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Dendrobium officinale flowers flavonoids enriched extract protects against acute ethanol-induced gastric ulcers via AMPK/PI3K signaling pathways

Zhiyu Zhang^a, Hualing Xie^a, Mohamed A. Farag^b, Zhenhao Li^c, Qingping Wu^d, Ping Shao^{a,e,*}^a Department of Food Science and Technology, Zhejiang University of Technology, Hangzhou 310014, China.^b Pharmacognosy Department, College of Pharmacy, Cairo University, Cairo 12613, Egypt.^c Zhejiang ShouXianGu Botanical Drug Institute Co., Ltd., Hangzhou 321200, China^d Guangdong Provincial Key Laboratory of Microbial Safety and Health, State Key Laboratory of Applied Microbiology Southern China, Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou 510070, China^e Eco-Industrial Innovation Institute Zhejiang University of Technology, Quzhou 324000, China

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ABSTRACT

Gastric ulcer is a widespread disease caused by various etiologies. *Dendrobium officinale* flowers exert several health benefits owing to their rich flavonoid content. In this study, protective effects and possible action mechanisms of *D. officinale* flowers' flavonoid enriched extract (DOFF) were assessed against gastric ulcer. The result of sodium nitrite-aluminum nitrate colorimetry showed that 52.34% of the total extractive was flavonoid, and ultra-high performance liquid chromatography time of flight mass spectrometer (UPLC-Q-TOF/MS) revealed the presence of 28 components in DOFF of which 14 belonged to flavonoids. In addition, *in vivo* assay revealed DOFF potential in reducing the formation of ethanol-induced gastric mucosal lesions, with drop-in ulcer index from 64.33 ± 8.76 to 32.00 ± 4.47 . Similar results were revealed in human gastric mucosal epithelia cells, with cells viability to increase from 27.2% to 61.6% post DOFF administration. To analyzed the protect effect of DOFF, we used Western blotting and immunofluorometric assay to revealed the expression levels of key proteins in cell pathways. The results showed that DOFF (320 $\mu\text{g/mL}$) could increase the level of oxidation marker protein (HO-1), apoptosis regulatory protein (Bcl-2) and autophagy marker (LC3 β) by 50.84%, 43.85%, and 59.21% compared with ethanol-treated group respectively. Further analyzed of the mitochondrial activity and apoptosis pathway, we found that DOFF appeared to mitigate against ethanol-induced gastric mucosal injury via AMPK/mTOR/ULK1 and PI3K/AKT autophagy signaling pathways.

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1. Introduction

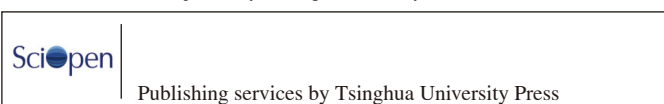
Gastric ulcer is a common gastrointestinal tract disease affecting approximately 5%–10% of humans worldwide^[1]. The main contributors to gastric ulcer include *Helicobacter pylori* infection, overuse of non-steroidal anti-inflammatory drugs, and other factors such as inadequate dietary habits, excessive ethanol consumption,

cigarette smoking, and stress^[2–3]. Upon stomach exposure to alcohol and acid, the dynamic protective process of gastric mucosa lining the stomach gets disrupted. The gastric wall will be further gradually damaged by the generated gastric acid, leading to the break of gastric mucosa and finally development of gastric ulcers and other diseases^[4]. The most commonly used drugs to treat and prevent gastric ulcers include H2 receptor antagonists (such as cimetidine, ranitidine), proton pump inhibitors (such as omeprazole (OME), lansoprazole), antacids and cytoprotective factors that provide mechanical protection to gastric mucosa^[5]. However, long-term use of these drugs may cause serious side effects^[6]. Therefore, search for safe and effective, especially extracting natural resources from plants as alternative

* Corresponding author at: College of Food Science and Technology, Zhejiang University of Technology, Hangzhou 310014, China.

E-mail address: pingshao325@zjut.edu.cn (P. Shao)

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drugs, still attracts a large number of researchers^[7]. Examples of effective anti-ulcer agents identified from plant extracts include *Adinandra nitida* and camellianin A^[8], *Osyris quadripartite*^[9], and *Cyperus conglomeratus*^[10] against the progression of acute gastric ulcer in mice.

With more than 1 500 species, *Dendrobium* is the second largest genus in Orchidaceae^[11-12], and its members are widely distributed throughout Asia, Europe, and Australia^[13]. Of these, 74 species were identified in China^[14]. *D. officinale* is one of the major traditional Chinese medicinal herbs with a wealth of medicinal properties and likewise used in many Asian countries for centuries. *D. officinale* is recognized as a high-quality healthy food in China and other Southeast Asian countries due to its wide range of pharmacological and clinical effects^[15]. Traditionally used organs are mainly the stems and to less extent its flower. Flowers are often though discarded directly as a by-product of the plant usage as nutraceutical. The chemical components of *D. officinale* stems mainly include polysaccharides, amino acids, alkaloids, etc.^[16], with multiple health effects, such as antioxidant, antitumor, and immunity enhancement^[17-19]. The chemical components of *D. officinale* flowers are similar to those of stems with flavonoids to amount for the main bioactive compounds in its extract^[20]. Zhang et al.^[21] reported that flavonoids could significantly attenuate ethanol-induced gastric injury mediated *via* their antioxidant, anti-inflammatory, and anti-apoptotic effects. *In vitro* and *in vivo* studies have shown that *D. officinale* plays a gastroprotective effect by inhibiting oxidative stress-induced apoptosis^[22]. However, no study has assessed out *D. officinale* flower flavonoids for its gastroprotective activity yet especially being enriched in that organ. This study main goal was to assess the protective effect of flavonoids from *D. officinale* flower (DOFF) on gastric ulcers and to identify the underlying action mechanisms.

Ethanol-induced gastric ulcer is a common animal model for the identification of new anti-ulcer drugs resembling the pathophysiology of human ulcer^[23]. Recent research shows that ethanol-induced damage to the gastric mucosa likely occurs as a result of oxidative stress due to excessive production of reactive oxygen species (ROS) during ethanol metabolism^[24-25]. To prevent the generation of ROS, mammalian cells have developed many antioxidant defense molecules, such as total antioxidant capacity (T-AOC), superoxide dismutase (SOD) and malondialdehyde (MDA), which scavenged oxygen-derived free radicals directly or increased levels of radical scavengers^[26]. Apoptosis is a programmed cell death pathway which has been linked to the pathogenesis of gastric mucosal insult triggered by ethanol^[27-29]. In addition, gastric mucosal cell apoptosis induced by excessive oxidative stress accompanies the mucosal injury and aggravates gastric ulcer^[22]. Although ethanol exposure can lead to cell death, cells can activate complex protective systems to minimize the damage, such as cell autophagy could degrade the damaged proteins or organelles to reduce immune response^[30]. There is an increasing evidence that appropriate level of autophagy can be a significant cellular protective mechanism that contributes to cell survival^[31], and further protects gastric mucosal cells from ethanol-induced damage *via* multiple signaling pathways^[32-34]. The regulation of autophagy involves various signaling pathways, including adenosine monophosphate-activated protein kinase (AMPK), phosphatidylinositol 3-kinase (PI3K), and mechanistic target of rapamycin (mTOR) pathways^[35]. Furthermore, PI3K/protein kinase B (Akt) and AMPK are the major upstream regulators of mTOR^[36-37]. AMPK and mTOR could regulate that initiation of autophagy by

direct phosphorylation of unc-51 like autophagy activating kinase 1 (ULK1) and ULK complex^[38]. Dysfunction of autophagy can lead to impairment of mitochondrial functions, accumulation of ROS, and oxidative stress in cells^[39]. Moreover, autophagy can be activated in response to a number of insults, including excessive ROS as a consequence of oxidative stress to eliminate toxic polymers and damaged organelles^[40]. According to previous reports, autophagy adaptively protects gastric mucosal epithelial cells from ethanol-induced oxidative damage and apoptosis through mTOR signaling pathway^[30]. Therefore, autophagy response was used mainly as a measure to assess DOFF protective effect on ethanol-induced gastric ulcers using *in vitro* cellular and *in vivo* animal models, and to further identify potential underlying signaling pathways.

2. Materials and methods

2.1 Chemicals and reagents

The flowers of *D. officinale* were obtained from the Yueqing Runfeng *Dendrobium* Co., Ltd. (Wenzhou, China). OME (O118724) and 3-methyladenine (3-MA, M129496) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Rapamycin (RAPA, R817296), AB-8 macroporous resin (A875381-500g) and *N*-acetylcysteine (NAC, N800425) were provided from Shanghai Maclin Biochemical Technology Co., Ltd. (Shanghai, China). RIPM 1640 medium was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Alcian blue (A15494) was purchased from Shandong Xiya Chemistry Industry Ltd. (Shandong, China). The mitochondrial membrane potential (MMP) detection kit (C2006) was purchased from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China). Sephadex LH-20 (S26929-25g) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Information about the antibody proteins used in this study is provided in Table 1.

Table 1
Antibody protein and manufacturing company.

Reagent name	Name of production company	Serial Number	Place of production
HO-1 antibody	Wanleibio	WL02400	Shenyang, China
LC3B II/I antibody	Wanleibio	WL01506	Shenyang, China
Bcl-2 antibody	Wanleibio	WL01556	Shenyang, China
p-PI3K p85 antibody	Affinity Biosciences	AF3241	Changzhou, China
PI3K p85 antibody	Wanleibio	WL02240	Shenyang, China
p-AKT antibody	Wanleibio	WLP001a	Shenyang, China
AKT antibody	Wanleibio	WL0003b	Shenyang, China
p-AMPK antibody	Wanleibio	WL05103	Shenyang, China
AMPK antibody	Wanleibio	WL02254	Shenyang, China
p-mTOR antibody	Wanleibio	WL03694	Shenyang, China
mTOR antibody	Wanleibio	WL02477	Shenyang, China
p-ULK1 antibody	Affinity Biosciences	AF4387	Changzhou, China
ULK1 antibody	Affinity Biosciences	DF7588	Changzhou, China
Beclin-1 antibody	Wanleibio	WL02508	Shenyang, China
p62 antibody	Wanleibio	WL02385	Shenyang, China
Goat Anti-Rabbit IgG/HRP	Wanleibio	WLA023	Shenyang, China
β-Actin	Wanleibio	WL01372	Shenyang, China

2.2 Preparation of DOFF

After harvesting in June 2021, *D. officinale* flowers were dried in an electric thermostatic drying oven at 50 °C, crushed with a grinder, and passed through a 20 mesh sieve to obtain fine powder that was stored at −20 °C. Extraction of flavonoids was based on previous studies^[41–42]. Briefly, powdered material (200 g) was soaked in 80% ethanol (7 000 mL) and extracted using ultrasonic water bath at 40 °C and 40 kHz for 50 min. The filtrate was obtained by suction filtration withing buchner funnel, and concentrated using a rotary evaporator (40 °C) to remove ethanol, cooled at room temperature and freeze-dried to obtain the crude extract. Dried residue (21 mg) was redissolved in 80% ethanol (30 mL), separated and purified by AB-8 macroporous resin to yield the aqueous fraction and ethanol extracted fraction. Finally, ethanol fraction was purified using Sephadex LH-20, and eluted successively with MQ water (200 mL), aqueous methanol (30%, 50%, 70% and 90% methanol, 200 mL for each) at room temperature, and the total flavonoid content was estimated at 52.34% using sodium nitrite-aluminum nitrate colorimetry and rutin as a standard product suggestive that this fraction was enriched in flavonoids^[43].

2.3 Structural characterization of DOFF using ultra-high performance liquid chromatography time of flight mass spectrometer (UPLC-Q-TOF/MS)

Flavonoids from *D. officinale* flowers were separated using Waters UPLC (ACQUITY UPLC I-Class, Waters Co., USA) coupled to an ultraviolet-visible (190–700 nm) and MS detectors. Analysis was based on the method of Zhao et al.^[44] with slight modifications. Separation was performed on Waters ACQUITY UPLC BEH-C₁₈ column (100 mm × 2.1 mm, 1.8 μm) at a flow rate of 0.3 mL/min and temperature of 40 °C. The injection volume was 5 μL. Eluent A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) respectively. Gradient elution system was as follows: 0–9 min, 2%–100% B; 9–12 min, 100% B; 12–13 min, 100%–2% B; 13–18 min, 2% B. MS analysis was performed on Waters XEVO-G2QTOF Mass Spectrometer (Waters Co., Milford, MA, USA) equipped with an electrospray ionization (ESI) system operated in a negative ion mode considering the improved sensitivity of flavonoids to ionize in that mode. The source voltage was set at 2.7 kV, and the source temperature was 450 °C, sheath gas flow rate of 30 arb, auxiliary gas flow rate of 10 arb, and scan range for both MS and MS/MS between *m/z* 200 and 1 000. MS spectra were processed for visualization using MassLynx Workstation Software, version 4.1.

2.4 Protective effect of DOFF on gastric mucosal injury mice model

2.4.1 Animal and experiment design

SPF grade male KM mice ((20 ± 2) g, 6–8 weeks) were obtained from Liaoning Changsheng Biotechnology Laboratory and given free access to food and water at (23 ± 2) °C with humidity set at (50 ± 10)% for 12 h of light/dark cycle. According to previous studies, Belayneh et al.^[45] found that the flavonoid extracts (200 and 400 mg/kg) from *Solanum incanum* L. (Solanaceae) leaves and roots had anti-gastric ulcer effects on albino mice, and found no significant side

effects in mice through acute toxicity studies. Therefore, this study selected flavonoid (DOFF) concentrations of 200 and 400 mg/kg for further experiments, and the dose of administered OME used in animal study was based on previous reports^[46]. All mice were randomly divided into 5 groups (*n* = 6): control group; model group; low dose of DOFF group (200 mg/kg); high dose of DOFF group (400 mg/kg); and OME group (20 mg/kg OME). After adaptive feeding for 1 week, mice were given a pretreatment by gavage. Mice in the control and model groups were administered 10 mL/kg of 0.5% sodium carboxymethyl cellulose by gavage, whereas other groups were administered same volume/weight of the drug for 7 days, once a day for 7 consecutive days, and then all mice were fasted for 24 h with free access to water prior to receiving the 7th-day treatment. 2 h post administration on day 7, 10 mL/kg 100% ethanol was given by gavage to induce gastric ulcer, and the control group was given an equal volume of normal saline^[47]. Blood was collected 2 h after last administration through the abdominal aorta (mice were treated with intraperitoneal injection of 4% chloral hydrate anesthesia according to the mice weight at 0.1 mL/10 g). Blood samples were centrifuged at 3 000 r/min for 10 min, and the supernatant was aspirated, for serum biochemical analysis of total gastric juice. After blood collection, mice were sacrificed by cervical dislocation, and stomach was removed, weighed, and its contents were collected, rinsed with cold saline, and then stored at −80 °C till further analysis. All experiments were performed by following the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China. This experiment was approved by the Ethics Committee of Zhejiang University of Technology (No. 20220516317).

2.4.2 Mice gastric injury evaluation

The gastric tissue was cut along the greater curvature and the inner wall was completely exposed, rinsed with normal saline to remove blood clots, spread on filter paper, and fixed with the filter paper. The size of the lesion was measured with a vernier caliper and magnifying glass, with ulcer index (UI) determined according to Guth's standard^[48]: no lesions (score 0); epithelial damage or lesion length < 1 mm (score 1); 1 mm ≤ lesion length < 2 mm (score 2); 2 mm ≤ lesion length < 3 mm (score 3); 3 mm ≤ lesion length < 4 mm (score 4); 4 mm ≤ lesion length (segmented score); if width > 1 mm, the score was doubled. The UI score and ulcer inhibition rate were photographed and calculated for each group. The formula used for calculation of inhibition rate was as follows:

$$\text{Ulcer inhibition rate (\%)} = \frac{\text{UI in model} - \text{UI in test}}{\text{UI in model}} \times 100$$

2.4.3 Determination of gastric acidity

Stomach was removed, and gastric contents was collected, centrifuged at 3 000 r/min for 10 min and the supernatant was aspirated to measure pH values with precision pH test paper (Sigma-Aldrich).

2.4.4 Measurement of gastric mucus content

Alcian blue binding assay was performed according to previous report^[49]. The glandular segments from the stomach was removed, weighed (0.1 g), and soaked for 24 h at 25 °C in a tube containing

10 mL of Alcian blue solution (0.02% alcian blue solution, 0.16 mol/L sucrose, 0.05 mol/L sodium acetate, pH 5.8). The Alcian blue complexed with mucus was centrifuged at $3\,000 \times g$ for 10 min at 4 °C. Absorbance of the supernatant was measured at 620 nm using a spectrophotometer (Synergy H1, BioTek Instruments, Inc., US). The quantity of mucus was calculated using standard calibration curve of Alcian blue and result was expressed in mg/g of glandular tissue.

2.4.5 Histological examination

For histopathological examination, stomach tissues were immersed in 10% buffered formalin. Dehydrated using 70% (2 h), 80% (overnight), 90% (2 h) and 100% (1 h) ethanol gradients and after fixation, stomach was cut open longitudinally along greater curvature, and pathological changes in the stomach lumen was observed. Take 2–3 mm thick sections from representative areas and processed to prepare 3 paraffin blocks. The section was cut at 5 μ m of thickness and stained with hematoxylin and eosin (HE)^[50]. Sections were then evaluated microscopically for histological changes including erosions, edema, hemorrhage, fibrinoid necrosis, and inflammatory cells infiltration. The glandular portion of the tissues was stained with periodic acid-Schiff (PAS) to visualize mucus and assess changes in basic and acidic glycoproteins^[51].

2.4.6 Estimation of non-protein sulfhydryl groups (NP-SH)

NP-SH content of gastric mucosa was estimated according to the method of Sedlak et al.^[52]. Briefly, aliquots of 200 μ L of the homogenates were mixed in centrifuge tubes with 160 μ L of distilled water and 40 μ L of 50% trichloroacetic acid. The tubes were centrifuged at $3\,000 \times g$ after being shaken intermittently for 5–10 min. A sample was aliquoted of 100 μ L and combined with 200 μ L of 0.4 mol/L Tris buffer, pH 8.9, and 5 μ L of 0.4% 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB). The absorbance of DTNB addition was measured at 412 nm for not more than 5 min. The blank group was made with same reagents except homogenate to eliminate the experimental errors.

2.4.7 Serum and gastric tissues parameters

Mice blood were centrifuged at 4 °C ($1\,000 \times g$, 20 min) to collect serum. Levels of tumor necrosis factor α (TNF- α), interleukin 6 (IL-6) and interleukin 1 β (IL-1 β) in serum were detected using enzyme-linked immunosorbent assay (ELISA) kits following manufacturer protocols (Wanleibio, Shenyang, China). Nitric oxide (NO) level was determined using procedure provided by manufacturer (Nanjing Jiancheng Bioengineering Institute, China). Gastric tissue homogenate was collected post centrifugation at 4 °C (2 500 r/min, 10 min) and homogenized in ice-cold saline (1:9, V/m) to give a 5% homogenate suspension. SOD activity in tissue was determined following manufacturer's instructions (Wanleibio, Shenyang, China). MDA and myeloperoxidase (MPO) activities in the supernatant were determined using manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Prostaglandin E₂ (PGE₂) serum level was measured in the supernatant using ELISA kit (MultiSciences, Hangzhou, China).

2.5 Protective effect of DOFF on human gastric epithelium (GES-1) apoptosis

2.5.1 Cell culture and determination of drug concentration

The GES-1 cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA) and purchased from Punuosai Biotechnology Co., Ltd. (Wuhan, China). GES-1 cells were cultured in a RPMI 1640 medium supplemented with fetal bovine serum (10%) and incubated in a humidified incubator atmosphere containing added 5% CO₂ at 37 °C. GES-1 cells in the logarithmic growth phase and well-growing were selected and cultured in a 6-well plate (2 mL/well) for 24 h, and then treated with 10, 20, 40, 80, 160, 320, 640 and 1 000 μ g/mL ethanol extract of *D. officinale* for a period of 24 h^[53–54] or 50 nmol/L RAPA (dissolved in DMSO), 2 mmol/L 3-MA (dissolved in sterile distilled water)^[34], 5 mmol/L NAC (dissolved in sterile distilled water) for 2 h^[55]. Viability of GES-1 cells was detected using Cell Counting Kit-8 (CCK-8) kit (Wanleibio, Shenyang, China) to determine toxic effects on cells. According to the viability value of GES-1 cells, 80, 160, 320 μ g/mL of *D. officinale* ethanol extract showed no toxicity effect in the cells. Consequently, the 3 doses were selected and further treated with 5% ethanol for 4 h to induce GES-1 cell damage mode^[56]. Ethanol-containing medium was removed after 4 h, and the viability of GES-1 cells was detected using CCK-8 kit to determine *D. officinale* extract gastro protective effect.

2.5.2 Cell apoptosis analysis

Apoptosis detection kit was used to evaluate apoptotic rate of GES-1 cells under different treatments. Briefly, cells from each group were centrifuged at $500 \times g$ for 5 min and collected, carefully aspirated from the supernatant, cells were washed in phosphate buffered saline (PBS), re-suspended by adding Annexin V binding buffer in each flow tube further incubated with Annexin V fluorescein-5-isothiocyanate (FITC) and propidium iodide (PI) for 15 min at room temperature. Finally, samples were analyzed using a flow cytometer (NovoCyte, ACEA, USA).

2.5.3 MMP determination

MMP was determined using the fluorescent dye JC-1. At high MMP, JC-1 accumulates in mitochondrial matrix to form a polymer, which yields a red fluorescence. Upon lowered MMP, JC-1 cannot aggregate inside the matrix. Briefly, 5×10^4 cells were collected and washed twice with 1 mL PBS and then incubated at 37 °C for 20 min with 0.5 mL JC-1 dye in the dark. Next, cells were collected by centrifugation at $500 \times g$ for 3 min, and discard the supernatants. Then, cells were washed twice with 0.5 mL JC-1 staining buffer (1 $\times$) (pH 7.4), centrifuged, and the supernatants were removed. Afterward, the cells were re-suspended in 0.5 mL JC-1 staining buffer (1 $\times$) and subsequently analyzed using flow cytometry (NovoCyte, Aceabio, USA).

2.5.4 Intracellular ROS measurement

ROS in GES-1 was measured using a commercial ROS detection 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method

according to the manufacturer's instruction (Wanleibio, Shenyang, China). In brief, after cell pretreatment with extracts for 24 h or with 5 mmol/L NAC for 2 h, GES-1 cells were incubated with 5% ethanol for 4 h at 37 °C followed by incubation with DCFH-DA in the dark for 20 min at 37 °C, then washed with serum-free medium, cells were digested and centrifuged at $300 \times g$ for 5 min. ROS level was determined using flow cytometry (NovoCyte, Aceabio, USA).

2.5.5 Measurement of T-AOC and SOD levels

T-AOC and SOD activity were determined using T-AOC and SOD detection kit according to manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Protein content was quantified using bicinchoninic acid (BCA) protein assay kit (Wanleibio, Shenyang, China).

2.5.6 Transfection with green fluorescent protein-light chain 3 (GFP-LC3) plasmids and detection of punctate LC3-positive structures

After cells reached 70% confluence, GFP-LC3 fusion protein were transfected into GES-1 cells using Lipofectamine 3000 (Invitrogen, USA) as the transfection reagent for 24 h. After that, the cells were pre-treated with DOFF (320 µg/mL) extract for 24 h and then treated with ethanol for 4 h to induce a GES-1 cell damage model. Punctate LC3-positive structures were visualized using fluorescence microscope (IX53, Olympus, Japan). GFP-LC3 puncta were calculated manually from at least 50 cells for each experiment for reproducibility.

2.5.7 Immunofluorescence imaging

Briefly, cell suspension (1×10^5 cells/mL) was inoculated on coverslips. GES-1 cells on the coverslips were fixed with 4% paraformaldehyde for 15 min, cells were washed 3 times with PBS, incubated with 0.1% Triton X-100 for 30 min, washed 3 times with PBS and dripped with 1% BSA at room temperature for 15 min. Cells were incubated with Bcl-2, light chain 3β (LC3β), and heme oxygenase-1 (HO-1) antibodies (Affinity Biosciences Ltd.). Afterward, cells were diluted at 1:10 with goat serum and stored overnight at 4 °C. Cells were subsequently washed three times with PBS, and the goat anti-rabbit antibody (1:200) was incubated at room temperature for 60 min, washed with PBS, and then stained with 4',6-diamidino-2-phenylindole (DAPI). Images were observed and collected under the fluorescence microscope (BX53, Olympus, Japan).

2.5.8 Western blot

GES-1 cells were homogenized using lysis buffer containing proteases and phosphatase inhibitors, with protein concentration determined using BCA assay. Protein (40 µg) was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes, blocked using 5% skimmed milk for 1 h at room temperature, and further incubated with primary antibody at 4 °C overnight. After washing with Tris-buffered saline containing 0.1% Tween-20 (TBST), membrane was incubated with a secondary antibody at 1:5 000 dilution and visualized using ECL chemiluminescent detection system (Wanleibio, Shenyang, China). Bands were analyzed using gel image

Table 2
MS data for characterization of compounds in the flowers of *D. officinale* by UPLC-Q-TOF/MS.

No.	Retention time (s)	[M-H] ⁺ (m/z)	Formula	Name	Class	Reference
1	0.78	293.096 8	C ₁₇ H ₂₆ O ₄	Unknown phenol	Phenol	
2	1.41	312.095 2	C ₁₆ H ₂₇ NO ₅	Heliotrine	Alkaloid	[89]
3	1.83	164.071 5	C ₉ H ₈ O ₃	3-Hydroxycinnamic acid	Phenolic acid	[90]
4	1.84	328.140 2	C ₁₆ H ₂₇ NO ₅	Heliotrine, N-oxide	Alkaloid	[89]
5	2.18	341.087 6	C ₂₀ H ₂₂ O ₅	Arisantetralone A	Phenol	[91]
6	2.48	325.092 2	C ₁₈ H ₁₄ O ₆	5-Hydroxy-7-acetoxy-8-methoxyflavone	Flavone	
7	3.00	577.154 3	C ₂₇ H ₃₀ O ₁₄	Violanthin	Flavone	[92-94]
8	3.03	609.143 0	C ₂₇ H ₃₀ O ₁₆	Rutin	Flavone	[63,92,93,95,96]
9	3.28	593.148 5	C ₂₇ H ₃₀ O ₁₅	Kaempferol-3-O-rutinoside	Flavone	[63,92]
10	3.30	271.024 7	C ₁₅ H ₁₂ O ₅	Naringenin	Flavone	[92,93,95,96]
11	3.39	563.139 0	C ₂₆ H ₂₈ O ₁₄	Schaftoside	Flavone	[92-96]
12	3.68	739.185 1	C ₃₂ H ₃₄ O ₂₀	Apigenin-6-C-pentosyl-8-C-(2''-O-quinoyl)-glucoside	Flavonoid	[95]
13	4.74	921.282 8	—	—	—	
14	4.93	905.285 6	—	—	—	
15	5.25	329.233 2	C ₁₉ H ₂₂ O ₅	Dihydroconiferyl-dihydro- <i>p</i> -coumarate	Phenolic acid	[95]
16	5.53	535.299 9	C ₃₀ H ₃₂ O ₉	Isolappaol A	Phenol	[97]
17	5.80	329.233 8	C ₁₉ H ₂₂ O ₅	Dihydroconiferyl-dihydro- <i>p</i> -coumarate	Phenolic acid	[95]
18	6.79	311.221 4	C ₁₈ H ₁₆ O ₅	3',4',7-Trimethoxyflavone	Flavone	
19	7.89	564.331 1	C ₂₆ H ₂₈ O ₁₄	Vicenin-3	Flavone	[98]
20	8.56	566.346 1	C ₂₆ H ₃₀ O ₁₄	Pyrroside B	Flavonoid	
21	8.63	347.170 4	C ₁₇ H ₁₆ O ₈	Hydroxysulochrin	Arene	
22	9.86	339.232 1	C ₁₉ H ₁₆ O ₆	Unknown flavonoid	Flavonoid	
23	10.11	379.232 1	C ₁₇ H ₁₆ O ₁₀	8-Hydroxy-5- <i>O</i> -β-D-glucopyranosylpsoralen	Phenolic glycoside	[99]
24	10.91	423.326 0	C ₂₅ H ₂₈ O ₆	Unknown flavonoid	Flavonoid	
25	11.29	327.289 2	C ₁₉ H ₂₀ O ₅	Unknown flavonoid	Flavonoid	
26	11.38	654.335 8	—	—	—	
27	12.82	433.264 3	C ₂₁ H ₂₂ O ₁₀	Isoengletin	Flavonoid	[100]
28	16.30	595.288 9	C ₃₂ H ₃₆ O ₁₁	9- <i>O</i> -Feruloyl-5,5'-dimethoxylariciresil	Lignan	

Note: — Means that the corresponding name was not found in the literature.

analysis system (Gel-Pro-Analyzer Software). All target proteins were normalized against the loading positive control β -actin.

2.6 Statistical analysis

All of the test values were expressed as means $\pm$ standard deviation (SD) in triplicate measurements. The results were analyzed with IBM SPSS Statistics 26.0. One-way ANOVA was used to make comparisons among groups, with P -values < 0.05 considered to indicate a statistically significant difference.

3. Results

3.1 Characterization of DOFF flavonoids composition using UPLC-Q-TOF/MS

As showed in section 2.2, flavonoids were estimated at 52.34% in the ethanol extract as measured using sodium nitrite-aluminum nitrate colorimetry suggestive for flavonoids enrichment. Further, UPLC-Q-TOF/MS was used to profile flavonoids in ethanol extract of *D. officinale* flowers to be used in biological assays. The chromatogram obtained for DOFF was shown in Fig. S1, a total of 28 chromatographic peaks were detected in negative ion mode, of which 25 were annotated (Table 2). Extractive components were analyzed by comparing retention times and MS data of detected compounds with those reported in the literature along with searching online public databases. Among peaks, 14 belonged to flavone or flavonoid compounds, other classes included alkaloids, arenes, phenolic glycosides, lignans, phenols, and phenolic acids.

3.2 DOFF mitigated ethanol-induced mucosal injury

First, DOFF protective effect on gastric mucosa in a mouse model of ethanol-induced gastric mucosal injury was assessed. As shown in Fig. 1A, severe tissue damage and visible hemorrhagic mucosal injuries were found in the stomach of ethanol-treated mice as manifested by irregular dark red hemorrhagic streaks and diffuse gastric edema with mucosal erythema and erosion. In contrast, positive drug control of OME group showed only small punctate type damage (Fig. 1A). As expected, ethanol caused ulcerative

bleeding on mice's gastric mucosal surface with an UI of 64.33 ± 8.76 ($P < 0.001$), while gastric mucosal surface of mice in the normal group was smooth and uninjured, with no bleeding spots or ulcers. Compared with the model group, UI and degree of gastric mucosal damage in DOFF group decreased gradually with increase in administered dose (44.33 ± 9.31 and 32.00 ± 4.47 , respectively) (Table 3). Such amelioration indicated that DOFF could improve gastric ulcer status in mice and to protect its gastric mucosa.

Mucus is one of the major components of the "gastric mucosal barrier", which covers the mucosal surface. Mucus contents assay was performed by Alcian blue binding assay to assess for antiulcer effect. Compared with the control group, mucus content of the model group was significantly reduced to reach 0.38 mg Alcian blue/g wet stomach ($P < 0.001$). The loss of mucus content was significantly reduced in case of OME group compared with model group, and DOFF exerted a dose-dependent protective effect on mucus content due to ethanol administration (Fig. 1C). Gastric acid is produced by gastric parietal cells and secreted into the stomach at a typical pH of 2–3 as maintained by the proton pump H^+ and K^+ -ATPase. Gastric pH of mice was detected at 3.0 (fed) and 4.0 (fasted), which was higher than that of humans^[57]. The pH of gastric fluid collected at the time of sacrifice were significantly decreased in the ethanol-induced gastric ulcer model group (2.25 ± 0.52) when compared with normal group (3.92 ± 0.58 ; $P < 0.001$), while mice pre-treated with high-dose DOFF group (2.92 ± 0.58) or OME group (3.33 ± 0.41) in combination with ethanol, showed a significantly increased pH when compared with the ulcer model group (Table 3).

Table 3

The effects of different dosages of DOFF and Omeprazole on ethanol-induced gastric ulcer.

Group	pH	UI	Ulcer inhibition rate (%)
Control	3.92 ± 0.58	–	–
Model	$2.25 \pm 0.52^{***}$	$64.33 \pm 8.76^{***}$	–
DOFF (200 mg/kg)	2.67 ± 0.61	44.33 ± 9.31	31.09 ± 14.47
DOFF (400 mg/kg)	$2.92 \pm 0.58^{\#}$	32.00 ± 4.47	$50.84 \pm 6.95^{\#}$
OME	$3.33 \pm 0.41^{##}$	$19.00 \pm 7.46^{##}$	$70.47 \pm 11.59^{###}$

Note: – Means that the data is not applicable. Data are presented as means $\pm$ SD of 6 mice per group. $^{\#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ vs. Model group; $^{***}P < 0.001$ vs. Control group.

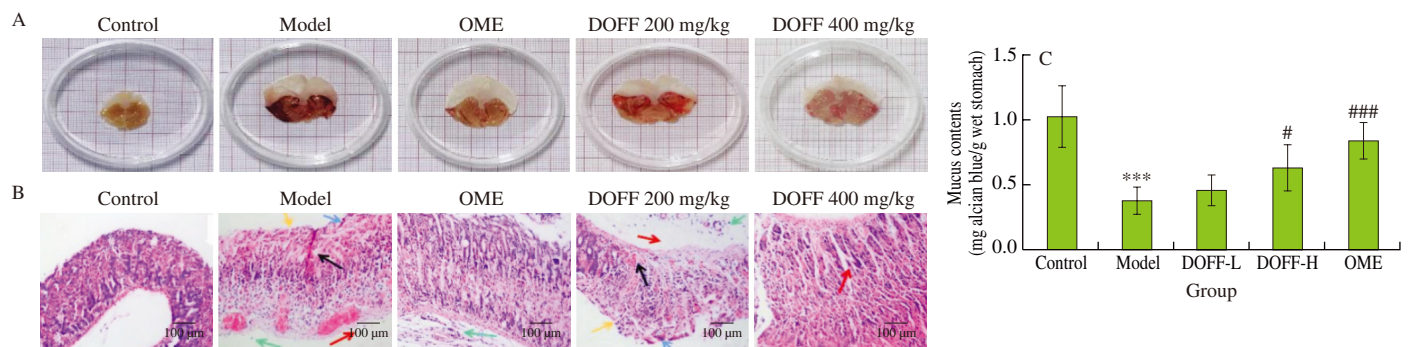


Fig. 1 Effect of DOFF on ethanol-induced gastric mucosal injury in mice. (A) Typical appearance of gastric mucosal tissue under different treatments. (B) Microscopic representative images of gastric mucosa under different treatments HE stained, 200 $\times$ magnification; Scale bar 100 μ m. White arrow: healthy appearance or with minimal changes in the coating structure and mucosal layer; Yellow arrow: superficial epithelial cell loss; Blue arrow: focal necrosis; Red arrow: edema; Black arrow: hemorrhagic injury; Green arrow: inflammatory cells infiltration. (C) Bar chart showing difference in mucus content among different treatments using alcian blue binding assay. Data are presented as mean $\pm$ SD of 6 mice for each group; $^{\#}P < 0.05$, $^{##}P < 0.01$ vs. model group; $^{***}P < 0.001$ vs. control group.

3.3 Histological examination of gastric lesions

Histological examination of the stained stomach sections revealed serious damaging effects on gastric mucosa integrity of the ulcer control group compared to a normal group as manifested by severe damage and superficial epithelial cell loss, focal necrosis in the mucosal layer with edema and infiltration of inflammatory cells in underlying submucosa. Notably, the pathological injury of gastric mucosa was reduced significantly by pretreatment with DOFF and OME compared with model group (Fig. 1B). Mucus secretion in the gastrointestinal tract is an important mucosal defensive factor during gastric injury, with mucopolysaccharide in mucus serves as the main protective layer on gastric epithelium. Herein, PAS staining was employed to examine acid mucopolysaccharide level on the surface of gastric mucosa as illustrated by the number of blue-violet spots. The ulcer model group exhibited a remarkable decrease in PAS staining intensity, compared with normal group. The loss in mucosa glycoprotein was significantly reduced by pre-treatment with OME (Fig. 2J). The DOFF group showed a significant protective effect in a dose-dependent manner compared with model ulcer group (Fig. 2J) and supportive of DOFF protective effect on gastric mucosa against ethanol-induced damage.

3.4 Effects of DOFF on PGE₂ and NO levels

NO is one of the reactive nitrogen species that acts as an important radical molecule in intracellular signaling pathway and to exert a protective role in vascular dilation. However, if excessive is generated this radical can be destructive^[58]. Considering its biological role, NO level was measured in the serum of experimental groups. NO level showed elevation in ethanol-induced gastric ulcer group. Compared

with the model group, NO level in the high-dose DOFF group was significantly reduced by 40.6% (Fig. 2A). PGs are one of the most fundamental gastroprotective factors, mainly PGE₂ with decreased level at the gastric mucosa associated with gastric ulceration. Compared with the control group, model group significantly decreased PGE₂ level by 60.1%. In contrast, compared with the model group, DOFF pretreatment at 200 and 400 mg/kg doses significantly improved PGE₂ level by 30.3% and 70.7%, respectively, versus 85.8% increase of PGE₂ in OME group (Fig. 2B).

3.5 Effects of DOFF on TNF- α , IL-6, IL-1 β and MPO levels

The inflammatory response was activated upon gastric mucosal damage, which might lead to secondary mucosal damage. TNF- α , IL-6, and IL-1 β were identified as the crucial cytokines involved in inflammation-related diseases^[59]. The production of these inflammatory factors was detected in serum levels including TNF- α , IL-6, and IL-1 β . Compared with the control group, TNF- α expression increased in the model group; on the other hand, compared with the model group, DOFF (200 and 400 mg/kg) showed decrease by 14.2% and 37.3%, respectively. Likewise, DOFF in the high-dose group showed decrease in IL-6 (30.0%, $P < 0.01$) and IL-1 β (39.9%, $P < 0.001$), ameliorating the symptoms of ethanol-induced gastric ulcer (Figs. 2C–E). To confirm results of gastric tissue invasion by inflammatory cells, gastric biochemical marker MPO was further assessed revealing increased activity at 4.0-fold versus control group. Relative to the model group, high-dose DOFF group alongside OME groups significantly reduced MPO activity, detected at 2.0- and 1.5-fold compared with the control group, respectively (Fig. 2F). In conclusion, DOFF appears to exert a protective effect in response to ethanol-induced neutrophils infiltration resulting in an imbalance of inflammatory cytokines.

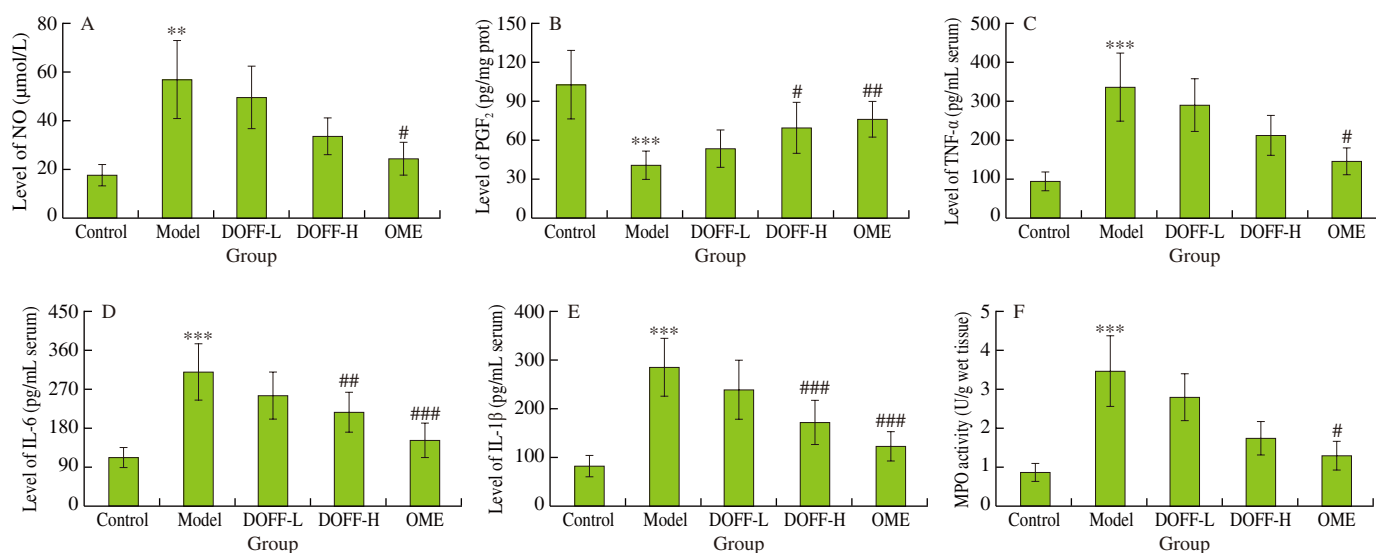


Fig. 2 DOFF protective action against ethanol-induced damage of mice gastric mucosa as revealed using inflammatory response for its different marker. Effects of DOFF (200 and 400 mg/kg bw) and OME (20 mg/kg bw) on levels of (A) NO, (B) PGE₂, (C) TNF- α , (D) IL-6, (E) IL-1 β , (F) MPO activity, and (G) NP-SH content against ethanol-induced gastric ulcer in mice. DOFF prevents against oxidant response in gastric ulcer tissue. Levels of (H) MDA and (I) SOD in the ethanol-induced gastric ulcer tissue at the time of 7 days after DOFF (200 and 400 mg/kg) and OME (20 mg/kg) treatment. (J) Microscopic representative images PAS stained, magnification 200 $\times$; scale bar: 100 μ m. The carmine color in the apical epithelial cells represents PAS-positive staining. The black arrows showed no fuchsia. Data are presented as means $\pm$ SD of 6 mice for each group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. model group; ** $P < 0.01$, *** $P < 0.001$ vs. control group.

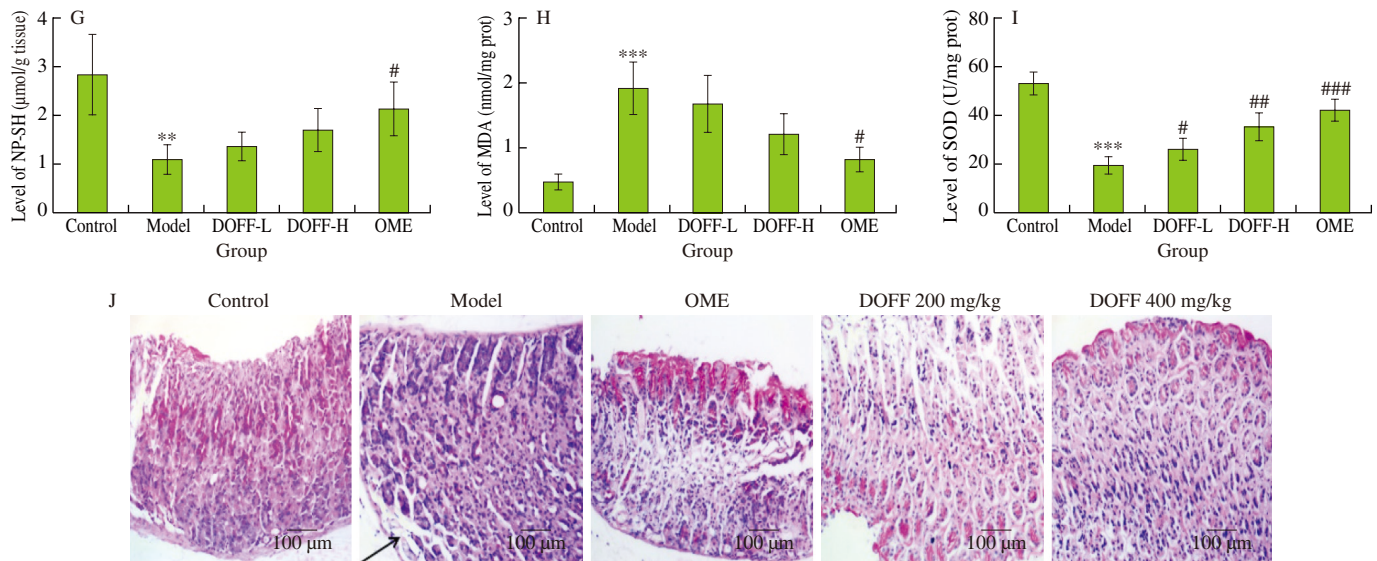


Fig. 2 (Continued)

3.6 Effect of DOFF on ethanol-induced mucosal NP-SH depletion

NP-SH plays an important role in tissue protection by scavenging prooxidants, endogenous NP-SH complex is a major component in mucosal protection against ethanol-induced stomach damage^[60]. Assay results showed that ethanol significantly decreased gastric mucosal NP-SH level compared to the control group ($P < 0.01$). In contrast, an increase in NP-SH level was observed in the OME positive drug group compared to the model group. Although that both low-dose (200 mg/kg) and high-dose (400 mg/kg) of DOFF showed improved NP-SH level, no statistical significance was observed (Fig. 2G). These findings suggested that NP-SH may be involved in the protective gastric action of DOFF.

3.7 DOFF improved gastric lesion via inhibition of oxidative stress

Oxidative stress is a critical pathogenic factor during gastric mucosal injury, MDA level is commonly employed as an indicator of lipid peroxidation and oxidative damage^[59]. In the current study, gastric MDA level was used to evaluate DOFF effect on oxidative damage. Intragastric ethanol administration induced a significant increase in MDA levels at 4.00-fold versus control group ($P < 0.001$) attributed to lipid peroxidation and oxidative damage due to ethanol administration. MDA level in mice treated with 400 mg/kg DOFF and OME ($P < 0.05$) showed decrease by 2.5- and 1.71-fold, respectively (Fig. 2H). In contrast, SOD activity was used to evaluate DOFF antioxidant effect in ethanol ulcer model. A significant reduction in ethanol-induced SOD enzymatic activity ((19.14 ± 3.73) U/mg prot, $P < 0.001$) was detected, and antagonized using OME ((42.03 ± 4.54) U/mg prot, $P < 0.001$) and 400 mg/kg DOFF ((35.24 ± 5.89) U/mg prot, $P < 0.01$) pretreatment (Fig. 2I). These findings suggested that DOFF extract may attenuate oxidative stress induced by ethanol in mouse ulcer model of acute gastric ulcer.

3.8 DOFF modulates GES-1 cell apoptosis via ROS-dependent pathway

GES-1 cells, one of the most used cell-lines in gastric mucosal injury^[61], was exposed to 5% ethanol for 4 h according to previous reports^[62]. To assess for any deleterious effect of DOFF on GES-1, CCK-8 assay was first performed. A dose-dependent significant decrease in GES-1 cells viability was observed, especially at the highest dose level (1 000 $\mu\text{g/mL}$) at cell viability level of 82.7% ($P < 0.01$; Fig. 3A). In contrast, no obvious toxicity was detected on GES-1 cells at 3 doses of 80, 160, and 320 $\mu\text{g/mL}$ selected for subsequent experiments. To determine whether DOFF could protect gastric epithelial cells from ethanol-induced viability loss, GES-1 cells were pretreated with DOFF (80, 160, 320 $\mu\text{g/mL}$), 50 nmol/L RAPA, 2 mmol/L 3-MA, and 5 mmol/L NAC prior to its incubation with 5% ethanol for 4 h. Results showed that cell viability in the ulcer model group (27.16%, $P < 0.001$) was significantly reduced relative to the control group. Pretreatment with DOFF at 80 (33.9%), 160 (50.4%), and 320 $\mu\text{g/mL}$ (61.7%) remarkably improved decrease in cell viability compared with the model group, which was consistent with prior reports^[63]. The best effect observed at a dose of 320 $\mu\text{g/mL}$ was thus selected for further *in vitro* assays. Moreover, administration of autophagy agonist RAPA and ROS scavenger NAC to cells pretreated with DOFF showed increased survival rate of GES-1 to reach 78.1% and 79.8% respectively, compared with model group. To confirm that autophagy contributed to DOFF cellular protection against ethanol, pretreatment with inhibitor autophagy, 3-MA was attempted and to exert suppressed cell viability lower than that of the model group. GES-1 viability treated with 3-MA and DOFF (44.9%, $P < 0.001$) was higher than that treated only with 3-MA (10.2%; Fig. 3B).

To further validate the effects of DOFF on ethanol-induced GES-1 cells and its antioxidant protective effects, GES-1 cells were pretreated with DOFF (320 $\mu\text{g/mL}$), 50 nmol/L RAPA (autophagy agonists), 2 mmol/L 3-MA (autophagy inhibitors), and 5 mmol/L NAC (ROS scavenger) prior to incubation with 5% ethanol for 4 h. In the control group, apoptosis rate was detected at $(4.51 \pm 0.68)\%$, and

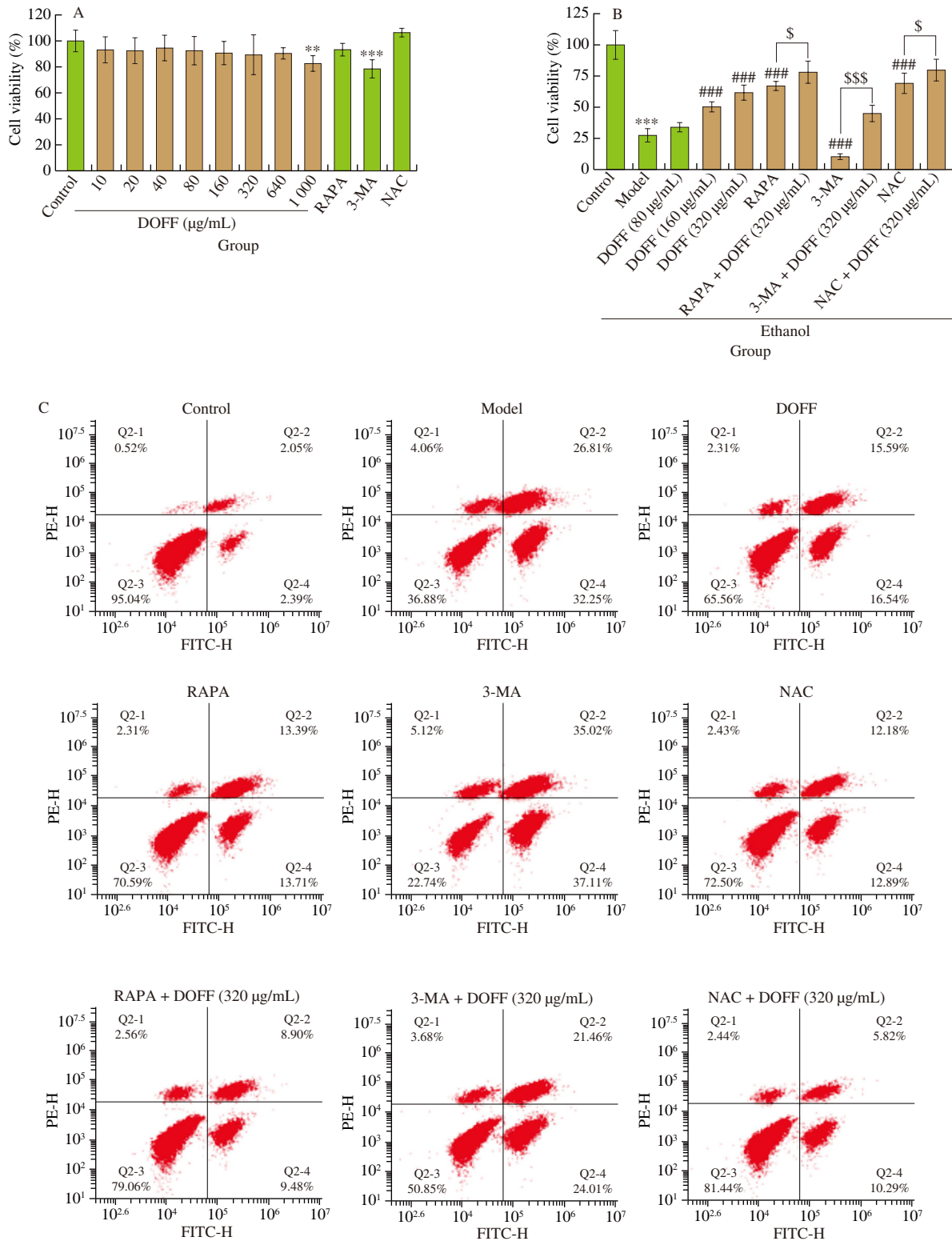


Fig. 3 Ethanol exposure and DOFF treatment regulated ethanol-induced GES-1 cell growth and mitochondrial apoptosis of GES-1. (A) The cell viability of GES-1 cells treated with DOFF (10, 20, 40, 80, 160, 320, 640 and 1 000 µg/mL) was detected. (B) CCK-8 assay kit used to detect DOFF (80, 160, 320 µg/mL), RAPA, 3-MA and NAC acted cell viability of ethanol-induced GES-1 cells. (C) Apoptotic cells analyzed using flow cytometry of Annexin V-FITC/PI staining assay. (D) Western blot for apoptosis-related proteins Bcl-2, Bax and cleaved Caspase-3 expression in GES-1 cells. Images of representative expression patterns are presented. (E) Quantification of apoptotic cells of the indicated groups. Quantification of relative expression level of (F) Bcl-2/Bax and (G) Caspase-3 in treatment groups. (H) Immunohistochemical image of Bcl-2 measured using immunofluorescence among different treatments. Error bars are presented as mean $\pm$ SD of 3 independent experiments. $^{###}P < 0.001$ vs. model group; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{\$}P < 0.05$, $^{sss}P < 0.001$ vs. RAPA group or 3-MA group or NAC group; $^{\&\&}P < 0.05$, $^{\&\&\&}P < 0.001$ vs. DOFF group.

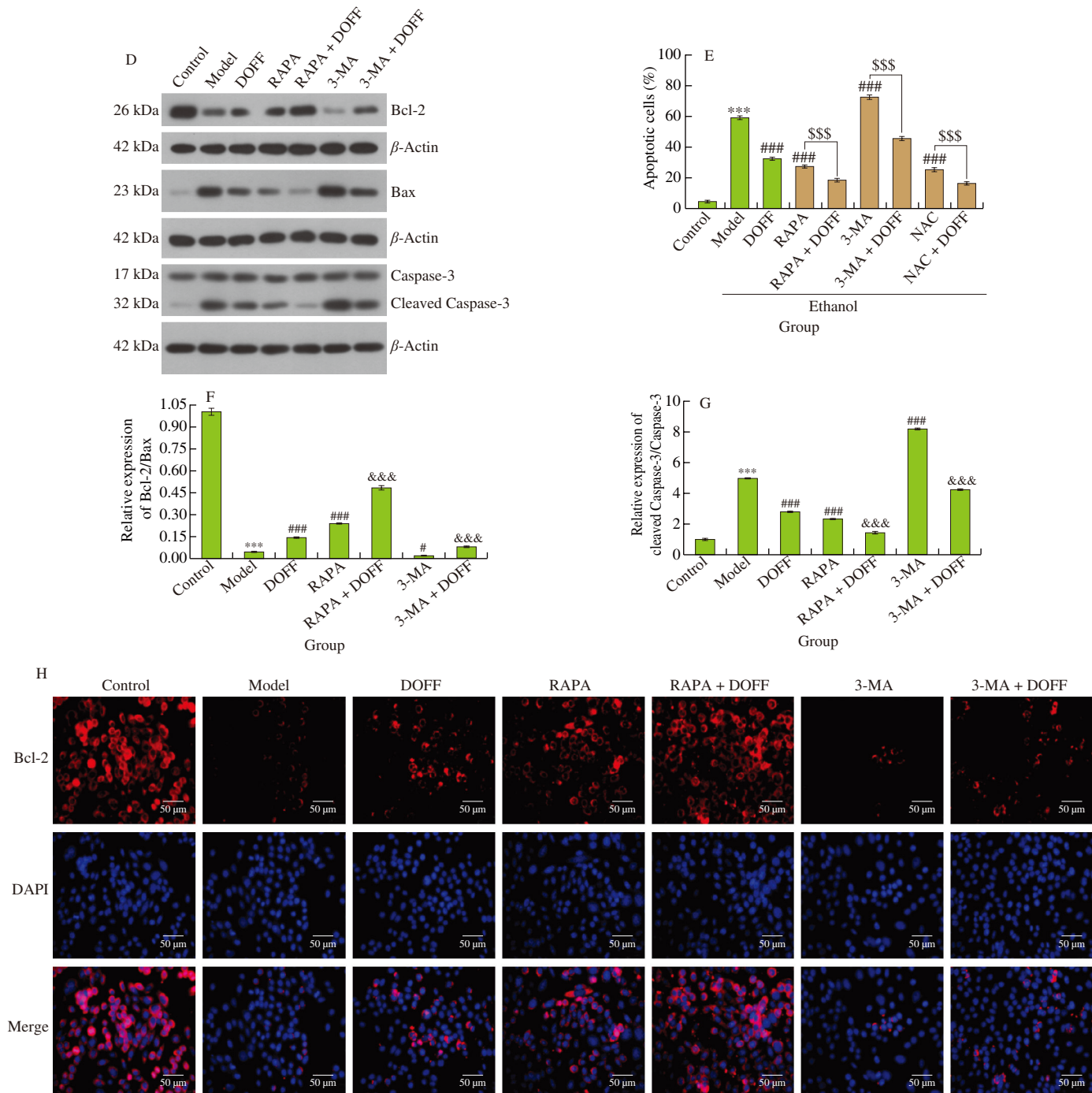


Fig. 3 (Continued)

DOFF ($32.28 \pm 1.13\%$) significantly reduced upregulated apoptosis rate induced by ethanol ($59.04 \pm 1.10\%$, $P < 0.001$) (Figs. 3C and E). Compared with RAPA ($27.18 \pm 1.04\%$), 3-MA ($72.26 \pm 1.43\%$), and NAC ($25.18 \pm 1.18\%$) alone, apoptotic rate was significantly reduced after the addition of DOFF ($P < 0.001$). Immunoblotting was used to detect the expression of apoptotic proteins at the targeted protein expression level (Fig. 3D). The apoptotic markers, cleaved Caspase-3 (4.95-fold) was significantly upregulated in GES-1 cells post ethanol treatment compared to negative control. It is noteworthy that expression of the antiapoptotic marker Bcl-2/Bax (0.05-fold) was downregulated by ethanol treatment. DOFF (2.80-fold), RAPA with DOFF (1.44-fold), and 3-MA with DOFF (4.25-fold) pretreatment

all significantly decreased upregulated expression level of cleaved Caspase-3 induced by exposure of cells to ethanol. More importantly, after DOFF and RAPA with DOFF pretreatment, expression level of Bcl-2/Bax was recovered to reach 0.15- and 0.48-fold, respectively (Figs. 3F and G). These results indicated that DOFF can alleviate ethanol induced GES-1 cell apoptosis. As shown in Fig. 3H, expression level of Bcl-2 detected using immunofluorescence was significantly increased in DOFF and RAPA groups as compared with ulcer model group. ROS can act as an upstream signaling molecules to trigger cell apoptosis. These results indicated that DOFF regulated ethanol-induced apoptosis in GES-1 cells mediated *via* ROS-dependent pathways.

3.9 Ethanol exposure-induced oxidative stress and mitochondrial dysfunction in GES-1 cells

In vitro assay revealed that DOFF attenuated oxidative stress, which was a critical index as determined from levels of SOD and T-AOC on GES-1. Antioxidant enzymes (such as SOD) function to detoxify ROS intermediates or repair damage when intracellular ROS levels are increased. Compared with the control group, ethanol significantly reduced T-AOC and SOD activities. Pretreatment of GES-1 cells with DOFF and DOFF + NAC further improved activities

of T-AOC and SOD to varying degrees concurrent with an inhibition of oxidative stress damage of ethanol to cells (Figs. 4F and G). In addition, HO-1 was retained in GES-1 of the model group as determined using immunofluorescence staining for the darker reddish-blue fluorescence. DOFF and RAPA treatments substantially improved HO-1 expression level, by increasing the intensity of the reddish-blue staining (Fig. 4I). Furthermore, Western blot results showed decrease in HO-1 protein expression in the ulcer model group (0.30-fold) compared with the control group, level of antioxidant enzyme expression was significantly increased after DOFF treatment

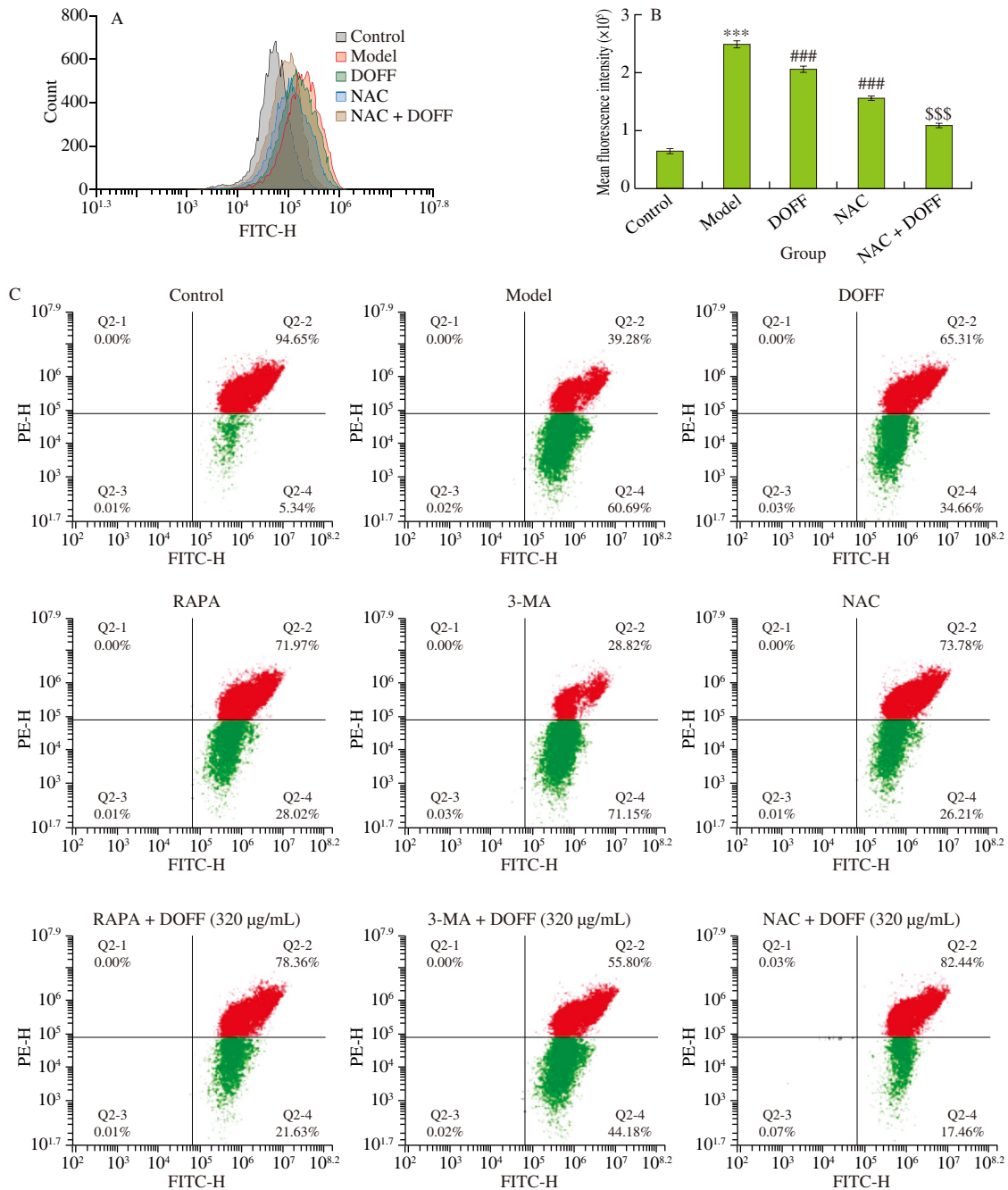


Fig. 4 DOFF modulates ethanol-induced oxidative stress and mitochondrial dysfunction in GES-1 cells. (A) ROS level as determined by DCFH-DA fluorescence probe. (B) Quantification of data in (A). (C) Mitochondrial membrane potential measured using JC-1 assay kit. (D) Quantification of JC-1 monomers. (E) The relative protein expression levels of HO-1 and β -actin in GES-1 cells were analyzed by Western blotting. Treatment of DOFF increased T-AOC and SOD levels in ethanol-induced GES-1 cells. (F) T-AOC activity. (G) SOD activity. (H) Statistical analysis on HO-1. (I) Immunohistochemical image of HO-1 expression measured using immunofluorescence. Results are presented as the mean $\pm$ SD of 3 independent experiments; ^{***} $P < 0.01$, ^{###} $P < 0.001$ vs. model group; ^{***} $P < 0.001$ vs. control group; ^{\$} $P < 0.05$, ^{\$\$\$} $P < 0.001$ vs. RAPA group or 3-MA group or NAC group; ^{&&&} $P < 0.001$ vs. DOFF group.

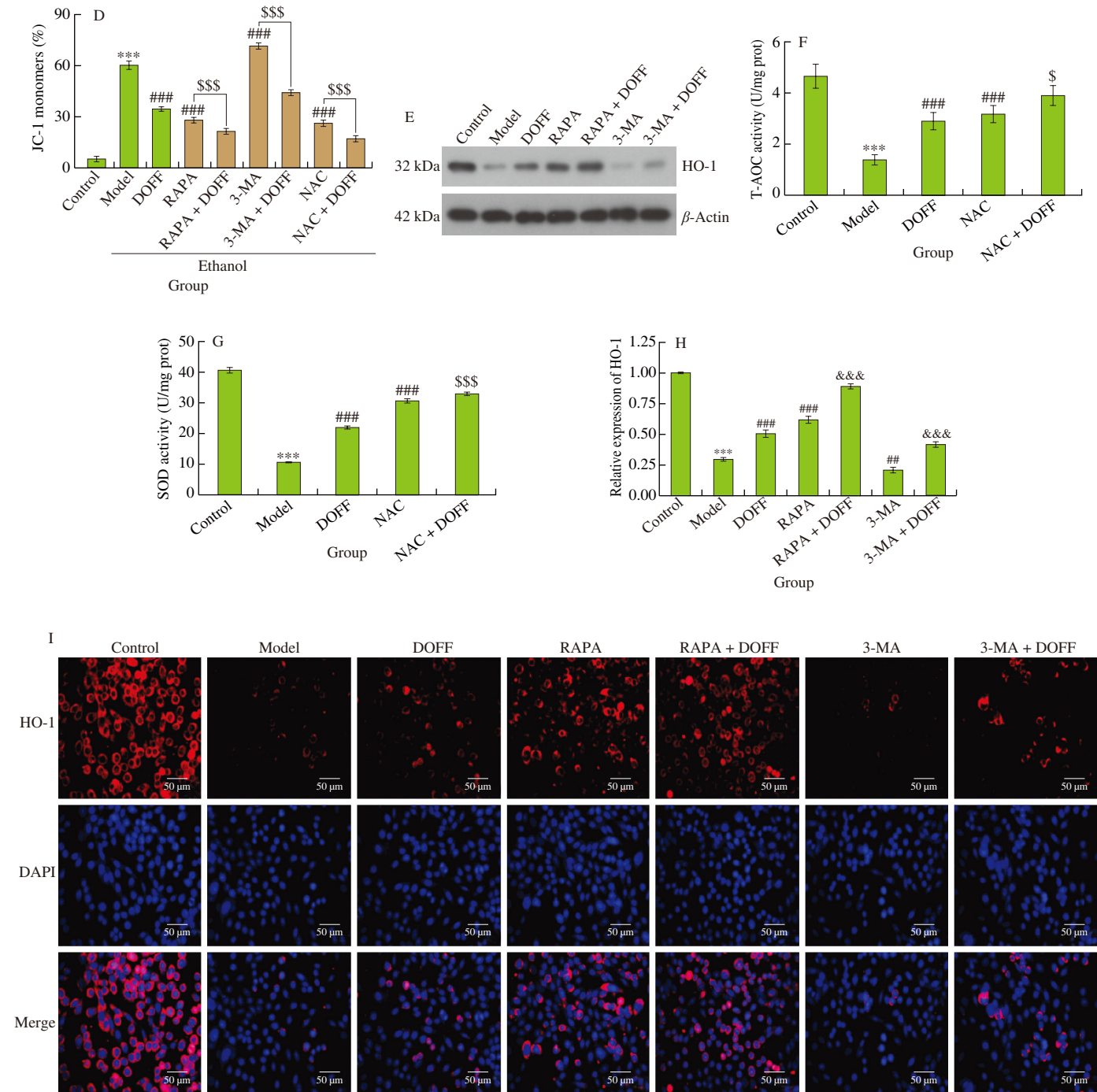


Fig. 4 (Continued)

(0.51-fold) (Figs. 4E and H) and confirming the hypothesis that DOFF exert potential protective effects against oxidative stress from ethanol in gastric mucosal cells likely attributed for its rich flavonoids composition.

MMP ($\Delta\Psi_m$) decreases early in apoptosis, and the loss of MMP can lead to the release of apoptotic-inducing factors and caspase activators^[64]. Upon change in MMP to high level, JC-1 accumulates inside the mitochondrial matrix to form a polymer. Whereas at low MMP JC-1 becomes a monomer. To further evaluate whether DOFF could alleviate ethanol-induced apoptosis, we used JC-1 to detect any alteration in the MMP. Flow cytometry assay results demonstrated

that in control cells, the descent rate of $\Delta\Psi_m$ was at $(5.36 \pm 1.41)\%$, while it was increased to reach $(60.49 \pm 2.28)\%$ post ethanol treatment. Results showed a decrease in MMP after pretreatment with DOFF, RAPA, and NAC ($P < 0.001$) and in agreement with *in vivo* results (Figs. 4C and D). Mitochondrial metabolism is the major source of ROS, and several studies have shown that ROS accumulation could cause cell apoptosis *via* mitochondria-dependent pathway. Intracellular ROS level appeared to be affected by DOFF using DCFH-DA. As shown in Fig. 4A, ROS levels showed increase in the model group, with shifts of the fluorescence peaks in the groups after pretreatment with DOFF was alleviated, and likewise in case

of NAC pretreatment. NAC is an antioxidant that can inhibit ROS generation. To further assess whether NAC inhibited ethanol-induced increase in ROS levels, GES-1 cells were pretreated with DOFF (320 $\mu\text{g/mL}$) for 24 h, cells were cocultured with NAC (5 mmol/L) for another 2 h. Co-incubation with NAC effectively blocked ROS production and demonstrating that DOFF could attenuate cellular ROS accumulation under *in vitro* conditions.

3.10 DOFF induced expression of autophagy-related proteins in GES-1 cells overstimulated with ethanol

As ROS is closely related to autophagy, assessment of whether DOFF induced autophagy in GES-1 cells was considered targeting LC3 protein as another specific marker for autophagosome formation visualized using the green fluorescent protein (GFP)-fused LC3. Autophagy induction was assessed by measuring GFP-LC3 puncta level formed in transformed cells. Under fluorescence microscope, in control, green fluorescence diffused regularly throughout the cytoplasm. Similarly, the numbers of GFP-LC3 puncta were not altered by ethanol treatment (control vs. ethanol: 6.40 ± 2.07 vs. 7.40 ± 4.27) (Figs. 5A and B). In contrast, numbers of GFP-LC3 spots showed increase post combined ethanol and DOFF treatment ($P < 0.001$) suggestive for autophagy induction. It was also suggested that DOFF may induce LC3-II production and autophagosome formation. Changes in autophagy flux was further determined as mTOR signaling is a vital upstream regulator of autophagy, RAPA

was used to induce autophagy, which served as the positive control, and used 3-MA to inhibit autophagy. As expected, LC3 β II/LC3 β I and Beclin-1 protein levels were significantly upregulated after RAPA treatment of GES-1 cells, while p62, a marker for autophagic flux showed decrease, with opposite results post 3-MA treatment. Results revealed the DOFF combined with RAPA significantly increased LC3 β II/LC3 β I and Beclin-1 protein levels, concurrent with decreased p62 protein levels compared with DOFF treatment alone. Co-administration of 3-MA and DOFF significantly increased p62 protein level ($P < 0.001$). In addition, Western blot was further employed to assess expression of autophagy markers at protein level in comparison to immunofluorescence results. Expression levels of LC3 β II/LC3 β I (0.43-fold), and Beclin-1 (0.48-fold) in the model group showed decrease, whereas higher expression level of p62 (3.75-fold) was detected compared with control group. In contrast, pretreatment with DOFF upregulated expression level of LC3 β II/LC3 β I (0.58-fold), Beclin-1 (0.69-fold) concurrent with downregulated expression level of p62 (2.14-fold), further suggesting that autophagy was induced by DOFF as one of its protective mechanisms against ulcer (Figs. 5C–F). The immunofluorescence results showed red fluorescence indicative of the positive expression of LC3- β in cells, being attenuated in ethanol-induced GES-1 cells compared with the control group. In contrast, both DOFF and RAPA pretreatments reversed such pattern. Red fluorescence was significantly attenuated in the 3-MA group as compared with DOFF

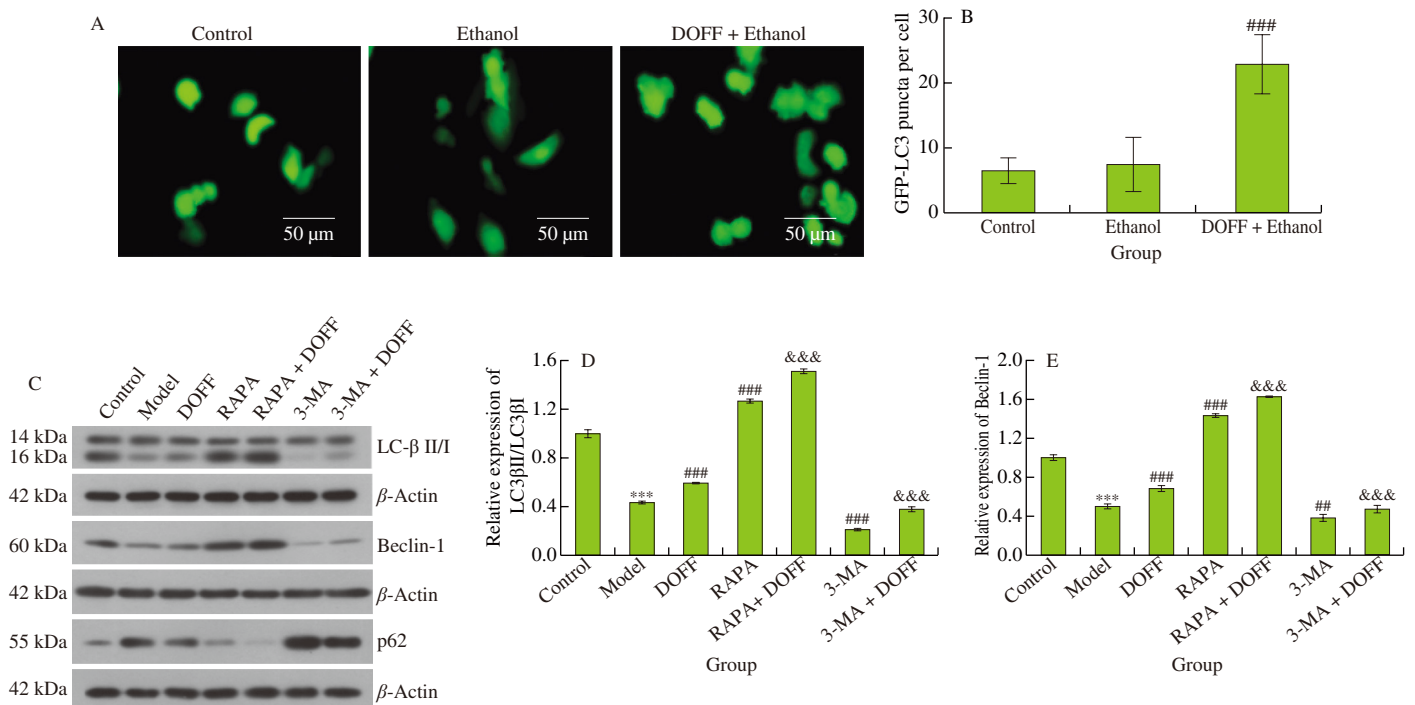


Fig. 5 DOFF activates autophagy *in vitro* via AMPK/mTOR/ULK1 and PI3K/Akt signaling pathway. GES-1 cells were transiently transfected with a GFP-LC3 plasmid and then treated with DOFF for 24 h, autophagosome formation (LC3 dots) was measured; (A) LC3 dots quantification images using fluorescence microscopy in control, ethanol and ethanol + DOFF groups. (B) Quantification of GFP puncta per cell of indicated groups. (C) Expression of LC3 β II/LC3 β I, Beclin-1, p62 detected using Western blot. Images of representative expression patterns are shown. Quantification of (D) LC3 β II/LC3 β I, (E) Beclin-1, (F) p62, (G) p-PI3K p85/PI3K p85 protein expression. (H) The Western blot results of p-PI3K p85, PI3K p85, p-Akt, and Akt. Quantification of (I) p-Akt/Akt, (J) p-AMPK/AMPK, (K) p-mTOR/mTOR, and (L) p-ULK1/ULK1 protein expression. (M–O) The Western blot results of p-AMPK, AMPK, p-mTOR, mTOR, p-ULK1, ULK1. (P) Immunohistochemical image of LC3 β measured by immunofluorescence technique. Data are presented as mean $\pm$ SD of 3 independent experiments; $^*P < 0.05$, $^{***}P < 0.01$, $^{****}P < 0.001$ vs. model group; $^{**}P < 0.001$ vs. control group; $^{\&\&}P < 0.05$, $^{\&\&\&}P < 0.001$ vs. DOFF group.

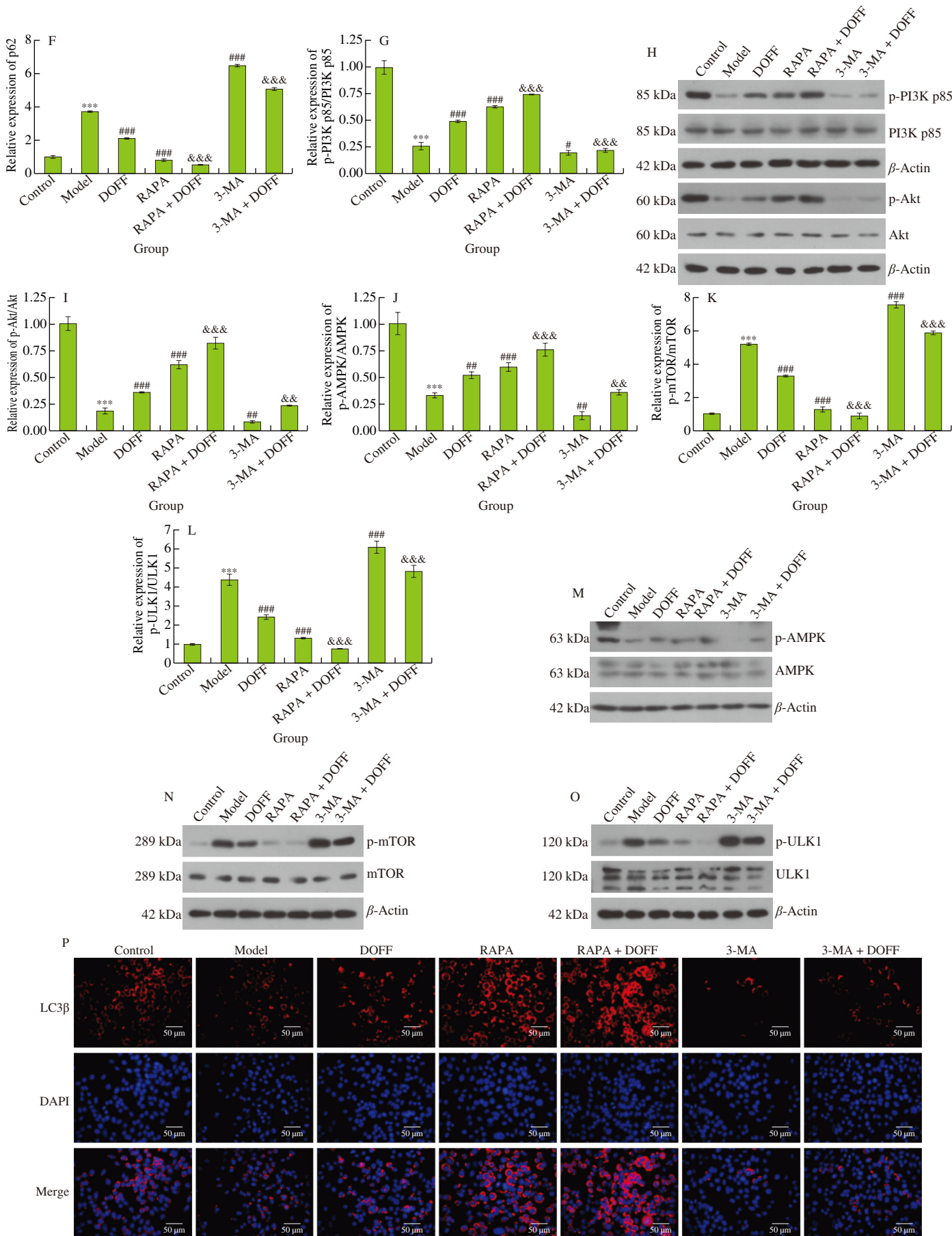


Fig. 5 (Continued)

group. After staining with DAPI, model group showed lower blue fluorescent responses from LC3- β than control group. Whereas, DOFF and RAPA improved blue fluorescence (Fig. 5P).

The mTOR is an essential regulator of autophagy^[65], and its well-established upstream regulator pathways including PI3K/Akt and AMPK. ULK1, a kinase essential for autophagy initiation, was controlled by phospho-mTOR. This study measured protein expression level of AMPK and PI3K/Akt pathways. In GES-1 cells, compared with the control group, expression levels of p-PI3K p85 (0.26-fold), p-Akt (0.20-fold) (Figs. 5G–I), and p-AMPK (0.33-fold) in the ulcer group showed decrease, concurrent with increased expression level of p-ULK1 (4.18-fold) and mTOR (5.22-fold; Figs. 5K, L and O). By contrast, pretreatment with DOFF combined with RAPA evidently upregulated expression level of p-PI3K p85 (0.75-fold), p-Akt (0.84-fold) and p-AMPK (0.80-fold), while downregulating expression level of mTOR (0.85-fold) (Figs. 5J, K, M and N). This result suggested that activation of AMPK/mTOR/ULK1 and PI3K/Akt pathways plays an important role in ethanol-induced autophagy response in GES-1 cells, and yet to be confirmed using *in vivo* model.

4. Discussion

Gastric ulcers are mucosal lesions that can be painful and even lead to gastric perforation and internal bleeding if left untreated. Ethanol can cause edema, erosion, hemorrhage and mucosal necrosis, and upon gastric mucosa dysfunction, it will interfere with its ability to mediate gastric acids and gastric juice release^[66]. According to previous studies, violanthin, rutin, and kaempferol-3-*O*-rutinoside exert anti-ulcer effects which could be detected using UPLC-MS as major flavonoids in *D. officinale* (Table 2)^[67–69]. This study revealed that DOFF exerted a preventive effect on ethanol-induced gastric ulcers. *In vivo* assay showed that DOFF exhibited potential antioxidant and anti-inflammatory activities, reduction of ethanol-induced gastric ulcer injury in mice by modifying the activity of MDA, SOD, NO, PGE₂, MPO, NP-SH, and inhibiting TNF- α , IL-6, IL-1 β . In addition, *in vitro* studies revealed that DOFF restored ethanol-induced GES-1 cell viability, reduced cell apoptosis, and increased total antioxidant capacity and SOD activity to prevent damage in GES-1 cells. Results also revealed that DOFF restored the balance between pro-apoptotic and anti-apoptotic proteins, which indicated that DOFF might be related to decrease in gastric mucosal cell apoptosis.

Increasing evidence revealed that stomach is stimulated after gastric mucosa is damaged, to affect gastric juice being excreted at large amounts leading to a reduction in pH value. These events worsen the gastric mucosal injury, thus intensifying gastric injury^[4]. It was manifested in this study that DOFF effectively increased gastric juice pH of ethanol-induced in mice, thereby protecting gastric tissues and preventing against gastric injuries (Table 3). In addition, macroscopic, microscopic inhibition percentage of ulcers also confirmed such effect. The gastric mucus layer is considered to be the most important defensive gastric factor that acts as the first line to protect gastric mucosa from bacterial infection as well as the diffusion of gastric acid and pepsin. Gastric mucus is composed of mucin glycoproteins, and loss of gastric mucus is considered to be closely related to the pathology of gastric ulcers^[70]. This study revealed that

DOFF retained and enhanced production of gastric-adherent mucus, which enabled viscous mucus layer to cover the gastric tissue against deleterious effect of ethanol.

Another fundamental step involved in the complex event of gastric ulcer progression lies in the expansion and accumulation of neutrophils, especially activated neutrophils that are responsible for the increase in oxidative stress and inflammatory response, which impair the cellular component^[71]. MPO as an index of inflammatory cell expansion and TNF- α , IL-6, IL-1 β levels of inflammatory cytokines were assessed revealing that DOFF exhibited a significant dose-dependent protective effect on neutrophil infiltration^[72]. NP-SH mediates its gastroprotective effect by maintaining integrity of the mucosal layer and antioxidant effect. According to this study, DOFF treatment significantly prevented increase of MDA and NO levels alongside depletion of SOD and NP-SH levels. Furthermore, DOFF improves antioxidant defense system (by participating in free radical scavenging). Previous studies revealed that gastric mucosa homeostasis is regulated by both the control of oxidative stress and by secretion of several mediators such as cytokines and prostaglandins. In particular, prostaglandins play a key role in regulating mucosal blood flow, inhibiting gastric acid secretion, and enhancing mucus and bicarbonate production to maintain mucosal integrity, making PGE₂ a gastric protective agent^[73]. Our results showed that the PGE₂ level of ethanol-induced gastric ulcer was significantly reduced, with DOFF in the high-dose group found comparable restoration of PGE₂ level as the antiulcer drug omeprazole. In conclusion, the protective effect of DOFF on gastric cells appears to function at multiple levels including gastric mucosa protection by increasing or preserving gastric mucus and reducing oxidative stress and inflammatory reaction.

Ethanol can activate cellular responses and increase ROS levels, which is a cytotoxic agent of the stomach mucosa^[74]. The activity of SOD, T-AOC, and HO-1 was consumed by ethanol upon damaging the gastric mucosa^[75]. The detailed reaction of DOFF to GES-1 cytotoxicity was evaluated by analyzing ROS generation, T-AOC, and SOD, HO-1 activity. In our study, DOFF pretreatment reduced production of ROS and improved level of SOD, T-AOC, and HO-1. NAC inhibited ethanol-induced increase in ROS levels in GES-1 cells, whereas both DOFF and NAC acted on the GES-1 cells, the intracellular ROS was decreased relative to DOFF group (Fig. 4). NAC could abolish ethanol-induced ROS accumulation and to block the inhibitory effect of ethanol on cell proliferation, which also shows that ethanol inhibits cell proliferation in a ROS-dependent manner. Mitochondria is not only the important cellular organelle of ROS, but rather the main target of ROS production^[76]. During the process of cell metabolism, mitochondrial electron transport chain continuously produces ROS, which acts as a second messenger alters the MMP, and then its environmental change leading to cell death^[77]. As shown in Fig. 4C, it was found a decrease of MMP level in the model group compared with normal group, while the loss of MMP is regarded as one factor for the stimulation of mitochondrial apoptosis pathway, after cellular stress, pro-apoptotic members of the Bcl-2 family alter the mitochondrial outer membrane permeability (MOMP), permitting the secretion of pro-apoptotic factors from mitochondria^[78], with initiation of caspases' cascade^[79], according to the above results, mitochondrial abnormalities caused by ROS further induced cell apoptosis. The flow cytometry assay obtained same results, as showed in Fig. 3C, which confirmed that ethanol could induce apoptosis of

GES-1 cells. In the meanwhile, the use of DOFF could reduce the apoptosis caused by ethanol, as shown in Fig. 3D, *in vitro* cell assays also revealed that DOFF reversed altered levels of Bcl-2/Bax and Caspase-3. As we know that the specific value of Bcl-2 and Bax equally shows the anti-apoptotic ability of cells, Fig. 3F showed that the use of DOFF increased the ratio of Bcl-2/Bax compared with model group, and the results of Fig. 3G displayed that the ratio of cleaved Caspase-3 to total Caspase-3 was increased in model group and decreased after DOFF treated, which indicated that DOFF might be related to decrease of gastric mucosal cell apoptosis. Combined with the results of the above experiments, it was proved that ethanol can increase ROS levels in cells, and damage the cells not only by physical interaction but also promoting the expression of apoptosis factors by changing MMP, which could lead to cell apoptosis. In addition, the use of DOFF could reverse such adverse changes by regulating the mitochondrial activity and inhibiting cell apoptosis caused by ROS.

Autophagy is an adaptive response to stimulation^[80], which recycles various damaged cellular components to create new cellular structures or provide energy sources^[81]. It has been reported that ROS can activate autophagy. Autophagy is generally confirmed by the accumulation of autophagosomes, conversion of LC3βI to LC3βII, and increased expression of autophagy-related marker^[82]. Thus, assessment of autophagic flux is an important tool to further confirm autophagy activation. The p62 is a ubiquitin-binding protein degraded by autophagy, p62 can be selectively incorporated into autophagosomes through direct binding with LC3 and efficiently degraded during autophagic process thus is widely used to assess autophagic flux^[83]. Beclin-1 as the mammalian ortholog of yeast Atg6, which plays a central role in autophagy. In this study, LC3βII/LC3βI and Beclin-1 protein expression levels were shown to be enhanced by DOFF pretreatment of ethanol-treated GES-1 cells indicating that DOFF induced autophagy of GES-1 cells (Fig. 5C). To further confirm that autophagic flux was not inhibited, p62 expression level was monitored, alongside the effect of autophagy agonist RAPA and the effect of autophagy inhibitor 3-MA. The results of Fig. 5F showed that the expression level of p62 in the cells was reduced after DOFF treated. Treatment with RAPA reversed the reduction in LC3βII/LC3βI and Beclin-1 protein expression levels concurrent with increased p62 protein expression level, opposite to 3-MA results, and to confirm that autophagic flux was activated post DOFF treatment. The results showed that DOFF activated ethanol-induced autophagy of damaged cells, thereby reducing the immune response and oxidative stress. The role of autophagy as a defense mechanism against ethanol toxicity was further confirmed using *in vitro* GES-1 cell ulcer model.

Deficiency in nutrition is often associated by AMPK activation, activated AMPK inhibits mTOR and reduces phosphorylation of ULK1 thus initiates autophagy^[84]. In the present study ethanol dramatically improved the expression level of p-mTOR and led to a decline in that of p-AMPK, while DOFF upregulated p-AMPK and downregulated p-mTOR expression (Figs. 5J and K). It is well known that AMPK and mTOR could regulate the initiation of autophagy by direct phosphorylation of ULK1 and ULK1 complex^[85]. The results of proteins level in the cells showed that ethanol inhibited the expression of autophagy proteins, which made these cells could not launch autophagy to recycle their own material and led

to their death of them. Akt downstream of PI3K activates mTOR leading to inhibition of autophagy. In fact, activation of PI3K/AKT pathway is crucial for cells to resist against oxidation and apoptosis^[86]. In this study, ethanol significantly reduced expression of p-AKT and p-PI3K p85, which is consistent with previous report^[87]. Ethanol inhibited PI3K/AKT pathway, which may be unfavorable to cell survival and proliferation, and simultaneously contributing to apoptosis, while DOFF reversed ethanol-induced reduction of p-AKT and p-PI3K p85, and up-regulated the expression of p-AKT and p-PI3K p85 in GES-1 cells. The results showed that DOFF effect on ethanol-induced GES-1 cells were mediated *via* PI3K/AKT signaling pathway. Consistent with results revealed from this study, Arab et al.^[88] also proposed a critical role for PI3K-Akt pathway in ethanol-induced gastric ulcers in rats, posing it as potential drug target for ulcer management in the future.

Taken together, pretreatment with DOFF exerted a protective effect on ethanol-induced acute gastric ulcers. The excessive production of ROS released inflammation and apoptosis signals, and inhibited antioxidant elements to promote the continuous ROS production. Our findings revealed that DOFF may protect against ethanol-induced gastric ulcer formation through its anti-inflammatory, antioxidant, anti-apoptosis and autophagy promotion effects mostly attributed to its rich composition in several flavonoids. Specifically, DOFF can reduce the expression of inflammatory factors and restore the immune response in mice with gastric ulcers. Otherwise, *in vitro* and *in vivo* studies had shown that ethanol damage can cause ROS to rise. On the one hand, high concentration of ROS directly damaged cells and caused the death of gastric tissue; on the other hand, ROS reduced the level of MMP and influenced mitochondrial activity, which inhibited the normal physiological metabolism of cells and promoted the expression of pro-apoptotic factors, and caused cell apoptosis finally. After that, as we found that ethanol-induced gastric ulcers were related to cellular energy metabolism, it was tried to verify if there were autophagy in this processing. After ethanol treatment, PI3K/Akt pathway in cells was inhibited, which reduced energy utilization of cells, thus inhibiting cell physiological metabolism. After that, the AMPK/mTOR/ULK1 pathway was activated in cells as energy was not enough. Firstly, AMPK was activated, and the activity of mTOR would be inhibited by inhibiting upstream proteins, which further affected the expression of ULK1 protein, finally inducing autophagy in cells. In this study, DOFF was proven to reverse the harmful effect caused by ethanol through mitochondrial pathway of PI3K/AKT and AMPK/mTOR/ULK1. The results showed that DOFF may be a better natural agent for the treatment of ethanol-induced gastric mucosal injury, thus enabling the development of new drug treatments that have yet to be tested using other ulcer models to be conclusive. Standardizing DOFF extract should be the next logical step for its most active agent if to be incorporated in future nutraceuticals used for ulcer management alongside identification of the main active ingredient in its extract.

Conflict of interests

The authors declare that there is no conflict of interest in relation to this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2023.9250048>.

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