Chinese Journal of Natural Medicines

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Citation: Zhiru Yang, Haolin Guo, Pengfei Zhang, Kairui Liu, Junli Ba, Xue Bai, Shiti Shama, Bo Zhang, Xiaoning Gao, Jun Kang, Capsaicin (CAP) exerts a protective effect against ethanol–induced oxidative gastric mucosal injury by modulating the chemokine receptor 4 (CCR4)/Src/p47phox signaling pathway both *in vitro* and *in vivo*, Chinese Journal of Natural Medicines, 2025, 23(2), 191–202. doi: 10.1016/S1875–5364(25)60823–5.

View online: https://doi.org/10.1016/S1875–5364(25)60823–5

Chinese Journal of Natural Medicines 23 (2025) 191-202

Contents lists available at ScienceDirect

Chinese Journal of Natural Medicines

journal homepage: www.cjnmcpu.com/

Original article

Capsaicin (CAP) exerts a protective effect against ethanol-induced oxidative gastric mucosal injury by modulating the chemokine receptor 4 (CCR4)/Src/p47phox signaling pathway both *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 22 March 2024
Revised 28 May 2024
Accepted 3 July 2024
Available online 20 February 2025

Keywords:
Capsaicin
Gastric mucosal damage
Chemokine signaling
NF-κB p65

ABSTRACT

Ethanol (EtOH) is a common trigger for gastric mucosal diseases, and mitigating oxidative stress is essential for attenuating gastric mucosal damage. Capsaicin (CAP) has been identified as a potential agent to counteract oxidative damage in the gastric mucosa; however, its precise mechanism remains unclear. This study demonstrates that CAP alleviates EtOH-induced gastric mucosal injuries through two primary pathways: by suppressing the chemokine receptor 4 (CCR4)/Src/p47phox axis, thereby reducing oxidative stress, and by inhibiting the phosphorylation and nuclear translocation of nuclear factor-κB p65 (NF-κB) p65, resulting in diminished inflammatory responses. These findings elucidate the mechanistic pathways of CAP and provide a theoretical foundation for its potential therapeutic application in the treatment of gastric mucosal injuries.

1. Introduction

Ethanol (EtOH), commonly known as alcohol and frequently encountered in daily life, poses significant health risks. EtOH-induced gastric mucosal diseases, including gastritis, gastric ulcer, and potentially gastric cancer, are among the most persistent and prevalent disorders [1, 2]. Upon ingestion, EtOH directly compromises the integrity of the gastric mucosal barrier, enhances mucosal permeability, impairs gastric acid cells, and induces gastric mucosal damage and hemorrhage [3]. Gastric mucosal injury triggers an imbalance in pro-inflammatory cytokines and prompts neutrophil deformation and migration toward the affected area. Elevated concentrations of reactive oxygen species (ROS) and other inflammatory mediators result in oxidative damage [4]. The accumulation of EtOH's downstream metabolites, acetaldehyde, and ROS progressively increases the risk of cytotoxic oxidation and DNA damage. A disrupted redox balance can lead to cell death, stimulating the proliferation of remaining cells. This process amplifies mutation potential, elevates oncogene expression, and may ultimately result in gastric cancer [5]. Therefore, preventing oxidative damage is crucial in reducing the incidence of gastric diseases.

The chemokine pathway coordinates various biological processes (BP) by activating downstream signaling molecules *via* the interaction of diverse chemokines with their corresponding receptors on the cell surface. Humans possess approximately 43 chemokines and 19 receptors [6]. Specifically, chemokine receptor 4 (CCR4) is primarily activated by its ligands, CCL17 and CCL22 [7]. Research indicates that CCL17-mediated activation of CCR4 in macrophages can facilitate bleomycin-induced oxidative lung injury. Notably, the absence of CCR4 attenuates the pathological effects of bleomycin-induced lung injury [8], suggesting that CCR4 expression may serve as a valuable biomarker for assessing oxidative damage in the lung.

Src, a non-receptor tyrosine protein kinase encoded by the human proto-oncogene, plays a crucial role in numerous cellular signaling cascades and functions as a downstream molecule in chemokine pathways. Studies have shown that Src regulates the activity of reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), a primary *in vivo* ROS source, thereby inducing oxidative stress in cells [9]. The activation of NADPH oxidase requires serine phosphorylation of its p47phox subunit [10]. Src promotes ROS generation through two mechanisms: acutely, by facilitating the phosphorylation and translocation of p47phox, and chronically, by increasing the levels of p22phox and p47phox [11]. Consequently, the assessment of Src and p47phox levels in cells can serve as an indicator of cellular oxidative stress.

Natural products have historically played a crucial role in hu-

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https://doi.org/10.1016/S1875-5364(25)60823-5

manity's efforts to combat diseases, serving as invaluable sources for drug discovery. The specific dominant structures within these products make them ideal candidates for lead compound derivation. CAP, the primary component responsible for the pungency of chili peppers, has demonstrated efficacy in addressing oxidative damage. Fibroblast pretreatment with CAP significantly reduces ROS production following ultraviolet irradiation, thereby mitigating ultraviolet-induced skin collagen loss and conferring anti-aging benefits [12]. In animal studies, low doses of CAP effectively attenuate oxidative damage to the gastric mucosa [13]. However, the precise mechanism by which CAP counters oxidative damage remains to be elucidated.

In this study, we employed network pharmacology to predict the molecular mechanisms by which CAP addresses gastric mucosal oxidative damage. Subsequently, we established oxidative injury models in GES-1 and RAW264.7 cells to verify the effects of CAP pretreatment on the chemokine pathway and NF-κB signaling pathway. Finally, we evaluated the protective role of CAP on rat gastric mucosa, analyzed changes in key proteins associated with the chemokine pathway, and assessed inflammatory factors (Fig. 1). This approach enabled a comprehensive examination of the molecular mechanisms underlying CAP's treatment of oxidative injuries.

2. Materials and methods

2.1. Reagents

CAP (Cat No. HY-10448), *N*-acetylcysteine (NAC) (Cat No. HY-B0215), and rebamipide (Cat No. HY-B0360) were acquired from MedChemExpress (Shanghai, China). Cell culture reagents, including RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin (PS), and L-glutamine, were obtained from Gibco (New York, USA). Reagents for western blotting and immunofluorescence were procured from Solarbio (Beijing, China). Antibodies for protein kinase C delta (PKCδ) (Cat No. 19132-1-AP), p21 protein activated kinase 1 (PAK1) (Cat No. 21401-1-AP), Src (Cat No. 11097-1-AP), p47phox (Cat No. 28187-1-AP), BCL2-associated X protein (Bax) (Cat No. 50599-2-Ig), nuclear factor-κB p65 (p65) (Cat No. 10745-1-AP), phospho-NF-κB p65 (P-p65) (Cat No. 82335-1-RR), and beta actin (ACTB) (Cat No. 81115-1-RR) were sourced from Proteintech (Wuhan, China). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cat No. KM90027), goat anti-rabbit IgG (H + L)-HRP antibody (Cat No. LK2001), and goat anti-rat IgG (H + L)-HRP antibody (Cat No. LK2009) were obtained from Sungene Biotech (Tianjin, China). Additionally, the CCR4 antibody (Cat No. ab59550) and p47phox antibody (Cat No. ab308256) were acquired from Abcam (Shanghai, China). Furthermore, the antibodies used for *in vivo* immunofluorescence experiments were as follows: myeloperoxidase (MPO) antibody (Cat No. Ab208670, Abcam, Shanghai China), cluster of differentiation 68 (CD68) antibody (Cat No. GB113109, Servicebio, Wuhan China), and P-p65 antibody (Cat No. AF2006, Affinity, Shanghai China).

2.2. Network pharmacology analysis

CAP was analyzed using four drug target databases: BATMAN, CTD, STITCH, and TCMSP, with the aim of identifying its molecular targets. To link these targets to gastric mucosal injury, we performed a targeted search in the GENECARDS disease using the term "gastric mucosal injury". By comparing the targets of CAP with those associated with gastric mucosal injury, we identified potential therapeutic targets for further investigation [14-18]. Enrichment analyses of these targets were then performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) frameworks *via* the DAVID database. The GO analysis provided insights into BP, cellular components (CC), and molecular functions (MF), while the KEGG analysis elucidated significant signaling pathways in BP. Data from both GO and KEGG were integrated into the Bioinformatics platform for visual interpretation. Based on these findings, specific signaling pathways of interest were selected for deeper investigation, focusing on those most likely to mediate capsaicin's therapeutic effects. This approach facilitated the identification of target proteins crucial for subsequent in-depth studies [19-22].

2.3. Cell culture and model construction

GES-1 cells and RAW264.7 cells, obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China), were cultivated in RPMI 1640 medium supplemented with 10% FBS and 1% PS. The cells were maintained in a controlled-environment incubator at 37 °C with 5% CO_2. For RAW264.7 cells, the medium was additionally supplemented with L-glutamine at a final concentration of 1–4 mmol·L^{-1}. Experimental procedures were initiated when cell confluence exceeded 80%.

The experimental procedure involved pretreating cells with CAP to establish a protective barrier, followed by EtOH exposure to induce oxidative stress. This approach aimed to elucidate the protective mechanism of CAP against oxidative damage. Cells were cultured in 24-well plates for 24 h, then pretreated with varying concentrations of CAP for 2.5 h, and subsequently exposed to 3.5% EtOH for 1.5 h. These cells were then harvested for further analysis to investigate CAP's protection against EtOH-induced oxidative stress. In RAW264.7 cells, to determine whether CAP's influence on the chemokine pathway is attributable to the intrinsic properties of the molecule rather than solely its antioxidant capability, another antioxidant, NAC, was introduced as a reference control.

2.4. Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA extraction from cells was performed using Qiazol (Qiagen, Hilden, Germany) and quantified using Nanodrop. The extracted RNA was reverse transcribed into cDNA using the TransScript First-strand cDNA Synthesis SuperMix (Transgene,

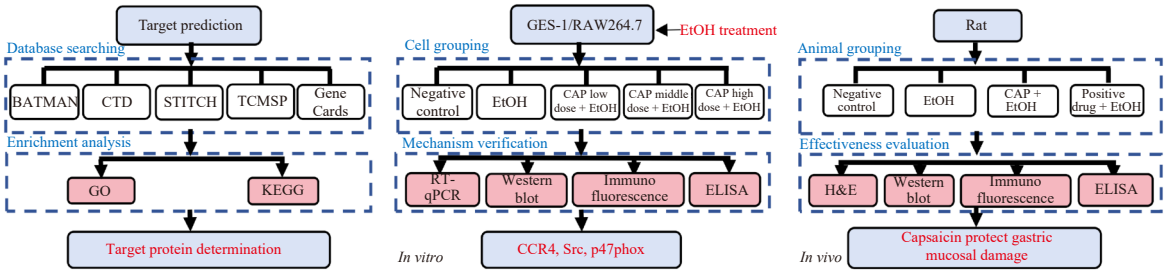


Fig. 1 Schematic diagram of the study design.

Beijing, China). Subsequently, quantitative real-time PCR was conducted on the cDNA using the TransStart Top Green qPCR SuperMix Kit (Transgene, Beijing, China) to measure mRNA levels. The PCR cycling conditions were as follows: 94 °C for 30 s, followed by 45 cycles of 94 °C for 5 s and 60 °C for 30 s, with final storage at 4 °C. mRNA expression levels were normalized to GAPDH [23]. Primers were designed based on the PrimerBank website (https://pga.mgh.harvard.edu/primerbank/), and their specificity was verified through NCBI-Primer Blast before synthesis (Table S1).

2.5. Western blotting analysis

Cells were harvested *via* centrifugation and lysed using radioimmunoprecipitation assay lysis buffer. Subsequently, they were heated in a metal bath at 99 °C for 10 min. Following protein sample preparation, electrophoresis was performed on a polyacrylamide gel. The proteins were then transferred to a nitrocellulose filter membrane and blocked with 5% non-fat milk. The membrane was incubated overnight at 4 °C with primary antibodies corresponding to proteins PKCδ (1∶1000), PAK1 (1∶1000), Src (1∶1000), p47phox (1∶1000), Bax (1∶1000), P-p65 (1∶1000), GAPDH (1∶20 000), and CCR4. This was followed by a 1-hour incubation with secondary antibodies (1∶5000). Protein bands were detected using a chemiluminescence kit (Epizyme, Shanghai, China). These bands were captured with a gel imaging system, and their intensities were analyzed in grayscale USA [24, 25].

2.6. Immunofluorescence

Cells were seeded in 48-well plates and cultured for 24 h. They were then pretreated with 8 μmol·L^{-1} CAP for 2.5 h, followed by a 1 h exposure to 2% EtOH. The EtOH concentration and exposure time were reduced to minimize cell death and prevent cell suspension. After treatment, cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. To prevent non-specific antibody binding, cells were treated with 1% bovine albumin (BSA). Subsequently, they were incubated overnight at 4 °C with the NF-κB p65 antibody diluted in BSA (1∶100). Following TBST washes, a secondary antibody was applied, and cells were incubated in darkness for 30 min. Cells were then washed with TBST, stained using 4',6-diamidino-2-phenylindole (DAPI, Cat No. 62248, Thermo, Shanghai, China), mounted on slides, and examined under a fluorescence microscope [26].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Following the model construction in RAW264.7 cells, the supernatants were collected, and the secretion level of cytokine interleukin-6 (IL-6) was quantified using an ELISA kit (Cat No. SEKM-0007, Solarbio, Beijing, China).

2.8. Animals

Twenty-four male Sprague-Dawley (SD) rats aged 6–8 weeks (200–250 g) were obtained from SpePharm Biotechnology Co., Ltd. (Beijing, China). The rats were housed in an environment with controlled temperature (25 ± 2 °C), relative humidity (50% ± 15%), and a 12 h light-dark cycle, with ad libitum access to water and food. The rats were randomly divided into four groups of six: (a) control group, (b) EtOH-impaired group, (c) CAP + EtOH-impaired group, and (d) rebamipide + EtOH-impaired group. Following a 24 h fasting period, rats in group c received CAP at a dosage of 1 mg·kg^{-1} (based on preexperimental effects), dissolved in a solvent containing 10% Tween 80 and 90% saline. Group d was administered the positive control drug rebamipide dissolved in saline at 100 mg·kg^{-1}. Groups a and b received equivalent volumes of saline. After 45 min, group a was administered saline, while groups b, c, and d were given 75% EtOH (Cat No. A171299, Aladdin, Shanghai, China) at 10 mL·kg^{-1}. CAP, rebamipide, and EtOH were administered *via* direct gavage. The dosages of rebamipide and EtOH were determined based on references [27, 28]. The rats' conditions were subsequently monitored. One hour later, the rats were euthanized for further data analysis. At the study's conclusion, rats were exposed to carbon dioxide under general anesthesia, followed by cervical dislocation and euthanasia. All animal research procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Faculty of Medicine, Tianjin University (SYXK - 20190002).

Following gas anesthesia of the rats, the abdominal cavity was accessed, and blood was extracted from the abdominal aorta. The pyloric end was ligated, and paraformaldehyde was administered *via* gavage. Once the gastric wall folds were fully extended, the cardia was clamped, and internal fixation was conducted for 5 min. The stomachs were then removed from the euthanized rats, incised along the greater curvature, rinsed to remove any remaining contents, and subsequently flattened and fixed. The number of folds and instances of hemorrhage were observed. The dimensions of any damaged areas were measured to assess the extent of injury to the rats' gastric mucosa [29]. A 0.5 cm × 0.5 cm tissue sample from the rat gastric antrum was extracted and fixed in 4% paraformaldehyde for 12–24 h. The sample then underwent dehydration, clarification, wax immersion, embedding, and sectioning. Following hematoxylin-eosin (H&E) staining, the sample was examined under a light microscope to evaluate histopathological changes in the stomach [30-32].

Paraffin sections underwent double staining using a 1∶100 dilution of MPO and a 1∶200 dilution of CD68 antibodies to identify neutrophils and macrophages, respectively. Immune cell infiltration was visualized through confocal microscopy, and the average fluorescence intensity was quantified using ImageJ software. Furthermore, paraffin sections were stained using a 1∶200 dilution of P-p65 antibody to evaluate the effect on the NF-κB signaling pathway.

The rat stomach tissue was pulverized in liquid nitrogen, then combined with loading buffer and heated at 99 °C for 10 min. The mixture was centrifuged at 12 000 r·min^{-1} for 2 min. The resulting supernatant was collected and subjected to polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to a nitrocellulose membrane and blocked with 5% skimmed milk. The membranes were then incubated overnight at 4 °C with primary antibodies specific to Src (1∶1000), p47phox (1∶1000), CCR4 (1∶1000), and ACTB (1∶5000) proteins. Following this, the membranes were incubated with a secondary antibody (1∶2000) for 1 h. Protein bands were visualized using a chemiluminescence kit, and their intensities were quantified by grayscale analysis using ImageJ software.

The expression levels of inflammatory markers in rat serum, including the chemokine (C-X-C motif) ligand 1 protein (CXCL1/KC), tumor necrosis factor α (TNFα), IL-6, IL-1β, IL-10, were quantified using ELISA kits (Cat Nos. SEKR-0014, SEKR-0009, SEKR-0005, SEKR-0002, SEKR-0006, Solarbio, Beijing, China).

2.9. Statistical analysis

Data were derived from three independent experiments and are expressed as the mean ± standard deviation (SD). The normality of data was assessed using the Shapiro-Wilk test in the Statistical Package for Social Sciences IBM (SPSS Inc., Chicago, Illinois, USA). Statistical differences between datasets were then evaluated using the Student's *t*-test in Prism 8 software (Graph-

Pad, San Diego, CA, USA). The levels of significance were denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Network pharmacological analysis of CAP's targets

Network pharmacology was utilized to investigate molecular mechanisms through which CAP mitigates oxidative stress-induced gastric mucosal injury (Fig. 2A). To identify its molecular targets, four drug target databases—BATMAN, CTD, STITCH, and TCMSP—were queried. After consolidating the data and eliminating redundancies, 604 non-redundant molecular targets of capsaicin were identified. Notably, the transient receptor potential vanilloid type 1 (TRPV1) protein emerged as the only common target across all four databases (Fig. 2B). Next, 2824 disease-associated targets linked to gastric mucosal injury were retrieved from the Genecards Disease Target Database. The intersection of these with the drug targets yielded 350 target proteins, suggest-

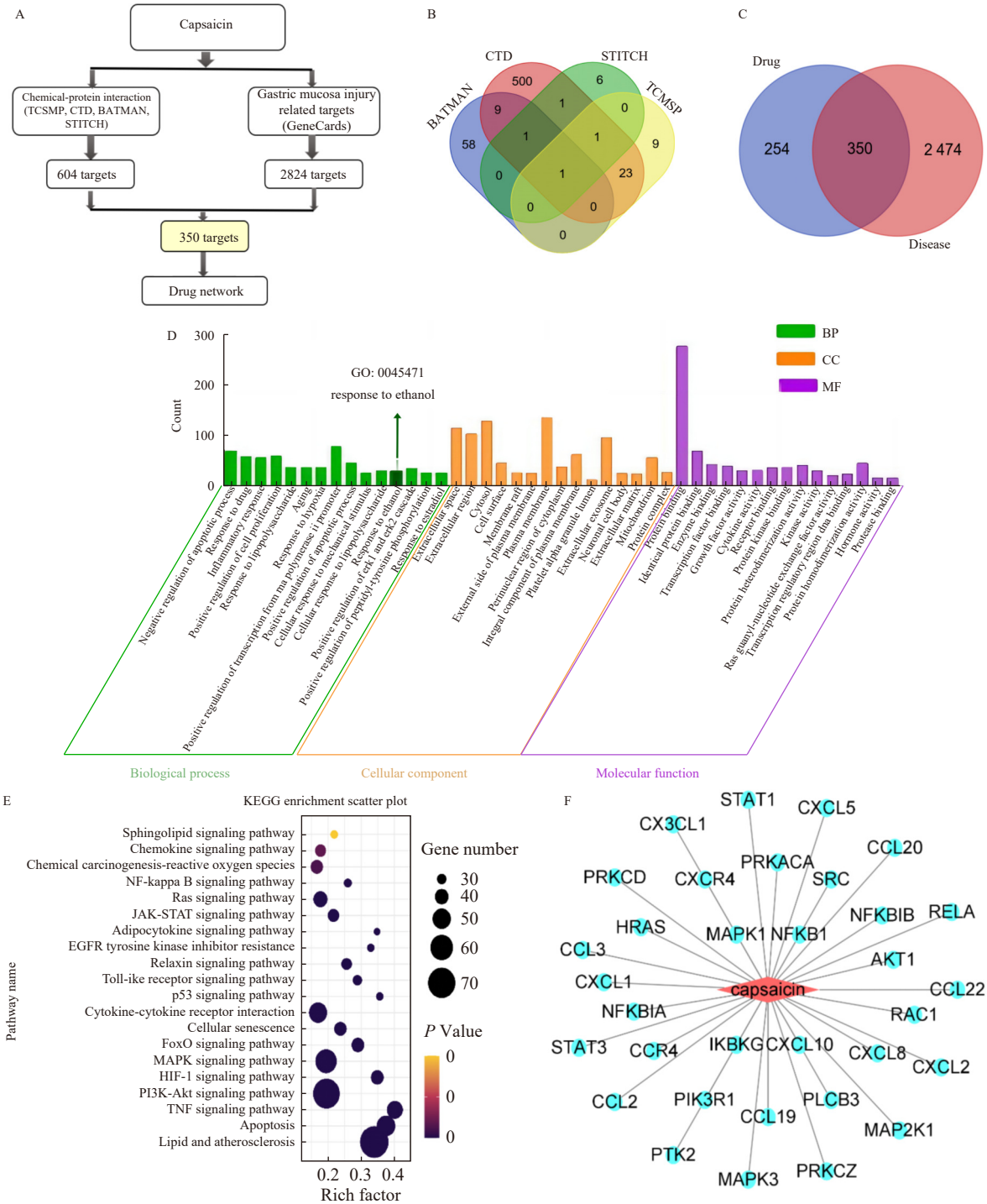


Fig. 2 Network pharmacological evaluation of CAP. (A) Flowchart illustrating the network pharmacological analysis of CAP's effect on gastric mucosal injury. (B) Analysis of CAP's comprehensive target profile. (C) Venn diagram depicting the intersection between CAP targets and disease-related targets. (D and E) GO and KEGG enrichment analyses highlighting potential targets for CAP's ameliorative effects on gastric mucosal injury. (F) Identification of target proteins within the chemokine pathway modulated by CAP.

ing potential mechanisms for CAP in mitigating gastric mucosal injury (Fig. 2C). GO and KEGG analyses elucidated the functional modules and signaling pathways of these 350 potential targets. In the BP enrichment, the entry GO: 0045471, corresponding to the response to EtOH, was prominently featured (Fig. 2D). Furthermore, KEGG analysis identified key signaling pathways influenced by capsaicin, including the chemokine signaling pathway, NF-κB signaling pathway and TNF signaling pathway (Fig. 2E). Despite 33 target proteins being mapped to the chemokine pathway, CAP's regulatory role in this pathway remains unreported (Fig. 2F).

3.2. Inhibition of Src/p47phox expression by CAP in GES-1 cells

KEGG analysis revealed key interconnections between the target proteins in the chemokine signaling pathway, highlighting CCR4, protein kinase A (PKA), and Src as the most promising targets of CAP (Fig. 3A). Additionally, p47phox, an essential component of NADPH oxidase, plays a crucial role in the formation of NADPH complexes and regulation of oxidative stress. Based on these findings, we hypothesized that CAP might modulate the chemokine pathway.

We first examined the transcript levels of kinases PKC, PKA, and p21 PAK1 within the chemokine pathway. Results indicated that CAP administration did not significantly affect the transcription of these proteins (Figs. 3B–3D). Subsequently, grayscale analysis at the translational levels of PKCδ and PAK1 revealed a slight decrease in PKCδ protein expression with 8 and 32 μmol·L^{-1} CAP treatment. In contrast, PAK1 protein expression increased with rising CAP concentrations (Fig. 3E).

In the subsequent phase, we evaluated changes in CCR4, p47phox, and Src at both transcriptional and translational levels. The results revealed that 8 μmol·L^{-1} CAP significantly reduced CCR4 transcription (Fig. 3F). While a trend toward Src transcriptional inhibition was observed, it did not reach statistical significance (Fig. 3H). However, CAP at concentrations of 8 and 32 μmol·L^{-1} demonstrated significant inhibitory effects on the expression of p47phox and Src (Figs. 3G and 3I).

3.3. Inhibition of Src/p47phox expression by CAP in RAW264.7 cells

While p47phox and CCR4 expressions were relatively subdued at the GES-1 cell level, and considering the chemokine pathway's primary role in immune cells, we extended our investigation to mouse macrophages RAW264.7 to validate key indicators. Consistently, transcription findings aligned with those observed in GES-1 cells. Both instances revealed significant suppression of CCR4 and p47phox transcription by CAP, while NAC showed no effect. Compared to the EtOH group, 32 μmol·L^{-1} CAP inhibited the transcription of Src in the RAW264.7 cell line, and NAC also had some effect, albeit less pronounced than CAP. However, PKCδ's transcriptional expression remained unaffected (Figs. 4A–4D). Grayscale analysis demonstrated that CAP significantly reduced Src and p47phox expressions. In contrast, NAC did not inhibit the expressions of Src and p47phox (Figs. 4E and 4G). These findings suggest that CAP's modulation of the chemokine pathway may differ from the typical effects of antioxidants.

The network pharmacology analysis highlighted Bax, a crucial protein in apoptotic processes, and related apoptotic pathways. Our findings indicated that both CAP and NAC significantly reduced Bax expression, suggesting their potential to mitigate EtOH-induced apoptosis (Fig. 4F).

3.4. Inhibition of P-p65 expression and p65 entry into the nucleus by CAP

RELA/NF-κB p65 protein is a crucial transcription factor involved in regulating inflammatory responses. It was among the 33 key proteins identified in both the chemokine and inflammatory response-associated pathways, specifically the NF-κB signaling pathway, as revealed by network pharmacology analysis. The active form of this protein is its phosphorylated modification. In GES-1 cells, we observed a significant reduction in P-p65 protein levels following CAP treatment (Figs. 5A and 5B). Simultaneously, immunofluorescence co-localization experiments demonstrated that in untreated GES-1 cells, NF-κB p65 was predominantly localized in the cytoplasm and did not co-localize with DAPI fluorescence. However, EtOH-treated cells exhibited prominent nuclear localization of NF-κB p65, aligning with DAPI fluorescence. Treatment with 8 μmol·L^{-1} CAP appeared to reverse this pattern, restoring control levels (Fig. 5C). Analysis of NF-κB p65 and DAPI co-localization across three distinct fields of view revealed significant differences (Fig. 5D). Further investigation of P-p65 expression in parallel experiments using RAW264.7 cells demonstrated that CAP significantly inhibited P-p65 levels in macrophages (Figs. 5E and 5F). Additionally, we examined the effect of CAP on the expression levels of cytokines downstream of the NF-κB signaling pathway. Using IL-6 as an example, ELISA results showed that CAP significantly inhibited IL-6 expression in RAW264.7 cells (Fig. 5G).

3.5. CAP's role in mitigating gastric mucosal damage and inhibiting CCR4/Src/p47phox in vivo

Utilizing a rat model of EtOH-induced gastric mucosal injury, we examined the protective effects of CAP (1 mg·kg^{-1}) and the positive control drug, rebamipide (100 mg·kg^{-1}), on the gastric mucosa under bright field conditions. These observations were consistent with previous literature reports (Fig. 6A). Furthermore, pathological section analyses demonstrated that pre-administration of these drugs effectively prevented glandular disorganization in gastric tissues, reduced inflammatory cell infiltration, and minimized epithelial cell detachment (Fig. 6B).

Immune cell infiltration was evaluated using immunofluorescence. MPO and CD68 antibodies were utilized to simultaneously stain neutrophils and macrophages in paraffin sections. Fluorescence intensity analysis was conducted on tissue samples from three rats per group using ImageJ software, with representative images provided (Fig. 6C). The EtOH group's images exhibited an abundance of both red and green fluorescent markers on inflammatory cells, highlighting EtOH's capacity to induce macrophage and neutrophil infiltration, resulting in an acute gastric inflammatory response. Notably, both CAP and the positive control drug treatments effectively reduced this inflammatory cell invasion. Statistical analysis revealed that CAP treatment decreased the EtOH-induced neutrophil infiltration from 2.40% to 0.41% and reduced macrophage infiltration from 12.12% to 2.17%. Rebamipide reduced immune cell infiltration to 0.50% and 2.40%, which was slightly less effective than CAP (Fig. 6D).

Western blotting analyses were conducted using proteins extracted from gastric mucosa. The results demonstrated that the three target proteins—Src, p47phox, and CCR4—displayed low expression in healthy gastric tissues, with CCR4 and p47phox being nearly undetectable. However, in gastric tissues from rats with EtOH-induced injury, a notable increase in the expression of these target proteins was observed. Importantly, this upregulation was significantly suppressed by both CAP and the positive control drugs. Grayscale analysis further emphasized CAP's substantial inhibitory effect on Src, p47phox, and CCR4 expression at the animal level, which was significantly more pronounced than that of rebamipide (Figs. 6E and 6F). These findings corroborate CAP's modulatory influence on the chemokine pathway in rats.

3.6. CAP's inhibition of NF-κB signaling pathway and inflammatory cytokine secretion in vivo

The presence of P-p65 in rat gastric mucosal tissues was assessed using immunofluorescence. The findings indicated that

EtOH administration significantly elevated P-p65 levels in gastric tissues. Both CAP and the positive control drug effectively reduced P-p65 content and inhibited the NF-κB signaling pathway, with comparable efficacy (Figs. 7A and 7B).

Furthermore, the levels of cytokine secretion downstream of the NF-κB signaling pathway were quantified in rat serum using ELISA. Both CAP and the positive control drug significantly reduced the levels of pro-inflammatory markers chemokine (C-X-C motif) ligand 1 [CXCL1/KC (IL-8)], IL-1β, IL-6, and TNF-α, while enhancing the expression of the anti-inflammatory cytokine IL-10. This contributed to the attenuation of the EtOH-induced inflammatory response (Figs. 7C–7F). However, the results indicated that CAP was more effective than rebamipide in upregulating the anti-inflammatory factor IL-10, while it was less effective

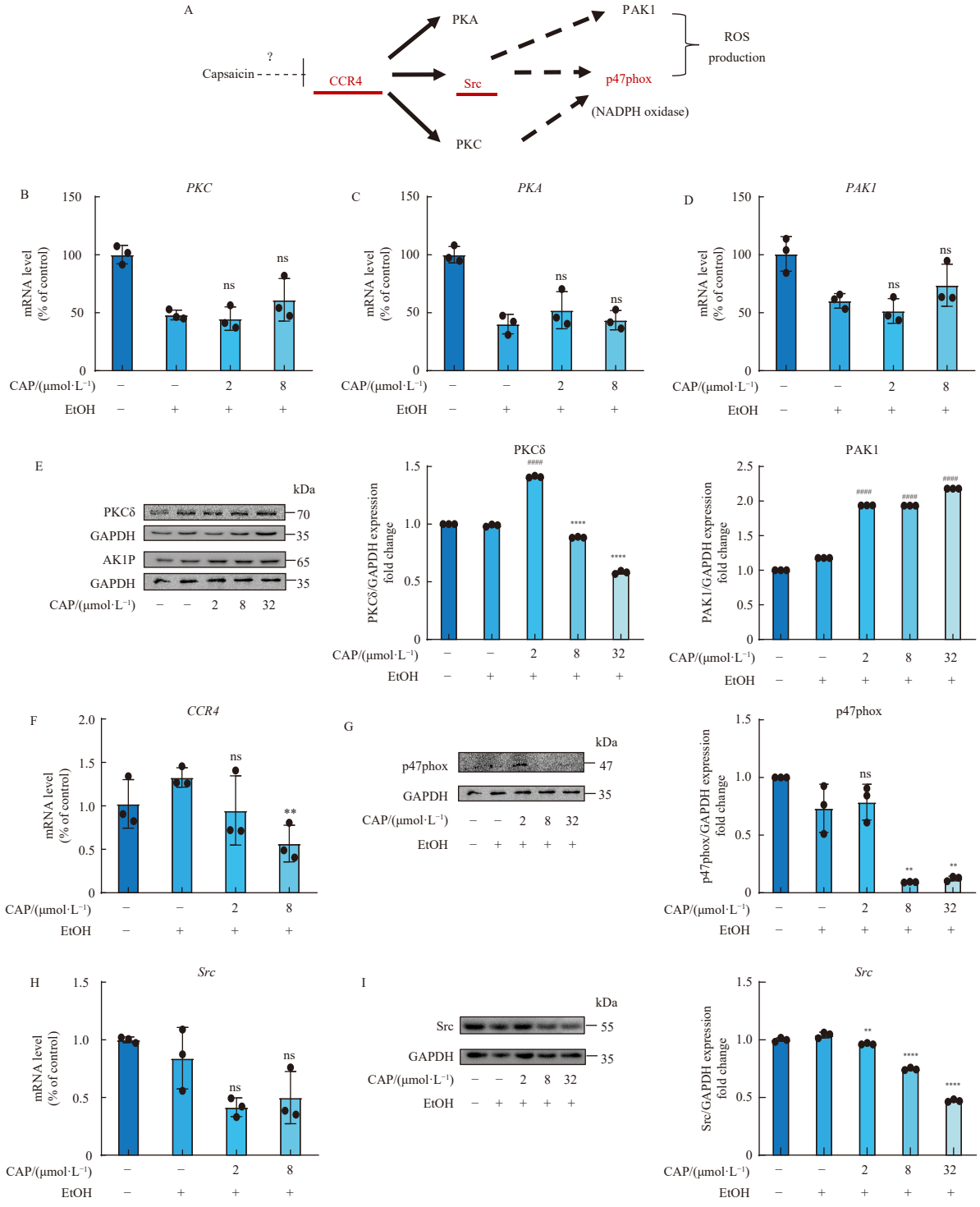


Fig. 3 CAP inhibits Src/p47phox expression in GES-1 cells. (A) Analysis of potential targets for CAP-mediated regulation of chemokine pathways to reduce ROS. (B–D) The regulatory effect of CAP on the transcriptional levels of protein kinase C (PKC), PKA, and p21 PAK1 within the chemokine pathway is examined using reverse transcription-quantitative PCR. (E) Western blotting analysis illustrating CAP's effect on PKCδ and PAK1 expression. (F) Evaluation of CAP's influence on the transcriptional activity of CCR4. (G) Investigation of CAP's impact on the expression of p47phox, a key subunit of NADPH oxidase. (H and I) Assessment of CAP's influence on the transcription and expression of Src, a central component in the chemokine pathway. Data are presented as mean ± SD for three independent experiments. The statistical significance of differences is evaluated by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****/\#\#\#\#}P < 0.0001$ *vs* only EtOH treated group. *Represents down-expression and $^{\#}$represents up-expression.

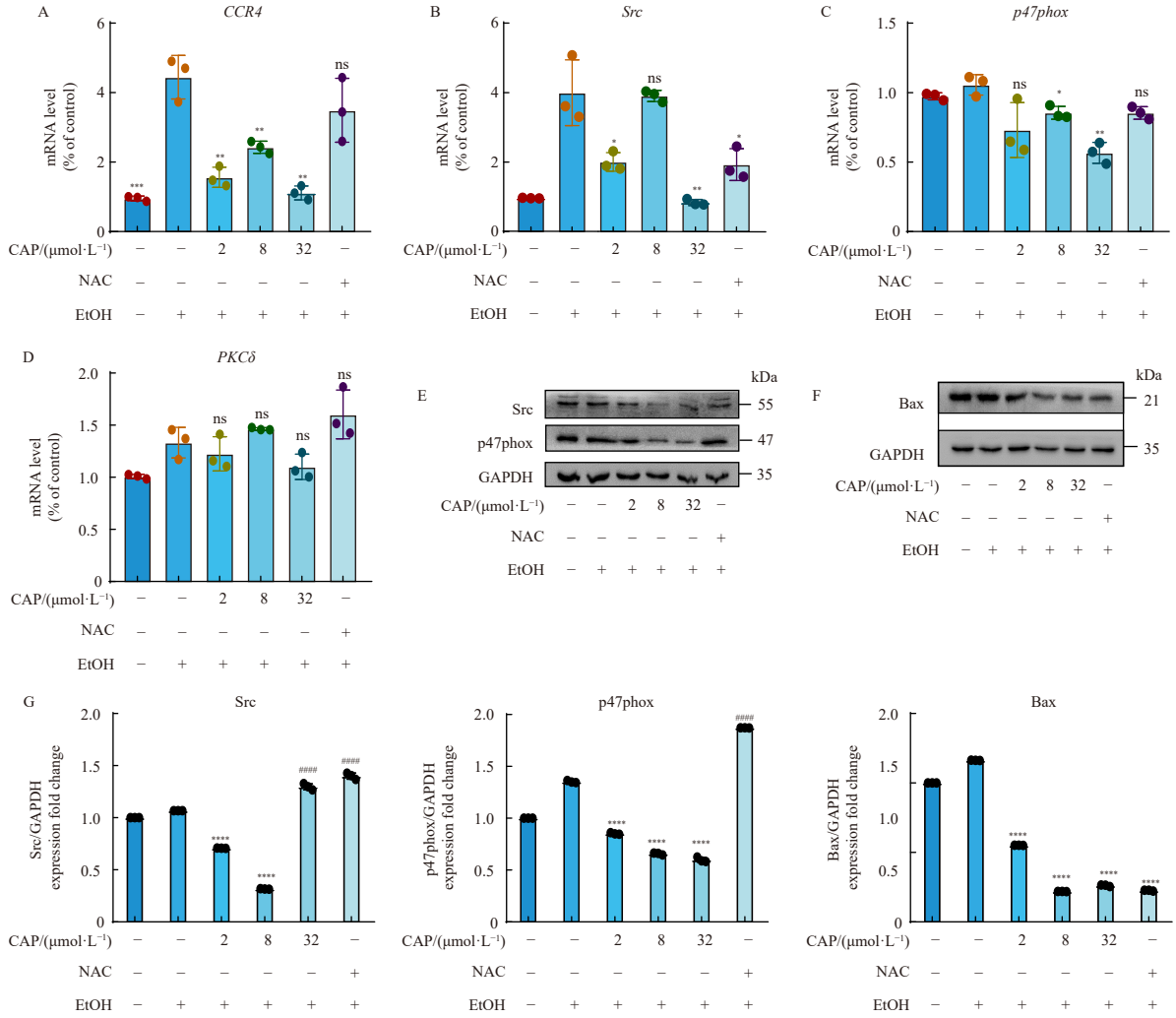


Fig. 4 CAP suppresses Src/p47phox expression in RAW264.7 cells. (A–D) The effect of CAP on the transcriptional activities of CCR4, Src, p47phox, and PKCδ is assessed using reverse transcription-quantitative PCR. (E–G) Western blotting analyses demonstrate the impact of CAP on the expression of Src, p47phox, and Bax, a key protein in the apoptotic process. Data are presented as mean ± SD from three independent experiments. The statistical significance of differences between data is determined by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****/\#\#\#\#}P < 0.0001$ *vs* only EtOH treated group. *Represents down-expression and $^{\#}$represents up-expression.

in inhibiting the remaining pro-inflammatory factors. This disparity may be attributed to pharmacokinetic factors, as the effect of CAP on blood factors may not have been optimal. Rebamipide might exert superior anti-inflammatory effects by targeting additional signaling pathways.

4. Discussion

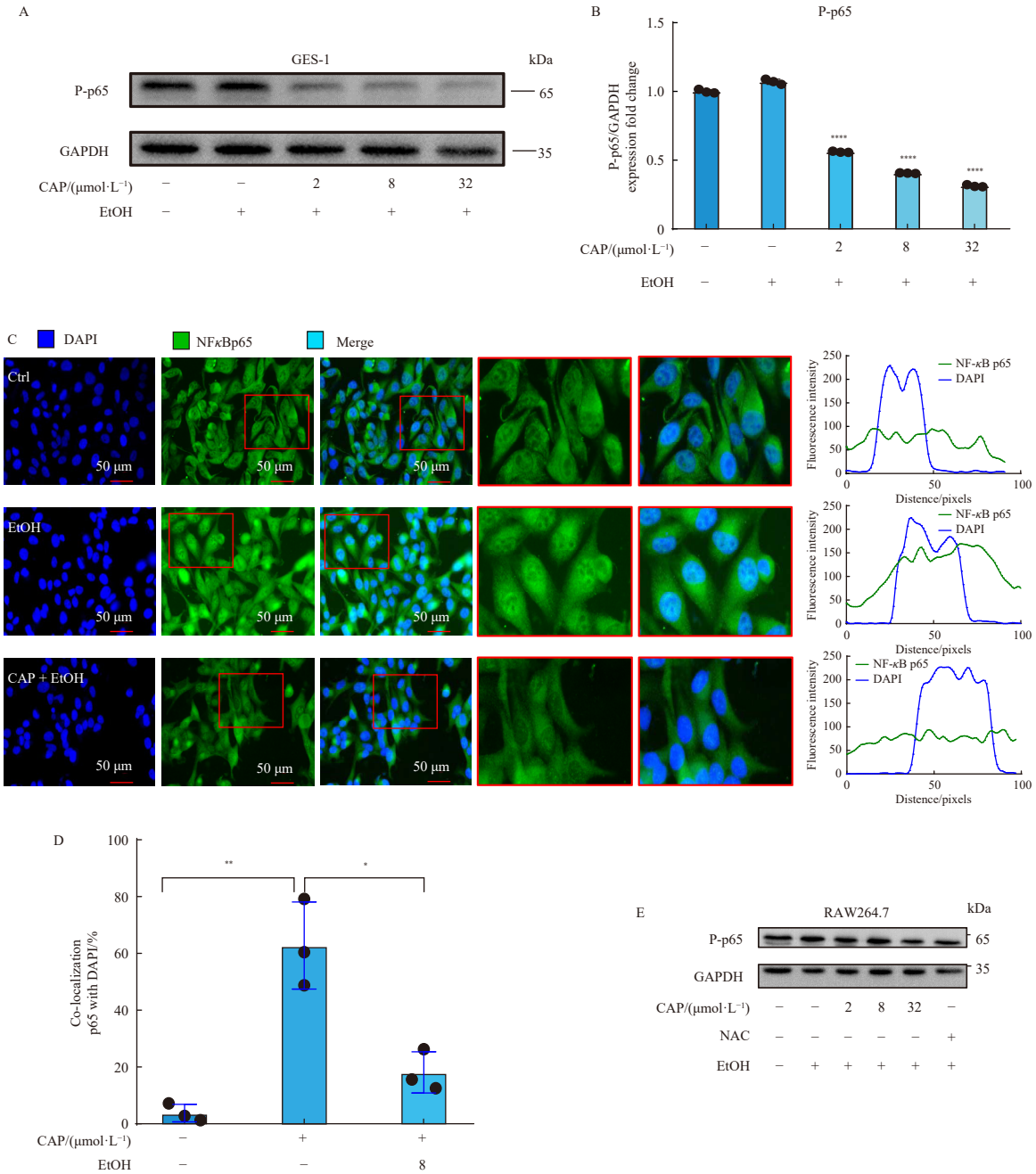
The development of various inflammatory gastrointestinal disorders, including gastritis, gastric ulcers, and gastric cancer, is closely associated with oxidative damage [33]. Therefore, preventing oxidative damage is crucial for maintaining health. CAP, the primary compound responsible for the pungency of chili peppers, is gaining attention due to its expanding range of biological activities. Given its natural origin from widely consumed chili peppers and its frequent use as a food flavor enhancer, CAP demonstrates excellent biosafety and shows promise as a therapeutic agent [34]. In this study, we employed network pharmacology to elucidate CAP's targets of action. We identified CCR4 and Src, two proteins within the chemokine pathway, as potential key mediators in CAP's mitigation of oxidative damage. *In vitro* tests confirmed that CAP effectively inhibited the transcription of CCR4 and the expression of Src, thereby reducing oxidative harm. Simultaneously, the expression of p47phox, a molecule downstream of Src, significantly decreased upon CAP administration, leading to a direct reduction in ROS generation. Furthermore, CAP inhibited the phosphorylation and nuclear translocation of NF-κB p65, an integral transcription factor associated with the inflammatory response. This action subsequently suppressed the release of pro-inflammatory agents such as TNF-α and IL-6, while enhancing the levels of the anti-inflammatory agent IL-10. These combined effects provide significant protection to the gastric mucosa, highlighting CAP's therapeutic potential (Fig. 8).

CAP inhibits the expression of Src, a key protein in the chemokine pathway, and suppresses p47phox, a crucial subunit of NADPH oxidase. This inhibition affects NADPH oxidase activation, resulting in reduced ROS production. Additionally, CAP attenuates cellular inflammatory responses by inhibiting the phosphorylation and nuclear translocation of NF-κB p65 protein, thus regulating cytokine expression.

Various medications are currently available for treating gastric mucosal injuries. Their primary mechanisms of action include the inhibition of gastric acid or pepsin erosion and the promotion of cellular regeneration [35,36]. Among those designed to protect the gastric mucosa by counteracting oxidative damage are magnesium aluminium carbonate and rebamipide [37]. Magnesium aluminium carbonate activates nitric oxide synthase to produce nitric oxide. This molecule then binds to superoxide anions, thereby reducing ROS production either by inhibiting xanthine oxidase or NADPH oxidase. However, a limitation of prolonged magnesium

aluminium carbonate use is its effect on serum electrolyte balance and the absorption of fat-soluble vitamins [37, 38]. In our animal experiments, rebamipide served as the positive control drug. According to Sakurai et al., rebamipide inhibits superoxide anion production in neutrophils and actively neutralizes free radicals, endowing it with potent antioxidant and anti-inflammatory properties [39]. However, studies by Zhang et al. and Dong et al. identified potential side effects, including dyspepsia, diarrhea, and abdominal discomfort, associated with rebamipide administration [40, 41]. Thus, the investigation of natural small molecules for treating gastric mucosal oxidative damage holds both clinical significance and commercial potential. CAP, a natural small molecule, has documented benefits against cancer and central nervous system disorders. Available formulations have shown promising therapeutic outcomes [42, 43]. The full medicinal potential of CAP remains to be elucidated.

Regarding oxidative stress resilience, Zhang et al. demonstrated that CAP reduced aminotransferase levels and mitigated concanavalin A-induced apoptosis and oxidative stress in hepatocytes [44]. Furthermore, Chen et al. established that CAP could inhibit ROS production in human umbilical vein endothelial cells, providing protection against cellular oxidative stress in human umbilical vein endothelial cells (HUVECs). This protective effect is mediated *via* a Caspase 3-dependent pathway, which shows promise in treating atherosclerosis [45]. Building on these findings, our study focused on the molecular mechanism underlying CAP's alleviation of gastric mucosal injury by combating oxidative stress. We identified Src as a crucial target in CAP's protective action against gastric mucosal damage. Previous research has highlighted the modulation of oxidative stress through Src regulation. For instance, Chu et al. revealed that *N*-*n*-butyl haloperidol iodide could inhibit the c-Jun *N*-terminal kinase/SH3 domain-binding protein 5/Src/ROS pathway, reducing ROS production and mitigating mitochondrial oxidative stress [46]. Additionally, Qu et al. elucidated that the 3*S*,3'*S*-astaxanthin isomer suppressed the activity of the Na/K-ATPase/Src/extracellular signal-regulated



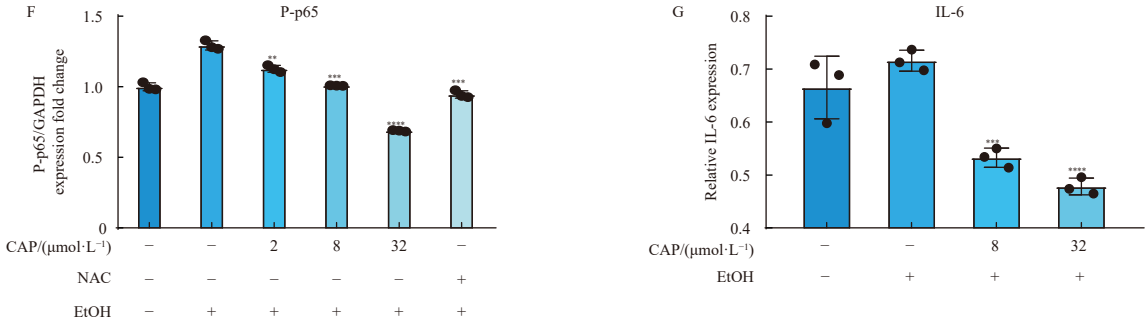
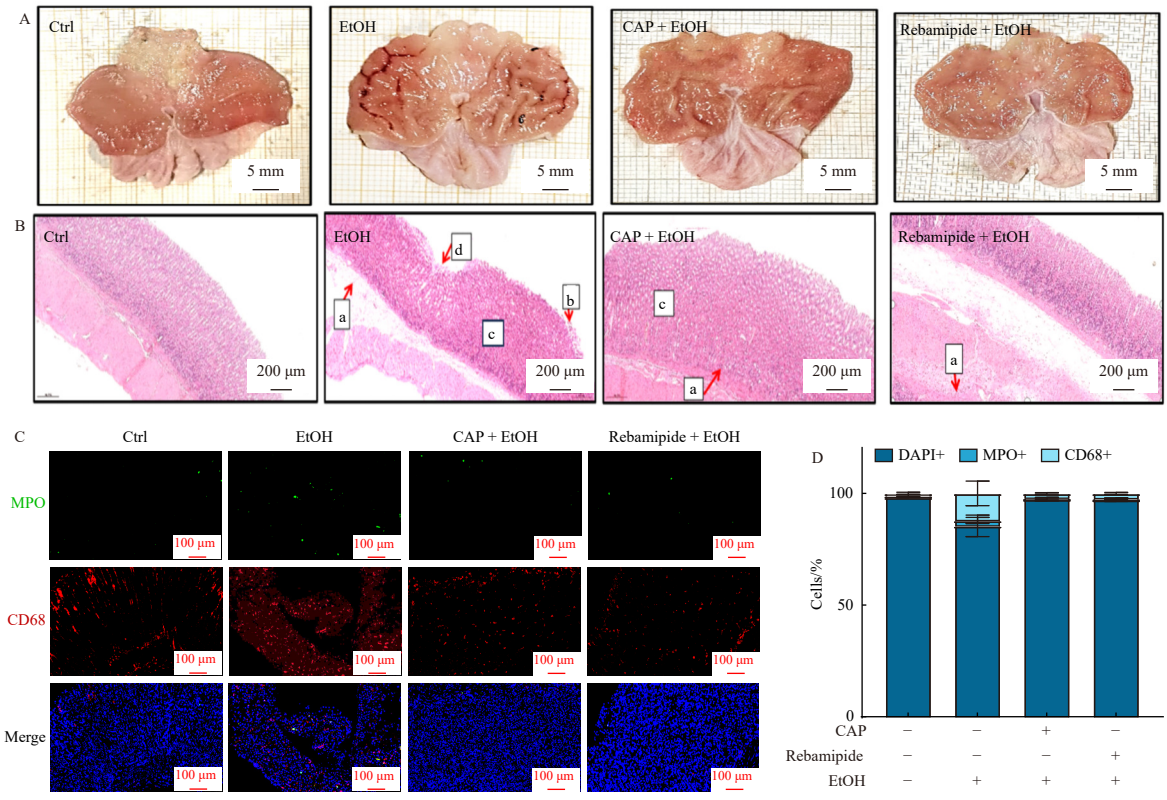


Fig. 5 CAP inhibits P-p65 expression and p65 nuclear translocation. (A and B) The effect of CAP on P-p65 expression is evaluated in the GES-1 cell model using Western blotting analysis. (C and D) Immunofluorescence co-localization experiments are performed to assess the influence of EtOH and CAP on NF-κB p65 protein nuclear translocation. (E and F) The modulatory effect of CAP on P-p65 expression is determined in the RAW264.7 cell model. (G) The impact of CAP on IL-6 expression levels is measured by ELISA in the RAW264.7 cell model. Data are presented as mean ± SD from three independent experiments. The statistical significance of differences between data is determined using Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ *vs* only EtOH treated group.

kinase 1/2(ERK1/2) signaling pathway, thereby preventing off oxidative stress-induced damage in cardiomyocytes [47]. Similarly, Wang et al. observed that caffeine can attenuate mitochondrial oxidative stress injuries. This effect is mediated through the adenosine A2A receptor/cyclic adenosine monophosphate/PKA/Src/ERK1/2/p38 mitogen-activated kinase-like protein pathway, leading to a reduction in oxidative stress and subsequent prevention of hyperoxia-induced lung damage [48]. In conclusion, our study highlights CAP's potential to counteract oxidative stress and inflammatory reactions. It achieves this by inhibiting the CCR4/Src/p47phox pathway, ultimately alleviating EtOH-induced gastric mucosal injuries.

The NF-κB signaling pathway plays a crucial role in regulating inflammatory responses. p65, a key component of the NF-κB protein family, can be activated through the classical signaling pathway, leading to the transcription of inflammatory mediators and apoptotic proteins [49]. Somensi et al. demonstrated that carvacrol inhibits the lipopolysaccharide-induced translocation of NF-κB p65 from the cytoplasm to the nucleus, highlighting its anti-inflammatory potential, which may be linked to its inherent antioxidant properties [50]. Additionally, Shen et al. observed that baicalein suppresses the expression of specific proteins, including NF-κB p65 and Bcl-2/Bax, thereby protecting against oxidative stress damage in ulcerative colitis rat models [51]. Furthermore, Hu et al. reported that salidroside reduces the activation of NF-κB p65 and NLR family pyrin domain containing three inflammasomes, alleviating endothelial inflammation and oxidative stress [26]. In RAW264.7 cells, our study revealed that CAP inhibits the phosphorylation of RelA/p65 protein at the Ser536 residue, thus suppressing NF-κB signaling and mitigating harmful inflammation. The Ser536 site is a critical phosphorylation locus on p65, which can be targeted by various kinases, such as IκB kinase, TANK-binding kinase 1, and ribosomal S6 kinase 1, to enhance the transcriptional activity of p65 [52-54]. Additional research is required to determine whether CAP also inhibits the activity of these protein kinases. In line with previous findings, our investigation demonstrates that CAP can alleviate EtOH-induced oxidative damage to the gastric mucosa by inhibiting both the activity and nuclear migration of NF-κB p65, ultimately suppressing inflammation and apoptosis mediated by the downstream p65



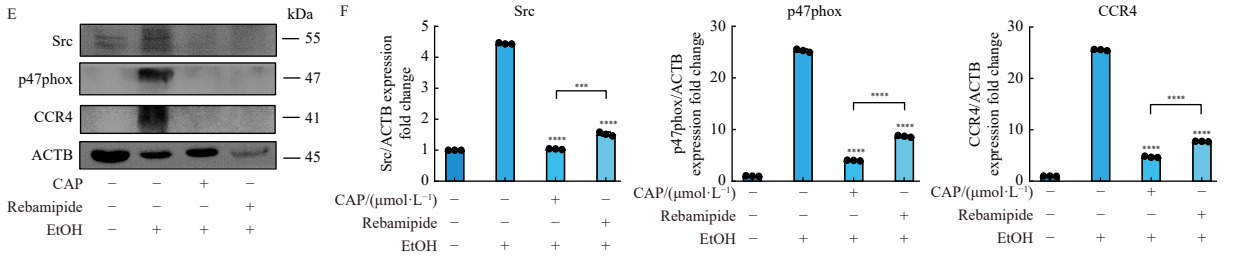


Fig. 6 CAP mitigates gastric mucosal injury and suppresses CCR4/Src/p47phox expression *in vivo*. (A) Gastric mucosal protection provided by CAP and the positive control drug, rebamipide. (B) H&E staining of pathological gastric mucosal sections. In the illustration, a indicates the condition of submucosal and muscular layers and inflammatory cell infiltration, b indicates mucosal epithelial detachment, c shows the overall gastric tissue structure and glandular arrangement, d highlights tissue edema. (C and D) Immunofluorescence analysis is performed to assess neutrophil (myeloperoxidase/MPO$^+$) and macrophage (cluster of differentiation 68/CD68$^+$) infiltration in rat tissue sections. (E and F) The rat model is employed to evaluate CAP's effect on Src/p47phox/CCR4 expression. Data are presented as mean ± SD for three independent experiments. The statistical significance of differences between data is analyzed by Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Symbols above the columns indicate results compared to the EtOH treatment group alone.

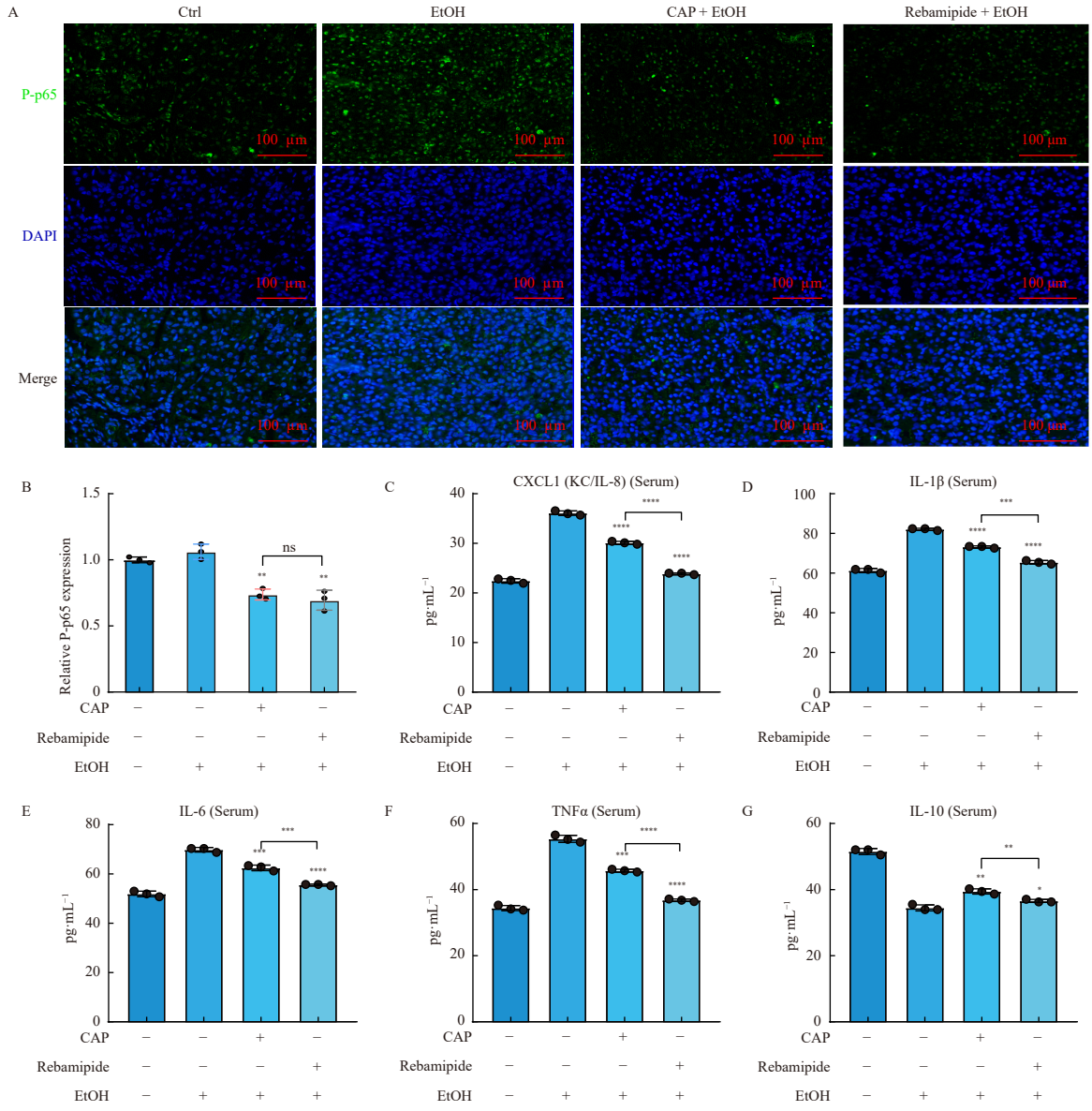


Fig. 7 CAP inhibits NF-κB signaling pathway and inflammatory factor secretion *in vivo*. (A and B) Immunofluorescence analysis of P-p65 expression in rat tissue sections. (C–G) ELISA measurement of chemokine expression levels of chemokine (C-X-C motif) ligand 1 [CXCL1/KC (IL-8)], IL-1β, IL-6, TNF-α, and IL-10 in rat serum. Data are presented as mean ± SD from three independent experiments. The statistical significance of differences between data is determined using Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Symbols above columns indicate comparisons to the EtOH treatment group alone.

pathway.

CAP, a known agonist of TRPV1, primarily exerts its effects through this receptor. The impact of CAP on TRPV1 is complex. While its activation of TRPV1 channels can induce chronic inflammatory pain [55], prolonged topical CAP application may lead to the degradation of autonomic nerve fibers in the skin, subsequently reducing pain perception [56]. Additionally, CAP has been associated with cardiomyopathy prevention by restoring vascular per-

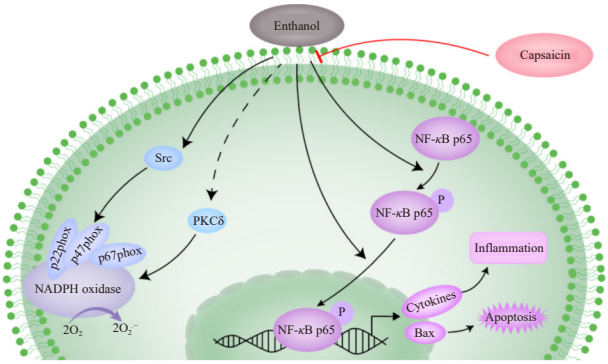


Fig. 8 Molecular mechanisms underlying CAP's prevention of gastric mucosal injury.

meability [34]. Conversely, it may also induce insufficient vasoconstriction, potentially leading to vasospasms and myocardial infarction [57]. Notably, Sharma et al. emphasized CAP's potential to mitigate immune oxidative and inflammatory signals to the brain through the TRPV1 pathway [56]. However, Chaudhary et al. observed that CAP might also possess antioxidant properties *via* mechanisms not mediated by the TRPV1 receptor [58]. In our study, it remains unclear whether the inhibition of Src and NF-κB p65 proteins by capsaicin, along with its protective effect on gastric mucosal integrity, is dependent on the TRPV1 pathway. This question warrants further investigation. While our research highlighted the expression levels of target proteins to elucidate the molecular mechanisms of CAP, future studies are needed to explore the specific molecular interactions between CAP and these proteins is a future research direction.

In conclusion, this investigation illuminates the molecular mechanisms underlying CAP's antioxidant and anti-inflammatory effects. CAP demonstrates efficacy in suppressing both the transcription and expression of Src, a key protein in the chemokine pathway, while also inhibiting the p65 pathway. These combined actions provide protection to the gastric mucosa against EtOH-induced oxidative stress. Considering its abundant availability and favorable biosafety profile, CAP presents significant potential for further research and development.

Funding

This work was supported by the High-level Talent Research Start-up Project Funding of Henan Academy of Sciences (No. 252028037).

Availability of supporting information

Supporting information for this study can be obtained by contacting the corresponding authors *via* E-mail.

Declaration of competing interest

These authors have no conflict of interest to declare.

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