

Original Article

miR2916-p5 and miR6478 from *Forsythia suspensa* fruit suppress inflammatory responses by targeting AKT signaling in human colorectal epithelial cells

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Abstract

Cross-kingdom regulation by microRNAs (miRNAs) has been widely observed across species. Plant-derived miRNAs can be absorbed through the mammalian gastrointestinal tract, subsequently enter the bloodstream, and reach various target tissues. However, the specific composition, types, and functions of miRNAs in traditional Chinese medicinal herbs, such as *Forsythia suspensa*, remain unexplored. In this study, we identify 225 conserved miRNAs and 121 novel miRNAs in *F. suspensa* fruit by high-throughput sequencing. Target gene prediction of abundant *Forsythia*-derived miRNAs reveals that multiple downstream targets of fsu-miR2916-p5 and fsu-miR6478 are components of the human PI3K/AKT pathway, suggesting this pathway may serve as a key regulatory axis co-modulated by *Forsythia*-derived miRNAs. Overexpression of fsu-miR2916-p5 and fsu-miR6478 in human colorectal epithelial cells DLD1 suppresses the expression of AKT1 and AKT3, as well as the levels of phosphorylated AKT (p-AKT), as demonstrated by western blot analysis. Furthermore, RNA sequencing reveals downregulation of pro-inflammatory genes. RT-qPCR confirms the presence of *Forsythia*-derived miRNAs in the mouse colon after oral administration of *F. suspensa* extract, and confocal microscopy analysis validates their accumulation in colonic cells following gavage of synthetic *Forsythia*-derived miRNAs. Moreover, uptake of synthetic fsu-miR2916-p5 and fsu-miR6478 in DLD1 cells attenuates LPS-induced mitochondrial reactive oxygen species (ROS) production and reduces the expression of inflammation-associated molecules, including ICAM1, TGFB1, and IL1R1, as assessed by immunofluorescence and western blot analysis. These findings identify miRNAs in *F. suspensa* and elucidate their regulatory influence on human gene expression and anti-inflammatory activity, thus offering a novel perspective on miRNA-mediated, plant-based therapeutic strategies for colitis.

Key words *Forsythia suspensa*, plant-derived microRNAs, cross-kingdom regulation, PI3K/AKT pathway, anti-inflammatory activity

Introduction

Forsythia suspensa is a widely used traditional Chinese medicinal herb with diverse pharmacological activities. It has been shown to exhibit a wide range of pharmacological activities, including anti-inflammatory [1], antioxidant [2], antibacterial [3], anticancer [4], antiviral [5], anti-allergic [6], and neuroprotective effects [7], which contribute to its broad clinical applications [8].

F. suspensa produces various bioactive compounds, including forsythiaside A, forsythin, chlorogenic acid, phillygenin, and naringenin [9]. Among these compounds, forsythiaside A is considered the primary bioactive marker [10]. Studies indicated that forsythiaside A modulates multiple signaling pathways, such as NF- κ B, Nrf2 [11], MAPK [12], JAK/STAT [13], RLRs [14], TRAF [15], and TLR7 [16], exhibiting anti-inflammatory, antiviral, and

neuroprotective effects [17–19]. Forsythin primarily suppresses LPS-induced inflammation by inhibiting TLR2 and TLR4 pathways, leading to downstream inhibition of NF- κ B and MAPK signaling, thereby reducing the production of pro-inflammatory cytokines [20]. The PI3K/AKT signaling pathway plays a critical role in regulating cell proliferation, metabolism, and immune responses. Several studies have reported that components of *F. suspensa* can modulate the PI3K/AKT pathway, thereby exerting effects such as inhibiting viral replication, inflammation, and apoptosis [21–23]. *F. suspensa* extracts have been shown to effectively alleviate inflammatory conditions associated with chronic diseases such as colitis, pelvic inflammatory disease, atopic dermatitis, and liver fibrosis [24–26]. However, the half-life of forsythiaside A after oral administration in rats is 1.91 ± 1.76 h, while that of forsythin is 3.52 ± 4.37 h [27,28]. Such a short half-life requires frequent dosing to maintain effective concentrations for the treatment of chronic diseases, thereby increasing the potential medication burden on patients. These findings highlight the importance of investigating other *Forsythia*-derived components with the potential to modulate chronic inflammation in a more stable, targeted, and safer manner.

Plant-derived miRNAs remain stable under harsh cooking conditions, such as steaming and boiling, and effectively resist salivary digestion [29]. Notably, they can survive for at least 75 minutes in a simulated intestinal environment with 3.2 mg/mL of pepsin at pH 1.2 [30,31]. Several studies have detected plant-derived miRNAs in the blood, stomach, and intestines of mammals following plant consumption [29,32,33]. The stability of plant miRNAs is attributed to their unique sequence features, such as high GC content, and structural modifications, including 2'-O-methylation at the 3' end [34,35]. Furthermore, plant miRNAs are often encapsulated in extracellular vesicles, and when consumed, they are mixed with food nutrients (starch, proteins, fats, etc.), which enhances their resistance to digestive degradation [35,36]. As a result, these plant-derived miRNAs exhibit a relatively long half-life and sustain their biological effects [29,37]. These unique characteristics of plant-derived miRNAs enable them to be absorbed by the human body, to enter mammalian cells, and to exert regulatory functions [34,37]. Meanwhile, the target specificity and functional range of these miRNAs can be predicted through bioinformatics tools, offering promising potential for future applications.

Cross-kingdom regulation of plant-derived miRNAs is a well-documented natural phenomenon. These miRNAs are primarily absorbed in the gastrointestinal tract via pH-dependent uptake mediated by the SIDT1 receptor. They are subsequently transported to various organs and exert their regulatory effects [38]. For instance, the rice miR168a-5p binds to the mRNA of human *LDLRAP1*, inhibiting its expression in the liver and thus regulating liver function [37]. Similarly, the honeysuckle miR2911 interacts with several target genes of influenza A virus and suppresses its replication [34]. These findings suggest that plant miRNAs can specifically target and silence genes in mammals and viruses, resembling RNA interference mechanisms [39]. However, there are few reports on the regulatory effects of Chinese herbal miRNAs on human genes. Unique miRNAs of *F. suspensa* have not been sequenced, and whether miRNAs are the key molecules responsible for the anti-inflammatory effects of *F. suspensa* remains to be investigated.

In this study, we integrate miRNA sequencing, target gene

prediction, and additional experimental approaches to explore the miRNAs expressed by *F. suspensa* and their potential pharmaceutical value.

Materials and Methods

Materials

F. suspensa fruits were purchased from Henan Province, China. Mouse anti-phospho-AKT antibody (Ser473) (66444-1-Ig), rabbit anti-AKT1 antibody (10176-2-AP), mouse anti- β -actin antibody (66009-1-Ig), and rabbit anti-AKT3 antibody (21641-1-AP) were purchased from Proteintech (Wuhan, China). Synthetic miRNA with or without Cy5-label was synthesized by Generay Biotechnology (Shanghai, China). pBDV, pCA-N, pCXN2-P, and pCA-L were synthesized by Dynegene Technologies (Shanghai, China). Human colorectal epithelial DLD1 cells (BNCC100190) were purchased from BNCC (Beijing, China). HEK293T cell line was obtained from early laboratory stocks.

Total RNA extraction, miRNA library preparation and sequencing

Total RNA was extracted from *F. suspensa* fruits using TRIzol reagent (Thermo Fisher Scientific, Waltham, USA), followed by treatment with DNase I (Thermo Fisher Scientific) to remove any contaminating genomic DNA. RNA integrity was evaluated with Bioanalyzer 2100 (Agilent, Santa Clara, USA), and the RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). All RNA samples had a concentration of 200 ng/ μ L and a total yield of 2 μ g. The QIAseq miRNA Library Kit (Qiagen, Hilden, Germany) was used to construct the miRNA sequencing library from total RNA. A pre-adenylated DNA adapter (5'-TGGAATTCTCGGGTGCCAAGG-3') was ligated to the 3' end of the miRNAs, and then an RNA adapter (5'-GTTCAGAGTTCTA CAGTCCGACGATC-3') was ligated to the 5' end. To generate cDNA, SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) was used, and products were further amplified using Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, USA) with an integrated unique molecular index. The PCR products were purified and enriched through PAGE electrophoresis. Libraries were sequenced on the HiSeq 2500 System (Illumina, San Diego, USA). A complete list of miRNAs identified from *F. suspensa* is provided in [Supplementary Table S1](#).

Identification of conserved and novel miRNAs in *F. suspensa*

Conserved miRNA in *F. suspensa* were identified by Blastn (<http://www.ncbi.nlm.nih.gov>), Rfam 9.1 (<http://rfam.xfam.org>) and miRBase 15.0 (<http://www.mirbase>) with default parameters. To define potential novel miRNAs, miRNA candidates were aligned with the genome sequence of *F. suspensa*, and reference genome was obtained from 1K Medicinal Plant Genome Database (<http://www.herbgenome.com>).

RT-qPCR

Expressions of *Forsythia*-derived miRNAs were validated by RT-qPCR. Total RNAs of *F. suspensa* fruits were extracted using TRIzol reagent. Reverse transcription and qPCR were performed using Hifair miRNA 1st Strand cDNA Synthesis and Hieff miRNA Universal qPCR SYBR Master (Yeasen, Shanghai, China), following the manufacturer's instructions. *U6* primers and universal reverse

primers were included in the kits, while forward primers were synthesized as follows: fsu-miR2916-p5/peu-miR2916-p5: 5'-GGGCTCGAAGACGATCAGAT-3'; fsu-miR6478/ptc-miR6478: 5'-CCGACCTTAGCTCAGTTGGT-3'; ath-miR166a-3p: 5'-TCGGAC CAGGCTTCATTCCCC-3'; and ath-miR159a: 5'-TTTGGATTGAAGG GAGCTCT-3'.

Quantification of fsu-miR2916-p5 in mouse colon and stomach after oral administration was performed as previously described [40]. Stem-loop qPCR was conducted using miRNA 1st Strand cDNA Synthesis Kit and miRNA Unimodal SYBR qPCR Master Mix (Vazyme, Nanjing, China). Briefly, total RNA from mouse colon and stomach was extracted using TRIzol reagent, and reverse transcription was carried out using an fsu-miR2916-p5 stem-loop-RT primer. The cDNA was then used as the template for real-time qPCR, and the Cq values of fsu-miR2916-p5 were determined by fixed threshold settings after the reaction. To quantify the amount of fsu-miR2916-p5, a standard curve correlating Cq values with miRNA concentrations was generated using synthetic fsu-miR2916-p5 at known concentrations. Specifically, 100 nM of synthetic fsu-miR2916-p5 was reverse-transcribed into cDNA as described above. The resulting cDNA was then serially diluted from 2.5 nM to 25 fM and subjected to qPCR to build the standard curve. The absolute amount of fsu-miR2916-p5 was calculated by interpolating its Cq value into the standard curve. The stem-loop assay primers were synthesized as follows: fsu-miR2916-p5 stem-loop-RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCCGACTG GATACGACTATCTG-3'; fsu-miR2916-p5 stem-loop-F: 5'-CGGGGCTCGAAGACGAT-3'; and fsu-miR2916-p5 stem-loop-R: 5'-AGTGCAGGGTCCGAGGTATT-3'.

Target genes and secondary structures prediction of miRNAs

Target genes of miRNA candidates were predicted using Miranda (<http://www.microrna.org/>) and Targetscan (<https://www.targetscan.org>). Secondary structures of miRNAs were predicted by mfold (<http://www.mfold.org/>), and mature miRNA sequences were highlighted in blue. The list of potential human target genes of fsu-miR2916-p5, fsu-miR2916-p3, and fsu-miR6478 is provided in [Supplementary Table S2](#).

Functional enrichment and pathway analysis of identified target genes

The previously predicted target genes were then annotated by performing similarity searches against protein databases using BlastX (<https://blast.ncbi.nlm.nih.gov/>). Functional clustering analysis was conducted by DAVID online analysis tool (<https://davidbioinformatics.nih.gov/>). KEGG analysis powered by omicstudio (<https://www.omicstudio.cn/>) was used to identify biological pathways and the involved human genes ([Supplementary Table S3](#)).

Transcriptome analysis

Total RNA was extracted from DLD1 cells using Trizol reagent. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies), and only samples with an RNA Integrity Number (RIN) > 7.0 were used for library construction. RNA-seq libraries were prepared using the NEBNext Ultra RNA Library Prep Kit (NEB) according to the manufacturer's protocol. Sequencing was performed by GENEWIZ (South Plainfield, USA) on an Illumina HiSeq platform using a paired-end 2× 150 bp configuration.

Dual-luciferase reporter assay

The predicted 3' untranslated regions (UTRs) or corresponding mutant sequences were cloned into the firefly luciferase reporter vector pGL-basic (MiaoLing Plasmid, Wuhan, China). HEK293T cells were seeded in 24-well plates and co-transfected with pGL-basic vectors containing either the predicted 3' UTRs or mutant 3' UTRs, together with the test miRNA and the pRL-TK plasmid (MiaoLing Plasmid) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The pRL-TK plasmid served as an internal control to normalize for transfection and harvesting efficiency. Cells were collected 24 h after transfection, and luciferase activity was measured using the Dual-Luciferase Assay Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The miRNA sequences used in this study are as follows: fsu-miR2916-p5: 5'-GGGCUCGAAGACGAUCAGAU-3'; fsu-miR6478: 5'-CCGACCUUAGCUCAGUUGGUA-3'; and scrambled miRNA: 5'-AUCGAUCGAUCGAUCGAUCG-3'.

Overexpression of fsu-miR2916-p5 and fsu-miR6478

Recombinant borna disease virus (BDV) persistently expressing fsu-miR2916-p5, rBDV-miR2916-p5, was constructed as previously described [41]. Briefly, the stem sequence of the miR-155 precursor was replaced by fsu-miR2916-p5, and the modified cassette was inserted into pBDV. rBDV-miR2916-p5 was rescued by co-transfecting HEK293T cells with the recombinant pBDV and helper plasmids expressing BDV N, P, and L genes (pCA-N, pCXN2-P, and pCA-L). DLD1 cells infected with rBDV-miR2916-p5 were selected based on persistent BDV infection and designated DLD1-miR2916-p5. DLD1 cell lines expressing fsu-miR6478 (DLD1-miR6478) or co-expressing both miRNAs (DLD1-miR2916-miR6478) were obtained as described above. DLD1 cells transfected with BDV empty vector were used as the negative control.

Validation of miRNA uptake

Synthetic miRNAs labeled with Cy5 were prepared at a final concentration of 100 pM. Cy5-labeled miRNAs were incubated with DLD1 cells in complete culture medium for 12 h. Cells were then washed three times with cold PBS to remove unbound Cy5-labeled miRNA. Hoechst (Thermo Fisher Scientific) was used to stain the nuclei for 5 min, followed by PBS washing. Cells were finally fixed with 4% paraformaldehyde for 15 min at room temperature. Confocal laser scanning microscopy was performed using an FV1200 confocal microscope (Olympus, Tokyo, Japan) to visualize the uptake of Cy5-labeled miRNAs in DLD1 cells. Cy5-labeled miRNAs were excited at 635 nm and emission was detected at 660–700 nm, while Hoechst was excited at 350 nm and detected at 460–490 nm. Images were acquired as single-plane scans and processed using Fluoview software (Olympus).

Western blot analysis

DLD1 cells, DLD1-miR2916-p5 cells, and DLD1-miR6478 cells were cultured separately. Additionally, LPS (1 µg/mL) was added to the fsu-miR2916-p5 + LPS and fsu-miR6478 + LPS groups. The cultured cells were collected after 24 h of stimulation and lysed using sodium dodecyl sulfate buffer containing a protease inhibitor cocktail (Selleck, Houston, USA). Protein samples were separated by 12% SDS-PAGE and blotted with antibodies against phospho-AKT (Ser473), AKT, AKT3, and β-actin. Proteins were visualized using HRP-conjugated anti-rabbit or anti-mouse IgG (Proteintech) and the

densities of the bands were quantified with Quantity one software (Bio-Rad, Hercules, USA).

To evaluate the anti-inflammatory effects of synthetic fsu-miR2916-p5 and fsu-miR6478 taken up by DLD1 cells, cells were transfected with either scrambled miRNA or fsu-miR2916-p5 and fsu-miR6478 (10 nM) using Lipofectamine 2000. LPS (1 µg/mL) was added 48 h post-transfection. Samples were collected 12 h later and subject to western blot analysis as described above.

ROS production

Mitochondrial ROS production was assessed in DLD1 cells transfected with either scrambled miRNA or fsu-miR2916-p5 and fsu-miR6478. LPS (1 µg/mL) was added 48 h after transfection. Twelve hours later, cells were stained with MitoSOX (Invitrogen), CellMask (Invitrogen), and Hoechst, and analyzed using the FV1200 confocal microscope.

Detection of fsu-miR2916-p5 in the mouse colon after oral administration of *F. suspensa* extract and synthetic miRNAs

The study was approved by the Institutional Review Board of the Institute of Biophysics, Chinese Academy of Sciences (SYXK2023201). All animal procedures and experiments were approved by the Institution Animal Care and Use Committee at the Institute of Biophysics, Chinese Academy of Sciences. Female C57BL/6 mice aged 6–8 weeks were purchased from Beijing Huafukang Biotechnology Co., Ltd (Beijing, China). All mice were maintained in a specific pathogen-free condition with a 12-h light/12 h dark cycle, an ambient temperature of 22–25°C, and a humidity of 40%–70%. *F. suspensa* extract was prepared by mincing *F. suspensa* fruits and mixing the extract with glucose. Mice were fasted overnight and fed with the *F. suspensa* extract, while mice fed with standard laboratory chow (SPFbiotech, Beijing, China) served as a negative control. To avoid miRNA degradation and contamination, fresh *F. suspensa* extract was prepared daily prior to administration. Two days later, colons and stomachs were harvested and washed with PBS. Total RNA extraction and RT-qPCR analysis were conducted as described above. In a separate experiment, mice were gavaged with synthetic Cy5-labeled fsu-miR2916-p5. Colons were excised 1 h after gavage, cryosectioned, and stained with DAPI.

Statistical analysis

Statistical parameters and significance are reported in figures and figure legends. Data are expressed as the mean ± SEM. Statistical analysis was performed with GraphPad Prism version 9.5. Data sets were tested for normality of distribution with the Kolmogorov-Smirnov test. Comparisons between two groups were assessed by Student's *t* test, while comparisons among multiple groups were performed using ordinary one-way ANOVA followed by Tukey's multiple comparisons test. No statistical method was used to predetermine the sample size.

Results

Identification of conserved and novel miRNAs in *F. suspensa*

F. suspensa is a key component in various traditional Chinese medicines and exhibits significant anti-inflammatory, neuroprotective, antioxidant, and other pharmacological properties [1,2,23].

The roots, stems, leaves, and flowers of *F. suspensa* are all used medicinally [9], with the fruit being the most commonly utilized part (Figure 1A).

In this study, we employed high-throughput sequencing to identify miRNAs in *F. suspensa* fruit for the first time. High-quality RNA samples were obtained from *F. suspensa* fruit, and the RNA exhibited a high level of completeness (5S, 18S, and 28S peaks were fully represented, RIN = 7.6) (Figure 1B). Sequences of miRNAs that showed significant alignment with entries in miRBase were classified as conserved miRNAs, and their corresponding species of origin were annotated. Sequences without matches were classified as novel miRNAs. Among the 346 mature miRNAs identified (Supplementary Table S1), 225 were classified as conserved. The five most abundant were peu-miR2916-p5, peu-miR2916-p3, ath-miR166a-3p, ptc-miR6478, and ath-miR159a. The mature sequences and corresponding abundance values of these miRNAs are shown (Figure 1C,E). Additionally, a total of 121 novel miRNAs were identified, each mapped to corresponding gene loci in the *F. suspensa* genome. The eight most abundant novel miRNAs are listed as follows: PC-3p-15363_373, PC-5p-26034_158, PC-3p-35908_94, PC-3p-27741_140, PC-3p-42926_71, PC-3p-104865_17, PC-5p-43644_66, and PC-5p-48094_57. Their mature sequences along with abundance values are shown (Figure 1D,F).

We then analyzed the species distribution of identified mature miRNAs in *F. suspensa*. Of the 346 mature miRNAs identified, 35.28% were unique to *F. suspensa* and exhibited no sequence homology to any known miRNAs in the miRBase (Figure 1G). In addition, several highly abundant miRNAs identified through high-throughput sequencing were validated by RT-qPCR (Figure 1H).

Compared to conserved miRNAs, the novel miRNAs in *F. suspensa* exhibited lower read counts, suggesting a relatively lower abundance. Given that the levels of these newly discovered miRNAs are significantly lower than those of conserved miRNAs, we conclude that the predominant miRNAs exerting biological functions in *F. suspensa* fruit are likely to be conserved miRNAs.

Conserved miRNA precursors in *F. suspensa* possess unique secondary structures and mature sequences

The mature sequences of peu-miR2916 and ptc-miR6478 obtained in this study differed from those reported in miRBase and previous research [42] (Figure 2A). We further compared their precursor sequences with corresponding reported sequences and predicted the corresponding secondary structures (Figure 2B,C). The sequence of *Forsythia* MIR2916 shared high similarity with peu-MIR2916 in miRBase (peu-MIR2916_ref). However, several nucleotide differences were observed at specific positions. These differences led to distinct secondary structures and cleavage sites in *Forsythia* MIR2916, resulting in unique mature sequence. Conversely, *Forsythia* MIR6478 was longer than ptc-MIR6478_ref, exhibiting different secondary structures but similar cleavage sites and mature sequences.

The secondary structures of two highly expressed conserved miRNAs (*Forsythia* MIR2916 and MIR6478) and one novel miRNA (PC-3p-15363_373) in *F. suspensa* are shown in Figure 2C. Other abundant miRNAs identified, such as ath-miR166a-3p and ath-miR159a, have relatively few predicted target genes and were therefore not the focus of this study.

These findings suggest that while *Forsythia* miR2916 and

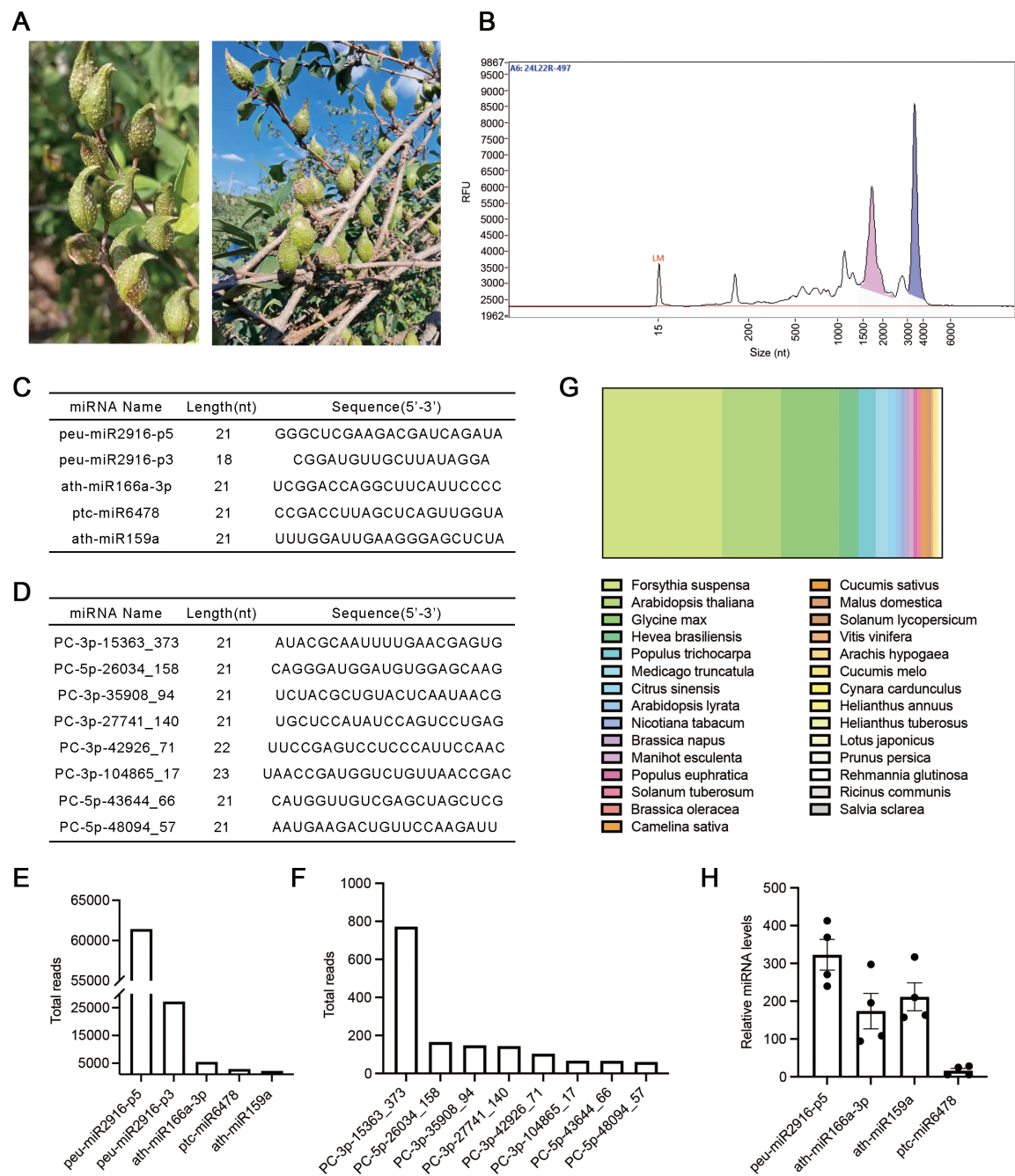


Figure 1. Conserved and novel miRNAs identified in *F. suspensa* (A) Representative image of *F. suspensa* fruit. (B) RNA sample quality control was assessed using the Agilent 2100 Bioanalyzer. The peak of 18S rRNA (Pink) and 28S rRNA (Purple) are shown. (C,E) Sequences, lengths, and net reads of highly expressed conserved miRNAs. (D,F) Sequences, lengths and net reads of highly expressed novel miRNAs. (G) Species distribution of identified mature miRNAs in *F. suspensa*. (H) RT-qPCR analysis of miRNA expression levels in *F. suspensa*.

miR6478 share sequence similarity with previously reported orthologs, their precursor secondary structures and mature sequences are distinct. Therefore, we named the two newly discovered conserved miRNAs in *F. suspensa* as fsu-miR2916 and fsu-miR6478.

Putative target genes of fsu-miR2916-p5 and fsu-miR6478 are involved in PI3K/AKT signaling pathway To explore the function of *F. suspensa* miRNAs in cross-species

regulation, we selected the highly abundant miRNAs, namely fsu-miR2916-p5, fsu-miR2916-p3 and fsu-miR6478, and predicted their potential target genes in humans (Supplementary Table S2). The homologous target genes predicted for plant miR2916-p5 and miR6478 are numerous. Specifically, the downstream target genes of miR2916-p5 include those involved in the PI3K/AKT signaling pathway (Figure 3A). KEGG analysis of the target genes revealed that miR6478 potentially influences the core PI3K/AKT pathway (Figure 3B). Additionally, miRNA binding site predictions indicate

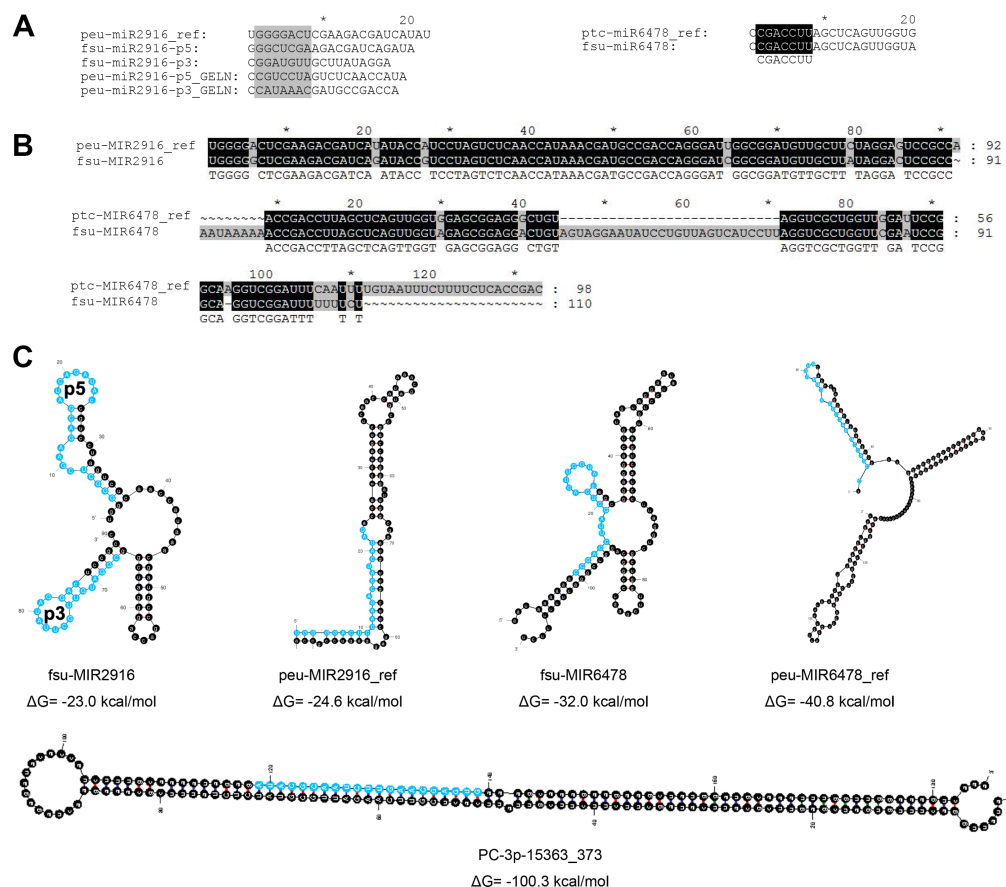


Figure 2. *Fsu-miR2916* and *fsu-miR6478* precursors possess unique secondary structures and mature sequences (A) Alignments of *fsu-miR2916* and *fsu-miR6478* seed regions (peu-miR2916_ref and ptc-miR6478_ref from miRBase; *fsu-miR2916*-p5, p3 and *fsu-miR6478* from our research; peu-miR2916-p5, p3_GELN from reported research). (B) Alignments of *fsu-miR2916* and *fsu-miR6478* precursor sequences with reference sequences from miRBase. (C) Putative secondary structures and their free energies of miRNA precursors predicted by mfold, and mature sequences are highlighted in blue.

that *fsu-miR2916*-p5 and *fsu-miR6478* share overlapping target genes, suggesting a possible synergistic effect in their gene regulation (Figure 3C). The seed sequence of *fsu-miR2916*-p5 predicted a binding site in the 3' UTR of human *AKT1* with high affinity, while the seed sequence of *fsu-miR6478* exhibited a binding site to the 3' UTR of human *AKT3* (Figure 3D). To further validate the direct interactions between the miRNAs and their predicted target genes, a dual-luciferase reporter assay was performed in HEK293T cells. The 3' UTRs of human *AKT1* and *AKT3*, each containing its putative binding site, were cloned downstream of the luciferase coding sequence. Compared to the scrambled miRNA control, both *fsu-miR2916*-p5 and *fsu-miR6478* significantly reduced luciferase activity, indicating their direct binding to the 3' UTR of *AKT1* and *AKT3*, respectively. Meanwhile, the failure of miRNAs to suppress luciferase expression when co-transfected with reporters containing mutated binding sites confirmed the specificity of these interactions (Figure 3E).

Based on these findings, we hypothesize that the PI3K/AKT pathway may serve as a core regulatory pathway modulated by the collective action of multiple *Forsythia*-derived miRNAs. In particular, the regulatory effects of *fsu-miR2916*-p5 and *fsu-miR6478* appear to be mediated through direct binding to their target genes.

Overexpression of *fsu-miR2916*-p5 and *fsu-miR6478* suppresses AKT expression and phosphorylation and attenuates inflammation in DLD1 cells

To validate the regulatory effects of *Forsythia*-derived miRNAs on the PI3K/AKT pathway, we used the human colorectal epithelial cell line DLD1 as a model. *fsu-miR2916*-p5 and *fsu-miR6478* were overexpressed in the DLD1 cell line to achieve stable expression and to preliminarily explore their regulatory functions. The expression levels of *fsu-miR2916*-p5 and *fsu-miR6478* were subsequently confirmed using RT-qPCR (Figure 4A).

Under both basal and LPS-induced inflammatory conditions, *fsu-miR2916*-p5 and *fsu-miR6478* specifically downregulated the expression of *AKT1* and *AKT3*, respectively, both also reduced AKT phosphorylation levels (Figure 4B,C). Transcriptome analysis further revealed that the mRNA levels of *AKT1* and *AKT3* were also reduced by *fsu-miR2916*-p5 and *fsu-miR6478*. Furthermore, co-expression of *fsu-miR2916*-p5 and *fsu-miR6478* in DLD1 cells significantly decreased the mRNA levels of several pro-inflammatory genes, including *IL1A*, *TRAF6*, *ICAM1*, and *NOS2*. Although the mRNA expression levels of *NFKB1*, *TGFB1*, and *TNFSF4* also showed a downward trend, the differences were not statistically significant (Figure 4D). These data suggest that overexpression of *fsu-miR2916*-p5 and *fsu-miR6478* in human colorectal epithelial

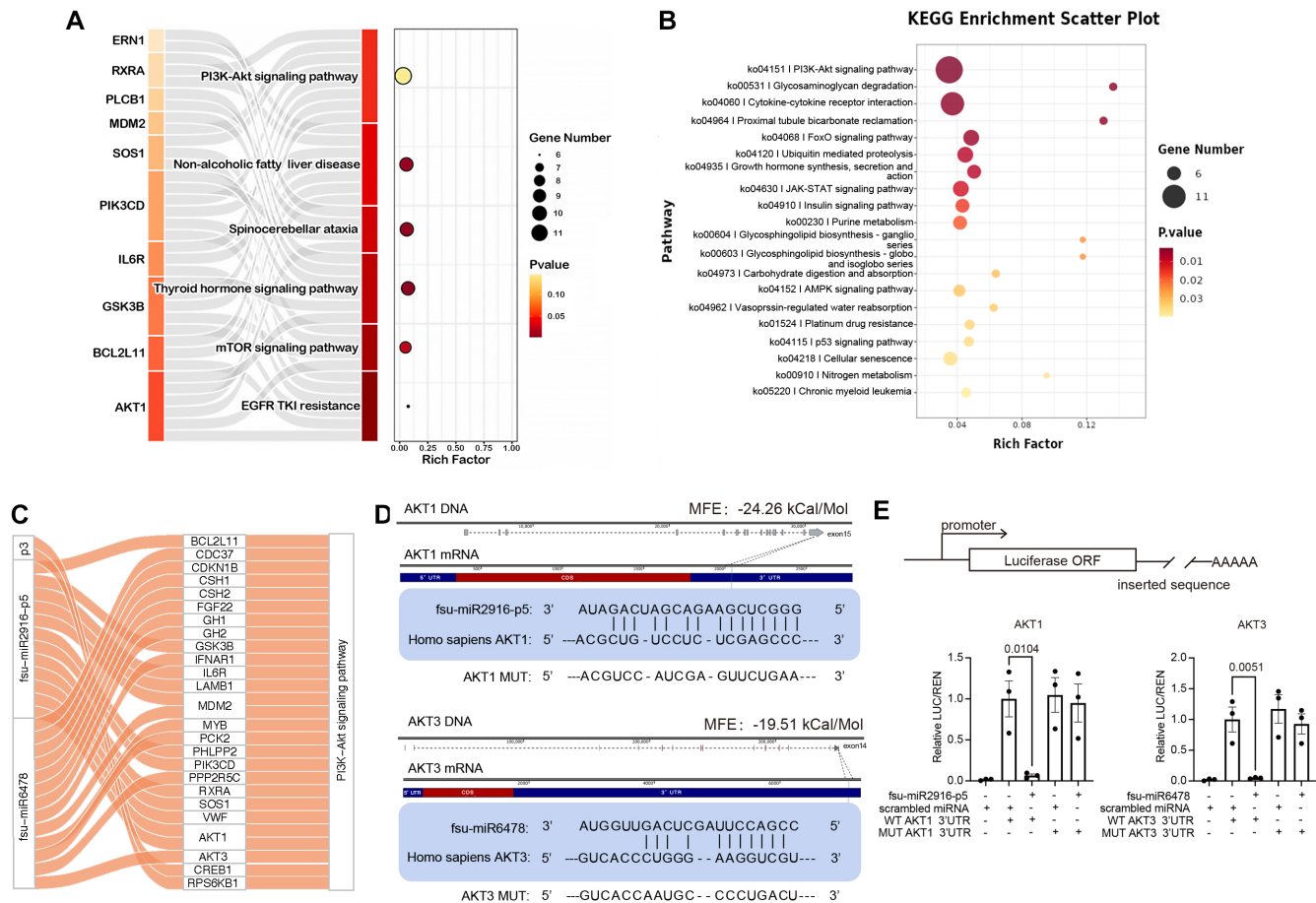


Figure 3. Putative target genes of fsu-miR2916-p5 and fsu-miR6478 regulating PI3K/AKT signaling pathway (A) Predicted target genes of fsu-miR2916-p5 and the signaling pathways in which they are involved. (B) KEGG enrichment analysis of predicted target genes for fsu-miR6478. (C) Analysis of predicted target genes of fsu-miR2916-p5, fsu-miR2916-p3 and fsu-miR6478 involved in PI3K/AKT signaling pathway. (D) Schematic diagrams of the putative binding sites of fsu-miR2916-p5 and fsu-miR6478 in human AKT mRNA (Red: coding site; Blue: untranslated region). Mutant binding sites of AKT1 and AKT3 for dual-luciferase reporter assay are shown below. (E) Relative firefly luciferase (LUC) to Renilla luciferase (REN) activity in AKT1 and AKT3 reporters following fsu-miR2916-p5 or fsu-miR6478 transfection, as determined by dual-luciferase reporter assay.

cells may effectively suppress LPS-induced pro-inflammatory genes, likely through silencing AKT genes.

fsu-miR2916-p5 and fsu-miR6478 reduce LPS-induced ROS production and inflammation in DLD1 cells

The presence of fsu-miR2916-p5 in mouse colon and stomach after oral administration of *F. suspensa* extract was confirmed (Supplementary Figure S1). Furthermore, after gavage with synthetic Cy5-labeled fsu-miR2916-p5, Cy5 signals were found to be enriched in the mouse colonic lumen and to accumulate in both the surface epithelial cells and the underlying lamina propria cells (Supplementary Figure S2).

To explore whether synthetic miRNAs could be absorbed by human colorectal epithelial cells and consequently downregulate inflammation-related genes, experiments were conducted by co-incubating synthetic miRNAs with DLD1 cells. First, the cellular uptake of fsu-miR2916-p5 and fsu-miR6478 was examined. Confocal microscopy showed that, without the use of any transfection reagent, substantial amounts of fsu-miR2916-p5-Cy5 and fsu-miR6478-Cy5 were internalized by DLD1 cells after 12 h of incubation, with fluorescent signals distributed throughout the

cytoplasm (Figure 5A). Whether the uptake of exogenous fsu-miR2916-p5 and fsu-miR6478 could mediate anti-inflammatory effects was explored next. It is well-known that excessive ROS production from damaged mitochondria leads to NLRP3 inflammasome activation [43]. To test the effect of fsu-miR2916-p5 and fsu-miR6478 on mitochondrial ROS production, confocal microscopy was employed. Results indicated that co-treatment with fsu-miR2916-p5 and fsu-miR6478 reduced mitochondrial ROS induced by LPS stimulation (Figure 5B). Additionally, inflammation-associated molecules induced by LPS, including ICAM1, IL1R1, and TGFBR1, were downregulated by fsu-miR2916-p5 and fsu-miR6478 (Figure 5C,D).

Together, these findings demonstrate that *Forsythia*-derived fsu-miR2916-p5 and fsu-miR6478 suppress LPS-induced inflammation on human colorectal epithelial cells, potentially by inhibiting the AKT signaling pathway (Figure 5E).

Discussion

F. suspensa is a widely used traditional herbal medicine renowned for its efficacy in treating inflammation through multiple pathways [24,44]. In an LPS-induced acute pneumonia model, *F. suspensa*

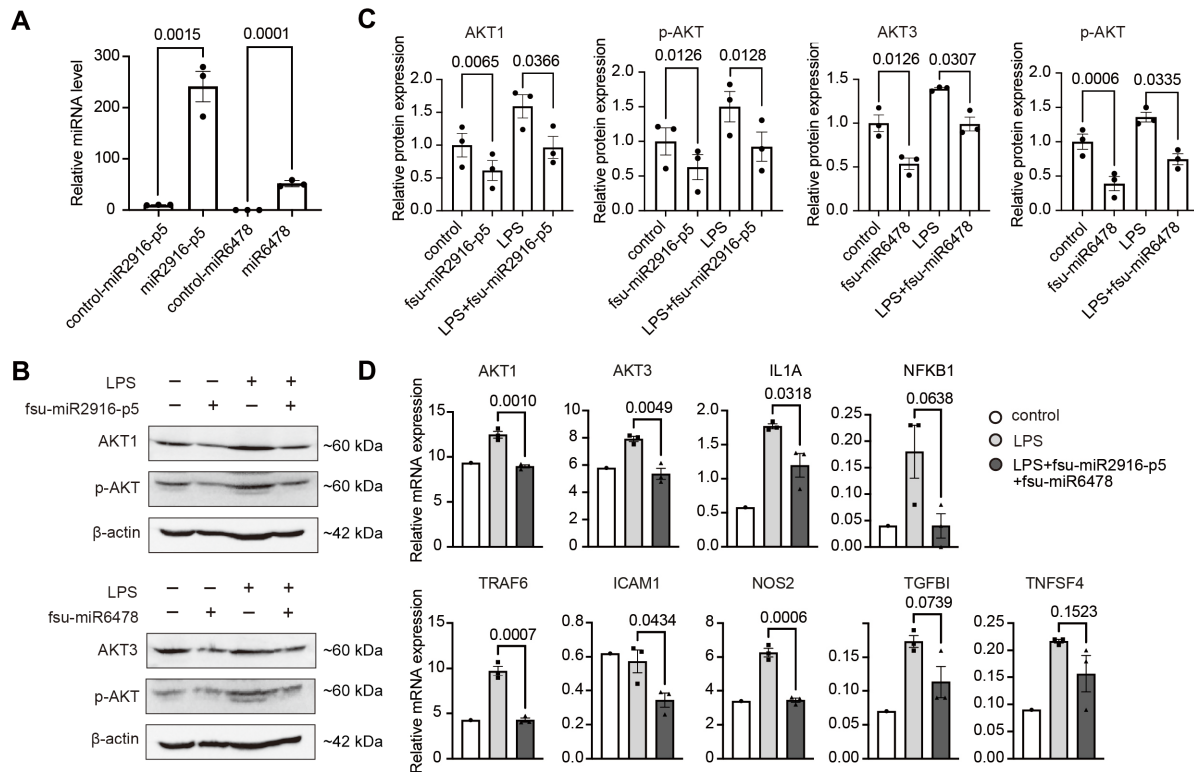


Figure 4. Overexpression of fsu-miR2916-p5 and fsu-miR6478 suppresses AKT expression and phosphorylation to attenuate inflammation in DLD1 cells (A) Validation of fsu-miR2916-p5 and fsu-miR6478 expression levels in DLD1-miR2916-p5 and DLD1-miR6478 cells. (B,C) Western blot analysis of total and phosphorylated AKT1 and AKT3 in DLD1-miR2916-p5 and DLD1-miR6478 cells. Inflammatory conditions were induced by treating cells with LPS. Samples were subsequently collected 24 h post-treatment. (D) Transcriptomic analysis of inflammation-related genes expression in DLD1-miR2916-miR6478 cells.

inhibited the activation of the TLR4/MAPK/NF- κ B inflammatory signaling pathway, downregulating the expression of pro-inflammatory cytokines such as TNF- α and IL-6 [45]. Another study indicated that *F. suspensa* inhibits the activation of the NLRP3 inflammasome, enhances cellular antioxidant activity, and suppresses pyroptosis [24]. *F. suspensa* also activated the anti-inflammatory factor Nrf2 and increased the expression of A20, disrupting the inflammatory signaling cascade [46]. Furthermore, *F. suspensa* reduced the production of pro-inflammatory mediators such as COX-2 and iNOS in the liver [26]. Preventive administration of *F. suspensa* increased the activity of antioxidant enzymes like SOD and GSH-Px, while decreasing ROS and MDA levels in serum and liver, thus alleviating inflammation induced by oxidative stress [37]. Current research suggests that Forsythoside A and Forsythoside are the main anti-inflammatory components, but their short half-life implies the potential involvement of other molecules in treating chronic diseases.

Accumulating evidence has revealed that plant-derived miRNAs can function in a cross-kingdom manner, targeting key mammalian genes to regulate processes such as metabolism, immune responses, vascular protection, cancer cell proliferation, and viral replication [37,44–47]. Through high-throughput sequencing, we identified several miRNAs in *F. suspensa*, with peu-miR2916-p5, peu-miR2916-p3, and ptc-miR6478 exhibiting high abundance. Alignment with the miRBase database and prior research revealed that the precursors of peu-miR2916-p5 and peu-miR2916-p3 in *F. suspensa* showed strong similarity to database sequences, but

their mature miRNA sequences differed, likely due to unique hairpin folding structures altering cleavage site specificity. In contrast, ptc-miR6478 demonstrated low similarity in precursor sequences but differed by only one nucleotide at the 3' end of the mature miRNA. Based on these unique processing characteristics, we reclassified these miRNAs as fsu-miR2916-p5, fsu-miR2916-p3, and fsu-miR6478.

Plant miRNAs typically exhibit high complementarity to their target gene sequences, with binding sites predominantly located within the coding sequences (CDS) of target genes, facilitating mRNA cleavage mediated by the RNA-induced silencing complex (RISC) [47]. In contrast, animal miRNAs require lower complementarity for target pairing, with multiple binding sites usually located in the 3' UTR of mRNAs. Instead of affecting mRNA stability, animal miRNAs mainly suppress translation [39,48]. Among studies reporting cross-kingdom regulation of mammalian genes by plant-derived miRNAs, most binding sites are located in the 3' UTR [49–51], although a few targeted the CDS [37]. Some studies have shown that plant miRNAs downregulate target genes at both mRNA and protein levels [51–53], while others have reported reduced protein expression without mRNA degradation [37,50]. The endonuclease activity of RISC typically relies on nearly perfect complementarity between miRNAs and mRNA, a phenomenon rarely observed in mammals [39,54]. However, exceptions exist, such as miR-122, which binds its target CAT-1 with mismatched nucleotides but still primarily suppresses gene expression by degrading mRNA [55]. In our study, target gene prediction and dual-luciferase reporter assay

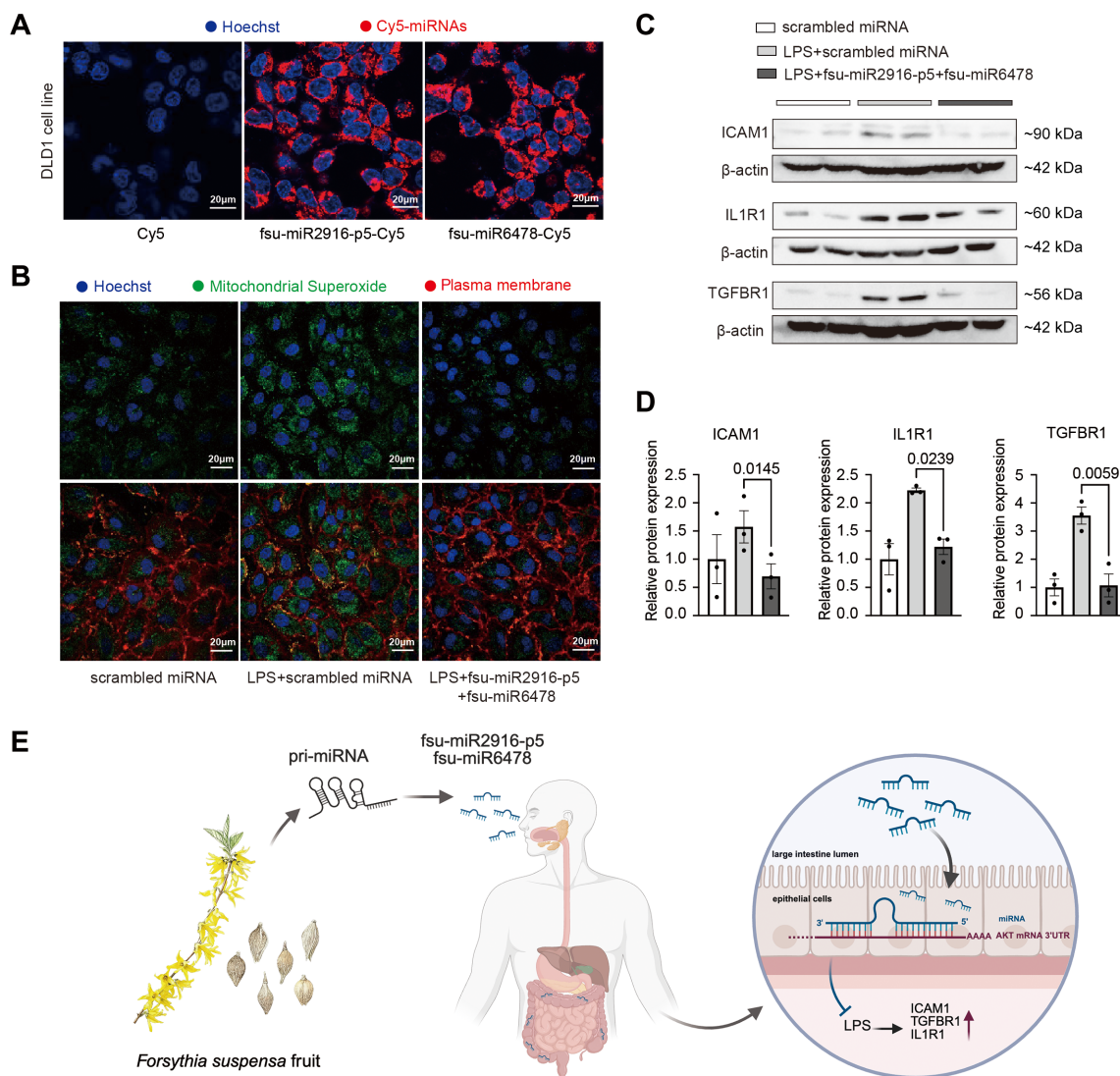


Figure 5. fsu-miR2916-p5 and fsu-miR6478 reduce LPS-induced ROS production and inflammation in DLD1 cells (A) Confocal microscopy analysis of the cellular uptake of synthetic fsu-miR2916-p5 and fsu-miR6478 in DLD1 cells. miRNAs were labeled with Cy5 and co-incubated with DLD1 cells at 37°C for 12 h (Blue: Hoechst; Red: Cy5). (B) Detection of mitochondrial ROS production in DLD1 cells. Cells were transfected with scrambled miRNA or fsu-miR2916-p5 and fsu-miR6478. LPS was added 48 h post-transfection, and cells were stained and analyzed 12 h after LPS treatment. Untreated cells served as a negative control (Blue: Hoechst; Green: mitochondrial superoxide; Red: plasma membrane). (C,D) Western blot analysis of ICAM1, IL1R1, and TGFB1 protein expression levels. (E) Schematic diagram illustrating the potential anti-inflammatory effects of dietary miRNAs derived from *F. suspensa* fruit in human colorectal epithelial cells. After ingestion of processed *F. suspensa* fruit, miRNAs such as fsu-miR2916-p5 and fsu-miR6478 may be absorbed by colonic cells. These *Forsythia*-derived miRNAs appear to bind to the 3' UTRs of their target genes and inhibit AKT signaling, thereby reducing LPS-induced inflammation by downregulating ICAM1, TGFB1, and IL1R1.

revealed that fsu-miR2916-p5 binds to the 3' UTR of human AKT1, while fsu-miR6478 interacts with the 3' UTR of human AKT3. Transcriptomic and western blot analyses demonstrated that target gene expression was reduced at both mRNA and protein levels. Further investigation is required to determine whether the miRNA-mediated suppression of target genes is primarily mediated by mRNA cleavage or translational suppression.

Although the stomach is considered the primary site for the absorption of free dietary miRNAs into the bloodstream [38], increasing evidence indicates that dietary miRNAs can also reach the colon, either in free form or encapsulated within microvesicles (MVs) or exosome-like nanoparticles (ELNs). Multiple studies have confirmed that orally delivered free plant miRNAs, including

miR168a, gma-miR159a, and miR172, can reach the gastrointestinal tract, including the colon, at biologically active concentrations to ameliorate colitis or prevent colon cancer [33,40,56]. A substantial proportion of plant miRNAs is encapsulated in ELNs, which protect them from degradation by digestive nucleases in the gastrointestinal tract [57]. ELNs derived from various plants have been shown to ameliorate colitis via their encapsulated miRNAs, such as osa-miR166d-5p, gma-miR396a-3p, miR159, and osa-miR164d [58–61]. Furthermore, exogenous miRNAs can be re-packaged into MVs in gastric epithelial cells, secreted into the circulation, and subsequently transported to distant tissues [37]. Consistent with these findings, our results demonstrated that synthetic Cy5-labeled fsu-miR2916-p5 and fsu-miR6478 could enter human colorectal

epithelial cells DLD1 without any transfection reagents. These observations suggest that *Forsythia*-derived miRNAs have the potential to reach the colon and exert local anti-inflammatory effects.

LPS is widely used to establish inflammation models. It is recognized by the pattern recognition receptor TLR4, which activates downstream MYD88-dependent and TRIF-dependent pathways. These pathways, in turn, stimulate key transcription factors such as NF- κ B and MAPK, leading to the release of cytokines, chemokines, nitric oxide, and reactive oxygen species [53,62]. The PI3K/AKT pathway modulates NF- κ B signaling through the phosphorylation of IKK α and Tpl2, influencing the expression of inflammatory mediators such as TNF- α , IL-1 β , and IL-6 [63,64]. Target gene predictions and KEGG pathway analysis revealed that both fsu-miR2916-p5 and fsu-miR6478 share common target genes involved in the regulation of the PI3K/AKT pathway. When DLD1 cells were stimulated with LPS, the phosphorylation levels of AKT1 and AKT3 were significantly increased. Overexpression of fsu-miR2916-p5 and fsu-miR6478 markedly reduced the expression levels of both AKT1 and AKT3, as well as the levels of phosphorylated AKT. Transcriptomic analysis further demonstrated that the overexpression of fsu-miR2916-p5 and fsu-miR6478 in DLD1 cells led to a downregulation of AKT1 and AKT3 transcription levels. Inflammatory cytokine IL-1 α typically binds to IL-1R1, activating the MyD88-TRAF6 signaling complex and triggering downstream NF- κ B and MAPK pathways to regulate inflammation-related genes [65,66]. Our findings revealed reduced expression levels of ICAM1, TGFBR1, and IL1R1, suggesting a potential alleviation of the inflammation in cells. fsu-miR2916-p3 is one of the most abundant miRNAs in garlic-derived extracellular vesicle-like nanoparticles (GELNs). It has been shown to significantly promote the growth of *Bacteroides thetaiotaomicron* in the gut, thereby maintaining intestinal homeostasis and ameliorating intestinal inflammation [42]. Our findings complement these studies by further elucidating the anti-inflammatory roles of the mature forms of fsu-miR2916. PI3K/AKT pathway inhibitors have been reported as potential treatments for inflammation by modulating multiple downstream signaling pathways. Elevated expression of p-AKT has been observed in the intestinal mucosa of colitis patients. Treatment with wortmannin, a PI3K inhibitor, significantly reduced p-AKT expression and TNF- α levels, alleviating inflammation [63]. AS605240 has been shown to protect mice against colitis by suppressing multiple inflammatory components through the NF- κ B pathway [67]. Oxymatrine has been reported to inhibit AKT phosphorylation, reduce pro-apoptotic and anti-inflammatory responses, and downregulate Th1 and Th17 cell differentiation, thereby mitigating colitis [68]. In this study, we demonstrated that both fsu-miR2916-p5 and fsu-miR6478 inhibited the PI3K/AKT pathway, highlighting their potential as therapeutic agents for colitis.

In summary, we identified conserved and novel miRNAs in *F. suspensa* and predicted their secondary structures and human gene targets. In human colorectal epithelial cells DLD1, overexpression and internalization of the two abundant *Forsythia*-derived miRNAs, fsu-miR2916-p5 and fsu-miR6478, suppressed AKT expression and phosphorylation and reduced the production of inflammatory mediators. This work provides new insights into the treatment of colitis and introduces a novel paradigm for exploring plant-derived miRNAs as innovative therapeutic agents for managing inflammatory disorders.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

The raw data of *F. suspensa* miRNA sequencing has been deposited in the NCBI Sequence Read Archive (SRR36661420). All other data can be accessed upon reasonable request from the corresponding authors.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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