Cancer Management and Research

Elevate Series

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ORIGINAL RESEARCH

Jujuboside B Inhibits the Proliferation and Migration of Non-Small Cell Lung Cancer H1299 Cells Through Inhibiting PI3K/Akt and Wnt/β-Catenin Pathways

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Background: Lung cancer has high incidence and mortality rates. In consideration of the high toxicity and side effects of traditional chemical drugs and drug resistance, researchers have substituted some natural products for anti-lung cancer drugs. Ziziphi spinosae semen (ZSS) is a famous traditional Chinese herb, and ZSS-based formulas have been used against lung cancer in clinic, such as Xiaoyi Sanjie Formula. Jujuboside B (JUB) is one of the main bioactive components in ZSS; however, JUB's roles in the treatment of lung cancer, and the mechanism have not been well reported.

Objective: This study intends to evaluate the effects of JUB on the proliferation and migration of non-small cell lung cancer H1299 cells (NC-1299 cells), as well as the potential related regulatory mechanism.

Methods: The viability, migration and invasive ability of NC-1299 cells were analyzed by CCK8, wound-healing, transwell invasion, and the expression of proteins was analyzed through Western blotting experiments.

Results: It showed that 160 and 320 μmol/L JUB significantly inhibited the proliferation of NC-1299 cells, reduced the cell migration in the wound healing test, and decreased the cell number of clonal assay ($P < 0.05$). JUB also significantly inhibited the expression of Vimentin, MMP2, and MMP9 proteins related to the migration and invasion of tumor cells, as well as inhibited the PI3K/AKT and Wnt/β-catenin pathways.

Conclusion: The results indicate JUB has a significant inhibitory effect on NC-1299 cells, and such inhibitory effect is associated with inhibiting the expression of migration and invasive proteins such as MMP2, as well as inhibiting the PI3K/AKT and Wnt/β-catenin pathways. The findings provide some references for the application of JUB in the substitution of anti-lung cancer drugs.

Keywords: Jujuboside B, non-small cell human lung cancer NC-1299 cells, proliferation, migration, PI3K/AKT, Wnt/β-catenin

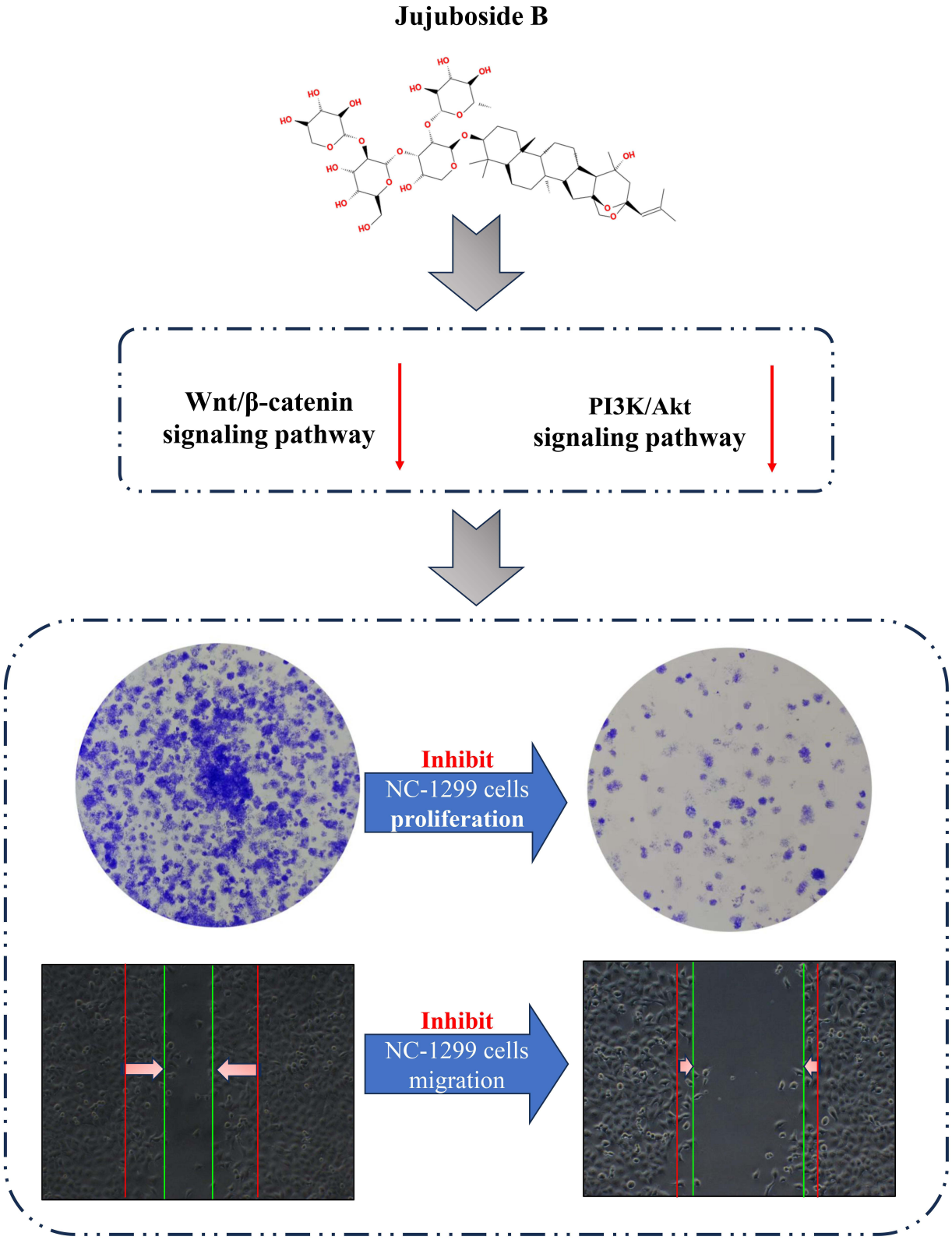
Introduction

Lung cancer is a common type of cancer with a high incidence and mortality rate.[1] In addition, lung cancer is often the direct cause of death when cancer from other organs spreads and metastasizes to the lungs.[2] In clinical practice, non-small cell lung cancer is the main type of lung cancer, and the common treatment options are radiotherapy,[3] chemotherapy,[4] immunotherapy[5] and surgical resection.[6] However, the above treatment options are highly restrictive to the patient's cancer stage and physical status, for example, surgery is not feasible for advanced or metastatic lung cancers, and radiotherapy and chemotherapy are too damaging to the body to be carried out if the patient is unable to withstand too much physical and mental pain. In addition, the drug resistance problem also restricts the efficacy of traditional anti-lung cancer drugs.[7] Therefore, there is an urgent need and practical significance to find new treatment strategies for new lung cancers. At present, targeted inhibition of pathways that are closely related to tumor cell migration and invasion and other vitality has become a popular solution to fight tumors.[8,9]

Studies have shown that lung cancer is closely related to the Wnt/β-catenin signaling pathway.[10] Key components of the pathway, Wnt1, β-catenin, and cell cycle protein D1 show obvious disordered expression, and abnormal hyperactivation of the Wnt/β-catenin pathway is associated with tumor metastasis, migration, invasion, and chemotherapy resistance, and it promotes the occurrence and development of lung cancer.[11] Meanwhile, PI3K/AKT pathway also plays a key role

Received: 4 March 2025
Accepted: 5 June 2025
Published: 16 June 2025

Graphical Abstract



in the development of lung tumors, and this pathway was often hyperactivated in lung cancer due to some components of it disordered.[12] For the above targeted pathways, the traditional strategy is to inhibit or block the pathway with chemical drugs such as betalain,[13] but chemical drugs have side effects such as high biotoxicity and drug resistance for prolonged use, which limits their use. Recent studies have shown that the use of natural products that can target the aforementioned pathways as an alternative to chemical drugs offers a new possibility against lung cancer, such as curcumin,[14] nobiletin,[15] and lycorine.[16]

Ziziphi spinosae semen is a traditional Chinese medicine, and it is one of the main ingredients of the Xiaoyi Sanjie formula that used for the treatment of lung cancer. Jujuboside B (JUB), a natural product saponin, is one of the main bioactive components in Ziziphi spinosae semen.[17] Studies have shown that JUB can exert anti-tumor effects through the PI3K/AKT and Wnt/β-catenin pathways,[18,19] but its resistance to lung cancer has not been reported. Human non-small cell lung cancer H1299 cells (NC-1299 cells) are often used as a cell model for in vitro investigation of non-small cell lung cancer. In this study, we proposed to treat NC-1299 cells with JUB firstly, and then comprehensively assess the inhibitory effect of JUB on NC-1299 cancer cells and related mechanisms by determining the tumor cell viability, migration and invasion ability, as well as the expression of Wnt/β-catenin and PI3K/AKT pathways, which may provide some reference bases for JUB application in the treatment of lung cancers.

Materials and Methods

Cell Culture and Reagents

NC-1299 cells were got from Peking Union Medical College Cell Bank (Beijing, China), and maintained at 37 °C, 5% CO_2 condition. JUB was got from Aladdin Biochemical Technology Co., Ltd (Shanghai, China), No. J114069. Cell Counting Kit-8 (CCK-8 Kit) was got from Beyotime Biotechnology (Shanghai, China), No. C0038. Antibodies for Cyclin D1 (No. ab16663) were got from Abcam company (Cambridge, UK); antibodies for E-cadherin (No. A20798), Vimentin (No. A19607) were got from ABclonal Technology (Wuhan, China); antibodies for MMP2 (No. bs0412R), MMP9 (No. bs4593R), p-PI3K (No. bs5570R), PI3K (No. bs10657R) were got from Bioss company (Beijing, China); antibodies for p-AKT (No. mAb 4060), AKT (No. mAb2118), β-catenin (No. mAb9562), c-Myc (No. mAb5605), GAPDH (No. mAb2118) were got from Cell Signaling Technology (Danvers, MA, USA).

Cytotoxicity Analysis Through CCK8 Assay

The cytotoxicity of JUB on NC-1299 cells was assessed using the CCK8 kit. Briefly, a density of 1×10^{3} cells were cultured in 96-well plates, per well, and treated with different concentrations of JUB (0, 10, 20, 40, 80, 160 and 320 μmol/L) for 24 or 48 h. Then, CCK8 was added in the wells and NC-1299 cells were incubated for another 4 h. Subsequently, the optical density (OD) value of each well was analyzed using a Varioskan™ LUX microplate reader (Thermo Fisher Scientific, Massachusetts, USA) at 450 nm. The cell survival rate and the cell inhibition rate were calculated based on the OD values. The half inhibitory concentration (IC50) of JUB on NC-1299 cells was analyzed using GraphPad Prism 8.0 software, based on the drug concentration and the corresponding calculated inhibition rate of each group. The IC50 value was 65.03 μmol/L and 55.27 μmol/L, for 24 h and 48 h, respectively.

$$\text{Survival rate}(\%) = \frac{\text{OD of drug group} - \text{OD of blank group}}{\text{OD of control group} - \text{OD of blank group}} \times 100$$

$$\text{Inhibition rate}(\%) = \frac{\text{OD of control group} - \text{OD of drug group}}{\text{OD of control group} - \text{OD of blank group}} \times 100$$

Wound Healing Assay

About 1×10^{5} cells per well were cultured in 6-well plates and treated with different concentrations of JUB (0, 80, 160 and 320 μmol/L). A 200 μL pipette tip was applied to create wound through scratching cells in a straight line, and an inverted microscope imaging system (IX-51, Olympus, Japan) was used to taken images of the wound scratch immediately. The

cells were cultured for another 48 h, and the wound scratch images at 48 h were taken with the aforementioned microscope imaging system again. The migration rate of cells was calculated with the following formula:

$$\text{Migration rate}(\%) = \frac{\text{Scratch width at 0 h} - \text{Scratch width at 48 h}}{\text{Scratch width at 0 h}} \times 100$$

Colony Formation Assay

About 5×10^2 cells per well were cultured in 6-well plates and treated with different concentrations of JUB (0, 80, 160 and 320 μmol/L). After 2 weeks culture, the cells were then fixed with 4% paraformaldehyde for 30 min, and they were further stained with crystal violet for 10 min. And the images of cells were taken with an inverted microscope imaging system (IX-51, Olympus, Japan), and further analyzed with ImageJ software (National Institutes of Health, Bethesda, USA).

Transwell Invasion Assay

The invasion assay was performed according to an earlier research.[20] Briefly, transwell chambers coated with Matrigel were used for invasion assays. NC-1299 cells were pretreated with JUB (0, 80, 160 and 320 μmol/L) for 48 h. Then, 5×10^4 NC-1299 cells that were resuspended with 100 μL serum-free medium were seeded in the upper chamber, and 600 μL of medium containing 10% FBS was added to the lower chamber as a chemoattractant. The cells then were incubated at 37°C for 24 h and invaded into the lower chamber, and they were fixed with 4% paraformaldehyde for 20 min, and stained with 0.1% crystal violet for 30 min. And 5 randomly selected fields were used to count the number of cells.

Western Blotting Assay

NC-1299 cells were cultured in 6-well plates and treated with different concentrations of JUB (0, 80, 160 and 320 μmol/L) for 48 h. Cells from all groups were collected and the total proteins of cells were obtained, and the concentration of protein samples was calculated with bicinchoninic acid method (BCA). The SDS-PAGE gel was then used to separate the proteins which were further transferred to PVDF membrane. After incubating with 5% nonfat milk for 1 h, the membranes were further incubated with primary antibody at 4°C overnight, secondary antibody for 1 h, respectively. The protein brands were visualized with ECL luminescent solution, and the brand density was evaluated with Image Lab software (Bio-Rad, Richmond, CA, USA).

Statistical Analysis

Data were statistically analyzed with SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance was used for comparison of difference, and $P < 0.05$ was considered as statistically significant difference.

Results

JUB Reduces the Cell Viability, Migration, Invasion, Proliferation of Human Lung Cancer NC-1299 Cells

The CCK-8, wound healing, and colony formation assays were used to evaluating the effects of JUB on the cell viability and proliferation of NC-1299 cells. Figure 1 illustrated that 20, 40, 80, 160 and 320 μmol/L JUB could remarkably inhibit NC-1299 cells both at 24 and 48 h, especially when the dose of JUB were 80, 160 and 320 μmol/L (Figure 1, $P < 0.05$). Hence, the aforementioned three doses were chosen for subsequent scratch wound healing, and colony formation assays. Figure 2 illustrated that the migration and invasion ability of NC-1299 cells was remarkably inhibited by JUB, as the scratch width (Figure 2A) was wider and the migration rate was remarkably reduced in the JUB groups (Figure 2B, $P < 0.05$), and the invasion rate was remarkably reduced in the JUB groups (Figure 2C and D, $P < 0.05$). Figure 3 illustrated that JUB could reduce the clonal formation of NC-1299 cells as the cell number was remarkably decreased in the JUB groups (Figure 3A and B, $P < 0.01$). Taken together, these findings indicated JUB could reduce the cell viability, migration, invasion, proliferation of NC-1299 cells.

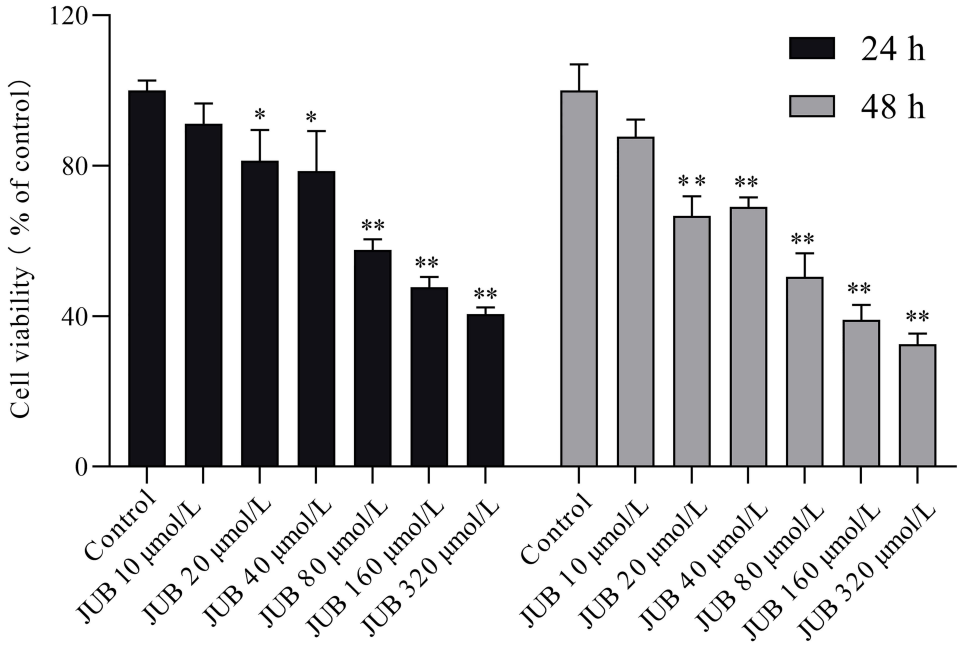


Figure 1 Effects of JUB on cell viability of NC-1299 cells. The relatively cell viability of NC-1299 cells after treated with different concentrations of JUB (0, 10, 20, 40, 80, 160 and 320 μmol/L) for 24 or 48 h. Data are represented as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ vs Control group.

JUB Inhibits the Protein Expression Associated with Tumor Cell Migration and Invasion in NC-1299 Cells

Tumor migration and invasion ability is an important indicator for assessing the viability and damage of tumor cells. Researches have shown that E-cadherin, Vimentin, MMP2, and MMP9 proteins are closely related to tumor migration and invasion.[21–23] Figure 4 illustrated that the expression of these proteins changed significantly when the JUB concentration was 160 or 320 μmol/L, especially at 320 μmol/L, as the level of Vimentin, MMP2, and MMP9 was remarkably down-regulated in the JUB 160 and 320 groups (Figure 4A–E, $P < 0.05$). Taken together, these findings indicated JUB could inhibit the protein expression associated with tumor cell migration and invasion in NC-1299 cells.

JUB Inhibits the PI3K/AKT Signaling Pathway in NC-1299 Cells

The abnormal activation of PI3K/AKT signaling pathway was a common phenomenon in tumors,[24] and high phosphorylation ratios of key proteins are important signs of activation of this pathway. Figure 5 illustrated that the phosphorylation ratio of PI3K and AKT proteins was remarkably down-regulated in the JUB 160 and 320 groups (Figure 5A–C, $P < 0.05$). Taken together, these findings indicated JUB could inhibit the PI3K/AKT signaling pathway in NC-1299 cells.

JUB Inhibits the Wnt/β-Catenin Signaling Pathway in NC-1299 Cells

The Wnt/β-catenin signaling pathway is closely associated with tumors, and suppression of this pathway was a common strategy against cancers.[25,26] And c-Myc, Cyclin D1, β-catenin are three key proteins in the Wnt/β-catenin pathway, Figure 6 illustrated that the level of the aforementioned key proteins was remarkably down-regulated in the JUB 160 and 320 groups (Figure 6A–D, $P < 0.05$). Taken together, these findings indicated JUB could inhibit the Wnt/β-catenin signaling pathway in NC-1299 cells.

Discussion

Consistent with the conjecture of this study, the results illustrated that JUB exhibited significant inhibitory effects on NC-1299 lung cancer cells, as 160 and 320 μmol/L JUB significantly inhibited the proliferation, invasive and migratory abilities of NC-1299 cells. In the present study, JUB, the main active ingredient in Ziziphi spinosae semen, was

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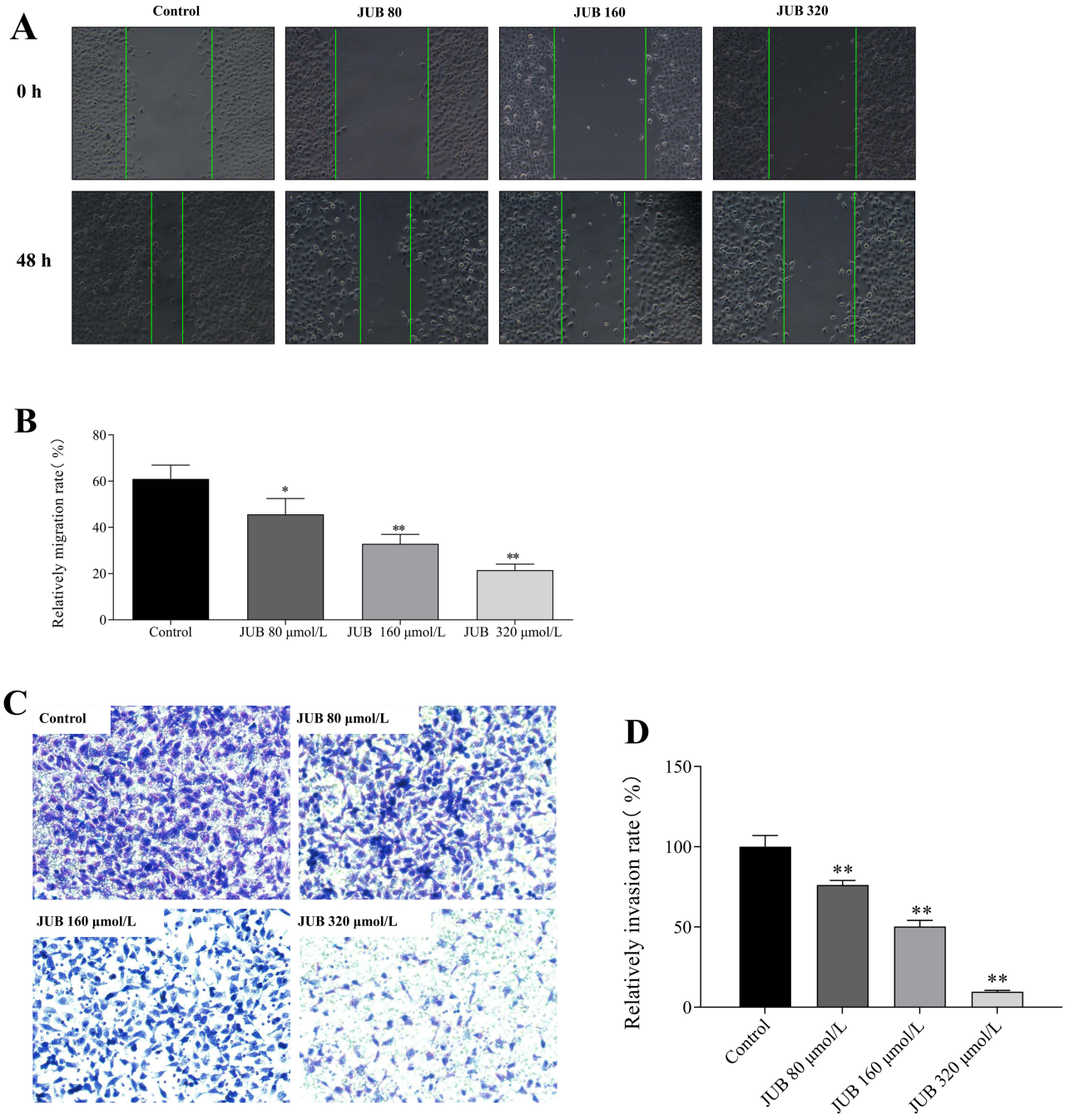

Figure 2 Effects of JUB on cell migration and invasion ability of NC-1299 cells. (**A**) Representative images of cell migration; (**B**) Relatively migration rate of NC-1299 cells; (**C**) Representative images of cell invasion; (**D**) Relatively invasion rate of NC-1299 cells. Data are represented as mean ± standard deviation. **P* < 0.05, ***P* < 0.01 vs Control group.

investigated, and its results indicated that JUB is one of the key components in the anticancer process of Ziziphi spinosae semen. These findings provide some reference bases and research directions for the subsequent in-depth investigation of the anticancer mechanism of Ziziphi spinosae semen.

The PI3K/AKT and Wnt/β-catenin pathways are two critical pathways that are frequently involved in cancer,[27,28] and they are often transitionally activated in cancer cells, resulting in enhanced proliferation, migration, and invasion of tumor cells.[29] Therefore, the development of novel anticancer drugs is often pre-screened on the basis of their ability to specifically target and inhibit these two pathways.[30,31] Based on this, many natural products with anticancer potential

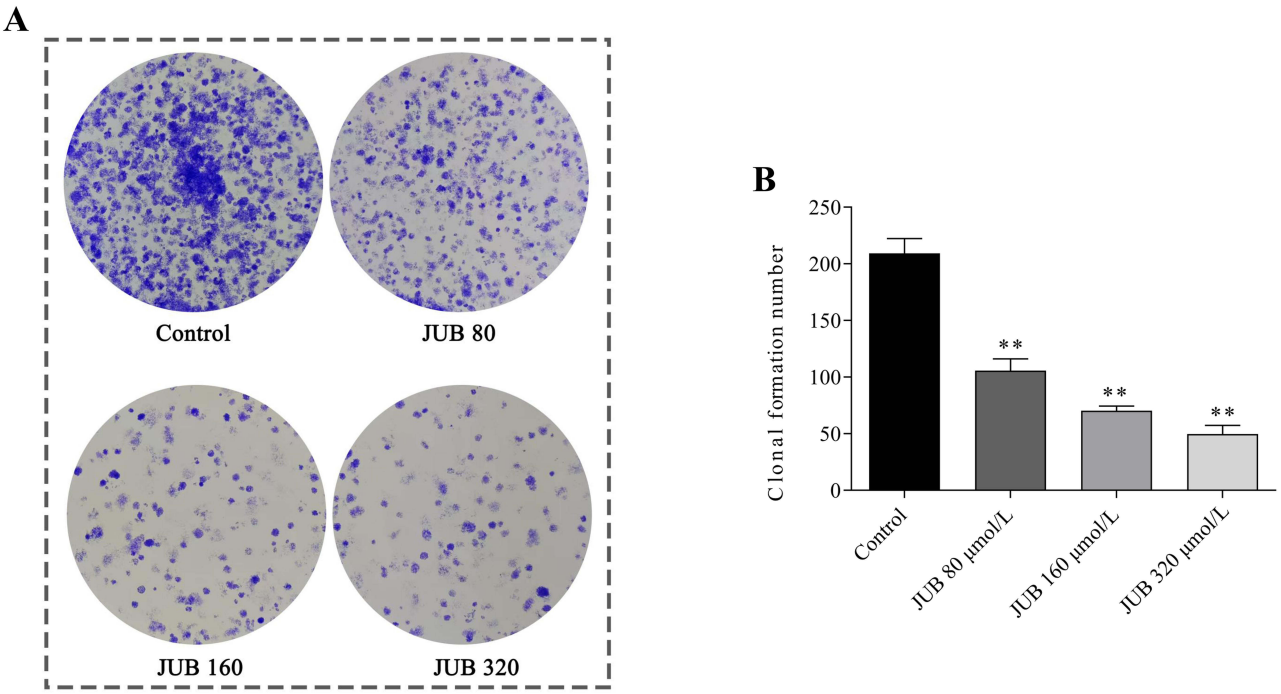

Figure 3 Effects of JUB on cell proliferation ability of NC-1299 cells. (**A**) Representative images of cell proliferation; (**B**) Clonal formation number of NC-1299 cells. Data are represented as mean ± standard deviation. $^{**}P < 0.01$ vs Control group.

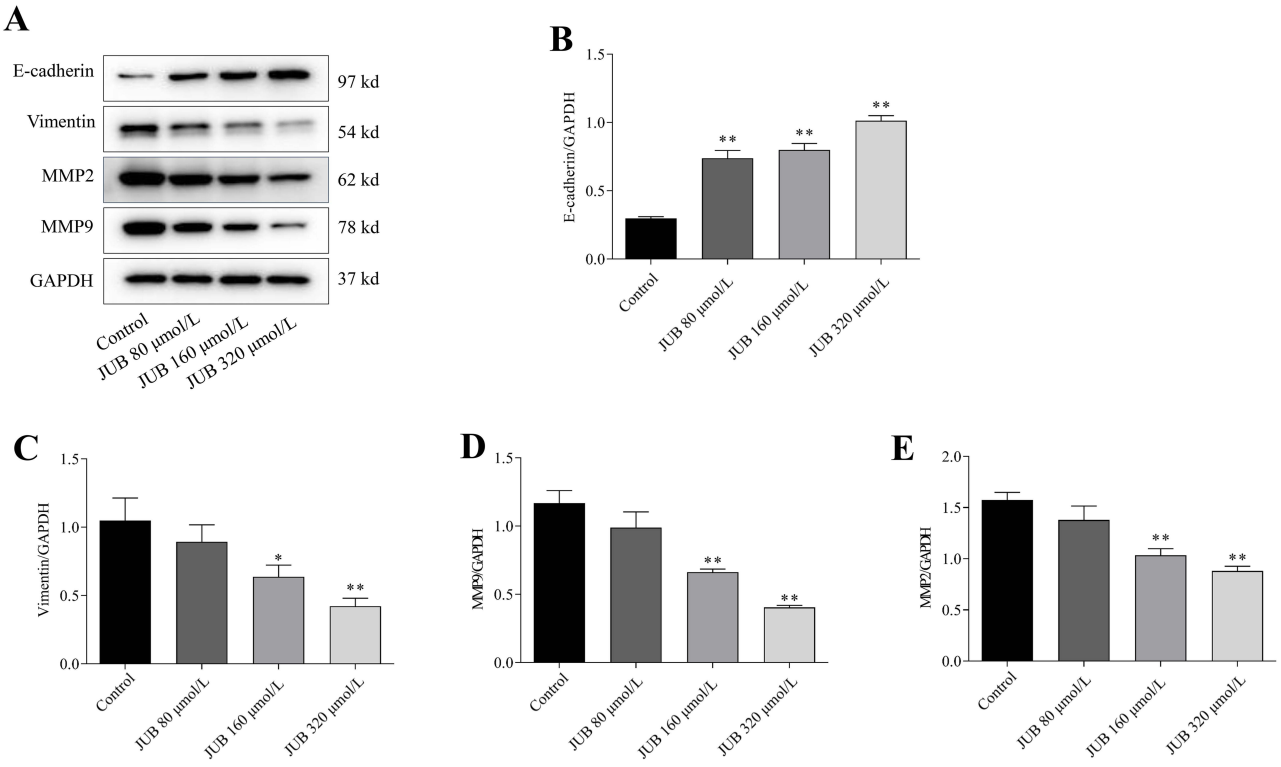

Figure 4 Effects of JUB on the protein expression associated with tumor cell migration and invasion in NC-1299 cells. (**A**) Protein band images of Western blotting experiment; Expression analysis of (**B**) E-cadherin, (**C**) Vimentin, (**D**) MMP2, and (**E**) MMP9 proteins. Data are represented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs Control group.

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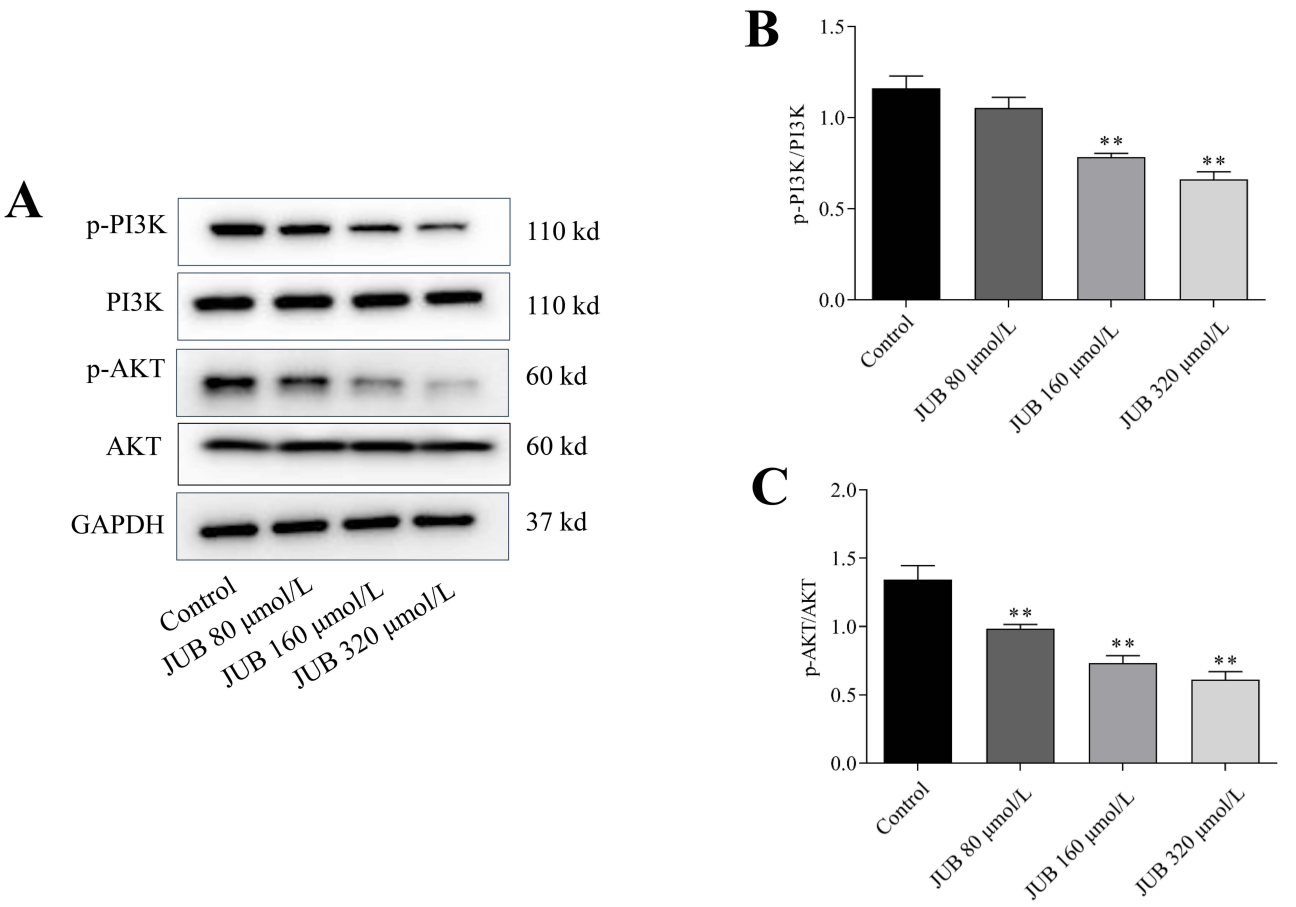

Figure 5 Effects of JUB on PI3K/AKT pathway in NC-1299 cells. (**A**) Protein band images of Western blotting experiment; Expression analysis of Phosphorylated (**B**) PI3K and (**C**) AKT proteins. Data are represented as mean ± standard deviation. $^{**}P < 0.01$ vs Control group.

have been discovered, and evidences showed that flavonoids berberine,[32] marmesin,[33] and dynactin[34] all can target the above two key pathways to inhibit cancer. The results of the present study showed that the anticancer effects of JUB were also associated with targeting and inhibiting the PI3K/AKT and Wnt/β-catenin pathways, which also laterally corroborates the value of PI3K/AKT and Wnt/β-catenin in screening for new anticancer drugs.

Notably, although the present study initially demonstrated the anti-lung cancer properties and potential mechanisms of JUB at the cellular level, there are still many limitations. First, the current works did not further explore which proteins in the pathways play the most critical regulatory functions. Subsequent studies will focus on evaluating the key factors in the PI3K/AKT and Wnt/β-catenin pathways with the help of pathway blockers,[35] protein inhibitors,[36] and protein overexpression systems,[37] in order to better utilize the anti-tumor properties of JUB. Secondly, this study did not conduct in vivo animal experiments, but only in vitro validation, which means that there is still a lot of work to be supplemented for the clinical application of JUB, such as clarifying the dosage, suitable dosage form, and route of administration in vivo, etc., and we will also focus on the related content in the subsequent work. In addition, there are other active ingredients in Ziziphi spinosae semen, eg, Sanjoinine,[38] etc., and their effects and mechanisms in the process of anti-tumor are also worth studying, which will provide more comprehensive references for the clinical application of Ziziphi spinosae semen in the treatment of lung cancer.

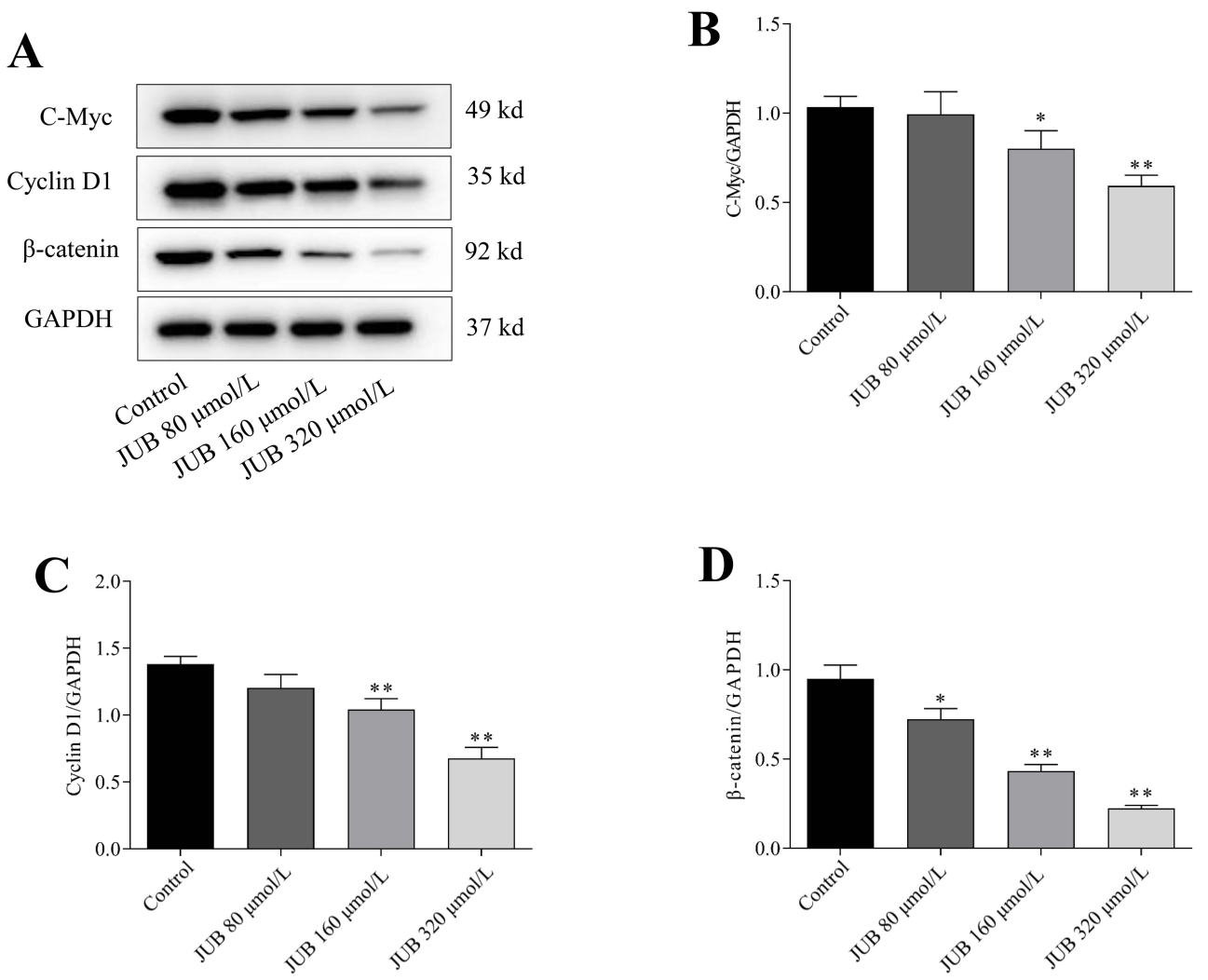

Figure 6 Effects of JUB on Wnt/β-catenin pathway in NC-1299 cells. (**A**) Protein band images of Western blotting experiment; Expression analysis of (**B**) c-Myc, (**C**) Cyclin D1 and (**D**) β-catenin proteins. Data are represented as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$ vs Control group.

Conclusion

In conclusion, this study revealed the inhibitory effect of JUB on the proliferation and migration of NC-1299 lung cancer cells and found that it was associated with the inhibition of PI3K/AKT and Wnt/β-catenin pathways. These findings offer a partial reference for the application of JUB and Ziziphi spinosae semen as alternative drugs against lung cancer in the clinic, as well as provide some evidence for the use of Ziziphi spinosae semen as a homology of medicine and food in the prevention and treatment of lung cancer.

Acknowledgments

The authors thank the Affiliated Hospital to Changchun University of Chinese Medicine for the supporting.

Funding

The present study was supported by the Science and Technology Development Plan item from the Science and Technology Department of Jilin Province (YDZJ202301ZYTS459).

Disclosure

There is no conflict of interest to declare.

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