WITHDRAWN:
The Molecular Mechanism of ESZM Extract on Treatment ER(-) Breast Cancer *in Vivo*: Application of "Combat Poison with Poison" Theory

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EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Background: Extract of *Euphorbia fischeriana* S. and *Ziziphus jujuba* M. (ESZM) is a formula preparation, was composed of *Euphorbia fischeriana* S. (*E. fischeriana*) and *Ziziphus jujuba* M. (Z. *jujuba*). The aim of the current research was to examine for the first time the pharmacological effects and the underlying molecule mechanism of ESZM extract on the growth inhibition and apoptosis in human ER(-) or ER(+) breast cancer *in vivo*, and enrich and innovate the theory of traditional Chinese medicine of combating poison with poison.

Methods: Growth inhibition, cell cycle arrest and apoptosis in tumor tissues were determined by tumor inhibition rates, cycle analysis kit, Annexin V-FITC/PI staining, Hoechst 33342/PI staining, TUNEL test and TEM observation. Related genes and proteins expression levels were identified by qRT-PCR assay, Western Blotting method, immunohistochemistry and immunofluorescence means. Drug toxicity was analysised by serum biochemistry detection and H&E staining.

Results: We found that ESZM extract can inhibite growth, block cell cycle at G_2/M phase and induce apoptosis of subcutaneously transplanted tumor, especially ER(-) breast cancer transplanted tumor. Furthermore, we demonstrated that the polyjuice can down-regulate or up-regulate the expression of Bcl-2 family and PI3k/Akt pathway -related signaling molecules proteins and genes *in vivo*. ESZM extract or *E. fischeriana* extract increased the levels of ALT, AST, Cr and BUN, and increased liver and kidney toxicity in tumor-bearing mice by serum biochemistry detection and H&E staining.

Conclusion: The pharmacological detection suggested that ESZM extract had significant anti-breast cancer effect, especially ER(-) breast cancer xenograft, and this process may be implement through the mitochondrion dependent pathway and the PI3k/Akt signaling pathway. Drug toxicity detection proved that ESZM extract because of compatibility with *Z. jujuba* was lower toxicity than *E. fischeriana* extract, was no significant difference in hepatorenal toxicity between ER(-) or ER(+) tumor-bearing mice.

Highlights

1. ESZM extract had significant anti-ER(-) breast cancer effect *invivo*.
2. ESZM extract can regulate PI3k/Akt pathway to inducing apoptosis *in vivo*.
3. ESZM extract have hepatorenal toxicity in tumor-bearing mice.
4. Compatibility with *jujuba* can reduce the toxicity of *E. fischeriana*.

Background

Global Cancer Statistics 2020: Breast adenocarcinoma has exceeded lung carcinoma as the most usually diagnosed carcinoma, with an predicted 2.3 million new cases (11.7%), followed by lung carcinoma (11.4%) [1]. With the technology progress of breast carcinoma treatment, the survival rate of breast carcinoma patients has been enhanced. However the mortality rate still ranks second among female

cancers, just behind the lung carcinoma [2–3]. So, it is the main task of the research that finding how to have a alternative treatment is a burning issue.

Chinese herb has become a rich source for finding new drugs, and more and more Chinese herb are found to be able to induce apoptosis [4]. Chinese herb may be one of the choices of anticancer drugs [5]. Traditional Chinese medicine (TCM) thought that the tumorigenesis is attributed to accumulation of pathogenic toxins in the body. On the treatment of malignant tumors, TCM uses a method of combating poison with poison in syndrome differentiation to treatment tumors. The literature reports that *E. fischeriana* is a traditional toxicity Chinese herbal of Family Euphorbiaceae plant, has many pharmacological activities for anti-tumor, insect repellent, inflammatory treatment and antiedematous [6]. It is reported that eating *Z. jujuba* steamed with *E. fischeriana* can cure tumors in the folk. It has been recorded in an ancient Chinese medical classic such as "*Prescriptions of the Golden Chamber"* and "*The Treatiseon Febrile Diseases*" that the toxicity of euphorbia family medicinal plants was reduced by compatibility with *Z. jujuba*. According to the theory of TCM, combination of various herbs at optimum ratios effectually improve the therapeutic action and decrease adverse reaction [7–8]. Referring to the prescription principle of Zhang Zhongjing's herbal formulae "*Shizao Tang*", when we use euphorbia herbs, we can use *Z. jujuba* to alleviate the adverse effects of drastically purgating water and gastrointestinal discomfort. So, ESZM extract was used as an experimental drug in our study.

In addition, endocrine therapy is the most important adjuvant treatment for hormone receptor-positive breast cancer patients and can significantly improve disease-free survival and reduce recurrence rates [9] with minimal side effects [10]. Then, the literature reports that 44% of all breast cancer patients with ER(-) breast cancer, which occurs mostly in young women, and has clinically more aggressive and worse prognosis [11–12]. Some ER(+) breast cancer patients can also turn negative due to drug resistance and other factors during the course of endocrine therapy [13]. Therefore, we think that ER(-) breast cancer is more "toxic", is an adaptive disease of "combat poison with poison" to *E. fischeriana*.

Promoting apoptosis of cancer cell has been the dominating goal of antineoplastic drug development in the areas of oncology for decades [14]. Researches have confirms that both cytotoxic medicines and targeted therapy medicines play a anti-tumor effects by inducing apoptosis of cancer cell. Lots of intracellular signaling pathways are involved in regulation of apoptosis. The PI3k/Akt pathway that is one of the vital intracellular signal transduction pathways is essential in regulation of cell growth, survival, differentiation, apoptosis and autophagy [15–16]. The literature reports that activation of PI3k/Akt signaling pathway in breast cancer is up to 70% [17].

Material And Methods

Reagents, experimental animals, cell and cell culture

Fetal bovine serum (FBS, cat. no. SV30087.02) and L15culture medium (cat. no. SH30525.01) were obtained from Hyclone (USA). Minimum essential medium (MEM, cat. no. 41500-034) were purchased

from Gibco company (USA). Cycle TESTTM PLUS DNA Resgent Kit and Annexin V-FITC/PI apoptosis kit (cat. no. 556547) were provided by BD Biosciences (USA). TUNEL kit (cat. no. c10618) and Antiquenching glue (cat. no. S36963) were obtained from Thermo Fisher Scientific (USA). RNAiso Plus (cat. no. 9108), SYBR Premix Ex Taq (cat. no. RR420A) and Prime Script RT reagent Kit (cat. no. RR037A) were purchased from TaKaRa Bio (Japan). Hoechst 33342/PI (cat. no. G023, Jiancheng Bioengineering Institute, China). The BCA kit (cat. no. CW0014) were obtained from ComWin Biotechnology (China). H&E kit (cat. no. C0105S) was provided by Beyotime Biotechnology (China).

BALB/c naked mice were acquired by Beijing Vital River Laboratory Animal Technology Co., Ltd., SCXK(Jing)2016-0011 and were kept in the experimental animal center of Qiqihaer Medical University (SYXK(hei)2016-001).

Human MCF-7 cells and MDA-MB-453 cells were purchase by the cell resource center of Shanghai institute of life sciences, Chinese academy of sciences. MCF-7 cells were fostered in MEM culture medium with 10% FBS, and maintained in an incubator for 37℃ and a moist environment of 5% CO_2. MDA-MB-453 cells were fostered in L15 culture medium with 10% FBS, and maintained in a moist incubator for 37℃. After about 1 to 2 days MCF-7 cells or MDA-MB-453 cells were digested and passed on when they filled the bottom of the cell culture dish. The cells at logarithmic growth stage were used for the test.

Experimental drug

The ingredients of ESZM extract formula include *E. fischeriana* 100 g, *Z. jujuba* 100 g, and distilled water 1000 mL. *E. fischeriana* was identified by professor Lina Guo of the natural pharmaceutical chemistry center of Qiqihar medical university, and was picked from Taikang county, Daqing city, Heilongjiang province, China. *Z. jujuba* were purchased from the wholesale market of medicinal materials in Qiqihar city. The formula were boiled for 120 min under sustaining stirring status. The procedures were repeated two times following that the polyjuices were collected centrifugation (Eppendorf, 5804R, Germany) at 8000×g. The supernatants were enriched and kept by low pressure for 70℃ to evaporation until 200 mL by rotary evaporator (Buchi, R-220, Switzerland). The same dosage of *E. fischeriana* extract and *Z. jujuba* extract were prepared by the above method.

HPLC analysis

HPLC analysis was executed via Waters 2489 system (Waters Technologies), which facility with a waters 2545 quaternary pump and a waters 2545 autosampler. ESZM extract samples were separated by Diamonil-C18 column (250×4.6 mm, 5 μm) at 35 ℃. The mobile phase system was comprised of (A) water with 0.1% formic acid and (B) methanol. 0–20 min, 98%A, 2%B; 20–32 min, 95%A, 5%B; 32–38 min, 90%A, 10%B; 38–39 min, 60%A, 40%B; 39–40 min, 100%B. The mobile phase flow rate was 1.0 mL per minute and the UV spectrum was 210 nm. 20 microlitre of volume was injected. Data acquisition was executed by Waters software system.

Establishment of xenograft nude mice

Five week-old BALB/c female naked mice were given 0.2 mL MCF-7 cells suspension (4×10^7 cell/mL) or 0.2 mL (containing 0.1 mL matrix adhesive) MDA-MB-453 cells suspension (6×10^7 cell/mL) to establish the nude mouse xenograft models of ER(-) or ER(+) breast cancer. The neoplastic mice were stochastically divided into treatment group that were given with intra-gastric administration of ESZM extract or *E. fischeriana* extract, and model group that were given with intra-gastric administration of saline or *Z. jujuba* extract once a day for 4 weeks or 8 weeks.

Inhibition of tumor growth

Tumor diameter was monitored weekly. The tumor volumes were computed by a formula, $V = (L \times S^2) /2$ (mm^3) (L: tumor long diameter, S: tumor short diameter). After 4 weeks of medication (model group, 2.5, 5.0, 10.0 g/kg groups of ESZM extract), nude mice were sacrificed using tumor inhibition rate detection. Tumor inhibition rate was computed by a formula: 1-mean tumor weight of drug treatment group / mean tumor weight of model group.

Hoechst 33342/PI staining analysis

Tumor tissues was dehydrated, deparaffinized and prepared into paraffin sections. Hoechst 33342 dye and PI dye were diluted with a buffer solution and severally dropped on the sections in 37℃ for 15 min, then the sections were rinsed with a buffer solution once. After slides with antiquenching glue were uesd to seal the sections. Last, the sections were observed and photographed with fluorescence microscope (Zeiss, Observer A1, Germany).

TUNEL assay analysis

Paraffin sections preparation were the same. TUNEL assay protocol as previously described [18]. The sections dropped antiquenching glue was encapsulated with slides, observed and photographed with an laser confocal microscopy (Zeiss, LSM710, Germany) to assess apoptosis index of TUNEL dyed tumour tissues.

TEM analysis

TEM protocol: A small piece of tumor tissue from each group were collected in containing 2.5% glutaraldehyde of EP tubes at 4°C for passthenight. Then, the ultrathin slices was prepared and fixed with 2% osmium tetroxide, soaked with Epon812, embedded, dyed with uranyl acetate and lead citrate, observed and photographed with a TEM (Hitachi Limited, HT7700, Japan) .

Detection of cell cycle and apoptosis in tumor tissues

Cell cycle and Apoptosis of tumor tissue was detected through flow cytometry (BD, FACSCalibur, USA) combined with PI or Annexin V-FITC/PI staining. Tumor tissue were made into cell suspensions. The suspensions were washed for 3 times with cooled PBS and collected in 12×75-mm capped polypropylene tubes. According to the manufacturer's instruction, add 250 μL of Solution A (trypsin buffer) to each tube and gently mix by tapping the tube, to react for 10 min at room temperature, and 200 μL of Solution B (trypsin inhibitor and RNase buffer) to react for 10 min at room temperature, and then add 200 μL of cold

Solution C (PI stain solution) to react for 10 min in the dark on ice. The cell cycle distribution were measured by using a flow cytometer.

The cell suspensions washed with cooled PBS for 3 times were resuspended with 300 μl buffer solution. Ten microlitre Annexin V-FITC and five microlitre PI were added to the suspensions. The samples was gentle mixed and fostered for 10 min at lightless room temperature. The apoptosis rate was evaluated by using a flow cytometer.

Immunohistochemical analysis

Paraffin sections preparation were the same. Immunohistochemical analysis on tumor tissue from mice treated with or without ESZM extract. The antigen was retrieved by a sodium citrate buffer. This was followed by endogenous peroxidase blocking and incubation in normal goat serum for 25 mins according to UltraSensitiveTM SP(Mouse/Rabbit) manufacturer's protocol (cat. no. KIT-9710, MaiXin Bio, China). These sections were incubated with Bcl-2 (1:400, Cell Signaling, USA), Bax (1:400,Cell Signaling, USA), caspase 3 (1:1000, Proteintech, USA), caspase 9 (1:400, Cell Signaling, USA) overnight at 4°C and then with the peroxidase-conjugated secondary antibody. Color development was performed with DAB, and slides were counterstained with hematoxylin. Neutral resin sealing was performed. The results of immunohistochemical staining were observed under a microscope (OLYMPUS, IX51, Japan).

Immunofluorescence analysis

The p-PI3k (1:250, Abcam, USA), p-Akt (1:50, Cell Signaling, USA) and p-FoxO3a (1:500, Abcam, USA) proteins expression levels were monitored with immunofluorescence method as previously described (Ma *et al*., 2021).

Western blot analysis

The tumor tissues were fetched from liquid nitrogen, and ground in lysis buffer with a pre-cooling EP tube until invisible precipitate. The suspension with buffer were enriched into 4 mL EP tubes for 30 min in ice bath, and then centrifuged at 12000 rcf for 15 min at 4℃ to acquire protein. The BCA kit was used to evaluate protein concentrations. The 20 μg of protein were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with BSA buffer solution at room temperature for 2 h, and fostered with antibodies of p-Her2 (1:500, Abcam, USA), p-PI3k (1:250, Abcam, USA), p-Akt (1:2000, Cell Signaling, USA), p-FoxO3a (1:500, Abcam, USA), p-FoxO1a (1:500, Abcam, USA) and GAPDH (1:1000, Cell Signaling, USA) passthenight at 4°C, and washed three times with trisbuffered buffer solution, and incubated with a horseradish peroxidase conjugated secondary antibody for 2.0 h. At last, the imaging system (Bio-Rad, ChemiDoc MP, USA) was used to detect and analyse the intensity of protein bands.

Quantitative real-time polymerase chain reaction (qRT-PCR) test

The tumor tissues were fetched from liquid nitrogen, and ground into RNAiso Plus with a pre-cooling EP tube until invisible precipitate to obtain total RNA. Ultraviolet spectrophotometry (Shimadzu, Biospecnano, Japan) was used to detect purity and concentration of RNA at A260 nm/A280 nm. All RNA were reverse-transcribed into cDNA by Prime Script RT reagent Kit, and the levels of Her2, Bcl-2, Bax, caspse 3, caspase 9, FoxO3a, FoxO1a and Bim gene expression were evaluated via qRT-PCR Detection System (ABI, Q5, USA) with Premix Ex Taq Kit. The sequences of primers for Bcl-2 (Forward: 5'-GTTGTCGACGACGAGCG-3'; Reverse: 5'-CTGAG TACCTGAACCGGCA-3', 106 bp), Bax (Forward: 5'-AGCTTCTTGGTGGACGCAT – 3'; Reverse: 5'-CAGAGGCGGGGTTTCATC-3', 101 bp), Bim (Forward: 5'-GATAGTGGTTGAAGGCCTGG – 3'; Reverse: 5'-CCTCCCTACAGACAGAGCCA-3', 102 bp), caspase 3 (Forward: 5'-TCGCTT CCATGTATGATCTTTG-3'; Reverse: 5'-CTGCCTCTTCCCCCATTCT-3', 110 bp), caspase 9 (Forward: 5'-CATGCTCAGGATGTAAGCCA-3'; Reverse: 5'-AGGTTCTCAGACCGGAAACA – 3', 116 bp), FoxO3a (Forward: 5'-AGTTCCCTCATTCTGGACCC-3'; Reverse: 5'-CTTCAAGG ATAAGGGCGACA-3', 102 bp), FoxO1a (Forward: 5'-GCACACGAATGAACTTGCTG-3'; Reverse: 5'-AAGAGCGTGCCCTACTTCAA-3', 106 bp), Her2 (Forward: 5'-AGCATGTCCAG GTGGGTCT-3'; Reverse: 5'-CTCCTCCTCGCCCTCTTG-3', 109 bp) and β-actin (Forward: 5'-G TTGTCGACGACGAGCG-3'; Reverse: 5'-GCACAGAGCCTCGCCTT-3', 93 bp). The cycling conditions for all genes were as follows: 95℃ for 30 s followed by 40 cycles of denaturation at 95℃for 5 s, annealing and extension at 65℃ for 34 s. The each candidate gene expression were normalized to β-actin. The fold change in gene expression was calculated according to the $2^{-\triangle\triangle Ct}$ method.

Drug toxicity analysis

After 8 weeks of medication (model group, 2.5, 5.0, 10.0g/kg groups of ESZM extract, 5.0 g/kg groups of *E. fischeriana* extract and *Z. jujuba* extract), the toxicity of the drug was examined in ER(-) or ER(+) breast tumor-bearing mice. The body weight, serum biochemical indexes, liver and kidney pathological indexes of the tumor-bearing mice were detected by an automatic biochemical analyzer or H&E staining analysis. H&E (cat. no. C0105, Beyotime Biotechnology, China) staining process follows. Paraffin sections of liver or kidney tissues preparation were the same. Hematoxylin staining, 1% hydrochloric acid alcohol differentiation, Eosin staining, gradient alcohol for dehydration, and seal slide. The sections pathological changes were assessed under a phase-contrast microscopy (OLYMPUS, IX51, Japan).

Statistical analysis

All data were presented as means ± standard deviation. SPSS19.0 statistical software was applied to evaluate statistical significance. Distinction between two groups was analyzed via two-tailed Student's T-Test, and distinction among three or more groups was analyzed with one-way ANOVA multiple comparisons. The distinction was conducted statistically significant when $P < 0.05$, $P < 0.01$ or $P < 0.001$.

Results

The quality control of ESZM extract

The quality control analysis of ESZM extract was performed by using a High Performance Liquid Chromatography (HPLC), which is stability and consummate repeatability (Fig. 1). Stored at -20 ℃ until use. To analyze stability of ESZM extract by HPLC, we diluted each batch of ESZM extract equally. Therefore, the fingerprint was obtained only suitable for quantitative observation and can't represent contents of each substance. Previous research confirmed that ESZM extract components contain Jokinolide B and 2,4-dihydroxy-6-methoxy-acetophenone through the UPLC-DAD method. Jokinolide B has been shown to have definite anti-tumor activity [19–22]. It has extracted several monomeric components from *E. fischeriana*, such as Jolkinolide A [23], 17-acetoxyjolkinolide B [24], 17-hydroxy-jolkinolide B [25], 12-Deoxyphorbol-13- palmitate [18, 26–27], and Jokinolide B, and proved to have significant anti-tumor activity from different directions. Therefore, in our study, herbal medicinal compound prescribe were selected as research object reflects the scientific connotation of Chinese herbal with multiple components, multiple effects and multiple targets.

Growth inhibition of transplanted tumor in nude mice

To demonstrate that ESZM extract has anti-tumor effect, especially ER(-) breast cancer *in vivo*, we established ER(-) or ER(+) breast cancer xenografts in nude mice and detected inhibition rate of tumor. The changes of tumor volume was recorded according to measured tumor diameter (Table. 1, Fig. 2). As showed in Table 2, from administration of ESZM extract 2.5 g/kg to 10.0 g/kg, the tumor inhibition rates were 18.5%, 35.7%, 41.3% in ER(-) breast cancer xenografts, and were 9.6%, 21.1%, 33.7% in ER(+) breast cancer xenografts.

Table 1
Changes of tumor volume. Tumor volume of nude mice that were treated with ESZM extract for 4 weeks were measured (mean ± SD, n = 10).

Groups	Sum	Tumor volume(mm^3)				
		0 d	7 d	14 d	21 d	28 d
ER(-) breast cancer bearing mice						
0 g/kg(model)	10	203.1 ± 14.7	384.5 ± 25.4	759.4 ± 36.5	990.9 ± 51.5	1299.9 ± 80.4
2.5 g/kg	10	214.5 ± 12.4	370.3 ± 24.1	713.4 ± 37.3	940.5 ± 61.4	1000.3 ± 76.6
5.0 g/kg	10	209.5 ± 14.4	333.6 ± 18.8	700.5 ± 44.5	839.1 ± 57.5*	935.9 ± 77.5*
10.0 g/kg	10	200.9 ± 10.1	321.1 ± 20.7	659.8 ± 36.9*	801.0 ± 52.8**	900.5 ± 78.4**
ER(+) breast cancer bearing mice						
0 g/kg(model)	10	316.9 ± 12.8	905.4 ± 35.6	2089.7 ± 296.2	4175.5 ± 558.6	4980.1 ± 724.4
2.5 g/kg	10	321.7 ± 13.5	900.1 ± 37.0	1872.0 ± 237.7	3854.0 ± 681.0	4535.0 ± 891.4
5.0 g/kg	10	310.8 ± 11.7	878.0 ± 50.8	1638.6 ± 259.5*	3407.4 ± 587.2*	4071.9 ± 927.2
10.0 g/kg	10	300.8 ± 13.4	856.0 ± 35.4	1480.0 ± 286.4**	2940.0 ± 393.5**	3446.5 ± 868.8**

*P< 0.05 or **P< 0.01 *vs*. model group.

Table 2
Tumor inhibition rate was measured in tumor-bearing mice treated with ESZM extract or paclitaxel (mean ± SD, n = 5).

Groups	Tumor weight(g)	Anti-tumor rate%
ER(-) breast cancer bearing mice		
0 g/kg(model)	4.64 ± 0.67	–
2.5 g/kg	3.78 ± 0.39	18.5
5.0 g/kg	2.98 ± 0.30*	35.7
10.0 g/kg	2.72 ± 0.25**	41.3
ER(+) breast cancer bearing mice		
0 g/kg(model)	8.97 ± 0.74	–
2.5 g/kg	8.11 ± 0.81	9.6
5.0 g/kg	7.08 ± 0.73*	21.1
10.0 g/kg	5.95 ± 0.85**	33.7
*$P < 0.05$ or **$P < 0.01$ *vs*. model group.		

Cell morphological changes in xenograft tumor tissues

Through morphological observation to illustrate the pharmacological effect of ESZM extract on growth inhibitor and induction apoptosis in tumor tissues. As shown in Fig. 3A-B, the tumor tissues dyed with Hoechst 33342/PI were observed with fluorescence microscope. Model group had dense cells, weak the blue and red fluorescence, while the tumor tissues treated with ESZM extract had decreased cell density and gradually prominent red fluorescence.

TUNEL fluorescence dyeing was applied to demonstrate effect of ESZM extract on induction apoptosis in tumor tissues of naked mice. In Fig. 3C-D, the nuclear morphology of tumor tissues were substantial consistent, and fluorescence intensity was weak for model group. For ESZM extract treatment group, TUNEL staining positive cells were dramatically increased, and cells morphology displayed shrinkage, cell density decreased, a little number of fragments.

The ultrastructure of cells was observed by using a TEM. In model group, the cell structure in tumor tissue was clear, the nuclear membrane was thin and complete, the nucleoli was obvious, chromatin was uniform, organelles were abundant, mitochondrial structure was complete, free ribosomes were abundant, these indicating the tumor cell were brisk growth, no apoptotic cells were seen. With the increase of ESZM extract dose, the microvilli of cells decreased or disappeared, and a large amount of lipid accumulated in the cytoplasm. Morphological characteristics of apoptotic were obvious, such as nuclear pyknosis,

nuclear membrane depression or disappearance, heterochromatin agglutination, mitochondrial swelling or disappearance and vacuolation, and endoplasmic reticulum dilation. (Fig. 3E-F).

Apoptosis rate of transplanted tumor

Flow cytometry was used to explore apoptosis and cell cycle distribution of tumor tissue treated with ESZM extract. As showed in Fig. 4(A-C), tumor tissues treated ESZM extract from 2.5 g/kg and 10.0 g/kg group were contained considerable number of apoptosis in ER(-) or ER(+) breast cancer bearing mice. As shown in Fig. 4(D-E), compared with the model group(3.16% or 6.50%), the treatment group showed a G2/M phase fraction of 9.17%, 19.97%, 25.78% or 12.84%, 25.64%, 28.45% in ER(-) or ER(+) breast cancer bearing mice with increased in a dose-dependent manner of ESZM extract, respectively. In addition, as those results, there were further apoptosis and G2/M phase arresting in tumor tissues of ER(-) breast cancer bearing mice.

Expression of apoptosis-related proteins in tumor tissue treated with ESZM extract

Immunohistochemical method was used to analyze the expression of apoptosis-related proteins in breast cancer bearing mice transplanted tumor tissues treated with ESZM extract. As shown in Fig. 5, the levels of Bcl−2 protein expression in ER(-) or ER(+) nude mice transplanted tumor tissue was down-regulated compared with the model group. The levels of caspase 3, caspase 9 and Bax protein expressions in transplanted tumor tissues of ER(-) or ER(+) nude mice were up-regulated compared with the model group.

Expression of PI3k/Akt signal pathway-related proteins in tumor tissue treated with ESZM extract

Immunofluorescence was used to detect whether PI3k/Akt signaling pathway was involved in regulating the growth and inducing the apoptosis of ER(-) or ER(+) breast cancer bearing mice. As shown in Fig. 6, fluorescence intensity (FI) of p-PI3k, p-Akt and p-FoxO3a proteins in transplanted tumor tissue of ER(-) or ER(+) breast cancer bearing mice treated with ESZM extract gradually decreased with the increase of dose, that is, the protein expression decreased gradually, compared with the model group, the difference was statistically significant .

Western blot to further detect PI3k/Akt signaling pathway-related proteins expression result showed that related proteins expression levels have a different degrees change in tumor tissue of nude mice treated with ESZM extract (Fig. 7). Compared with the model group, Levels of p-Her2, p-PI3k, p-Akt, p-FoxO3a and p-FoxO1a protein expression was down-regulated.

Expression of related genes in tumor tissue treated with ESZM extract

The qRT-PCT method to verify mitochondrial pathway and PI3k/Akt pathway related genes expression in tumor tissues treated with ESZM extract. Levels of caspase 3 (Fig. 8B), caspase 9 (Fig. 8C), Bax (Fig. 8E) and Bim (Fig. 8F) genes expression in each group of transplanted tumor tissues treated with ESZM extract was up-regulated. Levels of Her2 (Fig. 8A), Bcl-2 (Fig. 8D), FoxO3a (Fig. 8G) and FoxO1a (Fig. 8H) gene expression was down-regulated.

Toxic effect of E. fischeriana compatibility Z. jujuba on tumor-bearing mice

To evaluate the toxicity of ESZM extract, *E. fischeriana* extract and Z. jujuba extract on the body weight, serum biochemical indexes and histopathology of ER(-) or ER(+) breast cancer bearing mice, respectively.

As showed in Fig. 9(A-B), The body weight of ER(-) or ER(+) breast cancer bearing mice in the early stage (4 weeks before) of intragastric administration increased steadily or remained unchanged, and the body weight of mice in the late stage (4 weeks to 8 weeks) of intragastric administration decreased, especially in the *E. fischeriana* extract group. With the prolongation of administration time, mice in each group died, and the number of death in the *E. fischeriana* extract group was larger than 2.5, 5.0 g/kg of ESZM extract group.

As showed in Table 3, Serum biochemical results showed that after 8 weeks of continuous administration, ALT AST, Cr and BUN were changed to different degrees, and the *E. fischeriana* extract group was equivalent to the ESMC (10.0 g/kg) group, while there was no difference between the *Z. jujuba* extract group and the model group, between ER(-) and ER(+) breast cancer bearing mice.

As showed in Fig. 10(A-B), histopathology changes were surveyed by using a microscopy with H&E staining. There was no significant difference in histomathology of the liver and kidney between ER(-) and ER(+) breast cancer bearing mice. The hepatic sinuses were numerous and clear, the liver lobule were obvious boundary in liver tissues of the model groups or *Z. jujuba* group. The renal tubules were orderly, and the glomeruli were clear in kidney tissues of the model groups or *Z. jujuba* group. In 2.5, 5.0 g/kg groups of ESMC extract, the structure of the hepatic lobules were lost and the boundaries were blurred. There were more granular degeneration, cytoplasm loose, hepatic sinuses narrow and necrotic foci in the *E. fischeriana* extract and 10.0 g/kg groups of ESMC extract. The epithelial cells of renal tubules showed edema of different degrees, and volume of glomerular cells increased in kidneys of breast cancer bearing mice.

Table 3
Serum biochemical of ER(-) or ER(+) breast cancer bearing mice.

Groups		Animal amount	Serum biochemical			
			ALT IU/mL	AST IU/mL	Cr μmol/L	BUN mmol/L
ER(-) breast cancer bearing mice	ESZM 0 g/kg	8	31.7 ± 4.5	90.3 ± 7.9	32.5 ± 6.3	6.1 ± 0.59
	ESZM 2.5 g/kg	7	37.6 ± 9.3	101.2 ± 11.4	48.3 ± 5.9*	7.3 ± 0.99
	ESZM 5.0 g/kg	5	68.9 ± 5.8**	124.2 ± 5.7*	50.2 ± 10.4*	9.5 ± 0.73*
	ESZM 10.0 g/kg	4	83.4 ± 8.5**	158.1 ± 11.9**	58.2 ± 8.1***	10.2 ± 0.78**
	E. fischeriana extract	5	75.4 ± 10.1**	149.8 ± 12.1**	53.1 ± 7.2**	10.8 ± 1.09**
	Z. jujuba extract	9	34.8 ± 6.4	93.4 ± 10.6	33.5 ± 3.2	6.5 ± 0.65
ER(+) breast cancer bearing mice	ESZM 0 g/kg	9	29.3 ± 7.1	95.9 ± 8.6	39.1 ± 6.7	6.3 ± 0.95
	ESZM 2.5 g/kg	6	41.2 ± 6.1*	121.2 ± 14.7	45.3 ± 8.9	6.9 ± 0.82*
	ESZM 5.0 g/kg	4	67.4 ± 8.0**	133.2 ± 7.8**	46.8 ± 12.2*	7.4 ± 0.96*
	ESZM 10.0 g/kg	3	81.1 ± 8.4**	151.3 ± 12.9**	56.3 ± 7.2**	9.5 ± 1.45**
	E. fischeriana extract	3	80.3 ± 9.3**	153.1 ± 11.3**	55.3 ± 9.3**	8.8 ± 0.99**
	Z. jujuba extract	7	27.1 ± 8.8	101.7 ± 12.0	37.5 ± 9.1	6.1 ± 0.78

*$P < 0.05$, **$P < 0.01$ or ***$P < 0.001$ vs. model group.

Discussion

We previously found that ESZM extract can inhibit proliferation and induce apoptosis in breast cancer *in vitro*, especially in ER(-) breast cancer that has more aggressive and worse prognosis. The dialectical treatment of TCM for tumor thought that ER(-) was a poison of breast cancer, and that ESZM extract maybe have a better therapeutic effect on ER(-) breast cancer via the “combat poison with poison” theory.

To confirm the viewpoint, ER(-) tumor-bearing mice of breast cancer was established with MDA-MB-453 cell and treated with ESZM extract, and the same to established ER(+) tumor-bearing mice of breast cancer as control. The results showed that ESZM extract could inhibit growth of both ER(-) and ER (+) breast cancer xenograft nude mice, and has better therapeutic effect on ER(-) breast cancer bearing mice. However, inhibitory effect can be achieved by inducing apoptosis. Studies have shown that there are two pathways deemed to play important roles in regulating cells apoptotic in mammalian, which are the extrinsic and intrinsic pathways regulated with death receptors and mitochondria, severally [28–29]. The intrinsic pathway is mediated by Bcl-2 family proteins, including pro-apoptotic proteins (such as Bax, Bim and Bad) and anti-apoptotic proteins (such as Bcl-2 and Bcl-XL) [18]. It is generally adopted that Bcl-2 protein-mediated apoptosis blockade attribute to malignant transformation in many types of human neoplasms [30–31]. We therefore detected whether mitochondrial dependent apoptotic pathway were included in nude mice treated with ESZM extract. The results revealed that ESZM extract could down-regulate the gene and protein expression of Bcl-2, up-regulate the genes and proteins expression of Bax and Bim. Hence, we believe that ESZM extract might induce mitochondrial-mediated apoptosis via regulating Bcl-2 family members.

Besides, Her2 is one of the most studied biomarkers related to drug response in breast cancer. The expression level of Her2 in normal cells is extremely low, and Her2 gene amplification or overexpression is present in 25–30% of breast cancer [32]. Overexpression of Her2 may directly lead to malignant transformation and metastasis of cells. Inhibition of Her2 activity can inhibit growth and induce apoptosis of tumor cells [33]. Our results also confirmed that ESZM extract can reduce the expression of Her2 factor in breast cancer bearing mice, especially in ER(-) breast cancer.

Therewith, we further explored the mechanism of inducing apoptosis. Literature report that changes the PI3k/Akt pathway have been found in multiple neoplasms. And it may promote cell survival and proliferation [34–35]. FoxO3a and FoxO1a are important members of the Fox family, and are crucial factors downstream of the PI3k/Akt pathway [36]. In our study, those data also proved that ESZM extract might decrease expression of p-PI3k, p-Akt and p-FoxO3a proteins.

The 8 weeks of continuous administration of medication found that there were no differences in body weight and number of deaths between ER(-) and ER(+) breast cancer bearing mice. The degree of liver and kidney tissue damage in the 5.0 g/kg of *E. fischeriana* extract group was significantly higher than 5.0 g/kg groups of ESMC extract, was similar in 10.0 g/kg groups of ESZM extract group. These phenomena suggest that the compatibility of *Z. jujuba* can weaken the toxicity of *E. fischeriana*. It also enriches the scientific connotation of the anti-tumor principle of "combating poison with poison" in TCM, and provides a research ideas for anti-breast tumor method of "combating poison with poison".

Conclusion

Taken together, the results of our current study provide strong experimental evidence that ESZM extract can regulate the mitochondrial-dependent apoptotic pathway to achieve anti-breast cancer effects,

especially ER(-) breast cancer, and the PI3k/Akt pathway engaged in this regulation of apoptosis. ESZM extract was no significant difference in hepatorenal toxicity between ER(-) or ER(+) tumor-bearing mice. Compatibility of Z. jujuba has a certain detoxification effect on *E. fischeriana*. This study suggests that ESZM extract can be a potential novel therapeutic preparation for treatment of breast carcinoma.

Abbreviations

ESZM, Euphorbia fischeriana S. and Ziziphus jujuba M.; ER, estrogen receptor; TUNEL, Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling; TEM, Transmission Electron Microscopy; qRT-PCR, quantitative real time -polymerase chain reaction; H&E, Hematoxylin and eosin; ALT, alanine transaminase; AST, aspartate aminotransferase; Cr, creatinine; BUN, Blood Urea Nitrogen.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Liwei Ma wrote the paper draft. Zhe Chen corrected the draft. Wenbao Wang, Jinling Zhang and Hongtao Zhang performed the experiments. Jicheng Liu conceived the study. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Availability of data and materials

The datasets used or analyzed during the study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal care and experimental procedures used in the current study were permited by the experimental animal ethics committee of Qiqihaer Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

References

1. Sung H, Ferlay J, Siegel RL. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021; 0: 1-41. doi: 10.3322/caac.21660.
2. Jiang ST, Wang HQ, Yang TC. Expression of stanniocalcin 2 in breast cancer and its clinical significance. Curr Med Sci. 2019; 39(6): 978-983. doi: 10.1007/s11596-019-2131-2.
3. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2019. CA Cancer J Clin. 2019; 69(1): 7-34. doi: 10.3322/caac.21551.
4. Zhang N, Kong XL, Yan S, Yuan CZ, Yang QF. Huaier aqueous extract inhibits proliferation of breast cancer cells by inducing apoptosis. Cancer Sci. 2010; 101(11): 2375-2383. doi: 10.1111/j.1349-7006.2010.01680.x
5. Xu WF, Wang ZJ, Li K. Huai Qi Huang-induced apoptosis via down-regulating PRKCH and inhibiting RAF/MEK/ERK pathway in Ph+ leukemia cells. Curr Med Sci. 2020; 40(2):354-362. doi: 10.1007/s11596-020-2181-5.
6. Jian BY, Zhang H, Liu JC. Structural Diversity and Biological Activities of Diterpenoids Derived from Euphorbia fischeriana Steud. Molecules. 2018; 23(4): 935. doi: 10.3390/molecules23040935.
7. Song JL, Zhang FB, Tang SH, Liu X, Gao YB, Lu P, Wang YP, Yang HJ. A module analysis approach to investigate molecular mechanism of TCM formula: A trial on Shu-feng-jie-du Formula. Evid Based Complement Alternat Med. 2001; 2013: 731370. doi: 10.1155/2013/731370.
8. Xu ZH, Zhang F, Zhu YZ, Liu F, Chen X, Wei LY, Zhang N, Zhou Q, Zhong HR, Yao C, Zhu XW, Gong CY, Zhu SG, Zou CP. Traditional Chinese medicine Ze-Qi-Tang formula inhibit growth of non-small-cell lung cancer cells through the p53 pathway. J Ethnopharmacol. 2019; 234: 180-188. doi: 10.1016/j.jep.2019.01.007.
9. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, Peto R, Pritchard KI, Bergh J, Dowsett M, Hayes DF. 20-Year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. N Engl J Med 2017; 377:1836-46. doi: 10.1056/NEJMoa1701830.
10. Zhi XC, Yang XN, Pan T, Liu JJ, Chen X, Lou LP, Shi ZD, Zhang J. Correlation of radiotherapy with prognosis of elderly patients with hormone receptor-positive breast cancer according to immunohistochemical subtyping. Chin J Cancer Res. 2019; 31(3):471-480. doi: 10.21147/j.issn.1000-9604.2019.03.09.
11. Girgert R, Emons G, Grundker C. Estrogen signaling in ERα-negative breast cancer: ERβ and GPER [J]. Front Neuroendocrin. 2019; 9: 781. doi: 10.3389/fendo.2018.00781.
12. Mahdi AF, Malacrida B, Nolan J, McCumiskey ME, Merrigan AB, Lal A, Tormey S, Lowery AJ, McGourty K, Kiely PA. Expression of Annexin A2 promotes cancer progression in estrogen receptor

negative breast cancers [J]. Cells, 2020; 9:1582. doi: 10.3390/cells9071582.

13. Wang ZW, Katsaros D, Biglia N, Shen Y, Loo L, Yu X, Lin HY, Fu YY, Chu WM, Fei PW, Ni Y, Jia W, Deng XB, Qian BY, Yu H. ERα upregulates the expression of long non-coding RNA LINC00472 which suppresses the phosphorylation of NF-κB in breast cancer [J]. Breast Cancer Res Tr. 2019; 175(2): 353-368. doi: 10.1007/s10549-018-05108-5.
14. Shan XY, Yu WJ, Ni XJ, Xu TT, Lei C, Liu ZL, Hu XF, Zhang YD, Cai BC, Wang B. Effect of Chitosan Magnetic Nanoparticles Loaded with Ang2-siRNA Plasmids on the Growth of Melanoma Xenografts in Nude Mice. Cancer Manag Res. 2020; 12,7475-7485. doi: 10.2147/CMAR.S250479
15. Duan YF, Li JJ, Jing X, Ding XL, Yu YN, Zhao QX. Fucoidan Induces Apoptosis and Inhibits Proliferation of Hepatocellular Carcinoma via the p38 MAPK/ERK and PI3K/Akt Signal Pathways. Cancer Manag Res. 2020;12, 1713-1723. doi: 10.2147/CMAR.S243495
16. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003; 2(2003) , pp. 339-345. doi: 10.4161/cc.2.4.433
17. Zock JM. Applications of high content screening in life science research. Comb Chem High Throughput Screen. 2009; 12(9): 870-876. doi: 10.2174/138620709789383277.
18. Ma LW, Chen Z, Li J, Zhang HT, Jia YM, Liu JC. DP from Euphorbia fischeriana S. mediated apoptosis in leukemia cells via the PI3k/Akt signaling pathways. J Ethnopharmacol. 2021; 113889. doi: 10.1016/j.jep.2021.113889.
19. Gao C, Yan X, Wang B, Yu LN, Han JH, Li DF, Zheng QS. Jolkinolide B induces apoptosis and inhibits tumor growth in mouse melanoma B16F10 cells by altering glycolysis. Sci Rep. 2016; 6: 36114. doi: 10.1038/srep36114.
20. Sun C, Cui HX, Yang HY, Du XH, Yue LL, Liu JC, LinY. Anti-metastatic effect of jolkinolide B and the mechanism of activity in breast cancer MDA-MB-231 cells. Oncol Lett. 2015; 10(2): 1117-1122. doi: 10.3892/ol.2015.3310.
21. Yan YJ, Wang YF, Wang X, Liu DW, Wu XY, Xu C, Chen C, Li ZF. The effects of jolkinolide B on HepG2 cells as revealed by ^{1}H-NMR-based metabolic profiling. Eur J Pharmacol. 2015; 5(842): 10-19. doi: 10.1016/j.ejphar.2018.10.025.
22. Xu HY, Chen ZW, Hou JC, Du FX, Liu JC. Jolkinolide B induces apoptosis in MCF-7 cells through inhibition of the PI3K/Akt/mTOR signaling pathway. Oncol Rep. 2013; 29(1): 212-218. doi: 10.3892/or.2012.2113.
23. Shen L, Zhang SQ, Liu L, Sun Y, Wu YX, Xie LP, Liu JC. Jolkinolide A and Jolkinolide B Inhibit Proliferation of A549 Cells and Activity of Human Umbilical Vein Endothelial Cells. Med Sci Monit. 2017; 14(23): 223-237. doi: 10.12659/msm.902704.
24. Yan SS, Li Y, Wang Y, Shen SS, Gu Y, Wang HB, Qin GW, Yu, Q. 17- Acetoxyjolkinolide B irreversibly inhibits IkappaB kinase and induces apoptosis of tumor cells. Mol Cancer Ther. 2017; 7(6): 1523-1532. doi: 10.1158/1535-7163.
25. Wang Y, Ma XQ, Yan SH, Shen SS, Zhu HL, Gu Y, Wang HB, Qin GW, Yu Q. 17-hydroxy-jolkinolide B inhibits signal transducers and activators of transcription 3 signaling by covalently cross-linking

Janus kinases and induces apoptosis of human cancer cells. Cancer Res. 2009; 69(18):7302-7310. doi: 10.1158/0008-5472.

26. Yang Y, Cong H, Han CC, Yue LL, Dong HY, Liu JC. 12-Deoxyphorbol 13- palmitate inhibits the expression of VEGF and HIF-1α in MCF-7 cells by blocking the PI3K/Akt/mTOR signaling pathway. Oncol Rep. 2015; 34(4): 1755-1760. doi: 10.3892/or.2015.4166.
27. Xu HY, Pan, YM, Chen ZW, Lin Y, Wang LH, Chen YH, Jie TT, Lu YY, Liu JC. 12-Deoxyphorbol-13-palmitate inhibit VEGF-induced angiogenesis via suppression of VEGFR-2-signaling pathway. J Ethnopharmacol,2013, 146(3): 724-733. doi: 10.1016/j.jep.2013.01.007.
28. Hengartner MO. The biochemistry of apoptosis. Nature, 2000; 407: 770-776. doi: 10.1038/35037710.
29. Xu ZH, Zhang F, Bai C, Yao C, Zhong HR, Zou CP, Chen X. Sophoridine induces apoptosis and S phase arrest via ROS-dependent JNK and ERK activation in human pancreatic cancer cells. J Exp Clin Cancer Res. 2017; 36: 124. doi: 10.1186/s13046-017-0590-5.
30. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M, McHenry KT, Pinchback RM, Ligon AH, Cho YJ, Haery L, Greulich H, Reich M, Winckler W, Lawrence MS, Weir BA, Tanaka KE, Chiang DY, Bass AJ, Loo A, Hoffman C, Prensner J, Liefeld T, Gao Q, Yecies D, Signoretti S, Maher E, Kaye FJ, Sasaki H, Tepper JE, Fletcher JA, Tabernero J, Baselga J, Tsao MS, Demichelis F, Rubin MA, Janne PA, Daly PA, Nucera C, Levine RL, Ebert BL, Gabriel S, Rustgi AK, Antonescu CR, Ladanyi M, Letai A, Garraway LA, Loda M, Beer DG, True LD, Okamoto A, Pomeroy SL, Singer S, Golub S, Lander ES, Getz G, Sellers WR, Meyerson WR. The landscape of somatic copy-number alteration across human cancers. Nature. 2010; 463: 899-905. doi: 10.1038/nature08822.
31. Chetry M, Song YZ, Pan CY, Li R, Zhang JN, Zhu XQ. Effects of Galectin-1 on Biological Behavior in Cervical Cancer. Journal of Cancer. 2020; 11(6): 1584-1595. doi: 10.7150/jca.38538
32. Leung HWC, Leung JH, Chan ALF. Efficacy and safety of a combination of HER2-targeted agents as first-line treatment for metastatic HER2-positive breast cancer: a network meta analysis. Expert Opin Drug Saf. 2018, 17(1):1-7. doi: 10.1080/14740338.2018.1394454.
33. Rezaei Z, Sebzari A, Kordi-Tamandani DM, Dastjerdi K. Involvement of the Dysregulation of miR-23b-3p, miR-195-5p, miR-656-5p, and miR-340-5p in Trastuzumab Resistance of HER2-Positive Breast Cancer Cells and System Biology Approach to Predict Their Targets Involved in Resistance. DNA Cell Biol. 2019, 38(2):184-192. doi: 10.1089/dna.2018.4427.
34. Peltier J, Oneill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. Dev Neurobiol. 2007; 67(2007), pp. 1348-1361. doi: 10.1002/dneu.20506.
35. LaSarge CL, Pun RY, Muntifering MB, Muntifering SC. Disrupted hippocampal network physiology following PTEN deletion from newborn dentate granule cells. Neurobiol Dis. 2016; 96(2016) , pp. 105-114. doi: 10.1016/j.nbd.2016.09.004.
36. Fu Z, Tindall DJ. FOXOs, cancer and regulation of apoptosis. Oncogene. 2008, 27(16): 2312-2319. doi: 10.1038/onc.2008.24.

Figures

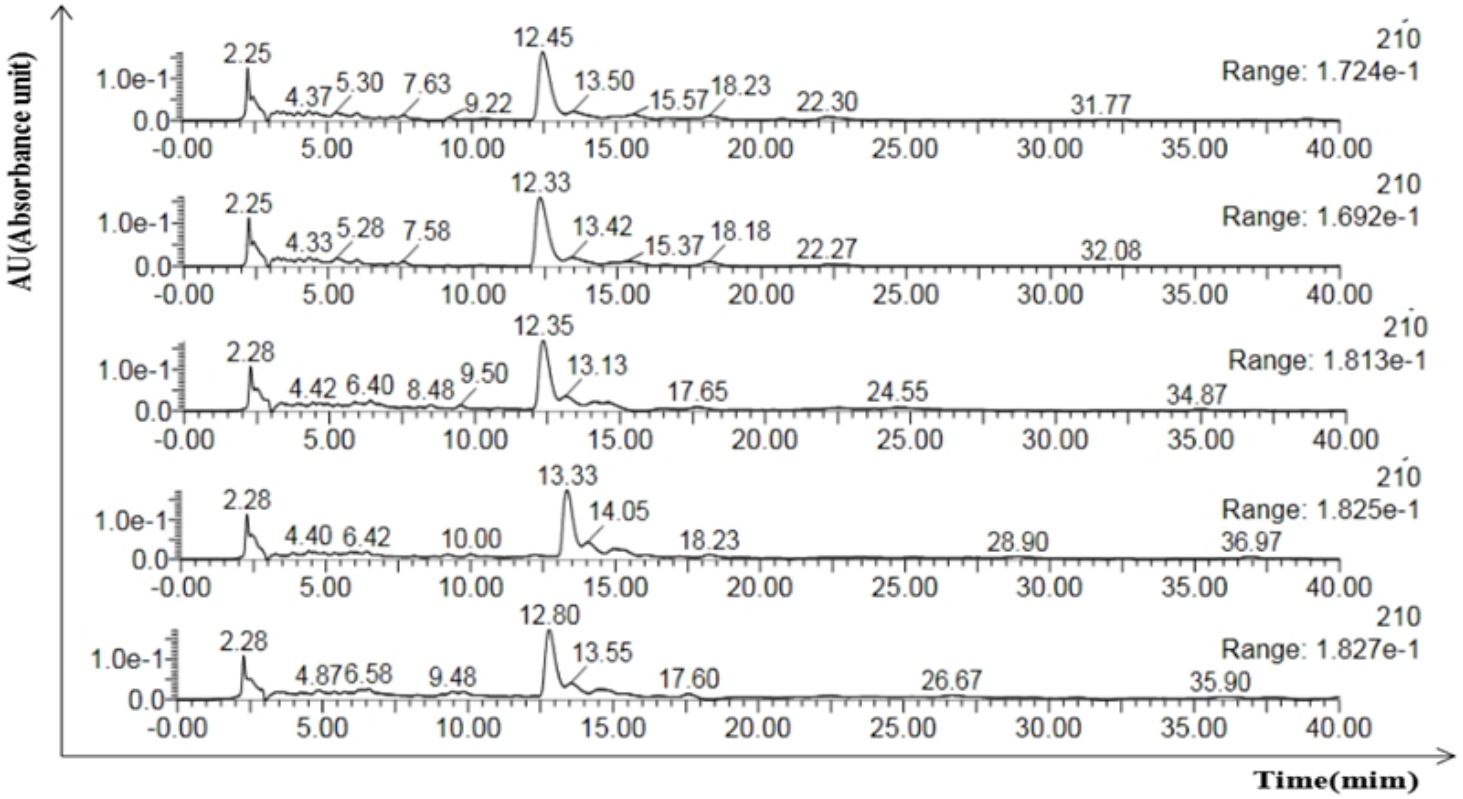

Figure 1

The chemical profile of ESZM extract with five different times were estimated with HPLC

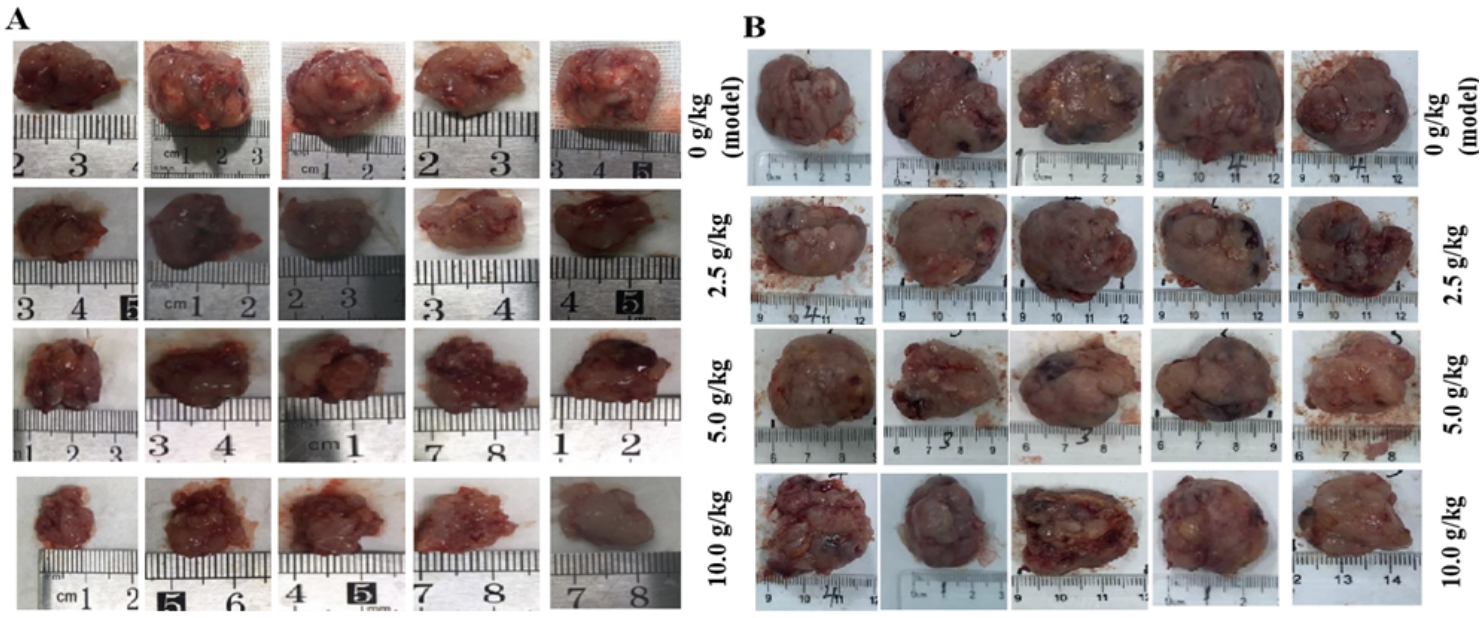

Figure 2

Tumor size in each groups. A: ER(-) breast cancer bearing mice; B: ER(+) breast cancer bearing mice.

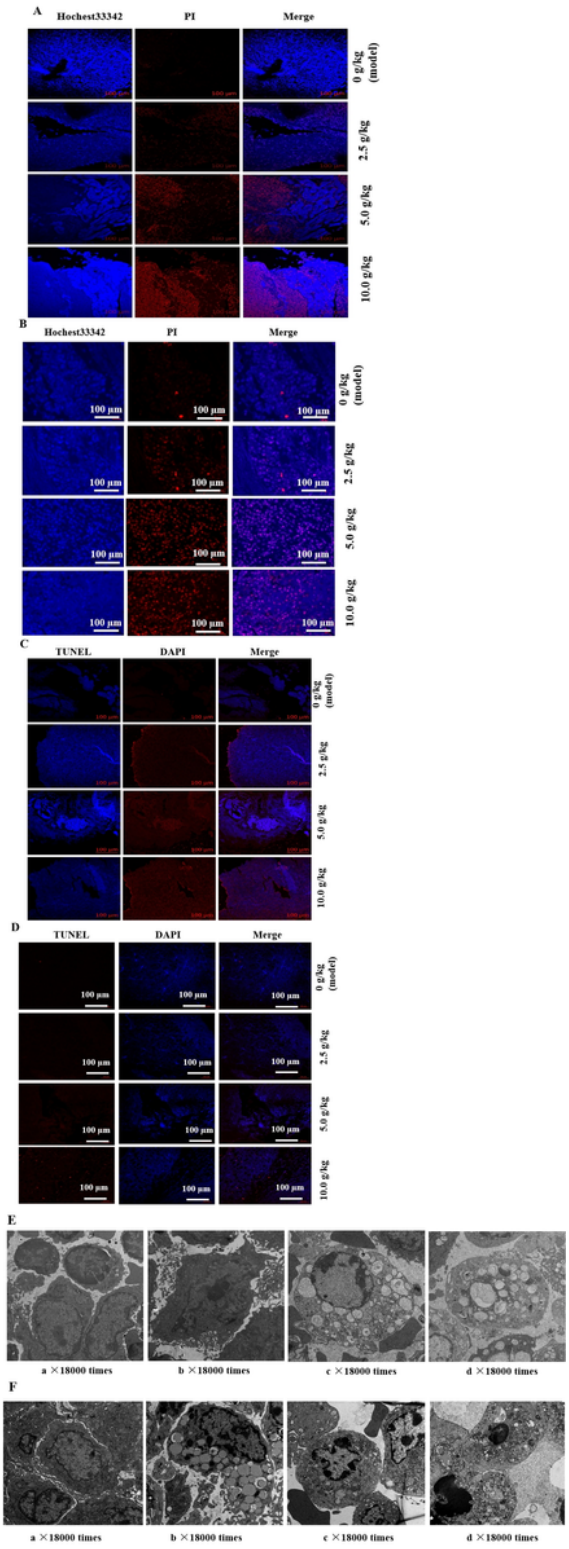

Figure 3

Effect of ESZM extract on morphological changes of tumor tissues. (A-B): Hoechst 33342/PI stain to observe morphological changes of tumor tissues. (C-D): Apoptosis of tumor tissue was observed by using a laser confocal microscope with TUNEL staining. (E-F): Ultrastructural changes in tumor tissue was explored with a TEM, a was model group, b-d was ESZM extract treatment groups. A, C, E: Tumor

histomorphology of ER(-) breast cancer bearing mice. B, D, F: Tumor histomorphology of ER(+) breast cancer bearing mice.

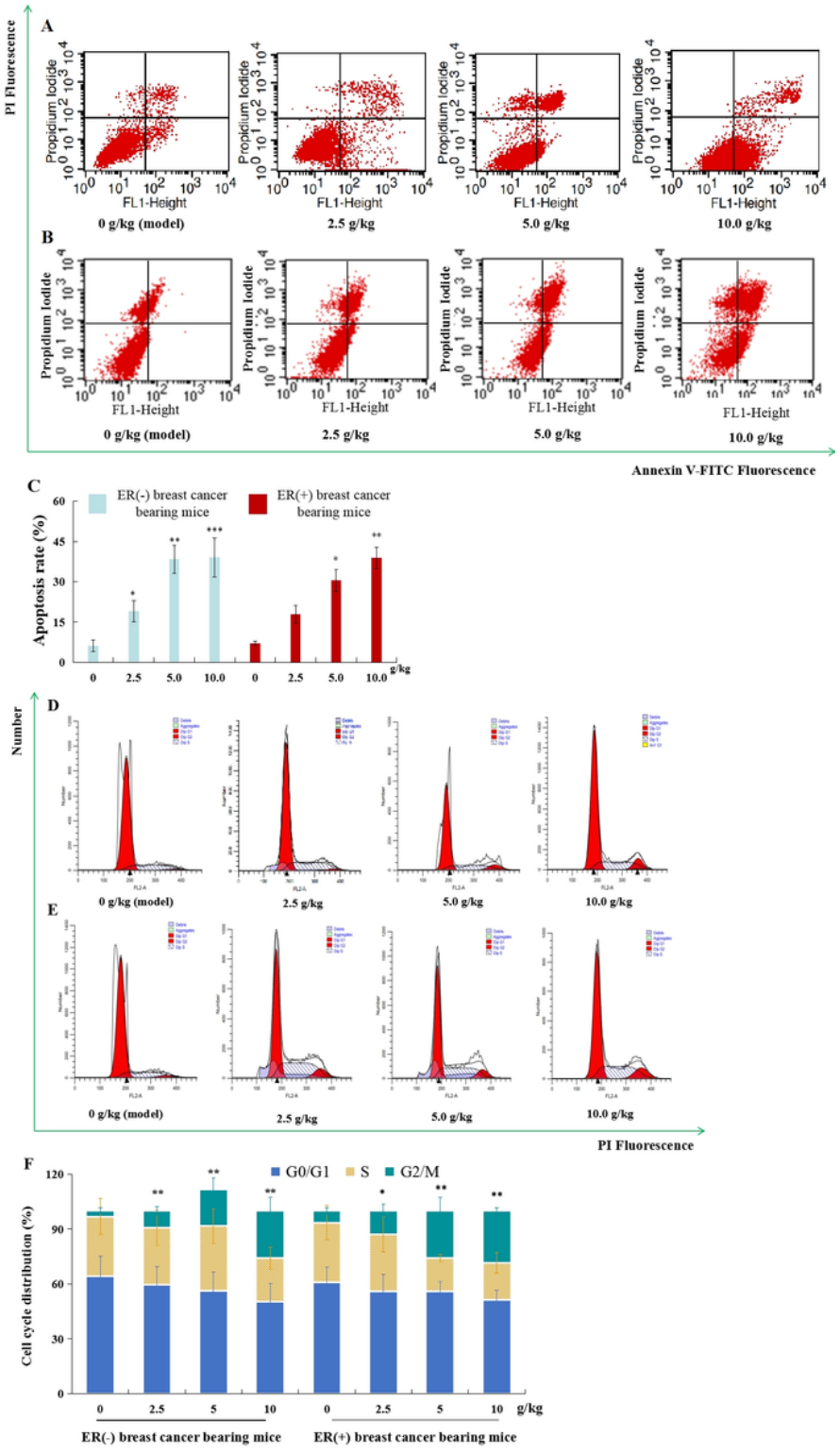

Figure 4

Effect of ESZM extract on apoptosis of tumor tissues. (A, B, D, E): Flow cytometry to detect apoptosis and cell cycle distribution of tumor tissue. A, D: Tumor histomorphology of ER(-) breast cancer bearing mice.

B, E: Tumor histomorphology of ER(+) breast cancer bearing mice. (C, F): The histogram show mean values are expressed in each group. *P<0.05, **P<0.01 or ***P<0.01 vs. model group.

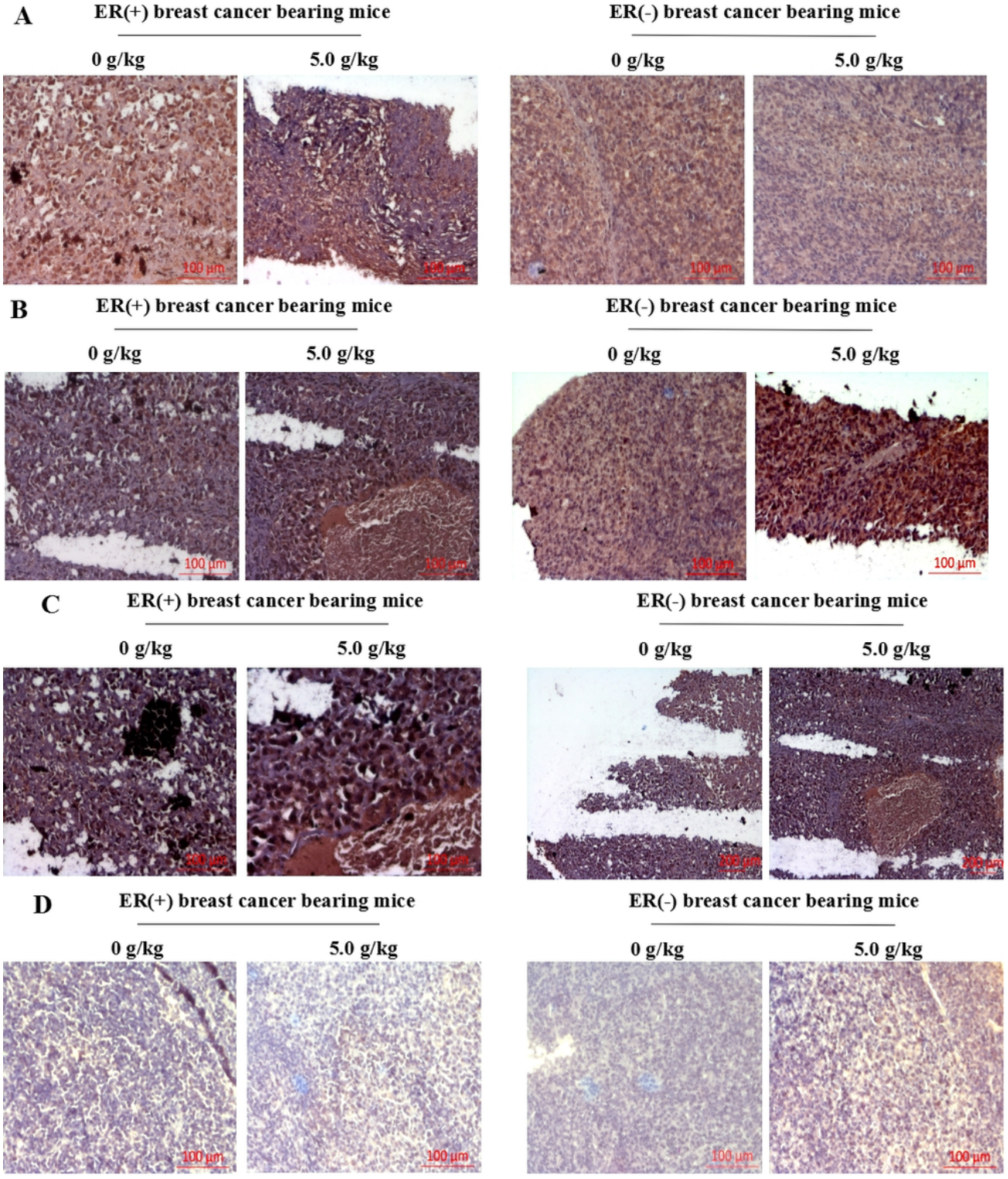

Figure 5

Immunohistochemical staining for Bcl-2, Bax, caspase 3 and caspase 9 protein in tumor specimens from ER(-) or ER(+) breast cancer bearing mice. A: Bcl-2 protein expression. B: Bax protein expression. C: caspae 3 protein expression. D : caspase 9 protein expression.

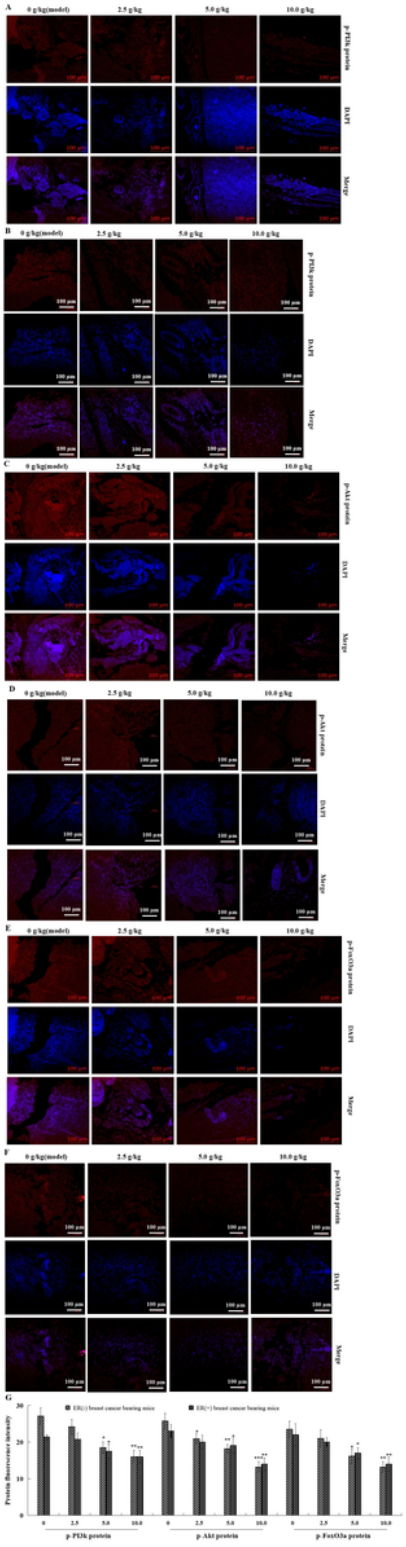

Figure 6

Immunofluorescence method was used to detect the expression levels of related protein. (A, C, E): p-PI3k, p-Akt and p-FoxO3a protein expression levels of ER(-) breast cancer bearing mice tumor tissue. (B, D, F): PI3k, p-Akt and p-FoxO3a protein expression levels of ER(+) breast cancer bearing mice tumor tissue. (G) The histograms show differences between groups, *P<0.05, **P<0.01 or ***P<0.001 vs. model group.

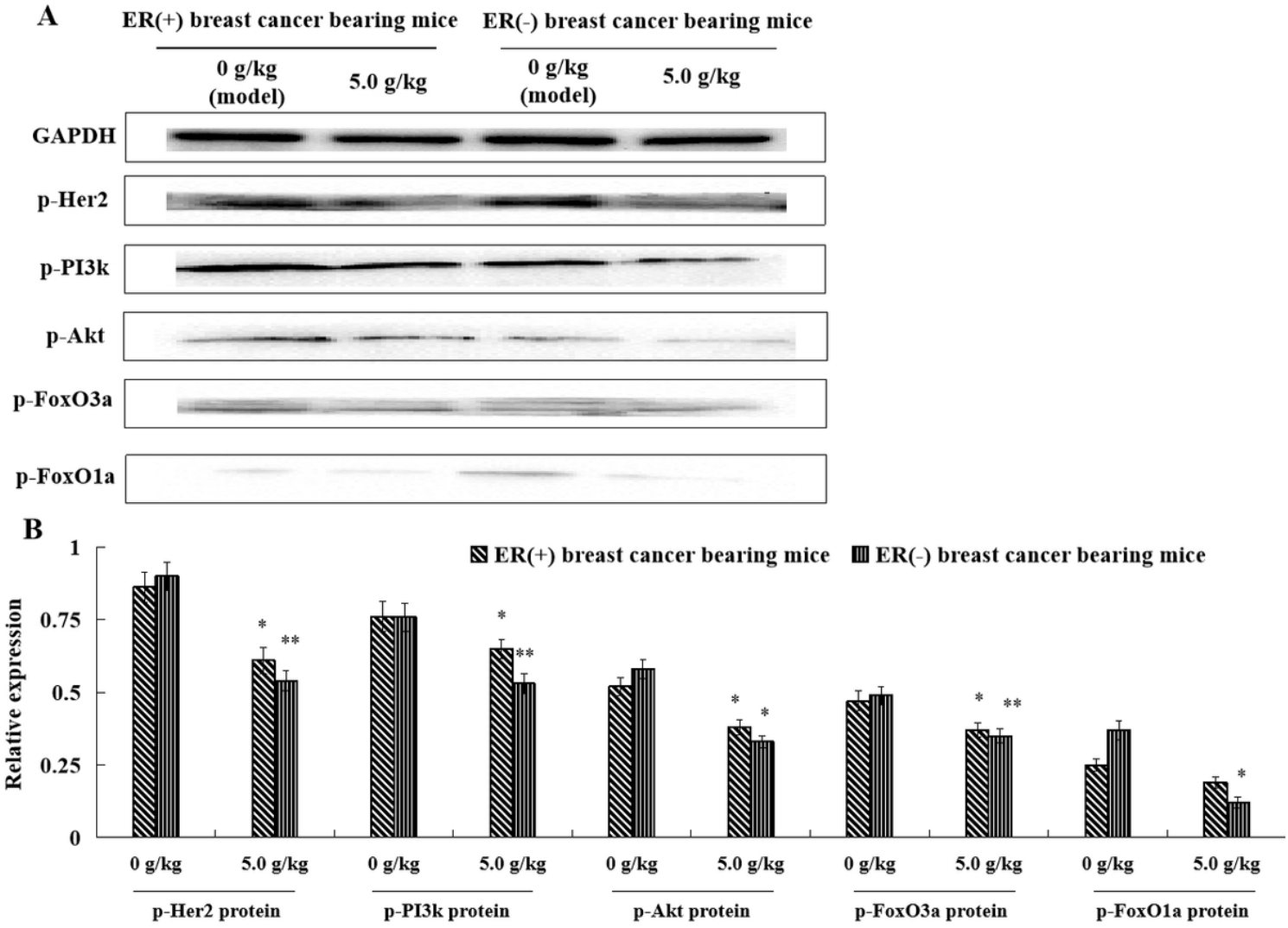

Figure 7

Effect of ESZM extract on related proteins expression of tumor tissue. (A): The visualization of related protein expression in transplanted tumor tissues of nude mice treated with ESZM extract were emerged by using a western blot analyse, and GAPDH was used as a housekeeping protein. (B): The histogram show mean values are expressed in each group. *P<0.05 or **P<0.01 vs. model group.

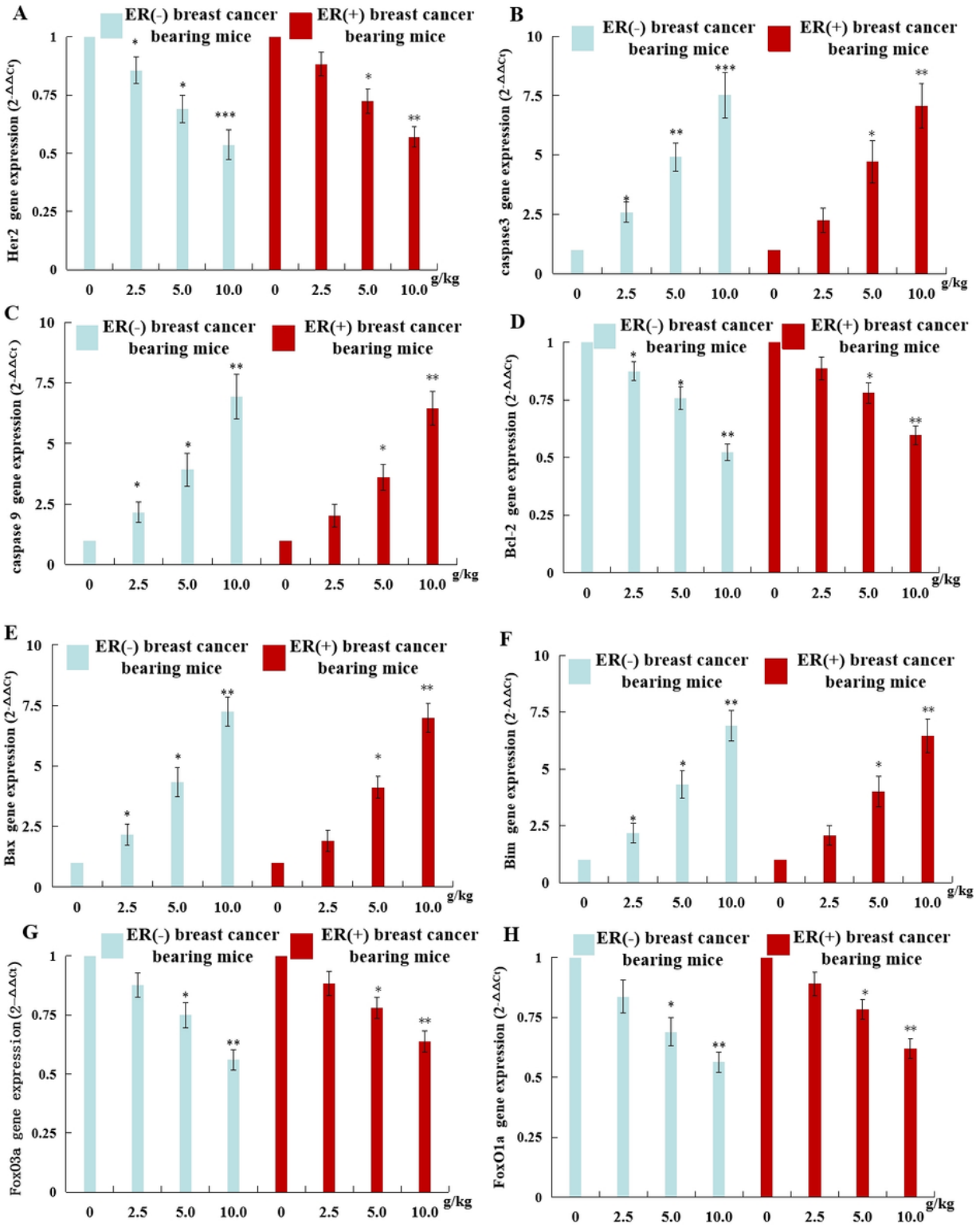

Figure 8

Effect of ESZM extract on related genes expression of ER(-) or ER(+) breast cancer bearing mice tumor tissue. (A-H): Expression of Her-2, caspase 3, caspase 9, Bcl-2, Bax, Bim, FoxO3a and FoxO1a gene in tumor tissues of nude mice treated with ESZM extract were tested by qRT-PCR assay. β-actin was used as a housekeeping gene. The histogram show mean values are expressed in each group. *P<0.05, **P<0.01 or ***P<0.01 vs. model group.

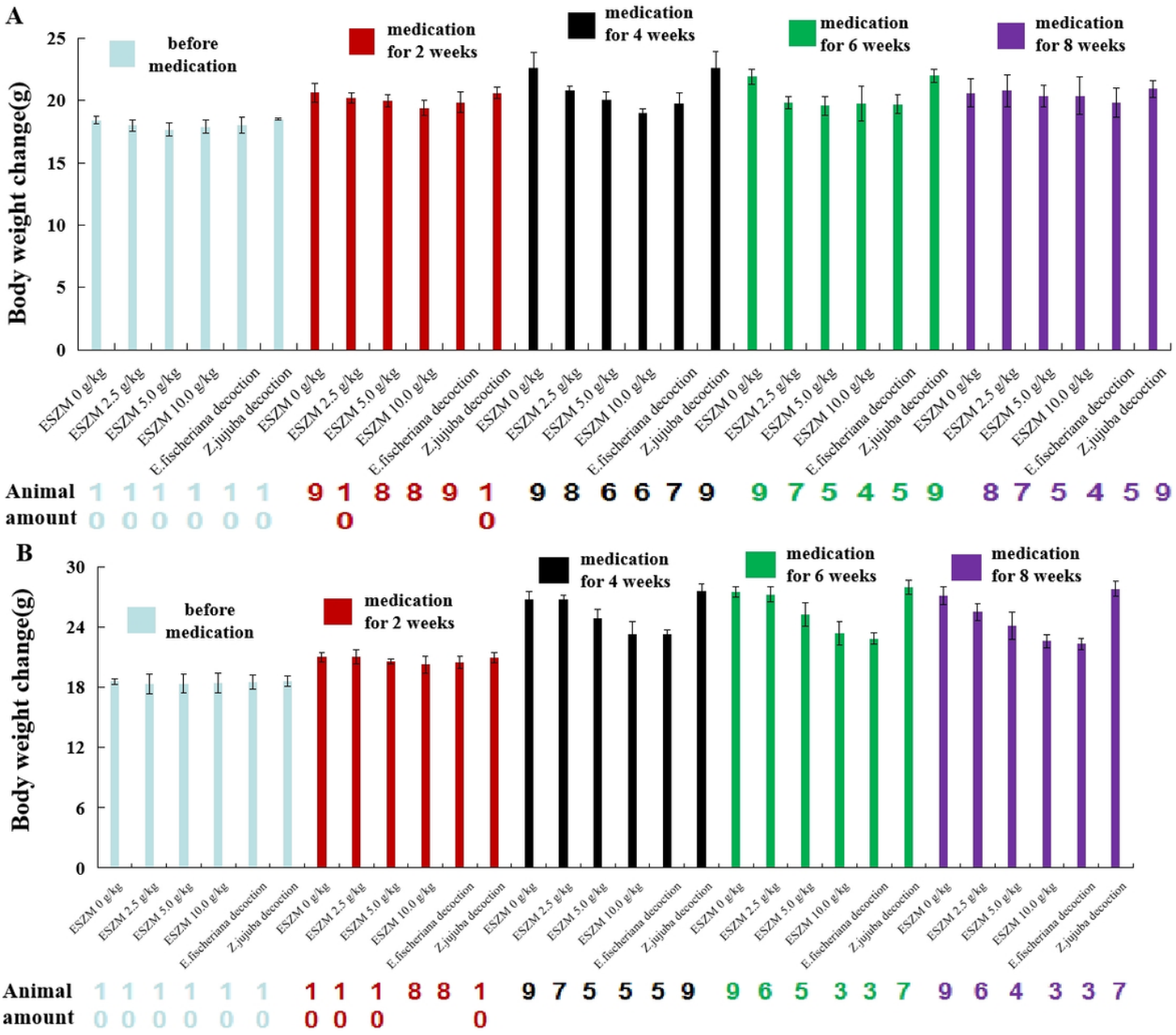

Figure 9

The body weight of bearing mice. A: ER(-) breast cancer bearing mice, B: ER(+) breast cancer bearing mice.

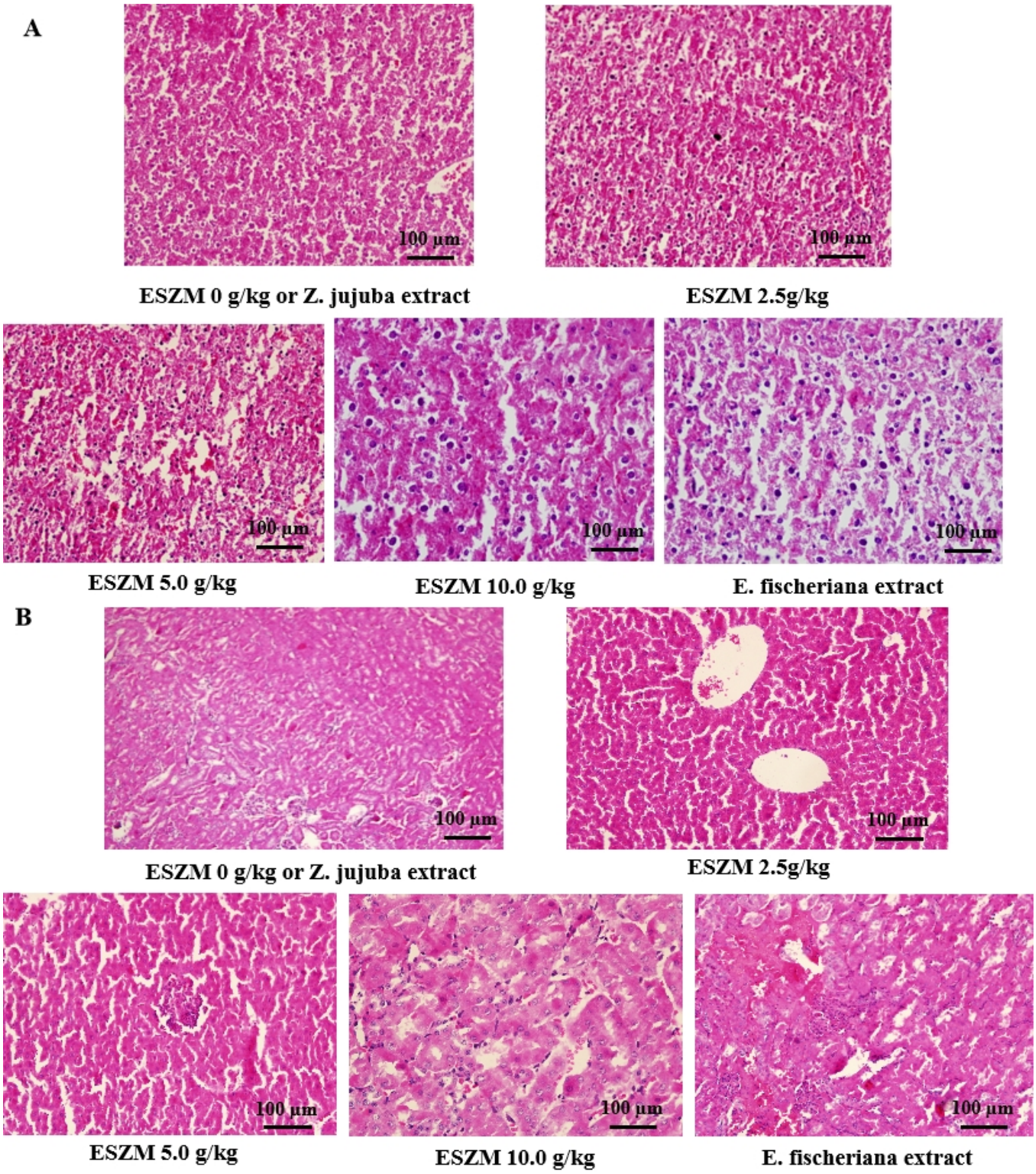

Figure 10

Liver and kidney histomorphology changes of breast cancer bearing mice. A: Liver histomorphology, B: kidney histomorphology.