

Investigation of *Moringa oleifera* Leaf Extract and Its Cancer-Selective Antiproliferative Properties

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Moringa oleifera is a tree native to a number of Asian, African, and Central American countries and has been used in traditional medicine for an assortment of medicinal uses for centuries. Due to bioactive compounds within *Moringa* leaves, it is believed that *Moringa* leaf extract may possess cancer-selective antiproliferative properties. Previous research has been conducted in regards to this topic, but poor experimental design due to lack of necessary controls limits the legitimacy of anticancer claims. While previous research has shown that *Moringa* leaf extract has the potential to kill cancer cells, the research fails to demonstrate the effects of *Moringa* leaf extract on healthy cells. In order for anticancer claims to be sufficient and yield the possibility of a future cancer treatment, *Moringa* leaf extract must not harm non-cancerous cells. This is essential in order to be considered a cancer-selective killing agent. The current study was designed using tissue type pairs including both cancerous and non-cancerous cell lines. These cell lines were treated with increasing concentrations of *Moringa* leaf extracts. After 48 hours, cell proliferation was measured with CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, and statistical analyses were completed. Results showed that the *Moringa* leaf extract had no statistically significant effect on either of the breast cell lines, cancerous or non-cancerous. However, the results suggest there is a statistically significant difference in cell proliferation between the lung cell lines. Low concentrations increased cell proliferation in the healthy lung cells while having no significant effect on the cancerous lung cells. The effects reversed at higher concentrations. This could be due to the difference in cell responses between cancerous lung cells and healthy lung cells. Microscopy findings provided qualitative results that helped determine the method of cell death occurring by cells treated with *Moringa* leaf extract. Ideally, an effective cancer treatment would cause cancerous cells to die by apoptosis, rather than necrosis. However, the findings suggest necrosis is the primary mode of death occurring in the cancerous lung cells, while the cancerous breast cells showed no signs of cell death. This research contradicts previous findings that *Moringa* leaf extract is a cancer killing agent; therefore, more research should be completed to understand these new findings.

Introduction

Moringa oleifera is a hardy tree with small, oval-shaped leaves and a thin trunk. It grows in dry places and hanging from its branches are large pods containing young seeds. *Moringa* is a tree of many names and uses, for its names range from the horseradish tree and drumstick tree in India, to the benzolive tree in Haiti; however, the name "Miracle Tree" has commonly been used¹. *Moringa* has been used in traditional medicine for centuries, dating back to being used by the ancient Romans and Greeks. Ayurvedic medicine, one of the world's oldest holistic healing systems, claims that *Moringa* can prevent up to 300 diseases, and aside from preventative measures, its leaves are capable of curative properties as well². The traditional uses of *Moringa* are great in number, for they include the treatment of bacterial, fungal, viral, and parasitic issues, along with asthma, circulatory, digestive, and inflammatory disorders³. Other targeted ailments include malaria, typhoid fever, arthritis, hypertension, and diabetes. Because of *Moringa*'s ability to improve the immune system, treatment of HIV and AIDS symptoms is also possible². All parts of *Moringa* have been utilized, for even the seeds are capable of purifying water³. With this vast list of traditional medicinal properties, it comes as no surprise that *Moringa* is packed with chemical components to give it an astounding phytochemistry. The familiar components that give *Moringa* its nutritional value are its high amounts of protein, calcium, iron, fiber, vitamin A, and vitamin B. Due to these components, *Moringa* is currently being advertised as a "super food," possessing higher nutritional value than other nutritious greens such as kale. A more in depth look at *Moringa*'s bioactive compounds in the leaves reveals what is responsible for its medicinal properties. *Moringa* contains glucosinolates and isothiocyanates, both of which have shown great potential in cancer prevention, for glucosinolates are the naturally occurring precursor of isothiocyanates. While the exact mode of action is unknown, research has shown that isothiocyanates are a cancer-killing agent^{4,5}.

A number of *Moringa* cancer studies have been published by researchers with experimental results that conclude by saying *Moringa* has anticancer activity^{6,7,8,2}. But after deeper review of the conducted studies, it is apparent that no negative controls were used^{7,8}. While it has been shown that *Moringa* leaf extract has anticancer activity, there is no evidence that the same leaf extract would not also kill healthy cells. The research is merely showing that at a specified concentration of *Moringa* leaf extract, cancer cells will not survive as well as if they were not

treated with the extract. This is an insignificant claim; if healthy cells are killed or damaged at the same rate, *Moringa* leaf extract has no potential of possibly being developed into a cancer drug. Also, controls must be conducted regarding the extract solvent used. The solvent without leaf extract must be applied to the cells to affirm that the solvent is not producing the anticancer effect. Another aspect to consider to determine the possibility of *Moringa* as a cancer treatment is the type of cell death that results, whether it be apoptosis or necrosis. Apoptotic cells undergo shrinking, nuclear condensation, and disconnect from neighboring cells or extracellular matrix. By achieving this sort of cell death, the outside environment is left unaffected. Necrosis, however, is considered to be an "accidental" cell death that is uncontrollable and lacks order. When cancer cells undergo necrosis, the cell membrane is disrupted in a way that results in the intracellular components being released into the surrounding cellular environment. This release of material will lead to an inflammatory response by immune cells, which could possibly lead to further tumor growth⁹. In light of this information, it is important to test the programmed cell death being achieved by the cancer cells in experimentation. More research is needed in determining whether or not *Moringa* leaf extract is cancer-selective in its antiproliferative properties and if it could serve as a possible cancer treatment in the future.

Methods

Plant materials

Moringa oleifera seeds were generously provided by Dr. Jed Fahey from Johns Hopkins University. The seeds were cultivated according to the standards recommended by his lab. The seeds were planted in a soil mixture containing a 2:1 ratio of potting soil to sand. The plants grew in an environmental chamber with 15 hours of light per day at 29°C. The plants were lightly sprayed with water once a day. Two weeks after planting, the sprouts were transferred to a larger container for optimal room for growth. Two months after planting, all viable, healthy leaves were stripped from the plants and placed in a specimen bag to dry in a vented chamber for one week. After drying, the leaves were ground with a mortar and pestle until reaching the consistency of a fine powder. A second source of *Moringa oleifera* leaf powder was obtained from Kuli Kuli, a *Moringa* provider with research-grade *Moringa* leaf powder.

Extraction

Moringa leaf powder (MOL) in the amount of 3.74 g was extracted by a Soxhlet apparatus with 95% ethanol. The extraction ran for 8 hours until all soluble constituents had dissolved in the solvent. A rotary evaporator was then used to evaporate the solvent at 60°C. *Moringa* leaf extract was collected at an amount of 0.4 g. The extraction procedure was repeated with the Kuli Kuli *Moringa* leaf powder (MOP). From the solid extract collected, a 5 mg/mL stock solution was made for each *Moringa* extract with a 95% ethanol solvent.

Cell culture: A549/NL20 and MDA-MB-231/MCF-10A

The human lung cell carcinoma cell line A549 (ATCC no. CRM-CCL-185) was cultured in DMEM, containing 10% fetal bovine serum (FBS) and 100 µg/mL gentamicin. The human lung cell line NL20 (ATCC no. CRL-2503) was cultured in Hams F12 media which includes 1.2 g/L sodium bicarbonate, 1.8 g/L glucose, and 1mM L-glutamine. Other added chemicals include 1.5g/L sodium bicarbonate, 2.7 g/L glucose, 200 mM L-glutamine, 10mM nonessential amino acids, 100mg/mL insulin, 10mg/mL transferrin, 100% FBS, 0.1 µg/µL epidermal growth factor, 5 µg/µL hydrocortisone, and 100µg/mL gentamicin. The human breast cell carcinoma cell line MDA-MB-231 (ATCC no. CRM-HTB-26) was cultured in RPMI, containing 10% FBS. The human breast cell line MCF-10A (ATCC no. CRL-10317) was cultured in DMEM, containing 5% horse serum, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/ml cholera toxin, 10 µg/mL insulin, and 5.0 mL pen/strep. All cell lines were grown at 37°C with 5% CO₂.

Dilution of extracts

The 5 mg/mL stock solution of MOL extract was diluted to achieve final concentrations of 300 µg/mL, 200 µg/mL, 100 µg/mL, and 50 µg/mL with A549, NL20, MDA-MB-231, and MCF-10A media. The 5 mg/mL stock solution from the MOP extract was also diluted with each cell media to the same concentrations. To serve as a negative control, similar dilutions with the cell types' respective medias were made using 95% ethanol solvent in place of *Moringa* extracts.

Proliferation assay

Four 96 well plates were used to plate 39 wells of each cell type per plate at 5000 cells/200 µL media. The plates were then placed in incubation overnight at 37°C. After incubation, the cells were treated in triplicates with each of the MOL and MOP extract concentrations and ethanol controls. The plates were left to incubate for 48 hours at 37°C.

After 48 hours of incubation, the 96 well plates were spun at 600g for one minute. Media was removed, and 100 µL of fresh media was added to each well to remove any spectrophometric differences due to the extract additions. The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay was used according to its protocol, and the plates were incubated for 3.5 hours. The 96 well plates were then read on a plate reader at 490 nm, data was collected, and background values were subtracted.

Microscopic imaging

Cells were passed and resuspended in media at a concentration of 10,000 cells/133 µL media. Cells were plated in an 8 well chamber slide and incubated for 24 hours at 37°C. Cells were then treated with 67 µL of 50 µg/mL and 300 µg/mL MOL extract concentrations and ethanol controls and left to incubate for another 24 hours at 37°C. MOL extract-treated wells and control wells were stained with Biotium's Apoptosis, Necrosis, and Healthy Cell Quantification Kit following the protocol given in the product information. Cell plates were then imaged with a Nikon TiEclipse Inverted Microscope.

Statistical calculation

Fold changes from the average control value were calculated from the raw data and used in all statistical calculations, where fold change was the absorbance of treatment divided by the average absorbance of the ethanol control. Fold change values accounted for the effects of the ethanol in the MOL and MOP extracts on cell proliferation, so that the effect of *Moringa* on cell proliferation could be observed, rather than the effect of *Moringa* and ethanol in combination. Fold change values also allowed for the direct comparison of cell lines that responded differently in degree of color change to the application of the CellTiter 96 Aqueous

Non-Radioactive Cell Proliferation Assay. A Levene's test was calculated using JASP, version 0.7.5.5. Two-way ANOVAs with Bonferroni posttests were calculated using GraphPad Prism. Differences were considered statistically significant when p values were ≤ 0.05 .

Results

The effects of *Moringa oleifera* leaf extract were tested on MDA-MB-231, MCF-10A, A549, and NL20 cell proliferation. An initial Levene's test showed there was unequal variance in the conditions. Because this violated an assumption of ANOVA, the ANOVA was run with the understanding that there was the potential for inflated type I errors.

Effects of MOL and MOP extracts on breast cell proliferation

The raw absorbance values of the MDA-MB-231 and MCF-10A cell lines treated with MOL and MOP extracts showed no identifiable trends in cell proliferation (Figure 1).

An ANOVA was completed on fold change values for treatment of MOL and MOP extracts on MDA-MB-231 and MCF-10A, showing no statistical significance at any of the extract concentrations ($p > 0.05$). There were also no identifiable trends in cell proliferation that could be observed in either breast cell line (Figure 2).

Effects of MOL and MOP extracts on lung cell proliferation

The raw absorbance values of the A549 and NL20 cell lines treated with MOL and MOP extracts showed that increasing amounts of ethanol had a negative effect on cell proliferation in A549 cells, but when in combination with *Moringa*, cell number increases (Figure 3). It was also evident that the NL20 cell line responded differently to the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay than the A549 cell line, by producing much lower absorbance values (Figure 3). The absorbance differences between cell lines can be accounted for by calculating fold change values.

An ANOVA for treatment of MOL and MOP extracts on fold change values of A549 cells revealed no statistical significance at any of the extract concentrations ($p > 0.05$). Although the A549 data lacked statistical significance, there was an upward trend in cell proliferation with increasing concentration of MOL and MOP extract. At 50 and 100 µg/mL of MOL and MOP extracts, the fold change of A549 cell proliferation did not differ from the control. However, at 200 and 300 µg/mL of MOL and MOP extracts, the fold change of A549 cell proliferation doubled that of the control (Figure 4).

An ANOVA for treatment on NL20 cells revealed significance at 50 µg/mL of MOL extract ($p \leq 0.001$), 50 µg/mL of MOP extract ($p \leq 0.05$), and 100 µg/mL of MOP extract ($p \leq 0.001$) with a fold change reaching heights of nine times the control. The two highest concentrations of MOL extract and MOP extract on NL20 cells showed no statistical significance with cell proliferation measuring below the control (Figure 4).

Evaluation of cell death

Cell staining and microscopic imaging allowed for cell death to be evaluated in each of the cell lines at the lowest and highest concentrations of MOL extract. Although these results are not quantitative, the information can help paint a larger picture of what may be happening to the cells after the addition of the MOL extracts. The MCF-10A cells appeared to die primarily by apoptosis at 50 µg/mL of MOL extract, while dying primarily by necrosis at 300 µg/mL of MOL extract (Figure 5). These findings suggest that although an overall change in cell number is not observed due to increasing concentrations of MOL extract (Figure 2), the method of cell death may be changing as the concentration of MOL extract increases. The MDA-MB-231 cells appeared healthy with limited cell death occurring at both concentrations of MOL extract (Figure 5).

At 50 µg/mL of MOL extract, the NL20 cells revealed a much smaller percentage of cell death occurrence than the NL20 cells treated with 300 µg/mL of MOL extract (Figure 6). These findings match the proliferation data that showed that NL20 cells had greater proliferation at low concentrations of MOL extract and less proliferation at high concentrations of MOL extract (Figure 4). The cell death experienced by NL20 cells at 300 µg/mL of MOL extract also appeared to be occurring

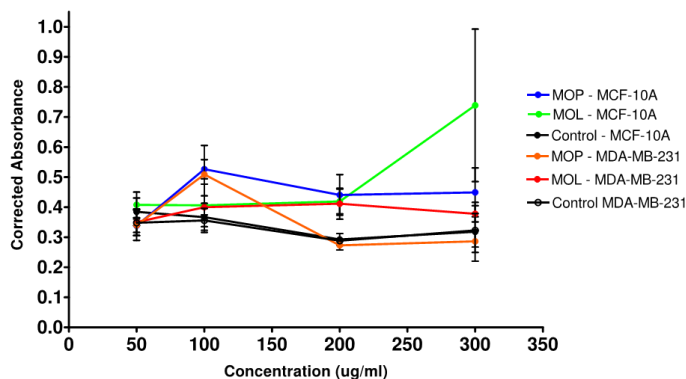


Figure 1. Raw absorbance values of MDA-MB-231 and MCF-10A with increasing MOL and MOP extract concentrations compared to ethanol controls.

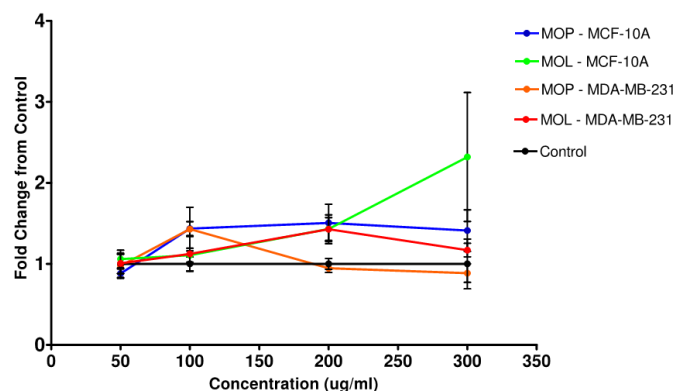


Figure 2. Fold change of MDA-MB-231 and MCF-10A with increasing MOL and MOP concentrations. * p<0.05, ** p<0.01, *** p<0.001

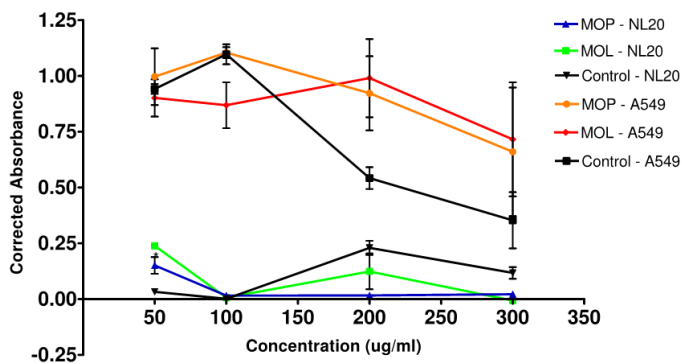


Figure 3. Raw absorbance values of A549 and NL20 with increasing MOL and MOP extract concentrations compared to ethanol controls.

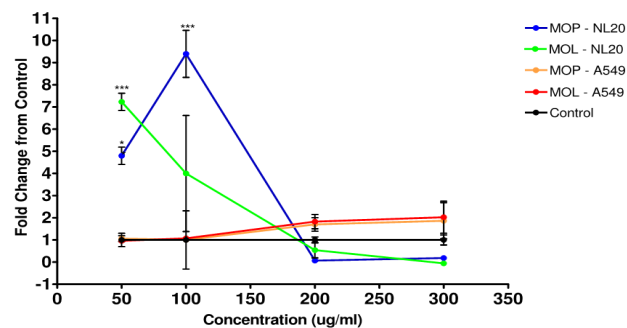


Figure 4. Fold change of A549 and NL20 with increasing MOL and MOP extract concentrations. * p<0.05, ** p<0.01,

by apoptosis, not necrosis (Figure 6). The A549 cells appeared to show increasing amounts of necrosis with increasing concentrations of MOL extract (Figure 6).

Discussion

A violation of the Levene’s test presents a possible limitation of this study’s findings, suggesting that the significance found by the ANOVAs could be a result of false positive results. However, data trends are evident, suggesting that the cell proliferation data collected in this study indicates that low concentrations of *Moringa* leaf extract increase healthy cell proliferation, especially evident in healthy lung cells (Figure 4). The images gathered by microscopic imaging showed that death in healthy cells at low MOL extract concentrations occurs by apoptosis or not all (Figures 5 and 6). In regards to method of cell death, apoptosis is preferred to necrosis because it prevents the outward environment of the dying cell from being affected. High concentrations of *Moringa* leaf extract decreased cell proliferation in healthy lung cells (Figure 4), suggesting that there may be a window of optimal concentration of *Moringa* leaf extract on healthy cells. In cancer cell lines, low levels of *Moringa* leaf extract did not have a significant effect on cancerous breast cells or cancerous lung cells (Figures 2 and 4). In fact, cancerous lung cells showed a steady increase in cell proliferation with increasing concentrations of MOL and MOP extracts (Figure 4), refuting the likelihood that *Moringa* could serve as a cancer-killing agent. Microscopic imaging of cancerous cells also did not show promising results for the use of *Moringa* as a cancer treatment option, for cell death did not appear to be occurring at all in the cancerous breast cells (Figure 5). These findings contradict a similar study that treated the same cancerous breast cell line, MDA-MB-231, with *Moringa* leaf extract and found “significantly low” cell survival above 250 µg/mL due to the induction of apoptosis⁷. Cell death in A549 cells suggests that necrosis is the primary mode of death with increasing concentration of MOL extract (Figure 6). This finding would also discredit the use of *Moringa* as a cancer-killing agent because cancer cell death achieved by necrosis would yield an inflammatory response and possible further tumor growth. When comparing the difference between MOL and MOP extracts on cell proliferation, no statistically significant differences were found (Figures 2 and 4).

Research previously reported has made contradictory conclusions to the ones made in this study. Although Al-Asmari et al.’s research using cancerous breast cells could not be reproduced, similar results were observed in the cancerous lung cells. However, different conclusions can be drawn because of the inclusion of ethanol controls. Corrected absorbance values of A549 cells showed that cell number decreased with increasing amounts of MOL and MOP extracts (Figure 3), which could suggest the conclusion that *Moringa* is resulting in decreased cancer cell proliferation. The inclusion of an ethanol control, however, showed that ethanol was also having an effect on cell proliferation. As ethanol percentage increased, cell number decreased (Figure 3). With the addition of *Moringa*, cell number increased above that of the control, a conclusion that could only be made with the use of fold change values. Al-Asmari et al. failed to account for the effects of ethanol in the *Moringa* extracts used, which led to the conclusion that *Moringa* was

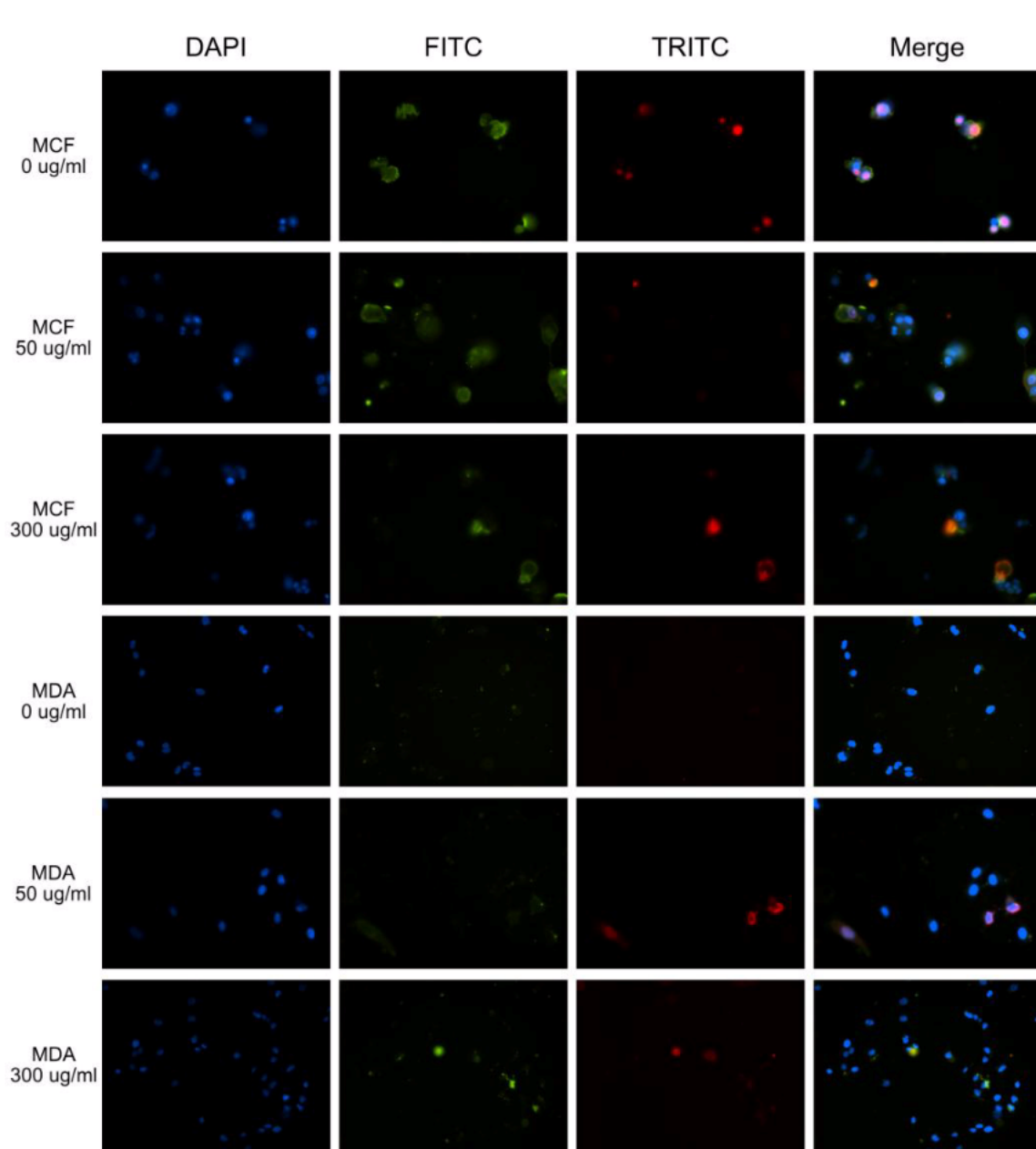


Figure 5. Effects of MOL extract addition to breast cell lines, MCF-10A and MDA-MB-231. DAPI - cell nucleus, FITC - apoptotic cells, and TRITC - necrotic cells.

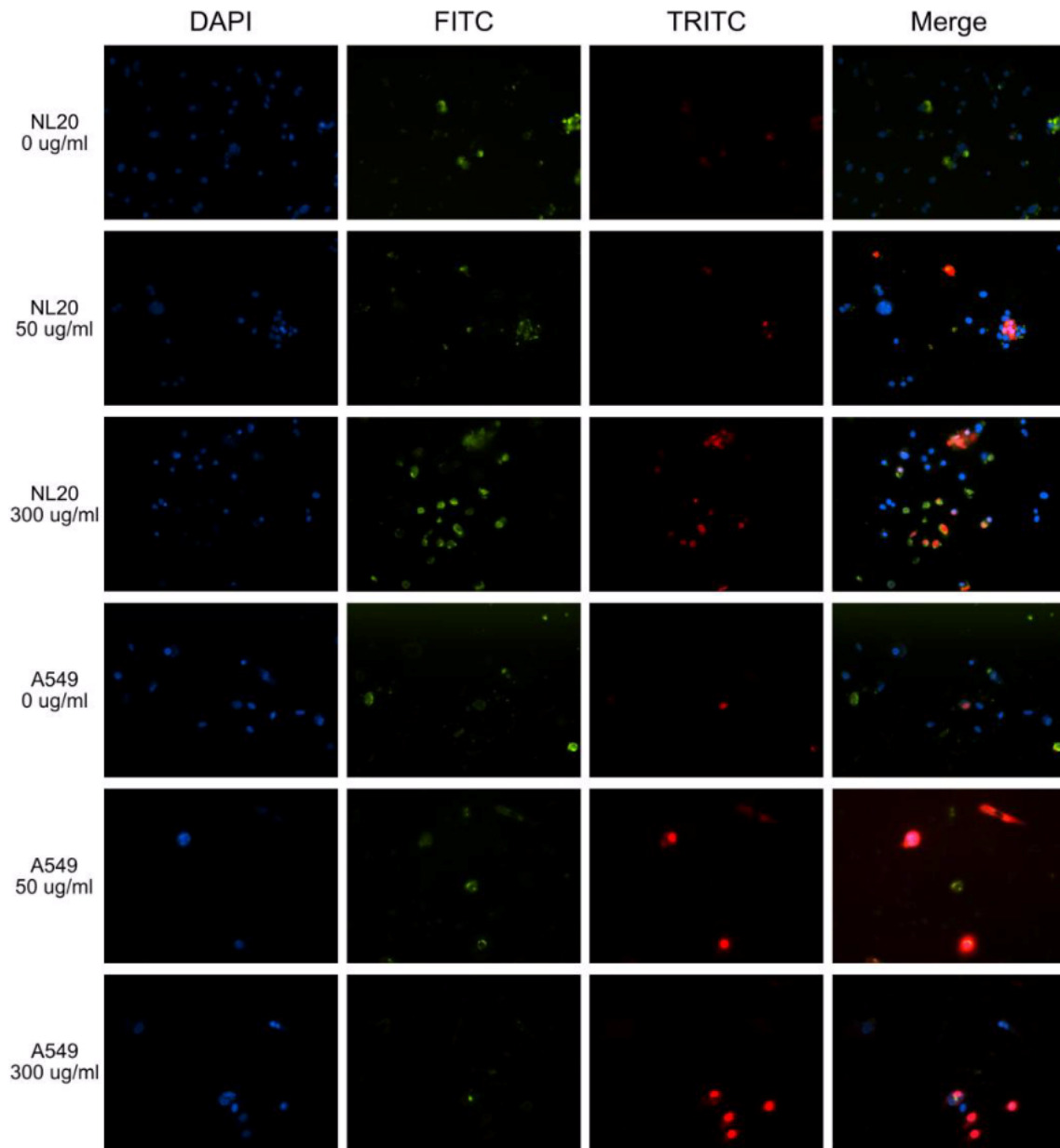


Figure 6. Effects of MOL extract addition to lung cell lines, NL20 and A549. DAPI - cell nucleus, FITC - apoptotic cells, and TRITC - necrotic cells.

killing cancer cells, when in reality, *Moringa* was slightly rescuing the cells from the harmful effects of ethanol. Future research should be conducted to determine why ethanol had such harmful effects on cancerous lung cells while breast cells were unharmed. The difference in proliferation response to ethanol between the lung and breast cell lines suggests a significant difference between the two cell lines.

Past research has also shown that *Moringa* leaf extract results in a reduction of cancer cell growth, while not comparing the treatment of *Moringa* leaf extract on normal cells^{7,8}. One study found that *Moringa* leaf extract “showed greater cytotoxicity for tumor cells than for normal cells”⁶, but this finding was also contradicted (Figures 2 and 4). One explanation lies in a dilemma that has been faced by the scientific community in recent years, a reproducibility crisis. An alarmingly high percentage of high-impact preclinical cancer papers have been unable to be replicated, and questions have begun to arise as to whether research experiments do not possess adequate controls or if the integrity of researchers should be in question. Although the purpose of this study is not to question the motives of other scientists, the contradictory results found may merit future attempts at reproducibility in research.

The results of this study do not support the use of *Moringa* leaf extract as a cancer-killing agent or future cancer treatment option; however, this does not discredit the use of *Moringa* for other medicinal uses. The increase in cell proliferation of normal lung cells at low concentrations of *Moringa* leaf extracts reinforces the healing properties that have been associated with *Moringa* for centuries, revealing that *Moringa* is beneficial to normal cells at appropriate concentrations. Although *Moringa* is unlikely to be a cancer-killing agent, it could still be an effective cancer preventative by increasing normal cell proliferation and preventing cancer cells from achieving metastatic growth. Future research in this area should be conducted to determine the true potential of *Moringa oleifera* in medicine.

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Notes and References

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