



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)

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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 isolate 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 IC₅₀ 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 IC₅₀s 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 while 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

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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 F₂₅₄) 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 (Lagon).

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 powdered 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 started 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 (¹H, ¹³C, ¹H–¹H COSY, DEPT ¹³C 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% N₂, 5% CO₂, and 5% O₂ 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% O₂, 5% CO₂ and 90% N₂ 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 μ L of each sample (concentration = 6.25 μ g/mL – 100 μ g/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 (CC₅₀ values) were determined by plotting concentration of extract on x-axis and percentage of cell viability on y-axis with dose-response curves.

$$\text{Cell viability (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A₀ = mean absorbance of wells with untreated cells (vehicle), A₁ = mean absorbance of test wells. CC₅₀ was then determined with respect to the control cultures.

2.5. Selectivity index (SI)

The selectivity index (SI) was calculated as the ratio of CC₅₀ to the IC₅₀ values. I.e.;

$$SI = \frac{CC_{50}}{IC_{50}}$$

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

IC₅₀ 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 ¹H 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 (–OCH₂–), four methylene (–CH₂–), and one primary methyl group (–CH₃), 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 ¹³C 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.

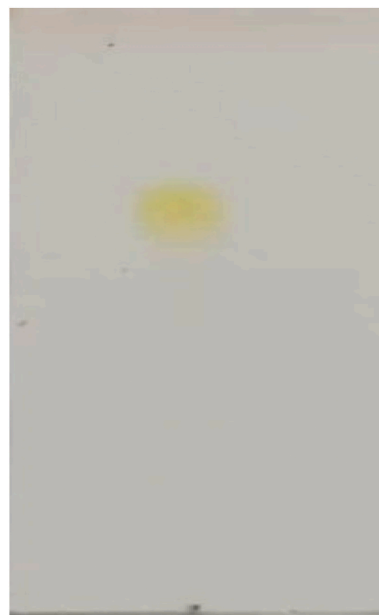


Fig. 1. TLC chromatogram of **AL1** in CF/EtOH (2:1).

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

Position	δ_H (ppm)		δ_C (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 – C₆H₁₂O₅ + H]⁺ and 149.2 [M – 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 IC₅₀ (5.83 (0.1137) nM) better than the chloroquine sensitive 3D7 *P. falciparum* strain.

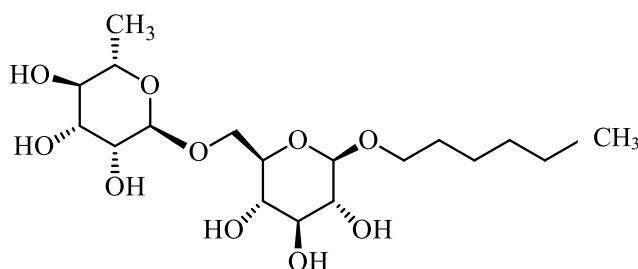


Fig. 2. Chemical structure of *n*-hexyl 1-O-rutinoside (AL1).

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 <i>P. falciparum</i>	
	3D7 strain	Dd2 strain
ALE	3.84 (0.03832)	4.255 (0.2245)
ALC	3.441 (0.3340)	3.505 (0.2053)
AL1 (<i>n</i> -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 CC₅₀s leading to cell survivals >50%.

The CC₅₀ of *n*-hexyl 1-O-rutinoside was higher than that of Artesunate (Table 3). However, this order was reversed for their SI because the IC₅₀ of the isolate was much lower than the IC₅₀ 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*-hexyl 1-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*-hexyl 1-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

CC₅₀ (μg/ml) and Selectivity indices produced by the compounds against human RBCs.

Compound	CC ₅₀ (μg/ml)	Selectivity Index (SI)	
		3D7 strain	Dd2 strain
ALE	89.28	23.25	20.98
ALC	98.82	27.98	28.20
AL1 (<i>n</i> -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
<i>N</i> -hexyl 1-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	−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
<i>N</i> -hexyl 1-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	−7.5	Chain A: Ile14 Cys15 Ala16 Cys17 Leu40 Leu46 Asp54 Met55 Phe58 Asn108 Ile112 Pro113 Phe116 Leu119 Ile164 Tyr170 Thr185
<i>N</i> -hexyl 1-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	−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.

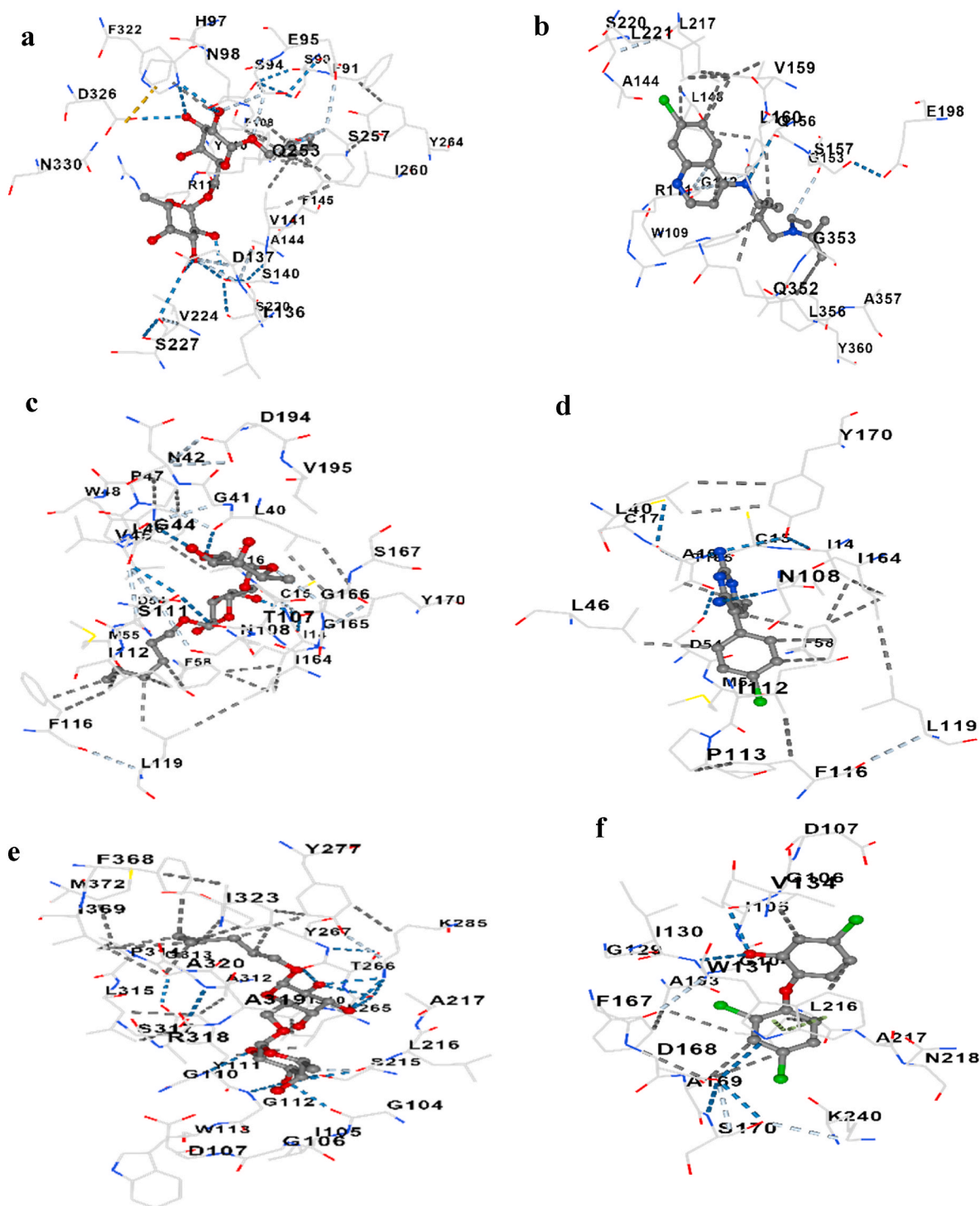


Fig. 3. The most stable conformations of the crystal structures produced by the ligands and their targets in the in-silico analysis. (a) NHR with PfCRT (C1, $\Delta G = -8.1$ kcal/mol); (b) Chloroquine with PfCRT; (c) NHR with PfDHFR; (d) PMA with PfDHFR; (e) NHR with PfPRN; (f) Triclosan with PfPRN. 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-hexyl 1-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 ($IC_{50} \leq 5$ $\mu\text{g/mL}$), promising ($IC_{50} = 5-15$ $\mu\text{g/mL}$), moderate ($IC_{50} 15-50$ $\mu\text{g/mL}$) or inactive ($IC_{50} > 50$ $\mu\text{g/mL}$) 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 $\mu\text{g/mL}$ (Table 2). Hence, ALE and ALC possessed high anti-malarial activity. Furthermore, there is no substantial difference in

the IC₅₀ 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 IC₅₀ value of a test substance, the higher the anti-malarial activity. In this study, the IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 comparison 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 IC₅₀ 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 IC₅₀ values (Table 2).

Selectivity index (SI) is the ratio of the 50% lethal concentration (CC₅₀) of a substance to its 50% pharmacologically active concentration (IC₅₀) (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 CC₅₀ and a very low IC₅₀ in order to produce a high SI of ≥10 to make the sample merit further investigation. The CC₅₀ values of ALE, ALC and *n*-hexyl 1-O-rutinoside were high and their IC₅₀ 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; Ehlgén 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 (Ehlgén 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 its 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 exogenous 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 PfENR 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 PfENR with 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 PfENR 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*-hexyl 1-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 PfENR. 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.

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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.

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Abbreviations

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

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