

Effect of *Senecio scandens* Ethanol Extract and SNHG16 on *Streptococcus pneumoniae*-Infected Alveolar Epithelial Cells

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Liu *et al.*: *Senecio scandens* Ethanol Extract Effect on Alveolar Epithelial Cells

To explore the effect of *Senecio scandens* ethanol extract on *Streptococcus pneumoniae*-evoked alveolar epithelial cell damage and its molecular mechanism. Alveolar epithelial cells and *Streptococcus pneumoniae* infected alveolar epithelial cells were incubated with *Senecio scandens* ethanol extract. Western blotting measured cleaved caspase-3 protein expression. Quantitative reverse transcription-polymerase chain reaction detected SNHG16 and microRNA-542-3p levels, and the dual-luciferase reporter assay investigated the targeting relationship between them. After treatment with different concentrations of *Senecio scandens* ethanol extract, alveolar epithelial cell survival rate did not change, while the survival rate of alveolar epithelial cells infected by *Streptococcus pneumoniae* was gradually decreased, and the decrease was stable at 32 µg/ml. After treatment with 8, 16, 32 µg/ml *Senecio scandens* ethanol extract, the apoptotic rate and inflammation of alveolar epithelial cells infected by *Streptococcus pneumoniae*, inflammation was repressed. *Senecio scandens* ethanol extract dose-dependently reduced SNHG16, but elevated microRNA-542-3p in cells. After SNHG16 knockdown, microRNA-542-3p levels were boosted, apoptotic rate was declined, and inflammation was inhibited ($p < 0.05$). Overexpression of SNHG16 could reverse the effects of *Senecio scandens* ethanol extract on the apoptosis and inflammatory factors of alveolar epithelial cells infected by *Streptococcus pneumoniae*. SNHG16 targeted microRNA-542-3p. *Senecio scandens* ethanol extract could impair *Streptococcus pneumoniae*-evoked alveolar epithelial cell injuries by regulating the SNHG16/microRNA-542-3p axis.

Key words: *Senecio scandens* ethanol extract, SNHG16, microRNA-542-3p, *Streptococcus pneumoniae*, alveolar epithelial cells, inflammation

Community Acquired Pneumonia (CAP) has a high mortality rate, with *Streptococcus pneumoniae* (*S. pneumoniae*) being its main pathogen. The main pathological changes of pneumonia caused by *S. pneumoniae* infection are apoptosis and inflammatory response in Alveolar Epithelial Cells (AECs)^[1,2]. Research has found that Traditional Chinese Medicine (TCM) is benefit to CAP treatment^[3,4]. *Senecio scandens* (*S. scandens*) (also known as Qianli Gunag) is a perennial climbing herb belonging to the family Compositae, and has excellent clinical therapeutic effects in various diseases. For instance, water extract of *S. scandens* could suppress Mas-Related G Protein-Coupled Receptor-B2 (MRGPRB2) receptor of mast cells to alleviate pruritus^[5]. *S. scandens* has known antibacterial properties, and exhibits

suppression on *S. pneumoniae*^[6]. In addition, the ethanol extract of *S. scandens* could elevate MUC2 and sIgA, and maintain the diversity of gut microbiota^[6]. However, the effects and mechanisms of *S. scandens* ethanol extract on the apoptosis and inflammatory response of *S. pneumoniae* infected AECs are still unclear.

SNHG16 is a functional long noncoding Ribonucleic Acid (lncRNA), and was discovered to promote

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Lipopolysaccharide (LPS)-evoked inflammation and apoptosis in acute pneumonia^[7]. SNHG16 could abolish microRNA (miR)-15a/16 cluster-induced down-regulation of inflammatory pathway^[8]. SNHG16 promoted inflammatory reaction and macrophage proliferation in atherosclerosis patients^[9]. Thus, these data suggested that SNHG16 was involved in regulating acute pneumonia and inflammatory response. However, whether *S. scandens* ethanol extract regulated CAP via SNHG16 remain unclear. Here, this work used *S. pneumoniae* infected AECs to mimic CAP *in vitro*, and investigated the action and relationship of *S. scandens* ethanol extract and SNHG16 in cell apoptosis and inflammation, and then explored their underlying mechanism.

MATERIALS AND METHODS

Experimental materials:

Pulmonary AECs (Hepapic), *S. pneumoniae* (American Type Culture Collection (ATCC), United States of America (USA)); Roswell Park Memorial Institute (RPMI)-1640 medium and Lipofectamine 3000 (Sigma, USA); *S. scandens* (Bozhou Pharmaceutical Market, China); SNHG16 siRNAs (si-SNHG16), overexpressing plasmids (plasmid cloning Deoxyribonucleic Acid (pcDNA)-SNHG16), miR-542-3p mimic, si-NC, miR-NC (RiboBio, China); 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyl Tetrazolium Bromide (MTT) kit and apoptosis detection assay kit (Shanghai Jingke Chemical Technology Co., Ltd.); protein extraction kit (Shanghai Beibo Biotechnology Co., Ltd.); cleaved caspase-3 (ab2302) and secondary antibodies (Abcam, USA); Tumor Necrosis Factor-Alpha (TNF- α), Interleukin (IL)-6 and IL-10 Enzyme-Linked Immunosorbent assay (ELISA) kits (Nanjing Saihongrui Biotechnology Co., Ltd.); Trizol and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) kit (Shanghai Caiyou Industrial Co., Ltd.); dual-luciferase reporter assay kit (Wuhan Purity Biotechnology Co., Ltd.).

Preparation of ethanol extract from *S. scandens*:

An appropriate amount of dried whole grass of *S. scandens* was took and crushed, the impurities were removed through a 20 mesh sieve. About 200 g samples were incubated with 2 l of 70 % ethanol for 1 h, and soaked using an ultrasonic instrument

to shake for 1 h. After heating in a microwave for 3 min, the mixture was soaked for additional 24 h. The residue was filtered, and the leachate was collected and added with 50 ml of anhydrous ethanol to the filtrate and filter again. Then the ethanol was recovered by reducing pressure, and the ethanol extract was obtained. The extraction was added with 1/3rd volume of ether, and refrigerated at 4° for 6 h, the extracts were took out and filtered again. Finally, the extracts were placed into -46° refrigerator for 12 h, and a dry powder was collected, and 1 g/ml of raw medicine was prepared for later use.

Cell culture, treatment and transfection:

Hepapic cells were maintained in 5 % Carbon dioxide (CO₂) at 37° with RPMI-1640 medium. Hepapic cells were infected with 1×10⁸ CFU/ml *S. pneumoniae* to mimic CAP *in vitro* (model group), with the untreated cells as control group. For transfection, Hepapic cells were transfected with oligonucleotides and/or plasmids based on the experimental design, followed by indicated treatment.

MTT assay:

Hepapic cells infected with *S. pneumoniae* or not were incubated with 0, 1, 2, 4, 8, 16, 32, 64, or 128 μ g/ml ethanol extract, respectively. 48 h later, cells at 3×10⁴ per well were inoculated on a 96 well plate overnight, followed by reacting with 20 μ l MTT (5 g/l) for 4 h. Where after, each well was incubated with 150 μ l Dimethyl Sulfoxide (DMSO) for 2 h lastly, the absorbance at 490 nm was examined.

Flow cytometry:

Hepapic cells were consecutively reacted with 5 μ l Annexin V-Fluorescein Isothiocyanate (FITC) and 5 μ l Propidium Iodide (PI) for 10 min avoiding light, followed by detecting cell apoptosis using the flow cytometry.

Western blotting:

Total extracted proteins were separated and underwent membrane transfer. Then cleaved caspase-3 (ab2302, 1:2000) was added and incubated with membranes at 4° for 12 h, and secondary antibody was used to incubate with members for 2 h. The gray values of the protein bands were analyzed.

ELISA:

After indicating treatment, Hepatic cell supernatant was taken from each group, and the Optical Density (OD) 450 nm value of each group was detected by enzyme label.

qRT-PCR:

The total RNAs of Hepatic cells were extracted using Trizol, and the NanoDrop 2000c spectrophotometer was applied for detecting the purity of RNAs. Then reverse transcription was conducted to generate complementary DNA (cDNA), which were then used for qRT-PCR reaction following the conditions; 95° 2 min, 95° denaturation 15 s, 60° 30 s, 72° 30 s, a total of 40 cycles. The fold changes were assessed using 2^{-ΔΔCt} method. The primers for qRT-PCR: SNHG16, forward 5'-CAGAATGCCATGGTTTCCCC-3'; reverse 5'-TGGCAAGAGACTTCTGAGG-3', Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH); forward 5'-CCACATCGCTCAGACACCAT-3'; reverse 5'-ACCAGGCGCCAATACG-3'; miR-542-3p forward 5'-TGTGACAGATTGATAACTGAAA-3'; reverse 5'-GTGCAGGGTCCGAGGT-3'; U6 forward 5'-CGCTTCGGCAGCACATATACTA-3' and reverse 5'-CGCTTCACGAATTTGCGTGTCA-3'.

Dual-luciferase reporter assay:

The predicted complementary sequences between SNHG16 and miR-542-3p and the corresponding

mutant sequence were amplified to establish Wild Type (WT)-SNHG16 and mut-SNHG16 luciferase reporter vector. All plasmids were co-transfected with miR-132-3p or miR-NC into Hepatic cells. 48 h later, the detection of the relative luciferase was conducted.

Statistical analysis:

The data were manifested by $\bar{x} \pm s$. The Analysis of Variance (ANOVA) or t-test was applied for the comparison in groups. $p < 0.05$ meant significant difference.

RESULTS AND DISCUSSION

As exhibited in fig. 1, the survival rate of Hepatic cells after treatment with different concentrations of *S. scandens* ethanol extract had no change; while Hepatic cells survival rate were decreased gradually by *S. pneumoniae* infection (model group), and the decrease was stabilized when the concentration of *S. scandens* ethanol extract was $> 32 \mu\text{g/ml}$.

The apoptosis rate, cleaved caspase-3 protein levels and TNF- α and IL-6 levels were rose, while IL-10 levels were declined in the model group ($p < 0.05$). Then cells in model group was treated with 8, 16, and 32 $\mu\text{g/ml}$ *S. scandens* ethanol extract, it was found cell apoptosis rate, cleaved caspase-3 protein levels, as well as TNF- α and IL-6 levels were reduced, and IL-10 levels were boosted in cells in a concentration dependent manner ($p < 0.05$) (fig. 2 and Table 1).

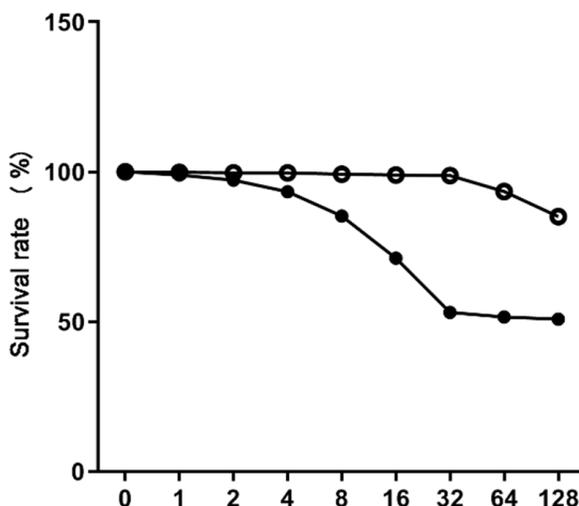


Fig. 1: Detection of Hepatic cell survival rate with ethanol extract of *S. scandens*
 Note: (○): Hepatic and (●): *Streptococcus pneumoniae*+Hepatic

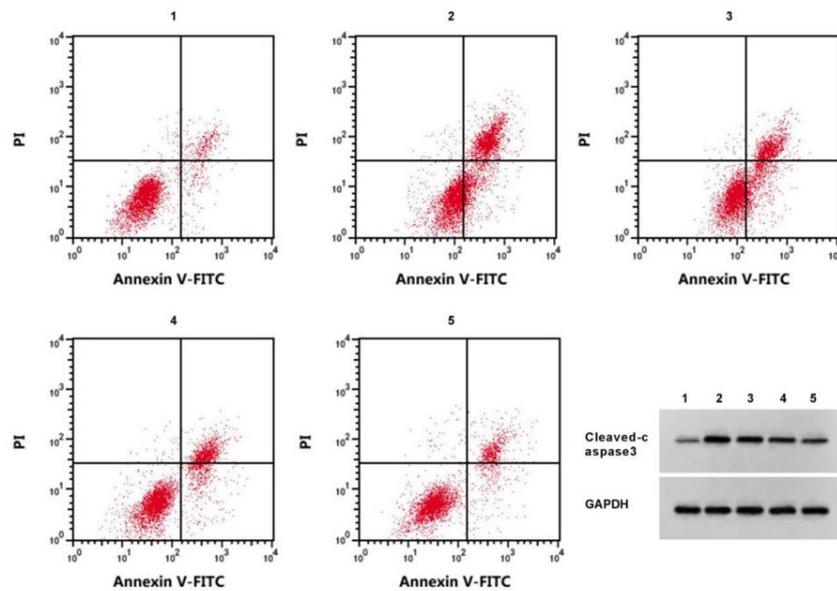


Fig. 2: The effect of *S. scandens* ethanol extract on cell apoptosis and cleaved caspase-3 protein level, (1): Control group; (2): Model group; (3): Model+8 µg/ml; (4): Model+16 µg/ml and (5): Model+32 µg/ml

TABLE 1: DETECTION OF APOPTOSIS AND INFLAMMATORY FACTORS BY *S. scandens* ETHANOL EXTRACT ($\bar{x} \pm s$, n=3)

Group	Apoptosis rate (%)	Cleaved caspase-3	pg/ml		
			TNF- α	IL-6	IL-10
Control	6.65 \pm 0.23	0.10 \pm 0.01	64.37 \pm 5.57	122.57 \pm 10.74	359.14 \pm 25.78
Model	26.40 \pm 0.92 ¹	0.79 \pm 0.06 ¹	302.21 \pm 17.13 ¹	541.81 \pm 25.77 ¹	46.20 \pm 5.64 ¹
Model+8 µg/ml	21.65 \pm 1.06 ²	0.60 \pm 0.05 ²	258.12 \pm 14.06 ²	480.19 \pm 17.40 ²	81.15 \pm 3.92 ²
Model+16 µg/ml	17.95 \pm 0.67 ^{2,3}	0.42 \pm 0.03 ^{2,3}	177.50 \pm 10.92 ^{2,3}	356.75 \pm 12.64 ^{2,3}	170.65 \pm 12.71 ^{2,3}
Model+32 µg/ml	13.94 \pm 0.35 ^{2,3,4}	0.25 \pm 0.03 ^{2,3,4}	115.78 \pm 5.92 ^{2,3,4}	230.76 \pm 12.05 ^{2,3,4}	285.37 \pm 10.00 ^{2,3,4}
F	375.907	140.569	213.291	323.494	271.683
p	0.000	0.000	0.000	0.000	0.000

Note: ¹p<0.05 vs. control group; ²p<0.05 vs. model group; ³p<0.05 vs. model group+8 µg/ml and ⁴p<0.05 vs. model group+16 µg/ml

The levels of SNHG16 were boosted and miR-542-3p contents were declined in the model group (p<0.05). After *S. scandens* ethanol extract treatment, SNHG16 level was decreased, and miR-542-3p level was elevated in the model+8, 16 and 32 µg/ml groups in a dose-dependent manner (p<0.05) (Table 2).

SNHG16 expression in the model+si-SNHG16 group was decreased, while miR-542-3p content was increased, the apoptosis rate and cleaved caspase-3 protein levels were reduced, and the contents of IL-6 and TNF- α were decreased, and IL-10 content was increased (p<0.05) (fig. 3 and Table 3).

Star base predicted the complementary sequences of miR-542-3p on SNHG16 (fig. 4). The luciferase

activity was notably declined in cells transfected with WT-SNHG16 and miR-542-3p (p<0.05) (Table 4).

SNHG16 expression level was decreased, miR-542-3p content was increased, apoptosis rate and cleaved caspase-3 protein expression levels were decreased, IL-6 and TNF- α levels were descended, and IL-10 content was boosted in model+ethanol extract (p<0.05). SNHG16 content was increased, miR-542-3p level was declined, the apoptosis rate, cleaved caspase-3 protein levels were increased, and IL-6 and TNF- α levels were boosted, while IL-10 content were descended (p<0.05) in the model+ethanol extract+pcDNA-SNHG16 group relative to the model+ethanol extract group, (fig. 5 and Table 5).

TABLE 2: MEASUREMENT OF SNHG16 AND miR-542-3P CONTENT ($\bar{x}\pm s$, n=3)

Group	SNHG16	miR-542-3p
Control	1.00±0.00	1.00±0.00
Model	4.29±0.07 ¹	0.12±0.01 ¹
Model+8 µg/ml	3.83±0.06 ²	0.24±0.02 ²
Model+16 µg/ml	3.09±0.03 ^{2,3}	0.45±0.03 ^{2,3}
Model+32 µg/ml	1.76±0.05 ^{2,3,4}	0.75±0.05 ^{2,3,4}
F	2422.221	506.037
p	0.000	0.000

Note: ¹p<0.05 vs. control group; ²p<0.05 vs. model group; ³p<0.05 vs. model group+8 µg/ml and ⁴p<0.05 vs. model group+16 µg/ml

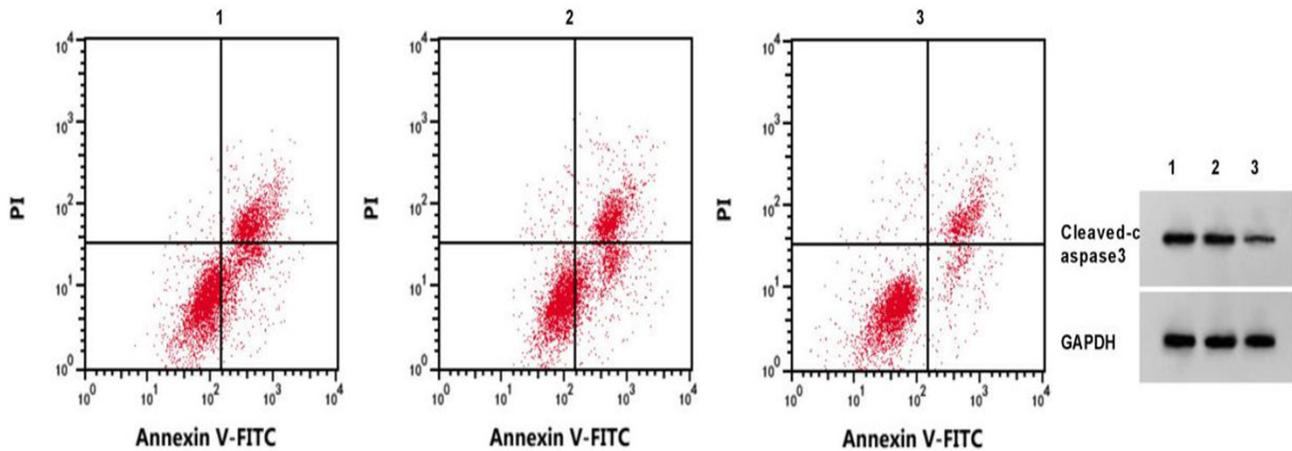


Fig. 3: The effect of inhibiting SNHG16 on cell apoptosis and cleaved caspase-3 protein expression, (1): Model group; (2): Model+si-NC group and (3): Model+si-SNHG16 group

TABLE 3: INHIBITION OF SNHG16 ON THE DETECTION OF CELL APOPTOSIS AND INFLAMMATORY FACTORS ($\bar{x}\pm s$, n=3)

Group	SNHG16	miR-542-3p	Apoptosis rate (%)	Cleaved caspase-3	pg/ml		
					TNF-α	IL-6	IL-10
Model	1.00±0.00	1.00±0.00	26.50±0.91	0.79±0.07	306.65±16.92	545.98±31.31	46.13±6.47
Model+si-NC	0.97±0.03	1.01±0.02	26.47±0.93	0.79±0.05	309.61±20.85	545.39±33.64	46.02±6.30
Model+si-SNHG16	0.24±0.02 ^{1,2}	7.28±0.22 ^{1,2}	9.21±0.33 ^{1,2}	0.15±0.01 ^{1,2}	84.82±5.68 ^{1,2}	167.07±10.21 ^{1,2}	319.96±17.57 ^{1,2}
F	1282.375	2420.637	496.853	163.840	198.620	194.047	576.645
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: ¹p<0.05 vs. model group and ²p<0.05 vs. model+si-NC



Fig. 4: The complementary sequence of SNHG16 and miR-542-3p

TABLE 4: DUAL-LUCIFERASE REPORTER ASSAY

Group	WT-SNHG16	MUT-SNHG16
miR-NC	1.03±0.07	1.01±0.08
miR-542-3p	0.24±0.02 ¹	0.98±0.05
t	18.795	0.551
p	0.000	0.611

Note: ¹p<0.05 vs. miR-NC group

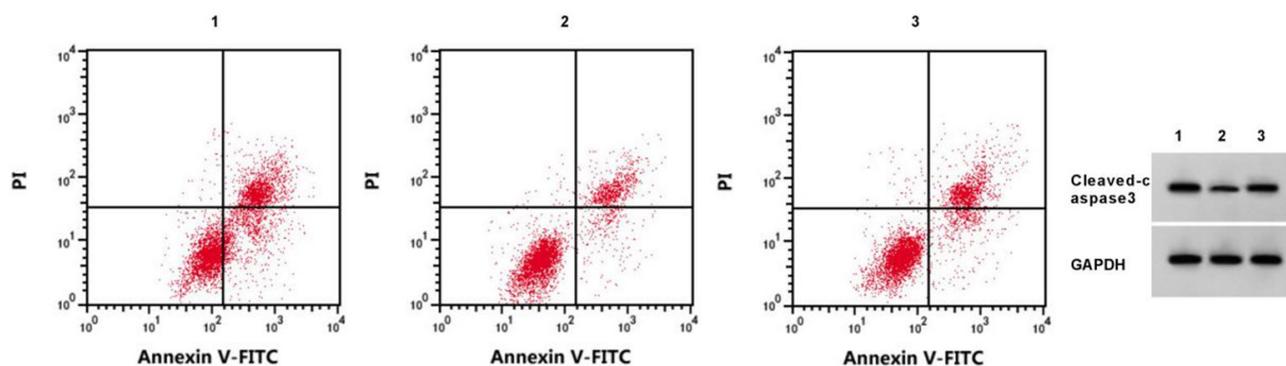


Fig. 5: Overexpression of SNHG16 could reverse the effect of *S. scandens* ethanol extract on cell apoptosis and cleaved caspase-3 protein expression, (1): Model group; (2): Model+*S. scandens* ethanol extract group and (3): Model+*S. scandens* ethanol extract+pcDNA-SNHG16 group

TABLE 5: OVEREXPRESSION OF SNHG16 COULD REVERSE THE EFFECTS OF *S. scandens* ETHANOL EXTRACT ON CELL APOPTOSIS AND INFLAMMATORY FACTORS

Group	SNHG16	miR-542-3p	Apoptosis rate (%)	Cleaved caspase-3	pg/ml		
					TNF- α	IL-6	IL-10
Model	4.33±0.07	1.00±0.00	26.58±0.92	0.78±0.05	306.15±18.08	546.44±30.43	47.58±5.68
Model+ethanol extract	1.77±0.08 ¹	6.26±0.08 ¹	13.89±0.33 ¹	0.24±0.02 ¹	114.85±6.75 ¹	239.24±14.05 ¹	286.29±9.06 ¹
Model+ethanol extract+pcDNA-SNHG16	4.10±0.10 ²	1.48±0.12 ²	23.73±0.68 ²	0.67±0.03 ²	282.44±13.27 ²	524.83±9.05 ²	65.94±5.44 ²
F	847.563	3659.596	281.425	192.868	178.412	219.533	1103.314
p	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Note: ¹p<0.05 vs. model group and ²p<0.05 vs. model+ethanol extract group

Currently, TCM treatment has great advantages and characteristics, providing new ideas for clinical treatment in CAP. As a common TCM, *S. scandens* and its extracts and compounds possess diverse pharmaceutical activities, like anti-inflammatory, antibacterial and anticancer activities^[6,10]. The petroleum ether extract of *S. scandens* could impede inflammatory response in LPS-stimulated RAW264.7 cells^[11]. In our work, we used *S. pneumoniae*-stimulated Hepatic cells to mimic pneumonia condition *in vitro*, followed by treating with different doses of ethanol extracts of *S. scandens*. We discovered that there was no significant change in the survival rate of Hepatic

cells after *S. scandens* treatment, while Hepatic cell survival rates were decreased by *S. pneumoniae* stimulation, but the decrease was suppressed when the concentration of *S. scandens* was greater than 32 μ g/ml, implying that *S. scandens* ethanol extract could affect *S. pneumoniae* infected AECs at a concentration of 32 μ g/ml, therefore, 8, 16, and 32 μ g/ml ethanol extract were selected for subsequent analyses. Functionally, we proved that *S. scandens* ethanol extract suppressed *S. pneumoniae* induced apoptosis and inflammation in AECs

In recent years, with the increasingly widespread bacterial resistance, the difficulty of treating

infectious diseases has increased dramatically. To address this issue, researchers have attempted a combination of TCM and Western medicine antimicrobial therapy. TCM can regulate the biofilm of drug-resistant bacteria by inhibiting quorum sensing system expression, bacterial adhesion, and other aspects. It does not directly kill bacteria, but inhibits communication between bacteria and reduces their infectivity. However, the antibacterial effect of TCM alone is limited, so in clinical applications, TCM is often applied in combination with antibiotics. An increasing number of studies have shown that the combination of TCM and Western medicine can better inhibit the quorum sensing system of drug-resistant bacteria, thereby reducing biofilm formation and improving the antibacterial effect of antibiotics. Thereafter, we also found that *S. scandens* ethanol extract led to a decrease of SNHG16 expression in model cells. SNHG16 deficiency impaired LPS-evoked apoptotic and inflammatory injuries by modulating miR-370-3p^[12]. SNHG16 deletion partially alleviated LPS triggered autophagy, apoptosis, and inflammation^[13]. These data suggested the potential involvement of SNHG16 in regulating alveolar epithelial cell function infected with *S. pneumoniae*. In this work, we proved that SNHG16 silencing could suppress the apoptosis and inflammatory reaction. Moreover, forced expression of SNHG16 could abolish the suppressing action of *S. scandens* ethanol extract on the apoptosis and inflammation evoked by *S. pneumoniae*. In addition, through bioinformatics analysis, we proved that SNHG16 bound to miR-542-3p. miR-542-3p is an important biomarker for CAP and pneumonia-associated sepsis^[14]. The extracellular vesicle miR-542-3p impaired the inflammation in ischemic glial cells^[15]. miR-542-3p functioned in regulating antiviral and inflammatory responses^[16]. Buxin Ruanmai granules could repress the inflammatory reaction and oxidative stress of myocardial cells through the miR-542-3p/GABARAP axis^[17]. All the results indicated the implication of miR-542-3p in CAP. Here, we also discovered that ethanol extract of *S. scandens* increased miR-542-3p expression in model cells. However, whether miR-542-3p was involved in the modulatory action of SNHG16 and *S. scandens* ethanol extract in *S. pneumoniae*-treated AECs needs further study.

In conclusion, *S. scandens* ethanol extract could

suppress *S. pneumoniae* evoked apoptotic and inflammatory injuries in AECs, suggesting the potential application of *S. scandens* ethanol extract in pneumonia treatment.

Authors contribution:

Yan Liu and Zhengya Wang have contributed equally to this study

Conflict of interests:

The authors declared no conflict of interests.

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