Antitumor Studies of Earthworm Fibrinolytic Enzyme Component A from *Eisenia foetida* on Breast Cancer Cell Line MCF-7

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Earthworm fibrinolytic enzyme from Chinese earthworm *Eisenia foetida* was isolated to investigate its antitumor activity in breast cancer cells. The protein isolated was characterised as earthworm fibrinolytic enzyme using the fibrin plate method. The molecular weight of earthworm fibrinolytic enzyme component was determined to be 25 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis assay using a standard protein ladder. Earthworm fibrinolytic enzyme can markedly inhibit the growth and migration of MCF-7 cells in a dose and time-dependent manner. In addition, MCF-7 cells treated with earthworm fibrinolytic enzyme (40 μg/ml) began to undergo apoptosis after 24 h. Expression of focal adhesion kinase and CD44v6 measured using reverse transcription polymerase chain reaction and Western blot was down-regulated in a concentration-dependent manner (20-80 μg/ml), resulting in the suppression of MCF-7 cells adhesion. The obtained earthworm fibrinolytic enzyme displayed an antitumor effect on MCF-7 cells *in vitro*, revealing the therapeutic potential of *E. foetida*.

Key words: Earthworm fibrinolytic enzyme component A, MCF-7 cells, migration, antitumor activity

Breast cancer is the most common malignancy among women presenting an emerging major health problem around the world and the number of cases of has increased dramatically in Asia over the past few decades[1-4]. The development drug resistance in breast cancer is a growing challenge in the field of cancer therapy. Thus, there is an urgent need to discover new therapeutic agents.

Earthworm is widely used in Chinese traditional medicine. Previous studies on earthworm extracts have demonstrated its ability to improve haemostatic disorders[5] and stimulate wound healing[6]. In addition, higher concentrations of earthworm extracts are nontoxic and have excellent antiinflammatory and antipyretic properties[7]. Further researches have revealed the potential for using earthworm extracts as antibacterial[8], antifungal[9], antiviral[10] and antitumor[8,9] agents. In this study, earthworms were studied to determine their active components used in the traditional prescriptions as starting materials.

Earthworm fibrinolytic enzyme (EFE, also known as lumbrokinase, a group of six serine proteases, was first isolated by Mihara and coworkers from *Lumbricus rubellus* who divided them into four groups: F-I-0, F-I-1/2, F-II and F-III-1/2[11,12]. To date, many other species of earthworm including *Eisenia foetida*[13], *Neanthes japonica*[14] and *Pheretima posthuma*[15] have been explored for several fibrinolytic enzymes. EFE has been applied as an oral thrombolytic agents for the prevention of cardiac and cerebrovascular diseases due to its strong fibrinolytic activity[16]. In recent times, the anticarcinogenic effect of EFE has drawn amounts of global research attention. A previous study has shown that EFE isolated from *E. foetida* has strong antitumor activity in human hepatoma cells *in vitro* and *in vivo*[17]. A novel acidic serine protease purified from *N. japonica* could inhibit the proliferation of cells, induce apoptosis and enhance chemo-susceptibility of acute promyelocytic leukemia cells NB4[14]. A recent study suggested that a serine protease from *P. posthuma*

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had potent cytotoxic activity against Vero cells and satisfactory antitumor activity in MCF-7 cells[3]. EFE component A (EFEA, EC#: 3.4.21), a protein, which is typical for an elastase-like enzyme and functions both as a direct fibrinolytic enzyme and a plasminogen activator, was purified from the earthworm *E. fetida*[18]. The crystal structure of the EFEa is the first reported for an EFE component and belongs to the trypsin-like serine protease family[19]. However, whether it has antitumor activity against mammary cancer is not yet clear.

In this study, EFEA was isolated and purified using size and charge based chromatographic separation techniques from *E. foetida*. MCF-7 is a type of cell line representing the breast cancer adenoma cells that can be expanded and differentiated in culture. Using different techniques, EFEA was determined that it could act as antiproliferative and antimetastatic factors, inhibiting the viability and migration of MCF-7 cells.

MATERIALS AND METHODS

The MCF-7, a human breast adenocarcinoma cell line, was obtained from Chinese academy sciences (Shanghai, China). Earthworms, *E. foetida* were provided by Huaneng Pharmaceutical Co. Ltd (Shandong, China). Columns of diethylaminoethanol (DEAE)-Sepharose FF, Sephacryl S-200 HR and Sephadex G-10 were purchased from GE (USA). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), focal adhesion kinase (FAK) and CD44v6 were purchased from GenScript (Jiangsu, China); antibodies for GAPDH and FAK were purchased from Santa Cruz (USA). Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS) and trypsin were all purchased from Gibco (USA); CCK-8 kit Beyotime (Shanghai, China); human fibrinogen and thrombin were purchased from Sigma (USA). Other reagents and chemicals used were of analytical grade and commercially available.

Isolation and purification of EFFA:

The preparation of EFEA was carried out as described earlier[19] with a slight change. Crude protein was isolated from 500 g homogenate of frozen *E. fetida* by ammonium sulphate precipitation. Afterwards, the precipitated protein was resuspended in 50 mM Tris-HCl buffer (pH 8.0) and subjected to successive DEAE-cellulose ion-exchange chromatography and Sephacryl S-200 HR gel filtration after dialysis. The eluates were loaded onto a Sephadex-G10 column for desalination. Finally, the active fractions were pooled, freeze-dried and stored at −20° for further study.

Enzyme assay on fibrin plate:

Enzyme activity was evaluated using the fibrin plate method[20] with some minor adjustments. Briefly, the solution composed of 6 mg/ml human fibrinogen in 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 0.8% agarose and 100 IU/ml thrombin was poured into a sterile petri dish. The solution in the plate was left still for 1 h to form fibrin clot and then 2 mm diameter wells were made in the plate for sample application. To observe the fibrinolytic activity, 10 µl of sample solution was carefully dropped into each well and incubated at 37° for 18 h.

Determination of molecular weight:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[21]. A 12% acrylamide matrix was prepared and protein samples (10 µl) were loaded with protein marker. After completion of electrophoresis, gels were stained with Coomassie brilliant blue G250 and destained with a destaining solution. The average molecular weight was determined with a standard protein ladder.

Liquid chromatography-mass spectrometry (LC-MS) analysis:

After SDS-PAGE, gel bands were stained with Coomassie brilliant blue G250 and destained with a destaining solution, and subsequently sent to Beijing Protein Innovation Co. Ltd for LC-MS analysis.

Cell culture:

Human breast adenocarcinoma cell line MCF-7 was cultured in DMEM, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated at 37° in a 5% CO_2 atmosphere. The medium was routinely changed every two days and at confluence, cells were subcultured by 0.25% trypsinisation. In all experiments, cells were used between P4 and P5 passage cultures.

Cell viability assay and morphology assay:

Cell viability was examined using a Cell Counting Kit-8 (CCK-8)[22]. The MCF-7 cells were seeded in 96-well plates (8×10^3 cells/well) and cultured at 37° in 5% CO_2 to adhere. After 24 h incubation, the supernatant was discarded and the cells were treated with a growth medium containing EFEa of varying

concentrations. Cells treated with paclitaxel (PTX) were adopted as positive controls. Cell morphology was observed under the invert microscope[23] after being treated for 12, 24 and 48 h. After incubation for 48 h, cell viability was tested by CCK-8.

Scratch-wound assay:

MCF-7 cells in logarithmic growth phase were plated in 24-well plates (5×10^5 cells/well) and grew until confluence. Thereafter, the procedure described[24] was followed. A straight scratch was made with a new 200 µl pipette tip in a monolayer of the cells to simulate a wound. The wound was exposed to EFEa or PTX and the cells without treatment were used as controls. After wounding for 4, 24 and 48 h, respectively, the scratches were observed under the invert microscope.

Reverse transcription polymerase chain reaction (RT-PCR):

The mRNA expression of related genes was investigated as previously described[22] with some modifications. Total RNA was isolated from cultured MCF-7 cells using TRIzol reagent according to the manufacturer's instructions. Isolated RNAs were reverse transcribed using Revert Aid™ First Strand cDNA synthesis kit. Amplification of the reverse transcription products by PCR with GAPDH serving as an internal control was carried out using Dream Taq™ PCR Master Mix (2×). The corresponding cDNA fragments were subjected to 35 cycles of annealing. After electrophoresis of the PCR products on agarose gel, which was stained with DuRed and visualised using Bio-Rad Gel Doc EQ, the results were analysed by Image J 1.47. The sequences of the PCR primers were: GAPDH (forward: 5'-GACA-CATATTGCTTCAATGCTTCAGC-3', reverse: 5'-GTCCACCACCCTGTTGCTGTAG-3'); FAK (forward: 5'-GGCAGCATCTATCCAGGTCAGG-3', reverse:5'-CAGGGCGAGGCGGTTTCTTT-3'); CD44v6 (forward: 5'-GACACATATTGCTTC AATGCT-TCAGC-3', reverse: 5'-TACTAGGAGTTGCCTGGAT-GGTAG -3').

Western blot analysis:

The protein expression of related genes was carried out as previously described[25]. Cells were homogenised in RIPA lysis buffer containing protease inhibitor phenylmethane sulfonyl fluoride (PMSF). Protein samples were loaded on an acrylamide/bisacrylamide SDS-PAGE gel, electrophoresed and transferred to polyvinylidene difluoride membranes. The polyvinylidene difluoride blots were blocked with 5% non-fat dry milk in tris-buffered saline tween 20 (TBST) buffer and then incubated with primary antibodies. The blots were then incubated with second antibody for 2 h. After three washes with TBST buffer, the resulting immunocomplex bands were developed with Immobilon Western Chemiluminescent HRP Substrate.

Statistical analysis:

Data were expressed as the means±standard deviation (SD) and paired t tests were used to evaluate statistical significance by the GraphPad Prism software package for Windows (v. 5.0 GraphPad Prism Software Inc, San Diego, CA). Values with 95% confidence ($P<0.05$) were considered significant.

RESULTS AND DISCUSSION

The protein isolated displayed strong fibrinolytic activity during testing by the fibrin plate method (fig. 1). Its molecular weight was 25 kDa, as determined by SDS-PAGE (fig. 2). The protein sequence was 67% identical to EFEA (Q8MX72) as determined by LC-MS (fig. 3) from Beijing Protein Innovation Co. Ltd. Therefore, it could be speculated that this protein was EFEA.

After incubation with PTX for 48 h, the cell growth was approximately 50% of the controls (fig. 4). EFEA caused a proliferation inhibition of MCF-7 cells in a dose-dependent manner, with significant reuction, which was comparable to PTX group at the highest tested dose (160 µg/ml). The IC_{50} value was 130.26 µg/ml. These data suggested that EFEA could effectively inhibit the proliferation of MCF-7 cells.
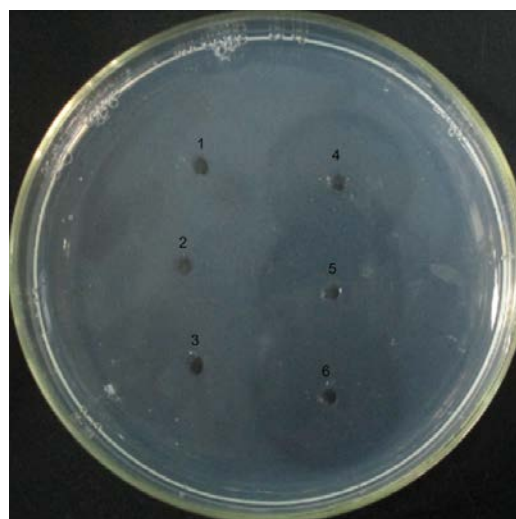

Fig. 1: Fibrinolytic activity of the protein isolated from *E. foetida* Fibrinolytic activity exhibited by 1-3: normal saline; 4-6: 100 µg/ml protein sample
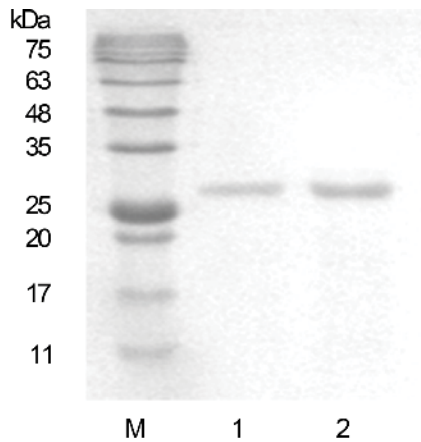
Fig. 2: SDS-PAGE of purified EFE
Lane M: molecular weight marker; lane 1: 1 mg/ml EFE; lane 2: 2 mg/ml EFE
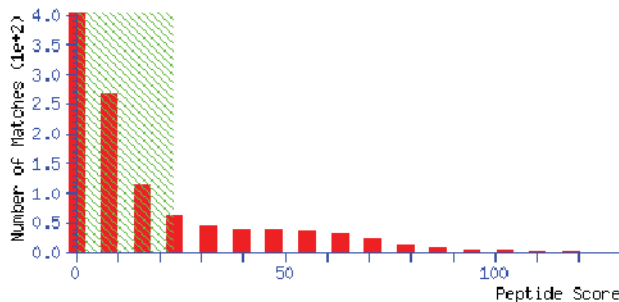
Fig. 3: Score analysis for peptide mapping of purified EFE
Individual ion scores greater than 23 can be considered to have extensive homology
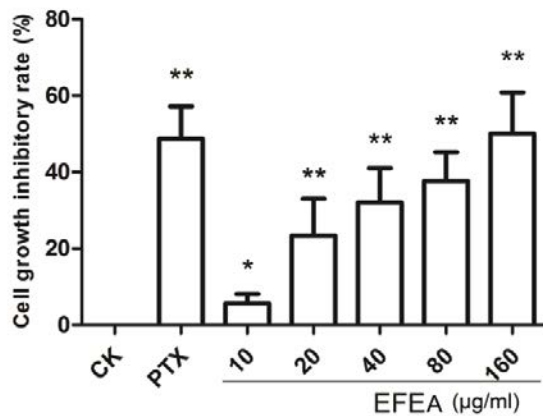

Fig. 4: The inhibitory effect of EFEA on MCF-7 cell growth
Cells were treated with PTX (0.5 µg/ml) or EFEA (10, 20, 40, 80 and 160 µg/ml) for 48 h; cell growth inhibition rate was identified by CCK-8 assay. CK: normal control group; PTX: paclitaxel group; EFEA: earthworm fibrinolytic enzyme component A group; n=5, **P<0.01 vs. CK

The majority of MCF-7 cells changed their morphology initially when they were exposed to 40 µg/ml of EFEA. As shown in fig. 5, after exposure to EFEA for 12 h,

spindle cells gradually became swollen compared to control group. Round cells gathered and some predisposed to undergo apoptosis after 24 h. These changes were more pronounced with the prolonged treatment time 48 h, which was consistent with the PTX (0.5 µg/ml) treatment group.

The ability of MCF-7 cells to migrate was examined at three time points (4, 24 and 48 h; fig. 6). The untreated control cells grew and migrated more rapidly compared to the EFEA treated group (40 µg/ml). The scratch band healed almost completely without EFEA treatment. Conversely, the migration of MCF-7 treated with EFEA were predominantly inhibited. These results showed that EFEA could suppress the migration of MCF-7 cells.
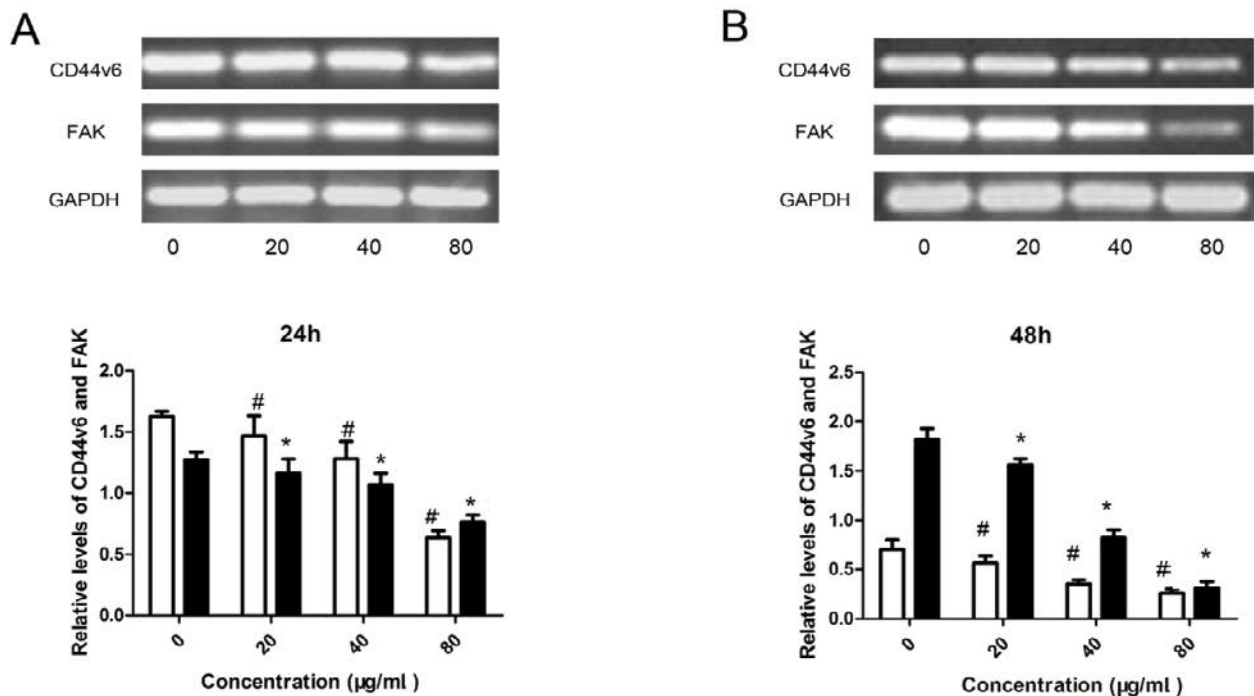
The mRNA expression of CD44v6 and FAK in MCF-7 cells was detected using RT-PCR assay at two time points (24 and 48 h). Exposure of MCF-7 cells to various concentrations of EFEA for 24 h (fig. 7A) decreased the expression of CD44v6 and FAK in mRNA level, with the greatest reduction at 80 µg/ml, the highest concentration ever measured. After 48 h (fig. 7B), the mRNA expression of CD44v6 and FAK was decreased further. It has been demonstrated that EFEA could resist cell adhesion by reducing the mRNA expression of CD44v6 and FAK in a concentration-dependent manner. The protein expression of FAK in MCF-7 cells treated with EFEA for 48 h was effectively attenuated in a concentration-dependent manner (fig. 8). The result, in accordance with fig. 7, showed that EFEA could inhibit cell adhesion by reducing the protein expression of FAK.
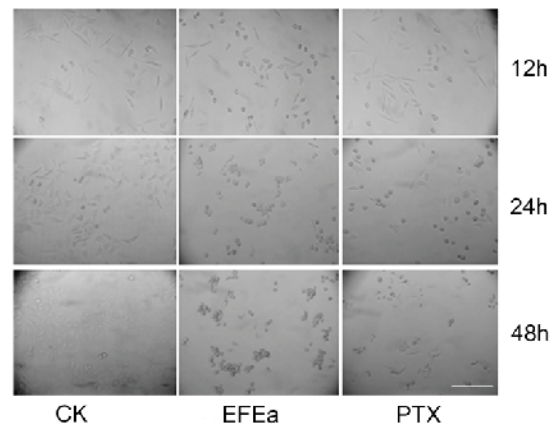
Fig. 5: The effect of EFEA on the morphology of MCF-7 cells
Cells were treated with PTX (0.5 µg/ml) or EFEA (40 µg/ml) for 12, 24, 48 h and morphological changes were observed with a microscope. CK: normal control group; EFEA: earthworm fibrinolytic enzyme component A group; PTX: paclitaxel group; scale bar=100 µm
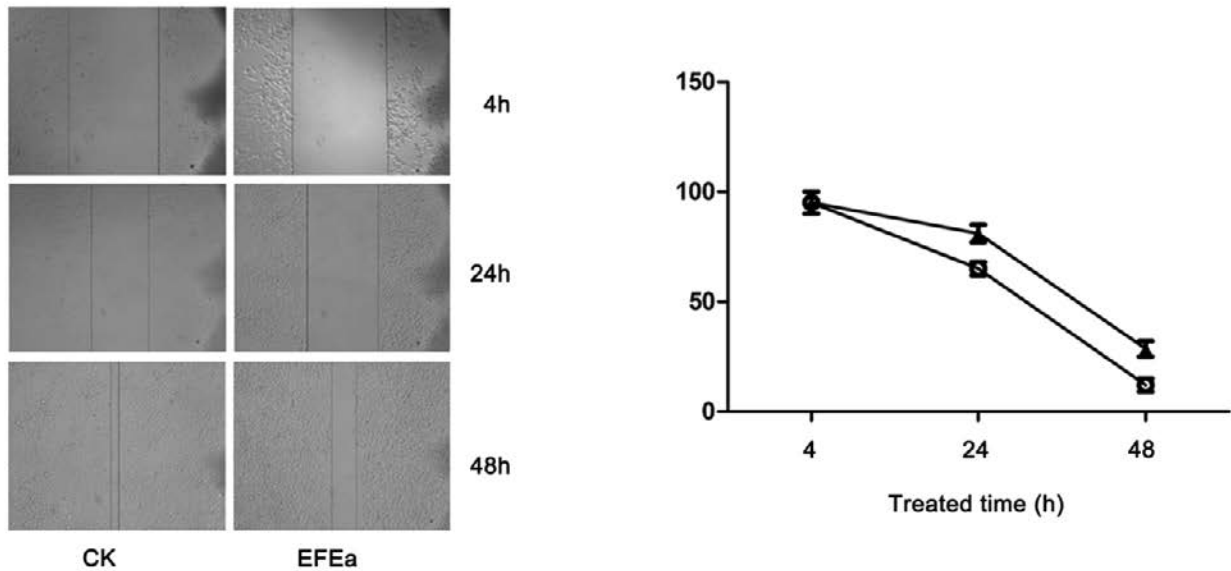
Fig. 6: The inhibitory effect of EFEA on the migration of MCF-7 cells
MCF-7 cells were treated with or without EFEA (40 μg/ml) for 4, 24 or 48 h. Wounds were created in the cultured cells and images were taken with a microscope. CK: normal control group; EFEA: earthworm fibrinolytic enzyme component A group; scale bar=100 μm. *P<0.01 vs. CK. —•— CK; —▲— EFEA

Fig. 7: Effect of EFEA on CD44v6 and FAK mRNA expression in MCF-7 cells
MCF-7 cells were treated with different concentrations of EFEA (0, 20, 40, 80 μg/ml) for 24 h (A), 48 h (B) and mRNA levels of CD44v6 and FAK were measured by RT-PCR. *,#P<0.01 vs. untreated group. □ CD44v6/GAPDH; ■ FAK/GAPDH

In recent years, among the diverse pharmceutical effects of EFE, its antitumor activity has attracted interest[3,14,17,26,27]. Using the CCK-8 assay, it could be inferred that EFEA could inhibit the proliferation of MCF-7 cells. The IC_{50} value was 130.26 μg/ml, which was lower than the protease purified from the Indian earthworm *P. posthuma* by Verma[3]. The antitumor machanisms of earthworm extract are complex. Some

studies verified it could enhance the swallowing ability of abdominal macrophage in tumor bearing mice, increase the spleen index and thymus index and activate the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in mice serum[28]. Therefore, one possible mechanism might relate to the enhancement in macrophage activity and antioxidative effect of earthworm extract. There
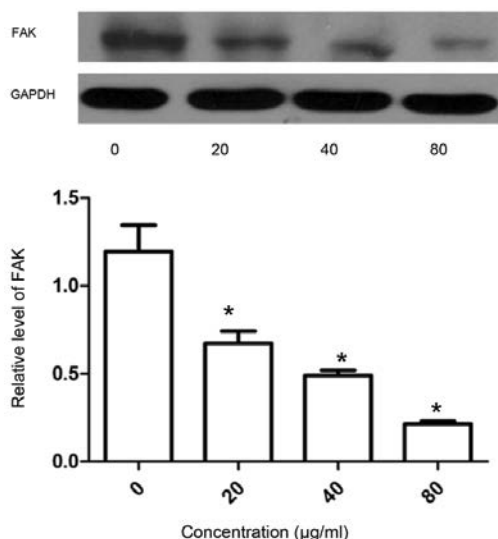
Fig. 8: Effect of EFEA on FAK protein expression in MCF-7 cells

MCF-7 cells were treated with different concentrations of EFEA (0, 20, 40 or 80 µg/ml) for 48 h. Protein expression of FAK was measured by Western blot. n=3, *P<0.01 vs. untreated group

are growing evidences that the antitumor effect of EFE is associated with the apoptosis of cells[14,17,29]. Consistent with these reports, our results indicated that cell apoptosis was induced by EFEA with significant morphological changes. However, we did not investigate apoptosis related proteins (caspases and Bax/Bcl-2) and signal pathways, which would become a focus of our subsequent research.

A wound scratch assay indicated that the migration of MCF-7, which was treated with EFEA (40 µg/ml) was inhibited when compared to the untreated control group. It could be inferred that the difference was a consequence of the diminished adhesion and movement of cells. The invasion, progression and metastasis of tumor cells was closely related to cell adhesion between the extracellular matrix and other cells[30]. Cell adhesion was dependent on cell adhesion molecules and kinase[31,32]. FAK, a non-receptor binding to tyrosine kinase, which could regulate focal adhesion formation and disassembly, is involved in tumour initiation and progression, including cell survival and apoptosis, adhesion, migration and invasion[33-36]. FAK is a key mediator of intracellular signaling by integrins, a major family of cell surface receptors in the extracellular matrix, in the regulation of different cellular functions in a variety of cells[37]. An earlier report illustrated that EFE showed significant antitumor activity in hepatoma cells and suggested this might be caused by EFE inducing apoptosis of hepatoma cells and inhibiting the expression of the

matrix metalloproteinase-2 (MMP-2)[17]. Our data showed that EFEA can down-regulate the expression of FAK in MCF-7 in a concentration-dependent manner. It is possible that there might be a change in MMP expression via the activation of FAK signalling. This warranted further investigation. A variant isoform of CD44, CD44v6 is a multi-structural and multifunctional transmembrane glycoprotein involved in cell-cell and cell-matrix adhesion interactions as well as cell migration[38]. Immunohistochemistry of CD44v6 has been performed on formalin-fixed paraffin wax-embedded breast cancer tissue samples by Chinese researchers. They demonstrated that CD44v6 played opposing roles in the development of breast cancer[39]. In addition, it has been reported that EFE could obviously inhibit the expression of CD44v6 in hepatocellular carcinoma mice[27]. In our present study, we found that EFEA decreased the expression of CD44v6 in a concentration-dependent manner compared with an untreated control group. The results showed that EFEA can resist the migration of MCF-7 cells. This might be due to the reductions in adhesion factors CD44v6 and FAK, which decrease in a dose-dependent manner at concentrations lower than IC_{50}.

In summary, EFEA was successfully isolated and purified, a distinct protein fraction from the crude extract, of which a 25 kDa fraction was found to possess a strong fibrinolytic function and aid in the suppression of the proliferation and migration of MCF-7 tumour cells. The present results suggested that EFEA could serve as a novel agent for the treatment of breast cancer. The anticancer effects *in vivo*, as well as the potential clinical effectiveness of EFEA is worthy of further investigation.

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Conflicts of interest:

There are no conflicts of interest.

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