RESEARCH ARTICLE

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Integrative Bioinformatics Analysis Reveals Potential Target Genes and PTEN Signaling in Breast Cancer and Effect of *Zingiber officinale* (Ginger) and *Allium sativum* (Garlic) extract on It

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Abstract

Introduction: Breast cancer is the most common type of cancer in women. The construction of a competing gene network is an important step in the identification of the role of hub genes in breast cancers. In the current research, we used a number of bioinformatics tools to construct this network in breast cancer and investigated the combined effect of garlic and ginger on mice model of breast cancer. **Materials and Methods:** We chose female mice weighing 18-20 g that were divided into 7 groups including; the cancer group receiving normal saline, different doses of ginger extract (100 and 500 mg/kg), different doses of garlic (50 and 100 mg/kg), tamoxifen (10 mg/ kg) and simultaneous garlic (100 mg/kg) and ginger (500 mg/kg) for 3 weeks intraperitoneal. Then we anesthetized the mice, isolated the tumor, and determined its size. Glutathione reductase and peroxidase levels and *HER2*, *PTEN*, and Cullin3 genes expression were measured. **Results:** We identified 20 hub genes for breast cancer. In animal phase we found that tumor size in all mice receiving garlic and ginger showed a significant decrease compared to the control. Glutathione reductase showed a significant increase in all groups, especially in ginger 500 and combined groups. Glutathione peroxidase increased almost in all groups, especially in ginger 500. Expression of *HER2* decreased in all treated groups. Expression of *PTEN* increased just in the combined group. **Conclusion:** Taken together, we introduce a number of novel promising diagnostic biomarkers for breast cancer. The use of garlic and ginger in the treatment of cancer can be useful. This action is probably through the antioxidant mechanism, and regulation of the expression of cancer related genes such as *PTEN*.

Keywords: Breast cancer- Garlic- Ginger- *PTEN*- Bioinformatics

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Introduction

Breast cancer is widely recognized as the predominant cancer in the female population, ranking as the second leading cause of death among women [1]. Chemotherapy is considered the primary therapeutic approach for breast cancer [2], yet its protracted administration can lead to adverse consequences, including cardiotoxicity [3] and immunosuppression [4]. Furthermore, the efficacy of chemotherapy in the treatment of breast cancer is diminished as a result of both intrinsic and acquired resistance to chemotherapy, resulting in the occurrence of recurrence and metastasis [5]. Surgery is commonly regarded as one of the preferred treatment options for breast cancer. Nevertheless, individuals with compromised systemic illnesses, such as severe ailments affecting vital organs, are contraindicated for surgical intervention [6]. Hence, targeted therapy assumes a paramount role in managing this condition to enhance the scope of beneficiaries and improve the effectiveness of treatment for individuals with breast cancer [7].

The procedure of aggression and metastasis in breast cancer cells is a complex phenomenon encompassing several genes and stages [8]. This process entails the activation of oncogene mutations and silencing tumor suppressor genes [9-11]. The finding of Phosphatase and tensin homolog (*PTEN*) in 1997 revealed its role as a tumor suppressor gene, possessing protein phosphatase and lipid phosphatase activities. As indicated by previous studies, *PTEN* is considered the second most significant tumor suppressor gene after *P53* [12, 13]. The *PTEN* protein has a significant role in several cellular processes, including cell proliferation, differentiation, adhesion, migration, metastasis, apoptosis, cell cycle regulation, energy consumption, genome stability, and other related mechanisms [12-14]. The *PTEN* protein

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has a significant role in several cellular processes, including cell proliferation, differentiation, adhesion, migration, metastasis, apoptosis, cell cycle regulation, energy consumption, genome stability, and other related mechanisms [14, 15]. The primary factors contributing to *PTEN* abnormalities in breast cancer cells include deletion mutations, inappropriate DNA methylation in the promoter region, and aberrant degradation or functional impairment leading to reduced production of the *PTEN* protein [12, 14-18]. The occurrence of loss of heterozygosity of *PTEN* has been documented in around 40% to 50% of individuals diagnosed with breast cancer. Additionally, *PTEN* mutations, primarily frameshift mutations, leading to the functional loss of *PTEN*, are observed in around 5% to 10% of breast cancer patients [15, 18].

PTEN, a crucial suppressor of PI3K signaling, has diminished expression across many cancer types and can potentially undergo downregulation via several pathways [8]. The *PTEN* gene plays a crucial role as an effector molecule in the signaling pathway of tumors, exerting its capacity to suppress the development of cancer cells [8]. The downregulation of *PTEN* expression could lead to inadequate suppression of Akt activation, leading to excessive activation of the pPI3/Akt signaling pathway. The progression of certain advanced human malignancies, including melanoma, breast, prostate, and kidney cancers, can be greatly influenced by the loss of *PTEN* and the overexpression of Akt [9]. Hence, it can be inferred that *PTEN* is a promising candidate for therapeutic intervention in the context of breast cancer treatment.

The creation of novel pharmaceuticals derived from natural sources has emerged as a potential technique for effectively combating the growth of breast cancer [10]. A molecule derived from Ginger (*Zingiber officinale* Roscoe), which belongs to the Zingiberaceae family, has been identified as a possible inhibitor of it [11]. Recent studies show ginger has demonstrated an inhibitory impact on breast cancer [12]. An inhibitory impact on proliferation and colony formation in MDA-MB-231 cells has been shown using the methanolic extract of ginger. The ginger extract has been observed to stimulate apoptosis in MCF-7 and MDA-MB-231 cells by upregulating Bax and downregulating Bcl-2, NF-κB, and survivin [13].

Garlic (*Allium sativum* L.), a member of the Amaryllidaceae family, is a fragrant herb and spice with a rich historical background, having been utilized for several generations [14]. The investigation of the correlation between the consumption of garlic and the risk of developing cancer is a topic of considerable interest [15]. The available evidence supporting the potential anti-cancer properties of raw garlic indicates that it may impact various pathways involved in cancer development. These pathways include alterations in enzymes responsible for metabolizing carcinogens, halting the cell cycle, triggering programmed cell death known as apoptosis, and inhibiting signal transduction pathways associated with cancer formation [16].

The role of oxidative stress in the initiation and advancement of cancer has been extensively studied [17], hence indicating the potential of antioxidant therapy in mitigating cancer risk [18]. Research findings indicate that both garlic and ginger possess antioxidant capabilities. According to Capasso et al. garlic has been found to exhibit a protective effect against oxidative damage by mitigating the impact of free radicals. [19]. Prior research has demonstrated that allicin had the ability to reduce the scavenging of free radicals, hence mitigating lipid peroxidation [20]. The study conducted by Liu et al. shown that the terpenoids included in ginger extract vapor induce apoptosis in endometrial cancer cells by the activation of p53 and Bax expression, while concurrently reducing Bcl-2 levels [21].

Human epidermal growth factor receptor 2 (*HER2*) overexpression is observed in around 20-30% of breast cancer tumors [22]. The overexpression of *HER2* is correlated with a more aggressive form of the illness, an increased risk of recurrence, and reduced overall survival [23]. According to the literature, *HER2* overexpression has been identified as a significant factor contributing to resistance to therapeutic interventions [24]. Shou et al. show that breast cancer cell lines, which initially respond to tamoxifen, develop resistance to the *HER2* oncogene. Consequently, several clinical studies are currently being conducted to evaluate novel potential therapeutic interventions [25]. Therefore, many clinical trials are testing new possible treatments.

Cullin-3 (CUL3) is a scaffold component within the CUL3-RING-E3 ubiquitin Ligase (CRL3) complex. The protein CUL3 facilitates the connection between the RING protein RBX1 and substrate adaptors, which deliver specific targets for ubiquitylation and subsequent proteasomal destruction [26]. The mechanism of ubiquitin-mediated protein degradation is of utmost importance in the regulation of several physiological processes, such as the cell cycle [27], immune cell formation [28], and maintenance of renal and redox homeostasis [29] among numerous others. Previous studies have demonstrated the overexpression of Cullin-3 in breast cancer cell lines and breast tissue samples [30].

This present study used an integrative bioinformatics approach to identify new targets and molecular mechanisms of breast cancer. We aimed to clarify the role of these target in breast cancer and effect of garlic and ginger on them. There have been many studies on the effect of garlic and ginger in the treatment of breast cancer. Since the simultaneous use of two drugs can have a synergistic effect in the treatment of cancer, we used a combination of garlic and ginger in the 4T1 model of breast cancer and evaluated its signaling pathways.

Materials and Methods

PPI Network Construction and Hub Genes Identification

The PPI network for pathway in breast cancer was built using the STRING database. The interactions between the proteins were examined using the Cytoscape software v3.9. Finally, the Cytohubba plugin of Cytoscape was used to calculate the degree of connectivity of nodes to find the top 20 DEGs as hub genes.

Gene-concept network of the top five GO terms

We used the clusterProfiler R package create the

relationships between the PPI hub genes and GO terms.

Gene Ontology (GO) Enrichment Analysis

We used the clusterProfiler R package to perform gene ontology (GO) enrichment analysis to investigate the functions of the remarkably hub genes that we discovered. The functional category criteria were established at an adjusted p-value of 0.05 or below.

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

To determine the potential roles of hub genes that took part in the pathways based on the KEGG database, KEGG pathway analyses of these genes were conducted.

Animals

In this experimental study, 48 healthy adult female BALB/C mice that weighed 20-22 g were kept on a 12-hour light/dark cycle at 22 ± 2°C and fed with standard pellets and water adlibitum. All animal experiments were conducted by national guidelines and protocols after approval from the Institutional Animal Ethics Committee. All experimental protocols were approved by the Iran University of Medical Sciences (IUMS) Ethics Committee.

Breast cancer model

To obtain a model of breast cancer in situ, female BALB/c mice were challenged with 4T1 cells (that were provided by the Pasteur institute). Briefly, 4T1 cancer cells were cultured in 12 mL DMEM supplemented with 10% FBS and 1× antibiotic-antimycotic (penicillin/streptomycin/fluconazole), using T75 culture flasks, and incubated (37°C, 5% CO2) until reaching 50-80% confluence. Cells were washed with 7 mL of serum-free medium (SFM), then dislodged using 3 mL of 0.25% trypsin/1 mM EDTA solution at room temperature for 2 min. Later, 7 mL of SFM was used to harvest the trypsinized cells from the flasks and then transferred into 15 mL conical tubes and centrifuged at room temperature (4 min; 112.7 g). The supernatant was then discarded and the pellet was resuspended with SFM.

5×10^5 4T1 cells were injected subcutaneously on the right lower flank. After one week, when the tumor mass was detectable, fennel treatment was started. Animals received daily fennel extracts via intraperitoneal (IP) injection for 2 weeks. Meanwhile, the tumor volume was measured every day using calipers and calculated using the following formula: (length × width2)/2 (mm^3).

Preparation of garlic and ginger extracts

Seeds were purchased from the Isfahan Seed Packers Company. They must be one-year-old and healthy. For preparing ginger aqueous extract; 2 g of ginger powder was added to 90 µl of heated distilled water on a hot plate. For aqueous extraction of garlic; 350 g of freshly crushed garlic with 250 ml of sterile distilled water was placed at laboratory temperature for 48 hours. Then the solutions were passed through sterile gas using a funnel to separate the impurities from the crushed plant. To precipitate the impurities, we put the extract solution in the centrifuge for 15 minutes at 4500 rpm. Then the aqueous extract was used for injection. The extract was prepared daily and fresh.

Treatment

Animals have divided 48 mice into six groups (n = 8 per group):

Female BALB/mice were divided into 7 groups. About 8 mice were selected for each group. Mice were weighed and the injection dose was calculated. Our selected doses were based on previous studies [31-43]. Groups included:

Sham

Mice received distilled water for 3 weeks

Treat 1

Mice were treated with tamoxifen (10 mg/kg; IP) for three weeks.

Treat 1

Mice were treated with an aqueous extract of garlic (50 mg/kg; IP) for three weeks.

Treat 1

Mice were treated with an aqueous extract of garlic (100 mg/kg; IP) for three weeks.

Treat 1

Mice were treated with an aqueous extract of ginger (100 mg/kg; IP) for three weeks.

Treat 1

Mice were treated with an aqueous extract of ginger (500 mg/kg; IP) for three weeks.

Treat 1

Mice were treated with an aqueous extract of garlic and ginger (100 & 500 mg/kg, respectively; IP) for three weeks.

At the end of the 3 weeks of the experiment, each animal was anesthetized with an IP injection of ketamine (45 mg⁄kg) and xylazine (35 mg⁄kg) mixture. Tumor tissues were dissected and subjected to further analyses.

Glutathione peroxidase and reductase assay

Glutathione peroxidase and reductase activity were also determined using a commercially available kit for GPx-1 activity (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions. The activity was measured spectrophotometrically by the reduction of glutathione disulfide (GSSG) recycled to reduced glutathione (GSH) by glutathione reductase using NADPH as a reducing agent. Enzymatic activity is quantitated by the change in NADPH absorbance at 340 nm over time.

Quantitative Reverse Transcription PCR (qRT-PCR) Analysis

Samples were weighed (range of 30-50 mg) and RNA was extracted using the RNx plus (sinaclon, Tehran, Iran), according to the manufacturer's protocol, and resolved in 50µl RNase-free water. RNA was analyzed by Nano-drop

to define their concentration and purity. The denaturing gel electrophoresis method was used to test the RNA integrity.

RNA was treated with DNase I (EN0521, Fermentas, Opelstrasse, Germany) to eliminate any DNA contamination. Purified RNA samples were converted into cDNA. cDNAs were synthesized with 1µg of RNA, 0.5 µL of oligo dTs, and 0.5 µL of random hexamer using cDNA Synthesis Kit (PrimeScript RT Master Mix, TAKARA, Kyoto, Japan). All procedures were based on the manufacturer's protocol. 1 µg of synthesized cDNA used for SYBR Green-based real-time RT-PCR via 2X qPCR kit (RR820L, Tli RNaseH Plus, TaKaRa, Kyoto, Japan). The primer pairs used in this study are indicated in Table 1. Thermocycling parameters were as follows: initial denaturation at 95°C for 30 s, 40 cycles at 95°C for 5 s, and annealing and elongation at 60°C for 30 s. Values from β-actin were used for loading normalization for each sample. Relative changes expression was determined using the ΔΔCt method relative to gene expression values for control mice.

Statistical analysis

All data were expressed as mean ± standard error (SEM). The one-way ANOVA test was applied to clarify significant differences between groups. When a significant effect was found, the Duncan post hoc U test was performed. All analyses were performed using SPSS version 16. The statistical significance level was set at $p \leq .05$

Results

PPI Network Construction and Selection of Hub Genes

To find the hub genes, a PPI network of DEGs with 146 nodes and 2412 edges created from STRING (Figure 1) was imported into the Cytohubba plugin of Cytoscape 3.9. *CTNNB1, AKT1, TP53, MYC, CCND1, GSK3B, EGFR, PTEN, JUN, ESR1, STAT3, EGF, KRAS, ERBB2, PIK3CA, NOTCH1, CDH1, MAPK3, IGF1R*, and MTOR were the 20 hub genes with the highest degree of connectivity. Figure 2 and Table 1 shows information about these hub genes. The greatest degree to the lowest degree is used to order these hub genes.

Gene-concept network of the top five GO terms

We used the clusterProfiler R package create the relationships between the PPI hub genes and GO terms. Figure 3 shows the gene-concept network of the top five GO terms and Figure 4 indicates a network of GO terms, genes and network plot for GO terms, genes. In Figure 5, the intersection of the top 10 GO phrases was represented by the UpSet plot. It highlights the gene overlap between several gene sets.

GO Enrichment Analysis of DEGs

For the analysis, the clusterProfiler package (version 4.4.4) was employed. In GO functional enrichment analysis, GO entries with an adjusted p value of less than 0.05, most of which were biological processes (BP), followed by cellular component (CC) and molecular function (MF). The first 10 entries are Wnt signaling pathway, cell-cell signaling by wnt, canonical Wnt signaling pathway, epithelial cell proliferation, cell fate commitment, positive regulation of MAPK cascade, regulation of epithelial cell proliferation, gland development, fibroblast growth factor receptor signaling pathway (BP), Wnt signalosome, beta-catenin destruction complex, endocytic vesicle membrane, endocytic vesicle, Golgi lumen, beta-catenin-TCF complex, RNA polymerase II transcription regulator complex, phosphatidylinositol 3-kinase complex, transcription repressor complex, endoplasmic reticulum lumen (CC),

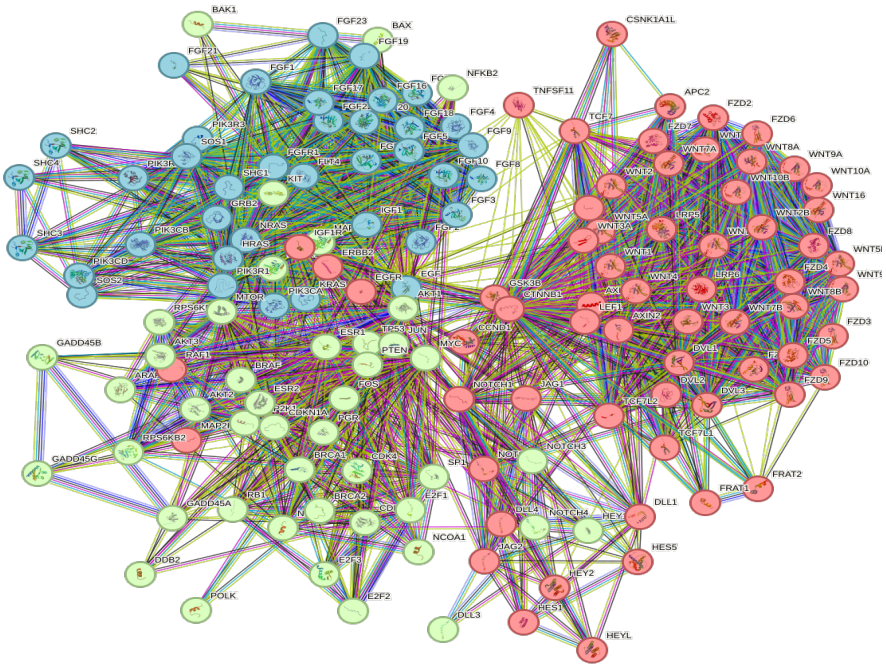

Figure 1. Protein-Protein Interaction Network Breast Cancer, Analyzed by STRING.

Table 1. Sequence of Specific Primers Used for Quantitative Real-Time PCR

bactin-F	TGAAGATCAAGATCATTGCTCCTC
bactin-R	TCAGTAACAGTCCGCCTAGAAG
cul3-F-Mice	AGGCAACATCTACAGGCAACG
cul3-R-Mice	ACTTTGGTGTGGCTGACTGAG
PTEN-F-Mice	CTGCTTTCCCATCTTCCAACAAC
PTEN-R-Mice	ACACTATCCTGCCATCTTCACAC
HER2-F-Mice	GGCTTGGTCTGTAACTCACTG
HER2-R-Mice	TTCCATACTCGGCACTCCTC

frizzled binding, fibroblast growth factor receptor binding, signaling receptor activator activity, receptor ligand activity, Wnt receptor activity, growth factor receptor binding, growth factor activity, G protein-coupled receptor binding, Wnt-protein binding, cytokine activity (MF). Figure 6 shows the bar plots of the top 6 enriched functions. The dot plot of the top 6 enriched functions visualized in Figures 7.

Pathway Analysis

the KEGG pathway analysis of hub genes was carried out (Table 2 and Figure 8).

Effects of Garlic and Ginger on Tumor Size

The tumor size was measured every week using calipers. The size of the tumor was significantly decreased in all treated groups. Garlic 100 showed more decrement relative to other groups. Tamoxifen 10 mg/kg (%59.80), Garlic 50 mg/kg (%65.62), Garlic 100 mg/kg (%79.31), Ginger 100 mg/kg (%52.23, Ginger 500 mg/kg (%47.68), Garlic 100 & Ginger 500 mg/kg (%51.54). The percentages of reduction in different groups are as follows: Tamoxifen 10 mg/kg (%59.80), Garlic 50 mg/kg (%65.62), Garlic 100 mg/kg (%79.31), Ginger 100 mg/kg (%52.23, Ginger 500 mg/kg (%47.68), Garlic 100 & Ginger 500 mg/kg (%51.54). (Table 3).

Table 2. Top 20 hub Genes based on Highest Degree Score, Analyzed by Cytohubba

Name	Betweenness Centrality	Closeness Centrality	Degree
CTNNB1	0.06851	0.787781	358
AKT1	0.052888	0.780255	352
TP53	0.05244	0.756173	332
MYC	0.033082	0.740181	318
CCND1	0.030495	0.727003	306
GSK3B	0.040937	0.714286	294
EGFR	0.022886	0.708092	288
PTEN	0.015641	0.704023	284
JUN	0.016609	0.698006	278
ESR1	0.015858	0.694051	274
STAT3	0.014603	0.67867	260
EGF	0.013577	0.669399	250
KRAS	0.009941	0.667575	246
ERBB2	0.011701	0.663957	244
PIK3CA	0.00754	0.651596	228
NOTCH1	0.010909	0.644737	224
CDH1	0.007753	0.643045	220
MAPK3	0.007129	0.641361	218
IGF1R	0.008262	0.639687	216
MTOR	0.013779	0.636364	212

Effects of Garlic and Ginger on Antioxidant Agent

The effect of garlic and ginger on antioxidant agents was evaluated. The amount of glutathione reductase as

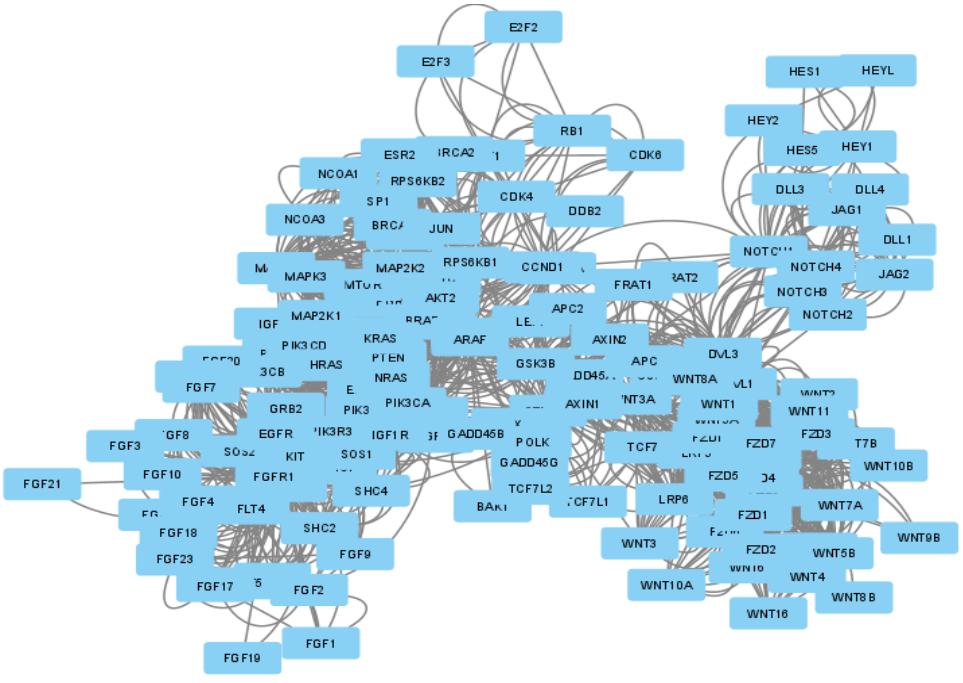

Figure 2. Hub Genes Selected based on the MCC Algorithm in CytoHubba.

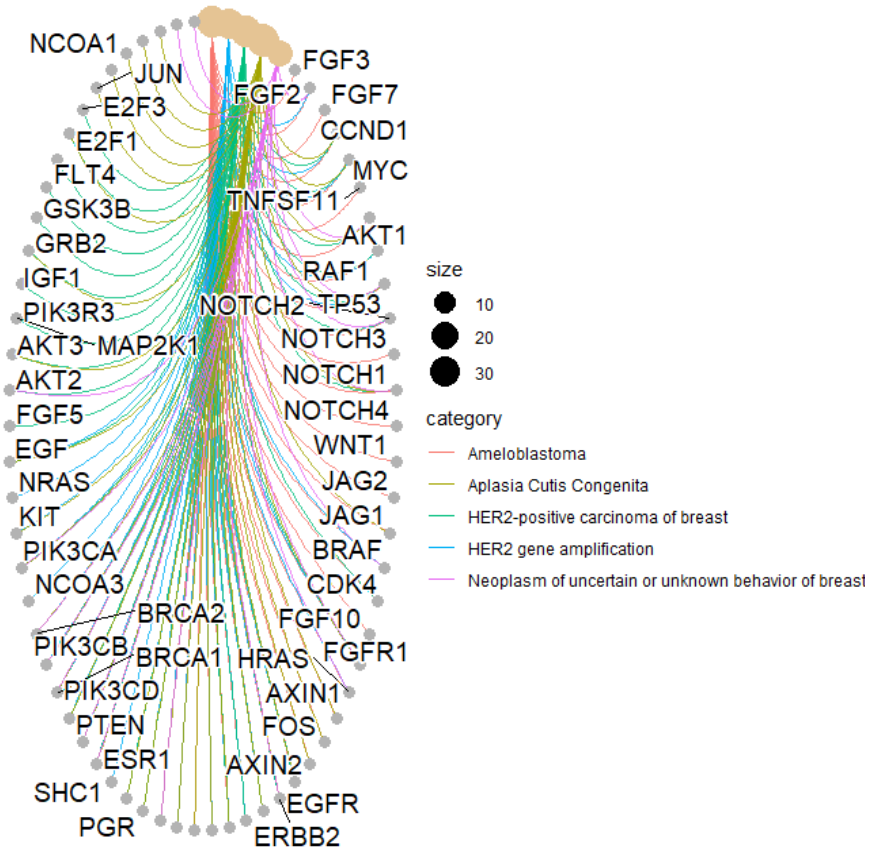

Figure 3. Top 5 GO Terms as a Network Plot. These GO terms are connected to genes in this graph. The connection of genes to the corresponding GO is marked with a special color; there are more genes for a specific GO term if the dot relating to it is bigger.

an antioxidant agent showed a significant increase in all groups, especially in ginger 500 and combined groups. Glutathione peroxidase increased in ginger 500 and combined groups (Figure 9 A&B).

Effects of Garlic and Ginger on Tumor Suppressor Agent

Effect of garlic and ginger on tumor suppressor agent; *PTEN* were evaluated. Expression of *PTEN* as a tumor suppressor increased just in the combined group (Figure 10).

Effects of Garlic and Ginger on Cancer Resistant Agent

Effect of garlic and ginger on the cancer-resistant agent; *HER2* was evaluated. Expression of *HER2* decreased in all treated groups (Figure 11).

Effects of Garlic and Ginger on Cullin3

The effect of garlic and ginger was evaluated. Expression of cullin3 did not show any difference in all groups (Figure 12).

Discussion

Breast cancer is the most common malignancy with a high mortality in females worldwide [44]. Therefor it is important to understand the mechanisms and genes involved in it, and find target genes for treatment. To identify breast cancer–related genes, we conduct analysis and prediction of breast cancer related genes based on the PPI network and KEGG pathway because PPIs are proven to be very useful in disease-gene prediction. Then we analyze breast cancer–related genes from other aspects:

Table 3. The Size of Tumor in Different Weeks

	Week 1	Week 2	Week 3
Control	12±0.7	19±1.2 *	23± 1.5 *
Tamoxifen 10 mg/kg	11.915± 0.712663	9.98± 0.716367	9.244± 1.109599
Garlic 50 mg/kg	8.83±1.129239	8.914444± 0.878549	7.906667± 1.411339
Garlic 100 mg/kg	10.219± 0.826006	8.109± 1.18897	4.757143± 2.153308
Ginger 100 mg/kg	9.92± 1	12.6± 0.89	10.985± 1.541532
Ginger 500 mg/kg	7.222857± 1.137268	11.31429± 1.090494	12.03333± 1.672193
Garlic 100 & ginger 500 mg/kg	9.557778± 0.905362	11.69222± 1.148117	11.145± 1.090612

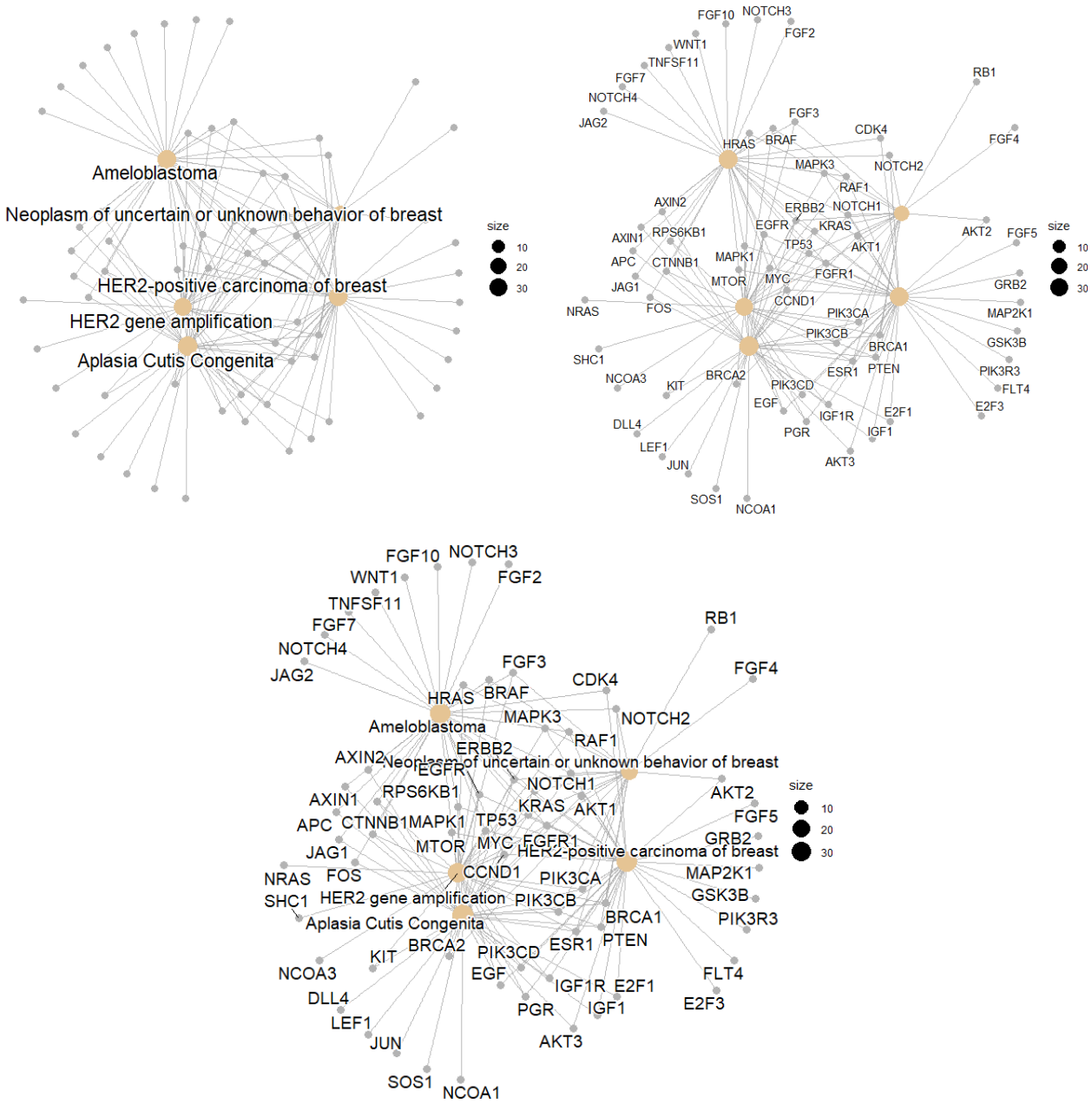

Figure 4. Top 5 GO Terms as a Network Plot. A) GO terms as a network plot. B) Genes as a network plot C) GO terms and genes as a network plot.

enrichment analysis. We labeled known breast cancer related genes in the networks of the KEGG pathways and found that *PTEN* was one of the most important gene in this pathway.

This study identified that *PTEN* is a targets gene in breast cancer and found molecular mechanisms of it using an integrative bioinformatics analysis. Then we evaluated effect of garlic and ginger on *PTEN*. With various dosages of ginger and garlic we were able to reduce tumor growth, increase the amount of glutathione reductase and peroxidase, decrease expression of *HER2*, and increase expression of *PTEN* as a tumor suppressor.

Tumor suppressor genes play a vital role in preserving the integrity of the genome and regulating the cell cycle. *PTEN*, a phosphatase, was the initial tumor suppressor to be discovered, exhibiting a wide range of activities such as controlling the cell cycle, apoptosis, and metastasis [45-48]. The presence of mutations or a decrease in the expression of the *PTEN* gene is linked to a diverse range of human tumors [49]. Prior research has established the significance of the PI3K/AKT/*PTEN* pathway in carcinogenesis. The PI3K signaling pathway is implicated in several facets of tumor biology, encompassing cell transformation, growth, proliferation, migration, evasion of apoptosis, genomic instability, angiogenesis, metastasis, and the care of cancer stem cells [50, 51]. The protein *PTEN* facilitates the degradation of the enzyme PI3K by catalyzing the removal of phosphate groups from phosphatidylinositol 3,4,5 trisphosphate and phosphatidylinositol 3,4 bisphosphate at the 3' position [52]. The impairment or decreased expression of *PTEN* results in the buildup of essential messenger lipids, thus

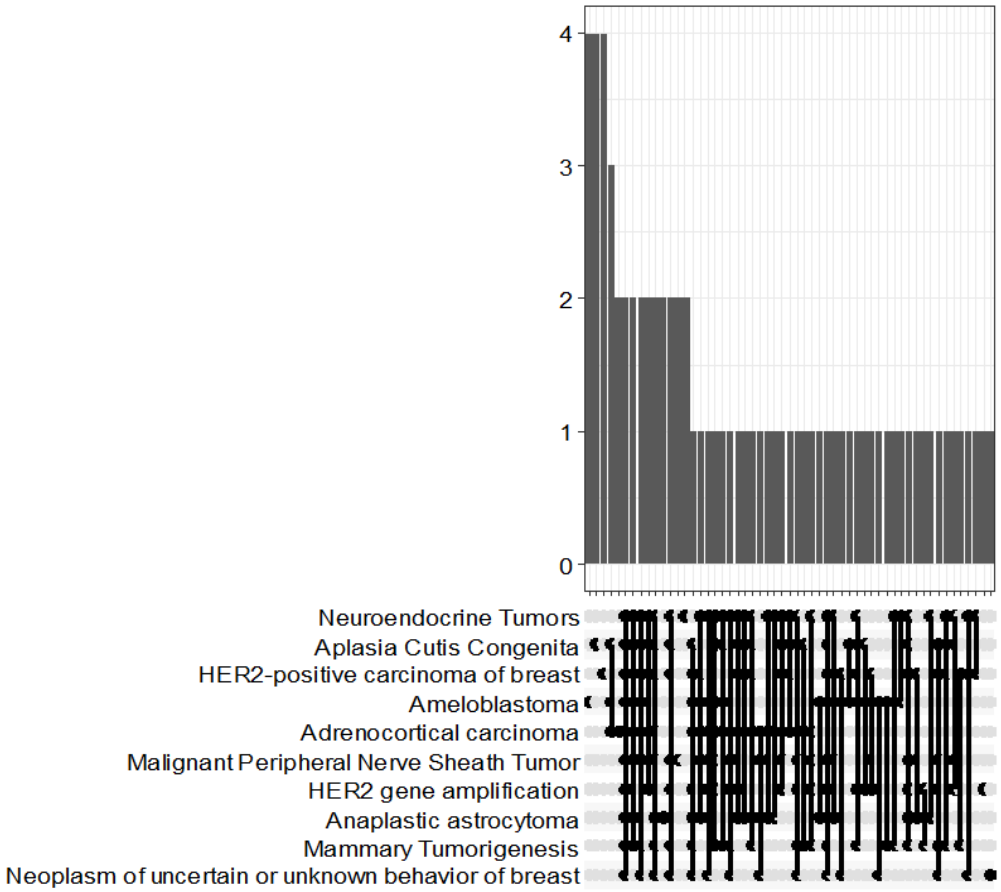

Figure 5. Upset Plot of 10 GO Terms.

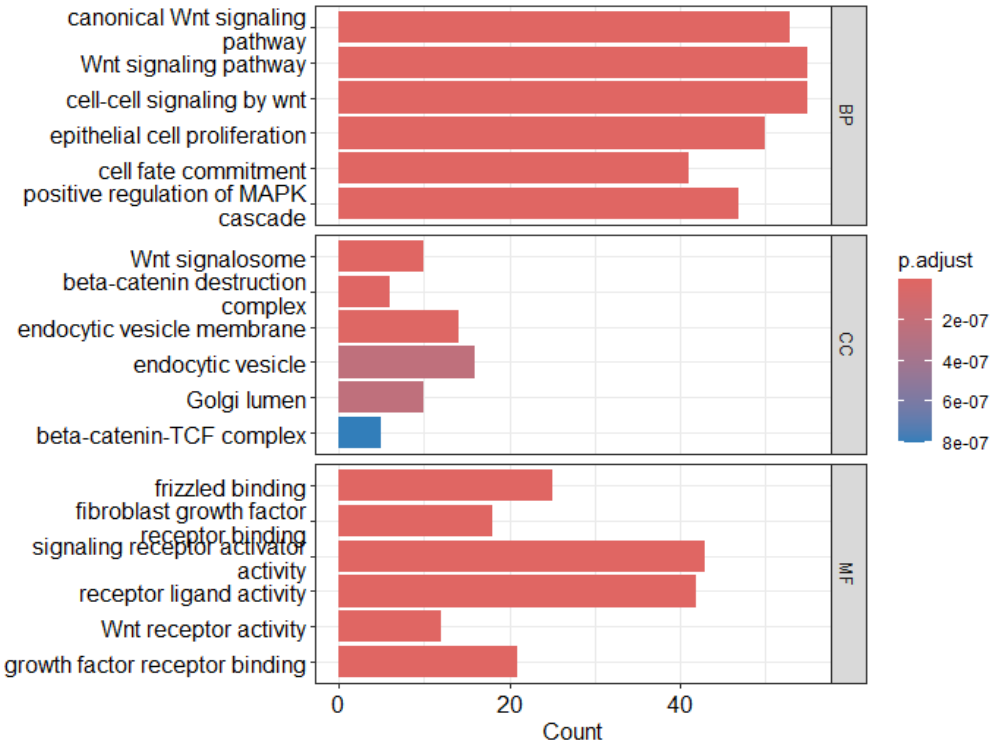

Figure 6. The Bar Plots of the Top 6 Enriched Functions Include BP (biological process), CC (cellular component), and MF (molecular function). The gene-set count is shown on the X axis, while the gene-set function is shown on the Y axis; the bar color, which ranges from red (most significant) to blue (least significant), indicates the adjusted p value.

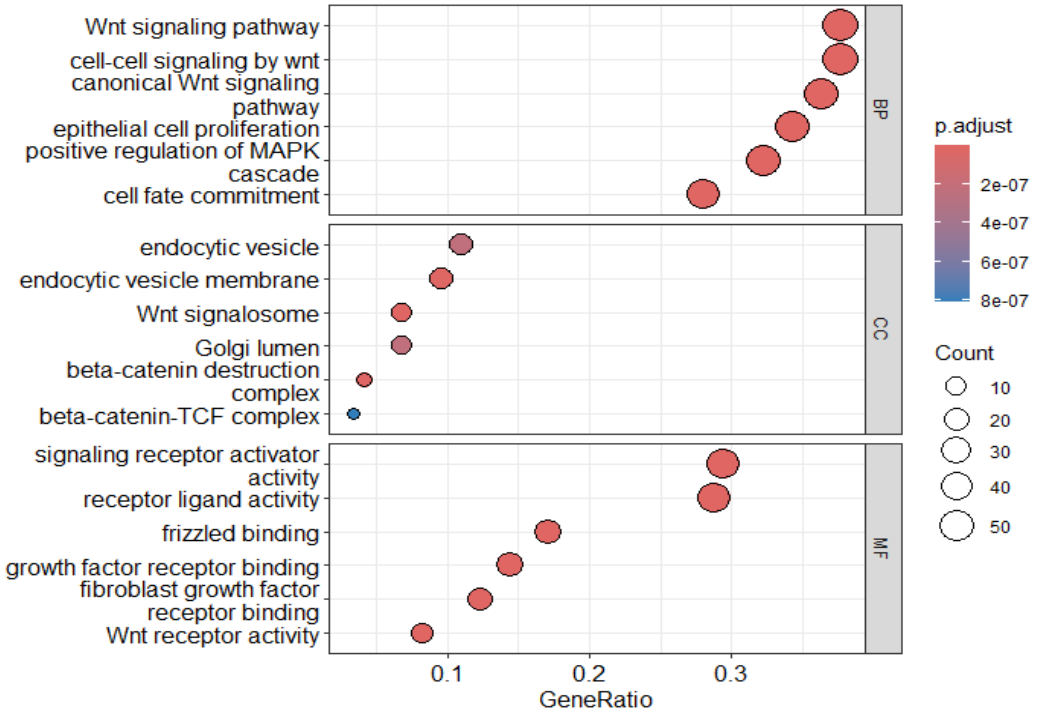

Figure 7. The Dot Plots of Top 6 Enriched Functions. The gene-set count is shown on the X axis, while the gene-set function is shown on the Y axis. The color of the dot reflects the adjusted p value, and ranges from dark blue (most significant) to red (least significant). GeneRatio is represented by the dot size.

augmenting AKT phosphorylation and activity. This, in turn, leads to a decrease in programmed cell death and/or an increase in mitogen signaling [53-57].

A further investigation demonstrated a correlation between the depletion of *PTEN* and the occurrence of metastasis in individuals diagnosed with endometrial cancer [58], and in prostate and breast cancer cell lines [59]. The study conducted by Zhang et al. provided evidence that *PTEN* impedes the invasion and metastasis of gastric cancer cells by hindering the PI3K/NFkB

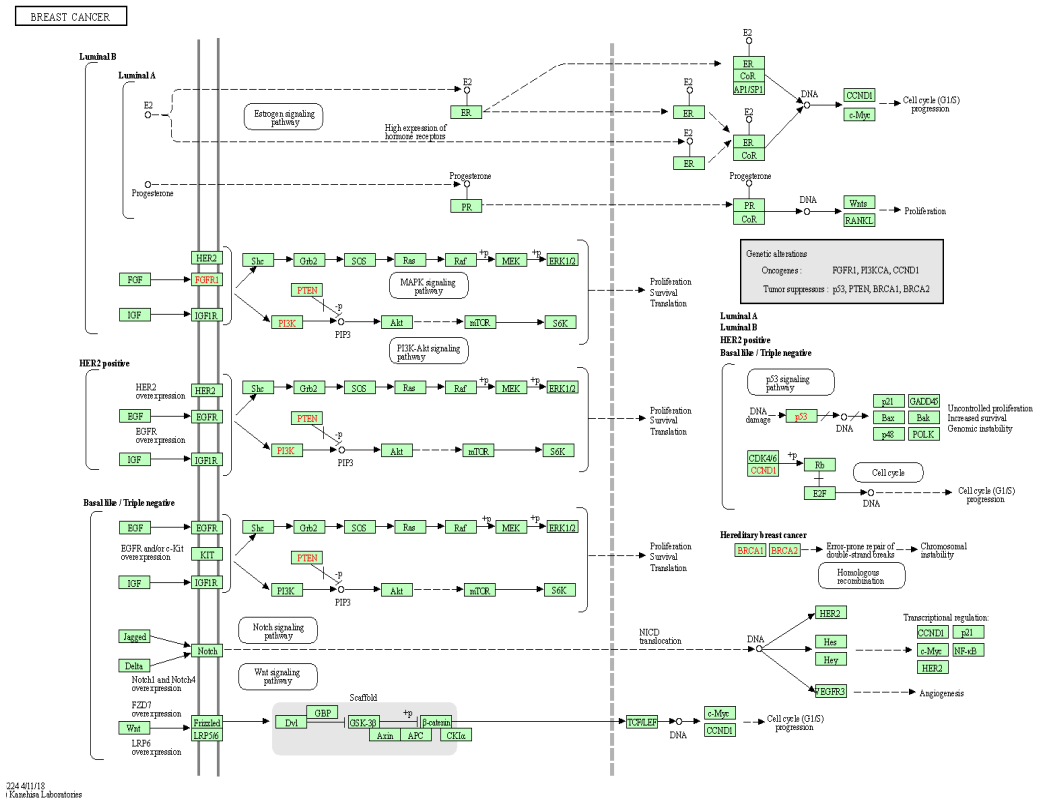

Figure 8. Visualization of the Pathways (Retrieved from KEGG).

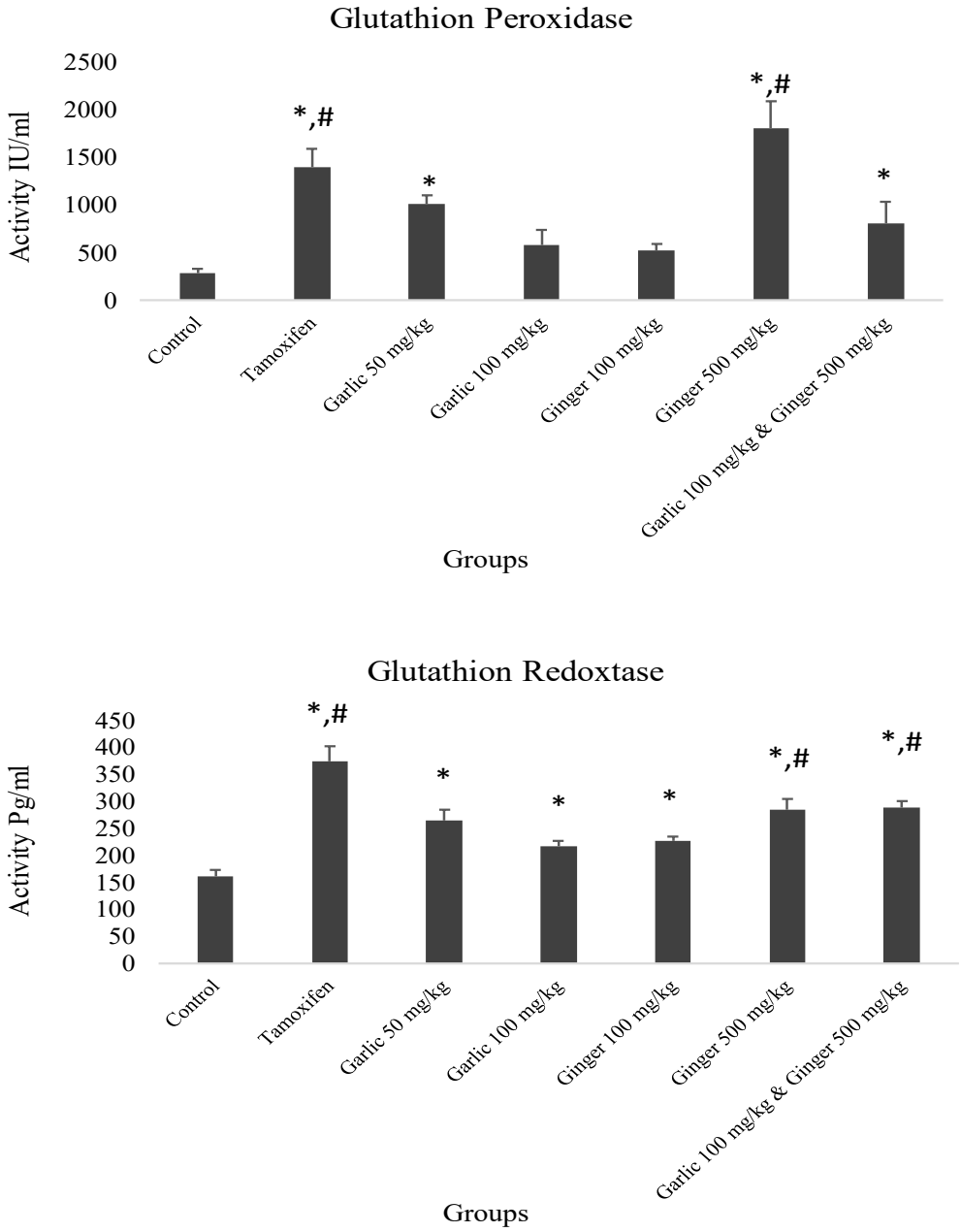

Figure 9. Glutathione Peroxidase Increased in Ginger 500 and Combined Groups. The amount of glutathione reductase as an antioxidant agent showed a significant increase in all groups especially in ginger 500 and combined groups. Data are presented as means±SEM. *P<0.05 compared to control group. #P<0.05 compared to other groups.

pathway and limiting the DNA binding of NFkB on the FAK promoter [60]. Nevertheless, some investigations indicate that *PTEN* effectively suppressed the PI3K/AKT/NFκB signaling pathway in human glioma cells [61], and prostate cancer cells [62]. According to the literature, *PTEN* has been identified as a tumor suppressor that triggers apoptosis by activating caspase-3 [63]. This entity functions as a suppressor of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway [64]. Wan et al. demonstrated a downregulation of *PTEN* expression in hepatocellular carcinoma (HCC) [65]. According to Yothaisong et al., it has been observed that the expression of *PTEN* is diminished or completely absent in roughly 70% of cholangiocarcinoma cases [66]. The *PTEN* gene has been observed to undergo frequent genetic changes and experience loss of expression in several types of human malignancies [67].

Several other researches have assessed the impact of herbal medicines on breast cancer and have demonstrated their potential to enhance the expression of *PTEN*. The study conducted by Li et al. showed that Berberine, when employed as an anti-tumor drug, resulted in an upregulation of *PTEN*, subsequently leading to a down-regulation of the PI3K/Akt/mTOR pathway [68]. This molecular cascade ultimately resulted in cell cycle arrest and the induction of autophagy in SW480 cells. The study conducted by Dong et al. showed that garlic extract exerted a regulatory effect on the pPI3/Akt signaling

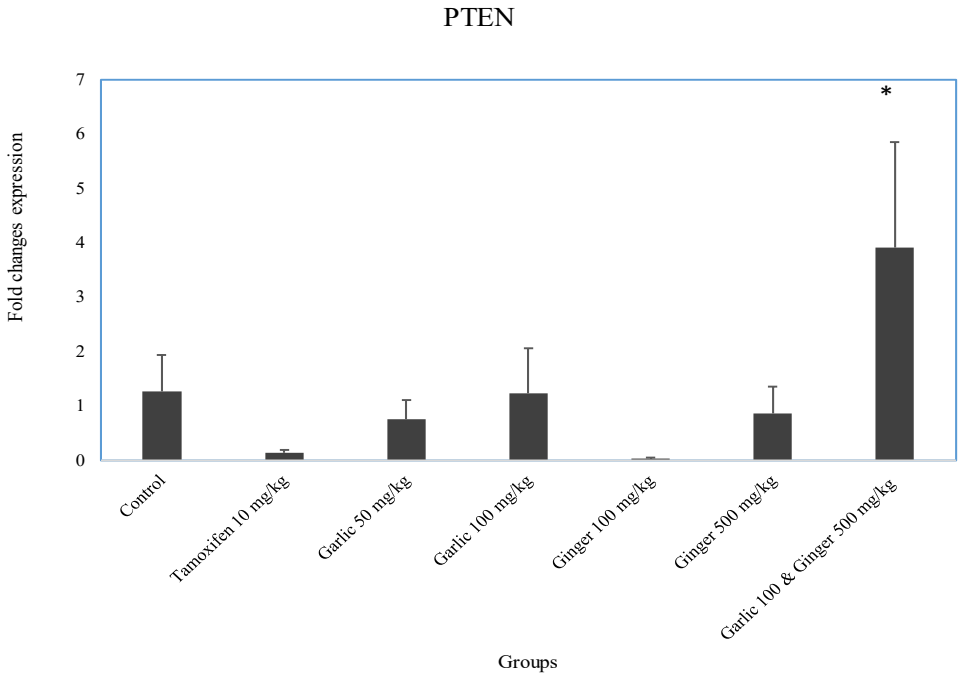

Figure 10. Effect of Garlic and Ginger on Tumor Suppressor Agent; *PTEN* were evaluated. Expression of *PTEN* as a tumor suppressor increased just in combined group. Data are presented as means±SEM. *P<0.05 compared to control group.

pathway in HT29 cells [8]. This effect was achieved by the overexpression of *PTEN* expression and the downregulation of Akt expression. According to Kiptiyah et al. ginger extract can trigger apoptosis and hinder the cell cycle progression in cumulus cells. This effect is achieved through the involvement of HTR1A, which leads to the inactivation of GSK3B and AKT-1 proteins and the activation of *PTEN*. [63]. In their study, Wang et al. demonstrated the anticancer effects of 10-Acetoxychavicol acetate (ACA), which was isolated from the rhizomes of tropical ginger. The researchers found that ACA exhibited efficacy against a diverse range of malignancies [69]. The study conducted by Aloliqi et al. showed that the compound 6-gingerol can reduce the development of colon cancer produced by AOM. This effect is achieved by reducing oxidative stress and inflammation, preserving

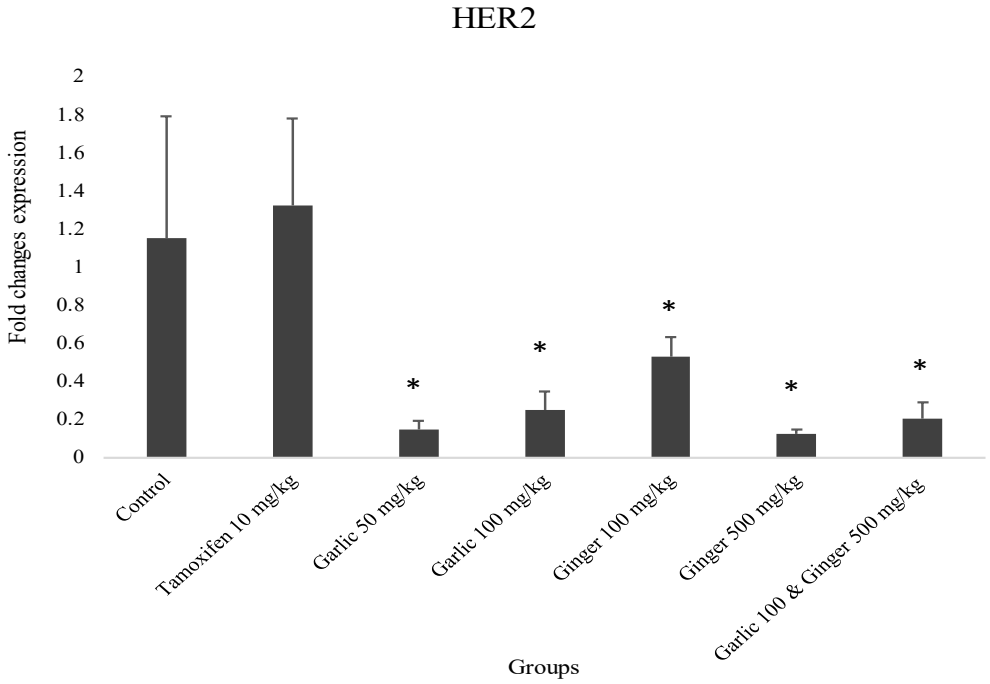

Figure 11. Effect of Garlic and Ginger on Cancer Resistant Agent; *HER2* were evaluated. Expression of *HER2* decreased in all treated groups. Data are presented as means±SEM. *P<0.05 compared to other groups.

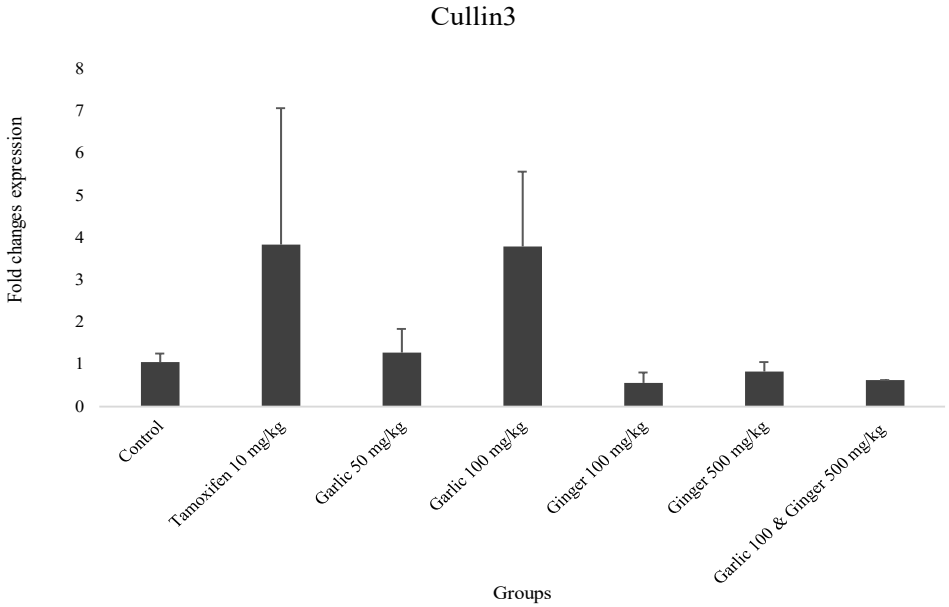

Figure 12. Effects of Garlic and Ginger on were Evaluated. Expression of cullin3 did not show any difference in all groups. Data are presented as means±SEM.

the structural integrity of the colon tissue and maintaining the expression of the *PTEN* protein [70].

Some studies evaluated the effect of herbal medicines on breast cancer alone, here we wanted to investigate the combined effect of garlic and ginger on breast cancer and expression of *PTEN*. Our results showed that the combination of garlic and ginger extract could significantly increase *PTEN* gene expression and suppress tumor growth. So they can have a synergistic effect.

Furthermore, there have been research highlighting the antioxidant capabilities of ginger, which therefore contribute to its potential anti-cancer effects. According to a study, individuals who consistently ingested ginger extract exhibited elevated levels of antioxidant enzymes and reduced levels of oxidative stress in their bloodstream [71]. The study conducted by the author demonstrates that allicin has the potential to mitigate the harmful effects of ethanol-induced liver damage by upregulating the expression of hepatic glutathione (GSH) and GSH-related enzyme systems [72]. According to the cited study, it can be inferred that allicin has the ability to augment antioxidation and detoxifying capacities in a manner that is dependent on the dosage [73]. Additionally, there have been research highlighting the antioxidant capabilities of ginger, which therefore contribute to its potential as an anti-cancer agent. Research findings indicate that the administration of ginger extract as a regular dietary supplement to individuals undergoing chemotherapy might lead to elevated concentrations of antioxidant enzymes in the bloodstream. These enzymes include superoxide dismutase, catalase, and glutathione peroxidase, as demonstrated by many studies [71]. The present study demonstrated a considerable rise in the levels of antioxidant enzymes across various treatment groups. The concurrent use of garlic and ginger exhibits significantly enhanced effects on glutathione reductase, although ginger 500 shown comparable efficacy. In the context of glutathione peroxidase, it was shown that the administration of Ginger 500 had a more pronounced impact compared to the combination group. Hence, it appears that the administration of a 500 dosage of ginger yields more efficacy. Consequently, further investigation is required to explore varying dosages of both garlic and ginger, necessitating additional experimentation.

The *HER2* receptor is classified as a transmembrane tyrosine kinase, which, upon activation, exerts an influence on cellular proliferation and viability [74]. The overexpression of *HER2* significantly contributes to the development and progression of breast tumors [74]. Resistance to therapy is a significant factor that influences treatment outcomes [24]. Polyphenols, a natural chemical with several advantageous qualities, might be a pivotal factor in surmounting this resistance [75]. The administration of apigenin at concentrations of 50-100 μM for 48 or 72 hours to *HER2*-positive breast cancer cells promoted G2/M cell cycle arrest and reduced cell proliferation [76]. Resveratrol at concentrations ranging from 4.4 to 50 mM for 4 hours effectively inhibited cell proliferation in human and animal *HER2*-positive breast cancer cells by downregulating the expression of *HER2* [77]. Quercetin-3 methyl ether was shown to significantly increase the apoptosis rate in SKBR3 breast cancer cells that overexpress *HER2* [78]. The *HER2*-positive MDAMB453 breast cancer cells exhibited cell cycle arrest and activation of apoptosis upon treatment with Kaempferol, a naturally occurring flavonol known for its anti-carcinogenic properties. This treatment also led to an up-regulation of p53 [79]. The findings of our study indicate that the administration of garlic and ginger extract resulted in a significant decrease in the expression of the *HER2* gene across all experimental groups. No significant differences were seen across various dosages of ginger, garlic, and their combination. All of the options demonstrated equal effectiveness. Nevertheless, it has

been suggested that ginger or garlic may be able to mitigate and overcome *HER2* as a tumor-resistant agent.

The expression of the Cullin-3 protein is correlated with the advancement of breast cancer [30]. The observed upregulation was linked with a reduction in the protein expression of Nrf2 [30]. It is well established that Nrf2 regulates many antioxidant enzymes, as evidenced by previous studies [65]. The inhibition of Cullin-3, leading to the stabilization of Nrf2, was associated with an elevated level of resistance to oxidative stress [80]. The study conducted by Haagenson et al. demonstrated an upregulation of Cullin-3 expression in both the xenograft breast cancer model and clinical samples, indicating its increased expression during disease development [30]. There were no observed differences in Cullin3 gene expression between the groups subjected to treatment with garlic and ginger extract.

In conclusion, this study has demonstrated the involvement of *PTEN* in breast cancer and its potential as a therapeutic target for this disease. Additionally, our research has shown that using garlic and ginger in managing cancer has potential benefits by modulating antioxidant levels and some associated genes in mice. This study demonstrated that the concurrent administration of a combination of garlic and ginger did not induce any further alterations in this domain, except an observed elevation in *PTEN* expression within the combination group. Further research is required to ascertain the optimal dosage levels and elucidate the underlying processes of this issue.

Author Contribution Statement

Conceptualization was conducted by Katayoon Sheybatzadeh, Seyed Ali Asghar Moshtaghie, Kahin Shahanipour; formal analysis was performed by Seyed Ali Asghar Moshtaghie, Kahin Shahanipour, Fereshteh Golab; funding acquisition was carried out by; Katayoon Sheybatzadeh, Seyed Ali Asghar Moshtaghie 1, Kahin Shahanipour , Fereshteh Golab; investigation was conducted by; Seyed Ali Asghar Moshtaghie, Kahin Shahanipour, Fereshteh Golab; resources were collected by Katayoon Sheybatzadeh, Seyed Ali Asghar Moshtaghie; writing of the original draft was performed by; Katayoon Sheybatzadeh 1, Seyed Ali Asghar Moshtaghie, Kahin Shahanipour, Fereshteh Golab. writing, review, and editing were conducted by Fereshteh Golab.

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Ethical Approval

All animal experiments were conducted in accordance with national guidelines and protocols after approval from the Institutional Animal Ethics Committee. All experimental protocols were approved by the Iran University of Medical Sciences (IUMS) Ethics Committee. The ethical number of this project is "IR.IAU.SRB.REC.1398.089".

Availability of Data

Data are available by request to the corresponding author.

Conflict of Interest

The authors declare no potential conflicts of interest.

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