Ind. J. Pharm. Edu. Res., 2024; 58(2s):s616-s622.
https://www.ijper.org

Original Article

Apoptotic Activity of Protopine against Human Breast Cancer Cell Lines: An *in vitro* Study

Shoujun Li[1], Gothai Sivapragasam[2], Palanisamy Arulselvan[3], Aimi Syamima Abdul Manap[4,*]

[1]Department of General Surgery, Songjiang Hospital Affiliated To Shanghai Jiaotong University School of Medicine, Zhongshan, Songjiang, Shanghai, CHINA.
[2]Department of Biological Sciences, School of Medical and Life Sciences, Sunway University, Petaling Jaya, MALAYSIA.
[3]Department of Chemistry, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu, INDIA.
[4]Department of Biomedical Science, College of Veterinary Medicine, King Faisal University, Al-Ahsa, SAUDI ARABIA.

ABSTRACT

Background: Breast Cancer continues to rank among all cancer types as the most dangerous malignancies, accounting for a significant portion of cancer-associated mortalities among women globally. Additionally, the number of breast cancer cases that are identified each year is rising globally. After surgery or radiation, the typical therapies entail chemotherapy which is followed by endocrine therapy. However, breast cancer is extremely resistant to therapies, which causes it to recur. As a result, emphasis is being placed on the development of complementary medicines with fewer adverse effects that are obtained from plants. An isoquinoline alkaloid, Protopine, possesses an array of biological properties, including anti-tumor efficacy against several malignancies. **Materials and Methods:** In this work, the anti-carcinogenic property of Protopine against the MDA-MB-231 cells has been investigated. The impact of Protopine on cell growth, apoptosis, ROS accumulation, and apoptotic marker levels was investigated. Doxorubicin was employed as the positive control drug in the studies. **Results:** The effect of Protopine on cell growth demonstrated its cytotoxic property was elevated dose-dependently and IC_{50} concentration was selected for additional work. The AO/EB and DAPI staining was performed to examine morphological alterations concerning apoptotic cell death. Additionally, Protopine augmented the ROS accumulation and induced DNA damage in Protopine-treated cells. The caspase levels were also increased upon Protopine treatment. **Discussion and Conclusion:** The outcomes highlight that Protopine remarkably inhibited cell growth by augmenting the ROS levels, inducing DNA fragmentation and apoptosis, leading to cell death by caspase-dependent pathway. Thus, Protopine could be possibly employed as an effective anti-cancer alternative for breast cancer treatment.

Keywords: Cytotoxicity, Anti-tumor, MDA-MB-231 cells, Breast Cancer, Apopotic Marker.

Correspondence:
Dr. Aimi Syamima Abdul Manap
Department of Biomedical Science, College of Veterinary Medicine, King Faisal University, Al-Ahsa, SAUDI ARABIA.
Email: amanap@kfu.edu.sa
ORCID ID: 0000-0003-2319-3047

Received: 31-10-2023;
Revised: 25-12-2023;
Accepted: 06-02-2024.

INTRODUCTION

Breast cancer is a prevalent and fatal tumor type that impacts women globally.[1] It is the second leading reason for cancer-related mortality and the most prevalent malignancy in women.[2] It is a lobule cancer of the breast tissues, milk ducts, and their inner lining. Based on the expression of the Estrogen Receptor (ER), the Progesterone Receptor (PR), and the Human Epidermal growth factor Receptor 2 (HER2), immunohistochemistry may classify invasive breast cancer into four main molecular subgroups.[3] Luminal A BC, which is characterized by ER+ and/or PR+ as well as HER2-, accounts for approximately 60% of breast cancer and is linked to a favorable prognosis.[4] Thirty percent of breast cancers are Luminal B (ER+ and/or PR+, and HER2+) and have a poor prognosis, elevated ki67 (>14%), a proliferation marker.[5] Ten percent of breast cancer have HER2 (ER-, PR-, and HER2+) and have a bad prognosis.[6] Finally, among the molecular subtypes of breast cancer, 15-20% are triple-negative (ER-, PR-, and HER2-). This subtype is more aggressive and has a worse prognosis than the others, and it typically affects younger women.[7]

According to estimates, wealthy countries identify breast cancers at a rate of 80%, compared to 40% in underdeveloped countries.[8,9]

Breast Cancer Care released statistics showing that 60,000 individuals are detected with breast cancer annually and that 1 in 8 people will suffer from it at some stage in their lifetime.[10] There are numerous alternatives for treating breast cancer patients as a consequence of ongoing advancements in surgical procedures. A significant variety of naturally derived products have been

DOI: 10.5530/ijper.58.2s.65

Copyright Information :
Copyright Author (s) 2024 Distributed under Creative Commons CC-BY 4.0

Publishing Partner : EManuscript Tech. [www.emanuscript.in]

associated with the prevention of breast cancer in the last few decades. Patients can experience the highest quality of life and the fewest side effects with efficient breast cancer treatment.[11]

Different types of variables (endogenous and exogenous) that have various effects induce breast cancer.[12] The capacity to control distant metastases may boost long-term survival, according to a substantial amount of research.[13] Treatment for breast cancer relies on one or more mechanisms that can be affected by substances that are currently in use as anticancer medicines, whether they are natural or synthetic. Breast cancer management comprises a variety of therapeutic modalities, including hormone therapy, surgery, radiation, and chemotherapy. Multidrug resistance continues to be a difficult barrier in the treatment of breast cancer, which ultimately causes mortality. As a result, it is important to fully comprehend and analyze the molecular causes of cancer resistance and develop new medications that are specifically tailored to accomplish it.[14]

In the northeastern region of Asia, some plants including those in the Papaveraceae and Fumariaceae families contain protopine, an isoquinoline alkaloid.[15] Protopine also known as corydinine or fumarine, has a role as a plant metabolite. Protopine shares several biological properties with other natural alkaloids, including anti-arrhythmic, anti-hypertensive, negative inotropic, vasodilator, anti-oxidative and neuroprotective, hepatoprotective, anti-microbial, anti-viral, cytotoxic and anti-proliferative.[16] Moreover, it has been proven to possess antitumor effects against a variety of malignancies, including pancreatic, prostate, and colon cancer.[17-19] Previous investigation has reported that the compound demonstrated anti-invasive and anti-adhesion impacts on MDA MB-231 cells and decreased the integrins expression as well as intercellular adhesion molecule-1.[20]

Phytochemicals have long been intriguing prospects for the formulation of new anticancer candidates. Recently, a study on Protopine exhibited anti-adhesive and anti-invasion effects in MDA-MB-231 cells where the expression of EGFR, ICAM-1, αv-integrin, β1-integrin and β5-integrin were remarkably reduced.[21] The study shed light on the inhibition of heterotypic cell adhesion, however, the mechanisms of Protopine against cancer metastasis remain an aspect to be elucidated.[21] Hence, the major purpose of the current investigation is to assess the anticancer potential of an alkaloid, Protopine in MDA-MB-231 cells. Further, its apoptotic process and ROS production ability were analyzed to evaluate its anticancer mechanism. MDA-MB-231 cell lines were chosen in this investigation as they represent triple-negative breast cancer (an aggressive subtype), providing valuable insights into potential therapeutic strategies.[22]

MATERIALS AND METHODS

Materials

Protopine and other chemicals were procured from Sigma Aldrich. The culture consumables as well as media were obtained from Sigma Aldrich. Abcam provided the ELISA kits.

Methods

Sample Preparation

For cytotoxic studies, 1% DMSO solution was added to the known amount of Protopine to make 1 mg/mL and stored at 4ºC until used.

In vitro Antitumor Activity of Protopine

MDA-MB-231 Cell Maintenance

MDA-MB-231 cells were maintained in DMEM medium with additives such as $Na_2 CO_3$ (1.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), amino acids (0.1 mM), HEPES buffer (10 mM), glucose (1.5 g/L), fetal bovine serum (10%) and 100 units/mL penicillin, and 100 μg/mL streptomycin (1%).

Cytotoxicity Estimation by WST-8 Assay

Cells (3×10^3 cells/mL) were seeded into each well of 96-well plates to examine the cytotoxicity of Protopine. The test compound was introduced at diverse concentrations (4, 8, 16, 32, 64, and 128 μg/mL). The mitochondrial activity in converting 2-(2-methoxy-4-nitrophenyl)-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan was evaluated to determine the cell viability 24 hr after incubation.[23] The concentration which resulted in a 50% (IC_{50}) growth decline was recorded.

Examination of Apoptosis by Acridine Orange/ Ethidium Bromide (AO/EB) Staining

To evaluate the apoptotic efficacy of Protopine against MDA-MB-231 cells, the dual staining method was employed following the previously published protocol.[24] The AO/EB staining approach is used to visualize nuclear changes and differentiate between viable, apoptotic (early or late stages), and necrotic cells.[24] The cells were added to a 6-well plate and subjected to Protopine treatment at IC_{50} concentrations for 24 hr. Immediately after the treatment, the cells were incubated for 5 min in a solution containing the dyes AO and EtBr. The cells were then rinsed with PBS. The cells were studied using a fluorescent microscope.

Investigation of Apoptosis by DAPI (4',6-diamidino-2-phenylindole) Staining

Using fluorescence microscopy, cell morphology was evaluated after DAPI labeling.[25] The cancer cells were exposed to the

compound Protopine (IC_{50}) and incubated for 24 hr. The cells were then rinsed with PBS, fixed with ice-cold ethanol resuspended in DAPI, and left for 15 min while being covered in aluminum foil. Then cells were washed with PBS, the cells were afterward studied using a fluorescent microscope.

Analysis of Reactive Oxygen Species (ROS) by Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) Staining

The analysis of intracellular Reactive Oxygen Species (ROS) was conducted using 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA).[26] Dichlorofluorescein is formed when an intracellular esterase hydrolyzes DCFH-DA, which permeates the cell membrane. DCF is quickly converted to dichlorofluorescein by peroxidase in the presence of intracellular ROS. MDA-MB-231 cells were inoculated and subjected to Protopine treatment (IC_{50}). The cells were collected after 24 hr, and three PBS washes were carried out. Cells were extracted (1-20×10^6/mL), combined with DCFH-DA (10 mmol/L), and then incubated for 20 min at 37ºC. The cells were washed thrice with medium to entirely neutralize DCFH-DA. After that, fluorescent microscopy was employed to quantify the levels of cellular ROS.

Examination of DNA Damage by COMET Assay

The comet assay was employed to quantify DNA damage, following the established technique outlined by Lu and Yang (2017).[27] Approximately, 10,000 cells from control, doxorubicin-treated, and protopine-treated cells (IC_{50} concentration) were separated into aliquots and put in tubes at first. After that, the tubes were centrifuged for 10 min at 800-g centrifugation at 4ºC. After discarding the supernatant, the cells were resuspended in agarose (0.7%), and 35 µL of this suspension was used to coat high-throughput comet assay slides. The silicon barrier between these slides allows for the concurrent layering of 10 various samples on each slide. The slides were then immersed in an ice-cold lysis buffer for an overnight period at 4ºC in the dark to lyse the adhering cells and permits DNA to unfold. After being immersed in the lysis buffer, the slides were located in a buffer for 30 min for DNA to unwind. After that, electrophoresis was conducted for 20 min at 1 V/cm. Then slides were stained with EtBr (15 µL), neutralized in a Tris solution, and dehydrated in 75% methanol before being seen under a fluorescence microscope.

Statistical Analysis

The values of each experiment were examined statistically using one-way ANOVA and a *post hoc* Tukey post-test after being conducted three times. The results are revealed as mean±SD, and the statistical criterion for significance was fixed at $p<0.05$.

RESULTS

Anticancer Activity of Protopine by WST-8 Cytotoxicity Assay

The cytotoxic ability of Protopine toward MDA-MB-231 cells was assessed via WST-8 assay, and the outcomes are given in Figure 1. Our findings indicated that Protopine treatment remarkably declined the proliferation of breast cancer cells dose-dependently. At the high dosage of Protopine, the cell growth was diminished to 25%. This outcome displays Protopine's substantial cytotoxicity towards MDA-MB-231 cells. The IC_{50} value for the cells was determined to be 32 µg/mL; therefore this concentration of Protopine was applied for further experiments.

Assessment of Apoptotic Induction by AO/EtBr Staining

In apoptosis, ROS buildup within the cells is responsible for cell membrane alterations. Dual AO/EtBr labeling was employed in the current study to assess membrane deformation related to apoptosis in MDA-MB-231 cells treated with Protopine as indicated in Figure 2. DNA of cells with intact cell membranes can be stained with the AO dye, rather than EB dye, which can only stain cells without complete cell membranes. Even though EtBr-positive cells were present in the treated groups, showing late apoptosis or dead cells following Protopine exposure (IC_{50}), AO-positive, yellowish, and greenish fluoresced cells were observed after Protopine treatment (IC_{50}).

Assessment of Nuclear Damage by DAPI Staining

Nuclear fragmentation as determined by DAPI staining. One of the distinctive characteristics of the apoptotic mechanism of cell death is nuclear fragmentation. Employing the DAPI dye, cell morphological alterations, and apoptosis-related cell death were investigated. Protopine's ability to cause apoptosis was revealed by the fragmented apoptotic bodies as well as smaller nuclei in

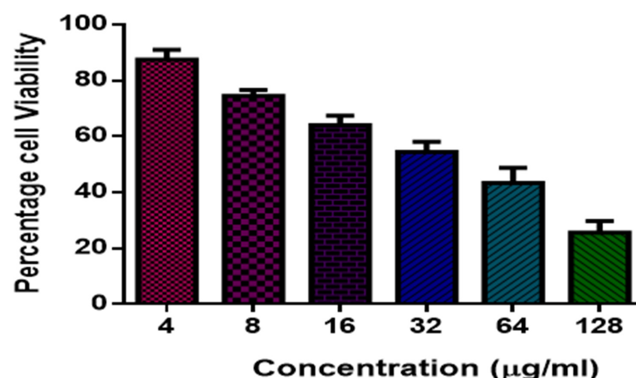

Figure 1: The experiment displays the outcomes of a cell cytotoxicity assay utilizing WST-8 and Protopine in MDA-MB-231 cells in 96-well plates at various doses. Experiments were performed in triplicate to calculate the IC50 value, and representative data for the dose that results in a 50% inhibition of cell growth are presented.

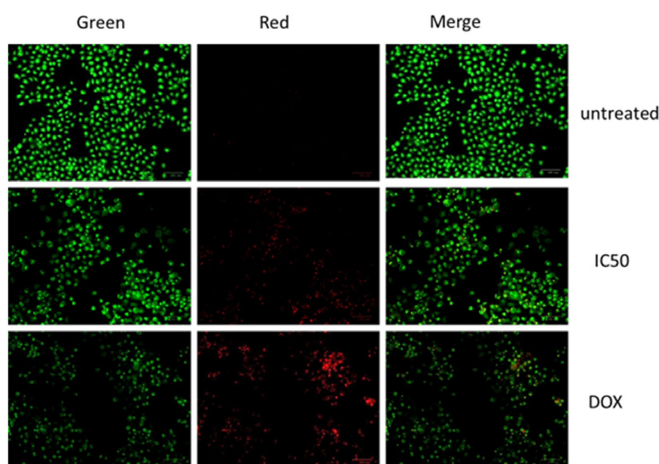

Figure 2: Effect of Protopine induces apoptotic induction in MDA-MB-231 cells. The cells were subjected to Protopine (IC50) and Doxorubicin (10 μM) treatment and untreated cells as controlled for 24 hr. The cells were then stained with dual-dye AO/EB and investigated using the fluorescent microscope. Red-stained cells represent the early stages of apoptosis, orange fluorescence reveals apoptotic cells and green fluorescence shows unaffected MDA-MB-231 cells.

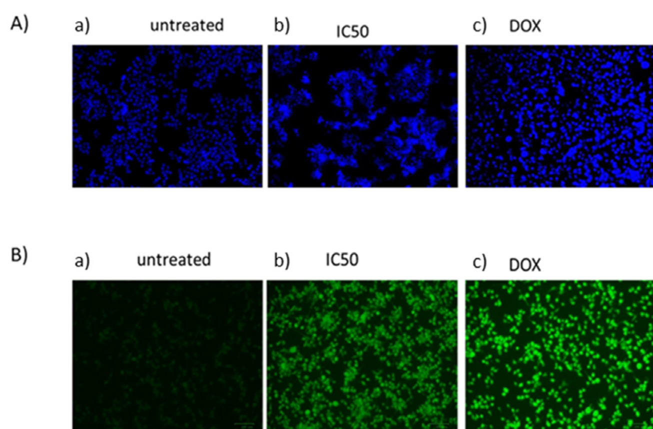

Figure 3: A) DAPI staining was employed to analyze apoptosis in MDA-MB-231 cells after 24 hr of treatment with Protopine (IC_{50}). Control cells (a), cells treated with IC_{50} of Protopine (b), and DOX (c). B) A fluorescence microscopic picture of intracellular ROS generation triggered by Protopine cells stained with DCF-DA dye. Control cells (a) cells treated with Protopine (IC_{50}), (b) and DOX (c). The representative microphotographs were captured during the study in triplicate and magnified 20X.

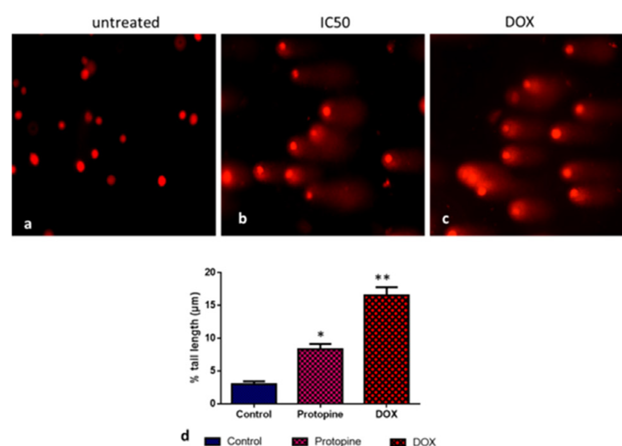

Figure 4: Effect of Protopine on DNA damage of MDA-MB-231 cells was analyzed by comet assay. The cells were subjected to Protopine (IC_{50}) and Doxorubicin (10 μM) treatment as a positive control for 24 hr. Results were computed using the mean±standard deviation of six distinct investigations. Treated groups vs. control group, *$p<0.05$ and **$p<0.001$.

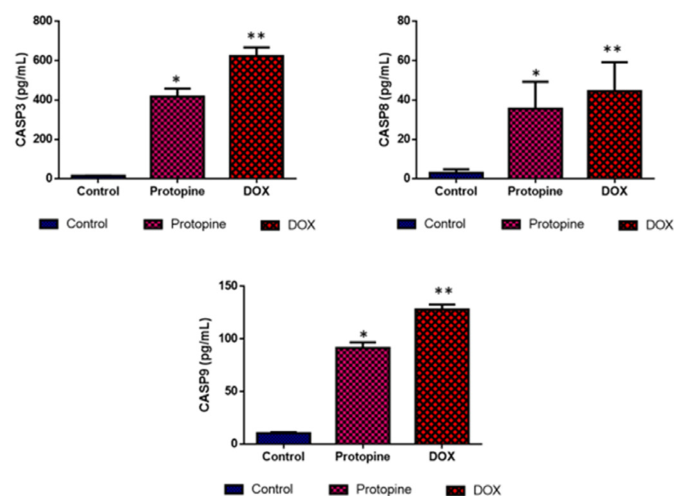

Figure 5: Protopine inhibits MDA-MB-231 cell growth and induces apoptosis. Levels of caspase-3, 8, and 9 in cells were measured via ELISA. Results were computed using the mean±SD of 3 distinct investigations. One-way ANOVA was employed to examine the data. Treated groups vs. control group, *$p<0.05$ and **$p<0.001$.

the Protopine-exposure cells as opposed to the healthy nucleus in the untreated cells (Figure 3A). The results therefore showed that Protopine increased the DNA damage in the cells and triggered cell death by apoptosis when compared with control.

Analysis of ROS Levels by DCFH-DA Staining

The ROS can damage cells in a variety of ways and affect both the apoptotic process and cell development. We thus investigated the ROS-generating capacity of Protopine by employing DCFH-DA labeling to ascertain the reason for the cytotoxicity noticed in MDA-MB-231 cells. The exposure to IC_{50} concentration of Protopine and Doxorubicin resulted in increased green fluorescence, which shows that the treated cells generated higher levels of ROS (Figure 3B). Therefore, it was demonstrated that Protopine generated ROS in breast cancer cells, suggesting that Protopine triggered oxidative stress-mediated cell death in these cells.

Assessment of DNA Damage Via Comet Assay

Figure 4 depicts the effect of Protopine and Doxorubicin treatment on the level of DNA injury by comet assay in MDA-MB-231 cells. The compound induced DNA alterations and significantly lengthened comet tails. The figure demonstrates that comet production increased in the treated cells while it was absent in

the untreated cells. According to these results, Protopine and Doxorubicin treatment appeared to have damaged the DNA of breast cancer cells.

Estimation Of Caspase-3, -8, And -9 Levels in Cells

To investigate whether promotion or inhibition of apoptotic protein expressions including caspase-3, 8, and 9 are involved in Protopine and Doxorubicin-induced apoptosis on MDA-MB-231 cells, the determination of these proteins was executed using the ELISA kit methods. The outcomes of the study into the influence of compound exposure on caspase 3, -8, and -9 levels are shown in Figure 5. Caspase levels were considerably elevated in treated cells after receiving an IC_{50} concentration of Protopine.

DISCUSSION

The most frequently occurring type of malignancy among women is breast cancer. Because researchers worldwide have been working on novel treatments, the most recent statistics on cancer reveal that women worldwide suffer from breast cancer at the highest incidences of morbidity and mortality.[28] Tumor formation is initiated by an imbalance between cellular proliferation and apoptosis. Extensive research has been conducted on inhibiting tumor cell growth and inducing cell death through the activation of the apoptotic pathway. This is because the apoptosis process is responsible for programmed cell death and is considered a promising approach for cancer treatment.[29,30] The stimulation of the apoptotic mechanism is employed in a range of cancer treatment options, according to several research. Several naturally occurring, plant-derived substances with exceptional apoptosis-based cancer prevention efficacy are among them.[31-33] This indicates that incorporating herbal substances to influence the apoptosis pathway can be an efficient strategy to develop anticancer treatments.[34] Consequently, the current study's goal is to provide a better understanding of cell death and the molecular mechanism by which Protopine initiates apoptosis in MDA-MB-231 cells.

The tetrazolium salt WST-8 has been commonly employed to assess cell viability. It is a water-soluble tetrazolium salt that, when analyzed, converts into a formazan product. As a result, it has been utilized to evaluate cell viability with the least possible margins for error.[35] It was established that Protopine exhibited a concentration-dependent tendency to reduce the growth of MDA-MB-231 cells. A similar dose-based growth reduction was reported when Protopine was tested against human osteosarcoma cells, cervical cancer cells, prostate cells, and breast tumor cells.[18,19,36,37]

The development of the immune system, cell homeostasis, and drug-stimulated cell death are all considered to be significantly influenced by apoptosis.[38] As an outcome of studies demonstrating that some chemo-preventive therapies function to prevent cancer by modulating the apoptotic process there is a chance for innovative medications that may be beneficial in managing cancer.[39] A better understanding of the Protopine-induced cell death process was established by employing the AO/EB staining method to examine morphological alterations in connection to apoptosis.[24] The viable cells fluoresced green owing to the ability of AO to penetrate the cell membranes, however, the apoptotic cells fluoresced orange because of shrinkage of the nucleus, demonstrating the initiation of apoptosis. A similar observation of cell shrinkage, nuclear fragmentation as well and condensation along with the appearance of apoptotic entities was found when protopine-containing plant extract was treated with lung tumor cells.[40] One of the most apparent indicators of the apoptotic cell death process is the fragmentation of the nucleus. The DAPI agent is usually utilized to assess apoptosis-linked cellular death and morphological alterations in cells.[41] The fragmentation of DNA and abnormal cell size, causing cells to constrict and shrink, are two traits that indicate the start of apoptosis in cancerous cells.[42] When compared to untreated cancer cells, cells with the IC_{50} concentration of protopine showed abnormal morphology, exhibiting shrinkage and an unclear nucleus shape. This is a result of DNA fragmentation of tumor cells, which leads to apoptosis. In previous studies, Protopine has been documented to induce similar chromatin condensation in prostate cancer cells.[18] Oxidative stress produced by excessive ROS production harms cell structures as well as may trigger cell death processes.[43-45] The amount of ROS produced within the cells was determined by utilizing the ROS-sensitive DCFDA probe to ascertain if ROS participated in the Protopine-induced cell death. Increased green fluorescence in the Protopine-treated cells suggested increased intracellular ROS generation.

The molecular basis for Protopine's apoptotic action was also revealed. The extrinsic and intrinsic pathways are the two main signalling pathways involved in apoptosis, and the important components involved are members of the caspase family. Representative caspases implicated in the extrinsic and intrinsic routes, respectively, include caspase-8 and caspase-9.[46,47] The active forms of procaspases, caspase-8, and caspase-9, and caspase-3 were elevated in Protopine treated breast tumor cells. These results suggest cell death via the caspase-dependent apoptotic route.

CONCLUSION

This study aimed to elucidate the anti-tumor effects of Protopine on MDA-MB-231 cells. An investigation was conducted to examine the effects of Protopine on cell proliferation, induction of apoptosis, buildup of ROS, and levels of caspase proteins. Our research indicates that Protopine's ability to kill cells is controlled by initiating apoptosis, a process that is supported by elevated levels of ROS and caspases, as well as the results of AO/EB and DAPI staining. An increased concentration of ROS leads to the disruption of cell membranes, ultimately ending in apoptosis.

In summary, the results demonstrate that Protopine effectively suppressed cell proliferation by increasing the amounts of ROS, triggering DNA fragmentation and apoptosis, ultimately resulting in cell death through a caspase-dependent pathway. Additional investigation into this theoretical framework, including the study of interactions between genes, the role of proteins, and signaling pathways has the potential to facilitate the development of a remarkably potent anti-cancer drug specifically designed for the treatment of breast cancer.

FUNDING

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Grant No. 5526).

ACKNOWLEDGEMENT

All authors would like to thank Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Grant No. 5526) for funding this project.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ROS: Reactive Oxygen Species; **ELISA:** Enzyme-Linked Immunoassay; **DMEM:** Dulbecco's Media; **DAPI:** 4′,6-diamidino-2-phenylindole; **DCFH-DA:** Dichloro-Dihydro-Fluorescein Diacetate.

SUMMARY

Protopine, an isoquinoline alkaloid derived from the Papaveraceae and Fumariaceae plant groups, exhibits potent anticancer activity against a wide range of cancer types, including breast cancer. This study provides evidence that Protopine effectively suppressed cancer cell growth by elevating levels of reactive oxygen species (ROS), inducing DNA breakage and apoptosis, ultimately leading to cancer cell death via a caspase-dependent pathway.

REFERENCES

1. Han Y, Fan S, Qin T, Yang J, Sun Y, Lu Y, *et al.* Role of autophagy in breast cancer and breast cancer stem cells (Review). Int J Oncol. 2018; 52(4): 1057-70. doi: 10.3892/ijo.2018.4270, PMID 29436618.
2. Burguin A, Diorio C, Durocher F. Breast cancer treatments: updates and new challenges. J Pers Med. 2021; 11(8): 808. doi: 10.3390/jpm11080808, PMID 34442452.
3. Joshi H, Press MF. Molecular oncology of breast cancer. In: The breast. Elsevier; 2018. p. 282-307.
4. Gao JJ, Swain SM. Luminal a breast cancer and molecular assays: a review. Oncologist. 2018; 23(5): 556-65. doi: 10.1634/theoncologist.2017-0535, PMID 29472313.
5. Ades F, Zardavas D, Bozovic-Spasojevic I, Pugliano L, Fumagalli D, De Azambuja E, *et al.* Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol. 2014; 32(25): 2794-803. doi: 10.1200/JCO.2013.54.1870, PMID 25049332.
6. Loibl S, Gianni L. LHER2-positive breast cancer. Lancet. 2017; 389(10087): 2415-29. doi: 10.1016/S0140-6736(16)32417-5, PMID 27939064.
7. Bergin ART, Loi S. Triple-negative breast cancer: recent treatment advances. F1000Res. 2019; 8. doi: 10.12688/f1000research.18888.1, PMID 31448088.
8. Akram M, Iqbal M, Daniyal M, Khan AU. Awareness and current knowledge of breast cancer. Biol Res. 2017; 50(1): 33. doi: 10.1186/s40659-017-0140-9, PMID 28969709.
9. Silvestri M, Cristaudo A, Morrone A, Messina C, Bennardo L, Nisticò SP, *et al.* Emerging skin toxicities in patients with breast cancer treated with new cyclin-dependent kinase 4/6 inhibitors: a systematic review. Drug Saf. 2021; 44(7): 725-32. doi: 10.1007/s40264-021-01071-1, PMID 33959899.
10. Pearce L. Non-alcoholic fatty liver disease. Nurs Stand. 2016; 30(52): 15. doi: 10.7748/ns.30.52.15.s16, PMID 27641564.
11. Fisusi FA, Akala EO. Drug combinations in breast cancer therapy. Pharm Nanotechnol. 2019; 7(1): 3-23. doi: 10.2174/2211738507666190122111224, PMID 30666921.
12. Fedele M, Cerchia L, Chiappetta G. The epithelial-to-mesenchymal transition in breast cancer: focus on basal-like carcinomas. Cancers. 2017; 9(10): 134. doi: 10.3390/cancers9100134, PMID 28974015.
13. Liu J, Ming B, Gong GH, Wang D, Bao GL, Yu LJ. Current research on anti-breast cancer synthetic compounds. RSC Adv. 2018; 8(8): 4386-416. doi: 10.1039/C7RA12912B.
14. Moo TA, Sanford R, Dang C, Morrow M. Overview of breast cancer therapy. PET Clin. 2018; 13(3): 339-54. doi: 10.1016/j.cpet.2018.02.006, PMID 30100074.
15. Nie C, Wang B, Wang B, Yu R, Zhang E. Protopine triggers apoptosis via the intrinsic pathway and regulation of ROS/PI3K/Akt signalling pathway in liver carcinoma. Cancer Cell Int. 2021; 21: 1-10.
16. Qing ZX, Huang JL, Yang XY, Liu JH, Cao HL, Xiang F, *et al.* Anticancer and reversing multidrug resistance activities of natural isoquinoline alkaloids and their structure-activity relationship. Curr Med Chem. 2018; 25(38): 5088-114. doi: 10.2174/0929867324666170920125135, PMID 28933285.
17. Son Y, An Y, Jung J, Shin S, Park I, Gwak J, *et al.* Protopine isolated from *Nandina domestica* induces apoptosis and autophagy in colon cancer cells by stabilizing p53. Phytother Res. 2019; 33(6): 1689-96. doi: 10.1002/ptr.6357, PMID 30932278.
18. Chen CH, Liao CH, Chang YL, Guh JH, Pan SL, Teng CM. Protopine, a novel microtubule-stabilizing agent, causes mitotic arrest and apoptotic cell death in human hormone-refractory prostate cancer cell lines. Cancer Lett. 2012; 315(1): 1-11. doi: 10.1016/j.canlet.2011.09.042, PMID 22033245.
19. Garcia-Gil M, Turri B, Gabriele M, Pucci L, Agnarelli A, Lai M, *et al.* Protopine/gemcitabine combination induces cytotoxic or cytoprotective effects in cell type-specific and dose-dependent manner on human cancer and normal cells. Pharmaceuticals (Basel). 2021; 14(2): 90. doi: 10.3390/ph14020090, PMID 33530428.
20. He K, Gao JL. Protopine inhibits heterotypic celladhesion in MDA-MB-231 cells through down-regulation of multi-adhesive factors. Afr J Trad Complement Altern Med. 2014; 11(2): 415-24. doi: 10.4314/ajtcam.v11i2.28.
21. Chen T, Jiang H, Hu G. FM, Huang B. Molecular mechanism analysis of protopine against triple negative breast cancer. In: Advances in biomedical and bioinformatics engineering. IOS Press; 2023. p. 480-6.
22. Simu S, Marcovici I, Dobrescu A, Malita D, Dehelean CA, Coricovac D, *et al.* Insights into the behavior of triple-negative MDA-MB-231 breast carcinoma cells following the treatment with 17β-ethinylestradiol and levonorgestrel. Molecules. 2021; 26(9): 2776. doi: 10.3390/molecules26092776, PMID 34066763.
23. Kamiloglu S, Sari G, Ozdal T, Capanoglu E. Guidelines for cell viability assays. Food Front. 2020; 1(3): 332-49. doi: 10.1002/fft2.44.
24. Nagabhishek SN, Madankumar A. A novel apoptosis-inducing metabolite isolated from marine sponge symbiont Monascus sp. NMK7 attenuates cell proliferation, migration and ROS stress-mediated apoptosis in breast cancer cells. RSC Adv. 2019; 9(11): 5878-90. doi: 10.1039/c8ra09886g, PMID 35517301.
25. Pal MK, Jaiswar SP, Srivastav AK, Goyal S, Dwivedi A, Verma A, *et al.* Synergistic effect of piperine and paclitaxel on cell fate via cyt-c, Bax/Bcl-2-caspase-3 pathway in ovarian adenocarcinomas SKOV-3 cells. Eur J Pharmacol. 2016; 791: 751-62. doi: 10.1016/j.ejphar.2016.10.019, PMID 27756602.
26. Rastogi RP, Singh SP, Häder DP, Sinha RP. Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2′, 7′-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937. Biochem Biophys Res Commun. 2010; 397(3): 603-7. doi: 10.1016/j.bbrc.2010.06.006, PMID 20570649.
27. Lu Y, Liu Y, Yang C. Evaluating *in vitro* DNA damage using comet assay. J Vis Exp. 2017; 128(128): e56450. doi: 10.3791/56450, PMID 29053680.
28. Yin M, Wang F, Zhang Y, Meng R, Yuan X, Wang Q, *et al.* Analysis on incidence and mortality trends and age-period-cohort of breast cancer in Chinese women from 1990 to 2019. Int J Environ Res Public Health. 2023; 20(1): 826. doi: 10.3390/ijerph20010826, PMID 36613148.
29. Obeng E. Apoptosis (programmed cell death) and its signals-A review. Braz J Biol. 2021; 81(4): 1133-43. doi: 10.1590/1519-6984.228437, PMID 33111928.
30. Park W, Wei S, Kim BS, Kim B, Bae SJ, Chae YC, *et al.* Diversity and complexity of cell death: a historical review. Exp Mol Med. 2023; 55(8): 1573-94. doi: 10.1038/s12276-023-01078-x, PMID 37612413.
31. Alam B, Javed A, Fanar A, Choi H, Lee S. Plant-based natural products for breast cancer prevention: A South Asian Association for Regional Cooperation (SAARC) countries perspective. Clin Surg. 2021; 6: 3047.
32. Tewari D, Patni P, Bishayee A, Sah AN, Bishayee A. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: A novel therapeutic strategy. Seminars in Cancer Biology. 2022; 80: 1-17. doi: 10.1016/j.semcancer.2019.12.008.

33. Ullah MF, Ahmad A, Bhat SH, Abuduhier FM, Mustafa SK, Al-Qirim T. Functional profiling of *Achillea fragrantissima* (a perennial edible herb) against human cancer cells and potential nutraceutical impact in neutralizing cell proliferation by interfering with VEGF and NF-κB signaling pathways. Ital J Food Sci. 2022; 34(3): 35-47. doi: 10.15586/ijfs.v34i3.2211.
34. An W, Lai H, Zhang Y, Liu M, Lin X, Cao S. Apoptotic pathway as the therapeutic target for anticancer traditional Chinese medicines. Front Pharmacol. 2019; 10: 758. doi: 10.3389/fphar.2019.00758, PMID 31354479.
35. Choi BH, Kim MR, Jung YN, Kang S, Hong J. Interfering with color response by porphyrin-related compounds in the MTT tetrazolium-based colorimetric assay. Int J Mol Sci. 2022; 24(1): 562. doi: 10.3390/ijms24010562, PMID 36614004.
36. Bae DS, Kim YH, Pan CH, Nho CW, Samdan J, Yansan J, *et al.* Protopine reduces the inflammatory activity of lipopolysaccharide-stimulated murine macrophages. BMB Rep. 2012; 45(2): 108-13. doi: 10.5483/BMBRep.2012.45.2.108, PMID 22360889.
37. Li L, Leung PS. Use of herbal medicines and natural products: an alternative approach to overcoming the apoptotic resistance of pancreatic cancer. Int J Biochem Cell Biol. 2014; 53: 224-36. doi: 10.1016/j.biocel.2014.05.021, PMID 24875648.
38. Rok J, Kowalska J, Rzepka Z, Stencel D, Skorek A, Banach K, *et al.* The assessment of anti-melanoma potential of tigecycline—cellular and molecular studies of cell proliferation, apoptosis and autophagy on amelanotic and melanotic melanoma cells. Cells. 2023; 12(12): 1564. doi: 10.3390/cells12121564, PMID 37371034.
39. Ahmed MB, Islam SU, Alghamdi AAA, Kamran M, Ahsan H, Lee YS. Phytochemicals as chemo-preventive agents and signaling molecule modulators: current role in cancer therapeutics and inflammation. Int J Mol Sci. 2022; 23(24): 15765. doi: 10.3390/ijms232415765, PMID 36555406.
40. Bougoffa-Sadaoui K, Maiza-Benabdesselam F, Ouadid-Ahidouch H. Antiproliferative activity of protopine extract from *Fumaria agraria* against human lung cancer cell lines. Phytothérapie. 2023; 21(2-3): 102-7. doi: 10.3166/phyto-2021-0293.
41. Velu P. TNF-α regulated inflammatory pathway by isopulegol in human lung adenocarcinoma (A549) cells through ROS generation. Aust J Sci Technol. 2020; 4: 348-52.
42. Gielecińska A, Kciuk M, Yahya EB, Ainane T, Mujwar S, Kontek R. Apoptosis, necroptosis, and pyroptosis as alternative cell death pathways induced by chemotherapeutic agents? Biochim Biophys Acta Rev Cancer. 2023; 1878(6): 189024. doi: 10.1016/j.bbcan.2023.189024, PMID 37980943.
43. Su LJ, Zhang JH, Gomez H, Murugan R, Hong X, Xu D, *et al.* Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. Oxid Med Cell Longev. 2019; 2019: 5080843. doi: 10.1155/2019/5080843, PMID 31737171.
44. Arfin S, Jha NK, Jha SK, Kesari KK, Ruokolainen J, Roychoudhury S, *et al.* Oxidative stress in cancer cell metabolism. Antioxidants (Basel). 2021; 10(5): 642. doi: 10.3390/antiox10050642, PMID 33922139.
45. Behl T, Makkar R, Sehgal A, Singh S, Sharma N, Zengin G, *et al.* Current trends in neurodegeneration: cross talks between oxidative stress, cell death, and inflammation. Int J Mol Sci. 2021; 22(14): 7432. doi: 10.3390/ijms22147432, PMID 34299052.
46. Abd-Rabou AA, Ahmed HH, Kishta MS. Implication of extrinsic and intrinsic apoptotic pathways in the targeted therapy of hepatocellular carcinoma using aptamer-labeled viramidine nanoparticles. BMC Cancer. 2022; 22(1): 1106. doi: 10.1186/s12885-022-10201-6, PMID 36309655.
47. Bhadra K. A mini review on molecules inducing caspase-independent cell death: A new route to cancer therapy. Molecules. 2022; 27(19): 6401. doi: 10.3390/molecules27196401, PMID 36234938.

Cite this article: Li S, Sivapragasam G, Arulselvan P, Manap ASA. Apoptotic Activity of Protopine against Human Breast Cancer Cell Lines: An *in vitro* Study. Indian J of Pharmaceutical Education and Research. 2024;58(2s):s616-s622.