In vitro and In vivo effects of Cucumis melo seeds extract and its purified trypsin inhibitor on angiogenesis and tumor characteristics in balb-c mice with breast cancer in comparison with tamoxifen

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Abstract

Background: Melon seeds as an excellent supply of protease inhibitors may have a protective role against tumor progression and angiogenesis. However, its anti-angiogenesis effects and related mechanism of action during cancer progression remain elusive. This study aimed to investigate the impact of bioactive compounds of melon seed on the expression of angiogenesis genes in breast cancer cell lines.

Methods: Trypsin inhibitor (TI) was purified from the seed powder of *Cucumis melo* (Muskmelon) Half-maximal inhibitory concentration (IC50) was determined for TI, extract of melon seed powder (EXT), and tamoxifen (TAM) by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Breast tumor was induced by subcutaneous injection of MC4-L2 cell line in blab-c inbreed mice breast tissue. After tumor growth, mice were treated with TI, EXT, and TAM in different doses and combinations to examine their effects on the tumor characteristics and the expression of angiogenesis-related genes including MMP-2, MMP-9, and VEGF using the RT-PCR method.

Results: TI, EXT, TAM, and adjuvant treatment of TI+TAM resulted in a reduction in expression of MMP-2, MMP-9, and VEGF. All treatments improved breast tumor characteristics and necrosis. The RT-PCR method verified the positive effects of treatments on breast cancer cell lines and tumors.

Conclusion: The results indicated that trypsin Inhibitor Purified from *Cucumis melo* seeds alone and in combination with tamoxifen might be a potential therapy with beneficial anti-angiogenesis and anti-tumor effects in breast cancer. Further studies are warranted.

Introduction

Breast cancer is the most typically diagnosed and the second leading reason for cancer-related mortality in women (1, 2). Like most solid tumors, the growth of new blood vessels is necessary for breast cancer. These new vessels not only help to meet the growing metabolic demands of the tumor by providing extra nutrients but also provide potential routes for tumor spread and metastasis (3). Tumor-induced angiogenesis is first evident in breast cancer at the pre-invasive stage of high-grade ductal carcinoma (4). It is currently apparent that tumors have a restricted capacity to grow while not having vascular support. Therefore, the formation of blood vasculature is an associate degree obligatory step to sustain the influx of essential nutrients to the cancer cells (5, 6).

Some proteases like matrix metalloproteinase (MMPs) are thought to play essential roles in immune responses, inflammatory reactions, and tissue remodeling (7, 8). Recent studies confirmed a closely correlated relationship between the expression of MMPs in tumor cells and their metastatic activity (9). The MMP axis has many areas of overlap with the inflammatory cytokines and cytokine network or growth factors that can regulate the expression of MMPs. Growth factors and cytokines play an important role in promoting the activation of MMPs from the inactive zymogens to the active enzymes. The current study demonstrates that vascular endothelial growth factor (VEGF) can promote

neovascularization (10). VEGF could be a key promoter of pathological and physiological angiogenesis and a specific survival factor and mitogen for endothelial cells (11-13).

Human epidermal growth factor receptor 2 (HER2) is a kind of protein found on the surface of breast cells. It is involved in the normal growth of breast cells and can promote the development of breast cancer cells. HER2-positive refers to breast cancer cells that have more than normal levels of HER2. In comparison to HER2-negative breast cancer, HER2-positives tend to grow and spread more quickly and also are much more likely to respond to treatment with drugs that target the HER2 protein. The advancement in HER2 targeted treatments, has altogether progressed the result for patient with HER2 positive breast cancer (14). Tamoxifen (TAM) is considered as a gold standard in the treatment of estrogen receptor-positive breast cancer (15). One of the most necessary targets in control of carcinogenesis and inhibition of tumor cell growth is estrogen receptors (15). However, long term therapy with TAM has contributed to side effects (16).

Some plant-derived compounds such as flavonoids, phytoestrogens, and protease inhibitors were reported to be able to prevent one-third of cancers (17, 18) and inhibit new vessel formation and tumor cell proliferation in tumors without major side effects and significant toxicity to normal tissues (17, 19). These natural compounds can have protective effects through the regulation of inflammatory pathways against inflammatory diseases(19). Some plants such as melon extract and its trypsin inhibitor protein has many biological functions including anti oxidative, anti-inflammatory, and anticancer effects (17). Melon seeds can be used as a source of nutrients, natural antioxidants, and bioactive compounds (20). Recent studies showed that different components of melon have anticancer role by affecting on a variety of different mechanisms including cell proliferation, autophagy, level of insulin-like growth factor 1 receptor and its downstream signaling pathways. However, the main mechanism and its antiangiogenic effects in breast cancer remains to be elucidated (21-24).

The present study aimed to investigate the effect of bioactive compounds of *Cucumis melo* seeds including TI protein and its EXT on the expression of angiogenesis genes including MMP-2&9 and VEGF *in vivo* and *in vitro*, in a mouse model of MC4-L2 breast cancer. We also assessed the changes in tumor tissue characteristics such as inflammation, necrosis, angiogenesis, cell proliferation, and tumor size.

Material And Method

Seed preparation of target plant

First, the seeds of the melon plant were prepared through washing it to remove any kind of contamination. The seeds were then dried indirectly using sunlight and the kernels were separated and crushed using a grinder. The resulting powder was used as a starting material to purify the target peptides by chromatography.

Preparation of affinity column with trypsin ligand and chromatography

After preparation of the specified seed powder, the chromatography method was done as previously described elsewhere (25). Our method was different just in the last step in which supernatant turned into loaded onto the column, and consequently the column turned into washed with deionized water till the absorbance of fractions at 280 nm got here to zero. Three column volumes of deionized water with PH=2.5 accustomed to wash sure proteins from the column (deionized water was adjusted to PH=1.5 with 0.1 N HCl).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate SDS-PAGE based on the Schagger and Von Jagow method (26) as previously explained (25).

Measurement of protein concentration

The final and quantitative protein concentrations were determined by the Bradford method as the standard procedure (27).

Assay of TI activity

The activity of the Trypsin inhibitor from *Cucumis melo* was determined by the residual trypsin activity following the method of Hajela (28). with slight modifications using N-α-benzoyl-DL-arginine-pnitroanilide (BApNA) as the substrate and bovine trypsin as the standard enzyme. The reaction mixture containing 50 µl TI (5 mg/ml), 50 µl trypsin (1 mg in 5 mL of 0.05 M Tris-HCl, pH 8.0, containing 0.03 M CaCl2) and 100 µl 0.05 M Tris-HCl (pH 8.0) containing 0.03 M CaCl2 was incubated at 37 °C for 10 min in a shaking water bath. The residual activity was measured by adding 1 mL of 0.8 mM BApNA (7 mg dissolved in a minimum volume of DMSO and adjusting its final volume to 20 mL with 0.05 M Tris-HCl, pH=8.0, containing 0.03 M CaCl2) to the reaction mixture followed by incubation at 37 ºC for 10 min in a shaking water bath. The reaction was stopped by adding 20 µl of 30% (v/v) glacial acetic acid. A blank and a trypsin control were run simultaneously. In blank, acetic acid was added prior to the addition of BApNA and in trypsin control, distilled water was added in place of the TI. The absorbance was recorded at 410 nm against the blank using a double beam UV-visible spectrophotometer (Model 2202, Systronics, India). An appropriate volume of the kidney bean extract, which was enough to give 40-60% inhibition of trypsin, was taken for the assay. One trypsin unit (TU) was defined as an increase of 0.01 absorbance units at 410 nm per 1.2 mL of the reaction mixture. TI activity was expressed as the number of trypsin units inhibited (TUI).

In vitro phase

Cell line and culture conditions

MC4-L2 mouse breast cancer cell line (National Center for Genetic and Biological Resources of Iran, Tehran) were maintained and grown in 25 and 75 cm2 flasks (SPL, Pocheon, Korea) in DMEM: Ham´s F12 + 2 mM L-Glutamine + 15 mM HEPES buffer, penicillin (100 µg/ml), streptomycin (100 µg/ml), and 10% (vol/vol) fetal bovine serum (FBS, Gibco BRL, Life Technologies, Grand Island, NY) in a 37°C incubator and 5% CO2. Cells were monitored by a phase-contrast microscope until they reached appropriate confluence. Once the cells reached 90% confluency, the MC4-L2 cells was harvested with 0.25% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA). Cell viability and numbers were determined by a hemocytometer and trypan blue exclusion. Cell viability was calculated to be greater than 98%.

Cell viability assay in vitro

Toxicity and cell proliferation were assessed using the MTT Sigma test. First, to determine and set up the exact number of cells required to perform the desired test in a 96-'s pellet in 8 rows of 12 wells, different values of $5 * 10^3$, $10 * 10^3$, $15 * 10^3$, $20 * 10^3$, $25 * 10^3$, $30 * 10^3$, $50 * 10^3$ and $100 * 10^3$ of MC4-L2 sol were poured into 10% FBS-enriched DMEM-F12 medium to evaluate cell growth. After 24 hours, the cell growth rate was examined using a microscope and the number of 10^4 cells per well of the pellet had the best response, which was selected as the number of cells approved for MTT testing. To perform the MTT test, 10^4 cells of MC4-L2 cell line were poured into each of 96 culture pellets and then 10% FBS enriched with 100 ml of DMEM-F12 culture medium per 100 ml was added. After 24 hours of incubation at 37 ° C with 5% CO2, different concentrations of TI (5, 10, 25, 50, 100, 200, 300, 400, 800, 1200 µg/ml), EXT (5, 10, 25, 50, 100, 200, 400, 800, 1200 µg/ml) and, TAM (0.01, 0.1, 1, 5, 10, 15, 20 µmoll) were added to each well and then 100 ml of the desired culture medium was added. The cells were incubated again for 48 hours and these steps were repeated 3 times for all concentrations. After 48 hours of incubation at 37° C with 5% CO2, equivalent to 10 microliters of 3- (4,5-dimethylthiazole-2) -2,5-diphenyltetrazolium bromide MTT solution (Sigma) (0.5 mg/ml MTT powder in PBS), was added to each of the culture medium houses and incubated again for 4 hours at 37 ° C with 5% CO2 and then centrifuged at 3000 rpm for 10 minutes. To dissolve the Formazan crystal, the supernatant containing MMT was completely removed and 200 ml of dimethyl sulfoxide (DMSO) was added to each well and kept at room temperature for 30 minutes to dissolve completely. The ELISA reader was read at 570 nm and 630 nm.

Examination of anti-angiogenesis effects of TI, EXT, and TAM

According to MTT results and after preparation of the MC4-L2 cell line, 10^4 cells were poured into each well of a 96-well plate and placed in an incubator for 24 hours. Then we emptied the medium on the wells and 500 µl of fresh medium with 10% FBS was added to the wells. The Control group received no treatment. Treatments groups were designed as BPS solution, 5 µmoll of TAM, 400 µg / ml of EXT, TI at concentrations of 200 and 300 µg/ml, and 300 µg/ml of TI + 5 µmoll of TAM. Then the plates were incubated at a CO2 incubator for 72 h. Treatments were carried out in five for each dose. Finally, the anti-angiogenesis effects were examined using fluorescent staining and the RT-PCR method.

Fluorescent staining method and viability test

PBS 1X solution, FDA (Fluorescein Diacetate) and, PI (Propidium Iodide) were used in a proportion of 1 ml, 10 µl, and 100 µl, respectively. Images were recorded using a microscope camera (Fig 1).

Animal Phase

Experimental animals and tumor model

The Ethics Committee at Shiraz University of Medical Sciences approved the experiments (IR.SUMS.REC.1398.950). Five to six weeks-old normal female BALB/c inbreed female mice were purchased from Pasteur Institute (Tehran, Iran). The mice were housed in an animal lab at a temperature of 22 -24°C and 65% humidity. Trypsinized MC4-L2 cells were then harvested and washed to induce tumor formation in the mice. Their concentration was adjusted to 3.5×10^6 cells/100 µl with phosphate-buffered saline (PBS) at less than 98% viability. Prepared cells were injected subcutaneously into the right upper thigh of each mouse. Approximately 7–10 days after injection of the cancer cells, the tumors were palpated in the injected areas (supp1). The BALB/c inbreed mice were randomly divided into six groups of five mice per group: controlled breast cancer mice without any treatment (normal control group), breast cancer mice treated with either 300 µgr/ml or 600 µgr/ml of TI, breast cancer mice treated with 800 µgr/ml of EXT, breast cancer mice group received 10 µmol TAM and the last group was breast cancer mice that received combination therapy of 600 µgr/ml of TI + 10 µmol TAM. The treatment period duration was 14 days. Finally, the mice were first anesthetized and then killed, and their tumor tissue was extracted and stored in 10% formalin.

Histological assessments

Tissue passage steps, preparation of paraffin blocks, and preparation of 5-micron sections were performed. H&E staining method was performed for histological assessments using undiluted Mayer's hematoxylin (Merck, Darmstadt, Germany) and 0.5% eosin (Merck). Evaluations were performed by light

microscope (Olympus cx31) for the intensity and scoring of inflammation (–, -/+, and +/+), necrosis (%), and peripheral vessels as angiogenesis (+, ++, +++) (Fig 2).

Molecular phase

RNA extraction and cDNA synthesis

Total RNA extraction was extracted from MC4-L2 cell line treated and tumor tissues of mice using TRIZOL reagent (Gene All, South Korea), according to the manufacturer's instructions. RNA concentrations were determined using the NanoDrop spectrophotometer (Thermo Scientific, Germany). The quality of extracted RNA was assessed by 1% agarose gel electrophoresis. After RNA extraction, the complementary DNA (cDNAs) were synthesized using a cDNA synthesis kit (EURx, Poland), according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was used to determine the expression levels of MMP-2, MMP-9, and VEGF genes in the MC4-L2 cell line and tumor tissue of mice.

Designing of primers used for RT-PCR were done by Allele ID 6 software and are listed in Table 1. Subsequently, the primer specificity was confirmed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and In-Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was considered as the housekeeping gene (internal control).

The real-time PCR reactions were performed in duplicates using the SYBR Green PCR master kit (EURx, Poland) in a real-time PCR instrument (Applied Biosystems, USA). The expression of interested genes (MMP-2, MMP-9, and VEGF) at transcript level were normalized to the GAPDH gene expression, and the standard deviations were calculated. Relative real-time PCR was performed in duplicates, and each experiment was repeated two times. The program for thermocycling was as follows: 1 cycle at 95°C for 2 min, followed by 40 cycles at 95°C for 30 seconds, and then 1 cycle at 65°C for 20 seconds. At last, by using the comparative Ct method the relative quantification of the gene expression was determined(29).

Table 1

Nucleotide sequences of the primers used for the gene expression analysis by real-time PCR.

Gene	Oligo Sequence 5'–> 3'	Aneling temperature
MMP-2 primers	Forward primer TGATGGCATCGCTCAGATCC Reverse primer TGTCACGTGGTGTCACTGTC	59
MMP-9 primers	Forward Primer CGCTCATGTACCCGCTGTAT Revers Primer GCCTTGGGTCAGGCTTAGAG	59
VEGFa primers	Forward Primer CTGGAAGAATCGGGAGCCTG Revers Primer ACCACCGTGTCTTCTCTTGC	58
GAPDH	Forward Primer ACTGAGCAAGAGAGGCCCTA Revers Primer TATGGGGGTCTGGGATGGAA	59

Statistical analyses

Histopathological factors and tumor characteristics was assessed using the Kruskal-Wallis test. Livak method ($2^{-\Delta\Delta CT}$) was used for comparing the statistical differences in the expression levels of genes and the fold changes in treated and control groups. One-way ANOVA was used for other parameters with LSD as the post-hoc test. Statistical analyses were performed using SPSS software (version 22.0; IBM Corporation, Armonk, NY, USA). The results were considered to be significant when the P-values were<0.05.

Results

Protein purification and electrophoresis

Electrophoresis analysis of purified protein Hejela method (28) identified a single band with a molecular mass of 3.4 kDa (Fig 3). The results are presented as a Supplementary file (supp2).

Anti-proliferative effect of TI, EXT, and TAM

The MTT results speculated that TI and EXT in doses of 5-1600 µgr/ml and TAM in doses of 0.01-20 µmoll induced a significant reduction in the proliferation of MC4-L2 breast cancer cells, which was dose-dependent with an IC50 value of about 300 µgr/ml, 400 µgr/ml, and five µmoll respectively (Fig 4).

Effect of TI, EXT and TAM on MMP-2, MMP-9 and VEGF secretion in vitro and in vivo

In vitro results on the MC4-L2 cell line showed a significant decrease in MMP-2 transcript gene expression in the groups receiving TI300 (99.9%, P-value< P<0.05) and TAM+TI300 (99.92%, P-value< P<0.01) compared to the control group and PBS. There was no significant difference among other groups (Fig 5-A). In addition, the expression of MMP-2 at the breast tumor tissue indicated a significant decrease in all groups including TAM, EXT800, TI300, TI600, and TAM+TI600 (98%, 97.12%, 97.93%, 99.94%, 99.99% respectively, P-value< P<0.001) compared to control group. There was also a significant decrease in the expression of the MMP-2 transcript gene in the TAM + TI600 group (99.76%, P-value< P<0.05) compared to the EXT800 group. There was no significant difference among other groups (Fig 6-A).

On the other hand, a significant reduction was observed in the expression of the MMP-9 transcript gene of the MC4-L2 cell line in the TAM+TI300 group (99.93%, P-value< P<0.01) compared to the control group. Also, a significant decrease was found in the expression level of the MMP-9 transcript gene in the TI300 (99.65%, P-value< P<0.05) and TAM+TI300 (99.93%, P-value< P<0.01) groups compared to the PBS group. There was no significant difference between the other groups (Fig 5-B). In addition, the expression of the MMP-9 transcript gene of breast tumor tissue was significantly decreased in all study groups, included TAM, EXT800, TI300, TI600, and TAM+TI600 groups (99.36%, 98.86%, 99.40%, 99.98%, ~100% respectively, P-value< P<0.001) compared to control group. There was also a significant decrease in the expression of MMP-9 transcript gene in TI600 (98.24%, P-value< P<0.05) and TAM+TI600 (99.74%, P-value< P<0.01) groups compared to EXT800 group. No significant difference was observed among other study groups (Fig 6-B).

Regarding VEGF transcript gene, the results of our study indicated a significant decrease in the expression of the VEGF transcript gene in both TI300 (99.87%, P-value<0.05) and TAM+TI300 (99.97%, P-value<0.01) groups compared to control group in MC4-L2 cell line. Also, the expression of the VEGF transcript gene in the groups receiving TAM+TI300 showed a significant reduction (99.96%, P-value<0.05) compared to PBS group. There was no significant difference among other groups (Fig 5-C). The results showed a significant decrease in the expression of the VEGF transcript gene at the tumor tissue level in all treated groups, TAM, EXT800, TI300, TI600, and TAM+TI600 (98.83%, 99.3%, 99.57%, 99.52%, 99.98% respectively, P-value<0.001) compared to control group. There was no significant difference among other groups (Fig 6-C).

Effect of TI, EXT, and TAM on angiogenesis, inflammation, and tissue necrosis

A significant decrease in the mean score of angiogenesis was observed in the groups receiving TI600 and TAM+TI600 compared to control group (P=0.018 and P=0.009, respectively). There was no significant difference among other groups (Fig 7-A).

The results showed a significant increase in the percentage of tumor tissue necrosis in the groups receiving TAM, EXT800, TI300, TI600 and TAM+TI600 compared to the control group (P <0.027, P = 0.016, P=0.009, P = 0.004, and P <0.001, respectively). Also, a significant increase was observed in TAM+TI600 group compared to the TAM and EXT800 groups (P = 0.024 and (P = 0.041, respectively). There was no significant difference among other groups (Fig 7-B).

The results showed a significant increase in the mean score of inflammation in all groups compared to the control group (P <0.001). There was no significant difference among other groups (Fig 7-C).

Effect of TI, EXT, and TAM on body weight and breast tumor tissue characteristics

The results indicated no significant difference in body weight between groups over time (Fig 8-A). There was a significant decrease in the mean tumor volume in all treated groups, TAM (131.94±6.83, P<0.001), EXT800(244.97±90.14, P<0.01), TI300 (205.08±54.79, P<0.001), TI600 (81.05±13.73, P<0.001), and TAM+TI600 (161.13±36.47, P<0.001) compared to the control group (558.35±26.68). No significant difference was observed between the other groups during the time (Fig 8-B).

The results showed a significant reduction in the mean tumor width in all treated groups including TAM (5.88±0.25, P<0.05), TI300 (5.62±0.49, P<0.01), TI600 (5.22±0.48, P<0.01), and TAM+TI600 (4.82±0.32, P<0.001) (except the group receiving EXT800) compared to the control group (9.20±0.36). There was no significant difference among other groups during the time (Fig 6-C).

A significant decrease in mean tumor length was observed in the groups receiving TI600 (4.72±0.28, P<0.01) and TAM (5.20±0.46, P<0.01) compared to control group (9.08±0.33) in second week of treatment. No significant difference was observed among other groups during the time (Fig 8-D).

There was a significant decrease in the mean tumor depth in the second week in all treated groups including TAM (3.78±0.26, P<0.05), EXT800 (3.16±0.45, P<0.01), TI300 (3.44±0.23, P<0.01), TI600 (2.57±0.34, P<0.01), and TAM+TI600 (3.84±0.76, P<0.05) compared to control group (5.76±0.21). No significant difference was observed among other groups during the time (Fig 8-E).

The results also show a significant decrease in the mean tumor weight in the groups receiving TI300 (0.124±0.015, P<0.01), TI600 (0.099±0.013, P<0.01), and TAM + TI600 (0.099±0.005, P<0.01) compared to control group (0.240±.0.022). Furthermore, a significant reduction was observed in the groups receiving TI300 (0.124±0.015, P<0.05), TI600 (0.099±0.013, P<0.01), and TAM+TI600 (0.099±0.005, P<0.01) compared to the TAM group (0.208±0.036). The groups receiving TI600 (0.099±0.013, P<0.05) and TAM+TI600 (0.099±0.005, P<0.05) showed a significant decrease compared to the EXT800 group. There was no significant difference among other groups (Fig 9).

Discussion

This study was the first to explore the anti-angiogenic potential of *Cucumis melo* TI, EXT, and combination therapy of TI and TAM in both *in vitro* and *in vivo* situations in the MC4-L2 breast cancer cell line and tumor tissue in mice. The results of the present study indicated that TI, EXT, TAM, and adjuvant treatment of TI + TAM resulted in a reduction in expression of MMP-2, MMP-9, and VEGF. All treatments improved breast tumor characteristics and necrosis. In general, the positive effects of our treatments on breast cancer cell line and mouse animal model was observed. Melon is one of the medicinal plants that have various antitumor and antioxidant compounds. It is also regarded as adjuvant therapy for anticarcinogenic drugs through various mechanisms. This plant has anti-inflammatory, anti-proliferative, anti-tumor, antioxidant effects and can regulate immune system. One of the most important components of this plant´s seed is protease inhibitors that are classified into cysteine protease inhibitors, serine protease inhibitors, and Metallocarboxy protease inhibitors (25, 30, 31) .

Previous studies were reported that protease inhibitors consumption can decrease the risk of cancer development by inhibition of angiogenesis (32). Since the formation of new blood vessels being one of the critical stages of tumor growth, angiogenesis inhibition can be regarded as an effective approach in cancer prevention (33). The results of this study identified that TI and EXT in doses of 5-1600 µgr/ml and TAM in doses of 0.01-20 µmoll can induce a dose-dependent reduction in the proliferation of MC4-L2 breast cancer cell line.

In this study, the effect of 200 and 300 µgr/ml of TI, 400 µgr/ml of EXT and 5 µmoll of TAM in MC4-L2 breast cancer cell line and 300 and 600 µgr/ml of TI, 800 µgr/ml of EXT and 10 µmoll of TAM in mouse animal model with breast cancer was assessed along with a combination therapy of TI + TAM. The results identified that these treatments had a beneficial effect on angiogenesis inhibition by reduction or inhibition of MMP-2, MMP-9, and VEGF transcript gene expression. These treatments also could improve the breast tumor characteristics and had a beneficial effect on the increase of tumor necrosis and reduction of peripheral vessels in comparison to the control group. These effects were also more pronounced in TI and TAM + TI treated groups.

Rasouli et al. reported similar results of TI from *cucumis melo* on the expression of angiogenesis-related gen such as VEGF, MMP-2&9 in breast cancer cells (25). In another study, a similar effect of trypsin inhibitor protein extracted from soybean on inhibition of angiogenesis was reported (34).

According to the previous studies, plant-derived compounds specifically inhibited tumor cell proliferation and new vessel formation in tumors without significant toxicity to normal tissues and major side effects (19, 35). Also, several studies have already reported the cytotoxic, antioxidant/anti-inflammatory, and immunomodulatory effects of *cucumis melo* extract (36).

Melon seeds were reported to be a good source of natural active components and have antioxidant properties (37–39). As a fact, the Kunitz and Bowman-Birk inhibitors as two major families of protease inhibitors in some plant seeds, have been earlier studied as anticancer agents(40–42). Some protease inhibitors from other sources have also been studied on cancer development. Since, angiogenesis-

mediated metastasis of the primary tumor is the most common cause of cancer death in humans, angiogenesis modulation can be a promising approach to treat cancer (43, 44).

Angiogenesis is a multistep process involving degradation of basement membrane and extracellular matrix components, proliferation, migration, and tubulogenesis of endothelial cells, and finally maturation of the neovasculature. In the present study, TI and melon seed extract inhibited expression of VEGF, MMP-2&9 from MC4-L2 cells, and breast tumor tissue in mice. VEGF, as the most important antiangiogenic factor, plays a key role during the angiogenesis process which involves induction of endothelial cell proliferation, migration, and MMP secretion (45). According to these results, TI and EXT's suppressive effect on the expression of VEGF, MMP-2&9 which affects other important events during angiogenesis, might be considered as one of the mechanisms of its anti-angiogenic activity. However, more studies are required to determine exact mechanisms underlying the anti-angiogenesis activity of TI and EXT.

Previous studies indicated that inflammation increased vascular permeability, in which the leukocytes migrate into the injured tissues. The inflammatory mediators like TNF-α, interferon-γ, interleukins as well as chemokines play an important role in inflammation (8, 13). However, dysregulation of the inflammatory response may result in many disorders including autoimmune diseases and cancer (46). In this regard our results found that all treatments led to an increase in necrosis and inflammation in tumor tissue and a significant decrease in peripheral vessels resulting in a reduction of angiogenesis in comparison to control group. The results also demonstrated that the TAM + TI600 treatment is more beneficial than either other treatments or control group. Similar results were reported in some studies about TI and TAM's beneficial effects on necrosis, inflammation, and angiogenesis (25, 34, 46).

Our results identified that there was a significant reduction in tumor characteristics such as weight, length, depth, and volume in all treated groups in comparison to control group. These changes may be due to the inhibition of angiogenesis and increases in tumor necrosis. Based on our recent search, no published study has been investigated these outcomes so far.

Our study has some limitations. It was better to evaluate effects of more variable doses on the study parameters. Additionally, our study could have been conducted on a larger sample size. A second protein based analysis could have been performed to validate RT-PCR results. In spite of these limitations, our study has several strengths. The study duration was relatively adequate, effects of interventions were evaluated both *in vivo* and *in vitro*. Furthermore, studies in this field have mostly studied extracts of different plant seeds. Our study was first to evaluate both TI and extract of *cocomis melo* seeds in comparison with tamoxifen in mouse model of breast cancer. RT-PCR was used to assess the gene expression of different anti-angiogenesis factors which is a precise and validated method. We have also examined a variety of doses and combinations of treatments on factors related to breast cancer.

Conclusion

TI, EXT, and TAM therapy could inhibit the expression of angiogenesis-related genes such as MMP-2, MMP-9, and VEGF, and increase tumor tissue necrosis. This intervention can also cause desirable

changes in tumor tissue parameters such as length, width, depth, and height of the tumor in a dose-dependent manner. Combination therapy by TI and TAM had the greatest effect on reducing tumor size, inhibiting the expression of angiogenesis-related genes and tissue necrosis. Further *in vivo* and *in vitro* studies will be warranted to confirm these results and to discover the molecular mechanisms.

Abbreviations

TI: Trypsin Inhibitor protein; EXT: Extract of *cucumis melo* seed powder; TAM: Tamoxifen; MMPs: Matrix metalloproteinases; VEGF: Vascular endothelial growth factor; ERs: Estrogen receptors ; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis; BApNA: N-α-benzoyl-DL-arginine-pnitroanilide; MTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; DMSO: Dimethyl sulfoxide; FDA: fluorescein diacetate; PI propidium iodide; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction; EDTA: ethylenediaminetetraacetic acid.

Declarations

Acknowledgments

The results presented herein were extracted from the thesis written by Ms. Shahla Rezaei.

Authors' contributions

ShR and ZM designed the study. ShR and FK were involved in the data collection and analysis. ShR prepared the drafting of the manuscript. ShR, MH and, SD prepared the final draft of the manuscript. ShR, FK, NA, MRH, RY were involved in the design of the study, analysis of the data, and critically reviewing the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The Ethics Committee at Shiraz University of Medical Sciences approved the protocol of the experiment (IR.SUMS.REC.1398.950).

Availability of data and materials

All the data used and/or analyzed during the current study are available from the corresponding author on eligible request.

Conflict of interest

The authors had no conflict of interest to declare.

Competing interests

The authors declare that they have no competing interests.

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Figures

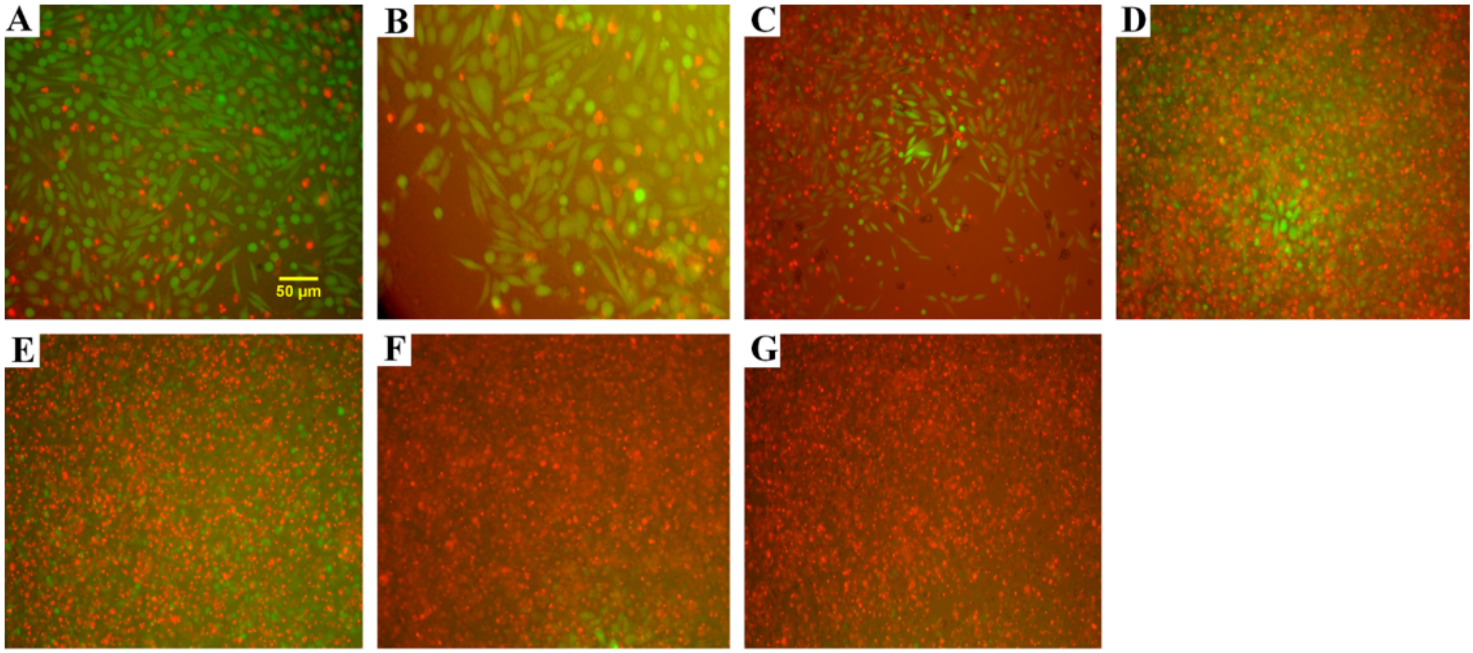


Figure 1

Evaluation of photomicrography by fluorescent staining method. A-G indicate control, tamoxifen 5µmol, extract 400µgr/ml, trypsin inhibitor 200 µgr/ml, trypsin inhibitor 300 µgr/ml and tamoxifen 5µmol+trypsin inhibitor 300 µgr/ml groups by inverted microscopy, respectively.

Green and red areas represent living and dead cells, respectively.

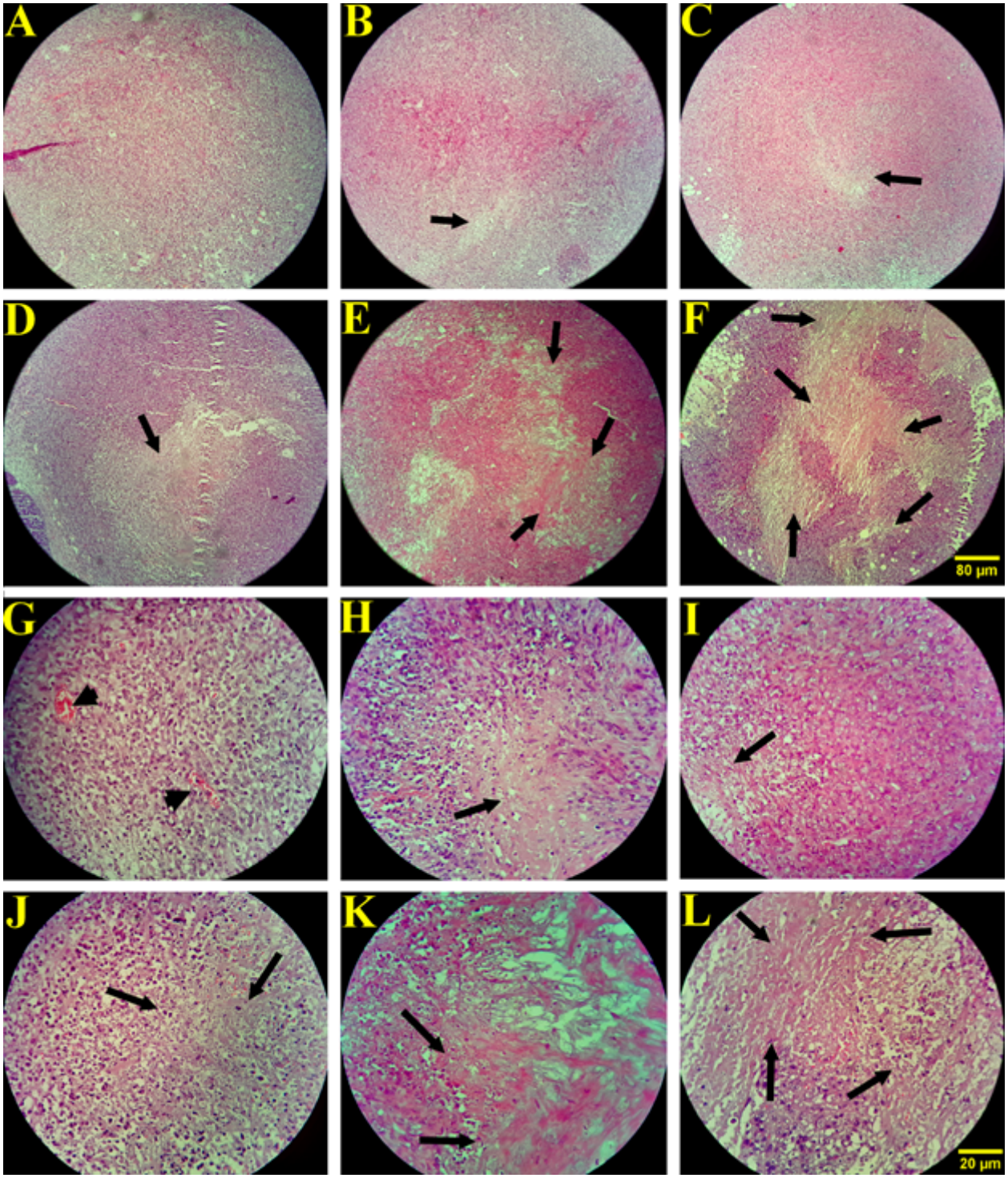


Figure 2

Histopathology evaluation of breast tumor tissue in experimental groups by H&E method (A-F*100, G-L*400 indicate magnification). B&H: breast cancer tamoxifen 10 µmol group; C&I: breast cancer extract 800 µgr/ml group; D&J: breast cancer trypsin inhibitor 300 µgr/ml group; E&K: breast cancer trypsin inhibitor 600 µgr/ml group; F&L: breast cancer tamoxifen 10 µmol+trypsine inhibitor 600 µgr/ml. The white areas represent the shattered nuclei. The arrow sign indicates necrotic areas and the arrowhead indicates blood vessels.

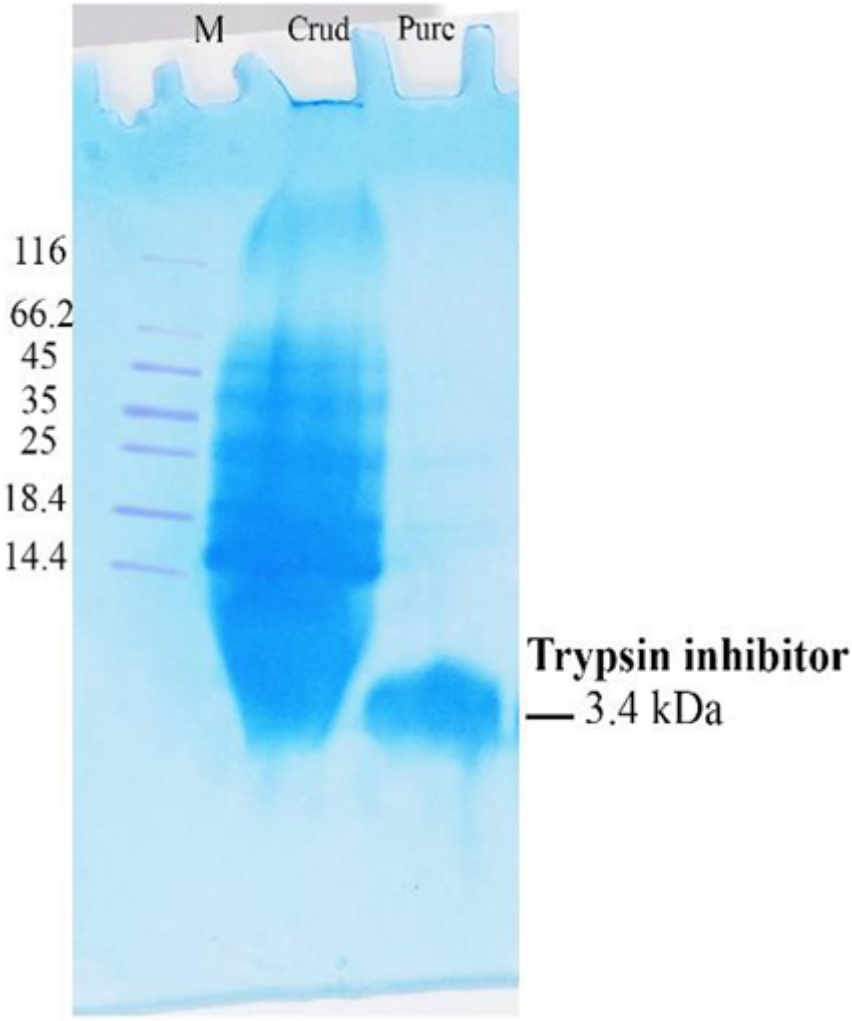


Figure 3

purified trypsin inhibitor from *cucumis melo*

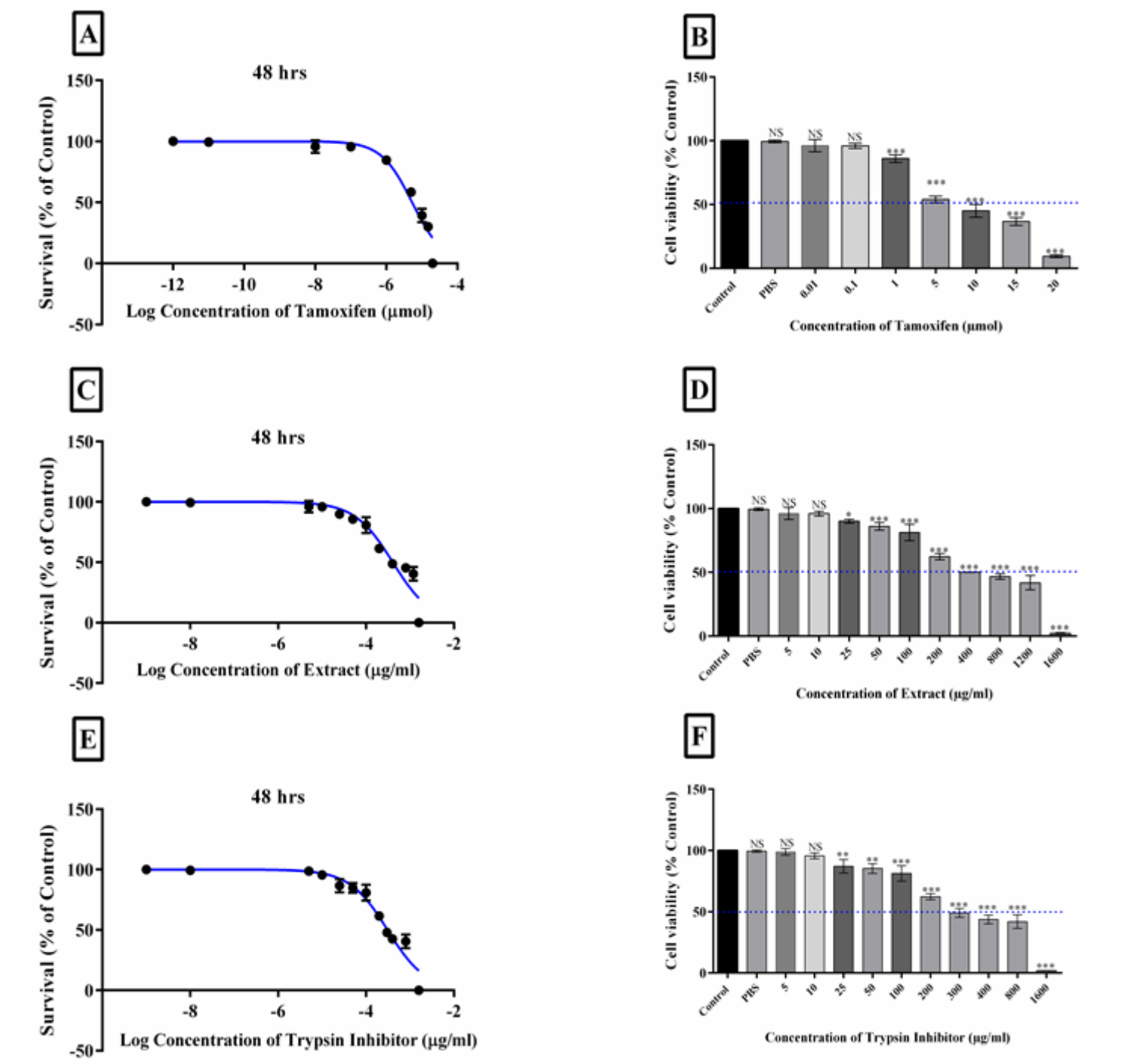


Figure 4

MTT assay of TAM, EXT, and TI on MC4-L2 cell line after 48h: (A, C, E) TAM, EXT and TI inhibited the growth of MC4-L2 cell line at 0.01-20 µmoll, 5-1600 µg/ml respectively. Data are presented as mean ± SD; B, D, F) TAM, EXT, and TI have no cytotoxic effect on MC4-L2 cell line at 0.01-20 µmoll, 5-1600 µg/ml, respectively.

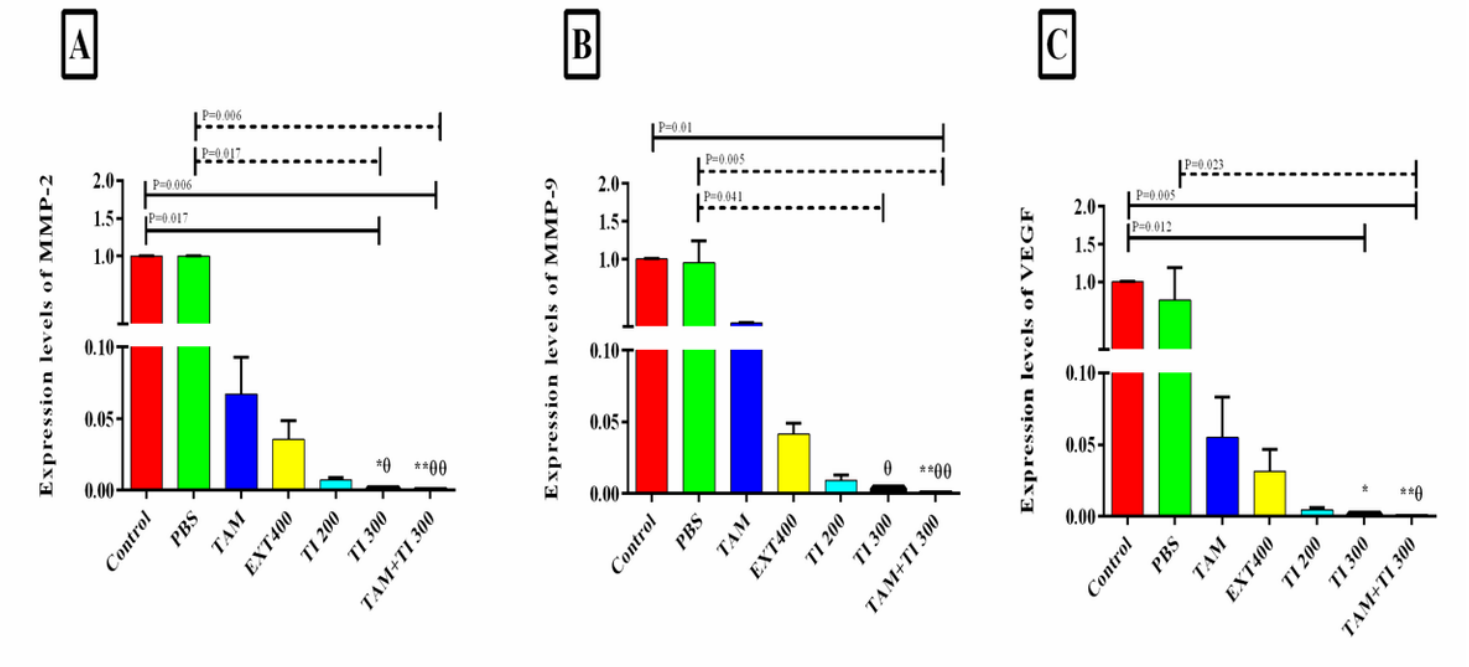
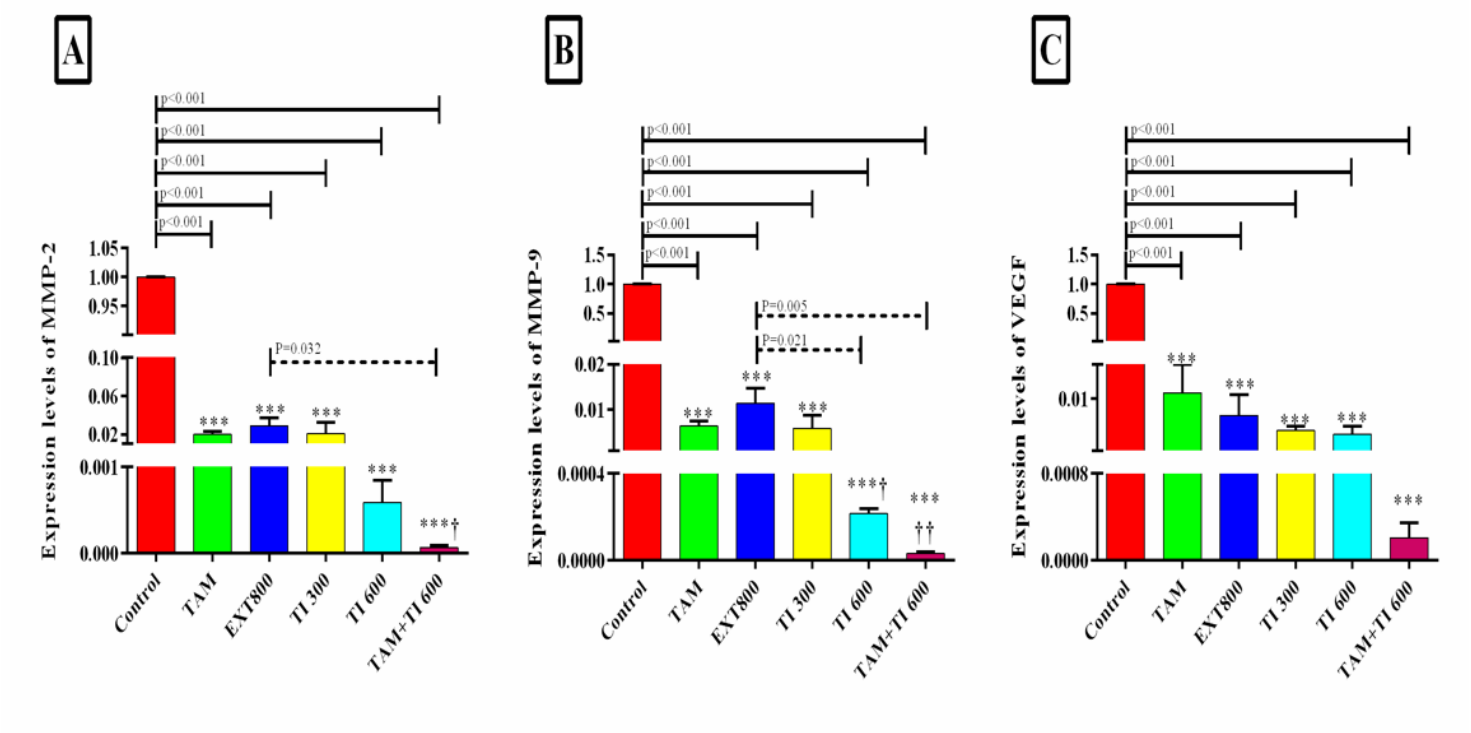


Figure 5

In vitro evaluation of MMP-2, MMP-9, and VEGF secretion by RT-PCR method on MC4-L2 cell line(A–C). Control: Medium culture+MC4-L2 cell line as control group; BPS: Medium culture+MC4-L2 cell line + PBS solution; TAM: Medium culture+MC4-L2 cell line+tamoxifen 5 µmol group; EXT400: Medium culture+MC4-L2 cell line+extract 400 µgr/ml group; TI200: Medium culture+MC4-L2 cell line+trypsin inhibitor 200 µgr/ml group; TI300: Medium culture+MC4-L2 cell line+trypsin inhibitor 300 µgr/ml group; TAM+TI600: Medium culture+MC4-L2 cell line+tamoxifen 5 µmol+trypsin inhibitor 300 µgr/ml. A) *, **: TI300 and TAM+TI300 vs. Con at P<0.05 and P<0.01, respectively; □& □ □: TI300 and TAM+TI300 vs. PBS. B) **: TAM+TI300 vs. Con at P<0.01; □& □ □: TI300 and TAM+TI300 vs. PBS. D) *, **: TI300 and TAM+TI300 vs. Con at P<0.05 and P<0.01, respectively; □: TAM+TI300 vs. PBS. Each data point was presented as mean ± SD.



cancer mice(A–C).

Figure 6

In vivo evaluation of MMP-2, MMP-9, and VEGF secretion by RT-PCR method in breast cancer mice(A–C). Control: breast cancer control group; TAM: breast cancer tamoxifen 10 µmol group; EXT800: breast cancer extract 800 µgr/ml group; TI300: breast cancer trypsin inhibitor 300 µgr/ml group; TI600: breast cancer trypsin inhibitor 600 µgr/ml group; TAM+TI600: breast cancer tamoxifen 10 µmol+trypsin inhibitor 600 µgr/ml. A) ***: All treated groups vs. Con at P<0.001; †: TAM+TI600 vs. EXT800. B) ***: All treated groups vs. Con at P<0.001; †, ††: TI600 and TAM+TI600 vs. EXT800. C) ***: All treated groups vs. Con at P<0.001. Each data point was presented as mean ± SD.

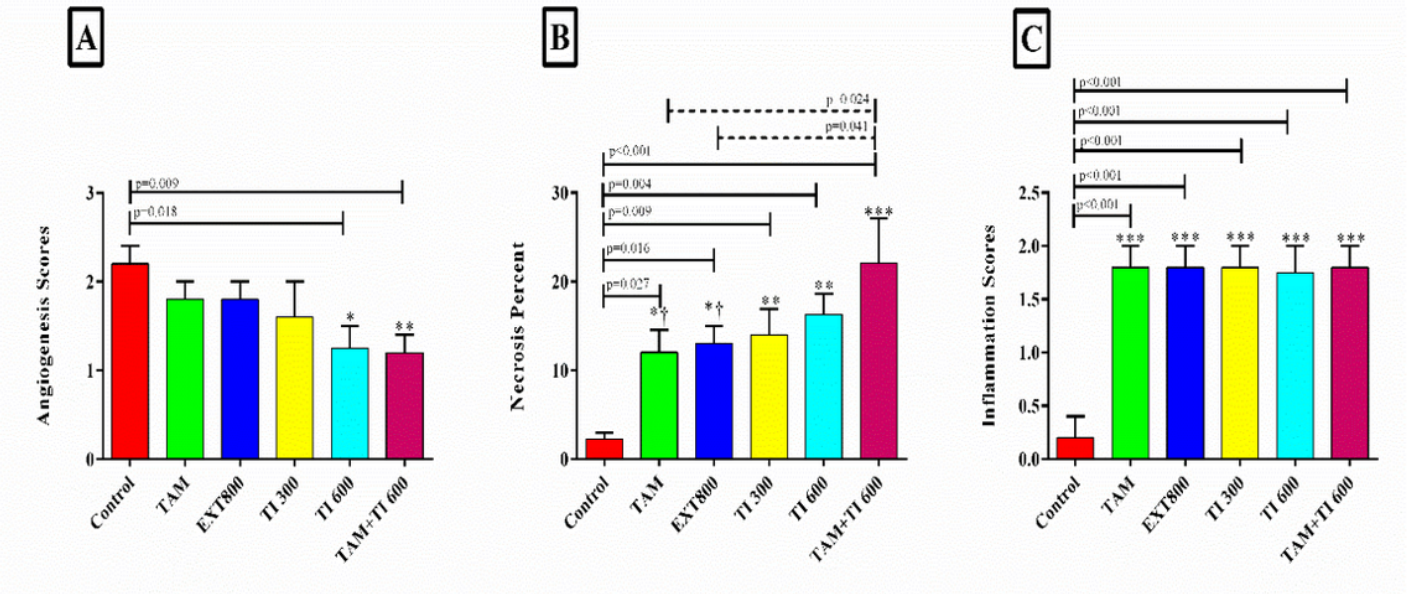


Figure 7

Evaluation of angiogenesis, necrosis, and inflammation of breast tumor tissue (A-C). Control: breast cancer control group; TAM: breast cancer tamoxifen 10 µmol group; EXT800: breast cancer extract 800 µgr/ml group; TI300: breast cancer trypsin inhibitor 300 µgr/ml group; TI600: breast cancer trypsin inhibitor 600 µgr/ml group; TAM+TI600: breast cancer tamoxifen 10 µmol+trypsin inhibitor 600 µgr/ml. A) angiogenesis: *, **: TI600 and TAM+TI600 vs. Con at P<0.05 and P<0.01, respectively; B) necrosis: *, **, ***: TAM, EXT800, TI300, TI600 and TAM+TI600 vs. Con at P<0.05, P<0.01 and P<0.001, respectively; †: TAM+TI600 vs. TAM and EXT800. C) inflammation: ***: All treated groups vs. Con at P<0.001. Each data point was presented as mean ± SD.

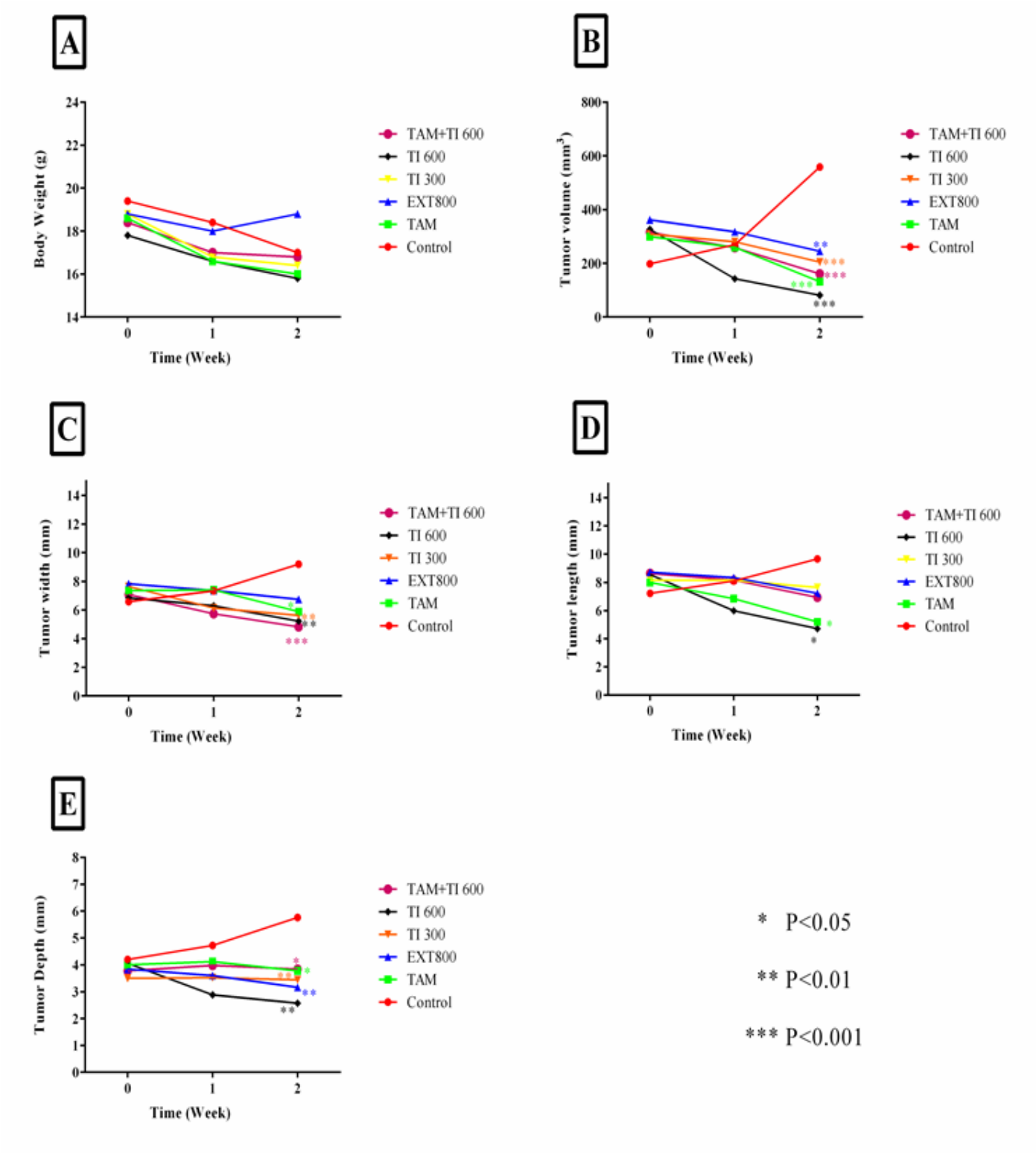


Figure 8

Evaluation of body weight and tumor characteristics in experimental groups. A-E: Control: breast cancer control group; TAM: breast cancer tamoxifen 10 µmol group; EXT800: breast cancer extract 800 µgr/ml group; TI300: breast cancer trypsin inhibitor 300 µgr/ml group; TI600: breast cancer trypsin inhibitor 600 µgr/ml group; TAM+TI600: breast cancer tamoxifen 10 µmol+trypsin inhibitor 600 µgr/ml. A) *: All treated groups vs. Con at P<0.05; B) **, ***: TAM, EXT800, TI300, TI600 and TAM+TI600 groups vs. Con at

P<0.01 and P<0.001, respectively; C) *, **, ***: TAM, TI300, TI600 and TAM+TI600 vs. Con at P<0.05, P<0.01 and P<0.001, respectively; D) *: TAM and TI600 vs. Con at P<0.05; E) *, **, ***: TAM, TI300, TI600 and TAM+TI600 vs. Con at P<0.05, P<0.01 and P<0.001, respectively. Data are presented as Mean ± SD.

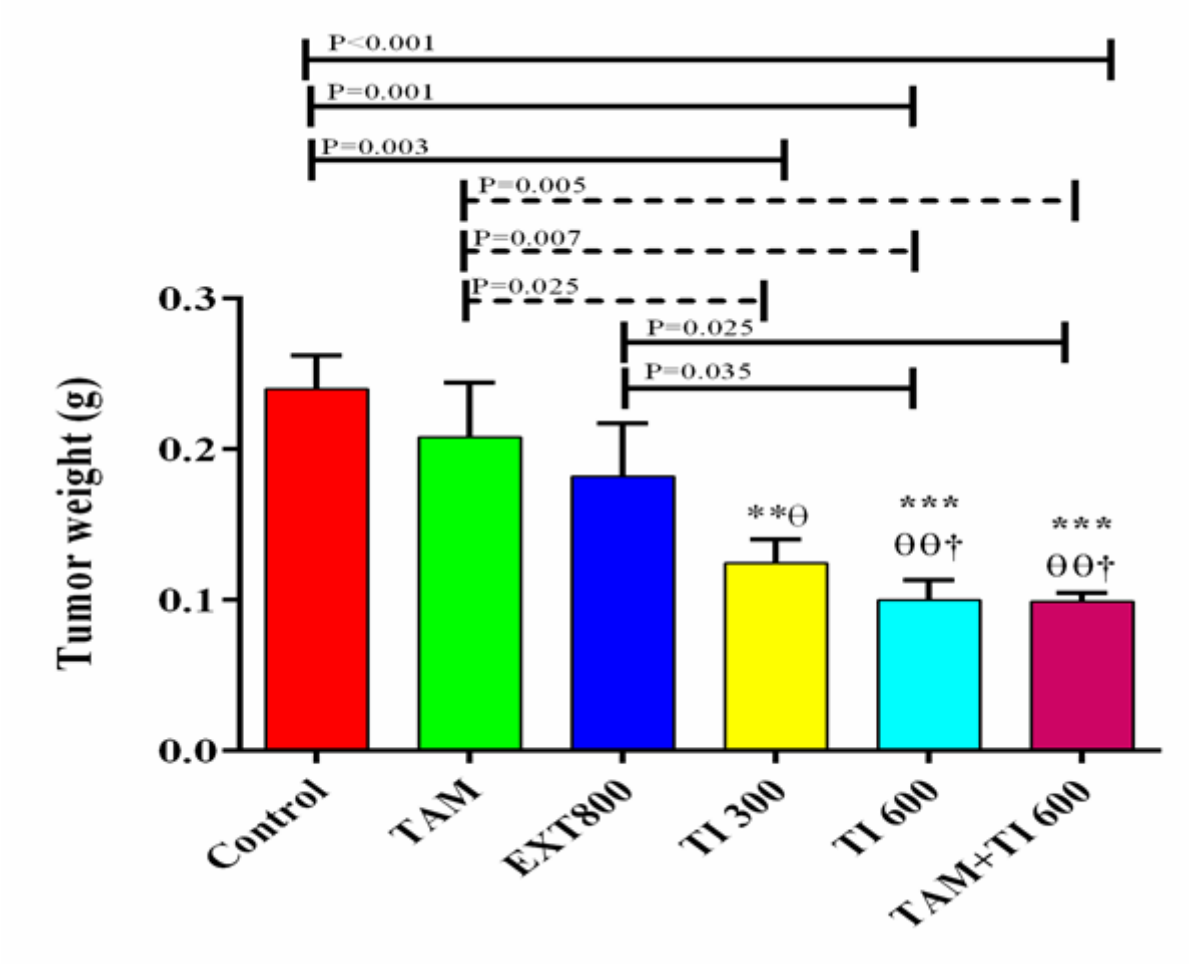


Figure 9

Evaluation of tumor weight in experimental groups at the end of the interventions. Control: breast cancer control group; TAM: breast cancer tamoxifen 10 µmol group; EXT800: breast cancer extract 800 µgr/ml group; TI300: breast cancer trypsin inhibitor 300 µgr/ml group; TI600: breast cancer trypsin inhibitor 600 µgr/ml group; TAM+TI600: breast cancer tamoxifen 10 µmol+trypsin inhibitor 600 µgr/ml. **: All treated groups vs. Con; □, □ □: TI300, TI600, and TAM 10µmol+TI600 groups vs. TAM; †: TI600 and TAM+TI600 groups vs. EXT800. Data are presented as Mean ± SD.

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