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ORIGINAL ARTICLE

Antimalarial flavonoid glycoside from *Carica papaya* with inhibitory potential against *Plasmodium falciparum* dihydrofolate reductase thymidylate synthase: an *in-silico* study

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ABSTRACT

BACKGROUND

Carica papaya is traditionally used to treat malaria. The mechanism of action of the active constituents may be determined by molecular docking. This study therefore examined the in silico antimalarial activity of selected compounds from *Carica papaya* using *Plasmodium falciparum* dihydrofolate reductase thymidylate synthase (PfDHFR-TS) as target protein.

METHODS

Antimalarial activity screening of *Carica papaya* compounds was done in silico using AutoDock 4.2 software which was equipped with Autodock tools 1.5.6 for preparation. Five compounds contained in *Carica papaya* leaves, i.e. quercitrin, isoquercitrin, carpaine, caricaxanthin, and violaxanthin were successfully docked with the target protein. The molecular docking method is declared valid if the RMSD obtained is not more than 2 Å. In vitro evaluation of the test compounds as antimalarials was accomplished by determining their inhibitory activity against dihydrofolate reductase thymidylate synthase (PfDHFR-TS) which plays a role in the synthesis of nucleotides needed by *Plasmodium falciparum*.

RESULTS

Validation of *Plasmodium falciparum* DHFR-TS with PDB ID 1J3I showed an RMSD value of 1.58 Å. The docking results showed that quercitrin, isoquercitrin, carpaine, and caricaxanthin showed negative energy values similar to the native ligand. Therefore the four compounds have good affinity for the target protein, while violaxanthin shows a positive energy value, indicating no affinity for the target protein.

CONCLUSION

Based on binding affinity values and molecular interactions, isoquercitrin and quercitrin have inhibitory activity against dihydrofolate reductase thymidylate synthase (PfDHFR-TS), such that they have potential as natural antimalarial candidates.

Keywords: *Plasmodium falciparum*, isoquercitrin, quercitrin, dihydrofolate reductase, molecular docking

INTRODUCTION

Currently malaria is a health problem in the world,[1,2] being a disease that could result in deaths, especially if caused by *Plasmodium falciparum*. It was estimated in 2022 that there were 249 million cases of malaria worldwide, with the number of deaths reaching 608 thousand people in 85 countries, of which the largest number was found in children.[1]

The challenge in the malaria elimination program is the discovery of resistance to both antimalarial drugs and insecticides which play a role in killing the parasites and the mosquito vectors, respectively.[3,4] Resistance to artemisinin, which is the first and best drug of choice for malaria, has begun to be reported in the Great Mekong River region of Southeast Asia and in East Africa.[5] With reports of artemisinin resistance being discovered since 2008, efforts need to be made to develop new antimalarial drugs.[6] Approaches that can be used in developing antimalarial drugs are conventional methods, repurposing drug development, and genomic techniques that provide new drug targets.[7] Molecular docking, a drug development method that is currently being implemented, is a virtual method that can be used to screen candidate compounds that have the potential to be developed to discover new drugs. Docking can also simulate the structural hypothesis of the mechanism by which the ligand inhibits the target.[8,9] Dihydrofolate reductase (DHFR), thymidylate synthase, and serine hydroxy methyltransferase are 3 important enzymes involved in the development of parasitic diseases, including malaria and trypanosomiasis, by acting in folic acid synthesis. Of thymidylate synthase, three inhibitors are known in humans, namely pemetrexed (PMX), raltitrexed (RTX), and nolatrexed (NTX).[10]

Flavonoids are a group of polyphenolic compounds found in many flowering plants and include quercetin and isoquercitrin. The latter flavonoid is a compound with a therapeutic profile similar to that of quercetin, but with better bioavailability. The isoquercitrin biological activities include antihypertensive, antioxidant, antidiabetic, antiproliferative, and anti-inflammatory. Several studies have examined the anticancer activity of quercetin and isoquercitrin.[11] The hydromethanolic extract of *Carica papaya* leaves has been proven to contain phytochemicals including (i) carpaine, (ii) methyl gallate, (iii) loliolide, (iv) rutin, (v) clitorin, (vi) kaempferol-3-O-neohesperidoside, (vii) isoquercetin, (viii) nicotiflorin, and (ix) isorhamnetin-3-O-β-d-glucopyranoside. The synonyms of isoquercetin are quercetin-3-O-β-D-glucopyranoside, isoquercitrin, and isotrifoliin.[12]

Drug discovery is a complex, lengthy process and requires large costs. However, the docking method shortens the time from a drug candidate to clinical trials.[13] Molecular docking is a virtual method that can be used to screen candidate compounds that have the potential to be developed into new drugs. Docking can also simulate the structural hypothesis of the mechanism by which the ligand inhibits the target.[9] The discovery of new drugs currently makes extensive use of molecular docking methods. Molecular docking was actually first developed in the 1980s, with advances in both hardware and software, easy access to proteins, and small and large molecular structures, making the development of docking methods very rapid and of interest to medical researchers and the pharmaceutical industry. Currently, docking is widely used in computer-aided drug design or CADD.[14] Hence, researchers have adopted multiple approaches to identify new antimalarial leads from medicinal plants.[15,16]

In-silico techniques such as high throughput screening, molecular docking, toxicity analysis, bioavailability estimation, and bioactivity prediction have played an important role in identifying and developing antimalarial leads.[17,18] With the aid of in-silico techniques, a library of prepared compounds may be virtually screened against dihydrofolate reductase thymidylate synthase (PfDHFR-TS) to identify a potential antimalarial lead. In-silico techniques provide valuable insights into the pharmacological profile of plant bioactive compounds.[19] Quercetin and quercitrin both have biological activities as anticancer, antioxidant, anti-inflammatory, and antifibrosis compounds,[20] while in addition quercetin may also be used as antidiabetic, anti-Alzheimer, antimicrobial, cardiovascular, and antiarthritic drug.[21] Quercetin is found mostly in the form of quercitrin glycoside, but can also be found in aglycone form and as quercetin glycoside. Research results show that quercetin/quercitrin has the ability to prevent cytokine-induced cell death, increase glucose-stimulated insulin secretion (GSIS), and inhibit the accumulation of ROS and NO. Quercetin and quercitrin can prevent β-cell death through mitochondrial pathways and NF-κB signaling.[22] Dihydrofolate reductase inhibitor (DHFR inhibitor) is a type of antifolate, and is a molecule that plays a role in inhibiting the function

of dihydrofolate reductase. Living cells that divide rapidly need folate to make the amino acid thymine, a principle that is exploited for therapeutic benefit. Bacteria also need DHFR to grow and reproduce, so inhibitors work selectively against bacterial DHFR but do not affect the host's DHFR and can be used as antibacterials.[23]

Various studies have shown that dihydrofolate reductase-thymidylate synthase (DHFR-TS) is one of the potential drug targets to be developed as a new antimalarial because DHFR-TS is a bifunctional enzyme required for folate biosynthesis in *P. falciparum*. Inhibition of DHFR has been shown to be effective against malaria. Research using the in-silico technique carried out by Zothantluanga et al.[24] showed that PfDHFR-TS is an antimalarial target of the flavonoid-glycoside components found in the *Acacia pennata* plant. The use of the in-silico technique can explain the pharmacology of the bioactive component profile of the plant.[24,25]

Flavonoid glycosides derived from plants have been proven to have various biological activities. The flavonoid quercetin has been proven to have anti-dengue activity by inhibiting the viral non-structural 2B and 3 (NS2B-NS3) protease complexes by the in-silico method.[26] The plant form of flavonoid C-glycosides has been proven to have antidiabetic activity by inhibiting protein tyrosine phosphatase 1B (PTP1B) which is a negative regulator of the insulin signaling pathway.[27] Quercitrin and myricitrin also showed in-vitro antimalarial activity against P. falciparum.[28] The present study aimed to identify flavonoids reported from *Carica papaya* as a possible antimalarial agent against PfDHFR-TS by in-silico investigations.

METHODS

Research design

The research was carried out exploratively through in silico molecular docking tests using the stages of preparation and optimization of test compounds and positive controls, target protein preparation, method validation, and docking, which was carried out using AutoDock 4.2 software equipped with AutoDock tools 1.5.6 for preparation. The study was conducted in the Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Udayana University, from July until October 2024.

Structure preparation

In the preparation of the isoquercitrin ligand molecular structure, the isoquercitrin protein structure with PDB ID number IJ31 was downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org). Parameters used in the molecular docking process with Autodock version 4.2 included an RMSD (Root Mean Square Deviation) of <2Å.

Analysis of molecular docking results

An analysis was carried out to compare the binding energy results/scores of molecular docking between the native ligand and the binding energy results of isoquercitrin. If the result or binding energy score obtained from isoquercitrin is smaller or lower than the binding energy value of the native ligand, then it can be concluded that the compound isoquercitrin can compete to bind to *Plasmodium falciparum*-dihydrofolate reductase thymidylate synthase (PfDHFR-TS) and is more potent. The best binding energy results/scores obtained from molecular docking results are displayed in table form and then the binding sites and types of molecular bonds are visualized in 3 dimensions (3D).

Statistical analysis

The data were analyzed descriptively and presented in the form of tables and graphs

Ethical clearance

No ethical clearance is required, because this research consisted of molecular docking using software.

RESULTS

One of the validated molecular drug targets in the treatment of malaria is *Plasmodium falciparum* thymidylate synthase-dihydrofolate reductase (PfDHFR-TS), which plays a role in nucleotide biosynthesis. The docking protocols have good accuracy for redocking the native ligands into *Plasmodium falciparum*- dihydrofolate reductase thymidylate synthase (PfDHFR-TS) and are declared valid based on the RMSD value. The redocking of native ligand into *Plasmodium falciparum*-dihydrofolate reductase thymidylate synthase (PfDHFR-TS) produced RMSD values of 1.58 Å.

Molecular docking results, as shown in Table 1, reveal the interactions between target proteins and ligands. This provides a quantitative prediction of binding energy and ranks compounds based on ligand-receptor binding affinities. The docking process begins with

validation of the docking method with RMSD parameters <2 Å. Validation is carried out by docking the native ligand back to the target protein. Validation results on the *Plasmodium falciparum* thymidylate synthase-dihydrofolate reductase (TS-DHFR) protein with PDB ID 1J3I show an RMSD value of 1.58 Å. A valid method is used for the docking process of the test compound. The docking results are shown by the binding free energy as listed in Table 1. The test results show that the 4 test compounds, namely quercetin, isoquercetin, carpaine, and caricaxanthin, have negative energy values, just as does the native ligand. This shows that the four compounds have good affinity for the target protein. The violaxanthin compound shows a positive energy value, which indicates that there is no affinity of the compound with the target protein.

Figure 1 shows the interactions that occur between the native ligand and 4 test compounds with the target protein. The two test compounds, namely quercetin and isoquercetin, produce hydrogen bonds with amino acid residues in the target protein, just as does the native ligand, while the compounds carpaine and caricaxanthin do not show hydrogen bonds. Apart from hydrogen bonds, the test compounds also show other types of interactions, namely hydrophobic and van der Waals interactions. From this docking test, it can be seen that only the caricaxanthin compound has better affinity for the target protein compared to the native ligand.

DISCUSSION

Our research found that flavonoid glycosides with inhibitory activity against PfDHFR-TS are quercetin, isoquercetin, carpaine, and caricaxanthin. Among these four compounds caricaxanthin has the highest affinity for PfDHFR-TS. Research by Rani et al.[29] found that *Carica papaya* contains the alkaloid compounds include caricaxanthin, violaxanthin, papain, saponins, flavonoids, and tannins. This research study has found that caricaxanthin has the lowest docked energy (-10.79 Kcal/mol) against *Plasmodium falciparum* DHFR-TS compared to another study that had found that a pyrimethamine analog screened from the literature against *Plasmodium falciparum* DHFR structure had the lowest docked energy (-11.48 kcal/mol).[30] Dasgupta et al. [31] discovered that the N-terminal tail of TS-DHFR of *P. falciparum* is a new highly selective target for the development of potential antifolates in malaria. Zothantluanga et al.[24] tested various flavonoid-glycoside components found in the *Acacia pennata* plant, including apigenin, kaempferol, quercetin, and isovitexin. Based on two docking simulations carried out, it was found that isovitexin and quercetin had high binding affinity for PfDHFR-TS but that pinocembrin was proven to have the best binding affinity, as well as molecular properties, bioavailability score, and synthetic accessibility score.

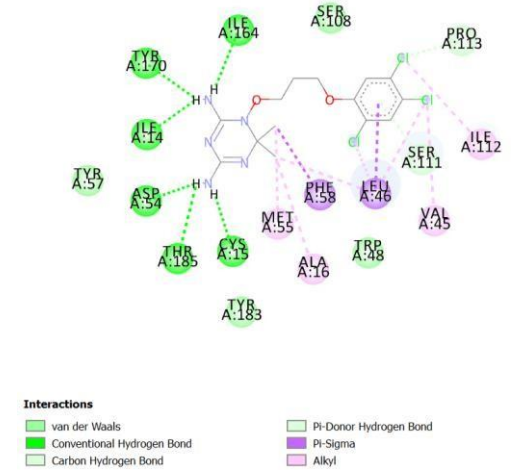
Luteolin from *Carica papaya* has better binding energies than artemether and lumefantrine against the PfDHFR-TS protein. This result suggested that luteolin from *Carica papaya* may possess antimalarial activity.[32] A study tested the comparative affinity of luteolin contained in *Citrus* for dihydroorotate dehydrogenase (PfDHODH), dihydrofolate reductase thymidylate synthase (PfDHFR-TS), and plasma membrane P-type cation translocating ATPase (PfATP4), and found that the best affinity of luteolin was for PfDHODH and PfATP4.[33]

Research conducted by Adams et al.[34] found that flavonoid compounds which have binding affinity against PfDHFR are flavodic acid and sakuranetin, a compound derived from naringenin. Their binding affinity was -8.9 and -8.6 kcal/mol respectively. The potential leads also interacted hydrophobically with the critical residue Phe58. A novel critical residue, Leu46 was identified as being essential for the catalytic activity of PfDHFR. The potential leads were also predicted to be anti-protozoal with a probability of active (Pa) values ranging from 0.319 to 0.537.

Table 1. Docking result of native ligand and test compound against protein

Protein	Ligand	Binding energy (Kcal/mol)	Ångstrom
PfDHFR-TS	Native	-8.42	1.58 Å
(PDB ID: IJ31)	Quercetin	-6.55	
	Isoquercetin	-7.94	
	Carpaine	-3.53	
	Caricaxanthin	-10.79	
	Violaxanthin	863138.51	

Note: PfDHFR-TS = *Plasmodium falciparum* dihydrofolate reductase thymidylate synthase



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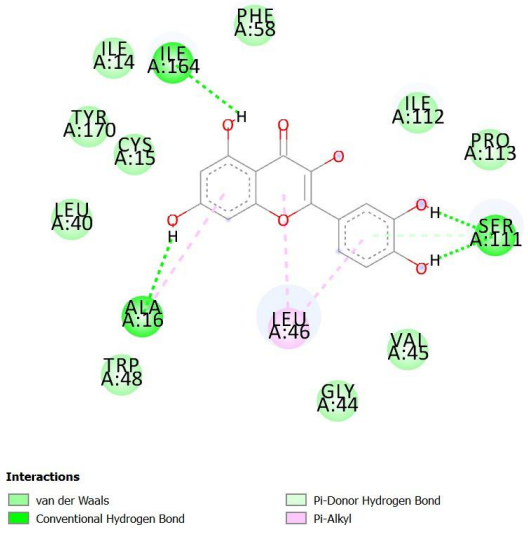
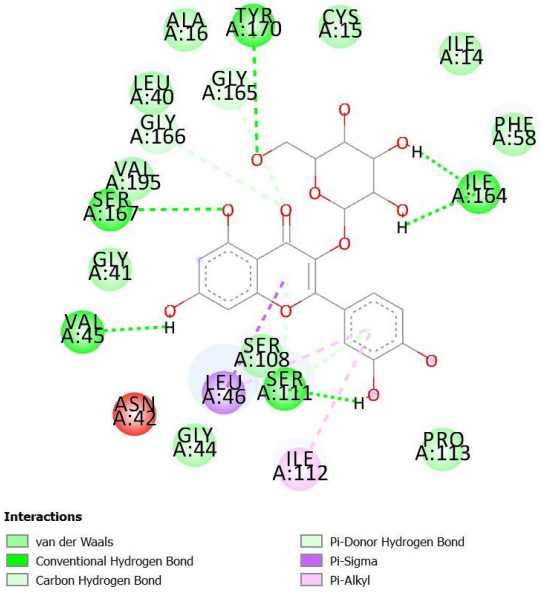
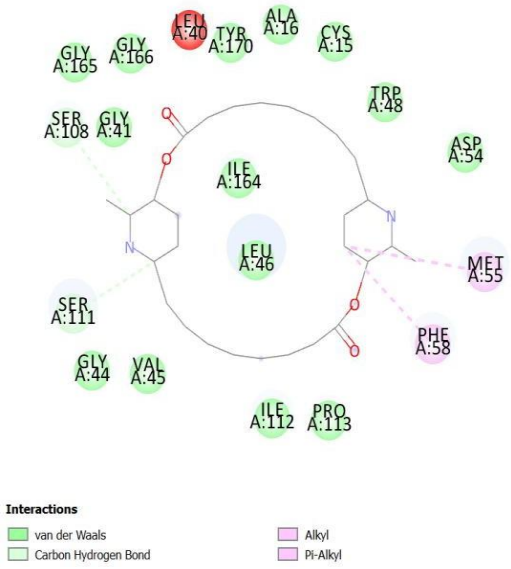


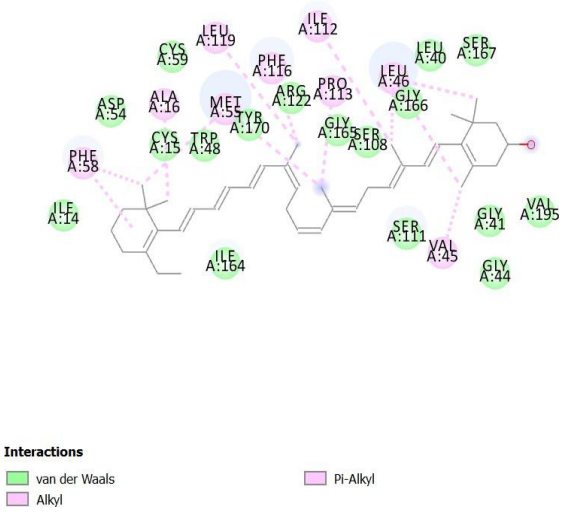
Quercetin



Isoquercetin



Carpaine



Caricaxanthin

Figure 1. Interaction between native ligand and test compound with target protein

Molecular docking research on quercetin shows that quercetin has the ability to act as an anti-tuberculosis, anti-malarial, anti-inflammatory, anti-neoplastic (breast cancer), anti-obesity, and anti-Alzheimer's drug compared to standard drugs. In addition, it has been proven that quercetin's acute toxicity is lower in mice, as well as having an acceptable level of oral acute toxicity in humans.[35]

Isoquercetin and quercetin have broad spectrum antiviral activity, namely against the influenza, Zika, Ebola, and dengue fever viruses. The safety profile of isoquercetin and quercetin is also very good and has been proven to be able to prevent infection with severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and prevent the disease from becoming severe. Various in silico studies have shown that isoquercetin and quercetin occupy the top position as inhibitors of SARS-CoV-2.[36]

Carpaine was shown to have a cardioprotective effect on embryonic cardiomyocytes of the H9c2 cell line. Carpaine's mechanism of action is by stimulating upregulation of the cell cycle marker proteins cyclin D1 and PCNA through activation of the FAK-ERK1/2 and FAK-AKT signaling pathways. In the setting of ischemia reperfusion injury (IRI), carpaine provides a significant protective role to restore the wound area subjected to hydrogen peroxide (H_2O_2) treatment. In addition, the reduction of mitochondrial membrane potential (MMP) caused by oxidative stress and excessive production of reactive oxygen species (ROS) was attenuated by carpaine treatment.[37]

Flavonoids are polyphenolic compounds that are widely distributed in plants and consist of various compounds; however, research regarding flavonoid-glycosides from *Carica papaya* targeting PfDHFR has been very limited. Toxicity tests of isoquercitrin, quercitrin, carpaine, and caricaxanthin were not conducted in this study. The safety profiles of isoquercitrin and quercitrin are generally well known. Unfortunately in vivo and in vitro research on the toxicity of carpaine and caricaxanthin has been very limited, therefore it is necessary to carry out further toxicity tests on these compounds, especially carpaine and caricaxanthin.

CONCLUSION

The study concludes that caricaxanthin of *Carica papaya* is the most promising flavonoid-glycoside that is effective against PfDHFR-TS. However, further experimental (in vitro/ in vivo) studies are required to fully understand the activity of caricaxanthin against PfDHFR-TS.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

DAASL conceptualized and designed the study, drafted the initial manuscript, obtained the funding, and reviewed and revised the manuscript; NMPS did the molecular docking, I DAIP, IWS, and PAAD collected data, drafted the initial manuscript, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

Declaration of the Use of AI in Scientific Writing

We declare that we do not use AI in our scientific writing.

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