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Anticancer and Antioxidant Effects of Ethanolic Extract of *Melilotus officinalis* on MCF-7 Cells via p53 Activation

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ABSTRACT

Background and Objective: The role of inherited and genetic factors has been approved as the predisposing to breast cancer. There have been different studies regarding breast cancer, but the cure for the disease has not been obtained yet. Therefore, the *in vitro* study aimed to investigate the effect of ethanolic extract of *Melilotus officinalis* on the growth of MCF-7 breast cancer cells as well as its effect on the P53 gene expression in MCF-7 cell line. **Materials and Methods:** The MCF-7 cell lines were cultured at 24, 48, and 72 hrs to study inhibition by dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay via ethanolic extract of *Melilotus officinalis* at different doses (62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.9, 0.4, 0.2, and 0.1 µg/mL). Subsequently, the gene expression of P53 by using Real-Time PCR (RT-PCR) was assessed. Group differences were analyzed using one-way ANOVA, RT-PCR results with paired-samples T-test, and $p<0.05$ were deemed significant. **Results:** The extract of *Melilotus officinalis* inhibited the MCF-7 cells and decreased cell growth. The extract inhibition rate was highest during 72 hrs at a dose of 15.6 µg/mL. The MCF-7 cancer cells that received the extract showed a higher expression of the P53 gene if compared to the control group ($p = 0.04$). **Conclusion:** The ethanolic extract of *Melilotus officinalis* has a positive effect on the inhibition of the growth of MCF-7 cancer cells with its anti-cancer effect via increasing p53 gene expression and apoptosis.

KEYWORDS

Breast cancer, *Melilotus officinalis*, ethanolic extract, P53 gene expression, MCF-7 cells

INTRODUCTION

Breast cancer encompasses almost 10% of all patients living with cancer in the world, making it the second most common type of non-skin cancer and the fifth most common cause of death due to cancer among women[1]. Breast cancer is the uninhibited growth of abnormal cells that occurs in various breast tissues.

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The proliferation of cells in the natural state is under the very strict control of mechanisms associated with the cell division cycle, and these mechanisms are controlled by several genes[2]. About 20-30% of breast cancers become metastatic and mainly are seen in the lungs, brain, bone, and liver[3]. About 90% of breast cancer patients die from metastatic disease[4]. Hereditary and genetic factors have been implicated as predictors of breast cancer. One-third of all patients have a positive history of breast cancer in one or more of their first or second-degree relatives. When people in their first-degree relatives have a history of breast cancer, they are at increased risk of cancer[5]. The incidence and mortality rate of breast cancer varies across different ethnicities and geographical locations, and different mechanisms play a role in its initiation and progression[6].

Melilotus officinalis (L.) Lam (yellow sweet-clover) belongs to the Leguminosae family. It is a perennial herb with three folium leaves and a very branching stem. The flowers are yellow and relatively small[7]. The flowering tops of this plant contain the main constituents of coumarins, flavonoids, phenolic carboxylic acid, vitamin C, allantoin, tannin, and mineral salts[8].

Several studies have shown that the high content of phenol and the high flavonoid constituents of *Melilotus officinalis* extract have an important role in its antioxidant function[9]. Also, coumarins in the extract of *Melilotus officinalis* affect lymphedema and its polysaccharides have immune-modulatory and anti-anemia effects[10].

The p53 gene is one of the most important target genes due to its contribution to a wide range of tumors (about 55%) because it is the most targeted mutation in human cells[11]. The p53 gene is located on the short arm of chromosome 17 (17p) and it has 11 exons that are involved in various cellular functions, such as apoptosis. The p53 gene contains information on the p53 nuclear phosphoprotein that expresses the target gene. The product of these genes stops the cell cycle at the G1 stage[12].

While breast cancer is treated with surgery, chemotherapy, or radiation with severe side effects, herbal medication can be an appropriate choice instead of the interventions[1]. Therefore, finding a novel medication with at least side effects is a key solution for treatment. Much research has been implemented on the cancer, but the complete treatment for this type of cancer has not yet been discovered. Therefore, this study aimed to investigate the effect of ethanolic extract of *Melilotus officinalis* on the growth of MCF-7 breast cancer cells as well as the effect of this extract on the P53 gene expression in the MCF-7 cell line.

MATERIALS AND METHODS

Study area: The aerial parts of *Melilotus officinalis* were collected from Lorestan Province, near Borujerd, Iran, in May, 2017. The total weight of the collected plant was 191 g. Identification of the plant species was approved by PMP-372 code number at the herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences (TUMS), Tehran, Iran.

Research methodology: The collected plant materials were air-dried in darkness at ambient temperature. Ethanolic extract was prepared from *Melilotus officinalis* according to the maceration method[13]. Briefly, 181.52 g of the dried plant was milled, then soaked with hydroalcoholic solvent (75% ethanol), and the total extract was achieved at 24, 48, and 72 hrs. The rotary (BUCHI, Switzerland) apparatus was used to extract concentration. The yield of obtained extracts based on dry weight was calculated from the following formula[14]:

$$\text{Yield (\%)} = \frac{W_1}{W_2} \times 100$$

where, W_1 was the weight of the extract after evaporation of the solvent and W_2 was the dry weight of the sample. The concentrated extract was dried using an oven (Memmert, Germany) at 40-45°C temperature picked up in a vial, and then refrigerated at 4°C until the test.

Cell culture: The human breast cancer cell line (MCF-7) was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The cells were cultured in RPMI-1640 (Bio idea, Iran) with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK) at a constant temperature of 37°C with a humidified atmosphere of 5% CO_2. At low density (1.0×10^4 cells/mL), allowed to attach and initiate log-phase growth for 24, 48, and 72 hrs, and were then exposed to ethanolic extract of *Melilotus officinalis* at different doses (62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.9, 0.4, 0.2, and 0.1 µg/mL).

Viability and IC_{50} calculation: Dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, American) assay was applied to evaluate cell proliferation where 5×10^4 cells were seeded in 96-well plates and incubated with ethanolic extract of *Melilotus officinalis* at different doses (62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.9, 0.4, 0.2, and 0.1 µg/mL) for 24, 48 and 72 hrs. When the incubation finished, 20 µL MTT (0.5 mg/mL) was added to each well and then incubated for 3 hrs; 150 µL of dimethyl sulfoxide (DMSO) (Scharlab, Spain) was added to each well, and the absorbance was recorded at with ELISA reader (SCO/DIAGNOSTIC, Russia). Finally, the viability and IC_{50} of each group were calculated (cell viability) by the following formula[15]:

$$\text{Cell inhibition} = 1 - \frac{\text{OD adjacent cells with cytotoxic composition}}{\text{OD control cells}} \times 100$$

$$\text{Cell viability} = 100 - \text{Cell inhibition}$$

IC_{50} was calculated by Excel 2010 software.

The MCF-7 cells were treated with ethanolic extract of *Melilotus officinalis*. Then, total cell RNAs were isolated by the EZ-10 Spin column total RNA mini-preps super kit (Bio Basic Inc., Canada) according to the manufacturer's recommendation. The total RNAs were eluted in 20 µL of RNase-free water and stored at -80°C for subsequent procedures. The quantity and quality of the purified RNAs were verified by the nanodrop spectrophotometer (Thermo, American). The purified RNA was used as a template for cDNA synthesis. Reverse transcription was carried out by prime script RT reagent kit (PRT) (Takara, Japan).

Primer's sequence and specifications: The RT-PCR mixture consisted of forward and reverse primer, P53 gene-specific and cDNA of samples, and Real Q Plus 2× master mix green without ROX™ (Ampliqon, Denmark), which included DNA polymerase, SYBR green I dye, dNTPs, and PCR buffer, in a total volume of 25 µL/mL. Amplification of GAPDH, a housekeeping gene, was used to normalize the efficiency of cDNA synthesis and the amount of RNA applied. Oligo 7 software designed specific primers based on sequences obtained from the GenBank database. The designed primers were subjected to BLAST (NCBI GenBank) to ensure complete homology with the genes mentioned in Table 1.

The first cycle in RT-PCR (Qiagen, Germany) activated the hot-start enzyme at 95°C for 15 min. Then, cDNA was subjected to 40 cycles of RT-PCR consisting of denaturation at 95°C for 30 sec and annealing and

Table 1: Forward and reverse primers were used in the real-time PCR analyses of the p53 and GAPDH genes

mRNA or gene	Oligonucleotide sequence (5'-3')	Annealing (°C)	Product length (bp)
P53	Forward: GGCTCTGACTGTACCACCATC Reverse: CTCAAAGCTGTTCCGTCCCAGTAG	60	138
GAPDH	Forward: TCACCATCTTCCAGGAGCGAG Reverse: CAGTTGGTGGTGCAGGAGG	60	247

extension at 60°C for 30 sec. The product was separated by electrophoresis in a 2.5% agarose gel (DNA biotech, Iran) to verify the successful amplification.

Statistical analysis: Statistical analysis of cells' differences between the groups was performed by one-way ANOVA with IBM SPSS Statistics software 22.0 and RT-PCR results were analyzed by paired-samples T-test and $p<0.05$ as significant.

RESULTS

The IC_{50} values of *Melilotus officinalis* extract in MCF-7 cells at 24, 48, and 72 hrs were 123 µM, 330 µM, and 340 µM, respectively. Figure 1(a) shows MCF-7 cells treated with the ethanolic extract of *Melilotus officinalis* and after 24 hrs, tetrazolium was added. Formazan crystals precipitated in the living cells after 3 hrs. Figure 1(b) introduced MCF-7 cells treated with colchicine as the positive control. Figure 2(a) illustrates the viability of MCF-7 cancer cells by applying the extract of the *Melilotus officinalis* with concentrations of 5.62, 25.31, 6.15, 8.7, 9.3, 9.1, 9.0, 9.0, 4.0, 2.0, 1.0 µg/mL which after 24 hrs (red line), the viability was 68.73, 94.68, 05.71, 94.78, 85.78, 6.79, 91.80, 87.77, 21.82, 96.96%, respectively, after 48 hrs (blue line), 15.66, 09.74, 37.63, 61, 76.64, 92.63, 71.03, 64.06, 64.2 and 68.53%, and after 72 hrs (yellow line), 51.63, 71.75, 59.23, 76.60, 64.42, 18.56, 59.54, 62.59, 23.59 and 12.73%. The effect of *Melilotus officinalis* extract on MCF-7 cancer cells depended on the dose and time of using the extract. The MCF-7 cancer cells that received the extract of *Melilotus officinalis* for 72 hrs at a dose of 15.6 µg/mL showed an inhibitory rate compared to the controls.

Figure 2(b) displays the viability of normal HEK-293 cells by applying the extract of *Melilotus officinalis* (no lethal effect) with concentrations of 5.62, 25.31, 6.15, 8.7, 9.3, 9.1, 9.0, 9.0, 4.0, 2.0, 1.0 µg/mL. After 24 hrs (red line), the viability was 103.12, 65.97, 79.94, 31.95, 03.07, 65.97, 18.92, 25.106, 9.93, and 100%, respectively, after 48 hrs (blue line), 69.6, 62.98, 33.104, 88.94, 63.97, 45.96, 9.105, 81.98, 54.103, 14.103%, and after 72 hrs (yellow line) shows 48.99, 74.99, 1.103, 12.96, 86.95, 7.98, 12.96, 38.96, 68.105, 93.97%, respectively. Cell viability increased from high to low concentrations and there was a meaningful difference at all concentrations with the control group. Ethanolic extract of *Melilotus officinalis* with different concentrations, as well as increased time of effectiveness, did not affect the controls (HEK-293 cells).

Figure 3 demonstrates the gene expression of p53 and GAPDH (internal control gene with constant expression) for the dose of 15.6 µg/mL after 72 hrs based on RT-PCR analysis. In the left side of the figure, the expression of the GAPDH gene in the first column is shown in HEK-293 cells without extract, HEK-293 with *Melilotus officinalis* extract, MCF-7 without extract, MCF-7 with *Melilotus officinalis* extract,

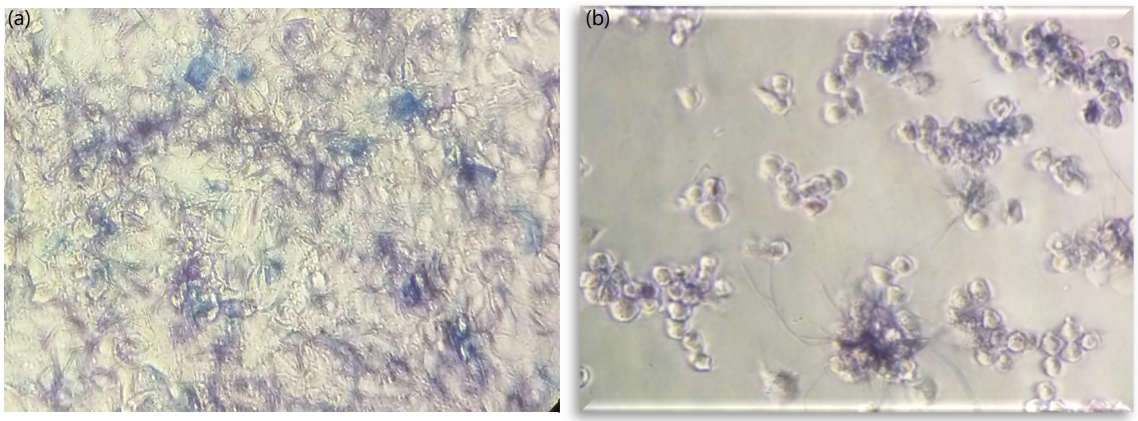

Fig. 1(a-b): Cells with formazan crystals, (a) MCF-7 cells with formazan crystals after 24 hrs and (b) Control group

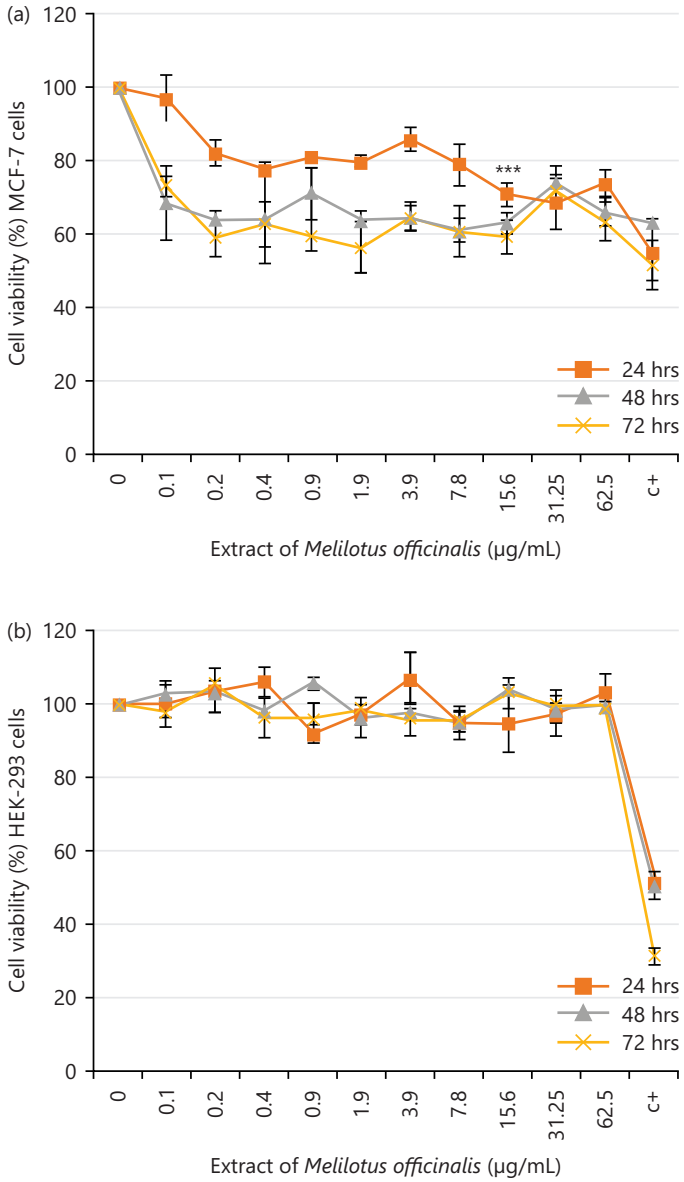

Fig. 2(a-b): Percentage of the viability of (a) MCF-7 cells and (b) HEK-293 cells after MTT assay via an ethanolic extract of *Melilotus officinalis* at different doses

***p<0.001, significant difference compared to the negative control group

respectively. The expression of the P53 gene in the second column is pointed in HEK-293 cells without extract, HEK-293 with *Melilotus officinalis* extract, MCF-7 without extract, and MCF-7 with *Melilotus officinalis* extract, respectively. The MCF-7 cancer cells that received the extract of *Melilotus officinalis* for 72 hrs at a dose of 15.6 µg/mL showed a significant difference in p53 gene expression compared to MCF-7 cells (the control group) and increased p53 gene expression.

The samples in Fig. 4 (from the left side) including negative p53 and negative GAPDH, indicate that there was no contamination. The MCF-7 and drug, MCF-7 without drug, HEK-293 and drug, and HEK-293 without drug demonstrate the expression of the P53 gene. The P53 gene is 138 nucleotides long and binds to the leader in the correct position. MCF-7 and drug, MCF-7 without drug, HEK-293 and drug, and HEK-293 without drug show the expression of the GAPDH gene. The GAPDH gene is 247 nucleotides long and binds to the leader in the correct position. These bands indicate that the primers are designed and work correctly, the RNAs are extracted correctly and the cDNAs are made correctly.

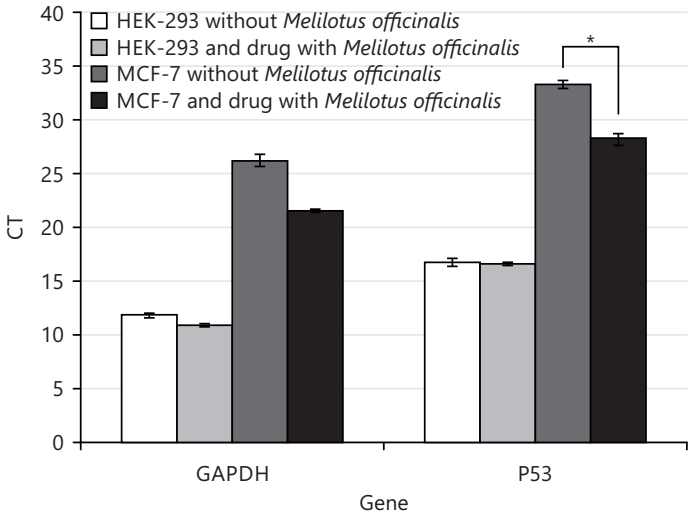

Fig. 3: RT-PCR analysis shows P53 and GAPDH gene expression

*p>0.05, significant difference compared to the negative control group, MCF-7 cancer cells that received the extract of *Melilotus officinalis* for 72 hrs at a dose of 15.6 µg/mL showed a significant difference (p≤0.05)

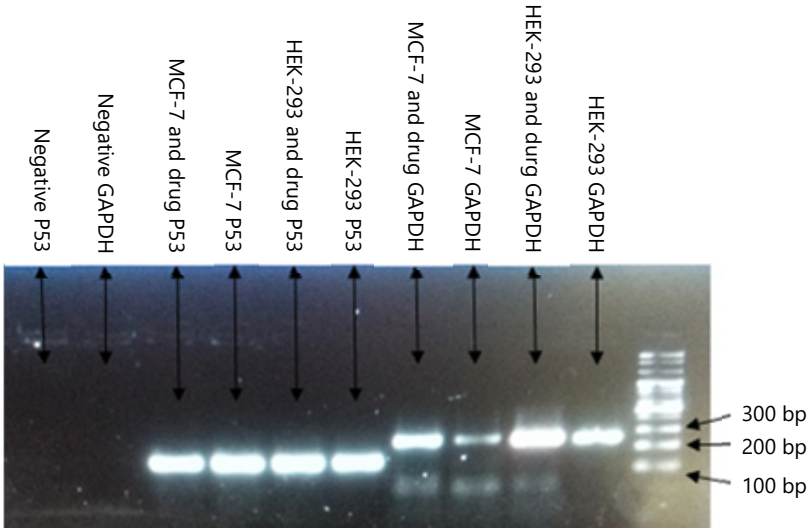

Fig. 4: Expression of the P53 in MCF-7 cells in the treated and control groups

MCF-7 cancer cells which received the extract of *Melilotus officinalis* for 72 hrs at a dose of 15.6 µg/mL, increased p53 gene expression

Figure 5 illustrates the amplification plot of the RT-PCR for P53 and GAPDH genes. Each sample was replicated three times for the P53 and GAPDH genes. The samples in Fig. 5 from the left for GAPDH gene expression include HEK-293 without drug, HEK-293, and drug, MCF-7 without drug, and MCF-7 and drug. Also, the samples for P53 gene expression include HEK-293 without drug, HEK-293, and drug, MCF-7 without drug, MCF-7 and drug. All three samples are identical to each other.

DISCUSSION

The present study showed that the extract of *Melilotus officinalis* had an inhibitory effect on MCF-7 cells, which reduced cell growth and increased programmed cell death. The inhibitory effect promoted by the extract of *Melilotus officinalis* was dependent on time and concentration. Extract of *Melilotus officinalis* at 15.6 µg/mL at 72 hrs inhibited growth and increased apoptosis in the MCF-7 cell line. In addition, the extract of *Melilotus officinalis* had good cytotoxicity on MCF-7 cancer cells.

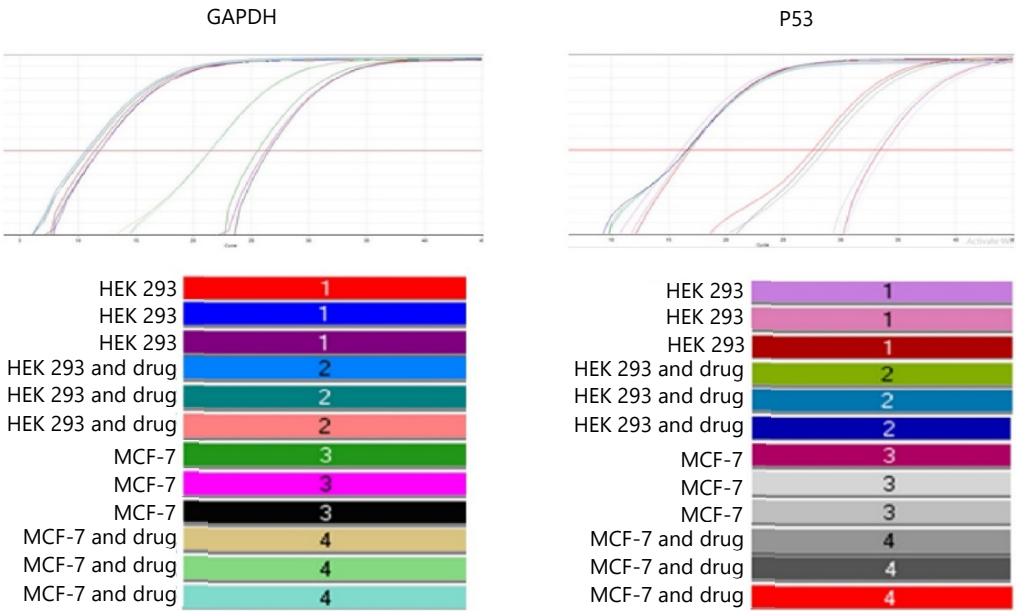

Fig. 5: Amplification plot of the RT-PCR for P53 and GAPDH genes

Based on the literature, the extract of *Melilotus indicus* in hepatocellular carcinoma has also been investigated through the mechanism of mitochondrial-mediated pathways. In a study with human liver carcinoma cells, *Melilotus indicus* extract induced apoptosis. However, this apoptosis has been attributed to the mechanism involved in mitochondrial-mediated pathways and reduced mitochondrial membrane potential and apoptosis-inducible factor translocation[16]. In a study conducted in China (2018) to isolate, purify, identify, and act on the monomeric compounds of *Melilotus officinalis* for clinical applications, monomeric compounds were used for clinical applications. The 70% ethanolic extract of *Melilotus officinalis* was isolated using chromatographic columns, for compounds 1 and 2, together with seven known compounds: Salicylic acid (compound 3), coumarin (compound 4), betaine (compound 5), fumaric acid (compound 6), and caffeine acid (compound 7), Luteolin (compound 8), quercetin (compound 9) were obtained. Each of these compounds affected MCF-7 cells separately. Results showed that compounds 1, 2, 3, 5, 7, 8, and 9 could inhibit MCF-7 tumor cell growth, but not compounds 4 and 6. Most of the compounds of *Melilotus officinalis* (L.) Lam have anti-oxidation, anti-tumor, and anti-inflammatory effects[17].

In the current study, there was a significant difference in the expression of the p53 gene compared to MCF-7 cells (without extract of *Melilotus officinalis*) ($p \leq 0.05$) and increased p53 gene expression. In a study in which *Melilotus officinalis* plant extract was used to treat multiple sclerosis in female mice, it reduced the clinical symptoms of the disease. It also increased the expression of anti-inflammatory genes and antioxidant enzymes in the treatment group[18]. Studies have shown that coumarin extracted from *Melilotus officinalis* has an antioxidant and anti-inflammatory effect, which removes free radicals from stressful organs[10]. Flavonoids are the major antioxidants found in ethanolic extracts of *Melilotus officinalis*. In addition to their antioxidant properties, flavonoids have anti-cancer properties, which may prevent the onset of cancer by strengthening the antioxidant system and eliminating carcinogens, and free radicals[19].

In a study, the researchers investigated the therapeutic effects of *Melilotus officinalis* on the healing of ulcerative colitis (UC) in male rats. The results have shown that the extract of *Melilotus officinalis* included large amounts of antioxidant compounds that can be attributed to the therapeutic effects of UC. This treatment resulted in increased colonic antioxidants and decreased inflammation and acute colon injury[19].

Quercetin is a polyphenolic product and is one of the most abundant and important constituents of the flavonoid family. Quercetin is a flavanol with antioxidant properties and can remove free radicals. Quercetin in extracts of *Melilotus officinalis* is capable of increasing acidic and cytotoxic stress in tumor cells, which, at concentrations above 40 μM, increases cellular injury and cell death through reactive oxygen species. Quercetin suppresses cell proliferation and increases apoptosis[20,21]. About 20-40% of breast cancers are caused by mutations in the p53 protein. A study has shown that mutations in the p53 gene produce mutant proteins that are more stable than normal proteins. Therefore, p53 protein levels are different in breast tissue tumors. One of the reasons seems to be the genetic differences[22,23]. In an investigation conducted with women with breast cancer, mutations in the p53 gene and overexpression of the p53 protein were associated with response to chemotherapy[23].

The central p53 region is of key importance in regulating apoptotic, transcription-dependent, or transcriptional functions. The P53 is a well-established tumor suppressor that plays an important role in genomic homeostasis, cell cycle regulation, and induction of apoptosis in response to various cellular pressures, particularly DNA damage. The P53 regulates the transcription of p21, Bax, and other main components in DNA repair, cell cycle arrest, and apoptosis[24]. Similarly, p53 mutations have been identified in most human cancers.

In normal situations in unstressed cells, p53 activity is very low. After stress, p53 is activated through post-translational modifications and can bind to specific DNA sequences. The p53 recognition sequence is very loose and is available in several hundred genes. They are differentially regulated depending on the cell type, the stress nature, and the damage extension. At low cellular levels, p53 is only a subset of the regulated genes that regulate higher levels. In addition to inducing genes that lead to apoptosis, p53 can also activate gene expression that suppresses survival signals such as PTEN, or apoptosis inhibitors including BIRC5[25,26]. The collection of *Melilotus officinalis* is only possible in May and June in Iran. This has been a limitation of the present study. So, if the extract expires it is no longer available until May and June. Moreover, a rise in the price of lab materials, as well as a shortage of materials. Likewise, with the introduction of counterfeit laboratory materials, the study has been dormant and prolonged.

CONCLUSION

It can be concluded that with the mechanism of increasing both p53 gene expression and apoptosis, the investigators could observe the anti-cancer effect of ethanolic extract of *Melilotus officinalis* on MCF-7 in breast cancer. Subsequently, the high expression of the p53 gene has led to decreased survival of breast cancer cells and no longer proliferation. It is recommended to investigate the effect of ethanolic extract or other fractions of the *Melilotus officinalis* on breast cancer *in vivo*, the expression of other genes involved in apoptosis, and other cancer cell lines. Finally, by more exact laboratory methods like using flow cytometry on MCF-7 and P53 cancer cells, the effect size of this extract on the amount of the P53 gene can be determined more precisely.

SIGNIFICANCE STATEMENT

This study identified the effect of ethanolic extract of *Melilotus officinalis* on the P53 gene expression in the MCF-7 cell line, which could be beneficial for preventing of the growth of MCF-7 breast cancer cells. This study will assist researchers in uncovering critical areas of breast cancer that have remained unexplored by many. Consequently, a new theory on breast cancer treatment may be developed.

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