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Original article

Vaccaria segetalis extract can inhibit angiogenesis

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Background: Study of antiangiogenic drugs is one of the cutting-edge fields in new antitumor drug development. However, system studies on *Vaccaria segetalis* extract as an inhibitor of tumor-induced neovascularization have not been published yet. In the current study, we performed a preliminary investigation of the antiangiogenic effect of *Vaccaria segetalis* extract on angiogenesis.
Objective: We studied the inhibitory effect of *Vaccaria segetalis* extract on neovascularization.
Methods: The effects of *Vaccaria segetalis* extract on in vitro proliferation, migration, and tube formation of human mammary endothelial cells (HMECs) were determined by 3 - (4,5) - dimethylthiahiazo (-z-y1) - 3, 5 -di-phenytetrazoliumromide (MTT) assay and Matrigel plug assay. The antiangiogenic effect of *Vaccaria segetalis* extract was observed on the chick chorioallantoic-membrane (CAM) angiogenesis model. The CD31 expression on HMECs was detected by immunohistochemical staining. The therapeutic effect of *Vaccaria segetalis* extract on the metastasis of Lewis lung cancer was also observed in the mouse model.
Results: The proliferation and migration of human mammary epithelial cells (HMECs) were inhibited significantly by *Vaccaria segetalis* extract in a dose-dependent manner (IC_{50} = 50 μg/mL). Angiogenesis was also inhibited in the Matrigel-plug mouse model and the chick chorioallantoic-membrane model. The *Vaccaria segetalis* extract treatment reduced the expression of CD31 in HMECs and inhibited the growth and metastasis of Lewis lung carcinoma cells in the mouse model.
Conclusion: The *Vaccaria segetalis* extract, which can obviously inhibit angiogenesis, could be developed as a promising new antiangiogenic drug.

Keywords: Angiogenensis, CD31, endothelial cell, human mammary endothelial cells, *Vaccaria segetalis*

The most significant biological characteristics of tumor cells are the malignant behaviors of invasion, metastasis, and abnormal proliferation, which have been shown to be highly associated with tumor-induced neoangiogenesis, resulting from the disruption of cellular regulatory mechanisms (including the cellular transmembrane signal transduction mechanism) [1]. During tumor progression, the newly formed tumor-associated blood vessels serve initially as feeding/nurturing tubes for the growing tumor and then as conduits for dissemination of tumor cells that escape from the existing primary tumor. Therefore, control of tumor angiogenesis has become a central issue in the fight against cancer progression because direct antitumor therapy may be ineffective once tumor cells reach target secondary organs and generate metastatic foci.

Since Folkman J. first proposed in 1972, that the growth and metastasis of tumors are dependent on angiogenesis [2], antiangiogenic therapy has been widely regarded in biological medicine research and pharmaceutical companies as a promising new strategy to inhibit tumor proliferation and metastasis. Antiangiogenic drugs, which target activated endothelial cells, could inhibit proliferation and metastasis of tumors by reducing the supply of nutrition, blood, and oxygen by new vessels. Thus, study of antiangiogenic drugs is one of the cutting-edge fields in new antitumor drug development [3]. The social and economic benefits of this type of drug are huge. For example, the new antitumor drug, Avasitin, of the American Genentech Company, which has been used in the late stage of rectal and breast cancers, can extend survival for four to six months. The annual turnover of Avasitin was over 3 billion dollars in 2008,

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and it has become one of the most important new antitumor drugs. In addition, several drugs targeting different genes, such as Angiostatin and Endostatin [4, 5], have been moved to clinical phase II and phase III trials, and they are predicted to have profits of more than 850 million dollars per year.

Although antiangiogenic drugs have been widely used in the clinical field, there are still many problems, such as the need for intravascular administration, high dosages, and lack of specificity for tumor blood vessels. These problems are due to most of the clinical antiangiogenic drugs being polypeptides, which are not stable in vivo and can only be administered by injection at high doses (50 mg/kg). Therefore, biological availability is low while expense is high. In addition, side effects with long-term treatment are still under evaluation. Consequently, it is crucial to develop drugs of high efficacy, high specificity for tumor angiogenesis, low toxicity, small molecular weight, and low cost.

Based on our previous work using the established in vivo and in vitro models for validating the antiangiogenic effect of drugs, we screened thousands of traditional Chinese medicines for an extract that can be administered orally—*Vaccaria segetalis* extract.

Vaccaria segetalis (Neck) Garcke (Caryophyllaceae) is widely distributed in China. In traditional Chinese medicine, the seeds of this plant, Wangbuliuxing, are used to activate blood circulation, to promote milk secretion and diuresis, and to treat amenorrhea and mastitis [6-9]. Phytochemistry studies have reported that the main components of this traditional medicine are flavonoid glycosides, cyclic peptides, triterpenoid saponins, lipoids, fatty acids, and monosaccharides. However, system studies on *Vaccaria segetalis* extract as an inhibitor of tumor-induced neovascularization have not been published yet. In the current study, we performed a preliminary investigation of its antiangiogenic effect.

Materials

Equipment

Model 3110 (Thermo-Forma, Burlingame, CA), MK3 (Thermo-Labsystems, the Republic of Finland), and BX-40 (Olympus, Japan)

Reagents and chemicals

Vaccaria segetalis was purchased from Shanhe Pharma in Wuxi, Jiangsu Province, and the botanical identification was performed by Professor Jianwei Chen (Nanjing University of Traditional Chinese Medicine).

Animals

BALB/c-nu/nu nude mice, C57BL/6 mice, and female Swiss Nude mice were obtained from the Institute of Zoology, Chinese Academy of Sciences. All experimental protocols were approved by our local Animal Ethics Committee

Cell lines

VSV40-transduced HMECs were provided by Professor He Lu from Institut National de la Sante et de la Recherche M dicale (INSERM) U553. C51 colon cancer cells and Lewis lung cancer cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences.

Methods

Preparation of Vaccaria segetalis and Glycyrrhiza extracts

Vaccaria segetalis was decocted twice in water for 1.5 hour and 1 hour, respectively. The extract liquids were combined, filtered, decompressed and condensed into concretes, and freeze-dried to *Vaccaria segetalis* extract powder. *Glycyrrhiza* extract was prepared according to the same procedure as described previously [10].

Cell culture

Monolayers of cells were grown in MCDB-131ÿmedium complete with trace elements ÿmedium consisting of MCDB-131 basic medium supplemented with 1 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor (EGF), 15% calf serum, and 0.01% L-glutamine. Cells were cultured in a 37°C incubator with a humidified atmosphere containing 5% CO_2. Media were changed every three days. Cells were detached from the tissue flasks by treatment with 0.05% trypsin/EDTA and passaged as usual as described [11]. For the assays, cells were monitored to maintain normal proliferation and collected during the logarithmic growth phase.

MTT assay

Cells were digested with 0.25% trypsin/EDTA and then seeded in a 96-well plate (about 6000 to 7000 cells/well). The plate was incubated overnight at 37°C in a humidified incubator supplied with 5% CO_2. The

cells were treated with compounds at different concentrations, with the cells in the medium as the baseline control and intact cells as the negative control of eight replicates in each group. After another 48-hour incubation, the MTT reagent (20 μL/200 μL per well of the 96-well plate) was added to a final concentration of 0.5 mg/mL, and the plates were incubated at 37°C for approximately four hours. The absorbance was read on a plate reader using approximately 570 nM [12]. The results were tabulated, and the viability was calculated as follows [13]: % Viability = (Mean absorbance of negative control - Mean absorbance of sample)/(Mean absorbance of negative control - Mean absorbance of baseline control) × 100%.

CAM assay

Fertilized White Leghorn chicken eggs were incubated in a humidified atmosphere and the CAM was prepared as previously described [14]. Six-millimeter filter disks prepared by stiletto and sterilized were used to confine the test material to a defined area of the CAM. Vaccaria segetalis extract was dissolved in distilled water (100 μg/mL) and 10 μL was placed on the membrane centers as the drug-treated group. The negative control group was treated with saline only. The disks were allowed to air dry under sterile conditions. After three days' incubation, the embryos of the different groups were placed with the air-dried disks directly attached on top of the CAM. After 48 hours, the vessels were observed with a stereomicroscope, and digitalized pictures were taken and recorded.

In vivo Matrigel plug assay

Matrigels (300 L each) in a liquid state at 4°C were injected subcutaneously into the abdominal region of 8-week-old female nude mice (Swiss Nude, 20 g) [15]. The mice were divided into four groups with five mice in each group. There were a baseline group (Matrigels mixed with 2% glucose solution), a control group (Matrigels mixed with 0.1 μg Basic fibroblast growth factor (bFGF)), a negative control group (Matrigels mixed with 0.1 μg bFGF and 100 μL 100 μg/mL *Glycyrrhiza* extract), and an experiment group (Matrigels mixed with 0.1 μg bFGF and 100 μL 100 μg/mL *Vaccaria segetalis* extract). All of the mice were treated on the same day and sacrificed on day 7. Matrigel plugs were harvested, xed in ethanol, and paraffin embedded for platelet-endothelial celladhesion molecule-1(PECAM-1) immuno-histochemistry. The slides were recorded under a stereomicroscope (200×), and the migrated vessels in the Matrigel plugs were calculated in the same view.

Analysis of the expression level of CD31 in vascular endothelial cells of the in vivo C51 tumor growth mouse model with different treatments

Two hundred million C51 colon cancer cells were suspended in 200 μL PBS and injected subcutaneously into the right thigh areas of 8-week-old BALB/c-nude mice. When the tumor volume reached about 5 mm × 5 mm, the mice bearing suitably sized tumors were randomly divided into three groups with five mice in each group. The baseline-control group was dosed with saline, the negative-control group was dosed with 0.4 mL *Glycyrrhiza* extract (100 μg/mL), and the experiment group was dosed with 0.4 mL *Vaccaria segetalis* extract (100 μg/mL). Oral administrations were performed using a gastric tube daily for 60 consecutive days, and the length and width of the tumors were recorded every day. Each mouse was observed for changes in general appearance and behavior, and no abnormal phenomena were recorded during the treatment period. After the study, the nude mice were sacrificed by CO_2 deep anesthesia [16], followed by cervical dislocation. The tumor tissues were resected and weighed. The results were tabulated, and the percent tumor inhibitory rate (IR) was calculated as follows, percent IR = ([Mean weight of control group - Mean weight of experiment group]/Mean weight of experiment group) × 100%. For histological examination of the tumor vasculature, the tumor tissues were quickly frozen in liquid nitrogen. Frozen sections of the tissues were cut at a thickness of 5 to 10 μm. For visualization of the blood vessels, immunohistochemical staining [17] for CD31 was performed, and the vessels were counted microscopically using 200 × magnification. For all sections, counting was done in three different parts of the sections, with the mean value recorded as microvessel density (MVD).

Analysis of the inhibitory effect of Vaccaria segetalis extract on the Lewis lung cancer metastasis mouse model

Fifteen male C57BL/6 mice with weights ranging from 14 to 18 g were used in this study. A subcutaneous injection of 106 Lewis lung cancer cells in 0.2 mL aliquots was administered into the right axillary area

of the mice through an incision with the animals under anesthesia. The mice were randomly divided into three groups with five mice in each group. The baseline-control group was dosed with saline, the negative-control group was dosed with 0.4 mL *Glycyrrhiza* extract (100 μg/mL), and the experiment group was dosed with 0.4 mL *Vaccaria segetalis* extract (100 μg/mL). Drug administration was initiated four days after Lewis cell inoculation. Mice were dosed by daily gastric infusion for four consecutive weeks and then sacrificed by cervical dislocation. The lungs and tumors were resected. For histological evaluation, part of the tumor tissue was prepared for paraffin and frozen sectioning followed by H&E staining [18]. The sections were observed under a microscope (×100) and three discontinuous sections of each sample were utilized to count the mean number of metastases to the lung.

Statistical analysis

The results are given as means±SD. Statistical significance was assessed with Student's *t* test for paired data ($p < 0.05$).

Results

The yield efficiency of Vaccaria segetalis extract

The yield of the *Vaccaria segetalis* extract powder was 12.14%, i.e., 121.4 mg extract powder from 1 g *Vaccaria segetalis*.

The effect of Vaccaria segetalis extract on the proliferation of HMECs and on the migration of HMECs

Vaccaria segetalis extract significantly inhibited proliferation of HMECs in a dose-dependent manner with an IC_{50} of 50 μg/mL as can be seen in **Figure 1**.

The number of cells migrating to the Matrigel was much higher in the control group (**Figure 2A**), while the *Vaccaria segetalis* extract inhibited the migration of HMECs in a dose-dependent manner (**Figure 2B, C, D**), suggesting that *Vaccaria segetalis* extract could inhibit the migration of vascular endothelial cells.

The effect of Vaccaria segetalis extract on angiogenesis in the CAM assay and in the Matrigel plugs

Quantitation was performed three days after implantation and involved counting the number of CAM vessels in the area of the filter disk (**Table 1**). In response to proangiogenic stimuli, the newly formed blood vessels converged toward the disk in a wheel-spoke pattern. The new blood vessel formation in *Vaccaria segetalis* extract group significantly reduced (**Figure 3B**) comparing the control group (**Figure 3A**), which suggested that inhibition of angiogenesis by antiangiogenic compounds resulted in the lack of new blood vessel formation and sometimes the disappearance of pre-existing vessel networks. Angiogenesis levels were also determined by counting branch points in the vessels, particularly adjacent to the disks.

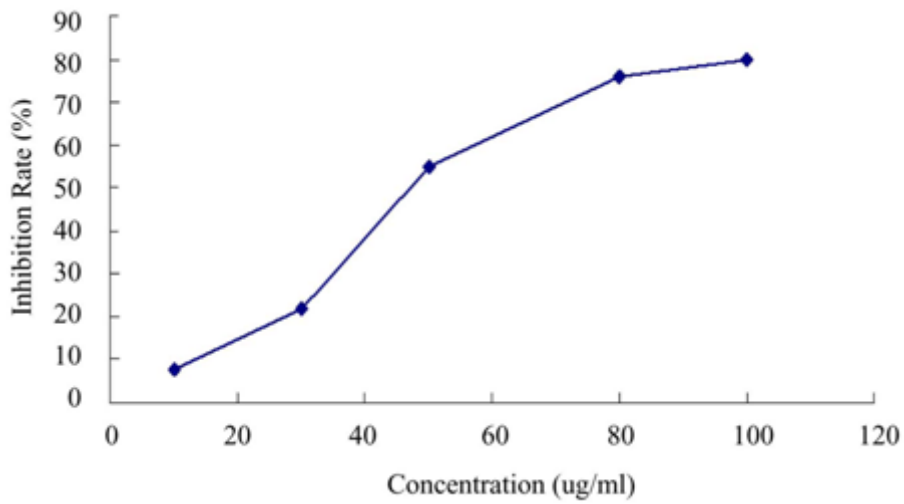

Figure 1. Effect of *Vaccaria segetalis* extract on proliferation of HMECs. HMECs were treated with compounds at different concentrations of 10ug/ml, 30 μg/ml, 50 μg/ml, 80 μg/ml, 100 μg/ml for 48 hours, and determined by MTT, with the cells in the medium as the baseline control and intact cells as the negative control of eight replicates in each group.

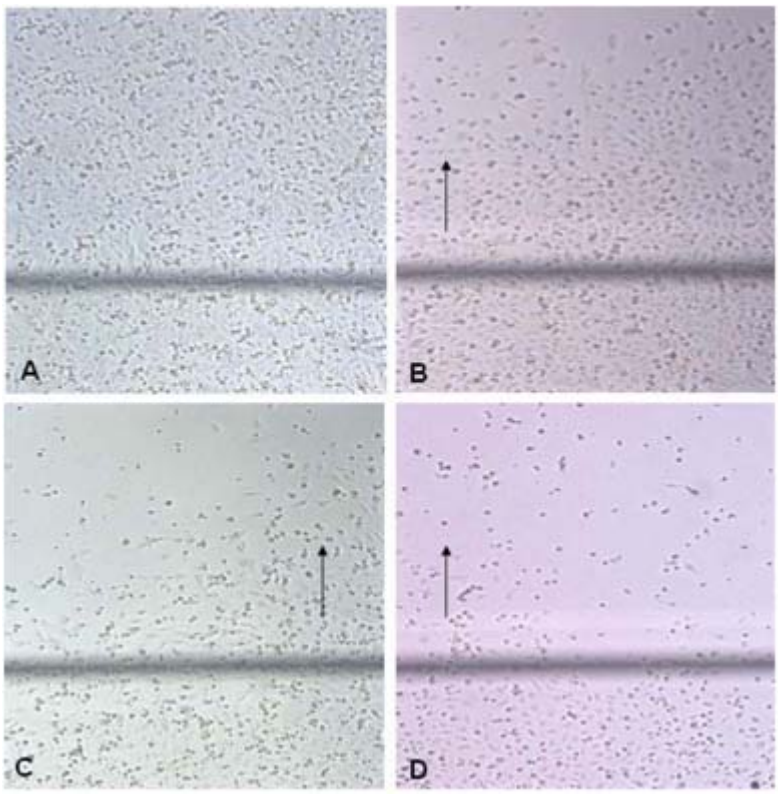

Figure 2. Effect of *Vaccaria segetalis* extract on the migration of HMECs (×200). **A:** Treated with saline only as control group. **B:** Treated with 10 μg/mL *Vaccaria segetalis* extracts. **C:** Treated with 30 μg/mL *Vaccaria segetalis* extracts. **D:** Treated with 50 μg/mL *Vaccaria segetalis* extracts. The arrows indicate the direction of migration. All groups were treated for 48 hours.

Table 1. Effect of Vaccaria segetalis extract on neovascularization in the CAM (cm2) (x±s, n = 12)

***Vaccaria segetalis* extract**	**Large vessel**	**Middle vessel**	**Small vessel**
0 μg/mL (control)	2.5±0.20	3.5±0.67	19.7±0.47
10 μg/mL	2.2±0.12	4.1±0.88	12.6±0.35
50 μg/mL	3.1±1.22	3.3±1.91	10.1±0.22*
100 μg/mL	2.7±0.53	2.5±1.11	6.3±0.32**

*p <0.05, **p <0.01 *vs*. Control

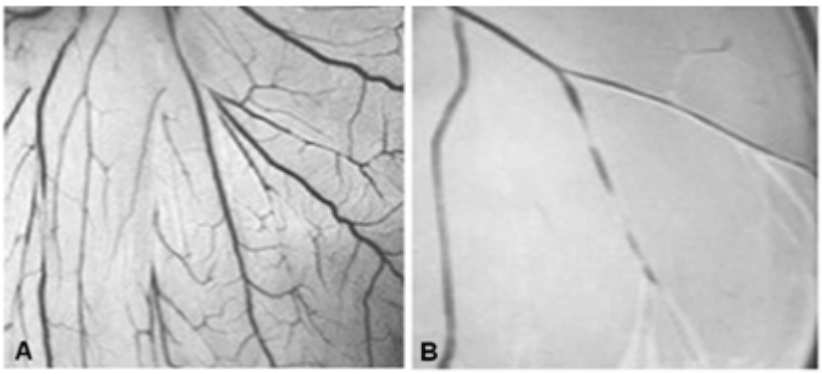

Figure 3. Effect of *Vaccaria segetalis* extract on neovascularization in the CAM (×10). **A:** Treated with saline only as baseline control group. **B:** Treated with 100 μg/ml *Vaccaria segetalis* extract as experiment group. All groups were treated for 48 hours.

We found that bFGF could significantly induce the formation of microvessels in the Matrigel plug (**Figure 4A and B**). There was no obvious difference in microvessel formation with the *Glycyrrhiza* extract (**Figure 4C**), but the *Vaccaria segetalis* extract significantly reduced the number of invasive endothelial cells in the Matrigel plug and inhibited microvessel formation (**Figure 4D**).

The effect of Vaccaria segetalis extract on the expression of CD31 on endothelial cells of the C51 tumor metastasis mouse model

The antitumor effect of the test drug was evaluated in the C51 colon cancer xenograft model. Treatment with *Vaccaria segetalis* extract resulted in 58.3% inhibitory rate of tumor growth, and this effect reached statistical significance when compared with the IR of the baseline-control group treated with saline (**Table 2**). However, there was no significant difference between the mean tumor weights of *Glycyrrhiza uralensis* extract-treated animals and saline-treated animals. For histological examination of the tumor vasculature, tumor tissues were stained for CD31. Comparing vessel densities in tumors of saline-treated animals with those of treated animals, a dramatic difference was apparent, reflecting the decreased vessel density with therapy (**Figure 5**).

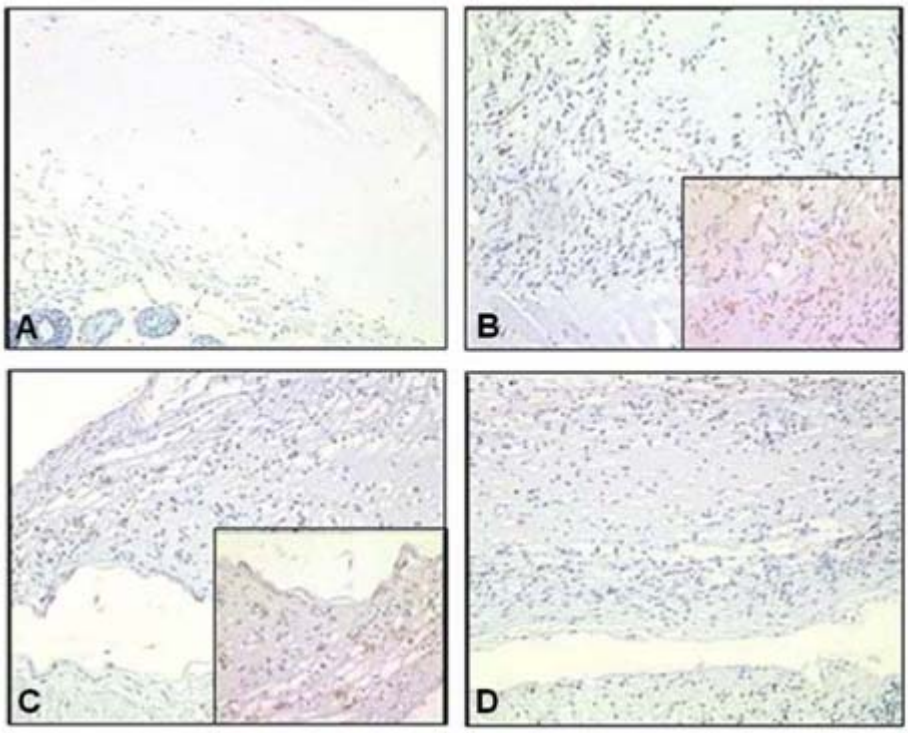

Figure 4. Effect of *Vaccaria segetalis* extract on angiogenesis by mouse Matrigel plug assay (×200). **A:** Baseline group. Matrigels mixed with 2% glucose solution. **B:** Control group. Matrigels mixed with 0.1 μg bFGF. **C:** *Glycyrrhiza uralensis* extracts group. Matrigels mixed with 0.1 μg bFGF and 100 μl 100 μg/ml *Glycyrrhiza uralensis* extract. **D:** *Vaccaria segetalis* extracts group. Matrigels mixed with 0.1 μg bFGF and 100 μl 100 μg/ml *Vaccaria segetalis* extracts. All of the mice were treated on the same day and sacrificed on day 7.

Table 2. The rate of inhibition (IR) of tumor growth and microvessel density (MVD) of different groups

Group	Animal number	IR (%)	MVD ($x \pm s$)
Saline	5	0	31.6±2.89
Glycyrrhiza uralensis extract	5	12.2	29.1±1.78
Vaccaria segetalis extract	5	58.3	11.2±0.54*

*p <0.01 *vs.* Control

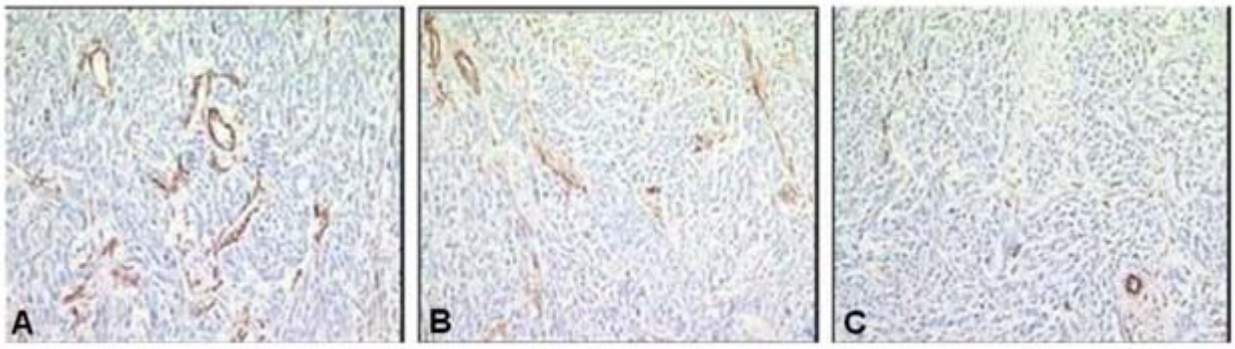

Figure 5. CD31 immunohistochemical staining in colon cancer cells of different groups (×200). A: Baseline control group was treated with 0.4 ml saline only. B: Negative control group was treated with 0.4 ml 100 μg/ml *Glycyrrhiza uralensis* extracts. C: Experiment group was treated with 0.4 ml 100 μg/ml *Vaccaria segetalis* extracts. All of the mice were treated with oral administrations using a gastric tube daily for 60 consecutive days.

The inhibitory effect of Vaccaria segetalis extract on tumor metastasis in the Lewis lung cancer mouse model

In the Lewis lung cancer metastasis model, the tumor region was wide, and the migration of tumor cells and metastatic foci were obvious with visible tumor giant cells in the saline-treated group (**Figure 6A**). Compared to the saline-treated group, no significant change was found in the group treated with *Glycyrrhiza uralensis* extract (**Figure 6B**). Notably, treatment with *Vaccaria segetalis* extract obviously ameliorated and stabilized the focus of infection and inhibited metastasis of tumor cells (**Figure 6C**), with scant visible pathological changes in the lung and normal structure of the alveoli (**Table 3**).

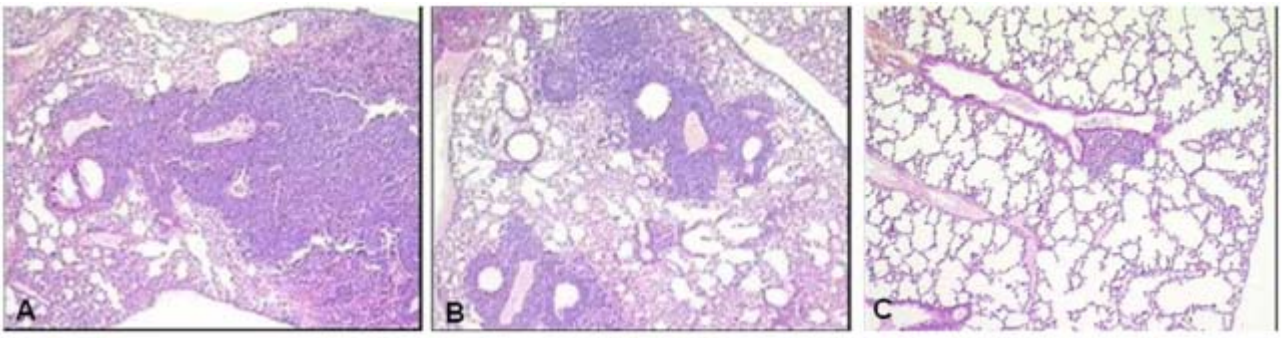

Figure 6. Antitumor effect of *Vaccaria segetalis* extract in mice implanted with Lewis lung carcinoma (×100). A: Baseline control group was treated with 0.4 ml saline only. B: Negative control group was treated with 0.4 ml 100 μg/ml *Glycyrrhiza uralensis* extracts. C: Experiment group was treated with 0.4 ml 100 μg/ml *Vaccaria segetalis* extracts. Drug administration was initiated four days after Lewis cell inoculation. All mice were dosed by daily gastric infusion for four consecutive weeks.

Table 3. Antitumor effect of Vaccaria segetalis extract in mice implanted with Lewis lung carcinoma (n = 3)

Group	Metastasis incidence ($x \pm s$)
Saline	4.5 6.6
Glycyrrhiza uralensis extract	3.6 1.31
Vaccaria segetalis extract	0.3 1.1*

*p <0.05, **p <0.01 *vs*. Control

Discussion

Studies have shown that angiogenesis inhibitors specifically inhibit new vessel formation and growth to block tumor metastasisb [19]. Compared to the conventional tumor-killing drugs and chemotherapeutics, the antiangiogenesis drugs have many advantages, such as a wide range of antitumor effects and avoidance of some drug-resistant side effects. Thus, it is imperative to investigate new anti-tumor drugs based on the antiangiogenesis effects. Because existing clinical drugs are too expensive for most patients, it is also very important to develop cheap angiogenesis inhibitors that are administered orally and are highly effective. Our traditional medicines have unique advantages, such as efficacy with oral administration and low toxicity, and have attracted the interest of medicine researchers to screen for the highly effective extracts. Based on various in-vivo and in-vitro models for validating drug efficacy of angiogenesis inhibitors, we have done ample work on new medicine screening, mechanism clarification, and drug development of our traditional medicines. Based on these studies, we found that the extract of *Vaccaria segetalis* significantly inhibited tumor angiogenesis by the oral route, which could solve the problem of intravenous injection of polypeptides. To date, we have not found any study of the effect of *Vaccaria segetalis* extract on angiogenesis. Therefore, we tried to describe the important influences of *Vaccaria segetalis* extract on tumor angiogenesis inhibition in this article. The proliferation and migration of vascular endothelial cells is the first step of angiogenesis, meantime the vascular endothelial growth factor (VEGF) is a highly specific mitogen for micro- and macrovascular endothelial cells derived from arteries, veins, and lymphatics [20]. VEGF is known as vascular permeability factor (VFP) based on its ability to induce vascular leakage [21, 22]. Furthermore, VEGF can promote the proliferation of the vessel itself and the tumor [23, 24]. Inhibition of the proliferation of vascular endothelial cells could decrease vessel generation and promote apoptosis of tumor cells, thereby inhibiting proliferation and metastasis of the tumor. Consequently, a therapeutic strategy that targets the endothelial cells by inhibiting their proliferation and migration has become a sought-after method. In this study, we found that in vitro treatment with *Vaccaria segetalis* extract at low dose (<100 μg/mL) showed a significant inhibitory effect on the proliferation and migration of HMECs. The inhibitory effect was dose dependent and the IC_{50} was 50 μg/mL. With a dose of 30 μg/mL, the migration of HMECs to the agarose was significantly reduced and even blocked. This suggested that the main function of *Vaccaria segetalis* extract is to block the proliferation and migration of endothelial cells. CAM assay is a widely accepted and applied in vivo angiogenesis model. The CAM data of *Vaccaria segetalis* extract application showed a strong inhibitory effect on angiogenesis in the early developmental stage of chicken embryos.

It is believed that tumor cells and infiltrative macrophages and mast cells in tumors can promote angiogenesis by paracrine secretion. These cytokines accelerate proliferation and migration of vascular endothelial cells to form new vessels, and increase the permeability of the vessels to cause aggregation of surrounding tissue fibrils and infiltration of monocytes, fibroblasts, and endothelial cells, which would further promote tumor-matrix formation and the metastasis of tumor cells along with the vessels [25]. bFGF is found in almost all tissues of mesodermal and neuroectodermal origin and in tumors derived from these tissues. It has been show to have mitogenic, chemotactic, and angiogenic activity, promoting cell growth, differentiation, and motility [26]. Many cells express bFGF only transiently and store it in a biologically inactive form [27-29]. According to our Matrigel plug assay, *Vaccaria segetalis* extract inhibited bFGF-induced endothelial cell-forming tubiform structures and blocked the signal transduction pathway of bFGF-induced angiogenesis, which might be one of the mechanisms of action of *Vaccaria segetalis* extract in antiangiogenesis. CD31 is a crucial molecule for cell adhesion. In the process of new vessel formation, CD31 might participate in signal transduction of cell adhesion. The homodimer of CD31 could promote the cell–cell connection of endothelial cells to generate new vessels [30, 31]. In the C51 colon carcinoma model, the application of *Vaccaria segetalis* extract reduced the tumor by 58.3%, decreased the expression of CD31 in the surrounding endothelial cells, and reduced the microvessel density of the surrounding tissue. The application of *Vaccaria segetalis* extract in the Lewis lung cancer metastasis model also showed its positive effect in inhibiting the cell transfer of Lewis cells to the lungs while improving the condition of the infective focus.

From the above pharmacodynamic assays, we deduced that the effects of *Vaccaria segetalis* extract

included not only inhibition of proliferation and migration of HMECs to block cytokine-induced vessel formation, but also reduction of angiogenesis in embryos and microvessel density in tumors to efficiently block the metastasis of tumor cells by the possible mechanism of decreasing the expression of CD31. It was suggested that *Vaccaria segetalis* extract was a potential new orally administered antiangiogenesis drug for tumor therapy.

The authors declare no conflict of interest to report.

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