Integrating Network Pharmacology, Molecular Docking, and Experimental Validation to Investigate the Mechanism of (-)-Guaiol Against Lung Adenocarcinoma

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Background: Lung adenocarcinoma (LUAD) is the most common type of lung cancer, which poses a serious threat to human life and health. (-)-Guaiol, an effective ingredient of many medicinal herbs, has been shown to have a high potential for tumor interference and suppression. However, knowledge of pharmacological mechanisms is still lacking adequate identification or interpretation.

Material/Methods: The genes of LUAD patients collected from TCGA were analyzed using limma and WGCNA. In addition, targets of (-)-Guaiol treating LUAD were selected through a prediction network. Venn analysis was then used to visualize the overlapping genes, which were further condensed using the PPI network. GO and KEGG analyses were performed sequentially, and the essential targets were evaluated and validated using molecular docking. In addition, cell-based verification, including the CCK-8 assay, cell death assessment, apoptosis analysis, and western blot, was performed to determine the mechanism of action of (-)-Guaiol.

Results: The genes included 959 differentially-expressed genes, 6075 highly-correlated genes, and 480 drug-target genes. Through multivariate analysis, 23 hub genes were identified and functional enrichment analyses revealed that the PI3K/Akt signaling pathway was the most significant. Experiment results showed that (-)-Guaiol can inhibit LUAD cell growth and induce apoptosis. Additional evidence suggested that the PI3K/Akt signaling pathway established an inseparable role in the antitumor processes of (-)-Guaiol, which is consistent with network pharmacology results.

Conclusions: Our results show that the effect of (-)-Guaiol in LUAD treatment involves the PI3K/Akt signaling pathway, providing a useful reference and medicinal value in the treatment of LUAD.

Keywords: Lung Neoplasms • Pharmacology • Signal Transduction

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DATABASE ANALYSIS

Background

According to the latest survey of American Cancer Society [1], lung cancer is still the leading cause of cancer-related death for both men and women (22% for men and 22% for women), with a high rate of metastases. This malignant disease, which has high morbidity and mortality rates, is classified into 2 subtypes: small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). Lung adenocarcinoma (LUAD), a subtype of NSCLC, is the most common subtype of lung cancer, accounting for 40% of all cases [2]. LUAD treatment differs depending on the stage of the disease. Surgical resection or radiotherapy is considered at the early stage [3]. Patients with advanced-stage disease are treated with chemotherapeutic medications such as platinum agents, taxanes, and therapies that target oncogenic mutation drivers [4]. Unfortunately, the clinical benefit of conventional therapy is limited by toxicity and acquired resistance, causing recurrence and distant metastasis. Patients are eventually susceptible to disease progression, with a poor prognosis. From 2010 to 2016, the 5-year survival rate for metastatic LUAD was only 6% [1]. It is essential to develop new drugs for combination therapy, which is considered a more effective and advanced treatment for overcoming resistance rather than monotherapies.

(-)-Guaiol, which had distinct antibacterial activity, is a natural compound found in medicinal plants. The formula of (-)-Guaiol is $C_{10}H_{16}O$, and the molecular weight is 222.37g/mol [5]. This sesqui-terpene alcohol with the guaiane skeleton has been used for thousands of years as a traditional Chinese medicine and is the main active ingredient found in Alisma orientale [6], Ferula ferioroides [7], Aloysia gratissima [8], and other traditional medicinal plants. (-)-Guaiol is a naturally occurring insecticidal product because of its mosquito larvicidal activity, anti-microbial activity, and anti-leishmania activity [9-11]. However, recent research indicates that (-)-Guaiol has a high potential for antitumor activity [12-14], although the molecular mechanisms are still unclear. In our previous study, the pharmacological investigation of antitumor mechanisms was focused on the treatment of lung cancer. (-)-Guaiol impairs mTOR signaling to induce autophagy in lung cancer cells [15,16], which should encourage more LUAD-related research. PI3K/Akt is one of the important signaling pathways in intracellular processes; it is known to inhibit apoptosis and to mediate cell survival. The literature has indicated that inhibiting the PI3K/Akt signaling pathway can reverse resistance caused by cisplatin in lung cancer [17], and it can activate downstream signaling molecules when activated inappropriately, affecting the occurrence and progression of lung cancer [18]. It is intriguing that (-)-Guaiol inhibits the proliferation of LUAD cells by specifically targeting mTOR, which is one of the downstream signaling molecules of the PI3K/Akt signaling pathway. Therefore, we conducted a series of investigations to further explore this topic.

In this study, a network was built using network pharmacology, weighted gene co-expression network analysis (WGCNA), and a system bioinformatics approach, which was then validated using molecular docking and experiments. The main goal of the current study was to determine the anticancer effect and molecular mechanism of (-)-Guaiol against LUAD, which complements those of earlier studies. A general study flow chart is shown in Figure 1.

Material and Methods

Identification of Differentially-Expressed Genes

The level 3 RNA sequencing (RNA-seq) profiles were downloaded from the The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) by searching the following keywords “bronchus and lung”, “adenomas and adenocarcinomas”, and “TCGA-LUAD”, consisting of 375 LUAD tissues and 32 adjacent normal tissues transcriptome profiles. After processing the available data with the limma [19] (v: 3.42.2) R package, differentially-expressed genes (DEGs) between tumor and normal tissues were specifically identified in the TCGA cohort, with a false-discovery rate of 0.05.

WGCNA Module Construction

Additionally, WGCNA, a systems biology modality for distinguishing the correlation patterns among genes across microarray samples [20], was used to screen the highly-correlated genes from RNA-seq profiles for summarizing such clusters and correlating modules to external sample traits. To create a scale-free network, the soft-thresholding parameter was defined as $=6$ (scale-free R2=0.85) using the soft function pickSoftThreshold. Integrating 2 systems biology approaches increased the discrimination ability of candidate genes selection.

Prediction of the Targets of (-)-Guaiol

The study used PubChem [21] (https://pubchem.ncbi.nlm.nih.gov/), the world’s largest chemical information collection website, to search for the SMILES structural formula of (-)-Guaiol. The obtained structure was then fed into ChemMapper [22] (http://llab.ecust.edu.cn/chemmapper/) to predict corresponding targets. The names of the corresponding targets were normalized in the Uniprot [23] database (https://www.uniprot.org/). Finally, using Cytoscape (v: 3.7.2) software, all attribute data were combined and the “disease-drug-target” network was visualized.

Co-Expression Network Construction and Screening of Hub Genes

After thoroughly preparing for data collection, a co-expression network was created, which connected 3 major gene cohorts...
Figure 1. In the flow chart, the systems pharmacology approach was used to determine the mechanism of (-)-Guaiol in the treatment of LUAD by integrating target identification, network construction, enrichment analysis, molecular docking and experimental validations.
(DEGs, WGCNA module genes, and (-)-Guaiol target genes). The network was displayed by a Venn diagram created with the R package VennDiagram [24] (v: 1.6.20). Following that, the co-expression genes were entered into Cytoscape (v: 3.7.2) software for protein–protein interactions (PPI) network construction. They were processed using “Biogenet” [25] (v: 3.0.0), an application for visualizing the relationships between multiple biomolecules, and CytoNCA [26] (v: 2.1.6) for topological analysis. In the network, only eligible genes can be selected. The selection criteria were set as: Interaction Degree (DC) >50 and Betweenness (BC) >100. After double standard screening, the genes that met the selection criteria were ultimately enrolled as key biological targets in the subsequent research.

**Enrichment Analysis**

System functional enrichment analyses were performed to elucidate the biological functions and signaling pathways associated with (-)-Guaiol against LUAD to gain more insights into the regulation of selected genes. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) are knowledge-base resources. GO provides information on biological processes (BPs), cellular components (CCs), and molecular functions (MFs), while KEGG connects genomic information with higher-order functional intelligence [27,28]. GO and KEGG pathway analyses with a P value of 0.05 were performed using 3 R packages: clusterProfiler [29] (v: 3.14.3), org.Hs.eg.db (v: 3.10.0), and enrichplot (v: 1.6.1). The R package ggplot2 (v: 3.2.1) were used to create the bubble diagrams for the GO enrichment and KEGG pathway analyses.

**Molecular Docking**

The current study constructed a KEGG pathway-target relation network based on the enriched pathways with related target proteins, with only the top-ranked pathways enrolled. In addition, Cytoscape (v: 3.7.2) was used for topological analysis with the “Network Analyzer” tools. The analysis revealed that targets with a high degree score were of interest and may participate in the molecular docking analysis. Molecular docking, an in silico structure-based approach that allows the identification of active ingredients of therapeutic interest [30], was used to estimate the molecular interactions between targets and (-)-Guaiol. In addition, the chemical formulas and 3D structures of the samples, CDK2 (PDB ID: 1B39: 2.1Å), FN1 (PDB ID: 2HAZ: 1.7Å), HSP90AA1 (PDB ID: 5NJX: 2.49Å), and HSP90AB1 (PDB ID: 1UYM: 2.45Å), were obtained from the PDB (https://www.rcsb.org/). www.rcsb.org/) and PubChem [31] (https://pubchem.ncbi.nlm.nih.gov/) databases. To improve the quality of molecular docking, AutoDockTools (1.5.6) was used to add charge and display rotatable keys to the compound structures, and Pymol was used to modify the protein crystal structures corresponding to the core target genes by removing water molecules and hetero-molecules. Moreover, the AutoDockTools (1.5.6) were applied to add hydrogen atoms and charge operations. It is necessary to transform the protein format from “pdb” to “pdbqt” format and search for active pockets via AutoDock software. After completing the preparation of the ligand (the compound) and receptors (the proteins corresponding to the core target genes), docking operations with AutoDock Vina could finally be initiated. The binding affinity assisted in measuring the conformational stability as a reference.

**Chemicals and Reagents**

(-)-Guaiol was commercially obtained from Sigma (448575, St. Louis, MO, USA). DMEM medium was purchased from KeyGEN BioTECH (KGM12800-500, Jiangsu, China). Fetal bovine serum (FBS) was provided by Gibco Life Technologies (10099-141, Grand Island, NY, USA). The reagent Z-VAD-FMK (Z-VAD) was obtained from Selleck (S7023, Shanghai, China). Annexin V-FITC/PI Apoptosis Detection Kits were purchased from KeyGEN BioTECH (KGA105-KGA108, Jiangsu, China). Cell Counting Kit-8 was purchased from YEASEN (40203ES76, Shanghai, China). LY294002 was purchased from MedChemExpress (HY-10108, Monmouth Junction, New Jersey, USA). SYTOX Green was purchased from Invitrogen (S7020, Carlsbad, California, USA). The following antibodies – Bcl-2(15071, 1: 1000), BAX (5023, 1: 1000), β-actin (4970, 1: 1000), p-Akt (4060, 1: 1000), Akt (2920, 1: 1000), PI3K (4255, 1: 1000), p-PI3K (4249, 1: 1000), and IgG secondary antibody (10700, 1: 2000) – were purchased from Cell Signaling Technology (Boston, Massachusetts, USA).

**Cell Culture and Drug Administration**

The LUAD cells A549 and Calu-1 and normal lung cells BEAS-2B were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. (-)-Guaiol was diluted in methanol at a primary stock concentration of 10 mM. The control group was DMEM with 10% FBS and methanol.

**Cell Survival Assay**

Cell survival was detected with a CCK-8 assay kit. Approximately 5000 cells were seeded into a 96-well culture plate, and after 24 h, the cells were exposed to various doses of (-)-Guaiol (60 μM or 150 μM) or Z-VAD (20 μM). The solution was then removed, and all wells were washed twice with PBS before adding 10 μl CCK-8 solution to the wells for incubation. After waiting 1h, a Multiplate Reader (Bio Tek, United States) was used to measure the OD value at 450 nm. The cell survival rate (%) was calculated as [(experimental group-blank group)/control group-blank group] 100% using the average OD value of 3 wells.
DATABASE ANALYSIS

Figures:

A: A scatter plot showing logFC values against log10(adj. P value). Points are color-coded to indicate significant, downregulated, and upregulated genes.

B: A heatmap displaying gene expression levels. The heatmap is color-coded to represent different types of expression levels, with a scale from -4 to +4.
Figure 2. (A) Volcano plot of DEGs in TCGA. (B) Heat map of DEGs in TCGA. The green parts present down-regulated genes, red parts present up-regulated genes, and black parts present non-differential genes. (C) The cluster dendrogram with different colors of TCGA. (D) Module-trait relationships. Each row corresponds to a color, while each column corresponds to the normal or tumor group.
SYTOX Green staining assay was performed according to the manufacturer’s protocol, visualizing cell death through imaging applications. The working concentration was 100 nM and experiments were carried out 3 times each.

**Apoptosis Analysis**

The cells were inoculated into 6-well plates and each well contained 2 mL of media with 10% FBS and approximately 2×10⁵ cells. They were cultured for 24 h at 37°C and 5% CO₂, then treated by (-)-Guaiol (60 μM or 120 μM) for 24 h. The cells were collected after drug treatment. We added binding buffer (500 μL), Annexin V-FITC (5 μL), and propidium iodide (5 μL) to each tube, followed by incubation at room temperature in the dark for 10 min. Finally, a fluorescence microscope was used to observe the cell morphology and flow cytometry was used to detect the apoptosis rate. The experiments were carried out 3 times each.

**Western Blot Analysis**

After treatment, the cells were collected and washed twice with PBS, then added to cell lysis buffer with protease and...
phosphatase inhibitors for 30 min at 4°C. The whole protein was quantified with a bicinchoninic acid kit (Beyotime, China). Samples were obtained after boiling within the 1% SDS loading buffer at 100°C for 8 min. Afterward, 20 μg protein was subjected to SDS-PAGE and subsequently transferred to a PVDF membrane. After immersing the membrane in BCA for blocking, the samples were incubated overnight with the following primary antibodies – β-actin, p-Akt (Ser473), Akt, PI3K, p-PI3K (110ko), BAX, and Bcl-2 – diluted 1: 1000. Next, we used an anti-rabbit IgG secondary antibody (1: 2000) to conjugate each primary antibody. Finally, the samples mixed with ECL (Tanon, Shanghai, China) solution were exposed and recorded by chemiluminescence reagent. The gray values of the bands, which were calculated automatically using Image J software, were normalized to β-actin as the internal control. The relative protein levels were calculated by normalizing the densitometry results of the experimental group to those of the control group. One-way ANOVA was used to statistically analyze the significance. The experiments were carried out 3 times each.

Data Analysis

All results were obtained from experiments independently repeated 3 times. The continuous variables of normal distribution or nearly normal distribution were displayed as average value±standard deviation. After homogeneity of variance test, one-way ANOVA was used for those meeting data requirements in SPSS 25.0. or GraphPad Prism 8.0. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of DEGs and WGCNA Module in TCGA Cohort

There were 15 208 RNA-seq genes of LUAD downloaded from the TCGA cohort, with 959 genes pertaining to DEGs and 6075 genes pertaining to WGCNA key modules. The 959 DEGs were described by the volcano plot and heat map, using the R package ggplot2 [32] (v: 3.2.1) for visualization (Figure 2A, 2B). The 6075 highly-related genes were classified into 15 color modules and are presented in Figure 2C, 2D.

Target Prediction and Analysis of (-)-Guaiol

A prediction network integrating targets of (-)-Guaiol against LUAD was obtained during this process. Figure 3 depicts the “disease-drug-target” network. As a result, 480 targets were identified and are represented by the green ovals in the plot; thus far, all of the target genes have been collected under the current conditions. The file containing total gene (DEGs, WGCNA module genes and drug target genes) information was included as Supplementary Table 1.

Identification of Hub Genes

The genes were then optimized and reduced to a few hub genes in the following step of the workflow. The selected co-expression genes among DEGs, WGCNA module genes, and (-)-Guaiol target genes were presented in a Venn diagram (Figure 4A), and only 17 genes were filtered out in the plot. The 17 co-expression genes were then analyzed by PPI network-to-topology analysis. The PPI network was established for topology feature analysis with the Cytoscape software platform based on degree and betweenness parameters for further investigation. There were 23 genes that met the degree >50 and betweenness >100 thresholds (Figure 4B), which meant these genes played an essential role in (-)-Guaiol treatment of LUAD. The specific gene information on 17 identified genes and 23 hub genes is shown in Supplementary Tables 2 and 3.

GO and KEGG Enrichment Analysis

The 23 genes that emerged were used in subsequent functional enrichment analyses. The GO and KEGG enrichment analyses identified a number of related biological activities that were statistically significant (Figure 5). In total, 883 significant GO terms and 27 significant pathways were enriched (P<0.05) (Supplementary Table 4). The BP aspect changes were clearly associated with 732 functional categories, including DNA metabolic process regulation, developmental cell growth regulation, protein binding regulation, and positive cell cycle regulation. Furthermore, there were 81 terms in the CCs analysis aspect, primarily vesicle lumen, secretory granule lumen, apical plasma membrane, and membrane microdomain. There were also 70 terms in MFs, such as ubiquitin protein ligase binding, cell adhesion molecule binding, MHC class II protein complex binding, and ATPase activity. The KEGG pathway enrichments revealed that the identified targets were mainly enriched in 27 pathways, including the phosphatidylinositol3-kinase/ProteinKinase-B (PI3K/Akt) signaling pathway, protein processing in the endoplasmic reticulum, cell cycle, and antigen processing and presentation. Of note, the PI3K-Akt signaling pathway was significant, which is listed at the top of the chart.

KEGG Pathway-Target Network and Molecular Docking

The pathway-target network for (-)-Guaiol and LUAD, which shows 19 targets and 20 pathways, was built to reveal potential mechanisms of action. Based on the calculated degree, each node pertaining to the pathway or target was displayed in an ordered arrangement (Figure 6). According to the network, the top 5 candidate targets (CDK2, EGFR, FN1, HSP90AA1, HSP90AB1) were investigated further for their interaction with (-)-Guaiol; notably, these genes were also involved in the PI3K/Akt signaling pathway in KEGG analysis. Each target was docked with the (-)-Guaiol 3D structure after
Figure 4. (A) Venn diagram was used to summarize the co-expression genes of DEGs, WGCNA module genes, and \((-\)-Guaiol target genes. (B) The 23 hub genes were predicted by topological screening of the protein–protein interaction (PPI) network with screening 2 times. DC – degree; BC – betweenness.
a series of modifications. As shown in Figure 7, CDK2, FN1, HSP90AA1 and HSP90AB1 displayed a favorable binding affinity, with ideal conjunction in general. The docking score in binding affinity represents the cohesiveness of the models. It is clear that the lower binding affinity score was preferred.

**(-)-Guaiol Can Suppress LUAD Cells and Induce Apoptosis**

The CCK-8 assay was used to assess the cytotoxicity of (-)-Guaiol in A549 cells and Calu-1 cells, taking normal lung cells BEAS-2B as a control. The survival rates of A549 and Calu-1 were concentration-dependently decreased when treated with different concentrations of (-)-Guaiol, as suggested by the CCK-8 assay (Figure 8A). According to the results of GraphPad Prism, (-)-Guaiol dramatically inhibited growth of A549 cells and Calu-1 cells, with IC50 values of 75.7 μM and 92.1 μM, respectively, whereas its IC50 value for BEAS-2B cells was 322.3 μM. To investigate the apoptosis action of (-)-Guaiol, the appropriate concentration (75 μM) of (-)-Guaiol and the apoptosis inhibitor Z-VAD were used in the cell death assays. When compared to the (-)-Guaiol single group, Z-VAD significantly reversed cell viability in A549 (p=0.048) and Calu-1 cells (p=0.033) (Figure 8B). Similarly, the SYTOX Green staining assay showed that (-)-Guaiol damaged LUAD cells. Under fluorescence microscopy, the exceptional brightness of the signal produced by the (-)-Guaiol group indicated more dead cells, whereas Z-VAD weakened the fluorescence intensity, counter-acting the effect of (-)-Guaiol, which was consistent with the CCK-8 assay results (Figure 8C).

**(-)-Guaiol Increased Early Apoptosis and Altered Expression of Related Proteins**

The Annexin V-FITC/PI dual-staining assay was used for quantitative analysis to gain more insight into the apoptosis induced by (-)-Guaiol. As shown in Figure 9A, the early apoptosis of the control group, 60 μM (-)-Guaiol group, and 120 μM (-)-Guaiol group for A549 was 17.75%, 25.9%, and 35.7%, respectively, and for Calu-1 it was 1.07%, 10.9%, and 13.9%, respectively. (-)-Guaiol induced apoptosis by increasing early apoptosis, as demonstrated by the detected green fluorescence indicating PS in apoptotic cells with low red fluorescence in the image.
and the proportion of cells with a higher level of green and lower level of red fluorescence was higher in the flow cytometry analysis (Figure 9B). According to western blot analysis, (-)-Guaiol inhibited the expression of the anti-apoptotic protein Bcl-2 (p<0.001) while increasing the expression of the pro-apoptotic protein BAX (p=0.028) (Figure 9C).

**(-)-Guaiol Regulates the Expression of Related Proteins in PI3K/Akt Signaling Pathway**

The PI3K/Akt signaling pathway, which includes classical proteins PI3K, phosphorylated PI3K, Akt, and phosphorylated Akt, was found to be highly likely to be involved in the mechanisms of (-)-Guaiol’s anti-LUAD activity. To validate the conclusion, western blotting was used to examine the protein expression of PI3K/Akt signaling in A549 cells treated with (-)-Guaiol (75 μM) and LY294002 (15 μM), a PI3K/Akt signaling pathway inhibitor. The expression of total PI3K and Akt was little changed (P>0.05). Treatment with (-)-Guaiol decreased p-PI3K (P=0.001) and p-Akt (P<0.001) levels and furthered the LY294002-induced decrease in p-PI3K (P<0.001) and p-Akt levels (P=0.004), as expected (Figure 10). These findings suggest that (-)-Guaiol inhibits the PI3K/Akt signaling pathway, which regulates apoptosis in A549 cells, thereby validating the network pharmacology result.

**Discussion**

Lung cancer is the most prevalent human malignancy worldwide; historically, LUAD became the most common subtype of lung cancer, outranking squamous cell carcinoma (SCC) by 1988 to 2002 [33]. As a result, numerous studies on LUAD treatment have been conducted. Medical research combined with network pharmacology is an appealing approach that is becoming increasingly popular in scientific research fields [34]. In any case, a better understanding of the underlying mechanism is essential for guiding effective and precise therapy.
(−)-Guaiol has shown potent antineoplastic activity, but the molecular mechanism has yet to be fully understood. To clarify the underlying mechanism, a network was constructed based on the significant genes, and through a series of network analyses, pivot targets and pathways were discovered. First, the gene information of LUAD patients was collected using the publicly available database TCGA, which was then processed using computational methods to generate 959 DEGs between tumor and non-tumor tissues, as well as 6075 genes highly correlated with WGCNA. At the same time, network pharmacology identified another 480 (−)-Guaiol target genes. These were extracted and shrunk to 17 co-expression genes, and they eventually yielded 23 hub genes through topological data analysis of PPI. Based on the findings, a GO biological process and KEGG pathway enrichment analyses were performed to investigate the potential mechanisms. However, there are some limitations to our study. As we know, bioinformatics has opened up a new field of pharmacological research that develops computing technology and networks public databases for analysis of biological data. Researchers use network databases for data collection, but some related genes may still have been missed, which is unavoidable. Only strongly correlated and significant genes can be used for subsequent analysis. Bioinformatics analysis provides significant options, although it may not be comprehensive. Based on the result of the enrichment analysis, we mainly focused on the PI3K/Akt pathway.

**Figure 6.** KEGG pathway-target network. The orange round nodes represent the top 19 related targets and the green round nodes represent the top 20 pathways.
Figure 7. Molecular docking diagrams of (-)-Guaiol with (A) CDK2, (B) EGFR, (C) HSP90AA1, (D) HSP90AB1, and (E) FN1. (F) The binding affinity for the 5 targets docked into the (-)-Guaiol crystal structure.

| Targets     | Binding affinity (Kcal·mol⁻¹) |
|-------------|-------------------------------|
| CDK2        | -8                            |
| EGFR        | -4.7                          |
| HSP90A      | -5.9                          |
| HSP90AB     | -6.9                          |
| FN1         | -7.3                          |

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**Figure 8.** (A) Cell survival rate was detected by performing a CCK-8 assay. The LUAD cells (A549 and Calu-1) and normal lung cells BEAS-2B cells treated with different concentrations of (-)-Guaiol. IC50 values were 75.7 μM for A549 cells, 92.1 μM for H1299 cells, and 322.3 μM for BEAS-2B cells. Data from 3 independent experiments were represented as means±SD. (B) A549 and Calu-1 were treated with (-)-Guaiol (75 μM) or (-)-Guaiol (75 μM) companied Z-VAD (20 μM) for 24 h. ** P<0.01 vs control in A549. # P<0.05 vs (-)-Guaiol in Calu-1. Data from 3 independent experiments were represented as means±SD. (C) A549 and Calu-1 were treated with or without (-)-Guaiol (75 μM) or (-)-Guaiol (75 μM) and Z-VAD (20 μM), and stained with SYTOX Green (100 nM), then observed under a fluorescence microscope.
signaling pathway, which was the most significant pathway in enrichment analysis.

The study was then completed with validation, which included molecular docking and experiments. The molecular docking study confirmed that the top 5 candidate targets from the KEGG pathway-target network (CDK2, EGFR, FN1, HSP90AA1, HSP90AB1), which also were found to be involved in the PI3K/Akt signaling pathway, had a high affinity for (-)-Guaiol, enhancing the results of network pharmacology. Specifically, Cyclin-dependent kinase 2 (CDK2) plays a vital role in cycle regulation and DNA damage response. In addition, CDK2 induces apoptosis by targeting FOXO1, a pivotal protein in triggering DNA-damage-induced apoptosis following dsDNA breaks [35], but CDK2 acts has opposite effects in cell apoptosis. In KRAS-mutant lung cancer models, inhibiting CDK2 kinase activity causes anaphase catastrophe and apoptosis [36]. The epidermal growth factor receptor (EGFR) is another cell growth regulator that belongs to the receptor tyrosine kinase (RTK) family, stimulating specific downstream signaling pathways such as PI3K/Akt, RAS/RAF/MAPK, and JAK/ATAT [37]. EGFR, which has been identified as an oncogenic driver of NSCLC, is associated with advanced disease stage and poor prognosis [38]. Coincidentally, fibronectin 1 (FN1), heat shock protein 90AA1 (HSP90AA1), and heat shock protein 90AB1 (HSP90AB1) are overexpressed in cancer, predicting a poor prognosis [39-41].

Furthermore, a series of experiments were carried out to investigate the PI3K/Akt pathway and effect of (-)-Guaiol in LUAD. The PI3K/Akt pathway, which had the lowest P value among the 27 candidate pathways in functional analysis, has a close relationship with malignant tumors because it regulates cell proliferation, differentiation, and metabolism [42]. One of the consequences of this process is apoptotic inhibition, which can lead to cell dysfunction and mutation. As a result, a number of PI3K and Akt inhibitors for lung cancer are being studied [43]. In LUAD cells, (-)-Guaiol also inhibits the PI3K/Akt pathway and regulates apoptosis.

RTK stimulation and somatic mutations in specific elements pathway elements are the 2 most common mechanisms of PI3K/Akt activation in human cancers [44]. However, somatic mutations and amplifications of the PIK3CA gene, which encodes the class I PI3K p110α, frequently occur in patients with NSCLC. Specifically, somatic mutations in PI3KCA are found in 3% to 10% of squamous cell carcinomas and 0% to 2.7% of adenocarcinomas, while PI3KCA amplification is found in 35% of squamous cell carcinomas and 7% of adenocarcinomas [45,46]. Moreover, laboratory research investigated various lung cancer cell lines and tumor tissues, including 86 NSCLC cell lines, 43 SCLC cell lines, 3 extrapulmonary small-cell cancer (ExPuSC) cell lines, and 691 resected NSCLC tumors, showing that 12.8% of cell lines and 19.1% of tumors were associated...
DATABASE ANALYSIS

B

A549

Control 60 µM 120 µM

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DATABASE ANALYSIS
with PI3KCA mutations or amplification [47]. Furthermore, high phosphorylated Akt expression was found in a significant proportion of NSCLC patients, which influenced PI3KCA mutations or amplification [43,47].

The aberrantly activated PI3K/Akt signaling pathway triggers a variety of molecular mechanisms, resulting in a series of changes in cell metabolism and cell growth. Active Akt has been shown to promote cell cycle progression and inhibit apoptosis via a variety of biological mechanisms, including phosphorylation of intermediate targets (GSK3, FOXO, BAD, and MDM2), inhibition of NFκB, and activation of mTOR [43]. The Bcl-2 family, a related downstream pathway component, is well known as an apoptosis regulator. The Bcl-2 family plays 2 roles in apoptotic regulation: anti-apoptotic executor and pro-apoptotic executor [48]. Bcl-2 and Bcl-xl are anti-apoptotic proteins that sequester...
caspases, a unique set of cysteine proteases that cleave critical cellular proteins to cause apoptotic changes or prevent mitochondrial apoptogenic factors from release into the cytoplasm. Other members of the Bcl-2 family, such as BAX and Bak, are responsible for pro-apoptotic functions such as facilitating caspase release and activation depending on mitochondrial outer-membrane permeabilization (MOMP) [49]. According to the findings, (-)-Guaiol inhibited Bcl-2 expression while increasing BAX expression, resulting in lung cancer cell apoptosis.

(-)-Guaiol was found to kill LUAD cells by inhibiting the PI3K/Akt signaling pathway and inducing cell apoptosis. However, (-)-Guaiol has little influence on normal lung cells. The apoptosis inhibitor Z-VAD was used to investigate the apoptosis effect of (-)-Guaiol in cell death assays. The CCK8 and SYTOX Green staining assay illustrated that the Z-VAD rescued the LUAD cells in (-)-Guaiol treatment, suggesting (-)-Guaiol causes apoptosis in LUAD. The apoptotic cells were mainly early apoptotic cells in the apoptosis analysis. Interestingly, previous research found that (-)-Guaiol activated double-strand breaks (DSBs)-induced cell apoptosis via caspase signaling and targeting RAD51, a key factor in homologous recombination repair [15]. Another study looked into the mechanism of autophagy and discovered that (-)-Guaiol targeted the mTORC1 and mTORC2 signaling pathways to cause autophagic cell death. Notably, the mTORC2-AKT signaling was blocked due to mTOR phosphorylation failure, and the mTORC1 signaling was impaired due to downstream factor inhibition [16]. In light of this, PI3K/Akt is regarded as a common upstream signaling pivot, initiating multiple downstream activities leading to cell death.

**Conclusions**

The purpose of this study was to assess the effects and mechanism of (-)-Guaiol against LUAD using network pharmacology and bioinformatics. As part of the validation process, molecular docking and experiments were carried out. The results showed that (-)-Guaiol targeted the PI3K/Akt pathway to exert its effects via the apoptotic pathway. These findings support the results of previous research on (-)-Guaiol, which indicate that (-)-Guaiol is a potential candidate for clinical application and promotion. Additional research on (-)-Guaiol, clinical trials, and more elaborate experiments are needed.

**Declaration of Figures' Authenticity**

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

**Supplementary Material**

Supplementary Table 1. The identified genes of DEGs, WGCNA module genes, and drug target genes.

| DEGs       | ADAMTS1 | ADAMTS8 |
|------------|----------|---------|
| A2M        | ADAMTS4  |         |
| AADAC      | ADAMTS5  |         |
| AATK       | ADR5     |         |
| ABCA3      | ADGR5    |         |
| ABCA8      | ADGRF5   |         |
| ABCC3      | ADH1B    | ADPRH   |
| ABCG1      |          |         |
| ABHD11     | ADRB1    | ADRB2   |
| ABI3BP     |          |         |
| AC007906.2 |          |         |
| ACADL      | AGER     |         |
| ACE        | AGR2     | AGR3    |
| ACKR1      | AGRP     |         |
| ACP5       |          | ANK1    |
| ACVRL1     | ADA2     | ANK2    |
| ADA2       |          |         |
| ADAM8      |          | AK1     |
|            |          |         |
|            |          | AKAP12  |
|            |          | AKR1B10 |
|            |          | AKR1C2  |
|            |          | ALDH1A1 |
|            |          | ALDH1B1 |
|            |          | ALDOA   |
|            |          | ALG1L   |
|            |          | ALOX15  |
|            |          | ALOX5   |
|            |          | ALOX5AP |
|            |          | ALPL    |
|            |          | AMOTL1  |
|            |          | ANGPT1  |
|            |          | ANKRD1  |
|            |          | ANKRD22 |
| Gene       | Gene       | Gene       |
|------------|------------|------------|
| ANKRD29    | BZW2       | CCL23      |
| ANLN       | C11orf96   | CCL24      |
| ANOS1      | C14orf132  | CCM2L      |
| ANXA3      | C15orf48   | CCN1       |
| AOC1       | C3orf69    | CCN2       |
| AOC3       | C1orf115   | CCN3       |
| APLN       | C1orf116   | CCN4       |
| APOBR      | C1orf162   | CCN5       |
| APOC1      | C1orf194   | CCNA2      |
| APOD       | C1orf198   | CCNB1      |
| APOL1D     | C7         | CCNB2      |
| AQP1       | C9orf24    | CCND2      |
| AQP2       | CA2        | CCT3       |
| AQP3       | CA4        | CCT5       |
| AQP4       | CA9        | CD101      |
| AQP9       | CA9orf20   | CD163      |
| CD24       | C2         | CDH1       |
| CDH18      | C2orf15    | CD30         |
| ARHGAP18   | C48PA      | CD300C     |
| ARHGAP31   | C5AR1      | CD300F     |
| ARHGAP44   | C5orf38    | CD300GL    |
| ARHGEF15   | C7         | CD34       |
| ARHGEF26   | C6         | CD36       |
| ARHGEF6    | C7         | CD44       |
| ARRB1      | C9orf24    | CD52       |
| ASF1B      | CA2        | CD53       |
| ATF3       | CA4        | CD55       |
| ATP13A4    | CA9        | CD74       |
| AURKA      | CA9orf20   | CD9A       |
| AURKB      | C2         | CD93       |
| B3GNT3     | C48PA      | CDC20      |
| B3GNT6     | C5AR1      | CDC5       |
| BASP1      | C7         | CDC5A      |
| BCHE       | C9orf24    | CDC7A      |
| BCL2A1     | CAV1       | CDC8       |
| BCL6B      | CA1V       | CDH3       |
| BEX4       | CAV2       | CDH5       |
| BIR5       | CAV1       | CDK1       |
| BMP2       | CAV1N      | CDK2B      |
| BMPR2      | CAV1N      | CDK3       |
| BOP1       | CAV1       | CDK4       |
| BPIFA1     | CCL18      | CEACAM1    |
| BTG2       | CCL21      | CEACAM5    |
| BTN1L9     |            | CELF2      |
|            |            | CEP25      |
|            |            | CES1       |
| CFD   | CTHRC1 | ECRG4   |
|-------|--------|---------|
| CGNL1 | CTNNAL1 | ECSR    |
| CHI3L2| CTSH   | ECT2    |
| CHRLD1| CTSS   | EDN1    |
| CITED2| CXCL1  | EDN8    |
| CKS1B | CXCL2  | EEF1A2  |
| CLDN18| CXCL12 | EFCC1   |
| CLDN3 | CXCL13 | EFEFMP1 |
| CLDN5 | CXCL14 | EFN3    |
| CLDN14A| CXCL16| EGF7    |
| CLECI4A| CYB5A | EGLN3   |
| CLECI3B| CYBB  | EGR1    |
| CLIC5 | CYBRD1 | EGR2    |
| CNN1  | CYP2A1A| EURH2   |
| COL10A1| CYP4B1| EIF4EBP1|
| COL11A1| CYR1R | EMCN    |
| COL17A1| DAPK1 | EMP1    |
| COL1A1 | DCN    | EMP2    |
| COL1A2 | DKK3   | EMP3    |
| COL3A1| DEPP1  | ENG     |
| COL5A1| DELR3  | ENO1    |
| COL5A2| DES    | EPA51   |
| COL6A6 | DHC24  | EPCAM   |
| COLEC12| DLC1  | EPHX3   |
| COMP  | DLGAP5 | ERG     |
| COX412| DMBT1  | ERO1A   |
| COX7A1| DNAJC12| ESAM    |
| CP    | DNTTIP1| ETS1    |
| CPB2  | DOK2   | ETV4    |
| CRABP2| DPEP2  | EVI2B   |
| CRIM1 | DPT    | FABP4   |
| CRLF1 | EHD2   | FABP5   |
| CR1C1 | DRAG1  | FAM162B |
| CRYAB | DSG1   | FAM167A |
| CSF3  | DSG2   | FAM183A |
| CSF3B | DSP    | FAM189A |
| CSRNP1| DUOX1  | FAM216B |
| CST1  | DUOX4  | FAM83A  |
| CSTA  | DUSP1  | FBLN1   |
| CT83  | DUSP4  | FBLN5   |
| Gene 1 | Gene 2 | Gene 3 |
|-------|-------|-------|
| FBP1  | GATA2 | HBEGF |
| FBXO32| GATA6 | HCAR2 |
| FCER1G| GBP4  | HCK   |
| FGR3A | GCNT3 | HEG1  |
| FCN1  | GIN1  | HK1   |
| FCN3  | GGT1  | HHIP  |
| FEN1  | GGTLC1| HIGD1B|
| FERMT1| GIMAP1| HIURP |
| FERMT2| GIMAP4| HK3   |
| FERMT3| GIMAP7| FGB   |
| FG5   | GIMAP8| FGF   |
| FGFBP2| GIAA  | FERMT1|
| FGFR2 | GIPR2  | FERMT2|
| FGFR4 | GJCI   | FERMT3|
| FGZL2 | GJ6    | FERMT4|
| FGR   | GKB1   | FFB   |
| FHL1  | GKN2   | FGD1  |
| FHL2  | GLIPR2 | GFB   |
| FHL5  | GMPFG  | FGD2  |
| FIB1  | GNG11  | FGD3  |
| FKBP10| GOLM1  | FGD4  |
| FKBP11| GPA33  | FGD5  |
| FLI1  | GPC3   | FGD6  |
| FLRT3 | GPD1   | FGD7  |
| FLRT6 | GPHBP1 | FGD8  |
| FOLR1 | GPM6A  | FGD9  |
| FOS   | GPM6B  | FGD10 |
| FOSB  | GPRC5A | FGD11 |
| FOXF1 | GPRIN2 | FGD12 |
| FOXM1 | GPT2   | FGD13 |
| FPR1  | GPX1   | FGD14 |
| FPR2  | GPX2   | FGD15 |
| FUT2  | GPX3   | FGD16 |
| FUT3  | GRAMD2A| FGD17 |
| FXYD6 | H1-0   | FGD18 |
| FZD4  | H1-1   | FGD19 |
| GABARAPL1| H1-2 | FGD20 |
| GADD45B| H1-3 | FGD21 |
| GALNT18| H1-4 | FGD22 |
| GAPDH | H1-5   | FGD23 |
| GAS6  | H1-6   | FGD24 |
| GCNT3 | H1-7   | FGD25 |
| GGCT  | H1-8   | FGD26 |
| GGT1  | H1-9   | FGD27 |
| GGTLC1| H1-10  | FGD28 |
| GIMAP1| H1-11  | FGD29 |
| GIMAP4| H1-12  | FGD30 |
| GIMAP7| H1-13  | FGD31 |
| GIMAP8| H1-14  | FGD32 |
| GIAA  | H1-15  | FGD33 |
| GJCI  | H1-16  | FGD34 |
| GJ6   | H1-17  | FGD35 |
| GKB1  | H1-18  | FGD36 |
| GKN2  | H1-19  | FGD37 |
| GLIPR2| H1-20  | FGD38 |
| GMPFG | H1-21  | FGD39 |
| GNG11 | H1-22  | FGD40 |
| GOLM1 | H1-23  | FGD41 |
| GPA33 | H1-24  | FGD42 |
| GPC3  | H1-25  | FGD43 |
| GPD1  | H1-26  | FGD44 |
| GPHBP1| H1-27  | FGD45 |
| GPM6A | H1-28  | FGD46 |
| GPM6B | H1-29  | FGD47 |
| GPRC5A| H1-30  | FGD48 |
| GPRIN2| H1-31  | FGD49 |
| GPT2  | H1-32  | FGD50 |
| GPX1  | H1-33  | FGD51 |
| GPX2  | H1-34  | FGD52 |
| GPX3  | H1-35  | FGD53 |
| GRAMD2A| H1-36| FGD54 |
| H1-0  | H1-37  | FGD55 |
| H1-1  | H1-38  | FGD56 |
| H1-2  | H1-39  | FGD57 |
| H1-3  | H1-40  | FGD58 |
| H1-4  | H1-41  | FGD59 |
| H1-5  | H1-42  | FGD60 |

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| Gene     | Gene     | Gene     |
|----------|----------|----------|
| INAVA    | KLF6     | LTBP4    |
| INMT     | KLF9     | LY86     |
| IQANK1   | KPNA2    | LYVE1    |
| IQGAP3   | KRT4     | LYZ      |
| IRAK1    | KRT4A    | M6A       |
| IRX2     | KRT8     | MAMDC2   |
| ITGA8    | KRT80    | MANF     |
| ITGB4    | KRTCAP3  | MAOA     |
| ITIH5    | LAD1     | MAOB     |
| ITIN1    | LAMC3    | MARCKS1  |
| ITL1     | LAMP3    | MARCO    |
| ITLN2    | LAPT5M4B | MATN3    |
| ITM2A    | LAPT5M5  | MCEMP1   |
| ITPKA    | LARGE2   | MCM2     |
| ITPR1D2  | LIPA     | MCM4     |
| JAM2     | LIP6     | MDK      |
| JAML     | LIPG     | MELK     |
| JCAD     | LIPA     | METTL7A  |
| JPT1     | LIPK1    | METTL7B  |
| JUN      | LIPK2    | MEX3A    |
| JUNB     | LIPK3    | MFAP4    |
| JUND     | LIPK4    | MFNG     |
| KANK2    | LGI1     | MFSD2A   |
| KANK3    | LGI3     | MGAT3    |
| KAT2A    | LGI3     | MGP      |
| KCNJ15   | LGI4     | MID1IP1  |
| KCN18    | LGI4     | MKI67    |
| KCNK3    | LGI5     | MME      |
| KCNN4    | LIG3     | MMEC     |
| KCTD12   | LIG5     | MMP1     |
| KDEL1R3  | LIG5     | MMP11    |
| KIAA0040 | LIG6     | MMP12    |
| KIAA1324L| LIG6     | MMP13    |
| KIF1     | LIG6     | MMP19    |
| KIF1A    | LIG6     | MMP28    |
| KIF2A    | LIG6     | MMP9     |
| KIF2C    | LHCP1    | MMRN1    |
| KIF4A    | LHCP1    | MMRN2    |
| KIF4C    | LHCP1    | MMN2A    |
| KIFC1    | LHCP1    | MRC1     |
| KL1     | LHCP1    | MROH6    |
| KLF2     | LHCP1    | MS4A15   |
| KLF4     | LHCP1    | MS4A4A   |
| KLF4     | LHCP1    |          |
| MS4A7 | MS4A8 | MSMO1 | MSR1 | MSRB3 | MT1A | MT1M | MTARC2 | MTHFD2 | MT-ND6 | MTURN | MUC13 | MUC20 | MUC21 | MUC5B | MYADM | MYBL2 | MYCT1 | MYH10 | MYH11 | MYL9 | MYRF | MY2AP | MZB1 | NAPSA | NCF2 | NCKAP5 | NDNF | NDRG2 | NDST1 | NEBL | NECAB1 | NECTIN4 | NEDD9 | NEK2 | NES | NET1 | NFIX | NHSN1 | NME1 | NME4 |
|-------|-------|--------|------|-------|------|------|--------|--------|--------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
|       |       |        |      |       |      |      |        |        |        |      |       |       |       |       |       |       |       |       |       |      |       |       |      |       |       |       |       |       |       |       |-------|-------|
|       | NOTCH4 | NPM3 | NPN1 | NPR1 | NR4A1 | NR4A2 | NR4A3 | NRGN | NRN1 | NTV4 | NUSAP1 | OCIAD2 | OGN | OLFL1 | OLML1 | OSCAR | OTUD1 | OTULIN | P3H2 | P4HB | P53 | PAEP | PAFAH1B3 | PAICS | PC5K9 | PCDH12 | PCOLCE2 | PCP4 | PDK1 | PDK2 | PDK4 | PDK5 | PDGFB | PDI4 | PDIM4 |
|       |       | PGC | PGGH | PHACTR1 | PHLDA2 | PIK3C1 | PIK3C2 | PIK3C3 | PIK3C4 | PIK3D | PIK3E | PIK3F | PIK3G | PIK3H | PIK3I | PIK3J | PIK3K | PIK3L | PIK3M | PIK3N | PIK3O | PIK3P | PIK3Q | PIK3R | PIK3S | PIK3T | PIK3U | PIK3V | PIK3W | PIK3X | PIK3Y | PIK3Z |
|       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| PRO2     | PROM1   | PROM2   | PROS1   | PSAT1   | PRX     | PTGDS   | PTGER4  | PTGES   | PTGIS   | PTGN2   | PTGPRB  | PTPRM   | PTG1    | PXDC1   | QKI     | QPCT    | RAB11FIP1 | RAC3    | RAI2    | RAMP1   | RAMP2   | RAMP3   | RARRES2  | RASGFI   | RASIP1   | RASL12  | RBMS2   | RBP4    | RCAN1   | RCC1    | RCN3    | RECQL4  | RETN    | RGG5    | RHOI    | RHORS   | RIPOR1  | RMI2    | RNASE1  | RNF144B  |
|----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|----------|---------|---------|---------|---------|---------|----------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|          |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |          |         |         |         |         |         |          |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| ROBO4    | RPL39L  | RROD    | RRA1    | RRAS    | RSS1    | RTK2    | S100A2  | S100A3  | S100A4  | S100A8  | S100P   | S1PR1   | S1PR4   | SAMHD1  | SCD     | SASH1   | SBCR1   | SCARA5   | SCD     | SCGB1A1 | SCGB3A1 | SCGB3A2 | SCGB3A3 | SGC1    | SGC2    | SGC3    | SGC4    | SGCE    | SGK1    | SGK2    | SGK3    | SGK4    | SGK5    |
| SERPING1 | SERTM1  | SESN1   | SEZEL2  | SFN     | SFTP1   | SFTP2   | SFTP3   | SFTP4   | SFTPB   | SFTPC   | SFTPD   | SFTPL   | SGCA    | SGCE    | SGK1    | SGK2    | SHMT1   | SHMT2   | SHMT3   | SHMT4   | SIRPA   | SIRPB1  | SLC1A1  | SLC1A2  | SLC1A3  | SLC1A4  | SLC1A5  | SLC1A6  | SLC1A7  | SLC1A8  |
|          |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |         |          |          |         |         |         |         |         |          |         |         |         |         |         |         |         |         |         |         |         |         |         |         |
| SLIT3 | SLPI | SMAD6 | SMIM22 | SMPLD3B | SNX25 | SOCS2 | SOCS3 | SOD3 | SORBS3 | SOSTDC1 | SOX17 | SOX4 | SOX7 | SPC4 | SPAG5 | SPARCL1 | SPDEF | SPI1 | SPN | SPOCK2 | SRP1 | SPTBN1 | SRD5A3 | SRGN | SRPK1 | SRPX | SSR4 | ST14 | STG6ALNAC1 | STG6ALNAC5 | STAC | STARD13 | STARD8 | STEAP1 | STOM | STX11 |
|-------|------|-------|--------|---------|--------|-------|-------|------|-------|---------|-------|------|------|------|------|--------|-------|------|-----|-------|------|--------|--------|------|------|------|------|------|------|------|------|
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
|       |      |       |        |         |        |       |       |      |       |         |       |      |      |      |      |        |       |      |     |       |      |        |        |      |      |      |      |      |      |      |      |
### Supplementary Table 2. The 17 co-expression genes.

| Co-expression genes |  |
|---------------------|-----------------|
| BCHE                | CA2             |
|                    | CA4             |
|                    | FABP4           |
|                    | FABP5           |
|                    | MMP13           |
|                    | PLA2G1B         |
|                    | VIM             |
|                    | SCD             |
|                    | SLC6A4          |

### Supplementary Table 3. The information on 23 hub genes.

**Supplementary Table 3** available from the corresponding author on request.

### Supplementary Table 4. The result of GO and KEGG enrichment analysis.

| ID      | Description                                      | Gene ratio | Bg ratio |
|---------|--------------------------------------------------|------------|----------|
| hsa04151| PI3K-Akt signaling pathway                       | 20.07.2021 | 354/8102 |
| hsa04141| Protein processing in endoplasmic reticulum      | 20.05.2021 | 171/8102 |
| hsa05215| Prostate cancer                                  | 20.04.2021 | 97/8102  |
| hsa04110| Cell cycle                                       | 20.04.2021 | 124/8102 |
| hsa04612| Antigen processing and presentation              | 20.03.2021 | 78/8102  |
| hsa04914| Progesterone-mediated oocyte maturation          | 20.03.2021 | 100/8102 |
| hsa04114| Oocyte meiosis                                   | 20.03.2021 | 129/8102 |
| hsa04915| Estrogen signaling pathway                       | 20.03.2021 | 138/8102 |
| hsa05418| Fluid shear stress and atherosclerosis           | 20.03.2021 | 139/8102 |
| hsa04120| Ubiquitin mediated protein degradation           | 20.03.2021 | 140/8102 |
| hsa05022| Pathways of neurodegeneration - multiple diseases| 20.05.2021 | 475/8102 |
| hsa03040| Spliceosome                                      | 20.03.2021 | 151/8102 |
| hsa05160| Hepatitis C                                      | 20.03.2021 | 157/8102 |
| hsa05165| Human papillomavirus infection                   | 20.04.2021 | 331/8102 |
| ID    | Description                        | Gene ID                                      | Count |
|-------|------------------------------------|----------------------------------------------|-------|
| hsa05134 | Legionellosis                      | Focal adhesion                              | 20.02.2021 | 57/8102 |
| hsa05140 | Leishmaniasis                      | Regulation of actin cytoskeleton             | 20.02.2021 | 77/8102 |
| hsa04512 | ECM-receptor interaction            | FN1/EGFR/HSP90AA1/YWHAZ/ITGA4/CDK2/HSP90AB1 | 20.02.2021 | 7/8102  |
| hsa05222 | Small cell lung cancer             | CDK2/HSP90AB1                               | 20.02.2021 | 92/8102 |
| hsa05131 | Shigelllosis                       | VCP/HSPA5/HSP90AA1/CUL1                     | 20.02.2021 | 246/8102 |
| hsa04657 | IL-17 signaling pathway            | EGFR/HSPA5/HSP90AA1/CUL1                   | 20.02.2021 | 94/8102 |
| hsa04933 | AGE-RAGE signaling pathway in diabetic complications | EGFR/HSP90AA1/CUL1 | 20.02.2021 | 100/8102 |
| hsa04659 | Th17 cell differentiation          | MCM2/YWHAZ/CDK2/CUL1                        | 20.02.2021 | 107/8102 |
| hsa04670 | Leukocyte transendothelial migration | HSP90AA1/HSP90AB1 | 20.02.2021 | 114/8102 |
| hsa04608 | FoxO signaling pathway             | HSP90AA1/VCAM1/HSP90AB1                   | 20.02.2021 | 131/8102 |
| hsa05135 | Yersinia infection                 | FUS/HNRNPU/U2AF2                            | 20.02.2021 | 137/8102 |

**DATABASE ANALYSIS**

Zeng Y. et al: Mechanism of (-)-Guaiol against lung adenocarcinoma © Med Sci Monit, 2022; 28: e937131

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| ID       | p value | p. adjust | q value | Gene ID | Count |
|----------|---------|-----------|---------|---------|-------|
| hsa04933 | 0.0248  | 0.120765971 | 0.096477 | FN1/VCAM1 | 2     |
| hsa04659 | 0.028122 | 0.131236173 | 0.104841 | HSP90AA1/HSP90AB1 | 2     |
| hsa04670 | 0.031614 | 0.141631452 | 0.113145 | ITGA4/VCAM1 | 2     |
| hsa04068 | 0.040766 | 0.175607639 | 0.140288 | EGFR/CDK2 | 2     |
| hsa05135 | 0.044211 | 0.183394284 | 0.146509 | FN1/ITGA4 | 2     |

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