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Original Article

The Role of Fennel Seed Extract on the Expression Pattern of Dysadherin, E-Cadherin and Ki67 in Metastatic Lung Cancer in BALB/C Female Mice

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Abstract

Background and Aim: Some herbs have anti-cancer properties such as the anticancer potential of fennel methanolic extract. Metastasis is an undesirable phenomenon that occurs in most cancers. Inhibition of metastatic proteins by fennel extract can increase survival chance.

Materials and Methods: Thirty-five adult BALB / C mice were divided into five groups. Four groups were infected with 4T1 cells and one group was considered as healthy control. After one week, three groups were treated with an aqueous extract of fennel seed. The mice were anesthetized after two weeks, and the lung tissues were removed**.** The expression of Ki67 and dysadherin was evaluated using the qRT-PCR. Moreover, we examined E-cadherin protein expression using the IHC method.

Results: Ki67 expression decreased at 100 mg/kg dose (Kruskal-Wallis test: $P<0.05$) but revealed a remarkable increase at a 200 mg/kg dose ($p<0.05$). Dysadherin expression significantly decreased at all the three doses (ANOVA test: $p <0.05$). E-cadherin showed non-significant expression (ANOVA test $P>0.05$).

Conclusion: A 100 mg/kg dose could noticeably decrease the expression of dysadherin and Ki67. The dose of 200 mg/kg can cause a bad prognosis.

Keywords: Ki67, Dysadherin, E-cadherin, Lung cancer, Fennel seed

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Introduction

Cancer is one of the most significant health problems throughout the world. In this second deadly disease all over the world, uncontrolled cellular proliferation leads to undesirable consequences (1, 2). Healthy cells are also damaged in chemotherapy treatment, which is also considered an expensive treatment (3). Some herbs have anti-cancer properties due to having some compounds such as steroids, amino acids, carbohydrates, and saponins with few side effects (4). The fennel herb, which is known scientifically as *Foeniculum vulgare*, belongs to the Apiaceae family. Moreover, it is one of the herbs evaluated in this research and previous studies (5). The seeds of this herb are used in processing and flavoring of food, beverages,

and drugs (6, 7). Another property of fennel is its ability in protecting the liver (8). As an aqueous extract of fennel seed which is used and consumed in many East Asian countries, it was studied in this research. It is also commonly used by women during the late days of labor and breastfeeding because of its ability in producing milk in breastfeeding mothers and facilitating the labor, both of which are due to its estrogenic properties (9). Given the possibility of mutagenicity and carcinogenicity of estragole in fennel, it should be used with caution (10). The presence of antioxidant compounds in fennel can disrupt the process of tumorigenesis by reducing the value of oxidants formed in the body while preventing DNA damages (11, 12). In this regard, we can refer to the results of studies conducted on the anticancer effect of methanol extract of fennel seed on breast, colon, and liver cancers, as well as its ability in protecting normal cells (13, 14). Metastasis is an undesirable phenomenon that occurs in most cancers, disrupts their treatment, and increases the likelihood of death in patients (15). 4T1 cells are highly invasive cells used in vivo studies to induce BALB / C mouse breast cancer. In most cases, this cell has metastases to other tissues. The lung is one of the target organs, and lung cancer is one of deadly cancers(16). Ki67 is a non-histone nuclear protein expressed at the cell proliferation phas (17). This protein has a half-life of about 1.5 hours and can be expressed at all phases of cell proliferation except the G0 phase. It is used as a marker of the expression of tumor proliferation (18). Most researchers believe that its increased expression in tumors suggests a poor prognosis. Furthermore, there is much evidence of its increased expression at the G1 phase and early stages of the S phase. A sudden decrease in its expression was seen at the onset of anaphase and telophase (18, 19). E-cadherin is a membrane glycoprotein molecule binding to adjacent cells in the presence of calcium ions, which contains cytoplasmic types with a molecular weight of 80 *kd* with proteolytic properties and membrane with the weight of 120 *kd* (20). Membrane E-cadherin function also causes adhesion of adjacent cells, and it is essential for the development of the embryo's growth factor. Moreover, it is also vital for the formation of epithelial cell structure in adolescence (21).

The binding of E-cadherin to a protein called catein and the formation of zenola bindings lead to the formation of cellular bindings, transmission of messages, and stability of tissues. E-cadherin plays a major role in preventing the metastasis of cancer cells, and reducing the expression or deficiency in its function that causes tumor invasion and metastasis (22, 23).

Reduction in E-cadherin expression or any disorder in its function is influenced by epigenetic processes. Processes such as CpG hypermethylation and slug transcription factor involvement are considered among these mechanisms (22, 24) . Dysadherin is a surface glycoprotein whose expression has been observed in a great number of tumors. However, it was found only in a small number of normal cells (25). Dysadherin belongs to the FXYD protein family. Passing through the cell membrane, it shows its activity through regulating the Na + / K + -ATPase function and modifying cell polarity. The extracellular domains of Dysadherin are highly glycosylated in tumor tissues, leading to an increase in its stability (26). It is involved in tumor metastasis due to different functions described earlier such as a reduction in E-cadherin expression reported in some articles. It also increases the production of chemokine compounds, which increase the stem cell properties of the tumor. Finally, the presence of this glycoprotein has been linked to tumor ability in tumor metastasis and proliferation (27). In this study, the impact of the extract on the expression of proteins involved in metastasis was evaluated. In the present research, Ki67 protein was used as a factor expressing tumor proliferation. In the majority of previous studies, the ability of the extract to kill cancer cells has been examined. The purpose of this selection is to evaluate the ability of the extract to reduce cell proliferation in the mass of cancer cells. The distinctive purpose of this study is to investigate the effect of the extract on metastasis inhibition. The study of Dysadherin expression as a major protein involved in metastasis is a feature of this article. Since Dysadherin is also expressed by many tumor stem cells, the decrease in its expression by the extract indicates a reduction in the activity of tumor germ cells. Moreover, in this research, an attempt has been made to identify the extract's possible and adverse effects. The choice of lung tissue is based on two pillars.

1. Lung metastasis is one of the most prevalent metastases in breast cancer patients.

2. Lung cancer is the most fatal cancer, and its metastasis leads to a decrease in survival.

Materials and Methods

This study was conducted after obtaining the ethical code of "IR.IAU.SRB.REC.1398.038".

Cell Line Preparation

4T1 line cells were purchased from the Iranian Pasteur Institute. Subsequently, the cells were grown in DMEM high glucose medium. The culture was carried out at 37 °C and 5% CO2. Given the cell growth, the medium inside the flask was replaced every 2 to 5 hours. These cells were removed from the flask to continue cell growth. There was no need to remove the whole medium. Also, 1ml to 4ml of media should remain in the flask due to the growth factors producing the cell (28).

Cell Count through Hemocytometer Slide

The cells were first removed from the bottom of the flask with trypsin, and then the solution containing culture medium was centrifuged for 4 minutes at room temperature (112.7 g). The supernatant was discarded and 1 ml of the flask was added to the sediments on the bottom of the flask. After pipetting, a uniform suspension of cells was created. After that, 10 µL of cell suspension was combined with 1 µL of 0.04% trypan blue solution. By pipetting, the cells were completely stained (30). Stained living cells were counted using a 10-lens microscope by hemocytometer slide, and the viability of the cells was also determined.

Via ability= number of living cellsxlOO
total count of cells

Animals and Their Cancer

Five groups (7 adult female BALB / C mice in each group) with the age range of 6-8 weeks weighing approximately 24-27 gr were selected and stored at 25 ° C for 12 hours in daylight and 12 hours in darkness. The mice were kept in the medium for a week before starting the work. In selecting their storage and feeding place, standard conditions were met by observing ethics and protecting the rights of laboratory animals. A total of 50,0000 living 4T1 cells were subcutaneously injected into the lumbar spine of the mice. After one week, the tumor was visible with the eyes. However, no tumor was observed in the healthy group that received no injection. Then, treatment with an aqueous extract of fennel seed was initiated in the studied groups, and treatment with distilled water was initiated in the sham group. The injection was intraperitoneally performed (IP) for two weeks.

Preparation Method of Fennel Aqueous Extract

The disinfected seeds of the fennel herb were purchased from Isfahan Pakan Bazr Company. The seeds were poured into the mill and powdered for 6 minutes. Two gr of powdered seeds were poured into 100 ml of distilled water and heated by the hot plate at 90-95 ° C until the powder was dissolved. The extract was prepared at a concentration of 2000 mg/dl.

The resulting solution was passed through the funnel. Moreover, the extracted solution was centrifuged (15 min, 112.7 g). From the concentrated extract, the required dilutions were prepared based on the weight of the mice. In this study, extraction was performed daily.

$\frac{weight\ of\ per\ muse \times Dose\ of\ The\ extract}{(concentration\ per\ one\ ml)20\times1000}$= Amount of extract to be injected into per mouse

Extract Injection

The mice were divided into 5 groups (7 mice in each group). The first group became cancerous mice and received distilled water daily. The other three groups were cancerous. An intraperitoneal injection of extracts at doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg was used to treat them. The last group was healthy mice without any injection. All the injections were performed in two consecutive weeks after the preparation of fresh fennel extract and the time of injections was between 13:00 - 14:30.

Killing the Animals and Isolation of Tissues

The animals were first anesthetized by chloroform by a veterinarian and the tissues were isolated. One lung lobe was placed inside the 10% formalin and the other lobe was placed inside the DNASe, RNASe Free microtube, and at a -80° C freezer. The lobes were randomly selected.

Paraffin Processing and H&E Staining

Lung tissues were divided for preparing paraffin blocks. The tissues were fixed in a 10% formalin solution and were moved into 70% ethanol after 48 h at room temperature. The tissues were dehydrated using a variety of growing graded ethanol baths. Then, they were cleared in xylene and embedded in paraffin. Finally, they were sectioned in 10µm slides that were stained by the hematoxylin-eosin stain.

Pathological Diagnosis

Lung metastasis was confirmed by the Pathological examination of tissues by the H&E method. It has been performed by a pathologist. The healthy lung has been shown in Figure 5-A and a metastatic lung has been indicated in Figure 5-B

DX of pathology (Microscopic& Macroscopic):

Diffuse patternless sheets and highly dysplastic cells with pleomorphic vesicular nuclei, prominent nucleoli, eosinophilic cytoplasm, High N/C ratio, and high mitotic activity in favor of high grade carcinoma.

Immunohistochemistry

After separating the mice's lungs, they were post-fixed overnight in 4% PFA in BPS at 4ºC. On the next day, the tissues were washed with ice-cold 30% sucrose in BPS**.** After cutting to the optimum temperature, the samples were kept at the appropriate temperature (-80°).

IHC Analysis and Staining

The tissues were serially sectioned at 5 μm in the coronal planes by the use of the floor that were standing fully automatic cryostat, MNT-SLEE (Mainz GmbH, Germany), and were then gathered onto Poly-L-lysine coated coverslips and utilized to analyze tissues. The samples were incubated at room temperature for 30 minutes until dryness. In the next step, the samples were rehydrated in 0.1 M PBS. This phase was performed twice, each time for 10 minutes. Then, they were incubated in a blocking solution (0.1 M PBS containing 2% bovine serum albumin (BSA), 1% normal goat serum, and 0.2% Triton X-100). Subsequently, the samples were placed in permeabilization buffer (10% goat serum, 0.1% Triton X-100 in PBS) for one hour. Primary antibodies were poured onto the samples and incubated at 4°C overnight**.** The next day after washing the samples, secondary antibodies were added and incubated for 2 hours in an antibody solution (5% goat serum, 0.05% Triton X-100 in PBS). Primary antibodies included mouse monoclonal antibodies to the E-cadherin (1:500; Bio-Rad) for E-cadherin IHC. E-cadherin immunostaining was FITC conjugated goat anti-mouse IgG (1:200). Depi *(2 µg/ml)* counterstained was used to visualize the nuclei. Negative control was performed, by deleting one of the primary or secondary antibodies and by observing no fluorescence (gave no signal). The results were analyzed by a fluorescence microscope (Nokia), and the images were taken with the help of a digital camera (Zeiss) (29-31).

Steps of Real-Time PCR (qRT-PCR)

RNx plus was used for RNA extraction. The extraction was used based on the manufacturer's protocol.

Nano-drop was used to determine the concentration and purity of RNA. The denaturing gel of RNA method was used to evaluate the integrity of the electrophoresis.

DNase I (EN0521, Fermentas, Opelstrasse, Germany) was used for RNA treatment. After obtaining pure RNA, it was converted into cDNA. cDNAs were synthesized with 1μg of RNA, *0.5* μL of oligo dTs, and 0.5 μL of random hexamer using a cDNA Synthesis Kit (Prime Script RT Master Mix, TAKARA, Kyoto, Japan). All the processes were performed in terms of the manufacturer's protocol. For SYBR Green-based real-time RT-PCR via 2X qPCR kit (RR820L, Tli RNaseH Plus, TaKaRa, Kyoto, Japan) 1µg of synthesized cDNA was used. Table 1 shows the pairs of primers used in the reactions of this study. Thermo cycling 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. We used values from β-actin to load normalization for every sample. Relative changes in expression were identified by the use of the $\Delta\Delta Ct$ method relative to gene expression values for controlling the mice.

Analysis of Statistical Data

Standard error, mean standard error, and standard deviation were calculated for all results. The normality of data distribution and equality of variance was tested at first. ANOVA was used to determine the remarkable distinctions between the groups (for normal distribution). Duncan's test was also used for separating the groups if ANOVA results were significant. Kruskal-Wallis test was used for non-normal distribution. SPSS version 23 and PRISM 8 software were used for calculations. $P < 0.05$ was used for a significant difference in means**.**

Results and Discussion

Examination of E-cadherin Expression by IHC Method

Table 1: Used Primers.

1	2	3	4	5	6	7	8	9	10
No.	**Name**	**Seq. (5-3)**	**MW**	**OD (1000µl)**	**nmol**	**Water/tube (µl)**	**TM**	**GC %**	**Mer**
1	Dysadherin-F	CCCTTCTACTACGATGATACTACC	7207.67	4	18.31	183.14	61.01	46	24
2	Dysadhein-R	GAGACAACTGCCTACACTTCC	6335.13	4	20.84	208.36	59.82	52	21
3	Betacatin-F	TGAAGATCAAGATCATTGCTCCTC	7311.76	4	18.05	180.53	59.30	42	24
4	Betaactin-R	TCAGTAACAGTCCGCCTAGAAG	6728.4	4	19.62	196.18	60.25	50	22
5	Ki67-F	GCAAGAGGCAGCAAAGGTC	5895.89	4	22.39	223.88	58.83	58	19
6	Ki67-R	ATTGTCCACTGTCACTGAATCC	6645.31	4	19.86	198.44	58.39	45	22

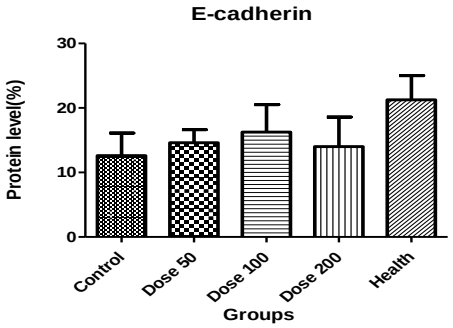

Figure 1. Expression of E-cadherin in Different Groups.

Figure 1 shows the expression of E-cadherin in different groups as a preliminary comparison. The examination of the chart showed a relative increase in E-cadherin expression at doses of 50 mg/kg and 100 mg/kg compared to sham (control). E-cadherin expression at a dose of 200 mg/kg was approximately equal to that of the sham group. E-cadherin expression was higher in the healthy group compared to all the groups. The fluorescence intensity was measured by a software program (image1.25h) and expressed as the percentage of expression after analysis (It has been shown in Figure 2A-2F)

The Fluorescence-containing antibody was bound to E-cadherin in healthy lung tissues (Figure 2-A). The fluorescence intensity was proportional to the level of protein expression in the cell membrane. The fluorescence intensity was analyzed by the software and expressed as a percentage of protein expression. In Figure (2-D), E-cadherin was detected in the cell membrane of the metastatic lung tissue by fluorescence antibody. The fluorescence intensity was proportioned to the level of protein expressed in the cell membrane. ANOVA statistical analysis was used to examine the significance of the differences in E-cadherin expression. ANOVA result with $p > 0.05$ ($p = 0.589$) indicated no significant difference among the treatment, sham, and healthy groups in terms of E-cadherin expression.

Ki67 Expression

Ki67 analysis was performed using qRT-PCR. Figure 3 shows the expression of Ki67 in different groups as a preliminary comparison.

The qRT-PCR method indicated a reduction in the expression of treatment groups with doses of 50 mg/kg and 100 mg/kg compared to the control group and indicated an increase in the expression of the *200* mg/kg dose group in comparison with the other groups (Fig. 3). Kruskal-Wallis analysis was used for the statistical analysis of Ki67 expression. The result with $P < 0.05$ (0.0387) at a dose of 100 mg/kg showed a significant decrease at this dose compared to the sham and 200 mg/kg groups. There was also a significant increase in the expression at a dose of 200 mg/kg compared to doses of 50 mg/kg ($p = 0.0218$) and 100 mg/kg at $P < 0.05$(0.0145) that was higher than that of the fluorescence antibody binding against Ki67 in the healthy group.

Dysadherin Expression

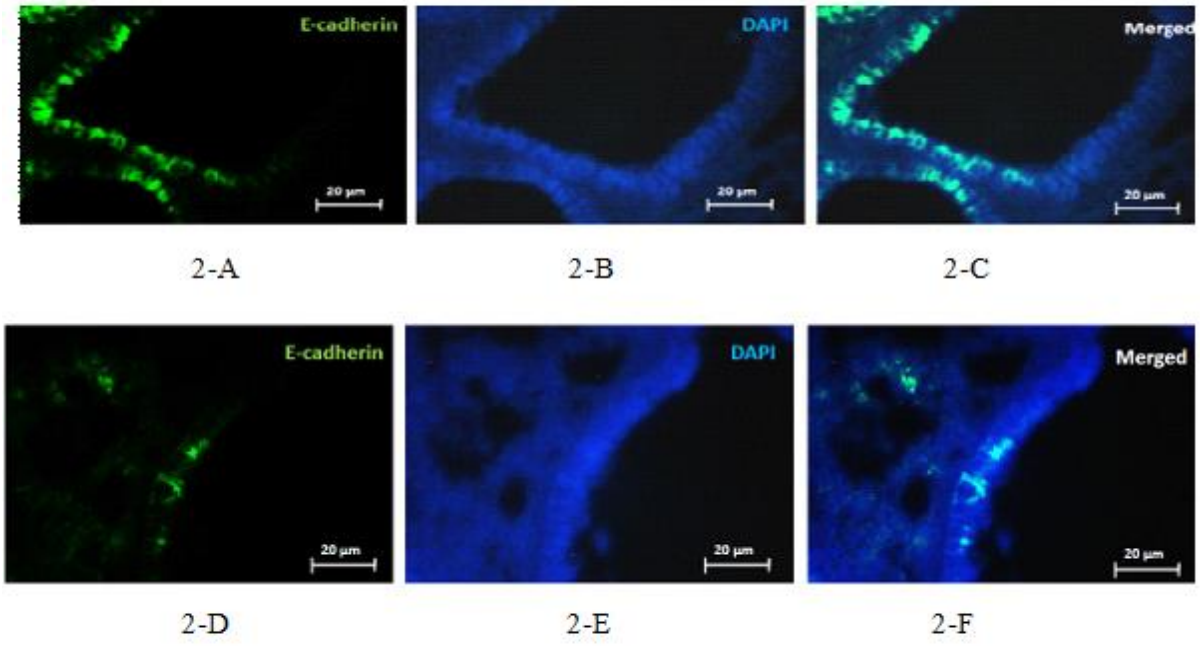

Figure 2. Immunohistochemical Images. Level of E-cadherin expression of the lung tissue was evaluated by IHC method and comparison of expression in normal and tumor tissues. A: E-cadherin expression in the membrane of healthy tissues and binding fluorescence-containing antibody to it; B: Dapi of cells in healthy tissues by IHC method; C: View of the cell membrane of healthy tissues in IHC method; D: E-cadherin expression in cell membranes in tumor tissues and antibody-containing fluorescence; E: Dapi of cells in tumor tissues in IHC method; and F: View of the tumor membrane of healthy tissues in the IHC method.

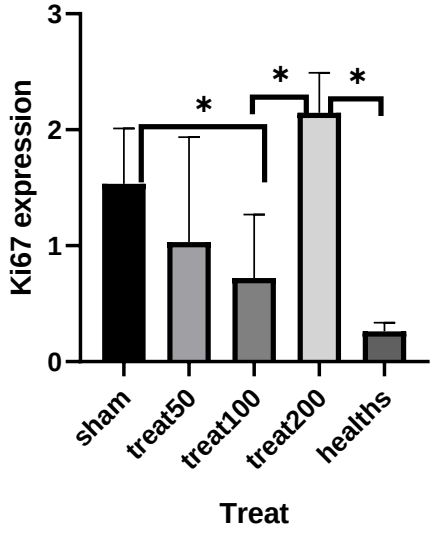

Figure 3. Expression of Ki67 in Different groups.

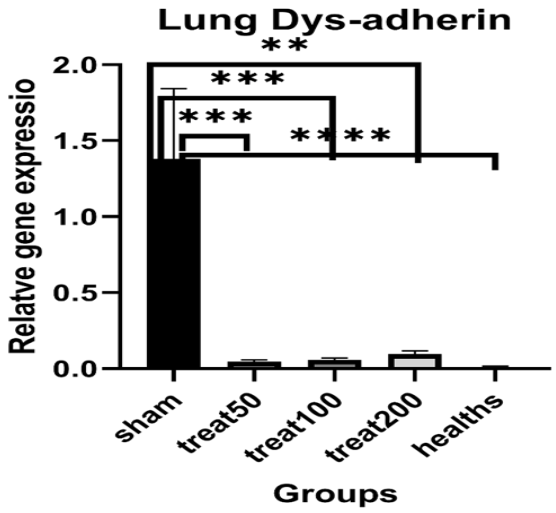

Figure 4. Expression of Dys-adherin in Different groups.

It was assessed by qRT-PCR. Initial results were plotted as a chart indicating a reduction in the expression in all treatment groups compared to the sham group (Figure 4). ANOVA test was performed and the non-equality of variances was confirmed with $p < 0.05$ (0.000). It was found that a change in the Dysadherin expression in the treatment group was noticeably different from that of the sham group. Duncan's test was used to determine the effective doses. Based on this test, two groups were formed.

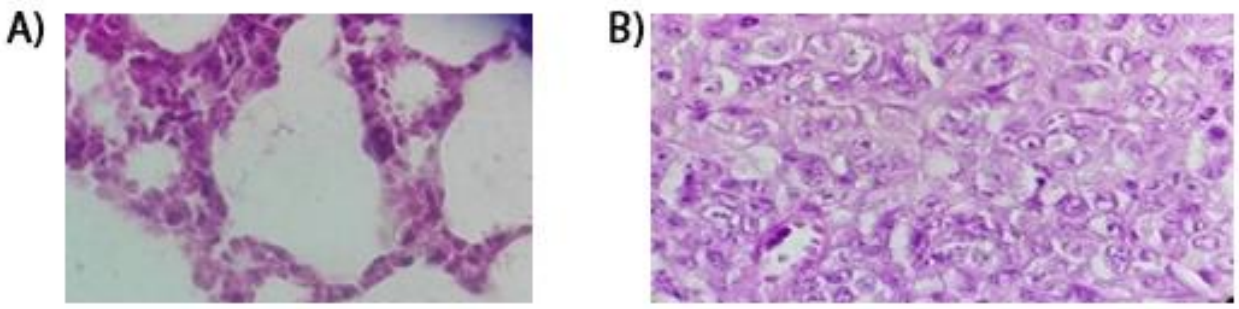

Figure 5. H&E Images; A) Healthy Lung with Normal Cell and Normal N/C Ratio, B) Metastatic Lung Cancer with High N/C Ratio.

Accordingly, the first group was non-treated control and the second group was treated with doses of 50 mg/kg, 100 mg/kg, 200 mg/kg, and a healthy group. Dysadherin expression was assessed by qRT-PCR. Initial results were plotted as a chart indicating a reduction in the expression in all treatment groups compared to the sham group (Figure 4).

Since many studies have been conducted done on the anti-cancer effect of fennel's non-aqueous extracts so far, in this research, the aqueous extract has been used. In most previous studies, the effectiveness of the extract in breast cancer has been considered. In contrast, the purpose of this research is lung cancer tissue. Moreover, since different parts of the fennel contain different and diverse amounts of compounds, fennel seeds have been used in this research. Breast cancer to lung metastasis is one of the most common metastases. Accordingly, the effect of the extract on lung metastasis and subsequent tumor proliferation in lung tissue has been investigated.

Lung metastasis has previously been observed in BALB / c mice with breast cancer with the 4T1 cell line (32). The chart of the results of E-cadherin expression using the IHC method showed a slight increase in the 100 mg/kg dose group compared to the other treatment groups and the Sham group. There was also a reduction in the expression of the 200 mg/kg dose group. The difference may be due to estrogenic compounds in the extract (6, 33). Alveolus generating power of estradiol has been shown in a study (34). Hence, reduction in E-cadherin expression in the *200 mg/kg* dose treatment group was slightly justified because the proliferation power of tumor cells was greater than that of the healthy cells and estrogenic components of the extract at 200 mg/kg concentration may stimulate tumor growth. It is suggested to compare the expression of estradiol beta receptors between tumors and healthy tissues. These receptors may be less in the tumor tissue, and may also require a higher dose to bind to them. However, this increase was not sufficient to cause a statistically significant difference. Two types of estrogen receptors, known as alpha and beta, have different distributions in different tissues(35) . Both alpha and beta receptors are present in breast tissue, and beta receptors in the lung tissue can form almost all estradiol receptors (36). The binding of estradiol agonists acetylates the histones and enhances the transcription of the E-cadherin gene by activating acetyltransferases (37). E-cadherin is known as an anti-metastatic factor and its expression is often reduced in cancers by the epigenetic pathway (22, 38). Since a change in expression was observed only at a dose of 200 mg/kg, it seems that it should be cautiously used for treatment. However, the change was not significant. It can be concluded that the extract at doses of 50 mg/kg and 100 mg/kg has no epigenetic effect on the reduction of E-cadherin expression but a dose of 200 mg/kg may have such an effect. This dose might exceed the permissible level of estrogen and may increase the proliferation of cancer cells compared to healthy cells. It has been found that estradiol has different effects on different cell lines in the lung tissue(39, 40). Since Ki67 is recognized as a proliferation stage protein, increasing its expression in lung tumors is not a good prognosis (41).

Ki67 expression increased at a dose of *200* mg/kg compared to the group of 100 mg/kg**.** This difference in expression was significant. It can be stated that the most

important point in the initial examination of the *qRT-PCR* and E-cadherin & Ki67 expression was an increase in the expression at a dose of 200 mg/kg for Ki67 and reduction in E-cadherin expression. Given the results of a change in E-cadherin expression at a dose of 200 mg/kg as well as the results of Ki67 expression at this dose, the toxicity of this dose was higher. Previous studies have also suggested a dose range for estragole, which is one of the main constituents of fennel (10). Another study by the authors found a decrease in Ki67gene expression in the breast and ovary (42). Dysadherin is another investigated protein, and the results of other studies have suggested that its expression can inhibit E-cadherin expression and metastasis (43). Several studies have reported a positive overlap between increased Dysadherin expression, and also metastasis, and a negative overlap between life expectancy and tumor prognosis (44). The creation of metastasis by Dysadherin has been described in several ways, ranging from decreased expression of E-cadherin to increased expression of chemokines that can enhance the ability of the tumor stem cell (45). Also, another study showed no negative overlap between Dysadherin and E-cadherin, and it was concluded that E-cadherin was first reduced in lung metastasis. However, its expression increased later, indicating that an increase in E-cadherin resulted in tumor growth (45). The extract has different effects on Dysadherin expression in different tissue (42). This difference seems to be related to different tissue receptors, indicating that an increase in E-cadherin could result in tumor growth (45). Aqueous extract of fennel seed could significantly reduce Dysadherin at doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg, which can be effective in reducing metastasis to other tissues and in proliferating tumor cells at the tumor site. Given the significant reduction in Dysadherin expression, it seems that the extract can have a beneficial effect on reducing proliferation and probably preventing other metastases. A dose of 100 mg/kg also significantly reduced ki67 and a non-significantly increased E-cadherin. Based on the charts showing increased expression of Ki67 at a dose of 200 mg/kg and also a slight increase in Dysadherin expression at this dose; however, this increase was not significant, and it can be stated that a dose of 100 mg/kg may be involved in tumor recovery. A dose of 100 mg/kg had favorable effects on all three proteins and the use of a dose of 200 mg/kg was not recommended as it might be carcinogenic

Conclusion

- The most important function of the extract was to reduce Dysadherin expression at all the three doses.
- The 50 mg/kg dose had almost no effect on Ki67 and E-cadherin expression.
- It was shown that the 200 mg/kg dose was not favorable and might be dangerous. It showed a remarkable increase in Ki67 expression.
- The 100 mg/kg dose significantly reduced Dysadherin and ki67 expression.
- The reduced expression at the 100 mg/kg gene level was about 50% compared to the sham group.
- The 100 mg/kg dose was recognized as the most effective dose.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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