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Project Name (Title): Investigation of the Effects of Oyster Mushroom (*Pleurotus ostreatus*) and Black Cumin Seed (*Nigella sativa*) Extracts on MOLT-4 Acute Lymphoblastic Leukemia

Main Project Area: BIOLOGY

Project Thematic Area: Public Health and Preventive Healthcare Services

Abstract:
ALL (Acute Lymphoblastic Leukemia) is a fatal disease that affects many people worldwide, especially children, and the root cause has not yet been found. The fact that chemotherapy, which is used as a medical treatment for leukemia, not only destroys cancerous cells but also damages healthy cells, creates very serious problems for patients. It even adversely affects healthy tissues. This is why scientists are looking for alternative treatment modalities to chemotherapy for the treatment of leukemia. The most significant characteristic of the active substance to be used in this treatment is that it destroys or stops malignant cancer cells without harming healthy cells. Based on this, we investigated the effect of the active substances in black cumin and oyster mushroom extracts, which are major nutraceutical nutrients in our country and Asian countries, on T-Lymphoblast Acute Lymphoblastic Leukemia (T-ALL) cells, a very dangerous type. In this context, black cumin seed and oyster mushroom extracts were applied to both T-ALL leukemia cells (Molt-4) and HEK293 (healthy embryonic kidney cells) cells, and cell survival was observed by spectrophotometer absorbance calculations. The results of the tests showed that high concentrations of black cumin seed and oyster mushroom extracts had cytotoxic effects on cancer cells, causing cell death, particularly at 48 and 120 hours. No cytotoxic effect of these extracts was observed in healthy cells. Based on the results of the controlled test, it was concluded that black cumin seed and oyster mushroom extract may be used for the treatment of ALL-type leukemia.

Keywords: T-ALL Leukemia, MOLT-4, HEK293, Black Cumin Seed Extract, Oyster Mushroom Extract, Thymoquinone, Ergothioneine

Objective:

Currently, some foods called nutraceuticals (non-pharmaceutical food supplements prepared in the form of medicines) are used in traditional and folk medicine for their curative effects, such as antibiotic, antitumor, anti-inflammatory, anticancer, antileukemic, and immunomodulatory effects. The extent to which these products designed for healthy living are effective is an important area of research. It is a matter of great curiosity in the scientific world whether it can also prevent cancer, one of the most important diseases of our age, and whether it can contribute to its treatment. The aim of our research was to apply the active substances found in black cumin seed and oyster

mushroom extracts to ALL leukemia (blood cancer) cells (Molt-4 T lymphoblast), a common and dangerous type of cancer, and observe the effects.

Introduction:

Cancer is one of the leading causes of death worldwide. Approximately 39.5% of individuals are expected to be diagnosed with cancer at some point in their lives. In 2018, there were 18.1 million new cases and 9.5 million cancer-related deaths globally. By 2040, the annual number of new cancer cases is expected to rise to 29.5 million and cancer-related deaths to 16.4 million. *(National Cancer Institute, 2020)*

Cancer is a disease in which there is an uncontrolled growth of certain cells in the body that spreads to other parts of the body. Normally, human cells grow and proliferate to generate new cells through cell division. When cells age or are damaged, they die and are replaced by new cells. Sometimes this order is broken, allowing aberrant or damaged cells to proliferate without dying. These cells can form tumors, which are clumps of tissue. Tumors may be benign or malignant (cancer). The cancer spreads and invades the surrounding area and may travel to distant places in the body to develop new cancerous tumors (metastases).

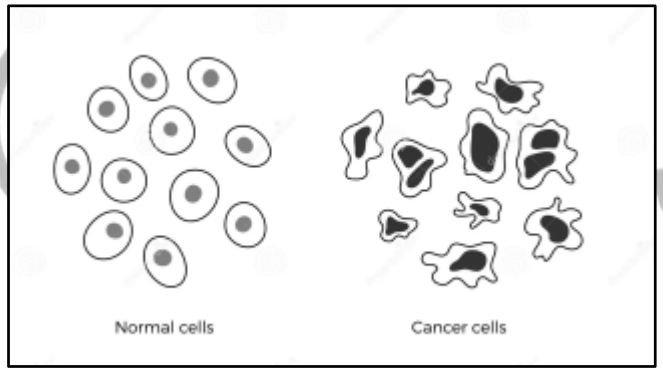


Figure 1 - Structural differences between cancerous and normal cells

Cancer is a genetic disease, meaning that it is caused by alterations in the genes that regulate how our cells work, especially how they grow and divide.
Normally, the body eliminates cells with damaged DNA before they become cancerous. But as we age, the body's ability to do so declines. This is one of the reasons for a higher risk of cancer later in life.

Cancer-causing genetic changes may result from defects that occur during cell division, DNA damage induced by environmental toxins (such as chemicals in tobacco smoke and UV rays from the sun), and genetic transmission from parents.

A type of cancer that is widely known and recognized by people in our country and around the world is leukemia. These are cancers that begin in the blood-forming tissue of the bone marrow.

These cancers do not produce solid tumors, but large numbers of aberrant white blood cells (leukemia cells and leukemic blast cells) accumulate in the blood and bone marrow, outnumbering normal blood cells. Low levels of normal blood cells can make it harder for the body to deliver oxygen to tissues, control bleeding, or fight infections

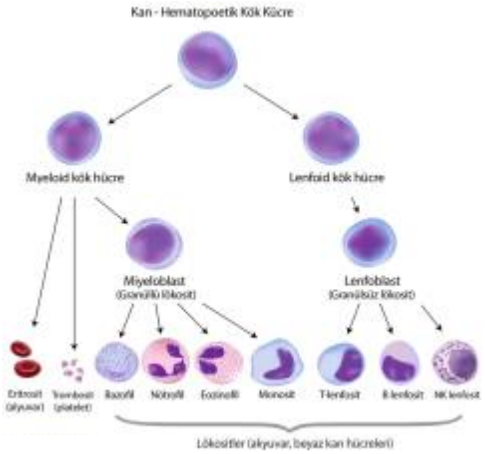


Figure 2: Red blood cells in the bone marrow *(Becton et al., 2024)*

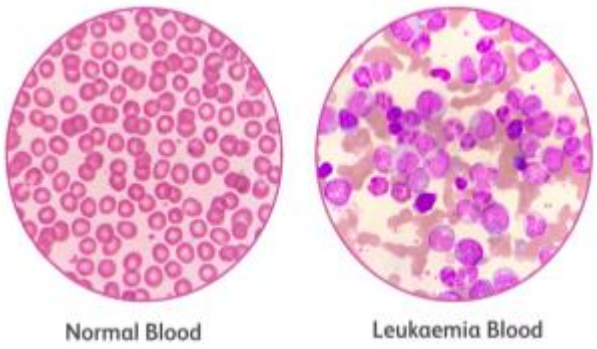


Figure 3: Structural differences between normal blood cells and cancerous blood

There are four common types of leukemia, classified by how quickly the disease worsens (acute and chronic) and the type of blood cell that develops cancer (lymphoblastic and myeloid). Acute lymphoblastic leukemia (ALL), acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), and chronic lymphocytic leukemia (CLL) are the names of four types of leukemia. *(National Cancer Institute, 2021) (Chennamadhavuni, A. et al., 2023)*

Blood cells proliferate and regenerate in a certain balance. Blood cells go through a complex maturation process during proliferation and regeneration; an out-of-control version of this process is seen in Acute lymphoblastic leukemia (ALL). White blood cells do not mature and proliferate in an uncontrolled manner. As a result, insufficient white and red blood cells are produced *(Kinderkrebsinfo, 2023)*. T-cell acute lymphoblastic leukemia (T-ALL), which we used in our project, is an aggressive cancer caused by the malignant transformation of immature T-cell precursors. It accounts for 15% and 25% of ALL cases in children and adults, respectively. *(P.M. Maciocia et al., 2022)*

In acute myeloid leukemia (AML), the maturation process is impaired. Many stem cells transform into young cells called myeloblasts or monoblasts, which fail to differentiate into mature cells and accumulate in the blood and bone marrow to form AML. Normal granulocytes and monocytes cannot form in AML. The life span of these dividing cells exceeds that of normal cells, and they

replace healthy cells. This leads to symptoms such as infection, fever, bleeding, and weakness. (Pizzo & Poplack, 2011, 566 in) *(H. Okutan, 17 Nisan).*

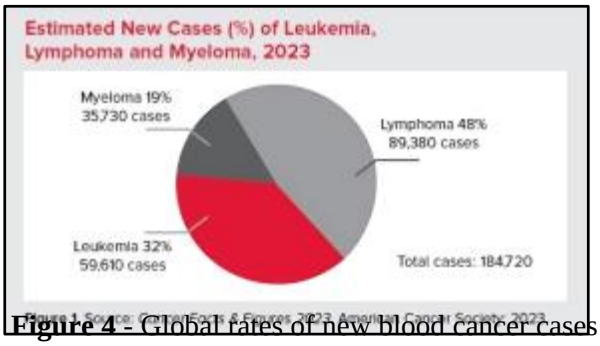


Figure 4 - Global rates of new blood cancer cases in 2023

The main types of blood cancer are leukemia, lymphoma, myeloma, myelodysplastic syndromes (MDS), or myeloproliferative neoplasms (MPN). The most common of these worldwide in 2023 are lymphoma (48%, 89.380 cases per year), leukemia (32%, 59,610 cases), and myeloma (19%, 35,730 cases per year), respectively.

The subtypes of leukemia cancer that we studied for the project, ALL, AML, CLL, and CML, are predicted to account for 59.610 cases globally in 2023. As mentioned above, the 5-year survival rate for leukemia, a highly fatal cancer, has increased significantly in recent years due to medical improvements. While there was a survival rate of 34% between 1975-1977, this rate doubled to 69% between 2012-2018. Survival rates of leukemia cancer types worldwide between 2012-2018 are provided below. *(LLS, 2023)*

- ALL – 70.8 percent overall, 92.1 percent for children and adolescents under 15, and 93.3 percent for children under 5
- AML – 30.5 percent overall and 69.0 percent for children and adolescents under 15
- CLL – 87.9 percent overall
- CML – 70.4 percent overall*

*(LLS, 2023)*The survival rate of CML in clinical trials is higher than stated above according to SEER data.*

Leukemia cancer accounts for 25-30% of childhood cancers, 97% of which are acute leukemia (ALL, AML). The annual incidence of ALL-type leukemia, which we used in the project, is 1.5/100,000 in Turkey. The leukemia survival rate, which was 10% in the 1960s in Turkey with the developments in the field of medicine in proportion to the development in the world, has increased to 80-90% today with new treatments. According to WHO data, the number of new leukemia cases in our country in 2020 was 7023, with a crude rate of 8.3 *(Thomas, 2020).*

There are many treatments for cancer today, but there are also nutraceutical foods that people think are good for cancer. Dr. Stephen DeFelice developed the phrase nutraceutical in 1989 by merging the words nutrition and pharmaceutical. As an extract or food, they exhibit a protective or physiological benefit against a chronic disease. This term refers to foods that have health benefits in addition to their fundamental nutritional qualities, according to the Merriam-Webster Dictionary. Products can be available in different dosage forms, such as tablets, capsules, soft gels, and capsules, but are not considered medicines *(BAŞARAN, 1970).* Whether these nutrients actually have an impact on the spread and development of cancer is a hotly debated topic. In Turkey, the foods considered to be good for cancer are thyme/thyme oil, onion and garlic, black/red

grapes, turmeric, broccoli, and black cumin (*Vázquez-Fresno, R. et al., 2019*)**.** In Asian countries, such plant extracts are also widely used by the public for the treatment and prevention of diseases. The herbs known in Asia are fenugreek (*Trigonella foenum-graecum*), Hedysarum (*L. Fabaceae*), the herbs known in Asia are fenugreek (*Trigonella foenum-graecum*), Hedysarum (*L. Fabaceae*), birthwort (*Aristolochia lona L.*), white horehound (*Marrubium vulgare L.*), and oyster mushroom (*Pleurotus ostreatus*). (J.M. Alves Silva, 2017)

In this project, one of the nutrients we tested on the leukemia cancer ALL cell line is black cumin (*Nigella sativa*). N. sativa seed contains 26-34% fixed oil, of which the major fatty acids are linoleic acid (64.6%) and palmitic acid (20.4%). The seed oil is comprised of 0.4%-2.5% essential oil. Among the different active substances reported so far, thymoquinone, found as the major component of the essential oil, is the most bioactive compound. It is believed to have wide-ranging therapeutic (curative) benefits. Thymoquinone (TQ), a substance isolated from black cumin seeds, is thought to inhibit the growth of cancer cells, and induce apoptosis. (E. Yimer, 2019) A. Soltani, 2017)

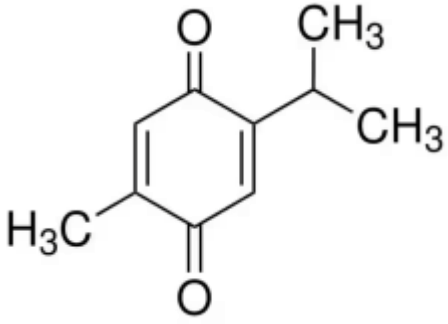


Figure 5: Thymoquinone formula

Figure 6: The black cumin plant (*Nigella sativa*) and its seed

Another substance we tested was oyster mushroom (*P. ostreatus*), which is commonly used in Asian countries to treat cancer. Antibiotic, antitumor, anti-inflammatory, anticancer, antileukemic, and immunomodulatory effects are among the actions of oyster mushrooms. Oyster mushrooms, phenolic compounds, flavonoids, terpenoids, polysaccharides, lectins, steroids, glycoproteins, various lipid components, and ergothioneine (Egt), vitamin C, beta-carotene, and selenium are some of the substances that act on leukemia cells. Dietary amino acid ergothioneine (Egt) functions as an antioxidant to protect against aging-related diseases. (Ebrahimi et al., 2018) (S.B.Rahimah, 2019)

In recent years, the popular ergothioneine has attracted the interest of scientists and encouraged them to investigate the anti-cancer properties of Egt. In particular, it was concluded that Egt may cause dose-dependent cytotoxicity in some cancer cells (e.g., colon cancer). (N. D'Onofrio et al.,2022)

Based on these studies, we considered investigating whether the high level of the active substance Ergothioneine in oyster mushrooms, one of the mushroom species, may be employed as an anticancer natural material against leukemia.

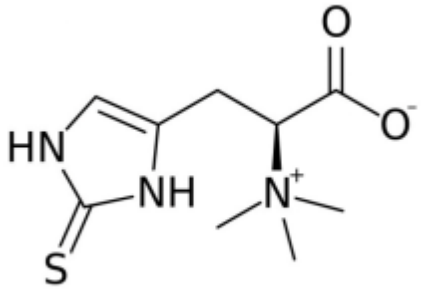


Figure 7: Ergothioneine formula

Figure 8: Oyster mushroom (*P. ostreatus*)

Based on the literature review, the objective of this project was to investigate the effects of the active substances in black cumin and oyster mushroom extracts (especially Thymoquinone and Ergothioneine), which are used for therapeutic purposes among people and are among the nutraceutical supplementary foods, on ALL-type leukemia cells (Molt-4), a common and very dangerous type of cancer.

Method:

Materials used:

Black cumin seeds (5g), oyster mushrooms (5g), filter paper, anhydrous sodium sulfate powder, spatula, drying oven, glass container, electronic scale, mortar, soxhlet, graduated cylinder, magnetic stirrer, ethanol, hexane, distilled water, magnetic stirrer, boiling stone, distillation apparatus, amber flask, fume hood, ultrasonic bath, centrifuge, beaker, nitrogen, lyophilizer, micropipette, 96-microplate, vortex, thoma, microscope, slide, incubator, cell medium, Molt4 and HEK293 Cells, microplate Varioskan Flash Multiplex Reader UV Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA)

Method Steps:
Our method consists of 3 steps:

1. **STEP: Preparation of ingredients:**

1- Preparation of black cumin seed and oyster mushroom extracts:

A. Preparation of oyster mushroom extract:

- Oyster mushrooms (*Pleurotus ostreatus*) were placed in a drying oven to dry for two hours.
- The dried mushrooms were removed from the drying oven and pulverized in a mortar.
- 5g of mushroom powder was weighed on an electronic scale.

- 100 ml of ethanol (C_2H_5OH) (96%) was added to 5g of mushroom powder.
- The solution was stirred in a magnetic stirrer for 1.5 hours.
- The mushroom solution was then extracted in an ultrasonic bath for 30 minutes.
- The resulting extract was centrifuged (6500 RPM, 15 minutes). It was observed to be divided into supernatant and mushroom powder.
- The supernatant was transferred to a beaker and allowed to dry under nitrogen in a fume hood.
- Finally, the resulting mushroom extract was lyophilized.

Figure 9- Drying mushrooms in a drying oven

Figure 10- Stirring in a magnetic stirrer

B. Preparation of black cumin seed extract:

- Black cumin seeds (*Nigella sativa*) were weighed at 5g on an electronic scale.
- After crushing the seeds in a mortar, 1g of anhydrous sodium sulfate powder was added.
- The crushed mixture was put into filter paper and placed in a soxhlet extractor at 73.6 °C.
- A graduated cylinder was used to measure 115 ml of hexane (C_6H_{14}) (98%) into the soxhlet extractor.
- We waited 2 hours for the extract.
- The extract was then distilled in a simple distillation apparatus.
- It was placed in an amber-colored bottle and left to completely volatilize under a fume hood.

Figure 11 - Extraction in the soxhlet extractor

Figure 12 - Preparation of black cumin

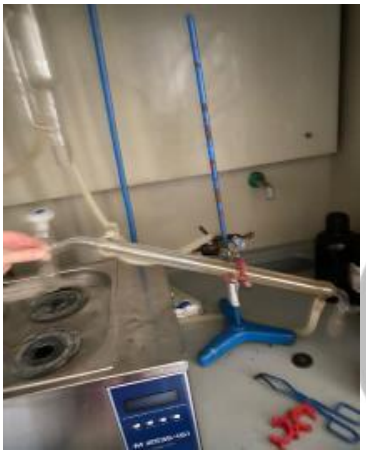


Figure 13- Simple distillation apparatus

Figure 14- Drying the extract in an amber glass bottle

2- Preparation of Molt-4 (T-lymphoblast cell line) and HEK293 cells:

- Human acute lymphoblastic leukemia cell line MOLT-4 and human embryonic kidney cell line HEK293 cells were cultured at 37°C in complemented RPMI-1640 medium (Gibco) containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 100 units/mL penicillin/streptomycin, and 1% non-essential amino acids in the presence of 5% CO2. Cells were seeded in 96-well petri dishes for the MTT assay and incubated for 24, 48, and 120 hours with or without varying concentrations of black cumin or oyster mushroom. After incubation, they were analyzed. All assays were prepared in triplicate within themselves and performed at least 3 times at different times.

Figure 15- Molt-4 cell count under a microscope

Figure 16- Microscopy view of Molt-4 cells

Figure 17- Loading cells onto Thoma

Figure 18- Adding cells into wells

3- Preparation of sample extracts to be applied to MOLT-4 cells:

- 5 milligrams of each extract were weighed and dissolved in 1ml of solvent. Oyster mushrooms were dissolved in ethanol and black cumin in hexane, respectively. Thus, a main stock was prepared from the extracts at a concentration of 5mg/ml. The extracts were pipetted to dissolve homogeneously in appropriate solvents, and a homogeneous solution was prepared by incubating at 50 degrees for 5 minutes.
- The master stock was added to the cells at concentrations of 10, 20, 50, 50, 100, 200, 500, and 1000 µg/mL.

4-Preparation and seeding of MOLT-4 T Lymphoblast cells:

- The suspended MOLT-4 cells were collected in a 50-ml vial.
- 10 µl of this cell suspension was then mixed with 10 µl of trypan blue dye.
- 10 µl of cell suspension diluted one-to-one and stained was loaded onto a thoma slide.
- The number of dead live cells was identified under a microscope.

- After cell counting, 96-well cell culture plates were seeded with 10,000 cells per well.
- Cells were incubated overnight at 37 °C.

STEP 2: Use of the extracts on Molt-4 cells and healthy cell samples

- After overnight incubation, Molt-4 cells were treated with 10 µg/ml, 20 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 500 µg/ml, and 1000 µg/ml of black cumin and oyster mushroom extracts separately.
- Triplicate replicates were performed for each sample.
- Cell plates to which the extracts were added were examined at the end of three different incubation periods of 24, 48, and 120 hours (see Table-1).
- The same procedure was performed on the healthy cell HEK293 (human embryonic kidney cell line).

Table-1: The application of extracts to ALL-type Molt-4 cells in 7 different samples at different concentrations

	GROUPS AND THE CONCENTRATION OF EXTRACT ADDED						
Type of extract added	**1. Group**	**2. Group**	**3. Group**	**4. Group**	**5. Group**	**6.Group**	**7.Group**
Black cumin extract	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL
Oyster mushroom extract	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL

Molt-4 cells were seeded in the 96-well microplate shown in **Figure 19**, and sample extracts were added as specified in **Table-2**.

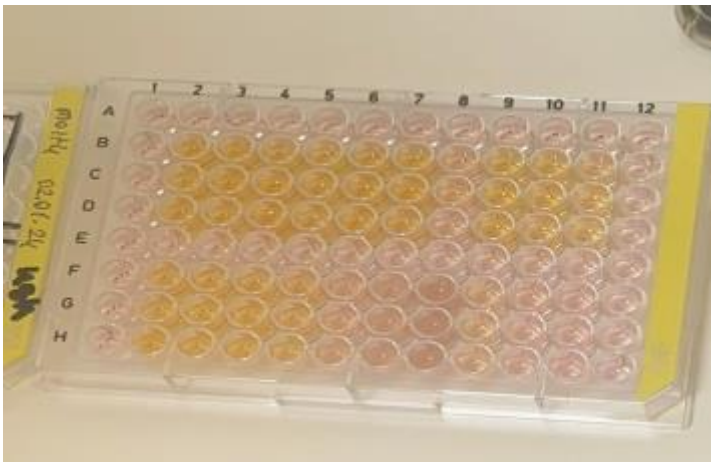
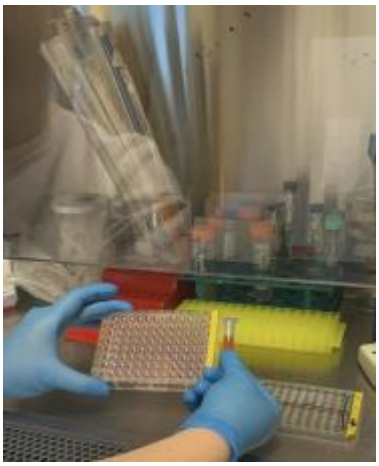



Figure 19 - Seeding of ALL Molt-4 type T lymphoblast cells and addition of different concentrations of sample extracts

Table-2: Representative demonstration of seeding Molt-4 cells in a 96-well Microplate

	K-1	10µg/ml	20µg/ml	50µg/ml	100µg/ml	500µg/ml	1000µg/ml		10% Hexane (black cumin/10% Ethanol oyster **(K-2)**	10	11	12
A		Initial values are not used										
B	Control	BCE	BCE	BCE	BCE	BCE	BCE		Hexane			
C	Control	BCE	BCE	BCE	BCE	BCE	BCE		Hexane			
D	Control	BCE	BCE	BCE	BCE	BCE	BCE		Hexane			
M		Initial values are not used										
F	Control	OE	OE	OE	OE	OE	OE		Ethanol			
G	Control	OE	OE	OE	OE	OE	OE		Ethanol			
H	Control	OE	OE	OE	OE	OE	OE		Ethanol			

*Black cumin extract=BCE
*Oyster Mushroom Extract=OE
*C=Control

STEP 3: Reading by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Analysis:

- 96-well microplates with cells were placed in a spectrophotometer. After incubation, 96-well microplates were read at 450 nm with a Varioskan Flash Multiplex Reader UV Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).
- The absorbance values of each of the wells were measured.
- Based on the absorbance results, the effect of the active substance in the extracts on cell viability was calculated as a percentage.
- The absorbance of MOLT-4 cells in the control group treated with hexane or ethanol and the percent viability of extract-treated cells were calculated and analyzed using Graphpad prism 5 and Two-way Annova.

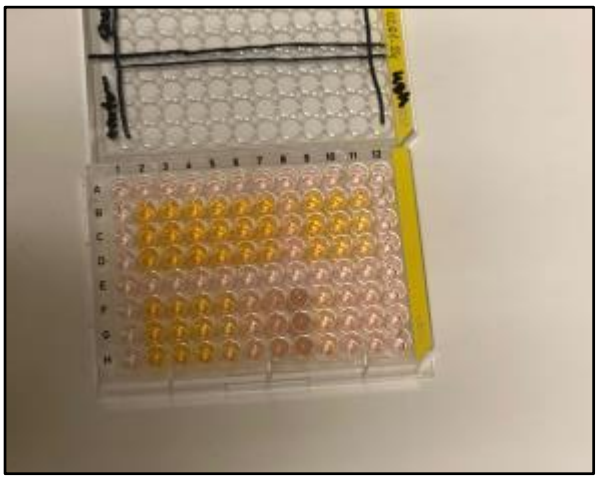


Figure 20 - Discoloration of microplates

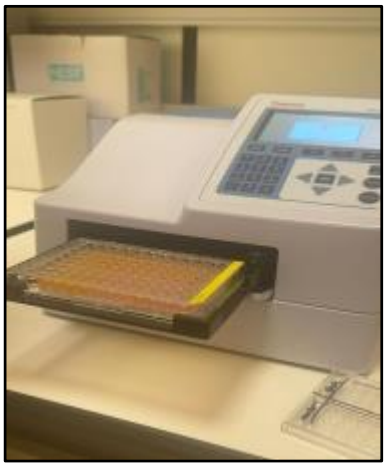


Figure 21 - Spectrophotometer used for measurement (Varioskan Flash Multiplex Reader UV)

Results

Under the project, the cells from the Molt-4 cell line of ALL, a subtype of leukemia, were treated with oyster mushroom and black cumin extracts, and the results demonstrated cell viability by absorbance measurement in a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total of Molt-4 cells (10,000 cells per well) were evenly transferred into a 96-well microplate. The data in the table show that the independent variables affecting cell viability are the type and amount of extract added, while the measured value, i.e., the dependent variable, is cell viability.

After removing the microplate containing the solution from incubation, 10µM MTT was added to each well. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used to monitor mitochondrial activity in cells. In the project, mitochondrial activity was examined to see if there was cell viability after the extracts were added. Any cell, healthy or unhealthy, has the enzyme reductase in its mitochondria. When MTT is introduced to the solution, the enzyme reductase, which is active in viable cells, converts MTT to formazan salt. The formazan salt is solid, so the formazan resulting from the reaction of MTT with mitochondrial reductase must be dissolved with dimethylsulfoxide (DMSO). The formazan salt dissolves to reveal the color.

Figure 22 - Color change upon dissolution of formazan salt indicates cell viability from mitochondrial activity

The solution in the wells containing black cumin (B 2-11, C 2-11, and D 2-11) became transparent with increasing amounts of extract. This discoloration indicates decreased mitochondrial activity. When MTT reacts with the reductase enzyme, the decrease in color release indicates that less formazan is synthesized, that is, less mitochondrial activity, that is, cell death occurs. The same is true for oyster mushroom extract. The discoloration indicates that cell death has occurred.
The absorbance values after black cumin extract and oyster mushroom extract were added to Molt-4 cells and kept for 24, 48, and 120 hours, respectively, were recorded as shown in Tables 3-4-5 (Values were recorded at 450nm in a Varioskan Flash Multiplex Reader UV Spectrophotometer).

Table 3 - Absorbance (A) values of the cell solution after 24 hours (at 450 nm)

	Concentration of extract applied								
	Control	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	**Control 2 10% hexane**
24 hours/Absorbance	1	2	3	4	5	6	7	8	10
A	~~0.1502~~	~~0.8953~~	~~0.6071~~	~~0.8565~~	~~0.1454~~	~~0.1450~~	~~0.1470~~	~~0.1435~~	~~0.1453~~
B Black cumin seed extract	0.1500	0.9664	0.9659	1.4340	0.9520	1.0682	0.8650	0.3045	0.7599
C Black cumin seed extract	0.1485	0.9954	1.7128	1.0671	1.0556	1.0543	0.8386	0.3023	0.7625
D Black cumin seed extract	0.1408	0.9377	1.6657	0.9668	0.9837	1.0920	0.7388	0.3289	0.6700
	Control	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	**Control 2 10% ethanol**
M	~~0.1487~~	~~0.6725~~	~~0.7062~~	~~0.9246~~	~~0.7443~~	~~0.1450~~	~~0.1466~~	~~0.1475~~	~~0.1457~~
F Oyster mushroom extract	0.1410	0.8932	0.8019	0.8449	0.8791	0.8913	0.7824	1.5865	0.4784
G Oyster mushroom extract	0.1413	0.8744	1.0283	1.1803	0.7282	0.9043	0.8204	1.5865	0.2095
H Oyster mushroom extract	0.1432	0.8372	0.8011	0.7332	0.8473	0.8745	0.8198	1.5707	0.2015

Table 4 - Absorbance (A) values of the cell solution after 48 hours (at 450 nm)

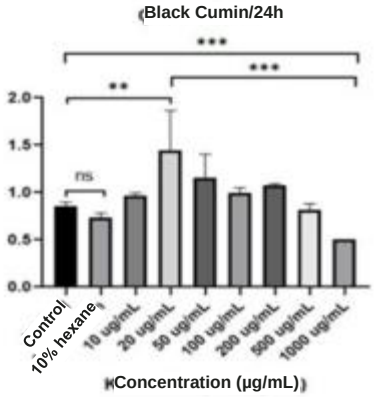
	Concentration of extract applied								
	Control -1	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	**Control 2 10% hexane**
48 Hours/Absorbance		1	2	3	4	5	6	7	
A	~~0.1943~~	~~0.1623~~	~~0.1574~~	~~0.1630~~	~~0.1589~~	~~0.1579~~	~~0.1542~~	~~0.1598~~	~~0.1554~~
B Black cumin seed extract	0.1686	1.8237	1.3732	1.1310	0.8890	1.1665	1.0255	0.4277	0.6806
C Black cumin seed extract	0.1579	1.7788	1.9726	0.8182	0.4374	0.5903	0.7164	0.5381	0.9199
D Black cumin seed extract	0.1530	1.7834	0.9302	1.4629	0.9196	0.5040	0.5068	0.2476	0.8615

	Control -1	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	Control 2 10% Ethanol
M	~~0.1556~~	~~0.1679~~	~~0.1629~~	~~0.1633~~	~~0.1547~~	~~0.1517~~	~~0.1492~~	~~0.1484~~	~~0.1458~~
F Oyster mushroom extract	0.1562	1.4995	1.9672	0.8415	0.9344	0.5056	0.8273	1.5941	0.2410
G Oyster mushroom extract	0.1565	1.8081	1.0401	1.0333	0.6100	0.3661	0.7705	1.6031	0.2134
H Oyster mushroom extract	0.1572	1.1669	0.9145	0.8858	0.6614	0.4381	0.8410	1.5340	0.1963

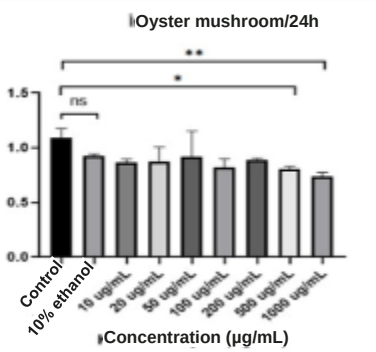
Table 5 - Absorbance (A) values of the cell solution after 120 hours (at 450 nm)

		Concentration of extract applied							
	Control -1	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	Control 2 10% hexane
120 hours/Absorbance		1	2	3	4	5	6	7	
A	~~0.3712~~	~~0.6717~~	~~0.6842~~	~~0.2879~~	~~0.2331~~	~~0.1855~~	~~0.2044~~	~~0.2004~~	~~0.2138~~
B Black cumin seed extract	0.2912	1.1921	1.5456	2.6096	1.7638	1.5487	1.4377	0.0481	1.5728
C Black cumin seed extract	0.2641	2.4082	2.9542	2.7096	1.8845	1.6196	0.7304	0.2552	1.1663
D Black cumin seed extract	0.2940	2.7414	2.8188	2.3624	1.5592	1.7155	0.2284	0.2589	0.3119
	Control-1	10µg/mL	20µg/mL	50µg/mL	100µg/mL	200µg/mL	500µg/mL	1000µg/mL	**Control 2 10% ethanol**
M	~~0.2883~~	~~0.2432~~	~~0.2395~~	~~0.1972~~	~~0.1654~~	~~0.1561~~	~~0.1551~~	~~0.1514~~	~~0.1585~~
F Oyster mushroom extract	0.2808	3.1319	2.7838	1.6283	0.7882	0.3605	0.7656	1.5695	0.1995
G Oyster mushroom extract	0.2514	3.3219	2.7865	2.7115	0.3952	0.3276	0.8195	1.2229	0.2234
H Oyster mushroom extract	0.3140	2.7411	2.5151	1.2868	0.3393	0.3195	0.8685	1.0268	0.2168

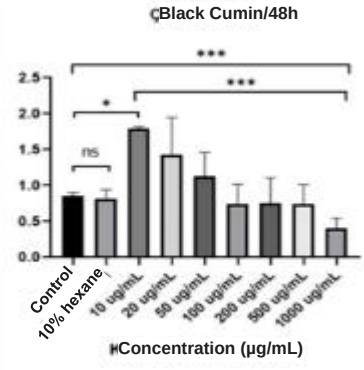
The graphs below were plotted using Graphpad Prism 5 and Two-way ANOVA for the absorbance values in Tables 3-4-5. (Graphs 1,2,3,4,5,6)



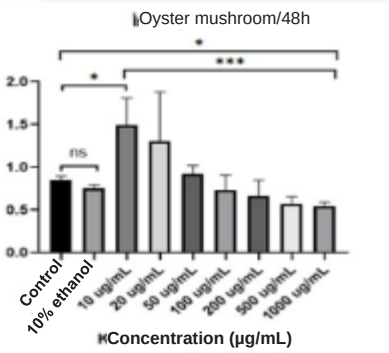
Graph 1: Application of black cumin seed extract to Molt4 cells for 24 hours



Graph 2: Application of oyster mushroom extract to Molt4 cells for 24 hours



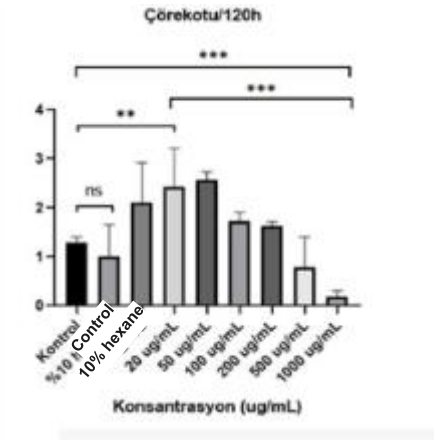
Graph 3: Application of black cumin seed extract to Molt4 cells for 48 hours



Graph4: Application of oyster mushroom extract to Molt4 cells for 48 hours

Black Cumin/120h

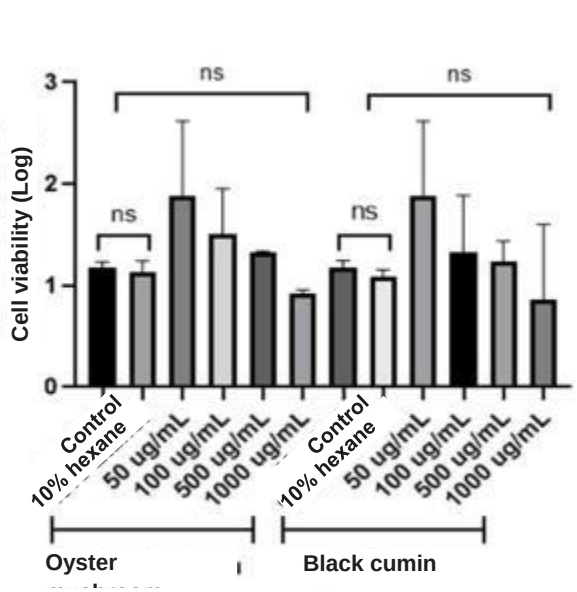
Oyster mushroom/120h



Graph 5: Application of black cumin seed extract to Molt4 cells for 120 hours

Graph 6: Application of oyster mushroom extract to Molt4 cells for 120 hours

In addition, to observe the effect of the extracts added on HEK293 (human embryonic kidney cell line), which we used as a healthy cell sample, Graph 7 was plotted with Graphpad prism 5 and Two-way Annova using the absorbance values obtained (This graph displays the mean values of 24-48-120 hours of application.



Graph 7-Effect of black cumin seed extract (right) and oyster mushroom extract (left) added to HEK293 (human healthy embryonic kidney cell line) on the viability of these healthy cells (%) (24-48-120 hours mean value)

Conclusion and Discussion:

As is well known, some dietary supplements have gained popularity in health and preventive medicine in recent years. They are products prepared using functional foods, also known as nutraceuticals (nutrition and pharmaceuticals), but offered for consumption in the form of pills,

powders, or other medicinal drugs (not in food form). The extent to which these products designed for healthy living are effective is an important area of research. It's also a topic of interest to scientists as to whether it can prevent cancer, one of the most significant disease of our age. Based on this, we investigated the effect of the active substances in black cumin and oyster mushroom extracts, which are major nutraceutical nutrients in our country and Asian countries, on T-Lymphoblast Acute Lymphoblastic Leukemia (T-ALL) cells, a very dangerous type. We experimentally investigated how the active substance of these foods would affect ALL leukemia, which is highly prevalent in children and adults in our country.

Considering our results, Graphs 1-3-5 show that the survival rate of Molt-4 cancer cells decreased as the concentration of black cumin seed extract increased. At a concentration of 1000 µg/ml, the percentage of cell survival was 55% at 24 hours and decreased to 40% at 48 hours. The best result was observed after 120 hours of waiting. About 15% of cancer cells have been shown to survive. Also, it was considered a good result that black cumin extract was also active at lower concentrations, especially during the 48-hour waiting period (see graph-3). Likewise, examining the results of oyster mushroom extract in Graphs 2-4-6, the survival rate of Molt-4 cancer cells was observed to decrease again with increasing extract concentration. At 120 hours, doses of 200µg/ml, 500µg/ml, and 1000µg/ml resulted in the highest cell death rates. At 120 hours, it was concluded that approximately 20% of cancer cells survived compared to the control group, with the remainder undergoing apoptosis. 100 µg/ml concentration also significantly reduced the number of cells (30%) (see Graph 6). At 24 and 48 hours, black cumin seed increased the apoptosis rate of cancer cells more than oyster mushroom. We believe that the cause of cell death is the toxic effect (cytotoxicity effect) of the active substance Thymoquinone in black cumin seed extract and the active substance Ergothioneine in oyster mushroom on cancer cells (see Figures 5-7). The most relevant data here is that our sample extracts applied to healthy cells (HEK293) had no significant toxic effects on these cells, and the survival rate of the cells was high (In both sample extract applications, values close to the control group were observed) (See Graph-7).

In conclusion, black cumin seed extract and oyster mushroom extract induced cytotoxicity in ALL leukemia cells, which has lethal effects and decreased mitochondrial activity of these cells (see Figure 22), which was interpreted as apoptosis of ALL cells. In addition, as the active substances in these extracts do not induce toxic effects in healthy cells, we think that they may be an alternative to chemotherapy, and we conclude that the extracts we investigated have significant effects.

Recommendations:

1-Investigating the effects of nutraceutical nutrients, which are not sold as drugs but are recommended as food supplements, and the active substances of these nutrients on cancer types with extensive studies

2-Investigation of the effects of mixtures of different concentrations of active substances

3-Investigation of the effects of active substances in the living organism as well as cell functioning

4-Detailed investigation of the antioxidant, antifungal, and antibacterial properties of active substances

5-Paying attention to the natural substances used by the people in our country and other countries in the treatment of some diseases, investigating them scientifically, and obtaining the active substances of these foods with the correct extraction method

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