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Neem Constituent as a Therapeutic Option for Prostate Cancer

Destiny Van
Chemistry and Biochemistry
Spelman College
350 Spelman Lane SW
Atlanta, Georgia 30314 USA

Faculty Advisor: Dr. Kimberly M. Jackson

Abstract

Prostate cancer, a multifocal disease, is initially responsive to therapies that inhibit androgen receptor (AR) signaling, but the disease eventually progresses to an androgen-independent or refractory state. Understanding and modulating AR function may be essential to the development of new treatment modalities. Native to the Indian subcontinent, the *Azadirachta indica* plant (Neem) possesses potent anticarcinogenic properties. Nimbolide is a bioactive constituent of the Neem leaves. Previous studies reveal that nimbolide inhibits androgen-independent prostate cancer cells survival and proliferation by modulating multiple pro-survival signaling pathways. In this study, we examined the effects of nimbolide on the cell growth of LNCaP human prostate cancer cells, an advanced and androgen-sensitive cell line. LNCaP cells were treated with different concentrations of nimbolide for 24-48 hours. Cell proliferation was dose-dependently inhibited at 1, 2, 5, 10 μM nimbolide with more than 50% growth inhibitory effects between 5 and 10 μM.

Keywords: Nimbolide, Prostate Cancer, LNCaP

1. Introduction

1.1. Prostate Cancer

Prostate cancer occurs in the prostate, a walnut-shaped gland below the bladder, found in men that produces seminal fluid and nourishes and transports sperm[1]. Prostate cancer is the third most prevalent cancer and second leading cause of cancer death in American men[2]. According to the Centers for Disease Control and Prevention (CDC), black men have the highest risk of getting prostate cancer, white men have the second highest risk, and Hispanic men have the third highest risk[3]. According to the American Cancer Society, about 1 man in 7 will be diagnosed with prostate cancer during his lifetime. Most cases of prostate cancer include men being diagnosed with the disease from the age of 65 and up[2]. However, most men diagnosed do not die from it unless it is advanced disease. Biochemical research has been conducted to detect new effective ways to prevent, detect, diagnose, and treat prostate cancer. Moreover, treatment depends on the stage of the cancer. There are four stages of prostate cancer reported. Stage I indicates the earliest stage of prostate cancer in which the cancer grows extremely slow and the tumor cannot be felt. Stage II indicates the second earliest stage of prostate cancer in which the tumor is solely found in the prostate. Stage III indicates the next advanced stage of prostate cancer in which the tumor has started to grow outside of the prostate gland. Stage IV indicates the most advanced prostate cancer stage in which the cancer has metastasized or spread outside the prostate to other areas such as distant lymph nodes[4]. There are various tests for doctors to determine whether one has prostate cancer. These tests include the digital rectal examination (DRE), a prostate-specific antigen (PSA) blood test, a prostate ultrasound, and a prostate biopsy. Treatments include alpha-blockers, 5-alpha-reductase

inhibitors, surgery, radiation therapy, and prostatectomy[5]. Although it is beneficial to have various therapeutic options for advanced prostate cancer, there are also various side effects and risks to potentially come along. This includes heart disease, problems from surgery, lung problems, and other forms of toxicity[6].

The Jackson Laboratory examines advanced or hormone-refractory prostate cancer. Hormone-refractory (also known as castration resistant) prostate cancer is when prostate cancer cells are unresponsive to further hormonal manipulation, usually seen in advanced prostate cancer[1]. The molecular mechanism in which prostate cancer progresses to a hormone refractory state is vague. It is important to identify a novel mechanism to initiate chemotherapeutic treatment that will prevent or delay the onset of hormone-refractory prostate cancer. The Jackson Laboratory continues to investigate various diet-derived agents as potential chemotherapeutic options for advanced or hormone-refractory prostate cancer. More recently, the laboratory has been investigating the effects of plant-based products from the Caribbean on the treatment of cancer.

1.2. Natural Products

Although there are several options for chemotherapy or chemopreventive agents to treat cancer, they have many disadvantages, such as causing toxicity that restricts further usage. Between the period of 1981 and 2002, about 74 percent of all drugs approved by the National Institute of Health (NIH) rapid screening program for clinical trials were either natural products, were based thereon, or mimicked them in one form or another[8]. Presently, natural products still serve a dominant role in the development of therapeutics and treatment for human disease such as cancer. In fact, within the last decade, about 83 percent of all drugs approved by the NIH rapid screening program were either natural products, were based thereon, or mimicked them in one form or another[7].

Natural products are considered secondary metabolites and are chemical compounds, substances, and small molecules produced by a living organism found in nature. Natural products are proven to be more beneficial as about 80 percent of the global population rely on medicines that include plant derived natural products[9]. Plants have been a beneficial source of anti-cancer agents and they come from bioactive compounds of plants from terrestrial and marine environments. Natural products (plants) with bioactive compounds that show anti-proliferative activity in prostate cancer cells will benefit the continuous search for therapies that will prevent or control cancer. Natural products are an excellent source for drug discovery. Newer improvements and developments from natural products are often used as therapies to prevent or control cancer.

1.3. Lymph Node Carcinoma Of The Prostate (LNCaP) Cell Line

Cellular models for prostate cancers can be distinguished by two categories: androgen receptor positive (i.e. MDA-PCa 2b and LNCaP) and androgen receptor negative (i.e. Du145 and PC-3). Specifically, the Jackson laboratory uses LNCaP cells as a prostate cancer model. The LNCaP clone FGC cell line was originated in 1977 and derived from the left supraclavicular lymph node of a 50-year-old Caucasian male. The cell line is considered epithelial, adherent, and androgen receptor positive[10]. Prostate carcinoma cell lines such as LNCaP, PC-3, and DU145 have been previously observed and experimented to display any growth inhibitory effects of dibenzoylmethane[11]. For over 40 years, these cell lines are now being experimented with natural products in hopes to display growth inhibitory effects.

1.4. Previous Work

The Jackson Laboratory has been working with dibenzoylmethane (DBM), a constituent of licorice root that has been verified to slow the growth of human LNCaP cells, a metastatic or advanced stage prostate cancer cellular model. The Laboratory was the first to report the antineoplastic effects of DBM in prostate cancer cells. DBM induced pronounced changes in LNCaP prostate cancer cell growth, causing an accumulation of cells in the G1 phase of the cell cycle and altered key proteins as seen by proteomics[12]. Carcinoma cell lines such as BeWo, HeLa, and U937 have been previously observed and experimented to report that nimbolide inhibits cell growth by interfering with cell cycle kinetics by inducing G_0/G_1 and S phase arrest. However, there is no report on the extensive study of the cell cycle arrest by nimbolide on the LNCaP cell line[13].

1.5. Plants in Antigua and Barbuda West Indies

The Jackson Laboratory has previously identified and characterized plants indigenous to the Caribbean nation of Antigua and Barbuda. This was done through hours of audio conversations between an Antiguan naturalist and the head of the laboratory (Dr. Kimberly Jackson) being transcribed and archived. Photography taken by Dr. Jackson of the various plants identified as "disease fighting plants" were meticulously matched by the characteristics and properties from the audio conversations, and literary sources such as PubMed were used to identify the medicinal plants that have been reported to act as potential therapeutic options for cancer. *Azadirachta Indica*, Neem, was identified as the medicinal plant of interest with the potential to decrease proliferation in prostate cancer cells.

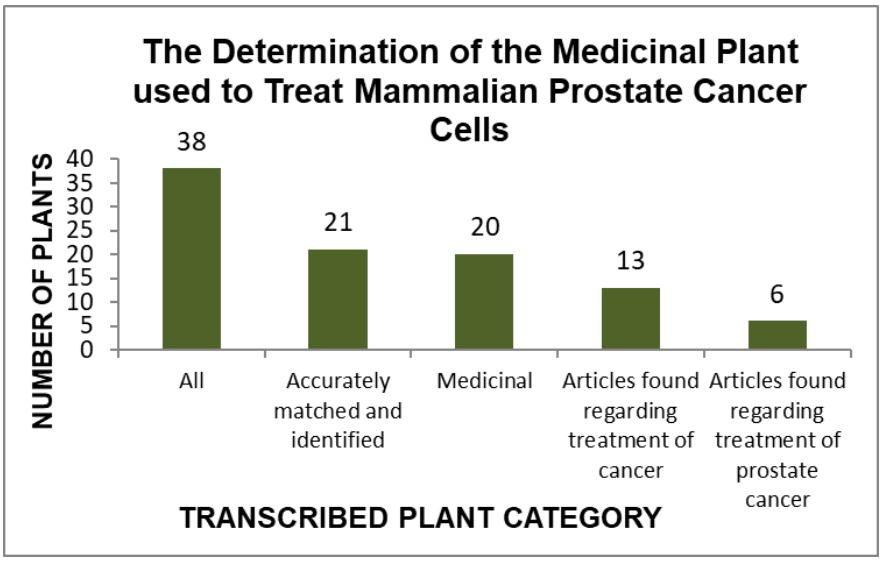

Figure 1. After hours of transcribing audio conversations and archiving photos of Antiguan plants, using literary databases, the plant chosen for further studies was scientifically and systematically identified and characterized.

1.6. Neem Plant And Nimbolide

Azadirachta Indica, a member of the Meliaceae family, has been commonly used in traditional Indian medicine to treat various diseases from prehistory to present time[14]. A previous study also shows that the neem plant inhibits cell proliferation in the prostate cancer cell lines PC-3 and LNCaP[15].

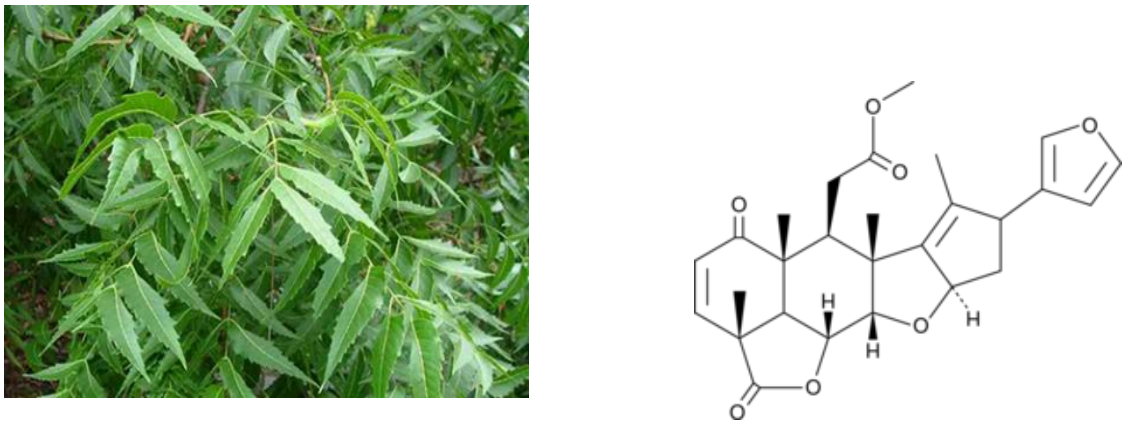

Figure 2. The *Azadirachta Indica* (Neem) plant in Antigua (photography by Dr. Kimberly Jackson, left). Right, the structure of nimbolide, a major active constituent of Neem[12]

Nimbolide, a bioactive constituent of the Neem plant, has reportedly prevented the proliferation of prostate cancer cell lines such as PC-3; therefore, nimbolide acts as a potential anti-cancer drug for prostate cancer[17]. In fact, cell growth-regulated genes involving signaling pathways that are active in cancer cells were inhibited by nimbolide. Recently, the Jackson laboratory has examined the effects of nimbolide on the cell growth of LNCaP human prostate cancer cells, an advanced and androgen-sensitive cell line. LNCaP cells were treated with different concentrations of nimbolide for 24-72 hours. Cell proliferation was dose-dependently inhibited at 1, 2, 5, 10 µM nimbolide with more than 50% growth inhibitory effects between 2 and 5 µM. Cell proliferation and viability were measured colorimetrically by reduction of MTT (a tetrazolium salt assay). These results further suggest that nimbolide may exhibit anti-proliferative activity in androgen-dependent prostate cancer cells, and progression of prostate cancer to metastatic or advanced disease may occur by modulating the AR signaling pathway.

2. Methodology

2.1. Reagents And Cell Culture

LNCaP cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 complete medium (ATCC) and grown in tissue culture treated T-75 flasks in 5% CO_2 at 37ºC. The medium contained 10% heat inactivated fetal bovine serum and 1% 100X concentration antibiotic-antimycotic solution (ThermoFisher Scientific, Waltham, MA, USA). The media was stored at 4ºC. Every two to three days the cells were subcultured in T-75 flasks and placed in the incubator. Using aseptic techniques for cell culturing, the media and 0.25% trypsin-ethylenediaminetetraacetic acid-EDTA, (ThermoFisher Scientific) was warmed in a water bath at 37ºC for 25 to 30 minutes. If the cell density was considered medium to highly confluent (between 60-80% confluency), they would be detached and passaged using the trypsin-EDTA. The 0.25% trypsin-EDTA was stored at -20ºC.

2.2. Trypan Blue Exclusion Assay

Cell viability (90% or greater) is required for the nimbolide treatments and the MTT proliferation assay. The cell viability of LNCaP cells was determined by a trypan blue exclusion test. To conduct this test, 10 µL of Trypan blue reagent were pipetted into an Eppendorf tube and 10 µL of the cells were pipetted into the same tube. After mixing both solutions, the total solution was pipetted (20 µL) onto a plastic Neubauer hemocytometer (chamber slide) and inserted in the Countess Automated Cell Counter (ThermoFisher Scientific) to read. Cell viability percentage, total number of cells, total number of living cells, and total number of dead cells were determined.

2.3. Preparation Of Drug Treatment

The cells were grown logarithmically, collected and prepared at a density of 1 x 10^5 cells/mL. Nimbolide concentrations were 0 µmol/L, 1 µmol/L, 2 µmol/L, 5 µmol/L, and 10 µmol/L respectively, as used in previous nimbolide treatment studies for cancer[17- 20]. These cells were incubated in 96-well plates with a volume of 100 µL per well and incubated for 24 hours and 48 hours in an incubator with 5% CO_2 at 37ºC.

Table 1. This figure displays the 96-well plate set-up. The experimental area of the plate contained the LNCaP cells that were treated with nimbolide and incubated for either 24 or 48 hours.

Design of the 96-Well Plate Set-Up for Nimbolide Treatment			
	1	2	3
A	0 µM w/ nimbolide	0 µM w/ nimbolide	0 µM w/ nimbolide
B	1 µM w/ nimbolide	1 µM w/ nimbolide	1 µM w/ nimbolide
C	2 µM w/ nimbolide	2 µM w/ nimbolide	2 µM w/ nimbolide
D	5 µM w/ nimbolide	5 µM w/ nimbolide	5 µM w/ nimbolide
E	10 µM w/ nimbolide	10 µM w/ nimbolide	10 µM w/ nimbolide

2.4. Cell Microscopy And Morphology

LNCaP cells were observed under the Zeiss Axio inverted light microscope under the ocular lens at 100X magnification. This was done to verify cell viability, to quickly verify cell detachment post trypsinizing, to determine confluency, and to also capture live photos prior and post drug treatment of cells.

2.5. Cell Viability – MTT Proliferation Assay

MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, is a tetrazolium salt is used as a colorimetric and proliferation assay to assess cell viability. If viable cells are present, the tetrazolium salt dye will be converted into formazan. Mechanistically, enzymes such as the NADPH-dependent oxidoreductase enzyme will be produced in the mitochondria in the cell. The tetrazolium salt dye is golden-yellow, and the formazan is purple. The number of viable cells is indicated by the degree of the purple dye[20]. Cells were removed from the incubator after the 24-hour or 48-hour treatment and 15 µL of MTT solution was added into each well (5 mg/mL, Sigma Aldrich). The cells were then incubated for 4 hours in a humidified, 5% CO_2 atmosphere at 37ºC. Then 100 µL of an organic stop solution was added to each well and the 96-well plate was wrapped with clear plastic and placed in a dark environment overnight. The cells were analyzed the next day by a BioTek Synergy HT spectrophotometer and the Gen5 computer software. Absorbance (OD_{570}) was measured at 570 nm. Microsoft Excel was used to graph the data with absorbance and concentration values.

2.6. Flow Cytometry

Flow cytometry, an immunophenotyping technique was used to analyze the characteristics of cells. It is most frequently used to distinguish cells in different phases (cell cycle analysis). It is also used as a part of protein analysis in which proteins involved with cell cycle regulation can be found[21]. It would be beneficial to use this technique to receive immunofluorescence data to analyze what phase (i.e. G_1/S phase, G_2 phase, M phase) displays any cell cycle arrest or deregulation in prostate cancer cells treated with nimbolide. Therefore, finding a signaling pathway that is altered by nimbolide can produce a better understanding of the growth and anti-growth of these cells. This in turn increases the possibility of more effective natural therapeutics toward advanced human prostate cancer.

For cell cycle analysis, after the LNCaP cells were treated for a 24-hour incubation period, cells were detached by scraping and placed in 15 mL conical tubes. These cells were then pelleted by centrifugation between five to ten minutes at 1000 revolutions per minute (rpm) and washed one time with phosphate-buffered saline (PBS). Furthermore, the LNCaP cells were fixed with one mL of cold 70% ethanol and stored for a minimum of 30 minutes on ice. After fixation, cells were pelleted by centrifugation, and then resuspended in one mL of PBS containing one mg/mL of glucose, 1 mg/mL RNase A (Sigma Chemical Co., St. Louis, MO), and 50 µg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). Using a BD Accuri C6 flow cytometer, flow cytometry was performed after a one-hour incubation in propidium iodide while on ice. Ten thousand events were recorded, stored, and analyzed and cell cycle phase analysis was conducted using histograms produced using Modfit version 5 software (Verity Software House, Topsham, ME).

3. Results

Nimbolide has been reported to display various biological activities including anti-cancer effects on prostate cancer cells[16-19]. In a previous study, it was observed that prostate cancer cell viability decreased with nimbolide concentration of 1 and 2 µM[19]. It was also observed in another study that nimbolide displayed anti-prostate cancer activity on LNCaP cells after being exposed to 0, 0.5, 1, 2.5, and 5 µM nimbolide concentration and incubated for four hours[18].

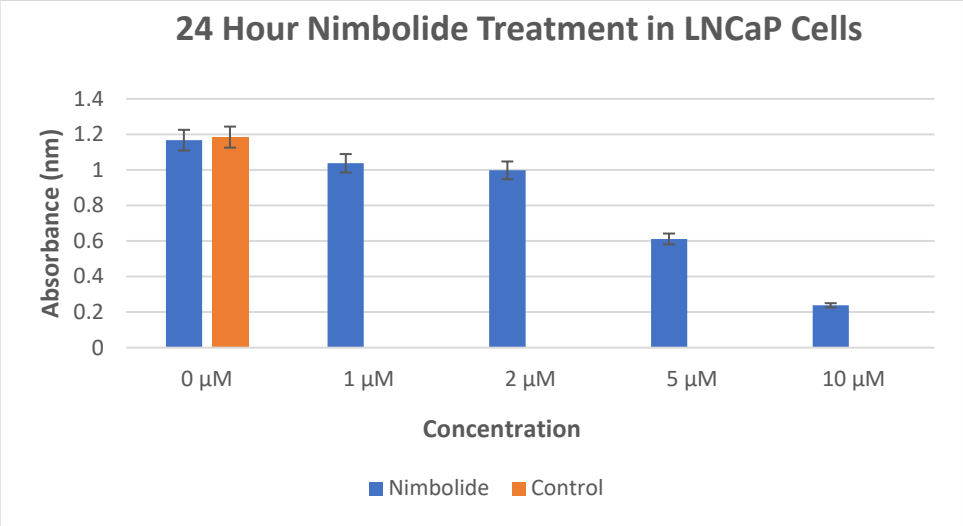

Figure 3. LNCaP cells were treated for 24 hours with increasing concentrations of nimbolide. Viable cells were measured using an MTT assay and expressed as corrected absorbance units at 570 nm (n= 3).

Absorbance (OD_{570}) of the cells at 0, 1, 2, 5, and 10 µM concentrations of nimbolide were measured at 570 nm (figure 4). There is a direct proportional relationship between absorbance and cell concentration while an inversely proportional relationship between absorbance and nimbolide concentration. This can be justified through the Beers-Lambert Law, which states that the absorbance is directly proportional to the concentration of the solution of the sample used in the experiment[22]. Furthermore, the data indicates that nimbolide is significantly inhibiting the cell viability.

The absorbance value of the same concentrations of the nimbolide was measured again at 570 nm (figure 5). Based on the numerical fluctuations in absorbance values as the concentrations of nimbolide increases, this can indicate that there is no clear relationship shown between the absorbance and the concentrations of nimbolide below 10 µM.

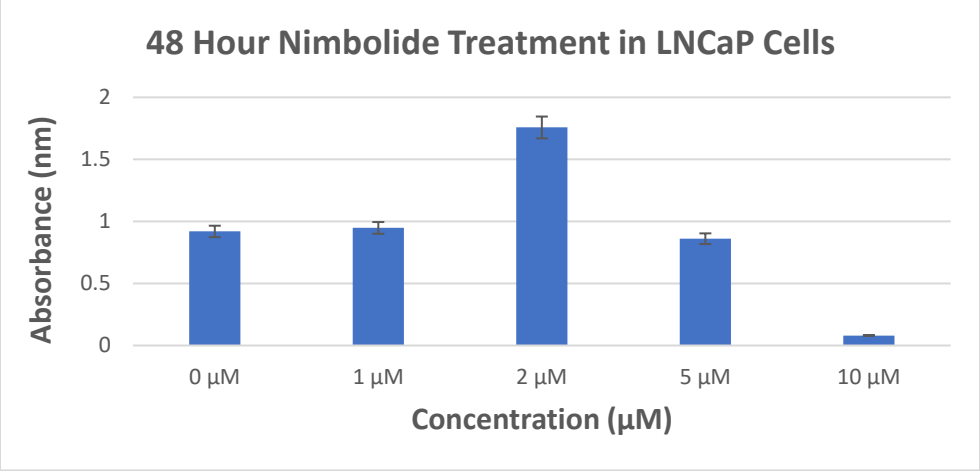

Figure 4. LNCaP cells were treated for 48 hours with increasing concentrations of nimbolide (0, 1, 2, 5, and 10 µM). Viable cells were measured using an MTT assay and expressed as corrected absorbance units at 570 nm (n= 3).

In this report, there is evidence of observed repression of the proliferation of LNCaP cells in both the 24-hour incubation period and the 48-hour incubation period between the nimbolide concentrations of 5 and 10 µM. After these cells were treated with the various nimbolide concentrations and incubated after 24 hours, the measured absorbance for the highest nimbolide concentration (10 µM) was the lowest value compared to the other nimbolide

concentrations. Interestingly, after these cells were treated with the various nimbolide concentrations and incubated after 48 hours, there was only in inhibition of cell growth shown between the nimbolide concentrations of 5 and 10 µM. Possible justification for these results include a preference of a 24-hour incubation period after nimbolide treatment due to possible cellular DNA repair during a lengthy incubation period. Studies have received promising data such as higher percentage in cell deregulation of LNCaP cells after a 24-hour incubation period[16, 17, 19].

The growth inhibitory effect of nimbolide was confirmed in microscopic images showing fewer cells present in nimbolide-treated cultures at a concentration of 5 and 10 µM (figure 5). After observing the cells, light microscopy further displayed the cells as long and thin opposed to the cells in 5d and 5e in which the cells look more circular shaped as though they were apoptotic bodies. The morphological changes induced by nimbolide were evident in both the 24-hour and 48-hour hour treatment.

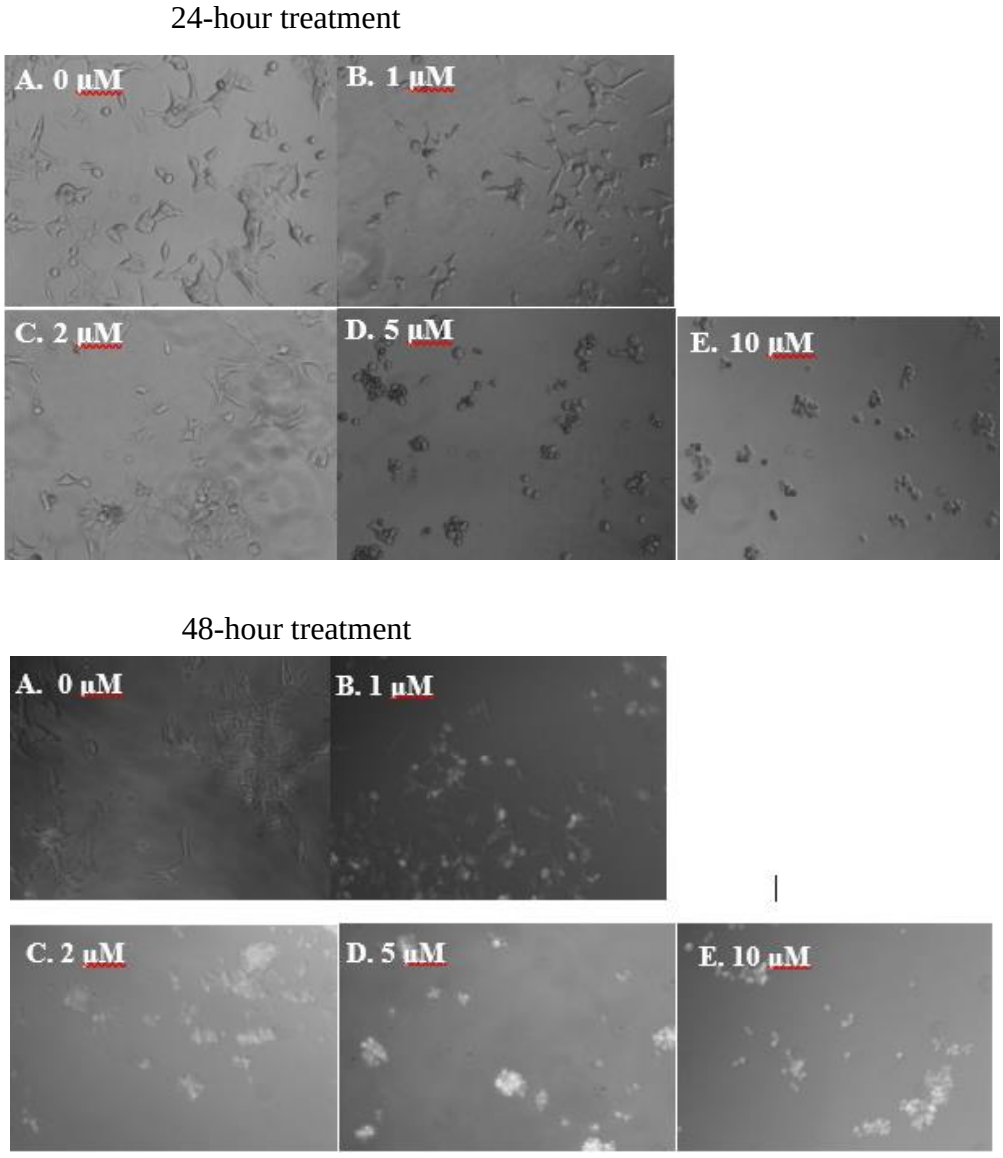

Figure 5. After the 24-hour or 48-hour incubation period of LNCaP cells post-nimbolide treatment, morphology images were taken using a Zeiss Axio inverted light microscope. Micrographs shown are of the 24 hour treatment (top) and 48 hour treatment (bottom).

4. Discussion and Conclusions

The goal of this research was to shed light on the biological activity of the Neem plant's bioactive compound, nimbolide, on the LNCaP cell line. Over the past decade, evidence of the inhibition of LNCaP cellular proliferation has been extensively studied. Nimbolide has been reported in previous studies to suppress the viability in multiple cancer cell lines, including other prostate cancer cell lines such as PC-3. Overall, the results of the nimbolide treatment indicated that nimbolide has suppressed the growth of LNCaP cells primarily between 5 and 10 µM of nimbolide. The results of the MTT proliferation assay also confirm that any decrease in cell viability from the 24-hour and 48-hour incubation period of nimbolide-treated LNCaP cells are evident through cell microscopy and morphology.

The goal of flow cytometry was to analyze the cell cycle of treated and non-treated human LNCaP cells and contain some immunofluorescence data that shows visual apoptosis. This visual would allow the laboratory to look at a signaling pathway involved in the cell cycle arrest of the LNCaP cells. Past research conducted on nimbolide had reported cell cycle deregulation of prostate cancer cell lines due to nimbolide in cell phase G_1 and S-phase in cancer cell lines such as HeLa and colon cancer cell lines[23]. It is imperative to conduct cell cycle analysis using flow cytometry for further investigation of DNA content in cells and to determine at which cell cycle phase displays cell deregulation. Preliminary results of the flow cytometry experiment revealed deregulation in cell growth in the G_1 phase (data not shown). However more experimentation is needed to confirm results and to assess treated LNCaP with nimbolide. Moreover, future analysis includes an investigation of possible signaling pathways that could be affected due to nimbolide treatment. Studies report that nimbolide prohibits cell survival through signaling pathways such as the PI3K/AKT, MAPK, and STAT3[16-18].

To clear the vagueness of the molecular mechanisms in this disease it is essential to observe multiple biochemical processes such as apoptosis and cellular proliferation. The use of a bioactive compound (nimbolide) of a Caribbean-cultivated plant (Neem) as a source of investigating the proliferation of the LNCaP cell line, has helped contribute to this goal.

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