

A polyphenol, pyrogallol changes the acidic pH of the digestive vacuole of *Plasmodium falciparum*

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ABSTRACT

Pyrogallol has a capability of generating free radicals like other antimalarial drugs such as artemisinin, which is thought to inhibit the proton pump located in the membrane of the *Plasmodium falciparum* digestive vacuole, thus alkalinising this acidic organelle. This study aimed to determine pH changes of the malaria parasite's digestive vacuole following treatment with pyrogallol. The antimalarial activity of this compound was evaluated by a malarial SYBR Green 1 fluorescence-based assay to determine the 50% inhibitory concentration (IC₅₀). Based on the IC₅₀ value, different concentrations of pyrogallol were selected to ensure changes of the digestive vacuole pH were not due to parasite death. This was measured by flow cytometry after 4-hour pyrogallol treatment on the fluorescein isothiocyanate-dextran-accumulated digestive vacuole of the mid-trophozoite stage parasites. Pyrogallol showed a moderate antimalarial activity with the IC₅₀ of 2.84 ± 9.40 μM. The treatment of 1.42, 2.84 and 5.67 μM pyrogallol increased 2.9, 3.0 and 3.1 units of the digestive vacuole pH, respectively as compared with the untreated parasite (pH 5.6 ± 0.78). The proton pump, V-type H⁺-ATPase might be inhibited by pyrogallol, hence causing the digestive vacuole pH alteration, which is similar with the result shown by a standard V-type H⁺-ATPase inhibitor, concanamycin A. This study provides a fundamental understanding on the antimalarial activity and mechanism of action of pyrogallol that has a potential to be the antimalarial drug candidate.

Keywords: *Plasmodium falciparum*; pyrogallol; antimalarial; pH changes; proton pump

INTRODUCTION

Malaria, a disease of humankind, occurs throughout most of the tropical and subtropical countries for thousands of years. The global cases of malaria in 2018 were reported to decrease to 228 million cases compared to 231 million cases in 2017 (World Health Organization, WHO, 2019). In the absence of effective vaccines, the treatment measure is strengthened by antimalarial drug therapies (Ashley & Phyo, 2018). However, the current effort to reduce the malaria burden has been threatened by the emergence and spread of *Plasmodium falciparum* resistance to artemisinin-based combination therapies (ACTs) (Ouji et al., 2018). In the Southeast Asia, the ring-stage parasites of *P. falciparum* had reduced the susceptibility toward artemisinin due to the point mutation in the "propeller region" of kelch protein K13 (Ashley et al., 2014). Therefore, new antimalarial drugs with high therapeutic value, rapid action and low cost are urgently needed to achieve a malaria-free world (Sharma & Awasthi, 2015).

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Pyrogallol (1,2,3-trihydroxybenzene) is a phenol compound, which has partial negative and positive charges that can be found in oak plants such as *Quercus infectoria* (Nik Mat Zin et al., 2019; Sutanto et al., 2019). Pyrogallol has been associated with many biological and antimicrobial activities such as antibacteria (Tinh et al., 2016) and anticandida (Baharuddin et al., 2015). Pyrogallol has been demonstrated to generate free radicals such as molecular oxygen (O₂), peroxide nitrate (ONOO⁻) and hydrogen peroxide (H₂O₂) since it can autoxidise rapidly in solutions ranging from pH 3.5-4.5 (Inui et al., 2004). This property increases the potential of pyrogallol to act as an antimalarial agent since free radicals have been shown to interrupt the function of parasite proteins (Percario et al., 2012). The natural host defence including macrophages and neutrophils induces oxidative alterations in response to pyrogallol treatment, causing generation of large amounts of reactive oxygen species (ROS) (Percario et al., 2012). The formation of oxidising species and the activity of antioxidants become imbalanced, thus triggering oxidative stress (Percario et al., 2012) and leading to parasite death (Bridgford et al., 2018; Gunjan et al., 2018). Pyrogallol has also been postulated to have an effect on the digestive vacuoles of *P. falciparum* function including haemoglobin degradation and haem detoxification (Alfaqih & Abu-Bakar, 2020).

The digestive vacuole is an organelle where haemoglobin digestion (Moura et al., 2009) and haemozoin formation (Wunderlich et al., 2012) take place, hence providing potential drug targets (Mohd-Zamri et al., 2017a; Kumar et al., 2007). The digestive vacuole contains a series of aspartic proteases (i.e. plasmepsins I and II) and cysteine proteases (i.e. falcipains) that have optimum pH range between 4.5 and 5.5 (Ibrahim et al., 2020; Ibrahim & Abu-Bakar, 2019; Abu Bakar, 2015). The acidic environment of this vacuole is maintained by two vacuolar-type proton pumps (i.e. V-type H⁺-PPase and V-type H⁺-ATPase) located on the digestive vacuole membrane. Direct action of the antimalarial drugs on the proton pumps might cause the pH alteration of the digestive vacuole, thus affecting haemoglobin metabolism of the parasite (Ibrahim et al., 2020; Gazarini et al., 2007). Therefore, this study aimed to determine pH changes of the digestive vacuole of *P. falciparum* following treatment with pyrogallol.

MATERIALS AND METHODOLOGY

In vitro culture of *P. falciparum*

3D7 parasites (MRA-102, Amsterdam) were maintained in culture flasks containing type O⁺ human erythrocytes and RPMI 1640 medium (Gibco, Illinois, 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% CO₂ at 37°C. The parasite growth was maintained at <10% parasitaemia (2% haematocrit). Asynchronous parasites were synchronised by 5% D-sorbitol (Sigma-Aldrich, Missouri, 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). Synchronised mature-stage parasites were enriched and purified by magnetic cell separation on the basis of the paramagnetic properties of haemozoin (Abu-Bakar, 2013; Ngerenna et al., 2019).

Malarial SYBR Green 1 fluorescence-based (MSF) assay

Synchronised ring-stage parasite suspensions (180 µL) (2% parasitaemia, 2% haematocrit) were added into 96-well microtitre plates containing different concentrations of pyrogallol (20 µL) (Nik Mat Zin et al., 2020). Artemisinin (Sigma-Aldrich, Missouri, USA) was used as a standard drug and parasite without treatment as a negative control. Parasites were incubated for 48 hours in normal parasite culture conditions. SYBR Green I (Thermo Fisher Scientific, Illinois, USA) (from a stock of 20×) was then added to a final concentration of 1× into each well. Plates were wrapped with aluminium foils and incubated for 1 hour at room temperature. The total fluorescence was measured with a microplate reader (SpectraMax M series) (the excitation λ = 490 nm and the emission λ = 530 nm) to analyse the parasite growth inhibition

(%) of each concentration from which IC₅₀ values of the drugs were determined by probit regression analysis with GraphPad Prism software (Version 8).

Preparation of FITC-dextran-containing resealed erythrocytes

Packed erythrocytes (600 µL) were lysed in haemolysis buffer (1350 µL) (5 mM sodium phosphate buffer, pH 7.5) in the presence of 1 mM Mg-ATP (Sigma-Aldrich, Missouri, USA) and 25 µM FITC-dextran (10 kDa, Thermo Fisher Scientific, Illinois, USA) (Ibrahim et al., 2020). Cell suspensions were incubated for 10 minutes to open the pores in the erythrocyte plasma membrane. Isotonic resealing buffer A (330 µL) (5 mM Na₃PO₄, 700 mM NaCl, 100 mM KCl, 27.5 mM glucose; Sigma-Aldrich; pH7.5) was added into cell suspensions and incubated for 20 minutes at 37°C. Cell suspensions were then added with isotonic resealing buffer B (12.75 mL of) (10 mM Na₃PO₄, 140 mM NaCl, 20 mM KCl, 5 mM glucose; Sigma-Aldrich; pH 7.4) and further incubated for 20 minutes to reseal the pores, thus entrapping FITC-dextran into erythrocytes. Resealed erythrocytes were resuspended in RPMI medium after washing twice with resealing buffer B and once with RPMI medium.

Generation of the pH calibration curve of FITC-dextran

FITC-dextran-containing resealed erythrocytes (2% haematocrit) were suspended in MES (Sigma-Aldrich, USA, pH 4.0, 5.5 and 6.0), NaH₂PO₄ (Sigma-Aldrich, Missouri, USA, pH 6.5, 7.0, 7.5 and 8.0) and TRIS (Sigma-Aldrich, Missouri, USA, pH 9.0) buffers in the presence of 10 µM of carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, Missouri, USA) (Ibrahim et al., 2020). This ionophore was used to equilibrate the pH of the erythrocyte compartments with the pH of the buffers. The 488-nm argon ion laser of FACS Canto II flow cytometer (BD Bioscience, USA) was used to excite the probe. The fluorescence intensity was collected at FITC/green (530 nm) and PE/yellow (585 nm) channels. The data were analysed by FCS Express 5 Flow Cytometry Software. Resealed erythrocytes were gated based on their side scatter (SSC) and forward scatter (FSC) profiles. An additional gate based on the peak of the fluorescence intensity collected at green and yellow channels from the histograms was established from which ratio of green/yellow fluorescence intensity (R_{gy}) was measured and plotted as a function of pH.

Determination of the digestive vacuole pH

Resealed erythrocytes containing FITC-dextran (2% haematocrit) were inoculated with synchronised schizont-stage parasites and grown in normal parasite culture conditions (Ibrahim et al., 2020). Different concentrations of pyrogallol (0.5 × IC₅₀ = 1.42 µM, 1.0 × IC₅₀ = 2.84 µM and 2.0 × IC₅₀ = 5.67 µM) were prepared. Concanamycin A (75 nm) (Sigma-Aldrich, USA), a proton pump inhibitor was used as a positive control and untreated parasites as a negative control. After 4-hour treatment, mid trophozoite-stage parasites in resealed erythrocytes were selectively permeabilised with 0.035% saponin (Sigma-Aldrich, Missouri, USA) for 10 seconds at room temperature to permeabilise the erythrocyte plasma membrane (EPM) and parasitophorous vacuolar membrane (PVM), thus leaving only the probe entrapped in the digestive vacuole for measurement of the fluorescence intensity by flow cytometry. Ratio of green/yellow fluorescence intensity (R_{gy}) was interpolated in the generated pH calibration curve of FITC-dextran to measure pH of the digestive vacuole (Ibrahim et al., 2020).

Statistical analysis

All experiments were conducted in triplicate on three independent occasions and analysed with GraphPad Prism software (Version 8). Mean values were expressed as mean ± standard deviation (SD). The data were tested for normality before proceeding to one-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons at 95%

confidence (comparison between treated groups and control groups) using IBM SPSS Statistics 20. Value of $p < 0.05$ was considered statistically significant.

RESULTS

The results of the MSF assay show that pyrogallol inhibited parasite growth with an IC_{50} value of $2.84 \pm 9.40 \mu\text{M}$, however, was less potent than that of artemisinin, a standard antimalarial drug ($IC_{50} = 2.13 \pm 9.68 \text{ nM}$). The R_{gy} in the pH calibration curve of FITC-dextran provides a measure of the pH of the digestive vacuole (Figure 1). The R_{gy} value was increased (ranged from 0.6-1.9) with increasing pH ranging from 4.0 to 9.0 and with pK_a of ~ 6.0 , indicating that FITC-dextran was able to distinguish between acidic and basic compartments.

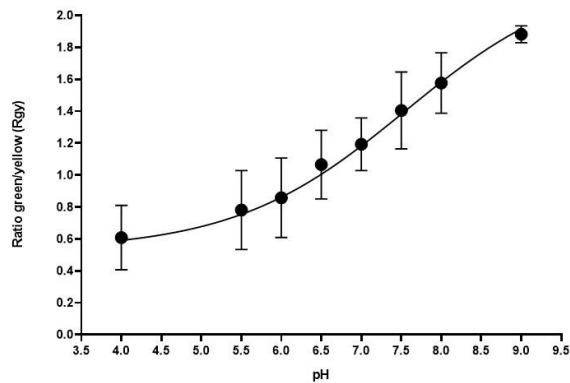


Figure 1: The standard pH calibration curve of FITC-dextran

Figure 2 shows the gating strategy to determine the population of saponin-permeabilised parasites in resealed erythrocytes containing FITC-dextran, which were treated with pyrogallol in the subsequent experiment. The population of the parasites (indicated as 'permeabilised cells') was gated based on the SSC/FSC plot (Figure 2A). The peak of the fluorescence intensity at green and yellow channels at the histograms (gated as 'M1') (Figure 2B-C) represents the signal from the digestive vacuole-associated FITC-dextran.

The non-treated digestive vacuole of saponin-permeabilised mid-trophozoite stage parasites (~ 34 -hour post invasion) showed the R_{gy} value of 0.78 ± 2.09 , which corresponds to $\text{pH } 5.6 \pm 0.60$ (Figure 3). Concanamycin A (75 nM), a standard V-type H^+ -ATPase inhibitor increased the R_{gy} value (1.87 ± 2.02) and alkalinised the digestive vacuole ($\text{pH } 8.8 \pm 0.10$). Treatment with 1.42, 2.84 and $5.67 \mu\text{M}$ of pyrogallol resulted in the increase of the digestive vacuole pH to 8.5 ± 0.20 ($R_{gy} = 1.76 \pm 1.88$), 8.6 ± 0.19 ($R_{gy} = 1.80 \pm 2.24$) and 8.7 ± 0.30 ($R_{gy} = 1.82 \pm 2.04$), respectively.

DISCUSSION

The capillary electrophoretic-based immunoassay method has gained interest in recent years compared to traditional methods for the detection and analysis of protein expressions due to the reliable results that are produced through standardized procedures and reagents.

Protein expression of HBEC lysates was measured using the capillary electrophoretic immunoassay, based on the size or charged separation system, which provides several benefits over conventional methods due to it being less time consuming, eliminate the need for gels for protein separation and require only a low sample amount (Moser & Hage 2013; Chen et al., 2015). In this study, HBEC samples that were treated with ammonium chloride and control (non-treated) were analyzed for Claudin-5 and ICAM-1 expression by Jess.

In this study, the 48-hour malarial SYBR Green I fluorescence-based assay was designed to determine the antimalarial activity of pyrogallol. This 48-hour drug inhibition assay has commonly been used to screen the activity of antimalarial drugs by determining the inhibitory concentration that kills 50% of the parasite population (IC_{50}) (Ibrahim et al. 2020; Mohd-Zamri et al. 2017b). Artemisinin with well-known mechanism of action was used as a standard control to validate the result of the antimalarial activity of pyrogallol. As erythrocytes do not contain any DNA, the technique using SYBR Green I, the DNA-specific fluorescent dye that binds directly to the nucleus of the parasites (Dery et al., 2015) was employed to discriminate between uninfected and infected erythrocytes based on the fluorescence intensity of probe (Jang et al., 2014).

Pyrogallol exhibited the antimalarial activity against the 3D7 parasite, however, was less potent as compared to artemisinin. This result is similar to that reported previously by Venancio et al., (2016) that pyrogallol inhibited the growth of the HB3 parasite strains with the IC_{50} values in the micromolar range (4.0 - $79.3 \mu\text{M}$). Pyrogallol is thought to possess oxidative properties, which is autoxidised in the pH ranging from 3.5-4.5 (Alfaqih & Abu-Bakar, 2020). Therefore, pyrogallol might have an effect on the parasite's digestive vacuole since this organelle has an acidic pH ranging from 3.7-6.5 (Hayward et al., 2006). Pyrogallol is thought to inhibit the parasite growth by producing free radicals (Alfaqih & Abu-Bakar, 2020; Inui et al., 2004), which could damage the proton pumps in the digestive vacuole's membrane. Reactive oxygen species (ROS) participation is crucial in the parasitaemia elimination process as the main mechanism through which most antimalarial drugs act. A study by van Schalkwyk et al., (2013) reported that H_2O_2 leads to lipid peroxidation in erythrocytes. Lipid peroxidation causes selective disruption of the membrane of the digestive vacuole (Wissing et al., 2002; van Schalkwyk et al., 2013). Addition of H_2O_2 to parasites also resulted in the acidification of the parasite cytosol, alkalisation of the digestive vacuole and decrease in the parasite ATP levels. The oxidising agent like pyrogallol, therefore, might disrupt the digestive vacuole pH regulation by direct inhibition of the V-type H^+ -ATPase. The change of the digestive vacuole pH caused by pyrogallol was further evaluated.

The digestive vacuole is an acidic compartment where physiological processes such as haemoglobin degradation (Moura et al., 2009) and haem detoxification (Wunderlich et al., 2012) take place. Both processes require optimal enzymatic pH, therefore, it is crucial to maintain an acidic pH in the digestive vacuole (Moura et al., 2009; Sandlin et al., 2016). In this study, we focused on the event in the mid-trophozoite stage of infection. The synchronised mid-trophozoite stage parasites were incubated for 4 hours with different concentrations of pyrogallol and isolated by saponin to measure the FITC-dextran fluorescence signal directly from the digestive vacuole for pH measurement.

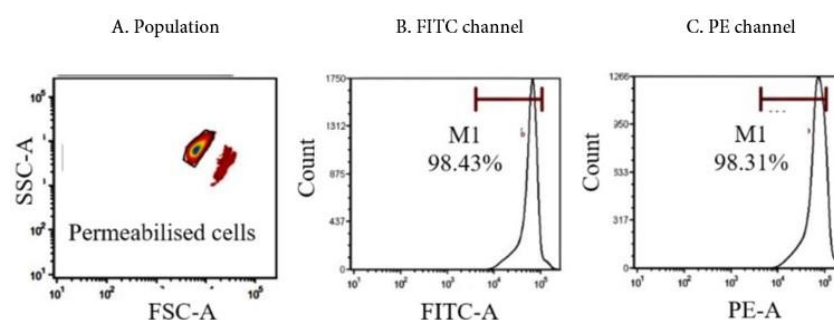


Figure 2: The gating strategy for determination of the population of saponin-permeabilised parasites in resealed erythrocytes containing FITC-dextran

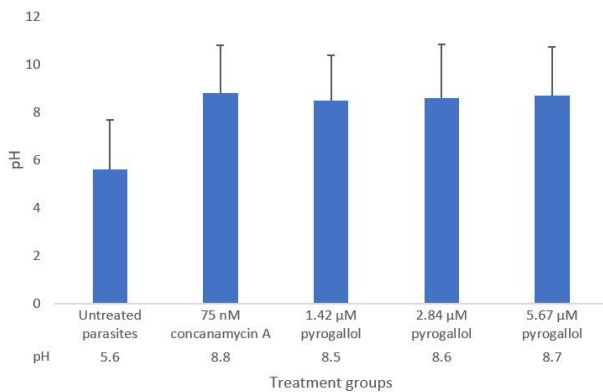


Figure 3: Analysis of the pH of the digestive vacuole of *P. falciparum* after treatment with different concentrations of the pyrogallol

The digestive vacuole pH of pyrogallol-treated parasites was changed in a concentration-dependent manner. Three concentrations of pyrogallol caused an increase of 2.9, 3.0 and 3.1 pH units, respectively as compared with the digestive vacuole pH of the untreated parasites. The digestive vacuole of the untreated chloroquine-sensitive (3D7) strain parasites had a steady-state pH of 5.60 ± 0.78 , which is approximately in the range of the physiological pH of the digestive vacuole previously reported (Abu Bakar, 2015; Hayward et al., 2006; Ibrahim & Abu Bakar, 2019). Similarly, the digestive vacuole of the parasites treated with concanamycin A, a specific inhibitor of V-type H^+ -ATPase showed an increase of 3.2 pH units as compared with the digestive vacuole pH of untreated parasites. This observation is consistent with the presence of the functional proton pump in the digestive vacuole's membrane that regulates the pH (Tang et al., 2019; van Schalkwyk et al., 2013). Concanamycin A has the capability of preventing the transportation of H^+ into the digestive vacuole and out of the parasite across the PPM, thus disrupting pH regulation and causing pH alteration (Ibrahim et al., 2020; van Schalkwyk et al., 2013; Saliba et al., 2003).

CONCLUSION

Pyrogallol showed the antimalarial activity against the malaria parasite and caused the alteration of the digestive vacuole pH similar like concanamycin A, indicating the presence of the active proton pump *in situ*. A further study must be done to confirm the inhibition of the V-type H^+ -ATPase via the action of pyrogallol. A competitive drug study, which involves the interaction of pyrogallol with concanamycin A can be performed to confirm the target of pyrogallol on the V-type H^+ -ATPase.

AUTHOR CONTRIBUTIONS

Nur Saidatul Aqilah Ja'afar: contributed to conception and design, analysis and interpretation of the data, drafting of the article, collection and assembly of data and statistical expertise; Nik Nor Imam Nik Mat Zin: contributed to analysis and interpretation of the data, collection and assembly of data and statistical expertise; Fatin Sofia Mohamad: contributed to analysis and interpretation of the data, collection and assembly of data and statistical expertise; Nurhidanatasha Abu-Bakar: contributed to conception and design, drafting of the article, critical revision of the article for important intellectual content, final approval of the article, statistical expertise, obtaining funding, collection and assembly of data.

ETHICS APPROVAL

Blood collection from healthy donors for malaria parasite *in vitro* culture was performed following the ethical approval obtained from the Human Research Ethics Committee, Universiti Sains Malaysia (HREC-USM) (USM/JEPeM/18050263).

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DISCLOSURES / CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

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