Evaluation of apoptosis and caspase-3 activity in EL4 cell line lymphoma using *Moringa oleifera* plant extract

Running title: apoptosis and caspase-3 activity in lymphoma using *Moringa elefera* extract

Abstract

Lymphoproliferative disorders are a group of disorders that involve lymphocytes. These disorders are generally classified into three groups: lymphoma, leukemia, and plasma cell tumors. Lymphomas are among the most common types of malignant tumors in veterinary medicine. They have a high prevalence rate in dogs and cats suffering from diseases such as FeLV. Due to the side effects of chemotherapy drugs, researchers have been exploring the use of plants with medicinal properties. *M. oleifera*, also known as the miracle tree, is a plant that contains anti-cancer compounds in all its parts, including the leaves, roots, and stems. It has been identified as having high antioxidant potential, making it a valuable plant for promoting health and preventing diseases. *M. oleifera* is a well-known plant of the *Moringaceae* family, and its therapeutic potential has been fully determined due to its high antioxidant power Recent studies on *Moringa oleifera* have shown many anti-inflammatory and anti-cancer properties. The present study aimed to investigate the toxicity effect of moringa extract on the EL4 lymphoma cell line. In the present study, EL4 cells were exposed to different concentrations of *M. oleifera* extract for 24, 48, and 72 hours. The survival rates of the different groups were first evaluated using trypan blue and MTT assay and then by real-time PCR. The data was analyzed using SPSS software and the one-way ANOVA test. The treatment of the cell line with *M. oleifera* powder extract at all concentrations inhibited cancer cell growth. The best result was observed at 10 µg/ml after 48 hours, and the IC 50 was higher than 50%. The study concludes that *M. oleifera* extract inhibits the growth of EL4 cells.

Key words: *Moringa oleifera,* lymphoma, EL4 cell line, caspase 3, apoptosis

1. Introduction

The studies conducted have clearly shown that most tumors are caused by a series of genetic disorders (3). Many of these genetic disorders that eventually lead to cancer have been identified. With the advancement of science, it is now possible to examine genomic sequences. To better understand the disease of cancer, we must first know that it is not a single disease, but a set of disorders that occur due to the uncontrolled growth of cells (20), (4). Cancer is primarily caused by genetic factors, but it is not always hereditary. Tumors develop due to the accumulation of mutations that inhibit the natural growth of somatic cells. Lymphoma is a malignant tumor despite its suffix "-oma." This type of tumor originates from lymphoblastic cells. One of the mouse lymphoma tumor cell lines is line EL4.

Apoptosis is a genetically regulated form of cell death that plays an important role in removing infected, damaged or other unwanted cells from the body.

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Caspases, a family of proteolytic enzymes, are the main executioners of apoptosis and involved in both the initiation and execution phases of cell death. Multiple pathological processes are associated with the alteration in the activities of different caspases or with the changes in the gene expression levels of these enzymes in various types of cancers (24). One of the most important proteases is caspase 3, which is involved in the well-known pathway of apoptosis. Deficiency and downregulation of caspase-3 is associated with carcinogenesis, indicating that caspase-3 can be a biomarker in cancer prevention and treatment. Actually, apoptotic and cell cycle signaling pathways are considered as specific molecular targets for anti-cancer therapy. (25)

Recent studies show that most lymphoma tumors in domestic animals can be treated if diagnosed in time (3). In some cases, advanced lymphomas can become resistant to chemotherapy, which can reduce the effectiveness of treatment (2). Additionally, despite treatment, many patients may experience long-term side effects from chemotherapy drugs.

Traditional medicine, which is used worldwide, often relies on plants as primary therapeutic drugs. Unlike chemotherapy drugs, herbal drugs are generally considered safe and have few side effects. However, due to their active biological compounds, they can interact with chemotherapy drugs. This interaction is particularly strong in plants that have antioxidant properties (17).

Moringa oleifera is a versatile plant that can thrive in different types of soil and withstand drought in both dry and humid regions, making it an ideal crop for many tropical areas. This plant has a long history of traditional use in treating a variety of health conditions, including inflammatory and infectious diseases, prostate issues, fungal infections, and even certain types of cancer (18).

M. oleifera, also known as the miracle tree, is a plant that contains anti-cancer compounds in all its parts, including the leaves, roots, and stems. It has been identified as having high antioxidant potential, making it a valuable plant for promoting health and preventing diseases. *M. oleifera* is a well-known plant of the *Moringaceae* family, and its therapeutic potential has been fully determined due to its high antioxidant power (19).

While the exact antitumor mechanism of *M. oleifera* is not yet fully understood, it is believed that the plant's antiproliferative effects are related to the reduction of the expression of IKβα and NF-κB proteins. Inappropriate activity of NF-KB is one of the mechanisms of diseases that are associated with apoptosis or inflammation. On the other hand, the antioxidant and anti-inflammatory effects of *M. oleifera* are caused by increasing the expression of Nrf2 genes (erythroid nuclear factor) by isothiocyanate present in the plant. Nrf2 genes are the key regulators of the body's defense systems in dealing with oxidative stress (21). Also, according to the evidence obtained from existing studies, the *M. oleifera* activates apoptosis by inducing caspases (5), (2).

In this study, we aimed to investigate the potential anti-cancer properties of *M. oleifera* on EL4 lymphoma. Specifically, we focused on the apoptotic effects of *M. oleifera* on the murine mouse lymphoblastic lymphoma cell line (EL4), as this area has not been extensively researched.

٧٨ ## 2. Materials and methods

٧٩ ### 2.1. Preparation of M. oleifera plant extract

٨٠ To prepare *M. oleifera*, plant powder from India (ORGANIC INDIA Pvt. Ltd, India) was used.
٨١ For the aqueous extract, 100 grams of the powder was soaked in one liter of distilled water. For
٨٢ the ethanolic extract, 200 grams of the powder was soaked in one liter of 96% ethanol and placed
٨٣ on a thermal shaker in 60 °C at 200 rpm for 48 hours (6) l Afterward, the extracts were filtered
٨٤ with filter paper (Whatman No. 1) and the excess solvent was evaporated and concentrated using
٨٥ a rotary evaporator. The resulting pure extract was collected in sealed closed glass containers and
٨٦ stored in the refrigerator (−19 °C to 4 °C) until analysis (less than 6 months). (6).

٨٧ ### 2.2. Dissolving the plant extract and preparing the desired concentrations

٨٨ To prepare the ethanolic extract, Dimethyl Sulfoxide (DMSO) was used. However, to avoid any
٨٩ cytotoxic effects from DMSO, the concentration of the substance in the final solution was kept at
٩٠ 0.2% which is non-toxic. Concentrations of 10, 20, 40, and 80 μg/ml were prepared from the
٩١ ethanolic extract. The range of concentrations was selected based on the results of similar
٩٢ studies.

٩٣ ### 2.3. Cell culture

٩٤ The EL4 cell line was obtained from the cell bank of Pasteur Institute in Iran and was cultured in
٩٥ RPMI-1640 cell culture medium. The medium contained 10% inactivated fetal bovine serum
٩٦ (FBS) and 500 microliters of antibiotics (penicillin and streptomycin solution). The cells were
٩٧ incubated at 37°C with 5% carbon dioxide and 95% humidity. Repeated passages were made to
٩٨ ensure that the cells reached the optimal level in terms of morphology and viability.

٩٩ Peripheral blood mononuclear cells (PBMC) were used as the control group (2). To isolate
١٠٠ PBMC cells, 15 ml of blood was taken from a healthy person in a heparin tube and diluted to an
١٠١ equal ratio with sterile PBS. 10 ml of Ficoll was poured into a falcon tube and the diluted blood
١٠٢ was slowly poured onto the Ficoll with a sampler. The mixture was then centrifuged at 600 g for
١٠٣ 25 minutes. The buffy coat containing mononuclear cells was separated and transferred to
١٠٤ another falcon tube. After PBS addition, it was centrifuged for 5 minutes at 300 g. The procedure
١٠٥ was repeated three times to wash and remove Ficoll completely.

١٠٦ ### 2.4. Determining the percentage of living cells by trypan blue staining

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١٠٨ Trypan blue is a dye that has been widely used for selective staining of dead tissues or cells.
١٠٩ After centrifuging the cells at 1600 rpm for 4 minutes, a hemocytometer was used to count and
١١٠ determine the percentage of living cells. To do this, 10 microliters of the cell mixture and trypan
١١١ blue dye in equal proportions were added to a haemocytometer. The cells were then counted
١١٢ using an inverted microscope. Live cells, which have a membrane that is impermeable to the
١١٣ trypan blue, remain colorless. On the other hand, dead cells, which have the dye in their
١١٤ cytoplasm, are observed in a violet-blue color.

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١١٧ The number of cells per milliliter was calculated as follows:
١١٨ Number of cells/ml = average number of counted cells x dilution factor x 10000
١١٩ Viable cells (%) = total number of viable cells divided by total number of cells x 100

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١٢١ **2.5. Cytotoxicity assay**

١٢٢ The MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)) colorimetric method
١٢٣ was used to investigate the cytotoxic effect of the extracts. The mitochondrial succinate
١٢٤ dehydrogenase enzyme, which is active only in living cells, creates a break in the MTT ring.
١٢٥ This results in the formation of purple crystals of formazan, which are dissolved by DMSO and
١٢٦ measured (10).

١٢٧ 20,000 cells were collected in a sterile 96-well plate and exposed to different drug
١٢٨ concentrations. The experiment was repeated in triplicate, with each concentration tested in three
١٢٩ separate wells. The control wells did not receive any drug. Wells containing different
١٣٠ concentrations of drugs and culture medium (cell-free wells) were used as the blanks. After 24
١٣١ hours of incubation in the incubator (37°C in 5% Co2), 10 μl of MTT solution (5 mg/ml in PBS)
١٣٢ was added to each well. After 4 hours of re-incubation in the incubator, all remaining supernatant
١٣٣ removed and 100 μl of DMSO was added to each well and incubated for 30 minutes in the dark
١٣٤ at room temperature to dissolve the resulting formazon crystals (11). An ELISA reader (Tecan,
١٣٥ Swiss) was used to read the optical absorbances at 570 nm. The absorbance of the blank well was
١٣٦ subtracted from the average absorbances of the replicates to obtain the OD for each
١٣٧ concentration. To convert OD to the percentage of live cells (cell viability), the following
١٣٨ formula was used to determine the IC50 values.

١٣٩ mean OD of treated cells / mean OD of control cells×100

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١٤١ **2.6. Investigating the gene expression level**

١٤٢ **2.6.1. PCR and Real-Time PCR tests**

١٤٣ In this study, we investigated the expression of caspase 3 gene to investigate the process of
١٤٤ apoptosis. Each sample was tested in triplicate for each gene. Total RNA Extraction kit
١٤٥ (A101231, UK) was used to extract RNA, which was then converted to cDNA using (K1622,
١٤٦ UK) kit. Caspase 3 primers were designed to study the expression of the target genes using a
١٤٧ Thermocycler (CFX96-Real-Time system, Bio Rad, USA). The GAPDH gene was used as the
١٤٨ housekeeping gene to assess the extraction process, cDNA, and gene expression.

١٤٩ **2.6.2. RNA extraction steps**

١٥٠ To extract cellular RNA, we used the Parstous Total RNA Extraction kit (Parstous, Iran). First,
١٥١ the concentration of all extracted RNA samples was determined using a nanodrop. Then, we
١٥٢ equalized the concentration of all RNA samples. To convert RNA to cDNA, RevertAid First
١٥٣ Strand cDNA synthesis kit (Thermo Scientific, USA) was used. Then 750 microliters of RL

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solution was added to the microtube containing cells and left it at room temperature for 5 minutes. After that, 150 µl of chloroform was added and placed on a shaker for 15 seconds until the alcohol and chloroform mixed together and the solution became milky in color.

After completing the previous step, two steps were performed. First 400 µl of the upper phase was removed and transferred to a RNAase-DNAase free microtube. Afterward, 400 µl of 70% alcohol was added and gently mixed. At this stage, the contents of the microtube were transferred to a filter tube that was included in the kit and centrifuged for one minute at 13000g and 4°C. The liquid in the lower tube was discarded and 700 µl of PW solution was added to it. It was then centrifuged again at 13000g for one minute at a temperature of 13000g. Then, 500 µl of PW solution was added, and it was centrifuged for one minute at the same speed. Centrifugation was continued for two more minutes, and then the bottom tube was replaced with a new RNAase-DNAse free microtube. To separate RNA from the filter, 500 µl of DEPC was added, and centrifuged for one minute at the same speed and 4°C. The resulting RNA was transferred and kept at -20 °C until conversion to cDNA.

2.6.3. *S*ynthesis *of* DNA from an RNA template

The EvertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., USA) was used to synthesize cDNA. First, 0.1 and 0.5 µl of template and final RNA were mixed with 1 µl of primer, and the microtube was filled with DEPC-treated water up to 12 µl. The mixture was then placed on a heat block at 65°C for 5 minutes and immediately transferred to an ice chamber. Next, 1 microliter of RNA inhibitor, 2 microliters of dNTP 10 Mm and 1 microliter of reverse transcription enzyme were added. The mixture was then centrifuged briefly. After that, the microtube was left at room temperature for 5 minutes, then at 42°C for one hour and finally at 70°C for 5 min (12), (13), (9).

2.6.4. Real-time PCR method

In order to check gene expression, we used relevant primers according to the Table 1 sequences.

Table 1: Oligonucleotide sequences primers targeting Caspase3 gene

Primer	٣´ ⟶ 5٥´
Caspase 3	Forward: CAG TGGAGGCCGACT TCTTG Reverse: TGGCACAAAGCGACTGGAT
GAPDH	Forward: AACGGATTTGGTCGTATTGG Reverse: TTTGGAGGGATCTCGCTCCT

The real-time PCR kit comprised of a 12.5 master mix solution, 5 microliters of distilled water, 0.5 microliters of forward primer, 0.5 microliters of reverse primer, and 1.5 microliters of cDNA.

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The final volume of the master mix was 20 microliters. The samples were then transferred to the ABI device, and the test was conducted following the temperature program of the thermocycler. The control sample consisted of untreated cells compared to the treated samples on 24, 48, and 72 hours. The negative control included all the items used in PCR except for cDNA.

The ABI device was operated in accordance with Table 2 protocol.

Table 2: Amplification of cDNAs by the real-time reverse transcription-polymerase chain reaction

Step	Time	Temperature
Initial Denaturation	2 minutes	95ºC
Denaturation	10 seconds	94ºC
Annealing	15 seconds	60ºC
Extension	30 seconds	72ºC
40 cycles		

After the completion of the thermocycler, the Real-Time PCR product was analyzed using Reset 2006 software.

To prepare the cells for analysis, the culture medium inside each well was emptied and the wells were washed with PBS solution. Then, trypsin solution was added with a 5 times dilution (1:5) to separate the cells from the bottom of the well. It is important to accurately time the use of trypsin, as a short time will cause the cells to not separate from the bottom of the wells, while a long time will cause the cells to die. After adding trypsin and waiting for 5 minutes, the wells were examined under a microscope (×10) to ensure that the cells were separated.

To neutralize the effect of trypsin, the culture medium was added to each well in four times the amount of trypsin consumed. All of these steps were performed under a laminar hood.

The resulting suspension was transferred to RNase/DNase-free microtubes and centrifuged at 1500 g for 5 minutes. The supernatant was discarded and the precipitate was kept at -70°C. On the day of extraction, the microtubes were removed from -70°C and placed in a water bath at 37°C. This is important because the cells should not remain at -4°C.

The concentration of the extract that exhibited the strongest inhibitory effect on the survival of treated cells was considered as the maximum dose of extracts.

2.7. Statistical Analysis

Data analysis was performed using SPSS software and one-way ANOVA test. The significance level of the test was considered as 0.05. The concentration of the extract that caused a 50% inhibition of cancer cell growth was considered as IC50.

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3. Results

3.1. Cell culture

Morphology of the cells adhered to culture flasks are shown in Figures 1 and 2.

Figure 1: Freshly cultured cells in a flask examined with an inverted microscope

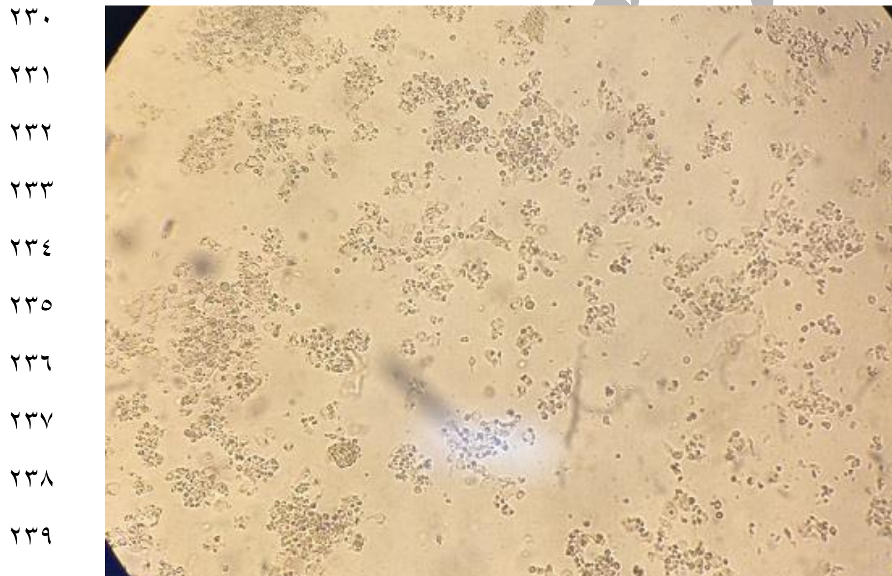

Figure 2: Colony formation, visible through an inverted microscope, indicates adhesion of cells to the plate.

3.2. Percentage of the living cells

Percentage of living cells: optical absorbance of treated cells / mean optical absorbance × 100.

Examining the percentage of live and dead cells shown in Figures 3, 4 and 5.

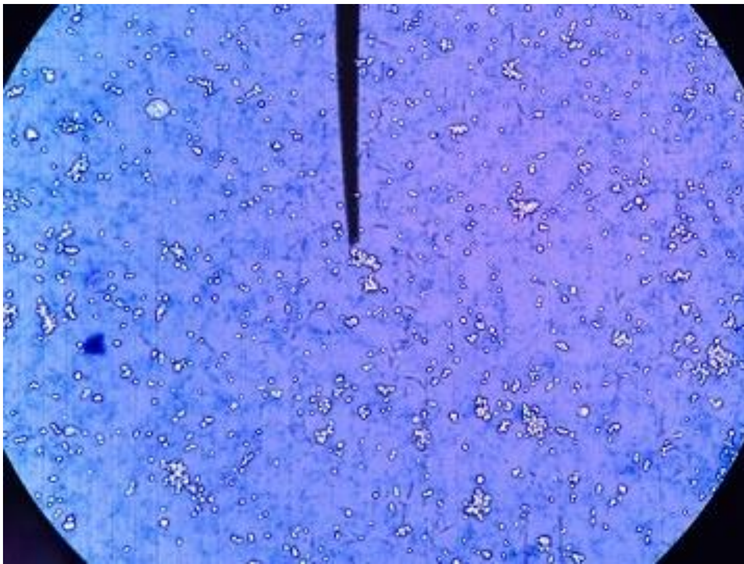

Figure 3: Examining the percentage of live and dead cells in a Neubauer chamber using a light microscope (×100) (trypan blue staining)

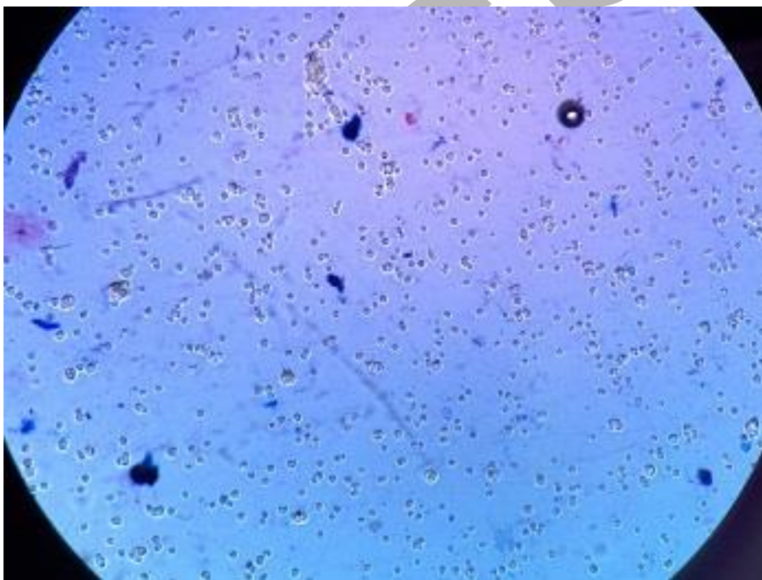

Figure 4: Calculation of the percentage of live and dead cells by trypan blue (×100)

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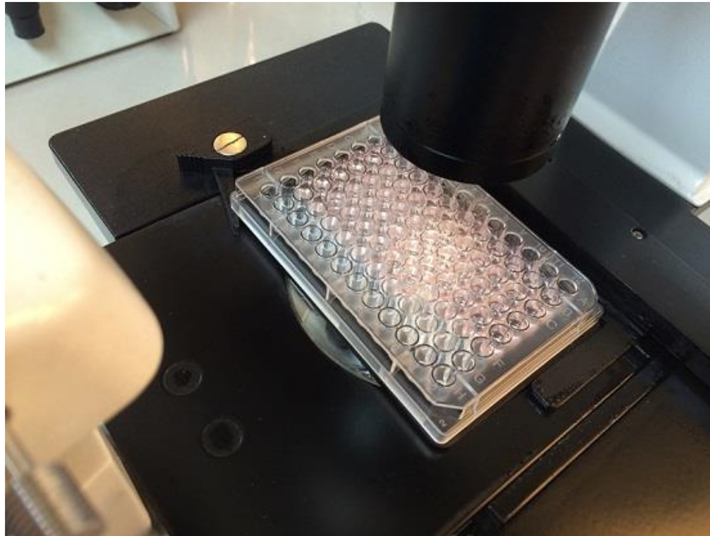

٢٧٥ **Figure 5:** Observing the establishment of cultured cells in the plate using an inverted microscope

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٢٧٧ According to the results of the MTT assay and gene expression analysis through real-time PCR,
٢٧٨ the inhibitory effects on cancer cells depend on both the duration of exposure and the
٢٧٩ concentration of *M. oleifera* extract. Higher concentrations and longer exposure times of the
٢٨٠ extract resulted in a significantly greater inhibitory effect. The best outcome was achieved at a
٢٨١ concentration of 10 µM and after 48 hours of exposure. However, the control group showed a
٢٨٢ lower number of cells at this concentration when compared to plates 24 and 48 hours. The
٢٨٣ inhibitory effects were observed at all three time points of 24, 48, and 72 hours, at concentrations
٢٨٤ higher than 10 µM.

٢٨٥ The results showed that different concentrations of *moringa* extract (10, 20, 40 and 80 µM)
٢٨٦ caused a decrease in the growth of cancer cells compared to the negative control. However, 40
٢٨٧ µM of *moringa* extract decreased the growth of cancer cells significantly ($P \leq 0.05$). (Figure 6).

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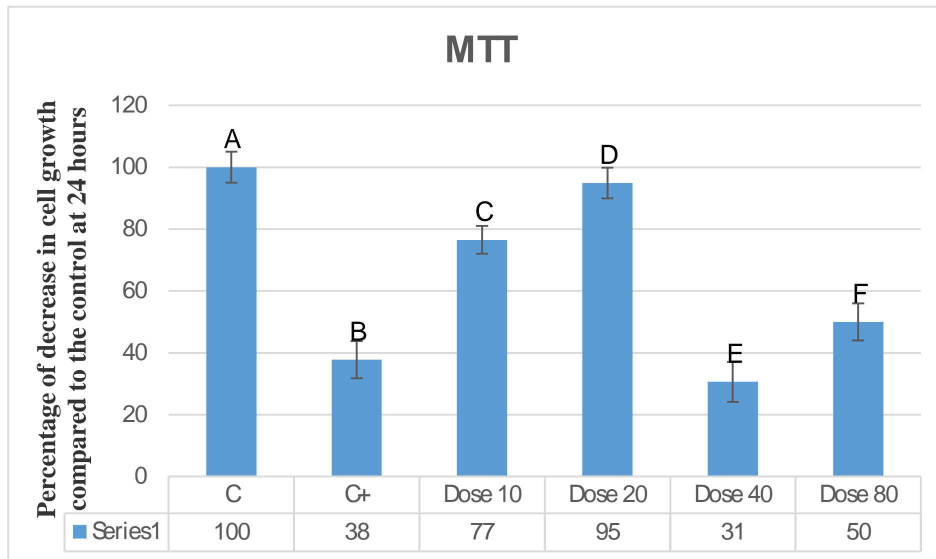


Figure 6: Percentage of cell growth reduction in MTT assay in 24 hours. The negative control (A), vincristine (B), and various concentrations of moringa extract concentrations (C-F) at 10, 20, 40, and 80 µM.

Different concentrations of *moringa* plant extract (10, 20, 40, and 80 µM) reduce cancer cell growth compared to the negative control. Concentrations of 20 and 40 µM significantly decrease the growth of cancer cells ($P \leq 0.05$) (Figure 7).

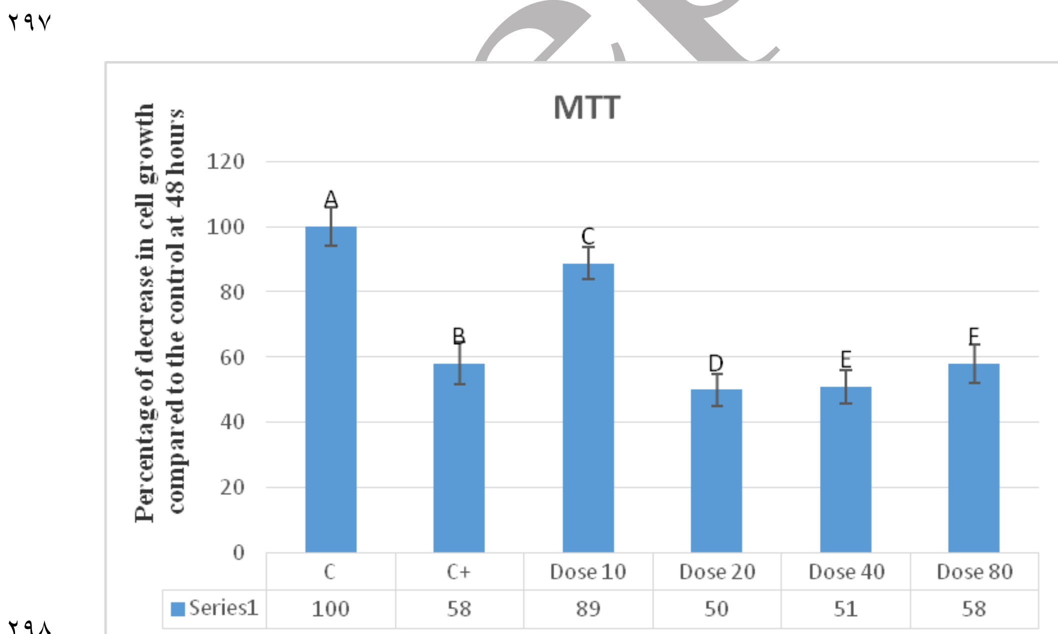


Figure 7: Percentage of cell growth reduction in MTT assay in 48 hours. The negative control (A), vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40, and 80 µM.

Various concentrations of moringa extract (10, 20, 40, and 80 µM) were tested for their impact on cancer cell growth. The results showed that moringa extract at concentrations of 20, 40, and 80 µM significantly decreased the growth of cancer cells compared to the negative control P≤0.05). The inhibitory effects of moringa extract on cancer cells were found to be both dose- and time-dependent. However, the best outcome was observed at a concentration of 80 µM and 72 hours of exposure. Therefore, the study suggests that moringa plant extract has the potential to inhibit cancer cell growth. (Figure 8).

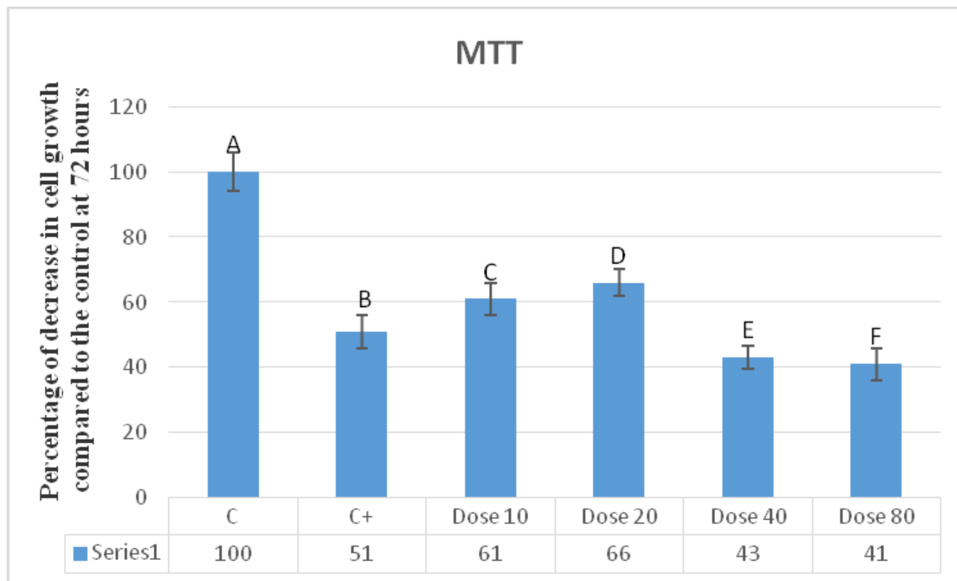
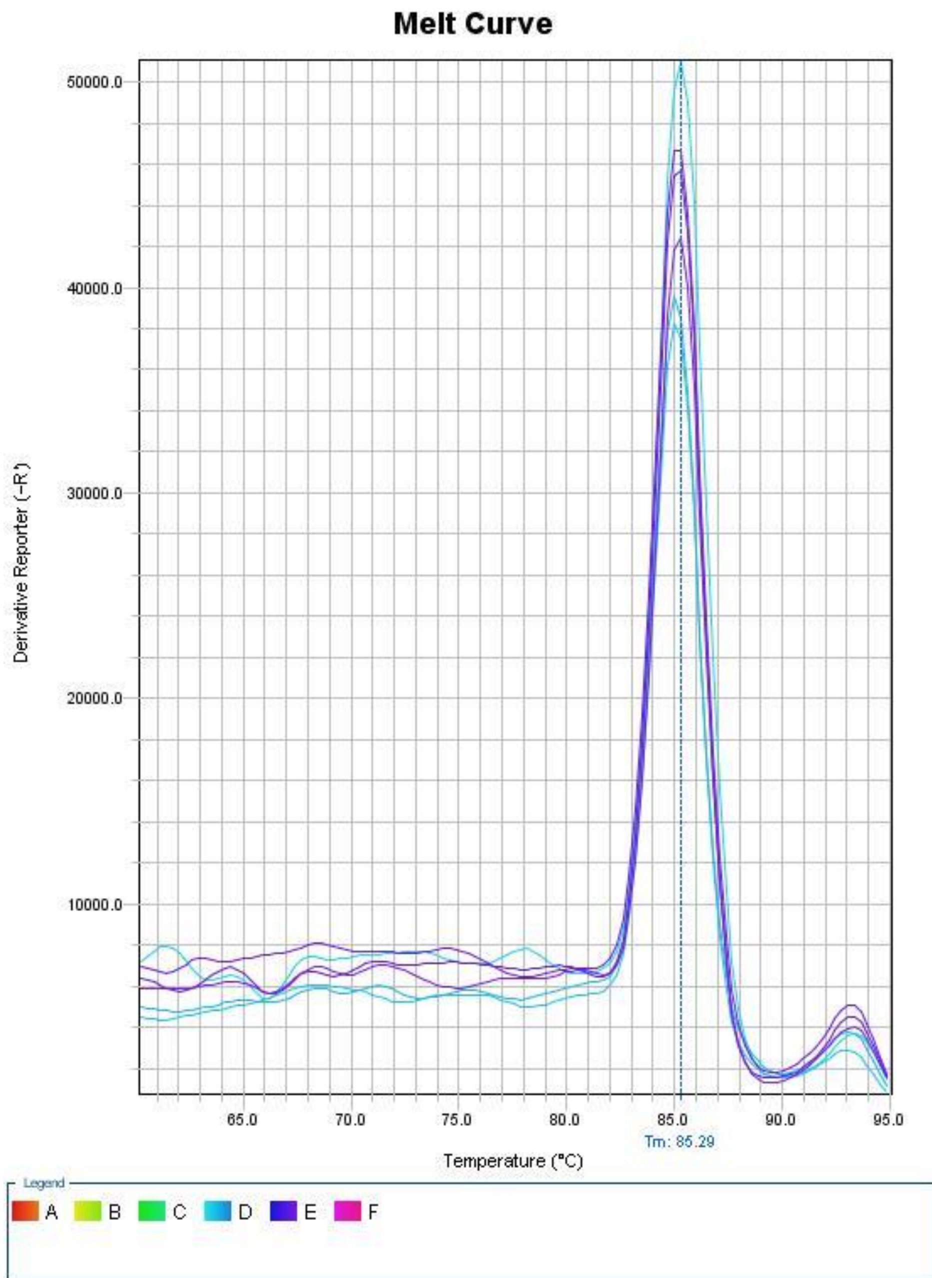


Figure 8: Percentage of cell growth reduction in MTT assay in 72 hours. The negative control (A), vincristine (B), and various concentrations of moringa extract concentrations (C-F) at 10, 20, 40, and 80 µM.

3.3. Expression of caspase 3 gene using real time PCR

At the melting point of a double-stranded DNA molecule, 50% of the hydrogen bonds break apart. This causes a sudden change in the amount of fluorescence. The peaks formed at low temperature are related to the amount of non-specific products that occur at the end of the PCR process. Each gene has its own melting curve, and the curves of one gene in all samples should match and have a single peak. In our study, the melt curve is single-peaked and coincides with each other (Figure 9).



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٣٢٢ **Figure 9:** The melt curve in our real-time PCR

٣٢٣ The study aimed to measure the expression level of the caspase 3 gene after exposing it to two
٣٢٤ different doses (40 and 80 µM) of moringa extract. The results revealed that after 24 hours of
٣٢٥ exposure, the gene expression level decreased in both doses in comparison to the positive
٣٢٦ control. However, the expression level reduced more significantly ($P \leq 0.05$) at the 40 µM dose
٣٢٧ with a 1.67-fold change. These changes were also significantly increased ($P \leq 0.05$) compared to
٣٢٨ the negative control. Additionally, it was observed that the moringa extract had the most
٣٢٩ inhibitory effect at a dose of 10 µM after 24 hours. The changes were significant and increased
٣٣٠ by 2.5-fold change compared to the negative control ($P \leq 0.05$). (Figure 10)

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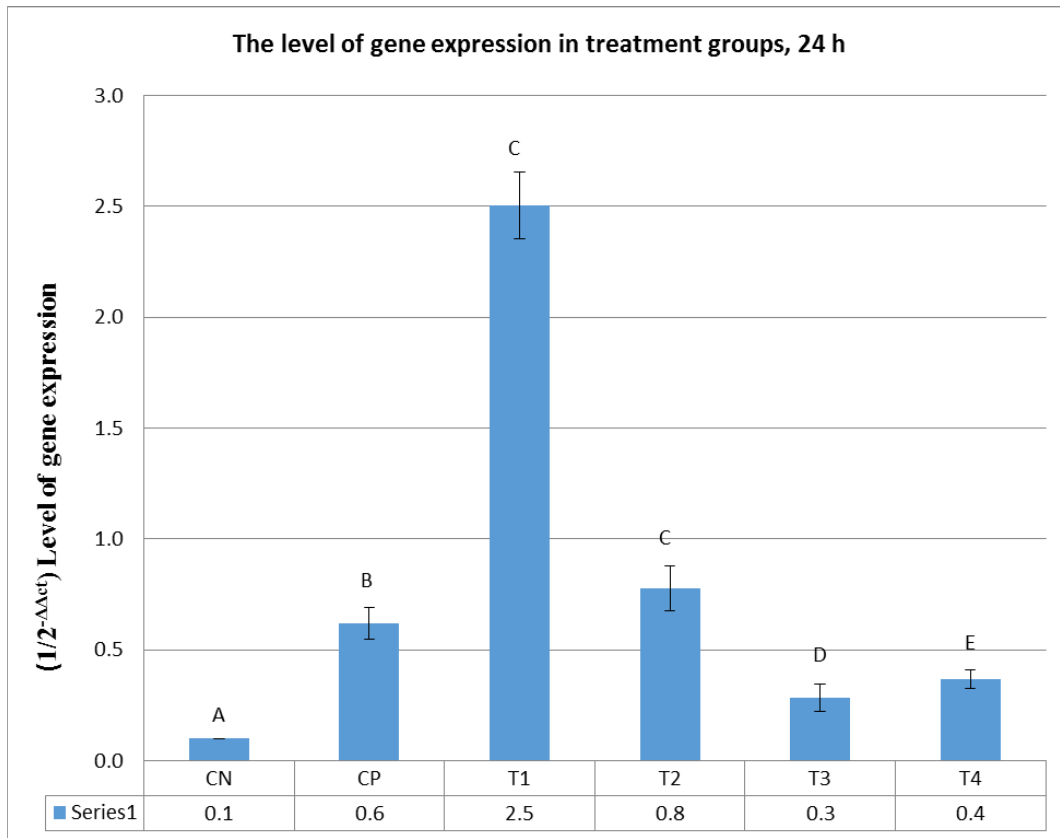


332 **Figure 10:** The amount of gene expression (caspase 3) in 24 hours. The negative control (A,
333 CN), vincristine (B, CP), and various concentrations of moringa extract concentrations (C-F, T_1-
334 T_4) at 10, 20, 40, and 80 µM.

335

336 The expression of the caspase 3 gene decreased significantly (P≤0.05) at a concentration of 80
337 µM of *moringa* extract after 48 hours, showing a 1.3-fold change compared to the positive
338 control. In comparison to the negative control, the expression of the gene increased significantly
339 (P≤0.05). The greatest inhibitory effect of *moringa* extract was observed at a concentration of 10
340 µM after 48 hours. These changes were significant (P≤0.05) and increased by 9.6-fold change
341 compared to the negative control (Figure 11).

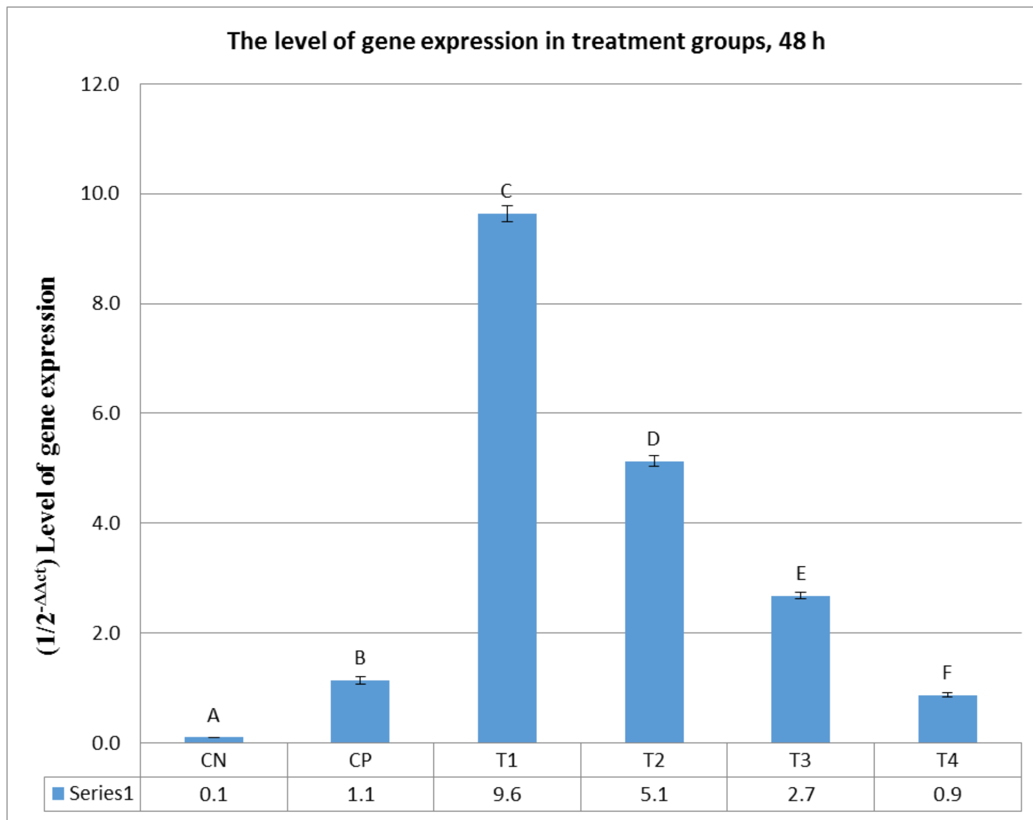
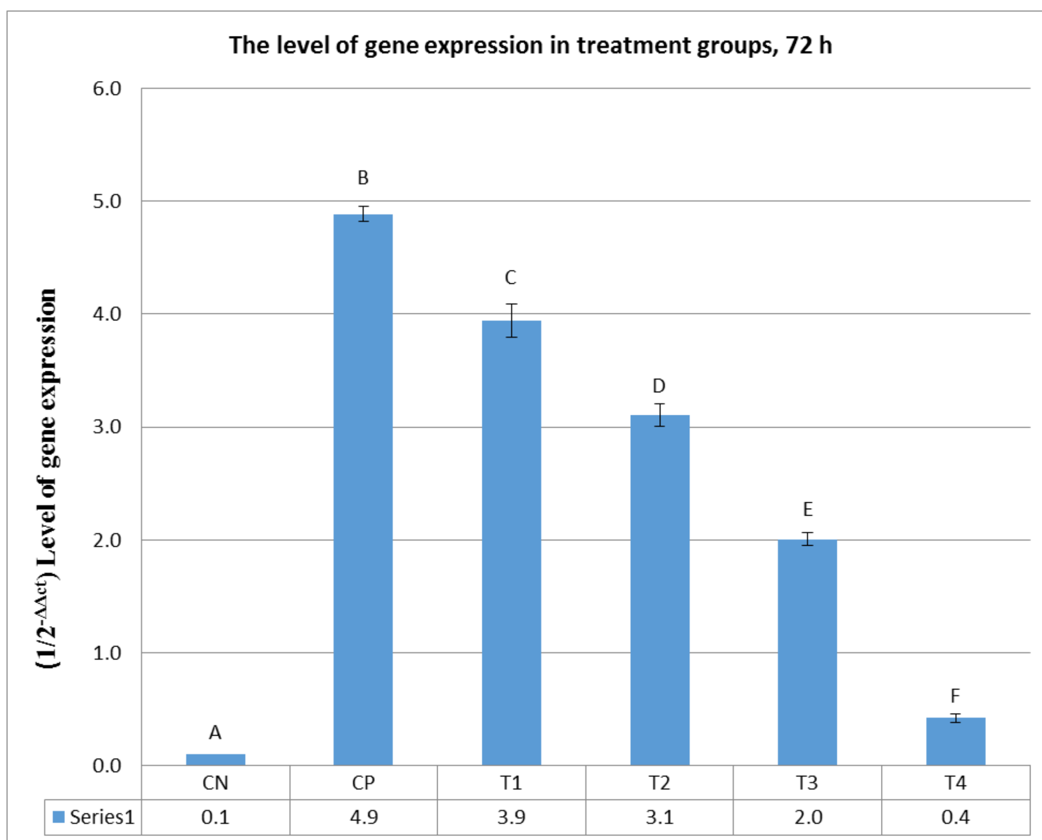


Figure 11: The amount of gene expression (caspase 3) in 48 hours. The negative control (A), vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40, and 80 μM.

The expression of the caspase 3 gene reduced after administering *moringa* extract at various doses (10, 20, 40, and 80 μM) for 72 hours, in comparison to the positive control. The expression at 80 μM decreased by 11.6-fold change ($P \leq 0.05$), which was more than the other doses. It increased significantly when compared to the negative control ($P \leq 0.05$). These changes were dependent on both the dose and time. The greatest inhibitory effect of the *moringa* extract was observed at 10 μM concentration and 72 hours, with significant changes ($P \leq 0.05$). It increased by 3.9-fold change compared to the negative control. Based on the results obtained, it was found that the *moringa* extract had a significant impact on reducing the growth of tumor cells. This effect was both time and dose-dependent. (Figure 12)



356

357 **Figure 12:** The amount of gene expression (caspase 3) in 72 hours. The negative control (A),
358 vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40,
359 and 80 µM.

360

361 **3.4. Optimum dose of the extract**

362 At a concentration of 80µM, the ethanol extract of *moringa* had the highest lethal effect on
363 lymphoma cells. The ethanolic extract at a concentration of 10 µM had the highest percentage of
364 growth inhibition of lymphoma cells, and the level of gene expression at this concentration was
365 reported as 9.6 in real-time PCR. The percentage of cell death at the mentioned dose was over
366 73%.

367 **3.5. IC50 of the extracts**

368 The IC50 value of *moringa oleifera* ethanoic extract on lymphoma cells was found to be 80 µM.
369 At a concentration of 40 µM, the lethality of lymphoma cells increased to more than 50%. The
370 study of different concentrations revealed that the effectiveness of *moringa* ethanol extract in
371 terms of cytotoxicity was dependent on the dose. When cells were treated with different
372 concentrations, the survival rates varied accordingly.

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4. Discussion

Chemotherapy is the most commonly recommended treatment for lymphoma, as it aims to eliminate cancer cells and prevent their further growth. However, the drugs used in chemotherapy can also harm healthy tissues and may not be effective against resistant tumor cells. Therefore, it is crucial to explore alternative or complementary treatments that can be used alongside chemotherapy to reduce its negative effects and increase its effectiveness.

Using herbal medicines is considered one of the least hazardous treatments available, as it has a minimal impact on natural cells. Medicinal plants, when used alone or in combination with chemotherapy, can reduce the side effects caused by chemotherapy. It has also been suggested that they may be able to overcome drug resistance and destroy resistant cells.(23)

Overall, there is a belief that medicinal plants can effectively overcome drug resistance and eradicate cancerous cells, making them a potential option for cancer treatment.

Natural compounds have been found to have potential in treating diseases, including cancer. The World Health Organization recognizes the use of herbal remedies as a form of medicine. In a recent study, *Moringa oleifera*, a plant with numerous medicinal properties and high antioxidant potential, was examined for its potential effect on lymphoma cells. Due to its drought resistance, *M. oleifera* can grow in different types of soils in dry and wet areas, and its antioxidants can help minimize the side effects of chemotherapy and reduce oxidative stress by strengthening the body's defense system. The plant's main phytochemical compounds, such as carotenoids, phenolic acids, flavonoids, and alkaloids, have beneficial effects on health and can help prevent malignancy. This particular plant has been traditionally used to treat a variety of inflammatory and infectious diseases, prostate problems, fungal infections, cancers, and more.

The research showed that the extract from this plant can inhibit the growth of lymphoma cells, and the effectiveness of the inhibition is dependent on both the dose and duration of exposure. This suggests that *M. oleifera* could be a potential source of natural compounds that could help in overcoming drug resistance and eradicate resistant cells. It is worth noting that some currently used anticancer drugs such as vincristine and vinblastine are derived from natural sources. (1,2)

The focus of the study is to investigate the potential of *M. oleifera* in inhibiting the growth of lymphoma cells due to its phenolic and flavonoid compounds, medicinal properties, and antioxidant potential. The study has found that the extract of *M. oleifera* has the ability to inhibit the growth of lymphoma cells in a time and dose-dependent manner. Many previous studies have also demonstrated the ability of *M. oleifera* to protect cells against oxidative stress by acting as a strong antioxidant, inhibiting the production of free radicals, and reducing oxidative stress caused by reactive oxygen species. (22)

Anwar et al. conducted a study on the leaves and stems of *M. oleifera*. They found that this plant contains various flavonoid and phenolic compounds which can help prevent and control oxidative damage, exhibit anti-cancer properties, and control inflammation (1). Later, Khallafa et al. conducted a study on the effect of *M. oleifera* on acute leukemia and liver carcinoma. They used cells collected from patients with leukemia and conducted an MTT assay to estimate the

٤١٤ percentage of cell viability. The results showed that *M. oleifera* extract can inhibit the release of
٤١٥ free radicals up to 77%. It also affected the growth rate and the percentage of cell death in acute
٤١٦ myeloid lymphoma cancer cells (2). In both studies, similarly with our results was observed.

٤١٧ The high antioxidant capacity of a plant is due to the accumulation of a large amount of phenolic
٤١٨ compounds. Berkovich et al. conducted a study on the impact of *M. oleifera* on the beta-kappa
٤١٩ receptor in pancreatic cancer. Pancreatic adenocarcinoma is one of the most common types of
٤٢٠ pancreatic tumors and is the fourth leading cause of cancer-related deaths worldwide.
٤٢١ Unfortunately, this disease is often diagnosed in advanced stages, which contributes to a low
٤٢٢ prognosis. Chemotherapy using platinum-based compounds is the primary treatment for this type
٤٢٣ of cancer. Factor kappa beta plays a crucial role in regulating the body's immunity by inhibiting
٤٢٤ cell proliferation. The study found that inhibiting the growth of cancer cells through the beta-
٤٢٥ kappa receptor could be a potential therapeutic approach for pancreatic cancer. It was also found
٤٢٦ that inhibiting the growth of cancer cells and the occurrence of apoptosis is a dose-dependent
٤٢٧ process, which is consistent with the results of our study. (5)

٤٢٨ Khazim et al. (2015) investigated the effect of extracts from *moringa* leaves, stems, and seeds on
٤٢٩ inhibiting the growth of colorectal cancer cells (6). The results indicated that curcumin regulates
٤٣٠ the expression level of apoptotic proteins such as caspases and BCL2. Additionally, anti-cancer
٤٣١ compounds such as lycopene suppress the PI3K/AKT signaling pathway in cancer apoptosis.
٤٣٢ *Moringa* leaf and stem extracts were shown to have significant effects on MDA-MB-231 and
٤٣٣ HCT cell lines, as they increased the rate of apoptosis by increasing G2/M in breast and
٤٣٤ colorectal cancer cells and confirms our findings.

٤٣٥ IL Lae et al. (2015) examined the effects of *moringa* on human liver cancer by analyzing the
٤٣٦ activity and expression of caspase and transcription factor. By examining apoptotic signals,
٤٣٧ including the induction of caspase activity or activation of the transcription factor, it was found
٤٣٨ that the extract of *moringa* has a high power in creating cytotoxicity against cancer cells. During
٤٣٩ experimental research, it was found that feeding *M. oleifera* extract to patients with liver cancer
٤٤٠ significantly reduces HePG2 cancer cell growth by about 44-50%, which is consistent with other
٤٤١ studies. (7).

٤٤٢ The effect of *moringa* root on HCT116 and Caco-2 cell lines from Colorectal cancer was first
٤٤٣ discussed in 2017 by Abdu Rabu et al (9). The antioxidant level of *moringa* varies depending on
٤٤٤ its different parts. Ethanol was used to extract from the root of the plant. The results of their
٤٤٥ study showed that the root of *moringa* significantly suppresses the proliferation of colon cancer
٤٤٦ cells. To investigate the cytotoxicity of *moringa*, the team exposed HCT116 and Caco-2 cells to
٤٤٧ different concentrations of 0, 20, 40, 60, 80, and 100, and assessed cell viability through MTT
٤٤٨ assay. Their findings showed that the effects of *moringa* are dose-dependent, meaning that the
٤٤٩ higher the concentration of *moringa*, the greater its cytotoxic effects.

٤٥٠ The research findings suggest that *moringa* has the ability to induce apoptosis, which is crucial in
٤٥١ stopping the growth of cancer cells. The effects of various doses and concentrations of *moringa*
٤٥٢ were studied to reinforce this discovery. A study by Wang et al. investigated the antioxidant and
٤٥٣ anti-proliferative properties of *M. oleifera* extract on head and neck cancer. *Moringa* is a rich

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source of flavonoids, anthocyanin, phenolic acids, alkaloids, and fatty acids, which are important in the treatment and prevention of head and neck cancer. The study tested the antioxidant properties of *moringa* using the 2,2-diphenyl-1-picrylhydrazyl and 2,2-azinobisbenzathiazonyl-6-sulfonic acid methods.

Moringa has been studied for its ability to combat breast, rectum, and colon cancers due to its anti-cancer properties. However, no research has been conducted on the effect of this plant on the treatment and overall healing process of head and neck cancer, so it can be said that this study is the first study recorded on this type of cancer.

It was shown that *Moringa* inhibits the growth of cancer cells in the first 24 hours of cell incubation and this effect is time and dose-dependent. Therefore, it can be said that *moringa* has strong antioxidant and anti-proliferative properties by causing apoptosis. So this study is also consistent with our and other studies in the field of plant effectiveness.

Rajabi et al., in 2022 studied the effect of aqueous and ethanol extracts of *M. oleifera* on inhibition of Jurkat and Raji cell lines of acute lymphoblastic leukemia. Acute lymphoblastic leukemia is a malignant hematological disorder of the lymphoid line. Acute lymphoblastic leukemia is a progressive hematological cancer, during which lymphoid progenitor cells multiply in the bone marrow and peripheral blood. Previous studies on the *M. oleifera* showed its anti-cancer properties on various cancer cells. After preparing aqueous and ethanolic extracts from *M. oleifera* leaves, Jurkat and Raji cells were treated with different concentrations of the extracts for 48 hours. Cell viability was evaluated by trypan blue staining and MTT assay. Peripheral blood mononuclear cells (PBMC) were used as the control group. Treatment of Jurkat and Raji cells with all concentrations of aqueous and ethanolic extracts led to inhibition of cell growth. The highest percentage of growth inhibition was obtained at a concentration of 150 µg/mL of aqueous extract, 48.5% and 47.4% for Jurkat and Raji cell lines, respectively. Also, the highest lethal percentage was obtained at a concentration of 50 µg/mL of ethanolic extract, 73.4% and 78.5% for Jurkat and Raji cell lines, respectively. The IC50 dose was obtained in all study groups at a concentration of 150 µg/mL, which was consistent with our study (16)
Dr. Balochi et al., (11), also studied the effect of the hydroalcoholic extract of *M. oleifera* in the mouse model of clone cancer induced with CacO-2 cell line. Flavonoids with antioxidant properties identified in this study included quercetin, gallic acid, and caffeic acid. MTT results showed that *moringa* at a dose of 1024 micromolar cannot kill 50% of cells, but when injected into the tumor, it has a positive effect in reducing the tumor volume. The effects of the extract on the tumor was time and dose-dependent. In the end, same as our study, it was concluded that *M. oleifera* extract with different flavonoids can be used as a useful compound in the treatment of colon cancer.

Kumar et al. (2010), investigated the effect of *Moringa* extract on Dalton's lymphoma. They showed 14 major bioactive compounds namely, Betulin, Gitoxigenin, 3- Bromopropyl phenyl ether, Lupeol, Cedran-diol, 8S-14, Olean-12-ene-3,28-diol, [3β], Lanosta-8,24-dien-3-ol, 1-monolinoleoylglycerol trymethylsilyl ether, βamyrintrimethylsilyl ether, 2-formyl-4-methylpentanoic acid, cyclopentaneundecanoic acid, 2-propyl-tetrahydropyran-3-ol, 2-octanone, 1-nitro, Isosorbidedintrate. The survival rate of cells and its toxic effects were evaluated using

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٤٩٦ the MMT assay. It was found that the occurrence of activation of oxygen radicals in the process
٤٩٧ of apoptosis plays an important role in the occurrence of toxic effects and suppression of tumor
٤٩٨ activity, which are all dose-dependent. The concentration of 450 mmol/ml *moringa* extract had
٤٩٩ the highest effect level. The study results confirm the dose- and time-dependent effect of
٥٠٠ *moringa* extract, which is consistent with our findings (12).

٥٠١ Krishnamurthy et al. investigated the anti-cancer properties of *moringa* leaf extract in 2015. In
٥٠٢ 2015, India reported 556,400 deaths due to this disease. In 2015, India recorded 556,400 deaths
٥٠٣ from this disease. Previous researches have shown that isothiocyanate compounds present in the
٥٠٤ *Moringa* plant have therapeutic potential for treating ovarian cancer. Studies have been
٥٠٥ conducted to investigate the potential preventative effects of dried *moringa* leaves on colon
٥٠٦ cancer. It was discovered that the plant releases sodium sulfate which has a positive impact on
٥٠٧ inhibiting cancer cells. In consistent with the findings, the concentration of the plant extract was
٥٠٨ found to be a key factor in determining the extent of the cytotoxic properties (8)

٥٠٩ Daghaghleh et al. (13) in 2021, evaluated different methods of extracting phytochemical and
٥١٠ antioxidant compounds from *M.oleifera* extract. The investigated cell line was A549, lung
٥١١ cancer. The results obtained from the preliminary phytochemical studies confirmed the presence
٥١٢ of flavonoids, tannins, alkaloids and simple sugars in the extract. While saponin compounds and
٥١٣ glycosides were not found in this plant. Examination of the morphology of the treated cancer
٥١٤ cells showed that there is a significant difference in the concentrations of 125 and 62.5 mg/liter
٥١٥ of the extract in 48 hours compared to the control group. The cells treated with the extract show
٥١٦ a state of shriveling and reduction in cell volume, loss of cell communication and interaction,
٥١٧ and the creation of a unique shape, which indicates that the cells are affected by the toxic effects
٥١٨ of the extract and they are removed. *Moringa oleifera* leaves are a rich source of phenolic
٥١٩ compounds, although the release rate of these compounds is largely influenced by the extraction
٥٢٠ method.

٥٢١ Badhresha et al. (2022), investigated the anti-cancer effects of *moringa* leaves on lung cancer
٥٢٢ through apoptosis. Lung cancer is one of the most dangerous and deadly cancers in the world.
٥٢٣ *Moringa* plant extract has significant effects in inducing apoptotic effects on lung cancer cell
٥٢٤ line. In addition, noticeable changes in the appearance of the cells were observed. It seems that
٥٢٥ this plant induces the effect of apoptosis and cell death by the occurrence of oxidative effects.

٥٢٦ The cells treated with *M. oleifera* extract appeared shriveled, with reduced volume and lost cell
٥٢٧ contact. This unique shape suggests that the extract has toxic effects and destroys the cells.
٥٢٨ *Moringa* leaves are a rich source of phenolic compounds, but the extraction method significantly
٥٢٩ affects their release rate. Badhresha and a group of researchers studied the anti-cancer effects of
٥٣٠ *moringa* leaves on lung cancer, one of the deadliest cancers, through apoptosis, they found that
٥٣١ *moringa* extract is effective in inducing apoptosis in the lung cancer cell line, along with
٥٣٢ noticeable changes in cell appearance. This indicates that the plant has apoptotic effects and can
٥٣٣ potentially be used in lung cancer treatment, which is consistent with our research. (14).

٥٣٤ It appears that this plant causes cell death and apoptosis by inducing oxidative effects. The toxic
٥٣٥ effects of the plant were studied at different concentrations ranging from 100 to 500 micrograms.

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After 24 hours of incubation, significant changes were observed in the cells. The results indicated that the toxic effects and reduced cell survival were time and dose-dependent. The highest effect was observed at doses of 400 and 500 micrograms, where the cells showed signs of cell wall breakdown and morphological changes in the nucleus.

Studies have shown that the BAX and BCL-2 genes undergo changes when exposed to *M. oleifera* extract. These genes are crucial in the process of apoptosis. As the dose of the extract increased, the level of gene expression also increased. This suggests that the extract plays a significant role in inducing cytotoxicity and apoptosis in the cancer cells. Additionally, a recent study by Phang Ly et al. explored the effectiveness of nanovesicles extracted from the *moringa* plant in treating cancer. Nanovesicles are membrane structures extracted from plant cells that play a vital role in this process (15).

The research utilized *Moringa* root extract, which was added to a breast cancer cell line. The cells were then incubated for 24, 48, and 72 hours. The study found that the longer the incubation time, the more nanovesicles were released by the cells. This resulted in the release of more apoptotic compounds in the medium, leading to an increase in the cytotoxicity of the cells. As a result, the plant extract was observed to have a direct effect on reducing the activity of cancer cells.

The results of the present study indicate that *moringa* extract has a deadly impact on the EL4 lymphoma cell line. This suggests that *M. oleifera* could be a potential anticancer, and its extract could be utilized as a compound for developing new drugs to treat lymphoma.

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Authors' contribution:

Study concept and design: S. S.

Acquisition of data: D. S

Analysis and interpretation of data: S. S, D. S.

Drafting of the manuscript: S. S, D. S, P. M

Critical revision of the manuscript for important intellectual content: S. S.

Statistical analysis: P. M

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Ethics:

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This article contains no studies with human participants or animals performed by any of the authors.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability:

The data that support the findings of this study are available on request from the corresponding author.

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