

Molecular docking study of ethyl acetate fraction of *Selaginella doederleinii* Hieron extract as anti-Warburg effect in breast cancer

[Estudio de acoplamiento molecular de la fracción de acetato de etilo del extracto de *Selaginella doederleinii* Hieron como efecto anti-Warburg en cáncer de mama]

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Abstract

Context: Aerobic glycolysis is a breast cancer hallmark that has become a novel target for anticancer therapy. GLUT1, HK2, PKM2, and LDHA are oncogenic proteins in the pathway. The compounds of ethyl acetate fraction of *Selaginella doederleinii* Hieron extract can be developed as aerobic glycolysis inhibitors by impeding the proteins' activity.

Aims: To analyze the anticancer activity of compounds of the ethyl acetate fraction of *S. doederleinii* via inhibiting oncogenic proteins in the aerobic glycolysis pathway.

Methods: The 3D protein structures were obtained from the RCSB database. Ligands were compounds of the ethyl acetate fraction predicted to have a good oral bioavailability based on our previous study. The ligand structures were downloaded from the PubChem database. Docking and analysis of chemical interactions were performed using Autodock Vina in Pyrx and Discovery Studio 2020, respectively.

Results: Some compounds interacted with the specific binding site of proteins with low binding affinity. Diosgenin interacted with GLUT1 and LDHA with the binding affinity of -11.8 and -9.6 kcal/mol. Frutinone A was found to bind PKM2 and LDHA with the binding affinity of -10.0 and -8.4 kcal/mol. Vitexin interacted with GLUT1 and PKM2 with the binding affinity of -10.4 and -10.0 kcal/mol. Valerodine and 5-hydroxyferulic acid methyl ester could bind HK2 with a binding affinity of -6.1 and -6.8 kcal/mol.

Conclusions: The compounds of ethyl acetate fraction of *S. doederleinii* extract were predicted to inhibit GLUT1, HK2, PKM2, and LDHA. Therefore, these compounds could be developed as anti-breast cancer agents targeting the aerobic glycolysis pathway.

Keywords: aerobic glycolysis; anticancer activity; binding affinity; binding site; *Selaginella doederleinii*.

Resumen

Contexto: La glucólisis aeróbica es una característica distintiva del cáncer de mama que se ha convertido en una nueva diana para la terapia contra el cáncer. GLUT1, HK2, PKM2 y LDHA son proteínas oncogénicas en esta vía. Los compuestos de acetato de etilo del extracto de *Selaginella doederleinii* Hieron pueden desarrollarse como inhibidores de la glucólisis aeróbica al impedir la actividad de las proteínas.

Objetivos: Analizar la actividad anticancerígena de los compuestos de la fracción de acetato de etilo de *S. doederleinii* mediante la inhibición de proteínas oncogénicas en la vía de la glucólisis aeróbica.

Métodos: Las estructuras tridimensionales de las proteínas se obtuvieron de la base de datos RCSB. Los ligandos fueron compuestos de la fracción de acetato de etilo que se predijo que tenían una buena biodisponibilidad oral basada en nuestro estudio previo. Las estructuras de los ligandos se descargaron de la base de datos PubChem. El docking y el análisis de las interacciones químicas se realizaron utilizando Autodock Vina en Pyrx y Discovery Studio 2020, respectivamente.

Resultados: Algunos compuestos interaccionaron con el sitio de unión específico de las proteínas con baja afinidad de unión. Diosgenina interactuó con GLUT1 y LDHA con una afinidad de unión de -11,8 y -9,6 kcal/mol. La frutinona A se unió a PKM2 y LDHA con una afinidad de unión de -10,0 y -8,4 kcal/mol. La vitexina interactuó con GLUT1 y PKM2 con una afinidad de unión de -10,4 y -10,0 kcal/mol. La valerodina y el éster metílico del ácido 5-hidroxiferúlico pudieron unirse a HK2 con una afinidad de unión de -6,1 y -6,8 kcal/mol.

Conclusiones: Se predijo que los compuestos de la fracción de acetato de etilo del extracto de *S. doederleinii* inhiben GLUT1, HK2, PKM2 y LDHA. Por lo tanto, estos compuestos podrían desarrollarse como agentes contra el cáncer de mama dirigidos a la vía de la glucólisis aeróbica.

Palabras Clave: actividad anticancerígena; afinidad de unión; glucólisis aeróbica; *Selaginella doederleinii*; sitio de unión.

ARTICLE INFO

Received: November 30, 2022.

Accepted: April 9, 2023.

Available Online: May 4, 2023.

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Abbreviations: 2-DG: 2-Deoxyglucose; 2-DG-6-P: 2-deoxy-D-glucose-6-phosphate; Akt: Protein kinase B; AMPK α : Adenosine monophosphate-activated protein kinase- α ; c-Myc: c-Myelocytomatosis; GLUT1: Glucose transporter 1; HIF-1 α : Hypoxia-inducible factor-1 α ; HK2: Hexokinase 2; LC-HRMS: Liquid Chromatography-High Resolution Mass Spectrometry; LDHA: Lactate dehydrogenase A; MCT4: Monocarboxylate transporter 4; mTOR: Mammalian target of rapamycin; NAD $^{+}$: Nicotinamide adenine dinucleotide $^{+}$; NADH: Nicotinamide adenine dinucleotide + hydrogen; NZT: N-(4-[[4-(pyrazin-2-yl)piperazin-1-yl]carbonyl]phenyl)quinoline-8-sulfonamide; PDB: Protein data bank; PI3K: Phosphatidylinositol 3 kinase; PKM2: Pyruvate kinase M2; RCSB: Research Collaboratory for Structural Bioinformatics.

INTRODUCTION

Breast cancer ranked first for the highest incidence of cancer and the leading cause of death in women due to cancer in most countries (Sung et al., 2021). The increased number of breast cancer cases represents a significant advance in the disease's early detection and diagnosis phase. However, the breast cancer mortality rate is still high, proving that some efforts are still required to increase the effectiveness of anti-cancer treatment through some research targeting potential oncogenic signaling pathways in breast cancer.

One of the breast cancer hallmarks is metabolic reprogramming. Cancer cells lose control of growth and proliferation and keep dividing indefinitely. Consequently, cancer cells develop an adaptation mechanism that differs from normal cells in how they consume glucose. These cells uptake a large amount of glucose and release a high lactate level under aerobic and anaerobic conditions (Fadaka et al., 2017). This phenomenon is known as the Warburg Effect or aerobic glycolysis. This hallmark is triggered by mutations in cancer cells' oncogenic signaling pathways, such as PI3K/Akt/mTOR (Magaway et al., 2019). The mutations affect the regulation of transcription factors, such as c-Myc and HIF1- α , causing increased expression levels of transporters and enzymes involved in aerobic glycolysis (Magaway et al., 2019).

Glucose transporter 1 (GLUT1), hexokinase 2 (HK2), pyruvate kinase M2 (PKM2), and lactate dehydrogenase A (LDHA) are proteins involved in aerobic glycolysis in cancer cells. GLUT1 is a member of the sodium-independent glucose transporter family, responsible for intracellular glucose transport. In cancer cells, the increased expression level of GLUT1 played an essential role in elevating glucose uptake, aerobic glycolysis, and chemo-radio-resistance through several oncogenic signaling pathways (Yang et al., 2019). Therefore, targeting GLUT1 could reduce the growth of tumors and even kill cancer (Pliszka and Szablewski, 2021). Meanwhile, HK2 is a hexokinase family member that catalyzes glucose phosphorylation at the beginning of the glycolysis process. In cancer cells, targeting this enzyme could decrease aerobic glycolysis metabolism, hinder tumor growth, promote apoptosis, and elevate cancer cells' sensitivity to irradiation therapy (Liu et al., 2017). Another glycolysis enzyme is PKM2, which converts phos-

phenolpyruvate to pyruvate by transferring a phosphate group from ADP to ATP. In cancer cells, PKM2 is majorly expressed in an inactive dimeric form (Mazurek et al., 2005). This dimeric PKM2 could regulate some gene expression involved in cancer progression and development (Sharma et al., 2021). Kung et al. (2012) identified that NZT compound could bind PKM2 to convert the conformation to one that reduced tumorigenicity. LDHA is an oxidoreductase enzyme catalyzing the conversion of pyruvate to lactate by transferring hydrate ions from NADH (Valvona et al., 2016). Therefore, the increased expression level of LDHA in cancer cells could facilitate excessive lactate secretion. Additionally, LDHA overexpression could promote proliferation, angiogenesis, invasion, and metastasis in cancer cells (Feng et al., 2018). These aerobic glycolysis proteins can be a promising anti-breast cancer target.

Because of their ability to target multiple oncogenic proteins in various pathways in cancer, natural products can be an alternative and complementary medicine to combat cancer. One of the medicinal plants used traditionally for generations in many parts of the world is *Selaginella doederleinii* Hieron (family *Selaginellaceae*) (Setyawan, 2009). This fern contains various bioactive compounds possessing anticancer activities (Li et al., 2014). The ethyl acetate fraction of this plant was reported to have the strongest anticancer activity (Li et al., 2014). This fraction could inhibit proliferation, trigger apoptosis, and induce autophagy in colorectal cancer cells via the PI3K/Akt/mTOR and AMPK α , two pathways regulating the aerobic glycolysis pathway in the cancer cell (Li et al., 2020). However, its inhibitory activity on transporters and enzymes in the glycolysis pathway has not yet been investigated.

In the previous study, we identified several compounds in the ethyl acetate fraction of *Selaginella doederleinii* Hieron extract (Pinanti et al., 2022). Among these compounds, nine obeyed Lipinski's rule, indicating that they could be active oral drugs. However, their ability to inhibit oncogenic proteins in the aerobic glycolysis pathway, such as GLUT1, HK2, PKM2, and LDHA, as an attractive anti-breast cancer target has never been explored. The study aimed to predict the possible inhibitory activity of some bioactive compounds in the ethyl acetate fraction of *Selaginella doederleinii* Hieron against the aerobic glycolysis pathway using the molecular docking method.

MATERIAL AND METHODS

Plant material

Selaginella doederleinii Hieron was collected from UPT. Balai Materia Medica, Batu, East Java, Indonesia (7°52'01.2"S and 112°31'13.2"E). The plant material was confirmed as *Selaginella doederleinii* Hieron and deposited by UPT. Balai Materia Medica, Batu, East Java, Indonesia, with determination number. 074/695/102.20-A/2022. The aerial part of the plant was used for this study (Pinanti et al., 2022).

Extraction and fractionation

The aerial part of the plant was grounded until it became 1 kg of powder and macerated twice using 96% ethanol for 24 h at room temperature. The total filtrate was concentrated using a rotatory evaporator. The solid extract was subsequently dissolved in the distilled water before being fractionated using n-hexane and ethyl acetate. Firstly, the extract-water mixture was added and homogenized with n-hexane in the separating funnel. Then, the water phase was separated from the n-hexane phase and homogenized with ethyl acetate. This process was carried out three times. The ethyl acetate phase was collected to be concentrated using a rotatory evaporator. A small amount of ethyl acetate fraction was used for Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) analysis (Pinanti et al., 2022).

LC-HRMS analysis and physicochemical properties prediction

The ethyl acetate fraction sample was diluted with its solvent until the volume became 1.5 mL. The sample was homogenized and filtered using a 0.22 µm syringe filter. After that, the sample was processed into an auto-sampler and injected into the Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) instrument. The analytical column in High-Performance Liquid Chromatography (Thermo Scientific Dionex Ultimate 3000 RSLC nano with micro-flow meter) used was Hypersil GOLD aQ (50 × 1 mm × 1.9 particle size). The mobile phases were solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The flow rate used in this study was 40 L/min for 30 minutes at 30°C. The polarity used in the High-Resolution Mass Spectrometer (Thermo Scientific Q Exactive) was positive and or negative. The raw data analysis and identification of compounds were performed using MZmine 2.53 software and the Kyoto Encyclopedia of Genes and Genomes (KEGG) online database, respectively (Pinanti et al., 2022). Subsequently, compounds used in this study were those which were predicted to have a good oral bioavailability based on Lipinski's rules

analysis from the ADME webserver (<http://www.swissadme.ch/>) (Pinanti et al., 2022). Lipinski's rules stated some criteria of physicochemical properties that had to be possessed by the orally active drug, such as MW ≤500, log P≤5, H-bond donors ≤5, and H-bond acceptors ≤10 (Lipinski et al., 2004). A compound was expected to be an orally active drug when it had at least one violation of Lipinski's rules.

Ligand preparation

Ligands used for this study were compounds predicted to be good oral drugs (Pinanti et al., 2022). The 3D structures of compounds as ligands were downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Energy minimization of these compounds was carried out using Open Babel on Pyrx. 8.0-Virtual Screening Tools software (Dallakyan and Olson, 2015). Files were saved in PDB format.

Protein preparation

The crystal structures of the protein complexes used in this study, namely GLUT1 (PDB. 5EQI), HK2 (PDB. 2NZT), PKM2 (PDB. 4G1N), and LDHA (PDB. 1I10), were downloaded from the RCSB PDB database (<https://www.rcsb.org/>). Unnecessary chains, water molecules, ion molecules, and native ligands were removed from the protein structure using the Biovia Discovery Studio 2020 software (Dassault Systèmes Biovia, San Diego, California, USA). Protein structures that were ready to be used for docking were saved in PDB format.

Molecular docking and the visualization

Molecular docking was performed using Auto-dock Vina in Pyrx. 8.0-Virtual Screening Tools software (Dallakyan and Olson, 2015). Docking was carried out at a specific site of each protein. In GLUT1, docking was conducted at the glucose-binding site, characterized by the presence of the native ligand, cytochalasin B, due to its overlapping binding mode with glucose (Kapoor et al., 2016). Cytochalasin B, known for inhibitory activity on GLUT1 was used as a positive control. In HK2, docking was also carried out at the glucose-binding site. Meanwhile, the positive control of the HK2 inhibitor was 2-deoxyglucose (2-DG), a competitive inhibitor of glucose in HK2, whose structure was obtained from the PubChem database (PubChem ID: 108223). In PKM2, docking was done at the binding site of the N-(4-[[4-(pyrazin-2-yl) piperazin-1-yl] carbonyl] phenyl) quinoline-8-sulfonamide (NZT), the native ligand. The activator was also used as a positive control for PKM2 docking. In LDHA, docking was performed at the pyruvate-

Table 1. The specific binding site of each protein and the grid setting for molecular docking.

No.	Protein	Grid	
		Center	Dimension
1.	GLUT1	X = -40.2014	X = 26.9522
		Y = 14.4641	Y = 22.6400
		Z = 10.1442	Z = 27.1300
2.	HK2	X = -2.4005	X = 31.2637
		Y = 1.8950	Y = 32.5487
		Z = 16.6305	Z = 31.0642
3.	PKM2	X = 1.7456	X = 30.3611
		Y = -13.6483	Y = 30.1714
		Z = 45.9439	Z = 32.7893
4.	LDHA	X = 10.3482	X = 24.7899
		Y = -17.6733	Y = 21.7418
		Z = 151.3142	Z = 30.0489

binding site, indicated by a native ligand, oxamate, a pyruvate mimic. Oxamate was also used as a positive control. Each docking site of the protein was used to determine the grid box position (Table 1). The docking results were visualized using Biovia Discovery Studio 2020 software (Dassault Systèmes Biovia, San Diego, California, USA).

Data analysis

The binding site and chemical interactions analysis between protein and compound was performed using Biovia Discovery Studio 2020 software (Dassault Systèmes Biovia, San Diego, California, USA). Compounds that bound the same binding site as the positive control of each protein were selected to be compared their binding affinity score with the positive control. The binding affinity score was obtained from Pyrx. 8.0-Virtual Screening Tools software (Dallakyan and Olson, 2015). The lower the binding affinity score, the more stable the protein and the compound interaction. Then, the compound with the lowest binding affinity score was further discussed in terms of the amino acid residues it binds to for determining its potential as an aerobic glycolysis inhibitor.

RESULTS

Chemical composition of ethyl acetate fraction of *S. doederleinii* obtained by LC- HRMS and the physicochemical properties prediction

The extraction and fractionation process of *S. doederleinii* produced 5.99 g (14.71%) of ethyl acetate fraction, 5.96 g (14.64%) of n-hexane fraction, and 28.76 g (70.65%) of the aqueous fraction. After that, a small amount of ethyl acetate fraction was further

analyzed using LC-HRMS to identify its compounds because, according to Li et al. (2014), the ethyl acetate fraction of this plant had the highest anticancer activity. Pinanti et al. (2022) revealed that the ethyl acetate fraction of *S. doederleinii* extract contained 12 phytochemical compounds with various pharmacological effects. These compounds were listed in Fig. 1 with their spectra (Pinanti et al., 2022). Then, these compounds were predicted in their physicochemical properties according to Lipinski's rule. Some compounds, which had potential as a good oral drug with the Lipinski violation no more than one were selected to be used in this molecular docking study, namely affinisine, diosgenin, 2 trans,6-trans-farnesal, 5-hydroxyferulic acid methyl ester, frutinone A, helveticoside, syringic acid, valeroidine, and vitexin (Pinanti et al., 2022). These compounds from LC-HRMS identification with their physicochemical properties' prediction were listed in Table 2.

Docking of GLUT1-compounds

The docking results of bioactive compounds against GLUT1 revealed that all these compounds, except frutinone A, could bind the same binding site as cytochalasin B, as the positive control. It was shown by the similarity of amino acid residues of GLUT1 interacting with them (Table 3). The binding mode similarity with the positive control indicated that these compounds were predicted to have the same biological function as the positive control (Kumar and Zhang, 2018). Among these compounds, diosgenin and vitexin displayed the lowest binding affinity scores, namely -11.8 and -10.4 kcal/mol, respectively (Table 4). Compared to the positive control, the scores were slightly higher (-12.0 kcal/mol).

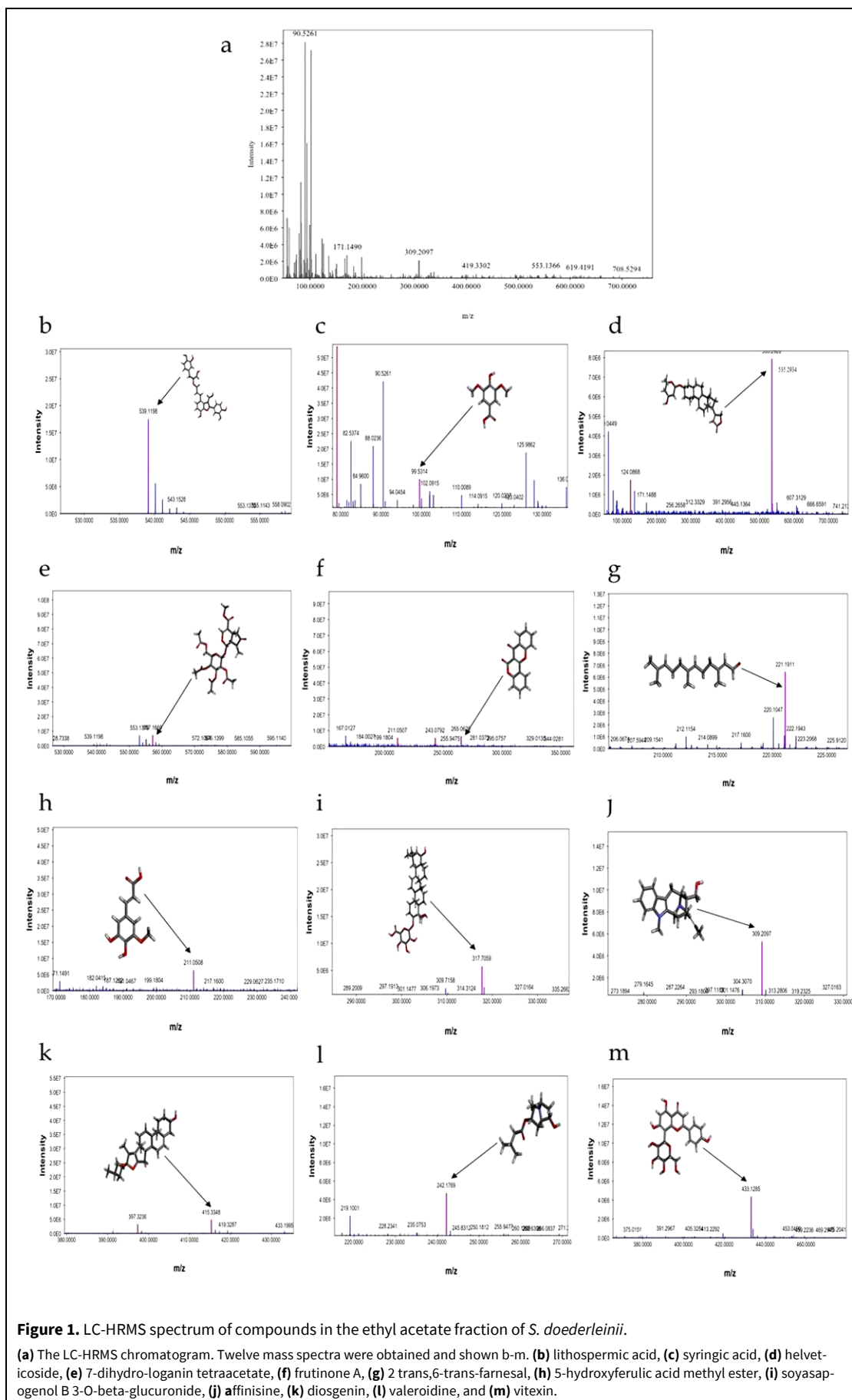


Table 2. Compounds of ethyl acetate fraction of *S. doederleinii*.

No.	Compound	Physicochemical properties				Lipinski violation	PubChem ID
		MW	mLogP	H-bond donor	H-bond acceptor		
1.	Lithospermic acid	538.46	0.45	7	12	3	4482010
2.	Syringic acid	198.17	1.20	2	5	0	10742
3.	Helveticoside	534.65	0.80	4	9	1	441860
4.	7-dihydro-loganin tetraacetate	556.51	-0.18	0	14	2	443349
5.	Frutinone A	264.24	3.55	0	4	0	441965
6.	2 trans,6-trans-Farnesal	220.36	5.50	0	1	1	5280598
7.	5-hydroxyferulic acid methyl ester	210.19	0.96	3	5	0	446834
8.	Soyasapogenol B 3-O-beta-glucuronide	634.84	2.48	6	9	2	13632905
9.	Affinisine	308.43	3.34	1	3	0	6325075
10.	Diosgenin	414.63	5.93	1	3	1	99474
11.	Valeroidine	241.33	1.40	1	4	0	443012
12.	Vitexin	432.38	0.52	7	10	1	5280441

The lower the binding affinity score, the stronger the interaction between protein and ligand. Nevertheless, the binding scores of these two compounds were still relevant to show the inhibitory effect on the protein (Dotolo et al., 2021). Diosgenin interacted with Glu380 and Thr137 through hydrogen bonds. Moreover, this compound was also found to bind Ile164, Ile404, Phe26, Trp388, and Trp412 through 10 hydrophobic interactions (Fig. 2a and Table 3). This compound could also bind similar amino acid residues with the positive control, namely Glu380, Trp388, and Trp412. These three residues were vital in ligand binding to GLUT1 (Almahmoud et al., 2019). Meanwhile, vitexin formed some chemical interactions that consisted of seven hydrogen bonds (Gln283, Asn288, Asn415, Gln282, Asn411, and Glu380), four hydrophobic interactions (Trp388 and Ile164), and an unfavorable bond (Asn288). This compound also could interact with the same amino acid residues as the positive control, such as Gln282, Glu380, and Trp388. These two residues, namely Glu380 and Trp388, were crucial for the interaction between the ligand and GLUT1.

Docking of HK2-compounds

The HK2 docking results showed only two compounds with the same binding site as 2-DG as the positive control, 5-hydroxyferulic acid methyl ester and valeroidine. The binding affinity score of 5-hydroxyferulic acid methyl ester and valeroidine was -6.8 kcal/mol and -6.1 kcal/mol, respectively (Table 4). Both compounds exhibited a lower binding affinity score than the positive control (-5.7 kcal/mol). The 5-hydroxyferulic acid methyl ester was found to bind

Asp84, Thr88, Ser415, Thr232, Thr213, Ser449, and Asp209 via eight hydrogen bonds (Fig. 2b and Table 3). This compound also interacted with Gly414 and Ile229 through two hydrophobic interactions. Asp209 was the only amino acid residue that this compound and the positive control could bind. Furthermore, this compound could bind Ser449, a residue that regulates N-terminal domain activity on HK2 through a hydrogen bond (Ferreira et al., 2021). Valeroidine formed three hydrogen bonds with Arg91, Ser155, and Asp209. Meanwhile, it interacted with Ile229 through hydrophobic interaction. Ser155 and Asp209 were two residues that could interact with valeroidine and the positive control.

Docking of PKM2-compounds

The PKM2 docking results exhibited that all these compounds, except diosgenin and helveticoside, had similar binding sites with the positive control, namely the NZT. All compounds showed a higher binding affinity score than the NZT (-13.0 kcal/mol) (Table 4). However, the binding affinity score of frutinone A and vitexin was -10.0 kcal/mol, respectively, the lowest value compared to other compounds. Frutinone A formed chemical interactions that consisted of two hydrogen bonds (Tyr390), six hydrophobic interactions (Phe26, Leu353, and Leu394), and the other type of bond (Met30) (Fig. 2c and Table 3). It interacted with the same amino acid residues with the positive control (Tyr390, Phe26, Leu353, and Leu394). Meanwhile, vitexin formed four hydrogen bonds (Lys311, Asp354, Leu353, and Ala388) and seven hydrophobic interactions (Phe26, Leu353, and Met30). It was found

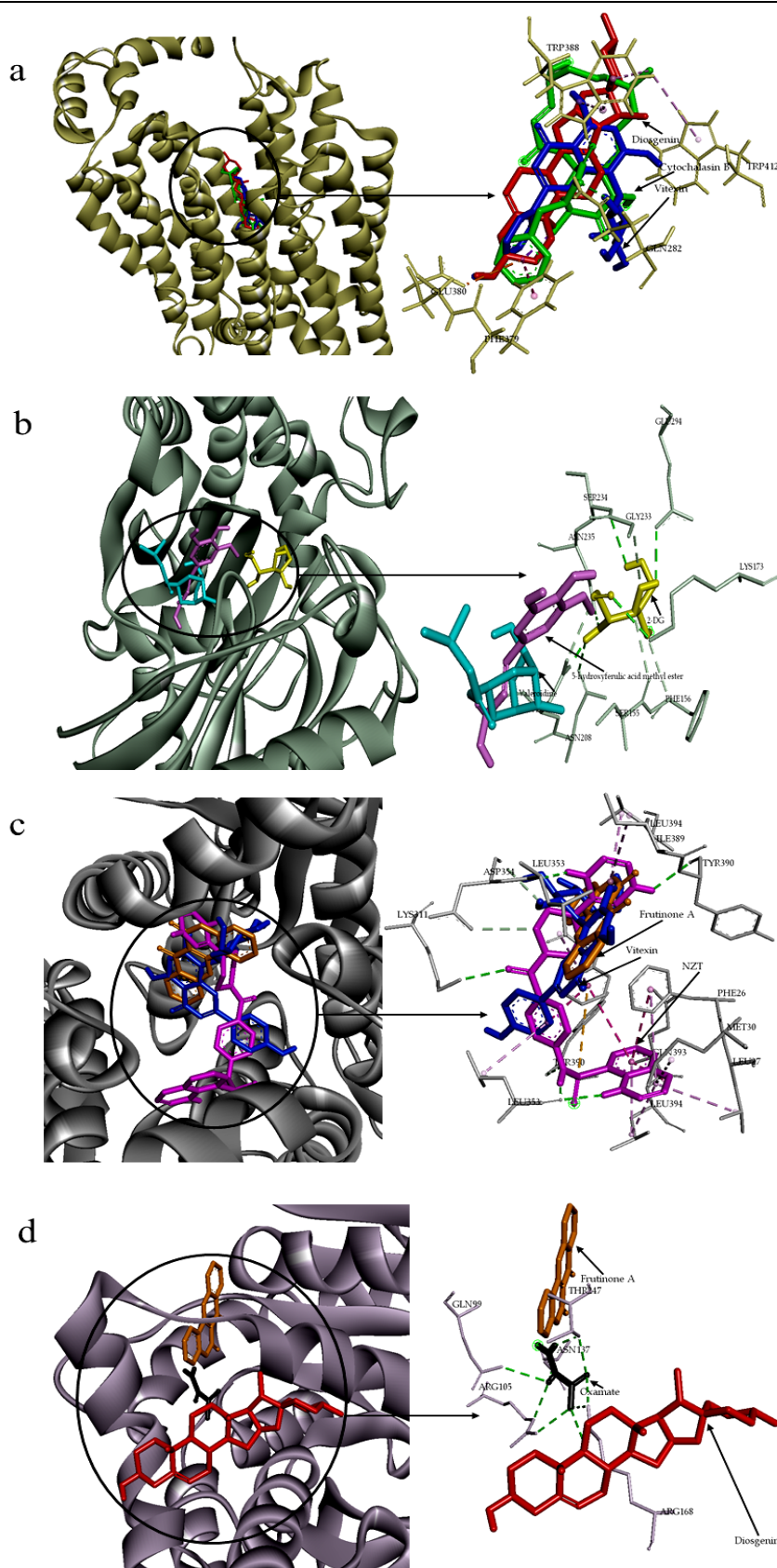


Figure 2. Bioactive compounds interact against aerobic glycolysis proteins.

(a) Cytochalasin B (green), diosgenin (red), and vitexin (blue) interacted with GLUT1; **(b)** 2-DG (yellow), 5-hydroxyferulic acid methyl ester (pink), and valeroidine (cyan) interacted with HK2; **(c)** NZT (purple), frutinine A (orange), and vitexin (blue) interacted with PKM2; and **(d)** Oxamate (dark green), frutinine A (orange), and diosgenin (red) interacted with LDHA.

Table 3. Chemical interactions between compounds and proteins.

Compound	Chemical interaction	Amino acid residues			
		GLUT1	HK2	PKM2	LDHA
Positive control (cytochalasin B for GLUT1, 2-DG for HK2, NZT for PKM2, and oxamate for LDHA)	Hydrogen Bond	TRP388, GLN282	GLU294, ASN208, ASP209(2), SER234, LYS173, ASN235, SER155, PHE156, GLY233	TYR390(2), LEU353(3), LYS311, ASP354(2)	THR247(2), GLN99, ARG105(2), ASN137, ARG168(3)
	Halogen				
	Other	-	-	PHE26	
	Electrostatic	GLU380	-	-	
	Hydrophobic	PHE379, TRP388(3), TRP412	-	PHE26(5), GLN393, LEU394(4), LEU27, MET30, ILE389, LEU353	
Affinisine	Hydrogen Bond	GLN283, ASN288(2)	THR336	-	ALA250
	Hydrophobic	TRP412(3), PHE26, ILE164, HIS160, PHE291, PHE379	MET300(2)	LEU353(3), PHE26(4), LEU27, LYS311	TYR171(3), LEU253, ALA167, ALA250
	Unfavorable	GLN283	GLY303	-	-
Diosgenin	Hydrogen Bond	GLU380, THR137	-	ARG399, GLU418	-
	Hydrophobic	ILE164(2), ILE404, PHE26, TRP388(4), TRP412(2)	MET300(2), LYS337(2), LYS419(2)	LEU392(2), ALA21, PHE421(2)	LEU172, ALA250(3), LEU253, ARG168(3) , TYR171
	Unfavorable	-	-	ARG447	-
2 trans,6-trans-Farnesal	Hydrogen Bond	ASN415	SER415, GLY231	-	GLN99, ARG105, ASN137
	Hydrophobic	ILE164, VAL165, PRO385(2), ILE287, PHE291(2), PHE379	ILE229(2), LYS418	PHE26(6), ILE389(2), LEU394, LEU353(3), LYS311, HIS29	VAL30(4), VAL135(2), ILE251(2), ALA29, LEU164, ILE251, ARG98, HIS192
5-hydroxyferulic Acid methyl ester	Hydrogen Bond	GLN282, GLN283(2), ASN288(2), TRP412, ASN415	ASP84, THR88, SER415, THR232(2), THR213, SER449, ASP209	ASN350(2), LYS311, LEU353	VAL135, THR247, SER160, ALA97, ASN137
	Hydrophobic	TRP412(2), PHE26, PHE72	GLY414, ILE229	LEU353, PHE26, ILE389	VAL30, ARG98
	Unfavorable	ASN411, GLN283	-	TYR390	THR247
Frutinone A	Hydrogen Bond	ASN288, ASN415	SER340, GLY299	TYR390(2)	ASN137(2), ARG98, THR247
	Other	-	-	MET30	-
	Electrostatic	-	LYS419	-	-
	Hydrophobic	PHE26(2), ILE164	THR88, THR336(3), LYS337(5), LYS419(2)	PHE26(3), LEU353(2), LEU394	VAL30(3), ARG98(4)

Table 3. Chemical interactions between compounds and proteins (continued...)

Compound	Chemical interaction	Amino acid residues			
		GLUT1	HK2	PKM2	LDHA
Helveticoside	Hydrogen Bond	ARG153, GLN161, THR137, SER80(2), GLY138	PHE90, GLU304, THR336(2), THR88	ASP24, GLU396, ARG400, GLU396	-
	Hydrophobic	TRP388(3), PRO141(2), ILE404, HIS160, PHE389	MET119, MET300(2), ILE114, ALA113	-	VAL240, LEU253, ALA167(2), ARG168, ALA250(3)
	Unfavorable	-	PHE90	ASP24, GLU396	-
Syringic acid	Hydrogen Bond	GLN283, TYR292, GLN282	ASP84, THR88(4), THR213, SER449, GLY231	TYR390	THR247(2), ALA97, ASN137
	Hydrophobic	TRP412(3), ILE287, PHE379	ILE229	LEU353(4), PHE26, ALA388, MET30, LEU33, LYS311	VAL30, ILE241, VAL135, ILE251, HIS192
	Unfavorable	THR30	-	LEU353	-
Valeroidine	Hydrogen Bond	ASN288, ASN415, GLU380	ARG91, SER155, ASP209	LEU353	GLN99(2), VAL135, THR94, ALA95
	Hydrophobic	TRP412	ILE229	MET30(2), LEU394, PHE26(4)	VAL30(2), ARG98(2)
	Unfavorable	TYR292	-	-	ASN137
Vitexin	Hydrogen Bond	GLN283, ASN288(2), ASN415, GLN282, ASN411, GLU380	ILE281, LEU286, GLY284	LYS311, ASP354, LEU353, ALA388	SER248, TRP249, ALA250
	Hydrophobic	TRP388(3), ILE164	-	PHE26(3), LEU353(3), MET30	ALA250(2), TYR171, ARG168(2)
	Unfavorable	ASN288	THR336	-	-

to bind the same residues as the positive control (Lys311, Asp354, Leu353, Phe26, and Met30).

Docking of LDHA-compounds

The LDHA docking results showed that all these compounds, except affinisine, occupied the same binding site as oxamate, the positive control. Diosgenin (-9.6 kcal/mol) and frutinone A (-8.4 kcal/mol) exhibited a lower binding affinity score than the positive control (-4.4 kcal/mol) (Table 4). Diosgenin was identified to bind Leu172, Ala250, Leu253, Arg168, and Tyr171 through 9 hydrophobic interactions (Fig 2d and Table 3). Arg168 was the only amino acid residue that interacted with diosgenin and the positive control. The residue formed three hydrophobic interactions with this compound. Meanwhile, Frutinone A interacted with Asn137, Arg98, and Thr247 through four hydrogen bonds. It also formed seven hydro-

phobic interactions with Val30 and Arg98. Asn137 and Thr247 also interacted with the positive control. Furthermore, frutinone A was found to bind Arg98, one of the residues in the mobile loop 96-107 crucial for the catalytic activity of LDHA (Read et al., 2001).

DISCUSSION

Aerobic glycolysis is a unique metabolic pathway that becomes a potential therapeutic target for breast cancer. This metabolic disorder is characterized by elevated expression levels of some oncogenic proteins involved in the pathway, consisting of transporters, such as GLUT1 and MCT4, and enzymes, such as HK2, PKM2, and LDHA (Magaway et al., 2019). Therefore, the glucose uptake, glycolysis rate, and lactate secretion of cancer cells increase. Inhibition of these proteins by small molecules can be one strategy for hindering the growth of cancer cells.

Table 4. Binding affinity scores of compounds interacted with proteins.

Ligand	Binding affinity (kcal/mol)			
	GLUT1	HK2	PKM2	LDHA
Cytochalasin B (positive control for GLUT1)	-12.0	-	-	-
2-DG (positive control for HK2)	-	-5.7	-	-
NZT activator (positive control for PKM2)	-	-	-13.1	-
Oxamate (positive control for LDHA)	-	-	-	-4.4
Affinisine	-9.1	-8.0	-9.4	-7.2
Diosgenin	-11.8	-9.0	-9.4	-9.6
2 trans,6-trans-Farnesal	-6.5	-5.2	-7.8	-6.9
5-hydroxyferulic Acid methyl ester	-6.8	-6.8	-6.8	-6.6
Frutinine A	-9.3	-8.0	-10.0	-8.4
Helveticoside	-10.0	-5.9	-6.3	-7.6
Syringic acid	-6.0	-8.9	-6.1	-6.1
Valeroidine	-6.7	-6.1	-6.9	-6.9
Vitexin	-10.4	-5.8	-10.0	-7.0

GLUT1 is a transport protein that is widely expressed in several cancer cases, including breast cancer. Cancer cells consume a high amount of glucose as their main energy source to support their unlimited proliferation. Glucose from the extracellular cannot enter the intracellular through the lipid bilayer due to its hydrophilic property (Pliszka and Szablewski, 2021). Therefore, glucose needs specific carrier proteins called glucose transporters. Glucose binds GLUT1 at the glucose-binding site in the C-domain (Almahmoud et al., 2019). When glucose binds the site, the GLUT1 conformation changes from outward-open to inward-open, releasing glucose into the cytoplasm to enter metabolic pathways (Almahmoud et al., 2019). This inward-open conformation is the favored conformation for ligand-protein interactions, so it is often utilized to design novel GLUT1 inhibitors (Almahmoud et al., 2019). Therefore, targeting the glucose-binding site is one of the strategies to inhibit GLUT1.

In this study, diosgenin and vitexin were the most potent compounds to inhibit GLUT1. These compounds had a close binding affinity with that of cytochalasin B, one of the GLUT1 inhibitors with an IC_{50} value of 0.110 ± 0.38 M (Kapoor et al., 2016). Cytochalasin B was identified to repress the growth of the murine B16F10 melanoma cell line (Bousquet et al., 1990). Moreover, some polyphenols, such as apigenin and naringenin could inhibit GLUT1, causing inhibition of cancer cell proliferation (Pliszka and Szablewski, 2021). Therefore, diosgenin and vitexin were also expected to interfere with the proliferation and

growth of cancer cells by inhibiting glucose transport in GLUT1.

Glucose entering cancer cells will undergo phosphorylation catalyzed by HK2. Glucose binds at the glucose-binding pocket of HK2 located in the N and C-domains, respectively (Nawaz et al., 2018). However, the catalytic activity of the N-domain of HK2 played a significant role in the cancer cell and contributed to the activity regulation of the whole enzyme (Nawaz et al., 2018). The glucose-binding pocket in the N-domain consists of several amino acid residues, namely Thr172, Lys173, Asn208, Asn235, Gln291, Glu260, Asp209, Ser155, and Pro157 (Nawaz et al., 2018). Meanwhile, Asp447, Ser449, and Lys451 are important amino acid residues in regulating N-domain activity (Ferreira et al., 2021). Therefore, one strategy to inhibit HK2 in cancer cells is targeting its glucose-binding pocket in the N-domain.

According to this study, 5-hydroxyferulic acid methyl ester and valeroidine were the best compounds as HK2 inhibitors. These compounds had a stronger binding affinity than 2-DG, one of the HK2 inhibitors, and displayed favored chemical interactions with HK2 residues. Inhibition of glucose phosphorylation by 2-DG accumulated the intracellular 2-DG-6-P, decreased ATP level, triggered cell cycle arrest, limited cell growth, induced apoptosis, and promoted autophagy (Pajak et al., 2020). Further studies need to be carried out to confirm the HK2 inhibitory and anti-cancer activities of 5-hydroxyferulic acid methyl ester and valeroidine against breast cancer.

Another well-known glycolytic enzyme as an anti-cancer target is PKM2. PKM2 catalyzes the terminal step of glycolysis, converting phosphoenolpyruvate to pyruvate by transferring a phosphate group to ADP. However, this enzyme also has a unique role in cancer cells, which can support their growth. PKM2 in cancer cells is mostly expressed as an inactive dimer, known to trigger glycolysis intermediates accumulation required for cancer cells to compensate for their high proliferation demands (Kung et al., 2012). Furthermore, the dimer conformation also allowed the enzyme to enter the nucleus and act as a nuclear protein kinase, contributing to the transcriptional regulation of genes involved in tumorigenesis and proliferation of cancer cells (Sharma et al., 2021). Therefore, one strategy to develop anticancer therapy targeting PKM2 is to alter the enzyme conformation to be an active tetramer using an activator. According to previous studies, the tetramer conformation of PKM2 converted the Warburg effect on cancer cells to a metabolism state close to normal differentiated cells and even reduced tumorigenicity (Boxer et al., 2010).

In this study, frutinone A and vitexin were expected to be the most potent compounds to inhibit PKM2. These compounds could bind the binding site of NZT, a PKM2 activator with a slightly weaker binding affinity. The activator has a unique allosteric binding mode on PKM2 where its quinoline moiety is located on the site's surface consisting of Phe26, Leu27, and Met30 residues of the A chain and Phe26, Tyr390, and Leu394 residues of the B chain (Kung et al., 2012). Moreover, the allosteric binding mode differs from the fructose 1,6-bisphosphate-binding pocket, thereby avoiding the negative regulation of PKM2 by phosphotyrosine peptides (Kung et al., 2012). The NZT decreased the amount of intracellular serine and inhibited tumorigenesis (Kung et al., 2012). Therefore, frutinone A and vitexin were predicted to have anticancer activities by inhibiting PKM2.

Pyruvate resulting from the catalytic reaction of PKM2 will be facilitated to convert into lactate by LDHA. The formation of large amounts of lactate in cancer cells results from the high rate of glucose uptake and aerobic glycolysis under both normoxic and hypoxic conditions in cancer cells. Pyruvate binds the substrate-binding site, whereas NADH binds the NADH cofactor-binding site of LDHA. The catalytic activity of LDHA occurs when NADH reduces pyruvate to produce lactate and NAD⁺. The pyruvate-binding site is often used as a target for designing novel LDHA inhibitors whose structures mimic those of the pyruvate. Small molecules binding the pyruvate-binding site could decrease LDHA activity and exhibit antiproliferative effects in cancer cells but did

not cause adverse side effects in normal cells (Shi and Pinto, 2014).

Diosgenin and frutinone A was predicted to be the most potent competitive inhibitors of LDHA, similar to oxamate. Oxamate, a well-studied LDHA inhibitor, could diminish lactate production and inhibit the proliferation of human gastric cancer SGC7901 and BGC823 cell lines (Zhao et al., 2015). However, oxamate had poor membrane permeability (Feng et al., 2018). The inhibitory activity and selectivity of diosgenin and frutinone A against LDHA in cancer cells require further research.

This study indicated that the bioactive compounds contained in the ethyl acetate fraction of *Selaginella doederleinii* Hieron were predicted to be aerobic glycolysis inhibitors in breast cancer cells by targeting the transporter proteins and the glycolysis enzymes. To verify the inhibitory activity of each compound as a potential Warburg effect inhibitor candidate and whether the inhibitory effect of each compound is simultaneous, further *in vitro* and *in vivo* research must be carried out. Previous studies revealed that the inhibition of glycolysis enzymes by natural compounds could be simultaneous to produce a promising anticancer effect. Naphthaquinones compounds, such as shikonin, vitamin K3, and vitamin K could suppress aerobic glycolysis in breast cancer MCF-7 cell lines by inhibiting the activity of several glycolytic enzymes such as hexokinase, phosphofructokinase-1, and pyruvate kinase simultaneously (Chen et al., 2018).

CONCLUSION

In the present study, we demonstrated that some bioactive compounds in the ethyl acetate fraction of *Selaginella doederleinii* Hieron might inhibit several aerobic glycolysis proteins in cancer cells, such as GLUT1, HK2, PKM2, and LDHA. This study predicted diosgenin as anti-GLUT1 and anti-LDHA, frutinone A as anti-PKM2 and anti-LDHA, valeroidine as anti-HK2, vitexin as anti-GLUT1 and anti-PKM2, and 5-hydroxyferulic acid methyl ester as anti-HK2. Therefore, the ethyl acetate fraction of *S. doederleinii* was expected to be an anti-breast cancer agent that acted as the Warburg effect inhibitor. Further research needs to be done to validate this *in silico* study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for funding this research through the PMDSU Batch V scholar-

ship (grant no: 015/E5/PG.02.00.PT/2022, research contract no. 612.6/UN10.C10/TU/2022).

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AUTHOR CONTRIBUTION:

Contribution	Pinanti HN	Christina YI	Rifa'i M	Widodo N	Djati MS
Concepts or ideas	x				
Design	x	x			
Definition of intellectual content	x				
Literature search	x				
Experimental studies	x	x			
Data acquisition					
Data analysis	x		x	x	
Manuscript preparation					x
Manuscript editing		x			x
Manuscript review	x	x	x	x	x

Citation Format: Pinanti HN, Christina YI, Rifa'i M, Widodo N, Djati MS (2023) Molecular docking study of ethyl acetate fraction of *Selaginella doederleinii* Hieron extract as anti-Warburg effect in breast cancer. *J Pharm Pharmacogn Res* 11(2): 354–366. https://doi.org/10.56499/jppres22.1549_11.2.354

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