Original Article

Evaluation of Apoptosis and Caspase-3 Activity in EL4 Cell Line Lymphoma Using *Moringa Oleifera* Plant Extract

Shekarabi, D[1], Safi, S[1*], Mortazavi, P[1]

1. *Department of Pathobiology, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.*

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ABSTRACT

Lymphoproliferative disorders are a group of hematological malignancies involving the proliferation of lymphocytes. These neoplasms are generally classified into three distinct groups: lymphoma, leukemia, and plasma cell tumors. Among these, lymphomas are among the most prevalent types of malignant tumors in veterinary medicine. These neoplasms exhibit a high prevalence rate in dogs and cats afflicted with diseases such as FeLV. In light of the deleterious side effects associated with chemotherapy drugs, there has been a surge of interest in exploring the use of medicinal plants. 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. Moringa oleifera, a well-known member of the Moringaceae family, has been extensively studied due to its high antioxidant capacity. Recent research has highlighted its significant anti-inflammatory and anti-cancer properties. The present study sought to investigate the toxicity effect of moringa extract on the EL4 lymphoma cell line. In this study, EL4 cells were exposed to varying concentrations of M. oleifera extract for 24, 48, and 72 hours. The survival rates of the different groups were initially evaluated using trypan blue and MTT assay methods, and subsequently, real-time PCR was employed for a more precise analysis. The resulting data were then subjected to thorough analysis using SPSS software and the one-way ANOVA test. The treatment of the cell line with M. oleifera powder extract at all concentrations resulted in the inhibition of cancer cell growth. The most significant outcomes were observed at a concentration of 10 µg/ml after 48 hours, and the IC50 value was found to be greater than 50%. The study concluded that M. oleifera extract inhibits the growth of EL4 cells.

Keywords: *Moringa Oleifera*, Lymphoma, EL4 Cell Line, Caspase 3, Apoptosis.

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Corresponding Author's E-Mail:
s.safi@srbiau.ac.ir

1. Introduction

The extant research has demonstrated that the majority of tumors are the result of a series of genetic disorders (3). A significant number of these genetic aberrations that ultimately result in cancer have been identified. The advent of sophisticated scientific instruments and methodologies has rendered the examination of genomic sequences a possibility. To comprehensively understand cancer, it is imperative to recognize that it is not a singular disease, but rather a constellation of disorders stemming from uncontrolled cell growth (20), (4). While genetic factors play a primary role in cancer development, the condition is not invariably hereditary. The development of tumors is primarily driven by the accumulation of mutations that impede the natural growth of somatic cells. Lymphoma, a type of cancer, is classified as a malignant tumor despite its suffix "-oma." Originating from lymphoblastic cells, this particular neoplasm is classified as a type of lymphoma. One notable example is the mouse lymphoma tumor cell line, designated as 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. 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). Caspases, a family of proteolytic enzymes, function as the primary effectors of apoptosis, contributing to both the initiation and execution phases of cell death. A multitude of pathological processes have been observed to be associated with alterations in the activities of different caspases or with changes in the gene expression levels of these enzymes in various types of cancers (24). Of particular significance is the role of caspase 3, a pivotal protease implicated in the well-defined pathway of apoptosis. Deficiency and downregulation of caspase-3 has been associated with carcinogenesis, suggesting that it may serve as a biomarker in cancer prevention and treatment. Indeed, the apoptotic and cell cycle signaling pathways are regarded as specific molecular targets for anti-cancer therapy (25). Recent studies demonstrate that the majority of lymphoma tumors in domestic animals are amenable to treatment if diagnosed in a timely manner (3). However, in certain instances, advanced lymphomas can exhibit chemoresistance, thereby diminishing the efficacy of chemotherapy (2). Moreover, numerous patients may encounter prolonged adverse effects from chemotherapy drugs, despite undergoing treatment. Traditional medicine, a global practice, frequently utilizes plant-based drugs as primary therapeutic agents. In contrast to the adverse effects associated with chemotherapy, herbal remedies are generally considered safe and have a low incidence of adverse effects. However, due to the presence of active biological compounds, herbal drugs may interact with chemotherapy drugs, which can complicate treatment regimens. This interaction is particularly pronounced in plants with antioxidant properties (17). Moringa oleifera, a highly adaptable plant, exhibits robust growth in diverse soil types and demonstrates resilience to arid and moist climates, rendering it a suitable candidate for cultivation in tropical regions. Moringa oleifera 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 referred to 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. Moringaceae, to which M. oleifera belongs, is a well-known plant family, and its therapeutic potential has been thoroughly determined due to its high antioxidant power (19). While the precise antitumor mechanism of M. oleifera remains to be fully elucidated, it is believed that the plant's antiproliferative effects are related to the reduction of the expression of IKβα and NF-κB proteins. The inappropriate activity of NF-KB is a mechanism that is associated with diseases that are linked to apoptosis or inflammation. Conversely, the antioxidant and anti-inflammatory properties of M. oleifera are attributed to the enhanced expression of Nrf 2 genes, which are induced by isothiocyanates present in the plant. Nrf 2 genes have been identified as pivotal regulators of the body's defense systems in dealing with oxidative stress (21). The present study is further supported by evidence from existing research, which demonstrates that M. oleifera activates apoptosis by inducing caspases (5, 2). The present study was undertaken to investigate the potential anti-cancer properties of M. oleifera on EL4 lymphoma. Specifically, we examined the apoptotic effects of M. oleifera on the murine mouse lymphoblastic lymphoma cell line (EL4), as this area has not been extensively researched.

2. Materials and Methods

2.1. Preparation of M. Oleifera Plant Extract

The preparation of M. oleifera involved the use of plant powder from India (ORGANIC INDIA Pvt. Ltd, India). For the aqueous extract, 100 grams of the powder was soaked in one liter of distilled water. For the ethanolic extract, 200 grams of the powder was soaked in one liter of 96% ethanol and placed on a thermal shaker in 60°C at 200 rpm for 48 hours (6). Thereafter, the extracts were filtered with filter paper (Whatman No. 1), and the excess solvent was evaporated and concentrated using a rotary evaporator. The pure extract was then collected in sealed, closed glass

containers and stored in a refrigerator set at temperatures ranging from −19°C to 4°C until analysis (less than six months) (6).

2.2. Dissolving the Plant Extract and Preparing the Desired Concentrations

The ethanolic extract was prepared using dimethyl sulfoxide (DMSO). However, to circumvent any potential cytotoxic effects that might be induced by the use of DMSO, the concentration of the substance in the final solution was maintained at 0.2%, a concentration that has been established to be non-toxic. Concentrations of 10, 20, 40, and 80 µg/ml were prepared from the ethanolic extract. The selection of this range was informed by the findings of analogous studies.

2.3. Cell Culture

The EL4 cell line was obtained from the cell bank of Pasteur Institute in Iran and was cultured in RPMI-1640 cell culture medium. The medium contained 10% inactivated fetal bovine serum (FBS) and 500 microliters of antibiotics (penicillin and streptomycin solution). The cells were then cultivated at a temperature of 37°C, in an atmosphere containing 5% carbon dioxide and 95% humidity. Repeated passages were made to ensure that the cells reached the optimal level in terms of morphology and viability. Peripheral blood mononuclear cells (PBMC) were utilized as the control group (2). To isolate PBMC cells, 15 ml of blood was obtained from a healthy donor in a heparin tube and subsequently diluted to an equal ratio with sterile phosphate-buffered saline (PBS). 10 ml of Ficoll was poured into a falcon tube, and the diluted blood was slowly poured onto the Ficoll with a sampler. The mixture was then subjected to centrifugation at 600 g for 25 minutes. The buffy coat, containing the mononuclear cells, was then meticulously separated and transferred to an alternative Falcon tube. Following the addition of Phosphate Buffered Saline (PBS), the mixture was subjected to a centrifugation step at 300 g for a duration of 5 minutes.This procedure was repeated on three separate occasions to ensure the complete removal of Ficoll.

2.4. Determining the Percentage of Living Cells by Trypan Blue Staining

Trypan blue is a dye that has been extensively utilized for the selective staining of dead tissues or cells. Following a centrifugation of the cells at 1600 rpm for 4 minutes, a hemocytometer was utilized to enumerate and ascertain the percentage of living cells. To this end, 10 microliters of the cell mixture and trypan blue dye, prepared in equal proportions, were added to a hemocytometer. The cells were then enumerated using an inverted microscope. The membrane of live cells, impermeable to trypan blue, remains colorless. Conversely, dead cells, which have the dye in their cytoplasm, are observed in a violet-blue color.

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

2.5. Cytotoxicity Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)) colorimetric method was utilized to examine the cytotoxic effect of the extracts. The mitochondrial succinate dehydrogenase enzyme, which is active only in living cells, creates a break in the MTT ring. This reaction consequently leads to the formation of purple crystals of formazan, which are then dissolved by DMSO and subsequently measured (10). A total of 20,000 cells were collected in a sterile 96-well plate and exposed to various concentrations of the drug. The experiment was replicated thrice, with each concentration being tested in three separate wells. The control wells were not administered any drug. The wells containing varying concentrations of drugs and culture medium (i.e., cell-free wells) served as blanks. Following a 24-hour incubation period in the incubator set at 37°C in 5% CO2, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well. Following an additional four hours of incubation, the remaining culture medium was removed, and 100 µL of DMSO was added to each well. The plates were then incubated for 30 minutes in the dark at room temperature to allow the resulting formazon crystals to dissolve (11). An ELISA reader (Tecan, Swiss) was utilized to measure the optical absorbances at 570 nm. Subsequent to this, the absorbances of the blank well were subtracted from the mean absorbances of the replicates, thereby yielding the OD for each concentration. To convert OD to the percentage of live cells (cell viability), the following formula was used to determine the IC50 values.

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

2.6. Investigating the Gene Expression Level

2.6.1. PCR and Real-Time PCR Tests

In this study, we investigated the expression of the caspase 3 gene to ascertain the process of apoptosis. Each sample was tested in triplicate for each gene. The Total RNA Extraction kit (A101231, UK) was utilized for RNA extraction, which was subsequently converted to cDNA using the K1622 kit (UK). The design of primer pairs specific to the target genes was accomplished using a Thermocycler (CFX96-Real-Time system, Bio Rad, USA). The GAPDH gene was utilized as the housekeeping gene to assess the extraction process, cDNA, and gene expression.

2.6.2. RNA Extraction Steps

The extraction of cellular RNA was accomplished through the utilization of the Parstous Total RNA Extraction kit (Parstous, Iran). Initially, the concentration of all extracted RNA samples was determined using a nanodrop. Subsequently, an equalization of the concentration of all RNA samples was performed. Subsequently, the conversion of RNA to cDNA was facilitated by the utilization of the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, USA). Subsequently, 750 microliters of RL solution was added to the microtube containing cells and left at room temperature for 5 minutes. Subsequently,

150 µL of chloroform was added and the mixture was agitated for 15 seconds in a shaker until the alcohol and chloroform amalgamated, resulting in a milky-hued solution. Subsequent to the execution of the aforementioned step, two additional steps were carried out. First, 400 µl of the upper phase was removed and transferred to a RNAase-DNAase-free microtube. Subsequently, 400 µl of 70% alcohol was added and gently mixed. Subsequently, the contents of the microtube were transferred to a filter tube that was included in the kit. The contents were then subjected to centrifugation at 13,000g and 4°C for one minute. The liquid in the lower tube was then discarded, and 700 µl of PW solution was added to it. The tube was then subjected to a second centrifugation cycle at 13,000g for one minute at a temperature of 13000g. Subsequently, 500 µl of PW solution was added, and the tube was subjected to another round of centrifugation for one minute at the same speed. Centrifugation was continued for two additional minutes, after which 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 the tube was subjected to centrifugation for one minute at the same speed and 4°C. The resulting RNA was transferred and stored at -20°C until conversion to cDNA.

2.6.3. Synthesis of DNA from an RNA Template

The EvertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc., USA) was utilized for the synthesis of cDNA. Initially, 0.1 and 0.5 µl of template and final RNA were mixed with 1 µl of primer. Thereafter, 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. Subsequently, 1 µl of RNA inhibitor, 2 µl of 10 mM dNTPs, and 1 µl of reverse transcription enzyme were added. Subsequently, the mixture underwent a brief centrifugation process. Subsequently, the microtube was left at room temperature for five minutes, then at 42°C for one hour and finally at 70°C for five minutes.

2.6.4. Real-time PCR method

In order to check gene expression, we used relevant primers according to the Table 1 sequences. The real-time PCR kit consists 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. The total volume of the master mix was 20 microliters. Subsequently, the samples were transferred to the ABI device, and the test was conducted in accordance with the temperature program of the thermocycler. The control sample consisted of untreated cells, which were then compared to the treated samples at 24, 48, and 72 hours. The negative control encompassed all the items utilized in PCR with the exclusion of cDNA. The ABI device was operated in accordance with Table 2 protocol. Subsequent to the completion of the thermocycler, the Real-Time PCR product was subjected to analysis using Reset 2006 software. The samples were then washed with a Phosphate Buffered Saline (PBS) solution. Subsequently, trypsin solution was introduced to the wells at a dilution of 1:5, with the objective of disintegrating the cells from the bottom of the well. It is imperative to meticulously calibrate the duration of trypsin application, as an insufficient duration will result in unsuccessful cell separation, while an excessive duration will lead to cell death. Following the addition of trypsin and a 5-minute waiting period, the wells were examined under a microscope (×10) to ensure that the cells had been separated. To counteract the effect of trypsin, the culture medium was added to each well in a quantity fourfold the amount of trypsin consumed. It is imperative to emphasize that all of these steps were meticulously executed under the protective barrier of a laminar hood. The resulting suspension was transferred to RNase/DNase-free microtubes and subjected to a centrifugation process at 1500 g for a duration of 5 minutes. The resultant clear layer was then discarded, and the precipitate was stored at -70°C. On the day of extraction, the microtubes were retrieved from the -70°C freezer and placed in a water bath at 37°C. It is imperative to note that the cells should not be exposed to temperatures lower than -4°C. The concentration of the extract that exhibited the strongest inhibitory effect on the survival of treated cells was designated 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.

Table 1: Oligonucleotide sequences primers targeting Caspase3 gene

Pri Primer	3` ⟶ 5`
C Caspase 3	For Forward: CAG TGGAGGCCGACT TCTTG Re Reverse: TGGCACAAAGCGACTGGAT
GAPDH	Forward: AACGGATTTGGTCGTATTGG Reverse: TTTGGAGGGATCTCGCTCCT

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		

3. Results

3.1. Cell culture

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

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. The results of the MTT assay and gene expression analysis through real-time PCR demonstrate that the inhibitory effects on cancer cells are contingent on both the duration of exposure and the concentration of M. oleifera extract. Higher concentrations and longer exposure times of the extract resulted in a significantly greater inhibitory effect. The optimal outcome was observed at a concentration of 10 µM and following a 48-hour exposure period. In contrast, the control group exhibited a lower number of cells at this concentration when compared to plates after 24 and 48 hours. The inhibitory effects were observed at all three time points (24, 48, and 72 hours) at concentrations higher than 10 µM. The findings indicated that varying concentrations of moringa extract (10, 20, 40, and 80 µM) led to a reduction in cancer cell growth compared to the negative control. However, a significant decrease in cancer cell growth was observed at a concentration of 40 µM of moringa extract ($P < 0.05$). (Figure 6). The findings of this study demonstrate that different concentrations of moringa plant extract (10, 20, 40, and 80 µM) have the capacity to reduce cancer cell growth in comparison to the negative control. Concentrations of 20 and 40 µM were found to be statistically significant in their ability to inhibit cancer cell growth ($P<0.05$) (Figure 7). A series of concentrations of moringa extract (10, 20, 40, and 80 µM) were examined to ascertain their effect on cancer cell growth. The findings demonstrated that moringa extract at concentrations of 20, 40, and 80 µM led to a substantial reduction in cancer cell proliferation, as indicated by a p-value less than 0.05 in comparison to the negative control. The inhibitory effects of moringa extract on cancer cells were found to be both dose- and time-dependent. The most significant outcomes were attained at concentrations of 80 µM and 72 hours of exposure. Consequently, the study posits that moringa plant extract possesses the potential to impede cancer cell proliferation. (Figure 8).

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 undergo dissociation. This results in a precipitous change in the amount of fluorescence. The peaks formed at low temperature are indicative of the amount of non-specific products that occur at the end of the PCR process. It is noteworthy that each gene possesses its own distinct melting curve, and the curves of a particular gene across all samples are expected to exhibit congruence, manifesting as a single peak. In the present study, the melt curve exhibited a single peak, which coincided with the other curves (Figure 9). The objective of the present study was to ascertain the expression level of the caspase 3 gene subsequent to exposure to two distinct doses (40 and 80 µM) of moringa extract. The results indicated that, after a 24-hour exposure period, the gene expression level decreased in both doses in comparison to the positive control. However, a more pronounced decrease in expression was observed at the 40 µM dose, with a 1.67-fold change, reaching a statistically significant level ($P\leq0.05$). Furthermore, these alterations were found to be considerably augmented ($P\leq0.05$) in comparison to the negative control. Furthermore, it was observed that the moringa extract exhibited the most pronounced inhibitory effect at a dose of 10 µM after 24 hours. The observed alterations were substantial, exhibiting a 2.5-fold increase compared to the negative control ($P\leq0.05$) (Figure 10). The expression of the caspase 3 gene demonstrated a significant decrease ($P<0.05$) at a concentration of 80 µM of moringa extract after 48 hours, exhibiting a 1.3-fold change compared to the positive control. In comparison to the negative control, the expression of the gene increased significantly ($P\leq0.05$). The most substantial inhibitory effect of moringa extract was observed at a concentration of 10 µM after 48 hours. These alterations were found to be statistically significant ($P\leq0.05$) and exhibited a 9.6-fold increase compared to the negative control (Figure 11). The expression of the caspase 3 gene was found to be reduced after the administration of moringa extract at various doses (10, 20, 40, and 80 µM) for a duration of 72 hours, in comparison to the positive control. The expression at the 80-µM dose exhibited a 11.6-fold decrease ($P\leq0.05$), which was more pronounced than the effects observed at the other doses. A significant increase was observed when compared to the negative control ($P\leq0.05$). The observed outcomes were found to be contingent upon both the administered dosage and the duration of the experiment. The moringa extract demonstrated its most significant inhibitory effect at a concentration of 10 µM and after 72 hours, exhibiting substantial changes ($P\leq0.05$). The moringa extract demonstrated a 3.9-fold increase compared to the negative control. The findings of this study indicate that the moringa extract exerts a substantial impact on the inhibition of tumor cell growth. This effect was found to be both time- and dose-dependent (Figure 12).

3.4. Optimum Dose of the Extract

At a concentration of 80 µM, the ethanol extract of moringa exhibited the most significant lethal effect on lymphoma cells. The ethanolic extract at a concentration of 10 µM exhibited the most significant growth inhibition of lymphoma cells, with a reported gene expression level of 9.6 in real-time PCR. The percentage of cell death at this concentration was reported to exceed 73%.

3.5. IC50 of the Extracts

The half-maximal inhibitory concentration (IC50) of moringa oleifera ethanoic extract on lymphoma cells was determined to be 80 µM. At a concentration of 40 µM, the lethality of lymphoma cells increased to more than 50%.

The study of varying concentrations demonstrated that the efficacy of moringa ethanol extract in terms of cell death was concentration-dependent.

The survival rates exhibited a corresponding variation when cells were subjected to varying concentrations.

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

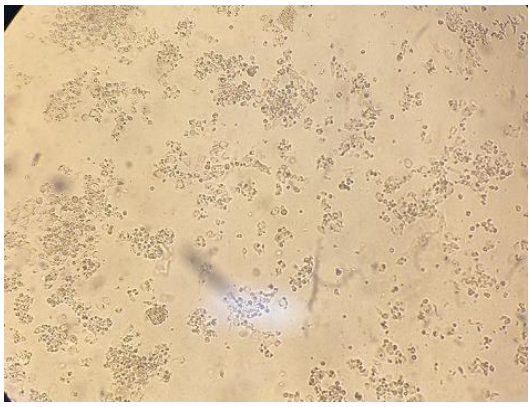

Figure 2: Colony formation, visible through an inverted microscope, indicates adhesion of cells to the plate.

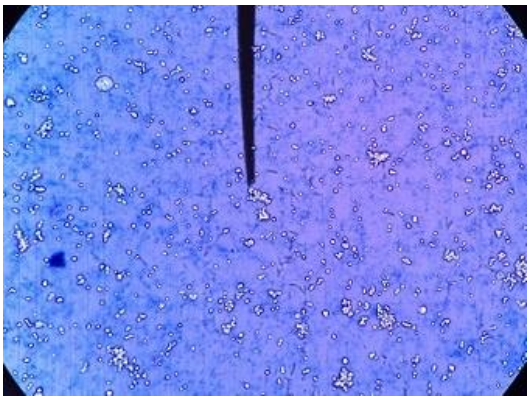

Figure 3: Examining the percentage of live and dead cells in a Neubauer chamber using a light microscope (×100) (trypan blue staining)

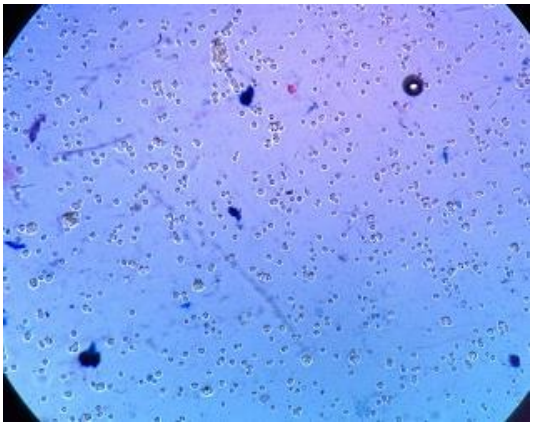

Figure 4: Calculation of the percentage of live and dead cells by trypan blue (×100)

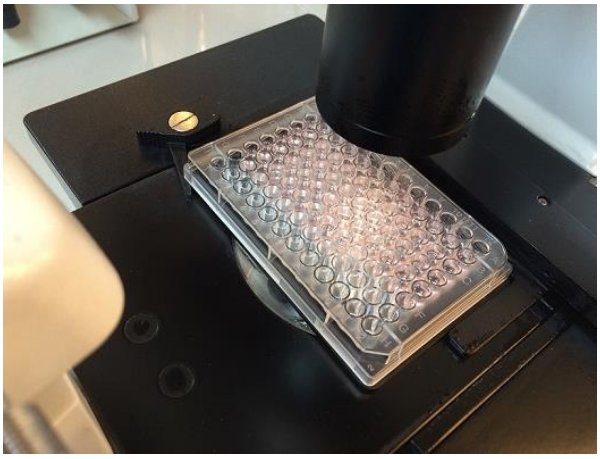

Figure 5: Observing the establishment of cultured cells in the plate using an inverted microscope

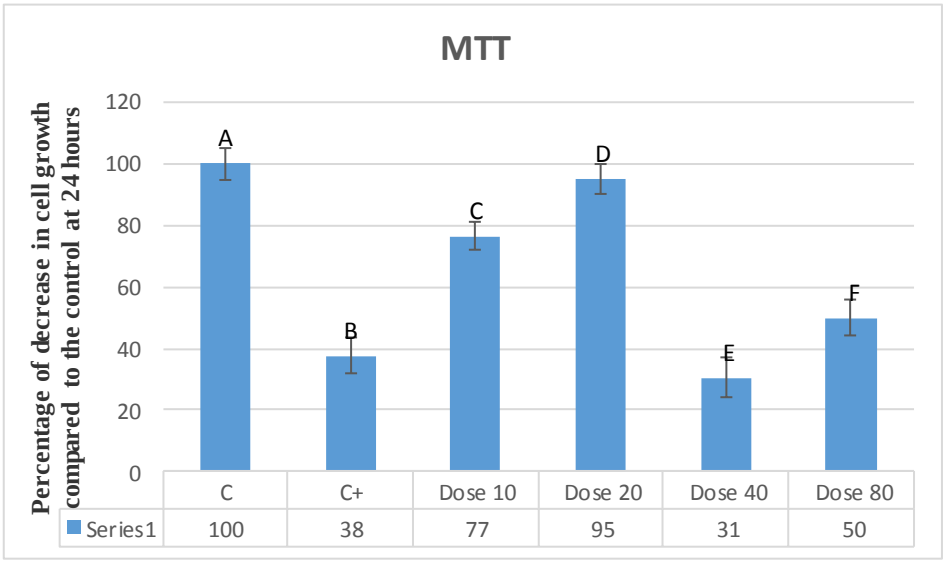

Figure 6: Percentage of cell growth reduction in MTT assay in 24 hours. The negative control (A), vincristine (B), and various concentrations of moringa extract concentrations (C-F) at 10, 20, 40, and 80 µM.

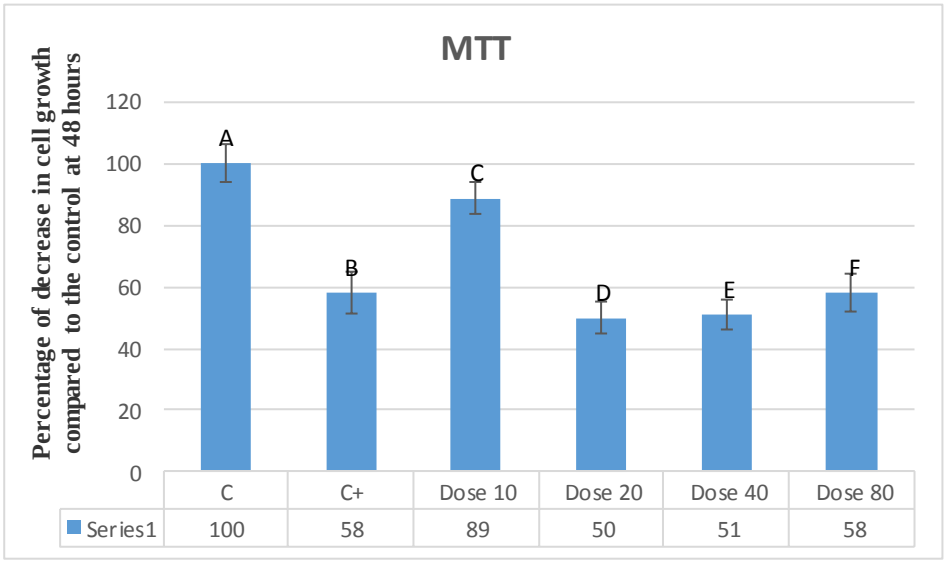

Figure 7: Percentage of cell growth reduction in MTT assay in 48 hours. The negative control (A), vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40, and 80 µM.

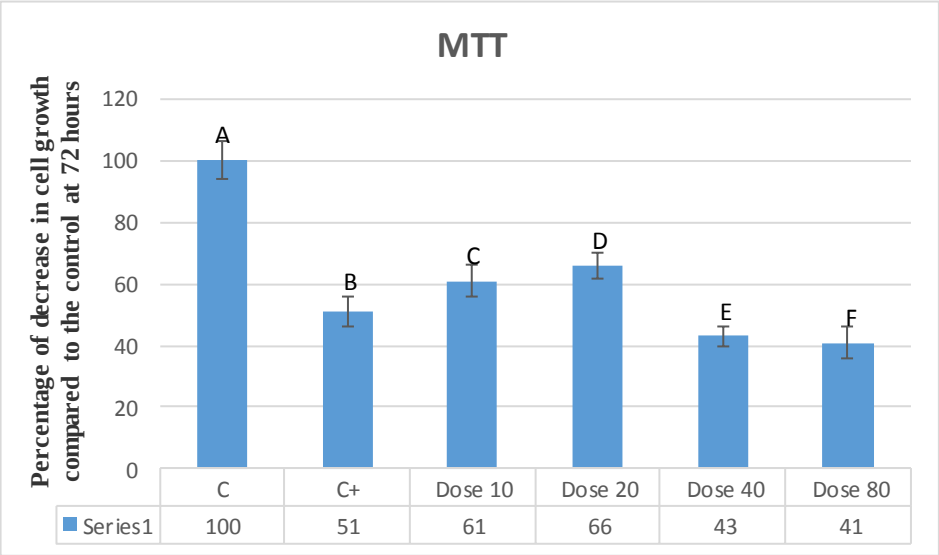

Figure 8: Percentage of cell growth reduction in MTT assay in 72 hours. The negative control (A), vincristine (B), and various concentrations of moringa extract concentrations (C-F) at 10, 20, 40, and 80 µM.

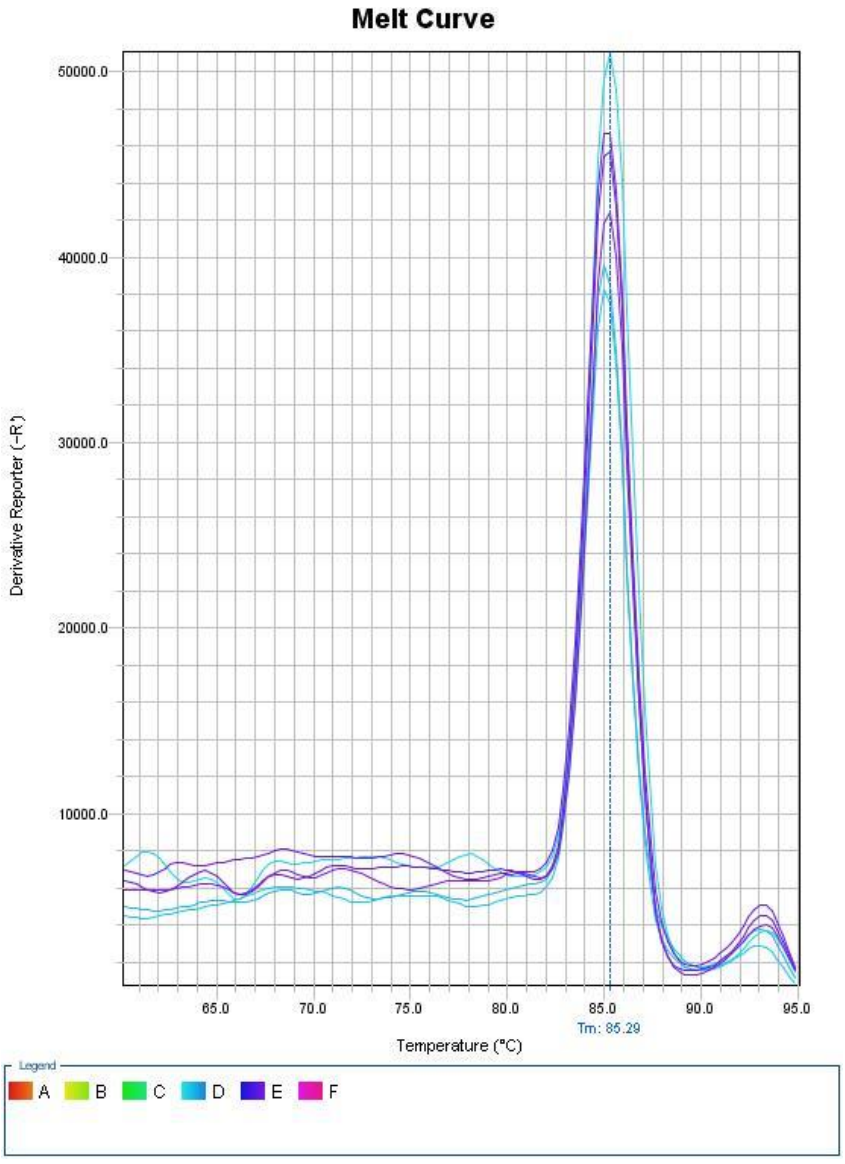

Figure 9: The melt curve in our real-time PCR

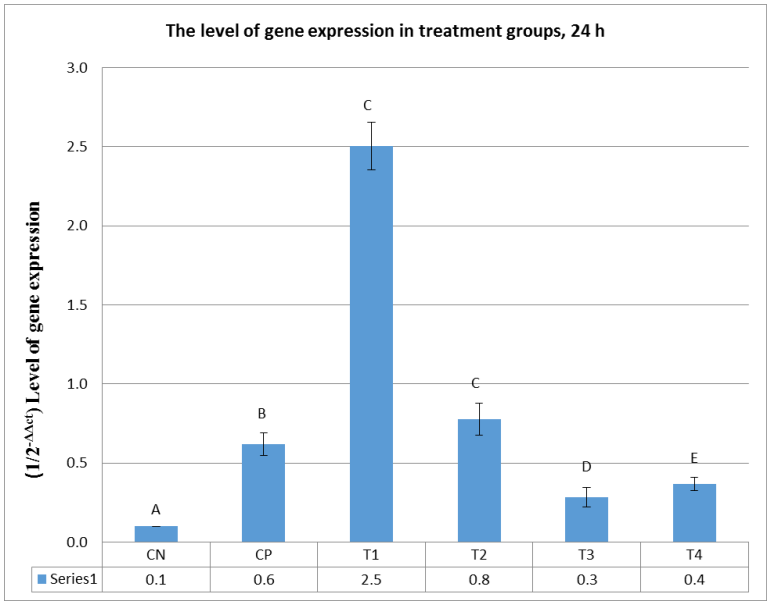

Figure 10: The amount of gene expression (caspase 3) in 24 hours. The negative control (A, CN), vincristine (B, CP), and various concentrations of moringa extract concentrations (C-F, T_1-T_4) at 10, 20, 40, and 80 µM.

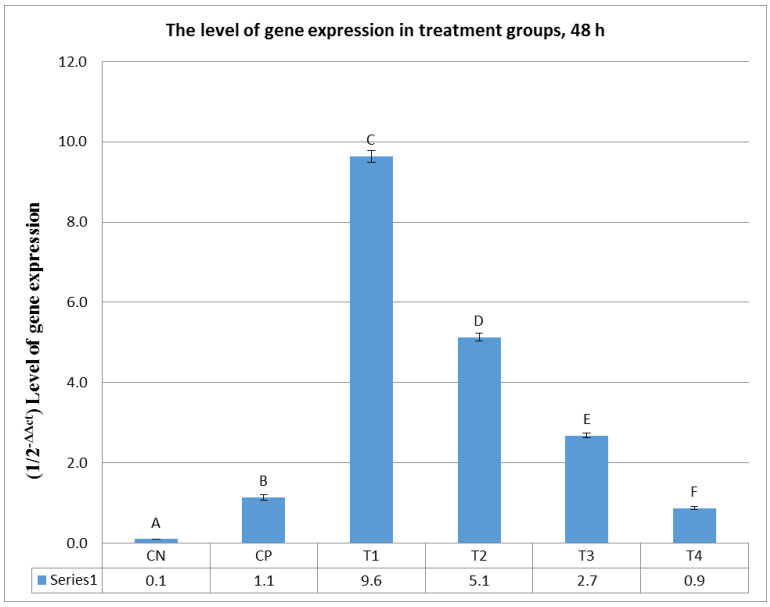

Figure 11: The amount of gene expression (caspase 3) in 48 hours. The negative control (A), vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40, and 80 µM.

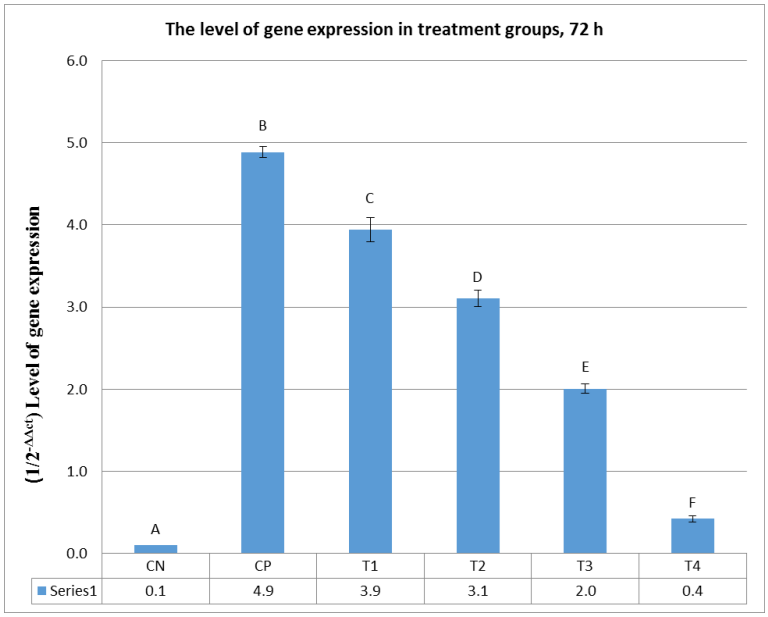

Figure 12: The amount of gene expression (caspase 3) in 72 hours. The negative control (A), vincristine (B), and various concentrations of *moringa* extract concentrations (C-F) at 10, 20, 40, and 80 µM.

4. Discussion

The objective is to eradicate cancerous cells and impede their proliferation. However, the pharmaceutical agents employed in chemotherapy can also compromise healthy tissues and may lack efficacy against resistant tumor cells. Consequently, there is an imperative to investigate alternative or complementary treatments that can be utilized in conjunction with chemotherapy to mitigate its adverse effects and enhance its efficacy. Among the available treatments, herbal medicines are regarded as one of the least hazardous options, given their minimal impact on natural cells. The use of medicinal plants, either as a standalone treatment or in conjunction with chemotherapy, has been proposed as a means of mitigating the adverse effects associated with chemotherapy. It has been proposed that they may be capable of overcoming drug resistance and eradicating resistant cells (23). In summary, 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 demonstrated potential in treating various diseases, including cancer. The World Health Organization acknowledges the utilization of herbal remedies as a form of medicine. A recent study examined Moringa oleifera, a plant with numerous medicinal properties and high antioxidant potential, for its potential effect on lymphoma cells. M. oleifera's capacity to thrive in diverse soil types, even in arid and semiarid regions, is a testament to its resilience. Its antioxidant compounds have been shown to mitigate the adverse effects of chemotherapy and enhance the body's antioxidant defense system, thereby promoting a state of enhanced resistance. The plant's main phytochemical compounds, such as carotenoids, phenolic acids, flavonoids, and alkaloids, have beneficial effects on health and can help prevent malignancy. Historically, M. oleifera has been utilized in traditional medicine for the treatment of various inflammatory and infectious diseases, prostate problems, fungal infections, and cancers. 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). The findings of the research indicated that the extract from this plant has the capacity to impede the proliferation of lymphoma cells. The efficacy of this inhibition is contingent upon both the dosage and the duration of exposure. This finding suggests that M. oleifera may serve as a potential source of natural compounds that could assist in overcoming drug resistance and eradicating resistant cells. It is noteworthy that several currently employed anticancer drugs, including vincristine and vinblastine, are derived from natural sources (1,2). The present study aims to examine the potential of M. oleifera in impeding the proliferation of lymphoma cells, a process that is hypothesized to be influenced by its phenolic and flavonoid compounds, medicinal properties, and antioxidant capacity. 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. Concurrently, numerous prior studies have corroborated the capacity of M. oleifera to safeguard cells against oxidative stress by functioning as a potent antioxidant, impeding the generation of free radicals, and curtailing oxidative stress triggered 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 percentage of cell viability. The results showed that *M. oleifera* extract can inhibit the release of free radicals up to 77%. It also affected the growth rate and the percentage of cell death in acute myeloid lymphoma cancer cells (2). In both studies, similarly with our results was observed. The high antioxidant capacity of a plant is due to the accumulation of a large amount of phenolic compounds. Berkovich et al. conducted a study on the impact of *M. oleifera* on the beta-kappa receptor in pancreatic cancer. Pancreatic adenocarcinoma is one of the most common types of pancreatic tumors and is the fourth leading cause of cancer-related deaths worldwide. Unfortunately, this disease is often diagnosed in advanced stages, which contributes to a low prognosis. Chemotherapy using platinum-based compounds is the primary treatment for this type of cancer. Factor kappa beta plays a crucial role in regulating the body's immunity by inhibiting cell proliferation. The study found that inhibiting the growth of cancer cells through the beta-kappa receptor could be a potential therapeutic approach for pancreatic cancer. It was also found that inhibiting the growth of cancer cells and the occurrence of apoptosis is a dose-dependent process, which is consistent with the results of our study (5). Anwar et al. conducted a study on the leaves and stems of M. oleifera. Their findings revealed the presence of various flavonoid and phenolic compounds within the plant, which have been shown to possess antioxidant properties, exhibit antineoplastic activity, and modulate inflammatory

responses (1). Subsequently, Khallafa et al. investigated the impact of M. oleifera on acute leukemia and liver carcinoma. They collected cells from patients with leukemia and used an MTT assay to estimate the percentage of cell viability. The results demonstrated that M. oleifera extract was capable of inhibiting the release of free radicals by up to 77%. The study also demonstrated a significant impact on the growth rate and the percentage of cell death in acute myeloid lymphoma cancer cells. The outcomes of these investigations are consistent with the results obtained in the present study. The substantial antioxidant capacity exhibited by this plant is attributable to its high phenolic compound content. Berkovich et al. conducted a study on the impact of M. oleifera on the beta-kappa receptor in pancreatic cancer. Pancreatic adenocarcinoma, a prevalent form of pancreatic malignancy, ranks as the fourth leading cause of cancer-related mortality worldwide. A notable challenge in addressing this condition is the tendency for diagnosis in advanced stages, which often portends a poor prognosis. Chemotherapy using platinum-based compounds is the primary treatment for this type of cancer. The kappa beta factor plays a pivotal role in regulating the body's immune system by impeding cell proliferation. The present study found that the growth of cancer cells can be inhibited through the beta-kappa receptor, which suggests that this could be a potential therapeutic approach for pancreatic cancer. The study further established that the inhibition of cancer cell growth and the induction of apoptosis are dose-dependent processes, aligning with the findings of our research (5). In their study, Khazim et al. (2015) examined the impact of moringa leaf, stem, and seed extracts on the proliferation of colorectal cancer cells (6). The results indicated that curcumin regulates the expression level of apoptotic proteins such as caspases and BCL2. Furthermore, the suppression of the PI3K/AKT signaling pathway in cancer apoptosis by anti-cancer compounds, such as lycopene, has been demonstrated. The study's findings, which were based on experiments with the MDA-MB-231 and HCT cell lines, revealed that moringa leaf and stem extracts significantly increased apoptosis by increasing G2/M in breast and colorectal cancer cells, thereby confirming the initial hypothesis. Furthermore, IL Lae et al. (2015) examined the effects of moringa on human liver cancer by analyzing the activity and expression of caspase and transcription factor. By examining apoptotic signals, including the induction of caspase activity or activation of the transcription factor, it was found that the moringa extract has a high degree of effectiveness in creating cancer cell death. The experimental research demonstrated that the administration of M. oleifera extract to patients with liver cancer led to a significant reduction in HePG2 cancer cell growth, ranging from 44% to 50%, which is consistent with the findings of other studies (7). The impact of moringa root on HCT116 and Caco-2 cell lines derived from colorectal cancer was initially examined in 2017 by Abdu Rabu et al. (9). The antioxidant level of moringa varies depending on the specific part of the plant being analyzed. The extraction of moringa's antioxidants involved the use of ethanol, a solvent, to extract the compounds from the root of the plant. The study's findings indicated that the root of moringa significantly suppresses the proliferation of colon cancer cells. To further investigate the cytotoxic potential of moringa, the team exposed HCT116 and Caco-2 cells to varying concentrations of the extract, ranging from 0 to 100 parts per million (ppm). Cell viability was then assessed through the MTT assay. The findings indicated that the effects of moringa are concentration-dependent, meaning that increasing the concentration of moringa results in increased cytotoxic effects. The study's findings suggest that moringa possesses the capacity to trigger apoptosis, a process vital for the inhibition of cancer cell proliferation. To further substantiate this finding, a comprehensive study was conducted, exploring the impact of varying doses and concentrations of moringa. A study by Wang et al. investigated the antioxidant and anti-proliferative properties of M. oleifera extract on head and neck cancer. Moringa is a rich source of flavonoids, anthocyanins, phenolic acids, alkaloids, and fatty acids, which are important in the treatment and prevention of head and neck cancer. The antioxidant properties of moringa were evaluated using two distinct methods: the 2,2-diphenyl-1-picrylhydrazyl and the 2,2-azinobisbenzathiazonyl-6-sulfonic acid techniques. Moringa has been the subject of research due to its potential to combat breast, rectum, and colon cancers, owing to its anti-cancer properties. However, to the best of our knowledge, no research has been conducted on the effect of this plant on the treatment and overall healing process of head and neck cancer. Therefore, it can be said that this study is the first study recorded on this type of cancer. The findings of the present study demonstrate that Moringa impedes the proliferation of cancer cells within the initial 24-hour period of cell incubation, an effect that is both time- and dose-dependent. Consequently, it can be posited that moringa possesses robust antioxidant and anti-proliferative properties, manifesting as apoptosis. This finding aligns with the findings of other studies that have examined the efficacy of plant-based interventions. In 2022, Rajabi et al. investigated the impact of aqueous and ethanol extracts of M. oleifera on the inhibition of Jurkat and Raji cell lines of acute lymphoblastic leukemia. Acute lymphoblastic leukemia (ALL) is a malignant hematological disorder of the lymphoid line. This form of cancer is characterized by the uncontrolled proliferation of lymphoid progenitor cells within the bone marrow and peripheral blood. Prior studies on M. oleifera have demonstrated its anti-cancer properties on various cancer cells. In the present study, aqueous and ethanolic extracts from M. oleifera leaves were prepared, and Jurkat and Raji cells were treated with different concentrations of the extracts for 48 hours. The assessment of cell viability was conducted through the utilization of trypan blue staining and the MTT assay. Peripheral blood mononuclear cells

(PBMC) were utilized 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, with 48.5% and 47.4% inhibition for the Jurkat and Raji cell lines, respectively. Conversely, the highest lethal percentage was observed at a concentration of 50 µg/mL of ethanolic extract, achieving 73.4% and 78.5% for Jurkat and Raji cell lines, respectively. The median inhibitory concentration (IC50) was consistently observed at 150 µg/mL across all study groups, aligning with the findings reported in our previous study (16). Dr. Balochi et al. (11) investigated the impact of the hydroalcoholic extract of M. oleifera in a mouse model of clone cancer induced with the CacO-2 cell line. The study identified flavonoids with antioxidant properties, including quercetin, gallic acid, and caffeic acid. The MTT results indicated that moringa at a dose of 1024 micromolar could not cause the death of 50% of cells. However, when injected into the tumor, moringa exhibited a positive effect in reducing the tumor volume. The observed effects of the extract on the tumor manifested as being time- and dose-dependent. Conclusively, analogous to the present study's findings, it was determined that M. oleifera extract, comprising diverse flavonoids, holds promise as a therapeutic agent for the management of colon cancer. Kumar et al. (2010) investigated the effect of Moringa extract on Dalton's lymphoma. Their findings revealed the presence of 14 significant bioactive compounds, including betulin, gitoxigenin, 3-bromopropyl phenyl ether, lupeol, cedran-diol, 8S-14, olean-12-ene-3,28-diol, [3β], and lanosta-8,24-dien-3-ol. 1-monolinoleoylglycerol trimethylsilyl ether, β-amyrintrimethylsilyl 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 the MTT assay. The results indicated that the activation of oxygen radicals during apoptosis plays a pivotal role in the induction of toxic effects and the suppression of tumor activity, which are both dose-dependent. The concentration of 450 mmol/ml moringa extract exhibited the most significant effect level. The study's findings corroborate the dose- and time-dependent effect of moringa extract, aligning with our prior observations (12). In 2015, Krishnamurthy and colleagues conducted a study to investigate the potential anti-cancer properties of moringa leaf extract. In the same year, India reported 556,400 deaths due to this disease. In the same year, India documented 556,400 mortalities due to this disease. Prior studies have demonstrated the therapeutic potential of isothiocyanate compounds present in the Moringa plant in the treatment of ovarian cancer. Concurrent studies have been conducted to investigate the potential preventative effects of dried moringa leaves on colon cancer. It was discovered that the plant releases sodium sulfate, which has a positive impact on inhibiting cancer cells. Concentration of the plant extract was identified as a pivotal factor in determining the extent of the cytotoxic properties (8).Daghaghleh et al. (13) in 2021, evaluated various methods of extracting phytochemicals and antioxidant compounds from M. oleifera extract. The investigation focused on the A549 cell line, which is associated with lung cancer. The preliminary phytochemical studies confirmed the presence of flavonoids, tannins, alkaloids, and simple sugars in the extract. However, the presence of saponin compounds and glycosides was not detected in this particular specimen. A subsequent examination of the morphology of the treated cancer cells revealed a significant difference in the concentrations of 125 and 62.5 milligrams per liter of the extract after 48 hours, as compared to the control group. The cells exposed to the extract exhibited a state of shrinkage and reduction in cell volume, loss of cell communication and interaction, and the formation of a unique shape, indicating that the cells were affected by the toxic effects of the extract and were eliminated. Moringa oleifera leaves are a rich source of phenolic compounds; however, the release rate of these compounds is largely influenced by the extraction method. In their study, Badhresha et al. (2022) examined the potential anti-cancer properties of moringa leaves in relation to lung cancer, focusing on the process of apoptosis. Lung cancer is among the most dangerous and deadly cancers worldwide. The study found that moringa plant extract has a significant impact on inducing apoptosis in a lung cancer cell line. Noteworthy alterations in cell morphology were also observed. The study suggests that the induction of apoptosis and cell death in lung cancer cells by moringa may be attributed to its oxidative effects. The cells exposed to M. oleifera extract exhibited a shrunken, diminished volume, and a disruption in cell-to-cell contact. This distinctive morphology indicates the potential for the extract to exert toxic effects and cause cell death. Moringa leaves are a rich source of phenolic compounds; however, the extraction method significantly affects their release rate. A study by Badhresha et al. investigated the anti-cancer properties of moringa leaves in the context of lung cancer, a particularly deadly form of cancer. Their findings, reported in the study, suggest that moringa extract exerts its anti-cancer effects through the induction of apoptosis in lung cancer cells. This observation is accompanied by significant alterations in the appearance of the cells. This finding aligns with the hypothesis that the plant possesses apoptotic properties, which could have therapeutic applications in lung cancer treatment, a finding that is consistent with the results of our own research (14). The available evidence suggests that this plant induces oxidative stress, which in turn leads to cell death and apoptosis. The toxic effects of the plant were studied at concentrations ranging from 100 to 500 micrograms. Following a 24-hour incubation period, substantial alterations were discernible within the cells. The findings indicated that the toxic effects and reduced cell survival were time- and dose-dependent. The most

pronounced effects were observed at concentrations of 400 and 500 micrograms, which resulted in indications of cell wall breakdown and nuclear morphological changes. Furthermore, studies have demonstrated that exposure to M. oleifera extract leads to alterations in the BAX and BCL-2 genes. These genes play a pivotal role in the process of apoptosis. As the dosage of the extract increased, so did the level of gene expression. This finding suggests that the extract plays a significant role in inducing cellular changes that lead to cell death and the process of apoptosis in cancer cells. A recent study by Phang Ly et al. explored the effectiveness of nanovesicles extracted from the moringa plant in treating cancer. These nanovesicles, which are membrane structures extracted from plant cells, have been shown to 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 subjected to an incubation period of 24, 48, and 72 hours. The study found that the longer the incubation time, the more nanovesicles were released by the cells. This phenomenon was found to be associated with an augmented release of apoptotic compounds within the medium, culminating in an escalation in the cellular toxicity. Consequently, the plant extract was observed to exert a direct effect on reducing the activity of cancer cells. The findings of the present study suggest that moringa extract exerts a lethal effect on the EL4 lymphoma cell line. This finding suggests that M. oleifera could be a promising anticancer agent, and its extract could serve 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
Administrative, technical, and material support: S. S, D.S., P.M.

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

Conflict of Interest
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 upon request from the corresponding author.

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