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Ziziphus mauritiana in Triple-Negative Breast Cancer: Integrating Network Pharmacology and *In Vitro* Evaluation

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ABSTRACT

New metabolites isolated from botanical sources are renowned for having traditional and therapeutic significance. According to ethnopharmacological studies, *Ziziphus mauritiana* (ZM) is a medicine effective against various diseases, including cancer. However, the underlying mechanism of ZM effectiveness remains unclear. Therefore, this study aimed to determine how ZM affects the treatment of Triple-Negative Breast Cancer (TNBC) using network pharmacology and in vitro validation. A protein interaction network was then constructed to screen hub genes for ZM and TNBC, using STRING database and Cytoscape software. Subsequently, protein module cluster analysis was performed using Cytoscape MCODE plugin. DAVID database was also carried out to assess gene ontology, Kyoto Encyclopedia enrichment, as well as gene and genome pathway analysis for targets common to both ZM and TNBC. CCK-8 assay was used to assess the cytotoxicity of ZM on MDA MB-231 cells. The results showed that there were 5 hub genes for ZM, along with 6 possible active compounds, 93 possible therapeutic targets, and 5 genes affecting TNBC cell death, growth, metastasis, angiogenesis, invasiveness, and chemical resistance. ZM was found to significantly reduce cell viability ($p < 0.001$). It produced a cytotoxic effect against MDA MB 231 cells and influenced AKT1, MYC, STAT3, as well as TP53, the primary targets in TNBC therapy. These targets and pathways provided a theoretical basis for the development of new drugs that impact drug resistance in TNBC.

***Keywords*:** *Ziziphus mauritiana*, Triple-negative breast cancer, Network pharmacology, Apoptosis, Cancer cell proliferation.

Introduction

Breast cancer is reportedly the most common malignancy found among women. According to GLOBOCAN 2020 data, the number of new cases in the world is estimated at 2.261.419.[1] Triple-Negative Breast Cancer (TNBC) is a specific type that has negative estrogen receptor (ER), negative progesterone receptor (PR) expression, and negative human epidermal growth factor 2 (HER2). Genetic expression suggests TNBC is a subtype of basal-like breast cancer.[2] Epidemiologically, it occurs in premenopausal women under the age of 40 years, which constitutes 15-20% of all breast cancer patients. Given the distinct molecular subtype, there is currently no specific treatment for TNBC, either through endocrine or molecular target therapy.[3] Chemotherapy is the primary therapy for TNBC, but the efficacy of adjuvant postoperative chemotherapy is very low.

Several countries widely use bevacizumab as a combination chemotherapy for TNBC, but it does not significantly increase patient survival time.[4,5] Therefore, developing new targeted therapy regimens for TNBC is essential.

In recent years, compounds derived from plants have often been developed as drugs for various diseases, including cancer. The wide application is due to the diverse pharmacological properties, including chemoprotective and cytotoxic effects.[6] Several well-known anticancer drugs, such as camptothecin, paclitaxel, and vinblastine, originate directly from natural plants and herbs.[7] *Ziziphus mauritiana* (ZM), included in *Rhamnaceae* group, is a flora often found in Asia and the Middle East, growing 400 meters above sea level with extreme and dry temperatures.[8,9] Several studies showed that ZM contains antioxidants,[10] antimicrobials,[11] and antileishmania.[12] It also contains aliphatic, phenolic, flavonoid, steroid, and triterpene compounds. Triterpenes in ZM, such as betulinic and ceanothenic acids, can increase anti-tumor, anti-inflammatory,[13,14] and antifungal activity.[15] Previous studies have suggested that ZM could serve as an anticancer agent.[16–18] However, no study has examined the potential use in TNBC. Further studies are needed to understand the potentially complex molecular mechanisms of the active antitumor compounds in ZM that could potentially treat TNBC.

Network pharmacology, a combination of pharmacology and pharmacodynamics is a new field of study that helps scientists understand how different drugs work together at various levels.[19] This study introduced the possibility of using ZM in TNBC therapy. Network pharmacology was used to investigate the potential molecular mechanism of ZM leaf extract as a therapeutic agent for TNBC. The results were then confirmed by using CCK8 assay to test the viability of TNBC cells.

Material and Methods

ZM Leaf Profile and Prediction of Bioactive Compound Activity

SMILE (Simplified molecular-input line-entry system) profile searches and 3D structures of each compound found in ZM leaf literature were obtained from the PubChem database

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(https://pubchem.ncbi.nlm.nih.gov/). The leaf profile of ZM was obtained from a study conducted by Mohankumar.[20]
The potential compounds in ZM leaf were analyzed using WAY2DRUG PASS prediction (http://www.pharmaexpert.ru/passonline/predict.php, accessed on December 7, 2023) as an anti-cancer treatment. WAY2DRUG PASS prediction used Structure Analysis Relationship (SAR) analysis to compare input compounds and those known to have particular potential, with a cutoff score of Pa 0.5. The prediction value increased with the similarity of the compound structure.[21]

Target Protein Prediction
Target analysis of ZM leaf was obtained from target SEA analysis (https://sea.bkslab.org/)[22] and the Comparative Toxicogenomics Database (https://ctdbase.org/).[23] Target predictions were obtained by entering SMILE data that had been obtained. The cut-off p-value was less than 0.01, and the maximum Tanimoto score was greater than 0.5. The Coefficient SEA Target score was found to be greater than 0.5 in the range of 0-1. Proteins and genes related to TNBC were obtained from the Genecards (http://www/genecards.org/) and Open Target database (https://www.opentargets.org/, accessed on January 1, 2024). Target genes related to TNBC and ZM leaf were then mapped using a Venn diagram (https://bioinfogp.cnb.csic.ec/tools/venny/) to determine the intersection. Annotation of ZM leaf targets was carried out using DAVID web server (https://david.ncifcrf.gov/)[24] with KEGG biological process and pathway terminology (accessed on January 9, 2024).

Network Pharmacology Analysis
The interaction of protein targets and TNBC was carried out using ZM v.11.5 STRING database.[25] The tsv format resulting from string analysis was then processed using Cytoscape V.3.10 for network analysis to determine the topological score. Furthermore, the Molecular Complex Detection (MCODE) plugin was used to determine the protein modules with the most dense interactions (accessed on January 1, 2024).

Collection and Authentication of Plant
Fresh ZM leaf was obtained from Malang, East Java, Indonesia (7° 59' 2.0688" S and 112° 37' 17.0076" E) in November 2023. Authentication was carried out by the Technical Implementation Unit of Materia Medica, Medical Laboratory Batu, East Java, Indonesia (Ref No. 000.9.3/3189/102.0/2023).

Preparation of Extract
The dried leaf was crushed and sieved with a mesh size of 80. The obtained powder (500 g) was extracted successively using nonpolar to polar menstruum with increasing polarity, starting from petroleum ether (60°C-70°C) to MeOH (65°C-75°C), followed by water (80°C-100°C) with a continuous hot extraction method for 6 hours. In MeOH extract, the menstruum was distilled and concentrated under reduce pressure to form a dark green mass. The concentrated ZM mass was stored at 2°C-4°C for further use.[16]

Cell line and Culture
MDA-MB 231 cells in this study were obtained from the American Type Culture Collection (ATCC; Rockville, USA). The cells were cultured in the biomedical laboratory culture division at the Faculty of Medicine, Universitas Pajajaran, Indonesia. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic solution (10 mg/ml streptomycin and 10,000 U/ml penicillin). Cell maintenance was carried out in an environment of 5% CO_2 and humidified air at 37°C.

Cell Viability Assay
Cytotoxic activity of ZM against MDA-MB 231 cells was determined using CCK8 assay (GLPBio, USA). Cancer cells were cultured in 96-well plates at a density of 100 µl per well in DMEM (100 µl). The cells were then incubated with 95% air humidity and 5% CO2 at 37°C for 24 hours. The doses of ZM used were 10, 25, 50, 100, 200, 300, and 400 µg/ml. Following the addition of CCK8 solution (10 µL) to each well of the plate with a repeating pipette, the plate was incubated for a duration of 1 to 4 hours. The absorbance was subsequently measured at 450 nm using a microplate reader, and the viability cell was calculated employing the formula:[26]

$$\text{cell viability (\%)} = \left[\frac{(\text{As} - \text{Ab})}{(\text{Ac} - \text{Ab})}\right] \times 100$$

Where;
As = absorbance of the experimental well
Ab = blank well absorbance
Ac = control well absorbance

Statistical Analysis
Data were expressed as the mean ± standard deviation (SD), and statistical analysis was conducted using SPSS 23.0 (SPSS, Chicago, IL) software. Statistically significant differences were identified using one-way analysis of variance (ANOVA). P-values were designated as follows *P < 0.05; **P < 0.01; ***P < 0.001.

Results and Discussion

Prediction of Potential Bioactive Compounds using the Structure Analysis Relationship (SAR) Approach
ZM leaf compounds were obtained from relevant studies, and then SMILE and 3D structure were carried out using the PubChem database, obtaining 18 potential compounds as antineoplastics. SAR analysis with Way2Drug Pass Online found that the bioactive ZM had quite a favorable potential as an antineoplastic agent. Oleanolic, Zizyberanalic, Alphitolic, Fluorocytidin, Betulinic, and Ceanothenic acids were the TOP 6 bioactives with the highest antineoplastic potential. C11 (betulinic acid), C12 (oleanolic acid), C16 (ceanothenic acid), and C18 (alphitolic acid) all had the potential to treat cancer in a similar manner (Figure 1). In several studies, these compounds play a role in apoptosis through the intrinsic pathway.[27] Additionally, betulinic acid contributes to breast cancer by inhibiting the NF-kB and topoisomerase pathways, as well as regulating protein transcription.[27]

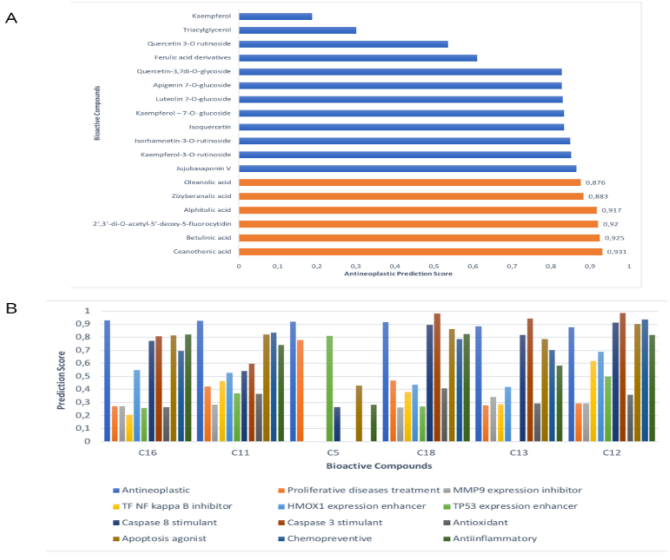

Figure 1: Prediction of the Potential of ZM using SAR Method as an Antineoplastic. (A) Based on bioactive compound; (B) Based on TOP 6 bioactive with the highest antineoplastik potential. Note: 2‘,3‘-di-O-acetyl-5‘-deoxy-5-fluorocytidine (C5); Betulinic acid (C11); Oleanolic acid (C12); Zizyberanalic acid (C13); Ceanothenic acid (C16); Alphitolic acid (C18)

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Target Protein Prediction

The Venn diagram identified 93 targets for ZM and TNBC (Figure 2). The Gene Ontology Biological Process showed that ZM target primarily contributed to the negative regulation of the intrinsic apoptotic signaling pathway (Figure 3a). Several genes that play a role in cell proliferation and apoptosis include p53, B-cell lymphoma (Bcl2), Nuclear Factor kappa-light-chain enhancer of activated B cells (NFkB), myc, Phosphatidyl-inositol 3-kinase (PI3K), and AKR mouse thymoma (AKT).

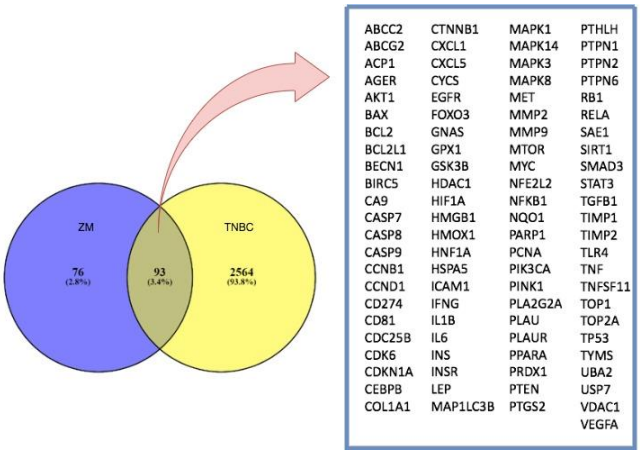

Figure 2: Venn Diagram of ZM Target and Target Association with TNBC

In KEGG pathways analysis, ZM plays a role in cancer and PI3K/Akt pathways (Figure 3b). The enzyme PI3K is found on the plasma membrane and activated by receptor tyrosine kinases (RTKs) as well as GPRs (G protein-coupled receptors). In general, GPRs, the most abundant type of receptor found on the surface of cells have a consistent structure, consisting of a single chain of protein that spans the cell membrane. These receptors use G proteins to relay signals to the cytosol, the fluid inside the cell. AKT is a key molecule downstream of PI3K cascade. When activated, AKT plays a role in regulating the cell cycle, promoting growth, facilitating proliferation, and controlling energy consumption. It has more than 100 substrates, which include transcription factors, cell cycle inhibitors, guanosin triphospatase (GTPase)-activating proteins, and apoptosis inducers.

The PI3K pathway undergoes multiple alterations in breast cancer, resulting from mutations or amplifications in the genes, which encode p110*α*, the catalytic subunits of PIK3CA and p110*β* (PIK3CB), along with the regulatory component p85α (PIK3R1). Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) is frequently mutated in human neoplasms. The p110*α* is encoded and is frequently found to be significantly elevated in head and neck, stomach, lung, cervical, and breast cancers. PIK3CA mutations are most frequently found in colon, prostate, endometrial, and breast cancers. Mutations occur in about 30 to 40% of breast cancer patients, resulting in the hyperactivation p110*α*.

Food Drug Association (FDA) has approved testing breast cancer patients using mutations of PI3K. This testing entails analyzing and isolating circulating tumor DNA from plasma specimens[28]. According to previous studies, ZM leaf extract using copper nanoparticles can induce anticancer effects in human renal cell carcinoma A498 cells through the mTOR/PI3K/Akt signaling pathway, which mediates apoptosis,[29] and modulates PI3K/Akt pathways in ER (-) xenograft mice.[30]

Network Pharmacology Analysis

The intersection target between ZM and TNBC targets was then analyzed by network pharmacology using STRING and CytoScape. STRING was used to observe protein interactions, while Cytoscape was used to determine the network topology score. The darker and wider the circle diameter, the higher the degree of centrality value (Figure 4a). Degree centrality describes the number of proteins, which interact with the node. TP53 and STAT3 were identified as the targets with the most significant degree values. Degree was used to determine the interactions between nodes to recognize the most pathway proteins. Furthermore, closeness centrality (CC) is a calculation of the average length of the shortest path to access all other proteins in the network, with high CC indicating the protein is simple to reach. Betweenness centrality (BC) compares the number of shortest paths that pass through a node with the total number of shortest paths. The higher the BC value, the more significant the role in information flow.

Molecular Complex Detection (MCODE) was used to identify the densest node interactions. Nodes included in the Top MCODE were filtered to determine the TOP 5 proteins, which were identified to be TP53, STAT3, AKT1, NFKB1, and MYC (Table 1). Therefore, these nodes were the ones that recognized the most other proteins in the pathway (Figure 4). Based on network analysis,

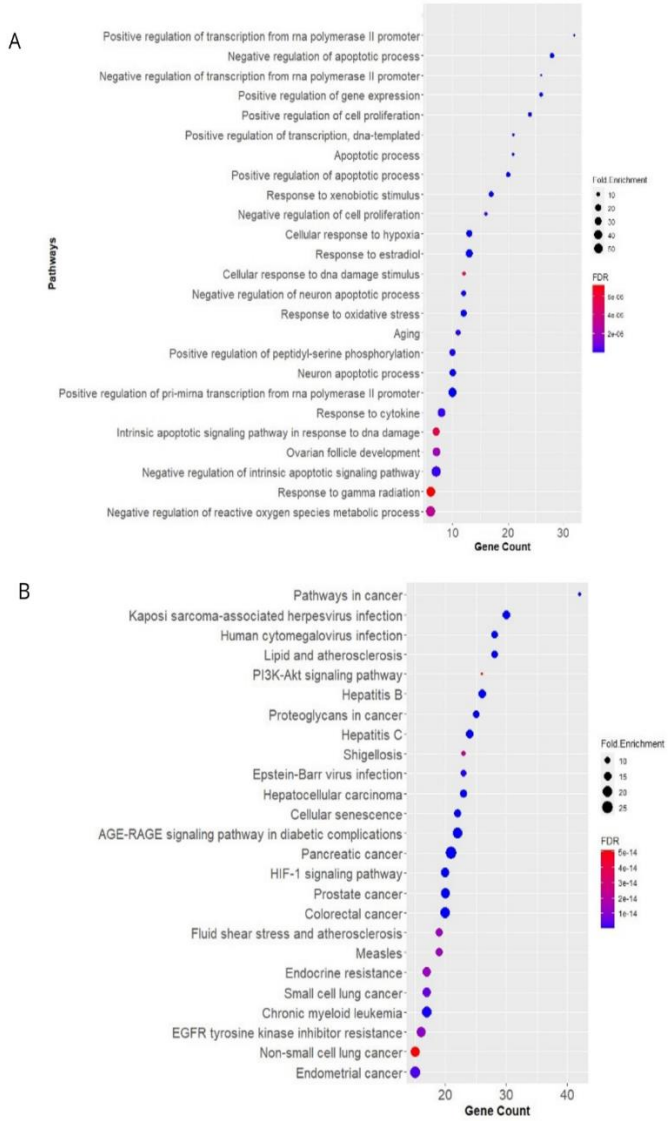

Figure 3: Target Protein Predicition. (A) Gene Ontology Annotation TOP 25 Biological Process Target ZM FDR < 0.01; (B) Annotation of TOP 25 KEGG Pathway Target ZM FDR < 0.05

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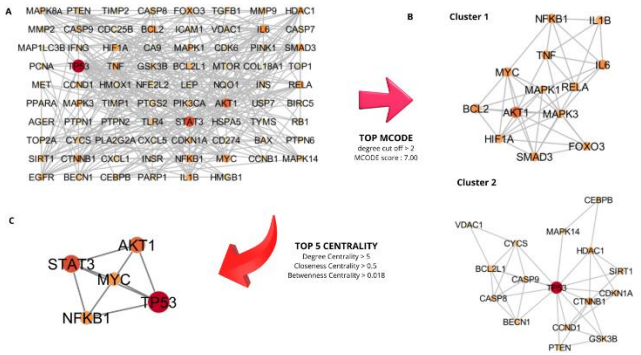

Figure 4: PPI Network (A) and Cluster analysis of 10 TOP protein modules in the PPI network (B)

TP53 was the node with the most potential.

P53 is a suppressor gene and contributes to tumor cell growth. TNBC has several P53 mutations, thereby making P53 pathway a viable therapeutic target. Normal cells do not activate P53 gene but several agents that cause DNA damage can stimulate cells and increase P53 expression. When DNA or chromosome damage occurs, P53 functions to initiate apoptosis.[31] In addition, cancer cells require a suitable environment for growth, which includes the need for factors caused by hypoxia, such as HIFα. Reactive Oxygen Species (ROS) production also activates the genes required for transcription of these factors. The enhanced expression of hypoxic genes further facilitates the development of cancer cells by increasing angiogenesis levels. The ROS production also leads to the activation of various oncogenes, including c-Myc and Akt, which are essential for cancer cell proliferation. Tumor suppressor genes, including P53, inhibit ROS production, resulting in decreased proliferation of cancer cells. Cytokine receptors can generate ROS that compromise the immune system, facilitating cancer cell metastasis.[32,33]

By examining the interactions among potential therapeutic targets, this study identified three distinct protein modules that each serve different biological functions. Module one includes topics such as cell apoptosis, hypoxia, and positive transcriptional regulation. Module two focuses on G1/S change in the cell cycle, the phosphorylation of proteins, and how p53-class mediators control signal transduction. Module three is related to the process of proliferation. These 93 putative therapeutic targets which have a close association with other targets are considered the "hub" of the therapeutic network. Furthermore, the five fundamental proteins, namely NF-kB, MYC, STAT3, AKT1, and TP53 have a collaborative function in the molecular processes of cell proliferation, metastasis, apoptosis, invasion, angiogenesis, and drug resistance in TNBC cells.

Nuclear factor kappa beta (NF-kB) is a major transcription factor crucial to inflammation, cell proliferation, differentiation, and various immunological responses. ROS are known to activate NF-κβ and NRF2 to avoid apoptosis.[34] Nashikari found NF-B activation in various breast cancer cell lines that were negative for estrogen receptors.[35] In approximately 30% of breast cancers, IKβKE was amplified or overexpressed to induce NF-kB and STAT, resulting in tumorigenesis. This makes NF-kB a potential therapeutic target for TNBC.[36] Furthermore, Sameem showed that anticancer extract from ZM leaf in hepatic carcinoma was proven to reduce oxidative stress and pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, interleukin-1β, and NF-kB.[18]

Signal transducer and activator of transcription (STAT) is a protein with seven members, namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Specifically, STAT3 plays a role in essential cell functions such as cell growth, cell survival, cell respiration, regeneration, differentiation, and immune response. It also functions in the regulation of Janus Kinase (JAK) and Epidermal Growth Factor (EGFR).[37] In TNBC patients, overexpression of JAK1 and JAK2 usually occurs. JAK/STAT pathway plays a crucial role in fundamental biological processes such as cell proliferation, differentiation, apoptosis, and immunological control. Over 50% of breast cancers contain phosphorylated STAT3, which is often associated with a poor prognosis due to the high invasiveness. Previous studies indicated that irregularities in IL6/JAK2/STAT3 pathway have a significant impact on TNBC. JAK/STAT3 might be a promising target for therapeutic intervention in TNBC.[38–40] Periasamy demonstrated that administering *Ziziphus jujuba* suppressed the activation of NFkB/IL-6/JAK1/STAT3 signaling pathways. Periasamy suspects that inhibiting this pathway can suppress colon tumorigenesis in mice.[41]

Table 1: TOP 5 Network Analysis PPI Ziziphus mauritiana

Parameter	Value	Closeness Centrality	Degree
TP53	0.308121917	0.636364	38
STAT3	0.181233685	0.566176	28
AKT1	0.092723754	0.553957	24
MYC	0.020430321	0.506579	17
NFKB1	0.045334095	0.503268	17

ZM Decreased The Viability of MDA MB-231 Cells

An inverted phase contrast microscope was used to observe significant morphological changes in MDA-MB 231 cells after 24 hours of exposure to ZM leaf extract at various concentrations. The results showed that MDA-MB 231 breast cancer cells were less likely to survive when exposed to extracts at different ZM concentrations compared to controls. This demonstrated that depending on the exposure dose, the administration of ZM significantly inhibited the viability of MDA-MB 231 cancer cells (Figure 5).

The viability of MDA-MB 231 cells when given the lowest dose of ZM (10 µg/ml) was 93.63% compared to the untreated group. Meanwhile, high doses of 200, 300, and 400 µg/ml could kill MDA-MB 231 cells with cell viability of 88.42%, 72.28%, and 29.77%, respectively. Statistically significant difference were found between dose groups ($p < 0.0001$) and administration times ($p < 0.05$).

The network pharmacology findings offer important insights into the possible anticancer mechanisms of ZM. The results indicate the plant's capacity to engage with multiple targets associated with cancer development and progression. Despite the results, this study remains subject to some limitations. Pathological processes are implicated in the development of the disease. The selection of treatment should consider the stage of TNBC since the therapeutic benefits of these substances varied dramatically at each stage. Consideration should also be given to the therapeutic effects of various ZM dosages while treating TNBC. The focus on database analysis and computational methodologies has not yielded any experimental results that confirm the results. Consequently, additional experimental studies are required to validate the mechanism of action for ZM against TNBC through the pathways and targets examined.

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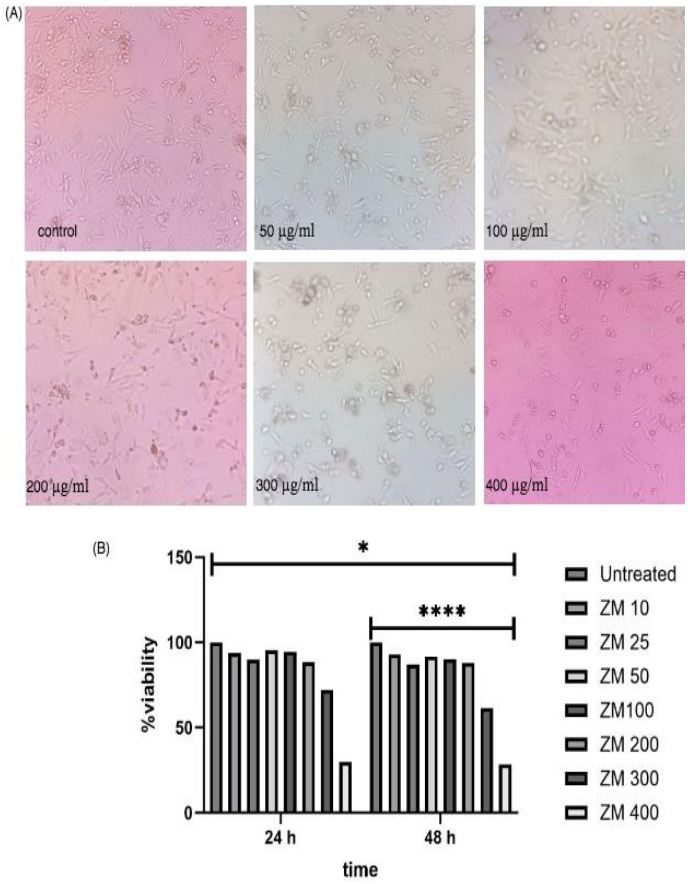

Figure 5: Effect of ZM leaf extract on MDA-MB 231 cell viability. (A) Morphological description of MDA-MB 231 cells after administration of ZM at concentrations of 50, 100, 200, 300, and 400 µg/ml (100x magnification). (B). Percentage of cell viability using CCK8 assay after 24 hours and 48 hours of administration of ZM leaf extract.

Conclusion

In conclusion, this study demonstrated that ZM significantly reduced the viability of MDA-MB 231 cells. Based on the pharmacological pathway, ZM controlled key targets, including MYC, AKT1, TP53, and STAT3, in the treatment of TNBC, implicated in apoptosis, cancer cell proliferation, invasion, metastasis, and resistance to chemotherapy through the PI3K/Akt and NFκB signaling pathways. These targets and pathways offer a theoretical basis for the development of new drugs that affect drug resistance in TNBC.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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