity of hydrogen peroxide.

In addition to being an important antioxidant pathway, Keap1-Nrf2-ARE is also closely related to inflammatory pathways. Since FDG has good protection against acute and chronic gastric mucosal damage, its intervention on keap1-Nrf2-ARE pathway deserves further investigation. First, although FDG's regulatory effect on the Keap1-Nrf2 pathway has been detected, the direct binding relationship between Keap1 and Nrf2, as well as the cytoplasmic-nuclear translocation process of Nrf2, have not been deeply analyzed through targeted experiments. Secondly, although the 50 core targets (such as AKT1) predicted by network pharmacology are enriched in oxidative stress-related pathways, there is a lack of *in vitro* experiments to further confirm their direct association with FDG's pharmacodynamic effects, and the 4 flavonoid components detected by HPLC, their direct roles and synergistic effects have not been confirmed through monomer intervention experiments. Subsequent studies will be carried out in a targeted manner to provide more sufficient support for FDG's clinical application.

Fresh products still have great potential and advantages to be discovered in the clinical application and research, especially *D. officinale*, in the prevention and treatment of gastric ulcer. There is a growing recognition that severe gastroduodenal diseases such as gastric ulcer and gastric cancer are not just the outcomes of *H. pylori* infection in the stomach. Rather, both diseases develop and progress due to the perfect storms created by a combination of multiple factors such as the expression of different *H. pylori* virulence proteins, consequent human immune responses, and dysbiosis in gastrointestinal microbiomes.[30] Therefore, fresh *D. officinale,* which has various biological activities including immune regulatory effects, may be an effective treatment for gastric ulcer.

5. Conclusion

Fresh Chinese herbal medicine has a unique curative effect in clinics and in the folk. This study suggested fresh *D. officinale* had great potential and advantages in the treatment of gastric ulcers. By combining modern pharmaceutical technology with molecular biology, the gastroprotective efficacy of FDG was proved, its mechanism may be related to the antioxidation activity and the regulation of Keap1-Nrf2 signaling pathway, which may bring advantages and convenience to the use of fresh herbal medicine in the future.

CRediT authorship contribution statement

Shuidi Zhang: Writing – review & editing, Investigation, Data curation. **Bei Gui:** Writing – original draft, Methodology, Formal analysis, Conceptualization. **Zijun Wu:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Yuanjun Wei:** Writing – review & editing. **Hong Deng:** Resources, Conceptualization. **Kunping Li:** Validation. **Caie Guo:** Resources. **Yanfen Chen:** Writing – review & editing, Validation, Project administration, Methodology, Conceptualization.

Ethical approval

This research was approved by the Animal Feeding and Use Ethics Committee of Guangdong Pharmaceutical University (No. gdpulacspf2017271).

Availability of data and material

The data used in this study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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