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Synergistic interaction of two antimalarial drugs, artemisinin and concanamycin A

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ABSTRACT

Artemisinin is a powerful drug that has been combined with other antimalarial drugs to combat malaria and it has been crucial to recent achievements in reducing malaria cases. However, the emergence of *Plasmodium falciparum* resistance against artemisinin has become a serious problem in malaria treatment. Our previous studies reported that artemisinin alkalinised the digestive vacuole of *P. falciparum* similarly to concanamycin A. Concanamycin A is a specific inhibitor of V-type H^+-ATPase, a proton pump located on the membrane of the digestive vacuole. A study also showed that a low concentration of concanamycin A is required to kill 50% of the parasites. Therefore, this study aimed to determine the interaction of artemisinin with concanamycin A by using the isobologram analysis of effects on parasite growth. The antimalarial activity (IC_{50}) of artemisinin and concanamycin A was evaluated by using a malarial SBYR Green I fluorescence-based (MSF) assay prior to isobologram analysis. Based on their IC_{50} values, six different combination solutions of the drugs were assigned and used in the isobologram analysis. The IC_{50} of artemisinin and concanamycin A was 13 ± 2.52 nM and 7 ± 1.15 nM, respectively. The interaction of artemisinin and concanamycin A was found to be synergistic, indicating that the combination of these drugs could kill the parasites more effectively. This study suggests that artemisinin and concanamycin A combination can be a new candidate in artemisinin-based combination therapies.

Keywords: *Artemisinin; concanamycin A; Plasmodium falciparum; proton pump and isobologram analysis*

INTRODUCTION

Although malaria is a disease that is preventable and curable, it remains a major and serious public health problem worldwide that leads to high morbidity and mortality (Centers for Disease Control and Prevention (CDC), 2021a). In 2019, the estimated number of malaria cases worldwide and in Malaysia was 229 million and 3 941, respectively (Ministry of Health, 2020; World Health Organization (WHO), 2021). The complexity of the malaria parasite's life cycle in the vertebrate and invertebrate hosts, the ability of the malaria parasite to evade the host's immune system, and the lack of understanding regarding the complex immune response

of the malaria infection have hindered the development of an effective malaria vaccine (Wykes, 2013; CDC, 2021b). Nevertheless, WHO has finally recommended Mosquirix™ (RTS,S/AS01) in 2021 as the first malaria vaccine for general use among children living in moderate to high malaria transmission regions. Further research on this vaccine is however being conducted to optimise its effectiveness, including the evaluation of alternate dosage regimens (CDC, 2021b; Laurens, 2020). Therefore, relying on antimalarial drugs remains the best option to control and treat malaria.

Artemisinin, derived from *Artemisia annua*, has a high antimalarial activity, quick onset of action and minimal adverse effects in clinical applications (Miller et al., 2013; Bakar, 2016). Due to its excellent efficacy, artemisinin has long been used in malaria treatment, which is effective against different blood stages of the malaria parasite and can suppress gametocyte formation (Heller & Roepe, 2019). Several mechanisms of action of this drug against the parasite have been hypothesised (Bhisutthibhan and Meshnick, 2001; Imwong et al., 2020; Li et al., 2005). A recent study by Ibrahim et al. (2020) suggested that alkalinisation of the *P. falciparum* digestive vacuole pH might be the major effect of artemisinin that disrupts the physiological process in the vacuole resulting in parasite death. Concanamycin A is a macrolide V-type H^+-ATPase inhibitor obtained from *Streptomyces* sp. (Auparakkitanon & Wilairat, 2006). It binds to proteolipid subunits of V-type H^+-ATPase located on the digestive vacuole membrane (Páli, 2017), resulting in the alteration of the vacuole pH (Ja'afar et al., 2021). The digestive vacuole in which proteolytic enzymes are involved in haemoglobin degradation and haemozoin formation is a possible drug target (Abu Bakar et al., 2010). The V-type H^+-ATPase is responsible for maintaining an acidic environment of the digestive vacuole (pH 5.0 - 5.5) (Shafik et al., 2020).

The emergence and unchecked spreading of artemisinin-resistant *P. falciparum* parasites in the Great Mekong subregion pose a significant danger to global malaria control and efforts to eradicate the disease. Artemisinin resistance jeopardises artemisinin combination therapies (ACTs), the first-line treatment for malaria. Artemisinin resistance raises the probability of ACTs therapy failure, favouring partner drug resistance. The artemisinin resistance is believed to be strongly associated with the *PfKelch13* mutations of *P. falciparum* (Imwong et al., 2020). A new study has proposed that artemisinin can alkalinise the digestive vacuole pH the same as concanamycin A. Therefore, the use of concanamycin A as a standard drug in the isobologram analysis was necessary. Besides facilitating the discovery of artemisinin and concanamycin A combination effects, the isobologram analysis of the interaction between these drugs could also provide a new idea for the scientist to produce a new ACT in malaria treatment.

METHODOLOGY

In vitro culture of *Plasmodium falciparum*

3D7 parasites (MRA-102, Amsterdam) were maintained in culture flasks containing type O^+ human erythrocytes and RPMI 1640 medium (Gibco, USA) supplemented with 25 mM HEPES, 0.2 % glucose, 50 µg/mL hypoxanthine, 25 µg/mL gentamicin and 0.25% Albumax II (Pua et al., 2020). Cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The parasite growth was maintained at < 10% parasitaemia (2% haematocrit). Asynchronous parasites were synchronised by 5% D-sorbitol (Sigma-Aldrich, USA) when the ring stage was ≥ 5% parasitaemia (Mohd Yasin et al., 2020) after confirmation by Giemsa-stained thin blood films (Babamale et al., 2020).

Malarial SYBR Green 1 fluorescence-based (MSF) assay

Synchronised ring stage parasite suspensions (180 µL) (2% parasitaemia, 2% haematocrit) were added into 96-well microtiter plates containing different concentrations of artemisinin (ART) and concanamycin A (CMA) (20 µL), respectively (Nik Mat Zin et al., 2020). Parasite suspensions (200 µL) without treatment (negative control) and complete culture medium (CCM) (blank) were added into respective wells. Parasite plates were incubated for 48 hours in normal parasite culture conditions. SYBR Green I (Thermo Fisher Scientific, USA) (from a stock of 20×) was then added to a final concentration of 1× into each well. Plates were wrapped in aluminium foils and incubated for 1 hour at room temperature. The total fluorescence was measured with a microplate reader (the excitation λ = 490 nm and the emission λ = 530 nm) to analyse the parasite growth inhibition (%) of each concentration from which IC_{50} values of the drugs were determined by probit regression analysis with GraphPad Prism software (Version 8).

Drug interaction assay: isobologram analysis

Isobologram analysis was conducted by using a modified protocol with the starting concentration of the compounds for different combination sets was assigned based on the IC_{50} values obtained previously (Kalkanidis et al., 2002; Abu Bakar, 2016). The drug solutions were made to allow the particular drug's IC_{50} to fall around the fourth two-fold serial dilution. Six different combinations of the compounds were prepared in fixed ratios and serially diluted in CCM across 96-well microtiter plates (Figure 1). For instance, solutions of one to six for ART and CMA in combination were prepared at 500:0, 250:5, 125:9, 63;19, 31:38, and 0:75, respectively (ART and CMA

concentrations in nM, with solutions 1 and 6 being each drug alone) (Table 1). Aliquots (20 µL) of drug-containing medium were added into individual wells containing parasite suspensions (180 µL) (2% parasitaemia, 2% haematocrit) in parasite plates and processed as for the standard MSF assay. The fractional inhibitory concentrations (FICs) of each drug in combination and the sum of fractional inhibitory concentrations (SFICs) of ART and CMA in combination were calculated. For instance, FIC of ART = IC_{50} of ART in combination / IC_{50} of ART alone. Similarly, the same equation was used to calculate the FIC of CMA. The isobologram was constructed by using FICs of ART and CMA. The nature of ART interaction with CMA either synergistic (SFIC < 1), antagonistic (SFIC > 1) or additive (SFIC = 1) was characterised based on the SFICs (SFIC = FIC ART + FIC CMA) (Bell, 2005).

Table 1

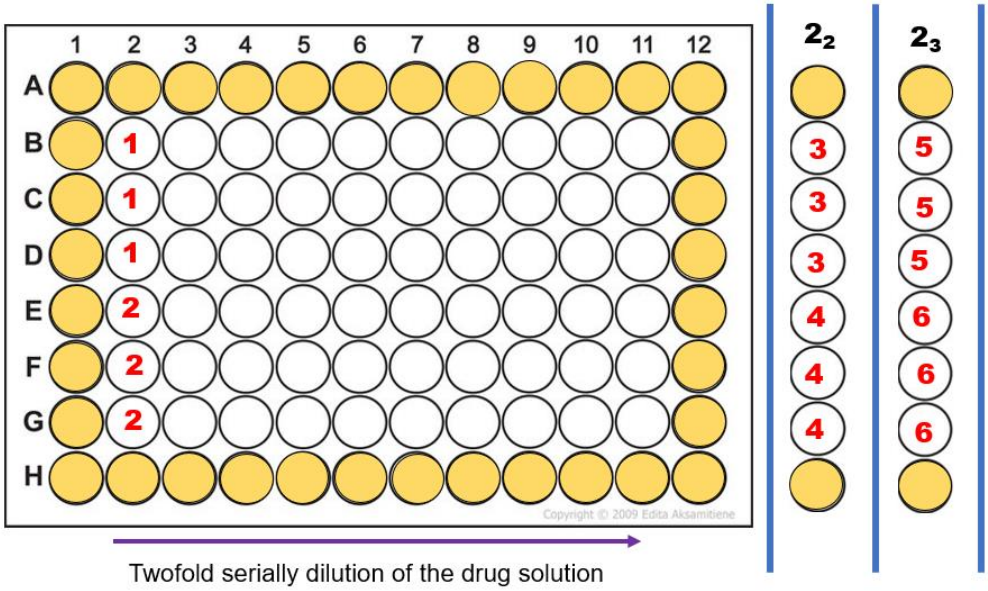
Combination solutions of artemisinin and concanamycin A in fixed ratios

Combination solution	Ratio of compound A & B		Concentration ratios of ART-CMA [nM]	
	A	B	ART	CMA
1	5	0	500	0
2	4	1	250	5
3	3	2	125	9
4	2	3	63	19
5	1	4	31	38
6	0	5	0	75

Note: Six different combination solutions with fixed ratios were assigned based on the IC50 obtained previously. A: Artemisinin (ART); B: Concanamycin A (CMA)

Figure 1

Design of 96-wells microtiter plates for combination assay of artemisinin and concanamycin A



Note: Each combination solution was placed into respective wells and performed in triplicate. Light orange colour wells for sterile distilled water were used to prevent the sample from evaporation. Another two plates were used with different combination solutions.

Statistical analysis

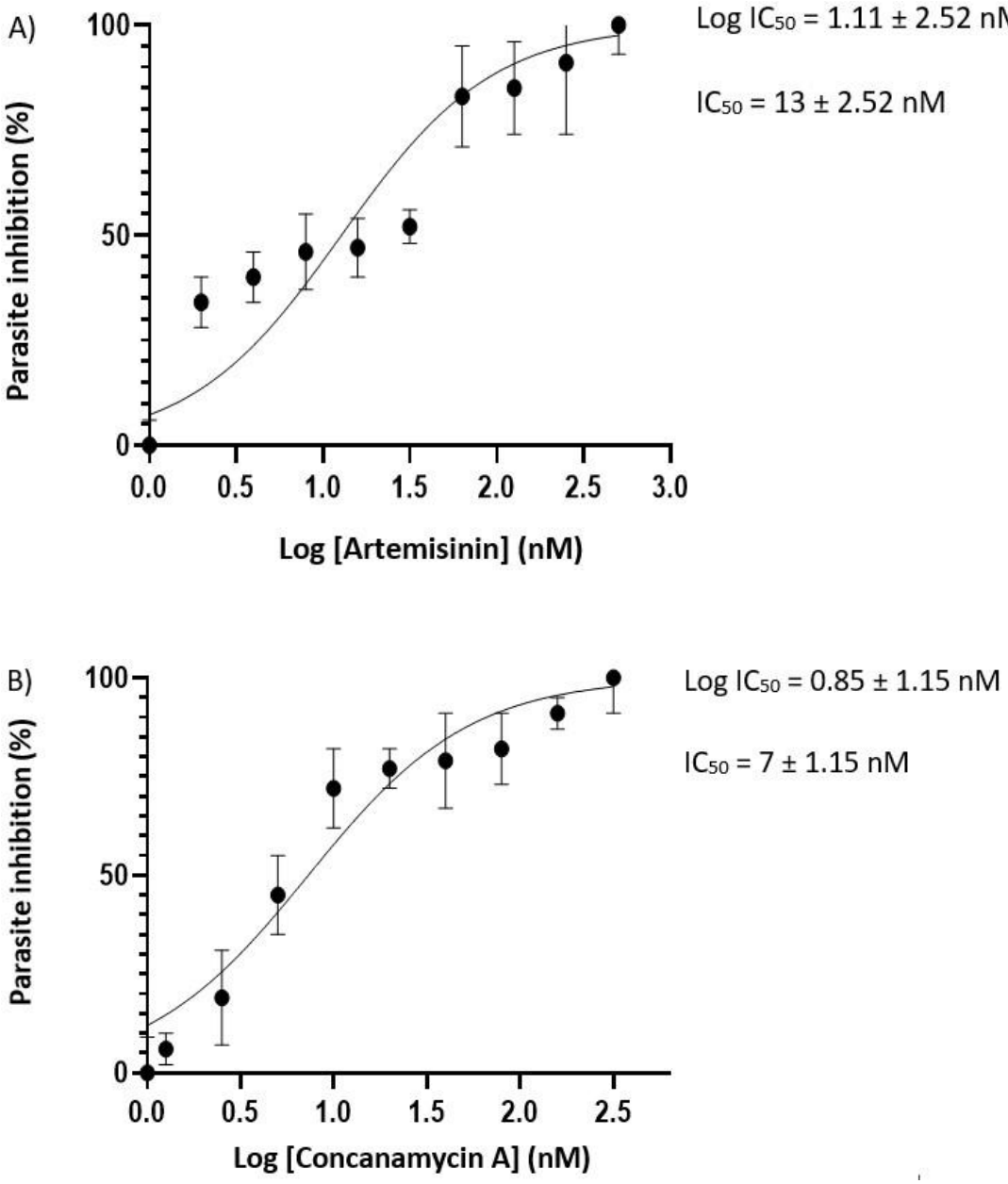
The half-maximal inhibitory concentration (IC_{50}) value of each drug in MSF and drug interaction assays was determined by using probit regression analysis [log(agonist) versus normalised response]. All experiments were conducted in triplicate (n = 3) on three independent occasions and analysed with GraphPad Prism software (Version 8). Values were expressed as mean (standard deviation, SD). The relationship between IC_{50} values of artemisinin and concanamycin A against parasites were analysed using independent t-test. The significance was accepted at $p < 0.05$. The graph of isobolograms was plotted by using Microsoft Excel spreadsheet.

RESULTS

The result of the MSF assay shows that concanamycin A has a more potent antimalarial activity with an IC_{50} value of 7 ± 1.15 nM against the 3D7 parasite than that of artemisinin (IC_{50} = 13 ± 2.52 nM) (Figure 2). Nevertheless, there is no significant difference between artemisinin and concanamycin A tested by using an independent t-test (*p*-value = 0.069).

Figure 2

Log concentration-response curve of (A) artemisinin and (B)concanamycin A against the 3D7 parasite



Note: The mean parasite growth inhibition ± standard deviation (SD) in percentage at different concentrations of artemisinin and concanamycin A were plotted on the log concentration-response curve obtained from three independent experiments performed in triplicate. The IC50 value of artemisinin and concanamycin A obtained from the curve is 13 ± 2.52 nM and 7± 1.15 nM, respectively.

The SFIC values were used to identify the interaction of the drugs in the combinations, which equal to 1 indicates additivity, < 1 indicates synergism and > 1 indicates antagonism (Bell, 2005; Matthews et al., 2017). Based on the calculated FIC values of the drugs, the SFIC values were 1.00, 0.75, 0.58, 0.83, 0.80, and 1.00 with individual data points ranging between 0.20 to 1.00 (Table 2). All of the SFIC values fell below 1, indicating the synergistic interaction between artemisinin and concanamycin A (Figure3).

Table 2

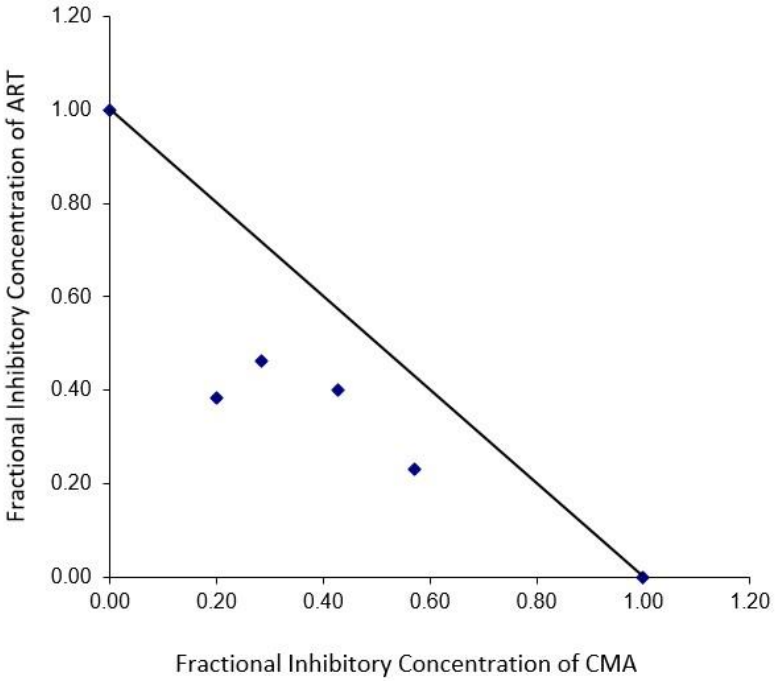
IC_{50} and SFIC values of artemisinin and concanamycin A alone and both drugs in combination

	[ART] (nM)	[CMA] (nM)	IC_{50} ART	IC_{50} CMA	FIC ART	FIC CMA	SFIC
ART	500	0	13	0	100	0.00	1.00
ART-CMA iso 1	250	5	6	2	0.46	0.29	0.75
ART-CMA iso 2	125	9	5	1	0.38	0.20	0.58
ART-CMA iso 3	63	19	5	3	0.40	0.43	0.83
ART-CMA iso 4	31	38	3	4	0.23	0.57	0.80
CMA	0	300	0	7	0.00	1.00	1.00

Note: SFIC values for the four drug combinations were 0.75, 0.58, 0.83 and 0.80, which are less than 1.00, indicating the synergistic interaction of artemisinin and concanamycin A.

Figure 3

Isobologram for combination treatment of artemisinin and concanamycin A



Note: The interaction between artemisinin and concanamycin A was classified as synergistic, since all values of drug combinations fell below the additive line.

DISCUSSION

In the present study, the malarial SYBR Green I fluorescence-based (MSF) assay was employed to determine the antimalarial activity of artemisinin and concanamycin A. This *in vitro* 48-hour drug sensitivity assay has commonly been used to measure the half-maximal inhibitory concentration of compounds that kills 50% of the parasite population (IC_{50}) (Ibrahim et al., 2020). SYBR Green I, a nucleic acid-intercalating dye, was used to identify the presence of the parasite's DNA in infected erythrocytes since mature uninfected erythrocytes have no DNA and are incapable of synthesising RNA (Vossen et al., 2010; Jang et al., 2014). The binding of SYBR Green I is therefore selective for malarial parasite DNA throughout its intraerythrocytic phases (Mohd-Zamri et al., 2017).

Artemisinin inhibited parasite growth with an IC_{50} value (13 ± 2.52 nM) lower than that demonstrated previously by Mohd-Zamri et al. (2017) (IC_{50} = 17.05 ± 0.93 nM) and Ja'afar et al. (2021) (IC_{50} = 134.90 ± 9.68 nM), which falls within the normal range of the IC_{50} values against the chloroquine-sensitive strain (3D7) of *P. falciparum* (2.2 - 124 nM) (Rebelo et al., 2015). Concanamycin A showed an IC_{50} value (IC_{50} = 7 ± 1.15 nM) consistent with that reported by van Schalkwyk et al. (2010) (IC_{50} = 7.76 ± 0.1 nM). There is no significant

difference in the IC_{50} value of artemisinin and concanamycin A, indicating that both drugs are powerful in inhibiting the malaria parasite *in vitro*.

Quantifying drug interactions in drug combinations and categorising the interactions into categories of either synergism, additivity or antagonism is of interest to many researchers (Kalkanidis et al., 2002). The isobologram analysis and the sum of fractional inhibitory concentrations (SFICs) are commonly used for determining drug interactions in the antimalarial drug combination. The investigation of artemisinin and concanamycin A interactions in combination is vital as the model can provide a strategy to predict the efficacy of these digestive vacuole's proton pump inhibitors through antimalarial drug combination approaches. The isobolograms were stimulated for artemisinin and concanamycin A with six fixed-ratio combinations for a particular effect level of IC_{50} values. The SFICs of these drugs in combinations were plotted in the isobologram graphs. A straight line, convex or concave denotes additivity, antagonism or synergism, respectively. The degree of divergence from the line of additivity (SFIC = 0) is used to determine the strength of the interaction between the drugs in the combinations. SFIC < 1.0 is defined as synergism, while SFIC > 1.0 is defined as antagonism (Bell, 2005; Matthews et al., 2017).

In the present study, it was observed that the interaction of artemisinin and concanamycin A was synergistic. According to the results, the best combination concentration is when 125 nM of artemisinin was combined with 9 nM of concanamycin A, resulting in SFIC = 0.58, which is the lowest value as compared with the other SFICs. SFIC <1 indicates synergism and the lower the SFIC, the better the synergistic activity of artemisinin and concanamycin A. A previous study showed that the parasites treated with artemisinin produced several parasite populations, which had a delayed development (Chotivanich et al., 2014). The delayed but viable parasite population showed an elevation in the pH of the digestive vacuole upon pulse treatment with artemisinin for 4 hours (Ibrahim et al., 2020; Klonis et al., 2011). Similarly, Ibrahim et al. (2020) reported that the pH of the digestive vacuole of the parasites treated with artemisinin was 1 pH unit higher than that of the pH of the digestive vacuole of the untreated parasites. An increase of merely a few tenths of the pH unit can significantly affect the lysosomal function since the organelle requires an acidic pH state similar to that found in the digestive vacuole for effective protease action (Eriksson et al., 2017).

Concanamycin A, the V-type H^+-ATPase inhibitor, is well known for alkalinising the parasite's digestive vacuole (Auparakkitanon & Wilairat, 2006; Ibrahim et al., 2020). A study by Ja'afar et al. (2021) reported that the digestive vacuole pH increased by 3.2 units when treated with concanamycin A, showing that the digestive vacuole was alkalinised. Concanamycin A is thought to alter the digestive vacuole pH by binding to proteolipid subunit c of the proton pump, preventing the proteolipid subunits from rotating (Páli, 2017). When proteolipid subunits stop rotating, another H^+ cannot bind to the Glu residues located in subunit c' and c'', making the proton pump fails to maintain the acidic pH of the digestive vacuole, leading to the alteration of the digestive vacuole pH (Forgac, 2007). Some synergistic drug combinations are known to arise from the activities against multiple pathways.

Artemisinin might bind to other proteolipid subunits (c' or c'') or other parts of the proton pump (ATP-proteolytic domain, V_1), which enhances the process of alkalinising the digestive vacuole. Both drugs could bind to the same target such that a conformational change caused by the binding of artemisinin improves the binding of concanamycin A or vice versa (Bell, 2005; Matthews et al., 2017). Other than that, artemisinin could stimulate the conversion of concanamycin A to a more active form or vice versa (Bell, 2005). Artemisinin could enhance the binding of concanamycin A to proteolipid subunit c resulting in a better alkalinisation of the digestive vacuole.

Many diseases necessitate the use of cocktails or combinations of many drugs taken concurrently (Roell et al., 2017). Malaria is one of the diseases which need this alternative as *Plasmodium* parasites have already developed resistance against certain antimalarial drugs such as artemisinin, chloroquine and quinine (Thu et al., 2017). Synergistic action is always preferred as it may reduce doses of drugs, increase therapeutic effect and reduce adverse effects or side effects upon treatments with the drugs (Bell, 2005).

Cultivation of the malaria parasite is the limitation addressed in this study. Conducting the experiment during the Movement Control Order (MCO) from August 2021 until January 2022 caused a difficulty in maintaining the parasite culture due to the problem to recruit blood and serum donors. The parasite would die easily if a sterilised culture medium and an optimised culture temperature (37°C) and environment (5% CO2) are not met. The parasite would also die due to microbial contamination if the aseptic technique is not applied throughout the process. The use of highly-nutrient culture medium tends to attract microorganisms and spores. Due to these problems, new batches of the parasite needed to be revived, and culture medium and blood as well as serum needed to be changed, which few days were wasted to allow the parasites to grow in the new culture.

CONCLUSION

The interaction between artemisinin and concanamycin A was synergistic. According to the graph, the best combination concentration is when 125 nM of artemisinin was combined with 9 nM of concanamycin A. ATP analysis and CRISPR are recommended to be conducted to determine the effectiveness and function of the artemisinin-concanamycin A combination against the parasite's digestive vacuole. ATP analysis is suggested because it relates to proton pump vacuolar type H^+-ATPase, while CRISPR helps to understand the drug function

and develop disease management approaches. Nevertheless, this study suggests that artemisinin and concanamycin A combination can be a new candidate in artemisinin-based combination therapies (ACTs).

AUTHOR CONTRIBUTIONS

Conception and design were carried out by Nurul Izzaty Najwa Zahari, Noor Fardziatun Ujal and Nurhidanatasha Abu-Bakar. Analysis and interpretation of the data were performed by Nurul Izzaty Najwa Zahari and Noor Fardziatun Ujal. The initial drafting of the article was done by Nurul Izzaty Najwa Zahari and Noor Fardziatun Ujal. Nurhidanatasha Abu-Bakar provided critical revision of the article for important intellectual content. The final approval of the article was given by Nurhidanatasha Abu-Bakar. Statistical expertise was provided by Nurul Izzaty Najwa Zahari and Noor Fardziatun Ujal. Nurhidanatasha Abu-Bakar was responsible for obtaining the funding for the research. The collection and assembly of data were conducted by Nurul Izzaty Najwa Zahari, Noor Fardziatun Ujal and Nurhidanatasha Abu-Bakar.

ETHICS APPROVAL

Not applicable.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in this work.

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