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Network Pharmacology Combined with Molecular Docking, Molecular Dynamics, and In Vitro Experimental Validation Reveals the Therapeutic Potential of *Thymus vulgaris* L. Essential Oil (Thyme Oil) against Human Breast Cancer

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ABSTRACT: Breast cancer is a major global health issue for women. Thyme oil, extracted from *Thymus vulgaris* L., has shown promising anticancer effects. In the present study, we investigated how Thyme oil can influence breast cancer treatment using a multimethod approach. We used network pharmacology to identify the active compounds of Thyme oil, their molecular targets, and the pathways involved in breast cancer. We found that Thyme oil can modulate several key proteins (EGFR, AKT1, ESR1, HSP90AA1, STAT-3, SRC, IL-6, HIF1A, JUN, and BCL2) and pathways (EGFR tyrosine kinase inhibitor resistance, prolactin signaling pathway, HIF-1 signaling pathway, estrogen signaling pathway, ERBB signaling pathway, AGE-RAGE signaling pathway, JAK-STAT signaling pathway, FoxO signaling pathway, and PI3K-AKT signaling pathway) related to breast cancer progression. We then used molecular docking and dynamics to study the interactions and stability of the Thyme oil−compound complexes. We discovered three potent compounds (aromadendrene, α-humulene, and viridiflorene) that can bind strongly to important breast cancer proteins. We also performed in vitro experiments on MCF-7 cells to confirm the cytotoxicity and antiproliferative effects of Thyme oil. We observed that Thyme oil can inhibit cancer cell growth and proliferation at a concentration of 365.37 μg/mL. Overall, our results provide a comprehensive understanding of the pharmacological mechanism of Thyme oil in breast cancer treatment and suggest its potential as a new or adjuvant therapy. Further studies are needed to validate and optimize the therapeutic efficacy of Thyme oil and its active compounds.
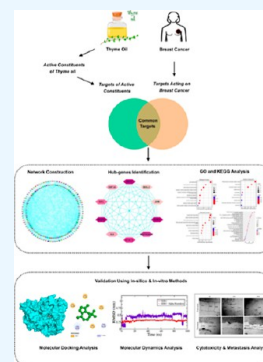
INTRODUCTION

Breast cancer is a widespread health concern. It is a widespread cancer that affects women all over the world.[1,2] In 2020, breast cancer was responsible for about 2.3 million new cases and 685,000 fatalities.[3] It is a cancer that begins in the cells of the breast and can move to other organs in the body. It can cause symptoms such as lumps, pain, swelling, nipple discharge, or changes in the shape or size of the breast.[4,5] It can be diagnosed by tests such as mammograms, ultrasounds, biopsies, or blood tests and can be treated by different methods depending on the stage, subtype, and personal factors of the patient.[6] Some of the usual treatments are removing the tumor by surgery, killing the cancer cells by drugs or radiation, blocking the hormones that fuel the cancer, or targeting specific molecules on the cancer cells.[5,7] However, there is still a need for more effective and less harmful treatment options.

Plant-based foods and their compounds have shown to affect cancer development and progression, including breast cancer.[8−12] They can prevent or reduce the damage caused by free radicals, inflammation, and immune system disorders that can promote cancer development and spread.[13] They also affect the behavior of cancer stem cells, such as their proliferation, apoptosis, and angiogenesis.[14] Another way that plant bioactive substances can act against cancer is by targeting the abnormal epigenetic changes that are characteristic of cancer cells. These changes include the silencing of tumor suppressor genes by methylation, the alteration of histone modifications, the dysregulation of noncoding RNA (ncRNA), and global DNA hypomethylation.[15−17] These epigenetic changes are reversible and can be influenced by dietary compounds, which is the focus of nutri-epigenetics. Many studies have shown that eating plant-based foods regularly and for a long period can reduce the risk of breast cancer.[18−21]

Thymus vulgaris (*T. vulgaris*) is a medicinal herb of the Lamiaceae family. It is an herb that has a lot of essential oil, and its main chemical components are oxygenated monoterpenes and monoterpene hydrocarbons. In particular, the highest concentrations are of thymol, carvacrol, *p*-cymene, borneol, *trans*-caryophyllene, and *cis*-sabinene hydrate.[22−25] Therefore, Thyme oil obtained from the plant has been reported for its diverse medicinal applications for treating acne

and skin infections, relieving coughs and respiratory infections, preventing hair loss, supporting heart health, and fighting cancer.[24,26,27] Considering that Thyme oil contains an inclusive phytochemical constituent and has a significant capability for bioactivity, it has triggered further exploration of the mechanisms by which these phytochemical constituents exert their effects, potentially offering novel approaches for breast cancer treatment.

Furthermore, advanced computational methods such as network pharmacology, molecular docking, and molecular dynamics approaches are important methods in drug discovery against complex diseases like breast cancer. Network pharmacology is a method that integrates the information on multiple biological networks, such as protein−protein interactions, gene expression, metabolic pathways, and drug−target interactions, to identify the potential mechanisms and effects of drugs on complex diseases.[28] Network pharmacology can help in the discovery of active components and targets of Thyme oil that are related to breast cancer and to analyze the biological activities and signaling pathways which are associated with its anticancer effects.[29] Molecular docking is a method that predicts the binding mode and affinity of a ligand to a receptor by using computational algorithms.[30] It can help to evaluate the association between the active components of Thyme oil and the key targets of breast cancer as well as to select the most promising candidates for further validation.[29,31] Molecular dynamics simulation is a method that simulates the movement and behavior of molecules over time by using physical laws and mathematical models.[30] It can help assess the stability and flexibility of the complexes formed by the active components of Thyme oil and the key targets of breast cancer to reveal the structural and functional differences which occur during the binding process.[29,31]

The rationale for this research is grounded in the need for novel therapeutic strategies that can enhance the efficacy and safety of breast cancer treatment. Thyme oil, as a natural compound, offers a potential alternative that may overcome the limitations associated with conventional therapies. By employing a combination of computational and experimental techniques, this study aims to provide a deeper understanding of the molecular interactions between Thyme oil and breast cancer-related targets as well as potential mechanism of action. The findings of this research have the potential to contribute to the development of targeted therapies for breast cancer, ultimately improving patient outcomes and the quality of life. Furthermore, the utilization of network pharmacology, molecular docking, and molecular dynamics simulations in this study demonstrates the power of integrative approaches in drug discovery and development. Therefore, the aim of the current study was to explore and identify the bioactive constituents of Thyme oil that have potential for treating breast cancer. A comprehensive approach was used to analyze the bioactive compounds of Thyme oil by combining network pharmacology with molecular docking and simulation methods. Furthermore, the anticancer and antiproliferative effects of Thyme oil were also tested using different in vitro methods.

■ MATERIALS AND METHODS

Finding the Potential Targets for Drug Discovery and Disease Treatment. Based on literature reports of Thyme oil compounds, the SMILES codes of a total of 118 active compounds were obtained via PubChem database. The cross-validation of the Smiles codes and the stereo configuration of the compounds were carried out using the ChemSpider database and using ChemSketch software. To find out the potential targets for the compounds, we used the SwissTargetPrediction database (http://www.swisstargetprediction.ch/, accessed on August 18, 2023)[32] to input the SMILES codes. The search for genes related to breast cancer was carried out using the OMIM databases (https://www.omim.org/, accessed on August 18, 2023), GeneCards database (https://www.genecards.org/, accessed on August 18, 2023), and DisGeNET (http://www.disgenet.org/, accessed on August 18, 2023) with the keyword "breast cancer". For DisGeNET, a cutoff of "score_gda > 0.1" was applied, while for GeneCards, a score of >30 was applied.

Estimating the Pharmacokinetic and Toxicological Parameters of Compounds. The compounds present in Thyme oil were filtered based on their ADMET properties in order to discover their potential. ADMET properties and PAINS patterns (Pan-assay interference compounds) were determined with assistance from the ADMETlab 2.0 server (https://admetmesh.scbdd.com/).[33] To predict the toxicity, PROTOX II server (https://tox-new.charite.de/protox_II/) was used.[34] The compounds with the best ADMET properties, PAINS patterns, and toxicity have been filtered out of the list. By use of the PAINS filter, compounds with specific patterns and a high affinity for multiple targets can be eliminated. An assessment of ADMET is conducted to determine if a compound has druglike physicochemical properties, and pharmacokinetic properties, lowering the probability of a clinical trial failing.

Finding and Securing Potential Targets. The FunRich tool version 3.1.3 was used to search for common targets between compounds of Thyme oil and breast cancer.[35] Further information about the probable protein targets was obtained from the Swiss target prediction database (https://www.http://www.swisstargetprediction.ch/error_page.php?error=1/search, accessed on August 19, 2023). To see the shared targets, we used FunRich to make Venn diagrams.[36]

Hub-Gene Identification and Analysis of Protein−Protein Interaction Networks. The cytoHubba plugin of Cytoscape (Version 3.10.1) identified the top 10 hub-genes in the network based on the degree method. The protein−protein interactions of potential targets were investigated using STRING (https://string-db.org/, accessed on August 19, 2023).[37] The analysis applied a confidence level of 0.700 and a false discovery rate (FDR) of 5%. The PPI network was built and analyzed using Cytoscape software (Version 3.10.1).[38] The network was imported from the STRING database to Cytoscape. Three topological measures characterized the network nodes: degree, betweenness, and closeness centrality. These measures selected a range of possible targets.

Functional Annotation and Pathway Analysis of Hub-Genes. The DAVID database was utilized to analyze the biological functions and pathways associated with the target proteins and disease (https://https://david.ncifcrf.gov/, accessed on August 19, 2023).[39] GO terms and pathways were used to visualize the enrichment analysis with a FDR of less than 0.05. The SRplot (https://www.bioinformatics.com.cn/) visualized the top 10 most relevant GO terms (BP, CC, and MF). A map of the top 30 KEGG pathways was created using ShinyGo 0.77 (http://bioinformatics.sdstate.edu/go/).

Virtual Screening of Compounds Using Molecular Docking. The binding affinity of compounds of Thyme oil to

breast cancer targets was evaluated using AutoDock v1.5.7.[40] The PubChem database provided the 3D structures of ligands and Open Babel v2.4.1 converted them from.sdf to.pdb.[41] Avogadro optimized the structures by reducing the energy using the MMFF94 force field and Steepest Descent algorithm for 5000 steps. The optimization stopped when the energy difference was less than 0.1, and the structures were stored as.pdb files. The 3D crystal structures of the receptors were obtained from RCSB-PDB database with the following PDB ID, EGFR (PDB ID: 4ZAU), ESR1 (PDB ID: 1A52), and AKT1 (PDB ID: 3OCB). The receptors had polar hydrogen and Kollman charge added after removing water and het atoms. The protein structures were saved as.pdbqt format. Biovia Discovery Studio and PyMOL was used to visualize protein−ligand complexes docked with AutoDock.[42]

Molecular Dynamics Simulation. Molecular dynamics (MD) was employed to study the conformational stability of ligands in receptor-binding pockets in response to changing time. GROMACS version 2019.4 was used for the MD simulations[43] using the CHARMM force field. In order to generate the force field parameters, the SwissParam server was utilized to obtain the topology of the ligand. The system was reduced in a vacuum for 1500 steps using the steepest descent algorithm. A TIP3P water model was used to immerse the protein−ligand complex. Na^+ and Cl^- ions were added to balance the system to a salt concentration of 0.15 M. With the leapfrog algorithm, the *NVT* and *NPT* equilibration steps were performed for 100 ps each. Solvated protein−ligand complexes were run for 100 ns in production MD. The trajectory file had the periodic boundary conditions removed in the MD simulation. The MD data were analyzed using Chimera, and the graphs were created using XMGRACE (https://plasma-gate.weizmann.ac.il/Grace/).

Cell Culture. A medium containing Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (10% FBS), penicillin (10,000 units), and streptomycin (5 mg) was used for breast cancer cell culture (MCF-7). To ensure the cells grew to their full potential, they were kept in a humid environment with 5% CO_2 at 37 °C.[44]

MTT Assay. Using an MTT assay, MCF-7 cells were exposed to Thyme oil for the determination of cell viability. The cells were removed from the T-25 flasks by trypsinization and aspirating them. They were then centrifuged at 3000 rpm in a centrifuge. The cells were resuspended in culture medium and adjusted to a density of 10,000 cells per 200 μL. The suspension (200 μL) was added to each well of a 96-well plate, and the cells were allowed to adhere by incubation for 24 h at 37 °C with 5% CO_2. Then, the cells were exposed to different concentrations of Thyme oil (Sigma-Aldrich) (200 μL) and incubated for another 24 h under the same conditions. After incubation, each well received 200 μL of fresh medium with 10% MTT reagent and was further incubated for 3 h at 37 °C with 5% CO_2. To dissolve the formazan crystals that formed, 100 μL of DMSO was added and mixed well. Absorbance was measured at 570 and 630 nm using a microplate reader. The background values and the blank values were subtracted from the results, and the IC_{50} value was calculated. The IC_{50} value is the concentration of Thyme oil that reduces 50% of cell growth.[45,46] The assay was performed in triplicate for each condition.

Scratch Assay. The effect of Thyme oil on the proliferation of MCF-7 cancer cells was determined via the scratch assay. Cells were seeded at 1×10^6 cells/mL in 3 mL of medium in six-well plates and grew to a confluent layer. A sterile 1 mL pipette tip made a scratch in the center of each well. The width of the initial scratch was measured and recorded by using an inverted microscope. The wells received Thyme oil at the IC_{50} concentration, and the plates were incubated for 44 h. The width of the final scratch was compared with the initial width. A smaller scratch width shows the wound healing ability of the cells.[47]

Statistical Analysis. The MTT assay was performed in triplicate for each condition. The IC_{50} value of Thyme oil for the inhibition of MCF-7 was calculated via regression equation derived from the logarithmic trendline of the percentage of inhibition versus the drug concentration in Excel. The graph was prepared using Graph Pad Prism software 8.0.

■ RESULTS

Screening of Phytochemical Compounds of Thyme Oil and ADMET Analysis. The literature search revealed 118 different phytochemical constituents of Thyme oil that were included in this study. The ADMET properties, PAINS patterns, and toxicity properties, of all the selected compounds were predicted using the ADMETlab 2.0 and PROTOX-II web server. Table S1 shows the ADMET properties of all of the compounds. The results showed that 107 compounds had no PAINS patterns and followed Lipinski rule with good ADMET properties. 11 compounds had toxicity properties and were removed from further analysis out of 118 compounds. Total 107 compounds had similar ADMET profiles without any toxic patterns. The ADMET properties indicated that these compounds could be safe and have potential leads for developing anticancer drugs.

Prediction and Screening of Potential Targets. The PubChem database was used to obtain detailed information about compounds, and the SwissTargetPrediction (STP) database was used to analyze their target classes. The Swisstarget prediction yielded 735 predicted targets for the phytochemical constituents of Thyme oil, while the Genecards (GDA cutoff of >20), DisGeNet (cutoff of >0.1), and OMIM databases yielded 1591 predicted targets for breast cancer after removing duplicates. The common targets between the compounds and disease were identified, resulting in 195 potential targets (Figure 1).

Finding of Hub-Genes and Construction of the Compound-Disease Common Target Network. The STRING database was used to obtain data on the potential target genes and their interactions. The data were visualized
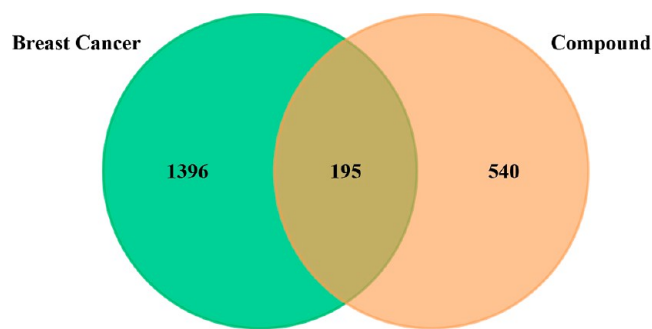

Figure 1. Venn diagram was created using the FunRich tool to show the common targets between the phytochemical components of Thyme oil and breast cancer.
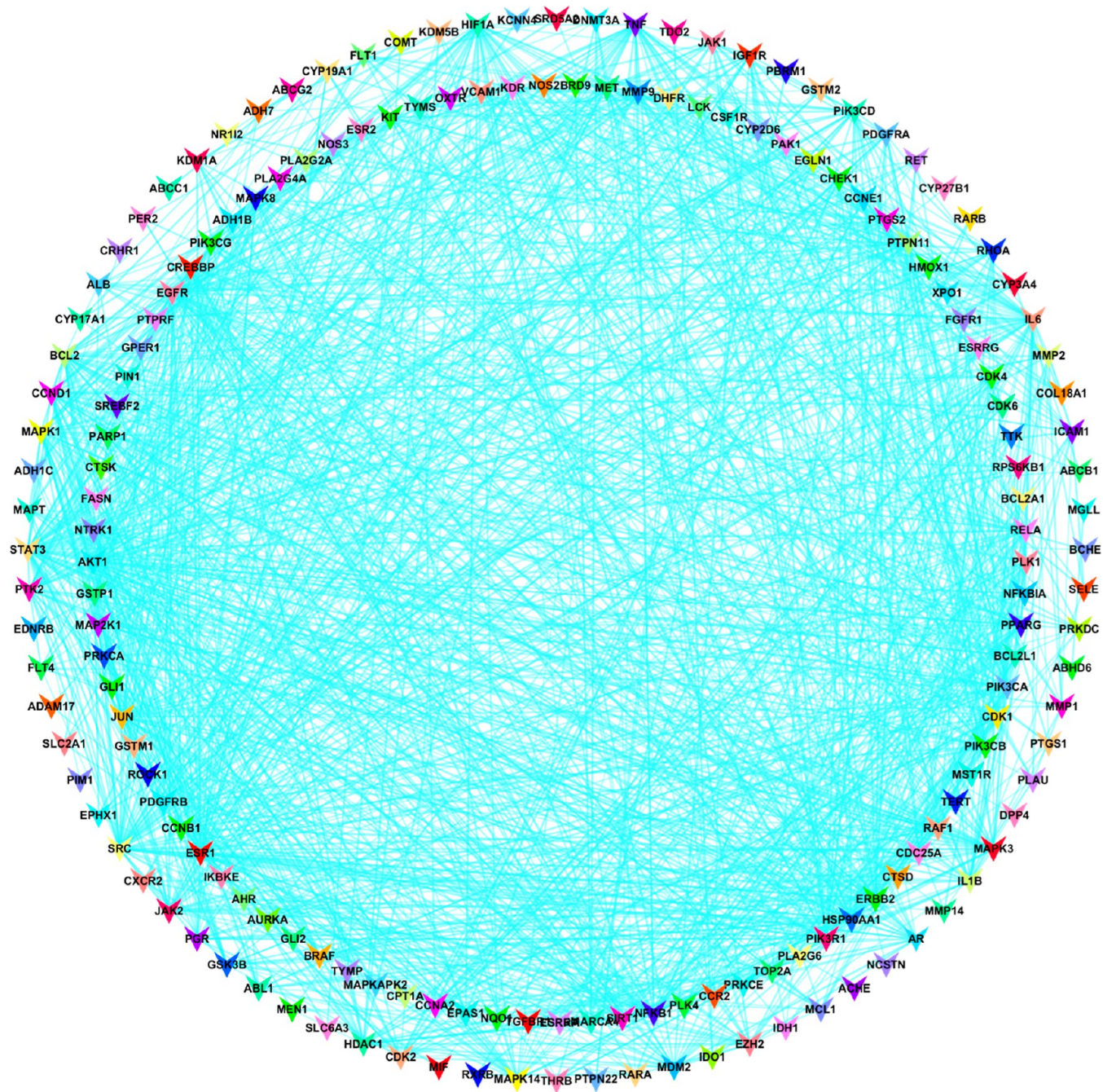
Figure 2. Common-protein target network of phytochemical components of Thyme oil and breast cancer constructed via Cytoscape software.

and analyzed as a PPI network using Cytoscape version 3.10.1. A network of common genes and compound targets was constructed (Figures 2 and 3). To evaluate the importance of each node in the network, three parameters such as degree, closeness, and centrality between the nodes were calculated using cytoNCA plugin (Tables S2 and S3). These parameters indicate the relative significance of respective node in the network. The target genes that were discovered were related to breast cancer development. These results imply that the anticancer activity of phytochemical constituents of Thyme oil may be mediated by these key targets. Using the degree method, the cytoHubba plugin in Cytoscape helped us find the 10 genes with the highest connectivity. The top 10 targets in the network that had the highest values of the parameters were

EGFR, AKT1, ESR1, HSP90AA1, STAT-3, SRC, IL-6, HIF1A, JUN, and BCL2 in the descending order (Figure 4A). These 10 targets may be the main targets that Thyme oil phytochemical constituents can modulate to prevent breast cancer. The relationships between the target genes and other genes in the network were further explored by using the GeneMANIA tool. The results show that the interactions in the network have different weights based on their types. The most common types of interactions were physical interactions (64.18%), coexpression (16.34%), genetic interactions (6.89%), colocalization (6.88%), and predicted (5.20%) (Figure 4B).

Functional and Pathway Enrichment Analysis. The identified hug-genes were further explored to understand their
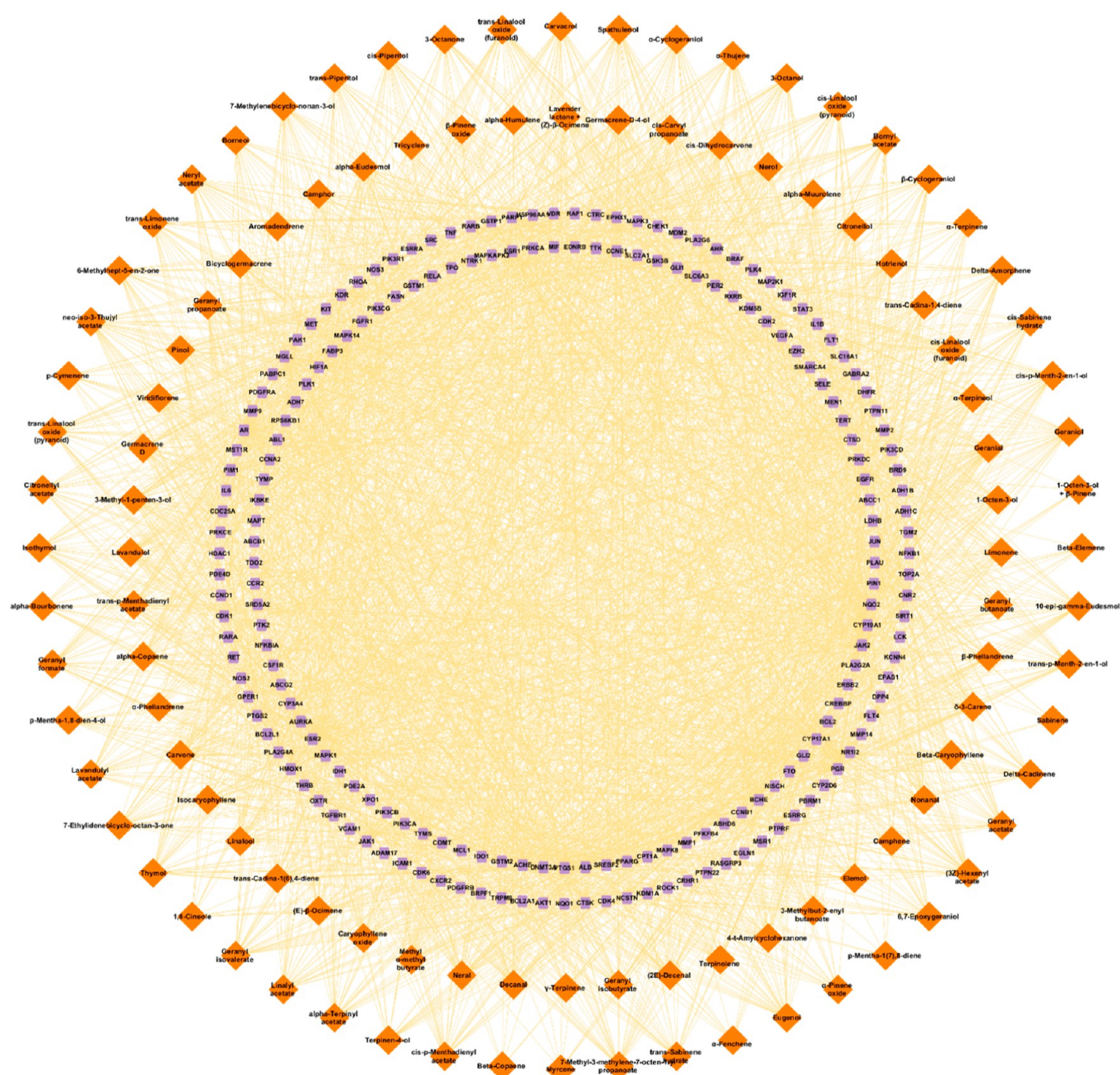
Figure 3. Common-protein targets for phytochemical constituents of Thyme oil (orange diamonds) and their associations (light yellow edges) with the genes involved in breast cancer (lavender square shape) were visualized using Cytoscape software.

functions and pathways, as well as how they are involved in the disease process via the DAVID database to gain more insights into the general mechanism of the disease. The GO annotations showed various GO enrichment terms related to the target genes. There were 1772 BPs, 81 CCs, and 108 MFs that were identified. The top 10 enriched terms for each GO category (Figure 5A–C) and each KEGG pathway (Figure 5D) were shown using a bubble chart. The identified targets were found to be associated with different biological processes such as cellular response to oxidative stress, epithelial cell proliferation, regulation of reactive oxygen species metabolic process, cellular response to chemical stress, positive regulation of peptidyl–serine phosphorylation via cellular components like transcription regulator complex, nuclear membrane, RNA polymerase II transcription regulator complex, vesicle lumen,

endocytic vesicle, dendrite terminus, and in molecular functions like ubiquitin protein ligase binding, nuclear hormone receptor binding, protein phosphatase binding, ATPase binding, RNA polymerase II specific DNA binding transcription factor binding. There were 154 KEGG pathways associated with these genes. Some of the most significantly enriched pathways were EGFR tyrosine kinase inhibitor resistance, prolactin signaling pathway, HIF-1 signaling pathway, estrogen signaling pathway, ErbB signaling pathway, AGE-RAGE signaling pathway, JAK-STAT signaling pathway, FoxO signaling pathway, PI3K-Akt signaling pathway, pathways in cancer, and C-type lectin receptor signaling pathway. Therefore, multiple signaling pathways in breast cancer can be modulated by the phytochemicals of Thyme oil, as per the obtained results.
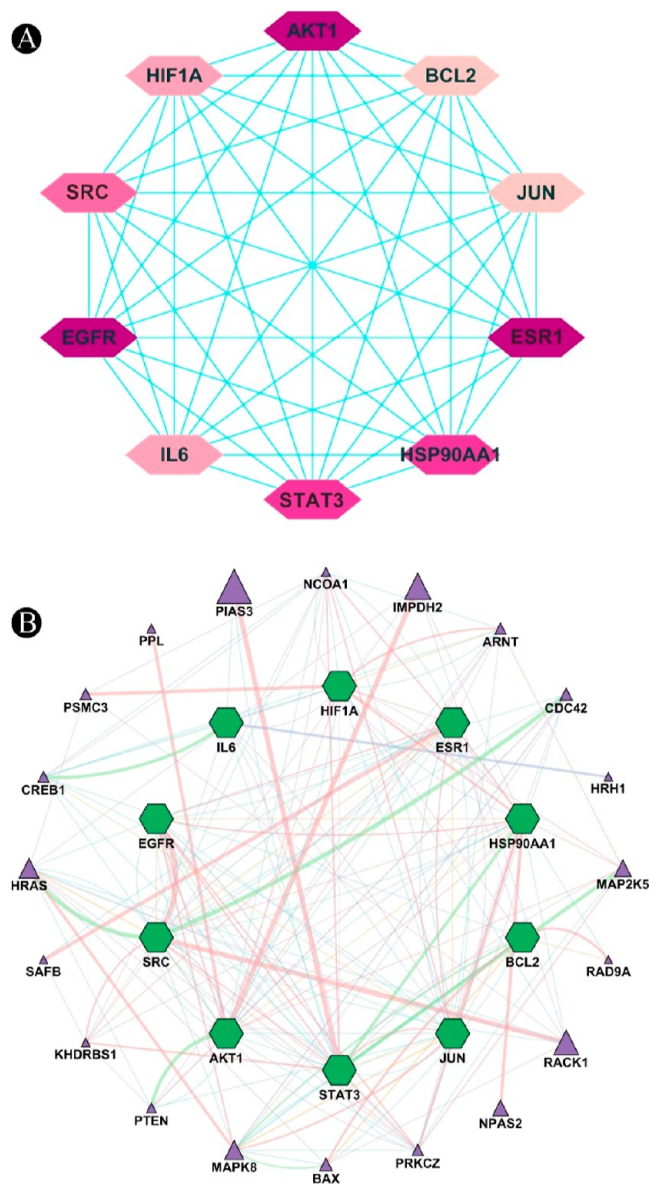
Figure 4. (A) Hub-genes in the PPI network were determined from the genes that were targeted by both the bioactives of Thyme oil and breast cancer. (B) Functional association of the hub-genes against breast cancer was examined by GeneMANIA. The connecting lines with various colors showed different types of correlations. The nodes on the outer ring indicated the genes that were associated with the query genes. The inner ring displayed the genes that were used as search terms to retrieve relevant information.

Molecular Docking Analysis. The molecular docking analysis of phytochemical constituents of Thyme oil against breast cancer targets is shown in Table S4. The interaction strength between the phytochemicals and target proteins is indicated by the binding energies. The interaction is stronger when the binding energy is lower. High affinity for their respective target proteins was shown by some phytochemicals in the docking analysis. The results of aromadendrene exhibited highest binding energy toward EGFR (−7.7 kcal/mol) showing eight alkyl bond (2*ALA743, LYS745, 2*VAL726, 2*LEU844, and MET766) and one π-alkyl bond (PHE723), α-humulene exhibited highest binding energy toward ESR1 (−8.4 kcal/mol) showing nine alkyl bonds (ALA350, LEU384, LEU391, LEU387, MET388, LEU391,

2*LEU346, and MET421), and viridiflorene exhibited highest binding energy toward AKT1 (−8.9 kcal/mol) showing nine alkyl bonds (2*LYS268, 2*VAL270, 3*LEU264, LEU210, and LYS268) and six π-alkyl bond (5*TRP80 and TYR272). The interaction analysis of phytochemical constituents of Thyme oil to target proteins is presented in Figures 6−8 and Table 1.

MD Simulation Analysis. MD simulation analysis was carried out to determine the stability and flexibility of the ligand−protein complexes of aromadendrene−EGFR, viridiflorene−AKT1, and α-humulene−ESR1. GROMACS software was used to perform the analysis in 100 ns. RMSD analysis was used to examine the structural deviation of proteins and ligand−protein complexes. The structural deviations of EGFR, AKT1, and ESR1 and their complexes with aromadendrene, viridiflorene, and α-humulene were investigated in the solvent environment during the simulation to determine their stability and movement. The simulation results showed that the RMSD values of the backbone of EGFR and its complex with aromadendrene were stable with little fluctuation in between (Figure 9A). The average RMSD of EGFR and its complex with aromadendrene were 0.50 and 0.66 nm, respectively. The RMSD values of the backbone of AKT1 and its complex with viridiflorene were stable with little fluctuation at 80−85 ns (Figure 10A). The average RMSD of AKT1 and its complex with viridiflorene were 0.44 and 0.37 nm, respectively. The RMSD values of the backbone of ESR1 and its complex with α-humulene were stable with little initial fluctuation at 0−10 and 90−95 ns (Figure 11A). The average RMSD of ESR1 and its complex with α-humulene were 0.44 and 0.37 nm, respectively. The distribution of the RMSD pattern did not show significant shifts in all protein ligand complexes during the simulation, which suggested that the complexes of ESR1−α-humulene, AKT1−viridiflorene, and EGFR−aromadendrene were stable with strong ligand-binding strength. The flexibility of each residue in a protein is indicated by the RMSF. The average fluctuation during the simulation was 0.15 nm for EGFR−aromadendrene (Figure 9B), 0.13 nm for AKT1−viridiflorene (Figure 10B), and 0.14 nm for the ESR1−α-humulene complex (Figure 11B). The fluctuations were stable and minimized after the binding of aromadendrene, viridiflorene, and α-humulene. The graph shows that EGFR, AKT1, and ESR1 interacted with remarkable constancy with aromadendrene, viridiflorene, and α-humulene, respectively. H-bonds are important for the stability and integrity of the protein structures. The time evolution of the formation and breakdown of H-bonds during the simulation time was examined to assess the structural integrity and stability of protein ligand complexes. The intramolecular hydrogen bonds formed within the docked complexes of EGFR-aromadendrene, AKT1-viridiflorene, and ESR1−α-humulene promoted the stability of the protein and its ligand. The average number of H-bonds was 189.03 for the EGFR−aromadendrene docked complex (Figure 9C), 261.77 for AKT1−viridiflorene (Figure 10C), and 196.44 for ESR1−α-humulene (Figure 11C). The time evolution of the H-bonds was examined during the simulation process. The compactness of protein molecules can be calculated from the molecular stability. The compactness measured in MD simulations is called R_g. The tertiary structure of a protein can be examined by using the compactness of a protein structure. The compactness of EGFR, AKT1, and ESR1 after aromadendrene, viridiflorene, and α-humulene binding was assessed using R_g values. The average R_g value was 1.98 for EGFR-aromadendrene (Figure 9D), 2.17 for AKT1−
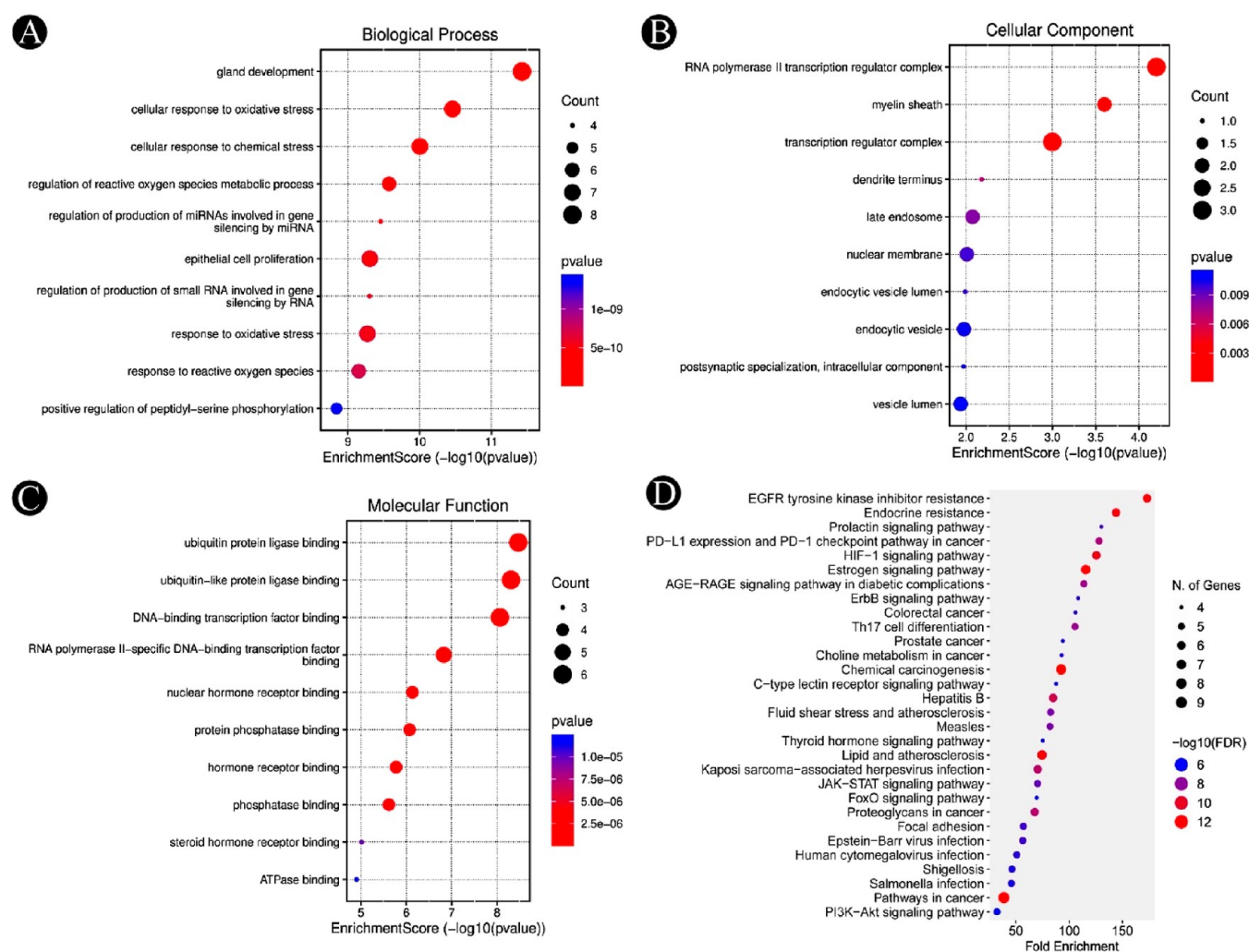
Figure 5. Hub-target proteins were subjected to GO enrichment and KEGG pathway analyses (p-value ≤ 0.05). (A) Top 10 biological processes that were involved, (B) top 10 cellular components that were involved, (C) top 10 molecular functions that were involved, and (D) top 20 KEGG pathways. The colors show the p-values for each term, with lower p-values having darker colors. The dot sizes show the number of genes that are associated with each term, with more genes having larger dots.

viridiflorene (Figure 10D), and 1.87 nm for ESR1–α-humulene (Figure 11D) complex. The R_g plot indicates that the protein–ligand complex remained compact throughout the simulation without any significant changes. SASA is the surface area of a protein molecule that is accessible to the neighboring solvent. SASA analysis is widely used to examine protein folding or unfolding and structural stability during simulations. There were no major peaks in SASA values based on the simulation, indicating that aromadendrene, viridiflorene, and α-humulene binding did not affect EGFR, AKT1, and ESR1 folding behavior. The average SASA value was 151.22 nm^2 for EGFR–aromadendrene (Figure 9E), 192.34 nm^2 for AKT1–viridiflorene (Figure 10E), and 132.87 nm^2 for ESR1–α-humulene (Figure 11E). SASA values showed that EGFR, AKT1, and ESR1 remained stable in the presence of aromadendrene, viridiflorene, and α-humulene.

Anticancer Activity of Thyme Oil. The MTT assay was used to measure the anticancer activity of Thyme oil against MCF-7 breast cancer cells. The viability of breast cancer cells was reduced by Thyme oil in a concentration-dependent manner, as the results indicated. The concentration of Thyme oil was higher when the viability of breast cancer cells was lower. The IC$_{50}$ value of Thyme oil against MCF-7 breast

cancer cells was 365.37 μg/mL, which indicated that this concentration of Thyme oil decreased the viability of breast cancer cells by 50% (Figure 12).

Effect of Thyme Oil on Wound Closure. The effect of Thyme oil on wound healing was tested on MCF-7 cells, which indicated the influence on cell proliferation and migration. The IC$_{50}$ concentration of Thyme oil which was derived from the MTT assay was used. The scratch assay measured the ability of the cells to fill a gap in a cell monolayer that was created by scratching in the presence or absence of Thyme oil. Figure 13 shows that wound closure was significantly inhibited by Thyme oil compared to the control group. The gap was progressively closed by the control group of untreated MCF-7 cancer cells after 19 and 44 h of scratching, whereas the IC$_{50}$ concentration of Thyme oil prevented the closure of the gap by blocking the movement of MCF-7 cancer cells. These results suggest that Thyme oil has an antiproliferative effect.

Discussion. Various natural products have demonstrated potential cancer treatments due to their beneficial effects.[48] Among these, terpenoids, a diverse group of compounds, including monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, and triterpenes, have shown anticancer activities such as inhibiting cancer cell growth and preventing their spread.[49−53]
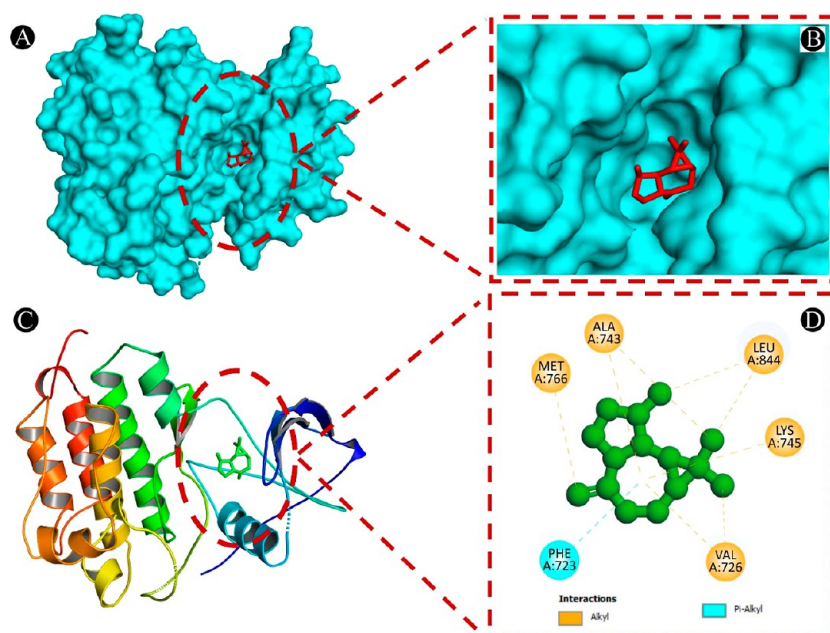
Figure 6. Binding mode of the EGFR protein with aromadendrene. (A) Hydrophobicity surface 3D representation of aromadendrene with EGFR protein, (B) enlarged view of the hydrophobicity surface 3D representation of aromadendrene with EGFR protein, (C) 3D interactions of aromadendrene with EGFR protein, and (D) 2D interactions of aromadendrene with the EGFR protein.
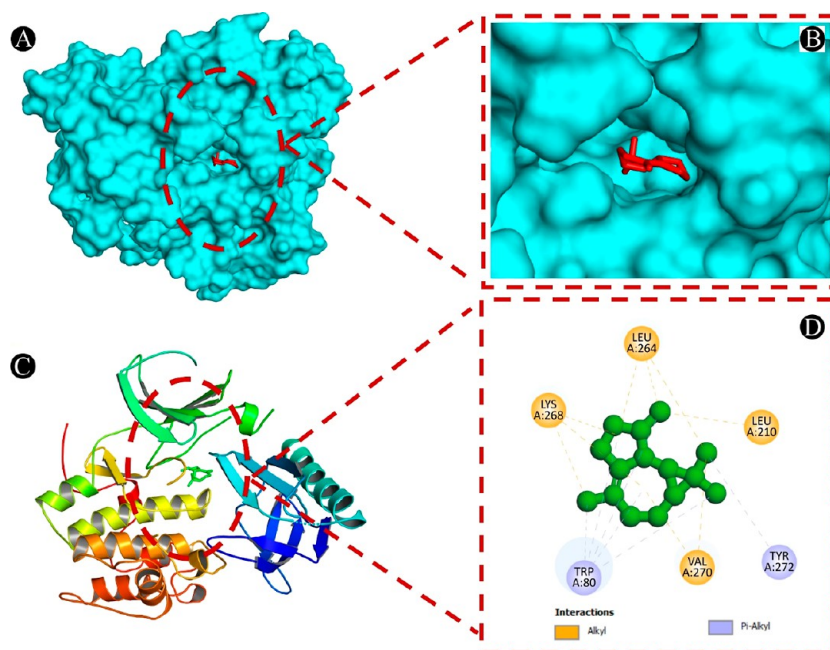

Figure 7. Binding mode of the AKT1 protein with viridiflorene. (A) Hydrophobicity surface 3D representation of viridiflorene with AKT1 protein, (B) enlarged view of the hydrophobicity surface 3D representation of viridiflorene with AKT1 protein, (C) 3D interactions of viridiflorene with AKT1 protein, and (D) 2D interactions of viridiflorene with AKT1 protein.

Compounds like artemisinin, thapsigargin, and parthenolide derived from natural sources are undergoing clinical trials to evaluate their potential as cancer drugs.[54] Thyme oil primarily contains oxygenated monoterpenes and monoterpene hydrocarbons, with thymol, carvacrol, *p*-cymene, borneol, *trans*-caryophyllene, and *cis*-sabinene hydrate as the major compounds.[14,15] The present study aimed to identify cancer-related targets and pathways of Thyme oil and its phytochemical constituents using network pharmacology-based analysis. In addition, we confirmed the anticancer

potential of Thyme oil against breast cancer cell lines via assessing cytotoxicity and antiproliferative activity in vitro.

We collected phytochemical data on Thyme oil from various sources, resulting in 107 compounds meeting our criteria. Target identification was carried out by using the SwissTarget-Prediction database. We then mapped these targets to breast cancer-related genes from OMIM, DisGeNET, and Gene-Cards, yielding 195 common targets. Functional enrichment analysis using the DAVID database revealed their association with various biological aspects and pathways related to breast
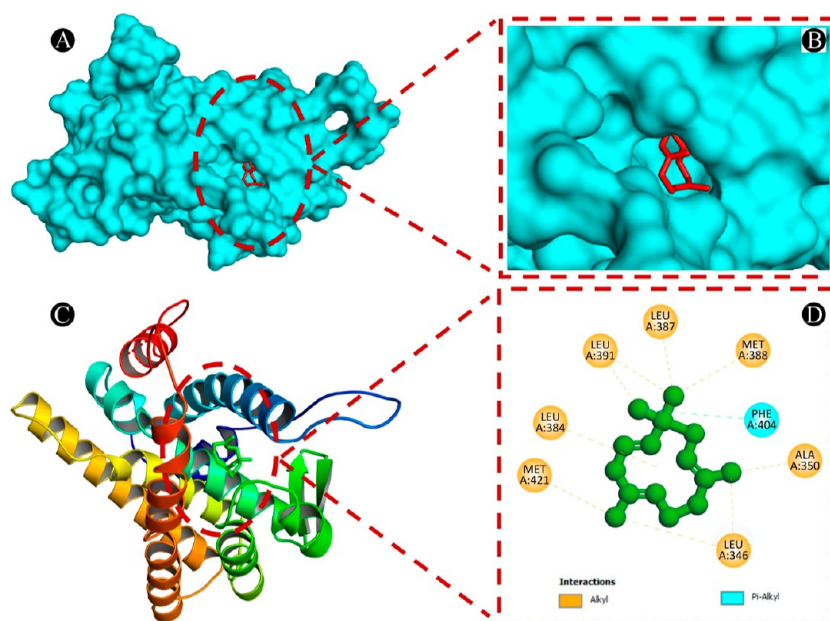
Figure 8. Binding mode of the ESR1 protein with α-humulene. (A) Hydrophobicity surface 3D representation of α-humulene with ESR1 protein, (B) enlarged view of the hydrophobicity surface 3D representation of α-humulene with ESR1 protein, (C) 3D interactions of α-humulene with ESR1 protein, and (D) 2D interactions of α-humulene with ESR1 protein.

cancer and other cancers. GO enrichment analysis highlighted their involvement in cellular response to oxidative stress, epithelial cell proliferation, regulation of reactive oxygen species metabolic process, cellular response to chemical stress, positive regulation of peptidyl—serine phosphorylation in cellular components like transcription regulator complex, nuclear membrane, RNA polymerase II transcription regulator complex, vesicle lumen, endocytic vesicle, dendrite terminus via molecular functions of ubiquitin protein ligase binding, nuclear hormone receptor binding, protein phosphatase binding, ATPase binding, and RNA polymerase II-specific DNA-transcription factor binding. Pathway analysis indicated connections with signaling pathways such as EGFR tyrosine kinase inhibitor resistance, prolactin signaling pathway, HIF-1 signaling pathway, estrogen signaling pathway, ERBB signaling pathway, AGE-RAGE signaling pathway, JAK-STAT signaling pathway, FoxO signaling pathway, PI3K-AKT signaling pathway, pathways in cancer, and C-type lectin receptor signaling pathway. These results suggest Thyme oil's potential in modulating multiple cancer-related pathways.

The top 10 targets included EGFR, AKT1, ESR1, HSP90AA1, STAT-3, SRC, IL-6, HIF1A, JUN, and BCL2 with EGFR, AKT1, and ESR1 being the top three highly involved in breast cancer. HER family is a group of receptor tyrosine kinases that includes EGFR, HER2, HER3, and HER4. These receptors are activated by ligand binding or heterodimerization with other members of the family. EGFR signaling regulates different cellular activities, such as cell proliferation, differentiation, migration, survival, and angiogenesis. It is overexpressed or amplified in about 15% of breast cancers, especially in the basal-like subtype.[55] EGFR overexpression is associated with poor prognosis, aggressive behavior, high grade, high proliferation rate, low hormone receptor expression, high HER2 expression or coamplification, and resistance to endocrine therapy and chemotherapy.[56,57] EGFR signaling can interact with ER signaling in breast cancer cells through various mechanisms. For example, EGFR can

phosphorylate ER at serine residues and modulate its transcriptional activity. EGFR can also induce the expression of ER coregulators or target genes. Moreover, EGFR can activate downstream pathways, such as MAPK or PI3K/AKT that can affect ER function or expression. Therefore, a therapeutic strategy for breast cancer has been to block EGFR signaling.[58]

The AKT family is a group of protein kinases that includes AKT1, AKT2, and AKT3. These proteins are associated with various biological processes such as cell survival, growth, metabolism, and migration. AKT1 is activated by the PI3K pathway, which is often deregulated in breast cancer due to mutations or amplifications of genes such as PIK3CA, PTEN, and AKT1.[59] Some studies have shown that AKT1 promotes breast tumor growth via enhancing cell proliferation and inhibiting apoptosis through the regulation of downstream targets such as S6 and cyclin D1.[59] Various aspects of tumor biology, such as growth, survival, metabolism, and resistance to treatment are influenced by the PI3K/AKT/mTOR pathway, which is a key target for breast cancer therapy.[60] However, the clinical efficacy of PI3K/AKT/mTOR inhibitors has been limited by several factors, such as heterogeneity, toxicity, feedback mechanisms, and crosstalk with other pathways.[61] Therefore, a better understanding of the specific roles and interactions of AKT isoforms in breast cancer may help in the development of more effective and personalized therapeutic strategies.

ESR1 is the gene that encodes the ER, a nuclear hormone receptor that mediates the activity of estrogen on breast cancer cells. Cell proliferation, differentiation, survival, and metabolism are affected by the expression of genes that are regulated by ER signaling.[62] ER is expressed in about 70% of breast cancers, and it is the most important prognostic and predictive factor for breast cancer patients. The production or action of estrogen on ER is blocked by endocrine therapy, which is a common treatment for ER-positive breast cancers.[63] Endocrine therapy consists of different types of drugs, such as aromatase

Table 1. Residues in the Target Proteins That Interact with the Phytochemical Constituents of Thyme Oil in Their Best-Fitting Pose

sr. no.	protein–compound	receptor–ligand	interaction type	distance
1	EGFR–aromadendrene	A:ALA743–N:UNK1	alkyl	5.31091
		A:ALA743–N:UNK1:C	alkyl	4.15241
		A:LYS745–N:UNK1	alkyl	4.81135
		N:UNK1:C–A:VAL726	alkyl	3.94245
		A:VAL726–N:UNK1	alkyl	4.84469
		N:UNK1:C–A:LEU844	alkyl	4.2384
		N:UNK1:C–A:LEU844	alkyl	4.36812
		N:UNK1:C–A:MET766	alkyl	5.14297
		A:PHE723–N:UNK1	π-alkyl	5.02553
2	AKT1–viridiflorine	A:LYS268–N:UNK1	alkyl	5.3074
		A:LYS268–N:UNK1	alkyl	4.81375
		A:VAL270–N:UNK1	alkyl	4.23575
		N:UNK1–A:LEU264	alkyl	5.3
		N:UNK1:C–A:LEU264	alkyl	4.3305
		N:UNK1:C–A:VAL270	alkyl	4.89921
		N:UNK1:C–A:LEU210	alkyl	4.49229
		N:UNK1:C–A:LEU264	alkyl	4.6689
		N:UNK1:C–A:LYS268	alkyl	3.87121
		A:TRP80–N:UNK1	π-alkyl	4.23846
		A:TRP80–N:UNK1	π-alkyl	4.09405
		A:TRP80–N:UNK1:C	π-alkyl	4.47542
		A:TRP80–N:UNK1	π-alkyl	4.81195
		A:TRP80–N:UNK1:C	π-alkyl	4.55521
		A:TYR272–N:UNK1:C	π-alkyl	4.84716
3	ESR1–α-humulene	A:ALA350–N:UNK1:C	alkyl	3.98765
		A:LEU384–N:UNK1	alkyl	5.32872
		N:UNK1:C–A:LEU391	alkyl	4.014
		N:UNK1:C–A:LEU387	alkyl	4.5872
		N:UNK1:C–A:MET388	alkyl	4.93984
		N:UNK1:C–A:LEU391	alkyl	4.26753
		N:UNK1:C–A:LEU346	alkyl	4.46104
		N:UNK1:C–A:LEU346	alkyl	4.71348
		N:UNK1:C–A:MET421	alkyl	4.20137
		A:PHE404–N:UNK1:C	π-alkyl	4.89694

inhibitors, selective estrogen receptor modulators, selective estrogen receptor downregulators, and estrogen receptor degraders. Patients with ER-positive breast cancer can benefit from endocrine therapy as it can improve their survival and quality of life significantly.[64]

In silico docking analysis revealed promising interactions between the 107 compounds and the top three targets. In the docking analysis, aromadendrene exhibited high binding energy against EGFR (−7.7 kcal/mol) through eight alkyl bonds and one π-alkyl bond; α-humulene showed good binding energy toward ESR1 with nine alkyl bonds, whereas viridiflorene showed good binding energy toward AKT1 with nine alkyl bonds and six π-alkyl bonds. MD simulations confirmed the stability of these interactions. The MD results were stable, with minor variations throughout the whole simulation period, as indicated by the RMSD plots. The RMSF graphs showed that the binding sites of all proteins had high flexibility to accommodate the ligand. The R_g plots revealed that all proteins stayed compact during the simulation. Aromadendrene is a plant-derived sesquiterpene that has anti-inflammatory, antioxidant, antibacterial, antidepressant, and anticancer properties, according to some reports. It is found in various plants, such as eucalyptus, guava, pineapple, and cannabis.[65,66] A study by Pavithra et al.[67] found that aromadendrene oxide 2, a derivative of aromadendrene, induced apoptosis (programmed cell death) in skin epidermoid cancer (A431) and precancerous (HaCaT) cells by activating caspases (enzymes that mediate apoptosis) and enhancing Bcl-2 family proteins expression (proteins that regulate apoptosis). The expression of MMPs, which are enzymes that break down the extracellular matrix and enable cancer metastasis, was suppressed by aromadendrene oxide 2, which also inhibited the migration and invasion of A431 cells. α-Humulene is a sesquiterpene that is found in various plants, such as hops, cannabis, sage, and ginger.[68] The reported properties of this compound include anti-inflammatory, antibacterial, antifungal, and anticancer effects. The anticancer activity of α-humulene on various types of cancer cells has been reported by modulating different molecular pathways involved in cell cycle regulation, apoptosis, inflammation, and angiogenesis.[69,70] Viridiflorol is a natural compound of the sesquiterpene class. It has various biological activities, such as killing cancer cells, reducing inflammation, scavenging free radicals, and fighting microbes.[71] The essential oil of a plant called *Senecio rowleyanus* has a high amount of viridiflorol (11%), and it can destroy brain cancer cells (U251) very effectively. Another plant that has viridiflorol in its essential oil (4.1%) is *Salvia leriifolia*. This plant's oil was tested toward various human cancer cells, such as lung, skin, kidney, prostate, and breast cancer cells, as well as normal human skin cells (142BR). The oil showed strong bioactivity against most of the cancer cells, and it was more powerful than the isolated viridiflorol alone.[72] Thus, targeting proteins identified via network pharmacology analysis with these compounds can be effective in the treatment of breast cancer.

Experimental results demonstrated that Thyme oil inhibited the growth and proliferation of breast cancer cells, with lower IC_{50} values, indicating its potential to interfere with cancer cell development. This may be because the compounds in Thyme oil had stronger interactions with proteins or pathways that are involved in tumor development. There are many reported studies that support the anticancer potential of Thyme oil and its components toward various types of cancer cells including
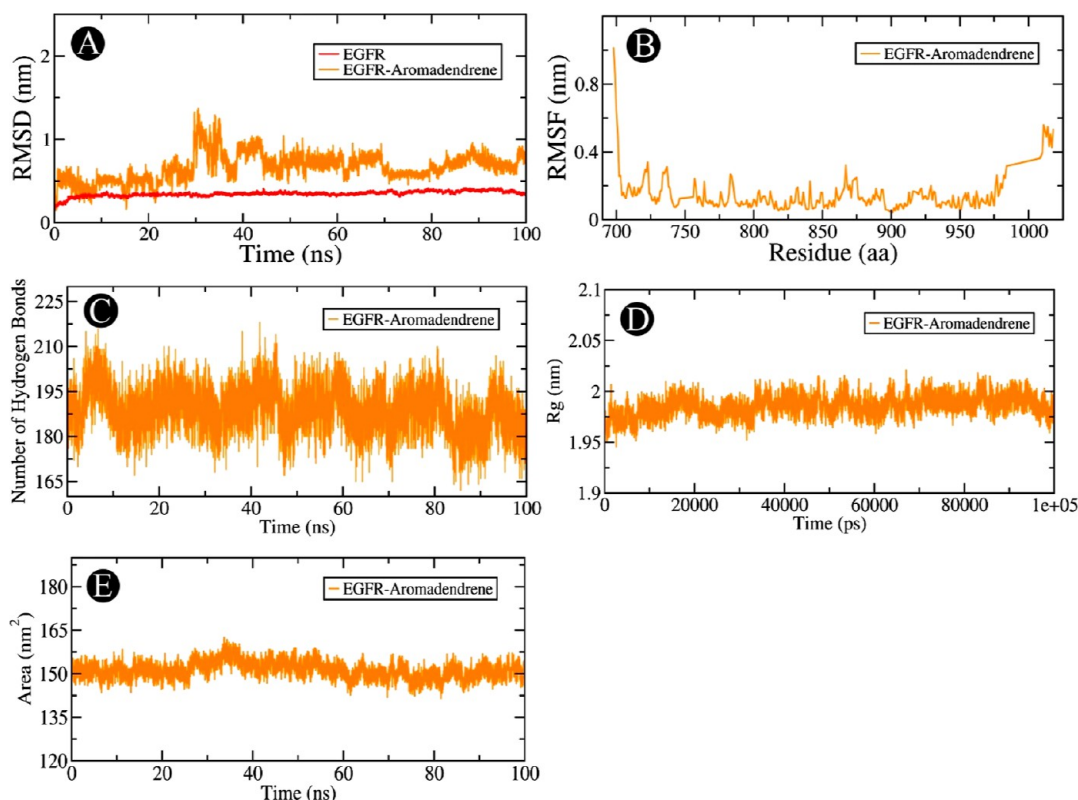
Figure 9. Molecular dynamics simulation analysis of the EGFR protein and aromadendrene molecule over time. (A) RMSD analysis of EGFR with and without aromadendrene binding, (B) RMSF analysis of the EGFR−aromadendrene complex, (C) EGFR−aromadendrene complex intramolecular H-bond time evolution, (D) R_g distribution of the EGFR−aromadendrene complex, and (E) SASA plot analysis of the EGFR−aromadendrene complex.
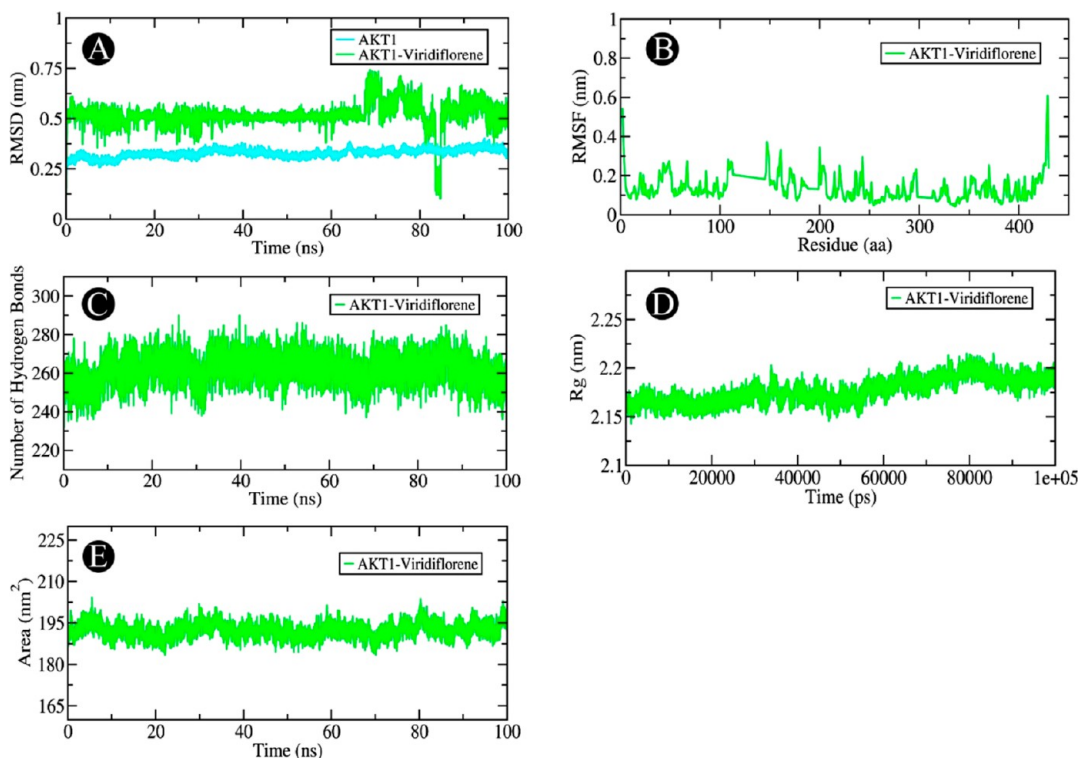

Figure 10. Molecular dynamics simulation analysis of the AKT1 protein and viridiflorene molecule over time. (A) RMSD analysis of AKT1 with and without viridiflorene binding, (B) RMSF analysis of the AKT1−viridiflorene complex, (C) AKT1−viridiflorene complex intramolecular H-bond, (D) R_g distribution of the AKT1−viridiflorene complex, and (E) SASA plot analysis of the AKT1−viridiflorene complex.
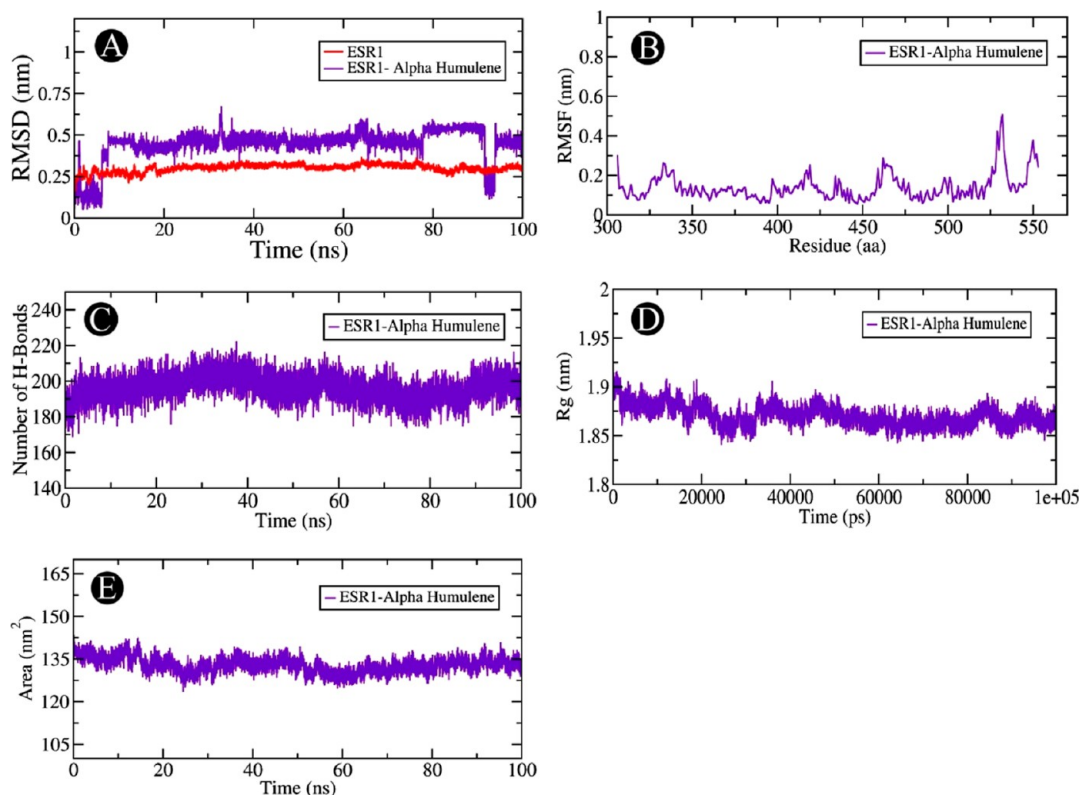
Figure 11. Molecular dynamics simulation analysis of the ESR1 protein and α-humulene molecule over time. (A) RMSD analysis of ESR1 with and without α-humulene binding, (B) RMSF analysis of the ESR1−α-humulene complex, (C) ESR1−α-humulene complex intramolecular H-bond, (D) R_g distribution of the ESR1−α-humulene complex, and (E) SASA plot analysis of the ESR1−α-humulene complex.
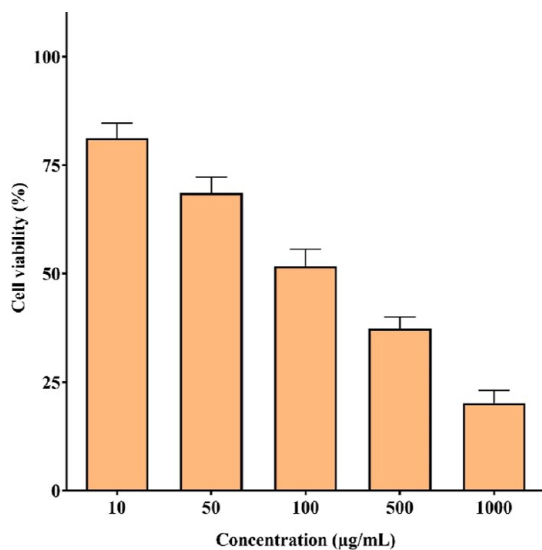

Figure 12. Effect of Thyme oil on the viability of MCF-7 cells determined via MTT assay. The cells were treated with different concentrations of Thyme oil for 24 h. Values are presented as the mean ± SD of three independent experiments.

lung, oral, ovarian, breast, colon, prostate, and leukemia. The mechanisms of action may involve inducing apoptosis (programmed cell death), inhibiting cell proliferation, modulating cell signaling pathways, and enhancing the immune system.[73−77] The potential to inhibit metastasis, a critical aspect of cancer,[78,79] was also explored. Thyme oil showed antiproliferative activity, suggesting its potential in preventing

metastasis. Overall, this study presents Thyme oil and its phytochemical constituents as potential natural antibreast cancer agents. More research and clinical trials are needed to further explore the therapeutic benefits and mechanisms of action. Thyme oil may offer new and effective treatments for breast cancer, providing hope for improved patient outcomes in the battle against this deadly disease.

■ CONCLUSIONS

The present study has demonstrated that Thyme oil, derived from *T. vulgaris*, possesses a promising antibreast cancer potential. Through an integrated approach involving network pharmacology, molecular docking, molecular dynamics, and in vitro experiments, we have identified 107 active compounds within Thyme oil that target 195 proteins associated with breast cancer. Furthermore, our analysis revealed that Thyme oil can modulate critical pathways, such as EGFR tyrosine kinase inhibitor resistance, prolactin signaling pathway, HIF-1 signaling pathway, estrogen signaling pathway, ERBB signaling pathway, AGE-RAGE signaling pathway, JAK-STAT signaling pathway, FoxO signaling pathway, PI3K-AKT signaling pathway, pathways in cancer, and C-type lectin receptor signaling pathway, all of which are implicated in breast and other cancers. Our molecular docking and dynamics simulations have provided strong evidence of the binding stability and affinity of key compounds, namely, aromadendrene, α-humulene, and viridiflorene to essential targets like EGFR, ESR1, and AKT1. The in vitro experiments further confirmed the efficacy of Thyme oil in inhibiting growth and proliferation of MCF-7 breast cancer cells at very low IC_{50} values. Therefore, these findings highlight the potential of
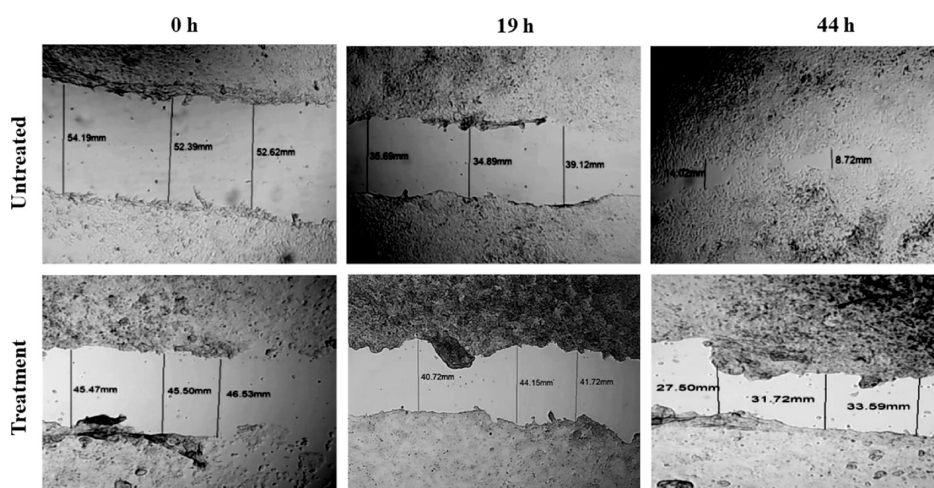
Figure 13. Wound closure assay of MCF-7 cells with and without treatment with IC_{50} concentration of Thyme oil. The cells were observed at 0, 19, and 44 h of incubation.

Thyme oil as a natural product with antibreast cancer properties. However, we acknowledge the need for further investigation, including animal models and clinical trials to translate these results into practical treatments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07782.

Phytochemical constituents of Thyme oil and their ADMET analysis (chemical absorption, distribution, metabolism, excretion, and toxicity); topological parameters of phytochemical constituents of Thyme oil; topological parameters of common genes related to Thyme oil and breast cancer; and binding affinity of the top-rated pose of a protein−ligand complex obtained after molecular docking analysis (PDF)

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