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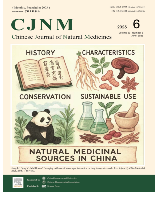

Original article

Deciphering the therapeutic potential and mechanisms of *Artemisia argyit* essential oil on flagellum-mediated *Salmonella* infections

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ABSTRACT

Salmonellosis represents a global epidemic, and the emergence of extensively drug-resistant (XDR) *Salmonella* and its sustained transmission worldwide constitutes a significant public health concern. Flagellum-mediated motility serves as a crucial virulence trait of *Salmonella* that guides the pathogen toward the epithelial surface, enhancing gut colonization. *Artemisia argyit* essential oil, a traditional herb extract, demonstrates efficacy in treating inflammation-related symptoms and diseases; however, its effects on flagellum assembly and expression mechanisms in anti-*Salmonella* activity remain inadequately explored. This study aimed to elucidate the mechanism by which *Artemisia argyit* essential oil addresses *Salmonella* infections. Network pharmacological analysis revealed that Traditional Chinese Medicine (TCM) *Artemisia argyit* exhibited anti-*Salmonella* infection potential and inhibited flagellum-dependent motility. The application of *Artemisia argyit* essential oil induced notable motility defects through the downregulation of flagellar and fimbriae expression. Moreover, it significantly reduced *Salmonella*-infected cell damage by interfering with flagellum-mediated *Salmonella* colonization. *In vivo* studies demonstrated that *Artemisia argyit* essential oil administration effectively alleviated *Salmonella* infection symptoms by reducing bacterial loads, inhibiting interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) production, and diminishing pathological injury. Gas chromatography-mass spectrometry (GC-MS) analysis identified forty-three compounds in *Artemisia argyit* essential oil, with their corresponding targets and active ingredients predicted. Investigation of an *in vivo* model of *Salmonella* infection using the active ingredient demonstrated that alpha-cedrene ameliorated *Salmonella* infection. These findings suggest the potential application of *Artemisia argyit* essential oil in controlling *Salmonella*, the predominant food-borne pathogen.

1. Introduction

Salmonella is a pervasive food-borne pathogen causing approximately 930,000 cases worldwide annually, presenting a significant threat to global health and safety [1], primarily transmitted through contaminated food and water. According to the European Food Safety Authority (EFSA), *Salmonella* is projected to become the second most prevalent zoonotic pathogen globally by 2022 [2], representing a substantial threat to human and animal health worldwide. While typical symptoms of *Salmonella* infection include fever, abdominal pain, vomiting, and diarrhea, the pathogen poses particular risks to children under five, elderly individuals, and immunocompromised adults, potentially causing systemic infections requiring antibiotic intervention [3]. The prevalence of salmonellosis continues to rise due to evolving food consumption patterns, an aging population with increasing numbers of immunocompromised individuals, and inappropriate antibiotic usage in food-producing animals [4].

S. typhimurium initiates infection by entering the intestinal tract, traversing the protective mucus layer, and colonizing the host's intestinal epithelium [5]. The virulence of *Salmonella* stems from multiple factors, including various secretion systems, plasmids, flagella, and fimbriae. During infection, *Salmonella* employs flagellum-mediated motility to traverse the intestinal mucus layer and reach the epithelial infection site [6]. The pathogen then invades nonphagocytic intestinal epithelial cells through interactions between bacterial fimbriae and host cell receptor proteins while stimulating fibroblasts to secrete extracellular matrix [7]. Subsequently, the type III secretion system secretes effector proteins that trigger inflammatory responses in neutrophils and facilitate intracellular *Salmonella* replication [8]. Among these mechanisms, intestinal colonization and adhesion are considered crucial factors in bacterial pathogenesis [9]. Consequently, flagella and fimbriae represent potential therapeutic targets against *S. typhimurium* infections.

The rise in bacterial resistance necessitates the development of novel anti-infection strategies for alternative treatments. Plant extracts have garnered significant scientific, industrial, and commercial attention due to their beneficial health effects [10]. Current

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research indicates that essential oils and their primary compounds merit investigation as potential antibiotic alternatives in poultry feed [11].

Artemisia argyit (Asteraceae, English name: mugwort, Chinese name: Aicao) represents a traditional medicinal plant with historical significance in medicine, particularly noted for its volatile oils. *Artemisia argyit* essential oil is derived through the processing and extraction of *Artemisia argyit*. Historical medical applications have revealed numerous therapeutic properties. Throughout millennia, *Artemisia argyit* has demonstrated consistent efficacy with minimal side effects in treating complex ailments. The plant exhibits multiple beneficial properties, including anti-oxidant [12], hepatoprotective [13], anti-spasmodic [14], anti-nociceptive [15], anti-bacterial [16], and anti-fungal [17] effects. In traditional medicine globally, *Artemisia argyit* serves to alleviate gastrointestinal discomfort and treat gynecological conditions, while its essential oil is prescribed for treating ulcers and diarrhea [18]. Additionally, *Artemisia argyit* essential oil is incorporated into skincare cosmetics, including creams, masks, and tonics [19]. These therapeutic properties are primarily attributed to various isolated secondary metabolites, particularly terpenoids such as terpineol, camphor, and eucalyptol [17]. These compounds hold Generally Recognized as Safe (GRAS) status. However, the anti-*Salmonella* properties of *Artemisia argyit* essential oil remain unexplored.

This investigation aims to elucidate the mechanisms by which the Traditional Chinese Medicine (TCM) *Artemisia argyit* treats *Salmonella* infection through its effects on flagella and fimbriae motility and adherence. To understand the underlying mechanisms of infection modification, this study employs a murine *Salmonella* infection model to examine the therapeutic effects of *Artemisia argyit* essential oil on *S. typhimurium* infection. This research presents an effective therapeutic approach and potential candidates for treating *Salmonella* infection.

2. Materials and methods

2.1. Bacterial strains, reagents, and materials

S. typhimurium SL1344, utilized in this investigation, was provided courtesy of Prof. Zhaoqing Luo from Purdue University. The bacteria were cultivated in Luria–Bertani (LB) medium (Qingdao Hope Biol-Technology Co., Ltd.) at 37 °C with agitation at 180 $r\cdot min^{-1}$. Microbiological procedures complied with established standards for managing biological risk in veterinary laboratory and animal facilities, ensuring the safety of laboratory personnel and environmental protection [20]. Prior to experimentation, personnel were required to don appropriate laboratory attire, including protective clothing, masks, and gloves. All procedures were conducted within an ultra-clean bench. Experimental waste disposal followed the "Regulations on the Management of Medical Waste" (State Council Decree No. 380) of Jilin University.

Caco-2, RAW264.7, and HeLa cells (human clonal colon adenocarcinoma cells) were maintained in Dulbecco's modified Eagle medium (DMEM)/high glucose supplemented with 10% fetal bovine serum, 100 $U\cdot mL^{-1}$ penicillin, and 100 $U\cdot mL^{-1}$ streptomycin. The cells were maintained at 37 °C in an atmosphere containing 5% CO_2.

2.2. Prediction of bioactive components and potential targets and enrichment of relevant pathways associated with *Artemisia argyit* essential oil

On the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database analysis platform (http://lsp.nwu.edu.cn/tcmsp.php), active chemical compounds of *Artemisia argyit* were screened based on oral bioavailability (OB) >20% and drug-likeness (DL) > 0.1 [21]. The Encyclopedia of Traditional Chinese Medicine database (ETCM, http://www.tcmip.cn/ETCM/) was employed for further refinement using a drug-likeness weight (quantitative estimate of drug-likeness, QED) > 0.49, and Wayne diagrams were generated through the SRplot online website (https://www.bioinformatics.com.cn/). The compounds' SMILES sequences were identified in the PubChem database (https://pubchem.ncbi.nlm.nih.gov) and entered into Swiss target prediction (http://www.swisstargetprediction.ch). The Swiss Target Prediction tool of the Swiss absorption, distribution, metabolism, and elimination (ADME) system complemented the TCMSP system components and their corresponding action targets [22]. Disease-related genes associated with *Salmonella* infection enteritis were obtained from genes (GeneCards) (https://www.genecards.org/). Cross-targets were analyzed in STRING (https://string-db.org/) to obtain protein-protein interactions (PPIs), visualized using Cytoscape 3.6. The Metascape database (https://metascape.org/) facilitated pathway enrichment analysis of target genes.

2.3. Swimming motility assay

Swimming assays were performed using the semisolid agar method, as previously described [23]. Various concentrations (0, 16, 32, 64, and 128 $\mu g\cdot mL^{-1}$) of *Artemisia argyit* essential oil were incorporated into 0.3% agar. *S. typhimurium* SL1344 cultured overnight was inoculated onto soft agar plates and incubated overnight at 37 °C. Colony ring diameters were measured across two intersecting planes.

2.4. Growth curve assay

The overnight cultured *S. typhimurium* SL1344, ATCC14028, and HYM2 were diluted 1 : 100 in LB medium, and at an optical density at 600 nm (OD_{600nm}) = 0.2, the bacterial solution was divided into 6 portions. *Artemisia argyit* essential oil was added to achieve final concentrations of 0, 16, 32, 64, and 128 $\mu g\cdot mL^{-1}$. OD_{600nm} values were recorded at hourly intervals over a 20-hour period.

2.5. Minimal inhibitory concentration (MIC) determination assay

The MIC was determined using the broth microdilution method, involving bacterial inoculation into an LB medium containing various concentrations of *Artemisia argyit* essential oil and the active ingredient, following established protocols [24].

2.6. Transmission electron microscope (TEM) observation

S. typhimurium SL1344 treated with *Artemisia argyit* essential oil was collected, washed twice in phosphate-buffered saline (PBS), and resuspended in PBS. A 10 μL aliquot of culture was placed on a carbon-coated nickel grid for 1 min and subsequently stained with 0.2% uranyl acetate for 30 sec. Sample visualization was conducted using a Tecnai Spirit (120 kV) TEM.

2.7. Purification of flagellar proteins

The flagellin of *S. typhimurium* SL1344 was purified following a previously established method [25]. Initially, the essential oil of *Artemisia argyit* was incubated with SL1344 at 37 °C for 4 h. Subsequently, the bacteria were collected in a suspension of 10 mL of sucrose solution. A solution containing 10 $mmol\cdot L^{-1}$ EDTA and 0.1 $mg\cdot mL^{-1}$ lysozyme was added to the suspension, which underwent stirring for 30 minutes at 4 °C. Subsequently, Triton-X (final concentration 1%) and 10 mmol·L^{-1} $MgSO_4$ were added, followed by stirring for 1 h at 4 °C. The suspension pH was adjusted to 11 using NaOH, followed by centrifugation to remove cellular debris. The resulting precipitate underwent ultracentrifugation (BACKMAN, USA) at 110 000 × g for 1 h at 4 °C. The final precipitate was resuspended in TE buffer, and flagellin detection was performed *via* sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after overnight incubation at 4 °C.

2.8. Western blotting

Various concentrations of *Artemisia argyit* essential oil were incubated with SL1344 at 37 °C for 4 h and centrifuged at 12 000 r·min^{-1} for 5 min to obtain the culture supernatant and bacterial precipitate. Bacterial protein samples were prepared by heating the bacterial precipitate with 1 × SDS–PAGE uploading buffer at 100 °C for 10 min. Secretion assays were performed as previously described [26]. Proteins in the culture supernatant underwent precipitation with 10% trichloroacetic acid overnight at 4 °C. The protein precipitate was collected, resuspended in SDS–PAGE loading buffer, and heated for 10 min at 100 °C. Following SDS–PAGE, immunoblotting was conducted using a polyclonal anti-flagellin (FliC) antibody (1 : 5000, Abcam, UK) as previously described [27]. Detection utilized the ECL Enhanced Immunoblotting Detection Kit (Biosharp, China), and blots were visualized using a Tanon-4200 imager. A minimum of three independent experiments were conducted.

2.9. Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) analysis

Gene expression analysis was performed using real-time quantitative polymerase chain reaction. The messenger ribonucleic acid (mRNA) expression levels of flagellar genes (*FlhC*, *FlhD*, *FliL*, *FliA*, *FliD*, *FliZ*, *FliK*, *FliC*, *FljB*, and *motA*) and fimbriae genes (*fimA*, *fimD*, and *stm0551*) were assessed in *S. typhimurium* SL1344. Total RNA extraction from bacteria was performed using TransZol Up (TransGen, China), and complementary deoxyribonucleic acid (cDNA) synthesis was accomplished through reverse transcription using a cDNA reverse transcription kit (Mei5bio, China). RT-qPCR was conducted using a QuantStudio1 detection system. The utilized primers are presented in Supplementary Table 1. As previously described [28], relative mRNA expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method.

2.10. Membrane permeability determination

S. typhimurium SL1344 in the logarithmic growth phase was harvested and washed three times with PBS buffer to achieve an OD_{600nm} 0.5. The membrane permeability assay was conducted as previously described [29]. Membrane permeability was assessed using propidium iodide (PI; Sigma–Aldrich) at a final concentration of 0.5 μmol·L^{-1} and *N*-phenyl-1-naphthylamine (NPN; Sigma–Aldrich) at a final concentration of 10 μmol·L^{-1}. The reaction mixture was initially incubated at 37 °C for 30 min, followed by the addition of varying concentrations of *Artemisia argyit* essential oil and further incubation at 37 °C for 4 h in dark conditions. Subsequently, the mixture was transferred to a 96-well black Petri dish for fluorescence intensity measurement. The PI fluorescence intensity was measured at an excitation wavelength of 535 nm and emission wavelength of 615 nm, while NPN fluorescence intensity was measured at 350 nm excitation and 420 nm emission wavelengths. The experiment included three biological replicates.

2.11. Cell viability and cytotoxicity assays

Caco-2, RAW264.7, and HeLa cells were seeded in 96-well plates at a density of 2 × 10^4 cells/well and incubated overnight at 37 °C. Various concentrations (16, 32, 64, and 128 μg·mL^{-1}) of *Artemisia argyit* essential oil were applied to the cells, followed by incubation at 37 °C for 4 h, with DMEM serving as the negative control. Subsequently, the cells were treated with cell counting kit-8 (CCK-8) working solution and incubated at 37 °C for 30 min before measuring absorbance at 450 nm using an enzyme marker (Tecan, CH).

Caco-2 cells at a density of 2 × 10^4 cells/well were exposed to different concentrations of *Artemisia argyit* essential oil (16, 32, 64, and 128 μg·mL^{-1}) and *S. typhimurium* SL1344 for 4 h at 37 °C. Control cells without *Artemisia argyit* essential oil received DMSO treatment, and cytotoxicity was evaluated using cytotoxicity assay kits according to the manufacturer's instructions (Beyotime, China). Lactate dehydrogenase (LDH) release in the supernatant was measured at 490 nm. The protective effect of *Artemisia argyit* essential oil against SL1344-induced cellular damage was evaluated using a LIVE/DEAD (green/red) reagent (Invitrogen, USA) following the manufacturer's protocols. Cell visualization was performed using an inverted fluorescence microscope (Olympus, Tokyo).

2.12. Cell adhesion assay, immunofluorescence, and invasion

The methodology was modified from previously described protocols [30]. Caco-2 cells were seeded in 24-well plates at 4 × 10^4 cells/well, and *S. typhimurium* SL1344 was treated with varying concentrations of *Artemisia argyit* essential oil (16, 32, 64, and 128 μg·mL^{-1}). Caco-2 cells were infected at MOI = 100 for 2 h. Nonadherent bacteria were removed by PBS washing, and cells were lysed with 0.1% Triton X-100. The lysate underwent serial dilution, and adherent bacteria were enumerated on streptomycin-resistant LB agar plates.

For immunofluorescence analysis, infected cells were fixed with 4% formaldehyde for 30 min and washed thrice with PBS as previously described [31]. Following blocking with 5% BSA for 30 min and subsequent washing, cells were incubated with *S. typhimurium* flagellin FliC antibody (1 : 100 dilution; Abcam, UK) for 1 h. Subsequently, cells were treated with secondary antibody Alexa Fluor 488 (1 : 1000 dilution; Invitrogen, USA) for 1 h. Nuclear staining was performed with DAPI, and immunofluorescence analysis was conducted using a fluorescence microscope.

Bacterial invasion was assessed using a gentamicin protection assay, modified from previously described methods [32]. Infected cells were washed thrice with sterile PBS and incubated with DMED containing 100 μg·mL^{-1} gentamicin for 1 h. Following incubation and PBS washing, cells were lysed using 0.1% Triton X-100. The lysates underwent serial dilution, and bacterial invasion was quantified by plating on streptomycin-resistant LB agar plates.

2.13. Animal experiment

The animal experiments in this study were conducted according to the recommendations and protocols approved by the Institutional Animal Care and Use Committee of Jilin University (permit number SY202306059). The experimental protocol followed the previously described method [33]. Disease models were established using 6-to 8-week-old (18–20 g) female BALB/c mice (Liaoning Changsheng Biotechnology Co., Ltd.) [34]. Following a one-week acclimatization period, all mice received 5 g·L^{-1} streptomycin in their drinking water for three days. *Artemisia argyit* essential oil therapeutic experiment: The mice were divided randomly into five groups (n = 6 per group): vehicle control group, SL1344-infected group, and groups treated with oral *Artemisia argyit* essential oil at 30, 60, and 100 mg·kg^{-1}·d^{-1} respectively. Therapeutic experiment on the active ingredients of *Artemisia argyit* essential oil: The mice were divided randomly into 6 groups: vehicle control group, SL1344-infected group, groups treated with oral alpha-cedrene (40 and 80 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively), and groups treated with oral Bornyl acetate (40 and 80 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively). Mice received oral administration of 5×10^6 CFU of *S. typhimurium* SL1344, followed by transoral administration 2 h later and every 12 h for 4 days. After the 4-day infection period, the mice were euthanized; liver, spleen, and cecum tissues were collected and homogenized in phosphate buffer. Serial dilutions of the homogenate were plated on XLD agar to determine CFU counts. The remaining tissues were fixed in 4% formaldehyde and underwent hematoxylin-eosin (H&E) staining for pathological analysis. Additionally, tissue homogenates were centrifuged at 4 °C and 12 000 $r \cdot min^{-1}$ for 10 min to measure cytokine levels [interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α)] using an ELISA kit (BioLegend, USA).

2.14. Analysis of the chemical composition of Artemisia argyit essential oil

Artemisia argyit essential oil obtained from Ji'an Zhongxiang Natural Vegetable Oil Co., Ltd. (Jilin, China) was stored in darkness at 4 °C and diluted to 10 $mg \cdot mL^{-1}$ with ethyl acetate. Analysis was conducted using a GC-MS (Agilent 8890–7000D, USA) equipped with a fused-silica DB-5 MS capillary column (30 m × 0.25 mm and 0.25 μm film thickness); mass spectra were scanned from 40–600 amu. Helium carrier gas flowed at 1.4 $mL \cdot min^{-1}$. A 1 μL splitless injection was performed. The EI energy was set at 70 eV, where maximum abundance was observed. The source temperature was maintained at 230 °C, with an interface temperature of 250 °C. The oven temperature program began at 45 °C for 2 min, then increased to 280 °C at 5.0 °C min^{-1}. The injector temperature was set to 250 °C.

2.15. Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test with GraphPad Prism 9.3 software (GraphPad Software, La Jolla, CA). The P values are indicated as follows: $P > 0.05$, $^{**}P < 0.01$, $^{*}P < 0.05$.

3. Results

3.1. Prediction of potential targets of Artemisia argyit against Salmonella infection and construction of PPI networks

Throughout Chinese medicine's extensive history, certain Traditional Chinese Medicines (TCMs) have demonstrated efficacy in treating bacterial infections. Consequently, TCMs present valuable candidates for investigating and validating anti-*Salmonella* properties. Network pharmacology serves as an effective tool for elucidating TCM treatment mechanisms [22]. Based on predictions from the TCMSP and ETCM databases, 521 targets of 13 chemical components were identified among the 31 primary screening compounds from *Artemisia argyit*. The GeneCards database yielded 1354 disease-related genes associated with *Salmonella* infection. Fig. 1A illustrates 123 overlapping targets between *Artemisia argyit* and *Salmonella* infection. These overlapping targets were analyzed through the STRING database, resulting in the construction of a PPI network (Fig. 1B and Fig. 1C). Within this network, AKT1 emerged as a key hub protein (degree > 74), which constitutes essential components of growth factor signaling pathways and regulates cell proliferation, migration, and invasion in antitumor studies [35]. Additionally, TNF (degree > 72) demonstrates a significant influence on cell proliferation, migration, invasion, and angiogenesis [36]. Research indicates that TNF-deficient patients exhibit increased mortality from *Sal-*

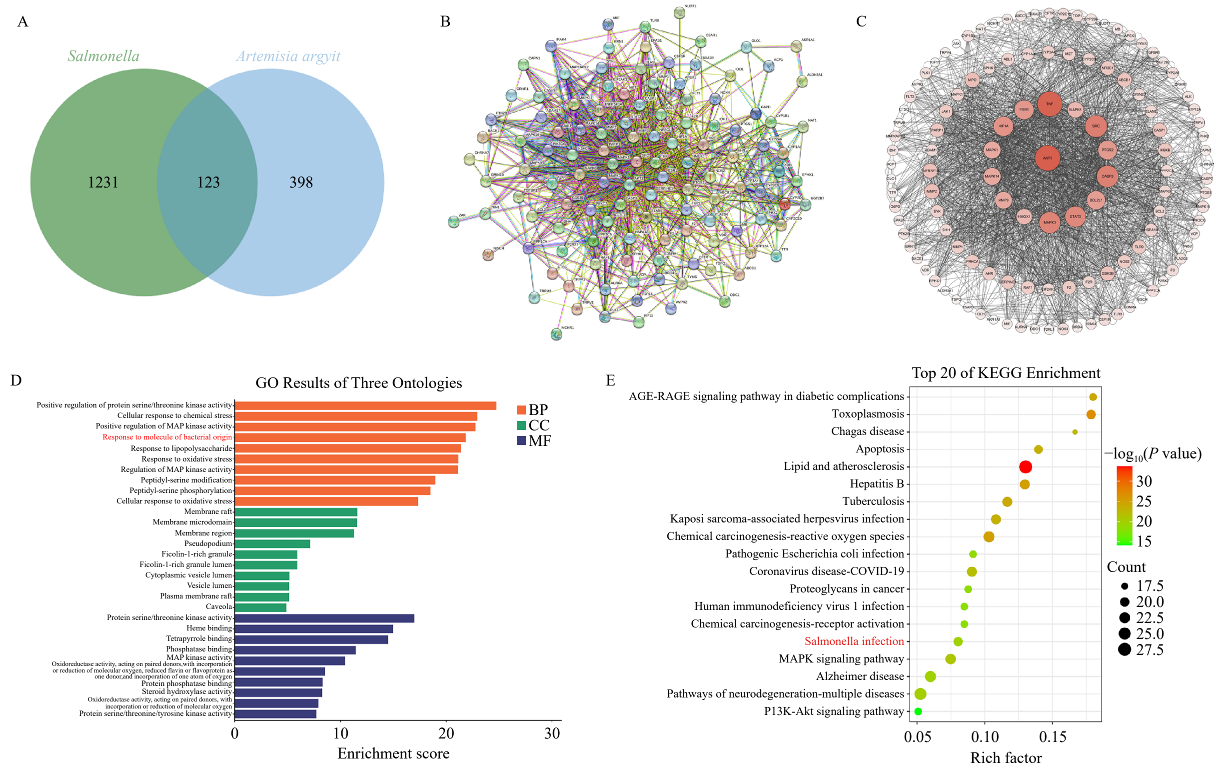

Fig. 1 Network pharmacological analysis of *Artemisia argyit* against *Salmonella* infection. (A) The 123 overlapping genes between the bioactive ingredients of *Artemisia argyit* obtained from the TCMSP and ETCM databases with ingredients (OB ≥ 20%, DL index ≥ 0.1, and QED > 0.49) and *Salmonella* infection. The related gene symbols. (B) and protein-protein interactions (PPI). (C) associated with *Artemisia argyit* against *Salmonella* infection. Circles represent target proteins. The greater the circle's diameter is, the higher the degree value and more intersections with other proteins. (D–E) Enrichment analysis of intersection targets between *Artemisia argyit* and *Salmonella* infection.

monella infections [37]. SRC (degree > 59), a nonreceptor protein tyrosine kinase, regulates various biological signaling pathways, immune responses, cell adhesion, cell cycle progression, migration, and transformation [38].

The principal anti-*Salmonella* infection targets of *Artemisia argyit* were analyzed using the Metascape system, revealing relevant pathways centered on cell membrane structure modifications, cell migration, and cell adhesion. KEGG pathway analysis indicated *Artemisia argyit* as a potential therapeutic agent for *Salmonella* treatment (Figs. 1D and 1E). These findings suggest that the relationship between *Artemisia argyit* intervention and *Salmonella* infection likely involves multiple coordinated targets.

3.2. Artemisia argyit essential oil inhibited the motility of S. typhimurium

Considering motility as a crucial virulence characteristic of *Salmonella*, motility assessments were conducted with and without *Artemisia argyit* essential oil. The investigation revealed that *Artemisia argyit* essential oil inhibited the motility of *S. typhimurium* SL1344, ATCC14028, and HYM2 in a dose-dependent manner (Figs. 2A–2B, 2C–2D, and 2E–2F).

Further evaluation of *Artemisia argyit* essential oil's antimicrobial properties through bacterial growth assays demonstrated that *S. typhimurium* SL1344, ATCC14028, and HYM2, when cultured with specified concentrations of the essential oil, showed no significant impact on bacterial growth (Supplementary Fig. 1A, 1B and 1C). The MICs of *Artemisia argyit* essential oil against *S. typhimurium* SL1344, ATCC14028, and HYM2 exceeded 512 μg·mL^{-1} (Supplementary Table 2). These findings demonstrate that while *Artemisia argyit* essential oil effectively inhibits motility, it does not impair *S. typhimurium* growth.

3.3. Artemisia argyit essential oil inhibited the assembly and expression of flagella and fimbriae of S. typhimurium

During the initial infection stage, *S. typhimurium* adherence to host tissue depends on proteinaceous filamentous surface structures, known as fimbriae, which identify and bind to specific receptors on host cells (Fig. 3A). The fimbriae not only serve a crucial role in adhesion but also interact with flagella to regulate bacterial motility. Consequently, fimbriae represent essential factors in understanding *Salmonella* pathogenesis [39]. The *fimA* promoter regulates the expression of multiple genes involved in fimbriae structure, while the proteins *fimW*, *fimY*, and the *stm0551* open reading frame modulate fimbriae transcription [40]. To elucidate these regulatory pathways, we confirmed significant downregulation of transcripts associated with fimbriae structural expression at 128 μg·mL^{-1} (Fig. 3B). To examine the effect of *Artemisia argyit* essential oil treatment on fimbriae expression, we assessed the fimbriae structure. As shown in Fig. 3C, the surface of bacteria treated with *Artemisia argyit* essential oil appeared smooth, with a disrupted membrane structure. These findings collectively demonstrated that, compared to the control treatment, *Artemisia argyit* essential oil treatment reduced surface fimbriae assembly, as evidenced by the absence of fimbriae genes.

The bacterial flagellum functions as a rotary nanomachine of the cytoplasmic membrane and comprises dozens of different proteins (Fig. 4A) [41]. We investigated whether *Artemisia argyit* essential oil's effect on *Salmonella* motility correlates with flagellar numbers using transmission electron microscopy. As illustrated in Fig. 4B, the control group of *Salmonella* bacteria exhibited complete flagellation. In contrast, the *Artemisia argyit* essential oil treatment group displayed significantly fewer flagella, explaining the substantially reduced motility. Notably, the filament consists of more than 20 000 subunits of a single protein [42]. Through purification and analysis of flagellin proteins in *S. typhimurium* SL1344, results indicated that the expression of flagellin FliC subunit (52 kDa), P ring FlgI (38 kDa), Rod FlgF (32 kDa), and FlgG (30 kDa) decreased progressively with increasing concentrations of *Artemisia argyit* essential oil compared to the positive control (Fig. 4C). Correspondingly, protein abundance analysis of FliC, FlgI, FlgF, and FlgG treated with *Artemisia argyit* es-

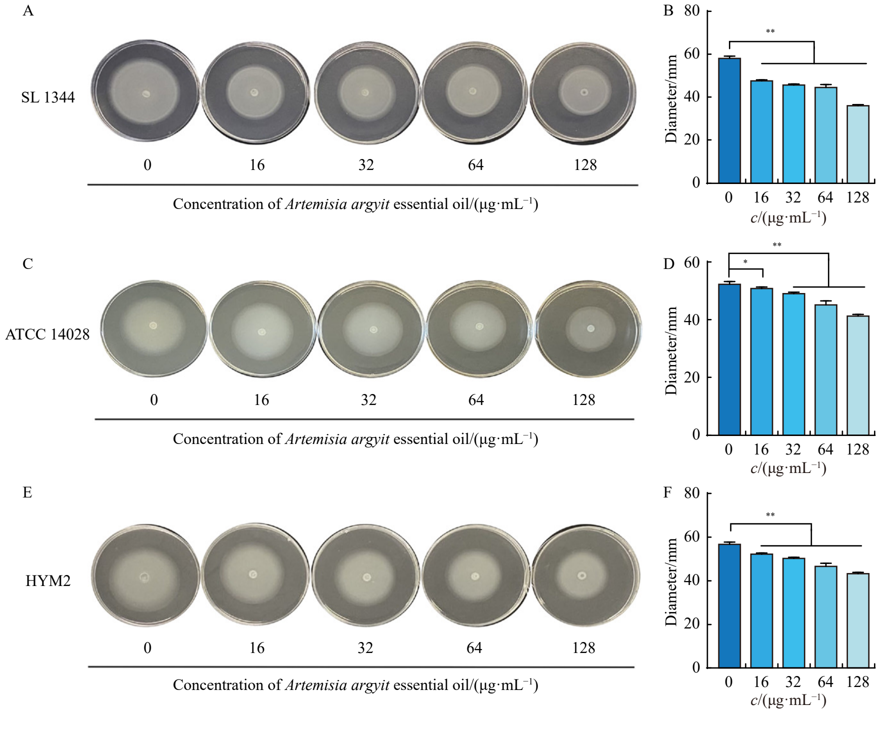

Fig. 2 *Artemisia argyit* essential oil suppresses *S. typhimurium* motility. Inhibition of *S. typhimurium* SL1344 (A–B), ATCC14028 (C–D), and HYM2 (E–F) motility by *Artemisia argyit* essential oil and the movement diameters measurements. All of the data shown are presented as the means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

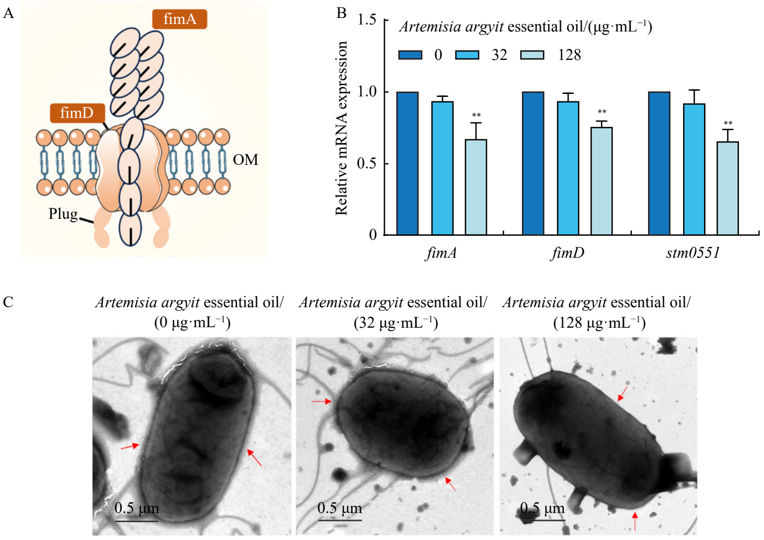

Fig. 3 *Artemisia argyit* essential oil improves the transcription of fimbriae genes and structure. (A) Schematic structure of *Salmonella* fimbriae. (B) Regulation of *Artemisia argyit* essential oil on SL1344 fimbriae regulatory genes. (C) The effects of *Artemisia argyit* essential oil on SL1344 fimbriae were observed using a transmission electron microscope (TEM). $^{**}P < 0.01$.

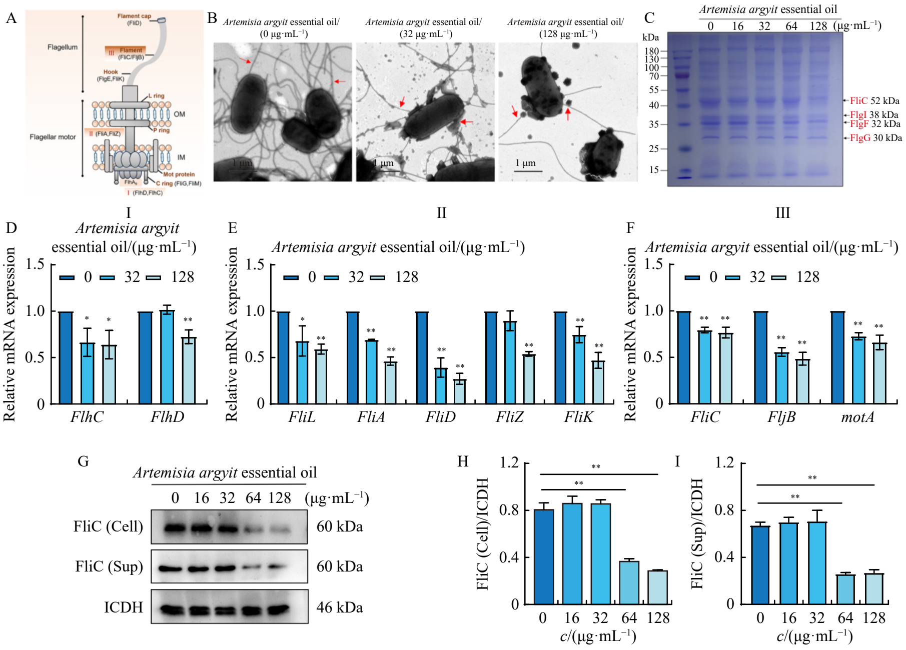

Fig. 4 Effect of *Artemisia argyit* essential oil on the assembly and expression of flagella. (A) Schematic structure of *Salmonella* flagella. (B) Effects of *Artemisia argyit* essential oil on SL1344 flagella were observed using a transmission electron microscope (TEM). (C) Purification of flagellin from *Artemisia argyit* essential oil-treated *S. typhimurium* SL1344. (D−F) Regulation of *Artemisia argyit* essential oil on flagellar regulatory genes of SL1344. (G) Western blotting showed that *Artemisia argyit* essential oil inhibited the expression of flagellin (FliC). (H−I) Western blot gray values were measured using ImageJ software. ICDH was used as tax control. $^{*}P < 0.05$; $^{**}P < 0.01$.

sential oil showed a reduction in a dose-dependent manner (Supplementary Fig. 2A, 2B, 2C and 2D). These studies demonstrated that *Artemisia argyit* essential oil inhibits several flagellin proteins' synthesis, potentially through suppression of upstream regulatory gene expression at the transcriptional level. We examined the transcriptional effects of *Artemisia argyit* essential oil on flagellar genes. Research indicates that flagellar genes undergo regulation at the transcriptional level through a hierarchical system comprising three gene classes [43]. We analyzed the expression of class I promoters *FlhC* and *FlhD*; class II promoters *FliL*, *FliA*, *FliD*, *FliZ*, and *FliK*; and class III promoters *FliC*, *FljB*, and *motA*. Results revealed a significant decrease in transcript levels of these flagellar genes upon *Artemisia argyit* essential oil treatment (Figs. 4D−4F). Western blotting analysis further confirmed the effect, showing a substantial reduction in FliC production and secretion in *Artemisia argyit* essential oil-treated bacteria (Figs. 4G−4I). These data collectively demonstrated that *Artemisia argyit* essential oil impairs *Salmonella* motility by disrupting flagellar and fimbriae expression and assembly.

3.4. *Artemisia argyit* essential oil enhanced cell membrane permeability

Network pharmacology analysis indicated that cell membranes represent potential targets for *Artemisia argyit* essential oil in combating *Salmonella* infections. Furthermore, flagella are structures integrated within the cell membrane. The integrity of the cell membrane correlates with flagellar status, and studies demonstrate that flagellum-deficient strains exhibit disrupted cell membrane structure [44]. NPN, a hydrophobic fluorescent probe that emits fluorescence upon interaction with the phospholipid bilayer's hydrophobic region, serves as an indicator of bacterial outer membrane permeability [45]. As illustrated in Fig. 5A, bacterial outer membrane permeability increased significantly in a dose-dependent manner when exposed to 16–128 μg·mL^{-1} *Artemisia argyit* essential oil. Additionally, an assessment of total bacterial membrane permeability using the fluorescent probe PI (Fig. 5B) revealed that cell membrane permeability increased proportionally with *Artemisia argyit* essential oil concentration.

3.5. *Artemisia argyit* essential oil treatment attenuated *S. typhimurium*-induced cellular injury

Considering the anti-virulence efficacy of *Artemisia argyit* essential oil, this study investigated its potential to mitigate *S. typhimurium*-mediated cell injury. The CCK-8 analysis presented in Fig. 6A demonstrated that the tested concentrations of *Artemisia argyit* essential oil exhibited no cytotoxic effects on RAW264.7, HeLa, and Caco-2 cells. Previous research indicates that *S. typhimurium* virulence can be assessed through cellular LDH release [46]. Subsequently, LDH levels in Caco-2 cell supernatants were examined to evaluate the protective effects of *Artemisia argyit* essential oil against *S. typhimurium* SL1344-induced cellular damage. While *S. typhimurium* stimulation resulted in Caco-2 cell injury, the addition of *Artemisia argyit* essential oil significantly reduced LDH release in cell supernatants (Fig. 6B). Live/dead cell staining confirmed that *S. typhimurium* exposure induced cell death, an effect that was mitigated by *Artemisia argyit* essential oil treatment (Fig. 6C). These findings demonstrate that *Artemisia argyit* essential oil provides effective cytoprotection against *S. typhimurium*-induced cellular damage.

3.6. *Artemisia argyit* essential oil inhibited *S. typhimurium*-mediated cell adhesion

Bacterial adhesion and colonization in the gut, facilitated by flagella and fimbriae during early infection stages, represent crucial steps in *Salmonella* pathogenesis [7]. Assessment of *Artemisia argyit* essential oil's effect on *S. typhimurium* SL1344 revealed that concentrations exceeding 32 μg·mL^{-1} significantly reduced SL1344 adhesion to Caco-2 cells (Fig. 7A). This finding was corroborated by immunofluorescence analysis (Fig. 7B). While the control group exhibited numerous bacterial adhesions surrounding the cells, treatment with *Artemisia argyit* essential oil notably decreased green fluorescence around Caco-2 cells.

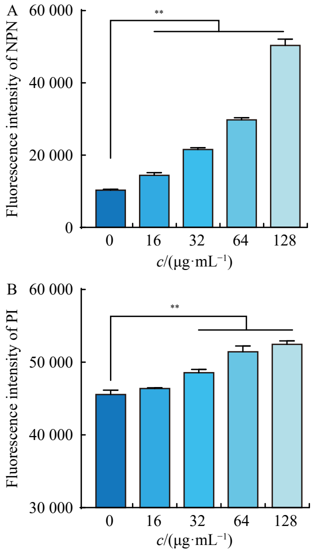

Fig. 5 *Artemisia argyit* essential oil significantly increases cell membrane permeability. Changes in the outer membrane permeability (A) and membrane permeability (B) of *S. typhimurium* SL1344 in the presence or absence of different concentrations of *Artemisia argyit* essential oil. $^{**}P < 0.01$.

Investigation of *Artemisia argyit* essential oil's impact on Caco-2 cell invasion demonstrated that concentrations above 32 μg·mL^{-1} significantly inhibited SL1344 invasion (Fig. 7C). These findings indicate that *Artemisia argyit* essential oil treatment effectively prevents both *S. typhimurium* adhesion and invasion of Caco-2 cells.

3.7. Oral administration of *Artemisia argyit* essential oil effectively attenuated *S. typhimurium* pathogenicity *in vivo*

During systemic infections in mice, *Salmonella* progresses from the intestines to lymph nodes before reaching organs such as the liver and spleen [47]. Previous results demonstrated that *Artemisia argyit* essential oil exhibits antitoxic effects by targeting bacterial flagella and fimbriae motility and adhesion properties. To evaluate the therapeutic efficacy of *Artemisia argyit* essential oil *in vivo*, its anti-infective effects were examined in SL1344-infected mice. Streptomycin treatment was administered to each mouse group 3 days prior to SL1344 infection (Fig. 8A). The *Artemisia argyit* essential oil-treated group showed significantly reduced bacterial numbers in the cecum, liver, and spleen, indicating decreased bacterial colonization (Figs. 8B, 8C, and 8D). These results demonstrate that *Artemisia argyit* essential oil treatment effectively reversed *Salmonella* infection. H&E-stained sections confirmed the therapeutic efficacy of different treatment regimens (Figs. 8E and 8F). Untreated infected liver and spleen exhibited congestion and enlargement. The cecum in the infection group and 30 mg·kg^{-1} treatment group contained minimal feces, while healthy and other treatment groups showed improved digestive tract absorption with small amounts of coagulated feces. SL1344 infection induced hepatocyte degenerative necrosis, inflammatory cell infiltration in splenocytes, and significant intestinal mucosal epithelial cell detachment. Treatment with *Artemisia argyit* essential oil substantially reduced histopathological damage, correlating with the colony colonization results. These findings demonstrate that *Artemisia argyit* essential oil treatment provides effective protection against *S. typhimurium* infection *in vivo*.

Salmonella infection triggers a cascade of immune responses, beginning with an innate immune response characterized by neutrophil and inflammatory monocyte convergence to eliminate bacteria, followed by T lymphocyte activation that initiates an adaptive immune response [48]. The analysis revealed significantly elevated levels of IL-6, IL-1β, and TNF-α in the cecum (Figs. 9A–9C), liver (Figs. 9D–9F), and spleen (Figs. 9G–9I) tissues of the SL1344 group compared to the control group. Conversely, mice treated with *Artemisia argyit* essential oil exhibited decreased levels of IL-6, IL-1β, and TNF-α in a concentration-dependent manner. These findings indicate that *Artemisia argyit* essential oil demonstrates potential as a therapeutic agent for treating *Salmonella* infections, effectively mitigating *S. typhimurium* SL1344 infection *in vivo* and reducing *Salmonella* pathogenicity.

3.8. Effect of the active components of *Artemisia argyit* essential oil on *S. typhimurium*

To identify the active compound from the *Artemisia argyit* extract that influences *Salmonella* flagellum assembly and expression, gas chromatography-mass spectrometry (GC-MS) analysis identified 43 pure compounds. The analysis revealed γ-terpinene as the predominant component (21.39%), followed by *β*-caryophyllene (8.84%), *α*-pinene (7.77%), m-cymene (7.06%), (+)-calarene (5.79%) and other compounds. The complete chemical composition is detailed in Supplementary Table 3, with chro-

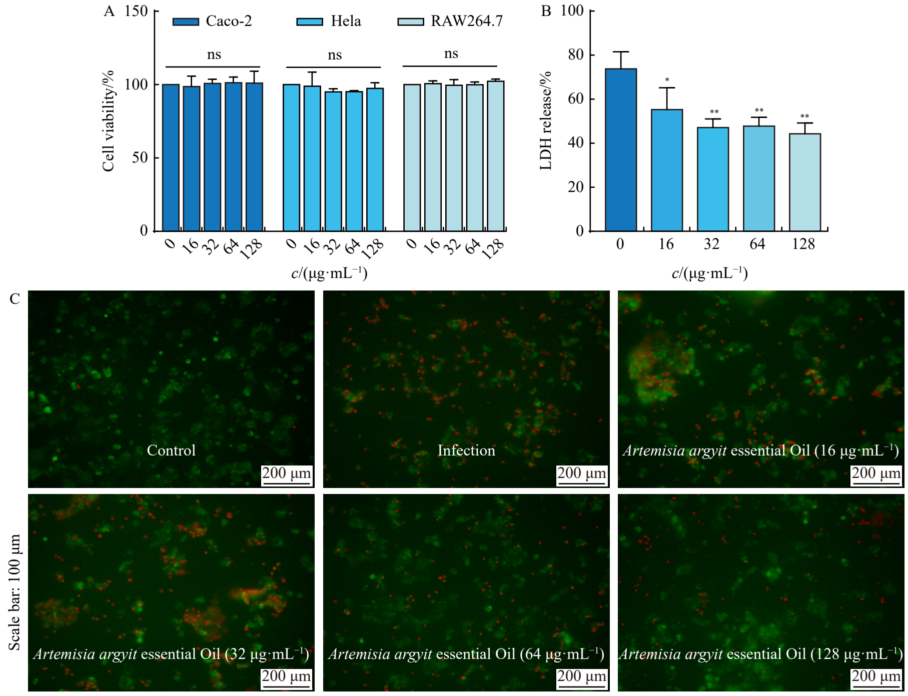

Fig. 6 *Artemisia argyit* essential oil effectively inhibits SL1344-induced cell injury. (A) Cytotoxicity of *Artemisia argyit* essential oil on Caco-2 cells, HeLa cells, and RAW264.7 cells. (B) Inhibitory effect of *Artemisia argyit* essential oil on SL1344-induced cell injury. (C) Caco-2 cells were infected with SL1344 and transfected with live/dead reagents (green/red). Live cells are stained green, and dead cells are stained red. $^{*}P < 0.05$; $^{**}P < 0.01$.

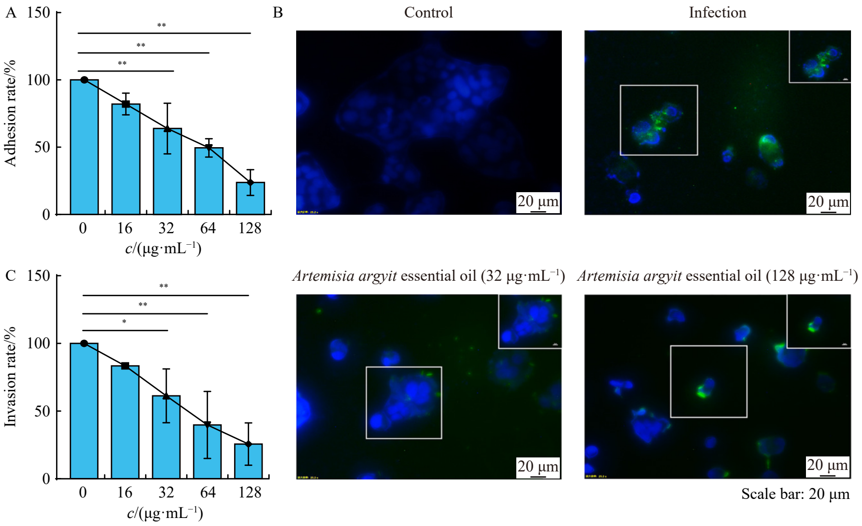

Fig. 7 *Artemisia argyit* essential oil inhibits SL1344-induced cell adhesion and invasion. (A) *Artemisia argyit* essential oil inhibits the adhesion of SL1344 to Caco-2 cells. (B) Representative images of bacterial adhesion observed by immunofluorescence microscopy. Bacteria are stained green, and nuclei are stained blue. The merged images acquired for the relevant immunofluorescence signals indicate the colonization of bacteria in host cells. (C) Protective effect of *Artemisia argyit* essential oil on the SL1344-induced invasion of Caco-2 cells. $^{*}P < 0.05$; $^{**}P < 0.01$.

matograms presented in Supplementary Fig. 3.

Analysis of 31 active chemical constituents of *Artemisia argyit* from the TCMSP and ETCM databases, compared with the essential oil composition, identified five active chemical constituents: alpha-copaene, cyperene, sabinene, Bornyl acetate, and (−)-alpha-cedrene (Fig. 10A). α-Copaene functions as an egg-laying promoter for Drosophila; however, human exposure to high concentrations can cause significant irritation to skin, eyes, and respiratory tract [49]. Current research on cyperene remains limited, with its presence primarily documented in essential oil extracts from various plant roots and stems. These characteristics constrain the therapeutic application of *Artemisia argyit* for *Salmonella* infection.

Previous research has demonstrated that [50 51 52], Bornyl acetate, sabinene, and (−)-α-cedrene are the principal bioactive constituents of *Artemisia argyit* essential oil, exhibiting potent antimicrobial effects against fungal pathogens, *Staphylococcus aureus*, and other organisms, while also showing anthelmintic and anti-oxidant properties. Bioinformatics analysis identified five active chemical pathways enriched in cell migration, cell adherence, T-cell immune effector processes, and regulation of inflammatory responses (Supplementary Fig. 4A and 4B). The active ingredients of *Artemisia argyit* essential oil, specifically Bornyl acetate at 4 μg·mL^{-1} and (−)-α-cedrene at 32 μg·mL^{-1}, effectively inhibited *S. typhimurium* motility. However, sabinene showed no significant inhibition of SL1344 (Figs. 10B–10G). Further assessment of anti-bacterial activity revealed that the MICs of Bornyl acetate, sabinene, and (−)-α-cedrene against *S. typhimurium* exceeded 512 μg·mL^{-1}, indicating that while Bornyl acetate and (−)-α-cedrene inhibited motility, they did not affect bacterial

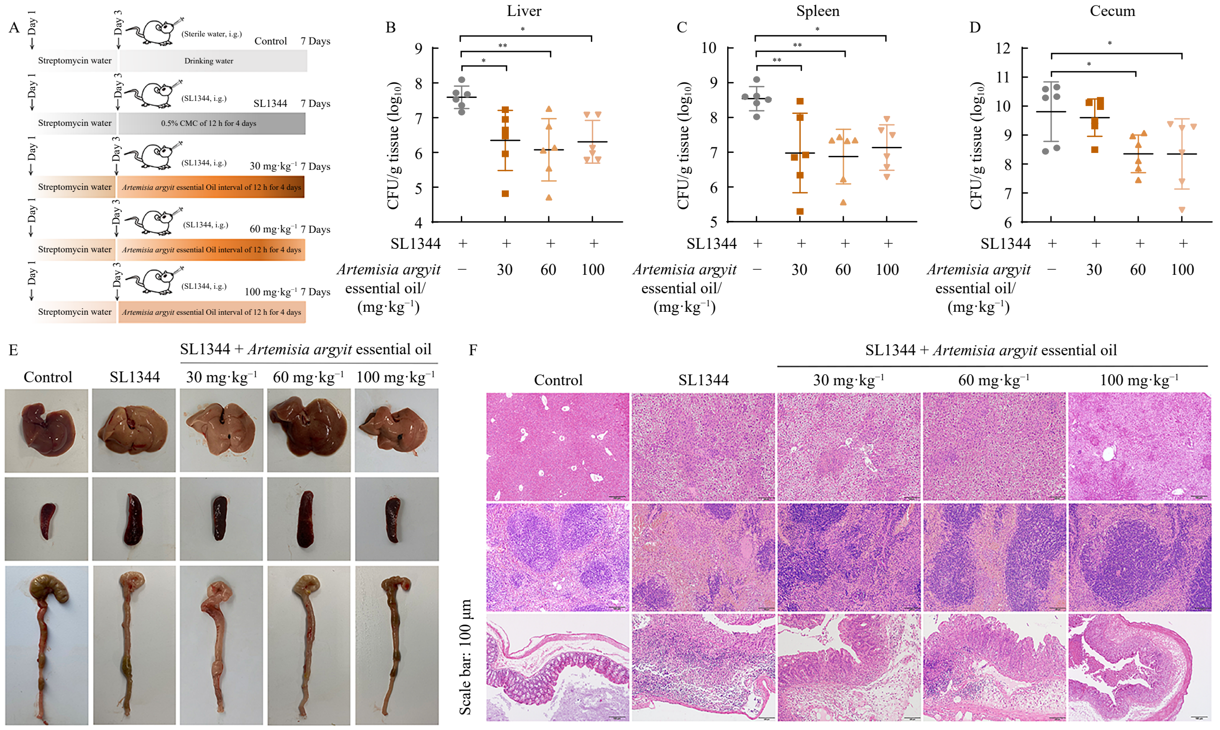

Fig. 8 Oral administration of *Artemisia argyit* essential oil protects mice from *S. typhimurium* infection *in vivo*. (A) Schematic diagram of the drug delivery treatments. (B–D) Bacterial loads in the liver, spleen, and cecum of *S. typhimurium*-infected mice after treatment with *Artemisia argyit* essential oil (n = 6 per group). (E–F) Histopathological injury of the liver, spleen, and cecum tissues treated with *Artemisia argyit* essential oil was observed. Scale bar: 100 μm. $^{*}P < 0.05$; $^{**}P < 0.01$.

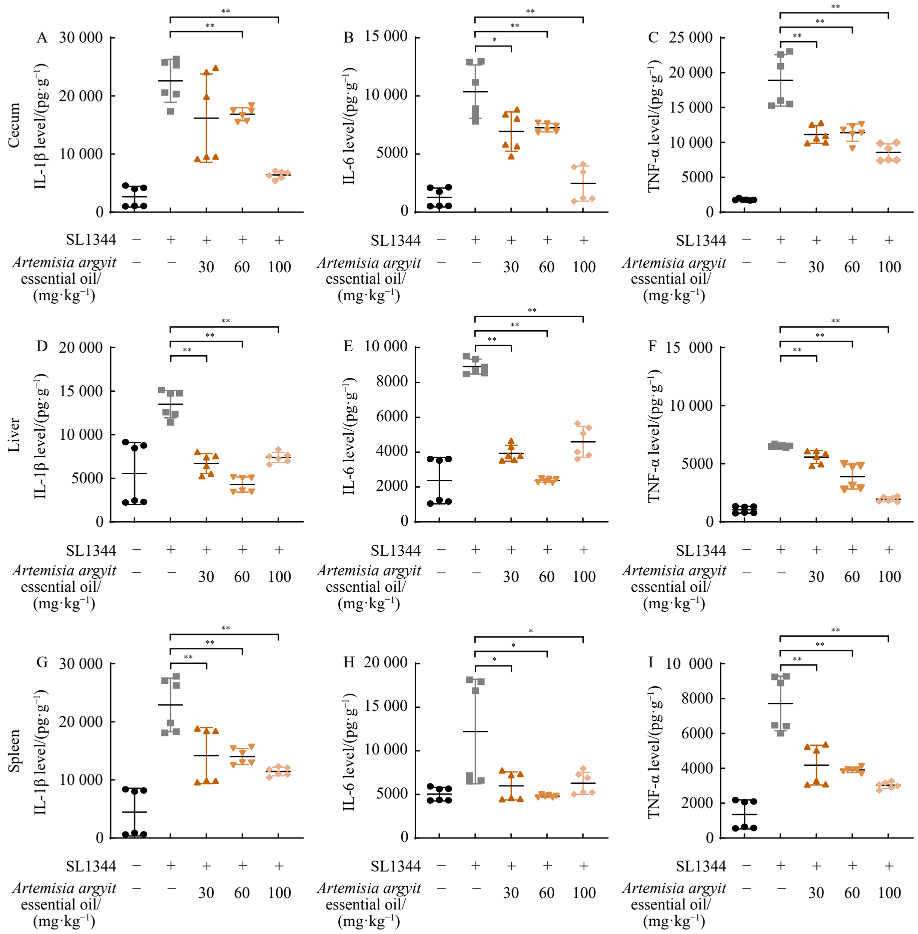

Fig. 9 *Artemisia argyit* essential oil treatment reduces inflammatory cytokines in mouse tissues. Levels of the inflammatory factors IL-6, IL-1β, and TNF-α in the cecum (A-C), liver (D-F), and spleen (G-I) of *S. typhimurium*-infected mice treated with *Artemisia argyit* essential oil (n = 6 per group). $^{*}P < 0.05$; $^{**}P < 0.01$.

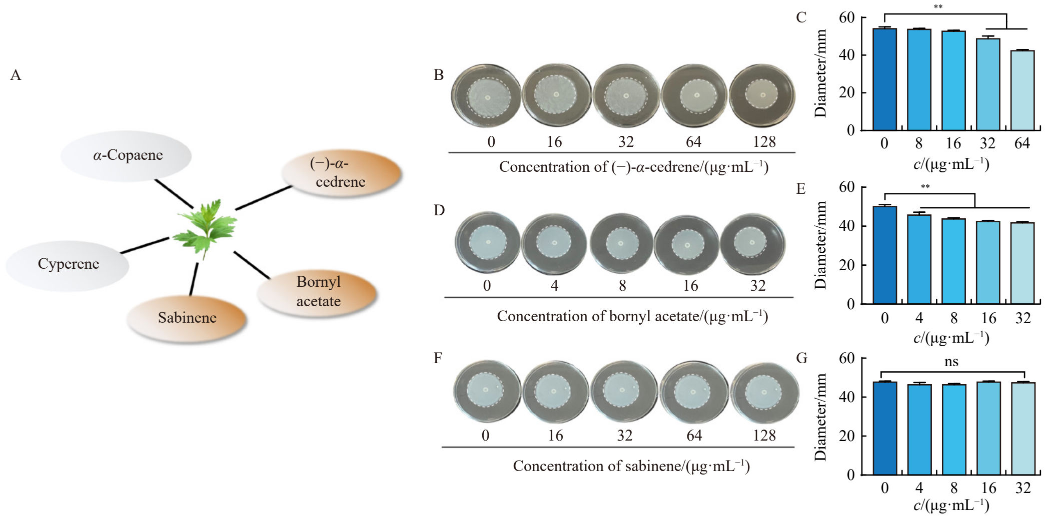

Fig. 10 Analysis of the active ingredients of *Artemisia argyit* essential oil. (A) Bioactive constituents of *Artemisia argyit* obtained from the TCMSP and ETCM databases based on GC-MS components. Effects of (−)-α-cedrene (B−C), Bornyl acetate (D−E), and sabinene (F−G), the active components of *Artemisia argyit* essential oil, on the motility of *S. typhimurium* SL1344. ns, $P > 0.05$; $^{**}P < 0.01$.

growth (Supplementary Table 4). These findings suggest that Bornyl acetate and (−)-α-cedrene are the primary components responsible for the anti-*Salmonella* motility of *Artemisia argyit*. Notably, sabinene concentration was tenfold higher than Bornyl acetate in the *Artemisia argyit* fraction. The effect of *Artemisia argyit* essential oil on *Salmonella* flagellum assembly and expression results from the synergistic interactions among all oil components rather than individual compound effects.

3.9. Alpha-cedrene is the active compound in Artemisia argyit essential oil that effectively attenuated S. typhimurium pathogenicity in vivo

While the inhibitory effects of *Artemisia argyit* essential oil extracts Bornyl acetate and alpha-cedrene on *Salmonella* motility were verified, their *in vivo* therapeutic effects remained unexplored. To evaluate the *in vivo* therapeutic efficacy of *Artemisia argyit* essential oil extract, the anti-infective effect was investigated in SL1344-infected mice (Fig. 11A). The alpha-cedrene treated group demonstrated significantly reduced bacterial loads in the liver, spleen, and cecum. In contrast, the Bornyl acetate treatment group showed no significant differences in tissue bacterial loads (Figs. 11B, 11C, and 11D). The images of intact organs and H&E-stained slices revealed varying therapeutic efficacy across treatment regimens (Figs. 11E and 11F). The SL1344 and Bornyl acetate groups exhibited nearly feces-free ceca, while the SL1344 + α-cedrene group showed healthy, solidified feces. Gray discoloration and enlargement were observed on liver and spleen surfaces in the SL1344 and Bornyl acetate groups, whereas the α-cedrene-treated group showed fewer lesions. Additionally, α-cedrene treatment improved hepatocyte degenerative necrosis, splenocyte inflammatory cell infiltration, and intestinal mucosa integrity. These findings indicate that α-cedrene may be a primary component responsible for the anti-*Salmonella* infection properties of *Artemisia argyit* essential oil.

4. Discussion

Salmonella represents a major zoonotic pathogen, with antibiotics serving as the primary treatment for *Salmonella* infections. However, mortality rates from *Salmonella* infections continue to increase due to emerging antibiotic resistance, necessitating the development of novel therapeutic approaches. Research demonstrates that during initial infection stages, *S. typhimurium* employs virulence factors, specifically flagella and fimbriae, for movement and gut colonization. These highly immunogenic structures trigger host immune responses [53]. Notably, therapeutic agents that reduce inflammation while inhibiting motility and adhesion demonstrate efficacy against *Salmonella* infection. Through bioinformatics analysis and experimental validation, this study identifies TCM *Artemisia argyit* essential oil as a promising therapeutic candidate targeting flagella and fimbriae in *Salmonella* infection.

Network pharmacology represents a contemporary analytical methodology incorporating networked databases. The analysis revealed that *Artemisia argyit* essential oil's active compounds interact with 123 targets related to *Salmonella* infection within the herb-component-target network. These findings indicate numerous potential motility and adhesion-related targets share similar binding patterns with *Artemisia argyit* essential oil compounds.

The flagella and fimbriae of *S. typhimurium* constitute primary virulence factors in infection progression. The flagellum, crucial in early infection stages, comprises a long, rotatable helical structure on the bacterial surface, enabling movement through liquid media and surface aggregation. The flagellum's structure encompasses three principal components: the basal body, hooks, and filaments [54]. Filament rotation facilitates bacterial movement, while hooks connect the basal body to filaments. Chemotaxis and motility proteins enable environmental sensing and directional movement. Membrane-bound transporter proteins utilize proton motive force (PMF) to export flagellin for hook and filament construction. The flagellin transport system comprises an exit gate complex (FlhA, FlhB, FliP, FliQ, FliR) and an ATPase ring complex (FliH, FliI, FliJ) [55]. Research indicates that flagellar class I promoter transcription inhibition reduces *S. typhimurium* motility and impairs colonization *in vivo* [56]. This study demonstrates that *Salmonella* responds to varying concentrations of *Artemisia argyit* essential oil, leading to flagellar synthesis downregulation and membrane permeability alterations. The essential oil significantly suppresses flagellar gene expression across class 1, 2, and 3 promoters.

Fimbriae are extracellular appendages primarily composed of FimA protein, measuring 0.5−10 μm in length and 2−8 nm in width. These structures facilitate adherence and bioepithelial membrane formation. During intestinal invasion, *Salmonella* utilizes fimbriae to bind to M cell mannose receptors, facilitating entry into intestinal epithelial cells. Subsequently, *Salmonella* enters the intestinal lumen, where replication leads to acute infection [57]. Type I fimbriae are encoded by the fim gene cluster, including *fimA*, *fimC*, *fimD*, *fimH*, *fimF*, *fimY*, *fimW*, and *stm0551*

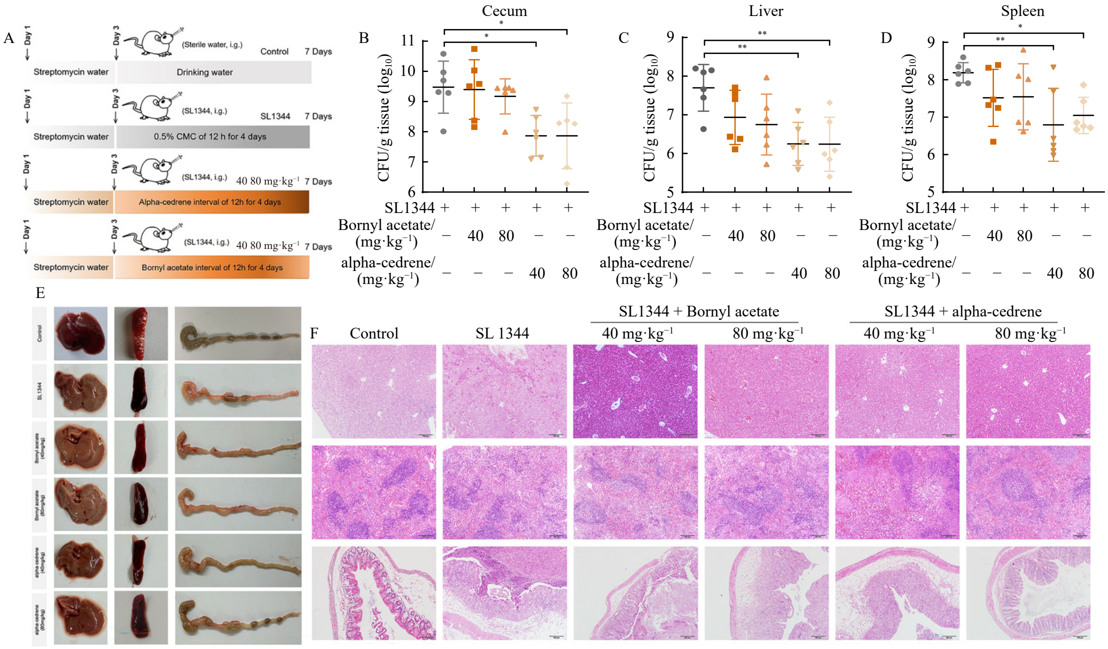

Fig. 11 Bornyl acetate and alpha-cedrene provide protection against *S. typhimurium* infection in mice. (A) Experimental design for the treatment of *S. typhimurium* infections with Bornyl acetate and alpha-cedrene. (B-D) Bacterial loading analysis in the liver, spleen, and cecum of *S. typhimurium* infection mice (n = 6 per group). (E-F) Representative photographs of the liver, spleen, and cecum with histopathology. Scale bar: 100 μm. *, $P < 0.05$; **, $P < 0.01$.

genes [58]. Studies demonstrate that *S. typhimurium* fimbriae inhibition reduces epithelial cell adhesion capability and pathogenicity [59]. Thus, the inhibition of adhesion and invasion represents a crucial therapeutic mechanism of *Artemisia argyit* essential oil against *Salmonella* infections.

Artemisia argyit has been widely recognized for its rich phytochemical composition and diverse bioactivities, particularly its medicinal efficacy and aromatic qualities. Research has demonstrated that *Artemisia argyit* extract exhibits anti-inflammatory and hypolipidaemic effects in hypercholesterolaemic rats. The extract demonstrated a significant reduction in the levels of NO, malondialdehyde (MAD), and TNF-α [60]. These therapeutic effects are potentially attributable to the inhibitory properties of *Artemisia argyit* secondary metabolites, particularly flavonoids and terpenoids, against inflammatory modulators. GC-MS analysis revealed that *Artemisia argyit* essential oil contains diverse terpenoids, including γ-terpinene, Bornyl acetate, alpha-cedrene, α-terpinene, and β-caryophyllene. Our research confirmed alpha-cedrene's capacity to inhibit *Salmonella* infections *in vivo*. Notably, besides alpha-cedrene (2.85%), the *Artemisia argyit* essential oil comprises γ-Terpinene, Borneol, camphor, and various other compounds in substantial quantities. Borneol, camphor, and Bornyl acetate represent promising monoterpenoids with extensive applications in medicine, flavor, food, and chemical industries. These compounds constitute the primary active components of volatile oils in numerous traditional Chinese medicines, exhibiting anti-inflammatory, anti-bacterial, and inflammatory properties. Consequently, we postulate that *Artemisia argyit* essential oil functions synergistically through these terpenoids against *Salmonella* infections. Despite numerous reported therapeutic effects of *Artemisia argyit* essential oil, no previous studies have demonstrated its efficacy in treating *Salmonella* infections. Our research reveals that *Artemisia argyit* essential oil administration significantly reduced the infiltration of cytokines IL-1β, IL-6, and TNF-α in colon tissues. These findings indicate that *Artemisia argyit* essential oil suppresses inflammation to mitigate *Salmonella*-induced colon injury.

In summary, this study investigated the mechanism underlying the inhibitory effects of *Artemisia argyit* essential oil on *S. typhimurium* flagella. The essential oil effectively suppressed the motility of *S. typhimurium*. Additionally, *Artemisia argyit* essential oil substantially reduced the pathogenicity of *S. typhimurium* in mice by inhibiting the transcription of relevant genes and protein synthesis, resulting in decreased flagella and fimbriae numbers, while inhibiting adhesion and colonization of epithelial cells by *S. typhimurium*. Moreover, active components of *Artemisia argyit* essential oil, particularly Bornyl acetate and (−)-alpha-cedrene, demonstrated inhibition of *Salmonella* motility. Alpha-cedrene specifically showed inhibitory effects against *Salmonella* infection *in vivo*. These findings suggest that *Artemisia argyit* essential oil achieves its anti-*Salmonella* virulence effects primarily through the combined action of alpha-cedrene with other active compounds. The research confirms that *Artemisia argyit* essential oil represents a promising traditional Chinese medicine extract with significant potential for treating *Salmonella* infections.

5. Conclusions

In summary, our research demonstrates that the TCM *Artemisia argyit* exhibits significant anti-*Salmonella* infection properties. *Artemisia argyit* essential oil and its bioactive components function as flagellum inhibitors by interfering with flagellum assembly and expression. The successful establishment of a *Salmonella*-infection model in mice validated the *in vivo* anti-infection and anti-inflammatory effects of *Artemisia argyit* essential oil and alpha-cedrene. This research highlights *Artemisia argyit* essential oil and its bioactive components as promising candidates for *Salmonella* infection treatment.

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Availability of supporting information

Supporting information for this work is available upon request from the corresponding authors *via* E-mail.

Declaration of competing interest

These authors have no conflict of interest to declare.

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