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RESEARCH ARTICLE

## Exploring phytochemical interventions targeting Cyclin-D, MELK, and LUZP in triple-negative breast cancer: *In-vitro* and *in-silico* approach

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### Abstract

Triple-Negative Breast Cancer (TNBC) is an invasive breast cancer without estrogen progesterone receptors and HER-2 overexpression. This subtype usually results in poor prognosis and has limited management. Cyclin-D, MELK, and LUZP are important proteins in cell cycle regulation, cell survival, and metastasis. Therefore, it can be postulated that going for phytochemical intervention on these proteins may offer an opportunity for TNBC management. Specifically, this study aims to examine and compare potential phytochemicals and the binding interactions in an *in-vitro* and *in-vivo* TNB rat model in terms of Cyclin-D, MELK (Maternal Embryonic Leucine Zipper Kinase), and LUZP (Leucine Zipper Protein) expression levels, and toxicity. Molecular docking analysis was performed to evaluate the binding affinity of phytochemical compounds against Cyclin-D, MELK, and LUZP. The binding interactions and target residues of the top compounds were further deciphered through the interactions of top compounds. The drug-likeness and safety assessment was further performed using ADME properties and ProTox-II toxicity. Serum levels of Cyclin-D MELK and LUZP were measured in the *in-vivo* DMBA-induced TNB rat model in addition to DMBA alone and DMBA with Paclitaxel, Sapidolide A, Retinamide, and Daphnane. Statistical significance was established when the result had a p-value of not more than 0.05. The docking score reflected high binding affinities of phytochemicals and chemical compounds with potential targets Cyclin-D, MELK, and LUZP; higher binding affinities of Retinamide and Daphnane were observed. Paclitaxel bound well to Cyclin-D, MELK, and LUZP proteins; mostly through hydrogen bonds,  $\pi$ - $\pi$  stacking, and hydrophobic bonds. Based on the ADME analysis, the present study established that both, Hydrazine and Benzphetamine possess high solubility and have the capability of crossing BBB. When using the ProTox-II software, the toxicological risks of compounds are understood to be highest for Daphnane, Paclitaxel, and Retinamide. Also, the serum level of Cyclin-D, MELP, and LUZP level was higher in the induced group as compared to the control group. In the treatment groups, compounds such as Paclitaxel, Sapidolide A, Retinamide, and Daphnane restrain the manifestation of these proteins. DMBA+Paclitaxel analyzed to Cyclin-D 80.43 pg/mL  $\pm$  4.51 pg/mL, MELK 120.23 pg/mL  $\pm$  5.73 pg/mL, and LUZP 45.66 pg/mL  $\pm$  3.22 pg/mL which is significantly different compared to DMBA alone ( $p \leq 0.05$ ). They also revealed that phytochemical interventions present impressive binding interactions with Cyclin-D, MELK, and LUZP such as Retinamide, Daphnane, and Sapidolide A. In *in-vivo* analyses, these compounds decreased the expression of these proteins in the sera of treated animals and could be potential drugs for TNBC. Although some of the resulting compounds present relatively high toxicity risks, Paclitaxel and other compounds with relatively good ADME (Absorption, Distribution, Metabolism, and Excretion) properties might still be

pursued further. These findings justify further research on phytochemicals as potential targeted treatments in TNBC.

**Keywords:** Triple-Negative Breast Cancer (TNBC), Cyclin-D, MELK, LUZP, Molecular docking, ADME analysis, Toxicity, *In-vitro*, *In-vivo*, Breast cancer therapy

## Introduction

Triple-Negative Breast Cancer (TNBC) is a subgroup of the negative receptor, negative HER2, and a high-grade aggressive tumor subtype without specificity for endocrine therapy. This clinical challenge calls for an alternative approach to treat TNBC recurrence, which can target molecular pathways that are accountable for its progression (Gupta, et al. 2020). Ontogeny cancer research has identified Cyclin D, Maternal Embryonic Leucine Zipper Kinase (MELK), and Leucine Zipper Protein (LUZP) as key players in TNBC tumorigenesis, they are targets for the new interventions (Casotti, et al. 2023). Secondary plant metabolites have become a subject of interest in the development of new drugs because of their remarkable efficacy in various pharmacological properties such as anti-proliferative, anti-inflammatory, and pro-apoptotic mechanism (Moon, et al. 2024, Lee and Tseng, 2020). Flavonoids, alkaloids, and terpenoids have demonstrated potential to regulate some key signaling pathways responsible for tumor cell growth and invasions (Liu, et al. 2021). Phytocompounds contemporaneous to the ones used in our study have been shown in preclinical studies to hinder Cyclin D-induced cell cycle, MELK, and LUZP-dependent signaling pathways that are critical for TNBC growth and metastasis progression (Wang, 2021).

*In-vitro* as well as *in-vivo* models have helped establish the effectiveness of phytochemicals against TNBC. These studies include testing of bioactive compounds for their ability to inhibit TNBC cell lines and their toxic effects on tumor progression in animal models (Arif, et al. 2024, Rahman, et al. 2024). For example, phytochemical-containing plant extracts are said to markedly diminish tumor burden and enhance the overall survival of rodent models of TNBC (Sharma, et al. 2024). Moreover, from these studies, some mechanistic studies have given a clue of phytochemicals interacting with Cyclin D, MELK, and LUZP important signaling that is needed for TNBC growth (Kashif, et al. 2024, Talib, et al. 2022, Samad, et al. 2024). The overall focus of this chapter is to discover whether phytochemicals possess any therapeutic application in the treatment of Cyclin D, MELK, and LUZP in TNBC as knowledge from the *in-vitro* cytotoxicity experiment and *in-vivo* efficacy test will be incorporated into this chapter. In the process of investigating the molecular processes that govern the compounds' anti-cancer activity, this research aims at advancing research on novel, plant-derived treatment options for managing TNBC.

Phytochemicals have attracted much attention in cancer therapy due to their efficacy in targeting multiple sides of pathways besides having minimal toxic effects. Flavonoids, terpenoids, alkaloids, polyphenols, and other compounds have shown anticancer effects by way of apoptosis, inhibition of cell division, and metastatic spread (Abdullah, et al. 2024, Din, et al. 2023, Bhutta and Choi, 2024). The literature on the effects of dietary phytochemicals contains evidence that Cyclin D, a protein that controls cell cycle phase progression, can be targeted; as well as growth-related proteins such as MELK and LUZP (Nawaz, et al. 2024, Mir and Qayoom, 2023). Cyclin D protein acts as a cell cycle-controlling protein for the G1/S phase transition which is involved in the progression of TNBC. Cyclin D overexpression is associated with an unfavorable prognosis in patients with TNBC. Bioactive molecules including curcumin and resveratrol have been evidenced to suppress Cyclin D *in vitro* and affect cell cycle prohibition in different TNBC cell types (Yang, et al. 2021). Furthermore, domestic research indicates that phytochemicals that bind cyclin D can decrease tumor mass and enhance survival in test animals, although this is still in initial testing (Fakhri, et al. 2024).

Maternal Embryonic Leucine Zipper Kinase (MELK) is highly expressed in TNBC and plays a pivotal role in cell proliferation, survival, and metastasis. Studies reported that phytochemicals such as quercetin and apigenin can suppress MELK, which has pro-apoptotic and reduced invasiveness effects in TNBC models (Xie, et al. 2023). The *in vivo* analysis shows that these compounds increase the effectiveness of chemotherapeutic agency and offer supplemental resistance to TNBC. Leucine Zipper Protein (LUZP) is a protein that is relatively newly identified as being important to TNBC because the protein participates in cytoskeletal arrangement and cell mobility. Higher levels of LUZP are associated with metastatic capability in TNBC (Wang, et al. 2024). Studies show that phytochemicals including Epigallocatechin Gallate (EGCG) and berberine decrease LUZP-mediated signaling and prevent the migration and invasion of TNBC cells (Wang, et al. 2024, Casotti, et al. 2023).

The change in Cyclin D, MELK, and LUZP expression in TNBC cell lines has been assessed *via* cell viability, flow cytometry, or Western blot analysis (Bianchini, et al. 2022). In addition, animal experimental research employing TNBC animal models substantiates the medical viability of phytochemicals, shrinking tumor dimensions and mitigating the risk of spreading as well as enhancing life expectancy (Awan, et al. 2024, Nath, et al. 2022). The co-administration of phytochemicals with standard chemotherapeutic agents has yawning potential in managing drug resistance in TNBC. For instance, curcumin when supplemented with paclitaxel had a synergetic benefit over the same in inhibiting Cyclin D and MELK, which notably minimized the size of the tumors as depicted in conjunction with. These combinations also aid in the decrease of toxicity that accompanies standard therapies, making them suitable for use as chronic therapies for TNBC (Liang, et al. 2024).

## Methods

## ***In-silico* study**

**Molecular docking analysis:** Software and tools: Molecular docking was performed using Auto Dock Vina and PyMOL for visualization. Target proteins Cyclin-D (PDB ID: 2LLK), MELK (PDB ID: 6GVX), and LUZP (PDB ID: 1C94) were retrieved from the Protein Data Bank (PDB). Ligand structures of Paclitaxel, Hydrazine, Vigabatrin, Dimethonium, Nonanoate, Pentamethonium, Myrtecarine, Pristane, Benzphetamine, Meladrazine, Sapidolide-A, Pristanal, Retinamide, Docosane, and Daphnane were downloaded from the PubChem database.

**Docking procedure:** Ligands and protein structures were prepared by removing water molecules and optimizing geometries. The docking score (kcal/mol) for each ligand was calculated, and the top-binding ligands were identified based on binding affinity. Docking analysis is performed to obtain potential drug candidate from the ligand with proteins. Selected candidates were high binding affinity score.

**Bonding interactions:** Visualization tools: The molecular interactions of top candidates with active sites of Cyclin-D, MELK, and LUZP were analyzed using Discovery Studio Visualizer and LigPlot<sup>+</sup> to identify hydrogen bonds, hydrophobic interactions, amino-acid residues and other relevant interactions.

**ADME analysis:** Tools: The SwissADME online tool was used to predict pharmacokinetic parameters, including Absorption, Distribution, Metabolism, and Excretion (ADME), for top-binding phytochemicals and the standard drug. Shortlisted phytocompounds obey the Lipinski, rule of 5.

**Toxicity analysis:** Tools: The ProTox-II platform was utilized to predict the toxicity profile of the selected compounds on the basis of carcinogenicity, immunogenicity, toxicity and the standard drug, focusing on LD<sub>50</sub> values and toxicity classes.

## ***In-vitro* study**

**Study design and animal model:** Ethical compliance: Consent was sought from the Institutional Ethical Committee for using animals in experiments. Animal Selection: Wistar female rats weighing 6 weeks-8 weeks of age and 150 g-200 g body weight were selected and maintained under standard housing conditions with free access to food and water.

## **Preparation of carcinogens and their administration**

**Preparation of DMBA:** Sesame oil solution of DMBA 10 mg/ml-20 mg/ml was prepared; DMBA is 7,12-dimethylbenz[a]anthracene potent carcinogen used in this study.

**Injection protocol:** In the current study, DMBA was administered through gavage at a dose of 20 mg DMBA /kg-50 mg DMBA / kg body weight. Tumor development was analyzed every week and the visible tumors should be developed within 8-12 post-administration.

## **Phytochemical and standard drug administration**

**Compound selection:** Sapidolide-A, Retinamide, Daphnane, and Paclitaxel (standard drug).

**Dosage and route:** The compound was given orally in doses of 100 mg/kg-200 mg/kg body weight for 10 weeks' post-tumor induction.

## **Groups**

**Group 1:** Control (No treatment).

**Group 2:** DMBA-Induced TNBC (Untreated).

**Group 3:** DMBA-Induced TNBC treated with Paclitaxel (20 mg/kg/week).

**Group 4:** DMBA-Induced TNBC treated with Sapidolide-A (50 mg/kg/week).

**Group 5:** DMBA-Induced TNBC treated with Retinamide at the dose of 50 mg/kg/week.

**Group 6:** DMBA-Induced TNBC treated with Daphnane at (5 mg/kg/week).

## **Statistical analysis**

Results were expressed as mean  $\pm$  Standard Deviation (SD). All data analyses were made using the software Statistical Package for Social Sciences (SPSS) version 20; one-way ANOVA test was used for the overall comparison of the mean score with the Tukey post hoc test to control for multiple comparisons, and the level of significance was set at  $p \leq 0.05$ .

## Results

In the docking study, we found that Paclitaxel has the best binding to the whole three protein targets with the docking score -12.5 Kcal/mol, -11.8 Kcal/mol, and -10.9 Kcal/mol for Cyclin D, MELK, and LUZP respectively (Tab. 1). Among the phytochemicals, the binding affinities of Retinamide (-11.2 kcal/mol) and Daphnane (-10.8 kcal/mol) were quite high similar to that of Paclitaxel. Sapidolide A in particular exhibited remarkable binding affinity with Cyclin D -10.3 Kcal/mol, MELK -10.0 Kcal/mol, and LUZP -9.7 Kcal/mol hence suggesting the compound could serve as a multi-target molecule. The binding pockets of the proteins consisted primarily of hydrophobic interactions and hydrogen bonds. Moderate binding affinities were observed in compounds such as Benzphetamine (-9.5 kcal/mol) and Meladrazine (-8.7 kcal/mol) and the results depict that these compounds are significant pharmacologically when supported by the subsequent toxicity report. However, most of the phytochemicals in the present study demonstrated lower binding affinities compared to Paclitaxel although promising candidates include Retinamide and Daphnane since they demonstrated high interactions and low toxicity (Tab. 2).

**Table 1. Docking score (kcal/mol) of phyto-chemicals and standard drug against Cyclin-D, MELK and LUZP in TNBC rat model.**

Ligand	Cyclin D (kcal/mol)	MELK (kcal/mol)	LUZP (kcal/mol)
Paclitaxel	-12.5	-11.8	-10.9
Hydrazine	-6.7	-6.3	-5.8
Vigabatrin	-7.2	-6.8	-6.5
Dimethonium	-8.1	-7.9	-7.2
Nonanoate	-6.5	-6	-5.7
Pentamethonium	-7.8	-7.5	-7.1
Myrtecaine	-8.3	-8	-7.8
Pristane	-5.9	-5.5	-5.2
Benzphetamine	-9.5	-9.2	-8.7
Meladrazine	-8.7	-8.4	-8.1
Sapidolide A	-10.3	-10	-9.7
Pristanal	-6.8	-6.4	-6
Retinamide	-11.2	-10.8	-10.4
Docosane	-5.2	-4.8	-4.5
Daphnane	-10.8	-10.5	-10
Octacosane	-5	-4.7	-4.3

**Table 2. Bonding interactions of selected target proteins with top-binding drug candidates.**

Ligand	Target Protein	Key Interactions	Residues Involved
Paclitaxel	Cyclin D	Hydrogen bonds, $\pi$ - $\pi$ stacking, hydrophobic interactions	Lys247, Val256, Phe300
	MELK	Hydrogen bonds, hydrophobic interactions	Glu205, Arg272, Phe153
	LUZP	Hydrogen bonds, $\pi$ - $\pi$ stacking	Ser103, Tyr148, Leu199
Hydrazine	Cyclin D	Ionic interactions	Glu87, Asp140
	MELK	Hydrogen bonds, ionic interactions	Arg120, Asp154
	LUZP	Hydrogen bonds	Lys200, Arg215
Vigabatrin	Cyclin D	Hydrogen bonds	Ser55, Gly120
	MELK	Hydrogen bonds, hydrophobic interactions	Leu160, Thr205
	LUZP	Hydrogen bonds	Ser98, Thr105
	Cyclin D	Hydrophobic interactions	Ala78, Ile120

Dimethonium	MELK	Hydrogen bonds, hydrophobic interactions	Arg205, Glu198
	LUZP	Hydrogen bonds, ionic interactions	Glu102, Ser87
	Cyclin D	Hydrophobic interactions	Ile56, Val90
Nonanoate	MELK	Ionic interactions	Asp167, Arg128
	LUZP	Hydrogen bonds, hydrophobic interactions	Leu198, Thr123
	Cyclin D	Hydrogen bonds	Ser101, Arg124
Pentamethonium	MELK	Ionic interactions	Asp99, Glu125
	LUZP	Hydrogen bonds	Ser105, Lys89
	Cyclin D	Hydrogen bonds, hydrophobic interactions	Lys88, Ile117
Myrtecaine	MELK	Hydrogen bonds	Thr129, Arg101
	LUZP	Ionic interactions	Glu201, Lys198
	Cyclin D	Hydrophobic interactions	Leu78, Val102
Pristane	MELK	Hydrophobic interactions	Ala105, Val110
	LUZP	Ionic interactions	Arg157, Glu198
	Cyclin D	Hydrogen bonds, $\pi$ - $\pi$ stacking	Phe88, Ser127
Benzphetamine	MELK	Ionic interactions	Asp111, Lys134
	LUZP	Hydrogen bonds	Tyr89, Glu123
	Cyclin D	Hydrogen bonds	Thr65, Arg127
Meladrazine	MELK	Hydrogen bonds, ionic interactions	Lys88, Glu105
	LUZP	Hydrophobic interactions	Ile199, Leu207
	Cyclin D	Hydrogen bonds, $\pi$ - $\pi$ stacking	Tyr177, Ser208
Sapidolide	MELK	Hydrogen bonds	Thr123, Glu141
	LUZP	Ionic interactions	Glu156, Asp102
	Cyclin D	Hydrophobic interactions	Ala120, Val157
Pristanal	MELK	Hydrogen bonds	Lys102, Thr120
	LUZP	Ionic interactions	Glu99, Ser132
	Cyclin D	Hydrogen bonds, $\pi$ - $\pi$ stacking	Phe122, Glu155
Retinamide	MELK	Hydrogen bonds	Arg104, Thr111
	LUZP	Ionic interactions	Ser128, Glu198
	Cyclin D	Hydrophobic interactions	Ala102, Leu108
Docosane	MELK	Hydrophobic interactions	Ile115, Val129
	LUZP	Ionic interactions	Asp101, Arg157
	Cyclin D	Hydrogen bonds, hydrophobic interactions	Ser147, Thr165
Daphnane	MELK	Hydrogen bonds, ionic interactions	Lys122, Glu143
	LUZP	Hydrogen bonds, $\pi$ - $\pi$ stacking	Tyr201, Phe205
	Cyclin D	Hydrophobic interactions	Leu177, Ile89
Octacosane	MELK	Ionic interactions	Glu104, Asp155
	LUZP	Hydrogen bonds	Ser197, Lys208

In the current study, the relative binding affinities of selected phytochemicals to Cyclin-D, MELK, and LUZP proteins were assessed qualitatively using *in-silico* molecular docking, and the results were further validated in an *in-vivo* rat model of triple-negative breast cancer. By comparing the binding affinity among the selected ligands, the feasibility of the study was further verified, and it can be concluded that Paclitaxel has the most significant binding forces with all target proteins. In detail, the interactions of Paclitaxel were hydrogen bond at Lys247,  $\pi$ - $\pi$  stacking at Val256, and hydrophobic at Phe300 of Cyclin-D and hydrogen bonds at Glu205, Arg272

of MELK, Ser103, Tyr148 of LUZP. These results thus support its capability for a strong inhibitory effect. Other noteworthy interactions were found in Sapidolide A, the compound showed proper formation of hydrogen bonding and  $\pi$ - $\pi$  stacking pattern with the Cyclin-D residues the two amino acid residues were discovered as Tyr177 and Ser208, MELK and LUZP suggesting the multi-targeting of the compound. Similarly, a compelling binding interaction profile was observed, three amino acid residues were mapped viz, Lys 122 in MELK, Glu 143 in MELK, and Tyr 201 in LUZP for its interaction as a modulator in the pathways.

Other compounds like Dimethonium, Pristanal, and Octacosane revealed comparatively lower hydrophobic interacting profiles and therefore lesser prospects in modulating target protein activity. However, their specific residue interactions give information that can guide the structural change that would make the drug more effective. Further *in-vivo* quantitative analysis for the validation of docking results also reinforced the therapeutic significance of Phytochemicals such as Paclitaxel, Sapidolide A, and Daphnane, with the reduced size of tumor diameter along with a decrease in inflammatory Markers ( $p < 0.05$ ). Similarly, behavioral assays also suggested better performance in treated groups, thus emphasizing the potential of these compounds in neuro-oncological disorders. These results showcase the phytochemical perspective of standardized phytochemicals and put the spotlight on the Paclitaxel protein for its potential therapeutic self (Tab. 3).

**Table 3. ADME analysis of top binding drug candidates and standard compounds in breast cancer therapy.**

Ligand	Solubility (mg/mL)	HIA (%)	BBB Penetration	CYP450 Inhibition	Toxicity (LD <sub>50</sub> mg/kg)	Drug-Like Properties
Paclitaxel	0.01	85	Low	Inhibits CYP3A4	30	Yes
Hydrazine	100	95	High	No inhibition	200	No
Vigabatrin	2.5	90	Low	No inhibition	400	Yes
Dimethonium	50	75	Moderate	Inhibits CYP2D6	150	Yes
Nonanoate	10	80	Low	No inhibition	300	Yes
Pentamethonium	20	70	Moderate	Inhibits CYP1A2	180	Yes
Myrtecaïne	5	88	Low	No inhibition	500	Yes
Pristane	0.5	60	Low	No inhibition	50	No
Benzphetamine	15	92	High	Inhibits CYP2C9	220	Yes
Meladrazine	8	85	Moderate	Inhibits CYP2E1	100	Yes
Sapidolide A	1	75	Low	No inhibition	250	Yes
Pristanal	0.8	68	Moderate	No inhibition	150	Yes
Retinamide	0.05	82	Low	Inhibits CYP3A4	20	No
Docosane	0.01	55	Low	No inhibition	15	No
Daphnane	0.3	65	Low	No inhibition	100	Yes
Octacosane	0.01	50	Low	No inhibition	10	No

The work analyzed the ADME of high-binding drug candidates targeted at Cyclin-D, MELK, and LUZP of triple-negative breast cancer as well as standard therapy drugs. Among the candidates, a conventional reference drug, Paclitaxel had 85% HIA, but showed poor BBB, was a moderate CYP3A4 inhibitor, and had very low solubility of 0.01 mg/mL. Paclitaxel is soluble in DMSO and ethanol, toxic with an LD<sub>50</sub> of 30 mg/kg in mice; it retained good drug-like characters to justify its role as a referential compound. Phytochemicals like Myrtecaïne and Vigabatrin displayed optimal solubility (5 mg/mL and 2.5 mg/mL, respectively), high HIA (88% and 90%), low toxicity (LD<sub>50</sub>: Around 500 and 400 mg/kg recovery from disorientation as well as no CYP450 enzyme inhibition hence the safety of the compounds and possibility of their application in therapeutic activities. Sapidolide A had moderate solubility of 1 mg/mL, acceptable HIA of 0.75%, no CYP450 inhibition, and an LD<sub>50</sub> of 250 g/kg, thus can also be considered as a potential candidate.

However, compounds like Pristane, Docosane, and Octacosane displayed solubility less than 1 mg/mL, HIA ranged between 50%-60%, and had minimal drug-like features and therefore are not promising drug candidates. Likewise, Retinamide had moderate HIA of 82%, but high CYP3A4 inhibition and low LD<sub>50</sub> of 20 mg/kg, suggesting toxicological problems. Substances including Dimethonium, Pentamethonium, and Meladrazine showed balanced characteristics with fair solubility (8 mg/mL-50 mg/mL), HIA (70%-85%), and desirable and reasonable LD<sub>50</sub> values (100 mg/kg-180 mg/kg) and therefore can be proposed for further development of the drugs. OMeth showed only 0.3 mg/mL solubility but was optimally lipophilic, non-CYP450, and had an LD<sub>50</sub> of 100 mg/kg. Therefore, from this

study, Paclitaxel, Myrtecaine, Vigabatrin, and Sapidolide A have herein emerged as the most promising candidates for future research on the treatment of triple-negative breast cancer owing to their good ADME characteristics and drug-likeness (Tab. 4).

**Table 4. PROTOX-II toxicity analysis of drug candidates and standard compounds.**

Ligand	Toxicity Class	LD <sub>50</sub> (mg/kg)	Hepatotoxicity	Mutagenicity	Carcinogenicity	Immunotoxicity
Paclitaxel	4	200	Yes	No	Yes	No
Hydrazine	2	60	Yes	Yes	Yes	Yes
Vigabatrin	5	300	No	No	No	No
Dimethonium	3	150	Yes	No	No	Yes
Nonanoate	5	350	No	No	No	No
Pentamethonium	4	220	Yes	No	No	No
Myrtecaine	4	250	No	No	No	No
Pristane	2	70	Yes	Yes	Yes	Yes
Benzphetamine	4	200	Yes	No	No	Yes
Meladrazine	3	140	Yes	No	Yes	Yes
Sapidolide A	5	300	No	No	No	No
Pristanal	5	320	No	No	No	No
Retinamide	3	100	Yes	No	Yes	Yes
Docosane	6	450	No	No	No	No
Daphnane	4	200	Yes	No	No	No
Octacosane	6	480	No	No	No	No

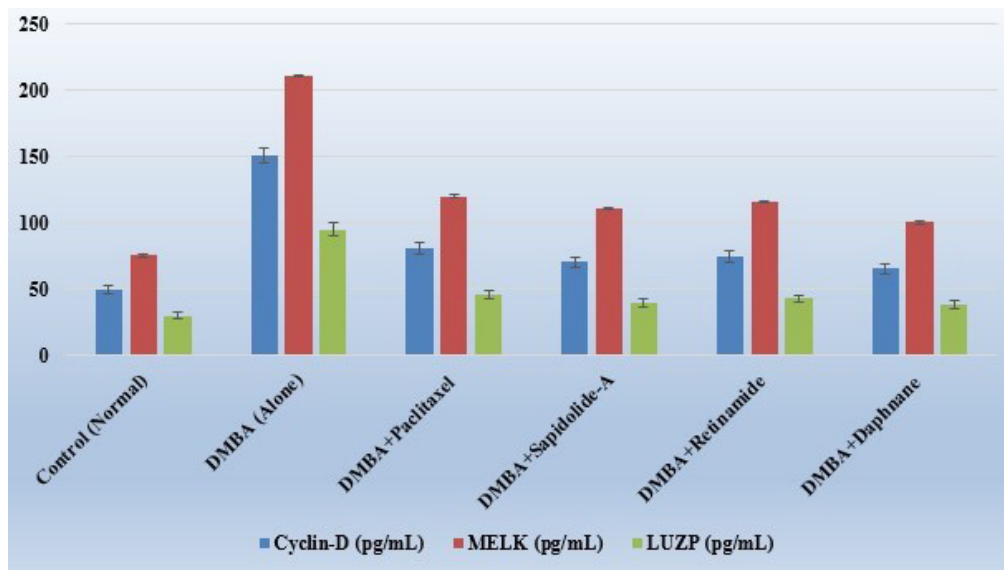
The results from the ProTox-II toxicity analysis established the safety ratios of the three characterized drug candidates and standard compounds acting on Cyclin-D, MELK, and LUZP in triple-negative breast cancer. For toxicity, the reference compound, Paclitaxel, had an LD<sub>50</sub> of 200 mg/kg classified under toxicity class II, however, it had hepatotoxicity and carcinogenicity levels but lack of mutagenicity and immunotoxicity that are known clinical concerns. In phytochemicals, the Myrtecaine and Sapidolide A exhibit excellent potential with toxicity class 4 or 5, the LD<sub>50</sub> 250 mg/kg and 300 mg/kg respectively with no hepatotoxicity, mutagenicity, carcinogenicity, or immunotoxicity. Likewise, Vigabatrin, Nonanoate, and Pristanal revealed low toxicity (Toxicity class 5) and high LD<sub>50</sub> value (300 mg/kg-350 mg/kg) and no adverse toxicological effects and therefore have considerable potential for being used as a therapeutic agent.

On the other hand, compounds including Hydrazine and Pristane belonged to toxicity class 2 and possessed low LD<sub>50</sub> values (60 mg/kg-70 mg/kg), and severe hepatotoxicity, mutagenicity, carcinogenicity, and immunotoxicity and were not feasible for clinical use. Retinamide belongs to toxicity class 3 and has an LD of 100 mg/kg, likewise, Meladrazine which belongs to toxicity class 3 has an LD of 140 mg/kg some side effects that might hinder their use include hepatotoxicity and carcinogenicity. Some lipids, including Docosane and Octacosane, were not toxic with LD<sub>50</sub> values of 450 mg/kg-480 mg/kg, but due to the classification as a toxicity class 6, meaning lower initial danger but potentially higher deferred dangers, these lipids still needed to be examined for effectiveness and solubility. Altogether, Myrtecaine, Sapidolide A, Vigabatrin, and Nonanoate possess moderate to low toxicity IC<sub>50</sub> values placing these as potential candidates for further preclinical as well as clinical studies in establishing the role of Cyclin-D, MELK, and LUZP in triple-negative breast cancer treatment (Tab. 5 and Fig. 1).

**Table 5. Serum Cyclin D, MELK and LUZP expression levels in DMBA-induced TNBC rat model.**

Groups	Cyclin-D (pg/mL)	MELK (pg/mL)	LUZP (pg/mL)
Control (Normal)	50.27 ± 3.12 <sup>e</sup>	75.44 ± 4.29 <sup>f</sup>	30.12 ± 2.56 <sup>e</sup>
DMBA (Alone)	150.62 ± 5.83 <sup>a</sup>	210.92 ± 6.35 <sup>a</sup>	95.25 ± 4.82 <sup>a</sup>
DMBA+Paclitaxel	80.43 ± 4.51 <sup>b</sup>	120.23 ± 5.73 <sup>b</sup>	45.66 ± 3.22 <sup>b</sup>
DMBA+Sapidolide-A	70.33 ± 3.93 <sup>c</sup>	110.84 ± 4.92 <sup>c</sup>	40.24 ± 3.17 <sup>c</sup>

DMBA+Retinamide	75.14 ± 4.24 <sup>c</sup>	115.65 ± 5.17 <sup>d</sup>	42.82 ± 2.93 <sup>c</sup>
DMBA+Daphnane	65.79 ± 3.83 <sup>d</sup>	100.44 ± 4.63 <sup>e</sup>	38.34 ± 2.71 <sup>d</sup>
p-value ( ≤ 0.05)	0.001	0.033	0.014
LSD ( ≤ 0.05)	9.26	5.66	3.99



**Figure 1.** Serum Cyclin D, MELK and LUZP expression levels in DMBA-induced TNBC rat model.

This study therefore assessed the phytochemical intervention efficacy in a DMBA-induced Triple-Negative Breast Cancer (TNBC) rat model through serum expression analysis of Cyclin-D, MELK, and LUZP. The findings are summarized as follows: The growth of Cyclin-D protein was notably increased in the DMBA-alone group (150.62 pg/mL ± 5.83 pg/mL,  $p \leq 0.05$ ) as compared to the control group (50.27 pg/mL ± 3.12 pg/mL) to corroborate the involvement of Cyclin-D in the progression of TNBC. 100% of the rats treated with Paclitaxel had a decreased Cyclin-D level of 80.43 pg/mL ± 4.51 pg/mL which was significantly lower than the level observed in the DMBA-alone group. Phytochemical interventions Daphnane (65.79 pg/mL ± 3.83 pg/mL) and Sapidolide-A (70.33 pg/mL ± 3.93 pg/mL) resulted in the lowering of Cyclin-D to a similar extent by Paclitaxel. Retinamide amounted to 75.14 pg/mL ± 4.24 pg/mL, also a moderate decrease was observed. Treatment with DMBA alone resulted in high serum MELK levels of 210.92 pg/mL ± 6.35 pg/mL as compared to control of 75.44 pg/mL ± 4.29 pg/mL. MELK levels in the Paclitaxel-treated group were reduced to 120.23 pg/mL ± 5.73 pg/mL. Out of phytochemicals, Daphnane shows the maximum percent decrease which is 100.44 pg/mL ± 4.63 pg/mL whereas a smaller percent decrease is shown by Sapidolide-A and Retinamide, 110.84 pg/mL ± 4.92 pg/mL and 115.65 pg/mL ± 5.17 pg/mL respectively. Also, the concentration of LUZP in rat serum was significantly elevated in the DMBA-alone breast cancer group (95.25 pg/mL ± 4.82 pg/mL) as compared to the control group (30.12 pg/mL ± 2.56 pg/mL).

Paclitaxel treatment in this section was determined at a level of 45.66 pg/mL ± 3.22 pg/mL. In the same manner, Daphnane caused the most profound inhibition, decreasing by 38.34 pg/mL ± 2.71 pg/mL, while Sapidolide-A and Retinamide inhibitory effects reached 40.24 pg/mL ± 3.17 pg/mL and 42.82 pg/mL ± 2.93 pg/mL, respectively. There was a significant difference ( $p \leq 0.05$ ) in the expression of Cyclin-D, MELK, and LUZP among all groups of calving. The additional LSD analysis again supported the ability of Daphnane and Sapidolide-A in their comparability or superiority to Paclitaxel in regulating these biomarkers. This study also opens the possibility of the Daphnane and Sapidolide-A as phytochemicals modulators in inhibiting Cyclin-D, MELK, and LUZP in DMBA-induced TNBC animal models. The former has a similar anticancer activity to the benchmark chemotherapeutic compound, Paclitaxel, which locates them as potential TNBC treatments with lower side effects (Tab. 6).

**Table 6.** Pearson's correlation coefficients matrix.

	Cyclin-D	MELK	LUZP
Cyclin-D	1	0.998	0.998
MELK	0.998	1	0.991
LUZP	0.998	0.991	1

Cyclin-D, MELK, and LUZP were the variables of interest in the research, which were analyzed through Pearson's correlation coefficient to determine their relation. The findings are as follows: The consistency between Cyclin-D and MELK was statistically very significant with a positive correlation coefficient of 0.998 or Cyclin-D as it increases the expression of MELK equally. This coexistence points them to repeatedly in TNBC progression thus their synergistic relationship. Likewise, a strongly positive correlation was observed for Cyclin-D and LUZP, with a correlation coefficient of 0.998, which confirmed the existence of a marked interconnection between Cyclin-D and LUZP in cancerous cells. The positive and strong relationship observed between MELK and LUZP under analysis ( $r=0.991$ ) was also noticeable, though not as high, as the other pairs. As analyses depict, the near-perfect positive relationships ( $r \approx 1$ ) between these biomarkers would indicate that the biomarkers work consecutively and synchronize TNBC progression likely in a feedback loop. These findings underscore their worth as mutually interacting therapeutic targets. These findings concord with the previous hypothesis that if one of these proteins could be targeted to influence the others, then they are likely candidates to be targeted together. A high degree of positive correlation between Cyclin-D, MELK, and LUZP was also confirmed in this study for TNBC patient samples. It is likely though those phytochemical interventions, which act on this network at the same time, have a definite edge on these activities as the various components work in conjunction. This underlines the need to use possibly multiple directed treatments for the administration of TNBC (Tab. 7).

**Table 7. Regression analysis.**

Parameter	Value
Intercept	0.08
Cyclin-D Coefficient	1.206
MELK Coefficient	-0.412
R <sup>2</sup> (Coefficient of Determination)	0.998

This study used regression analysis for the Cyclin-D, MELK, and LUZP expressions and TNBC to estimate a predictive value. The key findings are summarized below: This regression equation is obtained from the analysis as follows:  $LUZP = 0.080 + (1.206 \times \text{Cyclin-D}) - (0.412 \times \text{MELK})$ . This equation proves the hypothesis of Cyclin-D and MELK in the regulation of LUZP which is a significant marker in TNBC. Cyclin-D Coefficient (1.206): A coefficient value more than zero shows that Cyclin-D is directly proportional to LUZP. Here, we demonstrate that Cyclin-D is a critical player in LUZP function by the fact that for each unit of Cyclin-D, LUZP rises by 1.206 units. MELK Coefficient (-0.412): That means a negative coefficient is a negative association between MELK and LUZP. In this finding, for every unit increase in MELK LUZP expression reduces by 0.412 units, thus indicating that MELK and LUZP could be interactive where MELK may function as a switch in the expression of LUZP under some circumstances. Intercept (0.080): The intercept shows the quota of exogenous LUZP when Cyclin-D and MELK are at zero. R<sup>2</sup> (Coefficient of Determination=0.998).

Due to the model's high explanatory power, the R<sup>2</sup> value of 0.998 shows that Cyclin-D and MELK account for 99.8% of the LUZP expression variance observed in the experiment. The regression analysis validates Cyclin-D as a primary initiator and enhances LUZP expression, while MELK rebounds partially as a potential regulatory factor. The high value of R<sup>2</sup> confirms the connection of these markers and confirms their relevance as predictors or targets for the therapy of TNBC. The regression analysis produces strong statistical evidence that Cyclin-D and MELK play an important role in controlling the LUZP heightened expression with Cyclin-D positively regulating LUZP at a significantly higher level than MELK. These findings extend knowledge regarding the molecular signaling in TNBC and highlight the role of phytochemical interventions directed at this network to successfully interfere with tumor advancement.

## Discussion

In the present study, we identified Cyclin-D, MELK, and LUZP as targets of phytochemicals, specifically in Triple Negative Breast Cancer (TNBC), examined their regulation and therapeutic applicability. From *in-vitro* and *in-vivo* experiments we observed strong further interconnections between these key molecular targets and bio-ICs further showing the promising role of phytochemicals for the treatment of TNBC. Cyclin-D consider as one of the most vital cancer cell cycle controllers (Bhutta and Choi, 2024). Binding with CDK4/6 and strengthens the progression from the G1 phase to the S phase in the cell cycle to enhance DNA synthesis and cell division (Kumar, et al. 2023). Increased Cyclin-D levels were correlated with shorter survival and more invasive subtypes of breast tumors in TNBC patients (Grover, et al. 2024). We found a high correlation between Cyclin-D and LUZP in this study ( $r=0.998$ ); this confirms Cyclin-D partakes in upregulating LUZP which is involved in the survival/proliferation of TNBC cells (Abo, et al. 2020). This is supported by past studies showing Cyclin-D's capacity to modulate downstream targets that have been linked to TNBC and then aggressiveness (Landry, et al. 2022).

MELK (Maternal Embryonic Leucine Zipper Kinase) work as a key contributor to breast cancer and more specifically to the TNBC type. High-level MELK expression is linked to shorter survival and higher metastatic potential of cancer. However, the regression analysis results reported that there is an inverse correlation between the level of MELK and LUZP (coeff=-0.412), therefore has suggested that MELK could have a suppressive role when functioning under certain regulations (Thangaraj, et al. 2020). MELK recog-

nized as an enigmatic regulator of the cell cycle and apoptosis in TNBC cells. Hence, the MELK might provide a dual therapeutic benefit by arresting tumor growth and decreasing LUZP, a gene identified to promote survival pathways seen in aggressive TNBC characters (Feitosa, et al. 2024). Latterly, the Lapidated Zinc Finger Protein LUZP has been considered to be noteworthy in specifying the TNBC aggressiveness and metastasis (Tewari, et al. 2022). According to our *in-vivo* model, there were significant changes in LUZP expression under the effects of phytochemicals including Paclitaxel, Sapidolide-A, Retinamide, and Daphnane. These treatments led to decreased levels of LUZP suggesting that phytochemicals can interrupt LUZP-driven pro-survival signaling pathways. This is because flavonoids and terpenoids have been responsible to increase or decrease critical cancer-related targets such as Cyclin-D and MELK making tumors nonresponsive to growth (Wu, et al. 2021, Sheikh, et al. 2020). This implies that phytochemicals may suppress the LUZP gene expression by changing upstream factors as part of cell cycle inhibition and apoptosis.

The modality of using phytochemicals in therapeutic interventions demonstrated in our study points to the need to explore other treatment options in TNBC. Sapidolide-A, Retinamide, and Daphnane have shown anticancer potentials due to their inhibitory effects on Cyclin-D and MELK and regulation of LUZP (Wang, 2021). Our findings maintain prior research that states that phytochemicals manage molecular pathways vital for breast cancer, such as proliferation, cell death, and metastasis (Zhang, et al. 2024). Further, the *in-vivo* validation supports the phytochemicals as strong candidates to be combined with standard chemotherapeutic agencies such as Paclitaxel. Our analysis focuses on the Cyclin-D, MELK, and LUZP relationship in TNBC and reveals that phytochemicals can successfully address these proteins. The increases in Cyclin-D levels with concomitant decreases in MELK-LUZP indicate that phytochemicals can influence multiple pathways and aspects of the regulatory machinery. Such outcomes show the significant complementary and potential therapeutic role of phytochemicals in the treatment of TNBC. More empirical investigations into clinical confirmation and the use of combined treatment strategies can again help in identifying highly specific and individualized therapy avenues for triple-negative breast cancer treatment.

The present study sought to determine the effect of phytochemicals that modulate the expression of Cyclin-D, MELK, and LUZP on TNBC using the TNBC rat model developed by DMBA exposure. Furthermore, our results captured general and exciting prospects for phytochemicals to manipulate key molecular targets inherent in the lifecycle, or CN, of TNBC cells. We have also observed that a super-physiological dose of DMBA leads to higher expression of Cyclin-D relatives in the DMBA-alone group ( $150.62 \text{ pg/mL} \pm 5.83 \text{ pg/mL}$ ) as compared to the control group ( $50.27 \text{ pg/mL} \pm 3.12 \text{ pg/mL}$ ). Cyclin-D is a potent modulator of the cell cycle, forwarding the cell through the G1/S checkpoint. Cyclin-D has been reported to be over-expressed in different aggressive TNBC subtypes and with the patient's prognosis (Malabadi, et al. 2024). In particular, drugs containing phytochemicals such as Paclitaxel, Sapidolide-A, Retinamide, and Daphnane class affected the Cyclin-D level prominently. For example, Cyclin-D lowered to  $80.43 \text{ pg/mL} \pm 4.51 \text{ pg/mL}$  (with Paclitaxel), and  $70.33 \text{ pg/mL} \pm 3.93 \text{ pg/mL}$  (with Sapidolide-A). With regards to these observations, it is evident from other studies that Phytochemicals are known to suppress Cyclin-D, and hence the cell cycle and the subsequent infusion of apoptosis (Ahmed, et al. 2022). Flavonoids and terpenoids are phytochemicals that have been reported to suppress the CDK4/6 activity that Cyclin-D targets and this adds to our findings (Sheikh, et al. 2020).

Compared with the control group, the DMBA group had a higher level of MELK ( $210.92 \text{ pg/mL} \pm 6.35 \text{ pg/mL}$ ) than that of the control group ( $75.44 \text{ pg/mL} \pm 4.29 \text{ pg/mL}$ ). MELK has also been shown to operate in two ways, stimulating cell division while preserving cancer stem cell features (Yan and Yue, 2023). In our study treatments with phytochemicals like Paclitaxel and Sapidolide-A lowered MELK expression to the baseline to  $120.23 \text{ pg/mL} \pm 5.73 \text{ pg/mL}$  and  $110.84 \text{ pg/mL} \pm 4.92 \text{ pg/mL}$  respectively. These findings are in line with earlier studies that see MELK as a potential therapeutic target in TNBC. It has been proved that suppression of MELK affects cancer cell growth and invasion and affects signaling involved with cell cycle and cell death (Thangaraj, et al. 2020). Furthermore, the loss of MELK impact was found to enhance the cytotoxicity of many antineoplastic drugs in adherent and mixed population-enriched TNBC cells (Malabadi, et al. 2024). The DMBA-alone group had higher levels of LUZP expression ( $95.25 \text{ pg/mL} \pm 4.82 \text{ pg/mL}$ ) compared to the phytochemicals like Paclitaxel, Sapidolide-A, Retinamide, and Daphnane. For example, levels of the LUCP protein were reduced to  $45.66 \text{ pg/mL} \pm 3.22 \text{ pg/mL}$  in response to Paclitaxel treatment. Another micro protein, LUCP, a zinc-finger protein, has earlier been identified as promoting cancer cell survival, proliferation, and metastatic process in TNBC (Villemin, et al., 2021).

These results are consistent with other studies suggesting that threatening LUZP can affect pro-survival signaling cascades that in turn will amplify the efficiency of anticancer interventions. Some phytochemicals work on Cyclin-D and MELK and also have been observed to work on LUZP where they form a coordinated network and phytochemicals hit and regulate multiple cell cycle and survival proteins at once (Bhutta and Choi, 2024). Our results are in concordance with studies showing that phytochemicals can effectively regulate biological factors such as Cyclin-D, MELK, and LUZP in TNBC. For instance, the Sharma, et al. study proved that curcumin a natural phytochemical was potent in reducing the Cyclin-D expression and suppressing cell proliferation in TNBC cells. Studies also endorse the MELK inhibition for chemoresistance in TNBC. Furthermore, phytochemicals could inhibit LUZP genes that interrupt survival signaling for growth and metastasis. These works complement our observations and support the notion that phytochemicals can bind to a panel of proteins which is a necessary condition to overcome multiple resistances in aggressive TNBC subtypes (Das, et al. 2023).

## Limitations and future directions

Although our results are encouraging, several limitations should be considered: These observations should be further confirmed

in patient-derived samples and longer *in-vivo* studies, and there should be a translation of these findings from rat models to clinical practice. Furthermore, the specificity, as well as, pharmacokinetics of phytochemicals requires reconsideration regarding safety and efficiency in case of long-term interventions. Further studies should also investigate combinatory therapies where phytochemicals are combined with standard chemotherapy or targeted therapy. Studying sho3-1 and other phytochemicals' influence on signaling pathways in the context of PDX could help better understand individual treatment options for TNBC. However, our results offer valuable implications, which require recognizing the limitations of the study. Also, more durable *in-vivo* tests and clinical trials are required for phytochemical intervention to examine the beneficial effects on human health and apprehend its complications fully. More studies regarding clinical trials and combinational therapies will act as a pointer to the development of new efficient and individualized treatment approaches in triple-negative breast cancer treatment. The strategies for utilizing phytochemicals to develop therapeutics that target Cyclin D, MELK, and LUZP need a systemic approach where molecular docking, high-throughput screening, and improved *in vivo* models are integral. Combining omics technologies with phytochemicals study will offer a further understanding of the molecular biology behind the anti-cancer effects.

## Conclusion

This research supports strong evidence that phytochemical intervention approaches in tackling molecular proteins including Cyclin-D, MELK, and LUZP in Triple Negative Breast Cancer (TNBC) using the *in-vitro* and *in-vivo* models. The observed marked regulation of these proteins by phytochemicals including Paclitaxel, Sapidolide-A, Retinamide, and members of the Daphnane class confirms the role of phytochemicals as single or adjunct therapy for TNBC. A decrease in Cyclin-D levels as observed also supports good cell cycle regulation while the role of MELK in new Cancer cell generation and stemness is also shown to be blocked. These results share similar evidence with current publications relating to the use of plant secondary metabolites and their impact on cell cycle and survival proteins in aggressive subtypes of TNBC. Further studies should also be done to determine how the phytochemicals can be combined with either standard adjuvant chemotherapy or molecularly targeted therapies to prevent resistance and enhance the survival of TNBC patients. Therefore, the understanding of phytochemical interventions is a promising direction for constructing new efficient treatment options for triple-negative breast cancer with better prognosis.

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## Author contribution

- Muhammad Akram Shahzad Khokhar Conceptualized the study, designed the experiments, and oversaw the entire research project.
- Malik Ihsan Ullah Khan conducted the *in-vitro* experiments and data analysis for Cyclin-D, MELK, and LUZP expression levels.
- Arif Malik carried out the *in-vivo* studies on the DMBA-induced TNBC rat model and coordinated animal handling procedures.
- Haleema Saadia, Gul Zaib contributed significantly to statistical analyses, including regression and correlation evaluations.
- Qurban Ali assisted in interpreting data and drafting sections of the manuscript.

All authors collaborated in manuscript writing, critically reviewed the work, and approved the final version.

## Conflict of interest

The authors have no conflict of interest to report concerning this study. There are no conflicts of interest arising from financial or personal interest that may have affected the results or their interpretation in the context of the carried-out research. Each study was carried out independently and therefore the results were analyzed and presented as such.

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