RESEARCH ARTICLE

Editorial Process: Submission:04/11/2022 Acceptance:03/08/2022

Evaluation of the Anti-Metastatic Effect of Foeniculum Vulgare on the Protein Expression of HSP 70 & 90 in Balb/c Mice with 4t1 Model of Breast Cancer

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Abstract

Introduction: Breast cancer is one of the important factors of cancer-related deaths. Considering the drug resistance, special attention has been paid to natural compounds. This study aimed at evaluating the anti-metastatic activity of fennel in a breast cancer mouse model. **Methods:** A total of 35 adult female BALB/C mice were used in this study. Breast cancer was induced by subcutaneous injection of 4T1 cells in the right lower flank. The mice received fennel extracts daily via intraperitoneal injection for two weeks. Meanwhile, tumor volume was measured every day using calipers. After two weeks, each animal was anesthetized. The protein expression of HSP 70 & 90 was measured in liver tissue and ovary. The expression of her2 was measured in tumor tissue. The activity of Glutathione peroxidase and reductase as anti-oxidant agents were measured in serum. **Results:** Tumor size significantly decreased after nine days' treatment of the fennel. The expression of HER2 increased in the tumor tissue and decrease with different dose of fennel. Fennel treatment caused a decrease in the protein expression of HSP 70 & 90 in the liver tissues. **Conclusion:** Based on our findings, fennel has anti-tumor and anti-metastatic activities against aggressive cancers.

Keywords: Foeniculum vulgare- fennel- trans-anethole- breast cancer- HSP 70- HSP 90- Her2

Asian Pac J Cancer Prev, **24 (3)**, 833-840

Introduction

Breast cancer is one of the leading causes of cancer related mortalities noticed in women (Rampogu et al., 2019). Still, despite the wide clinical efficacy of current treatments, they're ineffective in cases that acquired remedial resistance and there's a clear need for the development of new remedial agents with new mechanisms of action(Mendis et al., 2015). A number of natural agents modulating different signal transduction pathways are presently in clinical evaluation (Zagouri et al., 2013).

Human epidermal growth factor receptor type2 (HER-2) was surfaced as a molecular

biomarker in breast cancer. It's located on chromosome 17q21.1 and is a member of the type I growth factor receptor family (Krishnamurti and Silverman, 2014) In breast cancer, HER-2 is over-expressed in 20 – 30 of primary malignancies (Ishikawa et al., 2014). According to the original report, HER-2 gene modification could singly prognosticate the time of complaint relapse and overall survival in breast cancer cases (Winstanley et al., 1991). Along with estrogen receptor (ER), HER-2 is an important gene for molecular purpose treatment of breast cancer. Therefore, as a predictor of the effectiveness of HER-2-targeted remedy, it is discussed in many studies (Ishikawa et al., 2014). HER-2 is useful in treatment of breast cancer; as a target of HER2 inhibitors and a predictor of the effectiveness of anti-cancer agents (Ishikawa et al., 2014). Inhibition of the expression of HER-2 is need for treating breast cancer in HER-2 subtype. Now medicines that target HER-2, like trastuzumab and lapatinib, are successfully used in clinical treatment, although the long term outgrowth is still unknown (Ishikawa et al., 2014).

Oxidative stress may play an important role in onset and progression of breast cancer (Lee et al., 2017). According to importance of subject that links reactive oxygen species (ROS) to the different phases of tumorigenesis, many studies have reported that ROS scavenging systems are important targets for cancer treatment (Hecht et al., 2016). Some of medicines approved for breast cancer that involved in ROS homeostasis, target glutathione (GSH) metabolism that plays a key role in preventing oxidative stress in human cells (Hecht et al., 2016). Glutathione peroxidase GPx utilizes GSH as a cofactor to reduce

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hydrogen peroxide, resulting in the formation of oxidized glutathione and protect against oxidative stress (Lubos et al., 2011). Glutathione Reductase (GR) is a NADPH-dependent flavoprotein that catalyzes the reduction of glutathione disulfide (GSSG) to GSH (Trivedi et al., 2013). GSH as a key signal transduction molecule is a controller of cell differentiation, proliferation, apoptosis and immune function and plays an important role in cancer cell metabolism and tumor microenvironment. So molecular changes in the GSH antioxidant system have been implicated in tumor initiation, progression, and treatment response (Kennedy et al., 2020). It is reported that GPx prevent oxidative DNA mutations (Baliga et al., 2007).

Heat shock proteins (HSP) are members of the molecular chaperones, that play important role in the folding of a large number of cellular proteins (Zagouri et al., 2012). They were originally discovered as moderators of resistance to hyperthermia (Calderwood and Ciocca, 2008). Also, they share directly in cell survival during hyperthermia by inhibiting apoptosis (Tang et al., 2005). Mitochondria retain a class of HSPs that all of them playing a crucial role in cancer progression (Leav et al., 2010). Hsp90 is an abundant protein in mammalian cells (Lai et al., 1984). Hsp90 interacts with other proteins that involve in breast neoplasia; including estrogen receptors (ER), tumor suppressor p53 protein, antiapoptotic kinase Akt, Raf-1 MAP kinase and a variety of receptor tyrosine kinases, like HER2 (Beliakoff and Whitesell, 2004).

HSP90 inhibitors suppress many oncogenic signaling pathways simultaneously, and reduce the possibility of mutations which lead to tumor resistance (Maloney and Workman, 2002; De Mattos-Arruda and Cortes, 2012). Specially, in the HER2-positive breast cancer type, data have showed that the anti-tumor activity of HSP90 inhibitors, has had promising results (Basso et al., 2002; Workman et al., 2007).

Foeniculum vulgare as the member of Apiaceae family with common name of fennel is well-known in traditional medicines (Mahboubi, 2019). Fennel with hot and dry nature is used as diuretic, stomachache, chronic fever, kidney stones, chronic diarrhea, vomiting, and urinary diseases (Amini et al., 2016). Studies have shown that various fennel extracts had anti-cancer effects on different kind of tumors such as skin and prostate cancer (Ke et al., 2021). However, the anti-tumor activity of Foeniculum vulgare and its underlying molecular mechanisms are unknown.

In this study we evaluated the anti-cancer property of fennel and its effect on oxidative stress and HSP proteins.

Materials and Methods

Animals

In this experimental study, 35 healthy adult female BALB/C mice that weighed 20-22 g were kept on a 12-hour light/dark cycle at 22 ± 2°C and fed with standard pellets and water adlibitum. All animal experiments were conducted in accordance with national guidelines and protocols after approval from the Institutional Animal Ethics Committee. All experimental protocols were approved by the Iran University of Medical Sciences (IUMS) Ethics Committee. The ethical number of this project is "IR.IAU.SRB.REC.1398.038".

Breast cancer model

To obtain a model of breast cancer in situ, female BALB/c mice were challenged with 4T1 cells (that were provided by Pasteur Institute). Briefly, 4T1 cancer cells were cultured in 12 mL DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic (penicillin/streptomycin/fluconazole), using T75 culture flasks, and incubated (37°C, 5% CO_2) until reaching 50-80% confluence. Cells were washed with 7 mL of serum-free medium (SFM), then dislodged using 3 mL of 0.25% trypsin/1 mM EDTA solution at room temperature for 2 min. Later, 7 ml of SFM was used to harvest the trypsinized cells from the flasks and then transferred into 15 mL conical tubes and centrifuged at room temperature (4 min; 112.7 g). The supernatant was then discarded and the pellet resuspended with SFM.

5×10^5 4T1 cells were injected subcutaneously on the right lower flank. After one week, when the tumor mass was detectable, fennel treatment was started. Animals received daily fennel extracts via intraperitoneal (IP) injection for 2 weeks. Meanwhile, the tumor volume was measured every day using calipers and calculated using the following formula: $(\text{length}\times\text{width}^2)/2$ (mm^3).

Preparation of fennel extracts

Fennel seeds were purchased from the Isfahan Seed Packers Company. Fennel seeds must be one-year-old and healthy. The seeds were ground into a powder by a grinder for 6 min. And 150 ml of distilled water was added to 2 gr of fennel powder and were placed on the heater. When the solution volume reached 90 ml, it was passed through sterile gas. In order to purify the extract, it was poured into falcon tubes in equal volumes and placed in a centrifuge machine for a period of 15 minutes with 4400 RPM. Then the aqueous extract was used for injection. The extract was prepared daily and fresh.

Treatment

Animals were divided 35 mice into five groups (n = 7 per group):

(i) Control: Cancer mice with no treatment.

(ii) Sham (Dw): Cancer mice that received solvent of fennel (Distilled water).

(iii) Fennel 50 (F50): Mice were treated with fennel (50 mg/kg; IP).

(iv) Fennel 100 (F100): Mice were treated with fennel (100 mg/kg; IP).

(v) Fennel 200 (F200): Mice were treated with fennel (200 mg/kg; IP).

At the end of 2-week period of the experiment each animal was anesthetized with an IP injection of ketamine (45 mg⁄kg) and xylazine (35 mg⁄kg) mixture. Tumor tissues and ovary were dissected and subjected to further analyses.

Measurement of Her2 gene expression by Quantitative Reverse Transcription PCR (QRT-PCR) Analysis

Samples were weighed (range of 30-50 mg) and RNA

was extracted using the RNx plus (Sinaclon, Tehran, Iran), according to the manufacturer's protocol, and resolved in 50µl RNase-free water. RNA was analyzed by Nano-drop to define their concentration and purity. The denaturing gel electrophoresis method was used to test the RNA integrity.

RNA was treated with DNase I (EN0521, Fermentas,Opelstrasse, Germany) to eliminate any DNA contamination. Purified RNA samples were converted into cDNA. cDNAs were synthesized with 1µg of RNA, 0.5 µL of oligo dTs and 0.5 µL of random hexamer using cDNA Synthesis Kit (PrimeScript RT Master Mix, TAKARA, Kyoto, Japan). All procedures were based on the manufacturer's protocol. 1 µg of synthesized cDNA used for SYBR Green-based real-time RT-PCR via 2X qPCR kit (RR820L, Tli RNaseH Plus, TaKaRa, Kyoto, Japan). The primer pairs used in this study are indicated in Table1. Thermocycling parameters were as follows: initial denaturation at 95°C for 30 s, 40 cycles of 95°C for 5 s and annealing and elongation at 60°C for 30 s. Values from β-actin was used to loading normalization for each sample. Relative changes expression was determined using the ΔΔCt method relative to gene expression values for control mice.

Measurement of GR & GPx Activities by Enzyme-linked Immunosorbent Assay (ELISA)

Activities of serum GR and GPx measurement were done based on colorimetric assay with microplate reader (Stat Fax, Awareness, USA) using ZellBio GmbH assay kits (Ulm, Germany), according to the manufacturer's protocol.

Immunofluorescence (IFS) Analysis

The embedded tissues in OCT, serially sectioned (10 µm thickness) in the coronal planes using the floor standing fully automatic cryostat, MNT-SLEE (Mainz GmbH, Germany), and collected onto poly-L-lysine coated coverslips and used for tissue analysis. The sections were air dried at room temperature for 30 min. The sections were then rehydrated in 0.1 M PBS twice (10 min each), incubated in blocking solution (0.1 M PBS containing 2% bovina serum albumin (BSA), 1% normal goat serum and 0.2% Triton X-100), and afterwards incubated for 1 h in permeabilization buffer (10% goat serum, 0.1% Triton X-100 in PBS).

Then sections incubated with the appropriately primary antibody at 4°C overnight followed by washing and further incubation with secondary antibodies for 2 hours in antibody solution (5% goat serum, 0.05% Triton X-100 in PBS). Primary antibodies included mouse monoclonal antibodies to the Hsp70 and 90 (1:500; bio-rad). The secondary antibodies (Santa Cruz Biotechnology) used to detect Hsp70 and 90 immunostaining was FITC conjugated goat anti-mouse IgG (1:200).

All sections were counterstained with DAPI (2 µg/ml) to visualize the nuclei. Negative controls were obtained by omitting either the primary or secondary antibody and gave no signal (data not shown). All analysis was examined using a fluorescence microscope (Nokia), and images were captured using a digital camera (Zeiss) (Sanadgol et al., 2010; Ramroodi et al., 2015; Sarvandi et al., 2015).

Statistical analysis

All data were expressed as mean ± standard error (SEM). The one-way ANOVA test was applied to clarify significant differences between groups. When a significant effect was found, the Duncan post hoc U test was performed. To compare tumor volume, Repeated Measures test was used to clarify significant differences between groups. All analyses were performed using SPSS version 23. The statistical significance level was set at $p \leq 0.05$.

Results

Effect of funnel Extract on tumor volume

The tumor volume was measured every day using calipers. The volume of tumor was significantly decreased in some different dose of fennel from ninth day to day twelfth (Figure 1).

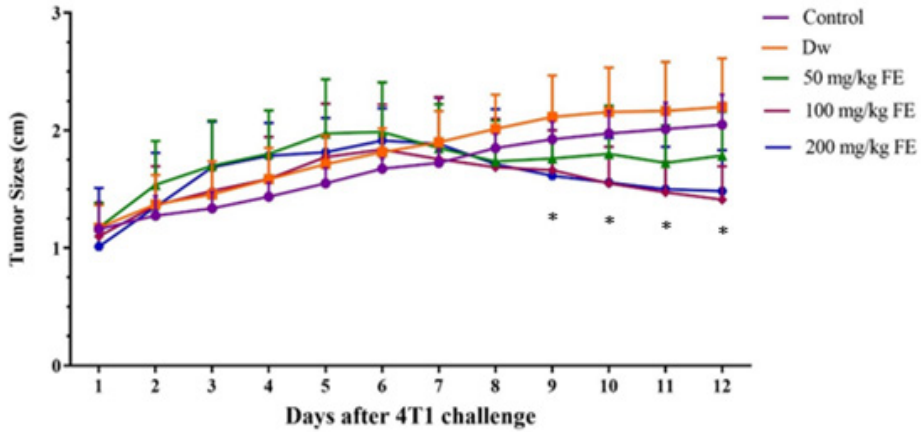

Figure 1. The Tumor Volume was Measured Every Days Using Calipers. The volume of tumor was significantly decreased in some different dose of fennel from ninth day to day twelfth. Data are presented as means ± SEM. *P < 0.05 compared to control.

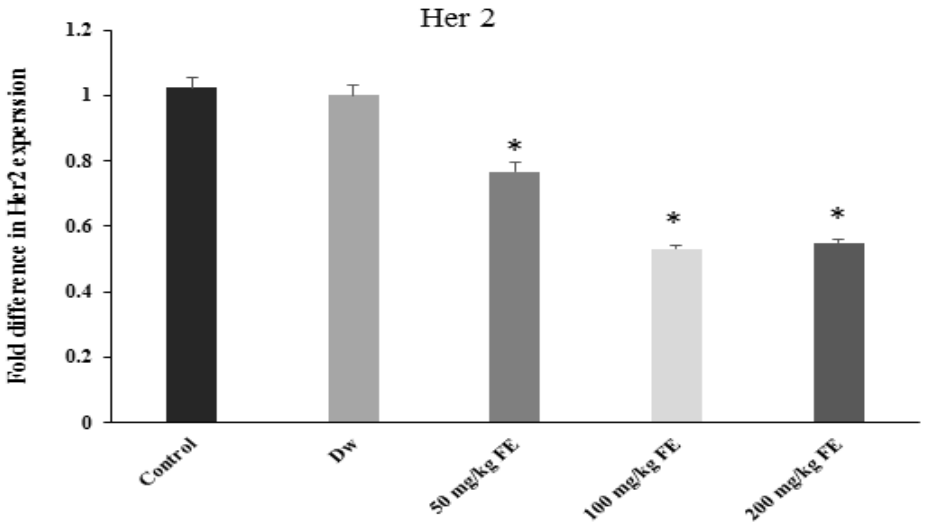

Figure 2. Evaluation of Effect of Fennel in mRNA Expression of Her2 by q-PCR Method in Tumor Tissue. Quantification of results shows that fennel significantly decreased mRNA expression of Her2 in 50,100 and 200 mg/kg of fennel. Data are presented as means ± SEM. *P < 0.05 compared to control.

Effect of funnel Extract on Her2 Gene Expression in tumor tissue

Effect of fennel on mRNA expression of HER2 by q-PCR method in in tumor tissue was evaluated. Quantification of results shows that fennel significantly decreased mRNA expression of HER2 in different dose of fennel (Figure 2).

Table 1. Sequence of Specific Primers Used for Quantitative Real-Time Revers Transcription PCR

Her2-F	GGCTTGGTCTGTAACTCACTG
Her2-R	TTCCATACTCGGCACTCCTC
bactin-F	TGAAGATCAAGATCATTGCTCCTC
bactin-R	TCAGTAACAGTCCGCCTAGAAG

Effect of funnel Extract on Serum Glutathione Peroxidase & Reductase

Evaluation of the effects of fennel on GPx & GR were evaluated. In our study the level of serum GR were significantly increase in dose of 100 and 200 mg/kg of fennel. But fennel couldn't increase GPx in all treated group (Figure 3).

Effect of funnel Extract on HSP70 & HSP90 protein Expression in Liver

Effect of fennel on HSP70 & HSP90 protein expression was investigated by IHC. It shows that fennel significantly decreased expression of HSP70 & HSP90 protein expression in 50, 100 and 200 mg/kg groups. A; Control, B; Sham, C; 50 mg/kg D; 100 mg/kg, E; 200 mg/kg. Magnification: 400× (Figures 4 and 5).

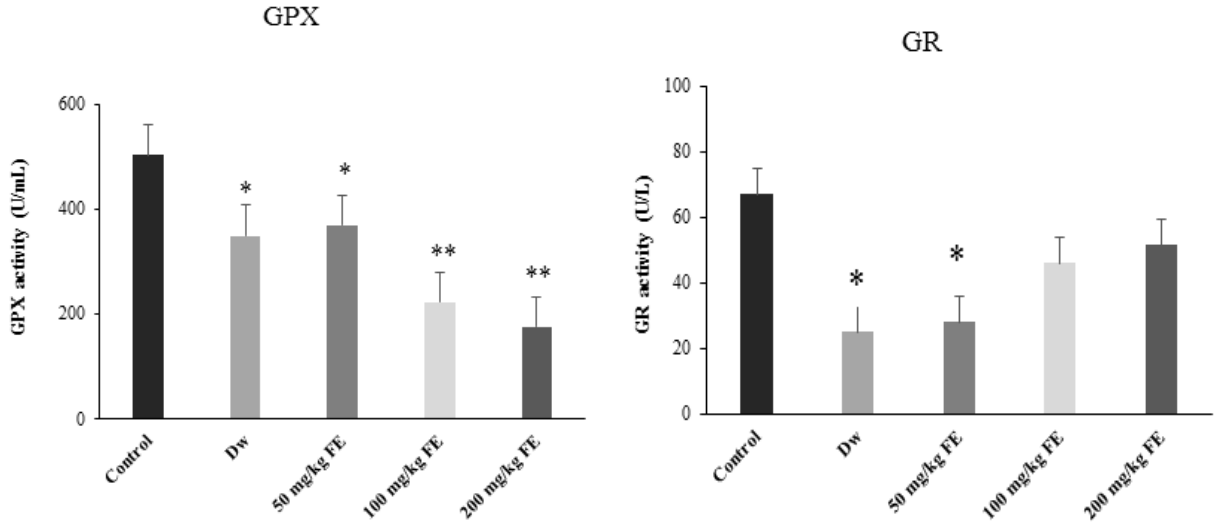

Figure 3. Evaluation of the Effect of Fennel on GPx & GR. Fennel couldn't increase GPx in all treated group. But after treatment of fennel, mean GR levels were significantly increased in dose of 100 & 200 mg/kg to control group. Data are presented as means ± SEM. *P < 0.05 compared to control.

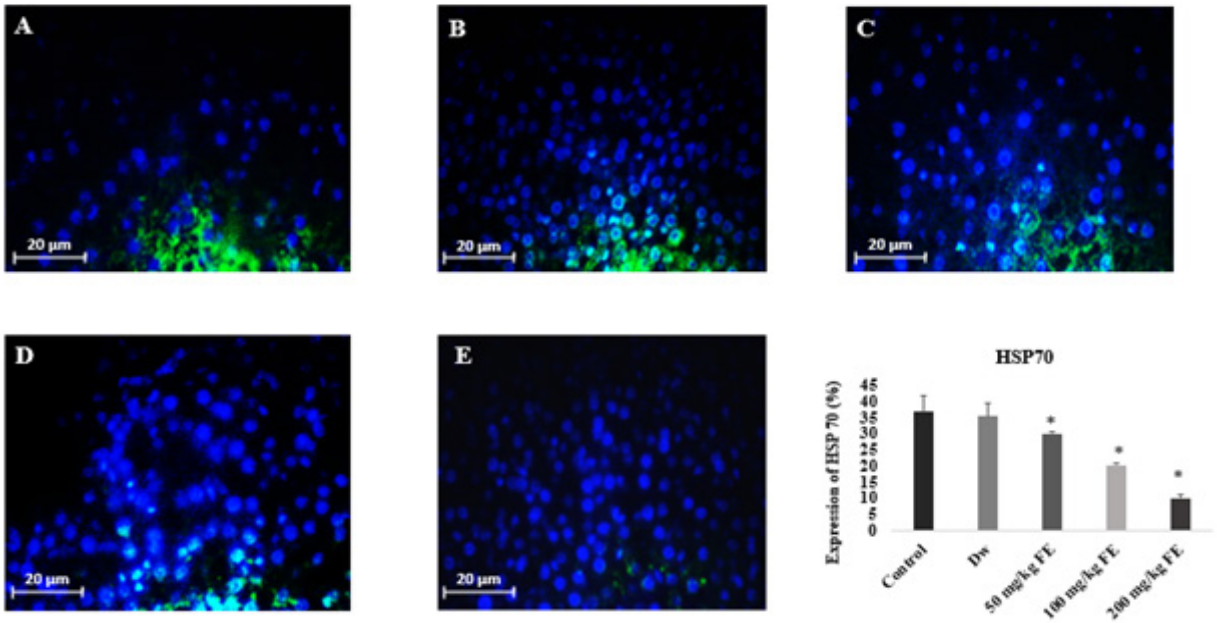

Figure 4. Evaluation of the Effect of Fennel on HSP70 Protein Expression. A significant decrease was observed in all treatment groups. Data are presented as means ± SEM. *P < 0.05 control.

Discussion

In the present study, treatment with different doses of fennel could decrease tumor volume in different dose of fennel from ninth day. The expression of Her2 increased in the group of cancer and decreased with different dose of fennel. In our study the level of serum GR were significantly increase in dose of 100 and 200 mg/kg of fennel. But fennel couldn't increase GPx in all treated group. The protein expression of HSP 70 & 90, significantly decreased with different dose of fennel compared to control group.

The present study confirmed the efficiency of fennel in improving breast cancer. The anti-proliferative effects of this plant were reported previously in breast cancer. Our results indicated the protective effects of fennel as an anti-cancer via decreasing oxidative stress and HSP proteins.

Breast cancer different subtypes, each with diverse behaviors, distinct genetic and various responses to treatment (Perou et al., 2000). Current therapies for breast cancer is on the basis of molecular tumor subtypes that have been defined by the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 (De Mattos-Arruda and Cortes, 2012). Breast cancer that overexpress HER2 are more aggressive and associated with poor

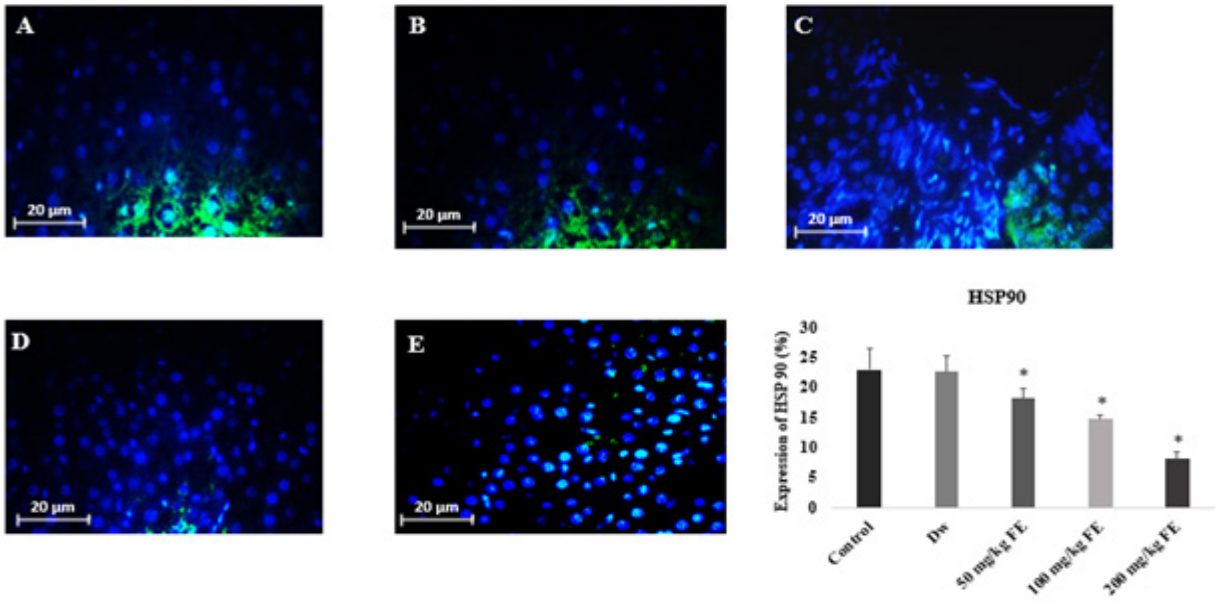

Figure 5. Evaluation of the Effect of Fennel on HSP70 Protein Expression. A significant decrease was observed in all treatment groups. Data are presented as means ± SEM. *P < 0.05 compared to control.

prognosis (Slamon et al., 1989). Multiple HER2 targeted drug have been in recent years. In this model of breast cancer that we induced with 4T1 cell line, the expression of Her2 was increased in tumor tissue. Treatment with different dose of fennel decreased the expression of Her2. In our previous study we showed that expression of Her2 decreased in ovarian tissue with the same doses (Mehralikhani et al., 2021). Chandrika et al reported that flavonoids such as Naringenin and Hesperetin inhibited in vitro HER2- Tyrosine Kinase activity; supporting that flavonoids can improve HER2 positive breast cancer (Chandrika et al., 2016). Babu et al showed that the compound ZINC67903192 can identify as HER2 inhibitor against gastric cancer (Babu et al., 2016).

Clinical studies have confirmed the involvement of ROS in carcinogenesis (Franco et al., 2008). Many naturally compounds from plant sources, have been identified as free radical scavengers (Duh, 1998; Oktay et al., 2003). Phenolic compounds and flavonoids have been shown that had antioxidant activity (Hussain et al., 2016; Crescenzi et al., 2021). In alternative medicine, fennel is recognized as a medicinal plant due to its bioactive constituents (Syed et al., 2018). Fatty acids, flavonoids, phenols, polyacetylenes, and metabolites have been isolated from fennel (Choo et al., 2011). Trans-anethole, fenchone, alpha-phellandrene, and estragole are the most abundant constituents of fennel. Trans-anethole is the most important component of this herbal medicine, accounting for almost 80% of its content (Cabral et al., 2017; Akhbari et al., 2018). In fact, the presence of anethole (an aromatic compound) may explain the anti-cancer potential of fennel seeds (Akhbari et al., 2018). The biological effects of anethole, including anti-platelet, antioxidant, and anti-tumor activities, have been confirmed in previous studies (Rajakumar and Rao, 1993; Ko et al., 1995; Nakagawa and Suzuki, 2003; Choo et al., 2011). Anwar et al., (2009) recorded that anethole, the main component of fennel essential oil, showed antioxidant, antibacterial, and antifungal activities. Ragaa et al., (2011) reported that fennel seed methanolic extract exhibited an antitumor effect by modulating lipid peroxidation.

In our study the level of serum GR were significantly increase in dose of 100 and 200 mg/kg of fennel. But fennel couldn't increase GPx in all treated group. The decrease in GSH concentration can be explained by a decrease in GSH synthesis or an increase in GSH consumption in the removal of peroxides and xenobiotics.

Pala et al showed that fennel essential oil could improve chlorpyrifos-induced toxicity in *C. carpio*, and increase catalase and GPx activities (Pala et al., 2021). Sadeghpour et al., (2015) reported that fennel extract at concentration of 100 and 200 mg/kg can significantly increase the serum level of antioxidant markers such as superoxide dismutase, Gpx and total antioxidant capacity in mice.

Heat shock proteins are known as important molecules in cancer development and are good targets in cancer therapy (Mendis et al., 2015). HSP 70 and 90 have been reported to be over expressed in different types of breast cancer cells (Rusak et al., 2002; Lee et al., 2006; Wei et al., 2011). Their expression often is accompanied by increased cell proliferation, lymph node metastasis, poor response to chemotherapy and poor survival (Ciocca and Calderwood, 2005). Due to the ability of HSP90 in prevention of signaling pathways involved in oncogenesis, inhibition of it has a great potential in breast cancer treatment (Zagouri et al., 2013). It is well documented that the Hsp90 inhibitors degrade the HER2 and regulates the signaling of estrogen and progesterone receptor signals (Bagatell et al., 2001; Zagouri et al., 2012). Several synthetic small molecule HSP90 inhibitors such as AUY922A, BIIB021, and SNX2112 are the more recent compounds studied (Zagouri et al., 2013).

Metastasis is a complex process, in which changes in some of genes are accompanied with the gain of a metastatic properties (Prensner et al., 2014). Elevated expression of each of the HSPs promotes metastasis strongly (Calderwood and Gong, 2016). An increase in Hsp90 is associated with metastasis largely due its ability to chaperone focal adhesion kinase, integrin linked kinase and the receptor tyrosine kinases ErbB2 and MET (Tsutsumi et al., 2009). It seems that MET is important for HSPs in cancer and Hsp70 inactivation causes decline in MET expression (Miyajima et al., 2013). Hsp27 can effect on the epithelial-mesenchymal transition in favors of metastasis, process in which cells switch from a compact shape to a spindle shape and gain enhanced motility (Gibert et al., 2012; Pavan et al., 2014).

In our study liver metastasis of breast cancer was observed. Liver HSP 70 & 90 protein expression were increased, and different dose of fennel could decrease them. It has been reported that natural anticancer compounds mediate their actions via several mechanisms including HSP inhibition (Amolins and Blagg, 2009). Amolins et al pursued that the phytochemical compounds are inhibitors for Hsp90 (Rampogu et al., 2019). Recent investigations have demonstrated that inhibition of breast cancer and cell proliferation by certain plant such as curcumin and quercetin (Rusak et al., 2002), may in part be mediated by inhibition of HSP expression. Mendis et al., (2015) reported that inhibition of HSP 70 and 90 expression by the F. leucopyrus decoction was observed in MCF-7 and MDA-MB- 231. Zagouri et al., (2013) reported that Hsp90 inhibitors include the natural products geldanamycin and radicicol, as well as their synthetic analogue 17-AAG, 17-DMAG, retaspimycin hydrochloride and KF58333 2 are the more recent compounds studied. Apoptosis is resistant to high expression of HSP (Mendis et al., 2015). HSP 70 or 90 binds to the Apaf-1, thereby inhibiting caspases activation and apoptosis (Mendis et al., 2015). HSP70 has also been shown to block caspase independent cell death through its association with apoptosis inducing factor (Jego et al., 2013). In this study different dose of fennel could decrease liver HSP 70 & 90 protein expression and improved breast cancer.

In conclusion, fennel extract in the treatment of breast cancer can be useful. This action is probably through the antioxidant mechanism, inhibition of the expression of cancer-resistant gene; Her2 and decrease in expression of HSP 70 & 90. Further studies of these pathways, may open avenues to explore novel drug targets to limit cancer progression.

Author Contribution Statement

Leila Sadegh Roudbari, Maryam Eslami, Monireh Movahedi conceived and designed the study. All authors contributed to performing the relevant experiments. Leila Sadegh Roudbari, Fereshteh Golab analyzed the data. Dr. Fereshteh Golab wrote the paper. All authors contributed to reviewing and editing the manuscript..

Acknowledgements

Funding Statement

This research was supported by grant No: "99-01-117-22043" from "Iran University of Medical Sciences". Tehran, Iran. It is part of an approved student thesis.

Ethical Approval

All animal experiments were conducted in accordance with national guidelines and protocols after approval from the Institutional Animal Ethics Committee. All experimental protocols were approved by the Iran University of Medical Sciences (IUMS) Ethics Committee. The ethical number of this project is "IR.IAU.SRB.REC.1398.038".

Availability of Data

Data are available by request to the corresponding author.

Conflict of Interest

The authors declare no potential conflicts of interest.

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