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Effectiveness of Crude Drug Extract of *Moringa Oleifera* on Hepato Carcinoma: A Cell Line Study

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Abstract: Hepatocellular carcinoma (HCC), one of the most prevalent forms of liver cancer, remains a significant global health challenge due to its aggressive nature and limited treatment options. This study explores the potential anticancer properties of crude extracts derived from *Moringa oleifera* a plant known for its rich phytochemical profile and medicinal properties on HepG2 liver cancer cells. The investigation employed in vitro assays, including MTT and cell migration assays, to evaluate cytotoxic and anti-metastatic effects. The MTT assay revealed a dose-dependent reduction in cell viability, with an IC_{50} value of 464 µg/mL, indicating cytotoxic potential of the crude extract. Additionally, the cell migration assay demonstrated that it significantly inhibited cell migration, a key process in cancer metastasis. These findings suggest that crude extract exhibits promising anticancer activity against hepatocellular carcinoma through inhibition of cell proliferation and migration, supporting its potential use as a natural therapeutic agent. Further studies involving purified constituents and in vivo models are warranted to validate and optimize its efficacy.

Keywords: Moringa oleifera, hepatocellular carcinoma, HepG2 cells, MTT assay, cell migration.

INTRODUCTION

The liver is an essential metabolic organ that is unique to vertebrates and is involved in many biological functions. It produces proteins, detoxifies the body, and synthesizes a variety of biochemicals necessary for development and digestion [1]. Over two million deaths worldwide are attributed to liver disease each year, making up 4% of all fatalities (1 in 25). Of them, females account for one in three liver-related fatalities. The number of fatalities from liver cancer alone ranges from 600,000 to 900,000 [2].

The hepatoprotective properties of Moringa oleifera (commonly known as the drumstick tree) have been widely researched due to its bioactive compounds. This plant is a rich source of antioxidants, vitamins, minerals, and phytochemicals, all of which play a role in its liver-protective effects [3].

The liver is highly susceptible to damage from various factors, including infections, excessive alcohol consumption, drug toxicity, and metabolic disorders, leading to conditions collectively known as liver diseases. These disorders, ranging from mild hepatic dysfunction to severe conditions like cirrhosis, hepatocellular carcinoma, and liver failure, hepatitis pose significant global health concerns [4][5].

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The ability of a substance (drug, herbal extract, or molecule) to either prevent liver damage or aid in the liver's recovery from injury is known as hepatoprotective action. Alcohol, drugs, illnesses, and pollutants can all have an impact on the liver, which is essential for digestion, metabolism, and detoxification [6].

Aqueous and alcoholic extracts (methanolic & ethanolic) of leaves and roots of *Moringa Oleifera* exhibit strong in-vitro anti-oxidant and radical scavenging activity[7]. Aqueous extract of *Moringa Oleifera* leaves shows anti-diabetic activity and controls diabetes and thus exhibit glycemic control [8]. Ethanolic extract of Moringa Oleifera leaves showed prominent anti-hypertensive or hypotensive activity[9]. Ethanolic extracts of leaves and seeds of Moringa oleifera shows potent anti–tumor activity[10]. The in-vitro anti-urolithiatic activity was performed in aqueous and alcoholic extract of bark of *Moringa Oleifera*. It showed reduction in weight of stone produced using ethylene glycol induced urothiasis. It also possesses both preventive and curative property[11]. Leaves, roots, bark and seeds of *Moringa Oleifera* show anti-microbial activity against bacteria and fungi[12]. Methanolic and aqueous extract of root and bark, methanolic extract of leaves and flowers and ethanolic extract of seeds of *Moringa Oleifera* possess anti-inflammatory activity[13]. *Moringa Oleifera* leaves extract restores mono amine levels of brain, which may be useful in Alzheimer's disease [14].

MATERIALS AND METHODS:

Cell lines

It is a population of cells that can be cultured and maintained in lab for an extended period often indefinitely under controlled conditions. Cell lines are derived from various sources including normal tissues, tumors, stem cell and they are commonly used in biological and medical research [15].

HepG2 Cell line:

Hep G2 is an immortal cell line that was derived in 1975 from the liver tissue of a 15–year–old Caucasian male from Argentina with a well-differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice [16]. HepG2 cells, a human hepatoma cell line, are used in research as in vitro model for studying liver-related functions, including drug metabolism, hepatotoxicity, and liver cancer, as well as viral infections and tissue engineering [17].

MTT Assay Experimental Procedures:

Preparation for Test Material -

All Test Samples were filter sterilized using 0.22µ filters and diluted by double dilution method in MEM with FBS [18].

PROCEDURE

Preparation of Cells

HepG2 cells were cultured in D-Modified Eagle Medium (DMEM) with NEAA media supplemented with 10% (v/v) fetal bovine serum. Cells were cultured at 370C and 5% CO2; the complete medium was changed every 2 to 3 days [19].

MTT Assay Procedure

Cells were seeded in 96-well plates at 1,00,000 cells per well (100 µl). The plates were incubated at 37°C and 5% CO2 atmosphere for 24 hr. After the incubation period cells were observed for half confluent monolayers. Culture medium was removed, and cells were treated with 9 different test item concentrations. Cells in cell culture medium without any Test Item incubated for 24 hr

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under the same condition served as Control. Plates were incubated at 37°C in a 5% CO2 incubator for 24 hrs. After 24 hr, cells were observed under an inverted microscope for any changes in morphology or death. After observation, t h e culture medium was removed, and 100 µl of fresh medium was added along with 10 µl of MTT reagent in each well. Plates were incubated for 4 hr at 37°C in 5% CO2 incubator. 100µl of the Solubilization solution was added into each well.
Plate was allowed to stand for 1 hr at 37°C in 5% CO2 incubator. After checking for complete solubilization of the purple formazan crystals, absorbance was measured at 570 nm using a microplate reader. IC50 values were calculated by plotting a log graph for the concentration of the test items vs. % cell survival [20]. Percentage Cell Survival was calculated using the formula:

$$\textbf{Percentage Cell Survival (\%)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \times 100$$

Cell Migration Assay Experimental Procedures

Preparation for Test Material -

All Test Samples were filter sterilized using 0.22µ filters and diluted by double dilution method in MEM with FBS.

PROCEDURE:

Cell Seeding:
Seed cells into a 6-well plate (or other suitable plates) at a density where they form a confluent monolayer after 24 hours. Incubate the cells overnight at 37°C in a CO_2 incubator.
Create the Scratch:
After the cells are confluent, use a sterile pipette tip or a mechanical scratching tool to create a single, straight scratch (or wound) across the cell monolayer. The width of the scratch should be uniform, but it's typically about 0.5 to 1 mm wide. Be gentle while scratching to avoid damaging the bottom of the well.
Gently wash the cells with sterile PBS to remove detached cells and debris. Add fresh culture medium (with or without serum depending on your experimental needs). If you want to assess migration in a controlled environment, you can use serum-free media.
Imaging (Initial Timepoint):

Take the initial image of the scratch area under a microscope to document the starting point (time 0). Ensure you capture the entire scratch area for later comparison.
Incubation:
Place the plate back in the incubator and allow the cells to migrate into the scratch area. Depending on your experiment, you can continue to observe the cells over a set period (e.g., 12, 24, or 48 hours).

Monitor and Take Images:

At regular time intervals, take images of the scratch area under the microscope to monitor cell migration.Ensure that you are capturing the same area each time for consistency.

Data Analysis:

Measure the gap (scratch area) using imaging software (e.g., ImageJ, CellSens, etc.) at each time point. Compare the width of the gap at different time points to assess the rate of cell migration. Migration is often quantified by calculating the percentage closure of the gap[21].

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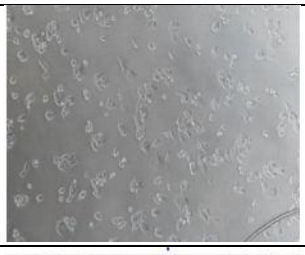
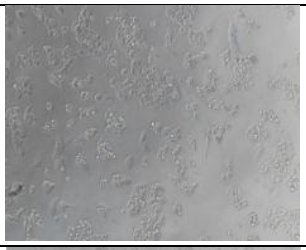
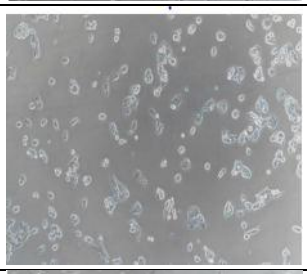
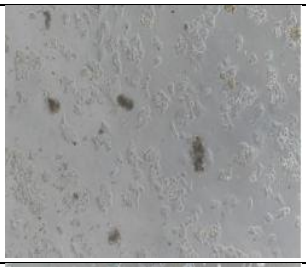
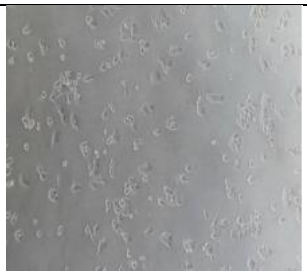
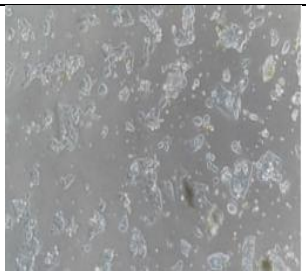
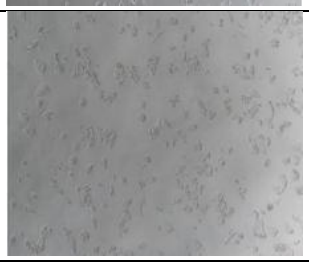
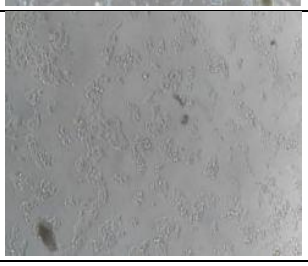
RESULTS AND DISCUSSIONS:

MTT Assay:

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NADPH-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT, which is chemically 3 (4,5dimethylthiazol2yl) 2,5diphenyltetrazolium bromide, to its insoluble formazan, which has a purple colour. The result of MTT Assay is given below [22].

MTT Result:

Dilutions	***Moringa Oleifera* Powder (0 hr)**	***Moringa Oleifera* Powder (24 hrs)**
controlled		
5µg/ml		
10µg/ml		
25µg/ml		
50µg/ml		

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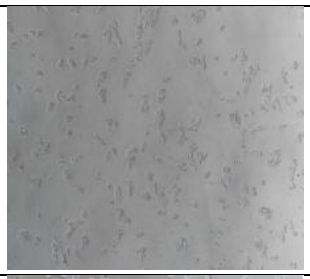
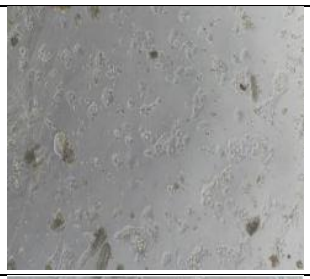
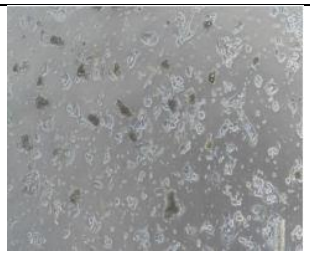
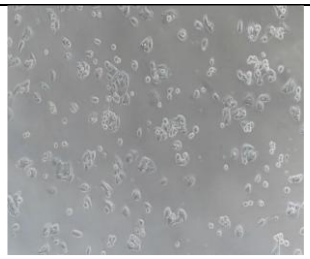
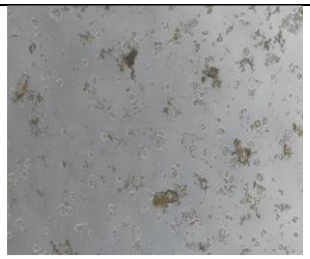
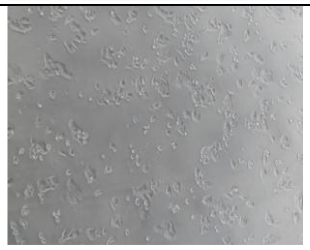
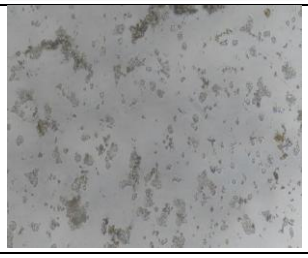
100µg/ml		
250 µg/ml		
500 µg/ml		
750 µg/ml		

Table No.01 Morphological Observations of *Moringa Oleifera* Powder

Table no. 01 shows the morphological changes in HepG2 cells treated with different concentrations of *Moringa Oleifera* powder ranging from 5 µg/ml to 250 µg/ml over a 24-hour period. The table includes images of the HepG2 cells at 0 hours and 24 hours for each concentration. The images help visualize the shape, size, and colour of the cells, which are indicators of cell viability and health. Control: Shows healthy, viable cells with no treatment. Treated Cells: As the concentration of *Moringa Oleifera* increases, the number of viable cells decreases, and cell morphology changes (e.g., cells may appear shrunken or fragmented), indicating cytotoxicity.

Concentration (µg/ml)	**Absorbance**			**Average**	**% Cell Survival**			**Average % Cell Survival**
CTRL	0.9	0.881	0.879	0.887	101.5	99.4	99.1	100.0
5	1.057	0.749	0.781	0.862	119.2	84.5	88.1	97.3
10	0.726	0.836	0.731	0.764	81.9	94.3	82.4	86.2
25	0.681	0.884	0.599	0.721	76.8	99.7	67.6	81.4
50	0.834	0.798	0.657	0.763	94.1	90.0	74.1	86.1
100	1.002	0.607	0.605	0.738	113.0	68.5	68.2	83.2
250	0.887	0.832	0.661	0.793	100.0	93.8	74.5	89.5
500	0.798	0.681	0.879	0.786	51.4	45.3	43.1	46.6
750	0.781	0.742	0.654	0.725	41.4	38.4	36.2	38.6
IC50 VALUE	**464 µg/ml**							

Table No.02 (MTT Results: *Moringa Oleifera* powder)

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Table no.02 provides quantitative data from the MTT assay, including absorbance values, average % cell survival, and the IC50 value. Concentration (µg/ml): The concentrations tested range from 5 µg/ml to 250 µg/ml. Absorbance: The absorbance values at 570 nm are recorded for each concentration, reflecting the amount of formazan produced by viable cells.
% Cell Survival: The percentage of cell survival is calculated by comparing the absorbance of treated cells to the control (untreated cells). IC50 Value: The IC50 value (464 µg/ml) is the concentration at which 50% of the cells are killed. This value indicates the potency of *Moringa Oleifera* in inhibiting HepG2 cell growth.

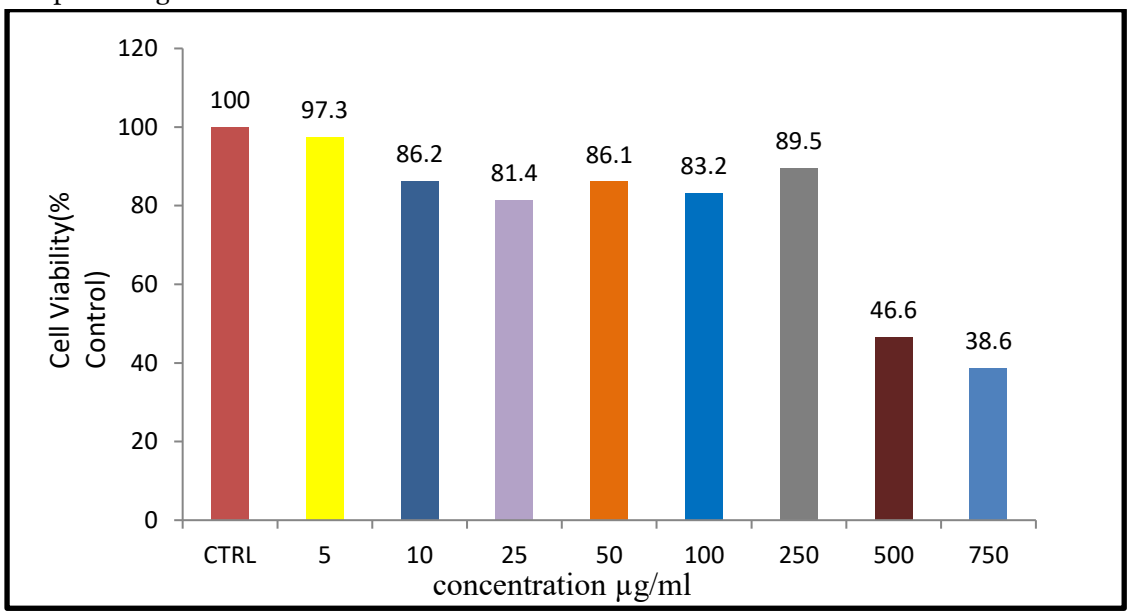
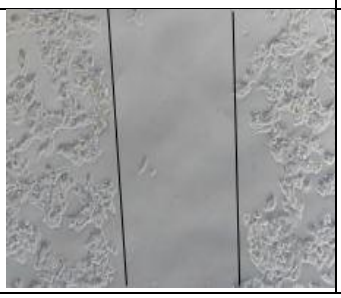
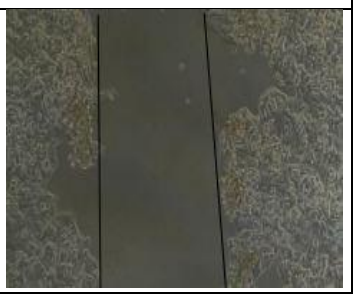


Figure No.01 Graph of conc. vs. % cell Viability

Fig no.01 is a column chart that further illustrates the % cell viability at different concentrations of *Moringa oleifera*. The X-axis represents the concentrations (Control to 250 µg/ml), and the Y-axis represents the % cell viability. The chart shows a clear trend of decreasing cell viability with increasing concentrations of *Moringa oleifera*, supporting the cytotoxic effect of the extract on HepG2 cells.

Cell Migration Result:

Dilutions	*Moringa Oleifera* Powder (0 hr)	*Moringa Oleifera* Powder (8 hrs)	*Moringa Oleifera* Powder (24 hrs)
Control			

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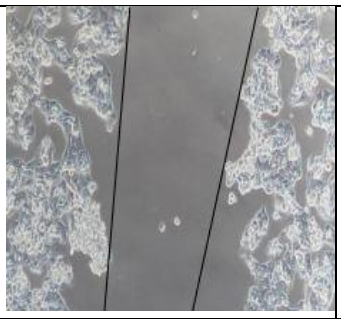
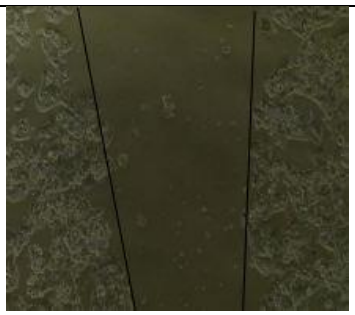
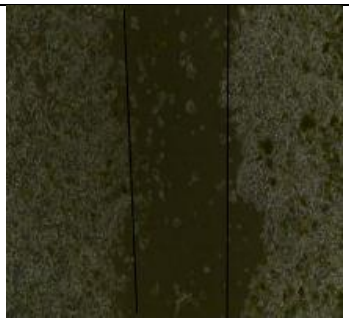
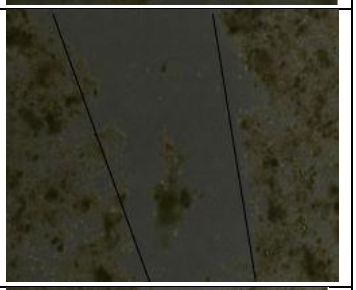
10µg/ml			
20µg/ml			
30µg/ml			
40µg/ml			
50 µg/ml			

Table No.03 Morphological Observations of *Moringa Oleifera* Powder Treated Wells

Table no.03 shows the wound healing process (cell migration) in HepG2 cells treated with *Moringa Oleifera* powder at concentrations ranging from 10 µg/ml to 50 µg/ml.

The table includes images of the HepG2 cells at 0 hours, 8 hours, and 24 hours for each concentration. Control: Shows the natural wound healing process over time.

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Treated Cells: The images show how *Moringa Oleifera* affects the migration of HepG2 cells into the scratch area. As the concentration increases, the rate of cell migration decreases, indicating that *Moringa Oleifera* inhibits cell migration, which is a critical factor in cancer metastasis.

Concentration (µg/ml)	Time	Area	Mean	Min	Max
Control	0 hr	680129	156.197	108	223
	8 hr	542320	36.252	19	186
	24 hr	688365	72.654	51	164
10	0 hr	658139	128.476	92	246
	8 hr	731880	50.375	31	115
	24 hr	705554	53.959	14	85
20	0 hr	764233	126.638	89	249
	8 hr	635508	30.803	14	134
	24 hr	760459	66.973	23	107
30	0 hr	713394	141.182	98	240
	8 hr	584251	50.592	28	152
	24 hr	539227	56.458	29	95
40	0 hr	854407	156.818	105	217
	8 hr	456760	110.283	45	161
	24 hr	616535	70.042	25	121
50	0 hr	691697	119.729	77	251
	8 hr	371565	93.68	31	143
	24 hr	614890	83.386	22	128

Table No.04 Cell migration Results: *Moringa Oleifera* powder

Table no.04 provides quantitative data from the cell migration assay, including the area covered by migrating cells, mean values, and minimum/maximum areas.
Concentration (µg/ml): The concentrations tested range from 10 µg/ml to 50 µg/ml.
Time: The observations are recorded at 0 hours, 8 hours, and 24 hours. Area: The area covered by migrating cells is measured at each time point. Mean, Min, Max: These values provide a statistical summary of the cell migration data, showing the variability in cell migration at different concentrations and time points. The data shows that as the concentration of *Moringa Oleifera* increases, the area covered by migrating cells decreases, indicating that *Moringa Oleifera* inhibits cell migration.

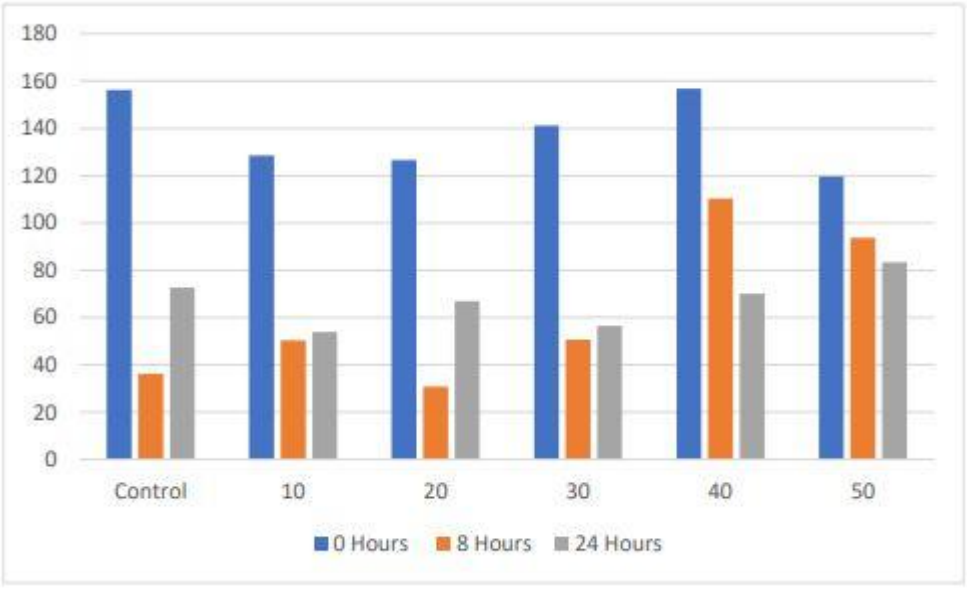

Figure No.02 Migration mean of *Moringa Oleifera* Powder

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Fig no.02 is a graphical representation of the cell migration data. It shows the mean area covered by migrating cells at different concentrations of *Moringa Oleifera* over time. The X-axis represents the concentrations (Control to 50 µg/ml), and the Y-axis represents the mean area covered by migrating cells.The graph shows a clear trend of decreasing cell migration with increasing concentrations of *Moringa oleifera*, supporting the anti-migratory effect of the extract on HepG2 cells.

CONCLUSION:

The results demonstrate that *Moringa Oleifera* has a cytotoxic effect on HepG2 cells, with an IC50 value of 464 µg/ml. The morphological images and graphs show a dose-dependent decrease in cell viability, indicating that *Moringa Oleifera* can inhibit the growth of liver cancer cells. Ic50 value of pure extract of *Moringa Oleifera* drug is 10 ± 1µg/ml but as we have used crude form of *Moringa Oleifera* the IC50 value was found to be 464µg/ml.

The results show that *Moringa Oleifera* inhibits the migration of HepG2 cells, which is a critical factor in cancer metastasis. The concentration of 30µg/ml showing the constant decrease in Area and Mean both. The images and graphs demonstrate that higher concentrations of *Moringa Oleifera* significantly reduce cell migration, suggesting its potential as an anti-metastatic agent.

These findings suggest that *Moringa Oleifera* has potential therapeutic effects against liver cancer by inhibiting cell proliferation and migration, making it a promising candidate for further research.

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