Journal of Ethnopharmacology 319 (2024) 117287

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

In vitro and in silico anti-malarial activity and cytotoxicity of n-hexyl 1-O-rutinoside (a glycoside) isolated from *Annickia polycarpa* (DC.) Setten and Maas ex I.M. Turner (Annonaceae)

Emmanuel Kofi Kumatia [a,b,*], Felix Kwame Zoiku [c], Alex Asase [d], Nguyen Huu Tung [e,**]

[a] Department of Phytochemistry, Centre for Plant Medicine Research, Mampong-Akwapim, Ghana
[b] Department of Quality Management, Centre for Plant Medicine Research, Mampong-Akwapim, Ghana
[c] Depaertment of Epidemiology, Nouguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana
[d] Plant Development Department, Centre for Plant Medicine Research, Mampong-Akwapim, Ghana
[e] Faculty of Pharmacy, Phenikaa University, Hanoi 12116, Viet Nam

ARTICLE INFO

Handling Editor: V Kuete

Keywords:
Malaria
Cytotoxicity
Molecular docking
Chloroquine resistance transporter
In silico mechanisms
Annickia polycarpa

ABSTRACT

Ethnopharmacological relevance: Annickia polycarpa leaf is an effective anti-malarial agent. However, its chemical constituents have not been isolated and assayed against any pathogen.
Aim of the study: To isolate and characterize anti-malarial compound(s) from the leaf of *A. polycarpa*.
Materials and methods: Bioassay-guided fractionation was employed to isolated the compound (AL1) from the chloroform fraction (ALCF) of the basified ethanol extract of *A. polycarpa* leaf (ALE). AL1 was characterized by LC-MS, 1D and 2D NMR spectroscopic analysis. Anti-malarial activity was evaluated against drug resistance Dd2 and drug sensitive 3D7 *Plasmodium falciparum* strains using the SYBR green assay. Cytotoxicity and mechanistic studies were determined using tetrazolium-based colorimetric assay and molecular docking respectively.
Results: AL1 was characterized as n-hexyl 1-O-rutinoside. The IC_{50} values of ALE and ALCF against 3D7 and Dd2 *P. falciparum* strains ranges from 3.441 (0.3389) - 4.255 (0.2246) μg/mL. The IC_{50}s obtained for n-hexyl 1-O-rutinoside and Artesunate (standard drug) were 7.71 (0.5473) and 0.001 (0.00008) nM against the 3D7 parasite strain respectively. Also, the efficacy of n-hexyl 1-O-rutinoside increased by 24.40% against the chloroquine resistance Dd2 *P. falciparum* strain whiles that of Artesunate decreased by 98.96%. Furthermore, ALE, ALCF and n-hexyl 1-O-rutinoside were weakly cytotoxic to human RBCs with high selectivity indices. N-hexyl 1-O-rutinoside inhibits *P. falciparum* chloroquine resistance transporter (PfCRT) and dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) better than chloroquine and pyrimethamine respectively. But, produced similar inhibition of *P. falciparum* 2-trans-enoyl -ACP-reductase (PfERN) as triclosan.
Conclusion: These results show that *A. polycarpa* leaf and n-hexyl 1-O-rutinoside possessed profound anti-malarial activity and are not cytotoxic. N-hexyl 1-O-rutinoside could therefore, be developed into a new anti-malarial medicine. This is the first study to report the anti-malarial activity of n-hexyl 1-O-rutinoside and its isolation from the genus *Annickia*.

1. Introduction

Malaria is a pathogenic disease caused by *Plasmodium* parasites (Especially, *Plasmodium falciparum* and *Plasmodium vivax*) which are transmitted to humans by some species of mosquitoes. The disease is a major cause of death and sickness in several developing countries. The World Health organization (WHO) reported approximately 241 million malaria cases including 627 thousand deaths in 2020, out of which 95% of the deaths occurred in the WHO African Region (WHO, 2021). Over the past two decades, improvements in malaria control and treatment led to reduced infection and deaths. However, current data shows that the decrease in transmission of the disease had either reversed or

* Corresponding author. Centre for Plant Medicine Research, P. O. Box 73, Mampong-Akwapim. Ghana
** Corresponding author. Faculty of Pharmacy, Phenikaa University, Viet Nam.
E-mail addresses: kofi2rhyme@yahoo.com, ekkumatia@cpmr.org.gh (E.K. Kumatia), fzoiku@noguchi.ug.edu.gh (F.K. Zoiku), alexasase22@gmail.com (A. Asase), tung.nguyenhuu@phenikaa-uni.edu.vn (N.H. Tung).

https://doi.org/10.1016/j.jep.2023.117287
Received 9 August 2023; Received in revised form 27 September 2023; Accepted 4 October 2023
Available online 10 October 2023

stagnate since 2016 (WHO, 2017).

Since there is no effective vaccine against malaria, the current treatment approach depends on chemotherapy. The WHO endorses artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum* malaria (WHO, 2018). However, the discovery of ACTs resistance *P. falciparum* in certain parts of the world posed a great challenge to the efforts being made to decrease malaria infections and deaths (Blasco et al., 2017). This has necessitated the search for novel bioactive anti-malarial compounds for development into anti-malarial drugs to curtail the disease.

Annickia polycarpa is a medicinal plant used to treat malaria and other infectious diseases in Africa (Irvine, 1961). Different classes of alkaloids were isolated from the stem bark of the plant (Kumatia et al., 2016; Jossang et al., 1977). The stem bark and its compounds exhibited anti-inflammatory, analgesic, anti-bacterial, anti-fungal, anti-malarial, anti-trypanosomal and anti-diabetic activities (Atindehou et al., 2004; Ali et al., 2013; Anosa et al., 2014; Kumatia et al., 2016). The leaf of the plant was also reported to possess anti-malarial activity in vivo but was inactive against *P. falciparum* (W2mef) and *T. brucei* (GUTat 3.1) strains (Kumatia et al., 2021; Dofuor et al., 2022). However, the chemical constituents in the leaf have not been isolated and assayed for any biological activity.

Additionally, increasing drug resistance in *P. falciparum* is global health burden since it reverses the achievements of the malarial control. Hence, understanding the molecular mechanisms of drug action and resistance is very crucial to the development of new anti-malarial agents (Antony et al., 2016). The aim of this study therefore, is to isolate and characterize the anti-malarial constituent(s) in *A. polycarpa* leaf against Dd2 chloroquine resistance and 3D7 chloroquine sensitive *P. falciparum* strains. And to elucidate the mechanism of anti-malarial action of the isolated compound by determination of its interactions with chloroquine resistance transporter (PfCRT), dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) and 2-trans-enoyl -ACP-reductase (PfERN) of the *P. falciparum* parasite using in silico studies.

2. Materials and methods

2.1. Chemicals and reagents

Petroleum ether (40–60 °C), chloroform and ethyl acetate were procured from Fisher Scientific (Loughborough, U.K). Silica gel (for CC, particle size - 40–60 μm) and TLC plate (aluminum sheets coated with silica gel 60 F_{254}) were also purchased from Sorbent Technologies (Atlanta, GA. USA) and Merck Chemicals (Damstadt, Germany) respectively. Ethanol (99%) was obtained from Midland Ghana Limited, Tema. 3D7 chloroquine-sensitive and Dd2 chloroquine *P. falciparum* strains were obtained from Department of Immunology, Noguchi Memorial Institute for Medical Research, University of Ghana (Legon).

2.2. Collection, processing, extraction of plant material and isolation of compound

2.2.1. Collection and extraction of A. polycarpa leaf

A. polycarpa leaf (FORIG 0012) was collected from Bobiri Forest Reserve (Kumasi, Ghana) and extracted according to our previous method (Kumatia et al., 2021). Briefly, 200 g of the air-dried powered leaf was extracted twice in 3 L of 99% ethanol (EtOH) each for 4 days and filtered with filter paper. The extract was dried into solid at 46 °C using a rotary evaporator (Eyeler N1110, Tokyo-Japan) and coded ALE (31.56 g; yield 15.78% w/w).

2.2.2. Isolation of compound(s)

Alkaloids in the leaf was extracted by reconstitution of 20.0 g of ALE in 400 mL of 20% ethanol-water, basified with 2.0 mL of ammonium hydroxide and extracted with chloroform (400 mL x 4). The chloroform fraction (CF) was dried at 46 °C in the rotary evaporator into solid and labelled ALC (17.66 g). ALC (12.0 g) was absorbed unto silica gel (20.0 g) and dried. It was then transferred unto a 350 g silica gel-petroleum ether (PE) slurry packed in a glass column. The column was eluted with PE-ethyl acetate (EA)-EtOH solvent systems. Elution was stared with 1.5 L of 100% PE, followed by introduction of 10% EA into every 0.5 L, PE-EA solvent system in order of increasing polarity until 100% EA was attained. It was then continued with 10% EtOH addition to every 0.5 L of EA-EtOH until 100% EtOH was attained. A total of 192 fractions were collected from the column and analyzed with TLC. Fractions with similar TLC chromatograms were combined to obtain 9 major fractions (F1–F9). F7 (1.019 g) eluted with EA-EtOH (70:30%) showed well resolve TLC chromatogram. It was absorbed unto 4 g of silica gel, dried and then re-chromatographed over 30 g silica gel slurry using a similar procedure described above for ALC. However, the solvent system was modified to PE–CF–EtOH at 0.1 L each. A total of 149 sub-fractions were eluted. Compound **1** (**AL1**) was obtained from sub-fraction 135 eluted with CF-EtOH (60:40%). Two other solids were obtained but, their qualities were about 1.0 mg each.

2.3. Structure elucidation using NMR and LC-MS analysis

The mass spectrum of AL1 was obtained by LC-MS analysis according to Kumatia et al. (2021b).

NMR (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, DEPT ^{13}C 135 and HMBC) experiments were ran on Bruker AM500 FT-NMR spectrometer (BrukerSpin, Germany) at room temperature using standard pulse program and tetramethylsilane as internal standard.

2.4. Evaluation of anti-malaria activity

2.4.1. Preparation of test samples

About, 1 mg of each sample was dissolved in 1 mL of 0.5% DMSO to prepare a stock solution of 1000 μg/mL which was filtered and diluted 10-fold to 100 μg/mL. Final working solutions of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 μg/mL were then prepared by 9-fold serial dilutions.

2.4.2. Parasite culturing

Anti-malarial activity of the samples was tested against the 3D7 chloroquine sensitive or the Dd2 chloroquine resistance strain of *P. falciparum* using a modified method of Trager and Jensen (1976). Briefly, continuous *P. falciparum* asexual cultures were maintained in vitro in an atmosphere of 90% N_2, 5% CO2, and 5% O_2 at 37 °C in CM (10.44 g/L RPMI 1640, 5.94 g/L HEPES, 5 g/L AlbuMAX II, 50 mg/L hypoxanthine, 2.1 g/L sodium bicarbonate). Parasites were cultured in O^{+} RBCs and maintained in the incubator with daily media change until a parasitemia of more than 5% ring stage was obtained. The culture then synchronized with 5% sorbitol to ring stage and allowed to grow to >5% parasitemia. Parasite suspension of 2% hematocrit from uninfected blood, 1% parasitemia and 11 mL CM was mixed for the plating.

2.4.3. Evaluation of anti-malarial activity

About 100 μL of each of the nine solutions (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 μg/mL) were plated in duplicates in a 96 well coastal plate. Artesunate (15 ng/mL, standard drug) was serially diluted and plated alongside. One hundred of parasite suspension with 2% hematocrit and 1% parasitemia of the parasite strain were added to each treated well starting from the 2nd well to the 10th well. The 11th well was the negative control (parasite suspension). The procedure was repeated each sample. The plates were arranged in a modular chamber and gassed for 5 min with gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and then kept at 37 °C for 72 h. The assay stopped by addition of 100 μL lysing buffer containing SYBR Green to each well with thoroughly and gently mixing. The plates were incubated in the dark for 30 min prior to the measurement of the excitation and emission wavelengths at 470 nm and 520 nm using Fluorometer (FLUOstar OPTIMA) (Kumatia et al.,

2023).

2.4.4. Determination of cytotoxicity of the compounds

Toxicity of the compounds on RBCs was evaluated using tetrazolium-based colorimetric assay (Indrayanto et al., 2021; Kumatia et al., 2023) with slight modifications. Approximately, 100 μL of each sample (concentration = 6.25 μg/mL – 100 μg/mL) were used to prepare the assay mixtures. The optical densities were measured at 570 nm in the microplate reader. The concentrations at which 50% cytotoxic effect occurred (CC_{50} values) were determined by plotting concentration of extract on *x*-axis and percentage of cell viability on *y*-axis with dose-response curves.

$$\text{Cell viability } (\%) = \frac{\text{Ao} - \text{A1}}{\text{Ao}} \text{ x } 100$$

where A_0 = mean absorbance of wells with untreated cells (vehicle), A_1 = mean absorbance of test wells. CC_{50} was then determined with respect to the control cultures.

2.5. Selectivity index (SI)

The selectivity index (SI) was calculated as the ratio of CC_{50} to the IC_{50} values. I.e.;

$$\text{SI} = \frac{\text{CC50}}{\text{IC50}}$$

2.6. In silico studies

In silico studies were performed using cavity-detection guided blind docking approach with CB-Dock2 (Liu et al., 2022). The target receptors were obtained from the protein data bank https://www.rcsb.org (RCSB PDB, 1971) with Biova Discovery Studio 2023 (Dassault systems, 2023) and cleaned off water molecules and het atoms.

2.7. Statistical analysis

IC_{50} values were calculated from dose-response curves by non-linear regression analysis using Graph pad Prism version 7.0 Software (USA). Data was presented as mean (standard deviation).

3. Results

3.1. Characterization of the compound

3.1.1. TLC and NMR analysis

The quantities of the other two solids were so minute. Hence, they could not be analyzed. The major constituent, **AL1** became the key focus of this study. **AL1** (376.2 mg; yield = 1.20% w/w) was obtained as a creamy granule. It was washed with PE/CF 1:1 mixture and dried. R_f = 0.625 (CF/EtOH 2:1); 0.188 (EA/EtOH 9:1), see the TLC chromatogram below in Fig. 1.

The NMR spectra of **AL1** revealed a glycoside structure (Table 1). The ^{1}H NMR data showed signals of the aglycone moiety at δ 3.90 and 3.50 (each 1H, m), 1.60 (2H, m), 1.40 (2H, m), 1.30 (4H, m), and 0.90 (3H, t, *J* = 6.7 Hz) accounting for one oxymethylene (–OCH_2–), four methylene (–CH_2–), and one primary methyl group (-CH_3), together with two anomeric protons at δ 4.20 (1H, d, *J* = 7.6 Hz) and 4.90 (1H, brs) attributable to one α-L-rhamnopyranosyl and one β-D-glucopyranosyl units. In addition, the ^{13}C NMR spectrum disclosed 18 carbon resonances including six carbons for the aglycone hexyl-1-ol and 12 carbons of the two arrays of six signals supporting the α-L-rhamnopyranosyl and one *β*-D-glucopyranosyl units. Based on the above evidence and comparison with literature data (Table 1), **AL1** was unambiguously identified to be *n*-hexyl 1-O-rutinoside.

Fig. 1. TLC chromatogram of **AL1** in CF/EtOH (2:1).

Table 1
^{1}H and ^{13}C NMR experimental and literature data of n-hexyl 1-*O*-rutinoside.

Position	δ_H (ppm)		δC (ppm)	
	Literature[a]	Experimental	Literature[a]	Experimental
1	3.56 m	3.50 m	71.00	70.52
2	3.87 m	3.90 m	–	–
3	1.64 m	1.60 m	30.80	29.35
4	1.41 m	1.40 m	26.60	25.94
5	1.35 m	1.30 m	32.90	31.65
6	1.35 m	1.30 m	23.70	22.32
7	0.94 t, 6.9	0.90 t, 6.7	14.40	13.10
1	4.25 d, 7.9	4.20 d, 7.6	104.40	102.81
2	3.18 m	3.20 m	75.10	77.63
3	3.35 m	3.30 m	78.20	78.39
4	3.28 m	3.20 m	71.80	71.34
5	3.40 m	3.40 m	76.90	77.99
6	3.99 m	3.90 m	68.20	68.04
7	3.63 m	3.60 m	–	–
1	4.76 brs	4.90 brs	102.30	102.49
2	3.86 m	4.00 m	72.30	70.69
3	3.39 m	3.40 m	74.10	72.67
4	3.69 m	3.70 m	72.40	70.78
5	3.69 m	3.70 m	69.80	68.91
6	1.28 d, 6.2	1.30 d, 6.3	18.10	16.62

[a] Darwish et al. (2016).

3.2. LC-MS fingerprinting analysis of AL1

The MS spectrum obtained from the LC-MS analysis of **AL1** reveal the parent ion at *m/z* 449.4 $[M + K]^+$ in addition to product ions at *m/z* 279.2 $[M - C_6H_{12}O_5 + H]^+$ and 149.2 $[M\text{- } OH + 2H]^+$. These MS features confirmed **AL1** as n-hexyl 1-O-rutinoside (Fig. 2).

3.3. Anti-malarial activity

The anti-malarial activity of ALE, ALC and *n*-hexyl 1-*O*-rutinoside is shown below (Table 2).

The crude extract (ALE) and its fraction (ALC) both strongly inhibited both strains of the *Plasmodium* parasite with similar IC50 values. However, *n*-hexyl 1-*O*- rutinoside, inhibited the chloroquine resistance Dd2 *P. falciparum* strain with low IC_{50} (5.83 (0.1137) nM) better than the chloroquine sensitive 3D7 *P. falciparum* strain.

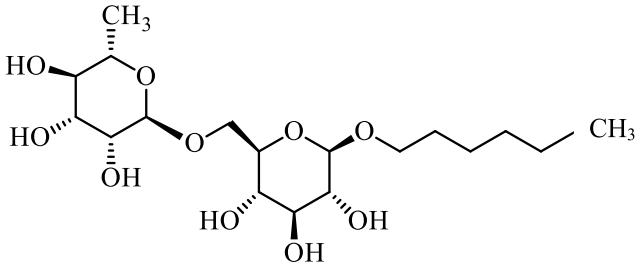

Fig. 2. Chemical structure of *n*-hexyl 1-O-rutinoside (**AL1**).

Table 2
Anti-malarial activity of ALE, ALC and *n*-hexyl 1-*O*-rutinoside (AL1) against 3D7 strain and Dd2 *P. falciparum* strains.

Compound	IC_{50} ± SEM (μg/mL) against *P. falciparum*	
	3D7 strain	Dd2 strain
ALE	3.84 (0.03832)	4.255 (0.2245)
ALC	3.441 (0.3340)	3.505 (0.2053)
AL1 (*n*-hexyl 1-*O*-rutinoside)	7.71 (0.5473) nM	5.83 (0.1137) nM
Artesunate	0.001 (0.00008) nM	0.0964 (0.0044) nM

3.4. Cytotoxicity and selectivity indices (SI) of the test samples

The results of the cytotoxicity test are shown in Table 3 below. All the samples showed weak cytotoxic effect on the human RBCs with high CC_{50}s leading to cell survivals >50%.

The CC_{50} of *n*-hexyl 1-*O*-rutinoside was higher than that of Artesunate (Table 3). However, this order was reversed for their SI because the IC_{50} of the isolate was much lower than the IC_{50} of Artesunate against both 3D7 and Dd2 *Plasmodium* strains tested (Table 2).

3.5. In silico studies

Results of the in silico studies are shown below in Table 4 and Fig. 3 respectively. N-hexyl 1-O-rutinoside produced a high binding affinity with the 3 targets receptors of the malaria parasite.

Namely; *P. falciparum* chloroquine resistance transporter (PfCRT), *P. falciparum* dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) and *P. falciparum* 2-trans-enoyl -ACP-reductase (PfERN). The binding affinities of n-hexy1-O-rutinoside to PfCRT (−8.1 kcal/mol) and PfDHFR (−8.3 kcal/mol) were higher than those of the standard anti-malarial inhibitors of these receptors such as chloroquine (−7.7 kcal/mol) and pyrimethamine (−7.5 kcal/mol) to PfCRT and PfDHFR respectively. However, the binding affinity of n-hexy1-O-rutinoside with PfERN (−8.0 kcal/mol) was slightly lower than that of the standard PfERN inhibitor, triclosan (−8.7 kcal/mol), Table 4. The various amino acids involved in the complex formation between the ligands and the receptors were also shown above (Table 4). And the best stable conformational poses of the crystal structures are shown below in Fig. 3.

Table 3
CC_{50} (μg/ml) and Selectivity indices produced by the compounds against human RBCs.

Compound	CC_{50} (μg/ml)	Selectivity Index (SI)	
	RBCS	3D7 strain	Dd2 strain
ALE	89.28	23.25	20.98
ALC	98.82	27.98	28.20
AL1 (*n*-hexyl 1-*O*-rutinoside)	243.90 nM	31.50	41.68
Artesunate	239.28 nM	>200 000	>2000

Table 4
Binding affinity and docking scores of n-hexyl 1-O-rutinoside and the corresponding standard drugs with PfCRT, PfDHFR-TS and PfERN.

Ligand	Target receptor	Highest ΔG (kcal/mol)	Amino acid residues
N-hexy1-O-rutinoside	PfCRT	−8.1	**Chain A**: Ser90 Phe91 Ser94 Glu95 His97 Asn98 Leu136 Asp137 Ser140 Val141 Ala144 Phe145 Ser220 Val224 Ser227 Gln253 Ser257 Ile260 Tyr264 Phe322 Asp326 Asn330. **Chain H**: Phe108 Tyr110 Arg111
Chloroquine	PfCRT	−7.7	**Chain A**: Ala144 Leu148 Gly153 Gln156 Ser157 Val159 Leu160 Glu198 Leu217 Ser220 Leu221 Gln352 Gly353 Leu356 Ala357 Tyr360 **Chain H**: Trp109 Arg111 Gly112
N-hexy1-O-rutinoside	PfDHFR-TS-K1 C59R + S108N) complexed with TOP, NADPH and dUMP	−8.3	**Chain A**: Ile14 Cys15 Ala16 Leu40 Gly41 Asn42 Gly44 Val45 Leu46 Pro47 Trp48 Asp54 Met55 Phe58 Thr107 Asn108 Ser111 Ile112 Phe116 Leu119 Ile164 Gly165 Gly166 Ser167 Tyr170 Asp194 Val195
Pyrimethamine	PfDHFR-TS-K1 C59R + S108N) complexed with TOP, NADPH and dUMP	−7.5	**Chain A**: Ile14 Cys15 Ala16 Cys17 Leu40 Leu46 Asp54 Met55 Phe58 Asn108 Ile112 Pro113 Phe116 Leu119 Ile164 Tyr170 Thr185
N-hexy1-O-rutinoside	PfERN	−8.0	**Chain B**: Gly104 Ile105 Gly106 Asp107 Gly110 Tyr111 Gly112 Trp113 Ser215 Leu216 Ala217 Leu265 Thr266 Tyr267 Tyr277 Lys285 Ile310 Ala312 Gly313 Pro314 Leu315 Ser317 Arg318 Ala319 Ala320 Ile323 Phe368 Ile369 Met372
Triclosan	PfERN	−8.7	**Chain B**: Ala103 Gly104 Ile105 Gly106 Asp107 Gly129 Ile130 Trp131 Val134 Phe167 Asp168 Ala169 Ser170 Leu216 Ala217 Asn218 Lys240

4. Discussion

Most alkaloids exist in plants as acid salts of tannates, citrate, oxalates etc. Hence, mixing a plant material with a basic solution converts the alkaloidal salts to their corresponding bases which are readily extracted by nonpolar organic solvents such as chloroform (https://www.uobabylon.edu.iq/eprints/publication_3_29572_6309.pdf). In this study, column chromatography of the alkaloid extract of the ethanol leaf extract of *A. polycarpa* led to isolation of a glycoside with a disaccharide glycon unit bonded to an aliphatic genin moiety as the major constituent of the leaf of *A. polycarpa*. This indicates that the harsh treatment required for isolation of alkaloids can also be used to expel other very polar phytoconstituents, such as glycosides, which are strongly bound to the plant matrix, before they can be isolated.

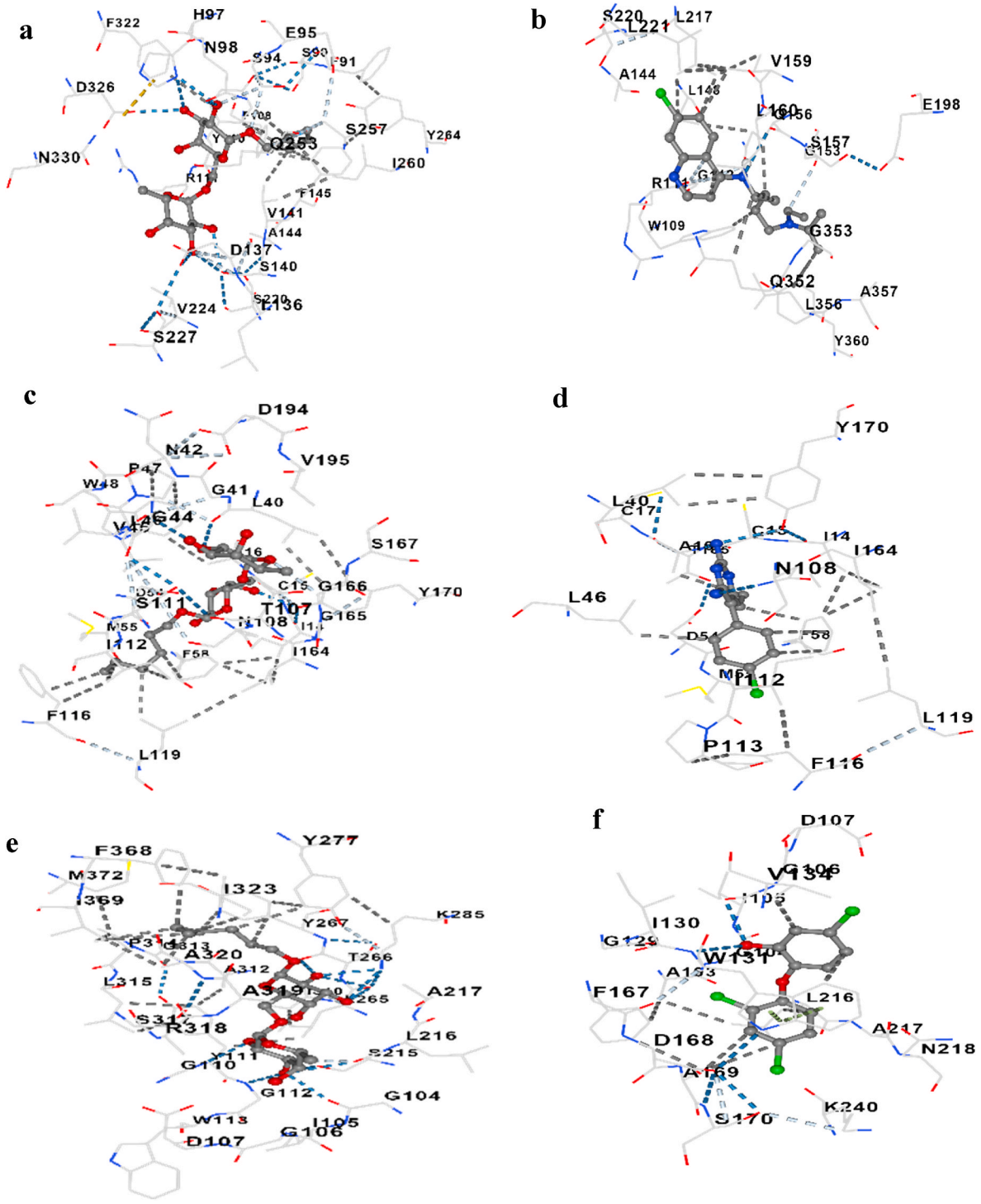

Fig. 3. The most stable conformations of the crystal structures produced by the ligands and their targets in the in-silico analysis. (**a**) NHR with PfCRT (C1, ΔG = −8.1 kcal/mol); (**b**) Chloroquine with PfCRT; (**c**) NHR with PfDHFR; (**d**) PMA with PfDHFR; (**e**) NHR with PfERN; (**f**) Triclosan with PfERN. The upper-case alphabets and numeric figures such as **A144, D326** and so on, in the figures indicate the amino acid and their codes involved in the interactions. NHR═N-hexyl1-O-rutinoside, PMA = Pyrimethamine.

Kumatia et al. (2021) showed that *A. polycarpa* leaf possessed significant anti-malarial activity and restored gross hematological and physical alterations induced by *P. berghei* in vivo and also, identified 3 alkaloids in the leaf. Nevertheless, they did not isolate and assay any individual compound from the plant's leaf. N-hexyl 1-O-rutinoside was first isolated from *Lumnitzera racemosa* and characterized as a hepatoprotective agent (Darwish et al., 2016). However, this is the first study to report the isolation of n-hexyl 1-O-rutinoside from another plant in addition to the description of its anti-malarial activity.

Anti-malarial activity of a crude extract is classified as high ($IC_{50} \leq 5$ μg/mL), promising ($IC_{50} = 5$–15 μg/mL), moderate (IC_{50} 15–50 μg/mL) or inactive ($IC_{50} > 50$ μg/mL) in in vitro assays (Jonville et al., 2008). In this study, the IC_{50}s of ALE and ALC against both chloroquine sensitive *P. falciparum* 3D7 and the chloroquine resistance *P. falciparum* Dd2 strains were >5 μg/mL (Table 2). Hence, ALE and ALC possessed high anti-malarial activity. Furthermore, there is no substantial difference in

the IC_{50} values of ALE and ALC. This shows that the active anti-malarial constituent of the crude leaf extract (ALE) is completely extractable with chloroform after basification. During in vitro anti-malarial studies, the lower the IC_{50} value of a test substance, the higher the anti-malarial activity. In this study, the IC_{50} obtained for *n*-hexyl 1-O-rutinoside (7.71 (0.5473) nM) and the standard anti-malarial drug, Artesunate (0.001 (0.00008) nM) were very low (i.e., in nanomolar or one-billionth of a molar concentration) against the 3D7 and Dd2 *P. falciparum* strains (Table 2). Hence, *n*-hexyl 1-O-rutinoside has a very high anti-malarial activity against these parasites. However, the anti-malarial activity of Artesunate was higher.

Furthermore, Katsuno et al. (2015), postulated that a validated hit drug candidate in anti-malaria drug discovery must produce an IC_{50} of less than 1.0 μM against both sensitive and drug resistance *Plasmodium* spp. and SI of greater than 10. Our results shows that n-hexyl 1-O-rutinoside produced IC_{50} of 7.71 and 5.83 nM against chloroquine sensitive 3D7 and chloroquine resistance Dd2 *P. falciparum* in addition to SI of 31.50–41.68 against the two parasite strains. The IC_{50} and SI of n-hexyl 1-O-rutinoside is far less than 1.0 μM and 10 respectively. Therefore, n-hexyl 1-O-rutinoside could be classified as a validated hit anti-malaria drug candidate.

A strain of microorganism such as a parasite, virus or bacterium is said to develop resistance to a drug when the organism is no longer susceptible to or effectively inhibited by the drug which was once effective against it or when a significantly larger amounts of the drug than the usual doses are required to achieve the desired effect (Lynch, 2022). On the other hand, a drug is said to be inactive when it is non-efficacious or does not produce a significant level of the desired biological activity when it is applied for the first time against a condition or a microorganism. Therefore, in order to determine whether a drug is resistance to a therapeutic agent, a quantitative baseline efficacy data is required for comparism with a later quantitative efficacy data. Additionally, the therapeutic agent can be tested against a genetically cloned resistant variant and non-resistance strains of the same organism and the results compared. Thus, in order to evaluate whether *P. falciparum* is resistance to the extracts (ALE and ALC) and *n*-hexyl 1-O-rutinoside, we tested them against the chloroquine sensitive and the chloroquine resistance strains of *P. falciparum*. ALE and ALC produced similar inhibition of both strains of the parasite with equivalent IC_{50} values. Surprisingly, the inhibitory effect of *n*-hexyl 1-O-rutinoside on the chloroquine resistant Dd2 strain of the parasite increased compared to that of the chloroquine sensitive strain. These findings indicates that the *P. falciparum* is not resistant to ALE, ALC or *n*-hexyl 1-O-rutinoside. However, the anti-malarial activity of Artesunate had decreased by 98.96% against the chloroquine resistance Dd2 strain of the parasite compared to the 3D7 drug sensitive strain based on the $1C_{50}$ values (Table 2).

Selectivity index (SI) is the ratio of the 50% lethal concentration (CC_{50}) of a substance to its 50% pharmacologically active concentration (IC_{50}) (Terefe et al., 2021). A high SI value indicates that a substance is potentially nontoxic and very efficacious. Indrayanto et al. (2021) proposed that, a model drug should therefore, have a relatively high CC_{50} and a very low IC_{50} in order to produce a high SI of $\geq$10 to make the sample merit further investigation. The CC_{50} values of ALE, ALC and *n*-hexyl 1-O-rutinoside were high and their IC_{50} values quite low such that the resultant SI were 2–4 times greater than 10, the proposed standard (Table 3). ALE, ALC and *n*-hexyl 1-O-rutinoside could therefore, be said to be safe and effective anti-malarial agents which requires further studies.

The food vacuole is a major digestive organelle of the *Plasmodium* parasite in which the parasite digests the host-acquired hemoglobin to obtain amino acids it required for protein and DNA synthesis (Winstanley, 2001; Ehlgen et al., 2012). Due to this vital role of the food vacuole, it has become a key target for many anti-malarial agents which disrupts the metabolic pathway of the parasite leading to its death. The food vacuole also contains two multiple-span membrane proteins namely; Multidrug Resistance Protein 1 (PfMDR1) and Chloroquine Resistance Transporter (PfCRT) which modulate resistance to anti-malarial drugs whose site of action resides in the food vacuole (Ehlgen et al., 2012). The result of the docking studies revealed that n-hexyl 1-O-rutinoside binds effectively to PfCRT with a binding affinity (−8.1 kcal/mol) which is better than that of the standard PfCRT inhibitor anti-malarial drug chloroquine (−7.7 kcal/mol) (Table 4; Fig. 3). This indicates that the anti-malarial activity of n-hexyl 1-O-rutinoside is due to is ability to inhibit PfCRT membrane protein in the food vacuole resulting in the parasite death.

Dihydrofolate reductase (DHFR) is a principal enzyme involved in the catalytic conversion of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). THF is an intermediate compound of the cofactors needed in the biogenesis of purine nucleotides and thymidine for DNA synthesis (Hussein et al., 2019; Raimondi et al., 2019). DHFR inhibitors stops proteins, RNA and DNA biosynthesis which leads to cell growth arrest. This makes the DHFR an important target for antimicrobial, anticancer and antiparasitic drug development (Wrobel et al., 2021). The docking results shows that n-hexyl 1-O-rutinoside inhibits the DHFR of *P. falciparum* better (with a 10.70% increase in the binding affinity (Table 4; Fig. 3) than pyrimethamine, a standard DHFR inhibitor in clinical use as anti-malarial drug. This suggest that n-hexyl 1-O-rutinoside also functions as DHFR inhibitor to produce anti-malarial activity.

Moreover, the *Plasmodium* parasite utilizes fatty acids for membrane biosynthesis (Qidwai and Khan, 2012). The parasite synthesizes its own fatty acids in the liver stage, but during the blood stage, it switches to the consumption of exogeneous fatty acids from its host. *P. falciparum* enoyl-acyl carrier protein reductase (PfENR), is the rate-limiting enzyme in type II fatty acid biosynthesis which converts trans-2-acyl-ACP to acyl-ACP via reduction in the parasite (Tallorin et al., 2014; Heath and Rock, 1995). Blocking PfERN in the liver stage of the parasite starve the parasite of fatty acids leading to its death. PfENR inhibitors were demonstrated to cause *P. falciparum* growth inhibition, hence making PfENR an attractive target for development of anti-malarial prophylactic agents (Schrader et al., 2013; Qidwai and Khan, 2012). N-hexyl 1-O-rutinoside also inhibited PfERN wth a binding affinity of −8.0 kcal/mol which is less than only 8.05% that of triclosan (−8.7 kcal/mol). This further suggest that n-hexyl 1-O-rutinoside is also a good PfERN inhibitor. And hence, could serve as a good prophylactic anti-malarial agent.

These results suggest that n-hexyl 1-O-rutinoside produce anti-malarial activity through multi-target inhibition of the *P. falciparum* malaria parasite. This will make it difficult for the *P. falciparum* to develop resistance against this compound as an anti-malarial agent.

5. Conclusion

This study has shown that the ethanol extract of *A. polycarpa* leaf, its basified chloroform fraction and isolated constituent, n-hexyl1-O-rutinoside possessed profound anti-malarial activity against chloroquine sensitive 3D7 and chloroquine resistance Dd2 *P. falciparum* strains with weak cytotoxicity and high SI. The mechanism of action of n-hexyl 1-O-rutinoside as anti-malarial agent is through the inhibition of PfCRT, PfDHFR and PfERN. These results indicate that n-hexyl 1-O-rutinoside is a new validated hit anti-malaria drug candidate which could be developed for the treatment of malaria.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Credit authorship contribution statement

Emmanuel Kofi Kumatia: conceived the research, isolated and purified the compounds, analyzed the NMR data, performed the molecular docking and wrote the manuscript. **Felix Kwame Zoiku:**

performed the antimalaria and cytotoxicity assays and analyzed the results. **Alex Asase:** was involved in the conception of the research idea and proofreading of the manuscript. **Nguyen Huu Tung:** elucidated the structures of the compound, All authors read and revised the manuscript and approved its publication in this journal.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors are very grateful to the technical staff of the Phytochemistry Department of the Centre for Plant Medicine Research, Mampong-Akuapim, Ghana for their support.

Abbreviations

ALE	Ethanol extract of *Annickia polycarpa* leaf
ALC	Chloroform fraction of ethanol extract of *A. polycarpa* leaf
PE	Petroleum ether
CF	Chloroform
EA	Ethyl acetate
EtOH	Ethanol
PfCRT	*P. falciparum* chloroquine resistance transporter
PfDHFR-TS	*P. falciparum* dihydrofolate reductase-thymidylate synthase
PfERN	*P. falciparum* 2-trans-enoyl -ACP-reductase
CM	Complete media

References

Ali, S., Igoli, J., Clements, C., Semaan, D., Alamzeb, M., Rashid, M.U., Shah, S.Q., Ferro, V.A., Gray, A.I., Khan, M.R., 2013. Antidiabetic and antimicrobial activities of fractions and compounds isolated from *Berberis brevissima* Jafri and *Berberis parkeriana* Schneid. Bangladesh J. Pharmacol. 8, 336–342.

Anosa, G.N., Udegbunam, R.I., Okoro, J.O., Okoroafor, O.N., 2014. In vivo anti-malarial activities of Enantia polycarpa stem bark against Plasmodium berghei in mice. J. Ethnopharmacol. 153, 531–534.

Antony, H.A., Pathak, V., Parija, S.C., Ghosh, K., Bhattacherjee, A., 2016. Transcriptomic analysis of chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*: toward malaria diagnostics and therapeutics for global health. OMICS 20 (7), 424–432.

Atindehou, K.K., Schmid, C., Brun, R., Kone, M.W., Traore, D., 2004. Antitrypanosomal and antiplasmodial activity of medicinal plants from Côte d'Ivoire. J. Ethnopharmacol. 90, 221–227.

Blasco, B., Leroy, D., Fidock, D.A., 2017. Anti-malarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. Nat. Med. 23 (8), 917–928.

Darwish, A.G.G., Samy, M.N., Sugimoto, S., Otsuka, H., Abdel-Salam, H., Matsunam, K., 2016. Effects of hepatoprotective compounds from the leaves of *Lumnitzera racemosa* on acetaminophen-induced liver damage in vitro. Chem. Pharm. Bull. 64, 360–365.

Dassault systems, BIOVIA Discovery studio visualizer: drive discovery with the leading molecular graphics environment 2023, accessed 15 May 2023, https://discover.3ds.com/discovery-studio-visualizer-download.

Dofuor, A.K., Kumatia, E.K., Chirawurah, J.D., Ayertey, F., 2022. Antiplasmodial, antitrypanosomal, and cytotoxic effects of *Anthonotha macrophylla, Annickia polycarpa, Tieghemella heckelii,* and *Antrocaryon micraster* extracts. Adv. Pharmacol. Sci. 9195753, 1–7.

Ehlgen, F., Pham, J.S., de Koning-Ward, T., Cowman, A.F., Ralph, S.A., 2012. Investigation of the *Plasmodium falciparum* food vacuole through inducible expression of the chloroquine resistance transporter (PfCRT). PLoS One 7 (6), e38781.

Heath, R.J., Rock, C.O., 1995. Enoyl-acyl carrier protein reductase (FabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*. J. Biol. Chem. 270, 26538–26542.

Hussein, E.M., Al-Rooqi, M.M., El-Galil, S.M.A., Ahmed, S.A., 2019. Design, synthesis, and biological evaluation of novel N4-substituted sulfonamides: acetamides derivatives as dihydrofolate reductase (DHFR) inhibitors. BMC Chem 13, 1–18.

Indrayanto, G., Putra, G.S., Suhud, F., 2021. Validation of in-vitro bioassay methods: application in herbal drug research. Profiles Drug Subst., Excipients, Relat. Methodol. 46, 273–307.

Irvine, F.R., 1961. Woody Plants of Ghana. Oxford University, London, pp. 8–9.

Jonville, M.C., Kodja, H., Humeau, L., Fournel, J., De Mol, P., Cao, M., Angenot, L., Frederich, M., 2008. Screening of medicinal plants from Reunion Island for anti-malarial and cytotoxic activity. J. Ethnopharmacol. 120 (3), 382–386.

Jossang, A., Leboeuf, M., Cave, A., 1977. Alkaloids of *Enantia polycarpa* Engl. And diels. Planta Med. 32 (7), 249–257.

Katsuno, K., Burrows, J.N., Duncan, K., Van Huijsduijnen, R.H., Kaneko, T., Kita, K., Mowbray, C.E., Schmatz, D., Warner, P., Slingsby, T.B., 2015. Hit and lead criteria in drug discovery for infectious diseases of the developing world. Nat. Rev. Drug Discov. 1–8.

Kumatia, E.K., Annan, K., Dickson, R.A., Mensah, A.Y., Aponsah, I.K., Tung, N.H., Ocloo, A., Habertamariam, S., 2016. Anti-inflammatory and analgesic activities of *Annickia polycarpa* stem back and its constituents. Der Pharm. Lett. 8 (13), 42–52.

Kumatia, E.K., Ayertey, F., Appiah-Opong, R., Bagyour, G.K., Asare, K.O., Mbatcho, V.C., Dabo, J., 2021. Intervention of standardized ethanol leaf extract of *Annickia polycarpa*, (DC.) Setten and Maas ex I.M. turner. (Annonaceae), in *Plasmodium berghei* infested mice produced antimalaria action and normalized gross hematological indices. J. Ethnopharmacol. 267, 1134.

Kumatia, E.K., Ofosu-Koranteng, F., Appiah, A.A., Barimah, K.B., 2021b. Standardization and quality control of the herbal medicine mist nibima, employed to treat malaria and COVID-19, using physicochemical and organoleptic parameters and quantification of chemical markers via UHPLC-MS/MS. Int. J. Anal. Chem. 6390481, 1–11.

Kumatia, E.K., Zoiku, F.K., Asase, A., Tung, N.H., 2023. Anti-malarial activity of the alkaloid, heptaphylline, and the furanocoumarin, imperatorin, from Clausena anisata against human Plasmodium falciparum malaria parasites: ex vivo trophozoitocidal, schizonticidal and gametocytocidal approach. Mala J. 22 (264), 1–8.

Liu, Y., Yang, X., Gan, J., Chen, S., Xiao, Z.-X., Cao, Y., 2022. CB-Dock2: improved protein– ligand blind docking by integrating cavity detection, docking and homologous template fitting. Nucleic Acids Res. 50.

Lynch, S.S., 2022. Tolerance and Resistance to Drugs. https://www.msdmanuals.com/home/drugs/factors-affecting-response-to-drugs/tolerance-and-resistance-to-drugs.

Qidwai, T., Khan, F., 2012. Anti-malarial drugs and drug targets specific to fatty acid metabolic pathway of *Plasmodium falciparum*. Chem. Biol. Drug Des. 80, 155–172.

Raimondi, M.V., Randazzo, O., La Franca, M., Barone, G., Vignoni, E., Rossi, D., Collina, S., 2019. DHFR inhibitors: reading the past for discovering novel anticancer agents. Molecules 24, 1140.

RCSB PDB: Enabling Breakthroughs in Scientific and Biomedical Research and Education. 1971, accessed 15 May 2023, https://www.rcsb.org.

Schrader, F.C., Glinca, S., Sattler, J.M., Dahse, H.M., Afanador, G.A., Prigge, S.T., Lanzer, M., Mueller, A.K., Klebe, G., Schlitzer, M., 2013. Novel type II fatty acid biosynthesis (FAS II) inhibitors as multistage anti-malarial agents. ChemMedChem 8, 442–461.

Tallorin, L.C., Durrant, J.D., Nguyena, Q.G., McCammona, J.A., Burkarta, M.D., 2014. Celastrol inhibits *Plasmodium falciparum* enoyl-acyl carrier protein reductase. Bioorg. Med. Chem. 22 (21), 6053–6061.

Terefe, E.M., Okalebo, F.A., Derese, S., Muriuki, J., Batiha, G.E.-S., 2021. In vitro cytotoxicity and anti-HIV activity of crude extracts of *Croton macrostachyus, Croton megalocarpus* and *Croton dichogamus*. J. Exp. Pharmacol. 13, 971–979.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture 2005. J. Parasitol. 91, 484–486.

Winstanley, P., 2001. Modern chemotherapeutic options for malaria. Lancet Infect. Dis. 1, 242–250.

World Health Organization, World Malaria Report 2017, accessed 10 May 2023, https://www.who.int/publications/i/item/9789241565523.

World Health Organization (WHO), 2018. Global Malaria Program, Artemisinin resistance and artemisinin-based combination therapy efficacy. Status report. https://www.who.int/docs/default-source/documents/publications/gmp/who-cds-gmp- 2018-26-eng.pdf.

World Health Organization (WHO), 2021. World Malaria Report. Geneva. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021.

Wrobel, A., Baradyn, M., Ratkiewicz, A., Drozdowska, D., 2021. Synthesis, biological activity, and molecular dynamics study of novel series of a trimethoprim analogs as multi-targeted compounds: dihydrofolate reductase (DHFR) inhibitors and DNA-binding agents. Int. J. Mol. Sci. 22, 3685.