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Antiproliferative Activity of Carnosic Acid is Mediated via Inhibition of Cell Migration and Invasion, and Suppression of Phosphatidylinositol 3-Kinases (PI3K)/AKT/ Mammalian Target of Rapamycin (mTOR) Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Lung cancer is one of the leading causes of cancer-related mortalities worldwide and majority of these deaths result from non-small cell lung cancer (NSCLC). The primary objective of this research was to determine the anticancer potential of carnosic acid, a plant derived abietane diterpene, against human lung cancer cells, as well as to determine its effects on cell migration and invasion, apoptosis, and the PI3K/AKT/m-TOR signaling pathway.

Material/Methods: Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay; fluorescence microscopy using acridine orange/ethidium bromide stain and Comet assay were used to study cellular apoptosis. *In vitro* wound healing assay was used to study effects on cell migration; Transwell assay was used to study cell invasion after drug treatment. Western blot assay was used to study effects of carnosic acid on the PI3K/AKT/m-TOR signaling pathway.

Results: It was shown that carnosic acid could inhibit the growth of A-549 human non-small cell lung carcinoma cells dose-dependently showing an IC_{50} value of 12.5 μM. This growth inhibition of A-549 cells was mediated via apoptotic cell death as observed by fluorescence microscopy showing nuclear fragmentation and chromatin condensation. Carnosic acid, dose-dependently, also inhibited cell migration and invasion. Finally, western blot assay revealed that carnosic acid also led to inhibition of the PI3K/AKT/m-TOR signaling pathway.

Conclusions: In conclusion, our results showed that Carnosic acid has the potential to inhibit cancer cell growth in A-549 lung cancer cells by activating apoptotic death, inhibiting cell migration and invasion and suppressing PI3K/AKT/m-TOR signaling pathway.

MeSH Keywords: Apoptosis • Cell Migration Assays • Lung Neoplasms • Phosphatidylinositol 3-Kinases

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Background

Lung cancer is ranked among the most frequent type of human malignancy affecting both women and men and its incidence is continuously rising. In China, the prevalence and mortality arising out of lung cancer has significantly increased recently making lung cancer in China the leading cancer in cancer-related deaths. Lung cancer has been shown to depend on gender as well as geographical differences arising out of different lifestyles and level of socioeconomic development. Several potential risk factors of lung cancer have been identified including air pollution, smoking, and asbestos exposure [1–3]. On the basis of histology, lung cancer can be divided into 2 types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The majority of the lung cancer cases (about 85%) are diagnosed as NSCLC. NSCLC can be further categorized into 2 subtypes: squamous cell carcinoma and adenocarcinoma [4,5]. In spite of the fact that various advancements have been recently achieved in early detection of the disease as well as treatment, there is still less than a 20% survival rate for NSCLC. The treatment of NSCLC includes use of chemotherapeutic drugs like cisplatin and oxaliplatin in tandem with paclitaxel or gemcitabine or vinorelbine. But in most patients, this treatment is effective only in earlier stages, and in the advanced stages, lung cancer cells acquire drug resistance which renders such treatments less effective. Furthermore, use of chemotherapy for lung cancer treatment is associated with severe side effects [6,7]. Plants have always played a critical role in reducing human diseases by furnishing anticancer, antibacterial, and antiviral drugs. The majority of the anticancer drugs that are currently used to treat cancer are either pure natural products or their synthetic derivatives [8,9]. Carnosic acid, mainly found in rosemary (*Rosmarinus officinalis*), is a naturally occurring key polyphenolic diterpene. It has been reported to possess several biological and pharmacological functions, including anticancer, anti-inflammation, and antivirus [10]. Keeping in view the outstanding potential of plant derived natural products as possible anticancer agents; the main objective of the current research work was to investigate the anticancer properties of carnosic acid on human lung carcinoma cells along with examining its effects on cancer cell migration and invasion, programed cell death and the PI3K/AKT/m-TOR signaling pathway.

Material and Methods

Evaluation of cell viability by CCK-8 (Cell Counting Kit-8) assay

The A-549 NSCLC cells and normal lung cells (MRC-5 cell line, fibroblasts derived from lung tissue) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells (both A-549 and MRC-5) were maintained in RPMI-1640 medium (Gibco, USA) at 37°C with 95% humidity and 5% CO_2. The cell viability of A-549 cells was examined by CCK-8 (Cell Counting Kit-8) assay (Dojindo Laboratories, Kumamoto, Japan). The A-549 cells were primarily treated with 0, 3.12, 6.25, 12.5, 25, 50, 100, and 200 µM dosages of carnosic acid following which 40 µL CCK-8 solution was gently added to the cell culture plates. The cell plates were kept for incubation for 12 hours at 37°C. The absorbance was measured at 450 nm wavelength using a microplate reader (BioTek Instruments, USA).

Colony formation assay

In order to examine effects of carnosic acid on the colony forming potency of A-549 cells, the method of Gupta et al. [11] was followed. In brief, A-549 lung cancer cells were treated with carnosic acid (with varying doses of 0, 12.5, 25, and 50 µM) for 24 hours. After treatment, the cells were seeded in the 6-well plates and again incubated for 6 days at 37°C. The cell colonies so developed were subjected to staining with crystal violet (0.3%) and counted under an inverted microscope (Olympus Corporation, Japan).

Acridine orange/ethidium bromide (AO/EB) staining assay and Comet assay for apoptosis

The A-549 human lung cancer cells at a cell density of 1×10^6 cells/mL were seeded into 6-well plates and cultured for 24 hours. The cells were treated with numerous concentrations of carnosic acid (12.5, 25, and 50 µM) and incubated for 12 hours. Subsequently, 20 µL of cell culture were placed onto glass slides and subjected to staining with acridine orange/ethidium bromide separately (20 µL). The glass slides were then covered with cover slips and examined under fluorescence microscope (Olympus Corporation, Japan) for evaluating apoptotic changes. DNA damage which accompanies apoptosis was measured by Comet assay which is an alkaline single cell gel electrophoresis assay and was performed as per the guidelines already mentioned in the literature [12].

Cell migration evaluation by *in vitro* wound healing method

The A-549 cells at a cell density of 1×10^5 cells/ml were plated in 6-well plates and for 12 hours these cells were cultured followed by treatment with increasing dosages of carnosic acid (12.5, 25, and 50 µM) for 24 hours. Then a sterile 20 µL pipette was used to make a straight cell-free wound in the wells and the suspended cells were thrown out. The A-549 lung cancer cells were cultured further and maintained in RPMI-1640 medium and lastly cancer cell migration of A-549 cells was observed by using optical microscope after every 48 hours using an inverted microscope system (Olympus Corporation, Japan).

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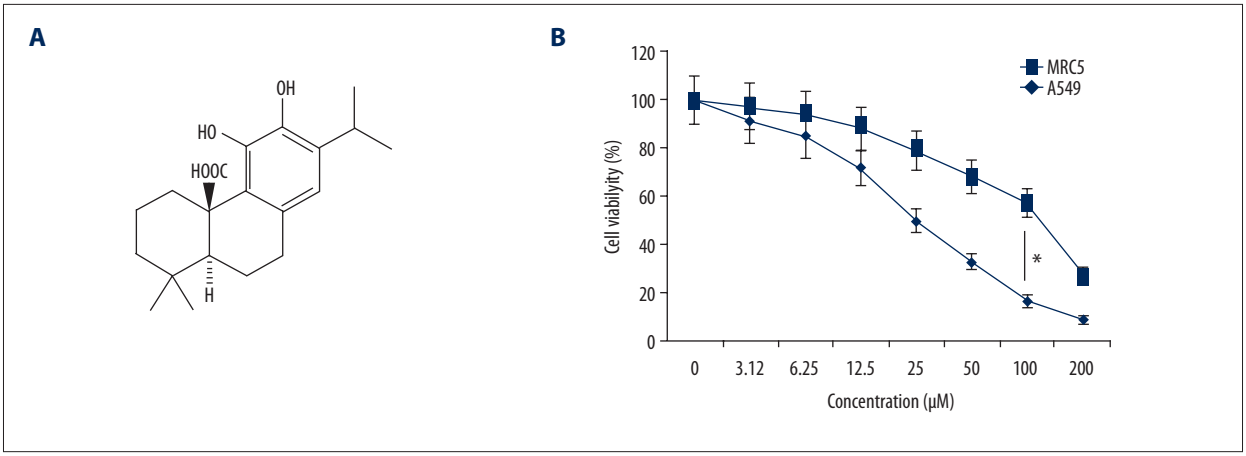

Figure 1. (**A, B**) Cell Counting Kit-8 (CCK-8) assay showing the effects of carnosic acid on the viability of the A-549 non-small cell lung cancer cells and normal lung cells (MRC-5 cell line). The experiments were performed in triplicate and shown as mean ± standard deviation (* *P*<0.05).

Transwell assay

The effects of carnosic acid on the invasion ability of A-549 cells, at varying doses (0, 12.5, 25, and 50 μM), was determined by Transwell chambers (8 mm pore size, Corning, NY, USA) with Matrigel (Millipore, Billerica, USA) The A-549 cells were transfected with miR-299 mimics and NC (control) and around 200 mL cell cultures were placed onto the upper chambers and only medium was placed in the bottom wells. After 24 hours of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were subjected to fixation with methyl alcohol and subsequently stained with crystal violet. Inverted microscope (Olympus Corporation, Japan) was used to count the number of invaded cells at 200× magnification.

Western blot analysis

The A-549 treated cells at varying concentrations (0, 12.5, 25, and 50 μM) were lysed in RIPA-lysis buffer containing the protease inhibitor. Around 45 μg of proteins from each sample were subjected to separation using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 hour. Thereafter, the membranes were treated with primary antibodies at 4°C for overnight. Subsequently, the membranes subjected to incubation with secondary antibodies. Finally, the signal was detected by Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Waltham, MA, USA). Actin was used as control for normalization.

Statistics

The results are presented as mean ± standard deviation values from 3 independent experiments. Differences between the groups were examined by Student's *t*-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). *P*<0.05 was considered to indicate a statistically significant difference.

Results

Carnosic acid selectively and dose-dependently inhibits the proliferation of lung cancer cells

CCK-8 assay for cell viability was carried out to examine the antiproliferative effects of carnosic acid in A-549 NSCLC cells as well as in MRC-5 cell line, (fibroblasts derived from lung tissue). The experiment was done at various doses of carnosic acid and results revealed that carnosic acid exerted dose-dependent and selective cytotoxicity towards A-549 lung cancer cells without exerting too much cytotoxicity towards the normal lung fibroblast cells (MRC-5). This was clearly visible from the IC_{50} values of carnosic acid against these cells; with lower value in A-549 lung cancer cells and a higher value in normal fibroblast cells. Figure 1A and 1B show the chemical structure of carnosic acid and cell viability graph induced by it. Further, colony formation assay revealed that carnosic acid exerted cytotoxicity in A-549 cells by decreasing the cell colony formation potential of these cells. This effect on cell colony inhibition was also found to depend on carnosic acid dose (Figure 2). The antiproliferative effects of the test molecule were unveiled by CCK-8 assay but still molecular mechanisms are not well known, needs further investigation.

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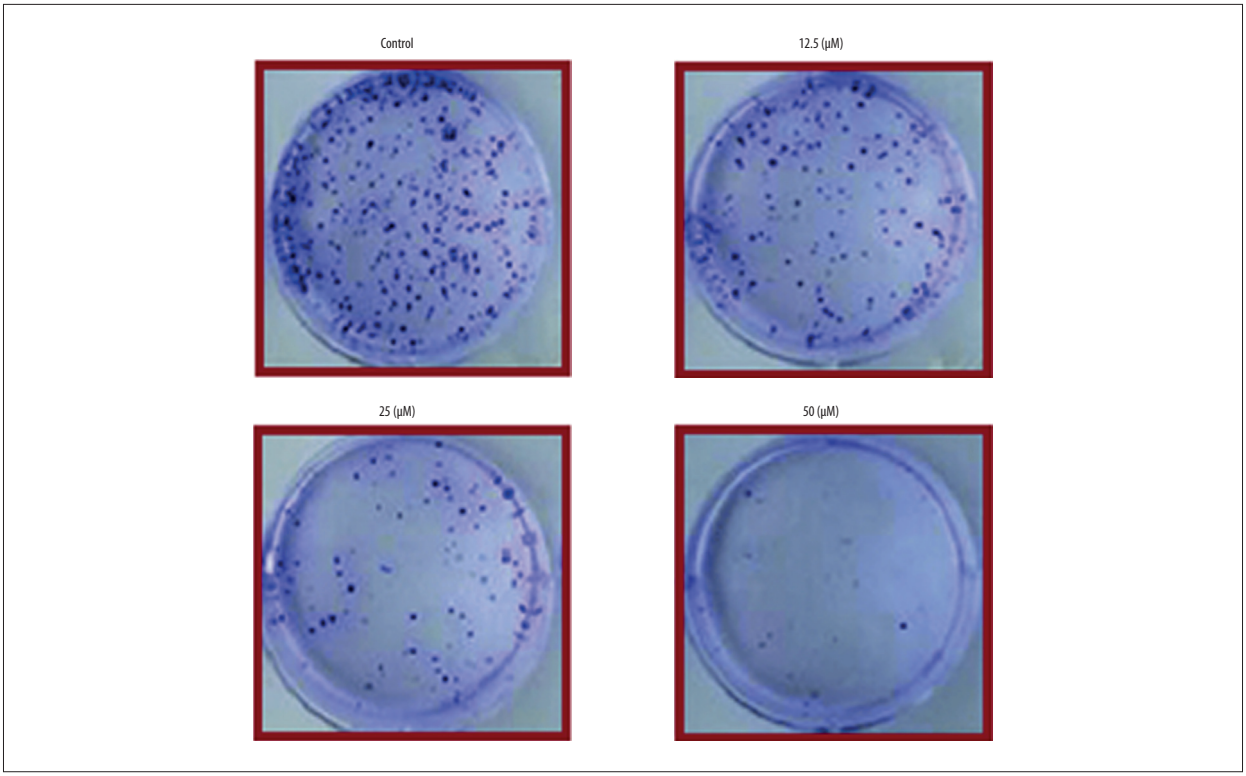

Figure 2. Effect of carnosic acid on the colony formation tendency of A-549 cancer cells as indicated by inverted microscopy using crystal violet staining.

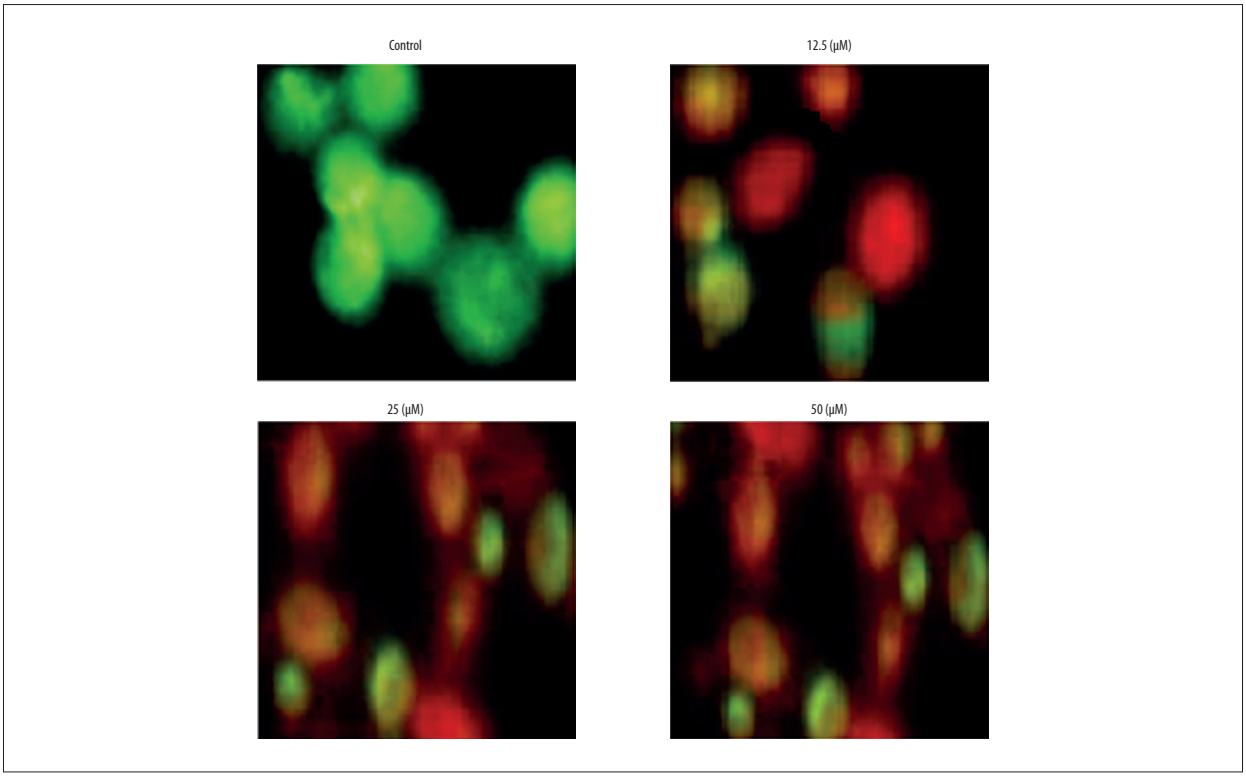

Figure 3. Apoptotic effects of carnosic acid in A-549 human lung cancer cells as indicated by fluorescence microscopy using acridine orange ethidium bromide staining. The experiments were repeated thrice.

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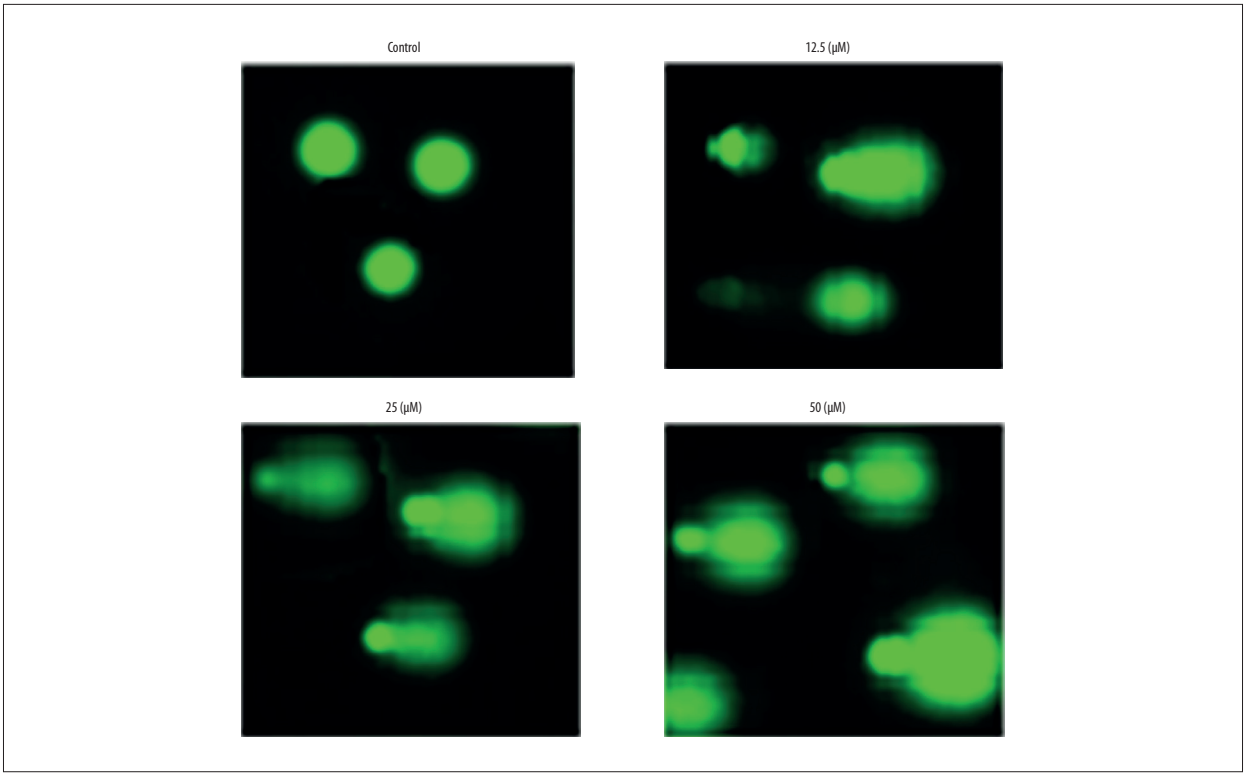

Figure 4. Comet assay using fluorescence microscopy showing the effects of carnosic acid on the DNA fragmentation in A-549 human lung cancer cells. The results showed increasing DNA fragmentation with the carnosic acid dose. The experiments were executed in triplicate.

Carnosic acid induced cellular apoptosis and DNA damage in A-549 cells

Figure 3 shows the results of acridine orange/ethidium bromide (AO/EB) staining results performed using fluorescence microscopy. These results indicate that with increasing doses of carnosic acid, the number of cells with red/orange fluorescence increases significantly reminiscent of programmed cell death. Untreated control cells showed no signs of apoptosis as no cells with orange fluorescence could be seen. This cellular apoptosis was mediated via DNA damage shown by Comet assay which indicated that as compared to the untreated control cells with zero dose of the drug showing intact DNA without any fragmentation. Carnosic acid-treated cells at increasing doses of 12.5, 25, and 50 μM exhibited considerable fragmentation of DNA in these cells looking like a comet in this assay. The percentage of these comet cells increased with increasing dose of carnosic acid and the results are shown in Figure 4.

Carnosic acid suppressed cancer cell migration as well as invasion in A-549 cells

In vitro wound healing assay for cell migration and Transwell assay for cell invasion were performed to evaluate the anti-metastatic effects of carnosic acid in A-549 lung cancer cells.

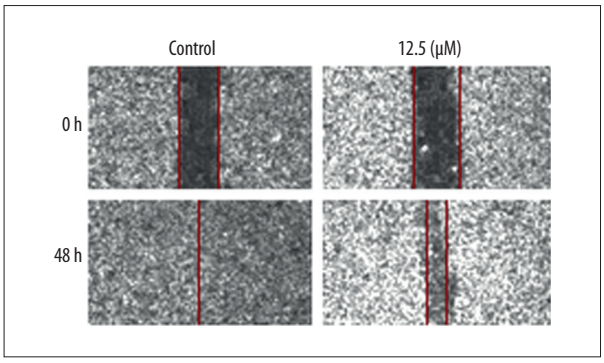

Figure 5. Carnosic acid induced substantial inhibition of cell migration in A-549 human lung cancer cells. The experiments were performed in triplicate.

The cell migration results are shown in Figure 5 and reveal that carnosic acid at a dose of 12.5 μM led to a significant suppression of cell migration after 48 hours of exposure. Figure 6 shows the effects of carnosic on cell invasion at 4 different doses of 0, 12.5, 25, and 50 μM and showed that carnosic acid led to inhibition of cell invasion in A-549 cells in a concentration-dependent manner. Both these assays indicate that carnosic acid might be a potential agent for curbing the cell metastasis in A-549 cells thus holding a huge potential. Additionally, western blot assay confirmed the results further by decreasing the

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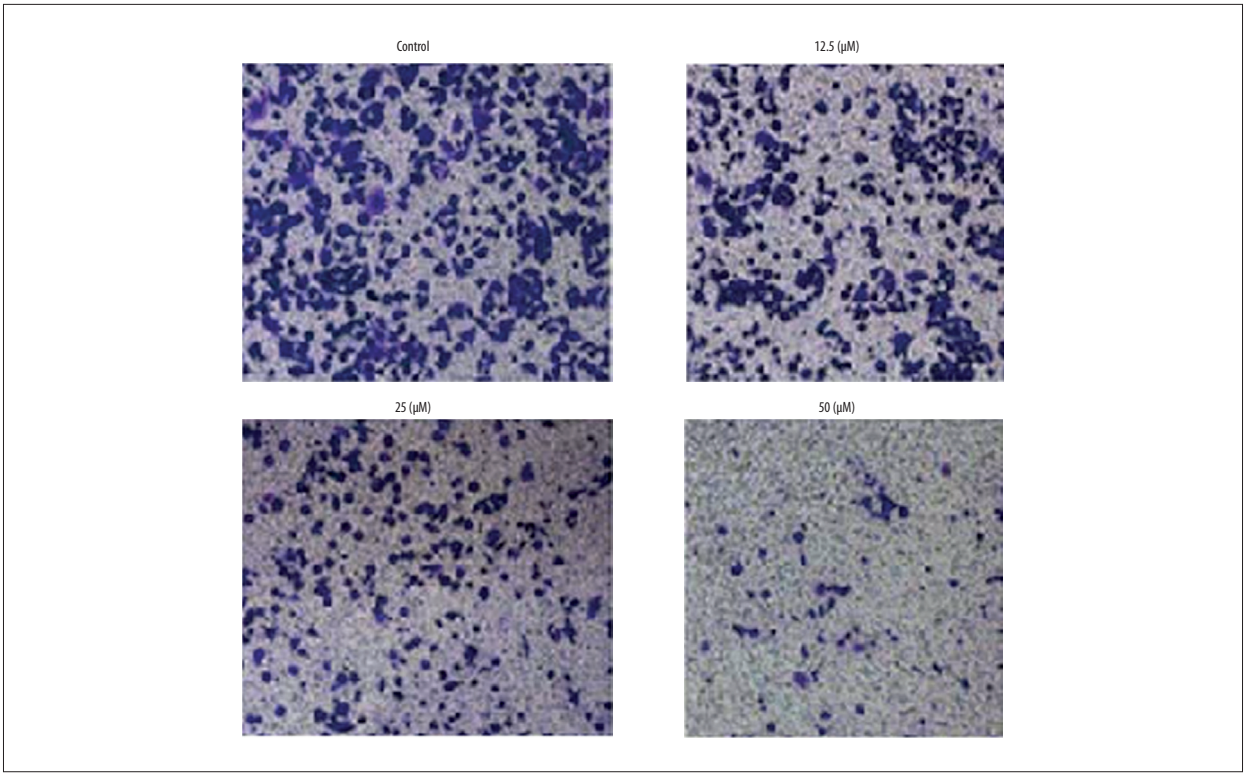

Figure 6. Carnosic acid induced substantial and concentration-dependent inhibition of cell invasion in A-549 human lung cancer cells. The experiments were performed in triplicate.

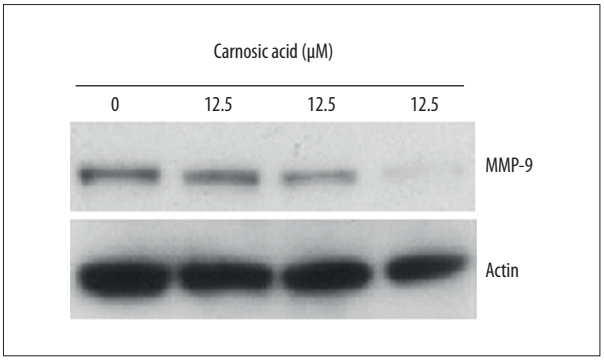

Figure 7. Carnosic acid led to a dose-dependent suppression of MMP-9 (metalloproteinase-9) using western blot assay. The experiments were performed in triplicate.

expression of matrix metalloproteinase-9 (MMP-9) dose-dependently (Figure 7); this protein expression has been reported to play critical role in cell migration.

Carnosic acid inhibited the m-TOR/PI3K/AKT signaling pathway

The m-TOR/PI3K/AKT signaling cascade is considered a therapeutic target for the treatment of various cancers as it has a crucial role to play in the process of carcinogenesis. The effects of carnosic acid at the concentrations of 0, 12.5, 25, and 50 μM on the phosphorylation state of the mTOR, PI3K and AKT are shown in Figure 8. The results of the western blot analysis showed that carnosic acid suppressed the phosphorylation of m-TOR, PI3K and AKT in a dose dependent manner. However, the total protein levels of m-TOR, PI3K and AKT remained seemingly unchanged.

Discussion

Lung cancer affects both men and women and is included among the most common types of human malignancies with an incidence rate that is continuously on the rise. In China, lung cancer prevalence is also on the rise and is a leading cause of cancer-related mortality. Natural products have always been at the forefront of anticancer drug discovery furnishing a number of drugs that are used even today to combat cancer. Many of these naturally occurring secondary metabolites have a tendency to induce apoptosis (i.e., programmed cell death) [13]. Apoptosis is a well-controlled biochemical process which plays a crucial role in tissue homeostasis by eradicating the damaged cells out of the body. The apoptosis process is one of the most well-studied biochemical processes primarily because of its role in normal as well as processes which cause numerous pathological conditions including carcinoma [14]. Structurally, carnosic acid is a polyphenolic

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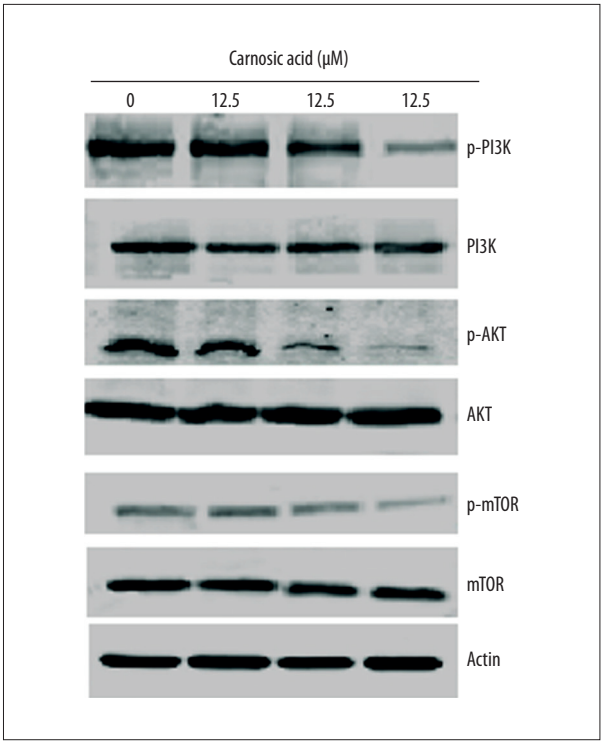

Figure 8. Effects of carnosic acid on the m-TOR/PI3K/AKT signaling pathway. Carnosic acid suppressed the phosphorylation of m-TOR, PI3K, and AKT in a dose dependent manner. However, the total protein levels of m-TOR, PI3K, and AKT remained seemingly unchanged.

diterpene isolated from various plants including *Rosmarinus officinalis*. This molecule has been previously reported to exhibit various pharmacological effects including antitumor, antiviral and anti-inflammatory effects [15-18]. It has been reported that carnosic acid inhibits cell growth and induces cell cycle arrest in B16F10 melanoma cells along with enhancing p21 expression [19,20]. Carnosic acid has also been reported to inhibit the growth of estrogen receptor (ER)-negative human breast cancer cells along with inducing G1 cell cycle arrest. Carnosic acid has also been shown to induce anticancer effects via inducing *in vitro* and *in vivo* cell growth inhibition, reactive oxygen species (ROS) generation and activating the JNK signaling pathway in human cervical cancer cells [21,22]. It has also been shown to inhibit cell proliferation and cell migration, along with reducing vascular endothelial growth factor expression. It has also been reported to downregulate cyclin A1 expression in leukemia and colon cancer cells. Carnosic acid was also reported to induce apoptosis in human prostate and human neuroblastoma cancer cells [23-26]. In the present study, its effects on A-549 NSCLC cells were examined along with a normal lung cell line-MRC-5. It was shown to induce a selective cytotoxic effect in A-549 cancer cells without too much affecting the normal lung cells. Further, carnosic acid was shown to induce apoptosis in these cells along with initiating DNA fragmentation. It also inhibited cell migration and invasion along with decreasing the expression levels of MMP-9. Western blot analysis showed that carnosic acid suppressed the phosphorylation of m-TOR, PI3K, and AKT in a dose dependent manner. However, the total protein levels of m-TOR, PI3K, and AKT remained seemingly unchanged.

Conclusions

Taken together, these results indicate that carnosic acid induced selective anticancer effect in A-549 lung cancer cells by inducing cellular apoptosis, suppressing cell migration and invasion, and inhibiting the m-TOR/PI3K/AKT signaling pathway.

Conflict of interest

None.

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