Unravelling Potential Mechanisms of *Piper retrofractum* Vahl. Bioactive Compounds as Cervical Cancer Chemopreventive Agent by Bioinformatic Approach

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Abstract

Cervical cancer is the leading cause of cancer death after breast cancer. HPV infection is the main cause of cervical cancer which will cause overexpression of E6 oncoprotein. E6 oncogene can cause cancer cells to continue to proliferate. Long pepper fruit (*Piper retrofractum* Vahl.) has antioxidant activity and contains piperine compounds that have anticancer activity. This study aims to assess the potential of long pepper fruit extract as a chemopreventive agent for cervical cancer. The research method was conducted by in silico assay with network pharmacology and molecular docking analysis. We found five hub genes including HSP90AA1, SRC, AKT1, STAT3, and PIK3CA. KEGG analysis showed that the most dominant signaling pathways involved are neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, MAPK signaling pathway, Calcium signaling pathway, and cAMP signaling pathway. Through molecular docking, it was found that long pepper fruit has the potential to inhibit the E6 oncogene of HPV. Based on the research results, long pepper fruit extract may has the potential as a chemoprevention agent for cervical cancer.

Keywords: *Cervical cancer, oncoprotein HPV16-E6,* Piper retrofractum *Vahl., molecular docking, network pharmacology.*

INTRODUCTION

Cervical cancer is one of the leading causes of cancer death in women besides breast cancer. In 2020, there were 604,127 new cases of cervical cancer with a death rate of around 341,831 cases (Sung, *et al*., 2021). In the same year, in Indonesia, the incidence of cervical cancer was reported at 36,633 cases with 21,003 fatal cases (Frianto, *et al*., 2022). HPV (Human Papilloma Virus) is the main cause of cervical cancer. HPV type 16 is one of the High-Risk HPV which is the main cause of cervical cancer with 60.5% of cervical cancer cases (Pal and Kundu, 2020). Of all the types of oncogene cellular interactions, E6 is the most significant interaction (Almeida, *et al*., 2019). Overexpression of E6 causes degradation of

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p53, which stops the cell cycle after the G1 phase, apoptosis, and DNA repair so that abnormal cells will continue to proliferate (Evriarti and Yasmon, 2019).

Cancer is a complex disease that is initiated and progresses due to dysfunction in the regulation of several proteins and related pathways, including cervical cancer. The presence of HPV in cervical cancer can regulate several signaling pathways such as PI3K-Akt signaling pathway, ERK-MAPK signaling pathway, and Wnt-β-catenin signaling pathway. (Bahrami, *et al*., 2017a, 2017b; Manzo-Merino, *et al*., 2014). Therapeutic opportunities are often lost when finding therapies that target only a single gene. To increase the therapeutic opportunity, therapies that can target multiple proteins and pathways are needed, in addition to potentially selectively addressing cancer cells (Sidhu, *et al*., 2023; Soave, *et al*., 2017).

Current treatments for cervical cancer are surgery, radiotherapy, and chemotherapy. However, these actions have side effects such as in radiotherapy there are side effects of vaginitis and vulvitis and chemotherapy has side effects of hair loss, low platelets, hemoglobin, erythrocytes, and leukocytes, and blackened and dry skin (Health Commission of the PRC, 2022; Setiawan, 2015). Therefore, research on alternative anticancer materials that have selective on cancer cell, no harmfull effect on normal cells, and minimum side effects continues to be developed, one of which uses materials from plants. Natural materials are now becoming a potential source of effective anti-cancer agents with low toxicity (Iqbal, *et al*., 2017). Many studies have stated that plants can act as anti-cervical cancer through various mechanisms, such as inhibition of cell proliferation, induction of apoptosis, reduction of telomerase activity, and inhibition of angiogenesis (Zhang, *et al*., 2023).

In Balinese culture, there is one manuscript known as *lontar usadha rukmini tatwa*. This manuscript explains the use of plants to maintain the beauty and health of female reproductive organs. One of the plants mentioned in the manuscript is long pepper (Rasna and Tantra, 2017; Suryadarma, 2010). Long pepper (*Piper retrofractum* Vahl.) is a plant that has potential compounds as chemopreventive in several cancer cell lines such as MCF-7, WiDr, Myeloma, SCLC-H22, and NCI-H187 (ATCC CRL-5840) (Buranrat, 2022; Ekowati, *et al*., 2012; Mitra, *et al*., 2022). Piperine, as phytochemical of long pepper has been show inhibitory activity proliferation and induced apoptosis of SNU-16 cells by downregulating PI3K-Akt signaling pathway (Chen, *et al*., 2020). However, the potential molecular mechanism of long pepper as cervical cancer chemopreventive agent remain incompletely defined.

Herbal plants have many compounds that have complex mechanisms of therapeutic activity. Likewise, cancer has a complex mechanism that supports the development of cancer cells. Network pharmacology, new paradigm that integrates bioinformatics and pharmacology that effectively interprets and predicts the interaction mechanism of herbal plants with various drug targets in a disease (Lee, *et al*., 2021; Zhou, *et al*., 2024). In addition, molecular docking can explain the potential interactions between targets and compounds in herbal plants (Iksen, *et al*., 2023). Through network pharmacology and molecular docking methods can help explain the potential molecular mechanisms of herbal plants to overcome various diseases such as cancer.

This study is intended to reveal the ability of compounds in long pepper fruit such as piperine, retrofractamide A/B/C/D, piperlonguminine, pipernonaline, dehydropipernonaline, pellitorine, germacrene D, and ar-turmerone as chemopreventive agents for cervical cancer using network pharmacology and molecular docking analysis.

MATERIALS AND METHODS

Bioavailability Analysis

Compounds of long pepper fruit were obtained by searching the literature on PubMed, Google Scholar, and Science Direct with the keywords "Piper retrofractum Vahl fruit compound". The compound data obtained was then predicted for bioavailability using the SCFBio web server (http://www.scfbio-iitd.res.in/software/drug-design/lipinski.jsp) based on the Lipinski rule of five (Ro5).

Identification Possible Target of Long Pepper Fruit and Cervical Cancer

Action targets of long pepper were obtained by using SwissTargetPrediction (http://www.swisstargetprediction.ch/). Disease-related targets were obtained from GenCards (http://genecards.org/) with the keyword "cervical cancer". The two results were intersected using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) to obtain targets that correlate between long pepper compounds and cervical cancer.

Protein-Protein Interaction (PPI) Network Construction

The PPI network was created by exporting the possible targets to the STRING database (https://string-db.org/). The species selected was "Homo sapiens" with a required score of 0.7 (high confidence). The results were then exported to Cytoscape 3.10.0 software. CytoHubba plugin was used to analyze hub genes based on degree values.

Gene Ontonlogy (GO) and KEGG Enrichment Analysis

Possible targets were entered into Shiny GO 0.77 (http://bioinformatics.sdstate.edu/go/) to further analyze the role of potential target proteins. Gene Ontology (GO) analysis includes biological processes (BP), cell components (CC), molecular functions (MF), and KEGG Enrichment to analyze their signaling pathways. Analysis was performed with a p-value ≤ 0.05, which is based on the FDR (False discovery rate) value.

Molecular Docking

The 3D structure of the E6 oncogene of HPV16 (PDB ID: 4GIZ) obtained through the RCSB PDB database (https://www.rcsb.org/) was prepared by removing other chains, water molecules, and adding hydrogen atoms using Chimera 1.11 software. The 3D structure of the long pepper fruit compound that has been obtained on PubChem (https://pubchem.ncbi.nlm.nih.gov/) was optimized by HyperChem with the AM1 semi-empirical computational method. Molecular docking was performed using PyRx 0.8 software. The analysis results will show the lowest binding affinity conformation to bind to the target protein.

RESULTS

Bioavailability Analysis

Based on the results of the drug-likeness test using Lipinski's Rule of Five, 11 active compounds met the drug-likeness rules (Table 1). Ro5 includes molecular weight, hydrogen bond donor, hydrogen bond acceptor, log$P$, and molar refractivity.

Identification Target of Long Pepper and Cervical Cancer

SwissTargetPrediction database search collected a total of 1,138 target genes from 11 compounds of long pepper. After gene duplication was removed, 478 targets were obtained. Searching for genes related to cervical cancer using the GenCards database, 10,452 genes were obtained. Then 367 genes overlap between test compounds with cervical cancer (Figure 1).

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Table 1. Bioavailability analysis based on Lipinski's rule of five.

Compounds	Lipinski's Rule of Five Criteria				
	MW (≤5)	HBD (≤5)	HBA (≤10)	Log*P* (≤5)	MR (40-130)
Piperine	285	0	4	2.997199	81.169983
Retrofractamide A	327	1	4	4.093299	96.960670
Retrofractamide B	355	1	4	4.873500	106.194672
Retrofractamide C	329	1	4	4.317299	97.054672
Retrofractamide D	341	1	4	4.483399	101.577667
Piperlonguminine	273	1	4	2.756900	78.586685
Pipernonaline	341	0	4	4.557599	99.637970
Dehydropipernonaline	339	0	4	4.333599	99.543976
Pellitorine	223	1	2	3.451299	70.269684
Germacrene D	204	0	0	4.891299	68.832985
ar-Turmerone	216	0	1	4.023919	68.648987

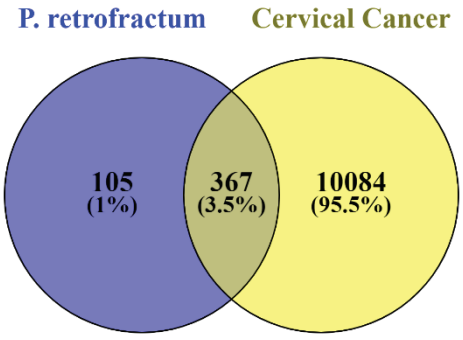

Figure 1. Venn diagram representing 367 possible targets between long pepper and cervical cancer.

PPI Network Construction and Analysis

The 367 possible targets were exported to the STRING database to construct and analyze the PPI network (Figure 2a). 367 nodes and 1,632 edges were obtained. The number of nodes depicts the gene and edges show the relationship between genes. The greater the number of lines, the stronger the association. The average value of nodes degree is 8.89 and the p-value of PPI enrichment is <1.0e-16. A *p*-value of less than 1 indicates that the resulting network has significantly more interactions than those excluded.

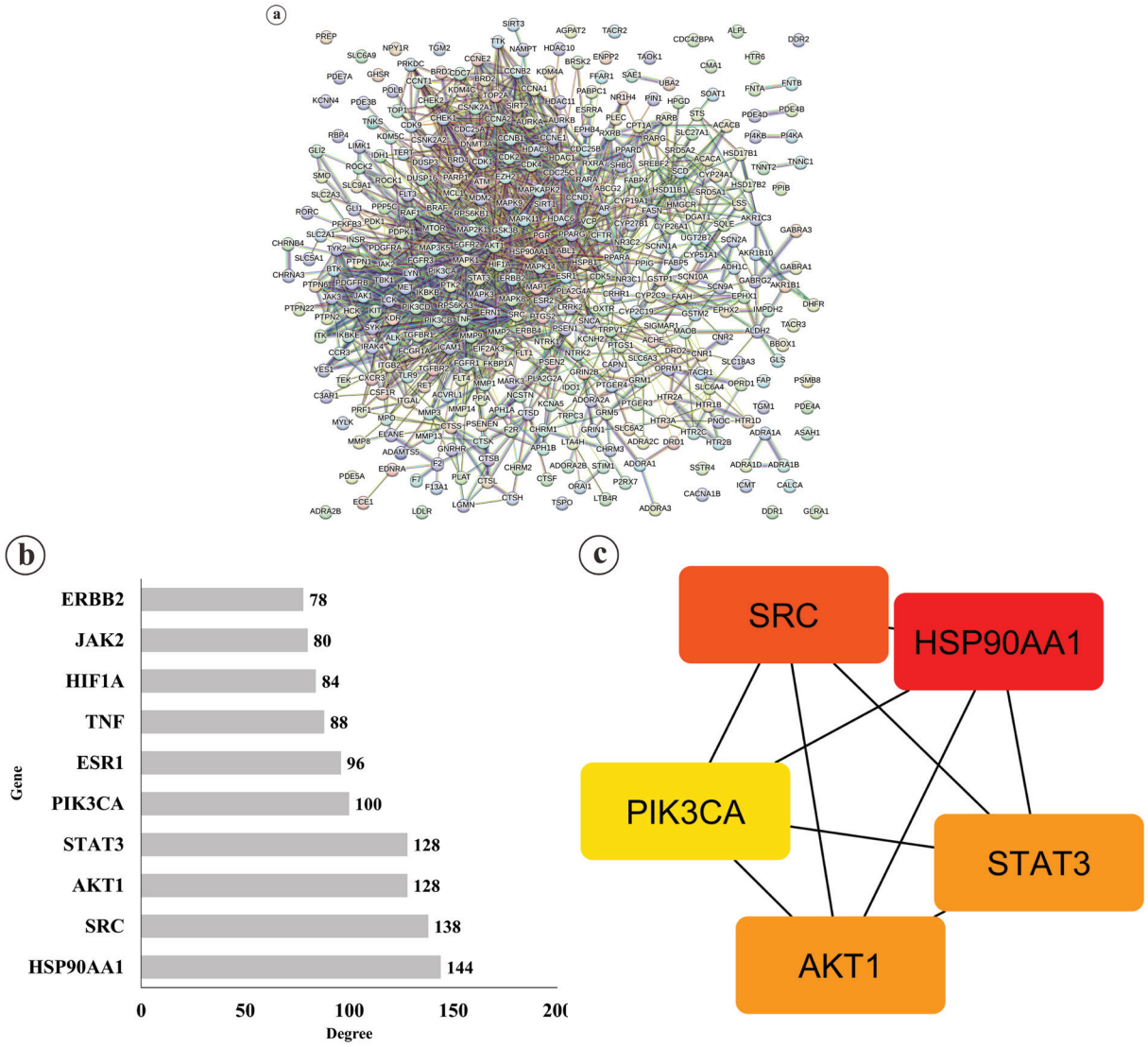

Figure 2. PPI analysis result. (a) Protein-protein interaction network of the 367 common target genes of long pepper and cervical cancer; (b) Bar chart of degree value for each genes; (c) Network interaction representing five hub genes.

The analysis results in the STRING database were then exported to Cytoscape 3.10.0 software to analyze the degree value of each node. The degree indicates the amount of direct correlation between one node and another. The higher the degree value, the more important the node (Zeng, *et al.*, 2021). It was found that HSP90AA1 (degree=144), SRC (degree=138), AKT1 (degree=128), STAT3 (degree=128), and PIK3CA (degree=100) were the 5 genes with the highest degree value (Figure 2b and 2c). This indicates that these genes have an important role and can be potential targets for further study related to the chemoprevention effect of long pepper fruit.

GO and KEGG Enrichment Analysis

The 367 possible targets were entered into the Shiny GO database for GO and KEGG analysis. GO analysis includes biological processes (BP), cellular components (CC), and molecular functions (MF) to predict the main biological functions that occur in the target protein. KEGG analysis aims to determine the signaling pathways that are dominantly involved in the mechanism of action of long pepper. Analysis was performed with a *p*-value ≤0.05, which is based on the FDR (False discovery rate) value. The analysis showed that the biological processes mostly involved response to oxygen-containing compound and protein phosphorylation. Cellular components mostly involve the intrinsic component of plasma membrane and neuron projection. Molecular functions mostly involve signaling receptor activity and molecular transducer activity (Figure 3a). The results of KEGG analysis showed that the dominant signaling pathways involved were neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, MAPK signaling pathway, Calcium signaling pathway, and cAMP signaling pathway (Figure 3b).

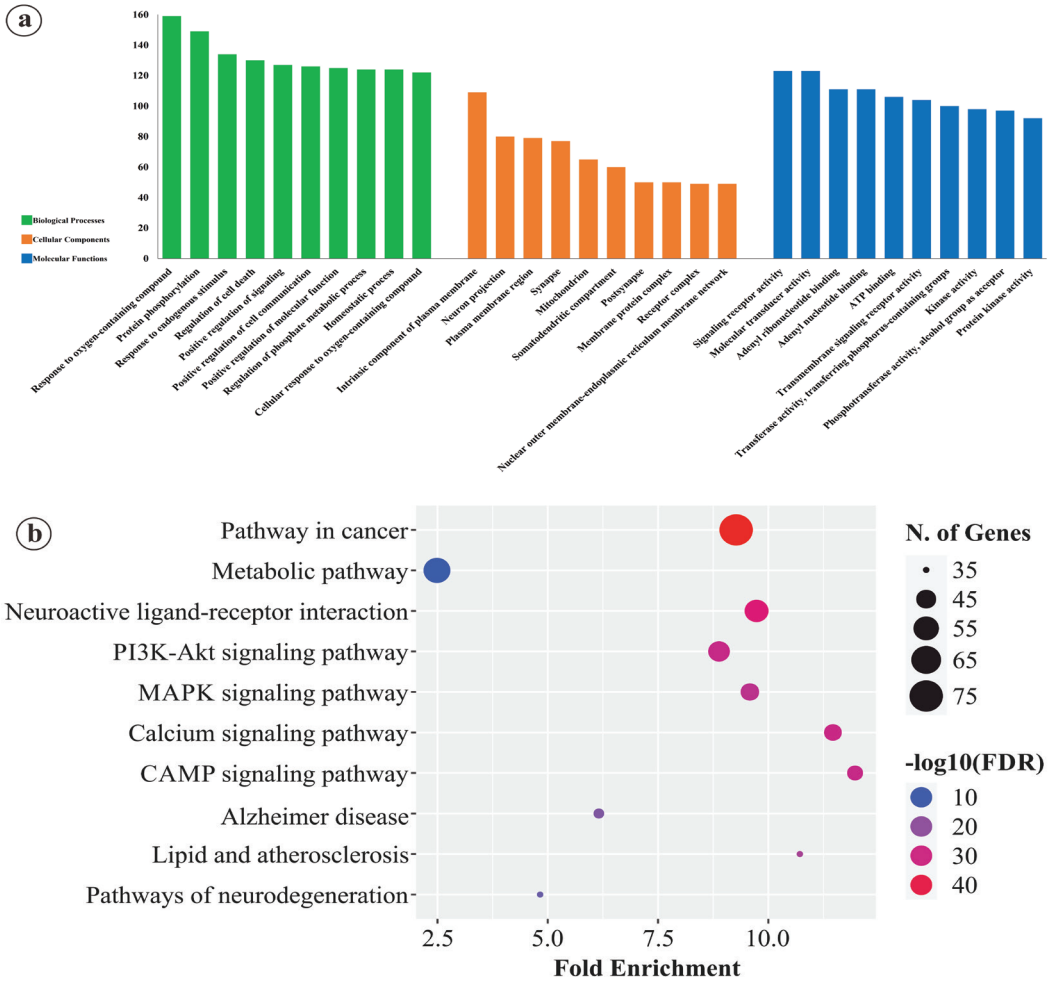

Figure 3. GO and KEGG enrichment analysis results. (a) Bar chart of GO enrichment analysis; (b) Dotplot of KEGG enrichment analysis.

Molecular Docking Analysis

Molecular docking was performed using PyRx 0.8 software on the binding site according to the native ligand location of each potential target. Because E6 oncogene doesn't have a native ligand, the binding site following the results of the study by Kolluru, *et al*. (2019) which examined the location of potential binding sites of the E6 oncogene. In this study, luteolin and jaceosidin were used as controls for the E6 oncogene (Cherry, *et al*., 2013; Lee, *et al*., 2005). The results show that all compounds have negative binding affinity values (Table 2 and Figure 4a-m).

Table 2. Binding affinity value of molecular docking result of long pepper fruit compounds with E6.

Compounds	Binding Affinity (kcal/mol)	Hydrogen Bonds	Other Interaction
Piperine -	6,7	-	Tyr32, Val31, Phe45, Cys51, Gln107, Leu67, His78, Arg77, Val62, Leu50, Val53, Ser74, Tyr70
Retrofractamide A	-7,2	Tyr30	Ser71, Gln107, Ser74, Tyr70, Leu67, Val53, Val31, Val62, Leu50, Phe45, Cys51, Arg102, Arg131
Retrofractamide B	-6,5	Arg131, Ser74, Gln107	Ser71, Cys51, Val53, Val31, Leu67, Tyr70, Thr133, Ile128, Phe45, Leu50, Val62, Tyr32
Retrofractamide C	-6,1	Arg131	Tyr32, Tyr70, Leu67, Gln107, Arg102, Phe45, Cys51, Val31, Val62, Val53, Leu50
Retrofractamide D	-6,5	Gln107	Leu67, Ser74, Ser71, Arg131, Arg102, Cys51, Leu50, Val62, Tyr32, Phe45, Val53, Val31
Piperlonguminine	-6,5	Ser74	Arg131, Glu75, Ser71, Thr133, Ile104, Tyr32, Val31, Phe45, Cys51, Gln107, Ile128, Leu50, Leu67, Val62, Val53
Pipernonaline	-6,4	Arg102, Arg131	Gln107, Leu67, Cys51, Leu50, Val62, Phe45, Val53, Tyr32, Val31
Dehydropipernonaline	- 7,2	Arg102, Arg131	Ala61, Ser71, Leu67, Ser74, Gln107, Tyr70, Val53, Leu50, Phe45, Val62, Val31, Tyr32, Cys51
Pellitorine	-5,5	Arg131	Cys51, Ala61, Ser71, Ser74, Gln, Thr133, Leu50, Phe45, Val31, Val53, Leu67, Val62, Ile128
Germacrene D	- 6,2	-	Arg55, Tyr32, Val31, Ala61, Phe45, Leu50, Leu67, Cys51, Val53, Val62
ar-Turmerone	- 6,2	-	Ser71, Leu67, Gln107, Ser74, Tyr70, Tyr32, Ala61, Cys51, Val53, Val62, Leu50, Phe45, Val31
Luteolin	-7,3	Arg131, Arg102, Gln107	Leu50, Cys51, Val62, Val53, Phe45, Tyr60, Ala61, Val31, Tyr32, Leu67, Arg131, Arg102
Jaceosidin	-7,0	Gln107	Arg102, Val62, Leu67, Leu50, Cys51, Phe45, Ala61, Val53, Val31, Tyr32, Ser74, Ile128, Ile128

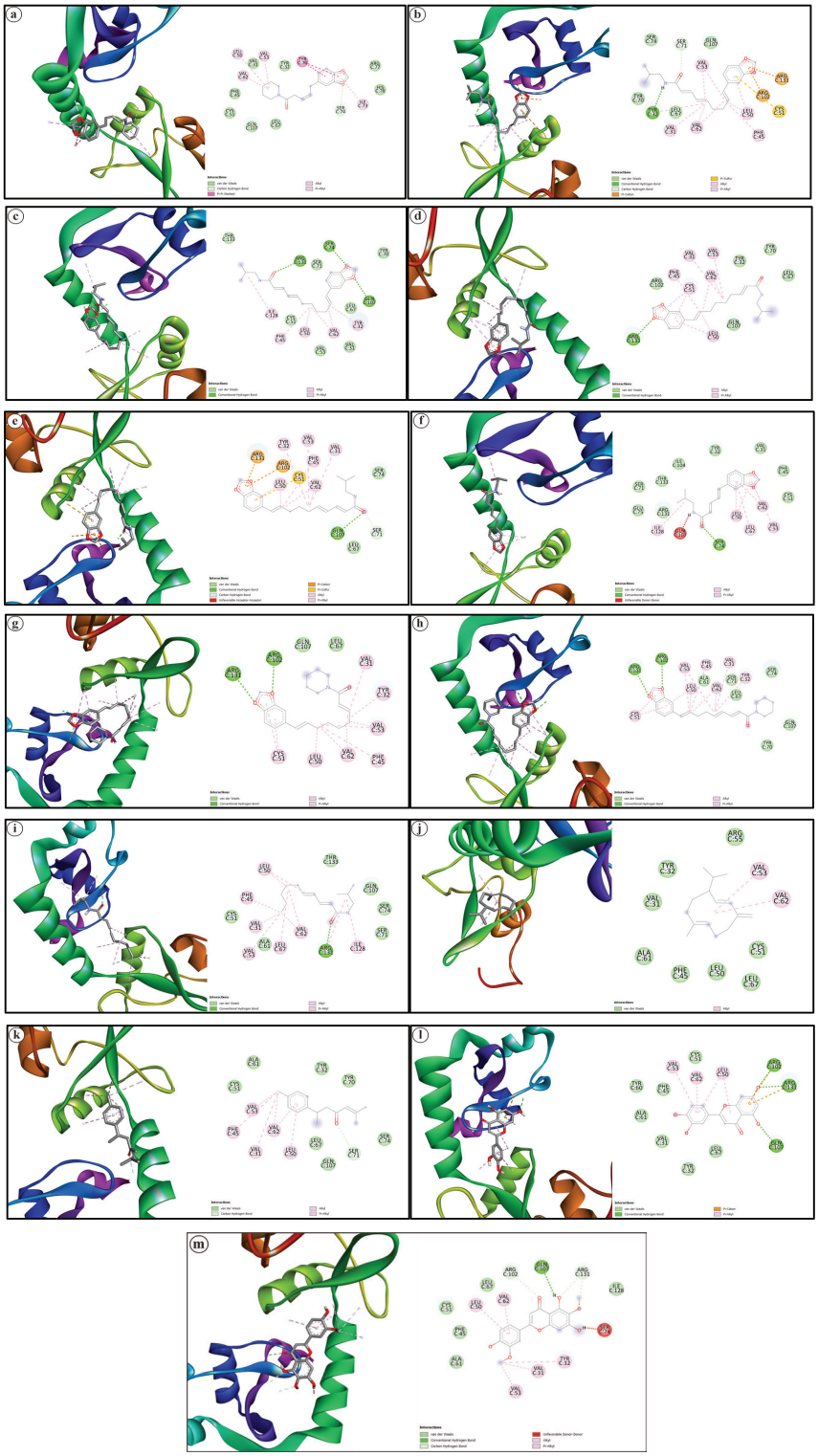

Figure 4. 3D and 2D of molecular docking result of long pepper fruit compounds with E6 (a) Piperine; (b) Retrofractamide A ; (c) Retrofractamide B; (d) Retrofractamide C; (e) Retrofractamide D; (f) Piperlonguminine; (g) Pipernonaline; (h) Dehydropipernonaline; (i) Pellitorine; (j) Germacrene D; (k) ar-Turmerone; (l) Luteolin; (m) Jaceosidin.

DISCUSSION

To predict bioavailability of long pepper fruit compound, SCFBio was used to assess the bioavailability of the compound based on Lipinski's rule of five. Bioavailability analysis aims to predict if a biologically active molecule is likely to have the chemical and physical properties to be orally bioavailable. The result showed that 11 compounds had good bioavailability. This can be seen from the results which show that the 11 compounds fulfill Lipinski's rule of 5. Compounds with a high probability of having drug-like properties must follow at least two rules. This rule consists of molecular weight (MW) ≤500, number of hydrogen bond donors (HBD) ≤5, number of hydrogen bond acceptors (HBA) ≤10, lipophilicity (log*P*) ≤5, and molar refraction (MR) between 40-130. (Lipinski, 2004). These compounds are predicted to have good bioavailability and can trigger a biological response when interacting with the target protein.

Cancer is a disease that has complex mechanisms that initiate and progress due to the dysfunction of several proteins and associated signaling pathways. Network pharmacology can help to elucidate drug targets and mechanisms to fight diseases such as cancer (Sidhu, *et al*., 2023). To obtain the potential target of long pepper fruit, the SwissTargetPrediction and GeneCards database database was used. SwissTarget and GeneCards database overlapped together and the intersection between the two was obtained with the help of Venny 2.1.0. A total 367 possible target between long pepper and cervical cancer was obtained. All 367 target was imported to STRING by selecting Homo sapiens as an organism. The PPI Network obtained was exported to CytoScape 3.10.0 and CytoHubba plug-in to obtain the top five hub genes and HSP90AA1, SRC, AKT1, STAT3, and PIK-3CA were obtained. Hub gene is the gene with the highest degree value of connection with other genes (Das, *et al*., 2023).

HSP90AA1, SRC, AKT1, STAT3, and PIK3CA were identified as major hub genes of long pepper on cervical cancer. Hub genes have a high association with nodes in a network and have been shown to have a high biological role in certain diseases (Yuan, *et al*., 2017). These hub genes may be a promising therapeutic target of long pepper fruit for cervical cancer. HSP90AA1 which is part of the heat shock protein 90 group is important in tumor cell proliferation and differentiation. Increased expression of HSP90AA1 is associated with poor survival of cervical cancer patients. HSP90AA1 inhibits the binding of caspase 9 to AP-1 and stabilizes the mutant p53 complex, thus inhibiting the initiation of apoptosis (Fan, *et al*., 2020). SRC is a non-receptor protein tyrosine kinase that will experience increased expression in several cancers, including cervical cancer. This increased expression causes activation of human cervical cancer tissue correlated with cell survival, proliferation, angiogenesis, and metastasis and contributes to the incidence of chemotherapy resistance. It was found that decreasing SRC expression can inhibit the proliferation process of HeLa and SiHa cells, so it can be a therapeutic target for cervical cancer (Kong, *et al*., 2011; Takiguchi, *et al*., 2017). Increased STAT3 phosphorylation (activated form of STAT3) that occurs in cervical cancer plays a role in stimulating cell proliferation and angiogenesis. STAT3 activation is associated with increased expression of antiapoptotic genes Bcl-xL, survivin, and Mcl-1 in tissues. This suggests that STAT3 contributes to cancer progression and tumor angiogenesis, so its inhibition could be a therapeutic target for cervical cancer (Chen, *et al*., 2007; Zhang, *et al*., 2014).

In this study, ShinyGO 0.77 was used to the association between GO and KEGG, respectively. The KEGG database can help in understanding the basic features and functions of biological systems that include cells, organisms, and the environment. KEGG can computationally

represent biological systems consisting of molecular building blocks, genes, proteins, and chemicals (Das, *et al*., 2023). KEGG analysis shows that the signaling pathways that are dominantly involved are neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, MAPK signaling pathway, Calcium signaling pathway, and cAMP signaling pathway. The PI3K-Akt signaling pathway plays a role in modulating mitogenic or oncogenic events leading to cancer development. The E6 oncogene activates the PI3K-Akt signaling pathway which will contribute to genetic instability, deregulation of proliferation, apoptosis resistance, and changes in metabolic characteristics, ultimately leading to malignancy in infected cells. One of the isoforms of E6, E6*, can modulate hDlg (human disc large) and can increase the expression of activated PTEN and Akt thereby increasing the expression of p-PI3K encoded by PIK3CA which activates MAPK and promotes cell proliferation (Zhang, *et al*., 2014).

Molecular docking in this study used PyRx 0.8 software which was carried out on binding sites in the following results of the research of Kolluru, *et al*. (2019), on amino acids Cys51, Leu50, Arg102, Arg131, Leu67, Val62, and Gln107. The molecular docking results show that the compound in long pepper has a binding affinity value with a negative value. It was found that retrofractamide A and dehydropipernonaline are compounds with the highest binding affinity values, which are -7.2; -7.2; and 6.7 kcal/mol, respectively.

These results also show that retrofractamide A and dehydropipernonaline have better binding affinity than jaceosidin but lower when compared to luteolin. The less energy obtained, the more stable is formed (Laksmiani, *et al*., 2016). Jaceosidin is a compound isolated from Artemisia argyi which has the ability as a natural inhibitor of the E6 oncogene of HPV 16 to binding with p53 so it is appropriate to be used as a control (Lee, *et al*., 2005). Luteolin is a flavonoid compound that also has the ability as a natural inhibitor of the E6 oncogene to bind to E6AP (Cherry, *et al*., 2013).

The results showed that long pepper compounds bind to the same amino acids of LxxLL binding pocket of E6 (Kolluru, *et al*., 2019; Soumia, *et al*., 2022). This suggests that the compounds in long pepper compound may have the ability to induce extrinsic and intrinsic apoptosis. E6 can bind to several proteins through the LxxLL binding motif, such as binding to p53 and FADD (Chitsike and Duerksen-Hughes, 2021; Kolluru, *et al*., 2019). E6 is capable of degrading the tumor suppressor p53. The E6 oncogene binds directly to E6AP through recognition LxxLL helix motif of the HECT domain of E6AP (Ricci-López, *et al*., 2019). Its causing its substrate specificity to be altered so that it stably associates with and polyubiquitylates p53, resulting in the degradation of p53 by the 26S proteasome (Beaudenon and Huibregtse, 2008). Tumor suppressor p53 which are degraded by E6 will allow the occurrence of upregulation of survival and proliferation signaling cascade causing abnormal cell growth (Das, *et al*., 2023).

In addition to interacting with p53, E6 can also interact with FADD (Tungteakkhun, *et al*., 2008). FADD plays an important role in influencing cancer cell development such as proliferation, apoptosis, cell cycle, autophagy, and drug resistance (Liu, *et al*., 2022). The E6 oncogene can interfere with the apoptotic pathway by interacting with FADD procaspase 8 in the extrinsic apoptotic pathway. Under normal conditions, FADD will induce death-inducing signaling complex (DISC) which will activate procaspase 8 and activate caspase 3 and 7 thus inducing apoptosis. However, when the E6 oncogene is expressed, E6 will bind to FADD and stop the activation process of procaspase 8 thus blocking apoptosis (Kolluru, *et al*., 2019; Pal and Kundu, 2020). Thus, the inhibitory effect of the E6 oncogene by long pepper fruit may can trigger apoptosis.

CONCLUSION

In silico analysis showed that long pepper fruit has compounds with good bioavailability. Through network pharmacology approach, it was found that HSP90AA1, SRC, AKT1, STAT3, and PIK3CA are the dominant hub genes in the mechanism of long pepper fruit on cervical cancer. The results of KEGG analysis showed that the results of molecular docking showed neuroactive ligand-receptor interaction, PI3K-Akt signaling pathway, MAPK signaling pathway, Calcium signaling pathway, and cAMP signaling pathway are the most dominant signaling pathways involved. Through molecular docking, it was found that long pepper fruit has the potential to inhibit the E6 oncogene of HPV so that it may trigger apoptosis. However, further studies such as *in vitro* and *in vivo* tests are needed to fully understand the potential of long pepper fruit as a chemopreventive agent for cervical cancer.

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