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ORIGINAL ARTICLE

Chinese Native Medicinal Plant *Euphorbiaceae* Show Antitumor and Anti-oxidant Features in Lewis Lung Cancer-Bearing Mice

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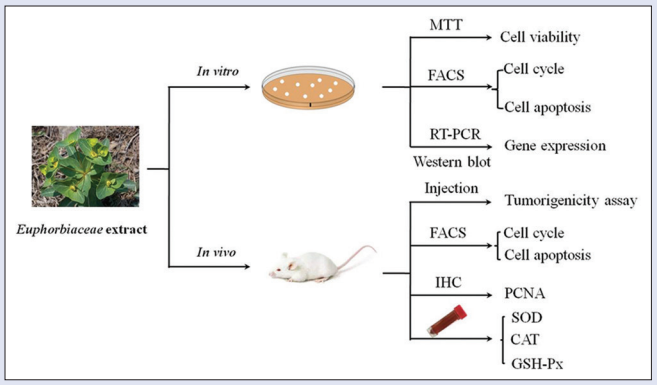
ABSTRACT

Background: As a promising means, natural products from Chinese herb provide valuable sources for cancer therapy. *Euphorbiaceae* has been used as a remedy for the treatment of many diseases, including cancer. However, studies about its effects on lung cancer are limited, and the mechanisms are not well established. **Objective:** The present study aims to investigate the anticancer effects of *Euphorbiaceae* extract (EE) *in vitro* and *in vivo*, as well as the potential mechanisms. **Materials and Methods:** In the present study, 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to investigate the effects of EE on Lewis lung adenocarcinoma cell (LLC) viability. Flow cytometric analysis was used to observe the changes of apoptosis and cell cycle of large cell carcinoma (LCC). The gene expressions were detected by real-time reverse transcription–polymerase chain reaction and Western blot. The tumorigenicity assay was performed to evaluate the inhibitory effects of EE *in vivo*. The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were also determined. **Results:** MTT assay demonstrated that EE significantly inhibited the growth of LLC in a dose-dependent manner *in vitro*. Flow cytometric analysis revealed that EE markedly induced apoptosis and G0/G1 arrest of the LCC cells. Furthermore, we found that EE led to the expression changes of apoptosis-related genes, with the decrease of Bcl-2 and the increase of Bax and caspase-9. The results from tumor-bearing animals further confirmed that the administration of EE significantly suppressed the tumor growth *in vivo*. Meanwhile, the activities of serum SOD, CAT, and GSH-Px increased significantly after the treatment of EE. **Conclusion:** These results suggested that the anticancer effects of EE may be involved in apoptosis induction, proliferation suppression, and oxidant scavenging.

Key words: Antioxidant, apoptosis, cell cycle arrest, *Euphorbiaceae*, lung cancer, tumor growth

SUMMARY

- The *Euphorbiaceae* extract (EE) exhibited significant anti-proliferative activities and pro-apoptotic functions in Lewis lung adenocarcinoma cell
- The administration of EE significantly suppressed the tumor growth in tumor-bearing mice
- The inhibitory effects of EE on lung cancer *in vitro* and *in vivo* may be involved in the abilities to proliferation suppression, apoptosis induction, and oxidant scavenging.



Abbreviations used: EE: *Euphorbiaceae* extract; LLC: Lewis lung adenocarcinoma cell; TCM: traditional Chinese Medicine; SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase; PCNA: Proliferating cell nuclear antigen; PCR: polymerase chain reaction.

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INTRODUCTION

Lung cancer is a growing problem worldwide due to the increasing incidences and devastating outcomes.[1,2] It has been estimated that about 1.3 million new cases will be diagnosed each year worldwide and 1.17 million will die of the disease. In China, lung cancer has also become the leading cause of cancer deaths for both men and women in the past 40 years.[3] Despite significant progress has been achieved in lung cancer biology and treatment, its recurrence and mortality still remain high, with 5-year survival rate <15%.[4,5] Thus, there is an urgent requirement

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to develop novel and effective anticancer candidates for the improvement of lung cancer treatment.

As a rich resource for anticancer drug discovery, medicinal plants from traditional Chinese medicine (TCM) are receiving increasing scientific attention. To date, numerous natural plant ingredients possessing anticancer effects have been translated from pharmacology investigations to clinical applications.[6,7] *Euphorbiaceae* is a perennial herbaceous plant distributed mainly in northern China.[8] Due to its effects on proliferation inhabitation, apoptosis induction, and detoxification, the medical herb has been applied to the treatment of various tumors over centuries, including leukemia and solid tumors.[9,10] Although results from preliminary studies *in vitro* indicated that *Euphorbiaceae* is a promising candidate for cancer therapy, its pharmacological action *in vivo* and related mechanisms are still obscure.

In the present study, the anticancer effect of prepared *Euphorbiaceae* extract (EE) was investigated using Lewis lung adenocarcinoma cells (LLCs). Furthermore, the influence of EE on tumor-bearing mice was observed to confirm its anticancer action *in vivo*. To explore the mechanisms induced by EE administration, molecular changes in cell proliferation, apoptosis, and antioxidant factors were detected.

MATERIALS AND METHODS

Ethics statement

All animal experiments were approved by the Binzhou Medical College Animal Care Committee (IACUC). Animal care protocols conducted were in accordance with the Animal Care Committee (IACUC) guidelines to minimize pain and discomfort to animals.

Preparation for *Euphorbiaceae* extract

The roots of *Euphorbiaceae* were purchased from Lunan Pharmaceuticals in Linyi, Shandong Province, China. The Lang-du extract was prepared according to the method in the previous literature.[11] The powdered roots of *Euphorbiaceae* were extracted with 88% ethanol at 50°C under mixing. After precipitation, the cooled solution was filtered and evaporated under reduced pressure to give a residue. Then, the extract was suspended in distilled water. After a second precipitation step using water, the supernatant was condensed as a Lang-du extract (EE) for the *in vitro* and *in vivo* experiments. The extract contained ~0.53% jolkinolide (A and B), 1.06% Euphorbia fischeriana (A and B), and flavonoids (1.75%).

Cell lines and treatments

The murine LLCs were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), glutamine (2 mM; GE Healthcare Life Sciences, HyClone Laboratories Inc.,), penicillin (100 U/ml; Sigma-Aldrich), and streptomycin (100 μg/ml; Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated under a humidified atmosphere of 5% CO_2 at 37°C. The cells in treatment groups were cultured in DMEM supplemented with 10% FBS containing different concentrations of EE (0.2, 0.4, 0.8, 1.6 mg/ml). The cells in the control group were treated with phosphate-buffered saline (PBS) vehicle.

Cell viability assay

Large cell carcinoma (LCC) cells were seeded in 96-well plates and cultured for 24 h. Then, the cells were exposed to different concentrations of EE (ranged from 0 to 1.6 mg/ml) for 24 h. Cell viability was assessed by 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, following incubation, 5 mg/mL MTT (10 μl/well) was added to the media, and the cells were further incubated in an atmosphere of 5% CO_2 at 37°C for 4 h. After the removal of the supernatant, 100 μl dimethyl sulfoxide was added to determine the optical density (OD) value at 570 nm using a microtiter plate reader (ELX800; USA). To calculate the percentage of cell viability, the means of the OD in the indicated groups from triplicate were used. Cell viability (%) = (OD treatment group/OD control group) ×100%.

The apoptosis and cell cycle analyses using flow cytometry

For cell apoptosis analysis, LLC cells were harvested and washed with ice-cold PBS, and then fixed with 70% ethanol (v/v) overnight at −20°C. Fixed cells were double stained with Annexin V-FITC (5 μg/ml) and PI (5 μg/ml). For cell cycle analysis, LCC cells were washed with ice-cold PBS twice and then resuspended in PBS containing PI (50 μg/ml)/RNase A (50 μg/ml) for 10 min. Finally, both the apoptosis and cell cycle were analyzed using a flow cytometer (FACS FC500; Beckman Coulter, Brea, CA, USA).

In vivo tumorigenicity assay

Twelve 5-week-old male BALB/C mice (from SLRC Laboratory Animal, Chinese Academy of Sciences, Shanghai, China) were given a standard laboratory diet (from the SLRC Laboratory Animal) and distilled water. The diet and water were available *ad libitum*. The mice were kept on a 12-h light/dark cycle at 22°C ± 2°C and allowed to adjust to their environment for 1 week before the initiation of experiments. The mice were injected s. c. in the right flank with 5×10^5 LLC cells in 100 μL serum-free medium. Two groups of mice (six mice per group) were then treated i. p. with EE (200 mg/ml/kg body weight) or with vehicle alone (PBS) every 2 days for 19 days. Animals were observed twice daily, and the body weight, as well as the food consumption of each mouse, was weighed every 2 days. Tumor size was measured every 2 days with calipers, based on the formula $L \times W^2/2$, where L is the length and W is the width of the tumor. At the end of the study, mice were sacrificed by cervical dislocation, and tumor masses were removed and weighed.

To explore the mechanisms of EE administration on inoculated cells *in vivo*, the apoptosis and cell cycle distribution were analyzed using flow cytometry. Briefly, fresh tumor samples (about 10 mg) from different groups were digested with trypsin containing EDTA for 30 min, and then washed with ice-cold PBS twice followed by centrifugation with 2000 r/min for 5 min. The cells were resuspended in 0.4 ml PBS and tested by flow cytometry as aforementioned. Meanwhile, the blood and tumor samples were collected and store at −80℃ for the subsequent detections.

Real-time reverse transcription–polymerase chain reaction

To explore the mechanisms of EE-induced apoptosis, the expressions of apoptosis-related molecules, such as Bax, Bcl-2, and caspase-9, were detected. Briefly, total RNAs from LCC cells or cancer tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed using SYBR®

Table 1: The primers used for quantitative real-time polymerase chain reaction

Gene	Forward primer	Reverse primer
β-actin	5'-aagccaccccacttctctct-3'	5'-gagaccaaaagccttcatacatct-3'
Caspase-9	5'-ccttgtgtcctactccaccttc-3'	5'-atctgcttgtaagtccctttcg-3'
Bax	5'-gagacacctgagctgaccttg-3'	5'-gaagttgccatcagcaaacat-3'
Bcl-2	5'-gattgtggccttctttgagttc-3'	5'-actcattcaaccagacatgcac-3'

Premix Ex Taq™ II (Takara Biotechnology co., Ltd., Dalian, China) according to the protocol provided by the manufacturer. The primers used for PCR are listed in Table 1. Human β-actin served as internal control. The cDNA was amplified under the following conditions: 95°C × 5 min; 30 cycles of 95°C × 30 s, 55°C × 30 s, and 72°C × 30 s and final extension of 72°C × 5 min. To control the PCR reaction components and the integrity of the RNA, 2 μl of each cDNA sample was amplified separately by β-actin-specific primers. The data were normalized to the β-actin expression value, and the relative expression levels were calculated using the $2^{-\Delta\Delta}$Ct method.

Western blot

The analyses of Western blot were performed to evaluate the protein expressions of apoptosis-related molecules, such as bax, Bcl-2, and caspase-9. Briefly, LCC cells with various treatments were washed twice in ice-cold PBS and lysed in 100 μl RIPA lysis buffer on ice. Cell lysates were then centrifuged at 14,000 g for 20 min at 4°C. The supernatant was recovered, and protein concentration was detected by Coomassie Blue Fast staining solution (Beyotime, China). Proteins of 20–40 μg were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane. After incubation in a blocking buffer (containing 10% nonfat milk and 0.1% Tween-20) for 2 h, the membranes were immunoblotted with specific antibodies overnight with gentle agitation at 4°C. The dilutions of primary antibodies used were caspase-9 (1:500), Bax (1:500), and Bcl-2 (1:500). β-actin (1:500) was used as a loading control. After washed for 5 min, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:3000) for detection. Finally, images were captured using a FluorChem FC2 gel imaging system (Alpha Innotech, San Leandro, CA, USA).

Immunohistochemistry

Tumor samples from animals were fixed for 24 h at 4°C in 4% formaldehyde, dehydrated, and embedded in paraffin sections (thickness, 5 μm) for immunohistochemical analysis of proliferating cell nuclear antigen (PCNA). Briefly, after deparaffinization and rehydration, the slides were washed and incubated with 2.5% H_2O_2 for 30 min to quench endogenous peroxide activities and then were blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. A monoclonal antibody against PCNA (1:150; Boster Biological Technology Co. Ltd, Wuhan, China) was used as the primary antibody for detecting protein expression. Immunodetection was performed by incubation with a specific biotinylated secondary antibody followed by use of the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA) was used as the developing reagent followed by a hematoxylin counterstain. Slides were examined under a light microscope (Olympus, Tokyo, Japan) with a × 200 magnification.

Activity detection of serum superoxide dismutase, catalase, and glutathione peroxidase in tumor-bearing mice

SOD activity was determined with the xanthine oxidase-cytochrome c method according to the previous study.[12] The cytochrome c reduction by superoxide anions generated by xanthine oxidase/hypoxanthine reaction was detected at 550 nm. Activity values were expressed in unit/ml, being 1 U of SOD defined as the amount of sample producing 50% inhibition under the assay conditions.

CAT activity was detected according to the manufacturer's instruction of the ELISA kit. The assay is based on the reaction of catalase to decompose hydrogen peroxide (H_2O_2). The excess H_2O_2 can form complexation with ammonium molybdate to produce a light yellow solution. The solution's adsorption at 450 nm can be used to calculate the concentration of H_2O_2 and therefore to indirectly indicate the CAT activities.

Measurement of GSH-Px activity was based on the method described by Kaynar.[13] GSH-Px catalyzes the oxidation of glutathione by cumene hydroperoxide for the whole activity of GSH-Px. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The reduction in the absorbance of NADPH at 340 nm was measured. One unit of GSH-Px activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of reduced glutathione (GSH) by cumene hydroperoxide or hydrogen peroxide to oxidize glutathione per min at pH 7 at 37°C.

Statistical analysis

A two-tailed Student's *t*-test and an analysis of variance were used to detect differences in the mean values of the variables. All statistical analyses were performed using SPSS 15.0 software package (SPSS, Inc., Chicago, IL, USA). For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Euphorbiaceae extract inhibits the growth of large cell carcinoma cell lines

After treated with various doses (0, 0.2, 0.4, 0.8, and 1.6 mg/ml) of EE for 24 h, MTT assays were performed to test the effect of EE on the viability of LCC cells. The results showed a significant concentration-dependent decrease in cell viability after 24 h and exposure to EE. In the control group, the rate of LCC viability was 92.1% ± 4.7%. In contrast, the rates of LCC viability after the treatments with EE for 24 h were 80.9% ± 3.9%, 66.7% ± 4.8%, 57.7% ± 3.8%, and 47.3% ± 6.2% for indicated concentrations, respectively [Figure 1].

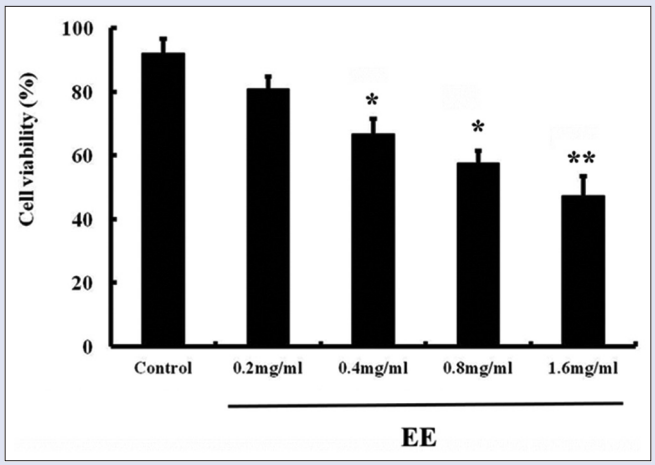

Figure 1: The effects of EE on LCC cells viability. Cell viability was tested using the MTT assay. The cell viability was significantly reduced during the treatment of EE with indicated concentrations (0.2, 0.4, 0.8, and 1.6 mg/ml) for 24 h. The group without EE exposure was a control. Data are expressed as the mean ± SD *$P < 0.05$, **$P < 0.01$ versus control. LCC: Large cell carcinoma; EE: *Euphorbiaceae* extract; MTT: 3-(4,5-dimethythiazol-. 2-yl)-2,5-diphenyl tetrazolium bromide

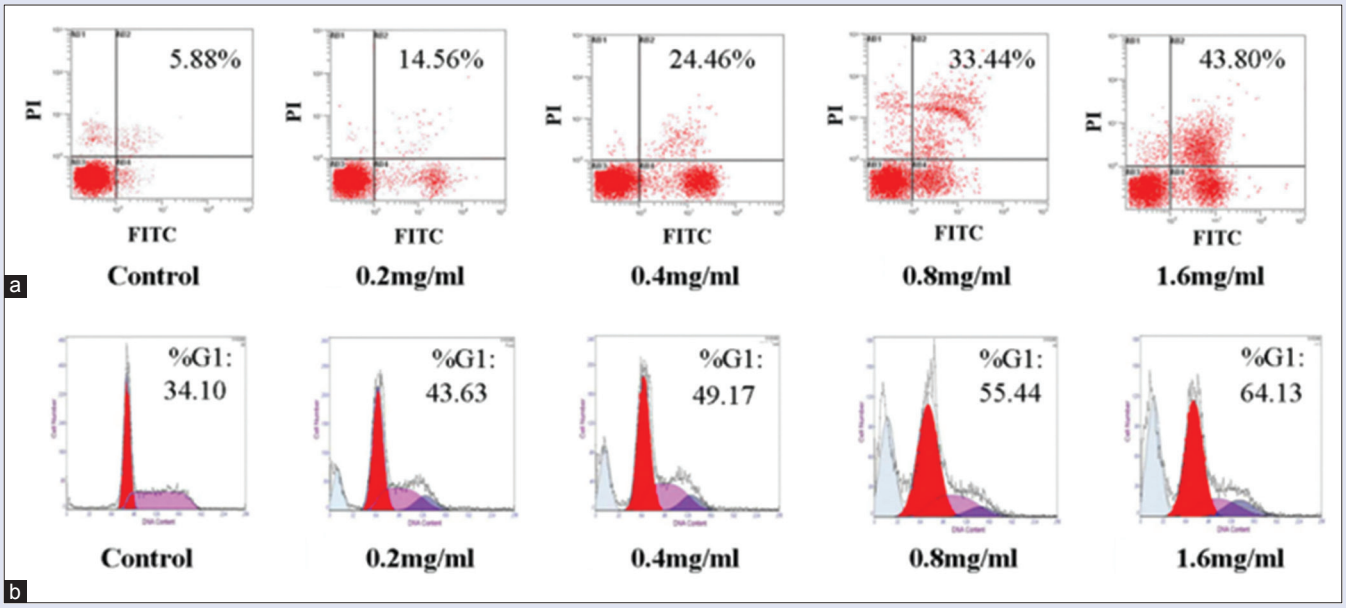

Figure 2: The effects of EE treatment on apoptosis and cell cycle distribution of LCC cells. (a) The apoptosis analyses in LCC cells treated with EE at the indicated concentrations for 24 h. (b) The proportions of LCC cells in the G0/G1 phase treated with EE are presented. The group without EE treatment was a control. LCC: Large cell carcinoma; EE: *Euphorbiaceae* extract

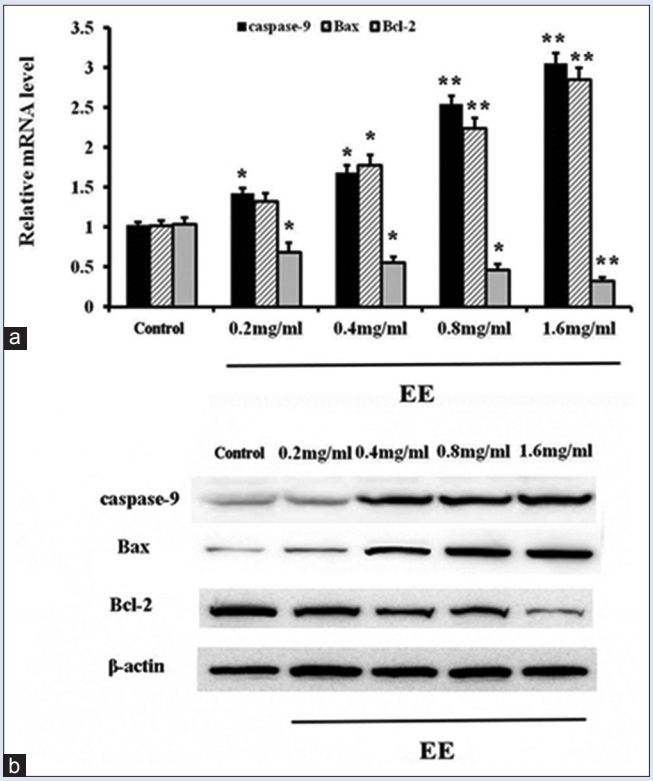

Figure 3: EE treatments influenced on the mRNA expression of apoptosis-related genes in LCC cells. The cells were treated with the indicated concentrations of EE for 24 h. (a) The mRNA expression levels of caspase-9, Bax, and Bcl-2 were analyzed by quantitative real-time PCR. (b) The protein expression levels of caspase-9, Bax, and Bcl-2 were analyzed by Western blot. β-actin was used as a control. Data were normalized to the mean value of the control group and presented as the mean ± SD *$P < 0.05$, **$P < 0.01$ versus control. PCR: polymerase chain reaction; SD: Standard deviation; LCC: Large cell carcinoma; EE: *Euphorbiaceae* extract

Euphorbiaceae extract induces apoptosis and G0/G1 cell cycle arrest in large cell carcinoma cells

To evaluate the influences of EE on the status of apoptosis and cell cycle distribution, flow cytometry was performed. The Annexin V-FITC/PI double staining assay revealed that apoptosis rate of LCC cells induced by EE increased in dose-dependent manners, with the average rates of 5.88%, 14.56%, 24.46%, 33.44%, and 43.80%, respectively [Figure 2a]. Furthermore, significant increases in the proportion of cell population in the G0/G1 phases were found, indicating the induction of G0/G1 arrest during EE treatments [Figure 2b].

To further delineate the mechanism by which EE-induced apoptosis in LCC cells, Realtime RT-PCR and Western blot analyses were performed to determine the mRNA and protein levels of caspase-9, Bax, and Bcl-2, respectively. As shown in Figure 3a and b, compared with the untreated control group, the levels of caspase-9 and Bax were considerably increased after EE treatment in a dose-dependent manner. However, Bcl-2 was markedly inhibited after EE incubation [Figure 3a and b].

Effect of *Euphorbiaceae* extract on inoculated tumors in BALB/c mice

Due to the immunologically compatible with LCC cells, BALB/c mice were used as an appropriate animal model to evaluate the inhibition efficiency of EE *in vivo*. From the injection of LCC cells (5×10^5) into the right axilla of mice to produce a tumor-bearing model, the tumor volumes of different groups at 1, 4, 7, 10, 13, 16, and 19 days, respectively. As shown in Figure 4a and b, the tumor volumes from EE-treated tumor-bearing mice were significantly reduced compared with those from the control group. At the endpoint (day 19), mice were sacrificed and tumors were removed and weighed. The data showed that the tumor weights of tumor-bearing mice in EE-treated groups were markedly decreased than those in the control group [Figure 4c and d]. Moreover, it should be noted that there is no difference in body weight between groups, indicating that no side effects were observed during EE treatment (data not shown).

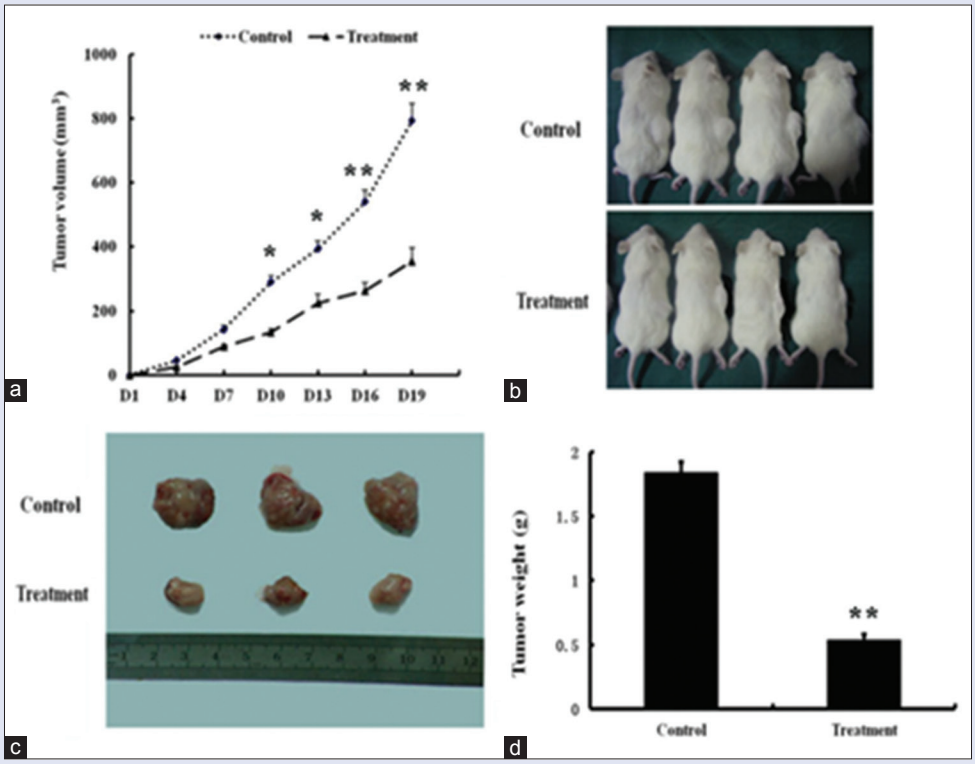

Figure 4: EE treatments inhibited tumor growth in tumor-bearing mice. (a) Comparison of tumor volume in tumor-bearing mice between groups with EE treatment and without EE treatment (control). The tumor volumes in different groups were measured on days 1, 4, 7, 10, 13, 16, and 19. The tumor volumes were calculated as described in materials and methods section. (b) The representative photos of tumor-bearing mice at day 19. (c) The values of tumor weight showing the inhibition effect of EE on tumor growth. (d) The histograms represent the quantitative data of tumor weights between groups. Data are expressed as the mean ± SD *P < 0.05, **P < 0.01 versus control. EE: *Euphorbiaceae* extract; SD: Standard deviation

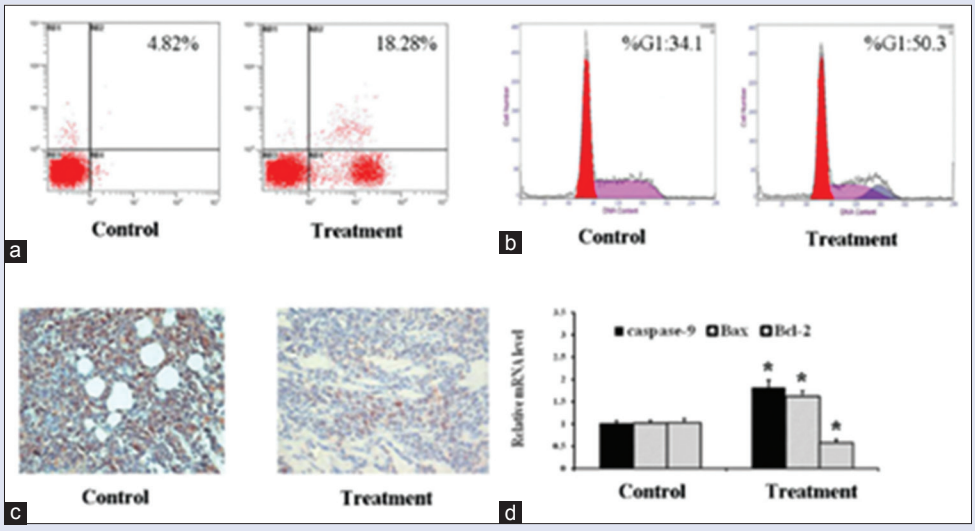

Figure 5: The effects of EE on the apoptosis and proliferation of LCC cells *in vivo*. (a) Flow cytometry analyses of apoptosis in LCC cells of tumor-bearing mice. (b) The G0/G1 proportions of LCC cells in tumor-bearing mice treated with EE are presented. (c) The immunochemistry detections of PCNA between groups. (d) The mRNA expression levels of caspase-9, Bax, and Bcl-2 were analyzed by quantitative real-time PCR in tumor tissues. The group without EE treatment was a control. Data are expressed as the mean ± SD *P < 0.05 versus control. SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase; SD: Standard deviation; LCC: Large cell carcinoma; EE: *Euphorbiaceae* extract; PCNA: Proliferating cell nuclear antigen; PCR: polymerase chain reaction

The proapoptotic and antiproliferative effects of *Euphorbiaceae* extract *in vivo*

The analyses of flow cytometry showed that, in LCC cells of tumor-bearing C57BL/6 mice, there was a marked increase of apoptotic rates after treating with EE. The apoptotic rates were 4.82% and 18.28% in control and EE-treated groups, respectively [Figure 5a]. At the meanwhile, Figure 5b demonstrated that the percentage of cells in G0/G1 phase increased obviously in the EE-treatment group compared with the control (34.1% and 50.3%, respectively). Furthermore, paraffin sections of tumor tissues were applied to the immunohistochemistry staining of PCNA, a well-known indicator for cell proliferation. As shown in Figure 5c, we found a decreased number of PCNA-positive cells in tumor tissue from the EE-treated mice. Similarly, RT-PCR analysis demonstrated that the mRNA levels of caspase-9 and Bax were upregulated in tumor tissues from the mice after EE treatment, as well as a significant downregulation of Bcl-2 [Figure 5d], which is consistent with the results from *in vitro* study. These observations took more insights into the roles of EE in tumorigenesis *in vivo*.

The activity of serum superoxide dismutase, catalase, and glutathione peroxidase in tumor-bearing mice

To further understand the possible mechanisms of action of EE against the LCC cell growth in mice, we investigated the effects of EE on the activities of antioxidant enzymes in tumor-bearing mice. Figure 6 showed the effect of EE on the activities of SOD, CAT, and GSH-Px in the serum. The analysis indicated that, compared with the control, the administration of EE could significantly increase the activity of SOD, CAT, and GSH-Px *in vivo* ($P < 0.05$).

DISCUSSION

As one of the most prevalent malignancies, lung cancer has always been a severe concern. It has been estimated that non-small-cell lung cancer accounts for about 85% of lung cancers, in which lung adenocarcinoma accounts for 40% of all the lung cancer cases.[14-16] Despite advances in the lung cancer biology and some improvements in treatment strategies, the therapeutic efficacy of lung cancer remains dismal.

The medical practice has illustrated that TCM possesses valuable material resources and provide a good foundation for developing novel agents for the management of lung cancer. Lines of studies have demonstrated that the administration of EE has benefit to various diseases including asthma, atherosclerosis, tuberculosis, and tumors.[17,18] However, little is known with regard to the therapeutic effects of EE on lung adenocarcinoma. In the current study, the effects of EE on the *in vitro* and *in vivo* growth of LCC cells and its molecular mechanisms of action were investigated. The results showed that EE induced the apoptosis of LCC cells in accordance with the changes of apoptosis-related proteins, such as Bax, Bcl-2, and caspase-9. Similarly, the analysis of cell cycle distribution indicated that the treatment of EE results in cycle arrest at the G0/G1 phase, implying its antiproliferative roles. Findings from *in vivo* demonstrated that the tumor growth of LCC-grafted mice could be suppressed significantly by EE supplement. Moreover, the immunohistochemistry detection revealed the declined expression of PCNA. In addition, the serum activities of SOD, CAT, and GSH-Px enhanced significantly. Based on these observations, the results indicated that the anticancer potentials of EE *in vivo* may be involved in the decrease of proliferation and angiogenesis, as well as in the increase of antioxidant capacity.

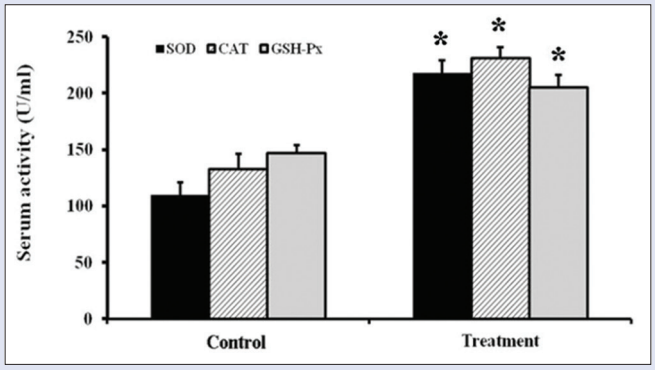

Figure 6: Activities of SOD, CAT, and GSH-Px in serum of LCC-inoculating mice. Values are presented as mean ± SD of six mice in relevant groups. The group without EE treatment was a control. Data were compared with the control and were analyzed using the Student's *t*-test. *$P < 0.05$ versus control. SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase; LCC: Large cell carcinoma; SD: Standard deviation

It is well known that the induction of apoptosis is one of the main mechanisms by which anticancer agents eradicate tumor cells.[18,19] This is also the case for extracts or preparations of TCM, including EE.[20,21] Considerable evidence confirmed that many compounds from *Euphorbiaceae*, such as diterpenoids, triterpenoids, and steroids, can act as potent apoptosis inducer in a wide spectrum of malignancies.[22,23] The EE-induced apoptosis in this study may be triggered by the classical mitochondrial pathway because of the increased expression of caspase-9 and the reduction of the Bcl-2/Bax ratio. Besides, the finding of the present study that EE treatment can lead to the prominent G0/G1 arrest in LLC cells is in accordance with that of the previous studies, in which G0/G1 regulation has been shown to play a key role in proliferation, differentiation, and apoptosis. These data *in vitro* preliminarily suggested that the pro-apoptotic and anti-proliferative actions of EE contributed to its anticancer effects.

Due to the complex constituents in natural products and their complicated interaction *in vivo*, ensuring the validity of the efficacy and safety in tumor-bearing animals is a critical step for the screening of active ingredients in TCM herbs.[24,25] Using LCC-bearing mice, our results demonstrated that the tumor volumes decreased markedly in the EE-treated group than those of the control group. In contrast, there is no difference in body weight between both groups. These data were in accordance with previous studies, indicating that some ingredients may have potential selectivity toward tumor cells.[26] To elucidate the underlying mechanisms about the protective effects of EE *in vivo*, the status of apoptosis and proliferation of implanted LCC cells was tested. Expectedly, EE showed an intensive suppression on transplanted LCC cells. Similarly, the proliferation can be blocked based on the findings from cell cycle analysis and PCNA staining. Subsequently, we try to determine factors related to the changes of apoptosis and proliferation. Considering the importance of oxidative stress on cell destination, the serum activities of enzymatic antioxidants including SOD, GPx, and CAT were further measured.[27,28] All enhanced activities of these antioxidative enzymes in the treated group indicated that the application of EE did have the impact of cellular redox status. This result is in line with previous reports that a reduction in cellular oxidants via supplementation with antioxidants can inhibit cell proliferation and promote apoptosis *in vitro*, as well as treatment of cancer cells with hydrogen peroxide lead to the enhancement of lung metastasis formation *in vivo*.[29-31]

Some limitations in the present study should be noted for future investigations. First, the extract of EE applied in this study is a compound containing a variety of active ingredients. The identification and isolation of single component with high target efficacy and the low side

effect will facilitate its clinical translation. Second, the exact mechanisms and signaling pathways involved in the anticancer actions of EE have not been fully elucidated. The more detailed understanding about the influences of cellular signal pathway induced by EE will be a benefit to its better applications in cancer therapy.[32,33] Third, further verification in patient samples should be warranted for the accumulation of clinical data to make the relevant findings more solid and convincing.

CONCLUSION

Our study revealed that EE from *Euphorbiaceae*, a medicinal plant from TCM, showed obvious anticancer effects both *in vitro* and *in vivo*. Moreover, its effects on cancer inhibition may be involved in the abilities to apoptosis induction, proliferation suppression, and the oxidant scavenging, indicating that EE may be a potential candidate for adjuvant or alternative therapy for lung cancer.

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Conflicts of interest

There are no conflicts of interest.

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