Original Article

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In Vivo and in Silico Antimalaria Activity of *Carica Papaya* Leaves from Bali

Dewa Ayu Agus Sri Laksemi, M.D., Ph.D.[1], Putu Indah Budi Apsari, M.D.[2], I Ketut Tunas, Ph.D.[3], I Made Sudarmaja, MD, Ph.D.[1], Putu Ayu Asri Damayanti, M.D, Ph.D.[1], Ni Made Pitri Susanti, Pharm.D., Ph.D.[4]

[1]Department of Parasitology, Faculty of Medicine, Udayana University, Denpasar, Bali 80234, Indonesia.

[2]Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universitas Warmadewa, Denpasar, Bali 80235, Indonesia.

[3]Health Information Management Study Program, Faculty of Health Sciences, Bali International University, Bali 80234, Indonesia.

[4]Pharmacy Study Program, Faculty of Mathematics and Natural Sciences, Universitas Udayana, Denpasar, Bali 80239, Indonesia.

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Abstract:

Objective: to determine the antimalarial effect of *Carica papaya* leaves and to examine the affinity and interaction models of selected *Carica papaya* compounds with a Plasmodium falcipain-2, which has an important role in parasite development.

Material and Methods: The design of the study was in vivo and in silico. This in vivo study used Peter's four-day suppressive test, and it was conducted using 18 mice to evaluate the antimalarial effect of *Carica papaya* leaves. Parasitemia levels were analyzed statistically using one-way analysis of variance (ANOVA). The screening of *Carica papaya* compounds was carried out in silico using AutoDock 4.2 software to examine their antimalarial activity. The molecular structure of the isoquercetin, falcipain-2, was downloaded from the Protein Data Bank (PDB ID: 3BPF). The docking method was valid if the root mean square deviation (RMSD) was less than 2 Ångströms.

Results: The in vivo results showed that the administration of ethanol extracts of *Carica papaya* leaves at doses of 100 and 1,000 mg/kg BW inhibited the increase in parasitemia levels, which were 82.1% and 83.8% on the second day and 95.9% and 95.9% on the third day, respectively. Meanwhile, in silico results showed that quercetin, isoquercetin, carpain, and caricaxanthin exhibited lower energies than the native ligand. Conversely, violaxanthin showed a positive energy value.

Conclusion: In vivo and in silico findings suggest that *Carica papaya* leaf extract at 100 and 1,000 mg/kg BW has antimalarial activity, and carpaine demonstrates promising in silico activity against falcipain-2, warranting further validation.

Contact: Dewa Ayu Agus Sri Laksemi, M.D.
Department of Parasitology, Faculty of Medicine, Udayana University,
Denpasar, Bali 80234, Indonesia.
E-mail: srilaksemi@unud.ac.id, srilaksmidps@gmail.com

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Keywords: antimalaria, carica papaya, falcipain, molecular docking, plasmodium

Introduction

The World Health Organization (WHO) reported an estimated 249 million malaria cases across 85 endemic countries in 2022, with a corresponding death toll of approximately 608,000. This represents an increase of 5 million cases compared to the previous year. Although global malaria incidence has remained relatively stable since the onset of the COVID-19 pandemic, both case numbers and mortality rates remain higher than those reported in 2019[1].

Efforts to eliminate malaria globally have been hampered by the emergence of artemisinin resistance, which was first reported in 2008. Artemisinin-based combination therapy (ACT) remains the first-line treatment for both severe and uncomplicated malaria. ACT consists of fast-acting artemisinin and its derivatives, combined with longer-acting partner drugs, such as piperaquine, lumefantrine, amodiaquine, and chlorproguanil-dapsone[2].

Carica papaya leaf extract has been used for a long time in traditional medicine to treat fever associated with various infectious diseases, including dengue fever, malaria, and chikungunya. The development of science and technology has enabled researchers to investigate and confirm the pharmacological and toxicological properties of this plant, providing evidence for its therapeutic potential beyond traditional medicine[3,4]. In addition to its antimalarial effects, the biological activities of *Carica papaya* leaves include gastroprotective, hypolipidemic, hypoglycemic, antibacterial, and wound-healing properties. These effects are attributed to phytochemicals contained in *Carica papaya* leaves, including papain, alkaloids, flavonoids, quinones, tannins, and steroids, which contribute to the plant's medicinal activity[5].

The doses of 100 and 1000 mg kg/BW were selected based on the results of previous studies and the LD50 of *Carica papaya* water extract 2000mg/kg BW[3]. There are several approaches to developing antimalarial drugs, including conventional methods, repurposing existing drugs to target new disease-related receptors, and using genomic techniques to identify new drug targets[6]. One alternative to conventional drug development is molecular docking, a computational technique widely used to identify potential drug candidates in vitro. Molecular docking predicts the interaction and binding affinity between ligands and receptor proteins. Nutraceuticals, bioactive compounds found in food sources, have attracted attention in drug development due to their potential to interact with molecular targets[7].

Plasmodium falciparum expresses falcipain-2, which is a cysteine protease. There are 4 groups of papain-family cysteine proteases, among which falcipain-2 and falcipain-3 are known to play an important role in the parasite's life cycle. Falcipain-2 and falcipain-3 are involved in hemoglobin hydrolysis and the development of parasites. Due to their essential biological roles, falcipain-2 and falcipain-3 are considered promising targets for antimalarial drug development[8].

The hydrolysis of human hemoglobin provides amino acids for the parasite to synthesize proteins and maintain osmotic stability. Additionally, *P. falciparum* cysteine proteases are implicated in chloroquine-mediated programmed cell death. The structure of falcipain includes a conserved active site composed of cysteine (Cys), histidine (His), and asparagine (Asn) residues, which belong to the papain group. Falcipain also possesses an N-terminal that functions in protein refolding, and a C-terminal that acts as a hemoglobin-binding domain.[9] A study by Pandey and Dixit has demonstrated that *P. falciparum* cysteine protease can be inhibited by vinyl sulphones. These inhibitors have been used in preclinical research targeting *Trypanosoma cruzi*[9].

Based on the aforementioned background, we hypothesized that the ethanol extract of Carica papaya leaves exerts antimalarial effects via inhibition of falcipain-2. This study aimed to investigate the antimalarial activity of *Carica papaya* in vivo and to evaluate the molecular binding of papain as a bioactive compound of *Carica papaya* against *Plasmodium falciparum* falcipain-2 using molecular docking methods.

Material and Methods

The equipment for in vivo included syringes, intragastric tubes, microscope, slides, Giemsa staining solution, oil immersion objective (100x), and hemocytometer. The equipment for in silico included a Lenovo laptop equipped with 4 GB RAM and an Intel Core i3 processor running on the Windows 11 operating system. The molecular docking procedure used the Protein Data Bank (PDB) to obtain the Plasmodium protein structure, while the active ingredients contained in *Carica papaya* leaves were downloaded from the PubChem database in GDP format.

Design

This study employed a randomized post-test only control group design to investigate the antimalarial activity of the ethanol extracts of *Carica papaya* leaves and an exploratory experimental design using molecular docking techniques; the in silico molecular docking procedures began with the preparation and optimization of test compounds and positive controls, followed by the preparation of target proteins and the validation of the docking method using AutoDock 4.2 software equipped with AutoDock Tools 1.5.6.

Experimental animals

The in vivo stage of this study involved the use of male BALB/c mice aged 8 weeks and weighing between 25 and 30 grams. The animals underwent a one-week acclimatization period under controlled temperature conditions with a 12-hour light and dark cycle. Sample size was determined using the resource equation method as proposed by Arifin and Zahiruddin, resulting in a total sample size of 18 mice allocated into 3 experimental groups[10].

In Vivo antimalarial tests

Plasmodium berghei was used for the in vivo antimalarial tests. Peter's four-day suppressive test was carried out after parasite stocks from the continuous reinfection of donor mice were sufficient. Mice were randomly assigned to 1 control group and 2 treatment groups, consisting of 6 mice each. The suppressive test commenced 3 hours after all the mice were infected with Plasmodium. The control group received distilled water (aquadest), while treatment groups I and II received ethanol extract of *Carica papaya* leaves at doses of 100 and 1,000 mg/kg BW, respectively. Daily blood smears were prepared from each mouse from the first to the third day of treatment. Parasitemia levels and percentage of the suppression of parasite growth were calculated according to established protocols. Microscopic examination of blood cells infected with malarial parasites was performed using a light microscope at a 100x oil immersion objective, observing 5 different fields of view per slide.

Structure preparation

The molecular structure of the isoquercetin ligand was prepared, and the structure of falcipain-2, was downloaded from the PDB (http://www.rcsb.org, PDB ID: 3BPF). The molecular docking process was conducted using AutoDock 4.2, employing parameters that included a root mean square deviation (RMSD) of less than 2 Ångströms.

Analysis of molecular docking results

The docking results were analyzed by comparing the binding energy scores of the native ligand with those of falcipain-2. If the binding energy score of falcipain-2

is lower than that of the native ligand, it indicates a higher binding affinity. This suggests that falcipain-2 can compete with the native ligand, thereby exhibiting greater antimalarial potency. The best binding energy scores were subsequently tabulated, and the binding sites, along with the types of molecular interactions, were visualized in a three-dimensional (3D) format.

Statistical analysis

Statistical analysis was conducted using SPSS software (v22.0). Data distribution was first assessed using a normality test. For normally distributed data, one-way analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Ethical clearance

This study received ethical approval from the Research Ethics Committee, Faculty of Medicine, Universitas Udayana, with a certificate number of 1896/UN14.2.2.VII.14/LT/2024.

Results

Antimalarial activity of the ethanol extract of *Carica papaya* leaves based on in vivo methods

The antimalarial activity of the ethanol extract of *Carica papaya* leaves was evaluated using Peter's four-day suppressive test. The parasitemia of the control group, treatment group I, and treatment group II on the first day was 14.2%, 14.5%, and 12.9%, respectively. The parasitemia of the control group, treatment group I, and treatment group II on the second day was 22.8%, 4.1%, and 3.7%, respectively. The parasitemia of the control group, treatment group I, and treatment group II on the third day was 40.8%, 1.7%, and 1.6%, respectively. Parasitemia on the first day after infection with plasmodium for the control group and treatment groups I and II, respectively: 19±1.04, 14.47±3.64, 12.98±1.88 (F-value:0.691; n:6). Parasitemia on the second day for the control group and treatment groups I and II, respectively 22.8±1.86, 4.08±0.88, 3.68±1.97 (F-value:<0.01; n:6). Parasitemia on second day for the control group and treatment groups I and II, respectively 40.8±2.55, 1.67±0.85, 1.64±0.99(F-value:<0.01; n:6)

It was concluded that there was no difference in mean parasitemia levels among the groups on day 1 (p-value 0.691), whereas on days 2 and 3, there was a significant difference in parasitemia levels (p-value<0.001).

Furthermore, post hoc tests using Tukey's HSD confirmed that on days 2 and 3, parasitemia levels in the treatment groups were significantly different from those of the control group (p-value<0.001). However, on day 1, there were no significant differences. This indicated that on day 1, all groups, both control and treatment, were infected with Plasmodium, resulting in no significant difference in plasmodium levels.

Based on the statistical analysis, it was found that the administration of ethanol extracts of *Carica papaya* leaves at doses of 100 and 1,000 mg/kg BW resulted in 82.1% and 83.8% inhibition rates on day 2, as well as 95.9 % and 95.9% on day 3, respectively.

Antimalarial activity of *Carica papaya* leaves based on molecular docking methods

The docking process began with validation of the docking method using an RMSD threshold of less than 2 Å. This validation was performed by redocking the native ligand into the target protein. The validation results for falcipain-2 (PDB ID: 3BPF) yielded an RMSD value of 1.54 Å, indicating that the method was valid and could be used for docking the test compounds. Docking results are presented in terms of binding free energy in Table 2.

The results showed that 4 test compounds, namely quercetin, isoquercetin, carpaine, and caricaxanthin,

Table 1 Comparison of parasite growth inhibition rates among groups on day 1, day 2, and day 3

Group	Day 1		% Inhibition Day 1	Day 2		% Inhibition Day 2	Day 3		% Inhibition Day 3
	Xp	Xk		Xp	Xk		Xp	Xk	
P1 (100 mg/kg BW)	14.47	14.19	−2.0%	4.08	22.78	82.1%	1.67	40.79	95.91%
P2 (1000 mg/kg BW)	12.98	14.19	8.5%	3.68	22.78	83.8%	1.64	40.79	95.98%

Xp=Mean of parasitemia level of the treatment groups, xk=Mean of parasitemia level of the control group

exhibited lower energy than the native ligand. This suggested that the 4 compounds had a stronger affinity for the target protein. In contrast, violaxanthin exhibited a positive energy value, indicating a lack of affinity with the target protein.

Table 2 Binding free energy of the native ligand and test compounds to the target protein

Ligand	Binding Energy (kkal/mol)	RMSD (Ångströms)
Native	−3.58	1,54 Å
Quercetin	−5.36	
Isoquercetin	−5.87	
Carpaine	−7.17	
Caricaxanthin	−5.40	
Violaxanthin	+863,138.51	

Figure 1–5 illustrates the interactions between the native ligand and the 4 test compounds with the target protein. Three of the test compounds, namely quercetin, isoquercetin, and carpaine, formed hydrogen bonds with residues similar to those involved in the native ligand binding. However, caricaxanthin did not form hydrogen bonds. In addition to hydrogen bonds, the test compounds also demonstrated other types of interactions, namely hydrophobic and van der Waals interactions. Among the tested compounds, carpaine exhibited the strongest affinity to the target protein, surpassing both the native ligand and the other test compounds.

Discussion

A study using control mice treated with DHP at 222 mg/kg body weight found that on the second day after administration, parasitemia was approximately 12%, and by the fourth day it had dropped to zero[11]. Another study observed control mice infected and treated with dihydroartemisinin–piperaquine (DHP) at 187.2 mg/kg body weight. Parasitemia levels on days 1 through 4 post-treatment were 6.2%, 8%, 6.5%, and 6%, respectively[12]. Factors influencing high parasite levels in the blood parasitemia include those related to the host, the parasite, and the environment. The primary determinants are Plasmodium species, parasite density, transmission intensity, and the host's immune response[13]. In this study, the variables of Plasmodium species, parasite density, and transmission intensity were all controlled. However, a key limitation is that we did not assess any parameters indicating the host's immune response.

Based on the in vivo findings, it can be concluded that the ethanol extract of *Carica papaya* leaves at doses of 100 and 1,000 mg/kg BW exhibits antimalarial activity, with parasite growth inhibition rates exceeding 80%. *Carica papaya* leaves are known to be antimalarial and anti-inflammatory. Taychaworaditsakul et al. conducted acute and chronic toxicity tests on Sprague Dawley rats using 10% *C. papaya* leaves. The results showed that the ethanol extract was non-toxic at an acute oral dose of 5,000 mg/kg body weight[14]. The administration of antimalarial drugs

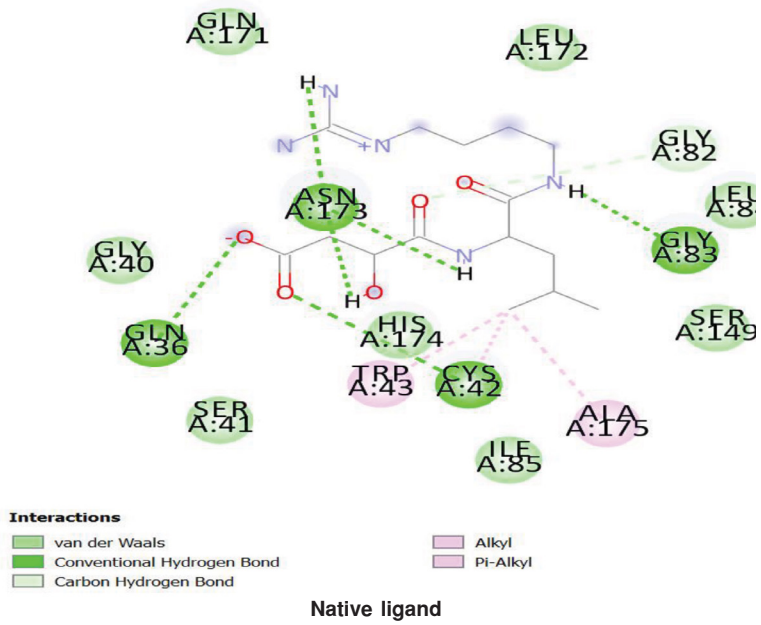

Figure 1 Interaction between the native ligand with the target protein, figure 1 shows the interaction between the native ligand and target protein resulting in Conventional Hydrogen Bonds (GLN A:36, ASN A:173, GLY A:83), Carbon Hydrogen Bonds (GLY A:82), Alkyl & Pi-Alkyl Interactions (ALA A:175, TRP A:43, CYS A:42), van der Waals Interactions (GLY A:40, LEU A:172, SER A:149)

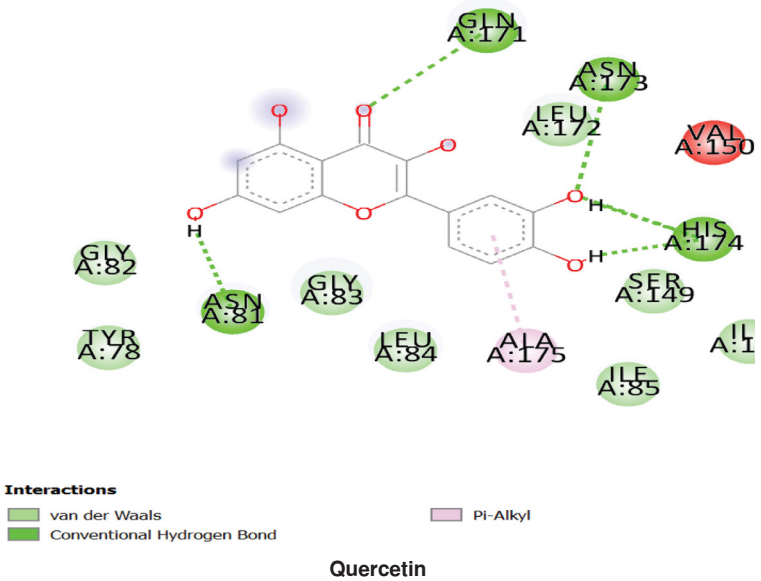

Figure 2 Interaction between quercetin with the target protein, figure 2 shows the interaction between quercetin and the target protein resulting in hydrogen bonds and Hydrophobic van der Waals bonds, and producing amino acid residues. This structure supports the potential of quercetin as an active ligand against falcipain-2, with various molecular interactions (hydrogen, π-alkyl, and van der Waals) playing an important role in the stability of the complex

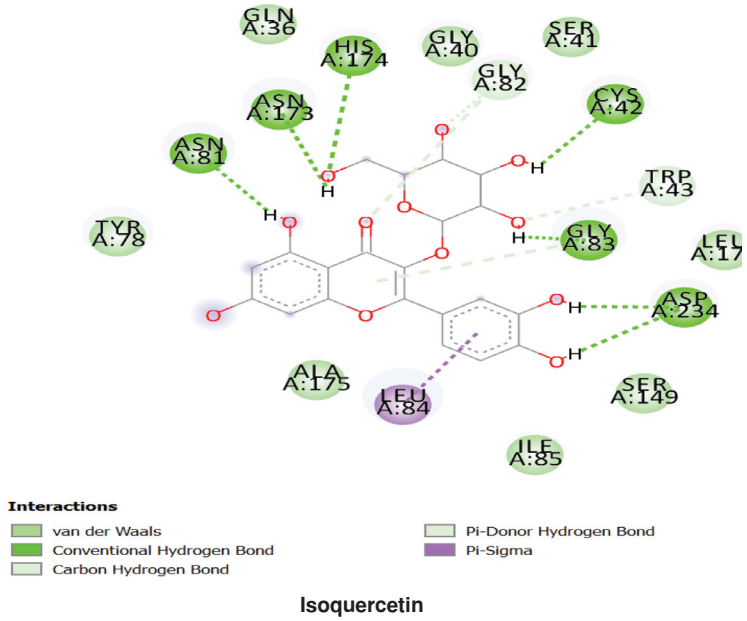

Figure 3 Interaction between Isoquercetin with the target protein, figure 3 shows the interaction between Isoquercetin and the target protein producing hydrogen bonds and Hydrophobic van der waals bonds, and producing amino acid residues

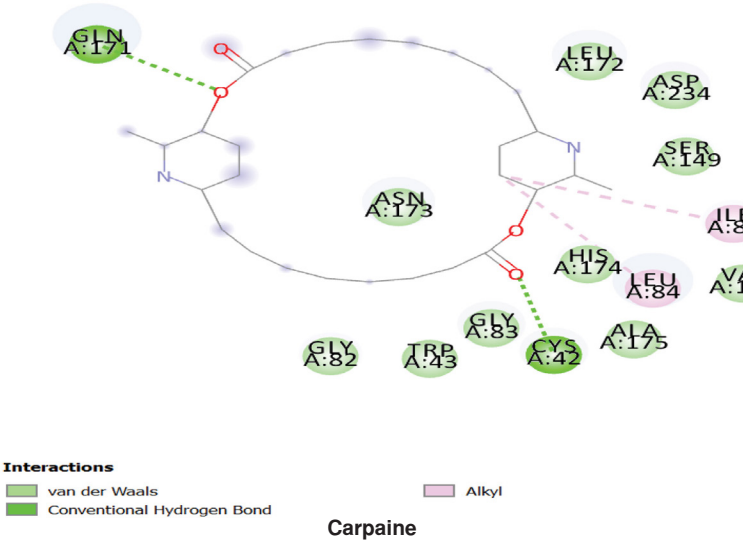

Figure 4 Interaction between Carpaine with the target protein, Figure 4 shows the interaction between the native ligand and target protein resulting in Conventional Hydrogen Bonds (GLN A:171 and CYS A:42)Alkyl & Pi–Alkyl Interactions (ILE A:85; LEU A:84; HIS A:174), van der Waals Interactions (LEU A:172; ASP A:234; SER A:149; ASN A:173; GLY A:83; TRP A:43; GLY A:82; ALA A:175; VAL A:150)

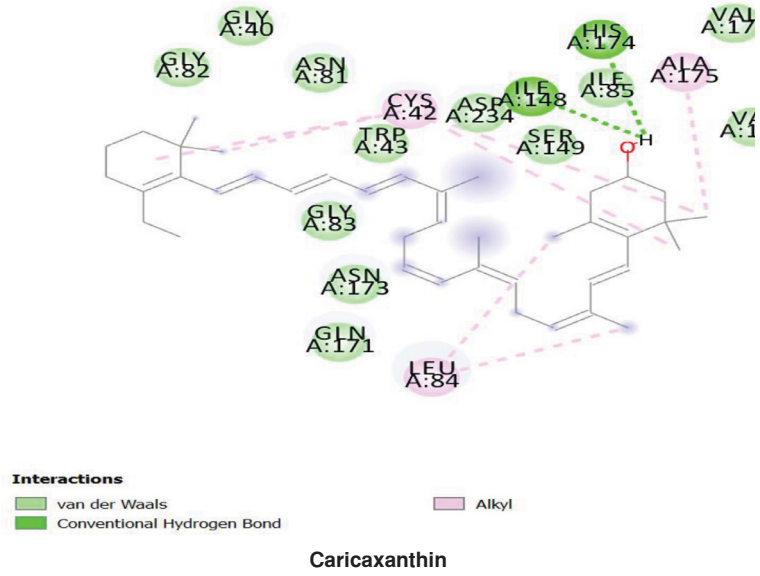

Figure 5 Interaction between Caricaxanthin with the target protein, Figure 5 shows the interaction between the native ligand and target protein resulting in Van der Waals Interactions (residues: GLY40, GLY82, ASN81, CYS42, TRP43, GLY83, ASN173, GLN171, HIS174, VAL150, VAL176); Conventional Hydrogen Bonds (ILE85 and SER149); Alkyl Interactions meaning hydrophobic interactions between nonpolar regions (LEU84, ILE85, ILE148, ALA175)

using the 4-day suppressive test method is carried out over a period of 3 days; therefore, the appropriate toxicity assessment is acute toxicity.

The findings of this study are contrary to those reported by other researchers. For example, Arifuddin's study found that the IC50 value of 70% ethanol extract of *Carica papaya* leaves as an antimalarial was 7.9 mg/ml[15]. Another study found that the most effective parasite growth inhibition was achieved with *Carica papaya* L at a dose of 9.7 mg/g[16].

A study by Atanu used a hydromethanol extract of *Carica papaya*. A chemosuppressive test was conducted to evaluate its preventive efficacy against *Plasmodium berghei* infection, using 3 doses of 100, 200, and 400 mg/kg BW. The parameters observed in the study included changes in body weight, parasitemia, packed cell volume (PCV), and mean survival time. In addition to the chemosuppressive test, the study also examined the curative effect of *Carica Papaya* using a 200 mg/kg BW dose administered for 3 days to mice infected with *Plasmodium berghei*. The parameters evaluated included parasitemia levels and biochemical indices of liver and kidney function, lipid metabolism, and oxidative stress[17].

The chemosuppressive test demonstrated that guava and *Carica papaya* leaf extracts reduced parasitemia levels, increased survival time, body weight, and PCV, while the curative test demonstrated that the hydromethanol extract of *Carica papaya* significantly suppressed parasitemia levels compared to the malaria-negative control group. Therefore, Atanu's study concluded that Alstonia boonei and *Carica papaya* extracts have antimalarial properties and are able to improve metabolic dysregulation caused by *Plasmodium berghei* infection[17].

The results of this study are slightly different from Atanu's study in terms of the effective dose. These variations in outcomes may be attributed to differences in test methods, dosages, extraction techniques, and types of extract used. Secondary metabolites, which serve as active ingredients in plants, are not only natural products but also play an important role in the plant's defense system against pathogens and environmental stressors. Yang highlighted that several factors cause the secondary metabolites in plants to vary, including genetic, ontogenic, morphogenetic, and environmental factors. The soil environment where the plant is cultivated also influences the biosynthesis and accumulation of secondary metabolites in the plant. These environmental factors include light, temperature, groundwater, soil fertility, and salinity[18].

In silico studies represent an initial step in the drug development process, allowing researchers to identify potential active compounds and assess their toxicity before proceeding to more advanced stages of research. This method also enables the determination of direct molecular mechanisms by which active compounds interact with specific molecular targets[19]. Based on the docking data, the binding energies between the test compounds, namely quercetin, isoquercetin, carpaine, and caricaxanthin, were all negative.

The affinity of the test compounds for the target protein is demonstrated by their negative binding energy values (ΔGbind) in kkal/mol[20]. The binding energies of quercetin, caricaxanthin, isoquercetin, and carpaine are −5.36, −5,40, −5.87, and −7.17 kkal/mol, respectively. The greater the negativity of the binding energy (ΔGbind), the stronger and more stable the interaction between the test compound and the target protein[21].

Based on the in silico findings, it can be concluded that quercetin, isoquercetin, and carpin have the potential to inhibit falcipain-2, which plays an important role in hemoglobin hydrolysis and the development cycle of the parasite. Nkungli's research in 2024 found that the free energy of binding the benzimidazole-thiosemicarbazone hybrid molecule to falcipain-2 was −30.32 to −17.17 kcal/mol, while the free energy of binding carpaine was −7.17 kcal/mol[22].

The most promising compound derived from *Carica papaya* demonstrated strong interaction and high affinity towards falcipain-2 (FP2). The contents of *Carica papaya* leaves include aalkaloids, carpaine, caricaxanthin, violaxanthin, papain, saponins, flavonoids, and tannin compounds[23]. A study identified several compounds, such as sitosterol, carpain, violaxanthin, pseudocarpain, Δ7-avenasterols, rutin, and cis-β-carotene, as having the highest binding affinity to the RdRp domain (5U04) and NS5-MTase domain (5WXB)[24].

A limitation of this study is that we did not consider including a comparative control or known inhibitor of falcipain-2 for further docking validation. Further research should include E-64, Suramin Quinoline–triazole hybrids, and Triazole–amino acid hybrids as known inhibitors of falcipain-2.

Conclusion

Based on the in vivo findings, it was concluded that the ethanol extract of *Carica papaya* leaves at doses of 100 and 1,000 mg/kg BW exhibited antimalarial activity, with parasite growth inhibition rates exceeding 80%. Based on the in silico findings, it was concluded that quercetin, isoquercetin, and carpin inhibited falcipain-2, which plays an important role in hemoglobin hydrolysis and the parasite development cycle. Future research should conduct toxicity tests for carpaine to help develop alternative drugs for malaria.

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Conflict of interest

No conflicts of interest.

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