# Terpenoid inhibition of DsbA; a periplasmic protein from the dysentery-causing bacterium, *Shigella sonnei*

**By**

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## Author's Declaration

This thesis is the result of the author's original research. It has been composed by the author and had not been previously submitted for examination which has led to the award of a degree.



Signed:

Date:

## Dedication

*To my late father for whom I shall always be indebted to. To my mother who pray for me at morning and evening and waiting me to back to her. Dad and Mum, you prepared me to face the challenges with faith and humility; always had confidence in me and offered me encouragement and support in all my endeavours. I know you would have been very proud to see this moment when I graduate with my doctorate degree. Dad I miss you too much, love you. Mum I'm going to back to you as soon as possible, love you.*

*I also dedicate this work to my lovely wife Hala, and also to my kids, Ahmed, Maram, Lara and the naughty little one Lina. I feel very grateful and humbled to have such a great family who supported and joined me throughout my journey.*

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**Abstract**

A major challenge faced by modern medicine is antimicrobial resistance that has become a major problem in recent year. As such, there is a requirement for the introduction of alternative approaches to treat infections of multi-resistant strains of bacillary dysentery-caused by *Shigella sonnei.* DsbA is a disulphide bond oxidoreductase enzyme and is a major contributor to virulence in *S. sonnei*. It is a periplasmic protein required for *Shigella* survival in the host cell cytosol. DsbA is found in all Gram-negative and some Gram-positive bacteria, therefore, targeting DsbA is a logical strategy to treat dysentery-causing *Shigella*. Here we show that terpenoids, natural substances present in the essential oils of plants, can reduce *S. sonnei* proliferation inside host cells. Terpenoids exert little cytotoxicity on the cell lines and protected *Galleria mellonella* larvae from killing by *S. sonnei*. Geraniol was the most potent compound from the twelve terpenoids tested in this study. In addition, the study confirmed that DsbA is vital for *S. sonnei* to survive and proliferate in a reducing environment. Geraniol was able to reduce the activity of purified DsbA protein on reduction of Di-E-GSSG in an *in vitro* assay. Importantly, this study also showed that *S. sonnei* could catalyse E-GSH conversion to Di-E-GSSG through DsbA activity. This finding explained for the first time a novel mechanism used by *S. sonnei* in recycling non-functional reduced DsbA to its functional oxidised form. Moreover, geraniol was also able to inhibit the catalysis of E-GSH to around half of the wild type DsbA activity in *S. sonnei*. Geraniol became completely inactive when its interaction sites were substituted on DsbA, and it was unable to protect *Galleria mellonella* from killing by *S. sonnei dsbA* mutants. These findings suggest that geraniol holds a great therapeutic potential against *Shigella* infection.

## Publication and Posters

### Publication

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### Posters

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**List of Abbreviations**

| | |
|---|---|
| ABM | Actin-based motility |
| ATG | Autophagy related genes |
| BfpA | Bundle-forming pilus A |
| CFU | Colony forming unit |
| Di-E-GSSG | Di-eosin isothiocyanate oxidised glutathione |
| DsbA | Disulphide bond A |
| DSB | Disulphide bond system |
| DMSO | Dimethyl sulfoxide |
| DTT | Dithiothreitol |
| EIEC | Entero-invasive *Escherichia coli* |
| EITC | Eosin isothiocyanate |
| EPEC | Enteropathogenic *Escherichia coli* |
| EcDsbA | *Escherichia coli* disulphide bond A |
| ER | Endoplasmic reticulum |
| Ero1 | Endoplasmic oxedoreductin 1 |
| Eos | Essential oils |
| EtBr | Ethidium bromide |
| FBDD | Fragment based drug discovery |
| FlgI | Flagellar P-ring motor protein |
| GSH | Reduced Glutathione |
| GFP | Green fluorescent protein |
| HeLa | Human cell line derived from cervical cancer |
| HEK293 | Human Embryonic Kidney 293 cells |
| IcsA | *Shigella* surface protein responsible for actin polymerisation |
| IcsB | *Shigella* protein responsible for evading autophagy |

| | |
|---|---|
| IL | Interleukin |
| Ipa | Invasion plasmid antigens |
| IPTG | Isopropyl-ß-D-1-thiogalactopyranoside |
| M cell | Microfold cell |
| MDR | Multidrug-resistant |
| MIC | Minimum inhibitory concentration |
| Mxi | Membrane expression of invasion plasmid antigens |
| MOI | Multiplicity of infection |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NK | Natural killer |
| NF-κB | Nuclear factor κB |
| Nod1 | Nucleotide-binding oligomerisation domain protein 1 |
| NDP52 | Nuclear dot protein 52kDa |
| NmDsbA | *Neisseria meningitides* DsbA |
| PAGE | Polyacrylamide gel electrophoresis |
| PAI | Pathogenicity Island |
| PDI | Protein disulphide isomerase |
| Pef | Plasmid encoded fimbriae |
| PMN | Polymorphonuclear cells |
| PI3K | phosphatidylinositol-3-kinase |
| p62 | Sequestosome 1 |
| PBS | Phosphate buffer saline |
| RAW 264 | Mouse leukaemic monocyte macrophage cell line |
| RFU | Relative fluorescence unit |
| SDS | Sodium dodecyl sulphate |
| SHI | Shigella Pathogenicity Island |

| | |
|---|---|
| Spa | Surface presentation of invasion plasmid antigens |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline tween |
| Tcp | Toxin co-regulated pilus |
| TEMED | N,N,N′,N′-Tetramethylethylenediamine |
| Trx | Thioredoxin |
| T3SS | Type three secretion system |
| VP | Virulence plasmid |
| UPEC | Uropathogenic *Escherichia coli* |

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# Chapter 1

# General Introduction

# 1 General Introduction

## 1.1 Introduction

Bacillary dysentery, also known as shigellosis is still a major health problem in the 21st century (Sansonetti et al., 2000). There are around 165 million cases of dysentery worldwide per year, causing over one million deaths, most of which occur in children under 5 years of age (Peirano et al., 2005). Shigellosis is prevalent in developing countries, with approximately 125 million cases occurring annually in Asia, of which, nearly 14,000 are fatal (Acharyya et al., 2015). On the other hand, there are up to 280,000 cases that occur every year in the USA (Bradley and Jackson, 2011).

The epithelium layer of the colon and the rectum is the site of infection (Ud-Din and Wahid, 2014), with *Shigella* being the main causative infectious agent of bacillary dysentery. Bloody diarrhoea, nausea, vomiting and abdominal pain are the principal symptoms for dysentery, with an underlying hyper-inflammation pathology in the colorectum. Bacteria of the genus *Shigella* are transmitted by the faecal-oral route via contaminated food or water, with humans being the main hosts of *Shigella,* although non-human primates are also susceptible to the infection (Acharyya et al., 2015, Rohmer et al., 2014). *Shigella* has a significant level of invasion with very low infectious doses (10–100 CFU) causing the aforementioned symptoms (Rohmer et al., 2014).

Previously, *Shigella sonnei* was thought to be responsible for dysentery in developed countries, but it is clear now that *S. sonnei* is replacing the more diverse *S. flexneri*, becoming problematic in the developing world, especially in places undergoing water quality improvements and economic development (Vinh et al., 2009, Kotloff et al., 1999). However, treatment and prevention of *Shigella* infection have proven to be difficult; mainly due to the low inoculum needed to cause the disease (Jennison and

Verma, 2004) and inadequate empirical therapy options secondary to antimicrobial resistance (Replogle et al., 2000). The world health organisation (WHO) called for the development of vaccines to prevent the morbidity and mortality resulting from *Shigella* infection in 1997 (WHO, 1997). However, despite international efforts over the last five decades (Camacho et al., 2013, Levine et al., 2007), there are still no effective vaccines. Currently, several vaccines are under development using bacterial components or killed or live attenuated bacteria; some of which are being tested in different clinical phase trials (WHO, 2006).

### 1.2 *Shigella* is resistant to antimicrobial therapy

The current treatments for shigellosis are fluoroquinolones for adults and cephalosporins for children (Kim et al., 2015). However, multidrug resistant (MDR) *Shigella* strains, particularly the *S. sonnei* lineage III, have become prevalent worldwide, especially in developing and newly-industrialised countries. In California in the United States, large outbreaks of shigellosis occurred in 2014 and 2015, which were later confirmed to be caused by *S. sonnei* lineage III. Most isolates from these outbreaks were resistant to fluoroquinolone and ciprofloxacin; and were related to a fluoroquinolone-resistance lineage from South Asia (Kozyreva et al., 2016). In 2015, another outbreak of dysentery caused by a *S. sonnei* strain resistant to extended-spectrum cephalosporins and fluoroquinolones was reported in the Republic of Korea (Kim et al., 2015). In India, De Lappe and his colleagues reported a *S. sonnei* strain resistant to ciprofloxacin (De Lappe et al., 2015). Yet, there is no vaccine available for shigellosis, therefore there is an urgent need to develop new anti-shigellosis drugs to combat this growing healthcare challenge.

## 1.3 Molecular and cellular pathogenesis of *Shigella* infection

The genus *Shigella* contains Gram-negative, non-motile, non-sporeforming and rod-shaped bacteria that were discovered in 1898 by the Japanese scientist, Kiyoshi Shiga (Trofa et al., 1999, Tran Van Nhieu et al., 2000, Peng et al., 2009). The main transmission route of *Shigella* spp. is the faecal-oral, whereby the bacterium enters the human body by ingestion of contaminated food and water. The genus *Shigella* is traditionally divided into four groups: *Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei*. It is now clear that all these types derive from multiple origins from the commensal bacterium *Escherichia coli* Figure (1.1) (Niyogi, 2005, Parsot, 2005). Each of these species belong to a specific subgroup: *Shigella dysenteriae* (subgroup A) has 15 serotypes; *Shigella flexneri* (subgroup B) has 14 serotypes; *Shigella boydii* (subgroup C) has 20 serotypes and *Shigella sonnei* (subgroup D) has one serotype (Peng et al., 2009). Apart from *Shigella*, the entero-invasive *Escherichia coli* (EIEC) can also cause dysentery. *Shigella* and EIEC possess the same virulence plasmids which confer primary determinants and can be differentiated by a few biochemical characteristics, including serological tests for O-antigens (Lan et al., 2001).

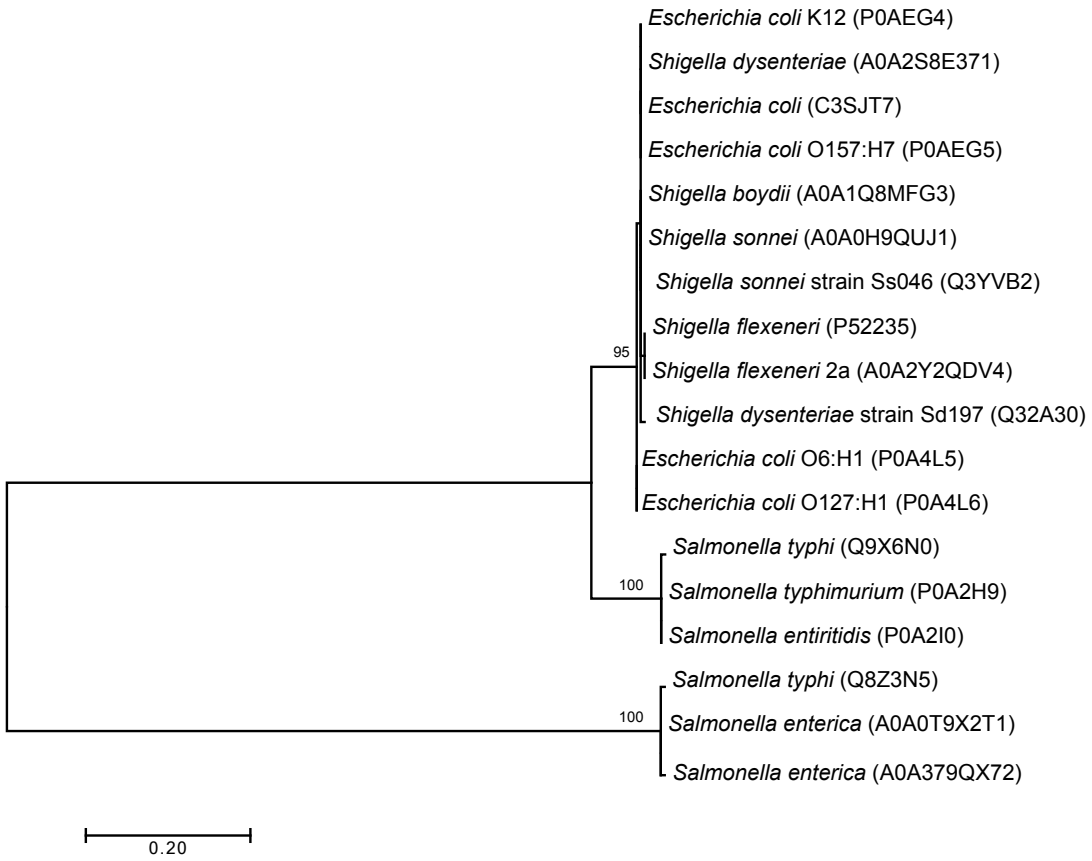



**Figure 1.1: Maximum Likelihood tree constructed using concatenated sequences of *dsbA* from *Escherichia coli* spp., *Shigella* spp. and *Salmonella* spp.**

Clustal X (2.0) was used to align the sequences. MEGA7 and model WAG+I (Hasegawa-Kishino-Yano) with 500 bootstraps was used to construct the phylogenetic tree.

Knowledge about *Shigella* pathogenesis largely comes from studies on *S. flexneri.* The *Shigella* genome is composed of a single circular chromosome, varying in size from 4.3 to 4.8 Mb, as well as a circular virulence plasmid (VP) of approximately 220 kb. The bacterial chromosome and the large VP encode the genetic information that generate the phenotype of *Shigella* spp. The VP is a fundamental determinant of *Shigella* spp. and encodes the molecular machinery necessary for tissue invasion and intracellular life (Yang et al., 2005, Sansonetti et al., 1983). The pathogenicity islands (PAI) are large mobile genetic entities located on the VP in the *Shigella*. PAI are characterised by their nucleotide composition and codon usage differing from those of the rest of the genome and they have a mosaic-like structure associated with mobile genetic elements and genetic instability (Schmidt and Hensel, 2004, Schroeder and Hilbi, 2008). In addition to PAI on the VP, *Shigella* pathogenicity islands (SHI) have been identified in the chromosome. The presence and genomic localisation of SHI differ between *Shigella* strains and may play a role in the variation of virulence phenotypes (Ingersoll et al., 2002, Schmidt and Hensel, 2004, Peng et al., 2006).

*Shigella* spp. are also able to manage and down-regulate the expression of antimicrobial peptides; important antibacterial effectors released from the mucosal surfaces of the intestinal tract. The bacteria reach the large intestine after passing through the stomach and small intestine, where they establish the infection (Islam et al., 2001). The intestinal epithelium layer functions as a physical and functional barrier, preventing invasion by pathogenic as well as commensal bacteria (Parsot, 2005). The first step of *Shigella* pathogenesis is to invade epithelial cells by passing through M cells because *Shigella* does not have the ability to invade the epithelial barrier from the apical side (Man et al., 2004). However, a recent study showed that *Shigella* could target colonic crypts in the early stage of invasion (Arena et al., 2015). Once reaching the underlying mucosal lymphoid tissue, *Shigella* triggers immune

responses by interacting with macrophages and can survive inside macrophages by inducing apoptosis (Zychlinsky et al., 1996, Islam et al., 1997). In the macrophages, IpaB binds to interleukin 1 (IL-1), activating apoptosis and macrophage death. The apoptotic macrophages release pro-inflammatory cytokines IL-1β and IL-18 (Sansonetti et al., 2000). IL-1β is a potent recruiter of neutrophils to the site of infection, while IL-18 is responsible for activating the natural killer (NK) cells and enhancing the production of interferon (IFN-γ) (Schroeder and Hilbi, 2008). After being released from dying macrophages, *Shigella* invades the epithelial cells from the basolateral side, escaping and proliferating in the epithelial cytoplasm (Parsot, 2005). By using the actin-based motility (ABM) system, *Shigella* can spread to the adjacent epithelial cells mediated by surface protein IcsA (VirG) (Monack and Theriot, 2001). *Shigella* can activate the nuclear factor κB (NF-κB) by interacting with the nucleotide-binding oligomerisation domain protein 1 (Nod1), which is an intracellular receptor, able to recognise the bacterial peptidoglycan segments and trigger the production and secretion of IL-8 (Pedron et al., 2003). The latter plays an important role in recruiting polymorphonuclear neutrophils (PMN) to the site of infection. PMN can infiltrate and destroy the integrity of the epithelial layer, allowing new bacteria to invade the sub-mucosa as shown in Figure (1.2) (Singer and Sansonetti, 2004, Pedron et al., 2003). By this mechanism, *Shigella* can weaken the tight junctions of the epithelial cell layer and change the protein composition of these junctions, also killing macrophages and disrupting the epithelial layer by massive PMN infiltration (Mandic-Mulec et al., 1997, Sakaguchi et al., 2002).

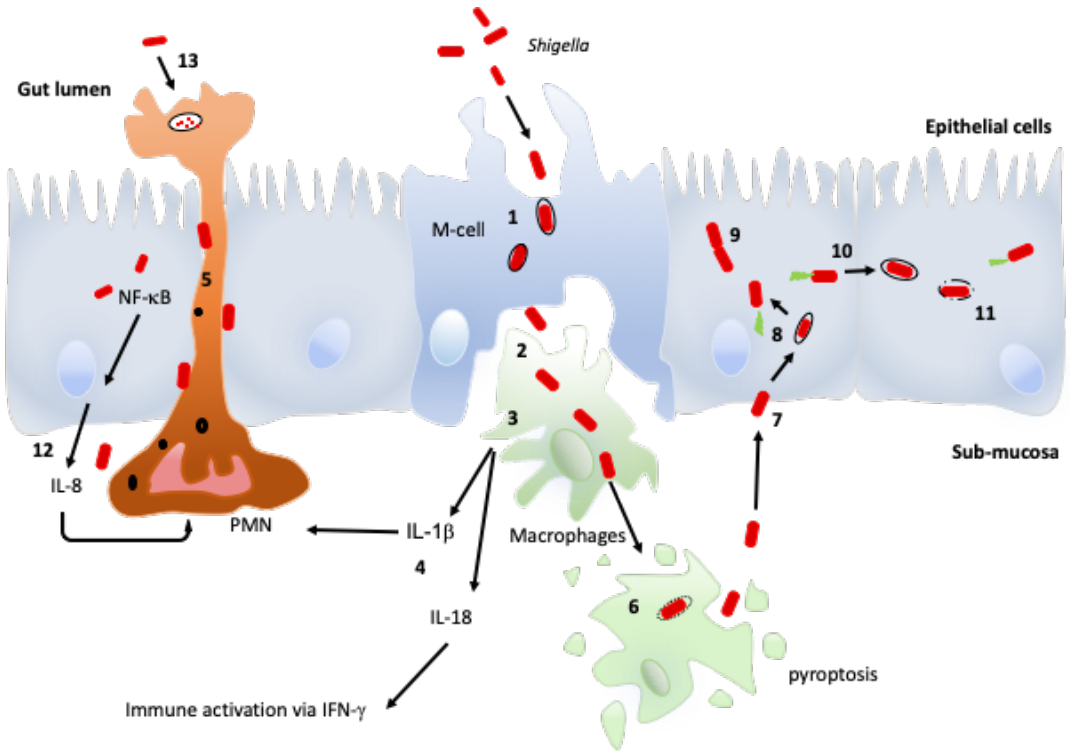



**Figure 1.2: Mechanism of *Shigella* invasion into the sub-mucosa and induced apoptosis inside macrophages.**

Entry into the colonic epithelium is mediated via M-cell membrane ruffling (1) and epithelial barrier destabilisation. The bacteria are then transported to the M-cell pocket, where they are endocytosed by resident macrophages (2). Epithelial barrier destruction is mediated by the pro-inflammatory IL-1 and chemotaxic IL-8 produced by neighbouring epithelial cells. PMN (12) are recruited and travel from the basolateral to the apical colonic epithelium, destabilising the junctions between the epithelial cells, thereby allowing further invasion of *Shigella* (5). Induction of pyroptotic macrophage death occurs after *Shigella* escape from the phagocytic vacuole (3 and 6). Activated caspase-1 cleaves and activates IL-1β and IL-18, leading to the release of these pro-inflammatory cytokines (4). Uptake of *Shigella* is a macropinocytic process at the basolateral membrane of epithelial cells (7). Stimulation of Rho-family GTPases triggers actin polymerisation and depolymerisation, forming filopodial and lamellipodial extensions of the epithelial membrane, leading to engulfment of the bacilli. Lysis of the macropinocytic vacuole allows *Shigella* to gain access to the epithelial cytoplasm, where it rapidly multiplies, escapes autophagy and fragments the Golgi (8 and 9). Exploitation of the epithelial actin assembly machinery allows *Shigella* to move both intra- and intercellularly (10). Protrusions mediated by bacilli are actively endocytosed by the clathrin-mediated endocytic pathways at intercellular junctions, and the double membrane vacuole is lysed to give *Shigella* access to the neighbouring cells cytoplasm (11). PMN leukocytes eventually eliminate *Shigella* infection from the colonic epithelium (13) (adapted from (Schroeder and Hilbi, 2008).

## 1.4 *Shigella* and the innate immunity

Microorganisms have evolved various strategies for survival and replication within host cells, with some residing in vacuoles, while others escape to the cytoplasm (Aggarwal et al., 2016). Many intracellular pathogens can avoid degradation by various methods, such as escaping from phagosomes, preventing lysosome fusion and delaying phagolysosome biogenesis (Luzio et al., 2007). For example, *E. coli* K1 prevents lysosome fusion by means of its capsule, while *Salmonella enterica* and *Mycobacterium tuberculosis* use a delay phagolysosome biogenesis to escape autophagy (Xu and Eissa, 2010). *Shigella* relies on type three secretion system (T3SS) to escape to the cytosol and on IcsB protein to evade autophagy, as IcsB prevents the bacterial colocalisation with the autophagosome. In addition, *Shigella* can move freely in the cytoplasm of the host cell (Ogawa et al., 2005). IcsA is responsible for actin polymerisation and autophagosome formation (Ogawa and Sasakawa, 2006). Another cellular mechanism, called septin cage formation, can work in conjunction with autophagy against *Shigella*. Septins are GTP-binding proteins, first discovered in *Saccharomyces cerevisiae,* which play a key role in cell division, cytoskeletal dynamics and membrane remodelling (Hall and Russell, 2004). Septin cage formation prevents intracellular *Shigella* from spreading and guiding bacteria to degradation by autophagy (Mostowy et al., 2010).

Autophagy is the process which delivers the cytoplasmic material to the lysosome for degradation. It consists of three types: macro-autophagy, micro-autophagy and chaperone-mediated autophagy (Lin and Baehrecke, 2015) and is a critical process in cellular functions, such as response to starvation, pathogens and cell death. The degradation of both intrinsic and exogenous components by autophagy occurs in lysosomes (Yang and Klionsky, 2010). Autophagy is selective and plays an important role in eliminating intracellular pathogens, either enhancing or limiting bacterial

replication *in vitro* and *in vivo* (Lin and Baehrecke, 2015). Bacterial autophagy is called xenophagy and the cytosolic sensors are activated by autophagy receptors such as sequestosome 1 (p62) and nuclear dot protein 52 kDa (NDP52). There are two pathways of autophagy, canonical and non-canonical: the canonical autophagy pathway process is dependent on the systematic recruitment of autophagy-related proteins ATG (Feng et al., 2014), whereas the non-canonical autophagy pathway is dependent on some ATGs to help in an autophagosome-like vacuole. Macro-autophagy is an important mechanism that cells use to control many different pathogens, using lysosomal enzymes to degrade the cytoplasmic components that the cells do not require (Luzio et al., 2007, Corrado et al., 2016). Three types of macro-autophagy have been recently discovered: mitophagy, which depends on mitochondria; xenophagy which is specific for bacteria, and pexophagy which depends on peroxisomes (Aggarwal et al., 2016).

### 1.5 *Shigella* virulence factors

The hydrophobic barrier in the cell envelope of Gram-negative bacteria acts against protein secretion on the bacterial cell surface. Therefore, the bacteria recognise substrates secretion on the inner membrane and actively transport it through the outer membrane using different conservative mechanisms called the secretin systems (designated types I to VI) (Schuch and Maurelli, 2001). These mechanisms are easily distinguished by different components consisting of more than forty elements. The T3SS is one of the most important specialised virulence mechanisms in bacterial pathogenesis in mammalian and plant hosts (Plano et al., 2001). The most important of these is the Mxi-Spa locus (also known as PAI), ~30 kb in size, which encodes the T3SS responsible for injecting effector proteins into host cells and initiating cell

invasion (Yang et al., 2005, Park et al., 2011). Under electron microscopy, T3SS resembles a needle-like structure, consisting of a neck and a bulb, which anchors onto the bacterial surface as shown in Figure (1.3) (Tran Van Nhieu et al., 2000). In addition to the T3SS system, the large VP and especially, the Mxi-Spa locus gene, also express several proteins (e.g. IpaA, IpaB, IpaC, IpaD and VirG or IcsA) which play essential roles in invading and spreading inside the host cells (Hesaraki et al., 2013).

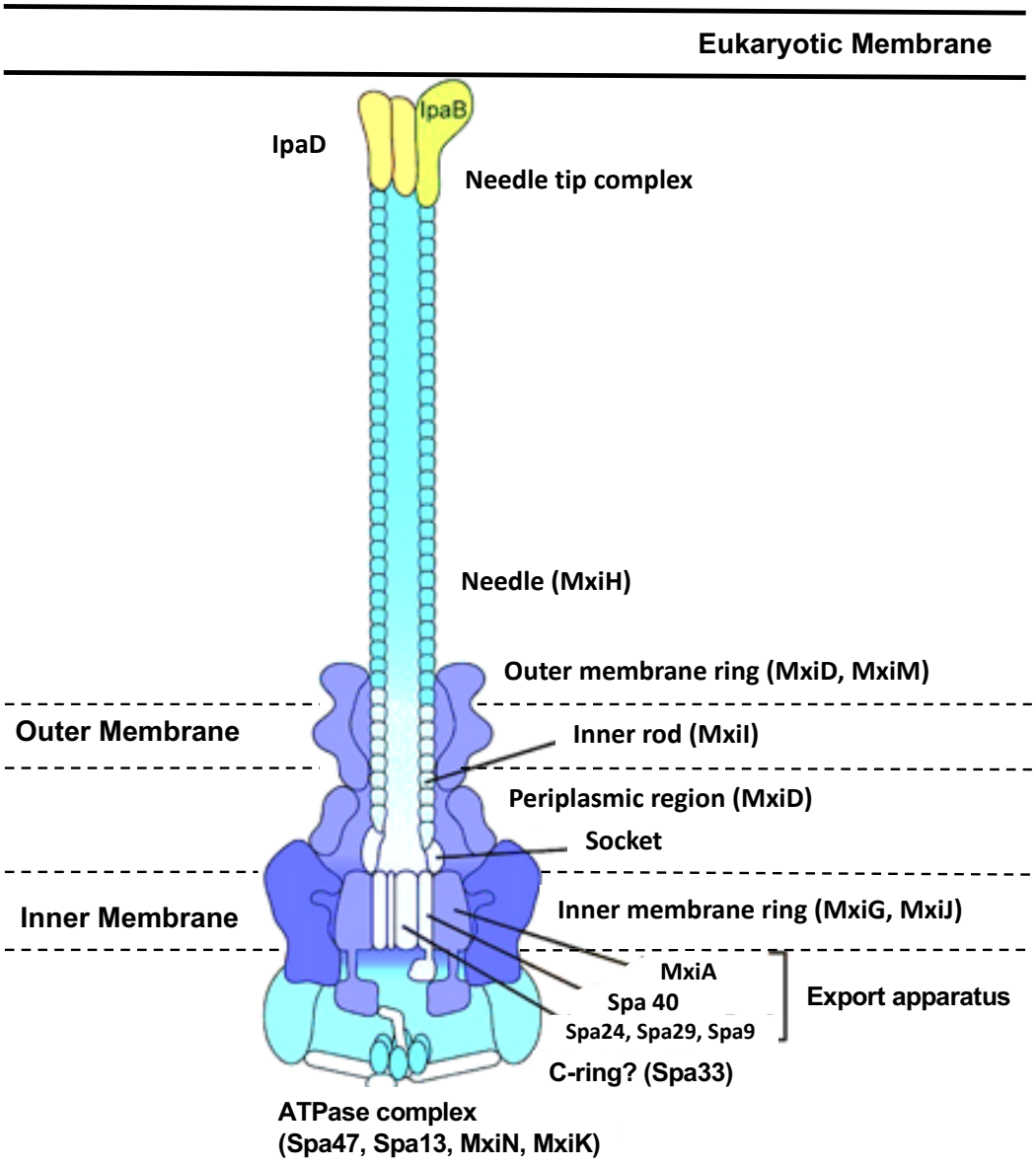



**Figure 1.3: Schematic drawing of the *Shigella* T3SS.**

The architecture of *Shigella* T3SS is based on the VP Mxi-Spa locus, which encodes proteins that produce the T3SS apparatus, a structure composed of a 60 nm hollow extracellular needle, a transmembrane domain, and a cytoplasmic bulb (adapted from (Mattock and Blocker, 2017).

### 1.5.1 *Mxi* genes

There are three major constituents of the base structure of *Shigella* T3SS, MxiD, MxiJ and MixG as shown above in Figure (1.2) (Blocker et al., 2001, Blocker et al., 1999). The main elements in the transenvelope base are these three ring-forming proteins that form the general structure of the T3SS needle complex (Schuch and Maurelli, 2001). MxiD is a member of secretin family, secretory proteins family, which polymerize into the bacterial outer membrane. MxiD has a large periplasmic protrusion that may extend into the peptidoglycan layer till the inner membrane (Nouwen et al., 1999, Nouwen et al., 2000). MxiG and MxiJ are integral inner membrane proteins that produce comparable membrane complexes (Allaoui et al., 1995, Allaoui et al., 1992). MxiM in *Shigella* spp. is anchored by a lipid moiety to the inner surface of the outer membrane. This protein can react with the MxiD secretin ring and has a cleavable signal sequence and is important for the secretion of T3SS (Schuch and Maurelli, 1999).

### 1.5.2 *Ipa* genes

The Ipa proteins play a vital role in bacterial entry and are secreted during contact with epithelial cell surface (Menard et al., 1994). IpaA proteins are necessary for invading the colonic and rectal epithelial cells and helping the bacteria to spread inside the host cell cytosol (Sansonetti, 2001). Another protein, IpaB, can induce apoptosis in macrophages and in dendritic cells as well as to cause inflammatory infection (Yang et al., 2005). IpaB binds to caspase 1 (IL-1$\beta$ converting enzyme) in the cytosol of macrophages and activates an apoptotic cascade (Chen et al., 1996, Thirumalai et al., 1997). IpaA is responsible for binding and activating the focal adhesion protein, vinculin, by cutting the intramolecular head-tail among the bundle conversion

mechanism (Park et al., 2011). After activation by IpaA, vinculin triggers actin depolymerisation, interacting with IpaB and IpaC to form a channel for effector translocation (IpaB and IpaC form the translocon) (Ramarao et al., 2007, Blocker et al., 1999). IpaC plays a role in actin polymerisation in addition to the formation of the translocon. The IpaD is on the tip of the needle in the T3SS and can bind to translocon proteins, IpaB and IpaC, which work together to connect the needle to the host cell (Parsot, 2005, Lee et al., 2014).

### 1.5.3 Intercellular spread gene (*IcsA*)

IcsA, also known as VirG, is an autotransporter with an atypical N-terminal signal sequence. It is a 120 kDa outer membrane protein and is not dependant on the T3SS for its secretion (Brandon et al., 2003). It was confirmed that *IcsA* is important for intercellular spread of *Shigella* strains as *IcsA* mutant cells were unable to spread within an epithelial monolayer and displayed a negative Sereny test (Bernardini et al., 1989). *Shigella* escapes to the host cell cytosol, proliferates and spreads intra- and intercellularly by hijacking the ABM system, which is mediated by the surface protein IcsA (Goldberg and Theriot, 1995). Recently, it was reported that IcsA plays two roles in *Shigella* pathogenicity, as the mediator of ABM and in the polar adhesion of *Shigella* to epithelial cells (Zumsteg et al., 2014).

## 1.6 Bacterial secretion pathways

One of the essential functions of the prokaryotic cell is proteins transport from cytoplasm into other compartments of the cell or to the environment, this process known as protein secretion. Prokaryotic organisms have developed many ways to transport the proteins between locations, which largely involve the assistance of dedicated protein secretion systems. The most commonly pathways used to transport proteins across the cytoplasmic membrane are the general secretion (Sec) and twin arginine translocation (Tat). These two pathways are the most highly conserved mechanisms of protein secretion (Natale et al., 2008, Robinson and Bolhuis, 2004). The Sec pathway primarily translocates proteins in their unfolded state and this system consists of three parts: a protein targeting component, a motor protein, and a membrane integrated conducting channel, called the SecYEG translocase (Papanikou et al., 2007). The Sec pathway relies on a hydrophobic signal sequence at the N-terminus for exporting the secreted protein. The hydrophobic signal sequence is typically 20 amino acids in length and contains 3 regions: a positively charged amino terminal, a hydrophobic core, and a polar carboxyl-terminal (Papanikou et al., 2007, Natale et al., 2008). The Sec pathway contains SecB-specific signal sequences which required for proteins that will be secreted into the periplasm or outside of the cell, while proteins meant to remain in the inner membrane contain a signal recognition particle (SRP)-specific signal sequence (Papanikou et al., 2007). Proteins that designed for transport to the periplasm or outside of the cell in many Gram-negative bacteria contain a removable signal sequence recognized by the SecB protein. This protein serves as a chaperone, binding to pre-secretory proteins and preventing them from folding (Randall and Hardy, 2002). SecA is a multi-functional protein recieves the transported protein from SecB and both work together to guide proteins to the SecYEG channel, and also serves as the ATPase that

provides the energy for protein translocation (Hartl et al., 1990). The protease protein cleaves off the SecB signal sequence from the protein prior to transport through the channel, and the secreted protein is then is folded upon delivery to the periplasm (Mogensen and Otzen, 2005). The SRP pathway is also used by the Sec system to transport proteins that are meant to remain in the inner membrane. Transmembrane proteins often contain hydrophobic domains, and thus are unstable when cytoplasmic, so, secretion by the SRP pathway utilizes a co-translational mechanism of export that couples translation of the protein by the ribosome with secretion through the SecYEG channel (Luirink and Sinning, 2004).

The Tat pathway primarily secretes folded proteins compared to the Sec pathway which is transported protein in their unfolded state. This pathway is critical because not all proteins can be secreted in their unfolded state, as certain proteins that contain post-translational modifications, such as redox factors, are synthesized in the cytoplasm. The materials required for these modifications would not be available extracellularly or in the periplasm and, thus, these proteins must be folded and modified in the cytoplasm prior to secretion in their 3-dimensional state (Berks et al., 2005). The Tat pathway consists of 2–3 subunits: TatA, TatB, and TatC (in Gram-positive bacteria, TatA and TatB are combined into one multi-functional protein). In E. coli, TatB and TatC bind the signal peptide of Tat-secreted proteins and then recruit TatA, which forms the membrane-spanning channel (Sargent et al., 1999, Pop et al., 2002, Muller, 2005). The signal sequence of Tat containes a pair of twin arginines in the motif S-R-R at the N-terminus of the folded protein. Whereas most proteins secreted by the Tat apparatus in Gram-positive bacteria are released extracellularly, Tat-secreted proteins in Gram-negative bacteria can either remain periplasmic or are transported out of the cell (Muller, 2005).

## 1.7 Systems and formation pathways of DSB

The structure of proteins supports their functions and this is turn means that immature or unfolded proteins may not be activated or are deactivated through the actions of proteases (Balch et al., 2008, Hutt et al., 2009). Clearly, the cell survival is depending on proteins survive and this is achieved when the protein reaches its full potential by rapidly and efficiently adopting its correctly folded structure. To control possible folding problems, there are an assortment of protein factors encoded by cells to help secreted proteins fold correctly or to prevent proteins unfolding under severe conditions, such as an increase in temperature. Some secreted proteins that function in the extracellular environment especially, need to be more stable so, a specialised modification such as protein disulphide bond is often introduced to the secreted proteins to maintain structure and function (Tutar and Tutar, 2010). Disulphide bonds (DSB) are covalent chemical bonds formed between sulphurs in thiol groups. The side chains of cysteine residues in the protein bind by disulphide bonds which form between the sulphurs. The oxidised form of protein is referred to the protein hold the disulphide bond between the cysteines and the reduced form to the protein with the dithiol groups on the cysteines (Shouldice et al., 2011). The disulphide bonds are important to protein function and consequently, to the proper functioning of the cell and organism; indeed all organisms encode proteins that catalyse the process of disulphide bond formation (Heras et al., 2007). Disulphide bonds formed between pairs of cysteines provide stability to secreted proteins, which include many bacterial virulence factors, and in Gram-negative bacteria, this process is mediated by the DSB family of proteins (Heras et al., 2009). DSB enzymes have been well studied in *E. coli* K12 (Smith et al., 2016, Depuydt et al., 2011). The periplasm of Gram-negative bacteria contains a set of DSB thioredoxin family proteins, DsbA, DsbB, DsbC, DsbD, DsbE, DsbF, and DsbG, which play important roles in disulphide bond exchange and

regulation of the redox potential (Missiakas et al., 1995, Andersen et al., 1997). Some of these proteins possess a Cys-X-X-Cys motif which is an active site and it's important for redox potential ;which is often embedded in a domain termed a thioredoxin-like fold (Katzen and Beckwith, 2000). The N-terminal cysteine being more active in folding than the C-terminal side and can comprise a disulphide bond. These DSB proteins are involved in two pathways: an oxidative pathway which introduces a disulphide bond into a folded protein, and an isomerase pathway which corrects non-native disulphide bonds as shown in Figure (1.3) (Kishigami et al., 1995, Hiniker and Bardwell, 2004).

### 1.7.1 DSB oxidative pathway

The oxidative pathway consists of two DSB catalysts, DsbA and DsbB in *E. coli* K-12. The redox active site of DsbA from *E. coli* (EcDsbA) has the characteristic CXXC motif flanked by a hydrophobic groove and a large hydrophobic patch (Martin et al., 1993, Guddat et al., 1997). EcDsbA possesses a disulphide bond between Cys30 and Cys33 in the oxidised state and the disulphide bond can be transferred to a substrate through bimolecular nucleophilic transfer Figure (1.4 A). EcDsbB is a membrane-bound protein consisting of four transmembrane helices linked to one cytoplasmic loop and two periplasmic loops (Inaba et al., 2006). There is one pair of cysteines in each periplasmic loop that are presumed to act in rhythm to reoxidise EcDsbA (Kadokura and Beckwith, 2002). EcDsbA is converted to the inactive form after oxidative folding of the substrate and can then interact with the periplasmic loop of the transmembrane partner of EcDsbB (Bardwell et al., 1993). The interaction between EcDsbA-EcDsbB regenerates the oxidised state of EcDsbA through the transfer of electrons to EcDsbB. The reducing equivalents are passed through the first periplasmic loop of EcDsbB to bound quinones or mena-quinones, which in turn are reoxidised by terminal oxidises. It is known that DsbB still functions efficiently to

promote disulphide bond formation under anaerobic growth conditions. The identification of menaquinone as an effective fide Bonds in *E. coli* recipient of electrons from DsbB, a pathway via mena-quinone to final electron acceptors. Thus, DsbB would switch its use of primary electron acceptors depending on the degree of aerobiosis (Debarbieux and Beckwith, 1999).

### 1.7.2 DSB isomerase pathway

In the isomerase pathway, DsbA can introduce a non-native disulphide bond into a protein that contains more than two cysteines. However, within the periplasm, EcDsbC can correct the disulphide bonds and isomerise it to the native form (Hiniker and Bardwell, 2004). In *E. coli* K-12, there are two proteins that work together to proofread and reshuffle non-native disulphides, EcDsbC and EcDsbD Figure (1.4 B). The soluble EcDsbC mediates the isomerisation of disulphide bonds in substrate proteins and it also possesses chaperone activity (Berkmen et al., 2005, Chen et al., 1999). EcDsbD is the second component of the isomerisation pathway and is an integral membrane protein that transfers electrons to EcDsbC to maintain the isomerase in the active reduced form. Therefore, in this bacterial disulphide isomerisation pathway, EcDsbC and EcDsbD are the counterparts of EcDsbA and EcDsbB of the oxidising pathway (Rietsch et al., 1997). Another homologues protein to EcDsbC, EcDsbG, is also found in the periplasm of *E. coli* K-12. The latter protein forms a homodimer with disulphide bond isomerase and molecular chaperone activity, sharing 24% sequence identity with EcDsbC. EcDsbG is expressed at lower levels than EcDsbC and exhibits more substrate specificity (Shao et al., 2000, Bessette et al., 1999).

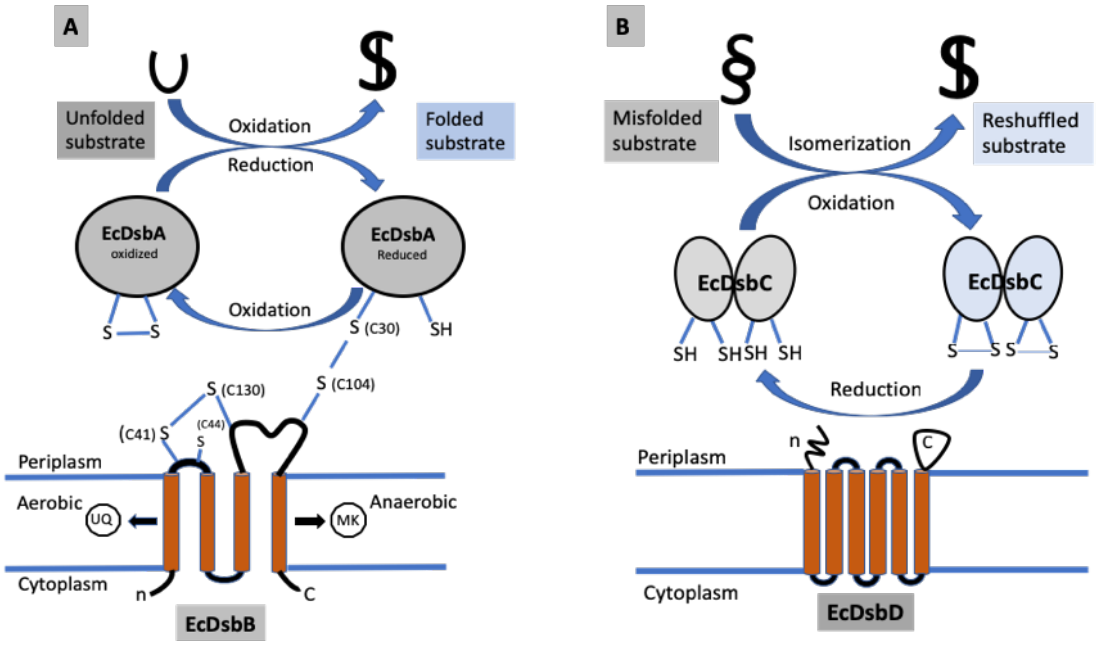



**Figure 1.4: The oxidative and isomerisation pathways in *E. coli* K-12.**

**(A)** Oxidised EcDsbA transfers its active site disulphide to an unfolded protein substrate, thus oxidising the cysteines to fold the protein. The active site cysteines C30 and C33 of EcDsbA become reduced and are reoxidised by C104 and C130 of EcDsbB. This cycle is repeated to maintain EcDsbA in the active oxidised form. Ubiquinone (UQ) is the recipient of electrons from EcDsbA and oxidises EcDsbB under aerobic conditions. Alternatively, menaquinone (MK) can oxidise EcDsbB under anaerobic conditions. **(B)** When EcDsbA catalyses incorrect disulphide bond formation in substrates, EcDsbC proofreads and shuffles the disulphide bonds until the correct connections are made. After introducing disulphides into the substrate, EcDsbC becomes oxidised and the disulphide at the active site of EcDsbC is reduced by the N-terminal domain of EcDsbD (nDsbD for N-terminal domain of DsbD) so that EcDsbC is converted into the active reduced form. adapted from (Shouldice et al., 2011).

### 1.7.3 The diverse DSB systems in bacteria

Our current knowledge of bacterial disulphide catalysis has derived from studies conducted on *E. coli* K-12. The *E. coli* K-12 paradigm of DSB folding enzymes that form two separate pathways is only conserved in $\gamma$ and $\beta$-Proteobacteria. The DSB pathway is conserved in these bacterial classes, while some variation has been observed in the number and types of DSB proteins (Denoncin and Collet, 2013). Several other organisms have been reported which contain a wide range of number of DSB proteins. *Salmonella enterica* serovars possess the prototypic *E. coli* K-12 oxidase and isomerase systems, as well as a pair of DsbL/DsbL and virulence plasmid-encoded DsbA-like protein, SrgA (Heras et al., 2010). *Neisseria meningitidis* also has both the oxidase and isomerase systems but without DsbG, as well as two additional DsbA-like lipoproteins anchored to the inner membrane. Gram-positive bacteria, such as *Staphylococcus aureus* and *Listeria monocytogenes,* only encode a DsbA but they do not encode any other DSB protein (Vivian et al., 2009, Kouwen et al., 2007). DsbA is the most taxonomically widespread of DSB proteins, and it is found in all classes of Proteobacteria and *Chlamydiales*. All DsbA proteins share a common three-dimensional architecture (Paxman et al., 2009).

### 1.7.4 DSB systems and bacterial virulence

Many bacterial virulence factors are dependent upon DsbA/DsbB mediated disulphide bond formation including toxins, secretion systems, adhesions and motility machines. The toxin subunits cannot fold into their native structures in the absence of DsbA catalysed disulphide bond formation, in most cases with reduced levels of toxin subunits (Smith et al., 2016). *Shigella flexneri*, *Yersinia pestis*, and *Salmonella typhimurium* use the T3SS to inject effector proteins into the cytosol of host cells to

modulate eukaryotic cell pathways and certain T3SS constituents require a disulphide bond formation for correct folding. In the absence of DsbA, the T3SS of these pathogens cannot transport effector proteins (Watarai et al., 1995, Jackson and Plano, 1999, Miki et al., 2004). In addition, the EPEC has a major structural subunit of the bundle-forming pilus (BfpA), which requires DsbA for the production or stability of adhesion important for bacterial colonisation. Moreover, uropathogenic *E. coli* has flagella mediated motility to spread by mediating DsbA catalysed disulphide bond formation. Consequently, mutations in the *dsbA* or *dsbB* means that *E. coli* and many other bacteria become non-motile (Zhang and Donnenberg, 1996, Dailey and Berg, 1993b). Importantly, the widespread distribution of the DsbA/DsbB redox system across different bacteria genera and the variety of virulence factors that depend upon this mediated disulphide bond formation, makes this system an attractive to target for the development of new anti-virulence therapeutic agents.

### 1.7.5 Targeting DSB proteins for the development of anti-virulence agents

The distribution of the DsbA-DsbB redox system across bacteria and the dependence of many virulence factors on DsbA-mediated disulphide bond formation, makes the oxidative system an attractive candidate to target for the development of anti-virulence therapeutics. However, there are numerous challenges in the inhibition of these enzymes such as identify suitable binding sites on these enzymes or their substrates. The DsbA forms a stable interaction with the most of its substrates, mediated by a disulphide bond formed by the nucleophilic cysteine (Cys30 in DsbA), but this bond is rapidly resolved as a result of the reactivity of this cysteine (Nelson and Creighton, 1994). There is also a significant challenge concerning the integral membrane protein DsbB due to technical difficulties related to working with a

membrane protein outside of its native environment. Nonetheless, in the last few years, there have been several attempts to target the DsbA-DsbB oxidative pathway. Fragment-based drug discovery (FBDD) first attempted to identify anti-virulence agents that inhibit DSB proteins and target the membrane disulphide oxidase EcDsbB. It has since emerged as an effective technique for developing a wide variety of drug targets and has already yielded compounds that have been approved for clinical use (Baker, 2013, Quintyne et al., 2012, Fruh et al., 2010). Another FBDD approach has also been used to identify fragments that bind to DsbA and inhibit it non-covalently in *E. coli*. This work provides the first example of a small molecule with dual activity on DsbA and DsbB, also showing a degree of selectivity as it was unable to inhibit human thioredoxin (Adams et al., 2015). Peptides and peptidomimetics are DsbA inhibitors that prevent the formation of the DsbA-DsbB redox complex, which is guided by the crystal structure of this complex. These inhibitors mimic the DsbB periplasmic loop involved in DsbA docking, binding to DsbA directly and preventing DsbB performing its function (Duprez et al., 2015, Kurth et al., 2014). Overall, no new approach targeting the DSB system has been developed in the isomerase pathway and interest in this has decreased as it appears to have an overall lesser role in bacterial infection.

### 1.7.6 DsbA is a master virulence regulator for Gram-negative bacteria

Protein disulphide isomerase (PDI) was the first oxidative folding catalyst protein identified in the late of 1960s. It is an eukaryotic endoplasmic reticulum (ER) protein which can introduce disulphide bonds into folding proteins (Fuchs et al., 1967). Surprisingly, the first bacterial oxidative folding catalyst was discovered in the early 1990s and it was a DsbA, which was first identified in *E. coli* and found to have a

signal sequence that localised it to the bacterial periplasm (Bardwell et al., 1991, Kamitani et al., 1992). DsbA is a bacterial periplasmic thiol disulphide oxidoreductase and the key member of the disulphide bond family of enzymes. DsbA catalyses the folding of secreted proteins, many of which are virulence factors. The structure of the thiol oxidant DsbA consists of a thioredoxin (Trx) domain, a common structural fold of thiol disulphide oxidoreductases with an inserted helical domain. Similar to the Trx fold oxidoreductases, DsbA has a pair of redox active cysteines in a CXXC motif ($C_{30}$-$P_{31}$-$H_{32}$-$C_{33}$; CPHC) and a *cis*-proline$^{150}$ residue that is near to the three-dimensional fold but is distant in the sequence, as well as playing a key role in the function of this protein (Martin et al., 1993, Kadokura et al., 2004). The *cis*-proline residue is a conserved feature of the Trx fold and, in DsbA, is located in a loop at the end of a long helix, which links the $\alpha$- helical and Trx domains. The structure of DsbA has been identified in a complex with DsbB, which showed that a periplasmic loop of DsbB is linked within the hydrophobic groove of DsbA. The latter has been widely estimated to be the substrate-binding site of DsbA (Inaba et al., 2006). DsbA enzymes display a wide range of substrate specificity. In *E. coli*, for example, the DsbA was estimated to oxidise hundreds of different substrate proteins. However, a number of DsbA enzymes have been reported recently that display a narrower substrate specificity (Dutton et al., 2008a). DSB proteins, especially DsbA, the most common oxidative folding catalyst, play a crucial role in the biogenesis of bacterial toxins and virulence factors. In general, mutations in DSB proteins makes the mutants defective in the DsbA-DsbB oxidation pathway, reducing fitness and attenuating virulence in infection models. Furthermore, mutations in DsbA alter several pathogenicity-associated phenotypes, either directly or because of pleiotropic effects (Yu and Kroll, 1999, Łasica and Jagusztyn-Krynicka, 2007). Here, some well-characterised examples of the role of DsbA in different stages of the infection process are described.

#### 1.7.6.1 Adhesion

Adhesion is an essential step for host colonisation for most bacterial pathogens and establishment of the disease. Adhesion is firstly mediated by fimbriae or pili (hair-like structure that radiate from bacterial cell surface and can reach up to several micrometres in length). Gram-negative bacteria have several types of fimbriae and pili which have been described extensively (Fronzes et al., 2008). These fimbriae are categorised related to their structure (type I, type P and type IV fimbriae) or mode of biogenesis (through the chaperone-usher pathway or type IV secretion). In the bacterial periplasm, it is essential for fimbrial biogenesis and function to correct the folding of fimbriae subunits and assembly proteins (Heras et al., 2009). A mutation in the gene encoding EcDsbA protein abolished the assembly of P fimbriae, which is a virulence factor in the UPEC. The defective assembly is due to the inability of the periplasmic chaperone PapD to adopt its native confirmation and bind P fimbrial subunits. DsbA is also required for the formation of a disulphide bond in the P fimbrial adhesion PapG, which is able to recognise the carbohydrate moieties on the surface of host cells in the urinary tract (Jacob-Dubuisson et al., 1994). *Proteus mirabilis* is an important uropathogen that requires DsbA. *P. mirabilis dsbA* mutant lacked expression of MrpA56, the major structural subunit of mannose-resistant Proteus-like fimbriae, which is required for colonisation of the murine urinary tract (Bahrani et al., 1991). The plasmid-encoded DsbA homologue SrgA is required for biosynthesis of plasmid-encoded fimbriae (Pef) in the *Salmonella enterica* subsp. *enterica* serovar Typhimurium and the Pef has been implicated in intestinal cell adhesion in a murine infection model (Bouwman et al., 2003). EPEC is required the DsbA-mediated formation of intramolecular disulphide bond in the bundle-forming pili (BfpA), the major subunit for fimbrial production, which mediates localised adherence to human epithelial cells. Furthermore, the type IV pili of *Neisseria meningitides* mediates

adhesion to human cells, whereas biogenesis of functional pili requires at least one of the two NmDsbA paralogues. DsbA is also required for the assembly of functional pili in *Vibrio cholerae* which is colonised by mediated the toxin co-regulated pilus (Tcp) (Zhang and Donnenberg, 1996, Tinsley et al., 2004, Peek and Taylor, 1992).

#### 1.7.6.2 Host cell manipulation

After adhesion-mediated colonisation by the bacteria, the successful infection of many bacterial pathogens depends on their ability to manipulate the host cell. For this manipulation, bacteria use a range of mechanisms from mass cell destruction induced by secreted proteins (toxins and proteases) to more specific host cell manipulations stimulated by type III secreted effectors. The DSB enzymes are required for correct folding of several secreted virulence factors, revealing the role of the DSB pathway in the advanced stages of bacterial pathogenesis. DsbA is required for the functional assembly of many proteins, including cholera toxin of *V. cholerae*, pertussis toxin of *B. pertussis* and heat-labile enterotoxin of enterotoxigenic *E. coli* (Peek and Taylor, 1992, Wülfing and Rappuoli, 1997, Stenson and Weiss, 2002). The type II secretion system pathway is secreted by many enzymes that are central to the ability of Gram-negative pathogens to cause disease, and several of these enzymes are constituted specific substrates for DSB enzymes. DsbA in *Pseudomonas aeruginosa* is required for the stability of optimal proteolytic activity of elastase, a highly abundant, secreted metalloprotease that enhances virulence in the lungs of patients with cystic fibrosis (Braun et al., 2001). There are other extracellular enzymes of *P. aeruginosa* that are important in opportunistic infection, such as lipase, which require DsbA and DsbC for their stability and secretion (Urban et al., 2001).

T3SS is an apparatus consisting of more than 20 proteins and it used by range of bacterial genera, such as *Shigella*, *Salmonella*, *Yersinia* and EPEC and EHEC. These pathogens use T3SS to inject the bacterial virulence proteins known as effectors into eukaryotic cells (Cornelis, 2006, Galán and Wolf-Watz, 2006). Interestingly, DsbA is required for correct folding of proteins that create the secretin complex of the T3SS in *S. flexneri*, *S. Typhimurium*, EPEC and *Y. pestis* (Watarai et al., 1995, Miki et al., 2004, Jackson and Plano, 1999). Furthermore, *S. flexneri* uses the Mxi-Spa encoded apparatus which is dedicated to the secretion of Ipa proteins that mediate invasion and virulence properties, such as the induction of apoptosis in macrophages, so *S. flexneri* needs DsbA to fold the secreted Ipa proteins (Yu and Kroll, 1999). Recently, it has been reported that two DsbA proteins in *Serratia marcescens*, an opportunistic pathogen, are required for virulence and proper deployment of the T6SS. In addition, DsbA plays a critical role in recipient cells, being required for the toxicity of certain incoming effector proteins (Mariano et al., 2018).

#### 1.7.6.3 Cellular spread and survival

Fimbriae-mediated adhesion allows bacteria to establish infection. However, the motility phenotype is crucial for bacterial convenience and virulence, as it permits the infecting bacteria to spread within the host. The mutations in either *dsbA* or *dsbB* in many genera of bacterial pathogens are non-motile because of their inability to produce functional flagella. This defect was detected to lack of *dsbA* in *E. coli*, which catalyses the formation of the intramolecular disulphide bond between two cysteines in the flagellar P-ring motor protein FlgI (Dailey and Berg, 1993a, Bringer et al., 2007). *S. flexneri* is an intracellular pathogen, and its ability to survive and spread across host cells is crucial for virulence. Ipa proteins are secreted by T3SS, acting primarily

as invasins to mediate bacterial uptake by epithelial cells. After intracellular proliferation, the Ipa protein secretion is vital for cellular spread. Mutations in *dsbA* result in intracellular spread and cell-to-cell invasion is prevented by defective Ipa secretion at the cellular protrusions formed by *S. flexneri* (Watarai et al., 1995, Dorenbos et al., 2002, Yu et al., 2000).

The competition for resources in the environment is crucial for some bacteria to enhance their fitness. Indeed, some bacteria eliminate their competitors by using strategies dependent on DsbA for survival. These survival strategies have been described in *B. subtilis*, a Gram-positive organism, which uses the DsbA homologue BdbB for production of a functional antibiotic peptide (lantibiotic sublancin 168). *B. subtilis* utilises this antibiotic for enhancing fitness at the expense of other organisms in its ecological niche (Dorenbos et al., 2002). The opportunistic human pathogen *P. aeruginosa* has been also described to use DsbA-dependent bactericidal activity, but the exact virulence pathway affected by loss of DsbA activity has not been identified (Park et al., 2005).

### 1.8 The mammalian cell cytosol is a highly reducing environment

The mammalian cell cytosol is a reducing environment due to the concentration of reduced glutathione (GSH) and thioredoxin (Trx). Cytosol contains approximately 10 mM of GSH and a small amount of oxidised glutathione (GSSG) (Hwang et al., 1992). GSH is a tripeptide compound synthesised in cell cytosol that has many activities in the cell. It consists of three amino acids, glutamate, cysteine and glycine linked together by γ peptide and α peptide. The GSH is a redox active molecule that can interact in a variety of antioxidant reactions, and GSH is an electron donor in the reduction of peroxides, and it is catalysed by glutathione peroxidases with relatively

slow kinetic (Winterbourn and Metodiewa, 1999). The intracellular glutathione concentration is in the millimolar range and the GSH-GSSG redox pair is considered a biological redox buffer (Hwang et al., 1992). GSSG is antioxidant which prevents damage of important cellular components caused by reactive oxygen species. GSH can reduce disulphide bonds by donating an electron to form GSSG (Appenzeller-Herzog, 2011). The maintenance of the glutathione redox state is highly controlled within the cell, which is defined by the squared GSH concentration divided by the GSSG concentration. The value of squared GSH/GSSG in the unstressed cytosol is kept constantly high by the NADPH-dependent activity of glutathione reductase and has also been determined in yeast (Østergaard et al., 2004).

GSH is synthesized in all cell types via two sequential reactions catalysed by $\gamma$ glutamyl cysteine synthetase ($\gamma$ GCS) and GSH synthetase (GS). The $\gamma$ GCS enzyme catalyses the formation of a peptide bond between the $\gamma$ carboxyl group of glutamate and the $\alpha$ amino group of cysteine. Both reactions depend on ATP, and their mechanisms are similar: both involve the formation of an acylphosphate intermediate (Meister and Anderson, 1983). GSH is described as antioxidant, a central source of cysteine amino acid first isolated from yeast, liver and muscle in 1922. GSH is major intracellular thiol compound that can involve in thiol redox state associated biochemical processes including protein synthesis, phosporylation, transport, stabilization of protein structures, folding, protection of cysteine residues, binding of DNA transcription factor and acceleration of H2O2 scavenging in redox pathway (Dhindsa, 1991, Davioud-Charvet et al., 2001). Most of the cellular GSH (85–90%) is present in the cytosol, with a little amount in other organelles including mitochondria, nuclear matrix, and peroxisomes (Wu et al., 2004). GSH is a primary substrate for glutathione peroxidase (GPx) and glutathione S-transferase (GST) which is involved

in the glutathione redox cycle. GSH plays a prominent role in the formation of microtubulin spindle in cell division (Szarka et al., 1995, Nath and Rebhun, 1976).

The ER forms the organelles of the secretory pathway, in which membrane proteins are synthesised and secreted by the introduction of disulphide bonds. The global GSH:GSSG ratio in the ER, Golgi bodies and the endosomal-lysosomal system was determined almost 20 years ago by rapid acidification-quenching of a glutathione-reactive peptide probe targeted to the exocytic compartment in mammalian cells (Hwang et al., 1992). The ratio of GSH to GSSG has been demonstrated between 1:1 to 3:1 in cell cytosol, and this ratio is important for the redox environment in the secretory pathway that is well-suited for disulphide bond formation (Gutscher et al., 2008). The conserved endoplasmic oxidoreductin 1 (Ero1) sulphydryl oxidases, of which there are two in humans known as ERO1A and ERO1B, and hereafter as Ero1$\alpha$ and Ero1$\beta$ (Sevier and Kaiser, 2008). These ER enzymes can oxidise the active site of the cysteine SH-group in PDI by reducing molecular oxygen, Ero1-dependent GSH to GSSG conversion (through PDI) in the yeast ER and bi-directional passage of GSH, but not of GSSG through rat liver microsomal membranes (Cuozzo and Kaiser, 1999, Bánhegyi et al., 1999). The cytosolic GSH, rather than cytosolic GSSG, contributes directly to ER redox regulation, as confirmed by combined data obtained from experiments that used living or semi-permeabilised mammalian cells (Jessop and Bulleid, 2004, Molteni et al., 2004). A model proposed by Cuozzo and Kaiser, by which cytosol-derived GSH can deliver reducing equivalents to the ER as well as balancing luminal thiol oxidation by Ero1 is shown in Figure (1.5). GSH is required during oxidative protein folding for the corrective reduction (or isomerisation) of non-native disulphide pairings (Cuozzo and Kaiser, 1999, Chakravarthi et al., 2006b). The membrane proteins (PSH) and GSH, which already contains thiol groups, enter the ER from the cytosol and react with $PDI^{ox}$. This interaction produces the disulphide-

bounded proteins (PSSP), GSSG and $PDI^{red}$, correctly folded products of which are exported to the Golgi complex by vesicular transport. PSSP proteins with non-native disulphides or misfolded can be reduced by $PDI^{red}$ or any other PDI family member, or by GSH directly to yield PSH and $PDI^{ox}$ (Wang et al., 2011). Another way to recycle the $PDI^{red}$ to $PDI^{ox}$ is by reaction with the active form of Ero1$\alpha$ which can pass the electrons on to molecular oxygen ($O_2$). Ero1$\alpha$ is regulated by a high concentration of $PDI^{red}$. The oxidation of $PDI^{red}$ by Ero1$\alpha$ generates a new $H_2O_2$ molecule, which is also converted into water by oxidation of $PDI^{red}$. When the proteins of $PDI^{ox}$, GSSG and PSSP become plentiful, the high ratio of $PDI^{red}$:$PDI^{ox}$ promotes the formation of regulatory disulphide in Ero1$\alpha$, which makes Ero1$\alpha$ inactive (Wang et al., 2011).

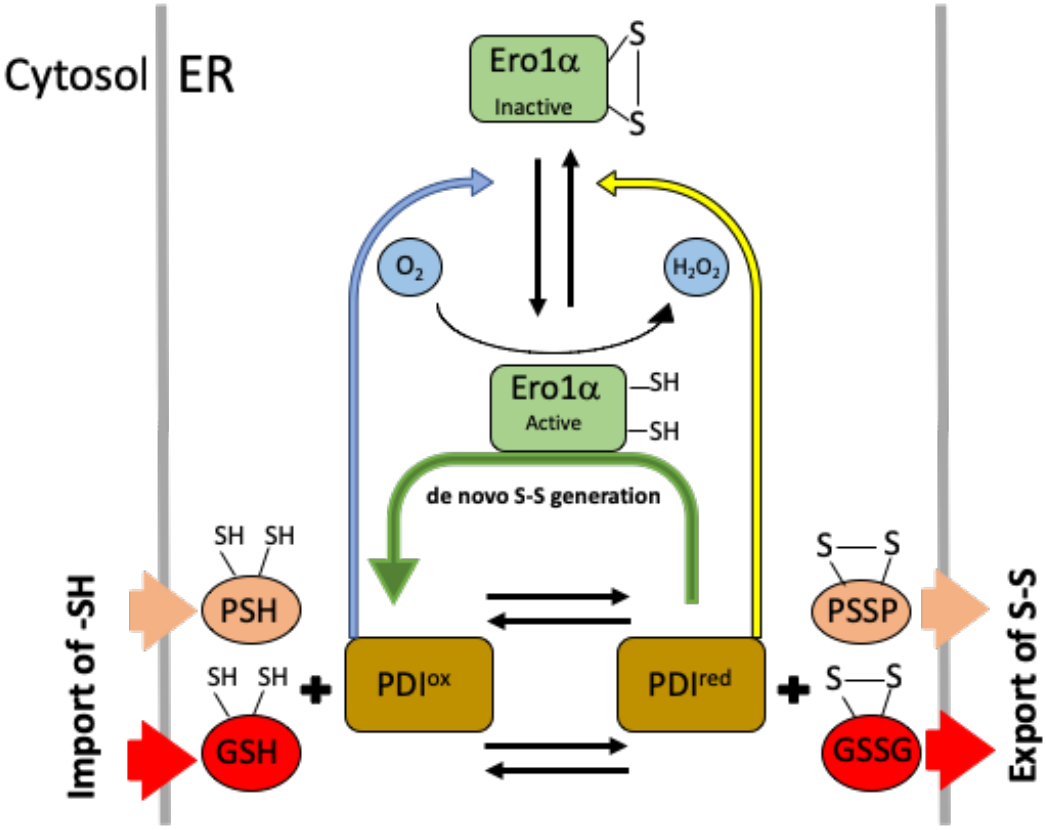



**Figure 1.5: Pathways of thiol disulphide exchange in the ER.**

Thiol groups (carbon-bonded SH-groups) in nascent secretory or membrane proteins (PSH) and GSH enter the ER from the cytosol, reacting with $PDI^{ox}$ to yield disulphide-bonded proteins (PSSP), GSSG, and $PDI^{red}$. Then, the correctly folded PSSP and GSSG are exported to the Golgi complex by vesicular transport. PSSP with non-native disulphide pairings (misfolded) can be reduced by $PDI^{red}$ either by other reduced PDI family members or by GSH directly (not depicted), yielding PSH and $PDI^{ox}$. Alternatively, $PDI^{red}$ is recycled to $PDI^{ox}$ by reacting with the active form of Ero1α that passes the electrons on to $O_2$ [referred to as Ero1α-driven oxidation of $PDI^{red}$ or de novo disulphide (S–S) generation; green arrow]. Ero1α is positively regulated by a high concentration of $PDI^{red}$ (yellow arrow). $H_2O_2$ generated by Ero1α for every $PDI^{red}$ molecule that is oxidised, is also converted to water by oxidising $PDI^{red}$. When $PDI^{ox}$, GSSG and PSSP become abundant, the high $PDI^{ox}$:$PDI^{red}$ ratio promotes the formation of regulatory disulphides in Ero1α (light-blue arrow) that render Ero1α inactive. In addition, a high concentration of GSSG facilitates GSSG-driven oxidation of $PDI^{red}$. In some tissues, Ero1α is complemented by Ero1β, which is also redox regulated (adapted from (Appenzeller-Herzog, 2011).

## 1.9 Bacterial Glutathione Transporter

The periplasm of Gram-negative bacteria is the site of elaborate and finely balanced redox control but, to date, the GSH/GSSG couple has not been considered a major player in that compartment. The periplasm is predominantly oxidising environment, where formation of disulphide bonds is controlled by thiol-disulphide oxidoreductases (O'Brian and Thöny-Meyer, 2002, Fabianek et al., 2000). The oxidising pathway comprises DsbA, a periplasmic, strongly oxidising, which is responsible for random formation of protein disulphide bonds in a rapid disulphide exchange reaction. DsbA is reoxidized by DsbB, an integral membrane protein that transfers electrons via quinones to the terminal oxidases and reductases of the respiratory chain (Bardwell et al., 1991, Kobayashi and Ito, 1999).

Evidence that GSH mediates oxidative stress tolerance in *E. coli* comes from the finding that a GSH-deficient strain is hypersensitive to hypochlorous acid, but GSH appears dispensable for bacterial growth under many conditions (Chesney et al., 1996, Greenberg and Demple, 1986). Five cotranscribed genes in *E. coli* have homology to the *dpp* genes and appear to encode a transporter required for utilization of GSH as sole sulphur source, suggesting a role in GSH transport. It has also been suggested that GSH might be secreted or leaked out into the periplasm (Parry and Clark, 2002, Dartigalongue et al., 2000). The ABC-type transporter, CydDC, is originally identified by its requirement for assembly of the cytochrome *bd*-type terminal oxidase of *E. coli*. And it is a transporter of cysteine outwards across the cytoplasmic membrane. However, it was reported that CydDC has higher transport activity with GSH than with cysteine as substrate (Poole et al., 1993, Pittman et al., 2002, Pittman et al., 2005). The CydDC transporter is an ABC-type transporter and is expected to derive the energy for transport solely from ATP hydrolysis; indeed, mutation of the

ABC domain abolishes function (Cruz-Ramos et al., 2004). GSH transport by CydDC is mechanistically similar to that of cystic fibrosis transmembrane conductance regulator and the yeast cadmium factor protein (YCF1). The bacterial CydDC system transports only the reduced forms of GSH and cysteine (Kogan et al., 2003, Li et al., 1997, Pittman et al., 2005).

GSH synthesised in the cytoplasm is exported by the CydDC ATP-dependent transporter to the periplasm Figure (1.6), where GSH is involved in reducing protein (p) thiols. Reoxidation of protein thiols is accomplished by DsbA, which is ultimately reoxidised by DsbB, with subsequent terminal electron transfer via menaquinone (MQ) and ubiquinone (UQ) to anaerobic reductases and via the aerobic oxidases cytochromes *bo* and *bd* to oxygen, respectively (Hoober et al., 1996). Transfer to the periplasm of reductant in the form of GSH can compensate for loss of DsbD function and transfer of reducing power from thioredoxin (TrxA), previously thought to be essential with DsbC for periplasmic cytochrome maturation, and other thiol reductase roles. The normal physiological role of GSH in the periplasm may be to protect against oxidative stress and consume excess oxidising equivalents or to facilitate correct protein-disulphide bond formation. The GSH and GSSG movement is spontaneously across the outer membrane (Pittman et al., 2002, Pittman et al., 2005).

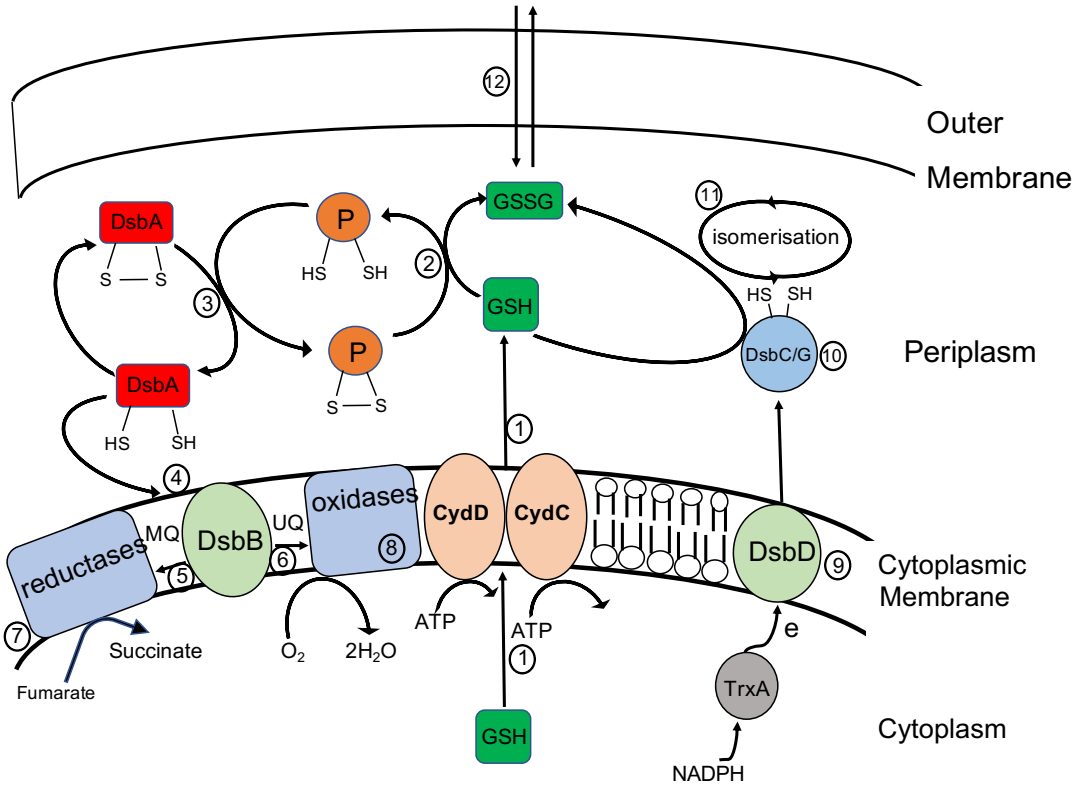



**Figure 1.6: Proposed role of GSH in periplasmic redox homeostasis.**

GSH synthesised in the cytoplasm is exported (1) by the CydDC ATP-dependent transporter to the periplasm, where GSH is involved in reducing protein (p) thiols (2). Reoxidation of protein thiols (3) is accomplished by DsbA, which is ultimately reoxidized by DsbB (4), with subsequent terminal electron transfer via menaquinone (MQ, 5) and ubiquinone (UQ, 6) to anaerobic reductases (7) and via the aerobic oxidases cytochromes bo and bd (8) to oxygen, respectively. Fumarate reductase is shown as an example of (7). Transfer to the periplasm of reductant in the form of GSH can compensate for loss of DsbD (9) function and transfer of reducing power from thioredoxin (TrxA), previously thought to be essential with DsbC (10) for periplasmic cytochrome maturation, and other thiol reductase roles (11). The normal physiological role of GSH in the periplasm may be to protect against oxidative stress and consume excess oxidising equivalents or to facilitate correct protein-disulphide bond formation. The GSH and GSSG movement is spontaneously across the outer membrane (12). Adapted from (Pittman et al., 2005)

## 1.10 Terpenoids, natural substances with diverse activity

Humans have exploited natural products for several centuries to treat infectious diseases. Traditional herbal medicine has long been used to treat infectious diseases and plants remain an alternative to conventional drugs in many developing countries. Medicinal plants contain a wide range of diverse natural products which could be used for their pharmacological activity (Rios and Recio, 2005). Some natural products can also be obtained from sources other than plants, for example, antimicrobial agents isolated from propolis, a resinous product from beehives (Seidel et al., 2008, Xu et al., 2011), as well as the a great many natural products derived from microorganisms (LaMarche et al., 2012, Schneditz et al., 2014). Many essential oils (EOs) can interact with bacterial cell membranes and inhibit bacterial growth *in vitro* (Solorzano-Santos and Miranda-Novales, 2012). For instance, EOs showed activity against *E. coli* (Sousa et al., 2010). Moreover, the effectiveness of antibiotics can be enhanced by EOs; co-application of EOs and antibiotics are more effect against pathogens (Ahmad and Viljoen, 2015). EOs are also commonly used to treat upper respiratory tract infection (i.e. inhalation therapy) (Hamid et al., 2011, Shubina et al., 1990).

Secondary metabolites produced by plants and microorganisms are the most interesting compounds due to their massive variety of structural and functional diversity. Among the various secondary metabolites, terpenoids represent one of the largest and most diverse classes (Demain and Fang, 2000). Terpenoids are found in 90% of EOs, specific scents produced by plants as chemo-attractants or repellents (McGarvey and Croteau, 1995, Crowell, 1999). Terpenoids are based on a hydrocarbon skeleton and have diverse structures (Bakkali et al., 2008), with most being highly volatile, lipophilic and colourless liquids with an aromatic scent. Many terpenoids are oxygenated and contain ketones, aldehydes, alcohols as well as carboxylic acid, the oxygenated forms of which are known as monoterpenes (Oz et

al., 2015). More than 55,000 terpenoids have been isolated, and this number has doubled each decade. Their functional roles are diverse, with some terpenoids characterised as hormones (gibberellins), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), mediators of polysaccharide assembly as well as communication and defence mechanisms (Breitmaier, 2006, Harrewijn et al., 2012).

Terpenoids, also known as terpenes, are the largest group of natural products, and they are plant secondary metabolites along with alkaloids and flavonoids. They are classified based on the number of carbons in their structure as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20) and triterpenes (C30) (Wang et al., 2005). Terpenoids display antimicrobial (antibacterial, antifungal and antiviral), anti-inflammatory, antioxidant, hypotensive, antipruritic and analgesic activities (Kordali et al., 2005, Guimaraes et al., 2013, Bastos et al., 2010). Twelve terpenoids were chosen in this study to test their activity against *S. sonnei* infection and are shown in Figure (1.7).

### 1.10.1 Geraniol as a promising drug

Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is an acyclic monoterpene with a water solubility of 100 mg/L at 25$^{o}$C and an *n*-octanol/water partition coefficient of 2.65 (Turina et al., 2006). Geraniol is a widespread in the EOs extracted from lemongrass, roses, lavender and other aromatic plants. Geraniol has a rose-like scent and taste described as sweet floral rose-like, citrus with fruit notes. It is a monoterpene alcohol and used widely as a in fragrance, being present in 76% of deodorants on the European market (Burdock, 2016).

It is possesses a wide spectrum of pharmacological activities, such as antimicrobial (Thapa et al., 2012), anti-inflammatory (Chen and Viljoen, 2010), antioxidant (Khan et al., 2013) and neuroprotective effects (Rekha et al., 2013). The oral administration of geraniol as antimicrobial agent effectively prevent colitis-associated dysbiosis and it reduces the systematic inflammatory profile of mice with colitis (De Fazio et al., 2016). Geraniol has been tested *in vitro* and *in vivo* against several cell lines and showed activity against cancerous cells (Carnesecchi et al., 2001, Yu et al., 1995, Burke et al., 1997).

Geraniol also demonstrated an activity against Gram-negative bacteria including multidrug resistant isolates, such as *Enterobacter aerogenes, E. coli, P. aeruginosa,* by targeting the virulence factors in these pathogens (Lorenzi et al., 2009). Geraniol is an active inhibitor of *E. coil* with bacterial activity value of (BA50 = 0.15), *L. monocytogenes* (BA50 = 0.28) and *S. enterica* (BA50 = 0.15). It can also inhibit the growth of both human and animal pathogens, including *S. typhimurium* and *E. coli* among 66 compounds tested (Si et al., 2006). Interestingly, the gaseous state of geraniol showed a significant activity against respiratory tract pathogens, including *Haemophilus influenza*, *Streptococcus pneumoniae*, *S. pyogenes* and *Staphylococcus aureus* (Inouye et al., 2001). Furthermore, geraniol, citronellol and nerol were active against *Mycobacterium tuberculosis in vitro*, showing MIC values between 64 and 128 µg/ml (Cantrell et al., 2001). Citral and linalool are two other monoterpenes active against Gram-negative bacteria including *E. coli* O157:H7 and *S. enterica* (Friedman et al., 2004). Another study demonstrated that citral, geraniol, and carvacrol were active against *Staphylococcus aureus* when combined with enterocin AS-48 (enterocin AS-48 is a circular bacteriocin produced by *Enterococcus faecalis*) (Grande et al., 2007).

### 1.10.2 Toxicity and allergenicity of geraniol

The toxicity of geraniol has been tested in various organisms. Rat studies reported that 1000 ppm of geraniol fed to rats for 28 weeks and even 10,000 ppm in the diet fed for 16 weeks did not show any adverse effects (Hagan et al., 1967). No mutagenic effects were observed in an Ames test conducted on *S. typhimurium* with 0.5 mg of geraniol in DMSO.

Furthermore, geraniol is not an electrophile and should not possess any sensitising capacity. However, there are few reports of allergic contact dermatitis resulting from sensitivity to geraniol (Ishidate Jr et al., 1984, Cardullo et al., 1989). Geraniol was listed on European Union's 26 fragrance allergens that must be identified on cosmetic and detergent product labels as contact sensitisation occurred when low molecular weight compounds were able to penetrate the skin and bind to proteins (Buckley, 2007, Hagvall et al., 2008).

Geraniol has the potential to autoxidise on air exposure or by metabolic activation in the skin to form highly allergenic compounds (Hagvall et al., 2008). Geraniol can metabolically decompose into geranial, neral, 2,3-epoxygeraniol, 6,7-epoxygeraniol and 6,7-epoxygeranial. Geranial, neral and 6,7-epoxygeraniol are considered as moderate sensitisers, while 6,7-epoxygarnial is a strong sensitiser (Hagvall et al., 2008). The risk assessment of the use of geraniol was comprehensively summarised in a safety data sheet, with studies listed on oral, dermal, intramuscular, subcutaneous, inhalation acute toxicity; skin irritation and sensitisation; mucous membrane (eye) irritation of geraniol. However, the scientific assessment focused on dermal exposure because the skin is the first exposure route for this fragrant material (Lapczynski et al., 2008).

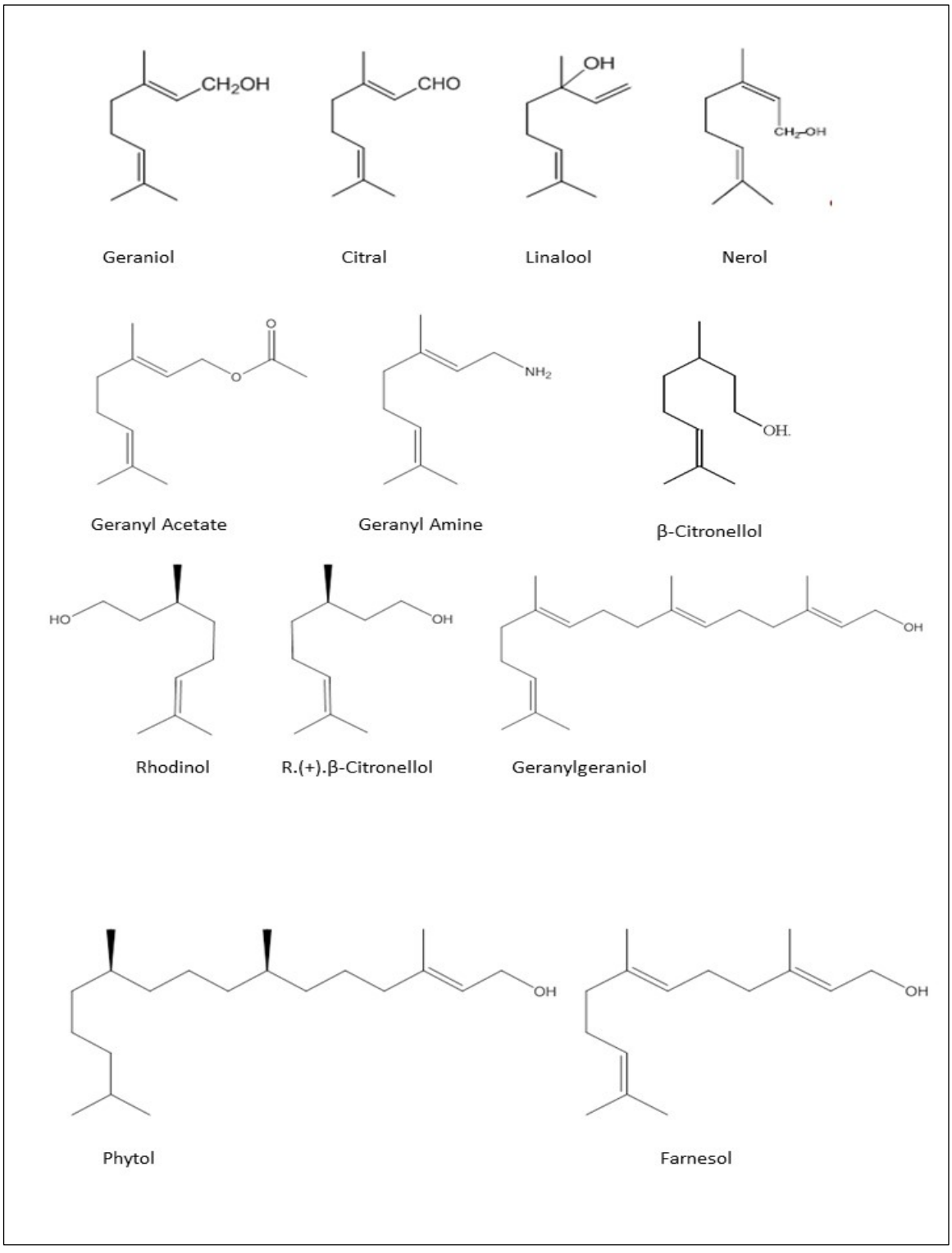



**Figure 1.7: Structures of selected terpenoids used in this study**

## 1.11 Research hypothesis and aims

DsbA is a thiol-oxidoreductase enzyme responsible for folding the secreted proteins in Gram-negative bacteria. Targeting this enzyme may lead to inhibition of the pathogenicity, proliferation and host survival techniques of *S. sonnei*.

It is hypothesised that selected terpenoids may have the ability to inhibit the *S. sonnei* protein, DsbA, so this study tested selected terpenoids depending on their structure relationship to propolin D as published by (Xu et al., 2011). The study aimed to unravel the mechanisms of how terpenoids could control *Shigella* intracellular proliferation as described by (Xu et al., 2011).

Furthermore, previous studies revealed that the *Shigella* periplasmic disulphide bond oxidoreductase, DsbA, is vital in supporting the survival of *S. flexneri* in the host cell cytosol (Yu, 1998). Therefore, *Shigella* needs a strategy to control the highly reducing environment in the cell cytosol, so it was hypothesised that *Shigella* may use DsbA to catalyse GSH to survive and proliferate in the cell. Therefore, this study also aimed to purify the DsbA protein, and at the same time, determine the role of DsbA on GSH. Selected terpenoids, especially geraniol, were tested *in vitro* using an enzymatic kinetic assay and *in vivo by* fluorescently labelling GSH and running the catalysis using different strains of *S. sonnei* to determine the role of geraniol in inhibiting this catalyst. Geraniol was shown previously to have the ability to inhibit the DsbA competitively (Mirza et al., 2018), therefore, we aimed to unravel the interaction sites between geraniol and DsbA to understand the mechanism by which geraniol affects DsbA. Lastly, an *in vivo* model of *Galleria mellonella* larvae was used to evaluate the pathogenicity of *S. sonnei* wild type and DsbA mutants, as well as activity and cytotoxicity of selected terpenoids.

The aims of this research were:

a- Evaluate the role of terpenoids in control of intracellular *S. sonnei* proliferation.

b- Evaluate the cytotoxicity of terpenoids *in vitro* and *in vivo*.

c- Determine the anti-DsbA activity of terpenoids using enzymatic kinetic assay.

d- Unravel the interaction sites between geraniol and DsbA to understand the mechanism by which geraniol affects DsbA.

# Chapter 2

# Material and Methods

# 2 Material and Method

## 2.1 General Material

### 2.1.1 Chemical and stains used in the present study

The different chemicals and stains used and their sources are presented in Table (2.1).

**Table 2.1: Chemicals and stains used in this study**

| Name | Source |
|---|---|
| Acrylamide 30% w/v | National Diagnostics, England, UK |
| Acetonitrile | VWR, chemical laboratories, France |
| Agarose A | Severn Biotech Ltd, UK |
| Ammonium chloride | Sigma Aldrich Co., USA |
| Ammonium per sulfate (APS)(ultra-pure) | AGTC Bioproducts, Global Life Science Supply |
| Bovine serum albumin 100X (purified) (BSA) | Bio Labs., New England |
| 5-bromo-4-chloro-3-indolyl-β-D- galacto-pyranoside (X-gal) | Thermo scientific, UK |
| Bromo-phenol blue | Sigma Aldrich Co., USA |
| Congo Red | Alfa Aesar, England |
| Dimethyl sulfoxide (DMSO) | Sigma Aldrich Co., USA |
| DL-Dithiothreitol (DTT) | Sigma Aldrich Co., Switzerland |
| Ethanol (99.5%) | Sigma Aldrich Co., USA |
| Eosin isothiocyanate (EITC) | Sigma Aldrich Co., USA |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma Aldrich Co., Germany |
| Ethidium bromide (EtBr) | Sigma Aldrich Co., USA |
| Fetal bovine serum (FBS) | Life technologies, USA |
| Glacial acetic acid | Fluka analytical, Sigma Aldrich Co., USA |
| D(+)-Glucose | AnalaR, BDH laboratory supplies, UK |

**Table 2.1: Continued**

| | |
|---|---|
| Glycerol 99% | Alfa Aesar, England, UK |
| Glycine | Fisher Scientific, UK |
| Reduced glutathione | Sigma Aldrich Co., USA |
| HisTrap FF column | GE Healthcare, Sweden |
| Imidazole | Sigma Aldrich Co., Germany |
| Isopropyl-ß-D-1-thiogalactopyranoside (IPTG) | Sigma Aldrich Co., USA |
| Luria Bertani (LB) with agar (Lennox) | Sigma Aldrich Co., USA |
| Luria broth base, Miller | Sigma Aldrich Co., USA |
| M9 medium | Fluka analytical, Sigma Aldrich Co., USA |
| Magnesium chloride | Sigma Aldrich Co., USA |
| Magnesium sulfate | Fisher Scientific, UK |
| Methanol | VWR Prolabo Chemicals, France |
| L-Methionine | Sigma Aldrich Co., USA |
| Nicotinic acid | Sigma Aldrich Co., USA |
| Non- essential amino acid | Sigma Aldrich Co., USA |
| Paraformaldehyde (PFA) | Sigma Aldrich Co., USA |
| Penicillin/Streptomycin (100X) Contains 10,000 units/mL of penicillin and 10,000 µg/ml of streptomycin in a 10 mM citrate buffer (for pH stability) | Life technologies, USA |
| Polyvinylidene difluoride (PVDF) | Millipore, UK |
| Potassium di-hydrogen phosphate | Sigma Aldrich Co., USA |
| Protein ladder Hyperpage (prestained protein marker) | Bioline Co., UK |
| Sodium chloride | Sigma Aldrich Co., USA |

**Table 2.1: Continued**

| | |
|---|---|
| Sodium dodecyl sulfate (SDS) | Sigma Aldrich Co., USA |
| Sodium phosphate dibasic dihydrate | Fluka analytical, Sigma Aldrich Co., USA |
| Sodium pyruvate | Sigma Aldrich Co., USA |
| N,N,N′,N′-Tetramethylethylenediamine (TEMED) | Sigma Aldrich Co., USA |
| Tris base | Fisher Scientific, UK |
| Triton X-100 | Sigma Aldrich Co., USA |
| L-Tryptophan | Sigma Aldrich Co., USA |
| Trifluoroacetic acid | Sigma Aldrich Co., Germany |
| Tween 20 | Usb Co., USA |
| TrypLE Express (Trypsin) | Life Technologies, USA |
| Wright-Giemsa stain, modified | Sigma Aldrich Co., UK |

### 2.1.2 Bacterial strains and plasmids.

All bacterial strains and plasmids and their origin that used in this study are listed in the Table (2.2). Bacteria were grown routinely on Lysogeny Broth (LB) agar containing 0.1% Congo red and incubated at 37°C overnight to isolate red colonies for subsequent experiments (Yu, 1998).

**Table 2.2: Abbreviations of bacterial strains, plasmids and their source**

| No | Name of strain | Characteristics | Reference |
|---|---|---|---|
| 1 | SSWT | *Shigella sonnei* (wild type) | Scottish *Salmonella* and *Shigella* Ref. Lab No. 86 (Glasgow, UK) |
| 2 | SSMD | *Shigella sonnei* (Δ*dsbA*) | (Yu, 1998) |
| 3 | SSPD | *Shigella sonnei* (Δ*dsbA/pDsbA*) | This study |
| 4 | SSPD-F29 | *Shigella sonnei* (Δ*dsbA/pDsbA-F29*) Phenylalanine 29 was substituted by Alanine in the *DsbA.* | This study |
| 5 | SSPD-H32 | *Shigella sonnei* (Δ*dsbA/pDsbA*-H32) Histidine 32 was substituted by Alanine in the *DsbA.* | This study |
| 6 | SSPD-Q35 | *Shigella sonnei* (Δ*dsbA/pDsbA*-Q*35*) Glutamine 35 was substituted by Alanine in the *DsbA.* | This study |
| 7 | SSPD-L68 | *Shigella sonnei* (Δ*dsbA/pDsbA*-L68) Leucine 68 was substituted by Alanine in the *DsbA.* | This study |
| 8 | BDPD3 | *E. coli, BL21(DE3)/pDsbA,* KanR | This study |
| 9 | BDPD2 | *E. coli, BL21(DE3)/pDsbA,* AmpR | This study |
| 10 | LDPD | *E. coli, Lemo21 (DE3)/pDsbA,* KanR | This study |
| 11 | DH5α | *E. coli,* Host for general cloning. $F^-$, *endA1*, *hsdR17* ($r_{K-}m_{K+}$), *supE44*, *thi-1* $\lambda^-$,*recA1,gyrA96,relA1, deoR*, Δ(lacZYA-araF)U169, φ80dlacZ ΔM15 | Bio Labs., New England, UK |

**Table 2.2: Continued**

| | | | |
|---|---|---|---|
| 12 | JM109 | *E. coli,* Host for general cloning. *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44, relA1, Δ(lac-proAB)/F' [traD36, proAB*$^{+}$*, lacI*$^{q}$*, lacZΔM15.* | Promega, USA |
| 13 | XL10-Gold | *E. coli,* TetrD(*mcrA*)183 D(*mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte* [F´ *proAB lacIqZDM15* Tn10 (Tetr) Amy Camr]. | Agilent, USA |
| 14 | pET26b(+) | pET26b(+) is a bacterial expression plasmid with T7 promoter and KanR | Lab stock |
| 15 | pTH102 | pMMB190/p*DsbA* is a bacterial expression plasmid with tac lacUV5 promoter region and AmpR | (Yu, 1998) |
| 16 | pTH103 | pET26b(+)/p*DsbA,* KanR | This study |
| 17 | pTH104 | pMMB190/p*DsbA-F29*, Phenylalanine 29 was substituted by Alanine in the *DsbA*. | This study |
| 18 | pTH105 | pMMB190/p*DsbA*-H32, Histidine 32 was substituted by Alanine in the *DsbA*. | This study |
| 19 | pTH106 | pMMB190/p*DsbA-Q35*, Glutamine 35 was substituted by Alanine in the *DsbA.* | This study |
| 20 | pTH107 | pMMB190/p*DsbA*-L68, Leucine 68 was substituted by Alanine in the *DsbA.* | This study |
| 21 | pTH108 | pGEM- T Easy/p*DsbA* | (Yu, 1998) |

### 2.1.3 Terpenoids used in the present study

Twelve terpenoids compounds were used in this study, all from Sigma-Aldrich Company, USA. The molecular weight, specific density and purity of each compound are listed in Table 2.3. An initial stock solution of each compound was prepared in dimethyl sulfoxide (DMSO) and then the working concentration was prepared by dissolving in the appropriate solution.

**Table 2.3: Terpenoids used in this study**

| No. | Terpenoids | Abbreviation | Molecular weight ( MW) in g/mole | Density in mg/mL | Purity % |
|---|---|---|---|---|---|
| 1- | Geraniol | G | 154.25 | 0.879 | 98 |
| 2- | Linalool | Li | 154.25 | 0.87 | 97 |
| 3- | Citral | Ci | 152.23 | 0.888 | 95 |
| 4- | Geranylgeraniol | GG | 290.48 | 0.88 | 85 |
| 5- | Rhodinol | Rh | 156.27 | 0.865 | 98 |
| 6- | Geranylacetate | GA | 196.29 | 0.916 | 97 |
| 7- | Geranylamine | GM | 153.26 | 0.829 | 90 |
| 8- | β-Citronellol | β-Ci | 156.27 | 0.857 | 95 |
| 9- | Nerol | N | 154.25 | 0.876 | 97 |
| 10- | Farnesol | F | 222.37 | 0.886 | 95 |
| 11- | Phytol | Ph | 296.53 | 0.85 | 97 |
| 12- | R.(+).β-Citronellol | R.(+).β-Ci | 156.27 | 0.851 | 98 |

### 2.1.4 Antibiotics and media used in this study

Antibiotics and media used in this study were listed in the table (2.4) and (2.5).

**Table 2.4: Antibiotic concentrations used in this study, all antibiotics stored at -20°C (Sambrook et al., 2001)**

| Antibiotic | Solvent used | Stock conc. (mg/ml) | Working conc. (µg/ml) |
|---|---|---|---|
| Ampicillin | SDW | 50 | 50 |
| Gentamicin | SDW | 50 | 50 |
| Chloramphenicol | Ethanol (100%) | 25 | 25 |
| Kanamycin | SDW | 50 | 25 |
| Polymyxin B | 1XPBS | 1 * | 1000U/mL |
| Penicillin/ Streptomycin | Citrate buffer | 100X | 100 U/mL, 400 µg/ mL |
| * 1 mg = 10000 U/mL | | | |
| | | | |

**Table 2.5: Component of different media used in this study**

| Media | Amount per litre |
|---|---|
| Lysogeny Broth (LB) **(Sambrook et al., 2001)** | 10 g tryptone, 5 g NaCl, 5 g yeast extract, 1 g glucose, 10 g agar (exclude for broth) add up to 1 litre with distilled water: adjust pH to 7.0 with NaOH |
| M9 minimal media | 30 g $Na_2HPO4$,15 g $KH_2PO4$, 5 g $NH_4Cl$, 2.5 g NaCl, 15 mg $CaCl_2$ (optional), 1 litre of high quality distilled water. After autoclaving, add sterilised components by filtration with 200 µl/100 mL of 1M MgSO4, 0.4g% glucose,12.5 µg/mL of Nicotinic acid, 45 µg/mL of L-methionine, and 20 µg/mL of L-Tryptophan. |
| SOC (Hanahan, 1983) | 20 g Tryptone, 5 g yeast extract, 10 mL 1M NaCl, 2.5 mL 1M KCl, add up to 1 litre with distilled water, After autoclaving, add sterilised components by filtration with a 0.22 µm filter: 1 M $MgCl_2.6H_2O$, 1 M $MgSO_4.7H_2O$, 1 M Glucose. |
| NZY+ ( Agilent, USA) | 10 g of NZ amine (casein hydrolysate), 5 g of yeast extract, 5 g of NaCl. Add deionized $H_2O$ to a final volume of 1 litre , adjust to pH 7.5 using NaOH, autoclave, add the following filter-sterilized supplements prior to use: 12.5 mL of 1 M $MgCl_2$, 12.5 mL of 1 M $MgSO_4$, 20 mL of 20% (w/v) glucose (or 10 mL of 2 M glucose). |

### 2.1.5 Antibodies and restriction enzymes that used in this work

Antibodies and restriction enzymes that used in this study and their source are listed in the Table (2.6).

**Table 2.6: Antibodies and restriction enzymes used in this work**

| Antibodies and restriction enzymes | Source |
|---|---|
| Alexa Fluor 680 goat anti-mouse IgG | Life technologies, USA |
| 6xHis epitope Tag antibody (His-H8) | Thermo Scientific, USA |
| BamHI restriction enzyme | Bio Labs., New England, UK and Promega, USA. |
| BglII restriction enzyme | Promega, USA |
| DpnI restriction enzyme | Agilent, USA |
| EcoRI restriction enzyme | Bio Labs., New England, UK and Promega, USA |
| EcoRV restriction enzyme | Promega, USA |
| NdeI restriction enzyme | Bio Labs., New England, UK and Promega, USA |
| SalI restriction enzyme | Bio Labs., New England, UK |
| SacI restriction enzyme | Promega, USA |
| SphI restriction enzyme | Promega, USA |
| XhoI restriction enzyme | Promega, USA |

### 2.1.6 Cell cultures.

HeLa (human epithelial cell-line) cells and Macrophages (RAW 264 mouse leukemic monocyte macrophage cell-line) cells were used in this study. The cell-lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 1% (v/v) sodium pyruvate (Sigma) and 1% (v/v) non-essential amino acid solution (Sigma) under 5% $CO_2$ atmosphere at 37 °C. Penicillin and streptomycin were added to DMEM to the final concentrations of 100 units and 400 μg per mL respectively. To keep cells fresh and with known passage, cells were split routinely by using trypsin (2 mL in 42 mm cell culture flask) for HeLa while scraping was used for RAW macrophages. After the cells reached 80-100 % confluency in the flask, they were split. For split cells, media and other reagents were pre-warmed to 37 °C in a water bath. The flasks were washed with 5 mL 1xPBS and then 2 mL trypsin was added for 1 min. 1.5 mL was removed and the flask was incubated for 5 min at 37 °C. Once the cells detached, they were re-suspended in 5 mL pre-warm DMEM and 1 mL from the cells suspension was transferred to 4 mL of fresh and pre-warmed DMEM medium and re-incubated under 5% CO2 atmosphere at 37 °C.

## 2.2 General Methods

### 2.2.1 Gentamicin protection assay (Xu et al., 2011).

HeLa (human epithelial cell-line) and Macrophages (RAW 264 mouse leukemic monocyte macrophage cell-line) cells were seeded and cultured until approximately 80% confluence in 24-well plates and bacteria strains were added to the cell monolayers at a multiplicity of infection (MOI) of 10. The plates were centrifuged at 2000 rpm for 10 min at 24 °C. The plates were incubated in cell culture incubator for 40 min at 37 °C under 5% $CO_2$ atmosphere to allow bacterial invasion into host cells. Thereafter, cells were washed twice with 1xPBS; DMEM containing gentamicin (50

μg/mL + 42 μM Terpenoids) were added, and the plates were incubated for two hours before terminating infection. Cells were washed 3 times using 1xPBS and lysed with Triton X-100 (0.1 % in $H_2O$) for 10 min. Cell lysates were serially diluted, plated on LB agar, incubated at 37 °C overnight and enumerated by colony count.

### 2.2.2 MTT assay for cytotoxicity of the Terpenoids (Xu et al., 2011).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a chemical that can be actively uptake by live cells and converted to purple precipitates. Cells were seeds into 96 well plates and cultured to about 60-80% confluency. The cells then were treated with each of the compounds at different concentration (1.31 μM to 2688 μM) and incubated overnight. 100 μL MTT was added to each well of the 96 well plates after removing the treatment media. The plate was incubated for 1-2 h until purple precipitation was visible. Thereafter, the MTT was removed and 100 μL of DMSO was added to each well to solubilise the purple precipitates. The absorbance of the plate at 540 nm (A540) was recorded with a Spectramax microplate reader (Molecular Device) as describes in (Xu et al., 2011).

### 2.2.3 Bacterial Growth Curve in M9 media.

Glutathione redox (GSH) was used in this protocol. Five different concentrations of GSH (2.5 mM to 30 mM) were prepared and added to M9 media. SSWT, SSDM and SSPD were grown overnight in LB media and for the strain SSPD, ampicillin 100 μg/mL was added. The bacteria were diluted 1:50 fold and added into M9 media and the range of GSH concentrations as indicated above were added to M9 broth. The bacterial cultures were incubated at 37 °C with 200 rpm shaking. The optical density (OD) was obtained each hour by using a (WPA) spectrophotometry at $OD_{600}$.

### 2.2.4 Fluorescent labelling of oxidise glutathione (GSSG) (Raturi and Mutus, 2007, Montano et al., 2014)

The labelling of Di-E-GSSG was prepared by incubating 100 μM from oxidised glutathione (GSSG) with 1 mM eosin isothiocyanate (EITC) overnight in potassium phosphate buffer (0.1 M potassium phosphate and 2 mM EDTA, pH 8.8) at 37 $^{o}$C with stirring.

For separating the unreacted EITC from the labelled Di-E-GSSG, precipitation by acetonitrile containing 0.1% trifluoroacetic acid was used. The volume ratio was 1:3 of Di-E-GSSG+EITC/acetonitrile (v/v). The mix was mixed by hand for 5 min and centrifugation was carried out for 10 min at 12000 rpm and the supernatant was removed carefully. The pellet was washed once more with acetonitrile and mixed up and down by hand for 5 min. The tubes were centrifuged again for 10 min at 12000 rpm. The supernatant was discarded and the pellet was lyophilized by using a DNA concentrator centrifuge for 30 min at 40 °C with negative vacuum. After lyophilization, the pellet was dissolved in DsbA buffer (0.1M potassium phosphate and 2 mM EDTA, pH 7.0) and stored at -20 °C. The concentration of Di-E-GSSG was obtained by molar absorption coefficient for EITC *e* = 56,000 $M^{-1}cm^{-1}$ using Beer-Lambert law at 525 nm in DsbA buffer (Montano et al., 2014).

### 2.2.5 Preparation of labelling reduced Glutathione (E-GSH)

The fluorescent reduced glutathione (E-GSH) was prepared by converting (10 μM) of Di-E-GSSG with (1 mM) DTT in DsbA buffer (0.1 M potassium phosphate and 2mM EDTA pH 7.0) overnight in the dark at room temperature. The separation of remaining DTT was carried out by the same precipitation of acetonitrile containing 0.1% (v/v) trifluoroacetic acid described in section 2.2.4. The concentration of E-GSH was

obtained by molar absorption coefficient *e* = 88,000 $M^{-1}$ $cm^{-1}$ using Beer-Lambert law at 525 nm in DsbA buffer (Raturi and Mutus, 2007).

### 2.2.6 Detection of the fluorescent labelling of Di-E-GSSG and E-GSH formation

The Di-E-GSSG and E-GSH were tested their ability to give fluorescent after labelling. Di-E-GSSG and E-GSH were diluted in DsbA buffer separately to get (150 nM) for each and then 200 μL of each one were placed in a 96 well plate in triplicate. 2 μL of DTT 1M was added to the reaction to get the final concentration of 10 mM. The increase of relative fluorescence unit (RFU) was monitored during 15 mins under the excitation 525 nm and emission 545 nm by using a Spectramax microplate reader M5 (Molecular Devices).

### 2.2.7 Test terpenoids by enzymatic kinetic assay

The enzymatic kinetic assay was set up in a 96 well plate using DsbA buffer (Mirza et al., 2018). Three solutions were prepared for monitoring DsbA inhibition. Mix one (150 nM Di-E-GSSG + 5 μM DTT + DsbA buffer), mix two (40 nM DsbA protein + DsbA buffer) and mix three (42 μM terpenoids + DsbA buffer) The three solutions were mixed before run using Spectramax microplate reader M5 (Molecular Device).The increase of relative fluorescence unit (RFU) was monitored for 15 min with excitation 525 nm and emission 545 nm.

### 2.2.8 Catalysis of E-GSH by growing *S. sonnei* bacteria in LB media (Mirza et al., 2018)

Strains were grown overnight in 5 mL LB media. The culture was diluted to 1:50 in fresh LB media and grown at 37 °C with shaking at 200 rpm. When the $OD_{600}$ reached 0.3, the E-GSH (75 nM) was added to the culture. At this point, both $OD_{600}$ and relative fluorescence unit (RFU) were recorded each hour for 12 to 13 hours. The fluorescent signal was monitored in Spectramax microplate reader M5 (Molecular Device) at 525 nm excitation and 545 nm emission and the $OD_{600}$ was obtained each hour using (WPA) spectrophotometry.

### 2.2.9 Use of *G. mellonella* larvae as a model for studying *S. sonnei* virulence (Mahmoud et al., 2016, Barnoy et al., 2017)

Larvae were obtained from Livefood, UK, and stored in the dark at room temperature. Larvae were selected based on absence of visible signs of pupation, slow motion, melanisation or any other illnesses before starting the infection. A small-rounded colony was picked from an LB agar plate and allowed to grow in 5 mL LB for 3 hours; bacteria were prepared at initial cell densities of $10^9$ CFU/mL and then serially diluted using 1xPBS into other 4 different cell densities $10^8$, $10^7$, $10^6$ and $10^5$ CFU/mL, and 10 μL were used for larvae injection. The larvae were separated into 3 groups with 10 larvae per each group. The mock infection control group were injected with 10 μL of 1xPBS, while the infection group received same volume of $10^5$ CFUs of SSWT or DsbA mutant strains. The treatment group were separated into four categories depending on the concentration of terpenoids used and injected with a suspension of $10^5$ CFU of live bacteria and terpenoids (10μL in total). The terpenoids were added to the suspension of bacteria prior to injection at a range of concentrations (4.6 μg, 12.8 μg, 25.6 μg and 51.2 μg) for infection, while, for cytotoxicity of terpenoids, the larvae

received 20.4 mg/kg, 204 mg/kg, 2.04 g/kg and 20.4 g/kg of terpenoids. The injection site for all larvae was above the last left proleg using a microliters syringe from Hamilton Microliter (Sigma Aldrich, UK). All larvae were incubated at 37 °C and were monitored for five days. Larval death was indicated by melanisation and loss of mobility. Survival data were analysed using the Kaplan-Meier estimator to compare between the mortality of larvae infected with wild type *S. sonnei* and different mutant strains or the ability of terpenoids to protect larvae from killing by bacteria.

## 2.3 Molecular Microbiology Methods

### 2.3.1 Isolation of plasmid DNA from different sources

All plasmids were extracted by using Isolate II plasmid mini kit from Bioline, UK. The protocol was carried out according to the company's procedure. Bacteria were inoculated into 5 to 10 mL LB with appropriate antibiotics for 16 to 18 hours at 37 $^{o}$C and shaking incubator at 200 – 250 rpm.

### 2.3.2 Oligonucleotides used in this study

Oligonucleotides for amplification of *dsbA* were designed using the SnapGene software and purchased dried from Integrated DNA Technologies (IDT), with concentrations provided by the manufacturer. NdeI, XhoI and SalI restriction sites were included in the primers to assist cloning. The primers used in the Site Directed Mutagenesis procedure were designed with the desired mutation in the middle of each forward and reverse primers using the SnapGene software and purchased dried from Integrated DNA Technologies (IDT), with concentrations provided by the manufacturer. The primers were reconstituted with the required amount of nuclease-

free water to an overall of 100 pmol/μL stock concentrations and stored at -20°C. The oligonucleotides used in this study are described in Table (2.7).

**Table 2.7: Oligonucleotides used in this study**

| Name | Sequence (5' to 3') | Site or Mutation |
|---|---|---|
| DsbA-F1 | ACAGCGCATATGGGAGAGAGTAGATCATGAAAAAG | NdeI |
| DsbA-R1 | ACAGCGCTCGAGGATCCTCTTTTTTTCTCGGACAG | XhoI |
| DsbA-F2 | CACGCGCATATGAAAAAGATTTGGCTGGCG | NdeI |
| DsbA-R2 | GCCGCAAGCTTGTCGACGGATCC | SalI |
| DsbA-F29F | AGTTTTTCTCTTTCGCGTGCCCGCACTGCTA | Phenylalanine to Alanine |
| DsbA-F29R | TAGCAGTGCGGGCACGCGAAAGAGAAAAACT | |
| DsbA-H32F | CTTTCTTCTGCCCGGCGTGCTATCAGTTTGA | Histidine to Alanine |
| DsbA-H32R | TCAAACTGATAGCACGCCGGGCAGAAGAAAG | |
| DsbA-Q35F | GCCCGCACTGCTATGCGTTTGAAGAAGTTCT | Glutamine to Alanine |
| DsbA-Q35R | AGAACTTCTTCAAACGCATAGCAGTGCGGGC | |
| DsbA-L68F | TCATGGGTGGTGACGCGGGCAAAGATCTGAC | Leucine to Alanine |
| DsbA-L68R | GTCAGATCTTTGCCCGCGTCACCACCCATGA | |
| DsbA-P174F | GCAATATGGATGTTGCGGTTCAGCAGTATGC | Phenylalanine to Alanine |
| DsbA-P174R | GCATACTGCTGAACCGCAACATCCATATTGC | |
| DsbA-SeqF | ATGAAAAAGATTTGGCTGGCG | sequencing |
| DsbA-SeqR | CGCCAGCCAAATCTTTTTCAT | sequencing |

### 2.3.3 Amplification of *dsbA* using PCR

Polymerase chain reaction (PCR) was used to amplify the *DsbA* gene using KOD Hot Start DNA polymerase Kit (Merck Millipore, UK). The primers of DsbA-F1, DsbA-R1, DsbA-F2 and DsbA-R2 Table (2.7) were used in this reaction and the DNA template is (pGEM T Easy) or (pMMB190) which has the *DsbA* gene already. The reaction mixture of PCR and the cycling conditions are shown in Table (2.8) and Table (2.9) respectively.

**Table 2.8: PCR reaction volume using KOD Hot Start DNA polymerase Kit (Merck Millipore, UK)**

| Components | Volume | Final Concentration |
|---|---|---|
| 10x Reaction Buffer | 5 μL | 1X |
| 25mM MgSo4 | 3 μL | 1.5 mM |
| dNTPs (2 mM each) | 5 μL | 0.2 mM each |
| Forward primer 10 μM | 1.5 μL | 0.3 μM |
| Reverse primers 10 μM | 1.5 μL | 0.3 μM |
| Template DNA | 1 μL | DNA plasmid (50ng – 100ng/μL) |
| KOD Hot Start DNA poly (1 U/μL) | 1 μL | 0.02 U/μL |
| Nuclease Free Water up to | 50 μL | 50 μL |

**Table 2.9: PCR cycling conditions using KOD Hot Start DNA polymerase Kit (Merck Millipore, UK)**

| Cycle | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|
| Polymerase activation | 95°C | 2 minutes | 1 |
| Denaturation | 95°C | 20 seconds | 30 |
| Annealing | 60°C | 10 seconds | |
| Extension | 70°C | 15 seconds | |
| Leaving | 5°C | 0 | 0 |

### 2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis at 0.7 (w/v) or 1% (w/v) were used to confirm the DNA size after amplification, digestion and colony PCR depending on expected size. To prepare a 0.7 or 1% (w/v) agarose gel, 0.7 g or 1 g of agarose were dissolved in 100 mL of 1x TAE buffer and mixed into a conical flask, and the agarose was melted by heating the solution in the microwaving oven for 2.5 min. The agarose solution was swirled to ensure the agarose dissolved completely, then the agarose mixture was allowed to cool slightly to 50°C and 7μL of ethidium bromide (EtBr) was added to the agarose solution to give final concentration of 10 mg/ml and swirled until the EtBr was dissolved completely. The agarose was poured slowly into the gel tray, removing any air bubbles and the comb was replaced on the gel tray about 1.5 cm from the edge of gel; the agarose gel was then left for 5 -15 minutes to solidify. After this, the comb was removed with a careful motion back and forth so as not to tear the gel. The electrophoresis buffer was added to the buffer chamber until it reached the maximum level above the gel surface. The DNA samples with loading dye were loaded, the lid was positioned on the gel box, and the electrodes attached. As a size marker, 1 kb DNA ladder (Bioline, UK) was used and the gel was run at 90 -100 V for 1:30 hours or until the dye front reached the last half of the gel. Ultimately, the gel's photography was captured by UV transilluminators VWR Genosmart system, UK.

### 2.3.5 DNA digestion with restriction enzymes

Restriction enzymes from (Promega or NEB) were used to digest all the plasmids that used in this study according to the enzyme manufacturer's instruction. The amount of enzyme required is calculated in units (1 unit of enzyme cuts 1 μg of DNA in 1 hour at 37 °C). The volume of restriction digestion reaction was set up as shown in Table (2.10), using 15 to 20 μL for analytical digests and 50-100 μL for preparative digests.

**Table 2.10: Restriction enzymes digest mixture was prepared as follows**

| Component | Volume (μL) |
|---|---|
| DNA sample (0.2 - 1.0 μg) | 1 μL |
| 10X restriction enzyme buffer | 2 μL |
| Restriction enzyme (5 – 12 U) | 1 μL |
| BSA (1mg/ml) (when required) | 0.2 μL |
| SDW made up to | 15 or 20 μL |

The incubation time of digestion varied depending on the amount of DNA need to be digested and the enzyme. Typically restriction digest reactions were carried out for 1 to 4 hours at the appropriate temperature. Digestion by two different enzymes was carried out, if a compatible buffer was available. The enzyme and the buffer were not compatible, sequential digestions were processed using the most acceptable level of the reaction buffer for each enzyme, and the DNA purified between reactions. Finally, at the end of digestion, the DNA samples were purified and used for further cloning or 15 μL was mixed with loading dye (3 μL) and analysed by gel electrophoresis for confirmation of sizes or either used to purify the fragment from the gel.

### 2.3.6 Purification of digested plasmids and amplification products

For continuation of cloning experiments, digestion products were purified using an Isolate II PCR and gel kit from (Bioline, UK) according to the manufacturers protocol provided with the kit.

### 2.3.7 Ethanol precipitation of DNA

Sodium acetate 3M, pH 5.2 was mixed at 1/10 volume with the DNA mixture. Then 2.5 volumes of ice-cold absolute ethanol was added, also mixed well and placed on 4 °C for 30 minutes to allow the DNA to be precipitated. The DNA was recovered by centrifugation at 4°C at 13000 for 30 minutes. The tube was half filled with 70% (v/v) ethanol after the supernatant was removed and subjected to further centrifugation at 12000 at 4°C for 15 minutes. The supernatant was removed and the tube was stored upside down on a tissue until the last fluid traces were removed. Afterwards the tube was dried at speed vacuum until the last traces of ethanol had evaporated. The DNA pellet was dissolved in the required volume of buffer (TE or sterile distilled water). Ultimately, the mixture was mixed well and stored at −20°C.

### 2.3.8 Ligation (Promega)

To determine the amount of insert DNA that need to be mixed with vector to get the correct ligation, the following formula was used for this purpose. A molar ration of 1:3 or 1:5 or 1:10 of vector: insert was used and an amount of vector between 50-100 ng was used in the cloning experiment.

$$\frac{ng\ of\ vector\ x\ kb\ size\ of\ insert}{kb\ size\ of\ vector}\ x\ \frac{molar\ ratio\ of\ \ insert}{vector} = ng\ of\ insert$$

The ligation reaction was assembled in a 1.5 mL tube as shown in Table (2.11), followed by the incubation at 4°C (16-18 hours) overnight before the recombinant DNA used to transform *E. coli* competent cells.

**Table 2.11: The ligation reaction solution**

| Ingredient | Volume (μL) |
|---|---|
| Vector DNA | Re-calculated according to the formula above |
| Insert DNA | Re-calculated according to the formula above |
| T4 DNA Ligase (3 Weiss units/μL) | 1 μL |
| 10X Ligation Buffer, T4 DNA Ligase | 2 μL |
| SDW to final volume of | 20 μL |

### 2.3.9 Transformation of bacterial strains with plasmid DNA

All plasmids were introduced into *E. coli* competent cells (DH5α, JM109, BL21(DE3) and Lemo21(DE3)) by chemical transformation and heat shocking. For example, 5 μL of ligation reaction were transferred into JM109 using the heat shock method. 50 μL of JM109 were thawed in ice for 5 min and mixed with 5 μL of ligation reaction and incubated for 10 min in ice. A heated block or water bath was set up at 42 °C and the cells were placed into the heated block for 45 sec and returned directly into ice for two minutes. LB media (950 μL) were added to the cells, which were then incubated at 37 °C and 200 rpm shacking incubator for one hour. Then, 100 μL from the culture were plated at LB agar plates with appropriate antibiotics and incubated at 37 °C for 16-18 hours.

### 2.3.10 Colony PCR

Putative recombinant plasmids were screened rapidly by colony PCR method. The colonies used for the rapid screening of insert DNA were about 1 mm in diameter at least. The PCR master mix reaction was prepared, and a colony was transferred to every tube containing PCR mix using toothpicks or pipette tips and mixed Table

(2.12). Then the PCR reactions were cycled using a thermocycler (BioRad) Table (2.13). Afterwards, the PCR products were tested by electrophoresis on agarose gel.

**Table 2.12: PCR reaction volume using MyTaq DNA Polymerase Kit, (Bioline)**

| Components | Volume | Final Concentration |
|---|---|---|
| 5x MyTaq Reaction Buffer | 10 μL | 1X |
| Forward primer 20 μM | 1 μL | 0.4 μM |
| Reverse primers 20 μM | 1 μL | 0.4 μM |
| Template DNA | 1 colony | 1 colony |
| MyTaq DNA Polymerase (5 units/μL) | 1 μL | 0.1 U/μL |
| Nuclease Free Water up to | 50 μL | 50 μL |

**Table 2.13: PCR cycling conditions using MyTaq DNA Polymerase Kit, (Bioline)**

| Cycle | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 1 minutes | 1 |
| Denaturation | 95°C | 15 sec | 25 |
| Annealing | 55°C | 15 sec | |
| Extension | 72°C | 10 sec | |
| Leaving | 4°C | 0 | 0 |

### 2.3.11 Site-directed mutagenesis (Agilent, USA)

Mutation in the *dsbA* gene was generated by using the QuikChange Lightning (QCL) Site-Directed Mutagenesis Kit from Agilent, USA. According to the manufacturer's instructions, the primers were designed with the desired mutation in the middle of the primer on *dsbA* for both the forward and reverse primers Table (2.7). The PCR

reaction was assembled as shown in the Table (2.14) and then the PCR reaction was cycled using a thermocycler (BioRad) Table (2.15).

**Table 2.14: PCR reaction volume for site-directed mutagenesis**

| Components | Volume | Final Concentration |
|---|---|---|
| 10x QCL Buffer | 5 μL | 1X |
| dsDNA template | 3 μL | (10 –100 ng) |
| Forward primer 10 μg | 1 μL | 0.2 μg |
| Reverse primer 10 μg | 1 μL | 0.2 μg |
| dNTP mix | 1 μL | |
| ddH2O to a final volume | 50 μL | 50 μL |
| QCL enzyme (2.5 U/μL) | 1 μL | 0.05 U/μL |

**Table 2.15: PCR cycling conditions for site-directed mutagenesis**

| Cycle | Temperature (°C) | Time | Number of Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 2 min | 1 |
| Denaturation | 95°C | 20 sec | 25 |
| Annealing | 60°C | 10 sec | |
| Extension | 68°C | 5 min * | |
| Leaving | 68°C | 5 min | 1 |
| * 30 seconds/kb of plasmid length | | | |

Following the PCR, the amplification reactions were placed in ice for 2 min to cool them to 37°C. Then 1 μL of Dpn I restriction enzyme was added directly to each amplification reaction to digest the parental nonmutated dsDNA. Following mixing by pipetting, and the reaction mix was centrifuged for 1 min at 11000 rpm and immediately incubated at 37°C for 5 min.

During the time of incubation; the XL10-Gold ultracompetent cells were prepared for transformation. Firstly, the XL10-Gold ultracompetent cells were thawed gently on ice and 45 μL were aliquoted in prechilled tube for each reaction. For the transformation efficiency, a 2 μL of the β-Mercaptoethanol (β-ME) mix were added to the cells and the tube of contents were swirled gently and incubated on ice for 2 minutes. Next, 1.5μL of the Dpn I treated DNA were transferred into the cells and the transformation reactions were swirled gently to mix and incubated on ice for 30 minutes. The tubes were heat-shocked at 42°C for 30 seconds and then incubated on ice for 2 min. Then 0.5 mL of preheated NZY+ broth media were added to the tubes and incubated at 37°C and 220-250 rpm shacking incubator for 1 hour. Finally, 100 μL of each reaction were plated on LB agar with appropriate antibiotic and incubated at 37°C for 16-18 hours. Several colonies were selected and used to extract the mutant plasmids afterwards and they were sent for confirmation of the mutant sequence.

### 2.3.12 Preparation and transformation of electro-competent SSDM (Sambrook et al., 2001)

A single colony of SSDM strain was streaked out on LB agar and incubated overnight at 37 °C. LB broth (5 mL) was inoculated by a single colony of SSDM strain and was incubated overnight at 37 °C with shaking at 225 rpm. Fresh LB medium (50 mL) was inoculated with 500 μL of the overnight SSDM culture and incubated at 37 °C with

shaking at 225 rpm for hours until the $OD_{600}$ reached 0.4 – 0.6. The culture was chilled on ice for 10 min. All reagents and equipment were chilled on ice and a cool centrifuge rotor and tips used prior to the start of the experiment. Then, the culture was transferred to a chilled falcon tube (50 mL) and centrifuged at 4000 rpm 4 °C for 5 min. The supernatant was removed carefully and the pellet was re- suspended gently in 25 mL ice-cold 10% (w/v) glycerol, followed by centrifugation at 4000 rpm 4 °C for 5 min and the supernatants removed carefully. Finally, the pellet was re-suspended gently in 0.5 mL ice-cold 10% (w/v) glycerol; 50 –100 μL of cell suspension was aliquoted quickly to chilled Eppendorf tubes and stored at –80 °C.

An electro-cuvette (0.2 cm) was pre-chilled on ice, and the Micropulser apparatus (Biorad) switched on. 50 μL of the SSDM competent cells were thawed on ice. Plasmid DNA (1 μL) was added to the competent cells and gently mixed by a yellow tip, and then incubated on ice for 1 min. The mixture (competent cells and DNA) was transferred to the bottom of the electro-cuvette. The cuvette top was closed and the cuvette was dried thoroughly. The cuvette was placed into the holder and inserted into the Micropulser. Then the pulse button was pressed until the instrument emitted its beeping sound. SOC media (1 mL) was immediately added to the cuvette, mixed, and transferred to a sterile universal 5 ml tube. The tube was incubated at 37°C for 1 to 1.5 hours with shaking at 200 rpm. 100 μL of the growth were spread out on the LB agar with a spreader and the plates containing an appropriate antibiotic and then incubated overnight at 37°C.

### 2.3.13 Preparation and transformation of chemically competent *E. coli*

A single colony of *E.coli* ( DH5α, BL21(DE3), Lemo21(DE3)) strain was streaked out on LB agar and incubated overnight at 37°C. 5 mL of LB broth was inoculated by a single colony of *E.coli* strain and was incubated overnight at 37°C with shaking at 225 rpm. Fresh LB broth (50 mL) was inoculated with 500 μL of the overnight *E.coli* culture and incubated at 37°C, with shaking at 200 rpm for hours until the $OD_{600}$ reached 0.4 – 0.6. All reagents and equipment were chilled on ice and a cool centrifuge rotor and tips used prior to the start of the experiment. The culture was centrifuged at 4000 rpm 4°C for 10 min. The supernatant was removed carefully and the pellet re-suspended gently on ice in 10 mL ice-cold 100 mM $MgCl_2$, for 3 min, followed by centrifugation at 4000 rpm 4°C for 10 min, and the supernatant was removed carefully. The pellet was re-suspended gently in 3 mL of ice-cold 100 mM $CaCl_2$, and incubated on ice for 20 min and then 22 mL of 100 mM $CaCl_2$ were added to the suspension. After this, the cell suspension was centrifuged at 4000 rpm 4°C for 10 min and re-suspended gently on ice in 1 mL of ice-cold sterile 100 mM $CaCl_2$ in 20% (w/v) glycerol. The cell suspension was aliquoted quickly to chilled Eppendorf tubes with 50–100 μL and stored at –80°C.

Competent cells 50 μL were thawed on ice for about 5 min and 1-5 μL of DNA was added to the competent cells by gentle mixing with a yellow tip or gentle flicking, and then incubated on ice for 20 min. The mixture was heat shocked at 42 °C in a heated block or heat water bath for 45-50 sec. Immediately, the mixture was incubated in the ice for 2 min. 950 μL of SOC media was added to the mixture and the mixture was incubated at 37°C for 1 or 1.5 hours, with shaking at 200 rpm. 100 μL of the culture were spread out on the LB agar with a spreader and the plates containing appropriate antibiotics and then incubated overnight at 37 °C.

### 2.3.14 DsbA proteins purification procedure

#### 2.3.14.1 Isolation of total periplasmic protein

DsbA wild type and mutants proteins were purified from BDPD2, BDPD3, SSPD-F29, SSPD-H32, SSPD-Q35 and SSPD-L68 strains. These strains were cultured overnight on LB agar plate at 37 °C. One small colony was cultured overnight in 5 mL LB broth supplemented with 100 μg/mL ampicillin or 25 μg/mL kanamycin depending on plasmid marker, in a shaking incubator at 37 °C and 220-250 rpm. The overnight cultures were subcultured 1:100 for 2 h in 200 mL LB broth, also supplemented with the same antibiotics in a shaking incubator at 37 °C and 200-250 rpm. Once the growth reached $OD_{600nm}$ 0.6 - 0.8, IPTG 1mM (range of IPTG concentrations from 50 μM to 2000 μM were tested) was added to the culture. Incubation was continued for a further 2 - 4 h. The cultures were transferred to 50 mL tubes for centrifugation at 4000 rpm, 4 °C for 10 min (from now the culture should be in ice and all components pre-chilled) . The supernatant was removed and the pellet was washed with pre-chilled 5-10 mL of 0.1 M sodium phosphate buffer, pH 7.0. The cultures were centrifuged again at 4000 rpm, 4 °C for 10 min. The supernatant was removed and the pellet was re-suspended in 2 mL 1x PBS containing 2000 U of polymyxin B (1000 U/mL). The suspension pellets were incubated on ice for 15 min. The suspension was centrifuged at 4000 rpm, 4°C for 10 min. The supernatant (periplasmic proteins) was transfer to a new tube after centrifugation.

#### 2.3.14.2 DsbA His-tag purification

To purify DsbA protein from the crude periplasmic proteins as described in section 2.3.14.1, a His-trap column was prepared (GE Healthcare). A syringe was filled with distilled water and the column was connected to the syringe and washed with 5

column volumes (CV) of distilled water. Then, the column was equilibrated with at least 5 CVs of binding buffer (20 mM sodium phosphate, 0.3 M NaCl, 20 mM imidazole, pH 7.4). The sample that contained DsbA protein was applied using a syringe to the top of the column and the eluate was collected in 15 mL tubes. The column was washed with 10 mL of binding buffer and the wash was collected in another 15 mL tube (flow rate of 1 mL/min). Finally, the protein was eluted with 2 mL of elution buffer (20 mM sodium phosphate, 0.3 M NaCl, 500 mM imidazole, pH 7.4) into 4 Eppendorf tubes (each tube 0.5mL).

#### 2.3.14.3 SDS-PAGE confirmation

SDS PAGE was set up and a polyacrylamide gels (14%) were prepared and mounted using a Mini-Protean (Bio-Rad) system. Clamp, gel plates and spaces of the system were assembled into the gel stand. The resolving gel mix Table (2.16) was poured directly into the assembly gel plates and distilled water was added above the resolving gel mix in plates to keep the surface wet and straight. The gel plates were left at room temperature for 40 min until they solidified. Next, the stacking gel mix was prepared Table (2.17). The mix was poured above the resolving gel (after removing the distilled water) and the appropriate comb was added. The gel was allowed to solidify at room temperature for 40 min.

**Table 2.16: Resolving gel (14%) preparation**

| Components | Volume |
|---|---|
| SDW | 9.95 mL |
| Resoling buffer | 8.75 mL |
| Acrylamide (30%) | 16.3 mL |
| Ammonium persulphate (APS 10%) | 105 μL |
| TEMED | 17.5 μL |

**Table 2.17: Stacking gel (12%) preparation**

| Components | Volume |
|---|---|
| SDW | 12 mL |
| Stacking buffer | 5 mL |
| Acrylamide (30%) | 3 mL |
| Ammonium persulphate (APS 10%) | 90 μL |
| TEMED | 30 μL |

A running buffer (1.44% glycine, 0.3% Tris, 0.1% SDS) was added to SDS-PAGE after the gel was setup. Protein samples, were prepared for SDS-PAGE by mixing protein loading buffer/protein (1:5) and incubating at 95 $^{o}$C for 10 minutes, samples were centrifuged at 11.000 rpm for 30 sec then incubated on ice for 5 min before loading in the gel. 10 μL of prestained protein HyperPAGE ladder (Bioline Co., UK) was added to one well of the gel. 20 μL of the protein samples on ice were loaded to the gel wells. The gel was subjected to electrophoresis for 1.30 h at 120 V with the protein running buffer. For SDS PAGE, the gel was released after the run was completed and stained with Coomassie blue stain for 20 min. The gel was washed three times with 20 mL of a de-staining solution (100 mL acetic acid, 200 mL methanol and 700 mL distilled water) until the bands appeared.

### 2.3.14.4 Western blotting confirmation

For Western blotting, the gel described in section 2.3.14. 3 was placed in a Mini Trans-Blot system (Bio-Rad) to transfer the protein bands. A polyvinylidene difluoride (PVDF) membrane was activated by immersion in 100% methanol for 3 min and the sandwich of transferring was performed. The gel was subjected to electrophoresis for 1 h at 0.4 Am in transfer buffer (1.44% glycine, 0.3% Tris, 0.1% SDS, and 20%

methanol), ice was supplemented to the container and put inside the tank before adding the transfer buffer. After completion of electrophoresis, the membrane was released and blocked by incubation in a TBS buffer (25 mM Tris, pH 8, 125 mM NaCl) containing 5% non-fat milk for 1 h at room temperature. Then, the membrane was incubated at 23 °C with shaking overnight with His epitope tag anti-mouse primary antibody diluted at 1:20,000 in TBS containing 5% non-fat milk. The membrane was washed three times with TBS-T (containing 0.1% tween 20). The membrane was incubated with ALEXA FLOUR 680 anti-mouse secondary antibody diluted at 1:10,000 in TBS containing 5% non-fat milk at 4°C with shaking for 2 hours. Finally, the membrane was washed three times with TBS-T and the fluorescent bands were detected in the membrane by Odyssey Infrared Imager, USA.

### 2.4 Statistical analysis

Data was analysed using GraphPad Prism Version 6.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. Normally distributed data was analysed using a student's t-test to compare two treatments or one-way analysis of variance (ANOVA), for 3 or more treatments a two-way (ANOVA) was used. For *in vivo* studies were analysed using a Kaplan–Meier survival curves to compare two treatments.

# Chapter 3

## Activity of natural products (terpenoids) in reducing *Shigella sonnei* infection

# 3 Activity of natural products (terpenoids) in reducing *S. sonnei* infection

## 3.1 Introduction

The rate of antimicrobial resistance (AMR) has dramatically increased in the last few decades and is a worldwide health concern. Meanwhile, the World Health Organisation (WHO) has placed *Shigella* spp. as a medium level of risk of the fluoroquinolone-resistance (WHO, 2017). Since the discovery of penicillin in the 1940s, most bacteria have developed resistance to all antibiotics introduced into clinical treatments (Clatworthy et al., 2007). By 2050, the annual mortality rate associated with multi-drug resistant (MDR) pathogens is predicted to exceed that of cancer at 10 million deaths annually if no immediate action is taken (de Kraker et al., 2016). One possible antibacterial strategy is to develop compounds that inhibit bacterial virulence rather than bacterial growth (Escaich, 2010). Bacterial pathogens depend on many virulence factors, such as toxins, Type 3 secretion systems (T3SS), fimbrial and non-fimbrial adhesins as well as motility systems, to attach and infect their host. As a result, anti-virulence strategies that concentrate on these systems might reduce or completely abolish the capacity of a pathogen to cause disease (Heras et al., 2015).

Adhesion is a crucial step for colonization by bacteria of host cells (Mahmoud et al., 2016). So, blocking this process may prevent establishment and maintenance of infection. Sortases are a family of cysteine transpeptidases and are responsible for adhesion in Gram-positive bacteria and have been targeted by several antibacterial drugs through screening against *Staphylococcus aureus* and *Bacillus anthracis* (Suree et al., 2009). For example, aaptamine is a natural product which can inhibit the sortase enzyme of *S. aureus*, while Pilicides inhibit the formation of virulence-associated pili and can block the chaperone/usher assembly pathway in UPEC (Jang

et al., 2007, Aberg and Almqvist, 2007). The T3SS machinery is present in many pathogens including *Escherichia, Shigella, Salmonella, Pseudomonas, Chlamydia* and *Yersinia* spp., so that targeting common elements could result in broad-spectrum T3SS inhibitors. Thiazolidinone derivatives were identified as an inhibitor that block T3SS from Gram- negative pathogens including *S. typhimurinm* and *Yersinia enterocolitica* (Felise et al., 2008). Recently, it was reported that Aurodox, a natural product from *Streptomyces goldiniensis* is able to downregulate the expression of the type III secretion systems of enteropathogenic and enterohemorrhagic *Escherichia coli*. (McHugh et al., 2019).

Bacillary dysentery is a global disease that is caused by *Shigella sonnei* in many newly industrialised countries and has replaced *Shigella flexneri* as one of most prevalent species globally as a causative agent for dysentery (von Seidlein et al., 2006). *S. sonnei* strains from lineage III are the most common epidemic-causing strains and exhibit multiple drug resistance due to the spread of resistance genes by transposons and independent point mutations in *gyrB* (Holt et al., 2012). In addition, there is no vaccine available against *Shigella* and developing an effective vaccine remains a challenge. The current treatment for shigellosis are fluoroquinolones for adults and cephalosporin for children (Kim et al., 2015).

The host cytosol is a reducing environment due to the concentration of reduced glutathione (GSH) and contains approximately 10 mM of GSH and a small amount of oxidised glutathione (GSSG)(Hwang et al., 1992). DsbA is a bacterial periplasmic thiol-disulphide oxidoreductase and is a key component of the disulphide bond family of enzymes (Denoncin and Collet, 2013). Up-regulation of DsbA expression is a specific response to reducing conditions as *S. sonnei* requires DsbA to survive in the reducing environment of the host cell cytosol (Yu, 1998). GSH is a tripeptide

compound synthesized in the cytosol and has many activities in the cell. GSH can reduce disulphide bonds by donating electrons to form GSSG (Appenzeller-Herzog, 2011). Dsb enzymes have been well studied in *E. coli* K12 (Smith et al., 2016, Depuydt et al., 2011) where they catalyse the folding of secreted proteins including many virulence factors.

Terpenoids are a class of natural products found in 90% of the essential oils (EO); specific scents produced by plants as chemo-attractants or repellents (McGarvey and Croteau, 1995, Crowell, 1999). Many EOs can interact with bacterial cell membranes and inhibit their growth *in vitro* (Solorzano-Santos and Miranda-Novales, 2012). Recent studies have demonstrated that terpenoids, especially monoterpenes, have antimicrobial, anti-inflammatory, anti-oxidant, hypotensive, antipruritic and analgesic activity (Kordali et al., 2005, Guimaraes et al., 2013, Bastos et al., 2010). It has been previously reported that a natural flavonoid called propolin D with a terpene side chain could reduce the production of the inflammatory cytokines IL-1 and IL-18 from infected macrophage-like U937 cells and inhibit the intracellular proliferation of *S. sonnei* in this cell line as well as in epithelial HEp-2 cells (Xu et al., 2011). In contrast, eriodictyol, a compound that shares identical flavonoid rings but lacks the prenylated (terpene-derived) side chain, showed very little intracellular accumulation. Not surprisingly, supplementation of 42 μM propolin D, but not of 42 μM eriodictyol, in the culture medium inhibited *S. sonnei* growth inside HEK 293 cells (Mirza et al., 2018). These results suggest that the terpenic side chain was responsible for the accumulation of propolin D as well as its activity on *Shigella*, inside host cells. As many terpenes are known to possess antimicrobial activity (Tsai et al., 2011), this led us to hypothesise that the terpenic side chain could be the key moiety responsible for the inhibition of SSWT intracellular growth. Furthermore, geraniol is a monoterpenes sharing similar structure to the side chain in the propolin D, and is able to reduce the numbers of

intracellular of *S. sonnei* and showed ability to inhibit the activity of *S. sonnei* DsbA to reduce GSSG and convert to GSH *in vitro* (Mirza et al., 2018). So, in this chapter we aimed to determine if geraniol and other selected terpenoids, that share similar features in their structure Figure (1.5), inhibit the *S. sonnei* proliferation.

The specific aims of this chapter were to;

1- Determine the effect of terpenoids on the intracellular proliferation of *S. sonnei*.

2- Evaluate the cytotoxicity of terpenoids on a range of cell lines.

3- Evaluate the activity and cytotoxicity of terpenoids using an *in vivo* larvae model.

### 3.2 Direct antimicrobial activity of terpenoids against SSWT bacteria

In order to determine the antimicrobial activity of terpenoids on SSWT in a reducing environment such as M9 media, an experiment was carried out in 10 mL tubes. The optical density (OD) was monitored for each hour up to 10h when the $OD_{600}$ reach a maximum. Geraniol showed antimicrobial activity by delaying the growth curve of SSWT (Figure 3.2.1 A). However, this activity was not statistically significant. Citral showed activity in delaying the growth of SSWT in Figure (3.2.1 B) but statistically remains not significant. The rest of terpenoids did not show any antimicrobial activity on SSWT and the growth curve was equal to the untreated (SSWT) at the 42μM concentration.

Despite this, the question remains; do these terpenoids have an antimicrobial activity if we increase the concentration? To answer this question, a minimum inhibition concentration (MIC) experiment was performed and different concentrations of terpenoids from (0.4 mg/L to 500mg/L) were tested. Most of the terpenoids showed no activity on SSWT even at the highest concentration of 500 mg/L or less. Figure (3.2.2) shows terpenoid activity at 500mg/L and it is clear that geranylamine has an ability to inhibit *Shigella* growth at this high level while it does not have this activity at concentration less 500mg/L. Geraniol, β-citronellol, rhodinol and R.(+). β-citronellol showed a little activity at this concentration towards SSWT when it compared to the untreated control. This confirmed that some of these compounds do not have antimicrobial activity and others may possess some activity on SSWT.

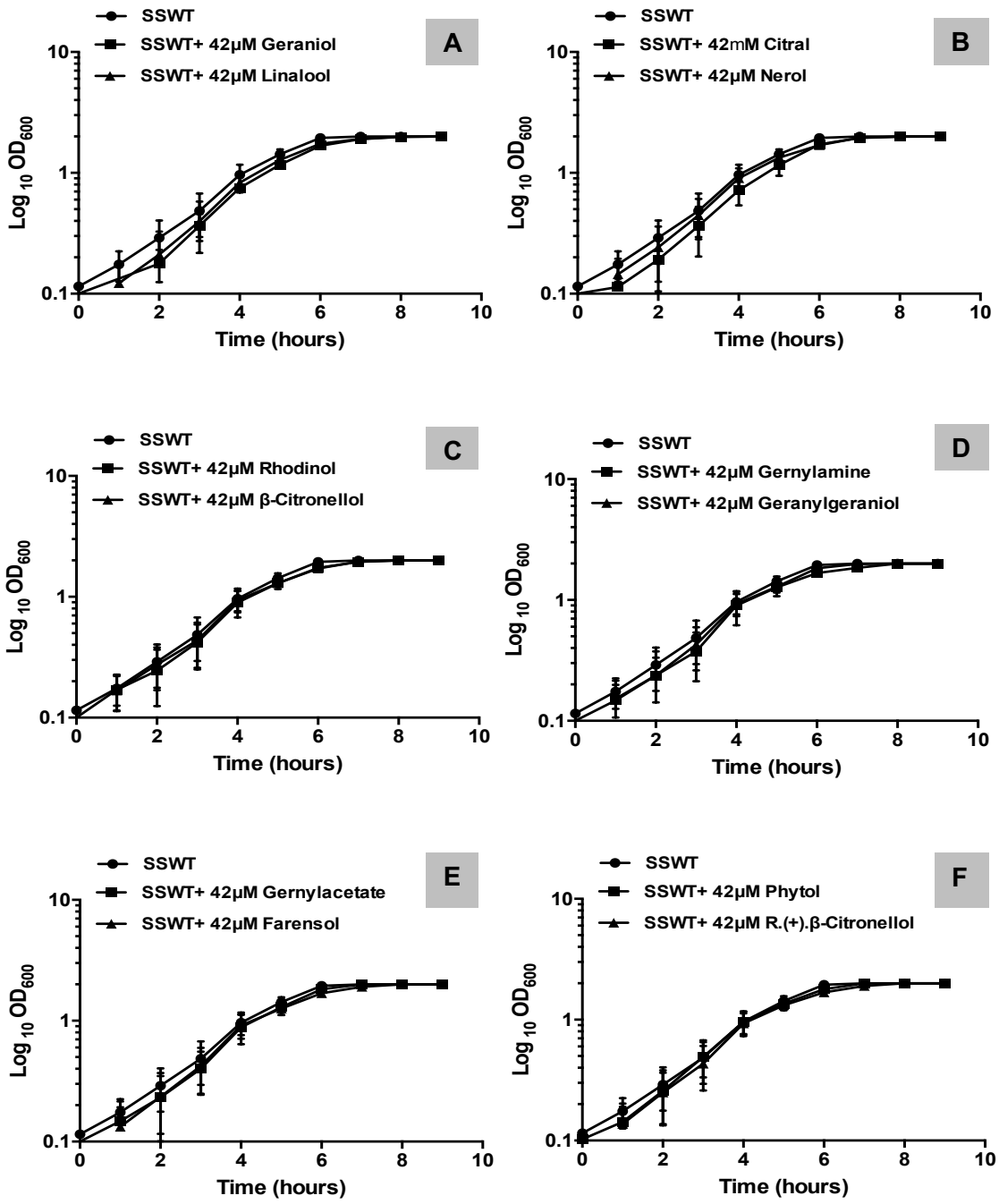



**Figure 3.2.1: Direct antibacterial activity of terpenoids against SSWT.**

**A-** Activity of geraniol and linalool against SSWT. **B-** Activity of citral and nerol against SSWT. **C-** Activity of rhodinol and β-citronellol against SSWT. **D-** Activity of geranylamine and geranylgeraniol against SSWT. **E-** Activity of geranylacetate and farnesol against SSWT. **F-** Activity of phytol and R.(+).β-citronellol against SSWT. All compounds did not show any significant activity against SSWT in the logarithmic growth, and the SSWT grew very well in the presence of terpenoids.

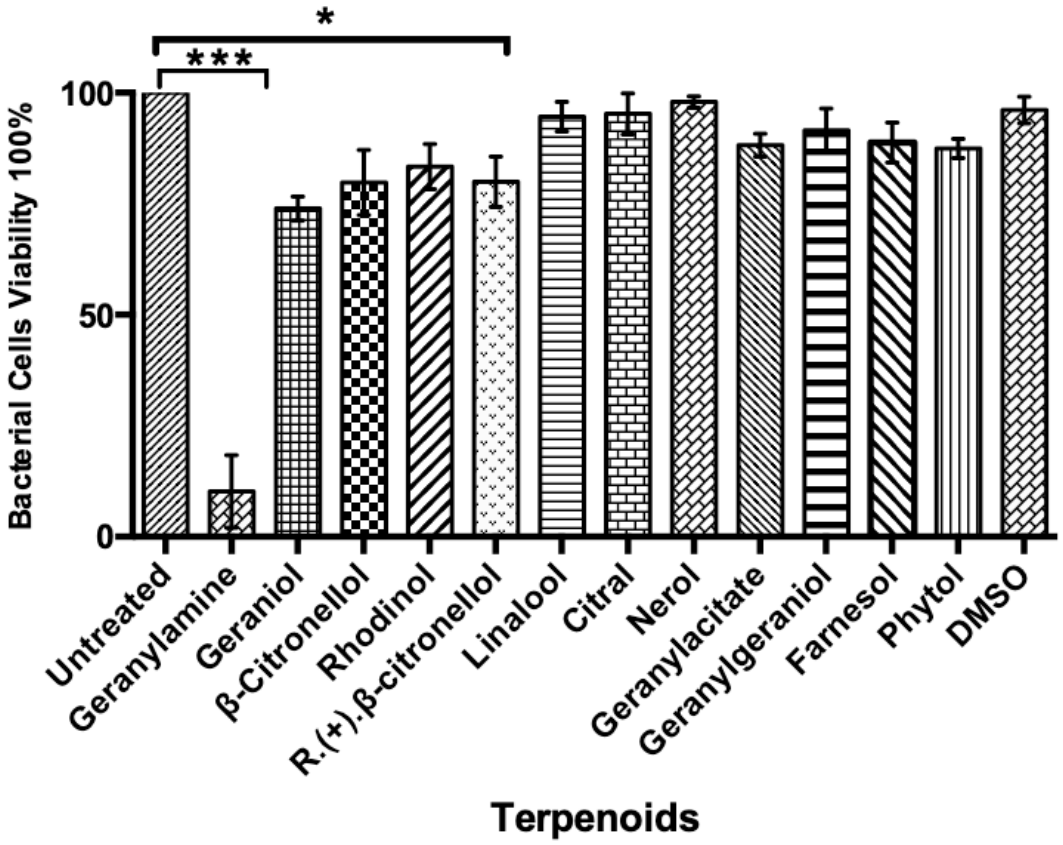



**Figure 3.2.2: Minimum inhibitory concentration (MIC) for terpenoids against *S. sonnei*.**

Columns represented the minimum inhibitory concentration for each selected terpenoid. Results assessed using the higher concentration of treatment (500mg/L) in triplicate and repeated three times. Error bars are mean + standard deviation calculated using One way ANOVA test. All terpenoids did not show any activity on SSWT except the geranylamine who was significant in inhibition the SSWT.

## 3.3 Cytotoxicity of terpenoids

It was clear that some tested compounds had an activity on SSWT, so it was important to know their cytotoxicity on the host cells. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a chemical which is actively taken up by live cells and converted to a purple precipitate (Stockert et al., 2012). This chemical was used to detect the cytotoxicity on both HeLa and RAW macrophage cell lines in the presence of terpenoids.

### 3.3.1 Cytotoxicity of terpenoids towards HeLa cells

In order to determine the cytotoxicity of terpenoids on HeLa cells at the target concentration of 42 µM, an MTT assay was performed (Stockert et al., 2012). The results showed that all terpenoids exerted little cytotoxicity on HeLa cells at the tested concentration (Figure 3.3.1). Rhodinol showed a 1 fold reduction in cell viability and this may explain the activity of Rhodinol on SSWT. The reduction in the number of intracellular CFUs was due to gentamicin that penetrated the dead cells after their exposure to rhodinol and killed all the intra- and extracellular bacteria. DMSO showed no toxicity towards HeLa cells at the concentration used.

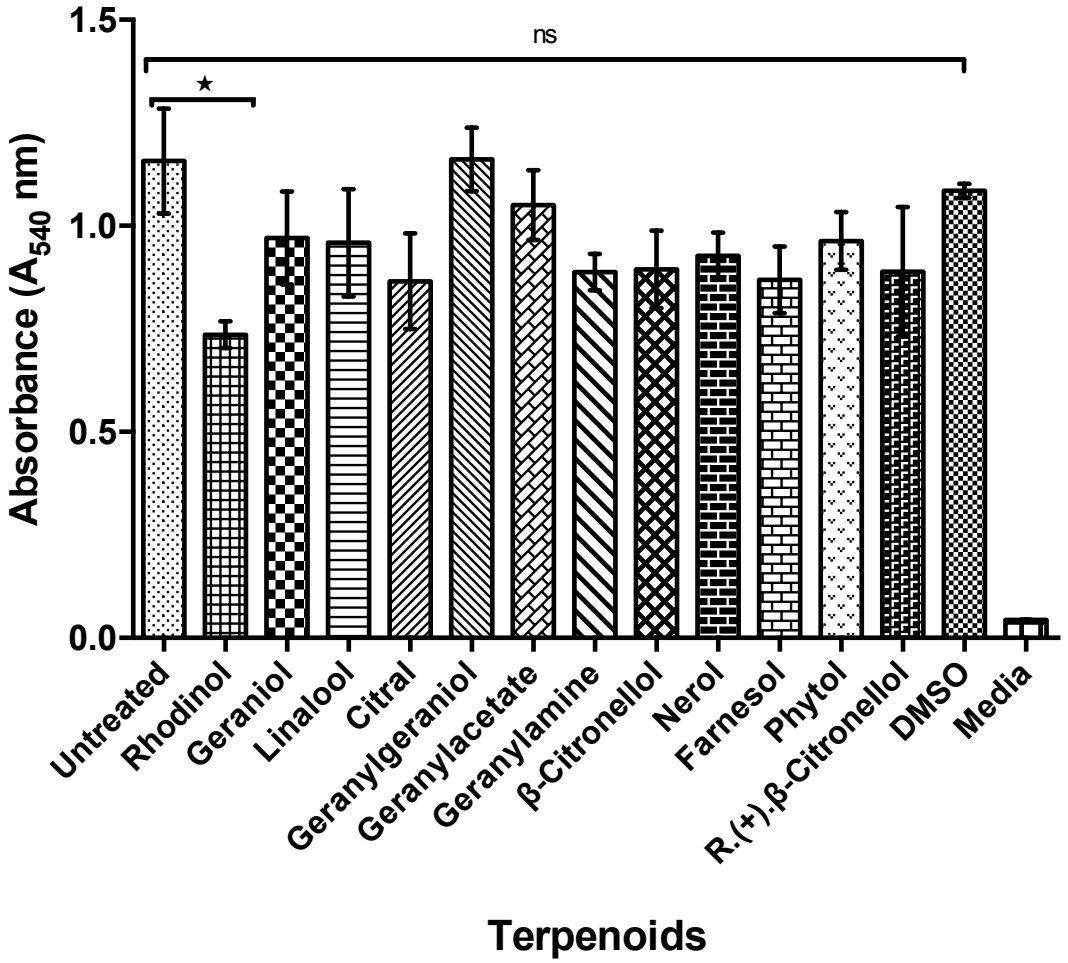


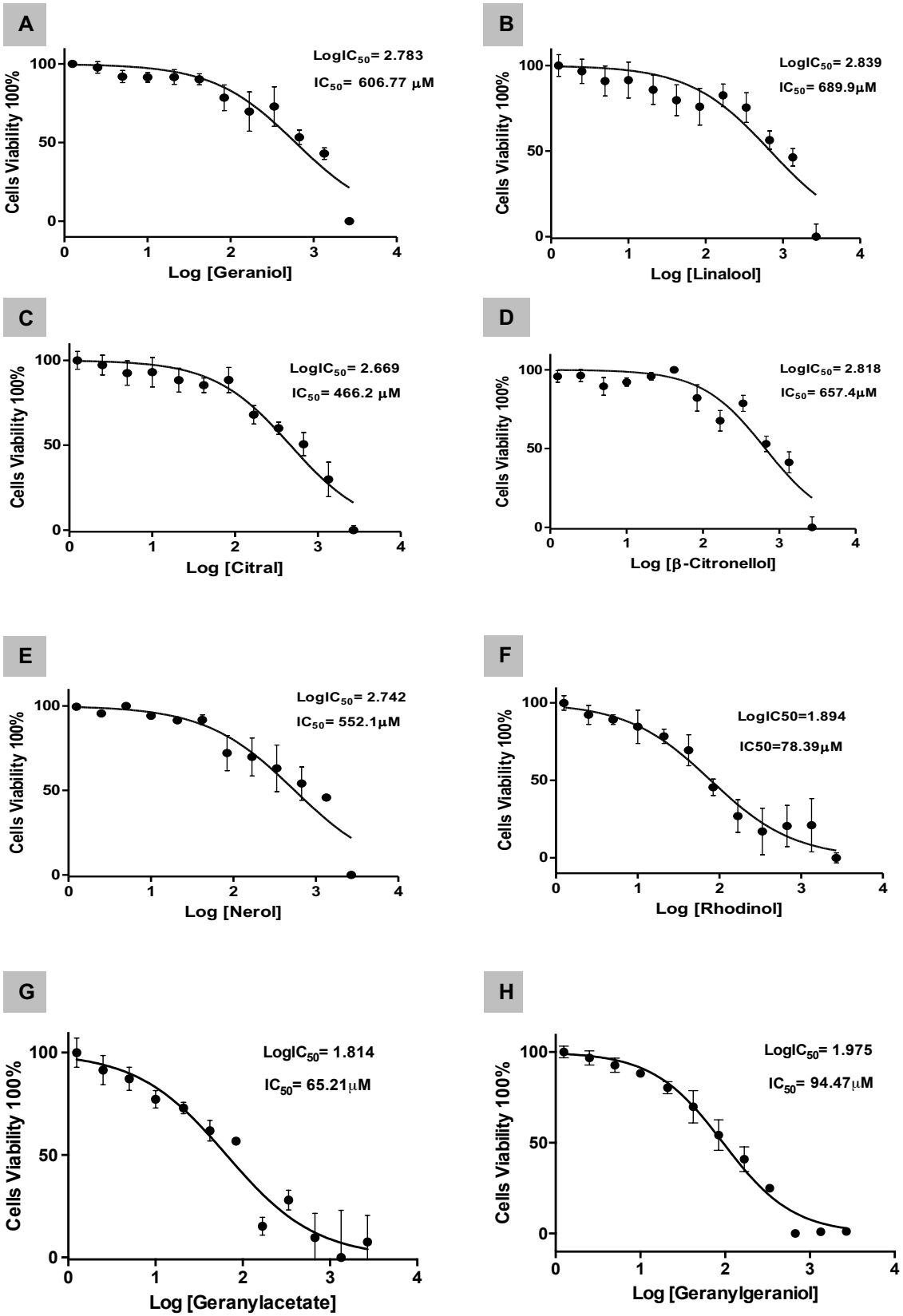

**Figure 3.3.1: Cytotoxicity of terpenoids on HeLa cells.**

Columns showed the variable viability of HeLa cells after exposure to 42 μM of selected terpenoids. Rhodinol showed ability to reduce the HeLa cell number but it still not significant. The other terpenoids did not show any significant ability to reduce the HeLa cell number. Experiments were carried out in triplicate and repeated three times. The results are shown as means ± SD. Differences between groups and control were assessed using One way ANOVA test.

The $IC_{50}$ for each terpenoid (i.e. the concentration of an inhibitor that gives a half-maximal response) were further calculated (Sebaugh, 2011). Different concentrations of terpenoids from 1.31 μM to 2.688 mM were prepared in the liquid medium and incubated with the cells overnight. The $IC_{50}$ values were calculated from the linear regression of the dose-log response curves. Rhodinol, geranylacetate, geranylgeraniol and farnesol (Figure 3.3.2 F, G, H and J) showed $IC_{50}$ values of 78.39 μM, 65.21 μM, 94.47 μM and 84.57 μM, respectively. When comparing these values with the target concentration (42 μM) in the Figure (3.3.1) these compounds showed no cytotoxicity except for rhodinol which was toxic at the target concentrations. Geranylamine in Figure (3.3.2 I) showed an $IC_{50}$ value of 147.8 μM, which is likely higher than the previous mentioned compounds. However, geranylamine showed nontoxic result to the HeLa cells at the target concentration. The rest of compounds used showed a high $IC_{50}$ when incubated with HeLa cells overnight and showed little or no cytotoxicity.

**Figure 3.3.2: $IC_{50}$ values of terpenoids on HeLa cells.**

**A-** Graph curve of $LogIC_{50}$ for geraniol on HeLa cells. Geraniol showed high $IC_{50}$ with 606.77μM. **B-** Graph curve of $LogIC_{50}$ for linalool on HeLa cells. Linalool showed high $IC_{50}$ with 689.9 μM. **C-** Graph curve of $LogIC_{50}$ for citral on HeLa cells. Citral showed high $IC_{50}$ with 466.2μM. **D-** Graph curve of $LogIC_{50}$ for β-citronellol on HeLa cells. β-citronellol showed high $IC_{50}$ with 657.4 μM. **E-** Graph curve of $LogIC_{50}$ for nerol on HeLa cells. Nerol showed high $IC_{50}$ with 552.1 μM. **F-** Graph curve of $LogIC_{50}$ for rhodinol on HeLa cells. Rhodinol showed low $IC_{50}$ with 78.39 μM. **G-** Graph curve of $LogIC_{50}$ for geranylacetate on HeLa cells. Geranylacetate showed low $IC_{50}$ with 65.21 μM. **H-** Graph curve of $LogIC_{50}$ for geranylgeraniol on HeLa cells. Geranylgeraniol showed low $IC_{50}$ with 94.47 μM. **I-** Graph curve of $LogIC_{50}$ for geranylamine on HeLa cells. Geranylamine showed low $IC_{50}$ with 147.8 μM. **J-** Graph curve of $LogIC_{50}$ for farnesol on HeLa cells. Farnesol showed low $IC_{50}$ with 84.57 μM. **K-** Graph curve of $LogIC_{50}$ for phytol on HeLa cells. Phytol showed medium $IC_{50}$ with 376.8 μM. **L-** Graph curve of $LogIC_{50}$ for R.(+).β-citronellol on HeLa cells. R.(+).β-citronellol showed high $IC_{50}$ with 849.8 μM. Cells were cultured in DMEM with or without terpenoids. Different concentrations of terpenoids from 1.31μM to 2.688mM were prepared in the liquid medium and incubated with the cells overnight. The $IC_{50}$ values were calculated from the linear regression of the dose-log response curves using GraphPad Prism 6. Error bars are mean ± S.D. The experiment carried out in triplicate and repeated twice.


A
LogIC50= 2.783
IC50= 606.77 μM
Cells Viability 100%
Log [Geraniol]
B
LogIC50= 2.839
IC50= 689.9μM
Cells Viability 100%
Log [Linalool]
C
LogIC50= 2.669
IC50= 466.2 μM
Cells Viability 100%
Log [Citral]
D
LogIC50= 2.818
IC50= 657.4μM
Cells Viability 100%
Log [β-Citronellol]
E
LogIC50= 2.742
IC50= 552.1μM
Cells Viability 100%
Log [Nerol]
F
LogIC50=1.894
IC50=78.39μM
Cells Viability 100%
Log [Rhodinol]
G
LogIC50= 1.814
IC50= 65.21μM
Cells Viability 100%
Log [Geranylacetate]
H
LogIC50= 1.975
IC50= 94.47μM
Cells Viability 100%
Log [Geranylgeraniol]

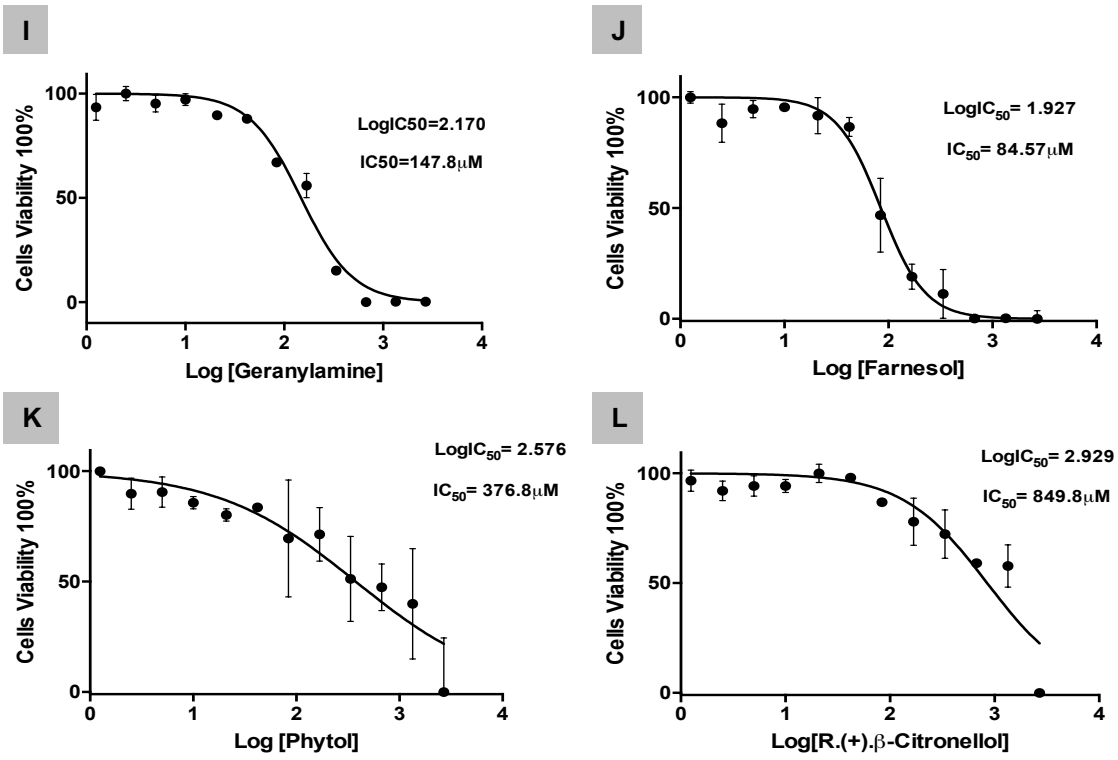



**Figure 3.3.2: $IC_{50}$ values of terpenoids on HeLa cells.**

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### 3.3.2 Cytotoxicity of terpenoids towards RAW macrophage cells

The terpenoids showed activity in reducing the number of intracellular CFUs of SSWT in RAW macrophage cells infected by gentamicin killing assay. So, it was necessary to test the cytotoxicity of terpenoids on these cells. Figure (3.3.3) showed that all twelve terpenoids were tested their cytotoxicity towards RAW macrophage cells at the target concentration. Geranylamine reduced the cell viability significantly to half when compared to the untreated column. This is revealing that geranylamine was toxic to the cells but was not active against SSWT. When comparing geranylamine in RAW macrophages to its treatment on HeLa cells, it is clear that this compound was not toxic to HeLa cells. At the same time, Rhodinol was toxic to HeLa cells in Figure (3.3.1), while it was not toxic to RAW macrophage cell lines. The rest of terpenoids showed no cytotoxicity or showed a little toxicity towards RAW macrophage cells at the indicated concentration. DMSO was used at the same concentration and it showed no cytotoxicity on this cell line.

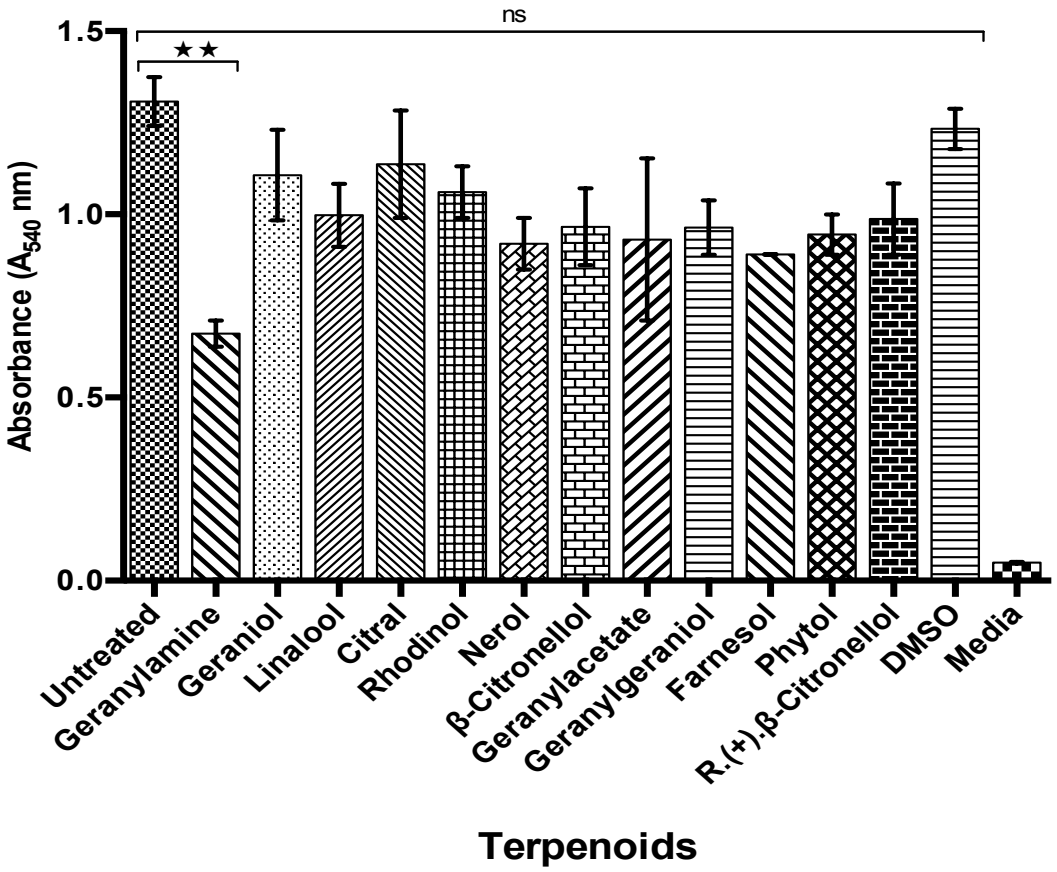


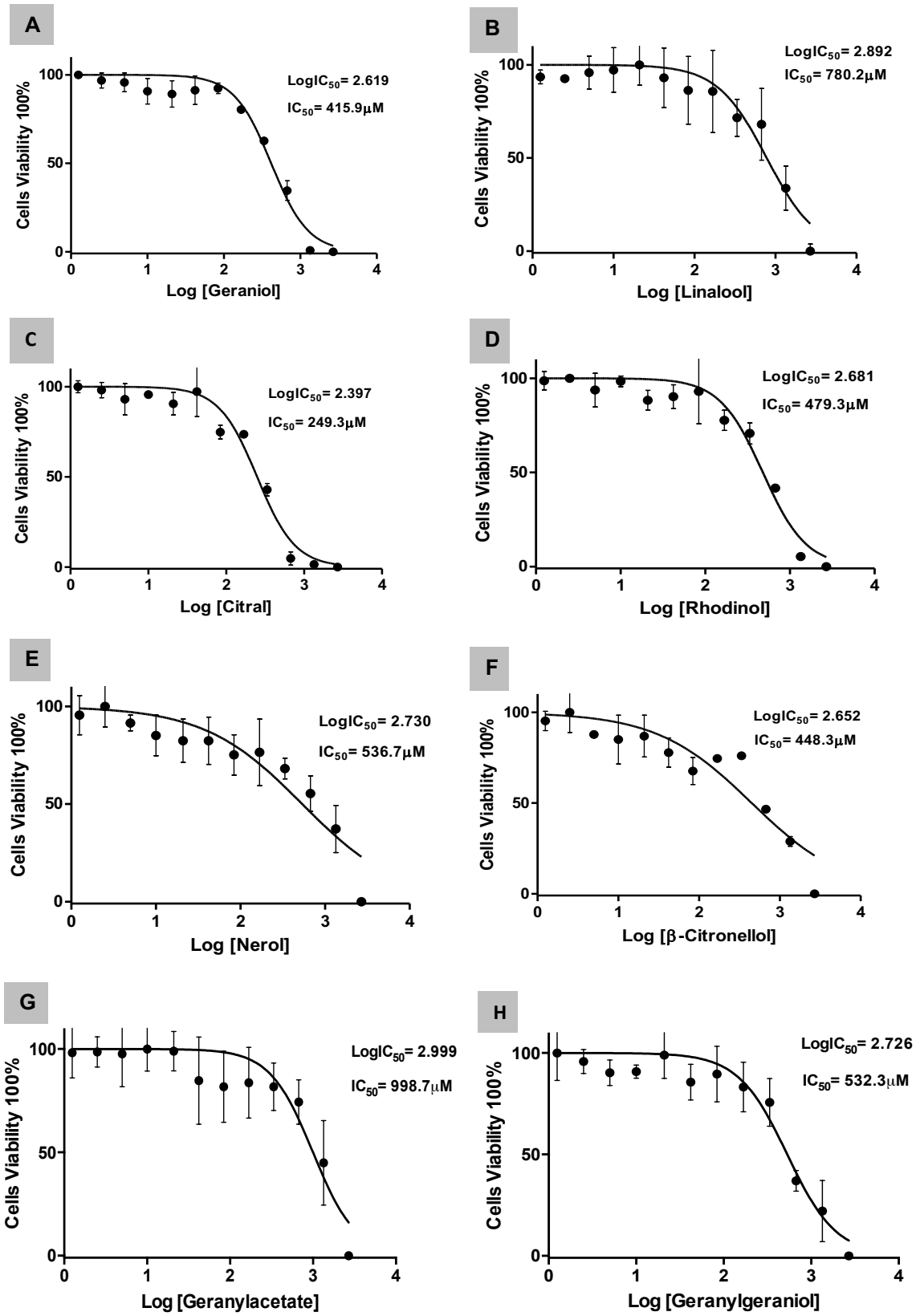

**Figure 3.3.3: Cytotoxicity of terpenoids on RAW macrophage cells.**

Columns showed the variable viability of RAW macrophage cells after exposure to 42 μM of selected terpenoids. Geranylamine was able to reduce the RAW cells significantly for one fold, while other compounds did not show any significant ability to reduce the RAW cells number. Experiments were carried out in triplicate and repeated three times. The results are shown as means ± SD. Differences between groups and control were assessed using One way ANOVA test.

The $IC_{50}$ values in RAW macrophage were determined as with HeLa cells in section (3.3.2). The terpenoids in Figure (3.3.4) showed a result different to their effect on HeLa cells. Geranylamine and farnesol in the Figure (3.3.4 I and J), both showed a lower $IC_{50}$ with 77.00 μM and 85.98 μM respectively. Geranylamine was toxic to RAW macrophage cells at the target concentration and this cytotoxicity confirmed when calculated the $IC_{50}$ for this compound. However, geranylamine was also a showed low $IC_{50}$ in HeLa cells. Farnesol was not toxic at target concentration but it showed here a low $IC_{50}$ on macrophage cells. Rhodinol, geranylacetate and geranylgeraniol in Figure (3.3.4 D, G and H) produced the lowest $IC_{50}$ on HeLa cells, while, they showed high $IC_{50}$ on RAW macrophage cells. The rest of compounds showed high levels of $IC_{50}$ in RAW macrophage cells and confirmed this compounds are nontoxic.

**Figure 3.3.4: $IC_{50}$ values of terpenoids on RAW macrophage cells.**

**A-** Graph curve of $LogIC_{50}$ for geraniol on RAW macrophages cells. Geraniol showed high $IC_{50}$ with 415.9 μM. **B-** Graph curve of $LogIC_{50}$ for linalool on RAW macrophages cells. Linalool showed high $IC_{50}$ with 780.2 μM. **C-** Graph curve of $LogIC_{50}$ for citral on RAW macrophages cells. Citral showed medium $IC_{50}$ with 249.3 μM. **D-** Graph curve of $LogIC_{50}$ for rhodinol on RAW macrophages cells. Rhodinol showed high $IC_{50}$ with 479.3 μM. **E-** Graph curve of $LogIC_{50}$ for nerol on RAW macrophages cells. Nerol showed high $IC_{50}$ with 536.7 μM. **F-** Graph curve of $LogIC_{50}$ for β-citronellol on RAW macrophages cells. β-citronellol showed high $IC_{50}$ with 448.3 μM. **G-** Graph curve of $LogIC_{50}$ for geranylacetate on RAW macrophages cells. Geranylacetate showed high $IC_{50}$ with 998.7 μM. **H-** Graph curve of $LogIC_{50}$ for geranylgeraniol on RAW macrophages cells. Geranylgeraniol showed high $IC_{50}$ with 532.3 μM. **I-** Graph curve of $LogIC_{50}$ for geranylamine on RAW macrophages cells. Geranylamine showed low $IC_{50}$ with 77.00 μM. **J-** Graph curve of $LogIC_{50}$ for farnesol RAW macrophages cells. Farnesol showed low $IC_{50}$ with 85.98 μM. **K-** Graph curve of $LogIC_{50}$ for phytol on RAW macrophages cells. Phytol showed high $IC_{50}$ with 1380 μM. **L-** Graph curve of $LogIC_{50}$ for R.(+).β-citronellol on RAW macrophages cells. R.(+).β-citronellol showed high $IC_{50}$ with 814.8 μM. The $IC_{50}$ values were calculated from the linear regression of the dose-log response curves using GraphPad Prism 6. Error bars are mean ± S.D. The experiment curried out in triplicate and repeated twice.


A
Cells Viability 100%
LogIC50= 2.619
IC50= 415.9µM
Log [Geraniol]
B
LogIC50= 2.892
IC50= 780.2µM
Log [Linalool]
C
LogIC50= 2.397
IC50= 249.3µM
Log [Citral]
D
LogIC50= 2.681
IC50= 479.3µM
Log [Rhodinol]
E
LogIC50= 2.730
IC50= 536.7µM
Log [Nerol]
F
LogIC50= 2.652
IC50= 448.3µM
Log [β-Citronellol]
G
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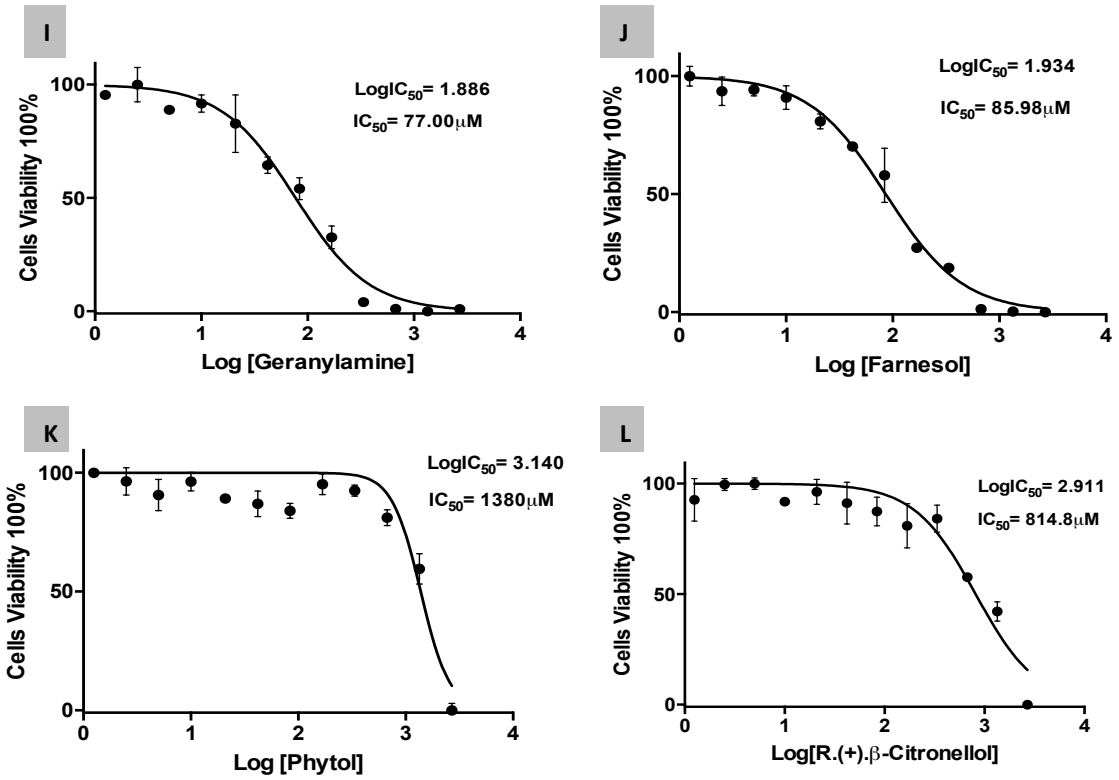



**Figure 3.3.4: $IC_{50}$ values of terpenoids on RAW macrophage cells.**

**A-** Graph curve of $LogIC_{50}$ for geraniol on RAW macrophages cells. Geraniol showed high $IC_{50}$ with 415.9 μM. **B-** Graph curve of $LogIC_{50}$ for linalool on RAW macrophages cells. Linalool showed high $IC_{50}$ with 780.2 μM. **C-** Graph curve of $LogIC_{50}$ for citral on RAW macrophages cells. Citral showed medium $IC_{50}$ with 249.3 μM. **D-** Graph curve of $LogIC_{50}$ for rhodinol on RAW macrophages cells. Rhodinol showed high $IC_{50}$ with 479.3 μM. **E-** Graph curve of $LogIC_{50}$ for nerol on RAW macrophages cells. Nerol showed high $IC_{50}$ with 536.7 μM. **F-** Graph curve of $LogIC_{50}$ for β-citronellol on RAW macrophages cells. β-citronellol showed high $IC_{50}$ with 448.3 μM. **G-** Graph curve of $LogIC_{50}$ for geranylacetate on RAW macrophages cells. Geranylacetate showed high $IC_{50}$ with 998.7 μM. **H-** Graph curve of $LogIC_{50}$ for geranylgeraniol on RAW macrophages cells. Geranylgeraniol showed high $IC_{50}$ with 532.3 μM. **I-** Graph curve of $LogIC_{50}$ for geranylamine on RAW macrophages cells. Geranylamine showed low $IC_{50}$ with 77.00 μM. **J-** Graph curve of $LogIC_{50}$ for farnesol RAW macrophages cells. Farnesol showed low $IC_{50}$ with 85.98 μM. **K-** Graph curve of $LogIC_{50}$ for phytol on RAW macrophages cells. Phytol showed high $IC_{50}$ with 1380 μM. **L-** Graph curve of $LogIC_{50}$ for R.(+).β-citronellol on RAW macrophages cells. R.(+).β-citronellol showed high $IC_{50}$ with 814.8 μM. The $IC_{50}$ values were calculated from the linear regression of the dose-log response curves using GraphPad Prism 6. Error bars are mean ± S.D. The experiment curried out in triplicate and repeated twice.

### 3.4 Activity of terpenoids on *Shigella*-infected cell lines

To test the hypothesis that terpenoids might inhibit DsbA, we examined twelve terpenoids for their potential in inhibiting intracellular SSWT growth using a gentamicin-killing assay. These compounds are plant derived natural products, mostly from lemongrass. These compounds were purchased from Sigma Aldrich USA and UK and were used to determine their activity in controlling intracellular bacteria. For each compound we considered the molecular weight, density and the purity Table (2.3). The compounds were dissolved in Dimethyl sulfoxide (DMSO) to make the 1$^{st}$ stock in molar, and the other working concentrations were then dissolved in the different media depending on the experiment. For consistency of results, the working concentration used in all experiments was 42 μM as previously used in (Xu et al., 2011).

#### 3.4.1 Activity of terpenoids in *Shigella*-infected HeLa cells

In order to determine the activity of terpenoids on *S. sonnei* (SSWT) in the host cells such as HeLa cells, a gentamicin killing assay was performed as described in section (2.2.1). HeLa cells (human cervical cancer derived) and macrophages (RAW 264 mouse leukemic monocyte macrophage cell-line) were used in the gentamicin killing assay to detect the activity of terpenoids. The compounds were added to DMEM 40 min after the bacterial invasion phase at the final concentration of 42 μM in the presence of gentamicin (50 μg/mL). The number of intracellular (Colony forming unit C.F.U) was determined 2h post infection.

The number of intracellular bacteria was significantly inhibited by 2.5 fold in the presence of geraniol, linalool, citral, rhodinol, geranyl amine, β-citronellol and nerol at

($p < 0.05$) when compared to the untreated wild type SSWT (Figure 3.4.1). All other terpenoids reduced the number of intracellular CFUs either by one or two fold, or not at all, compared with the untreated control. DMSO was used to dissolve all terpenoids, and it had little deleterious effect on the number of CFUs at the highest dose used (42μM). The number of CFUs observed for the *S. sonnei dsbA* mutant (SSDM) was low because such mutant, lacking *dsbA*, lost its ability to multiply inside the host cell due to misfolding of effector proteins (Yu, 1998). Geraniol was the most potent terpenoid tested. It significantly reduced the number of intracellular CFUs compared to an untreated control and to other terpenoids. The results also showed that geranylgeraniol and farnesol had no activity against intracellular SSWT compared with the untreated control and other terpenoids. R.(+).β-citronellol and phytol showed a small reduction in the number of intracellular CFUs compared to the untreated control. These results suggest that seven terpenoids have a promising activity, and further tests were undertaken to confirm this observation.

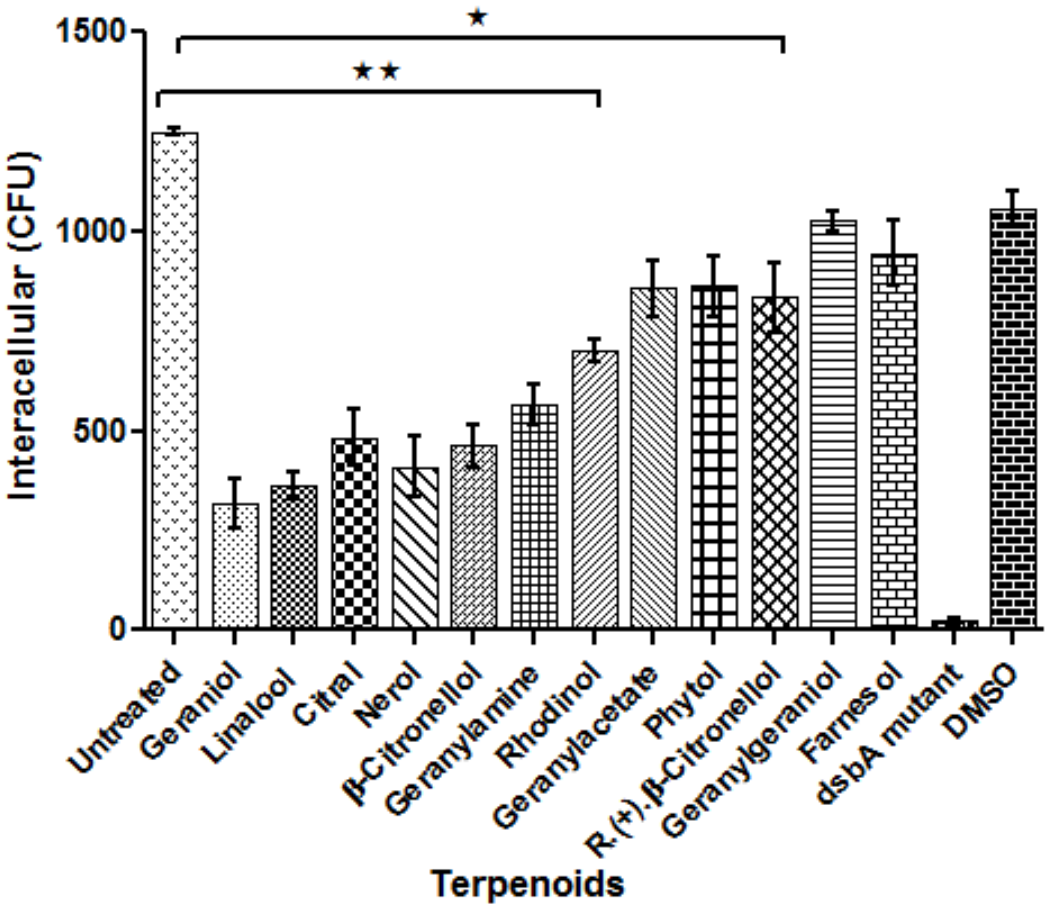



**Figure 3.4.1: Activity of terpenoids in *Shigella*- infected HeLa cells.**

Columns showed the activity of terpenoids on SSWT that infected the HeLa cells by using the gentamicin killing assay. Geraniol was the most active compound from the tested compounds and other terpenoids showed variable activity in reducing the intracellular SSWT. Cells were infected with SSWT for 40 minutes followed by 2h incubation in the presence of 50 µg/mL gentamicin and various terpenoids (42 µM). Differences between groups and control were assessed using the one way ANOVA test. by GraphPad prism 6. The statistical significance was at ($p < 0.05$).

### 3.4.2 Activity of terpenoids in *Shigella*-infected RAW 264.7 cells

In order to detect the activity of selected terpenoids in RAW macrophages a gentamycin killing assay was carried out following the same conditions as those used in HeLa infected cells (see section 2.2.1). It is clear from Figure (3.4.2) that only three terpenoids showed activity against SSWT in the RAW macrophage infected cells. Geraniol and geranylamine significantly reduced the number of intracellular CFUs 2.5 and 2 fold respectively compared to the untreated control. Rhodinol showed a 1 fold reduction in the number of intracellular CFUs compared to the untreated control. The compounds linalool, citral, nerol and β-citronellol did not show any activity towards SSWT in RAW macrophage-infected cells unlike that which was observed in HeLa-infected cells. DMSO (42 µM) had neither any detrimental effect on cells nor on SSWT bacteria.

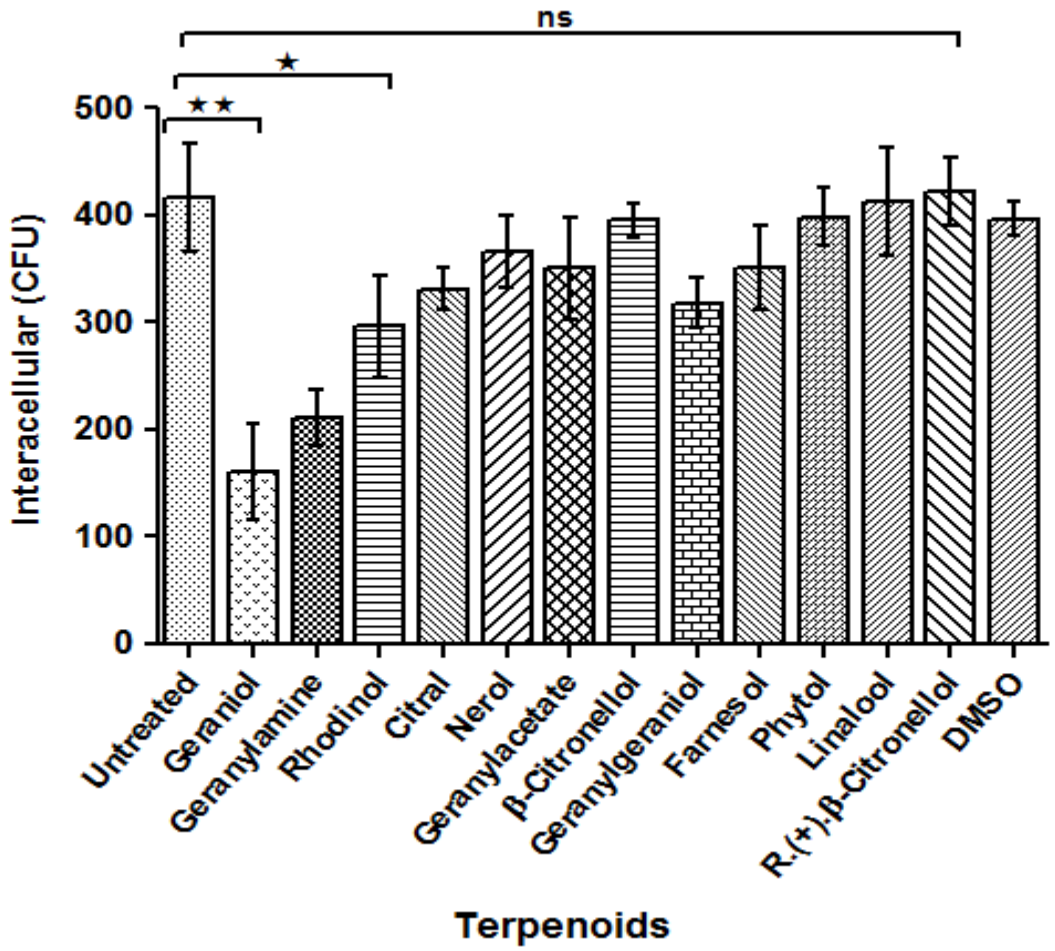



**Figure 3.4.2: Activity of terpenoids in *Shigella*-infected RAW 264.7 cells.**

Columns showed the activity of terpenoids on SSWT that infected the RAW cells by using the gentamicin killing assay. Geraniol was the most active compound from the tested compounds and other terpenoids showed variable activity in reducing the intracellular SSWT. Cells were infected with SSWT for 40 minutes followed by 2h incubation in the presence of 50 µg/mL gentamicin and various terpenoids (42 µM). The results were carried out in triplicate and experiment repeated twice. The results are shown as means ± SD. Differences between groups and control were assessed using One way ANOVA by GraphPad prism 6.

### 3.5 *In vivo* evaluation of the activity of terpenoids using a *G. mellonella* model

*Galleria mellonella* (also known as the wax worm) is a Lepidopteran greater wax moth. It is widely used as an *in vivo* model to study several human pathogens including *Shigella* (Barnoy et al., 2017). The life cycle of this organism consists of a larval stage that transforms within 6-8 weeks into pupae and finally into moth. The size of larvae is fairly large (about 2 cm long and 250 mg in weight) and it can tolerate various temperature (up to 42 $C^{o}$ ). To assess the ability of terpenoids to protect larvae from death following a *Shigella* infection, a set of *in vivo* experiments were carried out. It is known that *S. sonnei* can kill the larvae within one day at $10^5$-$10^6$ CFUs (Mahmoud et al., 2016, Mirza et al., 2018) and, as a result, all experiments were set up with this number of bacteria.

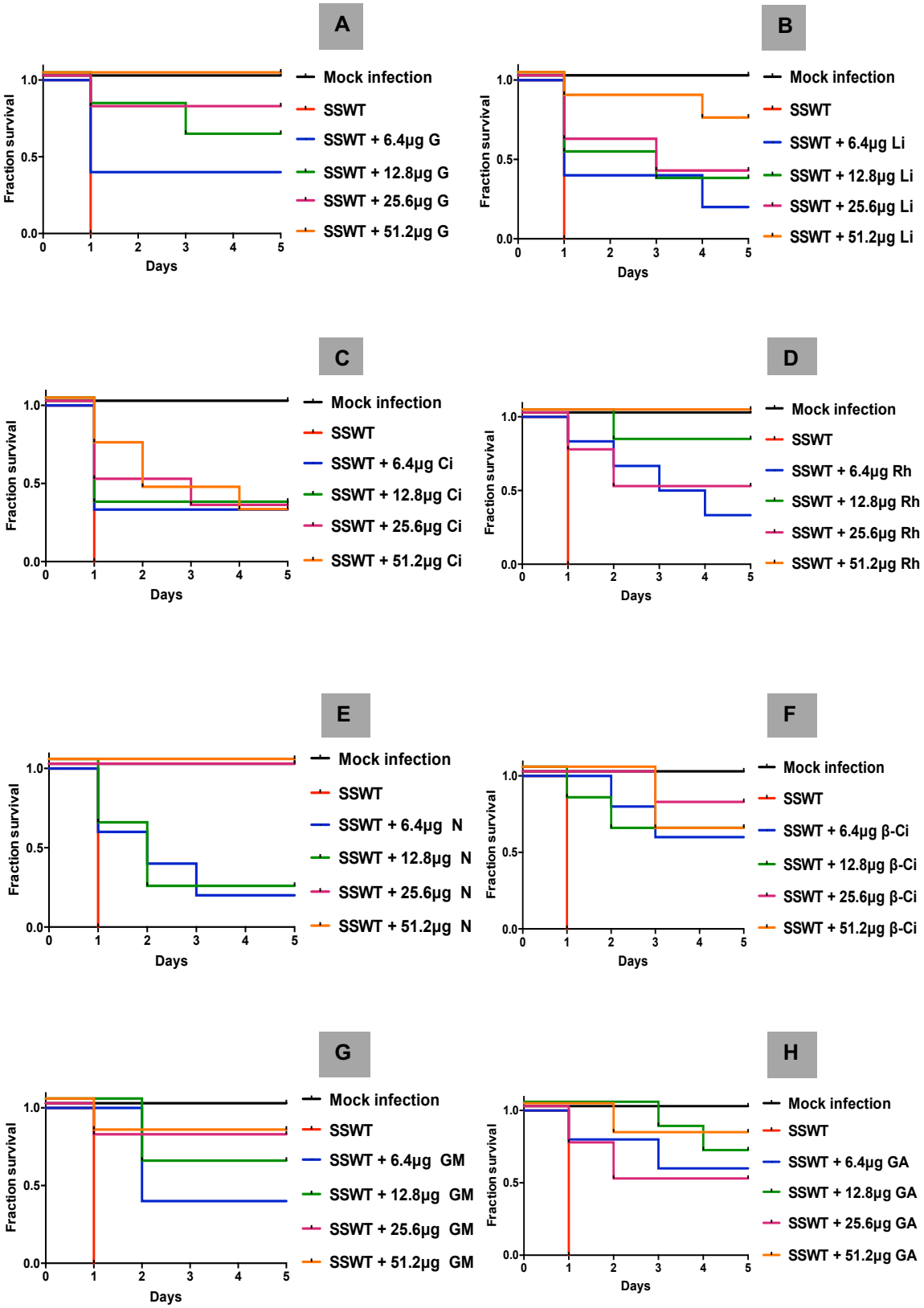
The larvae were separated into 3 groups with 10 larvae per each group. The mock infection control group larvae were injected with 10 μL of sterilized PBS, while the infection group also received the same volume of PBS, but containing $10^5$ CFUs of SSWT. The treatment group were separated into 4 categories depending on the concentration of terpenoids used and injected with a suspension of $10^5$ CFUs of live bacteria and terpenoids (10μL in total) with indicated concentrations in Figure (3.5).

SSWT killed all larvae in one day with$10^5$ CFUs. Some compounds showed ability to protect the larvae from killing Figure (3.5). 100% of the larvae in the treatment group at a dose of 51 μg of geraniol, rhodinol, nerol and farnesol were protected from SSWT killing (Figures 3.5 A, D, E, and J) respectively. Interestingly, 40% of larvae were protected by 6.4 μg to the end of the fifth day of incubation and the percent protection increased with the increase in geraniol concentrations. By using 25 μg of nerol, it's clear that nerol protects larvae with 100% along the incubation time Figure (3.5 E).

However, the lower concentrations of nerol only protected 20% of larvae during the incubation time. The rest of the compounds showed an ability to protect *Galleria mellonella* larvae at some concentrations, while failing in others.

**Figure 3.5: Protection of *Galleria* mellonella Larvae by terpenoids.**

**A-** Protection of Larvae by geraniol. Ten larvae were survived when treated with 51.2 μg of geraniol, while just 4 larvae were continuous a live until five day when treated with 6.4 μg. **B-** Protection of Larvae by linalool. Eight larvae were survived when treated with 51.2 μg of linalool, while just 2 larvae were continuous a live until five day when treated with 6.4 μg . **C-** Protection of Larvae by citral. Citral was very weak in protection larvae as jut 4 larvae were survived when treated with 51.2 μg and 6.4 μg. **D-** Protection of Larvae by rhodinol. Ten larvae were survived when treated with 51.2 μg of rhodinol, while just 4 larvae were continuous a live until five day when treated with 6.4 μg. **E-** Protection of Larvae by nerol. Ten larvae were survived when treated with 51.2 μg and 25.6μg of nerol, while just 2 larvae were continuous a live until five day when treated with 6.4 μg. **F-** Protection of Larvae by β-citronellol. Seven larvae were survived when treated with 51.2 μg of β-citronellol, while 6 larvae were continuous a live until five day when treated with 6.4 μg **G-** Protection of Larvae by geranylamine. Nine larvae were survived when treated with 51.2 μg of geranylamine, while just 4 larvae were continuous a live until five day when treated with 6.4 μg. **H-** Protection of Larvae by geranylacetate. Nine larvae were survived when treated with 51.2 μg of geranylacetate, while 5 larvae were continuous a live until five day when treated with 6.4 μg. **I-** Protection of Larvae by geranylgeraniol. Nine larvae were survived when treated with 51.2 μg of geranylgeraniol, while just 4 larvae were continuous a live until five day when treated with 6.4 μg. **J-** Protection of Larvae by farnesol. Ten larvae were survived when treated with 51.2 μg of farnesol, while all ten larvae were died since second day when treated with 6.4 μg. **K-** Protection of Larvae by phytol. Nine larvae were survived when treated with 51.2 μg of phytol, while 7 larvae were continuous a live until five day when treated with 6.4 μg. **L-** Protection of Larvae by R.(+).β-citronellol. Nine larvae were survived when treated with 51.2 μg of R.(+).β-citronellol, while 7 larvae were continuous a live until five day when treated with 6.4 μg. Experiments were repeated twice and pooled data were used to generate the graphs shown as Kaplan–Meier survival curves.


A
Fraction survival
Days
Mock infection
SSWT
SSWT + 6.4μg G
SSWT + 12.8μg G
SSWT + 25.6μg G
SSWT + 51.2μg G
B
Mock infection
SSWT
SSWT + 6.4μg Li
SSWT + 12.8μg Li
SSWT + 25.6μg Li
SSWT + 51.2μg Li
C
Mock infection
SSWT
SSWT + 6.4μg Ci
SSWT + 12.8μg Ci
SSWT + 25.6μg Ci
SSWT + 51.2μg Ci
D
Mock infection
SSWT
SSWT + 6.4μg Rh
SSWT + 12.8μg Rh
SSWT + 25.6μg Rh
SSWT + 51.2μg Rh
E
Mock infection
SSWT
SSWT + 6.4μg N
SSWT + 12.8μg N
SSWT + 25.6μg N
SSWT + 51.2μg N
F
Mock infection
SSWT
SSWT + 6.4μg β-Ci
SSWT + 12.8μg β-Ci
SSWT + 25.6μg β-Ci
SSWT + 51.2μg β-Ci
G
Mock infection
SSWT
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SSWT + 12.8μg GM
SSWT + 25.6μg GM
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SSWT + 25.6μg GA
SSWT + 51.2μg GA

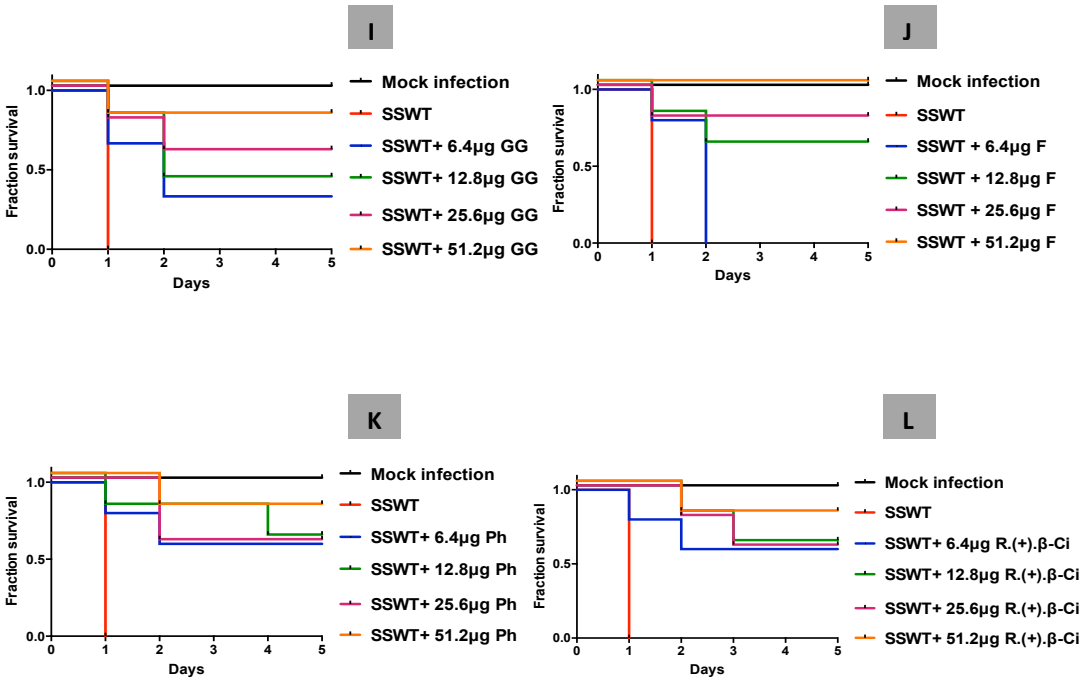



**Figure 3.5: Protection of *Galleria mellonella* Larvae by terpenoids.**

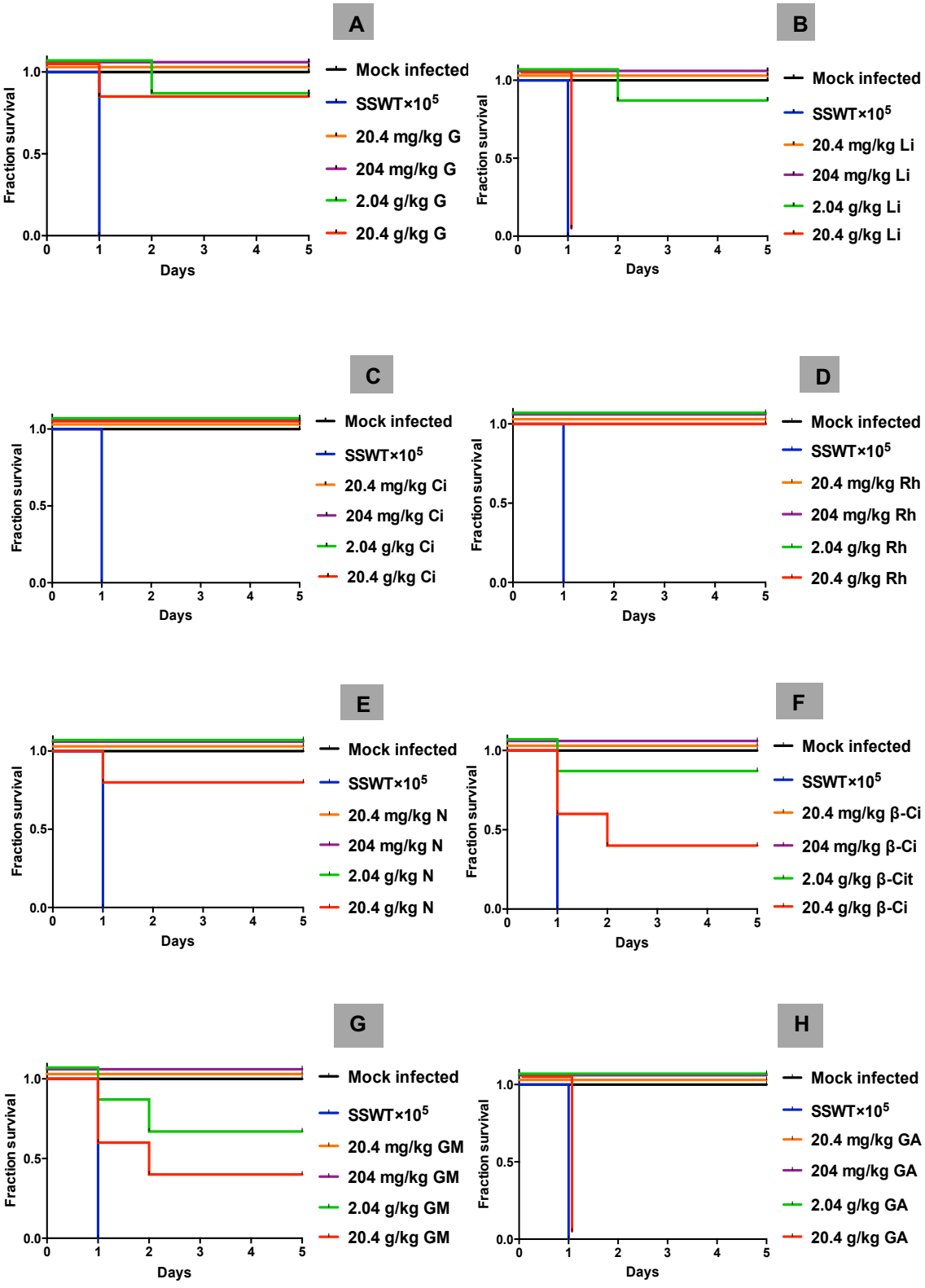
**A-** Protection of Larvae by geraniol. Ten larvae were survived when treated with 51.2 µg of geraniol, while just 4 larvae were continuous a live until five day when treated with 6.4 µg. **B-** Protection of Larvae by linalool. Eight larvae were survived when treated with 51.2 µg of linalool, while just 2 larvae were continuous a live until five day when treated with 6.4 µg . **C-** Protection of Larvae by citral. Citral was very weak in protection larvae as jut 4 larvae were survived when treated with 51.2 µg and 6.4 µg. **D-** Protection of Larvae by rhodinol. Ten larvae were survived when treated with 51.2 µg of rhodinol, while just 4 larvae were continuous a live until five day when treated with 6.4 µg. **E-** Protection of Larvae by nerol. Ten larvae were survived when treated with 51.2 µg and 25.6µg of nerol, while just 2 larvae were continuous a live until five day when treated with 6.4 µg. **F-** Protection of Larvae by β-citronellol. Seven larvae were survived when treated with 51.2 µg of β-citronellol, while 6 larvae were continuous a live until five day when treated with 6.4 µg **G-** Protection of Larvae by geranylamine. Nine larvae were survived when treated with 51.2 µg of geranylamine, while just 4 larvae were continuous a live until five day when treated with 6.4 µg. **H-** Protection of Larvae by geranylacetate. Nine larvae were survived when treated with 51.2 µg of geranylacetate, while 5 larvae were continuous a live until five day when treated with 6.4 µg. **I-** Protection of Larvae by geranylgeraniol. Nine larvae were survived when treated with 51.2 µg of geranylgeraniol, while just 4 larvae were continuous a live until five day when treated with 6.4 µg. **J-** Protection of Larvae by farnesol. Ten larvae were survived when treated with 51.2 µg of farnesol, while all ten larvae were died since second day when treated with 6.4 µg. **K-** Protection of Larvae by phytol. Nine larvae were survived when treated with 51.2 µg of phytol, while 7 larvae were continuous a live until five day when treated with 6.4 µg. **L-** Protection of Larvae by R.(+).β-citronellol. Nine larvae were survived when treated with 51.2 µg of R.(+).β-citronellol, while 7 larvae were continuous a live until five day when treated with 6.4 µg. Experiments were repeated twice and pooled data were used to generate the graphs shown as Kaplan–Meier survival curves.

### 3.6 Evaluation of the cytotoxicity of terpenoids on the *G. mellonella* model

The terpenoids tested showed some promising results in protecting *Galleria mellonella* from SSWT infection. In order to detect the cytotoxicity of terpenoids on larvae, an experiment was performed with the larvae divided in three groups. An infection group received 10μL of $10^5$ CFUs of SSWT, which killed all larvae within one day. A mock infection was also carried out using the same volume of sterilised PBS. Finally, a third group was injected with tenfold graduating increasing of terpenoids from 20.4 mg/kg to 20.4 g/kg. The results obtained showed that the larvae from the treatment group were able to tolerate high concentrations of terpenoids Figures (3.6). Interestingly, 100% of larvae were able to tolerate high concentrations (20.4 g/kg) of the citral and rhodinol as can be seen in Figures (3.6 C and D) respectively. On the other hand, all larvae were killed within one day by SSWT infection with $10^5$ CFUs or in the presence of 20.4 g/kg concentration of linalool, geranylacetate, geranylgeraniol, farnesol and phytol as shown in Figures (3.6 B, H, I, J and K) respectively. It has been reported that geraniol is toxic to larvae at 35 g/kg and can kill all larvae within one day as SSWT is able to do (Mirza et al., 2018). However, our results showed that 80% of the larvae were able to tolerate 20 g/kg of geraniol that we injected already Figure (3.6 A). Similar to geraniol, 80% of larvae also survived when treated with 20 g/kg of linalool and nerol as shown in Figure (3.6 B and E). While, β-citronellol, geranylamine and R.(+).β-citronellol were able to kill the larvae at 20g/kg and only 30% of larvae survived at this concentration. Finally, all compounds are not toxic at low concentrations such as 20.4mg/kg and 204mg/kg Figure (3.6).

**Figure 3.6: Cytotoxicity of terpenoids on the *Galleria mellonella* Larvae.**

**A-** Evaluate the cytotoxicity of geraniol on larvae**.** Ten larvae tolerated 20.4 mg/kg of geraniol, while 8 larvae tolerated high dose of it 20.4 g/kg until the fifth day of incubation. **B-** Evaluate the cytotoxicity of linalool on larvae. Ten larvae tolerated 20.4 mg/kg of Linalool, while all ten larvae died from the first day when treated with 20.4 g/kg. **C-** Evaluate the cytotoxicity of citral on larvae. All the ten larvae tolerated low and high dose of citral 20.4 mg/kg and 20.4 g/kg until fifth day of incubation. **D-** Evaluate the cytotoxicity of rhodinol on larvae. All the ten larvae tolerated low and high dose of rhodinol 20.4 mg/kg and 20.4 g/kg until fifth day of incubation. **E-** Evaluate the cytotoxicity of nerol on larvae. Ten larvae tolerated 20.4 mg/kg of nerol, while 8 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **F-** Evaluate the cytotoxicity of β-citronellol on larvae. Ten larvae tolerated 20.4 mg/kg of β-citronellol, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **G-** Evaluate the cytotoxicity of geranylamine on larvae. Ten larvae tolerated 20.4 mg/kg of geranylamine, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **H-** Evaluate the cytotoxicity of geranylacetate on larvae. Ten larvae tolerated 20.4 mg/kg of geranylacetate, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **I-** Evaluate the cytotoxicity of geranylgeraniol on larvae. Ten larvae tolerated 20.4 mg/kg of geranylgeraniol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **J-** Evaluate the cytotoxicity of farnesol on larvae. Ten larvae tolerated 20.4 mg/kg of farnesol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **K-** Evaluate the cytotoxicity of phytol on larvae. Ten larvae tolerated 20.4 mg/kg of phytol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **L-** Evaluate the cytotoxicity of R.(+).β-citronellol on larvae. Ten larvae tolerated 20.4 mg/kg of R.(+).β-citronellol, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. Experiments were repeated twice and pooled data were used to generate the graphs shown as Kaplan–Meier survival curves.


A
Fraction survival
1.0
0.5
0.0
0
1
2
3
4
5
Days
Mock infected
SSWT×10^5
20.4 mg/kg G
204 mg/kg G
2.04 g/kg G
20.4 g/kg G
B
Fraction survival
Days
Mock infected
SSWT×10^5
20.4 mg/kg Li
204 mg/kg Li
2.04 g/kg Li
20.4 g/kg Li
C
Fraction survival
Days
Mock infected
SSWT×10^5
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204 mg/kg Ci
2.04 g/kg Ci
20.4 g/kg Ci
D
Fraction survival
Days
Mock infected
SSWT×10^5
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20.4 g/kg Rh
E
Fraction survival
Days
Mock infected
SSWT×10^5
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20.4 g/kg N
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20.4 g/kg GA

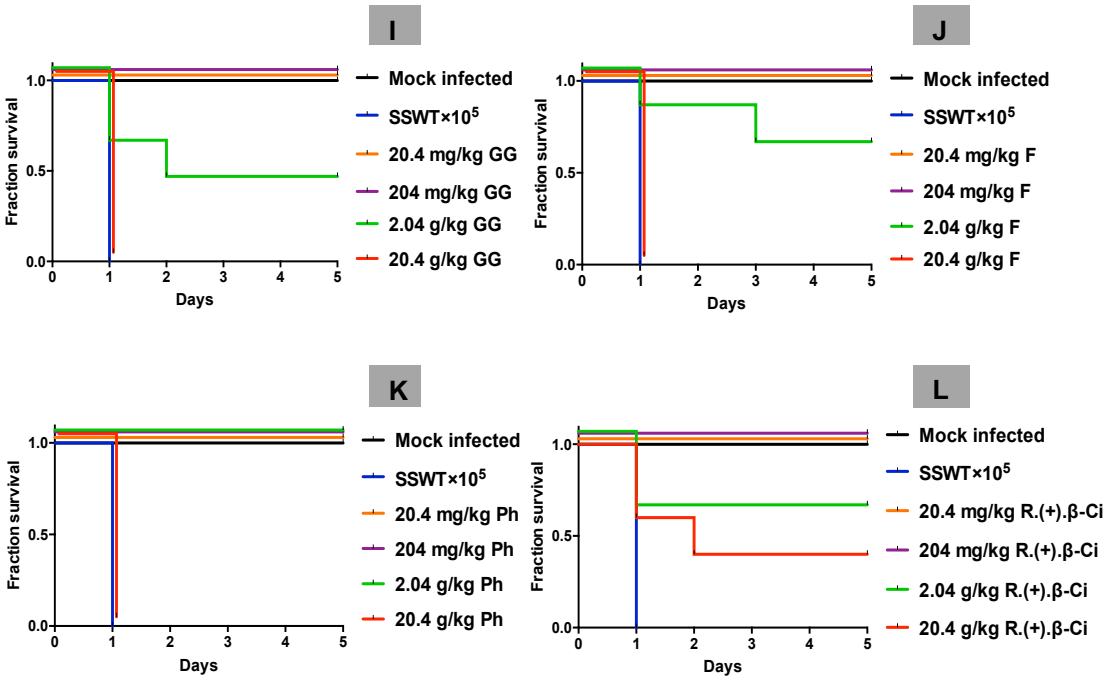



**Figure 3.6: Cytotoxicity of terpenoids on the *Galleria mellonella* Larvae.**

**A-** Evaluate the cytotoxicity of geraniol on larvae**.** Ten larvae tolerated 20.4 mg/kg of geraniol, while 8 larvae tolerated high dose of it 20.4 g/kg until the fifth day of incubation. **B-** Evaluate the cytotoxicity of linalool on larvae. Ten larvae tolerated 20.4 mg/kg of Linalool, while all ten larvae died from the first day when treated with 20.4 g/kg. **C-** Evaluate the cytotoxicity of citral on larvae. All the ten larvae tolerated low and high dose of citral 20.4 mg/kg and 20.4 g/kg until fifth day of incubation. **D-** Evaluate the cytotoxicity of rhodinol on larvae. All the ten larvae tolerated low and high dose of rhodinol 20.4 mg/kg and 20.4 g/kg until fifth day of incubation. **E-** Evaluate the cytotoxicity of nerol on larvae. Ten larvae tolerated 20.4 mg/kg of nerol, while 8 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **F-** Evaluate the cytotoxicity of β-citronellol on larvae. Ten larvae tolerated 20.4 mg/kg of β-citronellol, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **G-** Evaluate the cytotoxicity of geranylamine on larvae. Ten larvae tolerated 20.4 mg/kg of geranylamine, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. **H-** Evaluate the cytotoxicity of geranylacetate on larvae. Ten larvae tolerated 20.4 mg/kg of geranylacetate, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **I-** Evaluate the cytotoxicity of geranylgeraniol on larvae. Ten larvae tolerated 20.4 mg/kg of geranylgeraniol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **J-** Evaluate the cytotoxicity of farnesol on larvae. Ten larvae tolerated 20.4 mg/kg of farnesol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **K-** Evaluate the cytotoxicity of phytol on larvae. Ten larvae tolerated 20.4 mg/kg of phytol, while all ten larvae died since the first day when treated with high dose of it (20.4 g/kg). **L-** Evaluate the cytotoxicity of R.(+).β-citronellol on larvae. Ten larvae tolerated 20.4 mg/kg of R.(+).β-citronellol, while 4 larvae tolerated high dose of it (20.4 g/kg) until the fifth day of incubation. Experiments were repeated twice and pooled data were used to generate the graphs shown as Kaplan–Meier survival curves.

### 3.7 Role of DsbA on *Shigella* growth in the presence of glutathione (GSH)

In order to determine the role of DsbA in the intracellular redox environment and how *Shigella* cells survive and proliferate in this environment. A growth curve was obtained by growing *Shigella* in reducing media such as M9. Different concentrations of GSH were supplemented into media and three strains of *S. sonnei* tested: wild type (SSWT): a *dsbA* mutant (SSDM) and a strain complemented with an intact copy of *dsbA* (SSPD). As indicated previously, the cytosol contains approximately 10 mM of GSH, so, the concentrations of GSH used varied around this concentration. It is apparent from Figure (3.7) that SSWT grew very well in the presence of GSH even at 10 mM. However, *Shigella* spent more time to reach the stationary phase in the presence of 20 mM of GSH.

SSPD in Figure (3.7) grew better than SSWT even in the presence of various concentrations of GSH. SSPD grew well in the presence of 20 mM GSH and reached the peak of stationary phase faster than SSWT, presumably due to over expression of DsbA by the plasmid. In comparison, the SSDM in showed weak growth rate with and without GSH. It can be seen clearly that the bacteria spent a longer time to survive with low growth rate compared to SSWT and SSPD, to reach the peak and it were need more time to get the peak in the presence of 5 mM and 10 mM of GSH respectively. SSDM could not reach the peak in the presence of 20 mM of GSH extremely low growth rate due to missing *dsbA,* thus missing the folding of effector proteins. All strains grew in a slow rate when the media were treated with 30 mM of GSH.

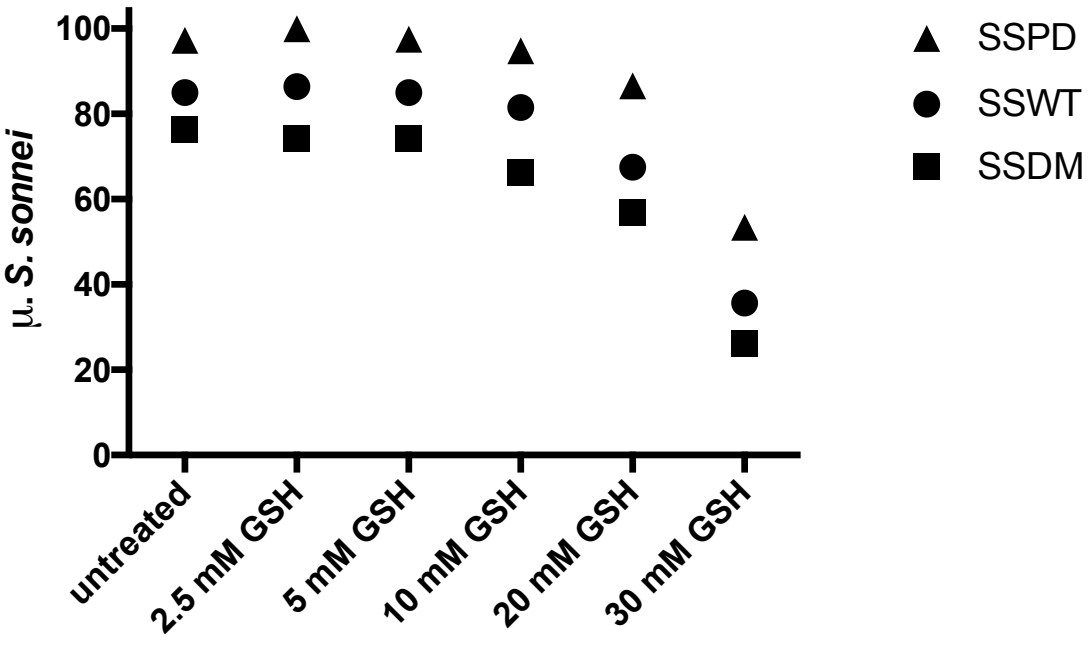



**Figure 3.7: Specific growth rates of *S. sonnei* growing in the presence of various concentrations of glutathione (GSH).**

The specific growth rate (μ) of the three strains of *S. sonnei* was very low in the presence of 30 mM of GSH and strains were struggling to survive in this high reducing media. *S. sonnei* wild type (SSWT), DsbA mutant (SSDM) and complementary with *dsbA* (SSPD) strains were grown in the M9 media with different concentration of GSH. The optical density was obtained each hour using (WPA) spectrophotometry at $OD_{600}$nm.

## 3.8 Discussion

A major challenge in modern medicine is that antimicrobial resistance has emerged as a global challenge to public health and multi-resistant *S. sonnei* are the cause of the current pandemic of shigellosis. The fact is that antimicrobial resistance will occur to any new antibiotic sooner or later. The demand for new strategies in treatment of MDR bacteria is became an urgent need (WHO, 2017). New approaches that may change the classical ideas in treatment bacteria may change the way that we targeting the disease. Therefore, the targeting of virulence factors potentially has a major advantage as a novel approach to control intracellular pathogens, as perhaps resistance mechanisms may take longer to develop.

Terpenoids, also known as terpenes, are the largest group of natural products, and they are plant secondary metabolites along with alkaloids and flavonoids. Terpenoids display antimicrobial (antibacterial, antifungal and antiviral), anti-inflammatory, antioxidant, hypotensive, antipruritic and analgesic activities (Kordali et al., 2005, Guimaraes et al., 2013, Bastos et al., 2010). Terpenoids were chosen for investigation into their potential therapeutic use against dysentery due to the related activity of propolin D, which has the ability to inhibit intracellular bacteria (Xu et al., 2011). Propolin D has a terpene side chain, which is responsible for its activity inside the host cells. The present study tested twelve terpenoids that shared a similar structure with the terpenic side chain in propolin D.

Terpenoids are known to exhibit antimicrobial activity against a wide range of bacteria and fungi. The antimicrobial activity of some terpenoids is due to their solubility in the phospholipid bilayer of cell membranes (Kalemba and Kunicka, 2003). It was also reported that the antibacterial activity of monoterpene alcohols including geraniol,

linalool, nerol and citronellol are more effective than their antifungal activity (Suppakul et al., 2003). Interestingly, these compounds have a little antimicrobial activity towards SSWT even when using high concentration with MIC equal to 500 mg/L. Geranylamine was the only compound who able to reduce the viability of SSWT ten-fold at 500mg/L but lost this activity when decreasing the concentration to 250 mg/L. Propolin D was previously shown to possess potent activity against *Staphylococcus aureus* but no direct activity against a Gram-negative bacterium such as *Pseudomonas aeruginosa* (Raghukumar et al., 2010). We found that was also true for geraniol, which had a little direct activity to inhibit *S. sonnei* growth when tested using a microdilution assay with MIC > 500 mg/L (Seidel et al., 2008). These facts strongly suggested that propolin D, as well as geraniol, could either enhance a host defence mechanism responsible for controlling intracellular bacteria or target a bacterial factor only active inside the host cell cytosol where *Shigella* thrives (Ray et al., 2009).

All terpenoids used in this study showed a little cytotoxicity in both HeLa and RAW macrophage cells when compared to untreated cells at a concentration of 42 μM. Rhodinol and geranylamine significantly reduced the viability of HeLa and RAW macrophage cells respectively, when compared to untreated cells and to treated cells with other compounds. This results may explain the activity of rhodinol and geranylamine on SSWT infected cells as these compounds may possess a toxicity towards these cells. A possible explanation for this might be these compounds may damage the cell membranes and the intracellular bacteria would be killed by gentamicin which would be able to penetrate inside the host cells. Xu and his colleagues confirmed that compound called totarol they used to inhibit *S. sonnei*

inside epithelial HEp-2 cells; totarol significantly reduced cell viability overnight (Xu et al., 2011).

$IC_{50}$ values were calculated from the linear regression of the dose-log response curves for both HeLa and RAW macrophage cells. Rhodinol, geranylacetate, geranylgeraniol and farnesol in Figure (3.3.2 F, G, H and J) showed a low concentration in the dose-response on HeLa. However, these compounds except farnesol showed high $IC_{50}$ on RAW macrophage cells, while farnesol exhibited a low $IC_{50}$ in both HeLa and RAW macrophage cells. Geranylamine was toxic at the target concentration (42 μM) on macrophage cells Figure (3.3.3) and showed low $IC_{50}$ concentration in both HeLa and RAW macrophage cell lines. As a result, geranylamine cannot be an eligible compound as an antimicrobial agent but it would be a good antitumor agent due to their activity on cancer cells. Derivatives of geranylamine were able to inhibit the cell growth *in vitro* in human hepatoma-bearing a thymic mice (Akiyama et al., 2005).

In order to investigate the activity of these compounds on *S. sonnei* infection, a gentamicin killing assay was performed to assess the role of terpenoids in reducing the number of intracellular bacteria. Most terpenoids in Figure (3.4.1) inhibited the number of intracellular bacteria (colony forming unit CFUs) when compared to the untreated control. Seven compounds showed a significant reduction of intracellular bacteria in which geraniol, linalool, citral, nerol, β-citronellol, geranylamine and rhodinol. The same compounds did not show any inhibition of RAW macrophage cells Figure (3.4.2) except geraniol and geranylamine. Geraniol was the most potent compound in reducing the intracellular bacteria cells significantly inside HeLa and RAW macrophage cells. All these compounds were tested at a concentration of 42

μM, although higher concentrations than 42μM may show more effect in controlling *Shigella* infection. Geranylgeraniol had a significant effect on intracellular parasites of *Leishmania amazonensis* (Lopes et al., 2012). However, the results showed that geranylgeraniol had no activity on SSWT in both HeLa and RAW macrophage cell lines. The results suggest that these terpenoids may work inside the cell cytosol and the reduction of intracellular bacteria may occur by terpenoids. Terpenoids were supplemented with gentamicin in the medium and the role of gentamicin is to kill all the extracellular bacteria and the terpenoids killed the intracellular bacteria.

An *in vivo* model of *Galleria mellonella* larvae was used to test the activity and cytotoxicity of these terpenoids. The important results that we obtained from this model confirm that some of these compounds can protected larvae from killing by SSWT and at the same time, the larvae can tolerate high concentrations of these terpenes. SSWT was able to kill the larvae within one day post infection with $10^5$ CFUs/larvae, this is consistent with what was reported in (Mirza et al., 2018). Interestingly, 40% of larvae were protected from killing by SSWT when treated with 6.4 μg of geraniol to the fifth day. Furthermore, geraniol with 51.2 μg can protect 100% of larvae from killing by SSWT until the fifth day of incubation. Rhodinol, nerol and farnesol also showed activity by protecting larvae from killing by SSWT with 100% at the concentration of 51.2 μg but it showed variable activity at low concentrations.

Larvae were able to tolerate high concentrations (20.4 g/kg) of the citral, rhodinol, geranylacetate and phytol and survive with 100% as can be seen in Figures (3.6 C, D, H and K) respectively. However, all larvae were killed within one day after infection with SSWT or in the presence of 20.4 g/kg concentration of linalool, geranylacetate, geranylgeraniol, farnesol and phytol as shown in Figures (3.6 B, H, I, J and K)

respectively. It has been reported that geraniol is toxic to larvae at 35 g/kg and can kill all larvae within one day as SSWT is able to do (Mirza et al., 2018). However, our results showed that 80% of the larvae were able to tolerate 20 g/kg of geraniol that we injected already Figure (3.6 A).

Previous studies revealed that the *Shigella* periplasmic disulphide bond oxidoreductase, DsbA, is vital in supporting the survival of *S. flexneri* in the host cell cytosol (Yu, 1998). The cell cytosol is a highly reducing environment with high concentrations of reduced glutathione (GSH) (Go and Jones, 2008). *Shigella* manipulate this environment by using DsbA to produce folded effector proteins that are involved in virulence and are essential to invade the targeted cells. Our results suggest that DsbA is important for *Shigella* to survive and grow in the reducing environment, which contains a high concentrations of GSH. SSWT grew very well in the presence of 5 mM and 10 mM of GSH, but it needs more time to grow with 20 mM. In comparison, SSDM struggled to grow in the presence of 10 mM of GSH, and it is never reached the peak of growth with 20 mM of GSH. Meanwhile, SSPD grew much better than SSWT in the presence of 20 mM of GSH, prsumably due to the over expression of DsbA in this complemented strain. This result is consistent with the previous finding which reported that *dsbA* mutant of *Shigella* loses its ability to survive and proliferate inside host cells (Yu, 1998).

From above results, we can conclude that geraniol was the best compound amongst the twelve terpenoids tested. It significantly reduced the number of intracellular CFUs in both HeLa and RAW macrophage cells. These terpenoids, especially geraniol, did not display any cytotoxicity to the cell lines tested or in the larvae model. Although, these compounds do not show any direct antimicrobial activity against *Shigella* infection, so it might be that they targeted the virulence system of the bacteria instead.

Importantly, some of these compounds and the most potent, geraniol, showed an ability to protect the larvae from killing by SSWT. As a result, this is suggests that these terpenoids may be a good agents against *Shigella* infection. Furthermore, these results confirmed that *Shigella* needs DsbA to survive in the reducing environment with high concentrations of GSH and DsbA may reduce the GSSG and convert it to GSH during infection to survive and proliferate inside the host.

# Chapter 4

## Kinetic investigations into terpenoid action on the *Shigella sonnei* protein, DsbA

# 4 Kinetic investigations into terpenoid action on the *S. sonnei* protein, DsbA

## 4.1 Introduction

Virulence factor proteins of Gram-negative bacteria possess many important features of which, perhaps the most important, are the disulphide bonds for structural stability. Disulphide formation between pairs of cysteine residues increases the chemical and physical stability of proteins and failure to form native disulphide bonds results in degradation of the secreted proteins and loss of their activity (Bardwell et al., 1993). The bacterial disulphide bond (DSB) machinery of *S. sonnei* comprises a soluble periplasmic enzyme known as thiol-disulphide oxidoreductase (DsbA) and the integral membrane protein DsbB. Oxidised DsbA introduces a disulphide bond directly into folding proteins and converts them to the reduced form. The reduced DsbA then regenerates by interaction with an integral membrane protein DsbB (Inaba et al., 2006).

DsbA is a bacterial periplasmic thiol-disulphide oxidoreductase and is the key component of the disulphide bond family of enzymes. The latter catalyses the folding of secreted proteins, many of which are virulence factors. Dsb enzymes are well-studied in *E. coli* K12 (Smith et al., 2016, Depuydt et al., 2011) and the periplasmic space of Gram-negative bacteria contains a set of Dsb thioredoxin family proteins such as DsbA, DsbB, DsbC, DsbD, DsbE, DsbF, and DsbG, which have important roles in disulphide bond exchange and in regulation of the redox potential (Missiakas et al., 1995, Andersen et al., 1997). These proteins possess a Cys-X-X-Cys active site. Dsb proteins operate through two pathways; an oxidative pathway which introduces disulphide bonds into folding proteins and an isomerase pathway that corrects non-native disulphide bonds (Kishigami et al., 1995, Hiniker and Bardwell, 2004). The oxidative pathway consists of two Dsb catalysts, DsbA and DsbB in *E. coli* K-12. The thioredoxin-like domain forms the main component of EcDsbA with an

inserted helical domain. The EcDsbA redox active site has the characteristic CXXC that is flanked by a hydrophobic groove and a large hydrophobic patch (Martin et al., 1993, Guddat et al., 1997). EcDsbA possesses a disulphide bond between Cys30 and Cys33 in the oxidised state that can be transferred to a substrate through bimolecular nucleophilic transfer. EcDsbA is converted to the inactive form after oxidative folding of the substrate and can then interact with the periplasmic loop of the transmembrane partner of EcDsbB (Bardwell et al., 1993). The interaction between EcDsbA-EcDsbB regenerates the oxidised state of EcDsbA through the transfer of electrons to EcDsbB. Therefore, inhibition of the EcDsbA-EcDsbB interaction may lead to blocking oxidative folding of virulence factors (Totsika et al., 2009). In the isomerase pathway, DsbA can introduce non-native disulphide bonds into a protein that contains more than two cysteines. DsbC, within the periplasm, can correct the non-native disulphide bonds in the secretion of folding proteins (Hiniker and Bardwell, 2004). A recent study using a mouse model infected with the causative agent of melioidosis, *Burkholderia pseudomalli* found that all mice died within 42 days, whereas the mice infected with same strain that lacking *dsbA* all survived (Ireland et al., 2014).

The cell cytosol is a reducing environment due to the concentration of reduced glutathione (GSH). Cell cytosol contains approximately 10 mM of GSH and a small amount of oxidised glutathione (GSSG) (Hwang et al., 1992). Previous studies revealed that the *Shigella* periplasmic disulphide bond oxidoreductase, DsbA, is vital in supporting the survival of *S. flexneri* in the host cell cytosol (Yu, 1998). The thioredoxin-1 and reduced glutathione are not in equilibrium and this results in a highly reducing environment with high concentrations of reduced glutathione (GSH) (Go and Jones, 2008). *S. sonnei* may benefit from this environment by using the GSH as a substrate for DsbA, thus requiring this to survive in this environment through regulation of the DsbA expression as a specific response to reducing conditions (Yu,

1998). GSH is a tripeptide compound synthesized in the cell cytosol that has many activities. It consists of three amino acids, glutamic acid, cysteine and glycine which are linked together by γ peptide and α peptide bonds. GSSG is an oxidant which prevents damage of important cellular components caused by reactive oxygen species. GSH can reduced disulphide bonds by donating electrons to form GSSG (Appenzeller-Herzog, 2011).

In the work described in Chapter Three, terpenoids were tested for their activity against *S. sonnei* and we showed that these compound could inhibit intracellular bacteria. Geraniol was the most potent of these, and possesses an activity to reduce the number of bacterial cells in addition to protection of *Galleria mellonella* from killing by *S. sonnei*. It was also reported that geraniol can inhibit the activity of DsbA in reducing GSSG and convert to GSH *in vitro* (Mirza et al., 2018). This results lead us to purify DsbA and test our terpenoids *in vitro*. Furthermore, we hypothesised, here, that *Shigella* uses DsbA to convert excessive GSH to GSSG, and geraniol may also have an ability to inhibit this activity.

The aims of this chapter were:

1- Cloning, expression and purification of DsbA.

2- Determine the anti-DsbA activity of a variety of terpenoids in the enzymatic kinetic assay.

3- Evaluate the anti-*Shigella* activity of geraniol using *in vivo* models.

## 4.2 Cloning of *dsbA* gene into pET26b(+) vector

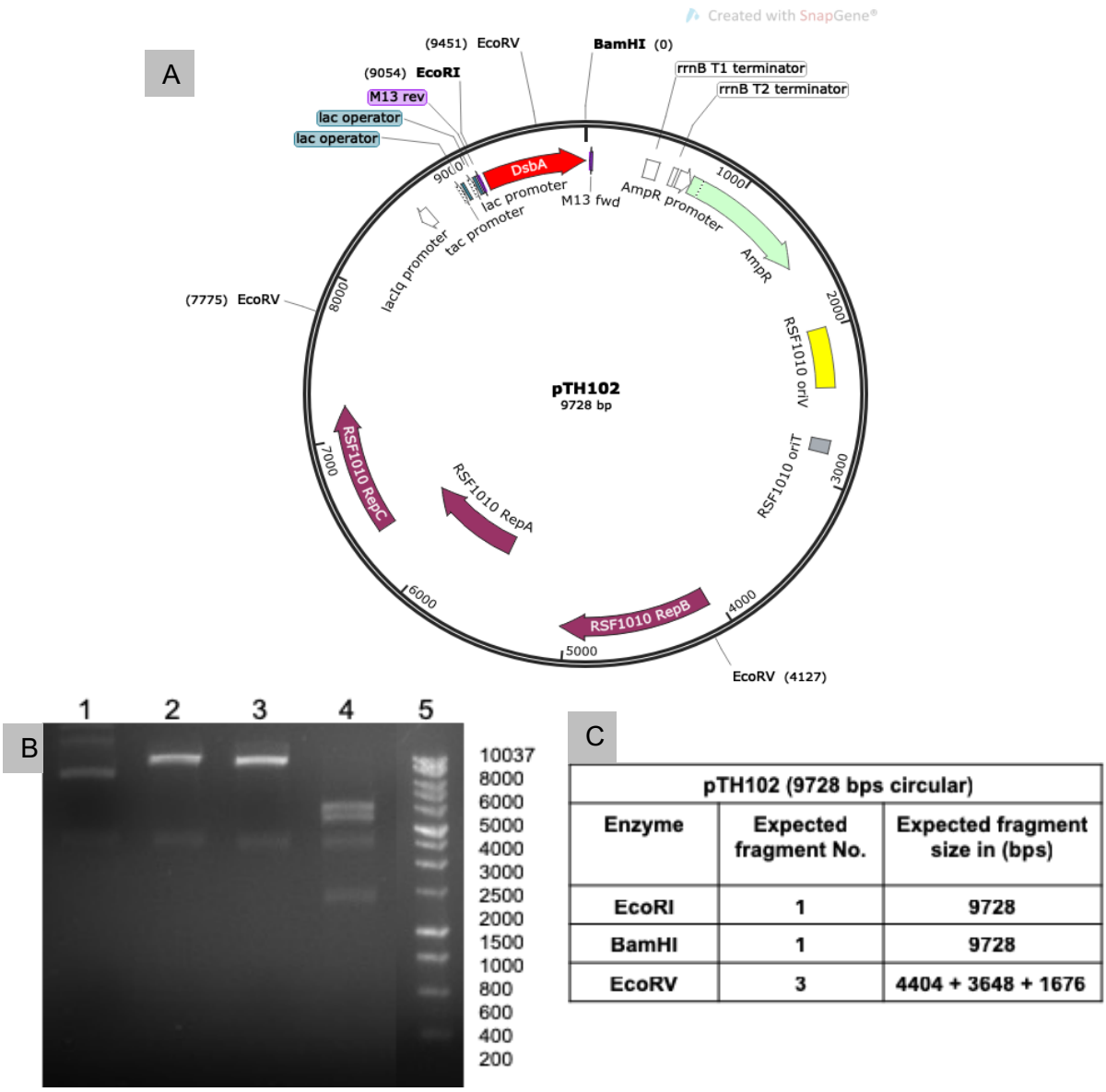
In order to purify the DsbA protein, we needed to clone the *dsbA* into pET26b(+). The latter is a plasmid designed for expression in *E. coli*. To purify DsbA easily, we needed to have 6xHis tag at the C terminus of DsbA to increase the affinity of the protein to bind to the purification column. Furthermore, DsbA is a periplasmic protein and needs a signal sequence; DsbA was previously shown to possess a signal sequence linked to the N terminus (Schierle et al., 2003). The signal sequence will separate from the protein at the inner membrane when the protein enters to the periplasm space and explains why we needed the 6xHis tag at the C terminus of the protein.

Several attempts to clone *dsbA* into pET26b(+) were carried out. One of the successful attempt was by insertion the gene into pET26b(+) using the NdeI and XhoI restriction enzymes. The product digestion confirmed the size of *dsbA*, but the sequencing tool confirmed that the *dsbA* contained a frame shift mutation within the region encoding the 6xHis in plasmid; this would make the affinity of protein very low and thus purifying the protein would be impossible. Another successful attempt was carried out by amplifying *dsbA* with 6xHis from pTH102 and insert it into pET26b(+), using NdeI and SalI restriction enzymes to produce pTH103, all details of this cloning mentioned in the section 4.2.1 and following.

The purification of DsbA protein from pTH103 was not easy, and several attempts to express the protein were carried out. Different concentrations of IPTG (50 μM – 2000 μM) were used and manipulation in the temperature (18-37 °C) also tested to express the protein. The successful attempt to express and purify the DsbA protein was carried out and mentioned in details in the section 4.3 and following.

### 4.2.1 Amplification of *dsbA*

To amplify the *dsbA* by PCR and insert it into pET26b(+); the pTH102 was used as a source of the gene. pTH102 is a 9.7 kb plasmid that carries the *dsbA* and expression is driven using the tac promoter. The pTH102 was confirmed by digesting using the EcoRI, EcoRV and BamHI enzymes Figure (4.1).The presence of *dsbA* was confirmed in the plasmid by using the standard M13 reverse primers which are the standard primers for this plasmid depending on manufacturer's instructions. To amplify *dsbA* with the 6xHis tag; primers were designed containing NdeI and SalI restriction sites for digestion of the forward (DsbA-F2) and reverse (DsbA-R2) primers respectively Table (2.7). The PCR product was analysed by 1% (w/v) agarose electrophoresis to confirm the *dsbA* size. Figure (4.2) shows that the primers successfully amplified the gene from pTH102. The amplified *dsbA* was purified and used for insertion into pET26b(+) to produce the pTH103.




C

| pTH102 (9728 bps circular) | | |
|---|---|---|
| **Enzyme** | **Expected fragment No.** | **Expected fragment size in (bps)** |
| **EcoRI** | **1** | **9728** |
| **BamHI** | **1** | **9728** |
| **EcoRV** | **3** | **4404 + 3648 + 1676** |

**Figure 4.1: Confirmation of pTH102 plasmid.**

**A**- pTH102 plasmid map, plasmid carried the *dsbA* gene which located after the lac promoter and it's ampicillin resistance plasmid. **B**-Confirmation agarose gel electrophoresis of pTH102 plasmid which digested with different restriction enzyme and the gel showed all the expected fragments. Lane 1: Uncut plasmid, Lane 2: EcoRI, Lane 3: BamHI, Lane 4: EcoRV, Lane 5: DNA Ladder 1kb (Bioline, UK). **C**- The different enzymes used to confirm the pTH102 and the expected fragment sizes.

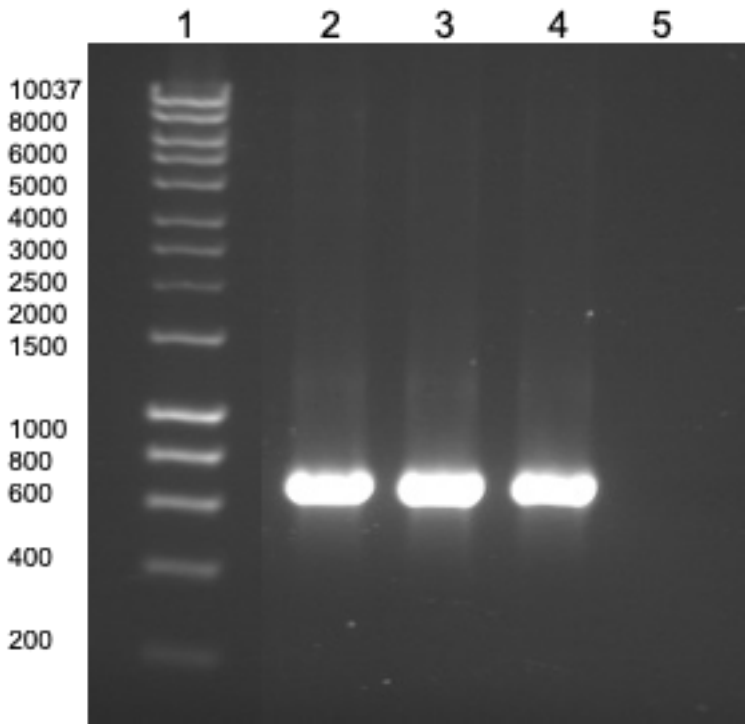
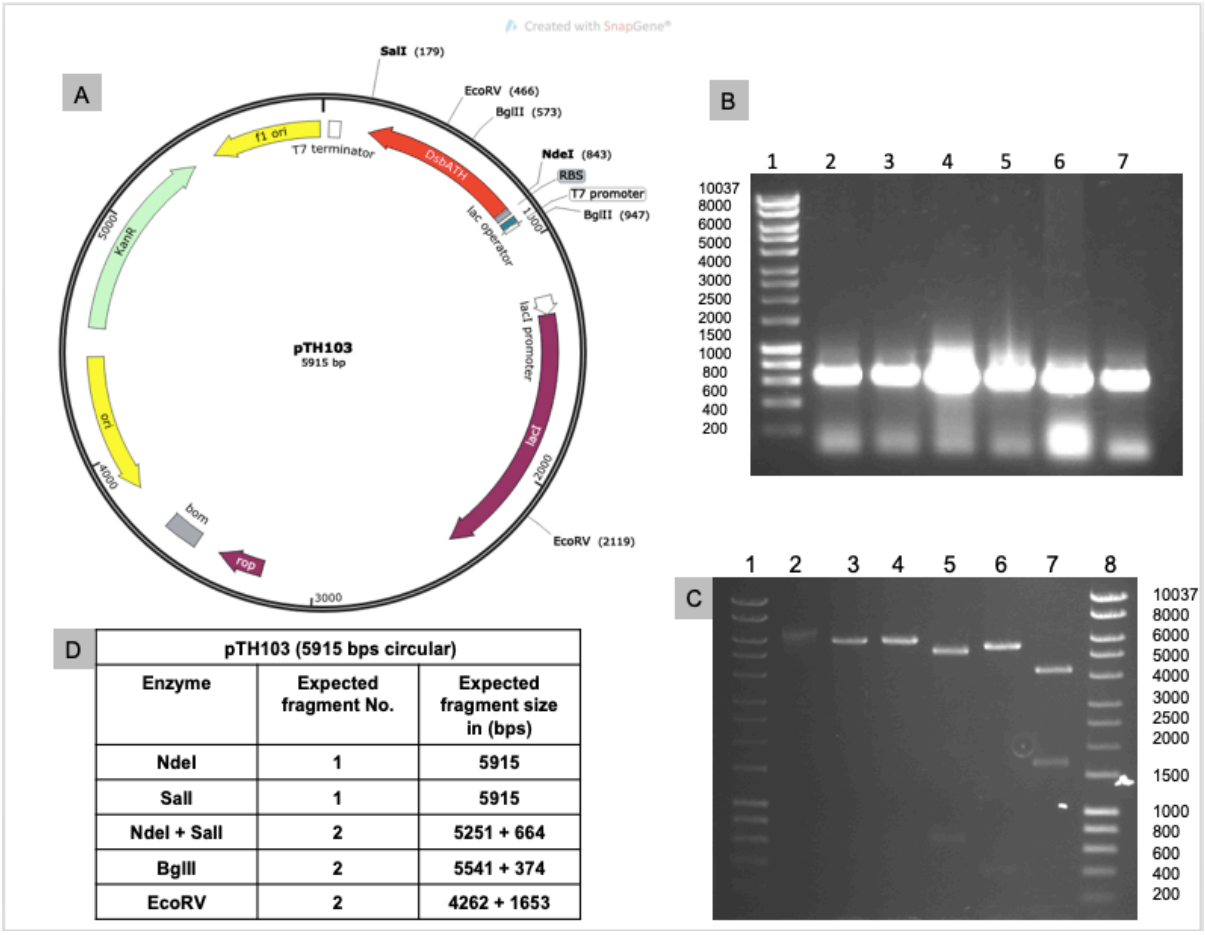



**Figure 4.2: Confirmation of PCR product of *dsbA* from pTH102.**

Lane 1: DNA ladder 1kb (Bioline, UK), Lane 2, 3 and 4: *dsbA* (PCR product), clear bands can be seen in the expected size domain (664 bp), Lane 5: control negative (all reaction contents except DNA template).

### 4.2.2 Insertion of *dsbA* into pET26b(+)

*DsbA* was successfully inserted into pET26b(+) to produce pTH103. The latter was confirmed by colony PCR using the same primers that were used in amplifying the gene and further confirmed by digestion with NdeI, SalI, BglII and EcoRV Figure (4.3). To confirm the sequence; the pTH103 was sent for sequencing, which confirmed that the gene was in the correct position with respect to the 6xHis tag and in frame with the promoter and ribosome binding site.




| pTH103 (5915 bps circular) | | |
|---|---|---|
| **Enzyme** | **Expected fragment No.** | **Expected fragment size in (bps)** |
| NdeI | 1 | 5915 |
| SalI | 1 | 5915 |
| NdeI + SalI | 2 | 5251 + 664 |
| BglII | 2 | 5541 + 374 |
| EcoRV | 2 | 4262 + 1653 |

**Figure 4.3: Confirmation of pTH103.**

**A-** pTH103 was constructed by ligation of the 664 bp amplicon of *dsbA* obtained from pTH102 using PCR amplification with 5360 bp of pET26b(+); the plasmid and fragment were digested by NdeI and SalI restriction enzyme. **B**- Confirmation agarose gel electrophoresis of colony PCR showed the expected size of *dsbA* from the cloning plasmid. Lane 1: DNA Ladder 1kb (Bioline , UK), Lane 2: colony 1, Lane 3: colony 2, Lane 4: colony 3, Lane 5: colony 4, Lane 6: colony 5, Lane 7: colony 6. **C**- Confirmation agarose gel electrophoresis of pTH103 by digestion of cloning; Lane 1 and 8: DNA Ladder 1kb (Bioline , UK), Lane 2: uncut, Lane 3: NdeI, Lane 4: SalI, Lane 5: NdeI + SalI, Lane 6: BglII and Lane 7: EcoRV. **D**- Different restriction enzymes used to confirm the pTH103 and the expected fragments size.

## 4.3 Expression of DsbA protein

The new construct of pTH103 plasmid was transferred into BL21(DE3) cells to produce a BL21(DE3)/pDsbA (BDPD3) for protein expression compared to the pTH102 which is in BL21(DE3)/pDsbA (BDPD2). BL21 (DE3) competent *E. coli* is a widely used T7 expression *E. coli* strain (NEB, UK). Since pTH103 carries a T7 promoter, it can be induced at different concentrations of IPTG (50 μM – 2000 μM ) for the protein expression. However, many studies reported that using the IPTG at 1mM to induce the expression by T7 promoter is often sufficient (Larentis et al., 2014, Rabhi-Essafi et al., 2007), so the expression was carried out with 1mM IPTG at 37 $^{o}$C.

The pTH102 and pTH103 were confirmed by sequencing to contain the *dsbA* gene for expression and purification of the DsbA protein. The SDS PAGE showed that the total lysate of BDPD2 cells were clearly expressed and gave a strong bands of DsbA protein with expected size (22 kDa), while, the BDPD3 did not show any bands of proteins in their lysate Figure (4.4). This result encouraged us to continue our work on DsbA protein by using the pTH102 plasmid as a source of protein and further purification will fellow.

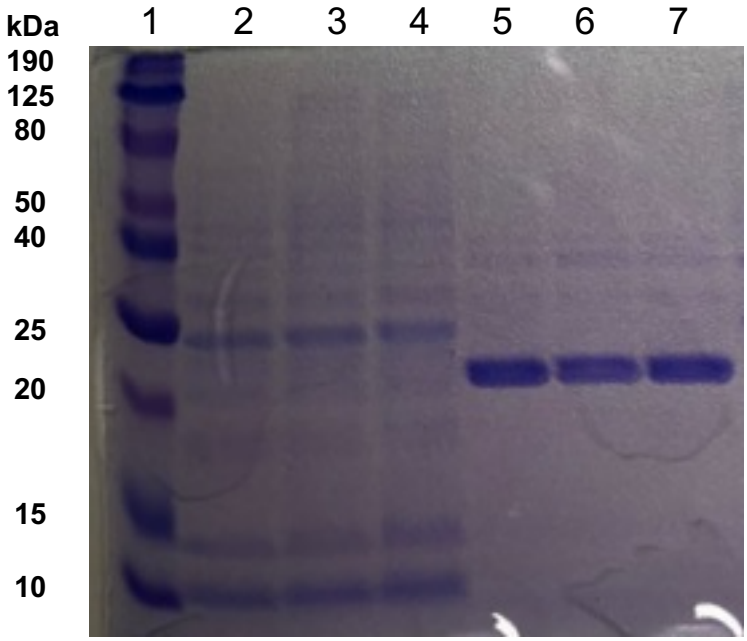



**Figure 4.4: Confirmation of DsbA protein expression in total lysate using SDS PAGE.**

Lane 1: Pre-stained protein ladder (HyperPAGE, Bioline, UK). Lanes 2, 3 and 4: total extracted proteins from BDPD3, which showed there is no DsbA expression; Lanes 5, 6 and 7: total extracted proteins from BDPD2 showed that clear bands of DsbA protein with expected size (protein bands estimated at 22 kDa).

### 4.3.1 Purification of DsbA protein from pTH102

To purify the DsbA protein, BDPD2 cells were used to purify the protein. After the incubation time, the cells were harvested and a cell lysate was obtained by using the antibiotic of polymyxin B. The target protein was purified with His-tag column (His-Trap columns are high binding capacity columns for his-tagged protein purification from GE Healthcare). The wild type DsbA protein was successfully expressed from pTH102 plasmid, purified and confirmed by SDS PAGE Figure (4.5 A).

The Western Blotting in Figure (4.5 B) confirmed that the purified protein possess the 6xHis tag, because it conjugated to the specific His epitope tag anti-mouse primary antibody. The purified protein was measured and aliquoted to small concentrations then stored into -80 freezer. This result opened the way to study the activity of our selective terpenoids on DsbA protein *in vitro*.

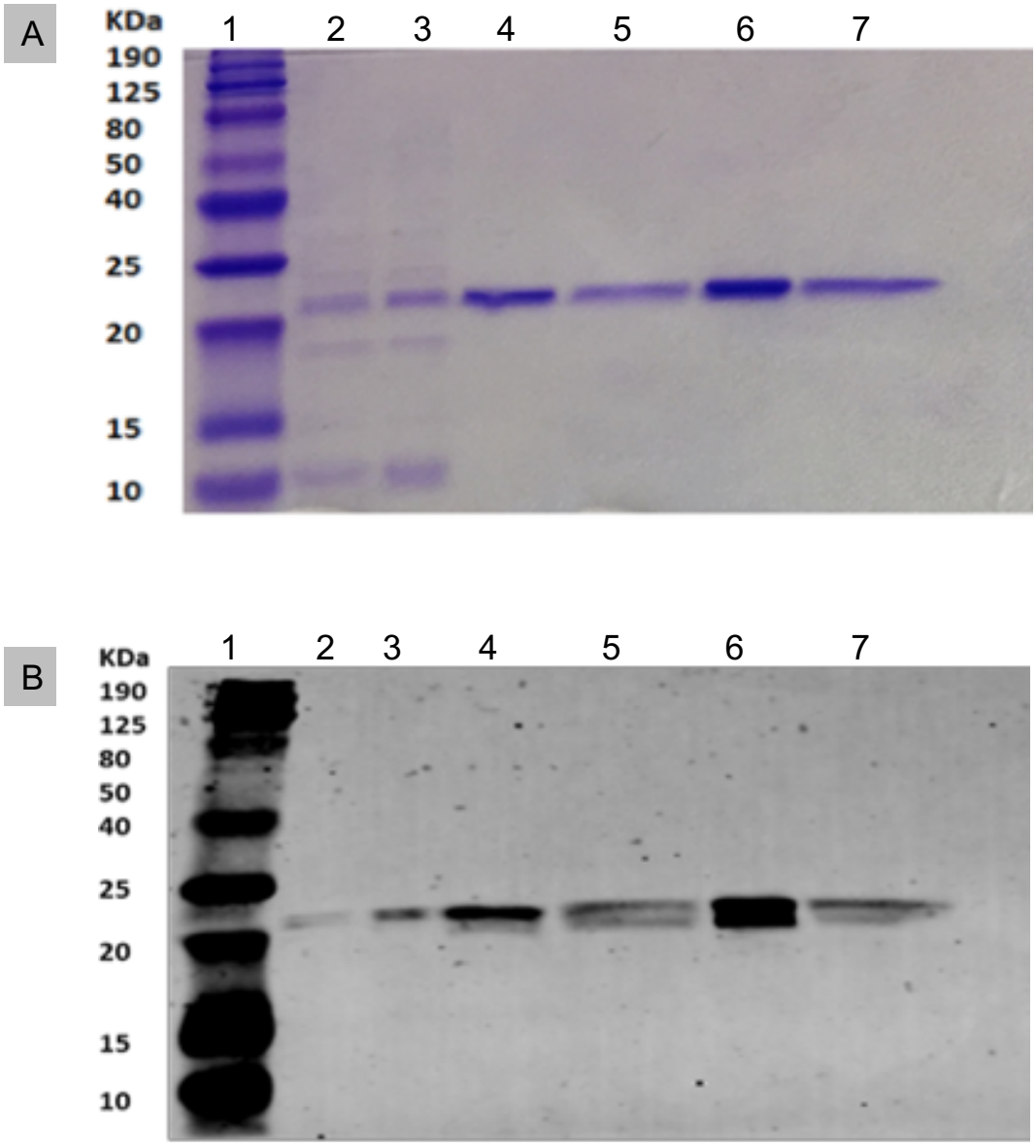



**Figure 4.5: Confirmation of the purified DsbA.**

**A-** SDS PAGE of DsbA protein. **B-** Immunoblotting confirmation of DsbA protein**.** Lane 1: Prestained protein ladder (HyperPAGE, Bioline, UK), Lane 2 and 3: Total extracted proteins from BDPD2 showed there is many protein bands plus the interested DsbA protein which located between 20 -25 kDa of the marker size. Lanes 4, 5, 6 and 7: Fractions of purified DsbA protein by His-Trap purification column, and it's clear there is no other proteins appeared with interested protein. As DsbA has two form, reduced and oxidised, there is double bands show in the immunoblotting figure due to the low amount of DTT in the loading dye.

## 4.4 Labelling of oxidised glutathione

In order to determine the anti-DsbA activity of a variety of terpenoids in the enzymatic kinetic assay labelling of Di-E-GSSG was performed. 1 mM eosin isothiocyanate (EITC) was incubated with 100 µM glutathione disulphide (GSSG) at pH 8.8 to enable the conjugation of the two free amino groups of GSSG to two molecules of eosin, forming Di-E-GSSG Figure (4.6). The product of Di-E-GSSG does not give fluorescence because fluorescence self-quenching (FSQ) occurs when eosin molecules are in close proximity which makes the molecule weakly fluorescent. When a reducing agent, such as dithiothreitol (DTT), is added to the solution, reduction of Di-E-GSSG occurs, which separates the two eosin molecules apart. This abolished FSQ results in a highly fluorescent molecule of E-GSH (Raturi and Mutus, 2007).

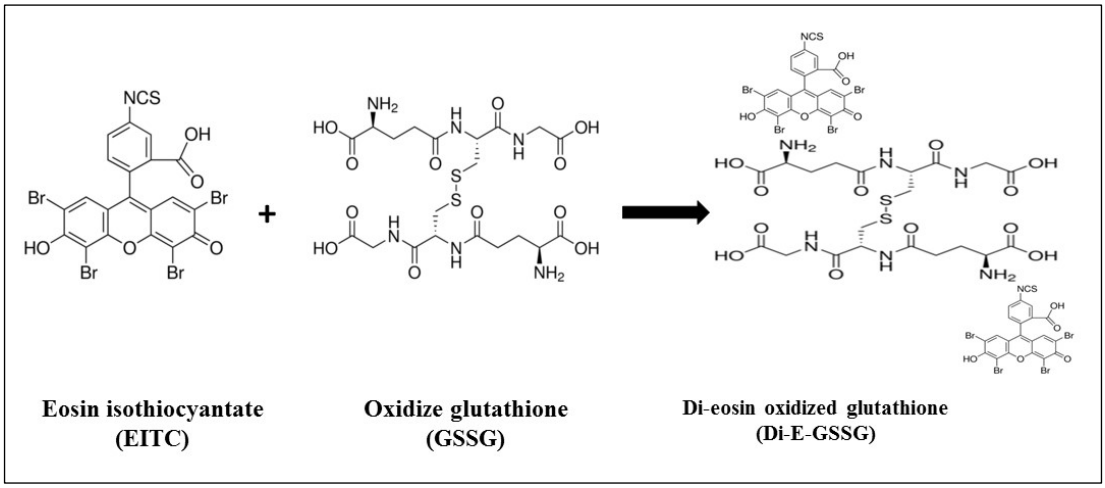



**Figure 4.6: Structure of mechanism of labelling oxidized glutathione.**

Two molecules of EITC were reacted with the free ammonia on both side of GSSG to produce the Di-E-GSSG. GSSG (100 µM) was incubated with 1mM eosin isothiocyanate overnight at 37 ºC and stirring. The Di-E-GSSG was separated by precipitation using acetonitrile with 0.1% trifluoroacetic acid.

To test that the labelling was successful, a 96 well plate assay was performed and a range of concentrations of Di-E-GSSG were reduced by 10 mM DTT in DsbA buffer (0.1 M potassium phosphate and 2 mM EDTA, pH 7.0) at excitation 525 nm and emission 545 nm for 15 min. The Figure (4.7) showed that all concentrations were reduced and converted from the oxidised glutathione form to the reduced glutathione form. The fluorescence fractions increased ~2 fold within 15 mins. The concentration of 150 nM has been used previously in redox state and reductase activity of protein disulphide isomerase (Montano et al., 2014, Raturi and Mutus, 2007). In this case, all the following work on enzymatic kinetic assay was carried out at a concentration of 150 nM Di-E-GSSG and will subsequently be used to test for terpenoids action *in vitro*.

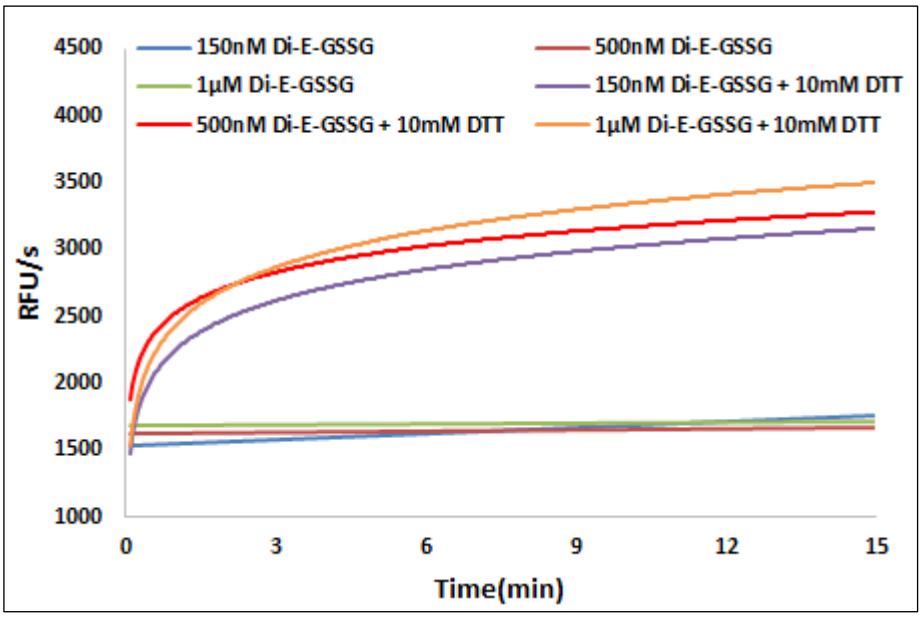



**Figure 4.7: Reduction of labelled Di-E-GSSG by 10 mM DTT.**

Three concentrations of Di-E-GSSG were reduced by 10 mM DTT in DsbA buffer (0.1 M potassium phosphate + 2 mM EDTA, pH 7.0). The rate of fluorescence that emit after reduction is depend on the concentration of Di-E-GSSG in reaction. The concentrations range of Di-E-GSSG (150nM-1μM) that reduced by DTT were gave a high fluorescent levels. The experiment was carried out in triplicate and repeated twice.

## 4.5 Reduction of labelled oxidised glutathione by PDI and DsbA.

PDI (protein disulphide isomerase) is a ubiquitously expressed enzyme that is widely distributed in eukaryotic tissues at relatively high concentrations. It can reduce labelled Di-E-GSSG and convert it to the reduced E-GSH form (Chakravarthi et al., 2006a). Therefore, it was used to test the labelling of Di-E-GSSG. 40 nM of PDI was incubated with 150 nM Di-E-GSSG and 5 µM DTT in DsbA buffer. The amount of DTT (5 µM) incorporated in the reaction did not have any effect on the reduction of PDI as showed in the control through Figures (4.7, 4.8 and related figures). In Figure (4.8 A), PDI clearly reduced the labelled oxidised glutathione and converted it to the reduced form within 15 mins when compared to DTT (10 mM) in the same graph. This result confirms that the new labelling was successful at this concentration and can be used at further reduction experiments.

DsbA displays a similar action on Di-E-GSSG and can convert it to the reduced form (Mirza et al., 2018). To confirm this on our labelled Di-E-GSSG, an enzymatic kinetic assay in 96 well plate format and with the same concentrations of Di-E-GSSG (150 nM) and DsbA (20 nM and 40 nM) were tested in DsbA buffer. Recombinant DsbA protein showed the same activity on the substrate of glutathione as PDI shown in Figure (4.8 A). DsbA protein in Figure (4.8 B) was able to reduce labelled oxidised glutathione and converted it to reduced glutathione within 15 min, which means this protein is active with this substrate. The $V_{max}$ was determined by incubating various concentrations of Di-E-GSSG (50 nM – 5 µM) with 40 nM of DsbA protein in DsbA buffer at room temperature. A theoretical hyperbolic curve allowed estimation of $V_{max}$ for DsbA to be 6.3 nmol/s, while the reductase activity of DsbA was monitored as function of Di-E-GSSG with an apparent $K_m$ value of 255.8 ± 33 nM as determined by means of a Lineweaver-Burk plot shown in Figures (4.9 A and B).

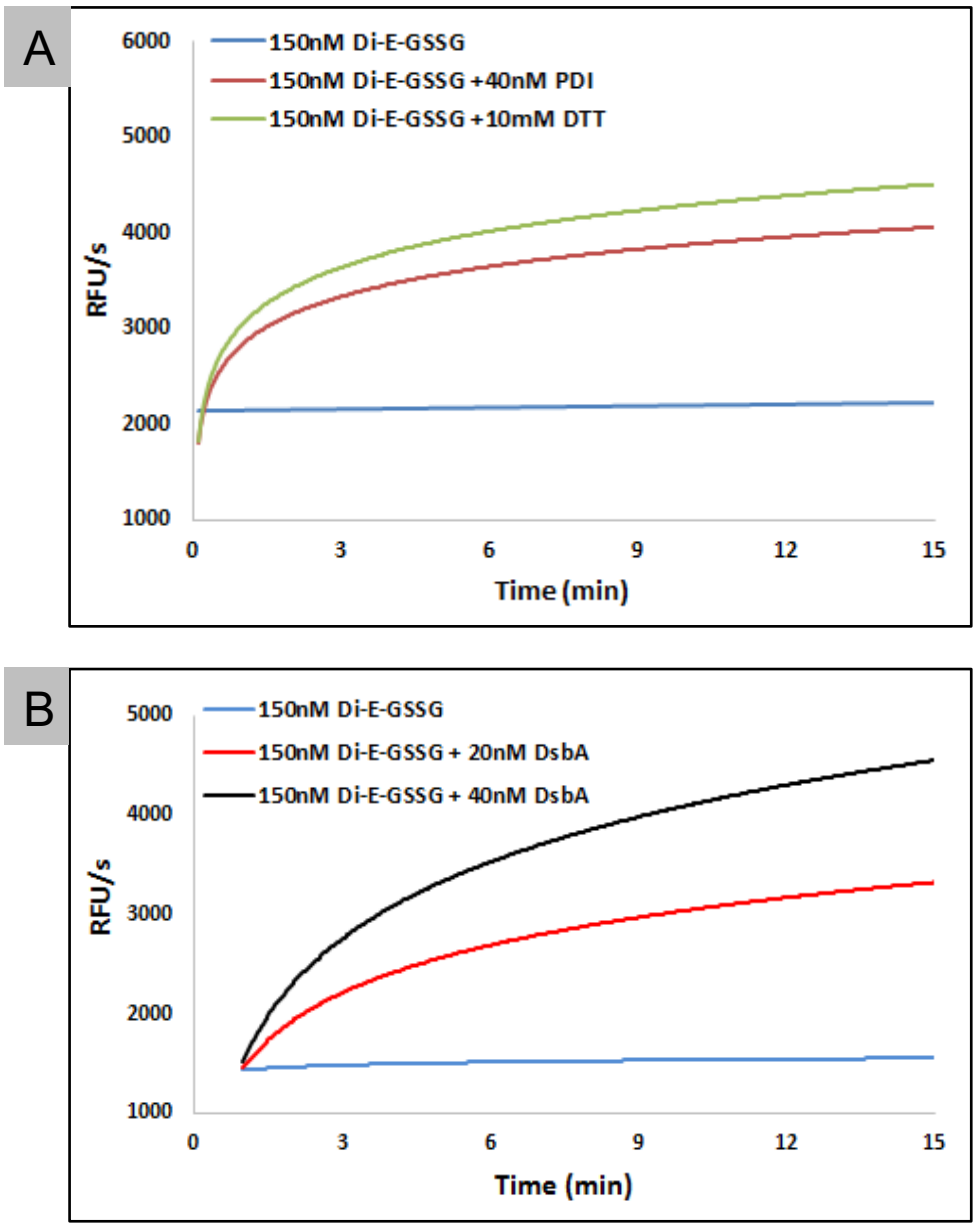



**Figure 4.8: Reduction of labelling Di-E-GSSG by PDI and DsbA proteins.**

**A**- PDI (40 nM) was incubated with 150 nM Di-E-GSSG in DsbA buffer **B**- Two concentrations of purified DsbA were incubated with 150 nM labelling Di-E-GSSG in DsbA buffer. DsbA was able to reduce the Di-E-GSSG and convert it to E-GSH as PDI. The fluorescent was increased directly after mix the DsbA with substrate and reach the high fluorescence level within 15 min.

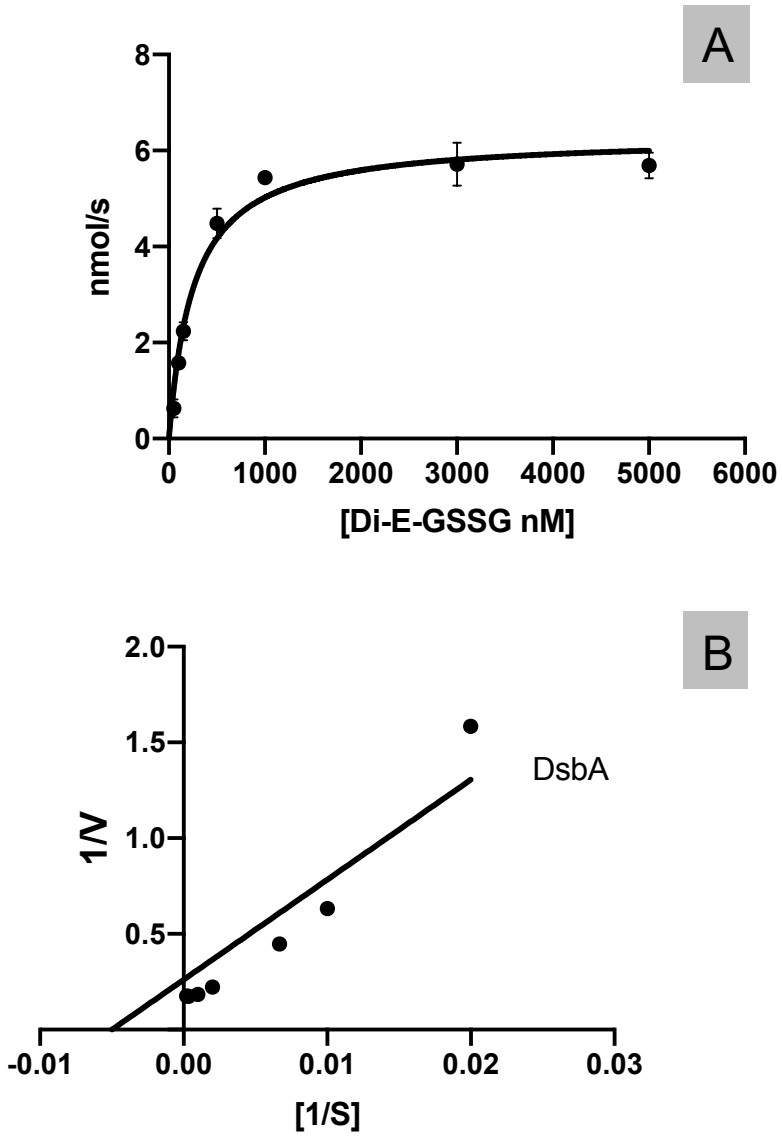



**Figure 4.9: Estimation of $V_{max}$ and $K_m$ for DsbA protein.**

**A-** Estimation of $V_{max}$ for DsbA by plotting theoretical hyperbolic curve. **B**- Estimation of $K_m$ for DsbA by Lineweaver-Burk plot. The $V_{max}$ was determined by incubating various concentrations of Di-E-GSSG (50 nM – 5 μM) with 40 nM of DsbA protein in DsbA buffer at room temperature. A theoretical hyperbolic curve allowed estimation of $V_{max}$ for DsbA to be 6.3 nmol/s, while the reductase activity of DsbA was monitored as function of Di-E-GSSG with an apparent $K_m$ value of 255.8 ± 33 nM as determined by means of a Lineweaver-Burk plot.

## 4.6 Inhibition of the reduction of DsbA activity by terpenoids in an enzymatic kinetic assay

Terpenoids were tested for their activity against *S. sonnei* in *in vitro* and *in vivo* in the previous results (chapter three). Terpenoids reduced the number of intracellular cells; especially geraniol significantly reduced the number of bacterial cells inside HeLa and RAW macrophages cells. Furthermore, geraniol was shown to possess an activity against *Shigella* DsbA (Mirza et al., 2018). Therefore, an enzymatic kinetic assay was performed with terpenoids in order to determine if there are other terpenoids work better than geraniol in *in vitro* against DsbA.

As shown in Figure (4.10), it is clear that all terpenoids display inhibition of E-GSH formation when compared with DsbA alone. The rate of inhibition was different depending on the terpenoid, although geraniol displayed the best inhibition activity (black line in Figure 4.10 A) when compared with the other terpenoids. Geraniol at 42 µM reduced the fluorescence from 5000 RFU/s to 3000 RFU/s when compared with DsbA reduction alone, while linalool 42 µM showed a significant inhibition but still less than geraniol Figure (4.10 A). Citral and nerol showed half of the reduction displayed by geraniol as can be seen in Figure (4.10 A and B) respectively. Most of the remaining terpenoids showed little inhibition equivalent to 1000 RFU/s (Figures 4.10 B, C and D).

Due to the activity showed by geraniol, it was used to determine the $V_{max}$ and $K_m$ of inhibition of enzymatic kinetic assay. Interestingly, geraniol showed an activity to be a competitive inhibitor by reducing the $V_{max}$ of DsbA from 6.3 nmol/s to 3.0 nmol/s with little as 42 µM. In the same time, the $K_m$ value was decreased dramatically from 255.8 $\pm$ 33 to 132.5 $\pm$ 39 and its clearly appears in the Figure (4.11 A and B).

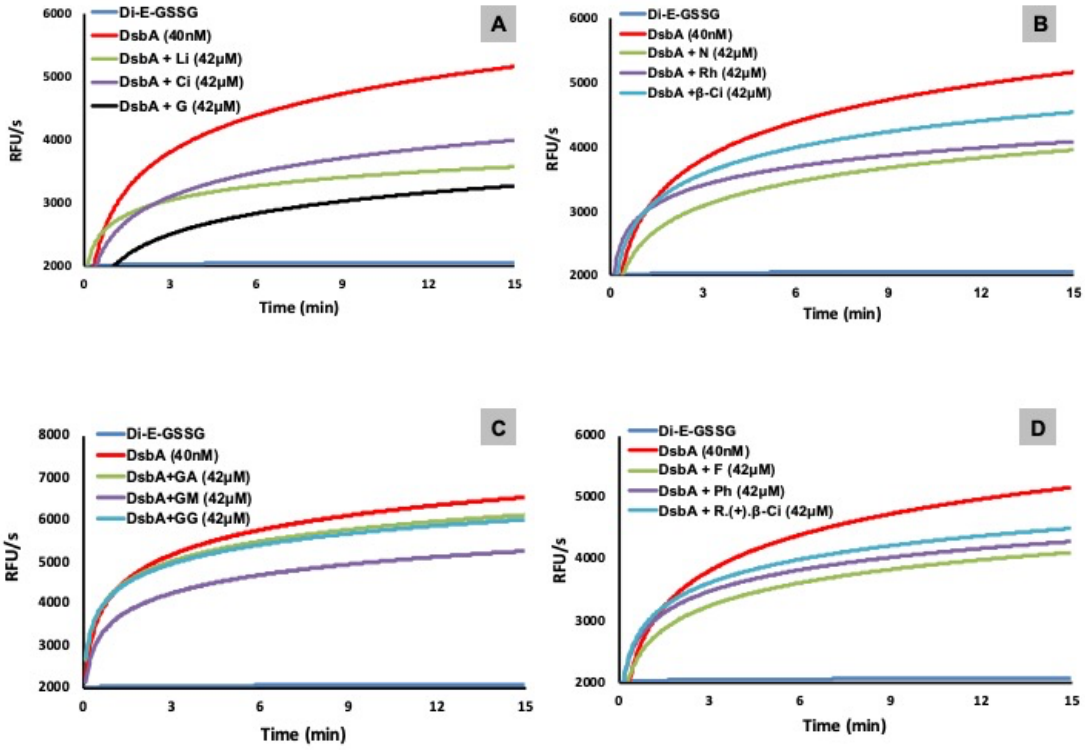
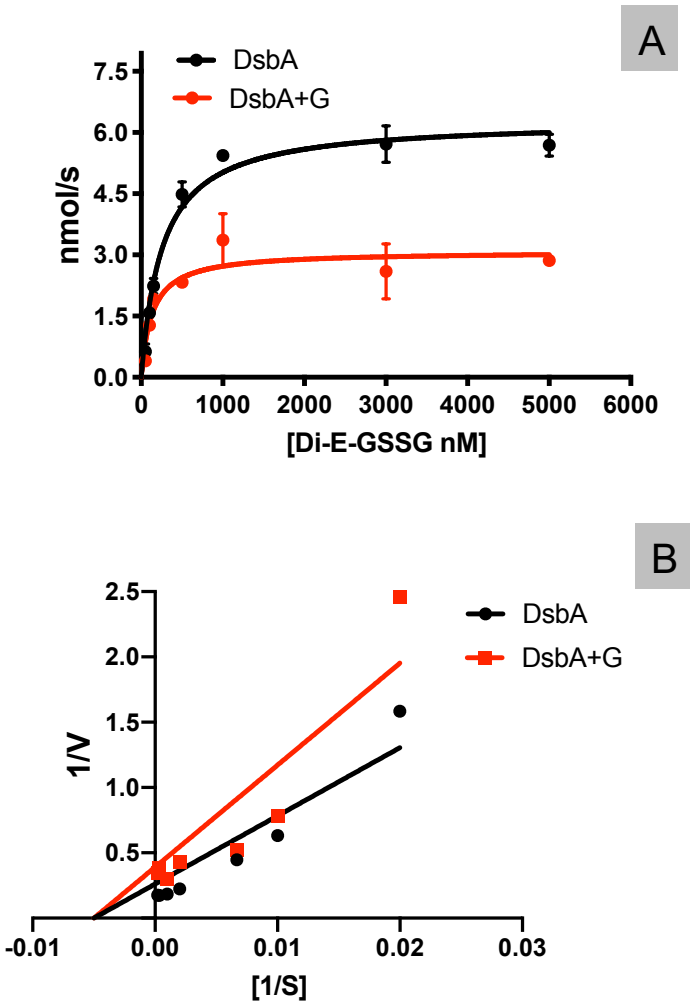



**Figure 4.10: Activity of terpenoids in inhibition of DsbA by reduction.**

**A-** Geraniol, linalool and citral inhibit the reduction of Di-E-GSSG by DsbA. **B-** Nerol, rhodinol and β-citronellol inhibit the reduction of Di-E-GSSG by DsbA. **C-** Geranylacetate, geranylamine and geranylgeraniol inhibit the reduction of Di-E-GSSG by DsbA. **D-** Farnesol, phytol and R.(+).β-citronellol inhibit the reduction of Di-E-GSSG by DsbA. The experiment was carried out in triplicate and repeated three times.




| | $V_{max}$ (nmol/s) | $K_m$ (nM) | $K_{cat}$ ($S^{-1}$) | $K_{cat}/K_m$ ($M^{-1}S^{-1}$) |
|---|---|---|---|---|
| **DsbA** | 6.3 | 255.8 | 0.004 | 19 x $10^{-4}$ |
| **DsbA+ G** | 3.0 | 132.5 | 0.002 | 18 x $10^{-4}$ |

**Figure 4.11: Estimation of $V_{max}$ and $K_m$ after inhibition the DsbA protein by geraniol.**

**A**- Theoretical hyperbolic curve allowed estimation of $V_{max}$ for DsbA after inhibition. **B**- Lineweaver-Burk plot for DsbA to estimate the $K_m$ after inhibition. Geraniol showed an activity to be a competitive inhibitor by reducing the $V_{max}$ of DsbA from 6.3 nmol/s to 3.0 nmol/s with little as 42 µM. In the same time, the $K_m$ value was decreased dramatically from 255.8 ± 33 to 132.5 ± 39.

## 4.7 Labelling of reduced glutathione (GSH)

The above data demonstrated that glutathione can be used as a substrate for *Shigella* DsbA. In order to see if the DsbA will be able to catalyse reduced GSH to oxidised GSSG, which is essential for *Shigella* to survive and proliferate in the host cell cytosol (Yu, 1998), the GSH was labelled with fluorescence EITC. As no *in vitro* assay has yet been described, a fluorescent E-GSH was set up as *in vivo* assay by converting of Di-E-GSSG by DTT in DsbA buffer overnight in the dark at room temperature. This reaction allowed DTT to convert all the low fluorescent Di-E-GSSG to high fluorescent E-GSH (Raturi and Mutus, 2007). The labelling of E-GSH showed high fluorescence with no reduction by DTT (Green line in Figure 4.12 A) which means all Di-E-GSSG was converted completely into E-GSH. In order to determine the smallest concentration of fluorescent E-GSH that can be detected by the machine in the medium, a different concentrations of E-GSH were performed in LB broth. The concentration of 75 nM was the smallest concentration that can be detected by molecular plate reader machine Figure (4.12 B), and it was used in the following experiments of catalysis.

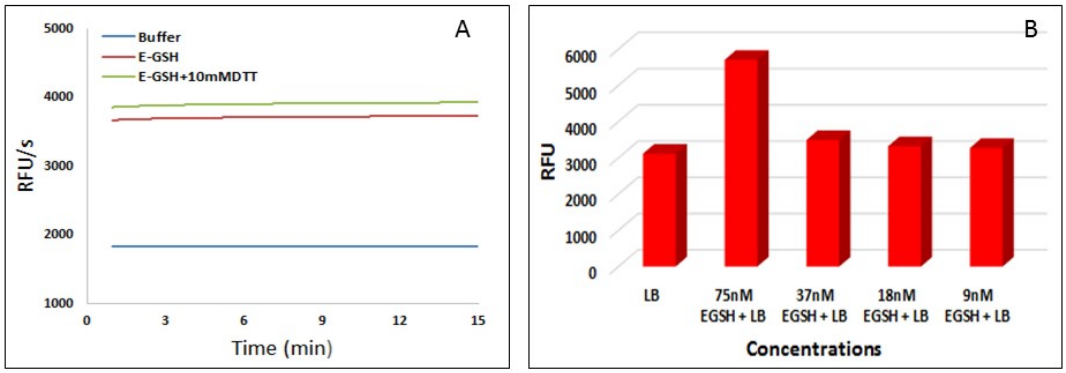



**Figure 4.12: Labelling and detection of concentration of E-GSH.**

**A**- Test reduction of E-GSH by 10 mM DTT after labelling with EITC. **B**- Detection of the fluorescent concentration of E-GSH with LB medium.

## 4.8 Catalysis of labelled reduced glutathione E-GSH (*In vivo*)

To answer the question, does *Shigella* DsbA catalyse reduction on GSH to oxidised GSSG, an *in vivo* experiment was set up and SSWT, SSDM and SSPD complemented strain were grown overnight in LB media. It can be seen clearly in Figure (4.13) that SSWT grew very well until the $OD_{600}$ reached 2.0 within 9 hours. During that time, the culture gradually became less fluorescent, from 5000 to 2000 RFU. Therefore, all the labelled reduced glutathione E-GSH was converted into the oxidised form, Di-E-GSSG.

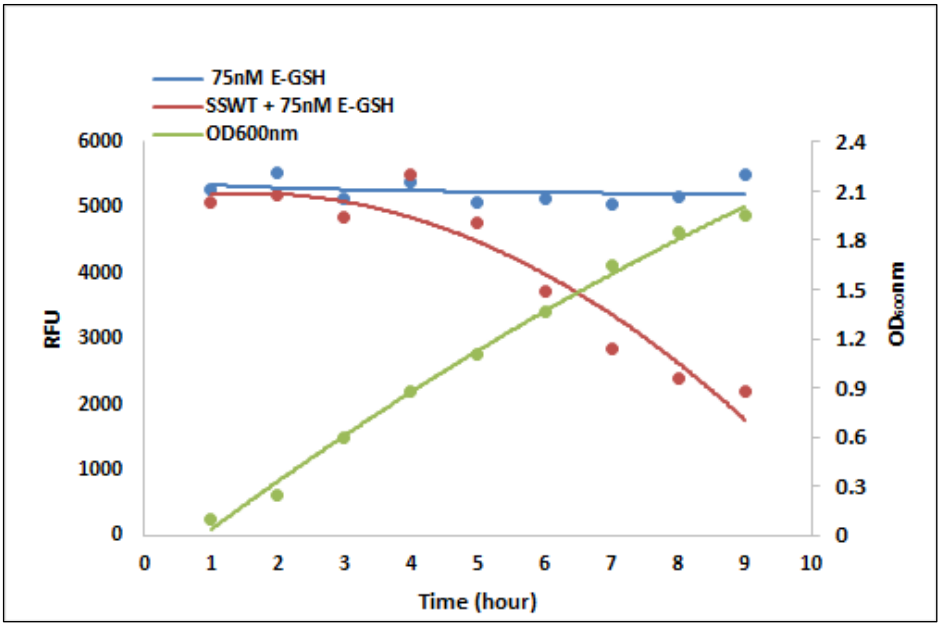



**Figure 4.13: *In vivo* catalysis of E-GSH by growing SSWT.**

Blue line is 75 nM of E-GSH in LB media. Red line is SSWT bacteria culturing in LB media and supplemented with 75 nM of E-GSH. Green line is the optical density of SSWT + E-GSH. The experiment was carried out in triplicate and repeated three times.

However, when the experiment was carried out with SSDM strain and with the same conditions as that used in wild type culture. It was clear that there was no reduction in fluorescence, although the mutant strain grew well under mildly reducing conditions Figure (4.14). Although SSDM grew slower than SSWT in compared (it eventually reached $OD_{600}$ 2 though), the fact that the SSDM cannot catalyse the E-GSH into Di-E-GSSG due to missing the *dsbA* and supports the role of this protein in *Shigella* virulence.

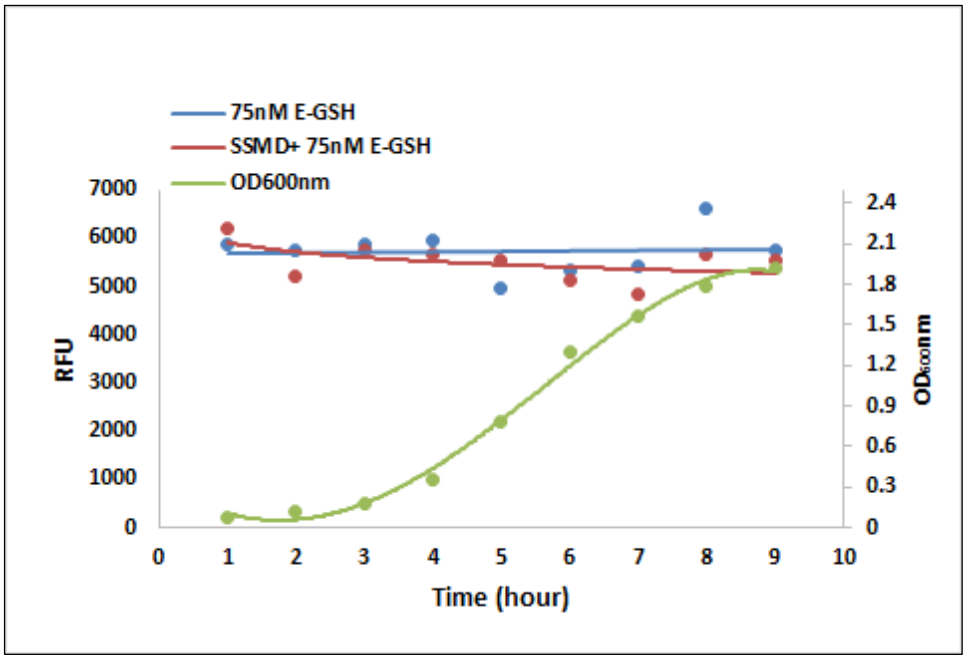



**Figure 4.14: *In vivo* catalysis of E-GSH by growing SSDM.**

Blue line is 75 nM of E-GSH in LB media. Red line is SSDM bacteria culturing in LB media and supplemented with 75 nM of E-GSH. Green line is the optical density of SSDM + E-GSH. The experiment was carried out in triplicate and repeated three times.

To confirm the role of DsbA in catalysis, a SSPD complemented strain was used in the culture with same method and conditions. A SSPD strain showed greater ability than wild type strain (SSWT) in reducing the cultures fluorescence, from 5000 to 1000 RFU, over time. Presumably this was due to the high copy of the plasmid encoded *dsbA* Figure (4.15). These results confirmed the role of DsbA in catalysis of GSH and its conversion it to GSSG in the cytosol. The catalysis of GSH to GSSG in the host cell cytosol is imperative for *Shigella* to survive, proliferate and establish infection. Furthermore, this process can regenerate the active DsbA during infection to do his job in the folding of secreted effector proteins.

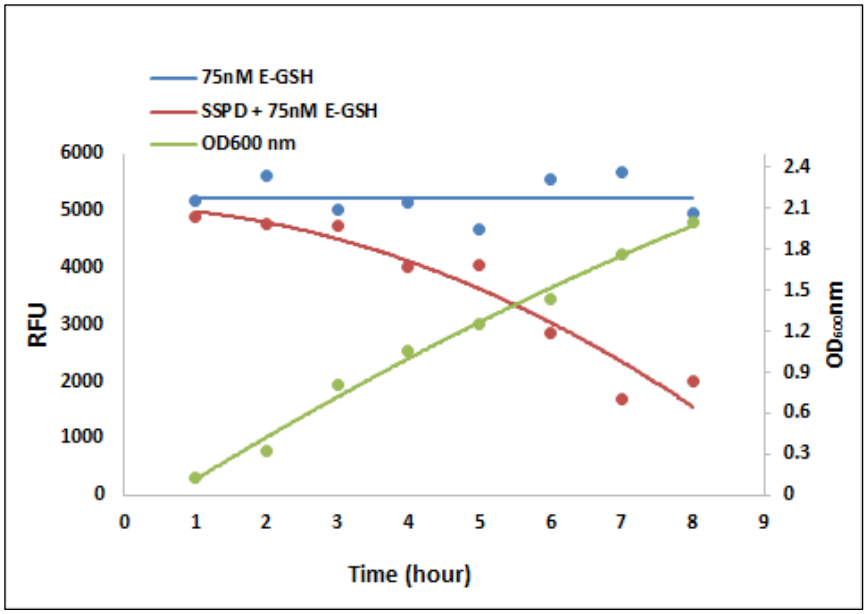



**Figure 4.15: *In vivo* catalysis of E-GSH by growing SSPD.**

Blue line is 75 nM of E-GSH in LB media. Red line is SSPD bacteria culturing in LB media and supplemented with 75 nM of E-GSH. Green line is the optical density of SSPD + E-GSH. The experiment was carried out in triplicate and repeated three times.

## 4.9 Inhibition of catalysis of reduced glutathione by geraniol

In the work described above we demonstrated that geraniol has the ability to inhibit the reduction of oxidised glutathione GSSG *in vitro* in an enzymatic kinetic assay. This allowed us to ask the question as to whether geraniol inhibits the catalysis of reduced glutathione *in vivo*. To answer this question, an experiment was carried out with two strains of *Shigella sonnei* (SSWT and SSPD) with the same conditions. Geraniol was used at concentrations of 42 μM in LB media. The $OD_{600}$ and related fluorescent unit (RFU) were obtained each hour using (WPA) spectrophotometry at $OD_{600}$ nm and Spectramax plate reader M5 (Molecular Devices) at excitation 525 nm and emission 545 nm respectively.

Geraniol showed inhibition of the culture fluorescence from 5000RFU into 4000RFU in both strains cultures when compared to the fluorescence in the untreated cultures as showed in Figures (4.16 A and B). Both strains that were treated or untreated with geraniol grew very well in the culture at the $OD_{600}$. The activity of geraniol was also tested against *S. sonnei* for antimicrobial activity and there is no directed activity has been observed when geraniol was supplemented with bacteria in the growth culture. This explain why both strains grew very well in the culture.

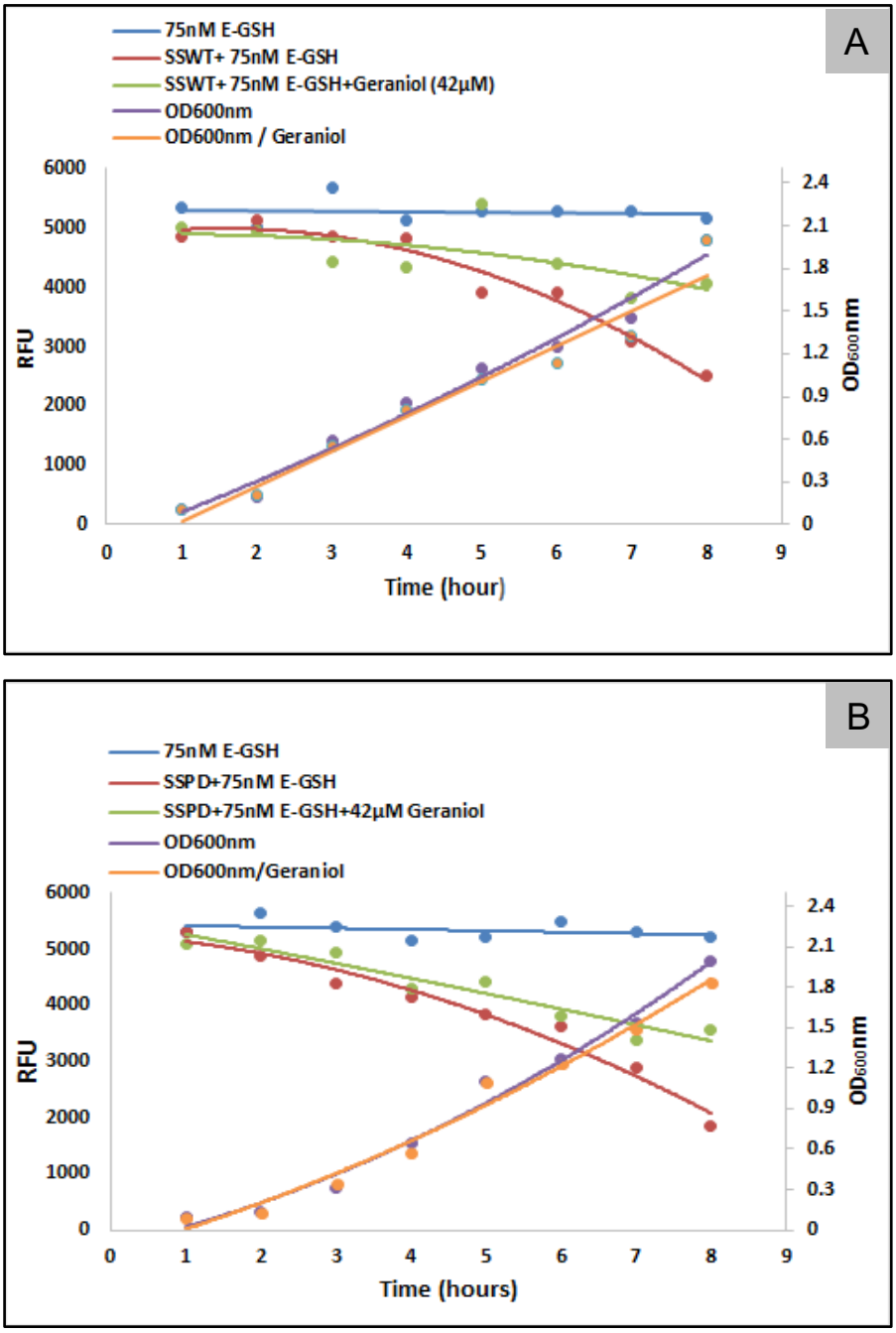



**Figure 4.16: Inhibition of catalysis of E-GSH by geraniol.**

**A-** Geraniol inhibits the catalysis of E-GSH during SSWT growth. **B**- Geraniol inhibits the catalysis of E-GSH during SSPD growth. The experiment was carried out in triplicate and repeated twice.

## 4.10 Discussion

DsbA is present in all Gram-negative bacteria and some Gram-positive bacteria, including many pathogens of human and animal. DsbA is considered as a master virulence factor as it catalyses the formation of disulphide bonds in exported proteins, of which many are virulence factors. Targeting Dsb proteins for anti-virulence therapy is not new but previous studies focusing on disrupting DsbA-DsbB interaction have yielded little progress; DsbB is required for recycling the non-functional reduced DsbA to its functional oxidised form (Smith et al., 2016).

Previous studies revealed that the *Shigella* DsbA, is vital in supporting the survival of *S. flexneri* in the highly reducing environment host cell cytosol (Yu, 1998). We found that true as *S. sonnei* missing the DsbA cannot grow well as the wild type in the presence of 10 mM of reduced GSH. The highly reducing environment of the host cell cytosol results from the concentrations of thioredoxin-1 and glutathione which are not in equilibrium. Meanwhile, the glutathione has two forms; reduced (GSH) and oxidised (GSSG), and the reduced form is existed in high concentrations in the cytosol compared to oxidised form (Go and Jones, 2008). *S. sonnei* may benefit from this environment by using GSH as a substrate for their DsbA protein and it requires this protein to survive in the reducing environment of the host cell cytosol by regulating the DsbA expression that is a specific response to reducing conditions (Yu, 1998). So, to understand the relationship between *Shigella* and GSH in *in vitro* and *in vivo* we aimed to clone the *dsbA* gene into a pET26b(+) to purify the DsbA protein for further tests.

The *dsbA* was cloned successfully into pET26b(+) to produce pTH103 from pTH102. Since DsbA is a periplasmic protein, so it needs to have a signal sequence to allow

the protein to move to the periplasmic space. Our *dsbA* already encodes the signal sequence at the N terminal of the gene. Therefore, we needed to have the 6xHis tag at the C terminal. The sequence tool confirmed that the cloning was corrected and the signal sequence was already located at the N terminal and the 6xHis at C terminal followed by stop codon. After confirmation of the new cloning plasmid, pTH103 with the tag and pTH102 was transferred into BL21(DE3) in order to purify the DsbA protein. IPTG was used at range of concentrations (50 – 2000 μM) and with different incubation temperatures using the LB media. Surprisingly, the pTH102 plasmid was expressed and gave a clear bands of DsbA protein with expected size when compared to the pTH103 plasmid who did not express the protein completely Figure (4.4).

The expression of genes in *E. coli* is influenced by a range of factors which make the protein expression and purification more difficult. The stability of the plasmid in the host is one of these factors, so that the plasmids are not always stable, especially in the cells grown for many generations in large-scale cultures, so that when a process is scaled up it is important that vector stability be addressed (Rai and Padh, 2001). Importantly, another factor is the location of the protein within the host cell, While *E. coli* proteins are synthesized in the cytoplasm, it is possible to direct a cloned gene product to the cytoplasm, the inner or outer membrane, or the periplasmic space (Baneyx, 1999). Secretion of a cloned gene product to the periplasmic space often allows for higher levels of expression of the foreign protein that might be degraded by proteases in the cytoplasm (Mergulhao et al., 2007). In addition, the signal sequences are important for *E. coli* to recognize and the secreted protein and export it correctly into the periplasmic space (Mergulhao et al., 2007). Some of culture conditions such as a decrease culture growth temperature has an advantages as growth at 37°C can

promote inclusion body formation for some proteins while growth at lower temperatures (e.g., 30°C, 25°C, 18°C) may not and lower temperature also decreases protease activity (Hunke and Betton, 2003). Finally, the recombinant DsbA protein was purified and confirmed by SDS PAGE and Western blotting using the His epitope tag anti-mouse primary antibody Figure (4.5 A and B). This result allowed to purify the DsbA protein and finish our objectives on determining its enzyme activity.

For the DsbA enzymatic assay; the GSSG was labelled successfully with eosin isothiocyanate (EITC) to give the Di-E-GSSG according to the method of (Montano et al., 2014, Raturi and Mutus, 2007). Di-E-GSSG possess a weak fluorescence because the fluorescence self-quenching occurs when eosin molecules are in close proximity which makes the molecule weakly fluorescent. When a reducing agent, such as dithiothreitol (DTT), is added to the solution, reduction of Di-E-GSSG occurs, which separates the two eosin molecules apart. This abolished FSQ results in a highly fluorescent molecule of E-GSH (Raturi and Mutus, 2007). A recombinant purified DsbA showed activity in the conversion of Di-E-GSSG to GSH within 15 min in DsbA buffer and this activity is similar to PDI activity *in vitro*. This activity is consistent with what a previous study that reported the activity of DsbA on Di-E-GSSG substrate (Mirza et al., 2018). The $V_{max}$ and $K_m$ of DsbA was determined and this enzyme showed $V_{max}$ of 6.3 RFUs and $K_m$ of 255.8$\pm$ 33.

Terpenoids were also tested for their activity in the enzymatic assay and geraniol proved to be the most effective compound in inhibiting the activity of DsbA enzyme. Linalool and citral also showed activity in the reduction of DsbA activity in an enzymatic kinetic assay. There are two major mechanisms for the inhibition of enzymatic activity; competitive and non-competitive. In competitive inhibition, the

substrate and inhibitor compete for binding to the same active site, while non-competitive inhibition occurs when the inhibitor binds to another site on the enzyme molecule, resulting in conformational change, with the result that the enzyme can no longer interact with its substrates (Cornish-Bowden, 2015). Interestingly, geraniol showed an activity as a competitive inhibitor by reducing the $V_{max}$ of DsbA from 6.3 RFUs to 2.9 RFUs with little of 42 µM. In the same time, the $K_m$ value was decreased dramatically from 255.8 ± 33 to 66.8 ± 39 and its clearly appears in the Figure (4.11 A and B).

In order to determine the activity of geraniol in inhibiting DsbA during catalysis of reduced glutathione *in vivo*, a labelling GSH with fluorescence eosin was incubated with *S. sonnei* strains. SSWT was able to convert reduced GSH into the oxidized form GSSG; this catalysis was monitored through reduced of RFU from 5000 RFU to 2000 RFU. To confirm the role of DsbA in this catalysis, a *dsbA* mutant strain (SSDM) was tested for its ability to reduce GSH; it showed no activity and the fluorescence level remains same even with *Shigella* growing very well. For additional confirmation, a complemented strain expressing *dsbA* (SSPD) was used that converted E-GSH even more than the wild type strain; presumably due to the high copy number of *dsbA* in this strain Figures (4.13, 4.14, 4.15). To the best of our knowledge, this is the first time that *Shigella* DsbA has been shown to catalase GSH to GSSG, which strongly supports the hypothesis that catalysis of GSH to GSSG in the host cell cytosol is important for *Shigella* to survive, proliferate and establish infection (Yu, 1998). At this point, geraniol was used with the SSWT and SSPD strains to determine their activity in the inhibition of DsbA catalysis. Geraniol reduced the activity of DsbA catalysis by approximately 50% in both the wild type and complemented strains Figure (4.16 A and B) respectively. In addition, geraniol showed no direct activity towards *Shigella*

*sonnei* at a minimum inhibitory concentration (MIC) 500 mg/L. This explains why the bacteria grow well with or without geraniol in the *in vivo* assays. All these observations indicate a potential therapeutic for geraniol in *Shigella* infection.

In summary, DsbA protein was successfully purified and used in the following tests. As DsbA is an active enzyme, it can reduce and catalyse an oxidised and reduced glutathione *in vitro* and *in vivo*. *In vitro*, the purified DsbA protein showed an ability to convert GSSG into GSH in DsbA buffer within 15 min with $V_{max}$ about 6.3 RFUs and $K_m$ around 255.8± 33. *In vivo*, *S. sonnei* grown in LB supplemented with fluorescence GSH, were able to catalyse the conversion of GSH to GSSG and confirmed the role of DsbA in this catalysis by the SSDM and SSPD strains. These findings explained for the first time another mechanism that is used by *S. sonnei* in recycling the non-functional reduced DsbA to its functional oxidised form. Geraniol was the best inhibitor against DsbA protein from the list of terpenoids on substrate (glutathione) and it can reduce the activity of DsbA. In addition, geraniol showed an activity to reduce the fluorescence of culture by half when supplemented in a culture of *Shigella*. Finally, we can confirm that terpenoids and especially geraniol hold a promising anti-virulence therapeutic in *Shigella* infection.

# Chapter 5

## Characterisation of DsbA interaction sites with geraniol

# 5 Characterisation of DsbA interaction sites with geraniol

## 5.1 Introduction

A disulphide bond is formed when an oxidation reaction occurs between two cysteine thiol groups, with the simultaneous release of two electrons and two protons (Denoncin and Collet, 2013). DsbA is a member of the thioredoxin superfamily and has a canonical CXXC catalytic motif located in the highly conserved fold. This catalytic motif maintains the DsbA to be more stable and enables it to react with proteins entering the periplasm to oxidise them. (Messens and Collet, 2006). This process is critical for the correct folding and structural stability of many secreted proteins. DsbA introduces disulphide bonds into envelope proteins; there are approximately 300 proteins in *E. coli* that are known or predicted substrates of DsbA (Dutton et al., 2008b, Vertommen et al., 2008). DsbA contributes to pathogenesis of numerous pathogenic bacteria and is necessary for the assembly and/or function of toxins and secreted enzymes (Heras et al., 2009). Inhibition of the Dsb system is not a new approach for antimicrobial chemotherapy, with many attempts to inhibit the interaction between DsbA-DsbB but with relatively little progress (Smith et al., 2016). Inhibition of these enzymes has a number of challenges; the finding of suitable binding sites on the DsbA–substrate binding interface presents the most significant issue. DsbA forms the most stabilizing interaction with its substrates, and is mediated via a disulphide bond formed by cysteine (Cys30); however, this bond is rapidly resolved as a result of the reactivity of this cysteine (Kurth et al., 2014) .

Polymerase chain reaction (PCR)-based site-directed mutagenesis is an essential technique in molecular, biochemical and genetic studies. The PCR progresses via linear amplification for the production of single-stranded DNA molecules that anneal to generate a circular plasmid with staggered single-stranded DNA breaks (Zheng et

al., 2004, Liu and Naismith, 2008). Mutagenesis is used in proteins studies to generate proteins which are more tractable for biophysical techniques. The ability to rapidly and specifically mutate residue(s) in a protein is important for mechanistic and functional studies (Xia et al., 2015). A number of strategies have been developed such as the QuikChange™ Site-Directed Mutagenesis System, which is probably the most favoured approach (Chapnik et al., 2008, Li et al., 2008). QuikChange™ works by using a pair of complementary primers with a specific mutation. In a round of PCR cycles, these primers anneal to the template DNA, replicating the plasmid DNA with the mutation. The resulting DNA pool (mutant and parental) is then treated with *Dpn*I to destroy the parental methylated DNA whilst the newly synthesized unmethylated mutant DNA remains intact before being used to transform *E. coli*. The two newly synthesised DNA strands are then ligated by host repair enzymes (Zheng et al., 2004, Tseng et al., 2008).

Geraniol is a monoterpene found in the essential oil of lemongrass (Cymbopogon) and other aromatic herbs. It has been tested *in vitro* and *in vivo* against several mammalian cell lines and showed activity against cancerous cells (Carnesecchi et al., 2001, Burke et al., 1997). Geraniol also demonstrated activity against Gram-negative bacteria including multidrug-resistant isolates such as *Enterobacter aerogenes*, *E. coli* and *Pseudomonas aeruginosa* by targeting the virulence factors in these pathogens (Lorenzi et al., 2009). Another study illustrated that geraniol, citral and carvacrol were active against *Staphylococcus aureus* when combined with enterocin AS-48 (Enterocin AS-48 is a circular bacteriocin produced by *Enterococcus* sp.) (Grande et al., 2007). Geraniol showed significant activity in inhibition of the reduction of oxidised glutathione (GSSG) by DsbA and the catalysis of reduced glutathione (GSH) *in vitro* and *in vivo*, respectively (Chapter 4). Furthermore, geraniol was the most effective compound tested against *Shigella sonnei* infection from the selected terpenoids

(Chapter 3). To understand the mechanism that enables geraniol to inhibit DsbA, geraniol was merged with DsbA by their structures using the Discovery Studio software. The software showed that there are five potential interaction sites that geraniol may bind to and inhibit the DsbA protein. We then tested these predicted interaction sites by altering the appropriate DsbA amino acid.

The aims of this chapter were:

1- Identify interacting amino acids in DsbA with geraniol.
2- Substitution of the amino acids that interact with geraniol.
3- Purify DsbA mutant proteins and test the activity of these proteins in reduction and catalysis of glutathione and the role of geraniol in this process.
4- Evaluate the DsbA mutant protein activity using an *in vivo* larval model.

## 5.2 Determination and substitution of amino acids at the interaction sites of DsbA protein and geraniol

In order to substitute the DsbA amino acids that may interact with geraniol, we need firstly to make prediction of these interaction sites. The Discovery Studio software revealed that there are five possible interaction sites between geraniol and the DsbA protein. When we merged geraniol structure with DsbA protein structure in the Discovery Studio software; five potential interaction sites were apparent: phenylalanine 29, histidine 32, glutamine 35, leucine 68 and phenylalanine 174 (Figure 5.1). These amino acids may be the links allowing geraniol to inhibit DsbA by blocking activity. Consequently, substitution of these amino acids with others may lead to geraniol becoming inactive. Alanine is the most commonly used amino acid for substitution of the original amino acid because it is neutrally charged and used in alanine-scanning mutagenesis methods (Pal et al., 2005). As a result, the amino acids that interact with geraniol were substituted with alanine by site-directed mutagenesis. The mutagenic primers used for the substitution were listed in Table (2.7). Each mutation was designed in the middle of forward and reverse primers. The pTH102 plasmid was mutated by the site directed mutagenesis (see section 2.3.11), and the new mutated plasmids were also listed in Table (2.2), and all new mutations being confirmed by sequencing of the novel plasmids. Four of five amino acids were successfully substituted (Figure 5.2), and the mutant plasmid was transferred into SSDM. Phenylalanine 174 appeared to be more difficult to be substituted by alanine and it could not be mutated after several attempts. Thus, the work on test the effect of substituted amino acids on geraniol was completed using the four successful mutations already obtained.

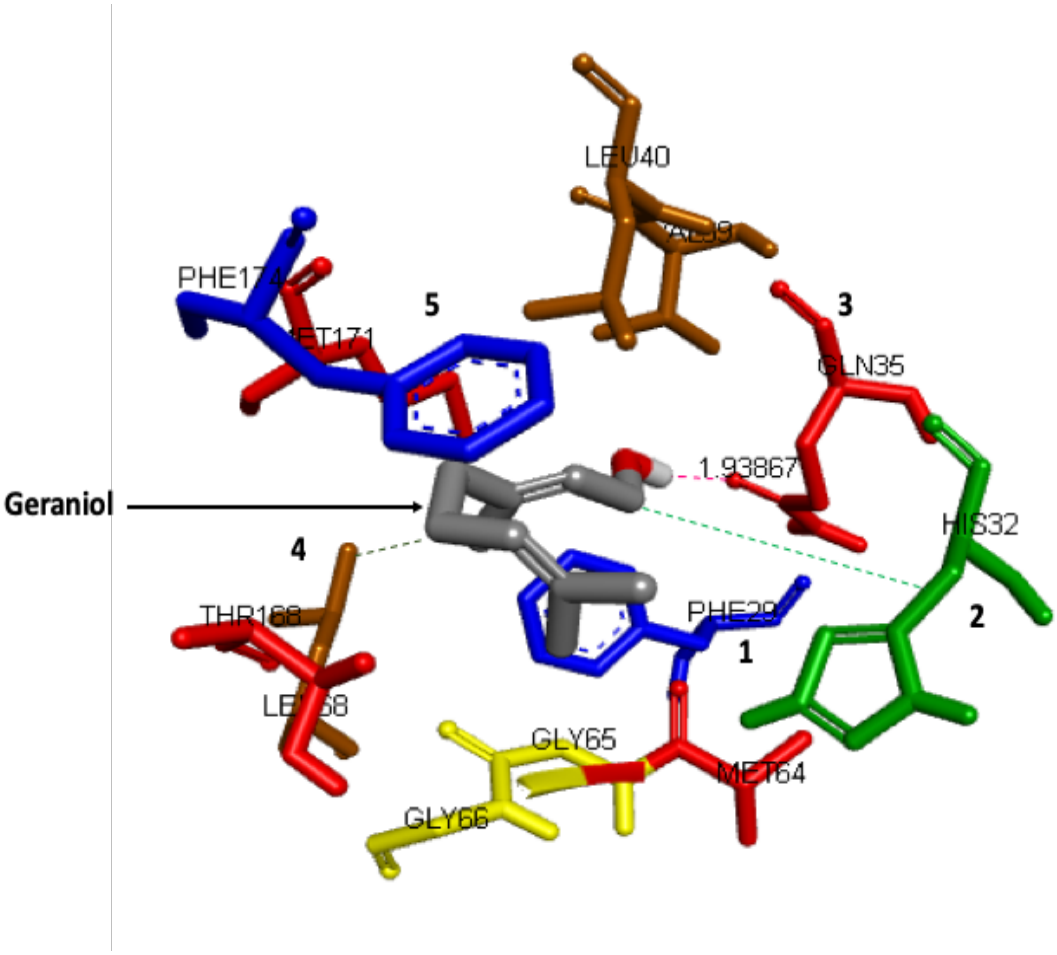



**Figure 5.1: Crystal structure of potential interaction sites between DsbA protein and geraniol.**

**1**- Phenylalanine 29, **2**- Histidine 32, **3**- Glutamine 35, **4**- Leucine 68 and **5**- phenylalanine 174. The software Autodock Vina was used to perform the molecular docking. The software Drug Discovery studio was used to visualise interactions, PDID of the protein was 4WF4. Parameters used were center_x = -6.6506, center_y = 21.2840, center_z = 11.9288, size_x = 22 Angstroms, size_y = 22 Angstroms and size_z = 22 Angstroms. The graph did not show the whole amino acids of protein and focused on the amino acids that located around the active site and have interaction with geraniol. Five amino acids appeared to have possible link with geraniol and three of them centred in and around the active site of the protein (Phenylalanine 29, Histidine 32 and Glutamine 35). Two amino acids (Leucine 68 and Phenylalanine 174) appeared to have connection with geraniol but were far from the active site CXXC motif.

**A**

| Name of protein | Amino acid substitution | Sequence before mutation | Sequence after mutation |
|---|---|---|---|
| DsbA-F29 | Phenylalanine 29 | FSF**F**CPHCYQFEE | FSF**A**CPHCYQFEE |
| DsbA-H32 | Histidine 32 | FSFFCP**H**CYQFEE | FSFFCP**A**CYQFEE |
| DsbA-Q35 | Glutamine 35 | FSFFCPHCY**Q**FEE | FSFFCPHCY**A**FEE |
| DsbA-L68 | Leucine 68 | TKYHVNFMGGD**L**G | TKYHVNFMGGD**A**G |

**B**

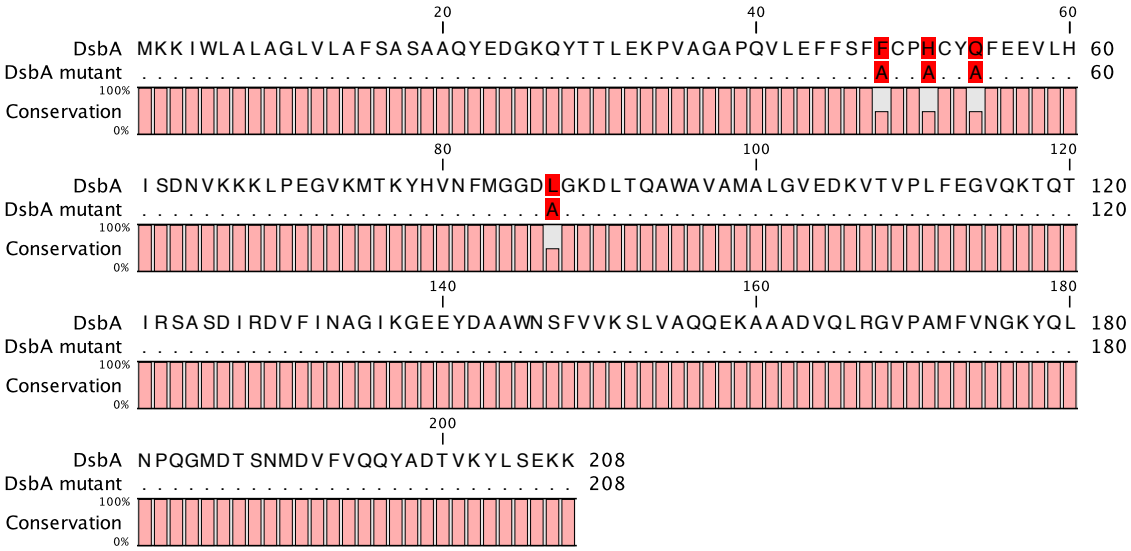



**Figure 5.2: Confirmation of the mutations in DsbA protein.**

**A-** Table of DsbA proteins after mutation**. B-** DsbA sequence alignment after mutation**.**

Each mutation was performed separately on the DsbA gene.

## 5.3 Pathogenicity of *S. sonnei* mutant strains

In order to determine the effect on pathogenicity of *S. sonnei* new mutations and their effect on DsbA activity then a new mutagenic plasmids were transferred into SSDM strains that lacked *dsbA* (Yu, 1998). The mutant strains SSPD-F29, SSPD-H32, SSPD-Q35 and SSPD-L68 were firstly tested with respect to their activity in infection of HeLa cells compared to the wild type strain (SSWT), with all mutations being observed to affect the activity of bacteria (Figure 5.3). The mutant strains reduced their activity by ~50% when compared to the wild type but were still more active than the *dsbA* deletion mutant (SSDM). Notably, geraniol did not show any reduction in the number of intracellular cells of mutant strains that infected HeLa cells in comparison to untreated mutant strains, except SSPD-Q35. Geraniol demonstrated low activity for SSPD-Q35 by reducing the number of intracellular cells compared to untreated SSPD-Q35. Furthermore, geraniol did not inhibit the number of intracellular CFUs in mutant strains when compared to the wild type SSWT, in which geraniol significantly reduced the number of intracellular cells ~2.5 fold. So, these mutations affected the DsbA activity and hence, the activity of *S. sonnei* to generate the infection.

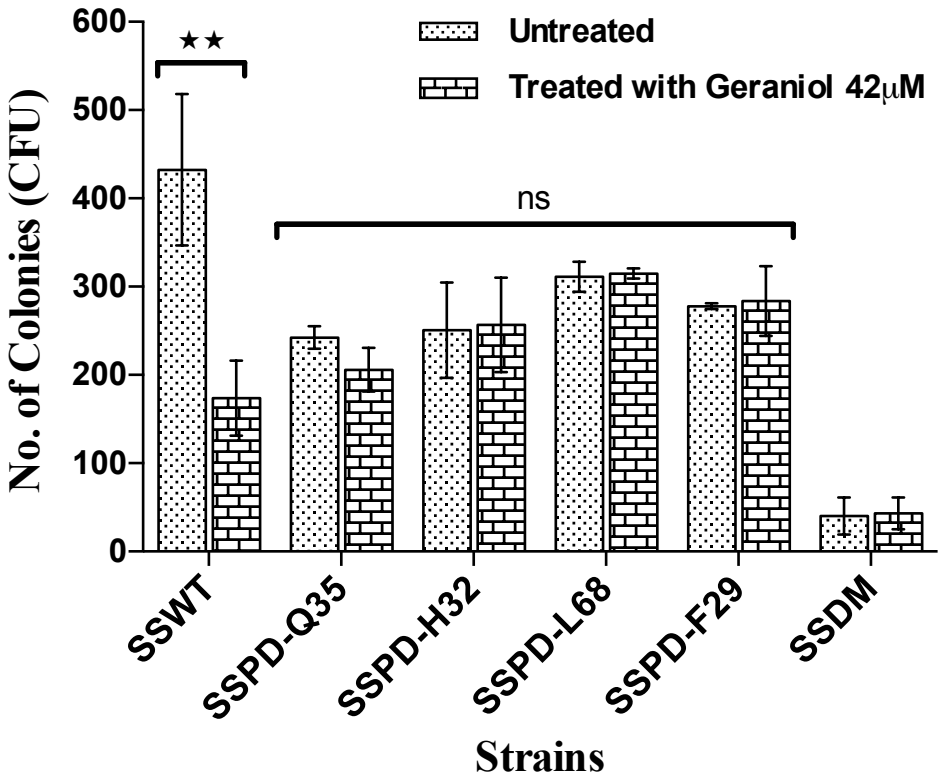



**Figure 5.3: Testing pathogenicity of mutant strains on HeLa cells by gentamicin killing assay.**

The experiment was carried out in triplicate and repeated three times. The results are shown as means ± SD. Differences between groups and control were assessed using a Student's *t* test in GraphPad prism 6. The statistical significance was set at $p < 0.05$.

## 5.4 Purified His-tagged mutant DsbA proteins

The aim of this experiment was to purify the mutant DsbA proteins in order to test the activity of geraniol on these proteins, as we used in an *in vitro* assay for the wild type protein. Purification of DsbA mutant proteins was performed by culturing SSPD-F29, SSPD-H32, SSPD-Q35 and SSPD-L68 which carry the pTH104, pTH105, pTH106 and pTH107 respectively, in LB medium with appropriate antibiotics. The DsbA mutants were tagged with 6XHis to increase the affinity of proteins to bind to the column as the process of purification was carried out using a HisTrap HP histidine-tagged protein purification column (see chapter 2 section 2.3.14). The DsbA mutant proteins were purified successfully from above strains and the SDS PAGE analysis illustrated in Figure (5.4) demonstrated that the mutant proteins were purified and gave the expected sizes. However, protein concentrations differed between DsbA-F29 and DsbA-L68 (Figure 5.4 A and D) and DsbA-H32 and DsbA-Q35 (Figure 5.4 B and C).

To confirm that the proteins obtained from SDS-PAGE were the specified His-tagged proteins, western blotting was performed with His-tag anti-mouse primary antibody. Figure (5.5) shows that all four mutant proteins are conjugated to the primary antibody and confirms the purification of these proteins.

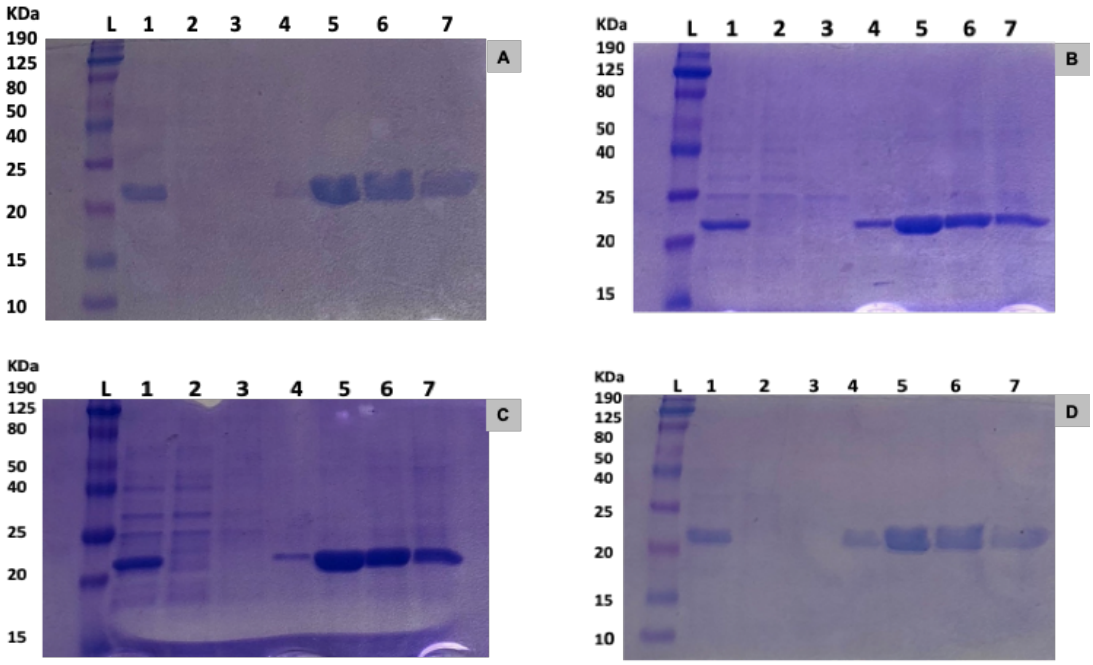



**Figure 5.4: SDS PAGE of purified mutant DsbA proteins.**

**A**- DsbA-F29 mutant protein, **B**- DsbA-H32 mutant protein, **C**- DsbA-Q35 mutant protein and **D**- DsbA-L68 mutant protein. **L**- pre-stained protein ladder (Bioline, UK), **1**- Total periplasmic proteins lysate, **2**- Flow through of total lysate in the column, **3**- Wash solution, **4,5,6** and **7**- Elution of purified proteins. proteins were purified using a HisTrap HP histidine-tagged protein purification column (GE Healthcare, Sweden).

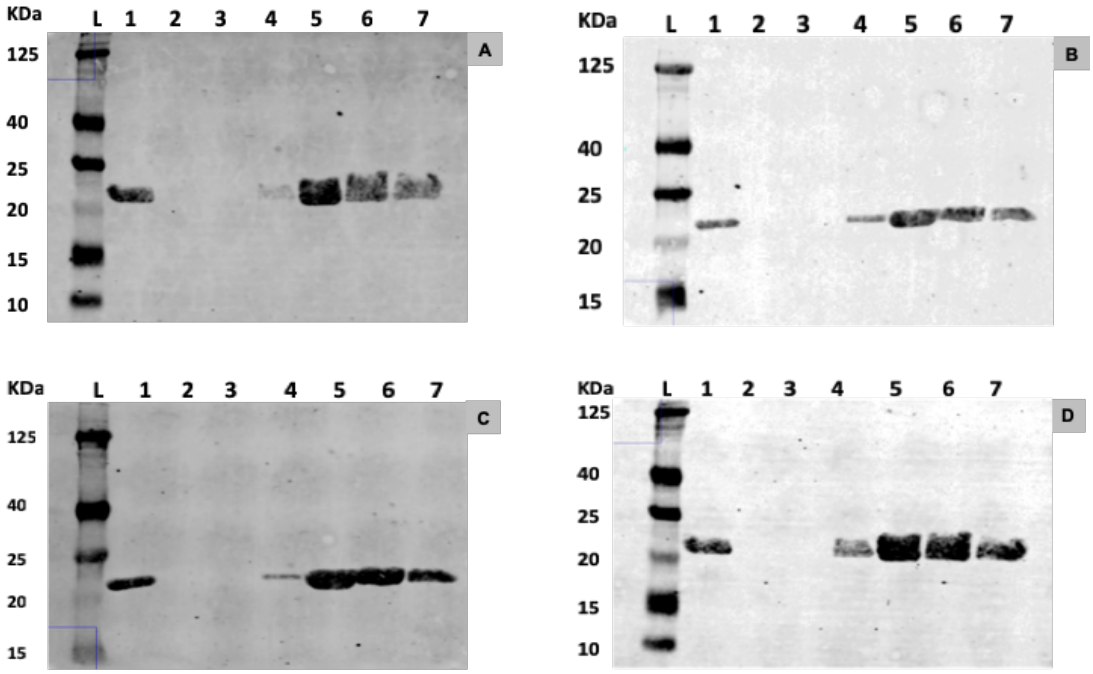



**Figure 5.5: Western blotting of purified His-tag mutant DsbA proteins.**

**A**- DsbA-F29 mutant protein, **B**- DsbA-H32 mutant protein, **C**- DsbA-Q35 mutant protein and **D**- DsbA-L68 mutant protein. **L**- pre-stained protein ladder (Bioline, UK), **1**- Total periplasmic proteins lysate, **2**- Flow through of total lysate in the column, **3**- Wash solution, **4,5,6** and **7**- Elution of purified proteins.

## 5.5 Activity of DsbA mutant proteins in reducing labelled glutathione (Di-E-GSSG)

DsbA wild type protein demonstrated activity in terms of reducing Di-E-GSSG and converting it into E-GSH within 15 min at a concentration of 40 nM (Chapter 4). To determine whether the DsbA mutants still had the same activity after the mutation or the activity was changed, an *in vitro* enzymatic kinetic assay was set up with mutant proteins and the Di-E-GSSG in the DsbA buffer and compared to the DsbA wild type protein. DsbA-F29 and DsbA-Q35 mutant proteins showed activity by reducing the Di-E-GSSG to reach 3000 RFU/s for both, but this result was less than for the wild type, which reached 4500 RFU/s Figure (5.6 A and C). While, both DsbA-H32 and DsbA-L68 were able to reduce the Di-E-GSSG to reach 2800 and 2500 RFU/s compared to DsbA wild type, respectively (Figure 5.6 B and D). In comparison, the activity of DsbA-H32 and DsbA-L68 proteins was less than the activity of DsbA-F29 and DsbA-Q35 protein in the enzymatic assay.

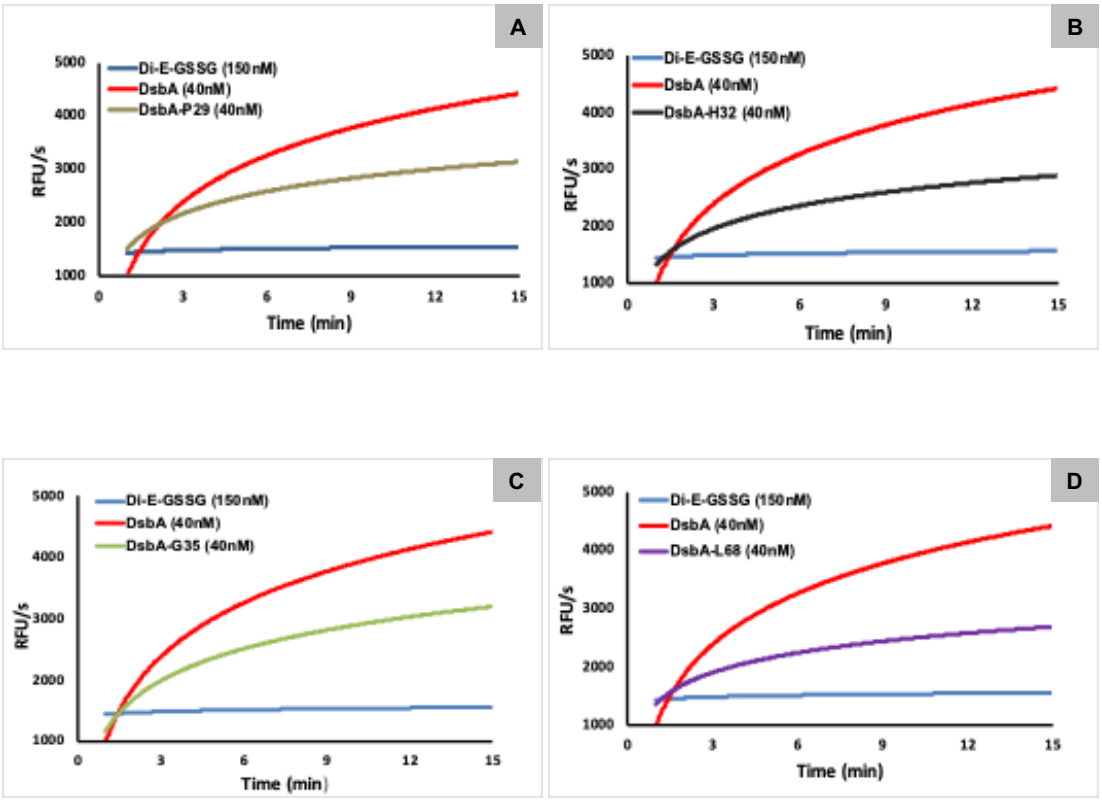



**Figure 5.6: Reduction of Di-E-GSSG by DsbA mutant proteins.**

**A-** DsbA-29**, B-** DsbA-H32**, C-** DsbA-Q35 and **D-** DsbA-L68. The increasing of related fluorescence was plotted against time for all figures. The experiment was carried out in triplicate and repeated three times.

## 5.6 Activity of geraniol in inhibition of reducing Di-E-GSSG by DsbA mutant proteins

To determine the activity of geraniol on the mutant proteins, an enzymatic kinetic assay was performed, and the geraniol was used at 42 μM. Geraniol demonstrated an ability to reduce the activity of wild type DsbA from 4500 RFU/s to 2500 RFU/s (Figure 5.7). In comparison, geraniol showed little or no activity on the mutant proteins. It was able to reduce the formation of E-GSH from 3000 RFU/s to 2700 RFU/s in the DsbA-Q35 mutant protein compared to the other mutant proteins (Figure 5.7 C), while geraniol did not show any inhibition of formation of E-GSH on the proteins of DsbA-F29, DsbA-H32 and DsbA-L68 during this time (Figure 5.7 A, B and D).

To calculate the $V_{max}$ and $K_m$ in terms of enzyme activity, a Michaelis-Menten and Lineweaver plot were carried out using 40 nM of wild type and mutant DsbA proteins incubated at a range of Di-E-GSSG concentrations (50 nM – 5 μM). Theoretical hyperbolic curves allowed the estimation of the $V_{max}$ for the mutant proteins and wild type DsbA (Figure 5.8 I, II, III and IV) and for the mutant proteins in the presence of geraniol (Figure 5.9 I, II, III and IV). The $K_m$ was calculated from the Lineweaver-Burk plot for the wild type and mutant DsbA proteins (Figure 5.10 I, II, III, and IV) and in the presence of geraniol (Figure 5.11 I, II, III and IV). The $V_{max}$ and $K_m$ of the wild type DsbA were significantly decreased in the presence of 42 μM geraniol Table (5.1). However, the $V_{max}$ and $K_m$ of the mutant proteins were also significantly decreased after mutation compared to the wild type DsbA. Interestingly, in the presence of 42 μM of geraniol there is no significant change in $V_{max}$ and $K_m$ (Table 5.1).

**Table 5.1: $V_{max}$ and $K_m$ for the wild type and mutant DsbA proteins**

| No. | Protein | Vmax (nmol/s) | $V_{max}$ + G | $K_m$ (nM) | $K_m$ + G | $K_{cat}$ ($S^{-1}$) | $K_{cat}/K_m$ ($M^{-1}S^{-1}$) |
|---|---|---|---|---|---|---|---|
| 1. | DsbA | 6.3 ± 0.21 | 2.9 ± 0.35 | 255.8 | 132.5 | 0.004 | 19 x10 $^{-4}$ |
| 2. | DsbA-F29 | 3.1 ± 0.08 | 3.1 ± 0.08 | 36.8 | 42.1 | 0.002 | 5 x 10 $^{-5}$ |
| 3. | DsbA-H32 | 2.9 ± 0.12 | 2.8 ± 0.13 | 16.2 | 23.3 | 0.002 | 32 x 10 $^{-3}$ |
| 4. | DsbA-Q35 | 3.2 ± 0.12 | 3.1± 0.14 | 38.5 | 42 | 0.0023 | 5 x 10 $^{-5}$ |
| 5. | DsbA-L68 | 3.4 ± 0.12 | 3.2 ± 0.11 | 52.9 | 51.6 | 0.0024 | 4 x 10 $^{-5}$ |

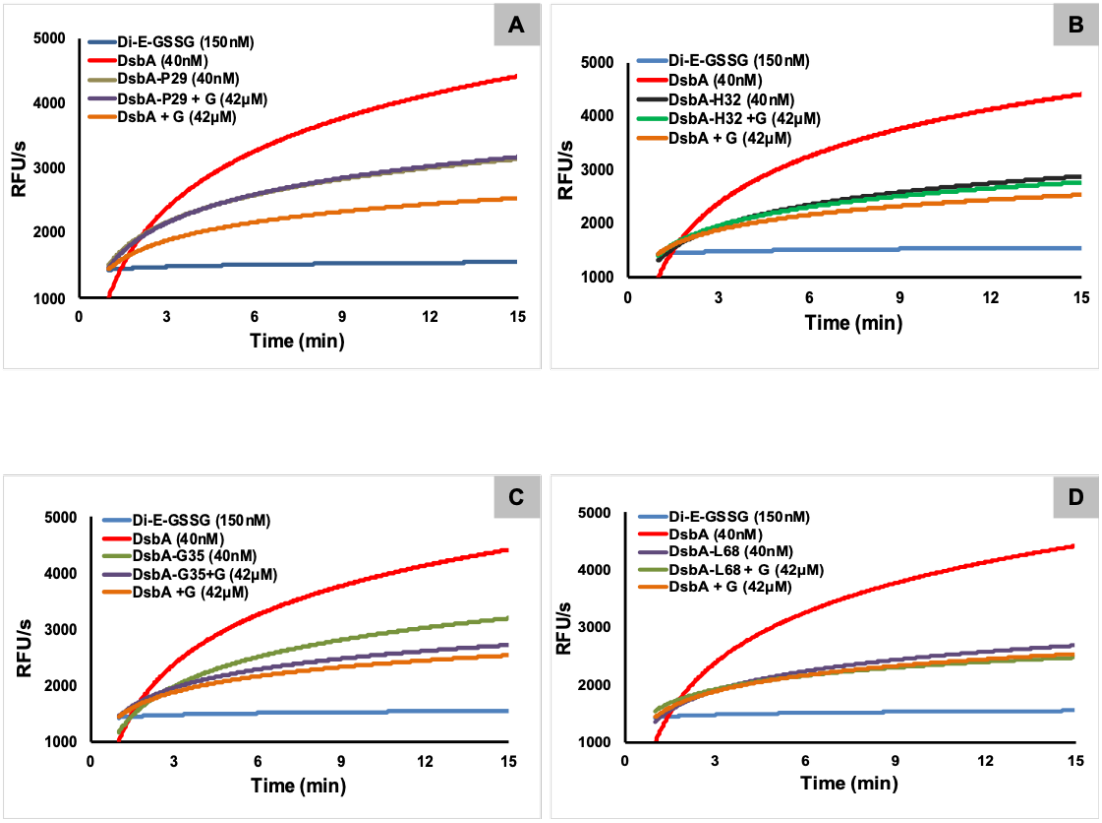



**Figure 5.7: Activity of geraniol in inhibition of reducing Di-E-GSSG by DsbA mutant proteins.**

**A**- Activity of geraniol on DsbA-F29 mutant protein. **B**- Activity of geraniol on DsbA-H32 mutant protein. **C**- Activity of geraniol on DsbA-Q35 mutant protein. **D**- Activity of geraniol on DsbA-L68 mutant protein.

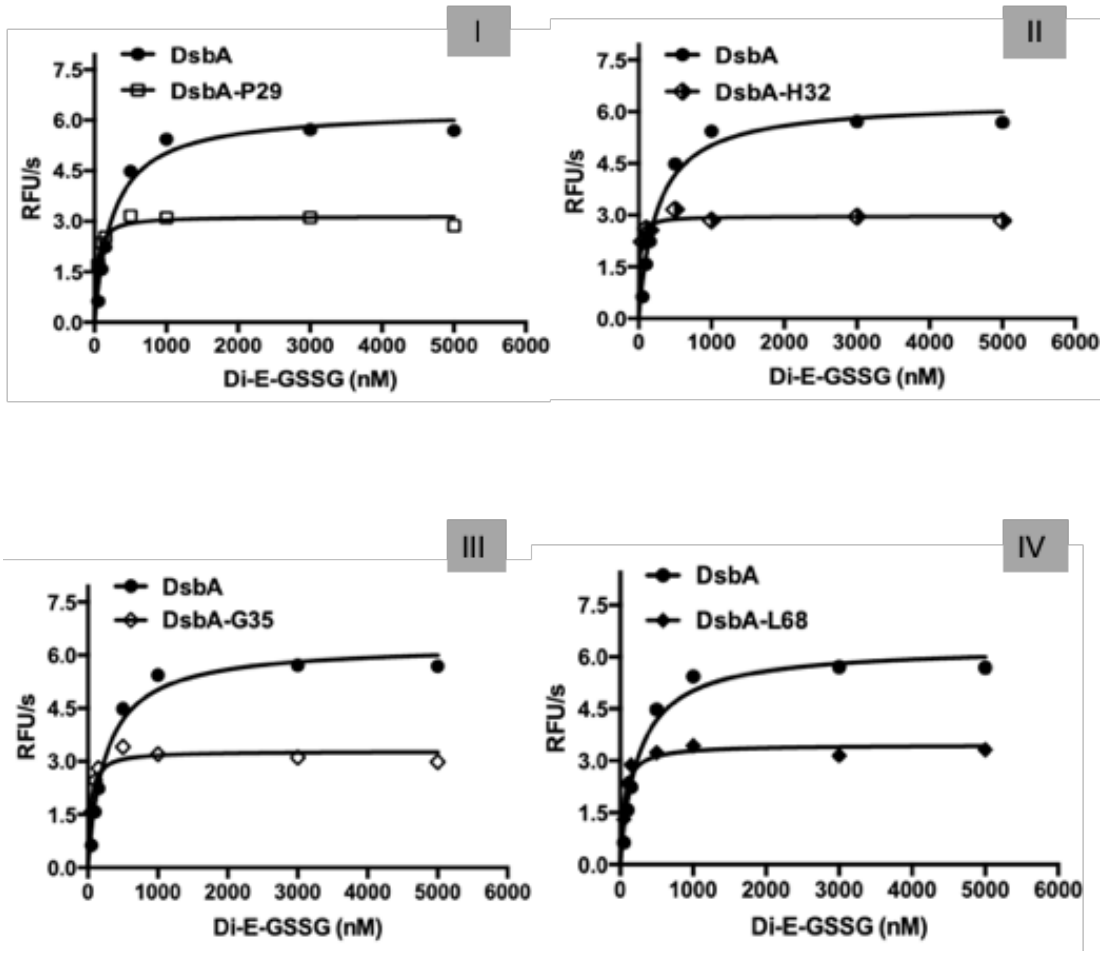



**Figure 5.8: Estimation of $V_{max}$ for DsbA mutant and wild type proteins.**

**I-** Estimation of $V_{max}$ for DsbA-F29 compared to DsbA wild type. **II-** Estimation of $V_{max}$ for DsbA-H32 compared to DsbA wild type. **III-** Estimation of $V_{max}$ for DsbA-Q35 compared to DsbA wild type. **IV-** Estimation of $V_{max}$ for DsbA-L68 compared to DsbA wild type. Theoretical hyperbolic curves allowed the estimation of the $V_{max}$ for wild type DsbA and mutant proteins. The experiment was carried out in triplicate and repeated twice.

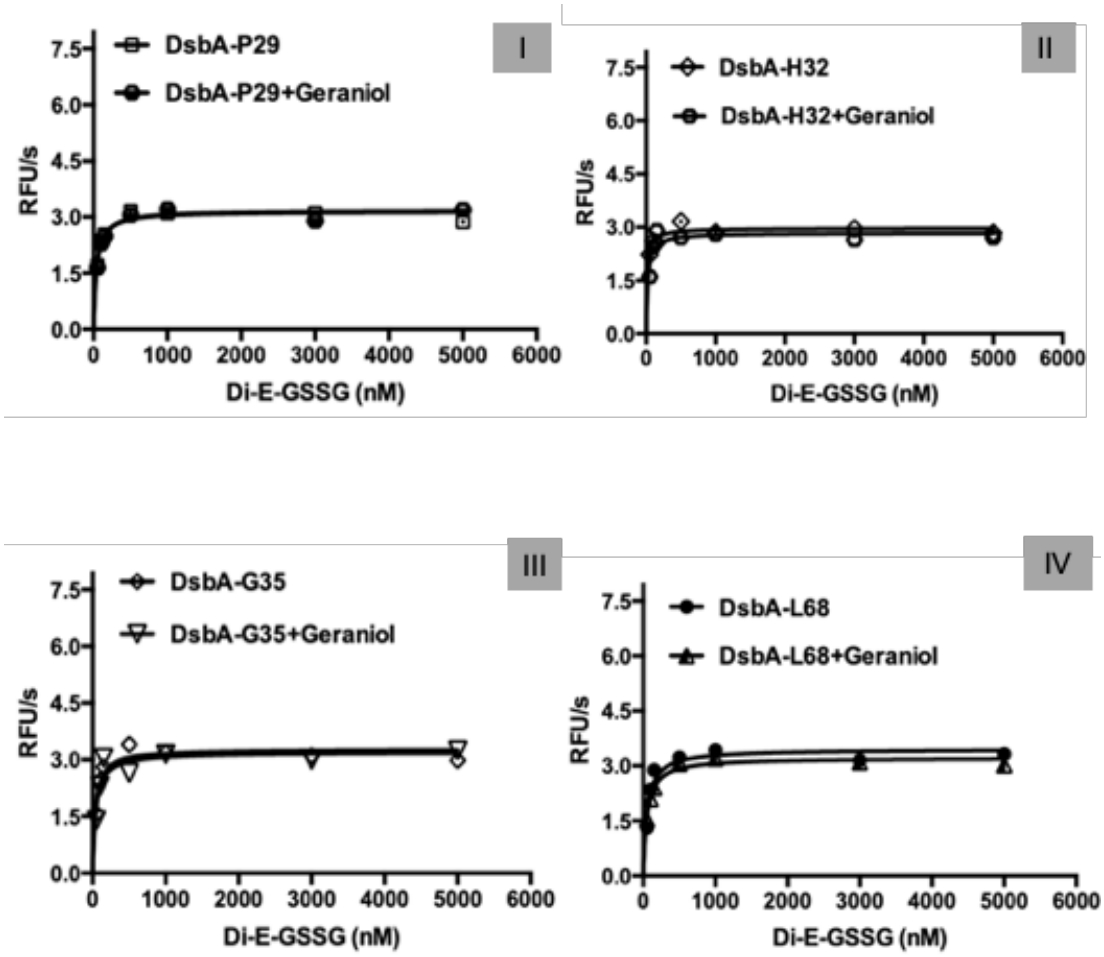



**Figure 5.9: Activity of geraniol in inhibition the estimation of $V_{max}$ for DsbA mutant proteins.**

**I-** Estimation of $V_{max}$ for DsbA-F29 after treated with geraniol. **II-** Estimation of $V_{max}$ for DsbA-H32 after treated with geraniol. **III-** Estimation of $V_{max}$ for DsbA-Q35 after treated with geraniol. I**V-** Estimation of $V_{max}$ for DsbA-L68 after treated with geraniol. Theoretical hyperbolic curves allowed the estimation of the $V_{max}$ for wild type DsbA and mutant proteins. The experiment was carried out in triplicate and repeated twice.

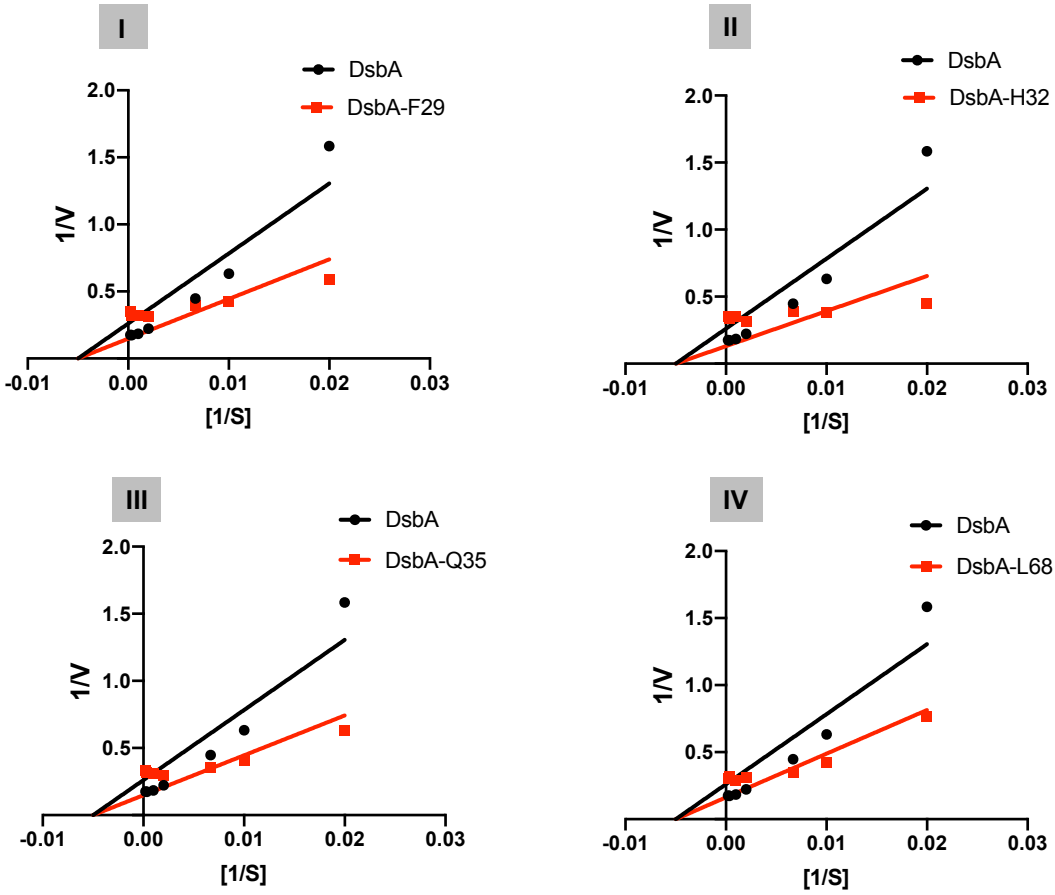



**Figure 5.10: Estimation of $K_m$ for DsbA mutant and wild type proteins.**

**I-** Estimation of $K_m$ for DsbA-F29. **II-** Estimation of $K_m$ for DsbA-H32. **III-** Estimation of $K_m$ for DsbA-Q35. **IV-** Estimation of $K_m$ for DsbA-L68. Lineweaver-Burk plots for DsbA wild type and mutants were calculated from the Michaelis-Menten curve in order to estimate the $K_m$ values. The experiment was carried out in triplicate and repeated twice.

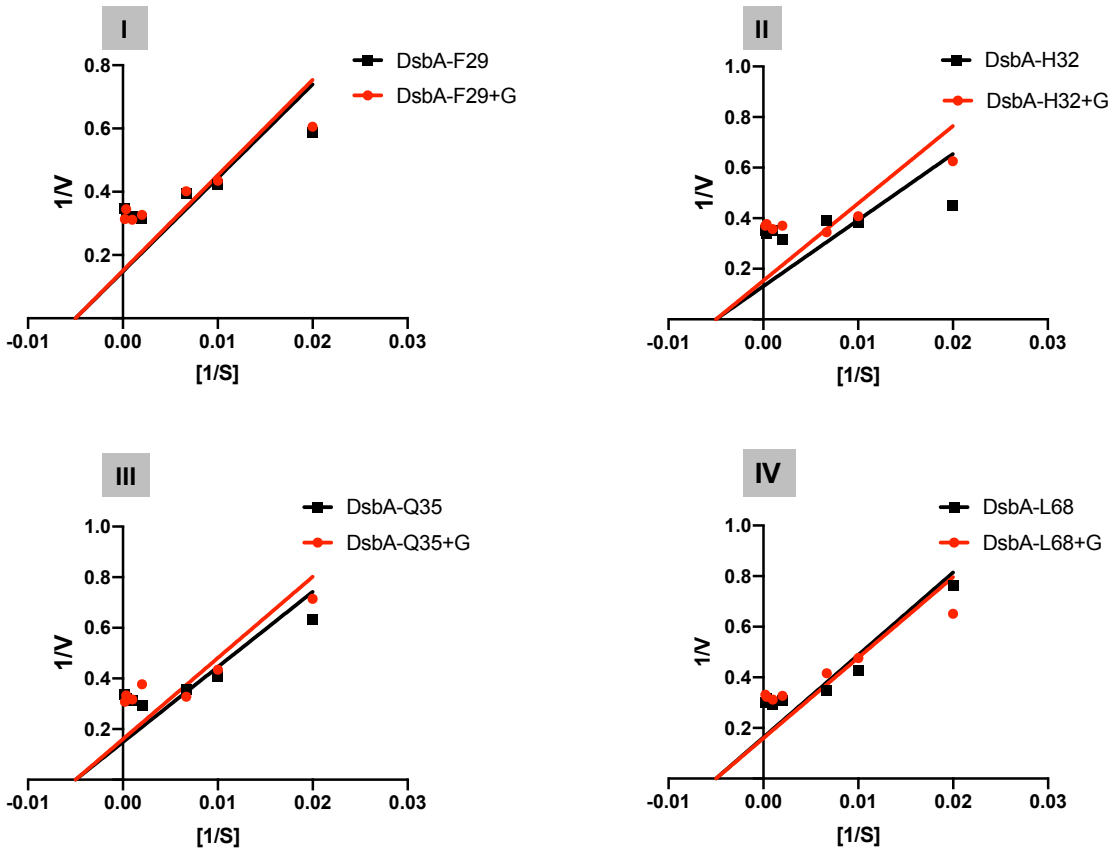



**Figure 5.11: Activity of geraniol in inhibition of estimation of $K_m$ for DsbA mutant and wild type proteins.**

**I-** Estimation of $K_m$ value of DsbA-F29 after treated with geraniol. **II-** Estimation of $K_m$ value of DsbA-H32 after treated with geraniol. **III-** Estimation of $K_m$ value of DsbA-Q35 after treated with geraniol. **IV-** Estimation of $K_m$ value of DsbA-L68 after treated with geraniol. Lineweaver-Burk plots for DsbA wild type and mutants were calculated from the Michaelis-Menten curve in order to estimate the $K_m$ values. The experiment was carried out in triplicate and repeated twice.

## 5.7 Catalysis of labelled reduced glutathione E-GSH (*In vivo*) by mutant strains

SSWT was able to catalyse the labelled E-GSH and convert it into Di-E-GSSG, as was confirmed from previous results in (chapter 4 section 4.8). To confirm this activity of catalysis by the strains of *S. sonnei* carrying the mutant proteins, an *in vivo* assay was performed with the mutant strains, which were grown overnight in LB media in order to reveal whether the strains still had the same activity of DsbA as the wild type strain. After the mutations in DsbA protein by substitution several amino acids section (5.2), the generated strains of SSPD-F29, SSPD-H32, SSPD-Q35 and SSPD-L68 appeared to become weak with respect to the DsbA activity. The result in Figure (5.12) demonstrated that all mutant strains lost their activity in terms of catalysis of the E-GSH at 85%, compared to the wild type in the same figure. However, all mutant strains demonstrated effective growth under mildly reducing conditions.

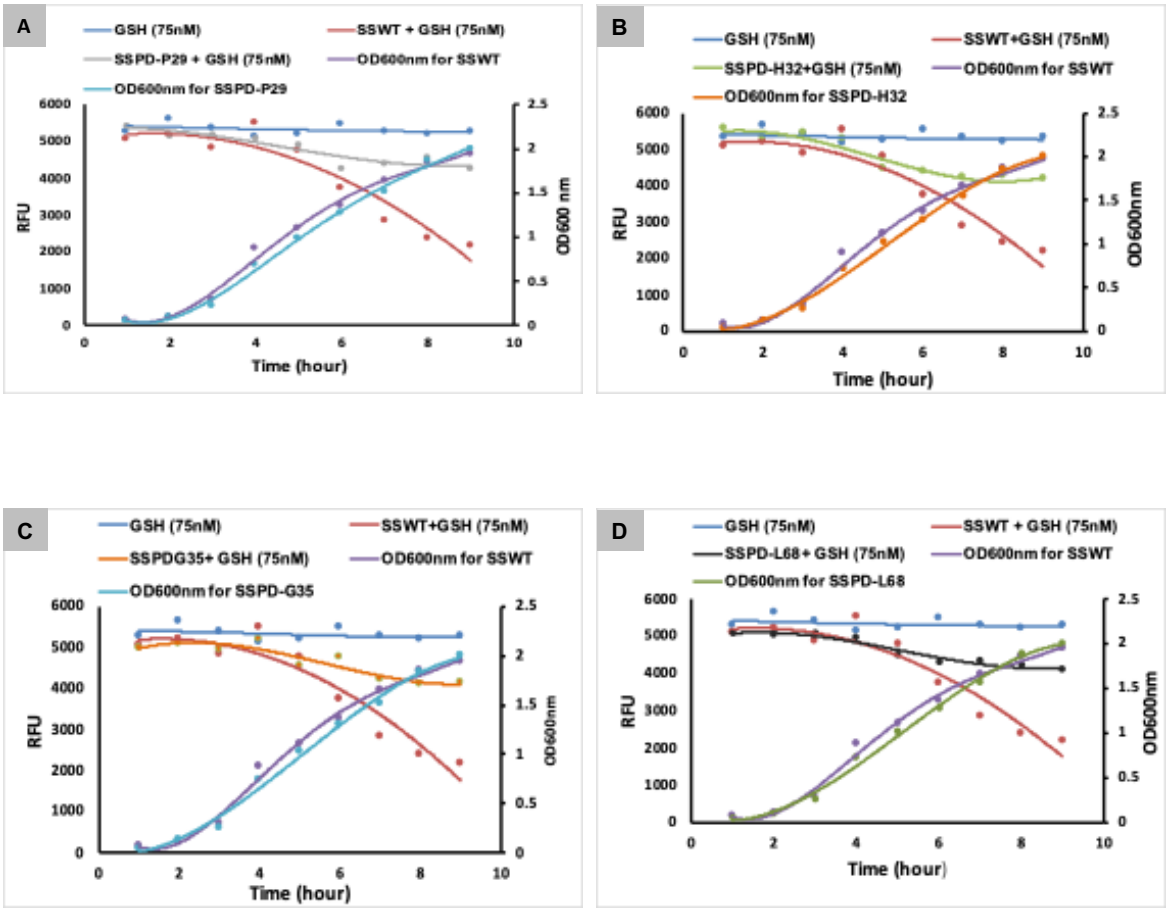



**Figure 5.12: *In vivo* catalysis of E-GSH by growing *S. Sonnei* DsbA mutants.**

**A-** Catalysis of E-GSH by growing SSPD-F29**. B-** Catalysis of E-GSH by growing SSPD-H32**. C-** Catalysis of E-GSH by growing SSPD-Q35**. D-** Catalysis of E-GSH by growing SSPD-L68**.** The experiment was carried out in triplicate and repeated three times.

## 5.8 Activity of geraniol in inhibition of catalysis of labelled E-GSH (*In vivo*) by strains carrying mutant DsbA

Geraniol showed little or no activity on the DsbA mutant proteins *in vitro*. To determine whether geraniol had the same activity as it did *in vitro*, an *in vivo* assay was set up overnight in the LB culture with the mutated DsbA proteins. Geraniol showed little activity with respect to the SSPD-Q35 and it reduced the catalysis from 5000 RFU to 4500 RFU. In comparison to the same strain without treatment, SSPD-Q35 catalysed the E-GSH from 5000 RFU to 4000 RFU (Figure 5.13 C). Furthermore, geraniol did not show any activity on other DsbA mutant strains (i.e. SSPD-F29, SSPD-H32 and SSPD-L68) (Figure 5.13 A, B and D). The results are consistent with the *in vitro* assay (Figure 5.7) that geraniol possesses activity on the DsbA-Q35 mutant protein, while it did not have any effect on the other mutant proteins (Figure 5.7 A, B and D).

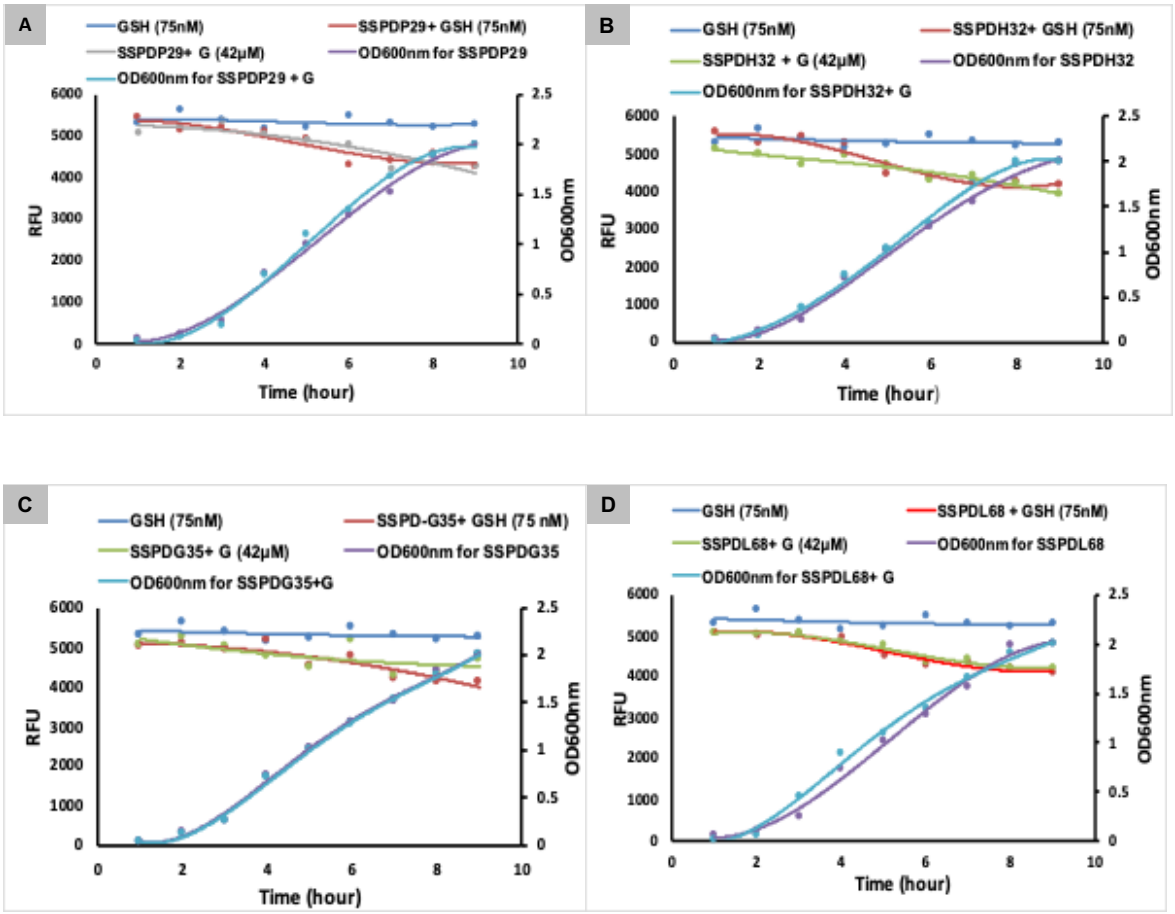



**Figure 5.13: Activity of geraniol in inhibiting catalysis of DsbA mutant strains.**

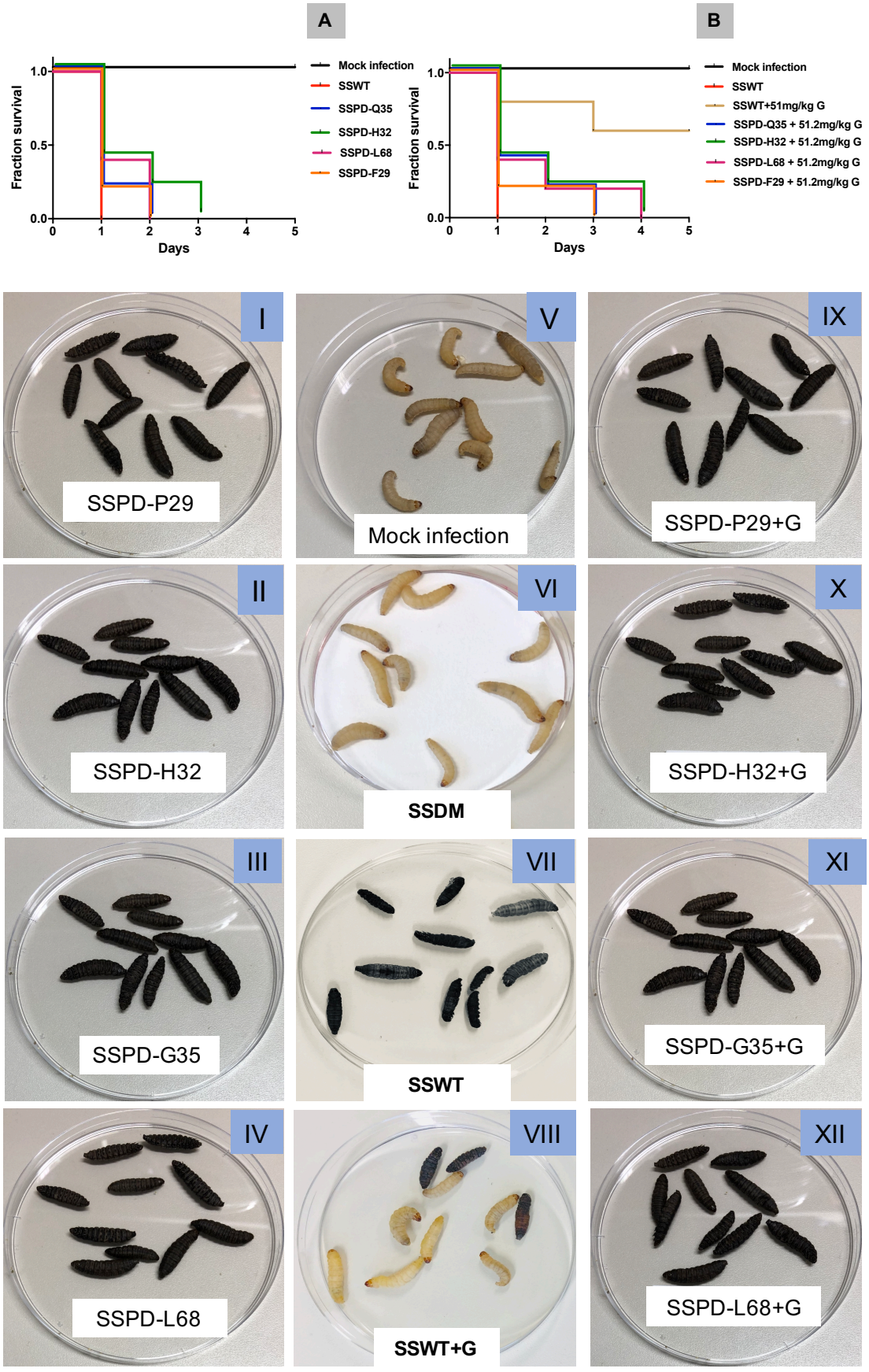
**A-** Activity of geraniol in inhibiting catalysis of SSPD-F29. **B-** Activity of geraniol in inhibiting catalysis of SSPD-H32. **C-** Activity of geraniol in inhibiting catalysis of SSPD-Q35. **D-** Activity of geraniol in inhibiting catalysis of SSPD-L68. The experiment was carried out in triplicate and repeated three times.

## 5.9 Evaluation of pathogenicity of DsbA mutant strains and the role of geraniol in protection of *G. mellonella* larvae

The *dsbA* mutant strains demonstrated an ability to infect HeLa cells; geraniol was able to reduce the number of intracellular CFUs of SSPD-Q35 strains and failed in other mutant strains (Figure 5.3). Therefore, in order to evaluate the pathogenicity of *Shigella dsbA* mutant strains in a larvae model, as well as the potential activity of geraniol inhibit these pathogens and protect larvae. As a result, the ability of *dsbA* mutant strains to infect *Galleria mellonella* larvae was determined. At the same time, the activity of geraniol in terms of protecting larvae from bacterial killing was also investigated. All strains carrying mutant *dsbA* were able to infect the larvae and 50–80% of larvae were died within the first day. The remaining 20–50% of larvae continued to survive only to the second day when they were infected with SSPD-F29, SSPD-Q35 and SSPD-L68, while 20% of larvae which were infected with SSPD-H32 continued to the third day compared to the SSWT, which were able to kill all larvae within one day (Figure 5.14 A).

However, geraniol at a 51.2 mg/kg concentration was able to protect or delay 20% of larvae from being killed by strains carrying mutant *dsbA* for one or two days more. However, the largest percentage (50–80 %) of larvae were killed on the first day compared with (50–80%) of larvae which were protected from being killed by wild type SSWT when treated with geraniol. Twenty percent of larvae continued to survive until the third day when infected with SSPD-Q35 and SSPD-L68 and treated with 51.2 mg/kg geraniol; similarly, 20% of larvae infected with SSPD-F29 and SSPD-H32 continued to live for four days when treated with 51.2 mg/kg geraniol (Figure 5.14 B). Photographs of larvae infected with *Shigella* mutant strains and wild compared to the larvae treated with geraniol appear in Figure (5.14, I - XII).

**Figure 5.14: pathogenicity of *dsbA* mutant strains and protection by geraniol.** **A-** Pathogenicity of *dsbA* mutant strains**. B-** Protection of larvae by geraniol**. I, II, III and IV-** Photographs of larvae infected with *dsbA* mutant strains**. V-** Photograph of mock infection**. VI-** Photograph of DsbA mutant SSDM**. VII-** Photograph of *S. sonnei* wild type SSWT**. VIII, IX, X, XI and XII-** Photographs of larvae treated with geraniol after being infected with SSWT and *dsbA* mutant strains**.** Larval death was indicated by melanisation and loss of mobility. Photographs of the larvae were taken after five days of incubation. Experiments were repeated twice, and pooled data were used to generate the graphs shown as Kaplan–Meier survival curves.


A
B
Fraction survival
Days
Mock infection
SSWT
SSPD-Q35
SSPD-H32
SSPD-L68
SSPD-F29
SSWT+51mg/kg G
SSPD-Q35 + 51.2mg/kg G
SSPD-H32 + 51.2mg/kg G
SSPD-L68 + 51.2mg/kg G
SSPD-F29 + 51.2mg/kg G
I
SSPD-P29
V
Mock infection
IX
SSPD-P29+G
II
SSPD-H32
VI
SSDM
X
SSPD-H32+G
III
SSPD-G35
VII
SSWT
XI
SSPD-G35+G
IV
SSPD-L68
VIII
SSWT+G
XII
SSPD-L68+G

## 5.10 Discussion

In this chapter, I demonstrated that geraniol may be an appropriate candidate to treat shigellosis and has potential specific interaction sites with the DsbA protein. By mutating all of the amino acids that showed a linking bridge with geraniol in the software, geraniol lost its ability to bind the DsbA after this change. The Discovery Studio software showed five possible amino acids in the DsbA protein which have a link with geraniol (Figure 5.1); in practice, however, we succeeded in substituting only four of them with alanine by site-directed mutagenesis of *dsbA*. The four amino acids that were substituted with alanine were phenylalanine 29, histidine 32, glutamine 35 and leucine 68 on the *dsbA* gene. All mutations were confirmed by sequencing using M13 reverse standard primers and alignment with *dsbA* using SnapGene software, USA (Figure 5.2). The amino acid phenylalanine 174 appears in Figure 5.1 with a linking bridge with geraniol which was impossible to substitute with alanine after several attempts by site-directed mutagenesis; this difficulty may be because this amino acid is located on the surface and is significantly more tolerant towards substitution (Pal et al., 2005).

The mutant plasmids were transferred into *S. sonnei* lacking *dsbA* and the new *Shigella* strains were tested for their pathogenicity using HeLa cells and a gentamicin killing assay. All strains showed a reduction in their ability to infect the HeLa cells in comparison to the wild type strain (SSWT) of 50% Figure (5.3). The decreasing mutant pathogenicity is due to the mutation which may decrease the rate of bacterial replication inside the host cells compared to the wild type (Yu et al., 2001). Geraniol was tested for its inhibition of the number of intracellular cells inside the host cells, with geraniol apparently losing activity when one of the interaction sites was substituted. However, altering amino acids in DsbA that have interaction links with

geraniol make the geraniol lose its binding sites on DsbA. One possible explanation would be that all amino acids that interact with geraniol are important and missing one of them makes the geraniol inactive with respect to inhibiting intracellular bacterial cells (Yu et al., 2001). Another explanation may be that DsbA alters its confirmation following the mutation, resulting in an alteration of the amino acid locations on the protein backbone (Kadokura et al., 2004).

To purify the mutant proteins, mutant *dsbA* genes were tagged with 6X Histidine to enable more efficient purification using the HisTrap column. Protein purification after mutation is relatively difficult because one cannot predict whether the protein will fold correctly or whether the mutation may change the protein structure. The successful purification of mutant protein depends on many factors, such as protein environment, structure and function. The protein purification may depend on the origin of the amino acid that is used in the mutation by considering the kind of amino acid to be substituted, its size and charge (Betts and Russell, 2003). Interestingly, the four mutation proteins were purified successfully and confirmed by SDS PAGE (Figure 5.4) and western blotting using His-tag anti-mouse primary antibody (Figure 5.5), as previously performed in (Chim et al., 2013, Tan et al., 2005, Kadokura et al., 2004).

The active site (Cys30-Pro31-His32-Cys33) is highly oxidised and is stable; the proline 31 and histidine 32 are important in terms of maintaining the active site in a highly oxidised form. A mutation in any of these two amino acids may decrease the oxidised form and thus general stability (Grauschopf et al., 1995). However, the mutant proteins of DsbA showed activity with respect to reducing Di-E-GSSG and converting it to E-GSH within 15 min, but this activity was less than for DsbA wild type (50%). A particularly interesting result was that these proteins were folded correctly after mutation and still had the ability to work as oxidoreductase enzymes (Figure 5.6).

Not all mutations will lead to a defective protein, and some are considered as a silent mutations in the gene. Kadokura and his colleagues determined that altering the proline 151 to threonine led to the DsbA being relatively slow with respect to oxidising substrates, allowing researchers to detect the potential substrates of DsbA (Kadokura et al., 2004). Geraniol demonstrated low activity in inhibiting the reduction of Di-E-GSSG on the DsbA-Q35 protein with a glutamine 35 mutation, suggesting that geraniol may still have activity on the DsbA-Q35 protein compared to the DsbA wild type (Figure 5.7 C). At the same time, geraniol lost activity on DsbA-F29, DsbA-H32 and DsbA-L68 mutant proteins, suggesting that all these amino acids are important for geraniol in terms of its function on DsbA (Figure 5.7 A, B and D).

The wild type DsbA protein was able to convert Di-E-GSSG to the more fluorescent E-GSH with an estimated $V_{max}$ of 6.3 $\pm$ 0.21 RFU/s and this result is consistent with (Mirza et al., 2018). Whereas the mutant proteins had a $V_{max}$ of 3.1 $\pm$ 0.08, 2.9 $\pm$ 0.12, 3.2 $\pm$ 0.12 and 3.4 $\pm$ 0.12 RFU/s for DsbA-F29, DsbA-H32, DsbA-Q35 and DsbA-L68, respectively. Importantly, in the presence of 42 $\mu$M geraniol, the wild type DsbA had significantly reduced $V_{max}$ values (2.9 $\pm$ 0.35 RFU/s), while the mutants had no reduction of $V_{max}$ ( 3.1 $\pm$ 0.08, 2.8 $\pm$ 0.13, 3.1$\pm$ 0.14 and 3.2 $\pm$ 0.11RFU/s for DsbA-F29, DsbA-H32, DsbA-Q35 and DsbA-L68, respectively).

The inhibition of DsbA by geraniol was previously demonstrated to be a competitive inhibition of the DsbA-catalysed reduction of Di-E-GSSG (Mirza et al., 2018) and this process was confirmed in our work. The $K_m$ was estimated for the wild type and mutant DsbA proteins and the Km was decreased five times in the presence of 42 $\mu$M of geraniol with wild type protein (Table 5.1). However, the change in the $K_m$ with mutant DsbA protein was not significant in the presence of geraniol, suggesting that these

mutations led to geraniol losing its activity with respect to inhibition of the DsbA enzyme.

DsbA is known as a catalysis enzyme and has a role in *Shigella* survival and proliferation inside host cells (Yu, 1998). In the previous chapter, we demonstrated that *in vivo*, DsbA can catalyse the high fluorescence E-GSH and convert it to low fluorescence Di-E-GSSG, with geraniol being able to decrease the catalysis by DsbA to half. The mutation in the DsbA with the sites that showed potential interactions with geraniol led to the DsbA being attenuated to 85% compared to the wild type (Figure 5.10). However, DsbA mutants had a medium activity in reduction of Di-E-GSSG and formed the E-GSH *in vitro* (Figure 5.6). The *S. sonnei* with DsbA mutants showed a low level of catalysis in the *in vivo* assay compared to the wild type, further confirming the weak activity of the mutant proteins *in vitro*. Furthermore, geraniol was unable to inhibit the catalysis of DsbA mutants in all four mutations and there was no significant change observed in this assay (Figure 5.11). Importantly, larvae injected with *S. sonnei* with DsbA mutants led to 80% of them dying on the first day, while only 20% continued to survive for three days, compared to larvae injected with wild type which began to die from the first day of incubation. As few as 20% of larvae infected with mutant strains continued to survive for four days when treated with 51.2 mg/kg geraniol (Figure 5.12).

In summary, this chapter has demonstrated that all amino acids that interacted with geraniol are important for this compound to be active in inhibition of DsbA protein. Four of five amino acids were successfully substituted by alanine using site-directed mutagenesis. The new mutations were confirmed by sequencing and transferred into *S. sonnei*, showing pathogenicity in infection of HeLa cells; geraniol was observed to be significantly poor in terms of reducing the number of intracellular bacterial cells.

Geraniol was not able to inhibit the reduction of labelled GSSG by mutant DsbA proteins *in vitro* after purification of the latter. Importantly, the $V_{max}$ and $K_m$ did not change after adding the geraniol to the reaction of the reduction of the Di-E-GSSG with mutant proteins compared to the wild type DsbA protein. Furthermore, geraniol was inactive in inhibiting catalysis of labelled GSH in the four mutated strains and was also not able to prevent the larvae from being killed, in comparison with *Galleria mellonella* larvae infected with wild type SSWT. All these results confirmed that these amino acids are important for geraniol to work and missing one of them leads to the geraniol becoming completely inactive.

# Chapter 6

# General discussion and future work

# 6 General discussion and future work

## 6.1 General discussion

Shigellosis, caused by members of the bacterial genus *Shigella*, is a severe and occasionally life threatening diarrheal infection. Worldwide, *Shigella* spp. are the most common cause of acute, bloody diarrhoea (dysentery) and are responsible for a significant proportion of the burden of morbidity and mortality associated with diarrheal disease (Sansonetti et al., 2000). A multi drug resistance of *S. sonnei* is now a huge problem. *S. sonnei* has become more resistance to current drugs that treat dysentery, such as fluoroquinolone and ciprofloxacin (De Lappe et al., 2015, Kim et al., 2015). Since there is no vaccine available against shigellosis, and the rapid development of antimicrobial resistance, it has become urgent to tackle this disease through the identification of new drug targets and agents.

DsbA is a periplasmic disulphide oxidoreductase enzyme, which is responsible for introducing the disulphide bond into secreted effector proteins. DsbA is present in all Gram-negative and some Gram-positive bacteria, including in many pathogens of humans and animals. DsbA catalyses the formation of disulphide bond formation in exported proteins, of which many are virulence factors (Martin et al., 1993, Kadokura et al., 2004). Targeting Dsb proteins for anti-virulence therapy is not new, but previous studies focused on disrupting DsbA-DsbB interactions have yielded little progress. DsbB is required for recycling the non-functional reduced DsbA to its functional oxidised form (Smith et al., 2016). Previous studies revealed that *Shigella* DsbA, is vital in supporting the survival of *S. flexneri* in the highly reducing environment of the host cell cytosol (Yu, 1998).

Terpenoids are natural products derived from plants and studies have demonstrated that terpenoids, especially monoterpenes, have antimicrobial, anti-inflammatory, anti-oxidant, hypotensive, antipruritic and analgesic activity (Kordali et al., 2005, Guimaraes et al., 2013, Bastos et al., 2010). Geraniol is a monoterpene that was used in our study along with eleven other terpenoids. Geraniol also demonstrated an activity against Gram-negative bacteria including multidrug resistant isolates, such as *Enterobacter aerogenes*, *E. coli*, *P. aeruginosa*, by targeting the virulence factors of these pathogens (Lorenzi et al., 2009). It was reported recently that geraniol can inhibit the DsbA protein competitively in the presence of GSSG and convert it to GSH *in vitro* (Mirza et al., 2018).

In this project we have focused on testing selected terpenoids in order to determine their ability to inhibit *Shigella* infection. More specifically, we focused on the disulphide bond protein, DsbA, which is a major contributor to virulence of *S. sonnei*. The activity of terpenoids, and especially geraniol to inhibit DsbA were investigated in both *in vitro* and *in vivo* model. We also studied the way that DsbA is interacted with geraniol by mutating the interaction sites using a site-directed mutagenesis process and studied these mutations *in vitro* and *in vivo* models.

Our initial results showed that geraniol was the best compound amongst the twelve terpenoids tested. It significantly reduced the number of intracellular bacterial cells in both HeLa and RAW macrophage cells. These terpenoids did not show any cytotoxicity towards the cell lines tested or in the larval model. In addition, these compounds have not shown any direct antimicrobial activity to *Shigella* infection even when it used with high concentrations. Terpenoids are known to exhibit antimicrobial activity against a wide range of bacteria and fungi. The antimicrobial activity of some terpenoids is due to their solubility in the phospholipid bilayer of cell membranes

(Kalemba and Kunicka, 2003). It was also reported that the antibacterial activity of monoterpene alcohols including geraniol, linalool, nerol and citronellol are more effective than their antifungal activity (Suppakul et al., 2003). Importantly, some of these compounds and the most potent, geraniol, showed an ability to protect the larvae from killing by SSWT, therefore, This is suggested these terpenoids may be a good agents against *Shigella* infection.

Previous studies revealed that the *Shigella* periplasmic disulphide bond oxidoreductase, DsbA, is vital in supporting the survival of *S. flexneri* in the host cell cytosol (Yu, 1998). The cell cytosol is a highly reducing environment with high concentrations of reduced glutathione (GSH) (Go and Jones, 2008). *Shigella* manipulate this environment by using DsbA to produce folded effector proteins that are involved in virulence and are essential to invade the targeted cells. Our results suggest that DsbA is important for *Shigella* to survive and grow in the reducing environment, which contains a high concentrations of GSH. SSWT grew very well in the presence of 5 mM and 10 mM of GSH, but it needs more time to grow with 20 mM. In comparison, SSDM struggled to grow in the presence of 10 mM of GSH, and it is never reached the peak of growth with 20 mM of GSH. Meanwhile, SSPD grew much better than SSWT in the presence of 20 mM of GSH, prsumably due to the over expression of DsbA in this complemented strain. This result is consistent with the previous finding which reported that *dsbA* mutant of *Shigella* loses its ability to survive and proliferate inside host cells (Yu, 1998). These results directed us to concentrate on DsbA and the activity of terpenoids on it.

After the confirmation of the relationship between DsbA and the way that *Shigella* strains survive in the reducing environment with high concentrations of GSH, we decided to purify DsbA protein for further investigation. The *dsbA* was cloned

successfully into pET26b(+) to produce pTH103 from pTH102. Since DsbA is a periplasmic protein, so it needs to have a signal sequence to allow the protein to move to the periplasmic space. Our *dsbA* already encodes the signal sequence at the N terminal of the gene. Therefore, we needed to have the 6xHis tag at the C terminal. The sequence tool confirmed that the cloning was corrected and the signal sequence was already located at the N terminal and the 6xHis at C terminal followed by stop codon. The expression of genes in *E. coli* is influenced by a range of factors which make the protein expression and purification more difficult. The stability of the plasmid in the host is one of these factors, so that the plasmids are not always stable, especially in the cells grown for many generations in large-scale cultures, so that when a process is scaled up it is important that vector stability be addressed (Rai and Padh, 2001). Importantly, another factor is the location of the protein within the host cell, While *E. coli* proteins are synthesized in the cytoplasm, it is possible to direct a cloned gene product to the cytoplasm, the inner or outer membrane, or the periplasmic space (Baneyx, 1999). Secretion of a cloned gene product to the periplasmic space often allows for higher levels of expression of the foreign protein that might be degraded by proteases in the cytoplasm (Mergulhao et al., 2007). In addition, the signal sequences are important for *E. coli* to recognize and the secreted protein and export it correctly into the periplasmic space (Mergulhao et al., 2007). Excitedly, DsbA was successfully purified and confirmed by SDS PAGE and western blotting and used in the *in vitro* tests. For the DsbA enzymatic assay; the GSSG was labelled successfully with eosin isothiocyanate (EITC) to give the Di-E-GSSG according to the method of (Montano et al., 2014, Raturi and Mutus, 2007). Di-E-GSSG possess a weak fluorescence because the fluorescence self-quenching occurs when eosin molecules are in close proximity which makes the molecule weakly fluorescent. When a reducing agent, such as dithiothreitol (DTT), is added to the solution, reduction of Di-E-GSSG occurs, which separates the two eosin molecules

apart. This abolished FSQ results in a highly fluorescent molecule of E-GSH (Raturi and Mutus, 2007).

DsbA can reduce and catalyse the conversion of oxidised to reduced glutathione *in vitro* and *in vivo*. *In vitro*, the purified DsbA protein showed ability to convert Di-E-GSSG into E-GSH in DsbA buffer within 15 min with a $V_{max}$ of 6.3 nmol/s and $K_m$ of 255.8 $\pm$ 33. There are two major mechanisms for the inhibition of enzymatic activity; competitive and non-competitive. In competitive inhibition, the substrate and inhibitor compete for binding to the same active site, while non-competitive inhibition occurs when the inhibitor binds to another site on the enzyme molecule, resulting in conformational change, with the result that the enzyme can no longer interact with its substrates (Cornish-Bowden, 2015). Interestingly, geraniol showed an activity as a competitive inhibitor by reducing the $V_{max}$ of DsbA from 6.3 nmol/s to 3.0 nmol/s with little of 42 µM. In the same time, the $K_m$ value was decreased dramatically from 255.8 $\pm$ 33 to 132.5 $\pm$ 39 and its clearly appears in the Figure (4.11 A and B).

*In vivo*, *S. sonnei* grown in LB supplemented with fluorescent E-GSH catalysed the conversion of E-GSH to Di-E-GSSG and confirmed the role of DsbA in this catalysis by the SSWT, SSDM and SSPD strains. SSWT was able to convert reduced GSH into the oxidized form GSSG; this catalysis was monitored through reduced of RFU from 5000 RFU to 2000 RFU. To confirm the role of DsbA in this catalysis, a *dsbA* mutant strain (SSDM) was tested for its ability to reduce GSH; it showed no activity and the fluorescence level remains same even with *Shigella* growing very well. For additional confirmation, a complemented strain expressing *dsbA* (SSPD) was used that converted E-GSH even more than the wild type strain; presumably due to the high copy number of *dsbA* in this strain. To the best of our knowledge, this is the first

time that *Shigella* DsbA has been shown to catalase GSH to GSSG, which strongly supports the hypothesis that catalysis of GSH to GSSG in the host cell cytosol is important for *Shigella* to survive, proliferate and establish infection (Yu, 1998). At this point, geraniol was used with the SSWT and SSPD strains to determine their activity in the inhibition of DsbA catalysis. Geraniol reduced the activity of DsbA catalysis by approximately 50% in both the wild type and complemented strains. In addition, geraniol showed no direct activity towards *Shigella sonnei* at a minimum inhibitory concentration (MIC) 500 mg/L. This explains why the bacteria grow well with or without geraniol in the *in vivo* assays. These findings explained for the first time a novel mechanism used by *S. sonnei* in recycling the non-functional, reduced DsbA to its functional oxidised form. Geraniol was the best inhibitor to the DsbA from the list of terpenoids that tested on substrate (glutathione). At this point, we can confirm that terpenoids and especially geraniol hold a promising anti-virulence therapeutic against *Shigella* infection.

Since geraniol was the best terpenoid in reducing the activity of DsbA, we needed further information about DsbA interaction sites with geraniol. Discovery Studio software showed that there are five possible interaction sites where geraniol might bind to DsbA. Four of five amino acids predicted to be situated at these interaction sites were successfully substituted by alanine using site-directed mutagenesis. The amino acid phenylalanine 174 appears in Figure 5.1 with a linking bridge with geraniol which was impossible to substitute with alanine after several attempts by site-directed mutagenesis; this difficulty may be because this amino acid is located on the surface and is significantly more tolerant towards substitution (Pal et al., 2005). These mutations to DsbA generated strains of *S. sonnei* with reduced pathogenicity of HeLa cells. All strains showed a reduction in their ability to infect the HeLa cells in comparison to the wild type strain (SSWT) of 50%. The decreasing mutant

pathogenicity is due to the mutation which may decrease the rate of bacterial replication inside the host cells compared to the wild type (Yu et al., 2001). Geraniol was observed to be significantly poorer in terms of reducing the number of intracellular bacterial cells.

Protein purification after mutation is relatively difficult because one cannot predict whether the protein will fold correctly or whether the mutation may change the protein structure. The successful purification of mutant protein depends on many factors, such as protein environment, structure and function. The protein purification may depend on the origin of the amino acid that is used in the mutation by considering the kind of amino acid to be substituted, its size and charge (Betts and Russell, 2003). Interestingly, the four mutation proteins were purified successfully and confirmed by SDS PAGE (Figure 5.4) and western blotting using His-tag anti-mouse primary antibody (Figure 5.5), as previously performed in (Chim et al., 2013, Tan et al., 2005, Kadokura et al., 2004). The active site (Cys30-Pro31-His32-Cys33) is highly oxidised and is stable; the proline 31 and histidine 32 are important in terms of maintaining the active site in a highly oxidised form. A mutation in any of these two amino acids may decrease the oxidised form and thus general stability (Grauschopf et al., 1995). However, the mutant proteins of DsbA showed activity with respect to reducing Di-E-GSSG and converting it to E-GSH within 15 min, but this activity was less than for DsbA wild type (50%). A particularly interesting result was that these proteins were folded correctly after mutation and still had the ability to work as oxidoreductase enzymes.

Geraniol was not able to inhibit the reduction of labelled Di-E-GSSG by mutant DsbA proteins *in vitro* after purification of the latter. Importantly, the $V_{max}$ and $K_m$ did not change after adding the geraniol to the reaction of the reduction of Di-E-GSSG by

mutant proteins compared to the wild type DsbA protein. Furthermore, the *S. sonnei* with DsbA mutants showed a low level of catalysis in the *in vivo* assay compared to the wild type, further confirming the weak activity of the mutant proteins *in vitro.* Geraniol was inactive in inhibiting catalysis of labelled E-GSH in the four mutated strains and was also not able to prevent larvae from being killed, in comparison with those *Galleria mellonella* larvae infected with SSWT. All these results confirmed that these amino acids are important for geraniol to work and missing one of them leads to the geraniol becoming completely inactive.

In conclusion, this study presents for the first time a way of controlling *S. sonnei* infection via inhibition of DsbA inside host cells. Geraniol was the best terpenoid tested among the twelve terpenes used in this study, and is a promising drug against dysentery. By *in vitro* and *in vivo* studies, geraniol was able to inhibit the activity of DsbA in the reduction and catalysis of GSSG and GSH respectively. Furthermore, this study confirmed that geraniol has specific binding sites on DsbA, and mutating these sites make the geraniol completely inactive.

## 6.2 Future work

This study can be extended in number of ways. Terpenoids are a wide range compounds that can be found in many animal and plant sources. Therefore, searching for new compounds is an interesting approach that may lead to find a compound that display same activity of geraniol or better than it against dysentery. Interestingly, geraniol may also be investigated for activity against other Gram-negative intracellular pathogens that require DsbA to establish infection. DsbA can be an important target for synthesizing drugs, due to its function, and these synthesized drugs can work on the GSH, a substrate to DsbA in the cytosol. In addition, further investigations for geraniol is required to understand the metabolism of geraniol in the *in vivo* models. Furthermore, animal models it would be a great confirmation for the role of geraniol in controlling the number of intracellular bacteria. As guinea pigs are using for confirmation the pathogenicity of Shigella by keratocunjunctivitis assay (Mahmoud et al., 2016), it can use mice models in confirmation of the role of geraniol in inhibition of S. sonnei infection.

Many of Gram-negative bacteria that relies on DsbA to establish their infection can be a good target to be treated by geraniol, such as entero-invasive *E. coli* (EIEC), adherence invasive *E. coli* (AIEC), *Salmonella* spp. and *Vibrio cholera* and many other (Skorko-Glonekv and Sobiecka, 2005, Yu and Kroll, 1999). These bacteria can invade the mammalian cells so it is possible to use the gentamicin killing assay to test the activity of geraniol on the intracellular bacteria using cell lines. Antimicrobial activity, cytotoxicity can also be invistigated for geraniol on these organisms.

# Chapter 7

# References

## 7 References


Aberg, V. & Almqvist, F. (2007). Pilicides-small molecules targeting bacterial virulence. *Org Biomol Chem,* 5**,** 1827-34.

Acharyya, S., Sarkar, P., Saha, D. R., Patra, A., Ramamurthy, T. & Bag, P. K. (2015). Intracellular and membrane-damaging activities of methyl gallate isolated from Terminalia chebula against multidrug-resistant Shigella spp. *J Med Microbiol,* 64**,** 901-9.

Adams, L. A., Sharma, P., Mohanty, B., Ilyichova, O. V., Mulcair, M. D., Williams, M. L., Gleeson, E. C., Totsika, M., Doak, B. C., Caria, S., Rimmer, K., Horne, J., Shouldice, S. R., Vazirani, M., Headey, S. J., Plumb, B. R., Martin, J. L., Heras, B., Simpson, J. S. & Scanlon, M. J. (2015). Application of fragment-based screening to the design of inhibitors of Escherichia coli DsbA. *Angew Chem Int Ed Engl,* 54**,** 2179-84.

Aggarwal, S., Mannam, P. & Zhang, J. (2016). Differential Regulation of Autophagy and Mitophagy in Pulmonary Diseases. *Am J Physiol Lung Cell Mol Physiol***,** ajplung.00128.2016.

Ahmad, A. & Viljoen, A. (2015). The in vitro antimicrobial activity of Cymbopogon essential oil (lemon grass) and its interaction with silver ions. *Phytomedicine,* 22**,** 657-65.

Akiyama, K., Kumamoto, T., Wada, S., Miura, H., Nomura, M., Yokogawa, K. & Miyamoto, K. (2005). Antitumor effect of geranylamine derivatives on human hepatoma. *In Vivo,* 19**,** 173-8.

Allaoui, A., Sansonetti, P. J., Menard, R., Barzu, S., Mounier, J., Phalipon, A. & Parsot, C. (1995). MxiG, a membrane protein required for secretion of Shigella spp. Ipa invasins: involvement in entry into epithelial cells and in intercellular dissemination. *Mol Microbiol,* 17**,** 461-70.

Allaoui, A., Sansonetti, P. J. & Parsot, C. (1992). MxiJ, a lipoprotein involved in secretion of Shigella Ipa invasins, is homologous to YscJ, a secretion factor of the Yersinia Yop proteins. *J Bacteriol,* 174**,** 7661-9.

Andersen, C. L., Matthey-Dupraz, A., Missiakas, D. & Raina, S. (1997). A new Escherichia coli gene, dsbG, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. *Mol Microbiol,* 26**,** 121-32.

Appenzeller-Herzog, C. (2011). Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *J Cell Sci,* 124**,** 847-55.

Arena, E. T., Campbell-Valois, F. X., Tinevez, J. Y., Nigro, G., Sachse, M., Moya-Nilges, M., Nothelfer, K., Marteyn, B., Shorte, S. L. & Sansonetti, P. J. (2015). Bioimage analysis of Shigella infection reveals targeting of colonic crypts. *Proc Natl Acad Sci U S A,* 112**,** E3282-90.

Bahrani, F. K., Johnson, D. E., Robbins, D. & Mobley, H. L. (1991). Proteus mirabilis flagella and MR/P fimbriae: isolation, purification, N-terminal analysis, and serum antibody response following experimental urinary tract infection. *Infection and immunity,* 59**,** 3574-3580.

Baker, M. (2013). Fragment-based lead discovery grows up. *Nat Rev Drug Discov,* 12**,** 5-7.

Bakkali, F., Averbeck, S., Averbeck, D. & Idaomar, M. (2008). Biological effects of essential oils--a review. *Food Chem Toxicol,* 46**,** 446-75.

Balch, W. E., Morimoto, R. I., Dillin, A. & Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science,* 319**,** 916-9.

Baneyx, F. J. C. O. I. B. (1999). Recombinant protein expression in Escherichia coli. 10**,** 411-421.

Bánhegyi, G., Lusini, L., Puskás, F., Rossi, R., Fulceri, R., Braun, L., Mile, V., Di Simplicio, P., Mandl, J. & Benedetti, A. (1999). Preferential Transport of Glutathione versusGlutathione Disulfide in Rat Liver Microsomal Vesicles. *Journal of Biological Chemistry,* 274**,** 12213-12216.

Bardwell, J. C., Lee, J. O., Jander, G., Martin, N., Belin, D. & Beckwith, J. (1993). A pathway for disulfide bond formation in vivo. *Proc Natl Acad Sci U S A,* 90**,** 1038-42.

Bardwell, J. C., Mcgovern, K. & Beckwith, J. (1991). Identification of a protein required for disulfide bond formation in vivo. *Cell,* 67**,** 581-9.

Barnoy, S., Gancz, H., Zhu, Y., Honnold, C. L., Zurawski, D. V. & Venkatesan, M. M. (2017). The Galleria mellonella larvae as an in vivo model for evaluation of Shigella virulence. *Gut Microbes,* 8**,** 335-350.

Bastos, J. F., Moreira, I. J., Ribeiro, T. P., Medeiros, I. A., Antoniolli, A. R., De Sousa, D. P. & Santos, M. R. (2010). Hypotensive and vasorelaxant effects of citronellol, a monoterpene alcohol, in rats. *Basic Clin Pharmacol Toxicol,* 106**,** 331-7.

Berkmen, M., Boyd, D. & Beckwith, J. (2005). The nonconsecutive disulfide bond of Escherichia coli phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. *J Biol Chem,* 280**,** 11387-94.

Berks, B. C., Palmer, T. & Sargent, F. (2005). Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. *Curr Opin Microbiol,* 8**,** 174-81.

Bernardini, M. L., Mounier, J., D'hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. (1989). Identification of icsA, a plasmid locus of Shigella flexneri that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc Natl Acad Sci U S A,* 86**,** 3867-71.

Bessette, P. H., Cotto, J. J., Gilbert, H. F. & Georgiou, G. (1999). In vivo and in vitro function of the Escherichia coli periplasmic cysteine oxidoreductase DsbG. *J Biol Chem,* 274**,** 7784-92.

Betts, M. J. & Russell, R. B. (2003). Amino Acid Properties and Consequences of Substitutions. *In:* BARNES, M. R. & GRAY, I. C. (eds.) *Bioinformatics for Geneticists.* West Sussex, England: John Wiley & Sons, Ltd.

Blocker, A., Gounon, P., Larquet, E., Niebuhr, K., Cabiaux, V., Parsot, C. & Sansonetti, P. (1999). The tripartite type III secreton of Shigella flexneri inserts IpaB and IpaC into host membranes. *J Cell Biol,* 147**,** 683-93.

Blocker, A., Jouihri, N., Larquet, E., Gounon, P., Ebel, F., Parsot, C., Sansonetti, P. & Allaoui, A. (2001). Structure and composition of the Shigella flexneri "needle complex", a part of its type III secreton. *Mol Microbiol,* 39**,** 652-63.

Bouwman, C. W., Kohli, M., Killoran, A., Touchie, G. A., Kadner, R. J. & Martin, N. L. (2003). Characterization of SrgA, a Salmonella enterica serovar Typhimurium virulence plasmid-encoded paralogue of the disulfide oxidoreductase DsbA, essential for biogenesis of plasmid-encoded fimbriae. *Journal of bacteriology,* 185**,** 991-1000.

Bradley, J. S. & Jackson, M. A. (2011). The use of systemic and topical fluoroquinolones. *Pediatrics,* 128**,** e1034-45.

Brandon, L. D., Goehring, N., Janakiraman, A., Yan, A. W., Wu, T., Beckwith, J. & Goldberg, M. B. (2003). IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. *Mol Microbiol,* 50**,** 45-60.

Braun, P., Ockhuijsen, C., Eppens, E., Koster, M., Bitter, W. & Tommassen, J. (2001). Maturation of Pseudomonas aeruginosa Elastase FORMATION OF THE DISULFIDE BONDS. *Journal of Biological Chemistry,* 276**,** 26030-26035.

Breitmaier, E. (2006). *Terpenes: flavors, fragrances, pharmaca, pheromones*, John Wiley & Sons.

Bringer, M.-A., Rolhion, N., Glasser, A.-L. & Darfeuille-Michaud, A. (2007). The oxidoreductase DsbA plays a key role in the ability of the Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 to resist macrophage killing. *Journal of bacteriology,* 189**,** 4860-4871.

Buckley, D. A. (2007). Fragrance ingredient labelling in products on sale in the UK. *British Journal of Dermatology,* 157**,** 295-300.

Burdock, G. A. (2016). *Fenaroli's handbook of flavor ingredients*, CRC press.

Burke, Y. D., Stark, M. J., Roach, S. L., Sen, S. E. & Crowell, P. L. (1997). Inhibition of pancreatic cancer growth by the dietary isoprenoids farnesol and geraniol. *Lipids,* 32**,** 151-6.

Camacho, A. I., Irache, J. M. & Gamazo, C. (2013). Recent progress towards development of a Shigella vaccine. *Expert Review of Vaccines,* 12**,** 43-55.

Cantrell, C. L., Franzblau, S. G. & Fischer, N. H. (2001). Antimycobacterial plant terpenoids. *Planta Med,* 67**,** 685-94.

Cardullo, A. C., Ruszkowski, A. M. & Deleo, V. A. (1989). Allergic contact dermatitis resulting from sensitivity to citrus peel, geraniol, and citral. *Journal of the American Academy of Dermatology,* 21**,** 395-397.

Carnesecchi, S., Schneider, Y., Ceraline, J., Duranton, B., Gosse, F., Seiler, N. & Raul, F. (2001). Geraniol, a component of plant essential oils, inhibits growth and polyamine biosynthesis in human colon cancer cells. *J Pharmacol Exp Ther,* 298**,** 197-200.

Chakravarthi, S., Jessop, C. E. & Bulleid, N. J. (2006a). The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO Rep,* 7**,** 271-5.

Chakravarthi, S., Jessop, C. E. & Bulleid, N. J. (2006b). The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO reports,* 7**,** 271-275.

Chapnik, N., Sherman, H. & Froy, O. (2008). A one-tube site-directed mutagenesis method using PCR and primer extension. *Anal Biochem,* 372**,** 255-7.

Chen, J., Song, J. L., Zhang, S., Wang, Y., Cui, D. F. & Wang, C. C. (1999). Chaperone activity of DsbC. *J Biol Chem,* 274**,** 19601-5.

Chen, W. & Viljoen, A. M. (2010). Geraniol—a review of a commercially important fragrance material. *South African Journal of Botany,* 76**,** 643-651.

Chen, Y., Smith, M. R., Thirumalai, K. & Zychlinsky, A. (1996). A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *Embo j,* 15**,** 3853-60.

Chesney, J. A., Eaton, J. W. & Mahoney, J. R. (1996). Bacterial glutathione: a sacrificial defense against chlorine compounds. *Journal of Bacteriology,* 178**,** 2131-2135.

Chim, N., Harmston, C. A., Guzman, D. J. & Goulding, C. W. (2013). Structural and biochemical characterization of the essential DsbA-like disulfide bond forming protein from Mycobacterium tuberculosis. *BMC Struct Biol,* 13**,** 23.

Clatworthy, A. E., Pierson, E. & Hung, D. T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol,* 3**,** 541-8.

Cornelis, G. R. (2006). The type III secretion injectisome. *Nature Reviews Microbiology,* 4**,** 811.

Cornish-Bowden, A. J. P. I. S. (2015). One hundred years of Michaelis–Menten kinetics. 4**,** 3-9.

Corrado, M., Mariotti, F. R., Trapani, L., Taraborrelli, L., Nazio, F., Cianfanelli, V., Soriano, M. E., Schrepfer, E., Cecconi, F., Scorrano, L. & Campello, S. (2016). Macroautophagy inhibition maintains fragmented mitochondria to foster T cell receptor-dependent apoptosis. *Embo j*.

Crowell, P. L. (1999). Prevention and therapy of cancer by dietary monoterpenes. *J Nutr,* 129**,** 775s-778s.

Cruz-Ramos, H., Cook, G. M., Wu, G., Cleeter, M. W. & Poole, R. K. (2004). Membrane topology and mutational analysis of Escherichia coli CydDC, an ABC-type cysteine exporter required for cytochrome assembly. *Microbiology,* 150**,** 3415-3427.

Cuozzo, J. W. & Kaiser, C. A. (1999). Competition between glutathione and protein thiols for disulphide-bond formation. *Nature cell biology,* 1**,** 130.

Dailey, F. E. & Berg, H. C. (1993a). Mutants in disulfide bond formation that disrupt flagellar assembly in Escherichia coli. *Proceedings of the National Academy of Sciences,* 90**,** 1043-1047.

Dailey, F. E. & Berg, H. C. (1993b). Mutants in disulfide bond formation that disrupt flagellar assembly in Escherichia coli. *Proc Natl Acad Sci U S A,* 90**,** 1043-7.

Dartigalongue, C., Nikaido, H. & Raina, S. (2000). Protein folding in the periplasm in the absence of primary oxidant DsbA: modulation of redox potential in periplasmic space via OmpL porin. *The EMBO journal,* 19**,** 5980-5988.

Davioud-Charvet, E., Delarue, S., Biot, C., Schwobel, B., Boehme, C. C., Mussigbrodt, A., Maes, L., Sergheraert, C., Grellier, P., Schirmer, R. H. & Becker, K. (2001). A prodrug form of a Plasmodium falciparum glutathione reductase inhibitor conjugated with a 4-anilinoquinoline. *J Med Chem,* 44**,** 4268-76.

De Fazio, L., Spisni, E., Cavazza, E., Strillacci, A., Candela, M., Centanni, M., Ricci, C., Rizzello, F., Campieri, M. & Valerii, M. C. (2016). Dietary geraniol by oral or enema administration strongly reduces dysbiosis and systemic inflammation in dextran sulfate sodium-treated mice. *Frontiers in Pharmacology,* 7**,** 38.

De Kraker, M. E., Stewardson, A. J. & Harbarth, S. (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? *PLoS Med,* 13**,** e1002184.

De Lappe, N., O'connor, J., Garvey, P., Mckeown, P. & Cormican, M. (2015). Ciprofloxacin-Resistant Shigella sonnei Associated with Travel to India. *Emerg Infect Dis,* 21**,** 894-6.

Debarbieux, L. & Beckwith, J. (1999). Electron avenue: pathways of disulfide bond formation and isomerization. *Cell,* 99**,** 117-119.

Demain, A. L. & Fang, A. (2000). The natural functions of secondary metabolites. *History of modern biotechnology I.* Springer.

Denoncin, K. & Collet, J. F. (2013). Disulfide bond formation in the bacterial periplasm: major achievements and challenges ahead. *Antioxid Redox Signal,* 19**,** 63-71.

Depuydt, M., Messens, J. & Collet, J. F. (2011). How proteins form disulfide bonds. *Antioxid Redox Signal,* 15**,** 49-66.

Dhindsa, R. S. (1991). Drought Stress, Enzymes of Glutathione Metabolism, Oxidation Injury, and Protein Synthesis in Tortula ruralis. *Plant Physiol,* 95**,** 648-51.

Dorenbos, R., Stein, T., Kabel, J., Bruand, C., Bolhuis, A., Bron, S., Quax, W. J. & Van Dijl, J. M. (2002). Thiol-disulfide oxidoreductases are essential for the

production of the lantibiotic sublancin 168. *Journal of Biological Chemistry,* 277**,** 16682-16688.

Duprez, W., Bachu, P., Stoermer, M. J., Tay, S., Mcmahon, R. M., Fairlie, D. P. & Martin, J. L. (2015). Virtual Screening of Peptide and Peptidomimetic Fragments Targeted to Inhibit Bacterial Dithiol Oxidase DsbA. *PLoS One,* 10**,** e0133805.

Dutton, R. J., Boyd, D., Berkmen, M. & Beckwith, J. (2008a). Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. *Proceedings of the National Academy of Sciences,* 105**,** 11933-11938.

Dutton, R. J., Boyd, D., Berkmen, M. & Beckwith, J. (2008b). Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. *Proc Natl Acad Sci U S A,* 105**,** 11933-8.

Escaich, S. (2010). Novel agents to inhibit microbial virulence and pathogenicity. *Expert Opin Ther Pat,* 20**,** 1401-18.

Fabianek, R. A., Hennecke, H. & Thöny-Meyer, L. (2000). Periplasmic protein thiol: disulfide oxidoreductases of Escherichia coli. *FEMS microbiology reviews,* 24**,** 303-316.

Felise, H. B., Nguyen, H. V., Pfuetzner, R. A., Barry, K. C., Jackson, S. R., Blanc, M. P., Bronstein, P. A., Kline, T. & Miller, S. I. (2008). An inhibitor of gram-negative bacterial virulence protein secretion. *Cell Host Microbe,* 4**,** 325-36.

Feng, Y., He, D., Yao, Z. & Klionsky, D. J. (2014). The machinery of macroautophagy. *Cell Res,* 24**,** 24-41.

Friedman, M., Henika, P. R., Levin, C. E. & Mandrell, R. E. (2004). Antibacterial activities of plant essential oils and their components against Escherichia coli O157:H7 and Salmonella enterica in apple juice. *J Agric Food Chem,* 52**,** 6042-8.

Fronzes, R., Remaut, H. & Waksman, G. (2008). Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. *The EMBO journal,* 27**,** 2271-2280.

Fruh, V., Zhou, Y., Chen, D., Loch, C., Ab, E., Grinkova, Y. N., Verheij, H., Sligar, S. G., Bushweller, J. H. & Siegal, G. (2010). Application of fragment-based drug discovery to membrane proteins: identification of ligands of the integral membrane enzyme DsbB. *Chem Biol,* 17**,** 881-91.

Fuchs, S., De Lorenzo, F. & Anfinsen, C. B. (1967). Studies on the mechanism of the enzymic catalysis of disulfide interchange in proteins. *J Biol Chem,* 242**,** 398-402.

Galán, J. E. & Wolf-Watz, H. (2006). Protein delivery into eukaryotic cells by type III secretion machines. *Nature,* 444**,** 567.

Go, Y. M. & Jones, D. P. (2008). Redox compartmentalization in eukaryotic cells. *Biochim Biophys Acta,* 1780**,** 1273-90.

Goldberg, M. B. & Theriot, J. A. (1995). Shigella flexneri surface protein IcsA is sufficient to direct actin-based motility. *Proc Natl Acad Sci U S A,* 92**,** 6572-6.

Grande, M. J., Lopez, R. L., Abriouel, H., Valdivia, E., Ben Omar, N., Maqueda, M., Martinez-Canamero, M. & Galvez, A. (2007). Treatment of vegetable sauces with enterocin AS-48 alone or in combination with phenolic compounds to inhibit proliferation of Staphylococcus aureus. *J Food Prot,* 70**,** 405-11.

Grauschopf, U., Winther, J. R., Korber, P., Zander, T., Dallinger, P. & Bardwell, J. C. (1995). Why is DsbA such an oxidizing disulfide catalyst? *Cell,* 83**,** 947-55.

Greenberg, J. T. & Demple, B. (1986). Glutathione in Escherichia coli is dispensable for resistance to H2O2 and gamma radiation. *Journal of bacteriology,* 168**,** 1026-1029.

Guddat, L. W., Bardwell, J. C., Zander, T. & Martin, J. L. (1997). The uncharged surface features surrounding the active site of Escherichia coli DsbA are conserved and are implicated in peptide binding. *Protein Sci,* 6**,** 1148-56.

Guimaraes, A. G., Quintans, J. S. & Quintans, L. J., Jr. (2013). Monoterpenes with analgesic activity--a systematic review. *Phytother Res,* 27**,** 1-15.

Gutscher, M., Pauleau, A.-L., Marty, L., Brach, T., Wabnitz, G. H., Samstag, Y., Meyer, A. J. & Dick, T. P. (2008). Real-time imaging of the intracellular glutathione redox potential. *Nature methods,* 5**,** 553.

Hagan, E. C., Hansen, W. H., Fitzhugh, O. G., Jenner, P. M., Jones, W. I., Taylor, J. M., Long, E. L., Nelson, A. A. & Brouwer, J. B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Food Cosmet Toxicol,* 5**,** 141-57.

Hagvall, L., Baron, J. M., Börje, A., Weidolf, L., Merk, H. & Karlberg, A.-T. (2008). Cytochrome P450-mediated activation of the fragrance compound geraniol forms potent contact allergens. *Toxicology and applied pharmacology,* 233**,** 308-313.

Hall, P. A. & Russell, S. E. (2004). The pathobiology of the septin gene family. *J Pathol,* 204**,** 489-505.

Hamid, A., Aiyelaagbe, O. & Usman, L. (2011). Essential oils: its medicinal and pharmacological uses. *Int J Cur Res,* 33**,** 86-98.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol,* 166**,** 557-80.

Harrewijn, P., Van Oosten, A. M. & Piron, P. G. (2012). *Natural terpenoids as messengers: A multidisciplinary study of their production, biological functions and practical applications*, Springer Science & Business Media.

Hartl, F. U., Lecker, S., Schiebel, E., Hendrick, J. P. & Wickner, W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane. *Cell,* 63**,** 269-79.

Heras, B., Kurz, M., Shouldice, S. R. & Martin, J. L. (2007). The name's bond.....disulfide bond. *Curr Opin Struct Biol,* 17**,** 691-8.

Heras, B., Scanlon, M. J. & Martin, J. L. (2015). Targeting virulence not viability in the search for future antibacterials. *Br J Clin Pharmacol,* 79**,** 208-15.

Heras, B., Shouldice, S. R., Totsika, M., Scanlon, M. J., Schembri, M. A. & Martin, J. L. (2009). DSB proteins and bacterial pathogenicity. *Nat Rev Microbiol,* 7**,** 215-25.

Heras, B., Totsika, M., Jarrott, R., Shouldice, S. R., Guncar, G., Achard, M. E., Wells, T. J., Argente, M. P., Mcewan, A. G. & Schembri, M. A. (2010). Structural and functional characterization of three DsbA paralogues from Salmonella enterica serovar typhimurium. *J Biol Chem,* 285**,** 18423-32.

Hesaraki, M., Saadati, M., Honari, H., Olad, G., Heiat, M., Malaei, F. & Ranjbar, R. (2013). Molecular cloning and biologically active production of IpaD N-terminal region. *Biologicals,* 41**,** 269-74.

Hiniker, A. & Bardwell, J. C. (2004). In vivo substrate specificity of periplasmic disulfide oxidoreductases. *J Biol Chem,* 279**,** 12967-73.

Hoober, K. L., Joneja, B., White, H. B. & Thorpe, C. (1996). A sulfhydryl oxidase from chicken egg white. *Journal of Biological Chemistry,* 271**,** 30510-30516.

Hunke, S. & Betton, J. M. J. M. M. (2003). Temperature effect on inclusion body formation and stress response in the periplasm of Escherichia coli. 50**,** 1579-1589.

Hutt, D. M., Powers, E. T. & Balch, W. E. (2009). The proteostasis boundary in misfolding diseases of membrane traffic. *FEBS Lett,* 583**,** 2639-46.

Hwang, C., Sinskey, A. J. & Lodish, H. F. (1992). Oxidized redox state of glutathione in the endoplasmic reticulum. *Science,* 257**,** 1496-502.

Inaba, K., Murakami, S., Suzuki, M., Nakagawa, A., Yamashita, E., Okada, K. & Ito, K. (2006). Crystal structure of the DsbB-DsbA complex reveals a mechanism of disulfide bond generation. *Cell,* 127**,** 789-801.

Ingersoll, M., Groisman, E. A. & Zychlinsky, A. (2002). Pathogenicity islands of Shigella. *Pathogenicity islands and the evolution of pathogenic microbes.* Springer.

Inouye, S., Takizawa, T. & Yamaguchi, H. (2001). Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *J Antimicrob Chemother,* 47**,** 565-73.

Ireland, P. M., Mcmahon, R. M., Marshall, L. E., Halili, M., Furlong, E., Tay, S., Martin, J. L. & Sarkar-Tyson, M. (2014). Disarming Burkholderia pseudomallei: structural and functional characterization of a disulfide oxidoreductase (DsbA) required for virulence in vivo. *Antioxid Redox Signal,* 20**,** 606-17.

Ishidate Jr, M., Sofuni, T., Yoshikawa, K., Hayashi, M., Nohmi, T., Sawada, M. & Matsuoka, A. (1984). Primary mutagenicity screening of food additives currently used in Japan. *Food and chemical toxicology,* 22**,** 623-636.

Islam, D., Bandholtz, L., Nilsson, J., Wigzell, H., Christensson, B., Agerberth, B. & Gudmundsson, G. H. (2001). Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. *Nature medicine,* 7**,** 180.

Islam, D., Veress, B., Bardhan, P. K., Lindberg, A. A. & Christensson, B. (1997). In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. *Infect Immun,* 65**,** 739-49.

Jackson, M. W. & Plano, G. V. (1999). DsbA is required for stable expression of outer membrane protein YscC and for efficient Yop secretion in Yersinia pestis. *J Bacteriol,* 181**,** 5126-30.

Jacob-Dubuisson, F., Pinkner, J., Xu, Z., Striker, R., Padmanhaban, A. & Hultgren, S. J. (1994). PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA. *Proceedings of the National Academy of Sciences,* 91**,** 11552-11556.

Jang, K. H., Chung, S. C., Shin, J., Lee, S. H., Kim, T. I., Lee, H. S. & Oh, K. B. (2007). Aaptamines as sortase A inhibitors from the tropical sponge Aaptos aaptos. *Bioorg Med Chem Lett,* 17**,** 5366-9.

Jennison, A. V. & Verma, N. K. (2004). Shigella flexneri infection: pathogenesis and vaccine development. *FEMS Microbiol Rev,* 28**,** 43-58.

Jessop, C. E. & Bulleid, N. J. (2004). Glutathione directly reduces an oxidoreductase in the endoplasmic reticulum of mammalian cells. *Journal of Biological Chemistry,* 279**,** 55341-55347.

Kadokura, H. & Beckwith, J. (2002). Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA. *Embo j,* 21**,** 2354-63.

Kadokura, H., Tian, H., Zander, T., Bardwell, J. C. & Beckwith, J. (2004). Snapshots of DsbA in action: detection of proteins in the process of oxidative folding. *Science,* 303**,** 534-7.

Kalemba, D. & Kunicka, A. (2003). Antibacterial and antifungal properties of essential oils. *Curr Med Chem,* 10**,** 813-29.

Kamitani, S., Akiyama, Y. & Ito, K. (1992). Identification and characterization of an Escherichia coli gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. *Embo j,* 11**,** 57-62.

Katzen, F. & Beckwith, J. (2000). Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade. *Cell,* 103**,** 769-79.

Khan, A. Q., Khan, R., Qamar, W., Lateef, A., Rehman, M. U., Tahir, M., Ali, F., Hamiza, O. O., Hasan, S. K. & Sultana, S. (2013). Geraniol attenuates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative stress and inflammation in mouse skin: possible role of p38 MAP Kinase and NF-kappaB. *Exp Mol Pathol,* 94**,** 419-29.

Kim, J. S., Kim, J. J., Kim, S. J., Jeon, S. E., Seo, K. Y., Choi, J. K., Kim, N. O., Hong, S., Chung, G. T., Yoo, C. K., Kim, Y. T., Cheun, H. I., Bae, G. R., Yeo, Y. H., Ha, G. J., Choi, M. S., Kang, S. J. & Kim, J. (2015). Outbreak of Ciprofloxacin-Resistant Shigella sonnei Associated with Travel to Vietnam, Republic of Korea. *Emerg Infect Dis,* 21**,** 1247-50.

Kishigami, S., Kanaya, E., Kikuchi, M. & Ito, K. (1995). DsbA-DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. *J Biol Chem,* 270**,** 17072-4.

Kobayashi, T. & Ito, K. (1999). Respiratory chain strongly oxidizes the CXXC motif of DsbB in the Escherichia coli disulfide bond formation pathway. *The EMBO Journal,* 18**,** 1192-1198.

Kogan, I., Ramjeesingh, M., Li, C., Kidd, J. F., Wang, Y., Leslie, E. M., Cole, S. P. C. & Bear, C. E. (2003). CFTR directly mediates nucleotide-regulated glutathione flux. *The EMBO journal,* 22**,** 1981-1989.

Kordali, S., Kotan, R., Mavi, A., Cakir, A., Ala, A. & Yildirim, A. (2005). Determination of the chemical composition and antioxidant activity of the essential oil of Artemisia dracunculus and of the antifungal and antibacterial activities of Turkish Artemisia absinthium, A. dracunculus, Artemisia santonicum, and Artemisia spicigera essential oils. *J Agric Food Chem,* 53**,** 9452-8.

Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., Adak, G. K. & Levine, M. M. (1999). Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull World Health Organ,* 77**,** 651-66.

Kouwen, T. R., Van Der Goot, A., Dorenbos, R., Winter, T., Antelmann, H., Plaisier, M. C., Quax, W. J., Van Dijl, J. M. & Dubois, J. Y. (2007). Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. *Mol Microbiol,* 64**,** 984-99.

Kozyreva, V. K., Jospin, G., Greninger, A. L., Watt, J. P., Eisen, J. A. & Chaturvedi, V. (2016). Recent Outbreaks of Shigellosis in California Caused by Two Distinct Populations of Shigella sonnei with either Increased Virulence or Fluoroquinolone Resistance. *mSphere,* 1.

Kurth, F., Duprez, W., Premkumar, L., Schembri, M. A., Fairlie, D. P. & Martin, J. L. (2014). Crystal structure of the dithiol oxidase DsbA enzyme from proteus mirabilis bound non-covalently to an active site peptide ligand. *J Biol Chem,* 289**,** 19810-22.

Lamarche, M. J., Leeds, J. A., Amaral, A., Brewer, J. T., Bushell, S. M., Deng, G., Dewhurst, J. M., Ding, J., Dzink-Fox, J., Gamber, G., Jain, A., Lee, K., Lee, L., Lister, T., Mckenney, D., Mullin, S., Osborne, C., Palestrant, D., Patane, M. A., Rann, E. M., Sachdeva, M., Shao, J., Tiamfook, S., Trzasko, A.,

Whitehead, L., Yifru, A., Yu, D., Yan, W. & Zhu, Q. (2012). Discovery of LFF571: an investigational agent for Clostridium difficile infection. *J Med Chem,* 55**,** 2376-87.

Lan, R., Lumb, B., Ryan, D. & Reeves, P. R. (2001). Molecular evolution of large virulence plasmid in Shigella clones and enteroinvasive Escherichia coli. *Infect Immun,* 69**,** 6303-9.

Lapczynski, A., Bhatia, S. P., Foxenberg, R. J., Letizia, C. S. & Api, A. M. (2008). Fragrance material review on geraniol. *Food and Chemical Toxicology,* 46**,** S160-S170.

Larentis, A. L., Nicolau, J. F., Esteves Gdos, S., Vareschini, D. T., De Almeida, F. V., Dos Reis, M. G., Galler, R. & Medeiros, M. A. (2014). Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in E. coli using shaking flasks and microbioreactor. *BMC Res Notes,* 7**,** 671.

Łasica, A. M. & Jagusztyn-Krynicka, E. K. (2007). The role of Dsb proteins of Gram-negative bacteria in the process of pathogenesis. *FEMS microbiology reviews,* 31**,** 626-636.

Lee, J. H., Park, H. & Park, Y. H. (2014). Molecular mechanisms of host cytoskeletal rearrangements by Shigella invasins. *Int J Mol Sci,* 15**,** 18253-66.

Levine, M. M., Kotloff, K. L., Barry, E. M., Pasetti, M. F. & Sztein, M. B. (2007). Clinical trials of Shigella vaccines: two steps forward and one step back on a long, hard road. *Nat Rev Microbiol,* 5**,** 540-53.

Li, X., Qiu, Y., Shen, Y., Ding, C., Liu, P., Zhou, J. & Ma, Z. (2008). Splicing together different regions of a gene by modified polymerase chain reaction-based site-directed mutagenesis. *Anal Biochem,* 373**,** 398-400.

Li, Z.-S., Lu, Y.-P., Zhen, R.-G., Szczypka, M., Thiele, D. J. & Rea, P. A. (1997). A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis (glutathionato) cadmium. *Proceedings of the National Academy of Sciences,* 94**,** 42-47.

Lin, L. & Baehrecke, E. H. (2015). Autophagy, cell death, and cancer. *Mol Cell Oncol,* 2**,** e985913.

Liu, H. & Naismith, J. H. (2008). An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnol,* 8**,** 91.

Lopes, M. V., Desoti, V. C., Caleare Ade, O., Ueda-Nakamura, T., Silva, S. O. & Nakamura, C. V. (2012). Mitochondria Superoxide Anion Production Contributes to Geranylgeraniol-Induced Death in Leishmania amazonensis. *Evid Based Complement Alternat Med,* 2012**,** 298320.

Lorenzi, V., Muselli, A., Bernardini, A. F., Berti, L., Pages, J. M., Amaral, L. & Bolla, J. M. (2009). Geraniol restores antibiotic activities against multidrug-resistant isolates from gram-negative species. *Antimicrob Agents Chemother,* 53**,** 2209-11.

Luirink, J. & Sinning, I. (2004). SRP-mediated protein targeting: structure and function revisited. *Biochim Biophys Acta,* 1694**,** 17-35.

Luzio, J. P., Pryor, P. R. & Bright, N. A. (2007). Lysosomes: fusion and function. *Nat Rev Mol Cell Biol,* 8**,** 622-32.

Mahmoud, R. Y., Stones, D. H., Li, W., Emara, M., El-Domany, R. A., Wang, D., Wang, Y., Krachler, A. M. & Yu, J. (2016). The Multivalent Adhesion Molecule SSO1327 plays a key role in Shigella sonnei pathogenesis. *Mol Microbiol,* 99**,** 658-73.

Man, A. L., Prieto-Garcia, M. E. & Nicoletti, C. (2004). Improving M cell mediated transport across mucosal barriers: do certain bacteria hold the keys? *Immunology,* 113**,** 15-22.

Mandic-Mulec, I., Weiss, J. & Zychlinsky, A. (1997). Shigella flexneri is trapped in polymorphonuclear leukocyte vacuoles and efficiently killed. *Infect Immun,* 65**,** 110-5.

Mariano, G., Monlezun, L. & Coulthurst, S. J. (2018). Dual role for DsbA in attacking and targeted bacterial cells during Type VI secretion system-mediated competition. *Cell reports,* 22**,** 774-785.

Martin, J. L., Bardwell, J. C. & Kuriyan, J. (1993). Crystal structure of the DsbA protein required for disulphide bond formation in vivo. *Nature,* 365**,** 464-8.

Mattock, E. & Blocker, A. J. (2017). How Do the Virulence Factors of Shigella Work Together to Cause Disease? *Front Cell Infect Microbiol,* 7**,** 64.

Mcgarvey, D. J. & Croteau, R. (1995). Terpenoid metabolism. *Plant Cell,* 7**,** 1015-26.

Mchugh, R. E., O'boyle, N., Connolly, J. P. R., Hoskisson, P. A. & Roe, A. J. (2019). Characterization of the mode of action of Aurodox, a type III secretion system inhibitor from Streptomyces goldiniensis. *Infection and immunity,* 87**,** e00595-18.

Meister, A. & Anderson, M. E. (1983). Glutathione. *Annual review of biochemistry,* 52**,** 711-760.

Menard, R., Sansonetti, P. & Parsot, C. (1994). The secretion of the Shigella flexneri Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *Embo j,* 13**,** 5293-302.

Mergulhao, F. J., Monteiro, G. a. J. J. O. M. & Biotechnology (2007). Analysis of factors affecting the periplasmic production of recombinant proteins in Escherichia coli. 17**,** 1236.

Messens, J. & Collet, J. F. (2006). Pathways of disulfide bond formation in Escherichia coli. *Int J Biochem Cell Biol,* 38**,** 1050-62.

Miki, T., Okada, N. & Danbara, H. (2004). Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the Salmonella pathogenicity island 2 type III secretion system. *J Biol Chem,* 279**,** 34631-42.

Mirza, Z., Hasan, T., Seidel, V. & Yu, J. (2018). Geraniol as a novel antivirulence agent against bacillary dysentery-causing Shigella sonnei. *Virulence,* 9**,** 450-455.

Missiakas, D., Schwager, F. & Raina, S. (1995). Identification and characterization of a new disulfide isomerase-like protein (DsbD) in Escherichia coli. *Embo j,* 14**,** 3415-24.

Mogensen, J. E. & Otzen, D. E. (2005). Interactions between folding factors and bacterial outer membrane proteins. *Mol Microbiol,* 57**,** 326-46.

Molteni, S. N., Fassio, A., Ciriolo, M. R., Filomeni, G., Pasqualetto, E., Fagioli, C. & Sitia, R. (2004). Glutathione limits Ero1-dependent oxidation in the endoplasmic reticulum. *Journal of Biological Chemistry,* 279**,** 32667-32673.

Monack, D. M. & Theriot, J. A. (2001). Actin-based motility is sufficient for bacterial membrane protrusion formation and host cell uptake. *Cell Microbiol,* 3**,** 633-47.

Montano, S. J., Lu, J., Gustafsson, T. N. & Holmgren, A. (2014). Activity assays of mammalian thioredoxin and thioredoxin reductase: fluorescent disulfide substrates, mechanisms, and use with tissue samples. *Anal Biochem,* 449**,** 139-46.

Mostowy, S., Bonazzi, M., Hamon, M. A., Tham, T. N., Mallet, A., Lelek, M., Gouin, E., Demangel, C., Brosch, R., Zimmer, C., Sartori, A., Kinoshita, M., Lecuit, M. & Cossart, P. (2010). Entrapment of intracytosolic bacteria by septin cage-like structures. *Cell Host Microbe,* 8**,** 433-44.

Muller, M. (2005). Twin-arginine-specific protein export in Escherichia coli. *Res Microbiol,* 156**,** 131-6.

Natale, P., Bruser, T. & Driessen, A. J. (2008). Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane--distinct translocases and mechanisms. *Biochim Biophys Acta,* 1778**,** 1735-56.

Nath, J. & Rebhun, J. I. (1976). Effects of caffeine and other methylxanthines on the development and metabolism of sea urchin eggs. Involvement of NADP and glutathione. *J Cell Biol,* 68**,** 440-50.

Nelson, J. W. & Creighton, T. E. (1994). Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. *Biochemistry,* 33**,** 5974-83.

Niyogi, S. K. (2005). Shigellosis. *J Microbiol,* 43**,** 133-43.

Nouwen, N., Ranson, N., Saibil, H., Wolpensinger, B., Engel, A., Ghazi, A. & Pugsley, A. P. (1999). Secretin PulD: association with pilot PulS, structure, and ion-conducting channel formation. *Proc Natl Acad Sci U S A,* 96**,** 8173-7.

Nouwen, N., Stahlberg, H., Pugsley, A. P. & Engel, A. (2000). Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy. *Embo j,* 19**,** 2229-36.

O'brian, M. R. & Thöny-Meyer, L. (2002). Biochemistry, regulation and genomics of haem biosynthesis in prokaryotes. *Advances in microbial physiology,* 46**,** 257-318.

Ogawa, M. & Sasakawa, C. (2006). Intracellular survival of Shigella. *Cell Microbiol,* 8**,** 177-84.

Ogawa, M., Yoshimori, T., Suzuki, T., Sagara, H., Mizushima, N. & Sasakawa, C. (2005). Escape of intracellular Shigella from autophagy. *Science,* 307**,** 727-731.

Østergaard, H., Tachibana, C. & Winther, J. R. (2004). Monitoring disulfide bond formation in the eukaryotic cytosol. *J Cell Biol,* 166**,** 337-345.

Oz, M., Lozon, Y., Sultan, A., Yang, K. H. & Galadari, S. (2015). Effects of monoterpenes on ion channels of excitable cells. *Pharmacol Ther,* 152**,** 83-97.

Pal, G., Ultsch, M. H., Clark, K. P., Currell, B., Kossiakoff, A. A. & Sidhu, S. S. (2005). Intramolecular cooperativity in a protein binding site assessed by combinatorial shotgun scanning mutagenesis. *J Mol Biol,* 347**,** 489-94.

Papanikou, E., Karamanou, S. & Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. *Nat Rev Microbiol,* 5**,** 839-51.

Park, H., Valencia-Gallardo, C., Sharff, A., Tran Van Nhieu, G. & Izard, T. (2011). Novel vinculin binding site of the IpaA invasin of Shigella. *J Biol Chem,* 286**,** 23214-21.

Park, S.-Y., Heo, Y.-J., Choi, Y.-S., Déziel, E. & Cho, Y.-H. (2005). Conserved virulence factors of Pseudomonas aeruginosa are required for killing Bacillus subtilis. *Journal of Microbiology,* 43**,** 443-450.

Parry, J. & Clark, D. P. (2002). Identification of a CysB-regulated gene involved in glutathione transport in Escherichia coli. *FEMS microbiology letters,* 209**,** 81-85.

Parsot, C. (2005). Shigella spp. and enteroinvasive Escherichia coli pathogenicity factors. *FEMS Microbiol Lett,* 252**,** 11-8.

Paxman, J. J., Borg, N. A., Horne, J., Thompson, P. E., Chin, Y., Sharma, P., Simpson, J. S., Wielens, J., Piek, S., Kahler, C. M., Sakellaris, H., Pearce, M., Bottomley, S. P., Rossjohn, J. & Scanlon, M. J. (2009). The structure of the bacterial oxidoreductase enzyme DsbA in complex with a peptide reveals a basis for substrate specificity in the catalytic cycle of DsbA enzymes. *J Biol Chem,* 284**,** 17835-45.

Pedron, T., Thibault, C. & Sansonetti, P. J. (2003). The invasive phenotype of Shigella flexneri directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. *J Biol Chem,* 278**,** 33878-86.

Peek, J. A. & Taylor, R. K. (1992). Characterization of a periplasmic thiol: disulfide interchange protein required for the functional maturation of secreted virulence factors of Vibrio cholerae. *Proceedings of the National Academy of Sciences,* 89**,** 6210-6214.

Peirano, G., Agerso, Y., Aarestrup, F. M. & Dos Prazeres Rodrigues, D. (2005). Occurrence of integrons and resistance genes among sulphonamide-resistant Shigella spp. from Brazil. *J Antimicrob Chemother,* 55**,** 301-5.

Peng, J., Yang, J. & Jin, Q. (2009). The molecular evolutionary history of Shigella spp. and enteroinvasive Escherichia coli. *Infect Genet Evol,* 9**,** 147-52.

Peng, J., Zhang, X., Yang, J., Wang, J., Yang, E., Bin, W., Wei, C., Sun, M. & Jin, Q. (2006). The use of comparative genomic hybridization to characterize genome dynamics and diversity among the serotypes of Shigella. *BMC genomics,* 7**,** 218.

Pittman, M. S., Corker, H., Wu, G., Binet, M. B., Moir, A. J. G. & Poole, R. K. (2002). Cysteine Is Exported from the Escherichia coliCytoplasm by CydDC, an ATP-binding Cassette-type Transporter Required for Cytochrome Assembly. *Journal of Biological Chemistry,* 277**,** 49841-49849.

Pittman, M. S., Robinson, H. C. & Poole, R. K. (2005). A bacterial glutathione transporter (Escherichia coli CydDC) exports reductant to the periplasm. *J Biol Chem,* 280**,** 32254-61.

Plano, G. V., Day, J. B. & Ferracci, F. (2001). Type III export: new uses for an old pathway. *Mol Microbiol,* 40**,** 284-93.

Poole, R. K., Hatch, L., Cleeter, M. W. J., Gibson, F., Cox, G. B. & Wu, G. (1993). Cytochrome bd biosynthesis in Escherichia coli: the sequences of the cydC and cydD genes suggest that they encode the components of an ABC membrane transporter. *Molecular microbiology,* 10**,** 421-430.

Pop, O., Martin, U., Abel, C. & Muller, J. P. (2002). The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of Bacillus subtilis form an autonomous Tat translocation system. *J Biol Chem,* 277**,** 3268-73.

Quintyne, K. I., Baker, S., Wallis, F. & Gupta, R. (2012). Good clinical and radiological response to BRAF inhibitor in patient with metastatic thin melanoma. *BMJ Case Rep,* 2012.

Rabhi-Essafi, I., Sadok, A., Khalaf, N. & Fathallah, D. M. (2007). A strategy for high-level expression of soluble and functional human interferon alpha as a GST-fusion protein in E. coli. *Protein Eng Des Sel,* 20**,** 201-9.

Raghukumar, R., Vali, L., Watson, D., Fearnley, J. & Seidel, V. (2010). Antimethicillin-resistant Staphylococcus aureus (MRSA) activity of 'pacific propolis' and isolated prenylflavanones. *Phytother Res,* 24**,** 1181-7.

Rai, M. & Padh, H. J. C. S.-B.-. (2001). Expression systems for production of heterologous proteins. 80**,** 1121-1128.

Ramarao, N., Le Clainche, C., Izard, T., Bourdet-Sicard, R., Ageron, E., Sansonetti, P. J., Carlier, M. F. & Tran Van Nhieu, G. (2007). Capping of actin filaments

by vinculin activated by the Shigella IpaA carboxyl-terminal domain. *FEBS Lett,* 581**,** 853-7.

Randall, L. L. & Hardy, S. J. (2002). SecB, one small chaperone in the complex milieu of the cell. *Cell Mol Life Sci,* 59**,** 1617-23.

Raturi, A. & Mutus, B. (2007). Characterization of redox state and reductase activity of protein disulfide isomerase under different redox environments using a sensitive fluorescent assay. *Free Radic Biol Med,* 43**,** 62-70.

Ray, K., Marteyn, B., Sansonetti, P. J. & Tang, C. M. (2009). Life on the inside: the intracellular lifestyle of cytosolic bacteria. *Nat Rev Microbiol,* 7**,** 333-40.

Rekha, K. R., Selvakumar, G. P., Sethupathy, S., Santha, K. & Sivakamasundari, R. I. (2013). Geraniol ameliorates the motor behavior and neurotrophic factors inadequacy in MPTP-induced mice model of Parkinson's disease. *Journal of Molecular Neuroscience,* 51**,** 851-862.

Replogle, M. L., Fleming, D. W. & Cieslak, P. R. (2000). Emergence of antimicrobial-resistant shigellosis in Oregon. *Clin Infect Dis,* 30**,** 515-9.

Rietsch, A., Bessette, P., Georgiou, G. & Beckwith, J. (1997). Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. *J Bacteriol,* 179**,** 6602-8.

Rios, J. L. & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *J Ethnopharmacol,* 100**,** 80-4.

Robinson, C. & Bolhuis, A. (2004). Tat-dependent protein targeting in prokaryotes and chloroplasts. *Biochim Biophys Acta,* 1694**,** 135-47.

Rohmer, L., Jacobs, M. A., Brittnacher, M. J., Fong, C., Hayden, H. S., Hocquet, D., Weiss, E. J., Radey, M., Germani, Y., Talukder, K. A., Hager, A. J., Kemner, J. M., Sims-Day, E. H., Matamouros, S., Hager, K. R. & Miller, S. I. (2014). Genomic analysis of the emergence of 20th century epidemic dysentery. *BMC Genomics,* 15**,** 355.

Sakaguchi, T., Kohler, H., Gu, X., Mccormick, B. A. & Reinecker, H. C. (2002). Shigella flexneri regulates tight junction-associated proteins in human intestinal epithelial cells. *Cell Microbiol,* 4**,** 367-81.

Sambrook, J., Russell, D. W. & Russell, D. W. (2001). *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press New York.

Sansonetti, P. J. (2001). Microbes and microbial toxins: paradigms for microbial-mucosal interactions III. Shigellosis: from symptoms to molecular pathogenesis. *Am J Physiol Gastrointest Liver Physiol,* 280**,** G319-23.

Sansonetti, P. J., Hale, T. L., Dammin, G. J., Kapfer, C., Collins, H. H. & Formal, S. B. (1983). Alterations in the pathogenicity of Escherichia coli K-12 after transfer of plasmid and chromosomal genes from Shigella flexneri. *Infection and Immunity,* 39**,** 1392-1402.

Sansonetti, P. J., Phalipon, A., Arondel, J., Thirumalai, K., Banerjee, S., Akira, S., Takeda, K. & Zychlinsky, A. (2000). Caspase-1 activation of IL-1beta and IL-18 are essential for Shigella flexneri-induced inflammation. *Immunity,* 12**,** 581-90.

Sargent, F., Stanley, N. R., Berks, B. C. & Palmer, T. (1999). Sec-independent protein translocation in Escherichia coli. A distinct and pivotal role for the TatB protein. *J Biol Chem,* 274**,** 36073-82.

Schierle, C. F., Berkmen, M., Huber, D., Kumamoto, C., Boyd, D. & Beckwith, J. (2003). The DsbA signal sequence directs efficient, cotranslational export of passenger proteins to the Escherichia coli periplasm via the signal recognition particle pathway. *J Bacteriol,* 185**,** 5706-13.

Schmidt, H. & Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clinical microbiology reviews,* 17**,** 14-56.

Schneditz, G., Rentner, J., Roier, S., Pletz, J., Herzog, K. A., Bucker, R., Troeger, H., Schild, S., Weber, H., Breinbauer, R., Gorkiewicz, G., Hogenauer, C. & Zechner, E. L. (2014). Enterotoxicity of a nonribosomal peptide causes antibiotic-associated colitis. *Proc Natl Acad Sci U S A,* 111**,** 13181-6.

Schroeder, G. N. & Hilbi, H. (2008). Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev,* 21**,** 134-56.

Schuch, R. & Maurelli, A. T. (1999). The mxi-Spa type III secretory pathway of Shigella flexneri requires an outer membrane lipoprotein, MxiM, for invasin translocation. *Infect Immun,* 67**,** 1982-91.

Schuch, R. & Maurelli, A. T. (2001). MxiM and MxiJ, base elements of the Mxi-Spa type III secretion system of Shigella, interact with and stabilize the MxiD secretin in the cell envelope. *J Bacteriol,* 183**,** 6991-8.

Sebaugh, J. L. (2011). Guidelines for accurate EC50/IC50 estimation. *Pharm Stat,* 10**,** 128-34.

Seidel, V., Peyfoon, E., Watson, D. G. & Fearnley, J. (2008). Comparative study of the antibacterial activity of propolis from different geographical and climatic zones. *Phytother Res,* 22**,** 1256-63.

Sevier, C. S. & Kaiser, C. A. (2008). Ero1 and redox homeostasis in the endoplasmic reticulum. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1783**,** 549-556.

Shao, F., Bader, M. W., Jakob, U. & Bardwell, J. C. (2000). DsbG, a protein disulfide isomerase with chaperone activity. *J Biol Chem,* 275**,** 13349-52.

Shouldice, S. R., Heras, B., Walden, P. M., Totsika, M., Schembri, M. A. & Martin, J. L. (2011). Structure and function of DsbA, a key bacterial oxidative folding catalyst. *Antioxid Redox Signal,* 14**,** 1729-60.

Shubina, L. P., Siurin, S. A. & Savchenko, V. M. (1990). [Inhalations of essential oils in the combined treatment of patients with chronic bronchitis]. *Vrach Delo***,** 66-7.

Si, W., Gong, J., Tsao, R., Zhou, T., Yu, H., Poppe, C., Johnson, R. & Du, Z. (2006). Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *J Appl Microbiol,* 100**,** 296-305.

Singer, M. & Sansonetti, P. J. (2004). IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of Shigella-induced colitis. *J Immunol,* 173**,** 4197-206.

Skorko-Glonekv, J. & Sobiecka, A. (2005). [Periplasmatic disulfide oxidoreductases from bacterium Escherichia coli--their structure and function]. *Postepy Biochem,* 51**,** 459-67.

Smith, R. P., Paxman, J. J., Scanlon, M. J. & Heras, B. (2016). Targeting Bacterial Dsb Proteins for the Development of Anti-Virulence Agents. *Molecules,* 21.

Solorzano-Santos, F. & Miranda-Novales, M. G. (2012). Essential oils from aromatic herbs as antimicrobial agents. *Curr Opin Biotechnol,* 23**,** 136-41.

Sousa, E. O., Silva, N. F., Rodrigues, F. F., Campos, A. R., Lima, S. G. & Costa, J. G. (2010). Chemical composition and resistance-modifying effect of the essential oil of Lantana camara Linn. *Pharmacogn Mag,* 6**,** 79-82.

Stenson, T. H. & Weiss, A. A. (2002). DsbA and DsbC are required for secretion of pertussis toxin by Bordetella pertussis. *Infect Immun,* 70**,** 2297-303.

Stockert, J. C., Blazquez-Castro, A., Canete, M., Horobin, R. W. & Villanueva, A. (2012). MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. *Acta Histochem,* 114**,** 785-96.

Suppakul, P., Miltz, J., Sonneveld, K. & Bigger, S. W. (2003). Antimicrobial properties of basil and its possible application in food packaging. *J Agric Food Chem,* 51**,** 3197-207.

Suree, N., Yi, S. W., Thieu, W., Marohn, M., Damoiseaux, R., Chan, A., Jung, M. E. & Clubb, R. T. (2009). Discovery and structure-activity relationship analysis of Staphylococcus aureus sortase A inhibitors. *Bioorg Med Chem,* 17**,** 7174-85.

Szarka, C. E., Pfeiffer, G. R., Hum, S. T., Everley, L. C., Balshem, A. M., Moore, D. F., Litwin, S., Goosenberg, E. B., Frucht, H., Engstrom, P. F. & Et Al. (1995). Glutathione S-transferase activity and glutathione S-transferase mu expression in subjects with risk for colorectal cancer. *Cancer Res,* 55**,** 2789-93.

Tan, J., Lu, Y. & Bardwell, J. C. (2005). Mutational analysis of the disulfide catalysts DsbA and DsbB. *J Bacteriol,* 187**,** 1504-10.

Thapa, D., Losa, R., Zweifel, B. & Wallace, R. J. (2012). Sensitivity of pathogenic and commensal bacteria from the human colon to essential oils. *Microbiology,* 158**,** 2870-7.

Thirumalai, K., Kim, K. S. & Zychlinsky, A. (1997). IpaB, a Shigella flexneri invasin, colocalizes with interleukin-1 beta-converting enzyme in the cytoplasm of macrophages. *Infect Immun,* 65**,** 787-93.

Tinsley, C. R., Voulhoux, R., Beretti, J. L., Tommassen, J. & Nassif, X. (2004). Three homologues, including two membrane-bound proteins, of the disulfide oxidoreductase DsbA in Neisseria meningitidis: effects on bacterial growth and biogenesis of functional type IV pili. *J Biol Chem,* 279**,** 27078-87.

Totsika, M., Heras, B., Wurpel, D. J. & Schembri, M. A. (2009). Characterization of two homologous disulfide bond systems involved in virulence factor biogenesis in uropathogenic Escherichia coli CFT073. *J Bacteriol,* 191**,** 3901-8.

Tran Van Nhieu, G., Bourdet-Sicard, R., Dumenil, G., Blocker, A. & Sansonetti, P. J. (2000). Bacterial signals and cell responses during Shigella entry into epithelial cells. *Cell Microbiol,* 2**,** 187-93.

Trofa, A. F., Ueno-Olsen, H., Oiwa, R. & Yoshikawa, M. (1999). Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. *Clin Infect Dis,* 29**,** 1303-6.

Tsai, M. L., Lin, C. C., Lin, W. C. & Yang, C. H. (2011). Antimicrobial, antioxidant, and anti-inflammatory activities of essential oils from five selected herbs. *Biosci Biotechnol Biochem,* 75**,** 1977-83.

Tseng, W. C., Lin, J. W., Wei, T. Y. & Fang, T. Y. (2008). A novel megaprimed and ligase-free, PCR-based, site-directed mutagenesis method. *Anal Biochem,* 375**,** 376-8.

Turina, A. V., Nolan, M. V., Zygadlo, J. A. & Perillo, M. A. (2006). Natural terpenes: self-assembly and membrane partitioning. *Biophys Chem,* 122**,** 101-13.

Tutar, L. & Tutar, Y. (2010). Heat shock proteins; an overview. *Curr Pharm Biotechnol,* 11**,** 216-22.

Ud-Din, A. & Wahid, S. (2014). Relationship among Shigella spp. and enteroinvasive Escherichia coli (EIEC) and their differentiation. *Braz J Microbiol,* 45**,** 1131-8.

Urban, A., Leipelt, M., Eggert, T. & Jaeger, K.-E. (2001). DsbA and DsbC affect extracellular enzyme formation in Pseudomonas aeruginosa. *Journal of bacteriology,* 183**,** 587-596.

Vertommen, D., Depuydt, M., Pan, J., Leverrier, P., Knoops, L., Szikora, J. P., Messens, J., Bardwell, J. C. & Collet, J. F. (2008). The disulphide isomerase DsbC cooperates with the oxidase DsbA in a DsbD-independent manner. *Mol Microbiol,* 67**,** 336-49.

Vinh, H., Nhu, N. T., Nga, T. V., Duy, P. T., Campbell, J. I., Hoang, N. V., Boni, M. F., My, P. V., Parry, C., Nga, T. T., Van Minh, P., Thuy, C. T., Diep, T. S., Phuong Le, T., Chinh, M. T., Loan, H. T., Tham, N. T., Lanh, M. N., Mong, B. L., Anh, V. T., Bay, P. V., Chau, N. V., Farrar, J. & Baker, S. (2009). A changing picture of shigellosis in southern Vietnam: shifting species dominance, antimicrobial susceptibility and clinical presentation. *BMC Infect Dis,* 9**,** 204.

Vivian, J. P., Scoullar, J., Rimmer, K., Bushell, S. R., Beddoe, T., Wilce, M. C., Byres, E., Boyle, T. P., Doak, B., Simpson, J. S., Graham, B., Heras, B., Kahler, C. M., Rossjohn, J. & Scanlon, M. J. (2009). Structure and function of the oxidoreductase DsbA1 from Neisseria meningitidis. *J Mol Biol,* 394**,** 931-43.

Wang, G., Tang, W. & Bidigare, R. R. (2005). Terpenoids as therapeutic drugs and pharmaceutical agents. *Natural products.* Springer.

Wang, L., Zhu, L. & Wang, C.-C. (2011). The endoplasmic reticulum sulfhydryl oxidase Ero1β drives efficient oxidative protein folding with loose regulation. *Biochemical Journal,* 434**,** 113-121.

Watarai, M., Tobe, T., Yoshikawa, M. & Sasakawa, C. (1995). Disulfide oxidoreductase activity of Shigella flexneri is required for release of Ipa

proteins and invasion of epithelial cells. *Proc Natl Acad Sci U S A,* 92**,** 4927-31.

Who, W. H. O. (1997). Vaccine research and development. New strategies for accelerating Shigella vaccine development. *Wkly Epidemiol Rec,* 72**,** 73-9.

Who, W. H. O. (2006). Initiative for vaccine research (IVR). New vaccines against infectious disesases: research and development status. *World Health Organization, Geneva, Switzerland. http://www. who. int/vaccine_research/en*.

Who, W. H. O. (2017). *WHO publishes list of bacteria for which new antibiotics are urgently needed* [Online]. GENEVA. Available: https://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed [Accessed 21.12. 2018 2018].

Winterbourn, C. C. & Metodiewa, D. (1999). Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radical Biology and Medicine,* 27**,** 322-328.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. & Turner, N. D. (2004). Glutathione metabolism and its implications for health. *J Nutr,* 134**,** 489-92.

Wülfing, C. & Rappuoli, R. (1997). Efficient production of heat-labile enterotoxin mutant proteins by overexpression of dsbA in a degP-deficient Escherichia coli strain. *Archives of microbiology,* 167**,** 280-283.

Xia, Y., Chu, W., Qi, Q. & Xun, L. (2015). New insights into the QuikChange process guide the use of Phusion DNA polymerase for site-directed mutagenesis. *Nucleic Acids Res,* 43**,** e12.

Xu, D., Saeed, A., Wang, Y., Seidel, V., Sandstrom, G. & Yu, J. (2011). Natural products modulate Shigella-host-cell interaction. *J Med Microbiol,* 60**,** 1626-32.

Xu, Y. & Eissa, N. T. (2010). Autophagy in innate and adaptive immunity. *Proc Am Thorac Soc,* 7**,** 22-8.

Yang, F., Yang, J., Zhang, X., Chen, L., Jiang, Y., Yan, Y., Tang, X., Wang, J., Xiong, Z., Dong, J., Xue, Y., Zhu, Y., Xu, X., Sun, L., Chen, S., Nie, H., Peng, J., Xu, J., Wang, Y., Yuan, Z., Wen, Y., Yao, Z., Shen, Y., Qiang, B., Hou, Y., Yu, J. & Jin, Q. (2005). Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. *Nucleic Acids Res,* 33**,** 6445-58.

Yang, Z. & Klionsky, D. J. (2010). Eaten alive: a history of macroautophagy. *Nat Cell Biol,* 12**,** 814-22.

Yu, J. (1998). Inactivation of DsbA, but not DsbC and DsbD, affects the intracellular survival and virulence of Shigella flexneri. *Infect Immun,* 66**,** 3909-17.

Yu, J., Edwards-Jones, B., Neyrolles, O. & Kroll, J. S. (2000). Key Role for DsbA in Cell-to-Cell Spread ofShigella flexneri, Permitting Secretion of Ipa Proteins into Interepithelial Protrusions. *Infection and immunity,* 68**,** 6449-6456.

Yu, J. & Kroll, J. S. (1999). DsbA: a protein-folding catalyst contributing to bacterial virulence. *Microbes and infection,* 1**,** 1221-1228.

Yu, J., Oragui, E. E., Stephens, A., Kroll, J. S. & Venkatesan, M. M. (2001). Inactivation of DsbA alters the behaviour of Shigella flexneri towards murine and human-derived macrophage-like cells. *FEMS Microbiol Lett,* 204**,** 81-8.

Yu, S. G., Hildebrandt, L. A. & Elson, C. E. (1995). Geraniol, an inhibitor of mevalonate biosynthesis, suppresses the growth of hepatomas and melanomas transplanted to rats and mice. *J Nutr,* 125**,** 2763-7.

Zhang, H. Z. & Donnenberg, M. S. (1996). DsbA is required for stability of the type IV pilin of enteropathogenic escherichia coli. *Mol Microbiol,* 21**,** 787-97.

Zheng, L., Baumann, U. & Reymond, J. L. (2004). An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res,* 32**,** e115.

Zumsteg, A. B., Goosmann, C., Brinkmann, V., Morona, R. & Zychlinsky, A. (2014). IcsA is a Shigella flexneri adhesin regulated by the type III secretion system and required for pathogenesis. *Cell host & microbe,* 15**,** 435-445.

Zychlinsky, A., Thirumalai, K., Arondel, J., Cantey, J. R., Aliprantis, A. O. & Sansonetti, P. J. (1996). In vivo apoptosis in Shigella flexneri infections. *Infect Immun,* 64**,** 5357-65.