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Pre-Treatment Of Erythrocytes With Garlic Or Tea Tree Oil Promotes Oxidation Of The Peroxiredoxin 2 Protein And Makes The Cells Less Susceptible To Infection By Plasmodium Falciparum

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ABSTRACT

Plasmodium falciparum, the causal organism of the most deadly form of human malaria, lacks catalase and glutathione peroxidase enzymes and thus is highly dependent on peroxiredoxin (Prx) enzymes for its defence against oxidative stress. In addition to its own five Prx enzymes, *P. falciparum* also uses the human Prx2 protein which it imports from the host erythrocyte. Here we have investigated the effects of pre-treatment of uninfected erythrocytes with increasing concentrations of garlic or tea tree oil on the redox/oligomerization state of the Prx2 protein and on the *P. falciparum* parasitemia in the erythrocytes. Both oils were shown to be able to disrupt the Prx2 redox state in pre-treated uninfected erythrocytes by promoting oxidised dimer formation. Garlic oil was a more potent promotor of the oxidation/inactivation of the Prx2 protein than tea tree oil. The results also showed that both oils promoted oxidation/inactivation of the Prx2 protein at the 2nd generation ring stage and also appeared to promote oxidation of other sulfhydryl group-containing proteins. Additionally, pre-treatment of uninfected erythrocytes with the test oils made the erythrocytes less susceptible to infection by *P. falciparum* at the 2nd generation ring stage (new infections). Garlic oil was more effective than tea tree oil in this respect. Thus, oxidation of the Prx2 protein might be involved in decreasing the susceptibility of pre-treated uninfected erythrocytes to infection by *P. falciparum*. These results suggest that garlic and tea tree oils could be used as antimalarial drugs.

Keywords: *Plasmodium falciparum*, *Peroxiredoxin 2*, *Garlic oil*, *Tea tree oil*

1. INTRODUCTION

Plasmodium falciparum is the causal organism of the most deadly form of human malaria. Increasing resistance to antimalarial drugs is the biggest problem currently facing malaria control [1]. The need for new antimalarial drugs less prone to the development of resistance is becoming increasingly urgent. Alternatively, some scientists have proposed a new strategy for malaria treatment called "host-directed therapy" which targets host proteins vital to the parasite instead of parasite proteins [2, 3]. This strategy may be a solution to treat malaria and to avoid antimalarial drug resistance.

Erythrocytes are exposed to a high level of oxidative stress from the continuous autoxidation of haemoglobin that produces reactive oxygen species (ROS) including hydrogen peroxide. Thus, they contain effective H_2O_2 removing enzymes including catalases, glutathione peroxidases and peroxiredoxins [4]. Peroxiredoxin (Prx) proteins belong to a family of thiol-specific antioxidant enzymes that catalyse the reductive decomposition of H_2O_2 and various organic hydroperoxides to less reactive products [5]. Human erythrocytes contain Prx1, Prx2 (Torin or Calpromotin) and Prx6 proteins [4]. In mammals, the Prx1 protein is located in the cytosol or the nucleus, the Prx2 protein is located in the cytosol or associated with the erythrocyte membrane and the Prx6 protein is located in the cytosol [5]. The Prx1 and Prx2 proteins belong to the typical 2-Cys Prx subfamily of Prx proteins and they are characterized by the presence of peroxidatic Cys (C_P) and resolving Cys (C_R) residues whereas the Prx6 protein belongs to the 1-Cys Prx subfamily and it has only C_P [5]. The Prx2 protein is the third most abundant protein in erythrocytes with an intracellular concentration of either 5.6 mg/ml (240 µM) or 26.8 µg/mg of packed erythrocytes, depending on the assay method [4, 6]. This compares with 0.31 µg/mg for the Prx1 protein and 0.14 µg/mg for the Prx6 protein [4]. Thus, the Prx2 protein concentration is 88 times greater than the concentration of the Prx1 protein and 192 times greater than the concentration of the Prx6 protein. The human erythrocyte Prx2 protein has been shown to react with H_2O_2 with a rate constant of 1.3×10^7 M^{-1} s^{-1} which is comparable with human catalase (3×10^7 M^{-1} s^{-1}) [7]. In the human typical 2-Cys Prx proteins, at low concentrations of H_2O_2, C_P in its reduced/thiol state (C_P-SH) reacts with H_2O_2 and becomes oxidised to a Cys sulphenic acid residue (C_P-SOH). C_P-SOH forms a disulphide bond with C_R on a separate subunit. The disulphide bond then is broken with the help of thioredoxin and thioredoxin reductase and C_P-SH is restored ready to begin another cycle of catalysis. However, at high concentrations of H_2O_2, C_P-SOH becomes hyperoxidised to a Cys sulphinic acid residue (C_P-SO_2H) that is catalytically inactive [8, 9]. In contrast to other human typical 2-Cys Prx proteins, the human erythrocyte Prx2 protein is relatively insensitive to hyperoxidation [10]. When the typical 2-Cys Prx proteins are trapped either in the oxidized or the hyperoxidised state, they are unable to react with and reduce H_2O_2 or any other hydroperoxides. Overall, the Prx2 protein plays a significant role in erythrocyte defence against oxidative stress [10].

The intraerythrocytic stage of the *P. falciparum* parasite is exposed to high levels of oxidative stress caused by host produced ROS and by byproducts of haem degradation which the parasite uses for its nutrition [11, 12]. Consequently, *P. falciparum* requires efficient antioxidant enzymes to survive under these conditions. Interestingly, *P. falciparum* lacks both catalase and glutathione peroxidase enzymes and thus is highly dependent on Prx enzymes for H_2O_2 detoxification and protection against oxidative stress [13]. Therefore, the Prx proteins in *P. falciparum* have been intensively investigated as important potential targets for antimalarial drug therapy. The *P. falciparum* parasite possesses five Prx isozymes which are Prx1a, Prx1m, PrxQ, Prx5 and Prx6 [14]. In addition to this, the human Prx2 protein is imported from the host erythrocyte into the cytoplasm of *P. falciparum* and the parasite uses this protein to protect itself against oxidative stress [15]. For example, it has been estimated that approximately half the peroxide detoxification activity of *P. falciparum* is catalyzed by the erythrocyte Prx2 protein [15]. Thus, the aim of the present study was to investigate the effects of pre-treatment of human erythrocytes with garlic or tea tree oil on the redox/oligomerization state of the Prx2 protein in the erythrocytes and on the *P. falciparum* parasitemia in the erythrocytes. It was predicted that treatment of uninfected erythrocytes

with garlic and/or tea tree oil would promote oxidation/inactivation of the Prx2 protein in these cells and reduce their infection by *P. falciparum*.

2. MATERIALS AND METHODS

2.1 *Plasmodium falciparum* culture

2.1.1 Preparation of the culture medium

All reagents were purchased from Life Technologies, unless otherwise stated. The *Plasmodium* culture medium [16] consisted of RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 0.4% (w/v) albumax II, 24 mM sodium bicarbonate, 0.184 mM hypoxanthine (Sigma-Aldrich) and 200 units ml^{-1} penicillin/streptomycin.

2.1.2 Preparation of the erythrocytes

Human erythrocytes suspended in an isotonic solution were obtained from the Australian Red Cross Blood Service. The erythrocytes were harvested by centrifugation at 500 *g* for 5 min and then washed twice with *Plasmodium* washing medium (*Plasmodium* culture medium without albumax II, sodium bicarbonate or hypoxanthine) [16]. The washed erythrocytes were harvested as described above and were then resuspended in an equal volume of *Plasmodium* culture medium.

2.1.3 General culturing techniques *for P. falciparum*

Plasmodium falciparum 3D7 strain parasites were used in this study. The parasites were maintained in human erythrocytes prepared as described above at 2% haematocrit in the *Plasmodium* culture medium. The parasites were cultured at 37^{o}C using the candle-jar technique as previously described [17]. The parasitemia level was maintained between 5 to 10% and the *Plasmodium* culture medium was replaced daily. Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee for the use of human blood to culture the *P. falciparum* parasite. Biosafety approval was obtained from the Flinders University Institutional Biosafety Committee (IBC) for the culture of the *P. falciparum* parasite.

2.1.4 Determination of parasitemia

A thin film of erythrocytes from a *P. falciparum* culture was smeared on a glass microscope slide and air-dried. The smeared slides were then fixed in methanol for 30 sec and air-dried again. Subsequently, the slides were incubated for 5 – 10 min in 10% (v/v) Giemsa stain solution (Sigma-Aldrich) diluted in phosphate buffered saline (PBS, pH 7.4) as previously described [18]. The stained slides were rinsed with deionized water and air-dried. The parasitemia levels were determined by counting the percentage of parasite-infected erythrocytes in 1,200 cells in Giemsa-stained thin blood smears using an Olympus CH-2 light microscope at 1000X magnification.

2.1.5 Stage synchronisation of parasites

P. falciparum cultures with a large number of young ring-stage parasites were selected for synchronisation [16]. Synchronisation was performed using 5% (w/v) sorbitol (Sigma-Aldrich) as previously described [18]. The principle behind the method is as follows. Unsynchronised *P. falciparum* cultures contain erythrocytes infected with ring-, trophozoite- and schizont-stage parasites as well as uninfected erythrocytes. The membranes of erythrocytes infected with trophozoite- and schizont-stage *P. falciparum* are permeable to sorbitol whereas uninfected erythrocytes and those infected with ring-stage *P. falciparum* are not. Thus, sorbitol treatment lyses erythrocytes infected with trophozoite- and schizont-stage *P. falciparum* but leaves those that are infected with ring-stage *P. falciparum* or those that are uninfected, intact. In brief, the method was as follows. Erythrocytes that had been cultured with *P. falciparum* were harvested by centrifugation at 250 *g* for 5 min. The harvested cells were then resuspended in 5% (w/v) sorbitol in PBS (pH 7.4) and incubated for 10 min at 37^{o}C. The sorbitol treated cells were washed twice with *Plasmodium* culture medium. The synchronised ring-stage parasites obtained in this way were resuspended in *Plasmodium* culture medium and cultured as described above or were used in parasitemia experiments (see below). Sorbitol synchronisation was performed twice a week to maintain synchronicity.

2.2 Pre-treatment of uninfected erythrocytes with garlic or tea tree oil

Stock solutions of garlic (*Allium sativum*) oil (100%, v/v; Mystic Moments, Fordingbridge, Hampshire, United Kingdom) and tea tree (*Melaleuca alternifolia*) oil (100%, v/v; Integria Healthcare Australia Pty Ltd) were prepared in dimethyl sulfoxide (DMSO). Following this, the

required dilutions of each of the oils were prepared in *Plasmodium* culture medium and the final concentration of DMSO was always 0.1% (v/v). Erythrocytes prepared as described above were exposed to various concentrations of either garlic oil or tea tree oil at 4% haematocrit in a total volume of 15 ml *Plasmodium* culture medium in 25 cm^2 cell culture flasks (SARSTEDT). The control cells were exposed to 0.1% (v/v) DMSO prepared in *Plasmodium* culture medium. The control and treated cells were incubated for 10 min at 37^oC using the candle-jar technique [17] and then harvested and washed twice with 25 ml of *Plasmodium* culture medium relative to 600 µl of pre-treated erythrocytes. The erythrocytes treated in this way were immediately used in either parasitemia experiments or protein experiments. There were three replicate flasks per concentration used in the parasitemia experiments and one flask per concentration used in the protein experiments.

2.3 Preparation of extracts of the pre-treated uninfected erythrocytes for the determination of the relative amounts of the reduced and oxidised forms of the Prx2 protein

The uninfected erythrocytes, pre-treated and washed as described above, were resuspended and then incubated for 15 min in a buffer containing 100 mM N-ethylmaleimide (NEM) to alkylate the sulfhydryl group (-SH) of C_P in the reduced monomer state of the typical 2-Cys Prx proteins [19]. The complete alkylation buffer contained 40 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.4), 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 mM phenylmethanesulfonyl fluoride (PMSF) and 100 mM NEM. Following this incubation, the cells were disrupted by the addition of 1% (w/v) 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS). Any insoluble material was removed by centrifugation at 16,000 *g* for 30 min at 4^oC and the supernatant was stored at -80^oC until it could be analysed. The protein concentration in the supernatant was determined using the BCA protein assay (Thermo Fisher Scientific). All experiments were performed three times and similar results were obtained in the replicate experiments. The results of a representative experiment are shown here.

2.4 Separation of the reduced and oxidised forms of the erythrocyte Prx2 protein using non-reducing polyacrylamide gel electrophoresis

When subjected to non-reducing polyacrylamide gel electrophoresis (NR-PAGE), the reduced form of the Prx2 protein runs as a monomer with a molecular weight of approximately 22 kDa whereas the oxidised form runs as a dimer with a molecular weight of approximately 44 kDa [20]. These different sizes can be resolved using NR-PAGE. Thus, the extracts prepared as described above were first of all mixed with an equal volume of loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol and 0.025% (w/v) bromophenol blue. These mixtures were then loaded into the wells of a NR-PAGE gel (60 µg protein per well). The NR-PAGE gels consisted of a stacking gel containing 0.625 M Tris-base (pH 6.8), 4% (w/v) acrylamide:N,N′-methylenebisacrylamide (37.5:1), 0.125% (v/v) N,N,N’,N’-tetramethyl-ethylenediamine (TEMED) and 0.05% (w/v) ammonium persulphate and a resolving gel containing 0.375 M Tris-base (pH 8.8), 10% (w/v) acrylamide:N,N′-methylenebisacrylamide (37.5:1), 0.1% (v/v) TEMED and 0.05% (w/v) ammonium persulphate. The gels were run in a buffer containing 25 mM Tris-base (pH 8.3), 192 mM glycine and 0.1% (w/v) SDS in a Bio-Rad Mini-PROTEAN®II electrophoresis apparatus set to deliver 200 V. At the end of the run, the gels were either stained for protein using Coomassie Blue or used for immunoblotting (see below). The Coomassie Blue protein staining solution contained 0.1% (w/v) Coomassie Brilliant Blue R-250 stain, 50% (v/v) methanol and 10% (v/v) glacial acetic acid. The destaining solution contained 50% (v/v) methanol and 10% (v/v) glacial acetic acid. Imaging of the gels was performed using a Bio-Rad Gel DocTM EZ Imager.

2.5 Detection of the reduced and oxidised forms of the erythrocyte Prx2 protein using immunoblotting

The reduced and oxidised forms of the erythrocyte Prx2 protein separated as described above were transferred to a nitrocellulose

membrane (Bio-Rad) using a Bio-Rad Mini Trans-Blot® apparatus set to deliver a constant current of 200 mA for 2 hours. The transfer buffer contained 50 mM Tris-base, 380 mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol. Following the transfer, the membrane was blocked for one hour at room temperature in a blocking buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% (v/v) Tween-20 and 5% (w/v) skim milk powder. Subsequently, the blocked membrane was incubated overnight at 4^{o}C in blocking buffer containing a 1:1000 dilution of anti-(Prx2) polyclonal antibodies produced by immunizing a rabbit with a full-length, recombinantly-expressed and highly purified Prx2 protein [21]. Following the overnight incubation with the anti-(Prx2) antibodies, the membrane was washed 5 x 5 minutes with washing buffer (blocking buffer minus the milk powder) and then incubated for 1-2 hours at room temperature with a secondary antibody preparation diluted 1:1000 in blocking buffer. The secondary antibody preparation contained goat anti-(rabbit IgG) conjugated to horseradish peroxidase (Rockland Immunochemicals for Research). Any cross-reacting proteins were detected using the SuperSignal® West Pico Chemiluminescent substrate kit (Thermo SCIENTIFIC) and images of the blots were made using a Bio-Rad ChemiDocTM MP imaging system.

2.6 Parasitemia experiments

Ring-stage parasites synchronised as described above were seeded into 96-well plates (Corning, round bottom) together with uninfected erythrocytes (pre-treated with DMSO or the test oils as described above) at a parasitemia level of 2% and a hematocrit of 4%, including ~3% (v/v) untreated erythrocytes and ~97% (v/v) pre-treated erythrocytes, in 200 µl of *Plasmodium* culture medium. There were 3 replicate wells for the control cells pre-treated with 0.1% (v/v) DMSO and for each oil concentration used to pre-treat the uninfected erythrocytes. The plates were incubated for 6 h (corresponding to the ring stage), 22 h (corresponding to the trophozoite stage), 38 h (corresponding to the schizont stage) or 52 h (corresponding to the second generation-ring stage) [16] at 37^{o}C using the candle-jar technique [17]. The culture medium was replaced every 24 hours. At each time-point, the parasitemia was determined by counting the percentage of infected erythrocytes in 1,200 cells according to the protocol described above. The experiments were performed twice and similar results were obtained in the replicate experiments. The results of a representative experiment are shown here.

2.7 Preparation of extracts of erythrocytes cultured with *P. falciparum* for the determination of the relative amounts of the reduced and oxidised forms of the erythrocyte Prx2 protein

Ring-stage parasites synchronized as described above were seeded into 25 cm^2 cell culture flasks (SARSTEDT) together with uninfected erythrocytes (pre-treated with DMSO or the test oils as described above) at a parasitemia level of 2% and a hematocrit of 4% in 15 ml of *Plasmodium* culture medium. The *P. falciparum* cells were cultured for 52 h (corresponding to the 2nd generation ring stage) at 37^{o}C using the candle-jar technique [17]. At the end of the culture period, the erythrocytes were harvested by centrifugation at 250 *g* for 5 min at 37^{o}C. The harvested cells were then resuspended and incubated for 15 min in NEM buffer. Following this incubation, cell extracts were prepared as described above. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific). The reduced and oxidised forms of the Prx2 protein were separated using NR-PAGE gels as described above and detected using immunoblotting as described above. All experiments were performed twice and similar results were obtained in the replicate experiments. The results of a representative experiment are shown here.

2.8 Statistical analyses

Statistical analyses were conducted using the IBM SPSS Statistics 19 software package. The data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* multiple comparisons test. Differences were considered to be significant when $p < 0.05$.

3. RESULTS

3.1 Effects of garlic and tea tree oils on the total soluble proteins extracted from uninfected erythrocytes and on the redox/oligomerization state of the Prx2 protein in these cells

The effects of pre-treatment of the uninfected erythrocytes with either garlic or tea tree oil on the total soluble proteins extracted from the cells and on the redox/oligomerization state of the typical 2-

Cys Prx2 protein in the cells are shown in Figure 1. When the uninfected erythrocytes were treated with increasing concentrations of either garlic or tea tree oil, a prominent smaller protein (~22 kDa) became less abundant whereas two prominent larger proteins (~44 kDa and ~58 – 60 kDa) became more abundant (Figures 1A-B). Immunoblotting analysis using anti-Prx2 antibodies showed that the antibodies reacted with the ~22 kDa and ~44 kDa proteins and therefore these must be the erythrocyte Prx2 proteins (Figures. 1C-D). In contrast, these antibodies did not react with the ~58 – 60 kDa protein and thus it was another protein. Thus, the ~22 kDa protein was the reduced monomer form of the Prx2 protein whereas the ~44 kDa protein was the oxidised dimer form of the Prx2 protein and the identity of the ~58 – 60 kDa protein is unknown (Figures 1A-B). When the uninfected erythrocytes were treated with increasing concentrations of garlic or tea tree oil, there was increasing conversion of the reduced monomer form of the Prx2 protein to the oxidised dimer form and there was also increasing abundance of the unknown ~58 – 60 kDa protein (Figures 1A-D). Both oil treatments induced oxidation of the Prx2 protein in erythrocytes. Garlic oil was more effective than tea tree oil in this respect. Overall, garlic and tea tree oil treatments were able to disrupt the redox state of the Prx2 protein in uninfected erythrocytes through promoting oxidised dimer formation.

3.2 Effects of pre-treatment of uninfected erythrocytes with garlic or tea tree oil on *P. falciparum* parasitemia

The effects of pre-treatment of uninfected erythrocytes with garlic or tea tree oil on the *P. falciparum* parasitemia at different stages of the parasite's lifecycle, the ring, trophozoite, schizont and 2nd generation ring (new infections) stages, were investigated. Pre-treatment of the uninfected erythrocytes with 0.01, 0.1 or 1.0% (v/v) garlic oil had no effect on the *P. falciparum* parasitemia at 6 h (ring stage), 22 h (trophozoite stage) or 38 h (schizont stage) after the erythrocytes had been inoculated with synchronised ring stage parasites (Figure 2). This suggested that the ring stage parasites used for the inoculation were well synchronised and that *P. falciparum* developed inside the infected erythrocytes until 44 – 48 hours (the last stage of the parasite's life cycle). In contrast, pre-treatment of the uninfected erythrocytes with garlic oil significantly decreased the *P. falciparum* parasitemia at 52 hours (corresponding to the 2nd generation ring stage) after the erythrocytes had been inoculated with synchronised ring stage parasites (Figure 2). This suggested that pre-treatment of the uninfected erythrocytes with garlic oil made them less susceptible to infection by *P. falciparum*. In addition, with increasing concentrations of garlic oil, there was also significantly reducing infection of the pre-treated uninfected erythrocytes with *P. falciparum* (Figure 2). Giemsa-stained thin blood smears showed that 0.01% (v/v) garlic oil had no effect on the total number of intact erythrocytes at all time points. However, 0.1 and 1.0% (v/v) garlic oil reduced the number of intact erythrocytes at all time points to approximately 83% and 25%, respectively, of the control value.

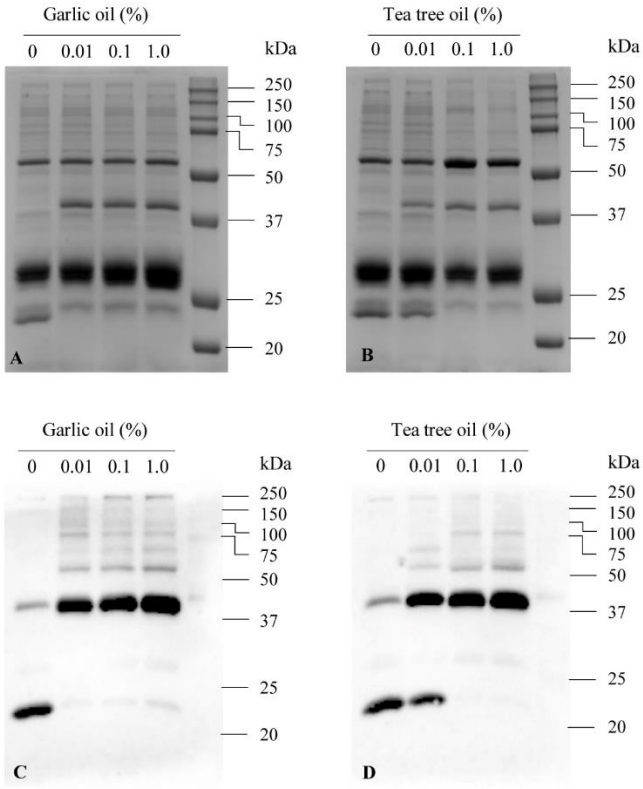

Figure 1. The effects of treating uninfected erythrocytes with garlic or tea tree oil on the redox/oligomerization state of the Prx2 protein in these cells. Each lane contains 60 µg of the total soluble proteins extracted from the erythrocytes. The proteins were separated using NR-PAGE. In panels A and B, the proteins were stained with Coomassie Blue. In panels C and D, the Prx2 protein was detected using anti-(Prx2) antibodies.

Thus, at the lowest garlic oil concentration, the decrease in parasitemia was due to an inhibitory effect of the oil on infection of the erythrocytes by the parasite. However, at 0.1 or 1.0% (v/v) garlic oil, increased erythrocyte fragility leading to a decrease in the total number of intact cells available for infection by the parasite was presumably the main reason for the decreased in *P. falciparum* parasitemia at these concentrations, in particular 1.0% (v/v). In conclusion, erythrocytes pre-treated with 0.01% (v/v) garlic oil became less susceptible to infection by *P. falciparum*.

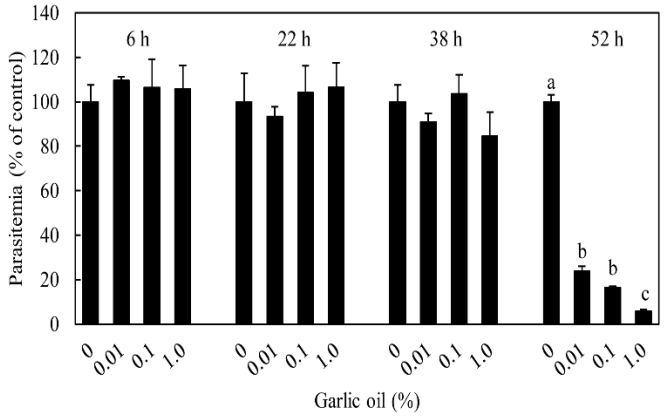

Figure 2. The effect of pre-treatment of erythrocytes with garlic oil on P. falciparum parasitemia. The time points correspond to the different intraerythrocytic stages of the parasite's life cycle. The data are the mean values for 3 replicates and the vertical bars represent ± standard error of the mean (n = 3). Values labelled with different letters are significantly different (p < 0.05).

Pre-treatment of the uninfected erythrocytes with 0.01, 0.1 or 1.0% (v/v) tea tree oil had no effect on the *P. falciparum* parasitemia at 6 h (ring stage), 22 h (trophozoite stage) or 38 h (schizont stage) after the erythrocytes had been inoculated with synchronised ring stage parasites (Figure 3). This supported our conclusion that the ring stage parasites used for the inoculation were well synchronised and that *P. falciparum* developed inside the infected erythrocytes until the last stage of the parasite's life cycle. In contrast, pre-treatment of the uninfected erythrocytes with tea tree oil significantly decreased the *P. falciparum* parasitemia at 52 hours (corresponding to the 2nd generation ring stage) after the erythrocytes had been inoculated with synchronised ring stage parasites (Figure 3). This suggested that erythrocytes pre-treated with tea tree oil became less susceptible to infection by *P. falciparum*. Furthermore, with increasing concentrations of tea tree oil, there was also significantly reducing infection of the pre-treated uninfected erythrocytes with *P. falciparum* (Figure 3). Giemsa-stained thin blood smears showed that 0.01% (v/v) tea tree oil had no effect on the total number of intact erythrocytes at all time points. However, 0.1 and 1.0% (v/v) tea tree oil reduced the number of intact erythrocytes at all time points to approximately 58% and 11%, respectively, of the control value. Thus, at the lowest tea tree oil concentration, the decrease in parasitemia was due to an inhibitory effect of the oil on infection of the erythrocytes by the parasite. However, at 0.1 or 1.0% (v/v) tea tree oil, the more likely reason for the decrease in parasitemia is that the medium was toxic to the extracellular merozoite as a result of cell lysis releasing toxic intracellular proteins (e.g. oxidised hemoglobin). Overall, erythrocytes pre-treated with 0.01% (v/v) tea tree oil became less susceptible to infection by *P. falciparum*.

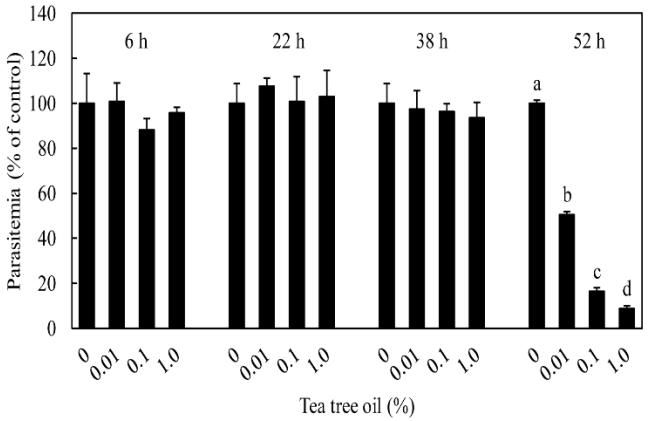

Figure 3. The effect of pre-treatment of erythrocytes with tea tree oil on P. falciparum parasitemia. The time points correspond to the different intraerythrocytic stages of the parasite's life cycle. The data are the mean values for 3 replicates and the vertical bars represent ± standard error of the mean (n = 3). Values labelled with different letters are significantly different (p < 0.05).

In summary, pre-treatment of uninfected erythrocytes with either 0.01% (v/v) garlic oil or 0.01% (v/v) tea tree oil made the erythrocytes less susceptible to infection by *P. falciparum* at the 2nd

generation ring stage and garlic oil was more effective than tea tree oil in this respect. As shown above, garlic and tea tree oil promoted oxidation/inactivation of the Prx2 protein in pre-treated erythrocytes (Figures 1C-D). Thus, we suggest that oxidation/inactivation of the Prx2 protein might be the cause of the reduced susceptibility of the erythrocytes to infection by *P. falciparum*. This hypothesis is further tested below by culturing *P. falciparum* with uninfected erythrocytes that had been pre-treated with garlic or tea tree oil.

3.3 Effect of pre-treatment with garlic or tea tree oil and culture with P. falciparum on the redox/oligomerization state of the Prx2 protein in erythrocytes at the 2nd generation ring stage

We have hypothesized that oxidation/inactivation of the Prx2 protein in uninfected erythrocytes (pre-treated with garlic or tea tree oil) might be involved in decreasing the susceptibility of these cells to infection by *P. falciparum* at the 2nd generation ring stage. Thus, in a follow-up experiment, we treated erythrocytes with either garlic or tea tree oil and then cultured them with *P. falciparum* to determine whether the effect on the erythrocyte Prx2 protein was the same in infected cells as it had been in uninfected cells. The results are shown in Figure 4. When the erythrocytes were pre-treated with increasing concentrations of either garlic or tea tree oil, prominent smaller proteins (~22 kDa and ~28 - 30 kDa) became less abundant whereas a prominent larger protein (~58 - 60 kDa) became more abundant (Figures 4A-B). Garlic oil was more effective than tea tree oil in this respect. Immunoblotting analysis using anti-Prx2 antibodies revealed that the antibodies reacted with the ~22 kDa protein (less abundant in the gels stained for total protein) and the ~44 kDa protein. Thus, these bands must be the erythrocyte Prx2 protein (Figures 4C-D). In contrast, these antibodies did not react with the ~28 - 30 kDa protein or the ~58 - 60 kDa protein and thus these were other proteins. Thus, the ~22 kDa protein was the reduced monomer form of the Prx2 protein whereas the ~44 kDa protein was the oxidised dimer form of the Prx2 protein and the identities of the ~28 - 30 kDa and ~58 - 60 kDa proteins are unknown (Figures 4A-D). With increasing concentrations of garlic or tea tree oil, there was increasing conversion of the reduced monomer form of the Prx2 protein to the oxidised dimer form and there was also increasing abundance of the unknown ~58 - 60 kDa protein and there was decreasing abundance of the unknown ~28 - 30 kDa protein (Figures 4A-D). Both oil treatments promoted oxidation of the Prx2 protein in the pre-treated erythrocytes at the 2nd generation ring stage but garlic oil was more effective than tea tree oil in this respect. In conclusion, garlic and tea tree oil induced oxidation/dimerization of the Prx2 protein in pre-treated erythrocytes, cultured with *P. falciparum*, at the 2nd generation ring stage.

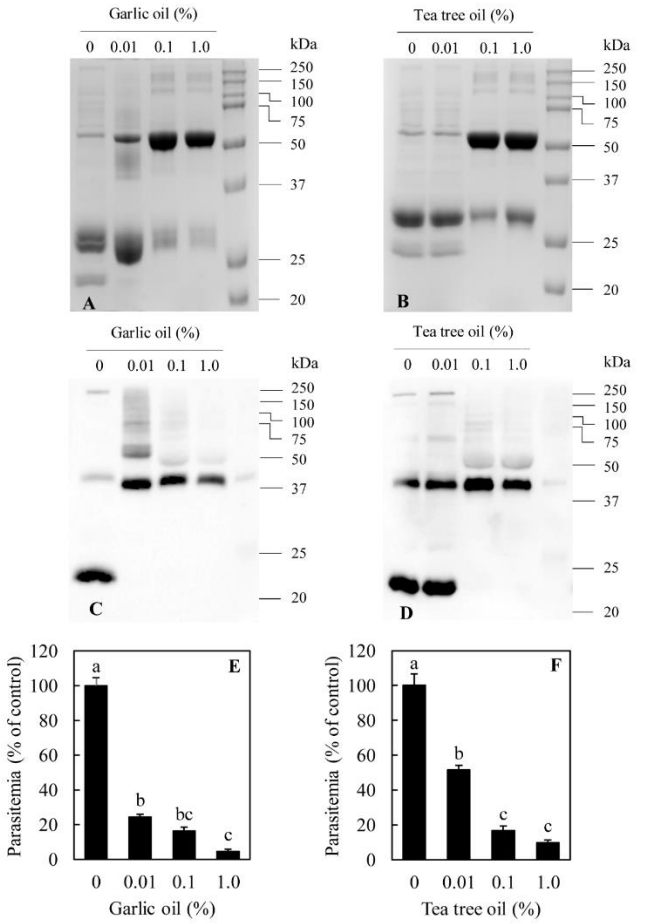

Figure 4. The effect of pre-treatment of erythrocytes with garlic or tea tree oil and their culture with P. falciparum on the redox/oligomerization state of the Prx2 protein in these cells at the 2nd generation ring stage of P. falciparum. Each lane contains 60 µg of the total soluble proteins extracted from the erythrocytes. The proteins were separated using NR-PAGE. In panels A and B, the proteins were stained with Coomassie Blue. In panels C and D, the Prx2 protein was detected using anti-(Prx2) antibodies. Panels E

and F show the effect on P. falciparum parasitemia at the 2nd generation ring stage. The data are the mean values for 3 replicates and the vertical bars represent ± standard error of the mean (n = 3). Values labelled with different letters are significantly different (p < 0.05).

In summary, both oils promoted oxidation of the Prx2 protein at the 2nd generation ring stage (52 h) and also appeared to promote oxidation of other sulfhydryl group-containing proteins. Garlic oil was more effective than tea tree oil at promoting these effects. In addition to this, garlic oil was more effective than tea tree oil in reducing the *P. falciparum* parasitemia in pre-treated erythrocytes at the 2nd generation ring stage (Figures 4E-F). This suggests that oxidation/inactivation of the Prx2 protein, and possibly other sulfhydryl group-containing proteins, might be involved in decreasing the susceptibility of pre-treated erythrocytes to infection by *P. falciparum*.

4. DISCUSSION

The human malaria parasite, *P. falciparum*, possesses multiple Prx enzymes but lacks any other antioxidant enzymes including catalase and GPx enzymes [13]. Therefore, the Prx enzymes are considered to play an important role in protecting the parasite against oxidative stress, especially during the intraerythrocytic stage of its life-cycle where there are high levels of ROS produced both by the host and as byproducts of the parasite's metabolism [13]. The human erythrocyte Prx2 protein is imported by the *P. falciparum* parasite and has been found to be responsible for ~50% of the total peroxide detoxification activity of *P. falciparum* during the intra-erythrocytic stage [15]. It is known that the human Prx2 protein undergoes oxidised dimer formation in erythrocytes exposed to high concentrations of H_2O_2 [10]. When the typical 2-Cys Prx proteins are in the oxidised dimer form, the –SH group of C_P is linked to C_R via a disulphide bond and thus it cannot link with H_2O_2, resulting in cessation of the reaction cycle [9]. Thus, here we have investigated the effects of pre-treatment of uninfected erythrocytes with garlic and tea tree oils on the redox/oligomerization state of the Prx2 protein in the erythrocytes and on the *P. falciparum* parasitemia in the erythrocytes. We predicted that treatment of uninfected erythrocytes with garlic and/or tea tree oil would promote oxidation/inactivation of the Prx2 protein in these cells and thereby reduce the infection of the erythrocytes by *P. falciparum*. We found that garlic and tea tree oils promoted oxidised dimer formation in the Prx2 protein in uninfected erythrocytes and made them less susceptible to infection by *P. falciparum* at the 2nd generation ring stage (new infections). Garlic oil was more effective at promoting these effects than tea tree oil. It is known that the *P. falciparum* parasite is able to complete the infection process of fresh erythrocytes in a relatively short period of time 30 – 90 sec [22]. In our experiments, the *P. falciparum* parasite was incubated with the pre-treated uninfected erythrocytes for 52 h but did not seem to be able to infect these cells at the 2nd generation ring stage. In addition to this, at the 2nd generation ring stage, the Prx2 protein was found to be oxidised in erythrocytes pre-treated with garlic or tea tree oil. This led to the supposition that the disruption of the redox state of the Prx2 protein in erythrocytes by garlic or tea tree oil might be involved in decreasing the susceptibility of erythrocytes to infection by *P. falciparum*. In a similar study to ours, human erythrocytes were pre-treated with up to 25 µM Conoidin A (2,3-bis(bromomethyl)-1,4-dioxide-quinoxaline) which is known to promote oxidised dimer formation in typical 2-Cys Prx proteins (Prx1 and Prx2) in epithelial cells [23, 24]. In that study it was observed that Conoidin A did not prevent *P. falciparum* parasite infection of pre-treated uninfected erythrocytes but it did prevent the development of the intraerythrocytic stage of the *P. falciparum* parasite to other stages [23]. Like Conoidin A, garlic and tea tree oils promoted oxidised dimer formation of the Prx2 proteins in the uninfected erythrocytes but unlike Conoidin A, both oils were shown to make the pre-treated uninfected erythrocytes less susceptible to infection by *P. falciparum*. Thus, this supports our conclusion that oxidation/inactivation of the erythrocyte Prx2 protein by garlic and tea tree oils in the pre-treated uninfected erythrocytes inhibits infection of these cells by *P. falciparum*.

Interestingly, we found that treatment with either garlic or tea tree oil increased the abundance of an unknown ~58 – 60 kDa protein in uninfected erythrocytes. This protein could be increasing in abundance because it is more stable in the

presence of garlic or tea tree oil than other proteins. Human erythrocytes have a cytosolic chaperonin with a molecular weight of 59.3 kDa [25]. This chaperonin protein belongs to a class of molecular chaperones that prevent aggregation of other proteins under normal and stress conditions [26]. Thus, the unknown ~58 – 60 kDa protein might be this chaperonin protein. Alternatively, human erythrocytes also possess a catalase protein with a molecular weight of 59.8 kDa [25], suggesting that the unknown ~58 – 60 kDa protein might be this catalase protein.

At the 2nd generation ring stage of the *P. falciparum* life cycle, we found that treatment with either garlic or tea tree oil increased the abundance of an unknown ~58 – 60 kDa protein in pre-treated erythrocytes, cultured with *P. falciparum*, but decreased the abundance of an unknown ~28 - 30 kDa protein (Figures 4A-B). These unknown proteins seemed to be associated with one another. We suggest that the unknown ~58 – 60 kDa protein may be a dimer of the unknown ~28 - 30 kDa protein. Human erythrocytes contain the Prx1 and Prx2 proteins [4], which have very similar amino acid sequences and molecular weights. Thus, the anti-Prx2 antibodies would have recognised the Prx1 protein as well as the Prx2 protein, indicating that the unknown proteins were not forms of the Prx1 protein. There are 182 unique proteins that have been identified in human erythrocytes [25]. It is known that out of the 182 unique erythrocyte proteins, twelve have molecular weights of 28.4 to 30 kDa [25]. Amongst these proteins, the carbonic anhydrase I protein is the first most abundant protein after hemoglobin in human erythrocytes [27]. This suggests that the unknown ~28 - 30 kDa protein is probably carbonic anhydrase whereas the unknown ~58 - 60 kDa protein could be albumin or a haemoglobin tetramer. It is possible that one or more of these proteins targeted by garlic oil or tea tree oil might be involved in reducing infection of the pre-treated uninfected erythrocytes by *P. falciparum*. This could be a subject for future investigation.

In conclusion, we have shown that treatment with garlic or tea tree oil promotes oxidised dimer formation in the erythrocyte Prx2 protein and that pre-treatment of uninfected erythrocytes with these oils makes them less susceptible to infection by *P. falciparum* at the 2nd generation ring stage. Oxidation/inactivation of the Prx2 protein might be involved in decreasing the susceptibility of pre-treated uninfected erythrocytes to infection by *P. falciparum*. Along with other studies investigating the antimalarial properties of garlic oil [28, 29], our results suggest that garlic oil as well as tea tree oil could be used as alternative antimalarial treatments.

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المعاملة المسبقة لكريات الدم الحمراء بزيت الثوم و زيت الشاي تحفز اكسدت انزيم البيروكسي ريدوكسن 2 وتجعل الكريات اقل تحسس للاصابة بطفيلي *Plasmodium falciparum*

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الخلاصة

يعتبر طفيلي الملاريا الخبيثة *Plasmodium falciparum* المسبب لاغلب وفيات ملاريا الانسان. يفتقد هذا الطفيلي الى انزيمات الكتليز و الكليتوثايون بروكسديز لهذا يعتمد بشكل رئيس على انزيمات البيروكسي ريدوكسن في دفاعاته ضد الاجهاد التأكسدي. بالرغم من امتلاك الطفيلي خمسة اشكال من انزيمات البيروكسي ريدوكسن فهو يستخدم انزيم البيروكسي ريدوسن 2 المتواجد في كريات الدم الحمراء في دفاعاته. لهذا فحصت الدراسة الحالية تاثير المعاملة المسبقة بالتراكيز المتزايدة لزيت الثوم والشاي لكريات الدم الحمراء غير المصابة على الحالة التاكسدية لانزيم البيروكسي ريدوكسن 2 وعلى قابلية اصابة طفيلي الملاريا لتلك الكريات الحمراء المعاملة مسبقا. اظهرت نتائج الدراسة الحالية قابلية زيت الثوم وزيت الشاي على تعطيل الحالة التأكسدية لانزيم البيروكسي ريدوكسن 2 في الكريات الحمراء المعاملة مسبقا بواسطة تكوين دايمر متأكسدة وكان زيت الثوم الاكثر امكانية في تحفيز هذا التاثير عند مقارنته بزيت الشاي. كما اظهرت نتائج الدراسة بان كلا الزيتين استمر في تعطيل انزيم البيروكسي ريدوكسن 2 اضافة الى انزيمات تحمل مجاميع SH في مرحلة الدور الحلقي الثاني لطفيلي الملاريا وكان ايضا زيت الثوم الاكثر امكانية في ابراز هذا التاثير عند مقارنتة بزيت الشاي. بالاضافة الى ذلك لوحظ ان كريات الدم الحمراء المعاملة مسبقا بالزيوت النباتية تصبح اقل تحسس للاصابة بطفيلي الملاريا وقد يعود ذلك لتعطيل انزيم البيروكسي ريدوكسن 2 في الكريات الحمراء المعاملة مسبقا. تشير نتائج الدراسة الحالية الى امكانية استخدام زيت الثوم وزيت الشاي كعقارات مضادة للملاريا.