Ind. J. Pharm. Edu. Res., 2025; 59(4):1483-1492.
https://www.ijper.org

Original Article

In vitro and *in silico* Analysis Protosappanin B Exerts Anti-Tumor Activity on Breast Cancer Cells via Activating Pro-Apoptotic Caspase Enzymes

Xia Zhang, Shuangnian Deng*

Department of Oncology, Linfen People's Hospital, Caihongqiao West, Binhe West Road, Linfen, CHINA.

ABSTRACT

Background: Breast cancer is the most common cancer and the second leading cause of cancer-associated mortality in women globally. Breast cancer can metastasize or recur because of drug resistance and toxicity. **Objectives:** The present work focuses on assessing the impact of *in vitro* and *in silico* analysis Protosappanin B on the growth and apoptosis of MDA-MB-231 cells. **Materials and Methods:** The effect of different concentrations of Protosappanin B on the MDA-MB-231 cells was investigated using the WST-1 assay. The Protosappanin B was assessed by analyzing the Lactate Dehydrogenase (LDH) amount that leaked into the growth medium. The analysis of apoptotic cells was done by using the dual staining method. The activities of the pro-apoptotic caspase-3, -8, and -9 enzymes in the cells were examined using respective assay kits and *in silico* Molecular docking studies. **Results:** The WST-1 assay results demonstrated that the Protosappanin B treatment at different dosages effectively inhibited MDA-MB-231 cell viability. The Protosappanin B effectively increased the LDH activity. The dual staining assay confirmed that Protosappanin B induced apoptosis in MDA-MB-231 cells. The activities of the caspase-3, 8, and -9 enzymes were remarkably elevated in the MDA-MB-231 cells after Protosappanin B treatment. **Conclusion:** The present study showed that Protosappanin B inhibits the viability of MDA-MB-231 cells and triggers their apoptosis via activating the pro-apoptotic caspase enzymes. Additionally, we confirmed the interactions of Protosappanin B with caspase enzymes through the molecular docking analysis. The computational study revealed that there are interactions between the Protosappanin B and caspase enzymes such as caspase-3 (-5.9 kcal/mol; RMSD = 1.762 Å), caspase-8 (-5.9 kcal/mol; RMSD = 2.805 Å), and caspase-9 (-6.4 kcal/mol; RMSD = 1.731 Å). Overall, the results from the *in vitro* and *in silico* analysis strongly described that Protosappanin B induced the caspase-3, -8, and -9 and increased the apoptosis which reduced the cell proliferation of MDA-MB-231 cells. These findings clearly show the anticancer activity of Protosappanin B against breast cancer. The study involves nursing care for laboratory animals, including ethical approval, health screening, tumor induction, administration of Protosappanin B, pain management, hydration, nutrition, and housing. Data collection involves accurate record-keeping, statistical analysis, and post-study care. Key nursing interventions include vigilant monitoring, ethical treatment, supportive care, and accurate data collection.

Keywords: Protosappanin B, Caspases, Apoptosis, Breast cancer, Molecular docking.

Correspondence:
Dr. Shuangnian Deng
Department of Oncology, Linfen People's Hospital, Caihongqiao West, Binhe West Road, Linfen-041000, CHINA.
Email: dengshuangnian@163.com

Received: 14-01-2025;
Revised: 07-03-2025;
Accepted: 30-06-2025.

INTRODUCTION

Breast cancer is the most common cancer and the second-leading cause of cancer-associated mortality in women worldwide. 2.1 million women were diagnosed with breast cancer and over 600,000 died from it globally in 2018, making it a significant issue in women's health.[1] It ranks fifth in causing tumor-related deaths, following lung, stomach, liver, and colorectal cancers. The available therapies for breast cancer encompass surgery, chemotherapy, and radiation therapy in clinical settings. These therapies are associated with several side effects and cancer can spread or recur because of drug resistance.[2] Breast cancer is categorized into many molecular subtypes depending on the presence or absence of three receptors, such as the Estrogen (ER), Progesterone (PR), and Human Epidermal growth factor Receptor 2 (HER2) receptors. Triple-Negative Breast Cancer (TNBC) is a distinct subtype of breast cancer characterized by the absence of ER, PR, and HER-2 expressions. This particular type of breast cancer is characterized by strong metastatic potential, aggressive characteristics, and a lower prognosis, resulting in adverse clinical results.[3] TNBC is distinguished by a considerably

DOI: 10.5530/ijper.20251723

elevated incidence of metastasis and mortality, causing 15-20% of all breast cancer cases.[4]

Patients with the TNBC subtype have a 10-20% chance of experiencing lower survival durations because of the high malignancy, recurrence rate, and metastasis. Tumors from TNBC can spread to internal organs within one to three years of diagnosis, with 40% of metastases occurring in the lungs.[5] This cancer type is linked to increased metastasis, higher relapse rates, and a poor survival rate. Breast cancer treatment typically involves a combination of therapies, including surgery, radiation therapy, and medicines such as hormone therapy and chemotherapy. TNBC tumors are unresponsive to endocrine therapy because of the absence of receptors, resulting in the absence of a standardized treatment for TNBC.[6] Thus, there is a pressing clinical need to explore new and efficient treatment methods for TNBC.

Apoptosis is a crucial therapeutic target that helps regulate the balance between cell life and death. A failure of the regulatory mechanisms of apoptosis can result in autoimmune diseases or the development of cancer. Tumor cells are typically responsive to apoptosis at first but can develop resistance to drugs due to dysfunction in apoptosis and the increased presence of anti-apoptotic genes.[7] Cancer cells cannot typically trigger apoptosis, leading to resistance to anti-cancer drugs. Inducing various apoptotic signalings in tumor cells could potentially address drug resistance and enhance responsiveness to chemotherapy.[8] Discovering and developing methods and drugs to treat drug-resistant tumors is crucial for protecting health as well as lives. Conventional chemotherapy is linked to significant toxicity, and tumor cells develop resistance to drugs. Natural products have several advantages over chemotherapy, such as chemical diversity, targeting various sites, low toxicity, and overcoming drug resistance. Plant-based compounds have demonstrated efficacy as novel anti-cancer medicines.[9]

Protosappanin B, a significant compound found in *Caesalpinia sappan*, is known for its diverse biological properties.[10] Currently, there is limited literature on the activities of Protosappanin B. Some studies have reported its antibacterial, antioxidative and antitumor properties. Additionally, research on its pharmacokinetics and bioavailability has been carried out in rodents.[11-15] However, only limited literature has explored the anti-cancer properties of Protosappanin B. The present work aimed to examine the impact of Protosappanin B on the viability and cell death of invasive breast cancer subtype MDA-MB-231 cells.

The research involves pre-study preparations, including ethical approval, baseline health assessments, tumor induction, administration of Protosappanin B, and general care and monitoring. Animals are monitored for adverse reactions, vital signs, behavioral changes, and tumor size measurement. Supportive care includes pain management, hydration and nutrition, and maintaining clean housing conditions. Blood and tissue samples are collected for analysis. Data collection and documentation involve accurate record-keeping and statistical analysis. Post-study care includes recovery, euthanasia, and ethical considerations. Results are compiled and analyzed, with findings prepared for publication to contribute to the scientific understanding of potential anti-cancer therapies targeting pro-apoptotic pathways. Key nursing interventions include vigilant monitoring, ethical treatment, supportive care, and accurate data collection

MATERIALS AND METHODS

Chemicals

The following chemicals, including Protosappanin B, Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and other materials, were purchased from Sigma-Aldrich, USA.

Cell culture

DMEM was utilized to culture the MDA-MB-231 cells. The medium was grown at 37°C in a humidified air environment with the addition of 10% FBS and 1% antibiotics. The cells were trypsinized after they reached 80% confluency and employed in the additional experiments.

WST-1 cell viability assay

Cells were grown in 96-well plates at 1×10^6 cells/well for the cell viability assay and then incubated for 24 hr. The cells were grown at 37°C for 24 hr and then treated with Protosappanin B at different dosages (2.5, 5, 25, 50, 100, 150, and 200 µM/mL). After 24 hr of treatment, fresh medium was used, and WST-1® (10 µL) reagent was mixed in each well for 3 hr at 37°C. The inhibition percentages were calculated by measuring absorbance with an ELISA reader at 460 nm to assess cell viability.

LDH activity assay

The cytotoxicity of Protosappanin B was assessed by analyzing the quantity that was released into the medium. After 24 hr growth of control and treated cells, the media was centrifuged at 5000 rpm to collect cell-free supernatants. A commercial kit was utilized to assess the LDH activity. The assay relies on the simultaneous decrease of NAD and the transformation of lactate into pyruvate with the help of LDH. The process outlined above leads to the generation of NADH, which then induces a modification in absorbance at 340 nm. Aliquots of medium were placed on a microtiter plate to determine the absorbance using a spectrophotometer.

Analysis of apoptosis by dual staining method

The cells with apoptosis were detected using the dual staining technique. Breast cancer cells were loaded in wells at 1×10^6 cells/well and allowed to proliferate for 24 hr. MDA-MB-231 cells

were treated with Protosappanin B at its IC_{50} and positive control cisplatin (cis) for 24 hr. The cells were then washed with PBS before being stained with a 1:1 mixture of AO and EtBr dye for 30 min and examined using a fluorescent microscope.

Measurement of Caspase-3, -8, and -9 activity

To confirm the capacity of Protosappanin B to induce apoptosis, various apoptotic effectors were examined. The commercial kits were employed to measure the caspase-3, -8, and -9 activities in control cells and Protosappanin B- at its IC_{50} and positive control cisplatin (cis) treated MDA-MB-231 cells.

Preparation of protein structure and Ligand

The 3-Dimensional structure (3D) of the described targets such as caspase-3 (PDB ID: 3DEI), caspase-8 (PDB ID: 3KJQ), and caspase-9 (PDB ID: 3V3K) were obtained from the Protein Data Bank. The water molecules and associated ligands were removed for the retrieved caspase enzymes using the Discovery Studio Visualizer v19.1.0.18287 (www.accelerys.com). The structure of the chemical compound Protosappanin B was downloaded from the PubChem compound database and saved in the structured data file format with the 3D atomic coordinates and converted into PDB format using Discovery Studio Visualizer v19.1.0.18287 (www.accelerys.com).[16-19]

Molecular docking and Visualization

We used PyRx software and implemented Autodock Vina after preparing Protosappanin B as ligand, and caspase-3 (PDB ID: 3DEI), caspase-8 (PDB ID: 3KJQ), and caspase-9 (PDB ID: 3V3K) as targets.[20] The prepared targets and ligands were converted into PDBQT format. The grid box size of caspase-3 (x = 57.15 Å, y = 49.97 Å, z = 49.35 Å), caspase-8 (x = 45.01 Å, y = 53.95 Å, z = 44.54 Å), and caspase-9 (x = 57.13 Å, y = 51.55 Å, and z = 57.16 Å) were assigned and identified the interactions between Protosappanin B and caspase enzymes. The Autodock Vina utilizes a new scoring algorithm for the docking process.[21]

New scoring mechanisms,

$$C = \sum_{i<j} f_{titj}(r_{ij}),$$

C- Sum of intermolecular and intramolecular distance; Σ - Over all of the pairs of atoms; f_{aith} - Symmetric set of interaction functions; r_{ij} - Interatomic distance

The results were visualized in 3D using Discovery Studio Visualizer v19.1.0.1828 (Dassault Systèmes BIOVIA, Rue Marcel Dassault, Vélizy-Villacoublay-78140, France, www.accelerys.com (accessed on 18 March 2024). Likely, the 2D diagram was visualized by importing the docked data into LigPlot+ v.2.2.

Statistical analysis

The mean±SD of three separate tests was utilized to depict the obtained data. A one-way ANOVA was utilized for the statistical study. Tukey's *post hoc* assay was utilized to assess the variability among the different sets of variables. A *p*-value below 0.05 was fixed and significant.

RESULTS

Protosappanin B inhibits the viability of breast cancer MDA-MB-231 cells

The effect of Protosappanin B on the growth of MDA-MB-231 cells was assessed by a WST-1 assay (Figure 1). The cells were treated with several dosages (2.5-200 µM/mL) of Protosappanin B for 24 hr. Protosappanin B treatment demonstrated dose-dependent inhibition of MDA-MB-231 cell viability, as indicated by the WST-1 assay results (Figure 1). The IC_{50} concentration of Protosappanin B was 50 µM/mL. The results indicated that Protosappanin B has a stronger inhibitory activity on the viability of MDA-MB-231 cells.

Protosappanin B promotes LDH enzyme release in MDA-MB-231 cells

The cells were exposed to several doses of Protosappanin B (5-200 µM/mL) for 24 hr to measure LDH activity. The results demonstrated that Protosappanin B significantly enhances LDH activity in a dosage-dependent manner, as illustrated in Figure 2. The LDH activity elevates in response to cell injury with 50 µM/mL and above concentrations of Protosappanin B. The present findings demonstrate that Protosappanin B and positive control can affect cell damage and induce cell death.

Protosappanin B induces apoptosis in the MDA-MB-231 cells

Cellular apoptosis is characterized by variations in cell morphological alterations. In this work, AO/EtBr dual staining was utilized to assess morphological alterations in the MDA-MB-231 cells caused by apoptosis (Figure 3). EtBr, a red fluorescent dye, can accurately penetrate the damaged nucleus of apoptotic cells. Only normal, non-apoptotic cells allowed the entry of green fluorescent AO dye. The results showed that non-apoptotic cells were identified as viable cells as they exhibited intense green nucleus fluorescence. Conversely, the MDA-MB-231 cells exposed to Protosappanin B for at dosages of 50 µM/mL value and positive control showed the presence of more apoptotic cells as they exhibited more orange and red fluoresced cell morphology (Figure 3). Orange fluorescence shows early apoptosis, while the red fluorescence of fragmented nuclei suggests late apoptosis.

Protosappanin B causes apoptosis in MDA-MB-231 cells by activating caspase expression

The pro-apoptotic enzymes caspase-3, -8, and -9 in MDA-MB-231 cells were tested, and the findings are described in Figure 4. The findings demonstrate the influence of Protosappanin B treatment on the activities of caspases in breast cancer cells. Levels of

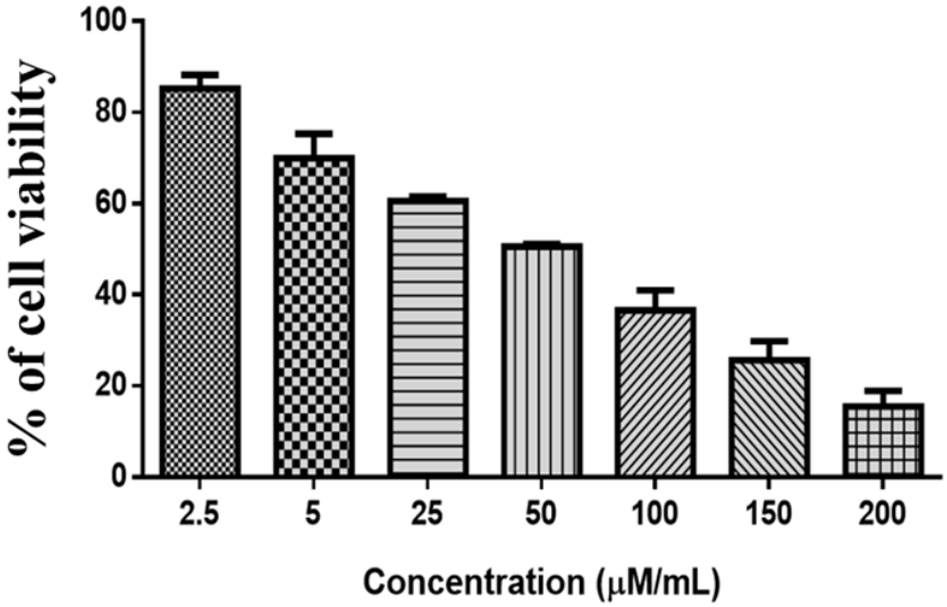

Figure 1: Effect of Protosappanin B on the viability of breast cancer MDA-MB-231 cells. The effect of Protosappanin B treatment on MDA-MB-231 cell viability was assessed by the WST-1 cell viability assay. The MDA-MD-231 cells treated with various dosages (2.5-200 μM/mL) of Protosappanin B for 24 hr showed a remarkable decrease in viability. Values are presented as the mean±SD of the triplicate assays. The data were analyzed by one-way ANOVA and Tukey's *post hoc* test. The values do not share a common superscript and significantly differ at $p<0.05$ from the control.

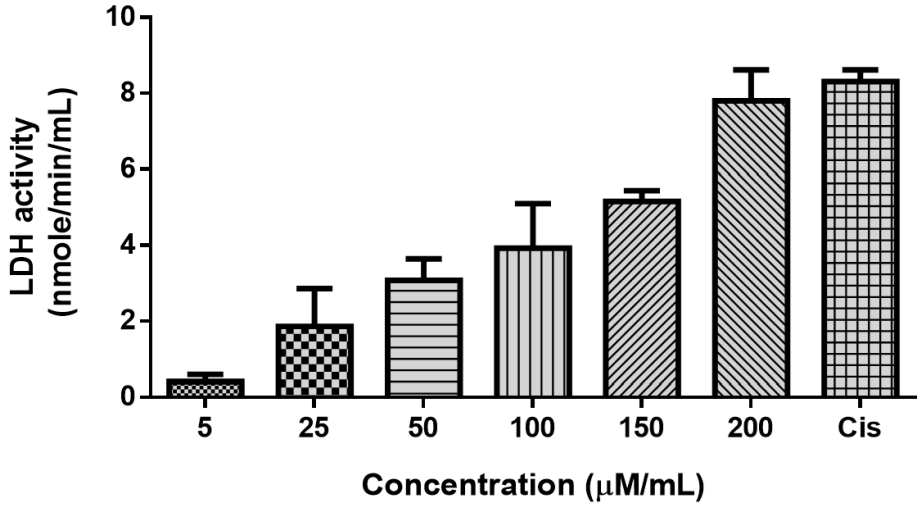

Figure 2: Effect of Protosappanin B on the LDH enzyme activity in the MDA-MB-231 cells. The results demonstrated that Protosappanin B and positive control for 24 hr treatment significantly increases LDH activity in a concentration-dependent manner. Values are presented as the mean±SD of the triplicate assays. The data were analyzed by one-way ANOVA and Tukey's *post hoc* test. The values do not share a common superscript and significantly differ at $p<0.05$ from the control.

caspase activities are significantly increased in cells after exposure to 50 μM/mL value of Protosappanin B and positive control drug (Figure 4). These findings proved that Protosappanin B can trigger apoptosis in breast cancer cells via activating caspase enzymes.

In silico analysis of Protosappanin B

The molecular docking analysis was performed for the Protosappanin B and caspase-3, caspase-8, and caspase-9 and the resulting binding affinity, RMSD, interacting residues, and its bond types were listed in Table 1.

Protosappanin B and Caspase-3

Protosappanin B formed two hydrogen bonds with the residues TRP214, and GLN217, and a pi-pi stacked bond formed with the PHE247. Also, the carbon-hydrogen bond formed with the GLU248 residue. Additionally, Protosappanin B - caspase-8 complex surrounded by hydrophobic residue GLU246 with

the binding affinity of -5.9 kcal/mol and the RMSD = 1.762 Å (Figures 5A, B, and Table 1).

Protosappanin B and Caspase-8

The caspase-8 residues such as ARG258, and GLY318 interacted with Protosappanin B via two hydrogen bonds respectively, and a pi-sulfur bond formed with the CYS360 residue. Also, the alkyl, Pi-alkyl, and pi-pi-T-shaped bond formed with the ILE257 and HIS317. Additionally, the hydrophobic residues such as LEU254, LYS253, HIS255, SER256, and TYR324 have surrounded the caspase-8-Protosappanin complex with the binding affinity of -6.7 kcal/mol; RMSD = 2.805 Å (Figure 5. C, D and Table 1).

Protosappanin B and Caspase-9

The Protosappanin B interacted with the residues such as ARG146, PHE399, and THR402 using three hydrogen bonds. The pi-alkyl and pi-cation bond formed with the residues ARG146 and LYS401. The hydrophobic residues such as GLY147, ASN148, ALA152, TYR153, ILE154, LEU155, SER156, and SER403. The estimated binding affinity and RMSD for the caspase-9 and Protosappanin B complex was -6.4 kcal/mol and 1.731 Å (Figures 5E, F and Table 1).

The LigPlot interactions also evidence that there were interactions between the Protosappanin B and caspase-3, -8, and -9 respectively (Figure 6). The results described that Protosappanin B interacted with the caspase-3 via four hydrogen bonds with the residues ASN208, TRP214, GLN217, and GLU248 (Figure 6A). Likely, Protosappanin B interacted with the caspase-8 via five hydrogen bonds with the four residues such as LYS253, SER256, ARG258, and GLY318(2) (Figure 6B). Also, the caspase-9 residues such as ARG146, TYR153(2), PHE399, and THR402 interacted with Protosappanin B using 5 hydrogen bonds (Figure 6C).

DISCUSSION

TNBC is an aggressive cancer that becomes resistant to therapy because it lacks ER, PR, and HER-2 receptors. Therapy resistance is the primary challenge in treating TNBC, despite the existence of several effective medicines.[22] Although survival rates have improved due to breakthroughs in early detection technologies, there is a strong focus on creating novel and potent

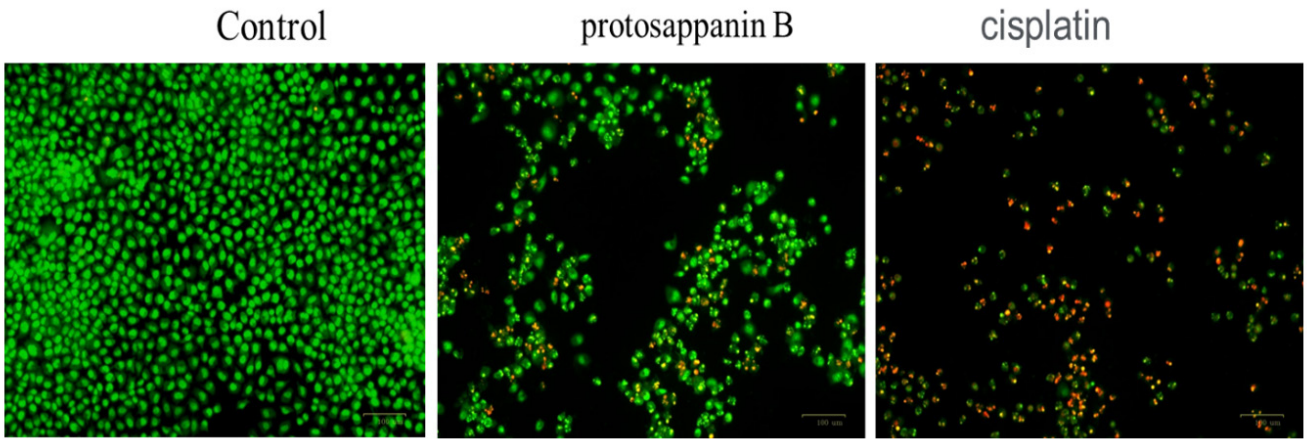

Figure 3: Effect of Protosappanin B on the apoptotic cell death in the MDA-MB-231 cells. The MDA-MB-231 cells treated for 24 hr with Protosappanin B at concentrations of IC_{50} and positive control showed the presence of more apoptotic cells in both early and late apoptotic stages, as they exhibited more orange and red fluoresced cell morphology.

Table 1: The list of caspase enzymes, PDB ID, binding affinity (kcal/mol), RMSD (Å), interacted residues, and their bond types were tabulated.

Targets Name	Targets PDB	Binding affinity (Kcal/mol)	RMSD (Å)	Type of interacted bonds		
				Hydrogen bond	Hydrophobic residues	Other bonds
Caspase-3	3DEI	-5.9	1.762	TRP214, GLN217	GLU246	PHE247^{pps}, GLU248^{ch}
Caspase-8	3KJQ	-6.7	2.805	ARG258, GLY318	LEU254, LYS253, HIS255, SER256, TYR324	CYS360^{ps}, ILE257$^{a, pa,}$ HIS317^{ppt}
Caspase-9	3V3K	-6.4	1.731	ARG146, PHE399, THR402	GLY147, ASN148, ALA152, TYR153, ILE154, LEU155, SER156, SER403	ARG146^{pc}, LYS401^{pa},

Note: pps – pi-pi-stacked; pa- pi alkyl; pc- pi cation; ch- carbon hydrogen bond; ppt -pi-pi-T-shaped; ps - pi-sulfurs.

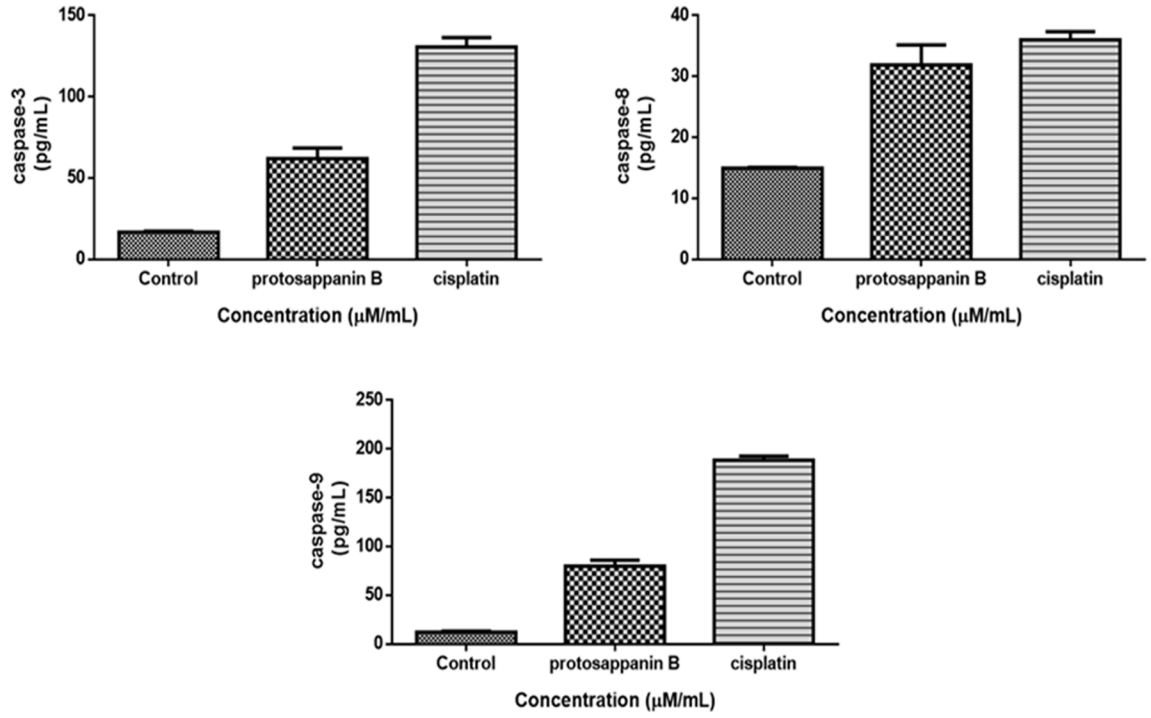

Figure 4: Effect of Protosappanin B on the caspase enzyme activities in the MDA-MB-231 cells. The results showed that the caspase-3, -8, and -9 activities were significantly increased in MDA-MB-231 cells after for 24 hr treatment with positive control and IC_{50} of Protosappanin B. Values are presented as the mean±SD of the triplicate assays. The data were analyzed by one-way ANOVA and Tukey's *post hoc* test. The values do not share a common superscript and significantly differ at *$p<0.05$ from the control.

chemotherapeutic agents for breast cancer treatment. New research should prioritize finding innovative and efficient treatments for TNBC. Apoptosis is a crucial cell-death mechanism that serves as a promising and optimal target for cancer treatment.[23] This work aimed to assess the impact of Protosappanin B on inducing apoptosis in invasive breast cancer cells.

Cell viability measurement is a crucial step in several cell culture methods that assess the quantity of healthy cells.[24] Cellular dehydrogenases can convert WST-1 into a water-soluble formazan. The formazan generated by WST-1 is highly soluble, resulting in an expanded linear range and increased sensitivity. Adding WST-1 once can demonstrate the impact of the sample agents at various time intervals.[25] Studying cellular viability is a crucial method for evaluating how cells respond to external stimuli. The WST-1 test allows for the evaluation of several samples without producing radioactive leftovers and demonstrates increased accuracy, which is now crucial in studying cellular viability.[26] In this work, the influence of Protosappanin B on the MDA-MB-231 cell growth was investigated by the WST-1 technique at 24 hr. The findings showed that Protosappanin B treatment demonstrated dose-dependent inhibition of MDA-MB-231 cell growth.

Apoptosis is a major cell death mechanism in multicellular species that can be utilized as a therapy option for cancer. Deviant changes in the usual function of this mechanism can lead to abnormal cell proliferation and unregulated cell division, potentially causing normal tissue to develop cancer.[27] Avoiding apoptosis is a critical characteristic of cancer development and leads to resistance against anticancer treatments. The molecular characteristics of apoptotic cells include cell shrinkage, chromatin aggregation, mRNA degradation, and the creation of apoptotic bodies. Excessive programmed cell death results in tissue shrinkage, while insufficient programmed cell death is linked to unregulated cell growth, as seen in malignancies.[28] Normal breast cells maintain cell homeostasis through a balance between cell growth and apoptosis mechanisms. Disruption of the equilibrium can result in unrestricted cell growth, therapy resistance, and tumor recurrence due to an overactive antiapoptotic pathway or deficiency in the Pro-apoptosis pathway.[29] In this work, the apoptosis in MDA-MB-231 cells was studied by an AO/EtBr dual staining assay. The results showed that Protosappanin B treatment resulted in more apoptotic cells in both early and late apoptotic phases, as they exhibited more orange and red fluoresced cell morphology. Hence, it was clear that Protosappanin B can trigger apoptosis in breast cancer cells.

LDH can be a target for cancer therapy. The LDH enzyme complex is made up of four subunits, including two distinct isoforms: LDH-A and LDH-B.[30] LDH-A is essential in the glycolytic process because of its increased attraction to pyruvate. It facilitates the pyruvate transformation into lactate by oxidizing NADH to NAD+. Conversely, LDH-B has a higher affinity for lactate and promotes the transformation of lactate to pyruvate by

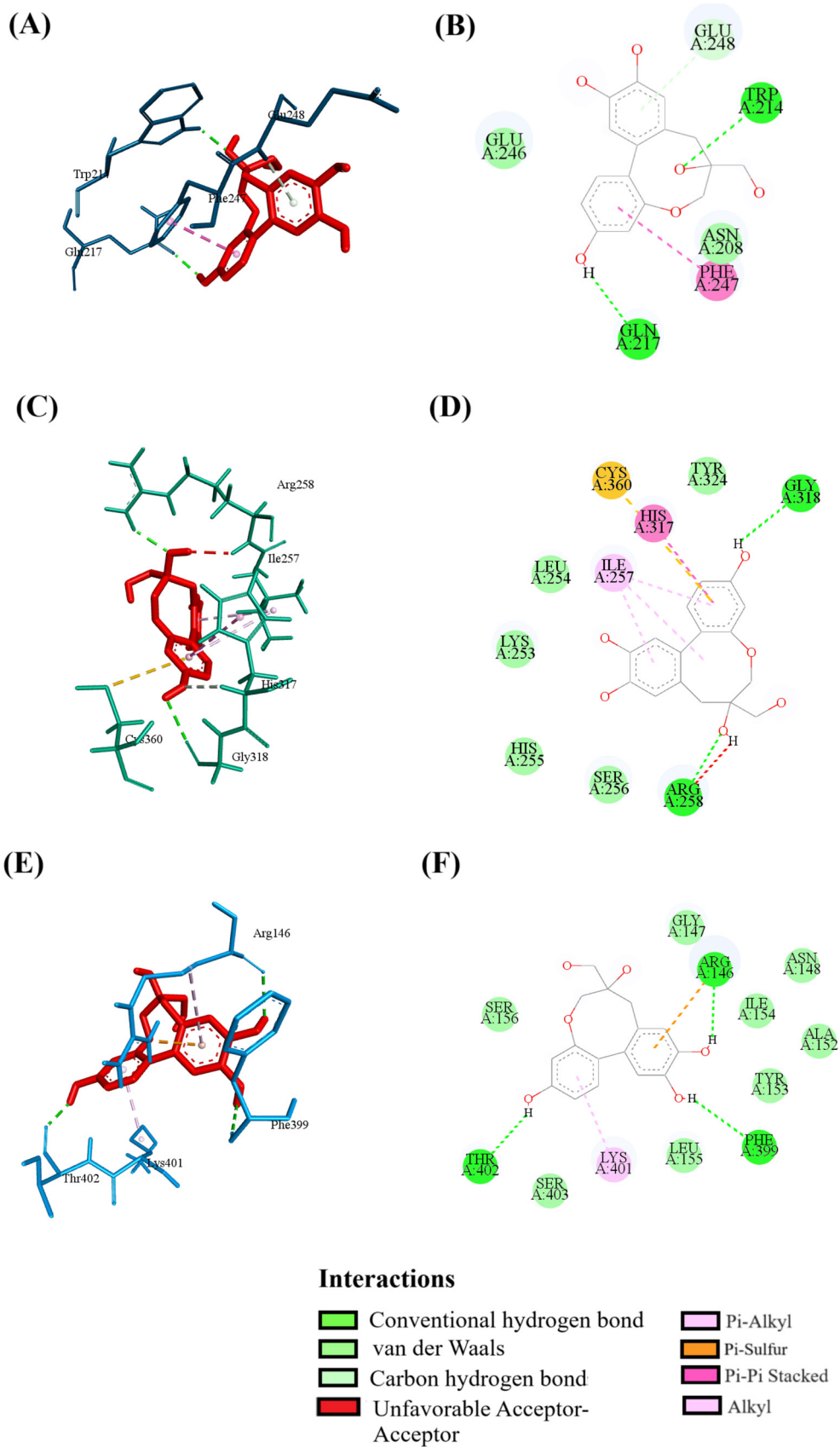

Figure 5: The docking pose of the determined targets caspase-3 (A, B), caspase-8 (C, D), and caspase-9 (E, F). The docking pose for the Protosappanin B and caspase enzymes complex was selected based on the binding affinity and RMSD ≤ 3.0 Å.

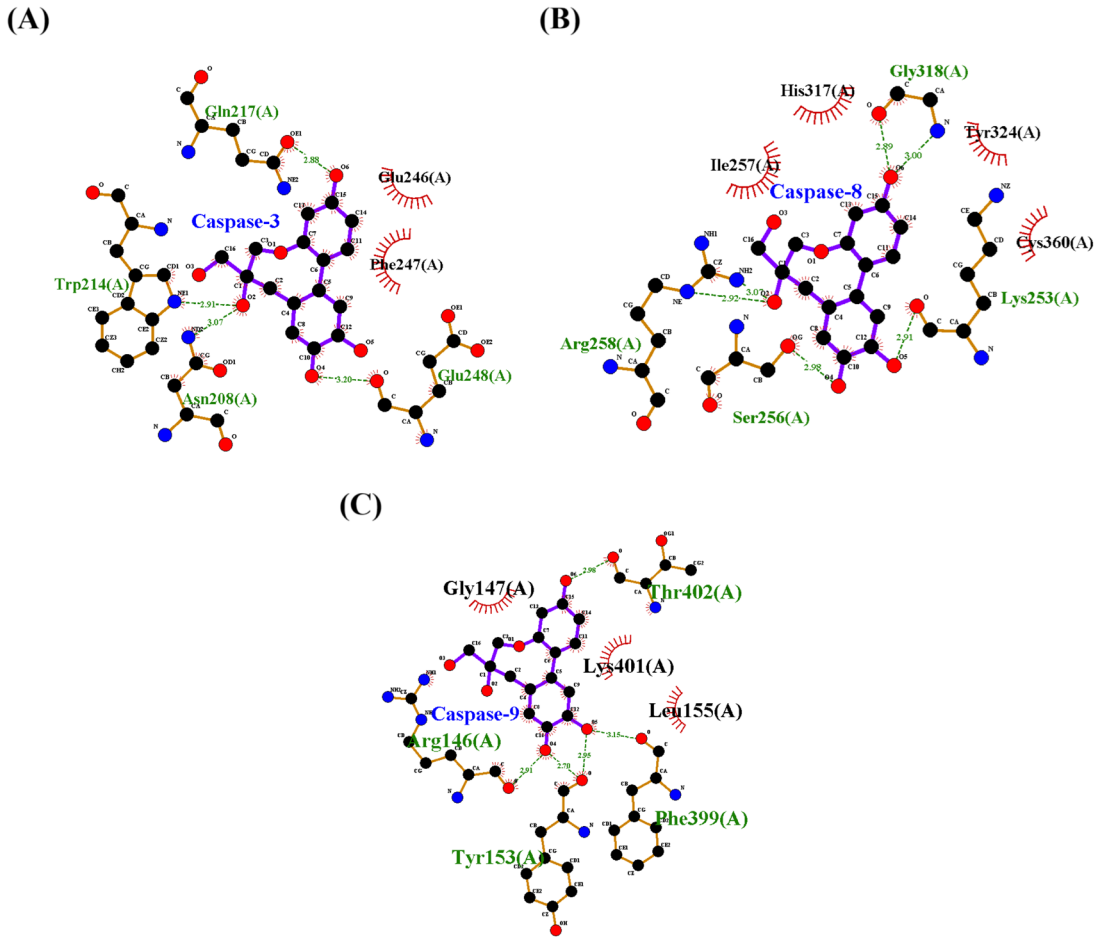

Figure 6: The LigPlot interactions for the chemical compound Protosappanin B with caspase enzymes. (A) caspase-3, (B) capase-8, (C) caspase-9.

converting NAD+ to NADH.[31] LDH activity is linked to several malignancies and increased enzyme activity is connected to a more advanced stage of the disease. Multiple pieces of evidence indicate a clear link between the various LDH isoenzyme activities and the advanced stage and prognosis of breast cancer.[32] In this work, the results demonstrated that Protosappanin B significantly enhances LDH activity in a dosage-dependent manner. LDH activity elevates in response to cell injury with Protosappanin B treatment. The present findings demonstrate that Protosappanin B can affect cell damage and induce cell death. Suppression of LDH-A and LDH-B leads to the diminished growth and invasion of breast cancer cells. This is likely caused by decreased lactate levels in the extracellular milieu around breast cancer cells.[33]

Targeting pathways that trigger apoptosis is a promising approach for tumor treatment.[34] Various cellular signaling pathways participate in controlling various stages of apoptotic cell death.[35] Apoptosis operates through two primary mechanisms: mitochondria-regulated (intrinsic) and death receptor-regulated (extrinsic) mechanisms. Proteases play a crucial role in the programmed cell death process by cleaving cellular substrates and linked with apoptosis.[36] The caspase-8 and -9 activation is associated with initiating the apoptotic pathways. Many drugs used to treat cancer trigger apoptosis via the mitochondrial mechanism.[37] Mitochondrial membrane potential alterations are a crucial sign of the beginning of apoptosis.[38] The changed membrane potential leads to the cytochrome c release, which then promotes the caspase-3 and caspase-9 activation. Activated caspases trigger caspase-3, which serves as the apoptotic executioner.[39,40] In this study, the activities of the pro-apoptotic enzymes caspase-3, -8, and -9 were assessed using assay kits. The findings clearly showed that Protosappanin B treatment effectively activated the caspase-3, -8, and -9 activities in the MDA-MB-231 cells. These findings proved that Protosappanin B can trigger apoptosis in breast cancer cells via activating caspase enzymes.

The nursing care and maintenance of laboratory animals include ethical approval, health screening, tumor induction, administration of Protosappanin B, general care and monitoring, pain management, hydration and nutrition, and housing. Blood samples and tissue samples are collected to monitor biochemical markers, liver and kidney function, and evaluate systemic effects of Protosappanin B. The study's findings will contribute to the understanding of potential anti-cancer therapies targeting pro-apoptotic pathways.

CONCLUSION

The present study proved that Protosappanin B inhibits the growth of MDA-MB-231 cells and triggers their apoptosis. Moreover, these activities are associated with increasing LDH release by cell damage and activating the pro-apoptotic caspase enzymes in the MDA-MB-231 cells. These findings prove the anticancer effects of Protosappanin B against breast cancer. Further research is still required to determine the pathways through which Protosappanin B produces these apoptotic effects. The current study indicates that it could be a promising and natural treatment candidate for breast cancer.

AKNOWLEDGEMENT

The authors would like to express their sincere gratitude to the Department of Oncology, Linfen People's Hospital, Caihongqiao West, Binhe West Road, Linfen, China, for their valuable support and contributions to this study. Their assistance and collaboration were instrumental in the successful completion of this work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LDH: Lactate dehydrogenase; **TNBC:** Triple-negative breast cancer; **FBS:** Fetal bovine serum; **DMEM:** Dulbecco's modified eagle medium; **TNBC:** Triple-negative breast cancer; **HER2:** Human epidermal growth factor receptor 2; **PR:** Progesterone; **ER:** Estrogen; **AO:** Acridine Orange; **EtBr:** Ethidium Bromide.

SUMMARY

The present work focuses on assessing the impact of Protosappanin B on the growth and apoptosis of MDA-MB-231 cells. Protosappanin B on the MDA-MB-231 cells was investigated using the WST-1 assay, LDH enzyme activity, dual staining AO/EB and the caspase enzyme activities in the MDA-MB-231 cells. Additionally, we interactions of Protosappanin B with caspase enzymes through the molecular docking analysis. Overall, the results from the *in vitro* and *in silico* analysis strongly described that Protosappanin B induced the caspase-3, -8, and -9 and increased the apoptosis which reduced the cell proliferation of MDA-MB-231 cells.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021; 71(3): 209-49. doi: 10.3322/caac.21660, PMID 33538338.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6): 394-424. doi: 10.3322/caac.21492, PMID 30207593.
3. Cuypers E, Claes BS, Biemans R, Lieuwes NG, Glunde K, Dubois L, *et al.* "On the spot" digital pathology of breast cancer based on single-cell mass spectrometry imaging. Anal Chem. 2022; 94(16): 6180-90. doi: 10.1021/acs.analchem.1c05238, PMID 35413180.
4. Marotti JD, de Abreu FB, Wells WA, Tsongalis GJ. Triple-negative breast cancer: next-generation sequencing for target identification. Am J Pathol. 2017; 187(10): 2133-8. doi: 10.1016/j.ajpath.2017.05.018, PMID 28734944.
5. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, *et al.* Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007; 13(15 Pt 1):4429-34. doi: 10.1158/1078-0432.CCR-06-3045, PMID 17671126.
6. Popolin CP, Reis JP, Becceneri AB, Graminha AE, Almeida MA, Corrêa RS, *et al.* Cytotoxicity and anti-tumor effects of new ruthenium complexes on triple negative breast cancer cells. PLOS One. 2017; 12(9): e0183275. doi: 10.1371/journal.pone.0183275, PMID 28898246.
7. Pommier Y, Sordet O, Antony S, Hayward RL, Kohn KW. Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. Oncogene. 2004; 23(16): 2934-49. doi: 10.1038/sj.onc.1207515, PMID 15077155.
8. Urtishak KA, Edwards AY, Wang LS, Hudome A, Robinson BW, Barrett JS, *et al.* Potent obatoclax cytotoxicity and activation of triple death mode killing across infant acute lymphoblastic leukemia. Blood. 2013; 121(14): 2689-703. doi: 10.1182/blood-2012-04-425033, PMID 23393050.
9. Dehelean CA, Marcovici I, Soica C, Mioc M, Coricovac D, Iurciuc S, *et al.* Plant-derived anticancer compounds as new perspectives in drug discovery and alternative therapy. Molecules. 2021; 26(4): 1109. doi: 10.3390/molecules26041109, PMID 33669817.
10. Mueller M, Weinmann D, Toegel S, Holzer W, Unger FM, Viernstein H. Compounds from *Caesalpinia sappan* with anti-inflammatory properties in macrophages and chondrocytes. Food Funct. 2016; 7(3): 1671-9. doi: 10.1039/c5fo01256b, PMID 26951869.
11. Zuo GY, Han ZQ, Han J, Hao XY, Tang HS, Wang GC. Antimicrobial activity and synergy of antibiotics with two biphenyl compounds, protosappanins A and B from *Sappan Lignum* against methicillin-resistant *Staphylococcus aureus* strains. J Pharm Pharmacol. 2015; 67(10): 1439-47. doi: 10.1111/jphp.12433, PMID 25920539.
12. Sasaki Y, Hosokawa T, Nagai M, Nagumo S. *In vitro* study for inhibition of NO production about constituents of *Sappan Lignum*. Biol Pharm Bull. 2007; 30(1): 193-6. doi: 10.1248/bpb.30.193, PMID 17202686.
13. Yang X, Zhao L, Zhang T, Xi J, Liu S, Ren L, *et al.* Protosappanin B promotes apoptosis and causes G1 cell cycle arrest in human bladder cancer cells. Sci Rep. 2019; 9(1): 1048. doi: 10.1038/s41598-018-37553-z, PMID 30705351.
14. Chen WY, Zhou XZ, Wu LL, Wu YS, Wang SM, Liu B, *et al.* A UPLC/MS/MS method for determination of protosappanin B in rat plasma and its application of a pharmacokinetic and bioavailability study. Biomed Chromatogr. 2017; 31(7). doi: 10.1002/bmc.3919, PMID 27976417.
15. Tong XZ, Zhu H, Shi Y, Xu HT, Wang B, Zhao JH. An LC/MS/MS method for simultaneous quantitation of two homoisoflavones: protosappanin B and brazilin with hypoglycemic activity in rat plasma and its application to a comparative pharmacokinetic study in normal and streptozotocin-treated rats. J Ethnopharmacol. 2013; 148(2): 682-90. doi: 10.1016/j.jep.2013.05.029, PMID 23707335.
16. Du JQ, Wu J, Zhang HJ, Zhang YH, Qiu BY, Wu F, *et al.* Isoquinoline-1,3,4-trione derivatives inactivate caspase-3 by generation of reactive oxygen species. J Biol Chem. 2008; 283(44): 30205-15. doi: 10.1074/jbc.M803347200, PMID 18768468.
17. Wang Z, Watt W, Brooks NA, Harris MS, Urban J, Boatman D, *et al.* Kinetic and structural characterization of caspase-3 and caspase-8 inhibition by a novel class of irreversible inhibitors. Biochim Biophys Acta. 2010; 1804(9): 1817-31. doi: 10.1016/j.bbapap.2010.05.007, PMID 20580860.
18. Moertl M, Maskos K, Steuber H. Human caspase 9 in complex with bacterial effector protein. PLOS One. 2013.
19. Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, *et al.* PubChem 2023 update. Nucleic Acids Res. 2023; 51(D1):D1373-80. doi: 10.1093/nar/gkac956, PMID 36305812.
20. Dallakyan S, Olson AJ. Small-molecule library screening by docking with PyRx. Methods Mol Biol. 2015; 1263: 243-50. doi: 10.1007/978-1-4939-2269-7_19, PMID 25618350.
21. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010; 31(2): 455-61. doi: 10.1002/jcc.21334, PMID 19499576.
22. Lyons TG. Targeted therapies for triple-negative breast cancer. Curr Treat Options Oncol. 2019; 20(11): 82. doi: 10.1007/s11864-019-0682-x, PMID 31754897.
23. Maniam S, Maniam S. Small molecules targeting programmed cell death in breast cancer cells. Int J Mol Sci. 2021; 22(18): 9722. doi: 10.3390/ijms22189722, PMID 34575883.
24. Keshelava N, Frgala T, Krejsa J, Kalous O, Reynolds CP. DIMSCAN: a microcomputer fluorescence-based cytotoxicity assay for preclinical testing of combination chemotherapy. Methods Mol Med. 2005; 110: 139-53. doi: 10.1385/1-59259-869-2:139, PMID 15901933.
25. Ngamwongsatit P, Banada PP, Panbangred W, Bhunia AK. WST-1-based cell cytotoxicity assay as a substitute for MTT-based assay for rapid detection of toxigenic *Bacillus species* using CHO cell line. J Microbiol Methods. 2008; 73(3): 211-5. doi: 10.1016/j.mimet.2008.03.002, PMID 18417231.
26. Guertler A, Kraemer A, Roessler U, Hornhardt S, Kulka U, Moertl S, *et al.* The WST survival assay: an easy and reliable method to screen radiation-sensitive individuals.

Radiat Prot Dosimetry. 2011; 143(2-4): 487-90. doi: 10.1093/rpd/ncq515, PMID 21183542.

27. Pistritto G, Trisciuoglio D, Ceci C, Garufi A, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. Aging (Albany, NY). 2016; 8(4): 603-19. doi: 10.18632/aging.100934, PMID 27019364.
28. Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett. 2013; 332(2): 237-48. doi: 10.1016/j.canlet.2012.01.007, PMID 22261329.
29. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu HY, Lin LT, *et al.* Broad targeting of resistance to apoptosis in cancer. Semin Cancer Biol. 2015; 35 Suppl:S78-S103. doi: 10.1016/j.semcancer.2015.03.001, PMID 25936818.
30. Ding J, Karp JE, Emadi A. Elevated lactate dehydrogenase (LDH) can be a marker of immune suppression in cancer: interplay between hematologic and solid neoplastic clones and their microenvironments. Cancer Biomark. 2017; 19(4): 353-63. doi: 10.3233/CBM-160336, PMID 28582845.
31. Ždralević M, Brand A, Di Ianni L, Dettmer K, Reinders J, Singer K, *et al.* Double genetic disruption of lactate dehydrogenases A and B is required to ablate the 'Warburg effect' restricting tumor growth to oxidative metabolism. J Biol Chem. 2018; 293(41): 15947-61. doi: 10.1074/jbc.RA118.004180, PMID 30158244.
32. Claps G, Faouzi S, Quidville V, Chehade F, Shen S, Vagner S, *et al.* The multiple roles of LDH in cancer. Nat Rev Clin Oncol. 2022; 19(12): 749-62. doi: 10.1038/s41571-022-00686-2, PMID 36207413.
33. Rizwan A, Serganova I, Khanin R, Karabeber H, Ni X, Thakur S, *et al.* Relationships between LDH-A, lactate, and metastases in 4T1 breast tumors. Clin Cancer Res. 2013; 19(18): 5158-69. doi: 10.1158/1078-0432.CCR-12-3300, PMID 23833310.
34. Bergamo A, Dyson PJ, Sava G. The mechanism of tumour cell death by metal-based anticancer drugs is not only a matter of DNA interactions. Coord Chem Rev. 2018; 360: 17-33. doi: 10.1016/j.ccr.2018.01.009.
35. Islam MS, Rahi MS, Jahangir CA, Jerin I, Hasan MM, Hoque KM, *et al.* Deciphering the molecular pathways of apoptosis using purified fractions from leaf extract of *Basella alba* through studying the regulation of apoptosis-related genes. Mol Biol Rep. 2021; 48(1): 85-96. doi: 10.1007/s11033-021-06136-z, PMID 33454909.
36. Aston WJ, Hope DE, Nowak AK, Robinson BW, Lake RA, Lesterhuis WJ. A systematic investigation of the maximum tolerated dose of cytotoxic chemotherapy with and without supportive care in mice. BMC Cancer. 2017; 17(1): 684. doi: 10.1186/s12885-017-3677-7, PMID 29037232.
37. Ryan L, O'Callaghan YC, O'Brien NM. The role of the mitochondria in apoptosis induced by 7beta-hydroxycholesterol and cholesterol-5beta,6beta-epoxide. Br J Nutr. 2005; 94(4): 519-25. doi: 10.1079/bjn20051524, PMID 16197575.
38. Doi S, Soda H, Oka M, Tsurutani J, Kitazaki T, Nakamura Y, *et al.* The histone deacetylase inhibitor FR901228 induces caspase-dependent apoptosis via the mitochondrial pathway in small cell lung cancer cells. Mol Cancer Ther. 2003; 9: 3052-7.
39. Rotem R, Heyfets A, Fingrut O, Blickstein D, Shaklai M, Flescher E. Jasmonates: novel anticancer agents acting directly and selectively on human cancer cell mitochondria. Cancer Res. 2005; 65(5): 1984-93. doi: 10.1158/0008-5472.CAN-04-3091, PMID 15753398.
40. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol. 2020; 17(7): 395-417. doi: 10.1038/s41571-020-0341-y, PMID 32203277.

Cite this article: Zhang X, Deng S. *In vitro* and *in silico* Analysis Protosappanin B Exerts Anti-Tumor Activity on Breast Cancer Cells via Activating Pro-Apoptotic Caspase Enzymes. Indian J of Pharmaceutical Education and Research. 2025;59(4):1483-92.