SHORT COMMUNICATION

MTT ASSAY EVALUATION OF *THEOBROMA CACAO* LEAF EXTRACT (COALEX-1) IMPACT ON BREAST CANCER CELL LINES ACROSS DIFFERENT SPECIES (4T1 AND MCF-7) *IN VITRO*

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ABSTRACT - Breast cancer remains one of the leading causes of cancer-related mortality worldwide, with ongoing research focused on identifying novel therapeutic agents. In this study, the cytotoxic effects of *Theobroma cacao* leaf extract (CoaLeX-1) were evaluated on two distinct breast cancer cell lines: 4T1 (murine-derived) and MCF-7 (human-derived). The cytotoxicity was assessed using the MTT assay to determine cell viability after treatment with various concentrations of CoaLeX-1. A dose-dependent reduction in cell viability was observed in both cell lines, with more pronounced effects in MCF-7 cells. The half-maximal inhibitory concentration (IC_{50}) was calculated to quantify the extract's potency. These findings suggest that *Theobroma cacao* leaf extract may exhibit anticancer properties with potential species-specific efficacy. The potential of CoaLeX-1 as a natural agent for further investigation in breast cancer treatment is highlighted, particularly considering its differential impact on cell lines from different species.

Keywords: *Theobroma cacao*, breast cancer, extract, MCF-7, 4T1

INTRODUCTION

Breast cancer, being the most prevalent form of cancer among women, represents a significant public health challenge on a global scale. This issue affects both developed and developing countries alike, highlighting the widespread impact and importance of addressing breast cancer across diverse populations and healthcare systems (Adham Foumani *et al.*, 2022).

Cancer cell lines, which are long-lived and derived from patient tumor cells, have historically been, and continue to be, extensively utilized to study the biology of breast cancer and to develop new therapies. These cell lines remain one of the primary models for breast cancer research (Pirsko *et al.*, 2018).

Cell viability refers to the number of living cells within a specific population. Assessing the quantity of proliferating cells serves as a crucial indicator of cellular response, providing insights into cell survival or death when exposed to drugs or chemical agents (Adan *et al.*, 2016). Cell cytotoxicity and proliferation assays are fundamentally employed to screen and evaluate how cells respond to a drug or any chemical agent.

The MTT assay, which stands for 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, relies on the conversion of MTT into formazan crystals by living cells. This process is used to assess mitochondrial activity, providing an indicator of cell viability (van Meerloo *et al.*, 2011). Because the total mitochondrial activity in most cell populations correlates with the number of viable cells, this assay is widely employed to measure the cytotoxic effects of drugs on cell lines in vitro.

In this research, we conducted a thorough evaluation of the cytotoxic and growth-inhibitory effects on both human breast cancer cell lines (MCF-7) and mouse breast cancer cell lines (4T1). By assessing these effects in two different species' cell lines, we aimed to gain a comprehensive understanding of the potential impacts of CoaLeX-1 on breast cancer treatment and progression

MATERIALS AND METHODS

Material and Chemical

Roswell Park Memorial Institute (RPMI) 1640, penicillin/streptomycin (100x), fetal bovine serum (FBS) and trypsin-EDTA (1x) and Thiazolyl Blue Tetrazolium Bromide (MTT) were obtained from Nacalai Tesque (Japan). Dimethyl sulfoxide (DMSO), Trypan blue (0.4 %) and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. USA.

Collection of Plant Material

During the peak fruiting season in mid-2019, fresh cocoa leaves were sourced from smallholder cocoa farms in different locations. A voucher specimen (SK 2434/14) was submitted to the Institute of Bioscience, Universiti Putra Malaysia.

Sample Preparation and Extraction Process

A total of fifteen kilograms of cocoa leaves were picked, washed, and air-dried in a shaded area. After drying, the leaves were ground into powder and kept in a cool, dark environment. This powder was then mixed with distilled water and subjected to a 2-hour extraction at 100°C using specialized equipment. The extract was filtered to eliminate impurities, concentrated, and spray-dried to produce cocoa leaf extract (CoaLeX-1), with a yield of 0.85%. The extract was then stored at 4°C for subsequent analysis.

Thawing Cell

The frozen cell stock was quickly thawed in a cryovial by rubbing it with hands until warm. The cells were then transferred to a cell culture flask with culture media. The cells were observed under an inverted microscope and then incubated for 24 hours at 37°C with 5% carbon dioxide.

Subculture Cell

The old media was discarded from the flask, and an appropriate volume of sterile PBS was added to rinse the cells and remove any residual medium and serum that could inhibit trypsin activity. The flask was gently swirled, and the PBS was aspirated. Enough trypsin-EDTA solution was added to cover the cell monolayer. Swirled gently to ensure even coverage. The flask was incubated at 37°C for 7 minutes, and the cells were observed under a microscope until they started to round up and detach. The solution was transferred, and the cell suspension was centrifuged at 12000 rpm for 5 minutes to pellet the cells.

Cell Counting

Culture media was added, and the cell pellet was resuspended. Mixed the cells and trypan blue in a 1:1 ratio on parafilm, then inserted the stained cells into a hemocytometer. The cells were counted using a hemocytometer or an automated cell counter to ensure the appropriate seeding density.

Cell Seeding

Prepared the stock culture for seeding. About 100 microliters were taken, and the cells were seeded in 96-well plates at the desired density, ensuring even distribution. Incubated for 24 hours at 37°C with 5% carbon dioxide.

Treatment

The stock extract was prepared with different concentrations. The media was removed from the 96-well plate. Treated the cells with 100 microliters of cocoa leaf extract in each well for each sample. Incubated the cells for the desired treatment period of 48 hours.

MTT Assay

At the end of the treatment period, 10 µL of MTT solution (final concentration typically 5 mg/mL) was added to each well. The plate was incubated at 37°C for 4 hours to allow the MTT to be metabolized by viable cells into formazan crystals. The culture medium was carefully removed without disturbing the formazan crystals. Then, 100 µL of DMSO was added to each well to dissolve the formazan crystals.

Data Analysis

The absorbance of each well was measured using a microplate reader at a wavelength of 570 nm (or 550 nm to 600 nm), with a reference wavelength of 630 nm to 690 nm to account for background. Calculated the percentage of cell viability relative to the untreated control cells.

RESULTS

From the results obtained, as shown in Table 1 and Figure 1, CoaLeX-1 enhanced the proliferation of the mouse breast cancer cell line used in this study (4T1). On the other hand, CoaLeX-1 reduced the proliferation of the human breast cancer cell line used in this study (MCF-7). However, even at the highest concentration of CoaLeX-1 (1000 µg/ml), it could not inhibit 50% of the MCF-7 cancer cell population (IC_{50}).

Table 1**.** Cell viability percentage of 4T1 (mouse breast cancer cell line) and MCF-7 (human breast cancer cell line) after 48 hours incubation with CoaLeX-1.

CoaLeX-1 concentration (µg/ml)	Cell viability (4T1) (%)	Cell viability (MCF-7) (%)
0.0	100.00	100.00
0.001	116.52	99.47
0.01	112.82	95.95
0.1	95.33	87.56
1	137.98	83.47
10	151.76	70.82
100	144.52	76.87
1000	134.76	56.60

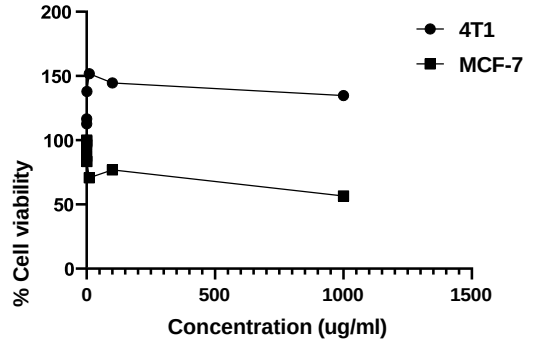

*Figure 1***.** Graph of cell viability percentage of 4T1 (mouse breast cancer cell line) and MCF-7 (human breast cancer cell line) after 48 hours incubation with CoaLeX-1 to show the possible IC_{50}.

DISCUSSIONS

The 4T1 cell line, derived from a mouse mammary tumor, serves as an invaluable model for studying the aggressive behavior of breast cancer due to its high proliferative and metastatic capabilities (Atiya *et al.,* 2019). These cells exemplify the hallmarks of cancer, including rapid and unchecked proliferation driven by multiple genetic and molecular alterations. Furthermore, 4T1 cells exploit various signal transduction pathways, particularly the PI3K/AKT/mTOR pathway, which supports their growth, survival, and metabolic needs. Their ability to evade apoptosis is facilitated by the overexpression of anti-apoptotic proteins and the suppression of pro-apoptotic factors, enabling sustained cell survival despite genomic insults that would typically trigger cell death (Fruman *et al.,* 2017).

The metabolic adaptations of 4T1 cells, such as the reliance on aerobic glycolysis (Warburg effect), provide the necessary energy and biosynthetic precursors to support their rapid growth (Heiden *et al.,* 2009). Additionally, the tumor microenvironment plays a crucial role in promoting 4T1 cell proliferation through interactions with stromal cells. The high degree of genomic instability in 4T1 cells further fuels their aggressive behavior, leading to the accumulation of mutations that confer additional growth advantages.

MCF-7 cells, a widely studied human breast cancer cell line, are pivotal in understanding the inhibitory effects on cancer cell growth under various conditions. Research on the potential inhibitory effects of cocoa leaf extract on MCF-7 cells, a widely studied human breast cancer cell line, highlights promising avenues in cancer therapy. Cocoa leaves contain various bioactive compounds such as polyphenols, flavonoids, and theobromine, which have been increasingly recognized for their health benefits, including potential anti-cancer properties (Cooper *et al.,* 2008). Studies have shown that these compounds can interfere with cellular processes crucial for cancer cell proliferation and survival.

Polyphenols and flavonoids, abundant in cocoa leaves, exhibit antioxidant properties that mitigate oxidative stress, a factor implicated in cancer development and progression. These compounds also modulate signaling pathways involved in cell growth and survival. For instance, they may inhibit the PI3K/AKT/mTOR pathway, which is often dysregulated in cancer cells like MCF-7, thereby reducing cellular proliferation rates (Davison *et al.,* 2008). Moreover, flavonoids in cocoa leaves can induce apoptosis (programmed cell death) in cancer cells by activating intrinsic pathways and enhancing the expression of pro-apoptotic proteins while inhibiting anti-apoptotic factors.

Furthermore, cocoa leaf extract has been studied for its ability to modulate estrogen signaling, crucial in ER+ breast cancers like MCF-7. By competitively binding to estrogen receptors or interfering with estrogen synthesis, cocoa leaf extract can reduce the proliferative effects of estrogen on MCF-7 cells, thereby inhibiting their growth.

CONCLUSIONS

In conclusion, while both 4T1 and MCF-7 cells are important models for breast cancer research, they represent different aspects of the disease spectrum.

4T1 cells are used to study aggressive, metastatic breast cancer behaviors and potential therapies to inhibit these processes. In contrast, MCF-7 cells are utilized to explore hormone-responsive breast cancer mechanisms and therapeutic strategies aimed at targeting estrogen signaling pathways. Understanding these cell lines' behaviors and responses is crucial for advancing breast cancer research and developing effective treatment approaches tailored to different subtypes and stages of the disease.

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REFERENCES

Adan, A., Kiraz, Y. & Baran, Y. (2016). The international international journal journal for timely for timely in-depth reviews in in-depth reviews in Pharmaceutical Pharmaceutical Biotechnology Impact Factor: 1.802 BENTHAM. *Current Biotechnology Pharmaceutical* **17(14)**: 1873–4316.

Adham Foumani, E., Irani, S., Shokoohinia, Y. & Mostafaie, A. (2022). Colchicine of Colchicum autumnale, A Traditional Anti-Inflammatory Medicine, Induces Apoptosis by Activation of Apoptotic Genes and Proteins Expression in Human Breast (MCF-7) and Mouse Breast (4T1) Cell Lines. *Cell Journal* **24(11)**: 647– 656.

Ahmadian, S., Barar, J., Saei, A.A., Fakhree, M.A.A. & Omidi, Y. (2009). Cellular toxicity of nanogenomedicine in MCF-7 cell line: MTT assay. *Journal of Visualized Experiments* (26).

Atiya, H.I., Dvorkin-Gheva, A., Hassell, J., Patel, S., Parker, R.L., Hartstone-Rose, A., Hodge, J., Fan, D. & Ramsdell, A.F. (2019). Intraductal adaptation of the 4T1 mouse model of breast cancer reveals effects of the epithelial microenvironment on tumor progression and metastasis. *Anticancer Research* **39(5)**: 2277–2287.

Baharum, Z., Akim, A.M., Taufiq-Yap, Y.H., Hamid, R.A. & Kasran, R. (2014). In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of *Theobroma cacao*. *Molecules* **19(11)**: 18317–18331.

Bahuguna, A., Khan, I., Bajpai, V.K. & Kang, S.C. (2017). MTT assay to evaluate the cytotoxic potential of a drug. *Bangladesh Journal of Pharmacology* **12(2)**: 115–118.

Cooper, K.A., Donovan, J.L., Waterhouse, A.I. & Williamson, G. (2008). Cocoa and health: A decade of research.

Davison, K., Coates, A.M., Buckley, J.D. & Howe, P.R.C. (2008). Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects. *International Journal of Obesity* **32(8)**: 1289–1296.

Ferhi, S., Santaniello, S., Zerizer, S., Cruciani, S., Fadda, A., Sanna, D., Dore, A., Maioli, M. & D'hallewin, G. (2019). Total phenols from grape leaves counteract cell proliferation and modulate apoptosis-related gene expression in MCF-7 and HepG2 human cancer cell lines. *Molecules* **24(3)**.

Fruman, D.A., Chiu, H., Hopkins, B.D., Bagrodia, S., Cantley, L.C. & Abraham, R.T. (2017). The PI3K Pathway in Human Disease. Cell Press.

Heiden, M.G.V., Cantley, L.C. & Thompson, C.B. (2009). Understanding the warburg effect: The metabolic requirements of cell proliferation.

Luis, C., Castaño-Guerrero, Y., Soares, R., Sales, G. & Fernandes, R. (2019). Avoiding the interference of doxorubicin with mtt measurements on the mcf-7 breast cancer cell line. *Methods and Protocols* **2(2)**: 1–5.

van Meerloo, J., Kaspers, G.J.L. & Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. *Cancer Cell Culture, Method and Protocols, Second Edition*, hlm. 237–245.

Pirsko, V., Cakstina, I., Priedite, M., Dortane, R., Feldmane, L., Nakazawa-Miklasevica, M., Daneberga, Z., Gardovskis, J. & Miklasevics, E. (2018). An effect of culture media on epithelial differentiation markers in breast cancer cell lines MCF7, MDA-MB-436 and SkBr3. *Medicina (Lithuania)* **54(2).**

Yahya, M., Ginting, B. & Saidi, N. (2022). Anticancer and antiretroviral activities of methanolic extract from *Theobroma cacao* L pod husk: focusing on the ethyl acetate partition. *F1000Research* **11**: 1395.