Evaluation of the Effect of Alfalfa Extract on Breast Cancer

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ABSTRACT

Introduction and Objective: Breast Cancer is the most common cancer among women. Isoflavones, as a subset of phytoestrogens (plant estrogens) have properties similar to mammalian estrogens. Alfalfa is rich in isoflavones. This study was conducted to evaluate the effect of alfalfa isoflavones on breast cancer and lipid profile in these patients.
Methods: Thirty BALB-C mice weighing 17±2 gr were selected. The mice were divided into four groups. The first and the second group were afflicted with breast cancer through cell implantation. The third and the fourth groups were healthy. The alfalfa extract was prepared through vacuum distillation. The first and the third groups received alfalfa extract. The second and the fourth groups (control groups) received no medication. After 6 weeks, the sera of the mice were tested for the concentration of estradiol, LDL-cholesterol, HDL-cholesterol, and total cholesterol. Statistical analysis was performed by t-test using Graphpad statistical software. The level of significance was considered P=0.05.
Results: The level of estradiol, total cholesterol, and LDL-cholesterol decreased significantly in the first group versus the second group (P<0.00 for all). The level of HDL-cholesterol increased insignificantly in the first group when compared to the second group (P=0.09).
Conclusion: Alfalfa extract may be useful in mice with breast cancer through affecting the level of estradiol and the profile of lipid.
KEY WORDS: Breast Cancer, Alfalfa, Isoflavin

INTRODUCTION

Breast cancer is the most common malignancy of women with an estimated 1.38 million new cases in 2008 (23% of all malignancies) and the second most common malignancy in the world (10.9% of all cancers). It has been recently discovered that the prevalence of breast cancer in developed and developing countries is about 690,000 cases in each region (population ratio 1:4). The prevalence of breast cancer varies from 19.3 in 100,000 women in East Africa to 89.7 in 100,000 women in the west of Europe with a higher prevalence in developed parts of the world (except for Japan) and lower prevalence (less than 40 in 100,000 women) in most of the developing countries. The higher prevalence of cancer in certain geographical locations highlights the role of environmental risk factors in the pathogenesis of breast cancer (1). Part of the increase in the prevalence of breast cancer can be attributed to the change of the reproduction pattern like later age at first pregnancy and having fewer children (2). Although the increase in the prevalence of breast cancer in Iran in the recent four decades has been less than other countries, it is still the most common malignancy in Iranian women (3).

Isoflavones are a subgroup of estrogens. Phytoestrogens are natural compounds found in plants. Isoflavones, comstans, and lignans are three major subgroups of phytoestrogens. Isoflavones have been studies more than other phytoestrogens and have properties similar to mammalian estrogens (4).

Isoflavones are abundant in soybean and its products and also in alfalfa. Many studies have evaluated the chemical compounds of alfalfa including flavones and isoflavones. Alfalfa isoflavones include tricin, genistein, diadzein, Biochanin A, formononetin, 5′-methoxysativan, coumarin derivatives [coumestrol, medicagol, sativol, trifoliol, lucernol, daphnoretin, and pectin methylesterase (5). The fasting plasma levels of isoflavones are about 500 nm/l in Asians with a half-life of 6-9 hours. Most isoflavones in the plasma are in the conjugated form bound to glucuronic acid and 3% circulates in the free form. Isoflavones become

active by the bacteria in the small intestine during digestion and absorption in the human and animal body. Then, the active form of isoflavones is absorbed in the small intestine. It is mostly taken up by the liver and a little amount of it is excreted in the urine (6-8). Phytoestrogens are similar to 17 β-estradiol functionally and structurally. Phytoestrogens bind to estrogen receptors in the blood. This bond is weaker than endogenous estrogens. However, these compounds are capable of producing estrogen effects. Considering the high isoflavones content of alfalfa, the present study was conducted to evaluate the effects of alfalfa isoflavones on the lipid profile in animals with breast cancer (9,10).

MATERIALS AND METHODS

In this study, 30 BALB-C mice weighing 17±2 gr were used as animal models. The mice were purchased from Pasteur Institute, Karaj, Iran. The animals were kept at 23±2°C with light/dark cycles of 12 hours and were fed with the special condensed food for rodents (Dam Pars, Tehran, Iran). The mice were randomly divided into four groups: breast cancer receiving medication (10 mice), breast cancer without medication (10 mice), control group receiving medication (7 mice), and control group without medication (3 mice).
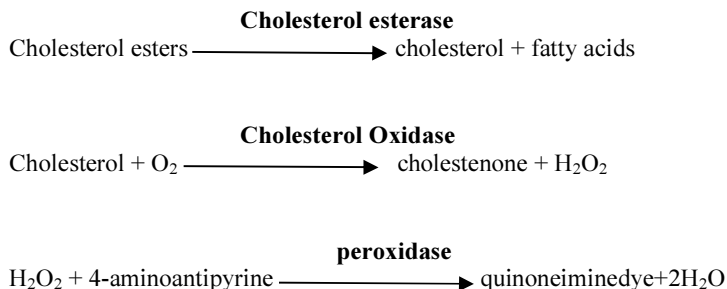
The first and the second group were afflicted with breast cancer through cell implantation (11). In this method, one BALB-C mouse with breast cancer was selected and after confirming the tumor and the spontaneous breast cancer model, the tumor was excised under sterile conditions and kept in sterile normal saline. In the solution, the tumor was cut into 3 mm^2 pieces. Then, the mice in the first and second groups were anesthetized with intraperitoneal injection of ketamine (10 mg/kg) and xylene (5 mg/kg). The tumor pieces were surgically implanted subcutaneously in the left flank and the site of operation was sutured with a special clips (12). The growth of the tumor was palpable 10 days after the procedure. The mice in the first (breast cancer receiving medication) and third (control receiving medication) groups were injected intraperitoneally with 0.1cc of the alfalfa extract daily for 6 weeks.

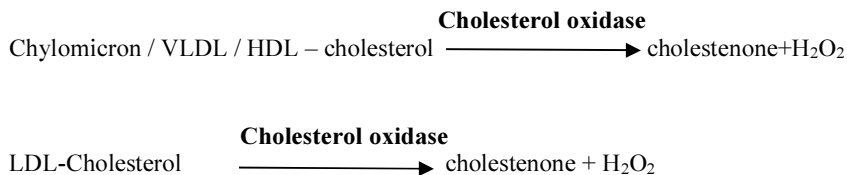Alfalfa was prepared from the garden of the Faculty of Agriculture of Tehran University.

Then, flowers and leaves was powdered by grinder in the laboratory of Pharmacognosy, Faculty of Pharmacy.
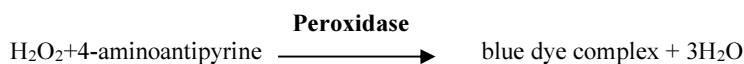
The powder was macerated in ethanol 70% for 48 hours. Ethanol was changed three times during maceration. Then, the extract was prepared using the vacuum distillation method in the chemistry laboratory of School of Allied Medical Sciences of Tehran University of Medical Sciences (13). The extract was injected daily to mice in groups one and three for six weeks. After that, 3cc of blood was drawn from the hearts of the mice and sera were separated through centrifugation at 3000 rpm. The sera were kept at -20°C for laboratory tests.

The amount of total cholesterol was measured based on cholesterol hydrolysis by enzyme.

$$\text{Cholesterol esters} \xrightarrow{\textbf{Cholesterol esterase}} \text{cholesterol + fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textbf{Cholesterol Oxidase}} \text{cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} \xrightarrow{\textbf{peroxidase}} \text{quinoneiminedye} + 2H_2O$$

The light absorbance of the color product was read at 520 nm. LDL-cholesterol was measured via the direct method, which requires no sample preparation.

$$\text{Chylomicron / VLDL / HDL – cholesterol} \xrightarrow{\textbf{Cholesterol oxidase}} \text{cholestenone} + H_2O_2$$

$$\text{LDL-Cholesterol} \xrightarrow{\textbf{Cholesterol oxidase}} \text{cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} \xrightarrow{\textbf{Peroxidase}} \text{blue dye complex} + 3H_2O$$

The light absorbance of the color product was read at 546 nm. HDL-cholesterol was measured via the direct method. The light absorbance of the color product was read at 600 nm. All three parameters were measured using the Zist Shimi kit.

Estradiol was measured using the DRG-Estradiol ELISA kit (DRG Instruments GmbH, Germany) (Cat. No. EIA-2693).

This kit is based on a solid-phase ELISA method.

The wells are coated with a rabbit polyclonal antibody. The endogenous estradiol of the mice competes with conjugated estradiol to bond with the antibody. After adding the substrate, the intensity of the color is inversely proportional to the concentration of estradiol in the sample.

RESULTS

Cholesterol, HDL, LDL, and estradiol were measured in four groups of mice.

Group 1 comprised 10 mice that were afflicted with breast cancer through cell implantation and received 50 mg of alfalfa extract daily for six weeks intraperitoneally. Group 2 comprised 10 mice that were afflicted with breast cancer through cell implantation but received no medication. Group 3 included 7 mice as the placebo group that received 50 mg of alfalfa extract daily for six weeks intraperitoneally. Group 4 included 3 mice as the control group.

In the first group, 2 mice on days one and five after implantation died due to transplant rejection. Moreover, in the second group, one mouse died on day 4 and one mouse died on day 15. The changes of estradiol, total cholesterol, LDL, and HDL are shown in Table 1, 2, 3, and 4, respectively.

	Esteradiol (pg/ml)			
	group 4	group 3	group 2	group 1
P-value	23.55	22.97	30.5	30.14
	15.79	26.08	31.01	28.33
	21.45	24.71	29.13	21.73
		25.49	27.31	20.79
P<0.00		28.58	31.3	19.49
		21.76	30.57	29.56
		23.54	30.21	27.68
			30.86	27.39
t=0.01				
تعداد	3	7	8	8

Table1: Changes of estradiol in different groups after 6 weeks

	Total Cholesterol (mg/dl)			
	group 4	group 3	group 2	group 1
P-value	112.8	166.4	231.2	167.2
	126.4	163.2	212	169.6
	147.2	219.2	285.6	96
		192.4	297.6	108
		136	250.4	208.8
		162.4	256.6	128.8
P<0.00		126.4	229.6	174.4
			236	125.6
t=0.01				
تعداد	3	7	8	8

Table 2: Changes of cholesterol in different groups after 6 weeks

	LDL (mg/dl)			
	group 4	group 3	group 2	group 1
P-value	117.56	127.78	234.76	161.11
	137.87	119.68	142.7	134.41
	118.38	138.05	164.53	138.1
		128.89	239.37	105.87
		174.92	276.19	103.36
		124.95	183.36	142.69
P<0.00		142.22	192.35	115.08
			174.81	129.75
t=0.001				
تعداد	3	7		8

Table 3: Changes of LDL-cholesterol in different groups after 6 weeks

	HDL (mg/dl)			
	group 4	group 3	group 2	group 1
P-value	35.5	30.19	53.92	66.86
	51.76	28.03	47.45	45.29
	47.45	776.45	69.01	58.23
		36.66	75.49	84.19
		47.45	40.98	90.58
		81.96	60.39	69.01
P<0.09		40.18	49.6	56.08
			34.5	76.64
t=0.3				
تعداد	3	7	8	8

Table 4: Changes of HDL-cholesterol in different groups after 6 weeks

*In the first group, 2 mice on days one and five after implantation died due to transplant rejection. Moreover, in the second group, one mouse died on day 4 and one mouse died on day 15

The difference in the mean estradiol between group one and group two was -4.473±1.574 which was significant (P<0.00). Therefore, t-test of group one and two was 0.01.

The difference in the mean total cholesterol between group one and group two was -103.7±17.21 which was significant (P<0.00).

The difference in the mean LDL-cholesterol between group one and two was -72.21±17.31 which was significant (P<0.00). T-test for group one and two was 0.001.

The difference in the mean HDL-cholesterol between group one and two was 14.44±7.247. According to Graph 4, HDL-cholesterol increased in group one but the difference was not significant (P=0.09). T-test of group one and two was 0.3.

Statistical analysis was performed by student t-test using Graphpad statistical software.

DISCUSSION

Breast cancer is the most common cancer in Iranian women and the second most lethal cancer in women after lung cancer. The incidence of this disease is increasing fast. Part of the increase can be attributed to the change of reproductive pattern like later age at first pregnancy or having fewer children (14,15,16). In total, 40,000 people are afflicted with breast cancer in Iran and more than 7,000 patients are added to this population annually (17).

The breast tissue is mainly regulated by estrogen and progesterone. Proliferation of the breast epithelial cells is widely used as a marker of exposure to hormones or their effects. Since the focus of the study was the estrogen-like properties of isoflavones, knowledge of the relationship between estrogen and

breast cancer may provide a perspective in this regard (18,19).

Many major epidemiological risk factors of breast cancer are associated with endogenous estrogen. For example, increased exposure to endogenous estrogen in conditions like the late onset of menopause or early onset of menstruation increases the risk of breast cancer (20-22). On the other hand, removal of the ovaries before menopause decreases the risk of breast cancer (23).

Isoflavones are active biological compounds that are found in plant-based food substances. Isoflavones are functionally and structurally similar to 17 β-estradiol and are capable of producing estrogen effects (24). They have drawn the attention of the researchers for the therapeutical properties like prevention and treatment of cancer, cardiac problems, and osteoporosis and relieving the menopausal symptoms (25). The role of isoflavones in cancer treatment, especially tumors controlled by endocrine glands (breast, prostate, etc.) is to protect against breast cancer, but whether they act like estrogen or have anti estrogen properties is unclear (26).

Isoflavones act like a estrogen hormone and can alter gene transcription or cell signaling through binding with estrogen receptors or act independent of the estrogen hormones. Since estrogen receptors play an important role in response to treatment and prognosis of breast cancer, it seems necessary to better evaluate the secondary effects of possible side effects of herbal anti cancer components. According to the review of the literature, no study has evaluated the effect of alfalfa extract on cancer improvement. In a study by Milan et al on the effect of red clover on decreasing the risk of breast and endometrial malignancy, an association was detected between high consumption of phytoestrogens and low risk of breast and endometrial cancer if these estrogens were administered in patients without hysterectomy, despite the conventional hormone replacement therapy (27-29).

This effect can be explained by the interaction of phytoestrogens and estrogen receptors. There are two types of estrogen receptors: α (ERα) and β (ERβ). Phytoestrogens bind with ERα weakly and with ERβ strongly and have organ-specific and anti estrogen properties in such a way that they have agonistic effects in some tissues and antagonistic effects in some other tissues. ERβ receptors are found in the wall of the blood vessels and bone cells while ERα receptors are found in the endometrium and breast tissue. Therefore, the benefits of phytoestrogens in women are:

1- Increase in HDL-Cholesterol
2- Down regulation of ERα receptors due to phytoestrogens bound to the ERβ receptors (30-32)

Therefore, the favorable effects of phytoestrogens on serum lipids are confirmed.

The present study was a investigation in which the effect of alfalfa extract and the risk of breast cancer were evaluated for the first time. Thirty BALB-C mice were used in this study. The mice in the first and second groups were afflicted with breast cancer. The mice in the first group that received the alfalfa extract continued to live after 6 weeks of the study while mice in the second group, which did not receive the extract, died after 6 weeks.

Investigations show that the biological effects of isoflavones depend on their source. Isoflavones derived from Black Cohosh have a weak potential to remove the signs and symptoms of menopause and have a weak effect on serum lipids. Research has shown that isoflavones of the red clover have beneficial biological effects (33). These derivatives have a positive effect on serum lipids in menopausal women. However, Rajpal et al reported that the isoflavones of the red clover were weak estrogens and were not capable of preventing breast cancer alone (34). Paul et al reported that daily consumption of 60mg isoflavones extracted from the red-clover had therapeutic properties with no side effects (35). Chedraui et al showed that isoflavones derived from the red clover were an appropriate treatment choice for high risk individuals such as postmenopausal women to prevent the production of lipids and BMI increase (36). Asgary et al reported that dietary red clover in hypercholesterolemic rabbits decreased triglyceride, total cholesterol, and LDL and increased HDL (37). Khan et al found that supplements containing soy isoflavones did not decrease cell proliferation in breast cancer (38). According to another study, consumption of soy containing foods does not increase the risk of breast cancer relapse or death (39).

The cancer protective effects of the alfalfa extract were evaluated in our study. Alfalfa contains a considerable number of isoflavones compounds including tricin, genistein, diadzein, Biochanin A, formononetin, 5´-methoxysativan, and coumarin derivatives (coumestrol, medicagol, sativol). Our study showed that the level of estradiol was significantly lower in mice with breast cancer who received the alfalfa extract due to its isoflavones compounds when compared to mice in the second group that did not receive the extract (P<0.00). The significant effect of the alfalfa extract on reducing the level of estradiol in the afflicted mice in our study is compatible with the results of studies conducted by Milan et al (10), Glazier et al (27), and Rajpal et al (34).

Milan et al reported that administration of red clover isoflavones decreased total cholesterol in postmenopausal women (10) and our study showed that the alfalfa extract significantly decreased total

cholesterol in mice in the first group that received the extract when compared to the mice in the second group that did not receive the extract. Our study showed that the alfalfa extract had a significant effect on serum lipids and their derivatives. The extract significantly decreased total and LDL-cholesterol which is in line with the findings of the studies performed by De klejn et al (33), Chedraui et al (36) and Asgary et al (37). The difference between our findings and the results reported by Asgary et al is that the alfalfa extract did not increase HDL-cholesterol significantly in our study.

Eldin et al reported that total and LDL-cholesterol significantly increased in patients with breast cancer while HDL-cholesterol did not show significant changes (40). When compared to the study performed by Eldin et al, our study showed that the alfalfa extract prevented the increase in total and LDL-cholesterol in mice afflicted with breast cancer while it had no effects on HDL-cholesterol in that study.

Yancey et al found that HDL-cholesterol decreased significantly in these patients (41). Therefore, the effect of the alfalfa extract on mice afflicted with breast cancer was investigated in this study. The extract can improve the conditions through affecting estradiol and lipid profile in the mice with breast cancer.

CONCLUSION

We investigated the effects of the alfalfa extract for the first time in Iran. This extract should be pharmacologically evaluated for its therapeutic and side effects in patients and administered upon the lack of contraindications. In this situation, the extract can serve as a good source of possible anticancer drugs. Moreover, this study can pave the way for the investigation of medicinal herbs in breast cancer.

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