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# *Celastrus orbiculatus* Extract Inhibits the Malignancy of Non-Small Cell Lung Cancer Cells Through PI3K/AKT/mTOR Pathway

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**Aim: This study is targeted at investigating the effects of *Celastrus orbiculatus* extract (COE) on non-small cell lung cancer (NSCLC) cells.**

**Methods: At 24, 48 and 72 h after different concentrations of COE were applied to treat NSCLC cells, MTT method, 5-Ethynyl-2′-deoxyuridine (EdU) assay, Transwell assay and flow cytometry were conducted to explore the effects of COE at different concentrations on the phenotypes of NSCLC cells. The expression of apoptosis-associated proteins and PI3K/AKT/mTOR-associated proteins were examined via Western blot.**

**Results: COE inhibited NSCLC cell multiplication, migration and invasion and facilitated the apoptosis, and this effect was dose-dependent. Additionally, also in a dose-dependent manner, COE suppressed Bcl-2 expression and facilitated Bax expression. After COE treatment, the phosphorylation levels of AKT, mTOR and PI3K were noticeably decreased.**

**Conclusion: COE suppresses the malignancy of NSCLC cells, probably via inhibiting PI3K/AKT/mTOR signaling.**

**Keywords:** *Celastrus orbiculatus* extract; PI3K/AKT/mTOR; NSCLC; proliferation

## Introduction

Lung cancer (LC) is a type of malignancy that seriously threatens human health, and 85% of LC patients are non-small cell lung cancer (NSCLC) cases [1,2]. At present, the main clinical treatment strategies include surgical excision, chemotherapy, molecular targeted therapy, radiotherapy and immunotherapy [3]. In China, traditional Chinese medicine is also applied for NSCLC patients as complementary/alternative medicine [4]. NSCLC patients' median survival time is far from satisfactory [5]. In this context, in a bid to improve the treatment efficacy, it is vital to decipher the mechanism underlying NSCLC tumorigenesis and find novel treatment methods.

Belonging to the genus Celastrus of the family Celastraceae, *Celastrus orbiculatus* is an important medicinal plant [6]. The stems, roots, fruits and leaves of *Celastrus orbiculatus* can be used as medicine in theory of traditional Chinese medicine, and showing pharmacological properties such as anti-inflammation and detumescence [7]. Previous studies have suggested that *Celastrus orbiculatus* extract (COE) contains various pharmacologically active ingredients, mainly terpenoids, aliphatics and glycosides; these natural compounds have varying pharmacological effects, such as anti-tumor, anti-inflammatory, immunosuppressive, antioxidant and anti-fibrosis effects [8,9]. Research has discovered that COE can restrain tumor growth and induce cancer cell apoptosis of multiple malignancies, such as hepatic carcinoma [10], gastric cancer [11], colorectal carcinoma [12], and nasopharyngeal carcinoma [13]. Nevertheless, the effects of COE on NSCLC cells and the potential mechanisms remain largely unknown.

Previous research has proven that PI3K/AKT/mTOR signaling features prominently in tumor cell proliferation and apoptosis [14]. For example, liquiritigenin induces autophagy-related cell death via suppressing this pathway, thus inhibiting the malignant progression of oral carcinoma [15]; in NSCLC, ROR1-AS1 knockdown inactivates this pathway, thereby suppressing NSCLC cell multiplication and invasion and promoting the apoptosis [16]. Nevertheless, it is not clear whether COE can play a role by modulating the PI3K/AKT/mTOR axis in NSCLC cells. We reports that COE shows the pharmacological property to inhibit the malignancy of NSCLC cells, probably via PI3K/AKT/mTOR axis.

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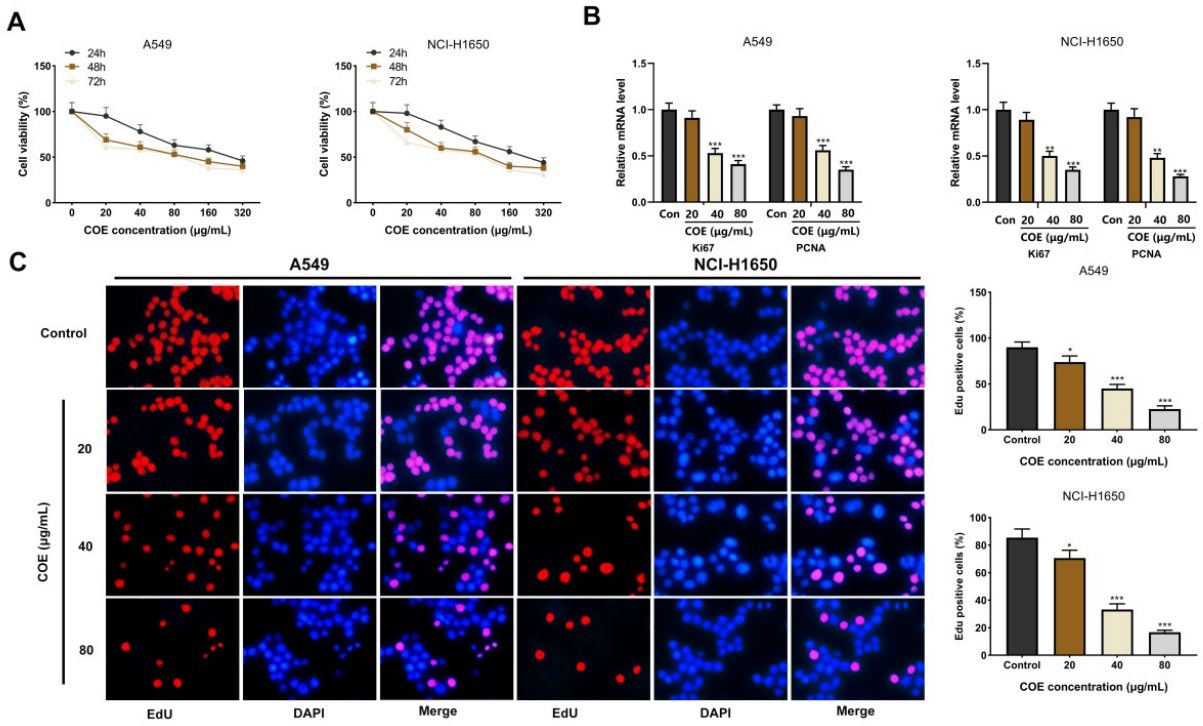


**Fig. 1. *Celastrus orbiculatus* extract (COE) inhibits non-small cell lung cancer (NSCLC) cell proliferation.** (A) After A549 and NCI-H1650 cells were treated with 0, 20, 40, 80, 160 and 320 μg/mL COE for 24, 48 and 72 h, A549 and NCI-H1650 cell viability were detected by the MTT method. (B) Detection via quantitative real-time PCR (qRT-PCR) of the relative expression of proliferation-related proteins Ki67 and PCNA. (C) 5-Ethynyl-2′-deoxyuridine (EdU) assay was conducted to detect NCI-H1650 and A549 cell proliferation capability. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, compared with the control group.

## Materials and Methods

### *Cell Lines*

From the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), NCI-H1650 and A549 cell lines were obtained. The cells were not contaminated, and authenticated with STR profile method. RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS; Gibco) was selected for culturing the NSCLC cell lines at 5% $CO_2$ in 37 °C. When the NSCLC cells grew to 90% confluence, they were trypsinized and passaged (at a ratio of 1:3).

### *Acquisition of COE*

*Celastrus orbiculatus* (Batch No. 070510) were purchased from Guangzhou Zisun Pharmaceutical Co., Ltd. The main process of the preparation of COE: cut the *Celastrus orbiculatus* into slices; crush them into powder; heat and reflux with 95% ethanol to extract 3 times; recover the solvent till it is dry; add water to disperse, and extract 3 times with petroleum ether and ethyl acetate; recover the solvent, concentrate under reduced pressure, and vacuum freeze and dry to obtain COE. Dimethyl sulfoxide (DMSO) was used to dissolve the extracts, and serum-free medium was used to prepare the working solution of the concentration required for the experiments. Before treating the cells, the working solutions were filtered and sterilized under the normal pressure.

### *MTT Assay*

The NCI-H1650 and A549 and cells were prepared into single-cell suspension ($3 \times 10^5$ cells/mL), and 100 μL of the suspension was added into each well of a 96-well plate. The wells were divided into COE treatment groups (20, 40, 80, 160 and 320 μg/mL) and a control group (added with DMSO). 6 replicate wells were set for each group. After culturing the cells for 24, 48 or 72 h in a 37 °C, 5% $CO_2$ incubator, 15 μL of 5 g/L MTT was added into each well. After culturing for another 4 h, the supernatant was discarded and 100 μL of DMSO was added into each well and the crystals were dissolved on a shaker. Finally, the absorbance of each well at 490 nm was determined on the microplate reader, and the cell viability was calculated.

### *Quantitative Real-time PCR (qRT-PCR)*

TRIzol reagent (Invitrogen) was adopted to extract the total RNA of the cells, and then the purity and concentration of RNA were evaluated on the spectrophotometer (ThermoFisher). A PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara) was adopted

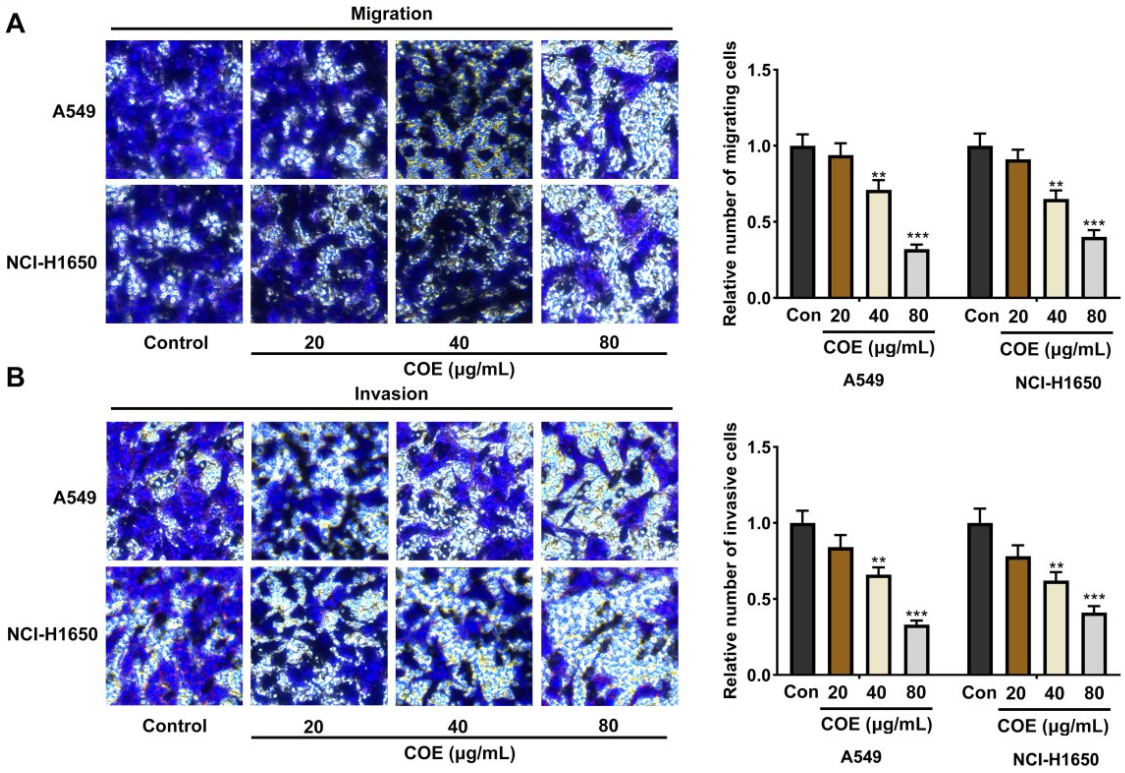


**Fig. 2. COE suppresses NSCLC cell migration and invasion.** (A,B) After A549 and NCI-H1650 cells were treated with 20, 40 and 80 μg/mL COE, A549 and NCI-H1650 cell migration and invasion abilities were detected by Transwell assays. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with the control group.

for the reverse transcription of the RNA into cDNA, and finally a SYBR Premix Ex Taq™ II Kit (Takara, Otsu, Japan) was employed for performing amplification. From Vazyme Biotech (Jiangsu, China), we obtained the primers for proliferating cell nuclear antigen (PCNA) and Ki67. The primer sequences (F for Forward; R for Reverse): PCNA: F: 5′-GTAATGACTCTATGTGATGCC-3′, and R: 5′-GATAAAAGGTTACAAACGATG-3′; Ki67: F: 5′-CTCCATCCTGGCCTCGCTGT-3′ and R: 5′-GCTGTCACCTTCACCGTTCC-3′.

### *5-Ethynyl-2′-deoxyuridine (EdU) Assay*

NSCLC cells ($5 \times 10^3$ cells/well, in a 96-well plate) were cultured for 24 h, and each well was added with 50 μmol/L EdU medium (RiboBio, 200 μL), incubated in the incubator for 2 h. After fixation and washing, each well was supplemented with PBS (100 μL) containing 0.5% TritonX-100, incubated for destaining for 10 min on a shaker, and subsequently rinsed twice with PBS for 5 min each time. Next, the cells were stained with Apollo (30 min, 37 °C, away from light) and DAPI (20 min, 37 °C, away from light) orderly. Eventually, the cell images were collected by a fluorescence microscope (Olympus). The nuclei of the positive cells were excited to show red fluorescence, and the DAPI-counterstained nuclei showed blue fluorescence after excitation.

### *Invasion and Migration Assays*

In cell invasion assay, the Transwell chamber (Millipore) membrane was precoated with Matrigel (BD Biosciences). The NCI-H1650 and A549 cells during logarithmic growth were prepared into single-cell suspension with serum-free medium, and inoculated in the upper Transwell chamber ($1 \times 10^6$ cells/well), and 10% FBS-containing medium (500 μL) was supplemented to each bottom compartment. The Transwell chambers were taken out 12 h later, and washed with PBS twice. Then, cotton swabs were applied to wipe the non-invaded cells off the membrane. The invaded cells were fixed and stained, and finally observed with an inverted microscope (Olympus, Tokyo, Japan). In the cell migration assay, the Matrigel was not used, and the other procedures followed the protocol of the invasion assay.

### *Flow Cytometry*

After COE treatment, NCI-H1650 and A549 cells were trypsinized, collected, and resuspended with 1 × binding buffer (1 mL), and mixed with annexin V-FITC (5 μL) and propidium iodide (PI) (5 μL) (Beyotime). After the mixture was shaken and mixed gently, it was incubated (37 °C, away from light, 15 min). Then, after diluting in 1 × binding buffer, we employed a flow cytometer (BD Biosciences) to evaluate the cell apoptosis rate.

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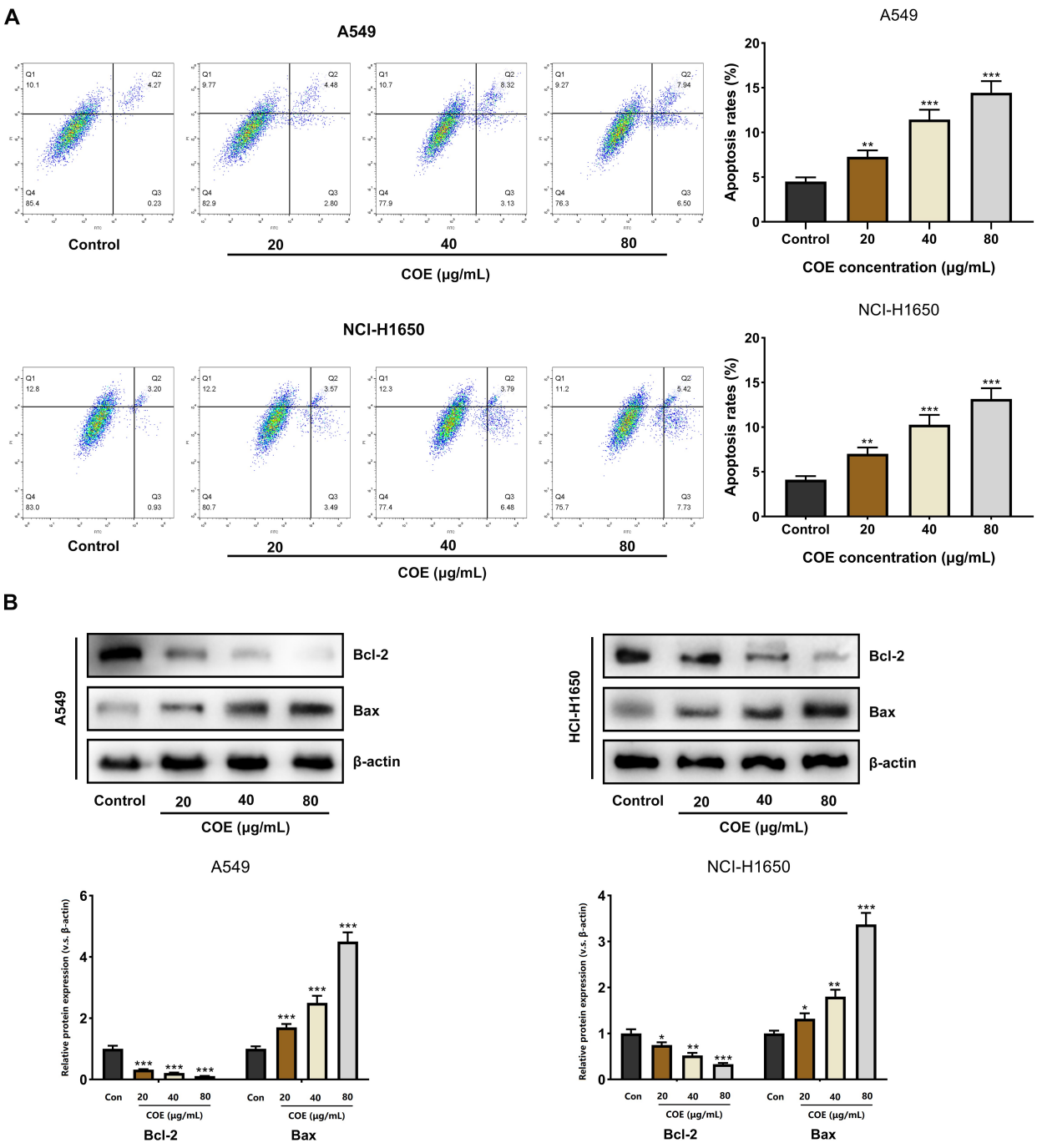


**Fig. 3. COE promotes NSCLC cell apoptosis.** (A) After A549 and NCI-H1650 cells were treated with 20, 40 and 80 μg/mL COE, the effects of COE on A549 and NCI-H1650 cell apoptosis were analyzed by flow cytometry. (B) Western blotting was conducted to detect the expression of Bcl-2 and Bax. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with the control group.

*Western Blot Assay*

The cells during logarithmic growth were added with different concentrations of COE. After 24 h of intervention, we extracted the total cell protein, and the concentration of the protein in each group was measured by the bicinchoninic acid kit (Peirce). After being separated by SDS-PAGE (Millipore), we transferred the protein onto the polypropylene fluoride (PVDF) membrane (Millipore), followed by membrane blocking with 5% skimmed milk (2 h, 37 °C, on a shaker). The membrane was subsequently incubated with primary antibodies (12 h, 4 °C, on a shaker) and, after membrane washing, incubated with secondary antibody (1:2000; ab205718; Abcam) (2 h, 37 °C, on a shaker). The ECL luminescence kit (ThermoFisher) was

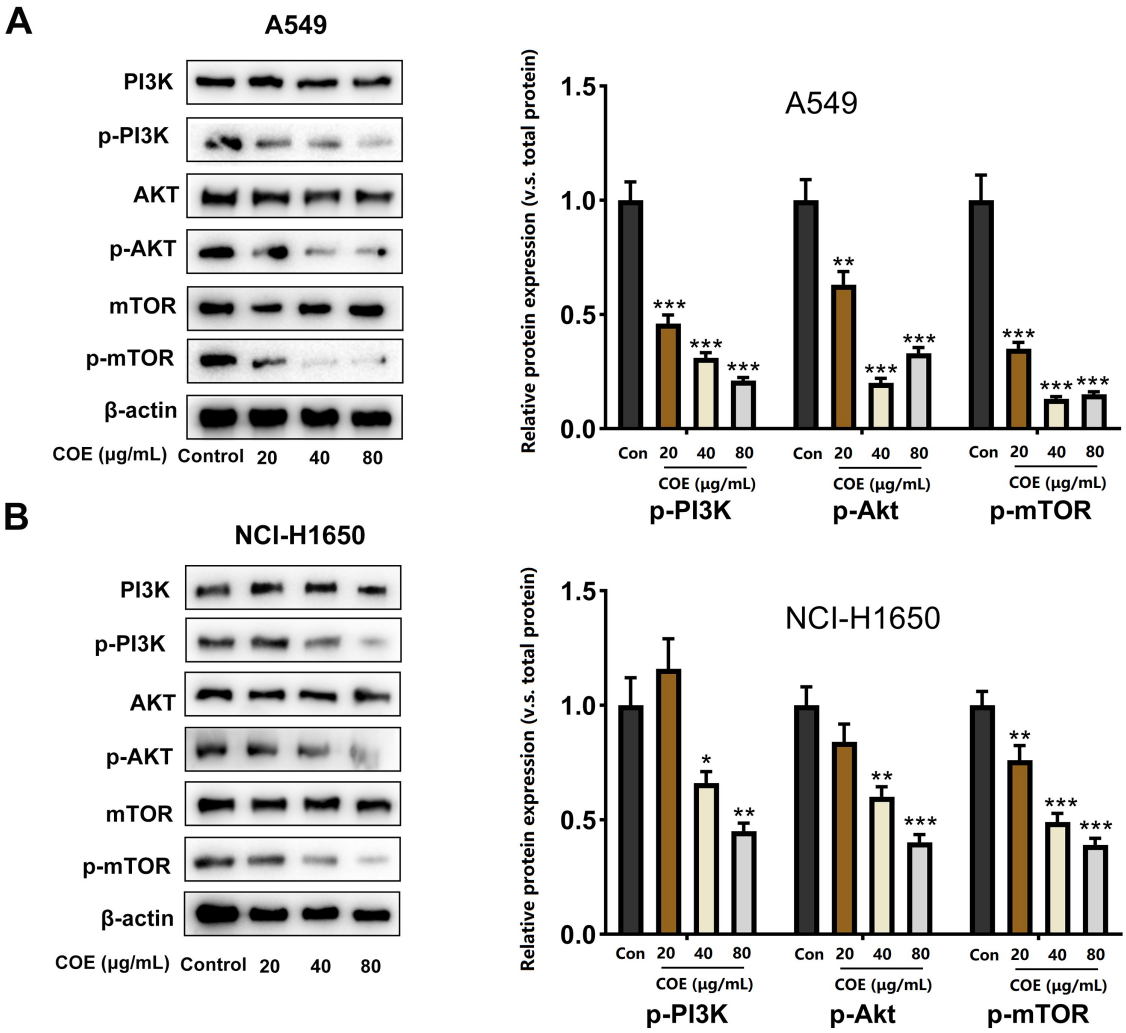


**Fig. 4. COE suppresses the activation of PI3K/AKT/mTOR pathway.** (A,B) After A549 and NCI-H1650 cells were treated with 20, 40 and 80 μg/mL COE, the expression levels of p-PI3K, p-AKT, and p-mTOR were detected via Western blot. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared with the control group.

utilized to develop the related protein bands. The protein bands were photographed and analyzed by a protein gel analyzer. From Abcam (Shanghai, China), we purchased the primary antibodies used in this study: anti-Bax (ab32503), anti-Bcl-2 (ab32124), anti-$\beta$-actin (ab8229), anti-PI3K (ab191606), anti-phospho PI3K (ab182651), anti-AKT antibody (ab8805), anti-phospho AKT antibody (ab38449), anti-mTOR antibody (ab32028), and anti-phospho mTOR (ab109268). The dilution ratio of the primary antibodies: 1:1000.

### *Statistical Method*

Mean ± standard deviation was the expression form of the measurement data, and the statistical processing tool was SPSS 24.0 statistical software (SPSS Inc.). One-way analysis of variance was performed for data comparison among multiple groups, followed by Tukey's post-hoc test. $p < 0.05$ implied being statistically significant.

## Results

### *COE Inhibits NSCLC Cell Proliferation*

To study whether COE affects NSCLC cell growth, the MTT method was utilized to examine NSCLC cell viability. The MTT results manifested that after treatment with COE (20, 40, 80, 160, and 320 μg/mL) for 24, 48 and 72 h, NSCLC cell growth was inhibited to varying degrees and in a certain time- and concentration-dependent manner (Fig. 1A). To exclude the cytotoxic effect of the drug, COE of 20, 40 and 80 μg/mL were employed for follow-up experiments. At the same time, COE largely inhibited the expression of the proliferation-associated genes PCNA and Ki67 in NCI-H1650 and A549 cells (Fig. 1B). EdU stain-

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ing indicated that the number of proliferating NCI-H1650 and A549 cells was reduced after intervention with COE as opposed to the normal group (Fig. 1C).

### *COE Inhibits NSCLC Cell Aggressiveness*

Through the Transwell cell migration assay, the effects of COE on NSCLC cell invasion and migration were analyzed, and it was unveiled that as against the control group, the invasion and migration capacities of NCI-H1650 and A549 cells intervened with COE were weakened as the drug concentration was increased and in a certain concentration-dependent manner (Fig. 2A,B).

### *COE Promotes NSCLC Cell Apoptosis*

To clarify whether COE induces the apoptosis of NSCLC cells, we treated NCI-H1650 and A549 cells with COE for 24 h, and then detected their apoptosis using the flow cytometer. Flow cytometry manifested that the apoptosis of COE-treated cells was enhanced relative to the control group (Fig. 3A). Consistently, Bcl-2 expression was decreased significantly with the increase of COE concentration, whereas Bax expression increased significantly (Fig. 3B).

### *COE Modulates PI3K/AKT/mTOR Axis*

To determine whether PI3K/Akt/mTOR signaling is implicated in NSCLC progression, we treated NCI-H1650 and A549 cells with COE for 24 h, and detected the phosphorylation levels of PI3K, AKT, and mTOR via Western blotting. As shown (Fig. 4A,B), with the treatment of COE, p-PI3K, p-AKT, and p-mTOR proteins in the NSCLC cells were markedly downregulated, which was concentration-dependent. This suggests that COE suppresses the malignancy of NSCLC cells by inhibiting the activation of the PI3K/Akt/mTOR axis.

## Discussion

Natural drugs have become a hot spot in anti-tumor research in recent years, and many herb extracts in traditional Chinese medicine have showed the potential to be new anti-cancer drugs, with the advantages of low side effects and low cost [17,18]. *Celastrus orbiculatus* is distributed mainly in the northeast and southwest of China [7,19]. Modern pharmacology finds that COE has an anti-tumor effect, inhibiting tumor invasion, metastasis and angiopoiesis, and promoting tumor cell apoptosis [20–23]. Reportedly, COE can reduce the expression levels of matrix metalloproteinases and repress epithelial-mesenchymal transition of gastric cancer cells, and restrain the aggressiveness of cancer cells through repressing the activation of the PI3K/Akt/Snail axis [24]. In liver cancer, mTOR inhibitor rapamycin and COE synergistically induces hepatocellular carcinoma cell apoptosis via modulating apoptosis-associated proteins and suppressing the mTOR pathway [25]. Moreover, another study reports that COE treatment may suppress liver cell viability and induce mitochondrial-mediated apoptosis [26]. Our study first found that COE can suppress NSCLC cell multiplication, migration and invasion and induce apoptosis in a dose-dependent manner, and suggests that COE is also potential to work as an anti-cancer drug for NSCLC.

The PI3K/AKT/mTOR axis is a pivotal pathway implicated in cell cycle, cell multiplication and energy metabolism, and accordingly it is crucial in tumorigenesis [27]. PI3K as a lipid kinase is a heterodimer made up of a catalytic subunit p110 and a regulatory subunit p85, and it is activated by many extracellular factors and participate in cell growth and survival [28]. AKT is an important downstream molecule of PI3K, and once AKT is hyperphosphorylated, it can activate multiple target proteins in the cytoplasm and nucleus to promote cell proliferation and survival [29]. mTOR is a key downstream molecule of AKT, and activated mTOR can activate the eukaryotic initiation factor E4 to facilitate cell survival by inhibiting autophagy [30]. In NSCLC, PI3K/AKT/mTOR signaling is in an activated state in about 90% of NSCLC cell lines, which can promote LC cell multiplication and inhibit cell apoptosis [31]. Drugs targeting PI3K/Akt/mTOR may induce NSCLC cell apoptosis, and this is considered to be a strategy to treat NSCLC [32]. Specifically, in adenocarcinoma patients carrying EGFR mutations, abnormal activation of PI3K/AKT/mTOR signaling is a mechanism of acquired resistance to EGFR-TK inhibitors [33]. Additionally, Vitexin can repress PI3K/AKT/mTOR signaling to suppress NSCLC cell proliferation and boost the apoptosis [34]. Betalain can increase NSCLC cell apoptosis and induce cell cycle arrest, and noticeably suppress PI3K/Akt/mTOR, and has anti-tumor activity [35]. This study indicated that the p-AKT, p-mTOR and p-PI3K levels were decreased in NSCLC cells treated with different doses of COE. The above-mentioned evidence demonstrates that COE facilitates NSCLC cell apoptosis by suppressing the PI3K/AKT/mTOR signaling activation in NSCLC cells.

Notably, some weaknesses exit in the present study. First and foremost, the detailed mechanism by which COE modulate the activation of PI3K/AKT/mTOR pathway is not investigated. Additionally, COE is a mixture of multiple bioactive constituents, and the dominant of its pharmacological effect on NSCLC cells needs to be identified in the subsequent studies. What's more, animal experiments are necessary to further verify the suppressive effects of COE on the malignancy of NSCLC cells, and this will be an important basis for its clinical application, to evaluate its safety and determine the dose. Last but not least, as an option of complementary/alternative treatment, the synergistic effects of COE and chemotherapy/radiotherapy to kill NSCLC cells await to be explored.

## Conclusion

To sum up, our study confirms that COE can inhibit NSCLC cell multiplication, migration and invasion, probably via repressing PI3K/AKT/mTOR signaling. In the following work, more data are needed to evaluate the potential of COE as the complementary/alternative drug to treat NSCLC in clinical practice.

## Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

## Funding

This research received no external funding.

## Conflict of Interest

The authors declare no conflict of interest.

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