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Original Research Article

LC-MS Chemical Profiling, *In Silico* Docking Studies to Unravel the Therapeutic Potential of *Streptomyces hygroscopicus* as a Source of Antimalarial Bioactive Compounds

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ABSTRACT

Malaria is a disease transmitted through the bite of female *Anopheles* mosquitoes carrying *Plasmodium* parasite. The resistance of the *Plasmodium* parasite against antimalarial drugs is a critical health concern necessitating the development of novel antimalarial treatments. Previous investigations reported that the crude extract of *Streptomyces hygroscopicus* Subsp. Hygroscopicus (*S. hygroscopicus*) possessed antimalarial properties, with *in vitro* assays confirming the ability to inhibit *Plasmodium* growth. Therefore, this research aimed to identify derivative compounds from *S. hygroscopicus* using Liquid Chromatography-Mass Spectrometry (LC-MS) and assess antimalarial activity by examining the binding site, pharmacokinetic profiles, and binding interactions through *in silico* analysis. *In silico* reverse molecular docking study was conducted with target proteins including *Plasmodium falciparum* malarial M1 aminopeptidase (PfA-M1), *Plasmodium falciparum* chloroquine resistance transporter (PfCRT), and falcipain-2 protease obtained from Protein Data Bank (PDB) and active compound ligands retrieved from PubChem. In addition, the analysis of pharmacokinetic profiles and bond interactions was performed using the SwissADME web tool and LigPlot software, respectively. The LC-MS analysis results showed that four compounds including dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate with high retention times met the drug similarity criteria and had binding affinity values comparable to control ligands of the respective target proteins. The four compounds may have antimalarial activity due to the formation of hydrogen and hydrophobic bonds identical to those found between the target proteins and control ligands. Specifically, dibenzyl amine expressed the highest binding affinity across all target proteins, suggesting it as a potential antimalarial candidate.

Keywords: Fraction 42, LC-MS, molecular docking, *Streptomyces hygroscopicus*

Introduction

Malaria is categorized as re-emerging and highly prevalent infectious disease transmitted through the bites of mosquitoes from the genus *Anopheles* carrying *Plasmodium* parasite which poses a significant public health challenge worldwide.[1,2] This parasite causes malaria infections that can lead to several life-threatening complications.[3] In 2019, approximately 229 million malaria cases were reported in 87 endemic countries, along with records of 409,000 deaths.[4] In Indonesia, the Annual Parasite Incidence (API) increased from 1.12 to 1.61 per 1,000 people in 2022 compared to 2021.[5]
The advent of *Plasmodium falciparum* parasites resistant to artemisinin-combination therapies (ACTs), a first-line therapies in Southeast Asia poses a challenge in regions where the disease is endemic,[6] as rising resistance rates contribute to increased illness and death.[7,8]

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In drug discovery efforts, pharmaceutical research incorporates various molecular modeling tools to facilitate medication development by capitalizing on technological advancements. Additionally, the synergy between computational and experimental approaches significantly aids in identifying and developing potential new compounds.[9]
Previous investigations using Liquid Chromatography-Mass Spectrometry (LC-MS) showed that 30 fractionated secondary metabolites derived from *Streptomyces hygroscopicus* subsp. Hygroscopicus *(S. hygroscopicus)* manifested inhibitory effects on *Plasmodium falciparum* growth. Furthermore, fractions 15 and 16 were found to inhibit the activity of the *Plasmodium falciparum* Malate Quinone Oxidoreductase (PfMQO) enzyme.[10] In fractions 41 and 44, *in silico* analysis identified tryptanthrin compounds meeting drug similarity criteria and presenting greater binding affinity values for *Plasmodium falciparum* malarial M1 aminopeptidase (PfA-M1), *Plasmodium falciparum* chloroquine resistance transporter (PfCRT), and falcipain-2 protease compared to control ligands.[11] Compounds derived from secondary metabolite extracts of *S. hygroscopicus* are promising for effective antimalarial drug candidates.[12] Therefore, this research aimed to identify derivative compounds from *S. hygroscopicus* using LC-MS and screen for its antimalarial activity by examining the binding site, pharmacokinetic profiles, and binding interactions through *in silico* analysis.

Material and Method

Design and Setting
An exploratory descriptive research design was used in identifying fractions of 42 secondary metabolites from *S. hygroscopicus* using LC-MS to determine possible active compounds contained based on detected mass numbers. *In silico* analysis was conducted to assess the antimalarial activity of compounds identified in fraction 42 using molecular docking for binding site and affinity prediction, while pharmacokinetic analysis was performed with SwissADME.

Culture of Streptomyces hygroscopicus
Streptomyces hygroscopicus were obtained from the Indonesian Institute of Sciences (LIPI) Microbial Collection in Cibinong, Indonesia, and subcultured in the Laboratory of Microbiology of the Faculty of Medicine, Universitas Brawijaya using International *Streptomyces* Project 4 (ISP4) media. Subsequently, *Streptomyces* colonies were confirmed with both macroscopic colony morphology and microscopic Gram staining.[11]

Inoculation of Streptomyces hygroscopicus
Bacterial inoculation was conducted under sterile conditions using 1000 mL of ISP4 media, with a pH ranging from 7.0 to 7.4. Bacterial colonies identified on ISP4 agar media were collected by scraping with a hose and then homogenized in a shaking incubator.[11]

Fermentation of Streptomyces hygroscopicus
Fermentation was initiated by adding an inoculum comprising 25.8×10^6 bacteria to ISP4 media at a ratio of 10:1. This mixture was covered with cotton wool and aluminum foil to eliminate air in the Erlenmeyer flask. Incubation was then performed for a period of five days in a shaking incubator at 28°C and a speed of 150 rpm.[11]

Extraction of Sterptomyces hygroscopicus Secondary Metabolites
To separate cells from debris, the fermented combination of inoculum and ISP4 media was centrifuged at 3000 rpm for 10 minutes. The resulting filtrate was stored in a sealed container for extraction, then combined with ethyl acetate solvent in a 1:1 (v/v) ratio and agitated in a separation funnel for one hour. The mixture was allowed to settle, forming two distinct layers including a solvent phase containing *S. hygroscopicus* secondary metabolites and a water phase. The extract was concentrated to dryness using a rotatory at 40-50°C.

Fractionation of Streptomyces. hygroscopicus Secondary Metabolite
Fractionation was conducted on the BUCHI Reveleris® PREP purification system using n-hexane and ethyl acetate solvents in isocratic mode. Silica gel 60 served as the stationary phase, and elution was performed with a mixture of n-hexane and ethyl acetate in a 1:1 (v/v) ratio. Wavelengths of the UV detector light applied were 254nm, 365nm, and 366nm, while the running process was carried out with a methanol gradient of 100% - 0% for 30 minutes at a flow rate of 5 ml/minute.

Identification of Secondary Metabolite Fractions of Streptomyces hygroscopicus Using Liquid Chromatography-Mass Spectrometry (LC-MS)
The analysis of the secondary metabolite fractions of *S. hygroscopicus* was performed using an LC-MS tool, specifically the Thermo Scientific Dionex Ultimate 3000 RSLCnano high-performance liquid chromatography (HPLC) LC system. HPLC Hypersil GOLD aQ column was used, and detection was conducted with a Thermo Scientific Q Exactive Mass Spectrometry detector at the Central Laboratory of Life Sciences, Universitas Brawijaya. Subsequently, the results obtained from this identification process were analyzed with the MassLynx application.

Pharmacokinetic Profiles
Pharmacokinetic profiles of compounds derived from *S. hygroscopicus* were evaluated using the SwissADME tool to test its drug-like properties and compare them with those of the control drug by entering the Canonical SMILES formula for analysis.

Target Protein Database Construction
The target protein database for this research was compiled through a literature search conducted using keywords such as "dibenzyl amine", "sedanolide", "levalbuterol", "dibutyl phthalate", "*Plasmodium*", "malaria", "*in silico*", "food vacuole", and "target protein". The selected target proteins were PfCRT (PDB ID: 6UKJ), PfA-M1 (PDB ID: 4ZW3), and falcipain-2 protease (PDB ID: 6SSZ) downloaded from the Protein Data Bank (PDB) through http://www.rcsb.org.pdb with a .pdb file extension. To enhance binding with ligands, residue separation and optimization of proteins were conducted. This optimization, executed through the PyMol 2.0 program, included the elimination of residual water, ligands from the crystallization process, and cofactors, followed by saving the results in a .pdb file format.

Ligands and Target Structures Preparation
The structure of ligands was initially identified from *S. hygroscopicus* derivatives through LC-MS analysis. The derivatives selected as active ligands included dibenzyl amine (ID: 7656), sedanolide (ID: 5018391), levalbuterol (ID: 123600), and dibutyl phthalate (ID: 3026) that were further searched in PubChem. The information about control ligands for the protein target was obtained from the RSCB Protein Data Bank and published literature, while the 3D structures were downloaded as .sdf files from https://pubchem.ncbi.nlm.nih.gov/. For example, the PfCRT protein had a control ligand represented by a protonated chloroquine structure generated with the MarvinSketch program and downloaded in a .pdb format.

Reverse Molecular Docking Simulation
Reverse molecular docking simulation was performed using Py Rx 0.9.5 software with grid box-based control ligands represented by original ligands of the target proteins. This featured interactions between active ligands including dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate compounds, with control ligands and *Plasmodium* target proteins. For PfA-M1, PfCRT, and falcipain-2 protease, docking grid box dimensions in the x, y, and z directions were configured as 12 x 12 x 12, 22 x 22 x 22, and 16 x 16 x 16, respectively, around the active site. The box center portions were respectively set to 89.960, 119.9592, and 12.7705, 22,809, 13,727, and 20,948, as well as 17.034, -40.547, and 5.048, while the exhaustiveness value was 8. For accuracy, docking process was repeated three to five times for each of the target proteins with control ligands and active compounds. Subsequently, the average binding affinity was calculated, and a comparison was made between the binding affinity values of active ligands derived from *S. hygroscopicus* and control ligands for the target proteins.

Visualization of Interactions
The LigPlot 2.2.8 software was used to visualize the binding affinity scores between the compounds and proteins compared to control ligands. This visualization process evaluated the consistency between residues formed and the resulting binding affinity scores. The analysis included assessing the similarity of the bonds connecting the drug and target proteins, along with those formed between control ligands and the target proteins. The objective was to identify the binding site of the compounds and examine interactions made with the target proteins.

Result and Discussion

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis
From the LC-MS analysis, the active compounds in fraction 42 of *S. hygroscopicus* were identified using the working principle of determining the mass (m) to charge (z) ratio and the mass spectrum fragmentation pattern (Figure 1). The results showed active compounds characterized by molecular weight and the presence of peak fragments at specific retention times (RT). Appearances of the active compounds were compared with available data sources to determine the respective identities, as presented in Table 1. Moreover, the completeness of the comparative data sources influenced the identification accuracy. Dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate were detected from the four strongest peaks observed in the RT range of 0-20 minutes and were discussed sequentially.

Dibenzyl amine ($C_{14}H_{15}N$) is a benzaldehyde derivative with a molar mass of 197.28 g per mole. Previous research reported this compound to possess anticonvulsant and antimalarial potential. Dibenzyl amine has a dual effect as an antimalarial through a mechanism similar to chloroquine (inhibiting hemozoin) and resistance reversal activity. Additionally, the 4-amino-7-chloroquinoline part connected to the N-atom of imipramine through an alkyl three-carbon link is active *in vitro* against both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains. This compound has a high lipophilicity value, leading to not being considered in drug developments. Subsequent research showed that dibenzyl amine as the head group had strong activity against parasites *in vitro* (IC50 <120 nM), where only small differences were found between chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains with low lipophilicity values.[13]

Sedanolide ($C_{12}H_{18}O_2$) is a natural phthalide compound first isolated from the seed oils of plants beloñging to the *Umbelliferae* family, including celery, and the 2-Benzofuran family.[14] It is recognized for its mechanism of action as an antibiotic-targeting bacteria, as well as for its mosquitocidal, nematocidal, and antifungal properties.[15] Sedanolide showed potential as an antibiotic based on the agar diffusion method inhibition test in research conducted on *Salmonella Typhi* and *Staphylococcus aureus* bacteria. This compound shows antagonistic activity against membrane permeability and acts as an inhibitor of protein synthesis by interacting with DNA ligase and DNA polymerase. *In silico* research performed using molecular docking showed that Sedanolide had a lower binding affinity value than the control compound. This observation suggested the potential to react with the target to produce an activation required in the antibiotic action process for bacterial growth suppression.[16]

Table 1: The results of fraction 42 secondary metabolites *S. hygroscopicus* identification using LC-MS

No	Retention Time	Compound	Molecular Formula	Molecular Weight (m/z)
1	7.45	Dibenzyl amine	$C_{14}H_{15}N$	197.12
2	13.59	Levalbuterol	$C_{13}H_{21}NO_3$	261.14
3	18.01	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15
4	20.64	Sedanolide	$C_{12}H_{18}O_2$	176.12
5	20.65	Acetophenone	C_8H_8O	120.06
6	20.69	Eicosapentaenoic acid	$C_{20}H_{30}O_2$	308.23
7	21.53	Oleyl analide	$C_{24}H_{39}NO$	363.31
8	21.69	Oleamide	$C_{18}H_{35}NO$	281.27
9	21.79	Oleoyl ethanol amide	$C_{20}H_{39}NO_2$	307.29
10	21.82	Hexadecanamide	$C_{16}H_{33}NO$	255.26
11	22.40	Stearamide	$C_{18}H_{37}NO$	283.29
12	23.23	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.28
13	25.63	Erucamide	$C_{22}H_{43}NO$	337.33
14	26.45	6-Aminocaproic acid	$C_6H_{13}NO_2$	131.09

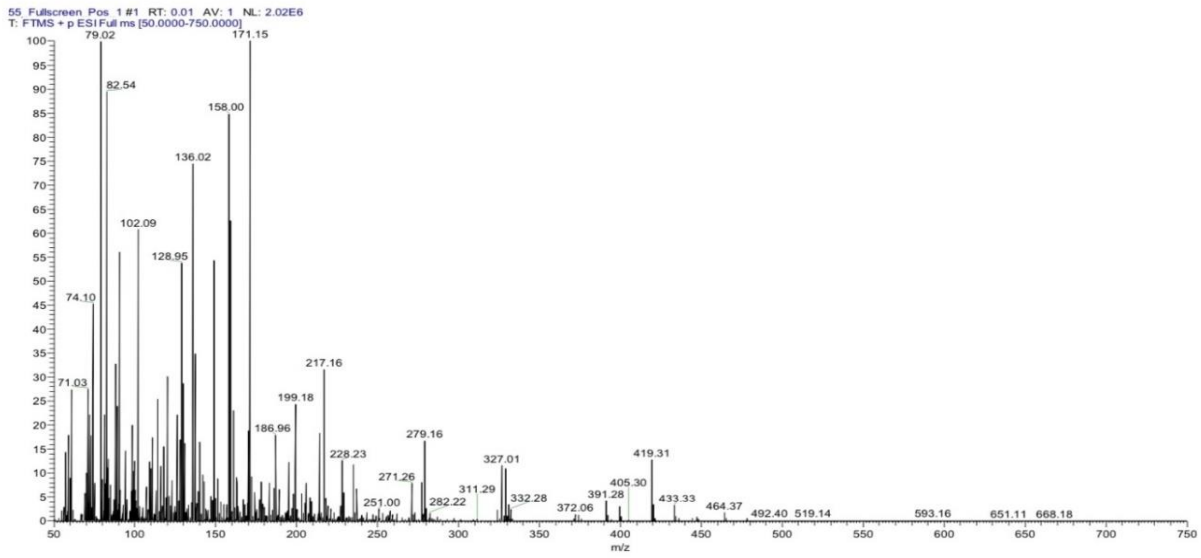

Figure 1: LC-MS Chromatogram result showing the major peaks of fraction 42 secondary metabolites *S. hygroscopicus*

Levalbuterol ($C_{13}H_{21}NO_3$), known as Levosalbutamol, is a potent bronchodilator with superior efficacy to albuterol in acute asthma treatment. Its molecular structure is similar to that of epinephrine and it binds selectively to beta2-adrenergic receptors in the airways. This binding induces relaxation of airway smooth muscle and dilation of the airway diameter by activating adenyl cyclase through a G-protein-coupled mechanism. Furthermore, increasing cAMP levels elevates the phosphorylation of some target proteins that depend on cAMP protein kinase affecting ion channels.[17,18] Levalbuterol serves as a potential drug for COVID-19 infection by disrupting the RNA methylation factor of coronavirus (Nsp16-Nsp10 complex), thereby circumventing the host immune system. Based on speculation, levalbuterol binds to catalytic residues and the Nsp10-binding pocket, preventing S-Adenosyl methionine (SAM) from delivering the methyl group to Nsp16, which is often transferred to viral RNA. This event would disrupt the methylation process, corresponding to the *in silico* research that showed the binding of levalbuterol with the Nsp16 active site in the Nsp16-SAM binding pocket (ASP133, GLU206, LYS49, and LYS173) where a relatively high binding affinity value of 72.88% was obtained.[19] Dibutyl phthalate ($C_{16}H_{22}O_4$) is a phthalate ester generally used in the chemical industry for manufacturing polymers and plasticizers. Various research isolated this compound from organisms, with antibiotic, anti-plasmodial, antifungal, and antidiabetic biological activity. Dibutyl phthalate is known for the ability to inhibit the human malaria parasite GSK-3β (Hs GSK-3β) and *Plasmodium falciparum* 3D7 (Pf 3D7). Dibutyl phthalate esters achieve this inhibition by establishing hydrogen bonds with the amino acid residues Arg96/Glu97 in pocket 2. In addition, *in vitro* enzymatic assays showed that dibutyl phthalate inhibited Hs GSK-3β activity through a combination of several inhibitory mechanisms with a moderate IC_{50} of 2.0 µM.[20]

Prediction of Pharmacokinetic Profiles

Pharmacokinetics is the quantitative research of absorption, distribution, metabolism, and excretion, commonly abbreviated as ADME. This constitutes the mechanism through which drug molecules are processed inside the body.[21] ADME prediction can provide information regarding absorption, distribution, metabolism, elimination, and toxicity comprising the pharmacokinetic and pharmacodynamic characteristics of drug molecules.[22] The evaluation of drug-likeness and oral bioavailability of test compounds plays an important role in drug development. The analysis of drug-like properties is performed according to Lipinski's Rule of Five, which includes the following criteria: (i) the molecular weight of the compound is < 500 mg/dL. (ii) the lipophilicity (clogP) is < 5, (iii) the number of hydrogen bond donors are < 5, and (iv) the number of hydrogen bond acceptors are < 10. A compound is predicted to have good absorption and permeability supposing two or more of the above criteria are met.[23] The oral bioavailability value can be evaluated using Veber's Rule with the criteria including rotatable bonds (ROTB) < 10 and topological polar surface area (TPSA) < 140 Å2.[24]

Table 2 shows that the active compounds of fraction 42 fulfill all the Lipinski and Veber's Rule criteria, signifying the possession of good absorption, permeability, and oral bioavailability as drug candidates administered through the oral route.

Reverse Molecular Docking Result

LC-MS analysis of fraction 42 yielded 14 compounds, among which four with the highest retention time were used, namely dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate. The structure of ligands was generated by retrieving the compounds from PubChem in .sdf format (Figure 2). The target proteins selected based on a search for *Plasmodium falciparum* food vacuole proteins were PfCRT, PfA-M1, and falcipain-2 protease. These three had respective control ligands which facilitated the comparison of binding affinity values for bonds formed between the target proteins and the compounds, as well as the target proteins and control ligands.

The modeling method utilized in this research was reverse molecular docking, which predicted interactions between a single ligand, specifically the drug, and multiple target proteins. Reverse molecular docking was conducted using PyRx software and the results showed binding affinity values and binding interactions between the compounds and target proteins.[25] The stronger the interactions, the greater the ligand's effect on the physiological function of the target proteins, hence, ligands that bind strongly to the target protein are chosen as therapeutic candidates.[26] Dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate, along with control ligands were installed and analyzed by comparing the binding affinity values to target proteins. The binding affinities observed between these four compounds and protein targets are presented in Table 3.

The binding affinity values of the four compounds were found to be similar to those of control ligands. The binding affinity of dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate to target proteins PfA-M1, PfCRT, and falcipain-2 protease was -7.4, -6.7, and 6.4 kcal/mol, -6.9, -6.4, and -5.5 kcal/mol, -6.9, -6.3, and -5.1 kcal/mol, as well as -6.8, -6.4, and 4.9 kcal/mol, respectively. Affinities of the target proteins for each control ligand were -8.1 kcal/mol, -7.3 kcal/mol, and -7.1 kcal/mol, respectively.

The hydrophobic interactions and hydrogen bonds also formed between the four compounds and target proteins were visualized using the software Ligplot 2.2 (Table S1). Specifically, hydrogen bonding is the attraction between the electronegative atoms of two molecules. An active chemical is predicted to have a strong interaction with the target receptor supposing it can bind tightly through hydrogen bonds and to the same amino acid residues as the active site.[27] Hydrophobic interactions play a key role in ligand-receptor binding affinities. Modifying hydrophobic interactions improves the binding affinity and biological activity of complex molecules and helps to stabilize the biochemical milieu of target-drug complexes.[28]

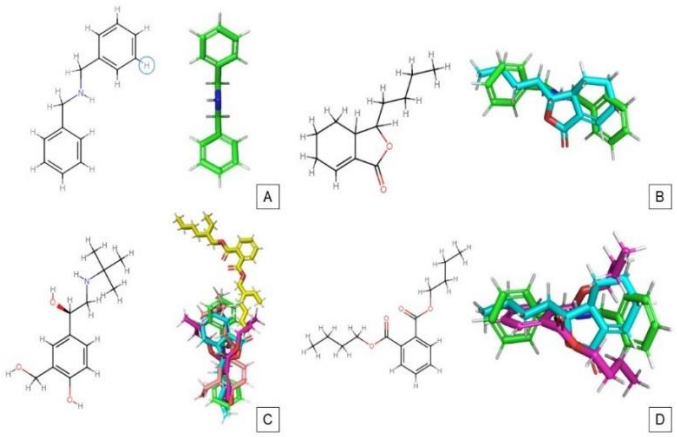

Figure 2: The chemical structure of fraction 42 active compounds in 2D and 3D. (A) Dibenzyl amine; (B) Sedanolide; (C) Levalbuterol; (C) Dibutyl phthalate. (Figure was obtained form https://pubchem.ncbi.nlm.nih.gov/)

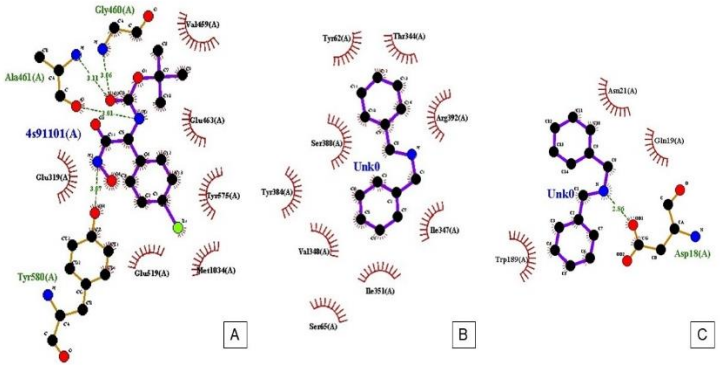

Figure 3: Binding interaction between Dibenzyl amine and protein target (A) Dibenzyl amine and PfA-M1; (B) Dibenzyl amine and PfCRT; (C) Dibenzyl amine and Falcipain-2 Protease (Figure was generated by LigPlot)

Table 2: Prediction of pharmacokinetic profile of fraction 42 active compounds

No	Ligand	Molecular Weight (g/mol)	Rotatable bonds	H-bond acceptor	H-bond donor	TPSA	cLogP
1	Dibenzylamine	197.28	4	1	1	12.03 Å	2.98
2	Sedanolide	194.27	3	2	0	26.3 Å	2.87
3	Levalbuterol	239.31	5	4	4	72.72 Å	1.22
4	Dibutyl Phthalate	278.34	10	4	0	52.6 Å	3.69

Table 3: Reverse Molecular Docking Result

No	ID PDB	Protein Target	Control Ligand	Active Compound	Binding Affinity (Kcal / mol)	
					Control	Compound
1	4ZW3	PfA-M1	tert-butyl [(1s)-1-(4-bromophenyl)-2-(hydroxyamino)-2-oxoethyl] carbamate,	Dibenzyl amine	-8.1	-7.4
				Sedanolide		-6.9
				Levalbuterol		-6.9
				Dibutyl phthalate		-6.8
2	6UKJ	PfCRT	Chloroquine^{2+}	Dibenzyl amine	- 7.3	-6.7
				Sedanolide		-6.4
				Levalbuterol		-6.3
				Dibutyl phthalate		-6.4
3	6SSZ	Falcipain 2 Protease	(~{e})-3-(1,3-benzodioxol-5-yl)-1-(3-nitrophenyl) prop-2-en-1-one).	Dibenzyl amine	-7.1	-6.4
				Sedanolide		-5.5
				Levalbuterol		-5.1
				Dibutyl phthalate		-4.9

Dibenzyl amine failed to share hydrogen bonds with PfCRT, but it bonded with PfA-M1 and falcipain-2 protease (Figure 3). This compound formed three hydrogen bonds with PfA-M1 through Ala461, Gly460, and Tyr580 amino acid residues, similar to the type formed between the target protein and the control ligand. Additionally, six hydrophobic bonds were formed with PfA-M1 through Glu319, Val459, Tyr575, Glu463, Met1034, and Glu519 where the first three bonds were the same as those between the target protein and control ligand. Nine hydrophobic bonds were formed with PfCRT through Ser65, Val348, Tyr384, Ser388, Tyr62, Thr344, Arg392, Ile347, and Ile351, all of which were the same as the bonds observed between the target protein and the control ligand. One hydrogen bond through Asp18 as well as three hydrophobic bonds through Trp189, Asn21, and Gln19 were formed with falcipain-2 protease.

Sedanolide did not produce hydrogen bonds with all target proteins (Figure 4). This compound formed six hydrophobic bonds with PfA-M1through Glu319, Val459, Tyr575, Glu463, Met1034, and Glu519, among which the first three were similar to hydrophobic bonds between the target protein and control ligand. Furthermore, 11 hydrophobic bonds were formed with PfCRT through Ser65, Tyr62, Val348, Ile61, Thr344, Arg392, Tyr391, Ser388, Ile347, Tyr384, and Asn395, of which 10 were the same as the hydrophobic bonds found between the target protein and control ligand. Five hydrophobic bonds were formed with falcipain-2 protease through Asp18, Gln19, His157, Trp189, and Ala140, among which one was identical to bonds found between the target protein and control ligand.

Levalbuterol formed hydrogen and hydrophobic bonds in all target proteins (Figure 5). This compound formed one hydrogen bond with PfA-M1 through Glu497 which was the same as the type found between the target protein and control ligand. Additionally, eight hydrophobic bonds were formed with PfA-M1 through Val493, Ala461, Glu319, Tyr575, Val459, Tyr580, Arg489, and His496, four of which were similar to the hydrophobic bonds observed between the target protein and control ligand. One hydrogen bond was formed with PfCRT through Ser388, similar to the type identified between the protein target and control ligand. Nine hydrophobic bonds were formed with PfCRT through Val348, Ser65, Tyr62, Ile61, Phe340, Thr344, Asn395, Tyr391, and Arg392 with the same seven bonds as the target protein and control ligand. Two hydrogen bonds through Trp189 and Asp18 as well as three hydrophobic bonds through Gln19, His157, and Lys20 were formed with falcipain-2 protease.

Dibutyl phthalate did not share hydrogen bonds with falcipain-2 protease, but it formed hydrogen and hydrophobic bonds with PfA-M1 and PfCRT (Figure 6). Dibutyl phthalate formed two hydrogen bonds with PfA-M1 through Gly460 and Ala461 as well as 10 hydrophobic bonds through Gln317(A), Met462, Tyr575, Tyr580, His496, Glu497, Val493, Thr492, Val459(A), and Glu319. Two hydrogen bonds were formed with PfCRT Tyr384 and Ser65 as well as eight hydrophobic bonds through Ile351, Ile66, Leu69, Leu385, Thr344, Ile347, Ser388, and Tyr391, of which four bonds were similar to the target protein and control ligand. Five hydrophobic bonds were formed with falcipain-2 protease through Asp18, Gln19, His157, Trp189, and Lys20. The prediction of strong binding interaction with protein targets was based on the observation of several similarities in the bonds formed. Therefore, dibutyl phthalate is expected to have strong binding properties for the effective formation of hydrogen bonds with the same amino acid residues, specifically when compared to the control ligand.[8]

Plasmodium M1 Metalloaminopeptidases (MAPs), also known as PfA-M1, are responsible for the last stages of intra-erythrocytic haemoglobin digestion. Selective inhibition of PfA-M1 has been reported to cause digestive vacuole (DV) enlargement and parasite death. Importantly, inhibition of this enzyme leads to an accumulation of undigested short peptide chains potentially derived from haemoglobin, thereby validating the *in vivo* activity.

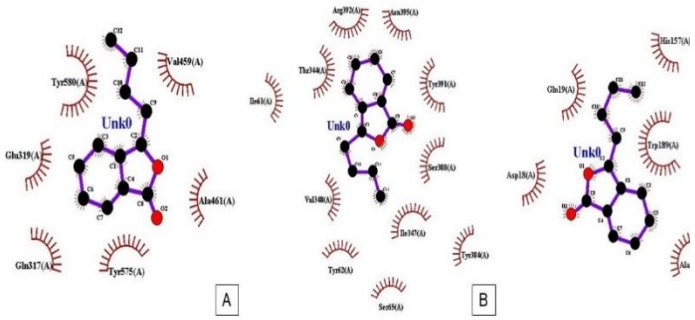

Figure 4: Binding interaction between Sedanolide and protein target (A) Dibenzyl amine and PfA-M1; (B) Dibenzyl amine and PfCRT; (C) Dibenzyl amine and Falcipain-2 Protease. (Figure was generated by LigPlot).

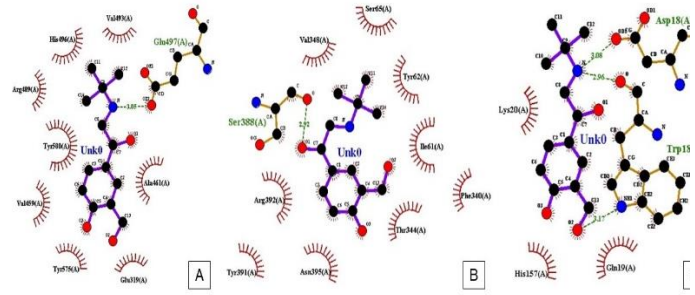

Figure 5: Binding interaction between Levalbuterol and protein target (A) Dibenzyl amine and PfA-M1; (B) Dibenzyl amine and PfCRT; (C) Dibenzyl amine and Falcipain-2 Protease. (Figure was generated by LigPlot)

The investigation conducted on mouse malaria models showed that the inhibition process resulted in parasitemia reduction.[29]

PfCRT is a transporter in the food vacuole organelle or DV of a parasite made up of 424 amino acids as well as 10 transmembrane helical domains organized into five pairs of helices. Two of the helices have an inverted antiparallel topology and the remaining two are juxta membranes. The entire PfCRT components form a core cavity with a negative charge of around 3,300A, which is open to the DV side. This hole allows positively charged drug molecules or metabolites concentrated in the DV to flow into the cytosol[30]. At the structural level, PfCRT can accommodate the binding of antibody fragments and active chemicals such as chloroquine, enabling the targeting of novel antimalarials.[31]

Falcipain-2, a cysteine protease found in *Plasmodium falciparum*, plays a crucial role in hemoglobin degradation during the blood stage, making it an important target for drug development. The process of hemoglobin degradation occurs in the food vacuole and is facilitated by a 200 kDa protein complex including cysteine (falcipain-2 protease), aspartate (plasmepsin II and IV), histo-aspartic proteases, and specialized enzyme (haem detoxification protein). Falcipain-2 is responsible for cleaving haemoglobin to tiny peptides, hence falcipain-2 suppression has been regarded an interesting technique for preventing parasite growth.[32]

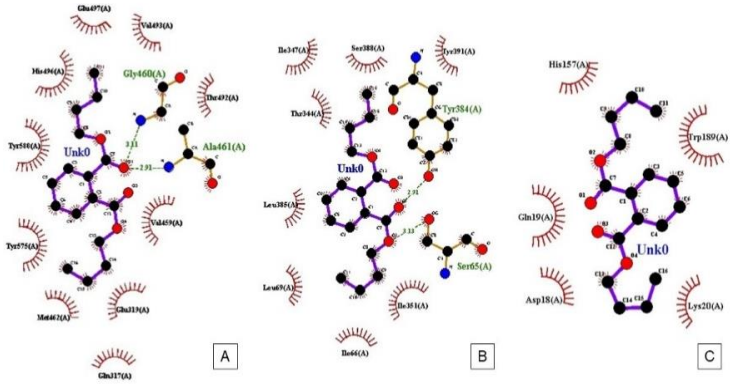

Figure 6. Binding interaction between Dibutyl phthalate and protein target (A) Dibenzyl amine and PfA-M1; (B) Dibenzyl amine and PfCRT; (C) Dibenzyl amine and Falcipain-2 Protease. (Figure was generated by LigPlot)

Table S1: Results of the interaction analysis of fraction 42 active compounds with protein target

No	ID PDB	Protein Target	Ligand	Interaction with the protein target	The Bond
1	4ZW3	PfA-M1	tert-butyl [(1s)-1-(4-bromophenyl)-2-(hydroxyamino)-2-oxoethyl] carbamate, (Control)	Hydrogen bond: Ala461(A), Gyl460(A), His496(A), His500(A), znll02(A), Glu497(A), Glu519(A), Tyr580(A) Hydrophobic bond: Arg489(A), Goll104(A), Val459(A), Tyr575(A), Glu319(A)	8 5
2	4ZW3	PfA-M1	Dibenzyl amine	Hydrogen bond: **Ala461(A), Gly460(A), Tyr580(A)** Hydrophobic bond: **Glu319(A), Val459(A),** Glu463(A), **Tyr575(A),** Met1034(A), Glu519(A)	3 6
3	4ZW3	PfA-M1	Sedanolide	Hydrogen bond: - Hydrophobic bond: **Tyr575(A),** Gln317(A), **Glu319(A)**, Tyr580(A), **Val459(A)**, Ala461(A)	0 6
4	4ZW3	PfA-M1	Levalbuterol	Hydrogen bond: **Glu497(A)** Hydrophobic bond: Val493(A), Ala461(A), **Glu319(A)**, **Tyr575(A)**, **Val459(A)**, Tyr580(A), **Arg489(A)**, His496(A)	1 8
5	4ZW3	PfA-M1	Dibutyl phthalate	Hydrogen bond: Gly460(A), Ala461(A) Hydrophobic bond: Gln317(A), Met462(A), **Tyr575(A)**, Tyr580(A), His496(A), Glu497(A), Val493(A), Thr492(A), **Val459(A), Glu319(A)**	2 10
6	6UKJ	PfCRT	Chloroquine^{2+} (Control)	Hydrogen bond: Hydrophobic bond: Ile61, Tyr62, Tyr391, Tyr384, Ile347, Ile351, Arg392, Leu385, Thr344, Val348, Ser65, Ser388, Ile389	0 13
7	6UKJ	PfCRT	Dibenzyl amine	Hydrogen bond: -	0

				Hydrophobic bond: **Ser65(A), Val348(A), Tyr384(A), Ser388(A), Tyr62(A), Thr344(A), Arg392(A), Ile347(A), Ile351(A)**	9
8	6UKJ	PfCRT	Sedanolide	Hydrogen bond: -	0
				Hydrophobic bond: **Ser65(A), Tyr62(A), Val348(A), Ile61(A), Thr344(A), Arg392(A)**, Asn395(A), **Tyr391(A)**, **Ser388(A), Ile347(A), Tyr384(A)**	11
9	6UKJ	PfCRT	Levalbuterol	Hydrogen bond: **Ser388(A)**	1
				Hydrophobic bond: **Val348(A), Ser65(A), Tyr62(A), Ile61(A),** Phe340(A), **Thr344(A),** Asn395(A), **Tyr391(A), Arg392(A)**	9
10	6UKJ	PfCRT	Dibutyl phthalate	Hydrogen bond: Tyr384(A), Ser65(A)	2
				Hydrophobic bond: **Ile351(A)**, Ile66(A), Leu69(A), Leu385(A), Thr344(A), Ile347(A), **Ser388(A), Tyr391(A)**	8
11	6SSZ	Falcipain-2 Protease	(~{e})-3-(1,3-benzodioxol-5-yl)-1-(3-nitrophenyl) prop-2-en-1-one). (Control)	Hydrogen bond: -	0
				Hydrophobic bond: Ala140(A)	1
12	6SSZ	Falcipain-2 Protease	Dibenzyl amine	Hydrogen bond: Asp18(A)	1
				Hydrophobic bond: Trp189(A), Asn21(A), Gln19(A)	3
13	6SSZ	Falcipain-2 Protease	Sedanolide	Hydrogen bond: -	0
				Hydrophobic bond: Asp18(A), Gln19(A), His157(A), Trp189(A), **Ala140(A)**	5
14	6SSZ	Falcipain-2 Protease	Levalbuterol	Hydrogen bond: Trp189(A), Asp18(A)	2
				Hydrophobic bond: Gln19(A), His157(A), Lys20(A)	3
15	6SSZ	Falcipain-2 Protease	Dibutyl phthalate	Hydrogen bond: -	0
				Hydrophobic bond: Asp18(A), Gln19(A), His157(A), Trp189(A), Lys20(A)	5

Note: Bonds in bold

Conclusion

In conclusion, the LC-MS analysis conducted in this research identified that fraction 42 of *Streptomyces hygroscopicus* Subsp. Hygroscopicus derivatives contained dibenzyl amine, sedanolide, levalbuterol, and dibutyl phthalate with a high affinity for PfA-M1, PfCRT, and falcipain-2 protease. Dibenzyl amine had the highest binding affinity towards all protein targets compared to other compounds, suggesting it as an effective antimalarial candidate.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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