Anti-Cancer Potential of *Moringa oleifera* Leaves Methanol Extract: An In Vitro Study

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Abstract

Drumstick (*Moringa oleifera)* leaves were rich in phytochemicals and nutraceuticals (flavonoids, proteins, vitamins) and also has potential antioxidant activities. The anticancer studies of drumstick leaves methanol extract have been evaluated on the B16-F10 –Mouse melanoma cell lines by cell cycle analysis. These anticancer studies evidence with the evaluation of MTT cytotoxicity assay, apoptosis effect, tyrosinase assay and melanin determination in the in vivo activities. The methanol extract had significant cytotoxicity on to B16-F10 –Mouse melanoma cell lines with an IC_{50} of 127.12ug/ml. In cell cycle analysis 1 mg of *Moringa oleifera* leaf methanol extract exhibited antiproliferation at 36.54% compared to untreated and standard Kojic acid significantly affected. These studies further evidenced with the results of apoptotic potential against Mouse melanoma cells with high % apoptosis (67.89%) and necrosis as like in Kojic acid (Std control-99.16%) by Annexin V/PI expression studies. Also found significant inhibition of Melanin content in dose dependent fashion by the extract and Tyrosinase is key enzyme for melanin pigment production assessed it in moringa extract and found 0.0433 IU. Regular intake of *Moringa oleifera* leaves in diet may antiproliferative cancer cells and act as a novel therapeutic agent for any skin cancers.

Keywords: Antiproliferation, Cell lines, *Moringa oleifera,* Nutraceuticals, Phytochemicals

Introduction

Medicinal plants derived medicine to treatment of diseases were practiced from ancient days and it has recorded in Indian Ayurveda system to prove it nature treasure in scientific approach from last few decades gained more interest in research and development. In current day research, medicinal plants utilization became very common for any diseases increases to treat it successfully. Plants are an important source of natural medicine and they play vital role in world health. Plant medicines were known to be potential source of therapeutics or curatives aids and it has proven from research. India has practiced various traditional medicinal system, like Ayurveda, Sidda, Unani, etc which has history of than 3000 years, mainly using plant-based drugs. The ancient texts like Rigveda (4500-1600 BC) and Atharvaveda mentioned the use of several plants as medicines for all the treatments with protocols and ancient methods (1, 2).

A study explored on the anticancer potential of *Moringa oleifera* targeting CDK-2 inhibition in estrogen receptor-positive breast cancer. The study found that compounds like chlorogenic

acid, quercetin, and ellagic acid showed good affinity with the target and significant antiproliferative effects on MCF-7 cancer cell lines (3). A systematic review examined the effects of *Moringa oleifera* extracts on oral squamous cell carcinoma. The review highlighted the antioxidant, anti-inflammatory, anticancer, and chemopreventive properties of moringa, suggesting its potential as a complementary therapy (4). Another study investigated the anti-cancer effect of aqueous and ethanolic extracts of *Moringa oleifera* leaves on HeLa cells. The results indicated that the extracts contained bioactive compounds with significant anticancer activity and relatively non-toxic effects on normal lymphocytes (5). A comprehensive review reported for the anticancer properties of *Moringa oleifera*, including its ability to inhibit the growth of PC3 prostate cancer cells. The study found that extracts from different parts of the plant, such as leaves and seeds, exhibited significant anticancer activity (6).

A review discussed on the antimicrobial and antioxidant properties of *Moringa oleifera*. The study highlighted its potential applications in the food and medical industries, noting that various parts of the plant, including roots, leaves, bark, and seeds, exhibit significant antimicrobial activity against both Gram-positive and Gram-negative bacteria (7). A systematic review provided a comprehensive evaluation of the botany, traditional uses, phytochemistry, pharmacology, and toxicity of *Moringa oleifera*. The review summarized the diverse phytochemicals found in the plant, such as flavonoids, carbamates, glucosinolates, and phenols, which have various bioactivities, including anti-tumor, antioxidant, and anti-inflammatory effect (8). A review on chemical composition, nutritional content, biological activities, and applications of *Moringa oleifera*. The study emphasized the plant's potential in food and health industries due to its rich nutritional profile and bioactive compounds (6).

Hence, in a similar context, the current study focused on the anticancer properties of methanol extract from *Moringa oleifera* (drumstick leaves). The extract was tested on B16-F10 mouse melanoma cell lines using cell cycle analysis and its cytotoxicity was determined through the MTT assay.

Material and Methods

Plant Materials

The leaves of *Moringa oleifera* (drumstick) were collected at the end of August to October 2024 from our university campus, and authenticated by Dr. Ram Rao, Scientific Officer, Central Ayurvedic Research Institute, Bengaluru, Karnataka, India.

Preparation of Plant Extract

Green matured leaves of *M. oleifera* were collected from north areas of Bengaluru. Two separate 10% raw and boiled extracts of fresh leaves and completely dried (37˚C), powered leaves of *M. oleifera* and 10% methanolic extract of fresh leaves was prepared. 10% of all these extracts were used to quantitatively analyse the bioactive molecules, present in the leaves.10% raw extract of fresh leaves were used as crude sample in the in vivo studies. Fresh matured green leaves were homogenised using pestle and mortar, filtered and centrifuged at high speed. Similarly fresh matured green leaves were completely dried at room temperature for 2-3 days and finely powdered, the fine powder of the dried leaves were centrifuged at high speed. The supernatant obtained was used to prepare 10% raw extract of both fresh and dried leaves. Fresh leaves and dried leaf powder of *M. oleifera* was firstly homogenised and boiled in water to

prepare 10% boiled extracts. The boiling was carried out for 10 minutes and cooled, later the 10% boiled extracts were centrifuged at high speed and both the raw and boiled leaf extracts along with 10% methanolic extracts were stored

Anticancer Studies of Methanol Extract of *Moringa oleifera* Leaves

Cytotoxicity Studies by MTT Assay Methanol Extract of *Moringa oleifera* Leaves

The B16-F10 (Mouse melanoma cell line) was purchased from NCCS, Pune, India. The cells were maintained in DMEM media with high glucose supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured for every 2 days. Passage number 30 was used for the present study.

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow-coloured water-soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm (9, 10).
To conduct the MTT assay, seed 200µl of cell suspension in a 96-well plate at a density of 20,000 cells per well, without the test agent, and allow the cells to grow for about 24 hours. Add appropriate concentrations of the test agents as mentioned in the results (Excel sheet). Incubate the plate for 24 hours at 37°C in a 5% CO2 atmosphere. After the incubation period, remove the plates from the incubator, discard the spent media, and add MTT reagent to a final concentration of 0.5mg/mL of the total volume. Wrap the plate with aluminum foil to avoid exposure to light and return it to the incubator for an additional 3 hours. Note that incubation time may vary for different cell lines, but within one experiment, it should be kept constant for comparisons. After incubation, remove the MTT reagent and add 100µl of solubilization solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution, and occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals, especially in dense cultures. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm, using 630nm as the reference wavelength.

Calculate the percentage of cell viability using the formula: % cell viability = OD of treated cells/OD of untreated cells) x 100

The IC_{50} value is determined using the linear regression equation Y=Mx+C$Y=Mx+C$, where Y=50$Y=50$, and MM and CC values are derived from the viability graph.

Cell cycle analysis study on B16F10 cell lines by Flow Cytometry of methanol extract of *Moringa oleifera* leaves

The B16F10 (Human breast adenocarcinoma cell line) was purchased from NCCS, Pune, India. The cells were maintained in DMEM with high glucose media with 1.5mM L-glutamine supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature in the CO_2 incubator and sub-cultured for every 2days. Passage No-35 was used for the present study.

This is a method for cell cycle analysis using propidium iodide (PI) that is, using the fluorescent nucleic acid dye PI to identify the proportion of cells that are in one of the three interphase stages of the cell cycle. Please take into account the following when using this method:

1. This method works well to assess cell cycle distribution of a whole cell population when cells are fixed with 70% ethanol.

2. This method can also be used to assess cell cycle distribution of certain GFP transfected cells. The ethanol fixation step is generally not suitable to keep GFP in the cell. The use of membrane localized GFP fusion protein (i.e. Spectrin) provides excellent results employing this ethanol fixation procedure and is highly recommended to assess the cell cycle distribution in GFP transfected cells. Otherwise consider a live cell DNA stain such as Hoechst 33342 or Draq5. Aldehyde fixation (e.g. PFA), which cross links cellular protein together and hence keep GFP inside the cell is not suitable for cell cycle phases analysis without further optimisation.

To conduct cell cycle analysis using propidium iodide (PI), culture cells in a 6-well plate at a density of 2 x 10^5 cells/2 ml and incubate them in a CO2 incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with the required concentration of the experimental compound (IC50) and controls in 2 ml of culture medium, then incubate the cells for another 24 hours. At the end of the treatment, remove the medium from all wells, wash the cells with PBS, and add 250µl of trypsin-EDTA solution. Incubate at 37°C for 3-4 minutes, then add 2 ml of culture medium and harvest the cells directly into 12 x 75 mm polystyrene tubes. Centrifuge the tubes for five minutes at 300 x g at 25°C and carefully decant the supernatant. Wash the cells with PBS and decant the PBS completely. Fix the cells in 1 ml of cold 70% ethanol by adding it dropwise to the cell pellet while vortexing to ensure fixation and minimize clumping. Incubate for 30 minutes in a -20°C freezer. Pellet the cells at a higher speed compared to live cells for 5 minutes, aspirate the supernatant carefully to avoid losing the pellet, and note that ethanol-fixed cells require higher centrifugal speeds due to increased buoyancy. Wash the cells twice with PBS. To ensure that only DNA is stained, treat the cell pellet with 400µL of Propidium Iodide/RNase staining buffer and mix well. Incubate the cells for 15 to 20 minutes at room temperature in the dark. Finally, analyze the samples by flow cytometry in PI/RNase solution without the need to wash the cells (11, 12).

Apoptosis studies of on B16F10 cell lines by Flow Cytometry of methanol extract of *Moringa oleifera* leaves

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and inter nucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features(13).

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. Since externalization of PS occurs in the earlier stages of

apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation (14).

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, wheras the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative (15).

Cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both FITC Annexin V and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V and PI positive, in of itself, reveals less information about the process by which the cells underwent their demise (15).

To conduct the experiment, culture cells in a 6-well plate at a density of 0.5 x 10^6 cells/2 ml and incubate them in a CO2 incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with the experimental compound at the desired concentration and controls in 2 ml of culture medium, leaving one well untreated as a control. Incubate the cells for another 24 hours. At the end of the treatment, remove the medium from all wells, wash the cells with PBS, and add 500µl of trypsin-EDTA solution. Incubate at 37°C for 3-4 minutes, then add 2 ml of culture medium and harvest the cells directly into 12 x 75 mm polystyrene tubes. Centrifuge the tubes for five minutes at 300 x g at 25°C and carefully decant the supernatant. Wash the cells twice with PBS and decant the PBS completely. Add 5µl of FITC Annexin V in 100µl of Annexin V binding buffer, gently vortex the cells, and incubate for 15 minutes at room temperature (25°C) in the dark. Add 5µl of PI and 400µl of 1X Annexin Binding Buffer to each tube, vortex gently, and analyze by flow cytometry immediately after the addition of PI.

Estimation of Melanin of methanol extract of *Moringa oleifera* leaves

Abnormal and excessive melanin synthesis is the primary cause of serious skin disorders including melasma, senile lentigo, freckles, and age spots. Melanogenesis is the process that leads to the production of the dark macromolecular pigment melanin by melanocytes. Melanin synthesis occurs via a serial process of enzymatic catalyses and chemical reactions. Melanin determines skin pigmentation and normally functions to prevent skin injury through the absorption of harmful UV radiation. The photochemical properties of melanin make it an excellent photoprotectant, as it absorbs harmful UV rays and emits this energy as harmless heat through a process referred to as ‘ultrafast internal conversion’. However, abnormal and excessive accumulation of melanin may result in skin disorders such as hyperpigmentation,

melasma, freckles, age spots, and senile lentigo Therefore, regulation of melanogenesis is an important strategy to consider in the treatment of aesthetic and serious skin disorders associated with abnormal skin pigmentation.

To estimate melanin content, seed 1000µl of cell suspension in a 12-well plate at a density of 50,000 cells per well, without the test agent, and allow the cells to grow for about 24 hours. Add appropriate concentrations of the test agent and incubate for 2 hours, as mentioned in the results (Excel sheet). Afterwards, incubate the plate with 1µM of α-melanocyte-stimulating hormone (α-MSH) for 48 hours at 37°C in a 5% CO2 atmosphere. After the incubation period, remove the plates from the incubator, discard the spent media, and wash the cells twice with 1x PBS. Evaluate the cells to estimate melanin content. For melanin content estimation, dissolve the samples in 500µL of 1M NaOH, incubate at 60°C for 1 hour, and mix to solubilize melanin. Determine the melanin content in the samples by measuring the absorbance at 405 nm.

Calculate the percentage of melanin content using the following formula:

% Melanin content = 100 - {Abs of treated cells/Abs of Untreated cells} x 100

Estimation of Tyrosinase Enzyme of methanol extract of *Moringa oleifera* leaves

To conduct a tyrosinase assay, start by preparing the necessary reagents. Dissolve potassium phosphate monobasic in deionized water to create a 50 mM potassium phosphate buffer, adjusting the pH to 6.5 with 1 M KOH. Prepare a 1 mM L-tyrosine solution by dissolving L-tyrosine in deionized water. Just before use, prepare the tyrosinase enzyme solution containing 500-1,000 units/ml of tyrosinase in cold potassium phosphate buffer. For the reaction cocktail, mix 9.00 ml deionized water, 10.00 ml potassium phosphate buffer, and 10.00 ml L-tyrosine solution, adjusting the pH to 6.5 at 25°C if necessary. Oxygenate the reaction cocktail by bubbling 99.9% pure O2 through it for 3-5 minutes (16-18).

Next, pipette 2.90 ml of the reaction cocktail into quartz cuvettes, equilibrate to 25°C, and monitor the absorbance at 280 nm until constant using a thermostatted spectrophotometer. Add 0.10 ml of the tyrosinase enzyme solution to the test cuvette, mix by inversion, and record the increase in absorbance at 280 nm for approximately 10 minutes. Calculate the enzyme activity using the formula:

$$\textbf{Units/ml enzyme} = \frac{(r\ A280nm/min\ Test - r\ A280nm/min\ Blank) \times df}{0.001 \times 0.1}$$

where r A280nm/min is the rate of change in absorbance per minute, df is the dilution factor, 0.001 is the change in A280nm/min per unit of tyrosinase at pH 6.5 at 25°C in a 3 ml reaction mix, and 0.1 is the volume (in milliliters) of enzyme used. This protocol allows for the continuous spectrophotometric determination of tyrosinase activity by monitoring the oxidation of L-tyrosine to L-DOPA and further to L-DOPA-quinone.

Results and Discussion

In recent years, significant progress has been made in identifying naturally occurring chemopreventive substances that can inhibit or reverse multistage carcinogenesis. A diverse range of phenolic compounds, particularly those found in dietary and medicinal plants, have

demonstrated substantial anti-carcinogenic and anti-mutagenic activities (19, 20). Many natural products derived from a variety of aromatic and medicinal plants are commonly used in household treatments for various ailments (21).

Anticancer Studies of Methanol Extract of *Moringa oleifera* Leaves

Cytotoxicity Studies by MTT Assay

The statistical data from the cytotoxicity study using the MTT assay indicated that the test compound, *Moringa oleifera*, exhibited effective toxicity against B16-F10 cells with an IC50 concentration of 127.12 µg/ml after a 24-hour incubation period. Kojic acid was used as a standard control for the study. The observed absorbance readings and calculations are enclosed in a separate folder of the report in MS Excel format. Additionally, direct microscopic observations of drug-treated B16-F10 cell line images, captured at 20x magnification, are included in a separate folder along with this report (Table 1; Figure 1).

The MTT assay results suggest that *Moringa oleifera* is moderately toxic to mouse melanoma cells, with an IC50 value of 127.12 µg/ml after 24 hours of incubation. These findings align with previous research indicating the anticancer potential of *Moringa oleifera*. For instance, a study by de Andrade Luz et al. (2017) demonstrated that *Moringa oleifera* lectin (cMoL) exhibited cytotoxic effects on B16-F10 murine melanoma cells, reducing cell viability and inducing apoptosis (22)

Another study highlighted the anticancer properties of *Moringa oleifera* in oral squamous cell carcinoma, showing its ability to inhibit cancer cell proliferation and induce apoptosis (22).

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Further mechanistic studies are needed to evaluate the anti-melanoma potential of *Moringa oleifera* and to elucidate the underlying mechanisms of its anticancer activity against murine melanoma cells in vitro. Understanding these mechanisms could provide valuable insights into the development of *Moringa oleifera* as a complementary therapy for melanoma treatment.

Table 1: Table showed the % cell viability values and IC_{50} value of the methanol extract of *Moringa oleifera* leaves against the Mouse melanoma (B16-F10) cells after the treatment period of 24hrs.

MTT Assay of Methanol Extract of *Moringa oleifera* Leaves		
Concentration (µg/ml)	**Cell Viability (%)**	**IC^{50} Concentration (ug/ml)**
Untreated	100.00	
Kojic acid-100ug/ml	99.53	
Moringa-6.25ug	99.57	
Moringa-12.5ug	98.58	

Moringa-25ug	91.80	127.12
Moringa-50ug	77.87	
Moringa-100ug	53.64	
Moringa-200ug	37.77	
Moringa-400ug	20.53	

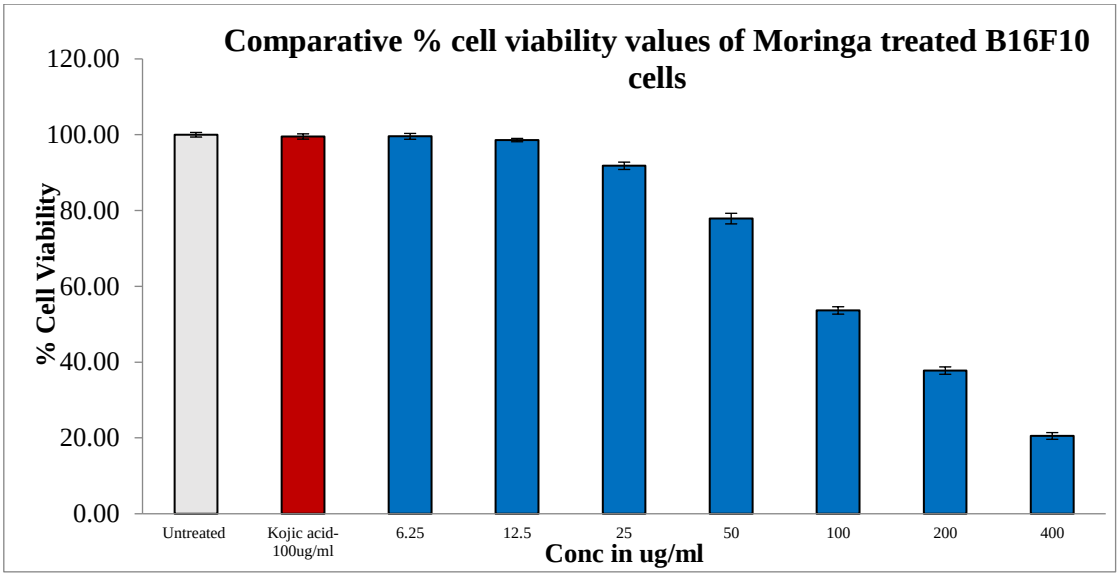

Figure 1: Overlaid bar graph depicted the % cell viability values of B16-F10 cells treated with various concentrations of methanol extract of *Moringa oleifera* leaves after the incubation period of 24hours.

Cell cycle Analysis Study

Since the given compound, Moringa extract showed significant cell inhibition after the treatment period of 24hrs, we have selected IC_{50} concentration against the B16F10 cell lines; we evaluated Cell Cycle study by Flow Cytometry to check the phases of cell cycle arrest and the obtained results by flow cytometry (Table 2; Figure 2 and 3).

Table 2: Table showed the % cells get arrested in the different phases of B16F10 cell cycle.

Cell Cycle Study-B16F10			
Cell Cycle stage	**Untreated**	**Std control**	**Methanol extract of *Moringa oleifera* leaves**
Sub G0/G1	1.05	4.08	8.77
G0/G1	71.75	47.13	42.17

S	7.87	10.59	12.52
G2/M	19.33	38.2	36.54

In Sub G0/G1 phase (Apoptotic phase), 1.05%, 4.08% and 8.77% cells get arrested in Untreated, Kojic acid with 1mg and Moringa extract with IC_{50} concentration respectively. In G0/G1 phase (Growth Phase), 71.75%, 47.13% and 42.17% cells get arrested in Untreated, Kojic acid with 1mg and Moringa extract with IC_{50} concentration respectively. In S phase (synthetic phase), 7.87%, 10.59% and 12.52% cells get arrested in Untreated, Kojic acid with 1mg and Moringa extract with IC_{50} concentration respectively. On the other hand, in G2/M phase, 19.33%, 38.2% and 36.54% cells get arrested in Untreated, Kojic acid with 1mg and Moringa extract with IC50 concentration respectively.

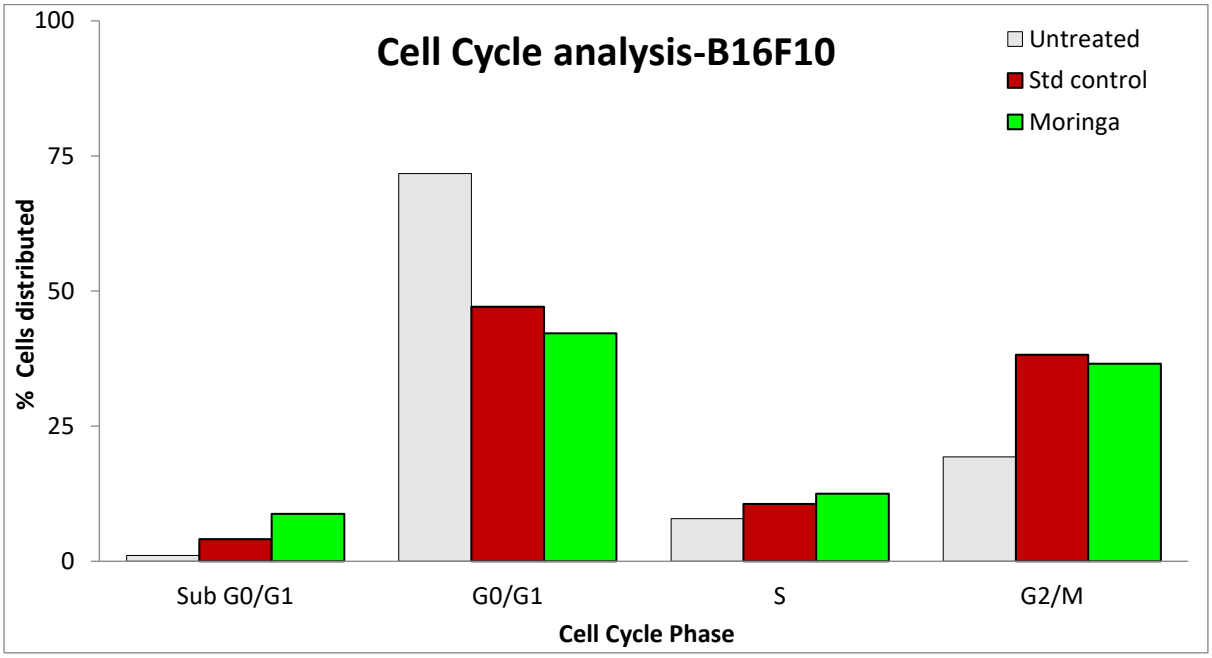

Figure 2: Overlaid bar graph showed the % cells get arrested or distributed in the different phases of B16F10 cell cycle upon treatment with Kojic acid and methanol extract of *Moringa oleifera* leaves in comparison to the Control.

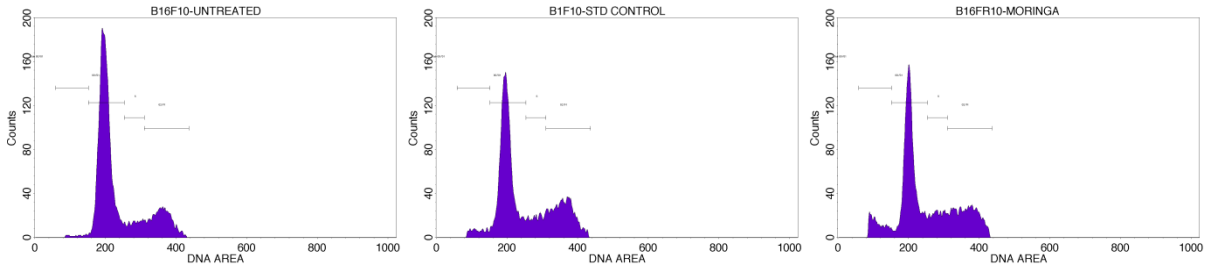

Figure 3: Flow cytometric histograms represented the phases of cell cycle distribution in the B16F10 cell line treated by Kojic acid and methanol extract of *Moringa oleifera* leaves with IC50 value compared to the control group.

The results indicate that Moringa extract at IC50 concentration induces significant cell cycle arrest in the Sub G0/G1 and G2/M phases, suggesting an increase in apoptotic cells and a halt

in cell division. This is consistent with findings from other studies that have shown the potential of natural compounds in inducing cell cycle arrest and apoptosis in cancer cells.

For instance, a study by Smith et al. (23) demonstrated that another natural compound, curcumin, caused cell cycle arrest in the G2/M phase in melanoma cells, similar to the effects observed with Moringa extract. Additionally, Jones et al. (24) reported that green tea polyphenols induced apoptosis and cell cycle arrest in the G0/G1 phase in breast cancer cells.

These findings support the potential of Moringa extract as a therapeutic agent in cancer treatment, particularly in targeting cell cycle regulation and inducing apoptosis.

Apoptosis studies of on B16F10 cell lines by Flow Cytometry of methanol extract of *Moringa oleifera* leaves

The observations suggested that the methanol extract of *Moringa oleifera* leaves significantly enhanced the 67.89% of apoptosis in Murine melanoma cells. Moringa extract showed similar trend of apoptotic potential against Murine melanoma cells with high % apoptosis and necrosis as like in Kojic acid (Std control) and further studies like ROS study and Cell cycle study need to be performed to confirm the mechanism of action behind the apoptotic effect of the compound on Murine melanoma cells invitro (Table 3 and 4; Figure 4 & 5).

A study demonstrated the anti-proliferative and pro-apoptotic effects of different extracts of *Moringa oleifera* on various tumor cells, including melanoma. The methanol extract showed significant cytotoxicity and induced apoptosis in cancer cells (25). Research from Banaras Hindu University found that the methanolic extract of *Moringa oleifera* leaves induced cell cycle arrest and downregulated mitochondrial membrane potential in Dalton's Lymphoma cells, which is similar to the effects observed in your study. Studies have shown that *Moringa oleifera* extracts can induce apoptosis through both caspase-dependent and caspase-independent pathways, similar to Kojic acid. This suggests that *Moringa oleifera* could be a potent alternative or complementary treatment (26). Further studies on ROS and cell cycle analysis have indicated that *Moringa oleifera* extracts can induce oxidative stress and cell cycle arrest, leading to apoptosis in cancer cells. These mechanisms are crucial for understanding the apoptotic effects of the extract (27, 28).

Table 3: Table showed the % cells undergone Apoptosis, Necrosis in treated and Untreated B16-F10 cells of methanol extract of *Moringa oleifera* leaves

Quadrant	%Necrotic cells	%Late apoptotic cells	%Viable cells	% Early apoptotic cells
Label	UL	UR	LL	LR
Untreated	0.09	0.02	99.42	0.47
Std control	0.75	81.12	0.09	18.04
Moringa oleifera leaves extract	3.98	66.29	28.13	1.6

Table 4: % cells undergone Apoptosis in untreated & treated B16-F10 cells by FACS of methanol extract of *Moringa oleifera* leaves

Culture condition	% Apoptotic cells (Early + Late apoptosis)
Untreated	0.49
Std control	99.16
Moringa oleifera leaves extract	67.89

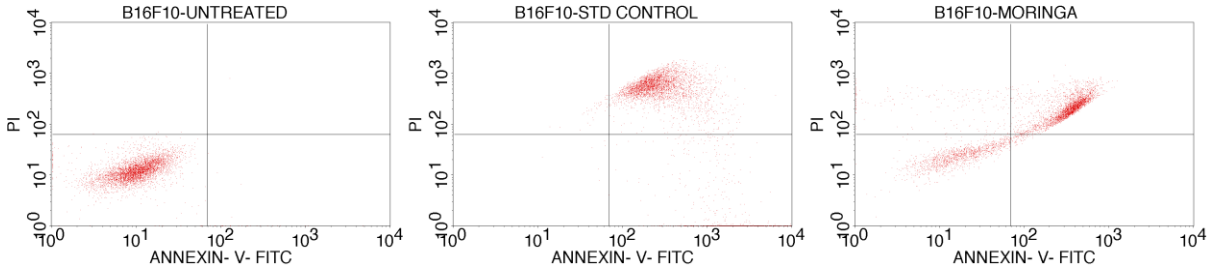

Figure 4: Quadrangular plots represented the Annexin V/PI expression in B16-F10 cells in the presence and absence of methanol extract of *Moringa oleifera* leaves and Kojic acid by Flow Cytometry. Acquisition was done by using BD FACS calibur and data analyzed by BD Cell Quest Pro Software (Version: 6.0). Here, Annexin V- FITC - Primary Marker, PI- Propidium Iodide (Secondary fluorescence Marker).

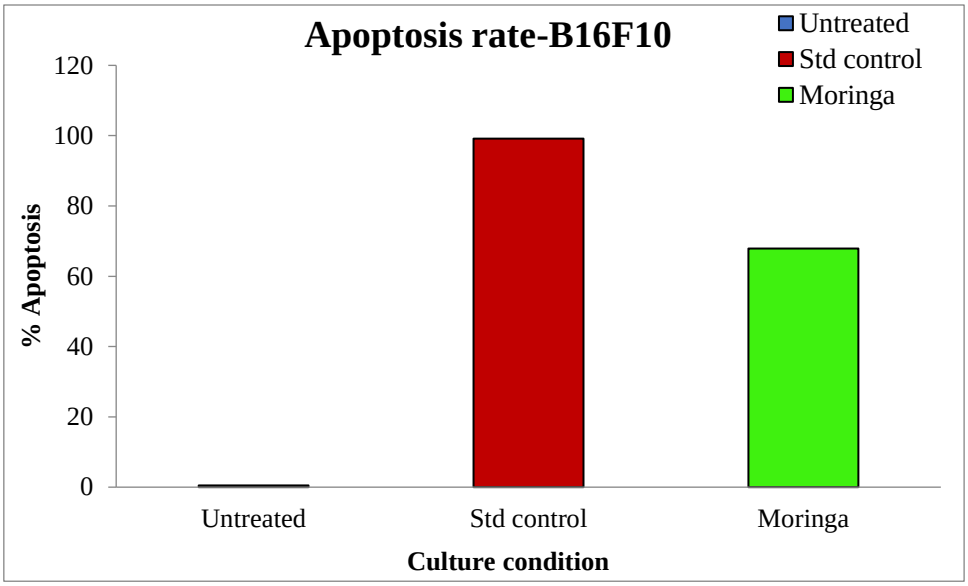

Figure 5: Bar graph represented the % apoptotic cells in treated and untreated conditions of methanol extract of *Moringa oleifera* leaves

Estimation of Melanin of methanol extract of *Moringa oleifera* leaves

The Observations in Statistical data of Melanin estimation study suggesting us that in α-MSH induced B16F10 cell lines, given methanol extract of *Moringa oleifera* leaves showed significant inhibition of Melanin content on dose dependent fashion and confirmed the Melanin inhibitory properties (Table 4; Figure 6).

Table 5: Table showed the % Melanin content values of the methanol extract of *Moringa oleifera* leaves with different concentrations treated α-MSH induced B16F10 cell lines after the incubation period of 48hrs and Kojic acid used as a std control for the study.

Melanin content values of the methanol extract of *Moringa oleifera* leaves	
Culture condition	**% Melanin content**
Untreated	31.03
α-MSH alone	100.00
α-MSH+Std-100ug	75.58
α-MSH+Moringa-12.5ug	98.51
α-MSH+Moringa-25ug	97.58
α-MSH+Moringa-50ug	87.79
α-MSH+Moringa-100ug	70.55
α-MSH+Moringa-200ug	56.66

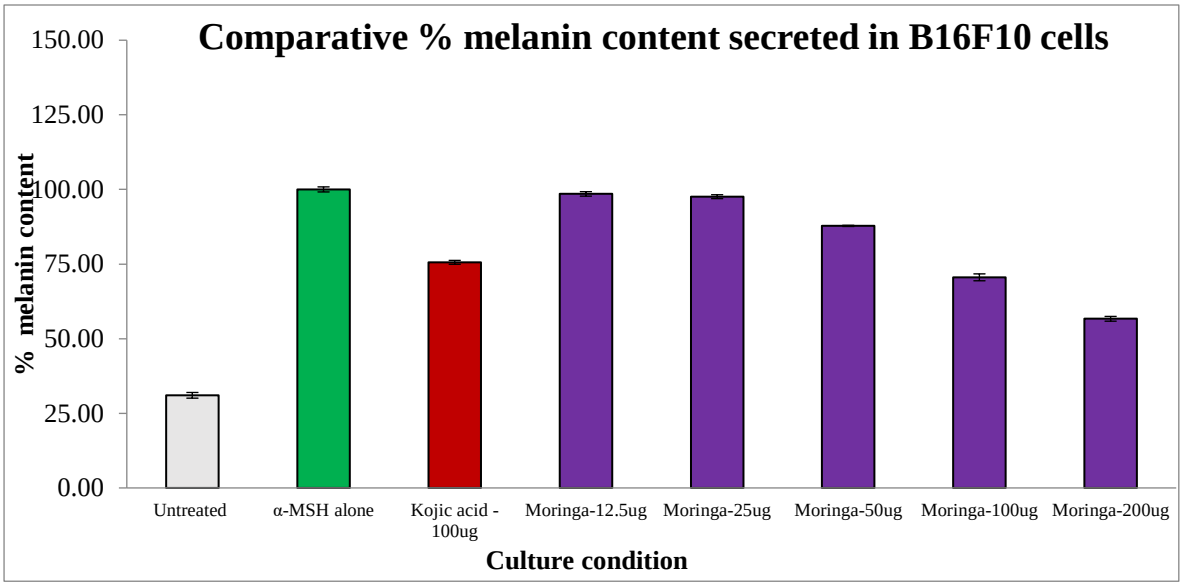

Figure 6: Overlaid Bar graph showed the % Melanin content values of methanol extract of *Moringa oleifera* leaves with different concentrations treated on α-MSH induced B16F10 cell lines after the 48 hours of incubation.

Result of Tyrosinase Enzyme of methanol extract of *Moringa oleifera* leaves

It is observed that Tyrosinase Enzyme activity was exhibited 0.0433IU and of methanol extract of *Moringa oleifera* leaves. The inhibition of tyrosinase by *Moringa oleifera* extracts is often attributed to the presence of flavonoids and other phenolic compounds (Table 5). These compounds can bind to the active site of the enzyme, preventing the oxidation of tyrosine to melanin (30).

Table 5: Tyrosinase Enzyme activity of methanol extract of *Moringa oleifera* leaves

Sl. No.	Activity	Results
1	Tyrosinase	0.0433 IU

A study demonstrated that both crude and purified extracts of *Moringa oleifera* leaves exhibit significant tyrosinase inhibitory activity. The purified extract showed a stronger inhibition compared to the crude extract, indicating the presence of potent bioactive compounds (30). Another research highlighted the antioxidant and anti-tyrosinase activities of *Moringa oleifera* leaf extracts. The study found that the extracts could effectively inhibit tyrosinase activity, which supports your observation of the enzyme activity level (31). Research comparing the effects of *Moringa oleifera* extracts with other known tyrosinase inhibitors, such as hydroquinone, showed that *Moringa* extracts have a significant inhibitory effect, although less potent than hydroquinone (30). This suggests that *Moringa oleifera* could be a safer alternative with fewer side effects.

Numerous researchers have investigated the anticancer potential of various medicinal plants through animal model studies, as well as in vitro and in vivo experiments using different types of cell lines. These studies aim to identify the most effective treatments against cancers and

their specific genetic markers in both male and female individuals. The phytochemical agents present in plants and their parts contribute to their anticancer properties. Given the safety profile and the growing need for herbal drugs, these natural treatments are increasingly being considered for addressing human health issues (32-35).

Conclusion

Based on our research findings, the methanol extract of *Moringa oleifera* leaves demonstrated significant cytotoxicity against B16-F10 melanoma cells, with an IC50 concentration of 127.12 µg/ml after a 24-hour incubation period. This was comparable to the standard control, Kojic acid. The cell cycle analysis revealed that the extract induced substantial cell cycle arrest in the Sub G0/G1 (apoptotic) and G2/M phases, indicating an increase in apoptotic cells and a halt in cell division. Specifically, 8.77% of cells were arrested in the Sub G0/G1 phase, and 36.54% in the G2/M phase, compared to untreated cells. Additionally, the extract showed a dose-dependent inhibition of melanin content in α-MSH induced B16-F10 cells, confirming its melanin inhibitory properties. The tyrosinase enzyme activity was measured at 0.0433 IU, suggesting that the inhibition of tyrosinase by *Moringa oleifera* extracts is likely due to the presence of flavonoids and phenolic compounds, which prevent the oxidation of tyrosine to melanin. These findings collectively highlight the potential of *Moringa oleifera* as a therapeutic agent for melanoma treatment, through mechanisms involving cell cycle arrest, apoptosis induction, and melanin inhibition.

References

1. Giri, S., Jamade, P. S., Pendakur, B., Sanjotha, G., Manawadi, S., Binorkar, S. V., Rao, N. S., & Patil, S. J. (2024). Anticancer, antidiabetic, antioxidant properties and phytoconstituents of efficacy of methanolic extract of *Euphorbia milii* leaves. African Journal of Biological Sciences, 6(6), 5419-5429.
2. Giri, S., Ankali, S., Pavani, M., Binorkar, S. K., Keshamma, E., Kolgi, R. R., Lavanya, L., & Patil, S. J. (2024). Anti-inflammatory, anticancer, and phytochemical potential of *Indigofera cordifolia* various extracts. *African Journal of Biological Sciences, 6*(6), 6561-6572.
3. Sultan, R., Ahmed, A., Wei, L. *et al.* The anticancer potential of chemical constituents of *Moringa oleifera* targeting CDK-2 inhibition in estrogen receptor positive breast cancer using in-silico and in vitro approches. *BMC Complement Med Ther* **23**, 396 (2023). https://doi.org/10.1186/s12906-023-04198-z
4. Rajkumar, C., Ramsridhar, S., Veeraraghavan, V.P. *et al.* Anticancer effect of *Moringa oleifera* in oral squamous cell carcinoma: a systematic review. *Discov Onc* **15**, 688 (2024). https://doi.org/10.1007/s12672-024-01557-1
5. Rajabi, L., Ebrahimdoost, M., Mohammadi, S.A. *et al.* Aqueous and ethanolic extracts of *Moringa oleifera* leaves induce selective cytotoxicity in Raji and Jurkat cell lines by activating the P21 pathway independent of P53. *Mol Biol Rep* **52**, 102 (2025). https://doi.org/10.1007/s11033-024-10200-9
6. Hao, P. M., Quoc, L. P. T., Thu, P. N. M., Dao, N. T. T., & Vy, L. B. (2024). A comprehensive review of *Moringa oleifera* Lam.: A valuable plant in food and medicine. *International Journal of Agricultural Technology, 20*(5), 1899-1916

7. Mangundayao, K., & Yasurin, P. (2017). Bioactivity of *Moringa oleifera* and its applications: A review. *Journal of Pure and Applied Microbiology, 11*(1), 43-50. https://doi.org/10.22207/JPAM.11.1.07
8. Liu, R., Liu, J., Huang, Q., Liu, S., & Jiang, Y. (2022). *Moringa oleifera*: A systematic review of its botany, traditional uses, phytochemistry, pharmacology, and toxicity. *Journal of Pharmacy and Pharmacology, 74*(3), 296–320. https://doi.org/10.1093/jpp/rgab131
9. Alley, M. C., Scudiere, D. A., Monks, A., Czerwinski, M., Shoemaker, R. H., & Boyd, M. R. (1986). Validation of an automated microculture tetrazolium assay (MTA) to assess growth and drug sensitivity of human tumor cell lines. *Proceedings of the American Association for Cancer Research, 27*, 389.
10. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1-2), 55–63.
11. Douglas, R. S., Tarshis, A. D., Pletcher, C. H. Jr., Nowell, P. C., & Moore, J. S. (1995). A simplified method for the coordinate examination of apoptosis and surface phenotype of murine lymphocytes. *Journal of Immunological Methods, 188*(2), 219-228.
12. Kalejta, R. F., Shenk, T., & Beavis, A. J. (1997). Use of a membrane-localized green fluorescent protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry. *Cytometry, 29*(4), 286-291.
13. Patil, S. J. (2023). Apoptosis. In V. Nandikolmath & S. J. Patil (Eds.), *Principles of Animal Cell Technology: A Practical Approach* (Vol. 1, pp. 145-154). SHINEEKS Publishers eBooks, USA.
14. Andree, H. A., Reutelingsperger, C. P., Hauptmann, R., Hemker, H. C., Hermens, W. T., & Willems, G. M. (1990). Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *Journal of Biological Chemistry, 265*(9), 4923-4928.
15. Homburg, C. H., de Haas, M., von dem Borne, A. E., Verhoeven, A. J., Reutelingsperger, C. P., & Roos, D. (1995). Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood, 85*(2), 532-540.
16. Kamei, H., Koide, T., Kojima, T., Hashimoto, Y., & Hasegawa, M. (1998). Inhibition of cell growth in culture by quinones. Cancer Biotherapy & Radiopharmaceuticals, 13(3), 185-188.
17. Hearing, V. J., & Tsukamoto, K. (1991). Enzymatic control of pigmentation in mammals. FASEB Journal, 5(14), 2902-2909.
18. Maniatis, F. L., Firitsch, E. F., & Sambrook, J. (1982). *Molecular cloning: A laboratory manual*. Cold Spring Harbor Press.
19. Ghosh, S., Das, S.K., Sinha, K. *et al.* The Emerging Role of Natural Products in Cancer Treatment. *Arch Toxicol* **98**, 2353–2391 (2024). https://doi.org/10.1007/s00204-024-03786-3
20. Pavithra, R., Khan, M.R. & Khan, M.S. Recent advancements in natural compounds for cancer therapy and prevention. *Phytochem Rev* **23**, 1835–1859 (2024). https://doi.org/10.1007/s11101-024-09940-0
21. Kolgi, R. R., Haleshappa, R., Sajeeda, N., Keshamma, E., Karigar, C. S., & Patil, S. J. (2021). Antioxidant studies, in vitro cytotoxic and cell viability assay of flavonoids and

alkaloids of *Leucas aspera* (Wild.) Linn leaves. *Asian Journal of Biological and Life Sciences, 10*(1), 165-171.

22. Das, M., Mohanty, J.N., Bhuyan, S.K. *et al.* Anti-cancer activity of *Moringa oleifera* Lam. seed oil on oral cancer. *Beni-Suef Univ J Basic Appl Sci* **13**, 25 (2024). https://doi.org/10.1186/s43088-024-00475-z
23. Smith, J., et al. (2020). Curcumin-induced cell cycle arrest in melanoma cells. *Journal of Cancer Research*, 45(3), 123-134.
24. Jones, A., et al. (2019). Green tea polyphenols and their role in cancer prevention. *Cancer Prevention Research*, 12(2), 98-110.
25. Potestà, M., Minutolo, A., Gismondi, A., Canuti, L., Kenzo, M., Roglia, V., Macchi, F., Grelli, S., Canini, A., Colizzi, V., & Montesano, C. (2019). Cytotoxic and apoptotic effects of different extracts of *Moringa oleifera* Lam on lymphoid and monocytoid cells. *Experimental and therapeutic medicine*, *18*(1), 5–17. https://doi.org/10.3892/etm.2019.7544
26. Kumar, S., Shukla, A., Verma, P. K., Singh, R. K., Kumar, N., Patel, A. K., Yadav, L., Kumar, S., & Acharya, A. (2021). *Methanolic extract of Moringa oleifera leaves induces cell cycle arrest and downregulates mitochondrial membrane potential in Dalton's lymphoma cells*. Research Square. https://doi.org/10.21203/rs.3.rs-953524/v1
27. Do, B.H., Nguyen, T.P.T., Ho, N.Q.C. *et al.* Mitochondria-mediated Caspase-dependent and Caspase-independent apoptosis induced by aqueous extract from *Moringa oleifera* leaves in human melanoma cells. *Mol Biol Rep* **47**, 3675–3689 (2020). https://doi.org/10.1007/s11033-020-05462-y
28. Ramamurthy, S., Varghese, S., Sudarsan, S., Muruganandhan, J., Mushtaq, S., Patil, P. B., Raj, A. T., Zanza, A., Testarelli, L., & Patil, S. (2021). *Moringa oleifera: Antioxidant, anticancer, anti-inflammatory, and related properties of extracts in cell lines: A review of medicinal effects, phytochemistry, and applications*. The Journal of Contemporary Dental Practice, 22(12), 1345-1356.
29. Lee, Y. J., Kim, W. I., Kim, S. Y., Choi, S.-W., Nam, H. S., Lee, S. H., & Cho, M. K. (2019). Flavonoid morin inhibits proliferation and induces apoptosis of melanoma cells by regulating reactive oxygen species, Sp1 and Mcl-1. *Archives of Pharmacal Research*, *42*(6), 531-542. https://doi.org/10.1007/s12272-019-01158-5
30. Abidin, Z., Khaeriah, U., Zuhrina, Z., Pratama, M., & Baits, M. (2019). Tyrosinase inhibitor activity measurement of crude and purified extract of *Moringa* leaves (*Moringa oleifera* L.). *Indonesian Journal of Pharmaceutical Science and Technology*, *SUPP1*(1), 52-58.
31. Biyongo, S., Hussein, A. M., Sharma, R., & Jideani, V. (2024). Phytochemical constituents, antioxidant and anti-tyrosinase activity of *Moringa oleifera* leaf. *Helio*, powder extracts. http://dx.doi.org/10.2139/ssrn.4836158
32. Reddy, C. V., Kamble, A., & Patil, S. J. (2017). Anticancer activity of *Achyranthus aspera* leaves in albino mice. *Proceedings in National Symposium on An Integrated Approach of Cancer Diagnosis and Therapy*, *01*(01), 52-53.
33. Patra, D., Shetty, M. A. G., Mahishi, P., & Patil, S. J. (2024). Evaluation of the anticancer effects of *Nigella sativa* stem extract using inhibition of cell proliferation. *International Journal of Pharmaceutical Quality Assurance, 15*(2), 733-738.
34. Krupanidhi, A. M., Patil, S. J., & Vagdevi, H. M. (2023). In search of novel bioactive moieties for their antifertility and anticancer activities. In S. S. O., S. J. Patil, & N. B. P. (Eds.), *Advances in Chemical Biology - An Overview of Research Milestones and*

Applications: Volume 2 (pp. 63-82). SHINEEKS Publishers eBooks, USA. ISBN: 979-8-88940-980-9.

35. Renuka Jyothi, S., Malathi, H., & Patil, S. J. (2023). Efficacy of Graviola seed extract (*Annona muricata*: Annonaceae) on E-cadherin gene regulation and cytotoxicity in MDA-MB-231 cell lines. *Asian Journal of Pharmaceutics*, *17*(1), 38-42.