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Research Article

CYTOTOXIC AND ANTITUMORAL EFFECT OF *CERATONIA SILIQUACTA* L. (CAROB) EXTRACT

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ABSTRACT. Carob (*Cerotonia siliqua* L.) is a plant with various uses in the field of health among the public. The present study aimed to evaluate carob fruit extract, syrup and essence cytotoxic effects using 3-(4.5-dimethylthiazol-2-yl) methyl thiazole tetrazolium (MTT) bromide test. The study also evaluated apoptotic and necrotic effect using a double staining method of Hoechst 33342 and propidium iodide. Carob fruit extract, syrup and essence were applied to adenocarcinomic human alveolar basal epithelial cells (A549) and mouse adipose tissue derived fibroblast (L929) cells. It was applied in 5 different doses (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL). According to the results carob extract were not found to be toxic against both L929 cells and A549 cells at administered doses. High degree of toxicity was detected in L929 and A549 cells which carob syrup and essence were directly applied. In high doses, necrosis was found to be high and decreased as it fell. The ratio of apoptosis in L929 cells was higher than that of A549 cells. Further studies should be conducted in different cell lines. Carob is a plant that can be easily cultivated and processed under the conditions of Turkey. Within the scope of the study, taking into consideration the effects on lung cancer cells; It can be expressed as an alternative approach in treatment with applications.

Keywords: *MTT, carob, toxicity, necrosis, apoptosis.*

INTRODUCTION

Carob (*Ceratonia siliqua* L.) is a plant of the Leguminosae family (Fabacea), which is a species of the Caesalpinioideae subfamily, and has an economic value due to its edible fruit [1]. Carob, have not yet been cultured in Turkey, but are bred in a natural environment. The amount of these trees in the natural flora in the forests and forest lands in few groups has not been determined. That consists of wild trees for fruit quality of 90% of the carob tree is low in Turkey [2]. Approximately 90% of the carob fruit is made with meat. In this part, it is very rich in terms of sucrose, glucose, cellulose and tannins. Due to these ingredients, it is consumed in various forms, in the form of molasses, both directly and in various forms, as a protective and energizing product in Turkey [3, 4].

Carob was used in gastrointestinal system disorders, gastritis, liver and especially lung diseases. In addition, it is used as a natural energy donor for dental and gum ailments, lowering cholesterol, muscle development and high energy potential [2, 5]. The most common use of this fruit in Turkey is its use as molasses. Carob molasses is a mineral source rich in iron and calcium. Therefore, it is a very good supplement to the body in

meeting calcium needs in diseases such as osteoporosis [6]. The effect and support of carob in lung edema is an important opportunity. Expectorant strength and treatment effect against asthma disease is very high. Smokers have great benefits because of their lung cancer preventive power [7].

Carob contains bacteriostatic and bactericidal effect against some cellulotic bacteria (*Celvibrio fulvus*) due to its tannins content as well as sugar content [8]. Plants and preparations prepared from them have emerged as an alternative treatment tool for many diseases including cancer, almost as old as human history. The active ingredient is purified and used as a commercial drug against various cancers such as ovaries, breasts, lungs, testicles. the like can be counted [9].

The cancer type with the highest mortality rate in the world is lung cancer. It ranks first in males and second in females after breast cancer [10]. Smoking, air pollution and radiation are the most important causes of lung cancer [11]. Lung cancer is a preventable disease. Considering the risk factors, it is thought that 85-100% of measures can be provided. The priority in this regard is to eliminate smoking [12]. Lung cancer is a lung tumor that is seen with uncontrolled cell growth in lung tissues. These cells can spread to other tissues in the body with metastasis [13]. Carob is a plant that can be grown and processed easily in the conditions of Turkey. The present study aimed to evaluate carob fruit extract, syrup and essence cytotoxic effects using 3-(4.5-dimethylthiazol-2-yl) methyl thiazole tetrazolium (MTT) bromide test.

MATERIALS AND METHODS

Extraction

Carob molasses (70 g carbohydrates, 0.1 g fiber) and carob extract (532.2 mg Mg, 12.135 mg K, 943.2 mg Ca, 805.82 mg P, 27.09 mg Fe, 14.81 mg Zn, 16.1 mg soluble dietary fiber) as an over-the-counter product in pharmacies are sold for commercial purposes. Locust bean molasses, locust extract and locust fruit obtained from Kırıkkale Pharmacy were weighed at 0.2 g / ml from samples as stated in TS EN ISO 10993-12 standard and incubated at 37 ° C for 24 hours. The extracts obtained were used in *in vitro* tests.

Determination of cytotoxicity using MTT test

MTT is a very sensitive test to determine cell proliferation and cell viability using 3.[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) salt. Adenocarcinomic human alveolar basal epithelial cells (A549) and mouse adipose tissue derived fibroblast (L929) cells were inoculated into 96 well plates at a concentration of $10x10^3$ per well. Cells were incubated for 24 hours in an incubator conditioned with 5% CO_2 at 37 ºC [14]. Carob fruit extract, syrup and essence were poured into separate tubes and 1 ml of 10% DMSO was added and sonicated afterwards. Then, 9 ml of DMEM medium was also added to each tube and vortexed. Then Carob fruit extract, syrup and essence were applied to A549 and L929 fibroblasts cells at various concentrations (200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL) and incubated for 24 hours. Samples were studied in triplicate. For control, only cell medium was used. At the end of 24 hours, cell medium was removed from the wells and 50 µL MTT solution was added to each well. Cells were incubated with MTT solution for 2 hours. Upon draining the MTT solution, 100 µl of isopropanol was added into each cells. Then, it was shaken gently

and read in an ELISA plate reader at 570 nm wavelength. The final data of cell viability (%) were obtained by comparing the results obtained from the ELISA reader to that of control.

Determination of Apoptosis and Necrosis using double staining method

A549 cell and L929 fibroblasts cell were inoculated into 48 well plates at a concentration of 10 $x10^3$ per well. Cells were incubated for 24 hours in an incubator conditioned with 5% CO_2 at 37 ºC. Then, melamine, cyanuric acid and their combination (1:1) were applied to L929 fibroblasts and A549 cancer cells at various concentrations (1000µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL) and incubated for 24 hours. Samples were studied in triplicate. For control, only cell medium was used. At the end of 24 hours, cell medium was removed from the wells and 79 µL of the working double staining solution was added to each well. The 48 well plates were incubated in an incubator for 15 min. For this 15 min incubation period, a completely dark environment is required. At the end of incubation, cells were evaluated in a fluorescence microscope. Apoptotic and necrotic cells were visualized using the DAPI and FITC (480-520nm wavelength) fluorescence filters, respectively.

Statistical analysis

For statistical analyses, the presence of difference was examined using the one way analysis of variance (ANOVA). In case of presence of a significant difference, the Tukey test was conducted for multiple comparisons. For comparison between A549 cell and L929 fibroblast for the same dose, the student's t-test was used. Data were expressed as mean ± standard deviation. A P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Within the scope of the study, 3 different forms of carob (*Ceratonia siliqua* L.); Cytotoxicity, necrosis and apoptosis assessments were reflected in cancer and fibroblast cells applied by dilution on carob molasses, carob extract and carob fruit extract.

MTT Test Findings (Cytotoxicity Findings)

Evaluation of the toxic effect was done in L929 fibroblast and A549 cancer cell line according to MTT cytotoxicity method and microscopic morphological analysis. When carob extract and molasses are directly applied to normal and cancer cells, high toxicity was detected. For this reason, the solutions obtained by diluting 1/10 was applied to the cells up to a concentration of 200 µg/ml.

Carob molasses was given to A549 cancer cells. As the amount of carob molasses given increased, vitality values decreased from 100 units to 59.4 units. When the toxic effect of carob molasses to A549 cancer cells was examined, it was observed that the toxic effect increased with increasing dose. Carob molasses was given to L929 fibroblast cells. As the amount of carob molasses given increased, vitality values decreased from 100 units to 41.61 units. The toxic effect of carob molasses on L929 cells increased with increasing dose (Table 1).

Table 1. *Toxic effects of Carob Molasses to A549 cancer cells and L929 fibroblast cells (mean±SE).*

	% Viability Values of A549 cancer cells	**% Viability Values of L929 fibroblast cells**
200 µg/mL	59.40±0.33*	41.61±0.19
100 µg/mL	63.65±0.24*	66.44±0.07
50 µg/mL	68.17±0.16*	77.39±0.12
25 µg/mL	75.81±0.09*	83.56±0.24
12.5 µg/mL	93.06±0.09*	84.24±0.14

*Presence of an asterisk (*) indicates a significant difference in the same row for the same dose (P<0.05).

Carob extract was given to A549 cancer cells. As the amount of carob extract given increased, vitality values decreased from 100 units to 46.39 units. The toxic effect of carob extract on A549 cancer cells increased with increasing dose. With the increase in the dilution rate at the same dose, the toxic effect is reduced. Carob extract was given to L929 fibroblast cells. As the amount of carob extract given increased, vitality values decreased from 100 units to 51.02 units. The toxic effect of carob extract on L929 cells increased as the dose applied increased (Table 2).

Table 2. *Toxic effects of Carob Extract against A549 cancer cells and L929 fibroblast cells (mean±SE).*

	% Viability Values of A549 cancer cells	**% Viability Values of L929 fibroblast cells**
200 µg/mL	46.39±0.09*	51.02±0.07
100 µg/mL	53.18±0.20*	63.69±0.11
50 µg/mL	66.76±0.09	65.41±0.31
25 µg/mL	76.94±0.17*	81.50±0.07
12.5 µg/mL	81.47±0.09*	92.12±0.17

*Presence of an asterisk (*) indicates a significant difference in the same row for the same dose (P<0.05).

Extracted carob fruit was given to A549 cancer cells. It has been observed that the amount of extracted carob fruit given. The liveliness values decreased from 100 units to 84.71 units. The toxic effect of extracted locust fruit on A549 cells was found to increase with increasing dose. Extracted carob fruit was given to L929 fibroblast cells. As the amount of extracted carob fruit given increased, vitality values first increased from 100 units to 170.97 units, but as the dose increased, it decreased to 124.09 units.

Table 3. *Toxic effects of Carob Fruit Extract against A549 cancer cells and L929 fibroblast cells (mean±SE).*

	% Viability Values of A549 cancer cells	**% Viability Values of L929 fibroblast cells**
200 µg/mL	84.71±0.16*	124.09±0.24
100 µg/mL	86.38±0.09*	135.63±0.16
50 µg/mL	90.63±0.05*	136.80±0.54
25 µg/mL	98.74±0.80*	157.57±0.09
12.5 µg/mL	102.02±0.15*	170.97±0.16

*Presence of an asterisk (*) indicates a significant difference in the same row for the same dose (P<0.05).

The toxic effect of extracted carob fruit on L929 cells increased with increasing dose. However, increasing vitality values showed that these cells develop better. Histopathological evaluation of cancer and normal cells with carob applied in different concentrations are given in Fig. 1. These images show the effect of carob forms on cell proliferations. Each image shows that cell proliferation decreases due to cytotoxicity.

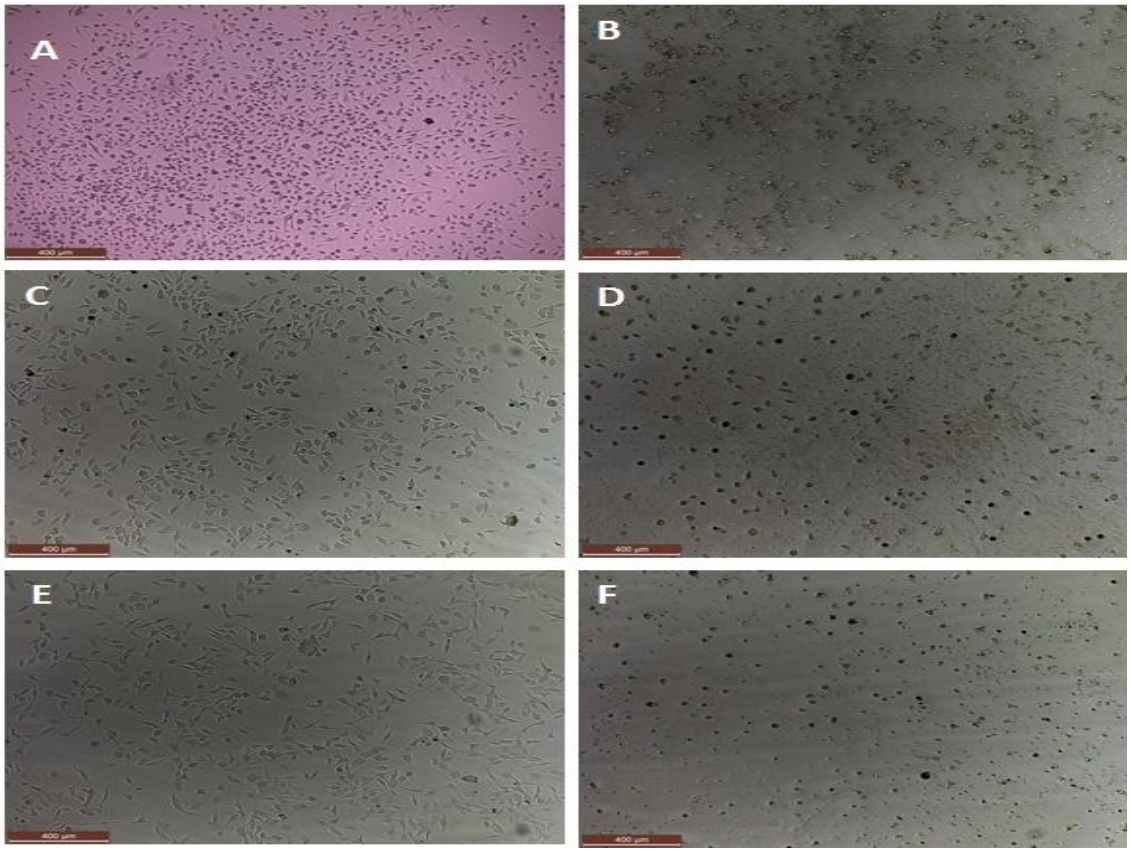


Fig. 1. *Photographs of cancer and normal cells treated with carob in different concentrations. A) Control fibroblast, B) The appearance of fibroblast cells with 200 µg / ml carob molasses, C) The appearance of 200 µg/ ml carob fruit extract treated fibroblast cells, D) The appearance of A549 cancer cells with 200 µg/ml carob extract, E) The appearance of A549 cancer cells with 200 µg/ml carob fruit extract, F) Appearance of A549 cancer cells with 200g/ml carob molasses.*

Apoptosis and Necrosis Findings

Three different pharmaceutical forms belonging to Carob (*Ceratonia siliqua* L.) were applied to cancer and fibroblast cells by diluting the carob molasses, carob extract and carob fruit extract.

Carob molasses was given to A549 cancer cells. It was observed that as the amount of molasses given increased apoptosis and necrosis. Carob molasses was given to L929 fibroblast cells. It was observed that as the amount of carob given increased apoptosis and necrosis (Table 4).

Table 4. *Percent Apoptosis-necrosis rates in A549 cancer cells and L929 fibroblast cells with carob molasses.*

	A549 cancer cells		**L929 fibroblast cells**	
	% Apoptosis	**% Necrosis**	**% Apoptosis**	**% Necrosis**
200 µg/mL	22.38±0.09	43.23±0.14	24.64±0.07	40.45±0.14
100 µg/mL	18.79±0.07	39.42±0.10	17.42±0.05	35.8±0.12
50 µg/mL	11.67±0.05	32.84±0.09	9.65±0.04	24.16±0.08
25 µg/mL	5.14±0.03	15.69±0.07	8.32±0.03	12.14±0.06
12.5 µg/mL	2.21±0.02	6.27±0.04	4.82±0.03	5.78±0.04

Carob extract was given to A549 cancer cells. It was observed that as the amount of carob extract given increased apoptosis and necrosis. Carob extract was given to L929 fibroblast cells. It was observed that as the amount of molasses given increased apoptosis and necrosis (Table 5).

Table 5. *Percent Apoptosis-necrosis rates in A549 cancer cells and L929 fibroblast cells with Carob extract.*

	A549 cancer cells		**L929 fibroblast cells**	
	% Apoptosis	**% Necrosis**	**% Apoptosis**	**% Necrosis**
200 µg/mL	20.13±0.07	45.46±0.17	23.01±0.05	43.91±0.18
100 µg/mL	15.47±0.05	38.71±0.15	14.65±0.04	37.63±0.15
50 µg/mL	11.61±0.05	35.36±0.13	10.71±0.04	30.32±0.12
25 µg/mL	6.87±0.04	19.9±0.10	9.49±0.02	18.43±0.07
12.5 µg/mL	1.53±0.01	5.46±0.04	3.76±0.01	7.89±0.01

Extracted carob fruit was given to A549 cancer cells. As the amount of extracted carob fruit given increased, apoptosis and necrosis increased but there was no significant increase. Extracted carob fruit was given to L929 fibroblast cells. As the amount of extracted carob fruit given increased, there was no significant change in apoptosis and necrosis values (Table 6).

Table 6. *Percent Apoptosis-necrosis rates in A549 cancer cells and L929 fibroblast cells with extracted Carob fruit.*

	A549 cancer cells		**L929 fibroblast cells**	
	% Apoptosis	**% Necrosis**	**% Apoptosis**	**% Necrosis**
200 µl	4.22±0.02	10.78±0.03	1±0.01	0
100 µl	2.32±0.01	9.86±0.01	1±0.01	0
50 µl	1±0.01	2.20±0.01	0	0
25 µl	1±0.01	1±0.01	0	0
12.5 µl	1±0.01	1±0.01	0	0

The images of cancer and normal cells with carob molasses apoptotic and necrotic in the microscope are shown in Fig. 2. In these figure, apoptotic cell nuclei appear bright and fragmented, and necrotic cell nuclei appear orange. Robust cell nuclei appear green. According to these images, the rates of apoptosis and necrosis of the cells were determined.

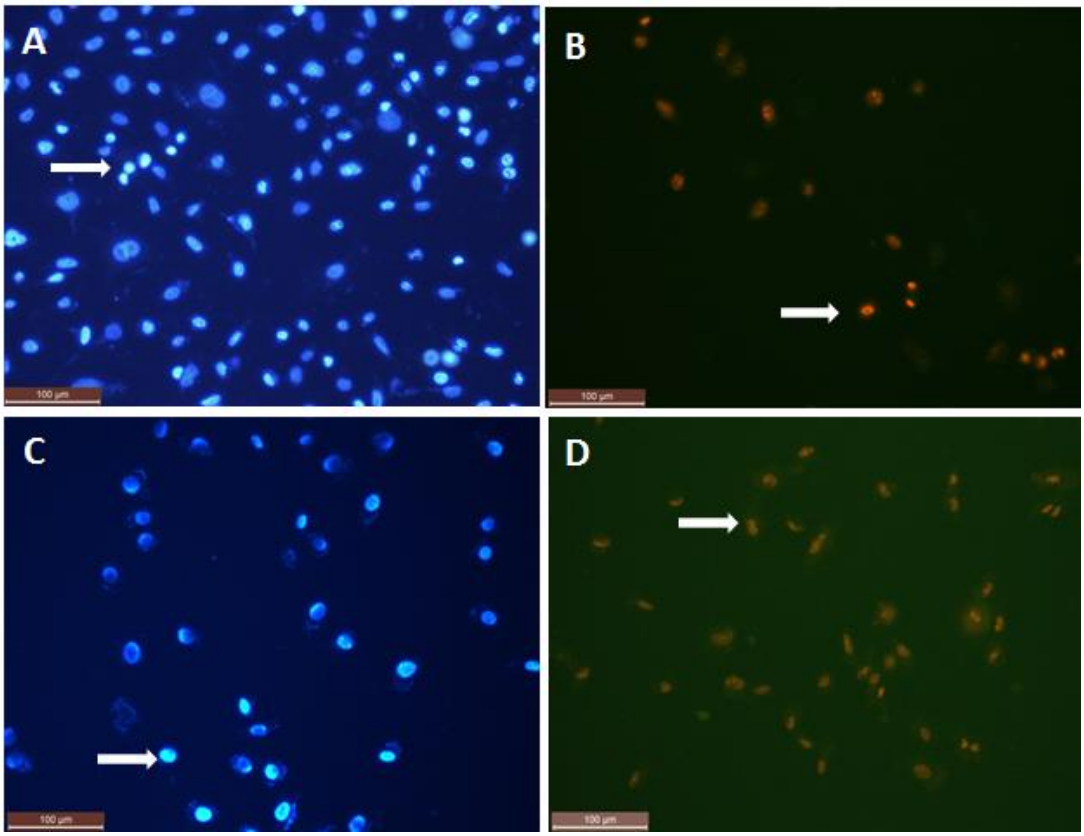


Fig. 2. *Cancer and apotosis necrosis of normal cells with carob molasses. A) Apoptotic fibroblast cells (arrow indicates apoptotic cells) with 200 µg/ml carob molasses, B) 200 µg/ml carob molasses necrotic fibroblast cells (arrow indicates necrotic cells), C) Apototic cancer cells with 200 µg/ml carob molasses (arrow indicates apoptotic cells), D) Necrotic cancer cells (arrow indicates necrotic cells) with 200 µg/ml carob molasses.*

CONCLUSION

It is stated that lung cancer is a malignant tumor with the highest incidence and mortality rate in the world. According to the data of the international cancer research agency, it was stated that there were approximately 1.82 million new lung cancer cases worldwide in 2012 and ranked first among all malignant tumors [15].

There are different approaches to drugs used for the treatment of cancer. The pharmaceutical industry is looking for natural products to treat cancer. Most herbal products that have reported anti-cancer efficacy have information that high levels of polyphenols or strong antioxidants are present. It has been interpreted that the antioxidant activity of the plant, especially polyphenols, should be the focus of anti-cancer drug discovery attempts [16].

In this study, three different pharmaceutical forms obtained from *Ceratonia siliqua* L. (harnup) plant were studied and their anticancer effect was investigated. Hsauna et al. [17] explained that *Ceratonia siliqua* L. has a protective effect against cancer. The data obtained shows that this herb can be used at certain doses, at certain intervals and at certain periods of cancer.

In a study in which *Ceratonia siliqua* L. was shown to have a cytotoxic effect against HeLa and MCF-7 cells, cytotoxicity increased as the concentration of carob product increased [17]. This study has been shown to show similar results with the current study. It is thought that *Ceratonia siliqua* L. can give similar results in different cancer cell lines.

Antiproliferative and cytotoxic effects of extracts prepared with methanol and ethanol from the fleshy parts of carob leaves and fruits were investigated using MTT method on HeLa cells. According to the findings obtained, carob's methanolic and ethanolic leaf extracts inhibit DNA synthesis of HeLa cells and suppress mitotic activity and proliferation, and ethanolic fruit extract reduces HeLa cells' DNA synthesis. As a result of studies with extracts prepared from *Ceratonia siliqua* L. leaves and fruits, it has been reported that the extract has antimicrobial, antifungal and cytotoxic activity, also induces apoptosis and inhibits cell proliferation. In his current work, it was observed that he was similar to these expressions. In addition, it is thought that new studies can be developed by evaluating the cytotoxic effect [17].

In another study conducted in Turkey, the antimicrobial and cytotoxic effects of carob extracts against microorganisms were examined. It is stated that the extract prepared with ethanol shows a strong antimicrobial effect. It has been interpreted that extracts prepared with ethanol, methanol and N-hexane show cytotoxic effect [18].

In this study, it was found that carob extract did not show a toxic effect at the doses applied to both normal cells and cancer cells. In carob extract and molasses, it was observed that each ratio used and different rates of these ratios were high toxicity against cancer cells and less toxic to normal cells. It has been determined that locust extracts increase cell proliferation and cell quality depending on the dose. When carob extract and molasses are directly applied to normal and cancer cells, a high level of toxicity has been detected. According to the results obtained, as the dilution rate increased, it was observed that toxicity decreased in both forms. In addition, while the toxic effect of carob in fibroblasts was higher than cancer cells, it was observed that as the concentration decreased, the viability of fibroblast cells increased and viability decreased in cancer cells.

According to the results obtained, necrotic death was higher in both normal cells and cancer cells than apoptotic death. In particular, apoptosis was found to be low in carob extract. Apotose ratios were obtained close to each other in carob extract and molasses.

It was also observed that the rate of necrosis varied depending on the dilution rate and the dose administered. While high rates of necrosis were obtained, it was found that necrosis decreased as the rate decreased. In normal cells, the rate of apoptosis was higher than cancer cells.

Consumers who are conscious about health in recent years, prefer food that has a special physiological effect in the body, prevents the occurrence of some diseases, protects, therapeutic, and also meets the basic nutrient requirements of the body [19].

Components such as important minerals such as potassium, phosphorus, calcium, nutritional fibers, tannins found in carob are functional components that provide functional properties to carob. Carob fruit is a functional food due to these properties and the beneficial components it contains. Considering the carob fruit grown completely naturally in Turkey, the fruit part remaining after the seeds have been separated does not have a very common usage area. Due to the easy growing and processing of carob plant in Turkey conditions; it can be evaluated as an alternative treatment approach especially in cases such as lung cancer; It is concluded that comprehensive studies on the subject should be done.

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