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RESEARCH ARTICLE

Antimalarial Assessment of Certain 1,2,4-Triazoles and Benzoquinolones against *Plasmodium knowlesi* A1H1

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ABSTRACT

Previous studies have reported the use of nitrogen-heterocyclic compounds to treat malaria. Interestingly, quinolone and 1,2,4-triazole scaffolds demonstrated an outstanding capacity to target at least two stages of the malaria parasite life cycle, especially when they are hybrid with other nitrogen heterocycles. Thus, the objective of this study is to assess the drug susceptibility of *Plasmodium knowlesi* A1H1 parasites to certain 1,2,4-triazoles and benzoquinones that bear pyrrolidinones using a plasmodium lactate dehydrogenase assay. All synthesized compounds showed moderate activity against *P. knowlesi* A1H1 with EC_{50} values in the range of 13.39-28.85 μM. Both 1,2,4-triazoles and quinolone derivatives were synthesized using commercially available pyrrolidinones.

Keywords: 1,2,4-Triazoles; quinolones; antimalarial, *Plasmodium knowlesi* A1H1

1. INTRODUCTION

Malaria is a major public health problem affecting more than 40% of the world population (Chin et al., 2020). This disease is caused by apicomplexan parasites of different species belonging to the genus *Plasmodium*. In the new millennium, *Plasmodium knowlesi* is the fifth medically important species of human malaria parasite found in parts of Southeast Asia (Lee et al., 2022). It has the shortest asexual replication cycle among all human malaria parasites giving rise to the level of demonstrable parasites present in the blood. Artemisinin-containing compounds are being used to treat uncomplicated and severe forms of malaria and it has caused high sensitivity to *P. knowlesi* (Chin et al., 2020). Meanwhile, there has been also reported that quinoline-containing drugs caused moderate and variably sensitivity to *P. knowlesi* (Chin et al., 2020; Olliaro, 2001). A previous study has reported the use of nitrogen-heterocyclic compounds to treat malaria such as piperazine and pyrrolidine derivatives (Mendoza et al., 2011). Interestingly, quinolone scaffold was reported as a promising key unit

for antimalarial drugs since it demonstrated excellent efficacy and selectivity towards various stages of the malaria life cycle (Beteck et al., 2014, Romero, 2019). On the other hand, the 1,2,4-triazole key unit was reported to serve as an antiparasitic agent, when linked to other nitrogen heterocycles such as pyrazole (Aggarwal et al., 2020) and pyrimidine (Aggarwal et al., 2020, Patel et al., 2021). Heterocyclic molecules containing nitrogen and oxygen atoms have shown the most potent biological activity among all heterocyclic compounds (Thakkar et al., 2017).

Considering the biological importance of nitrogen-based heterocyclic moieties, we have undertaken a challenge to synthesize 1,2,4-triazoles- and quinolone-containing pyrrolidine-2-one-ring moieties and to investigate their *in-vitro* antimalarial activities.

2. MATERIALS AND METHODS

2.1. Synthesis and characterization of compounds (2-6)

All compounds were prepared following previously reported methods: **2a**-**b** (Ahmad Zahir et al., 2022), **4**-**5** (Voskiene et al., 2012), (Riaz et al., 2020) and **6** (Popiolek et al., 2013) with slight modifications. All synthesized compounds were chemically characterized by spectroscopic methods which are Fourier Transform Infrared (FT-IR) and Nuclear Magnetic Resonance (NMR), as available in the in the Supporting Information. In addition, some synthesized compounds were characterized using Mass Spectrometry Instruments (MSI)-High Resolution Mass Spectrometer Model CO-1600 Autoconcept accommodated by Organic Synthesis Research Laboratory, Institute of Science, Universiti Teknologi MARA.

Synthesis of 1-(4-methoxyphenyl)-2-methyl-1H-benzo[g]pyrrolo[3,4-b]quinoline-3,11(2H,4H)-dione (2a) - The procedure for synthesizing 2,3-dioxo-5-(4-methoxyphenyl) pyrrolidine (**1a**) was based on the methodology described by (Mohammat et al., 2011). An aromatic amine (12.0 mmol) was added to compound **1a** (10.0 mmol) in ethanol (140 mL). The reaction mixture was then refluxed for 18 hours. The resultant solution was cooled at ambient temperature and heated before adding 10 mL of distilled water. After cooling, the solvent was removed in vacuo, and the crude extract was chromatographed with dichloromethane (100%) to obtain an intermediate compound. Later, this intermediate (0.6 mmol) was heated in diphenyl ether (5 mL) at 250°C under a nitrogen atmosphere for 30 minutes. After cooling the resultant solution to room temperature, petroleum ether was added, and the precipitate formed was filtered to procure compound **2a.**

Synthesis of 2-methyl-1-(thiophen-2-yl)-1H-benzo[g]pyrrolo[3,4-b]quinolone-3,11(2H,4H)-dione (2b) - The procedure for synthesizing 2,3-dioxo-5-(thiophen-2-yl) pyrrolidine (**1b**) was based on the methodology described by (Mohammat et al., 2011). An aromatic amine (12.0 mmol) was added to compound **1b** (10.0 mmol) in ethanol (140 mL). The reaction mixture was then refluxed for 18 hours. The resultant solution was cooled at ambient temperature and heated before adding 10 mL of distilled water. After cooling, the solvent was removed in vacuo, and the crude extract was chromatographed with dichloromethane (100%) to obtain an intermediate compound. Later, this intermediate (0.6 mmol) was heated in diphenyl ether (5 mL) at 250°C under a nitrogen atmosphere for 30 minutes. After cooling the resultant solution to room temperature, petroleum ether was added, and the precipitate formed was filtered to procure compound **2b**.

Synthesis of 1-benzyl-5-oxo-3-pyrrolidinecarbohydrazide (4) - The preparation method of the compound is based on the literature (Voskiene et al., 2012). Methyl-1-benzyl-5-oxo-3-pyrrolidine-carboxylate (**3**) (0.707 g, 1.0 mol), 2-propanol (50 mL) and 100% hydrazine hydrate (0.516 g, 3.4 mol) were mixed and refluxed for 5h. The mixture was cooled down. The

solution was spotted on thin-layer chromatography (TLC) plates with a solvent system of methanol: ethyl acetate: petroleum ether (1:3:6). The spot was observed under ultra-violet (UV) light to ensure all starting material has been reacted and a new compound has been formed. The solvent was evaporated by using a rotary evaporator. The crystal formed was dissolved in dichloromethane and transferred into a weighed beaker. The product **4** was air-dried overnight and weighed.

Synthesis of 2-[(1-Benzyl-5-oxo-3-pyrrolidinyl)carbonyl]-N-phenylhydrazinecarbothioamide (5) - *Method 1:* The preparation method of the compound is based on the literature (Voskiene et al., 2012). Compound **4** (0.400 g, 1.0 mol), methanol (50 mL) and phenyl isothiocyanate (0.232 g, 1.0 mol) were mixed and refluxed for 3h. The mixture was cooled down. The solution was spotted on a TLC plate with a solvent system of methanol: ethyl acetate: petroleum ether (1:3:6). The spot was observed under UV light to ensure all starting material has been reacted and a new compound has been formed. The solvent was evaporated by using a rotary evaporator. The product was dissolved in dichloromethane and transferred into a weighed beaker. The product was air-dried overnight and weighed. The solid was recrystallized by using dichloromethane. The crystal formed **5** was filtered off, air-dried, and weighed.

Method 2: The preparation method of the compound is based on the literature (Riaz et al., 2020). Compound **4** (0.300 g, 1.0 mol), ethanol (50 mL) and phenyl isothiocyanate (0.218 g, 1.0 mol) were mixed and refluxed for 4h. The mixture was cooled down. The solution was spotted on a TLC plate with a solvent system of methanol: ethyl acetate: petroleum ether (1:3:6). The spot was observed under UV light to ensure all starting material has been reacted and a new compound has been formed. The solvent was evaporated by using a rotary evaporator. The product was dissolved in dichloromethane and transferred into a weighed beaker. The product was air-dried overnight and weighed. The solid was recrystallized by using dichloromethane. The crystal formed was filtered off, air-dried and weighed. A crude compound **5** was obtained (0.35 g, 74%).

Synthesis of 1-benzyl-4-(4-phenyl-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)pyrrolidin-2-one (6) - The preparation method of the compound is based on the literature (Popiolek et al., 2013) with slight modification. Compound **5** (0.046 g, 1.0 mol) and 20–40 mL of 2 % aqueous solution of NaOH were refluxed for 6 h. After neutralization of the product with hydrochloric acid, the solution was spotted on a TLC plate with a solvent system of methanol: ethyl acetate: petroleum ether (1:3:6). The completion of the reaction was observed using TLC analysis. The solution was transferred to a separatory funnel. 50 mL of ethyl acetate were added, shaken vigorously and allowed to separate between the two layers. The solvent in the organic layer was evaporated by using a rotary evaporator. The crystal was dissolved in dichloromethane and transferred into a weighed beaker. The product **6** was air-dried overnight and weighed.

2.2. Antimalarial assessment against Plasmodium knowlesi A1H1

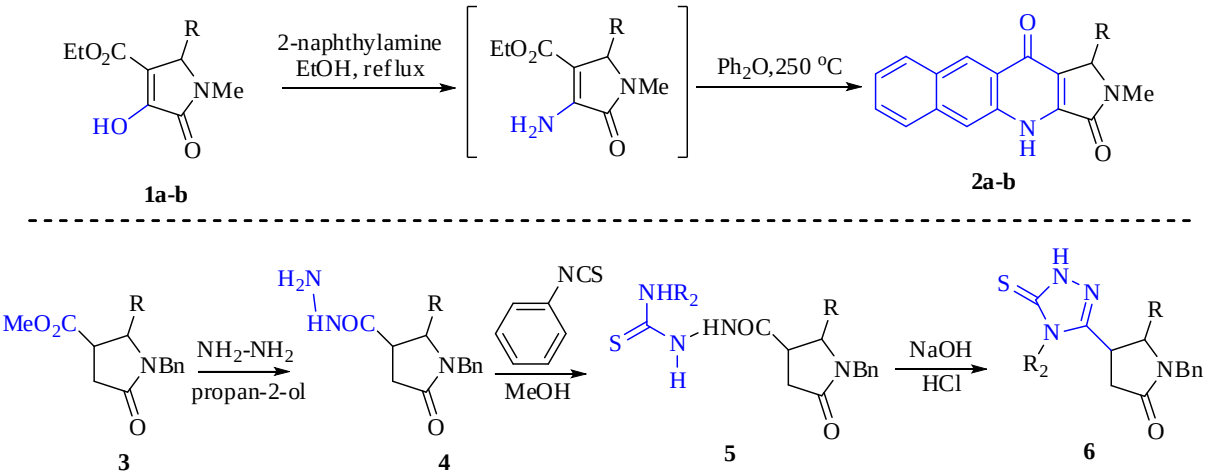
The parasites were cultivated according to Trager and Jensen method (1976) to determine the antimalarial activity of the compounds against *P. knowlesi* A1H1. The parasites were obtained from Malaria Laboratory, Universiti Malaya and maintained in continuous culture in fresh group O (Rhesus positive) human erythrocytes suspended in 2% hematocrit in RPMI 1640 containing 10% horse serum, glucose (3 g/L), hypoxanthine (45 μg/L) and gentamicin (50 μg/L). Plasmodium lactate dehydrogenase (pLDH) assays were performed as described by (Makler et al., 1993) with slight modifications. Parasites were plated in the asynchronized phase at 2% hematocrit and 2% parasitemia in 100 μL of the test compounds. Chloroquine diphosphate (CQ) was used as an antimalarial reference drug. Unparasitized erythrocytes

without the test compounds were blanked while parasitized erythrocytes without test compounds were the control for the assay. Plates were placed in an incubator supplied with 5% CO_2 and incubated at 37°C for 24 hours. After incubation, the plates were subjected to three 30 minutes of freeze-thaw cycles to lyse and resuspend the erythrocytes in culture. Subsequently, Malstat reagent (100 µL) and NBT/PES solution (25 µL) were added to each well of a new 96-well microtiter plate. The culture (25 µL) was then added to the corresponding well of the reagent plate to initiate the lactate dehydrogenase reaction. Colour development of the plate was monitored calorimetrically at 650 nm after an hour of incubation in the dark. Data obtained was analysed through non-linear regression via Graph pad prism software to obtain EC_{50} values (effective inhibitory concentration at 50% parasite growth).

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of compounds (2-6)

An intermediate enamine was formed in moderate yield via the nucleophilic addition of key precursor **1** with primary amines bearing aryl units. Compound **1** is available for keto-enol tautomerism. Since imine-enamine is analogous to the keto-enol tautomerism, a similar chemical environment offered by enolic **1** was expected to influence the formation of a stable enamine intermediate. This phenomenon was influenced by the conjugation and electronic effects of the aromatic rings. An exposure of an enamine to thermal intramolecular cyclization in diphenyl ether solution at elevated temperatures between 240–250°C via Conrad-Limpach synthesis successfully produced fused quinolone derivatives **2a-b** in 74–88% yields (Figure 1).



1a,**2a**: R=(*p*-OMe)(Ph)
1b,**2b**: R=C_4H_4S
3,**4**: R=H
5,**6**: R=H, R_2=Ph

Figure 1. Synthetic routes of 1,2,4-triazoles and quinolone derivatives

Diphenyl ether is commonly used as a heat transfer medium for any organic reactions that require high temperatures since its liquid has a relatively large temperature range. A plausible mechanism suggested that the nucleophilic C=C bond of the phenyl ring attacked the C=O ester to produce the 4-hydroxyquinoline. Subsequently, the product was targeted to undergo multiple tautomerizations to form its quinolone ring moiety.

On the other hand, compound **4** was prepared by a nucleophilic substitution reaction of commercially available **3** with hydrazine hydrate acting as the nucleophile in 2-propanol. The

amino group of compound **4** acts as the nucleophilic species towards the synthesis of thiosemicarbazide **5**. The proton from the nitrogen atom of hydrazide shifted to the nitrogen atom of phenyl isothiocyanate to form a stable compound **5** with 77% yield. Several attempts to synthesize 1,2,4-triazole ring moiety **6** was done. The method mentioned by Popiołek et al. (2013), however, failed to yield a targeted product. Thus, several modifications have been made in terms of reaction time and base concentration. To summarize, a combination of 6h reaction time and 2% NaOH solution managed to yield intramolecular cyclization's product **6** in 99% yield. Its IR spectrum indicates that there are two strong stretching bands at 3424 cm^{-1} and 1202 cm^{-1} that correspond to NH and C=S groups, accordingly. Meanwhile, in its ^{1}H NMR, all proton signals for the pyrrolidinone ring unit are moved to the upfield region compared to those in compound **5** due to the absence of the carbonyl amide. Besides, there is a broad signal observed at 10.41 ppm integrated for one proton confirming the presence of a single NH in the 1,2,4-triazole core ring unit. The analysis of its ^{13}C NMR confirmed the presence of all carbons in compound **6**. Interestingly, the C=O signal appeared in the downfield region at 171.0 ppm, followed by C=S at 154.5 ppm and C=N at 146.5 ppm. The formation of the 1,2,4-triazole ring unit makes the electron density of the remaining nitrogen atoms decreased, which subsequently effects the environment of C=S group.

Compound 2a - Brown solid; mp >350°C (dec.); yield 88%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.79 (s, 3H), 3.70 (s, 3H), 5.55 (s, 1H), 6.86-6.88 (d, 2H, *J* = 8.4 Hz), 7.15-7.18 (d, 2H, *J* = 8.4 Hz), 7.33-7.35 (t, 1H, *J* = 7.2 Hz), 7.52-7.63 (m, 2H), 7.86-7.88 (d, 1H, *J* = 8.8 Hz), 7.94-7.97 (d, 1H, *J* = 12.0 Hz), 8.13-8.15 (d, 1H, *J* = 9.2 Hz), 10.13-10.15 (d, 1H, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.7, 55.6, 63.2, 114.5, 119.1, 119.6, 124.0, 126.5, 126.5, 127.7, 128.6, 128.8, 129.5, 130.2, 130.6, 131.6, 134.3, 136.5, 139.0, 142.0, 159.6, 174.9. Exact mass: 370 (M^+) $C_{23}H_{18}N_2O_3$.

Compound 2b - Grey solid; mp >350°C (dec.); yield 74%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.86 (s, 3H), 5.97 (s, 1H), 7.02-7.04 (dd, 1H, *J* = 3.4 Hz, 5.4 Hz), 7.32-7.34 (d, 1H, *J* = 3.6 Hz), 7.44-7.46 (d, 1H, *J* = 5.2 Hz), 7.54-7.62 (m, 2H), 7.86-7.88 (d, 1H, *J* = 9.2 Hz), 7.96-7.99 (d, 1H, *J* = 12.0 Hz), 8.15-8.17 (d, 1H, *J* = 9.2 Hz), 10.17-10.19 (d, 1H, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 27.7, 58.9, 119.1, 119.5, 126.6, 126.7, 127.6, 128.1, 128.7, 128.8, 129.0, 130.3, 131.6, 134.5, 138.3, 139.4, 142.0, 163.1, 174.8. Exact mass: 346 (M^+) $C_{20}H_{14}N_2O_2S$.

Compound 4 - Yellow crystal; (0.706 g, 99%), m.p. 236-240°C; IR (KBr) (cm^{-1}): 3298, 3165 (NH, NH_2); 3029 (aromatic sp^2-H); 2930 (sp^3-H); 1669 (C=O); 1637, 754 (N-H bending); 1501 (aromatic C=C); 1452 (C-N); 699 (Ph-H). ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 2.63–2.75 (dd, 2H, CH_2, *J* = 8.3 and 9.5 Hz); 2.92–3.00 (m, 1H, CH); 3.33–3.47 (dt, 2H, CH_2, *J* = 7.5, 6.0, 9.0, and 9.5 Hz); 4.32–4.54 (dt, 2H, CH_2Ar, *J* = 14.5, 13.5, 15.0, and 15.0 Hz); 6.96–7.31 (m, 5H, Ar). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 34.7, 35.6, 46.6, 49.1, 127.8, 128.1, 128.8, 135.8, 172.6, 173.0.

Compound 5 - Yellow crystal; (0.49 g, 77%), m.p. 216-218°C; IR (KBr) (cm^{-1}): 3292, 3138 (NH); 3057 (aromatic sp^2-H); 2934 (sp^3- H); 1683 (C=O); 1627, 753 (N-H bending); 1496 (aromatic C=C); 1452 (C-N); 1177 (C=S); 703 (Ph-H). ^{1}H-NMR (500 MHz, DMSO-d_6), δ (ppm): 2.66–2.89 (dd, 2H, CH_2, *J* = 9.5 and 6 Hz); 3.42–3.70 (dd, 2H, CH_2, *J* = 8.4 and 4.8 Hz); 3.81–3.85 (m, 1H, CH); 4.29–4.51 (dd, 2H, CH_2Ar, *J* = 15.0 Hz); 6.97–7.48 (m, 10H, Ar) 10.40 (s, 1H, NH). ^{13}C-NMR (125 MHz, DMSO-d_6), δ (ppm): 28.2, 34.6, 45.9, 49.6, 117.4, 122.6, 128.0, 128.0, 129.1, 129.6, 160.6, 160.9, 173.0.

Compound 6 - Brown solid; (0.02 g, 99%); m.p. 128-130°C; IR (KBr) (cm^{-1}): 3424 (NH); 2928 (Csp^3-H); 1668 (C=O); 1496 (aromatic C=C); 1453 (C-N); 1202 (C=S) 700 (Ph-H). ^{1}H-NMR (500 MHz, $CDCl_3$), δ (ppm): 2.39–2.68 (dd, 2H, CH_2, *J* = 7.8 and 9.0 Hz); 3.18–3.47 (dd, 2H, CH_2, *J* = 6.8 and 9.0 Hz); 3.26–3.35 (m, 1H, CH); 4.30–4.39 (dd, 2H, CH_2Ph, *J* = 14.8

and 14.4 Hz); 7.08–7.44 (m, 10H, Ar); 10.41 (s, 1H, NH). ^{13}C-NMR (125 MHz, $CDCl_3$), δ (ppm): 28.7, 34.0, 45.5, 47.7, 126.3, 126.8, 127.1, 127.8, 128.8, 129.1, 146.5, 154.5, 171.0.

3.2 Antimalarial assessment against Plasmodium knowlesi A1H1

Based on the results, compounds **2a** and **3**-**6** exhibited moderate parasite inhibitory effects against *P. knowlesi* A1H1, while compound **2b** exerted weak antimalarial properties against A1H1 strain as shown in Figure 2 and Table 1.

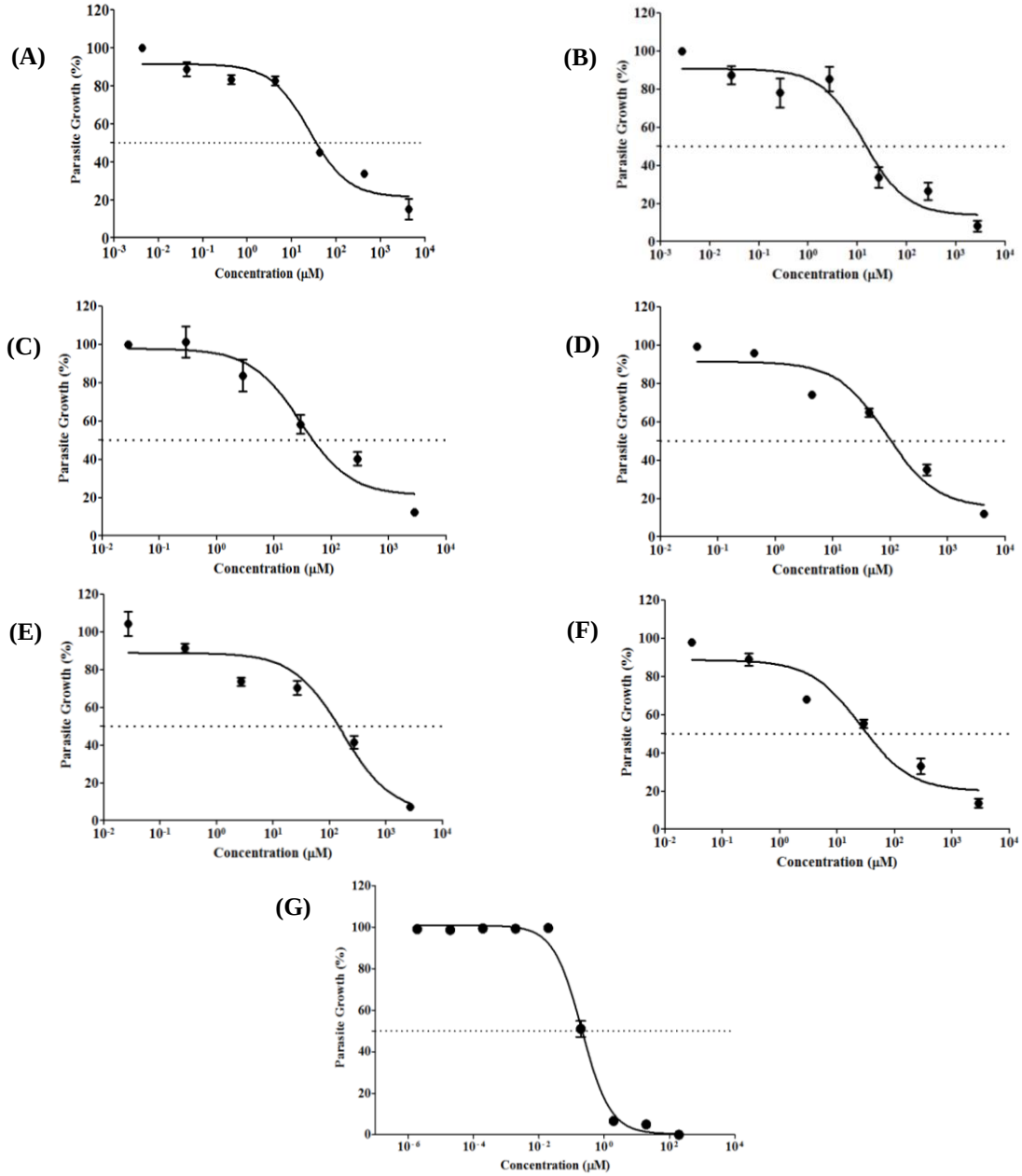


Figure 2. The *P. knowlesi* A1H1 parasite growth against concentrations of A = compound **4**, B = compound **5**, C = compound **6**, D = compound **2a**, E = compound **2b**, F = compound **3**, G = chloroquine.

Table 1. Antimalarial activities of compounds against *Plasmodium knowlesi* A1H1

Compound	*In vitro* antimalarial activity	
	***P. knowlesi* A1H1 EC_{50} (μM) ± SD[a]**	**Activit[b]**
2a	84.00 ± 3.11	Moderate
2b	173.25 ± 5.72	Weak
3	26.30 ± 0.91	Moderate
4	24.63 ± 2.21	Moderate
5	13.39 ± 1.55	Moderate
6	28.85 ± 2.47	Moderate
Chloroquine	0.21 ± 0.02	Potent

[a] SD: standard deviation, triplicate: n = 3; [b] Dolabela et al. (2008) and Basco et al. (1995)

Among all compounds, **5** showed the lowest EC_{50} value of which 13.39 ± 1.55 μM, indicating the most effective compound among others. Chloroquine exerted the most potent antimalarial effect against *P. knowlesi* in parallel with its role as antimalarial reference drug. The antimalarial effects of pure compounds were categorised based on the range, i.e., potent activity with $EC_{50} < 1$ μM, active with $1 < EC_{50} < 10$ μM, moderate with $11 < EC_{50} < 100$ μM, weak with $100 < EC_{50} < 200$ μM, and inactive with $EC_{50} > 200$ μM (Dolabela et al., 2008; Basco et al., 1995).

4. CONCLUSION

Synthesis of compounds containing1,2,4-triazole or quinolone ring moieties and pyrrolidine has been successfully designed, synthesized, and characterized. The *in vitro* antimalarial study against *P. knowlesi* H1N1 of all synthesized compounds (except **2b**) suggested that these molecules have the potential to be considered an antimalarial agent. All compounds showed moderate inhibitory activity against *P. knowlesi.* Compound **5** is the most effective antimalarial compound among the synthesized compounds with EC_{50} of 3.39 ± 1.55 μM. Further studies are recommended to modify the substituents on the ring to increase the potency of the compound. Besides, the structure-activity relationship (SAR) study of the substituted 1,2,4-triazole ring containing pyrrolidine-2-one ring moiety should be continued. In-depth understanding of the interaction of the compounds with the active sites of the DHFR enzyme are also suggested. 1,2,4-triazoles have potential as an antimalarial agent and could be used as a lead for a further guide in the research and development of antimalarial agents to overcome antimalarial drug resistance in the future.

Declaration of Interest
The authors declare that there is no conflict of interest.

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