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蘆薈水提物與吉非替尼聯合用藥對H1975細胞生長和遷移的抑制作用及其機制研究*

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摘要：肺癌是癌症相關死亡的主要原因，其中非小細胞肺癌（non-small cell lung cancer，NSCLC）發病率最高，約占肺癌的 80%。表皮生長因子受體（epidermal growth factor receptor, EGFR）突變是 NSCLC 最常見的驅動基因突變。EGFR 酪氨酸激酶抑制劑（TKI）吉非替尼（Gefitinib）可通過抑制 EGFR 的酪氨酸激酶活性，從而調節其下游信號通路並抑制腫瘤細胞的增殖和生長，顯著改善 NSCLC 患者的治療及預後。然而，多數接受吉非替尼治療的 NSCLC 患者最終會產生獲得性耐藥並導致治療效果降低。獲得性耐藥最常見的耐藥機制是 EGFR T790M 突變，在耐藥病例中占比大於 50%。研究表明，β-catenin 在 EGFR T790M 突變的 NSCLC 細胞中高表達，下調β-catenin 表達可增加 NSCLC 細胞對吉非替尼的敏感性，從而抑制 NSCLC 腫瘤的生長。因此，抑制 Wnt/β-catenin 通路可能為延緩或克服 EGFR-TKIs 耐藥提供新的治療策略。蘆薈水提物是一種中草藥提取物，在抗癌、抗病毒、免疫調節等方面均有顯著的生物活性，且毒副作用小。在前期實驗中發現蘆薈水提物對 Wnt/β-catenin 通路具有一定的抑制作用，但蘆薈水提物對 NSCLC 的藥理作用及其機制尚不明確。本研究旨在探討蘆薈水提物聯合吉非替尼在 NSCLC 耐藥細胞株中的作用及其機制。選取 NSCLC 耐藥的細胞模型為研究物件，分別給予吉非替尼、蘆薈水提物或二者聯合，進一步通過磺醯羅丹明 B（Sulforhodamine B, SRB）

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法、細胞劃痕實驗（Scratch wound healing assay）、流式細胞術（Flow cytometry）、蛋白免疫印跡法（Western blot）等方法，檢測 NSCLC 細胞增殖、遷移、凋亡、週期以及相關蛋白表達水準。結果發現，蘆薈水提物與吉非替尼聯合用藥後對 A549、PC-9 和 H1975 細胞的生長有明顯的抑制作用且呈濃度依賴性，尤其是對吉非替尼耐藥細胞株 H1975 細胞的抑制效果最顯著。相較于吉非替尼或蘆薈水提物單獨用藥組，聯合用藥組 H1975 細胞的遷移能力明顯降低。此外，蘆薈水提物能有效增加吉非替尼誘導的 H1975 細胞的凋亡率且聯合用藥可誘導 H1975 細胞發生 G2/M 期阻滯。進一步研究表明，高濃度聯合用藥組對 H1975 細胞中的 Wnt/β-catenin 通路具有抑制作用。因此，蘆薈水提物可能通過 Wnt/β-catenin 通路增強 H1975 細胞對吉非替尼的敏感性，抑制細胞增殖和遷移、促進細胞凋亡以及誘導細胞發生 G2/M 期阻滯，可能對延緩或克服吉非替尼耐藥有重要意義，為臨床治療 EGFR-TKIs 耐藥的晚期 NSCLC 患者提供理論基礎。

關鍵詞：蘆薈、吉非替尼、耐藥、EGFR T790M 突變、Wnt/β-catenin 通路

The Combination of *Aloe vera* and Gefitinib Effectively Suppresses Growth and Migration of Gefitinib-resistant H1975 Lung Cancer Cells and Inhibits Wnt/β-catenin Signaling

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Abstract: Lung cancer treatment has been revolutionized by the discovery of somatic mutations in epidermal growth factor receptors (EGFRs) and the development of EGFR tyrosine kinase inhibitors (TKIs). However, in most cases, non-small cell lung cancer (NSCLC) patients who are treated with gefitinib eventually develop resistance. The purpose of this study was to investigate the effect of Aloe vera (L.) Burm. f. (A. vera) extract combined with gefitinib on gefitinib-resistant NSCLC cell lines. The cytotoxicity assay showed that there was a minimal inhibitory effect of A. vera on cell proliferation in A549, PC-9, and H1975 cells, while the combined use of A. vera and gefitinib resulted in a significant inhibitory effect on cell growth in a concentration-dependent manner. Importantly, compared to other cell lines, H1975 proliferation was inhibited by the combined treatment more effectively. The IC50 values at 24 h decreased from 37.92 ± 1.19 to 20.09 ± 1.03 μM for H1975 cells. As a result of the combined treatment of A. vera and gefitinib, the migration ability of H1975 cells was decreased compared with that of the gefitinib group. Moreover, A. vera significantly increased the gefitinib-induced apoptosis in H1975 cells and the combination treatment induced G2/M phase arrest in H1975 cells. Furthermore, the Wnt/β-catenin pathway was inhibited in H1975 cells of the high-concentration combination group, suggesting that A. vera is a potential anticancer therapeutic adjuvant for gefitinib-resistant NSCLC with the EGFR-T790M mutation.

Keywords: *Aloe vera*; gefitinib; gefitinib-resistant; EGFR T790M; β-catenin

1. Introduction

Globally, lung cancer remains the leading cause of cancer-related death with high mortality and a poor prognosis.[1] It remains unsatisfactory to treat lung cancer using conventional chemotherapy.[2] Epidermal growth factor receptor (EGFR) mutations and EGFR tyrosine kinase inhibitors (TKIs) have revolutionized lung cancer therapy. It is the first-line treatment for non-small cell lung cancer (NSCLC) patients with EGFR mutations, which greatly improves their outcomes. However, due to continuous tumor drug selection pressure, in most cases, EGFR-TKIs will develop acquired resistance, resulting in a limited treatment effect.[3] There are two types of resistance observed in clinical trials: primary and acquired. Patients with primary resistance typically had mutations downstream of the EGFR signaling pathway, such as KRAS mutations.[4] The acquired resistance mainly occurs as a result of acquiring a single recurrent missense mutation within exon 20, the T790M mutation. Gefitinib and kinase domains can be blocked by this mutation, potentially triggering resistance, which has been identified as the major mechanism of resistance to gefitinib and erlotinib.[5] H1975 cells possess the L858R mutation along with a secondary mutation at T790M, leading to complete resistance to first-generation EGFR-TKIs. Afatinib, a second-generation EGFR TKI, was initially promising against T790M-mutant cells, but ultimately showed no additional

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2 Mark A Socinski, Tracey Evans, et al, "Hensing et al Treatment of Stage IV Non-small Cell Lung Cancer Diagnosis and Management of Lung Cancer, 3rd ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines," *The Journal of Circulation, Respiration and Related Systems* (Chest: 2013).

3 Susumu Kobayashi, Titus J Boggon, et al, "EGFR Mutation and Resistance of Non-Small-Cell Lung Cancer to Gefitinib," *The New England Journal of Medicine* 24, 352.8 (2005): 786-92.

4 Lihua Huang, Liwu Fu, "Mechanisms of Resistance to EGFR Tyrosine Kinase Inhibitors," A*cta Pharmaceutica Sinica B* 5 (2015): 390-401.

5 Kang-Yi Su, Hsuan-Yu Chen, et al, "Pretreatment Epidermal Growth Factor Receptor (EGFR) T790M Mutation Predicts Shorter EGFR Tyrosine Kinase Inhibitor Response Duration in Patients with Non-small-cell Lung Cancer," *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* (2012).

benefits over first-generation drugs.[6] The third-generation EGFR inhibitor AZD9291 is more effective at preventing tumor growth by circumventing drug resistance.[7] However, the high cost of the drug and its limited availability in a few countries constitute a major hurdle in clinical practice. Hence, exploring effective and feasible treatment strategies to prevent or delay the development of resistance to first generation EGFR-TKIs is still of significance for improving the prognosis of patients with NSCLC.[8]

More recent studies have concentrated on the combinatory usage of EGFR-TKIs for EGFR-mutant NSCLC treatment, which can improve the efficacy of EGFR-TKIs in EGFR-positive patients, delaying the occurrence of drug resistance.[9] Accordingly, several clinical trials have attempted to investigate the effects of gefitinib doublet regimens by combining chemotherapeutic agents with TKIs. Nevertheless, most of these trials are limited by negative results.[10] Clinical studies have shown that gefitinib in combination with paclitaxel and carboplatin can improve the OS (overall survival), and the combination with pemetrexed and carboplatin chemotherapy can promote survival in patients.[11] However, the simultaneous drug toxicity and high cost often compromise the

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6 Yi-Long Wu, Caicun Zhou, et al, "Afatinib Versus Cisplatin Plus Gemcitabine for First-line Treatment of Asian Patients with Advanced Non-small-cell Lung Cancer Harbouring EGFR Mutations (LUX-Lung 6): An Open-Label, Randomised Phase 3 Trial," *The Lancet Oncology* 15 (2014): 213-222; Youngwook Kim, Jeonghun Ko, et al, "The EGFR T790M Mutation in Acquired Resistance to an Irreversible Second-Generation EGFR Inhibitor," *Journal of Thoracic Oncology* 5 (2010): S421-S421.

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9 Curtis R Chong, Pasi A Janne, "The Quest to Overcome Resistance to EGFR-targeted Therapies in Cancer," *Nature Medicine* 19 (2013): 1389-1400.

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11 Herbst R S, Giaccone G, et al, "Gefitinib in Combination with Paclitaxel and Carboplatin in Advanced Non-small-cell Lung Cancer: A Phase III Trial-INTACT 2," *Journal of Clinical Oncology 22* (2004): 785-794; Hosomi Y, Morita S, et al, "Gefitinib Alone Versus Gefitinib Plus Chemotherapy for Non-Small-Cell

benefits of combination therapies.[12] Thus, it is a major clinical challenge to combine safe and cost-effective therapeutic modalities. During the last decade, various types of cancer have been frequently treated with naturally occurring compounds due to their anticancer efficacy and safety. Several preclinical studies have shown that a combination of natural compounds and conventional chemotherapy may be beneficial, which can enhance the anticancer therapeutic efficacy and reduce the toxicity, including curcumin, ursolic acid, and capilliposide from Lysimachia capillipes.[13] Therefore, researching natural products with minimal side effects might be a promising alternative strategy to prevent TKIs resistance or improve the prognosis of resistant patients.

Aloe vera (L.) Burm. f. is a species of medicinal plant belonging to the genus Aloe, which is widely used in the pharmaceutical, cosmetic, and food industries.[14] As a laxative, it is included in the Chinese Pharmacopeia for the treatment of constipation. In addition to its antitumor, antioxidant and anti-inflammatory properties, this species serves as a therapeutic agent in health management.[15] Furthermore, it provides a complementary

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12 Noronha V, Patil V M, et al, "Gefitinib Versus Gefitinib Plus Pemetrexed and Carboplatin Chemotherapy in EGFR-Mutated Lung Cancer," *Journal of Clinical Oncology* 38 (2020): 124; Cheng Y, Murakami H, et al, "Randomized Phase II Trial of Gefitinib With and Without Pemetrexed as First-Line Therapy in Patients With Advanced Nonsquamous Non-Small-Cell Lung Cancer With Activating Epidermal Growth Factor Receptor Mutations," *Journal of Clinical Oncology* 34 (2016): 3258.

13 Chen P, Huang H P, et al, "Curcumin Overcome Primary Gefitinib Resistance in Non-small-cell Lung Cancer Cells through inducing Autophagy-related Cell Death," *Journal of Experimental & Clinical Cancer Research* 38 (2019); Yang K Y, Chen Y, et al, "Ursolic Acid Promotes Apoptosis and Mediates Transcriptional Suppression of CT45A2 Gene Expression in Non-small-cell Lung Carcinoma Harbouring EGFR T790M Mutations," *British Journal of Pharmacology* 176 (2019): 4609-4624; Zhang S R, Xu Y S, et al, "Capilliposide from Lysimachia Capillipes Inhibits AKT Activation and Rrestores Gefitinib Sensitivity in Human Non-small Cell Lung Cancer Cells with Acquired Gefitinib Resistance," *Acta Pharmacologica Sinica* 38 (2017): 100-109.

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15 Yin T P, Yu Y, et al, "13C-NMR-based MixONat Strategy Coupled with 2D NMR for Rapid Dereplication and Identification of New Secondary Metabolites from *Aloe Vera*," *Journal of Food Composition and Analysis* 115 (2023); Nalimu F, Oloro J, et al, " Review on the Phytochemistry and Toxicological Profiles

alternative way to prevent and treat a variety of diseases. Additionally, *A. vera* selectively induces apoptosis in human cancer cells, with no significant effect on normal cells, reflecting its antitumor and cytotoxic potential.[16] Thus, with its numerous health benefits, *A. vera* makes it an excellent adjuvant for drug combination therapy. A clinical trial has shown that the combination of *A. vera* and chemotherapy drugs can enhance the anticancer therapeutic efficacy in terms of both tumor regression rate and survival time in metastatic cancer patients.[17] Experimental studies have also revealed that *A. vera* can reduce cell viability of human breast cancer and cervical cancer cells through apoptosis induction.[18] However, the efficacy of *A. vera* on TKI-resistant lung cancer cells and its molecular mechanisms of action remain elusive. Consequently, we hypothesized that the combination of *A. vera* and gefitinib could restrict the growth of primary TKI- resistant NSCLC cells. To address these issues, this study investigated the efficacy of the combination of *A. vera* and gefitinib, a first-generation EGFR-TKI, on H1975 cells, and the potential molecular mechanisms.

2. Materials and methods

2.1. *A. vera* preparation

A. vera was purchased from Yifang Pharmaceutical Co., Ltd. (Guangzhou, China), which were dried exudate of *A. vera*. Then, it was extracted ultrasonically in water at 80°C for 30 min before the extract was filtered. After that, the extraction solution was stored at -20°C until further use.

In this study, an LC-MS/MS assay was used in the quality control of *A. vera* extract.

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16 Radha M H, Laxmipriya N P, "Evaluation of Biological Properties and Clinical Effectiveness of Aloevera: Asystematic Review," *Journal of Traditional & Complementary Medicine* 5 (2015): 21-26.

17 Lissoni P, Rovelli F, et al, "A Randomized Study of Chemotherapy Versus Biochemotherapy with Chemotherapy Plus Aloe Arborescens in Patients with Metastatic Cancer," *Vivo* 23 (2009): 171-175.

18 Sanchez M, Gonzalez-Burgos E, et al, "Pharmacological Update Properties of Aloe Vera and its Major Active Constituents," *Molecules* 25 (2020).

By comparing the reference compounds, Aloin A, Aloin B, Aloesin, and Aloesin were identified according to their retention time and fragmentation pattern. Aloin A, Aloin B, Aloenin, and Aloesin were further quantified in Aloe as marker substances. The contents of Aloin A, Aloin B, Aloenin, and Aloesin were 1249.0, 1080.0, 23.8, and 4.3 μg/g, respectively. The data are detailed in the supplemental material **(Supplemental Material, Supplemental Figure 1)**.

2.2. Cell lines and cell culture

A549 (wild-type EGFR gene), PC-9 (EGFR exon 19 deletion), H1975 (EGFR exon 21 L858R and exon 20 T790M), and BEAS-2B (normal bronchial epithelial cell line) cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). BEAS-2B cells were maintained in Dulbecco's modified Eagle's medium (DMEM), and A549, PC-9, and H1975 cells were routinely cultured in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were maintained in a 37 °C humidified incubator with a 5% CO_2 atmosphere.

2.3. Sulforhodamine B assay for cytotoxicity screening

Cytotoxicity assays were performed according to the previously described sulforhodamine B (SRB) assay.[19] A total of 5×10^3 cells per well were seeded in a 96-well plate for 24 h and separately treated with various concentrations of *A. vera* and gefitinib for 24 h or 48 h. For the combination treatment, cells were incubated with *A. vera* extract at the indicated concentrations for 24 h and then washed with PBS before gefitinib was added for another 24 h or 48 h. Within 24 h of polysulfide exposure, HBSS was replaced by complete cell growth medium, and SRB assays were performed. Before fixation, the cells were rinsed with HBSS. A solution of 10% ice-cold trichloroacetic acid

[19] Balko J M, Jones B R, et al, "Combined MEK and EGFR Inhibition Demonstrates Synergistic Activity in EGFR-dependent NSCLC," *Cancer Biology & Therapy* 8 (2009): 522-530.

was added to each well, and then the cells were incubated for an hour at 4°C. Each well was incubated for 15 minutes at room temperature with 50 microliters of SRB stain (0.4% in 1% acetic acid). For each well, 1% acetic acid was rinsing four times and 10 mM Tris base solution (pH 10.5) was added, and then plates were shaken for 5 min to solubilize the protein-bound dye. An absorbance measurement of 570 nm was conducted on a plate reader (BioTek). A paired t-test was performed using Prism 8 software (GraphPad).

2.4. Scratch wound healing assay

H1975 cells were plated into 6-well plates at a density of 3×10^5 cells per well and incubated overnight. The confluent monolayer was scratched with the tip of a 200 μL sterile pipette when the cells were approximately 70% confluent. The detached cells were gently washed off twice, and the medium was replaced with 1% FBS complete medium. Cell monolayers were photographed under an inverted microscope after the exfoliated cells were washed away with PBS. The gap distance was quantitatively evaluated using ImageJ software.

2.5. Apoptosis assay

Cells were treated with the indicated concentrations of gefitinib, *A. vera,* or both for 24 h and centrifuged and collected after trypsin digestion. Apoptotic cells were identified using the FITC Annexin V Apoptosis Kit (BD Biosciences). Cells were gently resuspended in 200 μL binding buffer. Then, 5 μL Annexin V-FITC and 5 μL PI stains were added and incubated for 15 minutes in the dark. Apoptotic cells were detected using flow cytometry (BD Biosciences, CA, USA). Cell apoptosis rate was calculated with follows: cell apoptosis rate (%) = (early apoptotic cells + advanced apoptotic cells)/total cell number × 100%.

2.6. Cell cycle analysis

Cell cycle analysis was performed as previously described. In brief, cells were seeded at 3×10^5 cells/well in 6-well culture plates in duplicate and incubated with the indicated concentrations of gefitinib, *A. vera* or combination for 24 h. After harvesting and centrifuging the cells, they were suspended in PBS and fixed with 70% (v/v) cold ethanol solution overnight at 4°C. Cells were collected by centrifugation, resuspended in PBS, and incubated with 10 mg/mL RNase A (Sigma–Aldrich) and 5 mg/mL propidium iodide (Sigma–Aldrich) for 30 min in the dark at room temperature. Then, cells were analyzed on a BD LSRII flow cytometer (BD Biosciences). Finally, the percentages of cells in different phases (G0/G1, S and G2/M) were calculated using Modfit software (Verity Software House, USA).

2.7. Western blot analysis

Cells were plated in 6-well plates and treated as mentioned in the above section. Then, each sample was lysed with RIPA buffer and quantified with Bradford reagent (Bio-Rad Laboratory, Hercules, CA, USA). Cell lysates were mixed with 5×SDS loading buffer and denatured at 100°C for 10 min. Protein lysates (30 μg) were subjected to 10% SDS–PAGE and blotted onto nitrocellulose. Blots were blocked using 5% BSA and incubated overnight with primary antibodies at 4°C, followed by incubation with HRP conjugated secondary antibodies for 1 h. Blots were visualized on Amersham hyperfilm ECL or by AI800 Amersham Imager chemiluminescence. Bands were analyzed by densitometry using ImageJ software.

2.8. Statistical analysis

All the data were obtained from at least three independent experiments in a parallel manner and are presented as the mean ± standard deviation (SD). ANOVA was used to

analyze the statistical significance of any difference among different groups, while Student's *t*-test was used to evaluate the comparison between two groups. The level of significance is defined as 95% ($p < 0.05$) while $p < 0.01$ means very significant.

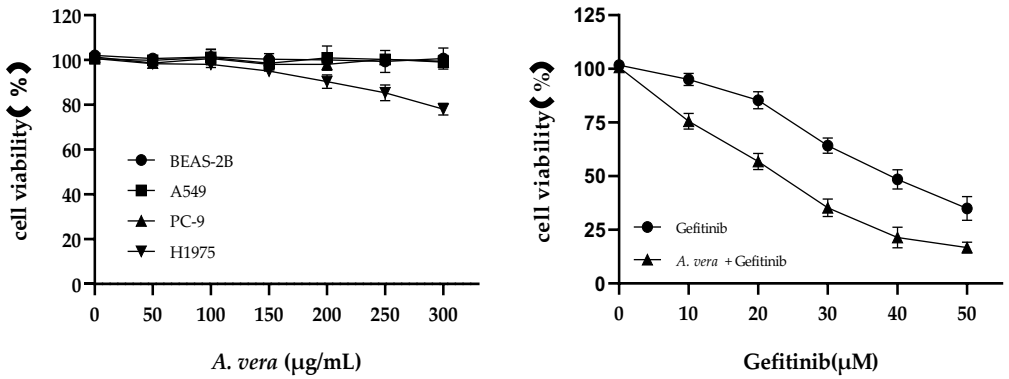
3. Results

3.1. Combination treatment with *A. vera* and gefitinib inhibits NSCLC cell growth

First, to evaluate the inhibitory efficacy of gefitinib or *A. vera*, cell proliferation was assessed in three human NSCLC cell lines. Among these cell lines, A549 cells have wild-type EGFR, PC-9 cells express the EGFR exon 19 deletion (19Del), and H1975 cells harbor the EGFR L858R/T790M mutation. Cells were treated with different concentrations of gefitinib (0-50.0 μM) for 24 h. The results showed that the 50% inhibitory concentration (IC50) values of A549 and H1975 cell lines ranged from near 17 to 27 μM, suggesting that these cells are relatively resistant to gefitinib treatment **(Table 1)**. In addition, after treating cells with different concentrations of *A. vera* (0-300.0 μg/mL) for 24 h, the results showed that *A. vera* ≤ 200.0 μg/mL had no obvious cytotoxic effect on human normal lung epithelial cells and NSCLC cells, and more than 90% of cells survived **(Figure 1a)**.

To assess the combination effects of *A. vera* and gefitinib, nontoxic doses of *A. vera* (200.0 μg/mL) were selected to combine with various doses of gefitinib. Compared to the gefitinib group, a significant loss of cell viability was observed after the combination treatment. *A. vera* increased the inhibitory efficacy of gefitinib with decreases in the IC50 values at 48 h from 8.45 ± 0.88 to 4.59 ± 0.96 μM for PC-9 cells, 17.52 ± 0.47 to 7.03 ± 0.53 μM for H1975 cells, and 26.25 ± 0.48 to 17.62 ± 0.65 μM for A549 cells **(Table 1)**. Of note, the combined treatment inhibited H1975 cell proliferation more effectively than other cell lines, and the IC50 values at 24 h decreased from 37.92 ± 1.19 to 20.09 ± 1.03 μM for H1975 **(Figure 1b and Table 1)**, confirming that *A. vera* could enhance the

inhibitory efficacy of gefitinib in gefitinib-resistant NSCLC cells. Hence, to further explore the combination efficacy, H1975 cells were treated with indicated 10 and 20 μM gefitinib for 24 h in the following experiments, selected based on the IC50 concentration of gefitinib **(Table 1)**.



(a) **(b)**

Figure 1. Cytotoxic effects of gefitinib and *A. vera* on NSCLC cells: (a) Cells were incubated with various doses of *A. vera* for 24 h and cell viability was analyzed using an SRB assay; (b) H1975 cells were treated with *A. vera* (200 μg/mL) combined with different doses of gefitinib for 24 h. Cell viability was examined by SRB assay and absorbance was read at 570 nm. The error bars represent the standard deviation of three independent measurements.

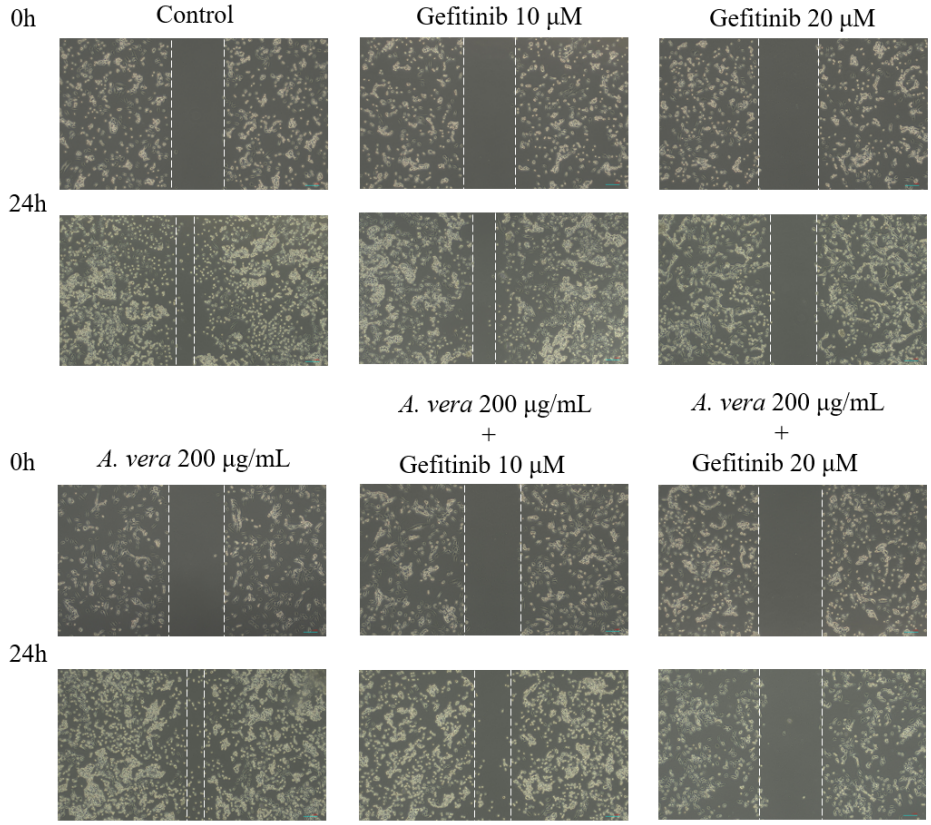
Table 1. Characteristics of NSCLC cell lines used in the study

		Gefitinib		*A. vera* + Gefitinib	
Cell lines	EGFR	IC50		IC50	
		(μM)		(μM)	
		24 h	48 h	24 h	48 h
A549	Wild type	N/A	26.25±0.48	N/A	17.62±0.65*
PC-9	19Del	N/A	8.45±0.88	N/A	4.59±0.96*
H1975	L858R/T790 M	37.92±1.19	17.52±0.47	20.09±1.03**	7.03±0.53**

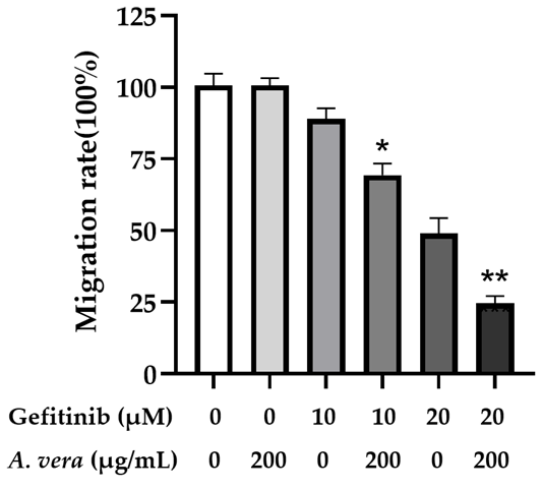
(* $p < 0.05$, ** $p < 0.01$, compared with the gefitinib group).

3.2. Combination treatment with *A. vera* and gefitinib decreases the migration ability of H1975 cells

To examine the inhibitory effect of *A. vera* or gefitinib on the migration of H1975 cells, a scratch wound-healing assay was performed in vitro. Cells were treated with 200 μg/mL *A. vera* alone, 10 or 20 μM gefitinib alone, or a combination treatment for 24 h. Compared to the gefitinib group, exposure to the combination treatment induced a higher percentage of wound closure **(Figure 2a and 2b)**, and the results were presented in a dose-dependent manner. It was indicated that *A. vera* could enhance the inhibitory efficacy of gefitinib on the migration of H1975 cells.



(a)

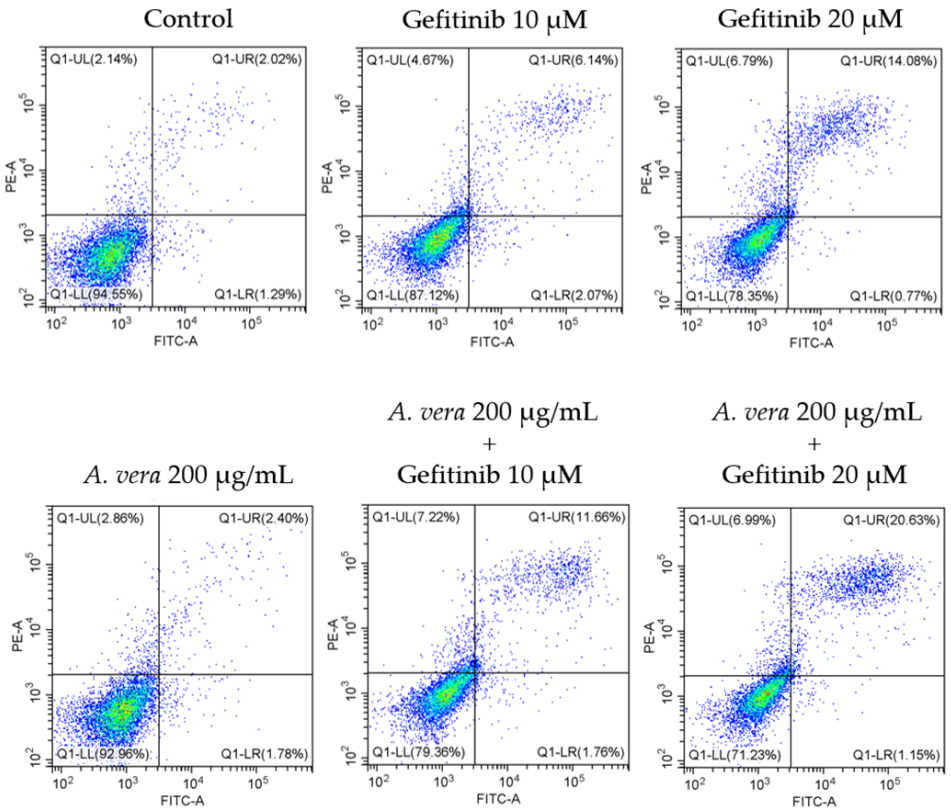


(**b**)

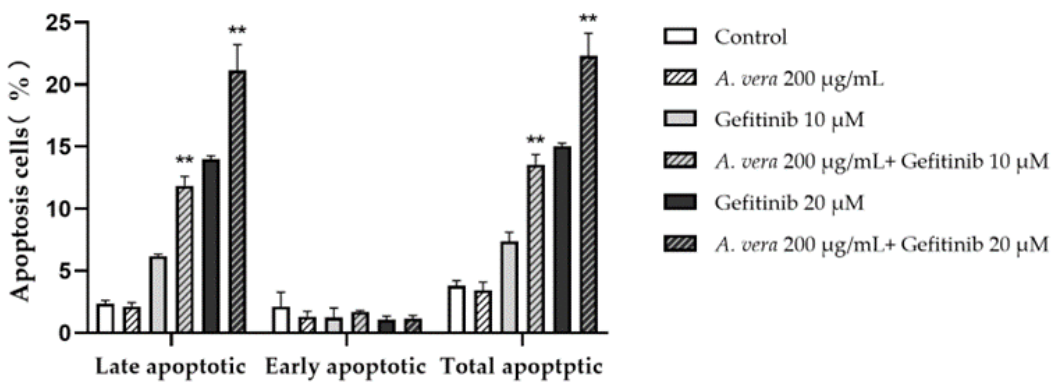
Figure 2. *A. vera* and gefitinib inhibit cell migration in gefitinib-resistant H1975 cells for 24 h. (a) Scratch wound healing assay. (b) Changes in cell motility were assessed by scratch wound healing assay. Error bars indicate the standard deviation of three independent measurements. (∗ $p < 0.05$, ∗ ∗ $p < 0.01$, compared with the Gefitinib group).

3.3. *A. vera* enhances gefitinib-induced apoptosis in H1975 cells

To clarify the mechanism of *A. vera*, cell apoptosis analysis was evaluated after treatment with 200 μg/mL *A. vera*, 10 or 20 μM gefitinib, or the combination. As shown in Figure 3a and 3b, a remarkable increase of total apoptosis was detected after treatment with the combination, compared to the gefitinib group (low-concentration group 8.21% vs. 13.42%, $p < 0.01$; high-concentration group 14.83% vs. 21.78%, $p < 0.05$). These results revealed that *A. vera* enhanced the apoptotic response to gefitinib treatment in H1975 cells.



(**a**)

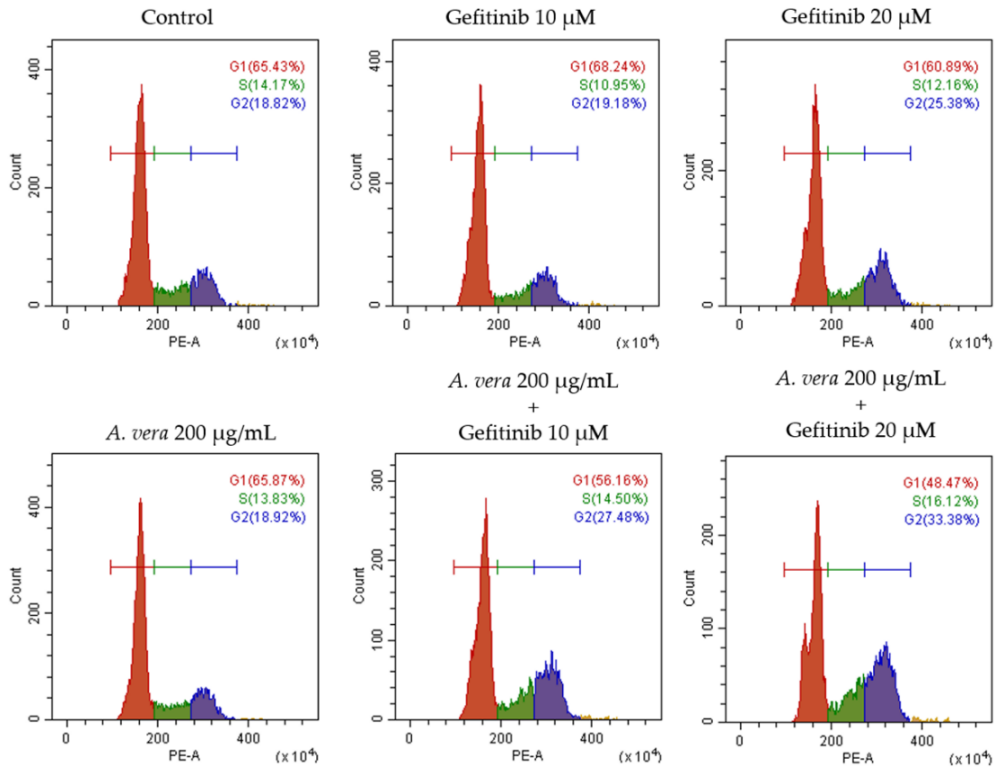


(**b**)

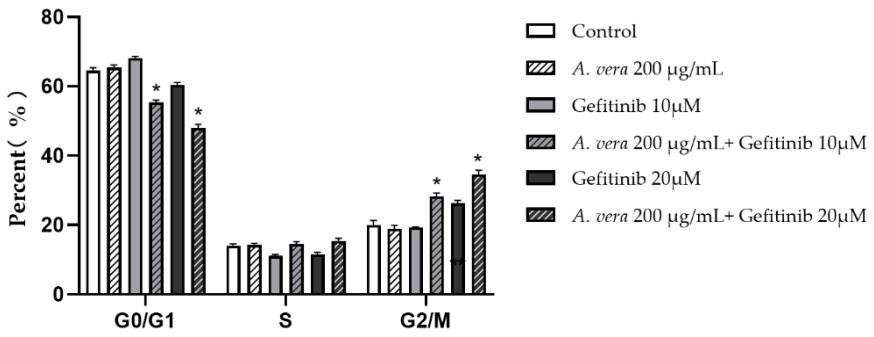
Figure 3. *A. vera* and gefitinib induce the cell apoptosis in gefitinib-resistant H1975 cells for 24h. (a) Representative images of cell apoptosis analysis after the indicated treatment. (b) The graph indicated the average ratio (column) ± SD of cell apoptosis (* $p < 0.05$, ** $p < 0.01$, compared with Gefitinib group).

3.4. Combination of *A. vera* and gefitinib induced G2/M phase arrest in H1975 cells

To investigate the anticancer efficacy of gefitinib on H1975 cells, cell cycle was measured by flow cytometry with propidium iodide (PI) staining. Cells were treated with *A. vera*, gefitinib or the combination for 24 h. As shown in Figure 4a and 4b, compared to the gefitinib group, the combination group in H1975 cells resulted in a marked decrease in the G0/G1 phase (low-concentration group 68.24% vs. 56.16%, $p < 0.05$; high-concentration group 60.89% vs. 48.47%, $p < 0.05$), and a remarkable increase in the G2/M phase (low-concentration group 19.18% vs 27.48%, $p < 0.05$; high-concentration group 25.38% vs 33.38%, $p < 0.05$). The results showed that the combination of *A. vera* and gefitinib induced G2/M phase arrest in H1975 cells, suggesting that *A. vera* enhanced the effect of gefitinib-induced cell cycle arrest in H1975 cells.



(a)



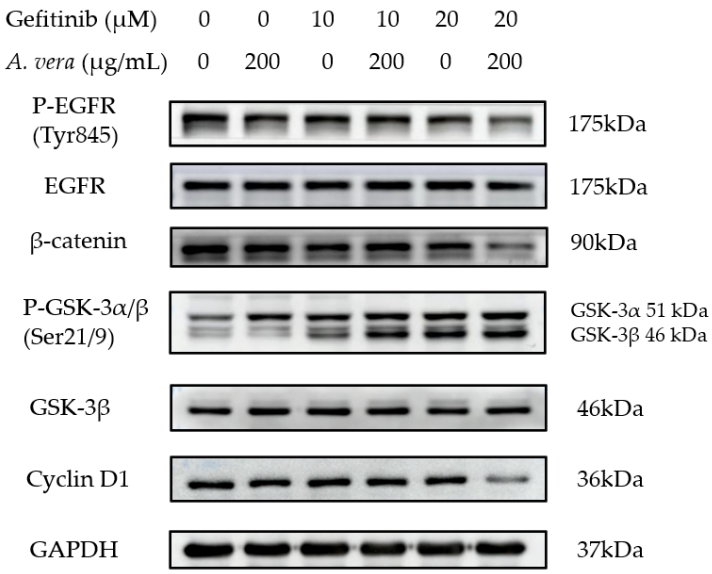
(**b**)

Figure 4. *A. vera* and gefitinib induce the G2/M phase cell cycle arrest in gefitinib-resistant H1975 cells for 24 h. (a) Representative images of cell cycle analysis after the indicated treatment in H1975 cells. (b) The graph demonstrated the average ratio (column) ± SD at different phases. (∗ $p < 0.05$, compared with Gefitinib group).

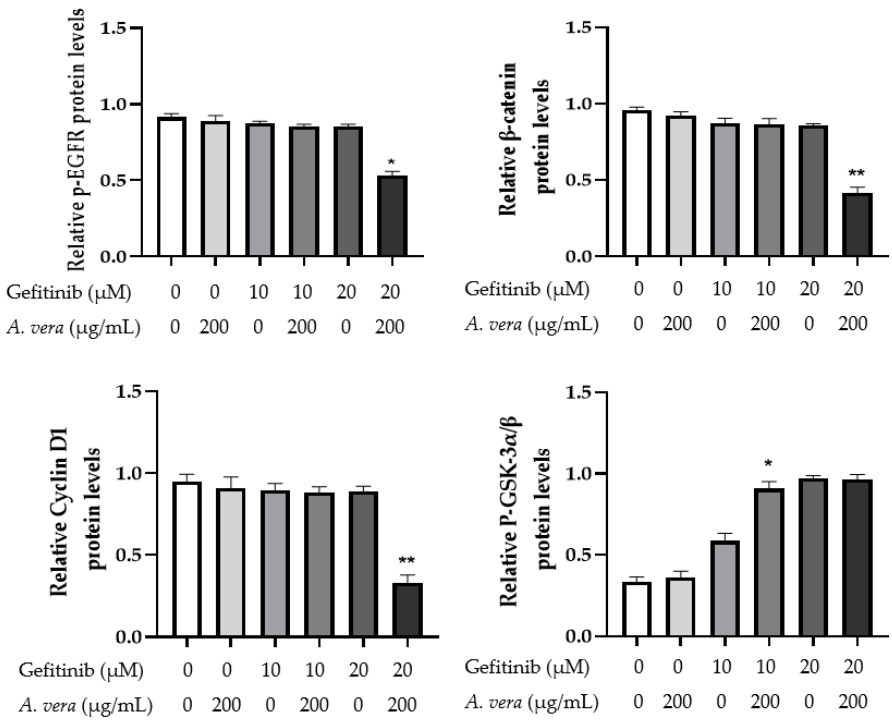
3.5. Combination of *A. vera* and gefitinib inhibits the Wnt/β-catenin signaling in H1975 cells

To further understand the mechanism of effect of the combination, the key genes of Wnt/β-catenin signaling were examined by Western blotting experiments. Cells were treated with 200 μg/mL *A. vera*, 10 or 20 μM gefitinib, or the combination. Figures 5a and 5b showed that the high-concentration (20 μM gefitinib) combination group effectively induced the downregulation of β-catenin signal, compared to gefitinib group ($p < 0.01$). Simultaneously, the expression level of phospho-EGFR protein was also downregulated after high-concentration combination treatment ($p < 0.05$). Furthermore, an increase in the level of phospho-GSK3β was observed in H1975 cells after exposure to the combination ($p < 0.05$). Cyclin D1 was detected, as an important downstream factor of the Wnt/β-catenin signaling, and an obvious decrease was observed in the high-concentration combination group ($p < 0.01$). It is worth noting that, compared to the low-concentration combination group (10 μM gefitinib), the high-concentration combination group (20 μM gefitinib) was significantly inhibited following exposure to the increasing concentrations of gefitinib. Taken together, these results demonstrated that the

combination of *A. vera* and gefitinib could inhibit the Wnt/β-catenin signaling in H1975 cells.



(**a**)



(**b**)

Figure 5. Detection of the Wnt/β-catenin pathway in H1975 cells. (a) The levels of Wnt/β-catenin pathway proteins were assessed by Western blotting, and GAPDH was used as a loading control. (b) Relative expression levels of Wnt/β-catenin pathway proteins. (* $p < 0.05$, ** $p < 0.01$, compared with the Gefitinib group).

4. Discussion

To date, the first-generation EGFR-TKI monotherapy is still the standard of first-line treatment for EGFR-mutated NSCLC, despite the third-generation TKIs being preferred. However, almost all patients who respond well to TKIs eventually develop acquired resistance to them, leading to the progression of their disease.

It is imperative that combination therapies continue to be utilized in light of the emergence and presence of drug resistance. In this study, our data demonstrated that the combination of gefitinib and *A. vera* could enhance the sensibility to gefitinib in H1975 cells. In the presence of *A. vera*, a reduced IC50 for gefitinib was observed in NSCLC cells expressing both wild-type EGFR and various types of EGFR mutations, suggesting that *A. vera* might make a wide range of NSCLC cells sensitive to gefitinib treatment. Furthermore, the suppression of cell proliferation and migration confirmed that *A. vera* could enhance the anticancer effects of gefitinib. Interestingly, despite the same concentration as the combination group, single *A. vera* treatment showed approximately non-toxic effects to cells, indicating that gefitinib may be the main force while *A. vera* was the assistant in combination treatment.

The molecular causes of first-generation EGFR-TKI resistance are only incompletely understood. EGFR activates several signal transduction pathways involved in cell survival and proliferation. EGFR-mutated cancers may become resistant to gefitinib or erlotinib if the activity of the downstream signaling pathways was maintained. Importantly, the major resistance mechanism is via the secondary mutation in EGFR at position 790 (T790M), which decreases the relative TKI binding to EGFR kinase domain,

leading to TKI resistance.[20] Therefore, developing inhibitors specific to EGFR-T790M or inhibitors of downstream targets of T790M may be a breakthrough point to study the resistance.

β-catenin is a key component of Wnt/β-catenin signaling, and is involved in malignant tumors pathogenesis and progression including in lung cancer.[21] It was found that NSCLC cell lines carrying EGFR mutations exhibited high levels of β-catenin expression in comparison with wild-type EGFR gene.[22] A previous article has reported that a pharmacological inhibition of T790M mutations of EGFR can suppress tumor growth in patients with EGFR-L858R, and a genetic deletion of the β-catenin gene reduces lung tumor formation in transgenic mice with EGFR-L858R-T790M.[23] These results suggest that β-catenin is closely related to the resistance induced by T790M mutation in NSCLC. Of note, several preclinical studies have shown that the inhibition of the β-catenin signal enhances the sensitivity to EGFR-TKIs in H1975 cells harboring the T790M mutation, which may influence curative effect of EGFR-TKI therapy through bypassing the EGFR stimulus or its downstream signaling.[24] Hence, there might be potential to prevent or overcome resistance to EGFR-TKIs by regulating β-catenin signaling.

Furthermore, it has been mentioned that the combination of β-catenin inhibitors and EGFR-TKIs significantly decreases the phosphorylation of EGFR and inhibits the β-

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20 Ariyasu R, Nishikawa S, et al, "High Ratio of T790M to EGFR Activating Mutations Correlate with the Osimertinib Response in Non-small-cell Lung Cancer," *Lung Cancer* 117 (2018): 1-6.

21 Anastas J N, Moon R T, "WNT Signalling Pathways as Therapeutic Targets in Cancer," *Nature reviews Cancer* (2013).

22 Nakayama S, Sng N, et al, "β-catenin Contributes to Lung Tumor Development induced by EGFR Mutations," *Cancer Research* 74 (2014).

23 Togashi Y, Hayashi H, et al, "Inhibition of β-Catenin Enhances the Anticancer Efect of Irreversible EGFR-TKI in EGFR-mutated Non-small-cell Lung Cancer with a T790M Mutation," *Journal of Thoracic Oncology* 10 (2015): 93-101.

24 Fang X, Gu P, et al, "β-Catenin Overexpression is Associated with Gefitinib Resistance in Non-small Cell Lung Cancer Cells," *Pulmonary Pharmacology & Therapeutics* (2014), 28: 41-48; Casás-Selves M, Kim J, et al, "Tankyrase and the Canonical Wnt Pathway Protect Lung Cancer Cells from EGFR Inhibition," *Cancer Research* 72 (2012): 4154-4164.

catenin signal in H1975 cells, compared with the use of EGFR-TKIs alone, suggesting that EGFR may interact with β-catenin signal.[25] Previous research has demonstrated that *A. vera* can modulate Wnt/β-catenin pathway with wnt3a ligands in human embryonic kidney HEK293 cells and colorectal cancer cells.[26] However, the impacts of *A. vera* on Wnt/β-catenin pathway in H1975 cells remain unclear. Thus, the efficacy of the combination with gefitinib and *A. vera* on β-catenin and EGFR protein levels was examined in this study, compared with the use of gefitinib or *A. vera* treatment alone. The results indicated that inhibition of either β-catenin or EGFR alone had a minimal influence, while the concurrent inhibition of both was obviously effective. It was also demonstrated that there might exist an interaction between EGFR and the β-catenin signal, which was consistent with the literature.

In addition, our study detected the expression levels of the key proteins of Wnt/β-catenin signaling and found that the phospho-GSK3β expression and Cyclin D1 expression were also downregulated. Remarkably, a significant decrease in protein levels was observed only in the combination group with high concentrations of the drug, while the low-concentration group had minimal effect, suggesting that the inhibitory effect of *A. vera* on β-catenin signal was enhanced with increasing gefitinib concentration. It is necessary to increase the concentration or extend the time of administration in further studies. Hence, *A. vera* could be a potential therapeutic adjuvant for gefitinib-resistant NSCLC with the EGFR-T790M mutation.

Recently, a growing number of studies have found that Chinese herbal medicine may

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[25] Togashi Y, Hayashi H, et al, "Inhibition of β-Catenin Enhances the Anticancer Effect of Irreversible EGFR-TKI in EGFR-mutated Non-small-cell Lung Cancer with a T790M Mutation," *Journal of Thoracic Oncology* 10 (2015): 93-101.

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be useful in cancer therapy for its effects on multidrug resistance.[27] Herbal medicines are generally inexpensive, plentiful, and show little toxicity or side effects in clinical application.[28] Traditional Chinese medicine therapy, as an adjunctive therapy, has been shown to significantly reduce adverse postradiation reactions and improve quality of life in NSCLC.[29] However, these results are based only on in vitro experiments in this research, and further studies are needed to clarify detailed regulatory mechanisms of NSCLC cells in relation to the therapeutic effects of combining a first-generation TKI with *A. vera*.

5. Conclusion

In summary, these results suggested a potential clinical impact of the therapeutic strategy with aresistance. These data showed that *A. vera* enhanced the anticancer activity of gefitinib by modulating Wnt/β-catenin pathways in H1975 cells, which indicated that *A. vera* might be a potential anticancer therapeutic adjuvant for NSCLC patients receiving TKI treatment as a first-line therapy.

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Visualization, Formal analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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