

Molecular docking and cytotoxicity interactions of naringenin and its nano-structured lipid carriers in ER α positive breast cancer

Priyadarshini S^{1*}, Akey Krishna Swaroop¹, Jubie S², Jawahar N³ & Vrajesh Divecha⁴

^{1&4}Department of Pharmacognosy; ^{1&2}Department Of Pharmaceutical Chemistry; & ³Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy Of Higher Education & Research, Ooty-643 001, Tamil Nadu, India

Received 26 December 2022; revised 11 January 2023

Phytoestrogens are known to have beneficial properties in various carcinomas. They exhibit its efficacy at cellular levels. Naringenin a flavonoid phytoestrogen is been explored for its antioxidant, cardio protective and cytotoxic function. The low absorption and poor bioavailability of naringenin makes it less efficient in targeting tumours at cellular levels. Due to the structural similarity of naringenin with estradiol and considering the affinity of naringenin with estrogen receptor, this study explores the interactions of naringenin on important signaling proteins involved in ER positive breast cancer through molecular docking studies and the prepared naringenin solid lipid nano particles were characterized and studied for its preventive potential against breast cancer cell lines. The lipidoid form of phytoestrogen shows promising cytotoxic potential compared with naringenin.

Keywords: Angiogenesis, Cytotoxicity, Ligand, Mannitol, Metastasis, Phytoestrogens, Surfactant

Breast cancer is known to be the second leading cause of cancer death in women and approximately 80% of the breast cancer shows over expression of estrogen receptor¹. A cascade of events occurs in ER signaling and the microenvironment within the cell such as corepressors, coactivators, genomic sequences of consensus binding sites affect the activity of ER². Moreover, E2 has a major etiological role in breast cancer development. Endocrine therapy is considered to be the major treatment strategy in er positive breast cancer which includes selective estrogen receptor modulators (SERM), selective estrogen receptor down regulators (SERD) and aromatase inhibitors (AI)¹. These hormonal therapies competitively bind to the estrogen receptors suppress the gonadal secretion and synthesis of estrogen. Endocrine therapies are known to have prominent side effects due to their interference with the estrogen. They are known to cause musculoskeletal disorder, hot flashes, lose of bone density, endometrial cancer and weight gain. These side effects can lead to non-adherence to treatment and increases the reoccurrence of breast cancer³. though ER positive breast cancer responds initially to endocrine treatment, about 20% of tumors

are resistant to treatment intrinsically and other 30-40% of tumors acquire resistance after many years. Resistance to endocrine therapy results in metastasis and relapse leading to death. It also results in need for newer endocrine drug combinations. Several studies suggest that the up regulation of pathways such as PI3K-AKT-Mtor interacts with the estrogen receptor signaling and confers endocrine resistance⁴. Certain preclinical studies shows that the response of resistant cells could be restored on treatment with PI3K and Mtor inhibitors and it could resensitize the breast cancer. Evidences from clinical and preclinical studies shows that the treatment targeting both growth factor pathway and ER can hold a promising effect in overcoming endocrine resistance. Hence there is a need for new treatment strategy with multitargeting potential.

Phytoestrogens have beneficiary effects to overcome this major challenge of endocrine resistance and also in treating ER α receptor positive breast cancer. It is predicted that these protective effects are due to the increase in estrogen catabolism that is induced by cytochrome p450 enzymes that in turn reduces the estrogen stress on mammary glands⁵. Phytoestrogens are bioactive compounds that structurally and functionally mimics mammalian estrogen 17 β estradiol. Reports suggests that phytoestrogens have inhibitory role in expression

*Correspondence:
E-mail: priya@jssuni.edu.in

of oncogenic Cyclin D1 and PI3KA⁴. They also exerts epigenetic property and regulates gene expression^{6,7}.

Naringenin, a flavonone phytoestrogen which is found abundantly in citrus and grape fruits found to have a range of therapeutic potential⁸. Extensive works are carried out on naringenin in respect to its cardioprotective effects⁹. Naringenin is less explored for its anti-proliferative property on breast cancer. In spite of its beneficial effects, naringenin is known to have low bioavailability^{10,11} and its rapidly converted to its crystalline form and has a short half-life with low absorption. To improve the bioavailability and tumour specific parameters, solid lipid nano carries of naringenin was prepared and explored for its interactions with oncogenic proteins of er positive breast cancer and cytotoxicity parameters.

Materials and Methods

Molecular docking studies of naringenin

In silico molecular docking studies were carried out using pyrex 0.8 version to predict the action of naringenin on oncogenic proteins involved in er positive breast cancer such as er receptor protein, Cyclin D1, PI3KA. The interaction of naringenin with the tumor suppressor protein PTEN were also studied¹².

Preparation of ligand

The ligand structure was constructed using chemsketch tool and its 3d structures are built using discovery studio visualizer.

Receptor protein preparation

The protein structures are downloaded from protein data bank and were analyzed using Swisspdb Viewer. Oncogenic proteins such as PI3K alpha (PDB ID: 5ITD), estrogen receptor protein (PDB ID: 4J26) (Fig. 1), Cyclin D1 (PDB ID: 5VZU)¹³ and tumor suppressor protein phosphatase and tensin homolog (PTEN)¹⁴ with PDB ID: 1d5r were prepared for the study^{15,16} (Fig. 2).

Molecular docking

The 2d and the 3d structures of ligands and the protein were prepared using discovery studio visualizer and predictions such as docking score, binding affinity and hydrogen bonding the amino acid pockets of the proteins were studied.

Preparation of solid lipid nano-particle

The solid lipid nano-particle of naringenin was prepared with reference to previous publication. The procedure was optimized by trial and error method^{17,18}. Glycerolmonostearate is used in oil phase and Tween 80 is used as a surfactant. The drug was dissolved in the lipid phase containing gms. The oil phase was slowly dispersed into the aqueous phase with continuous stirring at 1350 rpm for one hour at 50°C. Later the hot solution was immediately transferred to the beaker containing ice cold water. The complete mixture was stirred under cold condition for 4 h using magnetic stirrer at 2000 rpm. The solution was kept overnight and observed for phase¹⁹.

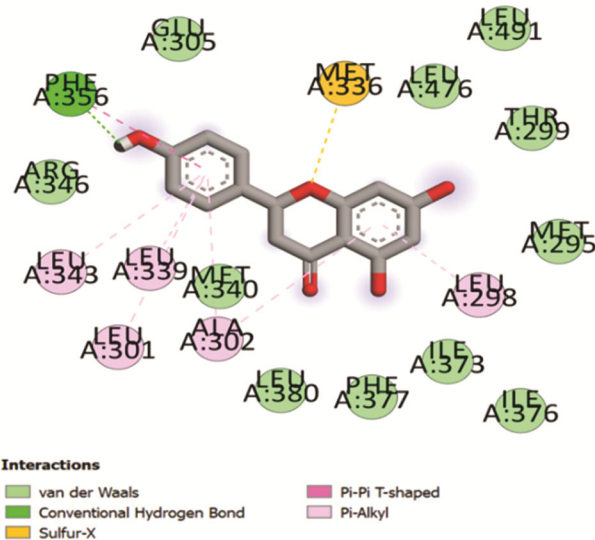


Fig. 1 — 2D Interaction of Naringenin and ER receptor protein (PDB ID: 4J26)

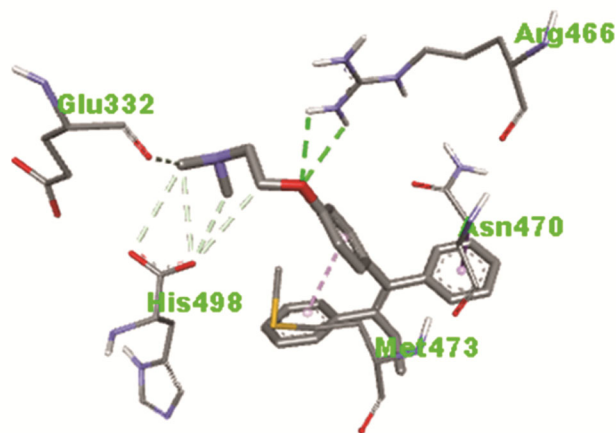


Fig. 2 — 3D Interaction of Tamoxifen and ER receptor protein (PDB ID: 4J26)

Conversion of solid lipid nano-particles to freeze dried particles

The prepared SLN of naringenin is converted to freeze dried particles to improve the stability. The prepared mixture was coated with mannitol by continuous stirring and was subjected to deep freezing for 8 h. The deep frozen product was further subjected to freeze drying technique.

Characterization of naringenin solid lipid nano-particles

Particle size determination and zeta potential

The particle size of the sample was determined after dilution containing concentration of 1% (w/v). Litesizer 500 was used to determine zeta potential. Particle size, polydispersity index and particle size distribution were analysed for the diluted sample by maintaining optimum temperature to 25°C.

Differential scanning calorimetry (DSC)

Naringenin solid lipid nano-particles were subjected to dsc analysis to determine its melting point, purity and transition. The temperature range was maintained between 200-300°C. The samples were observed in reflection mode by maintaining ambient conditions.

Cytotoxicity of naringenin solid lipid nanoparticles

The cell culture was centrifuged and the cell count was adjusted to 1.0×10^5 cells/mL using dmem medium containing 10% FBS. To each well of a 96 well flat bottom micro titre plate, 100 μ L of the diluted cell suspension (approximately 10,000 cells/well) was added. After 24 h, when the cell population was found adequate, the cells were centrifuged and the pellets were suspended with 100 of different test sample concentrations prepared in maintenance media. The plates were then incubated at 37°C for 48 h in 5% Co2 atmosphere, and microscopic examination was carried out and observations recorded every 24 h. After 48 h, the sample solutions were centrifuged and the pellets were re-suspended with MTT (2 mg/mL) in MEM-PR (MEM without phenol red). The plates were gently shaken and incubated for 2 h at 37°C in 5% CO2 atmosphere. The 100 μ L of dmsol was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage cell viability was calculated and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves.

Results

Molecular docking studies

Docking interaction of naringenin with estrogen receptor

Naringenin tend to have a strong binding potential *in silico* with ER receptor with binding affinity of -7.9 . The docking with ER reveals that naringenin have good interaction with ER receptor (Table 1).

Docking interaction of naringenin and tamoxifen with Cyclin D1 protein

Docking interaction of naringenin with PTEN protein

Results showing hydrogen bonding and vanderwaals interactions of naringenin and tamoxifen with PTEN protein (Fig. 3A-F).

Docking interaction of naringenin with PI3K protein

Characterization of naringenin solid lipid nano particles

Particle size determination

The prepared solid lipid nano-particles of naringenin was characterized for its particle size and purity using Litesizer 500 using X-ray diffraction (XRD). The particle size of the prepared SLN was found to be 273.4 nm and the polydispersity index was found to be 19.5% (Fig. 4A & B).

Zeta potential

The mean zeta potential of naringenin solid lipid nano particles was found to be -20.5 mV

Differential scanning calorimetry

The DSC analysis shows that there is a transition in the melting point of the prepared SLN which reveals that the compound's entrapment within the solid lipid nano particles. The purity of the prepared compound was found to be 99.22 mol % (Fig. 5).

Cytotoxicity assay

The cytotoxicity assay of naringenin solid lipid nano particles on MCF-7 was measured in accordance with the formazan levels. The percentage cell viability was measured and naringenin showed a dose dependent inhibition of cell viability in MCF-7 cell lines and IC₅₀ value of naringenin solid lipid nano particles was found to be 12.53903 μ g/mL. Naringenin also showed a dose dependent inhibition on MCF-7 cell lines and its IC₅₀ value was found to be 15 μ g/mL (Fig. 6A & B).

Table 1 — Comparative binding affinity Of Naringenin And Tamoxifen

Sl. No	Protein	Naringenin	Tamoxifen
1	PDB ID: 5VZU	-7.1 Kcal/Mol	-5.8 Kcal/Mol
2	PDB ID: 5ITD	-8.1 Kcal/Mol	-6.7 Kcal/Mol
3	PDB ID: 4J26	-7.9 Kcal/Mol	-6.0 Kcal/Mol
4	PDB ID: 1D5R	-8.8 Kcal/Mol	-4.7 Kcal/Mol

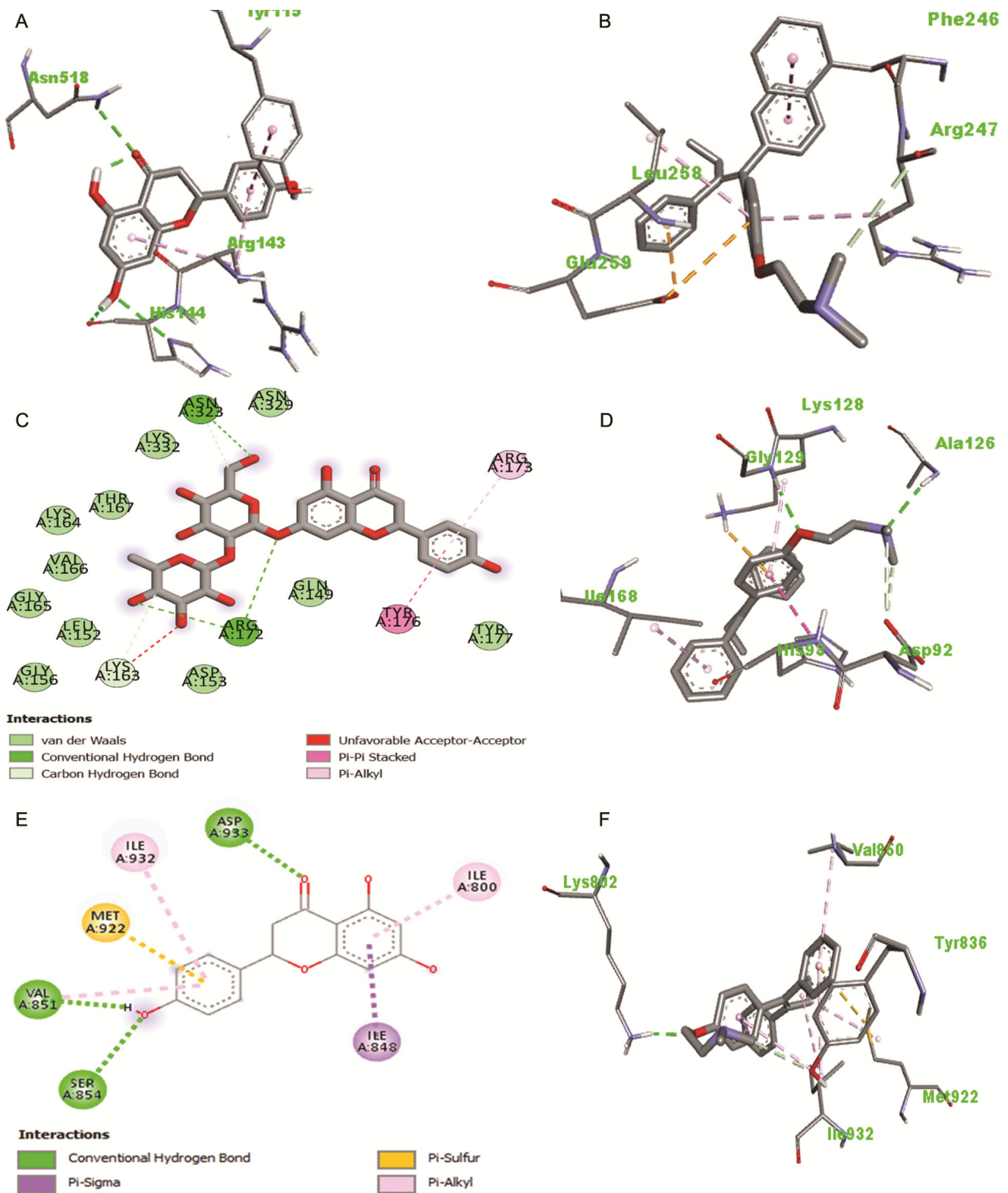


Fig. 3 — (A) Naringenin And Cyclin D1 Protein (PDB ID: 5vzu); (B) Tamoxifen And Cyclin D1 Protein (PDB ID: 5vzu); (C) Naringenin With PTEN; (D) Tamoxifen With PTEN; (E) Naringenin With PI3K; and (F) Tamoxifen With PI3K

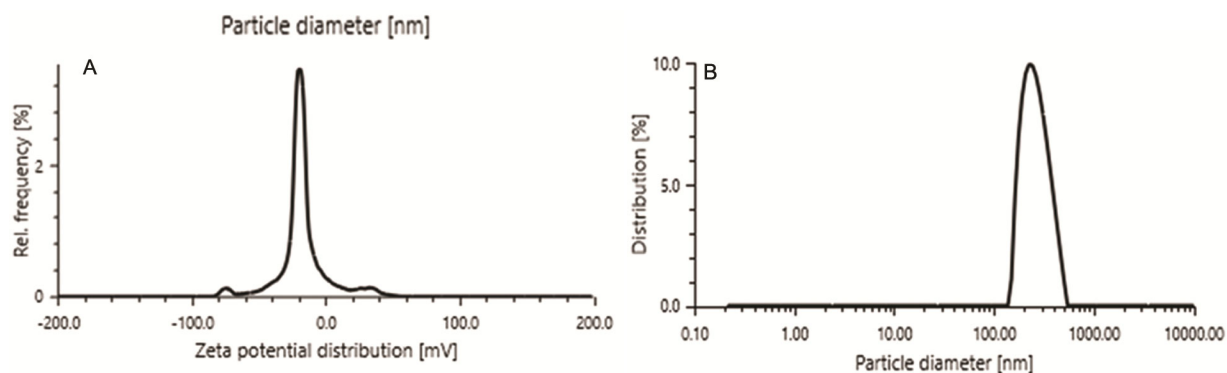


Fig 4 — Graphical representation of (A) Particle Size; and (B) Zeta potential distribution of naringenin solid lipid nano-particles

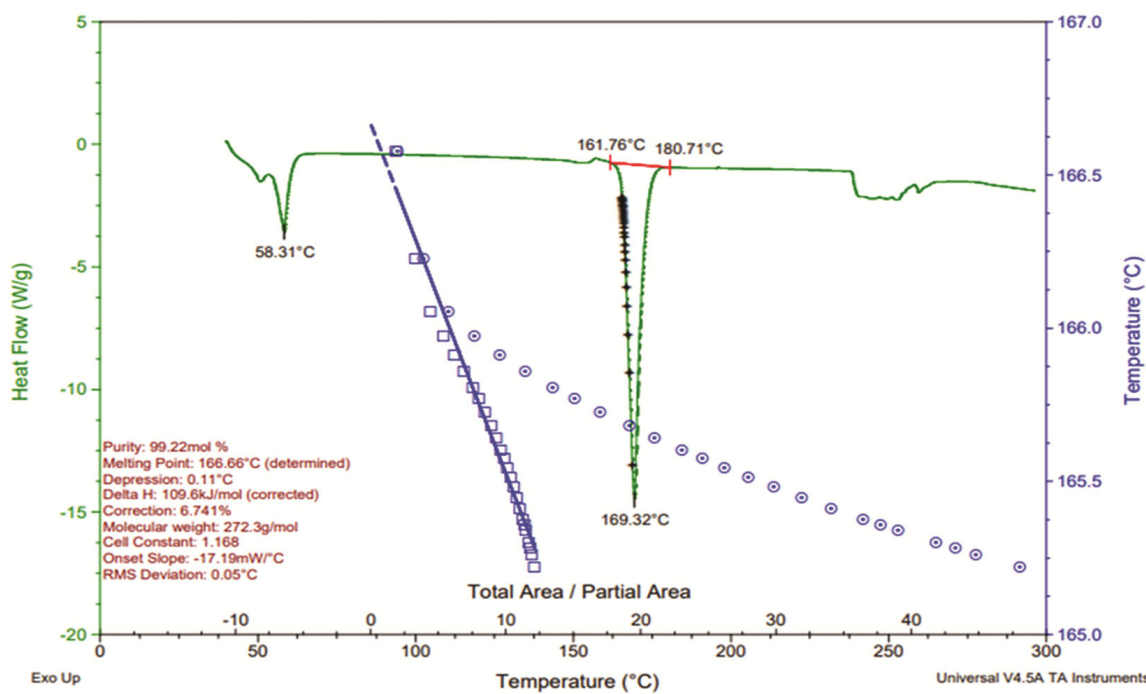


Fig 5 — DSC analysis of naringenin solid lipid nano-particles

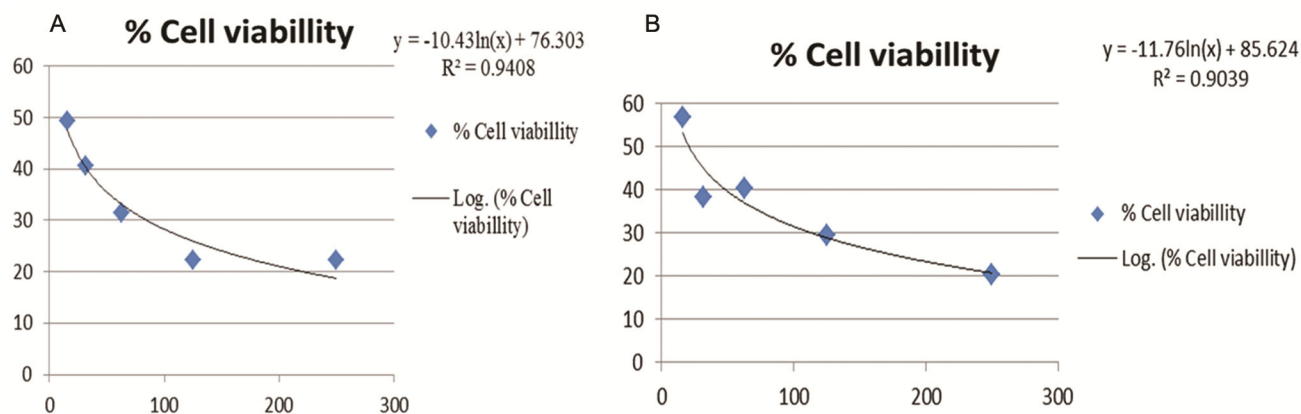


Fig. 6 — (A) Cytotoxic activity of naringenin; and (B) solid lipid nano-particles on MCF7 cell line

Discussion

The flavonone naringenin is known to have a wide pharmacological properties, however the poor bioavailability and weak affinity of naringenin on ER receptor is to be considered when estrogen responsive tumors. This study involves exploring the interaction of naringenin with ER positive breast cancer signalling elements, formulation of naringenin solid lipid nano-particles and to identify the cytotoxicity potential of formulated naringenin. Binding of a ligand to ER receptor and transcription are the two important factors that induces a cascade of cellular process. As a weak ER agonist, naringenin shares vanderwaals interaction and hydrogen bonding with the amino acids of ER receptor such as ARG A:346, PHE A:356, GLU A:305, LEU A:476, LEU A:491, THR A: 299, MET A:295, LEU A:380, PHE A:377, ILE A:378, ILE A:376, MET A:340.

Cyclin D1 is an important regulator of cyclin dependent kinases that is overexpressed in breast carcinomas²⁰. Cyclin D1 induces cell cycle transition from G1 to S phase which results in rapid cell growth and proliferation. The levels of Cyclin D1 kinases are maintained by phosphorylation dependent nuclear export²¹. Many flavonoids were reported to have inhibitory effect on Cyclin D1. The binding interaction of naringenin with Cyclin D1 was further explored through docking studies. The docking interaction revealed that naringenin shares hydrogen bonding interaction with HIS B:144, ASN B:518 and PI alkyl interaction with ARG B:143. Naringenin shares a good binding interaction with Cyclin D1 with affinity of -7.1 .

The phosphatase and tensin homolog PTEN is a tumor suppressor gene that are often deleted or mutated in a number of tumors that includes prostate cancer, breast cancer, lung cancer and endometrial cancer²². It is reported that PTEN induces apoptosis and controls cell migration, cell invasion and angiogenesis by interfering with various signalling pathways. PTEN dephosphorylates protein substrate on serine, threonine and tyrosine residues. It causes down regulation of Cyclin D1 and pi3k. The interaction of naringenin with PTEN protein was explored and it was found to have pi-alkyl interaction with ARG A:173 and hydrogen bonding interaction with ASN A:329, ASN A:328, THRA:167, LYS A:164, VAL A:166, ARG A:172. The binding affinity of naringenin on PTEN was found to be -8.8 .

The PI3K/AKT/Mtor pathway gets frequently activated in breast cancer leading to cell proliferation

and survival. Reports suggest that naringenin inhibits the expression of PI3K protein levels²³. The docking interactions in this study reveal that naringenin shares hydrogen bonding interaction with ASP A:933, VAL A:851, SER A:854 and PI alkyl interaction with ILE A:800. The binding affinity of naringenin with PI3K was found to be -8.1 .

Various phytochemicals and its nano-particles are been explored for its proliferative potential^{24, 25}. Studies suggest that nano particles play a promising role in targeting tumours that cellular levels^{26,27}. In this study naringenin nano particles was found to have highest binding affinity with PTEN protein and PI3K compared with tamoxifen. Binding affinity more than -8 kcal/mol are considered to be highest and the moderate affinity are found to be between -7 to -8 ^{28,29}. They show moderate interactions with ER receptor and Cyclin D1 protein. The prepared nano structured particles shows satisfying results. The particle size distribution and quality of the nano carriers were found to be within the acceptable range³⁰. The cytotoxicity interactions revealed that the nano structured naringenin shows improved activity compared with naringenin.

Thus various bioactives and their nano formulations investigated for its therapeutic effect shows promising activity against various diseases including cancer³¹. Exploring phytoconstituents and application of nano technology in improving their efficacy and bioavailability can play an important role in target specific drug discovery^{32,33}.

Conclusion

Our present data provides information on docking interactions of naringenin with the important cell signalling proteins involved in cell proliferation and survival of breast cancer. The interactions were compared with tamoxifen an estrogen receptor modulator. Naringenin shows good binding affinity with ER receptor protein due to its vanderwaals force and hydrogen bonding interactions compared with tamoxifen. Naringenin also shows satisfying interaction with PTEN protein, an important tumor suppressor that involves in dephosphorylation of PI3K. The bioavailability and the binding affinity of naringenin can be improved by converting to its solid lipid nano-particles. The prepared nano-particles are found to be efficient in controlling the proliferation of MCF-7 in a dose dependent manner. Thus the lipidoid form of phytoestrogens may have beneficial effects in

inhibition of cell growth and cell viability. Further studies on genomic level and protein levels can be explored to study the inhibitory effects of lipidoid phytoestrogens which maybe have promising role in various carcinomas.

Acknowledgement

The authors acknowledge JSS College of Pharmacy, Ooty for the facilities and support.

Conflicts of interest

All authors declare no conflicts of interest.

References

- Lumachi F & Santeufemia DA, Basso SM, Current medical treatment of estrogen receptor-positive breast cancer. *World J Biol Chem*, 3 (2015) 231.
- Fuentes N & Silveyra P, Estrogen Receptor Signaling Mechanisms. *Adv Protein Chem Struct Biol*, 116 (2019) 135.
- Strasser-Weippl K & Goss PE, Prevention of breast cancer using serms and aromatase inhibitors. *Mammary Gland Biol Neoplasia*, 8 (2003) 15.
- Yang J, Nie J, Ma X, Wei Y, Peng Y & Wei X, Targeting PI3K In Cancer: Mechanisms and advances in clinical trials. *Mol Cancer*, 18 (2019) 20.
- Basu P & Maier C, Phytoestrogens and breast cancer: *in vitro* anticancer activities of Isoflavones, Lignans, Coumestans, Stilbenes and their analogs and derivatives. *Biomed Pharmacother*, 107 (2018) 1650.
- Galluzzo P, Ascenzi P, Bulzomi P & Marino M, The nutritional flavanone naringenin triggers antiestrogenic effects by regulating estrogen receptor A-Palmitoylation. *Endocrinology*, 149 (2008) 2567.
- Latif AD, Gonda T, Vágvölgyi M, Kúsz N, Kulmány Á, Ocsosvzki I, Zomborszki ZP, Zupkó I & Hunyadi A, Synthesis and *in vitro* antitumor activity of naringenin oxime and oxime ether derivatives, *Int J Mol Sci*. 20 (2019) 2184.
- Salehi B, Fokou PV, Sharifi-Rad M, Zucca P, Pezzani R, Martins N & Sharifi-Rad J, The therapeutic potential of naringenin: A review of clinical trials. *Pharmaceuticals*, 12 (2019) 11.
- Zhao Z, Jin G, Ge Y & Guo Z, Naringenin inhibits migration of breast cancer cells *via* inflammatory and apoptosis cell signaling pathways. *Inflammopharmacology*, 27 (2019) 1025.
- Rajamani S, Radhakrishnan A, Sengodan T & Thangavelu S, Augmented anticancer activity of naringenin-loaded tpgs polymeric nanosuspension for drug resistive MCF-7 human breast cancer cells. *Drug Dev Ind Pharm*, 44 (2018) 1752.
- Bhia M, Motallebi M, Abadi B, Zarepour A, Pereira-Silva M, Saremnejad F, Santos AC, Zarrabi A, Melero A, Jafari SM & Shakibaei M, Naringenin nano-delivery systems and their therapeutic applications. *Pharmaceutics*, 23(2021) 291.
- Paplomata E & O'Regan R, The PI3K/AKT/Mtor pathway in breast cancer: Targets, trials and biomarkers. *Ther Adv Med Oncol*, 6 (2014) 154
- Qie S & Diehl JA, Cyclin D1, Cancer progression, and opportunities in cancer treatment. *J Mol Med*, 94 (2016) 1315.
- Zhang HY, Liang F, Jia ZL, Song ST & Jiang ZF, PTEN Mutation, Methylation And Expression In Breast Cancer Patients. *Oncol Lett*, 6 (2013) 164.
- Chen J, Sun J, Wang Q, Du Y, Cheng J, Yi J, Xie B, Jin S, Chen G, Wang L & Wang X, Systemic deficiency of PTEN accelerates breast cancer growth and metastasis. *Front Oncol*, 12 (2022) 825484.
- Barnes DM & Gillett CE, Cyclin D1 In breast cancer. *Breast Cancer Res Treat*, 52 (1998) 3.
- Akbarzadeh Z, Parvaresh F, Ghiasvand R & Miraghajani M, the effects of naringenin on some human breast cancer cells: A Systematic Review. *Arch Breast Cancer*, 10 (2016) 35.
- Ruh MF, Zacharewski T, Connor K, Howell J, Chen I & Safe S, Naringenin: A weakly estrogenic bioflavonoid that exhibits antiestrogenic activity. *Biochempharmacol*, 26 (1995) 1485.
- Bulzomi P, Bolli A, Galluzzo P, Leone S, Acconcia F & Marino M, Naringenin and 17 β -Estradiol coadministration prevents hormone-induced human cancer cell growth. *IUBMB Life*, 62 (2010) 55.
- Pestell RG, New roles of Cyclin D1. *Am J Pathol*, 183 (2013) 5.
- Diehl JA, Cycling to cancer with Cyclin D1. *Cancer Biol Ther*, 5 (2002) 226.
- Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaris C, Rodgers L, McCombie R & Bigner SH, PTEN, A putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, 275 (1997) 1945.
- Fruman DA & Rommel C, PI3K and cancer: Lessons, challenges and opportunities. *Nat Rev Drug Discov*, 13 (2014) 140.
- Yap TA, Bjerke L, Clarke PA & Workman P, Drugging PI3K In cancer: Refining targets and therapeutic strategies. *Curr Opin Pharmacol*, 23 (2015) 98.
- Kaan D, Assessment of cranberry bush on MCF-7 Human breast cancer cells. *Indian J Biochem Biophys*, 59 (2022) 985.
- Mehta S, Bakshi S, Choudhury S, Bose S, Nayak R, Hierarchical gold nanostructures based sensor for sensitive and fast detection of cancer biomarker. *Indian J Biochem Biophys*, 58 (2021) 135.
- Raja Namasivayam SK & Bharani RS, Silver nanoparticles loaded pyrrole based pesticidal metabolites (Agnps-PFM) nanoconjugate induced impact on the gut microbion and immune response against Lepidopteron Pest *Spodoptera litura* (Fab.). *Indian J Biochem Biophys*, 58 (2021) 478.
- Namini NM, Abdollahi A, Movahedi M, Razavi AE & Saghiri R, Association of SERPIND1 expression with grade, stage and presence of metastasis in breast cancer. *Indian J Biochem Biophys*, 58 (2021) 71.
- Jyothi K, Sivaranjani V, Pavithra U, Jayavel S & Muthulakshmi L, Computational studies on new leishmanial drug targets against quercetin. *Indian J Biochem Biophys*, 59 (2022) 909.
- Reena N, Babu BK & Biju AR, Evaluation of antiproliferative potential of manganese (II)-Dafone complex. *Indian J Biochem Biophys*, 58 (2021) 62.
- Jenifer DR, Malathy BR & Ariya SS, *In vitro* and *in silico* studies on the biochemistry and anti-cancer activity of phytochemicals from *Plumbago zeylanica*. *Indian J Biochem Biophys*, 58 (2021) 272.
- Selvam AK, Preparation and characterization of silver nanoparticle/aloe vera incorporated PCL/PEO matrix for wound dressing application. *Indian J Biochem Biophys*, 58 (2021) 35.