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Abstract **–**The cytotoxicity of various essential oils from plant extracts has been tested on two different breast cancer cell subtypes. An estrogen positive breast cancer, MCF7, and a triple negative breast cancer, Hs578T, were both used in this study. Concentrations (weight volume w/v) of test samples of 0.01%, 0.005%, 0.004%, 0.003% were used to determine the LC_{50} value for compounds determined to decrease the number of viable cells in a culture. Testing completed with MCF7 cells determined that the fraction 2C of *Terminalia catappa* (Teca) was the most efficient for cytotoxicity, with a percent kill of 80.56 at a concentration of 0.004% (w/v). Hs578t testing determined that fraction 3 of *Ipomoea tricolor* (IT) had the most efficient toxic effects, with a percent kill of 93.53 at a concentration of 0.003%(w/v), which was the lowest concentration tested.

I. Introduction

In the United States, one in every eight women is expected to be affected by breast cancer in her lifetime [1]. The problem with treating breast cancer is that it is not just one disease, and each form of the disease responds to treatments differently. Current chemotherapy protocols have several limitations, including the fact that these treatments do not only damage cancer cells but also the healthy cells. Additionally, cancer cells can develop mutations in their DNA, which allows them to become resistant to chemotherapeutic agents.

Breast cancer can be divided into different clinical subtypes. These subtypes are based on tests for three molecular markers: expression of estrogen receptor (ER), expression of progesterone receptor (PR), and amplification of HER-2/Neu. Estrogen receptor positive cells have receptors for estrogen, suggesting that their growth is promoted by signals it receives from estrogen [2]. HER2-positive cell types produce too much of the protein HER2, a mutated EGF receptor, making the cancer aggressive and fast growing [3]. Breast cancer cells that test negative for all three of the molecular markers are classified as triple negative. Triple negative cell types tend to have a less favorable prognosis than the hormone receptor expressing cell types [4]. Hormone receptor positive cell types are present in about two out of every three breast cancer diagnoses [2]. Triple-negative cell types occur at a much lower rate of about 10-15% [4].

In this study, we tested an estrogen-positive breast cancer cell type, MCF7, and a triple-negative breast cancer cell type, Hs578T. These cells were used in the cytotoxicity testing of various essential oils from plant extracts. Cytotoxicity testing is an important step in determining the effects of compounds on cell viability. In standard cytotoxicity testing methods, cell monolayers are grown to near confluence and then exposed to test samples directly by adding the compounds to the culture media. Cells may be observed for visible signs of toxicity, such as a change in size or appearance of cellular components [5]. Assays may be performed to determine the viability of the cells as well. One such assay is called a MTT assay, which is based on the selective ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into purple formazan crystals. The formation of these crystals is reliant on functioning metabolic activity, which makes it a useful method for testing for cytotoxicity [6]. The data collected from the MTT assay can be further analyzed to find the LC_{50} value for the compounds. This test is used to determine the lethal concentration of a compound required to kill fifty percent of the cells in a culture.

II. Materials and Methods

Removing Cells from Cryopreservation

The cryovial containing cells was removed from liquid nitrogen and immediately immersed in a 37°C water bath. The cells were quickly thawed by swirling in the bath. Then, the cells were transferred to a centrifuge tube and suspended in RPMI 1640 growth media supplemented with 10% fetal bovine serum. The cell suspension was then centrifuged for three to

four minutes, and the supernatant was removed using suction, being careful to not disturb the pellet. The cells were gently suspended in the growth medium once more and divided evenly between two T-25 flasks.

Cell Culture

Cells were maintained in a T-25 culture flask with RPMI 1640 growth media with 10% fetal bovine serum at a plating concentration of $2.5x10^5$ cells/mL per flask and 5 mL/ flask. The cells were incubated at 37°C and 5% CO_2 in a humidified chamber. The cells were grown in RPMI 1640 media with L-glutamine, 26 mL of 7.5% sodium bicarbonate per liter medium, 10,000 units of penicillin and 10,000 µg/mL streptomycin per liter of medium, and 15 mL of 1M HEPES per liter medium, buffered with 5.0 N NaOH to pH 7.35. The media was then sterile filtered using a Corning® bottle-top filter into two 500 mL bottles. The final growth media contained 10% fetal bovine serum. The growth media was exchanged every 48 hours, and cells were passaged once a week. Generally, two flasks of cells were maintained at a time.

Passaging Cells

Cells were removed from the flask using 1X Hank's Balanced Salts Solution, without calcium or magnesium, containing 5.0 g porcine trypsin, 2 g EDTA·4 Na per liter in 0.9% NaCl. The growth media was removed with suction, and the cells were washed with 5 mL of 1X Hank's Balanced Salts Solution. The Hank's solution was removed, and 5 mL of the trypsin-EDTA/Hank's solution mixture was added to the cells. After one to two minutes, some of the trypsin-EDTA/Hank's solution was removed so that the remaining liquid just covered the surface of the cells. To assist the cells' separation from the surface, the flasks could be placed in the CO_2 incubator for one to two minutes. After the cells could visibly be seen releasing from the surface, 5 mL of growth media was added to the flask. Any remaining cells on the flask surface were washed off with the growth media, and the contents of the flask were transferred to a centrifuge tube. The cells were then centrifuged for two to three minutes. The supernatant was removed using suction, being careful to not disturb the pellet. Then, the pellet was suspended in growth media. After the pellet was suspended, 0.1 mL of cells was added to a solution of 0.3 mL of 0.4% Trypan Blue Stain and 0.6 mL of 0.15 M NaCl. The cells were then counted using a hemocytometer. The appropriate number of cells were then distributed to the desired culture vessels.

MTT Assay

Cells were plated at a concentration of $1.44x10^6$ cells per well in a 96-well plate in a volume of 0.1 mL. Test samples were diluted in growth media to a concentration of 0.01% (w/v). Tingenone was used as a positive control, and it was prepared like the other test samples. The growth media and 0.01% (w/v) DMSO were used as negative controls, as the test samples were dissolved in DMSO. The media was removed from the cells using suction, and 100 µL of the test sample dilutions were added to the designated wells. The cells were incubated in these test samples at 37°C and 5% CO_2 for 48 hours. On the day of the assay, a 1:10 dilution of 5mg/mL of stock MTT in growth medium was made to give a final concentration of 0.5 mg/mL. The media on the cells was then removed using suction, and 100 µL of the MTT solution was added to each well. A 96 well plate SpectraMax spectrophotometer was used to immediately read the absorbance at 570 nm. The plate was then placed back in the incubator for three to four hours at 37°C and 5% CO_2. After the incubation period, the media was carefully aspirated from the wells, being careful not to disturb the purple formazan crystals. Then, 100 µL of ISO PBS, containing 100 mL isopropyl alcohol, 4.0 µL 5.0 N HCl, and 50.0 mL phosphate-buffered saline, was added to the wells, and the plate was gently shaken to dissolve the crystals. The post read was then completed on the spectrophotometer at 570 nm. This data was compiled in Microsoft Excel to calculate the percent of cells killed, the percent of viable cells, and the standard deviation for each.

LC_{50} Analysis

Compounds with a high percent of killed cells and a low standard deviation were chosen for the LC_{50} analysis. Concentrations of 0.01% (w/v), 0.005% (w/v), 0.004% (w/v), and 0.003% (w/v) were tested for each of the chosen samples. The same procedure for the MTT assay was followed.

III. Results

Table 1: MCF7 MTT Assay Results

Extract/ Sample	% Viable	% Kill	Std. Dev. of % Kill
ABBAEO	13.10	86.90	7.95
BOCAEO	-0.99	100.99	1.56
CAODEO	0.65	99.35	5.29
CILAEO	-1.45	101.45	1.54
COOFEO	4.78	95.22	2.75
CUSEEO	10.86	89.14	12.82
LAANEO	39.79	60.21	17.53
MYCOEO	66.08	33.92	13.48
SAAUEB	2.07	97.93	0.45
Teca 2C	2.13	97.87	1.24
Teca 3B	5.34	94.66	1.82

Table 1 shows the resulting data from MTT assays completed with MCF 7 cells with test samples at 0.01% (w/v). The percent of viable cells, percent of killed cells, and the standard deviation of the percent of killed cells are shown. Samples ending in "EO" are essential oils. Data from this assay was used to determine which Samples would be used for further analysis.

Table 2: LC_{50} Analysis of MCF7 Cells

Extract/ Sample	Conc. of Sample (w/v)	% Viable	% Kill	Std. Dev. of % Kill
Teca 2C	0.01%	-4.16	104.16	3.76
Teca 2C	0.005%	-1.13	101.13	2.76
Teca 2C	0.004%	19.44	80.56	6.83
Teca 2C	0.003%	58.42	41.58	8.30
Teca 3B	0.01%	-2.85	102.85	3.23
Teca 3B	0.005%	36.67	63.33	3.50
Teca 3B	0.004%	83.21	16.79	5.21
Teca 3B	0.003%	50.76	49.24	10.96

Table 2 shows the results from the LC_{50} analysis with MCF7 and Teca 2C and Teca 3B test samples. The concentration of the sample, percent viable cells, percent killed cells, and the standard deviation of the percent of killed cells are shown.

Table 3: Hs578T MTT Assay Results

Extract/ Sample	% Viable	% Kill	Std. Dev. of % Kill
ABBAEO	8.38	91.62	4.84
BOCAEO	6.21	93.79	6.95
CAODEO	4.17	95.83	2.52
CILAEO	-5.03	105.03	12.34
COOFEO	6.14	93.86	9.81
CUSEEO	2.98	97.04	1.74
LAANEO	5.45	94.55	1.49
MYCOEO	5.54	94.46	3.55
SAAUEB	4.30	95.70	1.19
IT 1	83.10	16.90	41.64
IT 2	1.03	98.97	1.73
IT 3	1.58	98.42	0.55
IT 4	1.30	98.70	2.68
IT 5	45.83	54.17	21.29
IT 6	21.81	78.19	8.81
IT 7	1.50	98.50	0.68
IT 8	15.94	84.06	6.25
IT 9	7.06	92.94	2.48
IT 10	19.63	80.37	3.65

Table 3 shows the results of the MTT assay on HS 578 T cells with various test samples at 0.01% (w/v). The resulting percent of viable cells, percent of killed cells, and the standard deviation of the percent of killed cells are shown.

Table 4: LC_{50} Analysis of Hs578 T Cells

Extract/ Sample	Conc. of Sample (w/v)	% Viable	% Kill	Std. Dev of % Kill
CAODEO	0.01%	3.68	96.32	2.11
CAODEO	0.005%	60.64	39.36	26.72
CAODEO	0.004%	58.99	41.01	22.83
CAODEO	0.003%	86.08	13.92	38.58
CILAEO	0.01%	7.16	92.84	6.37
CILAEO	0.005%	54.10	45.90	10.45
CILAEO	0.004%	58.06	41.94	3.78
CILAEO	0.003%	56.22	43.78	11.37
IT 2	0.001%	4.64	95.36	5.31
IT 2	0.005%	28.86	71.14	13.20
IT 2	0.004%	35.44	64.56	4.16
IT 2	0.003%	54.22	45.78	18.69
IT 3	0.01%	-1.06	101.06	0.48
IT 3	0.005%	-0.74	100.74	0.81
IT 3	0.004%	-3.44	103.44	6.59
IT 3	0.003%	6.47	93.53	3.52
IT 4	0.01%	4.43	95.57	3.39
IT 4	0.005%	34.58	65.42	20.76
IT 4	0.004%	30.03	69.97	14.15
IT 4	0.003%	61.99	38.01	20.78

Table 4 shows the LC_{50} analysis of Hs578 T cells with different test samples. The concentration of the test sample, the percent of viable cells, the percent of killed cells, and the standard deviation of the percent of killed cells are shown.

III. Discussion

Many compounds were tested on both the MCF7 and Hs578T cells. Some of the samples had about equal kill percentages, while others had different outcomes in the two cell types. For example, MYCOEO killed 94.46% of the Hs578T cells (Table 3), while it only killed 33.92% of MCF7 cells (Table 1). The sample LAANEO had a similar effect without as drastic of a difference, killing 94.55% of Hs578T cells (Table 3) and 60.21% of MCF7 cells (Table 1).

The MTT assay was performed using test samples at a 0.01% (w/v) concentration. Growth media and 0.01% (w/v) DMSO were used as negative controls, while 0.01% (w/v) Tingenone was used as a positive control for cytotoxicity. The data obtained from the MTT assay was used to decide which samples were used in further analysis. Samples with a percent kill of above 90% with a standard deviation less than 15 were used to complete an LC_{50} analysis. For MCF7 cells, the samples chosen were Teca 2C and Teca 3B. Teca 2C had a percent kill of 97.87% and a standard deviation of 1.24, while Teca 3B had a percent kill of 94.66% and a standard deviation of 1.82 (Table 1). For Hs578T cells, the samples chosen for the LC_{50} were CAODEO, CILAEO, IT2, IT3, and IT 4. The sample CAODEO had a percent kill of 95.83% with a standard deviation of 2.52, CILAEO had a percent kill of 105.03% with a standard deviation of 12.34, IT2 had a percent kill of 98.97% with a standard deviation of 1.73, IT3 had a percent kill of 98.42% with a standard deviation of 0.55, and IT 4 had a percent kill of 98.70% with a standard deviation of 2.68 (Table 3).

The LC_{50} analysis was performed to determine the lowest concentration at which the sample would still kill 50% of the cells tested. For MCF7 cells, Teca 2C could be used at 0.004% (w/v) to kill 80.56% of the cells, but at 0.003% (w/v) it only killed 41.58%. Teca 3B had a percent kill of 63.33% at a concentration of 0.005% (w/v). At 0.004% (w/v) Teca 3B, only 16.79% of cells were killed. The standard deviations for all concentrations tested were relatively low, which indicates that the results are accurate. For Hs578T cells, CAODEO had a percent kill of 96.32% at a concentration of 0.01% (w/v), but at a concentration of 0.005% (w/v) only 39.36% of cells were killed. For CILAEO, 92.84% of cells were killed at the 0.01% (w/v) concentration, but only 45.90% of cells were killed at the 0.005% (w/v) concentration. The compound IT2 had a percent kill of 64.56% at a concentration of 0.004% (w/v), and the percent kill at a concentration of 0.003% (w/v) was 45.78%. The compound IT3 had a percent kill above 90% at the lowest concentration tested of 0.003% (w/v). IT4 had a percent kill of 69.97% at a concentration of 0.004% (w/v), and it had a percent kill of 38.01% at 0.003% (w/v).

Overall, the fraction Teca 2C had the best cytotoxicity results for MCF7 cells, while IT3 had the best results for Hs578T cells. Going forward, the samples of IT1-IT10 should be tested on MCF7 cells, while the fractions Teca 2C and Teca 3B should be tested on Hs578T cells. Additionally, an LC_{50} should be performed with BOCAEO and CILAEO on MCF7 cells. The LC_{50} for the Hs578T cells should be repeated based on the results of the most recent MTT assay. The Hs578T MTT assay was repeated due to high standard deviations, and a new LC_{50} analysis should be performed to coincide with these results. All samples will be further fractionated to isolate new cancer drugs.

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