

Cyclo(Pro-Tyr) Suppresses Breast Cancer Cell Proliferation Through Modulation of Cyclin-Dependent Kinase Signaling: An *In Silico* and *In Vitro* Study

Shana Balachandran¹, Madan Kumar Arumugam^{1*}, Infanta Michael¹, Vijayavarsine Rajesh¹

¹Cancer Biology lab, Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai-600119, Tamil Nadu, India.

Abstract. Dysregulated cell-cycle progression is a central driver of breast cancer proliferation and is largely orchestrated by cyclin-dependent kinases (CDKs) and their regulatory cyclins. Although Cyclo(Pro-Tyr), a bioactive cyclic dipeptide, has been previously reported to induce apoptosis, its involvement in cell-cycle regulation and proliferative signaling remains insufficiently explored. Given that aberrant CDK activity is a hallmark of breast cancer, the present study investigated the interaction of Cyclo(Pro-Tyr) with key cell-cycle regulatory proteins using molecular docking and evaluated its anti-proliferative potential in breast cancer cells. Molecular docking was carried out using AutoDockTools against CDK2, CDK4, CDK6, and Cyclin D1, which collectively regulate G1-S phase transition. Cyclo(Pro-Tyr) demonstrated suitable binding affinities across all targets and formed stable interactions within functionally relevant regions of the proteins. Interaction analyses indicated a predominantly non-ATP-competitive binding mode, suggesting possible modulation of CDK-cyclin regulatory interfaces rather than direct enzymatic inhibition. Experimental validation using an MTT assay revealed a concentration-dependent reduction in MCF-7 breast cancer cell viability following 24 hours exposure, with an IC_{50} value consistent with suppression of proliferative capacity. Collectively, these findings suggest that Cyclo(Pro-Tyr) may exert anti-proliferative effects through modulation of CDK-driven cell-cycle regulation. This study supported computational evidence linking CDK-cyclin interaction with growth suppression and supports the further exploration of Cyclo(Pro-Tyr) as a multi-target peptide-based candidate for breast cancer therapy.

Keywords: Cyclo(Pro-Tyr), CDKs, breast cancer, cell cycle, molecular docking, MTT assay

*Corresponding author: madankumar@sathyabama.ac.in

1. Introduction

Breast cancer continues to be the one of the leading causes of cancer-related mortality within the women worldwide, with tumor progression largely driven by uncontrolled cellular proliferation [1]. Central to this process is dysregulation of the cell cycle, particularly the transition from the first interphase G1 to S phase, which is tightly controlled by cyclin-dependent kinases (CDKs) and their cyclin partners under routine physiological conditions [2]. Aberrant activation of CDK2, CDK4, and CDK6, often accompanied by overexpression of Cyclin D1, is frequently observed in estrogen-receptor-positive breast cancers and contributes to sustained proliferative signaling [3,4]. The clinical success of CDK4/6 inhibitors has validated cell-cycle kinases as effective therapeutic targets in breast cancer management [5]. However, resistance, toxicity, and pathway redundancy necessitate continued exploration of alternative or complementary modulators of CDK signaling. In this context, peptide-based compounds have attained attention due to their ability to engage regulatory and protein-protein interaction interfaces that may be inaccessible to conventional small-molecule inhibitors [6].

Cyclo(Pro-Tyr) is a cyclic dipeptide with reported biological stability and anticancer activity [7]. Previous studies have exhibited its capacity to suppress cancer cell viability and induce programmed cell death [8]. While these findings emphasize its cytotoxic potential, they do not address whether Cyclo(Pro-Tyr) can modulate proliferative pathways upstream of cell death. Given that growth inhibition often precedes apoptotic commitment, investigation of cell-cycle regulation provides a rational extension of its mechanistic profile. The current study was therefore designed to explore the interaction of Cyclo(Pro-Tyr) with key CDK-mediated cell-cycle regulators using molecular docking and to correlate these predictions with *in vitro* anti-proliferative effects in MCF-7 breast cancer cells. By focusing on proliferative signaling rather than apoptotic endpoints, this work aims to provide novel insight into the anticancer mechanism of Cyclo(Pro-Tyr).

2. Materials and Methods

2.1 Selection of molecular targets

Cyclin-dependent kinases play a crucial role in regulating cell cycle progression, particularly during the G1-S phase transition, which is frequently dysregulated in breast cancer. To explore whether Cyclo(Pro-Tyr) may exert anti-proliferative effects through interaction with cell cycle regulatory proteins, four key targets were selected: CDK2, CDK4, CDK6, and Cyclin D1. CDK2 is a major regulator of G1/S transition, while CDK4 and CDK6 function in complex with Cyclin D1 to promote early G1 progression. Cyclin D1 itself is commonly overexpressed in estrogen-receptor-positive breast cancer and is strongly associated with uncontrolled proliferation. Together, these proteins

represent the core proliferative machinery of breast cancer cells and provide a biologically relevant framework for docking-based prediction and mechanistic interpretation.

2.2 Protein and ligand preparation

Three-dimensional crystal structures of the target proteins were from the Protein Data Bank: CDK2 (PDB ID: 1HCK), CDK4 (PDB ID: 2W96), CDK6 (PDB ID: 1XO2), and Cyclin D1 (PDB ID: 2W9F). Protein structures were prepared by removing crystallographic water molecules and any co-crystallized ligands. Polar hydrogen atoms were added, and Kollman charges were assigned using AutoDock Tools (ADT). The structure of Cyclo(Pro-Tyr) was obtained from the PubChem database (CID: 119404) (<https://pubchem.ncbi.nlm.nih.gov/>) in SDF format and subsequently converted into PDB using Discovery Studio Visualizer (DSV) v19.1.0.18287 (www.accelerys.com). The ligand was energy-minimized and converted into PDBQT format prior to docking. Rotatable bonds were assigned to allow conformational flexibility during docking simulations.

2.3 Molecular docking using AutoDock Tools

Molecular docking was executed using AutoDock Tools to predict the binding affinity and interaction patterns between Cyclo(Pro-Tyr) and the selected cell cycle proteins. Grid boxes were defined to encompass the active or ligand-binding regions of each protein, based on reported functional domains and co-crystallized inhibitor sites. Docking parameters were kept consistent across all targets to ensure comparability. Multiple docking poses were generated for each protein-ligand complex, and the best-ranked conformation was selected based on the lowest binding energy and favorable orientation within the binding pocket. Protein-ligand interactions, including hydrogen bonding and hydrophobic contacts, were visualized and analyzed using Discovery Studio Visualizer v19.1.0.1828 (Dassault Systèmes BIOVIA, Rue Marcel Dassault, Vélizy-Villacoublay-78140, France, www.accel-erys.com).

2.4 In vitro validation

2.4.1. Cell culture and maintenance

The human breast cancer cell line MCF-7 was purchased from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (Cytiva, USA, Catalog# SH30243.01) supplemented with 10% fetal bovine serum heat-inactivated fetal bovine serum (FBS) (Himedia, Catalog# RM10432), 1% (v/v) penicillin-streptomycin solution (100 U/mL penicillin and 100 µg/mL streptomycin) (Cytiva, USA, Catalog# SV30010). Cell Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and subcultured upon reaching approximately 80% confluence.

2.4.2. MTT assay for anti-proliferative activity

The anti-proliferative effect of Cyclo(Pro-Tyr) was evaluated using the MTT assay in accordance with our lab prior publications [9, 10]. Briefly, MCF-7 cells were seeded at a density of 1×10^4 cells per well in 96-wellplate and allowed to adhere overnight at 37 °C. Cells were then treated with Cyclo(Pro-Tyr) at concentrations of 0, 50, 100, 150 and 200 μ M for 24 hours. Following the treatment, MTT solution was added and incubated at 37 °C for four hours to allow formazan crystal formation. The crystals were dissolved using DMSO, and absorbance formed was measured at 570 nm using a microplate reader (Enspire Perkin Elmer, USA). Cell viability were expressed as a percentage relative to untreated controls, and the IC₅₀ value was calculated from the dose–response curve. The MTT assay in this study was used exclusively to validate the predicted anti-proliferative effect of Cyclo(Pro-Tyr), independent of apoptotic endpoints previously reported.

2.5 Statistical analysis

All the experiments were carried out in triplicate, and data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism software. Differences between the treated groups and control were assessed using one-way ANOVA followed by Tukey’s post-hoc test. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Molecular docking analysis of Cyclo(Pro-Tyr) with cell-cycle regulatory proteins

To explore whether Cyclo(Pro-Tyr) could modulate breast cancer cell proliferation through cell-cycle regulation, molecular docking was carried out against key cyclin-dependent kinases and their regulatory partner. Docking analysis revealed that Cyclo(Pro-Tyr) exhibited favorable binding affinities toward CDK2, CDK4, CDK6, and Cyclin D1, suggesting stable and energetically favorable interactions within functionally relevant regions of these proteins. Predicted binding energies obtained using AutoDock Tools are summarized in Figure 1. Cyclo(Pro-Tyr) exhibited favorable binding affinities toward all four targets, with binding energy values ranging from -8.33 to -11.0 kcal/mol, indicating stable protein–ligand interactions. The grid box center coordinates, dimensions, and grid point spacing for all four protein–ligand complexes are summarized in Table 1

Among the proteins analyzed Cyclo(Pro-Tyr) exhibited favorable binding affinities towards all four targets, with binding energy values which were ranging from -8.33 to -11.0 kcal/mol, indicating stable protein–ligand interactions. These values suggest that Cyclo(Pro-Tyr) is capable of interacting with multiple components of the

CDK–Cyclin regulatory axis involved in G1 phase progression. Detailed residue-level interaction analysis revealed the formation of hydrogen bonds and hydrophobic contacts that contribute to the stability of the docked complexes. Predicted binding energies obtained using AutoDock Tools are summarized in Table 2. RMSD values observed were used for pose validation and visualization consistency rather than for cross-target affinity comparison.

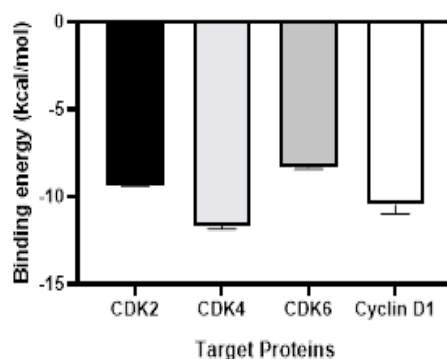


Fig.1. Molecular docking scores of Cyclo(Pro-Tyr) with cell-cycle regulatory proteins: showing the predicted binding energies (kcal/mol) of Cyclo(Pro-Tyr) docked with CDK2, CDK4, CDK6, and Cyclin D1 using AutoDockTools-1.5.6. Lower binding energy values indicates stronger predicted interactions.

Protein	Central Grid Points			Grid dimensional Points			Spacing (Å)
	Center X	Center Y	Center Z	X - points	Y - points	Z - points	
CDK2	103.545	87.462	79.834	102	126	126	0.464
CDK4	-0.238	10.940	14.239	126	124	126	0.375
CDK6	3.106	48.432	136.146	126	126	126	0.458
Cyclin D1	-0.238	12.138	14.798	126	126	126	0.375

Table 1: Grid box center coordinates and dimensions were used for AutoDock Tools docking of each target protein

S. No	Target	Binding Energy (kcal/mol)	RMSD (Å)	Hydrogen/ Conventional Hydrogen Bond Interaction	Hydrophobic Interactions	Other Interactions
1.	Cyclin D	-10.9	20.66	VAL261, GLU263, ARG209, LEU272	GLU265, ALA269, MET264, PRO262, LEU213, PHE214, LEU276, VAL260	PHE208, LEU273, LEU229
2.	cdk2	-9.34	166.75	ILE35, LEU76, ARG36	LYS75, PHE152, THR14, PRO45	VAL44, PRO155, TYR15
3.	Cdk4	-11.0	21.18	VAL72, GLU64	PHE159, PHE93, LEU74, LEU60, ALA65, GLU67, PRO69, LYS155, HIS68, PHE130, PHE66, GLU94, VAL71	LEU63, ARG73
4.	Cdk6	-8.33	143.73	LYS43, GLU61, VAL101	PHE164, ASP163, VAL27, GLN149, ASP104, GLN103, HIS100, GLU99	PHE98, VAL77, ALA162, ALA41, ILE19, LEU152

Table 2. Interaction profile of Cyclo(Pro-Tyr) docked with cell-cycle regulatory proteins.

3.2 Predicted binding poses of Cyclo(Pro-Tyr) within cyclin-dependent kinases

Docking conformations of Cyclo(Pro-Tyr) within CDK2, CDK4, and CDK6 are shown in Figure 2 (A-C). In all three complexes, the ligand was positioned within well-defined pockets of the kinase structures. The binding poses revealed that Cyclo(Pro-Tyr) occupies regions proximal to regulatory domains rather than deeply penetrating the canonical ATP-binding cleft. In CDK6, Cyclo(Pro-Tyr) demonstrated a stable orientation supported by hydrogen bonding and hydrophobic interactions with surrounding residues (Figure 2C). Although the predicted binding energy of CDK6 was modest compared to CDK4, the interaction geometry suggested a favorable and consistent binding mode. The visualization supports the potential of Cyclo(Pro-Tyr) to associate with CDK family members through non-classical binding mechanisms.

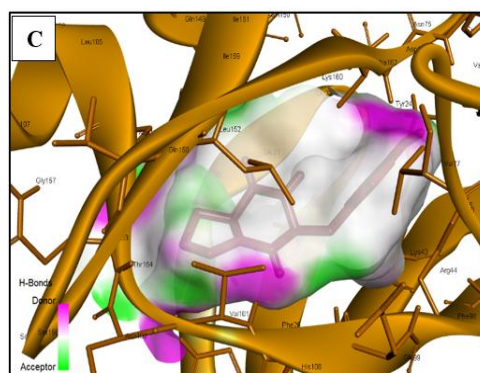
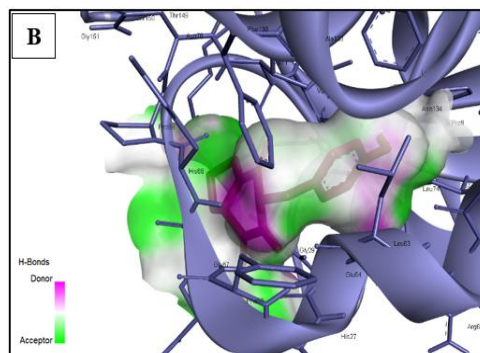
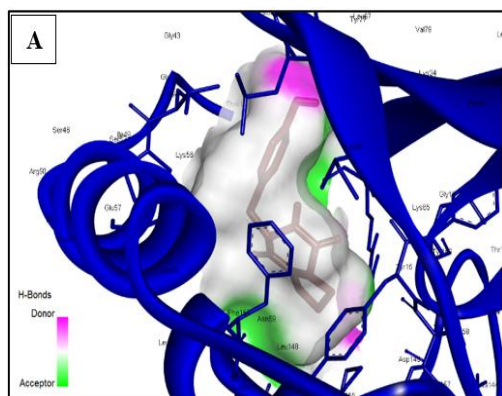


Fig 2. Predicted binding poses of Cyclo(Pro-Tyr) within cyclin-dependent kinases. Docking conformations of Cyclo(Pro-Tyr) within the binding pockets of (A) CDK2, (B) CDK4, and (C) CDK6. The ligand is shown in stick representation, while protein structures are displayed as ribbon or surface models. Key interacting residues are highlighted.

3.3 Interaction analysis of Cyclo(Pro-Tyr) with Cyclin D1

Docking against Cyclin D1 revealed that Cyclo(Pro-Tyr) could interact within regions involved in CDK binding, suggesting a potential to interfere with CDK–cyclin complex formation. Interaction analysis indicated that the binding was stabilized predominantly through hydrogen bonding and hydrophobic contacts rather than classical ATP-mimetic interactions. This pattern suggests that Cyclo(Pro-Tyr) may function as a non-canonical modulator of CDK activity, consistent with the behavior of small cyclic peptides. The two-dimensional interaction map highlighted specific amino acid residues contributing to ligand stabilization, reinforcing the predicted affinity observed in docking score analysis. These findings suggest that Cyclo(Pro-Tyr) may influence Cyclin D1 function by engaging regulatory interfaces involved in CDK complex formation rather than acting as a direct enzymatic inhibitor. Collectively, these molecular docking results predict that Cyclo(Pro-Tyr) can associate with multiple components of

the CDK–Cyclin D axis that governs breast cancer cell proliferation.

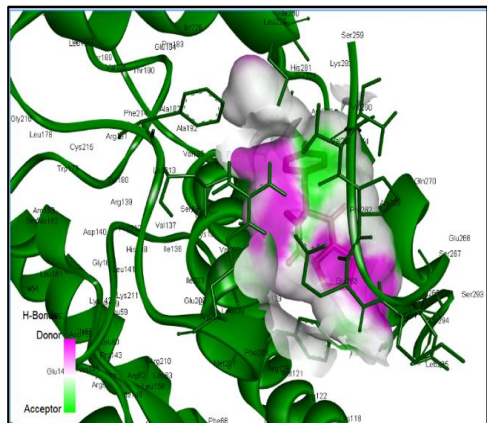


Fig 3. Interaction analysis of Cyclo(Pro-Tyr) with Cyclin D1.

3.4 Cyclo(Pro-Tyr) suppresses MCF-7 cell proliferation in a dose-dependent manner

The anti-proliferative effect of Cyclo(Pro-Tyr) was evaluated in MCF-7 breast cancer cells using the MTT assay after 24 hours of treatment. Consistent with the conditions used in the earlier our lab reports, Cyclo(Pro-Tyr) produced a clear, concentration-dependent reduction in cell viability across the tested range (0–150 μM) (Figure 4). A progressive decline in metabolic activity was observed with increasing concentrations of Cyclo(Pro-Tyr), resulting in a half-maximal inhibitory concentration ($\text{IC}_{50}=82.1\mu\text{M}$) comparable to our previous reports [9]. The reproducibility of the IC_{50} value under identical experimental conditions confirms the robustness of the compound’s anti-proliferative effect and allows direct correlation with the mechanistic predictions generated through docking analysis. In the framework of the present study, the reduction in cell viability is interpreted as suppression of proliferative capacity rather than induction of apoptosis, which has already been comprehensively characterized. The observed growth inhibition aligns with the predicted interaction of Cyclo(Pro-Tyr) with CDK-mediated cell-cycle regulatory pathways.

4. Discussion

Uncontrolled proliferation driven by dysregulated cell-cycle progression is a defining feature of breast cancer, particularly in estrogen-receptor-positive tumors such as MCF-7 cells [9, 10]. Cyclin-dependent kinases, especially CDK2, CDK4, and CDK6, together with Cyclin D1, constitute the core machinery governing the G1–S transition

and represent validated therapeutic targets in breast cancer [11]. Although CDK4 and Cyclin D1 exhibited lower predicted binding energy values compared to CDK6, the latter was prioritized for mechanistic interpretation due to its established role in sustaining proliferative signaling and therapeutic resistance in breast cancer. The predicted interaction of Cyclo(Pro-Tyr) with Cyclin D1 further supports the possibility of modulating CDK–Cyclin complex formation, a critical step in G1 phase progression.

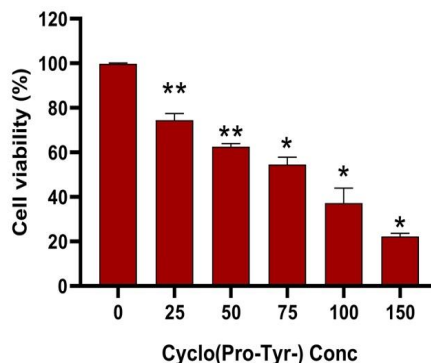


Fig.4. Anti-proliferative effect of Cyclo(Pro-Tyr) on MCF-7 breast cancer cells. Dose–response curve showing percentage cell viability of MCF-7 cells treated with increasing concentrations of Cyclo(Pro-Tyr) (0–150 μM) for 24 hours, as assessed by the MTT assay. Data are presented as mean \pm SEM (n = 3). Statistical significance was determined using one-way ANOVA followed by Tukey’s post-hoc test *($p \leq 0.05$) and **($p \leq 0.01$).

The interaction of Cyclo(Pro-Tyr) with Cyclin D1 further supports the possibility that this cyclic dipeptide may interfere with CDK–cyclin complex assembly, rather than acting solely as a classical ATP-competitive kinase inhibitor. Such non-canonical modes of interaction are increasingly recognized for peptide-based modulators and may offer advantages in terms of selectivity and reduced off-target effects [12]. The *in silico* predictions are supported by *in vitro* MTT data demonstrating consistent, dose-dependent suppression of MCF-7 cell proliferation following Cyclo(Pro-Tyr) treatment. Together, the present findings suggest that Cyclo(Pro-Tyr) exerts anti-proliferative effects that may precede or complement downstream cell death pathways described elsewhere.

Conclusion

This study positions Cyclo(Pro-Tyr) as a multifunctional anticancer peptide capable of targeting both proliferative signaling and apoptotic pathways. Cyclo(Pro-Tyr) exhibits stable interactions with key cell-cycle regulatory proteins Cyclin-dependent kinases including CDK4, CDK2, CDK6, Cyclin D1, and suppresses proliferation of breast cancer

cells *in vitro* suggesting dysregulation are linked to Cell proliferation [9 ,13]. Molecular docking and interaction analyses indicate that Cyclo(Pro-Tyr) engages regulatory regions of these proteins rather than acting as a classical kinase inhibitor making them target for further therapeutic studies.

References

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, Jemal A. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2024 May-Jun;74(3):229-263. doi: 10.3322/caac.21834. Epub 2024 Apr 4. PMID: 38572751.
2. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer.* 2009 Mar;9(3):153-66. doi: 10.1038/nrc2602. PMID: 19238148.
3. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 1999 Jun 15;13(12):1501-12. doi: 10.1101/gad.13.12.1501. PMID: 10385618.
4. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, Ginther C, Atefi M, Chen I, Fowst C, Los G, Slamon DJ. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines *in vitro*. *Breast Cancer Res.* 2009;11(5):R77. doi: 10.1186/bcr2419. PMID: 19874578; PMCID: PMC2790859.
5. Hortobagyi GN, Stemmer SM, Burris HA, Yap YS, Sonke GS, Paluch-Shimon S, Campone M, Blackwell KL, André F, Winer EP, Janni W, Verma S, Conte P, Arteaga CL, Cameron DA, Petrakova K, Hart LL, Villanueva C, Chan A, Jakobsen E, Nusch A, Burdaeva O, Grischke EM, Alba E, Wist E, Marschner N, Favret AM, Yardley D, Bachelot T, Tseng LM, Blau S, Xuan F, Souami F, Miller M, Germa C, Hirawat S, O'Shaughnessy J. Ribociclib as First-Line Therapy for HR-Positive, Advanced Breast Cancer. *N Engl J Med.* 2016 Nov 3;375(18):1738-1748. doi: 10.1056/NEJMoa1609709. Epub 2016 Oct 7. Erratum in: *N Engl J Med.* 2018 Dec 27;379(26):2582. doi: 10.1056/NEJMc180043. PMID: 27717303.
6. Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. *Drug Discov Today.* 2015 Jan;20(1):122-8. doi: 10.1016/j.drudis.2014.10.003. Epub 2014 Oct 17. PMID: 25450771.
7. Sirpu Natesh N, Arumugam M, Karanam G. Apoptotic role of marine sponge symbiont *Bacillus subtilis* NMK17 through the activation of caspase-3 in human breast cancer cell line. *Mol Biol Rep.* 2018 Dec;45(6):2641-2651. doi: 10.1007/s11033-018-4434-y. Epub 2018 Nov 9. PMID: 30414102.
8. Karanam G, Arumugam MK. Potential anticancer effects of cyclo(-Pro-Tyr) against N-diethyl nitrosamine induced hepatocellular carcinoma in mouse through PI3K/AKT signaling. *Environ Toxicol.* 2022 Feb;37(2):256-269. doi: 10.1002/tox.23395.
9. Karanam G, Arumugam MK. Reactive oxygen species generation and mitochondrial dysfunction for the initiation of apoptotic cell death in human hepatocellular carcinoma HepG2 cells by a cyclic dipeptide Cyclo(-Pro-Tyr). *Mol Biol Rep.* 2020 May;47(5):3347-3359. doi: 10.1007/s11033-020-05407-5
10. Sirpu Natesh N, Arumugam M, Karanam G. Apoptotic role of marine sponge symbiont *Bacillus subtilis* NMK17 through the activation of caspase-3 in human breast cancer cell line. *Mol Biol Rep.* 2018 Dec;45(6):2641-2651. doi: 10.1007/s11033-018-4434-y.
11. Glaviano A, Singh SK, Lee EHC, Okina E, Lam HY, Carbone D, Reddy EP, O'Connor MJ, Koff A, Singh G, Stebbing J, Sethi G, Crasta KC, Diana P, Keyomarsi K, Yaffe MB, Wander SA, Bardia A, Kumar AP. Cell cycle dysregulation in cancer. *Pharmacol Rev.* 2025 Mar;77(2):100030. doi: 10.1016/j.pharmr.2024.100030
12. Zabihi M, Lotfi R, Yousefi AM, Bashash D. Cyclins and cyclin-dependent kinases: from biology to tumorigenesis and therapeutic opportunities. *J Cancer Res Clin Oncol.* 2023 Apr;149(4):1585-1606. doi: 10.1007/s00432-022-04135-6
13. Zhang X, Wang Q, Luo Y, Song M, Zhou Z, Zeng L et al (2021c) Cyclin-dependent kinase 15 upregulation is correlated with poor prognosis for patients with breast cancer. *J Int Med Res* 49(6):300060521999552. 10.1177/0300060521999552