DOI: https://doi.org/10.54393/pbmj.v6i07.901

PAKISTAN BIOMEDICAL JOURNAL

https://www.pakistanbmj.com/journal/index.php/pbmj/index

Volume 6, Issue 7 (July 2023)

Original Article

Identification and Characterization of Sesquiterpene Lactones as Potential Falcipain-2 Inhibitors

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ARTICLE INFO

Key Words:

Sesquiterpene Lactones, Falcipain-2, Anti-Malarial Activity, Molecular Docking, Binding Energy, Inhibition Constant

How to Cite:

Rizwana, S., Maqbool, M. F., Maryam, A. ., Bashir, E. ., Khan, M. ., Nisar Khan, B. ., Shakir, H. A. ., & Irfan, M. . (2023). Identification and Characterization of Sesquiterpene Lactones as Potential Falcipain-2 Inhibitors: Sesquiterpene Lactones as Potential Falcipain-2 Inhibitors. Pakistan BioMedical Journal, 6(07). https://doi.org/10.54393/pbmj.v6i07.901

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Received Date: 6th July, 2023
Acceptance Date: 27th July, 2023
Published Date: 31st July, 2023

ABSTRACT

Drug resistance affects the most effective anti-malarial medications, hence finding new, unique bioactive compounds with strong anti-malarial activity is extremely desirable. Falcipain-2 (2GHU) is a protease of *plasmodium falciparum* and considered as an important target to design antimalarial drugs. **Objective:** To identify potential novel falcipan-2 inhibitor for effect treatment of malaria. **Methods:** Molecular docking analysis was performed by using different bioinformatic tools to check the interaction between the Alantolactone and Brevilin A and falcipain-2 (2GHU). **Results:** Alantolactone and Brevilin A show a strong affinity to bind with 2GHU with binding energy values -7.2kcal/mol and -8.1kcal/mol respectively. Moreover, results of ADMET and cytotoxicity analysis showed that both investigated compounds strongly followed the Lipinski rule of five for drug-likeness and are quite safe to be used as an antimalarial drug. **Conclusions:** Both of the studied sesquiterpene lactones may inhibit falcipain-2, according to the results of our molecular docking study, but Brevilin A is predicted to be the most effective inhibitor because it forms strong hydrogen bonds with the protein's amino acid residues and has lower values for binding energy and inhibition constant. Therefore, new anti-malarial medications can be created from these two bioactive sesquiterpene lactone molecules to overcome the resistance of *plasmodium falciparum* against already clinically approved drugs.

INTRODUCTION

Malaria is a well-known parasitic infection throughout the world. As it has been almost eradicated from temperate regions, many travelers from temperate zone each year visited tropical areas, where still malaria exists as a major cause of morbidity. Eukaryotic single-celled microorganisms of the genus Plasmodium are malarial parasites. Only four parasitic species of the genus Plasmodium including, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* may infect people. *Falciparum P.* is the main cause of malaria fatalities in young children in Africa and is the agent of severe, potentially fatal malaria. By being bitten by an infected female *Anopheles* mosquito, malaria is spread [1]. *Plasmodium falciparum* is a protozoan parasite that kills at least one million children each year. Simple malaria is associated with periodic fevers and chill that mirror the

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intraerythrocytic cycle. Multiple additional diseases, such as lactic acidosis, cerebral malaria (caused by infected erythrocytes adhering to the brain's endothelium), and severe anemia, are associated with severe malaria [2]. In 2010, malaria is thought to have caused 216 million illnesses and 655,000 mortalities in 108 countries with a combined population of about 3 billion people [3]. More than two billion people, or more than 40% of the world's population, is at risk of contracting malaria, and according to data collected by the World Health Organization (WHO) between 1999 and 2004, 1.1 to 1.3 million people die from malaria each year globally. Sixty percent of Pakistan's 161 million residents, or 95 million people, reside in areas where malaria is endemic [4]. Malaria re-emerged as an epidemic in the 1970s after being eradicated in the 1960s. In recent years, floods that affected almost 20 million people in over 60 districts have contributed to an increase in malaria cases [5]. The number of malaria cases reported nationwide in 2008 was 2.6 million, with a yearly fatality rate of 50,000 [4]. In 2010, the Eastern Mediterranean region reported over a million microscopy-confirmed malaria cases, 22% of which originated in Pakistan [4]. 50,000 people die from malaria-related causes each year in Pakistan, despite the existence of a well-established malaria control program, with 37% of cases reportedly occurring in areas near the Iranian and Afghan borders [6]. All the symptoms and pathologies connected with malaria are brought on by the asexual blood stage in which parasites infect the mature red blood cell [2]. Possible medications including Chloroquine and other related Quinolones (such as Hydroxychloroquine), Quinine, Primaquine, Mefloquine, Sulfonamides, and Artesunate & Artemether (Artemisinin analogs) is used to treat malaria to eliminate the parasite. But *Plasmodium falciparum* has become immune to all these medications. By dissolving erythrocyte proteins, most notably hemoglobin, Falcipain-2 (FP-2), a papain-family (C1A) cysteine protease of *Plasmodium falciparum*, greatly contributes to the development of the illness in the host. As FP-2 and its paralogs prevent parasite maturation, these proteins may make interesting targets for the development of brand-new anti-malarial drugs. The search for strong, focused, and efficient FP-2 inhibitors has been slowed down by a dearth of structural knowledge [7]. The plant has great medicinal importance due to the presence of secondary metabolites such as flavonoids, polyphenols, sesquiterpene lactones, alkaloids, etc. [8-10]. Sesquiterpene lactones are a group of secondary metabolites that are typical of the Compositae but sporadic in other angiosperm groups and even in some liverworts. Recent studies have proven that a prominent structural component of sesquiterpene lactones is an unsaturated lactone group, which has anti-tumor, cytotoxic, anti-microbial, and phytotoxic activities [11-15]. Drug designing, a branch of bioinformatics, uses molecular docking, which is the interaction of two or more molecules to create a stable adduct. Based on the ligand and target's binding properties, it can predict the three-dimensional structure of any complex. Virtual screening, bioremediation, drug discovery, protein de-orphaning, prediction of binding sites (blind docking), protein-protein interactions, mechanisms of enzymatic reactions, studies of structure-function, and protein engineering are all applications of molecular docking [16-18]. In our research, we used molecular docking to work on two sesquiterpene lactones; Alantolactone and Brevilin A, as falcipain-2 inhibitors to check whether they are effective anti-malarial drugs or not.

METHODS

Here we applied Molecular Docking analysis to find the interaction between falcipain-2 and two sesquiterpene lactones; Alantolactone and Brevilin A. AutoDock vina 4.2.1 was used for analyzing protein-ligand interaction. AutoDock mgl tool 1.5.6 and pyMOL2.4 were used for obtaining PDBQT format of protein and ligand. Finally, docking results were visualized by using Discovery Studio visualizer 2.5 and Ligplot+ 4.5.3. Molecular Docking (MD) involves the following steps. 1) Preparation of Protein Molecule. 2) Preparation of Ligand. 3) Molecular Docking protocol. 4) Visualization of Protein-Ligand complex. The following steps involve the preparation of protein for MD. The crystal structure of falcipain-2, with the PDB code of 2GHU at a resolution of 2.25 Å, was obtained from the RCSB PDB (https://www.rcsb.org/), the Research Collaboratory for Structural Bioinformatics Protein Data Bank. The structure was downloaded and saved in the protein data bank file format (PDB). Afterward, the protein was prepared by using "Autodock MGL tool (ADMGLT). Polar hydrogens were added, Kollman charges were injected, and water molecules were removed. Finally, the protein's PDB format was changed to the PDBQT format. The following steps involve the preparation of ligands for MD: The 3D crystal structure of two sesquiterpene lactones; Structure-data file (SDF) formats of alantolactone and brevilin A were downloaded from the Pubchem database on an online server (https://pubchem.ncbi.nlm.nih.gov/). For educational purposes, ligands were first translated from SDF format into PDB format using Pymol. Using ADVMGLT, the PDB format of the acquired ligands was converted into the PDBQT format required for MD. Molecular docking analysis of 2GHU with Alantolactone and Brevilin A was done by using AutoDock 4.2.1. The grid box was prepared by using 40 x 40 x 40 points in X, Y, and Z dimensions with center 51.151, 7.351, and -28.801 via ADMGLT. The ligand-

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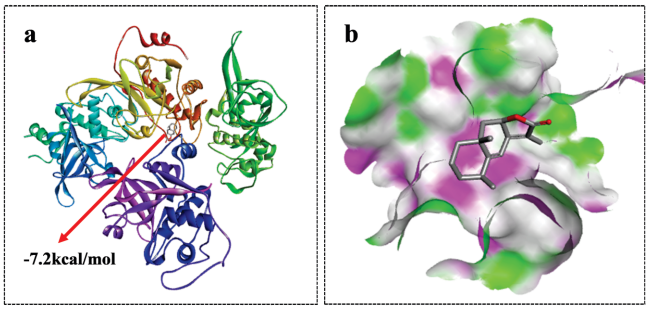
2GHU complex obtained because of MD was visualized by using Discovery Studio Visualizer. The conformation that had the lowest binding energy was considered the most stable conformation of the ligand with respect to the protein. In this work, medication similarity and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profile were predicted using a pkCSM web server. An open database server called pkCSM provides information on the pharmacokinetics of medicines. Ligand structures were retrieved from PubChem and was analyzed with pkCSMs. The toxicity ADMET module's server database was chosen to operate on [19].

RESULTS

Biova Discovery Studio Visualizer (Free verison) was used to examine the intricate interactions of the optimal conformation. The interaction of falcipain-2 and alantolactone showed that alantolactone showed hydrophobic interactions with three amino acids of falcipain-2; Pi-Sigma bond with TRP206 at a bond distance of 3.69181Å, Alkyl bond with CYS42 at a bond distance of 4.70387Å and Pi-Alkyl bond with HIS174 at a bond distance of 5.28753Å. For falcipain-2 and brevilin A interaction, we found that brevilin A showed hydrogen bonding with two amino acids of falcipain-2; ASN173 at a bond distance of 2.40334 Å and TRP206 at a bond distance of 2.50892Å as mentioned in Table 1 and shown in Figure 1 and Figure 2.

Table 1: Binding affinities, Inhibition constant, and interacting amino acid residues of 2GHU interacting with studied Sesquiterpene lactones

Ligands (Sesquiterpene Lactones)	Binding Energy	Inhibition Constant	Interacting Amino Acid Residues	
	(kcal/mol)	(μM)	H-bonds	Hydrophobic interactions
Alantolactone	-7.2	5.205	-	TRP206 (3.69181Å) CYS42 (4.70387Å) HIS174 (5.28753Å)
Brevilin A	-8.1	1.136	ASN173 (2.40334 Å) TRP206 (2.50892 Å)	-



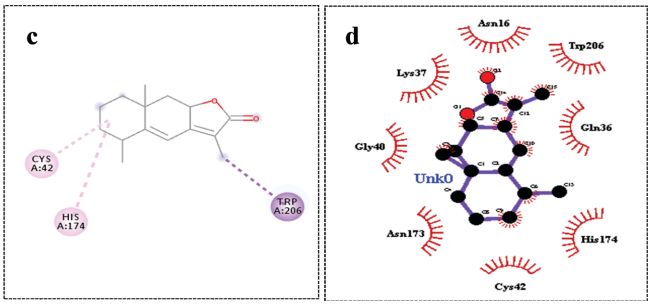


Figure 1: Alantolactone and 2GHUinteraction. (a) Output of AutoDock Vina visualized by Discovery Studio Visualizer shows the interaction of binding-site residues of chain A of 2GHU with Alantolactone. (b) Stick model showing Donor and acceptor regions of 2GHU interacting with Ligand (Pink color indicating H-bond donor region and green color indicating H-bond acceptor region of 2GHU). (c) Two-dimensional diagram showing the type of interaction formed between 2GHU and Alantolactone (the purple dotted line is representing the Pi-Sigma bond formed between TRP206 residue of 2GHU and Alantolactone, one light pink dotted line indicating Alkyl bond formed between CYS42 residue of 2GHU and Alantolactone, other light pink dotted line indicating Pi-Alkyl bond formed between HIS174 residue of 2GHU and Alantolactone. (d) Ligplot analysis of 2GHU –Alantolactone interaction mediated by the hydrophobic interactions (Hydrophobic interactions are shown by an arc with spokes pointing towards the ligand they are interacting with and interacting atoms of ligand with spokes radiating back)

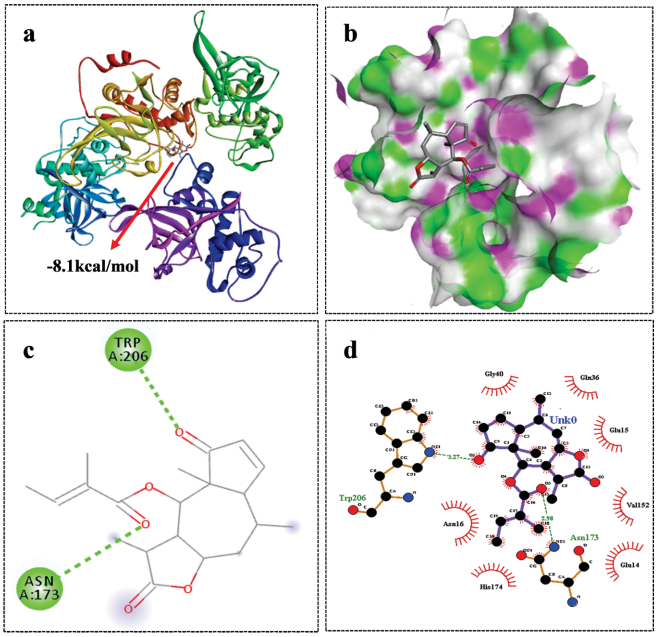


Figure 2: Brevilin A and 2GHU interaction. (a) Output of AutoDock Vina visualized by Discovery Studio Visualizer shows the interaction of binding-site residues of chain A of 2GHU with Brevilin A. (b) Stick model showing Donor and acceptor regions of 2GHU interacting with Ligand (Pink color indicating H-bond donor region and green color indicating H-bond acceptor region of 2GHU). (c) Two-dimensional diagram showing the type of interaction formed between 2GHU and Brevilin A (the green dotted lines are representing the hydrogen bonds formed

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between TRP206 and ASN173 residues of 2GHU and Brevilin A. (d) Ligplot analysis of 2GHU –Brevilin A interaction mediated by the hydrogen bonds (The hydrogen bond is represented by Green amino acid residue)

No matter how well a candidate molecule binds to the receptor, if it is poorly absorbed or is eliminated from the body too slowly, it is useless. Therefore, Lipinski's rule of five was applied to forecast drug-likeness, which is always necessary. This rule states that any ligand that satisfies the criteria of molecular weight 500 Dalton, number of H-bond acceptors 10, number of H-bond donors 5, and lipophilicity expressed as logP 5 is regarded to be drug-like [19]. Our both selected ligands obeyed this rule. Predicting human pharmacokinetic qualities is crucial for the drug-designing process since it aids in the identification and advancement of potential candidate molecules for the clinic [19]. One of the crucial steps for figuring out a drug's bioavailability after oral administration is absorption in the small intestine. It is important to note that sesquiterpene lactones have intestinal absorbance values of more than 30%, indicating their ease of absorption. Additionally, both compounds had water solubility (log S) greater than 5, reflecting their solubility in water at 25 °C (Table 2) (Muhammad *et al.*, 2021). Our findings also demonstrated that none of the compounds we chose are P-glycoprotein II inhibitors, despite this being a crucial component of pharmacokinetics research. Instead, they are P-glycoprotein I inhibitors. Therefore, it may be inferred that these medications may act as secure and efficient adjuvants to any pharmaceutical medication against falcipain-2. The dosage of drug needed for even distribution in blood and plasma is shown by the steady-state volume of distribution (VDss). If Log VDss is less than 0.5, it is considered low; if it is larger than 0.45, it is considered excessive. A higher VDss value indicates that the medication is distributed more widely in the plasma than in the tissue, while a lower VDss value indicates that the drug has a weak ability to diffuse or cross the cell membrane [19]. Our results show that alantolatone has a value near 0.45 and brevilin a has value of more than -0.5. alantolactone can be good candidate in respect to distribute uniformly in blood plasma (see Table 2). Drug metabolizing enzymes can also have an impact on pharmacokinetic interactions. The human body's key detoxification enzyme, cytochrome P450, is found mostly in the liver. The pharmacokinetics of medications that are processed by these enzymes can be disturbed by any change in their activity. Cytochrome P2D6 and cytochrome P3A4 are the two most significant isoforms of cytochrome P450 [19]. Brevilin A does not inhibit these enzymes, according to Table 2, and solely interferes with the CYP3A4 substrate. Therefore, it can be inferred that brevilin A is safe when used as an adjuvant with other medications. Total clearance measures the amount of medication removed from plasma or blood. Clearance (the process of removing drugs) is produced by both the kidney and the liver. Total clearance Log (CLtot) forecasts the sum of hepatic and renal clearance. It is crucial to establish steady-state concentrations by figuring out dosing rates and is related to bioavailability. When the medicine is taken at the right concentration and is bioavailable, a steady state level is reached. The greater the CLtot value, the faster the excretion process of the compound [19]. Table 2 shows the total clearance values from which their rate of excretion can be predicted. Moreover, it can be seen that brevilin A is not the substrate of renal OCT2. Protein transporter OCT2 (organic cation transporter 2) is essential for renal medication clearance. OCT2 substrates and OCT2 inhibitors can interact and cause negative effects [19]. Protein transporter OCT2 (organic cation transporter 2) is essential for renal medication clearance. OCT2 substrates and OCT2 inhibitors can interact and cause negative effects [19]. Table 2 reveals that both compounds do not have mutagenic effects. Determining a medication's capacity to cause liver damage is a crucial component of drug development research. From Table 2, both compounds are not hepatotoxic.

Table 2: Selected ligands' ADMET analyses utilizing the pkCSM online database server

ADMET	parameters	alantolactone	Brevilin a
Absorbance	Water solubility (log S) mol/L	-3.993	-4.007
	Intestinal absorption %	97.057	99.538
	P-glycoprotein I inhibitor	Yes	Yes
	P-glycoprotein II inhibitor	No	No
Distribution	Log VDss (L/kg)	0.361	0.066
Metabolism	CYP2D6 substrate	No	No
	CYP3A4 substrate	Yes	Yes
	CYP2D6 inhibitor	No	No
	CYP3A4 inhibitor	Yes	No
Excretion	Total clearance (log ml/min/kg)	1.076	1.174
	Renal OCT2 substrate	Yes	No
Toxicity	AMES	No	No
	Max. tolerable dose (human) (log mg/kg/day)	0.042	-0.081
	Hepatotoxicity	No	No

LIPINSKY'S rule of five is a rule designed to check the drug-likeness of any compound before purposing it as a drug molecule. Results of ADME analysis show that both selected molecules strongly obeyed the LIPINSKY'S rule of five without any violation as shown in Table 3. This suggested that both molecules can be used as a drug molecule against any disease.

DOI: https://doi.org/10.54393/pbmj.v6i07.901

Table 3: Drug linkess of Brevilin A and Alantolactone

Serial Number	Standard Values of LIPINSKY'S rule	ADMET values for Brevilin A	ADMET values for Alantolactone
H-bond donors	not over 5	0	0
H-bond acceptors	not over 10	5	2
Molecular weight	less than 500 g /mol	346.42 g/mol	232.32 g/mol
High lipophilicity	LogP not over 5	2.62	3.20
Molar refractivity range	40-130	93.47	67.95

DISCUSSION

The fundamentals of rational drug design involve using structural information and understanding lignad-protein attaching operations to investigate the possibility of discovering novel therapeutic targets. Therefore, having a thorough knowledge of the nature of recognitions and interactions at the molecular level are also very important because it will provide you insights into creating, developing, and discovering new medications. For investigating interactions and patterns of binding of proteins and ligands, MD is a widely used computer method. In the current investigation, MD was carried out using AutoDock Vina, a grid-based method. According to the docking analysis, the two sesquiterpene lactones under study alantolactone and brevilin A have a strong potential to attach to chain A of the 2GHU and thereby decrease its function. To achieve better outcomes, the docking was carried out three times. For each ligand and macromolecule, the molecular docking yielded nine postures, from which the optimal position was chosen based on the affinities of the binding partners. That protein-ligand complex is considered more stable whose binding energy value is less. 2GHU strongly binds with Brevilin A by making hydrogen bonds and with Alantolactone by making hydrophobic interactions with a binding affinity value of -8.1 and -7.2 kcal/mol respectively and inhibition constant (Ki) 1.136 and 5.205µM respectively. The Ki value reveals the amount of drug required to reach the 50% inhibition. Wang *et al.*, in 2014 screened 50 natural compounds as an inhibitor of 2GHU, out of these 50 compounds, FP-2 is shown to be moderately inhibited by 10 natural products with various scaffolds, with IC50 values ranging from 3.18 to 68.19 µM [20]. These inhibitors can be divided into three classes: caffeats (compounds 1, 2 and 8 having inhibition constant values 3.18, 3.77, and 53.12µM, respectively), flavonoid glycosides (compounds 5, 6 and 10 having inhibition constant values 15.74, 17.13 and 68.19µM, respectively) and flavonoids (compounds 3, 4, 7 and 9 having inhibition constant values 5.23, 9.12, 44.81 and 56.92µM, respectively) [8]. Out of these 10 compounds, not a single compound has an inhibition constant value less than that of our best-docked compound Brevilin A having an inhibition constant value of 1.136µM. Only two compounds from caffeats (compounds 1 & 2) have inhibition constant values more than that of our second docked compound Alantolactone having an inhibition constant value of 5.205µM. One of the sesquiterpene lactones we have investigated, alantolactone, interacts with 2GHU through hydrophobic interactions with the Pi-Sigma residues TRP206, CYS42, and HIS174 at bond distances of 3.69181Å, 4.70387Å, and 5.28753Å, respectively. Brevilin A interacts with 2GHU by making hydrogen bonds with ASN173 and TRP206 residues at a bond distance of 2.40334Å and 2.50892Å, respectively, while other investigated sesquiterpene lactones do not [14]. Findings of *in silico* study clearly show that both studied sesquiterpene lactone compounds are proved to be good inhibitors of falcipain-2. Among these two compounds, Brevilin A is the best inhibitor of falcipain-2.

CONCLUSIONS

Finding new anti-malarial medications is urgently needed in light of *Plasmodium falciparum's* evolving resistance to various anti-malarial medications. Falcipain-2 is crucial to the parasite life cycle. In the current study, we used molecular docking to study two sesquiterpene lactones, alantolactone and brevilin A, as falcipain-2 inhibitors. As a result of their interactions with several residues of 2GHU, including TRP206, CYS42, HIS174, and ASN173, our findings indicated that both of the compounds under consideration might interact with the binding pocket of that molecule. Alantolactone and Brevilin A's interactions with 2GHU resulted in binding energies of -7.2 and -8.1 kcal/mol, respectively. The binding energies of alantolactone and brevilin A in association with 2GHU yielded inhibition constant values of 5.205 and 1.136 M, respectively. Our findings demonstrated the effectiveness of both substances as potent falcipain-2 inhibitors, particularly Brevilin A, which is anticipated to be the most effective inhibitor of falcipain-2 among all the natural substances investigated to date. These results should be validated by additional in vivo and in vitro research before these two bioactive sesquiterpene lactone compounds are turned into brand-new anti-malarial medications.

Authors Contribution

Conceptualization: MK, EB
Methodology: MFM, SR
Formal analysis: SR, MFM
Writing-review and editing: MK, AM, BNK, HAS, MI

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

DOI: https://doi.org/10.54393/pbmj.v6i07.901

Source of Funding

The authors received no financial support for the research, authorship and/or publication of this article.

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