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

Nik Mat Zin, N.N.I. 1, Ibrahim, N. 1, Zakaria, Y. 1, Abu-Bakar, N. 1*

1School of Health Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
*Corresponding author: natashaa@usm.my

**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* Kelch13 (Pfk13) gene was involved in the treatment failure with respect to artesinin-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* (IC₅₀ = 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 quer cetin (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, antifungal and antifungalicidal 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
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^+\text-ATPase (V-type H^+\text-ATPase) and pyrophosphatase (V-type H^+\text-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 isotonic resealing buffer A (5 mM Na\text-_3PO_4, 700 mM NaCl, 100 mM (Ibrahim Fischer) were prepared by suspending washed cells in ice-cold PBS buffer containing 10 mM glucose, pH 7.4) before being used. The fluorescence intensity was collected by using NIH ImageJ (https://image..nih.gov/ij/). Mid trophozoite stage parasites (at~24-hour post-invasion) were treated with the acetone extract at three concentrations: 35.1 μg/mL (0.5 × IC\text{50-4-hour}), 70.2 μg/mL (1.0 × IC\text{50-4-hour}) and 140.4 μg/mL (2.0 × IC\text{50-4-hour}) based on the IC\text{50-48-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\text{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\text{gy} in the pH calibration curve of FITC-dextran provides a measure of the pH of the digestive vacuole. An increase in the R\text{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 pKₐ of ~5.8 (the inflection point in the curve) was depicted in Figure 2. The Rₘ 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 (Rₘ) 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 Rₘ 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 Rₘ. 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 (Rₘ) 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 (R² = 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 × IC50-4 hour (35.1 μg/mL), IC50-4 hour (70.2 μg/mL) and 2 × IC50-4 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 (Rgy) 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).
mechanisms impose the possibility of the antimalarial (Wunderlich hemoglobin digestion and subsequently heme detoxification occur in the digestive vacuole where it is central to clarified. However, it is important to note that these activities inhibit plasmepsin II), interfere with heme polymerisation and/or inhibition of plasmepsin II responsible for the digestion of 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.

ACKNOWLEDGEMENT

The authors wish to thank the School of Health Sciences, USM for providing the Research Incentive Grant (1001.PPSK. AUP5001) and the Ministry of Higher Education, Malaysia for providing the Fundamental Research Grant Scheme (FRGS: 203.PPSK.6171225). We also would like to thank Dr. Khairul Mohd Fadzli Mustaffa and Encik Badrul Mat Zain from the Institute for Research in Molecular Medicine (INFORMM), USM Kubang Kerian, Kelantan for giving the 3D7 parasite and access to the laboratory, equipment and cell culture facilities. We acknowledge the generous provision of the Immunology Department from the School of Medical Sciences for flow cytometry instrument throughout the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES


