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Anti-inflammatory effects of Weiyan I decoction against gastric ulcers in a rat model via inhibition of p38 mitogen-activated protein kinases signaling

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Abstract: Gastric ulcer (GU) is a common gastrointestinal disease that can lead to complications such as bleeding, perforation and even cancer. Weiyan I Decoction (WYI) is an effective Chinese medicine prescription against GU. This study aimed to explore the therapeutic mechanism of WYI in GU. WYI constituents were analyzed via ultra-high-performance liquid chromatography-mass spectrometry. SD rats were divided into control, model, lansoprazole (30mg/kg), SB203580 (2mg/kg), WYI (10.8g/kg, 5.4g/kg and 2.7g/kg) groups. GU was induced using ethanol or indomethacin post-WYI pre-administration. Ulcer area, histopathology, serum prostaglandin E2 (PGE2), nitric oxide (NO), gastric tissue cytokines and mitogen-activated protein kinases (MAPKs) were evaluated. Gastric mucus content and pH were determined in the pylorus ligation rat model. Administration of WYI reduced ulcer areas and inflammatory infiltration, elevated serum PGE2 and reduced NO. It decreased gastric tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 levels and inhibited p38 and JNK phosphorylation. The p38 MAPK inhibitor SB203580 significantly reduced the ulcer area, gastric cytokines (TNF-α, IL-1β and IL-6), serum NO and elevating serum PGE2. WYI had no significant impact on gastric acid and mucus secretion. WYI demonstrated gastroprotective effects in GU through anti-inflammatory actions and p38 MAPK pathway inhibition, providing insights for innovative GU therapies.

Keywords: Gastric ulcers. mitogen-activated protein kinases. SB203580, anhydrous ethanol. Weiyan I decoction.

INTRODUCTION

Gastric ulcers (GU) remain to be regarded as among the most prevalent gastrointestinal conditions. It is reported that the lifetime prevalence of peptic ulcer disease in the general population is anywhere between 5% and 10%, with an incidence of somewhere between 0.1% and 0.3% per year (Lanas and Chan, 2017; Zhang *et al.*, 2023). Despite the fact that its treatment has been more effective because of the subsequent implementation of proton pump inhibitors (PPIs) and the elimination of Helicobacter pylori (*H. pylori*), the disease is still relatively common (Khémiri and Bitri, 2019; Zhang *et al.*, 2023). There is a significant mortality rate as a consequence of complications like upper-gastrointestinal hemorrhage, perforation, intestinal obstruction and carcinoma (Chen *et al.*, 2015; Dunlap and Patterson, 2019; Sung *et al.*, 2009). Common etiological factors of gastric ulcers include *H. pylori* infection (Malfertheiner *et al.*, 2023), NSAIDs use (Sverdén *et al.*, 2019), as well as alcohol consumption (Zhou *et al.*, 2021). Disruption of the dynamic balance between gastric protective factors (gastric mucus and bicarbonate) and damaging factors (gastric acid) is an important cause of gastric ulcers (Khémiri and Bitri, 2019).

Currently, the drugs treatment for gastric ulcers includes acid suppressants, gastric mucosal protectors and antibiotics for eradication of *H. pylori* infection (Dunlap and Patterson, 2019). However, studies have shown that acid-suppressant PPIs and H_2-receptor antagonists can cause tolerance to build up rapidly in the stomach mucosa during treatment. This can result in gastric acid rebound after the drug is discontinued and increasing the risk of ulcer recurrence (Kangwan *et al.*, 2014). On top of that, the long-term use of PPIs has scientifically corresponded to an elevated susceptibility to gastric cancer (Cheung *et al.*, 2018; Seo *et al.*, 2021; Tran-Duy *et al.*, 2016). Therefore, the development of safer and more potent medications for the treatment of GU is urgently needed.

Traditional Chinese medicine (TCM) has long been utilized for GU treatment and it has been found to enhance gastric mucosal protective factors, strengthen immunity, regulate digestive disorders, enhance local microcirculation, safeguard mucosal barrier, have anti-inflammatory and antibacterial properties. TCM is considered safe and has few adverse effects during the treatment of gastric ulcers. Therefore, it could be a promising alternative to conventional drug therapy (Yao, 2015).

The Weiyan I Decoction (WYI) is composed of *Astragali Radix*, *Codonopsis Radix*, *Poria*, *Sepiae Endoconcha*,

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Sparganii Rhizoma and *Taraxaci Herba*. Although it is an empiric prescription of GU treatment for over two decades, the exact mechanism of its efficacy is not fully understood. The mitogen-activated protein kinases, often known as MAPKs, are a crucial component in the regulation of gastrointestinal diseases, inflammatory response in gastric tissues (Ko *et al.*, 2020; Shahin *et al.*, 2018), apoptosis (Chakraborty *et al.*, 2019; Harada *et al.*, 2015) and wound healing (Xia *et al.*, 2019). MAPKs consist of extracellular signal-regulated protein kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 MAPKs, which has essential function in cellular signaling (Zhang and Liu, 2002). Some of the components of WYI have been found to interfere with the MAPK signaling pathways (Chen *et al.*, 2021; Du *et al.*, 2022; Li *et al.*, 2017; Zhang *et al.*, 2021). As a result, we presumed that WYI's influence may be mediated over modulating the MAPK pathway.

MATERIALS AND METHODS

Animals

The Guangdong Medical Laboratory Animal Centre (SYXK (YUE) 2018-0085; Foshan, China) provided the male Sprague-Dawley (SD) rats that were used in the study. The rats ranged in weight from 200-250g. The animals were accommodated in a controlled environment animal facility, where the temperature was consistently maintained at 24±2°C. The humidity levels were kept within the range of 40-60% and a photoperiod cycle of 12 hours of light followed by 12 hours of darkness was implemented. All operations involving experimental animals were carried out in compliance with the ethical criteria established by Guangzhou University of Chinese Medicine and with approval number 20200826004. The committee makes sure that all experimental methods conform to ethical requirements.

Extraction and quality control of WYI

Extraction of WYI and lyophilized powder production

To prepare WYI, a mixture of *Astragali Radix*, *Codonopsis Radix*, *Poria*, *Sepiae Endoconcha*, *Sparganii Rhizoma* and *Taraxaci Herba* (Kangmei Pharmaceutical, Guangdong, China) was used in the ratio 2:1:1:2:1:1, respectively. The combination was immersed in distilled water for a duration of 30 minutes, subjected to boiling for a period of 30 minutes and afterwards the resulting filtrate was collected. The filtrate was then subjected to a second round of boiling with distilled water (5 times the volume of filtrate) for 30 minutes and the second filtrate was collected. The obtained filtrate was further concentrated using a rotary evaporator from Xiande company (Shanghai, China). A concentration of 1g/mL of crude drug solution was obtained and subjected to drying to obtain the dried powder with a yield of 5.3%. Quality control tests were conducted on the main components of WYI to ensure consistency and purity.

Chromatographic conditions

To analyze the five important components of WYI (the Chinese Pharmacopoeia 2020 version), namely astragaloside IV, calycosin-7-glucoside, syringin, lobetyolin and atractylenolide I, high-performance liquid chromatography-mass spectrometry (HPLC-MS) was used. An Acquity^{TM} UPLC system was employed for the separation step of chromatography. (Waters, Framingham, USA). A Waters ethylene-bridged hybrid (BEH) C18 column (250 × 4.6 mm^2, 1.7μm) was used. In the mobile phase, A: acetonitrile, B: 0.1% formic acid water, the program included the following: 0-5.5 min, 5-35% A; 5.5-9 min, 35% A; 9-12.5 min, 35-85% A; The temperature was 50°C, the volumetric flow was 0.4mL/min, whereas the injection volume was 3μL.

Mass spectrometric conditions

Mass spectrometric analysis was performed using a Waters Xevo G2-XS high-resolution time-of-flight (TOF) mass spectrometer. In the scanning mode, the capillary voltage was 2.5 kV (-2.5 kV), the cone-hole voltage was 30 V (-30 V), the ion source temperature was 120°C, the solvent gas (N_2) temperature was 550°C, the solvent gas (N_2) flow rate was 900 l/h, the cone hole gas (N_2) flow rate was 50 L/h and ranged in 100-1200 m/z. TOF-MS was used to collect the data and leucine enkephalin was used as an internal standard for real-time quality correction. The ions used for quantitative analysis were astragaloside A ($C_{41}H_{68}O14$; ESI: -829.4586; chemical abstracts service [CAS] registry number: 84687-43-4), calycosin-7-glucoside ($C_{22}H_{22}O_{10}$; ESI: -491.1191; CAS: 20633-67-4), atractylenolide I ($C_{15}H_{18}O_2$; ESI: +231.1385; CAS: 73069-13-3), syringin ($C_{17}H_{24}O_9$; ESI: -417.1397; CAS: 118-34-3) and lobetyolin ($C_{20}H_{28}O_8$; ESI: -441.1761; CAS: 136085-37-5); all the compounds were purchased from Chengdu Manster(Sichuan, China).

Provision of control solutions

Control solutions for astragaloside A, calycosin-7-glucoside, atractylenolide I, syringin and lobetyolin were prepared as follows: First, the compounds were weighed and then mixed with HPLC grade methanol from Merck (Darmstadt, Germany) to obtain control solutions of concentrations 1.15, 1.00, 1.11, 2.22 and 0.375mg/mL, respectively. These control solutions were stored until use. Working control solutions of concentrations 11.5, 10.0, 11.1, 10.0 and 3.75μg/mL, respectively, were then prepared by diluting the control solutions with HPLC grade methanol.

Preparation of test solution

To prepare the test solution, the lyophilized WYI powder was weighed (0.3g) and mixed with methanol (50mL) in a 125mL conical flask. The flask was then sealed and sonicated for 30 minutes before being cooled to normal temperature. Test solution was weight measured before and after sonication to calculate the difference in weight. Methanol was added to the solution to compensate for the

observed weight difference. A 0.22μm filter was used to filter the mixture and 1mL of the filtrate was mixed with 5mL of methanol to make the test solution.

System adaptability test

The ion flow diagrams of the control and test solutions were analyzed and presented in fig. 1. The chromatograms showed that astragaloside A, calycosin-7-glucoside, atractylenolide I, syringin and lobetyolin were well separated and the resolution between them and the adjacent peaks was greater than 1.5, meeting the quantitative criterion (the detection limit of the method was less than 5ng/mL).

Linear relationship test

The working control solutions were diluted to a series of concentrations (0 or 50 times the original concentrations) using HPLC grade methanol. The solutions were tested using the chromatographic and mass spectrometric conditions mentioned above and the peak areas were recorded. Linear regression was performed using peak area of the vertical ordinate (y) and concentration as horizontal coordinate (x,ng/mL). The linear regression equations used for the calculations were as follows: astragaloside A: y=0.71x + 140.02; calycosin-7-glucoside: y=0.73x - 30.76; syringin: y=0.47x - 23.13; lobetyolin: y=0.43x - 87.78; atractylenolide I: y=3.51x + 112.17.

Groups and drug administration

Male SD rats were distributed at random to different groups, including the control, (n=6), model (n=6), lansoprazole (30mg/kg, n=6), H-WYI (10.8g/kg, n=8), M-WYI (5.4g/kg, n=8) and L-WYI (2.7g/kg, n=8) groups, for the ethanol-induced GU experiment. In the indomethacin-induced gastric ulcer experiment, each group consisted of eight animals, while six animals were used for each group in the experiment of pylorus ligation. Equivalent dosage of WYI for rats was 5.4g/kg body weight (calculated based on the weight of the raw TCM decoction pieces, not the freeze-dried powder weight); The high and low doses of WYI used were 10.8g/kg and 2.7g/kg, respectively. In experimental groups, rats were pre-administered lansoprazole (Renhe Pharmaceutical, Guangdong, China) or WYI for 3 days. One hour after the last dose on the third day, equal amounts of pure water gavage and anhydrous ethanol (Tianjin Zhiyuan Chemical Reagent Factory, Tianjin, China) or indomethacin (Meilunbio, Dalian, China) were administered orally to the control and model groups, respectively, to induce ulcers. In experiments evaluating the activity of inhibitors, SD rats were distributed to control, model, SB203580 groups, n=6. Rats were injected with SB203580 (2mg/kg, dissolved in 4% dimethyl sulfoxide [DMSO] in purified water; Meilunbio) intraperitoneally in the SB203580 group, as described (Lv *et al.*, 2019; Mai *et al.*, 2022). After 1 hour of administration of SB203580, 1mL of anhydrous ethanol was administered orally to research p38 MAPK's potential connection to gastric ulcers.

Anhydrous ethanol- and indomethacin- induced gastric ulcers

The rats aside from control were given either 1mL anhydrous ethanol for 1 hour or indomethacin (100mg/kg) for 6 hours by oral gavage, according to a previously published procedure, in the anhydrous ethanol- and indomethacin-induced gastric ulcer investigations. (Mai *et al.*, 2022). An R550 Multi-output Anesthesia Machine was utilized to provide isoflurane to the rats as anesthesia. (RWD, USA). Serum was obtained by collecting blood using the abdominal aortic blood collection method and centrifuging at 3000 rpm for 10 minutes. The rats were then euthanized by an isoflurane overdose (≥5% for >1 min). The ulcer region was examined using ImageJ software (https://imagej.nih.gov/ij/) after the stomach tissue was separated, photographed and recorded after being cleaned with PBS. The gastric tissues were either stored in liquid nitrogen for testing or treated using 4% paraformaldehyde for paraffin embedding.

The calculation of the pH and volume of gastric mucus

After administration of the final dose of WYI and ethanol, the rats were anesthetized with isoflurane using the R550 Multi-output Anesthesia Machine, sterilized and then the mid-abdomens were incised. The pylorus was found and ligated using a 4-0 prolene suture for 4 h. Finally, the rats were euthanized. The quantity of mucus present was ascertained by rinsing the stomach with a sucrose solution, followed by an Alcian blue solution soak. Then, stomach tissue was immersed in a 0.5M solution of magnesium chloride while being vortexed for two hours. Then, diethyl ether (4mL) was added to the soaking solution (4mL). Finally, absorbance was measured and (A)/weight was calculated to obtain the volume of gastric mucus. In addition, the gastric fluid was collected to measure the pH values using pH agents.

Histopathology

The stomach samples were treated in paraformaldehyde overnight, then dehydrated using low to high concentrations of ethanol before being paraffin-embedded. Subsequently, the paraffin blocks were segmented by an RM2016 slicer into 4-μm slices. The sections were then dewaxed using TO type bioproduction transparency agent (Guangzhou Kejie Biotech Co., Ltd, Guangdong, China) and de-TO, taking high to low concentrations of ethanol and stained with hematoxylin-eosin (HE).

Prostaglandin E2 (PGE2), nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 assay

The gastric tissues were homogenized using an automated tissue lyser. The homogenates were collected and then centrifuged to obtain the total protein. Levels of PGE2

(Tianjin Anorikang Biotechnology Co., Ltd, China) in serum and TNF-α, IL-1β and IL-6 (4A Biotech Co, Ltd, Beijing, China) in gastric tissues were examined by ELISA kit. Standards, serum and tissue lysate samples were added (100μL per well) to a TNF-α, IL-1β and IL-6 or PGE2 (all 1:100 dilution) pre-coated plate and the corresponding biotinylated antibodies, enzyme-conjugated antibodies and tetramethylbenzidine solution were added. Subsequently, a volume of 100μL of termination solution was added to halt the progression of the reaction and the optical density value was measured. Nitric Oxide assay kit (Beyotime) was used to quantify serum NO levels in accordance with the instructions.

Western blotting

With a RIPA solution containing a protease inhibitor, tissues were then ground and ultrasonically lysed. By utilizing the BCA kit, the concentration was ascertained. SurePAGE (Genscript Biotechnology Co., Ltd., Piscataway, NJ, USA) was used for electrophoresis of protein. The proteins were subsequently translocated to PVDF membranes using a rapid wet transfer instrument (Genscript). The membrane was incubated for 2 hours at room temperature with 5% skim milk before being treated overnight at 4°C with primary antibodies: Phospho-p38 MAPK (1:500, #4511S), p38 MAPK (1:1000, #9212S), Phospho-p44/p42 (1:500, #4370S), p44/p42 (1:1000, #4695S), Phospho-JNK (1:500, #4671S), JNK (1:1000, #9251S) and β-actin (1:5000, #58169S). All the antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). After the secondary antibodies (1:5000) incubation the bands were identified by chemiluminescence and ImageJ was used to analyze the grayscale values.

STATISTICAL ANALYSIS

The data were analyzed utilizing the statistical software SPSS 19.0 (SPSS Inc., USA) and subsequently presented as mean±standard deviation. Differences between two groups were assessed using Student's *t-test*. The study employed a one-way *ANOVA* to assess the differences among several groups, followed by either the *LSD* or *Dunnett's* test for multiple comparisons. A significance level of $P<0.05$ was considered statistically significant.

RESULTS

Determination components of WYI

In fig. 1, the concentrations of astragaloside A, calycosin-7-glucoside, syringin, lobetyolin and attractylenolide I were 1.34, 0.59, 0.53, 0.59 and 0.44mg/g, respectively. The ion flow diagrams of both the control and test solutions are presented. The baseline separation of astragaloside A, calycosin-7-glucoside, atractylenolide I, syringin and lobetyolin was achieved and the separation between them and the adjacent peaks was no less than 1.5, which satisfied the quantitative analysis criterion (the detection limit of the method was less than 5ng/mL).

Protective impact of WYI on ulcer area of anhydrous ethanol- and indomethacin-induced GU rats

Fig. 2 and 3 demonstrate that, the ulceration of anhydrous ethanol and indomethacin rats are greater that control ($P<0.01$). Three days of lansoprazole medication avoided stomach ulcers and decreased ulcer area brought on by anhydrous ethanol ($P<0.05$) and indomethacin ($P<0.05$). Interestingly, H-WYI administration for three days also reduced the ulcer area induced by ethanol ($P<0.05$) and indomethacin ($P<0.05$), indicating that WYI has a preventive impact on rat gastric ulcers.

WYI administration alleviates the extent of pathological damage to gastric mucosa in ulcerated rats

The HE staining results indicated that the ulcerated rats induced by anhydrous ethanol exhibited significant gastric mucosal epithelial necrosis, detachment and hemorrhage, as well as a slight degree of inflammatory cell infiltration. As shown in fig. 4, however, the administration of lansoprazole and H-WYI effectively reduced gastric mucosal damage, hemorrhage and necrotic shedding of epithelial cells caused by anhydrous ethanol and decreased the level of inflammatory infiltration.

Effect of WYI on gastric tissue cytokines and serum NO and PGE2 levels in ulcerated rats

Gastric mucosal epithelial cells are vulnerable to harm from gastric acid or external agents, including alcohol, which can induce the aggregation of various immune cells to produce inflammatory cytokines, including TNF-α, IL-1β, IL-6 and NO. The quantification of cytokines in the stomach tissues was carried out using ELISA.

As per fig. 5, anhydrous ethanol ulcer rats had significantly higher levels of IL-1β ($P<0.05$), IL-6 ($P<0.01$) and TNF-α ($P<0.01$) in gastric tissue compared to the control group. WYI administration at all three doses contribute to a reduction in IL-6 in gastric tissues ($P<0.01$), while H-WYI administration reduced IL-1β levels ($P<0.05$). Furthermore, in the gastric tissues, TNF-α decreased after WYI (H- and L-, $P<0.01$) administration, as shown in fig. 5 (A-C).

As a protective factor for the stomach mucosa, the levels of serum PGE2 in the model group were shown to be considerably lower ($P<0.05$) than levels perceived in the control group. fig. 5D demonstrated that the increase in serum PGE2 levels after the pre-administration of all three dosages of WYI was statistically significant ($P<0.01$). Additionally, fig. 5E illustrated that the model group's serum NO levels were considerably greater than those of the control group's ($P<0.01$), the treatments of WYI (H-, $P<0.01$; L-, $P<0.05$) could also decreased the serum NO levels. These results suggest that WYI can alleviate the inflammation and NO release in ulcerated rats.

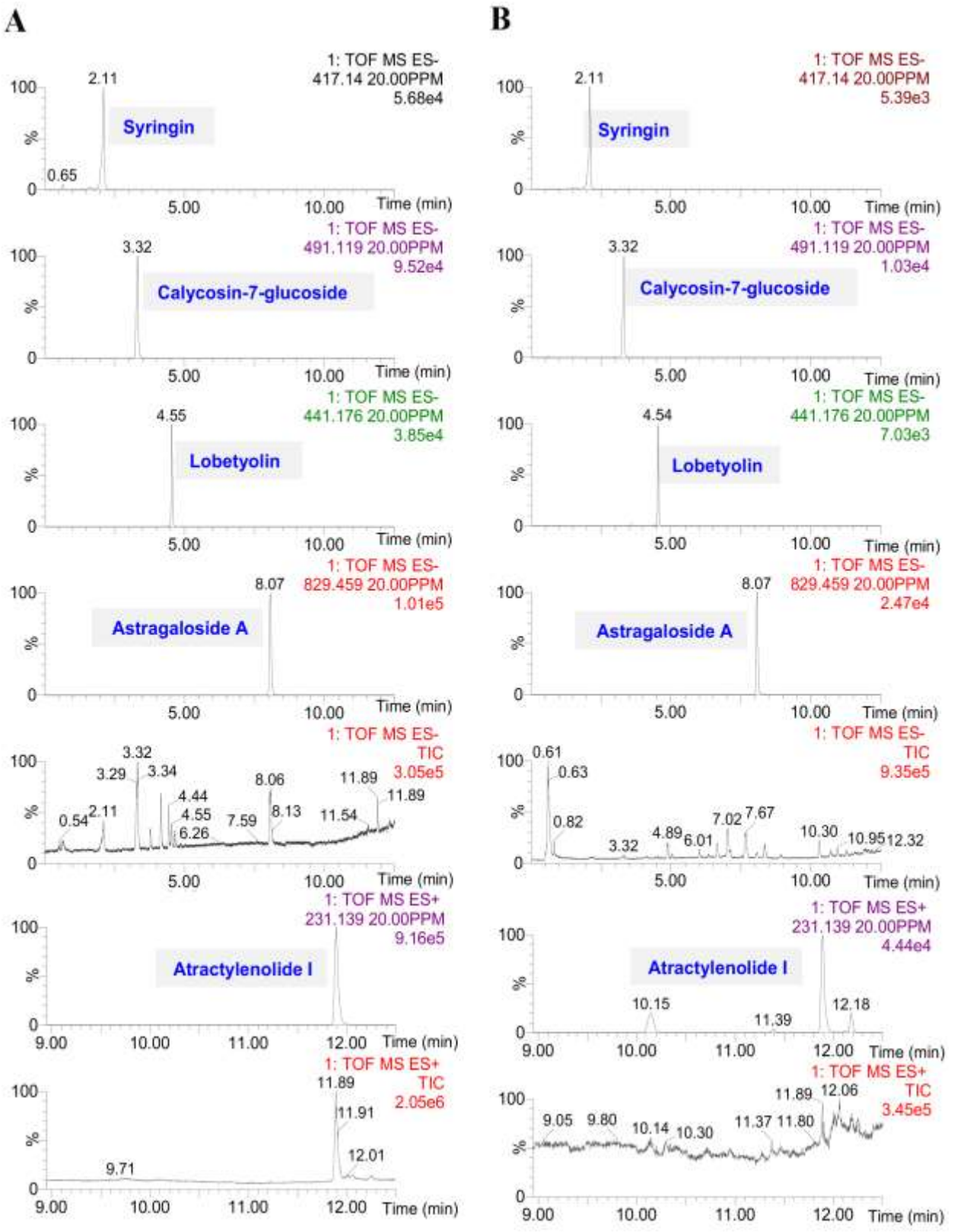

Fig. 1: Ion flow diagram of the 5 components present in the reference and test samples. (A) Reference substance, (B) Test substance.

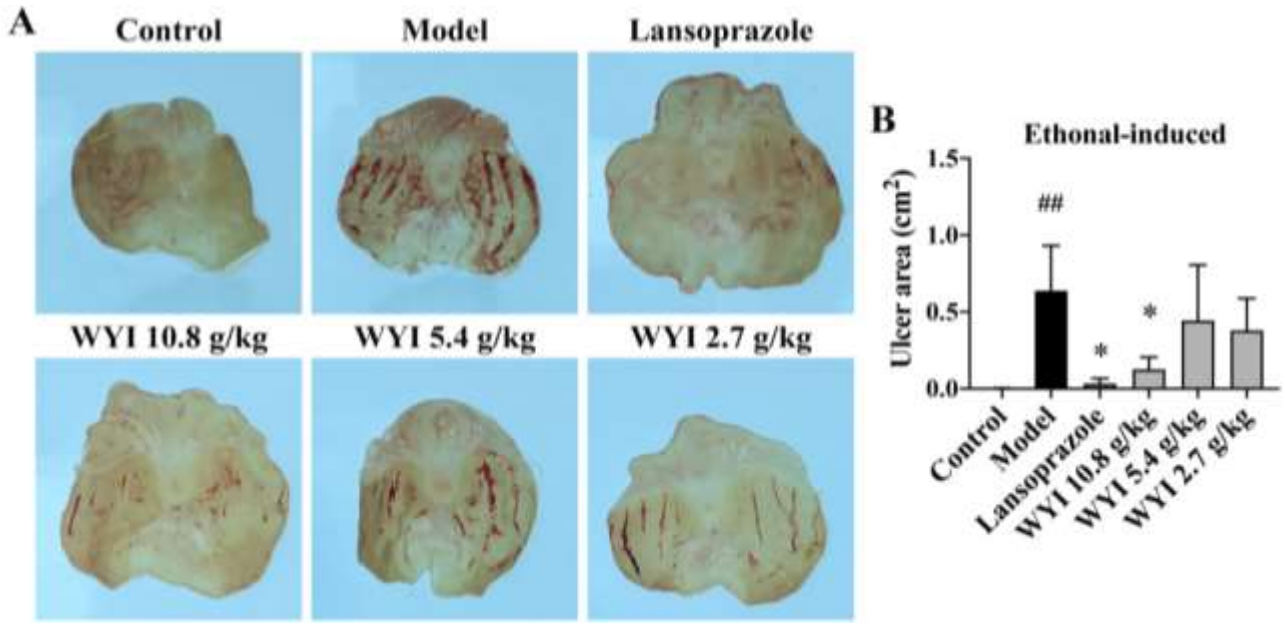

Fig. 2: Effect of WYI on ulcer area of ethanol-induced GU rats. (A) Representative views of rat stomach. (B) Statistical analysis of ulcer area. ## P<0.01, vs control; *P<0.05, vs model, n=6

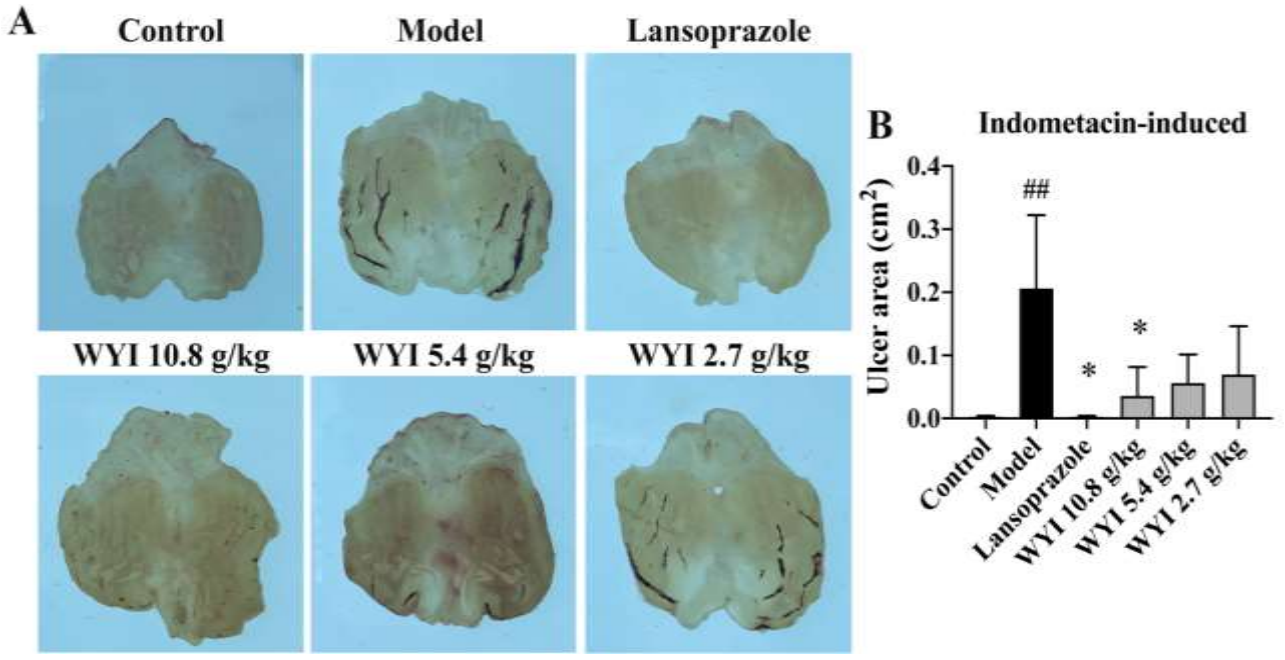

Fig. 3: Effect of WYI on indomethacin-induced gastric ulcer area in rats. (A) Representative images of rat stomach. (B) Statistical analysis of ulcer area. ## P<0.01, vs control; *P<0.05, vs model, n=8

Protein expression analysis of p38, Phosphorylated p38, p44, Phosphorylated p44, JNK and Phosphorylated JNK in gastric tissues of rats

The results in fig. 6, after anhydrous ethanol-induced ulceration, the model group exhibited significantly elevated P-p38/p38, P-p44/p44, P-p42/p42 levels compared to control group (P<0.05). However, treatment with any dosage of WYI resulted in a reduction in overall p38 expression levels (although this reduction was not statistically significant) and P-p38 expression were remarkably reduced (P<0.001). Furthermore, total p42 expression levels were visibly reduced in gastric tissue of model compared to control and the total p42 levels of M-WYI were also found decrease significantly (P<0.05). Additionally, the P-JNK levels in L-WYI were downregulated considerably (P<0.05). According to these results, WYI might differentially reduce the protein level or phosphorylation level of MAPKs, particularly p38 MAPK, which might play a significant part in WYI-mediated treatment of GU.

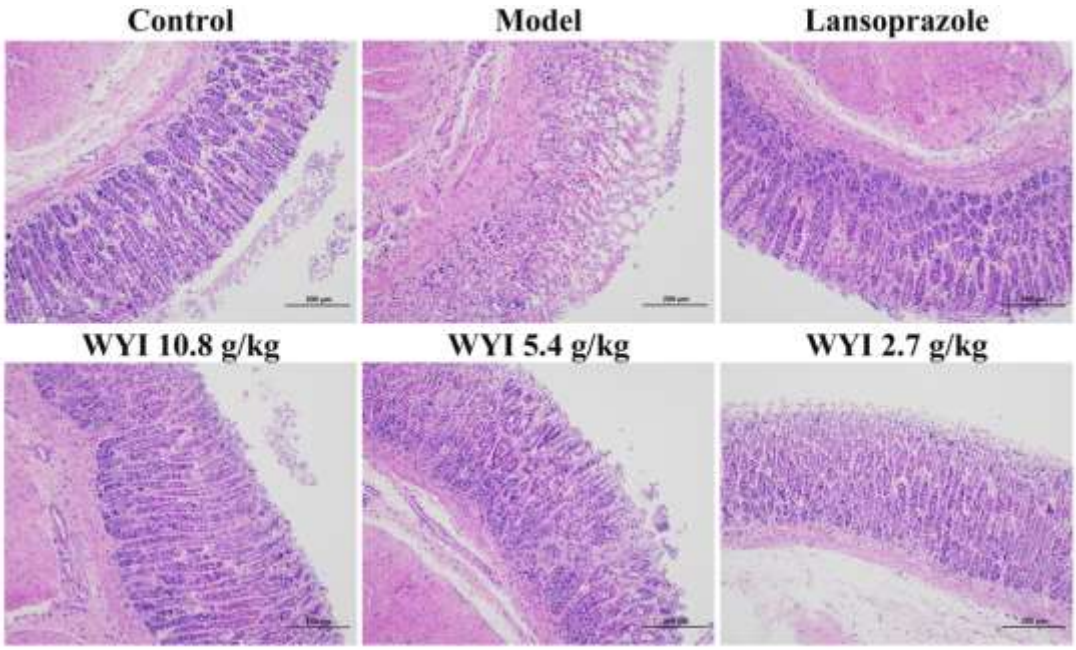

Fig. 4: Impact of Weiyan I Decoction (WYI) on ethanol-induced rats histopathology. The HE staining of Control, Model, Lansoprazole, H-WYI (10.8 g/kg), M-WYI (5.4 g/kg), and L-WYI (2.7 g/kg) groups, respectively, scale bar = 200μm, n=6.

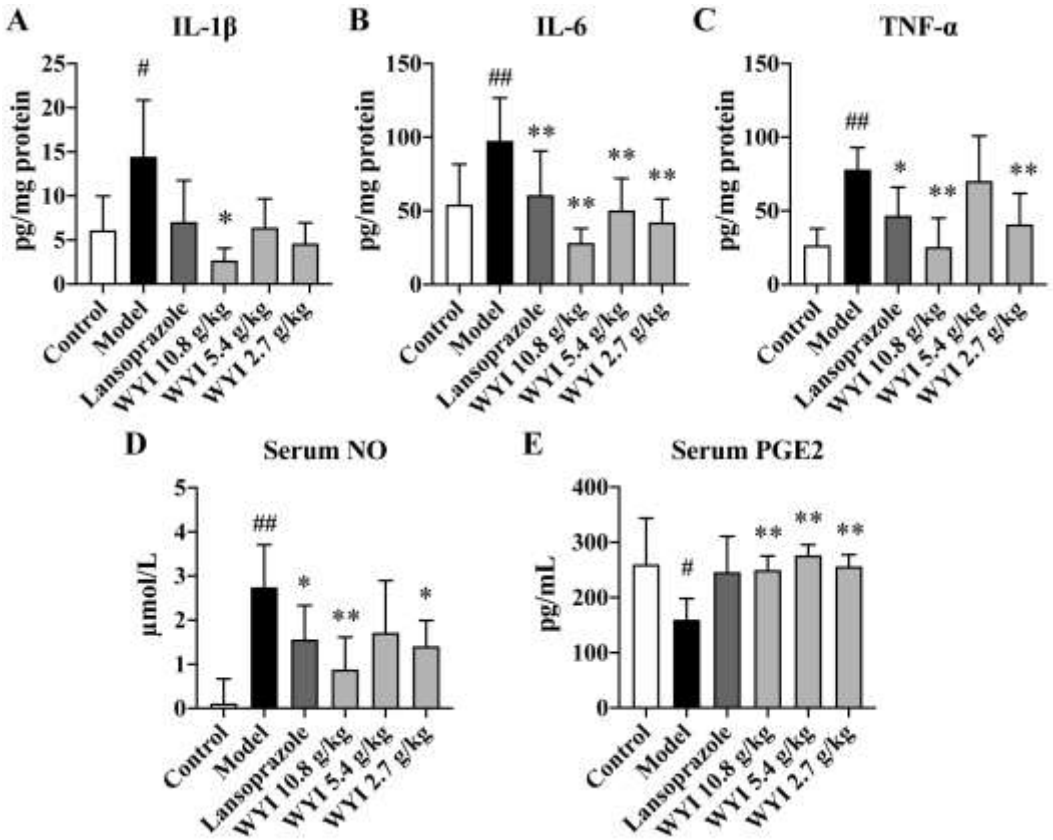

Fig. 5: Influence of WYI on Prostaglandin E2, Nitric Oxide, and gastric tissue cytokines in rats with ulceration. Level of IL-1β(A), IL-6(B), and TNF-α(C) in gastric tissue by ELISA. (D) Level of serum PGE2 by ELISA. (E) Level of serum NO by kits. #P<0.05 and ##P<0.01, vs control group. *P<0.05 and **P<0.01 vs model group, n=6.

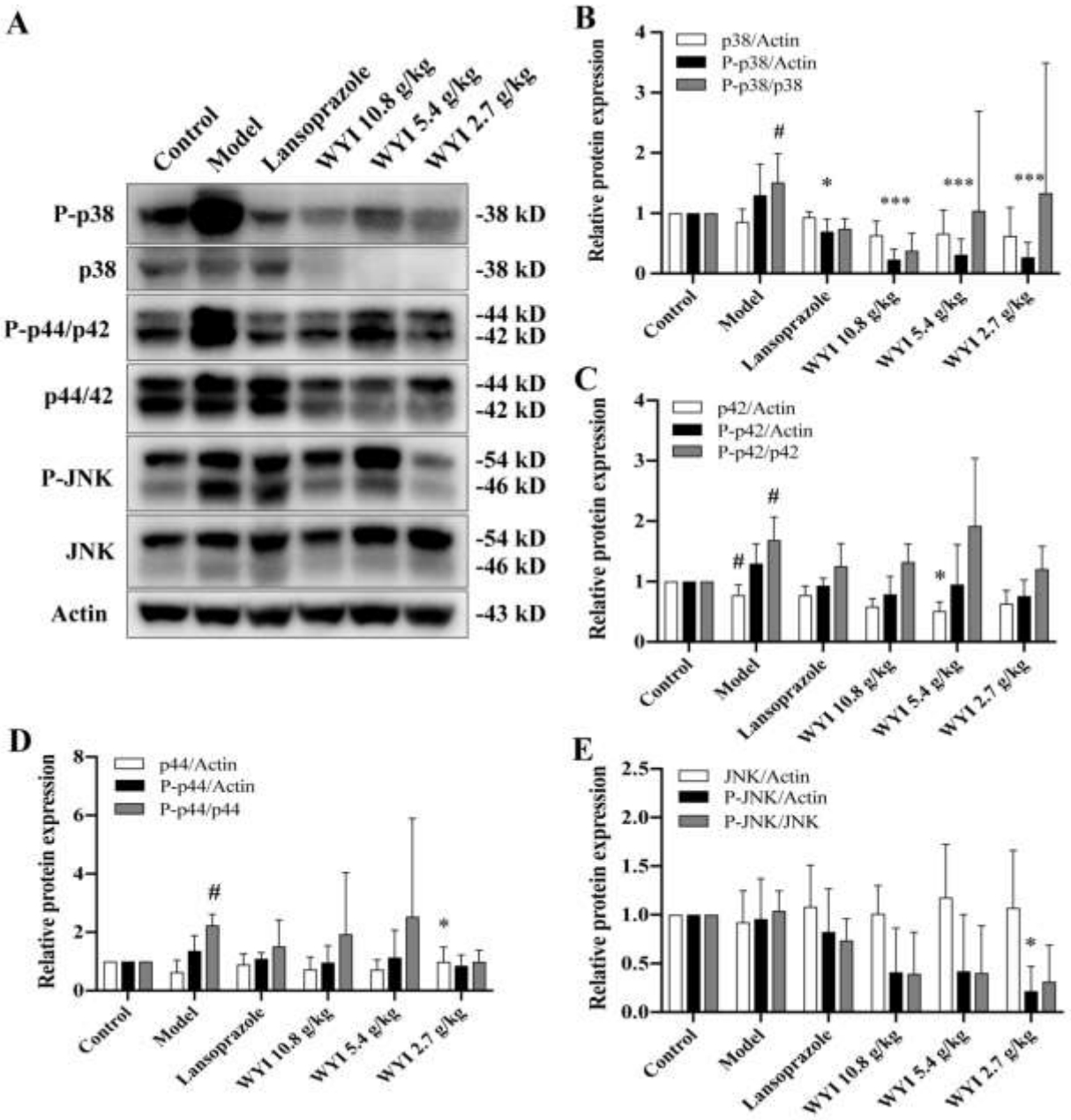

Fig. 6: Effects of WYI on expression and phosphorylation of the MAPKs pathway in ulcerated rats. (A) Western blot of MAPKs including P-p38/p38 , P-p44/42, p44/p42 , P-JNK/JNK, and β-Actin. (B-E) Statistical analysis of gray value of P-p38/p38, P-p42/p42, P-p44/p44, and P-JNK/ JNK, respectively. #P<0.05 and ##P<0.01, vs control group. *P<0.05 and **P<0.01 vs model group, n = 4.

Effect of p38 MAPK inhibition on ulcerated rats

Before ethanol modeling, SB203580 (2mg/kg) was intraperitoneally given to suppress the activation of p38 in the stomach mucosa to investigate the significance of p38 MAPK in forming gastric ulcers. As in fig. 7 (I and J), SB203580 remarkably reduced the p-p38/p38 in the SB203580 group than model group (P<0.05), showing that SB203580 has a constricting effect on the activation of p38 in ulcerated rats. Compared to model rats, the ulcer area was decreased in the SB203580 and the pathological appearance of the ulcer had improved (fig. 7C).

Furthermore, SB203580 resulted in a significant reduction in stomach TNF-α (P<0.01), IL-1β (P<0.01) and IL-6 (P<0.05) levels, as well as serum NO levels (P<0.01). Conversely, there was a notable increase in serum PGE2 levels (P<0.01) (fig. 7 [D-H]). This evidence indicates that p38 activation plays a significant part in GU development and that suppression of p38 phosphorylation may be an attainable therapeutic strategy for treating ulcers.

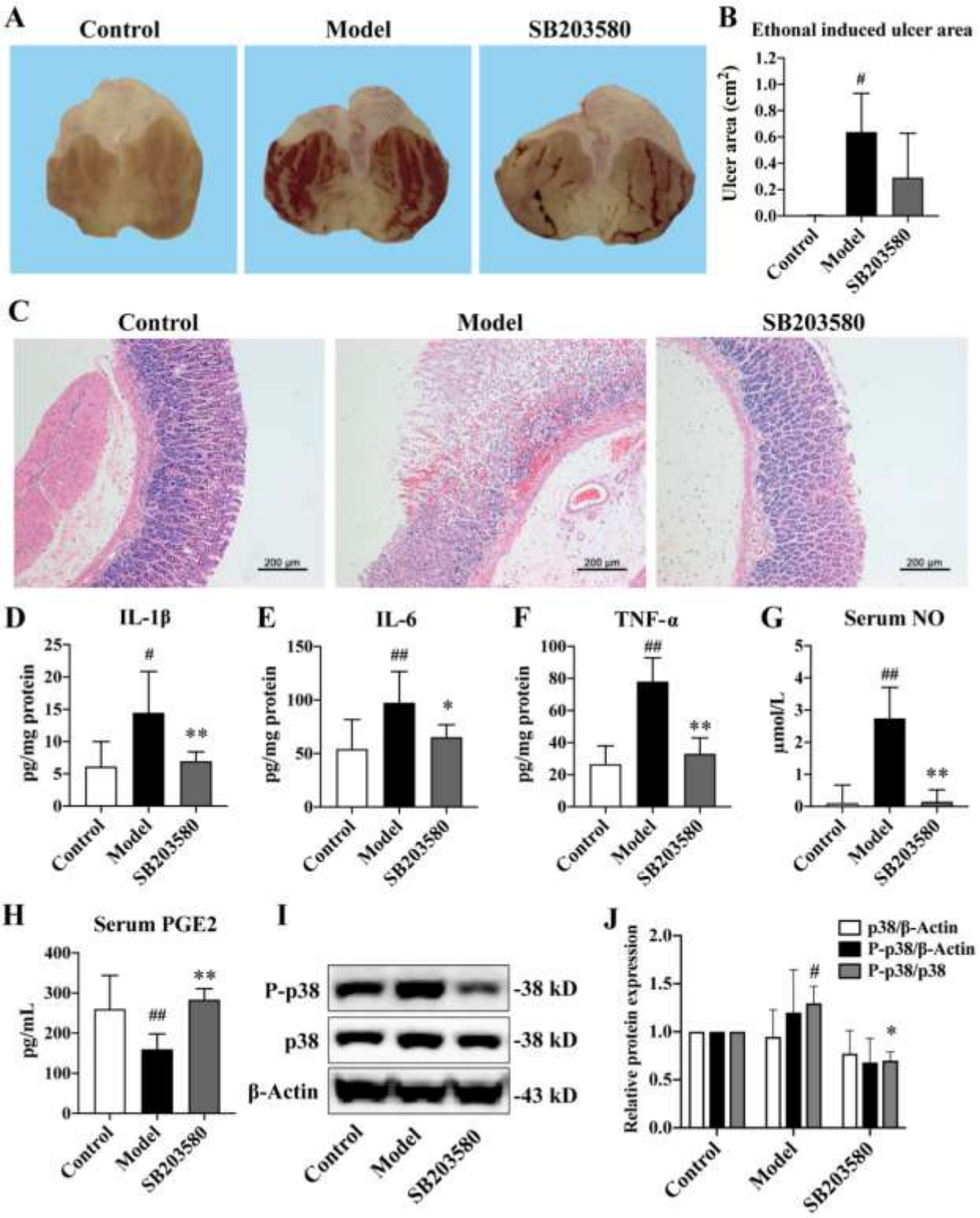

Fig. 7: Effect of p38 inhibitor on gastric ulcer in rats. (A) Rat's stomach representative images. (B) A statistical examination of the ulcer's area. (C) Representative photographs of the HE, scale bar = 200 μm, n=6. Level of IL-1β(D), IL-6(E), and TNF-α(F) in gastric tissue by ELISA, n =6. (G) Level of serum PGE2 by ELISA, n =6. (H) Level of serum NO by kits, n =6. (I) Inhibition of SB203580 on p38 MAPK in ethanol-induced GU rats. (J) Statistical analysis of gray value, n =4. # P<0.05 and ## P<0.01, vs control group. *P<0.05 and **P<0.01 vs model group.

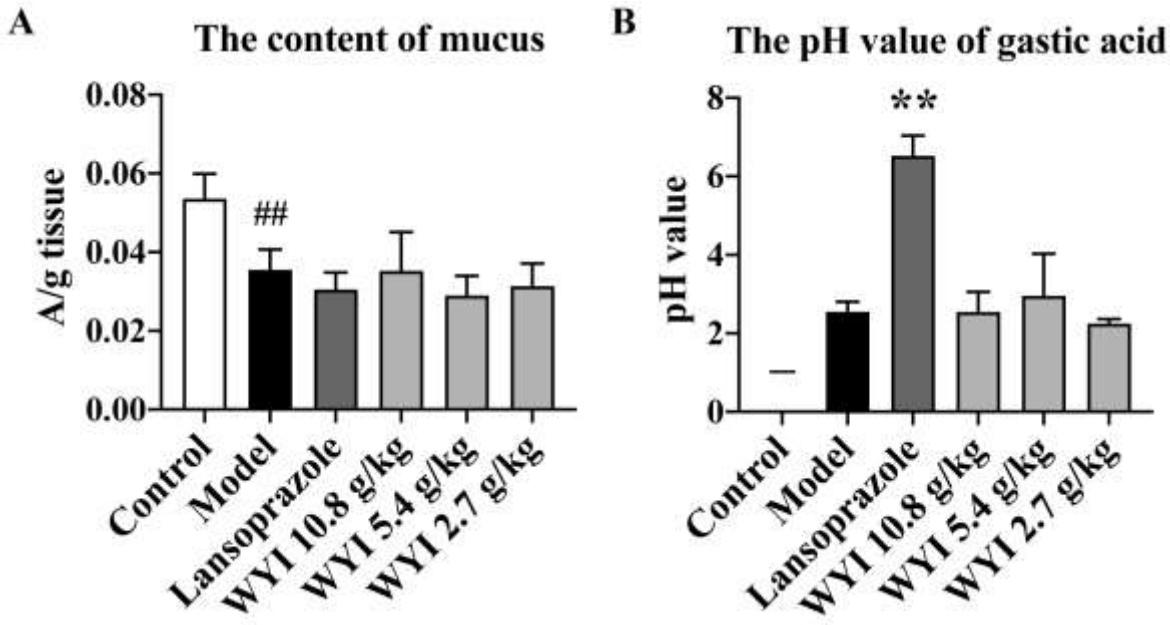

Fig. 8: Effects of WYI on gastric mucus and gastric acid pH in ulcerated rats. (A) Statistical analysis of the content of gastric mucus. (B) Statistical analysis of pH of gastric acid. ##P<0.01, vs control group; **P<0.01, vs model group, n=6.

Effect of WYI on gastric mucus and pH of gastric acid in rats

Pylorus ligation was conducted 1 hour after the last dose of WYI and ethanol models in order to determine the influence of WYI on the levels of mucous and gastric acid in the stomach. The model group rats exhibited a statistically significant reduction in the total quantity of mucus produced in their stomachs than control (P<0.01). Nevertheless, there was not a discernible impact of WYI on the total volume of stomach mucus. In a comparable way, WYI group did not have a significantly different impact on gastric acid and pH value. In contrast, as demonstrated in fig. 8, rats given lansoprazole had a pH level that was significantly more elevated than that of the control group (P<0.01).

DISCUSSION

Gastric ulcers are a prevalent digestive disease, characterized by the occurrence of disorders such as postprandial epigastric discomfort, hiccups, acid reflux, heartburn and other manifestations such as gastric perforation and bleeding. (Lv *et al.*, 2019; Shen *et al.*, 2017). Frequent occurrences of gastric ulcer episodes may result in severe consequences that pose a significant risk to an individual's life, including perforation, bleeding and the potential development of cancer. Current clinical treatments for gastric ulcers often have adverse side effects and high recurrence rates, hence necessitating the exploration of further alternative therapy modalities. In this work, we used various types of gastric ulcer models to investigate the anti-gastric ulcer activity of WYI, a traditional Chinese prescription drug. The findings of our study indicate that WYI had a statistically significant preventive effect against gastric ulcers resulting from ethanol and indomethacin in rats. The observed protective effect proved to be correlated with an improvement in the extent of the ulcer and an alleviation in the pathological characteristics of the ulcer.

The pathogenesis of gastric ulcers involves several factors, encompassing the impairment of the stomach mucosal barrier, inflammatory processes and the presence of oxidative stress (Hiruma-Lima *et al.*, 2009). The excessive consumption of ethanol and the utilization of NSAIDs are often identified as primary factors contributing to the development of gastric ulcers. Both ethanol intake and NSAID usage have the potential to induce harm to stomach mucosal barrier and alter the integrity of the gastric mucosal. NSAIDs inhibit cyclooxygenase activity and decrease PGE2 synthesis, reducing gastric mucus secretion and causing gastric ulcers (Tarnawski and Ahluwalia, 2021; Toda, 2019). The experimental model of gastric ulcers created by indomethacin has similarities to human actual peptic ulcers in pathological features, healing process and recurrence time. According to our findings, WYI significantly protected against gastric ulcers brought on by ethanol and indomethacin, suggesting that it would be an appealing therapeutic alternative for treating ulcers brought on by these factors.

The occurrence of gastric epithelium damage leads to the infiltration of inflammatory cells and the subsequent

generation of TNF-α, IL-1β and IL-6 (Augusto *et al.*, 2007). These cytokines are pivotal in sustaining the acute inflammatory process and modulating the severity of gastric ulcers (Luo *et al.*, 2018). TNF-α has pro-inflammatory properties and involved in the initial response to gastric mucosal injury. TNF-α can also cooperate with IL-1β to trigger an inflammatory cascade of reactions that leading to the synthesis of IL-6 and several other components associated with inflammation. IL-6 further activates macrophages, neutrophils and lymphocytes, implying that cytotoxic metabolites are produced and oxidative reactions are triggered, resulting in gastric injury (Luo *et al.*, 2018). Earlier studies have proven that IL-1β, IL-6 and TNF-α, elicit activation of neutrophils and are vital factors related to preserving and regulating gastric ulcers. Overall, our study demonstrates that the administration of WYI causes a considerable reduction in the amounts of these cytokines, suggesting that it exerts anti-inflammatory effects on ethanol-induced gastric ulcers. These findings support using WYI as a potential therapeutic option for treating gastric ulcers.

MAPKs regulate cellular responses to a wide spectrum of stimuli, including mitogens, osmotic stress, heat shock and pro-inflammatory cytokines (Kaminska, 2005). The MAPK family's major subgroups include p38, p44/p42 and JNKs. MAPKs become activated when they are phosphorylated by upstream kinases. Upon activation, MAPKs have the capability to phosphorylate transcription factors as well as downstream kinases, therefore exerting control over the pro-inflammatory cytokines (Chang *et al.*, 2015). Activated MAPKs can induce apoptosis, necrosis and ulcer formation (El-Din *et al.*, 2021). p44/p42, JNK and p38 MAPK are involved in gastric ulcerogenesis (Formiga *et al.*, 2021; Xie *et al.*, 2019; Zheng *et al.*, 2021). Ours results demonstrated that activation of p38, p44/P42 and JNK occurred after gastric impairment. These findings are in line with the results derived from several other studies (Fu *et al.*, 2021; Ma *et al.*, 2022; Zheng *et al.*, 2021). Pretreatment with WYI reduced the total p38 expression levels and significantly reduced the phosphorylation levels of p38. These results revealed that WYI interferes with the p38 MAPK pathway, which might subsequently reduce the release of pro-inflammatory cytokines.

p38 MAPKs are activated by various environmental stresses and inflammatory cytokines and their activation may cause apoptosis (Mantovani *et al.*, 2008). Furthermore, p38 MAPK engages in the process of regulating the activity and expression of inflammation-related mediators (Mantovani *et al.*, 2008). SB203580 is a selective inhibitor of p38 MAPK that competitively binds to its ATP pocket to inhibit its catalytic activity. Multiple investigations have verified its inhibitory impact across different disease models. (Sanit *et al.*, 2019; Xiao *et al.*, 2017). Indomethacin administration activates the MAPK pathway, while SB203580 administration improves the integrity of barrier function (Thakre-Nighot and Blikslager, 2016). In our study, SB203580 administration enhanced serum PGE2 levels in the WYI group. Moreover, WYI inhibited p38 MAPK phosphorylation, suggesting that the restorative effect of WYI on serum PGE2 levels might have been mediated through p38 MAPK. Research findings found that in gastric ulcers, activated MAPKs and phosphorylate nuclear factor-κB, hence regulating downstream factors such as TNF-α and IL-6 (Chang *et al.*, 2015). In our study, the inhibition of p38 by SB203580 effectively reduced the IL-1β, IL-6 and TNF-α and decreased the serum NO level, suggesting that WYI inhibited the local inflammatory and serum NO by inhibiting the p38 MAPK pathway.

Various studies have also shown that alcohol administration might result in NO overload in serum, leading to cytotoxic effects and free radical production (Amirshahrokhi and Khalili, 2015). In an acutely injured ulcer model, anhydrous ethanol stimulation led to elevated serum NO levels, activated inducible NOS (iNOS) and inhibited endothelial NOS (eNOS) expression (Bagyánszki *et al.*, 2011). In addition, after gastric mucosal injury, inflammatory cytokines activated iNOS to produce large amounts of NO, aggravating the gastric injury (Lucetti *et al.*, 2017; Wallace *et al.*, 2017). Besides, ethanol administration has been shown to reduce serum PGE2 levels. PGE2 supports the process of ulcer healing by stimulating the bicarbonate ions and mucus secretion (Amirshahrokhi and Khalili, 2017). In our study, serum PGE2 secretion was significantly decreased and NO levels were upregulated after ethanol administration. However, this effect was reversed by WYI pretreatment, indicating that WYI exhibits gastroprotective effects against ethanol-induced gastric ulceration.

Modern pharmacological studies have shown that several active components of WYI, such as astragaloside IV, calycosin-7-glucoside and atractylenolide III, show anti-inflammatory, antioxidant and ulcer healing effects (Pan *et al.*, 2021; Tsai *et al.*, 2019). The protective properties of Astragaloside IV on the gastric with aspirin-induced GU have been demonstrated. This protective mechanism is considered to be mediated by the modulation of many biochemical factors, including an increase in PGE2 levels, a reduction in superoxide dismutase activity and a decrease in NO production (Fan *et al.*, 2016). In the current investigation, after modeling GU by indomethacin, the rats exhibited an apparent degree of mucosal damage inside the ulcerated regions. Whereas the administration of high-dose WYI demonstrated notable gastroprotective properties via the reduction of the gastric ulcer area generated by indomethacin, consistent with the ethanol GU model. Furthermore, we gave the WYI to the control rats at clinically equivalent doses. No significant variations were seen in serum PGE2 and NO, as well as

the that of cytokines in gastric tissues after WYI administration (data not shown), indicating that WYI has no adverse effects. The mechanism by which WYI exerts gastroprotective effects in indomethacin-induced gastric ulcer models needs to be further investigated.

To further investigate the effect of WYI on gastric mucus and gastric acid secretion, we established a pylorus ligation model. The findings demonstrated that WYI had no discernible impact on either the amount of gastric mucus secreted or the pH of stomach acid, suggesting that WYI's anti-ulcer activity was no significantly related to mucus secretion and gastric acid. This observation indicated that the mechanism by which WYI exerts its anti-ulcer action differs from the inhibitory mechanism of proton pump inhibitors regarding gastric acid production. On the basis of these evidences, we suggest that the therapeutic benefits of WYI on ulcers do not involve suppression of gastric acid formation. While it has been shown that WYI may have an inhibitory effect on stomach ulcers generated by indomethacin, there is a need for additional investigation to determine the particular mechanism through which WYI protects against drug-induced ulcers.

CONCLUSION

In conclusion, our research highlights the potential therapeutic benefits of WYI as a practical therapy option for gastric ulcers. A standardized methodology for the extraction of WYI was developed and subsequently used to assess the quality of the extracted substances. Our findings indicate that WYI exhibits gastroprotective effects by inhibiting the p38 MAPK pathway and reducing inflammation. Overall, the research we conducted lays the foundations for WYI developing as a therapeutic alternative for gastric ulcers.

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