RESEARCH ARTICLE

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Metabolite Profiling and Network Pharmacology of Persimmon Vinegar (*Diospyros kaki*) Compounds, with in silico Validation for Non-Small Cell Lung Cancer Therapy

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Abstract

Objective: Non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer, with complex mechanisms, drug resistance, and significant side effects from conventional chemotherapy, all of which hinder effective treatment. *Diospyros kaki* (persimmon) vinegar, produced through spontaneous fermentation, has enhanced flavonoid content that may increase its pharmacological activity; however, its molecular mechanisms against NSCLC remain unclear. This is the first study to map the molecular mechanisms of persimmon vinegar compounds against NSCLC by integrating metabolite profiling, network pharmacology, and molecular docking validation. **Methods:** Compounds were identified using liquid chromatography–tandem mass spectrometry (LC-MS/MS) and screened for pharmacological properties, followed by network pharmacology and molecular docking to explore potential targets against NSCLC. **Result:** Twenty-two compounds were identified, with three key compounds benzoyl arginine amine, indoline, and exocarpic acid, implicated in the NSCLC pathway (hsa05223). Twenty-seven target genes were associated with this pathway. Benzoyl arginine amine exhibited a binding affinity of −86.9707 kcal/mol for CDK6 (PDB ID: 8I0M) and −120.017 kcal/mol for HRAS (PDB ID: 3K8Y), greater than their native ligand, suggesting its potential to inhibit CDK6 activity, block the G1/S phase transition, and suppress cancer cell proliferation via the E2F pathway. Its interaction with HRAS may inhibit the MAPK pathway, which is critical for cancer progression. **Conclusion:** These findings provide preliminary evidence for the potential of persimmon vinegar compounds as leads for NSCLC drug development, warranting further in vitro and in vivo studies.

Keywords: CDK6- HRAS- LCMS/MS- NSCLC- Persimmon

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Introduction

Non-small cell lung cancer (NSCLC) is the most prevalent form of lung cancer, comprising about 85% of cases [1]. NSCLC develops from lung epithelial cells and includes subtypes such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [2]. Globally, it remains a major cause of cancer-related deaths, with an estimated 1.8 million fatalities annually [3]. Key risk factors, including smoking, air pollution, and radiation exposure, continue to drive its high incidence [4]. These mutations are linked to drug resistance and side effects, posing challenges for treatment [5–7]. Despite progress in targeted and immunotherapies, the five-year survival rate for NSCLC is still low, at only 10–20%. While conventional treatments have evolved, drug resistance and significant adverse effects pose significant challenges in NSCLC management [5–7]. Consequently, developing novel, more effective, and safer therapeutic approaches is urgently needed. Over the past decade, molecular research has unveiled various oncogenic mutations that contribute to NSCLC, including KRAS (20–30%), EGFR (10–40%), and ALK translocations (3–5%), which activate pathways such as MAPK and CDK6, which play crucial roles in cell cycle regulation and proliferation [8, 9]. One promising approach to address these challenges is the utilization of natural compounds as adjunctive or alternative therapy. Phytochemicals found in medicinal plants have demonstrated the potential to inhibit cancer cell proliferation, induce apoptosis, and overcome drug resistance [10,11]. However, research on the use of medicinal plants in NSCLC therapy remains limited and

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warrants further exploration.

Natural products have emerged as promising alternatives due to their multi-targeted mechanisms and lower toxicity profiles. In particular, compounds derived from *Diospyros kaki* (persimmon) from the Ebenaceae family is one such plant that has shown promise as a chemopreventive agent. Persimmon contains secondary metabolites such as proanthocyanidins, epigallocatechin (EGC), epigallocatechin-3-O-gallate (EGCG), terpenoids, and naphthoquinones, which have been proven to possess anticancer activities [12, 13]. Flavonoid content from D. kaki have been reported to induce apoptosis in prostate cancer (PC-3) cells by increasing oxidative stress [14] and activate c-Jun N-terminal kinase (JNK) signaling [15]. polysaccharides isolated from persimmon have exhibited anticancer potential by suppressing Transforming Growth Factor-beta 1 (TGF-β1)-induced epithelial-to-mesenchymal transition (EMT) in A549 lung cancer cells [16]. Persimmon vinegar exhibits strong antioxidant potential, as evidenced by its ability to suppress lipid peroxidation and neutralize 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radicals, effects primarily attributed to its abundant phenolic compounds [15]. Spontaneous fermentation enhances its bioactive content, producing persimmon vinegar with higher levels of phenolics, flavonoids, gallic acid, and chlorogenic acid than fresh fruit or other vinegars, alongside stronger antioxidant activity [14, 15].

To date, no study has comprehensively characterized the molecular targets of persimmon vinegar against NSCLC using integrated metabolomics and network pharmacology approaches. Therefore, this study aimed to identify the bioactive compounds of persimmon vinegar and evaluate their potential to inhibit NSCLC pathways. The approach involves liquid chromatography-mass spectrometry (LC-MS/MS) analysis, network pharmacology, and in silico validation. Network pharmacology serves as a valuable tool for predicting biological targets and complex pathways influenced by natural compounds [17, 18], enabling the identification of potential synergistic effects between persimmon vinegar and conventional NSCLC therapies. This study is expected to significantly contribute to developing plant-based therapies for NSCLC by integrating these methods. The novelty of this research lies in the fermentation approach to enhance the pharmacological potential of persimmon and the exploration of molecular mechanisms through the analysis of signaling pathways involved in lung cancer cell proliferation and survival and its validation in silico.

The overall research design integrating LC-MS/MS metabolite profiling, target prediction, network pharmacology analysis, and molecular docking validation is summarized in Figure 1.

Materials and Methods

Fruit Determination

Diospyros kaki has been identified under the number 068/567/102.21/2023 and was collected from Tulungagung Regency, East Java, Indonesia at an altitude of 85 meters above sea level. The region has a tropical wet and dry climate (Aw) with two seasons: the rainy season (November–April) and the dry season (May–October). January is the wettest month (rainfall>270 mm), while August is the driest (rainfall<20 mm). The temperature ranges between 21°–32 °C, with an annual rainfall of 1.400–1.800 mm and 90–120 rainy days per year.

Fermentation of Diospyros kaki Fruit for Persimmon Vinegar Production

Persimmon fruits were selected and sorted based on ripeness level. Immature persimmons were washed, quartered, weighed, and placed in a tank for spontaneous fermentation at room temperature for 6 months. Peeled mature *Diospyros kaki* fruits were steamed, and dried using a hot oven approach before blended with the fermentation liquid from immature persimmons to form a homogeneous mixture. This mixture underwent another spontaneous fermentation for 6 months at room temperature to ensure a more complete alcohol conversion to acetic acid [19].

Metabolite Profiling Compounds Screening

Persimmon vinegar compounds were screened for their predicted physicochemical properties, toxicity, and bioactivity using SMILES codes obtained from PubChem. Physicochemical properties were evaluated with pkCSM (https://biosig.lab.uq.edu.au/pkcsm/prediction) to assess their suitability as orally administered drugs [20]. Screening followed Lipinski's rule of five, which specifies: molecular weight (MW) ≤ 500 g/mol, log P ≤ 5 (indicating hydrophobicity), hydrogen bond donors (HBD) ≤ 5, hydrogen bond acceptors (HBA) ≤ 10, polar surface area (PSA) ≤ 140 Å², and fewer than 10 rotatable bonds, reflecting drug permeability and molecular flexibility [21-23]. Compounds violating no more than one rule were considered suitable for oral administration [24].

Toxicity was assessed using Protox III (https://tox.charite.de/protox3/), which classifies compounds into six toxicity classes based on median lethal dose (LD50). Bioactivity was predicted using PASS Online (https://www.way2drug.com/passonline/predict.php), which generates a probability of activity (Pa) and probability of inactivity (Pi). Compounds with Pa > 0.7 were classified as highly promising anticancer agents, those with 0.5 < Pa < 0.7 as having moderate potential requiring further validation, and those with Pa < 0.5 as having low predicted anticancer activity [25–27].

LC-MS/MS experiment

The LC-MS/MS analysis was conducted using UPLC-MS systems with QToF analyzer and positive ESI as the ionization source with the Acquity C18 column 1.8 µm; 2.1 × 150 mm. The eluent applied was a mixture of (A) water (HPLC grade)/formic acid (Merck, Darmstadt, Germany) 99.9/0.1 [v/v]; (B) acetonitrile (Merck, Darmstadt, Germany)/formic acid 99.9/0.1 [v/v] with gradient elution. The source and desolvation temperatures were set at 100°C and 350°C, respectively. A 10 mg extract was dissolved in a 10 ml absolute methanol, and 5 µL was injected into the UPLC-MS system. The analysis parameters were set using positive ion mode, with spectra acquired over a mass range from m/z 120 to 1000. Data

processing and compound identification were conducted using Mass Lynx v4.1 (Waters, Massachusetts, USA) and PubChem (https://pubchem.ncbi.nlm.nih.gov/), generate the chromatogram and spectra of each observed peak. Additionally, chemical prediction was performed using the ChemSpider website with compound's confirmation based on MS/MS fragmentation and an inaccuracy of less than 5 ppm [18].

Identification of Potential NSCLC Targets

A crucial aspect of drug research is predicting whether a chemical interacts with the targets. The gene targets of active compounds from persimmon vinegar based on LC-MS/MS analysis were identified using the GeneCards database (https://www.genecards.org/, cut off>0.7). Conversely, NSCLC-related gene targets were retrieved using the DisGeNET database (https://www.disgenet.org). Subsequently, the network pharmacology using Cytoscape software 3.9.1 to visualize interaction between active compound-gene targets and disease-gene target [28].

Network Pharmacology and Protein-Protein Interaction Analysis

Network pharmacology visualization for overlapping target genes was performed using Cytoscape 3.10.1 (https://cytoscape.org/). Protein-protein interaction (PPI) analysis was conducted using STRING 12.0 (https://STRING-db.org/) with a minimum required interaction score of 0.400 26. The PPI analysis explored biological phenomena by annotating functional data related to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [29].

Gene Ontology (GO) dan KEGG Pathway Analysis

GO and KEGG pathway analyses were performed using SRPlot (https://www.bioinformatics.com.cn). The GO analysis covered biological processes, cellular components, and molecular functions affected by CLE compounds, while the KEGG analysis identified NSCLC-related signaling pathways. Both analyses were filtered with a p-value ≤ 0.05 and false discovery rate ≤ 0.05 [29].

The GO results were refined to the top ten terms for each category and visualized as bubble plots. KEGG pathway enrichment focused on signaling pathways relevant to NSCLC. The Kyoto Encyclopedia of Genes and Genomes (KEGG) provides integrated insights by linking genomic, chemical, and systems biology data to clarify key pathways involved [30].

Molecular Docking Analysis

The target genes identified through network pharmacology were validated using molecular docking with Molegro Virtual Docker 6.0 (http://www.molegro.com). Docking of benzoyl arginine amine was performed and visualized using ChemDraw Ultra 12.0 and Chem3D Pro. The two receptors used CDK6 (PDB ID: 8I0M) and HRAS (PDB ID: 3K8Y) were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). Initial validation involved re-docking each receptor with its native ligand to ensure accuracy, targeting an RMSD value below 2.0 Å. Docking results were assessed based on the rerank score and interactions with amino acid residues, and were compared with the binding of the native ligand [31].

Results

Secondary metabolites from LCMS/MS analysis of persimmon vinegar

LC-MS/MS analysis of persimmon vinegar identified 22 known compounds and 2 unknowns. The total ion chromatogram (TIC) obtained from mass spectrometry is presented in Figure 2, and detailed in Table 1, along with their respective elemental formulas. Data from mass spectrometry includes the detected and calculated m/z values, molecular formula, error rate in parts per million (ppm), retention time, and MS fragmentation pattern of the identified compounds.

Each peak in the chromatogram corresponded to a specific bioactive compound, which was categorized into one of the following classes: fatty acids/lipids and its derivatives, peptides, monosaccharide and its derivatives, flavonoids, phenol, saponin, amines, amides (Table 1). Notably, the primary component were capsaicinoids including capsaicin, dihydrocapsaicin, nordihydrocapsaicin and capsiamide.

Metabolite Profiling Compounds Screening

The physicochemical characteristics of persimmon vinegar compounds were assessed using Lipinski's Rule of Five. This rule outlines that for a compound to exhibit drug-like properties, it should fulfill the following criteria: molecular weight (MW) ≤ 500 g/mol, Log P ≤ 5 (reflecting hydrophobicity), no more than five hydrogen bond donors (HBD), up to 10 hydrogen bond acceptors (HBA), polar surface area (PSA) ≤ 140 Å^2, and fewer than 10 rotatable bonds, which relate to drug permeability and molecular flexibility [23]. Compounds that violate no more than one of these conditions are deemed viable for oral drug formulations. The screening process in this study identified 15 compounds that met the criteria (Supplementary material 1). Toxicity screening based on predicted LD50 values, confirmed that these compounds aligned with the established toxicity prediction parameters (Supplementary material 2). Among them, Tetrahydroharman-3-carboxylic acid was predicted to be the most toxic compound, with an LD_{50} value of 300 mg/kg, corresponding to toxicity class 3.

The PASS analysis identified 10 compounds with potential anti-NSCLC activity (Supplementary material 3). These compounds demonstrated positive physicochemical properties, low toxicity, and biological activity related to NSCLC.

Results of the analysis showed that 10 compounds fulfilled the predicted physicochemical properties, toxicity, and anticancer bioactivity based on PASS prediction. These compounds were selected for network pharmacology analysis to evaluate interactions and mechanisms in cancer therapy development.

Potential Target Genes and Protein-Protein Interactions

Target genes of ten persimmon vinegar compounds were identified using the GeneCards database with a relevance score above 0.5. Four compounds showed

Table 1. Interpretation of Metabolite Profiling Results of Persimmon Vinegar (*Diospyros kaki* Fruit)

No.	Rt	% Area	Measured mass	Calculated mass	Compound Formula	Compound Name	Compound Group
1.	0.53	0	3,713,158	3,713,161	C22H42O4	Diisooctyl adipate	Diester (adipic acid)
2.	1.74	2.27	2,210,426	2,210,423	C7H4N6O3	3-(3-Amino-4-cyano pyrazolyl)furazan-4-carboxylic acid	Heterocyclic (ether)
3.	2.2	1.03	1,890,771	1,890,776	C9H8N4O	N-Benzoyl-N'-cyanoguanidine	Guanidine derivative
4.	2.79	0.81	2,761,449	2,761,447	C12H21NO6	O-glutarylcarnitine	Acylcarnitine (glutaric acid)
5.	3.24	0.17	2,941,561	2,941,566	C13H19N5O3	Benzoyl arginine amine	Amino acid derivative
6.	3.92	1.15	1,200,823	1,200,813	C8H9N	Indoline	Alkaloid
7.	4.84	1.32	2,651,567	265,157	C13H25O3Cl	10-Chlorodecyl ethyl carbonate	Amino acid derivative
8.	5.19	2.44	2,311,137	2,311,134	C13H14N2O2	Tetrahydroharman-3-carboxylic acid	Alkaloid
9.	5.87	1.4	2,111,447	2,111,447	C11H18N2O2	1-Carboxyheptylimidazole	Carboxylic acid
10.	6.84	0.14	4,212,552	421,255	C19H36N2O8	methyl-2.3-di-O-l-leucyl-alpha-d-glucose	Glycoside
11.	7.17	1.23	275,132	2,751,317	C13H22O4S	Isopropyl (1S)-(+)-10-Camphorsulfate	Sulfate ester
12.	7.89	0.69	2,271,765	227,176	C12H22N2O2	Crotetamide	Amide
13.	8.18	0.45	3,072,016	3,072,013	C12H27N6OCl	Azepan-2-one;2-butyl-1-(diaminomethylidene) guanidine;hydrochloride	Heterocyclic
14.	8.88	1.34	2,752,013	2,752,011	C18H26O2	Exocarpic Acid	Fatty acid
15.	9.41	0.13	2,932,119	2,932,117	C18H28O3	Mueggelone	Lactone
16.	9.96	0.1	181,123	1,811,229	C11H16O2	Bovolide	Heterocyclic
17.	10.68	0.08	3,432,959	3,432,961	C19H38N2O3	Lauramidopropylbetaine	Quaternary amonium
18.	13.87	5.84	9,927,354	992.7357	C65H93N5O3	unknown	-
19.	14.33	3.81	9,927,359	9,927,362	C50H89N17O4	unknown	-
20.	15.03	10.96	9,927,407	9,927,402	C57H101NO12	CID : 172088879	Fatty acid
21.	15.54	5.57	9,927,327	9,927,322	C47H101N5O16	CID : 59117983	Polyquaternary ammonium
22.	17.03	42.71	8,797,441	8,797,442	C57H98O6	Trilinolein	Triglyserida
23.	18.53	16.35	6,634,527	6,634,571	C31H66O14	CID : 22051731	Polyether
24.	21.07	0.02	3,713,135	3,713,134	C18H38N6O2	CID : 10642926	Macrocyclic polyamine

target genes associated with NSCLC: O-glutarylcarnitine (1 target gene), benzoyl arginine amine (660 target genes), Indoline (109 target genes), and Exocarpic Acid (9 target genes) (Supplementary material 4). A comparative analysis revealed 191 overlapping target genes between the 718 genes linked to persimmon vinegar compounds and the 866 genes associated with NSCLC progression (CUI: C1737250) (Figure 3).

From 191 identified target genes, these genes were targets of several compounds that passed the selection process through the GeneCards platform. Among the four selected compounds (Supplementary material 4), three showed overlap with the target genes of NSCLC. These compounds were Benzoyl arginine amine, Indoline, and Exocarpic Acid. .

However, one compound, O-glutarylcarnitine, did not show any overlap with the target genes of NSCLC. This suggests that O-glutarylcarnitine may have a different mechanism of action or target different biological pathways that are not directly related to NSCLC. Nevertheless, the presence of this compound remains relevant for further investigation, as compounds without direct overlap may exhibit pleiotropic effects or contribute to overall health benefits through other mechanisms, such as antioxidant or anti-inflammatory effects.

Network analysis showed 189 nodes connected by 5,005 edges (Figure 4). This network comprises three bioactive compounds: benzoyl arginine amine, Indoline, and Exocarpic Acid, interacting with 191 NSCLC-related target genes (Figure 5). One additional node represents lung cancer as the network's central hub.

Gene Ontology (GO) dan KEGG Pathway Analysis

Gene Ontology (GO) analysis was visualized in a bubble chart (Supplementary Figure 1), showing the top ten GO terms covering biological processes, cellular components, and molecular functions affected by compounds in persimmon vinegar. The main biological process was lipopolysaccharide response, the key cellular component was the vesicle lumen, and the dominant molecular function was transmembrane receptor protein tyrosine kinase activity.

KEGG pathway analysis is shown in Supplementary Figure 2 as a dot plot, highlighting the top ten metabolic or signaling pathways potentially influenced by Benzoyl arginine amine, Indoline, and Exocarpic Acid found in persimmon vinegar. This diagram helps identify the biological pathways involved in the body's response to these compounds.

Supplementary Figure 3 illustrates the KEGG pathway

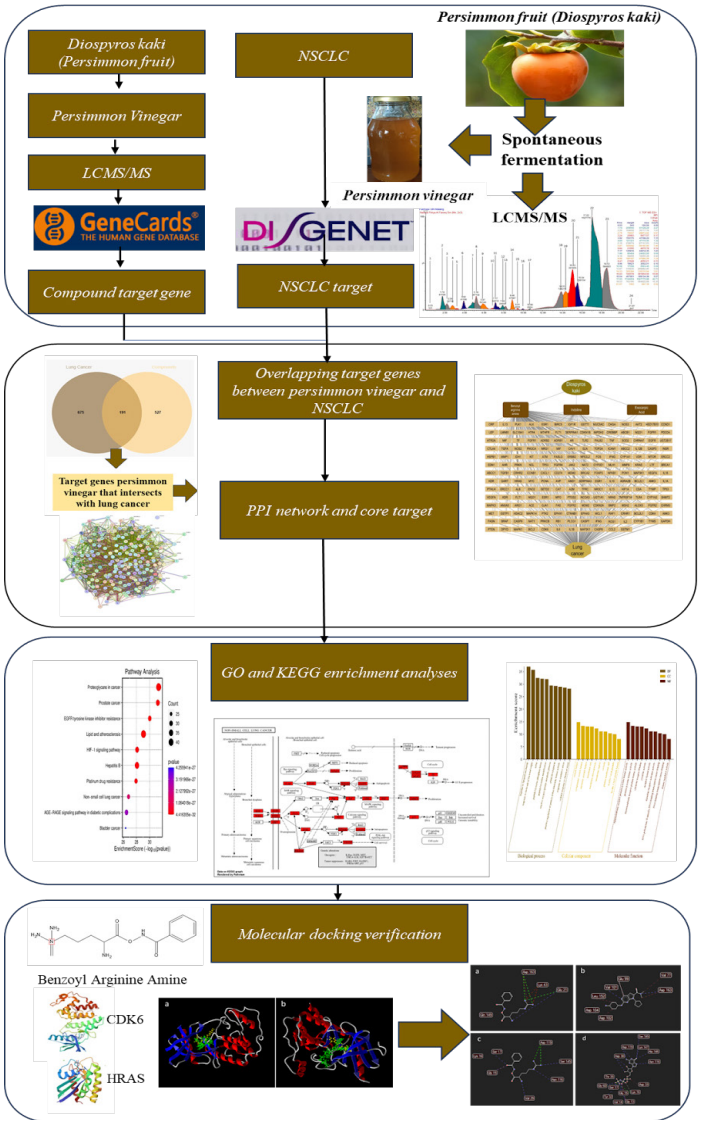

Figure 1. Research Flowchart.

analysis for NSCLC, showing that 27 out of 73 genes in this pathway interact with Benzoyl arginine amine, Indoline, and Exocarpic Acid. These genes, including *MET, PRKCB, ALK, KRAS, EGFR, TP53*, and others are involved in five signaling pathways: the *Erb, MAPK, PI3K-Akt, P53* pathways, and the cell cycle (hsa 5223).

Molecular Docking

This study screened 27 target gene receptors of compounds in persimmon vinegar to identify suitable receptor candidates for molecular docking. This screening step is crucial to ensure the reliability and validity of the in silico model. Receptor selection criteria included crystal structure resolution below 3 Å, no mutations, origin from Homo sapiens, presence of a native ligand, and determination by X-ray diffraction [32].

Based on these criteria, only two receptors qualified: CDK6 and HRAS (Harvey Rat Sarcoma Viral Oncogene Homolog) (Supplementary material 5). Both were selected for their high-resolution structures and key roles in cancer cell proliferation pathways, particularly in NSCLC. Receptor validation using the RMSD (Root Mean Square Deviation) parameter confirmed model accuracy, with RMSD values below 2 Å, indicating a reliable docking model [33].

Table 2 shows that benzoyl arginine amine exhibits greater affinity than the native ligand for CDK6 (PDB ID: 8I0M) and HRAS (PDB ID: 3K8Y), with rerank score

Table 2. Results of RMSD and Re-Rank Score of Test Compound with Receptor

Target Gene	Compound	Receptor (PDB ID)	RMSD (Å)	Rerank Score (kkal/mol)
CDK6	Benzoyl Arginine Amine	8I0M	1.46	-869,707
	Native Ligand	(NJ6 [A])		-849,192
HRAS	Benzoyl Arginine Amine	3K8Y	1.72	-120,017
	Native Ligand	(GNP [A])		-107,534

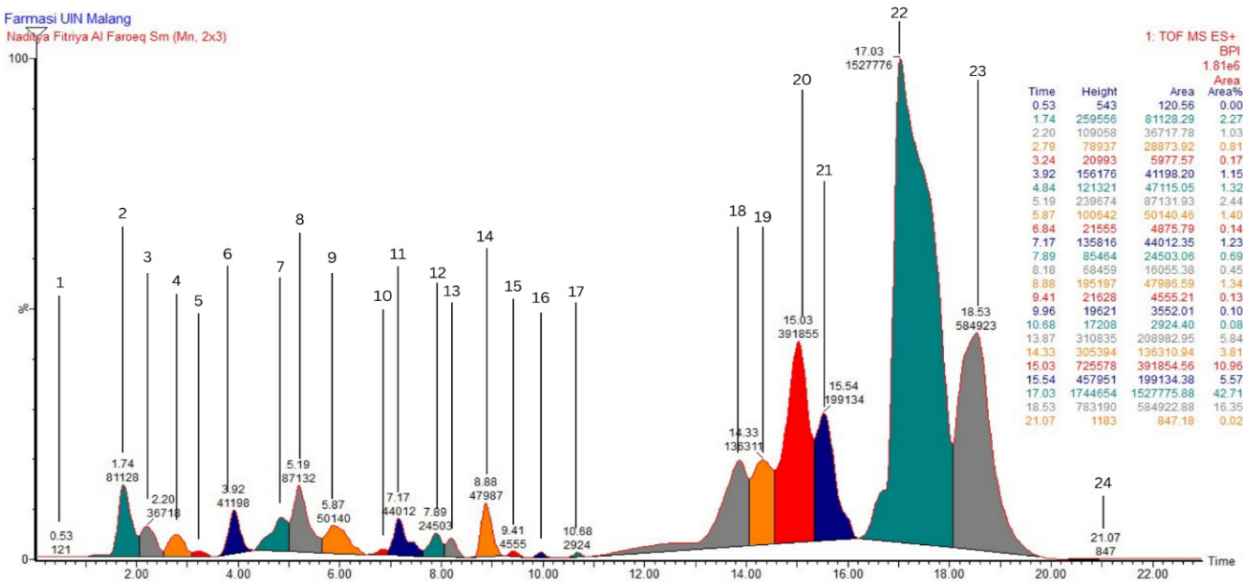

Figure 2. The Chromatogram of Persimmon Vinegar (*Diospyros kaki* fruit) was Analyzed Using the UPLC-QToF-MS/MS Method. A C18 column served as the stationary phase, while the mobile phases were composed of water with formic acid [99.9:0.1 (v/v)] and acetonitrile with formic acid [99.9:0.1 (v/v)]. Each peak observed in the chromatogram corresponds to an individual compound

-86.9707 kcal/mol and -120.017 kcal/mol, respectively. Docking results are shown in Supplementary Figure 4.

Amino acid residues interacting with benzoyl arginine amine within the CDK6 and HRAS catalytic sites (Supplementary Figure 5; Supplementary material 6). Hydrogen bonds are vital in docking studies because they stabilize ligand–protein complexes, increase ligand affinity through multiple interactions, and optimize ligand orientation within the active site for effective binding.

Discussion

Secondary metabolites from LCMS/MS analysis of persimmon vinegar

The results of this study highlight the promising potential of persimmon vinegar as an inhibitor of NSCLC. Metabolite profiling using LC-MS/MS successfully identified 22 bioactive compounds with physicochemical and toxicity profiles suitable for oral drug development, consistent with Lipinski's rule of five. Among these, three key compounds, which benzoyl arginine amine, Indoline, and Exocarpic Acid are predicted to exert significant pharmacological effects by targeting molecular pathways strongly associated with NSCLC progression.

Gene Ontology (GO) dan KEGG Pathway Analysis

The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed interactions with 27 target genes involved in the NSCLC signaling pathway (hsa05223) (Table 3). The

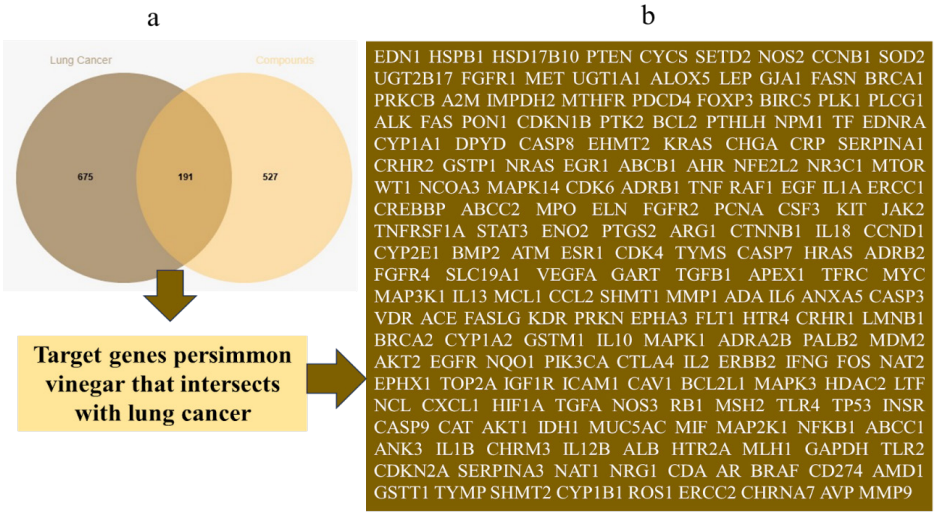

Figure 3. (a) Venn diagram results of 718 target genes from Diospyros kaki against 866 target genes with different disease types of lung cancer. (b) Target genes *Diospyros kaki* that intersects with lung cancer

Table 2. KEGG Pathway Target Genes of *Diospyros kaki* and Lung Cancer

ID	Pathway	Number of Genes	Genes	p-value	q-value
hsa01521	EGFR tyrosine kinase inhibitor resistance	30 of 80	PTEN/MET/PRKCB/PLCG1/BCL2/KRAS/NRAS/MTOR/RAF1/EGF/FGFR2/JAK2/STAT3/HRAS/VEGFA/IL6/KDR/MAPK1/AKT2/EGFR/PIK3CA/ERBB2/IGF1R/BCL2L1/MAPK3/TGFA/AKT1/MAP2K1/NRG1/BRAF	9.53E-26	8.58E-24
hsa05223	Non-small cell lung cancer	27 of 73	MET/PRKCB/PLCG1/ALK/KRAS/NRAS/CDK6/RAF1/EGF/STAT3/CCND1/CDK4/HRAS//AKT2/EGFR/PIK3CA/ERBB2/MAPKMAPK13/TGFA/RB1/TP53/CASP9/AKT1/MAP2K1/CDKN2A/BRAF	1.56E-22	1.79E-21
hsa04151	PI3K-Akt signaling pathway	48 of 362	PTEN/FGFR1/MET/BRCA1/CDKN1B/PTK2/BCL2/KRAS/NRAS/MTOR/CDK6/RAF1/EGF/FGFR2/CSF3/KIT/JAK2/CCND1/CDK4/HRAS/FGFR4/VEGFA/MYC/MCL1/IL6/FASLG/KDR/FLT1/MAPK1/MDM2/AKT2/EGFR/PIK3CA/IL2/ERBB2/IGF1R/BCL2L1/MAPK3/TGFA/NOS3/TLR4/TP53/INSR/CASP9/AKT1/MAP2K1/NFKB1/TLR2	2.97E-21	1.94E-20
hsa04010	MAPK signaling pathway	40 of 300	HSPB1/FGFR1/MET/PRKCB/FAS/KRAS/NRAS/MAPK14/TNF/RAF1/EGF/IL1A/FGFR2/KIT/TNFRSF1A/HRAS/FGFR4/VEGFA/TGFB1/MYC/MAP3K1/CASP3/FASLG/KDR/FLT1/MAPK1/AKT2/EGFR/ERBB2/FOS/IGF1R/MAPK3/TGFA/TP53/INSR/AKT1/MAP2K1/NFKB1/IL1B/BRAF	6.75E-17	2.38E-16
hsa04210	Apoptosis	28 of 136	CYCS/BIRC5/FAS/BCL2/CASP8/KRAS/NRAS/TNF/RAF1/TNFRSF1A/ATM/CASP7/HRAS/MCL1/CASP3/FASLG/LMNB1/MAPK1/AKT2/PIK3CA/FOS/BCL2L1/MAPK3/TP53/CASP9/AKT1/MAP2K1/NFKB1	1.16E-15	3.78E-15
hsa04012	ErbB signaling pathway	22 of 86	PRKCB/PLCG1/CDKN1B/PTK2/KRAS/NRAS/MTOR/RAF1/EGF/HRAS/MYC/MAPK1/AKT2/EGFR/PIK3CA/ERBB2/MAPK3/TGFA/AKT1/MAP2K1/NRG1/BRAF	1.33E-13	3.40E-13
hsa05222	Small cell lung cancer	20 of 93	PTEN/CYCS/NOS2/CDKN1B/PTK2/BCL2/CDK6/PTGS2/CCND1/CDK4/MYC/CASP3/AKT2/PIK3CA/BCL2L1/RB1/TP53/CASP9/AKT1/NFKB1	2.08E-10	3.97E-10
hsa04115	p53 signaling pathway	16 of 75	PTEN/CYCS/CCNB1/FAS/BCL2/CASP8/CDK6/CCND1/ATM/CDK4/CASP3/MDM2/BCL2L1/TP53/CASP9/CDKN2A	1.72E-07	2.46E-07
hsa04110	Cell cycle	16 of 158	CCNB1/PLK1/CDKN1B/CDK6/CREBBP/PCNA/CCND1/ATM/CDK4/TGFB1/MYC/MDM2/HDAC2/RB1/TP53/CDKN2A	1.48E-02	1.28E-02

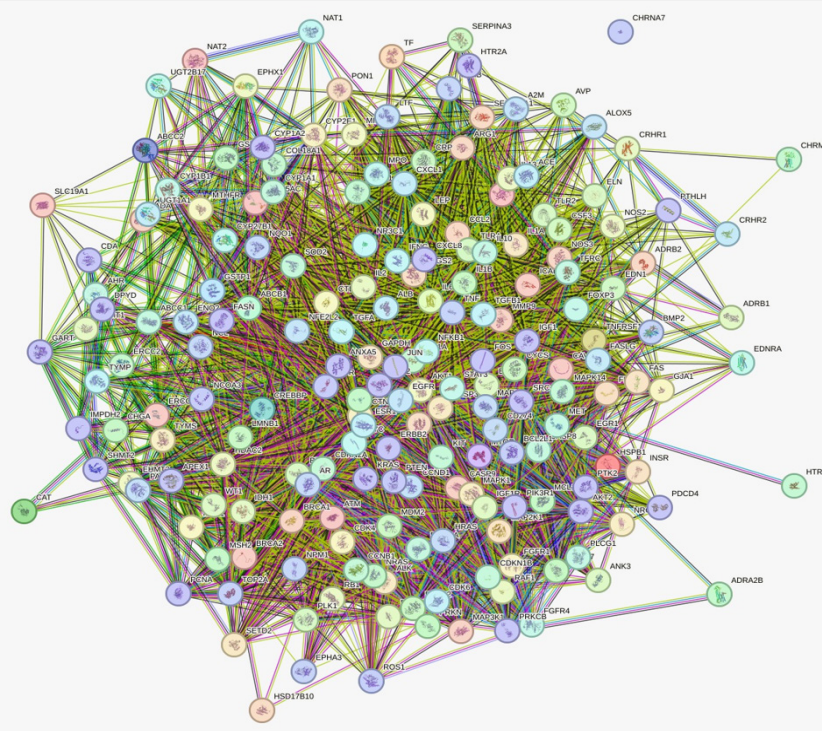

Figure 4. Protein-Protein Interaction Network with 189 nodes, 5005 edges, PPI enrichment p-value < 1.0e-16.

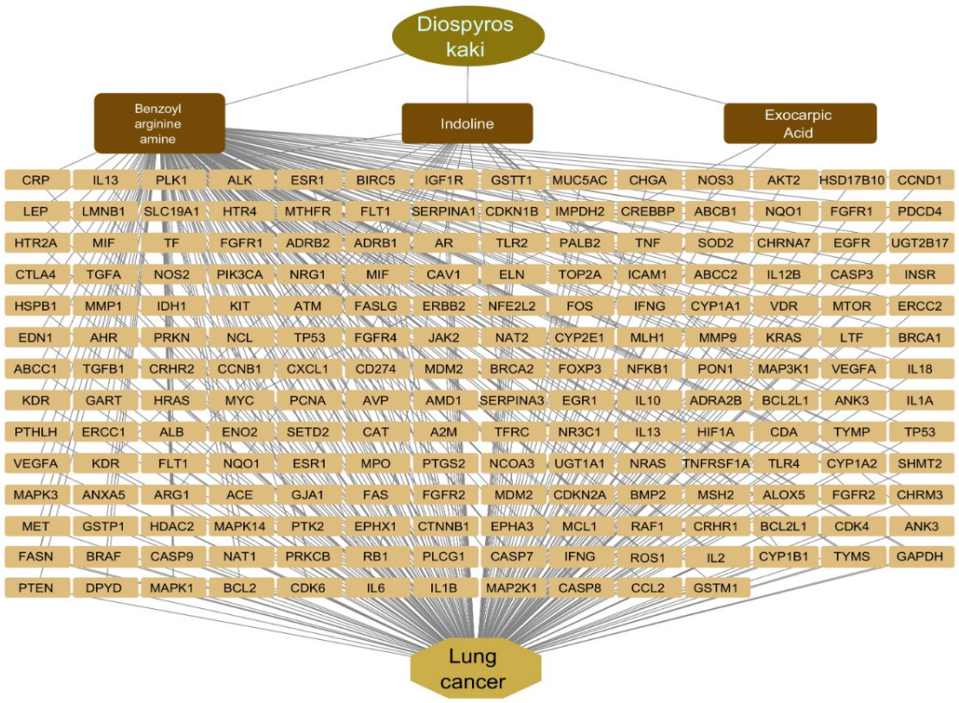

Figure 5. Visualization of Network Pharmacology Compounds in the Fermentation of *Diospyros kaki* (persimmon vinegar) using Cytoscape v3.7.2 Description: plant (green ellipse), bioactive compounds (dark brown round rectangle), target gene (light brown rectangle), and disease (light brown octagon).

main pathways potentially modulated include the EGFR, MAPK, PI3K-Akt, p53, and cell cycle regulatory pathways. The ErbB pathway is critical in activating tyrosine kinase receptors that stimulate cellular proliferation and differentiation [34]. The MAPK and PI3K-Akt pathways, meanwhile, are central to enhancing cancer cell growth, suppressing apoptosis, and contributing to therapeutic resistance [35]. Additionally, the p53 pathway plays a crucial role in regulating the cell cycle and apoptosis in response to genotoxic stress [36], while the cell cycle pathway precisely regulates phase progression that dictates cancer cell survival or elimination [37].

Estrogen receptor beta (ERβ) is also implicated in modulating oncogenic pathways by stimulating KRAS, PI3K, and PKB/AKT activation. This sequence suppresses caspase 9 activity, thereby enhancing anti-apoptotic processes that help cancer cells evade programmed cell death [38]. Notably, this study predicts that benzoyl arginine amine can inhibit KRAS, PI3K, and PKB/AKT, effectively restoring caspase 9 activity and triggering apoptosis. This suggests its role as a promising natural anticancer agent by promoting cell death pathways that are otherwise suppressed in malignant cells.

Molecular Docking

In the MAPK pathway, growth factors such as EGF (Epidermal Growth Factor) and TGF-α (Transforming Growth Factor-alpha) are known to activate EGFR (Epidermal Growth Factor Receptor) and ERBB2, which subsequently triggers the activation of the Ras-Raf-MEK-ERK cascade, playing a role in the regulation of DNA gene transcription and the increased expression of Cyclin D1, thereby causing uncontrolled cell proliferation [35, 39, 40]. Benzoyl arginine amine is predicted to inhibit EGF and TGF-α signaling, blocking EGFR and ERBB2 activation and consequently suppressing the Ras-Raf-MEK-ERK cascade. This may lead to reduced Cyclin D1 expression and help curb tumor growth by inhibiting cancer cell proliferation.

Additionally, the PI3K pathway is driven by JAK3 activation, which stimulates STAT3/5, further enhancing cancer cell survival and resistance to cell death [40]. The predicted action of benzoyl arginine amine includes STAT3 inhibition, which disrupts this pathway and sensitizes cancer cells to apoptosis.

Regarding the cell cycle, CDK4/6 and Cyclin D1 are crucial regulators of the G1/S phase transition. Inhibiting CDK4/6 activity maintains the retinoblastoma protein (Rb) in its active state, which binds to and inactivates the E2F transcription factor. This suppression prevents the expression of genes essential for DNA replication, effectively arresting cell cycle progression and limiting cancer cell growth [41, 42]. In this context, benzoyl arginine amine is predicted to function as a CDK6 inhibitor, reducing Rb phosphorylation and ensuring E2F remains inactive. This mechanism contributes to cell cycle arrest and promotes apoptosis, offering a promising route for halting NSCLC proliferation.

Moreover, preserving p53 function is vital, as mutations in this gene disrupt its role in initiating pro-apoptotic proteins like Bax and Bak [43]. Benzoyl arginine amine's antioxidant capacity may help prevent such mutations, maintaining p53's regulatory role and thus contributing to the suppression of tumor development.

Network pharmacology analysis, supported by in silico docking validation, confirmed the strong binding

affinity of benzoyl arginine amine to CDK6 (PDB ID: 8I0M) and HRAS (PDB ID: 3K8Y). The compound's lower binding energy compared to native ligands suggests stable interactions that may enhance its inhibitory effects on these critical molecular targets [44–46].

HRAS, an oncogene within the RAS/MAPK and PI3K-Akt pathways, also drives cell proliferation and blocks apoptosis [47]. By inhibiting HRAS, benzoyl arginine amine could disrupt this signaling, reduce Cyclin D1 expression, and allow caspase-9 activation to induce apoptosis [47, 48].

This study demonstrates that persimmon vinegar contains bioactive compounds with promising anticancer mechanisms. However, further in vitro and in vivo studies are necessary to validate these findings and confirm their safety and effectiveness as an adjuvant or complementary therapy candidate in the treatment for NSCLC.

Author Contribution Statement

Roihatul Mutiah contributed to the conceptualization and design of the study, provided supervision, and was responsible for funding acquisition. Ermin Rachmawati performed the data analysis and interpretation. Imam Taufik was responsible for drafting the manuscript, while critical revision of the manuscript was carried out by Alvi Milliana. Data acquisition was conducted by Nabila Rahmadani. Statistical analysis was performed by Riza Ambar Sari. Fitriyani contributed to administrative, technical, and material support, and provided final approval of the manuscript.

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General

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Approval

If it was approved by any scientific Body/ if it is part of an approved student thesis

Conflict of Interest

All the authors declare that there are no conflicts of interest.

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