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Research Article

Cell Cycle Target Protein Induced by Galangin Treatment in Luminal Cells Confirmed by Bioinformatics Analysis

Diyah Novi Sekarini[1,2], Yohanes Surya Jati[1,2], Nur Ayunie Zulkepli[5,6], Dyaningtyas Dewi Pamungkas Putri[2,3,4*]

[1]*Department of Biotechnology, Graduate School, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[2]*Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[3]*Pharmacology and Toxicology Laboratory, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[4]*Laboratory of Advanced Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[5]*Centre for Medical Laboratory Technology Studies, Faculty of Health Sciences, Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus, Selangor Malaysia*
[6]*Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Universiti Teknologi MARA, Selangor Branch, Puncak Alam Campus, Selangor Malaysia*

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ABSTRACT

Galangin has activity modulating cell cycle arrest on luminal cancer cells and has high selectivity and low cytotoxicity for normal cells. This research intends to know galangin's prospective targets for promoting cell cycle arrest in luminal breast cancer via experimental *in vitro*, network pharmacology, and bioinformatics validation. In this research, MCF-7, a luminal model cell, was treated with galangin dose-dependent. Consequently, galangin exhibited a cytotoxic impact, with IC_{50} values of 117.86 µM. After that, SwissTargetPrediction, UALCAN, ShinyGO, and OncoLnc were used for bioinformatics validations, and Cytoscape software and the STRING website were used for computational analysis. Eight overlapping galangin target genes against luminal breast cancer were found. According to the analysis of the protein-protein interaction (PPI) network, eight hub genes-including CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12-had the highest degree of freedom. Cell cycle regulation has been discovered to be tightly associated with overexpression of CDK1, PLK1, AURKB, and NEK2. By influencing the cell cycle, galangin inhibits the growth of luminal breast cancer, as determined by GO and KEGG enrichment analyses. In conclusion, by triggering cell cycle arrest, galangin may be used as a prospective chemotherapeutic treatment.

1. Introduction

One flavonoid that could be extracted from the root of *Alpinia officinarum* Hance is galangin (3,5,7-trihydroxyflavone) (Aloud *et al.* 2017). Galangin has demonstrated anticancer potential against a variety of cancer cell types via a variety of routes. By decreasing the amounts of phosphorylated Akt, galangin appears to cause ovarian cancer cells to undergo apoptosis (Huang *et al.* 2020). Additionally, galangin stops the advancement of the cell cycle by producing cell cycle arrest in S-phase, particularly in cells that are nasopharyngeal carcinomas (Lee *et al.* 2018). When galangin stopped the cell cycle in head and neck squamous cell carcinoma at the G0/G1 phase, it also decreased Akt phosphorylation, S6 kinase activation, and mammalian target of rapamycin (Zhu *et al.* 2014). Furthermore, galangin regulates cell cycle-associated proteins, which also helps MCF-7 cells enter cell cycle arrest (Song *et al.* 2017).

Galangin seems to possess the capacity to inhibit breast cancer progression, especially for luminal breast cancer subtypes. Treatment with galangin alone may suppress colony formation levels in T47D and MCF-7 in

* Corresponding Author
E-mail Address: dyaningtyas.dewi.p.p@ugm.ac.id

a dose-dependent way. Interestingly, the use of galangin had no effect on the cellular viability of normal cells, indicating that it is safe and not cytotoxic (Song *et al.* 2017; Fang *et al.* 2019). To the best of our knowledge, there has been little research into the effect of galangin on luminal breast cancer, and the potential of genes connected with its cell cycle has yet to be investigated using bioinformatics analysis.

This study attempts to evaluate the potential of galangin target genes in suppressing luminal breast cancer by employing bioinformatics-based analysis. Initially, galangin protein targets were obtained from the SwissTargetPrediction and STRING publicly available database, and galangin potential target genes were identified by analyzing Venn diagrams with genes that are overexpressed in luminal breast cancer. Bioinformatics analysis was also used to determine the protein targets of galangin in luminal breast cancer, as well as its expression and patient survival rate. The cytotoxic impact of galangin was then used to determine its inhibitory effect on luminal breast cancer protein targets. The findings of this study could provide a foundation for the development of targeted luminal breast cancer therapy that uses galangin to suppress cell cycle progression.

2. Materials and Methods

2.1. Materials

Material utilized in computational research was chemical structure or the SMILES code of galangin by accessing the website http://www.swisstargetprediction.ch/. Meanwhile, the genes, specifically overexpressed genes on Luminal Breast Cancer were acquired from the UALCAN database. Subsequently, materials employed in the in vitro investigation were galangin (#282200) (Sigma Aldrich, USA), MCF-7 (ATCC, USA), Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) enriched with 10% v/v Fetal Bovine Serum (FBS) (Sigma, USA) and 1% penicillin/streptomycin (Gibco, USA), Trypsin (Gibco, USA), DMSO 1% (Sigma, USA), MTT (Sigma, USA).

2.2. Methods

2.2.1. Computational Test

Figure 1 depicts the flowchart for this study. The targets were predicted using SwissTargetPrediction (http://www.swisstargetprediction.ch/) by inputting the chemical structure or the SMILES code of galangin. Following that, 100 target proteins are listed (Daina *et al.* 2019). The UALCAN database (http://ualcan.path.uab.edu/) presents a list of genes that are overexpressed in luminal breast cancer. The top 250 overexpressed genes are compiled into a list (Chandrashekar *et al.* 2017). Subsequently, Venn diagram slices representing 250 overexpressed genes and 100 target proteins are created to identify galangin as a possible target in Luminal Breast Cancer.

2.2.2. Construction of the Intersection Network and PPI Network

The Venn diagram slice findings are submitted to the STRING (https://string-db.org/) platform, which evaluates the interaction between targets. "Homo sapiens" was entered in the species column. The minimum required interaction score of 0.4 was entered, and the protein interaction PPI network was created. A TSV format file was created and imported into Cytoscape 3.10.1 (https://cytoscape.org/) to generate a visual representation of the PPI network.

2.2.3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analyses

The biological function of galangin was investigated utilizing GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses. The targets of galangin were uploaded into a ShinyGO 0.80 version web application (http://bioinformatics.sdstate.edu/go/) to retrieve the database of the GO function [cellular component (CC), biological process (BP), and molecular function (MF)] and the KEGG pathway. ShinyGO 0.80 was used to plot the top 20 GO analysis results and the top 6 KEGG analysis results, sorted by $-\log_{10}$ (FDR) and presented as an expanded dot bubble and bar plot (Ge *et al.* 2020).

2.2.4. UALCAN Analysis

The target protein of galangin in luminal breast cancer was examined using UALCAN (http://ualcan.path.uab.edu), an online, accessible resource of microarray profiles and next-generation sequencing. CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12 gene expression were assessed throughout breast cancers and normal tissues, as well as in other subgroups such as cancer subtypes. All statistical data were collected directly from the relevant database.

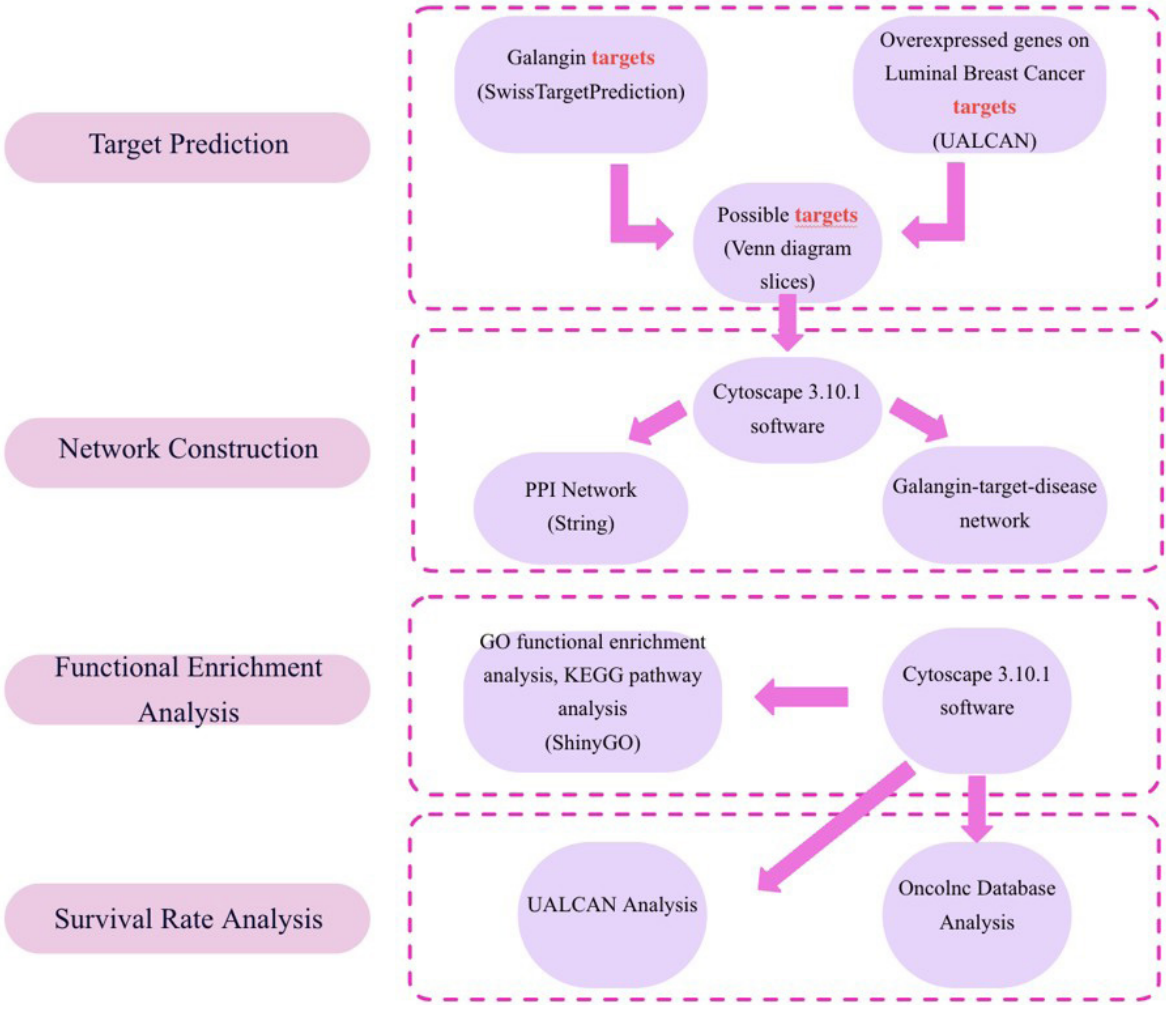

Figure 1. The flowchart of the bioinformatic analysis

2.2.5. Oncolnc Database Analysis

The Oncolnc database was used to evaluate the impact of overexpression of the galangin target protein in luminal breast cancer, as well as CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12, on overall survival. Based on the statistical computation the platform supplied, the significant variations in survival between the two distinct levels of expression were identified.

2.3. *In Vitro*

2.3.1. Cytotoxicity Activity of Breast Cancer MCF-7 Cells

A total, of 8×10^3 MCF-7 cells per well were planted on 96-well plates and treated with varying doses of galangin 10, 20, 80, 160, 320, and 640 µM for 24 hours in a 5% CO_2 incubator. Subsequently, cells were rinsed with phosphate buffer saline (PBS). Each well received 100 µL of 0.5 mg/ml MTT reagent and was incubated at 37°C for three hours. To stop the reaction, 10% sodium dodecyl sulphate (SDS) was added, and the mixture was incubated at room temperature overnight. The absorbance was determined at λ570 nm using a microplate reader, and the 50% inhibitory concentration (IC_{50}) value was obtained using a regression analysis as y = a +bx. By inserting 50 in variable y, the value of x was derived as the IC_{50} (He *et al.* 2016; Musyayyadah *et al.* 2021).

2.3.2. *In Vitro* Data Analysis

A one-way ANOVA with Bonferroni correction was used to determine cell viability. Statistical significance was established at $p<0.05$.

3. Results

3.1. Network Pharmacology Analysis of Galangin

Figure 2A depicts the chemical structure of galangin as taken from the PubChem NCBI database. The SwissTargetPrediction website revealed 100 potential galangin targets. Overexpressed genes in luminal breast cancer were identified using the UALCAN database, which contains 250 gene targets. Eight common targets of galangin against overexpressed genes in luminal breast cancer were discovered by intersecting 100 galangin targets with 250 overexpressed gene targets in luminal breast cancer (Figure 2B).

The target of galangin against overexpressed genes in luminal breast cancer was entered into the STRING database and processed, yielding a PPI network diagram. The data was uploaded into Cytoscape 3.10.1 software, and network topology analysis was done to filter the top core targets with the highest degree of centrality (Figure 2C, Table 1). Targets consist of CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12.

Eight common galangin targets and overexpressed genes in luminal breast cancer were evaluated for GO and KEGG enrichment. GO functional enrichment study demonstrated that the top pathways in the biological process (Figure 2D1) are chromosome condensation (Code GO), negative regulation of the G2/M transition of the mitotic cell cycle, mitotic spindle assembly, and protein localization to chromosomes. Subsequently, the top pathways in the cellular component (Figure 2D2) are the spindle midzone, mitotic spindle pole, spindle microtubule, and condensed nuclear chromosome. Also, the top pathways in molecular function (Figure 2D3) are histone kinase activity, protein serine kinase activity, protein serine/threonine/tyrosine kinase activity, and protein serine/threonine kinase activity. Moreover, KEGG enrichment analysis (Figure 2E) showed that galangin mainly regulates six signaling pathways, including endocrine resistance, progesterone-mediated oocyte maturation, cell cycle, oocyte meiosis, estrogen signalling pathways, and proteoglycans in cancer.

The protein levels of CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12 were assessed in different malignancies from TCGA using UALCAN analysis. Figure 3 demonstrates that the expression of these proteins was considerably higher in breast cancer. As a result, the expression of ESR1 and CA12 among diverse breast cancers reveals increased expression in breast cancers with the luminal subtype.

The prognostic value of each protein was analysed using a Kaplan-Meier plot using the Oncolnc database. Among these proteins, only PLK1 was significantly associated with the survival of patients (Figure 4). Patients with low levels of PLK1 had a greater overall survival than patients in groups with high expression ($p = 0.03$).

3.2. Cytotoxicity Activity of Breast Cancer MCF-7 Cells

The experiment was conducted to evaluate the cytotoxic activity of galangin in MCF-7 cells. Galangin was applied at 10 to 640 µM in a dose-dependent manner, $y = -0.0556x + 56.553$ with coefficient of correlation $(R2) = R^2 = 0.8141$ (Figure 5). The calculated IC_{50} value for galangin treatment was 117.86 µM, indicating that galangin exhibited a low level of cytotoxicity.

4. Discussion

Although a prior study revealed galangin's potential to suppress the proliferation of breast cancer cells, no investigation reported its therapeutic target protein in luminal breast cancer. Bioinformatics analysis and network pharmacology of galangin present a novel way to studying the mechanisms of therapeutic solutions for breast cancer treatment. Eight protein targets were found to be promising galangin targets for the inhibition of the luminal subtype of breast cancer. According to network pharmacology, galangin's protein targets against luminal breast cancer were CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12. PLK1, AURKB, NEK2, and CDK1 are among the most important cell cycle regulators. Pathway enrichment study revealed that galangin therapy in luminal breast cancer relies heavily on the cell cycle pathway. Targeting the cell-cycle signalling pathways is critically important due to cell-cycle proteins' role in regulating cell proliferation, which is often dysregulated in cancer (Otto & Sicinski 2017). This study also discovered that ESR1 and CA12 expression levels in luminal breast cancer are considerably greater than in normal cells. Subsequently, compared to groups with high expression, patients with low PLK1 levels had a higher overall survival rate. Therefore, the bioinformatics study shows that targeting ESR1, CA12,

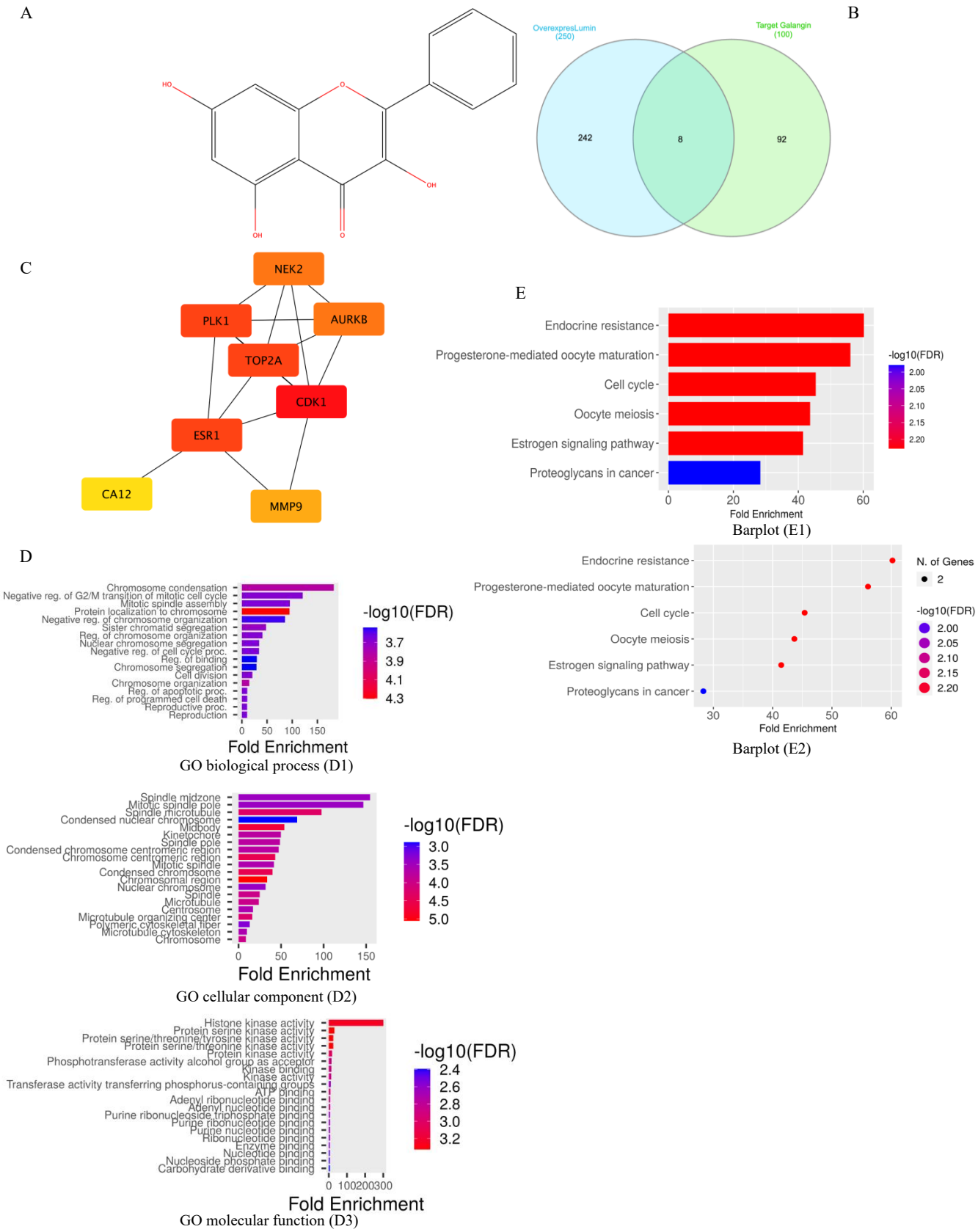

Figure 1. Network pharmacology predicted the potential mechanisms of PGV-1 or galangin for luminal breast cancer. (A) The chemical structure formula of galangin, (B) the Venn diagram of the targets shared by galangin and luminal breast cancer, (C) PPI network of galangin against luminal breast cancer, (D) GO enrichment analysis includes BPs, CCs, and MFs of galangin against luminal breast cancer, (E) KEGG enrichment analysis of potential therapeutic targets for galangin against luminal breast cancer

Table 1. Top 8 hub genes based on degree score 'Luminal and Galangin'

Gene symbol	Gene name	Degree score
CDK1	Cyclin-dependent kinase 1	6
PLK1	Polo Kinase 1	5
TOP2A	Topoisomerase II-alpha	5
ESR1	Estrogen receptor 1	5
AURKB	Aurora Kinase B	4
NEK2	Never in mitosis (NIMA) related kinase 2	4
MMP9	Matrix metalloproteinase-9	2
CA12	Carbonic anhydrase XII	1

and PLK1 could be an effective option for breast cancer treatment (Table 2).

Estrogen Receptor 1 (ESR1) holds an important role in breast cancer. ESR1 could promote tumor growth and the higher ESR1 expression is correlated with metastatic progress of cancer (Frannata *et al.* 2022). Mutations in ESR1 have been observed in 11-55% of metastatic ER-positive breast cancers and may activate other signaling pathways (Toy *et al.* 2013; Fanning *et al.* 2016). In addition, preclinical data suggests this mutation is a driver of metastasis and needs to be targeted to increase life

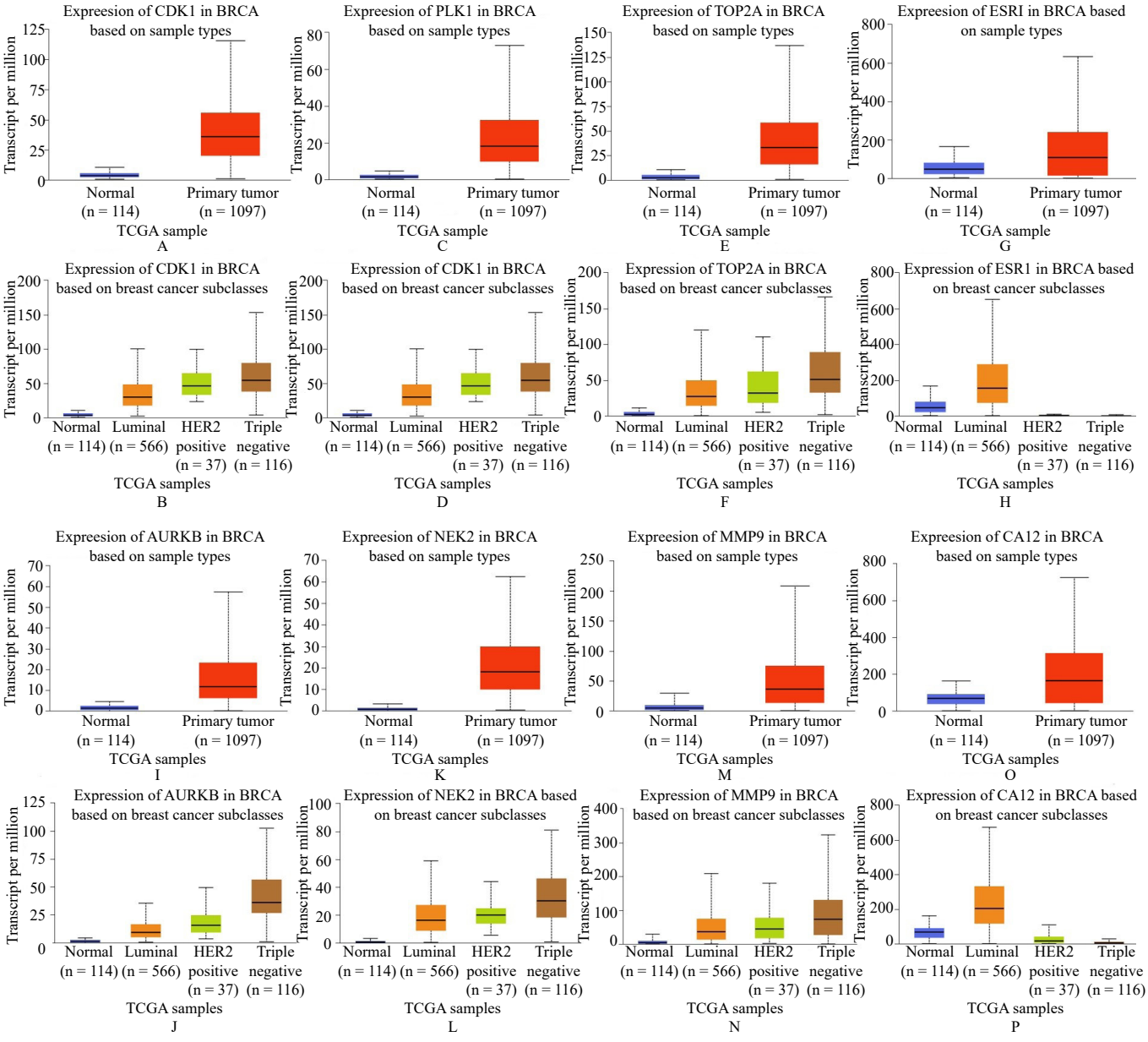

Figure 3. Expression of luminal breast cancer protein marker in normal cell, tumor cell, and various types of breast cancer cells, (A, B) CDK1 (p value 1×10^{-12}), (C,D) PLK1 (p value 1.62×10^{-12}), (E, F) TOP2A (p value 1×10^{-12}), (G, H) ESR1 (p value 1×10^{-12}), (I, J) AURKB (p value 1.62×10^{-12}), (K, L) NEK2 (p value 1.62×10^{-12}), (M, N) MMP9 (p value 2.09×10^{-5}), and (O, P) CA12 (p value 1.62×10^{-12}). The UALCAN website offers access to the TCGA database

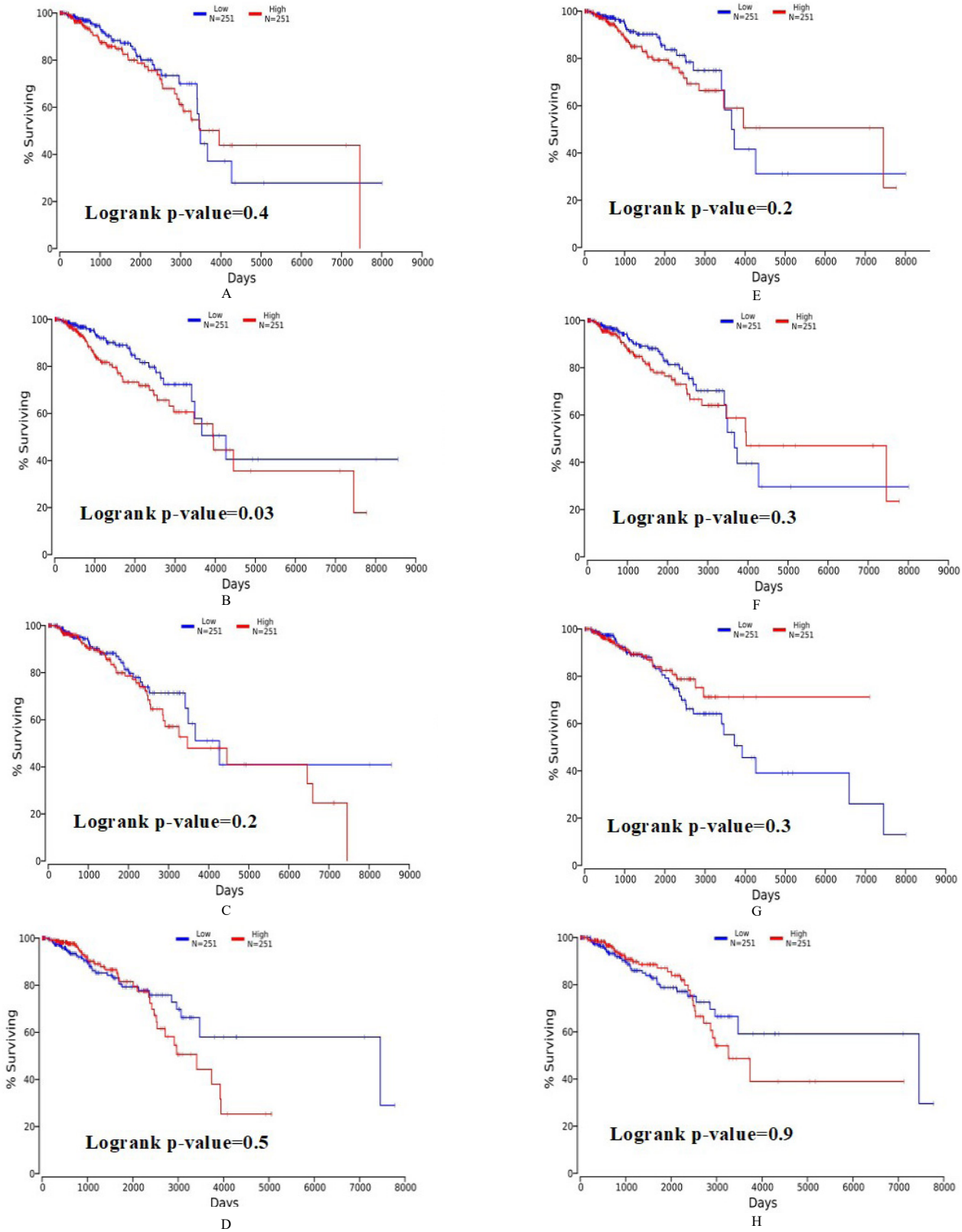

Figure 4. Overal survival in patients with breast cancer related to the protein levels of CDK1, PLK1, TOP2A, ESR1, AURKB, NEK2, MMP9, and CA12. The plot was considered significant if logrank was p<0.05. (A) Percent survival of patients with high and low CDK1 expression, (B) percent survival of patients with high and low PLK1 expression, (C) percent survival of patients with high and low TOP2A expression, (D) percent survival of patients with high and low ESR1 expression, (E) percent survival of patients with high and low AURKB expression, (F) percent survival of patients with high and low NEK2 expression, (G) percent survival of patients with high and low MMP9 expression, (H) percent survival of patients with high and low CA12 expression. The Oncoln website offers access to the TCGA dataset

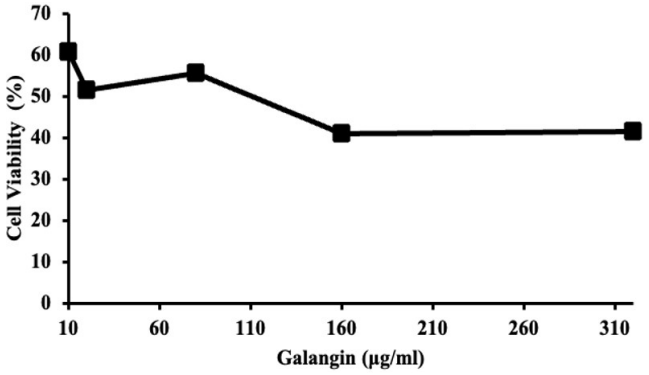

Figure 5. Cytotoxic effects of galangin on MCF-7. Cell viability was evaluated by MTT assay and was incubated with the compound for 24 hours. This showed the cell viability profiles after treatment with galangin

expectancy (Jeselsohn *et al.* 2018). Previously, galangin has been shown to induce S-phase cell cycle arrest in certain cancer cells through the suppression of the PI3K-AKT signaling pathway (Lee *et al.* 2018). Although there has been no direct evidence linking galangin to modulation of the ESR1 pathway, its effects on the PI3K-AKT pathway may indirectly influence ESR1 signaling. Mutations in ESR1 are known to activate alternative signalling pathways, including PI3K (Herzog and Fuqua 2022).

Meanwhile, carbonic anhydrases (CAs) are zinc metalloproteins engaged in catalysis (Mantovani *et al.* 2021). One of the CAs is Carbonic Anhydrase XII (CA12) is a transmembrane protein that helps catalyze

Table 2. Role and function of galangin target proteins

Protein name	Gene symbol	Role	Function	Reference
Never in Mitosis (NIMA) Related Kinase 2	NEK2	Kinase protein	Regulates the cell cycle and the duplication and separation of chromosomes	(Schultz *et al.* 1994; Fang and Zhang 2016)
Topoisomerase II-alpha	TOP2A	Catalysing enzyme	Cell cycle regulation, chromosome division, and apoptosis regulation	(Fountzilas *et al.* 2012; Wu *et al.* 2023)
Polo Kinase 1	PLK1	Kinase protein	Regulates stages of mitosis and meiosis, centrosome maturation, spindle assembly, and cytokinesis	(Liu 2015; Kalous and Aleshkina 2023)
Cyclin-dependent Kinase	CDK1	Kinase protein	Initiates mitosis, Regulates G2/M phase	(Ibar and Glavic 2017)
Aurora Kinase B	AURKB	Kinase protein	Regulates chromosome condensation and segregation	(Willems *et al.* 2018)
Matrix metalloproteinase-9	MMP9	Proteolytic enzyme	Degrades extracellular matrix (ECM) proteins, activates cytokines and chemokines to regulate tissue remodelling, and also promotes metastasis and angiogenesis	(Gialeli *et al.* 2011; Yabluchanskiy *et al.* 2013; Mehner *et al.* 2014)
Carbonic Anhydrase XII	CA12	Transmembrane protein	Catalyse carbon dioxide hydration and dehydration and is involved in cellular pH regulation of metabolically active cells/ tissues	(Silagi *et al.* 2021)
Estrogen Receptor 1	ESR1	Hormone receptor	Transcription factor in the metabolism of sex steroids	(Modugno *et al.* 2001; DeSantis *et al.* 2019)

a reversible reaction of carbon dioxide hydration and dehydration and is involved in cellular pH regulation of metabolically active cells (Silagi *et al.* 2021). CA12's most important tasks in intracellular pH homeostasis show how cancer cells adapt to the hazardous circumstances of the extracellular environment (Chiche *et al.* 2009). CA12 is overexpressed in human malignant tumours and has prognostic value, especially in pancreatic cancer, and helps promote tumor cell apoptosis via NF-κB signalling pathway (Damaghi *et al.* 2013; Du *et al.* 2021). CA12 is also implicated in invasion and metastasis, because genetic knockdown of CA12 could inhibit the invasion and migration of MDA-MB-231 breast cancer cells (Hsieh *et al.* 2010). This makes CA12 the most promising therapy supported by Li *et al.* (2021) who showed that CA12 is abundantly expressed in glial tumours relative to normal tissues and predicts a worse clinical fate in tumor patients. Inhibition of CA12 could potentially reduce cell proliferation, induce apoptosis in T-cell lymphomas, and also induce cell cycle arrest by disrupting the acidic microenvironment that cancer cells rely on leading to increased cellular stress and potential activation of cell cycle checkpoints (Lounnas *et al.* 2013; Mboge *et al.* 2018). Galangin interacts with the CA12 pathway in breast cancer cells, particularly through its effects on cell migration and invasion. Galangin inhibits the migration and invasion of breast cancer cells by reducing critical proteins such matrix metalloproteinases (MMP-2 and MMP-9) (Hsieh *et al.* 2010).

Furthermore, Polo-like kinases (Plks) are a family of serine-threonine kinases that play a variety of activities, particularly in intracellular processes like DNA replication, mitosis, and stress response. PLK1 is one of the Polo-like kinases (Plks) that regulates crucial roles of the cell cycle, such as mitosis, which includes functional centrosome maturation and bipolar spindle assembly, M phase entry, nuclear envelope breakdown (NEBD), sister chromatid cohesion, and the creation of kinetochore-microtubule attachments, and also cytokinesis (Liu 2015). According to recent investigations, PLK1 affects the motility and invasiveness of various cancer cells and has been discovered to be overexpressed in many malignancies (Lim *et al.* 2024). Because of its central role in cell cycle regulation, PLK1 has emerged as an appealing target for cancer therapy. PLK1 inhibition can cause cancer cells to undergo mitotic arrest and apoptosis. Inhibiting PLK1 induces apoptosis and DNA damage in glioma stem cells by controlling YBX1 nuclear translocation. (Li *et al.* 2023). Galangin inhibits PLK1 in HCC cells by directly downregulating its expression at both mRNA and protein levels, resulting in G0/G1 phase cell cycle arrest and apoptosis. (Li *et al.* 2024).

In addition to the results of bioinformatics analysis, *in vitro* study revealed that galangin with concentrations of 10 to 640 μM could inhibit the proliferation of one cell line subtype luminal, MCF-7 cells in a dose-dependent manner with the IC_{50} values of 117.86 μM (Figure 2). Therefore, the IC_{50} value of galangin in this study is potentially low cytotoxicity as compared to previously reported by Jaiswal *et al.* (2012).

The current study included a number of limitations. Initially, a specialized algorithm was employed to curate or anticipate galangin protein targets based on publically available data. Secondly, sophisticated clinical trials, as well as in vitro and in vivo experiments, are necessary to validate the results of the bioinformatics analysis. However, the findings of this study could accelerate the discovery of drugs for the treatment of luminal breast cancer.

Acknowledgements

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