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Methyleugenol Enhances the Anticancer Effect of Chemotherapeutic Drug and Boosts Chemotherapy Drug Tolerance

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ABSTRACT

Chemotherapy plays an important role in treating lung cancer. Chemotherapy drugs usually have good therapeutic effect, but due to the side effects, the dose needs to be considered. The use of non-toxic adjuvant natural product combined with chemotherapy drugs will be an important treatment mode in the future. The purpose of this study is to use non-toxic natural product (methyleugenol) to increase the therapeutic effect of chemotherapeutic agent (doxorubicin) on lung cancer and investigate the toxic effect of methyleugenol combined with chemotherapeutic agents on drug-resistant lung cancer cells. Methyleugenol combined with doxorubicin treated lung adenocarcinoma A549 cell line and established drug-resistant lung adenocarcinoma cell line (A549DoxR) were used in the study. The methods including proliferation assay, cell wound healing assay, colony formation assay, DNA fragmentation assay, gelatin zymography assay, comet assay, reverse transcriptional polymerase chain reaction (RT-PCR), and western blot were adopted. The results showed methyleugenol significantly enhanced doxorubicin inhibited the cell growth, the cell colony formation, the cell migration, and the metastasis of A549 cells and A549DoxR cells. Methyleugenol strengthened doxorubicin to induce apoptosis and autophagy of A549 cells and A549DoxR cells. Methyleugenol can significantly enhance the treatment effect of chemotherapy drugs in lung cancer and strengthened the toxic effect of chemotherapy drugs on drug-resistant lung cancer cells. Methyleugenol can be developed to be used as an adjuvant to assist Chemotherapy drugs s and is targeted to clinically treat patients with multidrug resistance.

Keywords: methyleugenol, doxorubicin, lung cancer cells, chemotherapy drugs, natural product

INTRODUCTION

Lung cancer is the major cause of cancer-related deaths in world (Siegel et al. 2021). There are two main types of lung cancer, 80% to 85% of lung cancers are non-small cell lung cancer (NSCLC), 10% to 15% of lung cancers are small cell lung cancer (SCLC) (Fadejeva et al. 2017; Testa et al. 2018). At present, the most commonly methods used for the treatment of lung cancer are surgery, radiotherapy, chemotherapy, targeted drug therapy, and immunotherapy. Chemotherapy, targeted drug therapy, and immunotherapy belong to systemic therapy (Ellis et al. 2017). Chemotherapy is the most commonly systemic treatment used in clinical (Chaft et al. 2021). Chemotherapy adopts drugs to destroy cancer cells, preventing cancer cells from proliferation. It has been proven to improve the lifetime and quality of lung cancer patients at all stages. Chemotherapy may also damage healthy cells in the body, including blood cells, skin cells and nerve cells. Most lung cancer patients with chemotherapy treated will develop a lot of side effects and resistance to chemotherapy drugs (Guo, et al. 2017; Sara et al. 2018). In recent years, the reports have pointed out that natural products have anti-cancer effects and will not cause complications. The treatment of cancer contains

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the adjuvant treatment of natural products, which forms a kind of "Complementary and Alternative Medicine therapy (CAM)" (Lin et al. 2017). Based on the lung cancer symptoms and comprehensive literatures, it is pointed out that factors including angiogenesis, cancer cell proliferation and migration, immune inflammatory response and epithelial-mesenchymal transition can induce tumor cells to metastasize and invade surrounding tissues (Ribatti et al. 2020). The study indicates that the autophagy protein beclin-1 is an important regulator in the process of promoting and anti-apoptotic (Booth et al. 2014). Beclin-1 content in early autophagy will increase significantly, which is one of the indicators of early autophagy (Parzych and Klionsky 2014). The content of LC3-II is positively correlated with the number of autophagic vesicles (Parzych and Klionsky 2014). The detection of LC3 protein can determine the autophagy of cell (Hamacher-Brady et al. 2006).

The studies have shown that during pregnancy, normal physiological conditions, the increase of matrix metalloproteinases MMP-2 and MMP-9 is related to vasodilation, embryo formation and uterine dilation (Chen and Khalil 2017). In pathological conditions, the increase expression of MMPs is related to tumor growth, migration and metastasis, which leads to the development of cancer (Rahat et al. 2016). MMP-2 and MMP-9 are specially two gelatinases in many kinds of matrix metalloproteinases, believed to be closely related to the occurrence of tumor metastasis and angiogenesis, which can help tumor cells decompose tissues to advance migration and metastasis (Gaffney et al. 2015). The molecular markers of normal epithelial cells include E-cadherin and α-catenin, the molecular markers of malignant mesenchymal cells include vimentin, N-cadherin, fibronectin and α-smooth muscle actin. The different characteristics of molecular markers between normal epithelial cells and malignant mesenchymal cells, the expression of these proteins can be used to observe the cells metastasized (Lu and Kang 2019). Epithelial-mesenchymal transition (EMT) is the important direction of clinical research, and these markers can be regarded as the index in cancer metastasis.

The study confirmed that activated transcription factor 3 (ATF3) is a stress-inducing transcription factor. ATF3 is a type of stress stimulus that has low expression in cells. The expression level of ATF3 mRNA will be rapidly induced in external stress stimuli, such as malnutrition, hypoxia, DNA damage and other stress signals (Yan and Boyd 2006). ATF3 participates the process of changes in inside and outside the cell with the various physiological stresses and regulate the balance between proliferation and apoptosis in tumor cells (Li et al. 2018).

Soluble resistance-related calcium-binding protein (sorcin) is a calcium-binding protein (Shabnam et al. 2018). The gene is located on the same homologous chromosome as the multidrug resistance gene (MDR-1) encoding P-gp and involved in the regulation of intracellular calcium ions. In drug-resistant tumor cells, the occurrence of P-gp can mediate the expression of sorcin and MDR-1 genes (Yu et al. 2018). The study reported that the increase of sorcin expression caused the changes in the regulatory function of P-gp or ABC transporter, resulting in tumor cells to develop drug resistance (Colotti et al. 2014). Sorcin is important factor in cancer progression. The literature shows that the increase of sorcin expression in cancer, increases the metastasis of tumor cells through the path of epithelial-mesenchymal cell transformation (Hu et al. 2014). The study confirmed the high affinity between sorcin and a variety of chemotherapy drugs. The high affinity leads to chemotherapy drugs resistance when processing tumor cells (Colotti et al. 2014; Genovese et al. 2017). In addition, many studies have shown that sorcin is involved in various processes, such as the regulation of drug efflux, the regulation of apoptosis and the regulation of epithelial-mesenchymal cell transformation through the expression of E-cadherin (Colotti et al. 2014). This shows that sorcin is an indicator of multidrug resistance MDR-1 and is a new target for tumor resistance diagnosis and treatment.

Combinations of two chemotherapy drugs are used to treat lung cancer in clinical. However, this combination treatment is accompanied by serious side effects. Some studies have shown that the combination of natural product and chemotherapy drug can be used to increase the therapeutic effect of chemotherapeutic agent and reduce the side effects by chemotherapy (Osman et al. 2012). Natural products are used clinically as an adjuvant cancer treatment, and it has been shown to have good efficacy and low toxicity. There are various natural product types, but methyleugenol is a new discovery we've made. In the study, we found effective natural products (methyleugenol) that enhance chemotherapeutic efficacy and reduce the effect of drug resistance in lung cancer cells.

MATERIALS AND METHODS

Materials

The natural ingredient (methyleugenol) was purchased from Sigma-Aldrich Corporation.

Cell culture

Lung adenocarcinoma A549 cell line and normal human embryonic lung fibroblast (MRC-5) cell line was maintained in DMEM/F12 (Gibco BRL, Grand Island, NY) medium supplemented containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. The process of drug-resistant lung adenocarcinoma cell line (A549DoxR) was established from A549 lung adenocarcinoma cell line treated with low concentration of doxorubicin to induce its drug resistance.

MTT analysis

Human lung adenocarcinoma cell line (A549), drug-resistant lung adenocarcinoma cells (A549DoxR), and normal human embryonic lung fibroblast cells (MRC-5) were cultured in 96-well culture plates. After 24 hours of culture, each group was treated with different concentrations of methyleugenol at 0, 5, 25, and 50 μM for 24 hours, and then doxorubicin 8 μM was subsequently added. After 24 hours of culture, add the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent and measure the absorbance of the solution at values of optical density at 570 nm (OD570).

DNA fragmentation assay

A549 and A549DoxR cells were cultured in doxorubicin 8 μM, and methyleugenol 50 μM combined with doxorubicin 8 μM separately. After treatment for 24-48 hours, cell was collected. Electrophoresis was performed using 2% agarose gel (100 mV, 30 min). In the late stage of apoptosis, cells shrink and the DNA in the cells is degraded by nuclease into 180bp-200bp fragments. These fragments are hierarchical and can be electrophoresed through 2% agarose gel. The ladder-shaped electrophoresis band is a clear feature of cell apoptosis.

Wound healing analysis

The A549 cells and A549DoxR cells were cultured in a 24-well culture plate at a cell density of $5x10^4$/ml. After 24 hours of culture, the cells filled the entire well plate, use the tip of a micropipette to draw a small groove in the center of the cells at the bottom of the plate, and then suck out the cell culture medium in the well plate.

Add 5 ml of sterile phosphate-buffered saline (PBS, pH 7.2) along the wall of the well, then aspirate the floating cells and wash the cells twice. O-Methyleugenol 50 μM, Doxorubicin 8 μM, and O-Methyleugenol 50 μM combined with chemotherapy drug Doxorubicin 8 μM were added separately. After 24 hours of treatment, the migration of cancer cells was observed using an inverted phase-contrast microscope and photographed for archiving and quantification.

Gelatin zymography assay

The A549 cells and A549DoxR cells were cultured in 10 cm culture dishes at a cell density of 1.5 $x10^6$/ml. After 24 hours of culture, 50 μM O-Methyleugenol, 8 μM Doxorubicin, and 50 μM O-Methyleugenol combined with 8 μM Doxorubicin were added. After 24-48 hours of treatment, the supernatant was collected and mixed with 5× non-reducing sample buffer at a ratio of 1:3 to prepare samples for electrophoresis (25°C, 80 V, 120 mA, 2 hr). After the SDS-PAGE electrophoresis, remove the slide from the glass slide and immerse it in 20 ml of washing buffer and shake it for 15 minutes. Repeat this step twice, then immerse it in 30 ml of incubation buffer and shake it for 30 minutes. Replace with fresh Incubation buffer and react in a 37°C incubator for 12-24 hours. Place in a staining solution containing Coomassie Blue. Evenly stain the film at room temperature using a horizontal oscillator for 30 minutes. Soak the film in destaining solution until a transparent band appears, then seal the film with cellophane, let it dry, scan and photograph it for archiving.

Colony formation assay

A549 and A549DoxR cells were transformed into suspension cells with a 0.25% trypsin-EDTA solution.-The cell volume is about 2-$5x10^4$ / mL of cells per well. Mix 0.5% agar solution and cell suspension at a ratio of 1 : 1. The cells were separately treated with doxorubicin 8 μM, and methyleugenol 50 μM combined with doxorubicin 8 μM. The reaction was followed by staining with crystal violet for 5 min, washing and de-staining with 1 × PBS, observing the cell growth under an inverted phase contrast microscope and counting the number of colonies.

RNA extraction and reverse transcriptional polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol reagent (Sigma, St. Louis, MO) and converted to complementary DNA (cDNA) was synthesized with SuperScript™ III reverse transcriptase (Invitrogen, USA), PCR conditions were set 30 amplification cycles consisting of 94˚C for 15 sec, 60˚C for 30 sec and 72˚C for 1 min for 30 cycles, then final 72˚C extension for 7 min. PCR products were electrophoresed in 2% agarose gel.

Comet assay

Pipette 10 µl of cell suspension and 100 µl of 0.5% agar solution into 1.5ml eppendorf tubes. Pipette the entire cell mixture and cover the glass slide with the bottom agar, cover with the cover glass, and place on ice for 3-4 min, waiting for it to solidify. Immerse the slides in the staining tank containing lysis solution, soak for 1 hour, pour alkaline electrophoresis solution into the electrophoresis tank. Set the electrophoresis tank to 15 Volts 100 mA. Place the slide in the electrophoresis tank for 20 min. Drying the slide at 37℃ for 10-15 min, stain at room temperature for 30 min, and observe under a fluorescent microscope.

Western blotting

Cell lysates were separated by 8% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and were transferred to nitrocellulose membrane (PVDF membrane) using a wet blot format. The membranes were blocked with blocking buffer at room temperature for 20 min and were incubated primary antibody for 24 h at 4℃. Next, the membranes were washed three times with TBST (Tris-HCl, pH 7.4, NaCl and 0.1% Tween 20) for 10 min and were incubated secondary horseradish peroxidase-conjugated goat anti-mouse antibodies for 1 h. After washing, the membrane was detected by enhanced chemilluminescence (ECL) system and was measured densitometry by Image J software.

Statistical analysis

Differences among groups were determined by were analyzed using one-way analysis of variance by the statistical analysis system (SAS) application package. All measurements are presented as means ± SD. It was considered statistically significant from a difference with $p < 0.05$.

RESULTS AND DISCUSSION

The effect of methyleugenol on the survival of normal human lung fibroblasts (MRC-5)

The results of MTT analysis showed that the survival rate of MRC-5 cells was 100±6 %, 99±5%, 97±6%, and 98±7% by treated with different concentrations of methyleugenol (0, 5, 25, and 50 µM), indicating that methyleugenol didn't result in cytotoxicity of normal human lung fibroblasts MRC-5.

Methyleugenol strengthening doxorubicin on the cytotoxic effect of A549 cells

The survival rates of A549 cells treated with doxorubicin (8 µM) were 69±4%, the survival rates of A549 cells treated with methyleugenol (5, 25, and 50 µM) combined with doxorubicin (8 µM) were 59±6%, 44±5%, and 18±4%. Methyleugenol strengthens the cytotoxic effect of doxorubicin on A549 cells in a dose-dependent manner. The results showed methyleugenol significantly enhanced doxorubicin inhibited the growth of A549 cells.

The change of cell shape of A549 cells with treated methyleugenol combined with doxorubicin

A549 cells were treated with 8 µM doxorubicin, 50 µM methyleugenol combined with 8 µM doxorubicin, and control (Figure 1A). Compared with doxorubicin 8µM, and control groups, the cell shape of A549 cells treated with 50 µM methyleugenol combined with 8 µM doxorubicin was obviously changed and showed vacuolation and shrinkage. During apoptosis, several biochemical events lead to characteristic cell morphological changes, including vacuolation and shrinkage. The results showed that methyleugenol can effectively enhanced doxorubicin to induce the apoptosis of A549 cells.

The effect of methyleugenol enhanced doxorubicin on the inhibition of migration in A549 cells

50µM methyleugenol combined with 8 µM doxorubicin significantly inhibited the migration of A549 cells, compared with 8 µM doxorubicin and control groups (Figure 1B). Because cancer cells have strong migration ability, methyleugenol can further enhance the inhibition of chemotherapy drugs on the migration ability of cancer cells. The results showed that methyleugenol can effectively enhanced doxorubicin to inhibit the cell migration of A549 cells.

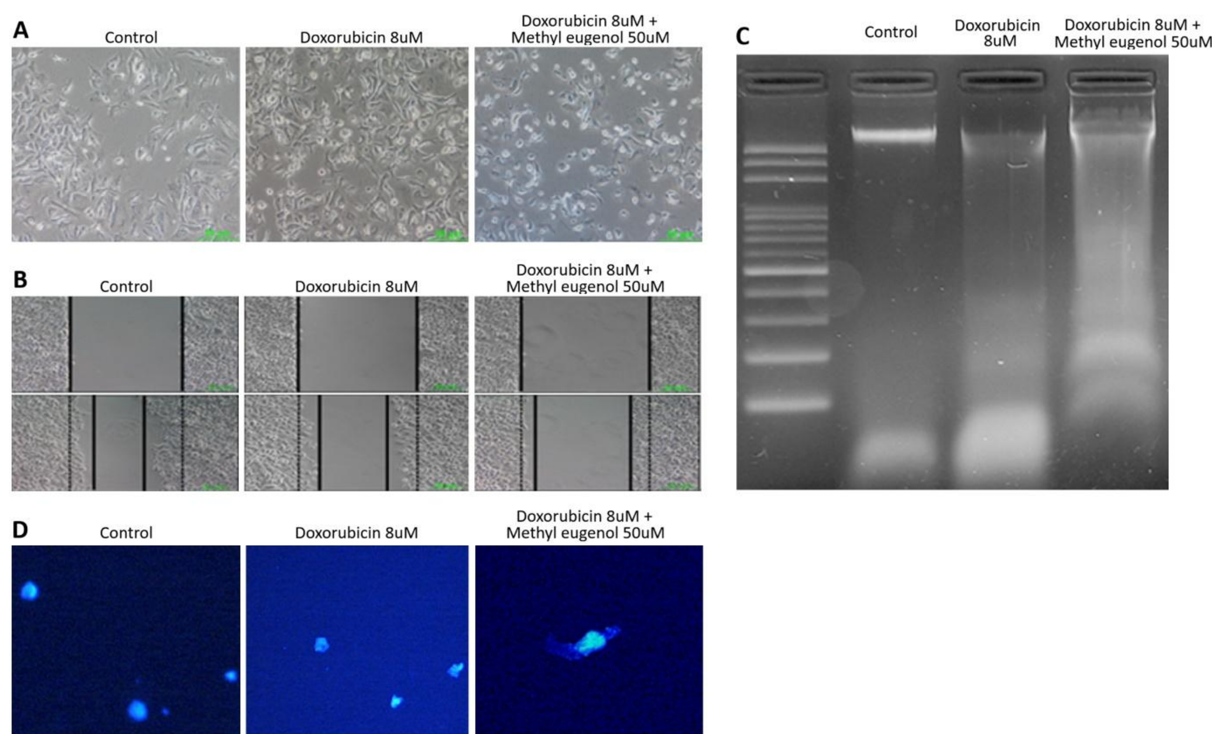

Figure 1. (A) Methyleugenol enhanced the effect of doxorubicin on the survival and morphological changes of A549 cells; (B) Methyleugenol enhanced the inhibition of migration of doxorubicin on A549 cells by wound healing assay; (C) Methyleugenol enhanced the effect of doxorubicin on the apoptosis of A549 cells by DNA fragmentation assay;(D) Methyleugenol enhanced the effect of doxorubicin on the DNA damage of A549 cells by comet image.

The apoptosis of A549 cells treated with methyleugenol combined with doxorubicin

In figure 1C, A549 cells were treated with 8 μM doxorubicin, and 50 μM methyleugenol combined with 8 μM doxorubicin. The phenomenon of DNA fragmentation of A549 cells treated with 50 μM methyleugenol combined with 8 μM doxorubicin was significantly changed, compared with other groups. During apoptosis, several biochemical events lead to cell death, including cell vacuolation, cell shrinkage and DNA fragmentation. The results showed that methyleugenol can strengthen doxorubicin to induce the apoptosis of A549 cells, leading to the death of A549 cells.

The effect of DNA damage of A549 cells treated with methyleugenol combined with doxorubicin

In figure 1D, compared with the control group and 8 μM doxorubicin group, the results of the comet assay showed that A549 cells treated with methyleugenol 50 μM combined with doxorubicin 8 μM had significantly longer comet tails and caused a large accumulation of fragmented DNA. Apoptosis involves a series of biochemical processes that result in cell death, such as the formation of vacuoles within the cell, reduction in cell size, fragmentation of DNA, and damage to DNA. The results indicated that methyleugenol can enhance the effect of doxorubicin poisoning A549 cells.

Methyleugenol enhanced doxorubicin on the inhibition of the cell colony formation of A549 cells

Compared with 8μM doxorubicin and control group, the cell colonies of A549 lung adenocarcinoma treated with methyleugenol 50μM combined with doxorubicin 8 μM were obviously inhibited (Figure 2A). The results showed methyleugenol 50μM combined with doxorubicin 8 μM significantly inhibit the formation of A549 cell colonies. According to the results of crystal violet staining, observed under an inverted phase-contrast microscope, methyleugenol 50 μM combined with doxorubicin 8 μM can effectively inhibited the colonization of A549 cells (Figure 2B). Colony formation assay is an indicator for analyzing the malignant transformation of cells.

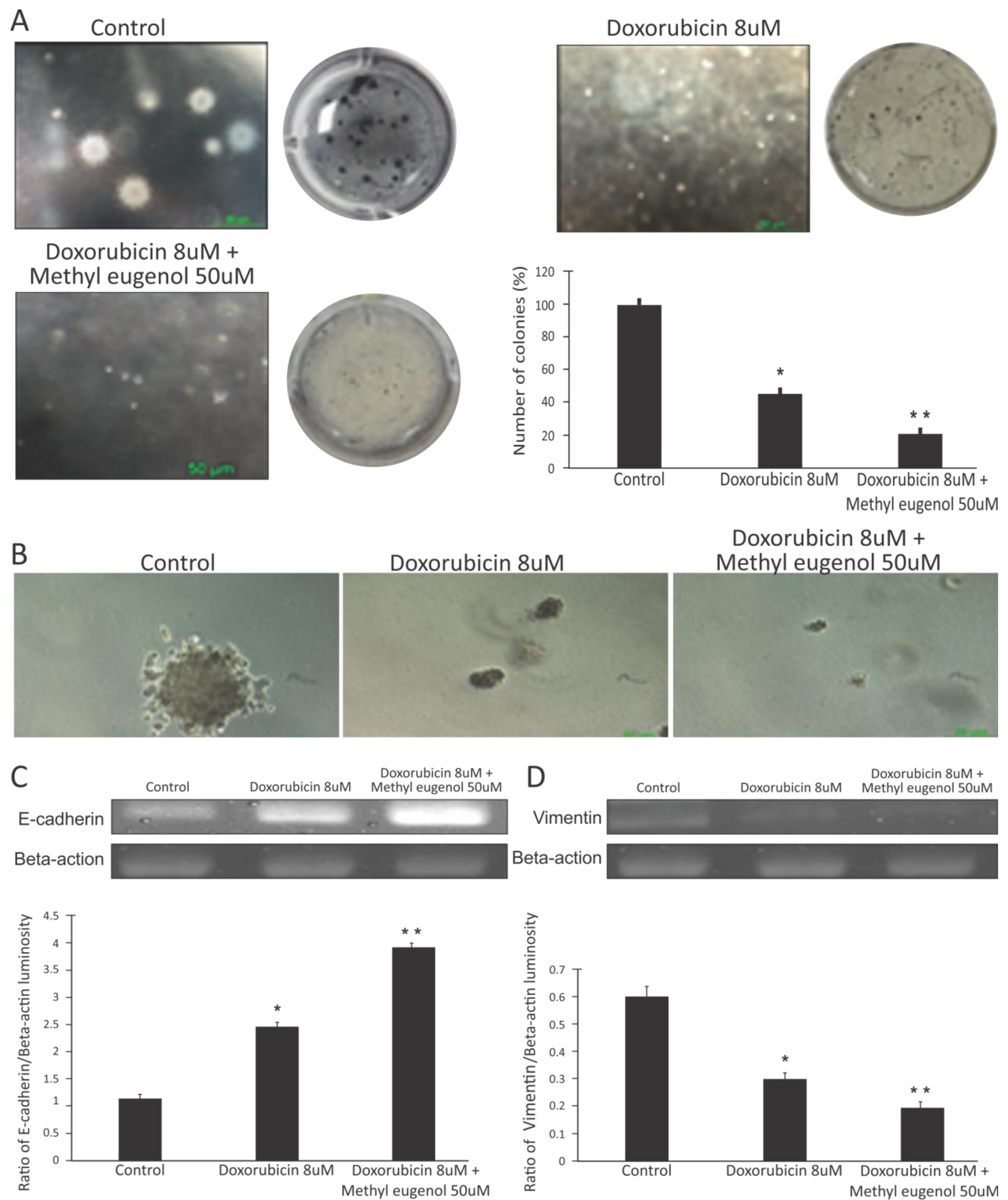

Figure 2. (A) Methyleugenol enhanced the effect of doxorubicin on the colony formation of A549 cells by colony formation assay; (B) The results of crystal violet staining under an inverted phase-contrast microscope; (C) The effect of methyleugenol enhanced doxorubicin on mRNA expression of E-cadherin in A549 cells by RT-PCR analysis; (D) The effect of methyleugenol enhanced doxorubicin on mRNA expression of vimentin in A549 cells by RT-PCR analysis.
* $P < 0.01$ vs control; ** $P < 0.01$ vs control, and doxorubicin (8 μM)
Values expressed as mean±SEM of three independent experiments.

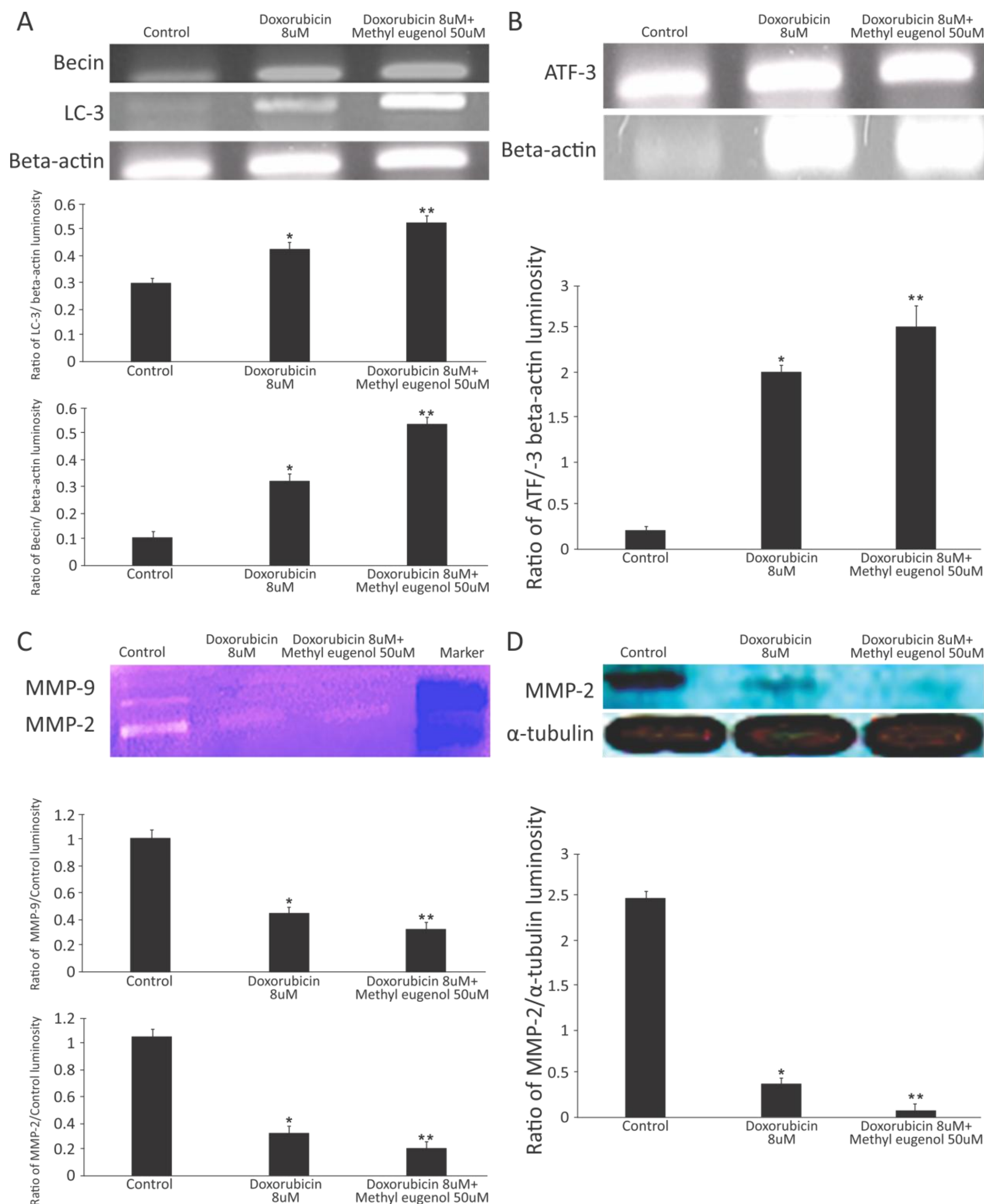

Figure 3. (A) The effect of methyleugenol enhanced doxorubon on autophagy of A549 cells; (B) The effect of methyleugenol enhanced doxorubicin on activated transcription factor 3 (ATF-3) of A549 cells; (C) Methyleugenol enhanced doxorubicin on the inhibition of secretion of MMP in A549 cells by gelatin zymography analysis; (D) Methyleugenol enhanced doxorubicin on the inhibition of MMP-2 protein in A549 cells.

* $P < 0.01$ vs control; ** $P < 0.01$ vs control, and doxorubicin (8 μM); Values expressed as mean±SEM of three independent experiment

A colony refers to a cell group formed after a single cell proliferates for more than 6 generations in vitro, with a diameter of about 0.3-1.0 mm. Normal cells need to adhere to the culture plate to grow when cultured in vitro, while malignantly transformed tumor cells can grow in colonies in semi-solid soft agar. The generation of colonies can help us understand the generation and proliferation capabilities of tumor cells. The results showed that methyleugenol can effectively strengthen doxorubicin to inhibit the cell colony formation on A549 cells.

The effect of methyleugenol enhanced doxorubicin on E-cadherin and vimentin in A549 cells

Compared with the control group and 8 μM doxorubicin group, the expression level of E-cadherin mRNA is significant increase (Figure 2C) and the expression level of vimentin mRNA decreased significantly (Figure 2D) in A549 cells treated 50μM methyleugenol combined with doxorubicin 8μM. The results showed that methyleugenol can effectively enhance doxorubicin in the inhibition of the metastatic ability of A549 cell.

Methyleugenol strengthen doxorubicin on the autophagy effect of A549 cells

A549 cells were treated with doxorubicin 8 μM, and 50μM methyleugenol combined with doxorubicin 8 μM. The expression of mRNA related to cell autophagy (Figure 3A). Compared with the control group and 8 μM doxorubicin group, the expression of beclin and LC-3 mRNA is increased in A549 cells treated with 50 μM methyleugenol combined with doxorubicin 8 μM. The results showed that methyleugenol combined with doxorubicin treated A549 cells, the expression of beclin and LC-3 increased to cause autophagy of A549 cells. Methyleugenol strengthened doxorubicin to induce death of A549 cells.

Methyleugenol strengthen doxorubicin on the activation of transcription factor 3 (ATF3) of A549 cells

According to the results (Figure 3B), the expression of activated transcription factor 3 (ATF3) mRNA was significant increase in A549 cells treated with 50 μM methyleugenol combined with doxorubicin 8 μM, compared with the control group and doxorubicin 8 μM group. The result confirmed that methyleugenol enhanced doxorubicin in the expression of the ATF3gene of A549 cells and increased the apoptosis of A549 cells.

Methyleugenol enhanced doxorubicin on the inhibition of metastasis in A549 cells

MMP (Matrix metalloproteinases) secreted during cell metastasis has a great relationship with tumorigenesis. Compared with 8 μM doxorubicin and control group, the content of MMP-2 and MMP-9 decreased significantly in A549 cells treated 50μM methyleugenol combined with doxorubicin 8 μM (Figure 3C). The result confirmed that methyleugenol combined with doxorubicin can effectively inhibit MMP-2 and MMP- 9 activity of A549 cells, which inhibits proteolytic enzymes secreted by cancer cells.

The result showed that the expression of MMP-2 protein was significantly inhibited in A549 cells treated with methyleugenol 50 μM combined with doxorubicin 8 μM, compared with control group and doxorubicin 8 μM group (Figure 3D). The results were consistent with gelatin zymography assay. Gelatin zymography assay is a method for testing matrix metalloproteinases (MMPs). Among them, MMP-2 and MMP-9 are two proteolytic enzymes that are believed to play an important role in the angiogenesis, invasion, migration and metastasis of cancer. Therefore, when cancer cells metastasize, they will release active gelatinases. In this experiment, the supernatant with O-Methyleugenol added was tested for its ability to digest and decompose gelatin on SDS-PAGE to determine whether Inhibits gelatinase activity. The results confirmed that methyleugenol can enhance doxorubicin on the inhibition of metastasis in A549 cells

Observation of mRNA expression of sorcin and MDR-1 in A549 cells and A549DoxR cells

In the figure 4A, the results showed that the expression level of sorcin mRNA in A549DoxR cells was higher than that in A549 cells. In the figure 4B, the expression level of MDR-1 mRNA in A549DoxR cells is higher than that in A549 cells. (Figure 4C), compare with A549 cells, expression of the sorcin protein in A549DoxR cells showed a significant overexpression. Sorcin is an indicator of multidrug resistance MDR-1 and can become a new target for diagnosis and treatment of tumor resistance. The results showed that overexpressing sorcin and MDR-1 genes and sorcin protein confirms drug-resistant lung adenocarcinoma cell line (A549DoxR) established in the study.

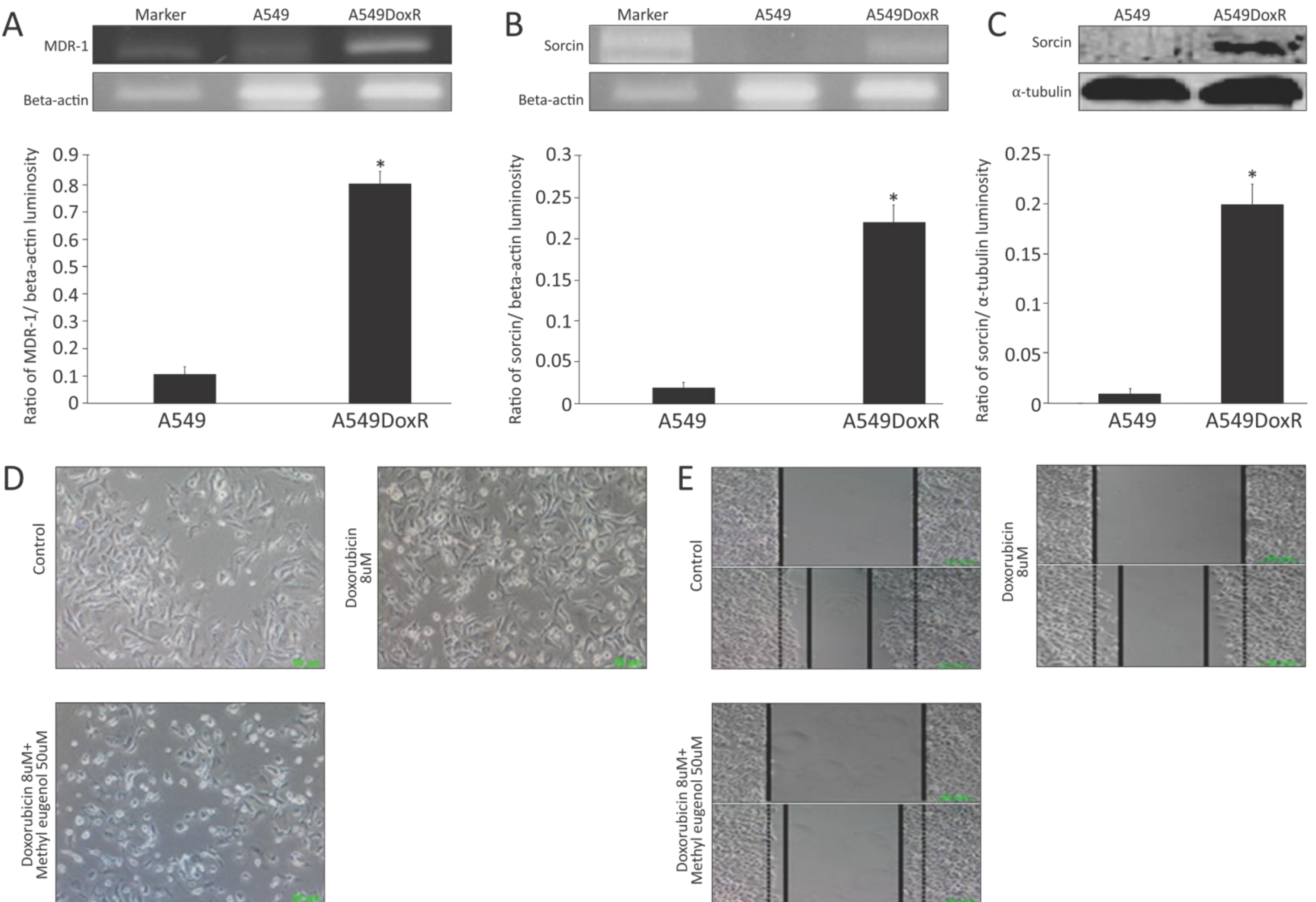

Figure 4. (A) The mRNA expression of sorcin in A549 cells and A549DoxR cells; (B) The mRNA expression of MDR-1 in A549 cells and A549DoxR cells;(C) The protein expression of sorcin in A549 cells and A549DoxR cells; (D) The effect of methyleugenol enhanced doxorubicin on the mortality and morphological changes of A549DoxR cells; (E) Methyleugenol enhanced doxorubicin on the inhibition of migration ability of A549DoxR cells by wound healing assay.
* $P < 0.01$ vs A549 cells. Values expressed as mean±SEM of three independent experiment

Methyleugenol strengthened doxorubicin on the cytotoxic effect of A549DoxR cells

Methyleugenol can enhance the cytotoxic effect of doxorubicin on A549DoxR cells in a dose-dependent manner. The survival rates of A549DoxR cells were 92±5%, 88±5%, 60±6% and 45±3% in a dose- dependent methyleugenol combined with doxorubicin 8 μM, respectively. O-Methyleugenol enhances the cytotoxic effect of Doxorubicin on drug-resistant lung adenocarcinoma cells (A549DoxR) in a dose-dependent manner, confirming that O-Methyleugenol has the effect of assisting Doxorubicin and thus enhancing the cytotoxic effect on drug-resistant lung adenocarcinoma cells (A549DoxR). The effect of methyleugenol enhanced the doxorubicin on the cytotoxic effect of A549DoxR cells.

The change of cell shape of A549DoxR cells with treated methyleugenol combined with doxorubicin

Observe the changes in cell morphology with an inverted phase contrast microscope. In figure 4D, the A549DoxR cells were treated with methyleugenol 50 μM combined with doxorubicin 8 μM and doxorubicin 8 μM alone. Compared with the control group and 8 μM doxorubicin group, A549DoxR cells treated with methyleugenol combined with doxorubicin obviously present shrinkage and vacuolization. Apoptosis is characterized by a series of biochemical processes that culminate in cellular demise, which includes phenomena such as cellular vacuolation, reduction in cell size, fragmentation of DNA, and the occurrence of DNA damage. The results showed that methyleugenol can effectively enhanced doxorubicin to induce the apoptosis of A549DoxR cells.

Methyleugenol enhanced the effect of doxorubicin on the inhibition of migration in A549DoxR cells

In wound healing assay (Figure 4E), A549DoxR cells were treated with methyleugenol 50 μM combined with doxorubicin 8 μM and doxorubicin 8 μM, respectively. Compared with the control group and doxorubicin 8 μM group, A549DoxR cells treated with methyleugenol 50 μM combined with doxorubicin 8 μM was significantly inhibited on the migration of A549DoxR cells. The results showed that methyleugenol can effectively enhance doxorubicin inhibiting the migration ability of A549DoxR cells.

Methyleugenol enhanced the inhibition of doxorubicin on the colony formation of A549DoxR cells

The colony formation of A549DoxR treated with methyleugenol 50 μM combined with doxorubicin 8 μM were significantly inhibited, compared with the control group and doxorubicin 8 μM (Figure 5A). The results of crystal violet staining showed that methyleugenol can effectively enhance doxorubicin to inhibit the colony formation of A549DoxR cells (Figure 5B). Observed under an inverted phase contrast microscope, the results confirmed that methyleugenol can effectively enhance doxorubicin to inhibit the colony formation of A549DoxR cells.

Methyleugenol strengthened the apoptosis of A549DoxR cells treated with doxorubicin

A549DoxR cells were treated with 8 μM doxorubicin, and 50 μM methyleugenol combined with 8 μM doxorubicin (Figure 6A). DNA fragmentation of A549DoxR cells treated with 50 μM methyleugenol combined with 8 μM doxorubicin was significantly changed, compared with other groups. During apoptosis, several biochemical events lead to cell death, including cell vacuolation, cell shrinkage and DNA fragmentation. The results showed that methyleugenol could enhance the apoptosis of A549DoxR induced by doxorubicin.

Methyleugenol combined with doxorubicin on the metastasis inhibition of A549DoxR cells

The MMP (Matrix metalloproteinases) secreted has a great relationship with the metastasis of tumors. The contents of MMP-2 and MMP-9 was significantly decreased in A549DoxR treated with methyleugenol 50 μM combined with doxorubicin 8 μM, compared with control group and doxorubicin 8 μM. Methyleugenol 50 μM combined with doxorubicin 8 μM effectively inhibit the activity of MMP-2 and MMP-9 in A549DoxR cells (Figure 6B) . The results showed that methyleugenol can enhance doxorubicin on the metastasis inhibition of A549DoxR cells.

Methyleugenol enhanced the effect of doxorubicin on autophagy of A549DoxR cells

Methyleugenol 50 μM was combined with doxorubicin 8 μM to treat A549DoxR cells, the expression levels of beclin and LC-3 mRNA were significantly increased, compared with control group and doxorubicin 8 μM group (Figure 5C).

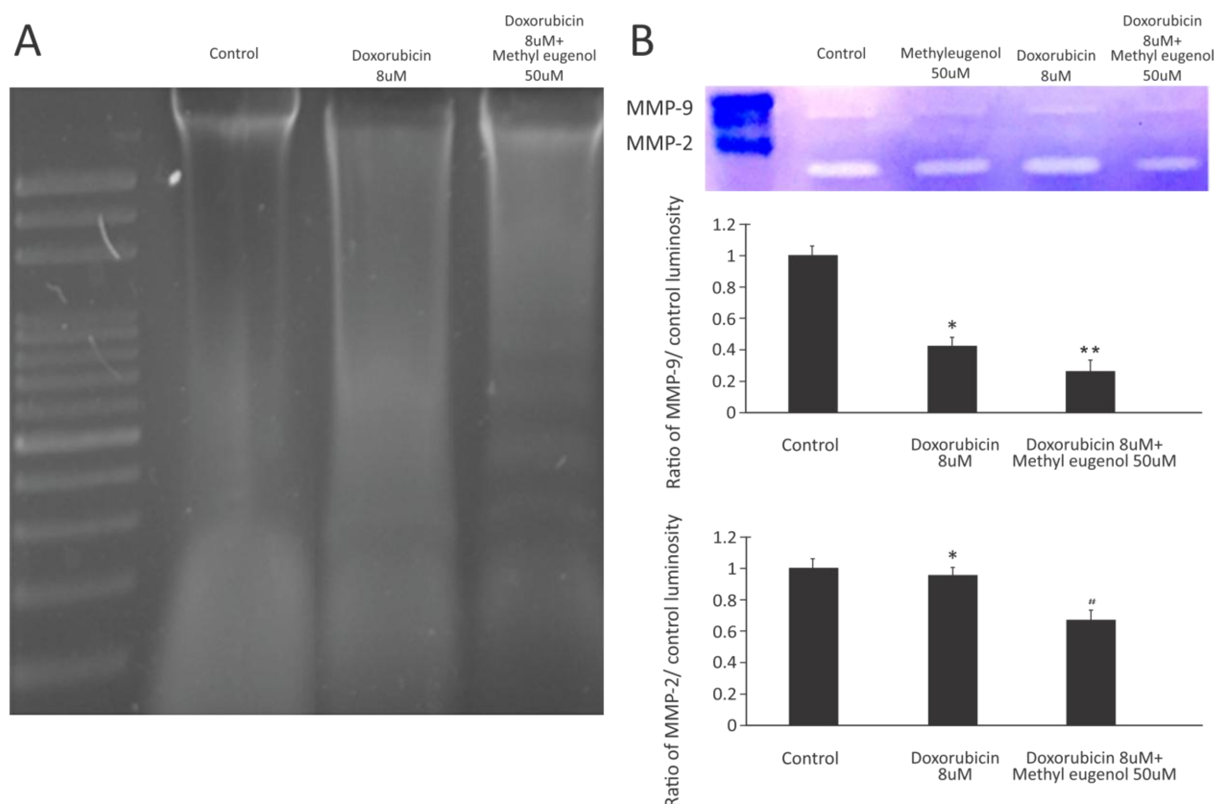

Figure 6. (A) Methyleugenol enhanced Doxorubicin on the apoptosis of drug-resistant lung adenocarcinoma cells (A549DoxR) by DNA fragmentation assay; (B) Methyleugenol enhanced doxorubicin on the inhibition of MMP secretion of A549DoxR cells by Gelatin zymography assay;
* $P < 0.01$ vs control; ** $P < 0.01$ vs control, and doxorubicin (8 μM); # $P < 0.01$ vs control, and doxorubicin (8 μM). Values expressed as mean±SEM of three independent experiments.

The results showed that methyleugenol 50 μM and doxorubicin 8 μM were combined to treat A549DoxR cells, the expression of beclin and LC-3 increased, resulting in the autophagy of A549DoxR cells. Beclin 1 and LC3 were observed to align with their established functions in the regulation of autophagy activation. The result confirmed that methyleugenol enhanced doxorubicin in the expression of beclin and LC-3 of A549DoxR cells and increased the autophagy of A549DoxR cells.

Methyleugenol enhanced the effect of doxorubicin on the activation of ATF3 in A549DoxR cells

Methyleugenol 50 μM and doxorubicin 8 μM were combined to treat A549DoxR cells, the expression of activating transcription factor 3 (ATF3) mRNA was significantly increased, compared with control group and doxorubicin 8 μM group (Figure 5D). ATF3 is involved in the cellular responses to various physiological stresses, both internally and externally, and helps regulate the balance between cell proliferation and apoptosis in tumor cells. The result confirmed that methyleugenol enhanced doxorubicin in the expression of the ATF3 gene of A549DoxR cells and increased the apoptosis of A549DoxR cells.

Lung cancer has a high mortality rate of human in the world (Siegel et al. 2019). The research and development of the treatment of lung cancer is very important. The best and fastest way is to improve the current treatment drugs. The side effects of chemotherapy drugs are serious, the dosage will be limited, so it is easy to affect the efficacy. Development of non-toxic adjunctive natural product for chemotherapeutic drugs is necessary. At present, the study used chemotherapy drug combined with non-toxic adjunctive natural product to enhance the treatment effect and reduce the resistance of chemotherapy drugs in lung cancer. Many literatures have confirmed that natural active

components can improve clinical therapeutic efficacy in the treatment of tumors, bacterial infections and cardiovascular diseases (Che et al. 2013). This study investigated the biological activity of methyleugenol on lung cancer cell lines, the results showed that methyleugenol 50 μM has a significant cytotoxic effect on A549 cells, so we use the value of 50 μM for subsequent experimental analysis, compared with the control group. In recent years, most patients with lung cancer have developed multi-drug resistance (MDR-1) in chemotherapy, which has led to limitations in the treatment of patients with lung cancer. The literatures have demonstrated that the use of natural compounds with low cytotoxicity has a certain effect on inhibiting cell proliferation, angiogenesis, metastasis, and can reverse the overexpression of P-glycoprotein in drug-resistant cancer cells (Kebsa et al. 2018; Khan et al. 2018). Our study hopes to solve the problem of drug resistance caused by chemotherapy. We established drug-resistant lung adenocarcinoma cells (A549DoxR) that were resistant to the chemotherapeutic drug doxorubicin.

Sorcin is a calcium-binding protein, which is related to the formation of multidrug resistance (MDR) and has high affinity for the chemotherapeutic drugs (Genovese et al. 2017). Drug-resistant cancer cells can be stimulated by chemotherapeutic drugs to express a large number of MDR-1. In our study, the expression of mRNA of MDR-1 of A549DoxR cells is higher, compared with A549 cells. Sorcin can directly bind to doxorubicin with high affinity and can inhibit the toxic effect of doxorubicin against lung cells. We confirmed A549DoxR cells had the obvious expression of sorcin by western blotting method, and the results were consistent with the results of RT-PCR experiments.

Autophagy involved in various physiological and pathological processes, including anti-aging, tumor suppression, protein quality control and regulation of cell death. The literature has pointed out that natural products can modulate the role between apoptosis, autophagy, and necrosis, and apoptosis and autophagy can play a synergistic effect in special condition (Radogna et al. 2015).

In this study, the results showed that methyleugenol effectively enhanced the Doxorubicin to induce autophagy and apoptosis in A549 cells and A549DoxR cells. The results of study observed that methyleugenol can enhance the sensitivity of doxorubicin to A549DoxR cells. The literature suggested that the expression of ATF-3 gene is related to the survival of cells under the stimulation of external stress (Liu et al. 2024). When methyleugenol combined doxorubicin to treat A549 cells and A549DoxR cells, the result showed methyleugenol enhanced the apoptosis of lung cancer cells induced by doxorubicin through the expression of ATF3 gene.

Malignant tumors such as lung adenocarcinoma will metastasize from the original site through invasion and metastasis and invade the stromal cell layer through the extracellular matrix (ECM) (He et al. 2017). For the metastatic changes of epithelial-mesenchymal cells, we investigated the role of methyleugenol in cell signal transduction during the metastasis of lung adenocarcinoma cells. The results showed that methyleugenol can enhance doxorubicin to inhibit the migration effect of A549 cells and A549DoxR cells.

The studies have shown that matrix metalloproteinases (MMPs) play a key role in cancer metastasis, especially in the process of destroying the extracellular matrix (ECM). MMP-2 and MMP-9 are key signaling pathways regulating cellular angiogenesis, invasion, migration, and metastasis (Webb et al. 2017). Numerous studies have used non-toxic natural products to prevent, delay, reverse or inhibit the development of cancer. Natural products can inhibit the activity of matrix metalloproteinases through different mechanisms to block the development of tumor cells (Huang et al. 2018). In the study, compared with control group and doxorubicin group, the expression levels of MMP-2 and MMP-9 of methyleugenol combined with doxorubicin treated A549 cells and A549DoxR cells were significantly reduced. Methyleugenol combined with doxorubicin can effectively reduce the activity and expression of MMP-2 and MMP-9 on A549 cells and A549DoxR cells. Methyleugenol can enhance doxorubicin to inhibit the structure of the extracellular matrix decomposed, reducing the effect of cancer cells invading the basement membrane, inhibiting the occurrence of tumor cell metastasis.

Based on the results, methyleugenol did not affect MRC-5 normal human lung fibroblasts, but also enhanced doxorubicin to inhibit the invasion, proliferation and migration, and inducing apoptosis and autophagy of A549 cells and A549DoxR cells. Methyleugenol enhanced doxorubicin inhibited the expression of vimentin in the interstitium of tumor cells and strengthened the expression of E-cadherin epithelial cell indicators.

CONCLUSION

In cancer treatment, adjuvant non-toxic natural product for chemotherapeutic drugs are necessary not only to enhance the therapeutic effect of chemotherapeutic drugs, but also to reduce the resistance of chemotherapeutic drugs. In this study, non-toxic natural product has been developed for adjuvant chemotherapy agents with good effects in the treatment of lung cancer. It is expected that methyleugenol can be clinically adjuvant chemotherapy drugs to be of great help to patients with lung cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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