AJMB
eISSN: 2600-8173
https://doi.org/10.37231/ajmb.2024.8.2.760
https://journal.unisza.edu.my/ajmb
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Asian Journal of Medicine and Biomedicine

Ellagic Acid, a Polyphenolic Compound Synergistically Interacts with Concanamycin A, an Inhibitor of *Plasmodium Falciparum* Proton Pump

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Received: 10th March 2024 **Accepted: 30th April 2024** **Published: 27th October 2024**

Abstract

Ellagic acid, a bioactive phenolic constituent of many plants, was found in our prior research to induce alkalinisation of the *Plasmodium falciparum*'s digestive vacuole, mirroring the effect of concanamycin A. Concanamycin A acts as a specific inhibitor of the V-type H^+-ATPase located on the digestive vacuole's membrane of the malaria parasite. This study aimed to investigate the interaction between ellagic acid and concanamycin A by employing isobologram analysis to assess their combined effect on parasite growth. The antimalarial activity (IC_{50}) of ellagic acid and concanamycin A was carried out using a malarial SBYR Green I fluorescence-based (MSF) assay before the isobologram analysis was conducted using six different drug combinations determined by their IC_{50} values. The IC_{50} of ellagic acid and concanamycin A was 34.0 ± 0.18 nM and 9.2 ± 0.93 nM, respectively, and their interaction was observed to be synergistic, indicating a more effective parasite-killing capability. This study suggests that ellagic acid and concanamycin A could serve as potential candidates for combination therapies with artemisinin, a standard antimalarial drug believed to affect the pH of the digestive vacuole microenvironment.

Keywords

Ellagic Acid, Concanamycin A, *Plasmodium Falciparum*, Digestive Vacuole, Proton Pump, V-Type H^+-Atpase, pH, Malarial SYBR Green 1 Fluorescence-Based Assay, Isobologram Analysis.

Introduction

Plasmodium falciparum (*P. falciparum*) is the primary cause of malaria and death in tropical and subtropical countries [1]. In 2021, it was estimated that there were 247 million malaria cases and 619,000 deaths worldwide [2]. The implementation of insecticide strategies targeting *Anopheles* mosquito breeding grounds, alongside the distribution of insecticide-treated nets (ITNs) and indoor residual spraying (IRS), has notably decreased malaria incidence. Furthermore, in 2021, the World Health Organization (WHO) endorsed Mosquirix™ (RTS,S/AS01) as the initial malaria vaccine for children residing in areas with moderate to high malaria transmission. However, ongoing research is underway to enhance the vaccine's efficacy [3]. Consequently, given the limited availability of the malaria vaccine, reliance on antimalarial drugs remains crucial for both controlling and treating malaria, particularly in regions with constrained vaccine access.

Concanamycin A, derived from *Streptomyces* sp., serves as an inhibitor of V-type H^+-ATPase [4]. This compound has been used in various studies to investigate autophagic responses [5] and transport mechanisms in plants [6] as well as to search for pH regulators as cancer biomarkers [7] and chaperones of lysosomal enzymes as therapeutic targets [8]. Concanamycin A has demonstrated an ability to bind to proteolipid subunits of the *P. falciparum* V-type H^+-ATPase located on the membrane of the digestive vacuole [9], resulting in a pH alteration [10], where this proton pump maintains an acidic environment (pH 5.0-5.5). This environment is crucial for the activity of proteolytic enzymes involved in haemoglobin degradation and haemozoin formation [11,12]. Ellagic acid, also known as 3,3',4,4'-tetrahydroxydiphenic acid, is a naturally occurring phenolic compound found in oak galls [13]. Apart from possessing various beneficial properties such as antioxidant, anti-inflammatory, anticancer, cardioprotective, hepatoprotective, nephroprotective, and neuroprotective effects [14], ellagic acid has exhibited promising antimalarial activity *in vitro* against *P. falciparum* [15-17]. This activity has been linked to its ability to target the parasite's digestive vacuole [18].

Artemisinin resistance, emerging in the Great Mekong subregion, poses a significant threat to artemisinin-based combination therapies (ACTs), which are vital for malaria treatment [19]. This resistance is believed to be linked with the *Kelch13* mutation of *P. falciparum* [20]. A previous study suggested that artemisinin may induce alkalinisation of the digestive vacuole pH, similar to concanamycin A [10]. Given this observation, an isobologram analysis was conducted to explore the interaction between concanamycin A and ellagic acid. The aim was to ascertain whether the combined effect of these drugs is synergistic or antagonistic, potentially aiding in the identification of suitable partner drugs for use in ACTs.

Methods

In vitro culture of *P. falciparum*

The 3D7 parasite (MRA-102, Amsterdam) was cultured in flasks containing type O^+ human erythrocytes and RPMI 1640 medium (Gibco, USA). The medium was supplemented with 25 mM HEPES, 0.2% glucose, 50 µg/mL hypoxanthine, 25 µg/mL gentamicin and 0.25% Albumax II [21]. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Parasite growth was controlled to remain < 10% parasitaemia with a 2% haematocrit. Asynchronous parasites were synchronised using 5% D-sorbitol (Sigma-Aldrich, USA) when the ring stage reached 5% parasitaemia [22], confirmed by Giemsa-stained thin blood films [23].

Malarial SYBR Green 1 fluorescence-based (MSF) assay

Aliquots (180 µL) of synchronised ring stage parasites (2% parasitaemia, 2% haematocrit) were dispensed into 96-well microtiter plates containing 20 µL of different concentrations of ellagic acid (EA) and concanamycin A (CMA), respectively, in each well [24,25]. Untreated parasite and complete culture medium (CCM) were used as negative control and blank (200 µL), respectively. After 48 hours of incubation under

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eISSN: 2600-8173
https://doi.org/10.37231/ajmb.2024.8.2.760
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standard parasite culture conditions, the plates were added with SYBR Green I (Thermo Fisher Scientific, USA) (1× final concentration), wrapped in aluminium foils, and further incubated for 1 hour at room temperature. Total fluorescence was measured using a microplate reader at 490 nm-excitation and 530 nm-emission wavelengths to determine the % of parasite growth inhibition for each concentration. IC_{50} values of the drugs were calculated using probit regression analysis using GraphPad Prism software (Version 8).

Drug interaction assay: isobologram analysis

Isobologram analysis was performed following a modified protocol, where the initial compound concentrations for various combination sets were determined based on the IC_{50} values (Table 1) [25-27]. Drug solutions were prepared to ensure that each drug's IC_{50} fell approximately within the fourth two-fold serial dilution. Six different combinations of the compounds were prepared at fixed ratios and serially diluted in CCM across 96-well plates (Figure 1). Subsequently, aliquots (20 µL) of the drug-containing medium were added to individual wells containing parasite suspensions (180 µL), following the same procedure as for the MSF assay. The fractional inhibitory concentrations (FICs) of each drug in combination and the sum of fractional inhibitory concentrations (SFICs) of EA and CMA in combination were calculated. For example, the FICs of EA was determined by dividing the IC_{50} of EA in combination with the IC_{50} of EA alone, and a similar equation was used to calculate the FIC of CMA. The isobologram was constructed using the FICs of EA and CMA, and the nature of their interaction (synergistic, antagonistic, or additive) was characterised based on the SFICs (SFIC = FIC EA + FIC CMA), where a SFIC < 1 indicated synergism, SFIC > 1 indicated antagonism, and SFIC = 1 indicated additivity [28].

Table 1: The combination solutions of ellagic acid (EA) and concanamycin A (CMA) in fixed ratios.

Combination solution	Ratio of EA & CMA		Concentration ratios of EA-CMA [nM]	
	EA	CMA	EA	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

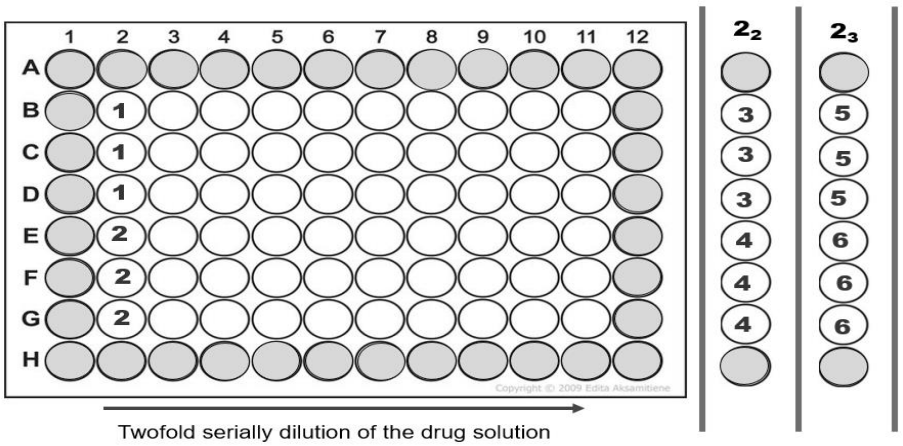

Figure 1: The design of 96-well microtiter plates for combination assay of ellagic acid and concanamycin A.

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https://doi.org/10.37231/ajmb.2024.8.2.760
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Analysis

The half-maximal inhibitory concentration (IC_{50}) for each drug in both MSF and isobologram assays was determined through probit regression analysis [log(agonist) versus normalised response]. All experiments were performed in triplicate on three separate occasions and analysed using GraphPad Prism software (Version 8). Results were presented as mean values with standard deviations (SD). The relationship between the IC_{50} values of ellagic acid and concanamycin A was assessed using an independent t-test with significance set at $p < 0.05$. Isobolograms were generated using a Microsoft Excel spreadsheet.

Results

The result of the MSF assay indicates that concanamycin A exhibits notably stronger antimalarial activity, demonstrating an IC_{50} value of 9.2 ± 0.93 nM ($p < 0.012$) against the 3D7 parasite compared to ellagic acid (IC_{50} = 34.0 ± 0.81 nM) (Figure 2).

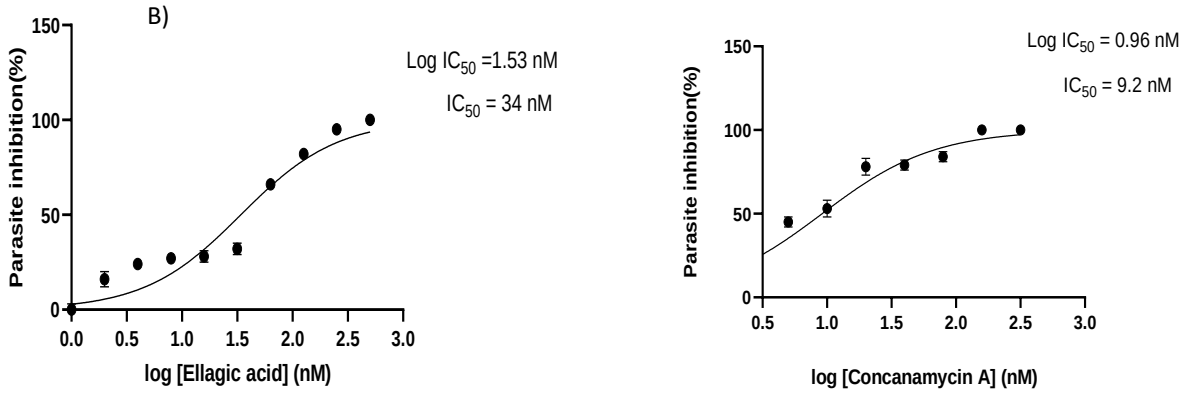

Figure 2: Log concentration-response curve of (A) ellagic acid and (B) concanamycin A against the *P. falciparum* 3D7 parasite.

The SFIC values were employed to identify the interaction between the drugs in the combinations. A value of 1 indicates additivity, while < 1 signifies synergism and > 1 denotes antagonism [28,29]. With reference to the calculated FIC values of the drugs, the SFIC values ranged from 1.00 to 0.37, with individual data points spanning from 0.07 to 1.00 (Table 2). Notably, all SFIC values were below 1, indicating a synergistic interaction between ellagic acid and concanamycin A (Figure 3).

Table 2: The IC_{50} and SFIC values of ellagic acid (EA) and concanamycin A (CMA) alone and both drugs in combination.

	[EA] (nM)	[CMA] (nM)	IC_{50} EA	IC_{50} CMA	FIC EA	FIC CMA	SFIC
EA	500	0	34	0	1.00	0.00	1.00
EA-CMA iso 1	250	5	26	2	0.76	0.20	0.96
EA-CMA iso 2	125	9	19	3	0.56	0.33	0.88
EA-CMA iso 3	63	19	9	4	0.26	0.43	0.70
EA-CMA iso 4	31	38	2	3	0.07	0.30	0.37
CMA	0	75	0	9	0.00	1.00	1.00

AJMB
eISSN: 2600-8173
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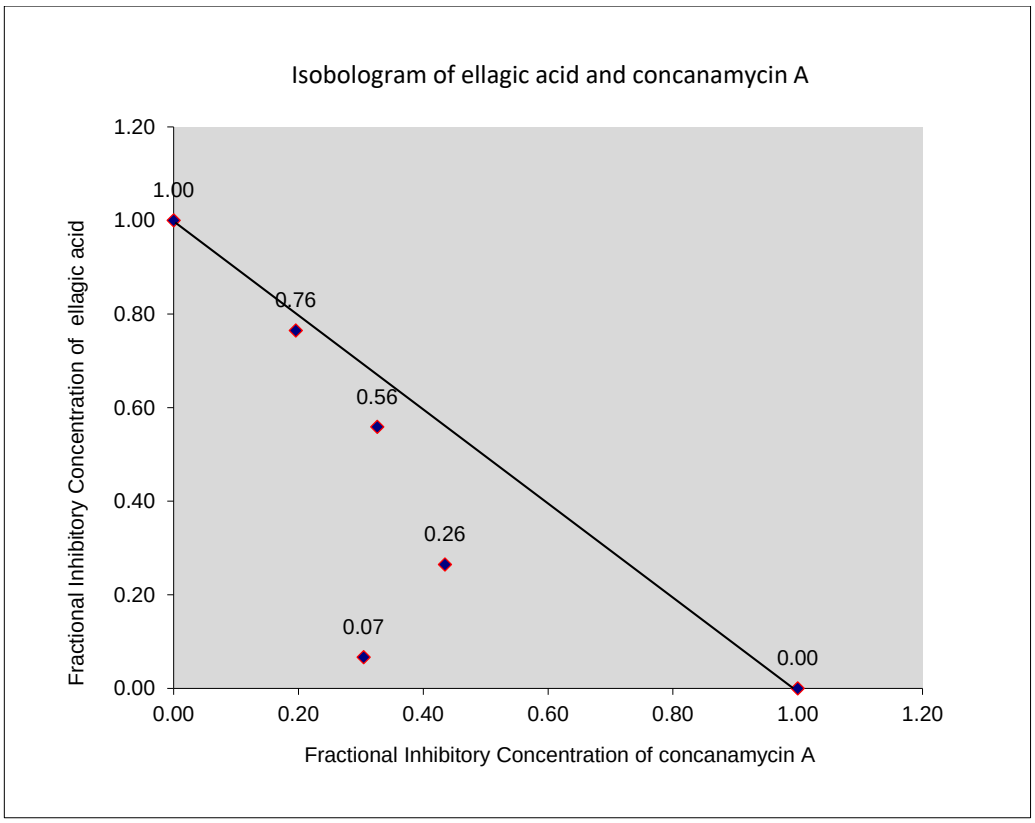

Figure 3: The isobologram for combination treatment of ellagic acid and concanamycin A.

Discussion

In this study, the antimalarial activity of ellagic acid and concanamycin A was evaluated using the MSF assay, a method commonly employed for determining the IC_{50} values [30]. SYBR Green I, a nucleic acid-intercalating dye, was used to detect the presence of parasite DNA in infected erythrocytes, as mature uninfected erythrocytes lack DNA and cannot synthesise RNA [31,32]. Therefore, the binding of SYBR Green I is specifically targeted towards malarial parasite DNA throughout its intraerythrocytic stages [33].

Ellagic acid exhibited inhibition of the 3D7 parasite growth with an IC_{50} value of 34.0 ± 0.81 nM, which is higher compared to that reported by Muchtar et al. [18] (IC_{50} = 1.9 ± 4.57 nM), falling within the typical range of the IC_{50} values against the chloroquine-sensitive strain (3D7) of *P. falciparum* (ranging 2.2 to 124 nM) [34]. Meanwhile, the findings of Soh et al. [17] showed even higher IC_{50} values of the same compound against chloroquine-resistant and mefloquine-resistant strains of *P. falciparum* (ranging 105 to 330 nM). Concanamycin A demonstrated an IC_{50} value of IC_{50} = 9.2 ± 0.93 nM, consistent with the findings of Zahari et al. [25] (IC_{50} = 7.0 ± 1.15 nM) and van Schalkwyk et al. [35] (IC_{50} = 7.8 ± 0.1 nM).

Many researchers are interested in quantifying drug interactions within drug combinations and categorising these interactions as either synergistic, additive, or antagonistic [27]. Isobologram analysis and SFICs are commonly employed for assessing drug interactions in antimalarial drug combinations. Investigating the interactions between ellagic acid and concanamycin A in combination is crucial, as this model can offer insights into predicting the efficacy of these proton pump inhibitors through antimalarial drug combination approaches. In this study, a synergistic interaction between ellagic acid and concanamycin A was observed, with the most effective combination concentration being 31 nM ellagic acid combined with 38 nM concanamycin A, resulting in an SFIC of 0.37, the lowest value compared to other

SFICs. An SFIC < 1 indicates synergism, and the lower the SFIC, the stronger the synergistic activity of ellagic acid and concanamycin A. Synergistic action is preferable as it may allow for reduced drug doses, increased therapeutic efficacy, and minimised adverse effects associated with drug treatments [28].

Dell'Agli et al. [15] showed that ellagic acid isolated from *Punica granatum* possessed a promising antimalarial activity *in vitro* against *P. falciparum*, which was hypothesised to involve in the process of haemoglobin digestion and detoxification of haematin into β-haematin in the digestive vacuole. Soh et al. [17] demonstrated that ellagic acid had a high level of prophylactic activity *in vivo* against the murine model without any toxicity. This study also showed that the compound was more active on mature-stage parasites of the intraerythrocytic cycle during which most of the haemoglobin-rich host cell cytoplasm is ingested and digested. Concanamycin A is renowned for its ability to alkalise the parasite's digestive vacuole [4,30]. A study by Ja'afar et al. [10] revealed that treatment with concanamycin A led to a 3.2-unit increase in the digestive vacuole pH, indicating alkalinisation. This effect is attributed to concanamycin A's interaction with the proteolipid subunit c of the proton pump, inhibiting the rotation of proteolipid subunits [9]. With the rotation of proteolipid subunits halted, H+ cannot bind to the Glu residues in subunits c' and c'', ultimately disrupting the proton pump's ability to maintain the acidic pH of the digestive vacuole, thereby altering its pH [36]. Ellagic acid may target other proteolipid subunits (c' and c'') or other regions of the proton pump (ATP-proteolytic domain, V1), enhancing the process of digestive vacuole alkalinisation. Both drugs might bind to the same target, whereby the binding of ellagic acid induces a conformational change that improves the binding of concanamycin A, or vice versa [28,29]. Additionally, ellagic acid could potentially catalyse the conversion of concanamycin A to a more active form, or vice versa [28]. Ellagic acid could also augment the binding of concanamycin A to proteolipid subunit c, resulting in enhanced alkalinisation of the digestive vacuole.

Conclusion

The synergy between ellagic acid and concanamycin A was evident. As per the graph, the most effective combination concentration was observed when 31 nM of ellagic acid was paired with 38 nM of concanamycin A. To comprehensively evaluate the effectiveness and mechanism of action of the ellagic acid-concanamycin A combination against the parasite's digestive vacuole, it is recommended to conduct ATP analysis and utilise CRISPR techniques. ATP analysis is pertinent as it provides insights into the activity of the vacuolar-type H^+-ATPase proton pump, while CRISPR facilitates a deeper understanding of drug function and aids in the development of disease management strategies. Despite these recommendations, this study underscores the potential of the ellagic acid and concanamycin A combination as a novel candidate in ACTs.

Competing Interests

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.

Authors' Contributions

Noor Fardziatun Ujal: Methodology, Formal Analysis, Software, Investigation, Writing - Original Draft. Nurul Izzaty Najwa Zahari: Methodology, Formal Analysis, Software, Investigation, Writing - Original Draft. Nurhidanatasha Abu Bakar: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

Acknowledgements

The authors express gratitude to the Ministry of Higher Education, Malaysia for their support through the Fundamental Research Grant Scheme (FRGS) (FRGS/1/2019/STG03/USM/03/03). Special thanks are

extended to Dr. Khairul Mohd Fadzli Mustaffa from the Institute for Research in Molecular Medicine (INFORMM), Health Campus, Universiti Sains Malaysia (USM) for providing the 3D7 parasite. Additionally, we appreciate Assoc. Prof. Dr. Aziah Ismail for granting access to the laboratory, equipment, and cell culture facilities at INFORMM.

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