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Synthesis, Activity Test and Molecular Docking of Novel Nitrophenylcalix[4]-2-methylresorcinarene Derivatives as Antimalarial Agent

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ABSTRACT. This research involved the synthesis, antimalarial assay and molecular docking of novel nitrophenylcalix[4]-2-methylresorcinarene derivatives. Calix[4]-2-methylresorcinarene derivatives, i.e., **2N**, **3N** and **4N**, were synthesized in a one-step reaction through the cyclo-condensation reaction between resorcinol and aldehydes, i.e., 2-nitrobenzaldehyde, 3-nitrobenzaldehyde and 4-nitrobenzaldehyde, respectively. The reaction was carried out through the reflux method with ethanol and hydrochloric acid 37% as the solvent and catalyst, respectively. The synthetic products were characterized using FTIR, ^{1}H-NMR, ^{13}C-NMR, and LC-MS spectrometers. Furthermore, the *in vitro* antimalarial assay was carried out against *Plasmodium falciparum* strain 3D7. The results showed that compounds **2N**, **3N** and **4N** were successfully synthesized in 86.4, 78.6 and 95.7% yield, respectively. Compounds **2N**, **3N** and **4N** showed active antimalarial activity with IC_{50} values of 2.35, 1.68 and 1.79 μM, respectively. The molecular docking studies revealed that nitrophenylcalix[4]-2-methylresorcinarenes gave the binding affinity in a range of -5.1 to -6.1 kcal/mol against the *Pf*LDH receptor through the hydrogen bonds to Arg109, Thr101 and Lys102 amino acid residues. The molecular docking results agreed with the experimental antimalarial assay demonstrating the mechanism of action of nitrophenylcalix[4]-2-methylresorcinarenes as active antimalarial agents happened through the inhibition of the *Pf*LDH receptor.

Keywords: antimalarial, calix[4]-2-methylresorcinarene, molecular docking, *Plasmodium falciparum* 3D7, synthesis

INTRODUCTION

Malaria is seen as a severe threat to humanity and a major public health issue. According to the World Health Organization (WHO) report in 2021, there were 241 million malaria cases worldwide in 2020 with 627,000 death cases. Blood parasites of the species *Plasmodium*, which cause malaria, infect humans and spread the disease when they bite infected female Anopheles mosquitoes (Fletcher & Beeching, 2013; Talapko et al., 2019). Five *Plasmodium* species infect humans, including *P. malariae, P. falciparum*, *P. vivax*, *P. ovale*, and *P. knowlesi* (Talapko et al., 2019). The majority of instances of malaria are caused by *P. falciparum*, the most deadly *Plasmodium* parasite (Dassonville-Klimpt et al., 2022).

Chloroquine and hydroxychloroquine have been known as the primary malaria treatments for decades because of their excellent efficacy, tolerance, and low cost (Lei et al., 2020). Unfortunately, chloroquine and hydroxychloroquine usage has been constrained due to the persistence of resistant *Plasmodium* strains and their development. The WHO also suggests artemisinin-based combination (ACT) therapy for treating malaria. However, reports of artemisinin resistance to antimalarial medications other than chloroquine have been also reported (Ashley et al., 2014; Sibley, 2015). The advent of parasite strains resistant to the medication has encouraged the design of efficient antimalarial medications.

Calixarene is a macrocyclic polyphenol of relevance in supramolecular chemistry. Calix[4]resorcinarene is one of the calixarene families that has undergone substantial biomedical research. Calix[4]resorcinarene has been linked to biomedical activities such as antioxidant, antiviral, and anticancer effects (Yamin et al., 2014; Sayekti et al., 2020; Jumina et al., 2022). Shah et al. (2016) have reported calix[4]arene derivatives with quinoline-pyrimidine substituents as effective antimalarial agents. However, the *in vitro* and *in silico* antimalarial activity of calix[4]resorcinarene derivatives remains unknown as of today. Consequently, it is crucial to conduct research on the functionalization of the calix[4]resorcinarene structure, which has the potential as a novel antimalarial contender.

Park et al. (2017) reported that triazolyl artemisinin derivatives substituted for electron-withdrawing groups (–F, –Cl, and $–CF_3$) had better antimalarial activity compared to artemisinin derivatives with

electron-donating groups (–H, $–CH_3$, $–C_5H_{11}$, and $–OCH_3$). Then, it is thought that another electron-withdrawing group, like nitro ($–NO_2$), increase antimalarial activity (Park et al., 2017). Nitro-based aromatic compounds have been widely reported as antimalarial agents (Cakmak et al., 2011; Grellier et al., 2001; Maroziene et al., 2019; Tukulula et al., 2013). Thus, the functionalization of calix[4]resorcinarene derivatives with nitro-based aromatics is expected to provide high antimalarial activity.

This research is aimed to synthesize three calix[4]-2-methylresorcinarene derivatives, which were *C*-2-nitrophenylcalix[4]-2-methylresorcinarene (**2N**), *C*-3-nitrophenylcalix[4]-2-methylresorcinarene (**3N**) and C-4-nitrophenylcalix[4]-2-methylresorcinarene (**4N**). All synthesized compounds were investigated for *in vitro* antimalarial activity assay against *P. falciparum* strain 3D7 and docked against *P. falciparum* lactate dehydrogenase (*Pf*LDH) receptor to predict the binding affinity and interaction between a ligand and receptor.

EXPERIMENTAL SECTION

Materials and Instrumentation

The materials that were used for synthesis in this research are 2-nitrobenzaldehyde, 3-nitrobenzaldehyde and 4-nitrobenzaldehyde from TCI chemicals, while 2-methylresorcinol, ethanol and hydrochloric acid (HCl) 37% from Merck in pro analytical grade. The materials that were used for antimalarial evaluation are RPMI-1640, serum-blood, RBC (Red Blood Cell), microwell plate 96, dimethyl sulfoxide (DMSO), and the three-dimensional structure of *Pf*LDH (PDB ID: 1U4O). The equipments used in this research are a Fourier transform infrared (FTIR) spectrophotometer (Shimadzu Prestige-21, KBr pellet), ^{1}H-NMR (JEOL 500 MHz), ^{13}C-NMR (JEOL 125 MHz), and Liquid Chromatography-Mass Spectrometry (Waters, Xevo G2-S QTof). In addition, the melting points were measured using Electrothermal-9100. The molecular docking was carried out using ASUS ROG Strix 15 GL503GE hardware with Intel Core i7-8750H/2.2 GHz, 8 GB of RAM, NVIDIA GeForce GTX 1050 Ti Graphics, 1 TB of SSHD and 128 GB of SSD. Gaussian 09W and GaussView programs were used to optimize calix[4]-2-methylresorcinarene derivatives. Meanwhile, the software programs used for molecular docking are Autodock Vina, AutodockTools 1.5.6, PyMOL, and Discovery Studio 2017.

Synthesis of Nitrophenylcalix[4]–2–methyl resorcinarene Derivatives

The synthesis was performed using a similar method as reported by (Yamin et al., 2014; Priyangga et al., 2020) with slight modifications. The synthesis of compounds **2N**, **3N** and **4N** was initiated by dissolving benzaldehyde derivatives (0.76 g; 5 mmol) i.e. 2-nitrobenzaldehyde, 3-nitrobenzaldehyde and 4-nitrobenzaldehyde in 15 mL of ethanol 98%. Afterward, hydrochloric acid 37% v/v (0,5 mL) was added dropwise and then the mixture was stirred for 30 minutes. The 2-methylresorcinol (0.62 g; 5 mmol) was dissolved in 15 mL of ethanol and then added to the mixture. The mixture was stirred and refluxed for 24 hours and then cooled off at room temperature. The mixture was poured into cold distilled water until a precipitate formed. The residue was filtered and washed with ethanol/water (2:3 v/v) until the pH reached neutral. Furthermore, the solid formed was dried in a desiccator. The products were characterized using FTIR (KBr pellet), ^{1}H-NMR, ^{13}C-NMR and LC-MS spectrometers. The NMR spectrum measurement was conducted by dissolving the product in deuterated methanol (CD_3OD).

C–2–nitrophenylcalix[4]–2–methylresorcinarene(2N)

Compound **2N** was obtained as a maroon solid at a yield of 86.4%, m.p 240-250 ºC decomposed. IR spectrum (KBr pellet method, *v*, cm^{-1}): 3462 (O—H stretching), 1608 and 1519 (C=C aromatic stretching), 1473 (—CH— methine bending), 1350 (N—O stretching), and 1188 (C—O stretching). ^{1}H-NMR (CD_3OD, 500 MHz) boat (C_{2v}) δ (ppm): 2.01 (s, 12H, —CH_3), 5.58 and 5.85 (s and s, 4H, —CH— methine), 6.38 (s, 4H, —CH— proton of aromatic resorcinol), 6.58-7.90 (m, proton of nitro aromatic). ^{1}H-NMR, chair (C_{2h}) conformation δ (ppm): 2.08 and 2.10 (s and s, 12H, —CH_3), 6.17 and 6.26 (s and s, 4H, —CH— methine), 6.46 and 6.50 (s and s, 4H, —CH— proton of aromatic resorcinol), 6.58-7.90 (m, proton of nitro aromatic). ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 8.47 (—CH_3), 39.52 (—CH— methine), 112.43, 121.93, 124.03, 126.40, 128.41, 130.28, 131.50, 138.10, 149.36, 150.98 (10 aromatic carbons). LC-MS: peaks at 10.20 and 10.55 min retention time with m/z = 1046 as [M+NH_4^+].

C–3–nitrophenylcalix[4]–2–methylresorcinarene (3N)

Compound **3N** was obtained as an orange solid at a yield of 78.5%, m.p 245-255 ºC decomposed. IR spectrum (KBr pellet method, *v*, cm^{-1}): 3448 (O—H stretching), 1604 and 1527 (C=C aromatic stretching), 1472 (—CH— methine bending), 1350 (N—O stretching), and 1188 (C—O stretching). ^{1}H-NMR (CD_3OD, 500 MHz) crown (C_{4v}) δ (ppm): 2.00 and 2.03 (s and s, 12H, —CH_3), 5.69 (s, 4H, —CH— methine), 5.87 (s, 4H, —CH— proton of aromatic resorcinol), 6.81-7.83 (m, proton of nitro aromatic), 7.46-7.51 (s, O—H proton of aromatic resorcinol). ^{1}H-NMR, chair (C_{2h}) conformation δ (ppm): 2.09 and 2.13 (s and s, 12H, —CH_3), 5.59 and 6.20 (s and s, 4H, —CH— methine), 6.01 (s, 4H, —CH— proton of aromatic resorcinol), 6.81-7.83 (m, proton of nitro aromatic) and 7.46-7.51 (s, O—H proton of aromatic resorcinol). ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 8.46 (—CH_3), 43.25 (—CH— methine), 112.72, 119.81, 122.60, 123.53, 127.80, 128.15, 134.25, 146.35, 147.61, 151.15 (10 aromatic carbons). LC-MS: peaks at 10.62 and 11.05 min retention time with m/z = 1046 as [M+NH_4^+].

C–4–nitrophenylcalix[4]–2–methylresorcinarene (4N)

Compound **4N** was obtained as a light orange solid at a yield of 95.6%, m.p 289-302 ºC decomposition. IR spectrum (KBr pellet method, *v*, cm^{-1}): 3464 (O—H stretching), 1604 and 1512 (C=C aromatic stretching), 1476 (—CH— methine bending), 1350 (N—O stretching), and 1188 (C—O stretching). ^{1}H-NMR (CD_3OD, 500 MHz) crown (C_{4v}) δ (ppm): 2.12 (s, 12H, —CH_3), 5.59 (s, 4H, —CH— methine), 6.02 (s, 4H, —CH— proton of aromatic resorcinol), 6.71 and 6.81 (d and d, 8H, —CH— proton aromatic of nitro), 7.52-7.88 (m, 8H, —CH— proton aromatic of nitro). ^{1}H-NMR, chair (C_{2h}) conformation δ (ppm): 2.01 and 2.02 (s and s, 12H, —CH_3), 5.90 and 5.94 (s and s, 4H, —CH— methine), 5.81 and 5.84 (s and s, 4H, —CH— proton of aromatic resorcinol), 6.87 and 6.95 (d and d, 8H, —CH— proton aromatic of nitro), 7.52-7.88 (m, 8H, —CH— proton aromatic of nitro). ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 8.46 (—CH_3), 43.65 (—CH— methine), 112.89, 121.31, 122.37, 127.87, 128.30, 129.65, 145.87, 151.57 (8 aromatic carbons). LC-MS: peaks at 10.25 and 10.68 min retention time with m/z = 1046 as [M+NH_4^+].

Antimalarial Activity Assay

The *in vitro* antiplasmodial activities of synthesized products were evaluated against chloroquine-sensitive *P. falciparum* strain 3D7. In this research, the antimalarial test used the microtechnique method reported by Congpuong et al. (1998) and combined it with the WHO micro-test method. The synthesized compounds were dissolved with DMSO and diluted with RPMI medium to obtain a variation of concentration 20; 10; 5; 2.5; and 1.25 μM. The 100 μL of sample solution was poured into a 96-well microplate and added with 100 μL of inoculum solution containing the *P. falciparum* 3D7 parasite with three repetitions. Subsequently, the microplate was kept for 72 hours at 37 °C in a 5% CO_2 incubator, using the *Candle jar* method. Observations were made by counting red blood cells infected with parasites from at least 1000 normal red blood cells. The percent inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{A-B}{A}\ x\ 100\%$$

where A and B were the percent parasitemia of the negative control and samples, respectively. These results were used to determine IC_{50} by probit analysis using SPPS Statistics 26 software.

Molecular Docking Study

The three-dimensional structure of all compounds was optimized using DFT/B3LYP method with a basis set of 3-21G. The crystal structure of *P. falciparum* Lactate Dehydrogenase (*Pf*LDH) protein (PDB ID: 1U4O.pdb) was chosen as the protein target. The protein and ligand were prepared using Discovery Studio Visualizer 2017 to remove residues such as water molecules and ions. Then, hydrogen and Kollman charges were added to the receptor using Autodock Tools. The redocking was conducted using Autodock Vina by setting the exhaustiveness value to 16. The grid box dimensions were set to 30 × 30 × 30 Å and coordinates were set to the center coordinates of the native ligand, where the x, y, z coordinates are 29.931, 18.481 and 5.207. The RMSD value in this study was 0.385 Å, which was less than 2 Å, indicating that the method was valid and could be used for molecular docking analysis (Sohilait et al., 2017). All synthesized compounds, as well as chloroquine as the positive control, were docked in the active site of the receptor using the same parameters as redocking. Then, the interaction was visualized by Discovery Studio Visualizer 2017.

RESULTS AND DISCUSSION

Synthesis and Characterization of Nitrophenylcalix[4]-2-methylresorcinarene Derivatives

The nitrophenylcalix[4]-2-methylresorcinarene derivatives were synthesized using a cyclo-condensation reaction under an acidic condition (Yamin et al., 2014). The cyclo-condensation reaction of 2-methylresorcinarene with, respectively, 2-nitrobenzaldehyde, 3-nitrobenzaldehyde and 4 nitrobenzaldehyde generated compounds **2N**, **3N** and **4N**. The reaction occurred through an electrophilic aromatic substitution mechanism with water molecule as a by-product. **Figure 1** presents the synthesis scheme and chemical structure of compounds **2N**, **3N**, and **4N**. The nitrophenylcalix[4]-2-methylresorcinarene derivatives, i.e., **2N**, **3N** and **4N**, were successfully synthesized with 86.4, 78.6 and 95.7% yield, respectively. All synthesized compounds were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, and LC-MS. Compounds **2N**, **3N** and **4N** were decomposition at > 240 ºC, indicating that these compounds had high thermal stability. The high melting point was due to the intramolecular and intermolecular hydrogen bonds of the hydroxy groups and the relatively high molecular mass (1028 g/mol).

Figure 2 shows the FTIR spectra of nitrophenylcalix[4]-2-methylresorcinarene derivatives. Compounds **2N**, **3N** and **4N** gave similar vibration signals because the same functional group was located in a different position on the ortho, meta and para of the aromatic ring. The FTIR spectrum showed a broad signal at 3448–3464 cm^{-1}, which is a stretching vibration of the O—H phenolic group. The absorption of C=C aromatic rings is observed at 1604-1608 and 1512-1527 cm^{-1}. The presence of characteristic absorption at 1472-1476 cm^{-1} is strongly indicated as absorption from C—H methine bending. These three compounds also show the N—O functional groups characteristic absorption at 1350 cm^{-1} and the C—O functional group absorption at 1188 cm^{-1}. These spectral patterns were similar to previously reported C-3-nitrophenylcalix[4] resorcinarene (Yamin et al., 2014; Priyangga et al., 2020).

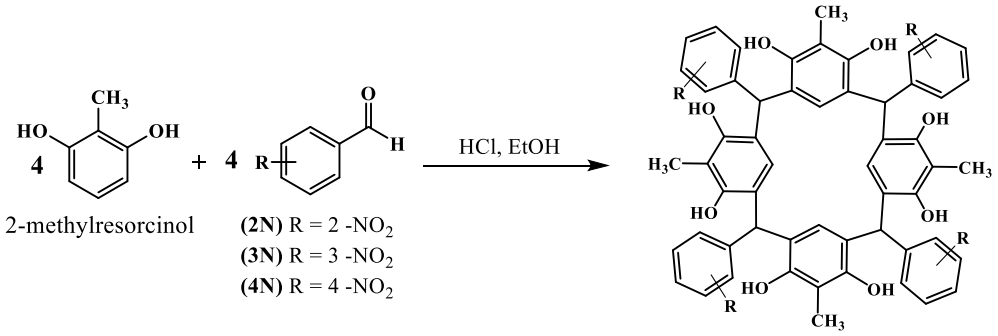

Figure 1. Synthesis scheme of nitrophenylcalix[4]-2-methylresorcinarene derivatives

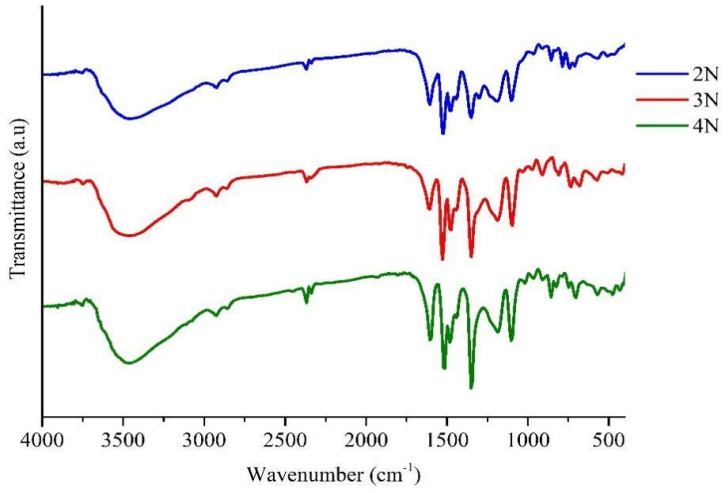

Figure 2. FTIR spectra of calix[4]-2-methylresorcinarene (**2N-4N**)

The successful cyclo-condensation reaction is indicated by the absence of an aldehyde signal on a ^{1}HNMR examination at a chemical shift of around 9 ppm. The ^{1}H-NMR spectra of compounds **2N**, **3N**, and **4N** show that two different conformations have formed. In general, two isomers of calix[4]resorcinarenes are produced during their synthesis, namely, cis-cis-cis (rccc) and cis-trans-trans (rctt), with crown (C_{4v}), chair (C_{2h}), or boat (C_{2v}) conformations (Sardjono & Rachmawati, 2017). Two conformations of compound **2N** exist, namely boat (C_{2v}) and chair (C_{2h}). The chair conformation produced two singlet peaks at 6.17 and 6.26 ppm from the -CH methine chemical shift, while the boat conformation produced two singlet peaks at 5.58 and 5.85 ppm. Compounds **3N** and **4N** produced the same conformations, namely crown and chair. A singlet peak at 5.69 ppm represents the proton methine peak in crown conformation of **3N**, and two singlet peaks at 5.97 and 6.20 ppm represent the chair conformation. The methine bridge proton crown conformation of compound **4N** was shown at singlet signal at 5.59 ppm and the chair conformation appears at 5.59 and 5.94 ppm with total integration of 4 protons. The crown conformation (C_{4v}) produces a singlet peak on the methine bridge, while the chair conformation (C_{2h}) produces two singlet signals, indicating that the protons on the methine bridge have two different directions, namely axial and equatorial (Utomo et al., 2011; Castillo-Aguirre et al., 2017).

The molecular mass of the target chemical is verified by LC-MS characterization. According to the LC-MS chromatogram, compounds **2N**, **3N** and **4N** formed several conformations. The peak retention times for compound **2N** are 10.20 and 10.55 minutes, those for compound **3N** are 10.65 and 11.05 minutes, and those for compound **4N** are 10.25 and 10.68 minutes. All of these compounds have the same MS, which is m/z = 1046 $[M+NH_4]^+$. LC-MS analysis results show that all three compounds verified have the same mass, m/z = 1028. Compounds **2N**, **3N**, and **4N** have been effectively synthesized based on FTIR, ^{1}H-NMR, ^{13}C-NMR, and LC-MS characterization.

Evaluation of Antimalarial Activity

The novel synthesized calix[4]-2-methylresorcinarene conjugates nitro aromatic derivatives were conducted for their antimalarial activity against chloroquine-sensitive *P. falciparum* strain 3D7. Incubation in the antimalarial test was carried out for 72 hours to make it easier to observe the schizont phase than the trophozoite phase under a microscope. **Table 1** displays the antimalarial activity results of the synthesized calix[4]-2-methylresorcinarene derivatives and their comparison to other antimalarial agents. These results

show that the IC_{50} values for compounds **2N**, **3N** and **4N** were 2.35, 1.68, and 1.79 μM, respectively. Antimalarial drugs were divided into four categories by Batista et al. (2009): very active (IC_{50} < 1 μM), active (IC_{50} 1–20 μM), moderate (IC_{50} 20–100 μM), and inactive (IC_{50} > 100 μM). Based on this classification, all synthesized compounds were considered active antimalarial agents.

Based on the IC_{50} values, the aromatic nitro group's position on the calix[4]-2-methylresorcinarene structure did not significantly affect the compound's effectiveness against *P. falciparum* strain 3D7. Compared to compounds **3N** and **4N**, the IC_{50} value of compound **2N** (2.35 μM) is the highest. The higher IC_{50} value is possible due to the steric effect of the **2N** structure, where the nitro group (NO_2) is in the ortho position adjacent to the aromatics of 2-methylresorcinol. The interaction of compounds with *P. falciparum* protein can be affected by this steric impact. The antimalarial activity of compound **3N** was slightly better compared to **4N**, with IC_{50} values that were not substantially different. This is conceivable because the nitro group in the para position makes the **4N** complex bulkier than the **3N** in the meta position, resulting in a lower specific contact with the receptor's active region. According to the research work of the antimalarial activity test, the calix[4]-2-methylresorcinarene derivatives with substituents of an electron-withdrawing group (–NO_2) had good antimalarial activity but were slightly inferior to the standard drug chloroquine.

Table 1 shows several research groups that have also reported the antimalarial activity of calix[4]arene and nitro-based compounds. Compounds **2N**, **3N**, and **4N** (IC_{50} = 1.68–2.35 μM) showed higher antimalarial activity compared to several reported nitro-aromatic compounds such as tetryl, trinitrotoluene, 4-nitrobenzaldehyde and nitrofuran derivatives (IC_{50} = 4.10–79.00 μM). Compared to other compounds, the calix[4]-2-methylresorcinarene derivatives exhibited lower antimalarial activity than *o*-, *m*-, and *p*-trinitro-tribenzylamine compounds (IC_{50} = 0.02–0.03 μM). However, as observed in this study, the addition of a nitro group to tribenzylamine skeletal in the meta position exhibits better activity against *P. falciparum* compared to the ortho and para positions (Kindala et al., 2016). Furthermore, the calix[4]-2-methylresorcinarene derivative also showed lower antimalarial activity compared to chloroquinoline–nitroimidazole and nitroimidazooxazine hybrids (IC_{50} = 0.01–0.09 μM). However, their synthesis procedure was more complicated than the one-pot synthesis of nitrophenylcalix[4]-2-methylresorcinarenes.

The antimalarial activity of calix[4]-2-methyl-resorcinarenes was compared with calix[4]arenes derivatives. Calix[4]resorcinarenes and calix[4]pyrogallolarenes have been evaluated as antimalarial agents for heme polymerization inhibitory assay (Putri et al., 2023; Sari et al., 2022). It was reported that calix[4]resorcinarene with nitro functional groups has stronger activity compared to the other substituents which agreed with this work (Putri et al., 2023). Compounds **2N**, **3N**, and **4N** showed lower antimalarial activity compared to calix[4]arenes conjugated quinolone-pyrimidine derivatives (IC_{50} = 0.04–0.07 μM) due to the more complicated chemical structure of calix[4]arenes conjugated quinolone-pyrimidine derivatives. Overall, the nitrophenylcalix[4]-2-methylresorcinarenes show remarkable antimalarial activity with a high synthesis yield (78.6–95.7%).

Table 1. Comparison of IC_{50} values of the reported antimalarial agents

Compounds	IC_{50} (μM)	References
2N	2.35	This work
3N	1.68	
4N	1.79	
Chloroquine	0.06	Septiana et al., 2022
Tetryl	4.10	Maroziene et al., 2019
2,4,6-Trinitrotoluene	9.40	
4-nitrobenzaldehyde	79.00	
Nitrofurantoin	12.90	Grellier et al., 2001
Nifuroxime	14.70	
Nitrofuran (a)	17.10	
Nitrofuran (b)	4.50	
o-trinitro-tribenzylamine	0.03	Kindala et al., 2016
m-trinitro-tribenzylamine	0.02	
p-trinitro-tribenzylamine	0.02	
Nitroimidazooxazine-chloroquinoline hybrid (9d)	0.01	Tukulula et al., 2013
Nitroimidazooxazine-chloroquinoline hybrid (9f)	0.02	
Methylnitroimidazole – chloroquinoline hybrid (14b)	0.09	
C-8-hydroxyquinolinecalix[4]arene	0.07	Shah et al., 2016
C-2-aminopyrimidinecalix[4]arene	0.04	

Molecular Docking Study

Molecular docking of compounds **2N**, 3**N** and **4N** against the *Pf*LDH receptor was conducted in order to elucidate the mechanism of action of these compounds as antimalarial agents. The *Pf*LDH receptor was chosen because of its important role in the final step of the glycolysis pathway, it can catalyze the conversion of lactate to pyruvate, which can regulate the production of adenosine triphosphate (ATP) (Singh et al., 2021). Inhibition of this receptor can cause death in parasites because ATP production does not work properly. The redocking process of native ligands was carried out to determine the validity of the docking method (Wati et al., 2020). The RMSD value for redocking in this study was 0.385 Å (which was less than 2.000 Å), indicating that the docking method used is acceptable. **Table 2** displays the binding affinity and interaction results of molecular docking.

The native NDD ligand has a binding affinity of -6.7 kcal/mol, which indicates the stability of the interaction with the *Pf*LDH receptor active site. Based on the redocking data, the native ligand forms hydrogen bonds with Ile31, Met30, and His195 at the active site of the *Pf*LDH receptor. The His195 amino acid residue, a specific amino acid residue in the active site of *Pf*LDH, is responsible for the catalytic proton transfer process during the reduction of pyruvate to lactate and then the substrate specificity loop opens to release lactate and NAD^+ (Tian et al., 2021). As shown in **Table 2**, in addition to hydrogen bonds, hydrophobic and van der Waals interactions play a role in stabilizing the interaction of the ligand with the receptor.

Chloroquine, as a positive control, has a binding affinity of -6.4 kcal/mol, which indicates the bond stability close to the native ligand. This result indicated that the interactions formed by amino acid residues were similar to the native ligand. Chloroquine formed hydrogen bonds with Thr97 and Gly99 amino acid residues. Other amino acid residues formed by chloroquine that have the same interactions as the native ligand were Ile31, Val138, Tyr247, Met30, and Gly29. The interactions with these amino acid residues indicated that chloroquine has a stable interaction with the NADH binding pocket on the *Pf*LDH receptor (Shadrack et al., 2016). This result showed that chloroquine has a strong activity as a competitive inhibitor at the cofactor (NADH) binding site of the crucial glycolytic enzyme (Oluyemi et al., 2022; Penna-Coutinho et al., 2011; Shadrack et al., 2016).

Compounds **2N, 3N** and **4N** have a binding affinity of -5.1, -6.1, and -6.0 kcal/mol, respectively. In comparison to compounds **2N** and **4N**, the binding affinity value suggests that compound **3N** has the most stable interaction with the active site of *Pf*LDH and is slightly lower than standard inhibitor chloroquine (-6.4 kcal/mol). In contrast to native ligand, interactions in compounds **2N, 3N** and **4N** demonstrate that these three compounds lack hydrogen bonds to His195.

Table 2. Binding affinity and interaction of native ligand, chloroquine and nitrophenylcalix[4]-2-methylresorcinarenes against *Pf*LDH

Compounds	Binding Affinity (kcal/mol)	Interactions			
		H-bond	Hydrophobic	van der Waals	Others
2N	-5.1	Arg109, Thr101, Lys102	Pro246, Val240, Met30	Ser245, Ala244	His243 (Carbon-H bond)
3N	-6.1	Met30, Ile31, Lys102, Arg109	Ala244, Val240, Ser245	Gly29, Tyr247, Pro246, His243, Thr101	Ser245 (pi-donor H bond)
4N	-6.0	Thr101	Met30, Met58, Val55	Gly99, Ala98, Gly27, Ser28, Lys62, Gly29, Ile31	Phe100 (Carbon-H bond), Asp53 (pi-anion), Lys102 (Pi-Cation)
NDD (native ligand)	-6.7	Ile31, Met30, His195	Pro250	Arg171, Pro246, Tyr247, Ser245, Leu167, Leu163, Val138, Gly29	-
Chloroquine	-6.4	Thr97, Gly99	Ile31, Val138, Pro250	Tyr247, Met30, Gly29, Gly27, Ser28, Phe100, Ile119, Asp53, Ile54, Ala98	-

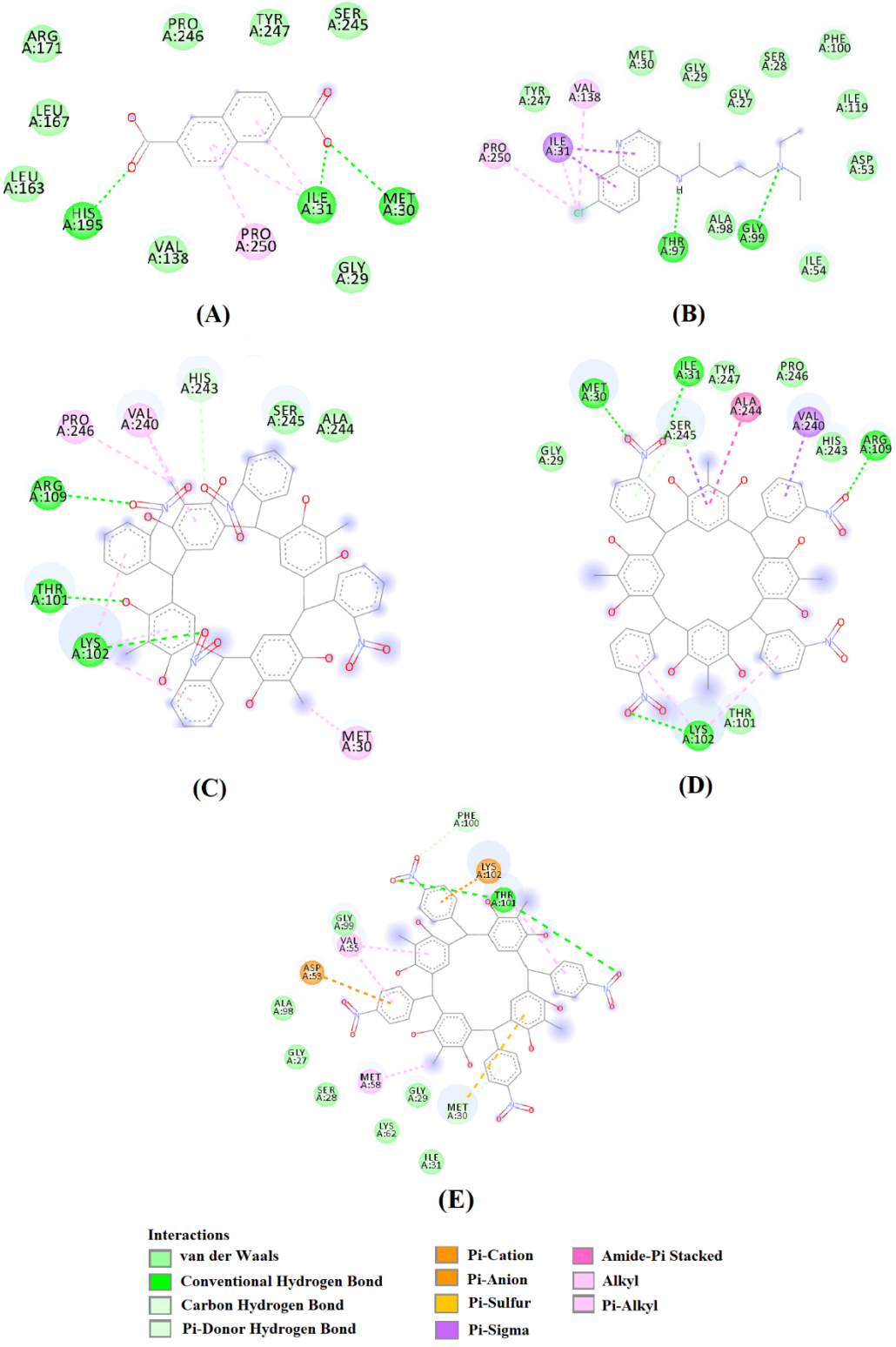

Figure 3. Two-dimensional visualization of molecular docking (A) Native ligand (NDD), (B) Chloroqunine, (C) **2N**, (D) **3N** and (E) **4N** toward the active site of *Pf*LDH

Although compounds **2N, 3N** and **4N** did not form interaction with the His195 residue, these compounds form hydrogen bonds with other crucial amino acid residues, namely Arg109, Thr101 and/or Lys102. According to Cameron et al. (2004), the inhibitor's carboxylic group interacts with the residue Arg109, mimicking the interaction of the pyruvate substrate of *Pf*LDH with oxamate as a substrate analogue. Pyruvate activity will be inhibited by binding to the Arg109 residue, which will prevent pyruvate from being used to produce ATP. The Thr101 and Lys102 amino acid residues, in contrast to the Arg109 amino acid residue, are commonly found on the side of the NADH cleft (Dunn et al., 1996; Khrapunov et al., 2021).

Moreover, this site is potential for the development of novel antimalarial agents (Dunn et al., 1996).

Figure 3 displays the molecular docking visualization outcomes for the native ligand, chloroquine and compounds **2N**-**4N**. At the active site of *Pf*LDH, it is shown that the amino acid residues Arg109, Thr101, and Lys102 form specific hydrogen bonds with compound **2N**. The Lys102 and Arg109 are the specific hydrogen bonds formed by compound **3N**. Meanwhile, compound **4N** has specific hydrogen bonds to Thr101 amino acid residues. In comparison to compounds **2N** and **4N**, compound **3N** forms a more stable interaction with the *Pf*LDH receptor as shown by a stronger negative bond affinity. This is due to the many hydrogen bonds it forms, which raises the bond stability with the receptor. Additionally, the steric effect of the nitro group in the ortho position close to the aromatic 2-methylresorcinol causes compound **2N** to have the lowest binding affinity, making it difficult to bind to the receptor. Compounds **2N**, **3N** and **4N** have higher binding affinity than the native ligands. This is possible due to the bulky structure of calix[4]resorcinarene, which causes a steric effect when docked to the active site of *Pf*LDH. The binding energy of compounds **2N**, **3N** and **4N** were in line with the experimental antimalarial assay. The order of binding energy was **3N** (-6.1 kcal/mol) < **4N** (-6.0 kcal/mol) < **2N** (-5.1 kcal/mol) agreed with the experimental IC_{50} value, i.e., **3N** (1.68 μM) < **4N** (1.79 μM) < **2N** (2.35 μM). The molecular docking results against *Pf*LDH supported the results of *in vitro* activity tests against *P. falciparum* strain 3D7, indicating that compounds **2N**, **3N**, and **4N** act as antimalarial agents through the inhibition of *Pf*LDH in the *P. falciparum* strain 3D7 parasites.

CONCLUSIONS

Three nitrophenylcalix[4]-2-methylresorcinarene derivatives have been successfully synthesized by the cyclo-condensation reaction of 2-methylresorcinol and nitro-based benzaldehyde derivatives under acidic conditions in 78.6–95.7% yield. Through *in vitro* antimalarial assay against *P. falciparum* 3D7, compounds **2N**, **3N**, and **4N** are classified as active antimalarial agents with IC_{50} values of 2.35, 1.68, and 1.79 μM, respectively. Molecular docking studies supported the experimental *in vitro* data by showing that all compounds had negative binding affinity values and specific interactions in hydrogen bonds with the amino acid residues Arg109, Thr101 and Lys102 in the active site of *Pf*LDH. The results indicated that nitrophenylcalix[4]-2-methylresorcinarenes serve as active antimalarial agents against *P. falciparum* 3D7 through the inhibition mechanism of the *Pf*LDH protein receptor.

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