Journal of Medicinal Plants and By-products

Original Article

Libanotis transcaucasica Schischk **Leaf and Seed Ethanolic and Methanolic Extracts: Cytotoxic Activity on MCF7 Cell Line and Antioxidant Properties**

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Article History: Received: 20 May 2023/Accepted in revised form: 02 July 2023

ABSTRACT

Libanotis is an important genus of the Apiaceae family with many aromatic species. These plants are widely used in traditional medicine, but there is little information about their phytochemical compositions and biological activities. An ecotype of *Libanotis transcaucasica* Schischk, about which there is little phytochemical information, grows in the Hezar-Masjed Mountains of northeastern Iran. The aim of this study was to investigate the anticancer activity and antioxidant properties of ethanolic and methanolic extracts from the leaves and seeds of this plant. Cytotoxic activity against MCF7 and HSkMC was determined by the MTT method, and the antioxidant properties of the extracts were evaluated by the DPPH and PFRAP methods. The results showed that the methanolic extract of seeds had the greatest effect on inhibiting the growth of cancer cells at 48 and 72 hours, with IC_{50} values of 54.35 and 50.52 µg/ml, respectively. Moreover, the methanolic extract of the seeds had the greatest effect on the degradation of DPPH free radicals with an IC_{50} value of 44.64 µg/ml, and this inhibitory effect was comparable to the effect of ascorbic acid. The antioxidant effect of iron reduction of the different extracts was significantly lower than that of ascorbic acid. However, among the extracts, the reducing power of the methanolic seed extract was better than the others. Considering that the methanolic seed extract had the most lethal effect on cancer cells, it seems that the extract that has stronger antioxidant power also has stronger anticancer properties.

Keyword: Apiaceae, Genus *Libanotis*, MTT Assay, DPPH, FRAP

INTRODUCTION

Cancer results from excessive and uncontrolled cell growth that can occur in any body tissue. Breast cancer is considered the most critical cancer in women, and is treated with various methods such as chemotherapy, radiotherapy, hormones, and surgery [1]. Recently, natural products have attracted the attention of many researchers due to their importance in preventing and treating cancer. Various medicinal plants have shown anticarcinogenic and antiproliferative effects [1] and some anticancer drugs are prepared based on compounds from medicinal plants [2]. For this reason, numerous studies have been conducted on the effects of medicinal plants on this disease.

Medicinal plants still play an important role in the treatment of various diseases in many countries, especially in developing and underdeveloped countries [3]. Medicinal plants contain antioxidant compounds that can be used to prevent cancer, aging, and other diseases caused by oxidants [4]. Indeed, free radicals and reactive oxygen species (ROS) react with macromolecules such as lipids, nucleic acids, pigments, and proteins, causing membrane damage, lipid peroxidation, and enzyme inactivation, thus affecting cell performance and viability [5]. In contrast, plant secondary metabolites, as a wide range of natural antioxidants, reduce oxidative stress in cells and are therefore very useful in the treatment of cancer [6].

Among plants, Apiaceae is one of the most important plant families with 3780 species and 434 genera, which are very important for the food, cosmetic, and pharmaceutical industries [7]. Many secondary metabolites such as terpenoids, phenylpropanoids, coumarins, furanocoumarins, sesquiterpene lactones, polyacetylenes (polyines), and other acetate unit derived compounds such as alkyl phthalides and toxic piperidine alkaloids are found in this family [8]. Various studies have demonstrated their antioxidant properties, as well as antimicrobial, apoptotic,

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hepatoprotective, vaso-relaxant, cyclooxygenase inhibitory, and antitumor activities [9-11] and in traditional medicine, plants of this family are used in the treatment of some human diseases [11]. In Iran, one of the most important diversity centers of this family, there are 121 genera and 360 species [12]. Due to the unique climatic conditions in Iran, 122 endemic taxa of the Apiaceae family have been listed in the Iranian flora [13].

The genus *Libanotis* (*Seseli*) belongs to the family Apiaceae and consists of about 125 to 140 species. The species of this genus are scattered in Europe, Africa, Asia, and Oceania [14, 15]. The species belonging to this genus are mainly rich in coumarins as well as terpenoids, essential oils, etc. [16] and have important medicinal activities with therapeutic effects such as inflammation, swelling, rheumatism, pain, and colds [14].

Libanotis transcaucasica Schischk is one of the most important plant species of the genus *Libanotis*, which is widely distributed from tropical to temperate regions [17]. In Iran, this species is found in northwestern Iran [18] and also in northeastern Iran in Hazar-Masjed Mountains. This species has medicinal properties and is used as a spice in some regions. Sesquiterpene hydrocarbons [19], monoterpene hydrocarbons [16], and coumarins [19] have already been isolated from this species.

However, there is no study on the effect of extracts of *L. transcaucasica* Schischk on breast cancer. Therefore, the aim of this study was to investigate the anticancer and antioxidant properties of ethanolic and methanolic extracts of seed and leaf tissues of *L. transcaucasica* Schischk from the Hazar-Masjed Mountains.

MATERIAL AND METHODS

Preparation of Alcoholic Extracts

The plant *L. transcaucasica* Schischk was collected from the mountains of Hezar-Masjed and subsequently identified by Dr. Mirtadzadini. After drying, 30 g of the seeds and leaves of this plant were ground into powder. In this step, 80% ethanol and methanol were used as solvents. The seed and leaf powder was placed in a Soxhlet extractor, each containing 500 ml of methanol or ethanol, and kept for 8 hours. This procedure was repeated three times. The extracts of *L. transcaucasica* Schischk were concentrated in a rotary evaporator for 75 min (vacuum, 45 °C) and then stored at 4 °C [20].

The MCF7 (Michigan Cancer Foundation-7) and Fibroblast Cell (Human Skeletal Muscle Cells (HSkMC)) Lines Culture

The MCF7 and fibroblast cell lines (HSkMC) were obtained from the National Cell Bank of Iran (Pasteur Institute, Iran, Tehran) and were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI1640 medium (Gibco, USA) supplemented with penicillin, streptomycin (100 U/ml and 100 µg/ml, respectively) and 10% fetal bovine serum.

Viability Assay

The inhibitory effect of ethanolic and methanolic extracts of *L. transcaucasica* Schischk on MCF7 and HSkMC was determined by MTT assay ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay) [20]. Cells were seeded at a density of 2×10^5 cells/well in a 96-well plate. To evaluate the growth inhibitory effect of *L. transcaucasica* Schischk, cells were treated with different concentrations (50, 100, 200, 300, 400, and 500 µg/ml) of ethanolic and methanolic extracts and incubated for one to three days at 37 °C in a humidified atmosphere containing 5% CO_2. One, two, and three days after the treatment, 20 µl of a 5 mg/ml MTT solution was added to the cells and incubated for 4 hours at 37 °C. After this time, the medium was removed and 200 µl of DMSO was added to each well to dissolve the formazan crystals. The absorbance of the formazan dye was measured using an ELISA plate reader at 490 and 630 nm and the optical density (OD) was recorded. All the above steps were performed three times. The following formula was used to calculate the cell growth inhibition rate:

$$\%\ \text{Cytotoxicity} = [1 - (\frac{\text{Abs 490 nm test - Abs 630 nm test}}{\text{Abs 490 nm control - Abs 630 nm control}})] \times 100$$

DPPH Radical Scavenging Activity

DPPH or 2,2-diphenyl-1-picrylhydrazyl is a stable violet- colored compound. This free radical is reduced in the presence of an antioxidant molecule, producing a colorless solution [21].

The DPPH assay was performed as follows: 1 mg of the methanolic and ethanolic extract powder was dissolved in 1 mL of absolute methanol (concentration 1000 µg/mL). Ascorbic acid was used as a positive control and was prepared at a concentration of 1000 µg/mL. For methanolic and ethanolic extracts, concentrations of 50, 100, 200, 300, 400, and 500 µg/ml were prepared from the above stocks. For ascorbic acid, similar concentrations were prepared for the extracts. 0.004% DPPH methanolic solution was also prepared. 100 µl of the prepared concentrations of the extracts were transferred to a Falcon containing 2.5 ml of methanolic DPPH solution. The solution was kept in the dark at room temperature for 30 min. The optical absorbance of the samples at a wavelength of 517 nm was measured using a spectrophotometer in comparison to the blank sample. The blank sample here is a 0.004% methanolic DPPH solution. The percentage of DPPH inhibition was calculated for each sample using the following equation, and the IC_{50}s were determined for the extracts:

$$\%\ Inhibition = \frac{\text{Absorbance } blank - \text{Absorbance } sample}{\text{Absorbance } blank} \times 100$$

Potassium Ferricyanide Reducing Antioxidant Power (PFRAP)

After the required concentrations of the extracts and ascorbic acid (as a positive control) were prepared, the antioxidant activity was measured by the PFRAP method as follows [22]:

1 ml of the sample was poured into a test tube. Then, 2.5 ml of 0.2 M phosphate buffer and 2.5 ml of potassium ferricyanide [$k_3Fe(CN)_6$] were added. The resulting solution was placed in an incubator at 50 °C for 20 minutes. Then, 2.5 ml of 10% trichloroacetic acid was added to the existing solution to stop the reaction. The flask containing the solution was centrifuged at 3000 rpm for 10 min. From the supernatant solution, 2.5 ml was carefully taken and added to the new falcon along with 2.5 ml of distilled water and 0.5 ml of ferric chloride [$FeCL_3$] (0.1%). The optical absorbance of the resulting solution was measured using a spectrophotometer at a wavelength of 700 nm compared to a blank sample. The absorbance values were plotted against concentration. A higher absorbance of the reaction mixture indicates a higher reduction capacity.

Statistical Analysis

MTT data and data from the measurement of antioxidant activity of methanolic and ethanolic extracts were analyzed using Excel and SAS software. Duncan multiple range test was used to compare the mean values. From the linear regression equations of the dose-response curve, the half-maximum inhibitory concentration (IC_{50}) and the concentration at which the scavenging activity was 50% (IC_{50}) were calculated.

RESULTS

MTTY Assays

Ethanolic Seed Extract

The results show that there is a significant difference between the effect of different concentrations of ethanolic seed extract at different times on MCF7 and HSKMC cell lines. To determine the differences between the different treatments, the Duncan mean comparison test was used. The results showed that the lethality of the extracts on the MCF7 and HSKMC cell lines increased with the increase in the concentration of the extract at each time point. With increasing time, the lethality also increased, but with a lower intensity compared with the concentration of the extract (Figure 1A). The lethality of HSKMC cells increased from a concentration of 300 µg/ml and peaked at a concentration of 500 µg/ml. Of course, the lethality of the extracts on MCF7 cells was generally higher than on HSKMC cells at most concentrations.

The IC_{50} of the effect of seed ethanolic extract on the MCF7 cell line was inversely related to increasing time and its value decreased with increasing time. This value in HSKMC cells also decreased with increasing time, but the decrease in IC50 in MCF7 cells was greater than that in HSKMC cells (Table 1).

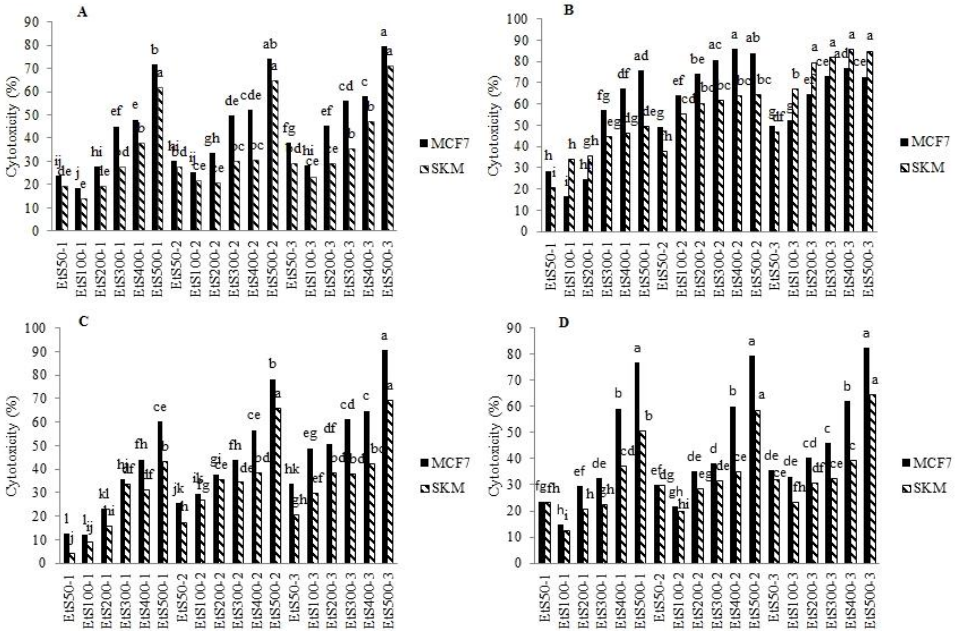

Fig. 1 The mean comparison of interaction between different times at different concentrations of ethanolic seed (A), methanolic seed (B), ethanolic leaf (C), and methanolic leaf (D) extracts (x-axis indicates different concentrations of extracts at different times (1, 2 and 3 indicating cytotoxity after 24, 48 and 72 hours) and the y-axis indicates the percentage of lethality in MCF7 and HSKMC cell lines)

Table 1 IC_{50} values of the ethanolic and methanolic extract of the seed and leaf at 24, 48 and 72 hours.

Extracts	Times	Mcf7	linear regression formula	HSKMC	linear regression formula
seed ethanolic extract	24 h	358.86	y=0.1086x+11.027	472.98	y=0.0934x+5.8228
	48 h	317.281	y=0.0993x+18.494	424.34	y=0.0817x+15.331
	72 h	249.21	y=0.097x+25.826	375.69	y=0.0918x+15.511
seed methanolic extract	24 h	296.31	y=0.1329x+10.62	464.35	y=0.0562x+23.903
	48 h	54.35	y=0.0734x+46.01	100.28	y=0.0461x+45.377
	72 h	50.52	y=0.0833x+45.791	76.90	y=0.0731x+55.614
leaf ethanolic extract	24 h	431.85	y=0.1079x+3.4031	574.45	y=0.0857x+0.7696
	48 h	301.19	y=0.1093x+17.079	418.20	y=0.0845x+14.662
	72 h	178.86	y=0.1047x+31.273	378.05	y=0.0858x+17.563
leaf methanolic extract	24 h	341.79	y=0.1274x+6.4558	528.04	y=0.0932x+0.7862
	48 h	309.85	y=0.1156x+14.181	481.48	y=0.0841x+9.5073
	72 h	259.13	y=0.1028x+23.361	429.43	y=0.0915x+10.707

Methanolic Seed Extract

The results of the mean comparison of the interaction of different concentrations of the methanolic seed extract at different times on the MCF7 cell lines showed that the lethality of this extract increases sharply with the increase of the concentration of the extract. Of course, the lethality of the extracts also increases sharply with increasing time (Figure 1B). The greatest effect of the extracts on the lethality of the MCF7 cell line occurred at a concentration of 400 and 500 µg/ml in 48 hours, at a concentration of 400 µg/ml in 72 hours, and at a concentration of 500 µg/ml in 24 hours. The highest lethal effect of the methanolic extract on the HSKMC cell line was also observed at concentrations of 300, 400, and 500 µg/ml in 48 hours, at 400 µg/ml in 72 hours, and at 500 µg/ml in 24 hours. As the concentration of the extracts increased, the lethality also increased. Time also had an important effect on lethality (Figure 1B).

The IC_{50} of the effect of the methanolic extract of the seeds on the MCF7 cell line also decreased dramatically with increasing time. Thus, this value reached 54.35 and 50.52 µg/ml after 48 and 72 hours, respectively (Table

1). In the HSKMC cell line, this value also decreased significantly with increasing time (Table 1). However, the decrease was greater in the MCF7 cell line than in HSKMC (Table 1).

Ethanolic Leaf Extract

A mean comparison of the interaction of different concentrations of ethanolic leaf extract at different times on MCF7 and HSKMC cell lines using Duncan's multiple range test showed that the effect of 500 µg/ml concentration had a greater lethal effect on MCF7 and HSKMC cell lines at 48 and 72 hours. However, MCF7 cells were more affected by the extract (Figure 1C). The least effect was observed at a concentration of 50 µg/ml of leaf ethanolic extract and on MCF7 and HSKMC cell lines at 24 hours (Figure 1C).

The IC_{50} effect of leaf ethanolic extract on the MCF7 cell line decreased with increasing time. In the case of the HSKMC cell line, this process was similar. However, the decrease in IC_{50} value was much higher in the case of the MCF7 cell line (Table 1).

Methanolic Leaf Extract

Duncan's multiple range test was used to compare the means of the interaction effects. The results showed that the lethality of the extracts on MCF7 and HSKMC cell lines was concentration- and time-dependent (Figures 1D). For both cell lines, the highest lethality occurred after 24, 48, and 72 hours and at a concentration of 500 µg/ml (Figure 1D). Naturally, the lethality of the extracts was higher in the MCF7 cell line.

The IC_{50} value of the effect of methanolic leaf extract on the MCF7 and HSKMC cell lines showed that it has an inverse relationship with time (Table 1), and with increasing time, its value decreases in both cell lines studied, although this decrease is greater in the MCF7 cell line.

DPPH Method

The results of the mean comparison of the effect of different concentrations of ethanolic and methanolic leaf and seed extracts on the inhibition of DPPH free radicals showed that in all treatments, the inhibitory effect of the extracts increased with the increase of the concentration of the extracts. Of course, the inhibitory effect of ascorbic acid was better than that of the extracts, especially at a concentration of 50 to 200 µg/ml (Figures 2A and 2B). However, the inhibitory effect of the methanolic extract of seeds was better than that of the other extracts and comparable to the inhibitory effect of ascorbic acid at a concentration of 300 to 500 µg/ml (Figures 2A and 2B). The IC_{50} of the ethanolic and methanolic extracts of seeds, ethanolic and methanolic extracts of leaves, and ascorbic acid were 310.5, 44. 64, 211.83, 111.44, and 34.47 µg/ml, respectively, with the lowest value for ascorbic acid and methanolic extract of seeds.

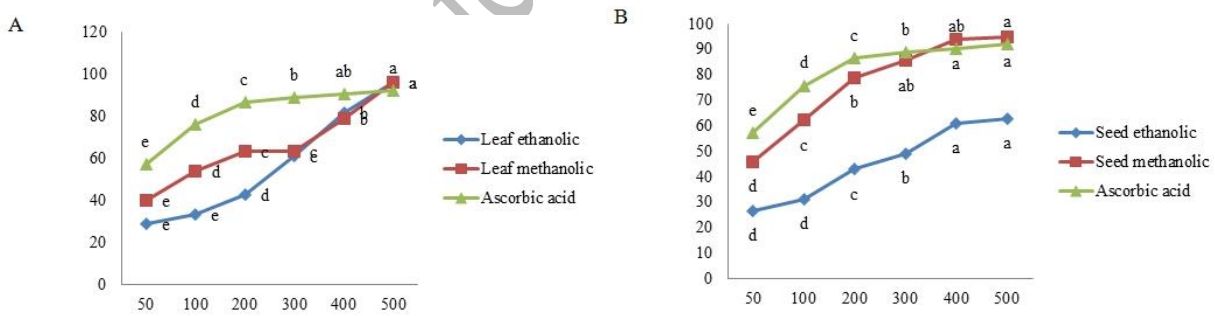

Fig. 2 The effect of DPPH free radical inhibition by different leaf (A) and seed (B) extracts; ascorbic acid was considered as control (x-axis indicates different concentrations of extracts and ascorbic acid (µg/ml) and y-axis indicates DPPH inhibition percentage)

Potassium Ferricyanide Reducing Power (PFRAP) Method

The result of the mean comparison showed that when the reducing power was measured, methanolic and ethanolic extracts had the same function at lower concentrations, but at higher concentrations, the methanolic extract showed higher reducing power than the ethanolic extract in both leaf and seed tissues (Figure 3A & 3B). Of

course, ascorbic acid showed a much stronger effect than all methanolic and ethanolic extracts. Among the extracts, the methanolic extract of seeds showed a stronger reducing effect (Figure 3A & 3B).

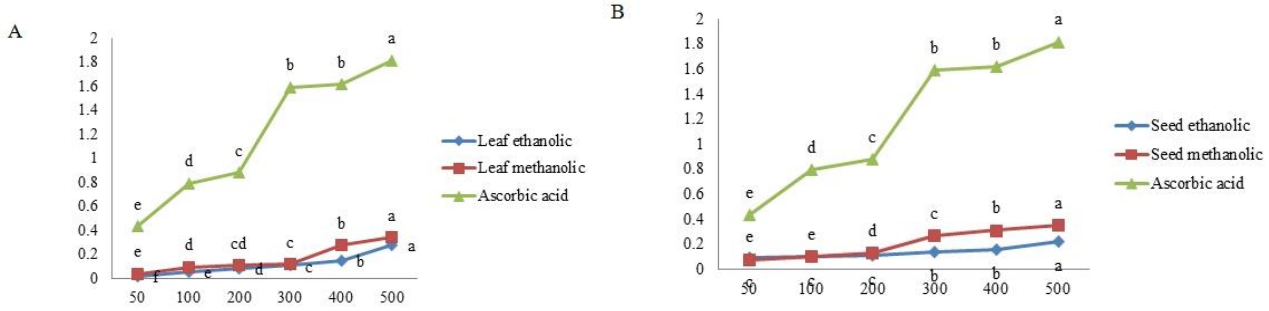

Fig. 3 Determining the antioxidant capacity of iron reduction by different leaf (A) and seed (B) extracts; ascorbic acid was considered as a control (the x-axis indicates different concentrations of extracts and ascorbic acid (µg/ml) and the y-axis indicates the absorbance values (Higher absorbance of the reaction mixture indicates a greater reducing power).

DISCUSSION

Natural products play an important role in the discovery of new drugs. More than half of newly approved drugs are of natural origin or have been developed based on natural product structures, although 40% of new drugs are synthetic compounds that mimic natural compounds [23]. Several clinical antitumor agents are also derived from plants, including paclitaxel, vincristine, and camptothecin [24]. The identification of medicinal plants with significant cytotoxic potential use for the development of cancer therapeutics has become increasingly important in the last decade, and research in this area is growing [25].

To date, there has been no study on the effect of *L. transcaucasica* Schischk extract on cancer cell lines such as MCF7. However, there are limited studies on the effect of extracts of other plants belonging to the Apiaceae family. The cytotoxic activity of *Seseli petraeum* essential oil showed potent cytotoxicity against treated MCF7 cells (IC_{50}=390.38 µg/mL) [26]. The essential oil of *Seseli tortuosum* also had cytotoxic properties on human cells at concentrations greater than 0.64 µl/mL [27]. The hexane extract of the root of *Seseli petraeum* M. Bieb. inhibited the growth of A549 cancer cells at the 24th hour with an IC_{50} value of 3.432 mg/mL [28].

In our study, the cytotoxic effect of ethanolic and methanolic extracts of seeds and leaves of *L. transcaucasica* Schischk was evaluated based on the minimum concentration of the extract that reduces the viability of cancer cells by at least 50% (IC_{50}). Four categories of extracts: highly active ($IC_{50} \leq 20$ µg/ml), moderately active ($20<IC_{50}<100$ µg/ml), less active ($100<IC_{50}<1000$ µg/ml), and inactive ($IC_{50}>1000$ µg/ml) was considered [29-31]. For a pure compound or drug, an IC_{50} value of less than 4 µg/ml is considered effective [32, 33].

In our study, the IC_{50} analysis showed that only the methanolic extract of the seed had a moderate effect on cancer cells at 48 and 72 hours, and the IC_{50} values were 54.35 and 50.52 µg/ml, respectively, and the rest of the extracts had a weak effect on the MCF7 cell line and their IC_{50} ranged from 178.86 to 431.85 µg/ml. In general, the methanolic extract of seeds showed the highest toxicity to the MCF7 cell line in 72 hours, and the ethanolic extract of leaves showed the least toxicity in 24 hours. In this study, the MCF7 cell line was found to respond very differently to ethanolic and methanolic extracts of seeds and leaves. It was already known that the differences in cell line, plant extract, solvent used and plant source contribute to the differences in the effect of plant cytotoxicity [34]. However, the results of our research have shown that in addition to the plant source and the type of extract, the type of tissue from which the extract is taken, the concentration of the extract, and also the duration of treatment can be very effective in determining the extent of toxicity. Similar to our results, Kavaz and Faraj (2023) showed that the application of increasing concentrations of *Juniperus sabina* and *Ferula communis* leaves methanolic extracts (10, 20, 50 and 150 µg/ml vs. control) showed a significant decrease in the cellular viability of breast cancer lines dependent on concentration. This may be due to increased bioactive compounds in the extract as the concentration increases [35].

Antioxidant properties can be very useful in preventing the harmful effects of free radicals in diseases such as cancer. DPPH radical scavenging is a recognized method for screening the antioxidant activities of plant extracts

[36]. It is believed that the effect of extracts on DPPH is due to their ability to donate hydrogen [37]. Many researchers have used this method to study the antioxidant properties of various plants. The methanolic extract of *Tabebuia pallida* leaves had similar radical scavenging activity compared to standard butylated hydroxytoluene (BHT) [36]. Önder *et al*., (2020) indicated that the ethyl acetate extract of *S. peucedanoides* (IC_{50}=0.49 mg/mL) and *L. transcaucasica* Schischk (IC_{50}=0.75 mg/mL) (Turkish *Seseli* species) had the strongest radical scavenging capacity in the DPPH assay. However, the methanolic extracts of *S. resinosum*, *S. gummifeum* subsp. gummiferum, and *S. peucedanoides* had the highest scavenging activity (IC_{50}=0.086, IC_{50}=0.088, and IC_{50}=0.091 mg/ml, respectively) [14]. IC_{50} of *L. transcaucasica* Schischk was 0.187 mg/ml. Also, by comparing three *Seseli* taxa, Matejić *et al*., (2012) showed that the extract of *S. libanotis* ssp. libanotis possessed the strongest antioxidant activities in the DPPH assay [38]. In the DPPH assay, the best properties were observed with the solvents ether, acetone, and methanol. Methanol leaf and fruit extracts of *Seseli rigidum* (SC_{50} = 159.20 and 116.96 mg/ml, respectively) showed the best activity [39]. The strongest antioxidant properties of *Seseli rigidum* Waldst. Et Kit. using the DPPH assay were exhibited by the aqueous (46.15 µg/ml) and methanolic (98.95 µg/ml) extracts [40]. In another study, the Highest radical scavenging activity using DPPH assay was observed in the *Euphrasiae stricta* (IC50 = 38.972 lg/mL), *Euphorbia platyphyllos* L. (IC_{50} = 40.817 lg/mL), and *Epimedium brevicomum* Maxim (IC50 = 46.265 lg/mL). Compared to ethanolic and methanolic extracts, the ascorbic acid scavenging activity was better (IC_{50} = 37.337 lg/mL) [41]. In our study, the inhibitory effect of the extracts increased with increasing concentration, with the lowest IC_{50} assigned to ascorbic acid (34.47 µg/ml) and methanolic extract of seeds (44.64 µg/ml). Based on the DPPH test, it seems that *L. transcaucasica* Schischk collected from the Hezar-Masjed Mountains has a high antioxidant capacity and can be considered a strong source of antioxidants.

In the PFRAP test, potassium ferrocyanide, formed by the reaction of antioxidant compounds with potassium ferricyanide, reacts with ferric chloride, and the resulting complex can be measured spectrophotometrically at 700 nm. According to the literature review, there is no study on the antioxidant properties of *Libanotis* species based on the PFRAP assay. The PFRAP activity of an extract depends on the ability of its antioxidants to reduce free radicals by donating an electron. The antioxidant activity of Rhizoma imperatae using the PFRAP assay increased with increasing extract concentration, which was dose-dependent and comparable to ascorbic acid [42]. Ethyl methyl ketone extract shows higher activity than methanolic extract using PFRAP assay in *Litsea quinqueflora* (Dennst.) Suresh [43]. The reducing power of *Euclea schimperi* leaf extracts in different concentrations of stock solutions for each solvent (methanol and chloroform) increased with increasing concentration. The reducing power of the methanolic extract was significantly higher than that of chloroform [44]. In our study, the antioxidant properties of methanolic and ethanolic extracts of *L. transcaucasica* Schischk leaves and seeds were dose-dependent. High concentrations had higher antioxidant properties. Of the extracts, the methanolic extract of seeds exhibited higher reducing power. It seems that depending on the plant species, plant tissue, the solvent used, and concentration of the extracts, the reducing power of the extracts in the PFRAP assay may be very different.

In this study, the methanolic extract of the seed had the greatest effect on inhibiting DPPH free radicals, with an IC_{50} of 44.64 µg/ml, and at a concentration of 300 µg/ml, this inhibitory effect was comparable to that of ascorbic acid. Although the reducing effect of the methanolic extract in the PFRAP assay was not comparable to that of ascorbic acid, it was relatively better than that of the ethanolic extract. Considering that the methanolic extract of the seed had the most lethal effect on cancer cells, it seems that the extract that has a stronger antioxidant power also has a stronger anticancer effect.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of institute of science and high technology and environmental sciences, Graduate University of Advanced Technology, Kerman, Iran, under grant number of 98.2214.

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