The Evaluation of Antioxidant and Anticancer Activity of Alfalfa Extract on MCF7 Cell Line

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Introduction: The Breast cancer is the second most common cancer in the women. Natural products extracted from medicinal plants can play an important role in cancer treatment. These compounds are probably effective in repairing damaged tissue in cancer. This study was designed to investigate antioxidant activity and cytotoxicity of ethanolic extract of alfalfa on breast cancer MCF7cell line. **Materials and Methods**: For this purpose, the MCF7 cells line was cultivated and proliferated. Then, the cells exposed to different concentrations of alfalfa (25, 50, 100, 200, and 400 µg/ml) and were incubated for 24, 48, and 72 hours. After the incubation period, the colorimetric MTT method was used to determine cytotoxicity. Also, the total amount of phenol, flavonoid, anthocyanin, and carotenoid as well as the antioxidant activity of the extract were determined. **Results**: The results showed that the different concentrations of ethanolic extract reduced significantly the growth of cells as compared to that of control (P<0.05). The most pronounced growth inhibition recorded as 83.74% at the concentration of 400 µg/ml. The extract of alfalfa is a rich source of antioxidant compounds, as phenolics and carotenoid contents of extract were 32.63±0.17 mgGAE/g DW and 33.41±0.22 mg/g FW respectively. Also, its different concentrations affected scientifically on DPPH radical inhibition as the highest inhibitory effect (53.4%) was recorded at concentrations of 3.4 mg of extract. **Conclusion**: These results suggest that the ethanolic extract of alfalfa have the most antioxidant activity and cytotoxicity against MCF7 cell line. It seems to come with further research, and utilizes its compound in cancer treatment.

Keywords: Alfalfa Extract; Antioxidant; Cancer; MCF-7 Cell line, Breast Cancer

Introduction

Today, cancer is considered the second reason for death after heart diseases, and breast cancer is the most common cancer in women between the ages of 40-44 years. Such cancer is a histologic diagnosis making standardized pathologic criteria and that develops from breast tissue (1). Breast cancer most commonly presents as a lump that feels different from the rest of the breast tissue. The earliest breast cancers are detected by a mammogram and Lumps finding in lymph nodes located in the armpits may also indicate breast cancer; however, more than 80% of cases are discovered when a person detects such a lump with the fingertips (4). There are some various methods to treatment such cancer including chemotherapy, radiotherapy, and surgical methods. However, these methods are not sufficient for treatment due to side effects and drugs that lower the immune system.

Conventional cancer treatments have serious side effects and the anticancer drugs used previously exhibited relatively high toxicity not only to the tumor cells, but also to the normal cells of the body part in which the cancer had developed (5). So one of the major concerns of the medical community in the world is to access to high efficiency, low toxicity, and inexpensive drugs that specifically affect calls. (6). One of the recommended methods to treat these diseases is to use medicinal herbs. Nowadays, in the process of many drugs, plants were utilized as one of the main sources of biological materials in treating cancers. In this regard herbal medicines are more important in preventing cancers because of the lack of side effects. Accordingly, medical plants are a

great source of hope for discovering new drags (7, 8). In fact, the presence of antioxidant compounds in plant can have anti-cancer effects relating to enzyme inhabitation, DNA reparation, stimulating antitumor enzymes in cells, regeneration of damaged tissue, and strengthening the immune system (9, 10).

Alfalfa (*Medicago sativa* L.) is a perennial herbaceous plant from the fabaceae family with various herbal chemicals such as alkaloids, amino acids, main enzymes, phytosterol, phytoestrogen, flavone, flavonoid, minerals, saponin and vitamins (11). Although, there are a few studies reporting the anticancer effect of alfalfa on various cancer cell lines, there are no comprehensive studies on the anticancer effects of alfalfa on breast cancer. Thus, the main purpose of this study was to investigate the antioxidant compound and the inhibitory effects of this plant on the growth of breast cancer cells, so that this plant may be economically exploited and hoped in the future as anticancer drug without side effects.

Materials and Methods

Plant collection and extraction

Alfalfa plant was collected from Lahijan region in Gilan province, Iran. Then, this plant dried at room temperature in dark and powdered. 30 gram of powder sample was extracted in 300 ml of ethanol (48h and 24h later) twice. Then, ethanolic extract was evaporated to dryness at 40°C using a rotary evaporator and the crude extract was kept at 4°C (12).

Measurement of anthocyanin

To measure the total anthocyanin content, 0.02 g of dried plant sample was extracted with 4 ml of hydrochloric acid containing 1% methanol in a porcelain mortar. The solution was kept in the refrigerator for 24 hours and then, centrifuged for 10 minutes at 13000 g. The supernatant was removed and absorbance of the extract was measured at 530 and 657 nm against the control (hydrochloric acid containing 1% methanol). The anthocyanin content of each extract was calculated using the following equation (13).

$A= A_{530}-(0.25\times A_{657})$

Where, A is absorbance of the solution (subscripts indicate the wavelength at which the absorbance is measured).

Measurement of total phenolic content

The total phenol content was determined by the Folin–Ciocalteu method (14). A volume of 2.8 ml of distilled water, 100 µl of Folin–Ciocalteu reagents and 2 ml of sodium carbonate 2% were added to 100 µl of supernatant and incubated for 30 minutes. The absorbance of sample was measured at 720 nm compared to the control. Gallic acid was used for the preparation of calibration curve (20-200 mg L^{-1}). The data was expressed as mg gallic acid equivalents (GAE) g^{-1} DW.

Measurement of total flavonoid content

For measurement of total flavonoid, 0.1 g of samples was homogenized in methanol 80 % using mortar and pestle. Homogenates were centrifuged at 10000 g for 5 min and then the 500 µL of supernatants, 1.5 ml of 80 % methanol, 100 µL of 10 % aluminum chloride solution, 100 µL of 1 M potassium acetate, and 2.8 ml of distilled water were added to 500 µL of each extract. After 40 minutes, absorbance of the mixture was measured at 415 nm compared to the control. Quercetin was used for the preparation of calibration curve (20-200 mg l-1). The total flavonoid content of the extract was reported as milligram quercetin equivalents (QE) g^{-1} DW (15).

Measurement of carotenoid content

0.05 gram of fresh plant tissue was homogenized with 5 ml acetone in a cold porcelain mortar and ice bath. Then, 1 g anhydrous sodium sulfate was added to the filtrated solution and was filtrated by filter paper. Solution was made to 10 ml volume by adding acetone and was centrifuged at 2600 g for 10 min. Supernatant was collected and solution absorbance was measured in 662, 645 and 470 nm against acetone blank. The amount of carotenoids was measured by following formulas for each extract (16):

$C_a=11.24\ A_{662}-2.04\ A_{645}$

$C_b=20.13\ A_{645}-4.19\ A_{662}$

$C_t=1000\ A_{470}-1.9\ A_{Ca}-63.14\ A_{Cb}/214$

In these formulas C_a, C_b, and C_t: the amount of chlorophyll a, b, and carotenoid, A_{470}: absorbance in 470 nm (for carotenoid), A_{645}: absorbance in 645 nm (for chlorophyll a), A_{662}: absorbance in 662 nm (for chlorophyll b).

Antioxidant activity assays by DPPH method

Measuring antioxidant activity by scavenging 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was done with slight modifications of Brand-Williams *et al.*, (1995) method (17). Briefly, the concentrations (100–2000 µL) of extracts were prepared in ethanol. DPPH solution (0.004%) was prepared in ethanol and 2 mL of this solution was mixed with the same volume of ethanol extracts and standard ascorbic acid solution separately. The mixture was incubated for 30 minutes in the

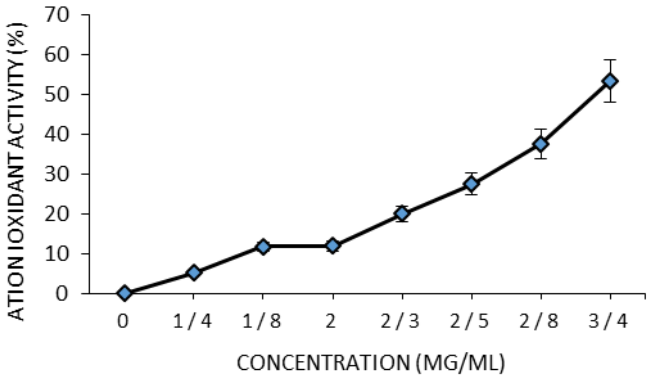

Figure 1. The effect of different concentration of alfalfa extract on antioxidant activity. The data represent the mean of three replications and error bars indicate SD.

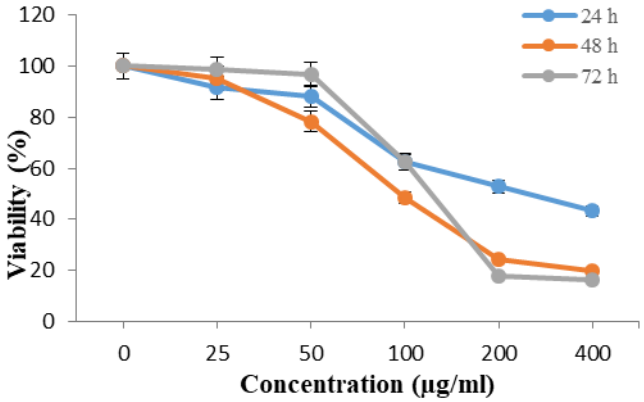

Figure 2. Effect of different concentration of alfalfa extracts on the viability of cancer cells within 24, 48, and 72 hours

dark at room temperature and the absorbance was measured at 517 nm. The results were expressed as inhibition of free radical by DPPH in percent (%I), and calculated by using the equation:

$A_{sample})/A_{control}] \times 100\text{-}\%I=[(A_{control}$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the plant extract), and A_{sample} is the absorbance of the sample.

Cell line

MCF7 cell line was purchased from Pasteur Institute of Iran. This cell line was cultured in a media containing enriched RPMI 1640 with 10% fetal bovine serum (FBS), streptomycin (100µg/ml) and penicillin (100 U/ml) and kept in an incubator at 37°C, 5% CO_2 and 95% humidity. The culture media were changed twice a week.

Assessment of cell viability and MTT assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide) assay was used to evaluate the effect of alfalfa extract on cell viability. Briefly, MCF7 cell line was seeded in 96-well plates (1×10^4 cells/well) and exposed to serial concentrations of Alfalfa extracts (25, 50, 100, 200, and 400 $\mu g^{-1}ml$) for 24, 48, and 72 h. MTT (0.5 mg/mL) was then added to each well, and cells were further incubated for 4 h at 37 °C. Supernatants were then removed, and 700 µL of DMSO were added to each well to dissolve the formazan product. Absorbance at 540 nm was measured using a microplate reader (BioRad, Hercules, CA). Optical density values of the control cells were calculated as 100% viability. Cell viability percentage was calculated by the following formula (18).

Cell viability (%)=sample OD/ control OD×100

Statistical analysis

All measurements were conducted with three replications and data were reported as mean±standard deviation (SD). The data were analyzed using GLM procedure by SPSS software (Ver.16) and Duncan test was used for mean comparisons at 1 % probability level. SPSS software was used to calculate the correlation coefficient (Pearson) between characteristics. Microsoft excel 2013 software was used for the preparation of figures.

Results

Antioxidant activity by DPPH

Antioxidant activity by DPPH is based on donating electron or hydrogen atom. The results indicated that different concentration of alfalfa extracts showed good antioxidant activity and the highest percentage of inhibition was observed in concentration of 3.4 mg/ml (53.4%) (Figure 1).

Phenol, flavonoid, anthocyanin and total carotenoid contents

The results indicated that alfalfa ethanol extract is a rich source of antioxidant compounds, as phenolics and carotenoid contents of extract were 32.63±0.17 mgGA E/g dw and 33.41±0.22 mg/g fw respectively (Table 1).

Anticancer potential of alfalfa extract in different concentrations and times

The resulted showed that different concentrations of alfalfa extract inhibited the growth of cancer cells at different times and the effect of different concentrations of the extract was significant so that by increasing the extract concentration at 24, 48, and 72 hours, the viability percentage decreased

Table 1. The effect of alfalfa on non- antioxidant compound. The data represent the mean of three replications ±SD

Sample	Total phenol (mgEGA/g DW)	Total flavonoieds (mgEQ/g DW)	Total anthocyanin (mg/g DW)	Carotenoids (mg/g FW)
Extract of alfalfa	32.63±0.172	13.58±0.641	1.11±0.008	33.41±0.223

mgEGA/g DW: milligram gallic acid equivalents per gram dry weight, mgEQ/g DW: milligram quercetin equivalents per gram dry weight, and mg/g FW: milligram per gram fresh weight

Table 2. Correlation between growth inhibition of cancer cells and some non-enzymatic antioxidant compounds of alfalfa extract

	Phenol content	Flavonoid content	Anthocyanin content	Carotenoid content	Inhibition of cancer cell growth
Inhibition of cancer cell growth	0.293 ^{ns}	0.802 **	0.662 *	0.568 ^{ns}	***1***
Carotenoid content	0.178^{ns}	-0.724 *	0.089 ^{ns}	1	
Anthocyanin content	0.155 ^{ns}	0.598 *	1		
Flavonoid content	0.257 ^{ns}	1			
Phenol content	1				

** Correlation is significant at 0.01 levels; * Correlation is significant at 0.05 levels; ^{ns} Correlation is not significant

(Figure 2). The results showed that in 24 hours the highest reduction of cancer cell, viability was observed in the concentration of 400 µg/ml of extract of 43.26%. Therefore, the highest inhibition of cancer cell growth was obtained at 56.74%. The results also showed that the highest percentage of inhibition of cancer cell in the concentrations of 200 and 400 µg/ml of extract was obtained in 75.77% and 80.09% in 48 hours, respectively. During 72 hours, the results indicated that different concentrations of alfalfa extract had a significant effect on the growth inhibition of cancer cells, so that the greatest inhibition of cell growth at concentrations of 200 and 400 µg/ml was obtained at 82.23 and 83.74%, respectively. The results also showed that the extract of this plant at concentrations of 25 to 50 µg/ml also decreased the inhibition of cancer cell growth with a slight decrease, but no significant difference was observed with the control sample.

Correlations among measured factors and inhibition of cancer cell growth

The results of correlation indicated that there was a positive and significant correlation between inhibition of cancer cells growth and flavonoid content at the level of 1% and anthocyanin content at the level of 5%. Therefore, increasing the contents of flavonoids and anthocyanin in this extract increased the inhibition of cancer cell growth. Also, the results shown that there was positive correlation between total phenolic content and cancer cells growth inhibition, but it was not significant (Table 2).

Discussion

In recent years, the use of natural compounds to fight cancer due to its low side and hopeful effects has been considered. Researches were conducted to investigate the anticancer effects of medicinal and native plants in different countries (19, 20). Medical therapy has been one of the useful methods to cure patients with cancers and different research centers in the world and in Iran try to provide effective drugs with selective impacts on cancer cells and less effect on healthy cells (20, 21). Medicinal plants are great and hopeful sources to detect new drugs. Therefore, alfalfa plants were collected from their natural habitats and then studied. The results of this study indicated that different concentrations of alfalfa ethanolic extract inhibited breast cancer MCF7 cell line and significantly decreased their growth in comparison with the control. It has also been shown that the extract of the alfalfa in a concentration-dependent manner inhibited the growth of these cells, so that by increasing the concentration of the extract, it increased the percentage of growth inhibition, and the highest percentage was 83.74% at the concentration level of 400µg/ml after 72 hours (Figure 2). Moreover, the findings indicated that alfalfa extracts contained antioxidant compounds like flavonoid, phenol, carotenoid, and anthocyanin and that there was a positively significant correlation among flavonoid and anthocyanin contents and the cancer cells growth inhibition; accordingly, the increases in their contents in plant enhanced cells growth inhibition (Tables 2). The compounds may inhibit cell cycle or activate

their checkpoints to prevent DNA replication and/or activate internal and external pathways of apoptosis (22, 23). Previous studies indicated that antioxidant compounds like phenolic acids, polyphenols, and flavonoids prevented or suppressed free radicals like hydroperoxide, superoxide, and hydrogen peroxide and also prevented oxidative processes resulting damage or mutation in genome. Flavonoids prevented immune and cell processes related to cancer development like cell proliferation, cell differentiation, and generating new vessels (9). Other reports stated that quercetin and poly hydroxulated flavonoids inhibited cancer cell growth in vitro and decreased DNA generation 14% comparing to control group and prevented passing cells from G_1 phase of cell cycle to S phase (24). Some researches stated that medicinal plants contained antioxidant compounds with cytotoxic effects these compounds could induce apoptosis pathway and decrease tumor cells growth by activating autophagy-related apoptosis pathway and transcription factor BOX_2 (25, 26). Although studying about the cytotoxic effects of alfalfa extracts is very low, some reports indicated that plant extracts displayed different anti-cancer effects in various cancers or even on same cell lines. In most studies, there was a correlation between phenolic contents and the extracts antioxidant capacities and their effect on cancer cells. The compounds affected cancer cells through different ways; while in most studies compounds of plant extract induced apoptosis in cancer cells and then increased *caspase3* gene expression. Some reports have also shown that extract compounds increased antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase and non-enzymatic antioxidant compounds removing free radicals and decreased new cancer cells generation (27, 28). Also, Natural products extracted from medicinal plants can play an important role in repairing damaged tissue. The biochemical pathways and extract mechanisms of alfalfa extracts for inhibiting breast cancer cells were not investigated by this study, but this study demonstrated that alfalfa extract contain high amount of antioxidant compounds and there was a positively significant correlation between antioxidant compounds and cancer cells growth inhibition. It indicated that the compounds may inhibit cancer cells growth through one of the mentioned pathways.

Conclusion

The results of this study indicated that ethanolic extract of alfalfa contains different antioxidant amounts. In addition, the extracts of this plant can inhibit cells growth because of their dose or concentration dependent effects on MCF7 cancer cell line. Therefore, future pharmacological studies can be useful for the inhibition of such cancer and finely, those may be hopeful for providing an anticancer drug to therapy that.

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Conflict of Interest: 'None declared'.

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