RESEARCH ARTICLE

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The Potential of *Eleutherine bulbosa* in Inducing Apoptosis and Inhibiting Cell Cycle in Breast Cancer: A Network Pharmacology Approach and *In Vitro* Experiments

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Abstract

Objective: The objective of this study was to evaluate the potential and mechanisms of phytochemicals in Eleutherine bulbosa (EBE) in inducing apoptosis and inhibiting the cell cycle in breast cancer through a network pharmacology approach and in vitro validation. **Methods:** This research employed a literature review approach to identify active anti-cancer compounds and utilized a network pharmacology approach to predict the mechanisms of action of EBE compounds in breast cancer. In addition, in vitro experiments were conducted using MTT method to evaluate the effects of EBE on the cytotoxicity of T47D cells, and the flow cytometry method was employed to determine the impact of EBE on apoptosis and the cell cycle. **Results:** The network pharmacology analysis revealed that EBE had an impact on 42 genes involved in breast cancer, including 23 important target genes implicated in the pathophysiology of breast cancer. Pathway analysis using the KEGG database showed a close association between EBE and crucial signaling pathways in breast cancer, including P53 signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, apoptosis and cell cycle. In vitro experiments demonstrated that EBE exhibited moderate anti-cancer activity. Furthermore, EBE demonstrated significant potential in inducing apoptosis in breast cancer cells, with a percentage of apoptotic cells reaching 93.6%. Additionally, EBE was observed to disrupt the cell cycle, leading to a significant increase in the sub G1 and S phases, and a significant decrease in the G2-M and G1 phases. **Conclusion:** EBE has the potential to be an anti-cancer agent through various mechanisms, including apoptosis induction and cell cycle inhibition in breast cancer cells. These findings provide new insights into the potential of EBE as an alternative treatment for breast cancer.

Keywords: Apoptosis- cell cycle- MAPK- PI3K- T47D

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Introduction

Breast cancer is a type of cancer that significantly impacts the health and quality of patient life (Montazeri, 2008). To address this challenge, research intended for the development of effective drugs for breast cancer treatment is very crucial. One promising approach in cancer drug development is network pharmacology, which is crucial in the development of natural compound-based cancer drugs. It provides a comprehensive understanding of the complex interactions between active compounds in natural ingredients and the biological targets involved in cancer development (Tang and Aittokallio, 2014). Therefore, by understanding the complex interactions between active compounds and biological targets within the cancer network, scientist can identify potential compounds, comprehensively evaluate drug effects, and optimize the use of drug combinations. This can help expedite the development of more effective cancer drugs and potentially reduce the global burden of the disease (Tang and Aittokallio, 2014; Zhang et al., 2019).

In this context, the extract of Eleutherin bulbosa (EBE) has been the focus of research as a potential candidate for breast cancer drug development. Previous studies have reported that EBE contains phytochemicals such as flavonoids, naphthalenes, and naphthoquinones (Insanu et al., 2014), which are known to have anticancer activities. To understand the mechanisms of action and effects of these compounds, a network pharmacology approach was applied in our study. This method allows researchers to understand the complex interactions between the compounds in EBE extract and the biological pathways

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involved in the growth and spread of breast cancer cells. It is essential to identify target compounds and understand their mechanisms of action in inhibiting cancer cell growth (Poornima et al., 2016).

In previous studies, there has been no reported evaluation of the molecular mechanisms, gene targets, and biological process pathway mechanisms of EBE phytochemicals using a network pharmacology approach integrated with in vitro validation. Therefore, this research aims to evaluate the potential, gene targets, and biological process pathway mechanisms of phytochemicals in Eleutherine bulbosa (EBE) in inducing apoptosis and inhibiting the cell cycle in breast cancer through a network pharmacology approach and in vitro validation.

Materials and Methods

Preparation of EBE Extract

The bulbs of EBE plants were collected from East Kalimantan, Indonesia. The sample was identified and authenticated at Materia Medika Laboratory Malang with collection number 074/348/102.7/2021. Specimens were stored in the Pharmacognosy Laboratory, Department of Pharmacy, Maulana Malik Ibrahim State Islamic University Malang. The powdered plant material was extracted using 70% ethanol as a solvent at a ratio of 1:20 (powder to solvent). The extraction method used was ultrasound-assisted extraction (UAE). The obtained filtrate was evaporated using a rotary evaporator. Subsequently, the extract was dried in an oven at 40°C before being used for further analysis.

Collection and Screening of Bioactive Components in EBE

The bioactive compound components of EBE were obtained based on a literature review from scientific studies using Google Scholar, PubMed, and ScienceDirect databases with the keyword "Eleutherine bulbosa compound."

Collection and Screening of Target Proteins

The target proteins and genes of the compounds were obtained from GeneCards. The results obtained from GeneCards were limited to targets with a relevance score of ≥10.00, which is considered to meet the database standards (Liu et al., 2018).

Collection and Screening of Disease-Related Target Proteins and Network Construction

The exploration of target genes associated with breast cancer for EBE compounds was conducted using DisGeNET (https://www.disgenet.org/). Next, a network of target proteins for the collected EBE compounds was constructed and visualized using Cytoscape v3.9.1., based on similarity networks. Target proteins and bioactive components of EBE were represented as "nodes," and the interactions between two proteins were represented as "edges." The more important proteins targeted by a particular component, the more significant the component was considered (Kong et al., 2021).

Construction of Protein-Protein Interaction (PPI) Network and Enrichment Analysis

Target genes at the intersection of active compounds and diseases were selected for further analysis using the STRING Version 12.0 (https://string-db.org/). The PPI network was constructed using common target proteins with a minimum required interaction score of 0.400. PPI network analysis was used to investigate biological activities by examining gene functional annotations through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to understand their role in signal transduction (Huang et al., 2020).

Cell culture

T47D breast cancer cells were obtained from Dr. Masashi Kawaichi at the Nara Institute of Science and Technology (NAIST), Japan. The cells were cultured as a monolayer in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma, USA), 150 IU/ml penicillin-150 µg/ml streptomycin (Gibco, USA), and 1.25 µg/ml amphotericin B (Gibco, USA). The cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere. For experiments, T47D cells were used at 80-90% confluence (Amalina et al., 2023).

Cell Viability Assay

The proliferation of T47D cells was studied using the MTT assay method. T47D cells (2×10^3 cells/well) were seeded in a 96-well micro-plate and allowed to attach overnight. The cells were incubated for 24 hours with EBE treatment, 1,4-naphthoquinone (Sigma-Aldrich, USA) (50–500 µM), and doxorubicin (DOX) (Sigma-Aldrich, USA) (0.01–10 µM). Untreated cells were used as a negative control. After treatment, 100 µL of MTT (Biovision) (0.5 mg/mL in medium) was added to each well and incubated for 4 hours at 37°C with 5% CO_2. Subsequently, the MTT formazan crystals were dissolved using a stop solution containing sodium dodecyl sulfate (SDS) and 0.01 N HCl and incubated overnight under dark conditions. Once the purple formazan was dissolved, the absorbance was measured using ELISA reader (Corona SH-1000) at a wavelength of 595 nm. Each treatment was performed in triplicate, and the cytotoxic activity was measured as the IC_{50}, which is the concentration required to reduce the cell population by 50% compared to the untreated cells (Mutiah et al., 2020).

Cell Cycle Analysis

Cell cycle analysis was performed using flow cytometry with propidium iodide (PI) staining. T47D cells with a density of 2×10^5 cells/well were cultured in a 6-well microplate. After treatment with EBE, 1,4-naphthoquinone (Sigma-Aldrich, USA) (50–500 µM), and DOX (Sigma-Aldrich, USA) (0.01–10 µM), all media were removed, and the cells were trypsinized and centrifuged at 2,000 rpm for 3 min. The collected cells were resuspended and fixed with ethanol for 30 min at 4°C. Subsequently, the cells were washed with cold PBS and centrifuged at 2,000 rpm for 3 minutes. The cell pellet

was resuspended in a solution of PI (50 µg/mL in PBS containing 1% Triton X-100 (Merck)) and DNase-free RNase A (20 µg/mL), and incubated for 30 min at 37°C. Finally, the cells were analyzed using Flow cytometry (FACS Calibur, BD Biosciences, USA). After electronic debris was discarded, red fluorescence was counted using the FL1 setting (log mode) (Mutiah et al., 2017).

Apoptosis Assay

Apoptosis assay was performed using Annexin V-FITC/PI staining and flow cytometry method for EBE, DOX, and 1,4-naphthoquinone treatments. Briefly, the collected cells were stained using the Annexin-V-FLUOS staining kit (Roche), consisting of 100 µL binding solution, 2 µL Annexin V, and 2 µL PI, for 10 min at ambient temperature in the dark. The cells were then measured using flow cytometry (FACS Calibur, BD Biosciences, USA). Fluorescence intensity was measured using the FL-1H parameter to detect FITC. Subsequently, the percentage of apoptosis was analyzed using the Cell Quest program (BD Bioscience) (Mutiah et al., 2018).

Results

Active compounds in EBE

From the literature review, a total of 16 active compounds (Table 1) with potential anticancer activity have been identified. These compounds belong to the groups of Naphtalene, Naphthoquinone, and Flavonoid.

Potential Breast-cancer target gene of the compounds

EBE contains 16 active compounds, and the target genes for breast cancer of these compounds were determined using the GeneCards database. The selected target gene relevance score was set to >7 (Zeng and Yang, 2017). Among them, four compounds showed target gene relevance scores >7, namely Eleutheroside B, C, D, and resveratrol. To determine the association between the compounds and breast cancer target genes, we constructed a compound-target interaction network (Figure 1). With an average number of neighbors of 2,291 and characteristic path length of 1835, this network has a density of 0.021. The results indicated that only Resveratrol compound has target genes related to breast cancer.

In this study, we used the Cytoscape application to visualize the interactions among compounds, active components, and target genes in breast cancer (Xiong et al., 2008). Through our analysis, we successfully constructed a pharmacological network consisting of 55 nodes and 65 edges connecting "compound-active component-target gene" (Figure 2). Our findings revealed that there are 16 EBE compounds specifically targeting 42 genes. Furthermore, we identified 23 important target genes that play a role in the pathophysiology of breast cancer. Regarding the resveratrol compound, out of the 27 target genes, 18 compounds were found to be associated with breast cancer target genes.

Construction of Protein-Protein Target Interactions

In this study, we utilized protein-protein interactions (PPI) selected from the STRING interactome database to depict biological processes, cellular components, molecular activities, and target protein pathways (Szklarczyk et al., 2017). The threshold value was determined based on a p-value of 0.05. As shown in Figure 3, the network was visualized using all target

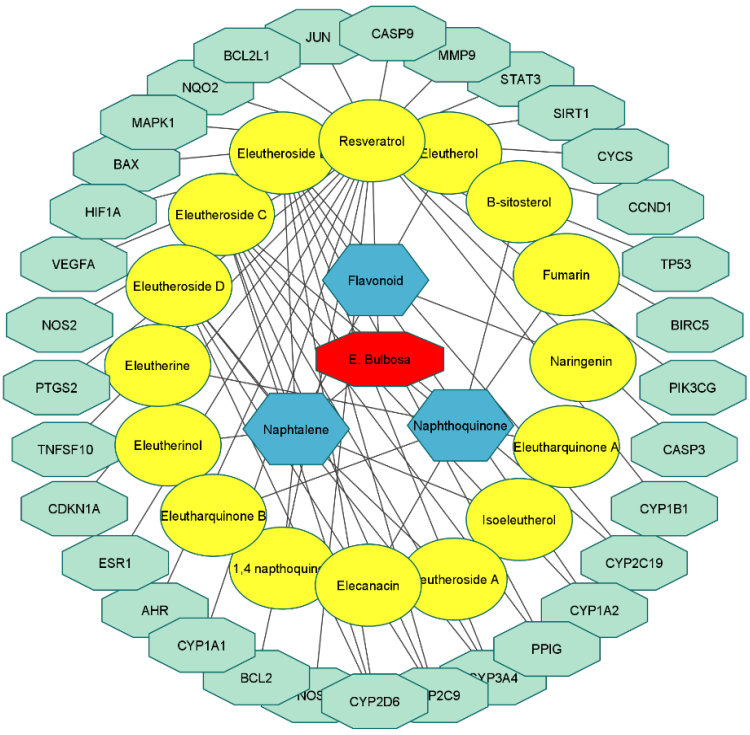

Figure 1. Network Topology of Compounds in *Eleutherine* Bulbosa with the Target Gene (Number of nodes: 55, Number of edges 63). The blue hexagons represent a group of compounds, yellow ellipses represent active components, and green heptagon represent gene targets. There are four compounds that can target multiple genes, namely resveratrol, Eleutheroside B, C, and D.

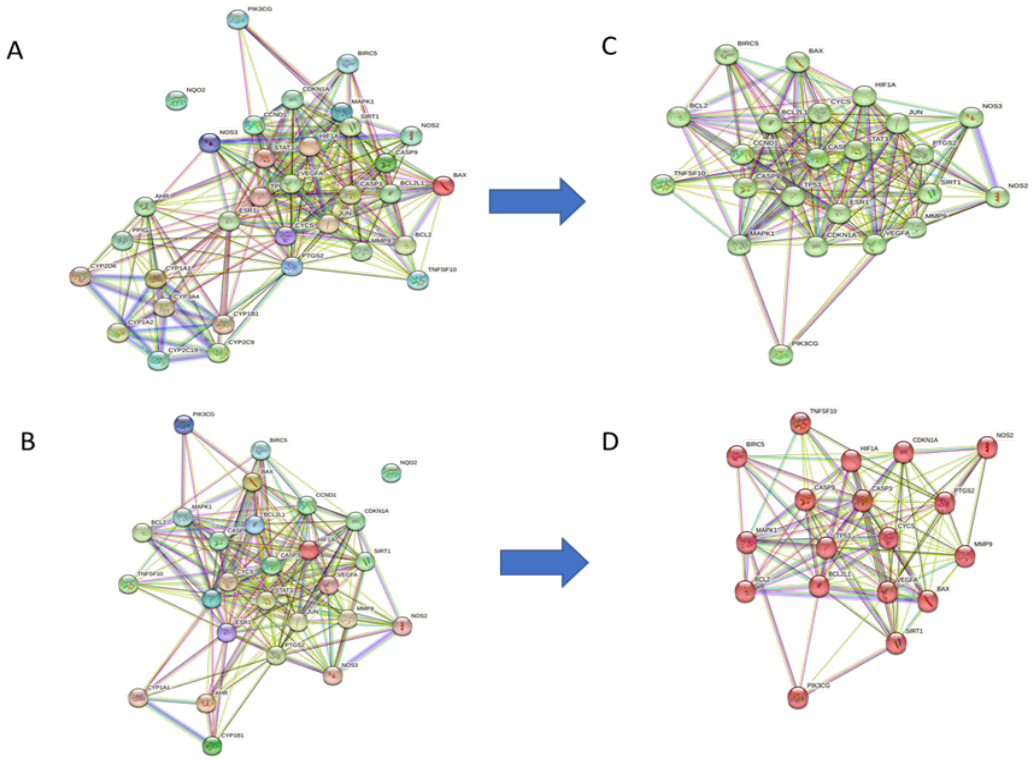

Figure 2. Main Target Genes of Compounds in EBE (*Eleutherine bulbosa*) for the Prevention and Treatment of Breast Cancer. Target genes of multi-component in EBE (*Eleutherine bulbosa*) (A); Breast cancer target genes of compounds in EBE (*Eleutherine bulbosa*) (B); Target genes of resveratrol compound (C); Breast cancer target genes of resveratrol compound (D).

proteins as input and generated a PPI network with various interactions. In this network, there are 36 target proteins connected by 253 interactions, with an average of each protein being connected to 15.3 other proteins and having an average local clustering coefficient of 0.764. The PPI enrichment results showed that the experimental p-value is less than $1.0e^{-16}$, indicating that these proteins have more interactions among themselves than expected for a set of randomly connected proteins of similar genome size. This suggests that these target proteins have significant biological relationships as a group, and the formed PPI network is not merely a random collection of connected proteins.

Table 1. Active Compounds in *Eleutherine bulbosa*

No	Active ingredient	Group	References
1	Eleutheroside A	Naphtalene	(Hara et al., 1997)
2	Eleutheroside B	Naphthalene	(Insanu et al., 2014)
3	Eleutheroside C	Naphthalene	(Insanu et al., 2014)
4	Eleutheroside D	Naphthalene	(Insanu et al., 2014)
5	Eleutherine	Naphthoquinone	(Hara et al., 1997)
6	Eleutherinol	Naphthalene	(Insanu et al., 2014)
7	Isoeleutherol	Naphthalene	(Hara et al., 1997)
8	Resveratrol	Flavonoid	(Mutiah et al., 2019)
9	Naringenin	Flavonoid	(Insanu et al., 2014)
10	Eleutherol	Naphthalene	(Hara et al., 1997)
11	Fumarin	Naphthoquinone	(Xu et al., 2006)
12	Elecanacin	Naphthoquinone	(Xu et al., 2006)
13	1,4 naphthoquinone	Naphthalene	(Annisa et al., 2020)
14	Eleuthraquinone A	Naphthoquinone	(Mahabusarakam et al., 2010)
15	Eleuthraquinone B	Naphthoquinone	(Mahabusarakam et al., 2010)
16	β-sitosterol	Naphthalene	(Insanu et al., 2014)

In the analysis of the PPI network, it was found that 23 compound target genes in EBE are involved in biological processes of breast cancer, including *SIRT1, TP53, CASP3, BCL2, ESR1, VEGFA, BAX, NQO2, JUN, MMP9, BIRC5, CDKN1A, NOS2, MAPK1, BCL2L1, CASP9, STAT3, CCND1, PIK3CG, NOS3, TNFSF10, PTGS2, CYCS* (Table 2). This indicates that the tested compounds in EBE have the potential as therapeutic agents or drugs in the treatment of breast cancer due to the protein-protein interactions between these compounds and the target genes.

Target Pathway and Enrichment Analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) are two databases used in biological studies to understand gene function and signaling pathways in organisms. KEGG provides information about signaling pathways and molecular interactions involved in biological processes, while GO classifies genes and their genetic products into categories based on function, cellular components, and biological processes (Berger and Iyengar, 2009; Xiang et al., 2022). EBE contains various active compounds and therefore has complex targets. During the analysis of potential target genes, we identified 156 pathways related to cancer, immune system, inflammation, and metabolism. Within

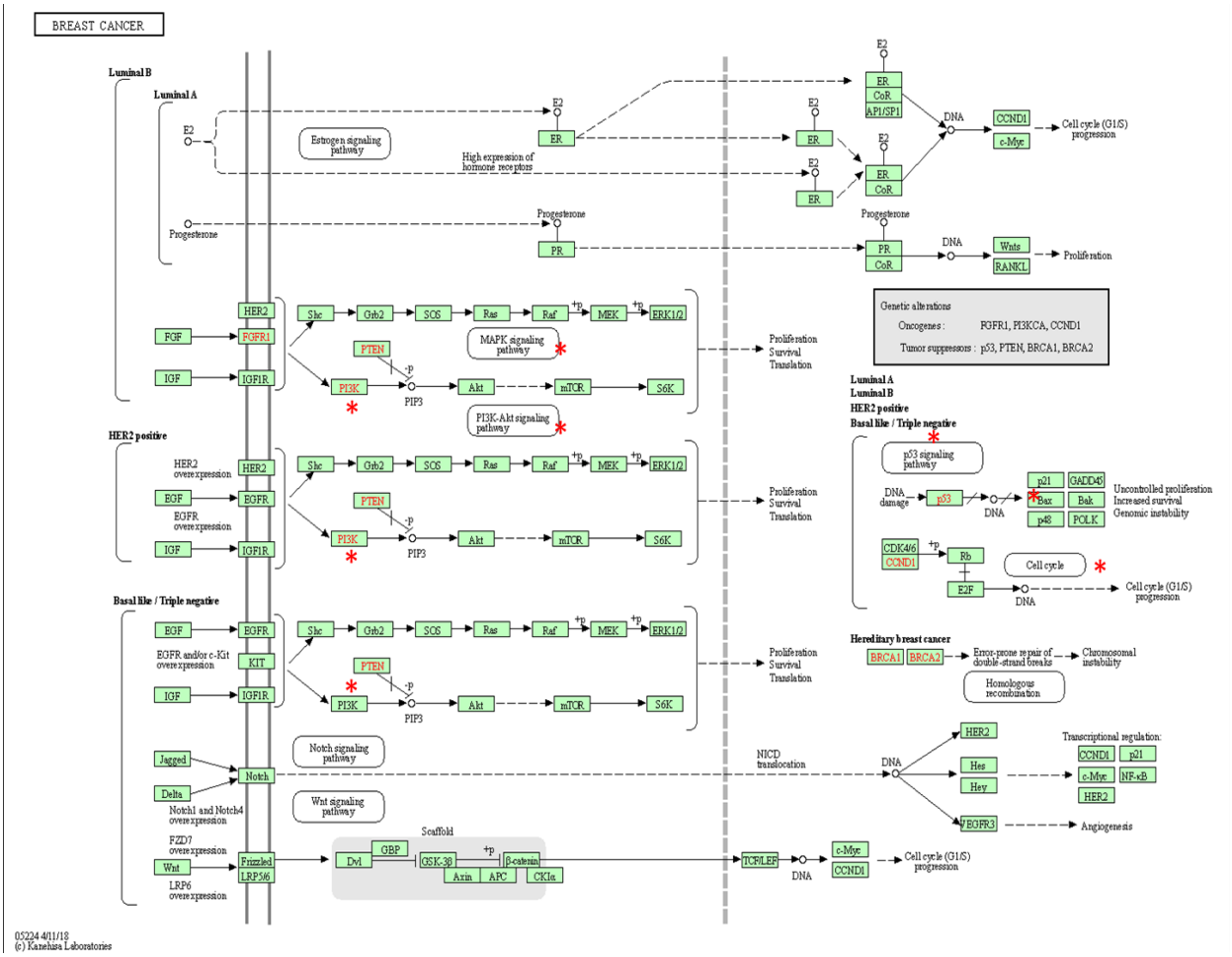

Figure 3. The Role of the Active Compound Resveratrol in *Eleutherine bulbosa* in the Breast Cancer Pathway

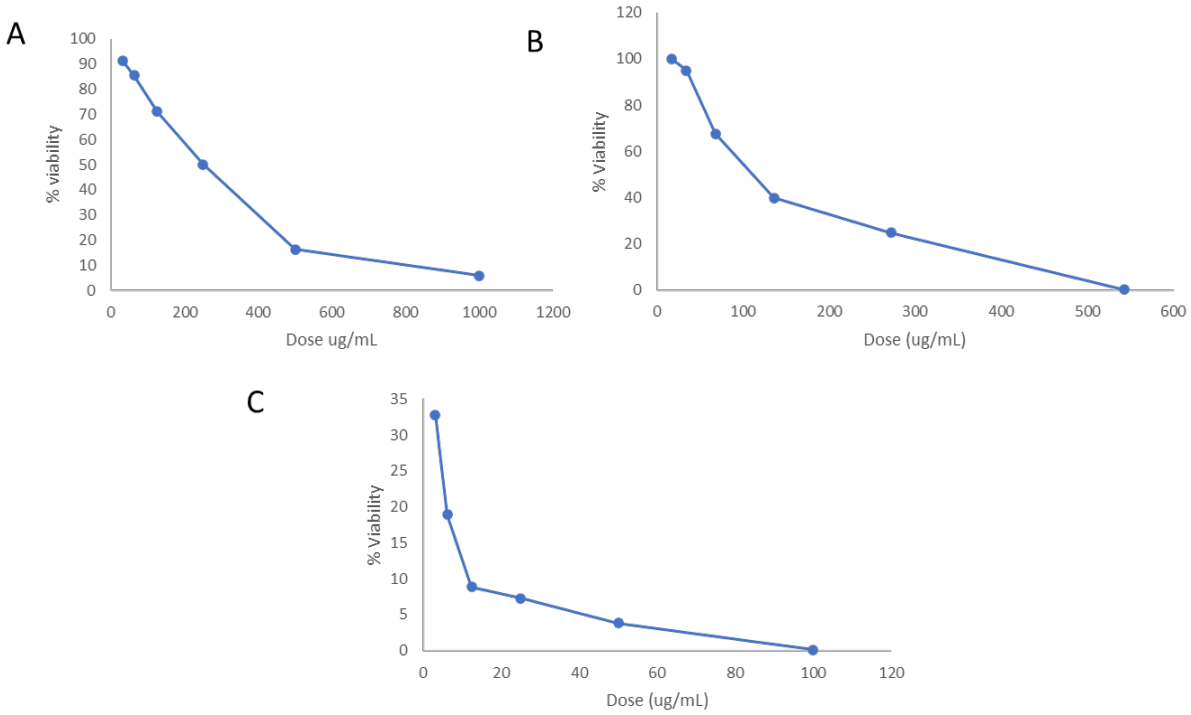

Figure 4. Viability of T47D Cells after Treatment with *Eleutherine bulbosa* (A), Doxorubicin (B), and 1,4 naphthoquinone (C). Over a period of 24 hours, T47D cells were treated with Eleutherine bulbosa, doxorubicin and 1,4 naphthoquinone at various concentrations, and cell survival was evaluated using the MTT assay. The cell viability profile is expressed as the mean ± SD of three independent experiments.

Table 2. The Target Gene of the Compound in *Eleutherine bulbosa* and Relevance Score of Target Gene (relevance score>7)

Compounds	Gene Symbol	Description	Gifts	Relevance score
Resveratrol	*SIRT1*	Sirtuin 1	52	1,655,425
Resveratrol	*TP53*	Tumor Protein P53	55	1,226,072
Resveratrol	*CASP3*	Caspase 3	52	1,068,921
Resveratrol	*BCL2*	BCL2 Apoptosis Regulator	53	1,033,782
Resveratrol	*ESR1*	Estrogen Receptor 1	56	1,000,319
Resveratrol	*PTGS2*	Prostaglandin-Endoperoxide Synthase 2	51	9,999,189
Resveratrol	*VEGFA*	Vascular Endothelial Growth Factor A	51	9,667,334
Resveratrol	*BAX*	BCL2 Associated X, Apoptosis Regulator	52	9,305,033
Resveratrol	*NQO2*	N-Ribosyldihydronicotinamide:Quinone Reductase 2	47	8,890,312
Resveratrol	*JUN*	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	50	8,709,743
Resveratrol	*MMP9*	Matrix Metallopeptidase 9	56	8,217,959
Resveratrol	*CYCS*	Cytochrome C, Somatic	51	8,145,127
Resveratrol	*BIRC5*	Baculoviral IAP Repeat Containing 5	48	7,831,698
Resveratrol	*CYP1B1*	Cytochrome P450 Family 1 Subfamily B Member 1	50	7,768,073
Resveratrol	*CYP1A1*	Cytochrome P450 Family 1 Subfamily A Member 1	50	7,761,432
Resveratrol	*CDKN1A*	Cyclin Dependent Kinase Inhibitor 1A	51	7,716,877
Resveratrol	*NOS2*	Nitric Oxide Synthase 2	51	759,807
Resveratrol	*HIF1A*	Hypoxia Inducible Factor 1 Subunit Alpha	51	7,587,591
Resveratrol	*MAPK1*	Mitogen-Activated Protein Kinase 1	54	753,255
Resveratrol	*BCL2L1*	BCL2 Like 1	50	744,572
Resveratrol	*CASP9*	Caspase 9	49	7,414,928
Resveratrol	*STAT3*	Signal Transducer And Activator Of Transcription 3	56	7,386,335
Resveratrol	*CCND1*	Cyclin D1	55	7,328,236
Resveratrol	*PIK3CG*	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma	50	7,168,639
Resveratrol	*NOS3*	Nitric Oxide Synthase 3	52	7,104,123
Resveratrol	*AHR*	Aryl Hydrocarbon Receptor	50	7,048,933
Resveratrol	*TNFSF10*	TNF Superfamily Member 10	47	7,038,575
Eleutheroside B	*CYP3A4*	Cytochrome P450 Family 3 Subfamily A Member 4	52	1,996,503
Eleutheroside B	*CYP1A2*	Cytochrome P450 Family 1 Subfamily A Member 2	48	1,801,899
Eleutheroside B	*PPIG*	Peptidylprolyl Isomerase G	43	1,522,853
Eleutheroside B	*CYP2C19*	Cytochrome P450 Family 2 Subfamily C Member 19	48	1,313,547
Eleutheroside B	*CYP2D6*	Cytochrome P450 Family 2 Subfamily D Member 6	49	9,098,722
Eleutheroside B	*CYP2C9*	Cytochrome P450 Family 2 Subfamily C Member 9	49	7,485,194
Eleutheroside C		Cytochrome P450 Family 3 Subfamily A Member 4	52	2,395,986
Eleutheroside C	*CYP2C9*	Cytochrome P450 Family 2 Subfamily C Member 9	49	2,049,925
Eleutheroside C	*CYP2C19*	Cytochrome P450 Family 2 Subfamily C Member 19	48	1,937,655
Eleutheroside C	*CYP1A2*	Cytochrome P450 Family 1 Subfamily A Member 2	48	1,904,041
Eleutheroside C	*PPIG*	Peptidylprolyl Isomerase G	43	1,602,481
Eleutheroside C	*CYP2D6*	Cytochrome P450 Family 2 Subfamily D Member 6	49	1,417,169
Eleutheroside D	*CYP3A4*	Cytochrome P450 Family 3 Subfamily A Member 4	52	3,497,591
Eleutheroside D	*CYP2D6*	Cytochrome P450 Family 2 Subfamily D Member 6	49	1,829,457
Eleutheroside D	*CYP2C9*	Cytochrome P450 Family 2 Subfamily C Member 9	49	7,978,017

these 156 pathways, there are seven pathways highly relevant to the development of breast cancer (Table 3), including the Breast cancer signaling pathway (hsa05224), pathway in cancer (hsa05200), apoptosis (hsa04210), P53 signaling pathway (hsa04115), MAPK signaling pathway (hsa04010), PI3K-Akt signaling pathway (has04151), and Cell cycle (hsa04110).

From the pathway analysis using KEGG, breast cancer (hsa05224) is found to involve components related to breast cancer pathway (hsa05224), which include the P53

Table 3. KEGG-based Signaling Pathway Analysis of Active Compounds in *Eleutherine bulbosa*

Pathway	Description	Count in network	strength	False discovery rate
hsa05224	Breast cancer	7 of 145	1.54	1.32E-08
hsa05200	pathway in cancer	18 of 517	1.40	6.74e-20
hsa04210	Apoptosis	8 of 132	1.51	6.7e-09
hsa04115	P53 signaling pathway	3 of 72	1.34	0.0016
hsa04010	MAPK signaling pathway	7 of 288	1.11	9.81e-06
has04151	PI3K-Akt signaling pathway	5 of 350	0.88	0.0021
hsa04110	Cell cycle	3 of 120	1.26	0.0022

signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and cell cycle (Table 3).

In the breast cancer pathway (hsa05224), the P53 signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and cell cycle interact with each other and play a role in controlling the growth, proliferation, and survival of breast cancer cells. Abnormal activation or mutations in these pathway components can lead to the development and progression of breast cancer (Paplomata and O'Regan, 2014; Braicu et al., 2019; Issaeva, 2019; Piezzo et al., 2020). From the results of our study, it is found that Resveratrol is involved in all four pathways, with PI3K, Bax, and P53 as the target genes (Figure 3).

The enrichment analysis of EBE compounds in breast cancer revealed several relevant findings in the context of the disease. These findings indicate the involvement of various biological processes relevant to breast cancer, such as cell proliferation, DNA damage response, apoptosis, cell cycle regulation, and related signaling pathways (Table 4).

In our study, after conducting the analysis of the anticancer potential of the compounds through Pharmacological Network, we further performed in vitro validation through cytotoxicity testing on breast cancer cell lines. Additionally, we also investigated the signaling pathways through apoptosis and cell cycle assays. In the cytotoxicity testing, it was found that all three samples, namely doxorubicin, EBE, and 1,4 naphthoquinone, exhibited moderate to very potential activity categories (Table 5, Figure 4).

Apoptosis Induction and Cell Cycle Arrest

As further evidence of the pharmacological network analysis results indicating the involvement of EBE

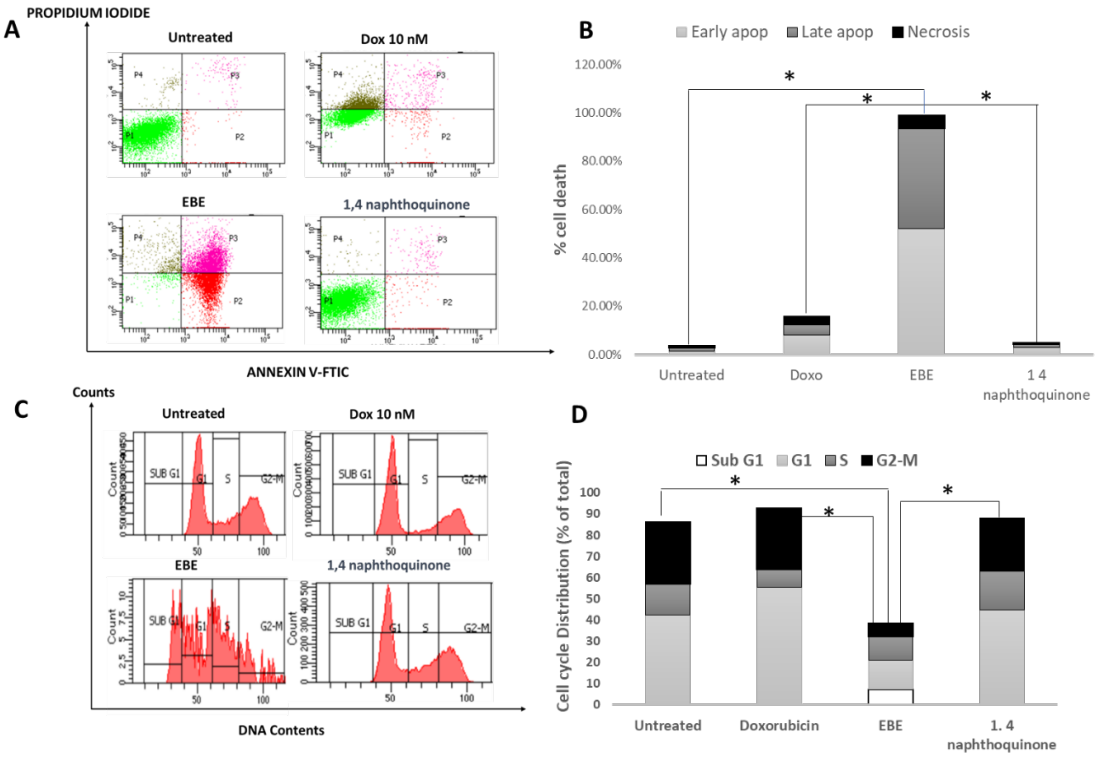

Figure 5. Apoptosis Induction and Cell Cycle Distribution by *Eleutherine bulbosa*, doxorubicin and 1,4-naphthoquinone Treatment in T47D Breast Cancer Cells. Cells were exposed to Eleutherine bulbosa, doxorubicin, and 1,4 naphthoquinone for 24 hours. The percentage of cell death (A, B) and the percentage of cell distribution in each phase (C, D) were measured using flow cytometry after staining with Annexin V-FITC/PI and PI, respectively. The lines indicate the mean ± SD values from three independent experiments. *p < 0.001 (post-hoc LSD test between each group) was considered statistically significant.

Table 4. Enrichment Analysis of Gene Ontology (GO) in *Eleutherine bulbusa* Compounds. In vitro cytotoxicity testing of EBE on breast cancer cell lines.

Go-Term	Description	Count In Network	Strength	False Discovery Rate
GO:0033598	Mammary Gland Epithelial Cell Proliferation	3 Of 12	2.26	4.18e-5
GO:0006978	DNA Damage Response, Signal Tarnduction By P53	2 Of 15	1.99	48
GO:1900119	Positive Regulation Of Execution Phase Of Apoptosis	2 Of 16	1.96	0.0052
GO: 0033599	Regulation Of Mammary Gland Epithelial Cell Proliferation	2 Of 16	1.96	0.0052
GO:0008631	Intrinsic Apoptotic Signaling Pathway	4 Of 17	1.93 1.96	0.00057
GO:2000045	Regulation Of G1/S Transition Of Mitotic Cell Cycle	5 Of 16	1.96	0.00067
GO:0007093	Mitotic Cell Cycle Checkpoint	2 Of 15	1.96	0.00059
GO:0097190	Apoptotic Signaling Pathway	2 Of 1	1.96	0.000087

Table 5. IC_{50} Value (50% Inhibitory Concentration) of *Eleutherine bulbosa* Extract against Cell Line T47D

	Sample	IC_{50} ±SD	category
A	Doxorubicin	115.1±0.54	Moderate
B	*Eleutherine bulbosa (EBE)*	202.37±2.29	Moderate
C	1,4-naphthoquinone	10.54±0.21	Very potential

compounds in apoptosis and cell cycle regulation in breast cancer cells, we conducted apoptosis and cell cycle assays in our study. Additionally, we compared the apoptosis and cell cycle activities of EBE compounds with the chemotherapy drug Doxorubicin and the compound 1,4-naphthoquinone. Our research findings in the apoptosis assay demonstrated that EBE compounds exhibited strong potential in inducing apoptosis in breast cancer cells, with a total of 93.6% of cells undergoing apoptosis and 5.5% undergoing necrosis (Figure 5). Furthermore, the statistical analysis of the number of cells undergoing both early and late apoptosis showed significant differences between the EBE-treated group and the control untreated group, as well as the Doxorubicin and 1,4-naphthoquinone groups ($p<0.001$).

In addition to inducing cell death, the increased anticancer effects can also occur through cell cycle modulation. Flow cytometry was used to determine the cell cycle distribution after treatment. T47D cells were treated with 10 nM DOX, IC_{50} EBE, IC_{50} 1,4-naphthoquinone for 24 hours. The results in Figure 2C, D showed a significant effect on the cell cycle distribution treated with EBE ($P < 0.0001$). EBE treatment resulted in an increase in the sub G1 and S phases, while there was a significant decrease in the accumulation in the G2-M and G1 phases. In contrast, treatment with DOX and 1,4 naphthoquinone did not affect the cell cycle. In Figures C and D, it is known that the cell cycle of DOX and 1,4 naphthoquinone is not different from the untreated group ($P > 0.0001$).

Discussion

In the study of developing breast cancer drugs using a network pharmacology approach from E. bulbosa extract as an anticancer agent, it is important to provide in vitro evidence through cytotoxicity assays, apoptosis assays, and cell cycle assays. Prior to conducting in vitro assays, a literature review revealed the presence of 16 active compounds in EBE that are suspected to have anticancer activity. These compounds belong to the Naphtalene, Naphthoquinone, and Flavonoid groups (Table 1). Furthermore, a network pharmacology analysis was conducted to evaluate the relationship between compounds in EBE extract and target genes involved in the pathophysiology of breast cancer. The results showed that there are 16 compounds from EBE specifically targeting 42 genes associated with breast cancer. Moreover, we also identified 23 important target genes that play a significant role in the pathophysiology of breast cancer. Additionally, it was found that resveratrol has a relationship with 27 target genes, and 18 compounds in EBE are associated with target genes involved in breast cancer. These findings provide valuable information regarding the potential mechanisms of action of these compounds in inhibiting the growth and spread of breast cancer cells.

Our research findings on enrichment analysis using KEGG indicate that various active compounds in EBE have complex target genes. In the potential target gene analysis, we identified 156 pathways related to cancer, immune system, inflammation, and metabolism. Out of these 156 pathways, there are seven pathways highly relevant to breast cancer development (see Table 3). These pathways include Breast cancer signaling pathway (hsa05224), pathway in cancer (hsa05200), apoptosis (hsa04210), P53 signaling pathway (hsa04115), MAPK signaling pathway (hsa04010), PI3K-Akt signaling pathway (hsa04151), and Cell cycle (hsa04110).

In the analysis of signaling pathways of compounds in the EBE extract using the KEGG database for breast cancer pathway (hsa05224), we identified components closely associated with this pathway. Our research findings show that the EBE extract contains compounds involved in several important signaling pathways in breast cancer, including P53 signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and cell cycle (Table 3).

The mechanism of the P53 signaling pathway in KEGG breast cancer (Figure 3) starts with DNA damage that activates the P53 gene. Under normal conditions, the P53 protein plays a role in maintaining genomic stability

and preventing uncontrolled cell proliferation. However, in the case of DNA damage, the P53 protein undergoes activation and induces various cellular responses (Issaeva, 2019). P53 activation results in increased expression of the Bax gene, which is a member of the pro-apoptotic protein family. Bax interacts with mitochondria and causes the release of cytochrome C, which triggers the apoptotic cascade (Gasco et al., 2002). The release of cytochrome C activates caspases, enzymes that mediate the cleavage of cellular proteins and stimulate apoptosis (Yang et al., 2015). In addition, P53 also inhibits the expression of the *Bcl-2* gene, which is a member of the anti-apoptotic protein family. Bcl-2 inhibits the release of cytochrome C and blocks the apoptotic pathway. By inhibiting Bcl-2 expression, P53 strengthens the apoptotic pathway induced by Bax (Issaeva, 2019).

At a more advanced level, P53 activation can influence the expression of various genes involved in cell proliferation and tumor formation. P53 regulates the expression of genes that inhibit the cell cycle and promote cell differentiation. Under normal conditions, P53 acts as a tumor suppressor and prevents the growth of cancer cells (Gasco et al., 2002; Turner et al., 2013).

EBE compounds are predicted to inhibit the P53 signaling pathway, which can potentially disrupt P53 activation and inhibit Bax gene expression. By inhibiting this pathway, EBE compounds can reduce apoptotic response and impact cancer cell proliferation. Our findings in in vitro tests on T47D cells show that EBE can induce apoptosis by 93.6%. Therefore, EBE may have the potential as an agent that inhibits tumor growth in breast cancer by involving the P53 signaling pathway mechanism.

The Cell cycle mechanism in KEGG breast cancer (Figure 3) begins with the activation of cyclin-dependent kinase 4/6 (CDK 4/6) (Piezzo et al., 2020). CDK 4/6 interacts with cyclin D, forming an active complex that phosphorylates retinoblastoma protein (Rb). Phosphorylation of Rb results in the release of the transcription factor E2F from the Rb-E2F complex (Caldon et al., 2006). Once released, the E2F transcription factor triggers the expression of genes involved in DNA synthesis and prepares the cell to enter the S phase (synthesis). The S phase involves DNA replication that occurs as the cell prepares for cell division (Johnson et al., 2016).

The activation of CDK 4/6 and E2F, as well as DNA replication, contribute to the transition of cells from the G1 phase (gap 1) to the S phase, known as Cell cycle progression. Cell proliferation in the G1/S phase is crucial for normal cell growth, but if disrupted, it can contribute to cancer development (Wang et al., 2011).

EBE compounds are known to inhibit the Cell cycle pathway in breast cancer can affect the activation of CDK 4/6 and the release of E2F from the Rb-E2F complex. By inhibiting this pathway, EBE compounds can disrupt the transition of cells from the G1 phase to the S phase and inhibit cancer cell proliferation. In other words, EBE compounds have the potential to inhibit the activity of CDK 4/6, maintain Rb in an unphosphorylated state, and prevent the release of E2F. This inhibits cell cycle progression in the G1/S phase and can inhibit tumor growth in breast cancer.

The MAPK (Mitogen-Activated Protein Kinase) signaling pathway mechanism (Figure 3) involves a series of reactions that start with the activation of growth factor receptors on the cell surface. Activation of these receptors triggers the activation of the protein PI3K (Phosphatidylinositol 3-Kinase). PI3K phosphorylates phosphatidylinositol biphosphate (PIP2) into phosphatidylinositol triphosphate (PIP3) (Braicu et al., 2019).

Furthermore, PIP3 recruits protein kinase B (Akt) to the cell membrane. Akt is then activated through phosphorylation by enzymes such as phosphoinositide-dependent kinase 1 (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2). Activated Akt influences various signaling pathways, including the MAPK signaling pathway and the PI3K-Akt signaling pathway (Zhu et al., 2011; Braicu et al., 2019). The activity of compounds in EBE known to work on PI3K is to inhibit the PI3K enzyme itself. By inhibiting PI3K, compounds in EBE can prevent the formation of PIP3 and the downstream activation of the PI3K-Akt pathway.

In the context of the MAPK signaling pathway, the inhibition of PI3K by compounds in EBE can also have an impact. The PI3K-Akt signaling pathway can interact with the MAPK signaling pathway through several connecting points. By inhibiting PI3K, compounds in EBE can inhibit the activation of the MAPK signaling pathway, which is usually triggered by the PI3K-Akt signaling pathway. Thus, compounds in EBE that work on PI3K can have inhibitory effects on both the MAPK signaling pathway and the PI3K-Akt signaling pathway, thereby disrupting signaling pathways that contribute to cancer cell proliferation and tumor growth. These findings provide a deeper understanding of the mechanisms of action of EBE extract in inhibiting breast cancer. This research reveals that EBE extract contains compounds that can influence signaling pathways and biological processes involved in breast cancer development. This understanding is crucial in the potential development of EBE extract as an anticancer agent that can specifically target important pathways in breast cancer.

In our study, after conducting an analysis of the potential anticancer compounds through a network pharmacology approach, we proceeded to strengthen the results by conducting cytotoxicity tests on breast cancer cells in vitro. We also performed apoptosis and cell cycle assays to verify the signaling pathways involved. In the cytotoxicity test, we obtained results indicating that the three tested samples, namely doxorubicin, EBE, and 1,4-naphthoquinone, exhibited activity ranging from moderate to highly potent. The IC_{50} values for anticancer activity in the development of herbal drugs according to the National Cancer Institute (NCI) can be interpreted as follows: IC_{50} > 100 µg/mL (no or very little anticancer activity); IC_{50} 50-100 µg/mL (low anticancer activity); IC_{50} 10-50 µg/mL (moderate anticancer activity); IC_{50} 1-10 µg/mL (high anticancer activity); IC_{50} < 1 µg/mL (very high anticancer activity). The lower the IC_{50} value, the stronger the ability of the compound to inhibit cancer

cell growth (Kroll, 2001).

These results indicate that the compounds in EBE have the ability to significantly inhibit the growth of breast cancer cells. The activity categories obtained from the cytotoxicity test indicate the potential of these compounds as effective anticancer agents (Kroll, 2001). This suggests that EBE has properties that can disrupt the proliferation of breast cancer cells and can be a potential candidate for the development of anticancer drugs. As supporting evidence for the results of the network pharmacology analysis, which showed the role of compounds in EBE in apoptosis and cell cycle in breast cancer cells, our study conducted apoptosis and cell cycle assays to directly prove their influence. Additionally, we compared the apoptosis and cell cycle activities of the EBE compounds with the chemotherapy drug Doxorubicin and the compound 1,4-naphthoquinone.

The results of our study on apoptosis assay showed that the compounds in EBE have strong potential in inducing apoptosis in breast cancer cells. The total percentage of cells undergoing apoptosis reached 93.6%, while the percentage of cells undergoing necrosis was 5.5% (Figure 5). Additionally, the statistical analysis results showed that EBE treatment significantly differed from the untreated control group, Doxorubicin, and 1,4-naphthoquinone in terms of the number of cells undergoing apoptosis, both in the early and late stages ($p<0.001$). Previous studies have also provided a strong foundation to support our research findings. Previous studies have shown that the compounds in EBE have significant anticancer activity, including the inhibition of breast cancer cell proliferation (Mutiah et al., 2019). By combining these findings with our research results, it can be strengthened that the compounds in EBE play an important role in inhibiting growth and inducing apoptosis in cancer cells (Mutiah et al., 2020; Mutiah et al., 2020).

In our study, in addition to inducing cell death, we also observed the effect of treatment on cell cycle distribution. Flow cytometry was used to analyze cell cycle distribution after treatment with specific compounds. The results obtained from the flow cytometry analysis in Figure 5 (C and D) showed a significant effect on cell cycle distribution after treatment with EBE ($P<0.0001$). EBE administration caused a significant increase in the sub G1 phase and S phase, while there was a significant decrease in the accumulation of cells in the G2-M phase and G1 phase. This indicates that EBE can disrupt the cell cycle process in T47D cells.

However, unlike EBE, the administration of DOX and 1,4-naphthoquinone did not show a significant effect on the cell cycle. The results in Figure 5 (C and D) showed that the cell cycle distribution in the DOX and 1,4-naphthoquinone treatment groups did not significantly differ from the untreated control group ($P>0.0001$).

These results indicate that EBE has the potential to influence the cell cycle in T47D cells, while DOX and 1,4-naphthoquinone do not have a significant effect on the cell cycle. These findings provide a better understanding of the mechanisms of action of these compounds in inhibiting the growth of breast cancer cells and provide a strong basis for further research and development of cancer drugs using EBE as a potential agent to regulate the cell cycle in breast cancer.

This study has several limitations as it was still tested in silico using a bioinformatics approach and in vitro using cell lines. Therefore, further scientific evidence is needed through in vivo testing on animal models and clinical trials in humans to prove the potential of Dayak onion as a candidate breast cancer drug.

In summary, our data indicate that EBE contains 16 potential anticancer compounds, including compounds from the Naphtalene, Naphthoquinone, and Flavonoid groups. Network pharmacology analysis reveals that EBE influences 42 genes involved in breast cancer, including 23 important target genes implicated in the pathophysiology of breast cancer. In the signaling pathway analysis using the KEGG database, EBE is closely associated with crucial signaling pathways in breast cancer, such as the P53 signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway,apoptosis, and cell cycle. In the in vitro cytotoxicity assay, doxorubicin, EBE, and 1,4-naphthoquinone could exhibit varying activities from moderate to highly potent in T47D cells. Moreover, EBE demonstrates strong potential in inducing apoptosis in breast cancer cells, with a percentage of cells undergoing apoptosis reaching 93.6%. Statistical analysis demonstrates that EBE treatment significantly differs from the control group in terms of the number of cells undergoing apoptosis. Additionally, EBE has been found to disrupt the cell cycle with a significant increase in the sub G1 and S phases, as well as a significant decrease in the G2-M and G1 phases. This indicates that EBE can inhibit the cell cycle process in T47D cells. Thus, the findings of this research suggest that EBE has the potential as an anticancer agent involving various mechanisms, including apoptosis induction and cell cycle inhibition in breast cancer cells.

Author Contribution Statement

All authors contributed equally in this study.

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General

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Ethical Declaration

This research was approved by the Research Ethics Committee on Health (KEPK) of the Faculty of Medicine at UIN Maulana Malik Ibrahim Malang with the number: No.139/EC/KEPK-FKIK/2022.

Data Availability

All data and materials are available if requested.

Conflict of Interest

The authors declare that they have no conflict of interest

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