# "Mechanisms associated with *Staphylococcus aureus* and *Acanthamoeba* Interactions, and Therapeutic applications"

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**A thesis submitted for the degree of Doctor of Philosophy**

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**April 2016**

**Authorship statement**

This thesis is the result of my own research studies, except where otherwise indicated. Some results of investigations have been presented at various international conferences and one paper is in the process of preparation for publication in the near future as a result of the findings in this thesis.

Signed…….(candidate)

Date ……..

Signed…….(supervisor)

Date ……

**Acknowledgements**

First and foremost I want to thank my supervisor Dr. Selwa Alsam. It has been an honour to be her Ph.D. student. She has taught me, both consciously and unconsciously, how good research experiment is done. I appreciate all her contributions of time and ideas to make my Ph.D. experience productive and stimulating. The joy and enthusiasm she has for her research was contagious and motivational for me, even during tough times in the Ph.D. pursuit. I am also thankful for the excellent example she has provided as a successful woman microbiologist.

I also would like to thank my co-supervisor Dr. Sinan Battah for all the support she has given throughout my years. Thank you for patiently helping to shape me into the man I am today, both in science and in life.

Special thanks to Prof. Graham Mitchell for his valuable suggestions, constructive comments and editorial input.

In microbiology lab my dear labmates, Mihaela, Osama, Bauyrzhan, Aiden, Kully, Alaa and Eniola have contributed immensely to my personal and professional time at Essex. This group has been a source of friendships as well as good advice and collaboration.

I am grateful to our laboratory technician Lynwen James who kept us organized and was always ready to help in the lab. I would like to give my thanks to every person in the school, whom I didn't mention, for help, assistance and support.

I gratefully acknowledge the funding sources that made my Ph.D. work possible. I was funded by the El-Baha University of the Kingdom of Saudi Arabia throughout 4 years.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who raised me with a love of science and supported me in all my pursuits. And most of all for my loving, supportive, encouraging, and patient wife and son whose faithful support during the all stages of this Ph.D. is so appreciated. Thank you.

**Abstract**

*Staphylococcus aureus is* a major cause of infections within hospitals. Its spread among patients in hospitals is one of the major problems that require urgent attention. *Staphylococcus aureus* has the ability to interact with other microorganisms such as the free-living amoebae *Acanthamoeba* which acts as a host for various pathogens. This interaction is highly complex and may be beneficial to one of them or both.

The main aims of this project were to study the interactions of *Acanthamoeba castellanii* with methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA), and to understand the molecular mechanisms involved. A large variety of molecules play a role in growth, function, migration and apoptosis. The role of Phosphoinositide 3-kinase (PI3K) in *Acanthamoeba*/bacteria interaction was studied through the use of inhibitors such as LY294002 and wortmannin, while the involvement of protein tyrosine kinase was studied through genistein and protein tyrosine phosphatase by sodium orthovandate ($Na_3VO_4$). The role of serine proteases of *Acanthamoeba* was investigated by using its inhibitor Phenylmethylsulfonyl fluoride (PMSF).

The results showed that MRSA exhibited more invasive abilities towards *Acanthamoeba*, when compared with MSSA. In addition, inhibition of PI3K and tyrosine phosphatase subsequently resulted in the reduction of MRSA and MSSA invasion. However, PMSF increased bacterial uptake by *Acanthamoeba*. Two dimensional gel electrophoresis was performed to separate and fractionate the *Acanthamoeba* conditioned medium and the Universal Protein Resource Uniprot database was used for the analysis and identification of protein for the forty three spots identified.

This study further stresses the need for antimicrobial drugs that are effective without inflicting damage upon the host, as a result of the resistant nature of MRSA towards a variety of antibiotics. One possible method that can be used is Photodynaic Therapy, due to its effectiveness against microorganisms in a localised infected area, through the use of local combined photosensitizers and light.

In addition, to simplify the interaction process, heat-killed fluorescein isothiocyanate-labelled (FITC)-labelled MRSA and MSSA were used. The findings suggest that trophozoites *Acanthamoeba* trophozoites uptake of MRSA was significantly higher than MSSA which may act as "vehicle" that facilitates the spread of MRSA to susceptible hosts.

This study showed that photodynamic therapy had a significant impact on bacterial survival. Photosensitizers m-Tetra (hydroxyphenyl) chlorin (*m*-THPC) and psoralen were very effective in eradicating bacteria. In antimicrobial assays, natural product eugenol derivatives demonstrated significant inhibitory activity on the growth of MRSA and exhibited dose-dependent cytotoxic effects on *Acanthamoeba* trophozoites.

Future work should continue to identify more molecular mechanisms of *Acanthamoeba* interaction with MRSA and finding new strategies for effective antimicrobial therapies.

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## List of Abbreviations

| | |
|---|---|
| 2D-PAGE | Two dimensional polyacrylamide gel electrophoresis |
| Ab-SnCe6 | Antibody- tin(IV) chlorin e6 conjugate |
| AGE | *Acanthamoeba* granulomatous encephalitis |
| AIDS | Acquired immune deficiency syndrome |
| AK | *Acanthamoeba* keratitis |
| AKT | Protein Kinase Thymoma |
| Akt | Protein Kinase B |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CA-MRSA | Community acquired methicillin-resistant *Staphylococcus aureus* |
| CFU | Colony-forming unit |
| CLED agar | Cysteine lactose electrolyte deficient |
| CM | Conditioned medium |
| CNS | Central nervous system |
| DEAE | Diethylethanolamine |
| DP | Deuteroporphyrin |
| DTT | Dithiothrietol |
| Ecto-ATPases | Ectoserine Triphosphate diphosphatase |
| EMRSA | Epidemic Methicillin-resistant *Staphylococcus aureus* |
| EN | Eugenol |
| FITC | Fluorescein isothiocyanate |
| HA-MRSA | Hospital-acquired methicillin-resistant *Staphylococcus aureus* |
| HBMEC | Human brain microvascular endothelial cells |

| | |
|---|---|
| HIV | Human immunodeficiency virus |
| IEF | Isoelectric focusing |
| IgG | Immunoglobulin G |
| IPG | Immobilized pH-gradient |
| kDa | kilo Daltons |
| LB | Luria-Bertani medium |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen-activated protein kinase |
| MBP | Mannose binding protein |
| MIC | Minimum inhibitory concentration |
| MIC | Minimum inhibitory concentration |
| MRI | Magnetic resonance imaging |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MS | Mass spectrometry |
| MSCRAMM | Microbial surface components recognising adhesive matrix molecules |
| MSSA | Methicillin-sensitive *Staphylococcus aureus* |
| *m*-THPC | *m*-tetra (hydroxyphenyl) chlorin |
| OD | Optical density |
| PARs | Protease-activated receptors |
| PBP2a | Penicillin-binding protein |
| PBS | Phosphate buffered saline |
| PDI | Photodynamic inactivation |
| PDK1 | Phosphoinositide-dependent kinase-1 |
| PDT | Photodynamic therapy |
| PI | Phosphatidylinositol |
| pI | Isoelectric point |

| | |
|---|---|
| PI3K | Phosphatidylinositol 3-kinases |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-trisphosphate |
| PMBN | Polycationic peptide polymyxin B nonapeptide |
| PMSF | Phenylmethylsulfonyl fluoride |
| PPc | Pyridiniumphthalocyanine |
| PS | Photosensitizer |
| Ps | Psoralen |
| PVL | Panton-Valentine Leukocidin |
| PYG | Peptone Yeast Glucose |
| Ras | Rat sarcoma |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RPM | Revolutions per min |
| RPMI | Roswell Park Memorial Institute |
| RTK | Receptor tyrosine kinase |
| SCC*mec* | Staphylococcal cassette chromosome (conferring methicillin resistance) |
| SCV | Small colony variants |
| SDS PAGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| SI | Singlet stage |
| SO | Singlet oxygen |
| ST | Sequence type |
| SWP | Southwest Pacific clone |
| UVA light | Ultraviolet light |

# Chapter 1. Introduction

### 1.1. *Staphylococcus aureus*

*Staphylococcus aureus (S. aureus)* is the major pathogen within the genus *Staphylococcus*. It is coagulase positive *Staphylococci* which causes a wide range of infections in both humans and animals (Ruzauskas *et al.*, 2015). *S. aureus* causes several diseases ranging from mild infections such as skin and soft tissue infections to potentially fatal infections such as endocarditis, bacteraemia and toxic shock syndrome. Depending on sensitivity to methicillin, there exist two types of *S. aureus* strains; the first strain is known as methicillin-sensitive *S. aureus* (MSSA) while the second strain is known as methicillin resistant *S. aureus* (MRSA) as they are resistant to methicillin and other β-lactam antibiotics. MRSA is also known as a 'superbug' due to its ability to resist many antibiotics (Okuma *et al.*, 2002). This resistance to antibiotics makes infections caused by MRSA more difficult to treat among others. UK Office for National Statistics bulletin presented the number of deaths caused by MRSA between 1993 and 2013 in England and Wales (Figure 1.1). It showed that the main cause of death is the presence of other underlying diseases rather than contracting MRSA at that time.

Figures are based on deaths registered in each calendar year rather than those occurring in each year. Since the majority of deaths involving MRSA registered in 2012 also occurred in the same year, registration delays are likely to have no impact on the findings in this bulletin. Please see the section on registration delays for further information. Furthermore, due to its occasional rapid spread and destruction of human flesh in necrotising fasciitis, it is also called 'flesh eating' bacteria (Foster, 2004). The ability to produce penicillin-binding protein 2a (PBP2a) molecules (encoded by the *mecA* gene) which have low affinity to β-lactam antibiotics explains the resistance of MRSA to methicillin and other β-lactams (Berger-Bachi and Rohrer, 2002). People with compromised immune systems are commonly infected by this bacterium (Gordon and Lowy, 2008) and MRSA causes infections in both hospital and community settings (Singh *et al.*, 2012).

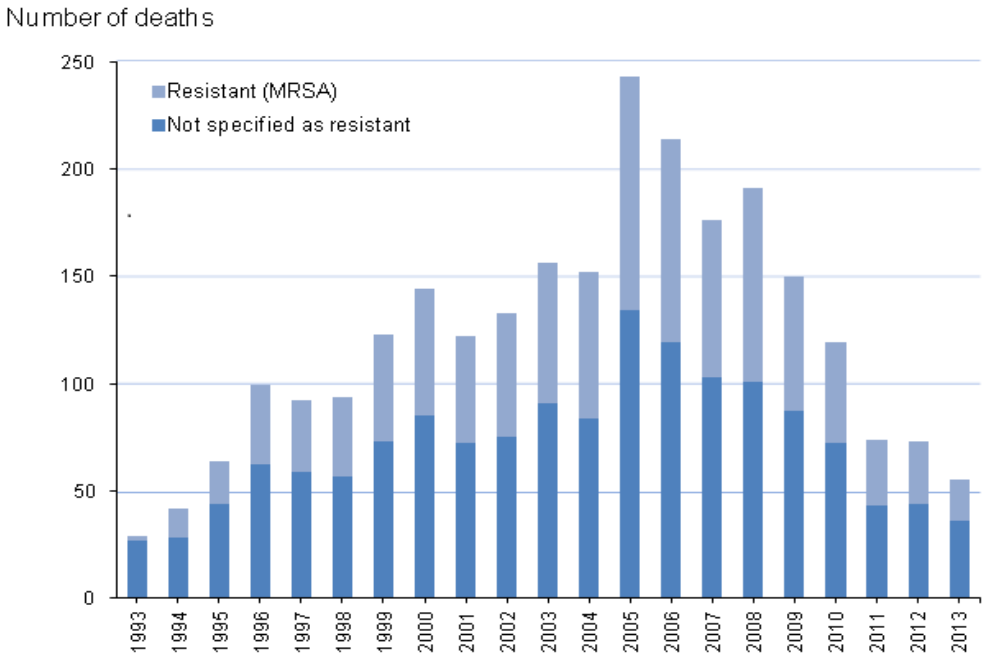



**Figure1.1. Number of deaths caused by MRSA between 1993 and 2013 in England and Wales.** These patients were already ill before contracting MRSA and it is their existing illness rather than MRSA which may be the underlying cause of death (Office for National Statistics, 2013)**.**

### 1.1.1. *Staphylococcus aureus* Colonisation

*Staphylococcus aureus* is a pathogen, as well as a commensal organism. The main ecological niches for *S. aureus* are the anterior nares. Persistent nasal colonisation of *S. aureus* occurs in approximately 20% of individuals, and intermittent colonisation is observed in approximately 30% (Gordon and Lowy 2008). It has been found, however, that several other sites can be subject to *S. aureus* colonisation, including the gastrointestinal tract, the axillae and the groin. In the event of colonisation, for example through catheterisation, surgery, aspiration or shaving, or when the immunity of the host is compromised, bacteria may be introduced from this pool of colonies, in turn increasing the risk of infection (Wertheim *et al.*, 2005). The colonising *S. aureus* strain is commonly responsible for subsequent infections (Ruzauskas *et al.*, 2015). This was shown in the study by Von Eiff *et al*., 2001 in which they found that blood *S. aureus* isolates were identical to nasal isolates. There is a need to further investigate the underlying causes for *S. aureus* colonisation, due to its complexity and a gap in our current knowledge as to why the host must have contact with *S. aureus* and why in the event of this contact, *S. aureus* is able to adhere to cells of a host and evade immunity of the host (Wertheim *et al.*, 2005).

### 1.1.2. Epidemiology and Prevalence of MRSA infection

The epidemiology of MRSA infections is complicated and involves several routes of transmission. The carriers of MRSA are at a higher risk of developing infections, and they serve as potential reservoirs for the subsequent transmission of the organisms. There are increasing reports of outbreaks of MRSA colonisation and infection through skin contact as in the cases of gyms and locker rooms in the US; it is thought that around 53 million people carry MRSA (Archibald *et al.*, 2008). Some scientists have estimated that 25-30% of the world's population carry MSSA bacteria (Islam *et al.*, 2008). MRSA infected patients in intensive care units increased from 35.9% in 1992 to 64.4% in 2003. In Europe, MRSA infections increased from 16% to 24% between 1999 and 2004. It was further found that the MRSA strain was more prevalent in southern parts of Europe than in the northern parts. The highest prevalence was found in Portugal (54%) and Italy (43-58%), but the prevalence in Switzerland and the Netherlands was very low (2%). In the UK, MRSA causes almost half of hospital infections. MRSA's

range is worldwide, with prevalence varying according to the type of clinical wards and geographical areas (Rello *et al.*, 2007). The highest rates of MRSA occurrence (>50%) are reported in North and South America, Asia and Malta. Intermediate rates (25–50%) are reported in China, Australia, Africa and European countries such as Portugal (49%), Greece (40%), Italy (37%) and Romania (34%), Netherlands (5%) and Scandinavia (1%) (Stefani et al., 2012) (Figure 1.2). It is clear that the MRSA incidence is correlated with the management of antibiotic consumption in different countries. Between 2000 and 2010, the consumption of antibiotics was amplified by 36% (from about 54 to 73 standard units). For example, Brazil, Russia, India, China, and South Africa accounted for 76% of this rise due to a significant variation in the number of MRSA infections. The overuse and misuse of antibiotics is more likely to happen in the developing compared with the developed countries. This is a significant factor in increasing antibiotic resistance. Therefore, researchers are more vigilant now to monitor and regulate antibiotic usage and it's resistance. A wider partnership between countries to help policy makers for the monitoring of antibiotic consumption is essential to control antibiotic resistance (Thomas et al., 2014).


**Figure1.2. The global map of the percentage of MRSA incidence. The darkest colour indicates high percentage (http://www.cddep.org).**

### 1.1.3. Disease progression as a result of virulence factors

*Staphylococcus aureus* has an extensive range of virulence factors; both secreted and structural products play a role in the pathogenesis of *S. aureus* infection (Patti *et al.*, 1994). *S. aureus* expresses numerous surface proteins called Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) which facilitate the adherence of connective tissue, such as fibrinogen, fibronectin and collagen, to the host tissue. Different strains of *S. aureus* have and express different arrays of MSCRAMMs, and so different strains cause particular kinds of infections (Patti *et al.*, 1994). MSCRAMMs play an important role in the initiation of bone, joint and endovascular infections (R J Gordon and Lowy, 2008).

*Staphylococcus aureus* can grow efficiently when adhered to medical prosthetic devices, or host organisms. On prosthetic materials, *S. aureus* forms biofilms which appear as a thin slime layer. In this way, it may persist and as a result evade antimicrobials and other host defence mechanisms (Donlan and Costerton, 2002). This is the reason for infections occurring in prosthetic devices being almost impossible to eradicate, without removing the device completely. On the other hand, *S. aureus* can survive in epithelial cells following invasion. *In vitro* studies have shown that they are able to evade host defence mechanisms by forming Small Colony Variants (SCVs) (Patti *et al.*, 2003). These may be the cause of infection persistence and recurrence, which has been found to be the case in endocarditis (Ogawa *et al.*, 1985). Furthermore, SCVs have been found to disguise themselves inside cells of a host without damaging the host cell significantly, and are less exposed to antibiotics. Being in host cells, they have the ability to proliferate and burst the cell, which in turn can lead to a recurrence of infection (Proctor and Peters, 1998).

Aside from the aforementioned contributors to the evasion of the immune system of the host, *S. aureus* has been found to exhibit further characteristics enabling this evasion. One of the main mechanisms studied, is the production of an antiphagocytic microcapsule (O'Riordan and Lee, 2004). Being zwitterionic, this capsule is able to induce the formation of abscesses (Weidenmaier *et al.*, 2010).

Infection is further caused by the binding of MSCRAMM protein A to the Fc portion of immunoglobulin (Foster, 2005). This can significantly reduce the chance of opsonisation occurring. Similarly, *S. aureus* can secrete extracellular adherence proteins and chemotaxis inhibitory proteins that inhibit chemotaxis and extravasation of neutrophils to the infection site (Foster, 2005). *S. aureus* also evade immunity of the host cell by producing leukocidins. This in turn results in the destruction of leukocytes, by forming cell membrane pores (Gladstone and Van Heyningen, 1957). This, in addition to the production of various enzymes including elastases, proteases and lipases, facilitate the destruction of host tissues and allow the metastasis of the infection to different sites. This can also induce a septic shock, through interaction with and activation of the immune system of the host and coagulation pathways. Contributors to this are α-toxin and Lipoteichoic acid (Wang *et al.*, 2007).

Certain strains of *S. aureus* are also capable of producing 'super antigens'. These can facilitate toxic shock syndrome and food poisoning (McCormick *et al.*, 2001). They can further cause a septic syndrome through the induction of a 'cytokine storm'. The capability of *S. aureus* to cause havoc in the cell is highlighted by the secretion of exfoliative toxins and epidermolysins. These can cause bullous impetigo and scalded skin syndrome (Prévost *et al.*, 2003). The expression of staphylococcal virulence factors needs to be highly regulated in order to reduce high demands on metabolism and has a key role in pathogenesis (Figure 1.3). For example, MSCRAMM expression only occurs when needed in bacteria; i.e. during the log phase of growth, or replication. Secreted proteins, on the other hand, are produced in the stationary phase of growth. The early expression of MSCRAMMs contributes to colonisation in tissue, and the later secretion of toxins contributes to the spread of bacteria(Rachel J Gordon and Lowy, 2008).

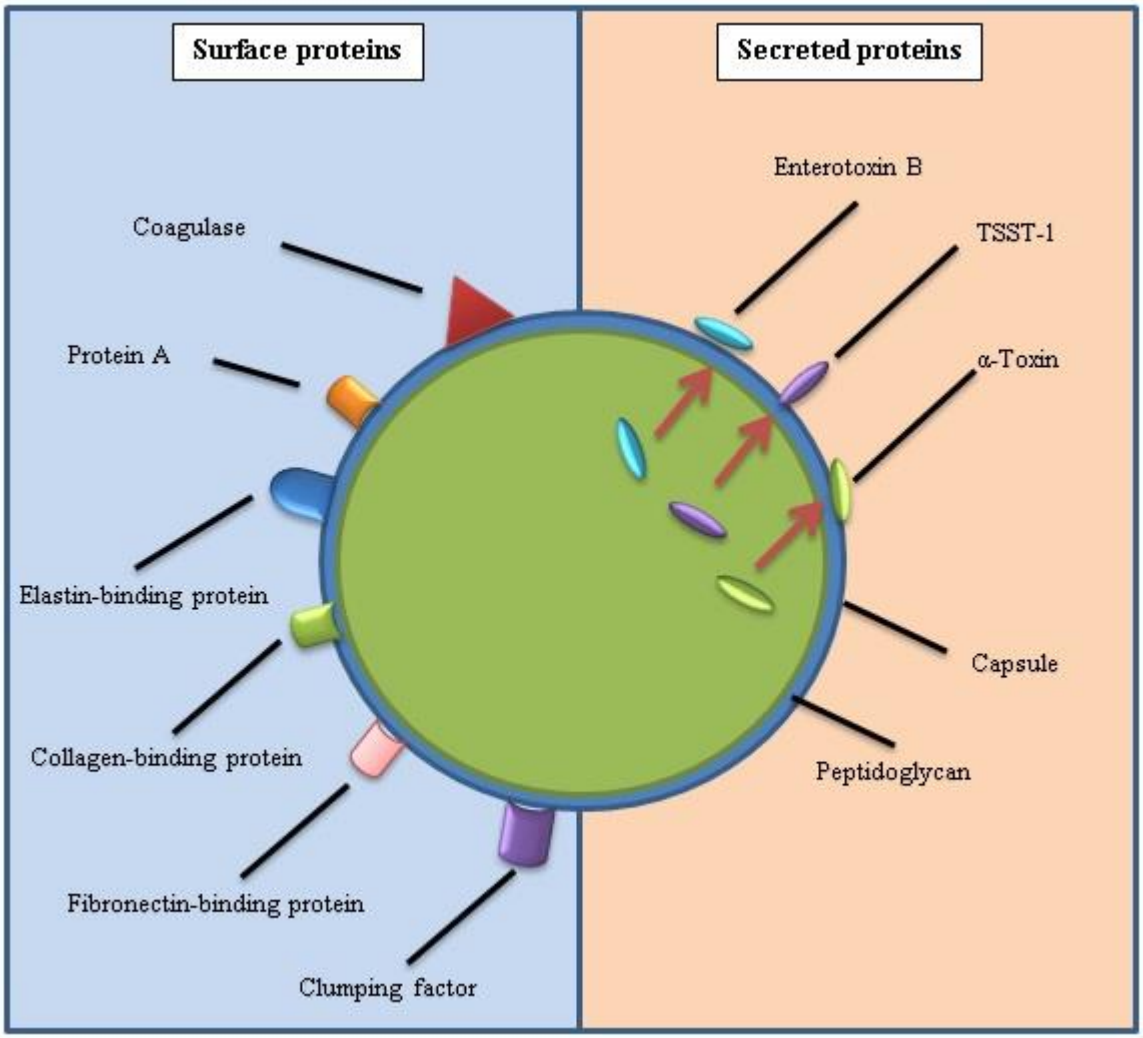



**Figure1.3. Pathogenic factors of *Staphylococcus aureus*, with structural and secreted products both playing roles as virulence factors.** A: Surface and secreted proteins. B and C: Cross-sections of the cell envelope (Gordon and Lowy, 2008).

The accessory gene regulator, *agr*, has a key role in regulation of the virulence of *Staphylococcus* (Yarwood and Schlievert, 2003). It is associated with some clinical syndromes; along with the 'staphylococcal accessory regulator' and *agr* mutants have been found to be low in virulence (Cheung *et al.*, 1992; Novick, 2003; Yarwood and Schlievert, 2003). A study has also shown that nasal carriers of *S. aureus* had the highest mortality rate compared with non-carriers (Wertheim *et al.*, 2004). This was the case in patients who were hospitalised in a nonsurgical setting. There is still much required in the way of understanding how the host may contribute to staphylococcal disease. Colonisation was also found to confer immunity in those who have developed a staphylococcal infection, due to the infection being induced by a colonising strain (Wertheim *et al.*, 2004). The occurrence of toxic shock syndrome can be reduced by antibodies. It was found that patients lacking antibodies of the toxins implicated during acute illness would almost certainly be subject to toxic shock syndrome (McCormick *et al.*, 2001). Finally, one must note that different strains of *S. aureus* have differing abilities to produce biofilms, and also differing abilities to evade the immune response mechanisms of their host. Virulence factors can be compared with the resulting clonal type, but can also be unrelated to genetic factors.

#### 1.1.4.Hospital-acquired (HA)-MRSA: History and pathogenesis

MRSA isolates were reported within a year of the discovery of methicillin in 1960 (Wu *et al.*, 1996). This resistance was observed in the penicillin-binding protein (PBP2A) encoding gene, *mecA.* This gene also confers a low affinity to β-lactam antibiotics. The genetic element 'the staphylococcal cassette chromosome (SCC*mec*) encodes the *mecA* gene as well as recombinase genes within the cassette chromosome (*ccrA/ccrB/ccrC*). These regulate SCC*mec* transmission: horizontally, intra and inter-species. SCC*mec* is absent in coagulase-negative staphylococcal species (Wu *et al.*, 1996). Few lineages of MRSA have been the product of SCC*mec* transfer into successful clones of MSSA.

Sequencing of a group of housekeeping genes (multilocus sequence typing), was used to demonstrate that MRSA clones are derived from 5 distinct groups of clonal complexes or related genotypes all arising from separate ancestral genotypes (Enright *et al.*, 2002). It was found that isolates of MRSA were derived from sequence type (ST) 8-MSSA, which became ST250-MSSA following a point mutation. ST250-MSSA is believed to be the first SCC*mec* recipient yielding MRSA, and termed ST250-

MRSA-I (Enright *et al.,* 2002). Following this study, it was then identified by Crisostomo *et al.*, in 2001, that there were other likely MSSA strain recipients in a separate group of isolates.

Historically, HA-MRSA infections were the result of five major clones disseminated internationally, including the Brazilian, New York/Japan, Hungarian, Pediatric and the Iberian clones (Oliveira *et al.,* 2002). As a result, drug resistant clones account for most HA-MRSA infections in various regions of the world, due to their global dissemination. An example of this dissemination is the spread of the Brazilian clone to other countries including Armenia, Chile, Portugal and Argentina (Oliveira *et al.,* 2002). The ease of this HA-MRSA transmission and establishment of particular strains remains subject to investigation.

One of the main issues faced today is the dominance of these strains in hospitals; commonly due to their antibiotic resistance. But aside from this, it has been also suggested that the increased virulence of clones has resulted in their enhanced colonisation of hosts, as well as their enhanced ease of transmission. Phage type 80/81 is an example of a clone that was responsible for the *S. aureus* pandemic of infections acquired in communities in the 1950s (Robinson *et al.,* 2005). After 1960, it became much less common due to the introduction of methicillin. Phage type 80/81 encodes the Panton-Valentine leukocidin gene (PVL) and is related to the southwest Pacific (SWP) clone. The SWP clone encodes the SCC*mec* IV in addition to the PVL gene. Due to the similarity in genetics of these two strains, it would be expected that the SWP clone would be extremely efficient in the spread of disease. It has been found in the UK and is thought to be closely related to the epidemic strain (ST3-MRSA-II) found in hospitals (Robinson *et al.*, 2005).

#### 1.1.5. Community acquired (CA)-MRSA Infections

Before 1990, it was rare for people who had no previous exposure to a health care setting to contract infection due to MRSA. Between 1989 and 1991, there was an outbreak of infections caused by CA-MRSA in Western Australia, among indigenous Australians, as well as members of neighbouring regions (MMWR Morb Mortal Wkly Rep 1999). During the 1990s, there was an aggressive outbreak of MRSA infections in the US, among individuals with no MRSA risk factors. During this time, four

children infected with CA-MRSA died in North Dakota and Minnesota between 1997 and 1999. Each reported case had associated sepsis from pulmonary abscesses or necrotizing pneumonia, and was fatal (MMWR Morb Mortal Wkly Rep 1999). It was subsequently found that the ST1 and PFGE strains (the MW2 strain) were the cause of these infections. Following this outbreak, CA-MRSA induced infection outbreaks in soft tissue and skin were reported; particularly in soldiers, prison inmates, men having same-sex intercourse and football players (Lina *et al.*, 1999). The ST8 and PFGE strains were found to be the cause of these outbreaks. Similar cases were also found to be the case internationally, additionally causing complications where *S. aureus* prevalence was low. Finally, cases were also found in which PFGE type USA300 caused the progression of pyomyositis and purpura fulminans with toxic shock syndrome (Lina *et al.*, 1999; Vandenesch *et al.*, 2003; Kravitz *et al.*, 2005).

### 1.1.6. Panton Valentine Leukocidin (PVL) as a determinant of Virulence

PVL is a cytotoxin—one of the β-pore-forming toxins. It has been the subject of studies due to its occurrence alongside CA-MRSA infections. The epidemiological association between the two, however, is not common in isolates of HA-MRSA or MSSA. A study carried out in France involved 593 HA-MRSA isolates, which were clear of PVL, however, PVL was found in all strains of CA-MRSA (Dufour *et al.*, 2002). A separate study showed that PVL was present in all CA-MRSA isolates, carried out in a large and international sample group (Vandenesch *et al.*, 2003). It has also been commonly found in both USA300 and USA400 (Saïd-Salim *et al.*, 2005). A study also revealed that co-transcribed PVL genes *lukS-PV* and *lukFPV*, were present in strains of *S. aureus* from patients exhibiting various symptoms of disease (Lina *et al.*, 1999). PVL has also been associated with 85% of strains from community-acquired pneumonia, in comparison with hospital-acquired pneumonia. PVL was also associated with strains causing skin infections such as furunculosis and cutaneous abscess, compared with superficial folliculitis. PVL was not observed in strains associated with infective endocarditis, toxic shock syndrome, or mediastinitis, although few strains were tested (Lina *et al.*, 1999). Another study, in which the role of PVL was investigated in patients with MRSA-induced soft tissue and skin infections, alluded to the fact that PVL is a CA-MRSA virulence factor (Diep *et al.*, 2004). PVL and other staphylococcal leukotoxins are secreted with S and F proteins (Menestrina *et al.*, 2003). The S and F protein combination dictates the

erythrocytolytic, dermonecrotic and leukocytolytic properties of the toxin (Menestrina *et al.*, 2003). PVL is made up of LukS-PV and LukF-PV. Four units of each form octameric b-barrel pores in leukocyte membranes, which contribute to cell lysis (Miles *et al.*, 2002). As a result, neutrophils release cytokines and other inflammatory enzymes (Colin *et al.*, 1994). Further, PVL can contribute to neutrophil apoptosis through interference in mitochondrial pathways, and at higher concentrations, can result in necrosis. It was also found that PVL was responsible for dermonecrosis in rabbits when injected intradermally (Genestier *et al.*, 2005). As a result, the association between strains of PVL CA-MRSA and infections of soft tissue and skin can be somewhat justified, and PVL can further be suggested to be the cause of increased virulence in CA-MRSA.

Contrastingly, studies have also compared the virulence of PVL-positive and PVL-negative strains of CA-MRSA in human polymorph nuclear cell lysis and did not find evidence of a difference in polymorph nuclear lysis (Saïd-Salim *et al.*, 2005; Voyich *et al.*, 2006). To further investigate this, PVL-positive and PVL-negative strains with strong similarity in genetic backgrounds in mouse abscesses were compared against PVL mouse knockouts for USA300 and USA400 strains. Again, no difference was observed in mouse survival in the mouse models. PVL-negative strains were, however, the cause of abscesses of larger sizes in mice. The isogenic PVL strains USA300 and USA400 contrastingly displayed no difference in *in vitro* polymorphonuclear lysis. In light of these data, it was concluded that PVL could not be the soul determinant of disease in CA-MRSA strains (Labandeira-Rey *et al.*, 2007). Further to this, a mouse pneumonia model was also studied. Non-CA-MRSA PVL-positive and PVL-negative strains were infected into mice. It was found that the PVL-positive strains gave rise to necrotizing pneumonia, while the PVL-negative strains showed a slight leukocytic effect. Severe tissue damage and mortality was seen to occur when PVL-negative mutants were complemented with plasmids containing the PVL operon. Further investigations in mice showed that LukS-PV and LukF-PV toxin exposure caused damage in mice lungs, as well as higher rates of mortality, which increased as the concentration of toxin increased. Another side effect was weight loss in mice (Labandeira-Rey *et al.*, 2007). It is important to note that in these experiments, a non-MRSA strain was implemented. To counter this, a separate study showed that PVL was not responsible for mortality in mice, and it was in fact α-hemolysin (Bubeck

Wardenburg *et al.*, 2007). This further suggested that there is little or no link between *S. aureus* virulence and PVL. To add to this, it was also documented that *S. aureus* secretes phenol-soluble modulins; which are often over expressed in strains of CA-MRSA when compared with their expression in HAMRSA strains. This can lead to the destruction of neutrophils, increase inflammation and further increase virulence in mice and bacteraemia models.

### 1.2. *Acanthamoeba*

*Acanthamoeba* is a genus of Free-Living Amoeba (FLA), first described by Castellani in 1930, when he reported the presence of an amoeba in *Cryptococcus pararoseus* cultures (Burger *et al.*, 1995). It is widely spread in the environment. Pathogenic Amoebae cause brain disease and death within days in monkeys and mice, and it first appeared in series of articles in Science (1958) and the Journal of Pathology (1959), written by Culbertson and collaborators. They first found and recognised amoebae accidentally in the lesions of animals that had died from inoculation with a tissue-culture fluid of trypsinised monkey kidney cells. It was assumed that this was caused by an unknown monkey virus, but it was later known to be *Acanthamoeba* (Culbertson *et al.*, 1958). *Acanthamoeba* species are bacteria-eating (bacteriovores), but some are opportunistic pathogens as well (Whan *et al.*, 2006).

#### 1.2.1. Ecology of *Acanthamoeba*

*Acanthamoeba spp.* is the most widespread protozoan in the environment, which have been found from natural and man-made environments as well as in vegetables, mushrooms, cultured cells, fish, reptiles, birds and mammals. (De Jonckheere, 1991).*Acanthamoeba* is ubiquitous in nature as it is found in the soil and different types of natural environments such as seawater, ocean sediments, beaches, pond water, stagnant water, soil, mud, shoreline soils, fresh water lakes, streams, frozen swimming areas, saltwater lakes, river water and even the air (Marciano-Cabral and Cabral, 2003).

In man-made environments, *Acanthamoeba* has been isolated from bottled mineral water, distilled water bottles, thermally polluted factory discharges, cooling towers of electric and nuclear power plants, jacuzzi tubs, hydrotherapy, ventilation ducts, humidifiers, air-conditioning units, sewage, compost and other sources (Marciano-Cabral and Cabral, 2003; Khan, 2009).

In human beings, they have been isolated from nasal cavities, throats, intestines and infected tissues, which include cerebral tissues, lung tissues, skin wounds, cornea and contact lenses (De Jonckheere, 1991).

### 1.2.2. Life cycle

The life cycle of *Acanthamoeba* consists of two stages: vegetative trophozoite, when *Acanthamoeba* is active, performs movement through actin polymerazation, phagocytosing and cellular division; dormant cyst, when it has rigid cell wall to survive undesirable conditions (Rondanelli, 1987).

#### 1.2.2.1. Trophozoite stage

When growth conditions are optimal, such as the organism's having sufficient food, a neutral pH, favourable temperatures and an osmolarity between 50-80 mOsmol/kg, the trophozoite stage starts (Band and Mohrlok, 1973). The trophozoite feeds on bacteria and varies in size from 25 to 40 µm (Marciano-Cabral and Cabral 2003). Organelles typically found in higher eukaryotic cells have also been identified in *Acanthamoeba*, such as: the Golgi complex, the smooth and rough endoplasmic reticula, free ribosomes, digestive vacuoles, mitochondria, and microtubules (Marciano-Cabral and Cabral, 2003). It also has spiny surface projections called Acanthopodia, distinguishing it from other organisms (Figure 1.4), prominent contractile vacuole in the cytoplasm that controls the water content of the cell, and a nucleus with a large central nucleolus.

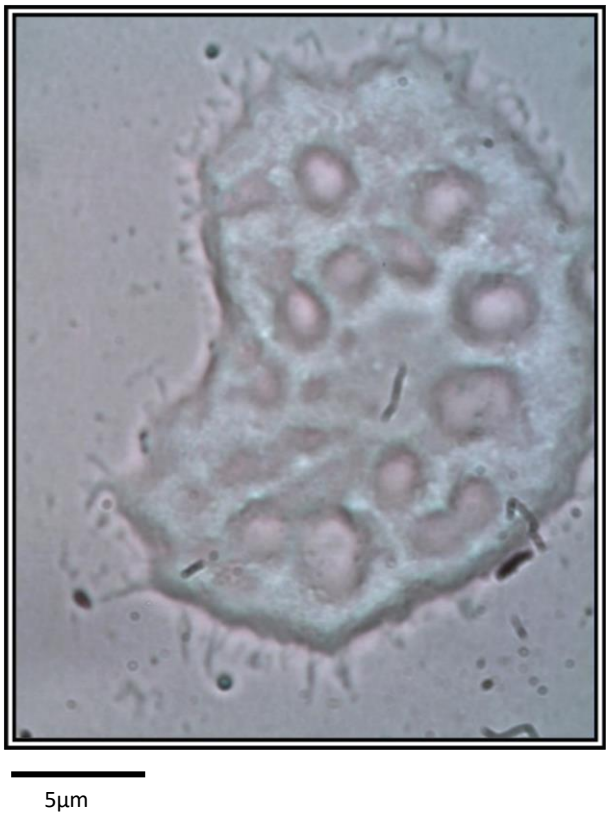



**Figure1.4.** ***Acanthamoeba*** **trophozoite under bright field microscope.**

*Acanthamoeba* has generation time is in the range of 8-24 h for isolates belonging to different species (Khan, 2009). During this generation time, the cell passes through a series of discreet stages, collectively called the growth cycle. *Acanthamoeba* reproduces asexually by binary fissions, resulting in two genetically identical daughter cells. Most of the cellular contents are synthesized continuously, so the cell mass gradually increases as the cell approaches division (Weisman, 1976). This is followed by cell division and involves two overlapping events in which the nucleus divides first, followed by cytokinesis i.e. division of the cytoplasm (Khan, 2009).

#### 1.2.2.2. Cyst stage

The cyst stage occurs under adverse environmental and nutritional conditions, such as insufficient food, unfavourable hypo- or hyper-osmolarity, extreme temperatures or pH, high cell densities, and chemicals so *Acanthamoeba* transform from trophozoites into the cyst stage by undergoing a process known as encystation (McClellan *et al.*, 2001). The combination of encystation and morphological changes cease the cell growth and biochemical differences (Abjani *et al.*, 2016). Decreased cellular levels of ribonucleac acid (RNA), protiens, triacyglycerides and glycogen occur in the process of encystation, which results in decreased volume and dry weight (Khan, 2009). In addition, during encystation, cytoplasmic mass is reduced by the shedding of excess food, cytoplasmic fluid and volumes of solutes, and the trophozoite condenses itself into a double-walled cyst, with the wall serving only as a shell to help survive hostile conditions (Marciano-Cabral and Cabral, 2003; Dudley *et al.*, 2005). There is a diagramatic representation of the life cycle of *Acanthamoeba* (Figure 1.5).

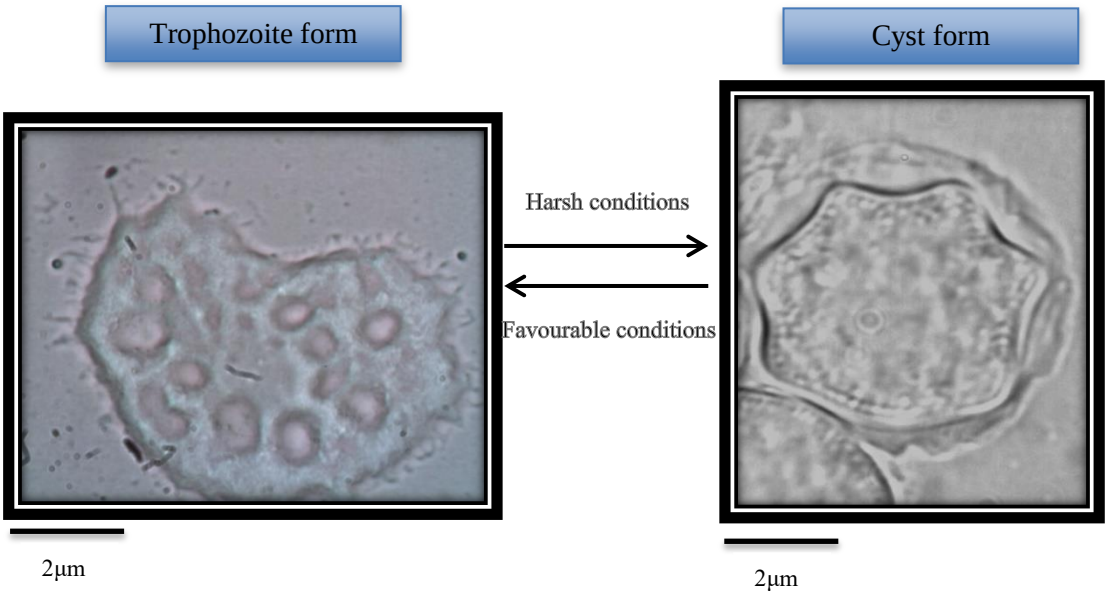



**Figure1.5. Diagram of the life cycle of *Acanthamoeba* under bright field microscopy.**

### 1.2.3. Feeding of *Acanthamoeba*

Environment microorganisms are the food source of *Acanthamoeba* (Weekers *et al.*, 1993). There are contractile vacuoles in cytoplasm of *Acanthamoeba* trophozoite and acanthopodia, emerging on its surface, are used for capturing food particles like bacteria, as well as tiny organic particles. Its feeding is done by pinocytosis and phagocytosis. Pinocytosis is when cell is uptaking liquid particles when phagocytosis is a process of uptaking of solid particles. At temperatures of 30°C, *Acanthamoeba* performs both phagocytosis and pinocytosis for consumig food particles and liquid (Figure 1.6) (Khan, 2009). During the process of phagocytosis, plasma membrane surrounds each particle and specific receptors mediate attachment. Afterwards, particles are taken up and surrounded by membrane inside the cytoplasm, forming new phagosomes. Following that, the phagosome combines with lysosomes containing hydrolytic enzymes, such as acid phosphatase, acid beta-glucosidase, n-acetyl glucosaminidase, alpha-glucosidase, acid beta-glucosidase etc. The outcome is formation of phago-lysosome ( Dudley, *et al.*, 2005) leading to acid hydrolysis over a 30 min period.

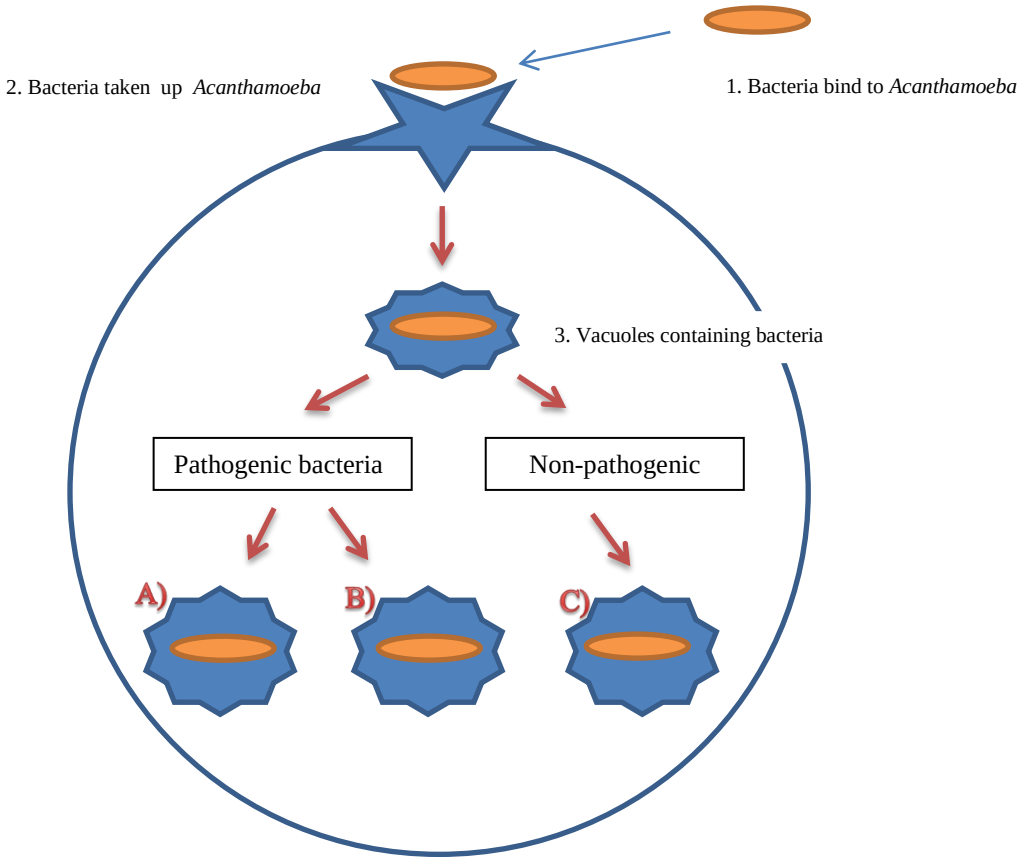



**Figure1.6.** ***Acanthamoeba*** **displaying the bacterial interactions within the cytoplasm.** (A) *Acanthamoeba* acting as a host for bacteria. (B) *Acanthamoeba* acting as 'Trojan horse' for bacteria transmission. (C) *Acanthamoeba* acting as predator of bacteria.

### 1.2.4. Classification

Identification of *Acanthamoeba* at the genus level is comparatively easy due to the occurrence of spiny superficial projections, named acanthopodia, on trophozoites. Nevertheless, using morphological criteria, identification of these amebae at the species level has been problematic. *Acanthamoeba* spp. have been positioned into three morphological groups (I, II, and III) based on cyst size and shape (Pussard and Pons, 2014). Species in group I were labelled on the basis of having a rigid cyst in comparison to that of species in the other groups. Species in group II were categorized as having a crinkly ectocyst and an endocyst which could be stellate, polygonal, triangular, or oval. Species in group III typically displayed a thin, smooth ectocyst and a round endocyst. However, classification of *Acanthamoeba* based on morphological characteristics of the cyst wall has been demonstrated as unreliable since cyst morphology can transform depending on environmental conditions (Daggett *et al.*, 1985). The genus *Acanthamoeba* was first established in 1931. The members of this genus were isolated very frequently from different environments. The taxonomical classification of the genus *Acanthamoeba* was confusing (Visvesvara, 1991). However, the traditional scheme of classification of *Acanthamoeba* based on ribosomal ribonucleic acid sequences is given below (Siddiqui and Khan, 2012). The discovery of progressive molecular techniques directed to the revolutionary work of the late Dr. T. Byers (Ohio University, USA) in the classification of the genus *Acanthamoeba* based on r-RNA gene sequences. Since life developed in the aquatic, most likely through self-replicating RNA as the genetic material or as a common ancestor and evolved into various forms, it is rational to investigate the evolutionary associations through r-RNA. Moreover, this is a highly accurate, consistent and instructive arrangement. Each base presents a single character providing a precise and various systematic. Based on r-RNA sequences (Figure 1.7), the genus *Acanthamoeba* is distributed into 20 different genotypes (T1–T20) (Adamska, 2016; Behera *et al.*, 2016). Every genotype displays 5% or more sequence deviation among diverse genotypes. With the increasing research interests in the area of *Acanthamoeba* and the universal accessibility to cutting-edge molecular techniques, undoubtedly additional genotypes will be identified. These studies will help to clarify the role of *Acanthamoeba* in the ecosystem, bacterial symbiosis, as well as in causing primary and secondary human infections (Siddiqui and Khan, 2012).

| Genotype Type | Name of *Acanthamoeba* with Accession number | Human disease association |
|---|---|---|
| T1 | *A. castellanii* V006 (U07400) | Encephalitis |
| T2 | *A. palestinensis* (U07411) | Keratitis, Encephalitis |
| T3 | *A. griffin* (S8137.1) | Keratitis |
| T4 | *A. castellanii* 9GU (Af251938) | Keratitis, Encephalitis |
| T5 | *A. leticullata* PD2S (U94741.1) | Keratitis, Encephalitis |
| T6 | *A sp. BR010* (JX683400.1) | Keratitis |
| T7 | *A. astronyxis* (AF019064) | NA |
| T8 | *A. tubiashi* (AF019065) | NA |
| T9 | *A comandoni* (AF019066) | NA |
| T10 | *A. culbertsoni* (CDCV409C) | Keratitis, Encephalitis |
| T11 | *A. sp. M-1* (HM778019.1) | Keratitis |
| T12 | *A. healyi* (AF019070) | Encephalitis |
| T13 | *A. sp.* UWC9 (AF132134) | NA |
| T14 | *A. sp.* PN 15 (AF333607) | NA |
| T15 | *A. jacobsi* AC080 (AY262361) | Keratitis |
| T16 | *A. sp. cvX* (GQ380408) | NA |
| T17 | *A. sp. TSP07* (JF325889) | NA |
| T18 | *A. sp. CDC:V621* (KC822461) | Encephalitis |
| T19 | *A. sp. USP-AWW-A68* (KJ413084) | NA |
| T20 | *A. sp. OSU 04-020* (DQ451161) | Keratitis, Encephalitis |

**Figure1.7. Comparison of *Acanthamoeba* spp. Isolates with reference strains available in GenBank.**

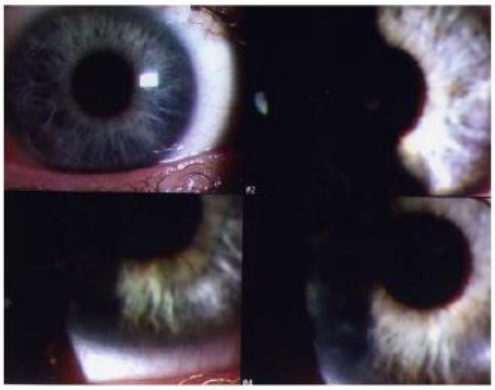
### 1.2.5. Human Infections

Both pathogenic and non-pathogenic strains of *Acanthamoeba* spread widely in the environment and cause different infections such as *Acanthamoeba* keratitis and encephalitis. Approximately, 50-100% of healthy people have serum antibodies which are hostile to *Acanthamoeba* (Chappell *et al.*, 2001). The following sections will describe some of the significant diseases caused by *Acanthamoeba*.

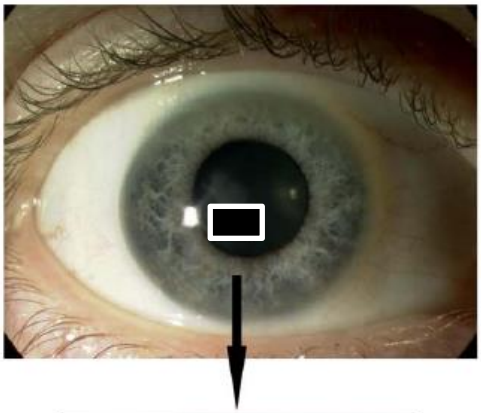
#### 1.2.5.1. *Acanthamoeba* keratitis

*Acanthamoeba* Keratitis (AK) is an eye infection, which is painful, progressive and sight-threatening infection (Alsam *et al.*, 2008). Although it is existing globally, *Acanthamoeba* keratitis (AK) is a rare condition. The first cases of ocular infection were identified in 1973 both in the UK and USA (Illingworth *et al.*, 1995), but the first case of AK was described in 1974 (Naginton *et al.*, 1974). AK was linked with five species of *Acanthamoeba*: *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, *Acanthamoeba hatchetii*, *Acanthamoeba polyphaga* and *Acanthamoeba rhysodes* (Logar and Kraut, 1997). It can arise in patients of any age, sex or race, but typically manifest in young, healthy adults. AK grows generally in contact lens wearers due to inadequate disinfection of contact lenses using non-sterile water, homemade saline solution, tap water, hard water, or splashing, swimming or bathing while wearing the contact lenses (Ibrahim *et al 2007*.). In non-lens users, it is related to trauma and exposure to contaminated water and soil (Dart *et al.*, 2009). It causes permanent visual impairment or blindness. The initial stages of AK infections are not different from the signs and symptoms of other common eye diseases. Main symptoms of AK include: eye pain, redness, blurred vision, sensitivity to light, excessive tears etc. Without proper treatment, AK can cause loss of vision. As shown in pictures below (Figure 1.8), the patient who suffered from AK, lost 80% of his vision in one month of infection (Alsam *et al.*, 2008). The treatment of AK is problematic due to its recurrence happens. Therapy usually includes a biguanide in combination with a diamidine. *In vivo* studies on rats demonstrated that miltefosine-polyhexamethylene biguanide combination was highly effective for treatment of AK (Polat *et al.*, 2014). Nevertheless, when available, confoscan and confocal scanning laser tomography (*in vivo* noninvasive screening tools) should be utilised before any type of corneal refractive surgery is carried out, so the latent protozoal infection can be diagnosed and treated (Al Kharousi and Wali, 2012).

A) Early epithelial infection



B) After 6 months of treatment



C) The affected area of the eye

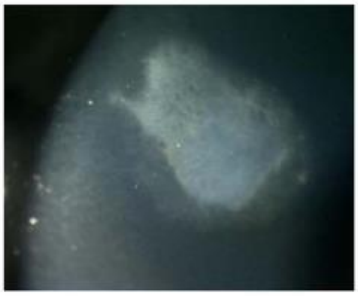
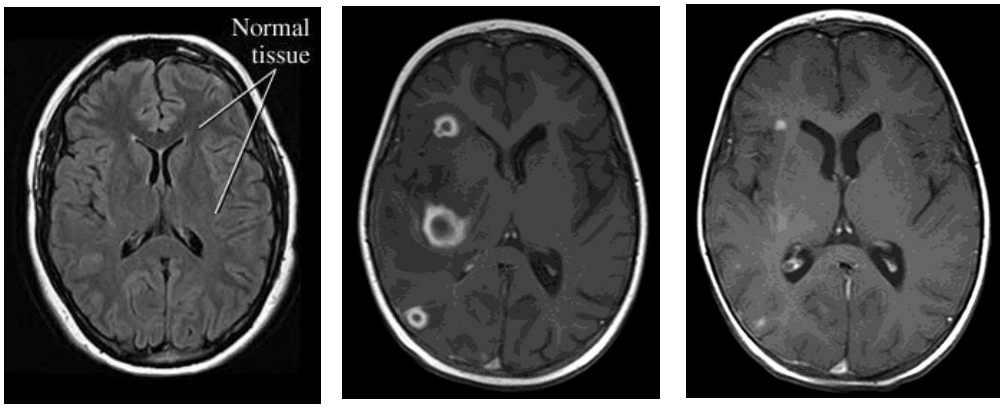


**Figure1.8. Eye infected with *Acanthamoeba* keratitis.** (A) Slit lamp picture of early epithelial infection with *Acanthamoeba* keratitis exhibiting a dendritiform epithelial pattern. (B) Slit lamp picture after 6 months of treatment with polyhexamethylene biguanide, propamidine- isethionate and neomycin. After the treatment the health condition of patient improved, but he lost 80% of his sight via corneal scarring. (C) Slit lamp picture displaying the affected area of the eye (Alsam *et al.*, 2008)**.**

#### 1.2.5.2.*Acanthamoeba* Granulomatous Encephalitis (AGE)

It is a serious infection of the brain and spinal cord that is typically caused by *Acanthamoeba* in persons with compromised immune system (Figure 1.9). The characteristic symptoms of AGE include headache, stiff neck, nausea, vomiting, tiredness, loss of balance and body control, seizure and hallucinations (Dudley, *et al.*, 2005). *Acanthamoeba* species can produce multifocal, chronic and subacute granulomatous encephalitis, with trophozoites and cysts presented in central nervous system (CNS) (Gardner *et al.*, 1991). Since haematogenous spread is a pre-requisite in AGE infection, the first line of defence is induced by complement initiation. Complement initiation occurs through the classical pathway triggered by antibody recognition of *Acanthamoeba* exterior antigens, the substitute pathway activated by C3 binding to the amoebic surface and mannose-binding lectin pathway triggered by *Acanthamoeba* surface saccharide configuration (Ferrante and Rowan-Kelly, 1983) Overall, these results display that the immune response of AGE is not clear. Additional studies are essential to categorize the factors that lead to this lethal infection. Since a high occurrence of *Acanthamoeba* antibodies is detected in both racially and ethnically diverse populations, it is not unexpected that this infection is typically limited to immunocompromised or debilitated patients (Pumidonming *et al.*). There is no effective treatment and neither a quick method to cure nor to diagnose this sickness. Existing therapeutic agents comprise a combination of ketoconazole, fluconazole, sulfadiazine, pentamidine isethionate, amphotericin B, azithromycin, itraconazole, or rifampicin that may be effective contrary to the central nervous system infections due to *Acanthamoeba*, but display severe side effects (Kaushal *et al.*). A fruitful result in a HIV-positive individual with AGE was detected following therapy with fluconazole and sulfadiazine (Seijo Martinez *et al.*, 2000). Additional studies are required to determine the mode of action against *Acanthamoeba* and to develop new methods of application and treatment. Treated patients may evolve disabilities such as hearing loss and vision damage (Akpek *et al.*, 2011).




(A) Normal brain tissue (B)Abscess formation (C) After treatment

**Figure1.9. Regression of brain tissues in patients with AGE.** (A) Magnetic resonance imaging (MRI) showing normal brain tissue. (B) MRI on day 44, showing multiple abscess formations with diameters of up to 20 mm. (C) MRI after 2 months, showing regression of abscess (Maritschnegg *et al.*, 2011).

#### 1.2.5.3. Disseminated infections

Disseminated infections affects skin, lungs, sinus and even the brain (Mortazavi *et al.* 2010). Disseminated *Acanthamoeba* infection is rare disease characterized by widespread granulomatous infiltration of the skin and extra-cerebral organs and usually occurs in immuno-compromised patients (Aichelburg *et al.* 2008).

### 1.2.6. Pathogenesis of *Acanthamoeba*

Environmental conditions factors as well as dependence factors upon host define pathogenesis of *Acanthamoeba* infections. The process through which amoebic killing starts include adhesion to the host cells, followed by phagocytosis through the secretion of extracellular proteases and the killing of the host cell. Pathogenic *Acanthamoeba* also possess greater adhesion properties, secreted cytotoxic products, invasion mechanisms and survival mechanisms than non-pathogenic *Acanthamoeba* (Alsam *et al.*, 2003). The pathogenesis of *Acanthamoeba* can be described using two mechanisms: contact-dependent and contact-independent mechanisms.

#### 1.2.6.1. Contact-dependent mechanisms

##### 1.2.6.1.1. Adhesion

Essential step of infection is adhesion. It is an important microbial property that facilitates colonisation and persistence. Anti-adhesion therapies could be helpful in the treatment of diseases caused by *Acanthamoeba*. Adhesion in *Acanthamoeba* seems to be a necessary process in its pathogenesis and is mediated by adhesion-receptors. Adhesion to host cells of *Acanthamoeba* is used during initial step when proceeding to the deeper tissue and to avoid being cleared out during the onset of the disease (Alsam *et al.*, 2005). A protein, known as Mannose Binding Protein (MBP) seems to play a role in adhesion of *Acanthamoeba* to the host. It is expressed on the surface of *Acanthamoeba*. Adhesion of *Acanthamoeba* leads to the next process, which is called phagocytosis (Alsam *et al.*, 2005).

##### 1.2.6.1.2. Phagocytosis

Phagocytosis is an actin-dependent process for *Acanthamoeba* because it leads to host cell damage. Although this process is associated with the uptake of food, it is still very important in

pathogenesis (Marciano-Cabral and Cabral, 2003). *Acanthamoeba* feed on bacteria using similar processes as outlined above (section 1.2.3).

The bacteria uptake by *Acanthamoeba* is a receptor-dependent process and is performed by MBP which is expressed on the surface of *Acanthamoeba* (Allen and Dawidowicz, 1990). This protein is also involved in the early events of *Acanthamoeba* binding to the host cells which indicates its important role in infection (Morton *et al.,* 1991; Yang *et al.*, 1997; Alsam *et al.*, 2003).

Physiological actions of serine proteases are brought about by the proteinase-activated receptors (PAR's) of host cells. These are responsible for bringing out cellular responses to infection, inflammation or injuries (Vu *et al.*, 1991). The active role of PAR's take place upon proteolytic cleavage of their N-terminus by serine proteases. This cleavage displays the ligand domain of the receptors which combine to the cleaved receptor and activate it.

#### 1.2.6.1.3. Ecto-ATPase activities

Ecto-ATPases (Ectoserine Triphosphate diphosphatase) are presented in plasma membranes. The active sites of Ecto-ATPase face the external environment which indicates that Ecto-ATPase may be involved in surface membrane interactions between parasites and their host cells. Ecto-ATPase are considered as necessary for safety from cytolytic effects of Adenosine triphosphate (ATP), cellular adhesion, the regulation of Ecto-kinase substrate concentration, the elimination of signalling and the environment involvement of signal conversion, showing that Ecto-ATPase may play crucial roles in the pathogenesis of *Acanthamoeba* (Sissons *et al.*, 2004).

### 1.2.6.2. Contact-independent mechanisms

Extracellular proteases of *Acanthamoeba* are one of the contact independent factors which target cells in close environment that leads to the death of the host cells as well as degradation of the epithelial basement membrane and underlying stromal matrix (Cao *et al.*, 2008).

Proteolytic enzymes or proteases are a group of enzymes which catalyze the degradation of peptide bonds. In parasitic protozoa the major pathogenicity determinants are proteases which damage the cell directly. There are three types of proteases present in *Acanthamoeba* such as extracellular serine

proteases, cysteine proteases and metalloproteases. *Acanthamoeba* proteases have been identified as harmful factors in AGE, AK and disseminated infections (Sissons *et al.*, 2006a).

To study *Acanthamoeba* extracellular proteases, zymography has been employed in several studies (Ferrante and Bates, 1988) where it has been found to contain elastase activity. It has been shown that elastase may play role in invasion of host tissue by destroying host protective barriers and tissues. They did not find any difference in levels of elastase between pathogenic and non-pathogenic strains, which suggests that it is not a virulence factor. It may play other important roles in *Acathamoeba* species. Cytopathic effects occur when the serine proteases of *Acanthameoba* have been involved in inducing host cell apoptosis (Garate *et al.*, 2004). Proteases from T1 genotype of *A. castellanii* have been found to play an important role in overcoming blood brain barrier during AGE when increasing the permeability by 45%. A 150 kD metalloprotease and 130 kD serine protease were found to be highly expressed in this strain and they were found to be capable of inhibiting collagen I and III, elastin, plasminogen, casein, and haemoglobin; but they failed to cause any cytotoxicity to Human Brain Microvascular Endothelial Cells (HBMEC) on their own (Alsam *et al.*, 2005).

Comparison of protease activity in pathogenic and non–pathogenic *Acanthamoeba* strains showed a significant difference. Pathogenic strains produce increased levels of protease activity and bring about higher levels of cytotoxicity. Extracellular proteases of pathogenic *Acanthamoeba* strains were found to be capable of disaggregating epithelial cells. One of the proteases identified was a 107 kDa serine protease (Lorenzo-Morales *et al.*, 2005).

### 1.2.7.*Acanthamoeba* used as a Trojan horse to carry MRSA

Historically, Greeks constructed a huge wooden horse, and hid a select force of men inside. In biological setting, a Trojan horse can be pro-drug delivering cytotoxins to tumour cells, and endocytic pathway ferring nanoparticles across a cell membrane into a target cell or cell carrying a pathogen, across a biological barrier. In this case, the last mentioned example coincides with the case of *Acanthameoba*. *Acanthamoeba* is considered to be the Trojan horse of the microbial world which is crucial clinically and environmentally (Siddiqui *et al.*, 2012). The presence of *Acanthamoeba* spp. in critically ill patients may

be advantageous to potentially pathogenic bacteria in urine, protecting them against antimicrobial drugs, disinfectants and host immunity. In this way, *Acanthamoeba* spp. may be a supply for pathogenic bacterial agents in severely ill patients or, as Khan *et al* described, a "Trojan horse for bacteria" (Santos *et al.*, 2009). *Acanthamoeba* is a single-cell amoeba, which commonly eats and digests environmental bacteria. Inside *Acanthamoeba*, the pathogenic multi-drug resistant bacteria MRSA survive and replicate (Cardas *et al.*, 2012). *Acanthamoeba* protects the pathogens; however, the bacteria survive and replicates inside *Acanthamoeba* instead of being digested (Alsam *et al.*, 2005). In harsh conditions, *Acanthamoeba* is more likely to form cysts which act as a carrier for spreading MRSA. It was discovered that the pathogens become more virulent and more resistant to antibiotics if they first infect an amoeba and then emerge from it (Moon *et al.*, 2011). It was also discovered that the amoebae and other protozoans help in persistence of MRSA in the hospital environment. The researchers found that in 24 hrs of its introduction, MRSA had infected around 50% of the amoebae in a sample (Saxena and Gomber, 2008). For MRSA, an amoeba acts like a site that helps it to become fitter and more pathogenic.

Generally, bacteria need to follow certain molecular pathways in order to breakthrough a living cell. The same method takes place when the MRSA invades *Acanthamoeba* (Alsam *et al.*, 2005). Thus it is essential to study the signalling pathways in order to identify the ones through which the MRSA invades the *Acanthamoeba*.

### 1.3. The role of signalling proteins in *Acanthamoeba* and bacteria interaction

#### 1.3.1. The role of Protein tyrosine kinase and Protein tyrosine phosphatase:

Tyrosine kinase, a subfamily of the protein kinase which is the protein that work as an "on" or "off" switch in many cellular functions by phosphorylating substrates at tyrosine, serine or threonine residues. These are mainly involved in cellular signal transduction. Tyrosine kinases consists of two major families: one is the trans-membrane receptor linked kinases and other one is cytoplasmic protein/ non-receptor proteins (Bhise *et al.*, 2004).

At the present time, approximately 2000 kinase proteins are known among which 90 protein tyrosine kinases are known to be found in human genome. Of the 90 protein tyrosine kinases, 58 are the

receptor tyrosine kinase (RTK) and 32 are cytoplasmic proteins. RTKs play crucial role in maintaining the cellular homeostasis; likewise in differentiation, cell survival, cell death and adhesion. There are three prominent domains in RTKs: one is the extracellular domain, required for the binding of specific ligand, a trans-membrane domain and an intracellular catalytic domain, which plays important role in activation of downstream signalling.

Most of the RTKs are involved in the activation of two major pathways, phosphoinositide 3-kinase (PI3K)/ AKT (Franke, 2008) and Rat sarcoma (Ras)/Mitogen-Activated Protein Kinase (MAPK) pathways, which play a pivotal roles in cell survival and cell proliferation. These receptors activate these two pathways through several signalling molecules (Kris *et al.*, 2003; Navis *et al.*, 2010).

**1.3.2. Phosphatidylinositol 3-kinase (PI3K):**

There are 15 kinase proteins in Phosphotidylinositol 3-Kinase and all of them have their own distinct substrates specificity, expression patterns and mode of actions. PI3 kinases mainly involved in the catalysing of the synthesis of phosphatidylinositol phosphate (PI), phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-trisphosphate (PIP3). These three lipid derivatives are involved in the cellular physiological processes like cell growth, survival, differentiation, and chemotaxis (Katso *et al.*, 2001).

The PI3K/Akt pathway gets started with the activation of PI3K by RTKs (Figure 1.10). Activated PI3K phosphorylates the PIP2 (3'-hydroxyl group of phosphatidylinositol 4,5 diphosphate) in PIP3 binds to Phosphoinositide-dependent kinase-1 (PDK1) and Akt, resulting in the autophosphorylation of PDK1. Phosphorylated PDK1 transphosphorylates Akt protein along with various proteins (Engelman *et al.*, 2006; Franke, 2008). It is highlighted that the pathway branches below the node occupied by Akt to influence many cellular processes promoting other pathways such as cycle progression, cell growth, energy metabolism and resistance to apoptosis (Knowles *et al.*, 2009).

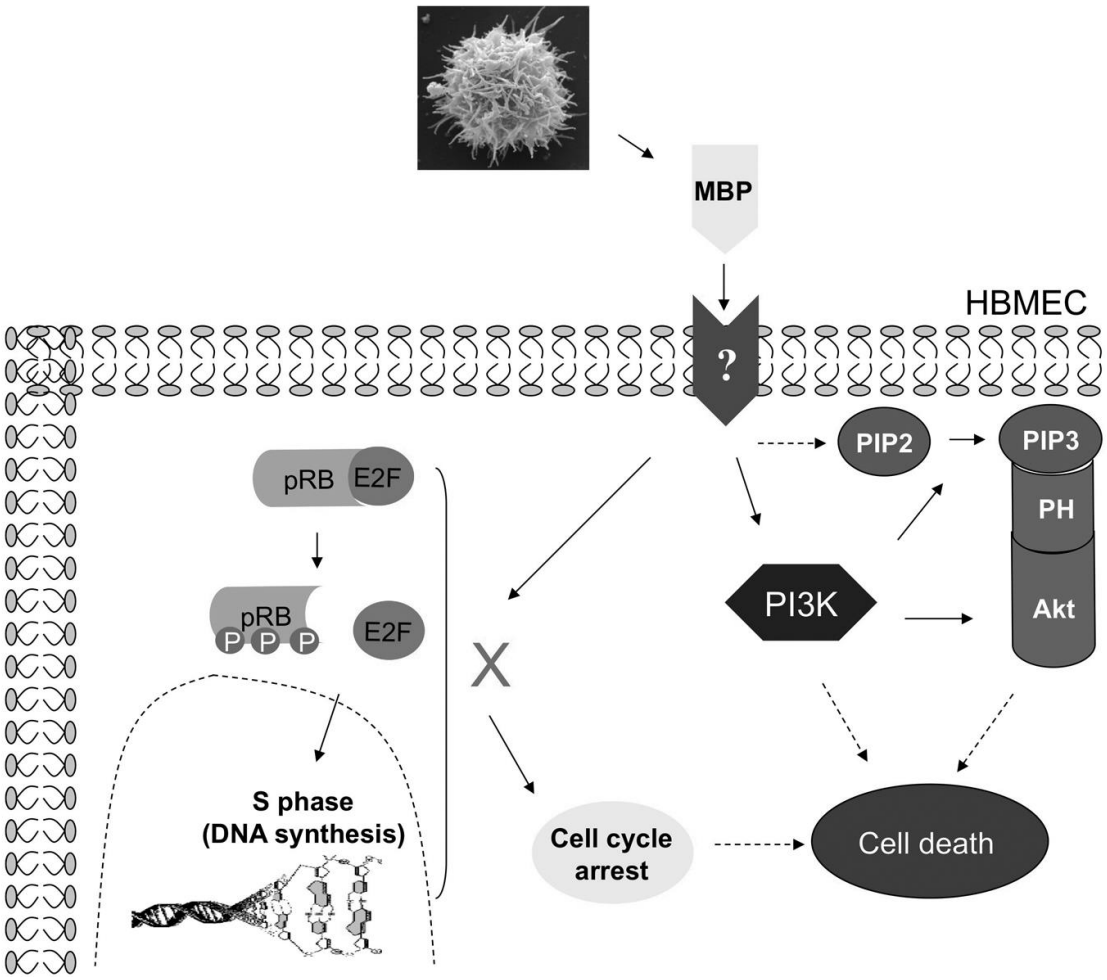



**Figure1.10. Signalling response of host to *Acanthamoeba*.** *Acanthamoeba* causes cell cycle arrest through disruption of gene regulation, pRB phosphorylation modulation and as part of the PI3K pathway. *Acanthamoeba* also inhibit tight junctions through interference with zonula-1 and occluding proteins. MBP (Mannose-Binding Protein) binds at the membrane causing inhibition of E2F (a proliferation-inducing transcription factor). PIP2 (phosphatidylinositol-4,5-bisphosphate) and PIP3 (phosphatidylinositol-3,4,5-trisphosphate) are recruited following membrane protein activation, further recruiting the PH domain of Akt (protein kinase B). Kinases are essential in this pathway for signal transduction to give rise to apoptosis, cell proliferation, and angiogenesis (Khan, 2009).

The PI3K enzymes have prominent involvement in various patho-physiology which makes them important therapeutic targeting sites. PI3K serves as the major component of insulin signalling and moreover has prominent roles in inflammation and autoimmune disease, thrombosis, viral infection, and cancer.

Protein tyrosine phosphatases are a cluster of enzymes that take away phosphate groups from phosphorylated tyrosine residues on proteins. Protein tyrosine (pTyr) phosphorylation is a common post-translational adjustment that can generate novel recognition themes for protein interactions and cellular localization, affect protein steadiness, and manipulate enzyme function. As a result, sustaining a suitable level of protein tyrosine phosphorylation is crucial for a number of cellular functions. Tyrosine-specific protein phosphatases catalyse the elimination of a phosphate group bound to a tyrosine residue, by means of a cysteinyl-phosphate enzyme intermediate. These enzymes are essential regulatory ingredients in signal transmission pathways, and are important in the control of cell growth, proliferation, differentiation, transformation, and synaptic plasticity (Navis *et al.*, 2010).

## 1.4. Antimicrobial agents and therapeutic strategies

### 1.4.1. Photodynamic Therapy (PDT)

#### 1.4.1.1. The principle of photodynamic therapy (PDT)

Photodynamic therapy (PDT) is used to treat a wide range of medical conditions including malignant cancers and it is a non-invasive and minimally toxic treatment strategy (Moan and Peng, 2003). PDT is a treatment that involves Photosensitizer (PS), light source and molecular oxygen within biological tissue. The wavelength of the light source needs to be fit for photosensitizer excitation and energy transfer to produce reactive oxygen species (ROS) such as radicals and singlet oxygen (Chen *et al.*, 2002; Moan and Peng, 2003). The combination of these three components leads to the chemical destruction of the tissues that have taken up the photosensitizer and have been locally exposed to light (Figure 1.11). Photosensitizers absorb the energy of light of specific wavelength, which excites the electrons from ground singlet stage (SO) to an excited singlet stage (SI). Photosensitizer can return to ground state through fluorescence or non-radioactive decay; it may change into intersystem crossing and enter excited triplet state (T1). Molecular oxygen is present in ground triplet state and so an energy transfer can occur between the triplet state of photosentiser and molecular oxygen; the energy transfer comes out in the generation of singlet oxygen (Tacelosky *et al.*, 2012).

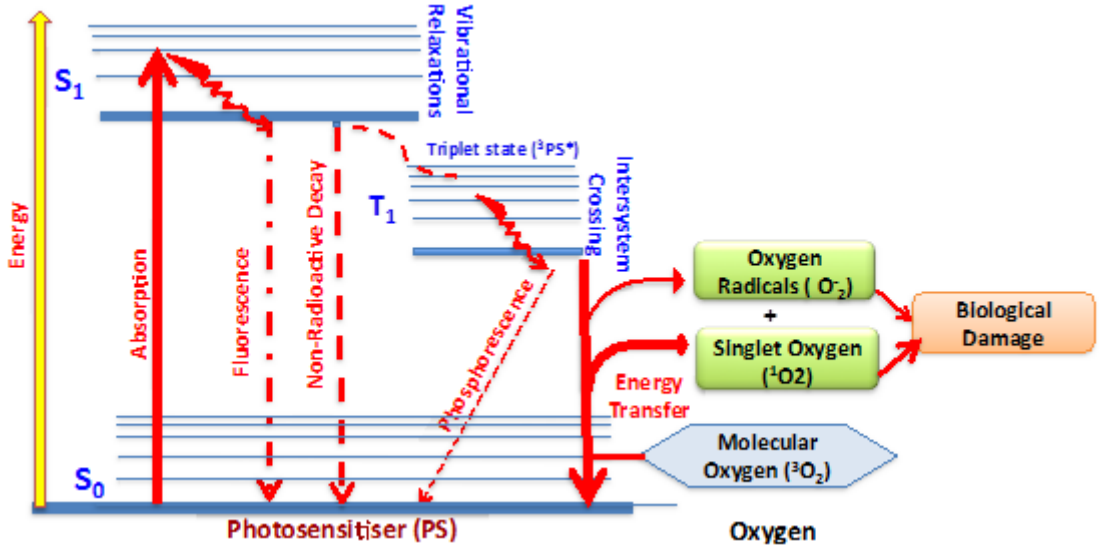



**Figure1.11. Jablonski diagram depicting changes in molecular electronic states associated with photodynamic therapy** (Tacelosky et al., 2012).

Photosensitizer molecules are usually excited from ground state to a short lived excited singlet state S1 and S2, on absorption of light. Following excitation, fast radiation less relaxation processes yield the excited triplet state T1 (Figure 1.10). It is considered there are two methods by which photosensitizers can act:

a) The radical-type hypothesis: Electron transfer processes are increased on excitation of a photosensitizer molecule and results in reducing an electron and oxidizing hole.

b) The singlet oxygen hypothesis: Singlet oxygen ($^1O_2$) is the cytotoxic agent responsible for the photo-inactivation of the tissue. The generation of $^1O_2$ results from energy transfer upon the interaction of excited photosensitizers with the molecular oxygen. The interaction of singlet oxygen with intracellular targets subsequently leads to the damage of cell membranes, mitochondria, lysosomes and nuclei, inducing tissue necrosis (Ochsner, 1997; Chen *et al.*, 2002).

#### 1.4.1.2. Applications of Photodynamic Therapy

One of the major PDT applications is the treatment of skin conditions notably, acne, psoriasis and also skin cancers. The PS can be applied topically and locally which is excited by a light source. In the local treatment of internal tissues, PS should be introduced to the targeted area first and then, light can be delivered using endoscope or fibre-optic catheter (Silva *et al.*, 2006). PDT has also previously been used to kill pathogenic microorganisms *in vitro*, but its use to treat infections in animal models or patients has not, as yet, been developed (Hamblin and Hasan, 2004; Wu *et al.*, 2006). PDT was also used to decontaminate blood and bone marrow samples before transfusion or transplant procedures (Boumédine and Roy, 2005). One study in Hong Kong confirmed that PDT was the tool to eradicate the rising incidence of multidrug-resistant (MDR) pathogens such as MRSA and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli*. The photodynamic inactivation (PDI) mediated by poly-L-lysine chlorine (e6) conjugate (pL-ce6) and toluidine blue O (TBO) showed a high efficacy against pathogens under investigation in a light- and drug dose-dependent matter. (Tang et al., 2007).

A great amount of photosensitizers have been assessed for possible clinical use. Numerous of these photosensitizers absorb light below 600 nm, thus restricting their therapeutic use to superficial lesions. The treatment of thicker tissue layers needs photosensitizers that absorb light above 600 nm. A huge number of photosensitizers with such a characteristic have been evolved in the last 20 years, and are presently being evaluated in clinical trials, largely for different cancers. The peculiarities of these photosensitizers to target lesions are still too low, current and future research is concentrated on technologies to unravel this problematic situation (*Photosensitizers in Medicine*, 2009).

#### 1.4.1.3. Photodynamic inactivation (PDI) of bacteria

There have been additional reports of bacteria being killed or inactivated by various combinations of PS and light, since many years have passed using PDT. It was discovered that there were fundamental differences in susceptibility to PDT between Gram (+) and Gram (-) bacteria. In the last decade, it has been found that, in general, neutral or anionic PS molecules are efficiently bound to and photodynamically inactivate Gram (+) bacteria, whereas they are bound only to the outer membrane of Gram (-) bacterial cells and do not inactivate them after illumination (Hamblin and Hasan, 2004). The cytoplasmic membrane of Gram (+) is surrounded by a porous layer of peptidoglycan and lipoteichoic acid that allows PS to cross and make it highly susceptible (Hamblin and Hasan, 2004). Whereas, the cell envelope of Gram (-) bacteria consists of an inner cytoplasmic membrane and an outer membrane, which are separated by the peptidoglycan-containing periplasm (Figure 1.12). In the outer membrane, several different proteins are present. Some of them function as pores to allow the passage of nutrients, whereas others have an enzymatic function or are involved in maintaining the structural integrity of the outer membrane and the shape of the bacteria. Therefore, the outer membrane forms a physical and functional barrier between the cell and its environment.

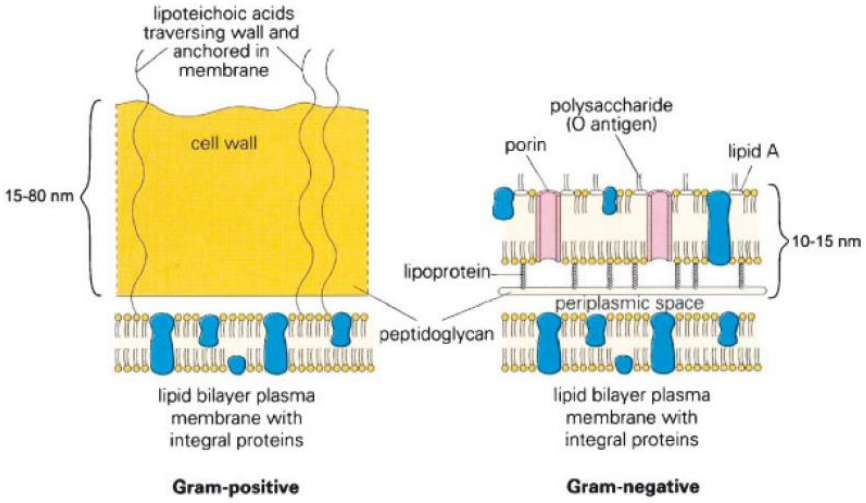



**Figure1.12. The cell walls diagram of Gram (+) and Gram (-) bacteria** (Jori *et al.*, 2006).

Several scientific groups investigated ways that would allow photodynamic inactivation (PDI) of Gram (-) species. Nitzan and co-workers conducted an experiment in which the permeability of the Gram (-) bacterial outer membrane was increased to allow PS that are usually excluded from cell to penetrate to a location where the reactive oxygen species generated upon illumination could cause fatal damage by using the polycationic peptide polymyxin B nonapeptide (PMBN). PMBN does not release lipopolysaccharide (LPS) from the cells, but 'expands' the outer leaflet of the membrane, allowing PS, such as deuteroporphyrin (DP), to penetrate and permitting the PDI of *E. coli* and *P. aeruginosa* (Ehrenberg *et al.*, 1992). They demonstrated an interaction between PMBN and DP in solution, and speculated that this binding assisted the penetration. In another technique adopted by some groups was to use a PS molecule with an intrinsic positive charge. Wilson and co-workers used the phenothiazine Toluidine Blue O to carry out PDI on a wide range of both Gram (+) and Gram (-) bacteria. The growth phase of the bacteria did not make a large difference to their susceptibility to the PDI, though the presence of serum in the medium decreased killing. A scientific group in Italy has used cationic porphyrins [meso-tetra (N-methyl)-4- pyridylporphine tetraiodide and tetra-4-(N,N,N trimethylanilinium) porphine (Figure 1.13) to photoinactivate Gram (-) species, such as *Vibrio anguillarum* and *E. coli* (Wilson, 1992, 1993).

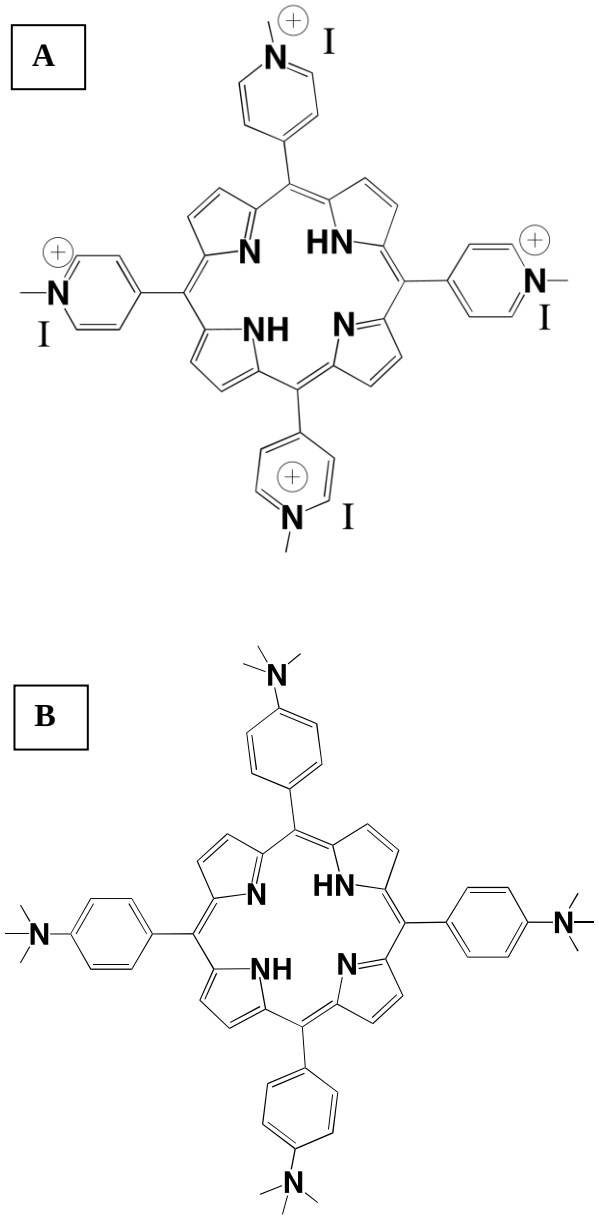



**Figure1.13. Molecular structure of porphyrins.**. A: meso-Tetra (N-methyl)-4- pyridylporphine tetraiodide. B: Tetra-4-(N,N,N trimethylanilinium) porphine (Wilson ., 1992 & 1993).

They deduced that washing the loosely-bound PS on the cells before illumination decreased the phototoxicity action of PS's. They explained this finding by proposing that the first dose of light on the PS bound to the outside of the outer membrane causes initial limited photo damage that then allows the further penetration of the PS (Fuortes *et al.*, 1996). The research group in the UK has used cationic phthalocyanines for PDI of Gram (-) bacteria (Minnock *et al.*, 1996).

An investigation on *E.coli* was performed in particular and the mechanism of uptake was studied. They found that incubation with zinc pyridiniumphthalocyanine (PPc) in the dark led to the increased sensitivity of the bacteria to hydrophobic antibiotics, such as erythromycin, (but not the hydrophilic). Incubation with PS also led to the increased uptake of radio-labeled protoporphyrin, a process that was reversed in the presence of up to 50 mM $Mg^{2+}$ ions. These observations are consistent with the uptake of PPc proceeding through the self-promoted uptake pathway (Dahl *et al.*, 1987, 1989). There are also reports of PDI of Gram (-) bacteria, clearly indicating that the PS does not have to penetrate the bacteria to be effective. Indeed, it does not even have to come into contact with the cells. According to this, if singlet oxygen can be generated in sufficient quantities near to the outer bacterial membrane, it will be able to diffuse into the cell and cause damage on vital structures (Dahl *et al.*, 1989).

Since MRSA infection is resistant to different types of antibiotics, PDT treatment could be used as an alternative strategy to treat the infectious diseases caused by MRSA. According to studies, the antibody immunoglobulin G (IgG)-SnCe6 conjugate activated with red light eliminated MRSA bacteria. However, it worked only against a particular strain and at a particular growth phase. Therefore, antibody-photosensitizer conjugate (Ab-SnCe6) was used, instead of IgG-SnCe6, due to its effectiveness on many strains of MRSA in all growth phases (Embleton *et al.*, 2004). In contrast to PDT for cancer, where the PS is usually injected into the bloodstream and accumulates in the tumour, they believe that PDT for localised infections will be carried out by local delivery of the PS into the infected area by methods such as topical application, instillation, and interstitial injection or aerosol delivery. Therefore, the effectiveness of the treatment in destroying sufficient numbers of the disease-causing pathogens would be effective selectivity of the PS for the microbes, thus avoiding an unacceptable degree of PDT damage to

host tissue in the area of infection, and the avoidance of regrowth of the pathogens from a few survivors following the treatment (Hamblin *et al.*, 2011a).

Several chemical natural products with photosensitizing effects have been discovered, and some of these molecules are commercially obtainable. Various photoactive principles are natural compounds. Many reviews recently have concentrated on photodynamic therapy, its effects and applications, but less attention has been paid to plant extracts or compounds of natural origin investigated for their phototoxic activity to date.

### 1.4.2. Natural products as antimicrobial agents

#### 1.4.2.1. Psoralen

*Psoralea corylifolia*, is a plant which has psoralen compound in its fruits. Psoralen (Figure 1.14) is a furanocoumarin which is the main active ingredient. A great number of biological properties are present in psoralen, such as preventing the proliferation of mucoepidermoid carcinoma, bladder carcinoma *in vitro* and mammary cancer cells (Liu *et al.*, 2015). It has also been previously used to treat a number of conditions such as vitiligo, alopecia areata and tinea. In addition, recent studies have identified a number of different plants that contain psoralen derivatives which were discovered to possess interesting biological properties (Liu *et al.*, 2015). Psoralen is a mutagen, and is utilized for this aim in molecular medicine research. Psoralen interpolates into the DNA and, on exposure to ultraviolet radiation, can form monoadducts and covalent interstrand cross-links (ICL) with thymines preferentially at 5'-TpA sites in the genome, stimulating apoptosis. Psoralen plus UVA (PUVA) therapy has shown considerable clinical efficacy (Wu *et al.*, 2005).

#### 1.4.2.2. Eugenol

Eugenol is a phenolic compound that is present in clove oil, bay leaves, cinnamon and basil (Figure 1.15). It has many uses and one of the most important application in medicine is as an antiseptic as well as a local anaesthetic (Liang *et al.*, 2015). A number of researchers have revealed the anti-Candida biofilm potential of plant-derived chemicals such as flavonoids (Cao *et al.*, 2008), and essential oils (Khan and Ahmad, 2012). Eugenol is the foremost active phenylpropanoid constituent of the

essential oil among various aromatic plants (De Vincenzi *et al.*, 2000). The inhibitory activity of eugenol alone and in combination with fluconazole and amphotericin B in contrary to planktonic cells of *Candida spp*. has been earlier informed. Moreover, eugenol can mediate with the initial stages of biofilm formation, along with the viability of developed biofilm of *Candida albicans* (Khan and Ahmad, 2012). Various antimicrobial agents can affect the manifestation of staphylococcal exotoxins, and eugenol, an essential oil ingredient in plants, has been stated to have activity against both Gram-positive and Gram-negative bacteria. It is crucial to study the effects of eugenol on the expression of the major exotoxins produced by both methicillin-sensitive and resistant *S. aureus* (De Vincenzi *et al.*, 2000).

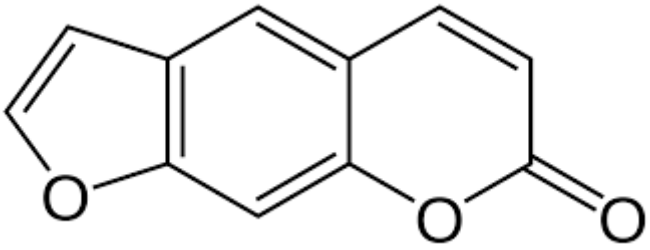



**Figure1.14. Molecular structure of Psoralen (PSO).**

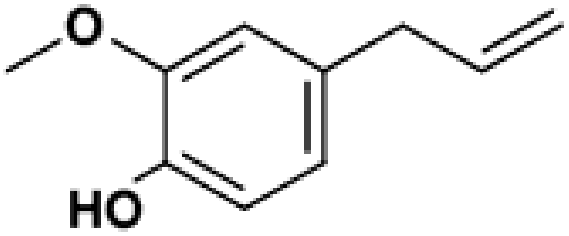



**Figure1.15. Molecular structure of Eugenol.**

**Hypothesis and Aims**

The interaction of *Acanthamoeba* with MRSA and MSSA is distinct as some bacteria phagocytised by *Acanthamoeba* survive intracellularly. This has clinical and environmental importance as *Acanthamoeba* has some similar phagocytic activities with macrophages. The molecular mechanisms which play a role in *Acanthamoeba* phagocytosis still remain unclear despite numerous studies on Mannose-Binding Protein and its role in *Acanthamoeba* interactions. The overall aim of this part of the project was to study the role of protein tyrosine kinases, protein tyrosine phosphatase, and phosphatidylinositol-3 kinase (PI3K), which modulate a variety of cellular events, including growth, structure, function, migration and cell death was investigated.

The overuse and misuse of antibiotics has led to the emergence of multi-drug resistant microorganisms such as MRSA. The subsequent constant demand for the exploration of novel strategies and antimicrobial agents for eradication is essential. To this end, photodynamic therapy was used as a novel promising approach against pathogenic bacteria which involves the usage of visible light, oxygen and a photosensitizer. The second aim of the present study was to demonstrate the ability of *m*-THPC and psoralen photosensitizers to bind to MRSA, MSSA and *Acanthamoeba*, and efficiently generate reactive oxygen species upon photostimulation.

Finally, natural products have attracted much attention as additional and/or replacement of antibiotics. It is difficult, but vital, to develop treatments capable of eliminating pathogenic organisms. Eugenols have attracted much attention and were documented to have antimicrobial properties. It is hypothesized that they could be effective against pathogenic microorganisms that are resistant to conventional drugs. Here, the efficacy of eugenols on the microorganisms under investigation was studied. Four novel eugenols (EN1-EN4) were tested against the survival of *Acanthamoeba castellanii* genotype T4 and the clinical MRSA and MSSA isolates.

# Chapter 2. *Acanthamoeba* interactions with *Staphylococcus aureus*

### 2.1. Background

*Staphylococcus* is a genus of bacteria that can cause diseases in the human body, including superficial infections such as boils, abscesses, and wound infections. In addition, the microbes can also cause septicaemia, endocarditis and pneumonia, skin exfoliation and toxic food poisoning (Greenwood *et al.*, 2007). Over 30 different species of Staphylococci can infect humans, but most infections are caused by methicillin-sensitive *Staphylococcus aureus* (MSSA) (Greenwood *et al.*, 2007). The application of multiple antibiotics led to the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA). Treatment is problematic because this microbe is sensitive to very few antibiotics.

The genus *Acanthamoeba* consists of both pathogenic and non-pathogenic strains. *Acanthamoeba* can cause *Acanthamoeba* Granulomatous Encephalitis (AGE), a fatal central nervous system (CNS) infection (R J Gordon and Lowy, 2008) and Amoebic Keratitis (AK), a painful sight-threatening disease of the eyes. *Acanthamoeba* species also have been associated with cutaneous lesions and sinusitis in AIDS patients and other immune-compromised individuals (Marciano-Cabral and Cabral, 2003).

In addition to its direct role in causing human and animal infections, it is now well established that *Acanthamoeba* acts as a host for various bacterial pathogens including *Legionella pneumophila, Coxiella burnetii, Pseudomonas aeruginosa, Vibrio cholera, Helicobacter pylori, Listeria monocytogenes* and *Mycobacterium avium* and may act as a vector to transmit these pathogens to susceptible hosts (Alsam *et al.*, 2006).

The feeding of *Acanthamoeba* on bacteria starts with the adhesion of bacteria to *Acanthamoeba* cell membrane. This leads to secondary processes such as phagocytosis and toxin secretion. The ecological significance of *Acanthamoeba* phagocytosis is in the uptake of food particles, such as bacterial and fungal cells. However, several studies have shown that phagocytosis plays an important role in the pathogenesis of *Acanthamoeba* (Niederkorn *et al.*, 1999). This is shown by the use of cytochalasin D, cell-permeable and potent inhibitor of actin polymerization. It dislocates actin microfilaments and stimulates the p53-dependent pathways triggering detention of the cell cycle at the G1-S transition. cytochalasin D inhibited *Acanthamoeba* -mediated host cell death, confirming that actin-mediated

cytoskeletal rearrangements play an important role in *Acanthamoeba* phagocytosis. The molecular mechanisms associated with *Acanthamoeba* phagocytosis still remain unclear despite a few studies on mannose-binding protein and its role in *Acanthamoeba* interactions (Alsam *et al.*, 2005; Khan, 2006). Bacteria, in general, need to follow certain molecular pathways in order to invade a living cell. Thus it is essential to study the signalling pathways of *Acanthamoeba* interaction with MRSA in order to identify the involvement of three major specific enzymatic pathways such as tyrosine kinase, PI3K, and tyrosine phosphatases.

Tyrosine kinase is the subfamily of the protein kinases. These are the proteins which are mainly involved in the switching on and off the protein by phosphorylating substrates at its tyrosine residues. These proteins are mainly involved in cellular signal transduction. Tyrosine kinases compromise of two major families: the transmembrane receptor linked kinase family and the cytoplasmic protein/non-receptor protein family (Bhise *et al.*, 2004). Among the 90 tyrosine kinase proteins, 58 are receptor tyrosine kinases (RTKs) and 32 are cytoplasmic proteins. RTKs play crucial roles in maintaining the cellular homeostasis; likewise in differentiation, cell survival, cell death and adhesion (Robinson *et al.*, 2000).

PI3K is a big family of 15 kinase proteins, and all of them having their own distinct substrate specificity, expression patterns and mode of action. PI3K is involved in cellular physiological processes such as cell growth, survival, differentiation, and chemotaxis (Figure 2.1) (Katso *et al.*, 2001). Assessment of PI3K's role in various processes is done using either PI3K mutants or chemical inhibitors of PI3K, such as LY294002 and wortmannin. The use of LY294002, which specifically attaches to the ATP binding site of PI3K and thereby selectively inhibits its catalytic activity, showing PI3K is required for phagocytosis. PI3K is known to be involved in actin reorganisation; a process that is required for phagocytosis, and most likely affects phagocytosis via this route (Alsam *et al.*, 2005).

*Acanthamoeba* is known to promote host cell death via the apoptotic pathway. It has been shown using LY294002 that host apoptosis is induced by *Acanthamoeba* in a PI3K dependent mechanism (Siddiqui and Khan, 2012).

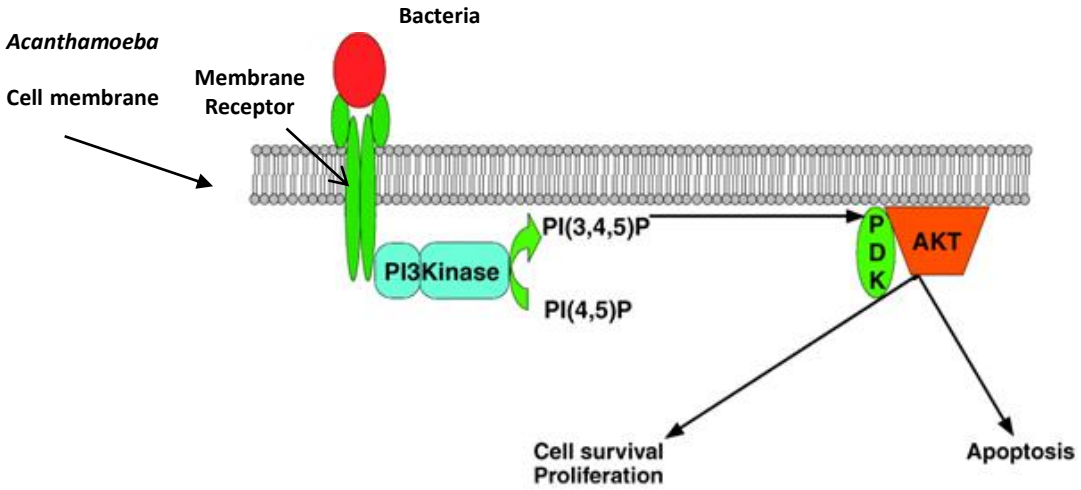



**Figure 2.1. PI3K signalling pathway.** Bacteria bind with *Acanthamoeba* cell membrane receptor, such as RTK (Receptor Tyrosine Kinase) which activates PI3K. Activated PI3K phosphorylates PI(3,4)P ($PIP_2$) to produce PI(3,4,5)P ($PIP_3$). $PIP_3$ in turn, recruits Phosphoinositide-dependent kinase (PDK1) to the *Acanthamoeba* cell membrane that phosphorylates and activates Akt (Planchon *et al.*, 2008).

Tyrosine phosphatases, alkaline phosphatases and certain ATP-ases can be inhibited by sodium orthovanadate ($Na_3VO_4$). It contains a vanadate ion ($VO_4^{3-}$) that is similar in structure to phosphate ions, therefore acting as their analogue. It competes with phosphate ions to bind to the active sites of tyrosine protein phosphatases, but unlike phosphate ions, the binding is irreversible for most tyrosine protein phosphatases (Figure 2.2). Inhibition of tyrosine protein phosphatase in *Acanthamoeba* has been shown to enhance the phagocytosis of *E. coli* (Khan, 2006).

Tyrosine kinase protein could be inhibited by a specific drug, genistein (4,5,7-Trihydroxyisoflavone). It is an isoflavone derivative and is a natural product obtained from plants. It has not yet been ascertained how genistein specifically blocks tyrosine kinases and not others, such as serine/threonine kinases (Alsam *et al.*, 2005).

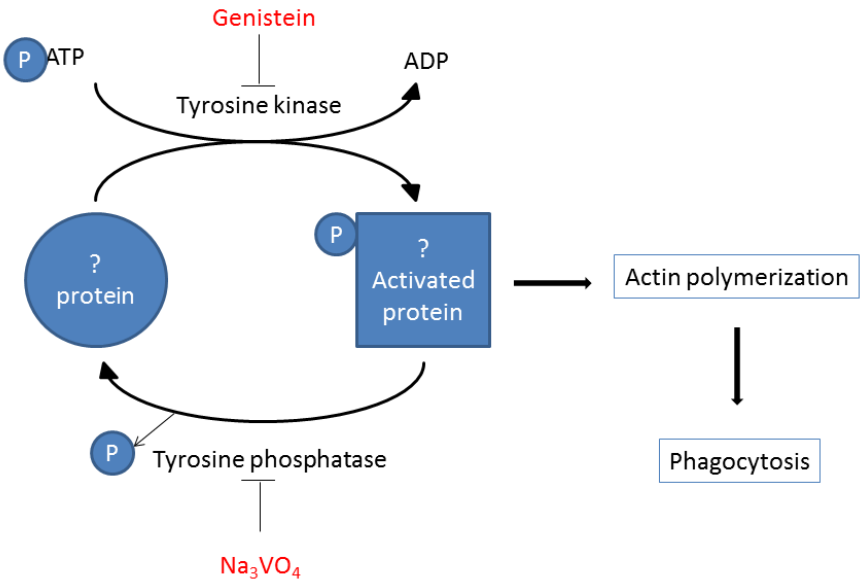



**Figure 2.2. Tyrosine kinase–mediated signal for actin polymerisation in *Acanthamoeba*.** The signal for actin polymerisation is given by an unidentified protein that is the target of phosphorylation/dephosphorylation by tyrosine protein kinases/phosphatases. Genistein, which is an inhibitor of tyrosine kinase, prevents activation of this protein and therefore stops the signal for actin polymerisation. $Na_3VO_4$, which is an inhibitor of tyrosine phosphatase, prevents dephosphorylation of the activated protein, thereby ensuring that actin polymerisation continues (Onsum *et al.*, 2007).

## 2.2. Materials and Methods

All the chemicals for this study were bought from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated.

### 2.2.1. *Acanthamoeba* culture

For this study, *Acanthamoeba castellanii* protozoan was used. It is a clinical isolate (AF239303) from a keratitis patient kindly provided by Dr Maciver, University of Edinburgh. *Acanthamoeba* was cultured without shaking in a PYG [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] medium. The PYG medium utilized for the growth of *Acanthamoeba* was obtained in T-75 tissue culture flasks incubated at 30°C (Sissons *et al.*, 2004).

### 2.2.2.MSSA and MRSA strains cultures

Two strains of each MRSA and MSSA were used in this project. MRSA and MSSA which had been isolated from blood samples of patients with septicaemia, kindly provided by Dr Tony Elston, Colchester NHS Trust Hospital. The bacterial isolates were grown weekly on Cysteine Lactose Electrolyte Deficient agar (CLED) plates. Prior to each experiment, bacteria were inoculated in a Luria-Bertani (LB) medium and incubated at 37°C overnight. For each bacterial strain, the optical density (OD) was measured at 595 nm.

### 2.2.3. Invasion assays

Invasion assays were performed to study the ability of bacteria to invade or be taken up by *A. castellani*. *Acanthamoeba* was cultured in 24-well plates to confluence ($5 \times 10^5$ amoebae or cells/well), followed by the addition of inhibitors and MRSA or MSSA ($1 \times 10^8$ CFU/mL) in a 0.5 mL final volume of Roswell Park Memorial Institute medium (RPMI). After 1 hr of incubation in RPMI, the wells were washed once with Phosphate Buffered Saline (PBS) and then vancomycin (100 µg/mL, final concentration) was added for 45 min to kill the extracellular bacteria. *Acanthamoeba* was washed with 500 µL (PBS) and lysed with 0.5% SDS and then cultured onto CLED plates, incubated at 37°C and bacterial colonies were counted. The proportion of intracellular bacteria was counted as follows: number

of bacteria recovered (CFU)/number of bacteria inoculated at the start of the experiment x 100 (Alsam *et al.*, 2006).

### 2.2.4.Labelling of MRSA and MSSA

MRSA and MSSA strains were labelled with fluorescein isothiocyanate (FITC) by adopting previous methods (Alsam *et al.*, 2005). MRSA and MSSA strains were grown in a shaker overnight at 37°C in one litre LB broth [Bactotryptone 1 % (w/v), 0.5% yeast extract (w/v), 0.5% NaCl (w/v)]. The following day, the bacteria were heat-killed at 65°C for 30 min. Cultures were then centrifuged at 10,000x *g* for 10 min and the pellet re-suspended in PBS. This process was repeated 3 times and finally the pellet was re-suspended in 1 mL PBS. The residue of MRSA and MSSA bacteria's substances were incubated with FITC (1 mg) in a carbonate-bicarbonate buffer, pH 9.4 (0.2 M $Na_2CO_3$ (w/v) mixed with O.2M $NaHCO_3$ (w/v) on a shaker for 30 min at room temperature in the dark. The cells were rinsed 4 times as above to remove unbound FITC. Finally, the pellet was re-suspended in one mL PBS and stored at - 20°C in 0.5 mL aliquots each containing 3.5 x $10^7$ FITC-labelled MRSA and MSSA (Rohloff *et al.*, 1994).

### 2.2.5.Phagocytosis Assays

#### 2.2.5.1.*Acanthamoeba* uptake of FITC-labelled heat killed bacteria

*Acanthamoeba castellanii* was incubated on sterile cover slips in 24-well plates in a PYG medium (5 x $10^5$ amoebae/mL PYG/well). Then plates were incubated for 17-20 h. The following day, cells were washed with PBS for 3 times to remove non-adherent *A. castellanii* and then incubated in 400 µl PBS. Phagocytosis was initiated by adding 1.5 µl containing $10^5$ FITC-labelled MRSA and MSSA to each well. Plates were incubated for 25 min at room temperature in the dark to maintain the integrity of the bound FITC. Next, *Acanthamoeba* cells were washed with PBS for 3 times to remove any non-adherent materials, this was followed by the addition of 400 µl of Trypan blue solution [final conc. 1%, (w/v)] for 5 min to quench the fluorescence of extracellular bacteria by binding to FITC-binding site and suppressing it. Finally, *Acanthamoeba* cells were gently washed with PBS for 5 times. *A. castellanii* was

fixed with 96% methanol at 20 °C for 15 min and observed under a fluorescent microscope (Alsam *et al.*, 2005).

#### 2.2.5.2. Invasion of live bacteria inside *Acanthamoeba*

A few colonies of each bacterium were inoculated into 10 mL LB medium and incubated at 37 °C overnight. Next day, bacteria were centrifuged at 3000 RPM for 10 min and the pellets were washed two times with PBS. Following centrifugation and washing, the pellets were resuspended in 1 mM (final concentration) Rose Bengal and incubated at 37 °C for 20 min. Next, $10^6$ *Acanthamoeba* trophozoites /mL were detached, centrifuged (3000 RPM for 3 min) and washed twice with PBS. Then, 10,000 bacteria per *Acanthamoeba* were added in a final volume of 500 µL RPMI 1640 and incubated for 1 h. Following 1 h of co-culture, cells were harvested by centrifugation (3000 RPM for 3 min). Then, the *Acanthamoeba* with bacteria were centrifuged and washed once with PBS. Cell suspensions with intracellular bacteria (10 µL) were pipetted on slides followed by coverslip application for 10 min at room temperature to allow the infected cells to settle and form a monolayer. The intracellular location of bacteria was assessed using the fluorescence microscope (Alsam *et al.*, 2005).

### 2.2.6. Determination of Phagocytic activity

The percentage of *Acanthamoeba* engulfing bacteria was determined in order to quantify the phagocytic activity. Briefly, using a 20X objective, seven fields were randomLy selected. Within these fields, all *Acanthamoeba*, with or without bacteria, were counted and their percentage was determined as follows: % phagocytosis = *Acanthamoeba* engulfing bacteria / total *Acanthamoeba* x 100

### 2.2.7. Statistical Analysis

The results are displayed graphically or tabulated with standard error representing the standard deviations of triplicate measurements. Differences are considered to be significant using the unpaired *t*-test with $P < 0.05$.

## 2.3. Results

### 2.3.1. Invasion assays

To determine the molecular mechanisms associated with the engulfment of bacteria by *Acanthamoeba*, several pathways were studied using different inhibitors as mentioned in materials and methods. Two strains of each MRSA and MSSA were used in these experiments.

#### 2.3.1.1. MRSA and MSSA Invasion Assays

Genistein, LY294002, wortmannin and protein tyrosine phosphatase inhibitor, $Na_3VO_4$ were used. All inhibitors were dissolved in vehicles with non- cytotoxic concentrations (Figure 2.3) *Acanthamoeba* T4 was pre-treated for 30 mins with these inhibitors before incubation with bacteria followed by incubation with MRSA1 for one hr. After this incubation, vancomycin was added to kill the extracellular bacteria, which were lysed and cultured as described in Methods. In general, use of the various inhibitors resulted in decrease of the level of invasion of MRSA1. The lowest level of MRSA1 invasion was noticed when wortmannin was used (0.036% compared with the control 0.118%) ($P<0.05$ using paired *t*-test, one-tail distribution). $Na_3VO_4$ reduced the bacterial invasion by 0.029% compared with T4 control ($P<0.05$ using paired *t*-test, one-tail distribution). LY294002 and genistein had minimal effect (0.014% and 0.015% reduction) (Figure 2.4A). Invasion experiments were repeated using MRSA2 strain in which a similar result pattern was noticed as shown in Figure 2.4B. The lowest level of MRSA2 invasion was noticed when wortmannin and $Na_3VO_4$ were used. In both cases the percentage was 0.028% compared with the control 0.083% ($P<0.05$ using paired *t*-test, one-tail distribution). Minimal effect was noticed when genistein and LY294002 were used (0.052% and 0.063% respectively). The lowest level of MSSA1 invasion was when protein tyrosine kinase inhibitor, genistein, was used: 0.042% in comparison with 0.135% the control sample (Figures 2.4C). Followed by $Na_3VO_4$, wortmanin and LY204002 (0.049%, 0.058% and 0.083% respectively) ($P<0.05$ using paired t-test, one-tail distribution). Simililarly, use of the various inhibitors resulted in decrease of the level of MSSA2 invasion. As indicated in Figure 2.4D, all inhibitors significantly reduced bacterial uptake by *Acanthamoeba* ($P<0.05$ using paired t-test, one-tail distribution). This further confirmed the dependency of *Acanthamoeba* phagocytosis on intracellular signalling pathways.

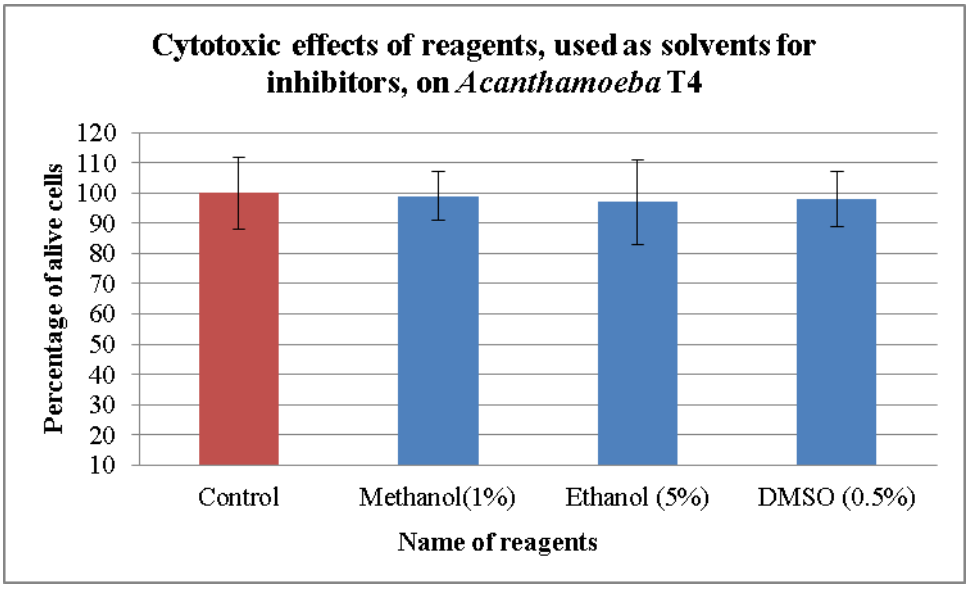
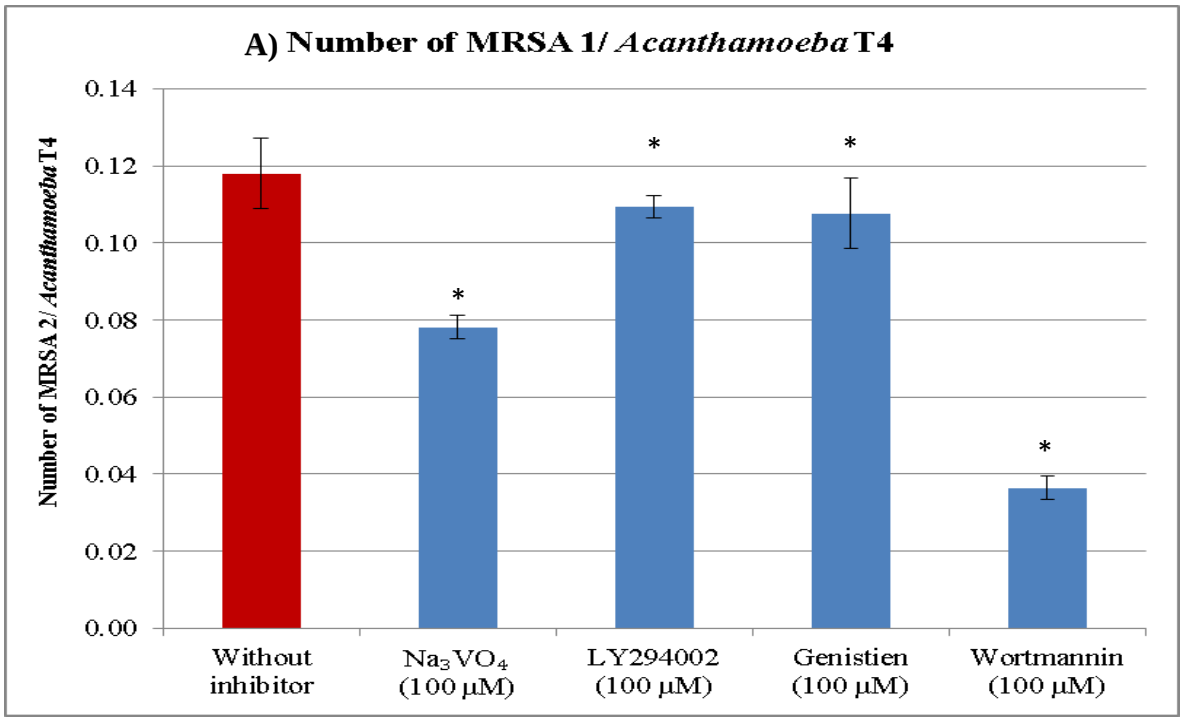
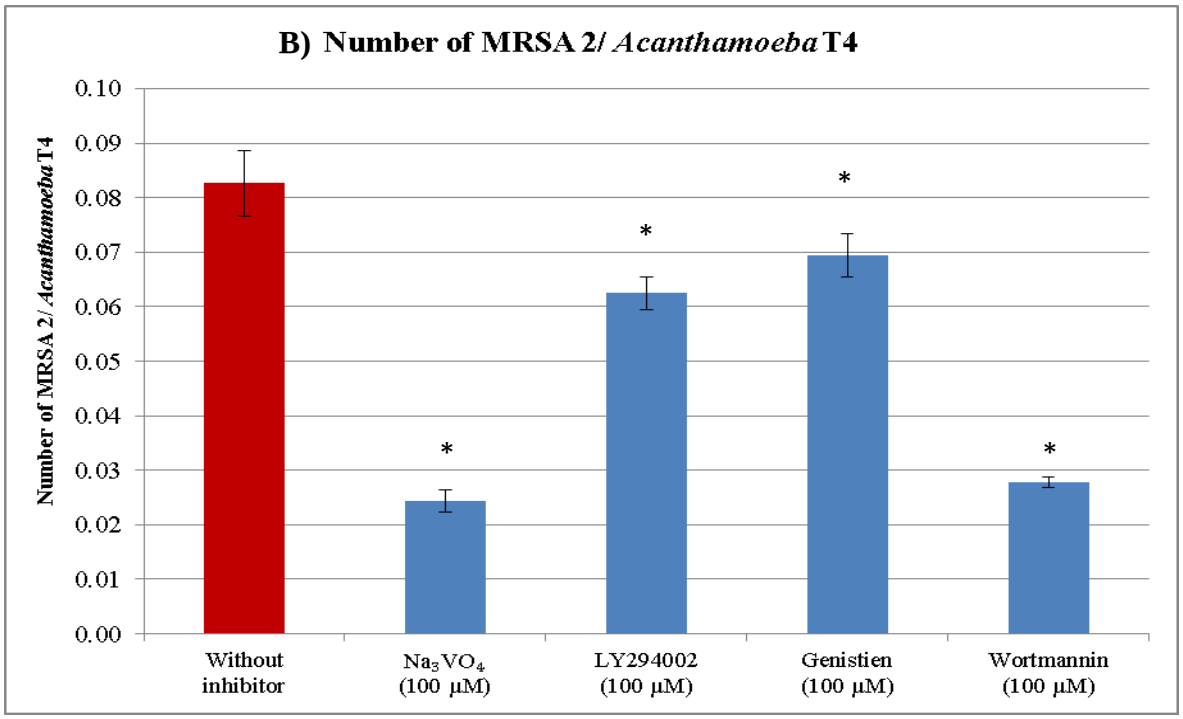
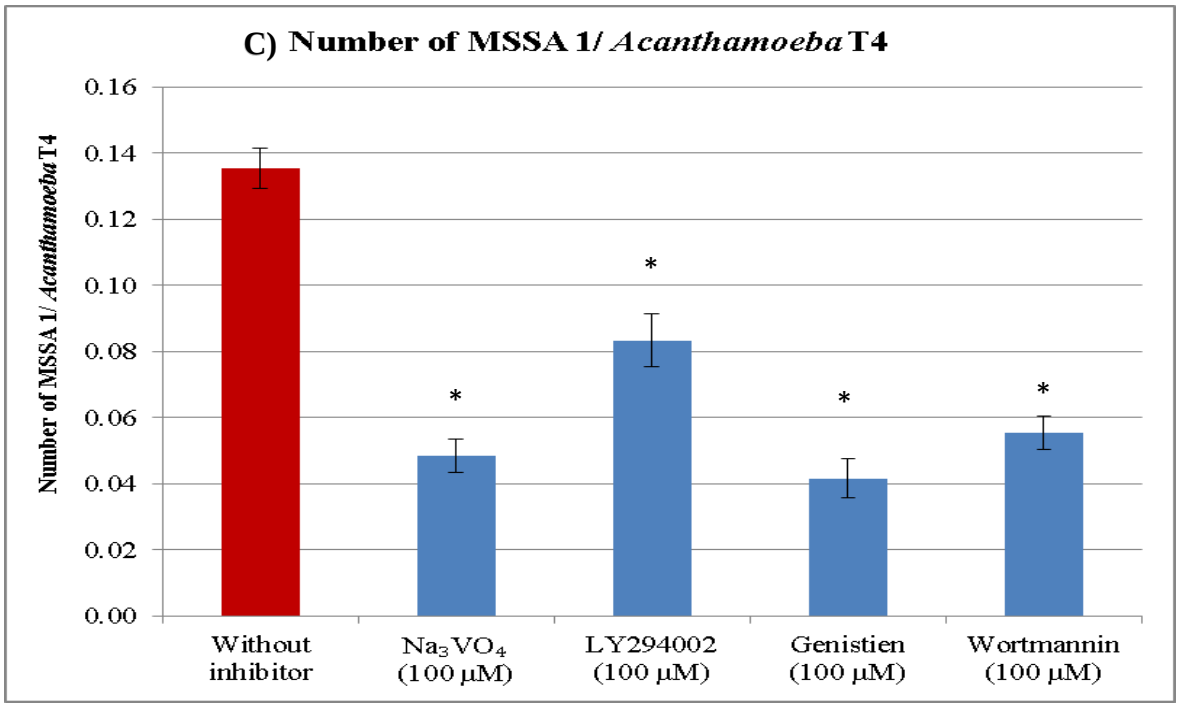



**Figure 2.3. Cytotoxic effects of vehicles (Methanol, Ethanol and DMSO) on *Acanthamoeba* T4.** Genistein inhibitor was dissolved in DMSO (0.5%); PMSF in Methanol (5%); LY294002 in DMSO (0.5%); Wortmannin in Ethanol (5%); Na3VO4 in distilled water. *Acanthamoeba* was grown until confluence in 24 well plates. All reagents with their final concentrations were added and incubated for 30 mins. The control wells were added RPMI. After incubation viability of cells were identified using Trupan Blue.


A) Number of MRSA 1/ Acanthamoeba T4
Number of MRSA 2/ Acanthamoeba T4
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
*
Without inhibitor
Na3VO4 (100 µM)
LY294002 (100 µM)
Genistien (100 µM)
Wortmannin (100 µM)


B) Number of MRSA 2/ Acanthamoeba T4
Number of MRSA 2/ Acanthamoeba T4
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
*
*
*
*
Without inhibitor
Na3VO4 (100 µM)
LY294002 (100 µM)
Genistien (100 µM)
Wortmannin (100 µM)



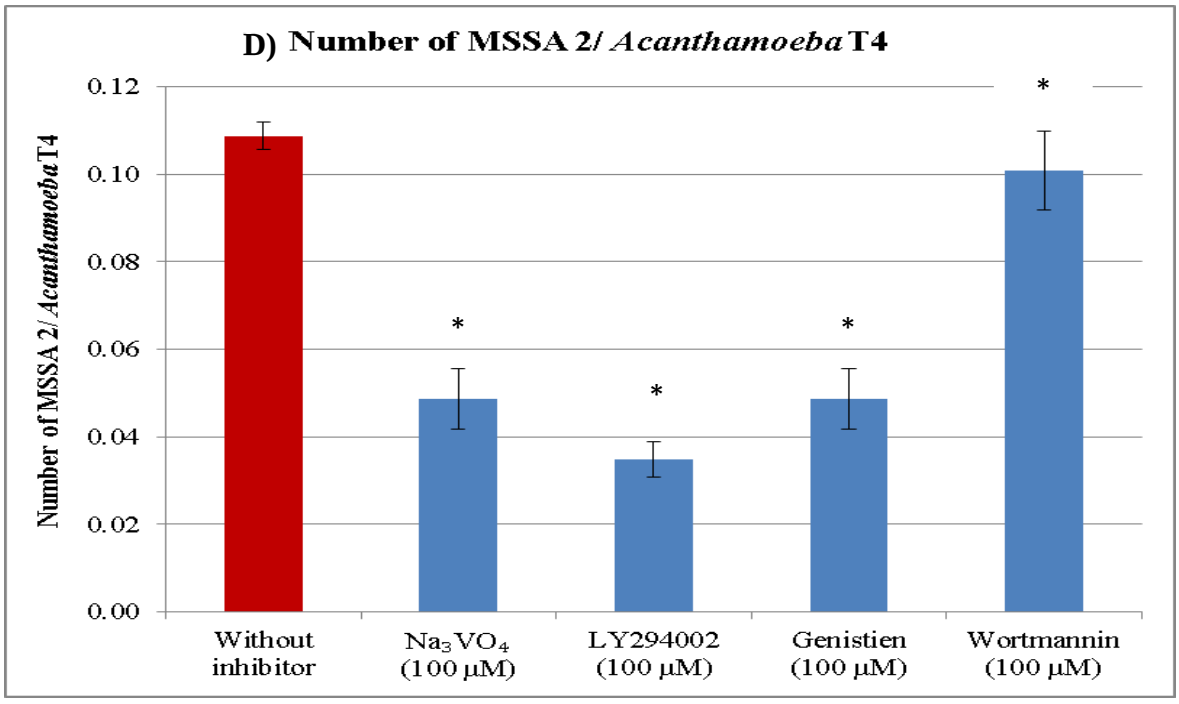
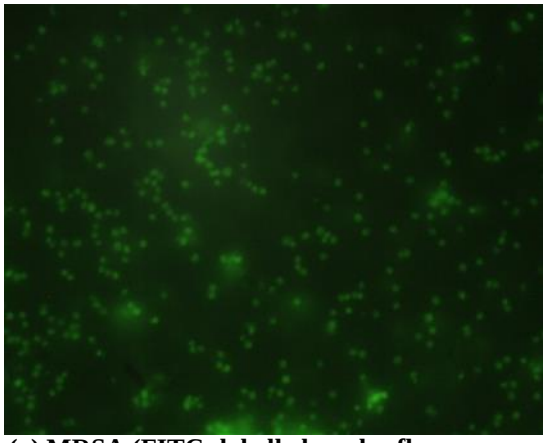



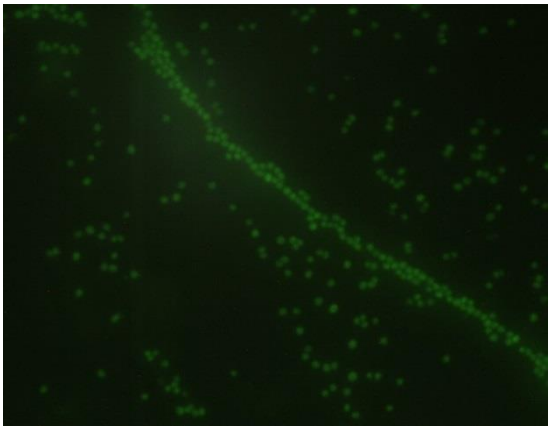

**Figure 2.4. Effect of four inhibitors on MRSA1 (A); MRSA2 (B); MSSA1 (C); MSSA2 (D); invasion in *Acanthamoeba*.** *Acanthamoeba* was cultured in 24-well plates and pretreated with inhibitors (100 µM of wortmannin, 100 µM of genistein, 100 µM of LY294002, and 100 µM of $Na_3VO_4$) for 30 mins before bacteria addition. After 1 hr of co-incubation, extracellular bacteria were killed. *Acanthamoeba* was washed, lysed and cultured onto CLED plates. The next day bacterial colonies (CFU) were counted. These results represent data of three independent experiments in duplicate. Error bars represent standard error. * $p < 0.05$

#### 2.3.1.3.*Acanthamoeba* engulfs heat-killed FITC-labelled MRSA and MSSA

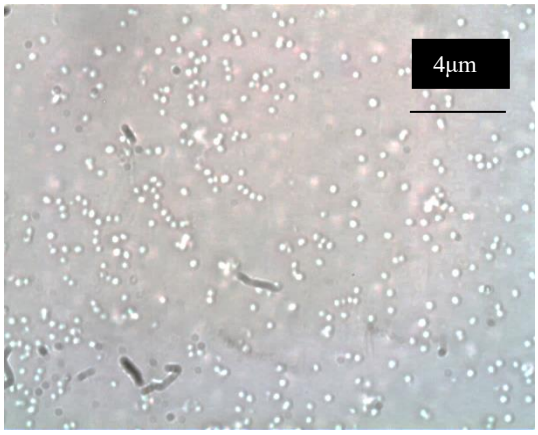
To simplify the phagocytosis process, assays were developed using heat-killed FITC-labelled MRSA and MSSA. The aim of using heat-killed bacteria was to identify the uptake of bacteria, excluding bacterial own invasion. Generally invasion process of bacteria inside the *Acanthamoeba* involves two processes in parallel. Bacteria is engulfed by *Acanthamoeba* as well as bacteria are penetrating themselves. To estimate and study the role of *Acanthamoeba* engulfment only by exclusion bacterial invasion gives the possibility to clarify phagocytosis process. In addition, we observed that trypan blue can be used to quench fluorescence of FITC (Figure 2.5). These bacteria were subsequently used in phagocytosis assays. It was observed that the *Acanthamoeba* uptake of MRSA was higher than the uptake of MSSA: 25 % and 14 % respectively (Figure 2.6) ($P<0.05$ using paired *t*-test, one-tail distribution).



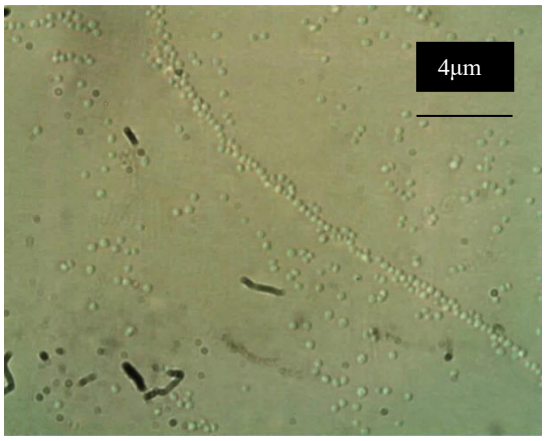
**(a) MRSA (FITC- labelled, under fluorescence microscope)**



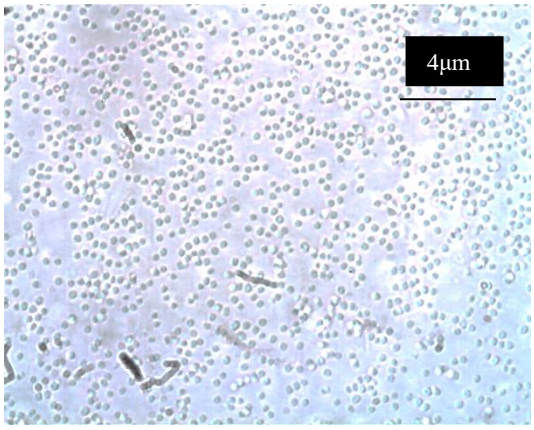
**(b) MSSA (FITC- labelled, under fluorescence microscope)**



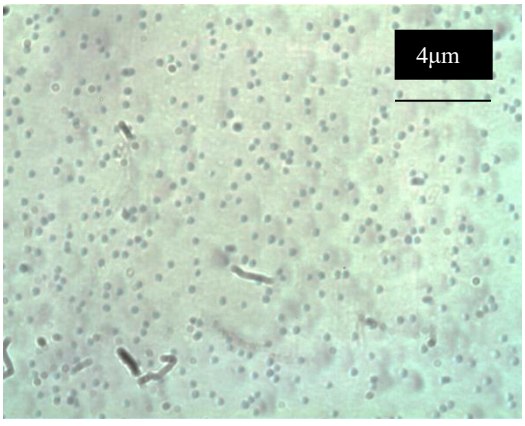

**(c) MRSA (FITC- labelled, under light microscope)**




**(d) MSSA (FITC- labelled, under light microscope)**

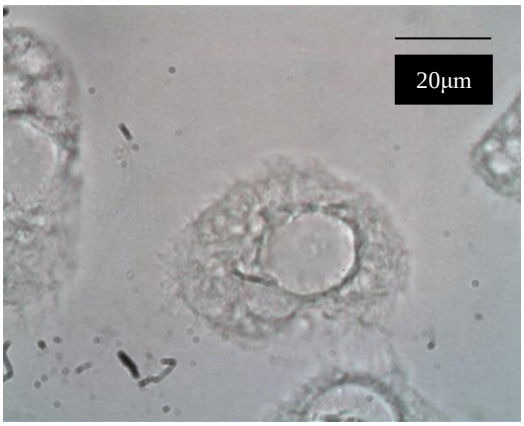



**(e) MRSA (FITC- labelled followed by Trypan blue treatment, under light microscope)**

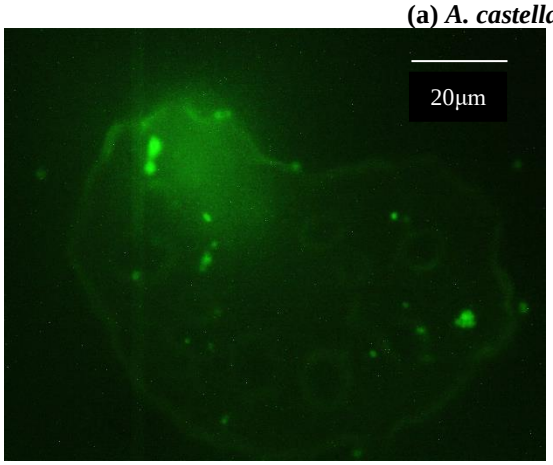



**(f) MSSA (FITC- labelled followed by Trypan blue treatment, under light microscope)**

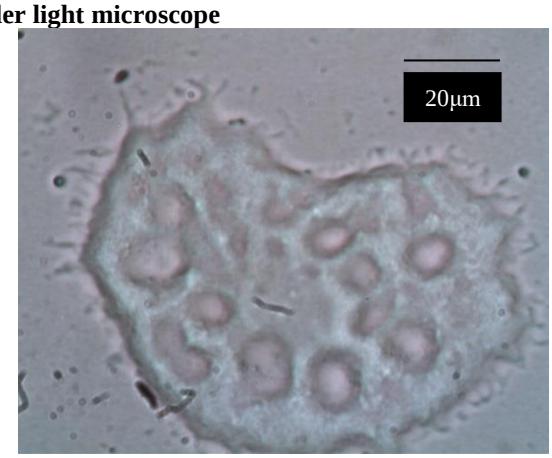
**Figure 2.5. MRSA and MSSA strains were successfully labelled with fluorescein isothicyanate (FITC). A, C, & E are MRSA; B, D, & F are MSSA.** Bacteria were grown overnight at 37°C in LB broth. The next day, bacteria were heat-killed at 65°C. Cultures were centrifuged at 10,000x g for 10 min. Bacteria were incubated with FITC on a shaker for 30 min at room temperature in the dark. The cells were rinsed 4 times to remove unbound FITC. Pellet was re-suspended in one mL PBS and stored at - 20°C.



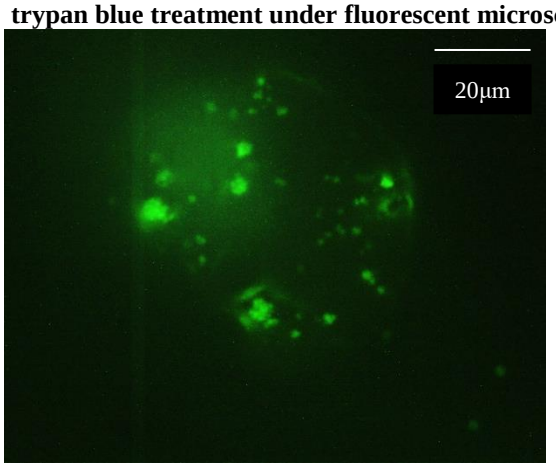

**(a) *A. castellani* under light microscope**



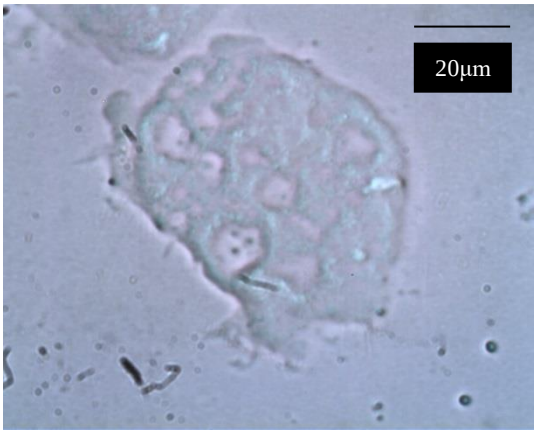

**(b) *A.castellanii* + MSSA (FITC-labelled) followed by trypan blue treatment under fluorescent microscope**.




**(c) *A. castellanii* with FITC labelled followed by trypan blue treatment under light microscope.**




**(d) *A. castellanii* +MRSA (FITC-labelled) followed by trypan blue treatment under fluorescent microscope.**




**(e) *A. castellanii* with FITC labelled followed by trypan blue treatment under light microscope.**

**Figure 2.6.** ***Acanthamoeba castellani* isolate phagocytosing FITC-labelled MRSA and MSSA.** *Acanthamoeba* was incubated on sterile cover slips in 24-well plates in a PYG medium (5 x $10^5$ amoebae /well). Plates were incubated for 17-20 h. The next day, cells were washed. Phagocytosis was initiated by adding 1.5 µl containing $10^5$ FITC-labelled bacteria for 25 min. Next, *Acanthamoeba* cells were washed, and followed by the addition of Trypan blue to quench the fluorescence of extracellular bacteria. Finally, *Acanthamoeba* cells were gently washed and fixed with 96% methanol at 20 °C and observed under a fluorescent microscope

#### 2.3.1.4. Invasion of live bacteria inside *Acanthamoeba*

To visualize whether the bacteria invaded or were phagocytised by *Acanthamoeba*, Rose Bengal-labelled MRSA and MSSA were used. As a result bacteria were able to invade or be engulfed by amoebae. Following 1 h co-culture at a multiplicity of infection of one cell: 10 bacteria, fluorescence microscopy showed that the MRSA presence in *Acanthamoeba* was higher than that of MSSA: 19 % and 11 % respectively (Figure 2.7) ($P<0.05$ using paired *t*-test, one-tail distribution).

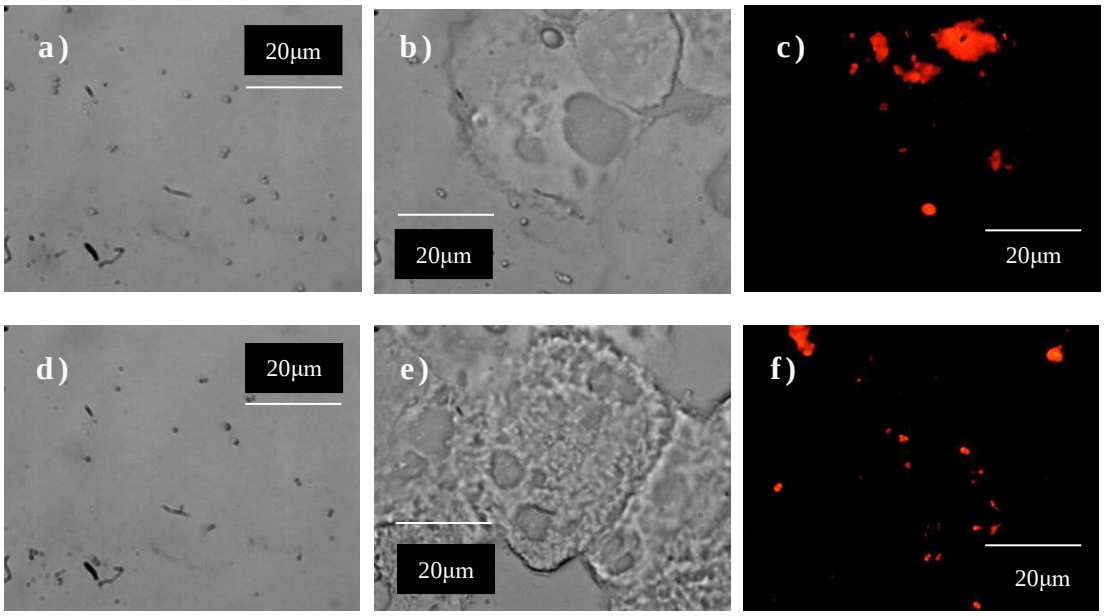



**Figure 2.7. MRSA and MSSA labelled with Rose Bengal.** MRSA under the light microscope alone (a), MRSA bacteria inside *Acanthamoeba* (b), under fluorescent microscope (c). Labelled with Rose Bengal MSSA under the light microscope alone (d), MSSA bacteria inside *Acanthamoeba* (e), and under fluorescent microscope (f). A few colonies of bacteria were inoculated in LB broth and incubated at 37 °C overnight. Next day, bacteria were centrifuged at 3000 RPM and pellets were re-suspended in 1 mM Rose Bengal and incubated at 37 °C for 20 min. Next, $10^6$ *Acanthamoeba* were detached, centrifuged and washed. Then, 10 bacteria per *Acanthamoeba* were added and incubated for 1 h. Following 1 h of co-culture, cells were harvested and the Acanthamoeba with bacteria inside were centrifuged and washed. Cell suspensions with intracellular bacteria (10 µL) were pipetted on slides followed by coverslip application. The intracellular location of bacteria was assessed using the fluorescence microscope

### 2.3.2. Phagocytic activity assays

Phagocytic activity assays were performed in the presence of various chemical compounds including LY294002, inhibitor of phosphoinositide 3-kinases (PI3Ks); $Na_3VO_4$, inhibitor of protein tyrosine phosphatases and alkaline phosphatases; Genistein, tyrosine kinase inhibitor; and PMSF, serine protease inhibitor. All of the agents inhibited bacterial uptake. LY294002 had the greatest inhibiting effect , which reduced uptake by 88%, and least effective was PMSF which decreased bacterial uptake by 53%. $Na_3VO_4$ and genistein reduced engulfing by 85% and 83% respectively (Figure 2.8).

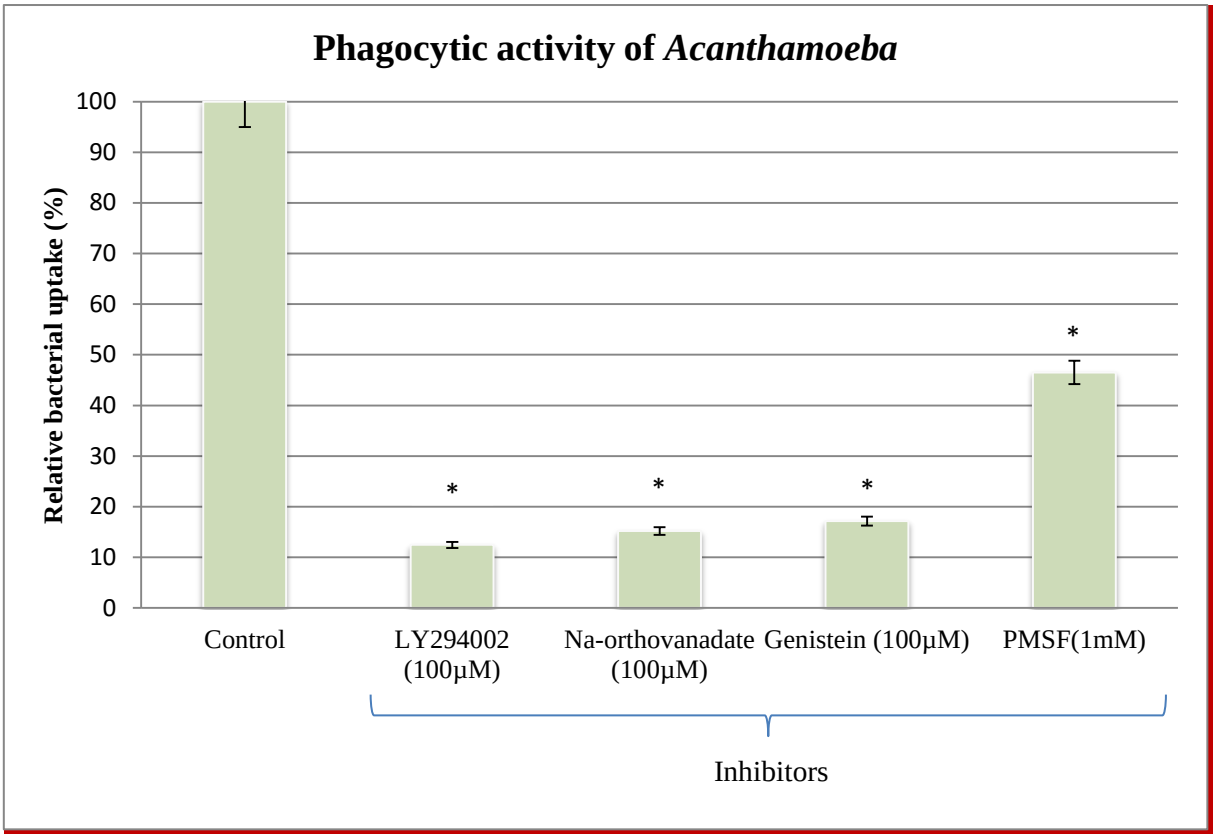



**Figure 2.8. Phagocytic activity of *Acanthamoeba* after treating with inhibitors: LY294002 (100µM), $Na_3VO_4$ (100µM), genistein (100µM) and PMSF (1mM).** Data is representative of three independent experiments ($P<0.05$ using paired t-test, one-tail distribution). The percentage of *Acanthamoeba* engulfing bacteria was determined in order to quantify the phagocytic activity. Briefly, using a 20X objective, seven fields were randomLy selected. Within these fields, all *Acanthamoeba*, with or without bacteria, were counted and their percentage was determined as follows: % phagocytosis = *Acanthamoeba* engulfing bacteria / total *Acanthamoeba* x 100.

## 2.4. Discussion

It is well known that *Acanthamoeba* acts as a host for several types of bacteria, and their ability to host bacterial pathogens, such as *Legionella pneumophila*, has gained specific attention. However, the detailed molecular mechanisms associated with *Acathamoeba*-bacteria interactions remain unclear. *Acanthamoeba* feeds on bacteria, but how these bacteria interact with *Acanthamoeba* needs to be elucidated. Here, MRSA and MSSA interactions with *Acanthamoeba* were studied. This might be the first study which focuses on *Acanthamoeba* and its interactions with MRSA and MSSA.

Several lines of evidence suggest that PI3K plays an important role in host cell cytoskeletal remodelling processes, such as phagocytosis, pseudopod formation and trafficking (Wymann and Pirola, 1998; Cox *et al.*, 1999). For example, it has been shown that PI3K controls Rho-mediated changes in actin cytoskeleton in fibroblasts (Reif *et al.*, 1996; Cantrell, 2001). Here it was observed that LY294002, a specific PI3K inhibitor, reduced *Acanthamoeba* invasion by MRSA and MSSA. This is in line with the findings of Alsam, *et al* (2006) who found that PI3K played an important role in *Acanthamoeba* interaction with *E. coli.* Similar results were found in the present study; LY294002 was used on *Acanthamoeba* and two strains of bacteria, MRSA and MSSA. The use of this inhibitor led to a reduction of the bacterial invasion of *Acanthamoeba*.

Other commonly used inhibitors of PI3K include wortmannin. Wortmannin inhibits all PI3-kinases but only at high concentrations at which it may affect PI3K-like enzymes, such as mTOR, MAP Kinase, PI4- kinases, and some other enzymes (Alsam *et al.*, 2005). This inhibitor was also tested in the present study on *Acanthamoeba* and the bacterial strains: MRSA and MSSA which resulted in a significant inhibition of the PI3K pathways; a reduction of the number of bacteria that invaded the *Acanthamoeba* was noted in comparison with the control sample.

Another inhibitor is $Na_3VO_4$ which is used to inhibit protein tyrosine phosophatase. It was found that $Na_3VO_4$ causes an increase in the *Acanthamoeba* uptake of *E.coli* (Sissons *et al.*, 2004; Alsam *et al.*, 2005). However, the present study used $Na_3VO_4$ to investigate the role of protein tyrosine phosphatase in

*Acanthamoeba* invasion by MRSA and MSSA which showed contradictory results. It was found that a significant reduction of the bacterial invasion was noted in comparison with the control sample.

Phagocytosis plays an important role in the pathogenesis of *Acanthamoeba* (Palusinska-Szysz *et al.*, 2014). Nevertheless, the detailed molecular mechanisms related to *Acanthamoeba* phagocytosis remain vague. In this study, we made use of FITC-, and Rose Bengal labelled MRSA and MSSA to study processes involved in the phagocytosis of *Acanthamoeba*. LY294002, inhibitor of phosphatidylinositol 3-kinase, inhibited phagocytosis of macrophages (Cox *et al.*, 1999). Pretreatment of *Acanthamoeba* with LY294002, resulted in reduction of bacteria (MRSA and MSSA) uptake by 88%. $Na_3VO_4$, protein tyrosine phosphatase inhibitor, decreased bacteria engulfment by 85%, genistein, protein tyrosine kinase inhibitor decreased by 83%. Among the inhibitors used the lowest inhibitory effect was of PMSF, serine protease inhibitor, which reduced phagocytosis by 53%. Overall our data shows that all these enzymes play role in phagocytosis. To conclude, these findings propose that particle uptake in *Acanthamoeba* is a multifarious process that could play a substantial role, both in food uptake as well as the pathogenesis of this protozoan. A comprehensive understanding of the molecular mechanisms related to this process might help identify therapeutic targets against this important pathogen.

# Chapter 3. Extracellular Proteases of *Acanthamoeba castellanii*

## 3.1. Background

### 3.1.1. Extracellular serine proteases

Extracellular serine proteases are known for various roles in a variety of organisms. In higher eukaryotes, serine proteases are involved with the blood coagulation pathway (thrombin, etc.) and digestive enzymes (chymotrypsin, trypsin, etc.), and other functions (Gensberg *et al.*, 1998; Thorburn *et al.*, 2003; Wang *et al.*, 2006; Kawabata and Muta, 2010). Extracellular serine proteases are believed to play a role in cell adhesion (Del Rosso *et al.*, 2002). Phenylmethylsulfonyl fluoride (PMSF) is a serine protease inhibitor. PMSF binds irreversibly to the serine residue present in the active sites of serine proteases by replacing the hydrogen present in the hydroxyl group of serine's side chain. PMSF does not bind to all the serine residues present in proteins, only to those present at the active site of serine proteases due to the hyperactive nature of these proteins. Serine proteases of *Acanthamoeba* are involved in various processes required for pathogenesis, such as making the Blood-brain barrier permeable and the encystment of cells (Figure 3.1) (Alsam *et al.*, 2005; Dudley *et al.*, 2008). Previous researches have shown that pathogenic strains of *Acanthamoeba* contain more serine proteases than non-pathogenic strains (Khan *et al.*, 2000).

Phagocytosis is one of the important factors that cause the pathogenesis of *Acanthamoeba*. The actual detailed mechanism responsible for the process of phagocytosis remains vague. In this study the role of extracellular serine proteases on *Acanthamoeba* phagocytosis was investigated.

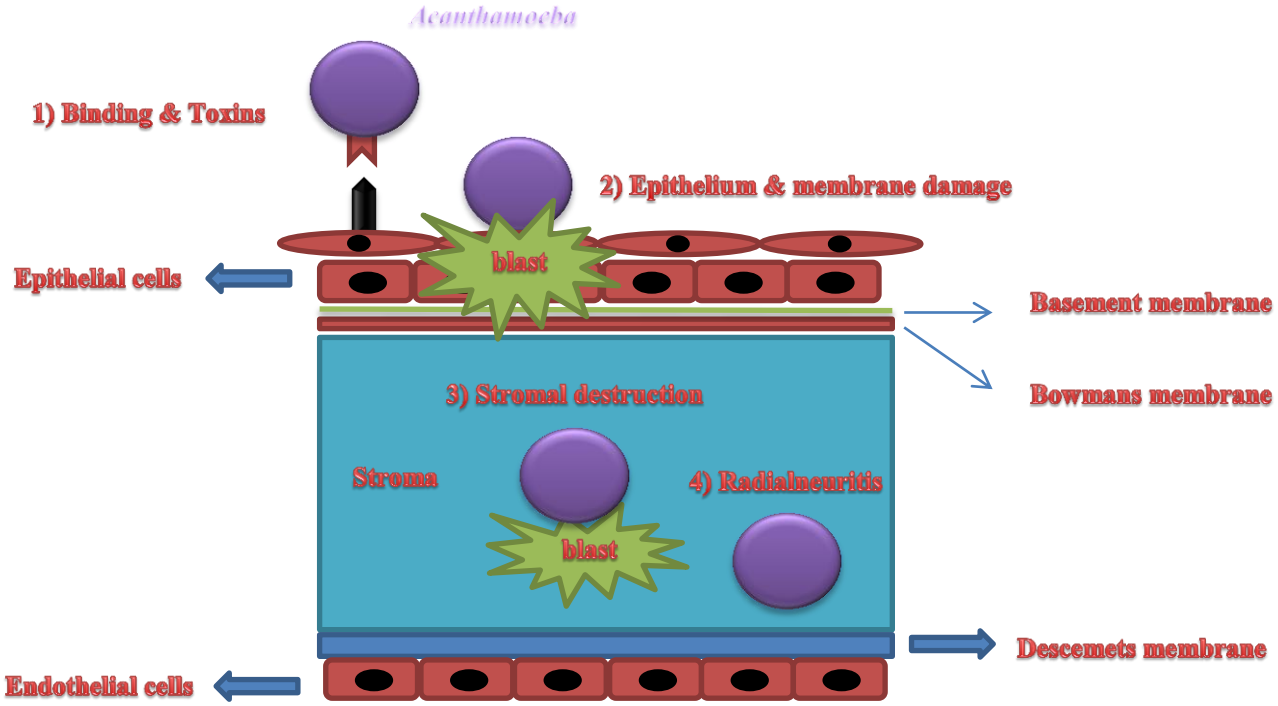



**Figure 3.1.** ***Acanthamoeba*** **secretes serine proteases that target tight-junction proteins between cells.** Parasites contribute to corneal epithelial cell damage, leading to *Acanthamoeba* entry into the cornea(Lorenzo-Morales *et al.*, 2015)**.**

### 3.1.2 Proteomics and 2D gel electrophoresis

Proteomics is defined as the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes and study the structure and function of proteins. A proteome is a complete set of proteins expressed by the genetic makeup of an organism in a specific set of environmental conditions. The application of mass spectrometry (MS) for analysing proteomics is a powerful tool for characterising proteins (Cravatt *et al.*, 2007). The classical method for analysing complex protein mixtures is the separation of the mixture by two dimensional gel electrophoresis of polyacrylamide (2D-PAGE) and the identification of proteins resolved by elution, digestion, and MS or tandem mass spectrometry (MS/MS) (Issaq and Veenstra, 2008). In contrast to the genome, the proteome of an organism is dynamic in nature due to post-translational protein-transcribed (Aebersold and Cravatt, 2002) changes. In addition genomics and transcriptomics, proteomics is important because proteins represent actual functional molecules in cells (Wilkins *et al.*, 2006). The identification of potential new drugs to treat diseases is one of the most promising developments to come from the study of human proteins (Abu-Farha *et al.*, 2009). This is based on information proteomics gather to identify proteins associated with a disease that computer software can then use as targets in the design of new drugs (Abu-Farha *et al.*, 2009). The entire set of proteins expressed by a genome, cell, tissue, or organism is a proteome (Andersen *et al.*, 2005). 2D-PAGE is a method which utilizes to separate complex protein samples that applies isoelectric focusing (IEF) using immobilized pH-gradient (IPG) dry strips in the first dimension and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The advantages of this method are: complete amount of proteins can be analysed at one time, high resolution of the protein by isoelectric point (pI) and molecular mass, comparison between resolved proteins spots for different conditions and use them for MS analysis. The disadvantages of this technique include difficulty detecting low molecular weight, as the time varies between the gels, the limited range (PI) proteins, and the unwieldy acidic, basic, and hydrophobic protein extremes (Aebersold and Cravatt, 2002; Zhu *et al.*, 2010). Recent studies have concluded that the key to improving recovery factors is hydrophobic proteins' presence of urea and thiourea as a chaotropic agent in the cell lysate buffer (Chevallet *et al.*, 1998; Rabilloud, 1998).

## 3.2. Materials and Methods

All the chemicals for this study were bought from Sigma Laboratories (Poole, Dorset, England) unless otherwise stated.

### 3.2.1. *Acanthamoeba* culture

The clinical isolate of *Acanthamoeba castellanii* (ATCC 50492) was grown in tissue culture flasks in PYG medium as described in section 2.2.1.

### 3.2.2. MSSA and MRSA strains cultures

Two strains of each MRSA and MSSA were used in this project. MRSA and MSSA were grown as described in section 2.2.2.

### 3.2.3. Invasion assays

Invasion assays of MRSA and MSSA strains with *Acanthamoeba* T4 (ATCC 50492) were performed as described in sections 2.2.3, 2.2.4 and 2.2.5.

### 3.2.4. *Acanthamoeba* Conditioned Medium (CM) preparation

T-75 cell culture flask of *Acanthamoeba* was put on ice for 30 mins. Detached *Acanthamoeba* was collected and centrifuged for five min at 3000 RPM. The supernatant was discarded and the pellet was re-suspended in the PYG medium and incubated at 30 °C overnight and the growth was observed. The next day supernatant was discarded and the pellet was re-suspended in RPMI. Additionally incubated overnight at 30 °C and centrifuged at 3000 x *g* for 5 mins. Finally, the supernatant (CM) was collected to be later purified.

### 3.2.5.*Acanthamoeba* protein purification

The extracellular protein samples of *Acanthamoeba* were purified with modification to the methods reported (Cho *et al.*, 2000). The precipitated debris was removed by centrifugation at 8500 x *g* for 15 mins. *Acanthamoeba* supernatant was concentrated with a concentrator GE healthcare Vivaspin 20. The fractionated supernatant solution was dialysed against the 50 mM Tris-HCl buffer for 12 hrs at 4 °C. The dialysate solutions were concentrated by lyophilisation then dissolved in 10 mL of 50 mM Tris-

HCl (pH 7.5). The protein culture samples were applied to an ion exchange chromatography column using Diethylethanolamine (DEAE) Sepharose Fast Flow Flash chromatography (1.6 x 15cm), equilibrated with a 50 mM Tris-HCl (pH 7.5) buffer, and washed with the same buffer at a flow rate of 45 mL/h. Fractions containing proteolytic activity were pooled, dialysed against distilled water at 4 °C, and lyophilised. The lyophilised preparation was re-dissolved in 50 mM Tris-HCl (pH 8.0) containing 1 M ammonium sulfate and applied to chromatography on Phenyl Sepharose CL-4B hydrophobic interaction chromatography (1.8 x 10 cm) equilibrated with the same buffer.

Adsorbed proteins were eluted by using a linear gradient from 1 M to 0.1 M ammonium sulfate. The active fractions were pooled, dialyzed against distilled water at 4 °C, and lyophilized. The lyophilized preparation was resolved in 50 mM Tris-HCl (pH 8.0) and applied to a chromatography on Sephacryl S-200 HR gel filtration (1.5 x 78 cm) equilibrated with the same buffer. Fractions (1.7 mL/fraction) of the eluate were collected and dialyzed against distilled water. Protein concentration was measured by the method of Lowry *et al*. (1951) with bovine serum albumin (BSA) as the standard (Cho *et al.*, 2000).

### 3.2.6. Zymographic assays

To study the effect of serine proteases on *Acanthamoeba* interaction with bacteria, zymographic assays were performed. *Acanthamoeba* CM was blended (1:1) with the sample buffer (0.5 M Tris, pH 6.8), glycerol 0.11 M, 0.2 M of 10% SDS, 0.05% (w/v) bromophenol blue, and electrophoresed on SDS-PAGE containing fish gelatine (4% w/v). After electrophoresis, the gels were soaked in 2% Triton x-100 (w/v) solution for 60 mins, incubated in a developing buffer (50 mM Tris-Hcl, pH 7.5 containing 10 mM CACL2) at 37 °C overnight, and then washed and stained with Comassie Brilliant Blue. In the gel, the non-staining parts represent bands of gelatine digestion. In some experiments, samples were treated with PMSF (an inhibitor of serine proteases; 1 mM final concentration), which was found to be the most effective in inhibiting *Acanthamoeba* extracellular proteases (Alsam *et al.*, 2005).

### 3.2.7.Two-dimensional (2-D) gel electrophoresis

#### 3.2.7.1.First-dimensional gel IEF

Immobiline 11 cm dry-gel strips with a pH range of 3 – 10 (GE Healthcare Bio-Sciences AB) were rehydrated in a dry-strip tray reswelling tray. The dried strip was rehydrated for 24 hrs at room temperature in 300 µl of a rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, protease inhibitor cocktail 1X, 20 mM DTT, 1 % ampholyte, Bromophenol Blue 0,1 %, 0.05 % SDS) containing 60 µg of the protein sample. The IPG strip was covered using ~3 mL immobiline dry-strip cover fluid, PlusOne (GE Healthcare Bio-Sciences AB). After rehydration, the IPG strip was gently rinsed with purite water to remove any crystallized urea. The strip subjected to isoelectric focusing was IPG Phor-cleaned with a cleaning solution Strip holder (GE Healthcare Bio-Sciences AB).The Ettan IPG Phor3 was switched on and connection with the IPG Phor3 control software was established. The IPG Phor mainfold was covered with 108 mL of Immobiline PlusOne dry-strip cover fluid and the rehydrated strips were then placed in individual lanes of the Ettan IPG strip holder (GE Healthcare Bio-Sciences AB) under the fluid using tweezers with the positive end towards the anode end of the main fold. Paper electrode wicks were placed between the IPG strips and the electrodes. The cathodic (negative) filter wick was rehydrated with 150 µL of 100 mM DTT. The anodic (positive) filter wick was hydrated with 150 µL Purite water.

The lid was closed and the IPG-Phor program was implemented according to the following schedule. (Görg *et al.*, 1988, 1995, 2000)**.**

Step 1. 500 V 6 hrs

Step 2. 1000 V 7 hrs 4 mins

Step 3. 6000 V 2 hrs

Step 4. 8000 V 2 hrs 22 mins

Step 5. 200 V 1 hr

At the end of the programme, the computer was disconnected and IPG-Phor was stopped. The paper electrode wicks were removed with tweezers and discarded. The IPG strips were placed in a Petri dish, rinsed briefly with deionized water, labelled, and stored at -80$^{o}$ C for later use.

#### 3.2.7.2. Equilibration of the IPG strips

Before the second dimension gel run, the IPG strips comprising isoelectrically focused proteins were equilibrated and reduced. First, the strips were incubated for 15 mins with 10 mL of equilibration buffer containing 100 mg of DTT. After that strip was incubated for another 15 mins with 10 mL of the equilibration buffer containing 250 mg of iodoacetamide, incubation time was at room temperature for both steps. The IPG strips were equilibrated, the equilibration buffer first containing 1% DTT and then containing 4 % iodoacetamide for 15 mins each at room temperature. Running buffer for SDS-PAGE (stock solution SX): Tris 15g, glycine (G8898) 72g, SDS 5g, make up to 1000mL with distilled water. Adjust pH to 8.3 with HCI. Dilute to 1X with distilled water prior to use. IPG strips were rinsed with 1X running buffer before placing on the second-dimension gel (Amersham Biosciences).

#### 3.2.7.3. Assembly and running of second-dimensional gel

An agarose sealing solution was heated to liquid status. IPG strips were cut from each end up to 0.6 cm, to a final length of 11 cm. A small square of felt paper electrode (2 x 3 cm of average thickness) was loaded with 10 µL of molecular weight marker and placed in the upper left corner of the gel. The IPG strip was placed in the well of 12 % SDS-PAGE gel with the acid side facing the glass plate of the hinge and sealed with the agarose solution, avoiding air bubbles. The electrophoresis tank was filled with 0.5 L of the gel running buffer. The gel strips were removed from the casting assembly and cut off on the protein core unit tank. The central unit was lifted into the tank. The cover was mounted in the tank and the cable connected to the power PAC (Bio-Rad, Power Pac 1000). Electrophoresis was carried out first at 50 V for 30 mins and then at 150 V for approximately 2 – 3 hrs or until the bromo-phenol blue dye front had reached the lower end. The core unit was then removed from the tank and gels were removed from the clamps. The spacers were loosened and an edge of the glass plate was lifted with a spatula. Gel was placed in a gel glass container containing fixative solution.

#### 3.2.7.4. Protein visualisation

For visualization of protein spots a modified silver staining protocol was constructed (Yan *et al.*, 2000; Gromova and Celis, 2006). Gels were washed with 20 % methanol for 20 mins with changing the wash buffer thrice and then sensitized with 0.02 % sodium thiosulfate and washed with purite water

twice, each for 1 min. Staining was performed with 0.2 % silver nitrate solution for 20 mins followed by a careful wash twice with ultra-pure water for a maximum of 1 min each time. The gels were developed with sodium carbonate 6 % and 0.0004 sodium thiosulfate for 2-5 mins until spots appeared, and process was terminated by adding 12 % acetic acid and shaked for 10 mins. The gels were stored in 12 % acetic acid at 4 $^{o}$C.

#### 3.2.7.5. Gel image capture and spot analysis

The gels were scanned using the Epson scanner image III with LabScan 6.0 software. First, the scanner was calibrated and configured to use the transparent setting to 300 dpi with a blue filter. The surface scanner was cleaned with 70% ethanol and some purite water that was poured on the surface. The gel was placed directly into the scanner preview and air bubbles were smoothed out. Then, the scanning area of the gel was selected and scanned. Gel images were saved as MEL and TIF files. Gel scanned images were characterised with the software package Progenesis SameSpot (Nonlinear Dynamics Limited, UK).

## 3.3. Results

### 3.3.1. Effect of *Acanthamoeba* serine proteases on bacterial uptake

To determine the effect of *Acanthamoeba* extracellular proteases on bacterial uptake, *Acanthamoeba* was treated with PMSF, serine protease inhibitor. Although, the results vary for the two types of bacteria, MRSA and MSSA, it is evident that the percentages of bacteria uptake by *Acanthamoeba* has increased in the absence of extracellular serine proteases (Figure 3.2). The highest increase of invasion was in the MRSA1 strain where the presence of PMSF inhibitor increased the invasion by two-fold in comparison with the control sample 0.118 % and 0.215 respectively ($P<0.05$ using paired *t*-test, one-tail distribution).

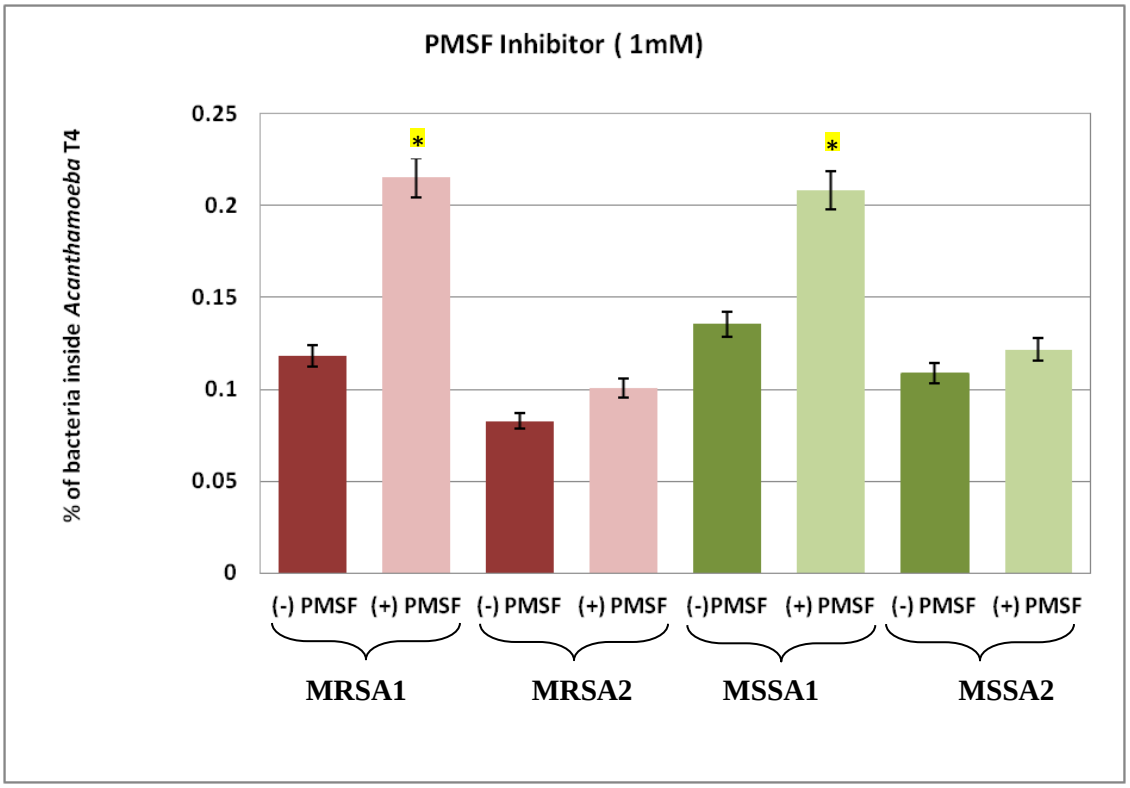



**Figure 3.2. Effect of serine protease inhibitor (1 mM of PMSF) on bacterial invasion in *Acanthamoeba*.** *Acanthamoeba* was cultured in 24-well plates and pretreated with inhibitors PMSF (final concentration 1mM) for 30 mins before bacteria addition. After 1 hr of co-incubation, extracellular bacteria were killed. *Acanthamoeba* was washed, lysed and cultured onto CLED plates. The next day bacterial colonies (CFU) survived were counted. These results represent data of three independent experiments in duplicate. Error bars represent standard error. * $p < 0.05$

To visualize *Acanthamoeba* uptake of bacterial strains, heat-killed FITC-labelled of MRSA and MSSA were used. *Acanthamoeba* was pre-treated with PMSF (1mM), serine protease inhibitor before performing interaction assays with bacteria. From images taken, it is evident that the number of both types of bacteria has increased when inhibition of extracellular serine proteases carried out (Figure 3.3).

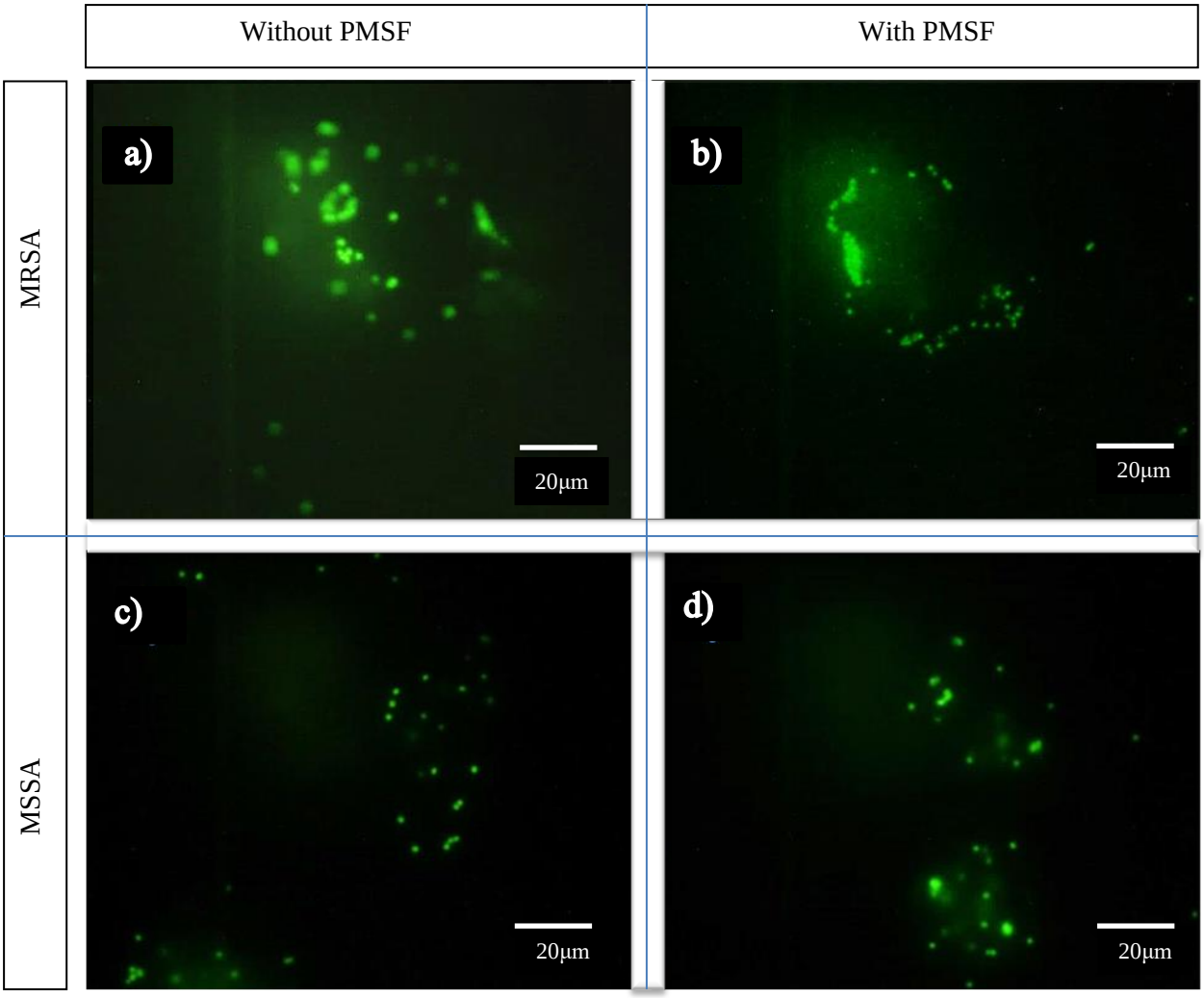



**Figure 3.3. Fluorescence of labelled MRSA without PMSF treatment (a), MRSA with PMSF treatment (b), MSSA without PMSF treatment (c) and MSSA with PMSF treatment (d).** *Acanthamoeba* was incubated on sterile cover slips in 24-well plates in a PYG medium (5 x $10^5$ amoebae /well). Plates were incubated for 17-20 h. The next day, cells were washed and pre-treated with 1mM PMSF. Phagocytosis was initiated by adding 1.5 µl containing $10^5$ FITC-labelled bacteria for 25 min. Next, *Acanthamoeba* cells were washed, and followed by the addition of Trypan blue to quench the fluorescence of extracellular bacteria. Finally, *Acanthamoeba* cells were gently washed and fixed with 96% methanol at 20 °C and observed under a fluorescent microscope.

### 3.3.2. Zymographic assays

The effect of serine protease inhibitor (PMSF) on expression level of T4 extracellular proteases is shown in (Figure 3.4) *Acanthamoeba* was incubated with and without the inhibitor in RPMI for 24h. Medium was collected, centrifuged and tested for the presence of proteases using zymography assays and fish gelatin as a substrate because *Acanthamoeba* produces little amount of extracellular serine proteases. Using 1 mM of PMSF was enough to inhibit the production of serine proteases totally. Analysis of proteases from keratitis-causing *Acanthamoeba* has shown that the presence of a 130 kDa protease was inhibited by PMSF. Two types of gel were used, one with fish gelatine and another without. The reason for using two different gels is to compare the sufficiency of conventional SDS gel to track bands from Zymographic gel. Gelatine was used since extracellular proteases degrade the compound allowing bands of degradation to be tracked on the gel. Further analysis with mass spectrometry was needed to determine the sequence and identity of the extracellular proteases. This would validate the findings and correlate with the existing proteins identified in databases. Using mass spectrometry at the School of Biological Sciences at Essex University was discussed however, that appeared to be too costly and therefore, it was decided to continue with the 2 D gel electrophoresis. Therefore, the 2 D gel electrophoresis was the only method used in this respect.

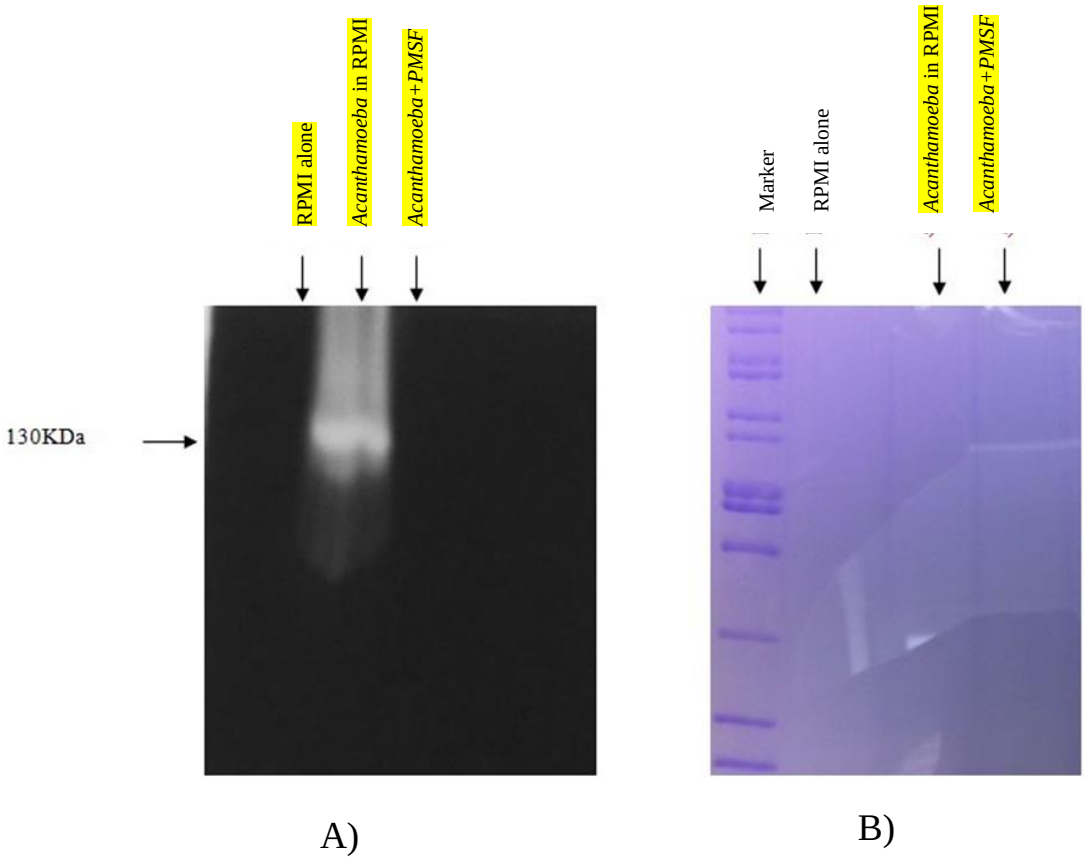



**Figure 3.4. A) *Acanthamoeba* T4 CM incubated with 1 mM of PMSF overnight at 30 °C. The non-staining parts represent bands of gelatine digestion. B) *Acanthamoeba* T4 CM incubated with 1 mM of PMSF overnight at 30 °C without fish gelatine.** *Acanthamoeba* CM was blended (1:1) with the sample buffer and electrophoresed on SDS-PAGE containing fish gelatine (4% w/v). After electrophoresis, the gels were soaked in 2% Triton solution for 60 mins, incubated in a developing buffer at 37 °C overnight, then washed and stained with Comassie Brilliant Blue. Samples were treated with PMSF, which was found to be the most effective in inhibiting *Acanthamoeba* extracellular proteases.

### 3.3.3. Two-dimensional gel analysis of serine protease inhibitor

*Acanthamoeba* has been used in this study as a model, and the role of serine protease inhibitor (1 mM of PMSF) has been investigated by two-dimensional gel electrophoresis followed by protein spot analysis.

Qualitative and quantitative comparison of proteomes in *Acanthamoeba* CM under different conditions (with and without PMSF) was studied using 2D electrophoresis. It is the classical method for analysing complex protein mixtures by separation of the mixture and the identification of proteins resolved by elution, digestion, and mass spectrometry. In this method every spot is scattered according to its Molecular Weight and pH, which is very helpful in identifying the protein.

The total proteome was separated by first-dimension isoelectric focusing on the basis of the proteins' isoelectric points (pI). The isoelectrically focused proteins were then resolved by second-dimension SDS-PAGE. Silver-stained protein spots in all cases challenged by 1 mM PMSF inhibitor (Figure 3.6) and an unchallenged control group which had 43 spots (Figure 3.5). Isoelectric point (pI) and molecular weight (MW) of 43 detected spots on 2-D gel were identified (Table 3.1). Gel images from all cases were then analysed using the Progenesis SameSpot software (Nonlinear Dynamics Limited), where the control untreated sample gel was used as a reference to find out differentially expressed proteins (Figure 3.7) (Table 3.2). Differentially expressed protein spots are circled with a blue colour. This data was used to gather preliminary results to discover the potential role of differential protein expression in response to a serine protease presence and the absence of a PMSF inhibitor. The PMSF inhibitor played an important role in inhibiting proteins (spots) in response to the serine protease.

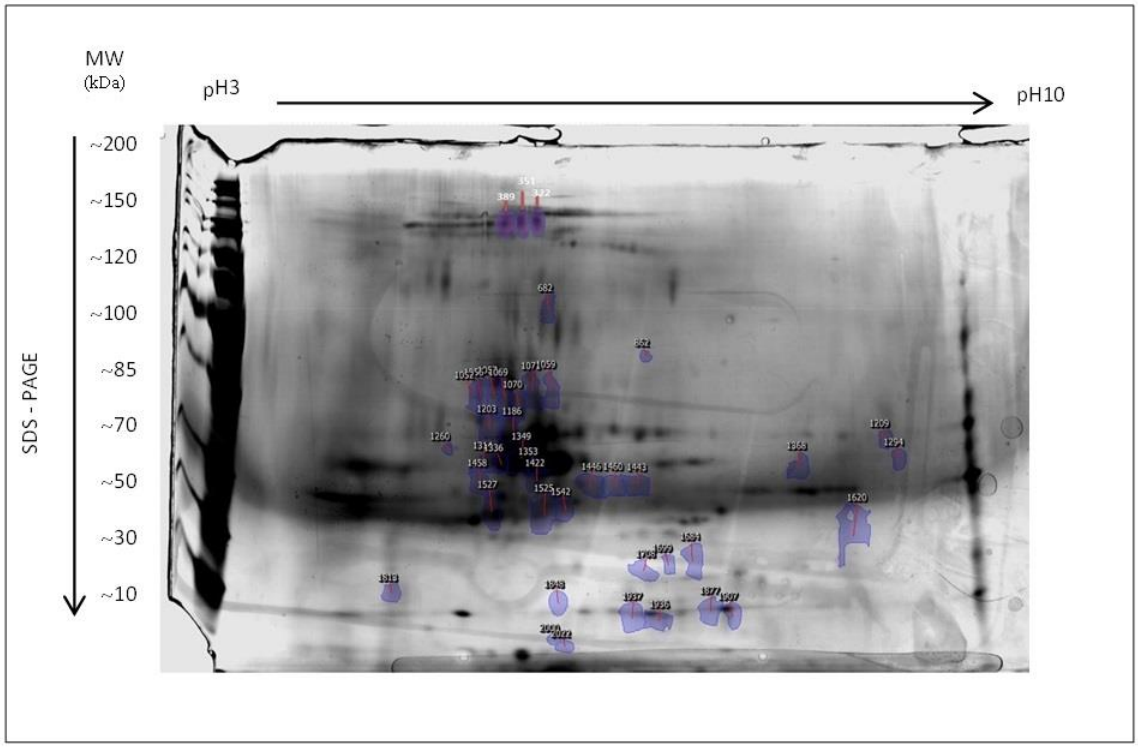
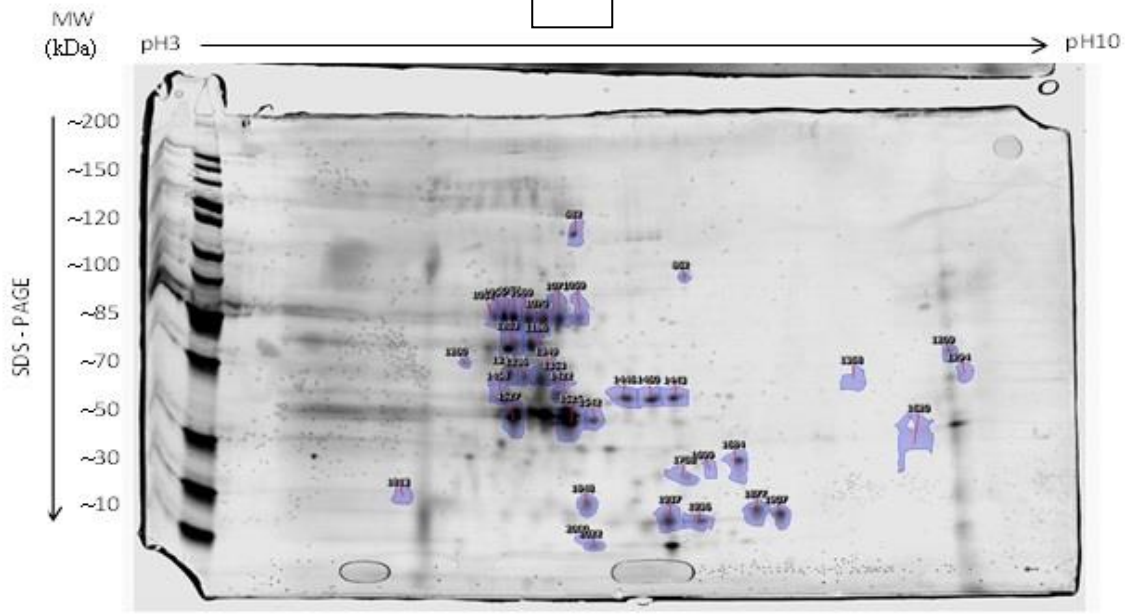
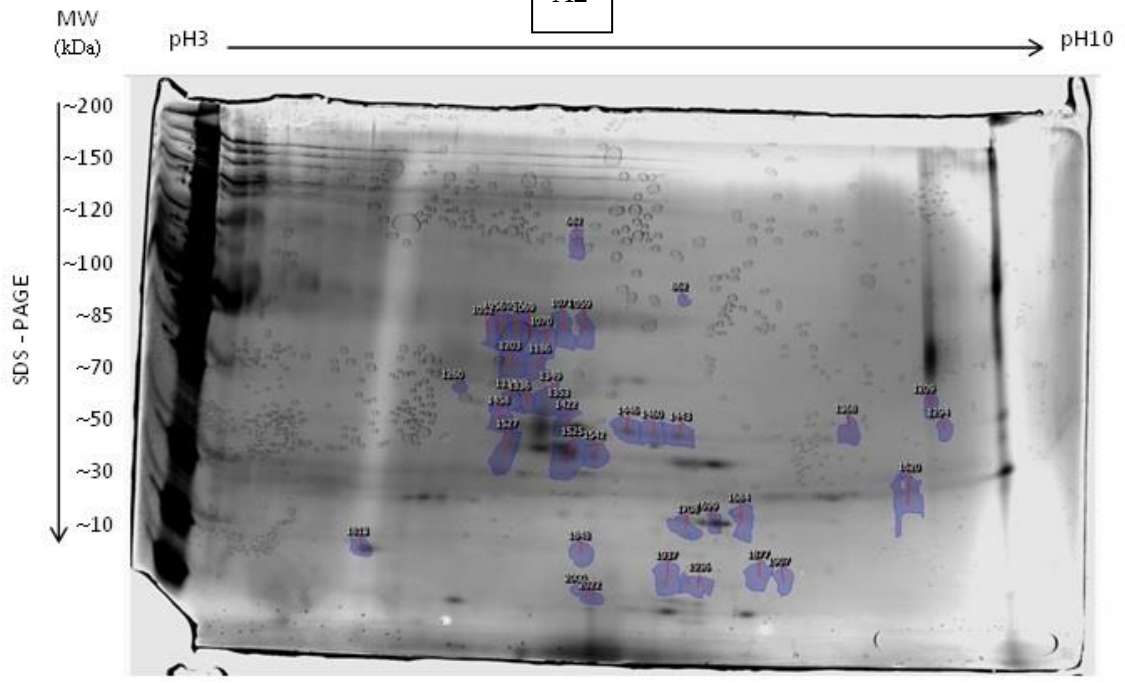



**Figure 3.5. Representation of a reference image showing all differentially expressed spots circled with the blue colour.** The reference image used for image analysis that was carried out with Progenesis SameSpot software. Isoelectric points pI range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.

A1


MW
(kDa)
pH3
pH10
~200
~150
~120
~100
~85
~70
~50
~30
~10
SDS - PAGE

A2


MW
(kDa)
pH3
pH10
~200
~150
~120
~100
~85
~70
~50
~30
~10
SDS - PAGE

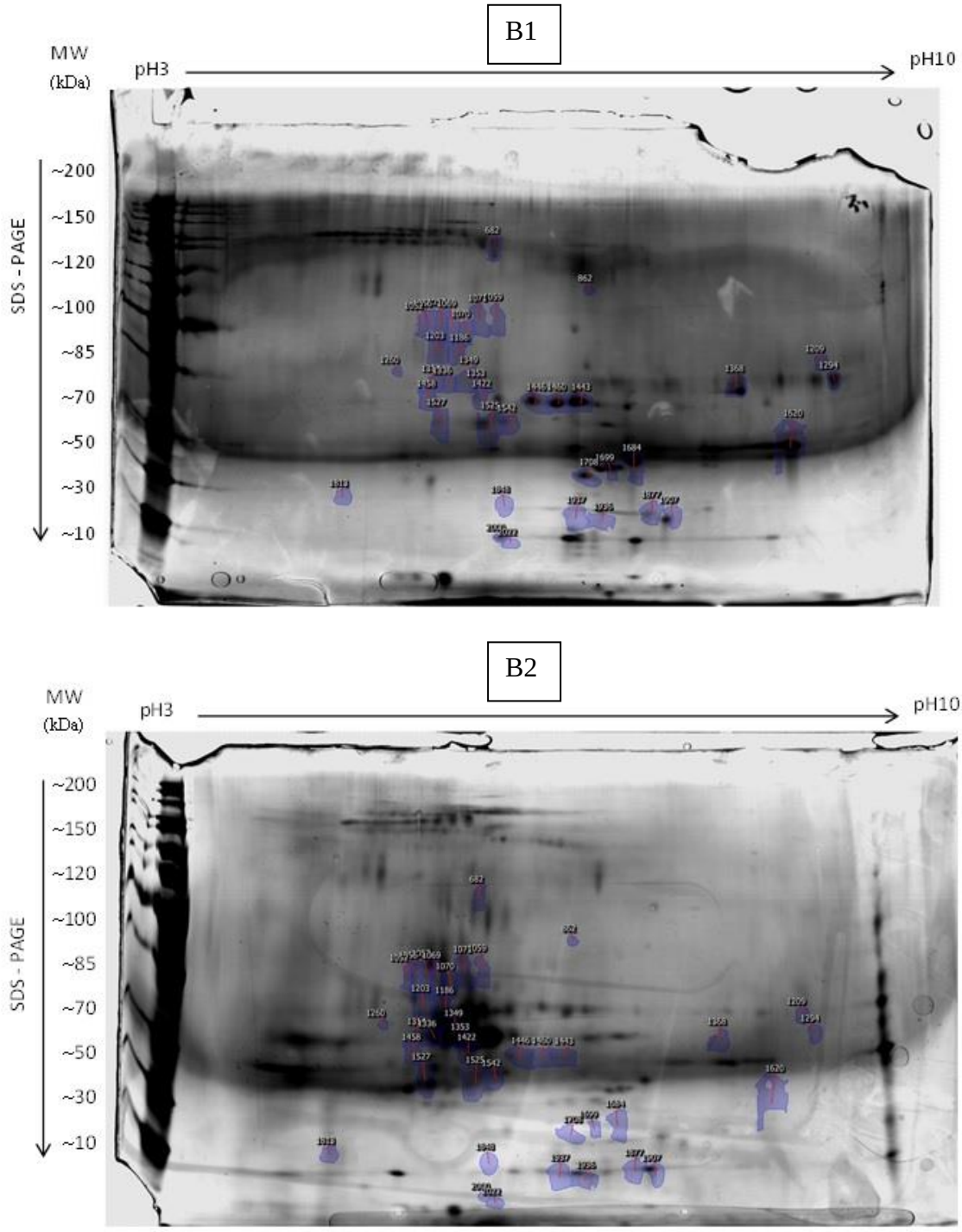



**Figure 3.6. Representative 2-D gel images of *Acanthamoeba* CM proteins untreated and treated with the PMSF inhibitor of serine proteases.** A1: untreated with 1 mM/mL of PMSF (0 – 100 kDa). A2: CM treated with 1 mM/mL of PMSF (0 – 100 kDa). B1: CM untreated with 1 mM/mL of PMSF (100 – 200 kDa). B2: CM treated with 1 mM/mL of PMSF (100 – 200 kDa). In the first dimension, 50 µg total soluble CM was separated on immobiline IPG strips (11 cm, pH 3 – 10). IEF was performed on the IPG-Phor unit. The second dimension was performed on 12% SDS-PAGE; gels were silver-stained and image

analysis was carried out with the Progenesis SameSpot software. M-molecular mass standards are shown on the right-hand side (kDa=kilo Daltons).

**Table 3.1 Specific protein spots identification.** Isoelectric point (pI) and molecular weight (MW) of detected spots

| Spot number | pI (pH) | MW (kDa) |
|---|---|---|
| 107 | 5.6 | 90 |
| 322 | 5.6 | 130 |
| 351 | 5.5 | 133 |
| 389 | 5.8 | 135 |
| 682 | 5.9 | 120 |
| 862 | 6.8 | 110 |
| 1052 | 5.2 | 95 |
| 1056 | 5.1 | 95 |
| 1057 | 5.4 | 95 |
| 1059 | 5.9 | 90 |
| 1069 | 5.5 | 95 |
| 1070 | 5.8 | 95 |
| 1186 | 5.7 | 85 |
| 1203 | 5.5 | 85 |
| 1209 | 9 | 70 |
| 1260 | 5 | 75 |
| 1294 | 9.1 | 60 |
| 1314 | 5.3 | 70 |
| 1336 | 5.5 | 70 |
| 1349 | 5.7 | 80 |
| 1353 | 5.9 | 70 |
| 1358 | 8.1 | 70 |
| 1368 | 8 | 60 |
| 1422 | 5.8 | 60 |
| 1443 | 6.8 | 65 |
| 1446 | 6.3 | 65 |
| 1458 | 5.1 | 60 |
| 1460 | 6.5 | 65 |
| 1525 | 6 | 50 |
| 1527 | 5.2 | 50 |
| 1542 | 6.1 | 50 |
| 1620 | 8.6 | 40 |
| 1684 | 7 | 35 |
| 1699 | 7 | 30 |
| 1708 | 6.9 | 25 |
| 1818 | 4.4 | 10 |
| 1848 | 6 | 10 |
| 1877 | 7.4 | 10 |
| 1936 | 6.8 | 10 |
| 1937 | 6.5 | 10 |
| 1907 | 7.6 | 10 |
| 2000 | 6 | 9 |

| | | |
|---|---|---|
| 2022 | 6.1 | 9 |

**Table 3.2 Representation of spot numbers identified by the Progenesis SameSpot software.** The spots up-regulated (Spot+) or down-regulated (Spot-) in treated and untreated CM.

| Spot number | (0 – 100) kDa Without inhibitor | (0 – 100) kDa With inhibitor | (100 – 200) kDa Without inhibitor | (100 – 200) kDa With inhibitor |
|---|---|---|---|---|
| 107 | Spot+ | Spot+ | Spot- | Spot- |
| 322 | Spot+ | Spot- | Spot+ | Spot- |
| 351 | Spot+ | Spot- | Spot+ | Spot- |
| 389 | Spot+ | Spot- | Spot+ | Spot- |
| 682 | Spot- | Spot+ | Spot+ | Spot- |
| 862 | Spot- | Spot+ | Spot- | Spot- |
| 1052 | Spot- | Spot+ | Spot- | Spot- |
| 1056 | Spot- | Spot+ | Spot- | Spot- |
| 1057 | Spot- | Spot+ | Spot- | Spot- |
| 1059 | Spot+ | Spot+ | Spot- | Spot- |
| 1069 | Spot+ | Spot+ | Spot- | Spot- |
| 1070 | Spot+ | Spot+ | Spot- | Spot- |
| 1186 | Spot+ | Spot+ | Spot- | Spot- |
| 1203 | Spot+ | Spot+ | Spot- | Spot- |
| 1209 | Spot- | Spot- | Spot- | Spot- |
| 1260 | Spot- | Spot- | Spot- | Spot- |
| 1294 | Spot- | Spot- | Spot+ | Spot- |
| 1314 | Spot+ | Spot+ | Spot - | Spot- |
| 1336 | Spot+ | Spot+ | Spot- | Spot- |
| 1349 | Spot+ | Spot+ | Spot+ | Spot- |
| 1353 | Spot+ | Spot+ | Spot- | Spot- |
| 1358 | Spot- | Spot+ | Spot- | Spot- |
| 1368 | Spot+ | Spot- | Spot+ | Spot- |
| 1422 | Spot+ | Spot+ | Spot- | Spot+ |
| 1443 | Spot+ | Spot+ | Spot+ | Spot+ |
| 1446 | Spot+ | Spot+ | Spot+ | Spot+ |
| 1458 | Spot+ | Spot+ | Spot- | Spot- |
| 1460 | Spot+ | Spot+ | Spot+ | Spot- |
| 1525 | Spot+ | Spot+ | Spot- | Spot+ |
| 1527 | Spot+ | Spot+ | Spot- | Spot- |
| 1542 | Spot+ | Spot+ | Spot- | Spot+ |
| 1620 | Spot- | Spot+ | Spot+ | Spot- |
| 1684 | Spot+ | Spot+ | Spot+ | Spot- |
| 1699 | Spot+ | Spot- | Spot+ | Spot+ |
| 1708 | Spot- | Spot- | Spot+ | Spot- |
| 1818 | Spot- | Spot- | Spot- | Spot+ |
| 1848 | Spot- | Spot+ | Spot- | Spot- |
| 1877 | Spot+ | Spot+ | Spot+ | Spot- |
| 1936 | Spot- | Spot+ | Spot- | Spot- |
| 1937 | Spot- | Spot+ | Spot- | Spot- |
| 1907 | Spot- | Spot+ | Spot+ | Spot- |
| 2000 | Spot- | Spot- | Spot+ | Spot- |
| 2022 | Spot+ | Spot- | Spot- | Spot- |

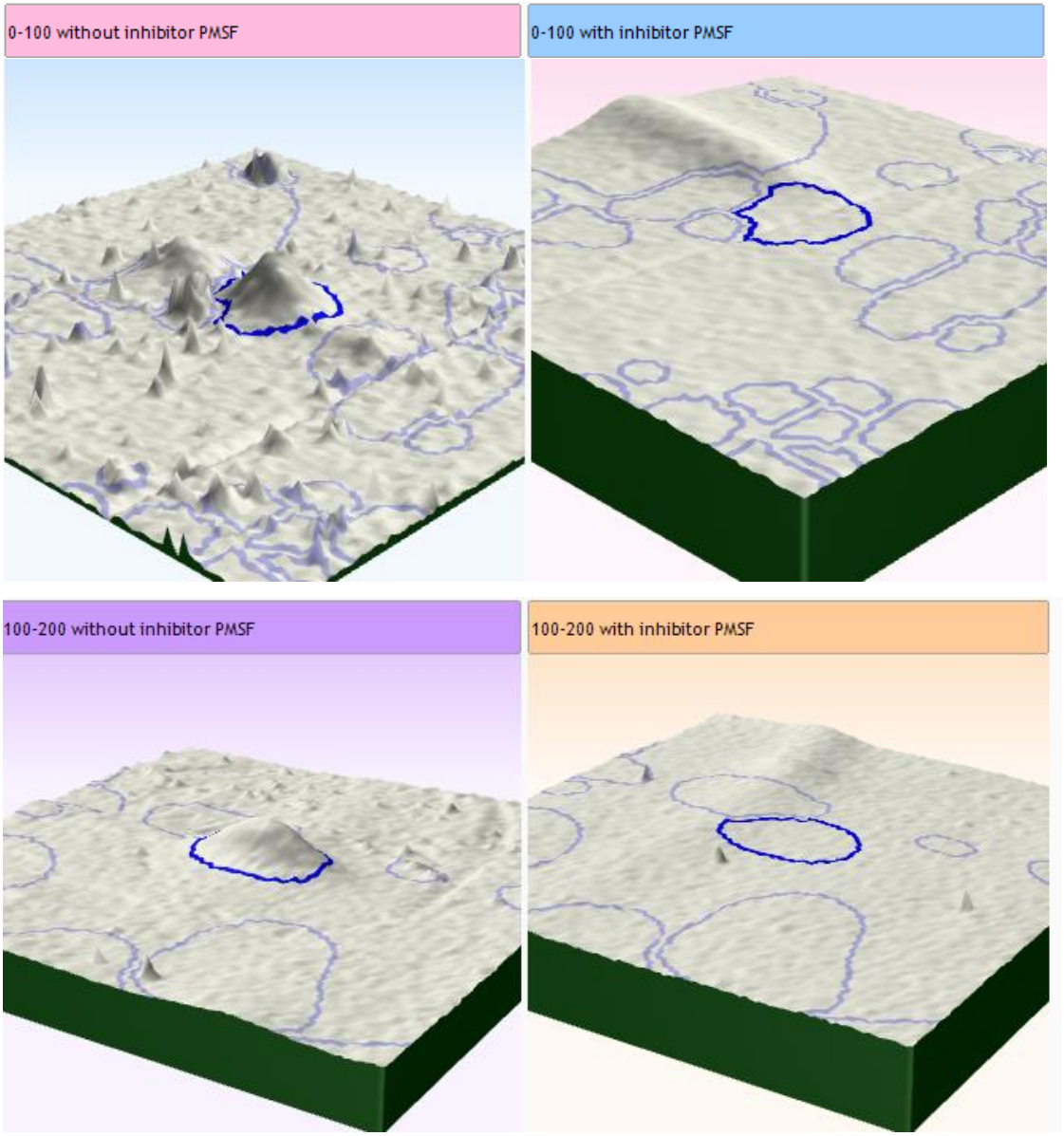



**Figure 3.7. 3D representations of spots identified by the Progenesis SameSpot software.** The results show spots up-regulated or down-regulated in treated and untreated CM stimulated with 1 Mm PMSF.

### 3.4. Discussion

Analysis of proteases from keratitis-causing *Acanthamoeba* shows that the presence of a 130 kDa protease was inhibited by PMSF, which is known to inhibit serine proteases involved in the degradation of the extracellular matrix required for structural and functional maintenance of brain tissue. Thus it has a role in permeabilising the blood-brain barrier (Sissons, Alsam, Stins, *et al.*, 2006). It is interesting that inhibition of serine proteases increased MRSA and MSSA uptake by *Acanthamoeba*. This is the first report to investigate this phenomenon, but it should be studied further.

On addition of 1 mM PMSF in these experiments, 130 kDa band representing serine proteases inhibited, indicating inhibition of serine proteases. This data supports work by Alsam *et al*. (2005), in which it was demonstrated that PMSF addition abolished permeability changes due to serine proteases. However, the role of serine proteases in *Acanthamoeba* interactions with MRSA and MSSA are yet to be investigated.

Phagocytosis is one of the most important factors that cause the pathogenesis of *Acanthamoeba* (Palusinska-Szysz *et al.*, 2014). The actual detailed mechanisms responsible for the process of phagocytosis remain vague. This study explores and describes these mechanisms by studying FITC-labelled bacteria phagocytised by *Acanthamoeba.* Those heat-killed FITC-labelled MRSA and MSSA bacteria were noticed inside the *Acanthamoeba* at 25% and 14%, respectively. Previous studies (Bowers and Olszewski, 1983) showed that *Acanthamoeba* uses two distinct pathways to uptake particles: pinocytosis and phagocytosis. The former is for the uptake of fluid particles and the latter is for the uptake of solid particles. The phagocytosis is receptor-mediated. The phagocytosis in *Acanthamoeba* is a complex process which might play an important role in both food uptake and the pathogenesis of this protozoan. Identifying the molecular mechanisms associated with phagocytosis may help identify therapeutic targets against this serious pathogen (Alsam *et al.*, 2005).

In an effort to investigate the expression of novel proteins, treated and untreated *Acanthamoeba* CM was investigated. Two-dimensional gel electrophoresis is employed to obtain preliminary data about potential differential protein expression. 2-D is considered one of the most powerful tools for protein

separation and fractionation since its introduction in 1975 (Görg *et al.*, 2000). The method has been improved over time to become a more robust tool for proteome analysis. At the same time, the robustness of 2-D is limited to samples with low complexity and subcellular fractions. Other limitations of 2-D include reproducibility, difficulty in separation of low molecular weight, and hydrophobic proteins. The method has a limited dynamic range (Issaq and Veenstra, 2008; Magdeldin *et al.*, 2012), however it has yielded many novel proteins and discoveries in protein chemistry. In this study, 2-D was used to separate and fractionate the whole *Acanthamoeba* CM (treated and untreated) stimulated with 1mM PMSF inhibitor.

Protein spots in the gels were made visible through silver staining and analysed by the Progenesis SameSpot software, as previously used in a number of studies (Amiour *et al.*, 2012; Brasier *et al.*, 2012; Brioschi *et al.*, 2013). Results obtained from this analysis were used as preliminary data to identify differences among treated and untreated *Acanthamoeba* CM treated with 1 mM PMSF inhibitor. The results of this proteomic analysis revealed the resolution of a number of polypeptide spots. Forty three protein spots resolved at a given pI and molecular weights were identified.

The protein search engine Uniprot was used for the analysis of the protein identity for a number of the forty three spots identified. This revealed potential useful results. For example, the polypeptide spot number 389 correlates with the receptor tyrosine-protein kinase erbB-2, which has a pI of ~5.8 and a MW of ~135 kDa. The spot number 1260 could be related to the serine/threonine-protein kinase Nek11, which has a pI of ~5 and a MW of ~75 kDa. The spot number 351 with a pI of ~5.5 and a MW of ~133 kDa (serine protease) could be related to the cell adhesion molecule, where oral immunisation reduced the severity of the corneal infection by modulating MMP-2 and MMP-3 expression (Siddiqui and Khan, 2012). The spot number 322 played a role in the parasite adhesion to the host cell as a primary step and was mediated by a pI of 5.6 and MW of 130 kDa mannose binding protein (MBP) expressed on the surface of *Acanthamoeba*, which was spot number 351 (Alsam, Sissons, Jayasekera, *et al.*, 2005). Finally, two superoxide dismutases have been identified in *Acanthamoeba*: an iron superoxide dismutase, which is spot number 1525 with a pI of 6 and an MW of ~50 kDa, and a copper-zinc superoxide dismutase, which is spot number 1699 with a pI of 7 and an MW of ~38 kDa (Siddiqui and Khan, 2012).

*Acanthamoeba* predominantly secrete serine proteases (Mitro *et al.*, 1994; Leher *et al.*, 1998; Khan *et al.*, 2000, 2001) of varying molecular weight: 12, 40, 42, 55, 70, 85, 97, 107, 130, 133, and 230 kDa reviewed in (Khan, 2006), all sensitive to the PMSF serine protease inhibitor.

Further analysis of mass spectrometry is needed to identify the sequence and identity of the places of differentially regulated polypeptides.

# Chapter 4. Photosensitizers as potential antimicrobial agents for Photodynamic Therapy applications

### 4.1. Background

*Acanthamoeba* are free-living protozoa widely distributed in the environment and recognised as amebic agents of fatal encephalitis and amoebic keratitis, especially in humans with a compromised immune system (Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004). Unfortunately, appropriate antimicrobial therapy for these infections is still lacking research and patient recovery is often problematic (Schuster and Visvesvara, 2004). *Acanthamoeba* protects and assists various bacteria in their dissemination in the environment, including MRSA (Cardas *et al.*, 2012). Intracellular survival and growth of bacteria within *Acanthamoeba* can affect bacteria, making them more pathogenic to humans and more resistant to extracellular hazards such as antimicrobials and disinfectants (Adékambi *et al.*, 2006; Ohno *et al.*, 2008).

*Staphylococcus aureus* is the most common bacterium isolated from both community-acquired and nosocomial infections. *S. aureus* is known to rapidly develop resistance to antibiotics (Wright, 2003; Deurenberg and Stobberingh, 2009). Staphylococcal disease has increased in many countries, in both healthcare and community settings. Currently, MRSA accounts for more than half of nosocomial *S. aureus* infections in most countries (Jean and Hsueh, 2011). Due to limited therapeutic strategies, staphylococcal infections are often difficult to treat. Therefore, there is a need to develop alternative therapeutic methods for the treatment and prevention of MRSA infections.

Because of the decreased number of antibiotics that can be used to treat MRSA infections, the need for new antimicrobial treatments has led to the investigation of photodynamic therapy (PDT). PDT is a treatment that utilizes a drug, called a photosensitizer or photosensitizing agent, and a specific type of light. When photosensitizers are exposed to a particular wavelength of light, they synthesize a form of oxygen that eradicates proximate cells (Hamblin *et al.*, 2011b). Basically, PDT involves treatment of the microorganisms with a chemical activated by light, known as a photosensitizer (PS). When the cells to be killed are pathogenic microorganisms the procedure is termed photodynamic inactivation (PDI) (O'Riordan *et al.*, 2006). The PS has no antimicrobial activity, after exposure to light of a wavelength that is capable of absorption; the PS undergoes a transition from a lower-energy "ground state" to a higher energy "triplet". In the triplet state, the PS can react with substrate molecules to produce free

radicals and free or molecular oxygen ions, resulting in the generation of singlet oxygen (Ochsner, 1997; Redmond and GamLin, 1999). Antimicrobial PDI may be a new approach to killing or eliminating pathogens that are infecting tissue. All studies that have examined the killing of antibiotic resistant bacteria by PDI have found them to be equally as susceptible as their native counterparts or even more susceptible (Tang *et al.*, 2007). Moreover it has not as yet been possible to artificially induce resistance to PDI in any microbes where it has been tested (Lauro *et al.*, 2002).

#### 4.1.1.Foscan

Foscan contains temoporfin (*meta*-tetrahydroxy phenyl chlorin, *m*-THPC), a photosensitising agent that is activated with non-thermal light at 652 nm several days after intravenous administration. It has an oncolytic effect, intended for palliative photodynamic treatment of head and neck squamous cell carcinoma lesions in patients not curable with surgery and/or radiotherapy. The therapeutic effect is mediated through the generation of Reactive Oxygen Species (ROS), a process dependent on the intracellular interaction of temoporfin with light and oxygen. *m*-THPC is a second generation photosensitizer, approved in the EU in 2001 for the diagnosis and management of head and neck cancers. It is the one of greatest powerful photosensitizer categorized by a low required light amount and reduced skin photosensitivity as compared to other PS (Figure 4.1).

In this study we tested a chlorin PS *m*-THPC, which is known as non-toxic to human cells compound .It has been shown to have antimicrobial efficacy against Enterococci (Ossmann *et al.*, 2015). The effect of chlorin *m*-THPC was studied with bacteria under three various conditions: a) PS in absence of light in order to establish the chemical toxicity of *m*-THPC alone; b) in the presence of only internalised (intracellular) PS and blue light to determine its ability to penetrate the cell membrane and cause biological damage; and c) in the presence of extracellular *m*-THPC without internalisation and blue light.

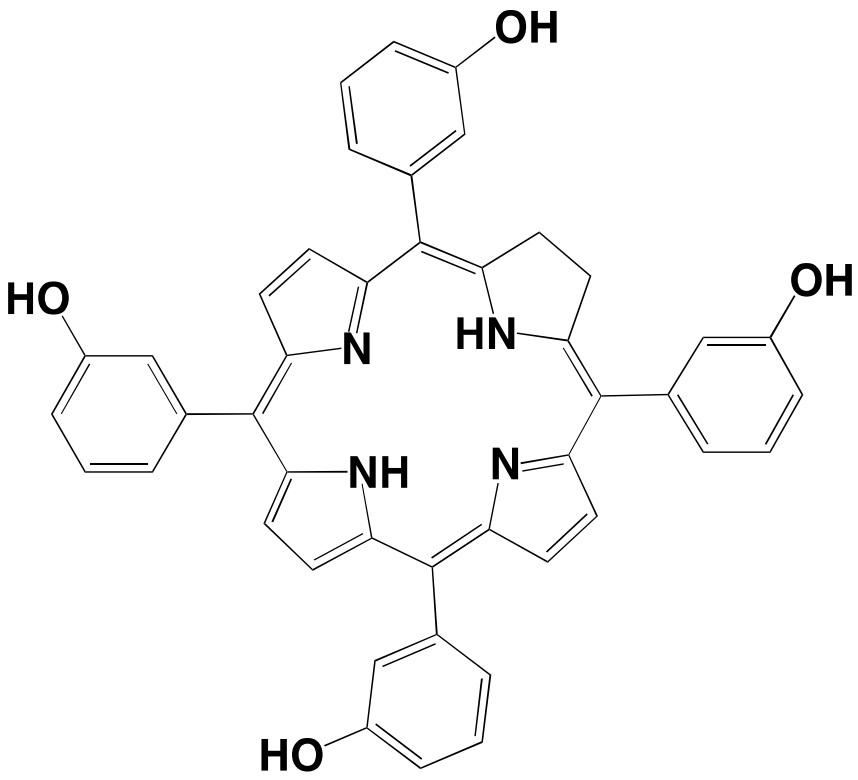



**Figure 4.1. The chemical structure of *meta*- Tetra (hydroxyphenyl) chlorin**

### 4.1.2. Psoralens

Psoralens are a class of planar aromatic molecules that can bind reversibly to single-stranded and double-stranded nucleic acids by intercalation (Figure 4.2). Psoralen illumination with long-wavelength ultraviolet light (UVA light, 320 – 400 nm), intercalated psoralens form mono adducts and interstrand crosslinks with RNA and DNA. In the absence of repair, the psoralen-modified genomes of viruses and bacteria are inactivated because transcription and replication cannot occur. Optimal amounts of psoralen and UVA illumination create sufficient nucleic acid alterations to overwhelm cellular repair capability. Additionally, it is reported to be preserved upon UVA illumination with psoralen (Abonnenc *et al.*, 2015). Another report, described an improved PCT process with psoralen derivatives application (Liu *et al.*, 2015). Different concentrations of psoralens have been used and synthesized to optimise aqueous solubility, cellular permeability, enhanced nucleic acid intercalative binding, and increased photo-reactivity with nucleic acids. Therefore, psoralen was exploited in this study to explore its inactivation potential against *Acanthamoeba* and bacteria with blue light illumination with the range of wavelength which is non-toxic to cells. *Acanthamoeba* and bacteria found to be inactivated with combination of psoralen and 12.5 J/ $cm^2$ of blue light, significantly.

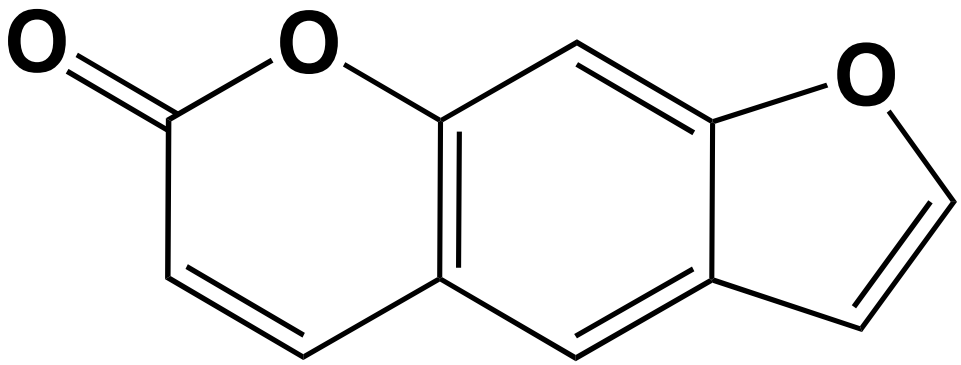



**Figure 4.2. Chemical structure of Psoralen**

## 4.2. Materials and Methods

### 4.2.1. PDT/PDI protocols for bacteria

#### 4.2.1.1. Dark toxicity

For the determination of the dark toxicity of *m*-THPC, MRSA and MSSA bacteria were incubated with and without the highest concentration used under investigation of PS, in order to examine the chemical toxicity of the drug without the light. A single type of bacteria was inoculated into the assigned wells in the dark with *m*-THPC (10 µg/mL concentration) in a 24-well plate, then wrapped with aluminum foil and incubated for 24 h at 37 °C. Bacteria were cultured on CLED agars and were allowed to stand for 20 h in the incubator at 37 °C. Then they were enumerated and the survival ratios of bacteria were calculated as a percentage of control (bacteria without, PS).

#### 4.2.1.2. Time course effect of the intracellular/internalized (*m*-THPC) only on MRSA and MSSA strains with light exposure

*m*-THPC was prepared in RPMI 1640 in a brown glass vial for each bacterial strain. Bacteria MRSA1&2 and MSSA1&2 strains (2 µg/mL, 4 µg/mL, and 6 µg/mL) with OD 0.22 5 x $10^6$, (5 x $10^8$ cfu/mL) were added separately to the above solution of *m*-THPC sample in the dark. The bacteria strains with *m*-THPC were added in Eppendorf tubes containing 500 µl RPMI in triplicate for each strain. In the meantime, bacteria without *m*-THPC were added as above and assigned as control samples. The bacterial samples with and without *m*-THPC were wrapped with aluminium foil and incubated at 37°C for MRSA1&2 and MSSA1&2 were incubated with PS (*m*-THPC) in RPMI 1640 (5 µg/mL) for 2 h, 6 h, 24 h. The samples were agitated gently after one hr to distribute the *m*-THPC. The bacteria were then centrifuged and washed with RPMI media after each incubation time and taken into 24-well plates. The 24-well plates were placed on top of a glass plate which was laid over the LumiSource™ light (PCI Biotech, Norway) source as above and light irradiation was applied for 30 min., corresponding to a total light dose of 12.5 J/$cm^2$. Once irradiated, the bacteria were cultured/counted after 2 h incubation of light exposure on agar plates. The numbers of viable bacteria with and without *m*-THPC and light treatment

were calculated and the results were presented as $Log_{10}$ the number of bacteria with (+) *m*-THPC and without (-) *m*-THPC (bacteria without PS and exposed to light).

The experiment was repeated and bacteria were incubated with *m*-THPC for several incubation periods (2 h, 4 h, 6 h, and 24 h) before light exposure. The bacteria were exposed to the blue light as above then cultured on agar plates after 2h of light exposure.

#### 4.2.1.3. The phototoxic/PDI effect of in presence of extracellular (*m*-THPC) on MRSA1 and MSSA1 strains with light exposure (short incubation)

*m*-THPC were prepared in RPMI 1640 (2 µg/mL) in a brown glass vial for each bacterial strains. Bacterial strains (MRSA1 and MSSA1) were incubated with *m*-THPC for 2 min. in 24 well plates. The plate was irradiated for 2 min., corresponding to a total light dose of 0.8 J/ $cm^2$ with LumiSource™ light in the presence of the extracellular *m*-THPC. Once irradiated, the bacteria were incubated for 24 h after light exposure at 37 °C. The bacteria then cultured on agar plates, as described above and the number of the percentages of viable bacteria were calculated against the control (bacteria without the *m*-THPC, and without light exposure).

#### 4.2.1.4. Determination of minimum inhibitory concentration (MIC) of *m* THPC for MRSA.

MRSA were incubated with and without a range of *m*-THPC concentration (0.2, 0.4, 0.6, 0.8, 1 µg/mL) for 2 min. at 37 °C in the dark. The 24-well plates were placed on top of a glass plate which was laid over the LumiSource™ light (PCI Biotech, Norway) source and light irradiation was carried out for 2 min., corresponding to a total light dose of 0.8 *J*/$cm^2$. Once irradiated, the bacterial samples with and without *m*-THPC were wrapped with aluminum foil and incubated for 24 h after light exposure at 37°C. The bacteria were cultured onto agar plates, as described above. The surviving bacteria with and without *m*-THPC and light treatment were calculated. The ratio between the numbers of viable bacteria and *m*-THPC concentration doses were displayed as a chart and the MIC value was determined. The MIC value is the concentration of *m*-THPC that eliminated MRSA1 bacteria totally after 24 h of light exposure.

### 4.2.2. Phototoxicity/PDI effect of *m*-THPC on *Acanthamoeba*

*Acanthamoeba* were incubated for 24 h in 24-well plates until reaching a confluent state. Next, the well plates were washed once with PBS. After washing, wells (in triplicates) containing *Acanthamoeba* were incubated with different concentrations of *m*-THPC (2, 4, 6 µg/mL) in RPMI 1640 were added and plates were incubated at 30 °C for 1h. Wells also incubated with *Acanthamoeba* without PS in the same plate to see the effect of blue light only on *Acanthamoeba*. Additional plate with *Acanthamoeba* only without PS or light were incubated in the same way and assigned as control (CRTL). The excess of *m*-THPC was discarded and the well plates with *Acanthamoeba* were washed with PBS. The well plates were exposed to blue light for 20 min. (8 $J/cm^2$), then were incubated overnight at 30 °C. Trypan blue (0.4 % concentration) was added and *Acanthamoeba* were counted. The experiment was repeated for longer incubation times (5 h and 14 h) instead of 1h with *m*-THPC (5 µg/mL). *Acanthamoeba* viability was calculated as the percentage of the control (*Acanthamoeba* only, without *m*-THPC or blue light but incubated at 30 °C for 1 h, 5 h, or 14 h).

#### 4.2.2.1. Phototoxicity/PDI effect of *m*-THPC on *Acanthamoeba* with the bacteria inside

*Acanthamoeba* were incubated for 24 hrs in 24-well plates until reaching a confluent state. Next, the well plates were washed once with PBS. After washing, 5 x $10^6$ bacteria were added for 1h. The well plates were washed again with PBS. Vancomycin (100 µg/mL, final concentration) was added for 45 min. to kill the extracellular bacteria. Wells containing *Acanthamoeba* with the intracellular/engulfed bacteria were incubated with/without *m*-THPC (2, 4, 6 µg/mL) for 1h, then repeated for longer 5h, or 14h with/without *m*-THPC (5µg/mL) at 30°C. The plates were exposed to blue light for 20 min (8 $J/cm^2$) and treated as above. Control (CRTL) *Acanthamoeba* with the intracellular/engulfed MRSA were considered and incubated in separate a plate without PS or light exposure.

#### 4.2.2.2. Phototoxicity/PDI effect of *m*-THPC on the intracellular/engulfed MRSA bacteria inside *Acanthamoeba*.

The intracellular MRSA within *Acanthamoeba* for the above plates, which incubated with/without m-THPC and with/without light exposure, were all examined. *Acanthamoeba* with the engulfed MRSA were lysed and (20 µl) of bacteria were taken on agar plate and counted as was explained in the experimental sections for bacteria.

#### 4.2.2.3. Effect of psoralen on *Acanthamoeba* and the bacteria inside *Acanthamoeba*

Methods were similar to section 4.2.2.1 but instead of *m*-THPC, psoralen was used with various concentrations (100, 200, 300, 400, 512, 768 µg/mL).

### 4.2.3. Irradiation light source

Light irradiation for PDT was carried out using a LumiSource™ lamp (Biotech), which had been turned on 30 min prior to use. The light source (Figure 4.3) contains a bank of four light tubes, emitting blue light with a peak at 420 nm and reflectors designed to provide homogenous illumination of the treatment area. The 24-well plates were placed on the removable glass plate on top of the light source for 30 min and covered with a dark sheet to shield against any other forms of present light. This light field was approximately 14 cm x 32 cm.

**Figure 4.3. LumiSource™ (PCI Biotech) lamp used for irradiation.**

## 4.3. Results

### 4.3.1. PDT assays

The effect of PDT on bacteria was examined using *m*-THPC drug as potential antimicrobial agent by three different sets of conditions. Firstly, the chemical toxicity of *m*-THPC uptake was determent after range of incubation periods between 2 h-24 h in the dark, without light exposure. Secondly, PDT effect of the internalized / intracellular *m*-THP only on bacteria strains after time-course incubation periods between 2h-24h, then exposed to blue light with energy level of 12.5 $J/cm^2$ (blue light for 30 min), in the absence of extracellular *m*-THPC. Thirdly, PDT effects of extracellular *m*-THPC on bacteria were investigated after very short incubation time (couple of min) then exposure to low energy level of 0.8 $J/cm^2$ (blue light for 2 min) in the presence of *m*-THPC. Number of viable bacteria was calculated after 24 h of light exposure.

#### 4.3.1.1. *m*-THPC Effect on MRSA and MSSA in the absence of blue light

To determine the dark toxicity of *m*-THPC on MRSA and MSSA strains without exposure to light, the experiment was carried out with *m*-THPC the highest concentration used for the phototoxicity (10 μg/mL). The results show the percentage of bacteria that survived after 24 h of incubation with the *m*-THPC in comparison to the control (bacteria without *m*-THPC) for each strain (Figure 4.4). In general, the chart showed an insignificant decrease in the number of bacteria with the PS (*m*-THPC) in the dark. The number of MRSA were even increased in the dark with PS slightly after 2 h and 4 h incubation. This indicated that *m*-THPC was not chemically toxic by itself for bacteria without light exposure.

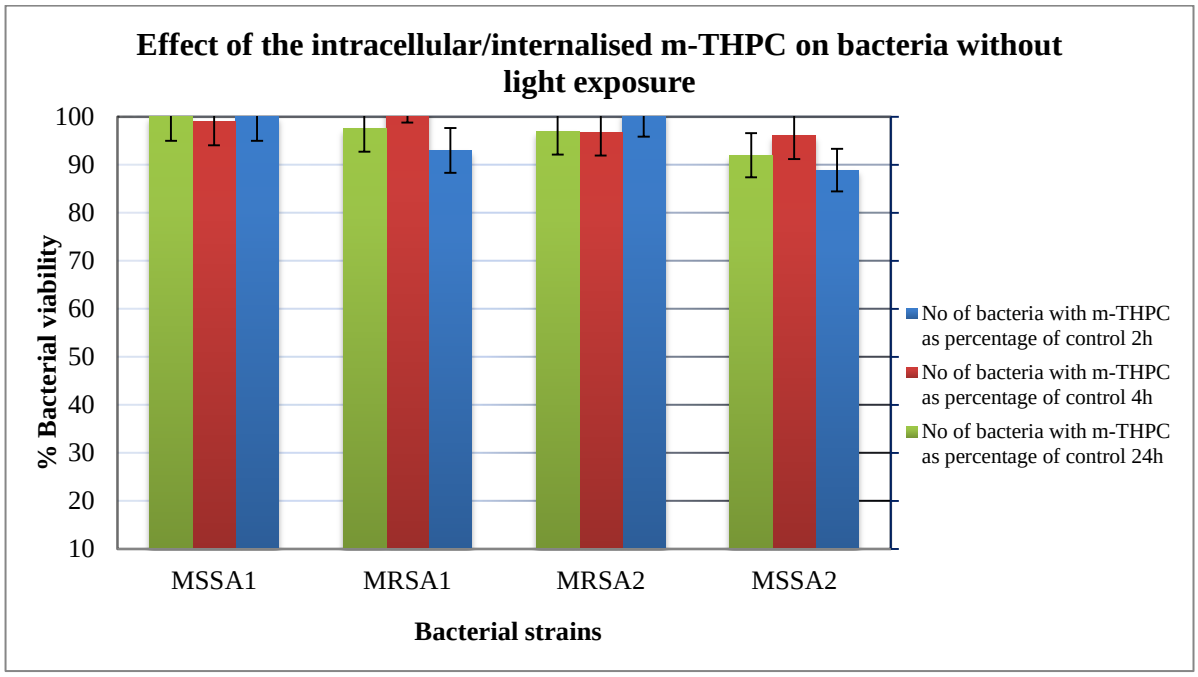



**Figure 4.4. Dark toxicity of *m*-THPC incubated with bacterial strains alone for 24 h in RPMI media, plates wrapped with aluminum foil to avoid light exposure at 37 °C.** Bacteria strains were washed and cultured on agar gel for determining the chemical toxicity. These results represent selected data of three independent experiments in duplicate. Error bars represent standard error (*P>0.5*). The standard error (SE) is ±1**.**

#### 4.3.1.2. Time-course effect of the intracellular / internalised (*m*-THPC) only on MRSA and MSSA strains with light exposure

The phototoxicity of *m*-THPC with a concentration of 5 μg /mL on MRSA and MSSA strains was examined during the time course experiments. Bacteria were incubated for several periods of time (2 h, 4 h, 6 h, and 24 h) and exposed to blue light 12.5 *J*/ $cm^2$ (wavelength band between 405 – 420 nm) in the absence of extracellular PS. The viabilities of bacteria were determined after 2 h of light exposure. The PS, *m*-THPC exhibited over 99 % reductions at that concentration for both MRSA and MSSA strains in each incubation period. Bacteria without *m*-THPC and exposed to light (control) also suffered significant reductions but continued the growth after light exposure (during 24 h). (Figure 4.5, A and B) The intracellular *m*-THPC molecules could not completely eradicate the bacteria 100%, bacteria growth was visible after several hrs of light exposure up to 24h. PDT using the above set of conditions showed a similar effect on both MRSA strains and MSSA strains; the viable bacteria were approximately less than 0.1% in both strains after the time-course of incubation with the PS between 2 h-24 h, (Figure 4.5 A, B).

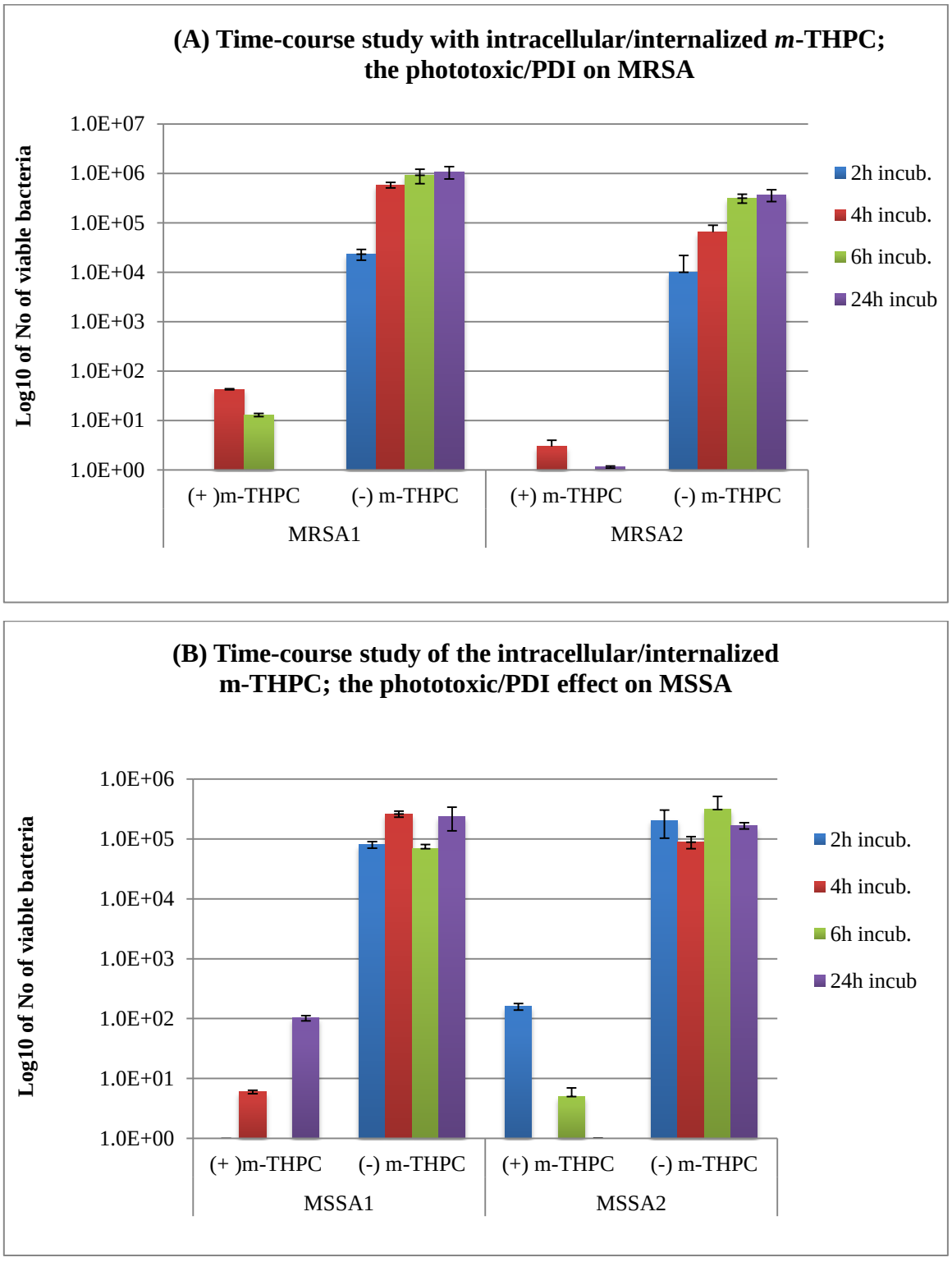
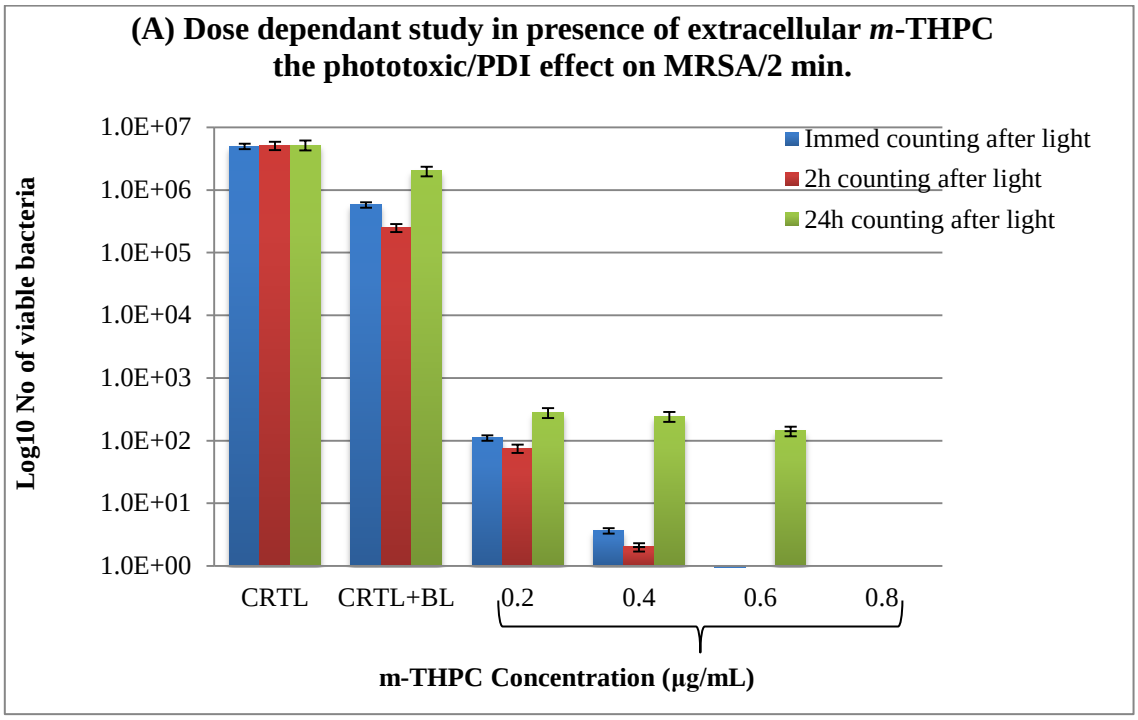



**Figure 4.5. PDT/Phototoxicity of intracellular/internalized *m*-THPC on MRSA1 and MRSA2 (A), , MSSA1 and MSSA2 (B) with light exposure.** *m*-THPC (5 μg/mL) incubated with bacteria of original number without light or PS (control, 5 x $10^6$) in RPMI medium for 2 h, 6 h, and 24 h at 37 °C, in the dark. The excess *m*-THPC was discarded and washed twice. Bacteria were exposed to blue light (12.5 J / $cm^2$ for 30 min.), then they were cultured after 2 h of the light exposure onto agar gel to calculate the viable bacteria. Data were analysed by *t*-test which showed (***P*<0.0005).

#### 4.3.1.3. Phototoxic/PDI effect in presence of extracellular *m*-THPC on bacterial strains

The following experiments attempted to determine whether the PS (*m*-THPC) was required to penetrate the cell membrane of bacteria in order to cause the damage, or bacteria eradication can occur without the internalisation. The results were relatively comparable for both strains; PDT effect was greater on MSSA than MRSA, where a total eradication of MSSA was accomplished at a lower concentration than the required one for MRSA strain.

The data (Figure 4.6) represents the experimental investigation of the phototoxicity of *m*-THPC on MRSA1 and MSSA1 bacteria during both PS and light exposure for very short incubation time. It showed the viabilities of bacteria after PS for a short period of time (2 min ) and with light energy (2 min, 0.8 $J/cm^2$) in the presence of *m*-THPC with a range of concentrations between 0.2 – 0.8μg/mL. Under the above set of conditions, MSSA1 showed better destruction than MRSA1 when bacteria cultured onto agar gel immediately after light exposure or after 24 h. MRSA1 survival was approximately 25 % using the concentrations of 0.2-0.6 μg/mL, while the total eradication was achieved with 0.8 μg/mL, Figure 4.6 (A). However, a complete elimination of MSSA1 and MRSA1 was recorded after 24 h light exposure with concentration less than 0.2 μg/mL for MSSA1 and 0.8 μg/mL for MRSA1(Figure 4.7). The MIC values for MRSA bacteria were determined at the above set of conditions (2 min incubation with *m*-THPC, 2 min of light exposure, and culturing to examine the bacterial survival after 24 h) using the range of concentrations between 0.2-1 μg/mL and 0.2 μg/mL (Figure 4.6). Bacteria growth from both strains did not continue after 24 h incubation under those conditions. Therefore, the MIC values of *m*-THPC were considered to be 0.8 $J/cm^2$ for MRSA and less than 0.2 μg/mL for MSSA1 (Figures 4.7 and 4.8).



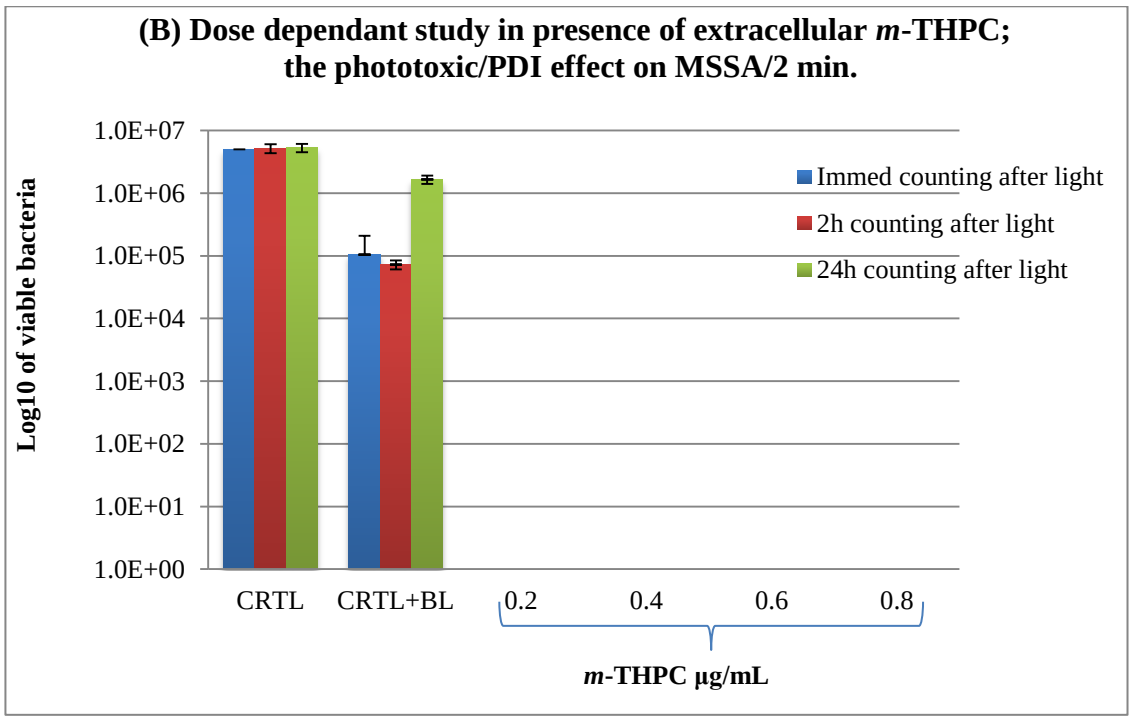




**Figure 4.6. PDT/Phototoxicity in the presence of extracellular PS under light exposure (A) for MRSA1 and (B) for MSSA1:** Bacteria were incubated with various concentrations of *m*-THPC at 37 °C, then exposed to blue light (0.8 $J/cm^2$, 2 min ) Bacteria then cultured/counted either immediately, 2 h, or after 24 h on agar gel and number of viable bacteria were calculated. (CRTL) represents the control for bacteria without light or PS, (CRTL+BL) represents bacteria exposed to light only without PS. (**$P < 0.0005$).

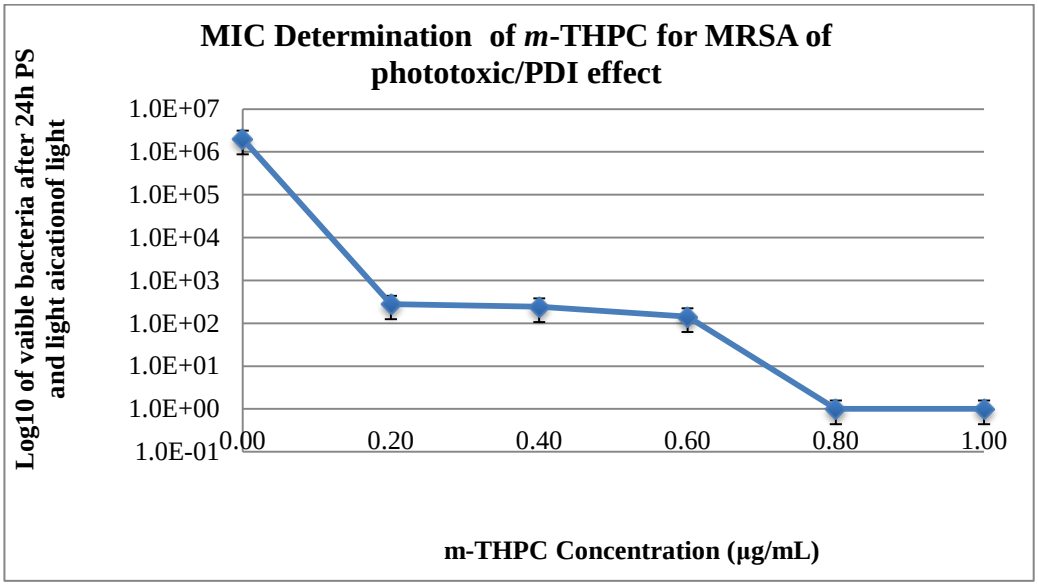



**Figure 4.7. Determination of MIC upon PDT treatment with presence of the PS for short incubation time and short light exposure**; MRSA1 incubated with various concentrations of *m*-THPC for 2 min at 37 °C, then exposed to blue light (0.8 J, 2 min.) in the presence of *m*-THPC. MRSA bacteria were cultured after 24 h from light exposure to measure MIC value (**$P < 0.0005$).

#### 4.3.1.4. The immediate effect of blue light on bacteria without PS

The experiment investigated the effect of blue light on MRSA and MSSA bacteria in the absence of PS *m*-THPC immediately after light exposure. It was found that blue light has a significant effect on bacteria. There were only 1% of both MRSA1 and MSSA1 bacteria surviving after exposure to blue light for 30 min (12.5 *J*), and cultured on agar gel, immediately. It was clear from the experimental data above, there were reductions of viable bacteria for both strains. The control (bacteria without *m*-THPC) which was exposed to light for 2 min (0.8 $J/cm^2$) was also considerably reduced, (Figure 4.8). It is worth recording that a prolonged light exposure time from 2 min to 30 min did affect the number of bacteria survived, on one hand. On the other hand, bacteria continued to grow to further after 24 h after light exposure.

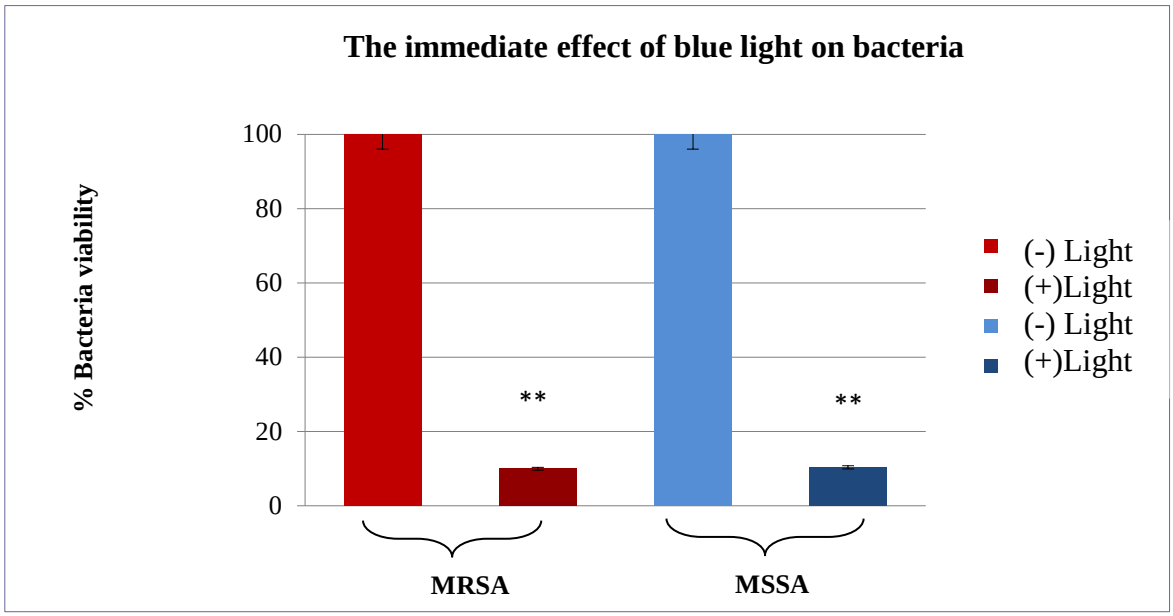



**Figure 4.8. The effect of blue light alone on bacteria.** The percentages of bacterial strains (MRSA, MSSA) survival after exposure to light for 2 min. (0.8 *J*) as the percentages of the original number of bacteria used without light, then cultured immediately afterwards (***P<0.0005*).

#### 4.3.1.5. Phototoxicity of *m*-THPC on *Acanthamoeba*

This experimental section explores the effect of PDT on *Acanthamoeba* trophozoites. PDT investigation was carried out first with incubating *Acanthamoeba* trophozoites with *m*-THPC with a range of concentrations (2 μg/mL, 4 μg/mL, 6 μg/mL) for one hr and exposed to blue light for 20 min (8.3 $J/cm^2$). The viability of *Acanthamoeba* trophozoites with or without *m*-THP*C* but exposed to the light were calculated after 24 h from their exposure to the light, againest the control (*Acanthamoeba* trophozoites that did not expose to light or *m*-THPC), (Figure 4.9 A). The blue light reduced the number of *Acanthamoeba* trophozoites to 78% without the PS effect. Further reduction of *Acanthamoeba* trophozoites were achieved with the range of *m*-THPC concentrations used and blue light together for 1 h, decreasing the numbers of *Acanthamoeba* trophozoites to 57%, 42%, and 36%, respectevely. When the experement was repeated with prolonging the incubation time for 5h and 14h, the number of *Acanthamoeba* trophozoites diminished to approximately 21% and 9.7%, respectively, (Figure 4.10, B). Whereas, the number of *Acanthamoeba* trophozoites incubated without PS for 5 h and 14 h and exposed then to blue light only were also reduced to 67 % and 83 %, respectively.

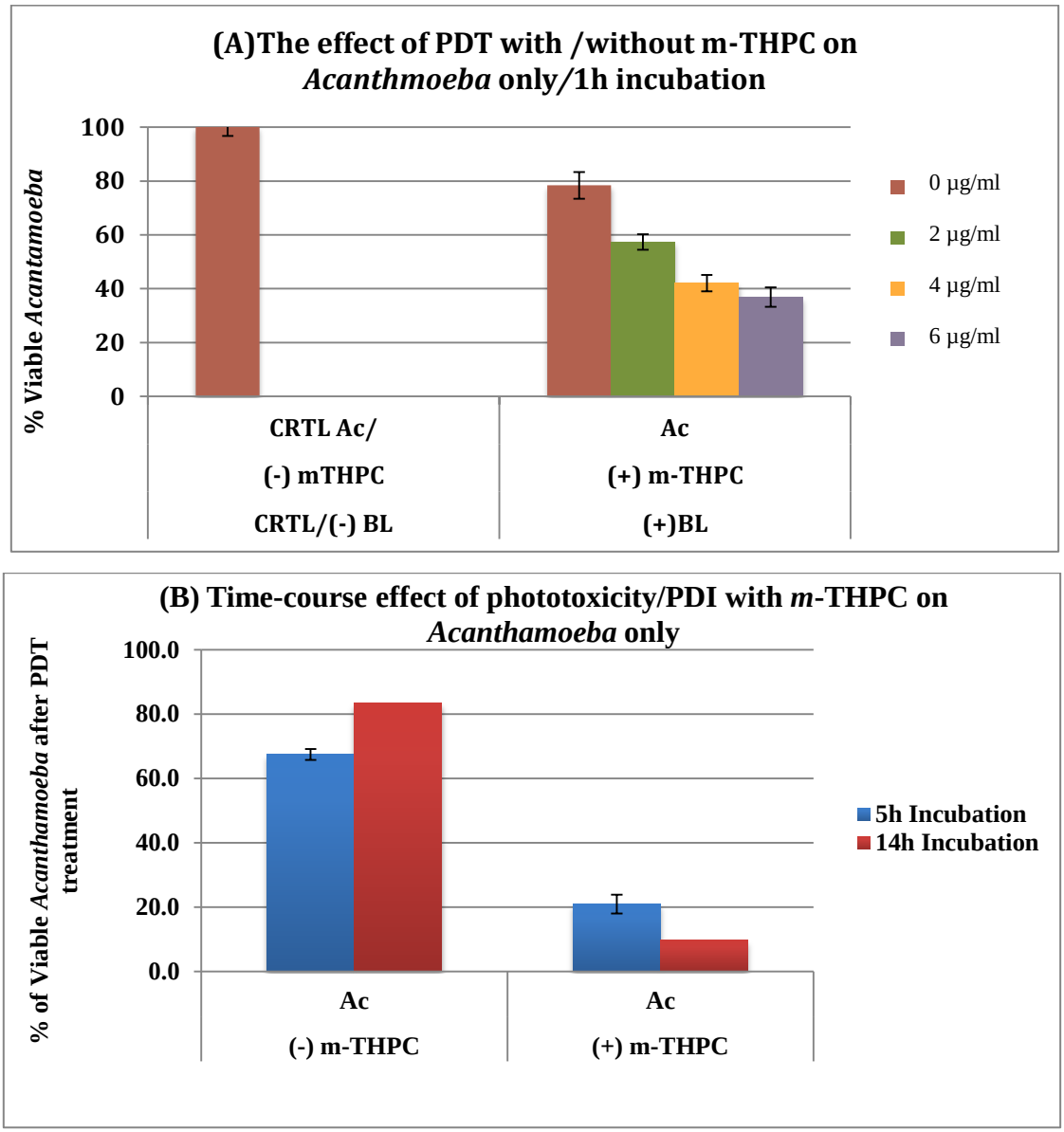
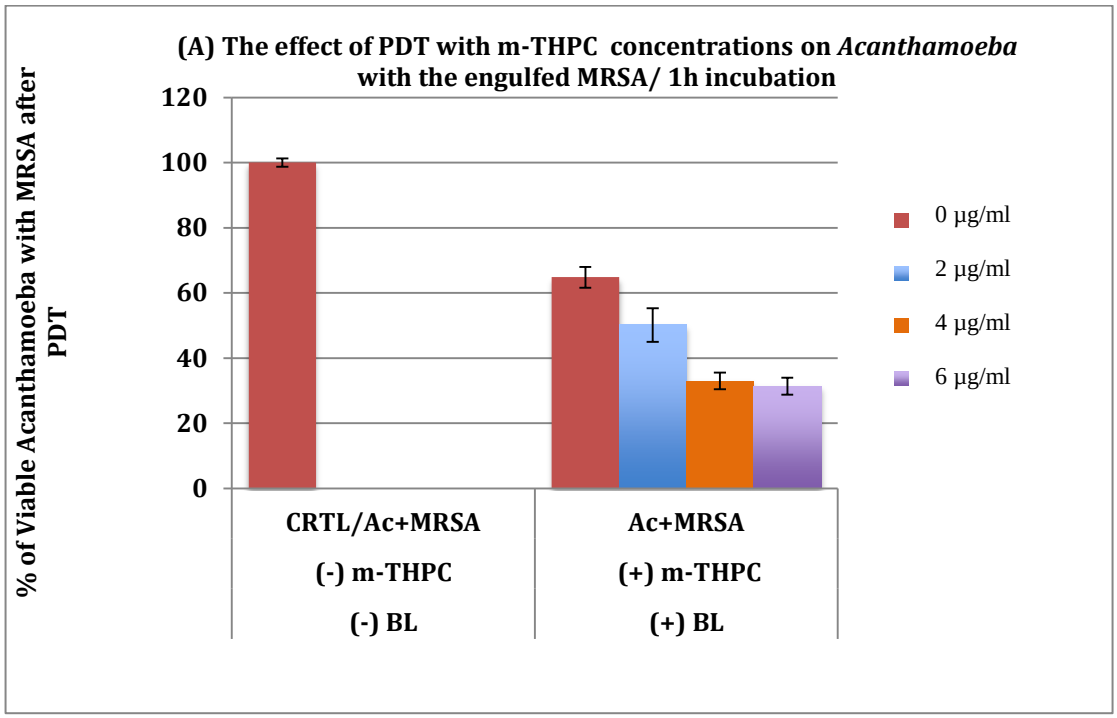



**Figure 4.9. PDT/Phototoxicity of PS m-THPC on (A) *Acanthamoeba* (Ac) *Acanthamoeba* incubated with *m*-THPC and exposed to light for 1 h. (B) *Acanthamoeba* incubated with *m*-THPC (2 µg/mL, 4 µg/mL, 6 µg/mL) and exposed to light for 5 h and 14 h.** The medium was discarded and *Acanthamoeba* was washed with PBS then exposed to light for 20 min (8.3 $J/cm^2$). The viability of *Acanthamoeba* was examined with trypan blue after 24 h of light exposure and the percentage was determined against CRTL (control: *Acanthamoeba* was not exposed to PDT). This result is a mean of three independent experiments in duplicate. Error bars represent standard error (**$P$<0.005).

#### 4.3.1.6. PDT Effect of *m*-THPC on *Acanthamoeba* with the intracellular/ingulfed bacteria

*Acanthamoeba* with the intracellular/engulf MRSA were also examined with three different concentrations of *m*-THPC (2, 4, 6 μg/mL) for 1 h, then exposed to light for 20 min (8.3 $J/cm^2$). There were significant reduction in the number of *Acanthamoeba* incubated with PS for 1 h, elevated with raising the dose of PS from 2 - 6 μg/mL. *Acanthamoeba* with MRSA reduced to a range between 50% - 31 %, applying the above concentration. Therefore, $IC_{50}$ (the concentration that eradicate *Acanthamoeba* with MRSA by 50 % would be approximatly 2 μg/mL, (Figure 4.10, A). It's worth noticing, the blue light alone eliminated around around 36% of *Acanthamoeba* with intracellular bacteria. The phototoxic effects of *m*-THPC with light exposure of 8.3 $J/cm^2$ on the viability of *Acanthamoeba* with engulfed bacteria were examined with increasing the incubation period to 5 h, then lysis after 24 h of light exposure. The phototoxicity of PS demonstrated higher reduction of around 80 %. A substantial effect has been found when longer incubation periods up to 14 h were applied on *Acanthamoeba* with engulfed bacteria. *Acanthamoeba* with intracellular/engulfed bacteria were reduced significantly by 93 % to reach approximately 7 % of the number of *Acanthamoeba* with engulfed MRSA (were not exposed to light and PS). *Acanthamoeba* with bacteria exposed to blue light, also decreased by approximately 44 % and 21 % without *m*-THPC incubation, (Figure 4.11, B).



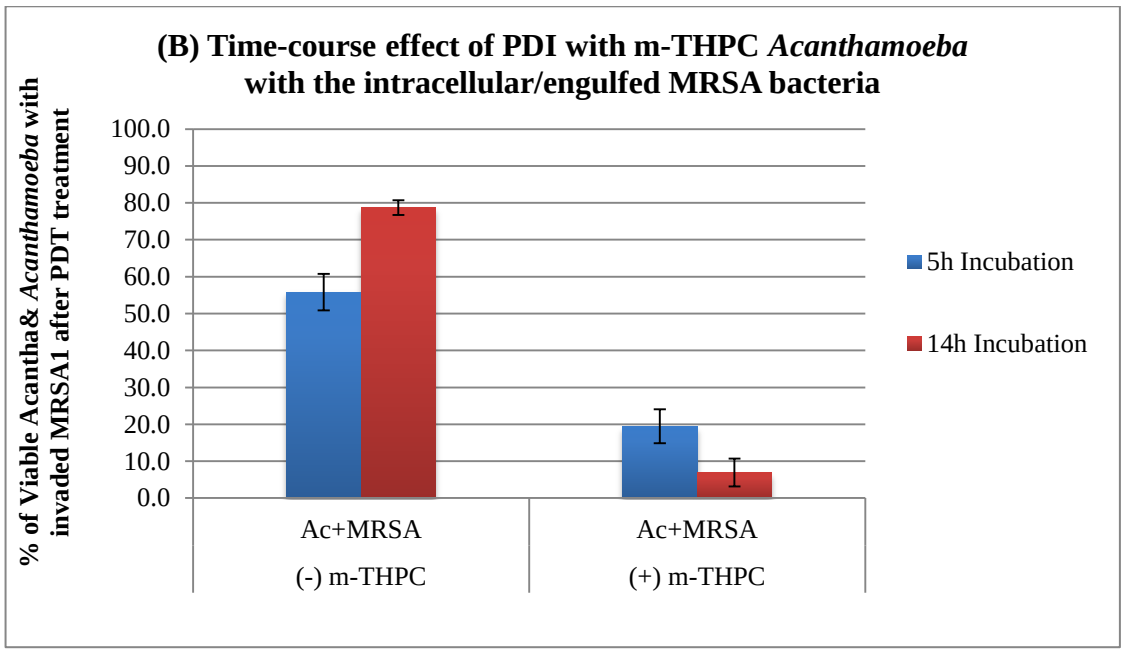




**Figure 4.10. PDT effect on *Acanthamoeba* with the intracellular/engulfed MRSA.** (A) The effect of *m*-THPC concentrations (2 µg/mL, 4 µg/mL, 6 µg/mL) for 1h on *Acanthamoeba* with the intracellular/engulfed MRSA. (B) Time-course effect on *Acanthamoeba* with the intracellular/engulfed MRSA incubated with/without 5 µg/mL *m*-THPC for 5 and 14h. *Acanthamoeba* was incubated with MRSA1 [10 x $10^6$] for 1h. The excess of bacteria was discarded and the membrane - associated bacteria were killed by vancomycin. *Acanthamoeba* with the intracellular/engulfed MRSA were incubated with or without *m*-THPC. The excess *m*-THPC was discarded. *Acanthamoeba* with the bacteria inside were washed and exposed to light for 20 min. (8.3 *J*/$cm^2$), The viability of *Acanthamoeba* was examined with trypan blue (0.4 %) after 24 h of light exposure and the percentage was determined against CRTL (control: *Acanthamoeba* with the intracellular/engulfed MRSA were not exposed to PDT). This result is a mean of three independent experiments in duplicate. Error bars represent standard error (***P*<0.005).

#### 4.3.1.7. The effect of *m*-THPC on the engulfed bacteria within *Acanthamoeba*

The role of *Acanthamoeba* in protecting bacteria against the PDI effect was also examined. Therefore, the viability of MRSA bacteria that invaded *Acanthamoeba* and subjected to PDT was inspected. *Acanthamoeba* were lysed and bacteria were cultured on agar gel after 24 h of light exposure and calculated. The viabilities of bacteria were reduced to 40.5 %, 26 % and 24 % for *m*-THPC concentrations applied (2, 4, 6 μg/mL) after 1h incubation and culturing after 24 h of light exposure, (Figure 4.11, A). Blue light alone reduced the number of intracellular bacteria by more than 45 %.

The engulfed MRSA bacteria strain inside *Acanthamobea* were diminished completely with longer incubation periods. There were no bacteria survived inside *Acanthamoeba* after PDT treatment (*m*-THPC, 5 μg/mL and blue light of 8.3 $J/cm^2$) for 5 h or 14 h, then cultured after 24 h of light exposure (Figure 4.11, B).

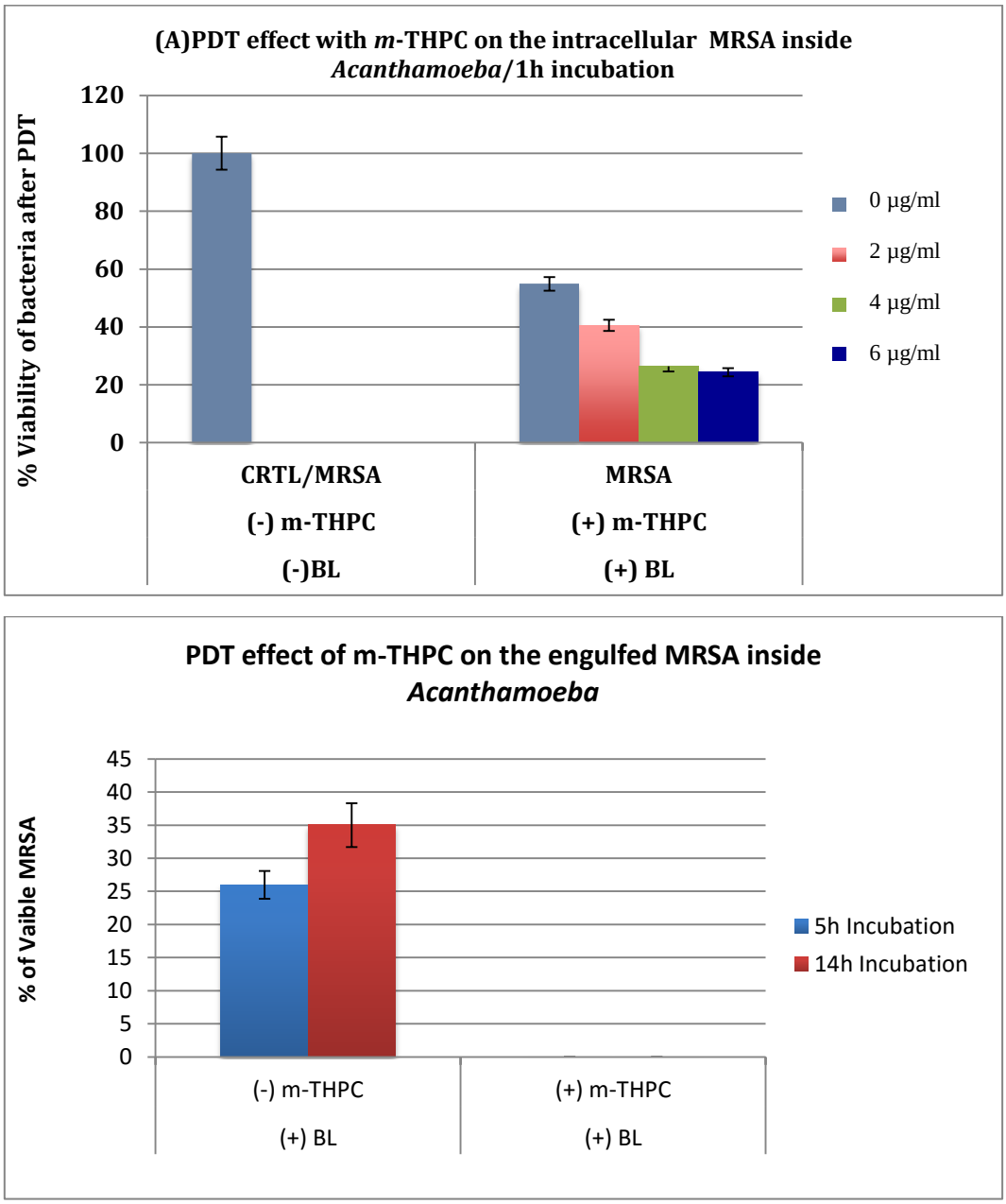



**Figure 4.11. PDI/Phototoxicity of MRSA inside *Acanthamoeba* incubated with *m*-THPC** (A) The effect of *m*-THPC concentrans (2 µg/mL, 4 µg/mL, 6 µg/mL) for 1 h and (B) Time-course effect on the intracellular/engulfed MRSA incubated with/without 5 µg/mL *m*-THPC for 5 h and 14 h and exposed to blue light (8.3 *J*/cm$^2$). The results were calculated after 24 h of light exposure as percentages of bacteria with/without PS against the percentage of control (bacteria without PS or light exposure). The results are the mean of three independent experiments performed in duplicate. Error bars represent standard error (**P<0.0005).

#### 4.3.1.8. Effect of psoralen on *Acanthamoeba* and bacteria inside *Acanthamoeba*

The effects of three different concentrations (100, 200 and 300 µg/mL) of psoralen on *Acanthamoeba* in the presence and absence of blue light were investigated. Blue light itself without psoralen was able to reduce the number of *Acanthamoeba* by 19 %. On the other hand, psoralen itself had dose dependent growth inhibitory effects against *Acanthamoeba*. 768 µg/mL of psoralen concentration eradicated 100% of trophozoites ($IC_{100}$). 300 µg/mL of psoralen without exposure to blue light reduced the number of *Acanthamoeba* by 23%. When the same concentration is used with blue light exposure, 100% reduction of *Acanthamoeba* number was found (Figure 4.12).

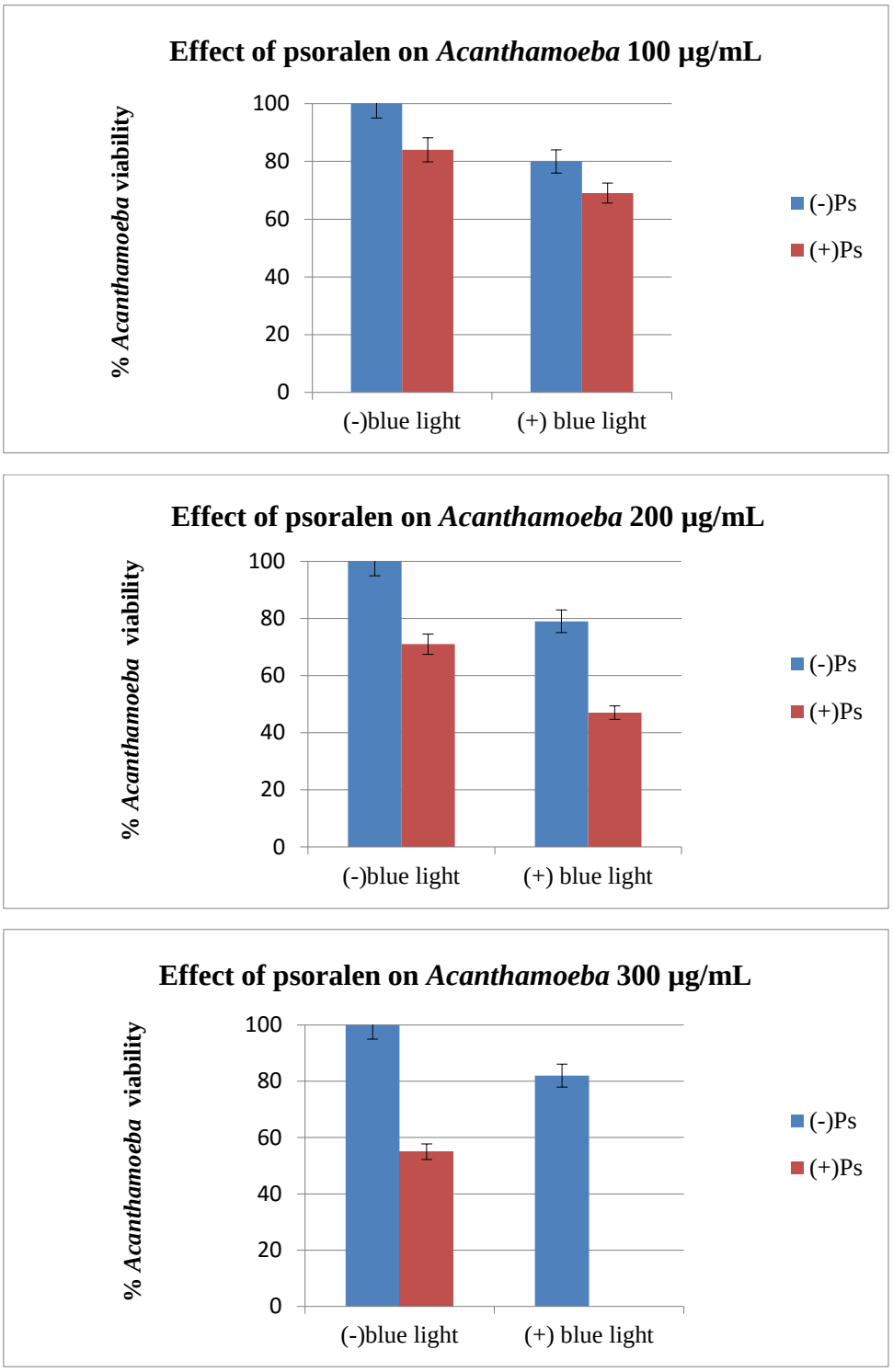
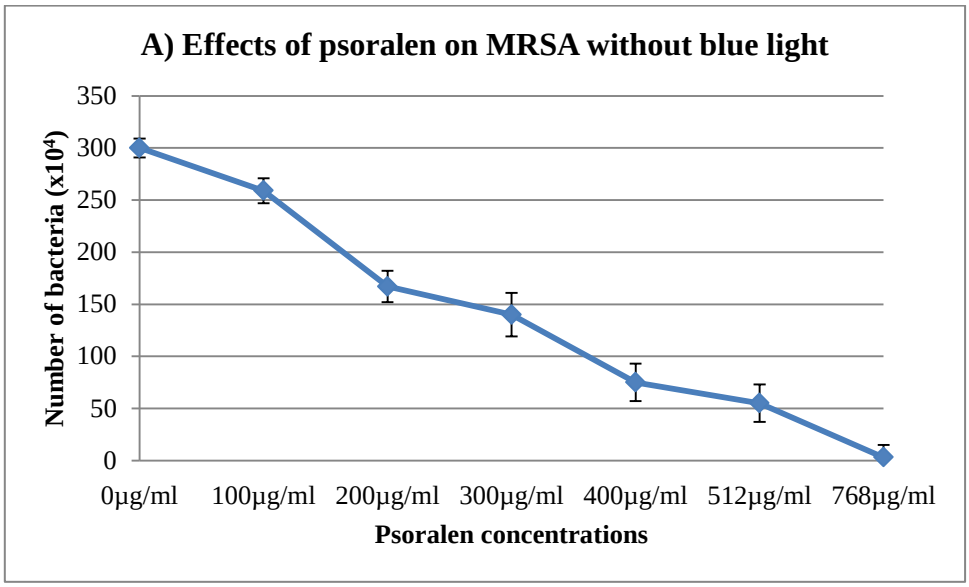
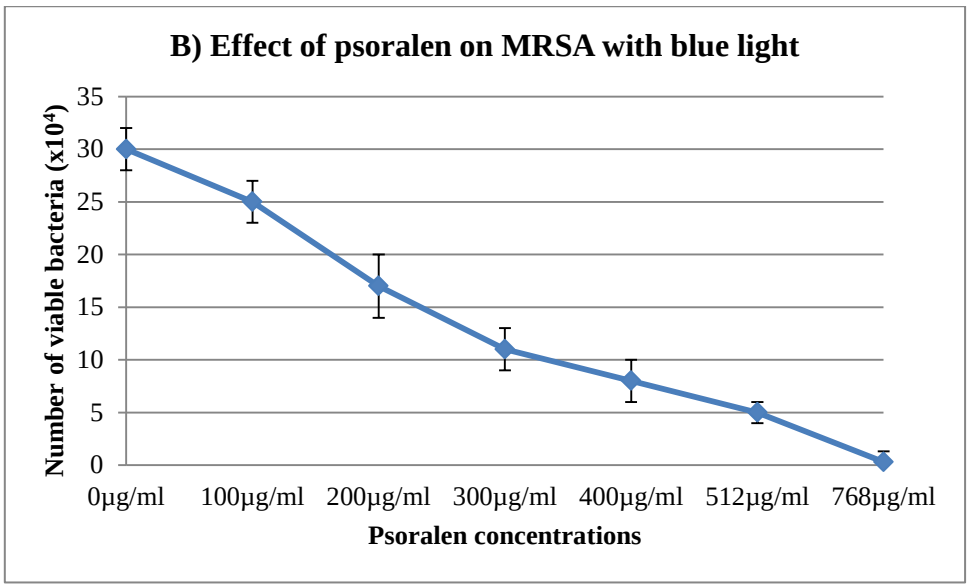



**Figure 4.12. Effects of psoralen (100 – 300 µg/mL) on *Acanthamoeba* in the presence of Ps with and without blue light exposure.** *Acanthamoeba* was incubated for 24 h and exposed to blue light (12.5 J, 30 min). Every result is a mean of three independent experiments performed in duplicate. Error bars represent standard error (*P<0.05).

#### 4.3.1.9. Effect of psoralen (Ps) on MRSA and MSSA bacteria

Bacteria with a starting number of 5 x $10^6$ colonies were incubated with various concentrations of psoralen for 24 h at 37 °C, then exposed to blue light (12.5 J, 30 min) in the presence and absence of psoralen, then cultured after 30 min of light exposure. The phototoxic effect on MSSA is much higher than that on MRSA under the same conditions (30 min incubation, 12.5 J) in the presence and absence of psoralen with a range of 100 – 768 μg/mL). The lowest concentration required to reach total destruction of both bacteria was 768 μg/mL with light exposure, while the concentration of 768 μg/mL was not enough to cause total damage of both bacteria without light exposure after 24 hrs (Figure 4.13).


A) Effects of psoralen on MRSA without blue light
Number of bacteria (x10⁴)
350
300
250
200
150
100
50
0
0μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml
512μg/ml
768μg/ml
Psoralen concentrations


B) Effect of psoralen on MRSA with blue light
Number of viable bacteria (x10⁴)
35
30
25
20
15
10
5
0
0μg/ml
100μg/ml
200μg/ml
300μg/ml
400μg/ml
512μg/ml
768μg/ml
Psoralen concentrations

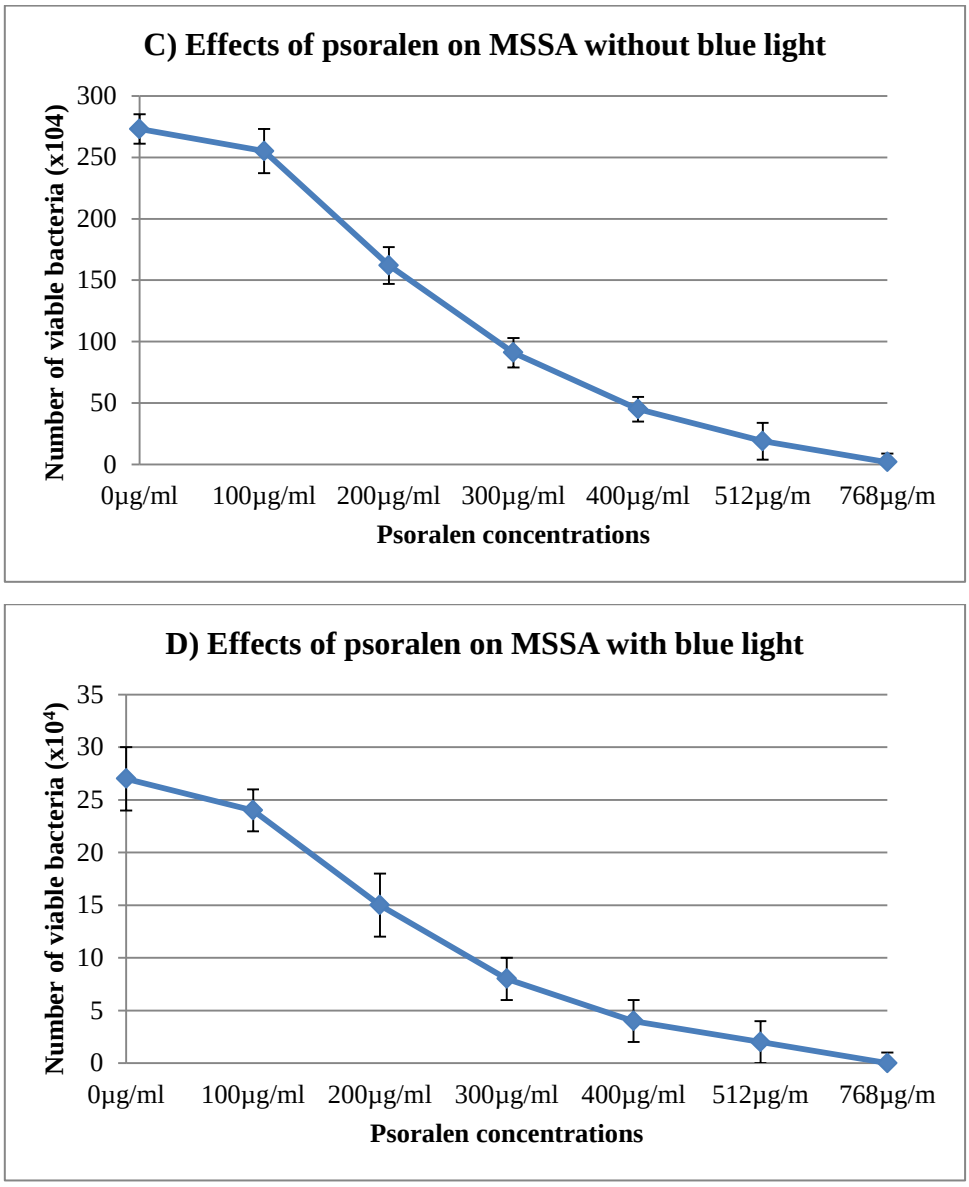



**Figure 4.13. Comparison of PDT/Phototoxicity of MRSA (A and B) and MSSA (C and D) in the presence and absence of the psoralen with and without light exposure.** This result is a mean of three independent experiments in duplicate. Error bars represent standard error; (***P<0.005*).

The effect of psoralen was tested against intracellular bacteria inside *Acanthamoeba* with blue light exposure. Invaded, untreated bacteria were accepted as a control. This was achieved by lyses of *Acanthamoeba* and culturing the lysate on plates. Another control was the intracellular bacteria exposed to blue light alone and intracellular bacteria exposed to psoralen alone. Blue light alone was able to reduce the number of intracellular bacteria by more than 70%. Moreover psoralen alone decreased the number of intracellular bacteria by more than 80%. 768 μg/mL of Ps with blue light exposure was lethal for both types of bacteria used in this study (Figure 4.14).

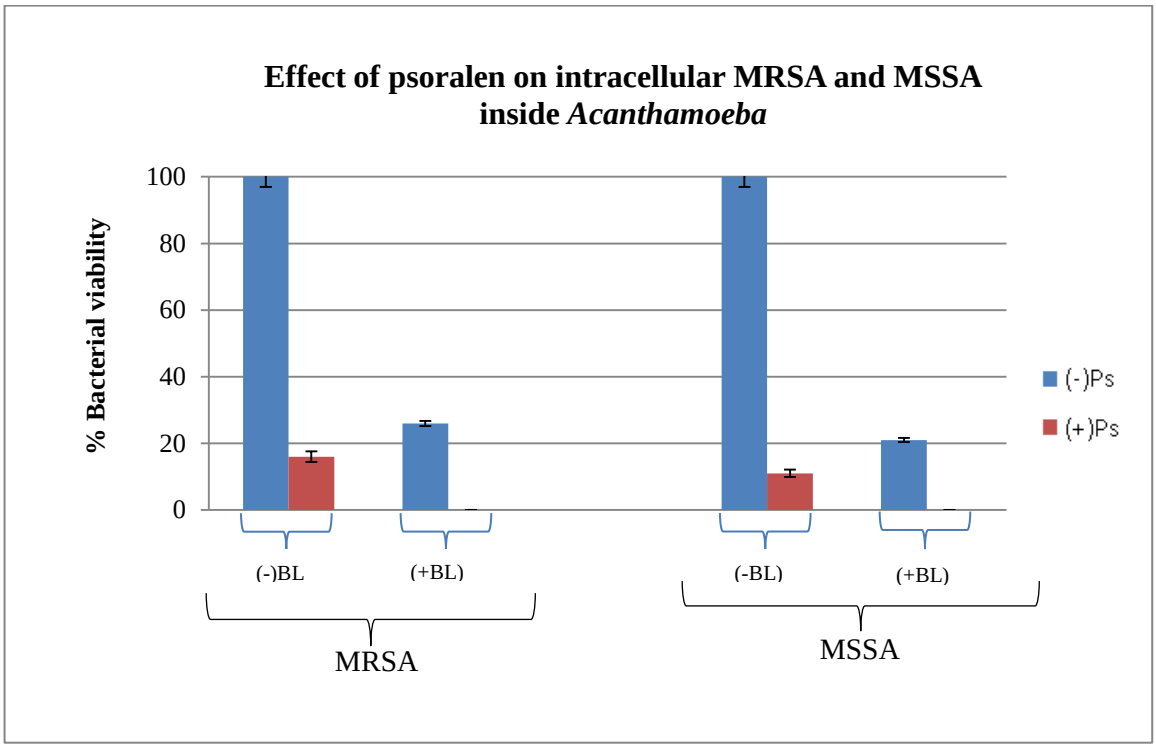



**Figure 4.14.** **PDT/Phototoxicity of intracellular MRSA and MSSA in the presence and absence of Ps (768 µg/mL ) with and without light exposure.** Intracellular/engulfed MRSA and MSSA were incubated with/without 768 µg/mL (psoralen) for 24 h and exposed to blue light for 30 min (8.3 J/cm$^2$). The results were calculated as percentage of bacteria with/without PS against the percentage of controls (bacteria without PS or light exposure). The results were the mean of three independent experiments performed in duplicate. Error bars represent standard error (***P<0.0005).

### 4.4. Discussion

All the available evidence suggests that multi-antibiotic resistant strains are as easily killed by PDT as native strains and that bacteria will not readily develop resistance to PDT (Maisch, 2007; Maisch *et al.*, 2011). It is known that Gram-negative bacteria are resistant to PDT, and commonly used PS will readily lead to phototoxicity in Gram-positive species. PS bearing a cationic charge or agents that increase the permeability of the outer membrane will increase the efficacy for killing Gram-negative organisms (Jori *et al.*, 2006; Rello *et al.*, 2007). Treatment of localised infections with PDT requires selectivity of the PS for microbes over host cells, delivery of the PS into the infected area, and the ability to effectively illuminate the infected lesion (Dahl et al. 1987; Dahl et al. 1989; Dai et al. 2009). When the cells to be killed are pathogenic microorganisms the procedure is termed photodynamic inactivation (PDI) (O'Riordan *et al.*, 2006).

This study explored the potential of one of the most powerful chlorins as antimicrobial PS agent, (*m*-THPC) with four hydroxy groups on four phenyl rings, which is considered a neutral compound. The effect of chlorin (*m*-THPC) on bacteria was examined under three sets of conditions: a) in the presence of a PS and absence of light, in order to establish the chemical toxicity of *m*-THPC; b) in the presence of only internalised (intracellular) PS and blue light to determine its ability to penetrate the cell membrane and cause biological damage; and c) in the presence of extracellular *m*-THPC and blue light without internalisation for very short incubation period and very short light exposure. The PS (*m*-THPC) proved to be not chemically toxic to the bacteria by itself at a concentration of at least twice the employed concentrations for the phototoxicity. The effect of *m*-THPC on MRSA and MSSA colonies was negligible in the dark without blue light irradiation. The chemical toxicity has a clinical relevance to any PS, taking into account the goal is to reduce the damage to the bacteria host (*High-Throughput Screening Methods in Toxicity Testing*, 2013). The PDT effect was significant on all four bacterial strains. *m*-THPC was capable of eliminating over 99 % of MRSA and MSSA bacteria after light irradiation after two hrs of light exposure. Nevertheless, in the second set of conditions, where the PS was eliminated during the light exposure, bacterial growth was visible after several hrs. This is likely due to the uptake of the PS

during the incubation time (up to 24 hrs) was not sufficiently yielding enough ROS to maintain the damage. These observations were reported for other PS that had penetration difficulties through bacterial cell membranes (Gomes *et al.*, 2013). In some cases, the combination of charged groups with certain porphyrine/chlorin derivatives resulted in increased cell recognition and water solubility, which improved cell membrane penetration (Nitzan *et al.*, 1992). In the third set of conditions, both MRSA and MSSA were exposed to very low concentrations of PS (*m*-THPC), following low-light energy exposure for a very short time to confer total and lasting destruction. This could be owing to the nature of PDT/PDI, where the initial damage was not the PS itself but the ROS moieties. Production of ROS could exert the desired damage in the cell membrane, which allowed the rest of the PS to pass through, as previously reported (Jori *et al.*, 2006).

There was a positive dose-dependent response of *m*-THPC phototoxic effects on both MRSA and MSSA, which was supported by several studies regarding PDT/PDI and different bacteria using a variety of PS (Di Poto *et al.*, 2009). The study of phototoxicity also sought to identify the MIC of the PS (*m*-THPC), which is sufficient to eradicate all bacteria after 24 h PDT application. The value of MIC for this compound on MRSA was found to be 0.8 μg/mL. This concentration achieved total eradication of bacteria after 24 h in the presence of PS after very short incubation time (couple of min.) and during very short light exposure. PDT efficacy in MSSA was much greater than MRSA, it required a concentration of less than 0.2 μg/mL to cause total destruction in MSSA. Therefore, MIC value for MSSA would be under the lowest concentration of the chosen range. The third set of conditions would consider ideal requirements as PDI concern, where in clinical field low concentration of PS and its short application are essential to avoid light sensitivity in human bodies that sometimes persists for days after PDT (Dai et al. 2009).

Following the work of (Elman *et al.*, 2003) on the use of high intensity light to treat acne, this study explores the use of blue light alone, without PS, to eradicate bacteria. Blue light had a significant effect on both MRSA and MSSA strains of bacteria, where about only 10% of the bacteria remained viable after exposure to blue light. The bacterial elimination by blue light alone, which has a wavelength range between 405 – 430 nm, can be attributed to the endogenous phorphyrins. Bacteria are known to

secrete amounts of endogenous heme precursors, such as uropophyrinogen, coproporphyrinogen, protoporphyrinogen, and protoporphyrin IX (Frankenberg *et al.*, 2003), which can be photochemically activated upon light exposure corresponding to the wavelength range of blue light. It's worth noticing the short lived effect of blue light didn't eradicate totally the bacteria after 24 h. This was clear from the control of all the experiments which contained bacteria without PS but exposed to blue light. Initially, the bacteria was reduced significantly after light exposure, then continued to grow back to a certain level in each case.

The experiments also investigated the effect of *m*-THPC on *Acanthamoeba* and the bacteria inside *Acanthamoeba.* One of the goals was to investigate whether *Acanthamoeba* might protect the bacteria inside them against the phototoxicity/PDI. One study with a similar scope (Chen *et al.*, 2008), concluded that *Acanthamoeba* keratitis is affected by photodynamic therapy when a PS was used. This study used a different PS than *m*-THPC, and found similar results, i.e. *Acanthamoeba* were reduced by approximately 20% upon internalized *m*-THPC with blue light exposure. Another finding was that the longer the period of PS incubation (1, 5, and 14 h), the higher the efficacy of the phototoxicity/PDI owing to the increased of *m*-THPC uptake by the tough *Acanthamoeba* cell membrane (Chen *et al.*, 2008). PDT/PDI effect on bacteria inside *Acanthamoeba* was examined and the results showed that there were reductons in the survival of the intracellular bacteria by 28 % - 44 %, with increasing *m*-THPC dosage from 2 μg/mL – 6 μg/mL after one-hr application. Nevertheless, bacteria inside *Acanthamoeba* were totally eliminated when *m*-THPC applied for longer periods (5h and 14h) on *Acanthamoeba* with engulfed bacteria. Therefore, we can confirm the substantial efficacy of PDI in preventing *Acanthamoeba* of shielding the intracellular bacteria inside and allowing their growth. The experiments investigated the effect of psoralen on *Acanthamoeba* and bacteria inside *Acanthamoeba*. There was positive dose-dependent response of psoralen phototoxic effects on *Acanthamoeba*, MRSA, and MSSA, which was supported by several studies regarding PDT and different bacteria using a variety of PS (Wainwright, 2009). The study of phototoxicity also sought to identify the MIC of the psoralen, which was sufficient to eradicate all bacteria after 24 h. The value of MIC for this compound on *Acanthamoeba* with light exposure was 300 μg/mL. This concentration achieved total eradication of bacteria after 24 h. The MIC

value with blue light for MRSA and MSSA was 768 μg/mL. However, the MIC value for MRSA and MSSA without blue light probably was over the highest concentration of the chosen range. One of the goals was to investigate whether *Acanthamoeba* might protect the bacteria inside them against phototoxicity. One study with a similar scope was that of (Solheim, 2008), who concluded that *Acanthamoeba* keratitis and bacteria were affected when psoralen was used. This study used psoralen and examined its effect on bacteria inside *Acanthamoeba.* The results show that there was a decrease in the survival of *Acanthamoeba* and *Acanthamoeba* with intracellular bacteria by 16 % and 11 %, respectively.

# Chapter 5.

# Effects of Eugenols on the survival of *Acanthamoeba* and internalised bacteria

### 5.1. Background

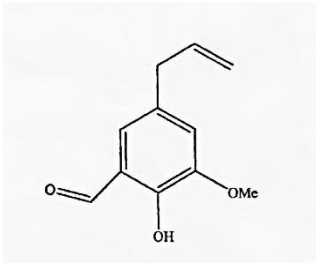
The antimicrobial effect of spices was first described by Anthony Van Leeuwenhoek. He described a reduction in the number and activity of animalcules in a sample of well water after addition of pepper in a letter dated 9 October 1676. In the 1980s, preservative agents were commonly being used but progress in the usage of spice derived components was very slow. The antimicrobial activity of spice oil has been found in a number of other aromatic molecules, one of those being eugenol.

Eugenol is a natural phenolic component of clove oil but it is also found in several other spices like basil, cinnamon, and bay leaves. It is the foremost active phenylpropanoid constituent of the essential oil among various aromatic plant (De Vincenzi *et al.*, 2000). Eugenol being a major component of clove oil is used primarily as a flavouring agent in food and cosmetic products. According to studies, eugenol possesses various biological abilities, including antimicrobial, antioxidant, anti-inflammatory, anticarminative, and antispasmodic activities (Hashimoto *et al.*, 1988; Nagababu and Lakshmaiah, 1997; Gill and Holley, 2004; Mohammed and Al-Bayati, 2009). In traditional Chinese medicine, eugenol has been used due to its analgesic, antiseptic, and antibacterial properties (Zheng *et al.*, 1992). Eugenol has antitumor properties causing destruction of various cancer cells. According to a number of researchers, the anti-*Candida* biofilm activity has potential of plant-derived chemicals such as flavonoids (Cao *et al.*, 2008), and essential oils (Khan and Ahmad, 2012). Moreover, eugenol also has anti-tumor potential related with apoptosis in different cancer cells. It has induced apoptosis in RBL-2H3 mast cells (Park *et al.*, 2005) and HL-60 human promyelocytic leukemia cells (Yoo *et al.*, 2005). Furthermore, eugenol has therapeutical application as an adjuvant in the management of neuropathy in rat brain.

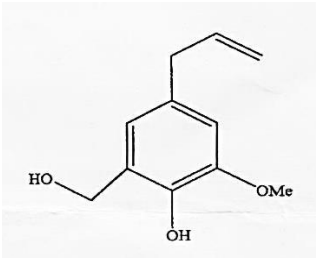
As claimed by two studies, eugenol affects $Ca^{2+}$ homeostasis in three cell types. Eugenol increased cytosolic free $Ca^{2+}$ concentrations ($[Ca^{2+}]$) in neonatal rat cardiac myocytes (Choudhary *et al.*, 2006), Jurkat T-cells and monocytic THP-1 cells (Chan *et al.*, 2005). Although, whether eugenol alters $Ca^{2}$+ movement and viability in cultured human glioblastoma cells is unclear. The decrease in levels of $Ca^{2}$+ in the endoplasmic reticulum would lead to activation of stress signals, which in turn switches on the genes associated with apoptosis (Nicotera & Orrenius 1998).

Certain antibiotics can influence the expression of staphylococcal exotoxins, and eugenol, an essential oil component in plants, has been reported to have activity against both Gram-positive and Gram-negative bacteria. This study aimed to determine the influence which sub-inhibitory concentrations of eugenol may have on the expression of the major exotoxins produced by MSSA, MRSA and *Acanthamoeba* (De Vincenzi *et al.*, 2000). Four novel eugenols were kindly provided by Professor Mark S. Baird from Bangor University. In this current project these eugenols were tested for their ability to inactivate a variety of *Acanthamoeba* and bacteria. By a differential concentration of eugenols, *Acanthamoeba* and bacteria inactivation can be achieved.

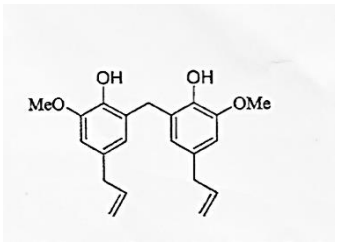
The experiments described were done to determine the effect of eugenols on the survival of *Acanthamoeba* and internalised bacteria. Structures of all eugenols used in the course of this study are shown (Figure 5.1).



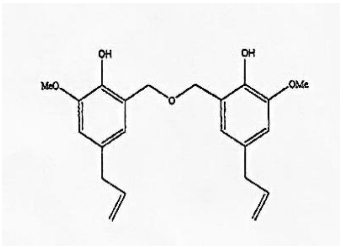

**EN1:** EN-1-(5-allyl-2-hydroxy-3-methoxybenzaldehyde)




**EN2:** EN-2- 4-allyl-2-(hydroxyl methyl)-6-methoxyphenol




**EN3:** EN-3- 6,6'-methylenebis(4-allyl-2-methoxyphenol)




**EN4:** EN-4 - 6,6'-(oxybis(methylene))bis(4-ally-2-methoxyphenol)methoxyph

**Figure 5.1. Structures of all eugenols used in the course of the study.**

## 5.2. Materials and Methods

### 5.2.1. The antimicrobial effects of eugenols on *Acanthamoeba*

*Acanthamoeba* belonging to T4 genotype was grown in PYG medium (5 mL) in T-25 tissue culture flasks until confluency. Before each experiment, 5 mL of PYG was added and the flasks were placed on ice for 20 – 30 min. The flasks were checked under the microscope to ensure that *Acanthamoeba* were floating and not adhered to the flask. The media containing the *Acanthamoeba* was then collected and placed in 50 mL Falcon tubes to be spun down for a count using a haemocytometer. After counting, 5 x $10^5$ *Acanthamoeba* were placed into each well of a 24-well plate and left overnight in 30 $^oC$ incubator. The following day, PYG medium was removed and wells were washed with PBS three times. Eugenols at volumes of 40, 30, 20 and 10 µg/mL were then added to specific wells and incubated overnight at 30 $^oC$. Each well was then treated with 100 µl of Trypan Blue (0.4 %) and left for 20 min following which medium was discarded and dead *Acanthamoeba* were counted. Same experiments were repeated to study the effect of eugenols on intracellular bacteria after doing the invasion assays. Dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% inhibition of growth were determined ($IC_{50}$) for every type of eugenol.

### 5.2.2. The antimicrobial effects of eugenols on intracellular bacteria

Invasion assays were performed to determine the ability of MRSA and MSSA to invade or be taken up by *Acanthamoeba*. *Acanthamoeba* was grown in 24-well plates to confluence followed by addition of bacteria. After 1 h of incubation, wells were washed once with PBS after which vancomycin (100 µg/mL, final concentration) was then added and incubated for 45 min to eradicate the extracellular bacteria. Wells were then washed once with PBS following which eugenols were added and incubated overnight at 37 $^oC$. *Acanthamoeba* was lysed by 0.05 % SDS, and number of bacteria released from lysed *Acanthamoeba* was enumerated using nutrient CLED agar plates.

### 5.2.3. Zymographic assays

To study the effect of eugenols on the production of proteases by *Acanthamoeba*, zymography assays were performed as described in section 3.2.6.

## 5.3. Results

### 5.3.1. Effects of eugenol derivatives on *Acanthamoeba* T4

Effects of four types of EN (EN1, EN2, EN3 and EN4) on *Acanthamoeba* T4 trophozoites were analysed by performing the survival assays. The survival assays showed dose dependent effects of EN on *Acanthamoeba* T4 survival (Figure 5.2). The doses of EN used for these assays were between 10 μg/mL - 64 μg/mL. Dead *Acanthamoeba* were confirmed by Trypan blue staining. At 40 μg/mL, EN1 and EN3 were seen to be more effective at inhibiting *Acanthamoeba* than EN2 and EN4. This is because 22.7 % and 23 % of *Acanthamoeba* remained viable after 24 h treatment with EN1 and EN3 respectively while 37 % and 27.7 % of *Acanthamoeba* remained viable after 24 h treatment with EN2 and EN4 respectively. Furthermore, at a higher concentration of 64 μg/mL, EN3 totally inhibited the growth of *Acanthamoeba* after 24 h while there were still 1.33 % of viable *Acanthamoeba* following treatment with EN1 after 24 h (Figure 5.3). In addition dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% inhibition of growth were determined ($IC_{50}$). For EN1, EN2, EN3 and EN4 $IC_{50}$ was found at 30, 35, 29 and 31 μg/mL respectively.

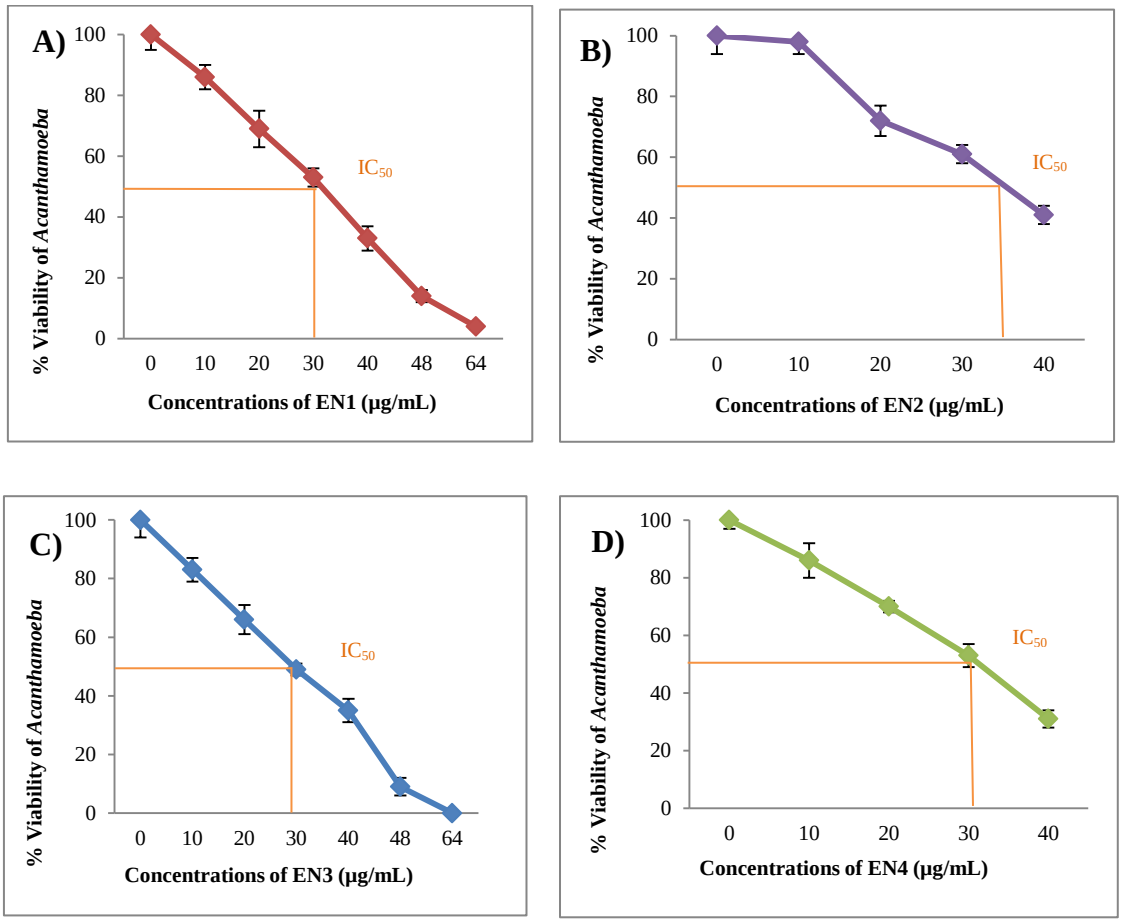



**Figure 5.2. The effects of Eugenols on *Acanthamoeba* T4 trophozoite after 24 h of exposure at 30 °C.** A. Effect of EN1 B. Effect of EN2 C. Effect of EN3 D. Effect of EN4. *Acanthamoeba* T4 was grown until confluency. After counting, 5 x $10^5$ *Acanthamoeba* were inoculated into each well of a 24-well plate and incubated at 30 °C. The following day, medium was removed and wells were washed. Eugenols at volumes of 40, 30, 20 and 10 µg/mL were then added to specific wells and incubated overnight at 30 °C. The next day each well was then treated with 100 µl of Trypan Blue (0.4 %) and left for 20 min following which, medium was discarded and dead *Acanthamoeba* were counted using haemocytometer. $IC_{50}$ (orange line) is point at which growth is inhibited by 50%. These results represent selected data of three independent experiments in triplicates. Error bars represent standard error, ($P<0.05$ using paired t-test, one-tail distribution).

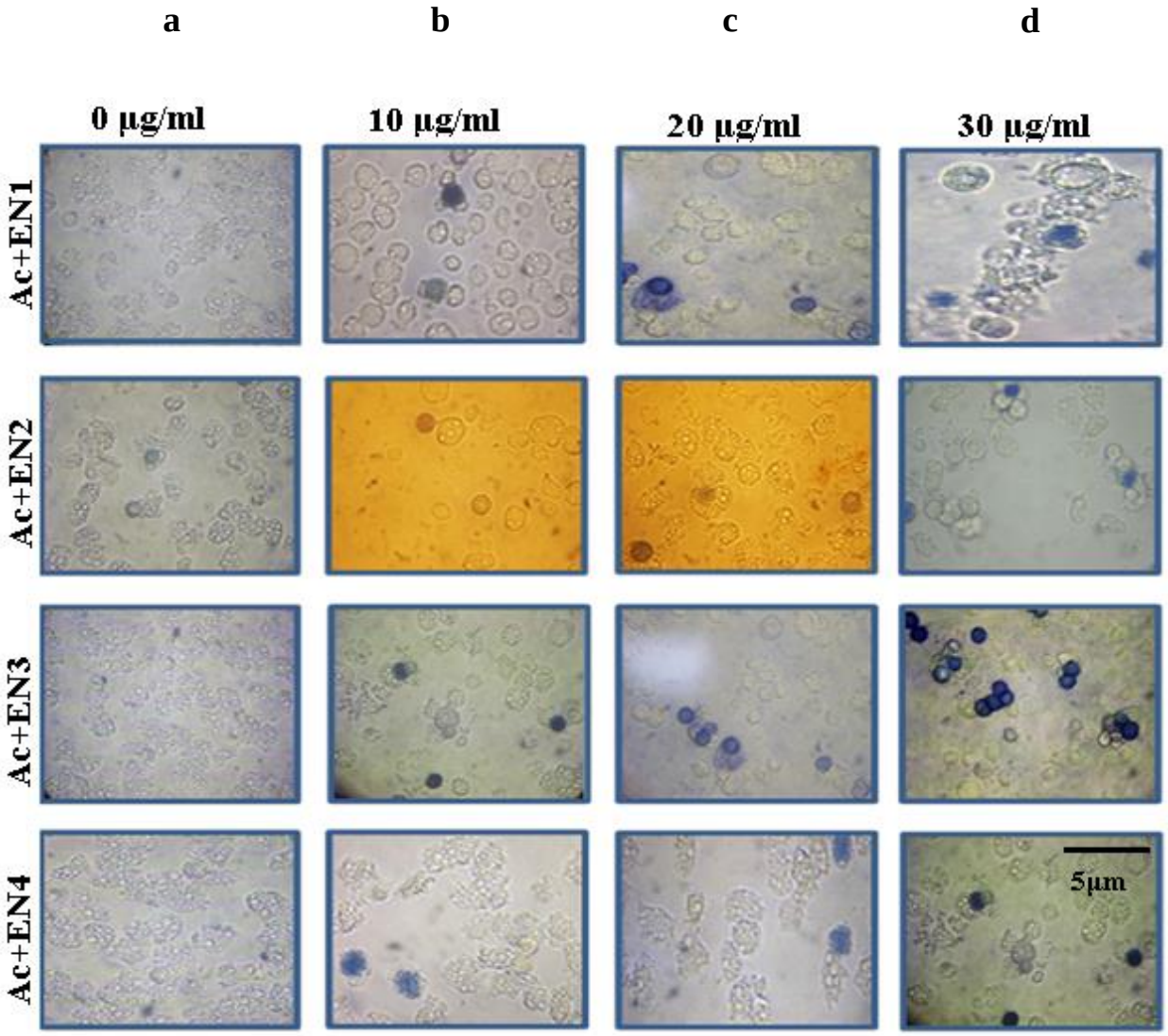
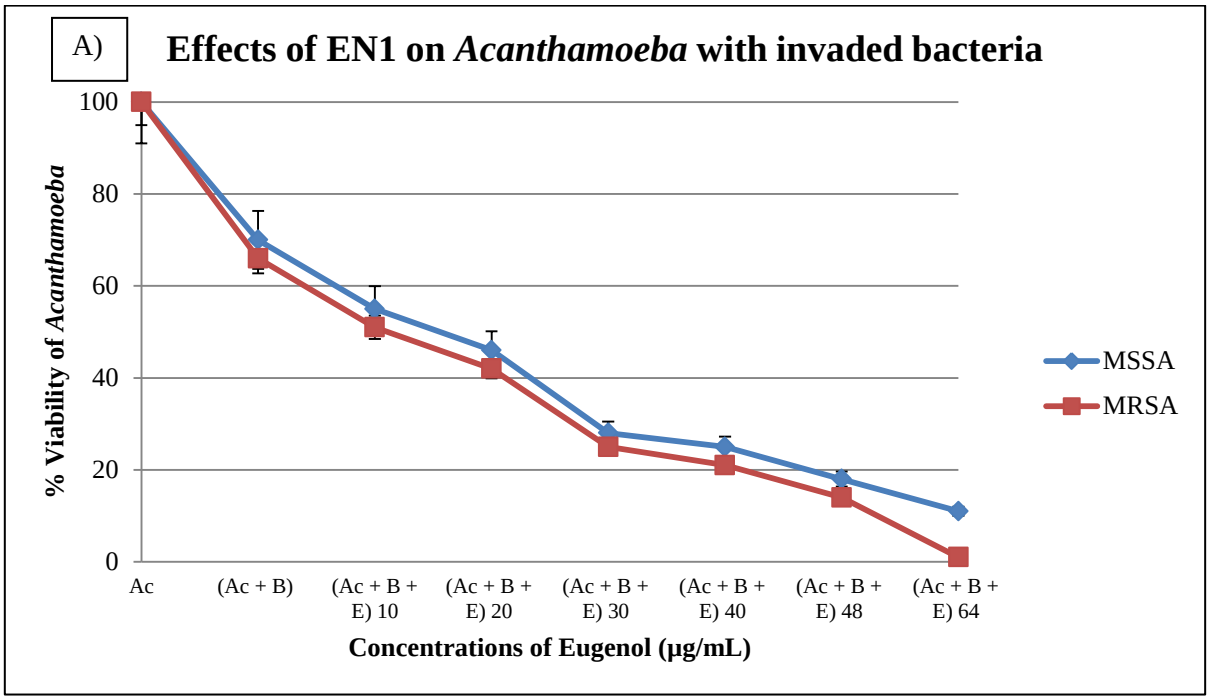
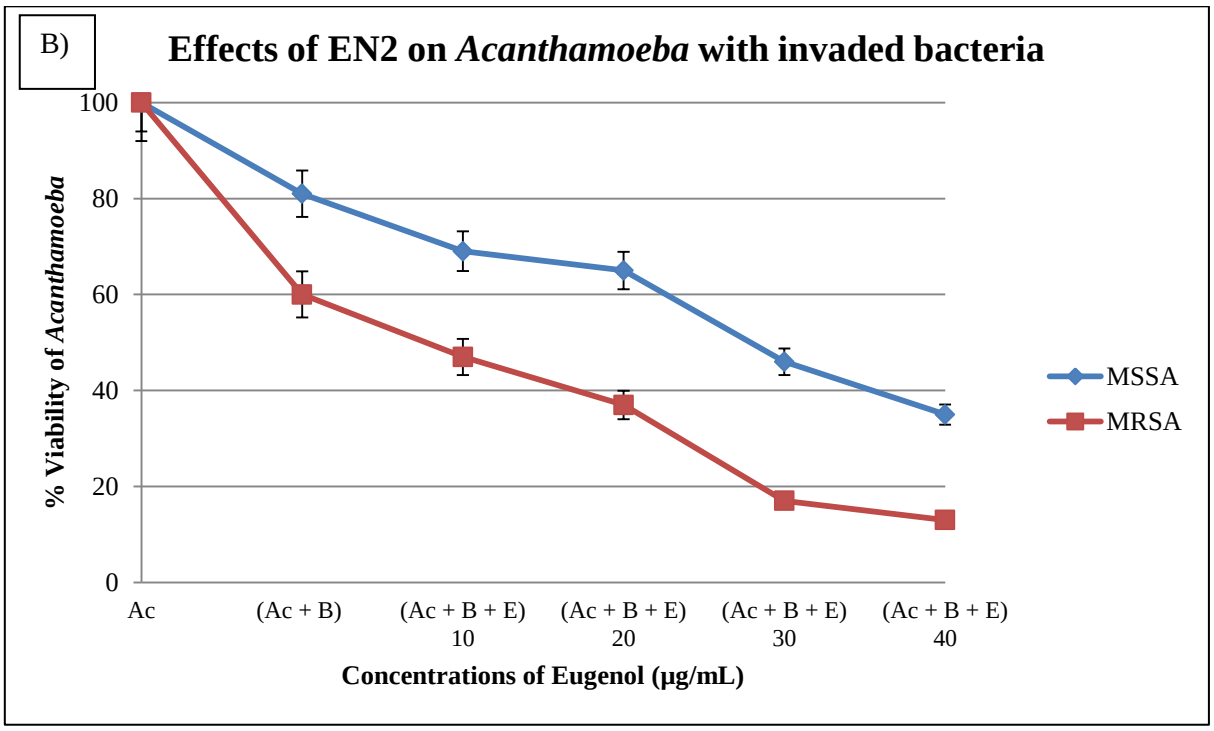


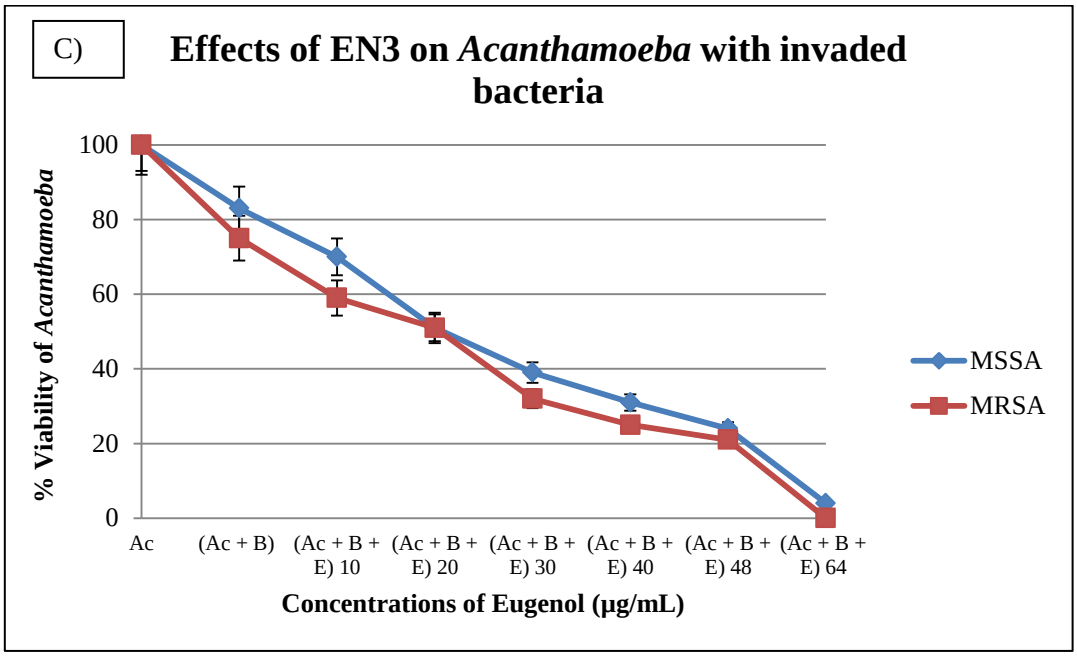

**Figure 5.3. Morphology of *Acanthamoeba* T4 trophozoite** (A) after 24 h: a) control, non-treated; b) 10 µg/mL EN; c) 20 µg/mL EN; d) 30 µg/mL EN with Trypan Blue. *Acanthamoeba* T4 was grown until confluence. After counting, 5 x $10^5$ *Acanthamoeba* were inoculated into each well of a 24-well plate and incubated at 30 $^oC$. The following day, medium was removed and wells were washed. Eugenols at volumes of 30, 20 and 10 µg/mL were then added to specific wells and incubated overnight at 30 $^oC$. The next day each well was then treated with 100 µl of Trypan Blue (0.4 %) and left for 20 min following which, medium was discarded and live/dead *Acanthamoeba* were photographed using bright field microscope.

### 5.3.2. Effects of eugenol derivatives on *Acanthamoeba* with intracellular bacteria

In these assays the effects of eugenols EN (1, 2, 3 and 4) on *Acanthamoeba* T4 trophozoites with invaded bacteria were studied. Treatments with all the ENs were for 24 h at 37 $^{o}$C. The bacteria used were MRSA and MSSA; death of *Acanthamoeba* with MSSA and MRSA was confirmed by trypan blue staining (Figure 5.4). At 40 μg/mL, 18 %, 33 %, 20 % and 20 % of *Acanthamoeba* with internalised MSSA survived following 24 h treatment with EN1, EN2, EN3 and EN4 respectively. On the other hand, 15 %, 9 %, 18 % and 4 % of *Acanthamoeba* with internalised MRSA survived after 24 h treatment with 40 μg/mL of EN1, EN2, EN3 and EN4 respectively. At 64 μg/mL, 8 % and 3 % of *Acanthamoeba* with internalised MSSA survived after treatment with EN1 and EN3 respectively. Moreover, EN3 was seen to totally inhibit the growth of *Acanthamoeba* with internalised MRSA at 64 μg/mL while 1 % of *Acanthamoeba* with internalised MRSA survived after treatment with EN1 at the same concentration. The effects of all ENs on *Acanthamoeba* with internalised MSSA and MRSA were determined after 24 h of exposure at 37 $^{o}$C (Figure 5.5).


A)
Effects of EN1 on Acanthamoeba with invaded bacteria
% Viability of Acanthamoeba
100
80
60
40
20
0
Ac
(Ac + B)
(Ac + B + E) 10
(Ac + B + E) 20
(Ac + B + E) 30
(Ac + B + E) 40
(Ac + B + E) 48
(Ac + B + E) 64
Concentrations of Eugenol (µg/mL)
MSSA
MRSA


B)
Effects of EN2 on Acanthamoeba with invaded bacteria
% Viability of Acanthamoeba
100
80
60
40
20
0
Ac
(Ac + B)
(Ac + B + E) 10
(Ac + B + E) 20
(Ac + B + E) 30
(Ac + B + E) 40
Concentrations of Eugenol (µg/mL)
MSSA
MRSA



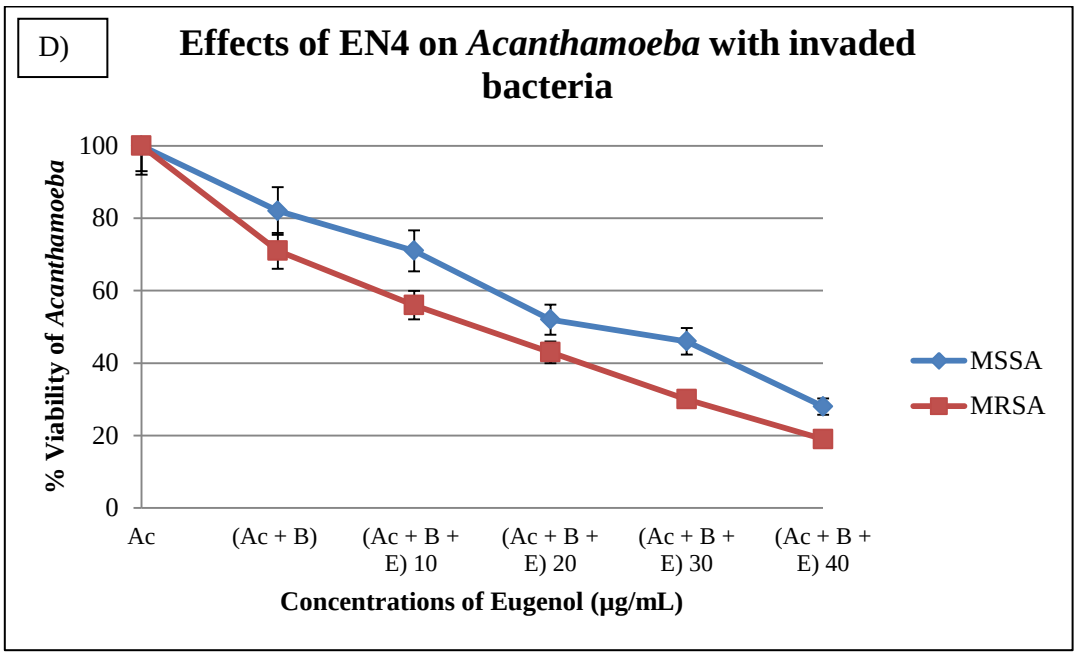




**Figure 5.4. The effects of ENs on *Acanthamoeba* T4 trophozoites with invaded MSSA and MRSA after 24 h of exposure at 37 °C.** A. Effect of EN1 B. Effect of EN2 C. Effect of EN3 D. Effect of EN4. *Acanthamoeba* T4 was grown until confluence. After counting, 5 x $10^5$ *Acanthamoeba* were inoculated into each well of a 24-well plate and incubated at 30 °C. The following day, medium was removed and wells were washed. Eugenols at volumes of 40, 30, 20 and 10 µg/mL were then added to specific wells and incubated overnight at 30 °C. The next day each well was then treated with 100 µl of Trypan Blue (0.4 %) and left for 20 min following which, medium was discarded and dead *Acanthamoeba* were counted using haemocytometer. These results represent selected data of three independent experiments in triplicates. Error bars represent standard error, ($P<0.05$ using paired t-test, one-tail distribution).

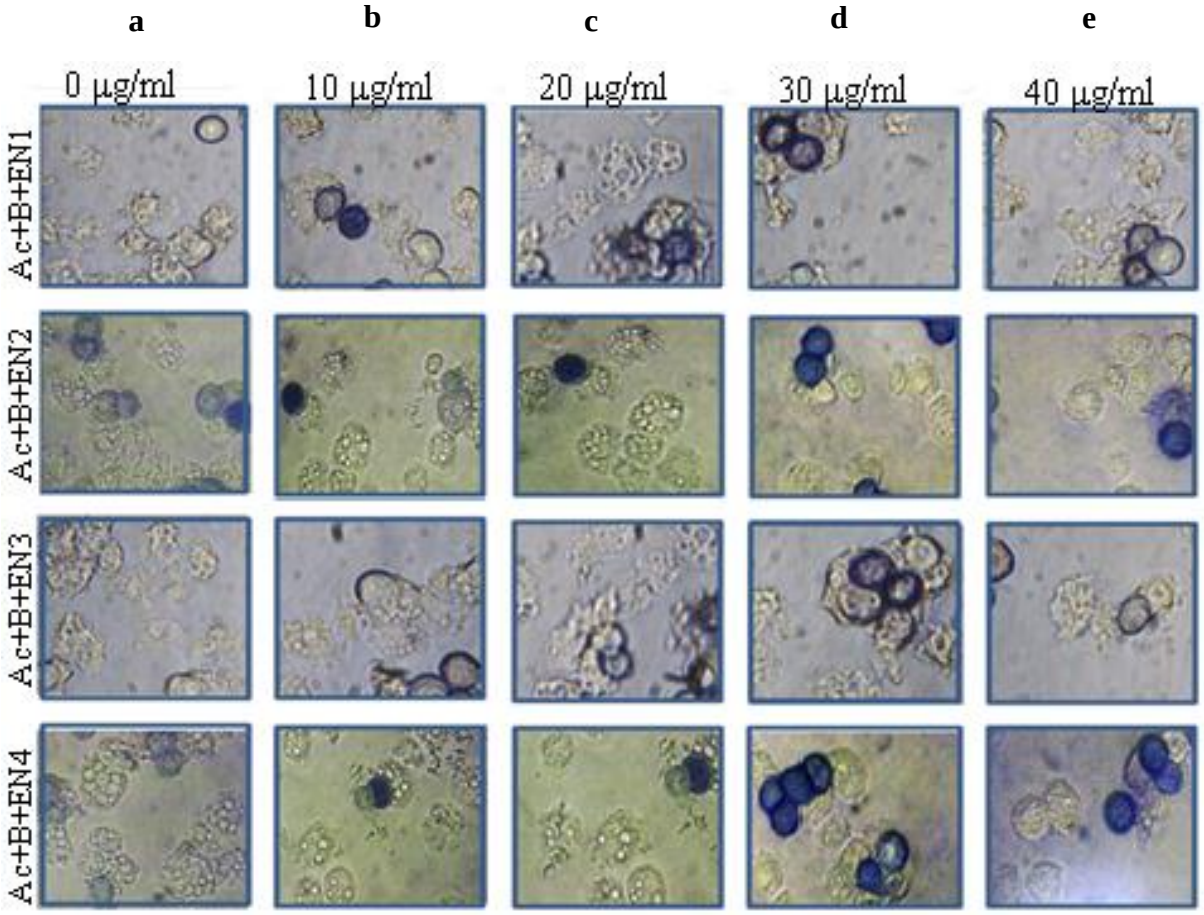
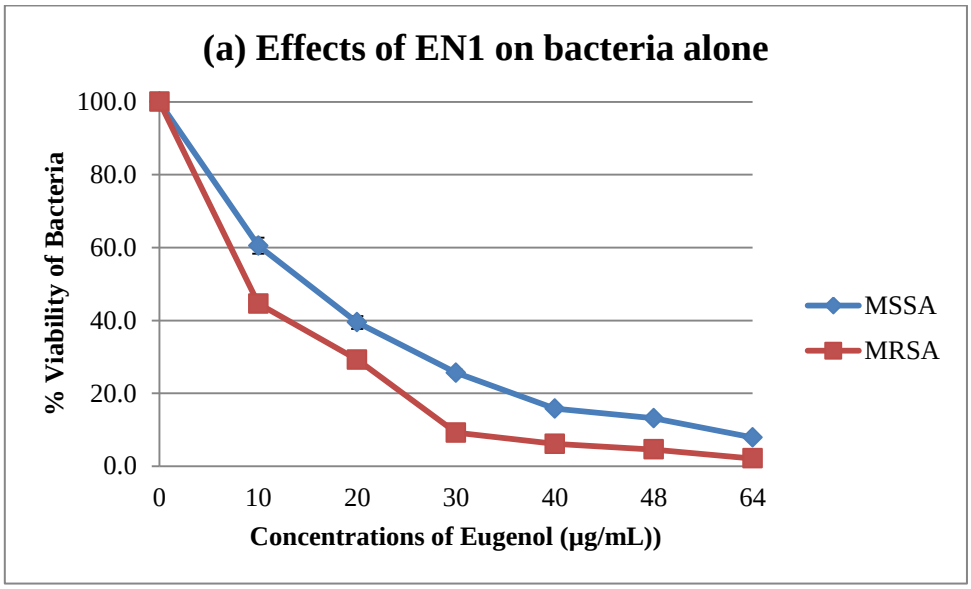
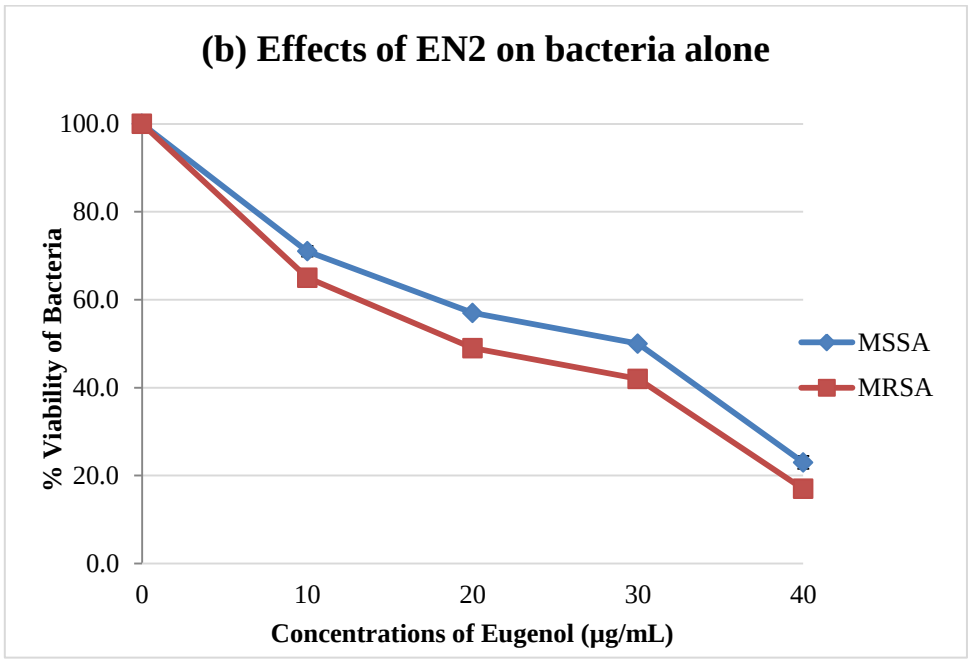


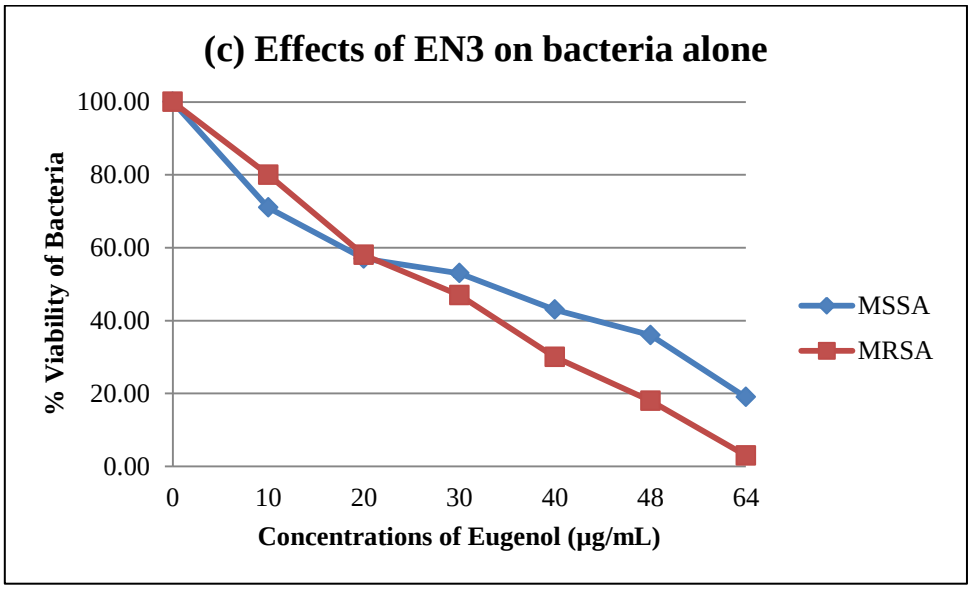

**Figure 5.5.** ***Acanthamoeba*** **T4 trophozoites with intracellular bacteria (A+B) after 24 h: a) control, non-treated; b) 10 µg/mL EN; c) 20 µg/mL EN; d) 30 µg/mL EN; e) 40 µg/mL EN with Trypan Blue.** *Acanthamoeba* T4 was grown until confluence. After counting, 5 x $10^5$ *Acanthamoeba* were inoculated into each well of a 24-well plate and incubated at 30 $^{o}$C. The following day, medium was removed and wells were washed. Eugenols at volumes of 40, 30, 20 and 10 µg/mL were then added to specific wells and incubated overnight at 30 $^{o}$C. The next day each well was then treated with 100 µl of Trypan Blue (0.4 %) and left for 20 min following which, medium was discarded and live/dead *Acanthamoeba* were photographed using bright field microscope.

### 5.3.3. Effects of eugenol derivatives on bacteria MRSA and MSSA

These assays were performed to investigate the effects of eugenols on survival of bacterial strains, MRSA and MSSA. EN1 was seen to be more effective than the other ENs at inhibiting MRSA and MSSA at 40 μg/mL. This is because only 12 x $10^4$ CFU/mL of MRSA and 3.5 x $10^4$ CFU/mL of MSSA survived after treatment with EN1 unlike in the case of the other ENs where it was seen that >15 % of MRSA and MSSA survived after 24 h treatment. At 64 μg/mL, only 6 x $10^4$ CFU/mL MRSA and 1.4 x $10^4$ CFU/mL of MSSA survived following 24 h treatment with EN1 while 14 x $10^4$ CFU/mL MRSA and 2 x $10^4$ CFU/mL MSSA survived following treatment with EN3. The effect of all the EN used in this study on MRSA and MSSA (Figure 5.6).


(a) Effects of EN1 on bacteria alone
100.0
80.0
60.0
40.0
20.0
0.0
% Viability of Bacteria
0
10
20
30
40
48
64
Concentrations of Eugenol (µg/mL))
MSSA
MRSA


(b) Effects of EN2 on bacteria alone
100.0
80.0
60.0
40.0
20.0
0.0
% Viability of Bacteria
0
10
20
30
40
Concentrations of Eugenol (µg/mL)
MSSA
MRSA



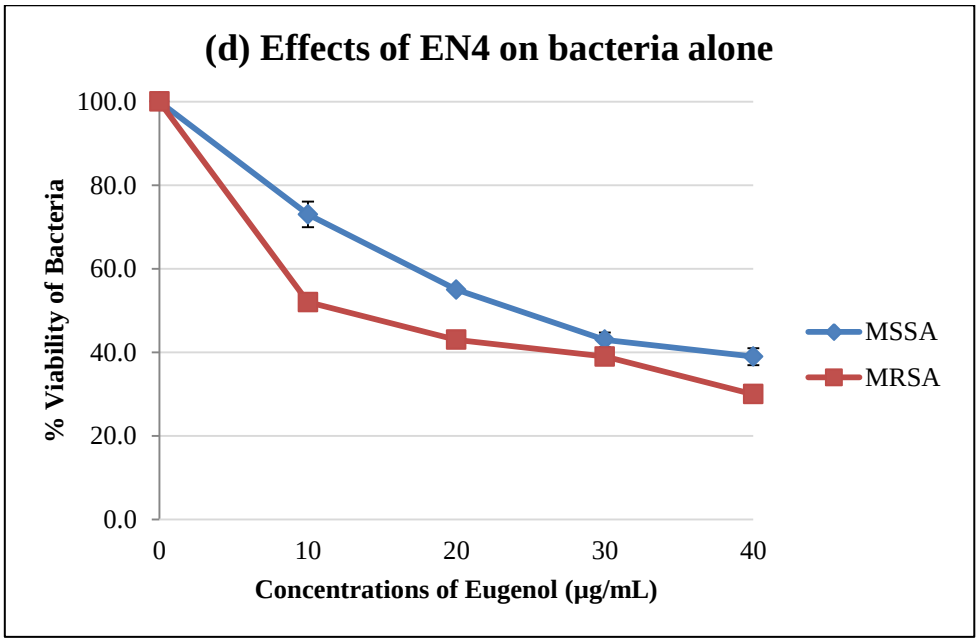
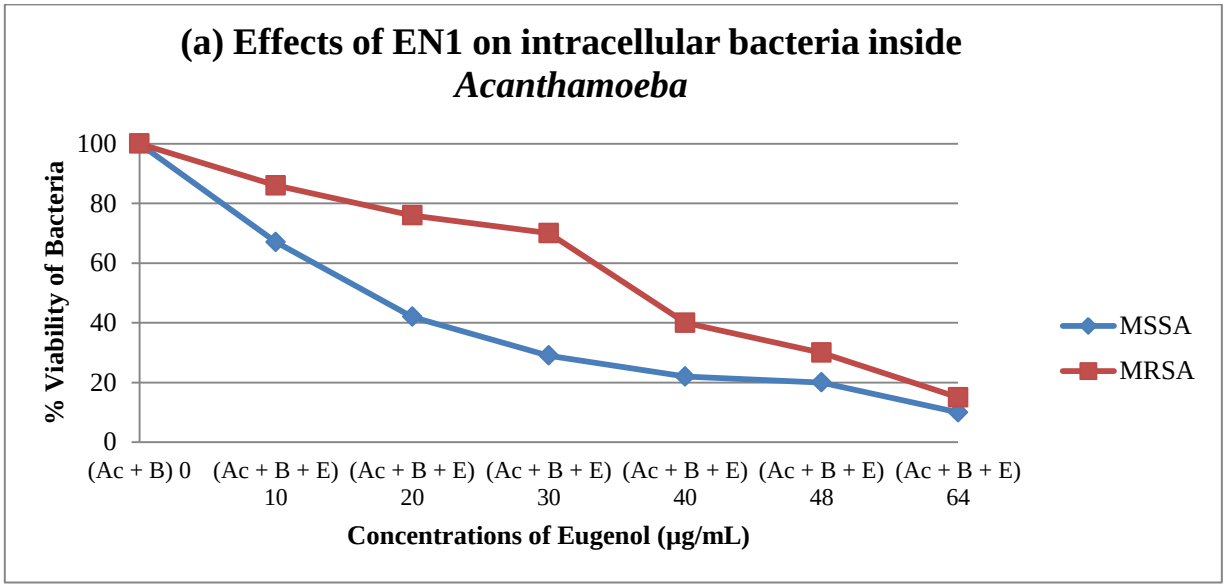
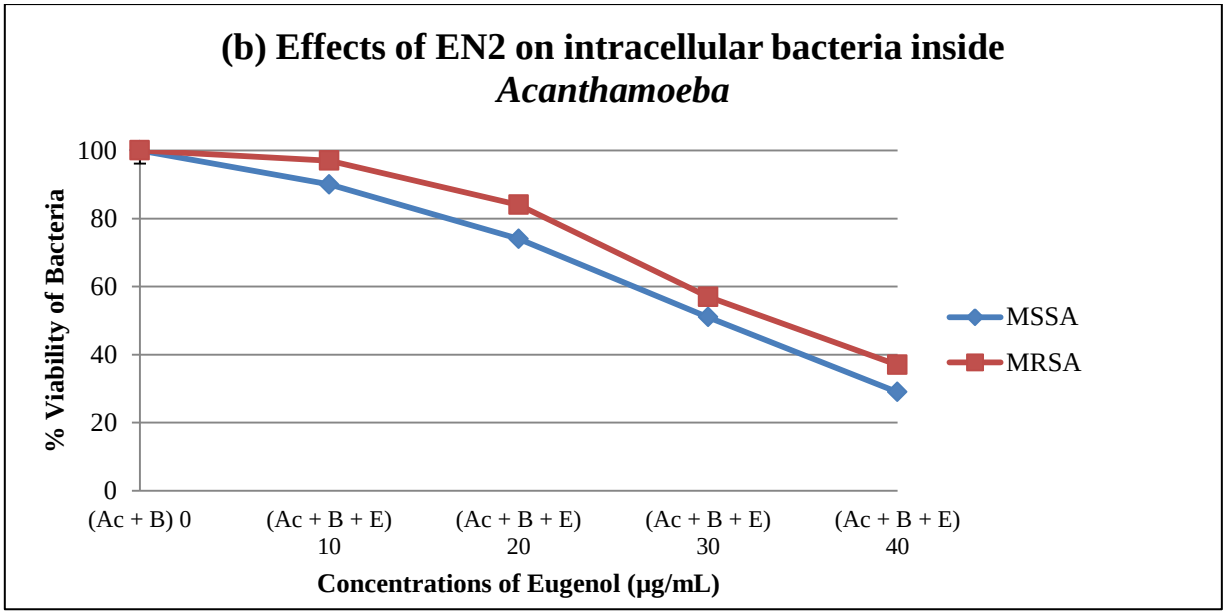



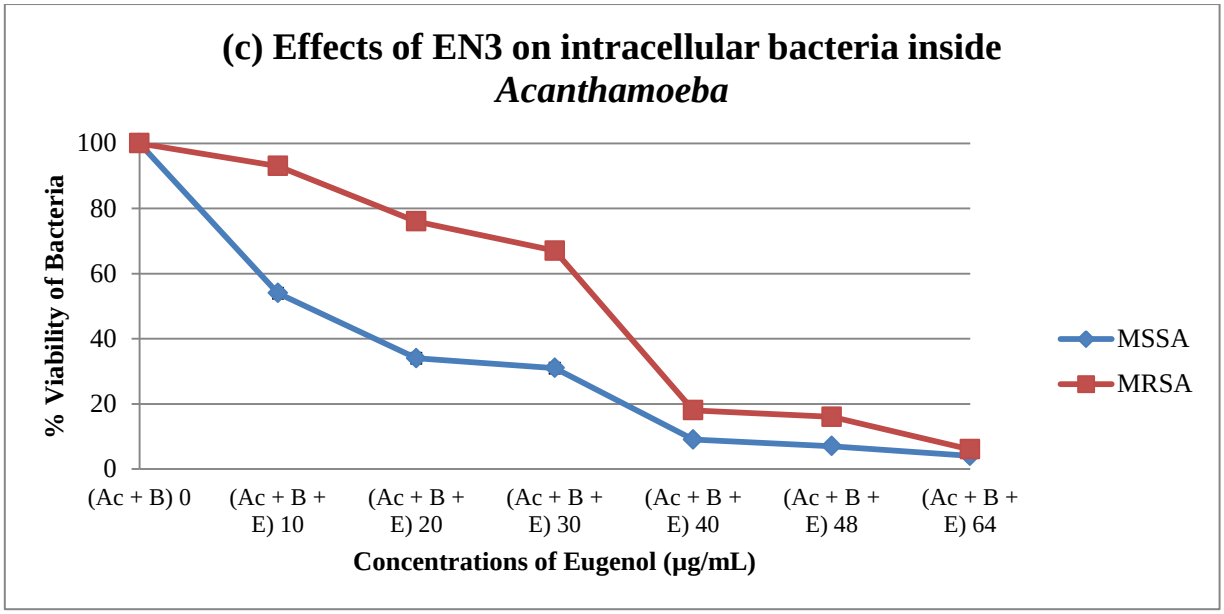

**Figure 5.6. The effects of ENs on MSSA and MRSA after 24 h of exposure at 37 °C.** a) Effect of EN1 b) Effect of EN2 c) Effect of EN3 d) Effect of EN4. Various concentrations of Eugenols were inoculated with bacteria (x $10^6$) overnight. The next day media were diluted and cultured into CLED agar plates. Plates were incubated overnight and colonies were counted. Percentage of bacterial viability was calculated. These results represent selected data of three independent experiments in triplicates. Error bars represent standard error, ($P<0.05$ using paired t-test, one-tail distribution).

### 5.3.4.Effects of eugenol derivatives on internalized bacteria inside *Acanthamoeba*

These assays were performed to investigate the effects of ENs on bacteria internalized by *Acanthamoeba*. EN3 was seen to be more effective than the other ENs at inhibiting MRSA and MSSA inside *Acanthamoeba*. < 20 % of both MRSA and MSSA inside *Acanthamoeba* survived after treatment with EN3 unlike in the case of other ENs where the surviving MRSA and MSSA were seen to be > 20 %. The effects of all ENs on MRSA and MSSA internalized by *Acanthamoeba* were determined after 24 h (Figure 5.7).


(a) Effects of EN1 on intracellular bacteria inside Acanthamoeba
% Viability of Bacteria
100
80
60
40
20
0
(Ac + B) 0
(Ac + B + E) 10
(Ac + B + E) 20
(Ac + B + E) 30
(Ac + B + E) 40
(Ac + B + E) 48
(Ac + B + E) 64
Concentrations of Eugenol (µg/mL)
MSSA
MRSA


(b) Effects of EN2 on intracellular bacteria inside Acanthamoeba
% Viability of Bacteria
100
80
60
40
20
0
(Ac + B) 0
(Ac + B + E) 10
(Ac + B + E) 20
(Ac + B + E) 30
(Ac + B + E) 40
Concentrations of Eugenol (µg/mL)
MSSA
MRSA



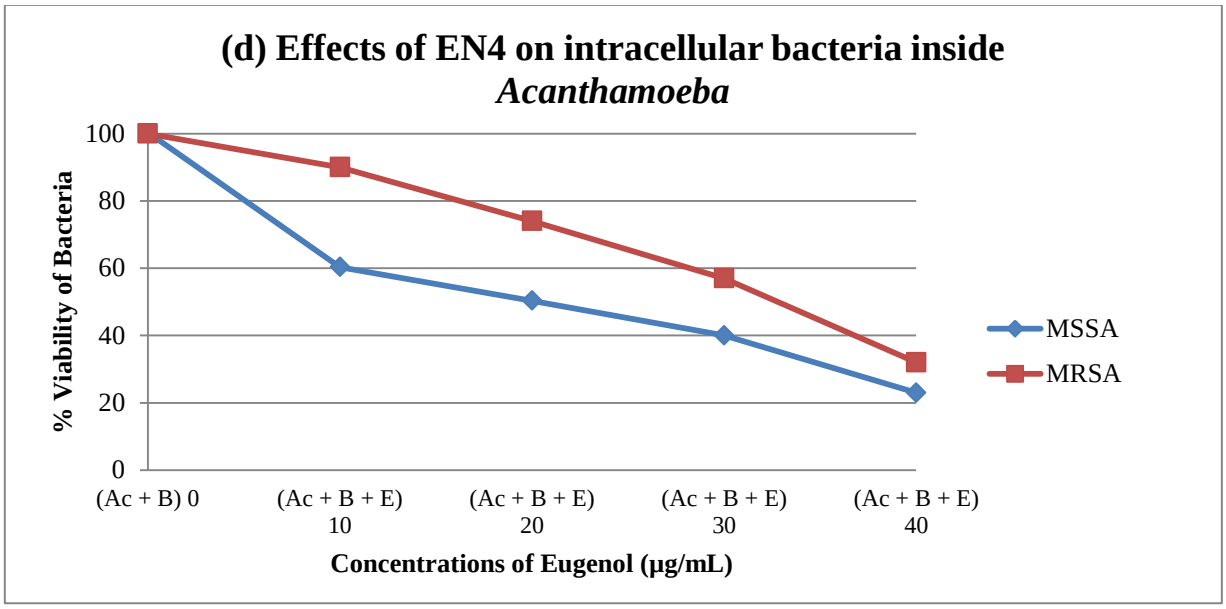




**Figure 5.7. The effects of ENs on MRSA and MSSA internalized *Acanthamoeba* after 24 h of exposure at 37 °C.** a) Effect of EN1 b) Effect of EN2 c) Effect of EN3 d) Effect of EN4. *Acanthamoeba* was grown in 24-well plates to confluence followed by addition of bacteria. After 1 h of incubation, wells were washed once with PBS after which vancomycin (100 µg/mL, final concentration) was then added and incubated for 45 min to eradicate the extracellular bacteria. Wells were then washed once with PBS following which eugenols were added and incubated overnight at 37 oC. *Acanthamoeba* was lysed by 0.05 % SDS, and number of bacteria released from lysed *Acanthamoeba* was enumerated using nutrient CLED agar plates.These results represent selected data of three independent experiments in triplicates. Error bars represent standard error, ($P<0.05$ using paired *t*-test, one-tail distribution).

### 5.3.5. Zymographic assays

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allows separating biological molecules such as proteins according to their molecular weight. Addition of SDS allows linearization and distribution of proteins according to their molecular weights/sizes. Zymography detects hydrolytic enzymes using a suitable substrate such as gelatin, which will be degraded when the enzyme is present.

This study was done to ascertain the effect of the ENs (EN1, EN2, EN3, and EN4) on the expression extracellular proteases by *Acanthamoeba*. At a concentration 40 μg/mL, EN1 and EN3 were seen to reduce the production of extracellular proteases when compared with the control and the effect of EN2 and EN4 (Figure 5.8). These results support outcomes obtained in section 5.3.1 where it was seen that EN1 and EN3 were more effective at inhibiting growth of *Acanthamoeba* at 40 μg/mL compared to EN2 and EN4.

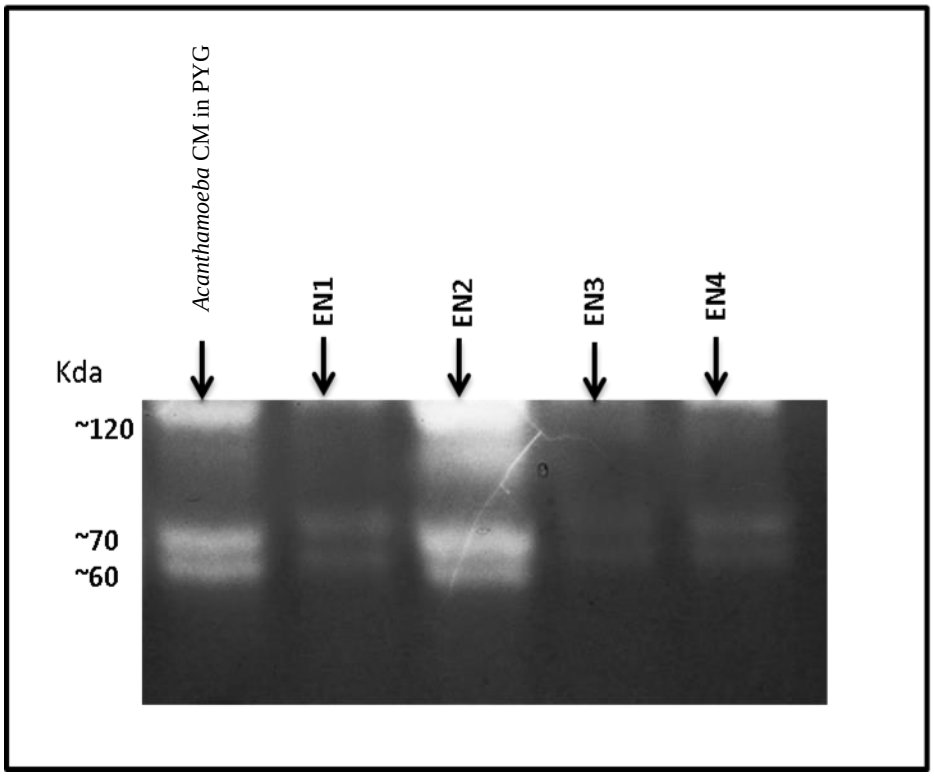



**Figure 5.8. Effects of ENs (EN1, EN2, EN3, and EN4) on the expression of extracellular proteases in *Acanthamoeba* T4** Conditioned Media. *Acanthamoeba* CM (only) and other samples (CM) with different concentrations of eugenols were blended (1:1) with the sample buffer and electrophoresed on SDS-PAGE containing fish gelatine (4% w/v). After electrophoresis, the gels were soaked in 2% Triton solution for 60 mins, incubated in a developing buffer at 37 °C overnight, then washed and stained with Comassie Brilliant Blue. Samples were treated with eugenols (EN1, EN2 EN3 and EN4), which was found to be the most effective in inhibiting *Acanthamoeba* extracellular proteases. At a concentration 40 μg/mL, EN1 and EN3 were seen to reduce the production of extracellular proteases when compared with the control and the effects of EN2 and EN4.

### 5.4. Discussion

*Acanthamoeba* is widely present in the environment (Khan, 2009) and can cause infections in humans. Common infections such as keratitis affect the cornea and can cause blindness. Another infection caused by *Acanthamoeba* is GAE, a rare serious infection that can end up fatal. Current treatments for *Acanthamoeba* keratitis include antibiotics and use of topical steroids, however the treatment for encephalitis, such as antimicrobial drugs, does not help as the diagnosis is made late (Siddiqui and Khan, 2012). The need for new treatments with broader anti-amoebic activities is driving the research into synthesis of new monoclonal antibodies and development of new drugs.

Eugenols are chemicals manufactured by plants, which possess numerous functions in plants. Researches have revealed that eugenol has various biological capabilities, including antimicrobial, antioxidant, anti-inflammatory, carminative, and antispasmodic activities (Qiu *et al.*, 2010).

It is broadly acknowledged that essential oils have antimicrobial activities, and recent investigations have revealed that these characteristics are attributable to the existence of various substituted aromatic molecules. These molecules comprise eugenol, cinnamaldehyde, and carvacrol (Jay and Rivers, 1984; Moleyar and Narasimham, 1992; Juven *et al.*, 1994). Eugenol, the main phenolic compound and antimicrobial constituent of clove oil, has been registered by the European Commission for use as a flavouring ingredient in foodstuffs and is identified to carry no risk to consumer health (Burt, 2004). Recent study specifies that eugenol ($IC_{50}$ 101 µg/mL) was identified to have antiprotozoal properties. The trophozoites adherence was decreased within the first hr of incubation. The major phenotypical changes were in the cell morphology, internalisation of flagella and ventral discs, membrane blends, and intracellular and nuclear clearing. (Machado *et al.*, 2011).

To investigate the possible anti-proliferative effects of eugenol derivatives as a first step on the way to the development of novel antimicrobial agents, we studied four types of eugenol derivatives (EN1, 2, 3 and 4) for their anti-amoebic potential to inhibit growth and viability of *Acanthamoeba* T4 trophozoites. This is the first study to investigate the effect of eugenol derivatives on the survival of *Acanthamoeba castellanii* T4 trophozoites, and its biological properties.

The outcomes of survival assays showed dose-dependent effects of EN3 and EN1 on *Acanthamoeba castellanii* T4 genotype trophozoites survival. The value of MIC for the most effective compound (EN3) on *Acanthamoeba* was found to be 64µg/mL. This concentration achieved total eradication of *Acanthamoeba* after 24 h. EN2 and EN4 had lower antimicrobial potencies. Additionally the 50 % growth inhibition was found ($IC_{50}$). The value $IC_{50}$ for eugenols EN1, EN2 EN3 and EN4 against *Acanthamoeba* trophozoites were 30, 35, 29 and 31 µg/mL respectively. Research study by Jaganathan et al., (2011) showed that eugenols have both anti-proliferative and apoptosis-inducing ability. In our study we investigated the phenotypic changes in *Acanthamoeba* trophozoites. The trypan blue staining assays showed that the non-treated control trophozoites were evenly distributed throughout the cell culture well and they were growing next to each other. Treatment with EN caused a significant decrease in the number of visible trophozoites present in the well. Furthermore, their structure changed to irregular shapes and there was some debris around them, which could be the trophozoites that underwent destruction after their membrane lysed, which could be because of the cellular stress caused by eugenols. This shows that the effect is not only on the limitation of the trophozoites metabolism, but it actually causes decrease in proliferation and increase in apoptosis by the possible lysis of trophozoites.

In parallel to this, effects of eugenol derivatives were investigated against *Acanthamoeba* with invaded bacteria in it. The expactations were to observe negative effects of intracellular bacteria on resistance of *Acanthamoeba* against eugenols. MRSA presence in *Acanthamoeba* was expected to have more negative results than when MSSA due to its greater virulence. Even though the MIC of eugenol against trophozoites alone was similar to that when it has bacteria inside, it was detected that the behaviour of *Acanthamoeba* has been changed. The lowest effective concentrations of eugenols were found to stimulate trophozoites to turn to cysts. This event was not seen when *Acanthamoeba* without intracellular bacteria was exposed to eugenols.

Eugenol has been documented to reduce the proliferation of *Escherichia coli*, *Listeria monocytogenes*, and *S. aureus* (Blaszyk and Holley, 1998; Ben Arfa *et al.*, 2006). In addition to antibacterial potencies, eugenol also has antifungal and antiviral potencies (Sissons *et al.*, 2006;

Schnitzler *et al.*, 2011). As of its antimicrobial characteristics, eugenol is the one of the primary candidates to be recognised as an antimicrobial agent.

To verify the antibacterial potential of eugenol derivatives, we tested eugenol compounds for their ability to inhibit growth and viability of bacteria such as MRSA and MSSA in intracellular and extracellular conditions.

Our results demonstrate significant inhibitory activity of eugenol derivatives on MRSA and MSSA in extracellular conditions. Eugenol predominantly damages the cell membrane and causes the leakage of the cell contents (Yadav *et al.*, 2015). EN1 and EN3 had the most antibacterial potent.

When eugenol derivatives were tested against MRSA and MSSA in intracellular conditions inside *Acanthamoeba*, it was expected to have less antimicrobial effect due to barriers of passing through *Acanthamoeba* membrane. Surprisingly, intracellular bacteria had lower MIC than that when they are in extracellular conditions. We hypothesize this is due to the less number of bacteria (less than x $10^6$) inside *Acanthamoeba*.

Previous studies showed that the mechanisms of tissue damage and invasion by amoeba involve the release of proteases, including cysteine proteases and serine proteases (Alfieri *et al.*, 2000; Alsam *et al.*, 2003). It was found that protease activity is much higher in pathogenic *Acanthamoeba* species as compared to non-pathogenic ones. The proteases from pathogenic *Acanthamoeba* were shown to cause cytotoxicity to endothelial cells by forming holes in their membranes (Hurt *et al.*, 2003). When Zymography was done on the extracellular proteases, it showed that the main protease released is a serine protease with a molecular weight of 107 kDa and inhibition potential by 1 mM aprotonin or 1 mM PMSF (Alsam *et al.*, 2003). In our study SDS-PAGE zymography was used to assess the effect of eugenol derivatives on proteases released from *Acanthamoeba castellanii* T4 (trophozoites). Our results displayed that when doses of EN were increased, the release of proteases from trophozoites was decreased. This indicates that EN can be potentially used in protecting against tissue damage. These proteases found were at a size band around 60 and 70 kDa, pointing out it was serine protease (Kim *et al.*, 2003).

# Chapter 6. General Discussion

## 6.1. Discussion

*Staphylococcus aureus* is one of the commonest human bacterial pathogens which cause both healthcare- and community acquired infections. In 1962, two years after the introduction of methicillin antibiotic for management of penicillin-resistant *S. aureus* infections, the first case of MRSA was detected. Despite use of control measures, methicillin resistance among *S. aureus* has gradually become greater than before. Approximately 30 % of the population carry *S. aureus* in the nares or on the skin (Wertheim *et al.*, 2005). Community-acquired MRSA (CA-MRSA) is more likely to cause soft tissue and skin infections in contrast with healthcare-acquired MRSA (HA-MRSA). The gene encoding methicillin resistance, *mecA*, is located on mobile chromosomal element called the staphylococcal cassette chromosome (*SCCmec*) (Ma *et al.*, 2002; O'Brien *et al.*, 2005; Tenover *et al.*, 2006). MRSA is the reason for a number of hospital-acquired infections, notably boils and abscesses, pneumonia, food poisoning, toxic shock syndrome and causes huge problems in diabetic patients.

One of the advantageous strategies to overcome treatments and cause reinfections for pathogenic bacteria such as *S. aureus* can be being able to survive in *Acanthamoeba* (Cardas *et al.*, 2012). *Acanthamoeba* is a free living protozoan which is greatly distributed in the environment. It was isolated from nasal cavities and pharynx of healthy humans. It is reported that on average every human inhales one *Acanthamoeba* cyst a day (Kingston and Warhurst, 1969). Generally, *Acanthamoeba* feeds on bacteria. Some of the phagocytized bacteria are digested but some bacteria are able to survive and multiply intracellularly, leading to new bacterial characteristics. The growth of bacteria inside *Acanthamoeba* has been related to enhanced environmental survival of bacteria, increased virulence and increased resistance to antimicrobial agents. This is of great importance from both clinical and environmental aspects.

The aims of this project were, to investigate *Acanthamoeba castellanii* (a keratitis isolate belonging to the T4 genotype) interactions with MRSA and MSSA; and factors affecting the molecular mechanisms involved in *Acanthamoeba* interaction with bacteria.

In this study, *Acanthamoeba* contact-dependent engulfment mechanism, phagocytosis, was studied. Phagocytosis plays an important role in the pathogenesis of *Acanthamoeba*. Nevertheless, knowledge of the detailed molecular mechanisms related to *Acanthamoeba* phagocytosis remain vague. FITC-, and rose Bengal labelled MRSA and MSSA were used to study processes involved in the phagocytosis of *Acanthamoeba*. Several lines of evidence suggest that PI3K plays an important role in host cell cytoskeletal remodelling processes, such as phagocytosis, pseudopod formation and trafficking (Wymann and Pirola, 1998; Cox *et al.*, 1999). Here it was observed that LY294002, a specific PI3K inhibitor, reduced the likelihood of *Acanthamoeba* invasion by MRSA and MSSA. This is in line with the findings of Alsam, et al (2006) who found that PI3K played an important role in *Acanthamoeba* interaction with *E. coli*. The use of this inhibitor led to a reduction of the bacterial invasion of *Acanthamoeba*. Another PI3K inhibitor, wortmannin, exhibited a similar effect on phagocytosis, suggesting that *Acanthamoeba* phagocytosis is dependent on PI3K-mediated intracellular signalling pathways. Na-orthovanadate was used to inhibit protein tyrosine phosophatase. Previous studies indicated that sodium orthovanadate causes an increase in the *Acanthamoeba* uptake of *E. coli* (Alsam *et al.*, 2005; Sissons *et al.*, 2006b). However, in our study, uptake of MRSA and MSSA was reduced unlike Alsam et al., (2005). Overall our data suggests that all enzymes investigated here play roles in phagocytosis, proposing that particle uptake in *Acanthamoeba* is a multifarious process that could play a substantial role, both in food uptake as well as the pathogenesis of this protozoan.

Binding of *Acanthamoeba* leads to another virulence factor such as production of extracellular proteases, one of the contact-independent mechanisms. The role of extracellular proteases in *Acanthamoeba* – bacteria interactions remains incompletely understood. To this end, inhibition of extracellular serine proteases was performed by PMSF inhibitor. Analysis of proteases from keratitis-causing *Acanthamoeba* shows that the presence of a 130 kDa protease was inhibited by PMSF. PMSF has a role in permeabilising the blood-brain barrier (Sissons *et al.*, 2006b). It is thought-provoking that inhibition of serine proteases increased MRSA and MSSA uptake by *Acanthamoeba*. In addition to that, zymography assays showed 130 kDa band representing serine proteases were not expressed, indicating

inhibition of these serine proteases. The similar results of 130 kDa band inhibition was confirmed by performing 2D gel electrophoresis. These data support work by Alsam *et al*, (2005b).

In an effort to investigate the expression of novel proteins, treated and untreated *Acanthamoeba* CM was investigated by two-dimensional gel electrophoresis. The results of our proteomic analysis revealed the resolution of a number of polypeptide spots. Forty three protein spots resolved at a given pI and molecular weights were identified. More analysis by mass spectrometry is needed to identify the sequence and identity of differentially regulated polypeptides observed. This could validate the findings and correlates with existing proteins identified in databases.

Bacterial infections have always been a persistent threat to human health. As a result of the unending rise of drug-resistant bacteria, both in healthcare and in the community, there is a necessity for novel strategies for antimicrobial therapy. PDT has recently been utilised to efficiently eradicate microorganisms. PDT turned out to be an effective strategy to kill multi- drug resistance microorganisms and there is no reported evidence for PDT resistant mechanisms as compared with antimicrobials. *In vitro* PDT experiments have demonstrated that *m*-THPC may be used as PS against *S. aureus* and MRSA (Sperandio *et al.*, 2013). According to these results, the effectivity of *m*-THPC mediated PDT was determined on internalized MRSA, MSSA and *Acanthamoeba* trophozoites. *m*-THPC mediated PDT was capable of eliminating 100 % the viability of internalised MRSA, MSSA and *A. castellani*i trophozoites proportionate to the increase of the PS concentration. Psoralen mediated phototoxic effect on *Acanthamoeba*, MRSA and MSSA was established as dose dependent, supporting several studies regarding PDT and different bacteria using a variety of PS (Wainwright, 2009). One of the goals was to investigate whether *Acanthamoeba* might protect the bacteria inside them against phototoxicity. Solheim *et al*. (2006) concluded *Acanthamoeba* keratitis and bacteria were affected when psoralen was used. The results of this study showed that psoralen is capable of eradicating even intracellular bacteria such as MRSA and MSSA inside *Acanthamoeba*.

Another set of experiments were carried out using natural product as antimicrobial agent. It has been revealed that eugenol has various biological capabilities, including antimicrobial, antioxidant, anti-

inflammatory, carminative, and antispasmodic activities (Qiu *et al.*, 2010). Recent study specifies that eugenol was identified to have antiprotozoal properties (Machado *et al.*, 2011). Four types of eugenol derivatives for their anti-amoebic potential to inhibit growth and viability of *Acanthamoeba* were studied. The outcomes of survival assays displayed dose-dependent effects. Treatment with eugenol derivatives caused a significant decrease in the number of visible trophozoites present in the well. Furthermore, their structure changed to irregular shapes and there was some debris around them, which could be the trophozoites that underwent destruction after their membrane lysed, which could be because of the cellular stress caused by eugenols. This shows that the effect is not only on the limitation of the trophozoites metabolism, but it actually causes decrease in proliferation and increase in cell death by the possible lysis of trophozoites. Our results demonstrate significant inhibitory activity of eugenol derivatives on MRSA and MSSA in intracellular as well as extracellular conditions. Eugenol predominantly damages the cell membrane and causes the leakage of the cell contents (Yadav *et al.*, 2015).

Previous studies showed that the mechanisms of tissue damage and invasion by amoeba involve the release of proteases (Alfieri *et al.*, 2000; Alsam *et al.*, 2003). The proteases from pathogenic *Acanthamoeba* were shown to cause cytotoxicity to endothelial cells by forming holes in their membranes (Hurt *et al.*, 2003). In our study SDS-PAGE zymography was used to assess the effect of eugenol derivatives on proteases released from *Acanthamoeba castellanii* T4 (trophozoites). Our results displayed that when doses of EN were increased, trophozoites released less proteases into environment. This indicates that EN can be potentially used in protecting against tissue damage.

In conclusion, this study has shown: MRSA and MSSA were able to invade, survive and multiply within *Acanthamoeba*; Inhibition of PI3K, tyrosine protein kinase and tyrosine protein phosphatase decreased bacterial uptake by *Acanthamoeba.* In contrast, inhibition of serine proteases resulted in greater uptake than control samples; It was detected that in the phagocytosis process some bacteria actively invade through the cell membrane while some of them are being taken up by *Acanthamoeba*. The number of internalised bacteria were mainly actively phagocytosed ones; PMSF was able to inhibit 130 kDa extracellular serine protease; Protein spot analyses indicated inhibition of 43 proteins on treated gel

which were expressed in untreated control gel; *m*-THPC and psoralen mediated PDT was effective against *Acanthamoeba* and bacteria both in intracellular and extracellular conditions; Among four types of eugenol, natural product derivatives utilised, two of them (EN1 and EN3) had higher antimicrobial effect than others.

### 6.2. Recommendations for future work

The findings derived from the work described in this thesis enlighten some aspects of *S. aureus* virulence, but also posed more questions that need to be answered. The focus of this study was on the ability of MRSA and MSSA to interact with *Acanthamoeba*. Also, it will be sensible to exploit the PDT approach and several antimicrobial agents against *Acanthamoeba*, MRSA and MSSA in intracellular and extracellular conditions for therapeutic use.

1. Understanding of metabolic pathways playing role in *Acanthamoeba*-bacteria interactions;

2. As keratitis is the most common infection caused by *Acanthamoeba*, it would be interesting to use in future human corneal epithelial cell line;

3. Identification of the gene products important for binding and intracellular survival of MRSA and MSSA within amoebae. One interesting method would be preparing mutants lacking the genes responsible for the expression of the corresponding virulence factors;

4. It is desirable to investigate drugs used against amoeba infections due to an increase in number of contact lens wearers. Most of the drugs given are mixed together which allow for an additive and synergistic effect but can also cause unwanted side effects and drug toxicity. A mixture of drugs is used because none of the available drugs is 100 % efficient;

5. The use of natural products, including eugenol derivatives as antimicrobial agents may have the potential to be one of the therapeutic protocols against *Acanthamoeba* and bacterial infections;

6. The use of PDT application as an alternative treatment for bacterial infection could overcome antibiotic drug resistance.

# Chapter 7. Appendices

## 7.1. Appendix one: *Acanthamoeba* storage, counting and viability

### 7.1.1. *Acanthamoeba* storage

*Acanthamoeba* trophozoites can be stored as axenic cultures for long term. Briefly, exponentially growing amoebae were re-suspended at a density of 5 x $10^6$ cells per mL in the PYG medium and placed into a cryotube. The cryotube was quickly placed in a box with ice and transferred to -20 °C for 1 h, followed by their storage at -80 °C overnight before permanent storage in liquid nitrogen.

### 7.1.2. Preparation of *Acanthamoeba* culture

Thawing and recovery of *Acanthamoeba* from liquid nitrogen was done quickly. Pre-warmed PYG medium was prepared in advance. The cryotubes were taken out from liquid nitrogen in dry ice and immediately placed in 37 °C incubator until 80 % has thawed. *Acanthamoeba* were transferred into a tube with pre-warmed medium and centrifuged at 1100 rpm for 3 min. After centrifugation the amoebae were gently re-suspended in growth medium (PYG) followed by their immediate transfer in a T-25 tissue flasks. Next, flasks with *Acanthamoeba* were incubated at 30 °C until they become confluent.

### 7.1.3. Counting *Acanthamoeba*

The number of *Acanthamoeba* was determined by using the haemocytometer. After the haemocytometer and the coverslip were cleaned using 70 % ethanol, 7 µl of cell suspension were placed on haemocytometer and the average number of *Acanthamoeba* in 1mL was calculated. The *Acanthamoeba* were diluted to the required number using appropriate medium and plated in flasks or plates as required by experimental design.

### 7.1.4. Determination of the viability of *Acanthamoeba*

To determine the viability of *Acanthamoeba* trophozoites, trypan blue exclusion test was used. *Acanthamoeba* was incubated at room temperature with 0.2 % trypan blue for 20 min and counted by using the haemocytometer.

## 7.2. Appendix two: Bacterial cell culture techniques, storage and counting

### 7.2.1. Bacterial strains

Clinical isolates of MRSA, MSSA were generously provided by Dr Tony Elston from Colchester hospital.

### 7.2.2. Media and growth conditions

For interaction assays, all staphylococcal strains were grown in Luria-Bertani (LB) broth at 37 °C and used at the logarithmic growth phase. All bacterial strains were cultured on nutrient agar plates and sub-cultured weekly.

### 7.2.3. Storage of bacterial cultures

Using a sterile loop roughly 20-30 isolated colonies of bacteria were picked up from the agar plate and inoculated in a 10 mL LB broth containing 30 % glycerol. Aliquots of 1 mL bacterial mixture were transferred in cryotubes and placed in a -20 °C freezer for permanent storage. To activate bacteria, a loop was dipped in the frozen cryotube and the streaking protocol was followed. The streaked agar plate was incubated in a 37 °C incubator overnight resting on its lid to prevent water condensation from falling onto the agar. After the incubation the plate was carefully examined if the colonies have similar morphologies such as textures, colours and border shapes and stored at 4 °C for up to a month.

### 7.2.4. Counting bacteria

The number of MRSA and MSSA before their incubation with *Acanthamoeba* was determined by spectrophotometric analysis, followed by serial dilution with PBS and plated on nutrient agar plates. After 48 h of incubation the number of colonies from a dilution plate showing isolated colonies was counted and according to the sample inoculated on each plate, the number of bacteria/mL was calculated.

## 7.3. Appendix three: Reagents and Inhibitors used in chapter two

### 7.3.1. Inhibitors used in phagocytosis assays

**Genistein (G6649):** 0.0027 g in one mL

**DMSO:** Concentrations used are 10, 50 and l00 µM.

**Sodium orthovanadate (S6508):** 50 mM stock was prepared by dissolving

0.0092 g in one mL distilled water. Concentrations used are 10, 50 and l00 µM.

**3.5 mM LY294002 (0012):** 3.5 mM stock was prepared by dissolving 5 mg in 5 mL of DMSO. Effective concentration is 50 µM.

**Phenylmethanesulfonyl fluoride (PMSF) (P7626-250 MG):** 0.017 dissolved in one mL methanol. For 1 mM final concentration, add 10 µl of 10 mM to 990 µl methanol.

### 7.3.2. Buffers used in phagocytosis assays

**Carbonate-bicarbonate buffer**: (9.5 mL 0.2 M $Na_2C0_3$ mixed with 41.5 mL 0.2 M $NaHC0_3$, solution made up to 200 mL, pH 9.4)

## 7.4. Appendix four: Reagents and Inhibitors used in chapter three

### 7.4.1. Reagents and buffers for Zymographic assays

1.5M Triethylamine hydrochloride (Tris) pH 7.4: (T8521) 18.1 g dissolved in 100 mL distilled water, adjust pH to 7.4 using hydrochloric acid (HCl). 0.5 M Tris pH 6.8: 6.057 g in 100 mL distilled water, adjust pH to 6.8 using HCL 10 % (w/v) SDS: (L3771) 10 g SDS make up to l00 mL with distilled water 30 % (v/v) Bis/ acrylamide solution for electrophoresis: (A3574) 4 % (w/v) gelatin: (9000-70-8) 4 g make up to 100 mL with distilled water. Running buffer for SDS-PAGE (stock solution SX): Tris 15 g, glycine (G8898) 72 g, SDS 5 g, make up to 1000 mL with distilled water. Adjust pH to 8.3 with HCI. Dilute to 1X with distilled water prior to use.

**Sample buffer (2X):** distilled water 4.0 mL; 0.5M Tris pH 6.8, 1.0 mL; glycerol 0.8 mL; 10 % SDS (w/v in distilled water) l.6 mL; 0.05 % (w/v in distilled water) bromophenol blue (B6131). To analyse protein samples using SDS-PAGE, 1:10 2-mercaptoethanol (M6250) to sample buffer is added, samples with sample buffer (1:1) are mixed, and heat at 95 °C for 5 min prior to electrophoresis. For zymography, no 2-mercaptoethanol was added to the sample buffer and the sample was not heated.

**Coomassie brilliant blue stain**: 0.5 % (w/v) Coomassie brilliant blue (B0149), 40 % methanol (v/v), 10 % acetic acid (v/v), 50 % distilled water (v/v). De-stain: 40 % methanol (v/v), 10 % acetic acid (v/v), 50 % distilled water (v/v). Temed solution: (T9281). Ammonium persulphate: (Sigma A3678), 4 % (w/v dissolved in distilled water) Tris buffer used to prepare triton and developing buffer: 50 mM Tris (6.057 g), adjust pH to 7.5.

**Triton:** 2 % Triton X-100 (v/v in Tris buffer) (X-100).

**Developing buffer:** 10 mM CaCl (1.4 g/l C1016).

10 % SDS-PAGE zymography running gels:

| | | |
|---|---|---|
| Distilled water | 4.2 mL | 31.89 % |
| Bis/acrylamide solution | 3.33 mL | 34.7 % |
| SDS 10 % | 100 µ1 | 0.1 % |
| Tris 1.5 M pH 7.4 | 2 mL | 20.8 % |
| Ammonium persulphate | 400 µ1 | 0.4 % |
| Temed solution | 6 µ1 | 0.06 % |

4 % Stacking gel

| | | |
|---|---|---|
| Distilled water | 4.3 mL | 6.9.77 % |
| Bis/acrylamide solution | 0.8 mL | 13 % |
| SDS 10 % | 0.75 mL | 12.16 % |
| Tris 0.5 M pH 6.8 | 60 µ1 | 0.97 % |
| Ammonium persulphate | 250 µ1 | 4.0 % |
| Temed solution | 3 µ1 | 0.048 % |

**7.4.2.Zymographic assays protocol**

The Biorads mini-protein electrophoresis unit (67512533, Biorad, Hemel Hempstead, UK) is assembled according to the manufacturers' instructions. The running gel reagents were mixed and loaded into the sealed gel caster (overlaid with 70 % ethanol to exclude oxygen), this is a vertical SDS-PAGE system that cast gels of 0.75 mm thickness. After polymerisation, the ethanol was decanted and the stacking gel was mixed and layered on top of the running gel. Plastic 10-well combs (holds up to 20 µl/well) were inserted into the stacking gel and polymerisation allowed to occur (~30 min). The combs were then removed and gels were assembled into the electrophoresis tank (Biorad 165-2949) and the tank filled with 1X running buffer as mentioned above. Samples mixed with sample buffer (1:1) were added to the wells and electrophoresed for ~2 hrs, 115V. After that gels were washed with 50 mL 2 % Triton X-100 in Tris buffer for 1 h, with gentle shaking to remove SDS and re-nature proteases. Then incubated overnight in 50 mL developing buffer at 37 °C without shaking. Next day, gels were stained with 50 mL coomassie blue stain for 1 h, followed by de-staining with 50 mL de-stain and the proteolytic activity was visualized as unstained regions. For SDS- PAGE only, gels were stained with 50 mL coomassie blue stain, with gentle shaking and then de-stained for ~30 min with gentle shaking to visualise protein bands on the same day.

### 7.4.3. Buffer Recipes for 2 DE

#### 7.4.3.1. Rehydration solution preparation

Just prior to use, the appropriate volume of stock solution is slowly thawed. Add the appropriate amount of IPG Buffer, if it is not already included in the rehydration stock solution. Add sample. The rehydration stock can be diluted no more than 1/8 by sample addition [eg. 62 ul of sample for each 0.5 mL rehydration stock]. The amount of protein that can be added is dependent upon the length of strip, the pH range and the detection method to be used. Up to 1 mg can be added to a wide pH range 17 cm strip.

Note: DTT and the sample must be added fresh, just prior to use.

| Strip length (cm) | total volume per strip[1] (ul) [min; max][2] |
|---|---|
| 7 | 125; 200 |
| 11 | 200; 280 |
| 13 | 250; 350 |
| 18 | 350; 500 |

[1] this volume includes the sample

[2] The minimum volume rehydrates the strip to a gel thickness of 0.5 mm and a gel composition of 4 % T/ 3 % C. The maximum volume allows for larger protein sample loads, facilitates entry of larger proteins and minimizes protein solubility problems.

For most samples it is recommended to stay as close to the lower volume limit as possible.

#### 7.4.3.2. SDS equilibration buffer

| Component | Final concentration | Amount |
|---|---|---|
| 1.5 M Tris-Cl, pH 8.8 | 50 mM | 6.7 mL |
| Urea (FW 60.06) | 6 M | 72.07 g |
| Glycerol (87 % v/v) | 30 % (v/v) | 69 mL |
| SDS (FW 288.38) | 2 % (w/v) | 4.0 g |
| Bromophenol blue | trace | (a few grains) |
| Double distilled $H_2O$ | | to 200 mL |

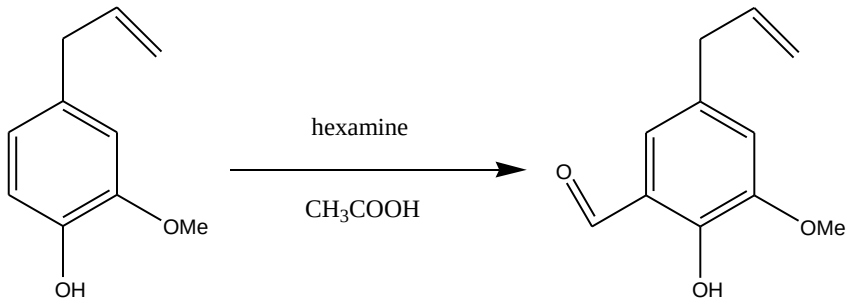
(50 mM Tris-Cl pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS, bromophenol blue, 200 mL)

Store in 40 mL aliquots at –20 °C.

## 7.5. Appendix five: Preparation of Eugenol Derivatives

### 7.5.1. Preparation of 5-allyl-2-hydroxy-3-methoxy-benzaldehyde AM2 ($C_{11}H_{12}O_3$)

4-allyl-2-methoxy-phenol (AM1, 10.66 g, 65 mmol) in glacial acetic acid (75 mL, 1312 mmol) was treated with hexamine (40 g, 285.7 mmol). The mixture was heated and stirred until a clear solution was obtained. After that, the reaction mixture was heated on oil bath at 105 ºC for 7 h. The dark brown-red solution was treated while hot with a boiling mixture of concentrated hydrochloric acid (50 mL) and water (100 mL). The reaction mixture was heated for another 5 min, then cooled slowly. The product was extracted with ether (3 x100 mL) .The combined organic layers were washed with water (3 x100 mL). The clear ether solution was then shaken with sodium hydroxide 30 mL (20 %). The lower aqueous layer was colourless showing that only acetic acid had been extracted. The organic layer was dried with magnesium carbonate, filtered and evaporated, to give a brown oil residue. Purified by column chromatography on silica gel and eluted with petroleum spirit: ether (1: 1) ( Rf = 0.57), to give 5-allyl-2-hydroxy-3-methoxy-benzaldehyde (AM2, 3.3 g, 30.9 % ), a yellow crystal. m.p.48-50. (Scheme: 1) (The Indian Academy of Sciences, 1949).

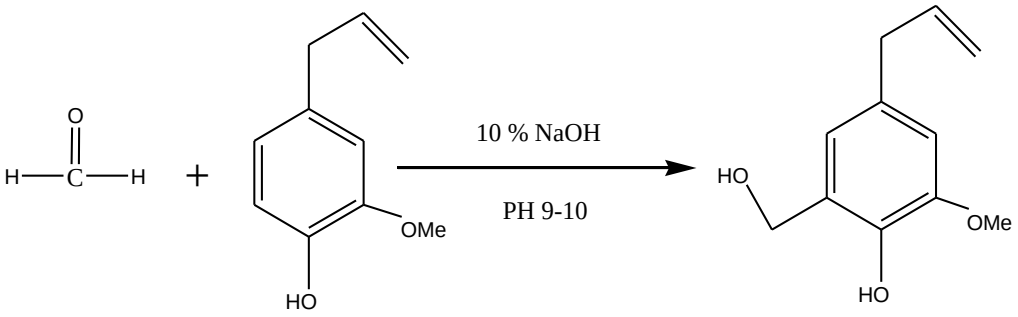



$AM_1$ $AM_2$

**Scheme (1): Preparation of $AM_2$ from $AM_1$**

### 7.5.2.Preparation of 4-allyl-6-hydroxy methyl-2-methoxy-phenol AM3 (C11H14O3)

4-allyl-2-methoxy-phenol (AM1, 14.76 g, 0.09 mol) and formaldehyde (8.1 mL, 0.1 mol) (37 % solution of formalin) are mixed in a round bottom flask at pH = 9-10 by adding 5 % NaOH solution, then stirred and refluxed at 100 ºC for 2.5 h. As a result, this yellow mixture was separated into two layers, the viscous yellow – brown liquid was dissolved in ethanol and neutralized with 5 % HCl solution, then diluted with water (100 mL). The hydroxy methyl eugenol was extracted with ether (2 x 100 mL), dried, evaporated to give a brown oil residue which was purified by column chromatography on silica gel eluted with petroleum spirit/ ether ( 1:1 )( Rf = 0.22), to give a 4-allyl-6-hydroxy methyl-2-methoxy-phenol (AM3, 3.7 g , 25.1 % ), as a yellow oil(Scheme :2) (The Indian Academy of Sciences, 1949).




$AM_1$ $AM_3$

**Scheme (2): Preparation of $AM_3$ from $AM_1$**

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| Poster and Oral Presentations | | | |
|---|---|---|---|
| **List of authors/Title/ Type of attendance** | **Conference** | **Location** | **Date** |
| **Participator** | **The Essex Biomedical Sciences Institute (EBSI)** | Biological Sciences Department | 28.09.2012 |
| **S. AlGhamdi, S. Battah, S. Alsam. "*Mechanisms associated with Staphylococcus aureus and Acanthamoeba interaction*". Oral presentation.** | **Graduate Forum, University of Essex** | Biological Sciences Department | 12.09.2013 |
| **Participator** | **The Essex Biomedical Sciences Institute (EBSI)** | Colchester General Hospital | 15.01.2014 |
| **S. AlGhamdi, S. Battah, S. Alsam. "*Molecular mechanisms of the interaction of MRSA with Acanthamoeba*". Poster presentation.** | **Seventh Saudi Student Conference (SSC2014)** | Edinburgh International Conference Center | 01.02.2014 |
| **S. AlGhamdi, S. Battah, S. Alsam. "*Photodynamic therapy and MRSA treatment*". Poster presentation.** | **M Annual Conference 2014** | Liverpool | 14.04.2014 |
| **S. AlGhamdi, S. Battah, S. Alsam. "*Effects of Eugenols on intracellular MRSA*". Poster presentation.** | **Eighth Saudi Student Conference (SSC2014)** | Imperial College - London | 02.02.2015 |
| **Participator** | **The Essex Biomedical Sciences Institute (EBSI)** | Colchester General Hospital | 01.07.2015 |
| **Participator** | **SGM Annual Conference 2016** | Liverpool | 22.03.2016 |