

Albizzia chinensis (Osbeck) Merr extract YS ameliorates ethanol-induced acute gastric ulcer injury in rats by regulating NRF2 signaling pathway

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Abstract

Background: Around the world, there is a high incidence of gastric ulcers. YS, an extract from the Chinese herb *Albizzia chinensis* (Osbeck) Merr, has potential therapeutic applications for gastrointestinal diseases. Here we elucidated the protective effect and underlying mechanism of action of YS on gastric ulcer in rats injured by ethanol.

Methods: The ethanol-induced gastric ulcer rat model was used to assess the protective effect of YS. A pathological examination of gastric tissue was performed by H&E staining. GES-1 cells damaged by hydrogen peroxide were used to simulate oxidative damage in gastric mucosal epithelial cells. Endogenous NRF2 was knocked down using small interfering RNA. Immunoprecipitation was used to detect ubiquitination of NRF2. Co-immunoprecipitation was used to detect the NRF2–Keap1 interaction.

Results: YS (10 and 30 mg/kg, i.g.) significantly reduced the ulcer index, decreased MDA level, and increased SOD and GSH levels in gastric tissues damaged by ethanol. YS promoted NRF2 translocation from cytoplasm to nucleus and enhanced the NQO1 and HO-1 expression levels in injured rat gastric tissue. In addition, YS regulated NQO1 and HO-1 via NRF2 in H₂O₂-induced oxidative injured GES-1 cells. Further studies on the underlying mechanism indicated that YS reduced the interaction between NRF2 and Keap1 and decreased ubiquitylation of NRF2, thereby increasing its stability and expression of downstream factors. NRF2 knockdown abolished the effect of YS on MDA and SOD in GES-1 cells treated with H₂O₂.

Conclusion: YS reduced the NRF2–Keap1 interaction, promoting NRF2 translocation into the nucleus, which increasing the transcription and translation of NQO1 and HO-1 and improved the antioxidant capacity of rat stomach.

KEYWORDS

antioxidative, ethanol, gastric ulcer, NRF2, YS

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1 | INTRODUCTION

Gastric ulcer is a type of peptic ulcer that in severe cases can cause bleeding in the stomach.^{1,2} Gastric ulcer is typically treated with a proton-pump inhibitor,³ an acid-inhibiting drug, but long-term use of proton pump inhibitors will change the pH of gastric juice and affect the absorption of nutrients.⁴

The causes of gastric ulcer include psychiatric factors, non-steroidal anti-inflammatory drugs and alcohol.⁵⁻⁷ Gastric mucosal injury is often caused by excessive alcohol intake, and therefore gastric ulcer rat models induced by ethanol are frequently used to evaluate anti-ulcer drugs. Heavy alcohol intake disrupts gastric secretory function, decreasing mucus production,⁸ and also generates excessive oxidative factors and inflammatory responses.^{5,9-11}

Oxidative stress is implicated in gastrointestinal mucosal injury.^{12,13} Controlling oxidative stress is essential for the treatment of this pathology. NRF2 is a crucial regulator of antioxidant factors and activation of NRF2 results in anti-ulcer effects.^{10,14} Keap1 is a predominant repressor of NRF2, facilitating the ubiquitination of NRF2, thereby promoting its degradation in a proteasome-dependent manner. Under oxidative damage condition, NRF2 disassociates from Keap1 and is translocated into the nucleus, initiating the expressions of downstream antioxidative factors.^{15,16}

More and more researches had shown that natural products extracted from Chinese traditional medicinal herbs can be used to treat gastric ulcer with fewer side effects.¹⁷ *Albizzia chinensis* (Osbeck) Merr belongs to the genus *Albizzia*. Chemical components in *Albizzia* bark are known to ease enteritis, diarrhea, and dysentery (Chinese Herbal Medicine Series, 2nd Ed. 1986, Vol. 2, p. 768). We have found an *Albizzia chinensis* extract (YS) displays anti-gastric ulcer activity. In this study, we determined the effect of YS on rat gastric ulcer induced by ethanol and studied its mechanism.

2 | METHODS

2.1 | Sources and preparation of YS

YS, an extract of *Albizzia chinensis* (Osbeck) Merr, was provided by Professors Shishan Yu and Shuanggang Ma from the Natural Medicinal Chemistry Department of our Institute. Its extraction process and the discovery of its anti-ulcer activity have been patented in China (ZL201280052172.2). The voucher specimen (No. 90269) has been deposited in the Herbarium of the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences.

An extraction procedure was employed to obtain the active fraction from *Albizzia chinensis* for gastric ulcer-related research. Initially, 20 kg of air-dried and ground *Albizzia chinensis* bark were extracted three times with 80 L of 95% EtOH under reflux conditions, and then concentrated under reduced pressure to obtain

2600 g of the extract. This extract was then dissolved in a 20% EtOH-H₂O solution, and the soluble fraction was subjected to polyamide column (30–60 mesh, 6.4 L) chromatography using gradient elution with EtOH-H₂O (20% EtOH and 60% EtOH). The YS was then obtained by eluting with 60% EtOH eluted and concentrated.

2.2 | Animals

Male SD rats weighing 180–200 grams were purchased from HFK BIOSCIENCE Co., Ltd (China, Beijing; license number is SCXK (Beijing) 2019-0023). The rats were accommodated under suitable environmental conditions with room temperature maintained at 25 ± 1°C. The experimental design was meticulously planned to minimize animal suffering and statistical analysis was conducted using only the minimum number of animals required for valid statistical evaluation. Animal procedures were conducted strictly according to the *Guide for the Care and Use of Laboratory Animals* as stipulated by Peking Union Medical College and the Chinese Academy of Medical Sciences. The humane treatment of all animals was a priority throughout the study.

2.3 | Gastric ulcer induction

Rats fasted for 18 h were randomly divided into different groups, respectively, distilled water or rabeprazole sodium (30 mg/kg body weight) or YS at doses of 3 mg/kg, 10 mg/kg, or 30 mg/kg administered by oral gavage. After 30 min, all animals except the control group were gavaged with 95% ethanol at 5 mL/kg b.w. to induce gastric damage. After 4 h, the rats were euthanized and the glandular portion of stomachs were taken to evaluate gastric lesions.¹⁸

2.4 | Tissue protein extraction and analysis

The gastric tissue was washed using phosphate-buffered solution (PBS, pH 7.4), then suspended in PBS containing protease inhibitor and homogenized. The tissue homogenate was centrifuged and the supernatant collected. SOD, MDA, GSH activity analysis kits purchased from Beyotime Biotechnology (China). The primary antibodies used in the Western blotting included anti-NRF2 (CST), anti-HO-1 (CST), anti-Keap1 (CST), anti-NQO1 (Santa Cruz Biotechnology) antibodies and polyclonal anti-Tubulin, anti-GAPDH (Proteintech) antibodies.

2.5 | Real-time qPCR

RT-qPCR was carried out in a reaction volume containing cDNA, SYBR green PCR master mix, and target gene primers. The NQO1

and HO-1 relative transcription levels were calculated as $2^{-\Delta\Delta Ct}$. The primers listed in Table 1 were used for RT-qPCR.

2.6 | RNA interference

GES-1 cells were cultured and then transfected with NRF2 siRNAs to knock down the NRF2 level for 24 h. They were then incubated with YS (10 $\mu\text{g}/\text{mL}$) for 24 h, followed by exposure to 750 micromolar (μM) hydrogen peroxide (H_2O_2) for 6 h. Finally the cells were harvested for analysis. The sequences of NRF2 siRNAs are listed in Table 1.

2.7 | Statistical analysis

Data analysis was by ANOVA and the data are presented as means \pm SD. A p value less than 0.05 was considered as indicating a significant difference.

TABLE 1 The primer sequences.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
NQO1	GAGGACATCATTCAACTA	ATAGCATAGAGGTCAGAT
HO-1	CCTGGTTCAAGATACTAC	CTACATGAGACAGAGTTC
GAPDH	TGCTGATGACTGGTTACAATA	GCTTGACTTACAGAAGAATCG
si-NRF2-1	GGUUGAGACUACCAUGGUUTT	AACCAUGGUAGUCUCAACCTT
si-NRF2-2	CCAGUAUCAGCAACAGCAUTT	AUGCUGUUGCUGAUACUGGTT
si-NRF2-3	GCCUGUAAGUCCUGGUCAUTT	AUGACCAGGACUUCACAGGCTT

3 | RESULTS

3.1 | YS mitigated ethanol-induced acute gastric ulcer and histopathological damage in rats

In the ethanol-induced model group, excessive ethanol led to hemorrhage and necrosis in the mucosal glandular area, with rats exhibited many bleeding strips in the glandular stomach, indicative of serious bleeding damage. In YS treatment groups (10 mg/kg and 30 mg/kg), the degree of damage was much lighter than that in the model group (Figure 1A). The protective effect of YS was evaluated by quantification of bleeding in the injury area using image J software. Pre-treatment with YS at doses of 3, 10, or 30 mg/kg or rabeprazole sodium (Rpz, 30 mg/kg) reduced the ulcer index (UI) percentage of (by 34.15%, 86.48%, 100.00%, and 46.88%, respectively) compared to the model group (Figure 1B). The histopathological characterizations revealed that YS effectively protects gastric tissues from ethanol damaged. The control group had intact gastric mucosa, while the ethanol-induced

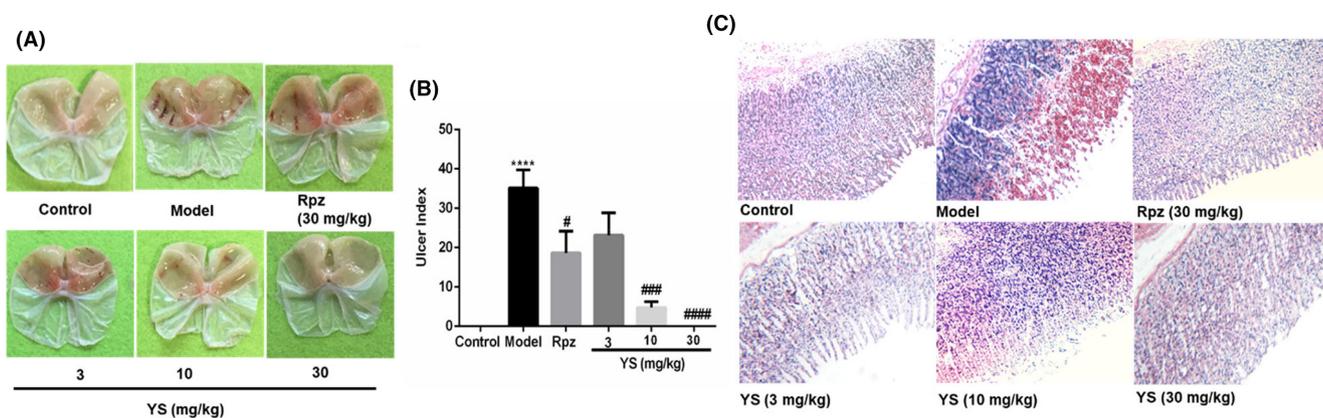


FIGURE 1 Effect of YS on ethanol induced gastric mucosa injury in rats. (A), Macroscopic appearance of gastric mucosa. (B), Ulcer index. (C), Microscopic appearance of gastric mucosa stained with H&E. Values are expressed as means \pm SD. **** p < 0.0001, compared with control group, # p < 0.05, ### p < 0.001 and #### p < 0.0001 compared with model group.

TABLE 2 The effect of YS on the levels of white cell subsets in ethanol-induced gastric ulcer rat blood.

Group	WBC ($\times 10^9/\text{L}$)	MON (%)	EOS (%)	BAS (%)	LYM (%)	NEUT (%)
Control	8.94 \pm 0.64	4.46 \pm 0.47	4.19 \pm 1.36	1.89 \pm 0.63	76.17 \pm 4.25	13.30 \pm 3.56
Model	8.58 \pm 1.51	14.64 \pm 2.30***	1.25 \pm 0.34***	0.85 \pm 0.45	64.92 \pm 4.95*	20.18 \pm 2.42**
YS (10 mg/kg)	5.92 \pm 0.46	5.64 \pm 1.64###	0.83 \pm 0.42	0.13 \pm 0.13	77.58 \pm 5.86#	13.22 \pm 3.12##

Note: Values are expressed as means \pm SD.

* p < 0.05; ** p < 0.01; *** p < 0.001, compared with control group;

p < 0.05; ## p < 0.01; ### p < 0.001, compared with model group.

group exhibited severe exfoliation and damage to the mucosal epithelium, and vascular congestion was observed. The damage was clearly attenuated when pretreated with oral administration of YS (10mg/kg, 30mg/kg) and rabeprazole sodium (30mg/kg) (Figure 1C).

3.2 | YS inhibited the inflammatory response and restored mucosal oxidative status in gastric tissue

Blood testing showed a change in the relative leukocyte composition in blood, with an increased percentage of monocytes and neutrophils and a reduction in lymphocytes in the model group relative to the vehicle group. After YS (10mg/kg) treatment, the levels of monocytes, neutrophils, and lymphocytes recovered to baseline (Table 2). Antioxidant enzymes protect tissues from oxidative damage under oxidative stress conditions. In our study, ethanol exposure extensively decreased SOD levels, while YS pretreatment significantly restored SOD activity and assisted the gastric mucosa to resist oxidative stress (Figure 2A). In addition, the antioxidant effects of YS were further verified by examining MDA and GSH levels. MDA is often used as an indicator of oxidative damage; ethanol led to an increase in MDA level while YS reduced the MDA level (Figure 2B). YS pretreatment increased the GSH level compared to the model group in gastric mucosa tissue (Figure 2C).

3.3 | YS promoted NRF2 translocation into the nucleus and activated its downstream factors

We used immunofluorescence to analyze the cellular distribution of NRF2. Pink fluorescence indicated the overlap between the nucleus and NRF2, representing NRF2 distributed in the nucleus. In the control group, the pink fluorescence signal in the gastric mucosa epithelium was very strong, indicating that there is more NRF2 distributed in the nucleus, while in the model ethanol injury group, the pink fluorescence signal was weaker in the damaged gastric mucosa epithelium, indicating that the distribution of NRF2 in the nucleus was greatly reduced. In the YS treatment group, a strong pink fluorescence signal was restored in the gastric mucosa epithelium, indicating that YS promoted the distribution

of NRF2 in the nucleus (Figure 3A). There was no difference in NRF2 total protein between different groups (Figure 3B). Ethanol led to a sharp increase of NRF2 in cytoplasm and a reduction in the nucleus, and YS promoted NRF2 translocation into the nucleus (Figure 3B,C). When the downstream signaling molecules of NRF2 were assessed, HO-1 and NQO1 levels were markedly decreased in the ethanol-induced ulcer group, but were restored to the control levels in the YS pretreatment groups (Figure 4A,B). In addition, the data showed that ethanol significantly reduced HO-1 and NQO1 mRNA levels, and YS elevated mRNA levels of both factors in injured tissue (Figure 4C,D).

3.4 | In NRF2 knockdown GES-1 cells YS had no effects on expressions of NQO1 and HO-1 in hydrogen peroxide damage model

To further determine whether YS exerts antioxidant activity through NRF2, NRF2 was knocked down by short interfering RNA (siRNA) in GES-1 cells. Firstly, the efficacy of three different siRNAs against NRF2 were examined via western blot, showing that expression of endogenous NRF2 was largely disrupted by siNRF2 (Figure 5A). GES-1 cells treated with H₂O₂ at 750μM for 6h were used as the cellular oxidative injury model. Expression of NQO1 and HO-1 was reduced in H₂O₂-treated GES-1 cells, while their expression was elevated in cells pretreated with YS (10μg/mL). YS lost its regulatory function on NQO1 and HO-1 when NRF2 was inhibited. (Figure 5B).

3.5 | YS increased the stability of NRF2 and promoted Keap1-NRF2 in rat gastric tissues

In a physiological state, Keap1 interacts with NRF2 as a repressor.¹⁹ Keap1 promotes proteasome-dependent degradation of NRF2, facilitating the polyubiquitination of NRF2 by acting as an E3 ligase adaptor component.^{15,20} In an injured condition, NRF2 and Keap1 dissociate, then NRF2 translocates into the nucleus and induces transcription of downstream factors. To investigate the protective mechanism of YS, Keap1 levels were examined in gastric tissues. YS did not affect the mRNA and protein levels of Keap1 in tissues damaged by ethanol

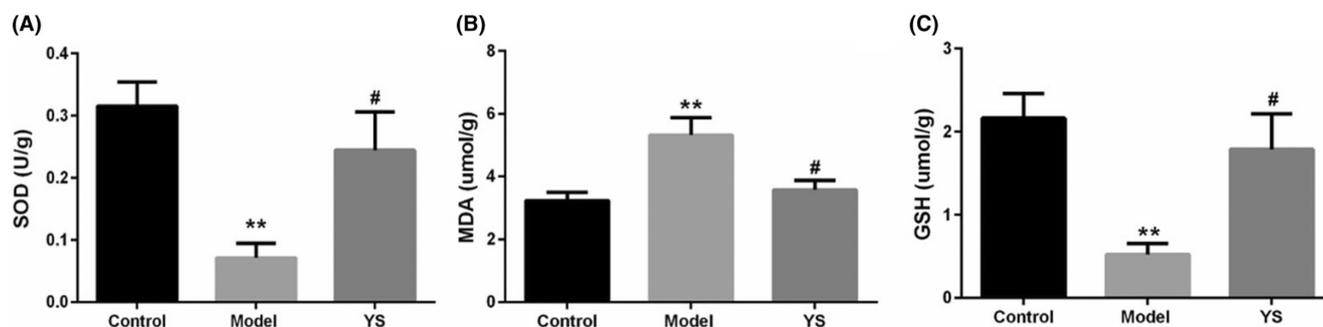


FIGURE 2 Effect of YS on oxidative stress factors in rat gastric tissues. (A), SOD level. (B), MDA level. (C), GSH level. The dose of YS is 10mg/kg. Values are expressed as means \pm SD. ** $p < 0.01$, compared with control group. # $p < 0.05$, compared with model group.

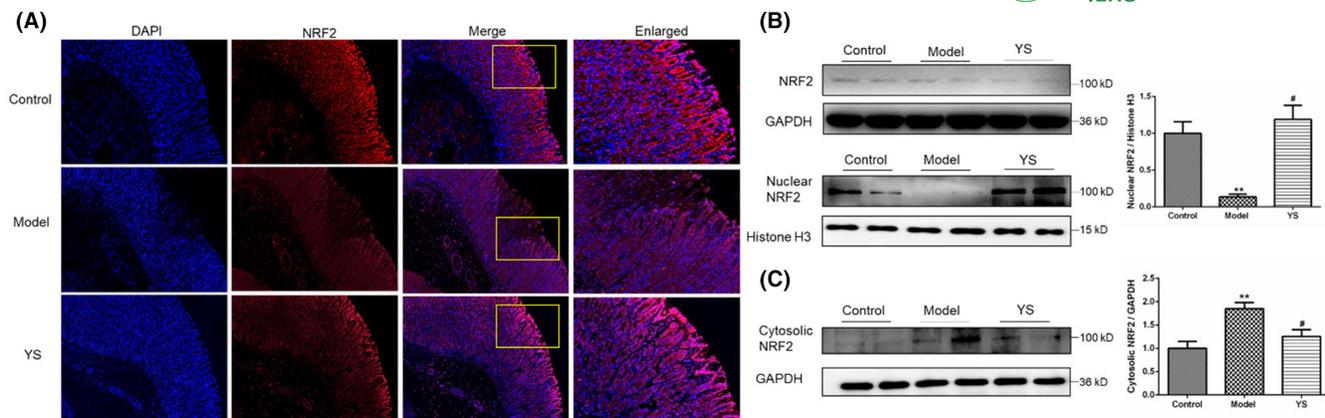


FIGURE 3 Effect of YS on NRF2 nuclear translocation in rat gastric tissue induced by ethanol. (A), Immunofluorescence staining. (B), Total and nucleus NRF2 protein levels. (C), Cytosolic NRF2 protein level. $**p < 0.01$, compared with control group. $\#p < 0.05$, compared with model group.

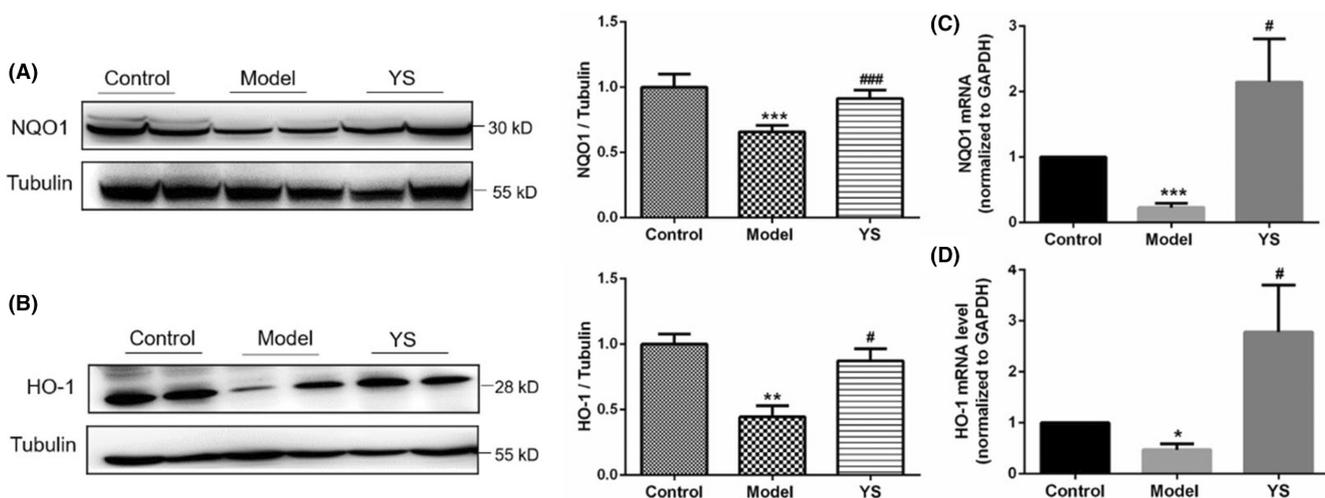


FIGURE 4 Effect of YS on downstream factors of NRF2 in rat gastric tissues injured by ethanol. (A), NQO1 protein level. (B), HO-1 protein level. (C), NQO1 mRNA level. (D), HO-1 mRNA level. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with control group. $\#p < 0.05$, and $###p < 0.001$ compared with model group.

(Figure 6A,B). In the model group, the interaction between NRF2 and Keap1 increased, while their interaction was reduced in the YS group, implying that YS can disassociate Keap1 and NRF2 (Figure 6C). The ubiquitination of NRF2 was then examined in different groups. As shown, alcohol injury increased NRF2 ubiquitination in rat gastric tissue, while YS treatment decreased NRF2 ubiquitination (Figure 6D). The above results demonstrated that YS can reduce the NRF2 and Keap1 interaction, decrease ubiquitination and increase the stability of NRF2, thereby enhancing the regulation of downstream factors.

3.6 | In NRF2 knockdown GES-1 cells YS had no effects on hydrogen peroxide induced oxidative damage

In order to further analyze the role of NRF2 in the function of YS, MDA and SOD levels were examined in H_2O_2 -treated GES-1 cells with NRF2

knockdown. MDA levels decreased and SOD increased when GES-1 cells damaged by H_2O_2 were pretreated with YS, while we found that NRF2 knockdown attenuated YS-dependent inhibition of MDA (Figure 7A). Moreover, we observed that the YS-dependent increase on SOD was abolished in NRF2 knockdown GES-1 cells (Figure 7B). In the summary, these observations imply that NRF2 serves as a critical signaling node in the antioxidant effect mediated by YS.

4 | DISCUSSION

Gastric ulcer is one of the commonest global illnesses. The prevention of gastric ulcers faces significant challenges due to the disadvantages of current gastric drugs available in the market, which show severe side effects, thereby limiting their efficacy against gastric diseases.^{21,22} Currently a large body of research has shown that natural products of herbal plants have been used for ulcer treatment, with

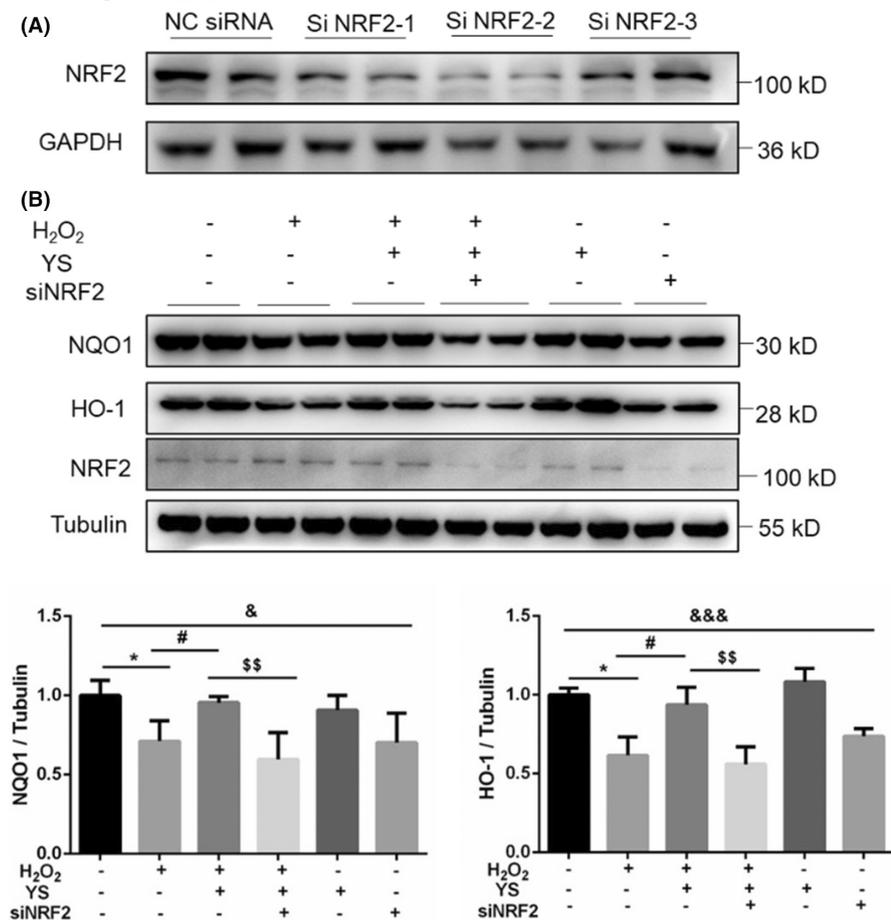


FIGURE 5 Effect of YS on NQO1 and HO-1 in H₂O₂-induced GES-1 cellular oxidative injury model. (A), NRF2 protein level. (B), NQO1 and HO-1 protein levels.

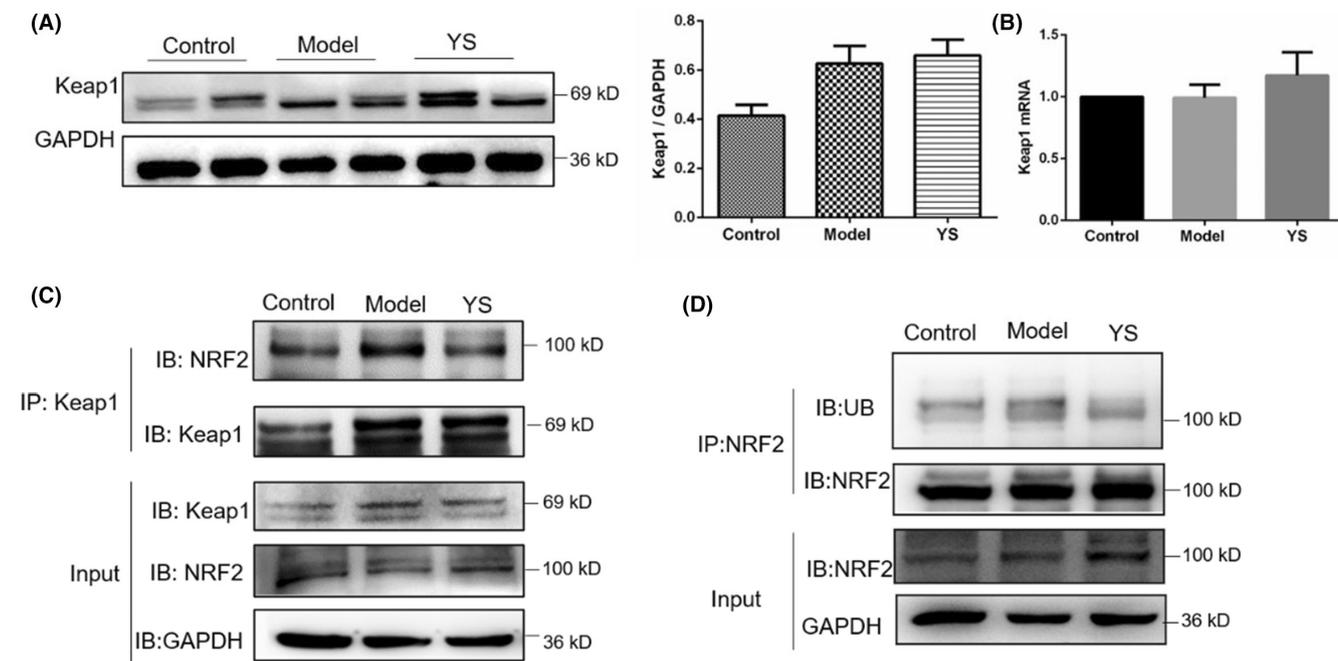
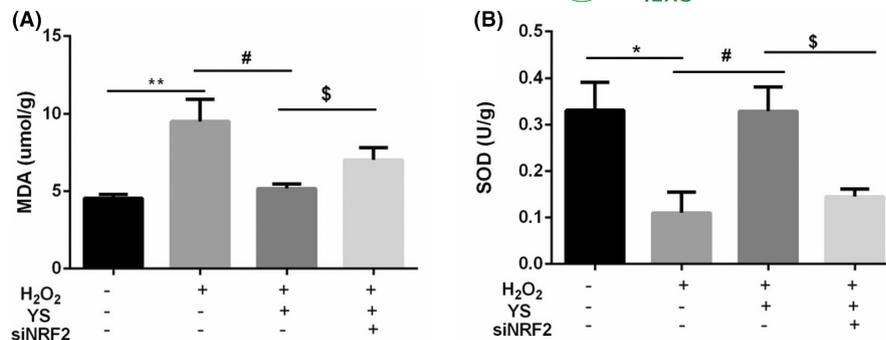


FIGURE 6 Effect of YS on the NRF2-Keap1 interaction and ubiquitylation of NRF2 in rat gastric tissue. (A), Keap1 protein level. (B), Keap1 mRNA level. (C), Co-immunoprecipitation analysis of the interaction between NRF2 and Keap1. (D), The ubiquitylation of NRF2.

fewer side effects.²³⁻²⁶ We have reported extracts of the Chinese herb *Albizia chinensis* (Osbeck) Merr with strong anti-gastric ulcer activity (ZL201280052172.2).

The ethanol-induced gastric mucosal injury model in rats has been widely used for assessing gastroprotective effect due to its similarity to human gastric ulcer.²⁷ However, the mechanism of

FIGURE 7 Effect of YS on oxidative stress factors in H₂O₂-induced GES-1 cellular oxidative injury model with NRF2 knockdown. (A), MDA level. (B), SOD level. Values are expressed as means ± SD. **p* < 0.05, ***p* < 0.01, compared with control group. #*p* < 0.05, compared with H₂O₂ treated group, \$*p* < 0.05, compared with H₂O₂ and YS co-treated group.



ethanol damages gastric mucosa is still unclear. Excessive ethanol intake directly leads to injury of gastric epithelial cells and the mucus layer.²⁸ It also damages gastric blood vessels, resulting in gastric mucosal bleeding,²⁹ infiltration of leukocytes and oxidative damage.^{30,31}

To determine whether YS promotes the metabolism of ethanol to ameliorate the damage it induces, the concentration of ethanol in blood was examined, and we found that the blood ethanol concentration (BEC) was greatly increased in model group, while YS had no effect on the BEC, implying that YS did not affect the metabolism of ethanol (data not shown). The catabolism of ethanol is mainly carried out in the liver. To confirm whether one-time excessive ethanol intake leads to liver injury and whether YS has a protective function, the index related to liver function was also examined. The data showed that there were no differences in the levels of aspartate transaminase (AST), albumin (ALB) and alanine aminotransferase (ALT) in the blood in all groups (data not shown).

Oxidative stress causes an imbalance between the oxidative and antioxidant systems, such as dysregulation of SOD, GSH and MDA levels.³² MDA is a commonly indicator of cell damage.³³ Interestingly, our results showed that YS has strong activity in the treatment gastric ulcer induced by ethanol. It reduced the ulcer index significantly and improved the pathology status of gastric mucosa. Ethanol intake triggered severe gastric oxidative stress by up-regulation of MDA, and down-regulation SOD and GSH. NRF2 is a regulator of the response to oxidative injury. Free NRF2 causes transcription activation of anti-oxidative factors.^{34,35} Our results showed that ethanol led to NRF2 being translocated from nucleus to cytoplasm, while YS strongly inhibited this process.

It had been reported that activation of NRF2 can ameliorate gastric ulcer in rats.³⁶ In this study, we also found that NRF2 was activated in the gastric tissue treated by YS. When NRF2 was silenced in GES-1 cells treated with H₂O₂, the function of YS was lost. We found that YS could upregulate the NQO1 and HO-1 levels, but when NRF2 was silenced, HO-1 and NQO1 protein levels reduced regardless of YS treatment.

NRF2 regulates the expression level of more than 100 oxidative related genes and it is therefore well studied as a target for disease treatment.³⁷ In normal conditions, NRF2 is regulated by Keap1, an adaptor of Cullin3 (Cul3) for proteasome degradation. Under oxidative stress, Keap1 inactivation leads to NRF2 translocation to the nucleus. Many strategies have been reported for regulating the

Keap1-NRF2 pathway.²⁰ In our study, YS did not affect the Keap1 level, but it weakened the NRF2-Keap1 interaction, implying that more NRF2 was released from NRF2-Keap1 complex. It also reduced the ubiquitylation of NRF2, decreasing the degradation of NRF2.

In summary, YS, extracted from *Albizia chinensis* (Osbeck) Merr, has a strong therapeutic effect on gastric ulcers. Its gastric protective effect is attributed to its ability to reduce the NRF2 and Keap1 interaction, causing NRF2 translocation into the nucleus, increasing the expression of NQO1 and HO-1, and improving the antioxidant capacity of rat stomach.

AUTHOR CONTRIBUTIONS

Jianjun Zhang conceived the study, Bo Tang designed all the experiments and performed the experiments. Liangning Li analyzed the data using software. Yuanzhi Yu constructed the rat model. Guibin Wang revised the article. Shuanggang Ma and Shishan Yu provided the YS materials.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

Animal procedures were conducted strictly according to the Guide for the Care and Use of Laboratory Animals as stipulated by Peking Union Medical College and the Chinese Academy of Medical Sciences.

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