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Cryotoxicity of Terminalia catappa on Cancer Cell Lines

Mary Natalie Davis

Cytotoxicity of *Terminalia catappa* on Cancer Cell Lines

by

Mary Natalie Davis

An Honors Capstone

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Dedication

I would like to dedicate this work to Dr. Moriarity, who gave me a chance in her laboratory, guided me with patience, trust, and endless amounts of knowledge, and helped to solidify my love for science and research. I owe my success, along with my ability to learn from my setbacks, to this inspiring mentor.

I would also like the thank all of the chemistry and biology departments who helped with this research, and David Cook and the Honors College for the opportunities presented to get involved with this research project and the guidance to complete it.

Abstract

Extracts from the plant *Terminalia catappa* were collected and tested for cytotoxic activity through the use of MTT assays. After the initial screening, the presence of cytotoxic activity of the crude extracts was found. This led to further fractionation of the sample, producing various samples. These fractions were screened against the estrogen receptor negative breast cancer cell line MDA-MB-231 and the prostate cancer cell line PC-3. Two samples, 2C and 3B, showed promising cytotoxic activity in both cell lines. The samples were found to have LC_{50} values of 73.5 µg/mL (±5.2 µg/mL) and 45.6 µg/mL (±7.1 µg/mL), respectively, in the MDA-MB-231 cell line. Further testing, along with structure elucidation, needs to be performed still, but these results show promise for possible future drug development.

Introduction

Since long before modern science, plants have been used for medicinal purposes to cure and treat a variety of ailments. From herbal teas to ointments and everything in between, nature has continually been the source for many treatments. As technology and understanding has progressed, specific compounds within plants have been isolated and medications have been developed. Whether they are collected directly from nature, or synthesized derivatives, these natural products have been a major part of drug discovery [1].

Natural Products Research Group

In the Natural Products Research Group, based at the University of Alabama in Huntsville, various plant extracts have been collected from locations locally and internationally in hopes of understanding the chemical basis for their medicinal use. Samples are usually prepared in the chemistry department, through various methods such as initial chloroform extraction and bioactivity-guided fractionation through column chromatography. Biological assays are performed in laboratories in the biology department. While there are many biological tests that can be performed to examine the abilities of the plants, this paper discusses the testing of cytotoxic activity of extracts from the plant *Terminalia catappa* against various human tumor cell lines. The discovery of new anticancer treatments is an important contribution to the scientific and medical world, as most current chemotherapeutic drugs carry many harmful side effects.

Terminalia catappa

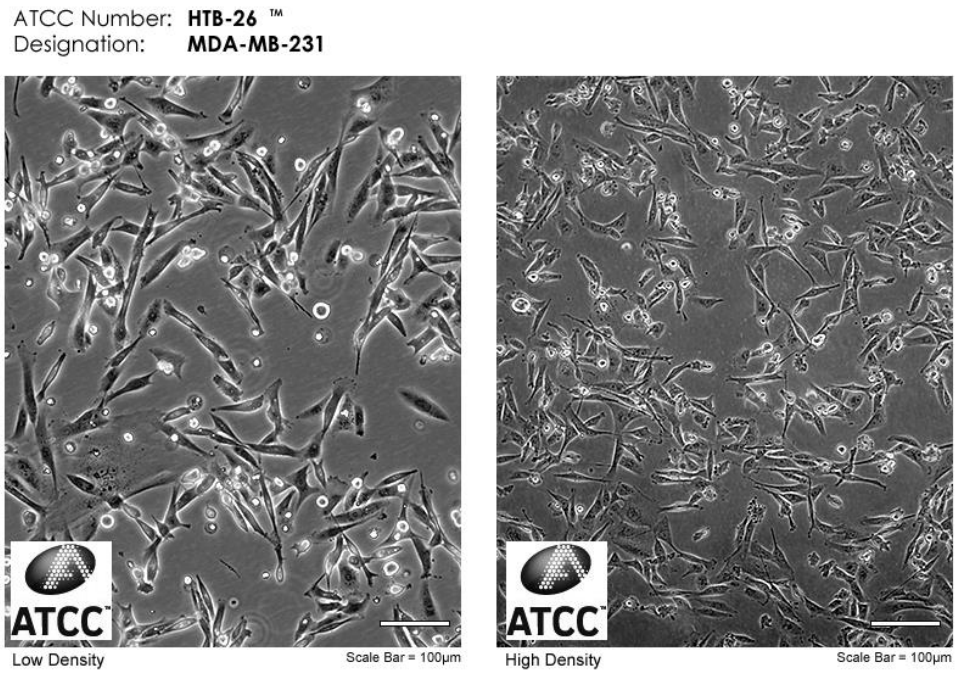
Terminalia catappa is a large tree that is native to Southeast Asia but grows in many tropical and subtropical climates [2]. The extracts tested in this study were gathered from Nigeria. The tree grows edible fruit and is known to serve several medicinal purposes. Various studies have shown *T. catappa* to have antimicrobial, anti-inflammatory, modulatory, analgesic, and many more healing properties [2]. Its high pharmacological usage led this research team to investigate its cytotoxic effects on several cancer cell lines. Various extracts from the leaves of *T. catappa* have shown anticancer effects on liver cancer, kidney cancer, and certain lymphomas through various biological mechanisms. Researchers in this study wanted to explore the cytotoxic effects of these extracts on breast and prostate cancers.

Figures 1 and 2: Terminalia catappa tree, leaves, and fruit [3],[2]

Cell Lines

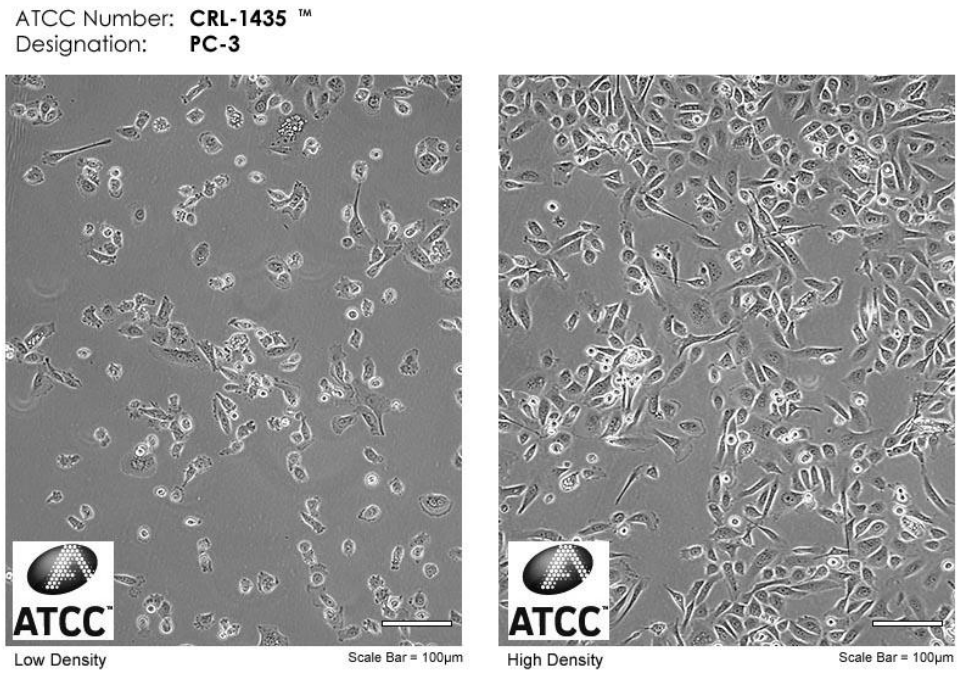
For this study, two different cell lines were used. Firstly, the MDA-MB-231 cell line (ATCC HTB-26) was used as a breast cancer model [4]. This adherent, epithelial cell line is estrogen receptor negative, but does express epidermal growth factor and transforming growth factor alpha receptors. The cells are tumorigenic and express the WNT7B oncogene.

Figure 3: Cultures of MDA-MB-231 cell line at low and high densities [4]



The natural products were also tested on cultures of PC-3 cells (ATCC CRL-1435) [5]. PC-3s are a mammalian cell line derived from a bone metastasis of a grade IV prostatic adenocarcinoma. They are an adherent cell line and epithelial in morphology.

Figure 4: Cultures of PC-3 cell line at low and high densities [5]



Methods

Media Preparation

MDA-MB-231

RPMI 1640 with L-Glutamine base media was used and made 1000-mL at a time. This was supplemented with 10 mL Penicillin-Streptomycin (10,000 units/mL Penicillin, 10,000 µg/mL Streptomycin), 15 mM HEPES buffer, and 0.195% sodium bicarbonate. The pH was balanced at 7.35 and sterile filtered using a 0.22-micron filter. A 100-mL aliquot was prepared with 10% fetal bovine serum before being used on the cells. The media was stored at 4°C and warmed to 37°C before being added to cells.

PC-3

A 500-mL bottle of F-12K media was combined with 0.195% sodium bicarbonate, and 5 mL Penicillin-Streptomycin (10,000 units/mL penicillin, 10,000 µg/mL Streptomycin). The pH was balanced at 7.35 and sterile filtered using a 0.22-micron filter. A 100-mL aliquot was prepared with 10% fetal bovine serum to before being used on the cells. The media was stored at 4°C and warmed to 37°C before being added to cells.

Thawing and Plating Cells

Cells were removed from cryopreservation in a liquid nitrogen tank and thawed in a 37°C water bath, keeping the cap out of the water to prevent contamination. The cells were carefully transferred to a 50-mL conical tube. 10 mL of pre-warmed media was added drop-wise to the cells, gently swirling to mix. The cell suspension was centrifuged for 2 minutes at 180 x g. The supernatant was aspirated from the tube and the pellet was resuspended in 10 mL of fresh media. The cell suspension was plated into two T-25 cm^2 flasks and incubated at 37°C at 5% CO_2 in a

humidified chamber.

Maintaining Cultures

Media was replaced every other day by aseptically aspirating the old media with a sterilized canula and replacing it with 5 mL of fresh, pre-warmed growth media. Cells were passaged once a week.

Subculturing

The old media was aseptically aspirated from the flasks, taking care not to disturb the cultures. The flasks of cells were then each rinsed with 5 mL of Hank's Balanced Salt Solution (HBSS) without calcium and magnesium to get rid of the serum, which contains a trypsin inhibitor. The HBSS was aspirated before 5 mL of a 1:10 dilution of 10X Trypsin-EDTA in HBSS solution was added to each flask to detach the cells from the flask wall. The solution was allowed to sit for 2 minutes at room temperature before 4 mL was aspirated. The flasks were moved to the 37°C incubator, in order to speed up the reaction, where they sat with the remaining 1 mL of solution for another 2 minutes before being removed from the incubator. The flasks were taken back to the biosafety hood, where 5 mL of fresh, pre-warmed growth media was added to each flask to inhibit the trypsin reaction. The cells were carefully moved from the flasks to one 50-mL conical tube. The solution was centrifuged for 2 minutes at 180 x g. The supernatant was removed, and the pellet was resuspended in 10 mL fresh growth media.

A mixture of 0.3 mL 0.4% Trypan Blue, 0.6 mL of normal NaCl solution, and 0.1 mL of the cell suspension was prepared in a small test tube. A small amount of the mixture was added to each side of a hemocytometer for cell counting. Using a light microscope, the viable cells were

counted, averaged, and the total number of cells were calculated using the dilution factor. The remainder of the cell solution was used to plate two more T-25 flasks at 1.75 x 10^5 cells per flask for MDA-MB-231s and 2.5 x 10^5 cells per flask for PC-3s, and a 96-well plate at 1.2 x 10^4 cells per well for the assay for MDA-MB-231s and 2 x 10^5 cells per well for PC-3s.

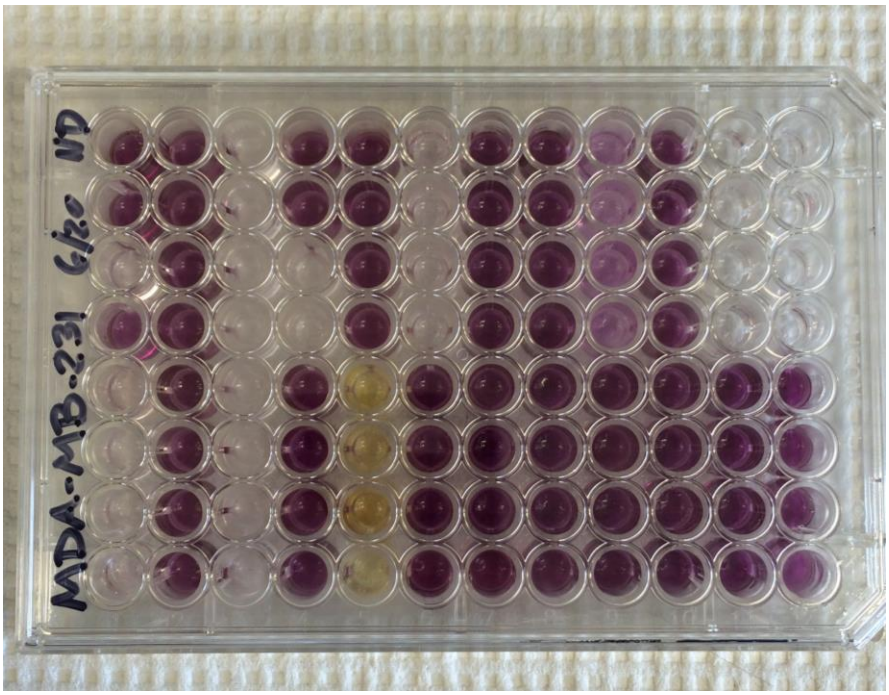
Test Compound Preparation

Preliminary tests were conducted with the extracts at 0.01% w/v by diluting the original samples (0.1% in DMSO) in complete growth media. Tingenone [6] was used as a positive control and DMSO was used as a solvent control, both at concentrations of 0.01%, diluted in complete growth media. Plain media controls were also used. The day before the assay, a 5 mg/mL dilution of MTT solution in complete growth media was prepared.

Cytotoxicity Screening

An MTT assay was used to screen for cytotoxicity [7]. This assay works by adding MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, to the cells of interest. The MTT solution is yellow but turns purple when reduced by metabolically active live cells. Therefore, a spectrophotometer can be used to measure the color change between the original MTT solution in the wells and the color of the wells after the reaction takes place to measure the amount of dead and live cells. A plate from a completed assay can be seen below in Figure 5.

Figure 5: 96-well plate after MTT solution is added



For the assay, the cell lines were plated in 96-well plates as described above in the subculturing procedure. The cells were allowed to grow for two days before the test compounds were added. The media was carefully aspirated before test compounds were added at a volume of 100 µL per well. The plates were set up to have quadruplicates of each compound, with eight replicates of the DMSO control. Wells A1-D1 were always used for a media control, wells E1-H1 were always used for the tingenone control, and wells A2-H2 were all used for the DMSO control. The remaining compounds were spread across the rest of the wells in groups of four. The cells with the test compounds added were incubated for another 48 hours before being assayed.

On the day of the assay, the MTT solution previously prepared was diluted 1:10 in complete growth media. The old solutions were carefully aspirated from each well and 100 µL of the 0.5 mg/mL MTT solution replaced them. The 96-well plate was inserted into the SpectraMax

spectrophotometer, and a pre-read was conducted at 570 nm. The plate was incubated for 3-4 hours in the 37°C incubator. After the time had elapsed, the media/MTT solution was carefully aspirated, being careful not to disturb the formazan crystals. Then, 100 µL of DMSO was added to each well and the post-read was conducted in the spectrophotometer at 570 nm.

The raw data were compiled in a Microsoft Excel spreadsheet, where the percent viability, the percent kill, and their standard deviations were calculated by comparing the pre- and post-reads and normalizing to the controls. The calculated percent kills of each compound show their effectiveness and allowed the researchers to choose which compounds to pursue and at what concentrations.

LC_{50} Determination

Compounds showing a high percent kill with a small standard deviation were pursued further. The compounds were screened at varying concentrations to determine their LC_{50} values, the lethal concentration required to kill 50% of the cells [8].

Results

Table 1: MDA-MB-231 MTT Assay Results

Compound	% Viable	% Kill	Std. Dev. Of % Kill
TeCa 1A	114.01	-14.01	12.08
TeCa 1B	76.79	23.21	22.43
TeCa 2B	95.23	4.77	4.01
TeCa 2C	-0.78	100.78	4.10
TeCa 3B	-0.98	100.98	3.57
TeCa 5B	7.68	92.32	3.48
TeCa 6A	83.29	16.71	28.06

Table 1 shows the initial screening of Terminalia catappa (TeCa) samples at 0.01% on MDA-MB-231 cells.

Table 2: LC_{50} Determination on MDA-MB-231 Cells

Compound	Concentration of Compound	% Viable	% Kill	Std. Dev. Of % Kill
TeCa 2C	0.01%	37.42	62.58	2.26
TeCa 2C	0.005%	67.42	32.58	6.67
TeCa 3B	0.01%	48.35	51.65	5.26
TeCa 3B	0.001%	98.62	1.38	40.24

Table 2 shows the concentrations that killed above and below 50% of the MDA-MB-231 cells in the assay. These values were used to determine LC_{50} values.

Table 3: Calculated LC_{50} Value of *TeCa* 2C and 3B in MDA-MB-231 Cells

Compound	LC_{50} (µg/mL)	Standard Deviation
TeCa 2C	73.5	5.2
TeCa 3B	45.6	7.1

Table 3 shows the calculated LC_{50} values of the 2C and 3B samples in MDA-MB-231 cell line, based on the data from the assay shown in Table 2.

Table 4: PC-3 MTT Assay Results

Compound	% Viable	% Kill	Std. Dev. Of % Kill
TeCa 1A	100.09	-0.09	10.98
TeCa 1B	82.13	17.87	4.69
TeCa 2B	110.99	-10.99	7.41
TeCa 2C	19.32	80.68	1.25
TeCa 3B	27.17	72.83	2.61
TeCa 5B	87.04	12.96	4.04
TeCa 6A	98.44	1.56	8.78

Table 4 shows the initial screening of Terminalia catappa (TeCa) samples at 0.01% on PC-3 cells.

Table 5: LC_{50} Determination on PC-3 Cells

Compound	Concentration of Compound	% Viable	% Kill	Std. Dev. Of % Kill
TeCa 2C	0.01%	19.55	80.45	4.29
TeCa 2C	0.005%	76.26	23.74	13.82
TeCa 3B	0.01%	60.34	39.66	8.36
TeCa 3B	0.005%	65.32	34.68	7.77

Table 4 shows the concentrations that killed around 50% of the PC-3 cells in the assay.

Discussion

Of all of the *Terminalia catappa* compounds tested, samples 2C and 3B were the only ones to show activity in both cell lines tested. The data show that between the experiments, a 0.01% concentration of *TeCa* 2C killed a higher percentage of PC-3 cells than MDA-MB-231 cells. Conversely, a 0.01% concentration of *TeCa* 3B killed a higher concentration of MDA-MB-231 cells than it did PC-3 cells. This shows that there may be cell line differences, in which the compounds affect different cancers in different ways. Further studies of the compounds could present an explanation for this difference by the mechanisms by which they act.

LC_{50} values were calculated for *TeCa* samples 2C and 3B tested on MDA-MB-231 cells and were found to be 73.5 µg/mL (±5.2 µg/mL) and 45.6 µg/mL (±7.1 µg/mL), respectively. Further testing needs to be performed in the PC-3 cell line in order to calculate an accurate LC_{50} value, as the assay did not produce a percent kill above 50% in the 3B sample. The similarities or differences in LC_{50} values between the two cell lines could tell more about the mechanistic differences previously discussed.

These calculated LC_{50} values can be compared to known chemotherapeutic drugs. Doxorubicin, for example, has an LC_{50} values of 25.9 µg/mL (±2.4 µg/mL). In theory, the lower the concentration of the drug used, the lesser the harm of other side effects may be. While still needed at a higher concentration than doxorubicin, compounds 2C and 3B are still mixtures, and the LC_{50} values may be lowered after activity-guided fractionation isolates these into individual compounds. Furthermore, it can be seen that these compounds have different effects in different cell lines, so the activity of these compounds may vary depending on the type of cancer.

Once more cytotoxicity assays are conducted with individual fractions, the bioactive fractions can undergo analyses of structure elucidation, such as x-ray crystallography and NMR

spectroscopy. This will tell exactly what the compounds are so that the mechanism of tumor inhibition can be understood, and new chemotherapeutic agents can be developed to specifically target certain biological pathways and treat cancers.

Many future experiments are required before the possibility of new chemotherapeutic drugs could be a reality. However, these experiments and collected data provide a strong basis of preliminary research to move towards this end goal. The pursuit of discovering and understanding natural products' role in medicine is critical to our development of the best treatment options there can be.

Conclusion

Natural products serve as a source for drug discovery. The Natural Products Research Group at the University of Alabama in Huntsville has strived to further the field with their mass collection and testing of plant extracts from all over the world. These experiments have produced many publications outlining the compounds found in these plants and their many medicinal purposes through various bioactivity screening.

In this study, the leaves of the *Terminalia catappa* tree were collected and tested. The tree's extracts have been used to treat various ailments for years and have recently been studied for the biological activity. This study examines collected extracts from the tree and tests their cytotoxic activity on two cancer cell lines through the use of MTT colorimetric assays. Two samples showed activity in both cell lines and will be further fractionated to find the exact compounds involved in order to move forward in the direction of drug development.

This study aides in the development of anticancer drugs, and their underlying mechanisms, while also demonstrating environmental importance. The discovery of natural products as medicinal sources aides in the justification of preservation of many plants and forests. This can provide an accessible source of drugs for medical providers, a source of income for local populations who may have access to these plants, and a cause to prevent worldwide deforestation, helping the entire planet. In conclusion, natural products provide many benefits and this study adds to our expanding source of knowledge on the subject.

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