

The effect of Wogonoside on the expression of pulmonary fibrosis factor in *Mycoplasma pneumoniae pneumonia*

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Abstract

BACKGROUND

Mycoplasma pneumoniae pneumonia (MPP) is an acute respiratory infectious pneumonia with pulmonary interstitial fibrosis. TGF- β 1 is well accepted as the central mediator for fibrosis which promotes the formation of tissue fibrosis factor. Qinbaiqingfei pellet (Qinbai) has already been approved as the first effective new traditional Chinese medicine which can delay activities of *Mycoplasma pneumoniae (M.pneumoniae)* and protect lung epithelial cells in clinical trials. However, the mechanism of Qinbai inhibiting the expression of TGF- β 1 is still unclear.

METHODS

The Chinese herbs in Qinbai were screened by surface plasmon resonance (SPR) and it was determined that *Scutellaria baicalensis* extracts in Qinbai showed the best binding with TGF- β 1. Then the active ingredient which can be bound with TGF- β 1 protein in the solution of Qinbai and *Scutellaria baicalensis* was isolated and analyzed by UPLC-Q-TOF-MS, which proved the active ingredient was *Wogonoside*. The affinity constant of *Wogonoside* and TGF- β 1 protein was measured by SPR affinity analysis. A549 cells infected with *M.pneumoniae* were intervened by *Wogonoside*, and then the expression of TGF- β 1 and Smad3 in A549 cells were analyzed by PCR and western blotting.

RESULTS

The results showed the bound effect of *Scutellaria baicalensis* and TGF- β 1 was effective. The active ingredients which can be bound with TGF- β 1 in the solution of *Scutellaria baicalensis* and Qinbai were obtained and analyzed to investigate the mechanism of Qinbai inhibiting the expression of TGF- β 1. UPLC-Q-TOF-MS results showed that the active ingredients was *Wogonoside*. SPR affinity analysis showed that the affinity constant was 21.71 μ M. Pharmacological experiments revealed that *Wogonoside* strongly inhibited the expression of TGF- β 1 and Smad3 in A549 cells infected by *M.pneumoniae*.

CONCLUSION

Wogonoside in Qinbai can be bound with TGF- β 1 and down-regulate the expression of lung fibrosis factors TGF- β 1 and Smad3. The finding may improve our understanding the molecular mechanism of Qinbai mediating *MPP* and provide new sights into the future pharmacological investigation of Qinbai.

Background

Mycoplasma pneumoniae (M.pneumoniae), a gram-stained negative microorganism, is a major cause for respiratory tract infections and community-acquired pneumonia^[1], accounting for approximately

10%-30% of all cases and as much as to 50% in outbreak years^[2]. *M.pneumoniae* is spherical or silk without cell wall, which makes it instinctually resistant to antimicrobials, such as β -lactams^[3], azithromycin and glucocorticoid^[4]. *M.pneumoniae* could attach to epithelial members, produce reactive oxygen species and injure epithelial cells. *Mycoplasma pneumoniae pneumonia (MPP)* is an acute respiratory infectious pneumonia with pulmonary interstitial fibrosis. *M.pneumoniae* invades the bronchioles, connective tissue around the bronchi and the alveolar septum, and eventually leads to interstitial fibrosis. Due to lung hypoplasia in children, *M.pneumoniae* is prone to cause interstitial fibrosis after repeated infections^[5]. TGF- β 1 is well accepted as the central mediator for fibrosis^[6]. It promotes the formation of tissue fibrosis factor in the aspect of strongest expression, increases in fibrosis diseases, promotes fibrosis foci inflammatory cells and fibroblast aggregation^[7, 8]

Qinbaiqingfei pellet (Qinbai) has already been approved as the first effective new traditional Chinese medicine which can delay activities of *M.pneumoniae* and protect lung epithelial cells in clinical trials. The previous studies have shown Qinbai can inhibit the expression of *M.pneumoniae* adhesion proteins P1 and P30 and promote the repair of airway tissues and epithelial cells^[9-11]. Yao *et al.* pointed that Qinbai inhibited epithelial-mesenchymal transition (EMT) of alveolar type II epithelial cells by reducing the content of TGF- β and restore the normal morphology and function of the lung by increasing the expression of SP-A^[12]. Wang *et al.* found that Qinbai decreased the expression of cytokines Wnt5a and α -SMA, and inhibit the cellulose deposition after *MPP*^[13].

The traditional Chinese medicine *Scutellaria baicalensis*, the dry root of *Scutellaria baicalensis Georgi*, is the most important herb in Qinbai. *Scutellaria baicalensis* has been used for the treatment of "Lung-Heat" syndrome in China for a long time. In *Xiaochaihu Decoction* and *Chaigejejei Decoction* which are the classical prescriptions for the treatment of pulmonary infectious diseases and initially recorded in Han Dynasty (20-220 AD), *Scutellaria baicalensis* is the main herb^[14]. In the traditional Chinese medicine practice, there have reported several researches on the pulmonary inflammation treatment by *Scutellaria baicalensis*^[15-17]. Xu *et al.*^[14] pointed that *Scutellaria baicalensis* may inhibit excessive release of pro-inflammatory cytokines, thereby inhibiting the systemic inflammatory response syndrome and promoting lung repair of tissue inflammatory lesions. *Scutellaria baicalensis* can suppress different tissue and organ fibrosis occurrence and development via various mechanisms, including down-regulating expression of promote-fibrosis cytokines, inhibiting pro-fibrogenic signaling pathways, anti-inflammatory and anti-oxidant effects^[19-21]. It's confirmed that the active components of *Scutellaria baicalensis* are *Wogonoside*, *Baicalin*, *Baicalein* and *Wogonin*^[22, 23]. *Wogonoside* is a flavonoid ingredient with various biological activities, such as anti-tumor, anti-inflammatory and anti-oxidation^[24-26]. Previous studies showed that *Wogonoside* inhibited the TLR4 expression and the phosphorylation of NF-kappa B p65 and I kappa B induced by LPS, exerting anti-inflammatory effects^[23, 27]. However, there are few reports on the treatment of *MPP* with *Wogonoside* and the mechanism of *Wogonoside* inhibiting the deposition of pulmonary interstitial cellulose to treat *MPP* needs to be further studied.

The surface plasmon resonance (SPR) biosensor is a refractometer that measures changes in the optical reflectivity of a thin metal film when species adsorb or bind to its surface or to any material coated onto its surface^[18, 28]. SPR has been applied in numerous fields including the researches of Chinese herbal medicine targets, medical diagnostics and pharmaceutical analysis^[29]. Based on SPR, Cao *et al.* firstly discovered that physcion-8-O- β -D-monoglucoside was a bioactive ingredient and the affinity to TNF-R1 was 376 nM^[30]. Tu *et al.* confirmed that protocatechualdehyde could control cardiovascular remodeling by binding with collagen I^[13]. In present study, it was found that *Scutellaria baicalensis* extract in Qinbai showed the best binding with TGF- β 1. On this foundation, SPR was applied to identify the active ingredient in *Scutellaria baicalensis* and reveal the mechanism of Qinbai in the treatment of MPP.

Materials And Methods

Materials

A549 cells were kindly provided by Chinese Academy of Sciences Stem Cell Bank (China). *Mycoplasma pneumonia* was purchased from DAAN GENE (China). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Hyclone (USA). PPLO broth was purchased from BD Biosciences (USA). Fresh yeast was purchased from Angel Yeast Company (China). Human TGF- β 1 protein was purchased from ACRO-Biosystems (USA). SDS-PAGE Gel Kit was obtained from Solarbio life sciences (China). Amine Coupling Kit (BR-1000-50), Acetate 4.0 and NaOH (50 mM) were purchased from GE Healthcare (Sweden). Mouse anti-TGF- β 1 antibody (ab64715) and Rabbit anti-Smad3 (ab40854) antibody were purchased from Abcam (USA). Rabbit anti- β -actin (bs-0061R) was purchased from Bioss (UK). Peroxidase-Conjugated Goat anti-Mouse IgG (H+L) and Peroxidase-Conjugated Goat anti-Rabbit IgG (H+L) were purchased from ZSGB-BIO (China). Trizol reagent was purchased from Life Technologies (USA). Go Taq 1-Step RT-qPCR System was purchased from Promega (USA). RIPA reagent was provided by Beyotime (China). Bradford method kit was purchased from Nanjing Jiancheng Biotechnology Co.Ltd (China). Polyvinylidene difluoride (PVDF) membranes were purchased from Biosharp (China). Hypersensitive electrochemiluminescence kit was obtained from Wanleibio (China). *Scutellaria baicalensis* was purchased from Beijing Tongrentng Pharmacy (China). Qinbai (Batch number: 130701) was obtained from Harbin tianheli Pharmaceutical Co., Ltd. *Wogonoside* was purchased from Shanghai Yuanye Biotechnology Co.Ltd, (20 mg, B20488, HPLC>98%). Formic acid was purchased from Fisher Scientific (USA). Methanol and acetonitrile were respectively purchased from Kermel (China) and Merck (Germany). ACQUITY™ UPLC system was obtained from Waters (USA). Triple-TOFTM 5600⁺ mass spectrometer was obtained from AB SCIEX (USA). Acquity UPLC BEH C₁₈ column (2.1 mm×100 mm, 1.7 μ M) and AQUITY UPLC BEH C₁₈ VanGuard Pre-Column (2.1 mm×5 mm, 1.7 μ M) were purchased from Waters (USA). Fluorescent Quantitative Detection System (Line Gene 9600) was purchased from BIOER (China). Biacore T200 was purchased from GE Healthcare (Sweden). Gel imaging system (Versa Doc MP 4000) was obtained from BIO-RAD (USA).

Preparation of six herbal extracts of Qinbai

Qinbai is composed of six Chinese herbal medicines including *Scutellaria baicalensis*, *Platycodon grandiflorus*, *Pheretima aspergillum*, *Stemona japonica*, *Aster tataricus* and *Ophiopogon japonicus*. According to Chinese Pharmacopoeia (2015 Edition), the six herbal extracts were prepared as follows:

Scutellaria baicalensis extract: 84g *Scutellaria baicalensis* was extracted with water for 4 h. The filtrate was concentrated to the relative density of 1.05-1.10 (80 °C), and pH was adjusted to 1.0-2.0 with 2 mol/L HCl at 80 °C. The solution was allowed to stand for 24 h. The supernatant was washed with water to pH 5.0, and then washed with 70% ethanol until pH reached 7.0. Finally, the solution was pulverized into fine powder at low temperature.

Pheretima aspergillum extract: 63g *Pheretima aspergillum* was crushed properly and extracted twice with 70% ethanol solution, the first time for 2 hours, the second time for 1.5 hours. After filtration, the filtrate was combined. *Platycodon grandiflorus* extract: 42g *Platycodon grandiflorus* was decocted twice with water for 1.5h each time, and the filtrate was filtered and combined. At 60 °C, the filtrate was concentrated into a transparent paste with a relative density of 1.30-1.35.

The extracts of *Stemona japonica*, *Aster tataricus* and *Ophiopogon japonicus* were prepared according to the extraction process of *Platycodon grandiflorus*.

SDS-PAGE

TGF- β 1 protein solution was diluted from 200 μ g/mL to 160 μ g/mL with the loading buffer and then heated at 100 °C for 5 min. SDS-PAGE was carried out with 12% polyacrylamide gel according to the molecular weight of TGF- β 1. The experimental conditions were as follows: the loading volume of TGF- β 1 protein was 5 μ L, the concentrated gel voltage was 70 V for 30 min and the separated gel voltage was 110 V for 1 h. The protein band was stained with Coomassie brilliant blue for 1 h and then eluted until the background was completely clean. The photograph was taken by the gel imaging system.

The binding of *Scutellaria baicalensis* and other herbal extracts with TGF- β 1 by SPR technology

SPR analysis was performed on the Biacore T200 system. Prior to the analysis, the whole flow path was primed by phosphate buffer saline (PBS) for three times and 70% bianormalizing solution was used for signal normalization. The CM5 sensor chip was activated by injecting the mixture of 100 mM N-hydroxysuccinimide and 400 mM N-ethyl-N'-(diethylaminopropyl)-carbodiimide (BR-1000-50, Uppsala, Sweden). The solution of TGF- β 1 protein was diluted in the pH 4.0 sodium acetate to 6 μ g/mL and immobilized on the second channel of the CM5 sensor chip for 600 s at a flow rate of 10 μ L/min. Finally, the CM5 sensor chip was blocked with ethanolamine. The first channel served as a negative control. The

extract of *Scutellaria baicalensis* and other herbal extracts were dissolved into 20 mg/mL solution with PBS solution respectively.

Every herbal extract was injected to TGF- β 1 sensor surface for 60 s at a flow rate of 30 μ L/min and then the chip surface was regenerated by glycine hydrochloric acid solution with pH 2.0 for 30s. The sensorgram was recorded and expressed in resonance units (RU).

Recovery of TGF- β 1 bound ingredients

TGF- β 1 protein was coupled to four channels of another CM5 chip with the final concentration of 6 μ g/mL. Similarly, 20 mg/mL *Scutellaria baicalensis* extract was injected into the chip surface at a flow rate of 5 μ L/min for 180 s. The system was then washed with PBS to thoroughly remove the remaining sample solution. Then a small volume of 2 μ L recovery solution (0.5% HCOOH) was injected into the flow cells and incubated for 20s to allow the bound ingredient to be dissociated into the recovery solution. The flow direction over the sensor surface was reversed and the recovery solution containing TGF- β 1 bound ingredients was deposited in 10 μ L ammonium bicarbonate (50 mM). In order to obtain sufficient samples, the total number of cycles was set to 20. 20 mg/mL Qinbai solution was also processed and recovered as the procedures above.

UPLC-QTOF/MS analysis

The SPR-recovered samples were dried under nitrogen and dissolved in 100 μ L methanol. The analysis of the SPR-recovered samples was performed on Waters ACQUITYTM UPLC system. The chromatographic columns were a Waters ACQUITY UPLC BEH C₁₈ column (2.1 \times 100 mm, 1.7 μ M) and a Van Guard Pre-Column (2.1mm \times 5mm, 1.7 μ M). The mobile phase consisted of 0.1% aqueous formic acid (A)-0.1% formic acid acetonitrile (B), using a gradient elution. The flow rate was 0.4 mL/min and the injection volume was 10 μ L. Elution procedure is 5% -100% B at 0-13 min, 100%-5% B at 13-13.10 min and 5% B at 13.10-15.00 min for SPR recovered samples. Mass spectrometry detection was performed by the AB SCEIX Triple-TOFTM 5600⁺ high resolution mass spectrometer. The product ion scan range was 50-1500 Da, enabling dynamic background deduction. The condition of ESI source was as follows: ion source voltage, 5500 V (positive ion mode), 4500 V (negative ion mode); ion source temperature, 550 $^{\circ}$ C; atomizing gas, N₂; auxiliary gas pressure, 379.17 kPa; air curtain gas pressure, 241.99 kPa; fragmentor voltage, 80 V; collision energy, \pm 35 eV; collision energy expansion, 15 eV. IDA was set to respond to the 8 highest peaks of more than 100 cps for secondary mass spectrometry scanning. All data was obtained by Analyst TF 1.6 software and analyzed by Peakview 2.0/masterview 1.0 software.

SPR affinity analysis

The system was primed by 10% methanol before experiments. *Wogonoside* was diluted in 10% methanol to 5 gradient concentrations (280 nM/mL to 17.5 nM/mL), then injected through the reference and active channels for 60 s at the rate of 30 μ L/min, and regenerated with pH 2.0 Glycine-HCL for 60 s. The experiments of 70 nM/mL were performed twice to assess the repeatability. The total number of cycles was 3. Finally, the results were analyzed by Biacore evaluation software (T200 Version 1.0) and fitted a steady-state affinity model to obtain the affinity constant (K_D). Equilibrium dissociation constant (K_D) was derived by fitting to a 1:1 Langmuir bound model.

***M.pneumoniae* and cell culture**

The *M.pneumoniae* cells (ATCC 15531) were cultured in PPLO broth (containing 20% fetal bovine serum, 10% yeast extract solution, 1% glucose, and 0.0002% phenol red), incubated at 37°C with 5% CO₂ and subcultured every 7 days. A549 cells were maintained in RPMI 1640 medium with 10% FBS at 37 °C and 5% CO₂, and subcultured every 3 days. 1×10^5 A549 cells were divided into infected cell group (infected by 10^6 CCU *M.pneumoniae* for 4 h), *Wogonoside* group (infected and given 40 μ M *Wogonoside* for 3 days) and blank group.

Real-time quantitative PCR

Total RNAs were extracted from A549 cells by Trizol. The level of RNAs was amplified by one-step Reverse Transcription-PCR kit and determined by Bioer Technology (China). The ratio for mRNA of interest was normalized to β -actin and presented as the mean \pm SD.

The primers used are as follows:

TGF- β 1 forward primer: 5'-AGC AAC AAT TCC TGG CGA TAC CTC-3'

TGF- β 1 reverse primer: 5'-TCA ACC ACT GCC GCA CAA CTC-3'

Smad3 forward primer: 5'-CAC AGC ATG GAC GCA GGT TCT C-3'

Smad3 reverse primer: 5'-AGG AGA TGG AGC ACC AGA AGG C-3'

β -actin forward primer: 5'-ACA GAG CCT CGC CTT TGC-3'

β -actin reverse primer: 5'-GCG GCG ATA TCA TCA TCC-3'

Western blotting

A549 cells were lysed by RIPA extraction reagent and then centrifuged (4°C) for 10 min at the rate of 12000 rpm/min. Total protein concentration was determined by the Bradford assay method. Samples

were subjected to 12% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in TBST (200 mM Tris buffer, pH 8.0, containing 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h. The membranes were incubated with primary antibodies overnight at 4°C. After incubated with HRP-conjugated anti-rabbit IgG or anti-mouse secondary antibodies, protein bands were visualized using enhanced chemiluminescence (ECL) substrate by Versa Doc Imaging Analysis System and quantified by densitometry using the Image J software.

Statistical analysis

All experiments were repeated at least twice. Data were presented as mean±SD and analyzed statistically with Dunnett's test. Differences were considered significant at $P<0.05$.

Results

SDS-PAGE

As shown in Fig.1, TGF- β 1 protein showed a clear band in the molecular weight range of 48 kDa and 35 kDa, which was in accordance with the experimental standard.

The bound results of *Scutellaria baicalensis* and other herbal extracts with TGF- β 1 by SPR analysis

According to Fig.2, TGF- β 1 protein solution diluted in pH 4.0 sodium acetate showed the best bound effect and the bound value reached 8894.9 RU. According to the results in Fig.3, the binding value of *Scutellaria baicalensis* extract to TGF- β 1 protein was significantly higher than that of other five herbs.

The results in Fig.4 showed that the bound curve of the second channel gradually increased and was higher than the first channel. The bound value of the 2-1 channel reached 159.2 RU in the steady state, indicating that the *Scutellaria baicalensis* extract showed a good binding with the target protein in vitro.

Recognition and recovery of the TGF- β 1 bound ingredients

TGF- β 1 protein solution was connected to four channels of CM5 chip for fishing ligand. The bound values in Fig.5 were 8192.9 RU, 6679.6 RU, 7687.1 RU, 5939.9 RU, indicating the protein was successfully bound with CM5 chip. *Wogonoside* was indentified as a recovery ingredient based on the retention time and m/z information in both positive and negative modes (Fig.6, Fig.7, Fig.8 and Table 1). The negative mode was employed as it showed better sensitivity of ion response than the positive mode. Fig.6A and Fig.7A showed that the fragmental ions of *Wogonoside* at m/z 285 $[M+H-C_6H_8O_6]^+$ and m/z 270 $[M+H-C_6H_8O_6-CH_3]^+$ were detected in the positive mode. In the negative mode (Fig.6B and Fig.7B), the fragment

ion was m/z 283 due to loss of 176($C_6H_8O_6$) and m/z 268 was from the loss of 15(CH_3). The retention time of *Wogonoside* was consistent with recovered ingredients and the error were less than 10 ppm.

Table 1 UPLC-Q-TOF-MS data in both positive and negative ion mode of *Wogonoside* and recovered samples.

The bound results of *Wogonoside* with TGF- β 1 protein by SPR analysis

SPR assay was conducted to verify the direct interaction of *Wogonoside* with TGF- β 1 protein. As shown in Fig.9A, serial concentrations of *Wogonoside* ranging from 17.5 nM/mL to 280 nM/mL were injected and bound with TGF- β 1 protein. It was found that the binding value increased gradually with the increase of the concentration. SPR analysis revealed that the dissociation constant (K_D) was calculated as 21.71 μ M (Fig.9B), indicating that *Wogonoside* was a potent bound ingredient with TGF- β 1 protein. The two curves were very close at 70 nM/mL which showed that the experiment was reproducible.

The decreased expression of TGF- β 1 and Smad3 in *M.pneumoniae*-infected A549 cells by *Wogonoside*

As shown in Fig.10, the mRNA levels of TGF- β 1 and Smad3 in the *M.pneumoniae* group were higher than those of the blank group ($P<0.01$). Meanwhile, it was found that the downregulation of TGF- β 1 and Smad3 was observed after treated with 40 μ M *Wogonoside*. Western blotting analysis (Fig.11) revealed that *Wogonoside* could decrease the protein expression of TGF- β 1 ($P<0.01$) and Smad3 ($P<0.05$). In conclusion, the experimental results indicated that *Wogonoside* could reduce the expression of TGF- β 1 and Smad3, thereby inhibiting the activity of *M.pneumoniae*.

Discussion

As we all know, it's a very difficult process to find the active ingredients from complex herbal medicines. SPR biosensors have become an important tool for characterizing and quantifying biomolecular interactions. In addition, considerable progress has been made in the development of SPR sensors for detecting chemical and biological species. Based on the SPR technology, the six herbal extracts of Qinbai were bound with TGF- β 1 protein respectively, and then the active ingredients which could be bound with TGF- β 1 protein were screened preliminarily. Nevertheless, SPR does not provide information for structural identification of protein-bound ingredients in an unknown mixture. UPLC-Q-TOF-MS was adopted to analyze the active ingredients. In this study, we described a method for screening the active ingredient from Chinese herbal medicines by SPR biosensor and UPLC-Q-TOF-MS technology.

The result showed that the bound value of *Scutellaria baicalensis* extract with TGF- β 1 protein reached 2881.9 RU, which was significantly higher than the other five herbs in Qinbai. The result laid a foundation

for the study of the effective components of Qinbai. The bound result of *Scutellaria baicalensis* extract and TGF- β 1 protein (Fig.4) showed that the bound value gradually increased with the prolongation of injection time and was significantly higher than that of the reference channel. In addition, the difference of bound values at stable state reaches 159.2RU, indicating certain binding of *Scutellaria baicalensis* extract with TGF- β 1 protein. UPLC-Q-TOF-MS results showed that the molecular formula, retention time, and fragmentation information of active ingredients that could be bound with TGF- β 1 in the solution of *Scutellaria baicalensis* and Qinbai were consistent with those of *Wogonoside* standard, indicating that *Wogonoside* in *Scutellaria baicalensis* is one of the major active ingredients in Qinbai. The affinity of *Wogonoside* and TGF- β 1 protein was determined based on SPR technology. 1: 1 bound model of small molecules and proteins was used for kinetic analysis. The affinity constant range of small molecules and proteins was 10^{-6} M \times 10^{-3} M. The bound values of *Wogonoside* with TGF- β 1 at different concentrations in Fig.9B showed that the K_D was 21.71 μ M which was consistent with the affinity characteristics of small molecules and proteins, indicating that there is a specific binding between *Wogonoside* and TGF- β 1.

Respiratory mucosal epithelial cells are particularly susceptible to pathogens. Repairing damaged respiratory mucosal epithelium is very important for the treatment of *MPP*. Therefore, A549 cells were selected for this study. *M.pneumoniae* stain assay was used to evaluate the cells with the infection of *M.pneumoniae*. It has been demonstrated that 10^6 CCU *M.pneumoniae* could lead to the presence of cloud-like stains around A549 cells^[31]. The result suggested that *M.pneumoniae* had been successfully adhered to A549 cells. In present study, 10^6 CCU *M.pneumoniae* was selected to build *M.pneumoniae* models.

The former studies indicated that A549 cells were obviously inhibited after the treatment with different doses of *Wogonoside*. The half-inhibitory concentration (IC_{50}) value was identified to be 46.1 μ M^[32]. *Wogonoside* of 40 μ M was selected in this study to detect the ability to inhibit the expression of TGF- β 1 and Smad3 after *M.pneumoniae* infection. Previous studies revealed that the effects of TGF- β are performed by the activation of downstream Smads, including Smad3 and Smad4^[33]. In addition, Smad3 mediated TGF- β 1-augmented contraction in HFL-1 cells^[34]. In present study, the results of PCR and Western blotting showed that 40 μ M *Wogonoside* markedly inhibited the impression of TGF- β 1 and Smad3 in *M.pneumoniae* models

Conclusion

In conclusion, the present study indicated *Wogonoside* in Qinbai can be bound with TGF- β 1 and down-regulate the expression of lung fibrosis factors TGF- β 1 and Smad3. The finding may improve our understanding the molecular mechanism of Qinbai mediating *MPP* and provide new sights into the future pharmacological investigation of Qinbai.

Abbreviations

MPP: Mycoplasma pneumoniae pneumonia; Qinbai: Qinbaiqingfei pellet; M.pneumoniae: Mycoplasma pneumoniae, SPR: surface plasmon resonance.

Declarations

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Authors' contribution

All the authors have contributed significantly. Huihui Wang for Conceptualization, data curation and writing—original draft. Yanli Meng and Weiming Wang for conceptualization, supervision and writing—review and editing. Guixin Tang and Xin Wang for methodology and resources. Xiaoxi Wang and Huixing Xu for the formal analysis and validation. Zhimin Yang for software. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All the data regarding the findings are available within the manuscript

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 UPLC-Q-TOF-MS data in both positive and negative ion mode of *Wogonoside* and recovered samples.

Name	Formula	Mass (Da)	Extraction Mass (Da)	Error (ppm)	Main ion mass peaks (m/z)	Found at RT [min]	Adduct
<i>Wogonoside</i>	$C_{22}H_{20}O_{11}$	460.10056	460.10784	0.7	461.1044 285.0722 270.0494	6.422	+H
			459.09329	7.3	283.7638 268.0377 163.0029	5.684	-H
<i>Scutellaria baicalensis</i> extracts			461.10784	0.6	285.0764 270.0531	6.420	+H
			459.09631	3	283.0622 268.0368 163.0024	5.689	-H
Qinbai solution			461.10784	0.8	285.0765 270.0528	6.231	+H
			459.09329	2.4	283.0612 268.0380 165.9895	5.517	-H

Figures

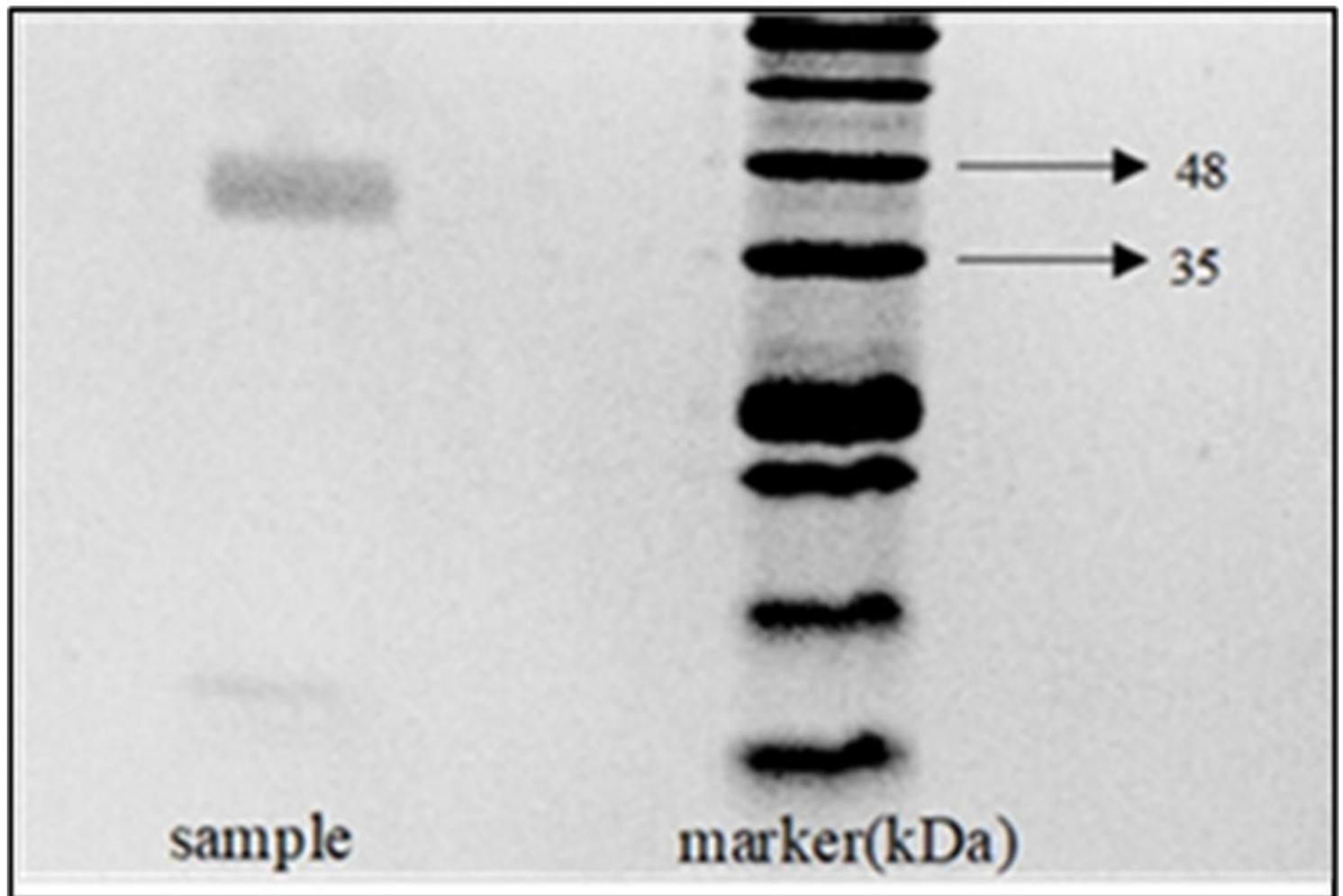


Figure 1

TGF-β1 protein purity detected by SDS-PAGE

Chip: CM5

Flow cell	Procedure	Method	Ligand	Response Bound (RU)
2	Time and flow	Amine	TGF-beta	8894.9

Figure 2

The bound result of TGF- β 1 protein with CM5 chip

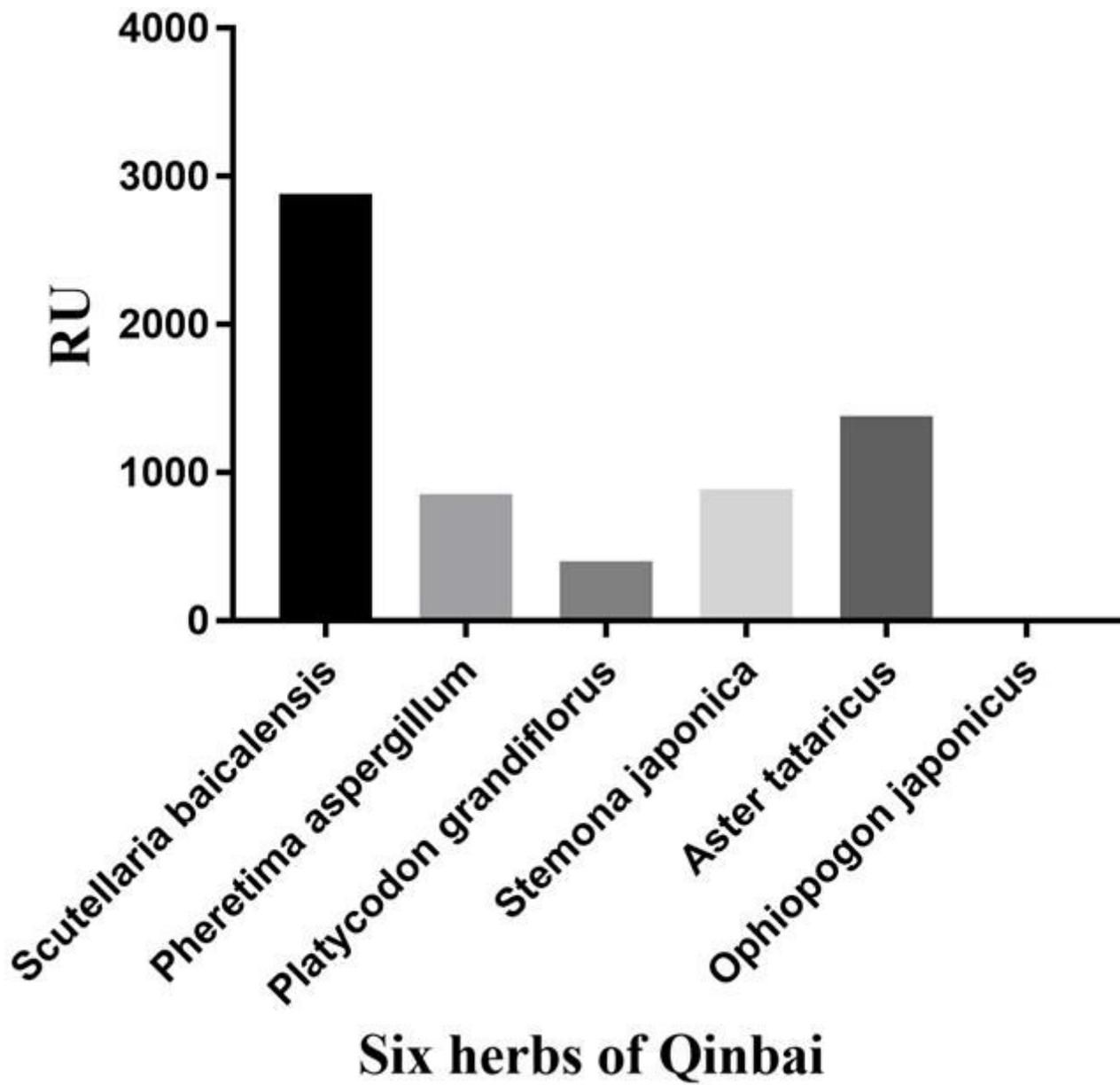
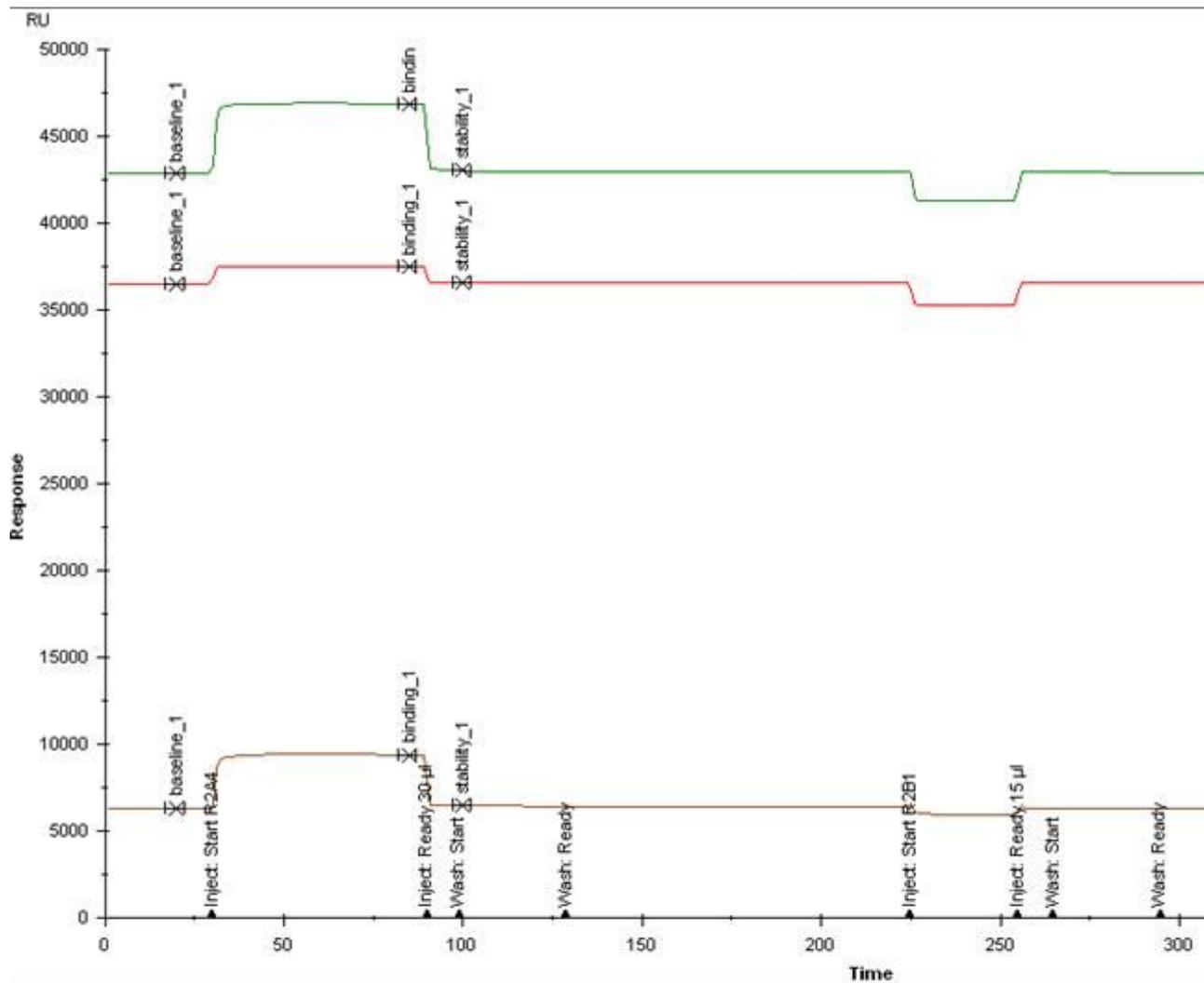


Figure 3

The bound results of six herbal extracts of Qinbai with TGF- β 1 protein



Fc	Time	Window	AbsResp	SD	LRSD	Slope	RelResp	Baseline	Id
1	20.0	5	36523.3	0.37	0.02	0.20	0.0	Yes	baseline_1
1	85.0	5	37458.4	0.15	0.01	-0.08	935.1	No	binding_1
1	100.0	5	36539.5	3.70	1.40	-1.86	16.1	No	stability_1
2	20.0	5	42837.2	0.27	0.02	0.14	0.0	Yes	baseline_1
2	85.0	5	46826.2	6.22	0.27	-3.32	3989.0	No	binding_1
2	100.0	5	43012.8	11.65	0.94	-6.21	175.6	No	stability_1
2-1	20.0	5	6313.9	0.10	0.01	-0.05	0.0	Yes	baseline_1
2-1	85.0	5	9367.8	6.07	0.28	-3.24	3053.9	No	binding_1
2-1	100.0	5	6473.1	8.21	0.55	-4.38	159.2	No	stability_1

Figure 4

The bound results of *Scutellaria baicalensis* extract with TGF- β 1 protein. The green line was the second channel, the red one was the first channel and the brown one was 2-1 channel

Chip: CM5

Flow cells per cycle: 4

Flow cell	Procedure	Method	Ligand	Response Bound (RU)
1	Time and flow	Amine	tgf	8192.9
2	Time and flow	Amine	tgf	6679.6
3	Time and flow	Amine	tgf	7687.1
4	Time and flow	Amine	tgf	5939.9

Figure 5

The bound results of TGF- β 1 ligand with four channels of CM5 sensor chip

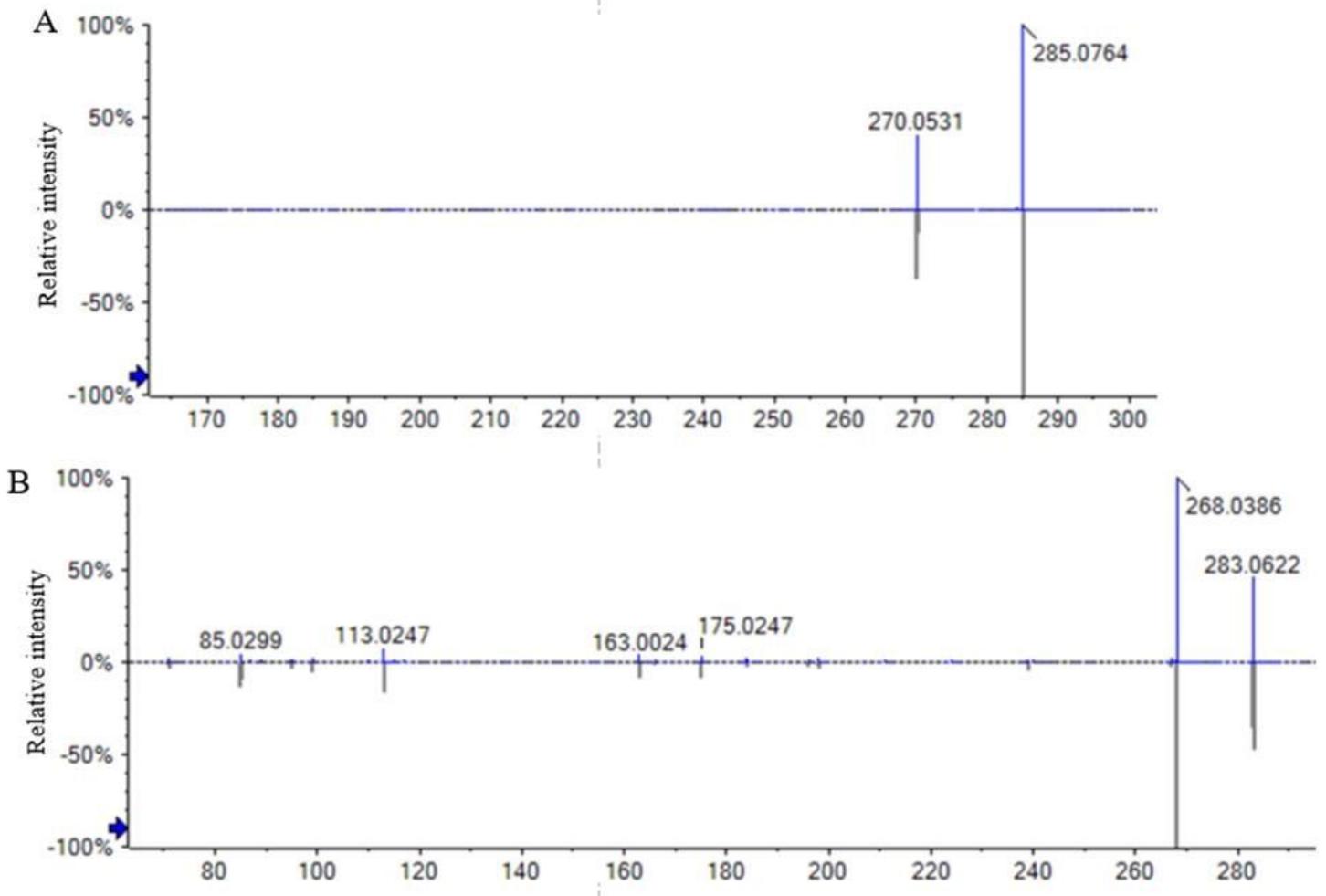


Figure 6

Secondary fragmentation information of *Scutellaria baicalensis* extract in positive ion mode (A) and negative ion mode (B).

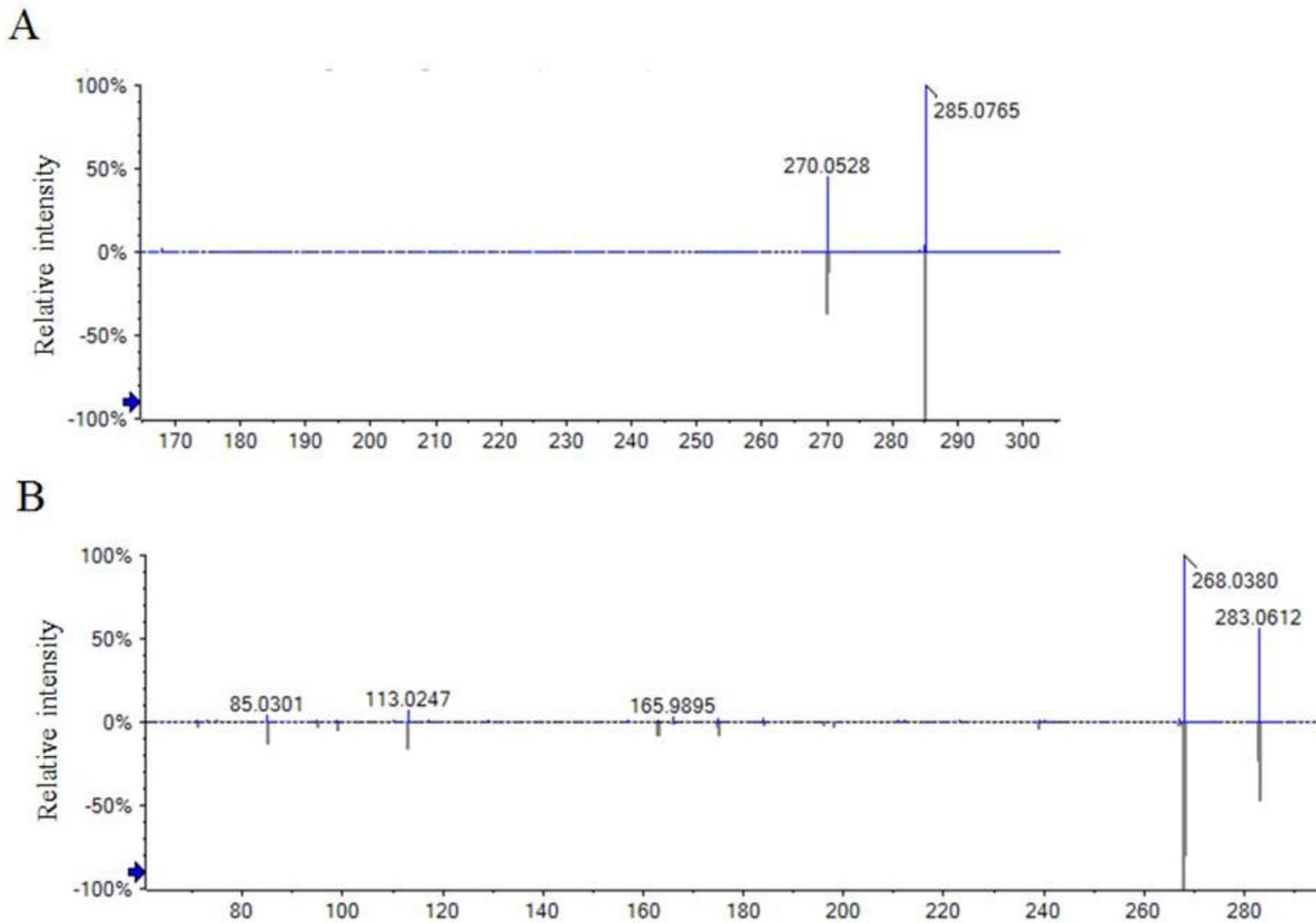


Figure 7

Secondary fragmentation information of Qinbai solution in positive ion mode (A) and negative ion mode (B).

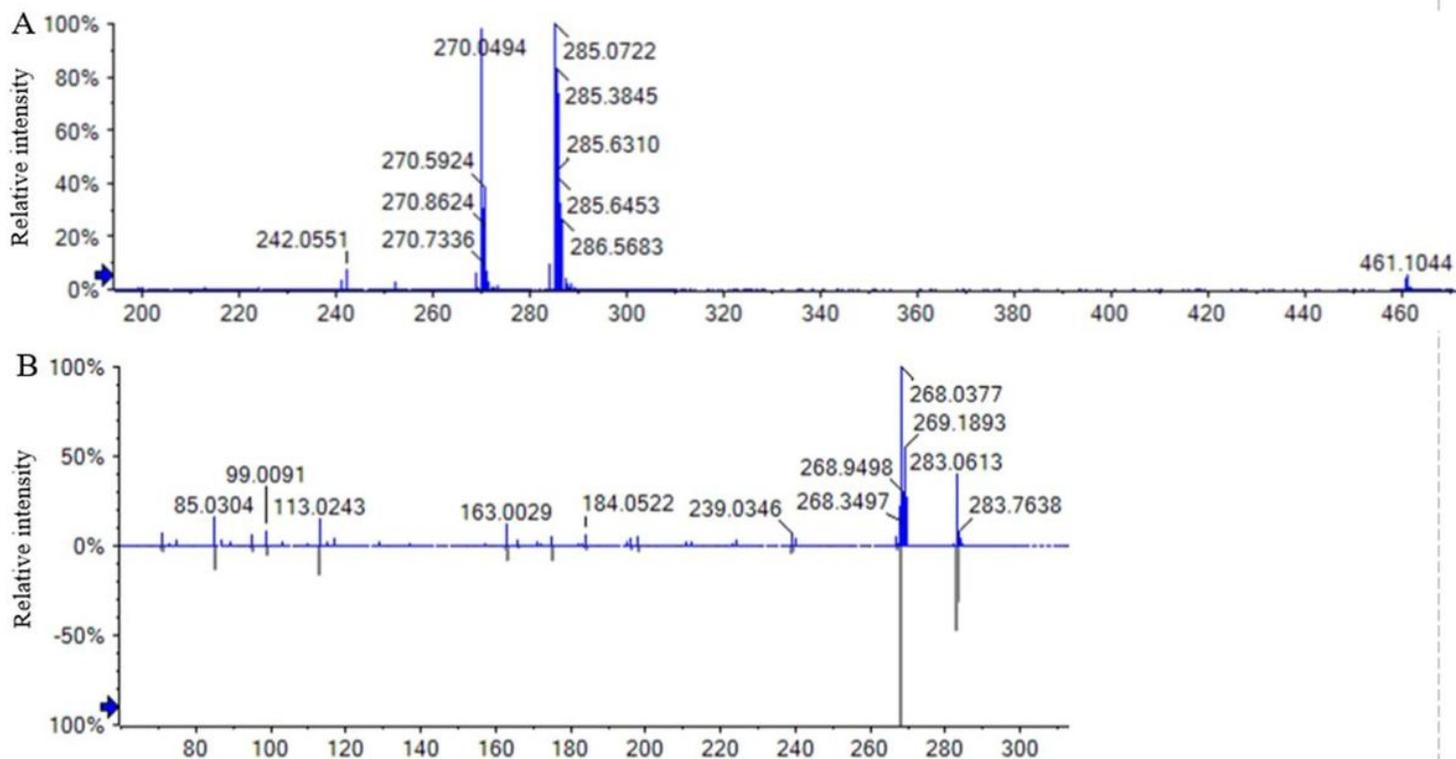


Figure 8

Secondary fragmentation information of Wogonoside in positive ion mode (A) and negative ion mode (B)

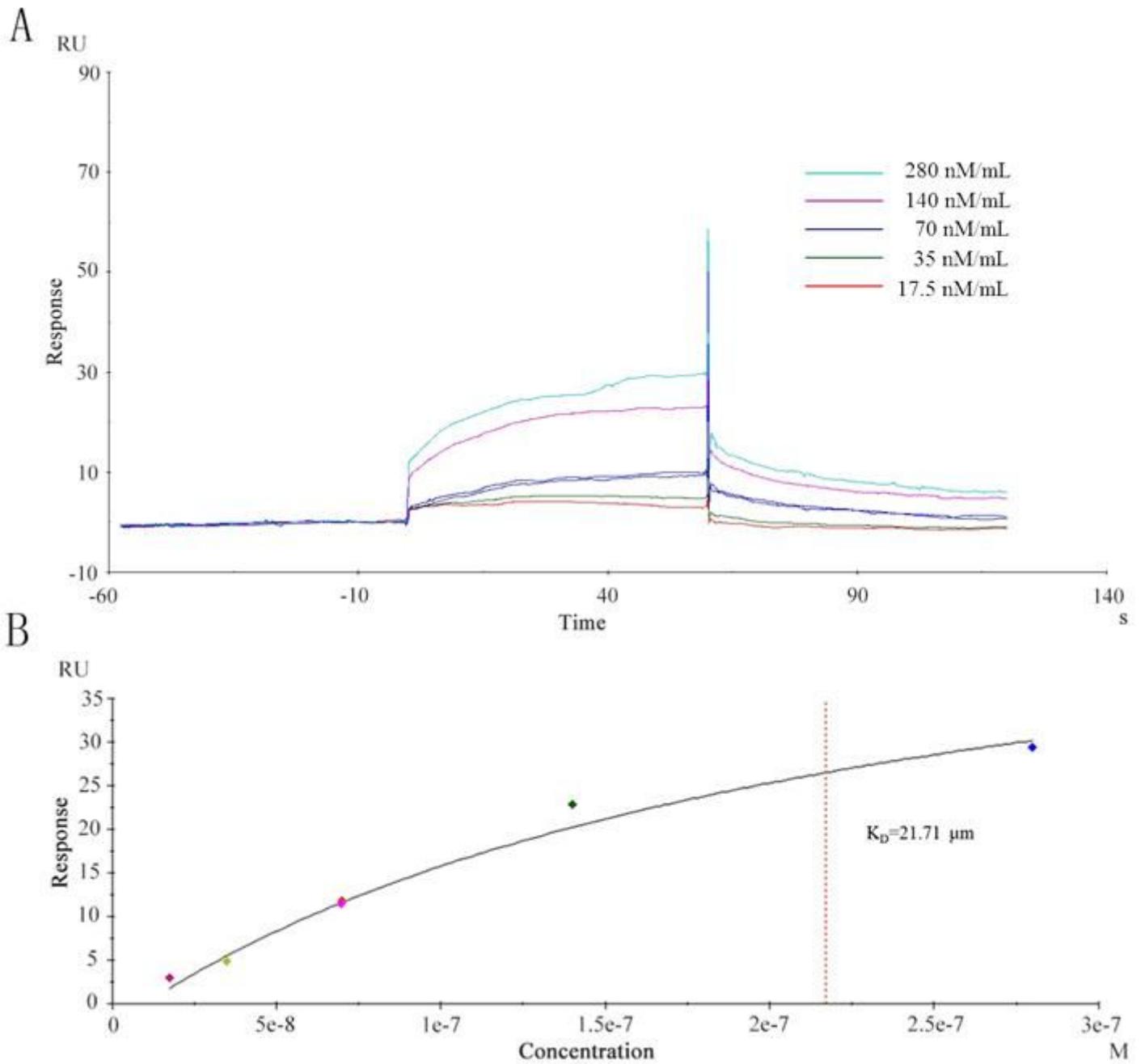


Figure 9

Measurement of affinity constant by SPR affinity analysis. (A) The bound values of different concentration Wogonoside with TGF- β 1 protein. (B) The affinity analysis of Wogonoside with TGF- β 1 protein (KD).

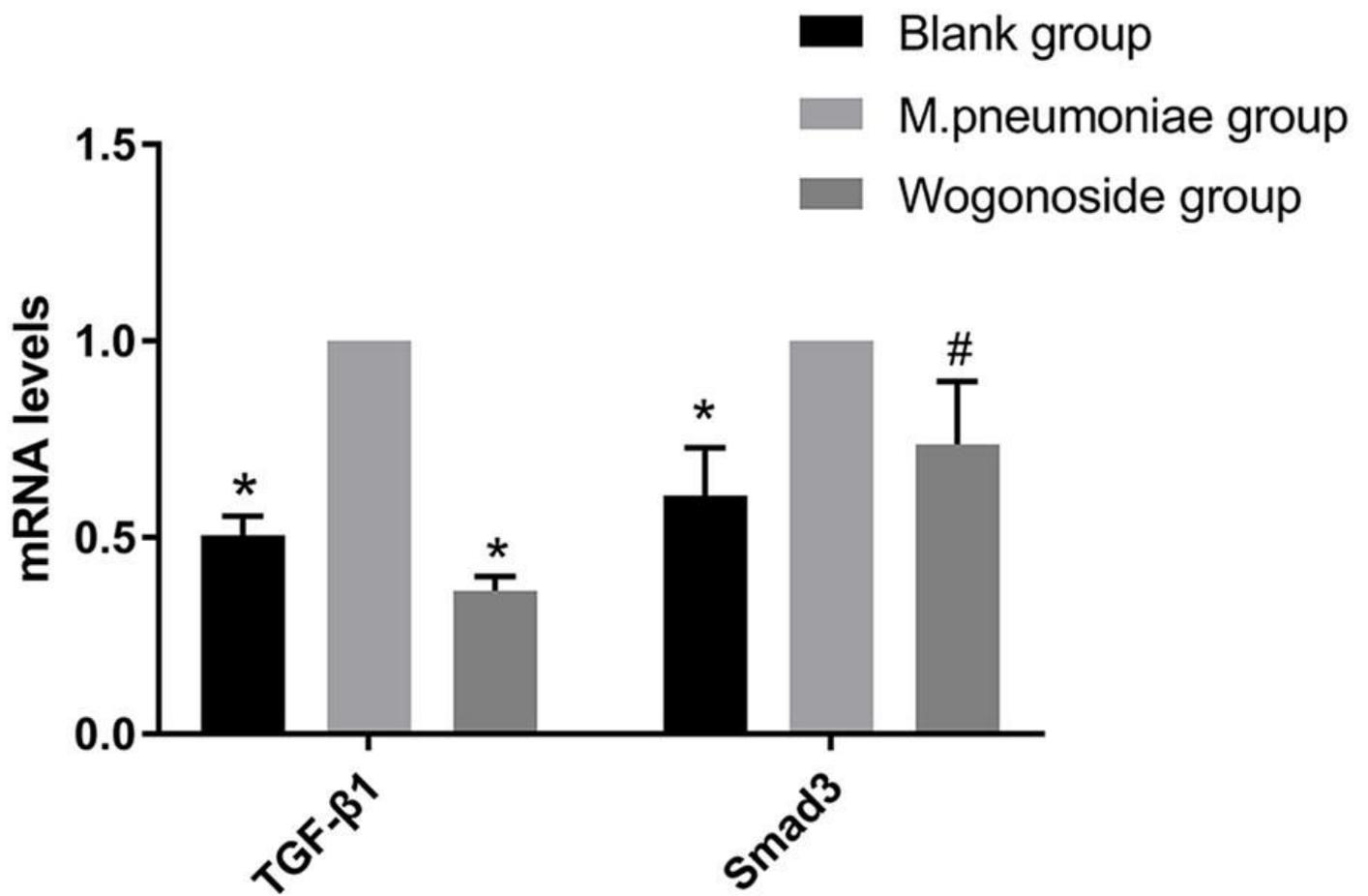


Figure 10

The mRNA levels of TGF-β1 and Smad3. RT-PCR analysis of TGF-β1 and Smad3 mRNA levels in M.pneumoniae infected A549 cells. Compared with M.pneumoniae group, *P<0.01, #P<0.05.

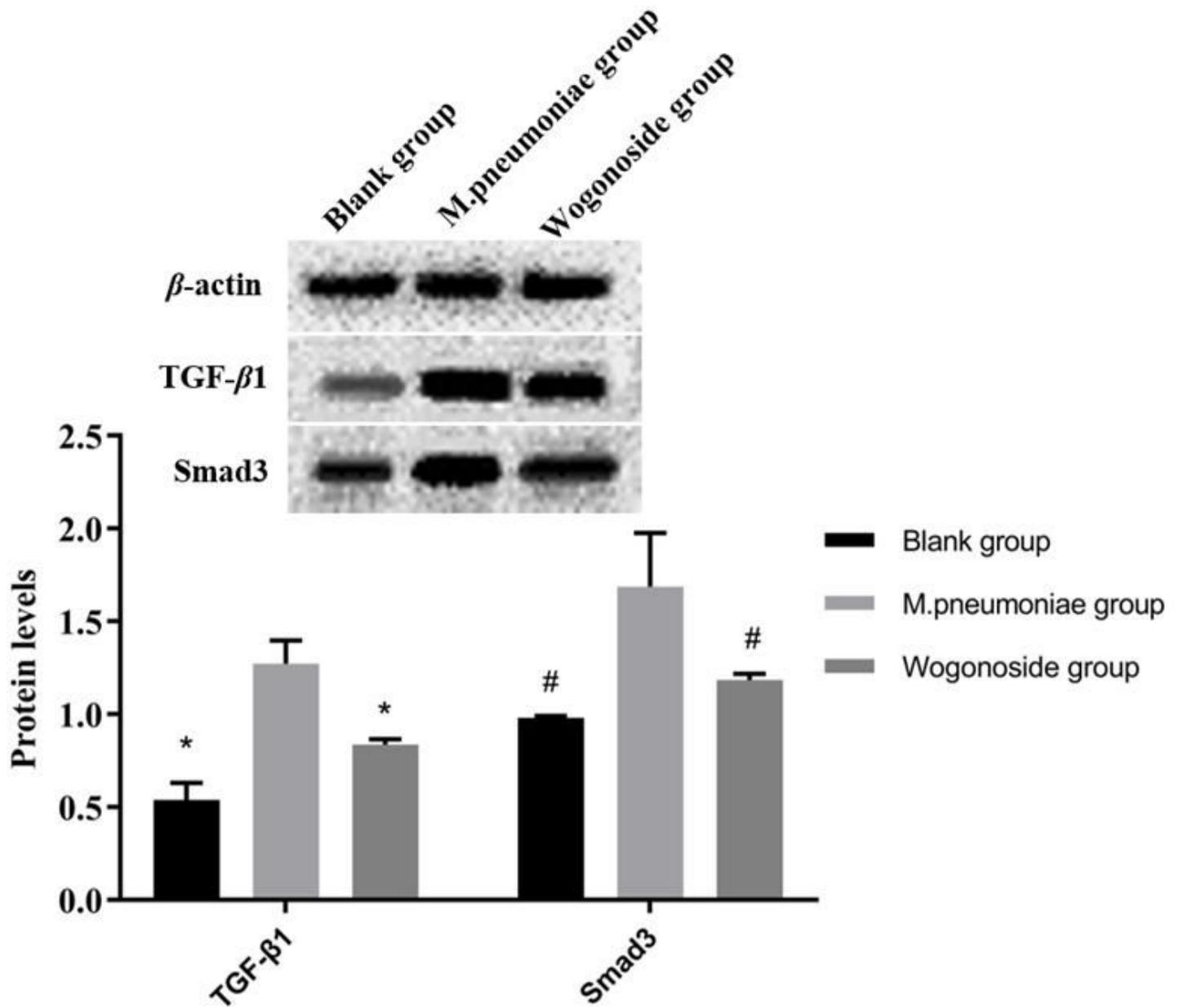


Figure 11

The protein levels of TGF- β 1 and Smad3. Western blotting analysis of TGF- β 1 and Smad3 protein levels in M.pneumoniae infected A549 cells. Compared with M.pneumoniae group, *P<0.01, #P<0.05.

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