Anticancer activity of eugenol from clove (Syzygium aromaticum L.) against MCF-7 breast cancer cells: in silico and in vitro evaluation

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SUMMARY

Breast cancer is a major cause of cancer-related deaths among women globally. Despite notable progress in the treatment options, the pursuit of effective natural-based therapeutic alternatives continues to be essential. Syzygium aromaticum L. (cloves) is rich in eugenol, a phenolic compound known for its antimicrobial, anti-inflammatory, and anticancer effects. This study aimed to assess the anticancer activity of eugenol extracted from cloves against MCF-7 breast cancer cells using in silico molecular docking and in vitro cytotoxicity assays. The cloves collected from Natuna District and Magetan District (Indonesia) were extracted through Soxhlet extraction with n-hexane and maceration with ethanol. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified eugenol as the primary component, making up 53.6% of the Soxhlet extract. The molecular docking results indicated that eugenol has significant binding affinities for AKT (-5.1kcal/mol) and ESR1 (-5.9kcal/mol), two essential proteins involved in the proliferation and survival of breast cancer cells. Cytotoxicity assessments using the MTT assay revealed a dose-dependent decrease in MCF-7 cell viability, with notable inhibition occurring at concentrations between 15.625 and 2.000ppm. These results suggest that the eugenol-rich clove extract demonstrates promising anticancer potential by exerting direct cytotoxic effects and influencing key molecular targets within the breast cancer signalling pathway.

Keywords: *eugenol, Syzygium aromaticum L., MCF-7, breast cancer, cytotoxicity, molecular docking*

INTRODUCTION

Breast cancer is one of the most prevalent and deadly malignancies affecting women worldwide. According to the World Health Organization (WHO), in 2020 there were approximately 2.3 million new cases of breast cancer and 685,000 deaths globally, making it the most common cancer in women and a leading cause of cancer-related mortality. Although breast cancer predominantly affects females, about 0.5–1% of all cases also occur in men (1,2). The increasing incidence in developed countries has been attributed to lifestyle factors, such as diet, reduced physical activity, reproductive patterns, alcohol consumption, and obesity (3). The risk factor of breast cancer involves both modifiable risk factors such as diet, alcohol consumption, physical inactivity, and non-modifiable factors such as age, genetics and radiation exposure (4–6).

Breast cancer progression is strongly influenced by the overexpression of estrogen receptors (ER/ESR1), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2), which are critical biomarkers and therapeutic targets for the disease (3,7). The PI3K/AKT pathway, particularly AKT1 pathway, plays a pivotal role in cell proliferation, survival, and resistance to therapy (8,9). The abnormal activation of this pathway occurs approximately 70% of breast cancer cases and is recognized as a significant factor contributing to resistance against treatments (8,10,11). Similarly, ESR1 serves as a key transcriptional regulator in hormone-dependent tumours, with its signalling playing a crucial role in cell proliferation and survival within the Luminal A subtype of breast cancer (12). Breast cancer is a heterogeneous disease classified based on the molecular subtype and receptor status. The most common classifications include Luminal A and B (ER+ and/or PR+, HER2−), HER2-enriched, and Triple-Negative Breast Cancer (TNBC). Luminal A tumors express estrogen receptors (ER) and/or progesterone receptors (PR) and show low proliferation activity, while Luminal B tumors tend to be more aggressive with higher Ki-67 levels (13,14). HER2-enriched tumors overexpress human epidermal growth factor receptor 2 (HER2), which leads to increased cell growth and genomic instability(15). TNBC lacks ER, PR, and HER2 expression, making it difficult to treat with hormone therapy and leading to a poorer prognosis (15).

The MCF-7 cell line, used extensively as an in vitro model in breast cancer research, is classified under Luminal A subtype and exhibits high ER and PR expression, making it suitable for evaluating the effects of hormone-targeted compounds (13,16). These cells are also characterized by their responsiveness to estrogen signaling and anti-estrogen drugs like Tamoxifen (13,17). Consequently, the utilization of this cell line is particularly pertinent to our research, which seeks to explore the interaction between eugenol and ESR1, as well as its effects on hormone-dependent cancer cells (12). One major hallmark of cancer is the evasion of apoptosis, which allows tumor cells to proliferate uncontrollably (18).

Eugenol, a natural phenolic compound extracted from clove (*Syzygium aromaticum L.*), has shown promising anticancer properties. Clove, a plant abundantly cultivated in Indonesia, contains eugenol at high concentrations ranging from 9,281.70 to 14,650.00mg/100g dry weight

(19–21) making it one of the most potent phytochemicals with reported antioxidant, anti-inflammatory, analgesic, and cytotoxic activities (20). Recent studies have shown that eugenol can induce apoptosis, arrest the cell cycle, inhibit angiogenesis and metastasis and modulate key signalling pathways in various cancer types including breast cancer (18). Despite its promising pharmacological effects, the molecular mechanism of eugenol, particularly its interaction with breast cancer-related proteins such as AKT1 and ESR1, remains underexplored. Furthermore, the cytotoxic potential of eugenol-rich clove extracts against hormone-dependent breast cancer cell lines such as MCF-7 has not been fully elucidated in a comparative in silico and *in vitro* model (18).

Therefore, this study aimed to investigate the anticancer potential of eugenol extracted from clove using Soxhlet and maceration techniques, by assessing its binding affinity toward AKT1 and ESR1 through molecular docking and evaluating its cytotoxic effect on MCF-7 breast cancer cells via MTT Assay. Through the integration of these in silico and in vitro methodologies, we aimed to clarify the potential of eugenol as a multitarget agent capable of disrupting both the PI3K/AKT and ESR1 signalling pathways, which are essential for the survival of Luminal A breast cancer cells.

METHODS

Sample collection and extraction

Fresh clove buds (*Syzygium aromaticum L.*) were obtained from Natuna (Riau Archipelago) and Magetan (East Java), Indonesia. The samples were dried either under sunlight or in an oven at 40°C and then ground into a fine powder using a blender. Two extraction methods were used: 1) Soxhlet extraction using *n-hexane* (non-polar solvent), with 23g of clove powder and 200mL of solvent for 7 extraction cycles; 2) Maceration using *ethanol* (polar solvent), with 100g of clove powder immersed in 300mL of ethanol, stirred using an orbital shaker for 3 days.

Based on the initial weight of the clove powder, the percentage yield of the extract was calculated. The major compound, eugenol, in each extract was quantified using the GC-MS method. The extracts were evaporated using a rotary evaporator (50°C, 120rpm), followed by oven drying at 40°C. Essential oils were stored in 10mL vials for further analysis.

GC-MS analysis

The chemical composition of the extracts was analyzed using Gas Chromatography–Mass Spectrometry (GC–MS) at the Laboratorium Penelitian dan Pengujian Terpadu (LPPT), Gadjah Mada University. The identification of major compounds was carried out based on their retention times and mass spectra. The analysis was performed under the following instrumental conditions: an HP-SMS UI column (30m $\times$ 0.25mm $\times$ 0.25μm film thickness) was used, with the oven temperature programmed at 325/350°C. Helium UHP (He) served as the carrier gas at a split flow of 50mL/min. The injector temperature was set to 300°C, while the mass spectrometry detector was operated at 280°C with a mass range of 40–500m/z.

In silico molecular docking

The target prediction of bioactive compounds was performed using *SwissTargetPrediction* and filtered for Homo sapiens proteins. The protein targets relevant to breast cancer were cross-referenced using the *Human Protein Atlas database*. The overlapping targets between the clove compounds and MCF-7 proteins were visualized using *Venny 2.1*, and protein-protein interaction networks were constructed using *STRING* and *Cytoscape v3.9.1.* The docking method was validated through the redocking of the native ligand to the active site of its receptor protein. This involved reintroducing the existing crystallographic ligand and the Root Mean Square Deviation (RMSD) was computed between the pose of the redocked ligand and that of the original crystallographic ligand. The docking method was deemed valid if the RMSD value was less than 2.0 Å (22,23). The ligand preparation process involved determining the appropriate protonation state at physiological pH and assigning Gasteiger Charges (24,25). The protein structures were retrieved from *the RCSB PDB*, and ligand structures were prepared using *PubChem*. Molecular docking was conducted using *PyRx 0.8*, *AutoDockTools 1.5.7.* and *UCSF Chimera*, with visualization using *PyMOL* and *Discovery Studio Visualizer*. Binding affinity was recorded in kcal/mol.

Cell cultures

This study was conducted under the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public. The MCF-7 human breast cancer cell line was obtained from the *Laboratorium Riset Terpadu* (LRT), Faculty of Medicine, Public Health, and Nursing, Gadjah Mada University. The cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). Cultures were maintained at 37°C in a 5% CO_2 incubator.

Cytotoxicity assay (MTT assay)

Cell viability was evaluated using the MTT assay (21). MCF-7 cells (1 $\times$ 10^4 cells/well) were seeded into 96-well plates and treated with serial concentrations of clove extract (15.625–2000ppm) for 24h. After incubation, 100μL of a 5mg/mL MTT solution was added to each well, followed by a further 4-hour incubation. Formazan crystals were solubilized using DMSO, and the absorbance was measured at 595nm using an ELISA reader. The control groups included untreated cells, solvent control, doxorubicin (positive control, 0.75 6μg/mL), and media-only wells. The experiments were conducted in triplicate. The IC_{50} value was determined using non-linear regression analysis with Google Collab software.

Statistical analysis

The results are expressed as mean $\pm$SD. One-way ANOVA followed by Tukey's HSD Test was used to determine the statistical significance between the treatment groups. The statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Phytochemical composition of clove extracts

The GC-MS analysis revealed that eugenol was the major compound in both the Soxhlet and maceration extracts. The Soxhlet extract (EC-N) showed a eugenol content of 53.6%, whereas the maceration extract (EC-E) contained 35.4%. This finding is consistent with prior studies that also indicate a high percentage of eugenol in clove extracts, frequently surpassing 50%, depending on the extraction method and solvent employed (20). Other significant compounds include β-caryophyllene, 3-allyl-6-methoxyphenylacetate, and ascorbic acid derivatives, all of which are known for their bioactive properties, including antioxidant and anticancer effects (19,26). The variation in eugenol concentration between the two extraction methods may be attributed to the differences in solvent polarity and extraction efficiency. *n-Hexane*, a non-polar solvent used in Soxhlet extraction, is more effective in isolating hydrophobic compounds such as eugenol. This finding supports the previous studies indicating that the solvent type significantly influences the yield and profile of essential oils in aromatic plants (27).

Table 1. Ten major compounds identified in the Soxhlet clove extract (EC-N).

Rel. area (%)	Compound name
53.60	3-Allyl-6-methoxyphenol
13.67	3-Allyl-6-methoxyphenyl acetate
7.16	cis-Vaccenic acid
6.57	2'3'4',Trimethoxyacetatophenone
3.26	Docosanoic acid, 1,2,3-propanetriyl ester
2.15	Caryophyllene
2.09	1-(+)-Ascorbic acid 2,6-dihexadecanoate
1.77	Caryophyllene oxide
1.43	Ethyl iso-allocholate
0.61	Glycidyl oleate

Table 2. Ten major compounds identified in the Soxhlet clove extract (EC-E).

Rel. area (%)	Compound name
35.4	Eugenol
19.04	cis-13-Octadecenoic acid
14.12	3-Allyl-6-methoxyphenyl acetate
8.3	l-(+)-Ascorbic acid 2,6-dihexadecanoate
5.65	Caryophyllene
2.75	Octadecanoic acid
0.78	Humulene
0.77	Glycidyl oleate
0.67	Palmitoleic acid
0.18	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester

Molecular docking and target prediction

The biological evaluation of eugenol extracted from *Syzygium aromaticum L.* against MCF-7 breast cancer cells was performed through a combination of in silico target prediction and molecular docking, followed by an *in vitro* cytotoxicity assessment. The findings of this study are presented in the following sections.

Target prediction and overlapping proteins

The target prediction of eugenol was conducted using *SwissTargetPrediction*, which yielded a total of 100 predicted human protein targets. These were compared with the list of proteins expressed in MCF-7 cells obtained from the Human Protein Atlas (HPA). The Venn diagram analysis showed that 44 proteins overlapped between the eugenol targets and MCF-7 expressed proteins (Figure 1), indicating the potential interaction sites for eugenol within the cellular environment of breast cancer.

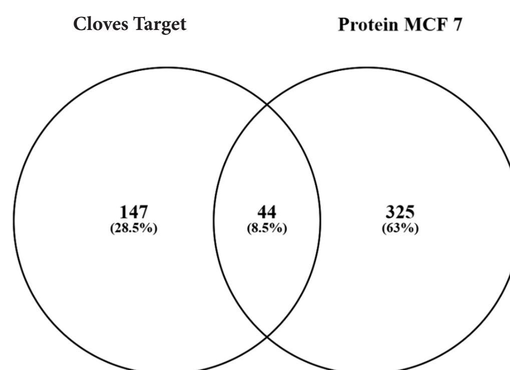

Figure 1. Venn diagram showing the overlapping protein targets between eugenol and MCF-7 expressed proteins groups.

This overlap highlights the significant potential for eugenol to interact with intracellular signaling mechanisms that are active in hormone-dependent breast cancer cells (18,28). Many of these intersecting proteins are involved in key processes, such as cell cycle progression, hormone response, signal transduction, and apoptosis regulation. This observation aligns with the previous reports that eugenol, as a phenolic compound, can modulate multiple oncogenic pathways, including ROS regulation, inhibition of cell cycle, apoptosis induction and suppression of metastasis (8,18). These multitarget properties underscore eugenol's potential to interfere with multiple hallmarks of cancer simultaneously, making it a promising candidate for therapeutic exploration in hormone-dependent breast cancer subtypes such as MCF-7 (16,21).

Protein-protein interaction (PPI) network analysis

The 44 overlapping protein targets were further analyzed using the *STRING* database to explore their functional associations. The resulting interaction data was imported into *Cytoscape* to visualize the protein-protein interaction (PPI) network, where each node represents a target protein, and the edges represent functional or physical interactions.

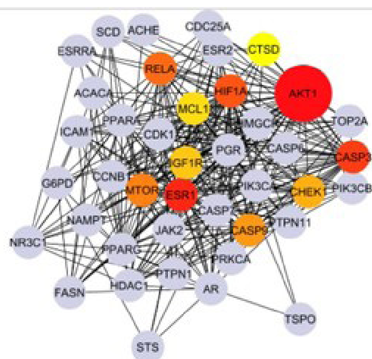

Figure 2. Protein-protein interaction (PPI) network of overlapping targets visualized in *Cytoscape*, highlighting AKT1 and ESR1 as central hub nodes.

Protein–protein interaction (PPI) network analysis was performed to explore further the interconnections among the 44 overlapping proteins between the predicted targets of eugenol and those expressed in MCF-7 cells (21). Using the *STRING* database (confidence score ≥ 0.7) and visualization via *Cytoscape v3.9.1*, a complex network representing the functional relationships of the protein targets potentially modulated by eugenol was generated. Hub gene identification was conducted using the *CytoHubba* plugin, which ranks nodes based on Maximal Clique Centrality (MCC), a topological algorithm that measures the centrality of a node by evaluating the number and density of maximal cliques it participates in. A maximal clique represents the largest subset of proteins, in which each node interacts directly with every other node in the clique. Therefore, proteins with high MCC scores are regarded as critical regulators or bottlenecks for maintaining network integrity (29).

MCC analysis identified AKT1 and ESR1 as the top-ranked hub genes in the network. AKT1, a serine/threonine kinase, is considered a critical kinase in the PI3K/AKT signaling pathway, which controls multiple processes related to oncogenesis, including cell survival, proliferation, metabolism, and resistance to apoptosis. The hyperactivation of AKT1 is frequently observed in breast cancer and contributes to endocrine therapy resistance and metastasis (8,18). On the other hand, ESR1 (estrogen receptor 1), encoding ERα, is a nuclear transcription factor that mediates the expression of estrogen-responsive genes. In luminal breast cancers such as MCF-7, ESR1 functions as a central transcriptional driver, and its expression is critical for hormone-dependent tumour growth and survival (12).

The high centrality of AKT1 and ESR1 within the PPI network reflects their essential roles in both the survival and hormonal signalling axes, underscoring their potential as core targets in breast cancer therapy. Eugenol's predicted interactions with these proteins suggest a dual mechanism of action, interfering with kinase-driven proliferative signals via AKT1 and modulating hormonal responses through partial inhibition or modulation of the ESR1 activity. Moreover, the network also highlighted other regulatory proteins, such as BCL2, which is involved in apoptosis inhibition, and MAPT, which is associated with cytoskeletal dynamics. This reinforces the hypothesis that eugenol exerts multifaceted biological effects by targeting the central nodes across multiple signalling pathways (18,30). Thus, the PPI network constructed using Cytoscape combined with MCC scoring provides strong computational evidence that eugenol may act through network central disruption, modulating highly connected nodes essential for cancer cell stability. These findings warrant further experimental exploration, including gene expression assays and pathway-specific reporter systems, to validate the extent of eugenol modulation of AKT1, ESR1, and the associated downstream effectors.

Molecular docking analysis

Molecular docking was performed to assess the potential of eugenol to interact directly with the active sites of AKT1 and ESR1. The docking scores and molecular interactions data were analyzed to predict the binding affinities and to visualize ligand-receptor interactions. The docking protocol was validated using a redocking procedure, in which the co-cyrstallized ligand was reintroduced into the active site of the protein. The method's accuracy was confirmed by calculating the Root Mean Square Deviation (RMSD) between the proses of the re-docked and original crystallographic ligands. A docking protocol is deemed valid if the RMSD value is below the 2.0 Å threshold (22). Before docking, a thorough ligand preparation was conducted, which involving adding all hydrogen atoms, determining the correct protonation state at physiological pH, and calculating the atomic charges (including Gasteiger charges) to ensure an accurate and realistic molecular representation.

Binding of eugenol to AKT1

Eugenol exhibited a binding affinity of -5.1kcal/mol with AKT1, indicating a moderate interaction that may still be biologically relevant. The 3D structure (Figure 3A) shows that eugenol is positioned within the ATP-binding pocket of AKT1, a region critical for its kinase activity. The binding is primarily facilitated by a network of hydrophobic interactions, which includes a crucial π-alkyl interaction with the side chains of Met105 and Ile65, along with van der Waals contacts with Phe109 and Ala70. Within this site, eugenol establishes multiple hydrophobic interactions, particularly π-alkyl interactions with Met105 and Ile65 and van der Waals interactions with Phe109 and Ala70. These hydrophobic residues form a part of the conserved catalytic domain, and their interaction with small-molecule inhibitors is often associated with the suppression of AKT1 phosphorylation and downstream signalling (8,18).

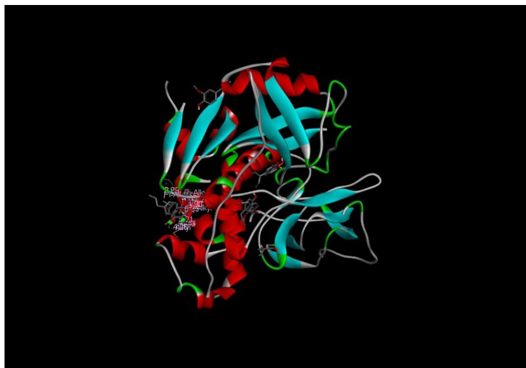

Figure 3. 3D Visualization of eugenol bound to AKT1 binding pocket.

The π-alkyl interaction between the aromatic ring of eugenol and the side chains of Met105 and Ile65 contributed significantly to ligand stabilization within the pocket. These interactions are non-directional, but energetically favorable, allowing eugenol to remain embedded in the hydrophobic cavity and potentially obstruct ATP access. The presence of van der Waals forces between Phe109 and the surrounding nonpolar residues further stabilizes the interaction. Notably, no conventional hydrogen bonds or electrostatic interactions were observed in this complex, indicating that hydrophobic packing is the main mode of binding.

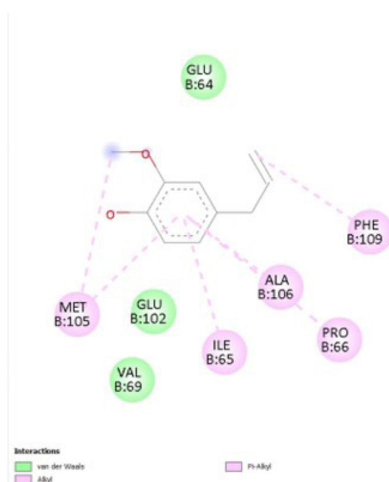

Figure 3b. 2D Interaction diagram showing π–alkyl and van der Waals interactions with Met105, Ile65, and Phe109.

The 2D interaction map (Figure 3B) confirms these findings. It shows the proximity of eugenol to residues Leu156, Val270, and Ala70, which, although not forming specific bonds, lies within the van der Waals interaction distance. The absence of unfavorable interactions (such as steric clashes or donor–donor repulsion) suggests a good spatial fit of eugenol in the AKT1 binding site, despite its relatively simple structure. These results imply that eugenol may act as a weak ATP-competitive inhibitor of AKT1, potentially altering the conformational dynamics of the kinase and inhibiting the downstream phosphorylation events. This mode of inhibition has been observed in other phenolic compounds such as curcumin and resveratrol, which similarly rely on π-interactions and hydrophobic occupancy rather than strong polar contacts (8,18). Furthermore, the ability of eugenol to engage with AKT1's catalytic site through non-covalent hydrophobic interactions supports its potential to attenuate the PI3K/AKT signalling pathway, which is commonly upregulated in breast cancer and contributes to chemoresistance and proliferation in MCF-7 cells (18,21).

Binding of eugenol to ESR1

The docking analysis of eugenol with estrogen receptor alpha (ESR1) resulted in a binding affinity of -5.9 kcal/mol, which, although lower than that of the native ligand (estradiol), suggests the potential for functional interactions. ESR1 is a key nuclear receptor that undergoes conformational changes upon ligand binding, leading to transcriptional activation of genes involved in cell proliferation and survival. In MCF-7 cells, which express high levels of ESR1, modulation of this receptor is a central therapeutic strategy (12,21). The 3D docking model (Figure 4A) shows that eugenol fits into the ligand-binding domain (LBD) of ESR1, which normally accommodates estradiol (12,31). The interaction is predominantly stabilized by hydrophobic forces, which include significant π-alkyl interactions between eugenol's aromatic ring and the aliphatic side chains of Leu384 and Leu525 (22). Hydrophobic interactions dominate the binding, with π-alkyl contacts formed between the aromatic ring of eugenol and the Leu384 and Leu525 side chains. These residues lie within the hydrophobic core of the LBD and contribute significantly to the stabilization of natural and synthetic ligands, as demonstrated in prior studies of SERM (Selective Estrogen Receptor Modulator) analogues.

However, the interaction map also revealed an unfavorable donor–donor interaction with His524 (Figure 4B). His524 is a critical residue in the ligand-anchoring pocket of the estrogen receptor and is often involved in hydrogen bonding with the hydroxyl groups of estradiol. Eugenol, lacking such functionality in the correct position, fails to form stabilizing polar interactions and instead may cause electrostatic repulsion, thereby reducing the overall binding affinity.

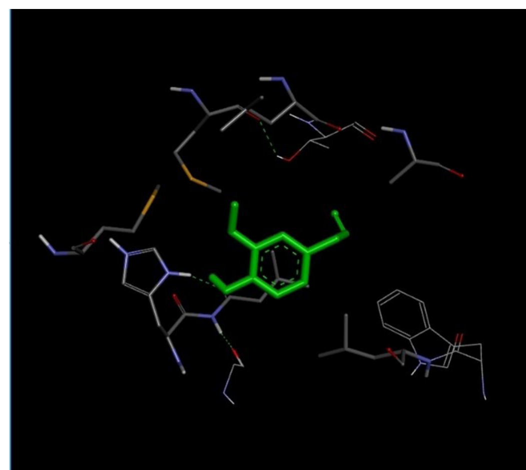

Figure 4a. 3D binding pose of Eugenol within the ESR1 ligand-binding domain.

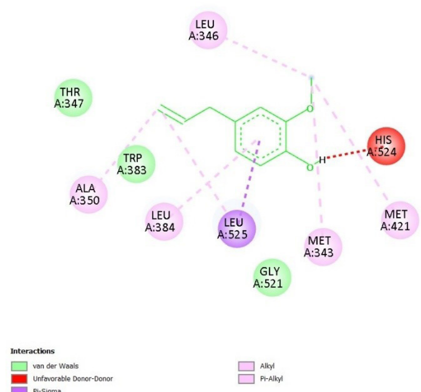

Figure 4b. 2D Interaction map showing π–alkyl contacts and donor–donor repulsion with His524.

Despite this, eugenol maintains several stabilizing van der Waals contacts with adjacent residues, such as Gly521 and Met421, which could provide sufficient stabilization for partial binding. The combined pattern of interactions indicated that eugenol may act as a weak partial agonist or antagonist of ESR1, potentially interfering with estrogen-mediated transcription. This behavior aligns with the known activities of polyphenolic compounds, many of which can modulate hormone receptors depending on structural compatibility, ligand orientation, and receptor subtype (12). The presence of both favorable and unfavorable interactions suggests that eugenol may not fully activate ESR1 but could compete with estradiol, thereby attenuating downstream gene expression involved in proliferation, which is a desirable outcome in ER-positive breast cancers, such as MCF-7.

Comparison of binding affinity

A comparative summary of docking scores is presented in Table 3. The reference ligand (0RF) showed a stronger binding affinity to AKT1 with -7.8kcal/mol, likely due to the formation of specific hydrogen bonds and improved steric complementarity with the protein's active site. In contrast, eugenol's binding affinity was lower for both AKT1 (-5.1kcal/mol) and ESR1 (-5.9kcal/mol), which can be attributed to its small size and lack of polar interaction groups.

Table 3. Binding affinity values (kcal/mol) of eugenol and 0RF against AKT1 and ESR1 targets.

EUG against AKT1 (kcal/mol)	EUG against ESR1 (kcal/mol)	ORF against AKT1 (kcal/mol)
-5.1 kcal/mol	-5.9 kcal/mol	-7.8 kcal/mol

Despite these modest scores, the ability of eugenol to engage with both proteins remains biologically relevant. In drug discovery, compounds with moderate affinity ($\geq$ -6.0kcal/mol) can exert meaningful biological effects, particularly if they act on multiple targets simultaneously (polypharmacology). This dual-target behaviour, combined with its low molecular weight and high natural abundance, makes eugenol a viable lead compound for further optimization and as an adjuvant therapeutic agent. Moreover, the natural origin of eugenol, coupled with previous reports of low toxicity and selective action on cancer cells, strengthens its candidacy for development as a multitarget agent in breast cancer therapy (16,18,32).

Cytotoxicity of clove extract on MCF-7 cells

The cytotoxic potential of the clove extract on breast cancer cells was evaluated using the MTT assay, a colorimetric method that measures mitochondrial metabolic activity as an indicator of cell viability. The assay was performed on MCF-7 cells treated with a range of extract concentrations (15.625–2000ppm) for 24h. Two types of extracts were tested: EC-N (Soxhlet extraction with n-hexane) and EC-E (maceration with ethanol). To ensure the integrity of the assay, the extracts were dissolved in a final DMSO concentration of 0.1%, which is well below the cytotoxic threshold for MCF-7 cells (14). The EC-N extract simultaneously showed higher cytotoxicity, which aligns with its higher eugenol content (53.6% vs. 35.4%), as determined by GC-MS.

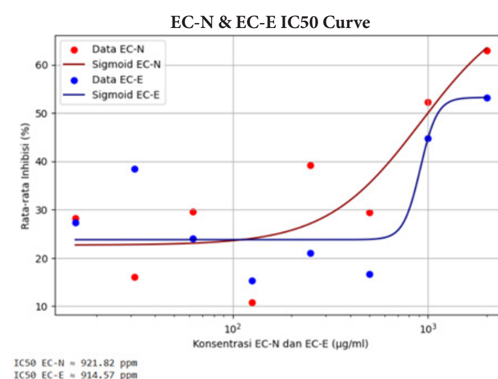

Figure 5. Dose–response curve showing MCF-7 cell viability after treatment with EC-N and EC-E clove extracts. The data indicate a dose-dependent cytotoxic effect, with EC-N exhibiting greater potency.

Figure 5 illustrates the dose–response relationship, where a progressive decline in MCF-7 cell viability was observed with increasing extract concentration. The inhibition was statistically significant ($p < 0.05$) at 125ppm and became more pronounced at 500–2000ppm. This pattern suggests a dose-dependent cytotoxic response, which is characteristic of compounds that induce programmed cell death rather than mere cytostatics (32,33). The calculated IC_{50} values the concentration required to inhibit 50% of cell viability were in the range of 400–600ppm for EC-N, which corresponds to previous findings on eugenol's cytotoxicity against MCF-7 cells (21,34) that have reported similar IC_{50} ranges for pure eugenol, suggesting that the biological activity in the extract is primarily attributable to its eugenol content.

Importantly, morphological changes in MCF-7 cells were observed under light microscopy following exposure to the extract. These changes included cell shrinkage and rounding, which indicate a loss of cytoskeletal integrity; membrane blebbing, a well-recognized hallmark of early apoptosis; and detachment from the substrate, consistent with the collapse of focal adhesion complexes. Collectively, these features are characteristic of apoptotic cell death rather than necrosis or

passive damage. These findings support the hypothesis that eugenol induces intrinsic apoptotic pathways, potentially through mitochondrial stress and the generation of reactive oxygen species (ROS). Several studies have proposed that eugenol triggers apoptosis via the disruption of mitochondrial membrane potential (8,35), release of cytochrome c (35), activation of caspases-3 and -9, and (36), Modulation of BAX/Bcl-2 protein ratios (35).

Given that AKT1 is known to inhibit apoptosis by phosphorylating and inactivating pro-apoptotic proteins, the interaction of eugenol with AKT1 (as shown in docking studies) may further contribute to the observed cytotoxicity. By occupying the ATP-binding site of AKT1, eugenol inhibits its kinase function, leading to increased apoptosis sensitivity. In addition, the extract contains other bioactive compounds, such as β-caryophyllene and caryophyllene oxide, which may exhibit synergistic effects with eugenol. These compounds have been reported to modulate oxidative stress, inhibit inflammation, and sensitize cancer cells to chemotherapeutic agents (18,32,35). Therefore, the observed cytotoxicity may not be solely due to eugenol but may result from the combined action of multiple constituents, which is a hallmark of phytochemical synergy.

From a therapeutic perspective, these findings suggest that eugenol-rich clove extract possesses measurable antiproliferative activity against hormone-sensitive breast cancer cells and has potential utility as a complementary or adjuvant agent. Compared with synthetic chemotherapeutics, natural extracts offer advantages in terms of biocompatibility, oral bioavailability, and lower systemic toxicity. Nevertheless, the in vitro cytotoxicity observed in this study was an initial indication.

Mechanistic implications for breast cancer therapy

The findings from this study, integrating both in silico molecular docking and in vitro cytotoxicity assays, provide compelling evidence that eugenol possesses anticancer activity through multiple converging molecular pathways. The dual interaction with key regulatory proteins AKT1 and ESR1 alongside significant cytotoxic effects on MCF-7 cells, indicates that eugenol acts not merely as a metabolic disruptor but as a mechanism-based modulator of breast cancer signalling.

This valuable multi-target characteristic of eugenol positions it as a promising candidate, akin to other well-studied phytochemicals such as curcumin and resveratrol, which are also recognized for their ability to modulate multiple oncogenic pathways (37). This multi-target approach offers a significant advantage over single-target drugs, which are often susceptible to resistance, as cancer cells can activate alternative signalling pathways (38). Thus, eugenol can be contextualized within its current stage of development, progressing toward a deeper understanding of its mechanism of action. The natural origin of eugenol and its extract offer advantages in terms of biocompability, lower systemic toxicity, and potential for synergistic effects with other bioactive compounds, a notable benefit compared to many synthetic chemotherapeutics.

Multitarget interaction: AKT1 and ESR1 as therapeutic hubs

The molecular docking results showed that eugenol interacted moderately with AKT1 (-5.1kcal/mol) and ESR1 (-5.9kcal/mol). While these binding affinities are not as high as those of synthetic inhibitors, they fall within a biologically meaningful range, particularly for natural compounds that exert effects through polypharmacology rather than high-affinity single-target inhibition (18). AKT1, a serine/threonine kinase, plays a central role in the PI3K/AKT/mTOR pathway and promotes cell proliferation, glucose metabolism, and antiapoptotic signaling. The inhibition of the AKT1 activity has been associated with increased apoptosis sensitivity, G1 phase arrest, and enhanced response to chemotherapy in MCF-7 and other breast cancer lines (18,38). On the other hand, ESR1 encodes the estrogen receptor alpha (ERα), a nuclear receptor that drives hormone-responsive gene transcription. The dysregulation of ESR1 leads to uncontrolled cell growth, endocrine resistance, and metastasis. Compounds that antagonize ESR1 or modulate its activity, such as tamoxifen, remain the cornerstone of treatment for ER-positive breast cancer (12). The ability of eugenol to occupy the functional domains of both AKT1 and ESR1 implies that it could interfere with estrogen-driven survival signals, as well as proliferative signaling cascades, which are rare but valuable traits for small, naturally derived molecules. The concurrent targeting of these two hubs could amplify the anticancer effects by disrupting the compensatory survival mechanisms that are often activated when only one pathway is inhibited.

Apoptosis induction and intracellular signaling modulation

The *in vitro* cytotoxicity results strongly suggest that eugenol triggered apoptotic cell death. This is supported by morphological observations, dose-response viability curves, and consistency with the published data (32). Mechanistically, eugenol has been shown to: 1) Induce mitochondrial dysfunction, leading to cytochrome c release(32); 2) Generate reactive oxygen species (ROS), contributing to oxidative stress (35,38); 3) Activate caspase-3, -7, and -9, initiating apoptosis cascade (38); 4) Downregulate Bcl-2 and upregulate Bax, shifting the balance toward cell death (35).

Notably, the AKT1 pathway normally inhibits apoptosis by phosphorylating and inactivating pro-apoptotic proteins (e.g., Bad, caspase-9) (18). Thus, the interaction of eugenol with AKT1 may alleviate this inhibition, thereby enhancing susceptibility to mitochondrial-mediated apoptosis. Moreover, the ESR1 activity has been linked to the expression of genes, such as Bcl-2 and cyclin D1. The partial antagonism of ESR1 by eugenol may downregulate these genes, leading to cell cycle arrest and mitochondrial destabilization. This coordinated disruption of both survival and hormonal axes highlights the potential of eugenol to bypass the mechanisms of hormonal resistance common in ER+ breast cancer.

Role of synergistic phytochemicals

GC-MS analysis revealed the presence of other bioactive compounds in the extract, including β-caryophyllene and caryophyllene oxide, which are known to modulate inflammation and oxidative stress. These compounds have demonstrated synergistic effects with eugenol in enhancing apoptosis and inhibiting tumour progression (32). Thus, the observed cytotoxic effect may result from the combined action of multiple constituents, not solely from eugenol. This reflects the concept of phytochemical synergy, in which weak interactions from several compounds converge on related signaling pathways to produce a network-level therapeutic effect.

Therapeutic potential and future directions

Based on these data, eugenol has promising potential as an adjunct or complementary agent for hormone-sensitive breast cancer therapy. Its natural origin, multi-target profile, and selective cytotoxicity support its advancement in preclinical development. The following research directions are recommended to confirm and expand these findings: 1) The gene expression analysis of AKT1, ESR1, BAX, Bcl-2, and Caspase-3 was performed to confirm the pathway involvement; 2) Flow cytometry was performed using Annexin V/PI staining to quantify the apoptotic populations; 3) ROS detection and mitochondrial membrane potential assays to validate stress-induced cell death; 4) *in vivo* efficacy testing in breast cancer xenograft models; and 5) combination assays with drugs, such as doxorubicin or tamoxifen, were used to evaluate synergy and chemosensitization.

Compared with synthetic chemotherapeutics, natural extracts offer advantages in terms of biocompatibility, oral bioavailability, and lower systemic toxicity. Moreover, the resistance to targeted therapies, such as lapatinib, in HER2+ breast cancer underscores the limitations of single-target approaches (18). Therefore, compounds such as eugenol, which acts on multiple nodes such as AKT1 and ESR1, may provide an advantage by mitigating compensatory survival signaling, suggesting that eugenol may act as a multitarget therapeutic compound capable of disrupting key molecular regulators in breast cancer cells. Further research involving gene expression analysis, pathway-specific inhibition, and *in vivo* validation is necessary to elucidate the clinical potential of eugenol in breast cancer treatment.

CONCLUSION

IIn conclusion, the findings from both in silico and in vitro analyses indicate that the eugenol-rich clove extract exhibits significant anticancer activity against MCF-7 breast cancer cells. Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified eugenol as the predominant component, with a concentration of 53.6% in the Soxhlet extract. Molecular docking studies revealed that eugenol interacts with key hub proteins, including AKT1 and ESR1, demonstrating binding affinities of -5.1kcal/mol and -5.9kcal/mol, respectively. These interactions suggest that eugenol has the potential to disrupt the PI3K/AKT signaling pathway and modulate estrogen receptor activity. The binding with AKT1 is primarily characterized by hydrophobic interactions, which may inhibit kinase activity and subsequent downstream signaling. Additionally, the interaction with ESR1 implies that eugenol may function as a weak partial agonist or antagonist, competing with estradiol for the ligand-binding domain and thereby reducing estrogen-mediated transcription. In vitro cytotoxicity assays confirmed a dose-dependent reduction in MCF-7 cell viability, with a low IC_{50} value, indicating that eugenol effectively induces cell death. Collectively, these results provide robust computational and experimental evidence that eugenol from cloves can disrupt critical signaling pathways and exert direct cytotoxic effects on Luminal A breast cancer cells. Consequently, eugenol emerges as a promising candidate for further development as a therapeutic agent or natural adjuvant in breast cancer treatment. Nonetheless, additional research, including *in vivo* studies and clinical trials, is essential to fully establish its efficacy and safety.

REFERENCES

1. Alfehaid M. Male Breast Cancer (MBC) - A Review. Pol Przeglad Chir Pol J Surg. 2023;95(6):24–30.
2. Antonio Pérez J, Salinas P, Poblete MT, Cardemil B. Breast cancer in males. Report of 4 cases. Rev Med Chil. 1995;123(10):1270–4.
3. Bardhan P, Bui MM, Minton S, Loftus L, Carter WB, Laronga C, et al. HER2-positive male breast cancer with thyroid cancer: An institutional report and review of literature. Ann Clin Lab Sci. 2012;42(2):135–9.
4. Li X, Zhang H, Yang H, Song Y, Zhang F, Wang A. Modifiable Risk Factors for Breast Cancer: Insights From Systematic Reviews. Public Health Nurs. 2025;42(2):1060–71.
5. Ang BH, Teo SH, Ho WK. Systematic Review and Meta-Analysis of Lifestyle and Reproductive Factors Associated with Risk of Breast Cancer in Asian Women. Cancer Epidemiol Biomarkers Prev. 2024;33(10):1273–85.
6. Vishwakarma G, Mehta A, Saifi M, Garg D, Paliwal D. Modifiable (Sleeping Pattern and Stress) and Non-Modifiable Risk Factors Associated with Breast Cancer: A Matched Case-Control Study in Delhi, India. Asian Pac J Cancer Prev. 2022;23(7):2469–76.
7. Budhwar M, Sharma M, Mehra S, Chopra M. Diagnostic-prognostic biomarkers and their clinical implication in breast cancer. In 2024. p. 493–511. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85203484391&doi=10.1007%2F978-981-99-6263-1_19&partnerID=40&md5=363e35fd5eb8bda2687be4e930df2e32
8. Park KR, Nam D, Yun HM, Lee SG, Jang HJ, Sethi G, et al. β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett. 2011;312(2):178–88.
9. Hellmann SS, Thygesen LC, Tolstrup JS, Grønbaek M. Modifiable risk factors and survival in women diagnosed with primary breast cancer: Results from a prospective cohort study. Eur J Cancer Prev. 2010;19(5):366–73.
10. Abrahao R, Ruddy KJ, Laurent CA, Chubak J, Haupt EC, Brunson AM, et al. Trastuzumab therapy and new-onset hypertension in adolescents and young adults with breast cancer. Breast Cancer Res Treat. 2025;213(1):81–92.

11. Modan B. Epidemiology of breast cancer. Preventive aspects. Isr J Med Sci. 1981;17(9–10):804–9.
12. Daraei A, Izadi P, Khorasani G, Nafissi N, Naghizadeh MM, Younosi N, et al. Epigenetic Changes of the ESR1 Gene in Breast Tissue of Healthy Women: A Missing Link with Breast Cancer Risk Factors? Genet Test Mol Biomark. 2017;21(8):464–70.
13. Ma H, Luo J, Press MF, Wang Y, Bernstein L, Ursin G. Is there a difference in the association between percent mammographic density and subtypes of breast cancer? luminal a and triple-negative breast cancer. Cancer Epidemiol Biomarkers Prev. 2009;18(2):479–85.
14. Atkinson RL, El-Zein R, Valero V, Lucci A, Bevers TB, Fouad T, et al. Epidemiological risk factors associated with inflammatory breast cancer subtypes. Cancer Causes Control. 2016;27(3):359–66.
15. John EM, Koo J, Phipps AI, Longacre TA, Kurian AW, Ingles SA, et al. Reproductive characteristics, menopausal status, race and ethnicity, and risk of breast cancer subtypes defined by ER, PR and HER2 status: the Breast Cancer Etiology in Minorities study. Breast Cancer Res [Internet]. 2024;26(1). Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85194884973&doi=10.1186%2Fs13058-024-01834-5&partnerID=40&md5=f9ce679cfcc0a265621ae730e96f527a
16. Derviş E, Yurt Kilcar A, Medine EI, Tekin V, Cetkin B, Uygur E, et al. In Vitro Incorporation of Radioiodinated Eugenol on Adenocarcinoma Cell Lines (Caco2, MCF7, and PC3). Cancer Biother Radiopharm. 2017;32(3):75–81.
17. Gupta N, Cohen JL, Rosenbaum C, Raam S. Estrogen receptors in male breast cancer. Cancer. 1980;46(8):1781–4.
18. Ma M, Ma Y, Zhang GJ, Liao R, Jiang XF, Yan XX, et al. Eugenol alleviated breast precancerous lesions through HER2/PI3K-AKT pathway-induced cell apoptosis and S-phase arrest. Oncotarget. 2017;8(34):56296–310.
19. Dwivedil V, Shrivastaval R, Hussain S, Ganguly C, Bharadwaj M. Comparative anticancer potential of clove (Syzygium aromaticum) - An Indian spice - Against cancer cell lines of various anatomical origin. Asian Pac J Cancer Prev. 2011;12(8):1989–93.
20. Kaur K, Kaushal S, Rani R. Chemical Composition, Antioxidant and Antifungal Potential of Clove (Syzygium aromaticum) Essential Oil, its Major Compound and its Derivatives. J Essent Oil-Bear Plants. 2019;22(5):1195–217.
21. Khan FA, Akhtar S, Almohazey D, Alomari M, Almofty SA. Extracts of Clove (Syzygium aromaticum) Potentiate FMSP-Nanoparticles Induced Cell Death in MCF-7 Cells. Int J Biomater [Internet]. 2018;2018. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85053042587&doi=10.1155%2F2018%2F8479439&partnerID=40&md5=3d8118c86fcbf59261ce3cb758b79fb7
22. Atmoko HD, Pangestuti IR, Maulina E, Kusuma TM, Wardani AK, Kaltsum U. In silico study to molecular docking study testing the activity of clove flower essential oil components (Syzigium aromaticum (L)) as anti-breast cancer on EGFR receptors. In Padang, Indonesia; 2023 [cited 2024 Feb 15]. p. 020128. Available from: http://aip.scitation.org/doi/abs/10.1063/5.0120359
23. Alonso H, Bliznyuk AA, Gready JE. Combining docking and molecular dynamic simulations in drug design. Med Res Rev. 2006;26(5):531–68.
24. Forli S, Olson AJ. A Force Field with Discrete Displaceable Waters and Desolvation Entropy for Hydrated Ligand Docking. J Med Chem. 2012;55:623–38.
25. Jendele L, Krivak R, Skoda P, Novotny M, Hoksza D. PrankWeb: a web server for ligand binding site prediction and visualization. Nucleic Acids Res. 2019 July 2;47(W1):W345–9.
26. Dwivedil V, Shrivastaval R, Hussain S, Ganguly C, Bharadwaj M. Comparative anticancer potential of clove (Syzygium aromaticum) - An Indian spice - Against cancer cell lines of various anatomical origin. Asian Pac J Cancer Prev. 2011;12(8):1989–93.
27. Idris H, Suryani E, Gustia H, Ramadhan AI. The effect of various essential oil and solvent additives on the botanical pesticide of Piper Aduncum essential oil on formulation antifungal activity. Results Eng [Internet]. 2022;16. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85138452619&doi=10.1016%2fj.rineng.2022.100644&partnerID=40&md5=3c3cfcf4a55a87f2875ed689fedc91a7
28. Akoko LO, Rutashobya AK, Lutainulwa EW, Mwanga AH, Kivuyo SL. The effect of reproductive, hormonal, nutritional and lifestyle on breast cancer risk among black Tanzanian women: A case control study. PLOS ONE [Internet]. 2022;17(2 February). Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85124327724&doi=10.1371%2Fjournal.pone.0263374&partnerID=40&md5=43a07f0850fa77c7e0b62931c0dc5c9b
29. Dierssen-Soto T, Gómez-Acebo I, Palazuelos C, Fernández-Navarro P, Altzibar JM, Gonzalez-Donquiles C, et al. Validating a breast cancer score in Spanish women. The MCC-Spain study. Sci Rep [Internet]. 2018;8(1). Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85042113618&doi=10.1038%2Fs41598-018-20832-0&partnerID=40&md5=f250e7d3fdc3e927b8d75d01d6305562
30. Mirabelli CK, Nusse R, Tuveson DA, Williams BO. Perspectives on the role of Wnt biology in cancer. Sci Signal [Internet]. 2019;12(588). Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85068912557&doi=10.1126%2fscisignal.aay4494&partnerID=40&md5=54a9d5771af3c146d9a7c3f1e41f1c87
31. Mukai K, Urai T, Asano K, Nakajima Y, Nakatani T. Evaluation of Effects of Topical Estradiol Benzoate Application on Cutaneous Wound Healing in Ovariectomized Female Mice. Plos One. 2016;11(9):1–15.
32. De-Sá-Júnior PLDS, Câmara DAD, Costa AS, Ruiz JLM, Levy D, De Azevedo RA, et al. Apoptotic effect of eugenol envolves G2/M phase abrogation accompanied by mitochondrial damage and clastogenic effect on cancer cell in vitro. Phytomedicine. 2016;23(7):725–35.
33. Debnath A, Lo YH, Bhattacharya M, Wen ZH, Chakraborty C, Das A. Eugenol's anti-cancer properties, its modulation of signalling pathways, and cascades across various cancers: A review. Curr Res Biotechnol [Internet]. 2025;10. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-105015035814&doi=10.1016%2Fj.crbiot.2025.100330&partnerID=40&md5=6c6b2a388f9ba6950a47f2daff3ddd3a
34. Fadhil ZA, Amin EEM, Mahmood SM, Hussein AH. Preparation of a nanoemulsion from clove oil extract containing phenol and studying its effect on a human MCF-7 breast cancer cell line. Salud Cienc Tecnol [Internet]. 2025;5. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-105006602104&doi=10.56294%2Fsaludcyt20251535&partnerID=40&md5=9078b75ef74cae321c69d822bb1dfd37
35. Debnath A, Lo YH, Bhattacharya M, Wen ZH, Chakraborty C, Das A. Eugenol's anti-cancer properties, its modulation of signalling pathways, and cascades across various cancers: A review. Curr Res Biotechnol [Internet]. 2025;10. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-105015035814&doi=10.1016%2Fj.crbiot.2025.100330&partnerID=40&md5=6c6b2a388f9ba6950a47f2daff3ddd3a

36. Sihombing INN, Arsianti A. Network pharmacology prediction and molecular docking analysis on the mechanism of eugenol as a candidate against estrogen receptor-positive breast cancer. J Pharm Pharmacogn Res. 2024;12(5):837–51.

37. Gonzales MC, Grayson J, Lie A, Yu CH, Shiao SYPK. Gene-environment interactions and predictors of breast cancer in family-based multi-ethnic groups. Oncotarget. 2018;9(49):29019–35.

38. Yan X, Zhang G, Bie F, Lv Y, Ma Y, Ma M, et al. Eugenol inhibits oxidative phosphorylation and fatty acid oxidation via downregulation of c-Myc/PGC-1β/ERRα signaling pathway in MCF10A-ras cells. Sci Rep [Internet]. 2017;7(1). Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85031046402&doi=10.1038%2Fs41598-017-13505-x&partnerID=40&md5=c0aade6313798cc7ce90268bc67db46a