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BIOMEDICAL SCIENCES

Trihydroxyflavanone isolated from *Dipteryx lacunifera* Ducke (Fabaceae) as a potential antimalarial drug: An *in silico* and *in vitro* approach

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Abstract: *Dipteryx lacunifera* is an example of the Brazilian cerrado flora which recently showed promising biological actions, such as antioxidant and antibacterial activities and a potential antimalarial effect. This study evaluated three flavonoids isolated from the fruits kernels of *D. lacunifera*, 7,3´, 4´-trihydroxyflavone (**1**), 7,3´,4´-trihydroxyflavanone (**2**), and (-)-eriodictyol (**3**). Virtual molecular modeling platforms (*in silico*), chemotherapy assays with *P. falciparum* W2 strain, and cytotoxicity assays (*in vitro*) were used to verify the antimalarial and cytotoxic potential of these molecules. Compound **2** showed the best results of inhibitory concentration against the *P. falciparum* W_2 strain (IC_{50} = 7.35 µM), with low cytotoxicity against the human fibroblast lineage (WI- 26-VA4) (LC_{50} > 100 µM) and high selectivity index (IS = 13.61). *In silico* analysis of the mechanism of action of **2** was performed by selecting the 5 crystallographic complexes with the best binding energy, namely: 1O5X, 2OK8, 2VFA, 4C81, and 4N0Z, suggesting more than one mode of action. The compounds met the ADMET criteria, indicating good bioavailability results. These results may lead this molecule to be submitted to further studies.

Key words: malaria, chemotherapy, docking, *Dipteryx lacunifera*, flavonoids.

INTRODUCTION

Malaria is an infectious disease caused by protozoa of the *Plasmodium* genus and transmitted by the bite of a female mosquito of the *Anopheles* genus infected with the parasite (WHO 2021a). It is still considered a global public health problem, as it causes numerous cases of infection and deaths (Cardoso et al. 2019). About 241 million cases of malaria were reported in 2020, and deaths reached 627 thousand worldwide (WHO 2021b).

Malaria control is a major challenge due to the presence of resistance to antimalarial drugs (Kojom Foko et al. 2019). Since 1960, the emergence of resistant *Plasmodium falciparum* strains to the main drugs used to treat humans is the biggest obstacle faced, and this biological event has been confirmed in several endemic areas around the world (White 2004, Dondorp et al. 2009, Sá 2011, Roux & Biot 2012). Therefore, the search for new drugs for treating malaria has been extensively studied. Natural products have played an important role in the discovery of molecules with chemotherapeutic activity (Harvey et al. 2015).

The use of natural products for developing therapies to treat diseases has been carried out for several years (Habibi et al. 2022). Great

examples are the artemisinin and quinine antimalarial drugs developed from natural products and widely used in antimalarial therapy (Kingston & Cassera 2022). Brazil has vast biodiversity in which the *Dipteryx* genus is an interesting target for research, as studies have shown the presence of secondary metabolites such as terpenoids, chalcones, aurones, isoflavonoids, fatty acids, coumarins and furanocassan diterpenoids in this genus (Vieira Júnior et al. 2008, Almeida et al. 2019, Mendes & Silveira 1994).

The *Dipteryx lacunifera* Ducke species belongs to the Fabaceae family, and its fruit is popularly known as chestnut-do-gurguéia, chestnut-de-burro, garampara or fava-de-morcego; however, it is still poorly studied (Vieira Júnior et al. 2007, Ribeiro et al. 2012). Compounds isolated from the fruit kernels of *D. lacunifera* have already demonstrated the presence of antioxidant, antibacterial, and more recently, antimalarial activity (Vieira Júnior et al. 2007, Ayres et al. 2008, Alexandre et al. 2020).

The identification of new compounds derived from *D. lacunifera* Ducke is necessary to better understand the profile and potential of compounds from *D. lacunifera* as antimalarial agents. *In silico* studies, employed as the virtual screening method in this research, represent a promising alternative in the drug development process. They predict molecules that are more likely to exhibit affinity for the target of interest (Cortopassi et al. 2018). It compares the binding energy generated with the binding energy of the crystallographic ligand, and its affinity has already been validated (Forli et al. 2016). These already described targets are deposited in databases such as the Brazilian Malaria Molecular Targets (BraMMT). BraMMT is a database of molecular targets created and validated by a research group that contains 35 pharmacological targets for *P. falciparum* (Table SI - Supplementary Material) (Nunes et al. 2019).

Thus, the present study aimed to evaluate the antimalarial activity of three flavonoids isolated from the fruit kernels of *D. lacunifera*, *in vitro* and *in silico*, aiming to obtain results which can be used to develop new drugs in the future.

MATERIALS AND METHODS

Compounds: isolation and characterization

First, *D. lacunifera* fruits (1.5 kg) collected in August 2003 in the city of Bom Jesus, Piauí, Brazil, as identified by Dr. Haroldo Cavalcante Lima, Instituto de Pesquisa Jardim Botânico do Rio de Janeiro, Brazil, and a sample was deposited at the Herbarium Graziela Barroso, Universidade Federal do Piauí, Brazil under reference TEPB 18246, SISGEN registration number AB 29255.

The compounds from the fruit kernels of *D. lacunifera* were provided by the Laboratory of Natural Products of the Federal University of Piauí, under the coordination of Prof. Dr. Gerardo Magela Vieira Júnior. The isolation of compounds **1** and **2** was performed according to the methodology described in the article by Alexandre et al. (2020). The methodology used for isolation of compound **3** is described in the article by Vieira Júnior et al. (2008). Chromatograms and peak concentration are in the Supplementary Material (Figures S1-S4).

The structure of the compounds were characterized by mass spectroscopy (MS) and Nuclear Magnetic Resonance (NMR) (Table SII) as described in references Alexandre et. al (2020) for **1** and **2**, and Vieira Junior et. al. (2008), for **3**.

Compound solubilization

Next, dimethylsulfoxide (DMSO) solvent was used to prepare the stock solution, resulting in an initial concentration of 10,000 µg/mL. This solution was kept in a refrigerator at

approximately 4° C. Dilutions were performed on the day of the tests using RPMI 1640 medium, resulting in the following concentrations from the stock solution: 1000, 100, 10, 1 and 0.1 µM. Serial dilutions were subsequently performed from these concentrations, where the final concentration range of the compounds in the cell plate were 100 - 0.01 µM. The final volume of DMSO concentration was 0.01%.

Biological assays

In vitro activity of the compounds

Chloroquine-resistant (W_2) strains of *P. falciparum* were cultured in human red blood cells in vitro under conditions established by Trager and Jensen (Trager & Jensen 1976). They were maintained in petri dishes at 5% hematocrit using complete culture medium (RPMI 1640 supplemented with 25 mM Hepes, 21 mM sodium bicarbonate, 300 µM hypoxanthine, 11 mM glucose, 40 µg/mL gentamicin and 10% (v/v) inactivated human plasma). The petri dishes containing the parasites were placed at 37ºC in desiccators, in which the appropriate concentration of gases was obtained by burning a candle, with daily changes of the medium.

Sorbitol-synchronized cultures of *P. falciparum* (Lambros & Vanderberg 1979) showing 0.5% ring-stage parasitaemia and 2% hematocrit were distributed in a 96-well plate (180 µL per well). The tested compounds were added (20 µL) to the test plate, in triplicate, and in different serial concentrations (50 to 0.78 µg/mL). The wells with the control contained infected red cells without the addition of test compounds (negative control). The standard antimalarial (chloroquine) was tested in parallel in all of the performed experiments in serial dilutions (500 to 7.8 µg/mL) (positive control). Next, 180 µL of non-parasitized erythrocytes were added into six wells to exclude their autofluorescence.

The test plates were incubated at 37ºC for 48h. After incubation, the supernatant was removed and 150 µL of 1X buffered saline (PBS) was added to each well. The plates were centrifuged at 700g for 5 minutes and the supernatant was again removed and 120 µL of lysis buffer with SYBR safe (20 mM TRIS-base, 5 mM EDTA, 0.008% (w/v) Saponin, 0.08% (v /v) Triton X-100, 0.2 µL/mL SYBR safe) were added.

After erythrocyte lysis, the wells were homogenized and 100 µL of the contents of each well were added to a new plate containing 100 µL of PBS. The fluorescence reading was performed after incubation for 30 minutes in the dark in a fluorimeter with excitation of 484 nm and emission of 535 nm.

Wells containing infected erythrocytes emit greater fluorescence than normal erythrocytes, and the response of the test compounds is inversely proportional to the emission of fluorescence compared to wells without the addition of compounds.

The results were expressed according to the drug concentration that reduced parasite viability by 50% (IC_{50}) in a nonlinear regression applied to fit dose-response curve.

In vitro cytotoxicity

In vitro cytotoxicity of each compound was assessed against WI-26VA4 (ATCC CCL-95.1, USA) human pulmonary fibroblast cells by MTT assay (Costa Júnior et al. 2020). The cells were cultured in RPMI-1640 (Sigma-Aldrich®, St. Louis, Missouri, USA) medium supplemented with 10% heat-inactivated fetal bovine serum in a 96-well plate (Denizot & Lang 1986). Compounds 1, 2 and 3 were diluted in different concentrations ranging from 0.2-200 µM and incubated with the cells for 24 hours in a 5% CO_2 atmosphere at 37ºC.

The optical density was determined at 540 nm to measure the signal and background, respectively (Spectra Max340PC384, Molecular

Devices, Sunnyvale, California, USA). The minimum lethal dose for 50% of the cells (LD_{50}) was determined as previously described (Valsalam et al. 2019).

Selectivity index

A selectivity index (SI) corresponding to the ratio between the cytotoxic and antiplasmodial activities of each compound tested was calculated. The values greater than 10 were considered indicative of lack of cytoxicity, whereas the substances with values below 10 were considered toxic (Espindola et al. 2022). The SI index was calculated as follows:

$$SI = \frac{IC50\ cells}{IC50\ P.\ falciparum}$$

Data analysis

IC_{50} values for *P. falciparum* were determined by making dose-response curves, being obtained by plotting data on the percent reduction in parasitemia versus the log of compound concentration. The concentration that inhibited 50% of cell growth (LD_{50}) in the presence of the test compounds was determined in comparison with cells grown without the presence of the compounds (considered 100% viability), with the preparation of dose-response curves. The regression coefficient of the curves was calculated by the method of least squares using the OriginPro 8.0 software program (OriginLab Corporation, Northampton, MA, USA).

In silico computer modeling tests

Inverse virtual screening

All compounds (ligands) were designed using the MarvinSketch software program. Then, they were refined with the help of the MOPAC software (Stewart 1990), using the semi-empirical method PM7 (Dutra et al. 2013). The Brazilian Malaria Molecular Targets (BraMMT) was used to perform the molecular docking (Trott & Olson 2009, Nunes et al. 2019), which is a platform of molecular targets built and validated by the Molecular Modeling Laboratory research group of the Federal University of São João Del-Rei, Centro Oeste Campus, Divinópolis, MG, Brazil.

Inverse virtual screening (Guido et al. 2008, Xu et al. 2018) of compounds **1**, **2** and **3** was performed against the 35 molecular targets of the BraMMT platform in order to determine the ideal target to be inhibited by each compound (Table SI).

The docking software used was AutoDock Vina, coupled to the Octopus platform (Maia et al. 2017), using its standard scoring function, which combines empirical and force field-based functions. For the docking box parameters, 20 Å was used for the X, Y and Z axes and conformational analysis was performed based on binding energy, in which the lowest energy conformation was used to generate the pharmacophoric maps.

Evaluation of physicochemical properties

The physicochemical properties were analyzed using the SwissADME webserver. SwissADME analyzes data that can be used to calculate a wide variety of properties: molecular mass, partition coefficient (cLogP), number of hydrogen donor groups and number of hydrogen acceptor groups. In addition to evaluating the toxicological characteristics of the ligand, the program also analyzes the mutagenicity, tumogenicity and irritability (Sander et al. 2015). Furthermore, it is possible to analyze the profiles of absorption, distribution, metabolism, excretion and toxicity of the tested compounds (Daina et al. 2017).

RESULTS

Biological assays

In vitro activity of the compounds and in vitro cytotoxicity

Compounds **1**, **2** and **3** (Figure 1) were tested for antiplasmodial activity against chloroquine-resistant *P. falciparum* W_2 and the IC_{50} value ranged from 7.35 µM to 29.09 µM (Table I). It can be seen that compound **2** presented the best result against the W2 strains of *P. falciparum* (IC_{50} = 7.35 µM) among the isolated compounds. The compound **2** had the IC_{50} values close to chloroquine, the antimalarial used as a positive control.

To evaluate the cytotoxic activity of the compounds **1**, **2** and **3**, the MTT assay was conducted in human pulmonary fibroblast cells (WI-26VA4). It was observed that none of the compounds showed cytotoxic activity when compared to the standard antimalarial chloroquine (LC_{50} >100) (Table I). Compounds **1**, **2** and **3** showed selectivity index values of 3.44, 13.61 and 5.01, respectively (Table I). Although compounds **1**, **2** and **3** have shown potentially active, only the compound **2** showed high selectivity for the parasites when analyzed by MTT assay (SI>10).

In silico computer modeling tests

Evaluation of physicochemical properties

Pharmacokinetic behavior of a compound can determine the success or failure of its biological activity (Sander et al. 2015). New potential antimalarial candidates must present good oral bioavailability and good membrane permeability as properties that can lead the *in vivo* experiments to reach success (Daina et

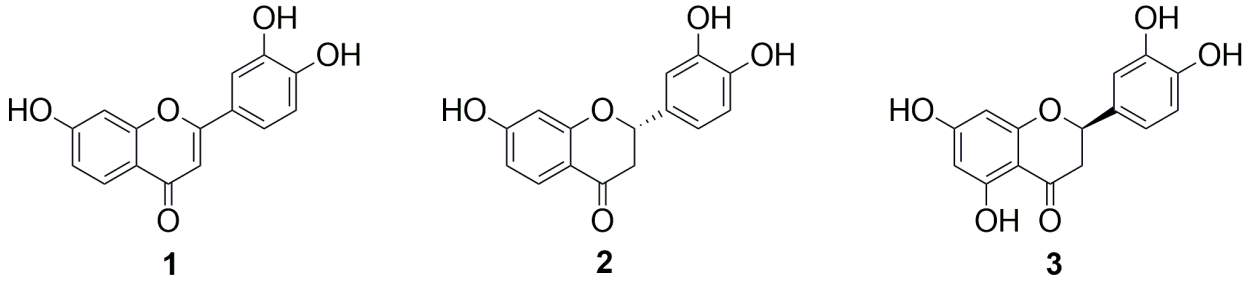

Figure 1. Compound molecular structures extracted from *Dipteryx lacunifera* Ducke. 7,3´,4´-trihydroxyflavone (1), 7,3´,4´-trihydroxyflavanone (2), and (-)-eriodictyol (3).

Table I. IC_{50} values obtained in *in vitro* tests against *Plasmodium falciparum* W2 strains; LC_{50} values in the WI-26VA4 cell line and selectivity index (SI) of *Dipteryx lacunifera* Ducke compounds.

Compound	W_2 IC_{50} (µM)*	WI-26-VA4 LC_{50} (µM)*	SI*
1	29.09 ± 0.015	>100	3.44
2	7.35 ± 0.013	>100	13.61
3	19.98 ± 0.024	>100	5.01
Chloroquine	0.92 ± 0.014	>100	108.70

***IC_{50} and LC_{50} values presented as means ± standard deviation of the mean; W_2: chloroquine-resistant *Plasmodium falciparum* strains; WI-26A4 - lung fibroblast cell line; SI: Selectivity Index.**

al. 2017). All calculated properties are shown in Table II.

Based on the physicochemical properties of compounds 1, 2, and 3, it was observed that all compounds exhibited a molecular mass below 500 g/mol (270.24, 272.25, and 288.25 g/mol, respectively), cLogP less than 5 (2.52, 2.05, and 1.56, respectively), number of hydrogen bond donors less than 5 (3, 3, and 4 groups, respectively), number of hydrogen bond acceptors less than 10 (5, 5, and 6 groups, respectively), wLogP less than 5.8 (2.58, 2.19, 1.89, respectively), number of rotatable bonds less than 15 (1, 1, 1), and polar surface area between 131 to 150 Å2 (90.90 Å2, 86.99 Å2, 107.22 Å2, respectively).

When analyzing the toxicological characteristics of the substances using the SwissADME program, factors such as mutagenicity, tumorigenicity, or irritability were not evidenced, as all substances exhibited cLogP values less than 3 (2.04, 1.64, and 1.45, respectively).

Therefore, it can be stated that all tested compounds exhibit a good profile for oral bioavailability, according to Lipinski, Gleeson, Hughes, Veber, Egan, Muegge and Ghose. The compounds were evaluated along with the standard antimalarial chloroquine.

Inverse Virtual screening

Virtual screening was performed against 35 molecular targets obtained from BRAMMT using the OCTOPUS software. According to Table III, the tested compounds showed interaction with 17 out of the 35 targets, exhibiting binding energy higher than the crystallographic data.

A possible mechanism of action for these compounds was explored using *in silico* approaches; thus, 5 targets which showed the greatest difference between the binding energy of compound **2**, which was the most active compound *in vitro*, and the binding energy of the crystallographic ligand (Table III). The five crystallographic structures selected were: 1O5X, 2OK8, 2VFA, 4C81 and 4N0Z (Figure 2).

DISCUSSION

The parasite resistance of available drugs limits the therapeutic arsenal for treatment, making the need to develop new effective antimalarial compounds essential (López-López et al. 2020).

The compounds tested in this work are one flavone (**1**) and two flavanones (**2** and **3**) (Figure 1), a subclass of flavonoids, secondary metabolites synthesized by plants that belong to the group of phenolic compounds (Nijveldt et al. 2001, Treutter 2005). Several studies have already demonstrated the antimalarial activity of flavonoids. Since the identification of chalcone licochalcone A as a main structure in the development of antimalarial drugs (Ziegler et al. 2004), several natural, semi-synthetic and synthetic flavones have been discovered and evaluated against *Plasmodium* species in *in vitro* and *in vivo* assays.

Substances with a high selectivity index (SI) are the most desired in developing new antimalarial agents. This indicates a more promising compound due to its selectivity against the malaria parasite (Bézivin et al. 2003). A drug with antimalarial potential must have an SI equal to or greater than 10 (SI ≥ 10) (Bézivin et al. 2003). Compounds **1**, **2** and **3** showed selectivity index values of 3.44, 13.61 and 5.01, respectively (Table I). Thus, in following the established criteria (Bézivin et al. 2003), only compound **2** proved to have low cytotoxicity, as it was the only one to present a value greater than 10 (IS = 13.61).

Table II. Physicochemical properties of the tested compounds and chloroquine obtained by the SwissADMET program.

PHYSICOCHEMICAL PROPERTIES	1	2	3	Chloroquine
Chemical Formula	$C_{15}H_{10}O_5$	$C_{15}H_{12}O_5$	$C_{15}H_{12}O_6$	$C_{18}H_{26}ClN_3$
Molecular Mass	270.24 g/mol	272.25 g/mol	288.25 g/mol	319.87 g/mol
Nº, heavy atoms	20	20	21	22
Nº, aromatic heavy atoms	16	12	12	10
Fractions Csp3	0.00	0.13	0.13	0.50
Nº, rotatable bonds	1	1	1	8
Nº, Hydrogen Acceptors **<10**	5	5	6	2
Nº, Hydrogen Donors **<5**	3	3	4	1
Molar Refractivity	73.99	71.57	73.59	97.41
Polar Surface Area	90.90 Å²	86.99 Å²	107.22 Å²	28.16 Å²
LIPOPHILICITY				
Log P_{olw} (ILOGP)	1.70	1.3	1.62	3.95
Log P_{olw} (XLOGP3)	2.91	1.94	2.02	4.63
Log P_{olw} (WLOGP)	2.58	2.19	1.89	4.62
Log P_{olw} (MLOGP)	0.52	0.71	0.16	3.20
Log P_{olw} (SILICOS-IT) **<5**	2.52	2.05	1.56	4.32
Log *P* olw	2.04	1.64	1.45	4.15
WATER SOLUBILITY				
Log S (ESOL)	-3.87	-3.13	-3.26	-4.55
Solubility	3.61 e-mg/ml; 1.33 e-04 mol/l	2.03 e-mg/ml; 7.44 e-04 mol/l	1.60 e-mg/ml; 5.54 e-04 mol/l	9.05e-03 mg/ml; 2.83e-05 mol/l
Class	Soluble	Soluble	Soluble	Soluble Moderately
Log S (Ali)	-4.48	-3.39	-3.90	-4.95
Solubility	8.95e-03 mg/ml ; 3.31e-05 mol/l	1.11e-01 mg/ml ; 4.06e-04 mol/l	3.64e-01 mg/ml ; 1.26e-04 mol/l	3.61e-03 mg/ml ; 1.13e-05 mol/l
Class	Moderate Solubility	Soluble	Soluble	Moderate Solubility
Log S (SILICOS-IT)	-4.40	-3.42	-2.84	-6.92
Solubility	1.07e-02 mg/ml; 3.94e-05 mol/l	1.04e-01 mg/ml; 3.82e-04 mol/l	4.19e-01 mg/ml; 1.45e-03 mol/l	3.86e-05 mg/ml; 1.21e-07 mol/l
Class	Moderate Solubility	Soluble	Soluble	Poorly Soluble
PHARMACOKINETICS				
Gastrointestinal Absorption	High	High	High	High

Table II. Continuation.

BBB *permeation*	No	No	No	Yes
P-gp substrate	No	Yes	Yes	No
CYP1A2 inhibitor	Yes	No	No	Yes
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	No
CYP2D6 inhibitor	Yes	No	No	Yes
CYP3A4 inhibitor	Yes	No	Yes	Yes
Log Kp (*skin permeation*)	-5.88 cm/s	-6.58 cm/s	-6.62 cm/s	-4.96 cm/s
DRUGLIKENESS				
Lipinski	Yes; 0 Violations	Yes; 0 Violations	Yes; 0 Violations	Yes; 0 Violations
Ghose	Yes	Yes	Yes	Yes
Veber	Yes	Yes	Yes	Yes
Egan	Yes	Yes	Yes	Yes
Muegge	Yes	Yes	Yes	Yes
Bioavailability *Score*	0.55	0.55	0.55	0.55
MEDICINAL CHEMISTRY				
PAINS	1 alert, catechol_A	1 alert, catechol_A	1 alert, catechol_A	0 alert
Brenk	1 alert, catechol_A	1 alert, catechol_A	1 alert, catechol_A	0 alert
Leandlikeness	Yes	Yes	Yes	No; 2 violations: Rotors>7, XLOGP3>3.5
Synthetic Facility	2.94	3.05	3.11	2.76

A phytochemical study of aerial exudates of *Polygonum senegalense* carried out by Midiwo et al. (2006) isolated a new flavanone (polygohomoisoflavanone) that together with other components of the air exudate (chalcones and dihydrochalcones) showed good antimalarial activity with values between 3.1 and 23.5 µM (*P. falciparum* strain D6) and 2.4 to 22.8 µM (*P. falciparum* strain W_2).

Yenesew et al. (2003) isolated several compounds from the stem bark of *Erythrina abyssinica*, such as chalcones, isoflavones, flavones, flavanones and pterocarpenes. All compounds showed moderate activity against *P. falciparum* (strain D6 - chloroquine sensitive and W_2- chloroquine resistant), with 10 flavonoid compounds active against both strains, with IC_{50} values ranging from 4.9 µM to 17.8 µM and 5.2 to 15.8 µM, respectively (Yenesew et al. 2003). Other promising *in vitro* results against *P. falciparum* D6 and W strains were also observed in another work by Yenesew et al. (2012). Crude flavonoid extracts of *Erythrina burttii* (isoflav-3-enes burttinol-A; burtinol-C., 2-arylbenzofuran

Table III. Binding energy values (kcal.mol^{-1}) of compounds extracted from the fruit kernels of *Dipteryx lacunifera* Ducke and *D*-Glucose with target molecules obtained with molecular docking assays.

TARGETS	CRYSTALLOGRAPHIC LIGANDS*	1	2	3	*D*-GLUCOSE
1LYX	-5.6	-6.3	**-6.5**	-5.9	-
1NHW	-8.3	-9.0	**-9.0**	-8.9	-
***1O5X**	-1.0	-6.1	**-6.2**	-5.7	-
1TV5	-9.3	-9.6	**-9.7**	-9.7	-
***2OK8**	-2.0	-5.6	**-5.3**	-5.3	-
2PML	-6.9	-9.0	**-8.6**	-8.5	-
***2VFA**	-1.0	-7.5	**-7.3**	-7.7	-
2YOG	-8.4	-9.5	**-9.6**	-9.6	-
3BPF	-6.3	-6.5	**-6.7**	-6.6	-
3K7Y	-7.7	-7.8	**-7.8**	-7.9	-
3PHC	-8.3	-10.3	**-9.9**	-9.7	-
***4C81**	-1.0	-6.3	**-6.5**	-6.6	-
***4N0Z**	-1.0	-6.0	**-6.1**	-6.2	-
4P7S	-6.0	-6.6	**-6.5**	-6.4	-
4QOX	-8.9	-9.2	**-9.3**	-9.5	-
PfATP6	-7.2	-7.8	**-7.5**	-7.5	-
PfHT	-5.7	-7.5	**-7.5**	-7.5	-4.3

Binding energy of the molecule in its lowest energy state (crystallographic conformation) when binding with the protein. * Five targets which showed the greatest difference between the binding energy of compound 2.

burttinol-D, as well as flavanone abyssinone V) showed some activity, with IC_{50} values lower than 10 µM.

The results published by Torres et al. (2013) from evaluating the antimalarial activity of alkaloids isolated from the plant *Aspidosperma ulei* Markgr indicated IC_{50} values close to 20 µM as moderately active. Based on these results, it can be considered that the compound 7,3´,4´-trihydroxyflavanone (**2**) was selective against *P. falciparum*, showing characteristics of a good antimalarial prototype.

The evaluation of the interaction profile of compound **2**, which presented the best result *in vitro*, allowed a potential identification of new antiplasmodial targets. We adopted a difference of -2kcal/mol in comparison with the energy binding values of the crystallographic ligands to select the best antimalarial targets (Alves et al. 2022).

The results of the *in silico* calculations indicated 5 possible targets of inhibition for this compound.

The first target analyzed was a *Plasmodium falciparum* Triosephosphate Isomerase (PfTIM) (PDB: 1O5X). Triosephosphate Isomerase (TIM) is a ubiquitous enzyme that catalyzes the isomerization of *D*-glyceraldehyde 3-phosphate

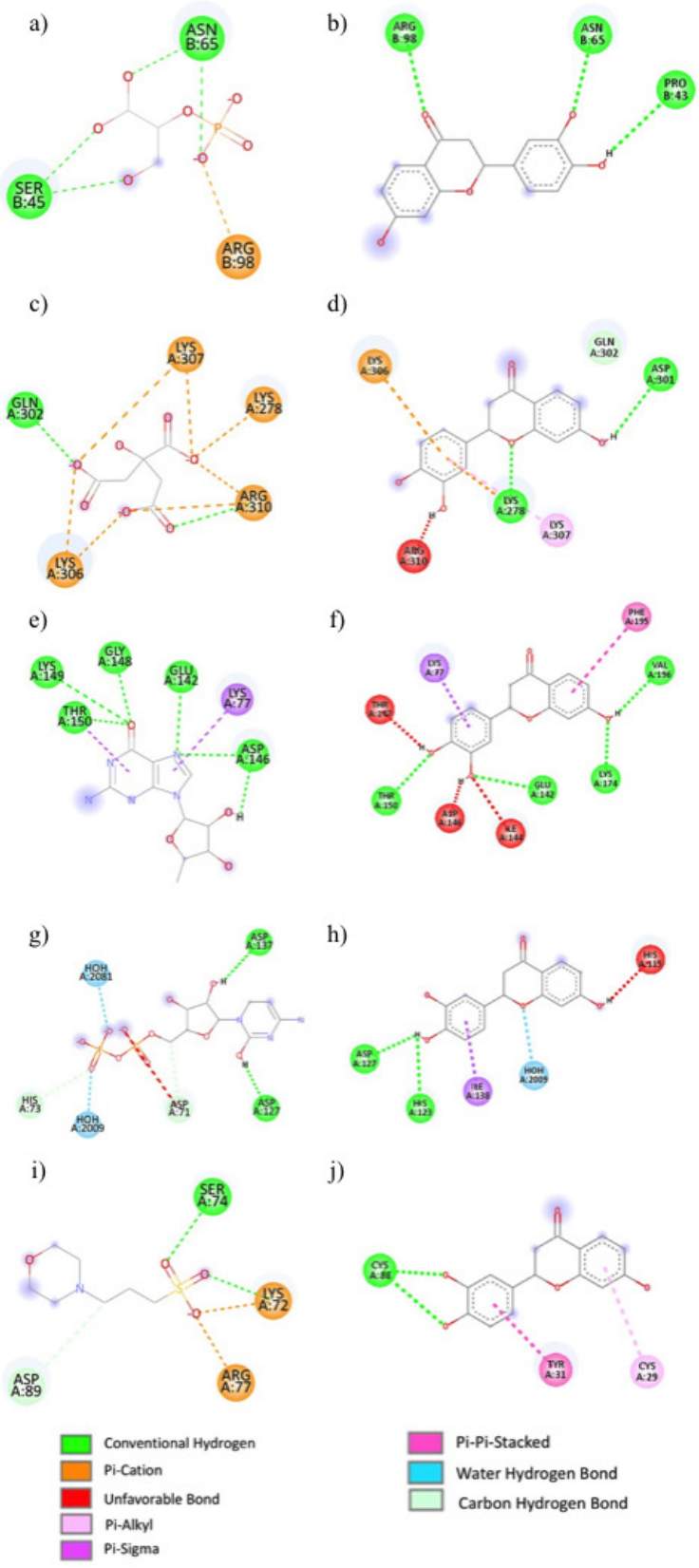

Figure 2. The pharmacophoric maps comparison between AB1 compound and crystallographic ligand. Complex 1O5X-Crystallographic Ligand (**a**); Complex 1O5X-AB1 compound (**b**); Complex 2OK8-Crystallographic Ligand (**c**); Complex 2OK8-AB1 compound (**d**); Complex 2VFA-Crystallographic Ligand (**e**); Complex 2VFA-AB1 compound (**f**); Complex 4C81-Crystallographic Ligand (**g**); Complex 4C81-AB1 compound (**h**); Complex 4N0Z-Crystallographic Ligand (**i**); Complex 4N0Z-AB1 compound (**j**).

(GAP) and dihydroxy acetone phosphate (DHAP) in the central step of the glycolytic pathway (Velanker et al. 1997). The pharmacological interest of *Pf*TIM stems from the fact that the parasite acquisition of ATP during the asexual stage in human red blood cells is exclusively performed by glycolysis (Pareek et al. 2016, Parthasarathy et al. 2002).

The 2-phosphoglycerate, crystallographic ligand of the protein 1O5X, is also a transition state analogue. It performs a non-polar bond with a Ser45 residue, in addition to 3 hydrogen bonds with Arg98, Asn65 and Ser45 residues (Figure 2a). Similarly, compound **2** forms 3 hydrogen bonds with 3 different residues, Arg98, Asn65 and Pro43 (Figure 2b). Although it does not have an ionic bond like the crystallographic ligand, compound **2** has a larger molecular size than 2-phosphoglycerate, which seems to promote a higher number of non-polar bonds at the site, giving greater affinity of the compound for *Pf*TIM.

Another target in which compound **2** has lower binding energy was *Plasmodium falciparum* ferredoxin-$NADP^+$-reductase (PfFNR), (PDB: 2OK8). The enzyme is located in the apicoplast and works by transferring a pair of electrons to the iron-sulfur protein-ferredoxin (Fd) (Milani et al. 2007). The PfFNR/Fd pair participates in several biosynthetic pathways in the apicoplast, performing electron transfer from NADPH to proteins participating in fatty acid synthesis pathways and mevalonate-independent isoprenoid pathways (Balconi et al. 2009, Lesanavicius et al. 2020).

The citrate anion, complexed to the 2OK8 protein, makes ionic interactions with the residues of Lys278, Lys306, Lys307, Arg310 and a hydrogen bond with a residue of Gln302 (Figure 2c) (Milani et al. 2007), while compound **2** forms a hydrogen bond with a residue of Lys278 and Asp301, a non-polar bond with Gln302, in addition to an ionic interaction with Lys306 and Pi-Akyl bond with Lys307 (Figure 2d). Although it presented a lower number of ionic bonds when compared to the crystallographic ligand, the greater amount of hydrogen and non-polar bonds caused a reduction in the binding energy compared to the citrate anion (Table III).

A third possible inhibition pathway suggested by the *in silico* data would be the synthesis of nucleotides, through the inhibition of hypoxanthine-guinine phosphoribosyltransferase (HGPRT) (PDB: 2VFA). This enzyme catalyzes the conversion of Guanine, Hypoxanthine and Xanthine in Guanine 5'-monophosphate (GMP), Inosine 5'-monophosphate (IMP) and Xanthosine 5'-monophosphate (XMP), respectively, through the transfer of the phosphoribosyl group from phosphoribosyl pyrophosphate (PRPP) (Gayathri et al. 2008). It is involved in the purine synthesis through the salvage pathway, mainly acquiring hypoxanthine from the host. Experiments carried out with hypoxanthine depletion led to its parasite death, suggesting the enzyme as a possible pharmacological target (Cassera et al. 2011, Gayathri et al. 2008, Keough et al. 2018).

The GMP, a product of this enzyme, was used as a crystallographic ligand for the complex, which could guide the interaction profile for the inhibitor. It performs hydrogen bonds with residues of Gly148, Lys149, Thr150, Glu142 and Asp146, in addition to a pi-sigma interaction with Lys77 (Figure 2e) (Gayathri et al. 2008). In a different way, compound **2** interacted with HGPRT through a pi-pi stacked interaction with Phe195 and a pi-sigma bond with Lys77, in addition to hydrogen bonds Glu142, Thr150, Lys174 and Val196 (Figure 2f). Although the compound showed an unfavorable bond with 3 residues (Asp146, Ile144 and Thr147), it was not enough to significantly affect the affinity, so that the other interactions

promoted an improvement in the binding energy of **2** in relation to the crystallographic ligand.

The 4C81 target was another enzyme found by the data as a possible target of compound **2** according to the *in silico* data. It represents a Zn^{2+}-dependent *Plasmodium falciparum* 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase (IspF), and it is the fifth enzyme of the 2*C*-methyl-*D*-erythritol-4-phosphate pathway (MEP). IspF participates in isoprenoid synthesis pathways located in the apicoplast of the parasite, and these isoprenoids participate in several biological functions of the parasite, from anchoring membrane proteins to enzymatic cofactors. Thus, the pharmacological interest in IspF comes from the fact that the inhibition of MEP would result in the absence of one of the types of universal isoprenoid precursors, isopentenyl pyrophosphate, resulting in protozoan death (O'Rourke et al. 2014, Rohdich et al. 2001).

The CDP, a ligand complexed to 4C81, exhibits non-polar interactions with a residue of His73 and Asp71, in addition to an unfavorable negative-negative interaction between Asp71 and the negatively charged oxygen of the phosphate group. The ligand is also stabilized by hydrogen bond with water molecules and with Asp127 and Asp137 (Figure 2g) (O'Rourke et al. 2014). In Figure 2h, the compound **2** performs one unfavorable bond with His115 residue, a hydrogen bonds with His123, Asp127 and with a water molecule, in addition to a pi-sigma bond with Ile138. Although they have more unfavorable binding than the crystallographic ligand, the presence of a greater number of interactions adjacent to the molecule, in addition to the pi-sigma bond with the benzene ring, seems to increase the affinity of the compound for IspF.

The last target evaluated was the *Plasmodium falciparum* Glutaredoxin-1 (*Pf*Grx1), (PDB: 4N0Z). Glutarredoxins are ubiquitous enzymes and use the reducing capacity of glutathione in the presence of NADPH and glutathione reductase to reduce protein-disulfides (Rahlfs et al. 2001). *Pf*Grx1 has several essential biological functions, such as protection against oxidative damage; it can supply hydrogen to ribonucleotide reductase, in addition to acting in transcriptional control (Rahlfs et al. 2001, Deponte et al. 2005). The pharmacological interest for enzymatic inhibition lies in the fact that it is exposed to high levels of Reactive Oxygen Species (ROS) during the erythrocytic phase of the parasite from both the erythrocytic environment and from the degradation of heme groups. Thus, the inhibition of proteins aimed at protecting against oxidative stress may become an important pharmacological strategy to eliminate the parasite (Deponte et al. 2005).

4N0Z is complexed with 3[n-morpholino] propane sulfonic acid, which interacts with the protein site through two ionic interactions, between the sulfonate group and the residues of Lys72 and Arg77, in addition to hydrogen bonds with Ser74 and a non-polar bond with Asp89 (Figure 2i) (Rahlfs et al. 2001). The compound **2** makes a pi-pi stacked bond with Tyr31, two hydrogen bonds with Cys88, in addition to a pi-alkyl interaction between Cys29 and the benzene ring (Figure 2j). PfGrx1 are characterized by having 2 conserved cysteine residues in the site originating from the N-terminal and C-terminal region. Both are closely linked to the protein reaction mechanism and act directly on the reduction of disulfides (Deponte et al. 2005). In addition to presenting lower binding energy than the crystallographic ligand, the interaction of compound **2** with these residues suggests directly interfering with the enzymatic activity of PfGrx1.

The oral administration route is recommended in developing drugs for tropical diseases, seeking better patient adherence to

treatment. Therefore, drugs must have good oral bioavailability, and this property can be predicted through *in silico* testing (Freire et al. 2006, Gelpi et al. 2015). Gatti et al. (2021) concluded that the *in vitro* and consequently *in silico* analyses show satisfactory results for the test compounds. Determining ADMET parameters through computational tools is a way to optimize the research of new drugs, serving to direct the following studies (Yousef et al. 2018).

All 3 compounds, along with the standard antimalarial chloroquine, were evaluated for their pharmacokinetic properties using the SwissADME program. All calculated properties are shown in Table II. Upon analyzing the physicochemical properties of the compounds, it is observed that all compounds (DFE 68-72, DFE 68-14-19, and DFE 80-14-01) presented a molecular weight below 500. Compounds with high molecular weights and an excessive number of hydrogen bond acceptor and donor groups face greater difficulty in crossing the lipid bilayer of cell membranes, as such characteristics increase the molecule's lipophilicity, hindering permeability (Lipinski et al. 2001). The analyzed compounds demonstrated a cLogP of less than 5. The partition coefficient (cLogP) is a measure of a compound's lipophilicity, which relates to the interaction of the compounds under study with the environment, serving as an important tool concerning absorption and transport. This measure is recommended for providing estimates of toxicological factors (Silva et al. 2005).

In a study conducted by Gleeson, it was demonstrated that compounds with a molecular weight below 400 g/mol and a cLogP below 4 have a more favorable ADMET profile (Gleeson 2008). According to Hughes et al. (2008), compounds with a cLogP below 3 present a low risk of being retained and stored in the lipid membrane, reducing the chances of toxicity and fewer side effects.

In addition to Lipinski's rule, other physicochemical parameters were added to better predict the oral bioavailability of a drug. For example, Ghose's rule defines that the molecular weight of a compound should be between 160 to 480 g/mol, the cLogP between -0.4 to 5.6, and the total number of atoms (acceptors and donors) should be between 20 to 70 (Ghose 1999). Veber's rule states that a compound should have a number of rotatable bonds less than or equal to 10 and a polar surface area (related to the sum of the surfaces of polar atoms) less than or equal to 140Å2 (Veber et al. 2002). Egan's filter includes that the wLogP (lipophilicity) should be less than or equal to 5.88 and the total surface area should be less than or equal to 131.6 Å2 (Egan et al. 2000). Finally, Muegge's rule asserts that the compound should have a molecular weight between 200 to 600 g/mol, a cLogP less than or equal to 5, the number of hydrogen bond acceptor atoms less than or equal to 10, the number of hydrogen bond donor atoms less than or equal to 5, the number of rotatable bonds less than or equal to 15, and a polar surface area less than or equal to 150 Å2 (Muegge 2003).

Analyzing the toxicological characteristics of the substances using the SwissADME program, factors such as mutagenicity, tumorigenicity, or irritability were not evidenced, which corroborates the literature, as all substances presented a cLogP of less than 3. Therefore, it is observed that all compounds have a good profile for oral bioavailability, i.e., sufficiently acceptable ADMET properties.

When studying a compound as a possible drug, it is necessary that the substance meets the ADMET pharmacokinetic parameters. They are important in determining the access and concentration of the compound in the

therapeutic target followed by its elimination by the body (Bojarska et al. 2020). Thus, it is observed that all compounds met the parameters of the established properties. This means they have the appropriate properties for this, such as absorption, toxicity, good solubility, low molecular weight and permeability. Therefore, these compounds may have good oral bioavailability (Tariq et al. 2016, Razzaghi-Asl et al. 2018).

It is important to note that the data collected in this study have certain limitations regarding the identification of potential targets. Furthermore, conducting large-scale molecular dynamics (MD) could be crucial in elucidating key insights, such as the dynamic behavior of molecules over time and various thermodynamic properties, including free energy landscapes, enthalpy, and entropy. MD can provide detailed atomic interactions that are essential for understanding the specificity and selectivity of molecular interactions, such as protein-ligand binding (De Vivo et al. 2016). Additionally, with the recent increase in computational capacity, machine learning techniques applied to drug development have enabled automated analysis of large and diverse datasets with high accuracy, employing computer algorithms (Carracedo-Reboredo et al. 2021). So, while the current study's data present limitations in identifying potential targets, leveraging large-scale molecular dynamics and advanced machine learning techniques holds promise in enhancing our understanding of molecular interactions and accelerating drug discovery processes

CONCLUSIONS

In performing the virtual screening of the 35 targets present in the BraMMT platform, the compounds tested showed binding affinity against 17 targets. Among them, 5 targets presented greatest difference between the binding energy of the tested compounds and the energy of the reference ligands, suggesting that these compounds interfere in pathways related to glucose metabolism, in the synthesis of isoprenoids, in the acquisition of nitrogenous bases or in the control of oxidative stress. All compounds showed good oral bioavailability in the evaluation of the physicochemical properties. When evaluating the antimalarial activity (IC_{50}) and the selectivity index, compound **2** showed better results against the *P. falciparum* W2 strain (chloroquine-resistant) and the SI value follows the criteria of Bézivin et al. (2003) (IS ≥ 10).

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SUPPLEMENTARY MATERIAL

Tables SI and SII.
Figures S1-S4.

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