# Jaceosidin inhibits cell viability and induces apoptosis in non-small cell lung cancer by inhibiting the Ras/Raf/MEK/ERK and Akt pathways

XIAODIE CAO[1,2*], CHONG MA[1*], XIN WANG[1*], CUI WANG[1], LANLIN SHEN[1], JINGRU WEI[1], RONGMIN YANG[1], YAN LI[2,3], XU HE[2,3] and LI CHEN[1,2]

[1]Department of Pathophysiology, School of Basic Medicine, Kunming University of Science and Technology, Kunming, Yunnan 650500, P.R. China; [2]The First People's Hospital of Yunnan Province, Kunming University of Science and Technology Affiliated Hospital, Kunming, Yunnan 650032, P.R. China; [3]Department of Geriatrics, The First People's Hospital of Yunnan Province, Kunming, Yunnan 650032, P.R. China

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**Abstract.** Non-small cell lung cancer (NSCLC) remains a major health challenge due to its poor prognosis and low 5-year survival rate; therefore, the development of efficient and less toxic anti-NSCLC therapies is of great importance. The present study aimed to investigate the anti-survival and pro-apoptotic effects of Jaceosidin, a flavonoid, on human NSCLC cells and to uncover its underlying mechanism. Cell viability, cell cycle progression and apoptosis were assess using the MTS assay and flow cytometry, and protein expression was analyzed by western blot analysis. The results showed that Jaceosidin significantly reduced A549 cell viability in a dose-dependent manner, whereas it exhibited significantly lower cytotoxicity against 293T cells. In addition, cell cycle distribution analysis demonstrated that A549 cell treatment with Jaceosidin induced S-phase cell cycle arrest, which was accompanied by p21 upregulation. Jaceosidin also enhanced cell apoptosis, and upregulated cleaved-poly-ADP ribose polymerase and cleaved-caspase-3 expression in a dose-dependent manner. Furthermore, Jaceosidin promoted the release of cytochrome *c* (cyto-c) from mitochondria into the cytoplasm, as detected by digitonin-based fractionation and western blotting, thus resulting in increased cytosolic cyto-c levels. Finally, Jaceosidin significantly reduced the expression of Ras and Raf, and the downstream signaling molecules ERK1/2 and Akt, which may suppress A549 cell viability and enhance apoptosis. Overall, the current study suggested that Jaceosidin exerts potential anti-NSCLC activity with tumor-selective cytotoxicity through inhibiting the Ras/Raf/MEK/ERK and Akt pathways, and activating both intrinsic mitochondrial and extrinsic apoptotic pathways, thus representing a promising natural product candidate for future NSCLC therapy.

*Correspondence to:* Dr Li Chen, Department of Pathophysiology, School of Basic Medicine, Kunming University of Science and Technology, 727 South Jingming Road, Chenggong, Kunming, Yunnan 650500, P.R. China
E-mail: atlanis@qq.com

Dr Xu He, Department of Geriatrics, The First People's Hospital of Yunnan Province, 157 Jinbi Road, Xishan, Kunming, Yunnan 650032, P.R. China
E-mail: 150396484@qq.com

*Contributed equally

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## Introduction

According to the World Health Organization, lung cancer is one of the most rapidly growing malignancies in terms of both morbidity and mortality worldwide (1). Histopathologically, lung cancer is mainly classified into small-cell lung cancer and non-small cell lung cancer (NSCLC). NSCLC accounts for 80-85% of all lung cancer cases, and lung adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma are the three main subtypes of NSCLC (2,3). Currently, surgical resection, radiotherapy and chemotherapy are the most common treatment options for NSCLC. Although advances have been made in the clinical diagnosis and treatment of lung cancer, early-stage lung cancer is commonly asymptomatic and ~75% of patients are diagnosed at advanced stages of the disease (2). Due to the lack of specific symptoms, limited therapeutic efficacy and poor prognosis, the global overall 5-year survival rate of NSCLC remains at ~15% (4-6). Therefore, the development of highly effective and low-toxicity chemotherapeutic agents for NSCLC is of urgent importance.

Due to their diverse pharmacological properties and low toxicity, natural products are considered important sources of anticancer compounds. Jaceosidin, a flavonoid active ingredient mainly derived from plants such as mugwort leaf and *Eupatorium lindleyanum*, exhibits several biological and pharmacological effects, including anti-inflammatory, antiviral, antioxidant, antidiabetic, antiallergic, antibacterial

and antitumor properties (7,8). *In vitro* studies have suggested that Jaceosidin exerts anticancer effects on several types of cancer (9,10). A previous study showed that Jaceosidin can induce apoptosis in Caki human kidney cancer cells by downregulating myeloid cell leukemia 1 and cellular FLICE-inhibitory protein at the transcriptional level (10). Jaceosidin can also induce apoptosis in Ras-transformed human mammary epithelial cells (MCF10A-ras) by upregulating Bax, downregulating Bcl-2 and enhancing reactive oxygen species (ROS) production by ≤40% (11). Jeong *et al* (12) revealed that Jaceosidin could restore TPA-induced cyclooxygenase 2 and matrix metallopeptidase 9 upregulation by inhibiting ERK1/2 phosphorylation in MCF10A cells, eventually inhibiting cell invasion and migration. In addition, Jaceosidin has been shown to induce $G_2$/M cell cycle arrest in U87 glioblastoma cells and Hec1A human endometrial cancer cells through upregulating p53 and p21, and modulating the ERK/ataxia-telangiectasia mutated kinase/checkpoint kinase 1/2 pathway, thereby attenuating cell proliferation (13,14). These previous findings indicated that Jaceosidin may exert potent anticancer effects; however, to the best of our knowledge, its pro-apoptotic and anti-survival effects in NSCLC cells have not been previously investigated. Therefore, the current study aimed to explore the capacity of Jaceosidin in inhibiting cell viability and inducing cell apoptosis in the human NSCLC cell line A549.

## Materials and methods

*Cell culture*. The A549 NSCLC cell line and 293T cells were purchased from Kunming Cell Bank of Type Culture Collection, Chinese Academy of Science. A549 and 293T cells were cultured in RPMI 1640 and DMEM (both from Gibco; Thermo Fisher Scientific, Inc.), respectively, supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin at 37˚C in a constant temperature incubator containing 5% $CO_2$. Jaceosidin (Fig. 1A) was obtained from Wuhan ChemFaces Biochemical Co., Ltd., and was prepared as a 100 mM storage solution in DMSO.

*Cell viability assay*. Cell viability was assessed using the MTS method (Promega Corporation). Briefly, A549 and 293T cells at a density of $1x10^4$ cells/well were inoculated in 96-well culture plates with three replicates per group, and the cells were cultured under saturated humidity at 37˚C and 5% $CO_2$. Following treatment of A549 cells with different concentrations of Jaceosidin (0, 40, 80, 160 and 320 $\mu$M) for 68 h at 37˚C, the cells were co-treated with 20 $\mu$l MTS/PMS (20:1 v/v) for an additional 4 h. For 293T cells, the treatment procedure was identical, and the cells were treated with difference concentrations of Jaceosidin (0, 20, 40, 80 and 160 $\mu$M). The optical density values in each well were measured at 490 nm using a microplate reader (Nanjing Detai Bioengineering Co., Ltd.). Finally, cell viability was calculated using the following formula: Cell viability (%)=(absorbance value of drug-treated group - absorbance value of blank group)/(absorbance value of control group-absorbance value of blank group) x100. The $IC_{50}$ values were calculated by non-linear regression curve fitting using Microsoft Excel (version 2016; Microsoft Corporation).

*Cell cycle distribution*. Following treatment of A549 cells ($2x10^5$ cells/well in 6-well plates, 2 ml medium/well) with different concentrations of Jaceosidin (0, 40, 80 and 120 $\mu$M) for 24 h at 37˚C, cell precipitates were collected by centrifugation at 300 x g for 5 min at 4˚C. Subsequently, DNA content was measured using a commercial cell cycle assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Briefly, cell pellets were fixed overnight in 75% ice-cold ethanol at 4˚C. Prior to staining, the cells were washed with PBS, resuspended in 100 $\mu$l RNase A solution and incubated for 30 min at 37˚C in a water bath, followed by staining with 400 $\mu$l propidium iodide (PI; MilliporeSigma) for 30 min at 4˚C in the dark. Cell cycle distribution was then analyzed by flow cytometry using a BD FACSCanto flow cytometer (BD Biosciences) with BD FACSDiva software (version 8.0; BD Biosciences).

*Flow cytometric analysis of apoptosis*. Apoptosis was assessed using the Annexin V-FITC/PI Apoptosis Detection Kit (Absin Bioscience Inc.). A549 cells ($2x10^5$ cells/well in 6-well plates, 2 ml medium/well) were treated with different concentrations of Jaceosidin (0, 40, 80 and 120 $\mu$M) for 24 h at 37˚C, and subsequently harvested by trypsinization. The cells were then stained with Annexin V-FITC and PI for 30 min at room temperature in the dark. The proportions of viable, necrotic, and early and late apoptotic cells were analyzed by flow cytometry using a BD FACSCanto flow cytometer with BD FACSDiva software (version 8.0).

*Western blot analysis*. Untreated control and Jaceosidin-treated A549 cells (treated with 40, 80 and 120 $\mu$M Jaceosidin for 36 h at 37˚C, or with 120 $\mu$M Jaceosidin for 12, 24 and 36 h at 37˚C) were collected by centrifugation at 300 x g for 5 min at 4˚C. The cells were then washed twice with PBS and lysed with RIPA lysis buffer (99 ml 1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with 1X Roche protease inhibitor cocktail, 1X PMSF, 0.05 M NaF and 0.05 M glycerophosphate) on ice for 30 min. After sonication (20 kHz; 3 pulses, 10 sec each, with 30-sec intervals on ice), the cell supernatants were collected by centrifugation at 12,000 x g for 10 min at 4˚C. The protein concentration was quantified using a BCA protein concentration assay kit (Beyotime Biotechnology), and equal amounts of protein (40 $\mu$g/lane) were mixed with loading buffer, denatured at 100˚C for 5 min and separated by SDS-PAGE on a 12% gel. Subsequently, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membranes (MilliporeSigma). After blocking in 5% skim milk in PBS-0.05% Tween-20 (PBST) for 1 h at room temperature, the membranes were incubated with primary antibodies against poly-ADP ribose polymerase (PARP; cat. no. 9542), caspase-3 (cat. no. 9662), caspase-8 (cat. no. 4790), caspase-9 (cat. no. 9502), cytochrome *c* (cyto-c; cat. no. 4280), p21 (cat. no. 2947), Ras (cat. no. 3965), Raf (cat. no. 9422), ERK1/2 (cat. no. 4695), phosphorylated (p)-ERK1/2 (cat. no. 4370), Akt (cat. no. 9272), p-Akt (cat. no. 4060), Bax (cat. no. 2772) and Bcl-2 (cat. no. 4223) (all from CST Biological Reagents Co., Ltd.) at 4˚C overnight. The primary antibodies were diluted in PBST to 1:250 (for anti-Raf), 1:8,000 (for anti-β-actin; cat. no. 4970; CST Biological Reagents Co., Ltd.) and 1:1,000 for the remaining antibodies. After washing three times with PBST, the PVDF

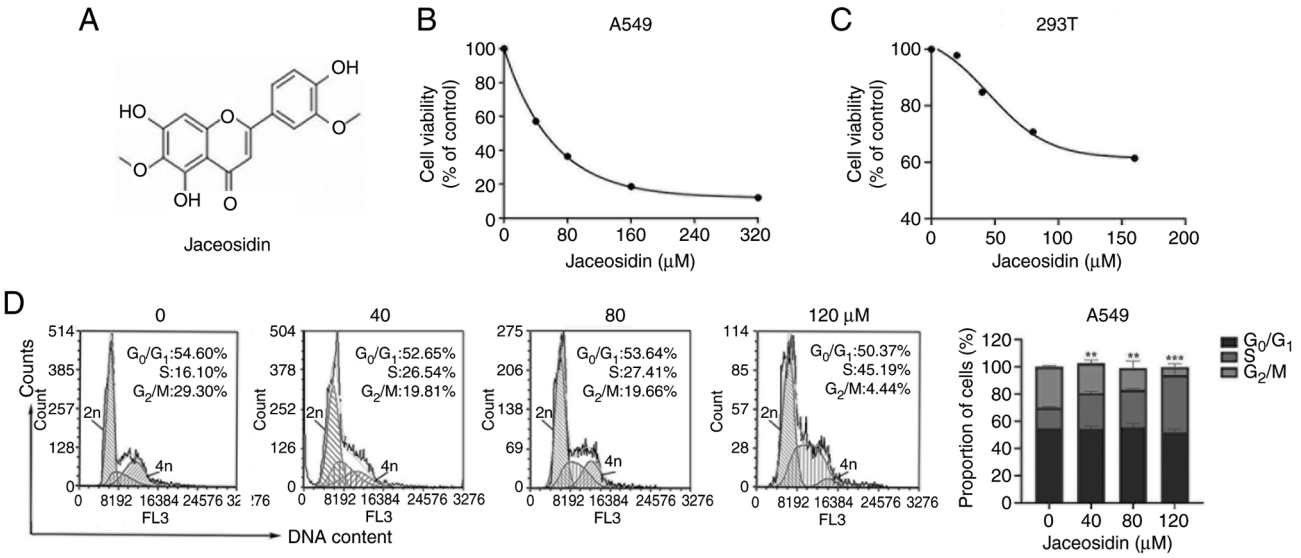


Figure 1. Jaceosidin inhibits viability and induces S-phase arrest in A549 cells. (A) Chemical structure of Jaceosidin. Viability of (B) A549 and (C) 293T cells treated with increasing concentrations of Jaceosidin for 72 h was assessed using MTS assay. (D) Flow cytometric analysis was performed to determine the cell cycle distribution of A549 cells treated with increasing concentrations of Jaceosidin for 24 h. As labelled directly on the histograms, the 2n and 4n labels mark the DNA content corresponding to the $G_0/G_1$ (diploid) and $G_2/M$ (tetraploid) phases, respectively. Data are presented as the mean ± SD of three independent experiments (n=3). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. $^{**}P<0.01$, $^{***}P<0.001$ vs. control.

membranes were incubated with the corresponding horseradish peroxidase-labeled anti-rabbit secondary antibodies (cat. no. 31460; Thermo Fisher Scientific, Inc.) diluted 1:5,000 in PBST at room temperature for 1 h. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500; MilliporeSigma). After incubation with ECL, the signals were detected by exposure to photographic films, which were subsequently developed, fixed and dried. Band intensities were semi-quantified and normalized to β-actin using ImageJ software (version 1.53t; National Institutes of Health).

*Cyto-c release assay*. Cyto-c is released from mitochondria into the cytosol during apoptosis (10); therefore, cytosolic cyto-c levels were analyzed in the present study. Following treatment with jaceosidin (0, 40, 80 and 120 $\mu$M for 36 h, or 120 $\mu$M for 12, 24 and 36 h at 37˚C), A549 were collected and centrifuged at 300 x g for 5 min at 4˚C to remove the culture supernatant and the pellet was washed twice with PBS. Subsequently, the cells were lysed with self-prepared 1.5% digitonin extraction buffer (containing 20 mM Tris-HCl, pH 7.4; 140 mM NaCl; 10 mM KCl; 1 mM $MgCl_2$; 1.5% digitonin) and incubated on ice for 10 min. The lysates were then centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant was aspirated as the cytosolic fraction. Finally, cyto-c levels were analyzed using the aforementioned western blotting protocol.

*Statistical analysis*. Each experiment was performed at least three times and all statistical analyses were performed using GraphPad 8.0 software (Dotmatics). Data are presented as the mean ± standard deviation, and differences among groups were assessed by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Jaceosidin inhibits the viability of A549 cells and induces S-phase arrest*. First, A549 cells were treated with increasing concentrations of Jaceosidin for 72 h and cell viability was determined by MTS assays. The results demonstrated that Jaceosidin effectively inhibited A549 cell viability in a concentration-dependent manner, with a half maximal inhibitory concentration ($IC_{50}$) value of 55 $\mu$M (Fig. 1B). However, Jaceosidin showed less toxicity in 293T cells, with an extrapolated $IC_{50}$ value of 248.5 $\mu$M (Fig. 1C). Since cell viability did not drop to 50% within the tested concentration range (0-160 $\mu$M), the $IC_{50}$ value was estimated by non-linear regression curve fitting using Microsoft Excel (Microsoft Corporation), and 248.5 $\mu$M represents an extrapolated value beyond the experimental range. Cell cycle arrest serves a key role in inhibiting cell proliferation; therefore, to reveal whether Jaceosidin could inhibit A549 cell viability via dysregulation of cell cycle distribution, PI staining followed by flow cytometry was carried out. Following cell treatment with 40, 80 and 120 $\mu$M Jaceosidin for 24 h, no significant changes were observed in the proportion of $G_0/G_1$-phase cells, compared with the control group (Fig. 1D). However, the ratio of S-phase cells increased from 16.10% to 26.54, 27.41 and 45.19%, respectively, whereas $G_2/M$-phase cells decreased from 29.30% to 19.81, 19.66 and 4.44%, respectively, compared with control cells. These results suggested that Jaceosidin may inhibit A549 cell viability via inducing dose-dependent S-phase arrest.

*Jaceosidin induces apoptosis in A549 cells*. To determine whether Jaceosidin exhibited pro-apoptotic effects on A549 NSCLC cells, flow cytometric analysis was conducted using Annexin V-FITC/PI double staining. As

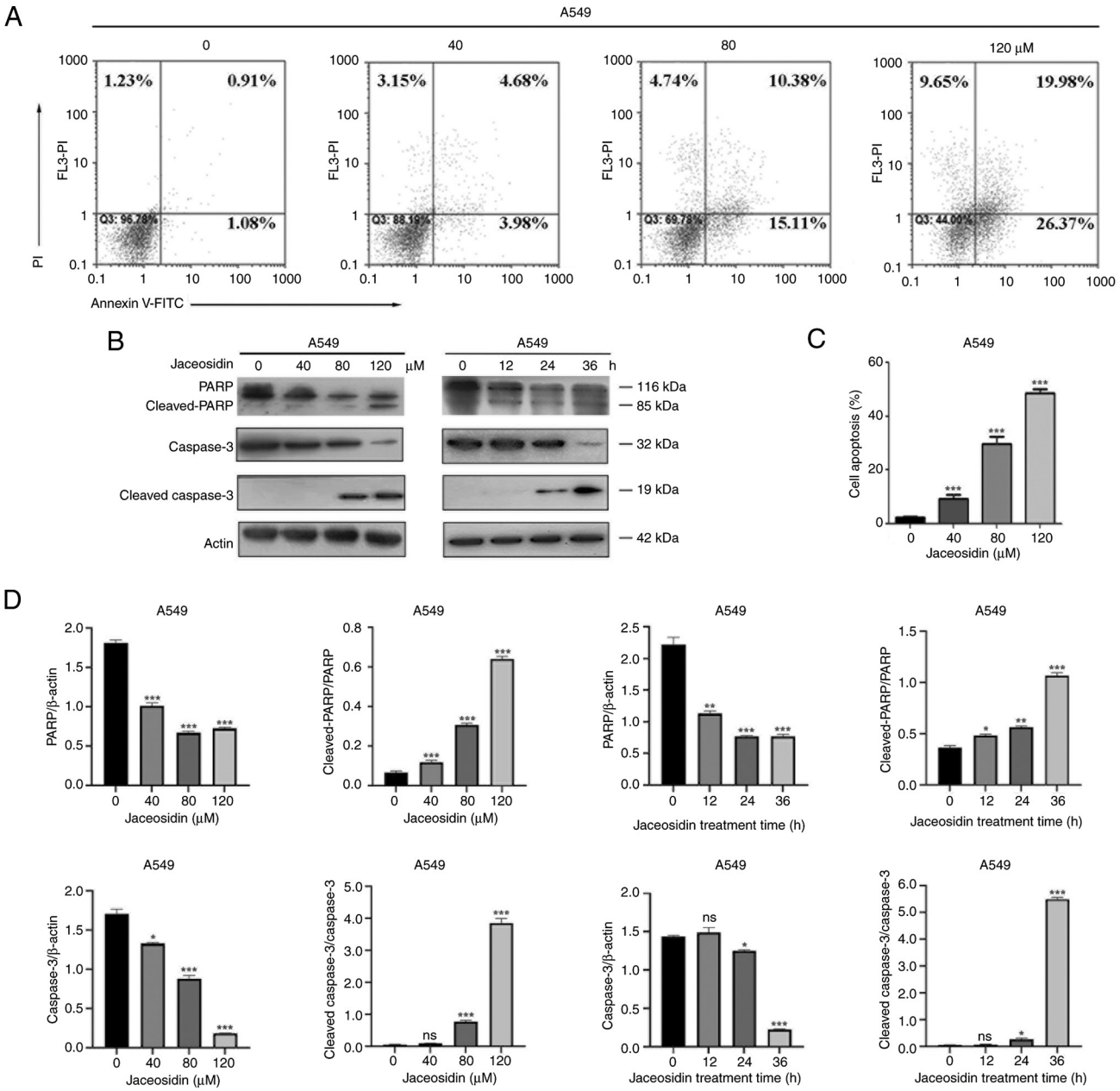


Figure 2. Jaceosidin induces apoptosis in A549 cells. (A) Annexin V-FITC/PI double staining was performed to detect apoptosis in A549 cells treated with Jaceosidin for 24 h. (B) Expression levels of the apoptosis-related proteins PARP and caspase-3 were detected in A549 cells treated with increasing concentrations of Jaceosidin for 24 h, or with 120 $\mu$M Jaceosidin for the indicated times, by western blot analysis. (C) Statistical analysis of apoptosis rates. (D) Semi-quantification of relative protein levels of PARP, cleaved-PARP, caspase-3 and cleaved-caspase-3. Data are presented as the mean ± SD of three independent experiments (n=3). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. control. ns, not significant; PARP, poly-ADP ribose polymerase; PI, propidium iodide.

shown in Fig. 2A and C, treatment with Jaceosidin for 24 h dose-dependently increased the percentage of apoptotic A549 cells compared with that in untreated cells, with marked induction of both early and late apoptosis at 120 $\mu$M. Subsequently, the expression levels of several apoptosis-related proteins, including those of PARP and caspase-3, were detected by western blot analysis. Notably, treatment of A549 cells with Jaceosidin resulted in a significant decrease in PARP and caspase-3 expression levels in a dose- and time-dependent manner (Fig. 2B and D). Consistently, the protein levels of both cleaved-PARP and cleaved-caspase-3 were markedly enhanced in a dose- and time-dependent manner. Together, these data verified that Jaceosidin could induce apoptosis in A549 cells.

*Mechanism underlying Jaceosidin-mediated inhibition of survival and induction of apoptosis*. To elucidate the mechanism underlying the effects of Jaceosidin on promoting A549 cell apoptosis, the protein expression levels of caspase-8, caspase-9, Bcl-2, Bax and cyto-c were detected. Compared with in the control group, the protein expression levels of cleaved-caspase-8 and Bax were significantly increased, whereas those of Bcl-2 were notably decreased in a dose- and time-dependent manner in Jaceosidin-treated cells

(Fig. 3A and C). Mitochondria play a key role in the regulation of apoptosis. To verify whether Jaceosidin could induce cell apoptosis via the mitochondrial pathway, the expression levels of cytoplasmic cyto-c and cleaved-caspase-9 were detected in A549 cells. The results demonstrated that Jaceosidin upregulated both cyto-c (Fig. 3B and C) and cleaved-caspase-9 (Fig. 3A and C) in A549 cells compared with those in the control group, which indicated release of cyto-c from mitochondria and activation of caspase-9. Furthermore, to investigate the mechanism involved in Jaceosidin-mediated cell cycle arrest in NSCLC cells, the protein expression levels of p21 were examined. Western blot analysis demonstrated that the expression levels of p21 were enhanced in a dose- and time-dependent manner in Jaceosidin-treated A549 cells (Fig. 3B and C). Overall, the aforementioned findings suggested that Jaceosidin could inhibit NSCLC cell viability and induce apoptosis by activating caspase-dependent mitochondrial signaling and upregulating p21.

*Jaceosidin inhibits the MAPK (Ras/Raf/MEK/ERK) and Akt signaling pathways in A549 cells.* Given the potential of MAPK and Akt signaling in treating NSCLC (4), as these pathways are frequently dysregulated in NSCLC and drive tumor cell proliferation and survival, representing key therapeutic targets, the effect of Jaceosidin on the expression levels of Ras, Raf, ERK1/2 and Akt in NSCLC cells were determined by western blot analysis. The results demonstrated that Jaceosidin dose- and time-dependently reduced the protein expression levels of Ras and Raf (Fig. 4A and B), and those of p-ERK1/2, ERK1/2, p-Akt and Akt in A549 cells (Fig. 4A and C), indicating that Jaceosidin may inhibit both the expression and activation of these key signaling proteins. These findings indicated that Jaceosidin could suppress A549 cell viability by inhibiting the MAPK and Akt signaling pathways.

## Discussion

Currently, chemotherapy remains the primary therapeutic strategy for NSCLC (15); however, conventional anticancer drugs commonly cause strong toxic side effects. Natural products have long been used in traditional medicine, and particular bioactive compounds derived from plants have been investigated as adjuvant agents in the treatment of cancer, capable of enhancing antitumor efficacy and alleviating chemotherapy-related side effects. Notable progress has thus been made in identifying novel compounds with potent and relatively low toxicity against NSCLC (16,17). Jaceosidin, a flavonoid extracted from plants of the genus *Artemisia*, exhibits potential antitumor activity against several types of cancer (8). The results of the present study demonstrated that Jaceosidin treatment could attenuate the viability of A549 cells compared with 293T cells, thus indicating that this compound could display tumor-specific cytotoxicity, further supporting its potential against NSCLC. 293T cells were used as a non-malignant control to evaluate the tumor selectivity of Jaceosidin.

Dysregulation of cell cycle progression is markedly associated with aberrant tumor cell proliferation (18). It has been reported that several anticancer drugs can trigger cell cycle arrest at $G_0/G_1$, S and $G_2$/M checkpoints, thus enabling DNA injury repair prior to mitosis (19,20); however, if the DNA damage cannot be repaired, the prolonged cell cycle arrest ultimately leads to cell apoptosis, thereby exerting an anticancer effect. The results of cell cycle analysis indicated that Jaceosidin promoted S-phase arrest, whereas no notable changes were reported in the $G_0/G_1$ phase. S-phase arrest has been identified as one of the primary molecular mechanisms underlying the action of several antitumor drugs. For example, Kim *et al* (21) demonstrated that dichloromethane fractions of *Calystegia soldanella* induced S-phase arrest in human colorectal cancer cells. Additionally, Li *et al* (22) reported that d-borneol could enhance the sensitivity of NSCLC cells to cisplatin via p21/p27-mediated S-phase arrest. In the current study, A549 cell treatment with Jaceosidin induced S-phase arrest, thus inhibiting cell viability.

p21, a cyclin-dependent kinase inhibitor downstream of p53, serves a key role in linking DNA repair and cell cycle regulation by suppressing cyclin-dependent kinase activity, thereby inhibiting cell cycle progression (23). In the current study, Jaceosidin upregulated p21 in a concentration- and time-dependent manner, thus suggesting that p21 induction could be involved in the observed Jaceosidin-mediated S-phase arrest in A549 cells. The induction of S-phase arrest by Jaceosidin is of particular biological importance for NSCLC therapy, as S phase is a critical stage for DNA replication and cell proliferation; blocking this phase effectively prevents the uncontrolled division of A549 cells (22). The upregulation of p21 not only mediates cell cycle arrest, but also acts as a bridge between cell cycle regulation and apoptosis (23), which may synergistically enhance the anti-survival effect of Jaceosidin. Additionally, Jaceosidin simultaneously activated the extrinsic (caspase-8-dependent) and intrinsic (mitochondrial/caspase-9-dependent) apoptotic pathways, which is a notable advantage for anticancer compounds, as it avoids the drug resistance caused by the single pathway activation in tumor cells (24). The crosstalk between the two apoptotic pathways further amplifies the apoptotic signal, leading to a more significant killing effect on A549 cells, which is consistent with the characteristics of effective natural anticancer products (8).

Apoptosis is closely associated with tumorigenesis. Therefore, the majority of anticancer drugs exert their effects by inducing apoptosis (25). Apoptosis can be triggered by the mitochondria (intrinsic pathway) or cell death receptors (extrinsic pathway), both promoting caspase activation (25,26). PARP is a DNA repair enzyme that serves as a cleavage substrate for caspases, key cystathionine aspartase proteases involved in apoptosis (27). Once PARP is cleaved during apoptosis, its DNA repair function is impaired. The caspase family serves a central role in mediating apoptosis, with cleaved-caspase-3 acting as the major effector downstream of both the intrinsic and extrinsic pathways, thus activating PARP and inducing apoptosis (28). Caspase-9 and caspase-8 are key proteins of the intrinsic and extrinsic pathways, respectively (25,29). In the present study, the experimental results showed that Jaceosidin significantly increased the cleaved forms of PARP, caspase-3, caspase-8 and caspase-9 during apoptosis. These findings indicated that Jaceosidin could induce apoptosis in A549 cells by activating both the intrinsic and extrinsic apoptotic pathways. Consistent

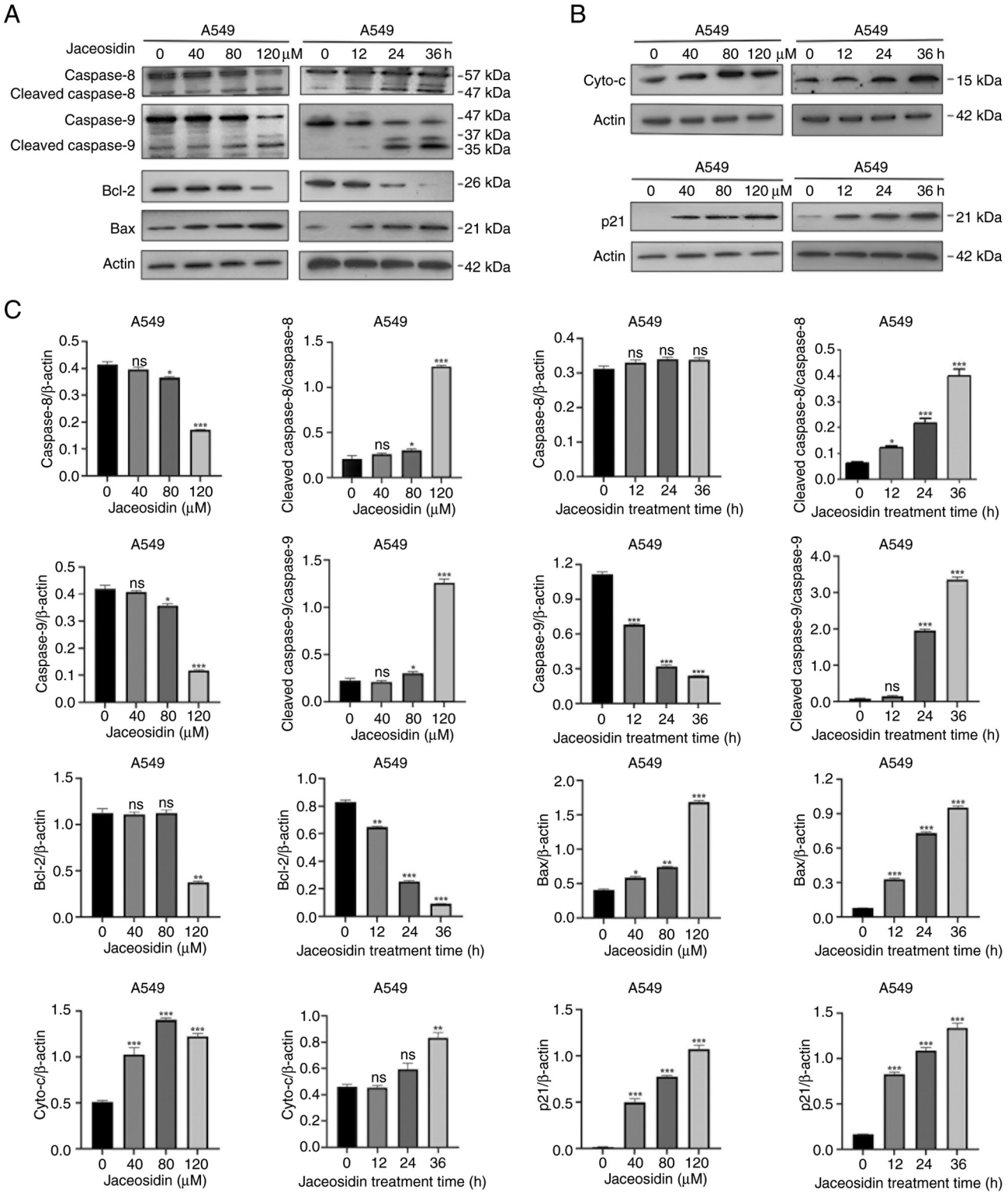


Figure 3. Effects of Jaceosidin on apoptosis-related signaling proteins and the cell cycle regulator p21. (A) Western blot analysis was performed to detect the expression levels of the apoptosis-related proteins caspase-8, cleaved-caspase-8, caspase-9, cleaved-caspase-9, Bcl-2 and Bax in A549 cells treated with the indicated concentrations of Jaceosidin for 36 h, or with 120 $\mu$M Jaceosidin for the indicated time points. (B) Western blot analysis was carried out to determine the expression levels of cyto-c in cytosolic fractions of A549 cells treated with the same concentrations of Jaceosidin. Western blot analysis of p21 was also performed under identical treatment conditions. (C) Densitometric analysis of caspase-8, cleaved-caspase-8, caspase-9, cleaved-caspase-9, Bcl-2, Bax, cyto-c and p21. Data are presented as the mean ± SD of three independent experiments (n=3). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. control. Cyto-c, cytochrome *c*; ns, not significant.

with the aforementioned results, a previous study reported that Jaceosidin could induce caspase-dependent apoptosis in the oral cancer cell lines HSC3 and Ca9.22 (30).

Mitochondria are notable regulators of cell death, and members of the Bcl-2 family, including the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2, serve key

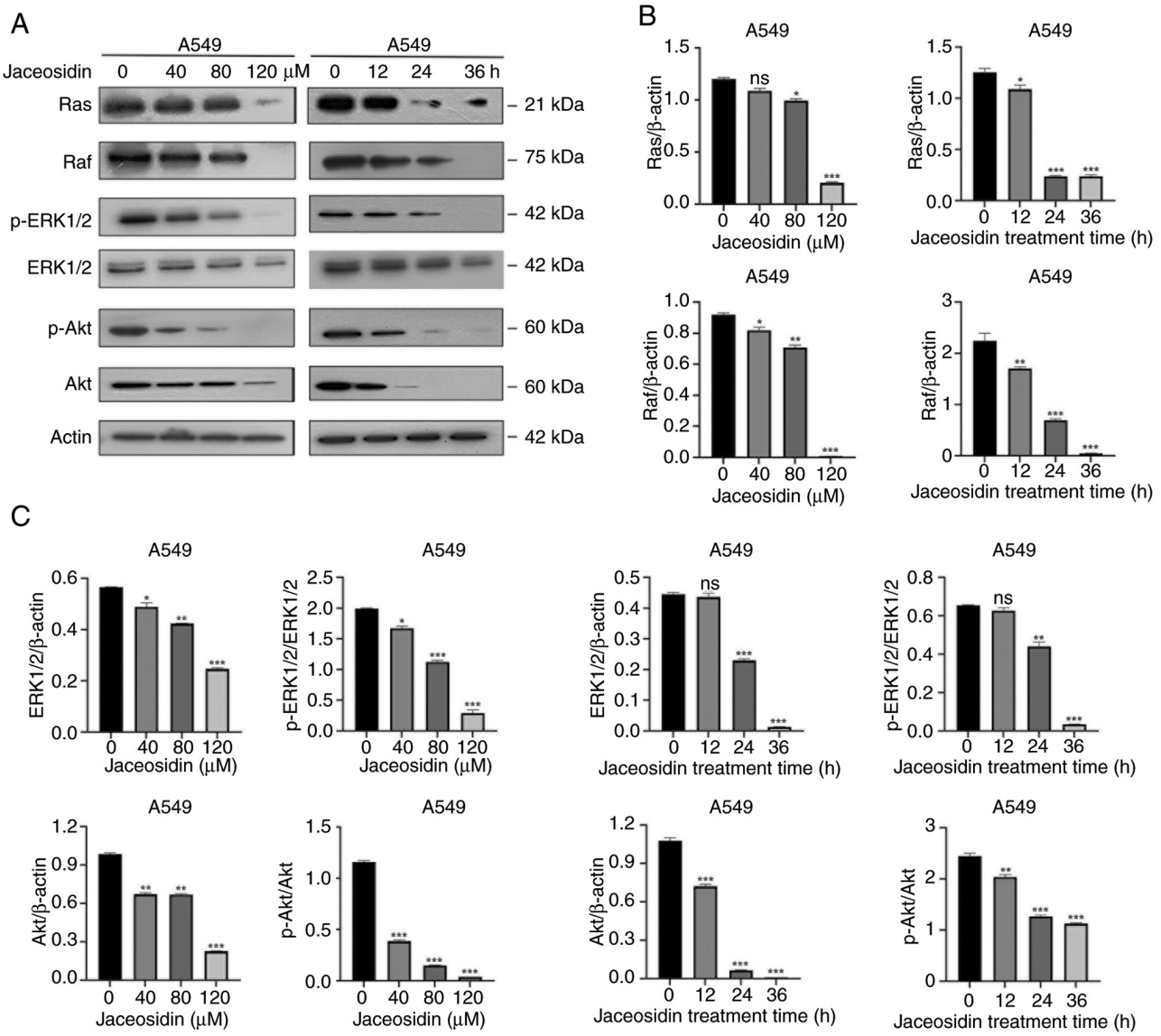


Figure 4. Jaceosidin inhibits MAPK (Ras/Raf/MEK/ERK) and Akt signaling pathways in A549 cells. (A) Western blot analysis of Ras, Raf, ERK1/2 and Akt in A549 cells treated with the indicated concentrations of Jaceosidin for 36 h, or treated with 120 $\mu$M Jaceosidin for the indicated time points. (B) Densitometric analysis of Ras and Raf. (C) Densitometric analysis of p-ERK1/2, ERK1/2, p-Akt and Akt. Data are presented as the mean ± SD of three independent experiments (n=3). Statistical differences were analyzed by one-way ANOVA followed by Tukey's post hoc test. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. control. ns, not significant; p-, phosphorylated.

roles in maintaining mitochondrial stability, and regulating apoptosis and tumorigenesis (31). Cyto-c is an apoptotic regulator found in mitochondria. Following apoptotic induction, cyto-c is released into the cytosol, where it binds apoptotic protease activating factor 1 and initiates the caspase cascade to induce apoptosis (32). In the current study, Jaceosidin treatment downregulated Bcl-2 and upregulated Bax expression in A549 cells in a dose- and time-dependent manner, thereby decreasing the Bcl-2/Bax ratio, and enhanced the release of cyto-c from mitochondria into the cytoplasm, thus leading to caspase cleavage. Therefore, it was hypothesized that Jaceosidin could induce apoptosis in A549 cells via a mitochondria-dependent pathway. These results were consistent with those reported by Kim *et al* (11), which demonstrated that Jaceosidin could induce apoptosis in Ras-transformed human mammary epithelial cells via the production of ROS, and those of Lv *et al* (33), which revealed that Jaceosidin could induce apoptosis in human ovarian cancer cells via the mitochondrial pathway. Although cytosolic cyto-c was significantly increased, the mitochondrial fraction was not assessed in the present study. Further experiments including mitochondrial separation and immunofluorescence staining for cyto-c will be performed to verify cyto-c release.

The MAPK (Ras/Raf/MEK/ERK) signaling pathway has a key regulatory role in the pathogenesis of NSCLC. The Ras gene encodes a family of proteins involved in the regulation of cell proliferation, differentiation and apoptosis (34). ERK is a downstream effector activated by several growth factors, including epidermal growth factor, nerve growth factor and platelet-derived growth factor. Activated ERK1/2 mediates and amplifies tumor-related signals during invasion and metastasis, and its phosphorylation triggers the activation of downstream substrates, thus regulating cell proliferation and apoptosis (35). In parallel, the Akt pathway has been shown to be associated

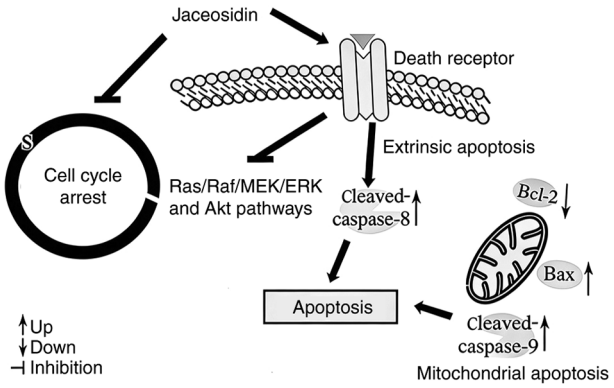


Figure 5. Proposed molecular mechanism of Jaceosidin-mediated inhibition of viability and induction of apoptosis in non-small cell lung cancer cells.

with cell survival and proliferation, and its activation has been involved in malignant growth and apoptosis escape in several types of cancer (36,37). The findings of the present study were consistent with those reported by previous studies, which showed that Jaceosidin can decrease p-ERK and p-Akt to induce apoptosis in Ras-transformed human mammary epithelial cells (11), and that Akt signaling is inhibited during Jaceosidin-mediated apoptosis in oral cancer cells (30). Collectively, these results suggested that Jaceosidin could inhibit A549 cell viability and promote apoptosis via inhibition of the Ras/Raf/MEK/ERK and Akt pathways. However, more extensive studies are needed to reveal the detailed molecular mechanisms underlying Jaceosidin-mediated pathway blockage. Additionally, Jaceosidin exhibited significant tumor-selective cytotoxicity in the present study, with an $IC_{50}$ value of 55 $\mu$M in A549 cells and 248.5 $\mu$M in normal 293T cells (a 4.52-fold difference), which provides a solid basis for its *in vivo* application and dose optimization (8). The 120 $\mu$M concentration used in the current study is an *in vitro* concentration ~2.2-fold higher than the $IC_{50}$ value determined (55 $\mu$M), which was used to clearly demonstrate the dose-dependent apoptotic effects and signaling pathway changes, a common experimental design for initial mechanistic research of natural products (17). Although the 120 $\mu$M concentration is higher than the $IC_{50}$, the significant tumor selectivity indicated that Jaceosidin has a wide therapeutic window. For *in vivo* application, the main challenge is the low bioavailability of flavonoids; future strategies, such as structural modification, nanocarrier delivery and combination therapy, may be used to improve the *in vivo* bioavailability of Jaceosidin and to reduce the effective therapeutic concentration (16). In addition, the inhibition of Ras/Raf/MEK/ERK and Akt pathways by Jaceosidin is particularly valuable for NSCLC, as these two pathways are frequently dysregulated in NSCLC and are key targets for clinical anti-NSCLC therapy (36).

It should be acknowledged that the present study has several limitations that need to be addressed in future research. First, the study was limited to the A549 lung adenocarcinoma cell line, a classical and well-characterized NSCLC cell line for mechanistic research; validation in other NSCLC cell lines (such as H1299 and H460 cells) with different genetic backgrounds is required to confirm the generalizability of the findings. Second, the mechanistic link between the inhibition of Ras/Raf/MEK/ERK and Akt pathways and apoptosis/cell cycle arrest is associative in the present study; future studies using specific pharmacological inhibitors (such as U0126 for MEK/ERK and LY294002 for PI3K/Akt) and genetic knockdown approaches will further confirm the causal relationship. Third, ROS, which has been implicated in Jaceosidin-induced apoptosis in other cancer models (11), was not explored in the current study; the role of ROS in Jaceosidin-mediated mitochondrial apoptosis and pathway inhibition in A549 cells will be investigated in subsequent work using DCFH-DA staining. Fourth, the present study only included *in vitro* experiments; *in vivo* xenograft models are needed to verify the anticancer effect of Jaceosidin and its pharmacokinetic characteristics. Fifth, the direct or indirect regulatory effect of Jaceosidin on the Ras/Raf/MEK/ERK and Akt pathways remains unclear; future biochemical experiments such as molecular docking will be performed to explore the potential direct binding of Jaceosidin to key proteins in these pathways (34).

In conclusion, given the high incidence and mortality rates of NSCLC, identifying effective therapeutic drugs is of great importance. Herein, the inhibitory effects of Jaceosidin on A549 NSCLC cells and its underlying mechanism of action were investigated. The results demonstrated that Jaceosidin upregulated p21 and induced cell cycle arrest, thus attenuating A549 cell viability. Furthermore, Jaceosidin promoted the cleavage of caspases and PARP, triggering apoptosis through both extrinsic death receptor and intrinsic mitochondrial pathways (Fig. 5). Notably, Jaceosidin may disrupt mitochondrial stability by downregulating Bcl-2 and upregulating Bax, thus facilitating cyto-c release from the mitochondria and the subsequent activation of apoptosis through the intrinsic mitochondrial pathway. Pathway analysis further indicated that the anti-survival and pro-apoptotic effects of Jaceosidin were mediated through inhibition of the Ras/Raf/MEK/ERK and Akt signaling pathways.

Overall, although additional experiments are required to fully uncover the regulatory network of Jaceosidin, the results of the current study suggested that it could represent a promising and innovative active agent for the treatment of NSCLC.

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**Availability of data and materials**

The data generated in the present study may be requested from the corresponding author.

**Authors' contributions**

XC, CM, XH and LC conceived and designed the study. Experiments were performed by XC, CM, XW, CW, LS, JW and RY. Data processing, analysis and interpretation were performed by XC, CM and XW. The manuscript was written by XC, CM and XW. Funding was obtained by XH and LC. YL

participated in study design, supervised the study and revised the manuscript. XH and LC supervised the study and revised the manuscript. XC and CM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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