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Pharmacophore Identification Through Molecular Docking of Scopoletin as a PFKFB3 Receptor Inhibitor for Anticancer Drug Development

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ABSTRACT

Cancer is one of the leading causes of death worldwide. Current therapies often have limited effectiveness and significant side effects. The ability of cancer cells to utilize glycolysis for their growth is one of the challenges in the development of cancer therapies. This study aims to identify the pharmacophore of scopoletin molecules and its derivatives as inhibitors of the PFKFB3 receptor, which plays an important role in cancer cell metabolism through the glycolysis process. The method used is in silico molecular docking. The results showed that of the 12 scopoletin derivative compounds tested, compound C1 (modified by removing double bonds in the carbonyl group and hydroxyl group) showed the best performance with a binding energy value of -7.07 kcal/mol, an inhibition constant of 5.35 μM, and the highest pharmacophore fit value of 48.63. Molecular interaction analysis identified amino acid residues TYR424, LYS168, ALA44, LYS47, and THR48 as important binding sites on the PFKFB3 receptor. All test compounds meet Lipinski's rule and show good ADMET profiles, with Human Intestinal Absorption (HIA) values of more than 89%. The removal of the hydroxyl group at the C-7 position provides better binding values for each class of scopoletin derivative compounds, while the methoxy group plays an important role in the interaction with the target protein.

Keywords: Cancer, Scopoletin, PFKFB3, Pharmacophore.

INTRODUCTION

Cancer is one of the leading causes of death worldwide. According to data released by the Global Cancer Observatory (2022), cancer is estimated to be one of the largest causes of death globally, with mortality reaching more than 9.7 million cases in 2022. Current therapies, such as surgery, radiotherapy, and chemotherapy, often have limited effectiveness and significant side effects (Ma et al., 2024). The ability of cancer cells to adapt to metabolic changes, especially increased metabolism, becomes one of the biggest challenges in developing cancer therapies.

Cancer cells utilize aerobic glycolysis to support their proliferation and anabolic growth, a phenomenon known as the Warburg effect (Pascale et al., 2020).

PFKFB3 is an enzyme that plays a role in the glycolysis process, where PFKFB3 is a bifunctional enzyme that reversibly regulates the synthesis and degradation of fructose 2,6-bisphosphate, which activates PFK1. PFK1 is a key molecule that regulates the activity of the glycolysis pathway, the main metabolic pathway used by cancer cells to meet their energy and biosynthesis needs (Da et al., 2023).

Previous research has shown that inhibition of PFKFB3 can inhibit cancer cell proliferation and induce apoptosis (Xiao et al., 2021). However, the main challenge in the development of PFKFB3 inhibitors is to design molecules with high selectivity toward PFKFB3 while minimizing adverse side effects (Liu et al., 2024). One compound of interest to study is scopoletin. Scopoletin is a coumarin compound found in various plants and is reported to have various biological activities, including anticancer effects by inhibiting proliferation and stimulating cancer cell apoptosis (Tian et al., 2019). However, the specific molecular mechanisms of its anticancer activity are not fully understood, particularly its potential to inhibit this glycolysis-regulating enzyme directly or indirectly through upstream signaling pathways.

Computational approaches of molecular docking and pharmacophore identification become highly relevant methods for designing potential PFKFB3 inhibitors. Molecular docking is a method in drug design that utilizes computers to analyze and understand the interactions between proteins and ligands. (Asnawi et al., 2023). Meanwhile, a pharmacophore is a feature of compounds that interact with target receptors. Using computational molecular modeling techniques, this research aims to explore the structural characteristics and molecular interactions of scopoletin with the PFKFB3 receptor. Through comprehensive pharmacophore analysis, it is expected that an interaction model can be found to explain the mechanism of enzyme activity inhibition and the potential development of more effective anticancer therapeutic strategies.

METHODOLOGY

Tools and Materials

The equipment used in this research includes a laptop with specifications of Windows 11 64-bit, Intel Core i3-1005G1 1.20 GHz, 8GB RAM. The Pharmacy Laboratory computer at Muhammadiyah University of Surakarta with specifications of Acer Veriton N46406, Windows 10 Pro Education 64-bit, Core i3 3.19 GHz, 4 GB RAM, 120 GB hard disk. The software used includes Discovery Studio 2024 Client,

PyMol, AutoDockTools, ChemDraw Professional 15.0, Chem3D 15.0, Protein Data Bank (PDB) website, PubChem website, and LigandScout 4.5.

The materials used include the structure of the scopoletin test ligand downloaded from the https://pubchem.ncbi.nlm.nih.gov/ website in (.sdf) format. The selected macromolecule is the PFKFB3 receptor (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3) with code: 2AXN downloaded from the Protein Data Bank (PDB) website. The reference ligands were downloaded from the https://www.bindingdb.org/ website.

Protein Ligand Preparation

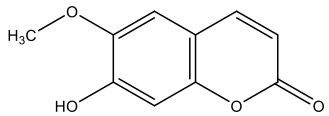
Preparation of the PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) protein ligand with code 2AXN was downloaded through the PDB (Protein Data Bank) website and saved in .pdb format. The preparation was performed by removing water molecules and molecular residues that could interfere with the docking process (Agu et al., 2023).

Docking Method Validation

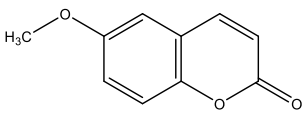
The protein, after being cleaned of water molecules, had its native ligand separated from the receptor. Docking method validation was performed by redocking the native ligand, with an RMSD criterion of less than 2.0 Å (Ramírez & Caballero, 2018) using AutoDockTools. The results were visualized using Discovery Studio software by comparing the redocked native ligand file with the original ligand structure, which showed significant overlap.

Test Ligand Preparation

Preparation of comparison compounds that have the same protein target were downloaded from the database site https://www.bindingdb.org, while the scopoletin compound was downloaded from the PubChem site in .sdf format (Rao et al., 2023) and modified by removing the functional groups of the scopoletin compound including the methoxy group, hydroxyl group, and lactone ring, which can be seen in the following figure.



A



A1

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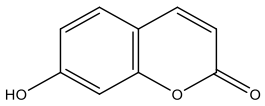

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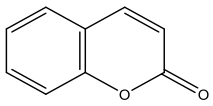
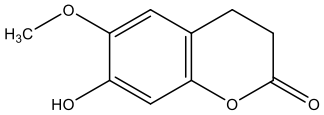
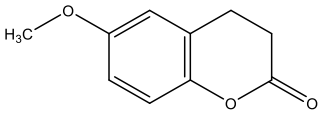


A3

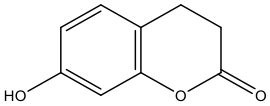
Figure 1. Modification of the scopoletin compound.



B



B1



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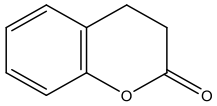
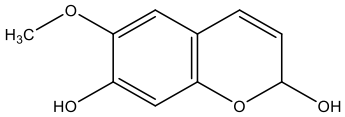
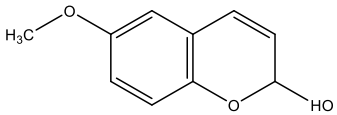


B3

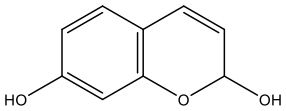
Figure 2. Modification of the scopoletin compound by removing the double bond in the lactone ring.



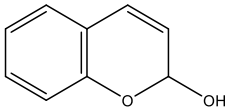
C



C1



C2



C3

Figure 3. Modification of the scopoletin compound by removing the double bond from the carbonyl group.

The modification of the scopoletin compound was performed manually using ChemDraw application and imported into Chem3D application to obtain the three-dimensional structure. Then, Lipinski's Rule of Five tests and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) predictions were conducted on all modified compounds as requirements for chemical compounds in the drug development process and discovery of drug candidates for oral administration.

Molecular Docking

Molecular docking was carried out using the same method as the validation process, employing the AutoDockTools application. Prior to docking, the required parameters were prepared, including the grid parameters based on the native ligand of the target protein. Grid settings involved defining the coordinates and volume of the grid box, with dimensions of 25×25×25 Å, in accordance with the recommended grid box range of 22.5–30 Å per axis (Feinstein & Brylinski, 2015). The primary parameters evaluated included binding free energy (ΔG), inhibition constant (Ki),

hydrogen bonding, and interaction profiles with amino acid residues at the receptor's active site (Rao et al., 2023).

Interaction Analysis

The docking results were analyzed using Discovery Studio Visualizer application to observe the interactions between the test ligands and amino acid residues on the target enzyme. This made it possible to identify which functional groups of the ligand would bind to amino acids at the active site of the enzyme (Syaqila et al., 2024).

Pharmacophore Analysis

Pharmacophores were built from the results of docking the test compounds with the target receptor using LigandScout 4.5 application. A pharmacophore is a structural feature of a compound that determines its specific interaction with the target protein in three-dimensional space to achieve optimal binding (Arba et al., 2020).

RESULTS AND DISCUSSION

The docking validation process was carried out by re-docking the native ligand that had been separated from the PFKFB3 enzyme, which was downloaded from the PDB (Protein Data Bank) website with code 2AXN. The re-docking of this native ligand used the AutoDockTools application to obtain the RMSD value, which was then visualized in the Discovery Studio Visualizer application.

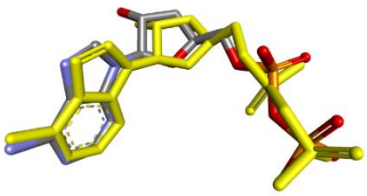


Figure 4. Visualization of the redocking of the native ligand alongside the redocked ligand (in yellow).

Table 1. Grid box parameters, RMSD value, and binding energy of the native ligand.

PDB code	Grid box	RMSD	Binding energy (kcal/mol)
2AXN	x : -9,829 Y : 47,892 Z : 9,136	0,73	-16,30

From the re-docking results shown in **Figure 4 and Table 1**, the native ligand and the re-docked ligand appear to overlap, and the RMSD value for the PFKFB3 receptor with code 2AXN is 0.73Å. The docking method is considered validated if the RMSD value is < 2 Å, allowing the method to be continued (Nugroho et al., 2023). RMSD (Root Mean Square Deviation) is a parameter used to measure the similarity of coordinates between two structures (Klara et al., 2023).

This research used scopoletin as the test ligand, which is a derivative of the coumarin compound. The molecular structure of scopoletin was then modified into three main series. Series A represents the basic modification of scopoletin, series B was created by removing the double bond in the lactone ring, and series C by removing the double bond in the carbonyl group. Each series consists of four variants (A-A3, B-B3, and C-C3), forming a total of 12 test compounds that were evaluated through various parameters. The modification of the scopoletin compound aims to determine which groups play a role and provide good binding and inhibition values with the target protein.

All test compounds underwent initial screening based on Lipinski's rule before the molecular docking process was carried out. In drug development and the discovery of drug candidates for oral use, there are five requirements that must be met, known as the "rule of five." These requirements include: the molecular weight of the candidate should not exceed 500, the number of hydrogen bond donors should not be greater than 5, the number of hydrogen bond acceptors should not be greater than 10, the log P value should be less than 5, and the molar refractivity value should range from 40 to 130 (Naveed et al., 2025).

Table 2. Physicochemical properties of the scopoletin compound based on Lipinski's rule of five.

Test compound	Hydrogen bond		Molecular weight (g/mol)	Log p	Molar Refractivity	Remarks
	Donor	Acceptor				
A	1	4	192	1,33	49,32	Meet
A1	0	3	176	1,62	47,66	Meet
A2	1	3	162	1,32	42,77	Meet
A3	0	2	146	1,61	41.11	Meet
B	1	4	194	1,25	48.61	Meet
B1	0	3	178	1,54	46.94	Meet

B2	1	3	164	1,20	42.06	Meet
B3	0	2	148	1,53	40.39	Meet
C	1	4	194	1,56	49.70	Meet
C1	1	3	178	1,41	48.66	Meet
C2	2	3	164	1,11	43.77	Meet
C3	1	2	148	1,41	42.11	Meet

Research on physicochemical properties shows that all tested compounds meet the Lipinski rule criteria. The molecular weight range of the compounds is between 146 - 194 g/mol, well below the maximum limit of 500 g/mol. The Log P values are in the range of 1.11 - 1.62, reflecting an optimal balance between lipophilic and hydrophilic properties to support membrane penetration ability. The number of hydrogen bond donors ranges from 0 - 2, and the number of hydrogen bond acceptors is in the range of 2 - 4, both of which are still below the recommended limits of Lipinski's rule. Additionally, the molar refractivity parameters of the compounds are in a good range, between 40.39 - 49.70. This indicates that all test compounds potentially have good oral bioavailability.

Further prediction of ADMET properties (Absorption, Distribution, Metabolism, Excretion, and Toxicity) provides information about the pharmacokinetic profiles of these compounds. Parameters analyzed in this study include Caco2 cell permeability (Human colon adenocarcinoma) and HIA (Human Intestinal Absorption) as indicators of intestinal absorption, as well as BBB (Blood Brain Barrier) as an indicator of brain absorption, plus Plasma Protein Binding as an indicator of distribution (Gabriel et al., 2024). This research was then continued with toxicity predictions on the same platform using mutagenic and carcinogenic parameters.

Table 3. ADMET testing (Absorption, Distribution, Metabolism, Excretion, and Toxicity) of test compounds

No	Ligand	Absorption			Distribution	mutagenic	carcinogenic
		Blood Brain Barrier	Caco-2 cell	HIA	Protein Plasma Binding		
1.	A	0,64	0,27	93,92	29,41	Mutagens	Negative

2.	A1	1,82	1,51	99,13	24,01	Mutagens	Negative
3.	A2	0,58	19,51	94,17	42,39	Mutagens	Positive
4.	A3	1,56	32,12	100	43,39	Mutagens	Positive
5.	B	0,64	0,27	93,11	36,73	Mutagens	Negative
6.	B1	1,82	1,61	99	29,64	Mutagens	Negative
7.	B2	0,59	19,52	93,66	46,08	Mutagens	Negative
8.	B3	1,55	32,76	100	37,32	Mutagens	Positive
9.	C	0,72	0,26	89,54	27,31	Mutagens	Negative
10.	C1	0,64	0,65	94,92	19,16	Mutagens	Negative
11.	C2	1,08	19,48	89,37	35,91	Mutagens	Negative
12.	C3	0,93	24,59	97,17	28,76	Mutagens	Positive

The majority of compounds showed varying Blood Brain Barrier and Caco-2 permeability. Compounds A, A1, B, B1, C, and C1 have low Caco-2 permeability (<4), indicating limited intestinal absorption. Nevertheless, all compounds have high Human Intestinal Absorption (HIA) values (more than 89%), which indicates good potential for oral bioavailability (Apriali et al., 2022).

Molecular docking was performed by preparing grid parameters to be used in the process of docking scopoletin compounds with the target receptor. The coordinates used in the docking were X: -9.829, Y: 47.892, and Z: 9.136. In the docking parameters, a change was made to the Number of GA Runs to become 100, indicating 100 trials of ligand interaction positions against the target protein in the molecular docking process (Dinata et al., 2023).

Table 4. Binding energy values of the native ligand and test compounds.

No	Ligand/ compounds	Binding energy (ΔG)	Number of hydrogen bonds	Amino acid residues	KI (μM)

1.	Natural ligands	-16,30	11	TYR424, LYS168, GLY46, THR48, LYS47, ALA44, ARG45, TYR49, VAL243, CYS154, VAL217	$1,13x10^{-4}$
2.	Control ligand	-7,66	5	**VAL243**, ASN163, **TYR424**, VAL167, **LYS168**	2,43
3.	A (Scopoletin)	-6,88	5	**THR48**,VAL167, **LYS47**, **ALA44**, **TYR424**	9,06
4.	A1	-6,91	6	**ALA44**, **LYS168**, **TYR424**, VAL167, **TYR49**, GLU166	8,63
5.	A2	-6,83	6	VAL167, **LYS168**, **GLY46**, **TYR424**, **LYS47**, **ALA44**	9,78
6.	A3	-6,67	4	**ALA44**, VAL167, **TYR424**, **LYS168**	12,88
7.	B	-6,45	5	**THR48**, **LYS47**, VAL167, **ALA44**, **TYR424**	8.46
8.	B1	-6,55	3	**ARG45**, **LYS47**, VAL167	8,91
9.	B2	-6,85	7	**TYR424**, **GLY46**, **LYS168**, **LYS47**, VAL167, **ALA44**, ASN163	9,81
10.	B3	-6,23	3	**ARG45**, **LYS47**, VAL167	9,94
11.	C	-6,93	4	**THR48**, **ALA44**, VAL167, **TYR424**	8,33
12.	C1	-7,07	4	**ALA44**, **LYS168**, VAL167, GLU166	5,35
13.	C2	-6,74	3	**ALA44**, **LYS168**, VAL167	9,45
14.	C3	-6,77	3	**LYS168**, **ALA44**, VAL167	10,91

The molecular docking results against the PFKFB3 receptor (PDB code: 2AXN) revealed variations in binding energies among the tested compounds. Among

the 12 scopoleptin derivatives evaluated, ligand C1 exhibited the best performance, with a binding energy of -7.07 kcal/mol and an inhibition constant (Ki) of 5.35 µM. The affinity of a test compound for the receptor is determined by the magnitude of its negative binding energy and its inhibition constant. A more negative binding energy and a lower inhibition constant indicate higher ligand affinity (Prasetiawati et al., 2022). In all series (A, B, and C), the removal of the hydroxyl group at position C-7 (A1, B1, and C1) resulted in favorable binding energies and inhibition constants. In contrast, the simultaneous removal of the hydroxyl group at C-7 and the methoxy group at C-6 (A3, B3, and C3) led to a decrease in both binding energy and inhibition constant. Interactions with amino acid residues TYR424, LYS168, ALA44, LYS47, and THR48 were consistently observed across several compounds, indicating the crucial role of these residues in the PFKFB3 inhibition mechanism.

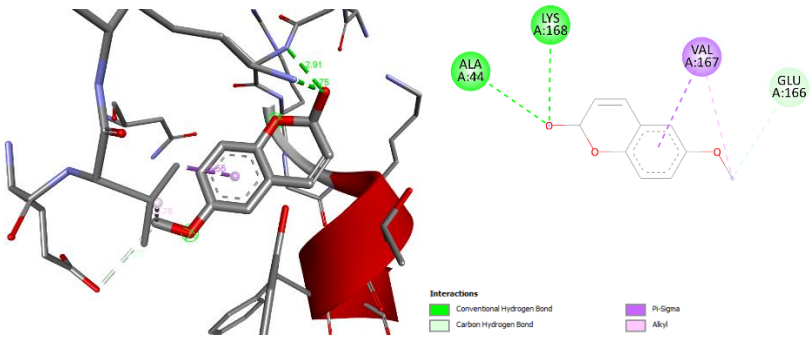


Figure 5. Visualization of the interaction between compound C1 and the target protein.

Following the visualization of ligand–target protein interactions, pharmacophore analysis was conducted using the LigandScout application. This analysis aimed to identify the pharmacophoric features of the ligands that contribute to interactions with the PFKFB3 receptor. Pharmacophores are essential structural features of a compound that play a critical role in optimizing specific binding to the target protein (Arba et al., 2020). Key pharmacophoric features include hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), hydrophobic regions (H), positively and negatively ionizable groups (PI/NI), and aromatic rings (AR) (Widyasari et al., 2022).

The pharmacophore fit score results indicated that compound C1 had the highest fit score (48.63), surpassing even the reference ligand (48.04), followed by compound A1 (48.02). A higher pharmacophore fit score correlates with greater

predicted effectiveness (Winardi et al., 2023). Both C1 and A1 share similar pharmacophoric features with the natural ligand, suggesting comparable inhibitory potential. These results indicate that compound C1, a scopoletin derivative modified by the removal of the double bond in the carbonyl and hydroxyl groups, exhibits the highest affinity toward PFKFB3, as evidenced by its binding energy of -7.07 kcal/mol, inhibition constant of 5.35 μM, and pharmacophore fit score of 48.63. This finding is consistent with the study by Xiao et al. (2021), which demonstrated that inhibition of PFKFB3 can suppress cancer cell proliferation and enhance chemosensitivity. The lowest fit score was observed for compound B3, which lacked the lactone ring double bond due to the removal of the methoxy group at C-6 and the hydroxyl group at C-7. This result highlights the importance of the lactone ring double bond as a key structural element in ligand binding.

Table 5. Results of the Pharmacophore Analysis of the Test Compound.

No	Compounds	Pharmacophore Fit Value
1.	Control ligand	48,04
2.	A (Scopoletin)	42,38
3.	A1	48,02
4.	A2	42,39
5.	A3	35,46
6.	B	32,12
7.	B1	39,96
8.	B2	39,89
9.	B3	32,46
10.	C	39,32
11.	C1	48,63
12.	C2	39,82
13.	C3	35,55

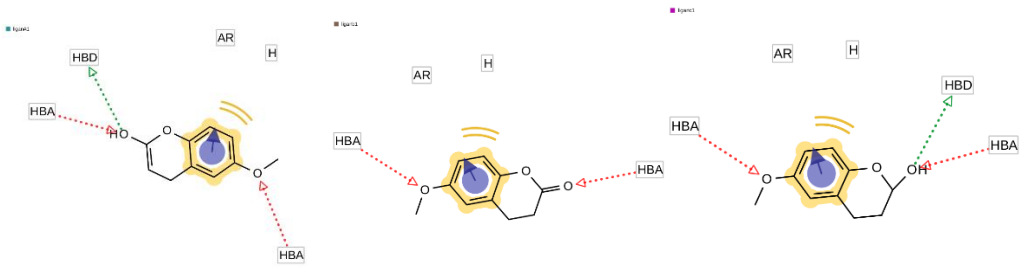


Figure 6. Pharmacophore depiction of scopoletin derivatives A1, B1, and C1, respectively.

Based on the pharmacophore analysis of all tested scopoleptin derivatives, the removal of the hydroxyl group at position C-7 resulted in favorable scores across all series. The hydroxyl group (-OH) functions as a hydrogen bond donor, meaning it

tends to donate a proton (H^+) to form a hydrogen bond with an acceptor (Zhang et al., 2025). The removal of hydroxyl groups that results in increased ligand affinity toward PFKFB3 is also consistent with the principles of hydrogen bonding in drug design. As explained by Chen et al. (2016), the presence of a hydrogen bond donor group (-OH) at an inappropriate position may interfere with optimal binding to the target protein.. In contrast, the removal of the methoxy group from scopoleptin derivatives led to poorer binding scores with the target protein, suggesting that the methoxy group plays a critical role in mediating effective interactions between the compound and the protein. odification of the carbonyl group into a hydroxyl group enhances the compound's affinity for the receptor. While the carbonyl group functions solely as a hydrogen bond acceptor, its conversion to a hydroxyl group allows the compound to act as either a hydrogen bond donor or acceptor, thereby facilitating more versatile and potentially stronger interactions with the receptor.

CONCLUSION

Based on the in silico study conducted, it can be concluded that among the 12 scopoletin derivatives, one compound demonstrated the best performance, characterized by the removal of the double bond in the carbonyl group and the hydroxyl group. This compound exhibited a binding energy of -6.93 kcal/mol, an inhibition constant of 5.35 μM, and a pharmacophore fit value of 48.63, which is higher than that of the reference ligand. Moreover, it was predicted to be non-carcinogenic. Therefore, the scopoletin derivative with the modified removal of the double bond in the carbonyl and hydroxyl groups may be considered a potential alternative in the development of anticancer drugs targeting the PFKFB3 receptor.

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