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RESEARCH ARTICLE

The acetone crude extract of *Quercus infectoria* (Olivier) galls alters pH of the digestive vacuole of the malaria parasite, *Plasmodium falciparum*

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ABSTRACT

The reduced efficacy of the mainstay antimalarial drugs due to the widespread of drug-resistant *Plasmodium falciparum* has necessitated efforts to discover new antimalarial drugs with new targets. *Quercus infectoria* (Olivier) has long been used to treat various ailments including fever. The acetone extract of the plant galls has recently been reported to have a promising antimalarial activity *in vitro*. This study was aimed to determine the effect of the *Q. infectoria* gall acetone crude extract on pH of the digestive vacuole of *Plasmodium falciparum*. A ratiometric fluorescent probe, fluorescein isothiocyanate-dextran (FITC-dextran) was used to facilitate a quantitative measurement of the digestive vacuole pH by flow cytometry. Mid trophozoite stage malaria parasites grown in resealed erythrocytes containing FITC-dextran were treated with different concentrations of the acetone extract based on the 50% inhibitory concentration (IC_{50}). Saponin-permeabilized parasites were analyzed to obtain the ratio of green/yellow fluorescence intensity (R_{gy}) plotted as a function of pH in a pH calibration curve of FITC-dextran. Based on the pH calibration curve, the pH of the digestive vacuole of the acetone extract-treated parasites was significantly altered (pH values ranged from 6.35-6.71) in a concentration-dependent manner compared to the untreated parasites (pH = 5.32) ($p < 0.001$). This study provides a valuable insight into the potential of the *Q. infectoria* galls as a promising antimalarial candidate with a novel mechanism of action.

Keywords: *Quercus infectoria*; crude extract; *Plasmodium falciparum*; pH; digestive vacuole.

INTRODUCTION

Malaria caused by *Plasmodium* spp. overwhelms humans in most tropical and subtropical countries, resulting in 409 000 deaths from 229 million cases reported in 2019 worldwide (World Health Organization, WHO, 2020). Although the global incidence rate has reduced since 2010 primarily because of the escalated investment and the massive scale-up effort of intervention and treatment, a malaria-free world is still failed to achieve due to the recurring antimalarial drug resistance (Gachelin *et al.*, 2018; Talapko *et al.*, 2019; WHO, 2020). It is observed that the parasite mutation in the region of *Plasmodium falciparum Kelchl 13* (*Pfk13*) gene was involved in the treatment failure with respect to artemisinin-based combination therapies (ACTs) (Menard & Dondorp, 2017; Grigg *et al.*, 2018; He *et al.*, 2019). In the absence of effective vaccines, treatment measures have therefore been reinforced by searching for new antimalarial agents especially from medicinal plants with new mechanisms of action.

Quercus infectoria (Olivier) (Fagaceae) is one of the medicinal plants traditionally used in folk medicines for post-partum medication and various ailments (Umachigi *et al.*, 2008; Vuthikunchai & Suwalak, 2008; Aroonrerk & Kamkaen, 2009; Jamal *et al.*, 2011). The galls of the plant have a broad spectrum of antimicrobial properties (Hussein *et al.*, 2000; Sawangjaroen *et al.*, 2004; Sawangjaroen & Sawangjaroen, 2005; Ozbilgin *et al.*, 2013; Baharuddin *et al.*, 2014; Kheirandish *et al.*, 2016; Mustafa *et al.*, 2018; Nik Mat Zin *et al.*, 2019). Our recent finding reported that the gall acetone crude extract possesses a promising antimalarial activity *in vitro* (IC_{50} = 5.85 ± 1.90 µg/mL) against the chloroquine-sensitive strain (3D7) of *P. falciparum* (Nik Mat Zin *et al.*, 2020). The non-toxic properties of the acetone extract on brine shrimps, normal erythrocytes and cell lines (NIH/3T3 and Vero) worthy for the extract to be further assessed its antimalarial effect.

The phytochemical constituents of the *Q. infectoria* galls highlight the richness of the phenolic compounds belonging to the classes of pyrogallol, ellagic acid, gallic acid, tannins and quercetin (Hamid *et al.*, 2005; Shrestha *et al.*, 2014; Tayel *et al.*, 2018). Pyrogallol has been associated with many biological and antimicrobial activities such as antibacterial, anticandidicidal and antifungicidal activities (Singh & Kumar, 2013; Baharuddin *et al.*, 2015). Ellagic acid has been shown to have a potent antimalarial activity (Dell'Agli *et al.*, 2003; Soh *et al.*, 2009; Muganga *et al.*, 2014). The phenolic compounds of the galls have been postulated to have their antimalarial

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effects on hemoglobin degradation and heme detoxification occurred in the digestive vacuole of *P. falciparum* (Tajuddeen & Van Heerden, 2019; Mamede *et al.*, 2020).

The digestive vacuole comprises a series of proteases such as plasmepsins I, II and falcipains that optimally function at an acidic pH, which are similar to the digestive vacuole pH (ranged from 4.0-5.5) (Moura *et al*., 2009; Machin *et al*., 2019; Nasamu *et al.*, 2020). The acidic digestive vacuole is not only crucial for hemoglobin degradation, but also vital for the conversion of heme into hemozoin (Toh *et al.*, 2010; Kapishnikov *et al.*, 2017). The proton pumps, the vacuolar H^+-ATPase (V-type H^+-ATPase) and pyrophosphatase (V-type H^+-pyrophosphatase) play a key role in maintaining the acidic pH of the digestive vacuole (Hayashi *et al.*, 2000; Saliba *et al.*, 2003; Spillman & Kirk, 2015; Weiner & Kooij, 2016). Given the important role of the digestive vacuole pH, this study aimed to determine the pH of the malaria parasite's digestive vacuole following treatment with the *Q. infectoria* gall acetone crude extract.

MATERIALS AND METHODS

Extraction of the plant material

The *Q. infectoria* galls were authenticated at the Natural Medicinal and Product Centre, International Islamic University Malaysia (IIUM) (voucher specimen: PIIUM 0229-1). The acetone extraction of the galls was performed as described previously (Baharuddin *et al*., 2015; Nik Mat Zin *et al.*, 2020).

***In vitro* culture of the malaria parasite**

The chloroquine-sensitive strain (3D7) of *P. falciparum* was cultured by using an established method (Mohd-Zamri *et al.*, 2017). Collection of blood from healthy donors for *in vitro* malaria parasite culture was performed following the ethical approval obtained from the Human Research Ethics Committee (HREC)-USM (USM/JEPeM/18050263). Synchronized schizont stage parasites were harvested by using the Vario MACS® magnetic cell separation technique (Mata-Cantero *et al.*, 2014).

Preparation of resealed erythrocytes containing FITC-dextran

Erythrocytes resealed with FITC-dextran (10 kDa, Thermo Fischer) were prepared by suspending washed cells in ice-cold hemolysis buffer in the presence of 25 µM FITC-dextran (Ibrahim *et al*., 2020). Cell suspensions were added with isotonic resealing buffer A (5 mM Na_3PO_4, 700 mM NaCl, 100 mM KCl, 27.5 mM glucose, pH 7.4) before incubation at 37°C followed by the addition of isotonic resealing buffer B (10 mM Na_3PO_4, 140 mM NaCl, 20 mM KCl, 5 mM glucose, pH 7.4). After centrifugation, resealed erythrocytes were stored at 4°C before being used.

Generation of the pH calibration curve of FITC-dextran

Resealed erythrocytes containing FITC-dextran (2% hematocrit) were suspended in buffers: MES (pH 4.0, 5.5 and 6.0), NaH_2PO_4 (pH 6.5, 7.0, 7.5 and 8.0) and TRIS (pH 9.0) (Abu-Bakar, 2015; Ibrahim & Abu-Bakar, 2019) in the presence of an ionophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP) (10 µM final concentration) to equilibrate the pH of the erythrocyte compartments with the pH of the external buffers. Cells were measured by using a flow cytometer at excitation of 488 nm argon ion laser and the fluorescence intensity was collected at FITC/green (530 nm) and PE/yellow (585 nm) channels. The data were analyzed by FCS Express 7 Flow Cytometry Software. The peak of the fluorescence intensity for green and yellow channels on histograms was measured to obtain a ratio of green/yellow fluorescence intensity (R_{gy}) plotted in a pH calibration curve of FITC-dextran.

Determination of the pH of the treated parasite's digestive vacuole

Resealed erythrocytes containing FITC-dextran (2% hematocrit) were inoculated with harvested schizont stage parasites (> 95% parasitemia), grown in normal culture conditions and monitored at 24- and 48-hour post-inoculations by epifluorescence microscopy. Images of the cells (8 bit) were collected and analyzed by using NIH ImageJ (https://imagej.nih.gov/ij/). Mid trophozoite stage parasites (~34-hour post-invasion) were treated with the acetone extract at three concentrations: 35.1 µg/mL (0.5 × $IC_{50\text{-}4\text{-}hour}$), 70.2 µg/mL (1.0 × $IC_{50\text{-}4\text{-}hour}$) and 140.4 µg/mL (2.0 × $IC_{50\text{-}4\text{-}hour}$) based on the $IC_{50\text{-}48\text{-}hour}$ (5.85 µg/mL) obtained from the previous study (Nik Mat Zin *et al*., 2020). Concanamycin A (75 nM), a proton pump inhibitor-treated and untreated parasite were used as positive and negative controls, respectively. Parasite suspensions (5% parasitemia, 2% hematocrit) were added to respective wells containing the extract or concanamycin in 48-well microtiter plates and incubated for 4 hours. The parasites were selectively isolated with 0.035% saponin (w/v) to permeabilize the erythrocyte plasma membrane (EPM) and the parasitophorous vacuolar membrane (PVM), releasing FITC-dextran in the erythrocyte cytoplasm without compromising the parasite plasma membrane (Saliba *et al.*, 2003; Abu-Bakar, 2015). The fluorescence intensity of FITC-dextran in the digestive vacuole of saponin-permeabilized parasites was measured by interpolating R_{gy} in the generated pH calibration curve of FITC-dextran.

Statistical analysis

All experiments were conducted in triplicate (n = 3) on three independent occasions and analyzed with GraphPad Prism 8.0 software. Mean values were expressed as mean ± standard deviation (SD). Mean values were tested for normality before proceeding to one-way analysis of variance (ANOVA) followed by Dunnett multiple comparisons at 95% confidence (comparisons between treated groups against a control group) using Minitab 17.

RESULTS

Morphology of the parasite in the resealed erythrocyte containing FITC-dextran

Figure 1 shows the developmental growth of the parasite in the resealed erythrocyte with FITC-dextran. The appearance of mid and late ring stage parasites (~24-hour post-invasion) was indicated by the indentation of the cytoplasm of the resealed erythrocyte where the parasite was observed as a small dark region in the fluorescence image of the resealed erythrocyte with FITC-dextran (Figure 1B-C, yellow arrows). As the parasite matures to the early trophozoite stage (~28-hours post-invasion), the appearance of hemozoin as fine granules was clearly visible (Figure 1D, red arrows in the bright field image). A small cluster of fluorescence signals appeared inside the parasite compartment (Figure 1D, red arrows), showing that hemoglobin as well as FITC-dextran was endocytosed by the parasite. At the mid trophozoite stage parasite (~34-hours post-invasion), the pigment-containing compartment increased in size (Figure 1E, red arrows), indicating the formation of the digestive vacuole.

Analysis of the digestive vacuole pH of the treated parasites

The R_{gy} in the pH calibration curve of FITC-dextran provides a measure of the pH of the digestive vacuole. An increase in the R_{gy} value (ranged from ~5.7-15.8) with increasing pH

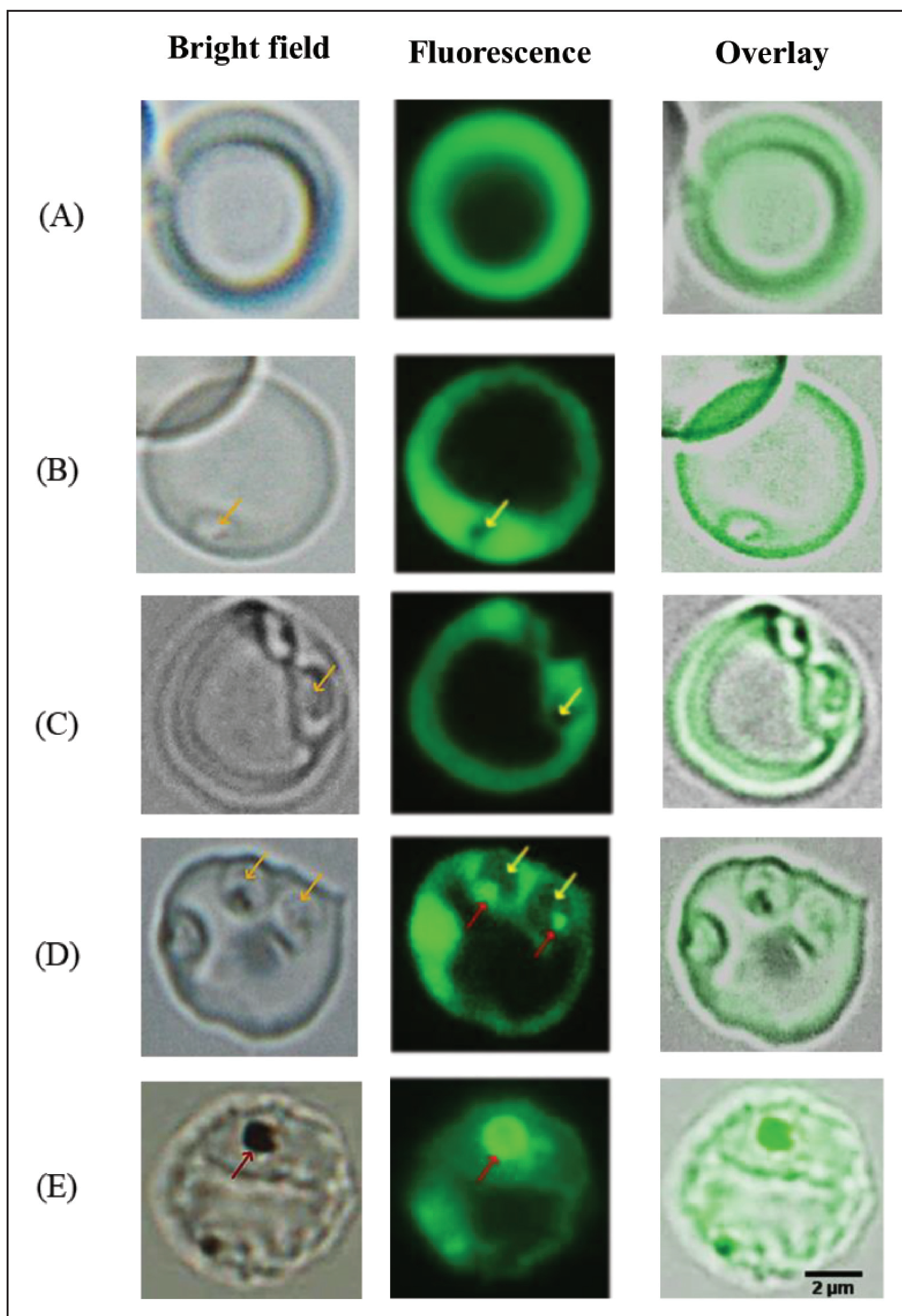

Figure 1. The morphology of *P. falciparum* grown in the resealed erythrocyte containing FITC-dextran (A) Uninfected (control), (B) mid ring stage parasite-, (C) late ring stage parasite-, (D) early trophozoite stage parasite- and (E) mid trophozoite stage parasite-infected resealed erythrocytes containing FITC-dextran. The yellow and red arrows indicate the parasite and the digestive vacuole, respectively. Results are representative of those obtained from three independent experiments done in triplicate. Scale bar: 2 µm.

(4.0-9.0) with pK_a of ~5.8 (the inflection point in the curve) was depicted in Figure 2. The R_{gy} value, however, remained plateau and maintained a steady-state at pH 7.5 and above. Following treatment of mid trophozoite parasites with different concentrations of the acetone extract, the parasites were isolated with saponin to avoid the interference of fluorescence signal from other compartments than the digestive vacuole. Hemozoin was accumulated in the digestive vacuole of the saponin-permeabilized parasite where the fluorescence signal of FITC-dextran was only detected (Figure 3A). Based on the established gating of saponin-permeabilized parasites (Figure 3B, left panel), the ratio (R_{gy}) of the fluorescence intensity of FITC-dextran at green and yellow channels was calculated (middle and right panels). The digestive vacuole pH value was determined by interpolating the R_{gy} with the pH calibration curve of FITC-dextran (Figure 2). The change of the digestive vacuole pH of the parasites treated with the acetone extract was observed and statistically significant in comparison with untreated parasites ($p < 0.001$) (Figure 3C). The digestive vacuole treated with 35.1, 70.2 and 140.4 µg/mL of the acetone extract resulted in an increase of 1.03, 1.23 and 1.39 pH units higher, respectively than those of the untreated parasite's digestive vacuole (pH = 5.32 ± 0.37). Concanamycin A, a V-type H^+-ATPase inhibitor, was able to neutralize the digestive vacuole pH (7.31 ± 0.13). An increase in the digestive vacuole pH of the acetone extract-treated parasites was a concentration-dependent manner.

DISCUSSION

To date, neither studies of the antimalarial activity nor the mechanism of the *Q. infectoria* galls are evident in the literature (Nik Mat Zin *et al.*, 2019). In this work, the evidence of the antimalarial effect of the acetone crude extract on pH of the malaria parasite's digestive vacuole was provided. The treatment with different concentrations of the acetone extract caused an increase in the digestive vacuole pH, which correlates with the high ratio of fluorescence intensities represented as R_{gy}. In contrast, untreated parasites showed an acidic digestive vacuole pH with a low ratio of fluorescence intensities, which is similar to other studies using the same probe (Hayward *et al*., 2006; Abu-Bakar, 2015; Ibrahim *et al.*, 2020). As expected, alkalinization of the pH of the digestive vacuole in concanamycin A-treated parasites was observed. This finding corresponds with the previous studies showing the capability of concanamycin A, a V-type H^+-ATPase inhibitor to disrupt pH regulation by preventing the transportation of H^+ into the digestive vacuole and out of the parasite across the parasite plasma membrane (Saliba *et al.*, 2003; Chan *et al.*, 2012; Pamarthy *et al.*, 2018). This also confirms the validity of the pH alteration of the digestive vacuole following treatment with the acetone extract.

The rise in pH has reduced the parasite's ability to degrade hemoglobin and subsequently detoxify heme in the digestive vacuole (Wunderlich *et al.*, 2012; Lee *et al.*, 2018). Many studies described that the malaria parasite death by

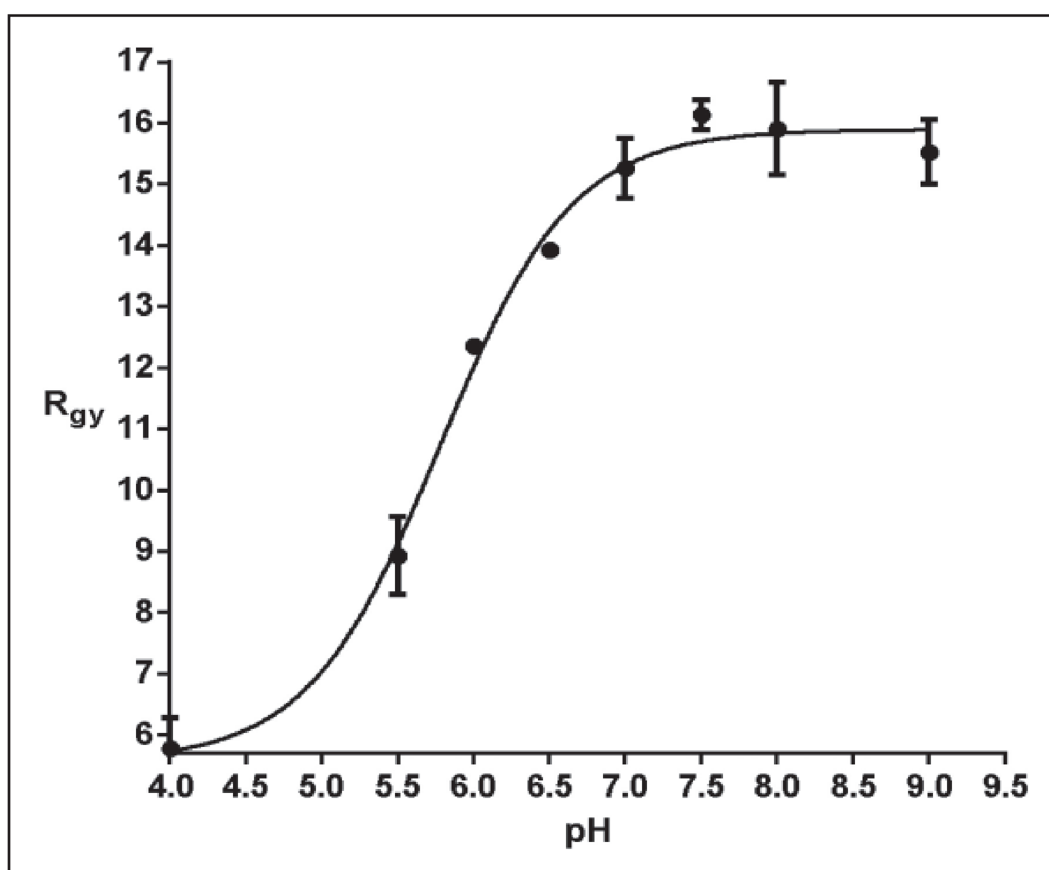

Figure 2. A standard pH calibration curve of FITC dextran A FITC-dextran pH calibration curve was constructed by suspending resealed erythrocytes in buffers of different pH (4.0-9.0) in the presence of CCCP, an ionophore. The fluorescence intensity was collected at green and yellow channels by flow cytometry, and the ratio of green/yellow fluorescence signals (R_{gy}) was plotted on the y-axis against the pH on the x-axis. The dose-response curves were fitted by non-linear regression with Graph Pad Prism ($R^2 = 0.9942$).

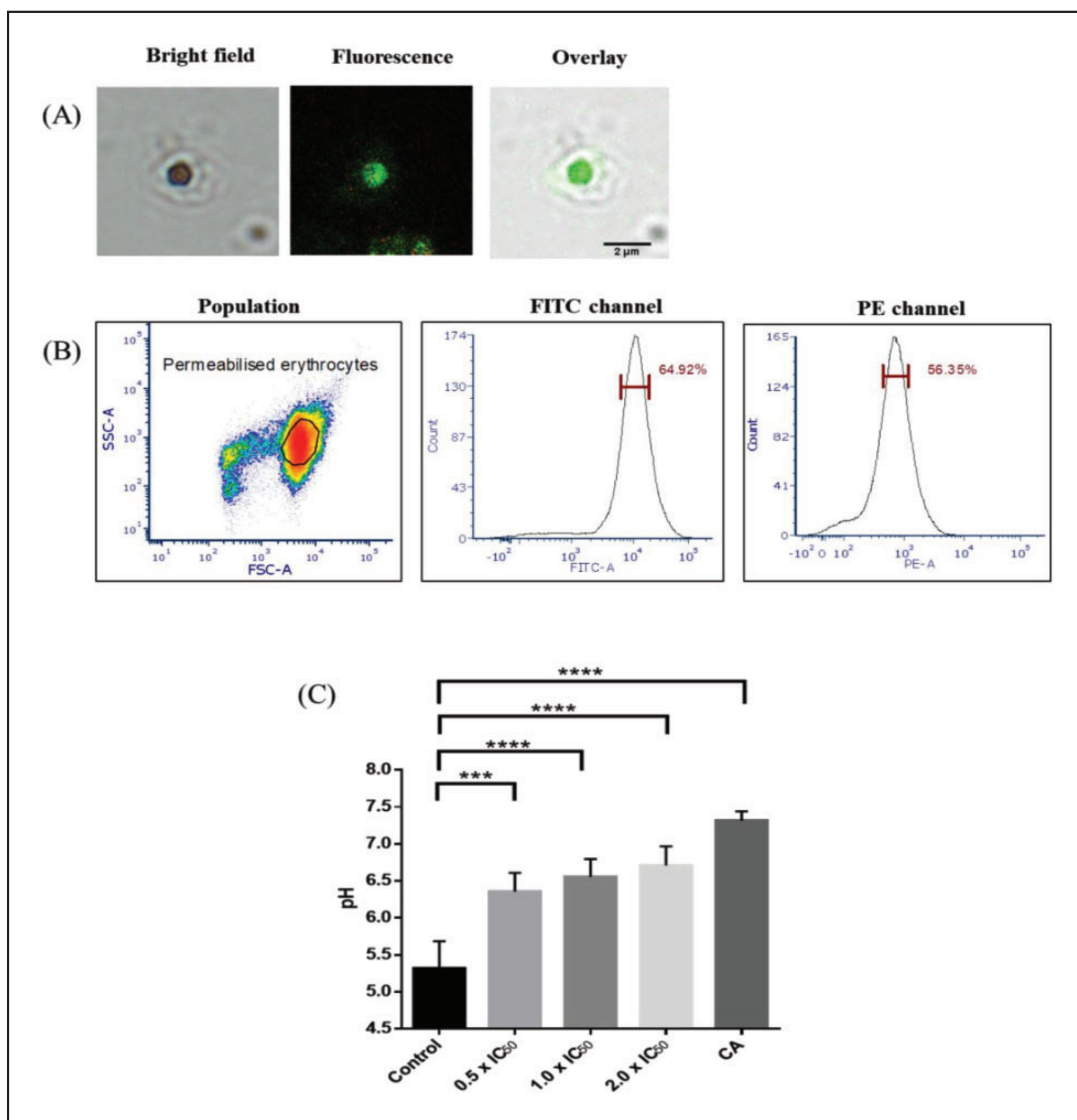

Figure 3. Analysis of the pH of the digestive vacuole of *P. falciparum* after treatment with different concentrations of the *Q. infectoria* acetone extract (A) The appearance of the fluorescence signal following EPM and PVM permeabilization by saponin was observed in the digestive vacuole. Scale bar: 2 μm. (B) Representative scatter and fluorescence intensity profiles of the saponin-permeabilised parasite population at FITC/green and PE/yellow channels. (C) The effect of the digestive vacuole pH after treatment with the acetone extract at three different concentrations: 0.5 × $IC_{50\text{-}4\ hour}$ (35.1 μg/mL), $IC_{50\text{-}4\ hour}$ (70.2 μg/mL) and 2 × $IC_{50\text{-}4\ hour}$ (140.4 μg/mL). Untreated mid trophozoite stage parasite was used as a negative control, while CA (Concanamycin A, 75 nM) was used as a positive control. The ratio (R_{gy}) was calculated and converted to a pH value by means of the standard calibration curve in Figure 1. ***$p < 0.001$ and ****$p < 0.0001$ were considered as statistically significant.

artemisinin treatment was associated with the altered pH of the digestive vacuole (Abu-Bakar *et al.*, 2013; Ibrahim *et al.*, 2020; Mohd-Zamri *et al.*, 2017). Subtle pH changes in other acidic organelles such as lysosomes (increase by 0.1-0.2 units) are known to cause a significant decrease in the lysosomal enzyme activity involved in the digestion process, eventually causing numerous pathological alterations (Xu & Ren, 2015; Colacurcio & Nixon, 2016; Lee & Hong, 2020). In addition, an increase in lysosomal pH could reduce the lysosomal degradation capacity and block autophagosome and endocytic maturation (Lee & Hong, 2020). Evidence has also shown that the depletion of parasite ATP through energy source removal required to pump H^+ altered the digestive vacuole and subsequently alkalized (Collins & Forgac, 2018; Pamarthy *et al.*, 2018).

One possible explanation for the pH alteration in the digestive vacuole upon treatment with the acetone extract could be related with the phenolic-rich compounds in the extract that disturbs the pumping function of the digestive vacuole membrane. Bioactive compounds such as phenolic compounds have been shown to inhibit microorganisms by disrupting cellular membranes, resulting in loss of cellular constituents and eventually death. It is possible due to presence of the hydroxyl (–OH) group in the phenolic compounds as one of the features that has been linked to antimicrobial properties, including antimalarial properties (Kumar & Goel, 2019; Othman *et al*., 2019; Tajuddeen & Van Heerden, 2019). Phenolic glycosides isolated from *Flacourtia indica* commonly known as governor's plum have been shown to act through the inhibition of heme polymerization and β-hematin formation (Singh *et al.*, 2017). The polyphenolic flavonoid, silymarin demonstrated antimalarial activity by forming soluble complexes with free heme, thereby blocking the formation of hemozoin (Mina *et al.*, 2020). Meanwhile, xanthones have been proposed to inhibit heme polymerization (Ke *et al.*, 2017). Ellagic acid has been shown to possess antimalarial activity, which has been linked to the inhibition of plasmepsin II responsible for the digestion of hemoglobin and impairment of β-hematin formation in the parasite (Dell'Agli *et al.*, 2003; Soh *et al.*, 2009; Sturm *et al.*, 2009).

The way phenolic compounds inhibit the enzymes (i.e. plasmepsin II), interfere with heme polymerisation and/or inhibit β-hematin/hemozoin formation is not entirely clarified. However, it is important to note that these activities occur in the digestive vacuole where it is central to hemoglobin digestion and subsequently heme detoxification (Wunderlich *et al*., 2012; Nasamu *et al.*, 2020). All presented mechanisms impose the possibility of the antimalarial activity through physiological alteration of the digestive vacuole such as pH which in turn causes the inhibitory reaction. Because the V-type H^+-ATPase regulating pH maintenance is located in the digestive vacuole, the phenolic-rich galls might probably disturb the pumping function and therefore alter the pH of the digestive vacuole, which was evidenced in this study. The crucial role that the V-type H^+-ATPase plays in maintaining the physiological pH of the digestive vacuole likely accounts for the array of mechanisms that allow a primary explanation for the antimalarial activity of the acetone extract of the *Q. infectoria* galls. The V-type H^+-ATPase is therefore considered as an attractive target for a new antimalarial candidate contained within the acetone extract.

CONCLUSION

The findings have unveiled important mechanism of the *Q. infectoria* galls on the malaria parasite, *P. falciparum* in which the pH of the digestive vacuole treated with the acetone extract increased with the concentration-dependent manner compared to the pH of the untreated digestive vacuole. As this study among the first to investigate the antimalarial effect of the galls in the context of pH alteration inside the digestive vacuole, a considerable amount of work in the molecular mechanisms underlying antimalarial effect is still needed to fully understand the potential of the galls. Knowing the effect of the gall extract on the digestive vacuole, therefore, further studies regarding on the inhibition of hemoglobin uptake, transport and digestion and heme detoxification *in vitro* could be carried out to provide detailed mechanisms of antimalarial action of the extract or isolated compound(s) of the galls.

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Conflict of interest
The authors declare that they have no conflict of interest.

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