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Original Research Article

Differentially Expressed Genes, and Molecular Docking and Dynamic Analysis Revealing the Potential of Compounds in *Zingiber officinale* Roscoe as Inhibitors of TP53- regulating Kinase (TP53RK) that Influence the p53 Signaling Pathway Related to Apoptosis and Cell Cycle

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ABSTRACT

Overexpression of CDKN1A—a downstream p53 gene—can trigger apoptosis in old cells but can also lead to chemoresistance and serve as a poor prognosis marker in cancer. This study explores apoptosis mechanisms via the p53 signaling pathway, focusing on the expression of CDKN1A (p21). Differential expression gene (DEG) analysis was performed on downstream p53 signaling genes using GSE23773 data from the Gene Expression Omnibus database, which includes whole human genome microarray data of colorectal cancer cells (HT-29) over time. Molecular docking simulations of compounds in ginger (*Zingiber officinale* Roscoe) ginger (*Zingiber officinale* Roscoe) against against TP53- related kinase (TP53RK), a protein that activates p53, were conducted to assess the potential of these compounds to influence downstream genes in the p53 signaling pathway. The DEGs were validated using machine learning and principal component analysis methods. Molecular docking and dynamic simulations of ginger compounds against TP53RK were performed on the crystal structure of the D-chain protein with PDB code 6WQX [D]. The docking scores of these compounds were then compared with pomalidomide, a known inhibitor of TP53RK. DEG analysis of GSE23773 data revealed that BAX, STEAP3 (TSAP6), AIFM2, SIVA1, GTSE1 (B99), and CDKN1A (p21) are downstream p53 genes whose expression undergoes regulatory changes in old HT-29 cells (day 7 cultures). Molecular docking and molecular dynamic simulation analysis indicated that gingerenone A, 6-shogaol, gingerol, and 6-paradol in *Z. officinale* Roscoe have the potential to down-regulate CDKN1A (p21) by inhibiting p53 activation through TP53RK and stable during 10ns.

Keywords: *Zingiber officinale* Roscoe, p53 signaling pathway, TP53-regulating kinase, differentially expressed genes, Molecular docking, Molecular dynamic simulation.

Introduction

Colorectal cancer (CRC) ranks as the third most common type of cancer for new cases and the second leading cause of cancer-related deaths worldwide.[1] The treatment of colorectal cancer faces several challenges. Conventional chemotherapy and radiotherapy have limited effectiveness, resulting in high mortality rates and low survival rates.[2] Ongoing research includes the exploration of new medicinal compounds, including those derived from plants. Ginger (*Zingiber officinale Roscoe*) is a widely used spice that contains various chemical compounds, including phenolic compounds, terpenes, polysaccharides, lipids, organic acids, and dietary fiber. The health benefits of ginger are primarily associated with its phenolic compounds, such as gingerol and shogaol.

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Ginger has been found to exhibit various biological activities, including antioxidant, immunomodulator, antibacterial, anti-inflammatory, anti-diabetic, anti-obesity, neuroprotective, anti-nausea, antiemetic, cardiovascular protective, respiratory protective, and anticancer activities.[3–5] The main phenolic compounds in ginger are gingerol, shogaol, and paradol. Gingerol is the major polyphenol in fresh ginger, while shogaol is formed through heat treatment or long-term storage.[6,7] Ginger has been studied for its potential in combating cancer through various mechanisms. One of the main ways ginger influences cancer is by inducing apoptosis, or programmed cell death. Compounds found in ginger, such as gingerol, shogaol, and zingerone, have been shown to trigger the apoptosis pathway in colorectal cancer cells. [8] Additionally, ginger extract has been proven to have anticancer properties by inducing cell cycle arrest, inhibiting ovarian cancer cell growth, and inducing apoptosis through the p53 pathway.[9] In CRC cells, ginger extract is known to inhibit proliferation, induce apoptosis, and halt the cell cycle in the G0/G1 and G2/M phases.[10] This provides additional control over tumor growth by preventing uncontrolled proliferation of cancer cells. Apoptosis is a crucial mechanism in anticancer therapy aimed at eliminating damaged cells without causing harm to surrounding tissues.[11] In cancer, cells exhibit a progressive decline in their ability to trigger apoptosis, while whereas in aging tissues, there is an accumulation of senescent cells.[12] Common anticancer drugs generally target apoptotic signaling pathways to induce cell death and prevent chemoresistance.[13] One signaling pathway that regulates

apoptosis is the p53 activity helps destroy cancer cells, while early senescence triggered by p53 promotes tumor regression in the environment.[14,15] p53 activation is carried out by TP53-regulating protein kinase (TP53RK), also known as PRPK, which is a major source of serine residue (Ser15) phosphorylation processes.[16,17] Activated p53 has various effects on gene expression. For example, in the cellular senescence process, p53 activation leads to an increase in p21 (CDKN1A). Increased p21 in aging cells causes cell cycle arrest.[18] On the other hand, cells that produce excess p21 have been known to exhibit chemoresistance.[19] Increased TP53RK expression in patients with multiple myeloma (MM) and colorectal adenocarcinoma is associated with a poor prognosis [20,21] Therefore, it is essential to explore TP53RK inhibitor compounds, including those derived from ginger.

This study analyzed differentially expressed genes (DEG) in GSE23773 data obtained from the Gene Expression Omnibus (GEO) database. In these data, genes expressed in the first-day cultures of CRC cells HT-29 compared with those of the seventh day after incubation. The study aims to explore the mechanism of apoptosis through the p53 signaling pathway, focusing on the expression of CDKN1A (p21) —a downstream gene of p53. The novelty of this research lies in its comprehensive approach to analyzing the differential expression of p53 downstream genes over time in HT-29 colorectal cancer cells, and in the unique application of molecular docking and molecular dynamic simulations involving ginger compounds. By focusing on the modulation of the p53 pathway through TP53RK, the study provides new insights into the potential therapeutic roles of ginger metabolites in regulating CDKN1A (p21) and inhibiting p53 activation, which may contribute to novel cancer treatment strategies.

The relevance of the research methods is highlighted by their ability to provide detailed insights into the p53 signaling pathway and its downstream effects in colorectal cancer cells. The use of GEO database data for differential gene expression analysis allows for the identification of significant changes in gene expression over time, which is crucial for understanding the progression and treatment of cancer. The application of molecular docking and molecular dynamic simulations to assess the interaction between ginger compounds- obtained from the 'Jamu of Knapsack' database,[22]- and TP53RK is pertinent for evaluating potential inhibitors that could modulate the p53 pathway. These methods collectively offer a robust framework for exploring new anticancer agents and therapeutic targets .

Materials and Methods

Analysis of Differentially Expressed Genes in GSE23773 of the GEO Database

GSE23773 data obtained from the GEO database underwent DEG analysis using GEO2R-an interactive web tool that allows users to compare two or more groups of samples in a GEO Series,[23] and the results were validated using machine learning methods in the Orange v3.37.0, University of Ljubljana, 2024.[24] Validation was conducted to obtain sensitivity, specificity, and the area under the curve (AUC) as measures of the DEG's ability to distinguish HT-29 cell cultures on day 1 (D1) and day 7 (D7) of incubation. The difference in the number of cells at D1 and D7 can depict cell proliferation. Differences in gene expression can illustrate the mechanisms of action of metabolite compounds present in ginger as anticancer agents regulating to the p53 signaling pathway. Validation was also performed using principal component analysis (PCA) in the Orange, to evaluate the clustering of D1 and D7 HT-29 cell cultures based on changes in downstream gene expression regulating to the p53 signaling pathway obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[25]

Molecular Docking and Molecular Dynamics Simulation

Preparation of *Z. officinale* Roscoe metabolite compounds involved predicting the druglikeness values of these compounds using DataWarrior v6.1.0, openmelocules, 2024.[26] Metabolite compounds with positive druglikeness values were then analyzed for their interactions with the TP53RK protein using Molegro Virtual Docker (MVD) v.7.0.0, Molexus Aps, 2019 (free trial).[27] The 3D structure or conformation of *Z. officinale* Roscoe metabolite compounds was searched in the Pubchem database,[28] and saved in a Structure Data File (SDF) format. Minimization of the 3D structure's energy was performed in Datawarrior, and the file was saved in SDF format. The PRPK-TP53RK binding protein (TPRKB)-AMPPNP (PDB ID: 6WQX) was downloaded from the RSCB PDB database (www.pdb.org) and saved as a protein data bank (PDB) file. [16] The computer used for the molecular docking process had Windows 11 Pro specifications, an Intel(R) Core(TM) i7-8665U CPU @ 1.90 GHz -2.11 GHz processor, and 16.0 GB of RAM.

The crystal structure of the protein downloaded from the RSCB PDB database was imported into the MVD. For molecular docking purposes, all water molecules were removed, and amino acid residue corrections were made. Ligand preparation was done with Datawarrior to find the most stable conformation or the lowest energy (Merck Molecular Force Field, MMFF94 value). MVD was used to predict potential cavities or binding sites on PRPK (PDB ID: 6WQX). The 6WQX protein is a complex consisting of 2 PRPK protein chains and two TP53RK binding protein (TPRKB) chains. The ligand in the crystal structure of the 6WQX protein is located on the PRPK protein chains identified as AMPPNP (adenyl-imidodiphosphate). A cavity with a volume of 193.024 Å^3 and a surface area of 593.92 Å^2 was identified as the binding site for ANP_301 (AMPPNP), a ligand found in chain D in the 6WQX crystal structure (6WQX [D]). The MolDock Score grid was set with a grid resolution of 0.30.

The interaction analysis between the protein and ligand through molecular binding was carried out using MVD on all 3D structures of ligands or *Z. officinale* Roscoe metabolite compounds in the Knapsack database. A re-docking process for 20 runs was performed on the 3D molecule ANP_301 (AMPPNP) in 6WQX [D] in the protein binding site region to validate the docking method used. The validity criteria for this method were set with a Root Mean Square Deviation (RMSD) value < 2 Å. Subsequently, molecular docking was applied to the 3D ligand structure against the crystal structure of the protein. Molecular docking was also performed on pomalidomide, a thalidomide analog known to interact directly with TP53RK protein and downregulate p21 mRNA expression.[21,29] In this docking process, measured parameters included the energy values involved, such as MolDock Score, Rerank Score, and Hbond. The Rerank Score is often used as a parameter to evaluate the strength of the binding between the ligand and the receptor protein.[30]

Molecular dynamic simulations were processed in 10 ns using YASARA version 23.12.24.W.64 licensed to Dr. Fadilah, S.Si., M.Si, Department of Medical Chemistry, Faculty of Medicine Universitas Indonesia with the Amber14 force field. The solvent system was set to cube form at 300 K and 1 atm of pressure. Trajectory analysis included Root Mean Square Deviation (RMSD) and root mean square fluctuation (RMSF).

Statistical Analysis

The p-values for the DEG analysis were calculated using GEO2R available on the GEO browser. The Area Under the Curve (AUC), Classification Accuracy (CA), Precision (Prec), Specificity (Recall), and Matthews Correlation Coefficient (MCC) were calculated using Orange.

Results and Discussion

Differentially Expressed Genes (DEG) Analysis

The results of the DEG analysis on the downstream genes of the p53 pathway in the GSE23773 data can be shown in Figure 1 (the complete DEG data is available in S1). In Figure 1, five genes, namely *BAX*, *STEAP3*, *AIFM2*, *SIVA1*, and *GTSE1(B99)*, evidently exhibited significant differences in expression between D1 and D7 HT-29 cells with adjusted p-value < 0.05, where the expression on D7 was downregulated relative to D1. The adjusted p-values were generated using the Benjamini–Hochberg correction. Additionally, the expression of CDKN1A also showed significant differences and was upregulated. The response to these significantly differentially expressed downstream target genes is shown in Table 1.

As shown in Table 1, among it was evident that among the p53 signaling pathway-regulating genes with significant differrences between D1 and D7, three genes were associated with apoptosis response (*BAX*, *AIFM2*,

SIVA1). Two other genes (*B99* and *CDKN1A*) were linked to cell cycle arrest, and one gene was associated with exosome-mediated secretion (TSAP6). This indicates that the mechanisms of apoptosis, exosome-mediated secretion, and cell cycle arrest can be targeted for therapy to promote the inhibition of HT-29 cell proliferation. The grouping of D1 and D7 based on the expression of the six genes (*BAX, STEAP3, AIFM2, SIVA1, GTSE1 (B99),* and *CDKN1A*) using PCA and machine learning analysis from Orange is presented in Figure 2 and Table 2 (data in S1). Figure 2, shows the PCA analysis using the gene expression data of *BAX, TSAP5, AIFM2, SIVA1, B99,* and *CDKN1A* (p21)., Relative clustering was evident between D1 and D7 HT-29 cells. Table 2 showcases the results of the validation using Support Vector Machine We We recorded an AUC value of 0.75, Classification Accuracy (CA) of 0.88, **Precision (Prec) of 0.9**, Specificity (Recall) of 0.88, and Matthews Correlation Coefficient (MCC) of 0.78.

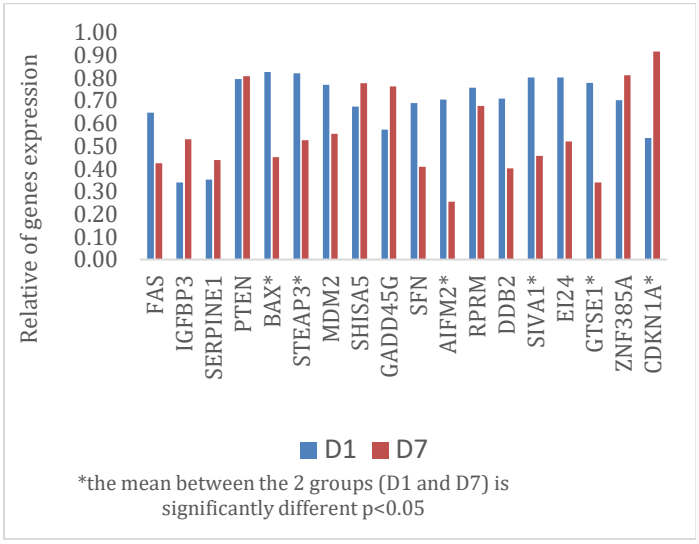

Figure 1: Downstream genes regulating the p53 signaling pathway that are significantly differentially expressed ($p < 0.05$).

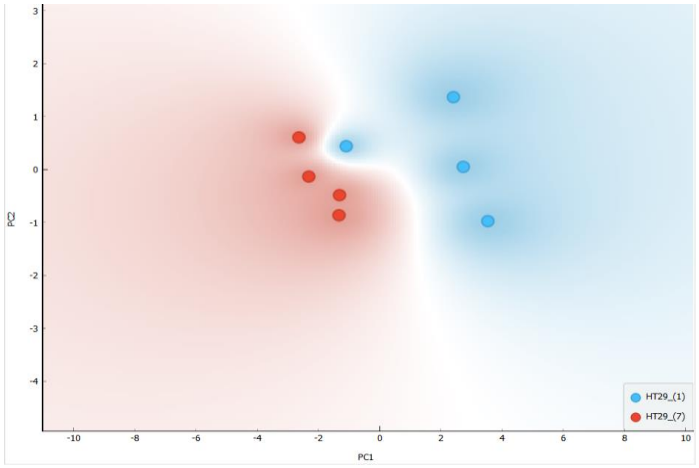

Figure 2: PCA Analysis Results Based on the Expression of BAX, TSAP5, AIFM2, SIVA1, B99, and CDKN1A. A relative clustering is observed between HT-29 cells at Day 7 compared to Day 1.

These values suggest that the six genes collectively were capable of effectively clustering D7 and D1 HT-29 cells. Therefore, these six downstream genes of the p53 signaling pathway can served as markers for observing the time-dependent proliferation of HT-29 cancer cells. *BAX, STEAP3 (TSAP6), AIFM2, SIVA1,* and *GTSE1 (B99),* as well as *CDKN1A* (p21), are downstream genes in the p53 protein signaling pathway that can be directly influenced by the regulation of the p53 protein, as depicted in Figure 3. Figure 3 presents the p53 signaling pathway diagram based on the KEGG database.[25] In Figure 3, it was evident that *GTSE1 (B99)* plays a role in influencing the cell cycle, while *BAX, SIVA1, AIFM2,* and *CDKN1A* are involved in the mechanisms of apoptosis. *GTSE1* is known to regulate the G1/S cell cycle transition and is reported to be overexpressed in human cancers, including CRC.[31,32] By contrast, *BAX, SIVA1,* and *AIFM2* are known pro-apoptotic proteins that play essential roles in the intrinsic apoptosis signaling pathway.[33–35] The downregulation of *BAX, SIVA1, AIFM2,* and *GTSE1* appeared to occur naturally in aging HT-29 cells (D7) and may be challenging to avoid. A different pattern is observed in the expression of CDKN1A, where in aging HT-29 cells (D7), its expression tends to be upregulated.

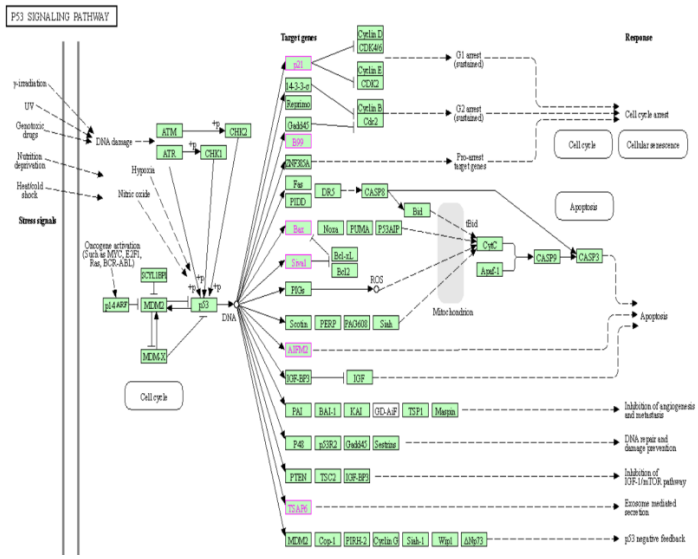

Figure 3: Genes associated with the p53 signaling pathway based on KEGG data [25]

According to the DEG analysis, downstream genes in the p53 signaling pathway, which initiate apoptosis and cell cycle arrest in HT-29 cells, exhibited decreased expression over time in culture . By contrast, CDKN1A (p21) showed increased expression in D7 compared to D1 HT-29 cells. As previously discussed, increased CDKN1A expression promotes cell cycle arrest but may lead to chemoresistance and a worsened prognosis in patients with CRC. Similarly, previous studies have associated the downregulation of p21 with longer survival among patients with CRC aged ≥60 years, while the opposite was observed in patients older than 60 of age.[36] Therefore, this study confirmed at the cellular level that the downregulation of p21 can be a therapeutic target, especially in older individuals with CRC. Downregulation of p21 in aging cells can be achieved by targeting the activity of p53 through the inhibition of TP53RK. Knockdown of TP53RK is known to inhibit p53 phosphorylation and trigger apoptosis in multiple myeloma cells. Furthermore, pharmacological inhibition of p53 phosphorylation through TP53RK can be achieved with immunomodulator drugs,such as pomalidomide. These drugs induce apoptosis by directly binding to TP53RK and inhibiting its kinase activity.[21] In wild-type p53 multiple myeloma cells, thalidomide and its analogs are also known to reduce p21 expression, facilitating the G1-to-S transition and increasing cell vulnerability to apoptosis.[29]

Molecular Docking and Molecular Dynamics Simulation

The DEG analysis revealed that CDKN1A (p21) was upregulated, making its downregulation a potential therapeutic target. In this study, the in silico downregulation of p21 was performed by inhibiting the activity of TP53RK using *Z. officinale* Roscoe. The inhibition effect was investigated in silico using molecular docking simulations with compounds present in *Z. officinale* Roscoe against the TP53RK protein.

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Table 1: Cellular Response to Changes in Downstream Target Genes of the p53 Signaling Pathway (D7 vs D1)

Target Gen	*BAX*	*STEAP3 (TSAP6)*	*AIFM2*	*SIVA1*	*GTSE1 (B99)*	*CDKN1A* (p21)
Responses (regulation)	Apoptosis (down)	Exosome mediated secretion (down)	Apoptosis (down)	Apoptosis (down)	Cell cycle arrest (down)	Cell cycle arrest (up)

Table 2: Validation of HT-29 cell clustering (D7 vs D1) based on the expression of BAX, TSAP5, AIFM2, SIVA1, B99, and CDKŃ1A

Model	AUC	CA	F1	Prec	Recall	MCC
SVM	0.75	0.88	0.87	0.90	0.88	0.78

A total of 635 compounds in *Z. officinale* Roscoe (data in S2) underwent drug likeness analysis based on Lipinski's rules,[37] and toxicology analysis based on the Registry of Toxic Effects of Chemical Substances (RTCES) database using Datawarrior. From this analysis, 40 compounds met the criteria—predicted to have relatively good bioavailability and relatively non-toxic (data in S3a and S3b)—were identified. These 40 compounds underwent in silico analysis using MVD to assess their interactions with the TP53RK protein. The three-dimensional structure of TP53RK and its native ligand are shown in Figure 4.

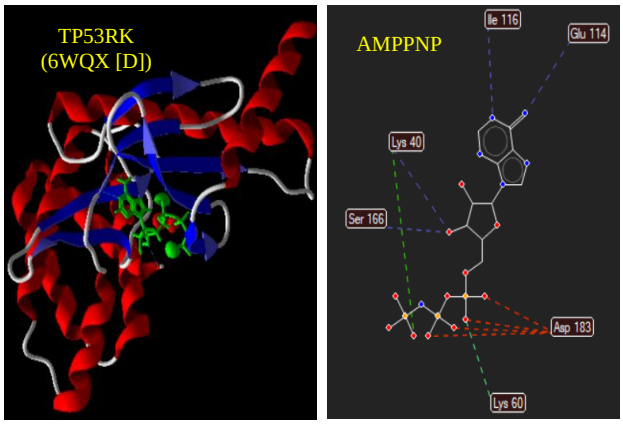

Figure 4: Interaction between TP53RK protein (6WQX [D]) and ligand (AMPPNP) in its crystal structure.

Before conducting molecular docking tests on the 40 compounds, validation was performed on the molecular docking model to be used. In this case, a re-docking process was carried out, where the molecular docking model was deemed valid if it produced an RMSD <2 Å. The RMSD value is generated from the distance between the native ligand (AMPPNP native) and the AMPPNP compound used in the re-docking process. The RMSD values shown in Table 3. One ligand posed with an RMSD < 1.59062 Å, indicating that the molecular docking simulation model to be used was sufficiently valid. The docking scores (Rerank score) resulting from the molecular docking test between compounds in *Z. officinale Roscoe* and pomalidomide with the TP53RK protein (6WQX [D]) shown in Table 4. Figure 5 shows the bonds or interactions that occur between the compounds in
Table 4 and the amino acid residues of the TP53RK protein (6WQX [D]). Based on the molecular docking simulation results, some compounds found in *Z. officinale roscoe* have the potential to be a better TP53RK inhibitors than pomalidomide (the complete molecular docking results is available in S4). In Table 4, it is evident that gingerenone A, 6-shogaol, gingerol, and 6-paradol have more negative Rerank scores than pomalidomide. MVD recommends using Rerank Score to evaluate the results of molecular docking, as it measured the binding affinity that combines the energy released from ligand-protein interactions (Einter) and the ligand's internal energy (Eintra).[30] The more negative the Rerank Score, the more stable the bond between the ligand and the receptor. More negative docking scores (such as Rerank Score) were also associated with increased compound or ligand activity. Activity coefficients can be directly linked to Gibbs free energy or the energy released due to ligand-receptor interactions.[38]

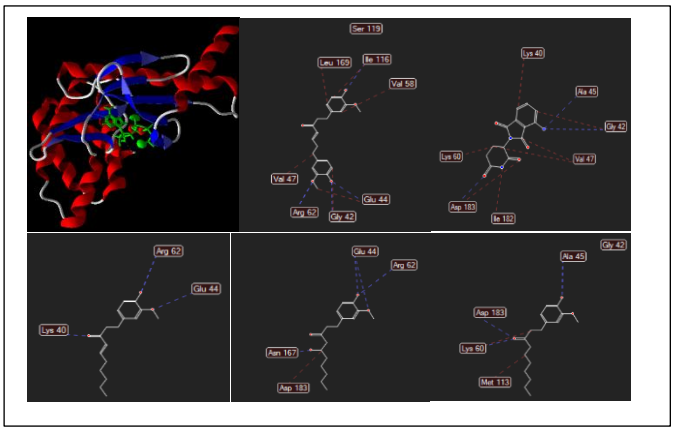

Figure 5: Interactions of TP53RK (6WQX [D]) with native ligand (AMPPNP/ANP_301) in its crystal structure (a), Gingerenone A (b), pomalidomide (c), 6-shogaol (d), gingerol (e), and 6-paradol (f). Legends: ---- Hydrogen Bonds; ---- Electrostatic Interactions; ---- Steric Interactions

Molecular dynamic simulations of all the compounds (shogaol, gingerenone A, paradol, and pomalidomide) were run in 10 ns. As shown in Figure 6, at 10 ns, all compounds were stable, with an average RMSD range of 1.894–2.194 Å, especially in the frame 67–100 ps. The interaction between shogaol, gingerenone A, paradol, and pomalidomide with TP53RK is also captured in Figure 7, with low fluctuation in some amino acids that play a role in the binding affinity, including Lys40, Lys60, Ile116, and Asp183.

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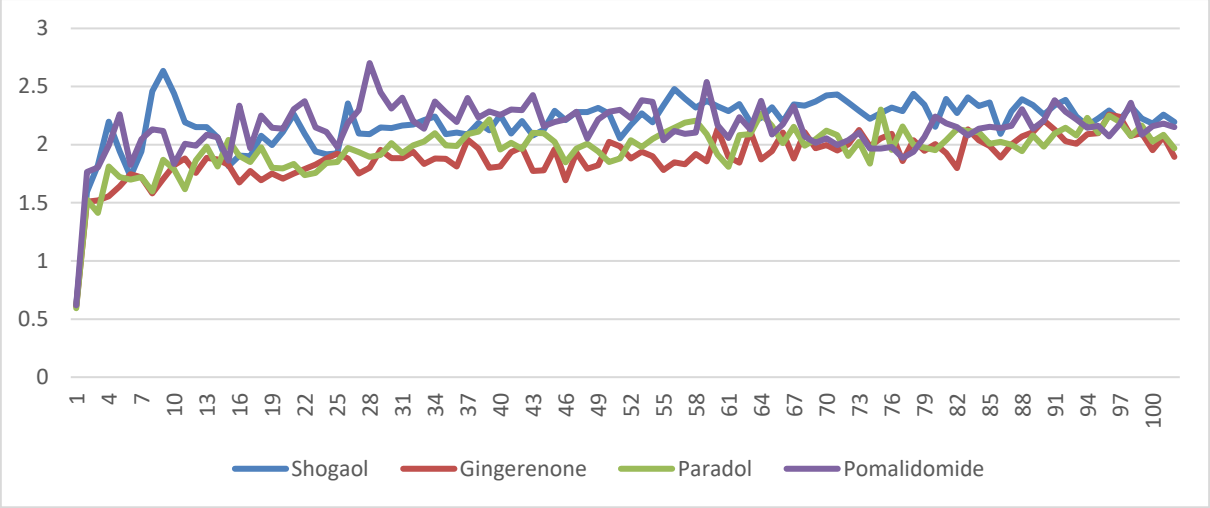

Figure 6: Root Mean Square Deviation (RMSD) of Shogaol, Gingerenone A, Paradol and Pomalidomide

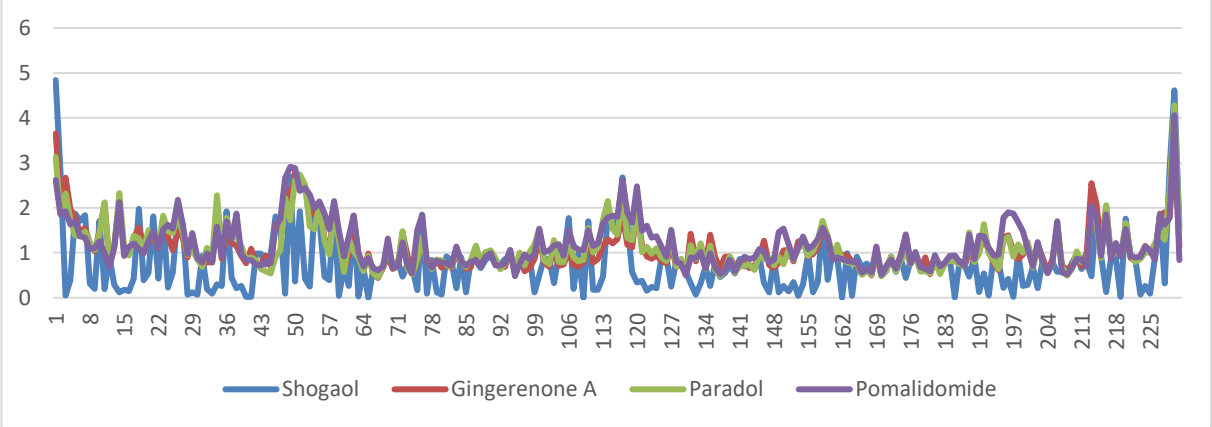

Figure 7: Root Mean Square Fluctuation (RMSF) of Shogaol, Gingerenone A, Paradol and Pomalidomide

Table 3: Re-docking process of the native ligand (AMPPNP native) with the resulting RMSD

Ligand Name	MolDock Score	Rerank Score	RMSD	HBond
[03]ANP_301 [D]	-152.96	-109.937	1.59062	-6.8951
[02]ANP_301 [D]	-144.068	-99.8166	4.11971	-9.15587
[04]ANP_301 [D]	-129.003	-100.202	6.59764	-5.83683
[00]ANP_301 [D]	-158.462	-118.108	8.00412	-7.67042
[01]ANP_301 [D]	-141.401	-113.942	8.76483	-6.44516

Table 4: Results of the molecular docking process of compounds in *Z. officinale* Roscoe against the TP53RK protein (6WQX [D])

Rank	Name Ligand	MolDock Score	Rerank Score	Torsions	HBond
1	[00]ANP_301 [D]	-157.328	-116.515	8	-9.8869
2	[01]Gingerenone A	-134.749	-109.241	9	-10.9433
3	[00][6]-Shogaol	-109.387	-84.1589	9	-5.7994
4	[00]gingerol	-109.172	-88.8661	10	-9.3906
5	[00][6]-Paradol	-103.989	-84.9867	10	-6.5615
13	[03]pomalidomide	-78.3933	-65.3638	1	-3.4985

Conclusion

This study indicated that downstream genes in the p53 signaling pathway related to cell cycle and apoptosis showed a natural decrease in expression in aging HT-29 cells (D7 cultures). However, CDKN1A (p21) exhibited upregulation in D7 cultures, indicating its potential as a therapeutic target at the cellular level in aging. The results of molecular docking studies suggested that compounds such as gingerenone A, 6-shogaol, gingerol, and 6-paradol found in *Z. officinale Roscoe* have the potential to down-regulate CDKN1A (p21) by inhibiting p53 activation through TP53RK. Molecular dynamic simulation also shows that all of compounds are stable during 10ns with low fluctuation in essential amino acid in binding site such as Lys40, Lys60, Ile116, and Asp183. These findings open the prospect of exploring other natural compounds using methods such as differential gene expression analysis, molecular docking and molecular dynamic simulation, and machine learning. However, further in vitro, in vivo, and clinical studies are necessary to validate the results and realize the prospect of developing *Zingiber officinale* Roscoe extract as an alternative cancer treatment.

Conflicts of interest

The authors have declared that there is no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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