Typical 2-Cys Peroxiredoxins in the Alveolata

by

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Summary

Typical 2-Cys peroxiredoxin (Prx) enzymes reduce H_2O_2 and various organic hydroperoxides to less reactive products. These enzymes are characterised by interacting active site peroxidatic Cys (C_P) and resolving Cys (C_R) residues located on separate subunits. C_P in its reduced/thiol state can become hyperoxidised by high concentrations of H_2O_2 and thus the hyperoxidised form of the enzyme loses its peroxidase activity which can be restored by sulfiredoxin proteins. There are "sensitive" (mostly eukaryote) and "robust" (mostly prokaryote) types of typical 2-Cys Prx enzymes. The category ("sensitive" or "robust") is not known for parasitic or non-parasitic alveolates. Thus, here we have used the well-studied *Tetrahymena thermophila* (a non-parasitic alveolate) to investigate this. This thesis focuses on the study of the typical 2-Cys Prx proteins in selected organisms of the clade of the Alveolata. It also makes comparisons with typical 2-Cys Prx proteins from humans and investigates the possible utility of garlic and tea tree oils, and their active constituents, as potential novel anti-malarial drugs. *T. thermophila*, as a representative for the alveolates, was highly resistant to the anti-proliferative effects of treatment with H_2O_2 and cumene hydroperoxide (CMHP) but it was susceptible to the anti-proliferative effects of treatment with garlic oil and its major constituents diallyl disulphide (DADS) and diallyl trisulphide (DATS) as well as tea tree oil and its major constituent terpinen-4-ol. This suggested that *T. thermophila* should have a highly active complement of antioxidant enzymes capable of removing peroxides from the cell. Indeed, *T. thermophila* had genes encoding four typical 2-Cys Prx proteins and these proteins are known, from other studies, to be highly efficient at using H_2O_2 and CMHP as substrates. We predicted that *T. thermophila Prx1a* and *Prx1b* are located in the cytosol or nucleus,

Prx1m in the mitochondria and *Prx1c* is likely to be secreted. The *T. thermophila* Prx deduced amino acid sequences contained C_P and C_R residues that are highly conserved among typical 2-Cys Prx proteins. They also contained the GGLG and Y*X* (where *X* is F or W) motifs that are highly conserved among the "sensitive" type eukaryotic typical 2-Cys Prx proteins, including those from humans. However, surprisingly, the *T. thermophila Prx1m* protein was insensitive to hyperoxidation despite containing the GGLG and Y*X* motifs. Thus it belongs to the "robust" type of typical 2-Cys Prx proteins. This indicates that the GGLG and YF (or similar) motifs might not be always associated with susceptibility to hyperoxidation. *T. thermophila* lacked any sulfiredoxin proteins, normally found only in organisms that have "sensitive" typical 2-Cys Prx proteins. Thus, the resistance of the *T. thermophila Prx1m* protein to hyperoxidation was associated with the absence of sulfiredoxin proteins. This thesis reports for the first time on the capacity of garlic oil and tea tree oil and their dominant constituents to disrupt the redox state of the typical 2-Cys Prx proteins in *T. thermophila* cells and Jurkat T-lymphocytes (human cancer cells). Inhibition of thioredoxin reductase activity by allyl sulphides (found in garlic oil) but not by terpinen-4-ol (found in tea tree oil) could, at least in part, explain the oxidation/inactivation of the Prx proteins in these cells. Expression analysis of the four *T. thermophila* Prx genes revealed that the *Prx1a* and *Prx1m* genes were the most highly expressed and also the most responsive to pro-oxidants and therefore the most important in protecting the organism against oxidative stress. Furthermore, this thesis reports that garlic oil and tea tree oil disrupt the redox state of the erythrocyte Prx2 protein and make treated erythrocytes less susceptible to infection by *P. falciparum*, suggesting that these oils could potentially be used as antimalarial treatments.

Declaration

'I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.'

Sarmad AL-Asadi

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Chapter 1 – General introduction

1.1 Reactive oxygen species

Aerobic organisms employ oxygen (O_2) as an essential molecule in many metabolic processes associated with their aerobic life (Halliwell, 1978). O_2 is a relatively unreactive molecule that serves as the terminal electron acceptor at Complex IV of the mitochondrial electron transport chain (Errede et al., 1978). It can be reduced by a total of four electrons to yield water. The incomplete reduction of O_2 to water during cellular respiration generates a variety of different oxygen intermediates (Li et al., 2016c). These reactive oxygen species (ROS) include the superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet OH$). ROS play significant physiological functions in certain circumstances, including in cell signalling and in defence against invading pathogens (Li et al., 2016c). In contrast, ROS can also rapidly react with important biomolecules such as proteins, nucleic acids and lipids leading to oxidative damage (Halliwell and Gutteridge, 2003).

1.2 Sources of ROS

1.2.1 Endogenous sources of ROS

ROS are produced by various endogenous sources including the mitochondrial electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase (Kostić et al., 2015; Li et al., 2016c). The mitochondrial electron transport chain is an important and significant endogenous source of ROS (Cochemé and Murphy, 2009). Early studies on sub-mitochondrial particles and intact isolated mitochondria indicated that they generated H_2O_2 (Chance and Oshino, 1971; Jensen, 1966). H_2O_2 generated by sub-mitochondrial particles was observed to be derived from the dismutation of $\bullet O_2^-$ (Loschen et al., 1974). The dismutation of $\bullet O_2^-$ to H_2O_2 and water can be catalyzed by superoxide dismutases (McCord and Fridovich,

1969). $\bullet O_2^-$ itself is generated from the interaction of O_2 with an electron leaking from the mitochondrial electron transport chain (Cochemé and Murphy, 2009). In isolated mitochondria, $\bullet O_2^-$ is formed by Complex I when the mitochondria have a high proton motive force and a highly reduced coenzyme Q pool when they are not creating ATP and also when there is a high NADH/NAD^+ ratio in the matrix (Kussmaul and Hirst, 2006). Investigation of isolated mitochondria from *Drosophila melanogaster* showed that in the presence of the cytochrome *c* reductase inhibitor antimycin, glycerol-3-phosphate dehydrogenase (GPDH) and center *o* of Complex III had the greatest capacity to produce $\bullet O_2^-$ on the cytoplasmic side of the inner membrane whereas on the matrix side of the inner membrane only Complex I produced a substantial amount of $\bullet O_2^-$ (Miwa et al., 2003). In contrast, in the absence of the inhibitor antimycin, GPDH and Complex I kept producing $\bullet O_2^-$ but centre *o* of Complex III did not. This indicated that Complex 1 and GPDH are significant endogenous sources of $\bullet O_2^-$ in mitochondria.

NADPH oxidase (NOX) proteins are membrane-bound enzyme complexes that are an important endogenous source of ROS. They are present in various cells including neutrophils, smooth muscle cells, endothelial cells, macrophages, monocytes and fibroblasts (Dhawan, 2014). The NOX protein family consists of seven isoenzymes which are referred to as NOX1 through 5, dual oxidase 1 and dual oxidase 2 (Dhawan, 2014). An early study of NOX proteins in neutrophils indicated that a NOX protein generated $\bullet O_2^-$ (Babior et al., 1973). This NOX protein is now known as NOX2 and it consists of 6 subunits which accumulate at the membrane of the phagosome after phosphorylation of the NOX2 subunits p67 phagocyte oxidase and p47 phagocyte oxidase by e.g. protein kinase C (Brown and Griendling, 2009). NOX2 assembly can also be activated by a number of stimuli such as growth factors, mechanical force and metabolic factors (e.g. insulin and interleukin-1). The phosphorylated NOX2 subunits

p67 phagocyte oxidase and p47 phagocyte oxidase translocate to the membrane and form a complex with the GTPase RAC, p40 phagocyte oxidase and the transmembrane subunits gp91 phagocyte oxidase and p22 phagocyte oxidase of the active NOX2. The active NOX2 complex oxidises NADPH to $NADP^+$ and reduces O_2 to $\bullet O_2^-$ which dismutates spontaneously to H_2O_2. The ability of host neutrophils and macrophages to kill foreign cells depends on a number of mechanisms including the production of ROS by NOX2 (Butler et al., 2010; Gough and Cotter, 2011). Thus, ROS are an important component of the innate immune system and cell signalling network.

Xanthine oxidoreductase (XOR), a metallo-flavoprotein, is another important enzymatic endogenous source of ROS (Nishino et al., 2008). This enzyme was first isolated from bovine milk by Schardinger (1902). In mammals, the highest levels of XOR activity are found in the liver and small intestine (Parks and Granger, 1986). The XOR protein has also been identified in other mammalian tissues, including bovine and rat hearts (Berry and Hare, 2004). The XOR enzyme has been demonstrated to be present in the cytoplasm and on cell membranes, with cell surface binding likely mediated by glycosaminoglycans (Adachi et al., 1993; Frederiks and Vreeling-Sindelárová, 2002; Radi et al., 1997; Rouquette et al., 1998). This enzyme is known to exist in two forms, xanthine oxidase and xanthine dehydrogenase, encoded by the same gene (Dhawan, 2014; Nishino et al., 2008). Xanthine dehydrogenase can be easily converted to xanthine oxidase. The XOR protein catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid in purine metabolism (Bray, 1975). The XOR protein reduces molecular oxygen in both reactions to form superoxide followed by the production of H_2O_2 (Nishino et al., 2008). There is also evidence that the XOR enzyme plays an antimicrobial role *in vivo* and *in vitro* (Hancock et al., 2002; Stevens et al., 2000). A role of XOR in other physiological processes including host

defense against oxidant-sensitive organisms and oxidant-mediated signal transduction has been suggested (Berry and Hare, 2004; Segal et al., 2000).

1.2.2 Exogenous sources of ROS

ROS are also generated from various environmental/exogenous sources including transition metals, pesticides and radiation (Al-Gubory, 2014; Li et al., 2016c). Transition metals, such as iron, copper, cobalt, vanadium and chromium, are able to promote the production of ROS (Valko et al., 2005). The common mechanism of ROS generation for these metals involves Fenton chemistry governed by the Haber-Weiss reaction (Valko et al., 2005). As noted by Fenton (1894), in the presence of H_2O_2 and a trace of a ferrous (Fe^{2+}) salt, tartaric acid was oxidised to produce a strong violet coloured solution on the addition of caustic alkali. It was later suggested that the ferric iron salts catalysed the decomposition of H_2O_2 to yield the hydroxyl radical in an overall reaction called the Haber-Weiss reaction (Kehrer, 2000). The Haber-Weiss reaction occurs in two chemical steps. The first step includes an interaction between the ferric (Fe^{3+}) ion and $\bullet O_2^-$ to yield Fe^{2+} and O_2 and the second step consists of an interaction between Fe^{2+} and H_2O_2 to yield Fe^{3+}, the hydroxyl anion (OH^-) and •OH. The Fe-catalyzed Haber-Weiss reaction, employing Fenton chemistry, is considered to be the main source of •OH in biological systems (Kehrer, 2000; Liochev, 1999).

In addition to transition metals, pesticides, including fungicides, herbicides, insecticides and rodenticides, widely used in agriculture, exhibit the ability to catalyse the generation of ROS (Slaninova et al., 2009). Pesticides such as organochlorines, organofluorines, organophosphates, chloroacetanilide and triazine, have been shown to induce oxidative stress in fish via the induction of lipid peroxidation and DNA damage (Al-Gubory, 2014; Slaninova et al., 2009). Similarly, exposure of rats and cultured rat PC-12 cells to the herbicide alachlor, polyhalogenated cyclic hydrocarbons

(endrin or chlordane) and organophosphates (chlorpyriphos or fenthion) has been shown to be able to cause oxidative stress via induction of lipid peroxidation and DNA damage in liver and brain homogenates and DNA damage in PC-12 cells (Bagchi et al., 1995).

Radiation, including low energy photons and ionising radiation, also participates in the production of ROS (Riley, 1994). Low energy photons, such as from visible and ultraviolet (UV) light, generate ROS via participation in photochemical reactions. Upon colliding with low energy photons, the energy levels of electrons in organic molecules are raised allowing subsequent chemical reactions to take place. Photochemical reactions may involve H-abstraction, resulting in the generation of a radical that can interact with O_2 to form an organic peroxyl radical (ROO•). Alternatively, these photochemical reactants may react immediately with O_2, producing singlet oxygen that can attack various substrates and form peroxides, hydroperoxides or endoperoxides (Riley, 1994). Ionising radiation, such as gamma rays, produces ROS through water radiolysis, direct ionisation of molecules and activation of NOX. In the absence of electron donors, radicals derived from ionised molecules can interact with O_2 to form ROO• that then participates in a variety of subsequent reactions (Riley, 1994). In addition, it has been observed that exposure of human lung fibroblasts to α-particles, using a collimated ^{238}Pu α-particle exposure system, significantly induces the production of $\bullet O_2^-$ and H_2O_2 in irradiated cells through the activation of plasma-bound NOX (Narayanan et al., 1997).

1.3 Oxidative stress

Oxidative stress occurs in biological systems when ROS generation exceeds the ability of non-enzymatic antioxidants (e.g. vitamin E and glutathione) and antioxidant enzymes to scavenge them (Halliwell and Gutteridge, 2003; Halliwell and Whiteman,

2004). It is associated with oxidative damage to important cellular molecules including lipids, proteins and nucleic acids. Moderate oxidative stress may lead to cell dysfunction and altered behavior such as dysregulated inflammatory responses, abnormal proliferation, accelerated senescence (cell cycle arrest) and cell tumorigenesis, whereas high levels of oxidative stress can cause cell death via apoptosis, necrosis, necroptosis, oncosis or autophagy (Buttke and Sandstrom, 1994; Chandra et al., 2000; Chen et al., 1998; Galluzzi and Kroemer, 2008; Higuchi, 2003; Li et al., 2016c; Martindale and Holbrook, 2002). In contrast, under certain circumstances, low levels of oxidative stress can be employed as a signalling mechanism leading to physiological cellular responses (Li et al., 2016c).

1.4 Antioxidant mechanisms in humans

1.4.1 Non-enzymatic antioxidants

Non-enzymatic antioxidants are low molecular weight scavengers that can be categorised as lipid-soluble or water-soluble antioxidants (Sies, 1997). The lipid-soluble antioxidants, including tocopherols (vitamin E compounds) and carotenes, are predominantly located in cell membranes. The water-soluble antioxidants, including ascorbate (vitamin C) and glutathione (GSH) are present in cellular fluids such as the cytosol. Tocopherols, intercepting radical chain-breaking compounds in biological membranes, scavenge ROO•. The scavenging activity of tocopherols stems from their chromanol nucleus, where a phenolic hydroxy group donates a hydrogen atom to an organic peroxyl radical to form hydroperoxide (ROOH) and a tocopheroxyl radical (Toc-O•) (Serbinova et al., 1991; Traber and Sies, 1996). The Toc-O• can be reduced to tocopherol by interaction with external reductants such as ascorbate serving as a hydrogen donor (Bendich et al., 1986). In addition to helping to preserve tocopherols, ascorbate scavenges free radicals. The scavenging activity of ascorbate is derived from

the great reducing potential of its C - C double bond (May, 1998). Ascorbate easily donates hydrogen atoms/electrons to a variety of oxidants including the O_2 free radical, peroxides, and $\bullet O_2^-$. The antioxidant function of carotenes such as β-carotene and lycopene stems from their capacity to physically quench singlet oxygen ($^1O_2$) and trap ROO• (Buettner, 1993; Edge et al., 1997; Stahl and Sies, 1996). The process of $^1O_2$ quenching has been shown to be very efficient and mainly depends upon an extended system of conjugated double bonds. In addition, the radical trapping activity of carotenes has been proposed to be mainly through $^1O_2$ quenching. Lastly, GSH is a tri-peptide consisting of glycine, cysteine and glutamate and it serves as a free radical scavenging antioxidant due to the presence of a sulfhydryl group (-SH). The scavenging activity of GSH depends upon transfer of a hydrogen atom from its –SH group, leading to the formation of a thiol radical (GS$^\bullet$) (Chaudière and Ferrari-Iliou, 1999). The GS$^\bullet$ is readily reduced back to GSH by ascorbate. All animal cells are able to synthesise GSH (Kosower and Kosower, 1978). GSH is synthesised in the cytosol by two enzymes, γ-glutamylcysteine synthetase (γGCS), catalysing the rate-limiting first step and GSH synthetase (GS) required for the second step (Lu, 2013; Meister, 1995).

1.4.2 Enzymatic antioxidants

1.4.2.1 Superoxide dismutases

Superoxide dismutase (SOD) enzymes are metalloproteins that catalyse the dismutation of $\bullet O_2^-$ to H_2O_2 and O_2 (McCord and Fridovich, 1969). These enzymes exist in three isoforms in mammals. The cytosolic and extracellular SOD isoenzymes contain copper (Cu) and zinc (Zn) ions in the active site whilst the mitochondrial SOD enzyme contains manganese (Mn) ions in the active site (Marklund, 1984; Marklund et al., 1982; Weisiger and Fridovich, 1973).

1.4.2.2 Catalases

Catalases are large enzymes consisting of four 60 kDa subunits that catalyse the reductive decomposition of H_2O_2 to water and O_2 in a two-step reaction (Chance and Oshino, 1971; Kirkman and Gaetani, 1984). Each subunit of a catalase enzyme contains a ferric-heme group in the active site employed to reduce the peroxide substrate. Three catalase isoenzymes have been identified in human erythrocytes (Thorup, 1967). Catalase has been shown to be one of the fastest enzymes with a rate constant of $kH_2O_2 \sim 1 - 2.6 \times 10^7\ M^{-1}s^{-1}$. Catalase cannot be saturated at high concentrations of H_2O_2 (Chance et al., 1979). It was believed to be the main enzyme required for the elimination of high H_2O_2 concentrations from cells until the discovery of peroxiredoxin enzymes (Gaetani et al., 1996; Johnson et al., 2005).

1.4.2.3 Glutathione peroxidases

Glutathione peroxidase (GPx) enzymes constitute a family of thiol peroxidases that can detoxify H_2O_2 and a broad range of organic hydroperoxides using GSH as their reducing agent (Arthur, 2001). These enzymes contain active site selenocysteine (Se-Cys) or cysteine (Cys) residues. They exist in eight isoforms in humans (Brigelius-Flohé and Maiorino, 2013). GPx1, 2, 3, 4 and 6 contain an active site Se-Cys residue whereas GPx5, 7 and 8 contain an active site Cys residue. The first member of the GPx family was identified in erythrocytes and is known as an antioxidant enzyme that protects erythrocytes against haemolysis by oxidation (Mills, 1957). This GPx enzyme is now called GPx1. The catalytic mechanism of GPx1, which is valid also for GPx3 and 4, involves oxidation of the active site selenol (Se-Cys-H) by ROOH to form the selenic acid intermediate (Se-Cys-OH) and the corresponding alcohol. Se-Cys-OH interacts with a molecule of GSH as the hydrogen donor to form Se-Cys-OH•GSH, which subsequently undergoes a condensation reaction to generate the half reduced

intermediate Se-Cys-SG and H_2O. The Se-Cys-SG reacts with a second molecule of GSH to form Se-Cys-SG•GSH, which subsequently rearranges to give Se-Cys-H•GSSG (Brigelius-Flohé and Maiorino, 2013; Toppo et al., 2009). Recycling of oxidised glutathione (GSSG) to reduced GSH, achieved by glutathione reductase (GR) in an NADPH-dependent reaction, yields Se-Cys-H that is now ready for the next cycle.

In the past, it was generally agreed that GPx scavenges low H_2O_2 concentrations whereas catalase is more effective to eliminate high H_2O_2 concentrations (Ho et al., 2004; Johnson et al., 2005; Makino et al., 1994). Within erythrocytes, this changed when peroxiredoxin (Prx) 2 was revealed to be essential in the model of peroxide metabolism (Johnson et al., 2005). Subsequently, it was demonstrated that Prx2 interacted faster with H_2O_2 than previously supposed (Low et al., 2007). In addition to this, Prx enzymes have been shown to be able to reduce peroxide substrates with rate constants exceeding 1×10^7 to 1×10^8 M^{-1} S^{-1} (Ferrer-Sueta et al., 2011; Manta et al., 2009; Ogusucu et al., 2007; Parsonage et al., 2005; Peskin et al., 2007).

1.4.2.4 Peroxiredoxins

Peroxiredoxin (Prx) enzymes are Cys-dependent peroxides that rapidly catalyse the reductive decomposition of H_2O_2, various organic peroxides and peroxynitrite to less reactive molecules (Trujillo et al., 2007; Wood et al., 2003b). The first member of the Prx enzyme family was identified in *Saccharomyces cerevisiae* and called thiol-specific antioxidant (TSA) since it was believed to scavenge sulphur species instead of ROS (Kim et al., 1989; Kim et al., 1988). A significant level of amino acid sequence identity was found to exist between the TSA proteins from rat and *S. cerevisiae* and the alkyl hydroperoxide reductase (AhpC) protein from *Salmonella typhimurium* (Chae et al., 1994b). Subsequent database searches showed >23 proteins from

organisms of all kingdoms of life that shared high sequence identity to TSA and AhpC, thereby defining a new family of antioxidant enzymes now known as the Prx family (Chae et al., 1994b). Meanwhile, three additional members of the AhpC/TSA family were identified at the cDNA level and they included natural killer enhancing factor (NKEF) A and B proteins from humans (Chae et al., 1994b). These proteins were identified based on their capability to enhance natural killer cell cytotoxicity towards cancer cells (Shau and Kim, 1994). An amino acid sequence alignment of the AhpC/TSA family members showed that the N-terminal Cys residue was conserved in all members and most also contained a conserved C-terminal Cys residue as well (Chae et al., 1994b). As a result, the AhpC/TSA family was separated into 2-Cys and 1-Cys sub-families.

The role of the conserved N-terminal and C-terminal Cys residues was examined in the yeast TSA protein by individual replacement with serine (Chae et al., 1994c). It was demonstrated utilising non-reducing polyacrylamide gel electrophoresis, that the wild type TSA protein existed as a dimer whereas both mutant TSA proteins existed as monomers. This led to the suggestion that dimer formation in the wild type TSA protein involved disulphide bond formation between the N-terminal Cys^{47} residue and the C-terminal Cys^{170} residue. The N-terminal Cys residue (but not the C-terminal Cys residue) was found to be essential for the antioxidant activity of the TSA protein (Chae et al., 1994c). Unlike SOD, catalase and GPx enzymes known to remove ROS, the yeast TSA enzyme was found to lack redox cofactors such flavin, heme and metal ions. Thus, it was proposed that the N-terminal Cys residue was responsible for the reduction of the various substrates of TSA-like enzymes.

The high level of amino acid identity between the TSA and AhpC proteins also led to the identification of the actual enzymatic function of the TSA protein (Chae et al.,

1994a). The reduced form of AhpC catalyses the reduction of alkyl hydroperoxides to alcohols via oxidation of its two conserved Cys residues and subsequent formation of a disulphide bond between them. The regeneration of the reduced form of AhpC is performed by alkyl hydroperoxide reductase component F (AhpF) which transfers reducing equivalents from NAD(P)H to the disulphide bond of AhpC (Jacobson et al., 1989). Thus, it was proposed that the yeast TSA protein may also act as a peroxidase like AhpC and be recycled by an enzyme with a similar role to AhpF. Subsequently, TSA from yeast was revealed to reduce H_2O_2 and alkyl hydroperoxides in the presence of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH (Chae et al., 1994a). The TSA name was replaced with thioredoxin peroxidase (TPx) based on the fact that this was the first peroxidase found to employ Trx as an intermediate hydrogen donor. The name of TPx was then replaced with Prx after it was discovered that a mammalian 1-Cys member of the AhpC/TPx family depended on GSH instead of Trx as the immediate hydrogen donor (Fisher, 2011). The new peroxiredoxin naming with abbreviations Prx or Prdx is now recommended by the "Nomenclature Committee of the International Union of Biochemistry and Molecular Biology" (Rhee, 2016).

Six isozymes of the Prx family have been identified in mammals (Wood et al., 2003b). They are: Prx1 (or NKEF-A) located in the cytosol or nucleus, Prx2 (or NKEF-B) located in the cytosol or associated with the erythrocyte membrane, Prx3 located in the mitochondria, Prx4 located in the cytosol or the endoplasmic reticulum, Prx5 located in the cytosol, mitochondria or peroxisomes and Prx6 located in the cytosol (Tavender et al., 2008; Wood et al., 2003b). Originally, as mentioned above, the Prx family was categorised into 2-Cys and 1-Cys Prx subfamilies, depending upon the number of Cys residues directly involved in the catalytic reaction. Mammalian Prx enzymes are now classified into three subfamilies, depending upon the catalytic mechanism. These three subfamilies are referred to as the typical 2-Cys Prx (Prx1 through 4), atypical 2-Cys

Prx (represented by Prx5) and 1-Cys Prx (represented by Prx6) sub-families (Wood et al., 2003b).

The catalytic cycle of the Prx subfamily members is as follows. All Prx subfamily members use their conserved active site peroxidatic Cys residue (C_P-SH) to attack the peroxide substrate and reduce it. In the process, C_P-SH becomes oxidised to a Cys sulphenic acid residue (C_P-SOH). C_P-SOH of the typical and atypical 2-Cys Prx enzymes then interacts with a second Cys residue referred to as the resolving Cys residue (C_R-SH) to form a disulphide bond. In the typical 2-Cys Prx enzymes, the interacting C_P-SOH and C_R-SH are located on separate subunits whereas in the atypical 2-Cys Prx proteins, they are located on the same subunit (Evrard et al., 2004; Wood et al., 2003b). Therefore, the typical 2-Cys Prx proteins form dimers between identical subunits whereas the atypical 2-Cys Prx proteins do not. The reaction cycle of the typical and atypical 2-Cys Prx proteins is completed with the reduction of the disulphide bond by an electron donor such as Trx (Chae et al., 1999; Seo et al., 2000). Trx is a 12 kDa protein with two -SH groups in its active site forming an intramolecular disulphide bond when oxidised (Lillig and Holmgren, 2007). This permits Trx to covalently bind to other oxidised proteins with thiol groups and yield a mixed disulphide intermediate. Trx then reduces the other protein by becoming oxidised. Oxidised Trx is recycled by TrxR in an NADPH-dependent reaction. In contrast to the 2-Cys Prx proteins, 1-Cys Prx proteins lack C_R-SH and thus, C_P-SOH is recycled back to its C_P-SH by GSH and π glutathione *S*-transferase (GST) in a two-step reaction (Manevich et al., 2004; Ralat et al., 2006; Zhou et al., 2013).

The catalytic mechanism of the typical 2-Cys Prx proteins is associated with conformational changes. Under reducing conditions, typical 2-Cys Prx head-to-tail homodimers assemble to form doughnut-shaped oligomers, including decamers (Prx1

and 2) and dodecamers (Prx3). This is called the fully folded conformation. Once C_P-SH becomes oxidised to C_P-SOH, the protein has to unfold locally to enable disulphide bond formation to occur with C_R-SH. These structural changes disorder the oligomer building (dimer-dimer) interface, breaking down the oligomer into its free dimers, which then shifts the protein structure from the fully folded to the locally unfolded conformation. In contrast to this, the reduction of the disulphide bond by Trx facilitates re-formation of the dimer-dimer interface, returning the protein to the fully folded conformation (Wood et al., 2003b). The underlying physiological role of the dimer-decamer conformational rearrangement is unknown. However, it has been shown that higher order oligomers (e.g. decamers) are better peroxidases while dimers are more easily reduced back to monomers by Trx as an electron donor (Hall et al., 2009). Under certain conditions, structural rearrangements slow down the disulphide bond formation (Wood et al., 2003a) and a second molecule of H_2O_2 is able to hyperoxidise the C_P-SOH residue to a sulphinic acid residue (C_P-SO_2H) (Baty et al., 2005; Koo et al., 2002; Rabilloud et al., 2002; Wagner et al., 2002; Yang et al., 2002). C_P-SO_2H cannot form a disulphide bond with the C_R-SH and thus the peroxidase activity of the typical 2-Cys Prx proteins is lost. Eukaryotic typical 2-Cys Prx proteins are, in general, more susceptible to hyperoxidation than prokaryotic typical 2-Cys Prx proteins (Wood et al., 2003a). Susceptibility of eukaryotic typical 2-Cys proteins to hyperoxidation is thought to be associated with the presence of a conserved GGLG motif located in the N-terminus of the protein and a conserved YF motif found at the C-terminus of the protein that are absent from most prokaryotic typical 2-Cys Prx proteins (Wood et al., 2003a). These conserved motifs are thought to slow down the fully folded to locally unfolded transition rate, thus favouring hyperoxidation. The formation of C_P-SO_2H was believed to be irreversible until it was observed that hyperoxidation of typical 2-Cys Prx proteins could be reversed by reductases from the sulfiredoxin family (Biteau

et al., 2003; Woo et al., 2003). Members of the sulfiredoxin family are small 13 kDa enzymes which were first discovered in yeast but they have now been detected in plants and mammals as well (Chang et al., 2004b; Liu et al., 2006). These enzymes specifically catalyse the reduction of C_P-SO_2H of the typical 2-Cys Prx proteins (excluding Prx5 and Prx6) to C_P-SOH, enabling Trx to reduce C_P-SOH to C_P-SH and restore catalytic activity (Woo et al., 2005). Sulfiredoxins employ ATP and intermediate hydrogen donors such as GSH or Trx in their four-step reaction cascade (Jeong et al., 2006; Jönsson et al., 2005).

In addition to their antioxidant function, the typical 2-Cys Prx proteins have diverse functions, including as cell signalling enzymes and chaperone proteins. The so-called "flood-gate hypothesis" describes the postulated role of the typical 2-Cys Prx enzymes in cell signalling (Wood et al., 2003a). According to this hypothesis, hyperoxidation/inactivation of the typical 2-Cys Prx proteins allows H_2O_2 to accumulate to act as a cell signalling molecule. In recent years, this hypothesis has been revised and refined and there is now compelling evidence that the typical 2-Cys Prx proteins act not only as "flood-gates" but also as important sensors and transducers in H_2O_2-mediated cell signalling (Rhee, 2016; Rhee et al., 2012). As signalling proteins, Prx proteins appear to be involved in the regulation of diverse biological processes. For example, mitochondrial Prx3 has been shown to be involved in the regulation of apoptotic signalling in mitochondria (Chang et al., 2004a) and in the differentiation of red blood cells as well (Yang et al., 2007). Prx proteins seem to be involved in the circadian rhythm in human/mouse erythrocytes (Cho et al., 2014; O'Neill and Reddy, 2011) and in mouse adrenal cortex cells as well (Kil et al., 2012). Alternatives for the physiological function of the typical 2-Cys Prx proteins also include chaperone activity. It was noted that the formation of stable high molecular weight structures of the yeast typical 2-Cys Prx protein due hyperoxidation was able

to prevent the aggregation of proteins in yeast cells during heat shock (Jang et al., 2004). This switch from peroxidase activity to chaperone activity can be reversed by sulfiredoxin enzymes. In human cells, the chaperone function of the typical 2-Cys Prx proteins appears to be able to prevent apoptosis (Moon et al., 2005). In contrast, however, it has been pointed out that in certain species, hyperoxidation and/or higher molecular weight oligomer formation were not essential for the chaperone action of the typical 2-Cys Prx proteins (Toledano and Huang, 2016).

1.5 *Plasmodium falciparum*

Plasmodium falciparum is the causal agent of the most serious form of human malaria. Malaria is transmitted to humans by infected *Anopheles* mosquito bites. The World Health Organization (WHO) reported that 3.2 billion people remain at risk of malaria and an estimated 214 million new cases of malaria occurred worldwide in 2015. In addition, there were 438,000 malaria deaths in 2015 (WHO, 2016). Thus, there is demand for reducing the burden of this disease but increasing resistance to antimalarial drugs is the biggest problem currently facing malaria control. *P. falciparum* has developed resistance to nearly all antimalarial drugs currently in use, including artemisinin and its derivatives that are highly recommended by the WHO (Fairhurst and Dondorp, 2016). Artemisinin and its derivatives are only active on blood-stage parasites (e.g. early trophozoite and ring stages) and do not affect parasites in the liver stage or within the mosquito. The need for new antimalarial drugs less prone to the development of resistance is becoming increasingly urgent.

P. falciparum has a complex life cycle, which involves two hosts, an invertebrate host (mosquito) and a vertebrate host (human). This life cycle is shared with other *Plasmodium* species (e.g. *P. ovale, P. malaria* and *P. vivax*) with only minor variations (Bannister and Sherman, 2009). In the *P. falciparum* life cycle, there are two stages,

which are the sexual stage (gametes) in mosquitoes and the asexual stage in humans (merozoite, ring, trophozoite and schizont phases) and mosquitoes (ookinete, oocyst, sporozoite phases). The infection is transmitted to humans when sporozoites are injected into the human skin with the saliva of a feeding *Anopheles* mosquito, preparing to take a blood meal (Bannister and Sherman, 2009; Cox, 2010) (Fig. 1.1). Sporozoites are transferred by the circulatory system to the liver and invade hepatocytes. In the hepatocytes, the parasite begins to develop and produce many merozoites in the process called exo-erythrocytic schizogony. When the merozoites have finished multiplying, they are released into the blood (Fig. 1.1). Merozoites invade erythrocytes and proliferate inside the host erythrocyte to form more merozoites that are able to repeat this stage many times and invade new erythrocytes in the process known as erythrocytic schizogony (Fig. 1.1). Erythrocytic schizogony is responsible for fever peaks and the malaria disease symptoms (Cox, 2010). Some merozoites develop inside the erythrocyte into a male microgametocyte and a female macrogametocyte (Fig. 1.1). Gametocytes in the peripheral blood of the infected person are taken up into the female mosquito gut when the mosquito feeds. Inside the mosquito, gametocytes develop to form female and male gametes and fertilisation occurs to produce a motile zygote (ookinete) which penetrates the gut wall and leads to the formation of an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites (sporogony). Sporozoites released from the mature oocyst rupture into the haemocoel of the mosquito and migrate to the salivary glands and then they are injected into the human body with the saliva of the mosquito (Bannister and Sherman, 2009). In order to survive in its human host and the malaria vector the *Anopheles* mosquito, *P. falciparum* depends on adequate antioxidant defences and effective redox equilibrium maintenance (Percário et al., 2012).

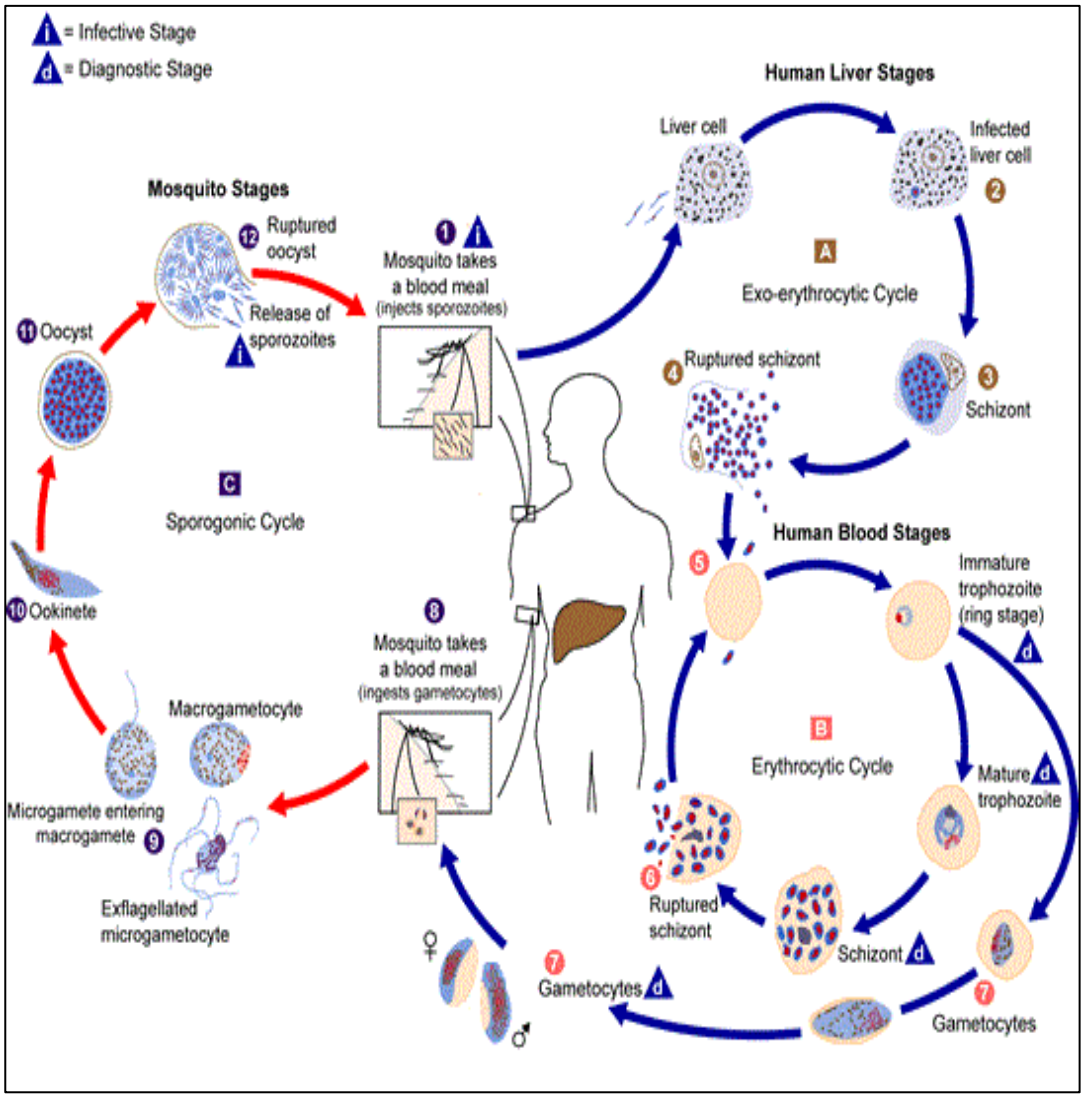

Fig.1.1. Life cycle of *P. falciparum* reproduced from CDC Malaria (https://www.cdc.gov/dpdx/malaria/).

1.6 Oxidative stress in *P. falciparum*

P. falciparum parasites are exposed to high levels of oxidative stress during the intra-erythrocytic stage of their life cycle. Oxidative stress is related to the production of ROS that cause damage to macromolecules in *Plasmodium* and other cells (Becker et al., 2004; Müller, 2004). The malaria parasite digests haemoglobin inside its food vacuole for nutrition because haemoglobin is the main amino acid source for the malaria parasite. Haemoglobin degradation contributes to the release of ROS ($\bullet O_2^-$, H_2O_2 and •OH) and toxic free haem (ferroprotoporphyrin IX; FP IX) (Tilley et al., 2001). The release of toxic free FP IX induces the haemoglobin prosthetic group to change from the Fe^{2+} to the Fe^{3+} state and form $\bullet O_2^-$ (Müller, 2004). The majority of FP IX is polymerised inside the food vacuole into haemozoin, known also as the malaria pigment, which is non-toxic to the parasite. However, a small amount of toxic free FP IX leaks to the *P. falciparum* cytosol where it results in membrane damage and can undertake redox reactions leading to the production of $\bullet O_2^-$ in the *P. falciparum* compartment. $\bullet O_2^-$ is either converted to H_2O_2 and molecular oxygen by the action of SOD or it can interact with H_2O_2, leading to the formation of •OH. In addition to this, •OH is also formed in the presence of Fe^{2+} through the Fenton reaction. ROS are toxic for *Plasmodium* ssp. because of their ability to lyse erythrocytes and damage parasite enzymes and membranes leading to the inhibition of *Plasmodium* proliferation. Although heme is the major source of ROS, they are also formed by the vertebrate immune system through the activation of the NOX2 enzyme in neutrophils (Postma et al., 1996).

1.7 Antioxidant mechanisms in *P. falciparum*

P. falciparum has very active antioxidant systems that contribute to the parasite's ability to resist oxidative stress and they are significant for the survival of the parasite through the intra-erythrocytic stage of its life cycle (Becker et al., 2004; Müller, 2004). There are two different types of antioxidant defence systems used by *P. falciparum* (Becker et al., 2004; Müller, 2004). One is the non-enzymatic antioxidant defence system, which includes vitamin C, vitamin E and GSH. The other is the enzymatic antioxidant defence system which includes SOD and Prx enzymes. Surprisingly, *P. falciparum* lacks classical GPx and catalase enzymes and it is highly dependent on Prx enzymes for H_2O_2 detoxification (Jortzik and Becker, 2012). Both systems work to protect the malaria parasite by converting ROS to less or non-reactive molecules. Moreover, the malaria parasite possesses an effective redox system that also equilibrates ROS levels utilising the reduced/oxidised ratios of thiols, GSH/GSSH and $Trx(SH)_2/Trx(S)_2$, and $NAD(P)H/NAD(P)^+$ (Bunik, 2003; Müller, 2004).

1.7.1 The GSH system in P. falciparum

GSH plays a vital role in the existence of most living organisms, including *P. falciparum* (Patzewitz et al., 2012). It is a tripeptide and is found within cells of most organisms at concentrations of 1 to 10 mM (Becker et al., 2003; Müller, 2015; Patzewitz et al., 2012). GSH is synthesised in the cytoplasm of *P. falciparum* by two enzymes which are GS and γGCS. It was observed that disruption of either GS or γGCS synthesis in the malaria parasite *P. falciparum* is unable to be performed (Patzewitz et al., 2012). This suggests that GSH synthesis is crucial for the development of *P. falciparum* in host erythrocytes. GSH serves as a substrate for detoxification catalysed by enzymes like GSTs, glyoxylases

and glutaredoxins. GSH-dependent enzymes are important in protecting *P. falciparum* against ROS, xenobiotics and toxic metabolic intermediates (Becker et al., 2003; Müller, 2015). GSH is maintained by NADPH-dependent GR. In addition to GSH function as a substrate for antioxidant and detoxification enzymes, it acts as an antioxidant either alone or in association with vitamin E and vitamin C (Galli and Azzi, 2010; Linster and Van Schaftingen, 2007).

1.7.2 The Trx system in P. falciparum

Trx proteins are important electron donors in antioxidant defence systems of all living organisms. These proteins are small disulphide-containing redox proteins and act as general protein disulphide oxidoreductases that associate with a wide range of proteins in a redox mechanism (Müller, 2004). *P. falciparum* lacks classical GPx and catalase enzymes but instead, it has Trx-dependent peroxidases (Prx proteins) (Jortzik and Becker, 2012). The different Prx proteins employ different Trx proteins as reducing substrates. Three different Trx proteins (Trx1 to 3) have been discovered in *P. falciparum* and they can be regenerated by TrxR (Becker et al., 2000; Nickel et al., 2006). *P. falciparum* Trx1 localised in the cytosol is the best studied Trx in *P. falciparum* and has the active site motif WCGPC, used to modify the redox state of protein targets through its Cys residues. *P. falciparum* Trx2 is localised in the parasitophorous vacuole and contains the active site motif WCQAC whereas *P. falciparum* Trx3 is localised in the endoplasmic reticulum and contains the active site motive WCKPC but is inadequately described (Kehr et al., 2010; Nickel et al., 2006). Trx functions mainly depend on the activity of TrxR. In all living organisms, TrxR is a disulphide oxidoreductase, existing in two types, low (35 kDa) and high (55 kDa) molecular weights (Becker et al., 2000; Hirt et al., 2002). The *P. falciparum*

TrxR is from the high molecular weight type, which is a 55 kDa homodimeric FAD-dependent oxidoreductase and is fundamental for *P. falciparum* survival in the erythrocytic life stage. *Plasmodium* TrxR enzymes contain two Cys residues, separated by four residues, within the active site motif at the C-terminus, whereas human TrxR proteins contain Cys and Se-Cys residues (Becker et al., 2000; Hirt et al., 2002; McCarty et al., 2015).

In addition to the main Trx proteins, the malaria parasite *P. falciparum* contains two types of Trx-like proteins (Tlp1 to 2) that are similar to other Trx proteins in their amino acid sequences (Nickel et al., 2006). However, they are not able to be reduced by TrxR. *P. falciparum* Tlp1 is localised in the cytosol and has the active site motif WCGPC whereas *P. falciparum* Tlp2 is localised in the mitochondrion and has the active site motif WCAPC (Kehr et al., 2010; Nickel et al., 2006).

1.7.3 SOD enzymes in P. falciparum

SOD enzymes play a vital role in the dismutation of superoxide radicals into H_2O_2 and molecular oxygen in all living organisms, including *P. falciparum*. $\bullet O_2^-$ is naturally generated when haemoglobin is digested in *P. falciparum*'s food vacuole. Furthermore, they can also be produced by the electron transport chain of the mitochondria due to incomplete reduction of O_2 to H_2O during cellular respiration (Bozdech and Ginsburg, 2004). *P. falciparum* has two kinds of SOD genes encoding a cytosolic Fe-dependent SOD protein (SOD1) and a mitochondrial Mn-dependent SOD protein (SOD2) (Bécuwe et al., 1996; Bozdech and Ginsburg, 2004; Gratepanche et al., 2002; Sienkiewicz et al., 2004). SOD1 is thought to scavenge $\bullet O_2^-$ in the parasite cytosol. In addition to the parasite SOD1, the host Cu/Zn-SOD is believed to detoxify $\bullet O_2^-$ generated during the digestion of

haemoglobin inside the food vacuole of *P. falciparum* (Fairfield et al., 1983). *P. falciparum* has an active respiratory chain that unavoidably leaks $\bullet O_2^-$ (Müller, 2004). Thus, SOD2 plays a vital role in the detoxification of $\bullet O_2^-$ generated via the highly active respiratory chain and blocks damage to nucleic acids, proteins, metabolic functions and mitochondrial membranes that would otherwise occur (Bozdech and Ginsburg, 2004).

1.7.4 Prx enzymes in P. falciparum

P. falciparum parasites are exposed to high levels of oxidative stress from host-defense ROS and by-products of haem degradation. Consequently, *P. falciparum* requires efficient antioxidant enzymes to survive in an environment rich with ROS, including H_2O_2. Interestingly, the parasite lacks catalase and GPx enzymes and thus it is highly dependent on Prx enzymes for H_2O_2 detoxification (Jortzik and Becker, 2012). Five members (*Prx1a*, *Prx1m*, *Prx5*, *Prx6* and *PrxQ*) of the Prx family have been identified in *P. falciparum* (Gretes et al., 2012). They are located in diverse cell compartments, with *Prx1a* located in the cytosol, *Prx1m* located in the mitochondria, *Prx5* located in the apicoplast and cytosol, *Prx6* located in the cytosol and *PrxQ* located in the nucleus (Boucher et al., 2006; Djuika et al., 2015; Kehr et al., 2010).

P. falciparum Prx1a is a typical 2-Cys Prx protein and is the best studied of the *P. falciparum* Prx protein family. It catalyses the reductive decomposition of H_2O_2, peroxynitrite, tert-butyl hydroperoxide (tBuOOH) and cumene hydroperoxide (CMHP) to less- or non-reactive molecules utilising electron donors such as Trx or plasmoredoxin (Jortzik and Becker, 2012; Nickel et al., 2005; Rahlfs and Becker, 2001). Plasmoredoxin was identified only in *Plasmodium* spp. The *P. falciparum Prx1a* gene is constitutively expressed through the erythrocytic stage (Yano et al., 2005). In *P. falciparum Prx1a*

knockout parasites, it was observed that the growth rate of the parasites was reduced in the presence of paraquat as a superoxide radical generating agent and sodium nitroprusside as a NO generating agent, thus reinforcing a role for *Prx1a* in protection against oxidative and nitrosative stresses (Komaki-Yasuda et al., 2003). Additionally, in the rodent malaria parasite *P. berghei*, a relative of *P. falciparum*, *Prx1a* is essential for the development of gametocytes (sexual blood stages) during the intra-erythrocytic stage in the vertebrate host (rodent) and is also required for the development of the sporozoites in the invertebrate host (mosquito) (Yano et al., 2006; Yano et al., 2008). Moreover, it might also be required for the development of merozoites during exo-erythrocytic schizogony (Usui et al., 2015).

P. falciparum Prx1m is a typical 2-Cys Prx protein as well and is approximately 52% identical with *P. falciparum Prx1a* in its amino acid sequence (Rahlfs and Becker, 2001). It detoxifies H_2O_2 and tBuOOH (but not CMHP) and prefers Trx2 over Trx1 as an electron donor (Boucher et al., 2006). The *P. falciparum Prx1m* gene is highly expressed during the trophozoite and schizont stages of the *P falciparum* erythrocytic life cycle (Yano et al., 2005). Recently, it was observed that disruption of the *Prx1m* (TPx2) gene in *P. berghei* had no effect on the development of the malaria parasite in mouse erythrocytes or mosquitoes. This led to the suggestion that *Prx1m* is not crucial for the development of the erythrocytic and insect/vector stages of *P. berghei* (Masuda-Suganuma et al., 2012).

P. falciparum Prx5, also known as antioxidant protein (AOP), is a 1-Cys Prx protein (Sarma et al., 2005). This protein is similar in its amino acid sequence to the human atypical 2-Cys Prx5. It prefers scavenging phosphatidyl hydroperoxide and tBuOOH over H_2O_2 and CMHP and it also prefers glutaredoxin (Grx) over Trx as an electron donor (Jortzik and Becker, 2012; Nickel et al., 2006; Sarma et al., 2005). The *Prx5* gene is

expressed during the erythrocytic stage and the highest expression is in the trophozoite stage (Nickel et al., 2006).

P. falciparum Prx6 is also a 1-Cys Prx protein. It reduces H_2O_2 and tBuOOH utilising Grx or Trx as the electron donor (Deponte et al., 2007; Nickel et al., 2006). Expression of *P. falciparum Prx6* is increased to high levels during the trophozoite and early schizont stages of the erythrocytic cycle of the malaria parasite (Yano et al., 2005). Kawazu et al. (2005) suggested that *P. falciparum Prx6* can react with FP IX to protect the malaria parasite against oxidative stresses. Nevertheless, a role for *P. falciparum Prx6* in the detoxification of FP IX appears to be improbable (Deponte et al., 2007; Jortzik and Becker, 2012).

PrxQ (also known as MCP1 and nPrx) is a 1-Cys Prx protein scavenging H_2O_2 and CMHP and preferring Grx over Trx as an electron donor (Richard et al., 2011). In addition to this, it has unusual biochemical features. For example, it combines with the malaria parasite chromatin in a genome-wide manner. Therefore, it seems to have a vital role in protecting the nuclear DNA and chromatin against oxidative stress. Unlike other *Plasmodium* Prx genes, the *PrxQ* gene could not be disrupted in *P. falciparum* and *P. berghei* and therefore it seems to be vital for these species (Richard et al., 2011).

As mentioned above, the malaria parasite *P. falciparum* lacks other H_2O_2 detoxification enzymes and thus it is highly dependent on Prx proteins for H_2O_2 detoxification. In addition to its Prx proteins, the human Prx2 is imported from the host erythrocyte into the cytoplasm of *P. falciparum* and the parasite uses it to protect itself against oxidative stress (i.e. approximately half the peroxide detoxification activity in *P. falciparum* is derived

from the erythrocyte Prx2) (Koncarevic et al., 2009). The imported human Prx2 reduces peroxides utilizing *P. falciparum* Trx1 as its electron donor.

1.8 *Tetrahymena thermophila*

T. thermophila is a free-living, ciliated protozoan that possesses two types of cell nuclei, a germinal micronucleus (MIC) and a somatic macronucleus (MAC), existing in a single cell (Prescott, 1994). The MIC has 5 pairs of chromosomes that are referred to as diploid and it is transcriptionally inactive during most of the *T. thermophila* life cycle. In contrast, the MAC is transcriptionally active and its length is ~104 Mb, consisting of ~225 chromosomes derived from the MIC chromosomes when the MAC develops from the MIC through the sexual mode of conjugation (Eisen et al., 2006). *T. thermophila* contains a genetically fully sequenced MAC with > 27,000 genes, ~ 55% of which are conserved between itself and higher eukaryotes including humans and as well it has an abundance of core processes shared with higher eukaryotes that are not found in other single-celled eukaryotic model organisms such as yeasts (Eisen et al., 2006*). T. thermophila* has been used extensively as a model organism to study eukaryote cellular and molecular biology and is also employed as a whole cell biosensor in eco-toxicological studies (Eisen et al., 2006; Gutiérrez et al., 2015). Its advantages as a model organism include its short generation time, rapid growth in inexpensive media, amenability to genetic manipulation and abundance of genes conserved between itself and higher eukaryotes, including humans (Collins, 2012; Eisen et al., 2006). Its advantages as a biosensor are that it is similar to humans and other mammals in lacking a cell wall to act as a barrier to environmental pollutants (Amaro et al., 2011; Gutiérrez et al., 2015). This contrasts with other organisms used as biosensors such as bacteria, microalgae and yeasts, all of which possess a cell wall. In addition, *T. thermophila* belongs to the clade Alveolata which also

includes such important parasites as *P. falciparum* (Dorrell et al., 2013). Within this clade, *T. thermophila* belongs to the phylum Ciliophora (ciliates) whereas *P. falciparum* belongs to the phylum Apicomplexa (apicomplexans). Most ciliates, including *T. thermophila*, are free-living whereas all apicomplexans are obligate parasites. Furthermore, *T. thermophila* shares some inherent features with *P. falciparum*, including an A+T-rich genome, similar codon usage preference and the production of a number of large and structurally complex proteins (Eisen et al., 2006; Gardner et al., 2002; Pollack et al., 1982; Wuitschick and Karrer, 1999). Therefore, *T. thermophila* is a useful model to study *P. falciparum* as well as other apicomplexan parasites without the need for a host to culture the cells or special laboratory facilities to protect research personnel against infection.

1.9 Oxidative stress in *T. thermophila*

T. thermophila cells are exposed to several sources of oxidative stress, including environmental pollutants. Some of the pollutants that have been shown to induce oxidative stress in *T. thermophila* cells include heavy metals and pesticides. For example, exposure of *T. thermophila* cells to heavy metals such as Cd, Zn and Cu was found to inhibit the proliferation of the cells (with an order Cd > Cu > Zn) as a result of the mitochondrial production of peroxides, in particular H_2O_2, leading to oxidative stress (Gallego et al., 2007). Moreover, a binary mixture of a selection of these metals was found to elevate ROS production, depending on the metallic ratio and the nature and concentration of the metals. Exposure of *T. thermophila* strain CU427 cells to silver nanoparticles (AgNPs) was found to significantly increase the expression of the catalase gene following a 2 h exposure and the expression of Mn-SOD, Cu/Zn-SOD and GPx2 genes following a 24 h exposure but not the GR gene (Juganson et al., 2017). In contrast, exposure of *T. thermophila* strain

CU428 cells to AgNPs was shown to significantly increase the expression of Mn-SOD and Cu/Zn-SOD genes following a 2 h exposure and the expression of GPx2 and GR genes following 2 and 24 h exposures but not the catalase gene (Juganson et al., 2017). The expression profiles of various oxidative stress-related genes were different in the two different strains. The authors suggested that the metabolic rate in the CU428 strain is higher than that in the CU427 strain. Therefore the production of ROS would be greater in the strain with the higher metabolic rate. Juganson et al. (2017) also observed that SOD and catalase activities were unaffected in the presence of AgNPs treatment in *T. thermophila* stains (CU427 and CU428). These results suggested that SOD and catalase activities and the expression of SOD and catalase genes are not directly related, particularly in the *T. thermophila* strain CU427. Furthermore, exposure of *T. thermophila* cells to the triazole fungicides myclobutanil and cyproconazole was found to decrease the GSH content, GST activity and the expression of SOD and GST genes but increase the expression of catalase (Huang et al., 2016). These results suggested that both fungicides induced oxidative stress in *T. thermophila* cells. Exposure of *T. thermophila* cells to the herbicide paraquat, known as a $\bullet O_2^-$ generator, was found to significantly increase the expression of five GPx genes, three GST genes and one gene each for TrxR and TPx (Prx) (Castello et al., 2007; Díaz et al., 2016). The significant increase in expression of all antioxidant genes tested was suggested to be an adaptive response to oxidative stress induced by the paraquat treatment (Díaz et al., 2016).

1.10 Antioxidant mechanisms in *T. thermophila*

There have been numerous studies employing *T. thermophila* in various biological fields, including the investigation of the capacity of certain compounds/chemicals to induce

oxidative stress in the cells (Díaz et al., 2016; Gao et al., 2013; Huang et al., 2016; Juganson et al., 2017). The majority of oxidative stress studies investigated the effects of compounds/chemicals on the GSH content of the cells and some antioxidant enzymes including SOD, catalase and GPx but there are no studies on the Prx enzymes in *T. thermophila*. Functional and structural characterizations of certain SOD proteins have been performed in *T. thermophila* (Ferro et al., 2015) and its relative *T. pyriformis* (Barra et al., 1990). In *T. thermophila*, there are three SOD genes encoding cytosolic Cu/Zn-SOD proteins (SOD1a, SOD1b and SOD1x) that have been identified (Ferro et al., 2015). SOD1x is expressed in the cells at a low level compared with SOD1a and SOD1b. This suggests that the *T. thermophila* cells depend predominantly on SOD1a and SOD1b to provide protection against oxidative stress by detoxifying superoxide. *T. thermophila* Cu/Zn-SOD proteins share most of the important catalytic residues with other Cu/Zn-SOD proteins from eukaryotes including humans and some ciliates. Exposure of *T. thermophila* cells to non-toxic doses of Cu for various time points (0.5, 1, 2, 4, 24 or 48 h), was found to significantly increase the expression of SOD1a following either 1 or 4 h exposure and then its expression was decreased following a 24 h exposure. Afterwards, the expression of SOD1a was returned to the control level following a 48 h exposure. Similarly, the SOD1b gene was significantly increased following a 1 h exposure and then it was decreased following a 24 h exposure. Afterwards, the expression of SOD1b was returned to the control level following a 48 h exposure. Ferro et al. (2015) also observed that SOD activity was increased in the presence of Cu treatment at various time points, in particular following 0.5 or 1 h exposure. These results suggested that SOD activity and the expression of SOD genes are not directly related. In *T. pyriformis*, a Fe-SOD protein was identified and was found to share ~ 42% identity with mammalian Mn-dependent SOD

proteins (Barra et al., 1990). In contrast to SOD proteins, other antioxidant enzymes, including Prx proteins, have not been characterised in *T. thermophila*. In addition to this, Prx proteins have not been characterised in any other non-parasitic alveolates.

1.11 Plant essential oils

Plant extracts and their active phytochemicals have been used for a long time as important sources to develop drugs against various diseases, including parasitic diseases (Anthony et al., 2005). The majority of parasitic diseases are treated with polar compounds from alcoholic and aqueous extracts (Anthony et al., 2005). In contrast, plant essential oils are considered to be an under-exploited resource in treating parasitic infections. Due to their lipid solubility, the non-polar compounds found in plant essential oils should diffuse more readily across cell membranes and thereby better target intracellular parasites. For example, garlic (*Allium sativum*) oil is well known for its antibacterial, antifungal, anticancer and antiviral properties and it has been shown to be highly effective against a wide range of human and non-human parasites, including *P. berghei* (Anthony et al., 2005; Li et al., 2016b; Perez et al., 1994). The allyl sulphides diallyl disulphide (DADS) and diallyl trisulphide (DATS) are the major constituents of garlic oil and they act as redox cycling agents by continuously generating ROS ($\bullet O_2^-$, H_2O_2 and $\bullet OH$) (Munday, 2012). Thus, these allyl sulphides are highly effective at inducing oxidative stress in cells. Like garlic oil, tea tree (*Melaleuca alternifolia*) oil is also well known for its antibacterial, antifungal, anticancer and antiviral properties (Carson et al., 2006; Li et al., 2016a) and more recently its active constituents (terpinen-4-ol, γ-terpinene and α-terpinene) have been shown to be toxic to the parasite *Trypanosoma evansi* which belongs to the phylum Kinetoplastida within the clade Euglenozoa, a sister clade to the Alveolata. (Baldissera et al., 2016). Tea tree oil is involved in inducing ROS-dependent responses, including apoptosis,

but the underlying mechanisms of action of tea tree oil and its constituents are not fully understood (Calcabrini et al., 2004; Kuttan et al., 2011; Shapira et al., 2016).

1.12 Aims of this study

The overall aim of this study was to investigate the structure/function relationships of the typical 2-Cys Prx proteins in parasitic and non-parasitic alveolates using *P. falciparum* and *T. thermophila*, respectively, as a representatives of these two different clades. In addition, this study aimed to compare these alveolate Prx proteins with similar proteins in humans using Jurkat T-lymphocytes as a model. Furthermore, this project aimed to investigate the possible utility of garlic and tea tree oils, and their active constituents, as potential novel anti-malarial drugs.

Chapter 2 - *Tetrahymena thermophila* is resistant to oxidative stress caused by peroxides but not redox cycling agents

Abstract

Tetrahymena thermophila has been used widely as a model organism for eukaryotes. However, it is not known whether or not *T. thermophila* is particularly susceptible or resistant to oxidative stress induced by peroxides. Here, we investigated the effects of H_2O_2, cumene hydroperoxide (CMHP), garlic oil and its main constituents diallyl disulphide (DADS) and diallyl trisulphide (DATS) as well as tea tree oil and one of its main constituents terpinen-4-ol on the proliferation of *T. thermophila* cells in comparison with Jurkat T-lymphocytes. The results showed that *T. thermophila* cells with IC_{50} values of 0.6 mM for H_2O_2 and 0.11 mM for CMHP were highly resistant to peroxides compared with Jurkat T-lymphocytes with IC_{50} values of 0.05 mM for H_2O_2 and 0.008 mM for CMHP. Interestingly, in contrast to this, *T. thermophila* cells with IC_{50} values of 0.004% for garlic oil, 0.55 mM for DADS, 0.017 mM for DATS, 0.036% for tea tree oil and 1.1 mM for terpinen-4-ol were similar in sensitivity to Jurkat T-lymphocytes with IC_{50} values of 0.003%, 0.37 mM, 0.013 mM, 0.016% and 0.42 mM, respectively. Thus, *T. thermophila* was resistant to oxidative stress induced by peroxides but not by plant oils/compounds, in particular garlic oil and its allyl sulphides that are known to act as redox cycling agents. These results suggest that the high resistance of *T. thermophila* cells to H_2O_2 and CMHP could be attributable to a high abundance and/or efficiency of antioxidant enzymes, in particular peroxiredoxins, for removing H_2O_2 and CMHP from the cell.

2.1 Introduction

Tetrahymena thermophila is a free-living, ciliated protozoan used extensively as a model organism to study eukaryote cellular and molecular biology (Eisen et al., 2006). Its advantages include its ease of culture in inexpensive media, its rapid proliferation to high cell densities, its amenability to genetic manipulation and its abundance of core processes shared with higher eukaryotes, including humans, that are not found in other single-celled eukaryotic model organisms such as yeasts (Collins, 2012; Eisen et al., 2006). It is best known for its contributions to the discoveries of ribozymes (Zaug and Cech, 1986), telomeric repeats (Blackburn and Gall, 1978; Yao and Yao, 1981), telomerase enzymes (Greider and Blackburn, 1985) and the function of histone acetylation (Brownell et al., 1996). In addition, it belongs to the clade Alveolata which also includes important parasites such as *Plasmodium falciparum*, the causal agent of the most serious form of human malaria (Dorrell et al., 2013). Within the clade Alveolata, *T. thermophila* belongs to the phylum Ciliophora (ciliates) whereas *P. falciparum* belongs to the phylum Apicomplexa (apicomplexans). All apicomplexans are obligate parasites whereas most ciliates, including *T. thermophila*, are free-living. Thus, *T. thermophila* is a useful model to study *P. falciparum* and other apicomplexan parasites without the need for a host to culture the cells or special laboratory facilities to protect research personnel against infection.

Resistance to current anti-malarial drug therapies is continuing to grow (Fairhurst and Dondorp, 2016). Thus, there is an ongoing need to develop new therapies. Historically, plants have been important sources of anti-parasitic agents with most focus being on relatively polar compounds from aqueous or alcoholic extracts (Anthony et al., 2005). It

has been argued that plant essential oils, containing relatively non-polar compounds, are an under-exploited resource (Anthony et al., 2005). Specifically, due to their lipid solubility, the non-polar compounds found in plant essential oils should diffuse more readily across cell membranes and thereby better target intracellular parasites. As an example, garlic (*Allium sativum*) oil has been shown to be highly effective against a broad range of parasites including *P. berghei*, a relative of *P. falciparum* that infects rodents (Anthony et al., 2005; Perez et al., 1994). Similarly, tea tree (*Melaleuca alternifolia*) oil is well known for its anti-bacterial and anti-fungal properties (Carson et al., 2006) and more recently its active constituents (terpinen-4-ol, γ-terpinene and α-terpinene) have been shown to be toxic to the parasite *Trypanosoma evansi* (Baldissera et al., 2016). Trypanosomes are protozoa belonging to the phylum Kinetoplastida within the clade Euglenozoa, a sister clade to the Alveolata.

Thus, the aim of the present study was to investigate the effects of garlic oil, and its major constituents diallyl disulphide (DADS) and diallyl trisulphide (DATS), and tea tree oil, and one of its major constituents terpinen-4-ol, on the proliferation of *T. thermophila* cells. For comparison, we also investigated the effects of these oils/compounds on the proliferation of Jurkat-T lymphocytes. These cells come from a human cancer cell line that grows in suspension like *T. thermophila* and responds sensitively to cancer chemopreventive phytochemicals especially the well-characterised isothiocyanates from cruciferous vegetables such as broccoli (Brown et al., 2010; Brown et al., 2008). In previous studies, the cytotoxicity of DADS and DATS has been linked to their ability to participate in redox cycling and thereby induce oxidative stress (Munday, 2012). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the cell's capacity to remove them (Halliwell and Whiteman, 2004). The most important

ROS are the superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\bullet$OH). ROS damage cells by reacting with cellular molecules such as proteins, lipids and DNA. Allyl sulphides, such as DADS and DATS, can be readily converted to the corresponding thiols in a thiol-disulphide exchange reaction catalysed by glutathione S-transferase and the resulting thiols can then undergo metal-catalysed oxidation by molecular oxygen to reform the original disulphides. This redox cycling leads to the formation of ROS, including the superoxide radical and H_2O_2. Superoxide dismutase can convert the superoxide radical to H_2O_2 and antioxidant enzymes, such as catalases, glutathione peroxidases and peroxiredoxins, can detoxify H_2O_2 by reducing it to water. Alternatively, H_2O_2 can be converted to the hydroxyl radical, the most damaging of the ROS. Thus, we compared the effects of garlic oil, DADS and DATS with those of H_2O_2 and cumene hydroperoxide (CMHP), an organic hydroperoxide. We reasoned that H_2O_2 and CMHP would act directly to cause oxidative stress whereas garlic oil, DADS and DATS would act indirectly by participating in redox cycling. Tea tree oil and its active constituent terpinen-4-ol were also tested as examples of lipophilic agents that do not participate in redox cycling.

2.2 Materials and methods

2.2.1 Oils and chemicals

Hydrogen peroxide (37%, v/v) was purchased from Thermo Fisher Scientific Pty. Ltd. and cumene hydroperoxide (CMHP; 80%, v/v) from Sigma-Aldrich. Garlic (*Allium sativum*) oil (100%, v/v) was purchased from Mystic Moments (Fordingbridge, Hampshire, United Kingdom) and diallyl disulfide (DADS; 80%, v/v) from Sigma-Aldrich. Diallyl trisulfide (DATS; 95%, v/v) was purchased from Cayman Chemical, tea tree (*Melaleuca alternifolia*) oil (100%, v/v) from Integria Healthcare Australia Pty. Ltd. and terpinen-4-ol (97%, v/v) from VWR International Pty. Ltd. (Australia).

2.2.2 Tetrahymena thermophila cell culture

Tetrahymena thermophila strain CU428.2 cells were obtained from the *Tetrahymena* Stock Centre (https://tetrahymena.vet.cornell.edu/) located at Cornell University, United States of America. For routine maintenance, the cells were cultured without shaking at 13^{o}C in Neff medium in 25-cm^2 cell culture flasks (Corning) with subculturing every 4 weeks. The Neff medium contained 0.25% (w/v) proteose peptone (Thermo Fisher Scientific Pty Ltd), 0.25% (w/v) yeast extract (Sigma-Aldrich), 0.5% (w/v) glucose (Ajax Finechem Pty Ltd) and 33.3 μM $FeCl_3 \cdot 6H_2O$ (BDH Chemicals, Australia Pty Ltd) (Collins, 2012). Subculturing involved the transfer of 5 x 10^2 cells into 10 ml of fresh Neff medium. Prior to the initiation of the experimental treatments, 5 x 10^5 cells maintained as described above were inoculated into 25 ml of SSP medium in a 250 ml Erlenmeyer flask and cultured, with shaking (100 rpm), at 35^{o}C for a period of approximately 20 h. The SSP medium contained 2% (w/v) proteose peptone, 0.1% (w/v) yeast extract, 0.2% (w/v)

glucose and 33 μM $FeCl_3.6H_2O$ (Collins, 2012). The number of viable cells was determined by first of all adding 0.3% (v/v) formaldehyde to an aliquot of the cells to slow their movement and then staining the cells with Trypan blue dye before counting them using a haemocytometer (Pappenheimer, 1917). Note that viable cells exclude the Trypan blue dye.

2.2.3 Jurkat T-lymphocyte culture

Unless otherwise stated, all cell culture reagents were purchased from Sigma-Aldrich. The cells used in this study were from the Jurkat T-lymphoma cell line. Unless otherwise stated, they were cultured at a temperature of 37^{o}C in an atmosphere of 5% (v/v) CO_2 in air in a medium consisting of RPMI 1640 culture medium supplemented with 10% (v/v) foetal bovine serum (FBS, Life Technologies), 100 units ml^{-1} penicillin/streptomycin and 4 mM L-glutamine. This medium is hereafter referred to as the RPMI culture medium. The number of viable cells was determined by staining the cells with Trypan blue dye and then counting them using a haemocytometer (Pappenheimer, 1917).

2.2.4 Establishing the best method to monitor the proliferation for the T. thermophila cells

Proliferation of the *T. thermophila* cells was monitored using three different methods, the direct cell counting method using a haemocytometer, the indirect cell counting method using the Resazurin dye and the indirect cell counting method using the absorbance of the cell culture at a wavelength of 600 nm. For the direct cell counting method using a haemocytometer, the *T. thermophila* cells were seeded into 24-well plates at a density of 4×10^4 cells in 1.5 ml of SSP medium per well. The cells were then incubated with shaking

at 100 rpm for 4, 8, 12, 16, 20, 24, 28 or 32 hours at 35ºC in ambient air. There were four replicate wells per time-point. At each time-point, an aliquot of the cells was treated with 0.3% (v/v) formaldehyde and then the cells were stained with Trypan blue dye before being counted using a haemocytometer. For the indirect cell counting method using the Resazurin dye, the *T. thermophila* cells were seeded into a 24-well plate at a density of 4 x 10^4 cells in 1.425 or 1.45 ml SSP medium per well. This was followed by 50 or 75 µl of 0.02% (w/v) Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt from Sigma-Aldrich) dye dissolved in SSP medium per well. The total volume of liquid in each well was 1.5 ml. Metabolically active cells reduce the non-fluorescent Resazurin to the fluorescent Resorufin (7-Hydroxy-3H-phenoxazin-3-one sodium salt). Thus, the Resazurin dye could be used to differentiate between metabolically active and metabolically inactive cells. There were four replicate wells containing cells and two blank wells without cells. The cells were incubated for 30 min at 35ºC with shaking at 100 rpm in ambient air in a Rosi 1000™ Thermolyne Reciprocating/Orbital Shaking incubator followed by 32 hours at 35ºC with shaking at 100 rpm in ambient air in a CLARIOstar® plate reader (BMG Labtech Pty Ltd). The plate reader was used to measure the fluorescence of the cells employing excitation and emission wavelengths of 530 and 590 nm, respectively. Readings were taken every 6 min. For the indirect cell counting method using the absorbance at a wavelength of 600 nm, the *T. thermophila* cells were seeded into a 24-well plate at a density of 4 x 10^4 cells in 1.5 ml of SSP medium per well. There were four replicate wells containing cells and two blank wells without cells. The cells were incubated for 32 hours at 35ºC with shaking at 100 rpm in ambient air in a FLUOstar® plate reader (BMG Labtech Pty Ltd). The plate reader was used to measure the turbidity of the cell suspension at a wavelength of 600 nm. Readings were taken every 6 min. Since

this method relies on turbidity, it does not differentiate between metabolically active or live and metabolically inactive or dead cells. All of the above experiments were repeated at least 5 times for both the direct and indirect cell counting methods.

2.2.5 Establishing the best method to monitor the proliferation for the Jurkat T-lymphocytes

Proliferation of the Jurkat T-lymphocytes was monitored using the same three methods as described above for the *T. thermophila* cells but with some modifications. For the direct cell counting method using a haemocytometer, the Jurkat T-lymphocytes were seeded into 24-well plates at a density of 3 x 10^4 cells in 1.5 ml of RPMI culture medium per well. The cells were then incubated for 12, 24, 36, 48, 60 or 64 hours at 37^{o}C in RPMI culture medium in 5% (v/v) CO_2 in air in a CO_2 incubator. There were four replicate wells per time-point. At each time-point, the cells were stained with Trypan blue dye and then the cell number was determined using a haemocytometer. For the indirect cell counting method using the Resazurin dye, the Jurkat T-lymphocytes were seeded into a 24-well plate at a density of 3 x 10^4 cells in 1.45 ml RPMI culture medium per well. This was followed by 50 µl of 0.02% (w/v) Resazurin dye dissolved in RPMI culture medium per well. The total volume of liquid in each well was 1.5 ml. There were four replicate wells containing cells and two blank wells without cells. The cells were incubated for 30 min at 37^{o}C in RPMI culture medium in 5% (v/v) CO_2 in air in a CO_2 incubator followed by 64 hours at 37^{o}C in ambient air in a CLARIOstar® plate reader. The plate reader was used to measure the fluorescence of the cells employing excitation and emission wavelengths of 530 and 590 nm, respectively. Readings were taken every 15 min. For the indirect cell counting method using the absorbance at a wavelength of 600 nm, the Jurkat T-

lymphocytes were seeded into a 24-well plate at a density of 3 x 10^4 or 1.6 x 10^5 cells per well in 1.5 ml of RPMI culture medium per well. There were four replicate wells containing cells and two blank wells without cells. The cells were incubated for 64 hours at 37^{o}C in ambient air in a FLUOstar® plate reader. The plate reader was used to measure the turbidity of the cell suspension at a wavelength of 600 nm. Readings were taken every 15 min. All of the above experiments were repeated at least 5 times for both the direct and indirect cell counting methods.

2.2.6 Effects of selected pro-oxidants on T. thermophila cell proliferation

The effects of H_2O_2, cumene hydroperoxide (CMHP), garlic oil, diallyl disulphide (DADS), diallyl trisulphide (DATS), tea tree oil and terpinen-4-ol on the proliferation of *T. thermophila* cells were investigated. Stock solutions of the test oils and compounds, except for H_2O_2 and CMHP, were prepared in dimethyl sulfoxide (DMSO). Prior to each experiment, the stock solutions and the H_2O_2 and CMHP were diluted in SSP medium and added to the appropriate wells of a 24-well plate. This was followed by the addition of 4 x 10^4 *T. thermophila* cells, in SSP medium, added to each well. The total volume of liquid in each well was 1.5 ml and the maximum concentration of DMSO was 0.15% (v/v). This concentration had been shown previously not to affect the proliferation of the cells (see Appendix A.1). For each concentration of each test substance, there were three replicate wells containing cells and one blank well without cells. The cells were incubated for 24 hours at 35^{o}C with shaking at 100 rpm in ambient air in a FLUOstar® plate reader. Cell proliferation was monitored according to the indirect cell counting method using the absorbance at a wavelength of 600 nm as described above (see Section 2.2.4.). This method was chosen as the most appropriate method (see Results section). The

concentration of each test substance required to inhibit cell proliferation by 50% (IC_{50}) was determined at 24 hours after the initiation of each treatment. The absorbance values at the end of the log (exponential) phase were used in the IC_{50} calculations.The IC_{50} values were calculated as previously described (Berthelier and Wetzel, 2006). In brief, a linear trend line was fitted to a plot of % inhibition of cell proliferation (relative to the control) against the concentration of the test substance and the equation of the line thus generated was used to determine the test substance concentration that gave 50% inhibition of cell proliferation. The experiments were repeated at least five times for H_2O_2, CMHP, garlic oil and DADS, three times for DATS, and five times for tea tree oil and terpinen-4-ol. The results for one representative experiment are presented here.

2.2.7 Effects of selected pro-oxidants on Jurkat T-lymphocyte proliferation

The effects of H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the proliferation of Jurkat T-lymphocytes were investigated. Stock solutions of the test oils and compounds, except for H_2O_2 and CMHP, were prepared as described above. Prior to each experiment, the stock solutions and the H_2O_2 and CMHP were diluted in RPMI culture medium and added to the appropriate wells of a 24-well plate. Subsequently, 3 x 10^4 Jurkat T-lymphocytes and 50 µl of 0.02% (w/v) Resazurin dye, both in RPMI culture medium, were added to each well. The total volume of liquid in each well was 1.5 ml and the maximum concentration of DMSO was 0.16% (v/v). This concentration had been shown previously not to affect the proliferation of the cells (see Appendix A.2). For each concentration of each test substance, there were three replicate wells containing cells and one blank well without cells. The cells were incubated for 30 min at 37ºC in 5% (v/v) CO_2 in air in a CO_2 incubator followed by 64 h at 37ºC in ambient air in a CLARIOstar® plate

reader. Cell proliferation was monitored according to the indirect cell counting method using the Resazurin dye as described above (see Section 2.2.5). The concentration of each test substance required to inhibit cell proliferation by 50% (IC_{50}) was determined at 64 hours after the initiation of each treatment. The fluorescence values at the end of the log (exponential) phase were used in the IC_{50} calculations. The IC_{50} values were calculated as previously described (Berthelier and Wetzel, 2006). In brief, a linear trend line was fitted to a plot of % inhibition of cell proliferation (relative to the control) against the concentration of the test substance and the equation of the line thus generated was used to determine the test substance concentration that gave 50% inhibition of cell proliferation. The experiments were repeated at least three times for H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol. The results for one representative experiment are presented here.

2.2.8 Gas chromatography/mass spectrometry analyses

The compositions of the garlic and tea tree oil preparations used in this study were determined using gas chromatography/mass spectrometry (GC/MS). The analyses were performed using an Agilent Technologies 5975C mass spectrometer equipped with an Agilent Technologies 7890A gas chromatograph and an Agilent Technologies 7683B auto-sampler. The GC column was a Restek Rxi-5Sil-MS capillary column of 30 m length and 250 µm diameter with a film thickness of 0.25 µm. Helium was used as the carrier gas at a constant flow rate of 1 ml min^{-1}. The injection port temperature was set at 280^{o}C. Aliquots of the garlic and tea tree oil preparations were diluted in heptane (1:50) and 1 µl of the diluted sample was injected into the GC with a split ratio of 50:1. The GC temperature was programmed to start at 40^{o}C for 4 min and then increase by 20^{o}C every

1 min before reaching a final temperature of 300^{o}C for 4 min. The GC/MS interface temperature was set at 280^{o}C and the source temperature was set at 230^{o}C. The MS quadrupole temperature was set at 150^{o}C. The mass spectrum was scanned from m/z 35 to m/z 550 using an electron ionisation system with the electron energy set to 70 eV. The compounds in the garlic and tea tree oil preparations were identified based on comparisons of their mass spectra with those of reference compounds. The relative abundances of the compounds were determined by calculating the area under the peak for each compound and then expressing this as a percentage of the total area for all peaks.

2.2.9 Statistical analyses

Statistical analyses were conducted using the IBM SPSS Statistics 19 software package. The data were analysed using either one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* multiple comparisons test or an independent-samples T-test. Differences were considered to be significant when $p < 0.05$.

2.3 Results

2.3.1 Chemical composition of the garlic and tea tree oil preparations used in this study

The chemical compositions of the garlic and tea tree oil preparations were determined using GC/MS and are reported as % of total compounds present based on the area under the peak for each compound (Tables 2.1). The major compounds present in the garlic oil were allyl sulphides. Diallyl disulphide (DADS) was the most abundant at 37.3% of the total followed closely by diallyl trisulphide (DATS) at 34.2%. The major compounds present in the tea tree oil were monoterpenes. Terpinen-4-ol was the most abundant at 39.7% of the total with lesser amounts of the other compounds.

2.3.2 Establishing the best method to monitor the proliferation for the T. thermophila cells

Fig. 2.1 shows proliferation curves for *T. thermophila* obtained using either the direct cell counting method employing a haemocytometer (Fig. 2.1A) or the indirect cell counting method based on the absorbance of the culture at 600 nm (Fig. 2.1B). The indirect cell counting method employing the Resazurin dye was abandoned because rapid reduction of the blue non-fluorescent Resazurin to the red fluorescent Resorufin occurred within a very short period of time giving fluorescence readings that exceeded the maximum range of the CLARIOstar® plate reader (data not shown). Regardless of which of the other two methods was used, the proliferation curves showed similar shapes with a lag phase at 0 – 4 h, a log/exponential phase at 4 – 20 h and a stationary/plateau phase at 20 – 28 h (Figs. 2.1A-B). Since it was less time-consuming than the direct cell counting method employing a haemocytometer and since cell proliferation could be continuously monitored, the

indirect cell counting method based on the absorbance at 600 nm, was chosen for the subsequent experiments. The number of cells determined using the absorbance at 600 nm was strongly positively correlated with the number of cells determined using a haemocytometer (Fig. 2.1C). Thus, this was evidence that the indirect cell counting method based on the absorbance of 600 nm was reliable.

2.3.3 Establishing the best method to monitor the proliferation for the Jurkat T-lymphocytes

Fig. 2.2 shows proliferation curves for the Jurkat T-lymphocytes obtained using either the direct cell counting method employing a haemocytometer (Fig. 2.2A) or the indirect cell counting method using the Resazurin dye (Fig. 2.2B). The indirect cell counting method based on the absorbance of the culture at a wavelength of 600 nm was abandoned because it required a much larger number of cells than either of the other two methods (data not shown). Regardless of which of the other two methods was used, the proliferation curves showed similar shapes with a lag phase at 0 – 16 h, a log/exponential phase at 16 – 60 h and a stationary/plateau phase at 60 – 64 h (Figs. 2.2A-B). Since it was less time-consuming than the direct cell counting method employing a haemocytometer and since cell proliferation could be continuously monitored, the indirect cell counting method using the Resazurin dye was chosen for the subsequent experiments. The number of cells determined using the Resazurin assay was strongly positively correlated with the number of cells determined using a haemocytometer (Fig. 2.2C). Thus, this was evidence that the indirect cell counting method using the Resazurin dye was reliable.

2.3.5 Effects of selected pro-oxidants on the proliferation of T. thermophila cells

Fig. 2.3 shows the effects of H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the proliferation of *T. thermophil*a cells. The proliferation curves for the control cells were similar between the different experimental treatments, with a short lag phase (0 to 4h), a long exponential phase (4 to 20h), and a short stationary phase (20 to 24h). All of the test oils/compounds inhibited the proliferation of the cells but some were more effective than others. The proliferation of the cells was completely inhibited at all time-points in the presence of 0.7 mM H_2O_2, 0.15 mM CMHP, 0.005% (v/v) garlic oil, 1.0 mM DADS, 0.020 mM DATS, 0.05% (v/v) tea tree oil and 2.0 mM terpinen-4-ol. Thus, garlic oil was more effective than tea tree oil and DADS and DATS were more effective than terpinen-4-ol. DATS was also more effective than DADS. This latter suggests that the number of sulphur atoms in the allyl sulphides was important in determining their anti-proliferative effect. CMHP was less effective than DATS but it was more effective than DADS or H_2O_2. In summary, DATS was highly effective. In addition, the IC_{50} values determined at 24 hours after the initiation of the treatments showed that CMHP was 5.5-fold more effective than H_2O_2 at inhibiting the proliferation of the cells whereas DATS was 6.5-fold more effective than CMHP (Table 2.2). DATS (IC_{50} = 0.017 mM) was much more effective than DADS (IC_{50} = 0.55 mM) or terpinen-4-ol (IC_{50} = 1.1 mM). DADS was more effective than terpinen-4-ol but it was similar in effectiveness to H_2O_2. Furthermore, garlic oil (IC_{50} = 0.004%) was more effective than tea tree oil (IC_{50} = 0.036%). DADS and DATS were approximately equally abundant in the garlic oil preparation used in this study (Table 2.1) but given that the IC_{50} value for DATS was less than 5% of that for DADS, it is likely that the anti-proliferative effect of garlic oil is mostly attributable to DATS.

2.3.6 Effects of selected pro-oxidants on the proliferation of Jurkat T-lymphocytes

Fig. 2.4 shows the effects of H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the proliferation of Jurkat T-lymphocytes. The proliferation curves for the control cells were similar between the different experimental treatments, with a short lag phase (~ 0 to 16), a long exponential phase (~ 16 to 60h) and a short stationary phase (~ 60 to 64). All of the test oils/compounds inhibited the proliferation of the cells but some were more effective than others. The proliferation of the cells was nearly totally inhibited at all time-points in the presence of 0.06 mM H_2O_2 and 0.016 mM DATS whereas it was completely inhibited in the presence of 0.04 mM CMHP, 0.005% (v/v) garlic oil, 0.8 mM DADS, 0.04% (v/v) tea tree oil and 2.0 mM terpinen-4-ol. Thus, garlic oil was more effective than tea tree oil and DADS and DATS were more effective than terpinen-4-ol. DATS was also more effective than DADS. Again, this latter suggests that the number of sulphur atoms in the allyl sulphides was important in determining their anti-proliferative effect. CMHP was similar in effectiveness to DATS but it was more effective than DADS or H_2O_2. In summary, DATS was highly effective. In addition to this, the IC_{50} values determined at 64 hours after the initiation of the treatments showed that CMHP was 6.3-fold more effective than H_2O_2 at inhibiting the proliferation of the cells (Table 2.2). DATS was similar in effectiveness to CMHP but it was much more effective than DADS (IC_{50} = 0.37 mM). Terpinen-4-ol (IC_{50} = 0.42 mM) was similar in effectiveness to DADS but was much less effective than H_2O_2. Furthermore, garlic oil (IC_{50} = 0.003%) was more effective than tea tree oil (IC_{50} = 0.016%). DADS and DATS were approximately equally abundant in the garlic oil preparation used in this study (Table 2.1) but given that the IC_{50} value for DATS was less than 5% of that for DADS, it is likely that the anti-proliferative effect of garlic oil is mostly attributable to DATS.

2.3.7 Comparison of IC_{50} values for T. thermophila and Jurkat T-lymphocytes

In general, the *T. thermophila* cells were less sensitive to the inhibitory effects of the pro-oxidants than the Jurkat T-lymphocytes but the effects were more or less pronounced depending on which pro-oxidant was being tested (Table 2.2). The differences between the two cell types were much greater for H_2O_2 and CMHP than they were for garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol. For H_2O_2 and CMHP, the IC_{50} values were in excess of 10-fold greater for the *T. thermophila* cells than they were for the Jurkat T-lymphocytes. In contrast, for all of the other test substances, the IC_{50} values were only 1.3- to 2.6-fold greater for the *T. thermophila* cells than they were for the Jurkat T-lymphocyttes. Thus, the *T. thermophila* cells were much less sensitive to the peroxides than the Jurkat T-lymphocytes but both cell types showed similar sensitivities to the oils and their dominant constituents.

2.4 Discussion

In this study, *T. thermophila* cells were found to be substantially more resistant to the anti-proliferative effects of H_2O_2 and CMHP than Jurkat T-lymphocytes but the two cell types responded similarly to garlic oil and its major constituents DADS and DATS as well as to tea tree oil and its major constituent terpinen-4-ol. This suggests that *T. thermophila* cells are more resistant than human cancer cells to oxidative stress but only when the stress is induced by H_2O_2 or CMHP. H_2O_2 and CMHP cause oxidative stress by reacting with cellular constituents including lipids, proteins and nucleic acids but they are relatively unstable (Ayala et al., 2014; Ayala et al., 1996; Halliwell and Whiteman, 2004). In contrast, allyl sulphides such as DADS and DATS can act as redox cycling agents continuously generating H_2O_2 and other ROS (Munday, 2012). The mechanism is as follows. Allyl sulphides are reduced to the corresponding thiols in a thiol-disulphide exchange reaction catalysed by glutathione *S*-transferase in the presence of glutathione, as the electron donor. Subsequently, the thiols undergo metal-catalysed oxidation by molecular oxygen to reform the original sulphides and perpetuate the cycle. In the process, ROS (including $\bullet O_2^-$, H_2O_2 and •OH) are continuously generated. This continuous generation of ROS, as opposed to a bolus supply, may explain why the *T. thermophila* cells were more resistant to H_2O_2 and CMHP than they were to DADS or DATS but it does not necessarily explain why the *T. thermophila* cells were more resistant to treatment with H_2O_2 and CMHP than were the Jurkat T-lymphocytes. A possible explanation as to why the *T. thermophila* cells were more resistant to the effects of H_2O_2 and CMHP than were the Jurkat T-lymphocytes could be that the *T. thermophila* cells have more effective antioxidant enzymes for removing the peroxides, H_2O_2 and CMHP. Known peroxide detoxifying antioxidant enzymes include catalases, glutathione peroxidases and

peroxiredoxins (Winterbourn, 2013; Wood et al., 2003a). All three enzyme families can use H_2O_2 as a substrate but only the glutathione peroxidases and peroxiredoxins can use CMHP with the peroxiredoxins being more efficient than the glutathione peroxidases in this respect (Nelson and Parsonage, 2011). Thus, our results suggest that *T. thermophila* has one or more very active peroxiredoxin enzymes. There is evidence that mammalian peroxiredoxins reduce more than 90% of the total cellular H_2O_2 (Adimora et al., 2010; Cox et al., 2010a; Karplus, 2015). Moreover, they are up to 100-fold faster at oxidising H_2O_2 than are the corresponding glutathione peroxidases (Huang and Sikes, 2014). Thus, it is plausible that *T. thermophila* may possess a particularly effective suite of peroxiredoxin enzymes. Future studies should test this hypothesis.

Previous studies have shown that relatively low concentrations of H_2O_2 (0.010 to 0.075 mM) can inhibit the proliferation of various human cancer cell lines. For example, the A549 human lung cancer cell line had an $IC_{50}(H_2O_2)$ value of 0.01 mM, the HepG2 human hepatocellular cancer cell line had an $IC_{50}(H_2O_2)$ value of 0.07 mM, the Calu-6 human lung cancer cell line had an $IC_{50}(H_2O_2)$ value of 0.075 mM and the HeLa human cervical cancer cell line had an $IC_{50}(H_2O_2)$ value of ~ 0.075 mM (Luo et al., 2016; Park, 2013; Park, 2014). These values are similar to the value obtained for the Jurkat T-lymphoma cell line (0.05 mM) in the present study. Thus, the Jurkat T-lymphoma cell line is similar to other human cancer cell lines in its response to H_2O_2. In contrast to the above, *T. thermophila* with an IC_{50} value for H_2O_2 of 0.6 mM was much more resistant to the effects of H_2O_2 than the human cancer cells. In a previous study, it was observed that treatment of *T. pyriformis*, a relative of *T. thermophila*, with 0.4 mM H_2O_2 totally inhibited the proliferation of the *T. pyriformis* cells (Fourrat et al., 2007). Thus, the value we obtained in the present study was similar to the value obtained in the previous study on a related

organism. This suggests that insensitivity to H_2O_2 may be a broader phenomenon affecting all *Tetrahymena* species and perhaps all ciliated protozoans. Very few studies have investigated this. We know of only one example. Like *T. thermophila*, the ciliates *Euplotes raikovi* and *E. nobilii* have been shown to be resistant to H_2O_2. *E. raikovi* required 0.75 mM H_2O_2 for the proliferation of the cells to be totally inhibited whereas *E. nobilii* survived in the presence of 2 mM H_2O_2 (Dobri et al., 2014).

Unlike their responses to H_2O_2 and CMHP, the *T. thermophila* cells were similar to the Jurkat T-lymphocytes in their responses to garlic oil, DADS and DATS. The HCT-15 and DLD-1 human colon cancer cell lines have been shown to be sensitive to DATS with IC_{50} values for DATS of 0.012 and 0.013 mM, respectively (Hosono et al., 2005). In contrast to this, DADS at 0.1 mM had no effect on the proliferation of either the HCT-15 or the DLD-1 human colon cancer cell line (Hosono et al., 2005). The values obtained with DATS for these two cell lines are very similar to those obtained with DATS for the Jurkat T-lymphoma cell line (0.013 mM) and the *T. thermophila* cells (0.017 mM). In addition, the sensitivity to DADS is similar amongst the *T. thermophila*, Jurkat T-lymphoma and colon cancer cells. Therefore, *T. thermophila* and human cells are equally sensitive to DADS.

Whereas garlic oil and its allyl sulphides are redox-cycling agents, tea tree oil and its monoterpene (terpinen-4-ol) are not redox cycling agents. *T. thermophila* cells were shown to be somewhat less sensitive to tea tree oil and terpinen-4-ol than Jurkat T-lymphocytes in the present study. Tea tree oil and terpinen-4-ol have been shown to inhibit the proliferation of a wide range of mammalian cancer cell lines. For example, the IC_{50} values for tea tree oil ranged from 0.012% for A549 human lung cancer cells to 0.031% for MCF-7 human breast cancer cells and 0.037% for PC-3 human prostate cancer cells

(Liu et al., 2009). In contrast, the IC_{50} values for terpinen-4-ol ranged from 0.36 mM for Jurkat T-lymphocytes in a previous study to ~ 1.9 mM for HeLa cervical cancer cells (Döll-Boscardin et al., 2012). Similarly, in mouse cancer cells, the IC_{50} values for tea tree oil ranged from 0.02% for AE17 malignant mesothelioma cells to 0.03% for B16 malignant melanoma cells (Greay et al., 2010). In contrast, the IC_{50} values for terpinen-4-ol ranged from 1.3 mM for AE17 malignant mesothelioma cells to 3.2 mM for B16 malignant melanoma cells (Greay et al., 2010). The values obtained with tea tree oil for human/mouse cancer cell lines are similar to the values obtained for the Jurkat T-lymphoma cell line (0.016%) and *T. thermophila* cells (0.036%) in the present study. In addition, the values obtained with terpinen-4-ol for human/mouse cancer cell lines are similar to the values obtained for the Jurkat T-lymphoma cell line (0.42 mM) and *T. thermophila* cells (1.1 mM) in the present study. Consequently, *T. thermophila* and various mammalian cells are equally sensitive to tea tree oil and terpinen-4-ol. Like garlic oil and allyl sulphides, tea tree oil and terpinen-4-ol act indirectly to induce oxidative stress in cells (Calcabrini et al., 2004; Greay et al., 2010). The exact mechanism of action of the terpenoids found in tea tree oil is not fully understood. Terpinen-4-ol is a monoterpene and therefore very different in its chemical structure to the sulfur-containing DADS and DATS (Kuttan et al., 2011; Santos et al., 2011). It is known that monoterpenes inhibit farnesyl transferases and that these enzymes catalyse the transfer of a 15 carbon isoprenyl lipid moiety onto a conserved Cys residue near to the C-terminus of various proteins including protein-tyrosine phosphatases (Basso et al., 2006). Protein farnesylation promotes membrane association and contributes to protein-protein interactions. Recently it was shown that the peroxidase activity of peroxiredoxin 1 is lost when it is phosphorylated by certain protein-tyrosine kinases and that this

phosphorylation/inactivation occurs only when peroxiredoxin 1 is associated with lipid rafts (Woo et al., 2010). The phosphorylation/inactivation of peroxiredoxin 1 can be reversed by protein-tyrosine phosphatases and it is proposed that inactivation of the lipid raft-associated peroxiredoxin 1 allows localised accumulation of H_2O_2 which in turn allows inactivation of the protein-tyrosine phosphatases.

In conclusion, we have provided evidence that *T. thermophila* cells are substantially more resistant to oxidative stress induced by peroxides (H_2O_2 and CMHP) than Jurkat T-lymphocytes but both cell types show similar sensitivities to oxidative stress induced by garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol. We suggest that the high resistance of *T. thermophila* cells to H_2O_2 and CMHP could be attributable to a high abundance and/or efficiency of antioxidant enzymes, in particular peroxiredoxins, for removing H_2O_2 and CMHP from the cell.

Table 2.1. Relative amounts of the major constituents of garlic (*A. sativum*) oil and tea tree (*M. alternifolia*) oil.

Garlic oil		Tea tree oil	
Compound	%	Compound	%
Diallyl disulfide	37.3	terpinen-4-ol	39.7
Diallyl trisulfide	34.2	γ-terpinene	19.2
Diallyl sulfide	14.4	α-terpinene	11.4
Methyl allyl trisulfide	5.4	1,8-Cineole	9.6
1,3-Dithiane	5.0	α-terpineol	6.5
Dimethyl trisulfide	2.7	α-terpinolene	5.6
3-Vinyl-3,4-dihydro-1,2-dithiine	1.0	p-Cymene	4.3
		α-pinene	3.7

Table 2.2. IC_{50} (mean (n = 3) ± standard error) values for the inhibition of the proliferation of *T. thermophila* and Jurkat T-lymphoma cells.

Treatment	IC_{50}		Fold difference
	T. thermophila	Jurkat T-lymphocyte	
H_2O_2* (mM)	0.6 ± 0.0039	0.05 ± 0.0011	12
CMHP* (mM)	0.11 ± 0.0012	0.008 ± 0.0001	14
Garlic oil** (%)	0.004 ± 0.0002	0.003 ± 0.00009	1.4
DADS* (mM)	0.55 ± 0.0016	0.37 ± 0.0012	1.5
DATS* (mM)	0.017 ± 0.00009	0.013 ± 0.0002	1.3
Tea tree oil** (%)	0.036 ± 0.00015	0.016 ± 0.0001	2.3
Terpinen-4-ol* (mM)	1.1 ± 0.0095	0.42 ± 0.0013	2.6

* indicates a significant difference among H_2O_2, CMHP, DADS, DATS and terpinen-4-ol ($p < 0.05$). ** indicates a significant difference between garlic and tea tree oils ($p < 0.05$).

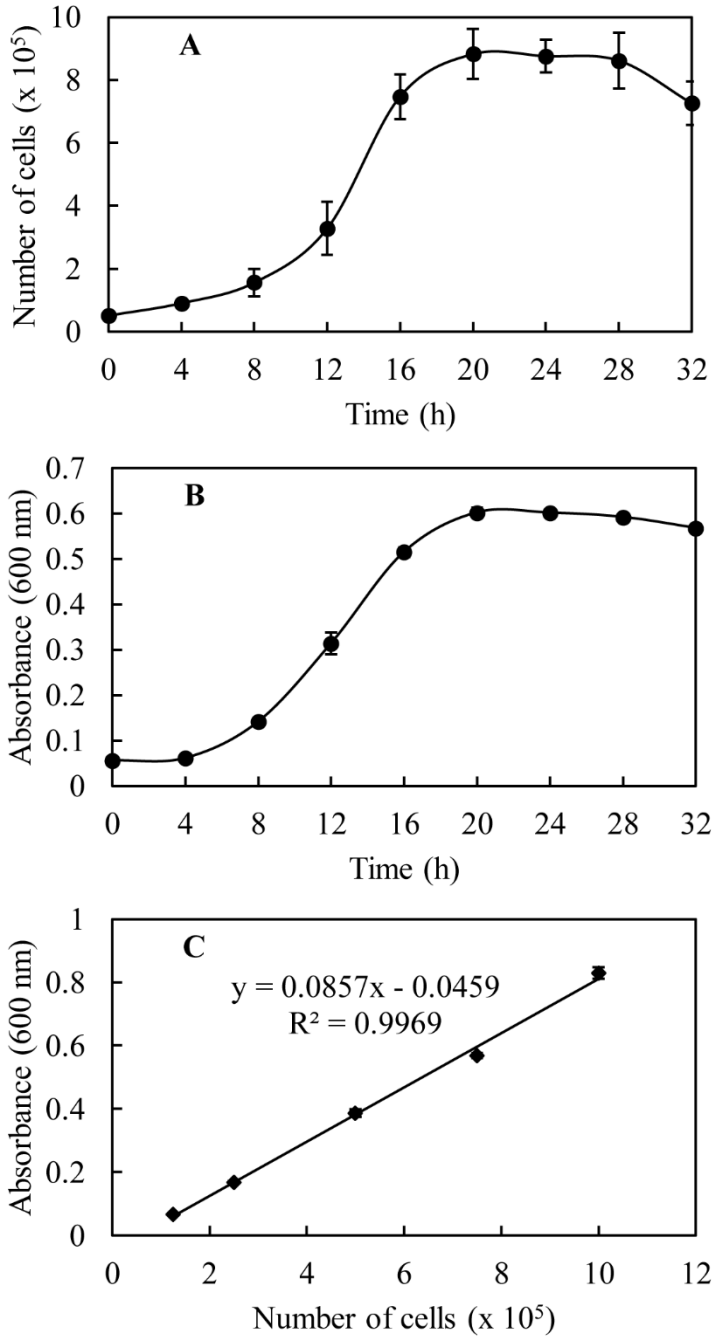

Fig. 2.1. Typical proliferation curve for *T. thermophila* cells. Cell proliferation was determined employing the direct cell counting method using a haemocytometer (A) and the indirect cell counting method using the absorbance at a wavelength of 600 nm (B). A standard curve shown in Panel C was determined using both direct and indirect cell counting methods shown in Panels A and B. The data points are the mean values for 4 replicate wells and the vertical bars represent ± standard error of the mean (n = 4, except for Panel C, n = 3). The sizes of the error bars in Panel C were smaller than the sizes of the data points in all cases.

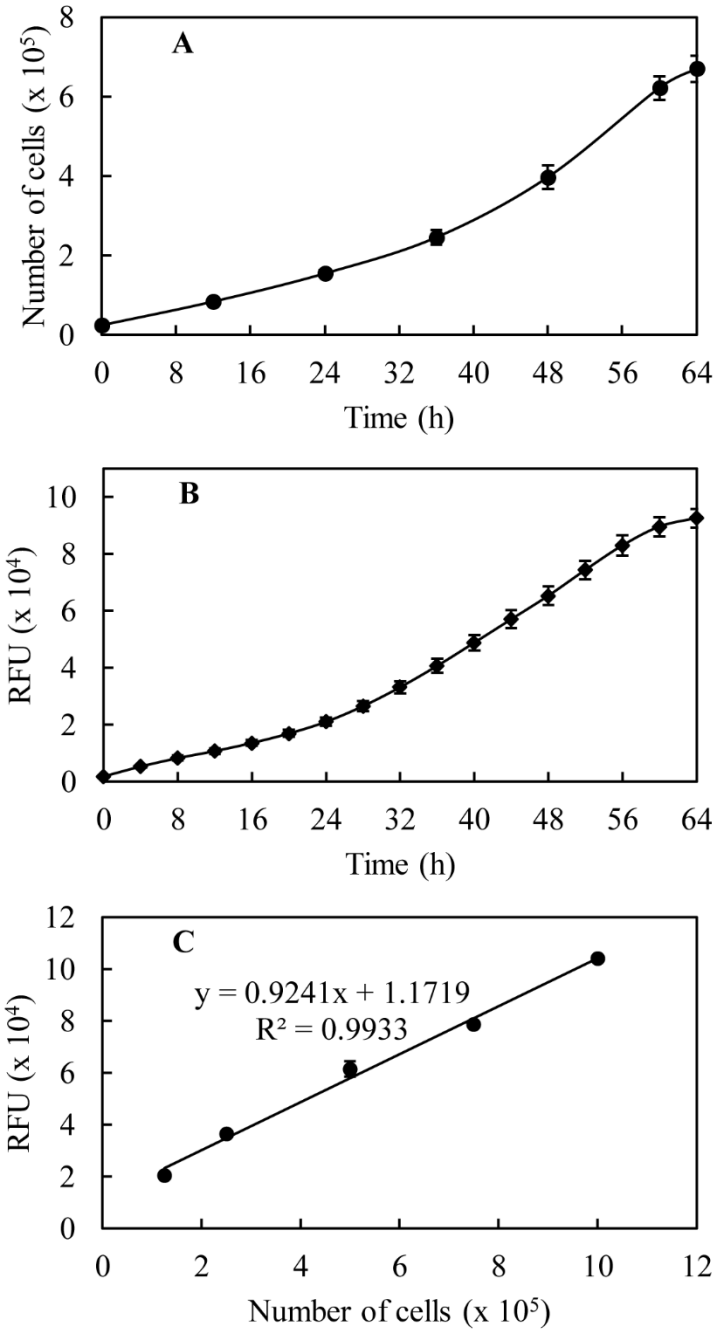

Fig. 2.2. Typical proliferation curve for Jurkat T-lymphoma cells. Cell proliferation was determined employing the direct cell counting method using a haemocytometer (A) and the indirect cell counting method using the Resazurin dye (B). A standard curve shown in Panel C was determined using both direct and indirect cell counting methods shown in Panels A and B. The data points are the mean values for 4 replicate wells and the vertical bars represent ± standard error of the mean (n = 4, except for Panel C, n = 3). The sizes of the error bars in Panel C were smaller than the sizes of the data points in all cases. RFU, Relative fluorescence units.

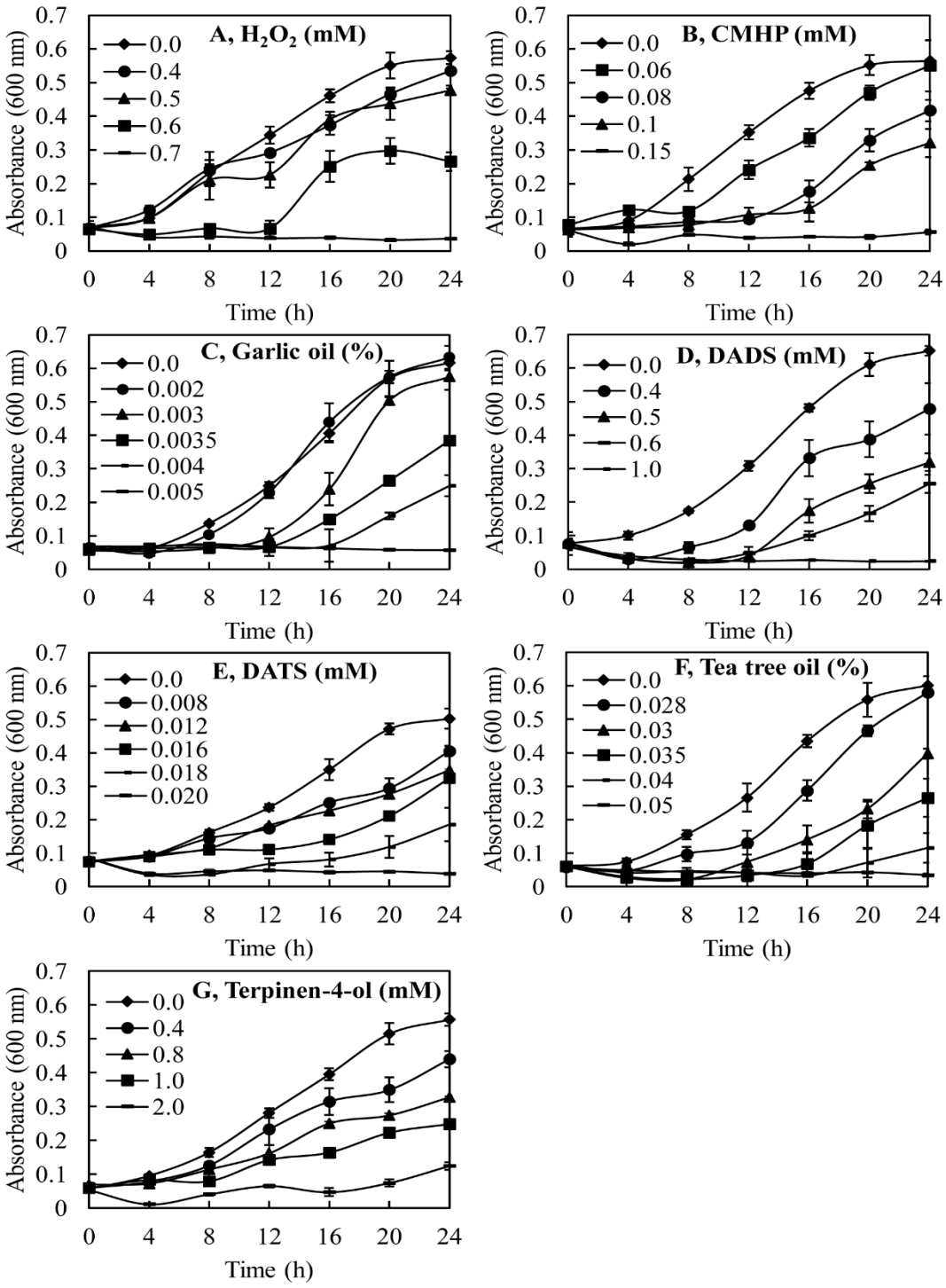

Fig. 2.3. Effects of pro-oxidant oils/chemicals on the proliferation of *T. thermophila* cells. The cells were exposed to various concentrations of H_2O_2 (A), CMHP (B), garlic oil (C), DADS (D), DATS (E), tea tree oil (F) or terpinen-4-ol (G). Cell proliferation was monitored employing the indirect cell counting method using the absorbance at a wavelength of 600 nm (see Materials and Methods). The data points are the mean values for 3 replicate wells and the vertical bars represent ± standard error of the mean (n = 3).

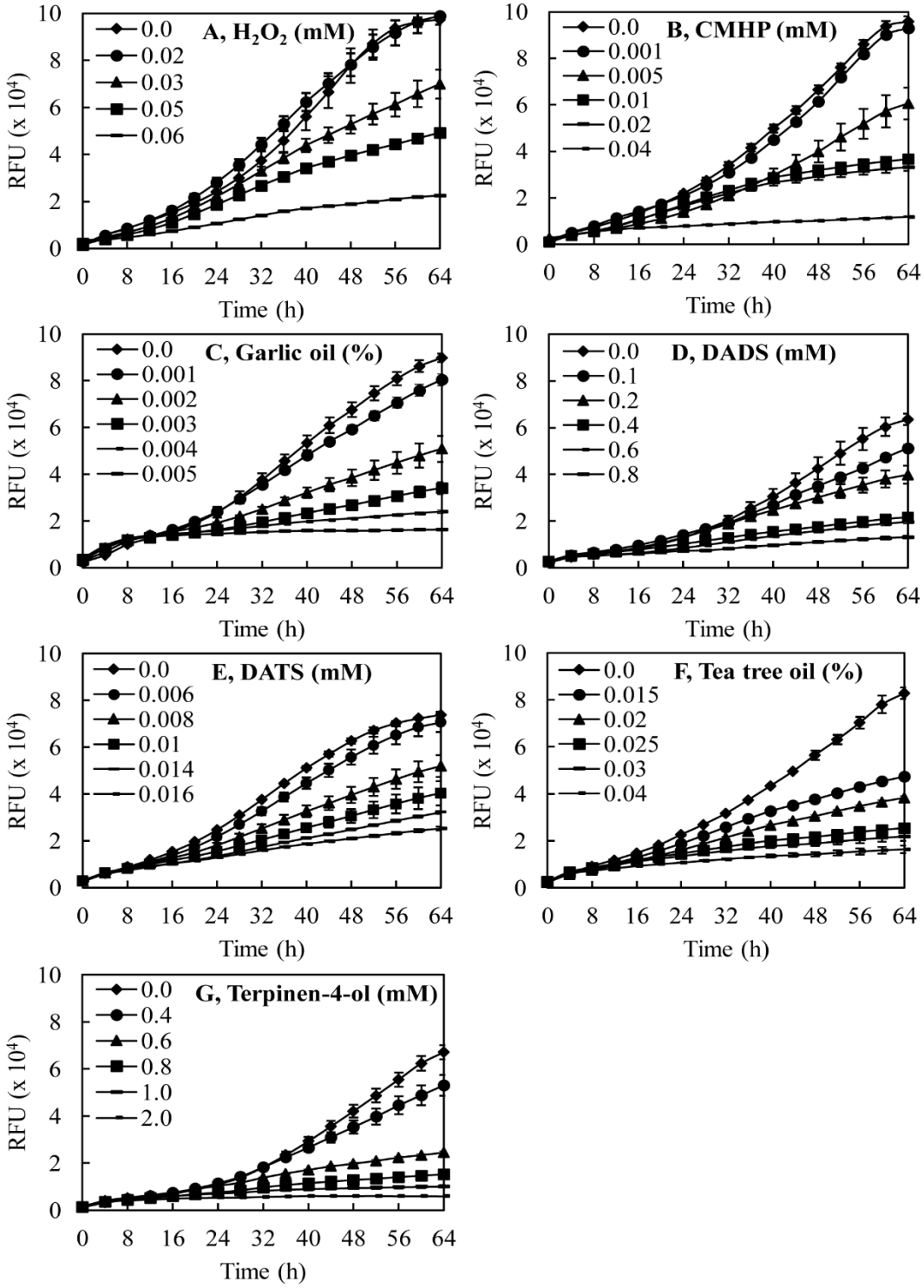

Fig. 2.4. Effects of pro-oxidant oils/chemicals on the proliferation of Jurkat T-lymphocytes. The cells were exposed to various concentrations of H_2O_2 (A), CMHP (B), garlic oil (C), DADS (D), DATS (E), tea tree oil (F) or terpinen-4-ol (G). Cell proliferation was monitored employing the indirect cell counting method using the Resazurin dye (see Materials and Methods). The data points are the mean values for 3 replicate wells and the vertical bars represent ± standard error of the mean (n = 3, except for DADS, n = 2). RFU, Relative fluorescence units.

Chapter 3 - *Tetrahymena thermophila* peroxiredoxin 1m protein is insensitive to hyperoxidation

Abstract

Peroxiredoxin (Prx) proteins are a family of thiol peroxidases that remove H_2O_2 and various organic hydroperoxides from the cell by reducing them to less reactive molecules. These enzymes have been studied in various species from prokaryotes to eukaryotes but not in *Tetrahymena thermophila* or any other non-parasitic alveolates. *T. thermophila* was found to contain four genes encoding typical 2-Cys Prx proteins but lacks any genes encoding atypical 2-Cys Prx or 1-Cys Prx proteins. It was predicted that *Prx1a* and *Prx1b* from *T. thermophila* were cytosolic or nuclear proteins whereas *Prx1m* was a mitochondrial protein. *Prx1c* was likely to be a secreted protein. The typical 2-Cys Prx proteins are known to exist as either reduced monomers, oxidised dimers or hyperoxidised monomers, depending upon the cellular H_2O_2 concentration and they have been classified into sensitive and robust types. Members of the sensitive type (mostly from eukaryotes) form hyperoxidised monomers and lose their peroxidase activity in the presence of high H_2O_2 concentrations whereas members of the robust type (mostly from prokaryotes) do not form hyperoxidised monomers. The four *T. thermophila* Prx proteins were shown to be closely related to the human sensitive typical 2-Cys Prx proteins in relation to their amino acid sequences. In particular, they possessed the GGLG and YF motifs that are a common feature of most sensitive eukaryotic typical 2-Cys Prx proteins. Unlike humans, *T. thermophila* lacks sulfiredoxin proteins, the enzymes required for the regeneration of the reduced monomer form of the typical 2-Cys Prx proteins from their hyperoxidised monomer form. The *in vivo* redox/oligomerization state of the *T. thermophila Prx1m* protein was investigated in the presence of increasing concentrations of H_2O_2, cumene hydroperoxide, garlic oil, diallyl disulphide (DADS), diallyl trisulphide (DATS), tea tree oil and terpinen-4-ol. All of the test substances, except for DADS, promoted oxidised dimer formation in the *Prx1m* protein but not hyperoxidised monomer

formation. Thus, the *T. thermophila Prx1m* protein was insensitive to hyperoxidation and therefore belonged to the robust typical 2-Cys Prx type. This indicates that the susceptibility of typical 2-Cys Prx proteins to hyperoxidation might not always be associated with the presence of the GGLG and YF motifs. The results also showed that DATS was more effective than DADS in inhibiting thioredoxin reductase (TrxR), the enzyme required for the regeneration of the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form. In addition, inhibition of TrxR activity by DATS was associated with oxidised dimer formation in the *T. thermophila Prx1m* protein. In contrast, terpinen-4-ol was a relatively strong promotor of oxidised dimer formation in the *T. thermophila Prx1m* protein despite being a relatively weak inhibitor of TrxR activity. We suggest that the resistance of the *Prx1m* protein to hyperoxidation could be associated with the absence of sulfiredoxin proteins in *T. thermophila*.

3.1 Introduction

T. thermophila is highly resistant to the inhibitory effects of H_2O_2 and cumene hydroperoxide (CMHP) on cell proliferation (Chapter 2). Thus, we hypothesised that it may have enzymes that are highly effective in the removal of these peroxides from the cell. In particular, we hypothesised that it may have highly effective peroxiredoxin (Prx) enzymes. Prx enzymes have been identified in species from all kingdoms of life including the parasitic protists *Plasmodium falciparum* and *Toxoplasma gondii* but they have not yet been studied in free-living protists such as *T. thermophila* (Gretes et al., 2012). Prx enzymes are thiol peroxidases that utilise a conserved active site cysteine (Cys) residue, known as the peroxidatic Cys or C_P, to catalyse the reductive decomposition of H_2O_2, and various organic hydroperoxides (e.g. CMHP), to less reactive products (Wood et al., 2003b). There is evidence that mammalian Prx enzymes can reduce more than 90% of the total cellular H_2O_2 and the mammalian mitochondrial Prx enzyme (Prx3) can reduce more than 90% of H_2O_2 originating in the mitochondria (Adimora et al., 2010; Cox et al., 2010a; Karplus, 2015). Thus, *T. thermophila* might have an effective mitochondrial Prx enzyme. Prx enzymes were first identified as antioxidant enzymes but are now thought also to be involved in H_2O_2-mediated cell signalling (Chae et al., 1994b; Kim et al., 1988; Latimer and Veal, 2016; Rhee, 2016; Rhee et al., 2005; Rhee et al., 2012). Thus, Prx proteins appear to be involved in the regulation of diverse biological processes from oxidative stress to cell signalling.

In mammals, there are six different Prx enzymes (Prx1 through 6), classified into three separate sub-families. These are the typical 2-Cys Prx sub-family (including Prx1 through 4), the atypical 2-Cys Prx sub-family (represented by Prx5) and the 1-Cys Prx sub-family (represented by Prx6) (Wood et al., 2003b). Members of the 1-Cys Prx sub-

family have only the C_P residue whereas members of the typical and atypical 2-Cys Prx sub-families also have a resolving Cys (C_R) residue. In the typical 2-Cys Prx proteins, C_P and C_R are located on separate subunits whereas in the atypical 2-Cys Prx proteins, they are located on the same subunit. The focus of this chapter is on the typical 2-Cys Prx proteins.

In the typical 2-Cys Prx proteins, the C_P residue in its reduced/thiol state (C_P-SH) reduces the peroxide substrate and is itself oxidised to a Cys sulphenic acid residue (C_P-SOH) (Wood et al., 2003b). C_P-SOH then forms a disulphide bond with C_R on a separate subunit and as a result, this form of the enzyme is detectable as a dimer utilising non-reducing polyacrylamide gel electrophoresis (NR-PAGE) (Cox et al., 2010b; Poynton and Hampton, 2014). Subsequently, the disulphide bond is disrupted and the reduced thiol form of C_P is restored by the thioredoxin (Trx)/Trx reductase (TrxR) system. The form of the enzyme with C_P in its reduced thiol state is detectable as a monomer, utilising NR-PAGE. The cycle described above occurs in the presence of low concentrations of H_2O_2 (< 20 µM in cultured human cells) but in the presence of high H_2O_2 concentrations ($\geq$ 20 µM in cultured human cells), C_P-SOH can react with a second molecule of H_2O_2 and become hyperoxidised to a Cys sulphinic acid residue (C_P-SO_2H) (Cox et al., 2009; Ikeda et al., 2011; Wood et al., 2003b). C_P-SO_2H cannot react with C_R and as a result, the hyperoxidised form of the typical 2-Cys Prx proteins loses its peroxidase activity. The peroxidase activity is only very slowly restored with the help of a family of enzymes known as the sulfiredoxins (Biteau et al., 2003; Woo et al., 2003). This unusual behaviour has been proposed to allow H_2O_2 to accumulate to act as a cell signalling molecule (Wood et al., 2003a). Thus, the typical 2-Cys Prx proteins are thought to be involved in cell signalling as well as acting as antioxidant enzymes.

Not all typical 2-Cys Prx proteins are sensitive to hyperoxidation (Baty et al., 2005; Dietz et al., 2002; König et al., 2002; Koo et al., 2002; Niimura et al., 1995; Park et al., 2000; Rabilloud et al., 2002; Ritz et al., 2001; Yang et al., 2002). Thus, these proteins have been further classified into sensitive and robust types. Members of the sensitive type lose their peroxidase activity in the presence of high concentrations of H_2O_2 whereas members of the robust type remain active. Most eukaryotic typical 2-Cys Prx proteins, including those from yeasts, plants and mammals (including humans) belong to the sensitive type whereas most prokaryotic typical 2-Cys Prx proteins, including those from bacteria (e.g. *Salmonella typhimurium*), belong to the robust type. There is evidence that once C_P-SH becomes oxidised to C_P-SOH, the typical 2-Cys Prx proteins have to undergo local unfolding to allow disulphide bond formation, otherwise C_P and C_R of the two monomers would be too far apart to interact (~14 Å separation) (Hall et al., 2009). In eukaryotes, unfolding is slower whereas in prokaryotes, unfolding is faster and thus prokaryotic typical 2-Cys Prx proteins are less susceptible for hyperoxidation (Wood et al., 2003b). The susceptibility of eukaryotic typical 2-Cys Prx proteins to hyperoxidation is thought to be associated with the presence of conserved GGLG and YF motifs that are absent from most prokaryotic typical 2-Cys Prx proteins (Wood et al., 2003a). These conserved motifs are thought to slow the transition from the fully folded to the locally unfolded state and thereby favour hyperoxidation. However, recently, Pascual et al. (2010) reported that the typical 2-Cys Prx proteins of some prokaryotes, including the cyanobacteria *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, contain the GGLG and YF motifs or motifs similar to these but yet the *Anabaena* Prx protein belongs to the sensitive type whereas the *Synechocystis* Prx protein belongs to the robust type. Additionally, *Anabaena* sp. PCC7120 has the sulfiredoxin enzyme found in most eukaryotes whereas *Synechocystis* sp. PCC6803 lacks this enzyme (Boileau et al.,

2011; Pascual et al., 2010). Thus, it is tempting to speculate that the sensitivity of the typical 2-Cys Prx proteins to hyperoxidation is associated with the presence of a sulfiredoxin enzyme in the organism rather than with the presence of the GGLG and YF motifs.

In the parasitic protists *P. falciparum* and *T. gondii*, the Prx proteins have been intensively investigated (Gretes et al., 2012; Jortzik and Becker, 2012) but this is not the case for free-living protists such as *T. thermophila*. *P. falciparum, T. gondii* and *T. thermophila* belong to the clade of the Alveolata (Dorrell et al., 2013). Within this clade, *P. falciparum* and *T. gondii* belong to the phylum Apicomplexa (apicomplexans) whereas *T. thermophila* belongs to the phylum Ciliophora (ciliates). The *T. thermophila* genome has been fully sequence and several genes encoding Prx proteins have been identified (Eisen et al., 2006). However, none of these proteins has been examined yet. Thus, the aims of the present study were to initiate the characterisation the Prx proteins in *T. thermophila* and to investigate, in particular, whether they were sensitive or robust to inactivation by H_2O_2. In *P. falciparum* and other parasitic protists, the susceptibility of Prx proteins to hyperoxidation has not been investigated. Thus, the non-parasitic ciliates, including *T. thermophila*, can be used also to understand the susceptibility of Prx proteins to hyperoxidation in parasitic protists. *T. thermophila* was chosen because it is by far the best studied of the ciliates and as a result techniques for its culture and genetic analysis are well developed (Collins, 2012; Eisen et al., 2006). The results of our investigations form the basis of future research into the roles of the Prx proteins in non-parasitic and parasitic members of the Alveolata.

3.2 Materials and methods

3.2.1 Bioinformatics

A search was done of the Ensembl Protists web site (http://protists.ensembl.org/index.html) to find all of the alveolate genomes for which sequencing and annotation had been completed. In the search results, there were six different genera from the phylum Ciliophora, eight different genera from the phylum Apicomplexa, one genus from the phylum Perkinsozoa and one genus from the phylum Chromerida. For further study, one species was chosen from each of the four ciliate genera, the six apicomplexan genera and the one perkinsozoan genus. The chosen species are listed in Table 3.1. The ciliate and apicomplexan species were chosen based on the fact that they have been extensively studied (Aeschlimann et al., 2014; Bosch et al., 2015; Collins, 2012; Gretes et al., 2012; Matthews, 2005; Verma and Singh, 2016; Zoller et al., 2012). *Perkinsus marinus*, from the phylum Perkinsozoa, was chosen because it is the closest relative to species within the phylum Dinoflagellata for which there were no genome sequence data available in the Ensembl Protists database. The phylum Dinoflagellata includes many ecologically important and well-studied species (Gómez, 2012).

Having chosen these species, searches were then done of the "Protein Database" on the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). The searches were done using the species name combined with the following enzyme names, superoxide dismutase, catalase, glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase and peroxiredoxin as well as the peroxiredoxin synonyms "thioredoxin peroxidase", "thioredoxin-dependent peroxide reductase", "thiol-specific antioxidant protein", "alkyl hydroperoxide reductase" and "peroxidoxin". The sequences obtained in this

way were aligned using Clustal X version 2.1 (http://www.clustal.org/clustal2/) (Larkin et al., 2007). Then the Neighbor-joining method (Saitou and Nei, 1987), accessed through Molecular Evolutionary Genetic Analysis (MEGA) version 6.06 (http://www.megasoftware.net/ (Tamura et al., 2013), was used to generate a phylogenetic tree. Highly similar sequences were aligned again and identity matrices were generated using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). Where sequences were ≥ 96% identical, and one sequence was longer than the other, the longer sequence was used for further analysis. All sequences were obtained either from the NCBI reference sequences or the GenBank database accessed through the "Protein Database" on the NCBI website.

3.2.2. Prediction of the subcellular locations of the typical 2-Cys Prx proteins

The subcellular locations of the typical 2-Cys Prx proteins from the four ciliate species, the six apicomplexan species and the one perkinsozoan species, were predicted using TargetP version 1.1 (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2007). As a comparison, the software was also used to predict the subcellular locations of the human typical 2-Cys Prx proteins. The software predicts first of all, whether the protein has an N-terminal pre-sequence and then whether this pre-sequence targets the protein to either the mitochondria or the secretory pathway. Proteins classified as being targeted to the secretory pathway could be located either in the endoplasmic reticulum, Golgi apparatus, plasma membrane, extracellular space, lysosomes or vacuoles (http://www.cbs.dtu.dk/services/TargetP/datasets/SP.715.rr.fasta). Proteins classified as lacking an N-terminal pre-sequence could be located either in the cytosol or the nucleus (http://www.cbs.dtu.dk/services/TargetP/datasets/datasets.php).

3.2.3 Molecular modelling of the structure of the T. thermophila Prx1m protein

Following the prediction of their subcellular locations, the alveolate Prx proteins were named according to a recently proposed new system designed to be more informative in terms of the structural and evolutionary relationships between Prx proteins (Gretes et al., 2012). Following this, it was decided to focus further study on *T. thermophila Prx1m*, a typical 2-Cys Prx protein predicted to be located in the mitochondria. A molecular model of the *T. thermophila Prx1m* protein was generated based on the crystal structure of the homo-36-dimeric human Prx3 (PDB: 5JCG) (Yewdall et al., 2016). The model was built using the SWISS-MODEL server (https://swissmodel.expasy.org/interactive) (Biasini et al., 2014). The suitable templates for the *Prx1m* protein were automatically identified based on searches of the BLAST (Altschul et al., 1997) and HHblits (Remmert et al., 2012) databases. For each identified template, the template's quality was predicted from features of the target-template alignment and the template with the highest quality was selected for model building. Various automated analyses at the SWISS-MODEL server indicated a high quality of the *T. thermophila Prx1m* model.

3.2.4 Production of anti-(T. thermophila Prx1m) polyclonal antibodies

A 14-amino acid peptide (EEYLRLVQAFQYAD) found in the predicted sequence of the *T. thermophila Prx1m* protein was chosen to immunise a rabbit (Fig. 3.4C). The peptide was selected using the Kolaskar and Tongaonkar Antigenicity method (Kolaskar and Tongaonkar, 1990), accessed through the "prediction of linear epitopes from protein sequence" of the B Cell Epitope Prediction Tools on the Immune Epitope Database and Analysis Resource (IEDB) website (http://www.iedb.org/). The peptide was analysed for charge and hydrophobicity as well as α-helix and β-sheet structures using the Peptide Analyser software (www.haubergs.com/peptide). Following this

analysis, we added a free amine group (H-) to the N-terminus of the peptide and a free acid group (-OH) to the C-terminus. We also added the cysteine residue required for conjugation to the N-terminus of the peptide. Thus, the final construct had the sequence H-CEEYLRLVQAFQYAD-OH. The final peptide sequence was synthesised and analysed for purity and molecular weight by Mimotopes Pty. Ltd. (Australia). The purity of the synthesised peptide (96%) was determined using reversed phase-high performance liquid chromatography (RP-HPLC) and the molecular weight (18,477 kDa) was determined using mass spectrometry (Appendix A.3). The synthesised peptide was then conjugated to keyhole limpet hemocyanin (KLH) as a carrier protein via a maleimidocaproyl-N-hydroxysuccinimide (MCS) linker. This was done by Mimotopes Pty. Ltd. (Australia). Polyclonal antibodies were then raised by immunising a New Zealand White rabbit with the conjugated peptide as follows. After pre-immune serum had been collected, a rabbit was immunised by subcutaneous injection of 200 µg conjugated peptide emulsified in complete Freund's adjuvant. This was repeated five times at intervals of three weeks except that incomplete Freund's adjuvant was used for the repeats. Following the third and fifth immunisations, serum from the immunised rabbit was collected for testing by bleeding the rabbit from its ear vein. Then antiserum was obtained by bleeding the rabbit via cardiac puncture under anaesthetic and the animal was immediately euthanised with 2.5 mL/kg body weight of a saturated 4 M potassium chloride solution injected intraperitoneally. Animal ethics approval was obtained from the South Australian Health and Medical Research Institute Animal Ethics Committee and all of the work met the requirements of the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition, 2013.

3.2.5 T. thermophila cell culture

T. thermophila strain CU428.2 cells were routinely maintained and cultured as described in section 2.2.2.

3.2.6 Investigating the effects of various pro-oxidant treatments on the in vivo redox/oligomerization state of the T. thermophila Prx1m protein

The effects of hydrogen peroxide (37%, v/v; Thermo Fisher Scientific Pty Ltd), cumene hydroperoxide (CMHP, 80%, v/v; Sigma-Aldrich), garlic (*Allium sativum*) oil (100%, v/v; Mystic Moments, Fordingbridge, Hampshire, United Kingdom), diallyl disulfide (DADS, 80%, v/v; Sigma-Aldrich), diallyl trisulfide (DATS, 95%, v/v; Cayman Chemical), tea tree (*Melaleuca alternifolia*) oil (100%, v/v; Integria Healthcare Australia Pty Ltd) and terpinen-4-ol (97%, v/v; VWR International Pty Ltd, Australia) on the redox/oligomerization state of the *T. thermophila Prxlm* protein were investigated. Stock solutions of the test substances, except H_2O_2 and CMHP, were prepared in dimethyl sulfoxide (DMSO). Prior to each experiment, the stock solutions, H_2O_2 and CMHP were diluted in SSP medium (Section 2.2.2) and added to the appropriate wells of a 6-well plate. This was followed by the addition of 4 x 10^6 *T. thermophila* cells per well. The cells (maintained as described in Section 2.2.2) had been pelleted by centrifugation at 2,500 *g* for 2 min and then resuspended in SSP medium and counted (Section 2.2.2) prior to being added to the wells. The total volume of liquid in each well was 4 ml and the maximum concentration of DMSO was 0.16% (v/v) which we had previously shown not to affect the proliferation of the cells (data not shown). The cells were incubated for 10 min at 35^{o}C and then harvested by centrifugation at 2,500 *g* for 2 min. Following this incubation, crude cell extracts were prepared as described below (Section 3.2.7). The experiments were repeated three times for H_2O_2, four times for CMHP, three times for garlic oil, DADS, DATS and tea

tree oil and four times for terpinen-4-ol. The results of representative experiments are shown here.

3.2.7 Preparation of cell extracts for the determination of the relative amounts of the reduced, oxidised and hyperoxidised forms of the T. thermophila Prx1m protein

The cell extracts were prepared essentially as described by Cox et al. (2010b) but with some modifications. As noted by Cox et al. (2010b), in order to prevent artefactual oxidation of the sulfhydryl (–SH) group of C_P during cell extraction, it is important to 'block' this group by using an alkylating agent such as N-ethylmaleimide (NEM). Oxidation of the –SH group of C_P in the typical 2-Cys Prx proteins occurs very rapidly even in the presence of only trace amounts of H_2O_2 (Cox et al., 2010b). Thus, the harvested cells were resuspended and incubated for 15 min in a buffer containing 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 the incubation in this buffer, 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 10 min at 4^{o}C and the supernatant, which contained the Prx proteins, was stored at -80^{o}C until it could be analysed. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific).

3.2.8 Separation of the monomer and dimer forms of the T. thermophila Prx1m protein using non-reducing polyacrylamide gel electrophoresis

The reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins were

separated essentially as described by Cox et al. (2010b). When subjected to non-reducing polyacrylamide gel electrophoresis (NR-PAGE), the reduced and hyperoxidised forms of the typical 2-Cys Prx proteins run as monomers with a molecular weight of approximately 20 kDa whereas the oxidised form runs as a dimer with a molecular weight of approximately 40 kDa. 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 (45 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), 15% (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 Doc™ EZ Imager.

3.2.9 Detection of the reduced, oxidised and hyperoxidised forms of the T. thermophila Prx1m protein using immunoblotting

The proteins 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-(*T. thermophila Prx1m*) antibodies produced as described above (see Section 3.2.3). Following the overnight incubation with the anti-(*T. thermophila Prx1m*) 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 ChemiDoc™ MP imaging system.

3.2.9 Thioredoxin reductase activity assay

Thioredoxin reductase (TrxR) is required to regenerate the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form (Rhee et al., 2012). For the TrxR assay, the *T. thermophila* cells were cultured in 500 ml Erlenmeyer flasks at

an initial density of 1 x 10^6 cells per flask in 50 ml SSP medium (Section 2.2.2) for a period of approximately 20 hours at 35^{o}C in air with shaking at 100 rpm. At the end of the incubation, the cells were pelleted by centrifugation at 2,500 *g* for 2 min and then washed twice in phosphate buffered saline (PBS, pH 7) before being disrupted in a lysis buffer containing 40 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (w/v) CHAPS and 2 mM PMSF. Insoluble material was removed by centrifugation at 16,000 *g* for 10 min at 4^{o}C and the supernatant was retained for analysis. Cell extracts prepared in this way were stored at -80^{o}C until they could be analysed for TrxR activity. TrxR activity was assayed, in the presence and absence of various concentrations of DADS, DATS or terpinen-4-ol, using a commercially available assay kit (Sigma-Aldrich®, catalogue number CS0170). The kit coupled the oxidation of NADPH to the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). The assays were run in triplicate at 25^{o}C in 96-well plates with a total assay volume of 200 µl per well and 120 µg of *T. thermophila* protein per assay. The increase in absorbance due to the production of TNB was monitored at 412 nm using a FLUOstar® plate reader (BMG Labtech Pty Ltd). TrxR enzyme activity was calculated by determining the difference in TNB production rate in the presence and absence of a specific inhibitor of TrxR activity supplied with the kit. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific). The concentration of each test substance required to inhibit TrxR activity by 50% (IC_{50}) was calculated as previously described (Berthelier and Wetzel, 2006). In brief, a linear trend line was fitted to a plot of % inhibition of the TrxR activity (relative to the control) against the concentration of the test substance and the equation of the line thus generated was used to determine the test substance concentration that gave 50% inhibition of the TrxR activity. All experiments were repeated twice, i.e., with different batches of cells.

3.2.10 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 $p < 0.05$.

3.3 Results

3.3.1 Antioxidant enzymes present in T. thermophila and other alveolates

The antioxidant enzymes identified, using bioinformatics tools, in *T. thermophila* and other alveolates (ciliates, apicomplexans and perkinsozoans) are shown in Table 3.1. Interestingly, most of the ciliate species examined had a greater diversity of antioxidant enzymes than any of the apicomplexan or perkinsozoan species. In particular, *T. thermophila* had four superoxide dismutase (SOD), one catalase, three glutathione peroxidase (GPx), nine phospholipid hydroperoxide glutathione peroxidase (PHGPx) and four Prx enzymes. *T. thermophila* contained only typical 2-Cys Prx enzymes and lacked atypical 2-Cys or 1-Cys Prx enzymes. This contrasted with the two SOD and five Prx enzymes (two typical 2-Cys and three 1-Cys) in *P. falciparum*. With a few exceptions, the apicomplexans lacked catalase, GPx and PHGPx enzymes but possessed several SOD and Prx enzymes. Similarly, *P. marinus* (a perkinsozoan) lacked catalase and PHGPx enzymes but possessed fourteen SOD, one GPx and eight Prx enzymes. Whereas the apicomplexans and perkinsozoans are exclusively parasitic, the ciliates are mostly free-living. Thus, it is tempting to speculate that the apparent reliance on Prx enzymes for peroxide detoxification is associated with the parasitic lifestyle.

3.3.2 Prediction of the subcellular locations of the typical 2-Cys Prx proteins from T. thermophila and other alveolates and a comparison with humans

The predicted subcellular locations of the typical 2-Cys Prx proteins from *T. thermophila*, other alveolates (ciliates, apicomplexans and perkinsozoans) and *H. sapiens* are shown in Table 3.2. It should be noted that the naming system recently recommended for the Prx proteins from *P. falciparum* and other parasites (Gretes et

al., 2012) has been adopted for all of the apicomplexans and extended to the non-parasitic alveolates as well. According to this system, all typical 2-Cys Prx proteins belong to the Prx 1 sub-family and different sub-family members are indicated using the letters, a, b, c etc. The exception is the mitochondrial typical 2-Cys Prx proteins which are indicated by the letter 'm'. To avoid confusion, the established nomenclature for the human Prx proteins has been retained, i.e., the human typical 2-Cys Prx sub-family members are designated Prx1, Prx2, Prx3 and Prx4.

The TargetP software predicted that the human Prx3, the *P. falciparum Prx1m* and the *T. gondii Prx1m* proteins, previously experimentally shown to be in the mitochondria, were indeed mitochondrial proteins with mitochondrial targeting peptide (mTP) scores of 0.90, 0.90 and 0.93, respectively. This compared with mTP scores of 0.73 for *T. thermophila Prx1m*, 0.50 for *T. thermophila Prx1c*, 0.90 for *S. lemnae Prx1m*, 0.6 for *I. multifiliis Prx1m*, 0.94 for *B. microti Prx1m* and 0.61 for *P. marinus Prx1m*. Consequently, the *S. lemnae Prx1m* and the *B. microti* Prx1m proteins were highly likely to be in the mitochondria whereas the likelihood was not so great for the other proteins from the other species. In the case of *T. thermophila*, *Prx1m* (mTP score = 0.73) was more likely to be in the mitochondria than *Prx1c* (mTP score = 0.50). However, *T. thermophila Prx1c* had a secretory pathway signal peptide (SP) score of 0.31 that was not so great compared with SP scores of 0.80 for human Prx 4, 0.92 for *S. lemnae Prx1a*, 1.0 for *I. multifiliis Prx1c*, 0.93 for *I. multifiliis Prx1e* and 0.91 for *O. trifallax Prx1a*. This suggested that *T. thermophila Prx1c* was not a secreted protein and perhaps it was located in the mitochondria. In contrast to this, the *T. thermophila Prx1a* and *Prx1b* proteins with mTP scores of 0.10 and 0.42, respectively, were both highly likely to be located in the cytosol or the nucleus.

The putative mTP lengths for the mitochondrial typical 2-Cys Prx proteins from *T. thermophila*, other alveolates (ciliates, apicomplexans and perkinsozoans) and *H. sapiens* are also shown in Table 3.2. They were 61 amino acids for the human Prx3, 19 amino acids for the *P. falciparum Prx1m*, 19 amino acids for the *T. thermophila Prx1m* and 64 amino acids for the *T. thermophila Prx1c* protein. Interestingly, the mTP length for the *T. thermophila Prx1m* protein was quite similar to that for the *P. falciparum Prx1m* protein but their mTP cleavage site residues, isoleucine (I) for the *T. thermophila Prx1m* protein and phenylalanine (F) for the *P. falciparum Prx1m* protein, were quite different (Fig. 3.1). Similarly, the mTP length for the *T. thermophila Prx1c* protein was similar to that for the human Prx3 protein but their mTP cleavage site residues, phenylalanine (F) for the *T. thermophila Prx1c* protein and histidine (H) for the human Prx3 protein, were quite different (Fig. 3.1). The *T. thermophila Prx1m* and Prx1c proteins were different in their mTP lengths and their cleavage sites. In particular, the *T. thermophila Prx1m* protein, differed from the other mitochondrial typical 2-Cys Prx proteins (different species) that had greater mTP scores. In addition to this, a mTP amino acid sequence comparison of the mitochondrial typical 2-Cys Prx proteins from *T. thermophila*, other alveolates and *H. sapiens* revealed little to no conservation (Fig. 3.1). Thus, the mTP sequences of the mitochondrial typical 2-Cys Prx proteins were unique to each species.

The predicted molecular weights of the typical 2-Cys Prx proteins from *T. thermophila*, other alveolates and *H. sapiens* are also shown in Table 3.2. The molecular weights of both putative mitochondrial and secreted proteins were calculated without including their targeting peptides (mTP or SP). The *T. thermophila* Prx enzymes with molecular weights of 21.4 to 28 kDa were similar to the Prx enzymes in the other organisms, including humans (21.5 - 26.6 kDa) (Table 3.2). Interestingly, unlike *O. trifallax Prx1a, Prx1b* and *Prx1e* and even other alveolate and human Prx

proteins, the molecular weights for *O. trifallax Prx1c* and *Prx1d* were 50.5 and 45.9 kDa, respectively. This is twice the commonly reported molecular weight for typical 2-Cys Prx proteins and it suggests that these proteins might be obligate dimers.

3.3.3 Phylogenetic analysis

Fig. 3.2 shows a phylogenetic analysis of the relationships between the typical 2-Cys Prx proteins from selected species from the clade Alveolata in comparison with the typical 2-Cys Prx proteins from humans. An *E. coli* typical 2-Cys Prx protein was used as the out-group to root the tree. The phylogenetic analysis produced five clearly separated clusters, one each for the typical 2-Cys Prx proteins from humans, apicomplexans and perkinsozoans, but two for the corresponding proteins from the ciliates. Both ciliate clusters contained the same organisms but different isoforms of the typical 2-Cys Prx proteins. Within one ciliate cluster, the *T. thermophila Prx1m* protein was grouped in a single clade with the *I. multifiliis Prx1m* protein whereas the *T. thermophila Prx1a* and *Prx1b* proteins were grouped together in a separate clade with the *I. multifiliis Prx1b* and *Prx1d* proteins. In the other ciliate cluster, the *T. thermophila Prx1c* protein was grouped in a clade with the *I. multifiliis Prx1c* and *Prx1e* proteins. The phylogenetic analysis also showed that the *T. thermophila Prx1m* protein was more likely to be a mitochondrial protein than the *T. thermophila Prx1c* protein as it was clustered with the other mitochondrial proteins whereas the *T. thermophila Prx1c* protein was clustered with the proteins predicted to have secretory pathways. This is further evidence that the *T. thermophila Prx1c* was not a mitochondrial protein. In addition, the *T. thermophila* Prx proteins were more similar to the *I. multifiliis* Prx proteins than to the *S. lemnae* Prx proteins or the *O. trifallax* Prx proteins. The phylogenetic analysis also revealed that the ciliate typical 2-Cys Prx

proteins were more closely related to the human typical 2-Cys Prx proteins than either the apicomplexan or perkinsozoan typical 2-Cys Prx proteins.

3.3.4 Amino acid sequence comparison of the typical 2-Cys Prx proteins from T. thermophila, P. falciparum and humans

An amino acid sequence comparison of the typical 2-Cys Prx proteins from *T. thermophila, P. falciparum* and humans revealed that the *T. thermophila* typical 2-Cys Prx proteins shared several highly conserved regions with the typical 2-Cys Prx proteins from humans and *P. falciparum* (Fig. 3.3). These included the F-motif (FTFVCPTEI) which contains the peroxidatic Cys (C_P) at residue 48 for *T. thermophila Prx1a*, at residue 71 for *T. thermophila Prx1b*, at residue 111 for *T. thermophila Prx1m*, and at residue 102 for *T. thermophila Prx1c* and the hydrophobic region (VCPXXW) which contains the resolving Cys (C_R) at residue 172 for *T. thermophila Prx1a*, at residue 195 for *T. thermophila Prx1b*, at residue 232 for *T. thermophila Prx1m* and at residue 228 for *T. thermophila Prx1c* (boxed in Fig. 3.3). Most of the *T. thermophila* Prx sequences also contained the highly conserved GG*X*G and YF motifs at residues 91-94 and 195-196 for *T. thermophila Prx1a*, at residues 114-117 and 218-219 for *T. thermophila Prx1b*, at residues 154-157 and 255-256 for *T. thermophila Prx1m* and at residues 145-148 and 249-250 for *T. thermophila Prx1c* (starred underlining and dotted underlining, respectively). Residue *X* can be either leucine (L), valine (V) or isoleucine (I). In human Prx 1, 2, 3 and 4 and in *T. thermophila Prx1a*, *Prx1b* and *Prx1m*, *X* is a leucine (L) residue whereas in *T. thermophila Prx1c*, *X* is a valine (V) residue and in *P. falciparum Prx1a* and *Prx1m*, it is an isoleucine (I) residue. In *T. thermophila*, the YF motif, which is present in all of the human typical 2-Cys Prx proteins, is replaced by a YW motif in *Prx1a* and *Prx1m* and a FW motif in Prx1b. Only *T. thermophila Prx1c* retained the YF motif

found in humans. Similarly, in *P. falciparum*, the YF motif was also replaced by either a YL motif (Prx1a) or a YM (Prx1m) motif. Thus, in the *T. thermophila Prx1a* and *Prx1m* proteins, a phenylalanine (F) residue was replaced by a tryptophan (W) residue whereas in the *T. thermophila Prx1b* protein, the tyrosine (Y) and phenylalanine (F) residues were replaced by phenylalanine (F) and tryptophan (W) residues, respectively. In contrast to this, in the *P. falciparum Prx1a* and *Prx1m* proteins, phenylalanine (F) was replaced with leucine (L) and methionine (M), respectively.

3.3.5 Is the T. thermophila Prx1m protein sensitive or robust?

Typical 2-Cys Prx proteins have been classified into sensitive and robust types based on their sensitivity to hyperoxidation (Wood et al., 2003a). Most eukaryotic typical 2-Cys Prx proteins belong to the sensitive type whereas most prokaryotic typical 2-Cys belong to the robust type. In yeasts and humans, the sensitive Prx proteins contain the GGLG and YF motifs whereas in bacteria, the robust Prx proteins lack these conserved motifs (Table 3.3). It has been hypothesised that these motifs are associated with sensitivity to hyperoxidation (Wood et al., 2003a). In *T. thermophila* and the other alveolates in this study, the Prx proteins had motifs similar to the GGLG and YF motifs which are a characteristic of the sensitive type of typical 2-Cys Prx protein (Table 3.3). Based on the presence of these sequence motifs, the alveolate proteins might be considered to be from the sensitive type but it is unknown whether indeed these proteins are from the sensitive or the robust type. In some organisms, including yeasts, humans and *Anabaena* sp. (NP_488681) (a cyanobacterium), the C_P in the hyperoxidised state can be restored to the reduced state by sulfiredoxin enzymes and as a result, the hyperoxidised/inactivated typical 2-Cys Prx enzymes can be reactivated. Thus, it would be expected that sulfiredoxin enzymes would be found only in organisms that have sensitive Prx proteins (Gretes and Karplus, 2013; Gretes et al.,

2012). Surprisingly, like most prokaryotes, including *Synechocystis* sp. PCC6803 (a cyanobacterium), *T. thermophila* and all other alveolates (ciliates, apicomplexans and perkinsozoans) in this study lacked sulfiredoxin enzymes (Table 3.3) and therefore their Prx proteins might not be considered to be from the sensitive type. These predictions will be tested experimentally in Section 3.3.8 (see below).

3.3.6 Prediction of the structure of the T. thermophila Prx1m protein

The predicted structural model of the *T. thermophila Prx1m* protein generated from the human Prx3 protein (Protein Data Bank accession number, PDB: 5JCG) is shown in Fig. 3.4. The monomer form of the *T. thermophila Prx1m* protein was predicted to have seven-β sheets and seven α-helices (Fig. 3.4A). It contained the two catalytic Cys residues, the C_P and the C_R. C_P was located in the α2 helix in the active site near the N-terminus of the protein whereas C_R was located in the coil near the C-terminus. The GGLG and YW motifs were located in the coil near the α4 helix and in the α7 helix near the C-terminus, respectively. The predicted structure also showed that the oligomeric state of the *T. thermophila Prx1m* protein was similar to that of the human Prx3 protein, forming a dodecamer (six homodimers) (Fig. 3.4B). The position of the C_P in one subunit was near the position of the C_R in a separate subunit (Fig. 3.4C). This is consistent with what is known about the catalytic mechanism of the typical 2-Cys Prx enzymes, i.e., during the catalytic cycle, C_P becomes oxidised to C_P-SOH which then forms an intermolecular disulphide bond with C_R from a separate subunit. The homology model of the *T. thermophila Prx1m* protein also revealed that a dodecamer of the *Prx1m* protein might assemble as a stack of three dodecameric rings with a high molecular weight (Fig. 3.4D).

3.3.7 Evaluation of the anti-(T. thermophila Prx1m) antibodies

Anti-(*T. thermophila Prx1m*) polyclonal antibodies were raised in a rabbit to recognise a peptide antigen chosen from the *T. thermophila Prx1m* protein sequence. The peptide was shown to be displayed on the surface of the predicted *T. thermophila Prx1m* dodecamer indicating that there would be a high chance of antibodies raised against this peptide cross-reacting with the *T. thermophila Prx1m* protein (Fig. 3.4C). Fig. 3.5 shows the evaluation of the anti-(*T. thermophila Prx1m*) polyclonal antibodies using immunoblotting analysis. Immunoblotting analysis showed that pre-immune serum did not detect any specific proteins to any significant extent in the extracts of the *T. thermophila* cells (Fig. 3.5C). This suggested that there was limited cross-reactivity between the pre-immune serum and any *T. thermophila* proteins. The serum collected following the fifth immunisation detected a protein with a molecular weight 2 - 3 kDa greater than 25 kDa which was similar to the predicted molecular weight (28 kDa) of the *T. thermophila Prx1m* monomer (Fig. 3.5D). This indicated that immunisation of the rabbit with the peptide antigen chosen from the *Prx1m* protein had triggered an immune response and consequent production of antibodies.

3.3.8 The effects of various pro-oxidants on the in vivo redox/oligomerization state of the T. thermophila Prx1m protein

T. thermophila cells were treated with various concentrations of H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol and the effects on the total soluble proteins extracted from the cells (Fig. 3.6) and on the *in vivo* redox/oligomerization state of the *Prx1m* protein (Fig. 3.7) were determined. Treatment of the cells with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol, regardless of the concentration, had no effect on the total soluble proteins extracted from the cells (Figs. 3.6A, C-G). However, at the highest concentrations tested (3 and 6 mM), treatment

with CMHP caused apparent protein denaturation. This was evidenced by the loss of most of the smaller molecular weight proteins (less than approximately 20 kDa) from the gels stained for total protein when the cells had been treated with 6 mM CMHP (Fig. 3.6B).

Fig. 3.7 shows the effects of the various pro-oxidant treatments on the redox/oligomerization state of the Prx1m protein. The protein was detected using the anti-(*T. thermophila Prx1m*) polyclonal antibodies. Immunoblotting analysis showed that the antibodies cross-reacted strongly with proteins that were 28 kDa and 56 kDa in the extracts of the *T. thermophila* cells. The cells had been treated with NEM to alkylate the sulfhydryl group of C_P-SH in the reduced state. Thus, the protein forms observed were the reduced monomer form containing C_P-SH (28 kDa) and the oxidised dimer form (56 kDa) in which C_P-SOH has formed a disulphide bond with C_R. In all cases, the control cells contained mostly only the reduced monomer form of the *Prx1m* protein. However, when the cells were treated with increasing concentrations of H_2O_2, there was increasing conversion of the reduced monomer form of the *Prx1m* protein to the oxidised dimer form (Fig. 3.7A). Like H_2O_2, except for the highest concentration, when the cells were treated with increasing concentrations of CMHP, there was increasing conversion of the reduced monomer form of the typical 2-Cys *Prx1m* protein to the oxidised dimer form (Fig.3.7B). At the highest CMHP concentration, the oxidised dimer form of the *Prx1m* protein disappeared. The loss of the oxidised dimer form in the cells treated with the highest CMHP concentration (6 mM) was probably due to the general loss of smaller molecular weight proteins that occurred in the presence of this high concentration (Fig. 3.6B). As stated above, we assume that this was due to protein denaturation at the high CMHP concentration. As was observed with

the H_2O_2 treatment, when the cells were treated with increasing concentrations of garlic oil, DATS, tea tree oil or terpinen-4-ol, there was increasing conversion of the reduced monomer form of the *Prx1m* protein to the oxidised dimer form (Figs. 3.7C, E-G). Garlic oil was more effective than tea tree oil in promoting oxidised dimer formation and DATS was more effective than terpinen-4-ol. These results suggested that the greater the number of sulphur atoms in the allyl sulphides, the greater their effectiveness in promoting the oxidation of C_P in the *Prx1m* protein. The blots (H_2O_2, CMHP and terpinen-ol) in Fig. 3.7 were also analysed with the specific anti-hyperoxidised Prx antibody but there was no cross reactivity seen on these blots (data not shown). In contrast to the above, DADS had no discernible effect on the redox/oligomerization state of the *Prxlm* protein (Fig. 3.7D). Interestingly however, with increasing concentrations of garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol, there was an apparent increase in the total amount of the *Prx1m* protein (both the reduced monomer form and the oxidised dimer form) (Figs. 3.7C-G). This suggested increasing synthesis of the *Prx1m* protein in response to the oxidative stress treatments. Terpinen-4-ol was considerably more effective than either of the oils or the allyl sulphides in this respect. These results suggest that there might be an increase *Prx1m* gene expression in response to garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol treatments.

3.3.9 The effects of selected pro-oxidants on TrxR enzyme activity

The regeneration of the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form requires TrxR activity. Thus, the differing effects of the various oils/compounds on the redox/oligomerization state of the *Prx1m* protein could be due to differing effects on TrxR activity. Greater inhibition of TrxR activity should lead to greater oxidation of the *Prx1m* protein and vice versa. Thus, we tested the

effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in crude extracts of the *T. thermophila* cells to determine whether there was a correlation between inhibition of TrxR activity and promotion of the oxidation of the *Prx1m* protein (Fig. 3.8). The most effective inhibitor of TrxR activity was DATS (IC_{50} = 0.2 mM) followed by DADS (IC_{50} = 0.67 mM) and then terpinen-4-ol (IC_{50} = 4.6 mM). This would suggest that DATS should be more effective than DADS in promoting oxidation of the *Prx1m* protein and indeed it was. On the other hand, it would suggest that terpinen-4-ol should be less effective at promoting oxidation of the *Prx1m* protein than either DADS or DATS. However, terpinen-4-ol was considerably less effective at promoting oxidation of the *Prx1m* protein than DATS but it was considerably more effective in this way than DADS. Thus, it appears that inhibition of TrxR activity could explain the differing effects of DATS and terpinen-4-ol but not DADS on the redox/oligomerization state of the *Prx1m* protein.

3.4 Discussion

It is known from genome sequencing projects, that ciliate species have a plethora of genes encoding typical 2-Cys Prx proteins but none of these proteins has been investigated in terms of their structure or function. Here we use *T. thermophila* as a model ciliate to begin such an investigation. We identified that *T. thermophila* has four typical 2-Cys Prx isozymes but it lacks either atypical 2-Cys Prx or 1-Cys Prx isozymes. The four *T. thermophila* typical 2-Cys Prx proteins were predicted to be found in different subcellular locations. *Prx1a* and *Prx1b* were predicted to be found in the cytosol or nucleus whereas *Prx1m* and *Prx1c* were predicted to be found in the mitochondria. *Prx1m* was more likely to be in the mitochondria than *Prx1c* because its predicted mTP score was higher than for *Prx1c*. In addition to this, a phylogenetic analysis revealed that *Prx1m* clustered together with a mitochondrial typical 2-Cys Prx protein from *I. multifiliis* whereas *Prx1c* clustered together with other proteins from *I. multifiliis* predicted to have secretory pathways. Thus, it is likely that the *T. thermophila Prx1m* protein is a mitochondrial protein whereas the *T. thermophila Prx1c* protein could be a secreted protein. The phylogenetic analysis also showed that the Prx proteins from *T. thermophila* and other ciliates were more closely related to the human Prx proteins than either the apicomplexan or perkinsozoan Prx proteins.

An amino acid sequence comparison of the *T. thermophila* Prx proteins with the Prx proteins from humans and *P. falciparum* confirmed that the *T. thermophila* proteins were isoforms in the typical 2-Cys Prx sub-family as they had the peroxidatic Cys residue within the highly conserved F-motif (FTFVCPTEI) and the resolving Cys residue within the highly conserved hydrophobic region (VCPAXW). These features are characteristic of typical 2-Cys Prx proteins from yeasts, plants, mammals, fish, parasitic protozoans (e.g. *Plasmodium* spp.) and bacteria (Dong et al., 2007; Gretes et

al., 2012; Hakimi et al., 2012; König et al., 2003; Sutton et al., 2010; Wood et al., 2003a). In addition to this, the *T. thermophila* Prx sequences also contained the highly conserved GG*X*G motif where *X* can be a leucine (L) or valine (V) residue. This motif is a common feature of most eukaryotic typical 2-Cys Prx proteins but it is lacking from most prokaryotic typical 2-Cys Prx proteins (Wood et al., 2003a). The *T. thermophila Prx1a*, *Prx1b* and *Prx1m* proteins retained the GGLG motif found in the human Prx proteins whereas the *T. thermophila Prx1c* had the GGLG motif replaced with a GGVG motif. The GGLG and GGVG motifs would be expected to participate in hydrophobic interactions as the amino acids leucine (L) and valine (V) are non-polar and have aliphatic R groups. In most eukaryotic typical 2-Cys Prx proteins, the GGLG motif is associated with a highly conserved YF motif (Wood et al., 2003a). Interestingly, in *T. thermophila,* the YF motif was only retained in *Prx1c* whereas it was replaced by a YW motif in *Prx1a* and *Prx1m* and a FW motif in *Prx1b*. However, YF, YW and WF should all participate in hydrophobic interactions because the amino acids tyrosine (Y), phenylalanine (F) and tryptophan (W) are non-polar and have aromatic R groups. From these observations, it can be predicted that the *T. thermophila* typical 2-Cys Prx proteins should belong to the sensitive type like other eukaryotic typical 2-Cys Prx proteins. Thus, we investigated whether or not this was the case.

Here we have investigated the effects of H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the *in viv*o redox oligomerization state of the *Prx1m* protein in *T. thermophila* cells. H_2O_2, tea tree oil and terpinen-4-ol promoted hyperoxidised monomer formation in the typical 2-Cys Prx proteins in Jurkat T-lymphocytes (see Sections 4.3.1 and 4.3.2). Our findings showed that none of these tested substances promoted hyperoxidised monomer formation in the *Prx1m* protein. We predicted that the *T. thermophila Prx1m* protein belongs to the sensitive type but instead we found that H_2O_2 and CMHP promoted only oxidised dimer formation of

the *Prx1m* protein in the *T. thermophila* cells and not hyperoxidised monomer formation. Thus, the *Prx1m* protein appears to belong to the robust type. Like H_2O_2 and CMHP, garlic oil, DATS, tea tree oil and terpinen-4-ol promoted oxidised dimer formation in the *Prx1m* protein in *T. thermophila* cells. These results suggest that the *Prx1m* protein is not susceptible to hyperoxidation and therefore belongs to the robust type. It has been hypothesised that the susceptibility of the Prx proteins to hyperoxidation is attributable to the presence of the GGLG and YF motifs in the protein sequence (Wood et al., 2003a). Although the *T. thermophila Prx1m* contained the GGLG and YF (or similar) motifs, typically found in the sensitive type Prx proteins, it was insensitive to hyperoxidation. Thus, it belongs to the robust type. This suggests that it is not universally true that the presence of the GGLG and YF motifs is always associated with sensitivity to hyperoxidation.

Based on the absence of sulfiredoxin enzymes from protozoan parasites that include the apicomplexans (e.g. *P. falciparum* and *Toxoplasma gondii*), the kinetoplastids (e.g. *Leishmania* ssp. and *Trypanosoma* ssp.), the metamonads (e.g. *Giardia lamblia* and *Trichomonas vaginalis*) and the amoebozoans (e.g. *Entamoeba histolytica*), it has been proposed that their typical 2-Cys Prx proteins might be resistant to hyperoxidation but this had not been investigated until the present study (Gretes and Karplus, 2013; Gretes et al., 2012). In mammals and yeast, sulfiredoxin enzymes catalyse the regeneration of C_P-SH in the reduced monomer form of the typical 2-Cys Prx proteins from C_P-SO_2H in the hyperoxidised monomer form (Biteau et al., 2003; Woo et al., 2003). Recently, it was observed in the cyanobacterium *Synechocystis* sp. PCC6803 which lacks any sulfiredoxin enzymes that its typical 2-Cys Prx protein was resistant to hyperoxidation despite containing the GGLG (or similar) and YF motifs found in the sensitive type Prx proteins (Pascual et al., 2010). In contrast, in the cyanobacterium *Anabaena* sp. PCC7120 which possesses a sulfiredoxin enzyme, its

typical 2-Cys Prx protein was sensitive to hyperoxidation as well as containing the GGLG (or similar) and YF motifs (Pascual et al., 2010). In the present study, we found that *T. thermophila* lacked any sulfiredoxin enzymes and its *Prxlm* protein was resistant to hyperoxidation despite containing the GGLG and YF (or similar) motifs found in most sensitive type Prx proteins. Hence, our results are similar to those previously obtained for the cyanobacterium *Synechocystis* sp. PCC6803. Thus, we conclude that the susceptibility of the typical 2-Cys Prx proteins to hyperoxidation might not always be associated with the presence of the GGLG and YF motifs but instead could be associated with the presence of sulfiredoxin enzymes. This might extend to the typical 2-Cys Prx proteins of other eukaryotes lacking sulfiredoxin enzymes, including *P. falciparum*.

Phytochemicals, such as the allyl sulfides found in garlic oil and the terpenoids found in tea tree oil, are known to have both pro- and anti-oxidant effects but it is unknown whether their mechanisms of action involve the Prx proteins (Kuttan et al., 2011; Munday, 2012; Wu et al., 2005). Here we have shown that garlic oil, DATS (but not DADS), tea tree oil and terpinen-4-ol can promote oxidation of the *Prx1m* protein in *T. thermophila* cells. Garlic oil was more effective than tea tree oil and DATS was more effective than terpinen-4-ol in this respect. In contrast, DADS had no effect on the oxidation of the *Prx1m* protein. This suggested that the number of sulphur atoms in the molecule is important. It is known that both DADS and DATS can promote redox cycling and that DATS is more effective than DADS in this respect (Munday, 2012). This redox cycling occurs because di-sulphides can be readily reduced to thiols in a thiol-disulphide exchange reaction with glutathione (GSH) and the resulting thiols can then undergo metal-catalysed oxidation by molecular oxygen to reform the original di-sulphides and perpetuate the series of reactions. In the process, reactive oxygen species (ROS), including H_2O_2, are produced. DATS is more active than

DADS in promoting redox cycling and the effects of both DADS and DATS in this respect can be diminished by the addition of catalase but not superoxide dismutase (Munday et al., 2003). Thus, both DADS and DATS can stimulate H_2O_2 production but DATS is more effective than DADS. This is consistent with our observation that DATS was more effective than DADS in promoting oxidation/dimerization of the *Prx1m* protein and it supports the conclusion that the effects of DATS are due to its ability to promote the production of H_2O_2 which reacts readily with C_P-SH in the Prx1m protein. Interestingly, DATS seemed to be more effective than H_2O_2 in promoting oxidation/dimerization of the *Prx1m* protein. Thus, the effects of DATS on this protein may not only be attributable to the production of H_2O_2. Additionally or alternatively, in theory, inhibition of TrxR activity should lead to greater oxidation/dimerization of the Prx1m protein. We tested this hypothesis by examining the effects of DADS, DATS and terpinen-4-ol on TrxR activity in crude extracts of *T. thermophila* cells. We found that DATS was a more effective inhibitor of TrxR activity compared with either DADS or terpinen-4-ol. Thus, the greater effectiveness of DATS in inhibiting TrxR activity was linked to its greater effectiveness in promoting oxidation of the *Prx1m* protein. It was known that DADS and DATS inhibit TrxR activity but it was not known how these phytochemicals affect the redox/oligomerization state of the typical 2-Cys Prx proteins (Hu et al., 2007). DADS was more effective than terpinen-4-ol in inhibiting TrxR activity but it did not seem to promote oxidation of the *Prx1m* protein. In contrast, terpinen-4-ol was a relatively strong promotor of oxidised dimer formation in the *T. thermophila Prx1m* protein despite being a relatively weak inhibitor of TrxR activity. Thus, this leads to the conclusion that DATS and terpinen-4-ol exert their effects on the typical 2-Cys *Prx1m* protein by inhibiting TrxR activity in *T. thermophila* cells. However, inhibition of TrxR may not be the only explanation for the differing effects of the different

phytochemicals on the redox/oligomerization state of the *Prx1m* protein in *T. thermophila*.

Interestingly, at higher concentrations, garlic oil and its most abundant constituents DADS and DATS, as well as tea tree oil and its most abundant constituent terpenin-4-ol appeared to increase the total amount of the *Prx1m* protein in *T. thermophila* cells. Terpinen-4-ol was more effective than its parent oil and even garlic oil, DADS or DATS in promoting this effect. It is possible that DADS, DATS and terpinen-4-ol increase the expression of the *Prx1m* gene in *T. thermophila* cells and as a result increase the total amount of the *Prx1m* protein. However, this needs to be investigated.

In conclusion, for decades, studies have investigated oxidative stress in various species of prokaryotes and eukaryotes and the role of antioxidant proteins in protecting these species against oxidative stress but none of these studies has included the *T. thermophila* Prx enzymes. Here we found that *T. thermophila* has four different typical 2-Cys Prx proteins but lacks any atypical 2-Cys Prx proteins or 1-Cys Prx proteins. *Prx1a* and *Prx1b* from *T. thermophila* are cytosolic or nuclear proteins whereas *Prx1m* is a mitochondrial protein. *Prx1c* is likely to be a secreted protein. The four *T. thermophila* typical 2-Cys Prx enzymes are closely related to the human typical 2-Cys Prx enzymes. Human Prx proteins are sensitive to hyperoxidation induced by H_2O_2 (Cox et al., 2009). However, we found that the *T. thermophila Prx1m* protein seemed to be insensitive to hyperoxidation. H_2O_2, CMHP, garlic oil, DATS (but not DADS), tea tree oil and terpinen-4-ol promoted oxidised dimer formation in the *T. thermophila Prx1m* protein but not hyperoxidised monomer formation whereas DADS did not seem to promote oxidation of the *Prx1m* protein. The greater effectiveness of DATS compared with DADS is probably attributable to its greater number of sulphur atoms and consequent greater capacity to produce H_2O_2. Additionally, DATS was a stronger

inhibitor of TrxR activity than DADS. Terpinen-4-ol might exert its effect on the *Prx1m* protein by inhibiting TrxR activity. At higher concentrations, these phytochemicals increased the total amount of the *Prx1m* protein. It is possible that *Prx1m* gene expression increased in response to these phytochemicals. This requires further investigation.

Table 3.1. Antioxidant enzymes found in alveolate organisms.

Alveolates	Habitat	SOD	Catalase	GPx	PHGPx	Peroxiredoxin		
						Typical 2-Cys	Atypical 2-Cys	1-Cys
Ciliates								
Tetrahymena thermophila	Free-living	4	1	3	9	4	0	0
Stylonychia lemnae	Free-living	7	4	11	0	3	1	0
Ichthyophthirius multifiliis	Free-living/ ecto-parasite	6	1	0	0	6	0	1
Oxytricha trifallax	Free-living	7	4	9	0	5	1	0
Apicomplexans								
Plasmodium falciparum	Parasite	2	0	0	0	2	0	3
Toxoplasma gondii	Parasite	3	1	0	0	2	1	1
Cryptosporidium parvum	Parasite	1	0	1	0	1	0	0
Eimeria tenella	Parasite	3	2	1	0	1	0	1
Theileria annulata	Parasite	2	0	0	0	2	1	0
Babesia microti	Parasite	2	0	0	0	2	0	0
Perkinsozoans								
Perkinsus marinus	Parasite	14	0	1	0	4	3	1

SOD, superoxide dismutase; GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase.

The accession numbers for the above proteins are: XP_001007667.2 (*T. thermophila_SOD1*); XP_001010506.1 (*T. thermophila_SOD2*); XP_001032187.1 (*T. thermophila_SOD3*); XP_001033543.1 (*T. thermophila_SOD4*); EAS06345 (*T. thermophila*_catalase); EWS73854.1 (*T. thermophila_GPx1*); EAR97894.2 (*T. thermophila_GPx2*); EAR82654.2 (*T. thermophila_GPx3*); XP_001022734.1 (*T. thermophila_PHGPx1*); EAR83553.3 (*T. thermophila_PHGPx2*); XP_001020136.1 (*T. thermophila_PHGPx3*); EAR83551.1 (*T. thermophila_PHGPx4*); EAR83552.1 (*T. thermophila_PHGPx5*); EAR83550.1 (*T. thermophila_PHGPx6*); XP_001014606.1 (*T. thermophila_PHGPx7*); XP_001014604.1 (*T. thermophila_PHGPx8*); EAR90809.2 (*T. thermophila_PHGPx9*); XP_001029987.1 (*T. thermophila_Prx1a*); XP_001031522.1 (*T. thermophila_Prx1b*); EAR92904.2 (*T. thermophila_Prx1m*); EAR84222.2 (*T. thermophila_Prx1c*); CDW86167.1 (*S. lemnae_SOD1*); CDW72913.1 (*S. lemnae_SOD2*); CDW80608.1 (*S. lemnae_SOD3*); CDW86249.1 (*S. lemnae_SOD4*); CDW84501.1 (*S. lemnae_SOD5*); CDW89968.1 (*S. lemnae_SOD6*); CDW78842.1 (*S. lemnae_SOD2*); CDW87159.1 (*S. lemnae*_catalase 1); CDW91299.1 (*S. lemnae*_catalase 2); CDW81170.1 (*S. lemnae*_catalase 3); CDW81232.1 (*S. lemnae*_catalase 4); CDW74426.1 (*S. lemnae_GPx1*); CDW77892.1 (*S. lemnae_GPx2*); CDW82235.1 (*S. lemnae_GPx3*); CDW83089.1 (*S. lemnae_GPx4*); CDW85782.1 (*S. lemnae_GPx5*); CDW86682.1 (*S. lemnae_GPx6*); CDW86833.1 (*S. lemnae_GPx7*); CDW89565.1 (*S. lemnae_GPx8*); CDW77314.1 (*S. lemnae_GPx9*); CDW89686.1 (*S. lemnae_GPx10*); CDW90044.1 (*S. lemnae_GPx11*); CDW79681.1 (*S. lemnae_Prx1a*); CDW72826.1 (*S. lemnae_Prx1b*); CDW79455.1 (*S. lemnae_Prx1m*); CDW72299.1 (*S. lemnae_Prx5*); XP_004034715.1 (*I. multifiliis_SOD1*); XP_004035843.1 (*I. multifiliis_SOD2*); XP_004037495.1 (*I. multifiliis_SOD3*); XP_004037445.1 (*I. multifiliis_SOD4*); XP_004036753.1 (*I. multifiliis_SOD5*); ABO69706.1 (*I. multifiliis_SOD6*); XP_004032246.1 (*I. multifiliis*_catalase); EGR27018.1 (*I. multifiliis*_Prx1a); EGR27550.1 (*I. multifiliis*_Prx1b); EGR30706.1 (*I. multifiliis*_Prx1c); EGR31994.1 (*I. multifiliis_Prx1d*); EGR29838.1 (*I. multifiliis_Prx1e*); EGR29584.1 (*I. multifiliis_Prx1m*); XP_004031009.1 (*I. multifiliis_Prx5*); EJY66799.1 (*O. trifallax_SOD1*); EJY64509.1 (*O. trifallax_SOD2*); EJY87105.1 (*O. trifallax_SOD3*); EJY85955.1 (*O. trifallax_SOD4*); EJY82908.1 (*O. trifallax_SOD5*); EJY81400.1 (*O. trifallax_SOD6*); EJY70280.1 (*O. trifallax_SOD7*); EJY86237.1 (*O. trifallax*_catalase 1); EJY81553.1 (*O. trifallax*_catalase 2); EJY78794.1 (*O. trifallax*_catalase 3); EJY69095.1 (*O. trifallax*_catalase 4); EJY87712.1 (*O. trifallax_GPx1*); EJY84266.1 (*O. trifallax_GPx2*); EJY83199.1 (*O. trifallax_GPx3*); EJY78445.1 (*O. trifallax_GPx4*); EJY77455.1 (*O. trifallax_GPx5*); EJY70022.1 (*O. trifallax_GPx6*); EJY67590.1 (*O. trifallax_GPx7*); EJY65021.1 (*O. trifallax_GPx8*); EJY65173.1 (*O. trifallax_GPx9*); EJY85734.1 (*O. trifallax_Prx1a*); EJY73729.1 (*O. trifallax_Prx1b*); EJY85449.1 (*O. trifallax_Prx1c*); EJY82209.1 (*O. trifallax_Prx1d*); EJY81461.1 (*O. trifallax_Prx1e*); EJY72164.1 (*O. trifallax_Prx5*); CAD51224.1 (*P. falciparum_SOD1*); CAG25047.2 (*P. falciparum_SOD2*); XP_001348542.1 (*P. falciparum_Prx1a*); XP_001350554.1 (*P. falciparum_Prx1m*); XP_002808799.1 (*P. falciparum_Prx5*); XP_001349492.1 (*P. falciparum_Prx6*); XP_001347552.1 (*P. falciparum_PrxQ*); EPR58843.1 (*T. gondii_SOD1*); EPR58845.1 (*T. gondii_SOD2*); EPR58829.1 (*T. gondii_SOD3*); EPR64252.1 (*T. gondii*_catalase); EPR63775.1 (*T. gondii_Prx1a*); EPR64157.1 (*T. gondii_Prx1m*); EPR59464.1 (*T. gondii_Prx5*); EPR60799.1 (*T. gondii_Prx6*); XP_001388283.1 (*C. parvum_SOD*); XP_626631.1 (*C. parvum_GPx*); ACV31867.1 (*C. parvum_Prx1a*); XP_013234977.1 (*E. tenella_SOD1*); XP_013232304.1 (*E. tenella_SOD2*); XP_013232031.1 (*E. tenella_SOD3*); XP_013234501.1 (*E. tenella*_catalase 1); XP_013231803.1 (*E. tenella*_catalase 2); XP_013231991.1 (*E. tenella_GPx*); XP_013230312.1 (*E. tenella_Prx1a*); XP_013233379.1 (*E. tenella_Prx6*); XP_952718.1 (*T. annulata_SOD1*); XP_953618.1 (*T. annulata_SOD2*); CAI76104.1 (*T. annulata_Prx1a*); CAI73861.1 (*T. annulata_Prx1b*); CAI73823.1 (*T. annulata_PrxQ*); XP_012648270.1 (*B. microti_SOD1*);

XP_012650427.1 (*B. microti_SOD2*); XP_012647735.1 (*B. microti_Prx1a*); XP_012647852.1 (*B. microti_Prx1m*); EER11967.1 (*P. marinus_SOD1*); EER05214.1 (*P. marinus_SOD2*); EER17601.1 (*P. marinus_SOD3*); EER01509.1 (*P. marinus_SOD4*); EER07754.1 (*P. marinus_SOD5*); EER01510.1 (*P. marinus_SOD6*); EER10693.1 (*P. marinus_SOD7*); EER15930.1 (*P. marinus_SOD8*); EER08187.1 (*P. marinus_SOD9*); EEQ98662.1 (*P. marinus_SOD10*); EER12622.1 (*P. marinus_SOD11*); EER12645.1 (*P. marinus_SOD12*); EER11969.1 (*P. marinus_SOD13*); EER20546.1 (*P. marinus_SOD14*); XP_002776786.1 (*P. marinus_GPx*); EER20376.1 (*P. marinus_Prx1a*); EER20377.1 (*P. marinus_Prx1b*); EEQ98046.1 (*P. marinus_Prx1c*); EER16147.1 (*P. marinus_Prx1m*); EER17891.1 (*P. marinus_Prx5a*); EER08481.1 (*P. marinus_Prx5b*); EER05353.1 (*P. marinus_Prx5c*); EER12446.1 (*P. marinus_Prx6*). Prx proteins were named according to the nomenclature proposed by Gretes et al. (2012). All sequences were obtained either from NCBI reference sequences or the GenBank database accessed through the "Protein Database" on the NCBI website (https://www.ncbi.nlm.nih.gov/).

Table 3.2. Predicted subcellular locations and molecular weights (MW) of the typical 2-Cys Prx proteins from *T. thermophila*, other alveolates (ciliates, apicomplexans and perkinsozoans) and *Homo sapiens*.

Species	Typical 2-Cys Peroxiredoxin	Score*			Location	Cleavage site	MW (kDa)#
		mTP	SP	other			
Human							
H. sapiens	Prx1	0.34	0.05	0.70	_	-	22.1
	Prx2	0.40	0.06	0.60	_	-	22
	Prx3	0.90	0.04	0.10	M	61	21.5
	Prx4	0.03	0.80	0.30	S	37	26.6
Ciliates**							
T. thermophila	*Prx1a*	0.10	0.32	0.60	_	-	23
	Prx1b	0.42	0.05	0.52	_	-	26
	Prx1m	0.73	0.24	0.10	M	19	28
	Prx1c	0.50	0.31	0.30	M	64	21.4
S. lemnae	*Prx1a*	0.11	0.92	0.03	S	17	22.9
	Prx1b	0.11	0.08	0.9	_	-	29.4
	Prx1m	0.90	0.05	0.10	M	29	24.6
I. multifiliis	*Prxla*	0.30	0.11	0.61	_	-	23.5
	Prxlb	0.20	0.11	0.70	_	-	23.1
	Prxlc	0.01	1.0	0.14	S	23	23.8
	Prxld	0.10	0.24	0.70	_	-	23.3
	Prxle	0.20	0.93	0.10	S	23	24.5
	Prx1m	0.60	0.05	0.42	M	17	21.8
O. trifallax	*Prx1a*	0.20	0.91	0.02	S	17	23.2
	Prx1b	0.34	0.10	0.44	_	-	27.5
	Prx1c	0.10	0.05	0.94	_	-	50.5
	Prx1d	0.10	0.05	0.94	_	-	45.9
	Prx1e	0.34	0.10	0.61	_	-	23.8
Apicomplexans**							
P. falciparum	*Prx1a*	0.14	0.10	0.82	_	-	21.8
	Prx1m	0.90	0.04	0.14	M	19	22.4
T. gondii	*Prx1a*	0.11	0.20	0.80	_	-	21.7
	Prx1m	0.93	0.03	0.11	M	83	22.1
C. parvum	*Prx1a*	0.23	0.11	0.60	_	-	21.7
E. tenella	*Prx1a*	0.20	0.10	0.80	_	-	21.4
T. annulata	*Prx1a*	0.11	0.12	0.80	_	-	21.8
	Prx1b	0.4	0.06	0.52	_	-	29.1
B. microti	*Prx1a*	0.10	0.20	0.80	_	-	21.9
	Prx1m	0.94	0.02	0.10	M	37	23.5
Perkinsozoans**							
P. marinus	*Prx1a*	0.08	0.12	0.82	_	-	21.6
	Prx1b	0.05	0.14	0.90	_	-	22.1
	Prx1c	0.08	0.10	0.90	_	-	21.6
	Prx1m	0.61	0.10	0.23	M	14	21.7

Abbreviations are mTP, mitochondrial targeting peptide; SP, secretory pathway signal peptide; M, mitochondrion; S, secretory pathway; _, any other location; -, absence. The predictions were made using TargetP version 1.1 software (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2007). * the scores indicate the likelihood of the predicted subcellular location. A score close to 1 indicates a high likelihood. ** indicates that the proteins were named according to the nomenclature proposed by Gretes et al. (2012). # indicates that the MW was calculated using the EXPASY Bioinformatics Resource Portal (http://web.expasy.org/). The accession numbers are shown in either table 3.1 or Fig. 3.2

Table 3.3. Bioinformatic analysis of the presence/absence of the GGLG and YF motifs and sulfiredoxin enzymes in various organisms and their association with biochemical data related to the sensitivity or robustness of the typical 2-Cys Prx proteins to hyperoxidation.

Species	Typical 2-Cys Peroxiredoxin	Motif [1]		Type	Sulfiredoxin
Human					
Homo sapiens	Prx1	GGLG	YF	Sensitive [2,3,4]	Present [9]
	Prx2	GGLG	YF	Sensitive [3,4,5]	
	Prx3	GGLG	YF	Sensitive [3,4,5]	
	Prx4	GGLG	YF	Sensitive [6]	
Yeast					
Schizosaccharomyces pombe	TPx1	GGLG	YF	Sensitive [7,8]	Present [9]
Saccharomyces cerevisiae	Tsa1	GGLG	YF	Sensitive [9]	Present [9]
	Tsa2	GGLG	YF	Sensitive [9]	
Ciliate*					
Tetrahymena thermophila	*Prx1a*	GGLG	Y*W*	Not studied	Absent
	Prx1b	GGLG	*FW*	Not studied	
	Prx1m	GGLG	Y*W*	This study	
	Prx1c	GG*V*G	YF	Not studied	
Apicomplexans*					
Plasmodium falciparum	*Prx1a*	GG*I*G	Y*L*	Not studied	Absent [10]
	Prx1m	GG*I*G	Y*M*	Not studied	
Toxoplasma gondii	*Prx1a*	GG*I*G	Y*L*	Not studied	Absent [10]
	Prx1m	GG*I*G	Y*L*	Not studied	
Cryptosporidium parvum	*Prxla*	GG*I*G	Y*L*	Not studied	Absent [10]
Eimeria tenella	*Prxla*	GGLG	Y*L*	Not studied	Absent [10]
Theileria annulata	*Prx1a*	*A*G*V*G	*HL*	Not studied	Absent [10]
	Prx1b	GG*VS*	Y*L*	Not studied	
Babesia microti	*Prx1a*	GG*I*G	*HL*	Not studied	Absent [10]
	Prx1m	GG*IP*	Y*L*	Not studied	
Bacteria					
Streptococcus mutans	Prx	-	-	Robust [1]	Absent [9]
Escherichia coli	Prx	-	-	Robust [1,11]	Absent [9]
Salmonella typhimurium	Prx	-	-	Robust [1,12]	Absent [9]
Cyanobacteria					
Synechocystis sp. (NP_442066)	Prx	GG*I*G	YF	Robust [13]	Absent [13]
Anabaena sp. (NP_488681)	Prx	GG*V*G	YF	Sensitive [13]	Present [14]

* indicates that the proteins were named according to the nomenclature proposed by Gretes et al. (2012). An italicised letter in the motif sequence indicates that a residue is different from the conserved GGLG or YF motif found in humans and yeast. All sequences were obtained either from NCBI reference sequences or the GenBank database accessed through the "Protein Database" on the NCBI website (https://www.ncbi.nlm.nih.gov/). [1](Wood et al., 2003a); [2](Yang et al., 2002); [3](Wagner et al., 2002); [4](Cox et al., 2009); [5](Rabilloud et al., 2002); [6](Wang et al., 2012); [7](Koo et al., 2002); [8](Vivancos et al., 2005); [9](Biteau et al., 2003); [10](Gretes et al., 2012); [11](Ritz et al., 2001); [12](Niimura et al., 1995); [13](Pascual et al., 2010); [14](Boileau et al., 2011).

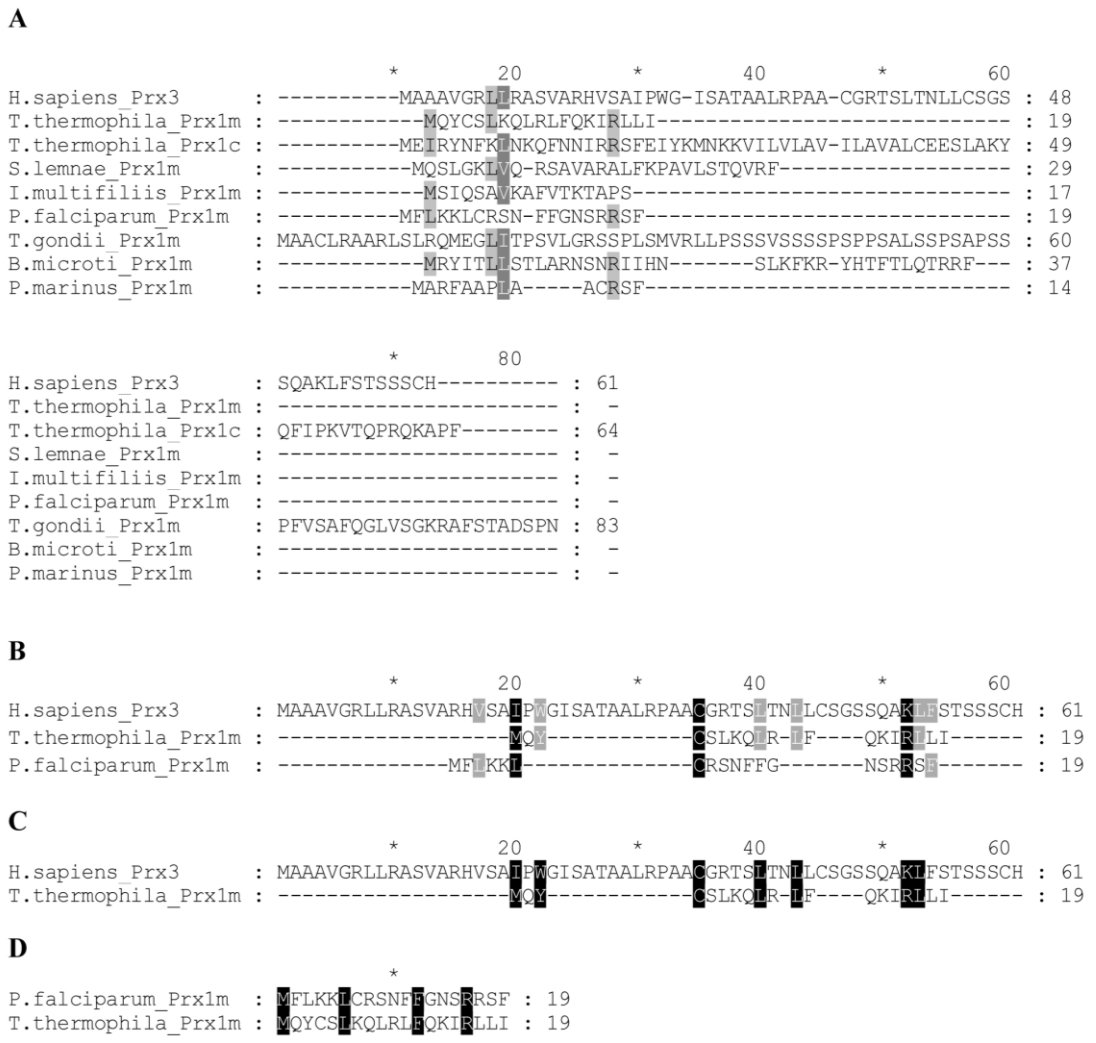

Fig. 3.1. An amino acid sequence alignment of the predicted and/or experimentally confirmed mTP sequences of the typical 2-Cys Prx proteins from *H. sapiens*, *T. thermophila* and other alveolates (A), *H. sapiens*, *T. thermophila* and *P. falciparum* (B), *H. sapiens* and *T. thermophila* (C) and *P. falciparum* and *T. thermophila* (D). The alignments were done using ClustalX version 2.1 (http://www.clustal.org) (Larkin et al., 2007). The degree of amino acid conservation is indicated by shading with black, dark grey and light grey indicating 100%, 80% and 60% conservation, respectively. All sequences were obtained either from NCBI reference sequences or the GenBank database accessed through the NCBI website (http://www.ncbi.nlm.nih.gov). The accession numbers are shown in Fig. 3.2.

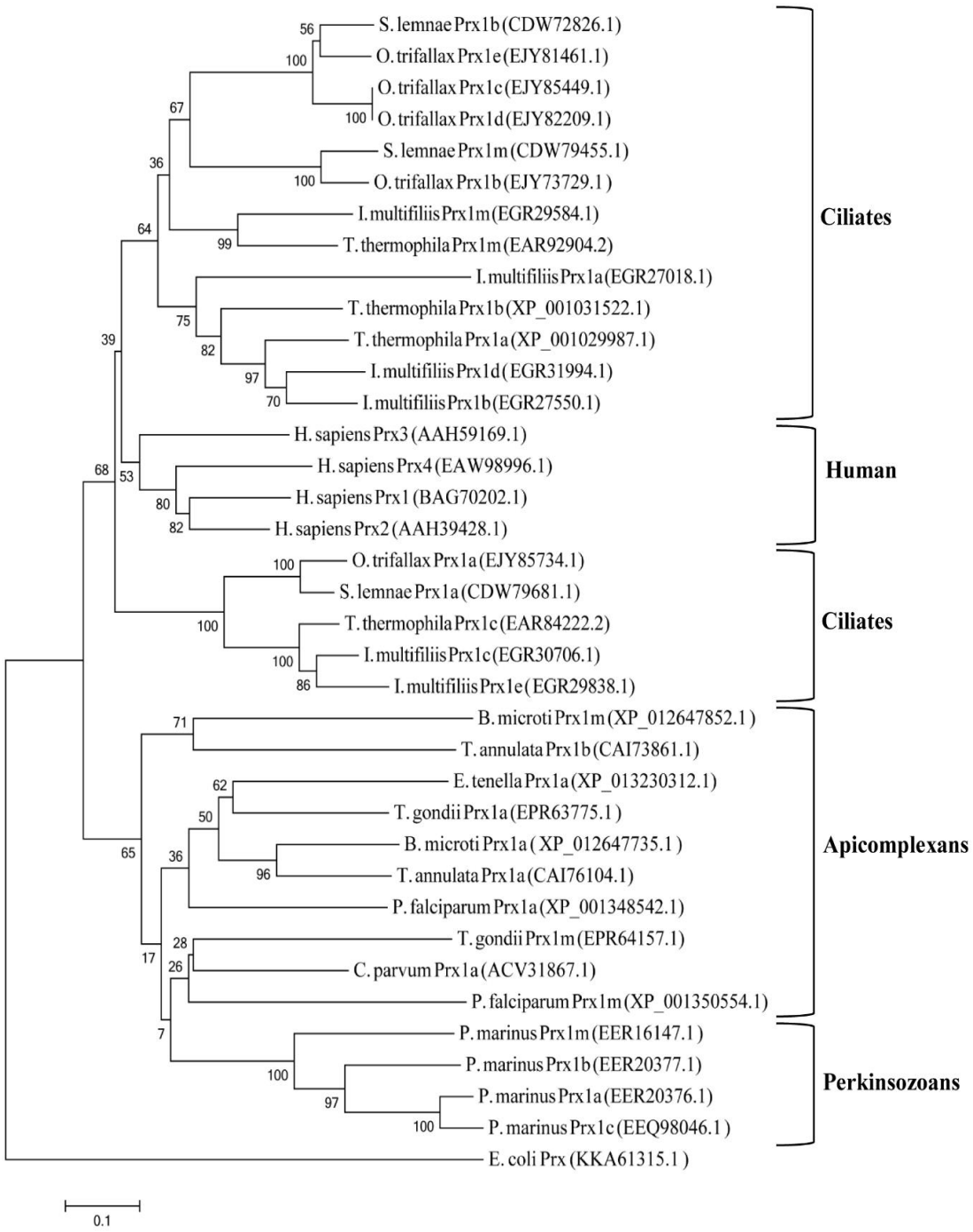

Fig. 3.2. A phylogenetic tree showing the relationships between the typical 2-Cys Prx proteins from *T. thermophila*, other alveolates (ciliates, apicomplexans and perkinsozoa) and *H. sapiens*. An *Escherichia coli* typical 2-Cys Prx protein was used as the out-group to root the tree. The sequences were aligned using ClustalX version 2.1 (http://www.clustal.org) (Larkin et al., 2007) and the tree was generated using the Neighbor-joining method (Saitou and Nei, 1987) accessed through Molecular Evolutionary Genetic Analysis (MEGA) version 6.06 software (http://www.megasoftware.net/) (Tamura et al., 2013). The horizontal branch length is proportional to the rate of amino acid substitution per site. The numbers represent bootstrap values calculated from 1000 replicates. All sequences were obtained either from NCBI reference sequences or the GenBank database accessed through the NCBI website (http://www.ncbi.nlm.nih.gov).

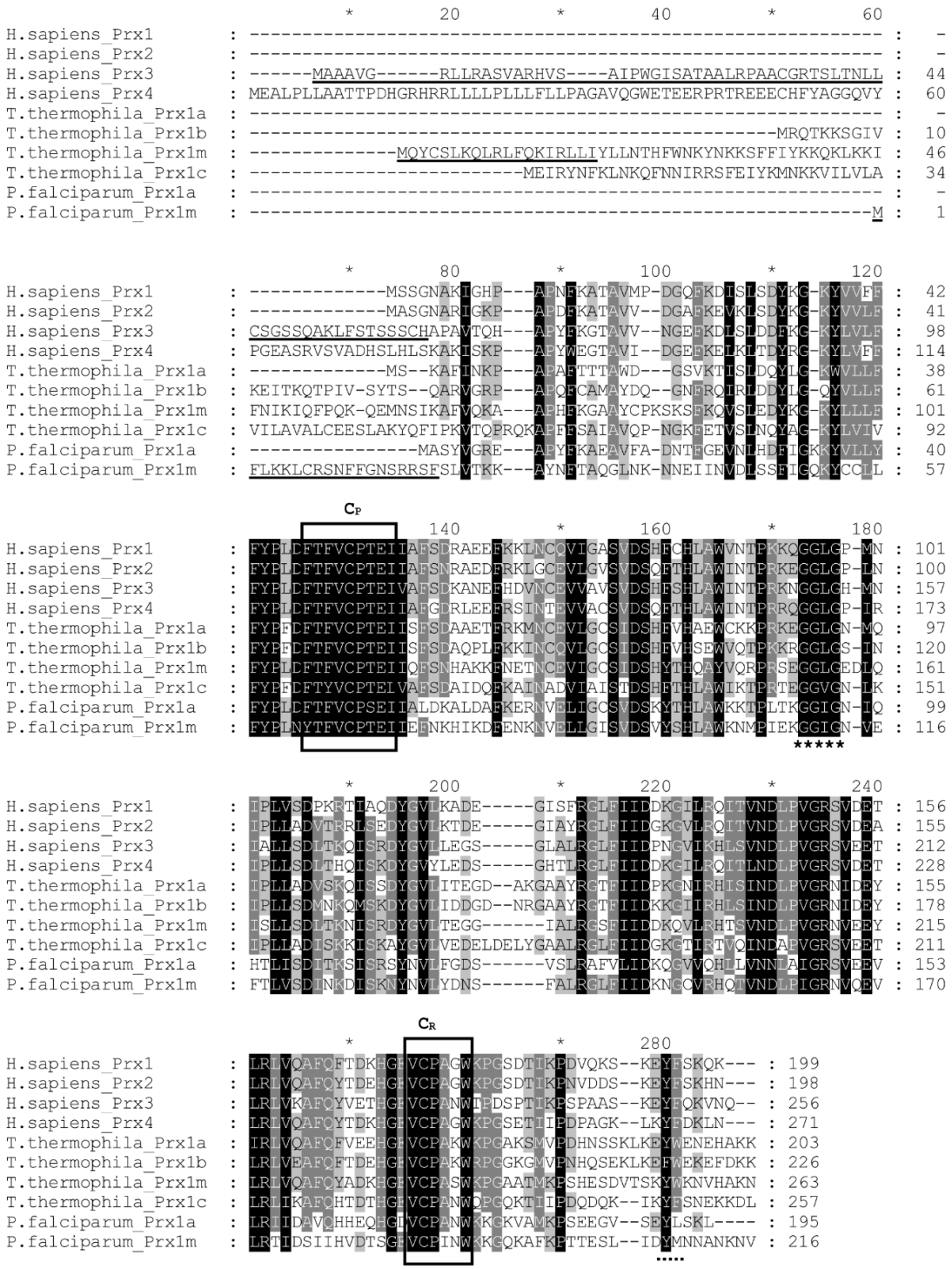

Fig. 3.3. An amino acid sequence alignment of typical 2-Cys Prx proteins from *H. sapiens*, *T. thermophila* and *P. falciparum*. The alignment was done using ClustalX version 2.1 (http://www.clustal.org) (Larkin et al., 2007). The degree of amino acid conservation is indicated by three levels of shading with black, dark grey and light grey indicating 100%, 80% and 60% conservation, respectively. The two active site motifs containing the redox-active Cys residues are boxed. The conserved GG*X*G and YF (or similar) motifs are highlighted with starred and dotted lines, respectively. The predicted and/or experimentally confirmed mTP sequences are underlined. All sequences were obtained either from NCBI reference sequences or the GenBank database accessed through the NCBI website (http://www.ncbi.nlm.nih.gov). The accession numbers are shown in Fig. 3.2.

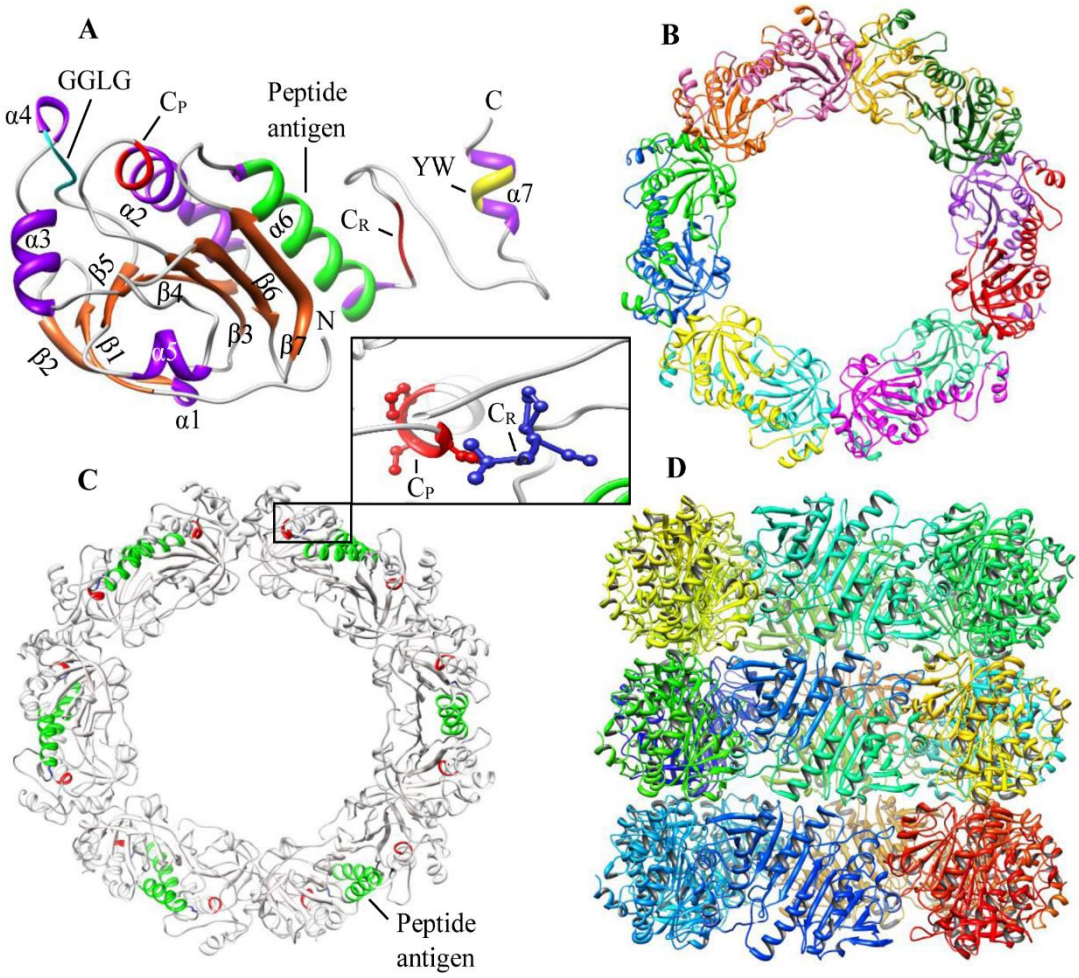

Fig. 3.4. Structural model of the *T. thermophila Prx1m* protein built from the crystal structure of the human Prx3 protein, (Protein Data Bank accession number, PDB: 5JCG). The images (A to D) were produced using UCSF Chimera version 1.10.1 (http://www.cgl.ucsf.edu/chimera/) (Pettersen et al., 2004). The VCP motifs containing the C_P or C_R residues in the Prx1m monomer are depicted in red and the GGLG and YW motifs are depicted in cyan and yellow, respectively (A). The individual monomers making up the dodecameric structure are indicated by different colours (B). On each subunit of the dodecameric structure, the VCP motif containing C_P is coloured red whereas the VCP motif containing C_R is coloured blue (C). The peptide antigen 'EEYLRLVQAFQYAD' is indicated in green (C). The different subunits making up a predicted stack of three dodecameric rings for the Prx1m protein are indicated in different colours (D).

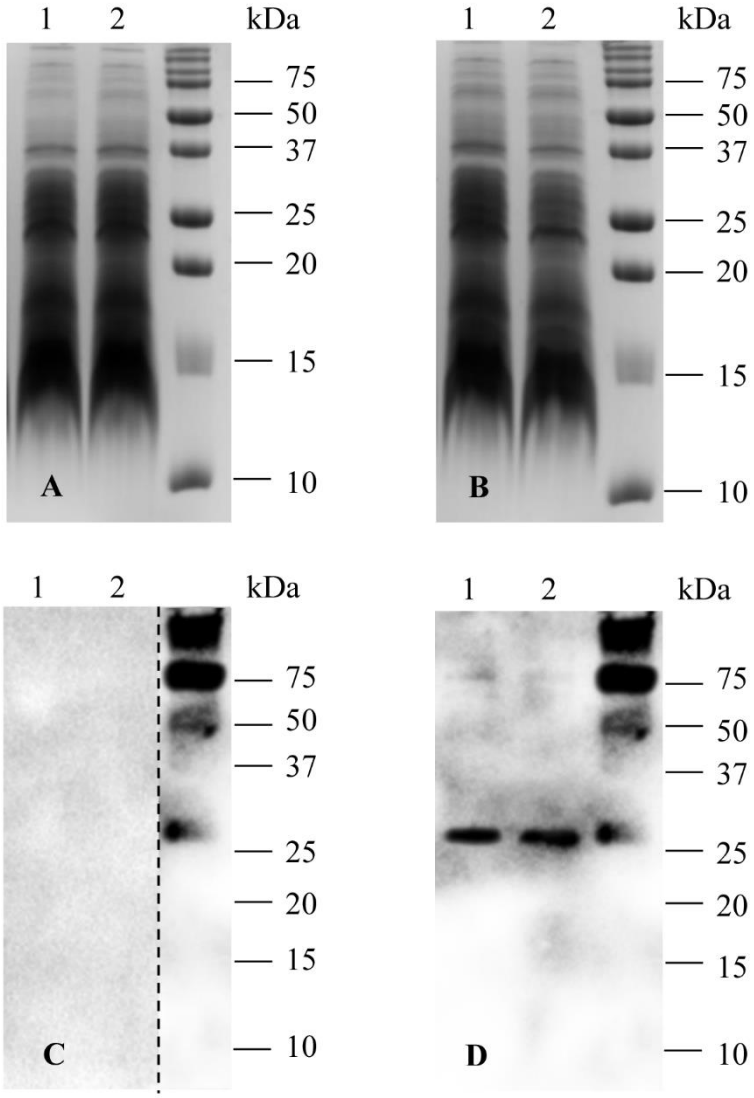

Fig. 3.5. Testing the anti-(*T. thermophila Prx1m*) polyclonal antibodies using immunoblotting. The total soluble proteins from *T. thermophila* cells (lanes 1 and 2) were extracted according to the protocol described in Section 3.2.7. The proteins were separated using NR-PAGE (Section 3.2.8). In panels A and B, the proteins were stained using Coomassie Blue. In panel C, the proteins were subjected to immunoblot analysis using pre-immune serum. In panel D, the proteins were subjected to immunoblot analysis using anti-(*T. thermophila Prx1m*) polyclonal antibodies produced according to the protocol described in Section 3.2.4. The experiment was repeated three times with similar results.

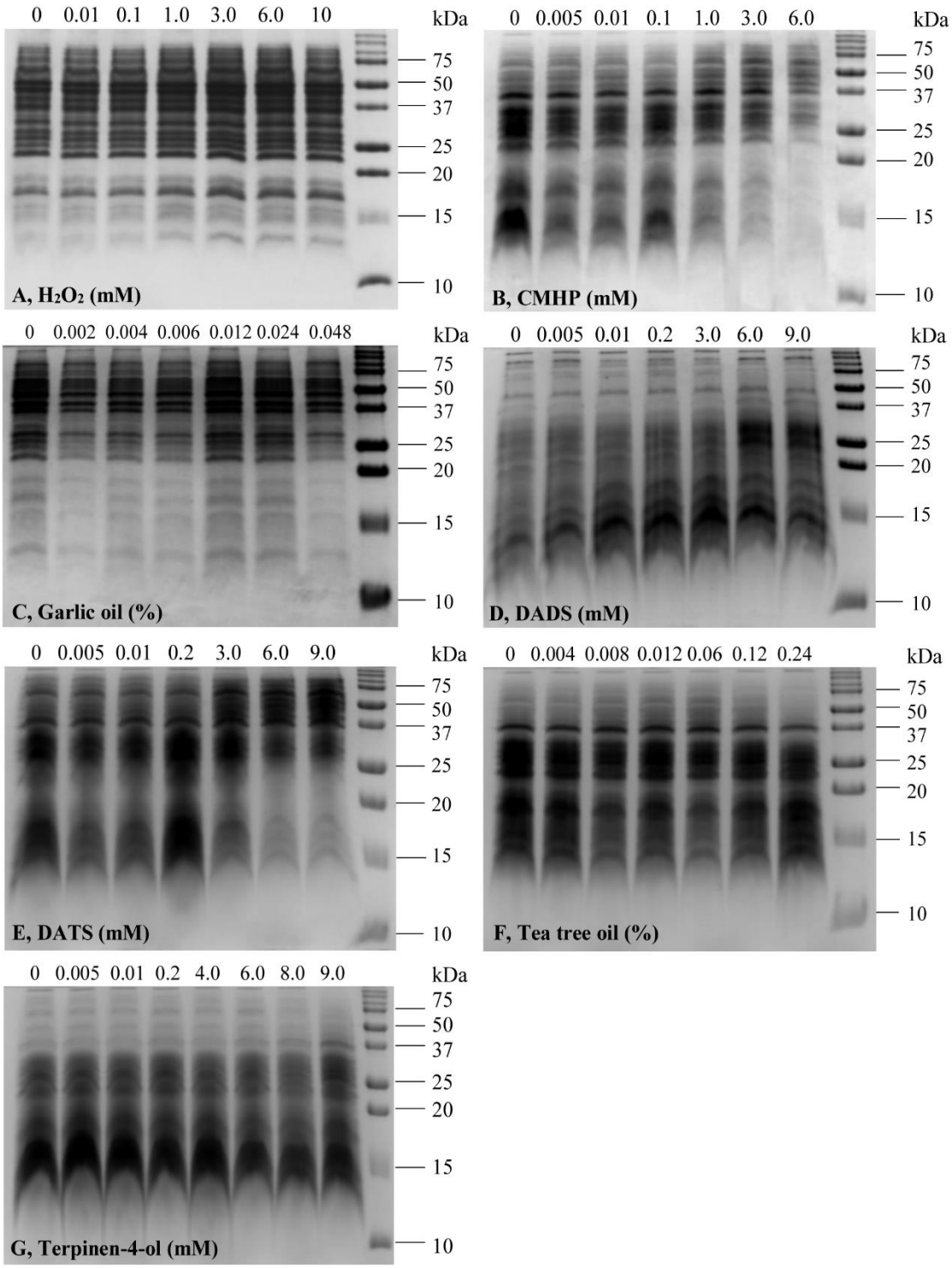

Fig. 3.6. The effects of treating *T. thermophila* cells with H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the total soluble proteins extracted from the cells. The proteins were separated using NR-PAGE and then stained using Coomassie Blue.

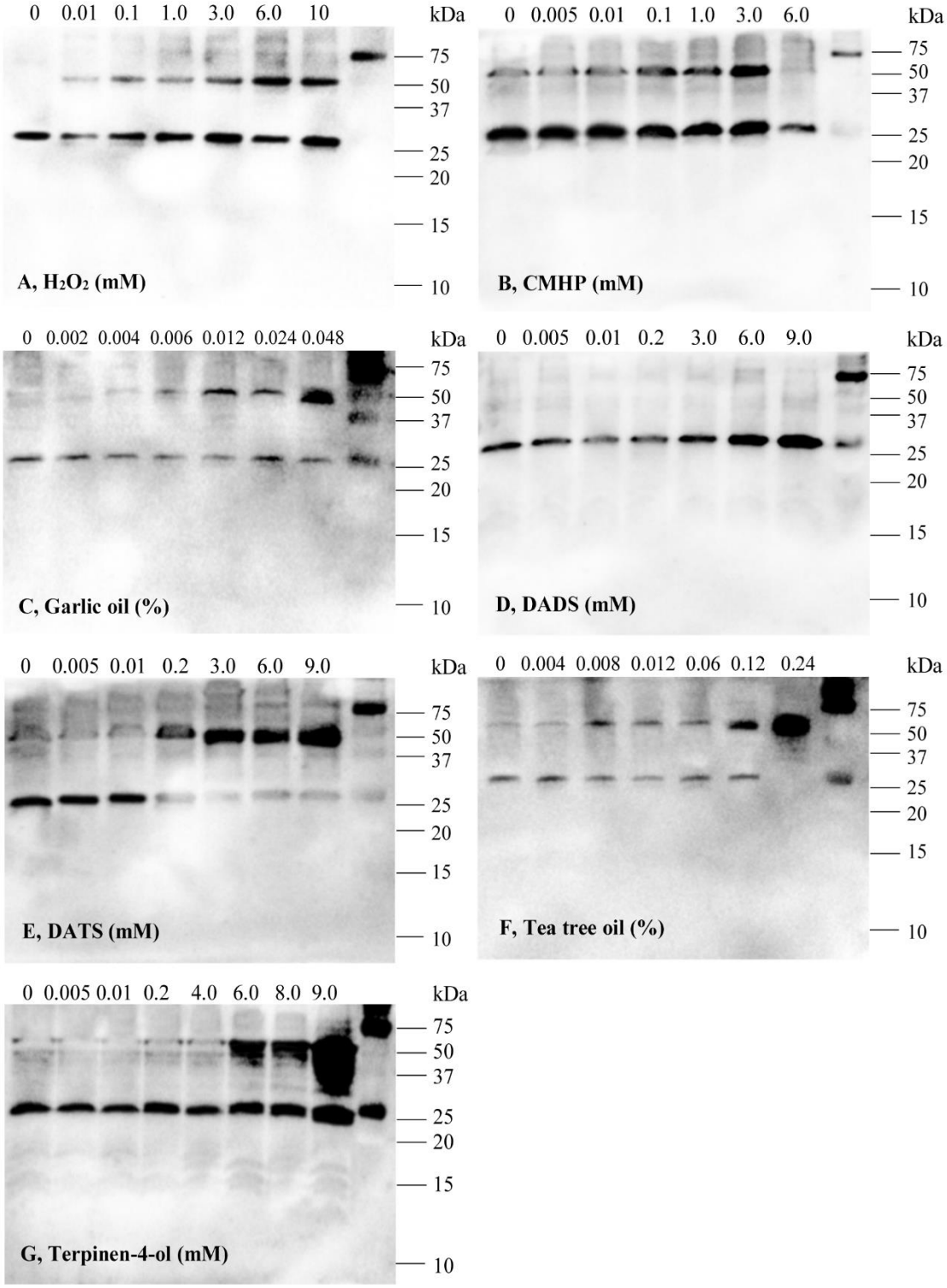

Fig. 3.7. The effects of treating *T. thermophila* cells with H_2O_2, CMHP, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the redox/oligomerization of the Prx1m protein. Gels identical to those shown in Fig. 3.6 were run and the proteins thus separated were subjected to immunoblot analysis using the anti-(*T. thermophila Prx1m*) antibodies raised against the peptide antigen "EEYLRLVQAFQYAD".

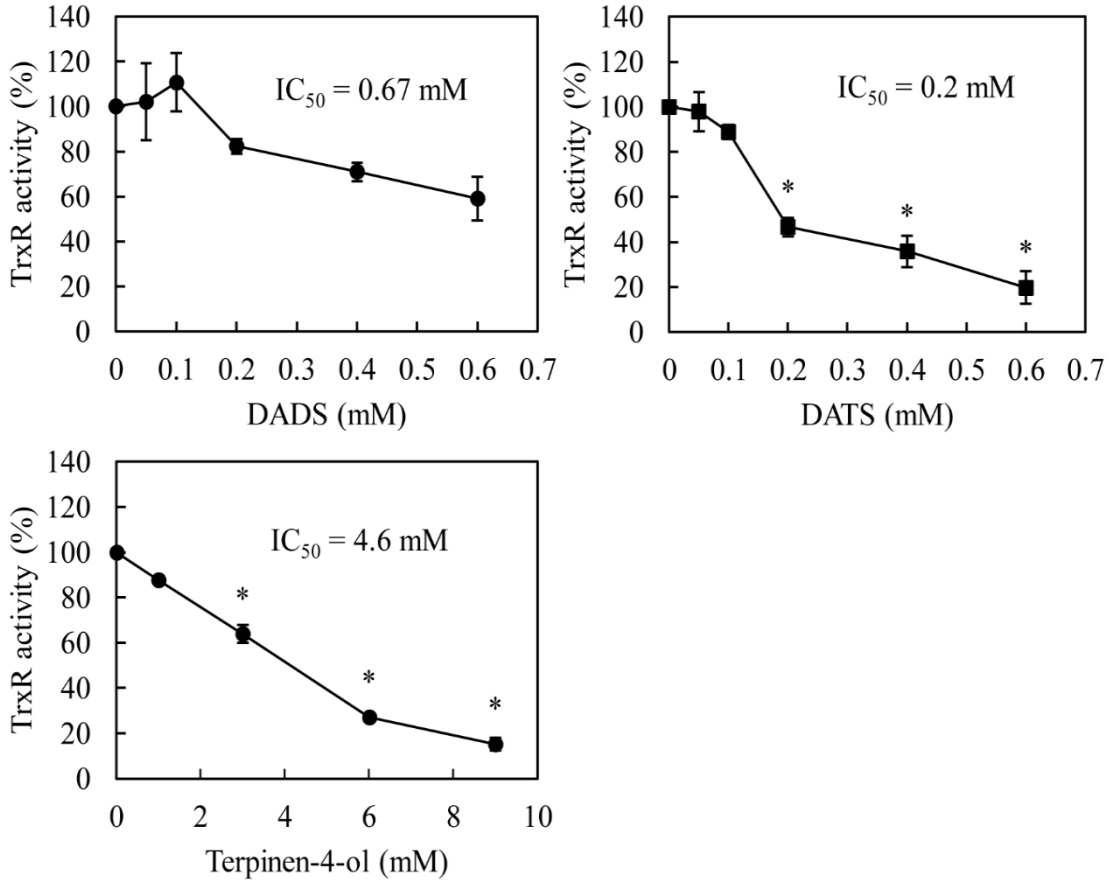

Fig. 3.8. The effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in crude extracts from *T. thermophila* cells. The data are expressed as % of the untreated control and each data point is the mean value for 3 replicate assays. The vertical bars represent ± standard error of the mean (n = 3). A star indicates a significant difference from the untreated control ($p < 0.05$).

Chapter 4 - Garlic and tea tree oil compounds promote oxidation of the typical 2-Cys peroxiredoxin proteins in Jurkat T-lymphocytes

Abstract

The mammalian typical 2-Cys peroxiredoxin (Prx) proteins have emerged as important sensors and transducers in the cellular response to oxidative stress. Plant natural products have been shown to have both pro- and anti-oxidant effects but their exact mechanisms of action are not fully understood. Here we show that garlic and tea tree oils and selected compounds from these oils promote either oxidised dimer formation or hyperoxidised monomer formation in the typical 2-Cys Prx proteins. Diallyl disulphide (DADS) and diallyl trisulphide (DATS), the main constituents of garlic oil, had similar effects to their parent oil but DATS was more effective than DADS indicating that the number of sulphur atoms is important. Tea tree oil was a less potent promotor of the oxidation of the Prx proteins than garlic oil. In contrast, terpinen-4-ol, the major constituent of tea tree oil, was a very effective promotor of the oxidation of the Prx proteins. We show that the results could partly, but not entirely, be explained by differences in the effects of the tested compounds on the activity of thioredoxin reductase (TrxR), the enzyme required for the regeneration of the reduced monomer form of the Prx proteins from their oxidised dimer form. The results are discussed in terms of the involvement of the typical 2-Cys Prx proteins in the cellular response oxidative stress.

4.1 Introduction

The typical 2-Cys peroxiredoxin (Prx) proteins are enzymes that catalyse the reductive decomposition of H_2O_2 and various organic peroxides to less reactive products (Wood et al., 2003b). They were first described as antioxidant enzymes that protect cells against oxidative stress but they are now thought to be involved in H_2O_2-mediated cell signalling as well (Rhee et al., 2012). In mammals, there are four different isoforms, Prx1 located in the cytosol or nucleus, Prx2 located in the cytosol or associated with the erythrocyte membrane, Prx3 located in the mitochondria and Prx4 located in the cytosol or the endoplasmic reticulum (Tavender et al., 2008; Wood et al., 2003b). All four isoforms are characterised by interacting active site Cys residues located on separate subunits known as the peroxidatic Cys (C_P) and the resolving Cys (C_R) (Wood et al., 2003b). During catalysis, C_P in its reduced/thiol state (C_P-SH) attacks the peroxide substrate and becomes oxidised to a Cys-sulphenic acid residue (C_P-SOH). C_P-SOH then forms a disulphide bond with C_R on a separate subunit. Subsequently, the disulphide bonded is broken with the help of thioredoxin (Trx) and Trx reductase (TrxR) and C_P-SH is regenerated ready to begin another cycle of catalysis. The cycle described above occurs in the presence of low concentrations of H_2O_2 but in the presence of high H_2O_2 concentrations, C_P-(SOH) can react with a second molecule of H_2O_2 and become hyperoxidised to a Cys sulphinic acid residue (C_P-SO_2H) (Ikeda et al., 2011; Wood et al., 2003b). C_P-SO_2H cannot react with C_R and as a result the hyperoxidised form of the enzyme has lost its peroxidase activity. The peroxidase activity is only very slowly restored with the help of a family of enzymes known as the sulfiredoxins (Biteau et al., 2003; Woo et al., 2003). This unusual behaviour has been proposed to allow H_2O_2 to accumulate to act as a cell signalling molecule (Wood et al., 2003a).

When C_P is in its reduced/thiol state (C_P-SH) or its hyperoxidised/sulphinic acid state (C_P-SO_2H), Prx1, 2 and 3 run as monomers (at approximately 22 kDa) on non-reducing polyacrylamide (NR-PAGE) gels whereas when C_P-SH becomes oxidised to C_P-SOH and forms a disulphide bond with C_R, these proteins run as dimers (at approximately 44 kDa) (Cox et al., 2009). The reduced and hyperoxidised monomer forms of these enzymes can be readily distinguished using alkylating agents that block the oxidation of the sulfhydryl group of C_P-SH during cell extraction and antibodies that recognise C_P in its hyperoxidised state (C_P-SO_2H). Thus, employing NR-PAGE and appropriate cell extraction conditions and antibodies, the redox/oligomerization state of the typical 2-Cys Prx proteins can be used as a sensitive endogenous indicator of oxidative stress (Poynton and Hampton, 2014).

Previous studies have shown that treatment of Jurkat-T lymphocytes, a model for human cancer cells, with low concentrations of H_2O_2 (< 20 μM) promotes conversion of the reduced monomer form of Prx1, 2 and 3 to the oxidised dimer form and that treatment with higher concentrations (≥ 20 μM) promotes conversion of the oxidised dimer form to the hyperoxidised monomer form (Cox et al., 2009). Studies have also shown that plant compounds such as phenethyl isothiocyanate, found in cruciferous vegetables (e.g. broccoli), can promote oxidised dimer formation in the Prx3 protein in Jurkat T-lymphocytes and promyelocytic leukemia cells (the human HL-60 cell line) (Brown et al., 2010; Brown et al., 2008). Allyl sulphides found in garlic (*Allium sativum*) oil, such as diallyl disulfide (DADS) and diallyl trisulfide (DATS), and terpenoids found in tea tree (*Melaleuca alternifolia*) oil, such as terpinen-4-ol, have both pro- and anti-oxidant effects but their exact mechanisms of action are not fully understood (Carson et al., 2006; Kuttan et al., 2011; Munday, 2012). Thus, the aims of the present study were to investigate the effects of garlic oil, DADS, DATS, tea tree oil and terpinen-4-ol on the *in vivo*

redox/oligomerization state of the Prx1, 2 and 3 proteins in Jurkat-T lymphocytes and also to investigate whether the oils/compounds exerted their effects on the typical 2-Cys Prx proteins by inhibiting the activity of TrxR, the enzyme required to regenerate the reduced/thiol state of C_P from its disulphide-bonded state.

4.2 Materials and methods

4.2.1 Jurkat T-lymphocyte culture

Jurkat T-lymphocytes were routinely cultured as described in Section 2.2.3.

4.2.2 Investigating the effects of various pro-oxidant treatments on the in vivo redox/oligomerization state of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes

The effects of hydrogen peroxide (37%, v/v; Thermo Fisher Scientific Pty Ltd), garlic (*Allium sativum*) oil (100%, v/v; Mystic Moments, Fordingbridge, Hampshire, United Kingdom), diallyl disulfide (DADS, 80%, v/v; Sigma-Aldrich), diallyl trisulfide (DATS, 95%, v/v; Cayman Chemical), tea tree (*Melaleuca alternifolia*) oil (100%, v/v; Integria Healthcare Australia Pty Ltd) and terpinen-4-ol (97%, v/v; VWR International Pty Ltd, Australia) on the redox/oligomerization state of the typical 2-Cys proteins in Jurkat T-lymphocytes were investigated. Stock solutions of the test substances, except H_2O_2, were prepared in dimethyl sulfoxide (DMSO). Prior to each experiment, the stock solutions and H_2O_2 were diluted in Jurkat cell culture medium (Section 2.2.3) and added to the appropriate wells of a 6-well plate. This was followed by the addition of 4 x 10^6 Jurkat T-lymphocytes per well. The cells (maintained as described in Section 2.2.3) had been pelleted by centrifugation at 1,500 *g* for 1 min and then resuspended in Jurkat cell culture medium and counted (Section 2.2.3) prior to being added to the wells. The total volume of liquid in each well was 4 ml and the maximum concentration of DMSO was 0.14% (v/v), which we had previously shown not to affect the proliferation of the cells (data not shown). The cells were incubated for 10 min at 37^{o}C in 5% (v/v) CO_2 in air and then harvested by centrifugation at 1,500 *g* for 1 min. Following this incubation, crude cell

extracts were prepared as described below (4.2.3). The experiments were repeated four times for H_2O_2 and garlic oil, three times for DADS, twice for DATS and three times for tea tree oil and terpinen-4-ol. The results of representative experiments are shown here.

4.2.3 Preparation of Jurkat T-lymphocyte extracts for the determination of the relative amounts of the reduced, oxidised and hyperoxidized forms of the typical 2-Cys Prx proteins

The cell extracts were prepared essentially as described by Cox et al. (2010b). As noted by Cox et al. (2010b), in order to prevent artifactual oxidation of the sulfhydryl (–SH) group of C_P-SH during cell extraction, it is important to 'block' this group using an alkylating agent such as N-ethylmaleimide (NEM). Oxidation of the –SH group of C_P-SH in the typical 2-Cys Prx proteins occurs very rapidly even in the presence of only trace amounts of H_2O_2 (Cox et al., 2010b). Thus, the harvested cells were incubated for 15 min in a buffer containing 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, the cells were disrupted by the addition of 1% (w/v) 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate (CHAPS) to the incubation mixture. The cell extract was then clarified by centrifugation at 15,000 *g* for 5 min at 4^{o}C and the supernatant, which contained the Prx proteins, was stored at -80^{o}C until it could be analysed. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific).

4.2.4 Separation of the reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins using non-reducing polyacrylamide gel electrophoresis

The reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins were separated utilizing non-reducing polyacrylamide gel electrophoresis (NR-PAGE) according to the protocol described in Section 3.2.8. When subjected to NR-PAGE, the reduced and hyperoxidised forms of Prx1, 2 and 3 run as monomers with a molecular weight of approximately 22 kDa whereas the oxidised form runs as a dimer with a molecular weight of approximately 44 kDa (Cox et al., 2009). These different sizes can be resolved using NR-PAGE.

4.2.5 Detection of the reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins using immunoblotting

The reduced, oxidised and hyperoxidised forms of the typical 2-Cys Prx proteins separated as described above were detected using immunoblotting according to the protocol described in Section 3.2.9 but with slight modifications. The modifications were that the blocked membrane was incubated overnight at 4^{o}C in the presence of one of three different kinds of primary antibodies diluted either 1:1000 or 1:2000 in blocking buffer. The primary antibodies, which were rabbit polyclonal antibodies, recognised different isoforms of the Prx proteins or the hyperoxidised state of C_P. The first set of antibodies, which we refer to as the anti-(typical 2-Cys Prx) antibodies, had been produced by immunizing a rabbit with a full-length, recombinantly-expressed and highly purified typical 2-Cys Prx protein which showed 81%, 78%, 64% and 72% identity at the amino acid level to the human Prx1, 2, 3 and 4 proteins, respectively (Sutton et al., 2010). We expected this set of antibodies to recognise all four isoforms of the typical 2-Cys Prx

proteins. The second set of antibodies, which we refer to as the anti-(Prx1) antibodies, had been produced by immunising a rabbit with the peptide PLVSDPKRTIAQDY which is unique to the human Prx1 protein (Antibody Technology Australia Pty Ltd). We expected this set of antibodies to recognise only Prx1. The third set of antibodies which we refer to as the anti-(peroxiredoxin-SO_3) antibodies had been produced by immunising a rabbit with a synthetic sulfonylated peptide with an amino acid sequence corresponding to that of the active site of the human typical 2-Cys Prx proteins in its hyperoxidised state (Abcam®, catalogue number ab16830). We expected this set of antibodies to recognise only the hyperoxidised form of the typical 2-Cys Prx proteins.

4.2.6 Thioredoxin reductase assay

Thioredoxin reductase (TrxR) is required to regenerate the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form (Rhee et al., 2012). For the TrxR assays, Jurkat T-lymphocytes were seeded into 75 cm^2 cell culture flasks (Corning) at a density of 5 x 10^5 cells per flask in 30 ml Jurkat cell culture medium (Section 2.2.3). Following this, the cells were cultured for a period of approximately 60 hours at 37^oC in 5% (v/v) CO_2 in air. At the end of the culture period, the cells were pelleted by centrifugation at 1,500 *g* for 1 min and then washed twice in phosphate buffered saline (PBS, pH 7) before being disrupted in a lysis buffer containing 40 mM HEPES (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (w/v) CHAPS and 2 mM PMSF. Insoluble material was removed by centrifugation at 15,000 *g* for 5 min at 4^oC and the supernatant was retained for analysis. Cell extracts prepared in this way were stored at -80^oC until they could be analysed for TrxR activity. TrxR activity was assayed, in the presence and absence of various concentrations of DADS, DATS or terpinen-4-ol using a commercially

available assay kit (Sigma-Aldrich®, catalogue number CS0170). The kit coupled the oxidation of NADPH to the reduction of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) to 5-thio-2-nitrobenzoic acid (TNB). The assays were run in triplicate at 25°C in 96-well plates with a total assay volume of 200 µl per well and 120 µg of Jurkat T-lymphocyte protein per assay. The increase in absorbance due to the production of TNB was monitored at 412 nm using a FLUOstar® plate reader (BMG Labtech Pty Ltd). TrxR enzyme activity was calculated by determining the difference in TNB production rate in the presence and absence of a specific inhibitor of TrxR activity supplied with the kit. The protein concentration in the cell extracts was determined using the BCA protein assay (Thermo Fisher Scientific). The concentration of each test substance required to inhibit TrxR activity by 50% (IC_{50}) was calculated as previously described (Berthelier and Wetzel, 2006). In brief, a linear trend line was fitted to a plot of % inhibition of the TrxR activity (relative to the control) against the concentration of the test substance and the equation of the line thus generated was used to determine the test substance concentration that gave 50% inhibition of the TrxR activity. All experiments were repeated twice, i.e., with different batches of cells.

4.2.7 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 $p < 0.05$.

4.3 Results

4.3.1 Effects of pro-oxidant treatments on the total soluble proteins extracted from the Jurkat T-lymphocytes and on the in vivo redox/oligomerization state of the typical 2-Cys Prx proteins in these cells

Jurkat T-lymphocytes were treated with various concentrations of H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol and the effects on the total soluble proteins extracted from the cells (Fig. 4.1) and on the *in vivo* redox/oligomerization state of the typical 2-Cys Prx proteins (Fig. 4.2) were determined. Treatment of the cells with H_2O_2, garlic oil, DADS or DATS, regardless of the concentration, had no effect on the total soluble proteins extracted from the cells (Figs. 4.1A-D). However, at the highest concentrations tested, treatment with tea tree oil or terpinen-4-ol caused apparent protein denaturation. This was evidenced by the loss of most of the smaller molecular weight proteins (less than approximately 40 kDa) from the gels stained for total protein when the cells had been treated with either 0.24% (v/v) tea tree oil or 9.0 mM terpinen-4-ol (Figs. 4.1E-F).

Fig. 4.2 shows the effects of the various oils/chemicals on the redox/oligomerization state of the typical 2-Cys Prx proteins. In all cases, the control cells contained approximately equal amounts of the reduced monomer and oxidised dimer forms of these proteins. When the cells were treated with increasing concentrations of H_2O_2, there was increasing conversion of the oxidised dimer form of the proteins to the hyperoxidised monomer form (Fig. 4.2A). In contrast, when the cells were treated with increasing concentrations of garlic oil, DADS or DATS, there was increasing conversion of the reduced monomer form to the oxidised dimer form (Figs. 4.2B-D). DATS was more effective than DADS in promoting oxidised dimer formation. This reinforces the conclusion that the greater the

number of sulphur atoms in the allyl sulphides, the greater their effectiveness.

Unlike garlic oil, tea tree oil had no discernible effect on the redox/oligomerization state of the typical 2-Cys Prx proteins (Fig. 4.2E). At all of the tea tree oil concentrations tested, except for the highest, there were approximately equal amounts of the monomer and dimer forms of the Prx proteins present. The loss of the monomer form in the cells treated with the highest tea tree oil concentrations (0.12 and 0.24% (v/v)) was probably due to the general loss of smaller molecular weight proteins that occurred in the presence of these high concentrations (Fig. 4.1E). As stated above, we assume that this was due to protein aggregation or denaturation at high tea tree oil concentrations.

In contrast to its parent oil, terpinen-4-ol had a very strong oxidising effect on the typical 2-Cys Prx proteins (Figs. 4.2F-G). With increasing concentrations of terpinen-4-ol, there was increasing conversion of the oxidised dimer form of the typical 2-Cys Prx proteins to a monomer form. Initially, we were unsure whether this was the reduced monomer or the hyperoxidised monomer. Thus, we tested this using antibodies that specifically recognise the hyperoxidised monomer (Fig. 4.3). As a control, we also tested the effects of exposing the cells to high concentrations of H_2O_2. This experiment showed that terpinen-4-ol, like H_2O_2, promoted conversion of the typical 2-Cys Prx proteins to their hyperoxidised monomer form. Thus, terpinen-4-ol had a stronger oxidising effect than either DADS or DATS and was similar in its effects to high concentrations of H_2O_2. In summary, whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation.

4.3.2 Effects of pro-oxidant treatments on the redox/oligomerization state of Prx1 in Jurkat T-lymphocytes

To confirm our results, we repeated a subset of the experiments described above but this time using antibodies that were specific for Prx1 (Fig. 4.4). For H_2O_2, garlic oil, DADS and DATS, the results were the same regardless of which type of antibodies were used. In contrast, for tea tree oil and terpinen-4-ol, the results were somewhat different. When using the antibodies expected to recognise all of the four different isoforms of the typical 2-Cys Prx proteins, tea tree oil appeared have no effect on the redox/oligomerization state whereas terpinen-4-ol promoted hyperoxidised monomer formation. In contrast, when using the antibodies that recognised only Prx1, both tea tree oil and terpinen-4-ol promoted hyperoxidised monomer formation. This is consistent with the data shown in Fig. 4.3 where terpinen-4-ol promoted hyperoxidised monomer formation. Thus, we conclude that tea tree oil and terpinen-4-ol promote hyperoxidized monomer formation in Prx1 whereas garlic oil, DADS and DATS promote oxidised dimer formation.

4.3.3 Effects of selected pro-oxidants on TrxR enzyme activity in Jurkat T-lymphocytes

The regeneration of the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form requires TrxR activity. Thus, the differing effects of the various oils/compounds on the redox/oligomerization state of the typical 2-Cys Prx proteins could be due to differing effects on TrxR activity. Greater inhibition of TrxR activity should lead to a greater relative abundance of the oxidised dimer or hyperoxidised monomer form of the typical 2-Cys Prx proteins. Thus, we tested the effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in extracts of Jurkat-T lymphocytes to determine whether there was a correlation between inhibition of TrxR activity and the promotion of

the oxidation/hyperoxidation of the typical 2-Cys Prx proteins (Fig. 4.5). The most effective inhibitor of TrxR activity was DATS (IC_{50} = 0.13 mM) followed by DADS (IC_{50} = 0.47 mM) and then terpinen-4-ol (IC_{50} = 3.0 mM). This would suggest that DATS should be more effective than DADS in promoting oxidation of the Prx proteins and indeed it is. In contrast, it would suggest that terpinen-4-ol should be less effective at promoting oxidation of the Prx proteins than either DADS or DATS. However, this was not the case. Instead, terpinen-4-ol was considerably more effective at promoting oxidation of the Prx proteins than either DADS or DATS. Thus, it appears that differential inhibition of TrxR activity is not the only explanation for the different effects of these various oil/chemicals on the redox/oligomerization state of the Prx proteins.

4.4 Discussion

The redox/oligomerization state of the typical 2-Cys Prx proteins can be used as a sensitive endogenous indicator of oxidative stress (Poynton and Hampton, 2014). It is known that treatment of Jurkat-T lymphocytes with H_2O_2, one of the first products of oxidative stress, results in either oxidised dimer formation or hyperoxidized monomer formation in the Prx proteins in these cells, depending upon the concentration of H_2O_2 (Rhee et al., 2012). At low concentrations (< 20 μM) H_2O_2 treatment results in oxidised dimer formation in Prx1, 2 and 3 whereas at high concentrations (≥ 20 μM) it results in hyperoxidised monomer formation (Cox et al., 2009). Here we have obtained similar results but the concentrations of H_2O_2 required were somewhat higher than those previously reported. This may be due to slight differences in experimental methods. Broadly speaking though, we have confirmed that low concentrations of H_2O_2 promote oxidised dimer formation in the typical 2-Cys Prx proteins whereas high concentrations promote hyperoxidised monomer formation.

Phytochemicals, such as the allyl sulfides found in garlic oil and the terpenoids found in tea tree oil are known to have both pro- and anti-oxidant effects but it is unknown whether their mechanisms of action involve the typical 2-Cys Prx proteins (Kuttan et al., 2011; Munday, 2012; Wu et al., 2005). Here we have shown that garlic oil and its major constituents DADS and DATS, as well as tea tree oil and one of its major constituents terpinen-4-ol, can promote oxidation/hyperoxidation of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes. Garlic oil was more effective than tea tree oil and DATS was more effective than DADS in these respects. This suggested that the number of sulphur atoms in the molecule is important. It is known that both DADS and DATS can promote H_2O_2

formation via redox cycling and that DATS is more effective than DADS in this respect (Munday, 2012; Munday et al., 2003). This is consistent with our observation that DATS was more effective than DADS in promoting oxidation/dimerization of the Prx proteins in Jurkat T-lymphocytes and it supports the conclusion that the effects of DADS and DATS on these proteins are due to their ability to promote the production of H_2O_2 which reacts readily with C_P in the Prx proteins.

The effects of tea tree oil and terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins are more difficult to explain. Terpinen-4-ol was a very effective promoter of the oxidation of the typical 2-Cys Prx proteins. Whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation. Thus, terpinen-4-ol was more effective at promoting oxidation of the typical 2-Cys Prx proteins than either DADS or DATS. This suggests that terpinen-4-ol is more effective at promoting H_2O_2 formation in the cells or alternatively that it is more effective at inhibiting TrxR. We tested this second hypothesis by examining the effects of DADS, DATS and terpinen-4-ol on TrxR activity in crude extracts of Jurkat T-lymphocytes. Contrary to our hypothesis, terpinen-4-ol was a relatively poor inhibitor of TrxR activity compared with either DADS or DATS. Thus, greater effectiveness in inhibiting TrxR activity does not seem to explain the greater effectiveness of terpinen-4-ol in promoting oxidation of the typical 2-Cys Prx proteins. On the other hand, DATS was more effective than DADS in inhibiting TrxR activity and it was also more effective in promoting oxidation of the typical 2-Cys Prx proteins. It was known that DADS and DATS inhibit TrxR activity but it was not known how these phytochemicals affect the redox/oligomerization state of the typical 2-Cys Prx proteins (Hu et al., 2007). Thus, ours is the first report that shows that garlic oil, DADS and DATS

promote the oxidation of the typical 2-Cys Prx proteins. Our observation that DATS is more effective than DADS in this respect and also that DATS is more effective at inhibiting TrxR activity suggests that in the case of these allyl sulphides, the capacity to inhibit TrxR activity is indeed linked to the capacity to promote the oxidation of the typical 2-Cys Prx proteins.

Previous studies have shown that phenethyl isothiocyanate (PEITC) and sulforaphane (SFN), cancer chemopreventive compounds found in cruciferous vegetables such as broccoli, are both very effective inhibitors of TrxR activity but yet PEITC is substantially more effective than SFN in promoting the oxidation of Prx3, a mitochondrial typical 2-Cys Prx protein (Brown et al., 2008). This led the authors to conclude that differential inhibition of TrxR activity does not completely explain the differences in the effects of these phytochemicals on the redox/oligomerization state of the typical 2-Cys Prx proteins. The results of our experiments with terpinen-4-ol support this conclusion. Subsequently, the same authors showed that a functional mitochondrial electron transfer chain is required for the oxidation of Prx3 in response to the treatment of cells with either PEITC or auranofin, the latter being a well established inhibitor of TrxR activity (Brown et al., 2010). The mitochondrial electron transfer chain is an important source of ROS, including H_2O_2. Thus, the results of these two studies support the conclusion that the oxidation of Prx3 in the presence of either PEITC or auranofin is due to perturbation of the mitochondrial electron transfer chain leading to increased H_2O_2 production which in turn leads to increased Prx3 oxidation. The results of our study suggest that this conclusion can be extended to apply to other typical 2-Cys Prx proteins and also other phytochemicals as well.

Whereas DADS and DATS promoted oxidised dimer formation in the typical 2-Cys Prx proteins, terpinen-4-ol promoted hyperoxidised monomer formation. This suggests that terpinen-4-ol is more effective at increasing the cellular H_2O_2 concentration than either DADS or DATS. Terpinen-4-ol is a monoterpene and therefore very different in its chemical structure to the sulfur-containing DADS and DATS (Kuttan et al., 2011; Santos et al., 2011). It is known that monoterpenes inhibit farnesyl transferases and that these enzymes catalyse the transfer of a 15 carbon isoprenyl lipid moiety onto a conserved Cys residue near to the C-terminus of various proteins including protein-tyrosine phosphatases (Basso et al., 2006). Protein farnesylation promotes membrane association and contributes to protein-protein interactions. Recently it was shown that the peroxidase activity of Prx1 is lost when it is phosphorylated by certain protein-tyrosine kinases and that this phosphorylation/inactivation occurs only when Prx1 is associated with lipid rafts (Woo et al., 2010). The phosphorylation/inactivation of Prx1 can be reversed by protein-tyrosine phosphatases and it is proposed that inactivation of the lipid raft-associated Prx1 allows localised accumulation of H_2O_2 which in turn allows inactivation of the protein-tyrosine phosphatases. Protein tyrosine phosphatases are known to be inactivated as a result of oxidation of their catalytic Cys residue by H_2O_2 and this allows their counterparts, the protein tyrosine kinases, to phosphorylate their targets and thereby propagate various intracellular signals. It could be that inhibition of protein-tyrosine phosphatase association with lipid rafts due to inhibition of their farnesylation by terpinen-4-ol disrupts the interaction between the protein-tyrosine phosphatases and Prx1 such that Prx1 is inactivated by protein-tyrosine kinases and cannot be reactivated. If this occurs, there would be increasing accumulation of H_2O_2 resulting in hyperoxidation of Prx 1, and possibly other typical 2-Cys Prx proteins. This remains to be investigated.

In conclusion, we have shown that garlic oil and its major constituents DADS, and especially DATS, are potent promotors of the oxidation of the typical 2-Cys Prx proteins. We propose that the greater effectiveness of DATS compared with DADS is due to its greater number of sulphur atoms and consequent greater capacity to promote redox cycling and associated H_2O_2 production. Alternatively, or additionally, the greater effectiveness of DATS compared with DADS may be due to its stronger inhibition of TrxR activity. In contrast to DADS and DATS, terpinen-4-ol was a relatively weak inhibitor of TrxR activity and yet is was the strongest of all of the tested chemicals in promoting the oxidation of the typical 2-Cys Prx proteins. Thus, an alternative mechanism needs to be invoked to explain the effects of terpinen-4-ol. This requires further investigation.

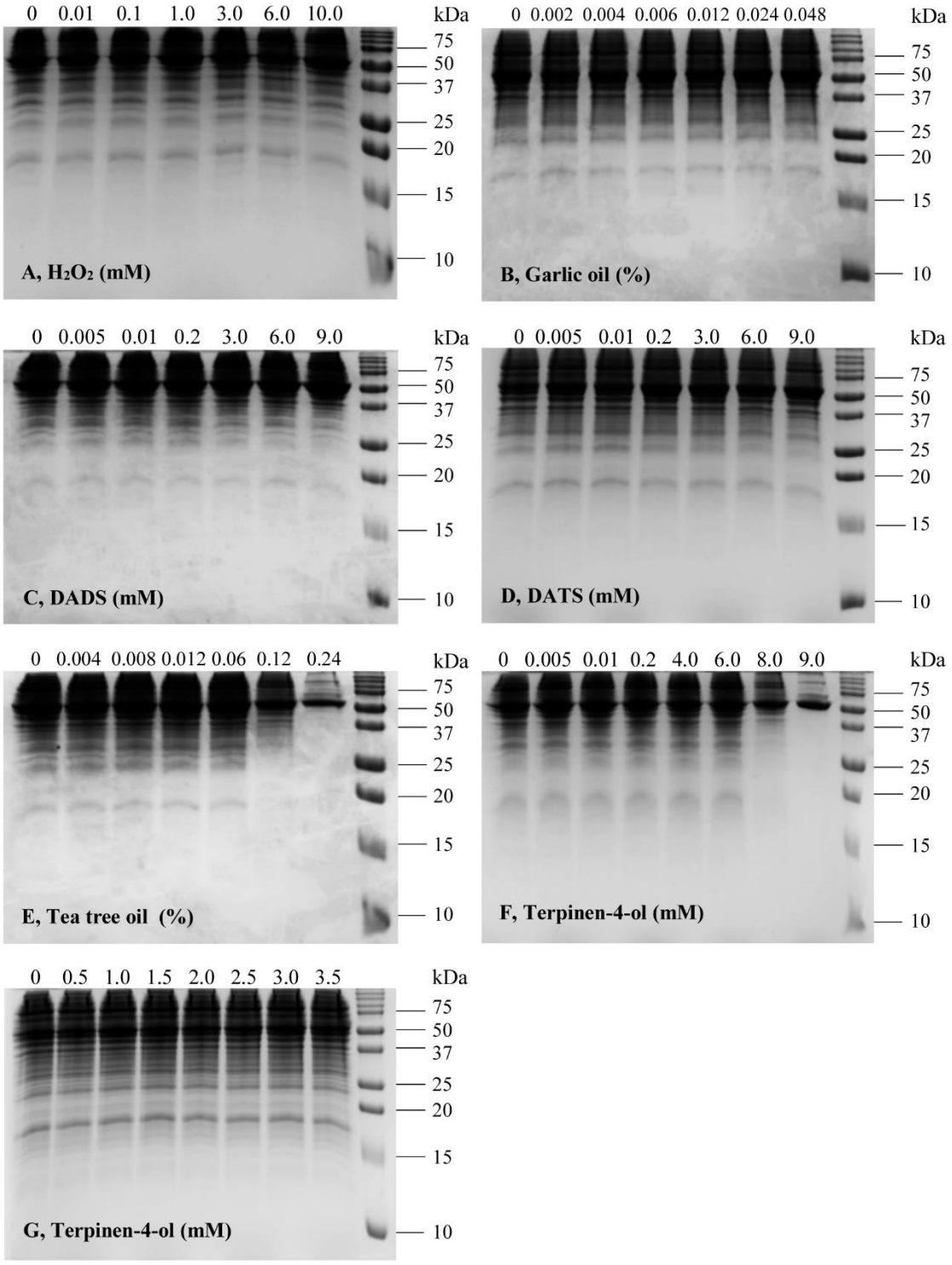

Fig. 4.1. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the total soluble proteins extracted from the cells. The proteins (30 µg protein per well) were separated using NR-PAGE and then stained using Coomassie Blue.

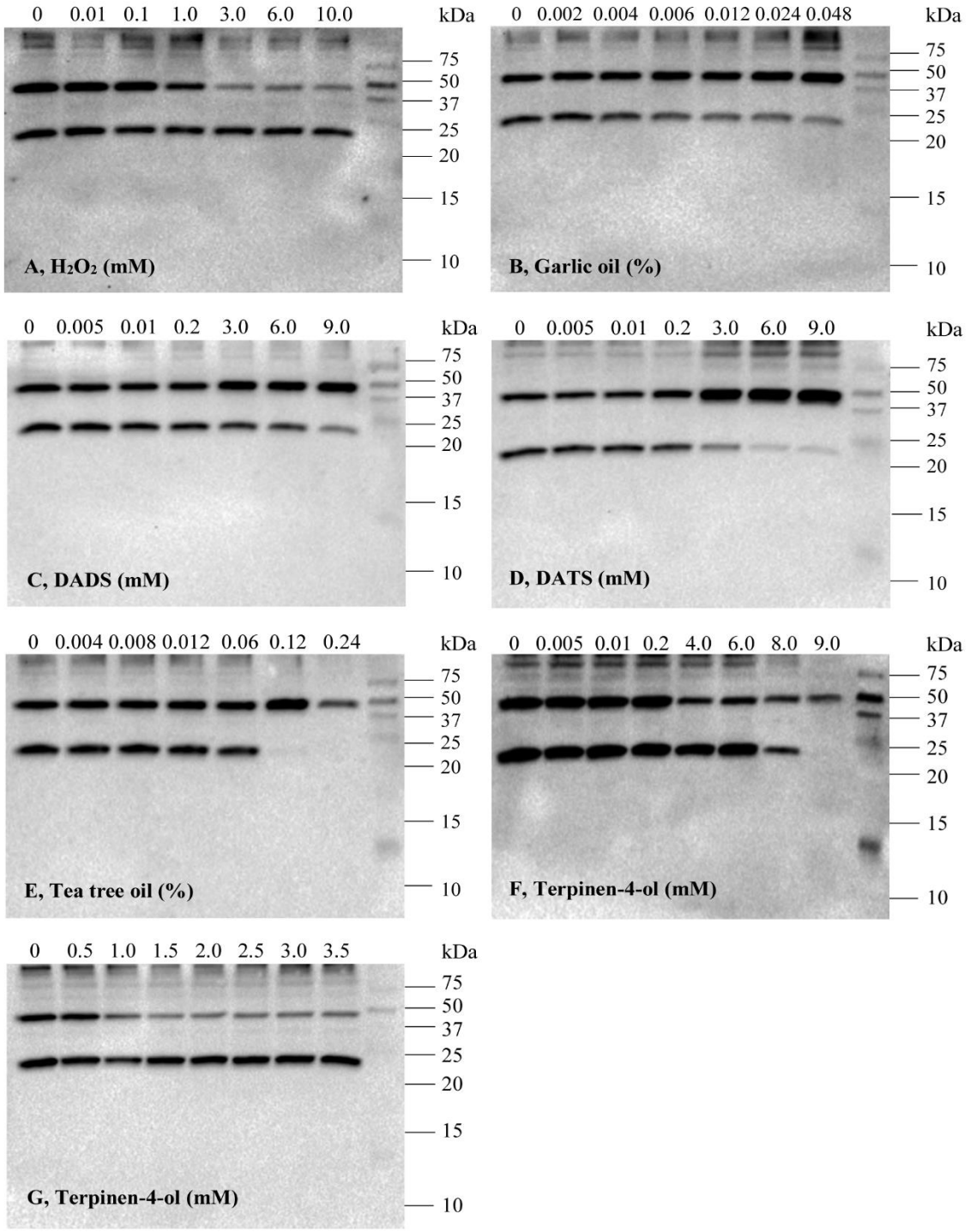

Fig. 4.2. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins. Gels identical to those shown in Fig. 4.1 were run and the proteins thus separated were subjected to immunoblot analysis using the anti-(typical 2-Cys Prx) antibodies (see Section 4.2.5 for antibody details).

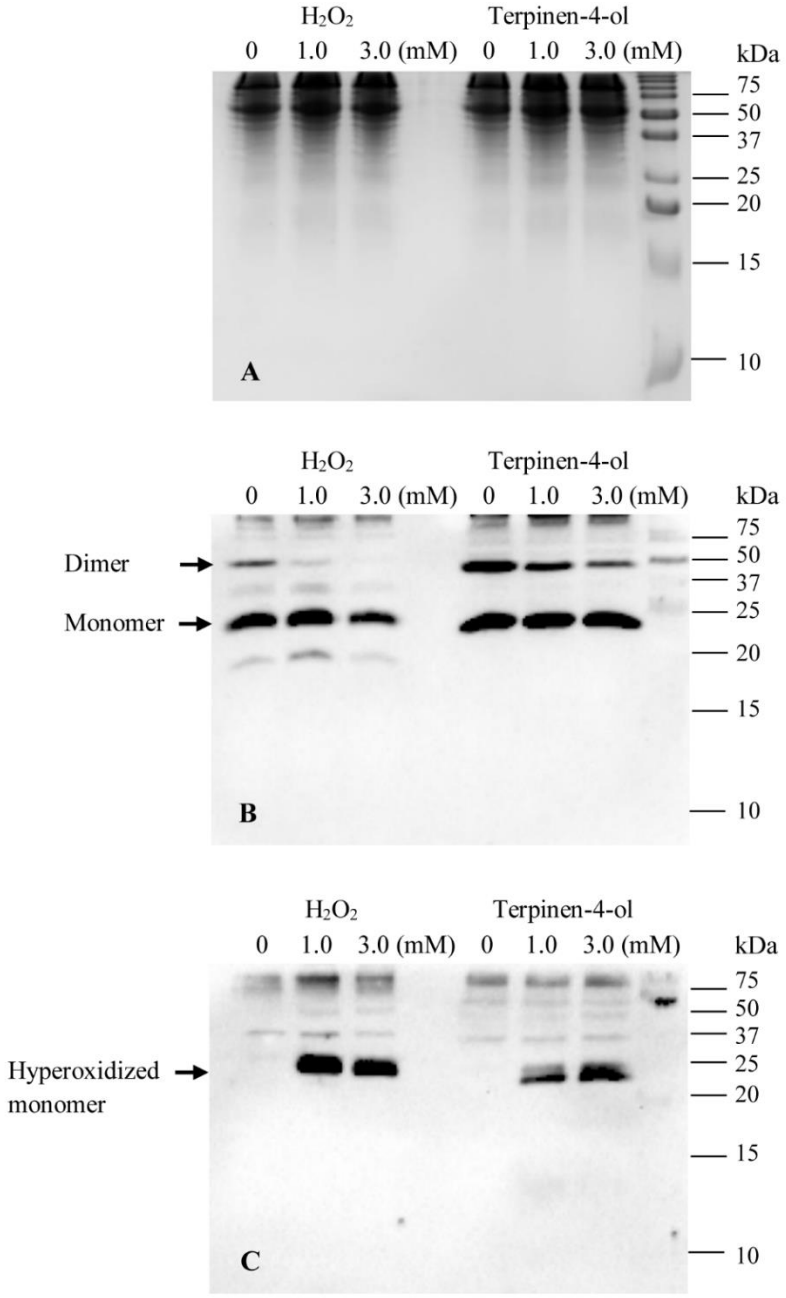

Fig. 4.3. The effects of treating Jurkat T-lymphocytes with H_2O_2 or terpinen-4-ol on the redox/oligomerization state of the typical 2-Cys Prx proteins. The total soluble proteins were extracted from the cells and separated using NR-PAGE. In panel A, the proteins were stained using Coomassie Blue. In panel B, the proteins were subjected to immunoblot analysis using the same antibodies as described in the caption to Fig. 4.2. In panel C, the hyperoxidised form of the typical 2-Cys Prx proteins was detected using commercially available anti-(Prx-SO_3) antibodies (see Section 4.2.5 for antibody details).

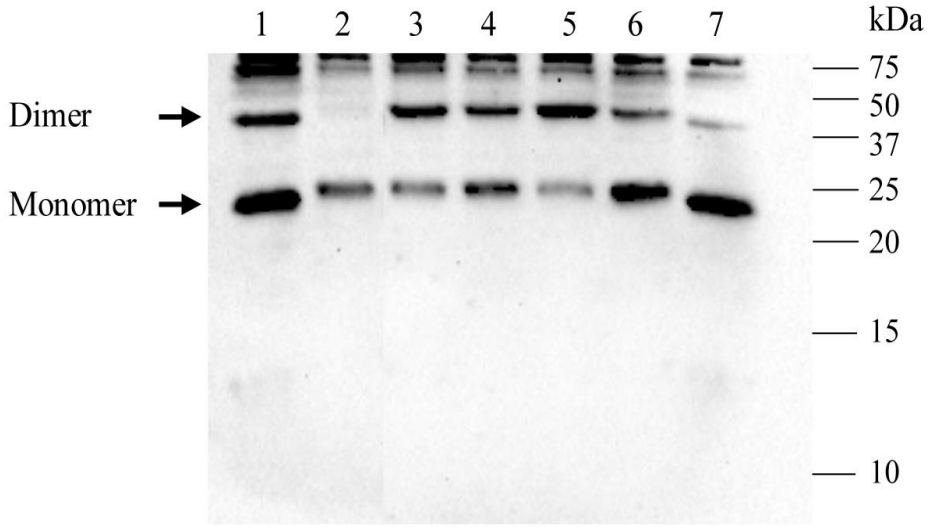

Fig. 4.4. The effects of treating Jurkat T-lymphocytes with H_2O_2, garlic oil, DADS, DATS, tea tree oil or terpinen-4-ol on the redox/oligomerization state of Prx1. The total soluble proteins were extracted from the cells and then separated using NR-PAGE. Prx1 was detected using antibodies raised against the peptide PLVSDPKRTIAQDY which is unique to human Prx1. Lane 1 contains the extract from the control cells. Lanes 2 through 7 contain extracts from cells treated with 3 mM H_2O_2 (Lane 2), 0.048% (v/v) garlic oil (Lane 3), 3 mM DADS (Lane 4), 3 mM DATS (Lane 5), 0.06% (v/v) tea tree oil (Lane 6) and 3 mM terpinen-4-ol (Lane 7).

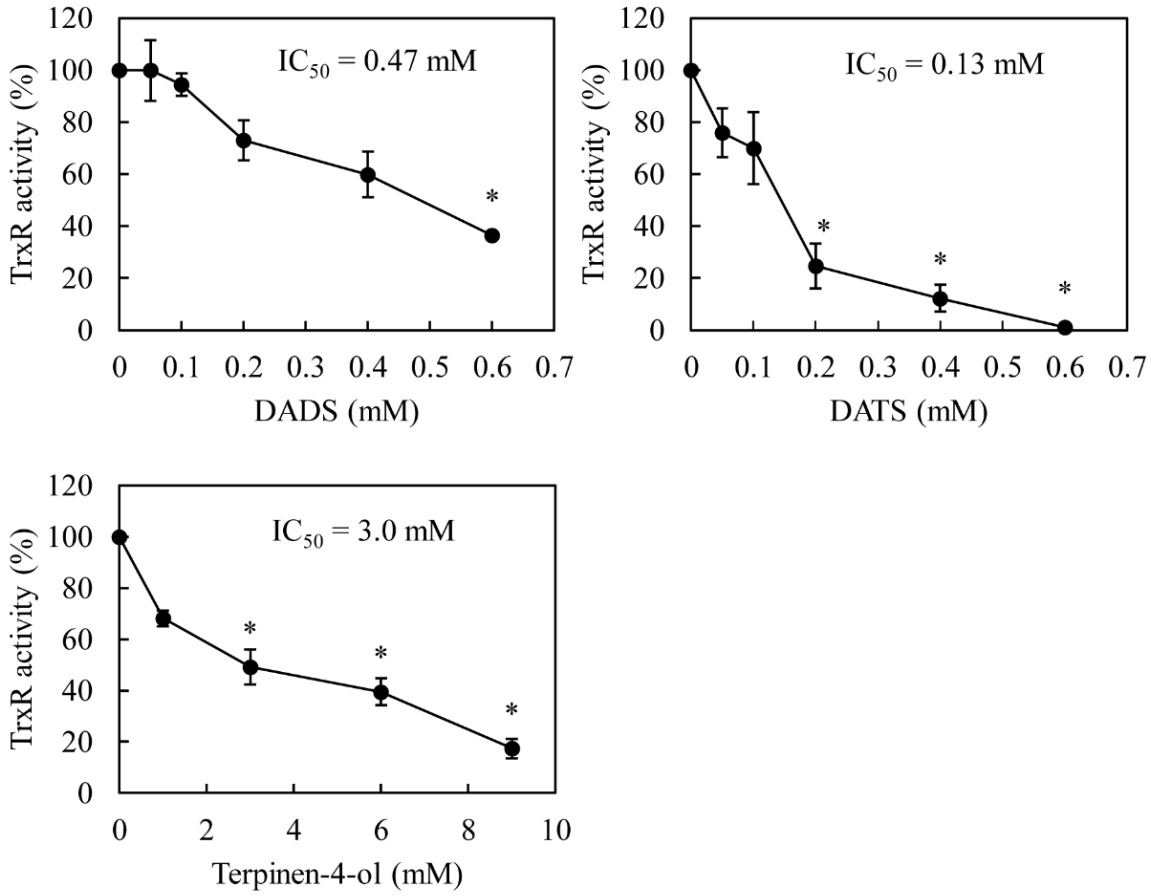

Fig. 4.5. The effects of DADS, DATS and terpinen-4-ol on TrxR enzyme activity in crude extracts from Jurkat T-lymphocytes. The data are expressed as % of the untreated control and each data point is the mean value for 3 replicate wells. The vertical bars represent ± standard error of the mean (n = 3). A star indicates a significant difference from the untreated control ($p < 0.05$).

Chapter 5 - Investigation of the expression of the typical 2-Cys peroxiredoxin genes in *Tetrahymena thermophila*

Abstract

Typical 2-Cys peroxiredoxin (Prx) genes encode enzymes that are involved in reducing H_2O_2 and various organic hydroperoxides to less reactive products. Ciliates, including *Tetrahymena thermophila*, have an abundance of typical 2-Cys Prx genes but none of these genes has been studied. Herein, we have investigated transcript abundances for these typical 2-Cys Prx genes in comparison with a selection of other peroxide detoxification genes in *T. thermophila.* In addition, we have investigated the responses of the typical 2-Cys Prx genes to oxidative stress. Our results showed that the catalase, *Prx1a*, *Prx1m* and phospholipid hydroperoxide glutathione peroxidase 9 (*PHGPx9*) genes were more highly expressed in the *T. thermophila* cells than the *Prx1b*, *Prx1c*, glutathione peroxidase 1 (*GPx1*), *GPx2* and *GPx3* genes. The transcript abundances for the catalase and *PHGPx9* genes were greater than those for the *Prx1a* and *Prx1m* genes and the transcript abundance for the catalase gene was greater than that for the *PHGPx9* gene. These results suggest that *T. thermophila* depends mainly on the enzymes encoded by the catalase, *Prx1a*, *Prx1m* and *PHGPx9* genes for peroxide detoxification. In addition to the above, the typical 2-Cys Prx genes responded differently to one another when the *T. thermophila* cells were subjected to oxidative stress. *Prx1m* gene expression was found to be increased by H_2O_2, tea tree oil and terpinen-4-ol whereas *Prx1a* gene expression was increased by H_2O_2 and garlic oil. The *Prx1a* gene was more responsive to H_2O_2 than the *Prx1m* gene. These results suggest that *T. thermophila* depends mainly on the enzymes encoded by the *Prx1a* and *Prx1m* genes for peroxide detoxification.

5.1 Introduction

Typical 2-Cys peroxiredoxin (Prx) genes encode enzymes that are notable for their high reactivity with and specificity for peroxides (Karplus, 2015). These enzymes are ubiquitous in prokaryotes and eukaryotes and are characterized by the conservation of their peroxidatic Cys and resolving Cys residues (Poole and Nelson, 2016; Wood et al., 2003b). The typical 2-Cys Prx enzymes were first identified as antioxidant enzymes but are now thought also to be involved in H_2O_2-mediated cell signalling (Latimer and Veal, 2016; Rhee, 2016). Mammals have four typical 2-Cys Prx genes (Nicolussi et al., 2017; Wood et al., 2003b) whereas apicomplexans have either one or two and ciliates have three to six (Chapter 3). Thus, ciliates have an abundance of typical 2-Cys Prx genes but they have not been studied beyond the annotation of their sequences in genome sequence databases. Most ciliates, including *Tetrahymena thermophila,* are free-living whereas all apicomplexans, including *Plasmodium falciparum,* are obligate parasites and both groups belong to the same clade, the Alveolata (Dorrell et al., 2013). In apicomplexans, there has been a great deal of interest in the Prx encoding genes from *P. falciparum* and *Toxoplasma gondii* (Gretes et al., 2012). This is firstly because these species are important human parasites and secondly because they appear to rely almost exclusively on Prx proteins to protect themselves against oxidative stress. Specifically, *P. falciparum* has five Prx enzymes, two of which are typical 2-Cys Prx enzymes, but no other peroxide detoxifying enzymes such as catalases or glutathione peroxidases (Jortzik and Becker, 2012). As a result, the *P. falciparum* Prx enzymes and the enzymes responsible for their regeneration, the thioredoxin reductases, have become attractive targets for the development of antimalarial drugs (Jortzik and Becker, 2012; McCarty et al., 2015).

T. thermophila, a useful model for *P. falciparum*, possesses four genes encoding the typical 2-Cys Prx enzymes but lacks any genes encoding any atypical 2-Cys or 1-Cys Prx enzymes (Chapter 3). The four *T. thermophila* typical 2-Cys Prx proteins were predicted to be found in different subcellular locations (Chapter 3). *Prx1a* and *Prx1b* were predicted to be located in the cytosol or nucleus, *Prx1m* was predicted to be located in the mitochondria and *Prx1c* was likely to be secreted. In addition, the *T. thermophila Prx1m* protein was shown to be resistant to hyperoxidation in the presence of increasing concentrations of H_2O_2, cumene hydroperoxide, garlic oil, diallyl trisulphide (DATS, the major constituent of garlic oil), tea tree oil and terpinen-4-ol (the major constituent of tea tree oil). Furthermore, at high concentrations of these oils and compounds, the total amount of the *Prx1m* protein seemed to increase. This led to the supposition that this increase might be a result of an increase in the expression of the *Prx1m* gene. Therefore, the main aim of the present study was to investigate the effects of various oxidative stressors (H_2O_2, garlic oil, DATS, tea tree oil and terpinen-4-ol) on the expression of the typical 2-Cys Prx genes in *T. thermophila* to determine whether oxidative stress did indeed increase the expression of these Prx genes. A secondary aim was to compare the transcript abundances for the typical 2-Cys Prx genes with those for other peroxide detoxifying genes.

5.2 Materials and methods

5.2.1 Cell culture

Untreated *T. thermophila* strain CU428.2 cells were cultured as described in Section 2.2.2 except that the cultures were initiated by inoculating 1 x 10^6 cells (pre-cultured in SSP medium as described in Section 2.2.2) into 50 ml SSP medium in 500 ml Erlenmeyer flasks. Treatment of the *T. thermophila* cells with hydrogen peroxide, garlic oil, diallyl trisulfide (DATS), tea tree oil or terpinen-4-ol was as described in Section 3.2.6.

5.2.2 RNA extraction

T. thermophila cells, cultured as described above (Section 5.2.1), were centrifuged at 2,500 *g* to obtain a cell pellet. The cell pellet was resuspended in 5 ml of SSP medium and the number of cells was determined as described in Section 2.2.2. Total RNA was extracted from 4 x 10^6 cells using a RNeasy® Mini kit (QIAGEN Pty Ltd, Australia) according to the manufacturer's protocol. This included on-column digestion with 27 Kunitz units/column of DNase I (QIAGEN Pty Ltd, Australia) to remove any potentially contaminating DNA from the sample. The concentration of the eluted RNA was determined using a Thermo Scientific NanoDrop® 1000 spectrophotometer. The RNA was stored at -80^{o}C until it was needed.

5.2.3 First strand cDNA synthesis

First strand cDNA synthesis was performed using 1 µg of *T. thermophila* RNA, extracted as described above (Section 5.2.2), together with a SuperScript® IV First-Strand Synthesis kit (Invitrogen Australia Pty Ltd), according to the manufacturer's instructions. The cDNA synthesis reactions were diluted 5-fold in nuclease free water

(Life Technologies Australia Pty Ltd.) and then stored at -20°C until they could be analysed.

5.2.4 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using the primers listed in Table 5.1. The primers were designed using Primer3 version 0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) with the default settings which included an optimal primer length of 20 nucleotides, an optimal melting temperature of 60°C and a GC content of between 20% and 80% (Koressaar and Remm, 2007; Untergasser et al., 2012). The primers were analysed for hairpin, self-dimer and hetero-dimer formation using OligoAnalyzer version 3.1 (https://sg.idtdna.com/calc/analyzer) (Integrated DNA Technologies). Each PCR reaction contained 10 µl Promega 5X Colorless Go*Taq*® Flexi buffer, 5 units of New England Biolabs Thermopol DNA Polymerase, 0.2 µM of each primer, 200 µM dNTPs, 500 µM $MgCl_2$ and 5 µl of the diluted first strand cDNA in a final volume of 50 µl. The PCR cycling conditions were a pre-denaturation step at 95°C for 2 min followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min plus a final extension at 72°C for 5 min. The products of the reactions were analysed using agarose gel electrophoresis as described below (see Section 5.2.5) and products of the expected sizes were purified from the PCR reaction mixtures using the Promega Wizard® SV Gel and PCR Clean-up System. Nucleotide sequencing of the purified PCR products was done in both the forward and the reverse directions using 50 – 80 ng of the purified PCR product together with 0.8 µM of the appropriate forward or reverse primer for each gene. The sequencing was done by the Australian Genome Research Facility. Each primer set yielded only one product as determined by agarose gel electrophoresis (Appendix A.4). The PCR products had the expected sequences (Appendix A.5).

5.2.5 Agarose gel electrophoresis

Agarose gels (1.5% (w/v)) were prepared by adding 450 mg of biotechnology grade agarose (Amresco) to 30 ml of Tris-acetate-EDTA (TAE) buffer containing 40 mM Tris base (Sigma-Aldrich), 0.11% (v/v) acetic acid (BDH Chemicals) and 1 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). The solution was heated using a microwave oven to dissolve the agarose. Once the molten agarose had cooled to approximately 50^{o}C, 3 µl of SYBR® Safe DNA Gel Stain (Invitrogen™) was added and a gel was poured in a Bio-Rad Mini-Sub® Cell GT agarose gel apparatus that had been fitted with a plastic comb to produce sample wells. Once the gel had set it was immersed in TAE buffer and the comb was removed. DNA samples to be analysed were mixed with 6X New England Biolabs Gel Loading Dye, Blue to give a final concentration of 1X. Five µ1 of each sample or the New England Biolabs 2-Log DNA Ladder (0.1-10.0 kb) (loading dye already added) were loaded into the appropriate wells in an agarose gel and the gel was run at 80 volts for 40 min. The DNA within the agarose gel was visualised using a Bio-Rad Gel Doc™ EZ Imager.

5.2.6 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a Platinum® SYBR® Green qPCR SuperMix-UDG kit and a Rotor-Gene® Q thermal cycler (QIAGEN Pty Ltd, Australia). Each reaction contained 12.5 µl Platinum® SYBR® Green qPCR SuperMix-UDG buffer, 1 mM $MgCl_2$, 0.1 µM of each primer (see Table 5.1) and 5 µl of the template cDNA (see Section 5.2.3) in a final volume of 25 µl. The cycling conditions, after a uracil-DNA glycosylase (UDG) incubation step at 50^{o}C for 2 min, were a pre-denaturation step at 95^{o}C for 2 min followed by either 45 or 50 cycles of denaturation at 95^{o}C for 15 s, annealing at 55^{o}C for 30 s and extension at 72^{o}C for 30 s. The fluorescence curves produced for each sample were

used to calculate the threshold cycle (Ct) value. The transcript abundance for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was calculated using the formula 2^{-Ct}. The transcript abundance for the gene of interest was then normalised to the transcript abundance for GAPDH using the formula $2^{-\Delta Ct}$, where ΔCt = Ct (gene of interest) – Ct (housekeeping gene (GAPDH)) (Livak and Schmittgen, 2001). Three or four biological replicates and two technical replicates for each biological replicate were analysed. Primer efficiencies were determined by producing standard curves using a series of 10-fold dilutions of the purified PCR products for each gene (produced as described in Section 5.2.4) as the template. The fluorescence curves for these analyses are shown in Appendix A.6. The primer efficiencies are shown in Table 5.1. For all genes, the primer efficiencies were $\geq$ 0.90 indicating that the primers were suitable for the analysis. Melt curve analysis was performed by heating the qRT-PCR reaction mixture to either 65 or 72^{o}C for 45 s and then raising the temperature by 1°C every 5 s until a maximum temperature of 95°C was reached while at the same time measuring the change in fluorescence. The resulting fluorescence curves are shown in Appendix A.7.

5.2.7 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 $p < 0.05$.

5.3 Results

5.3.1 Expression of selected antioxidant genes in T. thermophila

The transcript abundances for a selection of peroxide detoxifying antioxidant enzymes in untreated *T. thermophila* cells were investigated and the results are shown in Fig. 5.1. The catalase, *Prx1a, Prx1m* and phospholipid hydroperoxide glutathione peroxidase 9 (*PHGPx9*) genes were significantly more highly expressed in the cells than either the *Prx1b, Prx1c,* glutathione peroxidase 1 (*GPx1*), *GPx2* or *GPx3* genes. The *Prx1a* and *Prx1m* genes were expressed at 39% and 30%, respectively, of the level of the catalase gene. Thus, the *Prx1a* and *Prx1m* genes were expressed in the cells at similar levels. In contrast to this, the *Prx1b, Prx1c*, *GPx1, GPx2* and *GPx3* genes were expressed in the cells at very low levels. The *Prx1c* gene was expressed at 10% of the level of the catalase gene whereas the *Prx1b, GPx1, GPx2* and *GPx3* genes were expressed at 1%, 0.03%, 0.01% and 0.02%, respectively, of the level of the catalase gene. The expression of *Prx1c* was greater than that of *Prx1b, GPx1, GPx2* or *GPx3*. Overall these results suggest that among the enzymes studied, *T. thermophila* cells depend predominantly on catalase, *Prx1a, Prx1m, Prx1c* and *PHGPx9* to provide protection against oxidative stress.

5.3.2 Effects of selected pro-oxidants on the expression of the typical 2-Cys Prx genes in T. thermophila

The effects of H_2O_2, garlic oil, DATS (derived from garlic oil), tea tree oil and terpinen-4-ol (derived from tea tree oil) on the expression of the typical 2-Cys Prx genes in *T. thermophila* were investigated and the results are shown in Figs. 5.2 through 5.6. Treatment of the *T. thermophila* cells with 1 mM H_2O_2 increased the expression of *Prx1a* 7.5-fold and *Prx1m* 1.8-fold but it did not affect the expression of

either *Prx1b* or *Prx1c* (Fig. 5.2). The fold increase for the *Prx1a* gene was greater than for the *Prx1m* gene. This indicated that the *Prx1a* gene was more responsive to H_2O_2 treatment than the *Prx1m* gene. In contrast, treatment of the cells with 10 mM H_2O_2 had no effect on the expression of *Prx1a*, *Prx1m* or *Prx1c* but it decreased the expression of *Prx1b* to only 2% of the control value. The data obtained for *Prx1b* should be treated with caution, however, because the *Prx1b* transcript abundance was extremely low (Fig. 5.1). Regardless of the H_2O_2 concentration used, the transcript abundance for GAPDH (the normalisation gene) in the treated cells was the same as in the control cells, indicating that the H_2O_2 concentrations used were not generally toxic to the cells and therefore the relative expression results were reliable. Overall, the *Prx1a* and *Prx1m* genes were the most responsive to treatment with H_2O_2 and the *Prx1a* gene was more responsive than the *Prx1m* gene.

Treatment of the *T. thermophila* cells with 0.006% (v/v) garlic oil increased the expression of *Prx1a* 17-fold but it had no significant effect on the expression of either *Prx1b*, *Prx1m* or *Prx1c* (Fig. 5.3). Thus, the *Prx1a* gene was the most responsive to garlic oil treatment. In contrast, treatment of the cells with 0.048% (v/v) garlic oil had no effect on the expression of *Prx1a*, *Prx1m* or *Prx1c* but it decreased the expression of *Prx1b* to only 10% of the control value. Again, it is likely that the decrease in *Prx1b* gene expression was unreliable because the *Prx1b* transcript abundance was very low (Fig. 5.1). Interestingly, whereas the transcript abundance for GAPDH in the cells treated with 0.006% (v/v) garlic oil apparently decreased (but not significantly), the transcript abundance for GAPDH in the cells treated with 0.048% (v/v) garlic oil decreased significantly. This indicated that garlic oil was cytotoxic at 0.048% (v/v) but not at 0.006% (v/v). Thus, because the transcript abundances for the Prx genes were normalised to the transcript abundance for GAPDH, the data regarding the normalised transcript abundance for the Prx genes at 0.048% (v/v) garlic oil should be treated with

caution. In conclusion, the increase in the expression of *Prx1a* at 0.006% (v/v) garlic oil was the only effect that was biologically relevant. And again we can conclude that *Prx1a* was the most responsive to the oxidative stress treatment.

Treatment of the *T. thermophila* cells with 4 mM DATS apparently increased the expression of *Prx1a* 2.5-fold and *Prx1m* 1.7-fold but these differences were not statistically significant (Fig. 5.4). Similarly, treatment with 4 mM DATS had no significant effect on the expression of either *Prx1b* or *Prx1c*. In contrast, treatment of the cells with 9 mM DATS had no effect on the expression of *Prx1a* but it increased the expression of *Prx1b* 38-fold, *Prx1m* 2.6-fold and *Prx1c* ~3-fold. The data obtained for *Prx1b* and *Prx1c* should be treated with caution, however, because the transcript abundances for *Prx1c* were much higher than the transcript abundances for *Prx1b* (Fig. 5.1). Treatment of the cells with either 4 or 9 mM DATS apparently decreased the transcript abundance for GAPDH but this was not statistically significant, except in one case at 9 mM DATS (Fig. 5.4C). The GAPDH transcript abundance in the presence of 4 mM DATS was somewhat greater than in the presence of 9 mM DATS but this apparent difference was not significant. This indicated that DATS was toxic to the cells at the concentration of 9 mM. As a result, the transcript abundance estimates for the Prx genes in the presence of 9 mM DATS should be treated with caution. Although 4 mM DATS was not toxic to the cells, none of the Prx genes was responsive to DATS. Therefore, it can be concluded that DATS treatment did not induce the expression of the Prx genes. This was surprising given that garlic oil, which contains 34% DATS, increased the expression of *Prx1a* 17-fold.

Treatment of the *T. thermophila* cells with 0.012% (v/v) tea tree oil increased the expression of *Prx1m* ~1.3-fold and this was statistically significant but it had no statistically significant effect on the expression of *Prx1a*, *Prx1b* or *Prx1c* (Fig. 5.5).

The fold difference for the *Prx1m* gene was not large but it was significant. In contrast, treatment of the cells with 0.24% (v/v) tea tree oil had no effect on the expression of any of the Prx genes tested. Interestingly, whereas the transcript abundance for GAPDH in the cells treated with 0.012% (v/v) tea tree oil apparently decreased (but not significantly), the transcript abundance for GAPDH in the cells treated with 0.24% (v/v) tea tree oil decreased significantly. This indicated that tea tree oil was toxic to the cells at 0.24% (v/v) but not at 0.012% (v/v). Therefore, the transcript abundance estimates for the Prx genes in the presence of 0.24% (v/v) tea tree oil should be treated with caution. Thus, the increase in the expression of *Prx1m* at 0.012% (v/v) tea tree oil was the only effect that was potentially biologically relevant. However, it can be argued that because of the small size of the effect, it was not in actual fact of any relevance biologically.

Treatment of the *T. thermophila* cells with 4 mM terpinen-4-ol increased the expression of *Prx1m* 2.8-fold and *Prx1c* 4-fold but it had no significant effect on the expression of either *Prx1a* or *Prx1b* (Fig. 5.6). The fold increase for the *Prx1c* gene was greater than for the *Prx1m* gene. Thus, the *Prx1c* gene was more responsive to terpinen-4-ol than the *Prx1m* gene. However, as stated before, the *Prx1c* gene was expressed at a very low level and therefore any apparent effects on *Prx1c* expression should be treated with caution. Treatment of the cells with 9 mM terpinen-4-ol had no effect on the transcript abundances for *Prx1a* and *Prx1m* or *Prx1c* but it increased the expression of *Prx1b* 794-fold. Again the apparent effect on *Prx1b* should be treated with caution because the *Prx1b* transcript abundance was extremely low (Fig. 5.1). In the cells treated with 4 mM terpinen-4-ol, the transcript abundance for GAPDH somewhat decreased but this effect was not statistically significant. In contrast, in the cells treated with 9 mM terpinen-4-ol, GAPDH transcript abundance did significantly decrease. This indicated that terpinen-4-ol was toxic to the cells at 9 mM but not at 4

mM. Thus, because the transcript abundances for the Prx genes were normalised to the transcript abundance for GAPDH, the data regarding the normalised transcript abundances for the Prx genes at 9 mM terpinen-4-ol should be treated with caution. In conclusion, 4 mM terpinen-4-ol increased the expression of both *Prx1m* and *Prx1c*. The *Prx1c* gene was more responsive than the *Prx1m* gene but the data for the *Prx1c* gene should be treated with caution as explained above. This leaves the response of the *Prx1m* gene as being the only one that is potentially biologically relevant.

In summary, the responses of the *T. thermophila* typical 2-Cys Prx genes to oxidative stress induced by H_2O_2, garlic oil, DATS, tea tree oil or terpinen-4-ol varied from one Prx isoform to another. Expression of the *Prx1m* gene was found to be upregulated in the presence of 1 mM H_2O_2, 0.012% (v/v) tea tree oil and 4 mM terpinen-4-ol whereas expression of the *Prx1a* gene was found to be upregulated in the presence of 1 mM H_2O_2 and 0.006% (v/v) garlic oil. In contrast, none of the Prx genes was responsive to DATS. Thus, the *Prx1a* and *Prx1m* genes were the most responsive to H_2O_2 but the *Prx1a* gene was more responsive than the *Prx1m* gene. The *Prx1a* gene was the most responsive to garlic oil whereas the *Prx1m* gene was the most responsive to tea tree oil and terpinen-4-ol. Thus, the *Prx1a* and *Prx1m* genes were the most responsive to oxidative stress in *T. thermophila* cells but the *Prx1a* gene was in most cases more responsive than the *Prx1m* gene. Overall, these results suggest that *T. thermophila* depends predominantly on the enzymes encoded by the *Prx1a* and *Prx1m* genes for peroxide detoxification.

5.4 Discussion

Non-parasitic members of the Alveolata belonging to the clade of the ciliates have an abundance of typical 2-Cys Prx genes (Chapter 3). *T. thermophila* is one of these non-parasitic alveolates and it is often used as a model organism to study eukaryote cellular and molecular biology (Eisen et al., 2006). *T. thermophila* has four typical 2-Cys Prx genes but lacks any genes encoding either atypical 2-Cys or 1-Cys Prx enzymes (Chapter 3). The transcript abundances for the four typical 2-Cys Prx genes, as well as the transcript abundances for the catalase, *GPx1*, *GPx2*, *GPx3* and *PHGPx9* genes, were examined in *T. thermophila* cells cultured under control conditions. We found that the catalase, *Prx1a*, *Prxlm* and *PHGPx9* genes were more highly expressed in the cells than the *Prx1b*, *Prx1c*, *GPx1*, *GPx2* or *GPx3* genes. Thus, the functionality of the *Prx1b*, *Prx1c*, *GPx1*, *GPx2* and *GPx3* genes could be limited as their transcript abundances were relatively low in the cells but it is possible that these genes are activated under particular stress conditions. Consequently, *T. thermophila* cells depend predominantly on the enzymes encoded by the catalase, *Prx1a*, *Prx1m* and *PHGPx9* genes for reducing peroxides to less reactive molecules and thereby protecting the cells against oxidative stress. Our previous observations showed that the four *T. thermophila* typical 2-Cys Prx proteins are closely related to the human typical 2-Cys Prx proteins in terms of their amino acid sequences (Chapter 3). In human tissues, it was observed that the transcript abundances for Prx1 (encodes a cytosolic/nuclear protein) and Prx2 (encodes a cytosolic protein) were much higher than the transcript abundances for either Prx3 (encodes a mitochondrial protein) or Prx4 (encodes a secreted protein) (Cha et al., 2009; Tavender et al., 2008; Wood et al., 2003b). Based on their predicted amino acid sequences and subcellular locations, the *T. thermophila Prx1a* and *Prx1b* proteins are most closely related to the human Prx1 protein which is predominantly cytosolic, the *T. thermophila Prx1m* protein is most closely related to

the human Prx3 protein which is mitochondrial and the *T. thermophila Prx1c* protein is most closely related to the human Prx4 protein which is secreted (Chapter 3). However, the *T. thermophila Prx1a* and *Prx1b* amino acid sequences share only 58% and 59% identity, respectively, with the human Prx1 protein and the *T. thermophila Prx1m* and *Prx1c* amino acid sequences share only 51% and 49% identity with the human Prx3 and Prx4 proteins, respectively. Thus, the *T. thermophila* and human Prx enzymes are similar in their subcellular locations but not very similar in their amino acid sequences. Nevertheless, we are confident that *T. thermophila Prx1a* and *Prx1b* are predominantly cytosolic proteins, *T. thermophila Prx1m* is a mitochondrial protein and *T. thermophila Prx1c* is a secreted protein. Thus, we can compare these proteins and their genes with Prx1, Prx3 and Prx4, respectively, from humans. In a previous study with human tissues, Cha et al. (2009) found that the Prx1 gene was expressed at a higher level than either the Prx3 or Prx4 gene. The Prx3 and Prx4 genes were expressed at ~30 – 40% and ~6 – 20%, respectively, of the level of the Prx1 gene. Thus, the expression of Prx3 was greater than that of Prx4. In addition to this, in bovine tissues, the Prx1 and Prx3 genes seemed to be relatively highly expressed in all tissue types compared with other Prx genes, including Prx4 (Karplus, 2015; Leyens et al., 2003). The Prx3 gene was expressed at ~20 – 63% of the level of the Prx1 gene and thus, the expression of Prx1 was greater than that of Prx3. Similarly, in our study, we found that the *T. thermophila Prx1a* and *Prx1m* genes were expressed at higher levels than the either *Prx1b* or *Prx1c* gene. The *Prx1m*, *Prx1b* and *Prx1c* genes were expressed at 77%, 2.3% and 26%, respectively, of the level of the *Prx1a* gene. The expression of *Prx1a* was greater than that of *Prx1m* but this was not significant. Overall, like mammalian Prx1 and Prx3 genes, the *Prx1a* and *Prx1m* genes were the most highly expressed in *T. thermophila*.

In order to gain a better understanding of the functions of the four *T. thermophila* Prx genes, we also investigated the effects of various pro-oxidants (H_2O_2, garlic oil, DATS, tea tree oil and terpinen-4-ol) on their expression. Our findings revealed that the expression of the Prx genes seemed to be different from one isoform to another in relation to their responses to oxidative stress induced by the various pro-oxidants. The *Prx1a* and *Prx1m* genes were the most responsive to 1 mM H_2O_2 but the *Prx1a* gene was more responsive than the *Prx1m* gene. In mammals, there are only very few studies on the effects of H_2O_2 treatment on the expression of the typical 2-Cys Prx genes. In one study it was found that treatment of a human corneal epithelial cell line (HCE-2) with 0.2 mM H_2O_2 for 30 min significantly decreased Prx1 gene expression (Lee et al., 2017). Similarly, in another study, exposure of a mouse spermatocyte cell line (GC-2spd) to 0.2 mM H_2O_2 for 6 h was found to significantly down-regulate the expression of the Prx3 and Prx4 genes (Kopalli et al., 2016). In contrast in our study, we found that treatment of *T. thermophila* cells with 1 mM H_2O_2 for 10 min increased the expression of the *Prx1a* and *Prx1m* genes. Thus, in *T. thermophila*, *Prx1a* and *Prx1m* gene expression was increased in response to oxidative stress induced by H_2O_2 treatment whereas in mammalian cells, the expression of these genes was decreased in response to oxidative stress. It is unclear why the response in mammalian cells would be different from that in *T. thermophila* cells. However, one possible explanation could be that the mammalian Prx gene products are involved in activation of apoptosis signalling pathways in response to oxidative stress. For example, it has been proposed that the mammalian Prx1 protein can serve as a signal peroxidase in the transduction of peroxide signals to the apoptosis signalling kinase 1 (ASK1)/p38 mitogen-activated protein kinase (MAPK) pathway through transient formation of a Prx1 – ASK1 mixed disulphide intermediate (Jarvis et al., 2012). Thus, the mammalian Prx proteins appear to be involved in the regulation of apoptosis. Apoptosis is a logical strategy to dispose

of damaged cells in a multicellular organism (e.g. a mammal) but presumably not in a unicellular organism (e.g. *T. thermophila*). Thus, this may explain why mammalian cells respond differently from *T. thermophila* cells in terms of the responses of typical 2-Cys Prx gene expression to oxidative stress.

Comparing the *T. thermophila* Prx genes, we found that the *Prx1a* gene was the most responsive to garlic oil treatment whereas the *Prx1m* gene was the most responsive to tea tree oil or terpinen-4-ol treatment. Whereas treatment with H_2O_2 upregulated the expression of *Prx1a* 7.5-fold in the *T. thermophila* cells, treatment with garlic oil upregulated the expression of this gene 17-fold, indicating that the *Prx1a* gene was very responsive to garlic oil. It is known that the allyl sulphides found in garlic oil can act as redox cycling agents generating H_2O_2 as a byproduct (Munday, 2012; Munday et al., 2003). The greater the number of sulphur atoms in these allyl sulphides, the greater the capacity for H_2O_2 formation (Munday, 2012; Munday et al., 2003). Continuous generation of H_2O_2 by a redox-cycling agent may explain why garlic oil was a more potent inducer of *Prx1a* expression than H_2O_2 (single bolus addition). Recently, Díaz et al. (2016) reported that the herbicide paraquat increases the expression of thioredoxin peroxidase 30-fold in *T. thermophila* cells. Thioredoxin peroxidase is an old name for the Prx proteins. Díaz et al. (2016) randomly chose a Prx gene and there was no information given about its exact identity (e.g. accession number). Thus, we do not know whether the Prx gene chosen by Díaz et al. (2016) belongs to either the *Prx1a*, *Prx1b*, *Prx1m* or *Prx1c* gene families. However, according to our results, we can guess that the previously studied *T. thermophila* Prx gene might be either the *Prx1a* gene or the *Prx1m* gene because we found these to be the most highly expressed. The increase in transcript abundance for the previously studied Prx gene was 30-fold in the presence of 7.7 mM paraquat which was higher than the fold increase for either *Prx1a* or *Prx1m* in cells treated with 1 mM H_2O_2 (1.8- or 7.5-fold),

0.006% (v/v) garlic oil (17-fold), 0.12% (v/v) tea tree oil (1.3-fold) or 4 mM terpinen-4-ol (2.8-fold). Therefore, it can be concluded that the previously studied Prx (*Prx1a* or *Prx1m*) gene was very responsive to paraquat treatment. There are several possible reasons for the differences between our results and those of Díaz et al. For example, in our study, the *T. thermophila* cells were treated with the pro-oxidants for 10 min only whereas in the previous study of Díaz et al., they were treated with paraquat for 24 h. The difference in the exposure times as well as the type of pro-oxidant tested could explain the larger fold increase in the Díaz et al. study compared with our study. Like the allyl sulphides found in garlic oil, paraquat is a redox cycling agent producing superoxide which can be dismutated to produce H_2O_2 (Castello et al., 2007). Thus, paraquat might be a more effective redox cycling agent than the allyl sulphides DADS and DATS. Whereas treatment with DADS or DATS inhibited the proliferation of *T. thermophila* cells with IC_{50} (inhibitory concentration, 50%) values of 0.55 mM and 0.017 mM, respectively (Chapter 2), treatment with paraquat inhibited the proliferation of *T. thermophila* cells with a LC_{50} (lethal concentration, 50%) value of 32.2 mM (Díaz et al., 2016). Thus, it seems that the allyl sulphides are more effective redox cycling agents than paraquat.

Surprisingly, we found that garlic oil increased the expression of *Prx1a* but its major constituent DATS had no effect on the expression of any of the Prx genes, including *Prx1a*. In a previous study, it was found that treatment of Sprague Dawley rats with either 100 or 500 mg/kg body weight diallyl sulphide (DAS) per day for seven days increased the expression of catalase 1.5- or 2.1-fold, respectively, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) 1.2- or 1.9-fold, respectively, and GPx 1.8- or 2.2-fold, respectively, in lung tissue (Ho et al., 2011). DAS is another major constituent of garlic

oil. Thus, it is possible that the increase in *Prx1a* expression in response to garlic oil that we saw in our study was due to DAS or some other constituent of garlic oil rather than DATS.

Treatment with H_2O_2 increased the expression of *Prx1m* 1.8-fold whereas treatment with tea tree oil or terpinen-4-ol increased the expression of this gene 1.3- and 2.8-fold, respectively. Thus, the *Prx1m* gene was more responsive to terpinen-4-ol treatment than it was to treatment with either the parent oil (i.e. tea tree oil) or H_2O_2. However, the *Prx1m* gene was more responsive to H_2O_2 treatment than it was to tea tree oil treatment. This indicates that the *Prx1m* gene was very responsive to terpinen-4-ol treatment. A literature search showed that studies of the effects of either tea tree oil or terpinen-4-ol on the expression of antioxidant genes/enzymes are completely absent. However, we have proposed that terpinen-4-ol may induce the accumulation of H_2O_2 through the inhibition of certain proteins such as farnesyl transferases (Basso et al., 2006; Woo et al., 2010). Farnesyl transferases catalyse the farnesylation of various proteins including protein-tyrosine phosphatases (Basso et al., 2006). Protein farnesylation promotes membrane association and contributes to protein-protein interactions. In mammalian cells, it was found that Prx1 association with lipid rafts was inactivated by phosphorylation and the phosphorylation/inactivation of Prx1 could be reversed by protein-tyrosine phosphatases (Woo et al., 2010). Thus, it was proposed that inactivation of Prx1 allows localised accumulation of H_2O_2 which in turn allows inactivation of the protein-tyrosine phosphatases (Woo et al., 2010). Terpinen-4-ol is a monoterpene and farnesyl transferases are inhibited by monoterpenes (Mo and Elson, 2004). Thus, we suggest that a similar mechanism might be acting in *T. thermophila* cells.

Previous studies have investigated the expression of various antioxidant genes in *T. thermophila* cells exposed to oxidative stressors such as silver nanoparticles and the triazole fungicides myclobutanil and cyproconazole but these studies did not include any genes from the Prx family (Huang et al., 2016; Juganson et al., 2017). For example, treatment of two different strains of *T. thermophila* (CU427 and CU428) with silver nanoparticles for either 2 or 24 h was found to have different effects on the expression profiles of various antioxidant genes in the two different strains. In the CU427 strain, treatment with silver nanoparticles increased the expression of a catalase gene 36-fold following a 2 h exposure and the expression of a Mn-SOD gene 2-fold, a Cu/Zn-SOD gene 3-fold and a *GPx2* gene 4-fold (*PHGPx2*) following a 24 h exposure (Juganson et al., 2017). In contrast, in the CU428 strain, treatment with silver nanoparticles increased the expression of a Mn-SOD gene 2-fold, a Cu/Zn-SOD gene ~14-fold and a *GPx2* gene ~6- following a 2 h exposure but had no effect on catalase gene expression (Juganson et al., 2017). The authors suggested that the metabolic rate in the CU428 strain is higher than that in the CU427 strain and therefore the production of ROS would be greater in the strain with the higher metabolic rate and this could explain the different responses of the antioxidant genes between the two different strains. In another study, exposure of *T. thermophila* cells to myclobutanil and cyproconazole for 4 h was found to decrease the expression of Mn-SOD but increase the expression of catalase 3.3-fold for myclobutanil and 5-fold for cyproconazole (Huang et al., 2016). The authors of this study concluded that they were observing an adaptive response to oxidative stress. As can be seen from the above, ours is the first study to investigate the effects of oxidative stressors on Prx gene expression in *T. thermophila*. Most importantly, we have shown that the *Prxla* and *Prx1m* genes are the most responsive to oxidative stress and therefore, probably, the most important in protecting *T. thermophila* cells against oxidative stress. Furthermore, since the *Prx1a* and *Prx1m*

genes are expressed at nearly the same level as the catalase gene and at much higher levels that most of the GPx genes, these Prx genes must be very important in protecting *T. thermophila* cells against oxidative stress.

Previously we showed that at higher concentrations, garlic oil and its major constituents the allyl sulphides DADS and DATS as well as tea tree oil and its major constituent terpinen-4-ol, appeared to increase the total amount of the *Prx1m* protein in *T. thermophila* cells (Chapter 3.) Thus, we hypothesised that this increase might be due to increased expression of the *Prx1m* gene. However, the results shown here in this chapter do not support this hypothesis. Instead, in the present study, we found that at higher concentrations, which were the same as those used in Chapter 3, these oils/compounds were toxic to the *T. thermophila* cells as indicated by the significant decrease in the transcript abundance for the GAPDH (housekeeping) gene. Thus, it is unlikely that the apparent increase in abundance of the *Prx1m* protein in the *T. thermophila* cells exposed to oxidative stress (Chapter 3) is due to increased *Prx1m* gene expression. An alternative explanation for the increased abundance of the *Prx1m* protein could be that it is resistant to degradation in the presence of toxic concentrations of the plant oils/compounds tested. In a previous study it was shown that exposure of a human breast cancer cell line (MDA-MB-231) to 0.5 mM H_2O_2 for 30 min followed by recovery in fresh medium for various lengths of time (0 to 24 h), resulted in an oxidised Prx4 protein that was relatively resistant to degradation compared with hyperoxidised Prx2 and Prx3 proteins that were readily degraded (Song et al., 2016). The hyperoxidised Prx3 protein was degraded slower than the hyperoxidised Prx2 protein in these cells (Song et al., 2016). Additionally, Song et al. (2016) found that a lysine^{191} residue in the C-terminus of the hyperoxidised Prx2 protein was ubiquitinated and the ubiquitinated Prx2 protein was readily degraded via both the ubiquitin-proteasome system and the autophagy process. This lysine residue

is conserved in the human Prx1, Prx2 and Prx3 proteins whereas it is replaced by a leucine residue in the human Prx4 protein (Song et al., 2016). Thus, this lysine residue may be critical for determining the stability of the protein. In the *T. thermophila Prx1m* protein, the lysine residue found in the human Prx1, Prx2 and Prx3 proteins is replaced by a serine residue. Thus, this may explain why the *T. thermophila Prx1m* protein appears to be resistant to degradation. The amino acids leucine and serine might not participate in the degradation of the Prx proteins whereas the amino acid lysine might participate in the degradation of these proteins. These results support our conclusion that the *T. thermophila Prx1m* protein could be more stable in response to oxidative stress because it is insensitive to hyperoxidation as well as containing the serine residue rather than the lysine residue in the C-terminus of the protein. If this is the case then it suggests that the *Prx1m* protein may retain its activity when many other proteins have been inactivated and therefore the *Prx1m* protein may serve a protective function. For example, the *Prx1m* protein may gain chaperone activity. Chaperone activity has been found in a mitochondrial typical 2-Cys Prx protein from *Leishmania infantum* (a protozoan parasite) (Teixeira et al., 2015) as well as cytosolic typical 2-Cys Prx proteins from various organisms including humans, yeasts, bacteria and plants (Toledano and Huang, 2016). We propose that the *T. thermophila Prx1m* protein may also gain chaperone activity.

In conclusion, we found that the catalase, *Prx1a, Prx1m* and *PHGPx9* genes were more highly expressed in *T. thermophila* than the *Prx1b, Prx1c, GPx1, GPx2* and *GPx3* genes. Therefore, we conclude that *T. thermophila* depends mainly on the enzymes encoded by the catalase, *Prx1a, Prx1m* and *PHGPx9* genes for peroxide detoxification. Furthermore, we found that expression of the *Prx1a* and *Prx1m* genes was increased by H_2O_2 treatment but that the *Prx1a* gene was more responsive than the *Prx1m* gene to this treatment. The *Prx1a* gene expression was upregulated by garlic oil whereas the

Prx1m gene expression was upregulated by tea tree oil and terpinen-4-ol. Thus, we conclude that the *Prx1a* gene was in most cases more responsive than the *Prx1m* gene. In addition to this, we conclude that the increased abundance of the *Prx1m* protein in the *T. thermophila* cells exposed to oxidative stress (Chapter 3) is not due to increased *Prx1m* gene expression. Alternatively, we suggest that the increased abundance of the *Prx1m* protein is due to increased *Prx1m* protein stability.

Table 5.1. Oligonucleotide primers used for PCR in this study.

Target gene	Forward primer sequence (5´ → 3´)	Reverse primer sequence (5´ → 3´)	Amplicon size (base pairs)	Amplification efficiency
GAPDH	GTCTTGCTCCCGTTGCTAAG	GGTTGAGGCAGCTCTACCAG	152	0.94
Catalase	GAACGTGATCCTCGTGGTTT	TGTTAGCGCACTTGAGGTTG	166	0.94
Prx1a	ACCACTGCTTGGGATGGTAG	CTTTTCTGGGCTTCTTGCAC	226	0.93
Prx1b	GGAGCAGCGTATAGAGGAACA	GGACAAACTTCTCCGTGCTC	152	0.91
Prx1m	CAGAAGGTGGTTTGGGAGAA	TTCTACCGACTGGGAGATCG	177	0.92
Prx1c	CCCACTGAATTGGTTGCTTT	ATCTGCAAGGAGGGGAATCT	165	0.99
GPx1	ACAGGCCAAACCTCAAGAGA	TTCGGTTTCTCAAGCTACTGC	210	0.90
GPx2	GGAGCTGGCTTTACAGCATT	CTTTGGTTGCCACCTCCTTA	170	0.91
GPx3	CCATTTGATGAACCTGCAAT	AAACTGGTTTTCCCTCAGCA	199	0.98
PHGPx9	TCATGGGCTAAGAACCTTGG	CCATCGGGTCCAATTAAAAA	220	0.90

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Prx, peroxiredoxin; GPx, glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase

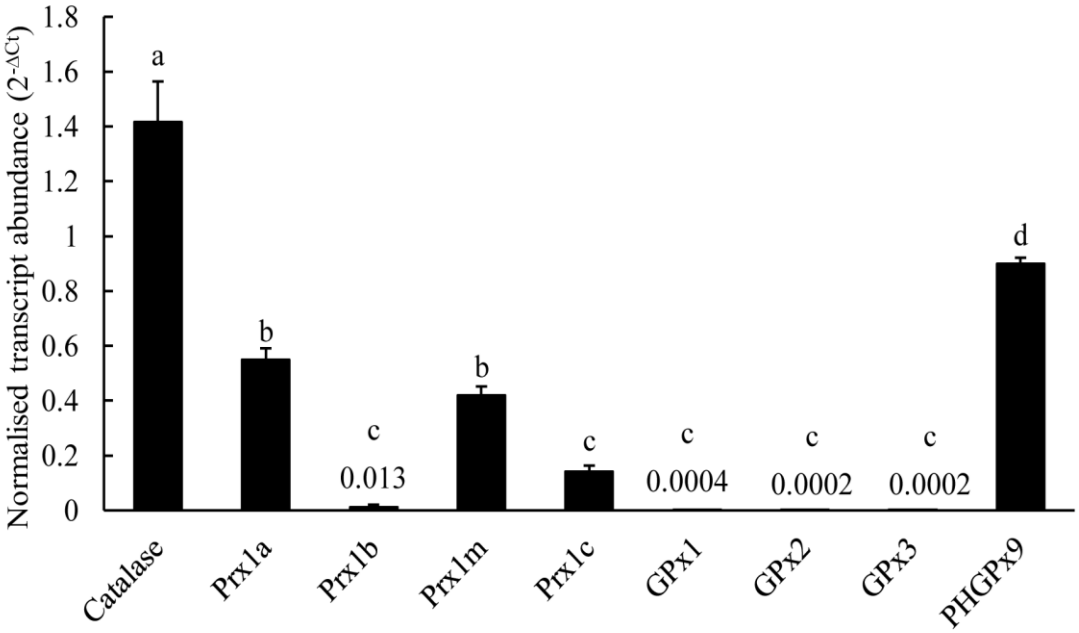

Fig. 5.1. Transcript abundances for *T. thermophila* catalase, *Prx1a*, *Prx1b*, *Prx1m*, *Prx1c*, *GPx1*, *GPx2*, *GPx3* and *PHGPx9* genes normalized to the transcript abundance for GAPDH (housekeeping gene). RNA was extracted from three replicate flasks. The normalized transcript abundances were calculated using the formula $2^{-\Delta Ct}$ where Ct is the threshold cycle and where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the gene of interest. The data are the mean ± the standard error of the mean (n = 3). Significant differences among the transcript abundances for *T. thermophila* genes are indicated by different letters ($p < 0.05$). This experiment was performed twice and similar results were obtained in the two experiments. The results of one experiment are shown here.

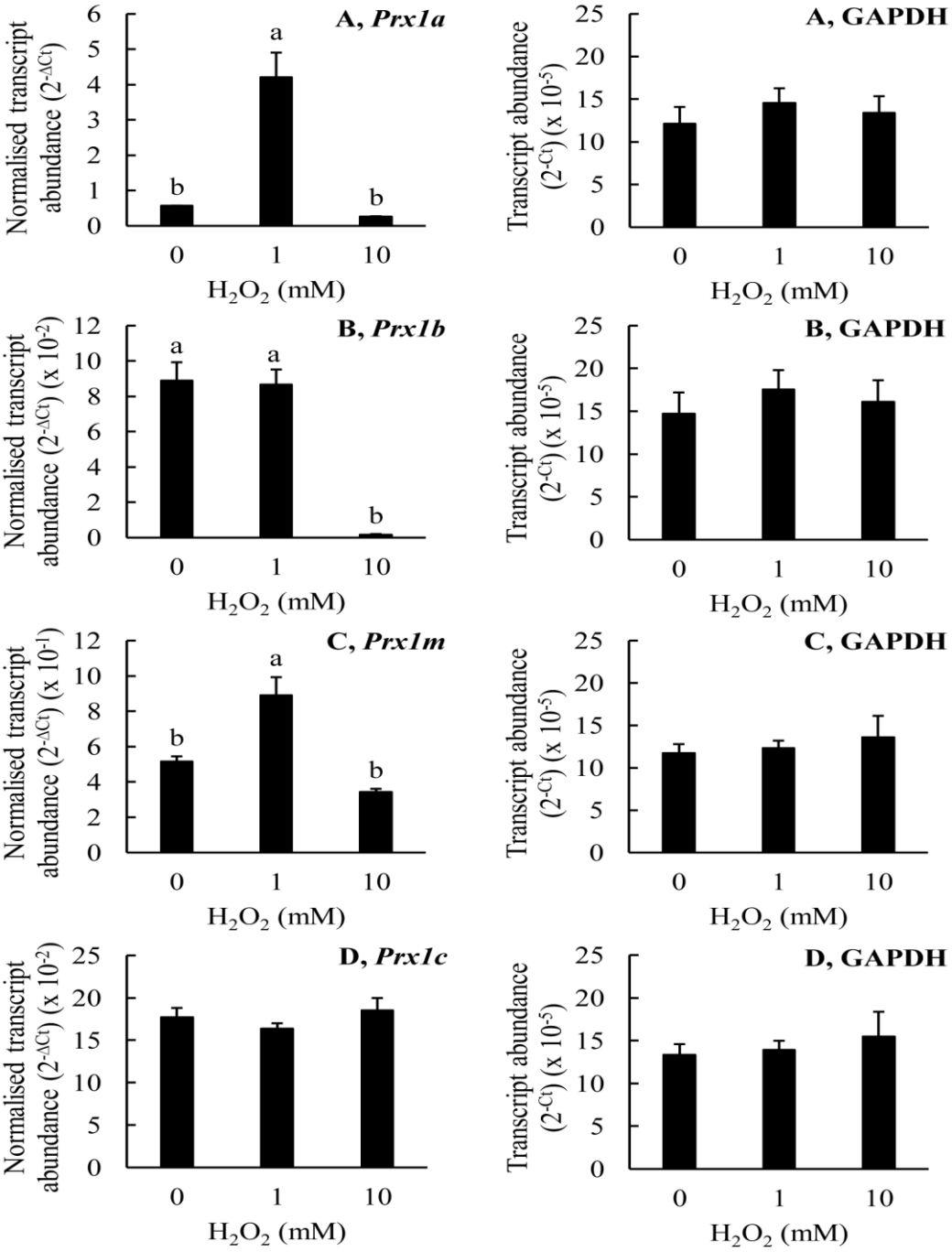

Fig. 5.2. Transcript abundances for *Prx1a*, *Prx1b*, *Prx1m*, *Prx1c* and GAPDH (housekeeping gene) following exposure of *T. thermophila* cells to H_2O_2. RNA was extracted from three replicate wells. The transcript abundances for GAPDH were calculated using the formula 2^{-Ct} where Ct is the threshold cycle, whereas the normalized transcript abundances for the Prx genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the Prx gene. The data are the mean ± the standard error of the mean (n = 3). Within each individual figure, significant differences for H_2O_2 treatment on the transcript abundances for *T. thermophila* Prx or GAPDH genes are indicated by different letters ($p < 0.05$). This experiment was performed three times and similar results were obtained in the three experiments. The results of a representative experiment are shown here.

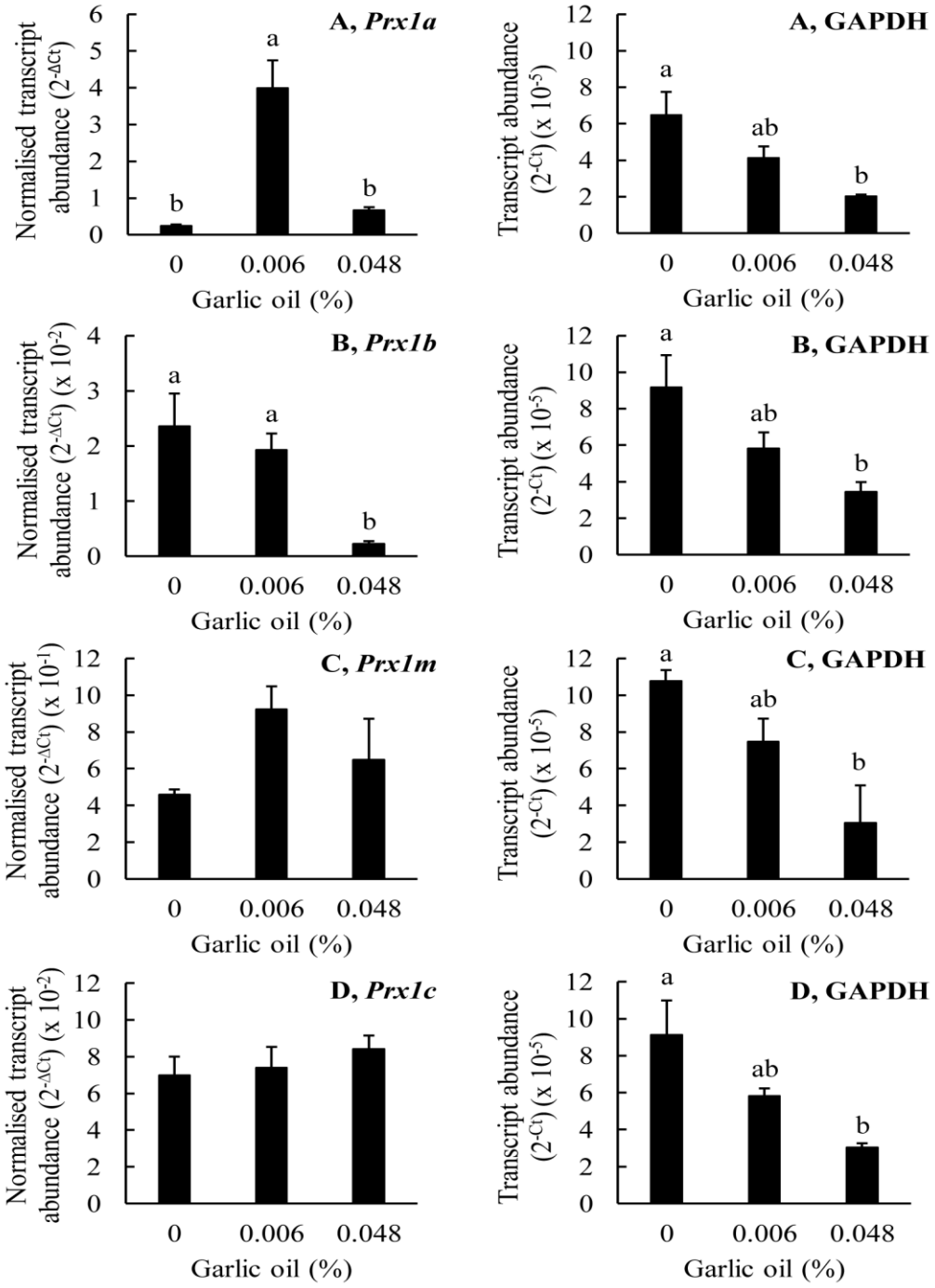

Fig. 5.3. Transcript abundances for *Prx1a*, *Prx1b*, *Prx1m*, *Prx1c* and GAPDH (housekeeping gene) following exposure of *T. thermophila* cells to garlic oil. RNA was extracted from three replicate wells. The transcript abundances for GAPDH were calculated using the formula 2^{-Ct} where Ct is the threshold cycle, whereas the normalized transcript abundances for the Prx genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the Prx gene. The data are the mean ± the standard error of the mean (n = 3). Within each individual figure, significant differences for garlic oil treatment on the transcript abundances for *T. thermophila* Prx or GAPDH genes are indicated by different letters ($p < 0.05$). This experiment was performed three times and similar results were obtained in the three experiments. The results of a representative experiment are shown here.

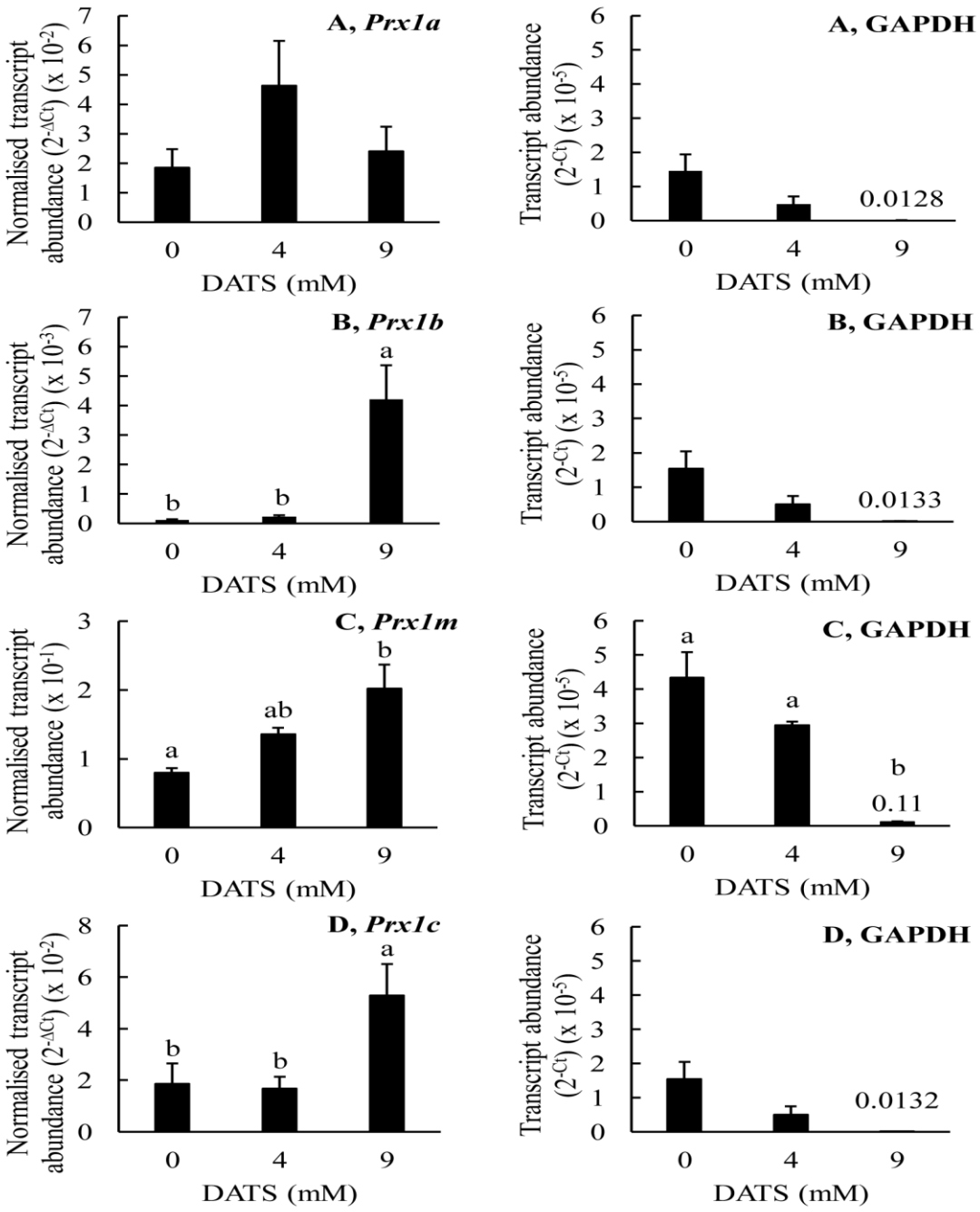

Fig. 5.4. Transcript abundances for *Prx1a*, *Prx1b*, *Prx1m*, *Prx1c* and GAPDH (housekeeping gene) following exposure of *T. thermophila* cells to DATS. RNA was extracted from four replicate wells. The transcript abundances for GAPDH were calculated using the formula 2^{-Ct} where Ct is the threshold cycle, whereas the normalized transcript abundances for the Prx genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the Prx gene. The data are the mean ± the standard error of the mean (n = 4). Within each individual figure, significant differences for DATS treatment on the transcript abundances for *T. thermophila* Prx or GAPDH genes are indicated by different letters ($p < 0.05$). This experiment was performed three times and similar results were obtained in the three experiments. The results of a representative experiment are shown here.

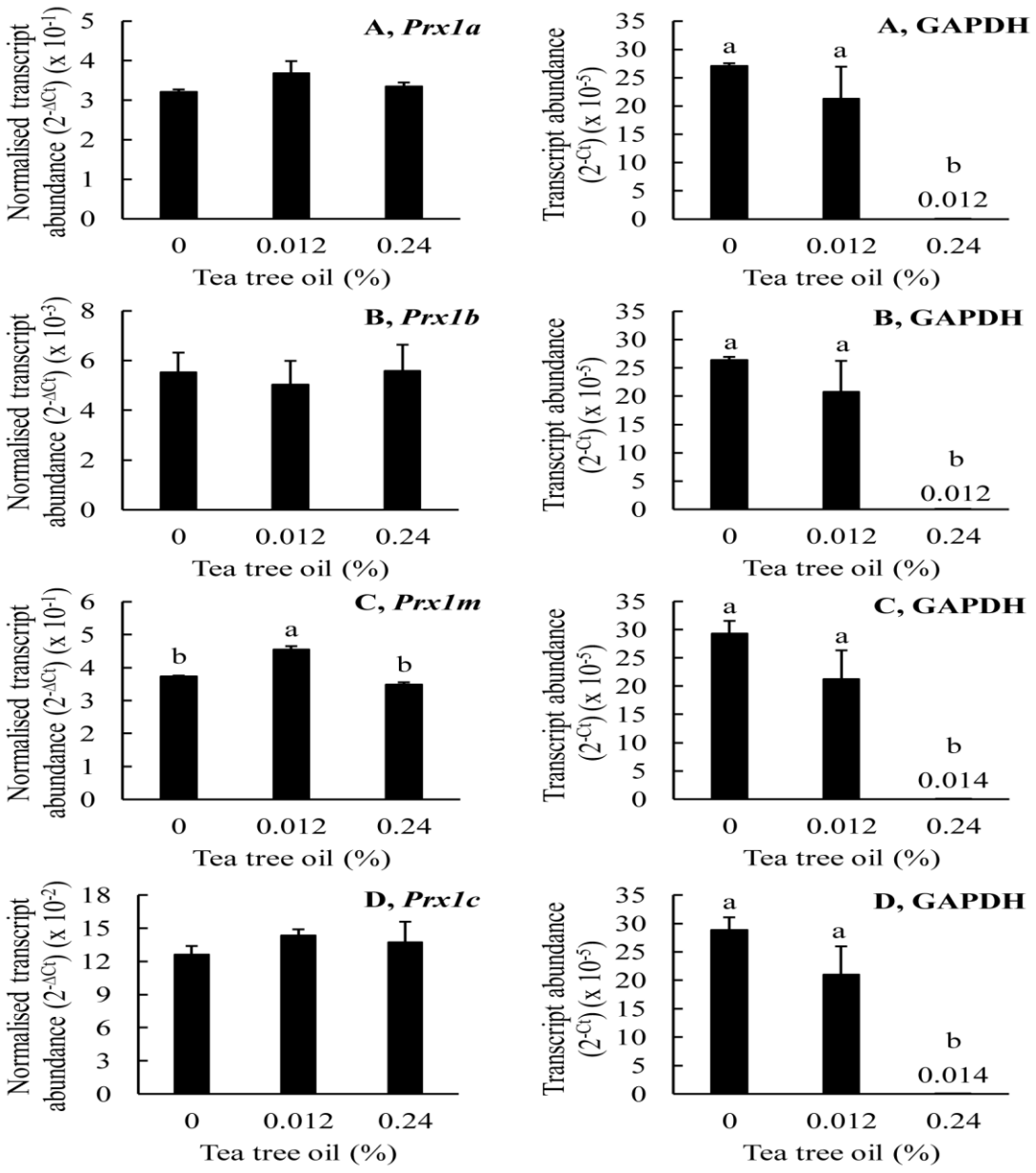

Fig. 5.5. Transcript abundances for *Prx1a*, *Prx1b*, *Prx1m*, *Prx1c* and GAPDH (housekeeping gene) following exposure of *T. thermophila* cells to tea tree oil. RNA was extracted from three replicate wells. The transcript abundances for GAPDH were calculated using the formula 2^{-Ct} where Ct is the threshold cycle, whereas the normalized transcript abundances for the Prx genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the Prx gene. The data are the mean ± the standard error of the mean (n = 3). Within each individual figure, significant differences for tea tree oil treatment on the transcript abundances for *T. thermophila* Prx or GAPDH genes are indicated by different letters ($p < 0.05$). This experiment was performed four times and similar results were obtained in the four experiments. The results of a representative experiment are shown here.

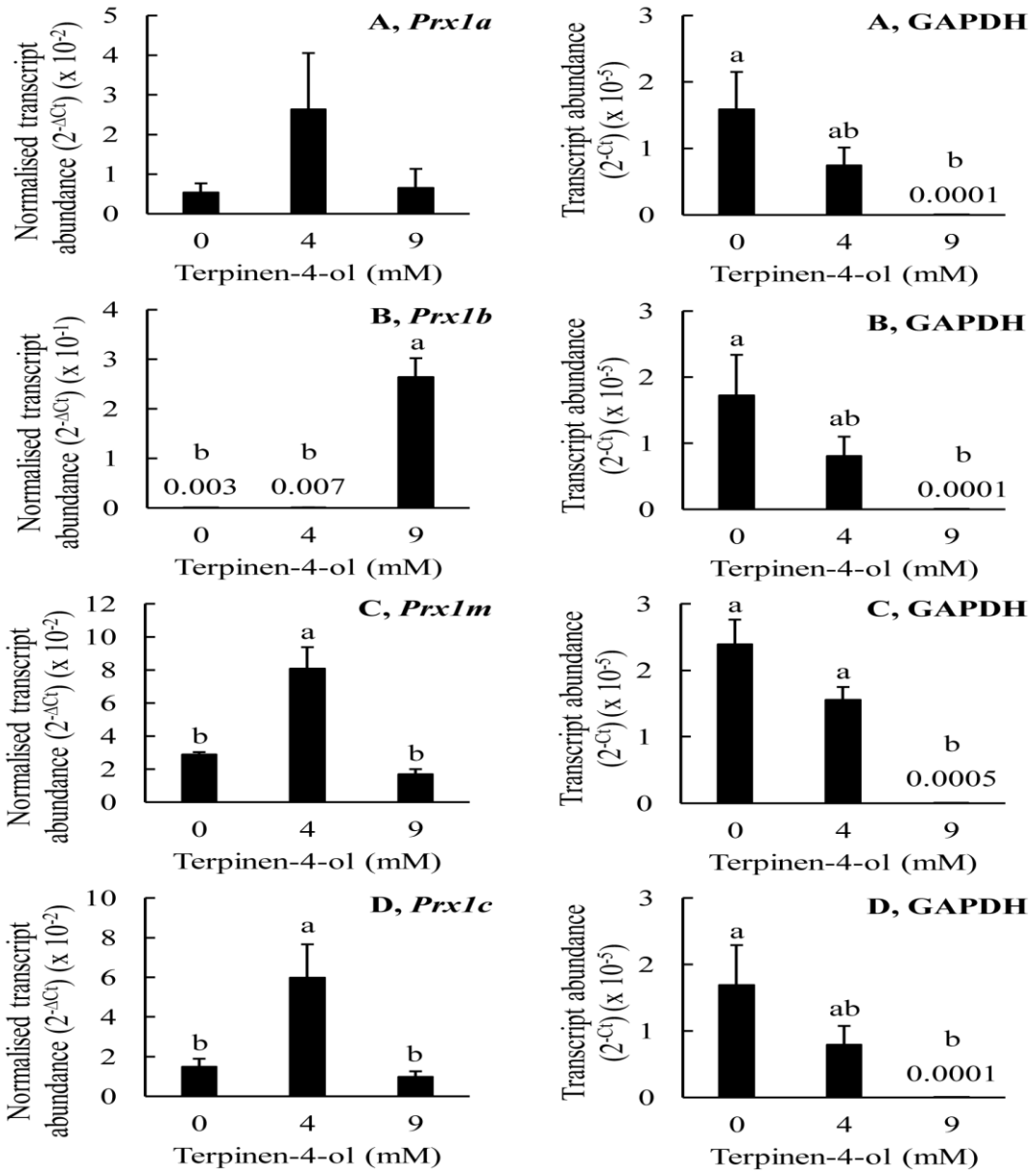

Fig. 5.6. Transcript abundances for *Prx1a, Prx1b, Prx1m1, Prx1c* and GAPDH (housekeeping gene) following exposure of *T. thermophila* cells to terpinen-4-ol. RNA was extracted from three replicate wells. The transcript abundances for GAPDH were calculated using the formula 2^{-Ct} where Ct is the threshold cycle, whereas the normalized transcript abundances for the Prx genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene subtracted from the Ct value for the Prx gene. The data are the mean ± the standard error of the mean (n = 3). Within each individual figure, significant differences for terpinen-4-ol treatment on the transcript abundances for *T. thermophila* Prx or GAPDH genes are indicated by different letters ($p < 0.05$). This experiment was performed three times and similar results were obtained in the three experiments. The results of a representative experiment are shown here.

Chapter 6 - 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*

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 treatments.

6.1 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 (Fairhurst and Dondorp, 2016). 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 (Foote, 2004; Prudêncio and Mota, 2013). 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 (Cho et al., 2010). 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 (Wood et al., 2003b). Human erythrocytes contain Prx1, Prx2 (Torin or Calpromotin) and Prx6 proteins (Cho et al., 2010). 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 (Wood et al., 2003b). 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 (Wood et al., 2003b). 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 (Cho et al., 2010; Moore et al., 1991). This compares with 0.31 µg/mg for the Prx1 protein and 0.14 µg/mg for the Prx6 protein (Cho et al., 2010). 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}) (Peskin et al., 2007). 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 (Ikeda et al., 2011; Wood et al., 2003a). In contrast to other human typical 2-Cys Prx proteins, the human erythrocyte Prx2 protein is relatively insensitive to hyperoxidation (Low et al., 2007). 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.

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 (Becker et al., 2004; Francis et al., 1997). 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 (Jortzik and Becker, 2012). 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 (Gretes et al., 2012). 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 (Koncarevic et al., 2009). For example, it has been estimated that approximately half the peroxide detoxification activity of *P. falciparum* is catalyzed by the erythrocyte Prx2 protein (Koncarevic et al., 2009). 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*.

6.2 Materials and methods

6.2.1 Plasmodium falciparum culture

6.2.1.1 Preparation of the culture medium

All reagents were purchased from Life Technologies, unless otherwise stated. The *Plasmodium* culture medium 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.

6.2.1.2 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). The washed erythrocytes were harvested as described above and were then resuspended in an equal volume of *Plasmodium* culture medium (Section 6.2.1.1).

6.2.1.3 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 (Section 6.2.1.2) at 2% haematocrit in the *Plasmodium* culture medium. The parasites were cultured at 37^{o}C using the candle-jar technique as previously described (Trager and Jensen, 1976). 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.

6.2.1.4 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 (Jensen, 2002). 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.

6.2.1.5 Stage synchronisation of parasites

P. falciparum cultures with a large number of young ring-stage parasites were selected for synchronisation (Radfar et al., 2009). Synchronisation was performed using 5% (w/v) sorbitol (Sigma-Aldrich) as previously described (Jensen, 2002). 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°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 (Section 6.2.1.3) or were used in parasitemia experiments (see below). Sorbitol synchronisation was performed twice a week to maintain synchronicity.

6.2.2 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 in Section 6.2.1.2 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°C using the candle-jar technique (Trager and Jensen, 1976) 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 (see Section 6.2.6) or

protein experiments (see Section 6.2.3). There were three replicate flasks per concentration used in the parasitemia experiments and one flask per concentration used in the protein experiments.

6.2.3 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 NEM to alkylate the sulfhydryl group (-SH) of C_P in the reduced monomer state of the typical 2-Cys Prx proteins (Cox et al., 2010b). The complete alkylation buffer contained 40 mM 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^{o}C and the supernatant was stored at -80^{o}C 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.

6.2.4 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 (Cox et al., 2009). These different sizes can be resolved using NR-PAGE. The reduced and oxidised forms of the Prx2 protein were separated utilising NR-PAGE according to the protocol described in Section 3.2.8 except that for the experiments described in this chapter, the NR-PAGE gels contained 10% (w/v) acrylamide:N,N′-methylenebisacrylamide (37.5:1).

6.2.5 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 (Section 6.2.4) were detected using immunoblotting according to the protocol described in Section 3.2.9 but with slight modifications. The modification was that 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 (Sutton et al., 2010).

6.2.6 Parasitemia experiments

Ring-stage parasites synchronised as described above (Section 6.2.1.5) 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) (Radfar et al., 2009) at 37^{o}C using the candle-jar technique (Trager and Jensen, 1976). 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 (Section 6.2.1.4). The experiments were performed twice and similar results were obtained in the replicate experiments. The results of a representative experiment are shown here.

6.2.7 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 (Section 6.2.1.5) 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 (Trager and Jensen, 1976). 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 (see Section 6.2.3). Following this

incubation, cell extracts were prepared as described in Section 6.2.3. 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 in Section 6.2.4 and detected using immunoblotting as described above in Section 6.2.5. All experiments were performed twice and similar results were obtained in the replicate experiments. The results of a representative experiment are shown here.

6.2.8 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 $p < 0.05$.

6.3 Results

6.3.1 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 Fig. 6.1. 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 (Fig. 6.1A-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 (Fig. 6.1C-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 (Fig. 6.1A-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 (Figs. 6.1A-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.

6.3.2 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 (Fig. 6.2). 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 (Fig. 6.2). 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* (Fig. 6.2). 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. 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*.

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 (Fig. 6.3). 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 2^{nd} generation ring stage) after the erythrocytes had been inoculated with synchronised ring stage parasites (Fig. 6.3). 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* (Fig. 6.3). 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*

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 in Section 6.3.1, garlic and tea tree oil promoted oxidation/inactivation of the Prx2 protein in pre-treated erythrocytes (Figs. 6.1C-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.

6.3.3 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 Fig. 6.4. 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 (Fig. 6.4A-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 (Fig. 6.4C-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 (Fig. 6.4A-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 (Figs. 6.4A-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.

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 (Figs. 6.4E-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*.

6.4 Discussion

The human malaria parasite, *P. falciparum*, possesses multiple Prx enzymes but lacks any other antioxidant enzymes including catalase and GPx enzymes (Jortzik and Becker, 2012). 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 (Jortzik and Becker, 2012). 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 (Koncarevic et al., 2009). It is known that the human Prx2 protein undergoes oxidised dimer formation in erythrocytes exposed to high concentrations of H_2O_2 (Low et al., 2007). 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 (Wood et al., 2003a). 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 2^{nd} 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 (Gilson and Crabb, 2009). 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 (Brizuela et al., 2014; Haraldsen et al., 2009). 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 (Brizuela et al., 2014). 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 (Kakhniashvili et al., 2004). This chaperonin protein belongs to a class of molecular chaperones that prevent aggregation of other proteins under normal and stress conditions (Motojima, 2015). 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 (Kakhniashvili et al., 2004), 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 (Figs. 6.4A-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 (Cho et al., 2010), 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 (Kakhniashvili et al., 2004). It is known that out of the 182 unique erythrocyte proteins, twelve have molecular weights of 28.4 to 30 kDa (Kakhniashvili et al., 2004). Amongst these proteins, the carbonic anhydrase I protein is the first most abundant protein after hemoglobin in human erythrocytes (D'amici et al., 2012). 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 (Coppi et al., 2006; Govindan et al., 2016), our results suggest that garlic oil as well as tea tree oil could be used as alternative antimalarial treatments.

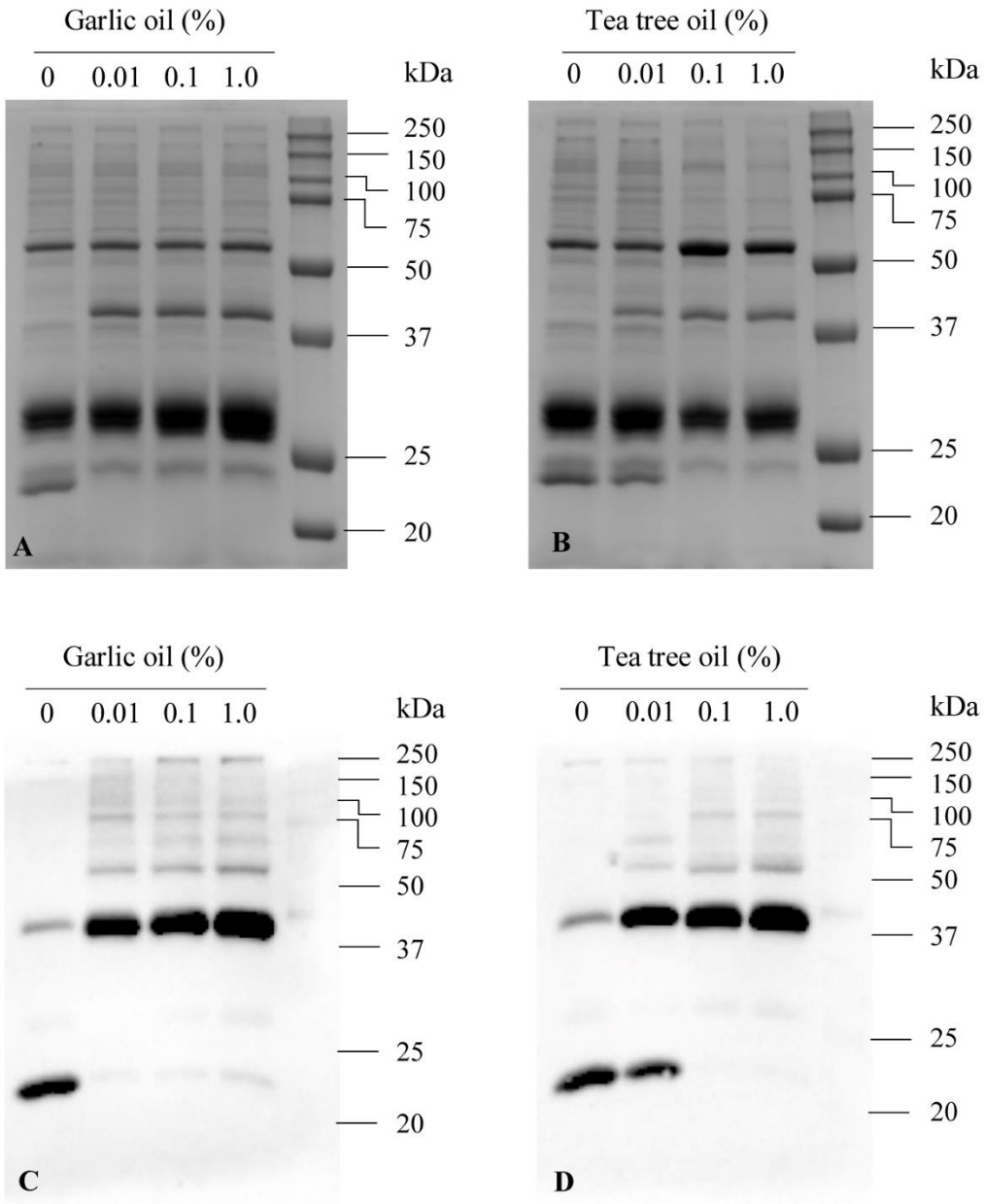

Fig. 6.1. The effects of treating uninfected erythrocytes with garlic or tea tree oil on the redox/oligomerization state of the Prx2 protein in these cells. Each lane contains 60 µg of the total soluble proteins extracted from the erythrocytes. The proteins were separated using NR-PAGE. In panels A and B, the proteins were stained with Coomassie Blue. In panels C and D, the Prx2 protein was detected using anti-(Prx2) antibodies.

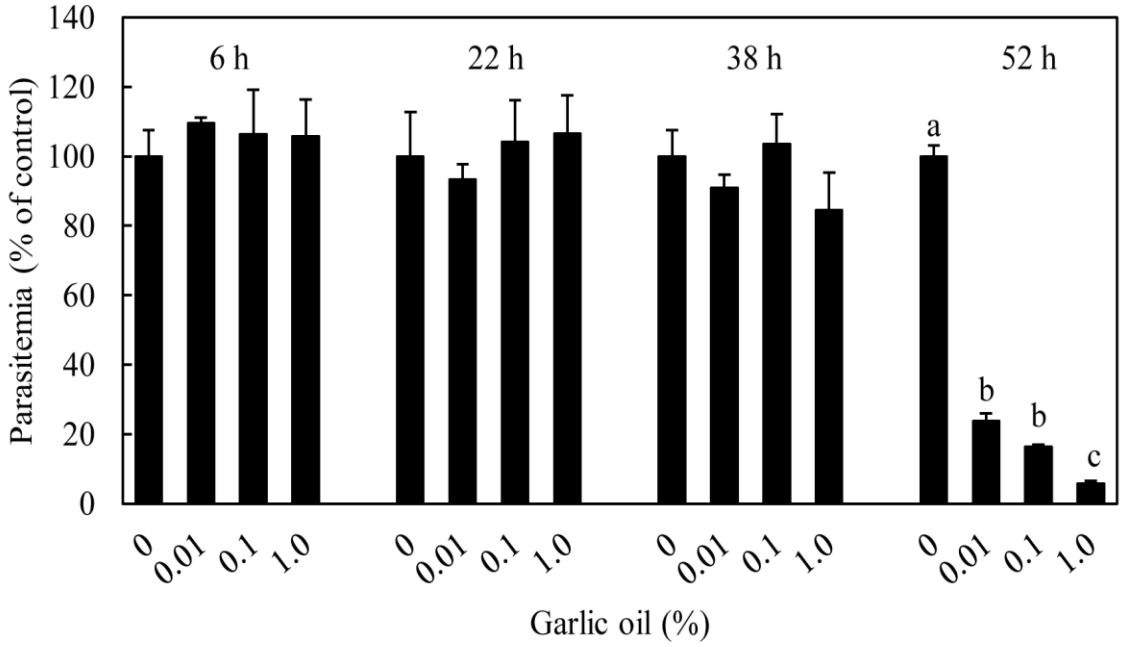

Fig. 6.2. The effect of pre-treatment of erythrocytes with garlic oil on *P. falciparum* parasitemia. The time points correspond to the different intraerythrocytic stages of the parasite's life cycle (see Section 6.2.6). The data are the mean values for 3 replicates and the vertical bars represent ± standard error of the mean (n = 3). Values labelled with different letters are significantly different ($p < 0.05$).

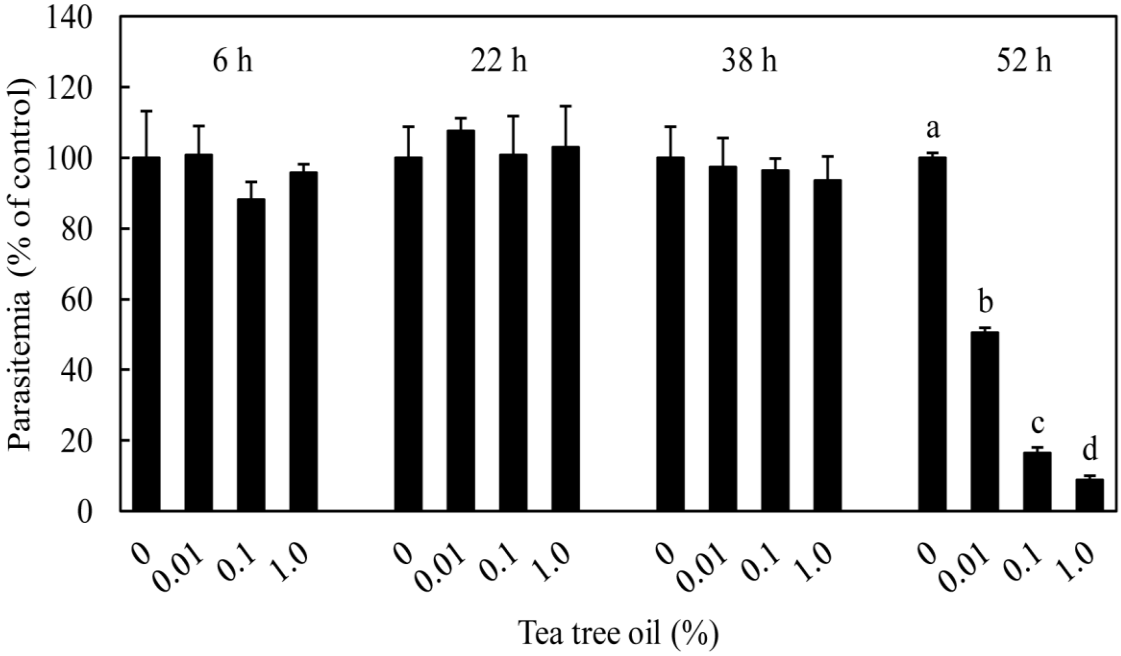

Fig. 6.3. The effect of pre-treatment of erythrocytes with tea tree oil on *P. falciparum* parasitemia. The time points correspond to the different intraerythrocytic stages of the parasite's life cycle (see Section 6.2.6). The data are the mean values for 3 replicates and the vertical bars represent ± standard error of the mean (n = 3). Values labelled with different letters are significantly different ($p < 0.05$).

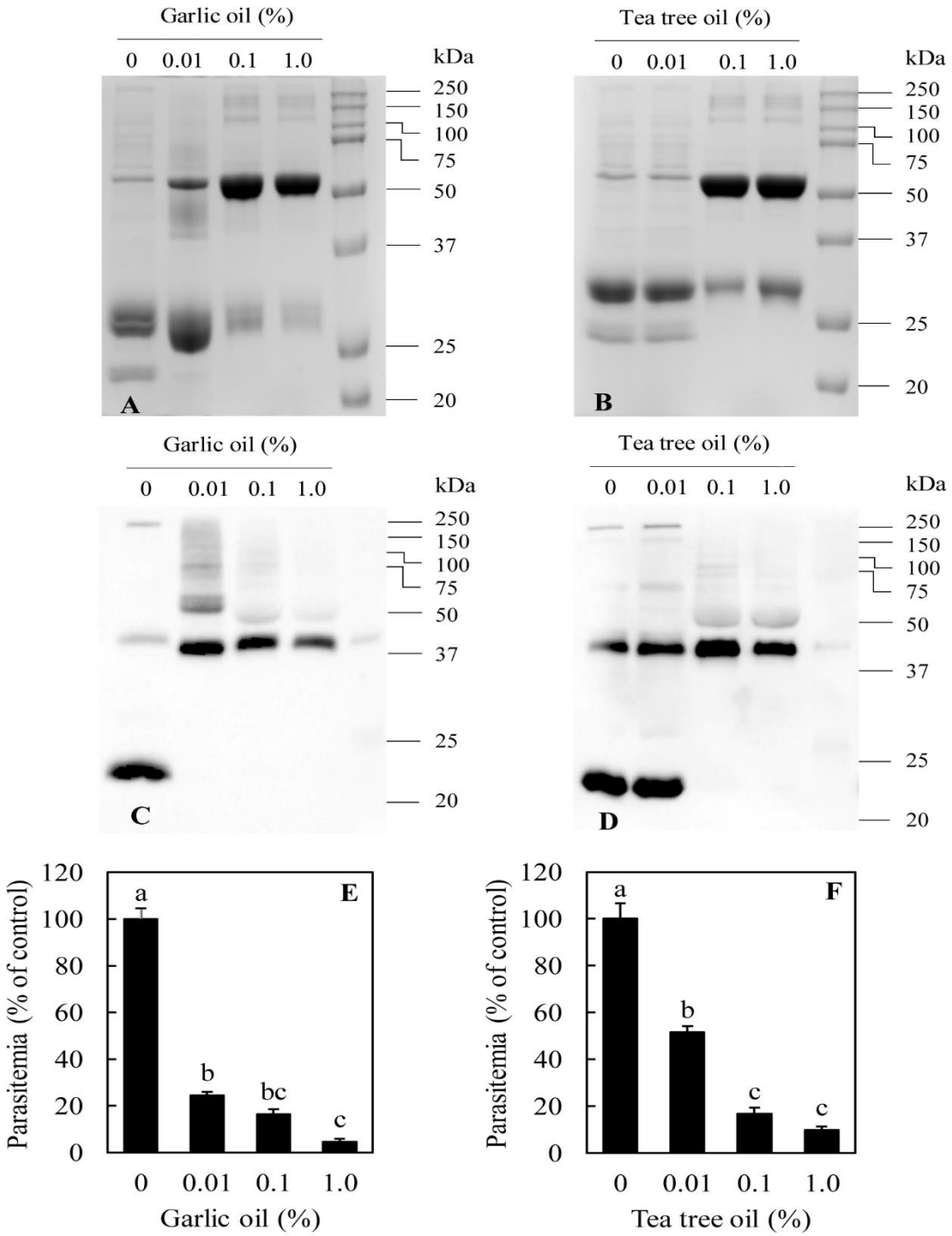

Fig. 6.4. The effect of pre-treatment of erythrocytes with garlic or tea tree oil and their culture with *P. falciparum* on the redox/oligomerization state of the Prx2 protein in these cells at the 2nd generation ring stage of *P. falciparum*. Each lane contains 60 µg of the total soluble proteins extracted 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 ($p < 0.05$).

Chapter 7 - General Discussion and Conclusions

7.1 Major findings

This thesis has reported on the structure and function of the typical 2-Cys peroxiredoxin (Prx) proteins in the clade of the Alveolata using the non-parasitic *Tetrahymena thermophila* and the parasitic *Plasmodium falciparum* as representative organisms for this clade. It has also reported on the effects of various pro-oxidants on the proliferation of *T. thermophila* cells and Jurkat T-lymphocytes and on the redox state of the typical 2-Cys Prx proteins in these cells and in human erythrocytes. In addition to this, this thesis has reported on the effects of various pro-oxidants on the activity of thioredoxin reductase in *T. thermophila* cells and Jurkat T-lymphocytes and on the expression of Prx genes in *T. thermophila* cells. It has also reported on the possible utility of garlic and tea tree oils, and their active constituents, as potential novel anti-malarial drugs. This chapter presents a summary of the major discoveries reported in this thesis.

7.1.1 T. thermophila is highly resistant to oxidative stress induced by peroxides but not by plant oils/compounds

T. thermophila has been employed in various biological studies, including investigations of the capacity of certain chemical compounds to induce oxidative stress (Díaz et al., 2016; Gao et al., 2013; Huang et al., 2016; Juganson et al., 2017). Recently, it has been found that *T. thermophila* is resistant to the anti-proliferative effects of the herbicide paraquat, heavy metals and other chemicals that are known to produce ROS (Díaz et al., 2016; Juganson et al., 2017; Kurvet et al., 2017). However, it is not known whether or not *T. thermophila* is particularly susceptible or resistant to oxidative stress induced by peroxides. In this thesis, *T. thermophila* cells were found to be much less sensitive to the anti-proliferative effects of H_2O_2 and cumene hydroperoxide (CMHP) than were Jurkat

T-lymphocytes (a human cell line) (Chapter 2). This could indicate that *T. thermophila* cells have a high abundance and/or efficiency of antioxidant enzymes, in particular Prx enzymes, for removing H_2O_2 and CMHP from the cells. This hypothesis is further discussed below. In contrast to H_2O_2 and CMHP, both cell types showed similar sensitivities to the anti-proliferative effects of garlic oil and its major constituents the allyl sulphides DADS and DATS as well as to tea tree oil and its major constituent terpinen-4-ol (Chapter 2). *T. thermophila* cells were more susceptible to the inhibitory effects of garlic oil and its allyl sulphides than to tea tree oil or terpinen-4-ol. These findings indicated that *T. thermophila* cells are highly resistant to oxidative stress induced by peroxides but not by plant oils/compounds. The non-polar compounds found in plant essential oils would be expected to diffuse more readily across cellular membranes. Recently, it has been demonstrated, using transmission electron microscopy, that garlic oil can penetrate easily the cellular membrane of the fungus *Candida albicans* as well as organellar membranes, including those of the mitochondria and that this results in organelle disruption (Li et al., 2016b). In addition, there is evidence that DADS and DATS found in garlic oil can act as redox cycling agents, continuously generating H_2O_2 and other ROS (Munday, 2012; Munday et al., 2003). Thus, this continuous generation of ROS as well as easy movement across cellular and organellar membranes may explain why the *T. thermophila* cells were more resistant to H_2O_2 and CMHP than they were to DADS or DATS. Terpinen-4-ol is involved in inducing ROS-dependent responses, including apoptosis, but the underlying mechanism of action is not fully understood (Calcabrini et al., 2004; Han and Parker, 2017; Kuttan et al., 2011; Shapira et al., 2016). We have proposed that terpinen-4-ol may induce accumulation of H_2O_2 through inhibition of

certain proteins such as farnesyl transferases (Basso et al., 2006; Woo et al., 2010) but this has not been investigated.

7.1.2 T. thermophila has a large number of antioxidant genes

T. thermophila is highly resistant to the anti-proliferative effects of H_2O_2 and CMHP (See Section 7.1.1). Thus, we expected that *T. thermophila* would have a large number of antioxidant genes that encode enzymes for removing peroxides and indeed it has. Using bioinformatics tools, we found that ciliates, including *T. thermophila*, have a greater diversity of antioxidant genes compared with other alveolates (apicomplexans and perkinsozoans) (Chapter 3). *T. thermophila* was found to have one catalase, three glutathione peroxidase (GPx), nine phospholipid hydroperoxide glutathione peroxidase (PHGPx) and four Prx genes. Interestingly, it had several typical 2-Cys Prx genes (*Prx1a*, *Prx1b*, *Prx1m* and *Prx1c*) but lacked atypical 2-Cys or 1-Cys Prx genes. There is evidence that all three antioxidant enzyme families, the catalase, GPx and Prx enzyme families, can use H_2O_2 as a substrate but only the GPx and Prx enzyme families can use CMHP, with the Prx enzyme family being more efficient than the GPx enzyme family in this respect (Nelson and Parsonage, 2011). It is known that typical 2-Cys Prx proteins are highly effective at removing peroxides and hydroperoxides from cells (Peskin et al., 2007; Rhee, 2016; Winterbourn and Peskin, 2016). Additionally, there is also evidence that mammalian typical 2-Cys Prx enzymes can reduce more than 90% of the total cellular H_2O_2 and the mammalian mitochondrial typical 2-Cys Prx enzyme (Prx3) can reduce more than 90% of H_2O_2 originating in the mitochondria (Adimora et al., 2010; Cox et al., 2010a; Karplus, 2015). As stated above, we found that *T. thermophila* is highly resistant to the inhibitory effects of H_2O_2 and CMHP on cell proliferation, which could indicate that *T.*

thermophila contains one or more highly effective typical 2-Cys Prx enzymes, in particular the mitochondrial typical 2-Cys Prx enzyme (Prx1m) which we have investigated.

7.1.3 T. thermophila Prx1m protein is resistant to hyperoxidation

A literature search revealed that in alveolates, including *T. thermophila* and *P. falciparum*, the susceptibility of the typical 2-Cys Prx proteins to hyperoxidation has not been investigated. In addition to this, we hypothesised that *T. thermophila* could have a highly effective mitochondrial typical 2-Cys Prx protein (See Section 7.1.2). For these reasons, we decided to investigate the susceptibility of the putative mitochondrial *Prx1m* protein to hyperoxidation in *T. thermophila* cells (Chapter 3). Treatment of *T. thermophila* cells with increasing concentrations of pro-oxidants (H_2O_2, CMHP, garlic oil, DATS, tea tree oil or terpinen-4-ol) was found to promote oxidised dimer formation in the *Prx1m* protein but not hyperoxidised monomer formation. This indicated that the *T. thermophila Prx1m* protein was insensitive to hyperoxidation and therefore it belongs to the "robust" type of typical 2-Cys Prx protein which remains active in the presence of mM concentrations of H_2O_2 (Wood et al., 2003a). It has previously been shown that treatment of Jurkat T-lymphocytes with low H_2O_2 concentrations ($\leq$ 20 μM) results in oxidised dimer formation in the Prx3 (mitochondrial) protein, whereas treatment of these cells with high H_2O_2 concentrations (>20 μM) results in hyperoxidised monomer formation (Cox et al., 2009). Unlike what was observed for the human mitochondrial Prx3 protein, we found treatment of *T. thermophila* cells with up to 10 mM H_2O_2 did not promote hyperoxidised monomer formation in the mitochondrial *Prx1m* protein. The deduced amino acid sequence of the *T. thermophila Prx1m* protein revealed that this protein contained the highly conserved

GGLG and Y*X* (where *X* is F or W) motifs, which are a common feature of most of the "sensitive" type eukaryotic typical 2-Cys Prx proteins, including those from humans (Wood et al., 2003a). These so-called "sensitive" types form hyperoxidised monomers in the presence of H_2O_2 concentrations as low as ~20 or 40 µM (Cox et al., 2009). The *T. thermophila Prx1m* protein was resistant to hyperoxidation despite containing the GGLG and Y*X* motifs found in the "sensitive" type typical 2-Cys Prx proteins. This indicated that the presence of the GGLG and YF motifs might not always be associated with susceptibility to hyperoxidation. Similarly, it was observed in the cyanobacterium *Synechocystis* sp. PCC6803 that its typical 2-Cys Prx protein was insensitive to hyperoxidation despite containing the GGLG (or similar) and YF motifs (Pascual et al., 2010). In contrast, in the cyanobacterium *Anabaena* sp. PCC7120, its typical 2-Cys Prx protein was sensitive to hyperoxidation as well as containing the GGLG (or similar) and YF motifs (Pascual et al., 2010). Unlike humans and *Anabaena* sp. PCC7120, *Synechocystis* sp. PCC6803 lacks any sulfiredoxin enzymes (Boileau et al., 2011; Pascual et al., 2010). We found that like *Synechocystis* sp. PCC6803, *T. thermophila* and all other alveolates (ciliates, apicomplexans and perkinsozoans) included in this study lacked any sulfiredoxin proteins (Chapter 3). These proteins have been reported to be found only in organisms that have "sensitive" typical 2-Cys Prx proteins (Gretes and Karplus, 2013; Gretes et al., 2012). Thus, this thesis has shown that the resistance of the *T. thermophila Prx1m* protein to hyperoxidation is associated with the absence of sulfiredoxin enzymes rather than with the absence of the GGLG and YF (or similar) motifs. Thus absence of sulfiredoxins might be a better predictor of whether a typical 2-Cys Prx protein is "sensitive" or "robust" to hyperoxidation. This finding might extend to the typical 2-Cys

Prx proteins of other alveolates (ciliates, apicomplexans and perkinsozoans) as well as other eukaryotes lacking sulfiredoxin enzymes.

7.1.4 Garlic and tea tree oils and their major constituents promote oxidation of the "sensitive" typical 2-Cys Prx proteins in Jurkat T-lymphocytes

Prx1, Prx2 and Prx3 proteins in Jurkat T-lymphocytes have been shown to belong to the "sensitive" type of typical 2-Cys Prx proteins (Cox et al., 2009). Previous studies have also shown that plant compounds such as phenethyl isothiocyanate, found in cruciferous vegetables (e.g. broccoli), can induce oxidised dimer formation in the Prx3 protein in Jurkat T-lymphocytes and promyelocytic leukemia cells (the human HL-60 cell line) (Brown et al., 2010; Brown et al., 2008). Thus, the *in vivo* redox/oligomerization states of the "sensitive" typical 2-Cys Prx proteins (Prx1, Prx2 and Prx3) were investigated in Jurkat T-lymphocytes treated with increasing concentrations of garlic and tea tree oils and their major constituents (Chapter 4). This was done to determine whether these plant oils/compounds could promote oxidised dimer formation or hyperoxidized monomer formation in these "sensitive" type typical 2-Cys Prx proteins. Garlic oil and its constituent allyl sulphides DADS and DATS were found to promote oxidised dimer formation in the typical 2-Cys Prx proteins in Jurkat T-lymphocytes but they did not promote hyperoxidised monomer formation. DATS was more effective than DADS in promoting oxidized dimer formation. Thus, these results indicated that garlic oil exerts its effects mainly through its constituent allyl sulphides, in particular DATS, known to act as a H_2O_2 and other ROS generator (Munday, 2012; Munday et al., 2003). In contrast to this, like H_2O_2, terpinen-4-ol and its parent tea tree oil were shown to promote hyperoxidised monomer formation in the Prx proteins. These results indicated that tea tree oil exerts its

effects mainly through terpinen-4-ol. Terpinen-4-ol was more effective at promoting hyperoxidised monomer formation in the typical 2-Cys Prx proteins than either DADS or DATS. This could indicate that terpinen-4-ol is more effective at promoting H_2O_2 formation in the cells. As pointed out in Section 7.1.1, it could be that terpinen-4-ol increases accumulation of H_2O_2 in the cells through inhibition of certain proteins such as farnesyl transferases (Basso et al., 2006; Woo et al., 2010).

7.1.5 Inhibition of thioredoxin reductase activity by allyl sulphides but not by terpinen-4-ol explains, at least in part, the oxidation of the typical 2-Cys Prx proteins

In mammalian cells, thioredoxin reductase (TrxR), together with thioredoxin (Trx), is responsible for regenerating the reduced monomer form of the typical 2-Cys Prx proteins from their oxidised dimer form (Park et al., 2016; Poynton et al., 2016; Rhee et al., 2012). Allyl sulphides (DADS and DATS) were known to inhibit TrxR activity but it was not known how these allyl sulphides affected the redox/oligomerization state of the typical 2-Cys Prx proteins (Hu et al., 2007).We have shown that the capacity of DADS and DATS to inhibit TrxR activity is positively associated with their capacity to promote the oxidation of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes (Chapter 4). Similarly, inhibition of TrxR activity by DATS was associated with its capacity to promote the oxidation of the *Prx1m* protein in *T. thermophila* cells (Chapter 3). This indicated that in addition to promoting H_2O_2 formation, allyl sulphides exert their effects on the typical 2-Cys Prx proteins by inhibiting TrxR activity (Munday et al., 2003). In contrast to DADS and DATS, terpinen-4-ol was a relatively poor inhibitor of TrxR activity but was a strong promotor of the oxidation of the typical 2-Cys Prx proteins in Jurkat T-lymphocytes (hyperoxidised monomer formation) and in *T. thermophila* cells (oxidised dimer

formation) (Chapter 3 and 4). These results suggest that terpinen-4-ol exerts its effects on the typical 2-Cys Prx proteins by inducing accumulation of H_2O_2 in the cells and not by inhibiting TrxR activity.

7.1.6 Treatment of T. thermophila cells with various pro-oxidants results in increased expression of the Prx1a and Prx1m genes but not the Prx1b and Prx1c genes

The four *T. thermophila* Prx genes were found to be differently expressed in *T. thermophila* cells (Chapter 5). The *Prx1a* and *Prx1m* genes were more highly expressed than either the *Prx1b* or *Prx1c* genes. Thus, *T. thermophila* depends mainly on the *Prx1a* and *Prx1m* gene products for protection against oxidative stress. We observed in *T. thermophila* cells treated with various oxidative stressors that *Prx1a* and *Prx1m* were the most responsive to H_2O_2 but that the *Prx1a* gene was more responsive than the *Prx1m* gene. Similarly, treatment with H_2O_2 has been shown to induce the expression of typical 2-Cys Prx genes in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and in the cyanobacterium *Synechocystis* Sp. PCC6803 (Chen et al., 2003; Gasch et al., 2000; Li et al., 2004; Vandenbroucke et al., 2008). Thus, these findings indicate that the Prx genes of unicellular organisms, including *T. thermophila*, are part of an inducible oxidative stress response. Furthermore, the *Prx1a* gene was the most responsive to garlic oil whereas the *Prx1m* gene was the most responsive to tea tree oil and terpinen-4-ol. Díaz et al. (2016) recently reported that transcript abundance for a thioredoxin peroxidase gene (likely *Prx1a* or *Prx1m* because of their high abundance) was increased in *T. thermophila* cells exposed to the herbicide paraquat. Thus, there are other corroborating reports that the Prx genes in *T. thermophila* are responsive to oxidative stress but our work is the first

to demonstrate which of the Prx genes is the most responsive and therefore the most important in protecting the organism against oxidative stress. We also observed that unlike H_2O_2, treatment of the *T. thermophila* cells with higher concentrations of garlic and tea tree oils and their abundant compounds had toxic effects on the cells as indicated by the significant decrease in the transcript abundance for GAPDH (housekeeping gene). Interestingly, these treatment concentrations had no significant effects on the total cellular RNA yields (data not shown). As a result, the transcript abundance estimates for the Prx genes in the presence of these treatment concentrations should be treated with caution.

7.1.7 Oxidation/dimerization of the Prx2 protein in uninfected erythrocytes pre-treated with either garlic or tea tree oil results in reduced infection of the erythrocytes by P. falciparum

The malaria parasite *P. falciparum* lacks catalase and GPx enzymes and thus is highly dependent on Prx enzymes for H_2O_2 detoxification (Jortzik and Becker, 2012). In addition to its five Prx enzymes, the human erythrocyte Prx2 protein, imported by *P. falciparum* from its host, has been found to participate in ~50% of the total peroxide detoxification activity in *P. falciparum* (Koncarevic et al., 2009). The human erythrocyte Prx2 protein has been found to be relatively insensitive to oxidative inactivation in the presence of high H_2O_2 concentrations and thus it is very efficient at removing H_2O_2 (Low et al., 2007; Peskin et al., 2007). We expected that treatment of uninfected erythrocytes with garlic or tea tree oil would promote oxidation/inactivation of the Prx2 protein in these cells (Chapter 6). As expected, garlic and tea tree oil were found to promote oxidised dimer formation in the Prx2 protein in uninfected erythrocytes. Thus, these oils were able to disrupt the redox state of the Prx2 protein in erythrocytes. We also predicted that pre-

treatment of uninfected erythrocytes with garlic or tea tree oil would reduce infection of the erythrocytes by *P. falciparum*, presumably because of the oxidation/inactivation of the erythrocyte Prx2 protein and consequent reduced capacity of the parasite to combat oxidative stress (Chapter 6). As predicted, our results revealed that *P. falciparum* parasitemia was indeed decreased in erythrocytes pre-treated with garlic or tea tree oil at the 2nd generation ring stage (new infections). This indicated that pre-treatment of uninfected erythrocytes with garlic or tea tree oil made the erythrocytes less susceptible to infection by *P. falciparum* and therefore these oils could potentially be used as antimalarial treatments. Additionally, both oils promoted oxidation of the Prx2 protein at the 2nd generation ring stage as well as appearing to promote oxidation of other sulfhydryl group-containing proteins. Thus, inactivation of the erythrocyte Prx2 protein appears to reduce the protection of the malaria parasite against oxidative stress. In addition, we suggest that the other proteins that became oxidized in our study could be carbonic anhydrase I and albumin or haemoglobin based on their abundance and sensitivity to oxidation as revealed in other studies (D'amici et al., 2012; Nagumo et al., 2014). One or more of these proteins might be essential for completion of the *P. falciparum* life cycle. Thus, they could be good targets for anti-malarial drug therapies. This should be a fruitful subject for future investigation.

7.2 General conclusion and future directions

This thesis has focussed on the study of the typical 2-Cys Prx proteins in selected organisms of the clade of the Alveolata and has also presented *T. thermophila* as a useful model for preliminary screening of plant oils/compounds for anti-malarial activity. This study has advanced the current understanding of the structure and function of the typical

2-Cys Prx proteins in *T. thermophila* and has provided a foundation for further investigations of the role of the typical 2-Cys Prx proteins in non-parasitic alveolates. Future work on the *T. thermophila* Prx proteins could involve investigation into sub-cellular localisation and chaperone activity of these proteins as has been shown in humans, *S. cerevisiae* (a yeast), *Arabidopsis thaliana* (a plant), *Pseudomonas aeruginosa* (a bacterium), *Anabaena* PCC7120 (a cyanobacterium), *Schistosoma mansoni* (a trematode metazoan) and *Leishmania infantum* (a kinetoplastid protozoan) (An et al., 2010; Angelucci et al., 2013; Banerjee et al., 2015; Jang et al., 2004; König et al., 2013; Moon et al., 2005; Pan et al., 2014; Teixeira et al., 2015; Toledano and Huang, 2016). Furthermore, the work with pre-treatment of erythrocytes with plant oils/compounds has presented an alternative way to prevent/reduce *P. falciparum* infections and has also provided a foundation for further investigation of the role of plant essential oils in the prevention of malaria. Future work on *P. falciparum* could involve investigation into whether or not the other erythrocyte located sulfhydryl group-containing proteins targeted by garlic and tea tree oil are essential for completion of the *P. falciparum* life cycle.

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Appendices

Appendix A.1 – Effect of DMSO on the proliferation of *Tetrahymena thermophila* cells

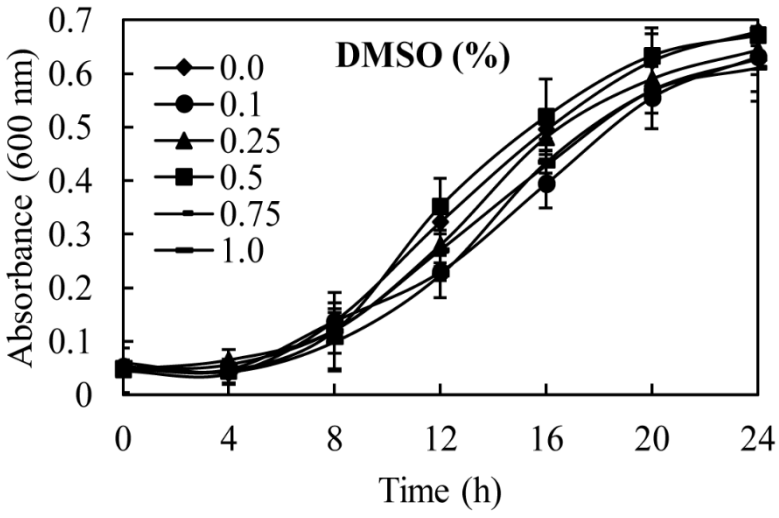

Fig. 1. Effect of DMSO on the proliferation of *T. thermophila* cells. The cells were exposed to various concentrations of DMSO. Cell proliferation was monitored employing the indirect cell counting method using the absorbance at a wavelength of 600 nm (see Section 2.2.6). The data points are the mean values for 3 replicate wells and the vertical bars represent ± standard error of the mean (n = 3). The experiments were repeated twice. The results for one representative experiment are presented here.

Appendix A.2 – Effect of DMSO on the proliferation of Jurkat T-lymphocytes

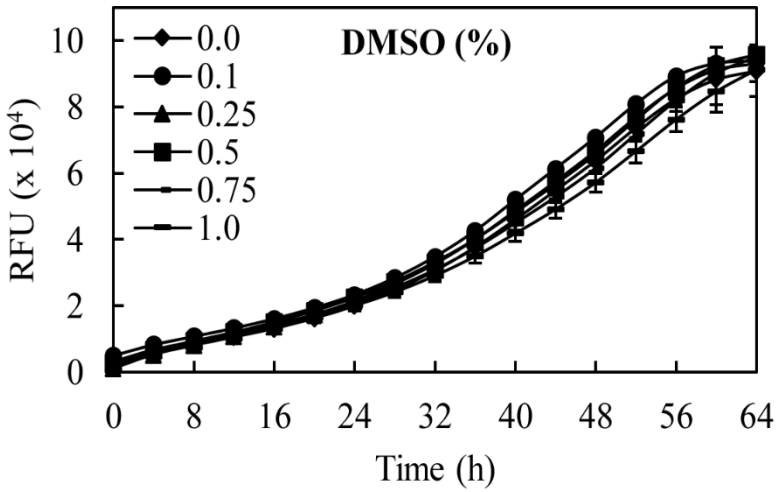

Fig. 2. Effect of DMSO on the proliferation of Jurkat T-lymphocytes. The cells were exposed to various concentrations of DMSO. Cell proliferation was monitored employing the indirect cell counting method using the Resazurin dye (see Section 2.2.7). The data points are the mean values for 3 replicate wells and the vertical bars represent ± standard error of the mean (n = 3). The experiments were repeated twice. The results for one representative experiment are presented here.

Appendix A.3 – Synthesis of the peptide antigen to use in producing the anti-(*T. thermophila Prx1m*) polyclonal antibodies.

MIMOTOPES

The Peptide Company

Mimotopes Pty Ltd ABN 90 090 841 286

11 Duerdin Street
Clayton Victoria 3168
Australia
Tel +61 3 9565 1111
Fax +61 3 9565 1199
mimotopes@mimotopes.com
www.mimotopes.com

Certificate of Analysis

Custom Synthesized Peptide

Order Number : 25154

Peptide Number : 2515403

Theoretical Molecular Weight : 1,848.07

Observed Molecular Weight : MS Analysis detected molecular ions consistent with the parent

Sequence : H-CEEYLRLVQAFQYAD-OH

At the N-terminus, H- means Free amine. At the C-terminus, -OH means Free acid.
Sequence is in standard single letter code (unless otherwise specified), amino terminus is on the left.

Requested : 19mg at 90% by HPLC

Supplied :

Tube	Mass (mg)	Minimum Purity (%)	Solubility Information Solubility	mg/mL	Buffer
1	9.3	96	Soluble	7.5	0-20% Acetonitrile in 100Mm Ammonium Bicarbonate
2	10.1	96	Soluble	7.5	0-20% Acetonitrile in 100Mm Ammonium Bicarbonate

* Solubility information provided was as used in the purification processes and is for information only. Many other solubilization conditions may be effective and we advise trying simpler methods first. (See 'A Guide to Handling and Storing Peptides').

Comments :
Amino Acid Code Sequence : Cys-Glu-Glu-Tyr-Leu-Arg-Leu-Val-Gln-Ala-Phe-Gln-Tyr-Ala-Asp
Peptide sensitive to oxidation.

Quality Assured by : _____ Tuesday, 5 August 2014

C. Pham - Quality Assurance Department

For any technical enquiries please contact: peptide_support_group@mimotopes.com
For Material Safety Data information please email :
mimotopes@mimotopes.com, requesting "Custom Synthesized Peptide" MSDS

MIMOTOPES
The Peptide Company

Mimotopes Pty Ltd ABN 90 090 841 286
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Conjugated Peptide Product Report

Conjugate Number :	2515402

Peptide Details :

Peptide Number :	2515403
Sequence :	H-CEEYLRLVQAFQYAD-OH
Molecular Weight :	1848
Purity of Peptide Coupled :	96%

Conjugate Details :

Carrier Protein :	Keyhole Limpet Hemocyanin
Linker :	Maleimidocaproyl-N -Hydroxysuccinimide (MCS)
Mass of Carrier Protein :	23.5mg
Mass of Peptide Used :	10.1mg

Comments :

Final form of conjugate as supplied : Freeze dried.
Diluent suitable for reconstitution : Purified water.
If the conjugate has not been completely dissolved then sonicate briefly or shake to obtain a uniform suspension.

Date Released : 31-Jul-2014

Signed : _____

Quality Assurance Department

For Material Safety Data information please email:
mimotopes@mimotopes.com, requesting "Conjugated Synthetic Peptide" MSDS.

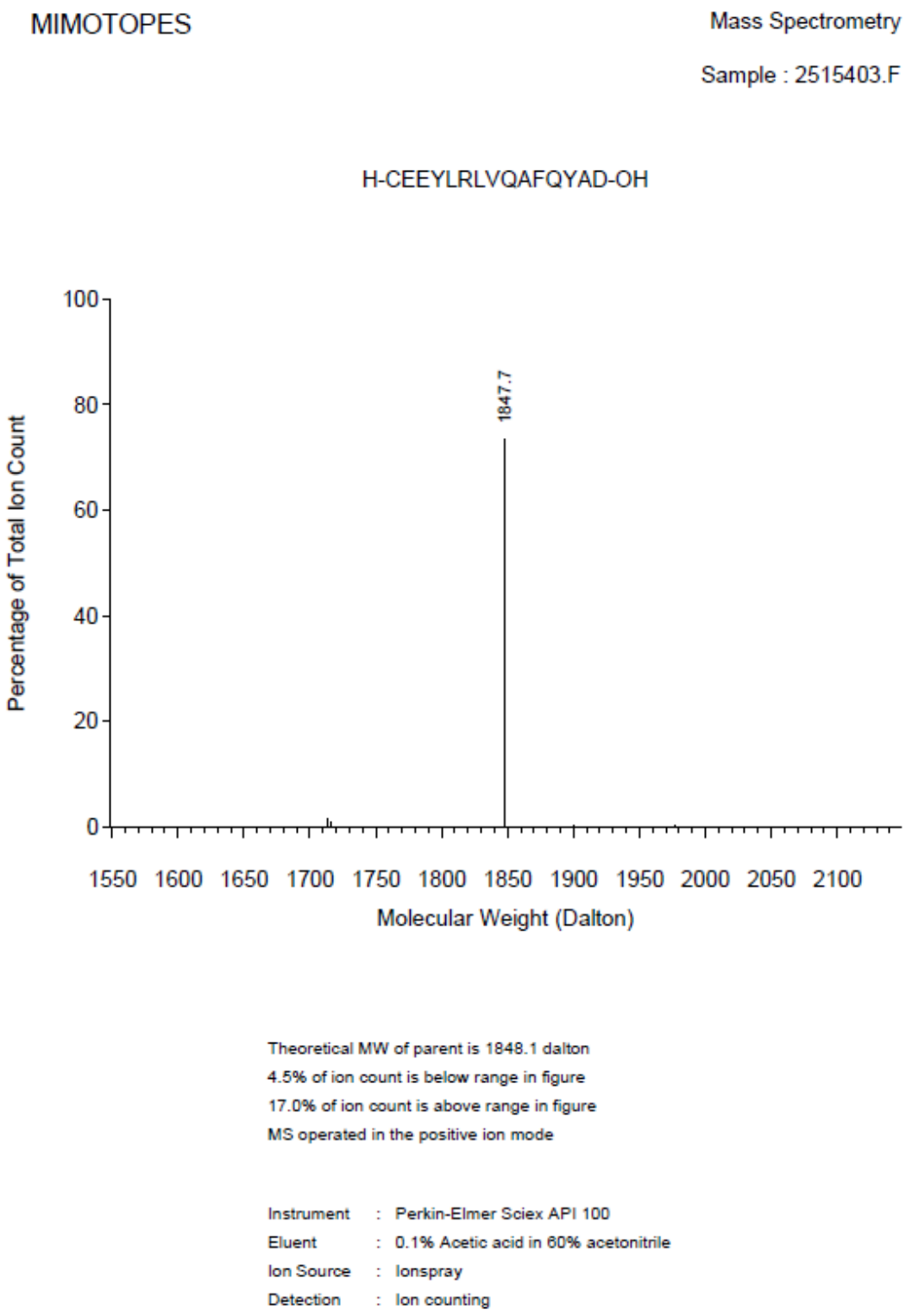
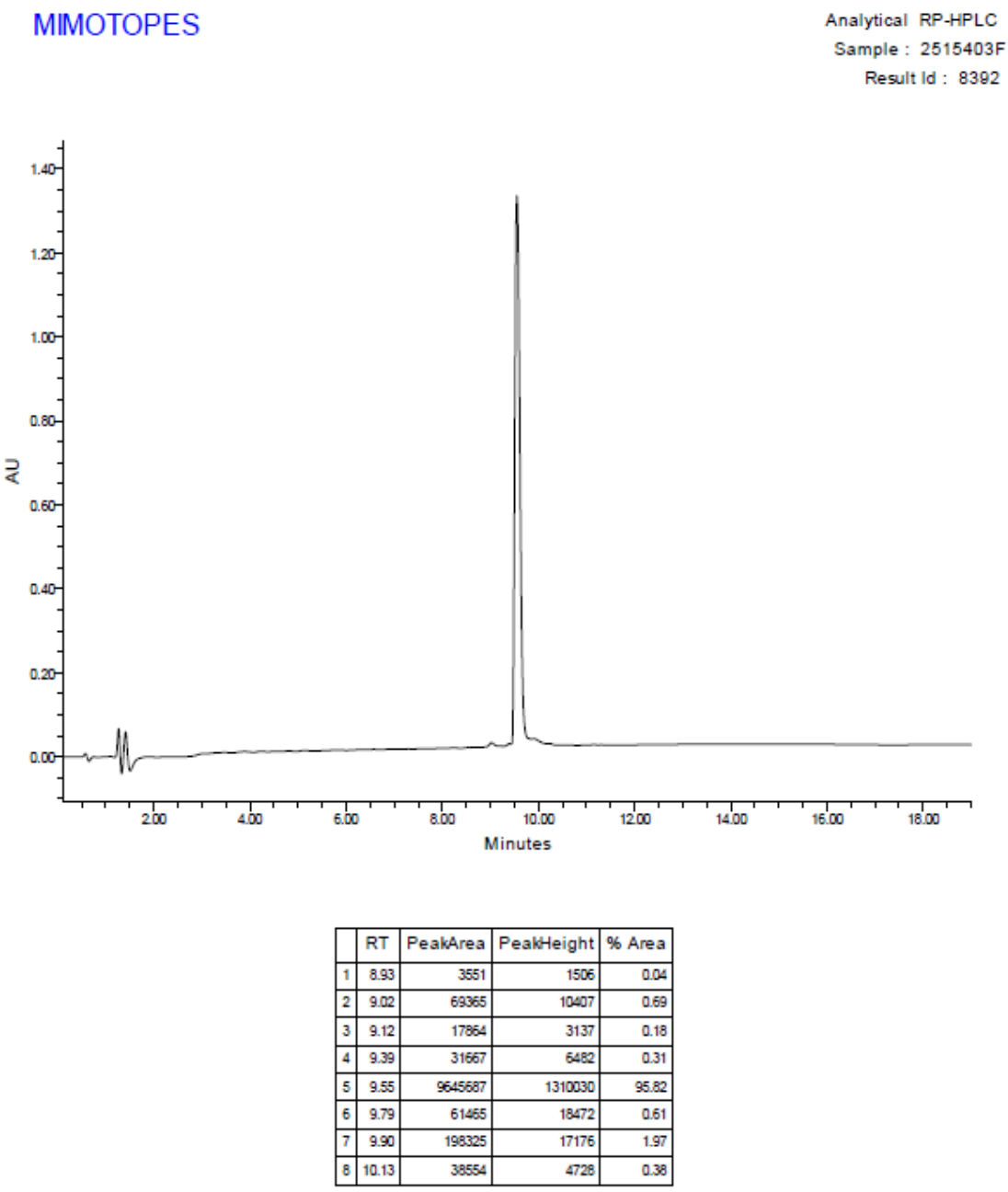


Fig. 3. The molecular weight of the peptide antigen as determined by mass spectrometry.



	RT	PeakArea	PeakHeight	% Area
1	8.93	3551	1506	0.04
2	9.02	69365	10407	0.69
3	9.12	17864	3137	0.18
4	9.39	31667	6482	0.31
5	9.55	9645687	1310030	95.82
6	9.79	61465	18472	0.61
7	9.90	198325	17176	1.97
8	10.13	38554	4728	0.38

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

Date Acquired: 24-Jul-2014 15:36:04 EST
Acq Method Set S6_15cm_25min__TFA
Analysis using Empower 2 Software Build 2154

Fig. 4. The purity of the peptide antigen as determined by reverse phase-high performance liquid chromatography (RP-HPLC).

Appendix A.4 – Testing the PCR primers for their capacity to amplify the correct PCR product, i.e., one product, expected size

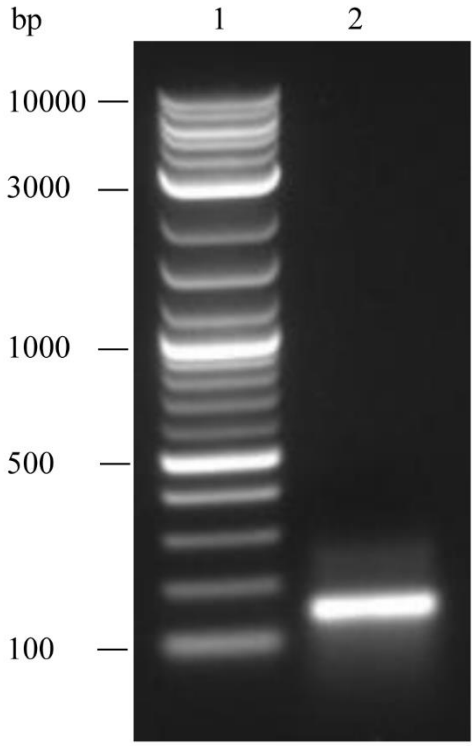


Fig. 5. Testing the *T. thermophila* GAPDH PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

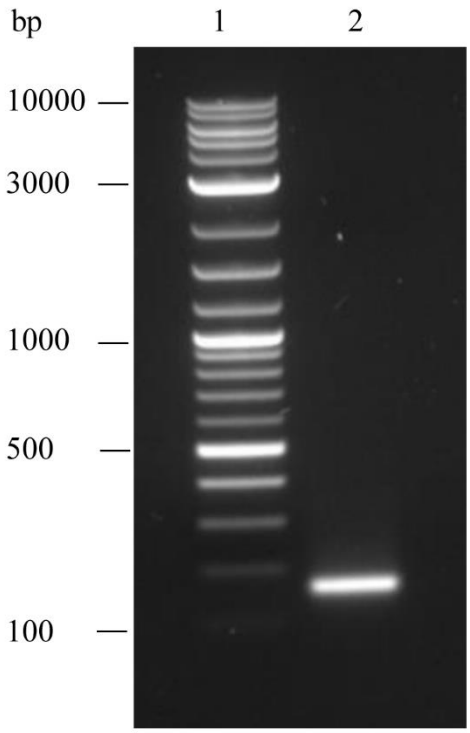


Fig. 6. Testing the *T. thermophila* catalase PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

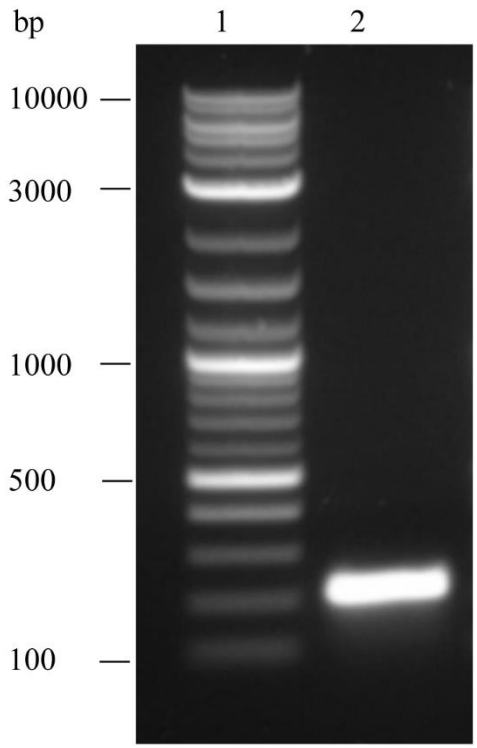


Fig. 7. Testing the *T. thermophila Prx1a* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

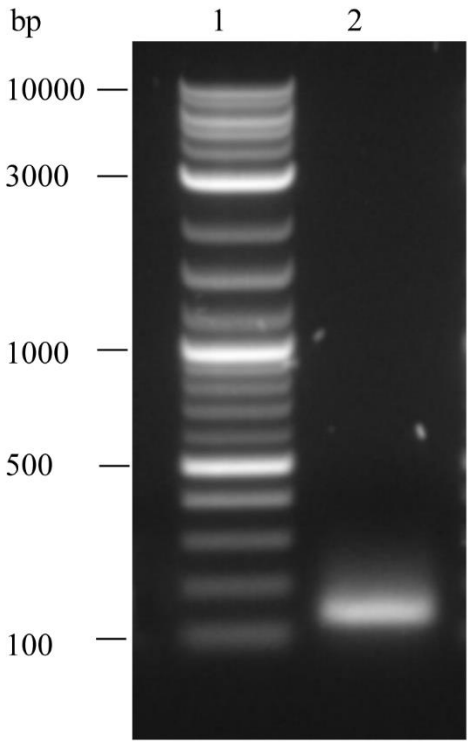


Fig. 8. Testing the *T. thermophila Prx1b* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

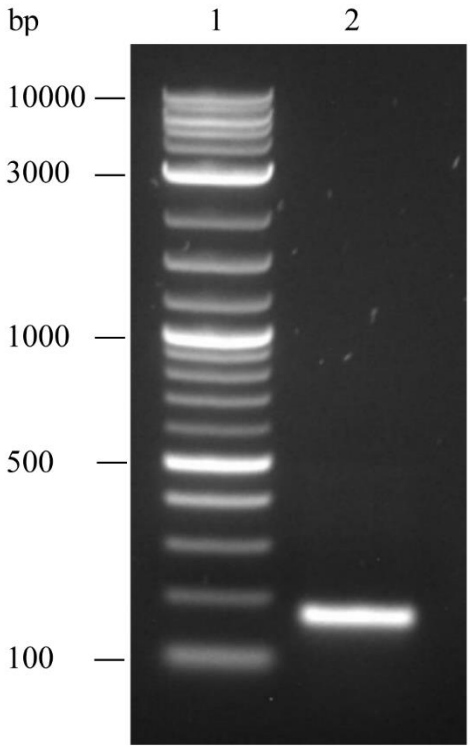


Fig. 9. Testing the *T. thermophila Prx1m* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

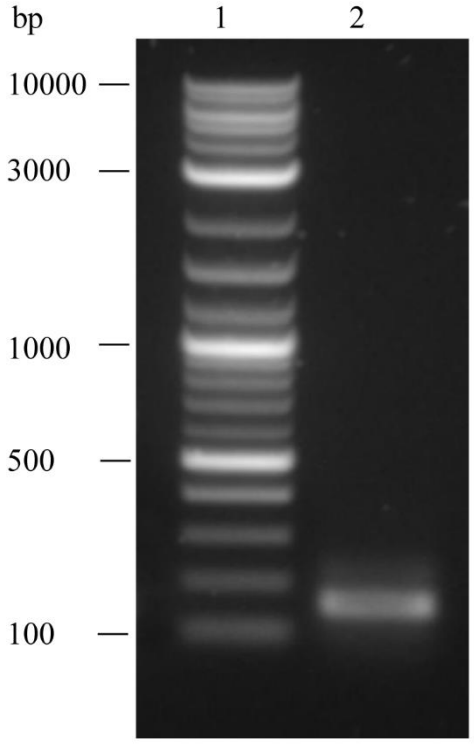


Fig. 10. Testing the *T. thermophila Prx1c* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

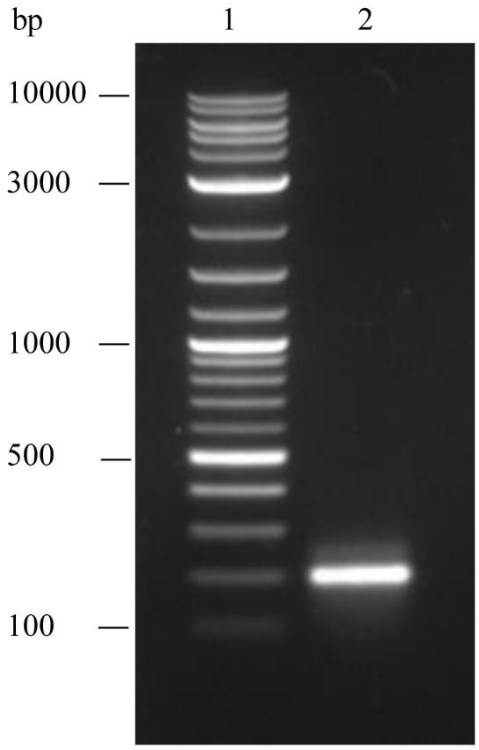


Fig. 11. Testing the *T. thermophila GPx1* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

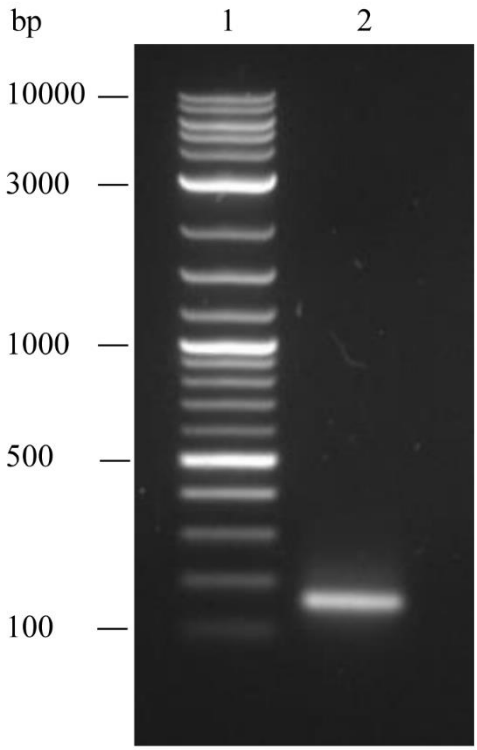


Fig. 12. Testing the *T. thermophila GPx2* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

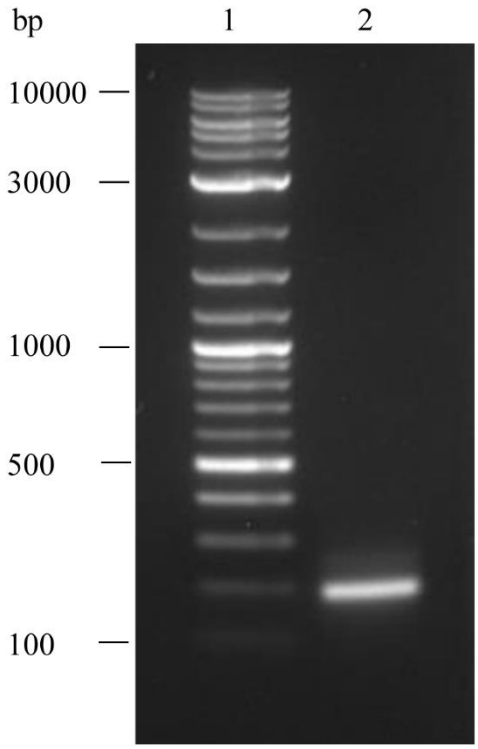


Fig. 13. Testing the *T. thermophila GPx3* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

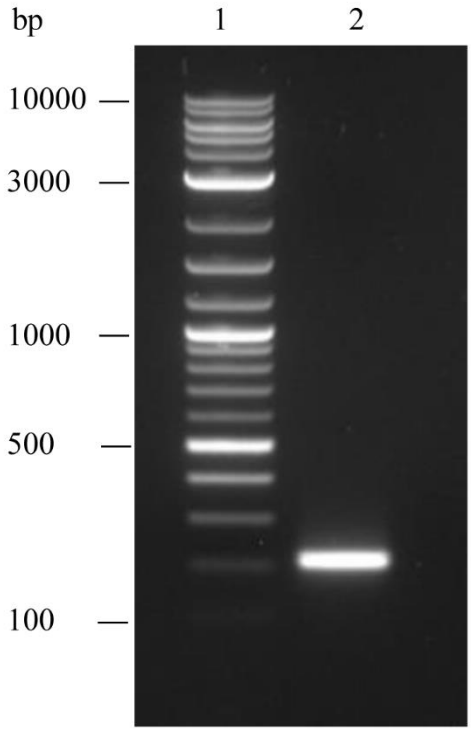


Fig. 14. Testing the *T. thermophila PHGPx9* PCR primers. The PCR product was separated on a 1.5 % (w/v) agarose gel as described in Section 5.2.5. Lane 1 contains a DNA ladder and lane 2 contains the PCR product.

Appendix A.5 – Sequencing of PCR products to determine whether the PCR primers amplified the correct products

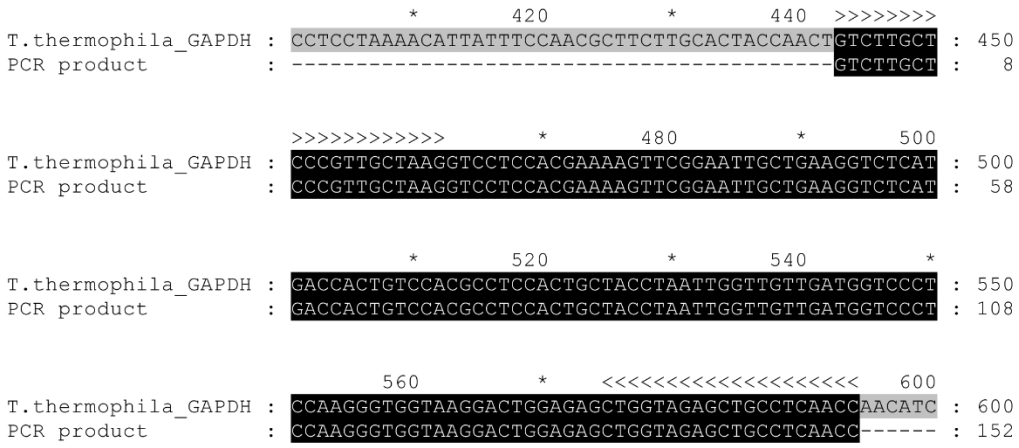


Fig. 15. A nucleotide sequence alignment of the *T. thermophila* GAPDH sequence (accession no. XM_001009203.3) with the *T. thermophila* GAPDH PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

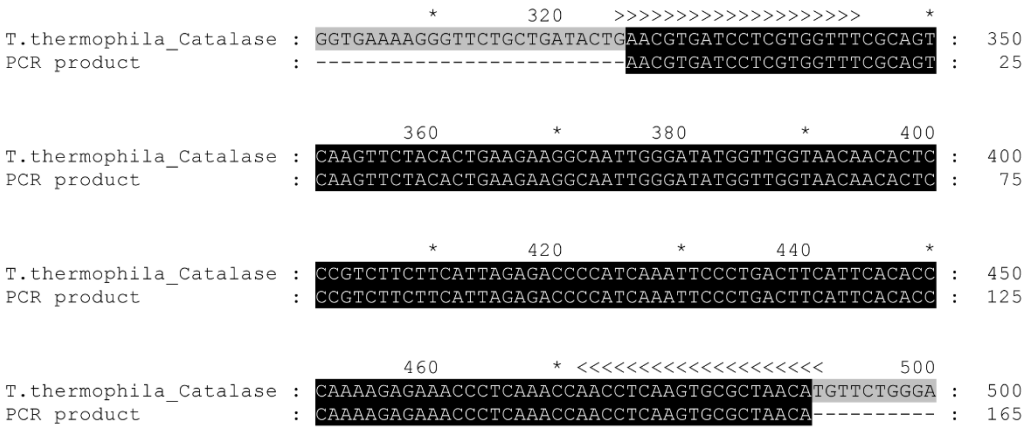


Fig. 16. A nucleotide sequence alignment of the *T. thermophila* catalase sequence (accession no. XM_001026590.3) with the *T. thermophila* catalase PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

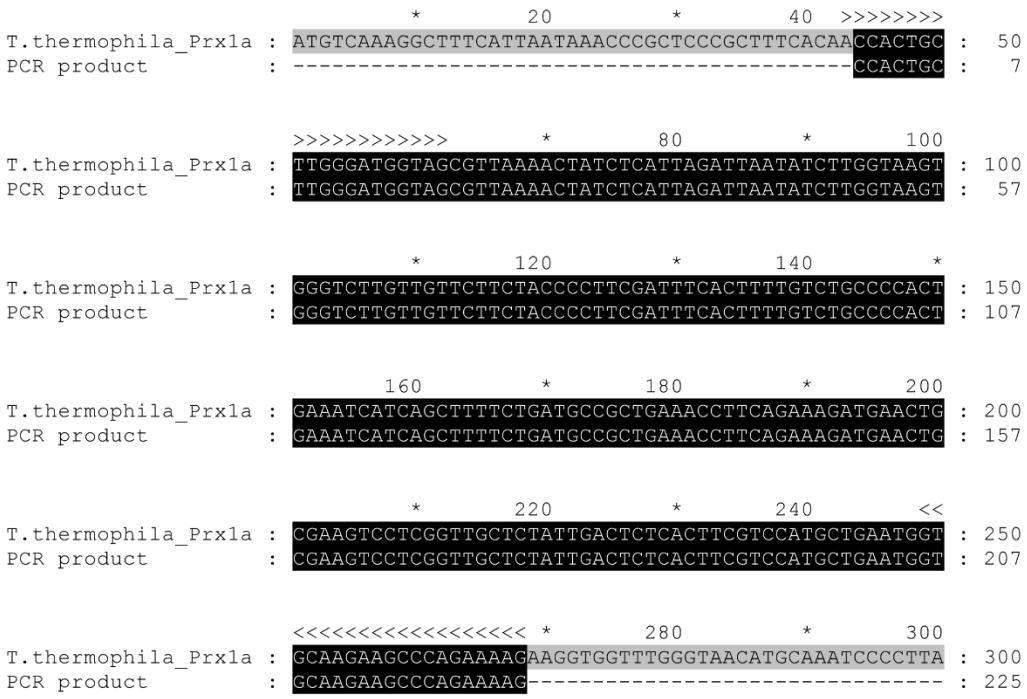


Fig. 17. A nucleotide sequence alignment of the *T. thermophila Prx1a* sequence (accession no. XM_001029987.1) with the *T. thermophila Prx1a* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

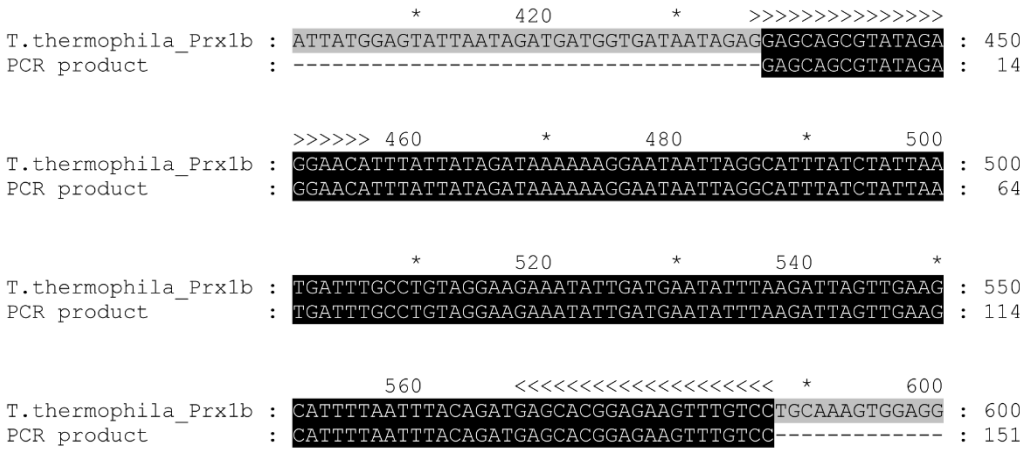


Fig. 18. A nucleotide sequence alignment of the *T. thermophila Prx1b* sequence (accession no. XM_001031522.2) with the *T. thermophila Prx1b* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

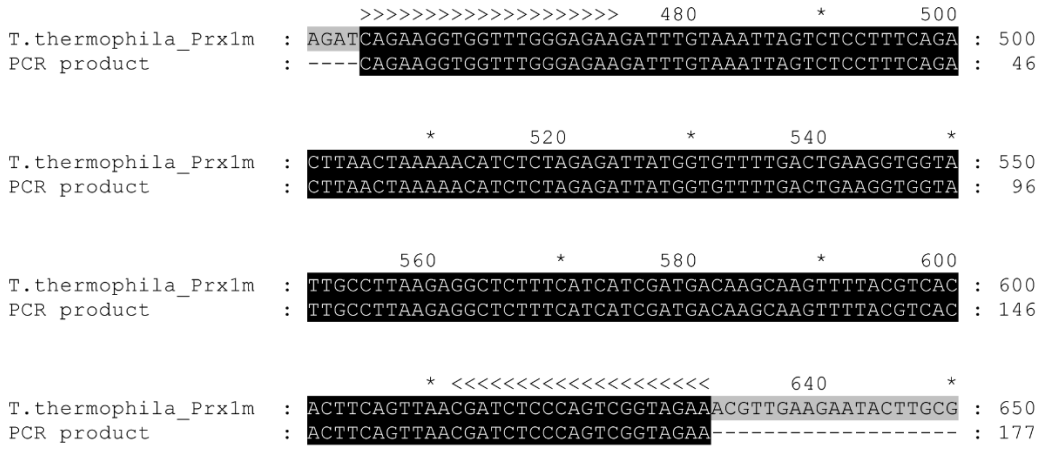


Fig. 19. A nucleotide sequence alignment of the *T. thermophila Prx1m* sequence (accession no. XM_001013149.3) with the *T. thermophila Prx1m* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

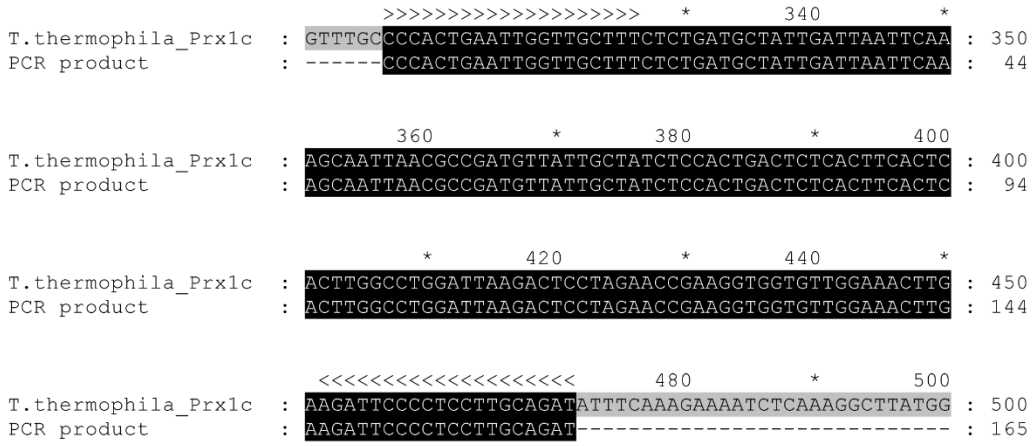


Fig. 20. A nucleotide sequence alignment of the *T. thermophila Prx1c* sequence (accession no. XM_001031885.3) with the *T. thermophila Prx1c* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

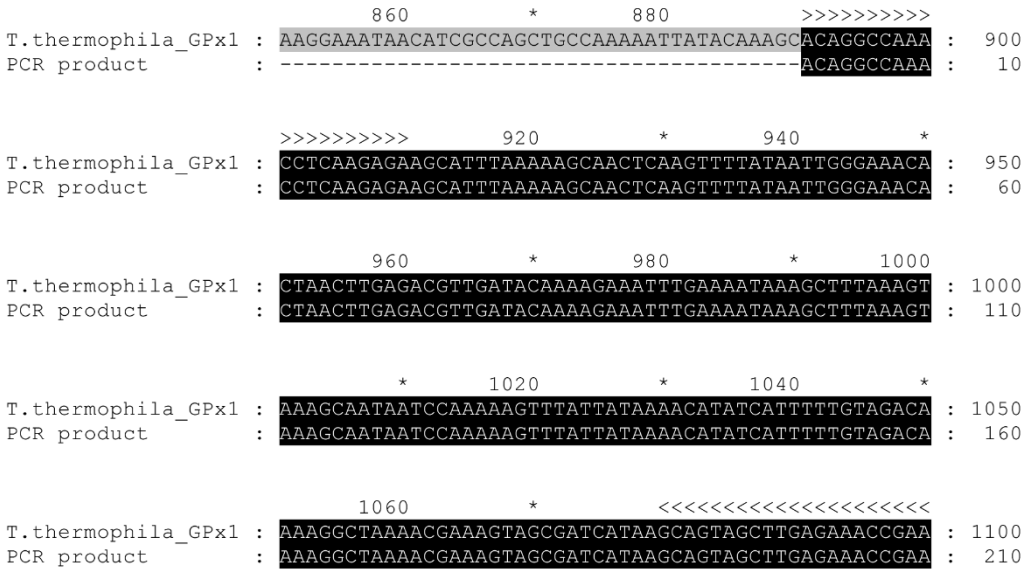


Fig. 21. A nucleotide sequence alignment of the *T. thermophila GPx1* sequence (accession no. XM_012798147.1) with the *T. thermophila GPx1* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<<, respectively.

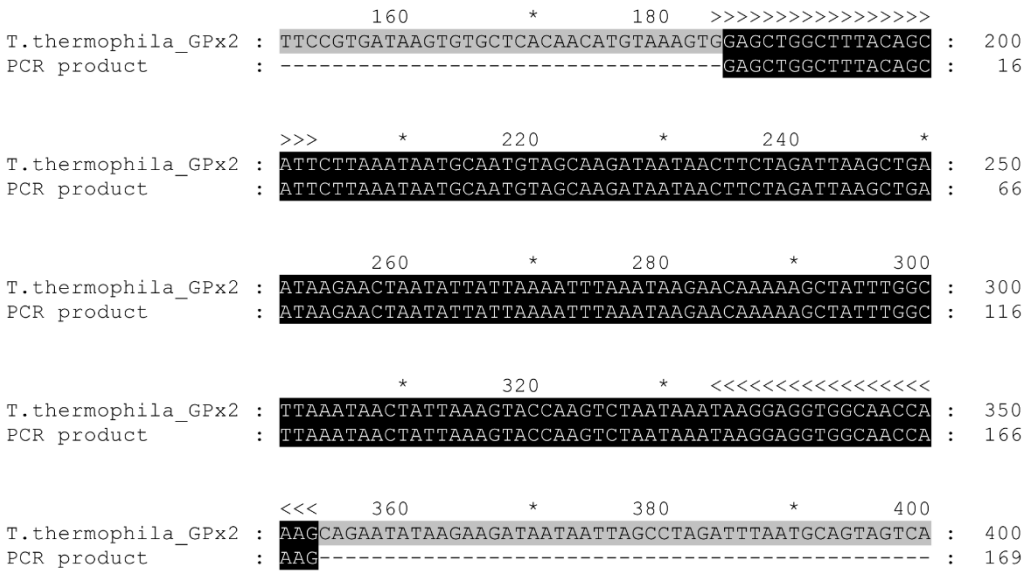


Fig. 22. A nucleotide sequence alignment of the *T. thermophila GPx2* sequence (accession no. XM_001018139.2) with the *T. thermophila GPx2* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<< respectively.

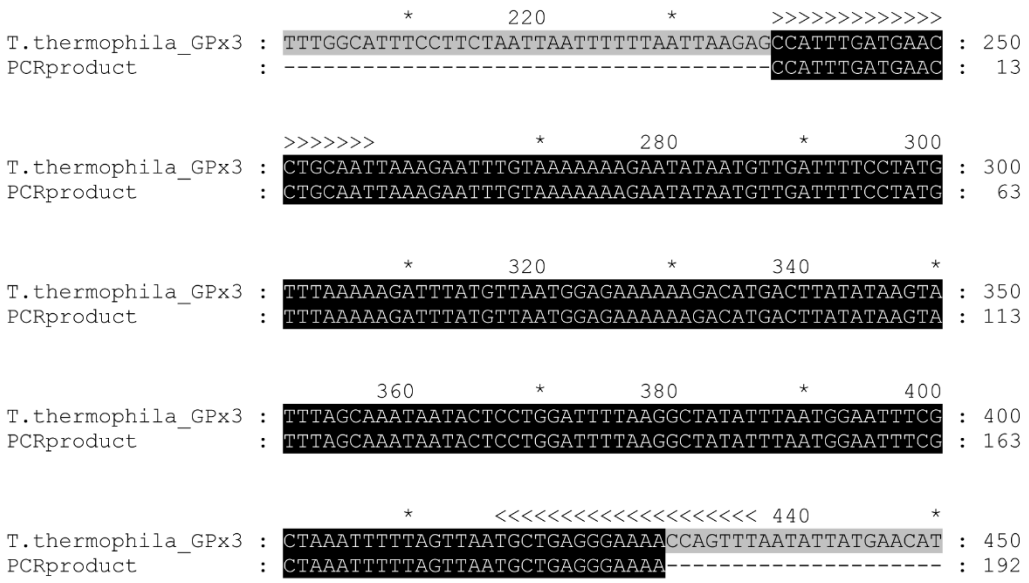


Fig. 23. A nucleotide sequence alignment of the *T. thermophila GPx3* sequence (accession no. XM_001030317.2) with the *T. thermophila GPx3* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<<, respectively.

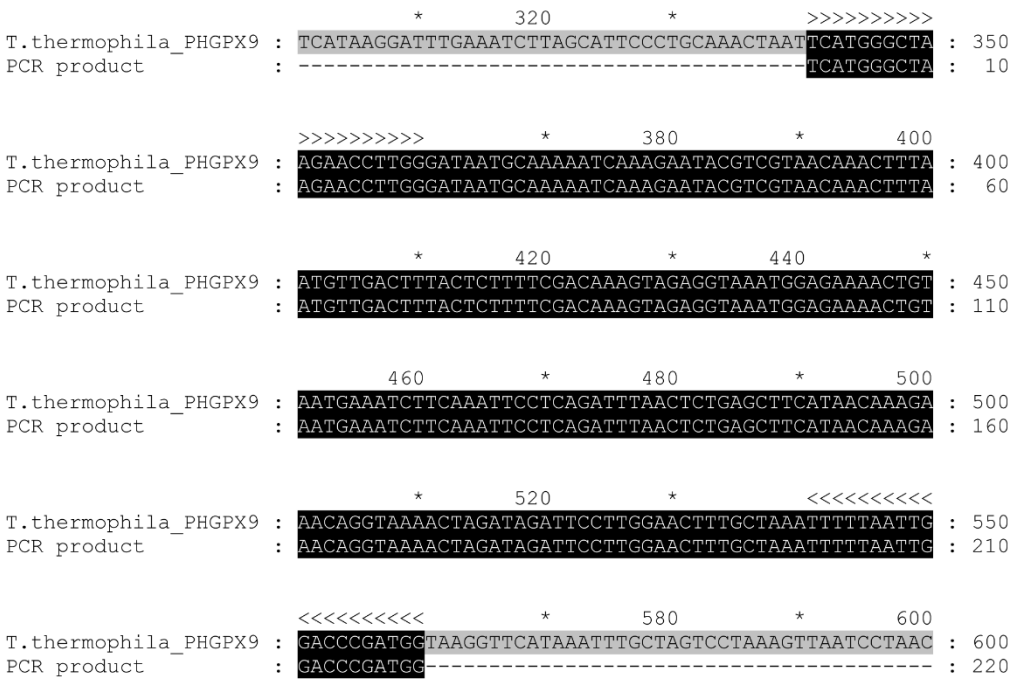


Fig. 24. A nucleotide sequence alignment of the *T. thermophila PHGPx9* sequence (accession no. XM_001011054.3) with the *T. thermophila PHGPx9* PCR product sequence obtained using the relevant primer set in Table 5.1 (see Section 5.2.4). The alignment was performed using ClustalX software (http://www.clustal.org) (Larkin et al., 2007). The forward and reverse primers are indicated by >>>>>> and <<<<<<, respectively.

Appendix A.6 – Determining the efficiency values for the PCR Primers

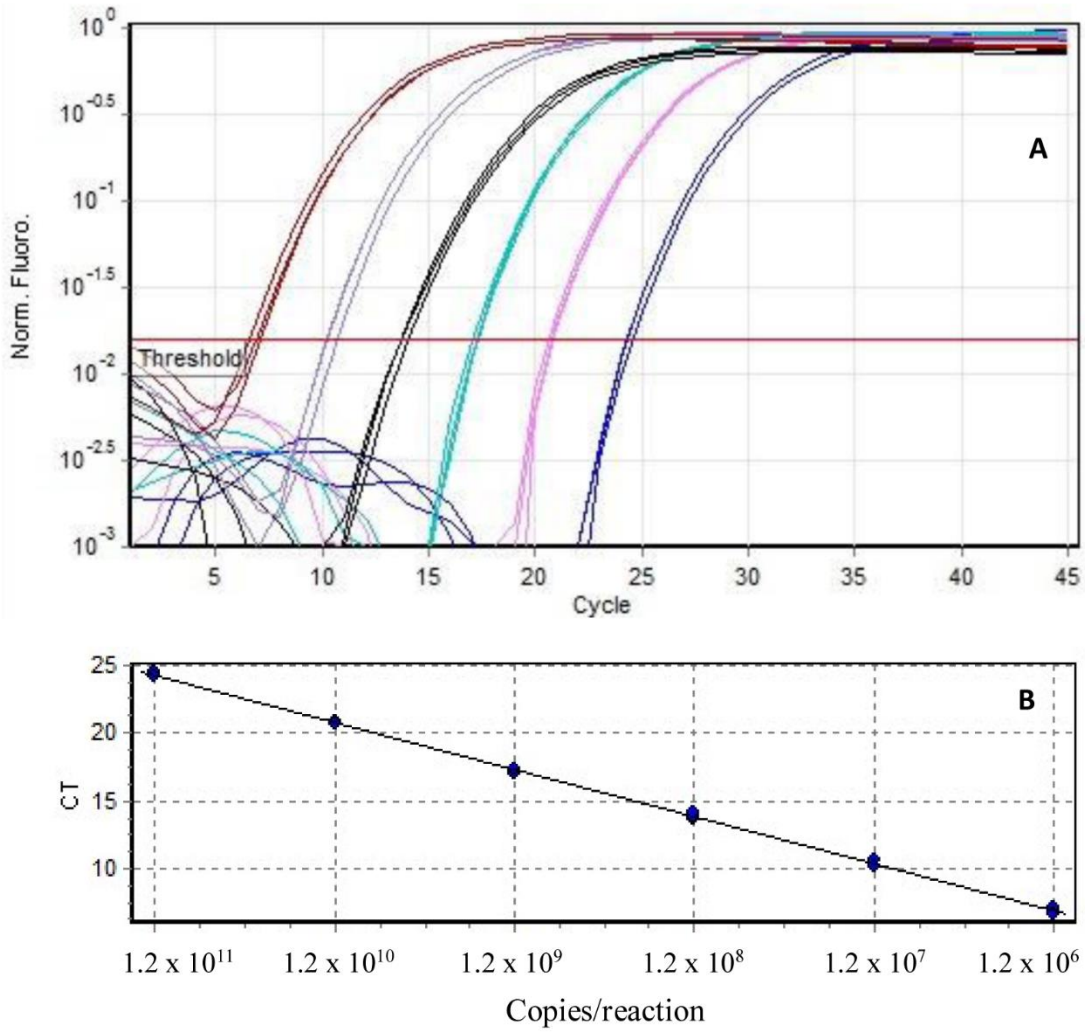


Fig. 25. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified GAPDH PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.485x + 34.726$. The slope of the line corresponded to an amplification efficiency of 0.94. Norm. Fluoro. = normalised fluorescence.

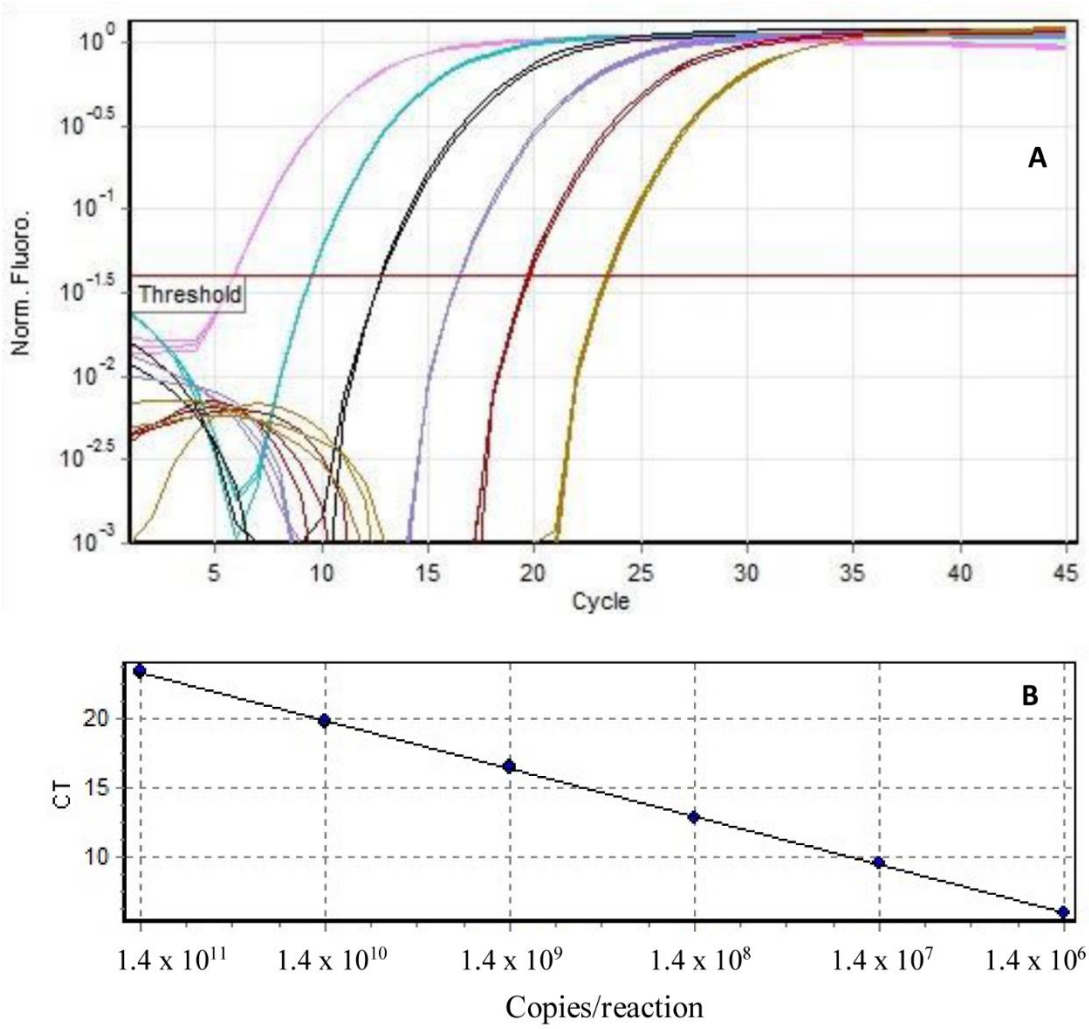


Fig. 26. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified catalase PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.484x + 33.765$. The slope of the line corresponded to an amplification efficiency of 0.94. Norm. Fluoro. = normalised fluorescence.

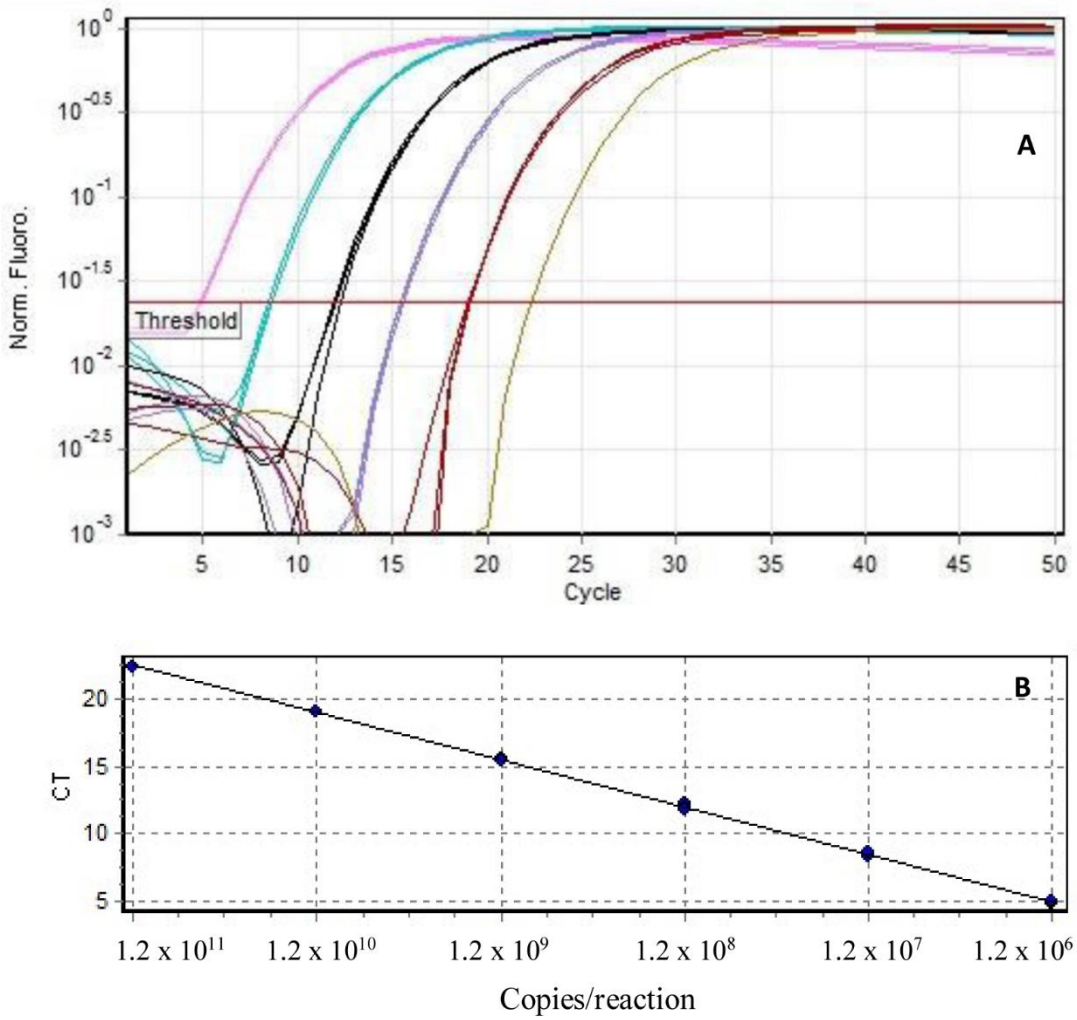


Fig. 27. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *Prx1a* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.493x + 32.941$. The slope of the line corresponded to an amplification efficiency of 0.93. Norm. Fluoro. = normalised fluorescence.

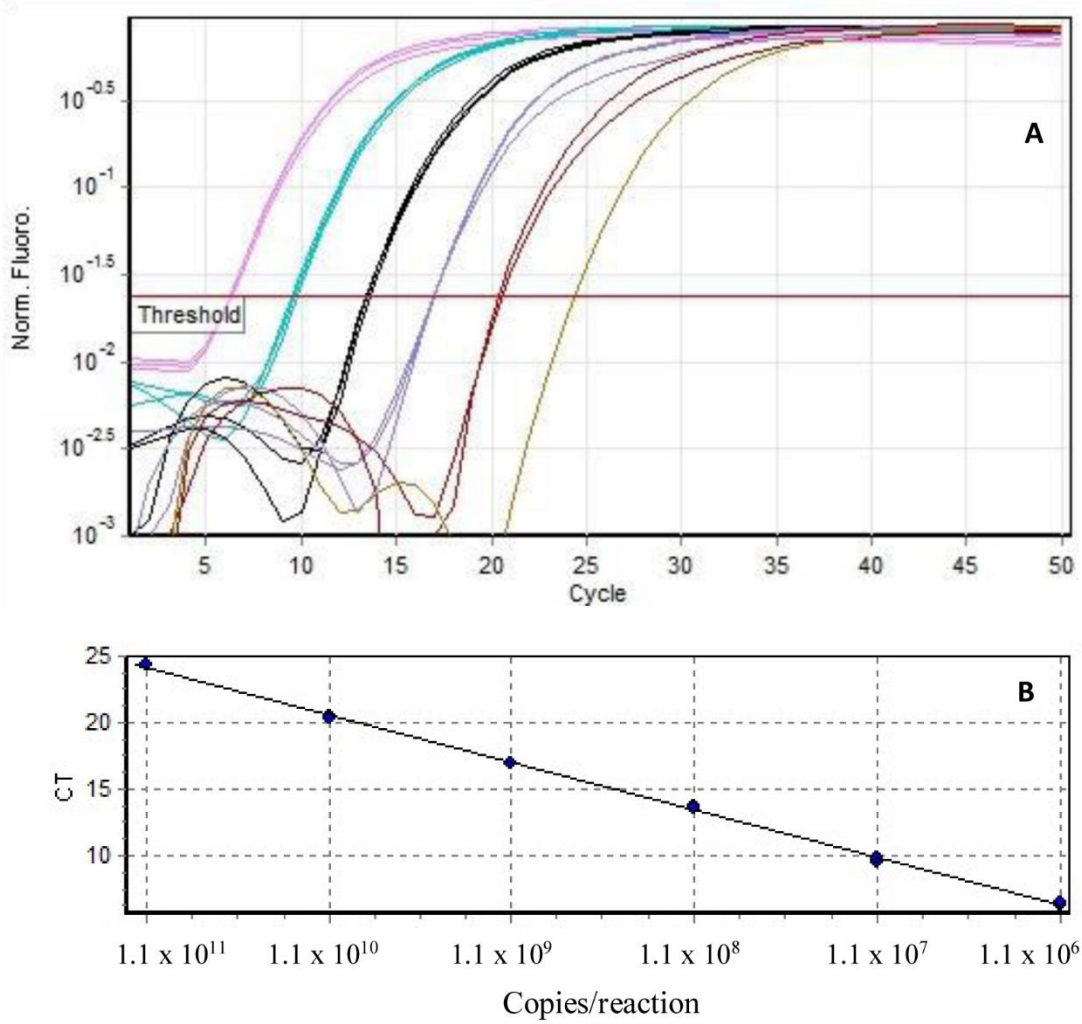


Fig. 28. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *Prx1b* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.593x + 34.958$. The slope of the line corresponded to an amplification efficiency of 0.91. Norm. Fluoro. = normalised fluorescence.

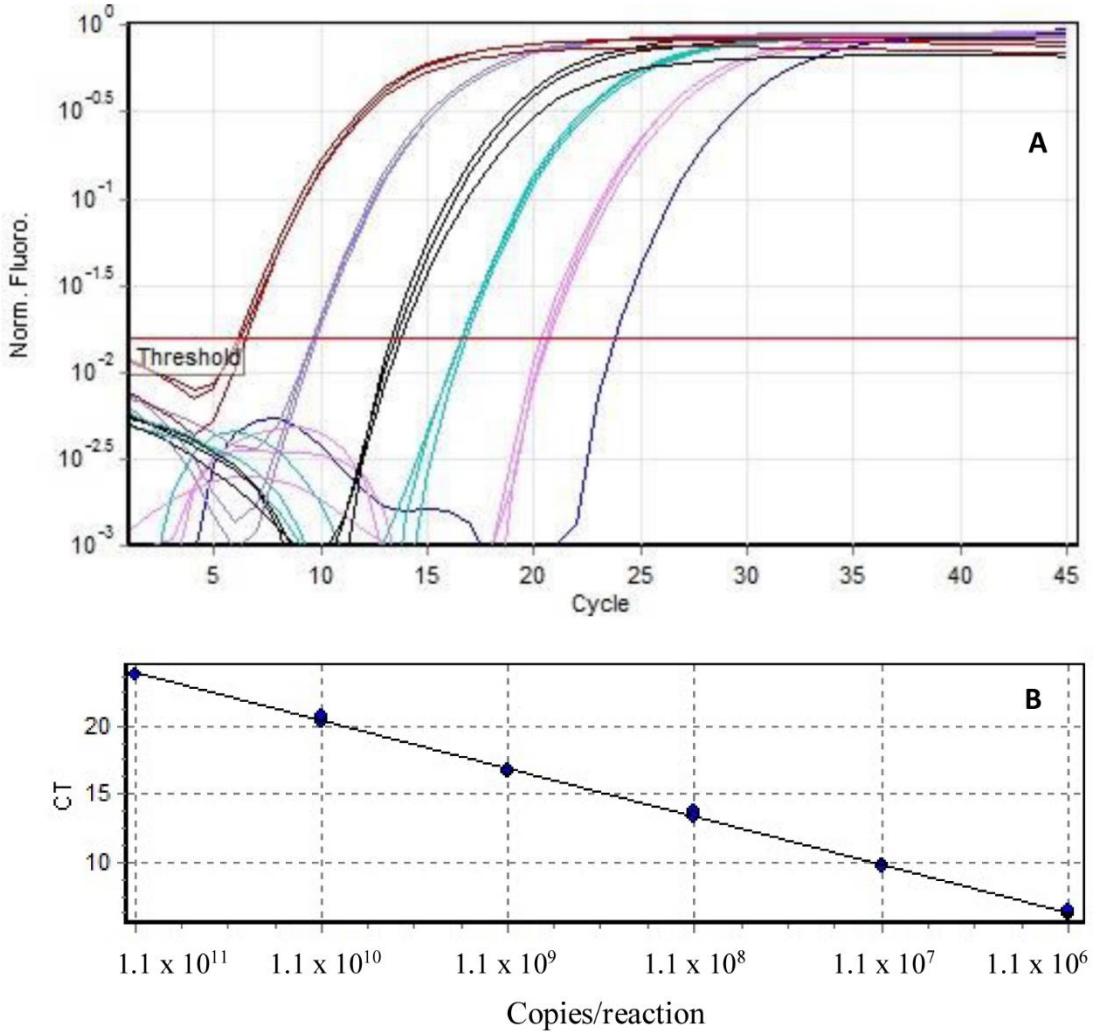


Fig. 29. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *Prx1m* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.473x + 35.772$. The slope of the line corresponded to an amplification efficiency of 0.92. Norm. Fluoro. = normalised fluorescence.

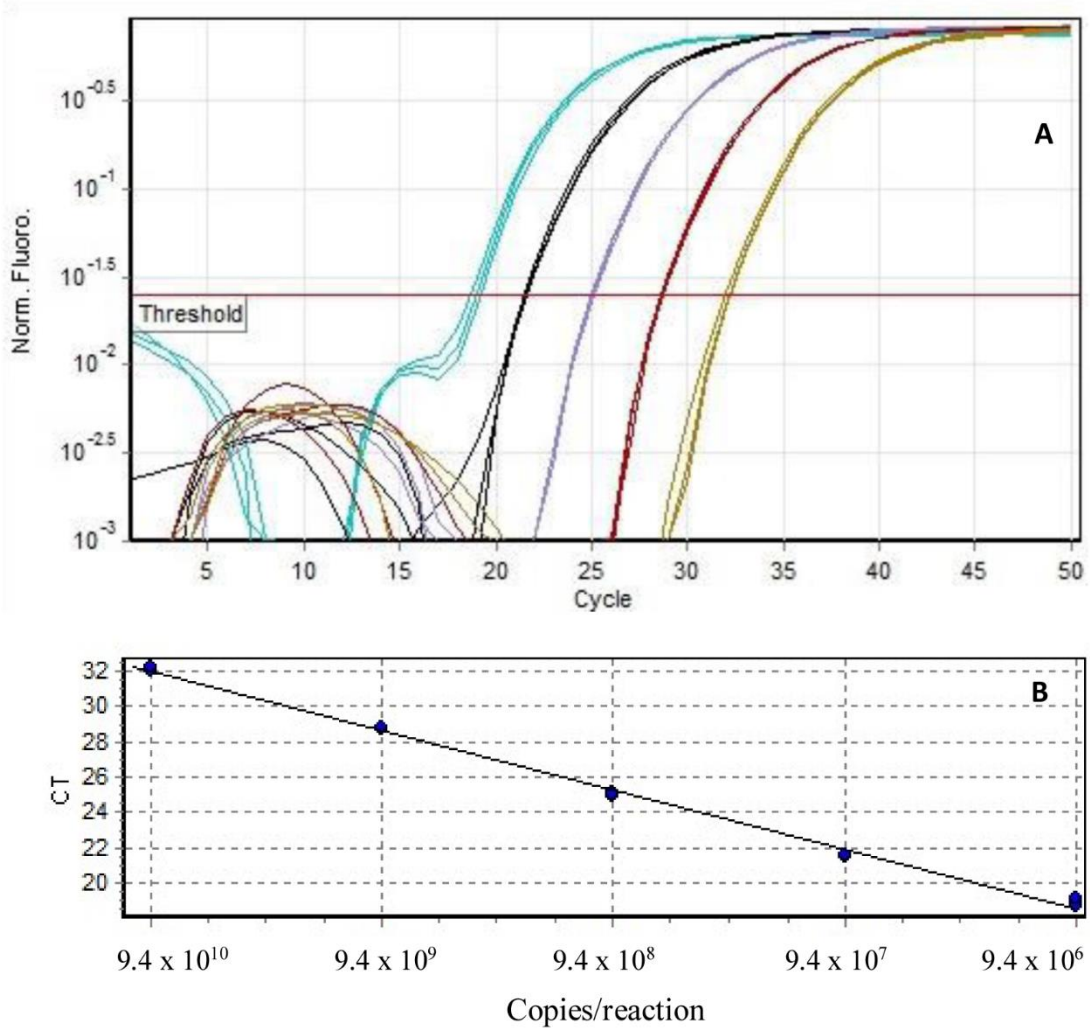


Fig. 30. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *Prx1c* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.351x + 42.032$. The slope of the line corresponded to an amplification efficiency of 0.99. Norm. Fluoro. = normalised fluorescence.

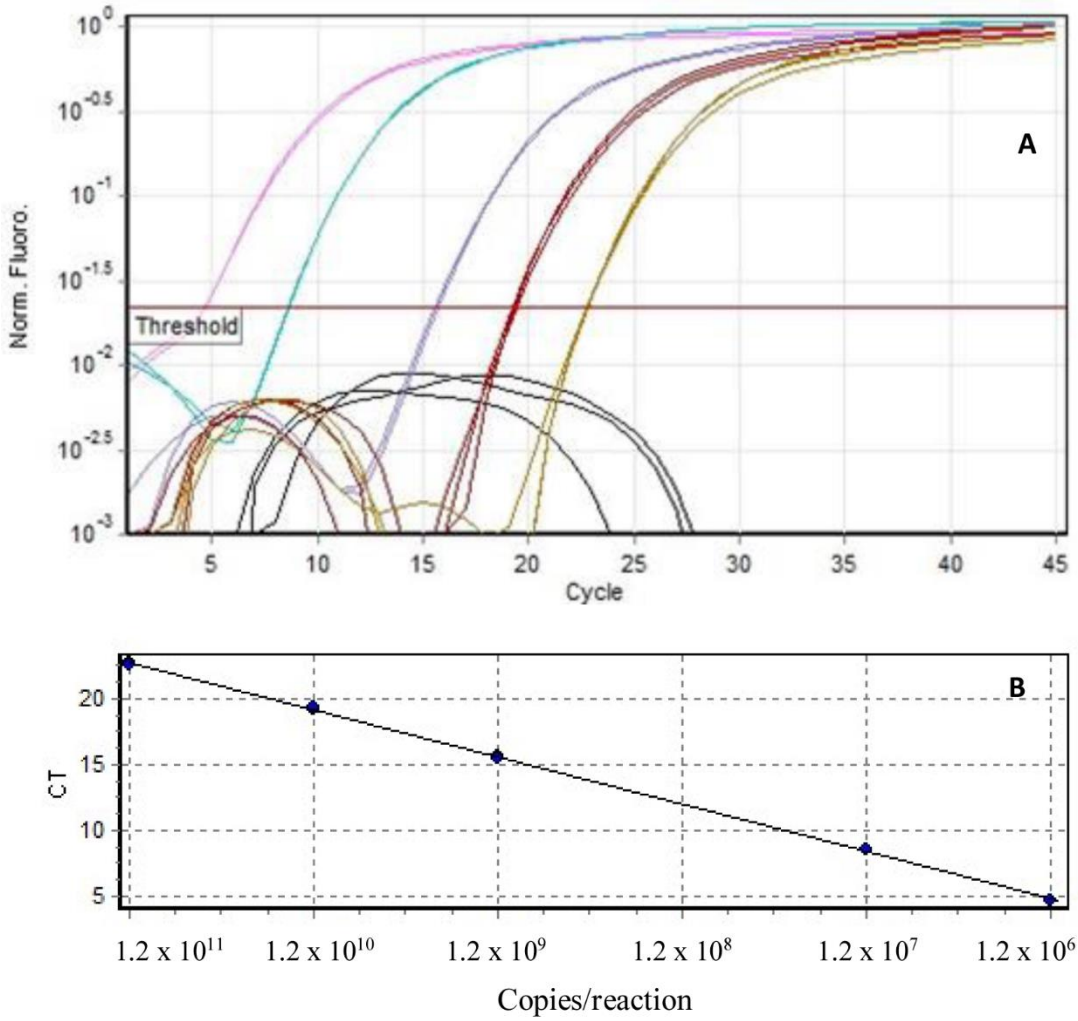


Fig. 31. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *GPx1* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.599x + 33.620$. The slope of the line corresponded to an amplification efficiency of 0.90. Norm. Fluoro. = normalised fluorescence.

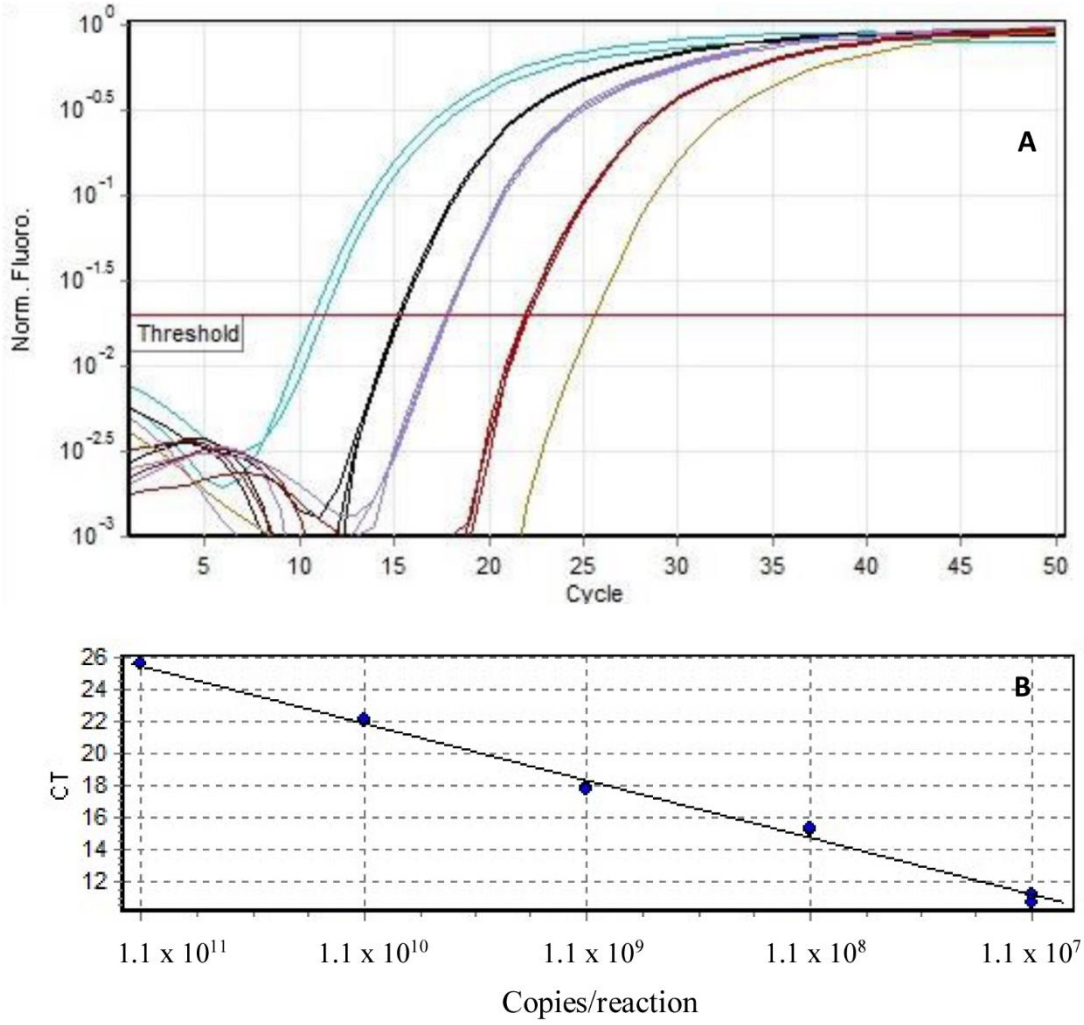


Fig. 32. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *GPx2* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.559x + 36.136$. The slope of the line corresponded to an amplification efficiency of 0.91. Norm. Fluoro. = normalised fluorescence.

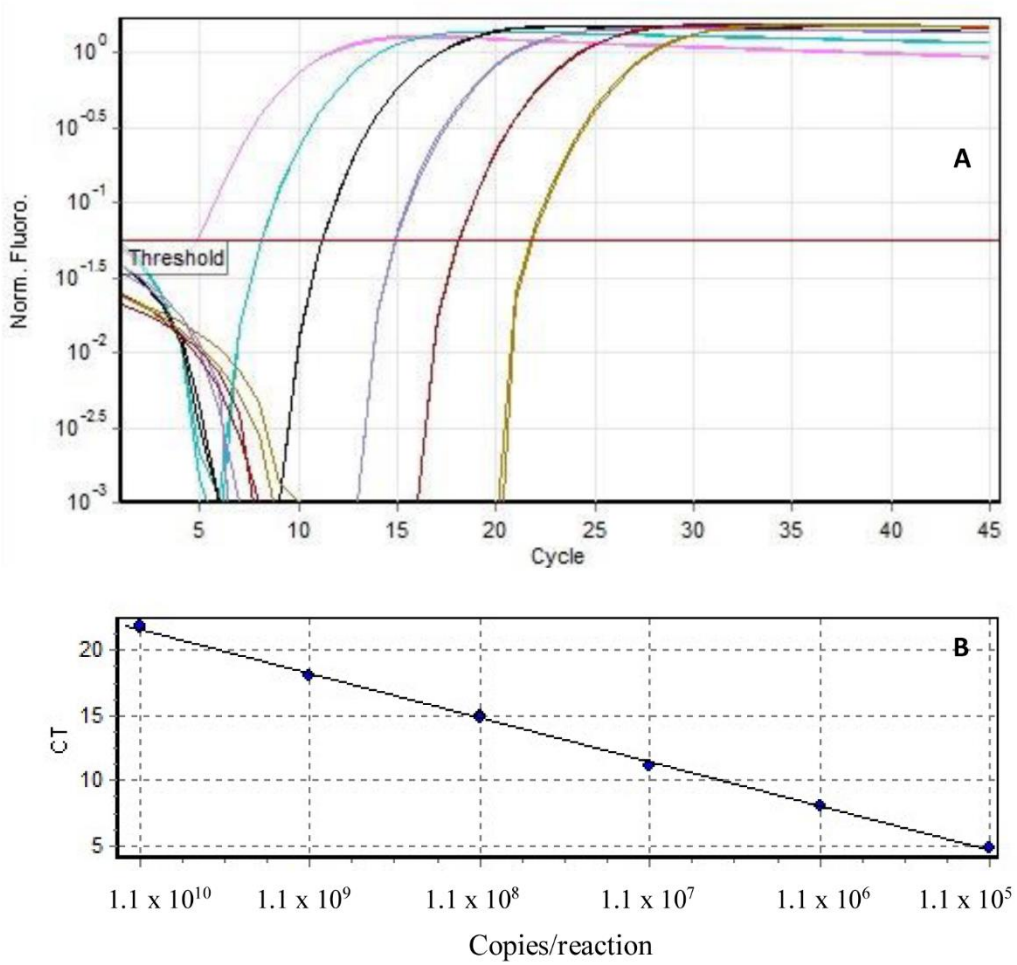


Fig. 33. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *GPx3* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.374x + 35.077$. The slope of the line corresponded to an amplification efficiency of 0.98. Norm. Fluoro. = normalised fluorescence.

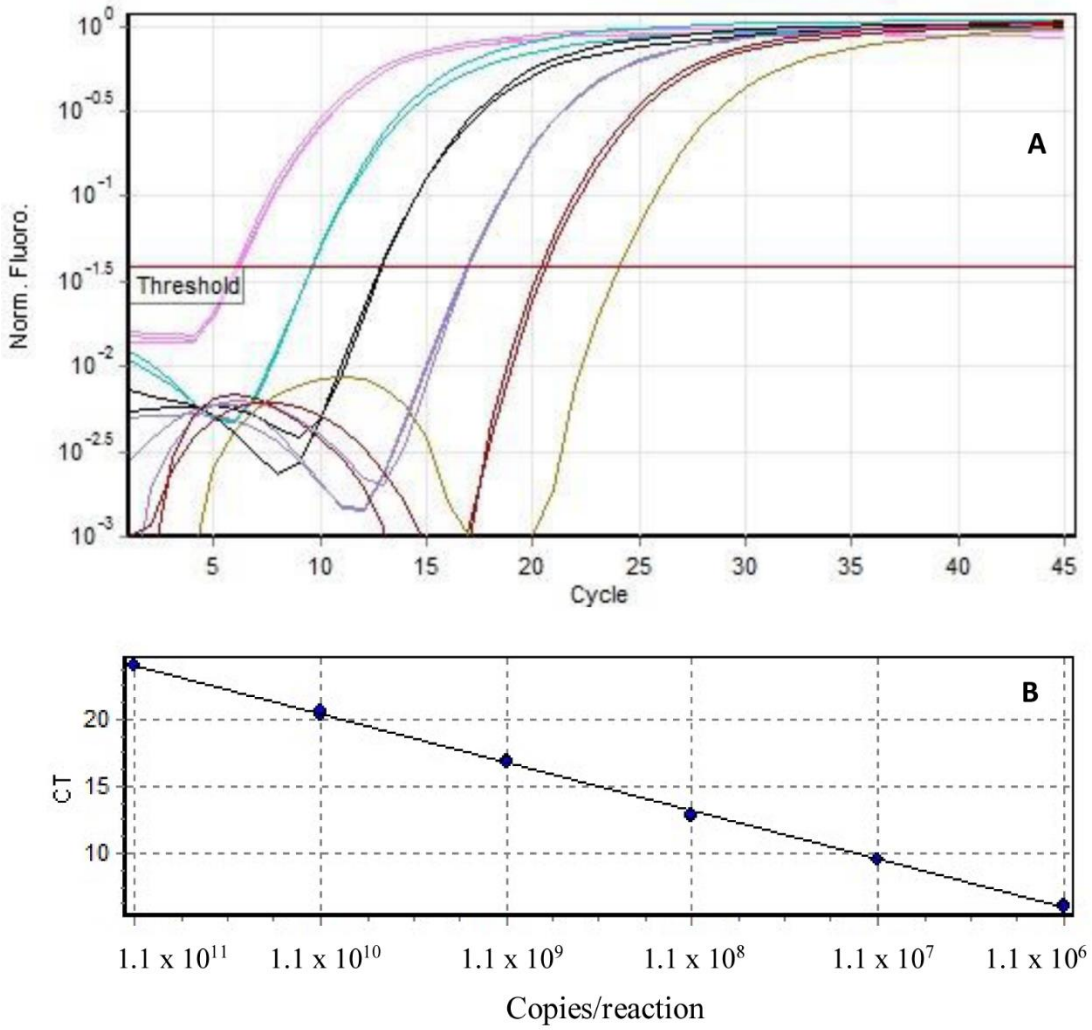


Fig. 34. Fluorescence curve (A) and standard curve (B) obtained using serial dilutions of a purified *PHGPx9* PCR product from *T. thermophila* cells as the template for qRT-PCR (Section 5.2.6). The cycle at which the fluorescence intensity crossed a defined threshold (Ct value) was calculated using the fluorescence curve. The standard curve was constructed by plotting the Ct values against the copy numbers. The equation for the standard curve was $y = -3.602x + 34.828$. The slope of the line corresponded to an amplification efficiency of 0.90. Norm. Fluoro. = normalised fluorescence.

Appendix A.7 – Melt curve analysis used to determine whether there was only one PCR product for each set of primers

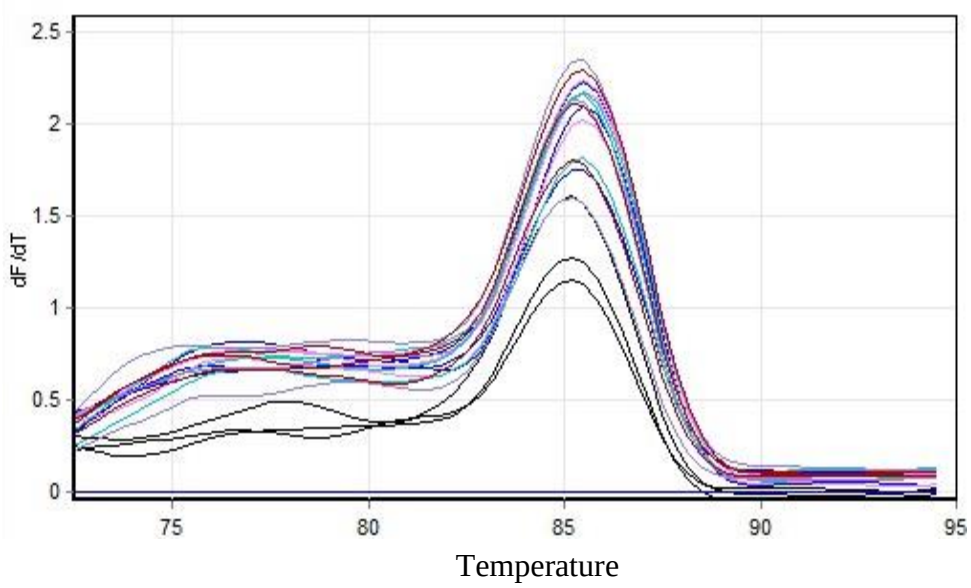


Fig. 35. Melt curve for GAPDH amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 86°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

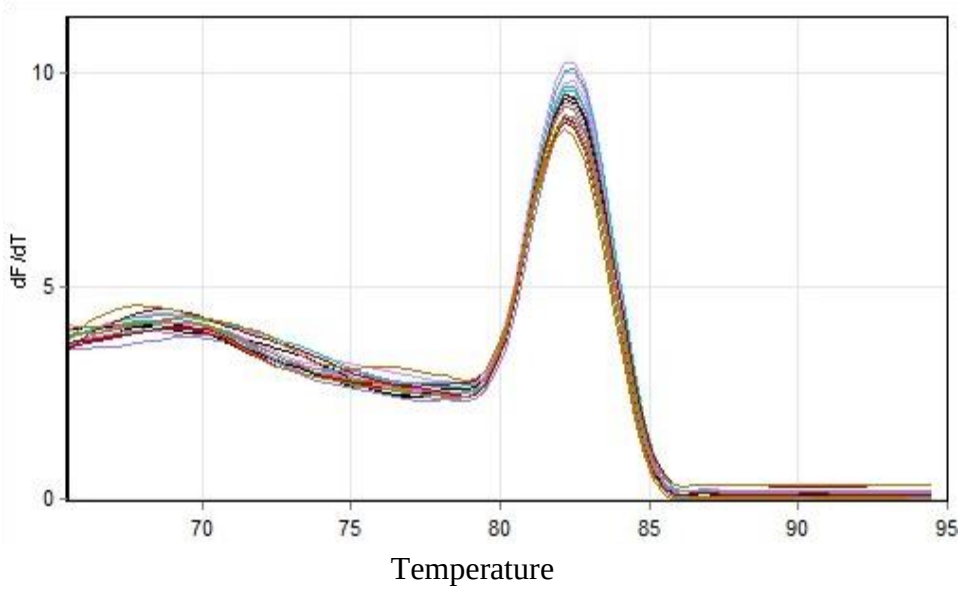


Fig. 36. Melt curve for catalase amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 82.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

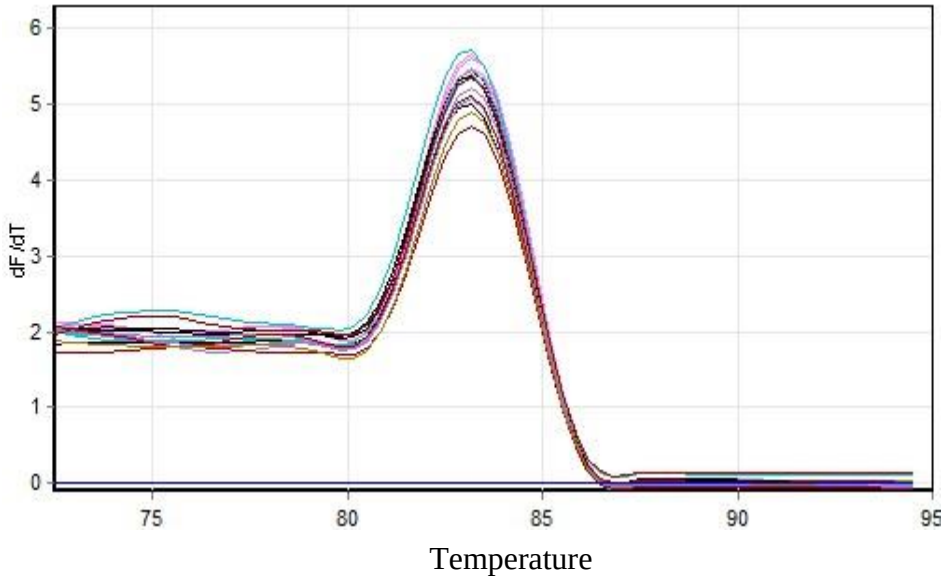


Fig. 37. Melt curve for *Prx1a* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 83°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

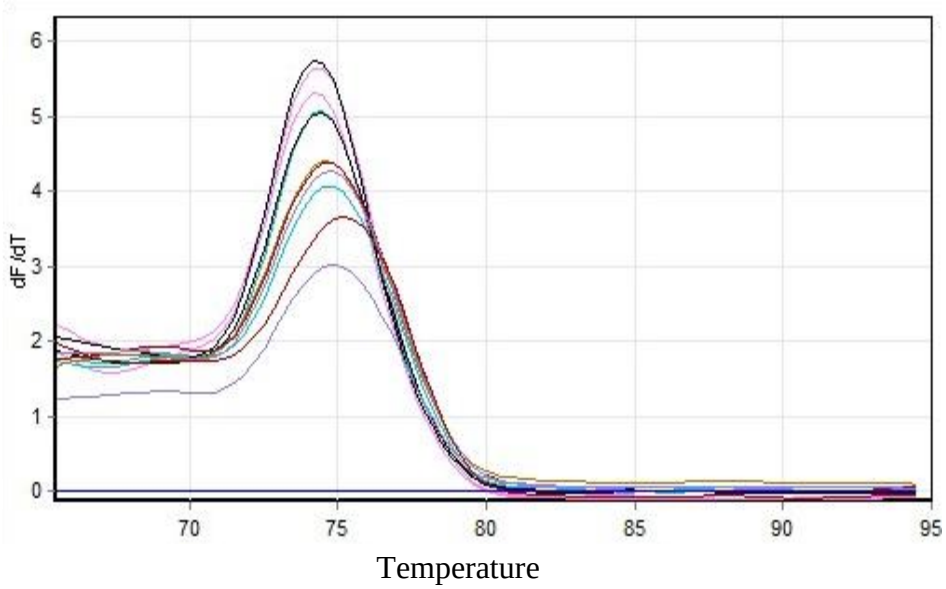


Fig. 38. Melt curve for *Prx1b* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 74.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

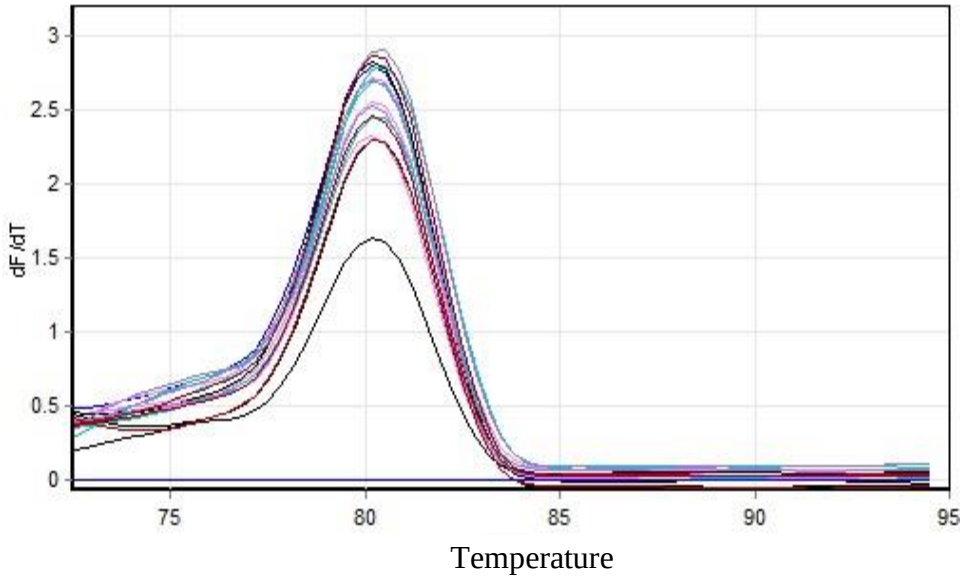


Fig. 39. Melt curve for *Prx1m* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 81°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

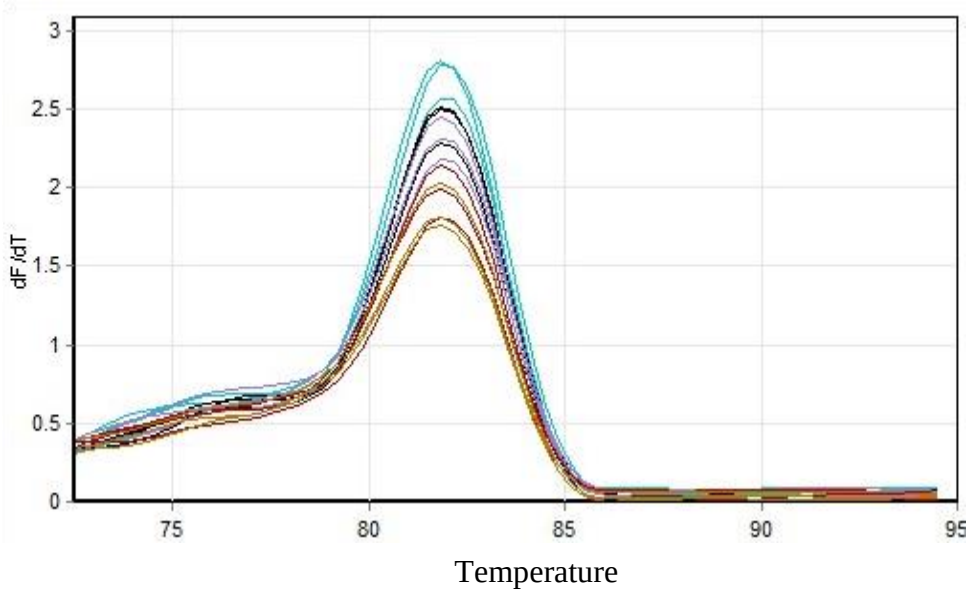


Fig. 40. Melt curve for *Prx1c* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 82°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

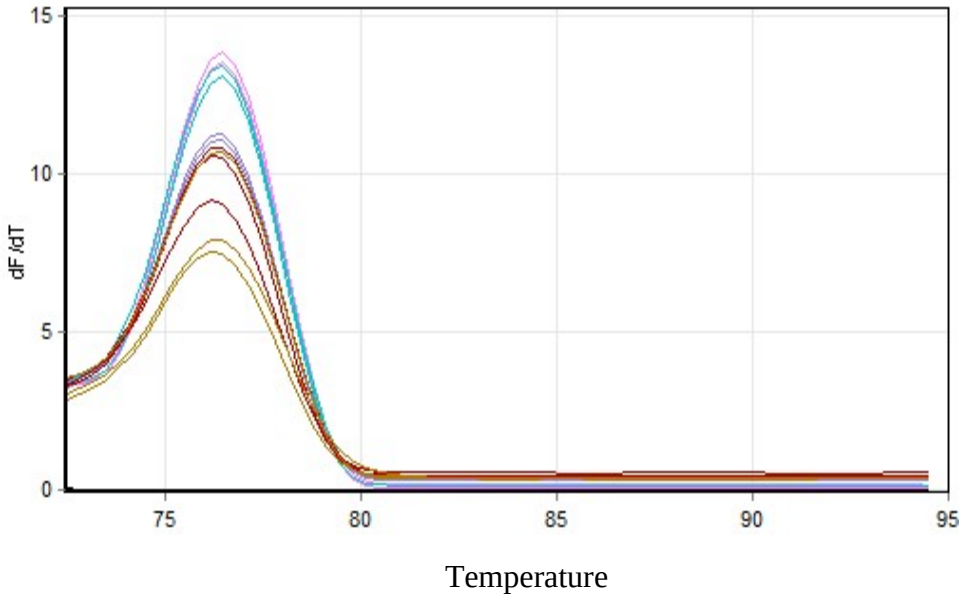


Fig. 41. Melt curve for *GPx1* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 76.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

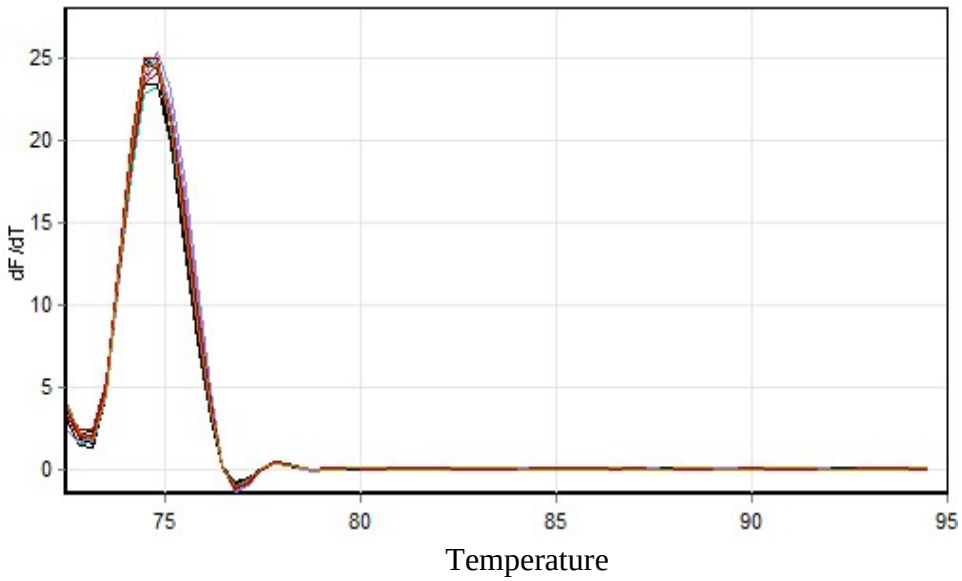


Fig. 42. Melt curve for *GPx2* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 74.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).

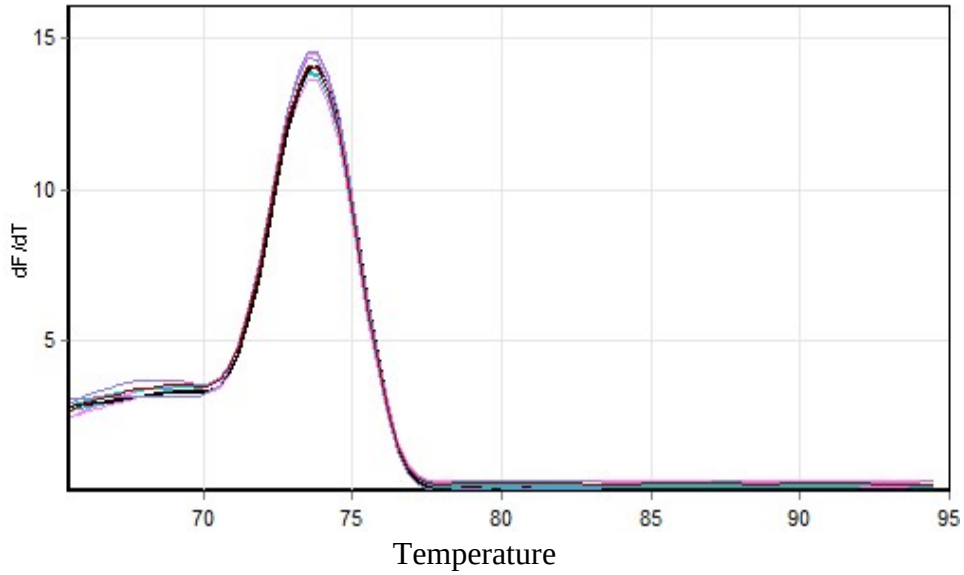


Fig. 43. Melt curve for *GPx3* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 73.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (°C).

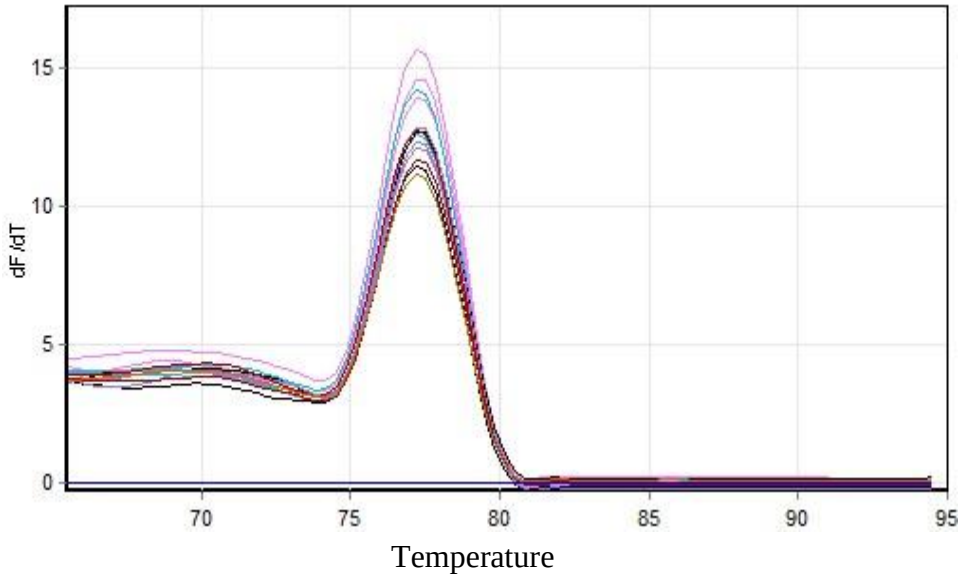


Fig. 44. Melt curve for *PHGPx9* amplicons produced using qRT-PCR (Section 5.2.6). Peaks on the graph correspond to the dissociation of different double-stranded DNA products. The graph shows a single dominant peak at approximately 77.5°C, which shows that a single dominant product was produced by the qRT-PCR reaction. dF/dT = the change in relative fluorescence (RFU) per unit temperature (^{o}C).