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Study the inhibitory effect and mechanism of the ethanol extract of deziyangxin on LLC cells

Yi Yun [a], Jiang Yahui [b], Bai Bobo [a], Zhang Caifeng [a], Zhao Yanli [a,*]

[a] Medical College, Qinghai University, No. 29 Tongren Road, Xining, 810001, Qinghai, China
[b] Ocean University of China Infirmary, Qingdao, 266100, Shandong, China

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ABSTRACT

Background: Chinese Tibetan medicine plays a crucial role in complementary anti-tumor treatments. This article aims to investigate the inhibitory effect of the alcoholic extract of Tibetan medicine Deziyangxin (DZYX) on the proliferation and migration of non-small cell lung cancer (NSCLC) cells, specifically LLC cells, as well as explore its potential mechanism of action.
Methods: The effect of the alcoholic extract on LLC cell viability was assessed using the CCK-8 method. The proliferation of LLC cells was evaluated using the EdU (5-Acetyl-2'-deoxyuridine) assay. Transwell assays were conducted to measure cell metastasis. Western blot analysis was performed to assess the expression of Cleaved Caspase-3, Bcl-2, Beciln-1, indicating the impact of DZYX on apoptosis and autophagy in LLC cells. Furthermore, the anti-tumor mechanism of DZYX was explored through transcriptome research and detection of Akt, p-Akt, p-mTOR protein levels.
Results: The ethanol extract of DZYX exhibited a concentration-dependent and time-dependent inhibitory effect on LLC cell viability, with an IC50 of 406.1 μg/ml. Moreover, the ethanol extract of DZYX significantly reduced the migration ability of LLC cells. Additionally, the alcoholic extract of DZYX upregulated the expression of Cleaved Caspase-3 and Beciln-1 proteins, while downregulating the expression of Bcl-2 in LLC cells. Importantly, DZYX ethanol extract down regulated the expression of Akt and p-mTOR proteins in LLC cells, which combined with transcriptome results indicated that the drug exerted a multi-target and multi-pathway effect, primarily related to inhibiting the activation of the PI3K/AKT/m-TOR signaling pathway.
Conclusion: The alcoholic extract of DZYX demonstrates inhibitory effects on LLC cells, promoting apoptosis and autophagy. It is hypothesized that its anti-tumor mechanism is associated with the PI3K/AKT/m-TOR pathway.

1. Introduction

According to 2020 statistics, lung cancer remains one of the most common causes of cancer death worldwide, accounting for 11.4% of all cancer cases, and the incidence and mortality of lung cancer among all types of cancer remain high [1]. There are two main types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Non-small-cell lung cancer is the predominant pathological type, accounting for 80%–85% of total lung cancers [2,3]. According to the international TNM staging criteria and clinical

* Corresponding author.
E-mail addresses: Yiyunaaaa@163.com (Y. Yun), jiangyh6668@163.com (J. Yahui), baibobo057@163.com (B. Bobo), 15033601950@163.com (Z. Caifeng), yanli_2000@163.com (Z. Yanli).

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guidelines for lung cancer, NSCLC is classified as stage 0 to IV. Patients with stage III-IV NSCLC diagnosed by clinical cytology or pathology are mainly classified as squamous, adenocarcinoma and mixed adenosquamous carcinoma using WHO pathological staging criteria [4,5].

Tibetan medicine, as the second major branch of Chinese medicine, boasts a rich history and mature development. Ancient Tibetan medical texts, such as "The Moon King's Medical Diagnosis," contain descriptions of diseases with symptoms similar to tumors. These texts propose that the imbalance of the three major factors, namely "Chiba" (fire), "Bacon" (soil and water), and "Long" (gas), is the key factor contributing to the development of tumors. Other classical treatises describe conditions such as "lung cancer with sore throat and difficulty swallowing" and "difficulty in breathing with wheezing." These records regarding "lung diseases" and "pulmonary tumors" affirm the experience of Tibetan medicine in treating lung cancer [6]. Numerous studies have highlighted the advantages of Traditional Chinese Medicine (TCM) in the treatment of lung cancer. TCM offers multiple targets, has a wide range of sources, and is cost-effective. It has also demonstrated advantages in preventing tumor recurrence and metastasis, enhancing treatment efficacy, and reducing toxicity [7–9].

DZYX, a Tibetan medicine, is a classic formula derived from the Tibetan Hospital of Hainan Tibetan Autonomous Prefecture in Qinghai Province, China. This formula is the result of the excavation and compilation of ancient Tibetan medical texts. DZYX is known for its reported effects, including promoting blood circulation, resolving blood stasis, dispersing lumps and tumors, healing ulcers, and reducing swelling. This study aims to explore the anti-tumor effect and possibly mechanism of this Tibetan medicine using in Lewis lung cancer as a basis, providing further theoretical support for its future clinical application.

2. Materials and methods

2.1. Cells and cell culture

The Lung adenocarcinoma cell line LLC cells and normal Gastric mucosa epithelial cells GES-1 were purchased from Wuhan Procell Life Science &Technology Company. LLC cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution, and cells at a logarithmic growth stage were taken for the experiment. It was propagated once every 3–4 days during the culture period. During subculture, the cells were first washed 3 times with PBS and digested by trypsin for 30 s. The cells were collected from the culture bottle into a centrifuge tube, then centrifuged at 1000 r/min for 5 min, the supernatant was discarded, 1 ml of culture medium was added, gently mixed, added to a new bottle and finally cultured in an incubator (37 °C, 5% CO_2).

2.2. Drugs and reagents

DZYX was provided by the Tibetan Hospital of Hainan Tibetan Autonomous Prefecture in Qinghai Province. The experimental drug was the ethanol extract of DZYX. It was obtained by grinding and weighing the DZYX pill, soaking it in 95% ethanol overnight, refluxing and extracting it three times, filtering twice, rotating evaporation and concentration, and vacuum freeze-drying. Then the DZYX alcoholic extract was dissolved in Dimethyl sulfoxide (DMSO) solvent and prepared to a maximum concentration of 600 μg/ml, filtered at 0.2 μM and stored at 4 °C in the refrigerator. The high sugar DMEM medium and fetal bovine serum were from the Corning Incorporated, USA. Shanghai Yeasen Biotech Co., Ltd. produced the CCK-8 Kit (batch number C6102030). The EdU Cell Proliferation Assay Kit was a product of Guangzhou Ruibo Biotechnology Co. β-Actin, Cleaved Caspase-3, Bcl-2, and Beclin-1 antibodies were products of Cell Signaling Technology, USA.

2.3. CCK-8 assay

Cell viability and cytotoxicity were assessed using the CCK-8 assay. LLC cells in the logarithmic growth phase were plated in 96-well plates at (5–8) $\times$ 10^3 cells per well and cultured in incubator for 24 h. After the cells were fully plastered, the medium was replaced with different concentrations of drug-containing medium according to the grouping design (Control group: 1‰ DMSO; Positive control group: Cisplatin (DDP), 5 μg/ml; Drug group: 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, 600 μg/ml), incubated for 12 h, 24 h, 36 h and 48 h respectively, then 10 μl of CCK-8 solution was added to each well and incubated for 1 h, protected from light, and the absorbance value at 450 nm was measured by enzyme marker to calculate the cell survival rate. The IC50 values were calculated by GraphPad Prism 8.0.2 statistical software, using the curve fitting method. Six replicate wells were set up for each group, and the experiment was repeated three times.

Cell viability = [OD (dosing group) - OD (blank group)]/[OD (control group) - OD (blank group)] x 100%

2.4. EdU cell proliferation assay

The cells in the logarithmic growth phase were seeded in 6-well plates with 5 $\times$ 10^5 cells per well, then changed into the medicated medium after 24 h of culture. After 24 h of treatment, 500 μl of 50 μM EdU medium was added to each well and incubated for 2 h, then the medium was discarded and washed twice with PBS for 5 min each time. Then prepared 1X Apollo staining reaction solution, added 500 μL of reaction solution per well, incubated for 30 min at room temperature in a shaker protected from light, then discard the reaction solution. Added another 100 μl of permeate (0.5% TritonX-100 in PBS) shall be cleaned three times with a shaker for 10 min each time, the permeate was discarded and the wells were washed with PBS for 5 min. Afterwards, 500 μl of 1X Hoechst 33342 reaction

solution was added to each well and incubated for 30 min at room temperature, protected from light, in a decolorized shaker, and then the staining reaction solution was discarded and washed three times with PBS. Finally, the results were observed under a fluorescent microscope, and three randomly selected fields of view were analyzed.

2.5. Transwell assay

Transwell chambers were placed in 24-well culture plates. First, 750 μl of DMEM culture medium containing 10% FBS was added to the bottom well. The upper chamber was added with 200 μl of serum-free cell suspension containing different concentrations of drugs at a cell density of 2.5 × 10^6/ml. Then, the cells were incubated in a cell incubator with saturated humidity at 37 °C with 5% CO_2 for 24 h. Next, the cells were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystalline violet for 15 min. After washing in PBS, the cells were observed under a microscope and 3 fields of view were randomly selected. Finally, Image J software was used to calculate the number of migrating cells.

2.6. Western blot assay

The proteins of LLC cells were extracted by using RIPA Lysis Buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing protease and phosphatase inhibitors after the treatment of alcohol extracts of various concentrations of DZYX or after treatment at different time points. After performing SDS-PAGE according to the instructions of FuturePAGE™ Protein Prep Gel (ACE Biotechnology Co., Ltd.), the protein samples were transferred to PVDF (polyvinylidene fluoride) membrane and then blocked with 5% skimmed milk at 25 °C for 1 h. PBST (Phosphate buffered solution containing 0.05% Tween-20) was used to wash the membrane twice. The protein samples were then incubated with primary antibodies in a refrigerator at 4 °C overnight. Next, the protein was incubated with secondary antibodies for 2 h at room temperature. After washing 3 times, the luminescent solution was then added for development. The inhibitory effect of DZYX alcohol extract on LLC cells was investigated by the expression of apoptosis proteins Cleaved Caspase-3 and Beciln-1, as well as autophagy-related protein Bcl-2, using β-Actin as an internal reference. The Image J software was used for analysis

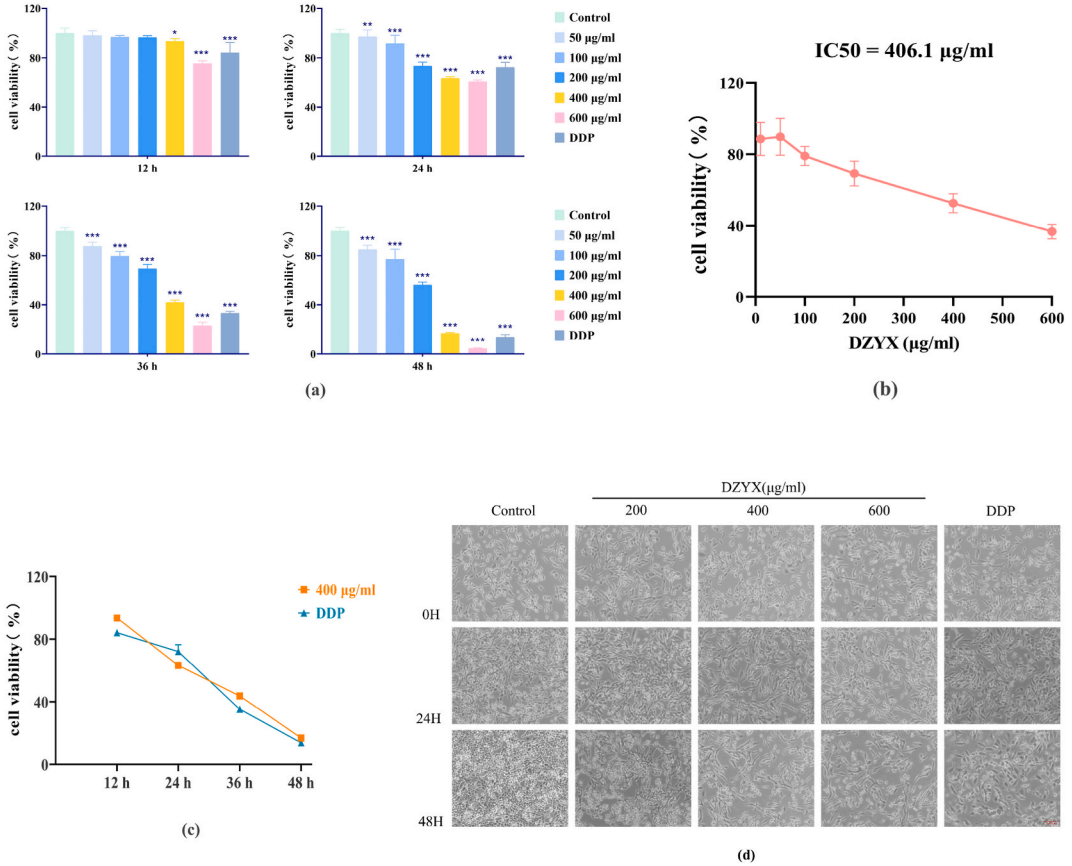


Fig. 1. Cellular activity of LLC cells inhibited by DZYX alcohol extract. (a) Cell survival after 12 h, 24 h, 36 h and 48 h incubation with different concentrations of drug-containing medium. (b) IC50 of LLC cells under the effect of DZYX alcoholic extract. (c) Time-survival rate of LLC cells under the effect of 400 μg/ml of DZYX alcoholic extract. (d) Cell morphology after 24 h and 48 h of the action of 200, 400 and 600 μg/ml DZYX alcohol extract (× 100). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

and quantification, and the mean gray scale values were counted. The relative expression of the target protein (IOD) = the gray value of the target protein/the gray value of the internal reference β-Actin.

2.7. RNA-seq and transcriptome analysis

RNA was extracted from control cultured LLC cells and LLC cells treated with 400 μg/ml DZYX alcohol extract for 24 h. The purity, concentration and integrity of RNA samples were examined using advanced equipment in molecular biology to guarantee the use of qualified samples for transcriptome sequencing. After enrichment of mRNA, the cDNA library was constructed, followed by bulk RNA sequencing and transcriptome analysis on the Illumina Novaseq system, entrusted to Biomarker Technologies Inc. (Beijing, China). Differential gene expression analysis was performed using FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) as a measure of gene expression levels. Gene functions were annotated based on the following databases: COG, KOG, GO, KEGG, Swiss-Prot, and eggnog. Finally, GO enrichment analysis and KEGG pathway enrichment analysis were performed for differentially expressed genes (DEGs).

2.8. Statistical analysis

GraphPad Prism 8.0.2 and SPSS 23.0 software were used for graphing and data analysis. Each experiment was repeated three times. Results are expressed as relative change compared with DMSO controls and presented as means ± SD. One-way ANOVA was used for comparison between multiple groups, and the LSD-t test was used for two-way comparison within groups. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. CCK-8 assay for the effect of DZYX alcohol extract on the activity of LLC cells

The results of the CCK-8 cytotoxicity assay demonstrated that the alcoholic extract of DZYX exerted an inhibitory effect on the proliferation of LLC cells. In all groups treated with different concentrations of DZYX alcoholic extract, except for the 12 h group, cell proliferation was significantly inhibited compared to the Control group ($P < 0.01$). The cell viability decreased significantly with increasing concentrations of the drug (Fig. 1(a)). The IC50 of LLC cells treated with DZYX alcohol extract for 36 h was determined to be 406.1 μg/ml (Fig. 1(b)). The inhibitory effect of DZYX alcohol extract on LLC cell proliferation was time-dependent, and the effect of the 400 μg/ml DZYX alcohol extract was comparable to that of the positive control (DDP 5 μg/ml) (Fig. 1(c)). Additionally, the effect of DZYX alcohol extract on LLC cell proliferation was visually observed using an inverted microscope. Compared to the control group, the number of LLC cells decreased after treatment with different concentrations of DZYX alcohol extract for 24 h and 48 h. The inhibitory effect of the 400 μg/ml and 600 μg/ml groups on proliferation was similar to that of the positive control group (DDP 5 μg/ml), indicating that DZYX alcohol extract could effectively inhibit the proliferation of LLC cells (Fig. 1(d)).

3.2. CCK-8 assay for the effect of DZYX alcohol extract on the activity of GES-1 cells

The results showed that there was no significant change in cell viability of GES-1 cells after treatment with different concentrations of DZYX alcohol extract for 24 h and 48 h as compared with the control group. It was suggested that DZYX alcohol extract has no inhibitory effect on normal gastric mucosal epithelial cells (Table 1, Fig. 2).

3.3. EdU assay for inhibition of proliferation of LLC cells by DZYX alcohol extracts

To further examine the impact of DZYX alcohol extract on the proliferation of lung cancer cells, the cellular DNA replication activity was assessed using a specific reaction based on EdU (5-Acetyl-2'-deoxyuridine) with Apollo fluorescent dye, employing the thymine (T) alternative doping method. In comparison to the Control group, LLC cells treated with DZYX alcohol extract displayed a significantly lower proportion of EdU-positive cells, indicating reduced DNA replication activity. This difference was statistically significant ($P < 0.05$) and exhibited a concentration-dependent trend (Fig. 3(a and b)).

3.4. Transwell assay showed that DZYX alcohol extract inhibited the migration of LLC cells

The migration ability of LLC cells in lung adenocarcinoma decreased as the concentration of DZYX alcohol extract increased. The

Table 1
Detection results of GES-1 cells after the action of DZYX alcohol extract.

	OD			
Time	Control	200 μg/ml	400 μg/ml	600 μg/ml
24 h	1.0961 ± 0.0159	1.0983 ± 0.0814	1.0645 ± 0.0600	1.0821 ± 0.0155
48 h	1.2587 ± 0.0584	1.2774 ± 0.0432	1.2268 ± 0.0298	1.2750 ± 0.4778

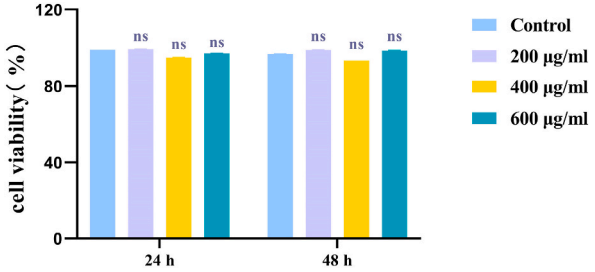


Fig. 2. Effect of DZYX alcohol extract on the activity of GES-1 cells. ns: P > 0.05 vs. control.

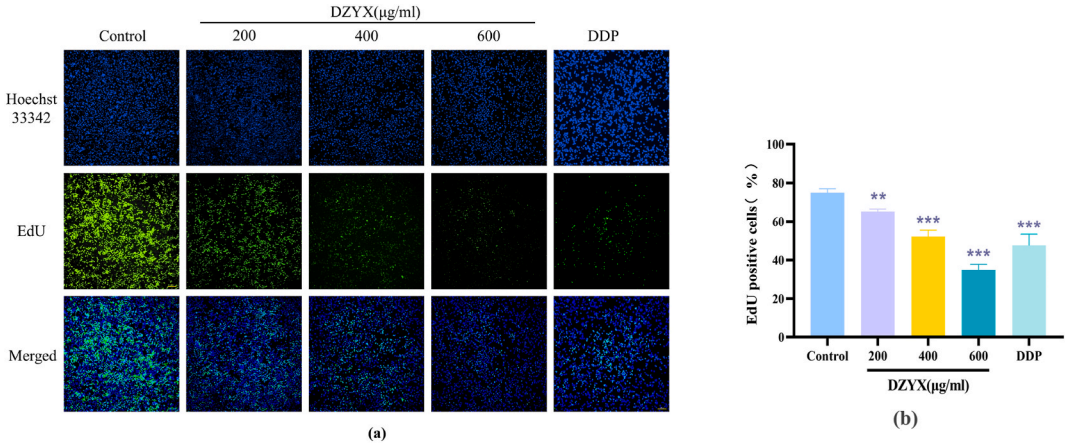


Fig. 3. The ethanol extract of DZYX inhibits the proliferation of LLC cells. (a) Microscopic results of EdU-positive LLC cells after 24 h of action of 200, 400 and 600 μg/ml DZYX alcohol extracts (× 100). (b) Histogram of the EdU-positive cell rate. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

number of migrating cells of LLC cells treated with 200 μg/ml, 400 μg/ml, and 600 μg/ml of DZYX alcohol extract gradually decreased compared to the control group. This difference was statistically significant (P < 0.001), indicating that DZYX alcohol extract effectively reduced the migration ability of LLC cells (Fig. 4(a and b)).

3.5. *Western blot assay to detect the Expression of Cleaved Caspase-3, Bcl-2, Beciln-1*

The results demonstrated a significant upregulation of the apoptosis-related protein Cleaved Caspase-3 in LLC cells treated with different concentrations of DZYX alcohol extract (400 and 600 μg/ml) compared to the Control group, with a statistically significant difference (P < 0.001). This indicates that the alcoholic extract of DZYX may inhibit the proliferation of LLC cells by promoting apoptosis (Fig. 5(a)). Specifically, at a concentration of 400 μg/ml, DZYX alcohol extract showed the highest expression of Cleaved Caspase-3 at 36 h, along with a significant decrease in the expression of Bcl-2 protein at 12 h, 24 h, and 48 h, all of which exhibited statistically significant differences (P < 0.001) (Fig. 5(b and c)).

3.6. *Results of transcriptome analysis*

Through RNA-seq and transcriptome analysis, Set |log2(Fold Change)| ≥2 and FDR<0.01 as the screening criteria, a total of 757 differentially expressed genes (DEGs) were identified in LLC cells treated with 400 μg/ml DZYX alcoholic extract for 24 h compared to the control group. Among these DEGs, 177 were found to be up-regulated, while 580 were down-regulated (Fig. 6(a)). Functional annotation of these DEGs using the mentioned databases revealed that DZYX alcohol extract had a significant impact on the biological processes, cellular components, and molecular functions of LLC cells (Fig. 6(b)). Notably, the alcoholic extract of DZYX exhibited significant regulation of cell migration, angiogenesis, and other angiogenesis-related processes in terms of biological processes (Fig. 6 (c)). Furthermore, KEGG pathway enrichment analysis indicated that DZYX alcohol extract could influence various signaling pathways, including pathways in cancer, human papillomavirus infection, PI3K-Akt signaling pathway, MicroRNAs in cancer, and MAPK signaling pathway, among others. These findings suggest that the effect of DZYX alcohol extract on lung cancer LLC cells may primarily involve the modulation of the PI3K/AKT signaling pathway, impacting molecular functions and biological processes (Fig. 6(d)). The PI3K-AKT clustering heat map (Fig. 6(e)) and expression results (Table 2) showed that the top ten genes with significant differences in this pathway were *Ngfr, Areg, Myc, Col6a1, Efna3, Col9a3, Pik3r3, Tnc, Itga4, and Fgf21*, suggesting that the DZYX alcohol extract may

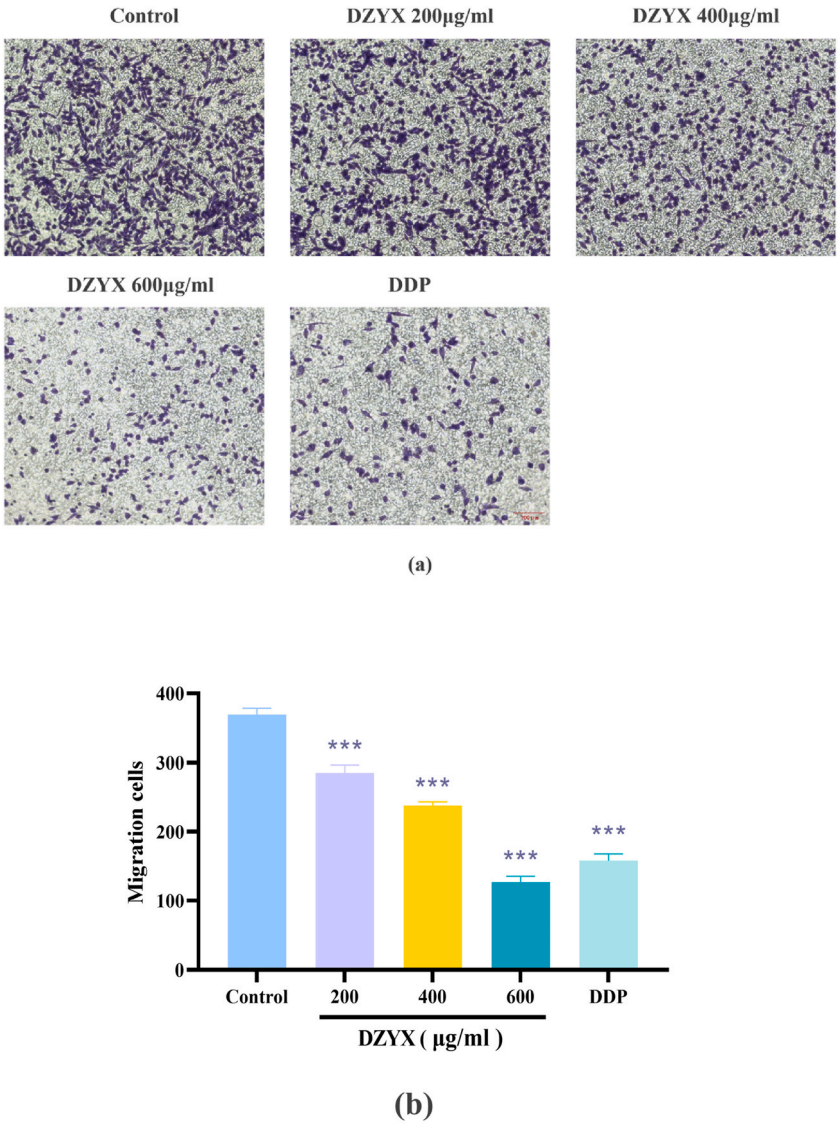


Fig. 4. Alcoholic extracts of DZYX inhibits the migration of LLC cells. (a) Results of LLC cell migration (× 100) after 24 h of action of 200, 400 and 600 μg/ml DZYX alcoholic extracts. (b) Histogram of the number of migrated cells. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

affect the activation of this pathway through the above genes.

3.7. Western blot assay to detect the expression of Akt、p-Akt、p-mTOR

To get some insight into the molecular mechanisms of inhibition of LLC cells by DZYX alcohol extract, expression of Akt、p-Akt、p-mTOR were analyzed by Western Blotting after 24 h treatments (Fig. 7). As shown in Fig. 7, expression of both Akt and p-mTOR strongly decreased after 200, 400 and 600 μg/ml DZYX alcohol extract intervention with respect to control cells. Moreover p-Akt levels notably increased in LLC cells in response to DZYX alcohol extract treatments starting from 400 to 600 μg/ml DZYX ($p < 0.05$).

4. Discussion

The Tibetan medicine DZYX, in the form of formula pills, exhibits anti-tumor effects through multiple pathways and targets. Its effectiveness and advantages in the adjuvant treatment of lung cancer are highly valuable and have been implemented in clinical practice. The findings of this study, utilizing the CCK-8 assay, EdU proliferation assay, and Transwell assay, provide evidence that DZYX alcohol extract can effectively inhibit the proliferation activity and migration ability of LLC cells. Moreover, the inhibitory effect of DZYX alcohol extract on proliferation was found to be both time- and concentration-dependent, further confirming its potential therapeutic impact on lung adenocarcinoma.

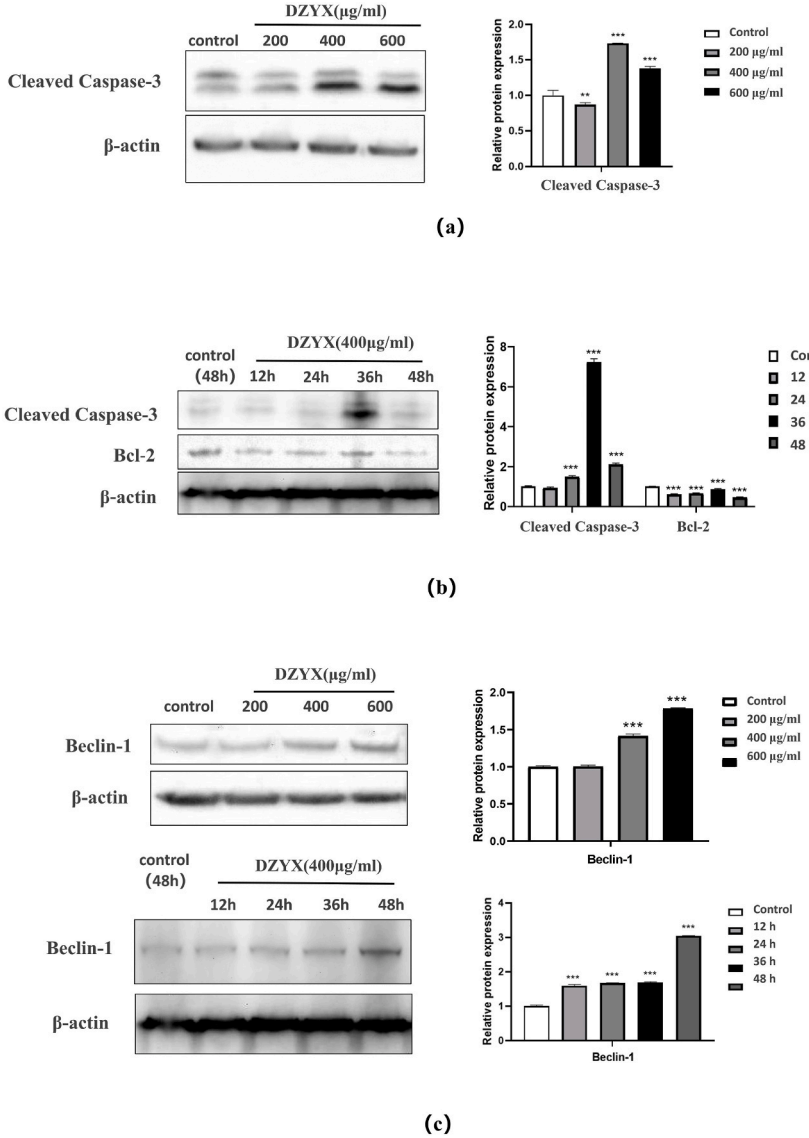
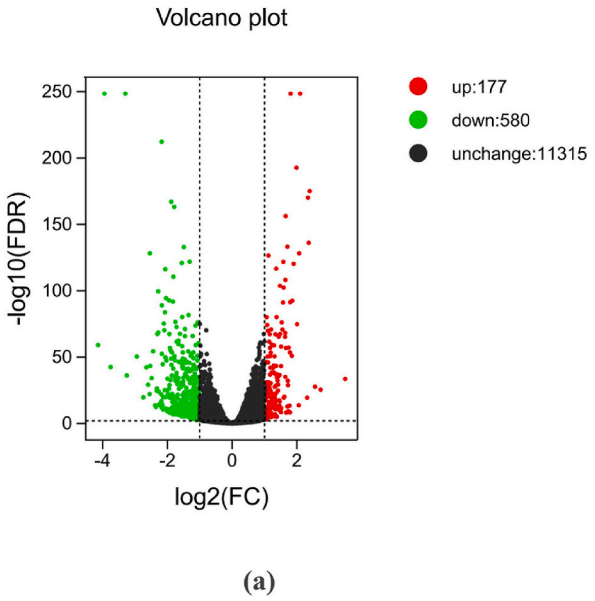


Fig. 5. The expression of Cleaved Caspase-3, Bcl-2 and Beciln-1 in LLC cells was regulated in vitro by the alcoholic extract of DZYX. (a) Expression of Cleaved Caspase-3 in LLC cells 24 h treatment with 200, 400, 600 μg/ml ethanol extract of DZYX. (b) Expression of Cleaved Caspase-3 and Bcl-2 in 400 μg/ml drug-containing medium after 12 h, 24 h, 36 h, and 48 h incubation respectively. (c) Enhanced expression of autophagy-related protein Beciln-1 with time and concentration. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

Apoptosis and autophagy are two distinct mechanisms involved in programmed cell death and regulate important intracellular physiological processes. Studies have shown that herbal components can effectively induce apoptosis and autophagy in the treatment of non-small cell lung cancer [10–12]. Caspase is a type of cysteine protease, and its activation serves as an important biochemical marker of apoptosis. Caspase-3, in particular, plays a critical role in the apoptotic cascade and is involved in the activation of inflammatory mediators [13]. The B-cell lymphoma-2 (Bcl-2) family of proteins is key in regulating apoptosis, and aberrant expression or activation of Bcl-2 is directly involved in promoting the survival and growth of cancer cells. Elevated Bcl-2 levels are associated with the development, progression, metastasis, and recurrence of various cancers [14,15], while a reduction in Bcl-2 protein levels can enhance the sensitivity of malignant cells to apoptosis. The results of this study revealed that DZYX alcohol extract upregulates the expression of Cleaved Caspase-3 protein and downregulates the expression of Bcl-2 protein, indicating its potential to promote apoptosis in LLC cells. Autophagy, on the other hand, is a process in which intracellular components undergo lysosomal degradation, and excessive autophagy can induce autophagic cell death in tumor cells [15–17]. Beclin-1 is a well-known autophagy-related protein that participates in autophagy activation and autophagosome formation. It acts as a subunit of the class III PI3K complex and functions as a scaffold in both the C1 and C2 complexes, allosterically regulating their associated lipid kinase activity. The C1 complex primarily governs autophagy, particularly in the nucleation of autophagosome bilayers, while C2 is involved in endocytic trafficking, lysosomal



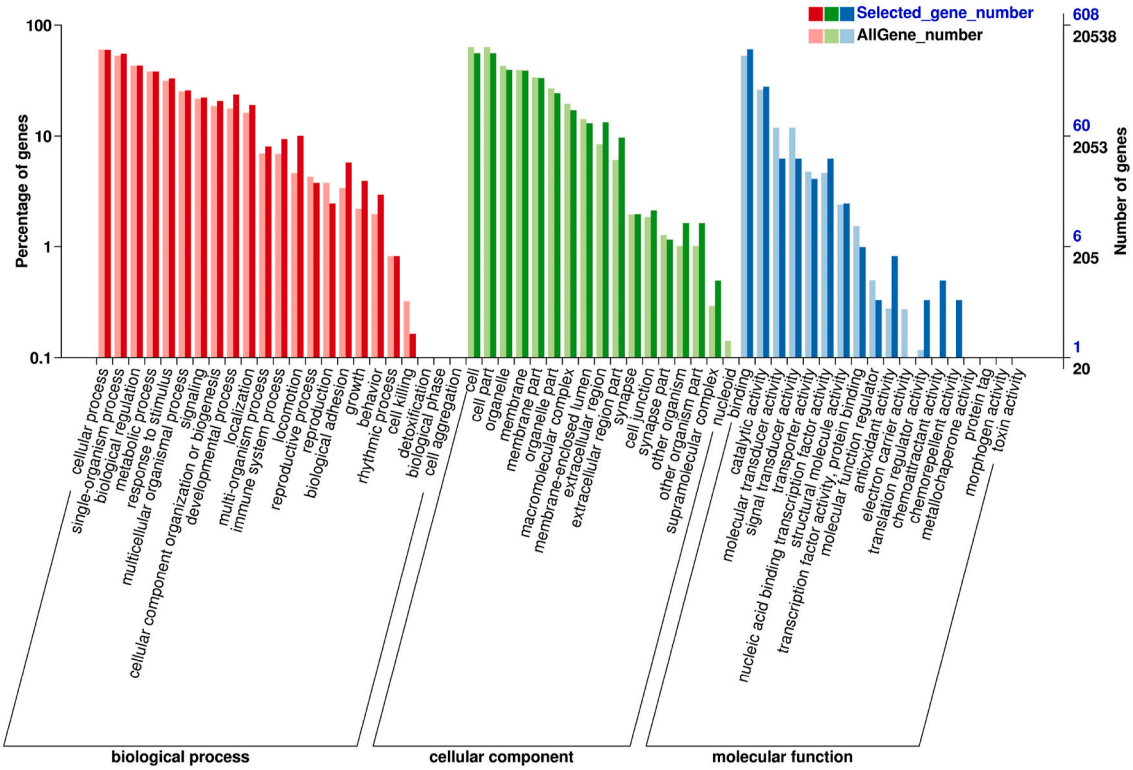
(a)

Fig. 6. Differential gene analysis. (a) The volcano plot. (b) GO enrichment analysis. (c)The biological processes by GO enrichment analysis. (d) KEGG pathway enrichment analysis. (e) PI3K-Akt pathway heatmap.

tubulation, and autophagy. Additionally, studies have demonstrated that Bcl-2 exerts an inhibitory effect on Beclin-1, and their interaction reduces the availability of Beclin-1 for C1 and C2 complex formation, consequently suppressing autophagy [15]. The upregulation of Beclin-1 protein expression and the downregulation of Bcl-2 protein expression induced by DZYX alcohol extract suggest its potential to induce autophagy in lung adenocarcinoma LLC cells.

The integration of Chinese and Tibetan medicine with single-cell transcriptome sequencing technology has provided a comprehensive and systematic analysis of the complex components and mechanisms involved. This approach has facilitated a more scientific understanding of drug efficacy, contributing to the development of Chinese and Tibetan medicine towards modern precision medicine. In the context of tumor development, it is recognized that it extends beyond the mere proliferation and metastasis of tumor cells. The tumor microenvironment, comprising the extracellular matrix (ECM) and neovascularization, plays a crucial role. Neovascularization, a characteristic feature of malignant tumors, is closely associated with tumor migration, invasion, and progression [18,19]. The ECM, composed of various polysaccharide components and secreted protein molecules, regulates cell adhesion behavior and influences the trajectory and speed of cell migration [20,21]. Through Gene Ontology (GO) enrichment analysis, it was revealed that DZYX alcohol extracts primarily influence the positive regulation of cell migration, extracellular matrix organization, and regulation of cell migration. The inhibition of LLC cell migration confirmed through Transwell assay further supports the ability of DZYX alcohol extracts to impede LLC cell migration. Considering that lung cancer is highly vascularized, inhibiting neovascularization holds significant importance in its treatment and prognosis [22]. The observed negative regulation of angiogenesis and the alterations in the biological function of angiogenesis resulting from the intervention of DZYX alcohol extract on LLC cells suggest its potential to inhibit angiogenesis in lung adenocarcinoma. In summary, the analysis of biological processes, combined with experimental validation, highlights that DZYX alcohol extract has the capability to inhibit LLC cell migration and angiogenesis. This signifies its potential as a therapeutic agent for lung adenocarcinoma, addressing critical aspects of tumor microenvironment dynamics.

The PI3K/AKT signaling pathway plays a crucial role in regulating signal transduction and various biological processes, including cell proliferation, migration, apoptosis, angiogenesis, glucose metabolism, and DNA repair [23]. Abnormal activation of this pathway is commonly observed in malignant tumors. Currently, the study of inhibitors targeting PI3K and mTOR in this pathway has become an important direction in the treatment of malignant tumors [24]. In the present study, the enrichment analysis of KEGG pathways revealed that the ethanol extract of DZYX primarily influenced cancer-related pathways and associated biological processes. Furthermore, multiple targets and pathways were involved in the anti-cancer mechanism of DZYX, with differential gene expression primarily enriched in the PI3K/AKT signaling pathway. The most significant differentially expressed gene *Ngfr* in the PI3K/AKT signaling pathway is a member of the tumor necrosis factor receptor family and one of the driving genes for lung cancer. It plays a role in regulating cancer cell proliferation, migration, invasion, apoptosis, and autophagy [25–27]. *Pik3r3*, as a classic PI3K regulatory subunit, plays an important role in tumor occurrence and metastasis [28,29]. The downregulation of the expression levels of both *Ngfr* and *Pik3r3* genes can to some extent inhibit the activation of the Akt pathway. Meanwhile, Western blot results showed that DZYX ethanol extract significantly downregulated the expression of Akt and p-mTOR proteins in LLC cells, achieving the effect of inhibiting this pathway. The upregulation of p-Akt protein expression is speculated to be related to the survival feedback mechanism of mTOR inhibition, and suggests the possibility of excessive generation of ROS [30]. The specific mechanism needs further research. The above



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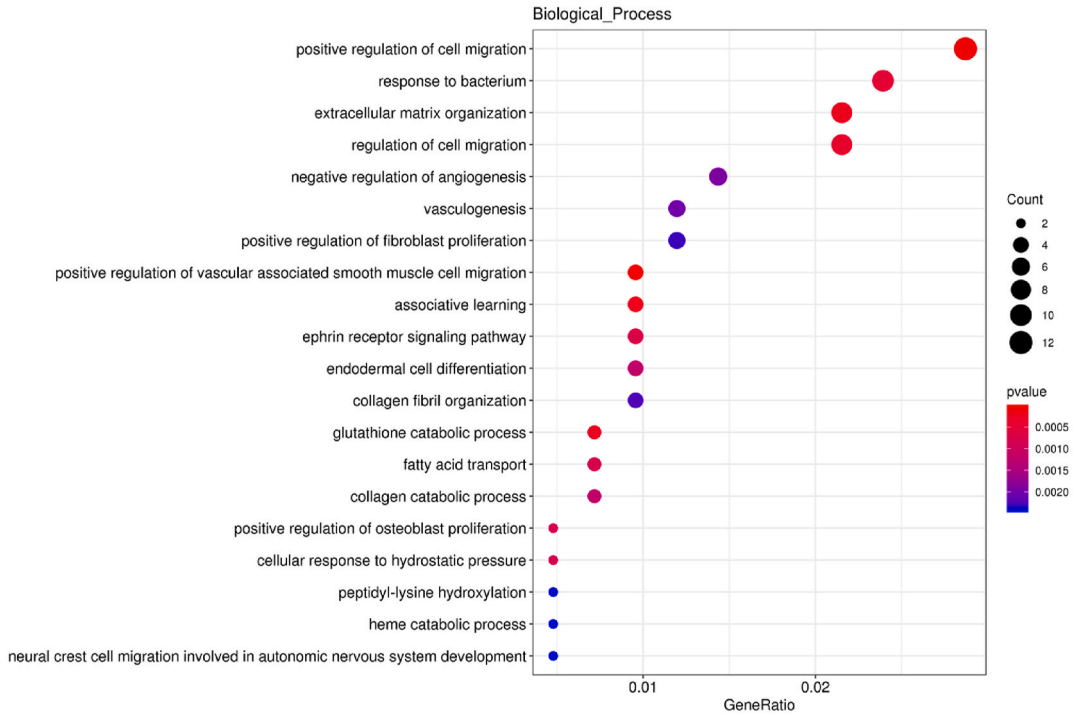
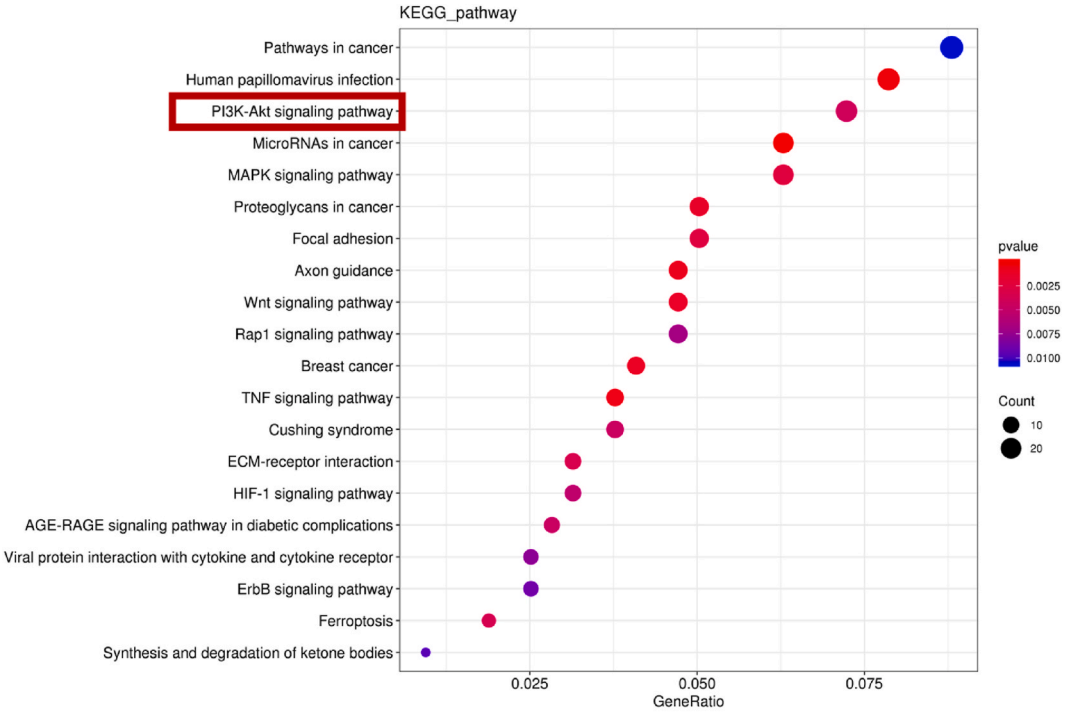


(c)

Fig. 6. (*continued*).



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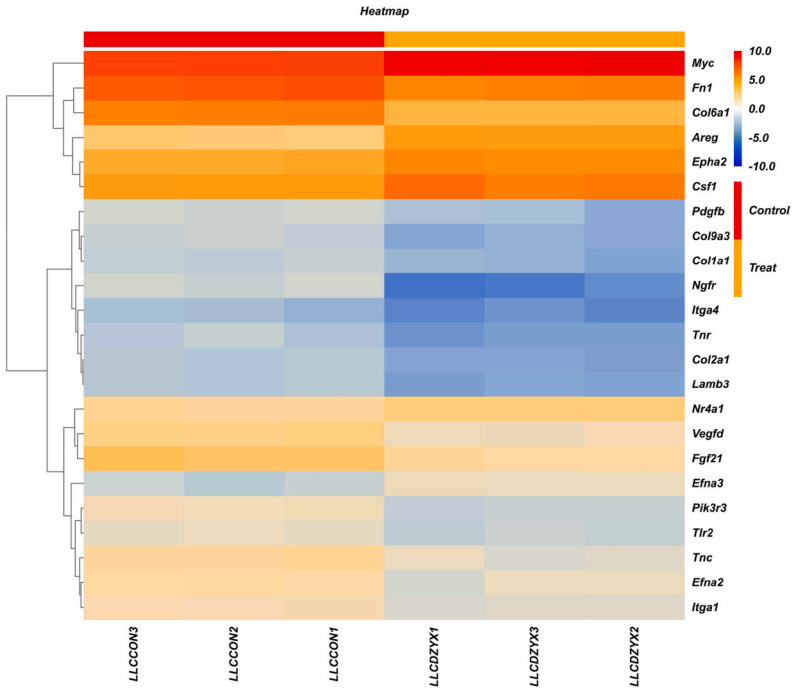

(e)

Fig. 6. (*continued*).

Table 2
PI3K-Akt pathway-related differential genes.

ID	Gene name	log2 (fold change)	P value	regulation
ENSMUSG00000000120	*Ngfr*	−2.743783378	2.34E-20	down
ENSMUSG00000029378	*Areg*	2.398642243	8.34E-176	up
ENSMUSG00000022346	*Myc*	1.897518108	5.83E-121	up
ENSMUSG00000001119	*Col6a1*	−1.880280738	8.08E-168	down
ENSMUSG00000028039	*Efna3*	1.51374646	2.83E-12	up
ENSMUSG00000027570	*Col9a3*	−1.49579846	1.10E-07	down
ENSMUSG00000028698	*Pik3r3*	−1.487910787	9.83E-24	down
ENSMUSG00000028364	*Tnc*	−1.487707514	1.14E-16	down
ENSMUSG00000027009	*Itga4*	−1.483199796	1.48E-07	down
ENSMUSG00000030827	*Fgf21*	−1.448162322	7.64E-20	down

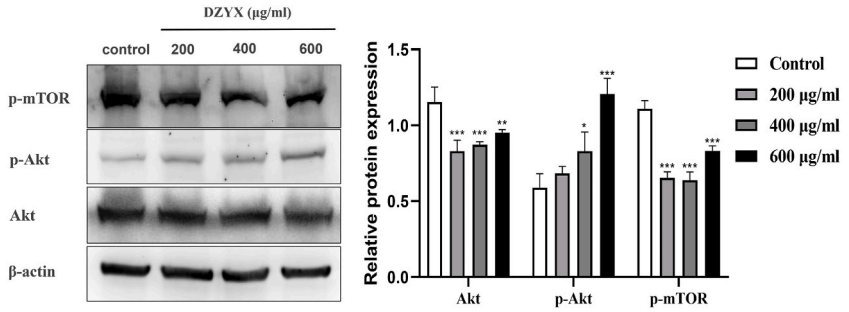

Fig. 7. The expression of Akt、p-Akt、p-mTOR in LLC cells was regulated in vitro by the alcoholic extract of DZYX. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

results suggest that the anti-tumor mechanism of DZYX alcohol extract is closely related to the activation of PI3K/AKT/m-TOR signaling pathway and may be mediated by *Ngfr* and *Pik3r3* genes.

These findings collectively demonstrate the potential of DZYX as an anti-tumor agent in the treatment of non-small cell lung cancer (NSCLC).

5. Conclusion

The alcoholic extract of DZYX has demonstrated the ability to inhibit the proliferation of LLC cells and induce apoptosis and autophagy. Additionally, it has been observed to reduce the migration ability of LLC cells. The anti-lung adenocarcinoma mechanism of DZYX alcohol extract was related strongly to the inhibition of the activation of PI3K/AKT signaling pathway.

Author contribution statement

Yun Yi: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yahui Jiang: Conceived and designed the experiments.

Yanli Zhao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18712.

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