International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 15, Issue 6, 2023

Original Article

NETWORK PHARMACOLOGY, APOPTOSIS, AND CELL CYCLE INHIBITION OF SESQUITERPENE COMPOUNDS FROM QUSTHUL HINDI ROOT EXTRACT (*SAUSSUREA LAPPA*) IN BREAST CANCER: AN *IN SILICO* AND *IN VITRO* APPROACH

ROIHATUL MUTIAH[1], SUKARDIMAN[2], ALVI MILLIANA[3], ERMIN RAHMAWATI[3], AVIN AINUR FITRIANINGSIH[3], YUYUN YUENIWATI[3]*

[1]Department of Pharmacy, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim Malang, East Java, Indonesia. [2]Department Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Airlangga, Indonesia. [3]Department of Medicine, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim Malang, East Java, Indonesia
*Corresponding author: Yuyun Yueniwati; *Email: yuyun@uin-malang.ac.id

Received: 27 Jul 2023, Revised and Accepted: 28 Aug 2023

ABSTRACT

Objective: The objective of this study was to evaluate the potential and mechanisms of compounds in Qusthul Hindi extract in inhibiting proliferation, cell cycle, and inducing cell death in breast cancer through a network pharmacology approach, *in silico* validation, and *in vitro* experiments.

Methods: This research employed a literature review approach to identify anti-cancer compounds and utilized a network pharmacology approach to predict the mechanisms of action of the compounds. Insilico docking was performed on the HER2 receptor (PDB: 3PP0) using Molegro Virtual Docker 6.0. Furthermore, the MTT method was used to evaluate the cytotoxic effects of Qusthul Hindi extract on T47D cells, and Flow cytometry was employed to determine the effects of the extract on apoptosis and cell cycle.

Results: The network pharmacology analysis revealed that Qusthul Hindi interacted with 66 genes related to breast cancer. Pathway analysis showed a close association between Qusthul Hindi and important signaling pathways such as P53, MAPK, PI3K-Akt, and the cell cycle. Molecular docking results showed better affinity of Saussureamine B and C towards the HER2 receptor compared to trastuzumab. *In vitro* cytotoxicity assays demonstrated the potential activity of Qusthul Hindi extract against T47D cells (IC50: 32.81 µg/ml). Qusthul Hindi also effectively induced apoptosis in breast cancer cells with a high percentage (85.3%), and inhibited the cell cycle by reducing the G2-M and S phases. Statistical analysis revealed significant differences between the Qusthul Hindi treatment group and the control group in terms of apoptotic cell count ($p<0.001$).

Conclusion: These findings suggest that Qusthul Hindi has potential for development as an anti-cancer agent through the inhibition of proliferation, induction of apoptosis, and cell cycle inhibition in breast cancer.

Keywords: Qusthul Hindi, Network pharmacology, HER2, T47D, Apoptosis, Cell cycle

DOI: https://dx.doi.org/10.22159/ijap.2023v15i6.48798. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Breast cancer is a leading cause of illness and death from cancer among women worldwide. Over the past 25 y, there has been a significant increase in breast cancer-related mortality globally, primarily due to the rising incidence and prevalence of breast cancer [1]. One of the reasons for the high mortality rate is the failure of current therapies such as chemotherapy, surgery, and radiation therapy. Furthermore, chemotherapy and radiation therapy cause serious side effects and demonstrate limitations in improving the quality of life for patients [2].

In recent decades, the development of more effective and safe cancer therapies has become a major focus in health research. One promising approach is the use of natural compounds derived from plants as potential therapeutic agents. Medicinal plants or herbal medicines have long been used in traditional medicine to treat various diseases, including cancer.

Medicinal plants or herbal medicines contain various components that exert therapeutic effects through multiple targets and pathways. Consequently, detecting the mechanism of action of herbal medicines solely through conventional experiments has become challenging. Therefore, a new systematic and comprehensive approach is needed to understand the mechanisms of action of herbal medicines. One effective modern approach in unraveling the molecular and pharmacological mechanisms of herbal medicines is network pharmacology [3]. This approach differs from the previous reductionist approach that assumed a single drug has a single target. In network pharmacology, it is recognized that many active compounds can interact with diverse genes or proteins, allowing for a more comprehensive understanding of the holistic mechanisms involved [4] [5]. Furthermore, network pharmacology can depict the interactive relationships among multiple active compounds, multiple targets, and multiple diseases. By visualizing these relationships in network models, network pharmacology can systematically explain the actions of drugs on the human biological system [6].

Saussurea lappa/*S. lappa* (also known as *Saussurea costus*) is traditionally known as Qust (Costus) and qusthul hindi. It is a perennial effective root found extensively in the Himalayan region. *Saussurea lappa* is one of the plants mentioned in the Sunnah of Prophet Muhammad (SAW) as a treatment for headaches and upper respiratory tract infections. The root is known to have tonic, appetite-stimulating, carminative, stimulant, and aphrodisiac properties. It is used as an antispasmodic for asthma, cough, and cholera, as well as an alternative treatment for chronic skin diseases and rheumatism. Cupping and sea costus are considered the best remedies [7].

Currently, Qusthul Hindi has been widely used to treat various diseases for its anti-ulcer, anticonvulsant, anticancer, hepatoprotective, anti-inflammatory, anti-arthritic, and antiviral activities. Phytochemical analysis of Qusthul Hindi reveals a diverse range of metabolites, such as sesquiterpenes, flavonoids, phytosterols, lignans, and terpenes. Among the sesquiterpenes, dehydrocostus lactone, dihydrocostunolide, costunolide, and lappadilactone have been isolated as the main chemical constituents. Sesquiterpenes and flavonoids are the main active constituents responsible for various pharmacological activities [8, 9]. Additionally, other glycoside compounds have been identified, including chlorogenic acid, β-costic acid, daucosterol, β-sitosterol, and saussureamines A-E [10].

This research has not been reported by previous researchers. In this study, the research focuses on the sesquiterpene compounds found in the root extract of Qusthul Hindi (*Saussurea lappa*) and their potential as therapeutic agents for breast cancer. Apoptosis, known as programmed cell death, and cell cycle inhibition are two important mechanisms in controlling the growth and spread of cancer cells. Therefore, this study aims to investigate the potential of these sesquiterpene compounds in inducing apoptosis and inhibiting the cell cycle in breast cancer through *in silico* and *in vitro* approaches.

MATERIALS AND METHODS

Plant material

Qusthul Hindi roots are obtained from Saudi Arabia in the form of costus root powder. The identification of the simplisia is conducted at the Pharmaceutical Biology Laboratory, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim Malang, using both macroscopic and microscopic approaches to ensure the accuracy of the simplisia's name. This specimen is labeled with the number 075/212/102 20-A/2022.

Preparation of qusthul hindi extract

The powdered plant material was extracted with 95% ethanol 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 [11].

Collection and screening of bioactive components of qusthul hindi

The bioactive compound components of Qusthul Hindi were obtained based on a literature review of other scientific studies from databases such as Google Scholar, PubMed, and ScienceDirect, using the keywords "Qusthul Hindi compound".

Collection and screening of target proteins

The target proteins and genes for the compounds were obtained from GeneCards. The results obtained from GeneCards were limited to targets with a relevance score of 7.00, which is considered to meet the database standards [12].

Creation of target network

The main steps in creating a target network using Cytoscape involve several procedures. Firstly, molecular target data is imported into the Cytoscape application, such as relevant protein targets. Each target is represented by a node, and the interactions between these targets are connected by edges. Node and edge attributes and properties are set according to relevant information, such as target names, interaction types, or interaction strengths. Next, the network display is organized using available layout algorithms for better understanding. The resulting target network is then annotated and interpreted based on patterns, connectivity, or other important features. Thus, understanding of the molecular target interactions involved in a biological process or disease is enhanced, and valuable insights for research or therapeutic development are gained [13].

Creation of protein-protein interaction (PPI) network and enrichment analysis

The procedure for creating a Protein-Protein Interaction (PPI) network and conducting enrichment analysis using STRING involves several important steps. Firstly, relevant protein data is imported into the STRING platform. This data includes information on physical interactions and predicted protein-protein interactions. The PPI network is then constructed based on this data, with nodes representing proteins/genes and edges connecting interacting proteins. Subsequently, using available layout algorithms, the network display is organized for better understanding. Next, enrichment analysis is performed using the integrated enrichment tools in STRING. In this analysis, the network data is compared to various reference sources to identify groups of proteins associated with specific biological functions, metabolic pathways, or cellular processes. The enrichment results provide further insights into the roles and interrelationships of proteins in relevant biological contexts. Finally, the results of the enrichment analysis are interpreted by examining the generated protein groups, observing the abundance of discovered features, and identifying statistically significant features. This process helps gain a deeper understanding of protein function and relationships within the studied biological system [14].

Molecular docking of sesquiterpene compounds in qustul hindi with HER2 receptor

In this study, the HER2 receptor (Human Epidermal Growth Factor Receptor 2) downloaded from the PDB (https://www.rcsb.org/structure/3pp0) with protein code 3PP0 was used, which contains the ligand 2-{2-[4-({5-chloro-6-[3-(trifluoromethyl)phenoxy]pyridin-3-yl}amino)-5H-pyrrolo[3,2-d]pyrimidin-5-yl]ethoxy}ethanol. The receptor preparation was performed by removing water molecules and reference ligands and adding hydrogen atoms using the Molegro Virtual Docker 6.0 application. The molecular docking process was carried out by taking the three-dimensional structures of the compounds, namely Saussureamine A-D, Costunolide, and Dehydrocostus Lactone, as well as the three-dimensional structure of the HER2 receptor. Virtual docking using Molegro was then used to model the interactions between these compounds and the HER2 receptor. The results of molecular docking will provide information about the binding affinity of these compounds with the HER2 receptor. This can be used to estimate the potential of these compounds in inhibiting cell proliferation or having pharmacological effects related to the HER2 receptor [15].

Cell culture

The T47D breast cancer cell line was 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% CO2 in a humidified atmosphere of 100% humidity. For experiments, T47D cells were used at 80-90% confluency [16-17].

Cell viability assay

The proliferation of T47D cells was studied using the MTT assay. T47D cells (2 × 10^3 cells/well) were seeded in a 96-well microplate and allowed to attach overnight. The cells were incubated for 24 h with treatments of Qustul Hindi extract (Sigma-Aldrich, USA) (50–500 µM) and DOX (Sigma-Aldrich, USA) (0.01–10 µM). Untreated cells were used as a negative control. After treatment, 100 µl of MTT (Biovision) solution (0.5 mg/ml in medium) was added to each well and incubated for 4 h at 37 °C with 5% CO2. Subsequently, the MTT formazan crystals were dissolved using a stop solution of sodium dodecyl sulfate (SDS) containing 0.01 N HCl and incubated overnight in the dark. Once the purple formazan was solubilized, absorbance was measured using an 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 untreated cells [18].

Apoptosis assay

Apoptosis assay was performed using Annexin V-FITC/PI staining and flow cytometry analysis on the treatment of Qustul Hindi extract. Briefly, cells that had been collected were stained using the Annexin-V-FLUOS staining kit (Roche), which consisted of 100 µl binding buffer, 2 µl Annexin V, and 2 µl PI for 10 min at room temperature in the dark. The stained 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 software (BD Bioscience) [19].

Cell cycle analysis

Cell cycle analysis was performed using flow cytometry and 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 Qustul Hindi extract (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 2000 rpm for 3 min. The collected

cells were resuspended and fixed with ethanol for 30 min at 4 °C. Next, the cells were washed with cold PBS and centrifuged at 2000 rpm for 3 min. The cell pellet was resuspended in a PI solution (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 the electronic debris was discarded, red fluorescence was quantified using the FL1 setting (log mode) [20].

Table 1: The target gene of the compound in qusthul hindi and relevance score of target gene (relevance score>7)

Bioactive	Prot-target	Description	Score
Saussure amine C	CCR5	C-C Motif Chemokine Receptor 5	40.77598
Saussure amine C	CXCR4	C-X-C Motif Chemokine Receptor 4	29.31928
Saussure amine B	CXCR4	C-X-C Motif Chemokine Receptor 4	17.73028
Saussure amine B	CCR5	C-C Motif Chemokine Receptor 5	11.50371
Stigma sterol	ABCG5	ATP Binding Cassette Subfamily G Member 5	9.638834
Stigma sterol	DYNC2LI1	Dynein Cytoplasmic 2 Light Intermediate Chain 1	8.976475
Stigma sterol	ABCG8	ATP Binding Cassette Subfamily G Member 8	8.265738
Saussure amine D	CXCR4	C-X-C Motif Chemokine Receptor 4	7.320037
Saussure amine D	CCR5	C-C Motif Chemokine Receptor 5	7.040105
Dehydrocostus Lactone	ABCB1	ATP Binding Cassette Subfamily B Member 1	7.508283
Dehydrocostus Lactone	ABCG2	ATP Binding Cassette Subfamily G Member 2 (Junior Blood Group)	7.362502
Costunolid	JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	7.291094
Xantosine	ITPA	Inosine Triphosphatase	8.755403
Cinnamic acid	TP53	Tumor Protein P53	27.13774
Cinnamic acid	PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha	20.97114
Cinnamic acid	CASP3	Caspase 3	20.06087
Cinnamic acid	MYC	MYC Proto-Oncogene, BHLH Transcription Factor	19.96577
Cinnamic acid	MAPK1	Mitogen-Activated Protein Kinase 1	19.36962
Cinnamic acid	AKT1	AKT Serine/Threonine Kinase 1	17.6771
Cinnamic acid	BCL2	BCL2 Apoptosis Regulator	17.27011
Cinnamic acid	JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	15.87453
Cinnamic acid	CASP8	Caspase 8	15.56787
Cinnamic acid	MAPK14	Mitogen-Activated Protein Kinase 14	15.49751
Cinnamic acid	CDKN3	Cyclin Dependent Kinase Inhibitor 3	14.87435
Cinnamic acid	MAPK3	Mitogen-Activated Protein Kinase 3	14.432
Cinnamic acid	JAK2	Janus Kinase 2	14.04231
Cinnamic acid	CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	13.70782
Cinnamic acid	CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	13.69777
Cinnamic acid	FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	13.53609
Cinnamic acid	BAX	BCL2 Associated X, Apoptosis Regulator	13.52594
Cinnamic acid	MAPK8	Mitogen-Activated Protein Kinase 8	13.44455
Cinnamic acid	MMP2	Matrix Metallopeptidase 2	13.37967
Cinnamic acid	MMP9	Matrix Metallopeptidase 9	13.07066
Cinnamic acid	STAT3	Signal Transducer And Activator Of Transcription 3	12.83352
Cinnamic acid	MAP2K1	Mitogen-Activated Protein Kinase Kinase 1	12.7167
Cinnamic acid	MAPK10	Mitogen-Activated Protein Kinase 10	12.00774
Cinnamic acid	BCL2L1	BCL2 Like 1	11.74514
Cinnamic acid	PIK3CG	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma	11.12555
Cinnamic acid	MMP13	Matrix Metallopeptidase 13	9.397499
Cinnamic acid	CFLAR	CASP8 And FADD Like Apoptosis Regulator	8.6861
Cinnamic acid	LCK	LCK Proto-Oncogene, Src Family Tyrosine Kinase	8.297522
Cinnamic acid	PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1	8.155284
Cinnamic acid	CASP2	Caspase 2	7.549953
Alpha terpineol	TNF	Tumor Necrosis Factor	77.1216
Alpha terpineol	IL1B	Interleukin 1 Beta	30.11614
Alpha terpineol	IL10	Interleukin 10	29.86718
Alpha terpineol	CXCL8	C-X-C Motif Chemokine Ligand 8	29.74007
Alpha terpineol	NFKB1	Nuclear Factor Kappa B Subunit 1	16.93485
Alpha terpineol	BCL2	BCL2 Apoptosis Regulator	15.33992
Alpha terpineol	XIAP	X-Linked Inhibitor Of Apoptosis	11.95834
Alpha terpineol	LINC01672	Long Intergenic Non-Protein Coding RNA 1672	9.165056
Alpha terpineol	NFKB2	Nuclear Factor Kappa B Subunit 2	7.619581
Betulin	BCL2	BCL2 Apoptosis Regulator	8.199333
Betulin	BCL2L1	BCL2 Like 1	8.115455
Betulin	CYCS	Cytochrome C, Somatic	7.482983
Diethyl di maleate	TP53	Tumor Protein P53	9.838255
Diethyl di maleate	IL6	Interleukin 6	9.011388
Diethyl di maleate	NFE2L2	NFE2 Like BZIP Transcription Factor 2	8.849256
Diethyl di maleate	CCND1	Cyclin D1	8.081886
Diethyl di maleate	TNF	Tumor Necrosis Factor	7.630271
Diethyl di maleate	BCL2	BCL2 Apoptosis Regulator	7.233994
Ethyl linoleate	JUNB	JunB Proto-Oncogene, AP-1 Transcription Factor Subunit	8.052756
Ethyl linoleate	CTNNB1	Catenin Beta 1	7.864361
Ethyl linoleate	ERBB3	Erb-B2 Receptor Tyrosine Kinase 3	7.566376
Ethyl linoleate	BRCA1	BRCA1 DNA Repair Associated	7.435139
Ethyl linoleate	TNF	Tumor Necrosis Factor	7.311637

RESULTS AND DISCUSSION

Components and protein targets

Based on the literature review and GeneCards analysis, 12 compounds were found in Qustul Hindi (*Saussurea lappa*) with a gene target relevance score greater than 7.00. These compounds belong to several groups, including sesquiterpenoids, terpenoids, flavonoids, and phenolics (table 1).

Among these compounds, three compounds showed the highest gene target relevance scores: Saussureamine C, Cinnamic acid, and Alpha terpineol. Saussureamine C is a sesquiterpenoid compound identified in Qustul Hindi. Cinnamic acid, on the other hand, belongs to the phenolic compound group. Alpha terpineol is a terpenoid compound found in Qustul Hindi [21, 22].

These findings indicate that these compounds have a strong correlation with specific gene targets, which may play a role in various biological activities. This information can provide important insights for research and development of the therapeutic potential of Qustul Hindi and its constituent compounds.

Protein target network

In the context of pharmacological networks, the topology of compound networks refers to the relationships between compounds (nodes) and their interactions (edges) in regulating the activity of target genes. Fig. 1 shows that there are 11 compounds that have 66 protein targets (nodes) and 68 interactions (edges) in the network. In this network topology, each node represents a protein, while each edge represents the interaction between these proteins. These interactions can involve compound binding to target genes, regulation of gene expression, or other interactions involved in biological regulation [14].

Fig. 1 also demonstrates that 10 compounds (90% of the total compounds) in Qustul Hindi have more than two target genes (multiple targets), indicating that the majority of compounds in the network have effects on several target genes in breast cancer.

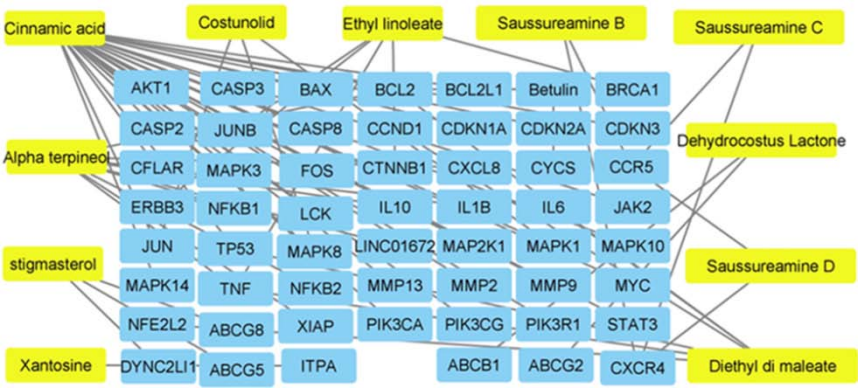

Fig. 1: Compound network topology in qustul hindi with gene targets (Number of nodes: 66, Number of edges: 68). Blue hexagons represent gene targets, yellow hexagons represent compounds in qustul hindi. 90% of the compounds have multiple target genes

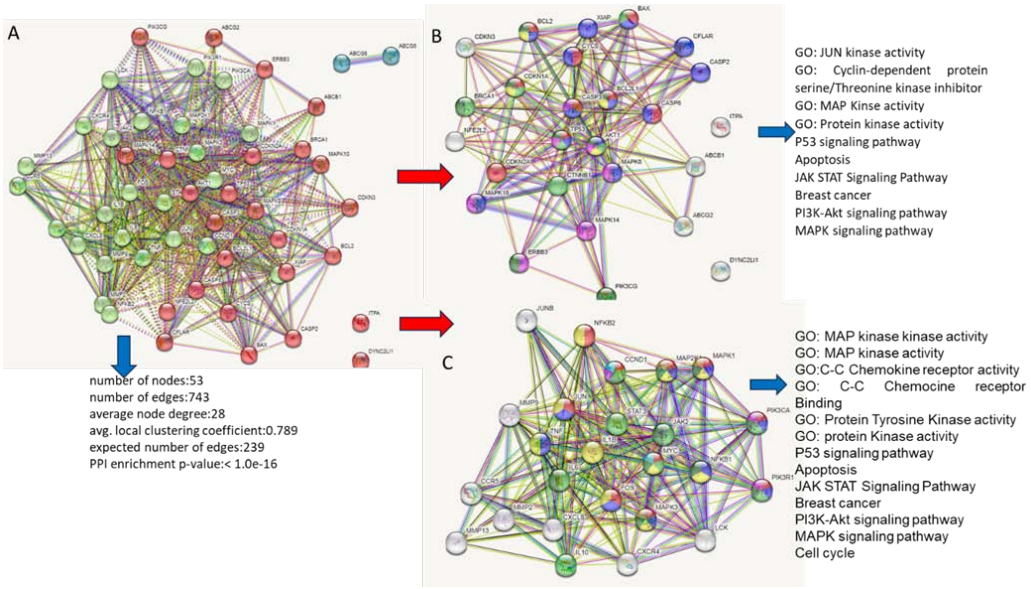

Fig. 2: The protein-protein interaction (PPI) of compounds in qustul hindi on the biological processes of breast cancer

Protein-protein interaction (PPI)

Protein-protein interaction (PPI) analysis of the gene targets of compounds in breast cancer aims to identify and understand the protein interactions involved in the biological processes of this disease. PPI refers to the physical or functional interaction between two or more proteins that play important roles in various cellular processes.

In the context of breast cancer, PPI analysis can provide insights into signaling pathways involved in the development and progression of the cancer. Signaling pathways are a series of molecular processes

that occur within cells and regulate various cellular functions, including growth, proliferation, differentiation, and apoptosis.

Fig. 2 provides an illustration of the protein-protein interaction (PPI) network analysis of the compounds found in Qustul Hindi and their involvement in the biological processes of breast cancer. The listed compounds include Saussureamine B, stigmasterol, Saussureamine D, Dehydrocostus Lactone, Costunolide, Xanthosine, cinnamic acid, Alpha terpineol, Betulin, Diethyl di maleate, and Ethyl linoleate.

The compounds involved are Saussureamine B, stigmasterol, Saussureamine D, Dehydrocostus Lactone, Costunolid, Xantosine, Cinnamic acid, Alpha terpineol, Betulin, Diethyl di maleate, and Ethyl linoleate. A) PPI on all target genes of Qustul Hindi compounds shows 53 nodes and 743 edges. B) Cluster 1 represents the PPI of target genes in cancer with 26 nodes and 169 edges. C) Cluster 2 represents the PPI of target genes in cancer with 25 nodes and 231 edges.

In fig. 2A, the PPI of all target genes of Qustul Hindi compounds in breast cancer is depicted. This fig. illustrates the overall PPI network involving all target genes of Qustul Hindi compounds in breast cancer. It shows 53 nodes, which likely represent the proteins produced by the target genes, and 743 edges, representing the interactions between these proteins. The nodes and edges in this network depict the physical or functional associations between these proteins, indicating their potential roles in breast cancer.

Fig. 2B shows Cluster 1 PPI of target genes in cancer, which indicates a specific cluster within the PPI network. This cluster represents a subset of target genes involved in processes related to breast cancer. It consists of 26 nodes, likely representing proteins, and 169 edges, indicating interactions between these proteins. This cluster highlights a sub-network of proteins that have significant functional associations related to breast cancer.

In the Gene Ontology analysis, it was found that compounds in Qustul Hindi are involved in GO terms such as JUN kinase activity, Cyclin-dependent protein serine/threonine kinase inhibitor, MAP Kinase activity, and Protein kinase activity. Meanwhile, the KEGG analysis indicates that the compounds in Qustul Hindi play a role in inhibiting breast cancer through the P53 signaling pathway, Apoptosis, JAK STAT Signaling Pathway, Breast cancer, PI3K-Akt signaling pathway, and MAPK signaling pathway.

In fig. 2C, Cluster 2 represents the PPI of target genes in cancer, which represents a different subset of target genes involved in processes related to breast cancer. This cluster consists of 25 nodes and 231 edges, indicating interactions between the proteins produced by these genes. Additionally, fig. 2C shows that compounds in Qustul Hindi are involved in the biological processes of breast cancer, including MAP kinase kinase activity, MAP kinase activity, C-C Chemokine receptor activity, C-C Chemokine receptor binding, Protein Tyrosine Kinase activity, and Protein Kinase activity.

Furthermore, in the KEGG analysis, it is shown that the compounds in Qustul Hindi are involved in the P53 signaling pathway, Apoptosis, JAK STAT Signaling Pathway, Breast cancer, PI3K-Akt signaling pathway, and MAPK signaling pathway.

Target pathway and enrichment analysis

In this study, a KEGG-based pathway analysis was conducted on the active compounds found in Qustul Hindi. This analysis is presented in table 2, which provides information about the signaling pathways influenced by the compounds in Qustul Hindi, as well as the related biological processes. The table reveals that these compounds affect several signaling pathways associated with breast cancer, such as the MAPK signaling pathway, PI3K signaling pathway, P53 signaling pathway, and cell cycle pathway. Additionally, there are several influenced biological processes, including cell proliferation regulation, apoptosis, and cell cycle.

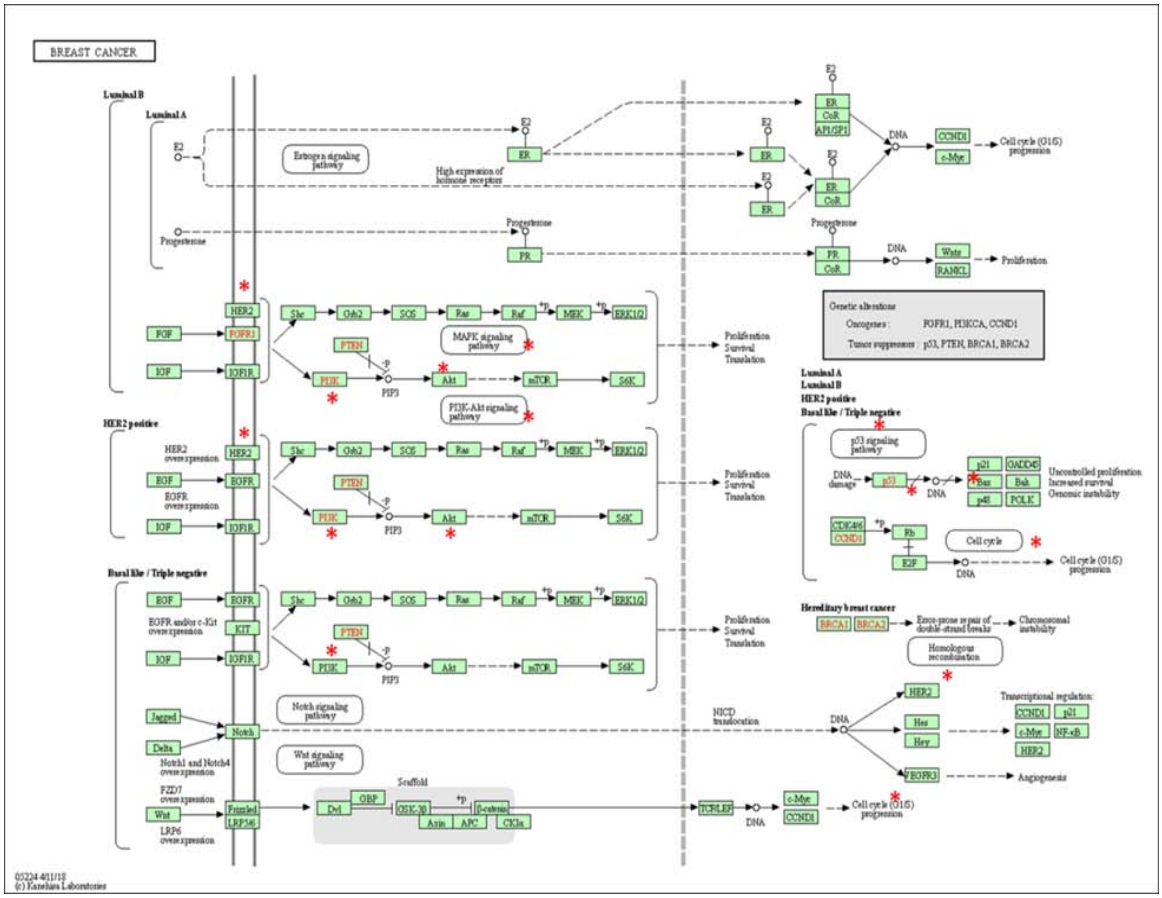

Fig. 3: The role of compounds in qusthul hindi in the breast cancer pathway

Table 2: KEGG-based signaling pathway analysis of active compounds in *S. lappa*

ID	Number of genes involved	Category	Description	FDR
hsa04115	6	KEGG Pathways	P53 signaling pathway	5.23e-9
hsa04210	6	KEGG Pathways	Apoptosis	1.00e-7
hsa05224	7	KEGG Pathways	Breast cancer	1,32e-08
has04151	7	KEGG Pathways	PI3K-Akt signaling pathway	8.9e-7
hsa04010	7	KEGG Pathways	MAPK signaling pathway	0.0014
GO: 0016493	2	GO Biological Process	C-C chemokine receptor activity	0.0339
GO: 0019957	2	GO Biological Process	C-C Chemokine binding	0.0373
GO: 0004705	2	GO Biological Process	JUN kinase activity	0.0022
GO: 0004861	3	GO Biological Process	Cyclin-dependent protein serine	0.00023
GO 0004707	2	GO Biological Process	MAP Kinase activity	0.0279
GO: 0004672	7	GO Biological Process	Protein kinase activity	0.0138
GO: 0004708	3	GO Biological Process	MAP kinase kinase activity	0.00078
GO: 0004713	3	GO Biological Process	Protein Tyrosine Kinase activity	0.0456
GO: 0097192	6	GO Biological Process	Extrinsic apoptotic signaling pathway	5.3e-09
GO: 1902510	2	GO Biological Process	Regulation of apoptotic DNA fragmentation	0.0040
GO: 1900119	2	GO Biological Process	Positif regulation of the execution phase of apoptosis	0.0061

In fig. 3, the KEGG pathway analysis of Breast Cancer pathway (has04151) reveals that the compounds in Qusthul Hindi inhibit proliferation in breast cancer through four main pathways: MAPK signaling pathway, PI3K signaling pathway, P53 signaling pathway, and cell cycle pathway. These compounds target several genes involved in these pathways, including HER2, PI3K, Akt, P53, Bax, and Cyclin-Dependent protein serine.

In the KEGG analysis shown in fig. 3, the MAPK signaling pathway indicates that the compounds in Qusthul Hindi target three genes to inhibit cell proliferation in breast cancer. These three genes are HER2, PI3K, and Akt. Additionally, these three genes are also targeted in the PI3K signaling pathway. By inhibiting these genes, excessive growth signals can be halted, and the proliferation of breast cancer cells can be reduced.

The mechanisms of the MAPK (Mitogen-Activated Protein Kinase) and PI3K (Phosphatidylinositol 3-Kinase) signaling pathways involving HER2, PI3K, and Akt genes play a crucial role in regulating cancer cell proliferation [23, 24]. Growth factors such as epidermal growth factor (EGF) interact with HER2 receptors on the surface of breast cancer cells. This interaction activates HER2 and generates signals that are transmitted to activate PI3K [25]. PI3K then produces a second molecule called phosphatidylinositol-3,4,5-trisphosphate (PIP3), which triggers the activation of protein kinase Akt. Akt activation can also occur through another pathway involving the insulin-like growth factor 1 receptor (IGF-1R) [26]. Activated Akt then plays a role in regulating cellular processes such as cell growth, proliferation, and survival [27]. Our research indicates that Saussureamine A-D, Costunolide, and Dehydrocostus Lactone (from *Saussurea lappa*) affect the MAPK signaling pathway and PI3K signaling pathway by targeting the HER2, PI3K, and Akt proteins, thereby reducing cancer cell proliferation and promoting cell survival.

In fig. 3, it can be observed that the compounds in Qusthul Hindi also have an impact on the P53 signaling pathway and the cell cycle at the G1/S phase. The P53 signaling pathway is an important pathway in regulating cell growth and development [28]. The P53 gene is one of the genes involved in this pathway. P53 is a tumor suppressor protein that plays a role in maintaining genome stability and preventing cancer cell growth. Activation of P53 can induce the expression of the Bax gene, which then triggers the apoptotic pathway and leads to cancer cell death [29]. Our research shows that the compounds in Qusthul Hindi can affect the P53 signaling pathway through the activation of the P53 and Bax genes, thereby enhancing their ability to induce apoptosis and inhibit cancer cell proliferation.

In addition to its influence on the P53 pathway, the compounds in Qusthul Hindi also affect the cell cycle at the G1/S phase [30-31]. The G1/S phase is an important phase in the cell cycle where cells prepare for DNA replication and cell division [32]. The compounds in Qusthul Hindi can target Cyclin Dependent protein serine, which is involved in the regulation of the cell cycle, particularly in the G1/S phase. By inhibiting the activity of Cyclin-Dependent protein serine, these compounds can slow down or halt the cell cycle at the G1/S phase, thereby inhibiting cancer cell proliferation.

Molecular docking of sesquiterpene compounds in qusthul hindi with the HER2 receptor

Previous studies have reported that the major compounds found in Qusthul Hindi are sesquiterpene lactones, including Saussureamine A-D, Costunolide, and Dehydrocostus Lactone. These compounds are responsible for the pharmacological effects of Qusthul Hindi, particularly in inhibiting cancer cell proliferation [22]. In this study, the activity of these compounds against the HER2 receptor was evaluated through *in silico* molecular docking using virtual docking molegro. Additionally, the drug trastuzumab, an anti-HER2 drug, was used as a comparative reference.

Table 3: The results of the reranked score, steric interactions, and hydrogen bonding of the compounds in qusthul hindi with the 3PP0 receptor

Compound	Rerank score	Steric interactions	Hydrogen bonds
Native ligand	-165.038	Ala 751, Met 801, Asn 850, Asp 863, Thr 862, Arg 849	Asp 863,Thr 862, Met 801, Asn 850
Saussureamine A	-75.1218	Thr 798, Ala 751, Leu 852, Leu 726, Asp 863, Gly 729, Val 734, Thr 862, Ser 783, Lys 753, Leu 796	Asp 863, Ser 783, Thr 862
Saussureamine B	-107.67	Ala 751, leu 852, Ser 783, Ile 752, Val 734, Thr 798, Leu 796, Lys 753, Thr 862	Ala 751, Leu 796
Saussureamine C	-122.924	Ser 783, Thr 798, Arg 784, Thr 862, Leu 796, Ala 751	Ser 783
Saussureamine D	-95.8749	Ser 783, Thr 862, Leu 796, Thr 798, Ala 751, Val 734, Asn 850, Ser 728, Arg 849, Asp 863	Arg 849, Asn 850, Ser 728, Asp 863, Thr 862, Ser 783
Costunolide	-79.7519	Met 801	Met 801
Dehydrocostus Lactone	-84.2819	Met 801	Met 801
Trastuzumab	-98.3893	Ser 783, Phe 864, Asp 863, Ile 752, Lys 753, Leu 785, Leu 796, Ala 751, Ile 767, Met 774	Asp 863, Ile 767, Ser 783

HER2 (Human Epidermal Growth Factor Receptor 2) is a protein found on the cell surface that plays a role in regulating cell growth and division. Mutations or amplification (increase in the number of copies) of the HER2 gene can lead to overproduction of the HER2 receptor, which can trigger the rapid growth of cancer cells [33-35].

Therefore, in this study, the HER2 receptor was used as the primary target for the sesquiterpene compounds in Qusthul Hindi. Trastuzumab, a commonly used drug for the treatment of HER2-overexpressing breast cancer, was used as a comparator. Trastuzumab is a monoclonal antibody that binds to HER2, inhibiting growth signals, stimulating immune responses, and inducing cancer cell death [36]. The use of trastuzumab has brought significant benefits in the prognosis and survival of patients with HER2-positive breast cancer.

In fig. 4 and table 3, the interactions between HER2 and sesquiterpene lactone compounds (Saussuramine A-D, Costunolide, Dehydrocostus Lactone) with the reference compound trastuzumab are shown. The docking results indicate that Saussureamine B and C have lower rerank scores compared to other sesquiterpene compounds and Trastuzumab. This indicates that these compounds have lower energy when binding to the HER2 receptor, resulting in a more stable binding. A lower rerank score indicates higher affinity and a more stable binding of the drug to the receptor.

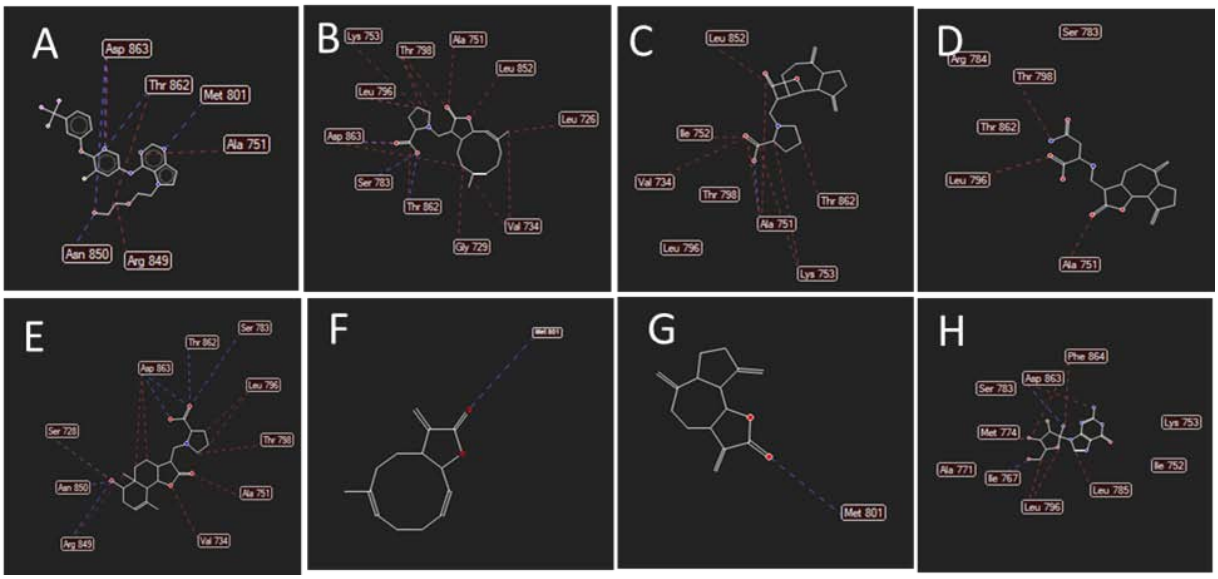

Fig. 4: Amino acid interactions with native ligand (A), Saussuramine A (B), Saussureamine B (C), Saussureamine C (D), Saussureamine D (E), Costunolide (F), Dehydrocostus Lactone (G), Trastuzumab (H), where blue lines represent hydrogen bonds and red lines represent steric interactions

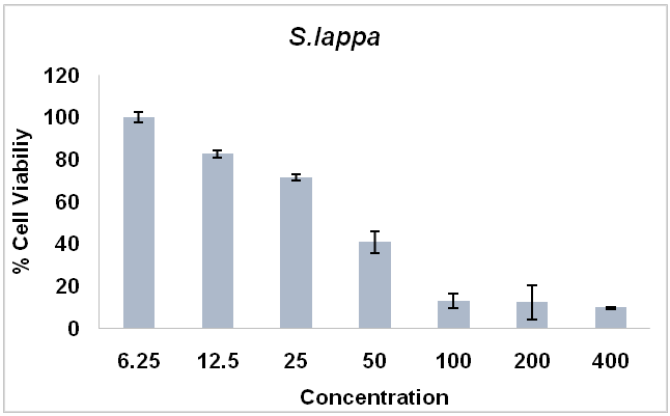

Fig. 5: Viability of T47D cells after treatment with qusthul hindi extract. For 24 h, T47D cells were treated with various concentrations of qusthul hindi extract, and cell viability was evaluated using the MTT assay. The cell viability profile is presented as the mean±standard deviation (SD) of three independent experiments.

The *in vitro* cytotoxicity test of qusthul hindi on T47D cell line

In our study, after analyzing the potential of anticancer compounds through pharmacological network analysis and subsequent *in silico* testing, we conducted *in vitro* validation through cytotoxicity testing on breast cancer cell lines. Additionally, we also investigated signaling pathways through apoptosis and cell cycle assays. The cytotoxicity test results revealed that the Qusthul Hindi extract exhibited moderate anticancer activity against T47D cells.

Our research shows that in the viability percentage graph (fig. 5), the IC_{50} value of Qusthul Hindi extract on T47D cells has been determined to be 32.81 µg/ml. Judging from the level of anticancer activity, the anticancer activity of Qusthul Hindi is classified as moderate activity. The IC_{50} values for anticancer activity in herbal drug development, 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 [37].

Apoptosis induction and cell cycle arrest

As further evidence of the pharmacological network analysis and *in silico* results indicating the involvement of Qusthul Hindi compounds in apoptosis and cell cycle regulation in breast cancer cells, we conducted apoptosis and cell cycle assays. Our research results on the apoptosis assay demonstrated that Qusthul Hindi compounds have a strong potential in inducing apoptosis in breast cancer cells, with a total of 85.3% of cells undergoing apoptosis and 5.8% undergoing necrosis (fig. 5). Furthermore, the statistical analysis of the number of cells undergoing both early and late apoptosis showed a significant difference between the Qusthul Hindi extract-treated group and the untreated control group (p<0.001).

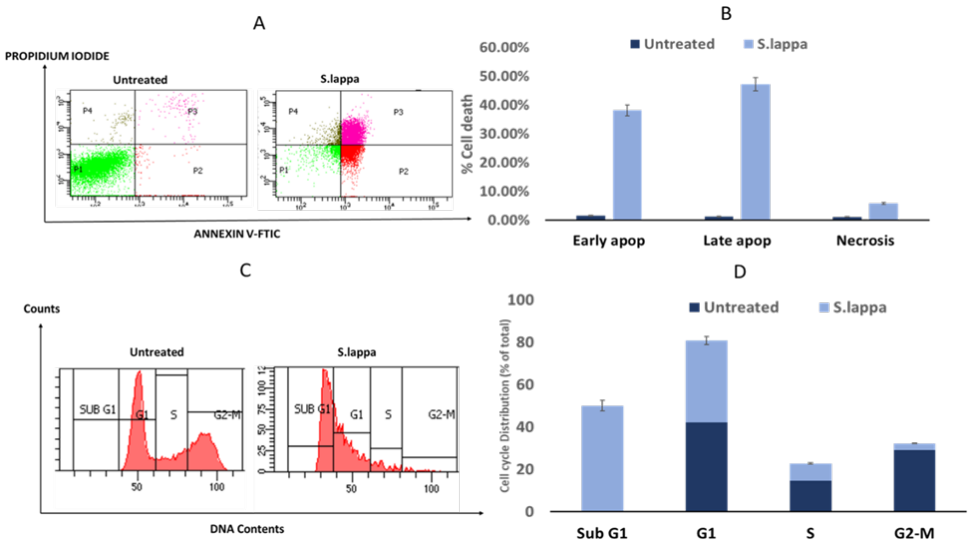

Fig. 6: Apoptosis induction and cell cycle distribution by qusthul hindi extract in T47D breast cancer cells. Cells were exposed to qusthul hindi extract for 24 h. Cell death percentage (A, B) and cell distribution in each phase (C, D) were measured using flow cytometry after staining with Annexin V-FITC/PI and PI, respectively. The lines represent the mean±standard deviation (SD) values from three independent experiments. *p<0.001 (post-hoc LSD test between each group) considered statistically significant

In addition to cell death induction, 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 Qusthul Hindi extract for 24 h. The results in fig. 2C, D show a significant influence on the cell cycle distribution treated with Qusthul Hindi extract (P<0.0001). Administration of Qusthul Hindi extract led to increased cell accumulation in the sub G1 phase, along with a significant decrease in accumulation in the G2-M and S phases. The increased number in the G1 phase indicates an increased number of apoptotic cells (P>0.0001).

The sub G1 phase in the cell cycle analysis indicates the accumulation of cells with reduced DNA content. The sub G1 phase is usually associated with cell death or apoptosis [38-40]. The finding of increased cell accumulation in the sub G1 phase after Qusthul Hindi extract administration indicates its potential to enhance apoptosis in cancer cells. Apoptosis is an important mechanism for eliminating abnormal or damaged cancer cells.

In our study, the decrease in cell accumulation in the G2-M and S phases after Qusthul Hindi extract administration suggests that the extract can inhibit or delay DNA replication and cell division. The G2-M phase is the preparation phase for cell division, while the S phase is where DNA replication occurs. By reducing cell accumulation in the G2-M and S phases, Qusthul Hindi extract can inhibit the proliferation of cancer cells.

Overall, these findings indicate that Qusthul Hindi extract administration has effects on cell cycle and apoptosis in cancer cells. The extract may enhance cancer cell apoptosis (as seen from increased accumulation in the sub G1 phase) and inhibit cell proliferation (as seen from decreased accumulation in the G2-M and S phases). The increased number of cells in the G1 phase indicates a delay in the cell cycle and may lead to inhibition of cancer cell growth. However, further research is needed to understand the mechanism of action of Qusthul Hindi extract contributing to these findings and to clarify its potential use as a cancer therapy.

CONCLUSION

Qusthul Hindi contains 11 active compounds that have the potential as anticancer agents, including compounds from the Sesquiterpene, Terpenoid, and phenolic groups. Network pharmacology analysis revealed that qusthul hindi influences 66 genes involved in breast cancer. Through KEGG pathway analysis, it was found that qusthul hindi is closely associated with important signaling pathways in breast cancer, such as the P53 signaling pathway, MAPK signaling pathway, PI3K-Akt signaling pathway, and cell cycle. Molecular docking results showed that Saussureamine B and C have better affinity towards the HER2 receptor compared to the reference drug trastuzumab. *In vitro* cytotoxicity testing demonstrated that qusthul hindi extract exhibited highly potent activity against T47D cells. Furthermore, qusthul hindi extract showed strong potential in inducing apoptosis in breast cancer cells, with a percentage of cells undergoing apoptosis reaching 85.3% and necrosis at 5.8%. Statistical analysis revealed that the treatment with qusthul hindi extract significantly differed from the control group in terms of the number of cells undergoing apoptosis. Additionally, qusthul hindi extract was found to affect the cell cycle by significantly increasing the number of cells in the sub-G1 phase while decreasing the number of cells in the G2-M and S phases. This indicates that the extract can enhance apoptosis in cancer cells through increased accumulation of cells in the sub G1 phase and inhibit cell proliferation by reducing the accumulation of cells in the G2-M and S phases.

ACKNOWLEDGEMENT

We would like to express our gratitude to the Department of Pharmacy, Faculty of Medicine and Health Sciences, UIN Maulana Malik Ibrahim Malang, for providing the laboratory facilities to complete this research.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All authors contributed equally to this work.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A. Global cancer statistics 2020: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209-49. doi: 10.3322/caac.21660, PMID 33538338.
2. Kayl AE, Meyers CA. Side-effects of chemotherapy and quality of life in ovarian and breast cancer patients. Curr Opin Obstet Gynecol. 2006;18(1):24-8. doi: 10.1097/01.gco.0000192996.20040.24, PMID 16493256.
3. Hopkins AL. Network pharmacology: the next paradigm in drug discovery. Nat Chem Biol. 2008;4(11):682-90. doi: 10.1038/nchembio.118, PMID 18936753.
4. Fang J, Wang L, Wu T, Yang C, Gao L, Cai H. Network pharmacology-based study on the mechanism of action for herbal medicines in Alzheimer treatment. J Ethnopharmacol. 2017;196:281-92. doi: 10.1016/j.jep.2016.11.034, PMID 27888133.
5. Zhang W, Chen Y, Jiang H, Yang J, Wang Q, Du Y. Integrated strategy for accurately screening biomarkers based on metabolomics coupled with network pharmacology. Talanta. 2020;211:120710. doi: 10.1016/j.talanta.2020.120710, PMID 32070601.
6. Boezio B, Audouze K, Ducrot P, Taboureau O. Network-based approaches in pharmacology. Mol Inform. 2017;36(10):1700048. doi: 10.1002/minf.201700048, PMID 28692140.
7. Al-Jauziya IIQ. Healing with the medicine of the prophet (PBUH). Darussalam; 1999.
8. El-Far A, Shaheen H, Alsenosy A, El-Sayed Y, Al Jaouni S, Mousa S. Costus speciosus: traditional uses, phytochemistry, and therapeutic potentials. Phcog Rev. 2018;12(23). doi: 10.4103/phrev.phrev_29_17.
9. Hassan R, Masoodi MH. Saussurea lappa: a comprehensive review on its pharmacological activity and phytochemistry. Curr Tradit Med. 2020;6(1):13-23. doi: 10.2174/2215083805666190626144909.
10. Abdallah EM, Qureshi KA, Ali AMH, Elhassan GO. Evaluation of some biological properties of Saussurea costus crude root extract. Biosci Biotech Res Comm. 2017;10(4):601-11. doi: 10.21786/bbrc/10.4/2.
11. Mutiah R, Listiyana A, Indradmojo C, Griana TP, Dwi HH, Atmaja RRD. Induction of apoptosis and phase-cell cycle inhibition of G0-G1, S, G2-M of T47D breast cancer cells on treatment with ethyl acetate fraction of jackfruit parasite leaves (Macrosolen cochinensis). J Appl Pharm Sci. 2017;7(10):138-43.
12. Liu X, Wu J, Zhang D, Wang K, Duan X, Zhang X. A network pharmacology approach to uncover the multiple mechanisms of *Hedyotis diffusa* willd. on colorectal cancer. Evid Based Complement Alternat Med. 2018;2018:6517034. doi: 10.1155/2018/6517034, PMID 29619072.
13. Jiang L, Lu J, Qin Y, Jiang W, Wang Y. Antitumor effect of guava leaves on lung cancer: a network pharmacology study. Arab J Chem. 2020;13(11):7773-97. doi: 10.1016/j.arabjc.2020.09.010.
14. Zhou Z, Chen B, Chen S, Lin M, Chen Y, Jin S. Applications of network pharmacology in traditional Chinese medicine research. Evid Based Complement Alternat Med. 2020;2020:1646905. doi: 10.1155/2020/1646905, PMID 32148533.
15. Mutiah R, Hariz MF, Indrawijaya YYA, Ma'arif B. *In silico* prediction of isoliquiritigenin and oxyresveratrol compounds to BCL-2 dan VEGF-2 receptors. Indones J Cancer Chemoprevent. 2019;10(2):51-9. doi: 10.14499/indonesianjcanchemoprev10iss2pp51-59.
16. Mutiah R, Sukardiman WA, Widyawaruyanti A. Cytotoxic effect of crude extract and fraction from calotropis gigantea Leaves O. Human C. Cancer Widr cell lines. Int J Pharm Pharm Sci. 2017;9(1). doi: 10.22159/ijpps.2017v9i1.9022.
17. Amalina ND, Salsabila IA, Zulfin UM, Jenie RI, Meiyanto E. *In vitro* synergistic effect of hesperidin and doxorubicin downregulates epithelial-mesenchymal transition in highly metastatic breast cancer cells. J Egypt Natl Canc Inst. 2023;35(1):6. doi: 10.1186/s43046-023-00166-3, PMID 36967442.
18. Hastuti HEA, Adisusilo MRP, Ashar YA, Meiyanto E, Jenie RI. Revealing the potential of compounds in sappan wood as cervical cancer metastasis chemopreventive agent with MMP9 target. Indones J Cancer Chemoprevent. 2022;13(1):22-32. doi: 10.14499/indonesianjcanchemoprev13iss1pp22-32.
19. Mutiah R, Minggarwati TS, Kristanti RA, Susanti E. Compound identification and anticancer activity of ethyl acetate fraction from bawang sabrang (Eleutherine palmifolia (L.) Merr.) on HeLa cervical cancer cell line. Indones J Cancer Chemoprevent. 2019;10(3):131-9. doi: 10.14499/indonesianjcanchemoprev10iss3pp131-139.
20. Sun H, Yin M, Hao D, Shen Y. Anti-cancer activity of catechin against A549 lung carcinoma cells by induction of cyclin kinase inhibitor p21 and suppression of cyclin E1 and P–AKT. Appl Sci. 2020;10(6):2065. doi: 10.3390/app10062065.
21. Ansari S, Maaz M, Ahmad I, Hasan SK, Bhat SA, Naqui SK. Quality control, HPTLC analysis, antioxidant and antimicrobial activity of hydroalcoholic extract of roots of *qust* (*Saussurea lappa*, C.B Clarke). Drug Metab Pers Ther. 2020:20200159. doi: 10.1515/dmdi-2020-0159, PMID 33780194.
22. Sen S, Chakraborty R. editors. Herbal medicine in India: indigenous knowledge, practice, innovation and its value. Springer; 2020. doi: 10.1007/978-981-13-7248-3.
23. Braicu C, Buse M, Busuioc C, Drula R, Gulei D, Raduly L. A comprehensive review on MAPK: a promising therapeutic target in cancer. Cancers. 2019;11(10):1618. doi: 10.3390/cancers11101618, PMID 31652660.
24. Zhu C, Qi X, Chen Y, Sun B, Dai Y, Gu Y. PI3K/Akt and MAPK/ERK1/2 signaling pathways are involved in IGF-1-induced VEGF-C upregulation in breast cancer. J Cancer Res Clin Oncol. 2011;137(11):1587-94. doi: 10.1007/s00432-011-1049-2, PMID 21904903.
25. Issinger OG, Guerra B. Phytochemicals in cancer and their effect on the PI3K/AKT-mediated cellular signalling. Biomed Pharmacother. 2021;139:111650. doi: 10.1016/j.biopha.2021.111650, PMID 33945911.
26. Paplomata E, O'Regan R. The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. Ther Adv Med Oncol. 2014;6(4):154-66. doi: 10.1177/1758834014530023, PMID 25057302.
27. Yang L, Hou Y, Yuan J, Tang S, Zhang H, Zhu Q. Twist promotes reprogramming of glucose metabolism in breast cancer cells through PI3K/AKT and p53 signaling pathways. Oncotarget. 2015;6(28):25755-69. doi: 10.18632/oncotarget.4697, PMID 26342198.
28. Issaeva N. p53 signaling in cancers. Cancers. 2019;11(3):332. doi: 10.3390/cancers11030332, PMID 30857153.
29. Gasco M, Shami S, Crook T. The p53 pathway in breast cancer. Breast Cancer Res. 2002;4(2):70-6. doi: 10.1186/bcr426, PMID 11879567.
30. Lee SJ, Moon GS, Jung KH, Kim WJ, Moon SK. C-Jun N-terminal kinase 1 is required for cordycepin-mediated induction of G2/M cell-cycle arrest via p21WAF1 expression in human colon cancer cells. Food Chem Toxicol. 2010;48(1):277-83. doi: 10.1016/j.fct.2009.09.042, PMID 19833164.
31. Caldon CE, Daly RJ, Sutherland RL, Musgrove EA. Cell cycle control in breast cancer cells. J Cell Biochem. 2006;97(2):261-74. doi: 10.1002/jcb.20690, PMID 16267837.
32. Zainuddin NASN, Zakaria Y, Sula MD. Dendrophthoe pentandra induced apoptosis and cell cycle arrest at G1/S in human breast adenocarcinoma cells, MCF-7 via Upregulation of p53. J App Pharm Sci. 2018;8(9):130-41. doi: 10.7324/JAPS.2018.8919.
33. Yarden Y. Biology of HER2 and its importance in breast cancer. Oncology. 2001;61Suppl 2:1-13. doi: 10.1159/000055396, PMID 11694782.
34. Loibl S, Gianni L. LHER2-positive breast cancer. Lancet. 2017;389(10087):2415-29. doi: 10.1016/S0140-6736(16)32417-5, PMID 27939064.

35. Zeng L, Yang K. Exploring the pharmacological mechanism of Yanghe Decoction on HER2-positive breast cancer by a network pharmacology approach. J Ethnopharmacol. 2017;199:68-85. doi: 10.1016/j.jep.2017.01.045, PMID 28130113.
36. Baselga J, Perez EA, Pienkowski T, Bell R. Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer. Oncologist. 2006;11Suppl 1:4-12. doi: 10.1634/theoncologist.11-90001-4, PMID 16971734.
37. Kroll DJ. Natural compounds in cancer therapy: promising nontoxic antitumor agents from plants and other natural sources John Boik (University of Texas). Oregon Medical Press, Princeton. J Nat Prod. 2001;64(12).521. doi: 10.1021/np000765k.
38. Demidenko ZN, Kalurupalle S, Hanko C, Lim CU, Broude E, Blagosklonny MV. Mechanism of G1-like arrest by low concentrations of paclitaxel: next cell cycle p53-dependent arrest with sub-G1 DNA content mediated by prolonged mitosis. Oncogene. 2008;27(32):4402-10. doi: 10.1038/onc.2008.82, PMID 18469851.
39. Shu CH, Yang WK, Shih YL, Kuo ML, Huang TS. Cell cycle G2/M arrest and activation of cyclin-dependent kinases associated with low-dose paclitaxel-induced sub-G1 apoptosis. Apoptosis. 1997;2(5):463-70. doi: 10.1023/a:1026422111457, PMID 14646529.
40. Preethi S, Kumar H, Rawal VB, Ajmeer R, Jain V. Overview of mitoxantrone-a potential candidate for the treatment of breast cancer. Int J Appl Pharm. 2022;14:10-22.