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Antimycobacterial, immunomodulatory and hepato-protective activities of quinones and organosulphur compounds

By

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ABSTRACT

Tuberculosis is one of the world's deadliest diseases. It is caused by the bacterium *Mycobacterium tuberculosis*. Two groups of samples: Quinones and Garlic polysulfide mixtures were assessed for their antituberculosis activity.

The microtitre alamar blue (MABA) and microtitre presto blue assays (MPBA), on *Mycobacterium tuberculosis* were conducted to determine the minimum inhibitory concentrations (MICs). Twenty quinones and six garlic oil polysulfide mixtures were tested for their *in vitro* antimycobacterial activity and cytotoxicity. The antimycobacterial activity was tested on the H37Rv *Mycobacterium tuberculosis* strain and the cytotoxicity was tested on primary peripheral blood mononuclear cells and secondary U937 human monocytes. Six quinone samples showed antimycobacterial activity with minimum inhibitory concentrations less than 3.125 µg/ml and one garlic sample had a minimum inhibitory concentration of 2.5 µg/ml. Samples KM 108-1 and IL 143 had selective indices above 8 and KM 140 had a selective index of 55.

Twenty quinone samples and six garlic polysulfide mixtures were also evaluated for their immune stimulatory effects as well as their liver protective activity. The immune response was assessed by evaluating the Th1/Th2 cytokine production levels by the peripheral blood mononuclear cells on exposure with various concentrations of the samples. The induced toxicity on Liver cells (C3A - hepatocytes), by acetaminophen, were used to assess the hepatoprotective efficacy of the samples. Three quinone compounds showed immune stimulatory effects (KM94, KM108-1 and KM117), and five showed significant hepatoprotective effects of 60-100% (KM108, KM140, $AqsNH_4$, CB5 and IL107) at ¼ Inhibitory Concentration of 50%. Garlic samples G5 and G6 showed slight immune stimulatory effects, while G6 was found to be the only sample to have hepatoprotective activity of 22%, probably due to the higher amount of diallyl tetrasulfide within the mixture.

All the quinone samples were evaluated for their subversive substrate activities on three flavoprotein disulfide reductases: Glutathiol, Mycothiol and Thioredoxin reductase. Glutathiol

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reductase is the human analog while Mycothiol and Thioredoxin are bacterial analogs. Substrate binding affinity was determined and it was found that quinones act as substrates for all three enzymes with the highest affinity for Thioredoxin reductase. Samples; Aqs and KM80 had half the maximum velocity (Km-value) of lower than 30 µM. It can also be concluded that anthraquinones had a higher affinity to these disulfide enzymes as compared to naphthoquinones.

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LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome

ATCC: American Type Culture Collection

CFU: Colony forming units

DMSO: Dimethyl sufoxide (CH_2Cl_2)

DNA: Deoxirybonucleic acid

EDTA: Ethylene-diamene-tetra-acetic acid

ELISA: Enzyme-linked immunosorbent assay

EMB: Ethambutol

ETH: Ethionamide

EtOH: Ethanol

FDA: Food and drug Administration

GSH: Glutathiol

GSSG: Glutathione disulfide (oxidized)

Gtr: Glutathiol disulfide reductase

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: Human immunodeficiency virus

IC_{50}: 50% inhibition concentration

INH: Isoniazid

Km: Michaelis constant

LJ: Löwenstein-Jensen

MDR: Multiple drug-resistant

MIC: Minimum inhibitory concentration

MSH: Mycothiol

MSSM: Mycothione or Mycothiol disulfide

Mtr: Mycothiol disulfide reductase

NADPH: Nicotinamide adenine dinucleotide phosphate

OADC: Oleic acid, albumin, dextrose, catalase

OD: Optical density

PANTA: Polymixin B, amphotricin B, nalidixic acid, trimethoprim, azlocillin

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RIF: Rifampicin

RPMI: Roswell Park Memorial Institute

SD: Standard deviation

SOD: Superoxide dismutase

STR: Streptomycin

TB: Tuberculosis

TM: Traditional medicine

Txr: Thioredoxin reductase

Vmax: Maximum velocity

WHO: World health organization

WWW: World Wide Web

XDR: Extensively drug-resistant

XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2-H-tetrazolium hydroxide

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Chapter 1

Introduction, Problem statement and Scope

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Chapter 1 – Introduction, Problem Statement and Scope

1.1 Background

1.1.1 Ethnopharmacology and phytomedicine

Throughout history, the human civilization has been depending and relying on natural products to treat their ailments. These products were obtained not only from flora, fauna and mineral sources in the indigenous people's immediate surroundings and also from remote areas. Thus nature has been the source of medicinal agents for thousands of years, and a staggering number of modern drugs have been isolated from natural sources, especially plants (Ghorbani and Mosaddegh, 2006).

These modern drugs are either lead compounds directly from plants or plant-derived compounds. Considering the impact of penicillin, obtained from micro-organisms, on the development of an anti-infectious agent, natural products seem to be highly important. It is estimated that about 25% of the prescribed drugs available, are isolated from plants. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin and a significant number are synthetic drugs from plant-based precursors (Rates, 2000). Some of the most well-known examples of drugs obtained from plants are digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristine and vinblastine from *Cantharanthus roseus*, atropine from *Atropa belladonna*, morphine and codeine from *Papaver somniferum*, artimisinin from *Artimisia anua* and aspirin from *Filipendula ulmaria.* Furthermore, it is also estimated that 60% of anti-tumour and anti-infectious drugs already in the market or under clinical trials are obtained from natural sources (Yue-Zhong Shu, 1998). The vast majority of these cannot yet be synthesised economically and are still obtained from wild or cultivated plants (Rates, 2000).

Over the last decade there has been a significant increase, and a growing interest, in alternative medicines, especially those from plant origin (Rates, 2000). This green revolution can be

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attributed to several reasons, namely; conventional medicine can be inefficient, abusive and incorrect use of these drugs can result in adverse side-effects. A large percentage of the world's population does not have access to conventional pharmacological treatment and indigenous medicine, and lastly ecological awareness suggests that "natural" products are safe and harmless (Rates, 2000). However, this is not always the case and the use of these natural products is not always authorized by legal authorities or practitioners who are familiar with the efficacy and safety of the phytomedicines. There is an abundance of articles and published papers that report on the lack of quality in the production, trade and prescription of phytomedical products (Rates, 2000).

Thus, it is clear that the modern society's general view on of health services has changed. The need for a novel pharmaceutical drug development from an ethnopharmacological milieu, as an alternative, represents a viable and suitable approach for new drug development and research. The private sector and government institutions are now financially supporting research programs aiming to elucidate and unravel the world of plant based medicines (Rates, 2000).

There is still a huge potential in higher plants as a source of new drugs. Only a few of the estimated 250,000 plant species have been investigated phytochemically, and only a small percentage of these have been studied and screened for bioactivity (Payne *et al*., 1991).

1.1.2 Natural products from plants used against Tuberculosis

Many plants have been used traditionally to treat the symptoms of Tuberculosis. According to previous research done, some of the plants from the southern parts of Africa have shown significant activity against *Mycobacterium* in *in-vitro* studies, with a minimum inhibitory concentration (MIC) of lower than 50ug/ml. These plants include; *Berchemia discolor* with a MIC of 12.5ug/ml, *Warbugia salutaris* (25ug/ml), *Terminalia sericea* (25 µg/ml), *Bridelia micrantha, Euclea natalensis* (8 µg/ml) (Fig. 1.1) and *Dyospyros mespiliformis* (Fig. 1.2) (Lall and Meyer, 2001, Mahapatra *et al*., 2007, Green *et al*., 2010). Pure compounds 7-methyl Juglone and Diospyrin, isolated from *Euclea natalensis* showed significant inhibitory properties, exhibiting minimum inhibitory concentrations (MIC's) of 0.5 ug/ml and 8.0 ug/ml respectively

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(Fig. 1.1 and 1.2) (Mahapatra *et al*., 2007). The inhibitory activity of these compounds against *M. tuberculosis* is very significant and comparable to the existing conventional Tuberculosis drugs. Considering South Africa's rich plant diversity, it is imperative to explore plants indigenous to South African plants for antituberculosis activity.

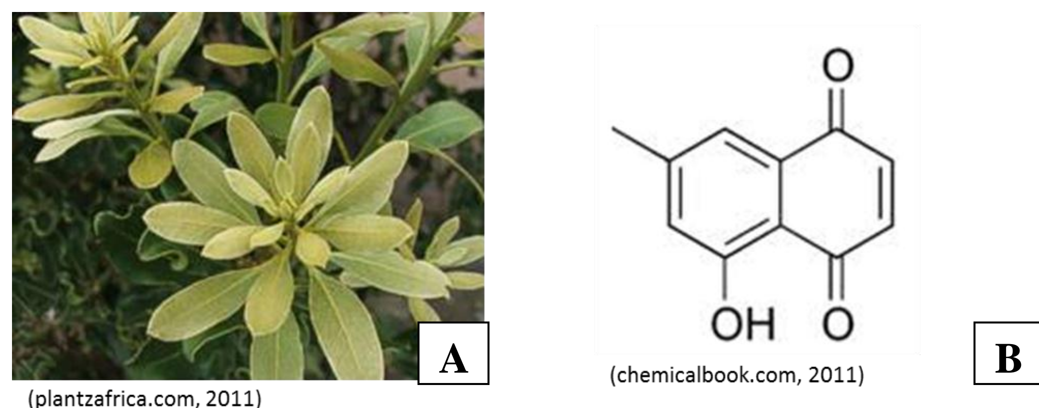

Figure 1.1: *Euclea natalensis*: (A) Leaves and shoots; (B) Isolated compound, 7 Methyl Juglone (MIC 0.5µg/mL).

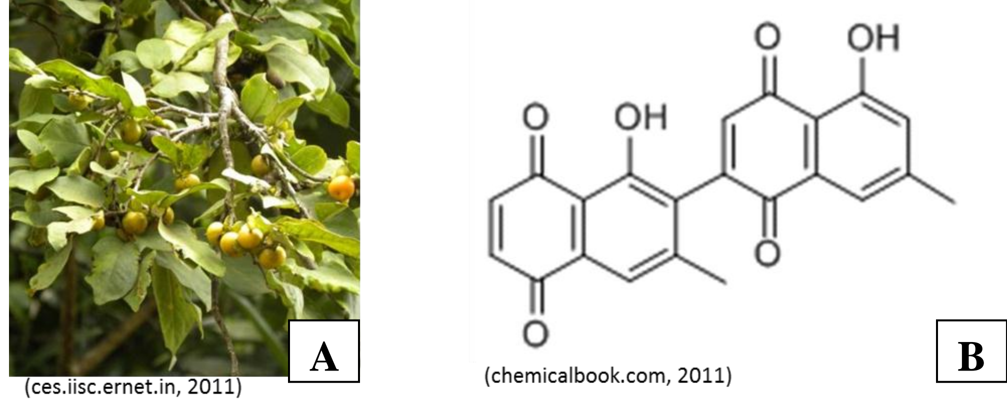

Figure 1.2: *Diospyros* sp: (A) Friuts and leaves of the tree; (B) Isolated compound, Diospyrin (MIC 8ug/ml).

1.1.3 Tuberculosis

1.1.3.1 The Mycobacterium tuberculosis bacterium

The *Mycobacterium tuberculosis* complex (MTC; *M. tuberculosis, M. bovis, M. microti, M. africanum, M. pinnipedii, M. avium, M. intracellulare, M. scrofulaceum* and *M. caprae* species)

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is a clonal progeny of a single ancestor that resulted from an evolutionary bottleneck about 30,000 years ago (Brosch *et al.*, 2002; Gutacker *et al.*, 2002; Hughes *et al.*, 2002). The *M. tuberculosis* bacterium is a gram-positive, rod shaped, aerobic, slow growing organism that was first isolated by Robert Koch in 1882 (Fig. 1.3).

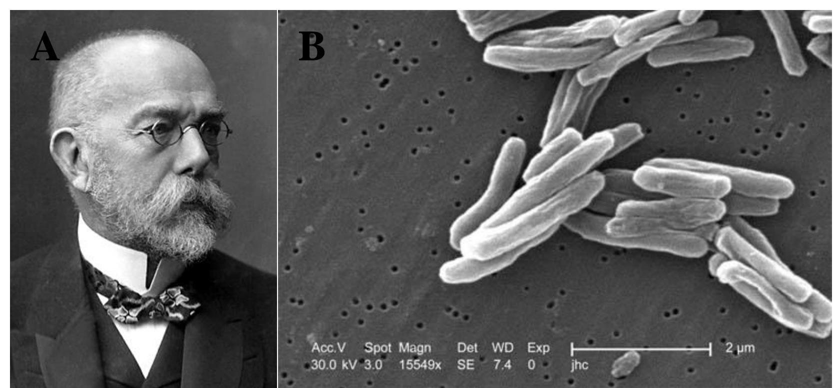

Figure 1.3: *Mycobacterium* bacilli (B) first isolated by Robert Koch (A) (embryology.med, 2012; nobelprize.org, 2012).

It is adapted to withstand weak disinfectants and relatively long periods in the open air, due to the thick bacterium-cell wall. This cell wall is one of the most complex structures, comprising of peptidoglycan covalently attached *via* a linker unit to a linear galactofuran which in turn is attached to several strands of a highly branched arabinofuran, which are attached to mycolic acids. This multilayered cell wall structure is approximately 20 nm thick (Brennan, 2003). The bacteria can spread easily through the air if an infected person with active TB sneezes, coughs, speaks or spits (Fitzgerald *et al.*, 2009). In 75% of the cases, the bacteria infect the lungs, termed pulmonary tuberculosis. In the other 25%, the bacteria infect other sites throughout the body. These sites include bones, joints, the central nervous system and the lymphatic system. In these cases it is termed miliary tuberculosis or extra-pulmonary tuberculosis (Fitzgerald *et al.*, 2009).

1.1.3.2 *Mycobacterium tuberculosis* prevalence

Mycobacterium tuberculosis (MTB), the etiological agent of Tuberculosis (TB) is one of the world's major epidemics, and is second only to HIV/AIDS, as the biggest killer due to a single infectious organism. In 2010 there was an incident rate of 8.8 million and a death rate of 1.4

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million per year, mostly in developing low-income countries (95%). To put this in perspective, it is close to 3900 deaths a day, or 3 people dying of TB per minute. TB is also the leading cause of death in patients with HIV/AIDS. One-third of the world's population is infected with TB (WHO, 2012). Also, an infected patient with untreated active TB will infect on an average, 10-15 people per year. Fortunately, the TB death rate has declined since 1990 by 40%, but with the increase in population and resistance, it is still not as significant as it ought to be.

Tuberculosis is contagious and airborne, affecting mostly young adults and the elderly in developing low-income countries. Figure 1.4 illustrates the TB incidence rate per 100,000 population (figures 1.5 – Multidrug resistance cases, 1.6 – Extensively drug resistance cases). It is also termed the disease of poverty. Although the death rate and incident rate is high, it is estimated that only 10% of the people who are infected with the *Mycobacteria* develop active TB. The active disease usually occurs in an infected person when the body's resistance or immunity is low, or if there is a prolonged exposure to the bacteria that overcome the body's natural defenses (National Jewish Medical and Research Center, 1994). The use of an immune modulator drug or herbal formulation, that increases the immune response in immune deficient patients, can decrease the risk factor.

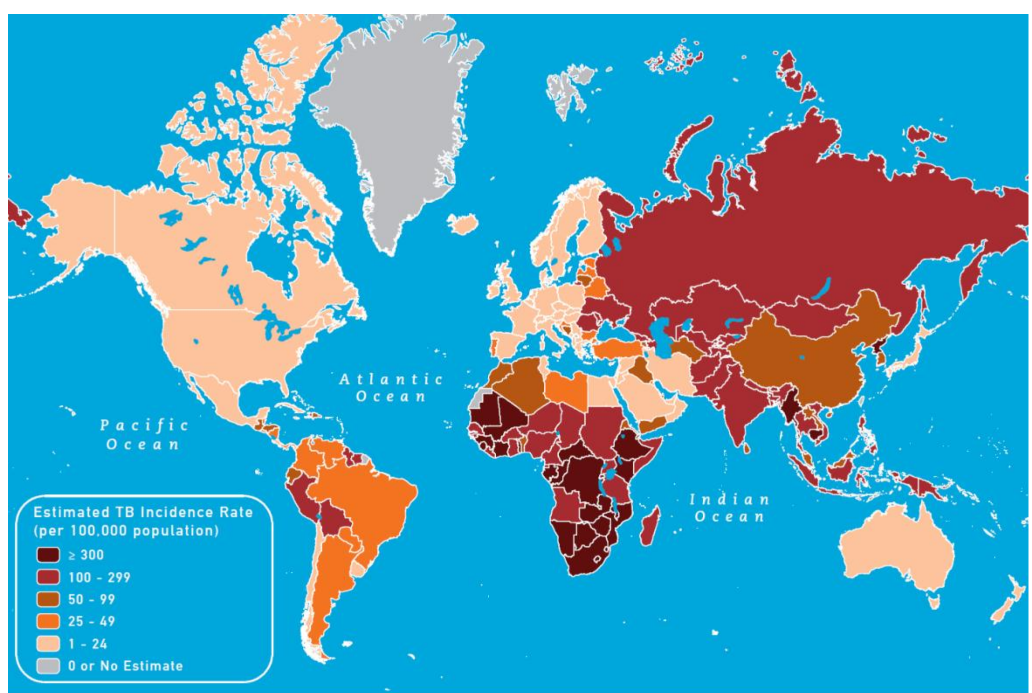

Figure 1.4: Estimated cases of TB per 100,000 population (WHO report, 2012-2013).

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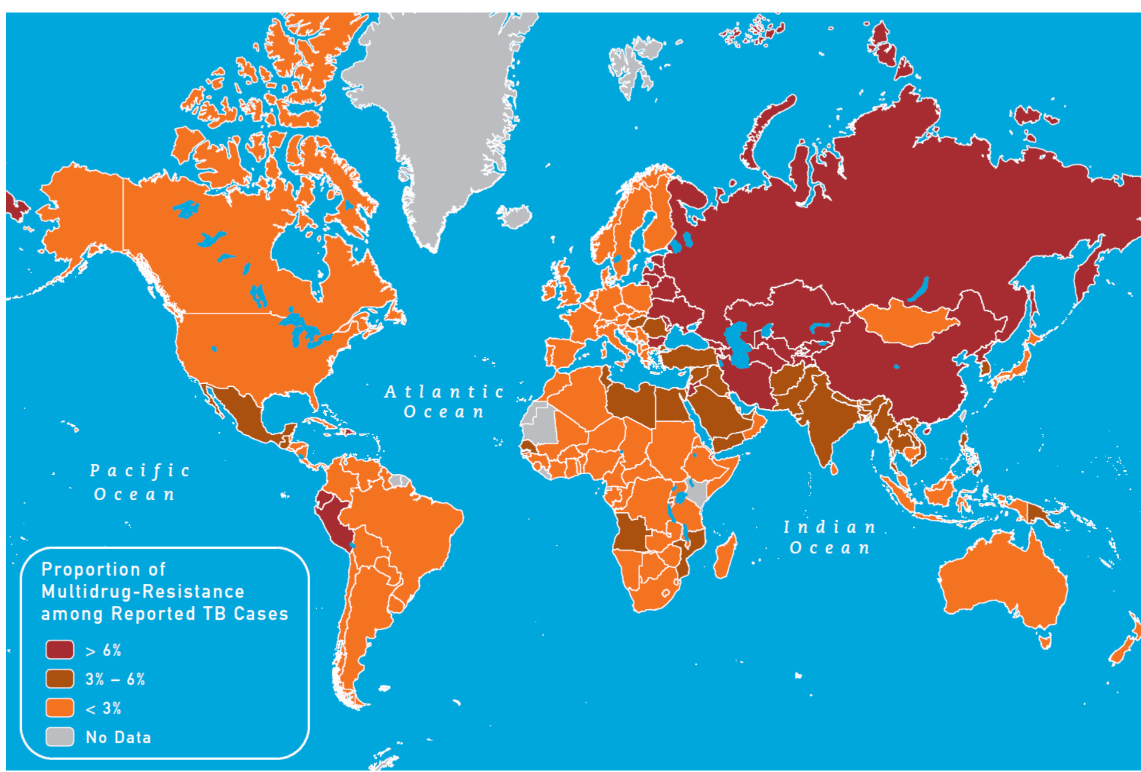

Figure 1.5: Proportion of reported multidrug resistance – TB amongst TB cases (WHO report, 2012-2013).

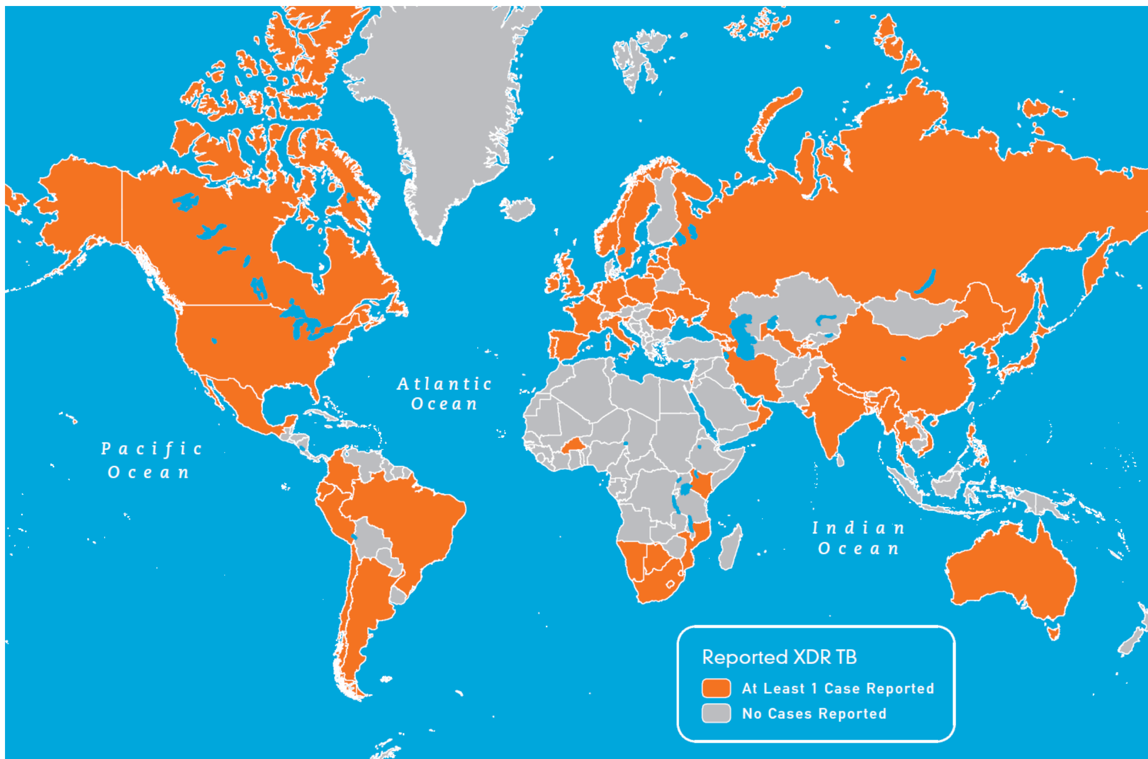

Figure 1.6: Reported extensively drug resistance – TB cases (WHO report, 2012-2013).

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1.1.3.3 Tuberculosis prevention and cure

Streptomycin was the first antibiotic agent against *Mycobacterium tuberculosis,* introduced in 1946 and isoniazid the second, introduced in 1952. Presently the first line of defense against TB, or first line drugs, consists of a combination of drugs, including streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide at different dosages for a period of six to nine months, depending on the severity of the infection (CDC, 2000). These drugs and their mechanism of actions are illustrated in figure 1.7. Although these first line drugs are very effective, they are only effective against certain strains of *M. tuberculosis*. A different drug regime needs to be administered if the patient is infected with a drug resistant strain (Multidrug resistant TB - MDR-TB). Tuberculosis drugs are also accompanied by hepatotoxicity (liver damage), which cause a loss of appetite; this effect is very undesirable in HIV/AIDS and TB co-infected patients (Tostmann *et al*., 2008). This is discussed further in section 1.1.3.5 and chapter 3. The length of treatment results into two problems. Firstly, the patients do not comply with the time schedule of treatment, thus allowing the bacteria to develop resistance to the first line drugs. Secondly, untreated patients and treated patients with active TB will increase the spread and rate of infecting healthy individuals (CDC, 2000). Thus a drug regime requiring a shorter time period could be a more effective treatment, than the one currently in place.

The symptoms of Tuberculosis include the following:

- Coughing for longer than a month
- Chest pain
- Fever
- Breathing difficulty e.g. shortness of breath
- Weakness or fatigue
- Loss of weight and appetite
- Chills and fevers (the fever may be low, and may be intermittent)
- Joint pain
- Excessive sweating, including sweating at night
- Hearing loss

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- Diarrhea
- A persistent lump or lesion

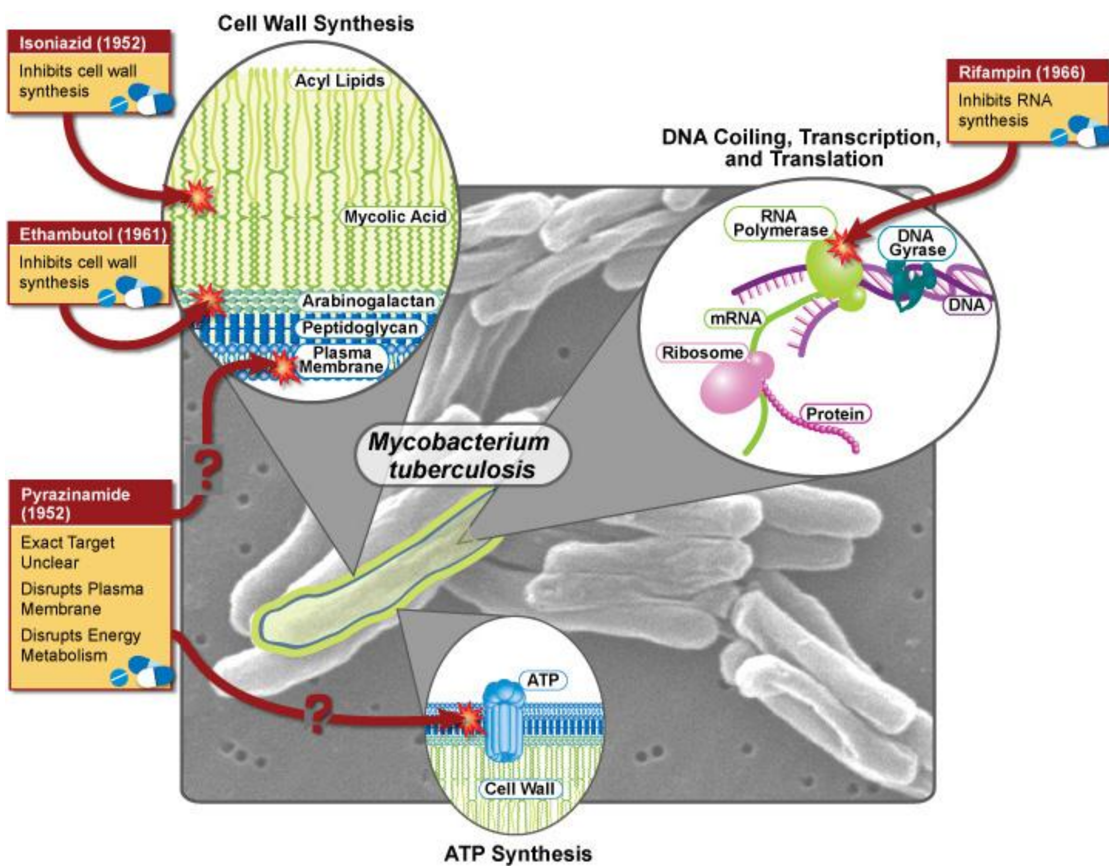

Figure 1.7: The four first-line drugs for tuberculosis in the standard regimen showing their diverse mode of actions as well as the year these four drugs were discovered (niaid.nih.gov, 2011).

1.1.3.4 Drug resistance

One of the biggest challenges associated with Tuberculosis, is the occurrence of multidrug-resistant (MDR) and extensively drug resistant (XDR) Tuberculosis. The response to this in countries with a high prevalence of TB is in general slow. In a study by Gandhi *et al*., in 2010, it was estimated that only 7% of the new cases of MDR-TB were reported to the World Health Organisation. Of these seven percent only 5% were clinically treated. The diagnosis and

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treatment regimen of drug resistant strains is substantially more costly and laborious, with a higher rate of failure.

1.1.3.5 Hepatotoxicity related to Tuberculosis drugs

During TB infection, drug induced liver toxicity, is a major problem, and may occur with all the current treatment regimens. The liver plays the central role in metabolism and detoxification of drugs, and in doing so, it is susceptible to injury. Isoniazid (6-9 months), rifampicin (4 months), isoniazid/rifampicin (4 months) as well as the two-fold treatment with pyrazinamide with either ethambutol or a flouroquinolone has shown hepatotoxicity (American Thoracic Society, 2000; Younossain *et al*., 2005). There are many factors that influence the severity of the toxic effect; these include, age, sex, cofactors, regimen and the occurrence of HIV/AIDS as well as Hepatitis B and C (Saukkonen *et al*., 2006). Although many studies have been conducted on TB drug induced injury, the precise mechanisms are not clear and more knowledge can be obtained.

1.1.3.6 Life cycle of *M. tuberculosis* and pathogenesis

The tuberculosis bacterium has evolved to cause infection in many patients but disease only in a few. As mentioned above, the majority (90 – 95%) will never develop any clinical illness or infectious sign. The human innate and acquired immunity is capable and well adapted to combat this pathogen. There are several medical conditions that increase the risk of progression to tuberculosis disease; these include: HIV/AIDS, diabetes mellitus, renal failure etc. Tuberculosis can also develop in a healthy person who doesn't have these risk factors. This is probably due to a genetic susceptibility of the infected person (Antas *et al*., 2006; Young, 1993).

M. tuberculosis is an airborne disease and the bacilli can be present in the air as droplets or nuclei, which are discharged by a person with an active infection. These bacilli can be stable and active in the atmosphere for several hours. This causes a major problem as the infection dose being as low as one to ten bacilli. Thus, transmission is an extremely efficient and effective process (Bloom, 1994). Unless an infected person receives prophylactic drugs, symptomatic disease will ultimately occur in 5-10% of infected patients. This depends on the ability of the

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Mycobacterium to survive within the macrophages, and in doing so, it is camouflaged within the host cell and evades the extracellular host defense system.

Pulmonary tuberculosis can be divided into four stages (Lurie, 1964). The infection is initiated after the inhaled *Mycobacterium* bacillus is phagocytized by the alveolar macrophages (Fig. 1.8). The primary site of infection is in most cases the middle or lower zones of the lung, although exceptions do occur. The destruction of the bacteria that have been phagocytized by the macrophages, depends on the inherent microbicidal ability of the host macrophage, as well as the virulence factor of the bacilli (van Crevel *et al*., 2002). Once the bacterium has been phagocytized, it modulates the behavior of its phagosome, not to merge with acidic, hydrolytically-active lysosomes, and in the process evades defense by the host (Armstrong and Hart, 1971; Rohde *et al*., 2007). The end of stage one culminates in the disruption of the macrophages and the attraction of monocytes and other inflammatory cells to the infection site (van Crevel *et al*., 2002).

In the second stage the attracted monocytes will differentiate into macrophages that still ingest bacilli but do not kill the bacteria. While more blood derived macrophages accumulate at the infection site, the bacteria continue to multiply. At this stage little to no tissue damage occurs (van Crevel *et al*., 2002).

The third stage begins two to three weeks after infection with the development of T-cell immunity. Antigen specific T lymphocytes arrive and proliferate within the tubercles (early lesions). These antigen specific cells activate macrophages to destroy intracellular bacteria. The logarithmic growth of the bacteria also comes to a halt in stage three (van Crevel *et al*., 2002).

The fourth stage in pathogenesis involves necrotic lesion formation and the development of the granuloma, which inhibits extracellular growth of *Mycobacteria*, resulting in stationary or dormant infection. Tuberculosis may progress under decreased immune surveillance and response. Progressive disease can happen directly after primary infection or after months to years designated as post-primary infection. Extracellular growth can also take place within liquefied

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caseous foci. Bronchi rupture can take place allowing the bacilli to spread within the lung as well as to the outside environment (van Crevel *et al*., 2002).

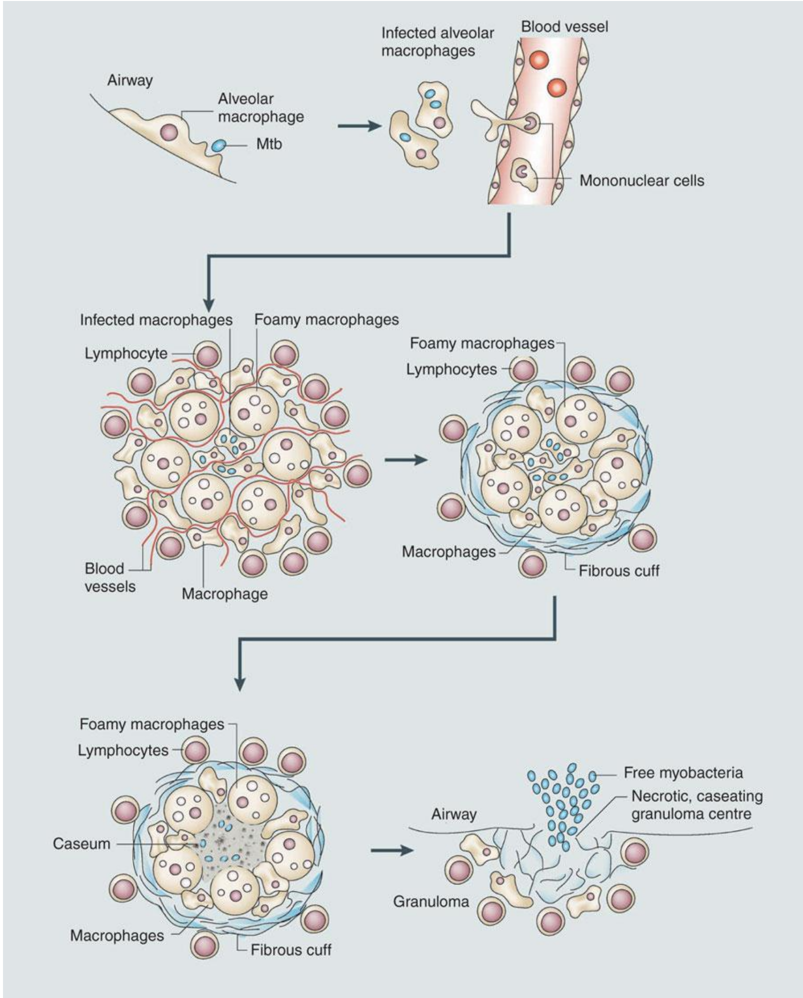

Figure 1.8: The life cycle of *Mycobacterium tuberculosis* (Russel *et al*., 2010).

There is a need to find a balance between outgrowth and killing of the bacteria to determine the outcome of the infection. The amount of tissue damage in the form of necrosis and fibrosis as well as the regeneration of tissue also influences the outcome of the infection (van Crevel *et al*., 2002).

1.1.4 Human Immunology

Although it is generally believed that the innate immune response does not play a big role in the progression of Tuberculosis, there are many evidence to support the importance of the innate

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defense mechanism. Studies have indicated the genetic susceptibility of different ethnic groups, which suggest that some individuals' innate response is more adaptable to mycobacterial infections with a higher bactericidal virulence (Stead *et al*., 1990). The innate response is also crucial for the initiation of the acquired immunity. The phagosomes play a critical role in the initiation and signaling of the T-cell response, by presenting the mycobacterial antigens to the T-cells, as well as, the expression of signal cytokines and chemokines (Lurie, 1964; van Crevel *et al*., 2002).

The containment and elimination of *Mycobacteria*, as well as, infection progression depend on the effective and efficient interaction between infected macrophages and the T lymphocytes. The occurrence of HIV and other immune suppressing diseases have shown the importance of an effective cellular immunity. One of the T lymphocyte subset, CD4+ cells, exerts its protective effect through the production of cytokines, after the stimulation and presentation of the antigen by the macrophage (Lurie, 1964). The CD8+ T lymphocytes are also involved with cytokine production with the added role of lysing infected cells (Geluk *et al*., 2000). The T cell response is for the most part antigen specific. The entire acquired response develops in the context of the major histocompatibility complex (MHC). Studies have shown that polymorphisms in the MHC may have an influence in the susceptibility of individuals to disease (Bothamley *et al*., 1989; van Crevel *et al*., 2002)

Within the T cell family there exists a functional diversity. The helper T lymphocytes can be divided into two groups: Th1 and Th2. In Tuberculosis infections, Th1 cytokines are crucial for protective immunity, while the Th2 cytokines are involved with the inhibition of activating macrophages and associated with disease progression (Fig. 1.9) (Cooper *et al*., 1997). Both Th1 and Th2 cells develop from naïve T cells, where the differentiation depends on the environment. Interleukin (IL) 12 stimulates the differentiation into Th1, while IL4 stimulates the differentiation into Th2 (Abbas *et al*., 1996). Th1 cells are characterized by the production of Interferon gamma (IFN-α) and Th2 produces IL4, but a subset of pro- and anti-inflammatory cytokines are linked to both Th1 and Th2 responses (Mosmann *et al*., 1986; van Crevel *et al*., 2002). The Th1/Th2 cytokines have been discussed in more detail in Chapter 3.

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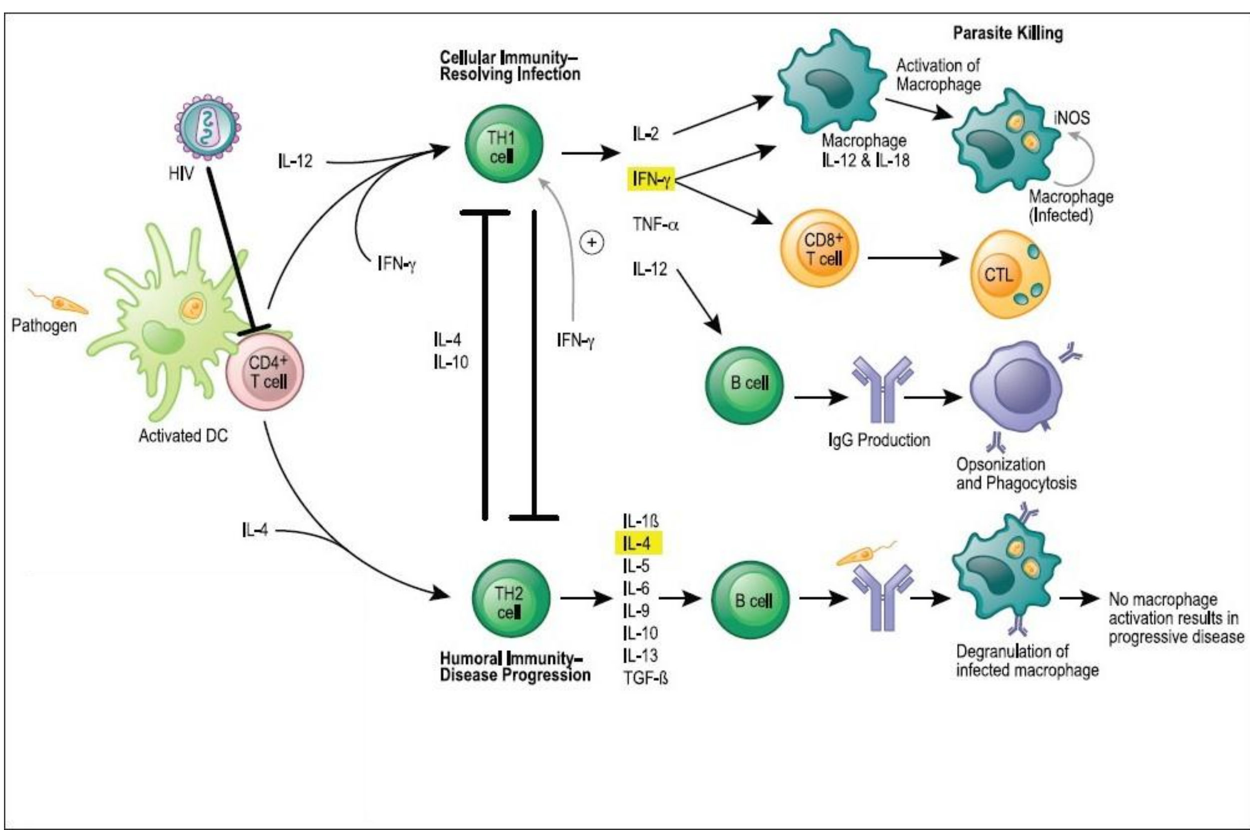

Figure 1.9: Th1 vs. Th2 response in infection. For both T helper 1 and T helper 2-cell differentiations, antigens are presented to naive CD4+ T cells by dendritic cells. In Th1-cell development, certain pathogens or pathogen-associated molecular patterns trigger antigen-presenting cells, through toll-like receptors, to secrete IL-12, which promotes the differentiation of naive T cells into Th1 cells. (Ezra *et al*., 2010).

Due to the problems identified above two sets of compounds were selected to assess their antituberculosis activity. *Allium sativum* polysulfide mixtures and quinone compounds were investigated further for their cytotoxicity, hepatoprotective and immunomodulatory activity.

1.1.5 Sample selection: *Allium sativum*

Allium sativum, commonly known as garlic belongs to the Alliaceae family. The plant forms a bulb that is consumed as food and as a medicine. The flowers are hermaphrodite and are pollinated by bees and other insects. The plant prefers well drained acidic soils, with medium light to perform at its best (Huxley, 1992). All the parts of the plant are edible, but the bulb is mostly consumed, raw or cooked with as flavoring. Garlic also has a very high nutritional value,

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and the *Allium* species are one of the world's oldest cultivated plants due to its long storage life (Fig 1.10).

Garlic has a wide range of actions; not only has it been reported that it contains antibacterial, antiviral, antifungal and antiprotozoan properties, but it also has beneficial effects on the cardiovascular and immune systems (Harris *et al*., 2001). Garlic essential polysulfide oils are the active oils of garlic which contribute towards the aforementioned biological properties. Essential polysulfide oil mixtures from *Allium sativum* were isolated and donated by Dr. Chris Hamilton, University of East Anglia, which were investigated for its biological activity in the present study.

Figure 1.10*: Allium sativum* with flowers and distinctive bulb (meemelink.com, 2012).

1.1.5.1 Essential oils of *Allium sativum*

The major amino acid present in garlic is Alliin (S-allylcysteine sulphoxide). By cutting or crushing the cloves, the enzyme allinase is released and the Alliin is reduced to a product called Allicin (diallyl thiosulphinate) (Fig. 1.11) which is a very unstable compound (Block, 1985).

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Figure 1.11: The reduction of Alliin (a) to Allicin (b) by the Allinase enzyme (Accelrys draw 4.0, 2012).

Allicin immediately starts to break down naturally. This breakdown process results in the formation of various diallyl sulphides, depending on the amount of sulfur in the structure. The chemical structures of the oil mixtures are presented in Table 1.1 (Block, 1985).

1.1.5.2 Garlic samples

All the garlic oil samples were obtained from Dr. Chris Hamilton at the University of East Anglia. Six samples were tested, named G1 to G6.

G1: Diallyl disulphide >95% Pure

Diallyl disulphide purified from "Garlic oil synthetic" by distillation (Fig, 1.12 and Fig. 1.13)

Figure 1.12: Chemical structure of diallyl disulphide (Accelrys draw 4.0, 2012).

G2 – G6: Garlic oil Synthetic

Figure 1.13: Reaction mechanism of synthesized oil (G6) containing different diallyl polysulfides, mainly diallyl monosulphide to diallyl hexasulfide (Accelrys draw 4.0, 2012).

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Garlic oils: Naturex (G2), Treatt (G3), Octavus (G4) and Stringer (G5) are all commercial garlic oils containing mixtures of diallyl mono to hexasulfide in slightly varying proportions. The main components are diallyl polysulfides for all the samples.

Table 1.1: Garlic samples showing approximate percentages of different diallyl polysulfides in the different garlic oils (based on HPLC analysis). The sum of all diallyl polysulfides was assumed to be 100% of the garlic oils.

Garlic Oils	**Naturex** (G2)	**Treatt** (G3)	**Octavus** (G4)	**Stringer** (G5)	**Synthetic oil** (G6)
DAS1	**<5**	**<5**	**<5**	**<5**	**<5**
DAS2	**30**	**20**	**15**	**25**	**5**
DAS3	**35**	**50**	**60**	**55**	**20**
DAS4	**20**	**20**	**20**	**15**	**45**
DAS5	**5**	**<5**	**<5**	**<5**	**20**
DAS6	**<5**	**<5**	**<5**	**<5**	**5**

1.1.6 Sample selection: Quinones

Quinones are a class of natural occurring phenols based on the C4-C6 skeleton. The derivatives of this compound have significant pharmacological properties. They have shown antibacterial, antifungal, antiviral, insecticidal, anti-inflammatory, and antipyretic properties (Babula *et al.*, 2007). Plants with quinones are widely used in traditional medicine to treat malignant and parasitic diseases. It has also been shown that some naphthoquinones are very effective in inhibiting and killing *Mycobacterium tuberculosis,* such as the isolated compound from *Euclea natalensis*, 7-methyl juglone (Mahapatra *et al.*, 2007).

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The quinones can be sub-divided further into different classes based on their chemical structure. These classes are the benzoquinones, naphthoquinones and the anthraquinones (Fig. 1.14).

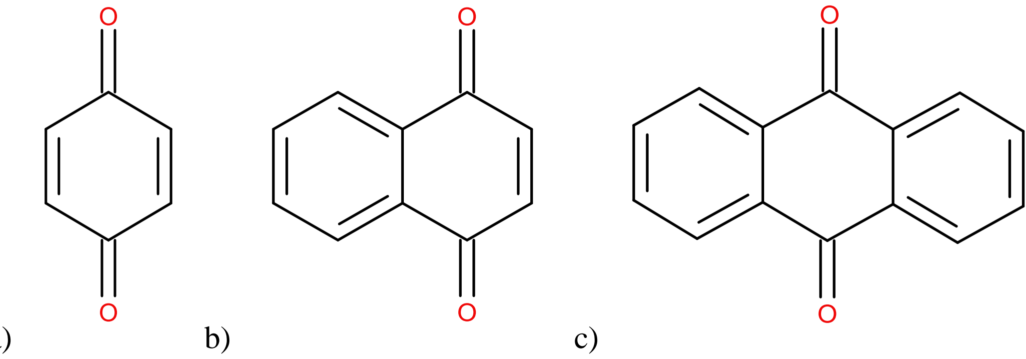

Figure 1.14: Classes of Quinones: a) Benzoquinone b) Naphthoquinone c) Anthraquinone (Babula *et al*., 2007; Accelrys draw 4.0, 2011).

The following synthesized quinone compounds were obtained from Dr. Chris Hamilton, University of East Anglia and were tested for antimycobacterial activity, cytotoxicity, immunomodulation, hepatoprotective activity and mechanism/mode of action (Table. 1.2).

Table 1.2: The chemical structures of synthesized compounds received from Dr. C. Hamilton, used in the present study.

Sample Code	Cation	Anion
IL-114	O, O, C_8H_{17}, $^{+}P-C_8H_{17}$, C_8H_{17}	N, - N, N
IL-113	O, O, C_8H_{17}, $^{+}P-C_8H_{17}$, C_8H_{17}	$F_3C-S(=O)_2-N^{-}-S(=O)_2-CF_3$

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Sample Code	Cation	Anion
IL-135	H_3C, $C_{10}H_{21}$, N^+, H_3C, C_4H_9	O, SO_3^-, O
KM-80	O, O, C_8H_{17}, N^+, CH_3, CH_3	N, N^-, N
KM-140	O, O, i-C8H17, N^+, i-C8H17, i-C8H17	F_3C, O, O, S, N^-, S, O, O, CF_3
KM-86	H_9C_4, C_4H_9, P^+, H_9C_4, C_4H_9	O, SO_3^-, ^-O_3S, O
KM-139	F, F, F, F, F, F, F, F, F, F, F, F, F, i-C8H17, N^+, i-C8H17, i-C8H17	O, SO_3^-, O
KM-60	H_3C, $C_{10}H_{21}$, N^+, H_3C, C_8H_{17}	O, SO_3^-, O

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Sample Code	Cation	Anion
KM-41	H_9C_4, C_4H_9, ^{+}N, H_9C_4, C_4H_9	O, SO_3^-, O
KM-108-1	H_3C, C_8H_{17}, ^{+}N, $H_{17}C_8$, C_8H_{17}	O, SO_3^-, O
AQS NH_4	NH_4^+	O, SO_3^-, O
KM-46	^{+}N, H_3C, C_8H_{17}	O, SO_3^-, O
IL-143	H_9C_4, $C_{14}H_{29}$, ^{+}P, H_9C_4, C_4H_9	O, SO_3^-, O
KM-52	$H_{13}C_6$, C_6H_{13}, ^{+}P, $H_{29}C_{14}$, C_6H_{13}	O, SO_3^-, O

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Sample Code	Cation	Anion
IL-142	H_9C_4, C_4H_9, ^+P, H_9C_4, C_4H_9	O, SO_3^-, O
CB-5	H_3C–N, +, N–C_4H_9	O, SO_3^-, O
KM-117	$H_{13}C_6$, C_6H_{13}, ^+P, $H_{29}C_{14}$, C_6H_{13}	O, SO_3^-, O
IL-107	O, O, i-C8H17, $^+$N—i-C8H17, i-C8H17	O, O, F_3C—S—N—S—CF_3, -, O, O
KM-108	H_3C, C_8H_{17}, ^+N, $H_{17}C_8$, C_8H_{17}	O, SO_3^-, O
KM-94	$H_{13}C_6$, C_6H_{13}, ^+P, $H_{29}C_{14}$, C_6H_{13}	O, SO_3^-, O

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1.2 Problem statement and research question

Mycobacterium tuberculosis causes Tuberculosis disease, and new drug discovery and development is of vital importance in the fight against this deadly disease. It is important to state that Tuberculosis is a worldwide problem, and an epidemic in most low-income countries. The first line drugs, although effective, cause some major problems. The Mycobacteria is showing resistance in some cases, and this will only increase with time if a solution is not brought forward. The patients, especially in developing countries, do not comply with the current regimen, thus spreading the infection and increasing resistance. Most of these first-line drugs are also accompanied and associated with liver toxicity and damage.

Many plants have been used traditionally for TB symptoms and some plants and compounds from plants, have been found to show activity against *Mycobacteria*. Some of these compounds are from the natural quinone group. Garlic is another plant that has been shown to have many biological applications. South Africa is privileged to have a massive indigenous flora, and the potential of finding new compounds in these plants is endless. Some lead compounds have already been isolated and identified as very active agents against TB. It is also known that plants have various positive effects when used as medicines; one of these effects is that plants work as immune stimulants.

The immune system of a healthy person is more than capable of handling a *Mycobacterium* infection. The development of active TB only initiates when the defenses are down or the patient has a genetic susceptibility towards the bacteria. Some plants contain the active compounds to stimulate the immune system, thus increasing the defense of the infected person.

Thus, if a compound or mixture of compounds can be identified that can treat the three problems of tuberculosis infection, patients can have a lower relapse and eventually a lower death rate (Fig. 1.15)**. The focus was to identify compounds that not only has antimycobacterial activity, but also stimulates the right immune response and protects the liver from the toxic effect of the first-line drugs.**

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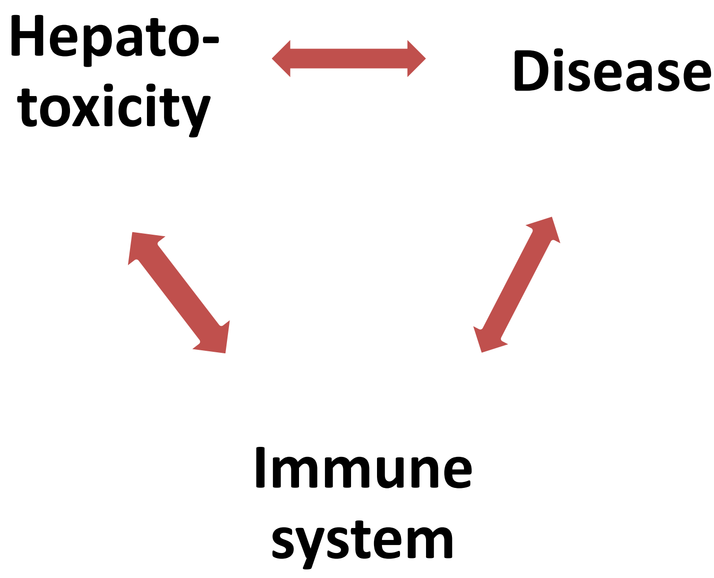

Figure 1.15: Three legs of a holistic Tuberculosis infection and disease.

1.3 Objectives

- To assess the antimycobacterial activity of the quinone compounds and garlic polysulfide mixtures.
- To investigate the toxicity of the samples on primary peripheral blood mononuclear cells and secondary human monocytes U937.
- To determine if the samples have any immune stimulatory effect based on its Th1/Th2 cytokine response.
- To investigate if the samples have any hepatoprotective activity to aid in the metabolism of toxic compounds.
- To establish if the compounds have any activity on Glutathiol/Mycothiol reductase and Thioredoxin reductase enzymes as part of a mechanism study.
- To determine if there is any relation between the structure and the activity of the sample.

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1.4 Methodology

1.4.1 Antimycobacterial activity

The antimycobacterial assays were done according to the microtitre alamar blue assay (MABA) (Franzblau *et al*., 1997) and a new adapted method utilizing Presto blue was also developed (Lall *et al*., 2013). The *M. tuberculosis* H37Rv strain was utilized. The assay is based on the reduction of resazurin, a blue dye to the red/pink resorufin dye by viable bacteria. The amount of converted red dye is proportional to the amount of living bacteria in the assay.

1.4.2 Cytotoxicity

Peripheral blood mononuclear cells (PBMC's) as primary cells and U937 monocytes as secondary cells were used to test the cytotoxic effect of the samples. Presto blue and 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) viability assays were conducted to determine the inhibitory concentration where 50% of the cell growth was inhibited(IC_{50}).

1.4.3 Immunomodulatory activity

The healthy human immune system is more than capable of combating a *Mycobacterium* infection. The immune response, relating to the cell signaling, occurring after the infection, is well adapted. Cytokines are produced by different cells in the immune arsenal. These cytokines are involved not only in signaling an inflammatory innate immune response, but also in an adaptive acquired immune response.

The samples were tested for their ability to activate the production of cytokines. Peripheral blood mononuclear cells were treated with the samples and the cytokine production was measured using cytometric bead array (CBA) as well as an enzyme-linked immunosorbent assay (ELISA). The (CBA) assay is specifically developed to measure Th1/Th2 cytokine production.

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1.4.4 Hepatoprotective activity

The first line Tuberculosis drugs, although effective, have certain limitations; one of them is the toxicity related to the metabolism of the antibiotics in the liver. Major liver damage can be seen in patients currently on the conventional treatment.

The samples were evaluated for their hepatoprotective activity on toxicity-induced liver cells. A toxic environment was induced using acetaminophen.

1.4.5 Enzymology – Mode of action

Enzyme investigation was done to assess the potential of the above mentioned compounds' possible mode of action or mechanism by which they affect the bacteria.

Glutathiol is the major thiol present in almost all eukaryotes. The reduced form of Glutathiol disulfide is a low molecular weight thiol, and it is mainly concerned with the protection of cells against oxidative stress and reactive electrophiles (Newton and Fahey, 2002). Mycothiol is the bacterial analog, with the same activity in *Mycobacteria* as the glutathiol in other organisms (Newton and Fahey, 2002; Rawat and Av-Gay, 2007). Thioredoxin is a major enzyme reducer, and is present in most living organisms. Two classes of thioredoxin reductase enzymes have been identified, one present in animals and the other present in bacteria and in some other eukaryotes.

The samples were tested on these enzymes, for subversive substrate activity. In the case of subversive substrate activity, the compound/substrate will bind to the enzyme and produce a product known as a semiquinone superoxide radical that is harmful to the bacteria.

1.5 Scope of dissertation

Chapter 1: Introduction, Problem statement and Scope

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Chapter 2: Antimycobacterial Activity and Cytotoxicity of Quinones and Garlic polysulfide samples

Chapter 3: Immunomodulation and Hepatoprotective activity of Quinones and Garlic polysulfide samples

Chapter 4: Mechanism of action on Glutathiol, Mycothiol and Thioredoxin reductase of Quinones and Garlic polysulfide samples

Chapter 5: Final Conclusion and Recommendations

1.6 Aim

The aim of the project was to study these compounds and polysulfide oil mixtures on a broad spectrum of biological activities related to Tuberculosis infection. To identify if these compounds can be considered as antituberculosis drugs, indicating hepatoprotective activity and a good immune modulating agent in addition to their significant inhibitory activity against *Mycobacterium tuberculosis* via targeting mycothiol reductase and thioredoxin reductase.

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Chapter 2

Antimycobacterial Activity and Cytotoxicity of Quinones and Garlic polysulfide samples

Twenty quinones and six garlic oil polysulfide mixtures were tested for their *in vitro* antimycobacterial activity and cytotoxicity. The antimycobacterial activity was tested on the H37Rv *Mycobacterium tuberculosis* strain and the cytotoxicity test was done on primary peripheral blood mononuclear cells and secondary U937 human monocytes. Six quinone samples showed antimycobacterial activity, with a minimum inhibitory concentration less than 3.125 µg/ml and one garlic sample had a minimum inhibitory concentration of 2.5 µg/ml. Samples KM 108-1 and IL 143 had a selective index above 8 and KM 140 had a selective index of 55.

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Chapter 2 - Antimycobacterial Activity and Cytotoxicity of Quinones and Garlic polysulfide samples

The two groups of compounds Quinones and Garlic oils were tested for their efficacy against *Mycobacterium tuberculosis* and toxicity on primary and secondary human cells.

2.1 Anti-mycobacterial activity against *Mycobacterium tuberculosis* H37Rv using MABA and MPBA

2.1.1 Test organism

The *Mycobacterium tuberculosis* (MTB) organism was first discovered and isolated in 1882 by Robert Koch. It lives in aggregate layers of cells attached to each other in a community called a biofilm (Brown-Elliot and Wallace, 2002). The bacilli shaped bacterium belongs to the family Mycobacteriaceae and the genus *Mycobacterium*, and like other *Mycobacteria* it is also classified as a Gram-positive bacterium (Megehee and Lundrigan, 2007).

Figure 2.1: Colonies of *Mycobacterium tuberculosis* grown on Lowenstein-Jensen media for 3-4 weeks. (http://www.bacteriainphotos.com/Mycobacterium%20tuberculosis.html, 2013).

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Although *M. tuberculosis* is considered as a gram-positive bacterium, it has some unique qualities that are divergent from most gram-positive bacteria. Its cell wall contains waxy mycolic acids; long, branched, fatty acids that are normally present in acid-fast bacteria, making the cells impervious to a gram stain. These mycobacteria are the etiological agent causing mainly tuberculosis in mammalian respiratory systems. The disease can also spread away from the main infection site (lungs) and cause extra-pulmonary tuberculosis or miliary tuberculosis.

This bacterium can be grown in any laboratory in a liquid (Middelbrook 7H9) or on a solid media (Middelbrook 7H11, lowenstein-jensen medium) (Fig. 2.1).

2.1.2 Materials and methods

The microtiter alamar blue assay was first described by Scott G. Franzblau in 1997, and is a colorimetric assay. It is based on the reduction of the blue resazurin salt by viable bacteria to a red resorufin dye. The intensity of the red dye can be correlated back to the cell concentration within the assay. This assay has been used to determine the minimum inhibitory concentration (MIC) of the samples on *M. tuberculosis*. During the course of the study a new alternative method was developed using Presto blue as the viability indicator (Appendix 1). Presto blue is also a resazurin based compound that can be used to indicate viable cellular growth (Fig. 2.2). The time required to convert resazurin to resorufin in Presto blue, by viable bacteria, is much faster than in Alamar blue (Lall *et al*., 2013)

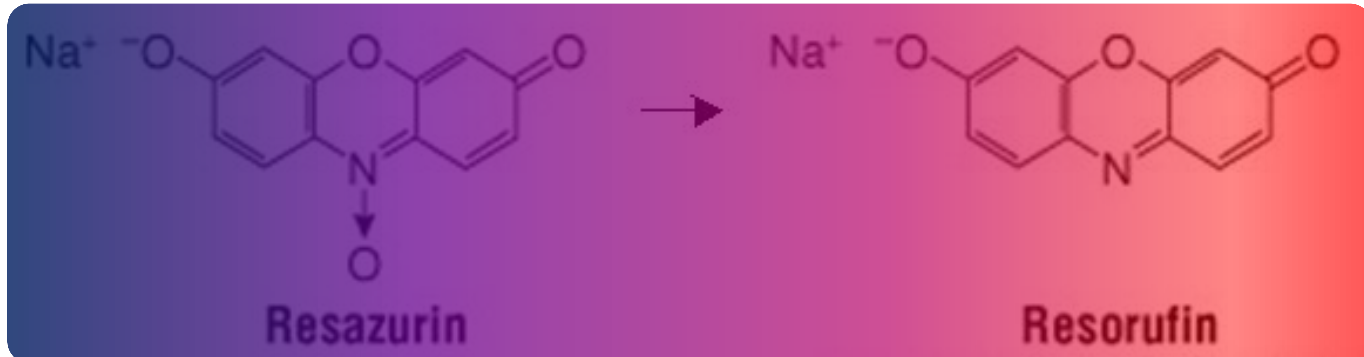

Figure 2.2: Conversion of resazurin to resorufin by viable bacteria (bmglabtech.com, 2011).

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2.1.2.1 Microbial strain: *Mycobacterium tuberculosis* H37Rv

The microorganism, maintained in complete 7H9 broth (2% Glycerol, 10% OADC and 2% PANTA), was supplied by the national health laboratories at the university. The *Mycobacterium tuberculosis* H37Rv (ATCC 27264) strain was used in the study. H37Rv is a non-clinical, non-resistant laboratory strain used worldwide in TB research.

2.1.2.2 Preparation of inoculum

Bacteria inocula were cultured on LJ media as well as in 7h9 broth supplemented with 10% OADC, 2% Glycerol and 2% PANTA (antibiotics to prevent the growth of other micro-organisms), for two to three weeks at 37°C until logarithmic growth phase was reached. From the three week old active culture, a McFarland standard # 1 was prepared in 7h9 broth, and diluted 100 times to reach a colony forming unit (CFU) of $3x10^6$ cfu/ml. The growth of *M. tuberculosis* was confirmed using a TB Ag MPT64 device obtained from KAT laboratory and medical Pty ltd (Fig. 2.3) (cat# SD-MPT64). Briefly, the test device was removed from the foil pouch and 100 µl of the cultured broth was transferred into the sample well, located on the strip. The test device was left at room temperature in a biosafety cabinet for ten minutes for the reaction to take place. Two lines indicate a positive reaction, while one line indicates a negative reaction. The culture was only used if a positive reaction was obtained.

Figure 2.3: The TB Ag MPT64 device showing a positive indication of *Mycobacterial tuberculosis* growth.

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2.1.2.3 Preparation of samples and controls

The samples were dissolved in 100% dimethyl sulfoxide (DMSO) and maintained at room temperature for at least one hour. These samples were further diluted to their final concentration with fresh culture broth. The final concentration of DMSO in all the assays was 2.5% or less, which is nontoxic to mycobacteria (Molina-Salinas *et al*., 2006.)

A DMSO solvent control was added to the assay. Solvent controls were prepared fresh for each experiment. Bacteria and media controls were also included in the assay.

The antituberculosis drugs isoniazid (INH) and ethambutol (EMB) were obtained from Sigma and used as the positive controls. Stock solutions of INH and EMB were prepared in sterile distilled water. The stock solutions were diluted in complete 7H9 broth to four times the maximum desired final testing concentrations prior to the addition to the 96-well microplates. Drug controls were prepared fresh before each experiment on the day of the experiment.

2.1.2.4 Experimental Method

The compounds were assayed in triplicate. All tests were carried out in sterile flat-bottomed 96-well microplates. Perimeter wells were filled with sterile distilled water to prevent or limit evaporation of the media during incubation. To the remaining test wells, 100 µl of freshly prepared 7H9 medium, supplemented with 2% glycerol, 10% OADC and 2% PANTA, was added. The working compound mixtures (100 µl) were added to the first well in each row, from which twofold serial dilutions were made in 7H9 broth. The final concentrations of these preparations ranged from 0.781 to 400 µg/ml for the samples, 0.125 to 4.0 µg/ml for INH and 0.3125 to 10 µg/ml for EMB (Appendix 2).

One hundred microliters of the prepared *M. tuberculosis* culture inoculum ($3x10^6$ cfu/ml) (section 2.1.2.2) was added to each well, yielding a final volume of 200 µl. A drug free inocula

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control was also included in the experiment as well as a medium, drug and solvent control. The plates were sealed with parafilm, and incubated for 7-10 days at 37°C on a rotation table to limit the formation of bacteria biofilm complexes.

After 10 days of incubation, 40 µl of a 1:1 alamar blue:10% Tween 80 or 40 µl of PrestoBlue:10% Tween 80 (1:1) solution was added to one growth control well. Tween 80 was used to limit the clumping effect as this has been shown to inhibit biofilm production. The plates were re-sealed and incubated for 24 hours at 37°C. Once the colour change was observed from blue to pink in the growth control sample after 24 hours, the reagent mixture was added to all the remaining wells (if it remained blue the plate would be incubated for another 24 hours). The plates were resealed and incubated for an additional 18-24 hours for the colour change to occur. A blue colour indicated no growth and a pink colour indicated growth of the bacteria (Fig 2.4). The MIC was described as the lowest concentration where no colour change from blue to pink occurred.

All the experiments were done in a Bio-safety level 2 laboratory at the National Health Laboratory Services at Prinshof Campus, University of Pretoria. Four independent studies in triplicate were performed and three out of the four experiments were used to calculate the average activity.

2.1.3 Results and Discussion

Good growth of *M. tuberculosis* (H37Rv) was observed in the 7H9 broth media within 4-6 weeks. Seven compounds out of 26 samples inhibited the growth of *M. tuberculosis* at a concentration lower than 5 µg/ml. Sample KM-140 (quinone sample) showed the highest activity with an MIC of 0.7 µg/ml, while G4 (garlic sample) showed the highest activity, with an MIC of 2.5 µg/ml.

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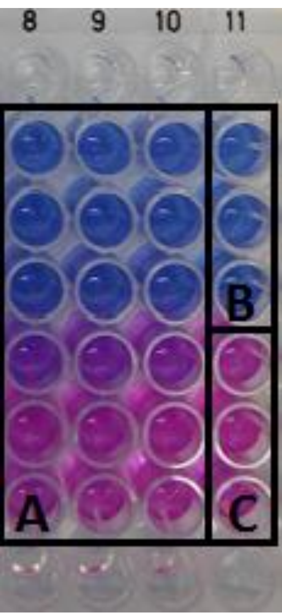

Figure 2.4: Presto blue as a growth indicator of *M. tuberculosis* (H37Rv) A: Color change of presto blue into the red/pink color indicating viable metabolically active mycobacterium cells. B: Media control containing only 7H9 media, no color change indicates no metabolically active cells and no contaminating microorganisms. C: bacteria control indicating viable bacteria.

Table 2.1: Minimal inhibitory concentrations (MIC in µg/ml) of the studied compounds and reference antibiotics, on *M. tuberculosis* H37Rv.

Code	MIC [a] (µg/ml)
Quinone samples	
AQS-NH_4	25
CB-5	200
IL-107	25
IL-113	400
IL-114	400
IL-135	50
IL-142	200
IL-143	3.125
KM-108	1.562
KM-108-1	1.562
KM-117	6.25
KM-139	6.25
KM-140	0.781
KM-41	200

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KM-46	200
KM-52	6.25
KM-60	3.125
KM-80	1.562
KM-86	200
KM-94	6.25
Garlic oils	
G1	1000
G2	6.25
G3	6.25
G4	2.5
G5	20
G6	50
Isoniazid*	0.25
Ethambutol*	1.25
Media	+
Bacteria	+

[a]Minimum inhibitory concentration on *M. tuberculosis*
[*]Reference antibiotics, Isoniazid and Ethambutol

The best antimycobacterial samples were found to be IL-143, KM-108, KM-108-1, KM-140, KM-60, KM-80 and G4 with MICs less than 3.125 µg/ml. This is comparable to the MICs of the some existing TB drugs, such as ethambutol that showed a MIC of 2 – 8 µg/ml (Franzblau *et al*., 1998). In a previous study the activity of 7-methyljuglone, a naphthoquinone isolated from *Euclea natalensis*, was reported to have an MIC of 0.5 µg/ml, which correlates with the KM samples (Lall *et al*., 2005). The KM samples showed better activity than the IL samples and the Garlic polysulfide oils showed relatively good inhibition with G4 showing the lowest MIC of 2.5 µg/ml. Polysulfide oils extracted from Alliaceae plants also showed a significant decrease in bacterial numbers in previous studies (Yin *et al*., 2003). In another study done by Munchberg *et al*. in 2007 it was also observed that diallylsulfide, sample G1 in the present study, showed the lowest antibacterial activity. This directly correlates with the results in Table 2.1.

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Results obtained in the present study correlated with quinone derivatives that were tested for their antimycobacterial activity, where it was reported that the quinones had MICs as low as 12.5 µg/ml (Tran *et al*., 2004). In the same study by Tran *et al*., (2004), benzoquinone showed a higher antimycobacterial activity as compared to anthraquinone and naphthoquinone.

In the present study it was found that the anthraquinone derivative KM-140 showed higher activity than the benzoquinones KM-117, with MICs of 0.789 µg/ml and 6.25 µg/ml respectively. This could probably be explained by the difference in attached moieties, with the anthraquinone KM-140 having a nitrogen-sulphur group attached that could increase the activity of the anthraquinone.

The size of the alkyl groups on the phosphonium salts is important (IL-143, IL-142, KM-52, KM-117, KM-94). It appears that the longer the alkyl chains on the phosphonium cation, the higher the potency (MIC of KM-52 < IL143 < IL-142). Similar trends also appeared for the quaternary ammonium cations (MIC of KM108 = KM108-1 and KM41 = KM 86, but longer alkyl chains are more potent. The relative potency trend on *M. tuberculosis* is generally consistent (Table 2.1).

It appears that the cation of the compound is responsible for the antimycobacterial activity of this series of compounds as there are several samples where the cation has been shown to have activity regardless of counter-anion. There are several samples where compounds that have the same anion displayed very large differences in MICs due to the presence of the specific cation.

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2.2 Cytotoxicity on primary and secondary cell lines

Toxicity testing is a laboratory practise necessary to determine if a compound has any *in vitro* toxicity. This is also an aspect of the preliminary safety evaluation. Cytotoxicity for many different compounds has been tested using certain types of cell lines. The cells used in these assays are either primary or secondary cell lines, including liver, skin, blood and many other types of cells (Fellows and O'Donovan, 2007).

These toxicity assays are conducted to determine the susceptibility and threshold of the cell-line to the foreign or xenobiotic substance. It should be able to provide information on the dose response of the compound and the possible dose range for human exposure. It is important to establish the toxic threshold, if further studies are required at pre-clinical and clinical levels. Cytotoxicity testing is a valuable and inexpensive alternative approach for short term toxicity testing.

The cytotoxic values were used in the Immunomodulatory as well as the Hepatoprotective experiments to carry out the assays at concentrations that will not have any lethal effect on the cells used.

2.2.1 Primary cell line – Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation using the methods; "Helmholtz Zentrum Munich CCG Immune Monitoring Protocol: PBMC Isolation, cryopreservation and thawing". The blood samples were collected from a healthy volunteer and the same volunteer was used in the replicate blood collection and testing.

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2.2.2 Secondary cell line – U937 monocytes

Monocytes are small nuclear blood cells that play a crucial role in the immune response, and monocyte cell lines are available to test the toxicity profile of compounds. One of these cell lines is designated as U937 human monocytes isolated from a histiocytic lymphoma. These cells are activated by a specific molecule and differentiate from monocytes to macrophages. This assay involves the incubation of the viable macrophages test model, with the test compound, and it is based on a tetrazolium colorimetric assay (Mosman, 1983).

2.2.3 Mechanism of toxicity – Quinones & Garlic compounds

The group of compounds called Quinones can be found in respiring eukaryotic animals as well as in plants. Many of the dyes used traditionally are natural quinones (Romanova *et al*. 1977). These compounds are used in the pharmaceutical industry for various purposes, for example as anticancer, antifungal and antibacterial drugs. The toxicity of these compounds can be caused directly by the compounds or in many cases by the metabolism of/with other drugs and environmental toxins. The mechanism of toxicity has been identified in rapidly dividing cells as well as non-dividing cells.

In cancerous cells it is believed that toxicity is due to the DNA alterations and modifications. In the non-dividing group of cells, toxicity can be attributed to the alkylation of essential proteins; especially targeting the thiol or amine groups. The toxicity can also be brought about by the increase in oxidative stress due to the formation of activated oxygen species (O'Brien, 1991).

These compounds are reduced by either reductase enzymes or by auto-oxidation and result into semiquinone radicals which in turn reduce oxygen into superoxide radicals, causing a redox cycling oxidation within the cells (O'Brien, 1991; Shultz and Bearden, 1998).

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Diallyl disulfide, from garlic, is an efficient agent for detoxication of the cells. It significantly increases the production of the enzyme glutathione S-transferase (GST), which binds to toxins in the cell. Therefore, garlic supports the detoxification function of liver cells *in vitro* and protects nerve cells from oxidative stress (Germain *et al*., 2003). The other diallyl sulfides have not been studied as intensely for toxicity as their disulfide counterpart.

2.2.4 Materials and methods

2.2.4.1 Isolation of Peripheral blood mononuclear cells

Peripheral blood mononuclear cells, or PBMCs, were isolated from whole blood samples using Ficoll with a density gradient of 1.077g/ml (Appendix 3). Blood was collected from a healthy volunteer. The blood from the EDTA tubes was transferred into a 50ml falcon tube, and diluted 1:1 with incomplete RPMI media. Half of the volume of blood was determined and that amount of Histopaque®-1077 (Obtained from Sigma Aldrich cat# 10771) was added to a fresh sterile 50ml falcon tube. The diluted blood was layered on top of the Histopaque®-1077 and centrifuged at 3000rpm for 30min at room temperature. The central cloudy layer of PBMCs was carefully removed using a pipette, and transferred into a new 50ml falcon tube (Fig 2.5). The removed cells were washed with incomplete RPMI media and centrifuged again at 2200rpm for 10min at room temperature. The supernatant was removed and approximately 5ml of ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA at a pH of 7.2) was added to lyse the contaminating erythrocytes. The ACK was deactivated by the addition of 15-20ml of incomplete RPMI and centrifuged at 1200rpm for 10min at room temperature. The supernatant was removed and the cells were resuspended in 5ml complete RPMI (RPMI + 10% FBS) and counted using a hemocytometer with a light microscope.

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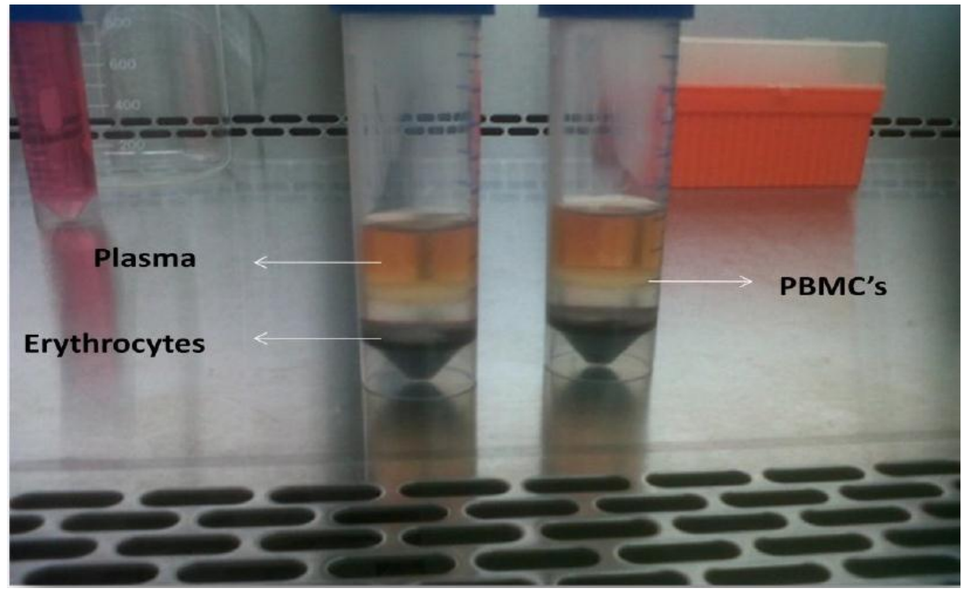

Figure 2.5: Histopaque®-1077 layered blood after centrifugation at 3000rpm, showing the white cloudy layer in the middle.

2.2.4.2 Differentiation of U937 cells to activated macrophages

The cytotoxicity assays were conducted with the help of Ms. Smeetha Singh at the University of Pretoria

The U937 cells (undifferentiated, ATCC obtained from Highveld Biological (Pty) Ltd, Sandringham, South Africa) were maintained in RPMI 1640 medium, with a pH of 7.2, supplemented with 10% heat activated foetal bovine serum (FBS), 2mM L-glutamine and a 0.1% antimicrobial solution (penicillin, streptomycin, and an antifungal, fungizone). Reagents were obtained from Highveld Biological (Pty) Ltd. The sub-culturing of the cells was done every 2-3 days after a confluent monolayer had formed. During the sub-culturing cells that had attached to the culture flask, were trypsinized (0.25% trypsin, containing 0.01% EDTA) for 10 min at 37°C, and stopped by the addition of complete medium. Cells were grown to a density of $5x10^8$ cells/ml, centrifuged and washed with phosphate buffer saline (PBS) solution. The cells were counted with a hemocytometer using a light microscope, to establish the cell concentration and were re-suspended in the correct amount of supplemented RPMI 1640 medium. A set of cells were treated with phorbol 12-myristate 13-acetate (PMA, 0.10ug/ml; Sigma), and 200 µl were plated on a 96-well plate and incubated for one day at 37^0C in a humidified atmosphere of 5%

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CO_2 to stimulate differentiation of the monocytes to mature macrophages (Passmore *et al*., 2001; Hosoya and Marunouchi, 1992).

2.2.4.3 Cytotoxicity assay

A series of two-fold dilutions (200 µg/ml to 0.78125 µg/ml) of the compounds were prepared in DMSO, and the cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-H-tetrazolium hydroxide (XTT) method. The dilutions were added to the inner wells of the microtitre plate and incubated for 72 hours. The outer wells contained 200 µl of sterile distilled water to limit evaporation of the medium in the test wells.

After 72 hours, 50 µl of XTT reagent (1.0 mg/ml XTT with 0.383 mg/ml PBS) was added to the wells and the plates were incubated for 1-2 hours. A positive drug, Actinomycin D (Sigma), at a final concentration range of $5.0x10^{-2}$ to 3.9×10^{-4} µg/ml was included. After the incubation of 1-2 hours, the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader (PowerWave XS, Bio-Tek), which measured the OD at 450 nm with a reference wavelength of 690 nm. DMSO (0.04%) was added to serve as a solvent control. GraphPad Prism software (4.03) was utilized to statistically analyse and determine the 50% inhibitory concentration (IC_{50}) values.

The toxicity assay was repeated using Presto blue as the viability indicator and similar results were obtained.

2.2.5 Results and Discussion

Cytotoxicity of the compounds was evaluated in cultured macrophages and isolated PBMCs by the XTT (for U937 monocytes) and Presto blue assay (for PBMCs).

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Selective index is the representation of the selectivity of the samples for antibacterial activity over toxicity. Selective index was determined as follows:

$$SI = \Delta IC50/\Delta MIC$$

Table 2.2: Cytotoxicity activity of the compounds against U937 cell line and isolated PBMCs

Code	IC_{50} PBMC [a]	IC_{50} U937 [b]	±SD [c]	SI (PBMC)[d]	SI (U937)[e]
		Quinone samples			
AQS-NH	3.23	5.6	1.66	0.129	0.224
CB-5	54.21	61.35	7.05	0.271	0.306
IL-107	2.815	6.81	0.164	0.112	0.272
IL-113	45.4	78.36	6.03	0.113	0.195
IL-114	24.93	21.68	0.73	0.062	0.054
IL-135	4.436	28.67	0.32	0.088	0.573
IL-142	84.81	105.36	3.06	0.424	0.526
IL-143	1.322	26.35	0.11	0.423	8.432
KM-108	0.9075	3.113	0.12	0.58	1.992
KM-108-1	0.1164	12.55	0.11	0.074	8.032
KM-117	NT *	1.65	0.09	NT *	0.264
KM-139	7.443	23.21	0.51	1.19	3.713
KM-140	0.366	43.01	0.47	0.469	55.05
KM-41	117.9	120.5	10.89	0.589	0.602
KM-46	87.99	92.35	11.74	0.439	0.461
KM-52	1.088	5.63	0.24	0.174	0.9
KM-60	1.561	2.89	0.02	0.499	0.924
KM-80	12.33	19.7	0.67	7.891	12.6
KM-86	114.3	180.3	8.16	0.571	0.901
KM-94	NT *	3.56	0.98	NT *	0.569
		Garlic oils			

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Code	**IC_{50} PBMC [a]**	**IC_{50} U937 [b]**	**±SD [c]**	**SI (PBMC)[d]**	**SI (U937)[e]**
G1	37.97		11.33	0.03797	
G2	4.276		0.33	0.68416	
G3	6.037		0.17	0.96592	
G4	0.2929		0.25	0.11716	
G5	1.392		0.13	0.0696	
G6	1.807		0.16	0.03614	

[a]Fifty percent inhibitory concentration on PBMCs
[b]Fifty percent inhibitory concentration on U937
[c]Standard deviation
[d]Selective index on PBMCs = IC_{50}(PBMC)/MIC
[e]Selective index on U937 = IC_{50}(U937)/MIC
[*]Not determined, due to too high toxicity

The highest cell survival rates were observed for cultures treated with KM-80, KM-140, KM-108-1 and IL-143.

Most of the samples showed an IC_{50} of medium to high toxicity, and in some cases, very high toxicity to the tested cells. An IC_{50} of lower than 20 µg/ml is considered medium toxic and an IC_{50} of lower than 5 µg/ml is considered toxic. From Table 2.2 it is clear that some of the samples did not show toxicity while others had high toxic effects. When comparing the toxicity to the activity, a relation can be established. The selectivity of the compounds is a comparison of activity over toxicity. The higher the SI, the more the sample targets bacteria as compared to cells. A selectivity of 10 and higher is desired.

Of all the samples, only one had a selectivity of higher than ten. KM-140 showed a selectivity of 55 on U937 cells. Three samples had a selectivity index between 5 and 10, IL-143 and KM-108-1 with a selectivity of 8 on U937 cells and KM-80 with an SI of 7.8 on PBMCs. Previous studies done on quinones have also indicated a medium to high cellular toxicity with some samples showing a higher selectivity than others (Beall *et al*., 1995). The garlic samples did not show

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promising results, with selective indexes below 1 for all the samples tested. Diallyl sulfides have shown moderate to low toxicity in previous studies (Germain *et al*., 2003).

The results in the present study also relate to previous toxicity reports. A study conducted by Mavundza *et al*., in 2010 found that the IC_{50} values of the tested compounds isolated from *Athrixia phylicoides* ranged from 2.6–81.38 µg/ml. The ranges of the IC_{50} values are similar between the two studies. In a similar study, the Isoquinolinequinone compounds show lower IC_{50} values. In this study the toxicity ranged from 0.26-10.67 µg/ml on primary and secondary cell lines (Delgado *et al*., 2012). Thus, compounds have a range for toxicity levels, and it is the selectivity of the compound that distinguishes a good sample from a bad one.

2.3 Conclusion

Seven compounds showed good antimycobacterial activity. Among the quinones, six samples showed an MIC less than 3.125 µg/ml. The others showed higher MICs between 6.25 to 1000 µg/ml. One garlic sample showed an MIC of 2.5 µg/ml, the others showed much higher activities with MIC's at 1000 µg/ml.

The samples with good antimycobacterial activities showed relative toxicity on PBMCs and U937 cells. The selective index was found to be 7.8 for KM-80 on PBMCs and 8 for KM-108-1 and IL-143 on U937 and 55 for KM-140 on U937 cells.

The samples were further tested for their hepatoprotective and immune modulatory activities in order to substantiate their potential as antimycobacterial agents.

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2.4 References

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Chapter 3

Immunomodulation and Hepatoprotective activity of Quinones and Garlic polysulfide samples

Twenty quinone samples and six garlic polysulfide mixtures were evaluated for their immune stimulatory effect as well as their liver protective activity. The immune response was assessed by evaluating the Th1/Th2 cytokine production levels by peripheral blood mononuclear cells on exposure with various concentrations of the samples. Liver cells (C3A hepatocytes) were used to assess the hepatoprotective efficacy of the samples on the toxic effect of acetaminophen. Three quinone compounds showed an immune stimulatory effect (KM94, KM108-1 and KM117), and five showed a hepatoprotective effect (KM108, KM140, AqsNH4, CB5 and IL107) of 60-100%. Garlic samples G5 and G6 showed a slight immune stimulatory effect, while G6 was the only sample to have hepatoprotective activity, probably due to the higher amount of diallyl tetrasulfide within the mixture.

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Chapter 3 – Immunomodulation and Hepatoprotective activity of Quinones and Garlic polysulfide samples

A few samples showed good selectivity for *Mycobacterium tuberculosis* and an attempt was made to establish if the samples can also be useful in stimulating the immune response an protecting the liver from toxicity. The quinones and garlic oil mixtures were assessed for their immune modulatory effects as well as their hepatoprotective activities.

3.1 Cytokine production using Peripheral blood mononuclear cells

3.1.1 Immune response – TB patients

It is estimated that a third of the world's population is infected with TB. Of these individuals only a small percentage develops active Tuberculosis. This suggests that the human immune response is in general successful in containing the pathogen. The percentage of people who do get active Tuberculosis probably get it due to either, the lack of initiation of an appropriate immune response or genetic susceptibility. Patients with latent infections can also develop active Tuberculosis if the immune system is compromised due to another disease or condition. Other factors such as, drug and alcohol abuse, aging and treatment with cortico-steroids, can also bring about the reactivation of latent infections (Flynn and Chan, 2001).

The complex immune reaction of the human body to the *Mycobacterium* bacilli is regulated by various factors. These include the cellular immune system and the communication between them through a variety of inflammatory mediators. The better this system works the better the body is capable of containing and fighting the infection. The immune cellular response encompasses the activation of T-helper cells, and the major subsets of CD4+ cells, Th1/Th2 cells. These subsets play different roles and produce different signals in the immune response for the recruitment and activation of other monocytes and macrophages (Mortellaro *et al*., 2009; Schluger and Rom, 1998).

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Th1 cellular response is important for macrophage stimulation and activation, cell mediated reactions for resisting infection, as well as delayed-type hypersensitivity and cytotoxicity. The cytokines most often associated with the Th1 response are Tumor necrosis factor-α (TNF-α), Interleukin 12 (IL 12) and Interferon-γ (IFN-γ). IFN-γ specifically activates and stimulates macrophages to phagocytize and destroy *Mycobacteria*. Th2 response on the other hand is more related with the production of antibodies, and antibody responses in the extracellular fighting arsenal. The cytokines associated with the Th2 response include Interleukins 4, 5, 6, 10, and IL 13 (Coffman and Romagnani, 1999).

Th1 and Th2 work on an inhibitory basis on each other. Examples of this are, IL 10 (Th2) that inhibits the activation and development of Th1 cells, by acting on antigen-presenting cells. IFN-γ, which is a Th1 cytokine, inhibits the activation of Th2 cells (Mosmann and Sad, 1997). It has been established that there exists an imbalance in Th1 and Th2 in Tuberculosis patients and that defects in the production and activation of Th1 cell effectors resulted in susceptibility to mycobacterial infections (Sieling and Modlin, 1994).

The CD4+ T-helper cells are very important in the immune response, but other T-cell subsets are also of importance. This includes gamma and delta T cells, the Th17 subset as well as the conventional CD8+ cells (Barker *et al*., 2009). CD8+ cytotoxic T lymphocytes are also important and necessary for crucial antimycobacterial mechanisms for the destruction of infected cells (Brighenti and Andersson, 2010). CD4+ cells have also shown a cytolytic activity in an *in vitro* assay on peripheral blood mononuclear cells (Mutis *et al*., 1993). Gamma/delta T cells are also important in the lysing of infected macrophages and production of IFN-γ, they play a role in the innate immunity (Dieli *et al*., 2000).

There have been studies indicating, that in TB patients there exists a decreased Th1 response but not always an increased Th2 response (Lin *et al*., 1996). Thus assessing the Th1 response is more appropriate than the Th2 response, although both are important in a healthy balanced immune response.

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The focus of an immune modulatory effector is to develop a balance between Th1 and Th2 responses, where one response or both are regulated to an extent where they are balanced.

3.1.2 Cytokines

Cytokines, originally called lymphokines, are hormone-like signaling protein produced by antigen stimulated T-lymphocytes. Cytokines is the broad term used and the different kinds of interleukins are designated by a number. The role of cytokines in the immune response is immiscible. Cytokines are soluble proteins that carry a signal. Three types of signaling can be distinguished: autocrine, a message delivered to same cell, paracrine, a signal to an adjacent cell or endocrine, where the signal is carried to a distant cell. Receptors for cytokines can be found on cells and if a cytokine binds it causes a change in the function of the development of the cell (Clark, 2013).

These small messenger proteins are produced by the wide variety of immune cells to communicate a certain response. Cytokines can be divided into six groups: interleukins (IL), interferon (IFN), tumor necrosis factor (TNF), colony-stimulating factor (CSF), growth factors and chemokines. The cytokines investigated in this study are discussed in the following section.

3.1.2.1 Pro-inflammatory cytokines (Th1)

Interleukin 2

Interleukin 2 is involved in the stimulation of the development of cytotoxic T lymphocytes and activation of B lymphocytes for the excretion of antibodies. This cytokine is primarily produced by T lymphocytes and T helper cells , although, macrophages, B cells, natural killer cells and oligodendrocytes have also been shown to produce this cytokine. IL 2 is also one of the few signals that can activate non-specific cytotoxicity in natural killer cells to combat the infection at a very early stage (Johnson *et al*., 1996).

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Interleukin 12

Interleukin 12 is one of the first cytokines induced after the phagocytosis of *Mycobacteria* bacilli by macrophages. This cytokine drives the development of a Th1 response and also induces the production of IFN-γ. *Mycobacteria* are a very good inducer of IL 12 (Sano *et al*., 1999). This is a critical cytokine in controlling Tuberculosis and it has been shown that increased numbers of IL 12 resulted in a decrease in bacterial count and increased survival rate in BALB/c mice (Flynn *et al*., 1995). Another study by Lowrie *et al*., in 1999 indicated that the administration of IL 12 mRNA to mice with chronic Tuberculosis infection caused a significant reduction in bacterial count. This could indicate a possible focus for a vaccine that induces the production of IL 12.

Tumor necrosis factor-α

This cytokine has been the focus of many studies related to Tuberculosis immunology. It is also believed that TNF-α has a variety of roles to play in the TB immune response and is a mediator in macrophage activation. TNF-α is produced by macrophages, dendritic cells as well as T cells (Sano *et al*., 1999). It is also associated with the maintenance and control of acute infections. TNF-α or TNF receptor deficient mice have shown a high susceptibility to infection and also a higher rapid mortality rate than control mice (Flynn *et al*., 2000). This cytokine has been shown to be an important signal in granuloma formation. It also affects the migration and localization of cells to the infected area and influences expression of adhesion molecules. The down regulation of TNF-α can lead to increased infection and necrosis (Moreira *et al*., 1997). The role of this cytokine is complex and multifaceted but in general it modulates inflammation and the disease cannot be combated without it.

Interferon-γ

Just as all the other Th1 cytokines, IFN-γ is important for controlling Mycobacterial infections. CD8, CD4 and Natural killer cells produce IFN-γ. IFN-γ production in infected macrophages has been shown to be partially dependent of IL 12 (Lyadova *et al*., 1998). IFN-γ knockout mice showed the highest susceptibility to infection (Wang *et al*., 1999). Patients with defective genes for IFN-γ are prone to serious infection and the bacteria grow unchallenged resulting in quick granuloma formation and eventually, necrotic lesions. IFN-γ on its own cannot combat the

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disease but is required for protective response. There is also evidence that Mycobacterial infection can alter the adequate response of IFN-γ of macrophages (Ting *et al*., 1999)

3.1.2.2 Anti-inflammatory cytokines (Th2)

Interleukin 4

Interleukin 4 is subjected to many different controversial ideas. It has been established that this Th2 cytokine acts as an inducer of Interleukin 12, which is a Th1 cytokine. IL 4 promotes the differentiation of basophils, increases antibody responses by B lymphocytes, activates natural killer cells and also contributes to granuloma formation. The production of this cytokine cannot directly be associated with an increase in Tuberculosis infection or disease, but rather, it is the lack of the production of IFN-γ that showed the biggest influence (North, 1998).

Interleukin 6

Interleukin 6, also a Th2 cytokine, plays many different roles in the immune response. These roles include the differentiation of T cells, increased inflammation and increased hematopoiesis. There has also been evidence that IL 6 suppresses the response of T cells (Van Heyningen *et al*., 1997).

Interleukin 10

Interleukin 10 is probably the biggest down regulator of Th1 cytokines. It acts as a strong anti-inflammatory cytokine. It is produced by macrophages and T cells and brings about the deactivation of macrophages as well as the down regulation of IL 12, which in turn decreases IFN-γ production. IL 10 also directly decreases CD4+ T cells' response to infection as well as the ability to inhibit APC function (Gong *et al*., 1996). More studies can be done to describe the role of IL 10 better.

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3.1.3 Materials and methods

Peripheral blood mononuclear cells (PBMCs) were used and were treated with the samples. Supernatants were collected and cytokine levels were evaluated using two methods, Enzyme-linked immune-sorbent assay (ELISA – Human IL 12 ELISA set cat#555171) and Cytometric bead array (CBA - Human Th1/Th2 cytokine kit cat# 551809) using flow cytometric methods, to assess the base immune modulatory effect. The flow cytometry work was done at the Department of Biochemistry (University of Pretoria) with the help of Ms. Sindi Nondaba.

3.1.3.1 Peripheral blood mononuclear cell isolation and treatment

PBMCs were isolated as described in chapter 2, section 2.2.4.1.The cells were counted and 1000 µl were plated at a density of 100,000 cells/well in a 24-well plate. The cells were treated with 1000 µl of the compounds prepared at ½IC_{50} as determined by the cytotoxicity assay. A media control, solvent control as well as a cell control was also added in triplicate. A viability test was also conducted, after incubation, to determine the viability of the cells when supernatants were collected.

3.1.3.2 Supernatant collection

All the treated cells were incubated for 18-24 hours at 37°C and 5%CO_2. The plates were removed and sealed with parafilm and centrifuged for 5 min at 800g. Supernatants were collected in150 µl aliquots and either used directly or stored at -72°C.

3.1.3.3 Enzyme-linked immune-sorbent assay experimental procedure

The procedure was done according to the manufacturers' protocol. The BD OptEIA™ Human IL12 (p40) ELISA set kit and Reagent set B were used (Cat # 555171 and 550534). Briefly; 18-24 hours before the experiment started, the 96-well ELISA plates were coated with the primary capture antibody, according to the lot-specific recommendations. The plates were blotted dry and

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200 µl of assay diluent were added to block the plates. The plates were incubated for one hour and washed again as mentioned before. The standards and samples were prepared as described in the kit brochure and 100 µl of each supernatant sample and standard (in triplicate) were added to the plates and incubated at room temperature for 2 hours. After sample/standard incubation the plates were washed five times and 100 µl of working detector was added to each well. The plates were sealed and incubated for another hour, followed by aspiration and seven washes. One hundred microliters of the substrate solution (1:1 Substrate A (hydrogen peroxide in buffer solution): Substrate B (3, 3', 5,5' Tetramethylbenzidine)) was added to each well, and incubated for 30min at room temperature. After 30min, 50 µl of stop solution (1M phosphoric acid) was added and the absorbance was read directly afterwards at 450nm (primary) and 570nm were used as the reference wavelength (Appendix 4). The basic principal of an ELISA experiment is shown in Figure 3.1.

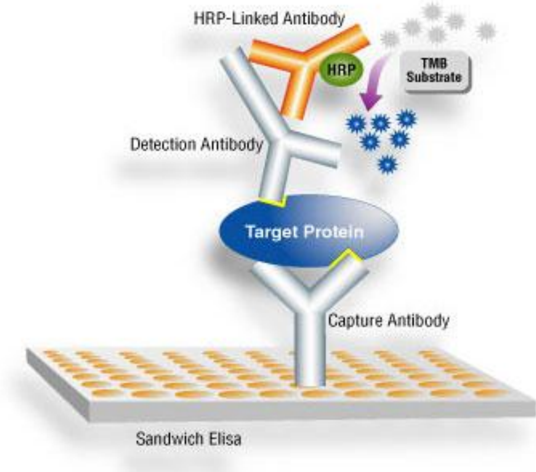

Figure 3.1: Principal of ELISA - Enzyme-linked immunosorbent assay (iaszoology.com, 2013).

3.1.3.4 Cytometric bead array experimental procedure

The BD CBA Human Th1/Th2 cytokine kit (cat# 551809) was used to determine the production and secreted amounts of IL 2, IL 4, IL 6, IL 10, TNF-α and IFN-γ in the supernatant samples. The procedure was done according to the manufacturers' protocol (Appendix 5). The basic principal of a bead array is shown in Figure 3.2.

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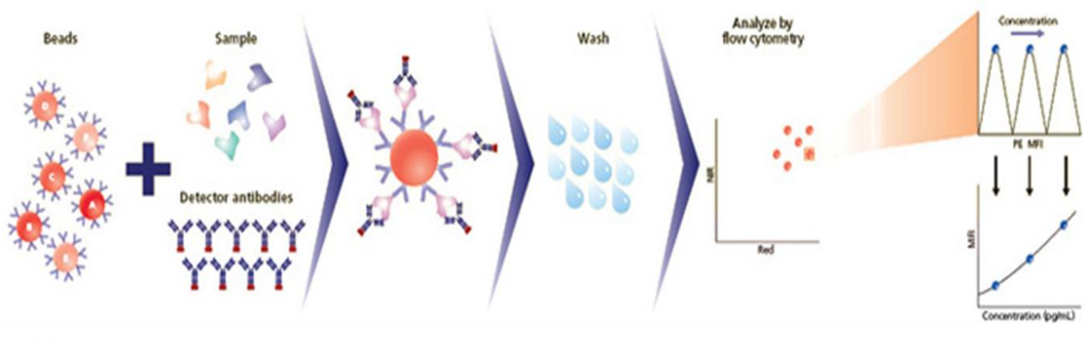

Figure 3.2: Principal of CBA - Cytometric bead array (bdbiosciences.com, 2013).

All the cells were treated with various concentrations of the compounds and the levels of cytokines were evaluated using the methods described above.

3.1.4 Results and Discussion

The immune response of Peripheral blood mononuclear cells was assessed after the treatment with the compounds. To eliminate false negatives, the viability of the cells was measured at the same time point as supernatant collection (Table 3.1).

Table 3.1: Viability of treated PBMC's with ½IC_{50} of samples after 24 hours incubation.

Chemical compounds	% viability [a]	± SD [b]
Quinone samples		
AQS-NH_4	78.16	9.81
CB5	73.91	0.56
IL-107	78.31	0.53
IL-113	80.80	6.41
IL-114	101.47	3.27
IL-135	83.15	5.23
IL-142	96.92	3.64
IL-143	62.92	3.62
KM-108	67.02	10.82
KM-108-1	85.78	4.59
KM-117	64.39	0.97
KM-139	73.18	4.25
KM-140	92.23	1.57
KM-41	109.23	2.09
KM-46	102.93	5.64

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Chemical compounds	% viability [a]	± SD [b]
KM-52	64.09	1.62
KM-60	78.90	1.58
KM-80	90.77	1.14
KM-86	134.15	0.46
KM-94	62.34	2.99
Garlic oils		
G1 (Diallyl disulphide)	76.37	6.36
G2 (Garlic oil OCTAVUS)	86.26	8.37
G3 Garlic oil (TREATT)	94.36	9.25
G4 Garlic oil (Synthetic)	88.27	1.26
G5 Garlic oil (Stringer)	90.14	3.69
G6 Garlic oil (Naturex)	89.37	1.04
Media + cells	100.00	0.13

[a] Viability was tested using the presto blue viability assay, and calculated as a percentage from 100% viability of untreated cell.
[b] Standard deviation.

All the cells had a viability of 70% or higher, thus indicating that the samples did not inhibit the growth of the cells at the tested concentrations.

Conditional formatting was used to designate a color to a value, where red indicates a high production of cytokines and blue indicates a low production of cytokines. Appendix 6 and 7 indicates the analysis and standardization of data.

Table 3.2 Cytokine production levels of Th1/Th2 response of PBMCs at 24 hours incubation with sample.

	TH1/TH2 Cytokines						
	Th2			**Th1**			
	IL 4	**IL 6**	**IL 10**	**IL 2**	**IL 12**	**TNF**	**IFN**
Sample	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml
Quinone samples							
IL107	2.19	282.93	1.74	4.57	42.70	3.92	2.23
IL113	0.00	191.32	0.00	0.00	21.35	1.62	0.00
IL114	0.00	236.28	0.00	0.00	54.10	0.00	0.00
IL135	0.00	180.09	0.00	0.00	54.46	1.62	0.00
IL142	0.00	294.47	1.18	2.92	35.87	2.17	3.19
IL143	0.00	241.06	0.00	2.50	41.82	0.00	0.00
KM41	0.00	188.46	0.00	0.00	36.04	0.00	0.00

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	TH1/TH2 Cytokines						
	Th2			Th1			
	IL 4	IL 6	IL 10	IL 2	IL 12	TNF	IFN
Sample	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml	pg/ml
KM46	0.00	285.77	0.00	0.00	53.20	0.00	0.00
KM52	0.00	342.05	0.00	0.00	88.67	1.62	0.00
KM60	1.82	166.10	0.00	0.00	30.33	0.00	0.00
KM80	0.00	127.47	0.00	0.00	38.31	0.00	0.00
KM86	0.00	224.72	0.00	3.95	58.43	1.86	1.61
KM94	0.00	377.95	0.00	0.00	117.68	3.05	0.00
KM108	0.00	306.47	0.00	0.00	72.86	3.39	1.94
KM108-1	0.00	385.56	0.00	0.00	117.38	1.31	0.00
KM117	0.00	409.35	0.00	0.00	114.11	3.65	0.00
KM139	0.00	141.19	0.00	2.72	12.01	2.02	1.47
KM140	0.00	300.41	0.00	0.00	73.23	0.00	0.00
CB5	2.61	303.43	1.94	5.73	34.30	3.43	3.77
AQS	0.00	325.40	0.00	0.00	84.74	1.70	0.00
Garlic oils							
G1	3.02	434.61	1.99	5.73	83.25	1.86	4.02
G2	0.00	401.27	0.00	0.00	83.25	0.00	0.00
G3	0.00	417.61	0.00	0.00	71.20	0.00	0.00
G4	0.00	325.40	0.00	0.00	71.20	0.00	0.00
G5	1.82	517.61	1.64	4.27	31.36	2.21	1.84
G6	3.66	616.64	2.84	9.11	31.36	3.92	5.21
Media + cells	0.00	525.43	1.74	4.87	103.60	4.20	0.00

Indicates production of cytokines

Indicates no production of cytokines

Red values indicate higher production of the specific cytokine. When comparing the values of untreated cells (Media + Cells control), with the samples, some did show an increase in Th1 cytokines and a decrease in Th2 cytokines. KM94, KM108-1 and KM117, increased the production of IL 12, which is one of the most important cytokines and also the first cytokine in the response against Tuberculosis infection. These three samples also showed a decreased production in IL 6 and IL 10 (Th2 cytokines). Some of the samples showed a null effect and more studies will need to be conducted to determine the full extent of the effect of these compounds on the immune response. Previous studies have indicated the immunomodulatory

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effect of quinones and garlic polysulfide extracts by assessing the hemagglutination and mannose binding activities of garlic as well as the effects on neutrophil and T cell activity by quinones. Both have shown to some extent a positive immune stimulatory effect other than a cytokine response (Chandrashekar and Venkatesh, 2010; T Hart, 1991).

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3.2 Hepatoprotective activity

3.2.1 Liver function

The liver is the most important organ involved in the metabolism reactions within the body. It is situated between the alimentary tract and the systemic circulation. This position maximizes processing of absorbed substances and also minimizes the exposure of toxins and xenobiotics to the body (Saukkonen *et al*., 2006).

Drugs taken up by the body are transported through the blood and the splanchnic circulation directly to the liver: this is called the “first pass”. There are three phases in the metabolism of a toxic chemical or drug. Phase 1 uses oxidation, reduction or hydrolysis via metabolic enzymes to convert the substances. The enzymes used are the cytochrome P450 class. The second phase is involved with producing conjugates, via glucuronidation, sulfation, acetylation and glutathione conjugate to form compounds that are easily excreted. There are many drugs where the liver uses different mechanisms of metabolism and excretion to detoxify the compounds, which explain the variety of toxic effects. The third phase involves the excretion of the newly formed compound mainly into the bile or in some instances the systemic circulation. This function is brought about by transporter proteins, which can be affected by circadian rhythms, hormones, drugs, cytokines, disease stages, genetic factors as well as other endogenous factors (Lee and Boyer, 2000).

3.2.2 Hepatotoxicity in TB patients

During TB infection, drug induced liver toxicity, is a major problem, and may occur with all the current treatment regimens. Drug induced Hepatotoxicity can be fatal if left untreated. This adverse effect has a negative impact on the outcome of treatment and can result in treatment failure and relapse (Totsmann *et al*., 2008).The liver plays the central role of metabolism and detoxification of drugs, and in doing so, it is susceptible to injury. Isoniazid (6-9 months), rifampicin (4 months), isoniazid/rifampicin (4 months) as well as the two-fold treatment with pyrazinamide with either ethambutol or a flouroquinolone has shown hepatotoxicity (American

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Thoracic Society, 2000; Younossain *et al*., 2005). There are many factors that influence the severity of the toxic effect; these include, age, sex, cofactors, regimen and the occurrence of HIV/AIDS as well as Hepatitis B and C (Saukkonen *et al*., 2006). Although many studies have been conducted on TB drug induced injury, the precise mechanisms are not clear and more knowledge can be obtained.

3.2.3 Hepatoprotective activity

Many different substances have been identified that can aid the liver in a toxic event. One of these substances that have been studied intensely is Silymarin. Silymarin is extracted from the plant *Silybum marianum*, or commonly known as milk thistle, and has been used to treat liver disorders from as long ago as the Greeks (Saller *et al*., 2007). Silymarin is a mixture of flavonoids and polyphenols, which protects the liver against reactive oxygen species via the scavenging properties and has the potential to reduce the toxic effect of other drugs (Tomankova *et al*., 2009). It was decided to test the twenty quinone derivatives and six garlic oils to assess if they have any hepatoprotective activity.

3.2.4 Materials and methods

C3A liver cells were used in the hepatoprotective assay and acetaminophen (paracetamol) was used as the toxic inducer. The samples were tested to assess if they show any protective effect against the toxicity of acetaminophen. The experiment was conducted at the Onderstepoort Research Institute of the University of Pretoria, under the supervision of Dr. Lyndy McGaw.

3.2.4.1 C3A cell culture

C3A liver cells (ATCC HB-8065) are a derivative of HepG2 human liver cells. They are an adherent cell line from a *Homo sapiens* origin. These cells were first isolated from a hepatocellular carcinoma of a 15 year old Caucasian male. The cells were cultured in 10% FBS in Eagle's Minimum Essential Medium. After cells formed a confluent layer, they were washed,

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trypsinized, counted and 100ul were seeded at a concentration of 10,000 cells/well in a 96-well plate.

3.2.4.2 Sample preparation

Samples were tested at ½IC_{50} (a concentration half of that of the 50% inhibition concentration) and ¼IC_{50} (a concentration quarter of that of the 50% inhibition concentration) and were prepared in the appropriate media (10%FBS, EMEM). The concentrations were selected based on the IC_{50} values to test at concentration ranges that will not affect the viability of the cells but still have a therapeutic effect. From the prepared concentrations, 50 µl was added to the already seeded plates.

The toxic inducer used in the assay was Acetaminophen (Paracetamol, obtained from Sigma Aldrich cat# 1001325327) at a concentration of 2mM. Acetaminophen was dissolved in the appropriate media at a concentration of 8mM and 50µl was added to each well.

Acetaminophen gets converted to a toxic N-acetyl-p-benzo-quinone imine by the P450 cytochrome enzymes present in the cells (Figure 3.3). This causes the toxic effect in the cells. The protective effect of the samples was measured using viability results, comparing the samples with the controls and untreated cells.

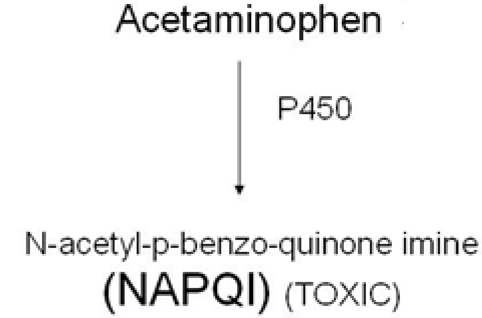

Figure 3.3: Conversion of acetaminophen, via the P450 cytochrome enzyme to N-acetyl-p-benzo-quinone imine. (Derived from: http://www.bmj.com/content/342/bmj.d2218).

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A positive control, Silymarin (obtained from Sigma Aldrich cat# S0292), was used to assess the hepatoprotective effect. Sylimarin is a known hepatoprotectant (see section 3.2.3). Sylimarin was prepared in the appropriate medium at a concentration of 400 µg/ml. Just as with the samples, 50 µl was added in triplicate to the plated cells. The test concentration of Sylimarin was 100 µg/ml.

3.2.4.3 Experimental procedure

Cultured C3A cells were counted, adjusted and 100 µl were plated at a density of 10,000 cells/well. Fifty microliters of the prepared samples were added to each well in triplicate. Fifty microliters of the toxic inducer, acetaminophen, was added to all the wells and the plates were incubated at 37°C, 5% CO_2 for three hours. After incubation, 20 µl of Presto blue was added to all the wells and incubated for another 0.5-1 hour. The fluorescence was measured at 535-560⁄590-615 nm and the protective effect was determined by comparing the samples to the untreated cells and cells treated with acetaminophen only.

3.2.5 Results and Discussion

Protective effect was determined by comparing the viability of the samples to the viability of the toxic inducer (0% protected) and the untreated cells (100% protected) (Table 3.3). Conditional formatting was used to designate a color to a value, where red indicates a protective effect and blue indicates no protective effect.

Table 3.3: Percentage Hepatoprotection of samples on toxic induced C3A cells.

Samples		% Hepatoprotection	± SD_a
Quinone samples			
KM60	1/2 IC_{50}	0.95	0.09
	1/4 IC_{50}	-110.48	13.42
KM80	1/2 IC_{50}	-303.81	22.56
	1/4 IC_{50}	-116.19	5.17
KM86	1/2 IC_{50}	-217.14	3.37
	1/4 IC_{50}	-116.19	4.06
KM94	1/2 IC_{50}	-345.71	29.25
	1/4 IC_{50}	-664.76	15.38
KM108	1/2 IC_{50}	61.90	3.87

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Samples		% Hepatoprotection	± SD_a
	1/4 IC_{50}	57.14	3.30
AQS NH_4	1/2 IC_{50}	38.10	12.36
	1/4 IC_{50}	115.24	23.66
CB5	1/2 IC_{50}	-40.95	14.13
	1/4 IC_{50}	119.05	7.55
IL107	1/2 IC_{50}	-80.00	2.52
	1/4 IC_{50}	155.24	7.55
IL113	1/2 IC_{50}	-167.62	2.30
	1/4 IC_{50}	-128.57	6.98
IL114	1/2 IC_{50}	-245.71	8.71
	1/4 IC_{50}	-240.95	1.92
IL135	1/2 IC_{50}	-20.95	2.43
	1/4 IC_{50}	-36.19	3.50
IL142	1/2 IC_{50}	-65.71	8.30
	1/4 IC_{50}	-340.00	16.68
IL143	1/2 IC_{50}	-448.57	16.86
	1/4 IC_{50}	-371.43	4.48
KM108-1	1/2 IC_{50}	-340.00	3.60
	1/4 IC_{50}	-136.19	6.83
KM117	1/2 IC_{50}	-207.62	8.86
	1/4 IC_{50}	-373.33	11.21
KM139	1/2 IC_{50}	-260.00	11.37
	1/4 IC_{50}	-142.86	2.26
KM140	1/2 IC_{50}	-290.48	3.88
	1/4 IC_{50}	114.29	2.07
KM41	1/2 IC_{50}	-35.24	8.48
	1/4 IC_{50}	-499.05	12.77
KM46	1/2 IC_{50}	-455.24	2.89
	1/4 IC_{50}	-445.71	1.67
KM52	1/2 IC_{50}	-340.95	1.31
	1/4 IC_{50}	-70.48	3.40
Garlic samples			
G1	1/2 IC_{50}	-297.14	10.54
	1/4 IC_{50}	-354.29	3.09
G2	1/2 IC_{50}	-163.81	3.44
	1/4 IC_{50}	-57.14	1.60
G3	1/2 IC_{50}	0.00	2.57
	1/4 IC_{50}	-72.38	1.25
G4	1/2 IC_{50}	-414.29	13.34
	1/4 IC_{50}	-390.48	0.91
G5	1/2 IC_{50}	-32.38	2.62
	1/4 IC_{50}	-233.33	31.82
G6	1/2 IC_{50}	-11.43	0.34
	1/4 IC_{50}	22.86	0.17
Silymarin	100 µg/ml	30.00	10.40
Acetaminophen	2mM	0.00	5.56
Medium		100.00	5.55

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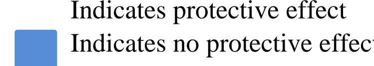
Indicates protective effect
Indicates no protective effect

[a] Percentage standard deviation

A significant decrease in cell viability was observed upon treatment of C3A cells with acetaminophen (2 mM). Five of the Quinone samples (KM108, KM140, AqsNH4, CB5 and IL107), and one Garlic sample (G6) exhibited a significant protection at a concentration of ¼IC_{50} towards cell toxicity of acetaminophen.

The positive control Sylimarin had a protective effect of 30%. KM180, $AqsNH_4$, CB5, IL107 and KM140 showed better protective effect than the positive control, with IL107 showing a protective effect of 100%. Samples showing a protection of above 100% were indicative of stimulating cell growth higher than the baseline growth. Quinones isolated from *Auxemma oncocalyx* have been reported to have hepatoprotective activity, *in vivo*, reducing the hepatotoxic related enzymes; alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin. This can be attributed to the antioxidant properties of the quinones (Ferreira *et al*., 2003). It can also be concluded that lower test concentrations showed better protective effect than higher concentrations, indicating the toxicity of these compounds on cells at higher concentrations.

One garlic sample (G6) showed some hepatoprotective effect. Previous studies indicated that Garlic homogenates showed hepatoprotective activity on mice treated with Isoniazid (Nasiru *et al*., 2012). Diallyl sulfides were also reported to have a protective effect, *in vivo*, on carbon tetrachloride induced liver injury in mice (Hosono-Fukao *et al*., 2009). Thus it was expected the diallyl garlic mixtures to show hepatoprotective effects against acetaminophen toxicity. This could indicate, either that the samples do not protect the cells against the toxic effect of acetaminophen, or that in an *in vitro* assay, it is difficult to assess the protective effect and that a more complex eukaryotic system is required to identify the right response.

In a previous study conducted by Hassanein *et al*., in 2011, it was found that phospholipids isolated from *Cyperus esculentus* had a significant hepatoprotective effect, against the toxicity of acetaminophen, at a concentration of 25 µg/ml. This correlates with the results in Table 3.3, with

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the samples KM108, KM140, Aqs-NH4, CB5, IL107 and G6 showing a significant protective effect at a much lower concentration.

The other samples showing a % hepatoprotection lower than 0% are considered as non hepatoprotective and had an antagonistic effect together with the acetaminophen, increasing the toxic effect. It could be speculated that the test concentrations were high, and more assays (preferably *in vivo*) should be conducted using the samples at lower concentrations to investigate the hepatoprotective effect.

3.3 Conclusion

Out of all the samples three quinone compounds showed immune stimulatory effect (KM94, KM108-1 and KM117), and five showed hepatoprotective effect (KM108, KM140, AqsNH4, CB5 and IL107). Out of the six garlic oil mixtures, G5 and G6 showed a slight immune stimulatory effect, while G6 was the only sample to have hepatoprotective activity, probably due to the higher amount of diallyl tetra sulfide in the mixture.

The mechanism of immunomodulatory and hepatoprotective activity, of how these two groups of compounds work, is still poorly understood. The hepatoprotective effect can mainly be attributed to antioxidant properties. From the composition of the garlic oils, it can be concluded that diallyl tetrasulfide might be responsible for the hepatoprotective effect.

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Chapter 4

Mechanism of action on Glutathiol, Mycothiol and Thioredoxin reductase

All the quinone samples were evaluated for their subversive substrate activity on three flavoprotein disulfide reductases: Glutathiol, Mycothiol and Thioredoxin reductase. Glutathiol reductase is the human analog while Mycothiol and Thioredoxin are bacterial analogs. Substrate binding affinity was determined and it was found that quinones act as substrates for all three enzymes with the highest affinity for Thioredoxin reductase. Samples; Aqs and KM80 had Km-values of lower than 30 μM. It can also be concluded that anthraquinones had a higher affinity to these disulfide enzymes as compared to naphthoquinones.

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Chapter 4 – Mechanism of action on Glutathiol, Mycothiol and Thioredoxin reductase of Quinones and Garlic polysulfide samples

The mechanism of action of the quinones and garlic oil mixtures were evaluated by assessing the enzyme activity on three flavoprotein enzymes, Glutathiol reductase, Mycothiol reductase and Thioredoxin reductase.

4.1 Introduction

Flavoprotein disulfide reductases (FDR) are a group of enzymes within the flavoprotein enzyme family. The enzymes in this FDR family include the following: Glutathione reductase (Gtr), Lipoamide dehydrogenase (LipDH), and thioredoxin reductase (Txr) (Williams, 1976). Recently, two new enzymes were added to this class of disulfide reductases, these include trypothione reductase (Ttr – discovered in the 1980's from trypanosome spp.) (Williams, 1992) and Mycothione reductase (Mtr – from *Mycobacterium tuberculosis*) (Patel and Blanchard, 1999). All these homodimeric flavoproteins, consist of a flavin-adenine-dinucleotide (FAD) and one redox-active disulfide per polypeptide chain. These are tightly but not covalently bound and they catalyze the pyridine-nucleotide-dependent (NADPH) reduction of their disulfide oxidized substrates.

The similarity of these enzymes was confirmed after extensive DNA cloning and gene sequencing experiments, as well as, the three-dimensional determination by X-ray crystallography (Williams, 1992). The enzymes studied in this project included the human analog – Glutathione reductase (Gtr) and the *Mycobacterium* analog – Mycothione reductase (Mtr), and Thioredoxin reductase (Txr), for assessing the inhibitory and subversive substrate activity of the selected samples.

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4.1.1 Function of Mycothiol and Glutathiol reductase enzymes

Both these enzymes are active (two identical active sites) in the homodimer form, consisting of two identical monomers. The monomers of Gtr are composed of FAD-binding, pyridine-nucleotide-binding, central and interface domains (Argyrou and Blanchard, 2004). The crystal structures are available for Gtr (Kuriyan *et al*., 1991), but not for the recently discovered Mtr. The interaction of two monomers results in two identical active sites. These active sites contain residues from the first three domains of one monomer and the interface domain of the other and this is where the His-Glu pair is located, the quaternary structure of the enzyme can be observed (Fig. 4.1). Both Gtr and Mtr are proposed to have the same affinity with regard to FAD- and pyridine-nucleotide binding (Argyrou and Blanchard, 2004), but when studying the disulfide substrate-binding site, it was found that the binding are different. Each enzyme is well developed to provide specificity for its own substrate. The transfer of electrons in the reaction is from NADPH $\rightarrow$ FAD $\rightarrow$ redox-active disulfide $\rightarrow$ disulfide substrate (Argyrou and Blanchard, 2004).

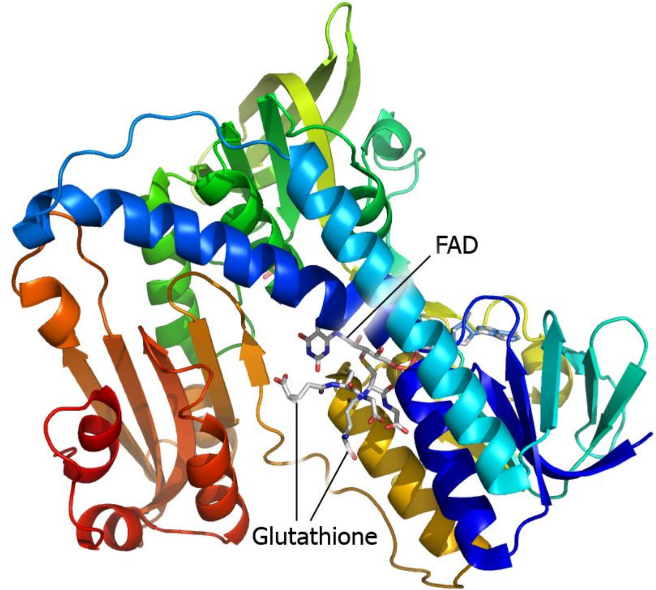

Figure 4.1: Structure of Glutathione reductase dimer (Human analog), with bound FAD and the natural substrate Glutathione (Mittl and Schulz, 1994). This enzyme catalyzes the NADHP-dependent reduction of GSSG to GSH.

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Within the cell the levels of Glutathiol (GSH) is higher than the levels of glutathione/glutathiol disulfide (GSSG), and this is due to the reaction of the Gtr enzyme (Williams, 1992). The whole reaction can be seen in equation 1 (Eq. 1)

$$\mathbf{NADPH + H^+ + GSSG \rightarrow NADP^+ + 2GSH} \qquad \textbf{(Eq. 1)}$$

Most living organisms contain a thiol group, and a functional homolog of Gtr has been identified from *Mycobacterium tuberculosis*. This thiol producing enzyme was named mycothione reductase (Mtr). This enzyme catalyzes the reduction of mycothione (also known as mycothiol disulfide) to mycothiol (Patel and Blanchard, 1999). The reaction can be seen in equation 2 (Eq. 2)

$$\mathbf{NADPH + H^+ + MSSM \rightarrow NADP^+ + 2MSH} \qquad \textbf{(Eq. 2)}$$

4.1.2 Function of Thioredoxin reductase

The thioredoxin system is one of the major lines of defense against oxidative damage (Hirt *et al*., 2002). Thioredoxin reductase is a member of the same pyridine nucleotide-disulfide oxidoreductases family as the Gtr and Mtr (Williams, 1992). Two classes of this enzyme can be found in nature: a high molecular weight eukaryotic and human analog as well as a low molecular weight bacterial analog (Hirt *et al*., 2002). With NADPH acting as the cofactor in the reaction, thioredoxin reductase catalyzes the reduction of the disulfide of thioredoxin. The reduced form of thioredoxin is important in many different thiol-dependent reduction systems within the cell (Holmgren, 1985). Some of the roles of Txr includes, defending the cell and deoxyribonucleotides against oxidative stress, regulating gene expression through reduction and oxidation mechanisms and transduction of signals through thiol redox control (Fig. 4.2) (Holmgrem, 1989; Nakamura *et al*. 1997). Txr with its disulfide active site is also the major cellular protein reductase (Arner and Holmgren, 2000). Txr also share many structural similarities with Glutathiol reductase due to their common ancestry, thus compounds affecting

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Gtr and Mtr will also have an effect on Txr (Hirt *et al*., 2002). The low molecular weight Thioredoxin reductase, isolated from *M. tuberculosis*, was used during the present study.

4.1.3 Function of low molecular thiols – Glutathiol and Mycothiol

Glutathiol (GSH) is the major thiol present in almost all eukaryotes and many bacteria. The reduced form of GSSG is a low molecular weight thiol, and it is mainly concerned with the protection of cells against oxidative stress and reactive electrophiles (Newton and Fahey, 2002). These thiols can react non-enzymatically with the reactive species, and with this mechanism, protect the cell against injury by acting as a buffer. After the reduction of GSSG to 2GSH, GSH can act as a substrate for two enzymes; glutathione S-transferase and glutathione peroxidase (Fig. 4.3). The former detoxifies a number of injurious electrophiles, while the latter reduces hydrogen peroxide to water (Ursini *et al*., 1995). Furthermore, reduced glutaredoxin together with reduced thioredoxin serves as a source of reducing equivalents for ribonucleotide reductase. GSH is also important in deoxyribonucleotide biosynthesis (Holmgren, 1989). Glutathiol disulfide reductase helps to maintain the intracellular reducing environment with a high level of GSH, by the NADPH-dependent reaction reducing GSSG back to GSH.

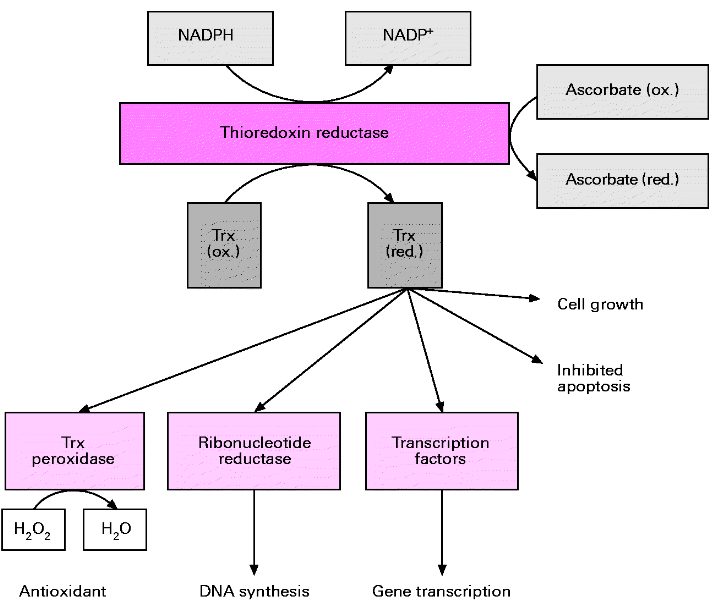

Figure 4.2: Function of Thioredoxin reductase in the cell (Mustacich and Powis, 2000).

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It was generally thought that GSH is present in all living organisms, until the discovery of mutant *Escherichia coli* lacking GSH, which was viable and also the finding that many other gram-positive bacteria do not produce GSH. The scientist hypothesized that this lack of GSH producing mechanisms in many aerobes, prompted a search for other thiols, which might play a parallel role to GSH (Newton and Fahay, 2002)

During the discovery of new thiols, a variety of previously unknown thiol structures were identified. One of these thiols was found in streptomycetes and other actinomycetes such as *Mycobacterium tuberculosis* and *Streptomyces coelicolor*, and was designated U17. This compound was later described as mycothiol (MSH). In recent studies, mycothiol was identified to play a similar role in many gram-positive bacteria as GSH in GSH-producing organisms (Newton and Fahey, 2002; Rawat and Av-Gay, 2007). Mycothiol is a structurally unique low-molecular-weight thiol, and it was found to be the most abundant thiol in the actinomycetes (including the mycobacteria and streptomycetes).

The oxidized form was given the trivial name mycothione (MSSM), while the reduced form is known as mycothiol (MSH) (Spies and Steenkamp, 1994). *Mycobacterium tuberculosis*, which lacks glutathione, was found to maintain millimolar concentrations of the structurally distinct low-molecular-weight mycothiol, and is thus the dominant thiol in these bacteria (Mahapatra *et al*., 2007).

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2 x reduced glutathione (GSH)

$NADP^+$ — Glutathione reductase — NADPH

H_2O — Glutathione peroxidase — $H_2O + ½ O_2$

Oxidized glutathione (GSSG)

Figure 4.3: Chemical structures of glutathione and schematic representation of the reactions catalyzed by glutathione reductase and glutathione peroxidase, which maintain the intracellular reducing environment (david-bender.purplecloud.net, 2011).

Mycothiol is maintained in the reduced form, by the reaction catalyzed by mycothiol disulfide reductase (Mtr), which also utilizes NADPH as a reducing cofactor (Fig. 4.4) (Patel and Blanchard, 1999). Mycothiol is believed to play the same role as glutathiol, in the inactivation of potentially damaging radicals and reactive oxygen species. In this process mycothiol is oxidized back to the symmetrical mycothiol disulfide

Not only does mycothiol play a role in oxidative stress, but it has also been shown that it plays a role in nitrosative stress. Mycothiol acts as a substrate for mycothiol-dependent formaldehyde dehydrogenase which, in addition to detoxify formaldehyde, also has S-nitrosomycothiol reductase activity (Newton and Fahey, 2002; Vogt *et al*., 2003). The mechanism of detoxifying electrophiles on the other hand, is by the formation of mycothiol S-conjugates. These conjugates are then hydrolyzed by an amidase to generate N-acetyl-cysteinyl S-conjugates, and the mycothiol precursor, glucosamine-myoinositol. The precursor re-enters the mycothiol biosynthetic pathway, while the S-conjugates are excreted (Newton and Fahey, 2002).

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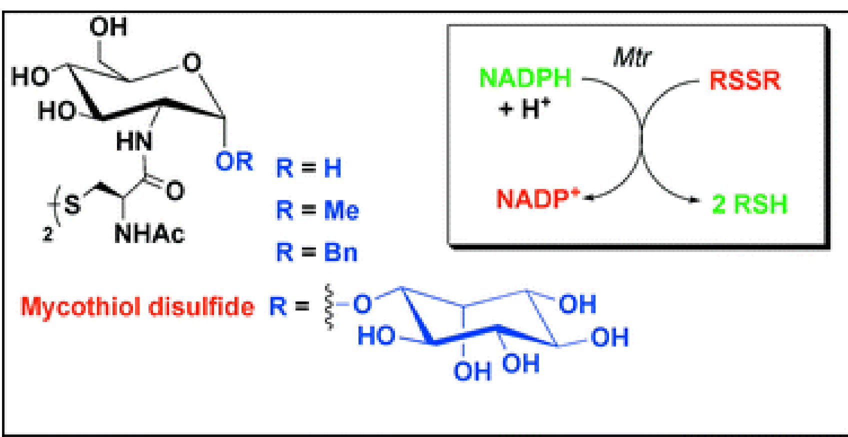

Figure 4.4: Chemical structure of mycothiol and schematic representation of mycothiol disulfide reductase (rsc.org, 2011).

Due to the fact that MSH has the ability to detoxify a variety of toxic compounds and has antioxidant capabilities, it is a candidate for protecting *M. tuberculosis* from inactivation by the immune response of the mammalian host during infections as well as for resisting antituberculosis drugs. Studies in the drug target field has identified that *Mycobacteria* deficient in MSH production, becomes hypersensitive to most currently used antitubercular drugs (Rawat *et al*., 2002, 2004).

It was also found that the exposure to the antibiotics resulted in an up regulation of the MSH-biosynthesis genes (Hayward *et al*., 2004). It was established that MSH is indeed essential for viability of *M. tuberculosis* (Buchmeier and Fahey, 2006; Bzymek *et al*., 2007; Sareen *et al*., 2003; Sassetti *et al*., 2003).

Considering the above statement, it can be suggested that substances or compounds that interfere with MSH-assisted detoxification, can be used as therapeutic agents and possible new type of antibiotic. One of the shortcomings in the Mtr enzyme studies is the scarcity of the natural substrates MSH and MSSM, which are very difficult to prepare in large quantities. Small quantities of MSH are routinely isolated by whole cell synthesis, which yield around 1mg per liter of *M. smegmatis* cell culture (Stewart *et al*., 2008).

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4.1.4 Inhibitory vs. Subversive substrate activity

A compound that inhibits the action of Gtr, Mtr and Txr, inactivates the enzyme for a period of time or indefinitely, in the case where a covalent bond has formed. There are three main types of enzyme inhibition: competitive, noncompetitive and uncompetitive, and these are used to describe the binding of an inhibitor to the target enzyme. Other factors can also influence the inhibition and severity of inhibition of an enzyme. These factors or events include allosteric, partial, tight-binding and time-dependent inhibition (nih.gov, 2010). None of the compounds tested in this study showed inhibition of Gtr, Mtr and Txr.

Many quinone compounds have been shown to have subversive substrate activity on flavoprotein disulfide reductases, such as glutathione reductase, mycothione reductase and Thioredoxin reductase. This causes an enzyme-mediated toxicity, due to the production of semiquinone radicals from the reduction of the quinone. The parent quinone is regenerated via the concomitant reduction of oxygen to toxic superoxide anion radicals (figure 4.5). In this manner the quinone substrate is regenerated and the futile redox cycle continues (Mahapatra *et al*., 2007), making this process a very plausible drug target for new drug development and antibiotic activity.

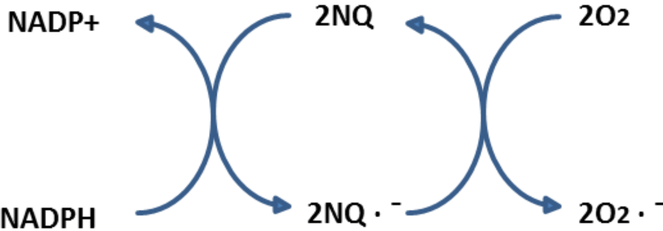

Figure 4.5: Schematic representation of a naphthoquinone acting as a subversive substrate on Mtr, producing semiquinone radicals, which in turn increase the oxidative stress due to the production of superoxide anion radicals (Mahapatra *et al*., 2007).

Thus in the process, the quinones eliminate the low-molecular weight thiol that protects the intracellular oxidative stress, and at the same time, increases the oxidative stress due to the production of the semiquinone radicals. Some quinone derivatives were tested for subversive

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substrate activity on Gtr and Mtr. Due to the similarity in binding sites of Gtr/Mtr and Txr, the compounds were also tested for subversive substrate activity on Txr.

4.2 Materials and methods

Thioredoxin reductase enzyme studies was conducted at the School of Chemical Sciences and Pharmacy (University of East Anglia, Norwich, England, UK)

4.2.1 Buffer preparation

The buffer was made up in aliquots of 500ml, using molecular grade water (de-ionized, nuclease free – obtained from Zigma). The buffer contained 50mM HEPES solution and 1mM EDTA. The buffer was adjusted to a pH of 7.6, by the addition of 5M NaCl solution, drop by drop, until the right pH was reached.

4.2.2 Screening of compounds and Km-value determination

Recombinant mycothiol disulfide reductase (Mtr) and mycobacterial thioredoxin reductase (Txr) were obtained from Dr. Chris Hamilton. Glutathione disulfide reductase, NADPH, Dimethyl-sulfoxide (DMSO) and Ellman's (DTNB) reagent were purchased from Sigma. The assay was carried out in a 96-well plate, at a final assay volume of 200 µl per well. The plates were read by a microplate reader (340nm – NADPH detection wavelength), and the data were processed using two computer programs: KC junior™ for data capturing and Graphpad prism™ for analysis. The enzymes were added at different concentrations, Gtr/5000 Mtr/4000 Txr/80, adding a volume of 20 µl. NADPH was added at a concentration of 140 µM at a volume of 20 µl. Ellman's reagent (DTNB) was added at 600 µM for the cycling of the substrate at a volume of 20ul.

After the addition of the enzyme and the NADPH, the plates were left to incubate for 5min at 35° C.

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The test compounds were dissolved in DMSO to a final concentration of 20mM. Six dilutions were prepared for each sample and 20 µl was added in triplicate for each dilution and each sample. The concentrations ranged from 0 to 1320 µM. The final volume of 200 µl was prepared by adding 130 µl of the prepared buffer. Table 4.1 shows the reagent concentrations and the volumes. The plates were read in a micro plate reader with the wavelength set at 340nm. The kinetic intervals were set to the lowest possible time and the assays were left to run for 15min at a set temperature of 35^0C.

Table 4.1: Experimental setup for Gtr, Mtr and Txr subversive substrate activity.

Stock [ ]	Reagent	Assay [ ]	Volume
GR/5000	Gtr/Mtr/Txr	**GR/50000**	20 µl
1.4mM	NADPH	**140uM**	20 µl
6mM	DTNB	**600uM**	20 µl
	Sample		10 µl
	Buffer		130 µl
		Final vol	200 µl

The Km-value of a substrate is the concentration where the enzyme is 50% active. This assay was carried out using various concentrations of the substrate. The sample concentrations ranged from 0–1320 µM. The enzyme velocity data were captured using KC junior™. Graphpad prism™ and Grafit were used for analysis and determination of the Vmax (maximum velocity of enzyme converting substrate to product – uM/min) and the Km-values on Gtr, Mtr and Txr.

4.3 Results and Discussion

The Km-values were determined from the subversive substrate activity experiments. The Km-value is essentially the binding affinity of the sample to the substrate. A low km-value indicates a better binding affinity and the low Km-value samples are a better substrate than samples with a higher Km-value.

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Km-values should not be confused with the quinone samples with the code KM. Due to solubility and precipitation problems, some of the quinone samples could not be tested. The garlic samples did not show any activity on Gtr, Mtr or Txr (Appendix 8).

4.3.1 Glutathiol reductase and Mycothiol reductase

The samples were first assayed for their subversive activity on Glutathiol and Mycothiol reductase. The Km-values were determined and are represented in Table 4.2.

Table 4.2: Substrate properties of quinones with Gtr and *M. tuberculosis* Mtr.

Sample	**Km (uM) Gtr [a]**	**Km (uM) Mtr [b]**
Aqs	6.726	9.815
CB5	69.36	57.36
IL 107	30.25	46.36
IL 113	480.36	479.36
IL 114	SP	SP
IL 135	654.36	523.36
IL142	356.36	289.39
IL 143	487.39	496.36
KM 41	SP	SP
KM 46	368.78	389.36
KM 60	SP	SP
KM 80	10.893	10.1336
KM 86	236.23	215.26
KM 94	SP	SP
KM 108	369.236	448.25
KM 108-1	199.6	104.7
KM117	816.36	798.36
KM139	897.36	903.25
KM140	605.03	645.7

[a]Half maximum activity of subversive substrate on Glutathiol reductase
[b]Half maximum activity of subversive substrate on Mycothiol reductase
SP–Solubility problems

Samples showing a lower Km-value on Mtr than on Gtr suggest that they act as better substrates on Mtr as compared to Gtr. In general there is no big difference between the subversive substrate

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activities on the two different enzymes. Two samples; $AqsNH_4$ and KM80, showed low Km-values of 6.7 µM and 10.8 µM on Gtr, suggesting that these samples had a higher binding affinity than the others. When comparing these results with previous studies conducted, it was found that these results correlated with other quinones tested against Mtr. In a study done by Mahapatra *et al*. (2007) it was found that some naphthoquinone derivatives had Km-values ranging from 36 to 483 µM, which is comparable with the Km-values for the tested quinone samples. The samples tested in the abovementioned study were all naphthoquinone derivatives, which correlate with the naphthoquinone samples in the present study, and these show similar Km-values. It can be concluded that anthraquinones had a higher affinity o the binding site as compared to the naphthoquinones. As mentioned above, some samples could not be tested due to solubility problems.

4.3.2 Thioredoxin reductase

The samples were evaluated for their subversive substrate activity against Thioredoxin reductase.

Table 4.3: Substrate properties of quinones with *M. tuberculosis* Txr.

Sample	Km (uM) [a]	std error
Aqs	93.1879	30.795
CB5	84.5742	44.4912
IL 107	19.3866	26.9266
IL 113	199.0154	135.4072
IL 114	21.5458	11.9962
IL 135	399.6545	218.9365
IL142	127.1886	215.1536
IL 143	27.0389	28.2178
KM 41	218.2336	142.8585
KM 46	92.5338	27.7837
KM 60	SP	SP
KM 80	9.4089	2.8276
KM 86	73.0759	46.2051
KM 94	SP	SP
KM 108	1521.442	488.134
KM 108-1	163.046	44.8219
KM117	1488.335	1942.031
KM139	2.388	3.1549
KM140	62.7374	7.7318

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[a]Half maximum activity of subversive substrate on Thioredoxin reductase
SP–Solubility problems

It is clear that these quinones do act as subversive substrates for Thioredoxin reductase. The Km-values were slightly lower on Txr in comparison to Gtr and Mtr, suggesting that these compounds have a higher affinity for thioredoxin reductase. This can also be seen in previous studies conducted, where it was found that quinones had a one order of magnitude higher affinity for Txr than Gtr and Trypanothione reductase (Tyr – also a pyridine nucleotide-disulfide oxidoreductase) (Cenas *et al*., 2004). Some samples had a lower Km-value, thus higher binding affinity. The samples with the best activity were found to be KM80 and KM139. Reaction catalytic velocity studies will be the next step to determine how fast the substrate is converted to a product. The Km-values only indicated the binding affinity to the enzyme and not the reaction rate.

4.4 Conclusion

It is clear that the quinone samples do act as substrates for all three enzymes. The affinity of these quinone samples is higher for Txr than Mtr and Gtr. These enzymes are viable drug targets that need to be investigated further. More quinone derivative can be produced/synthesized/isolated that could possibly show better activities on the bacterial enzyme analogs than the human analogs.

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Chapter 5

Final Conclusion and Recommendations

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Chapter 5 - Final Conclusion and Recommendations

5.1 Conclusion

Of all the quinone and polysulfide garlic samples tested, only a few showed promising results during the antimycobacterial and cytotoxicity assessments. Four samples: IL143, KM108-1, KM80 and KM140, showed very good activities with the lowest toxicities. The structures of IL143, KM108-1, KM80 and KM140, contain a chemical identity that are more selective in killing bacteria than killing human cells.

The samples showing the highest activity were; KM94, KM108-1 and KM117 as immune modulators, stimulating Interleukin 12 production, and KM180, $AqsNH_4$, CB5, IL107 and KM140 as hepatoprotectors.

It is clear that the quinone samples do act as substrates for all three enzymes tested. Mycothiol reductase and Thioredoxin reductase are viable drug targets that need to be investigated further. Anthraquinones were found to have a higher affinity to the flavoproteins than naphthoquinones.

Conclusion overview

- The tested quinones and polysulfide garlic mixtures acted as potent antimycobacterial agents.
- The samples showed medium to high toxicities on primary and secondary cell lines with some exceptions; where samples showed selective indices above 5.
- Three quinone compounds showed immune stimulatory effects (KM94, KM108-1 and KM117), and five showed hepatoprotective effects (KM108, KM140, AqsNH4, CB5 and IL107) at a concentration of ¼IC_{50}. Garlic samples: G5 and G6 showed slight immune stimulatory effects, while G6 was the only sample to have hepatoprotective activity at a concentration of ¼IC_{50}, probably due to the higher amount of diallyl tetrasulfide within the mixture.

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- The quinone samples showed subversive substrate activities on glutathiol, mycothiol and thioredoxin reductase enzymes.

5.2 Recommendations for further studies

- Metabolomics and NMR could be used to identify the specific entity that gives these compounds their antituberculosis activity over the others.

- More *in vivo* studies can be conducted on selected samples for its immune stimulatory and hepatoprotective effect.

- More quinone derivative can be produced/synthesized/isolated that that may show activity on bacterial enzyme analogs than human analogs.

- The enzymes assessed in this study can be studied further to identify the appropriate drug for these unique targets.

5.3 Manuscripts and presentations

5.3.1 Published article

Lall, N., Henley-Smith, C.J., De Canha, M.N., **Oosthuizen, C.B.**, Berrington, D. (2013). Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays. *International Journal of Microbiology*. Volume 5.

5.3.2 Manuscripts under preparation

- Oosthuizen, C.B., Hamilton, C., Lall, N. (2014). Selected quinone activity on disulfide reductase enzymes: Mycothiol and Thioredoxin reductase. *Journal of Biological Chemistry* (Under preparation)

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- Oosthuizen, C.B., Hamilton, C., Lall, N. (2014). Antimycobacterial activity of polysulfide mixtures. *BMC complementary and alternative medicine* (Under preparation)

5.3.3 Conference presentations

- The work represented in this dissertation was presented and the Fanie de Meilon post-graduate symposium at the Department of Plant Science, University of Pretoria. Awarded the prize for MSc best presentation 2013.

- The work will be presented at the 2014 Indigenous Plant Use Forum (IPUF) conference.

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Appendix 1

Manuscript published in the international journal of microbiology on the methods developed during this study

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Research Article

Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays

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This study compared different commercially available viability reagents. The growth indicator reagents include *p*-iodonitrotetrazolium violet (INT), PrestoBlue, and Alamar Blue which were used for antimicrobial analysis against *Streptococcus mutans*, *Prevotella intermedia*, *Propionibacterium acnes*, and *Mycobacterium tuberculosis*. PrestoBlue and Alamar Blue are resazurin based reagents that resulted in a quick and easily distinguishable colour change that allowed for visual readings. INT and Sodium 3′-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate (XTT) are tetrazolium based reagents which are converted to a formazan dye in the presence of metabolically active mitochondria enzyme. For cell viability analysis, reagents XTT and PrestoBlue were compared. PrestoBlue was able to clearly indicate the minimum inhibitory concentration (MIC) of various positive drug controls on various microbial strains. PrestoBlue was also a good indicator of the 50% inhibitory concentration (IC_{50}) of positive drug controls on various cell lines.

1. Introduction

PrestoBlue and Alamar Blue reagents are resazurin based, membrane permeable solutions that upon reduction form resorufin, a red fluorescent compound which can be quantitatively measured to determine viability. Initially developed as a cell viability indicator, PrestoBlue has been indicated for use on nonmammalian cells, such as bacteria, yeast, and eukaryotic cells. The variable reading methods of PrestoBlue makes this reagent an attractive alternative in cellular and microbiology. PrestoBlue can be measured either visually, using absorbance or utilising the fluorescent outputs of the reduced resorufin [1].

p-iodonitrotetrazolium violet (INT) is a tetrazolium dye precursor that once reduced forms a purple formazan dye (Sigma-Aldrich). Sodium 3′-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate (XTT) is also a tetrazolium based reagents which in the presence of metabolically active cells reduces the tetrazolium salt to an orange coloured formazan compound. The reduction of the tetrazolium salt is due to the activity of the mitochondria enzyme in the active cells. The intensity of the formazan compound can be measured using absorbance where the intensity of the compound is directly proportional to the number of metabolically active cells [2].

The aim of this study was to validate the use of PrestoBlue as a growth indicator and cell viability reagent by comparing it to other similar commercially available reagents.

2. Materials and Methods

2.1. Chemicals and Reagents. All microbial strains were purchased from American Type Culture Collection (ATCC), MD, USA. The A431 cell line and HeLa cell line were purchased from ECACC and Highveld Biological (Pty) Ltd., respectively. INT was obtained from Sigma-Aldrich, South Africa. PrestoBlue and Alamar Blue were both purchased

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from Invitrogen Corporation, San Diego, USA. XTT cell proliferation Kit II was obtained from Roche Applied Sciences, South Africa. The cell culture medium, trypsin-EDTA, fetal bovine serum (FBS), phosphate buffer saline (PBS), and antibiotics were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA). All other reagents were of analytical grade.

2.2. Determination of the Minimum Inhibitory Concentration (MIC) for the Microbial Strains

2.2.1. Culturing of Microorganisms. The microorganisms used in this study included *Prevotella intermedia* (ATCC 25611), *Streptococcus mutans* (ATCC 25175), and *Propionibacterium acnes* (ATCC 11827). *S. mutans* and *P. intermedia* were grown on Casein-peptone Soymeal-peptone Agar medium (CASO) enriched with 1% sucrose (Merck Chemicals (Pty) Ltd., Wadeville, South Africa) under anaerobic conditions in an anaerobic jar with Anaerocult A (Merck KGaA, Darmstadt, Germany) at 37°C for 48 hrs. Subculturing was done every second week. Inoculants were prepared by suspending bacterial test organisms in quarter strength sterile Ringer solution (Merck KGaA, Darmstadt, Germany) until turbidity was compatible with McFarland Standard 1 (Merck Chemicals (Pty) Ltd., Wadeville, South Africa). Furthermore a bacterial concentration of 4×10^8 (colony forming units) CFU/mL for *P. intermedia* and 3×10^8 CFU/mL for *S. mutans* was used [3].

P. acnes was cultured on Tryptone Soy Agar (Merck Chemicals (Pty) Ltd., Wadeville, South Africa) under anaerobic conditions in an anaerobic jar with Anaerocult A at 37°C for 72 hrs. Subculturing was done every 4th week as the doubling time of the bacterium is slow. Inoculants were prepared by suspending the bacterial colonies in Nutrient Broth (Merck Chemicals (Pty) Ltd., Wadeville, South Africa) until turbidity was compatible with a McFarland Standard 0.5 at a bacterial concentration of 1.5×10^8 CFU/mL [4].

2.2.2. Evaluation of MIC Values for Microorganisms. The microdilution technique using 96-well microtitre plates, as described by Eloff [5], was used to obtain the MIC values of the positive drug controls against the various microorganisms. The positive controls for oral bacteria, chlorhexidine gluconate (CHX) (Dental Warehouse, Sandton, South Africa) at 12.5 μg/mL, and Tetracycline (Sigma-Aldrich, 3050 Spruce Street, St. Louis) at 200 μg/mL for *P. acnes* were serially diluted in relevant broth medium adding 48 h old oral bacteria and 72 h old *P. acnes* and were incubated at 37°C in anaerobic conditions. The final concentration of CHX, ranged 3.13 μg/mL–2.44×10^{-2} μg/mL and Tetracycline ranged 100 μg/mL–0.781 μg/mL. *S. mutans* and *P. intermedia* were incubated for 24 hrs and *P. acnes* for 72 hrs at 37°C.

To indicate bacterial growth, 40 μL of (0.2 mg/mL) INT, 20 μL PrestoBlue, and 20 μL Alamar Blue was added to microplate wells and reincubated until a colour change occurred. The MIC was defined as the lowest concentration that inhibited the growth of the bacteria [6].

2.2.3. Evaluation of MIC against Mycobacterium tuberculosis. For the antimycobacterial assay the H37Rv (ATCC 27264) *M. tuberculosis* strain was used. The bacteria were cultured on Löwenstein-Jensen (LJ) medium (SA Medical Research Council, Pretoria) for three weeks (37°C, 5% CO_2). One colony was transferred under sterile conditions to 50 mL of 7H9 broth media (Sigma-Aldrich, South Africa), 10% OADC (Sigma-Aldrich, South Africa) and 2% PANTA (Becton, Dickinson and Company, USA). The bacteria were further cultured for one week. After the final incubation period, the bacteria were adjusted in 7H9 broth media to a turbidity of a McFarland Standard 1. Bacterial suspensions were further diluted 1 : 25 to obtain a concentration of 2×10^8 CFU/mL [7].

The Microtitre Alamar Blue assay (MABA) and Microtitre PrestoBlue assay (MPBA) were used to determine the MIC of positive drug controls on the bacteria [7]. Standard antituberculosis agents, INH (Sigma-Aldrich, Becton, Dickinson and Company), and RIF (Sigma-Aldrich, Becton, Dickinson and Company) were used. Stock solutions were diluted in 7H9 broth media to a final assay concentrations which ranged 5 μg/mL–0.078 μg/mL and 6 μg/mL–0.1875 μg/mL for INH and RIF, respectively. A 2% dimethyl sulfoxide (DMSO) solvent control, media control, and bacterial control was included in the assay. One hundred microlitres of bacteria was added to the inner wells of the 96-well plates. The outer perimeter wells were used to compensate for evaporation by adding 200 μL of dH_2O. The plates were incubated for 5 to 7 days at 37°C, 5% CO_2. After 5 days 20 μL of Alamar Blue/PrestoBlue indicator solution was added to one of the bacterial control wells. If a colour change was observed, all the subsequent wells received 20 μL of indicator solution. If no colour change was observed the plates were incubated for further 24 hrs.

2.3. Comparison of Growth Indicators for Cytotoxicity Analysis.

The HeLa and A431 cell lines were maintained in Eagle's Minimum Essential Medium supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) and 250 μg/mL fungizone. The cells were grown at 37°C in a humidified incubator set at 5% CO_2. Cells were subcultured by treating them with trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 minutes.

Cytotoxicity was measured using the XTT cell proliferation Kit II and MPBA. The method described by Zheng et al. [8] was used to perform the assay. Both cell lines were seeded in a 96-well microtitre plate at a concentration of 1×10^5 cells/mL. Cells were allowed to attach for 24 hrs at 37°C and 5% CO_2. The cells were exposed to the positive drug control Actinomycin D (Sigma-Aldrich, South Africa) with concentrations ranging between 0.5 μg/mL and 0.002 μg/mL. The microtitre plate was incubated for further 72 hrs and thereafter 50 μL XTT was added to a final concentration of 0.3 mg/mL to one set of plates and 20 μL PrestoBlue was added to another set of plates. The plates were incubated for further 2 hrs where after the absorbance of the colour complex was read at 490 nm with a reference wavelength set at 690 nm for XTT and at 570 nm with a reference wavelength set at 600 nm for PrestoBlue, using a BIO-TEK Power-Wave XS multiwell plate reader.

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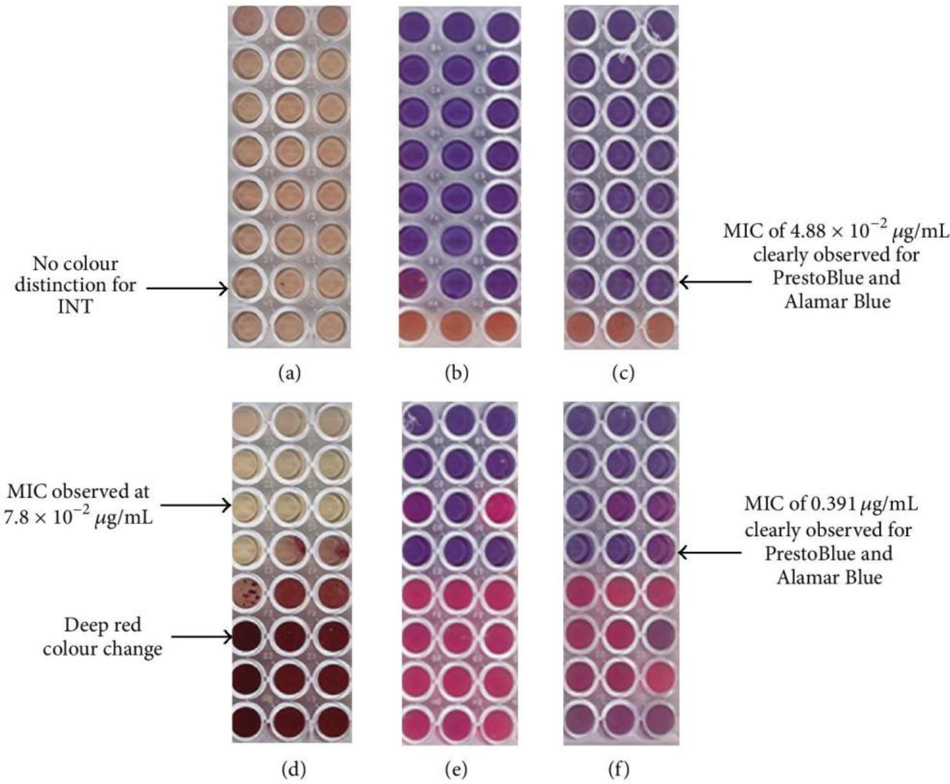

Figure 1: Comparison of different growth indicators on oral microorganisms, (a) INT on *S. mutans,* (b) PrestoBlue on *S. mutans,* (c) Alamar Blue on *S. mutans,* (d) INT on *P. intermedia,* (e) PrestoBlue on *P. intermedia* and (f) Alamar Blue on *P. intermedia.*

3. Results and Discussion

3.1. Determination of MIC for Microbial Strains

3.1.1. Evaluation of MIC against Oral Microorganisms. The colour change of the growth indicators INT, PrestoBlue, and Alamar Blue for *S. mutans* and *P. intermedia* is depicted in Figure 1.

The development time for each of the oral bacteria differed. With the addition of INT, *P. intermedia* developed within 40 minutes; however, *S. mutans* development took over two hours and it was difficult to distinguish where the MIC occurred. Conversely with PrestoBlue or Alamar Blue, *S. mutans* developed immediately and the MIC was easily distinguishable, with pink indicating viable bacteria and blue indicating nonviable bacteria. *P. intermedia* developed within 20 minutes, although the colour change took slightly longer with Alamar Blue. The MIC values obtained for the inhibition of *S. mutans* and *P. intermedia* by CHX showed the same values using both PrestoBlue and Alamar Blue at 4.88×10^{-2} μg/mL and 0.391 μg/mL, respectively. *P. intermedia* showed a slightly higher MIC with the development of INT (at 0.78 μg/mL). It was almost impossible to determine the MIC value for the inhibition of *S. mutans* using INT. There appeared to be a distinctive colour change for each of the bacteria whether using PrestoBlue or Alamar Blue.

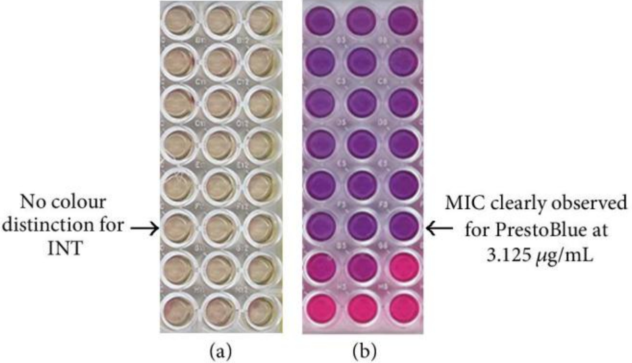

Figure 2: Comparison of different growth indicators on *P. acnes*: (a) INT and (b) PrestoBlue on *P. acnes*.

3.1.2. Evaluation of MIC against Propionibacterium acnes. The colour change of the growth indicators INT and PrestoBlue for *P. acnes* were observed after addition of the reagents as seen in Figure 2.

The comparison between INT and PrestoBlue for determination of MIC values for bacterial cells treated with tetracycline was clearly indicated with the use of PrestoBlue when compared with INT. The *P. acnes* bacteria had a tendency to coagulate at the bottom of the wells during the

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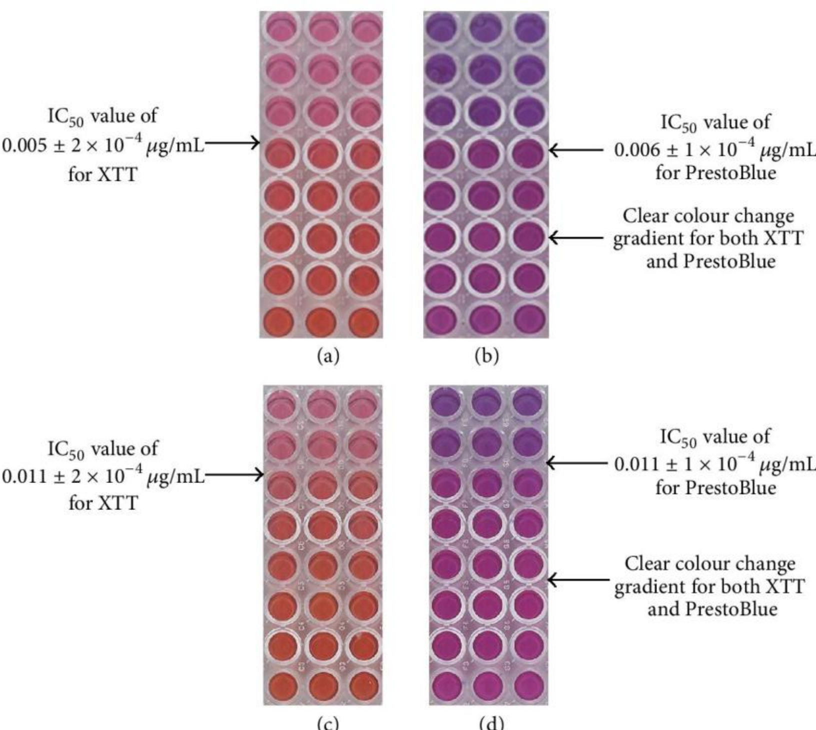

FIGURE 3: Comparison of different growth indicators on various cell lines (a) XTT on HeLa cells, (b) PrestoBlue on HeLa cells, (c) XTT on A431 cells, and (d) PrestoBlue on A431 cells.

incubation period and this was where the INT was reduced to the violet colour. The PrestoBlue provided an advantage in that the entire well was coloured, making visualisation of MIC values easier. Tetracycline treated cells in the presence of INT showed no colour change as seen in Figure 2(a). However in the presence of PrestoBlue, there was a clear distinction between wells with metabolically active bacterial cells (pink) and those which had been inhibited (blue) by the tetracycline control drug. Although the incubation period of the PrestoBlue plates was slightly longer (±3 hrs) compared with INT (±1 h) the determination of MIC values was much clearer. The MIC of tetracycline was determined to be 3.125 μg/mL with PrestoBlue and could not be visually determined with INT.

3.1.3. Evaluation of MIC against Mycobacterium tuberculosis. The health risk involved in working with *M. tuberculosis* meant no clear pictures of the colour changes could be obtained.

Due to the slow growth rate of *M. tuberculosis*, the conversion of the Alamar Blue and PrestoBlue into a red colour took up to 24 hrs. Although a colour change could be observed after 3 hrs, the longer incubation period resulted in a more distinguishable separation between viable pink bacterial wells and blue inhibited wells. There were no differences observed between the MIC values of the positive controls when comparing Alamar Blue and PrestoBlue. INH showed an MIC of 0.13 μg/mL whereas Rifampicin showed an MIC of 0.75 μg/mL for both PrestoBlue and Alamar Blue. The PrestoBlue had a slightly faster conversion rate compared to the Alamar Blue, but at 24 hr incubation the readings of the MICs were the same.

3.2. Comparison of Growth Indicators for Cytotoxicity Analysis. XTT and PrestoBlue were used to determine the antiproliferative effect of Actinomycin D on HeLa cells and A431 cells as seen in Figure 3.

For both growth indicators a colour change was observed after approximately 2 hrs. Both cell lines were exposed to Actinomycin D and the IC_{50} values were determined.

The IC_{50} values observed when comparing XTT to PrestoBlue were similar. On the HeLa cells the positive control showed IC_{50} values of $0.005 \pm 2 \times 10^{-4}$ μg/mL and $0.006 \pm 1 \times 10^{-4}$ μg/mL for XTT and PrestoBlue, respectively, whereas on the A431 cells the positive control showed IC_{50} values of $0.011 \pm 2 \times 10^{-4}$ μg/mL for both growth indicators. The colour change was observed at approximately the same time and both growth indicators showed a distinguishable colour change.

PrestoBlue showed great potential as a growth indicator for various microorganisms as well as a cell viability. It has minor limitations such as light sensitivity and the varied time taken for colour development, which is dependent on the metabolic rates of various bacteria and cell cultures. Although these are potential drawbacks the results remain conclusive.

4. Conclusion

PrestoBlue and Alamar Blue give clear and easily determined visual MIC values for *S. mutans*, *P. intermedia*, *P. acnes*, and

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M. tuberculosis. However, *S. mutans*, *P. intermedia*, and *M. tuberculosis* developed a colour change more rapidly in PrestoBlue than in Alamar Blue. Furthermore Alamar Blue and PrestoBlue as growth indicator reagents have an advantage over other reagents, such as INT, in that the whole assay well changes colour instead of only the bacteria. Therefore PrestoBlue may be the better indicator for these microorganisms.

Comparing the reagents XTT and PrestoBlue as cell viability indicators it was observed that both growth indicators showed clear distinction between viable cells and nonviable cells and the IC_{50} values obtained from both reagents were similar.

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Appendix 2

Antimycobacterial activity protocol developed and used during this study

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Microtitre Alamar Blue Assay Drug susceptibility assay	Document No.	*SOP-1-1*
	Version No.	001

BACKGROUND

This assay is used for the screening of extracts and pure isolated/synthetic compounds for activity against *Mycobacterium* species. It's based on the reduction of blue resazurin salt to a red/pink resorufin salt, by viable bacteria.

PURPOSE

This procedure is being standardized and implemented to achieve the following purposes:

To observe if the sample show any growth inhibition of the bacteria.

To determine the MIC of the sample.

APPLICABILITY

This procedure is applicable to all samples tested for susceptibility against *Mycobacteria*.

RESPONSIBILITIES AND PREPERATION

What will be needed:

96 well plates

Alamar blue

Bacterial inoculum

Biohazard disposable container

DMSO

Eppies

Glass beads (2mm)

INH

McFarland standard solution

PBS

Pipettes (10, 200, 1000ul and multichannel)

Sterile 7H9 broth media

Sterile distilled water

Sterile empty agar plates (reservoir)

Tips

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Preparation:

2 weeks before – Start culturing the bacteria by inoculating fresh 7H9 media (40ml) with a colony of H37Rv *Mycobacteria*. Incubate at 35 ◦C until the day of the experiment.

One day before – Autoclave all the tips, consumables and reagents. Weigh out sample and collect all the material needed for the experiment.

DEFINITIONS

CFU's – Colony forming units

MABA – Microtitre alamar blue assay

MIC – Minimum inhibitory concentration

Mtb – *Mycobacterium tuberculosis*

OADC – Oleic acid, albumin, dextrose, catalase

OD – Optical density

PANTA – Antibiotic mixture/supplement

TB – tuberculosis

PROCEDURE

Inoculum preparation

Add glass beads (approximately 20 beads per 40ml)

Vortex bacterial culture with glass beads for 5 min

Set aside to rest for 5 min

Adjust culture to the correct inoculation size ($1.5x10^{8}$CFU's/ml, 0.5 McFarland)

Sample preparation

Dissolve sample in 100% DMSO to the sought after concentration (20x higher than test concentration)

Further dilute sample by adding 100ul to 400ul 7H9 media

Add 100ul to the first well in duplicate/triplicate

Plate preparation

Label plates

Add 200ul of sterile distilled water in all the outer wells

Add 100ul of sterile 7H9 broth media to all the inner wells

Control preparation

DMSO solvent control – Add 100ul of 100% DMSO to 400ul of 7H9 media. Add 100ul to the first well, duplicate/triplicate.

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INH positive control – Weigh out 2mg of INH and dissolve in 1ml of sterile distilled water (2000ug/ml). Dilute further by adding 50ul to 950ul of water (100ug/ml). Dilute further by adding 50ul to 1200ul of 7H9 media (4ug/ml). Add 100ul to the first well. Highest concentration will be 1ug/ml.

Bacterial control – 100ul of media and 100ul of prepared bacterial inoculums

Media control – 200ul of 7H9 media

Experimental procedure

After all the samples and controls have been added, all the first wells will contain 200ul.

Prepare a serial two-fold dilution downwards for six concentrations, and discard the last 100ul.

Add 100ul of the prepared bacterial inoculums to all the inner wells, except the media control wells.

Seal with parafilm and incubate for 10 days at 35 C.

Addition of Alamar blue

After 9 days of incubation, add 40ul of 1:1 alamar blue: 10% Tween 80 solution. If a color change is observed after another 24hour incubation step, add 40ul of the same solution to all the wells.

The MIC is described as the highest concentration where no colour change was observed.

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Appendix 3

Peripheral blood mononuclear isolation protocol used during this study

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Appendix 3

PBMC's isolation & Separation of Lymphocytes from Monocytes

BACKGROUND

This assay is used for the isolation of Peripheral Blood Mononuclear Cells and the separation of the white blood cells into the lymphocyte and monocytes populations.

PURPOSE

This procedure is being standardized and implemented to achieve the following purposes:

To isolate the PBMC's

To separate the lymphocytes from the monocytes

To harvest cells for subsequent experiments (cytotoxicity & immunomodulation)

APPLICABILITY

This procedure is applicable for the isolation of PBMC's or for lymphocyte and monocytes.

RESPONSIBILITIES AND PREPERATION

What will be needed:

Falcon tubes (1.8ml, 15ml, 50ml)

Racks for tubes

Tips (Yellow, blue, 10ml, 50ml)

Waste beaker (with conc. Bleach)

Beaker with autoclave bag

Haemocytometer

RPMI

FBS

DMSO

Ficoll

ACK

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PBMC's isolation & Separation of Lymphocytes from Monocytes

PREPARATION:

Preheat media and ACK to 37°C before starting with the experiment.

Collect blood from clinic.

Spray all the consumables with 70%EtOH before putting it in the flow hood.

All liquid waste goes in the beaker with bleach. In a 1L beaker, pour concentrated bleach to 100ml. Add dH_2O to 500ml. Used tips and supernatant from centrifugation must go into this container but should not exceed the 1L mark such that the final concentration of bleach is 10% prior to autoclaving.

DEFINITIONS

ACK – Lysis buffer to lyse erythrocytes

CFU's – Colony forming units

DMSO – Dimethyl sulphoxide

FBS – Foetal bovine serum

Ficoll – density gradient of 1.077g/ml

PROCEDURE

PBMC's isolation

- Transfer the blood from the EDTA tubes to 50ml tubes.
- Take note of the volume transferred, and dilute the blood 1:1 with RPMI media (incomplete).
- Turn off the light (Ficoll is very sensitive).
- Add ficoll to a clean 50ml falcon (volume: half the final volume of the diluted blood).
- Layer the blood on to the ficoll at a 45° angle very slowly but steady.
- Centrifuge at 3000rpm, room temperature for 30min.
- Pipette PBMC's (white cloudy layer) into new falcon tube.
- Add RPMI media to the cells (approx. 30ml).
- Centrifuge at 2200rmp, room temperature for 10min.
- Pour of supernatant.
- To get rid of contaminating red blood cells add 5ml ACK, mix and wait for 5min.
- Add approximately 15ml RPMI media.
- Centrifuge at 1200 rpm, room temperature for 10min.
- Pour of supernatant.

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PBMC's isolation & Separation of Lymphocytes from Monocytes

- Re-suspend the cells in approximately 5ml of RPMI media.

PBMC STIMULATION

- PHA at a concentration of 2ug/ml can be used to stimulate the cells.
- As a positive control 5ug/ml can be used to stimulate cytokine production.

SEPARATION OF MONOCYTES FROM LYMPHOCYTES

- Plate the re-suspended cells on to two sterile petri dishes (spread over the whole plate).
- Incubate the plates at 37°C for two hours (the monocytes will adhere to the plastic).
- After incubation pour of the top layer of cells still in suspension (lymphocytes) and wash the excess cells of with RPMI carefully not to disturb the monocytes.
- The lymphocytes can be used in subsequent experiments
- Using a sterile cell scraper, scrape the cells from the plastic. Media can be added to help in the scraping process.
- Collect the cells by pipetting the cells up and transferring them to a new falcon tube.
- The scraping process can be repeated until most of the cells have been collected.
- Centrifuge the cells at 1200rpm for 10min.
- Pour of the supernatant and re-suspend the cells in a known volume of 10% RPMI media.
- Count and adjust the cells to the right concentration for the subsequent experiment.

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Appendix 3

PBMC's isolation & Separation of Lymphocytes from Monocytes

Differentiation of Monocytes to Macrophages

- Make up a 100ug/ml stock solution of PMA.
- For every 1ml of cells, add 1ul of the 100ug/ml PMA solution.
- Plate the cells and incubate for 7 days at 37°C, replacing the media at day 4 if necessary.
- After 7 days of incubation wash cells and run subsequent experiment.

REFERENCES

Helmholtz Zentrum Munich CCG Immune Monitoring Protocol: PBMC Isolation, cryopreservation and thawing

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Appendix 4

Enzyme-linked immune sorbent assay protocol used during this study

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Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance from each.

Plot the standard curve on log-log graph paper, with IL-12 concentration on the x-axis and absorbance on the y-axis. Draw the best fit curve through the standard points.

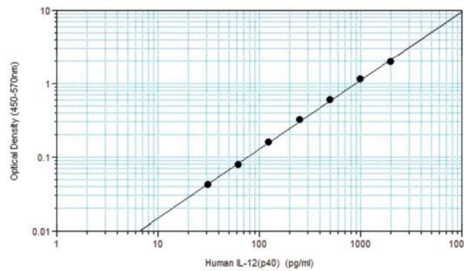
To determine the IL-12 concentration of the unknowns, find the unknown's mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the IL-12 concentration. If samples were diluted, multiply the IL-12 concentration by the dilution factor.

Computer data reduction may also be employed, utilizing log-log regression analysis.

Typical Standard Curve

This standard curve is for demonstration only. A standard curve must be run with each assay.

BD OptEIA™ Human IL-12 (p40) Set



Specificity

Cross Reactivity: The following factors were tested in the BD OptEIA™ assay at ≥ 10 ng/mL and no cross-reactivity (value ≥ 15 pg/mL) was identified.

Recombinant Human
IL-1α, IL-1β, IL-2, IL-3 IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, IL-15, G-CSF, GM-CSF, IFN-γ, CD23, Lymphotactin, MIP-1α, MIP-1β, MCP-1, MCP-2, NT-3, PDGF-AA, SCF, TNF, LT-α (TNF-β), VEGF

Recombinant Mouse
IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), IL-15, IFN-γ, GM-CSF, MCP-1, TCA3, TNF

Recombinant Rat
IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF

Other:
Viral IL-10 (1 ng/mL), Rabbit TNF

2621KI_555171 Rev.2

Standardization

This immunoassay is calibrated against purified Baculovirus-expressed recombinant human IL-12 (p40).

Assay Optimization

1. BD OptEIA™ Sets allow flexible assay design to fit individual laboratory needs. To design an immunoassay with different sensitivity and dynamic range, the following parameters can be varied: Capture, Detection Antibody titers, Incubation time, Incubation temperature, Assay Diluent formulation, Buffer pH, ionic strength, protein concentration, Type of substrate, Washing technique (i.e., number of wash repetitions and soak times)
2. "Typical Standard Curve" and 20-plate yield were obtained in the BD Biosciences Pharmingen laboratory, using the recommended procedure and manual plate washing.

Troubleshooting

Poor Precision

Possible Source	*Corrective Action*
• Inadequate washing/ aspiration of wells	• Check function of washing system
• Inadequate mixing of reagents	• Ensure adequate mixing
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Incomplete sealing of plate	• Ensure complete seal on plate

Poor Standard Curve

Possible Source	*Corrective Action*
• Improper standard handling/ dilution age of standards	• Ensure correct preparation, stor
• Incomplete washing/ aspiration of wells	• Check function of washing system
• Imprecise/ inaccurate pipetting	• Check/ calibrate pipettes
• Improper buffer/ diluent used pH	• Check buffer/ diluent preparation,

Low Absorbances

Possible Source	*Corrective Action*
• Inadequate reagent volumes added to wells	• Check/ calibrate pipettes
• Incorrect incubation times/ temperature times/reagents warmed to RT	• Ensure sufficient incubation
• Incorrect antibody titration Detector preparation	• Check Capture Ab and Working
• Improper buffer/ diluent used pH	• Check buffer/ diluent preparation,
• Overly high wash/aspiration pressure from automated plate-washer	• Utilize manual washing

Limitations of the Procedure

• Samples that generate absorbance values higher than the standard curve should be diluted with Standard Diluent and re-assayed.
• Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded.
• BD OptEIA™ Sets are intended for use as an integral unit. Do not mix reagents from different Set batches. Reagents from other manufacturers are not recommended for use in this Set.

BD OptEIA™

Technical Data Sheet

Human IL-12 (p40) ELISA Set

Cat. No. 555171

Materials Provided

The OptEIA™ Set for human interleukin-12 (IL-12 (p40)) contains the components necessary to develop enzyme-linked immunosorbent assays (ELISA) for natural or recombinant human IL-12(p40) in serum, plasma, and cell culture supernatants. Sufficient materials are provided to yield approximately 20 plates of 96-wells if the recommended storage, materials, buffer preparation, and assay procedure are followed as specified in this package.

Capture Antibody

Anti-Human IL-12 monoclonal antibody

Detection Antibody

Biotinylated Anti-Human IL-12 monoclonal antibody

Enzyme Reagent

Streptavidin-horseradish peroxidase conjugate (SAv-HRP)

Standards

Recombinant human IL-12 (p40), lyophilized

Instruction / Analysis Certificate

(lot-specific)

United States
877.232.8995

Canada
888.259.0187

Europe
32.53.720.211

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9995

BD
BD Biosciences

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10975 Torreyana Road
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Recommended buffers, solutions

Note: Do not use sodium azide in these preparations. Sodium azide inactivates the horseradish peroxidase enzyme.

The BD OptEIA™ Reagent Set B (Cat. No 550534) containing Coating Buffer, Assay Diluent, Substrate Reagents A and B, Stop Solution and 20X Wash Buffer Concentrate is recommended.

1. **Coating Buffer** - 0.1 M Sodium Carbonate, pH 9.5
   7.13 g $NaHCO_3$, 1.59 g Na_2CO_3; q.s. to 1.0 L; pH to 9.5 with 10 N NaOH.
   Freshly prepare or use within 7 days of preparation, stored at 2-8°C.
2. **Assay Diluent**- PBS* with 10% FBS#, pH 7.0. The BD Pharmingen™ Assay Diluent (Cat. No. 555213) is recommended.
   *Phosphate-Buffered Saline: 80.0 g NaCl.11.6 g Na_2HPO_4, 2.0 g KH_2PO_4, 2.0 g KCL, q.s. to 10 L; pH to 7.0.
   #Fetal Bovine Serum: Hyclone Cat. No. SH30088 (heat-inactivated) recommended.
   Freshly prepare or use within 3 days of preparation, with 2-8°C storage.
3. **Wash Buffer** - PBS* with 0.05% Tween-20. Freshly prepare or use within 3 days of preparation, stored at 2-8°C.
4. **Substrate Solution** - Tetramethylbenzidine (TMB) and Hydrogen Peroxide. The BD Pharmingen™ TMB Substrate Reagent Set (Cat. No. 555214) is recommended.
5. **Stop Solution** - 1 M H_3PO_4 or 2 N H_2SO_4

Additional Materials Required

1. 96-well Nunc-Immuno™ polystyrene Maxisorp ELISA flat bottom plates (ThermoFisher Scientific Cat. No. 442404) are recommended
2. Microplate reader capable of measuring absorbance at 450 nm
3. Precision pipettes
4. Graduated cylinder, one liter
5. Deionized or distilled water
6. Wash bottle or automated washer
7. Log-log graph paper or automated data reduction
8. Tubes to prepare standard dilutions
9. Laboratory timer
10. Plate sealers or parafilm

Storage Information

1. Store unopened reagents at 2-8°C. Do not use reagents after expiration date, or if turbidity is evident.
2. Before use, bring all reagents to room temperature (18-25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until expiration date. See below for reconstituted standard storage information.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic.

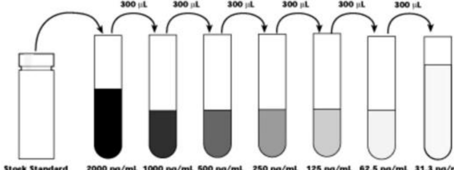
Cell culture supernatants: Remove any particulate material by centrifugation and assay immediately or store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using citrate, EDTA, or heparin as anticoagulant. Centrifuge for 10 minutes at 1000 x g within 30 minutes of collection. Assay immediately or store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Standards Preparation and Handling

1. **Reconstitution:** After warming lyophilized standard to room temperature, carefully open vial to avoid loss of material. Reconstitute lyophilized standard with 1.0 mL of deionized water to yield a stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex gently to mix.
2. **Storage/ handling of reconstituted standard:** After reconstitution, **immediately** aliquot standard stock in polypropylene vials at 50 µl per vial and freeze at -80°C for up to 6 months. If necessary, store at 2-8° C for up to 8 hours prior to aliquotting/freezing. Do not leave reconstituted standard at room temperature.
3. **Standards Preparation for Assay:**
   a. Prepare a 2000 pg/mL standard from the stock standard. Vortex to mix. (See dilution instructions on Instruction/Analysis Certificate.)
   b. Add 300 µL Assay Diluent to 6 tubes. Label as 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, and 31.3 pg/mL.
   c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. Assay Diluent serves as the zero standard (0 pg/mL).



Serial dilutions within the plate may also be performed by pipetting 100 µL of Assay Diluent into each standard well except the highest (2000 pg/mL), then adding 100 µL of the 2000 pg/mL standard to both that well and the 1000 pg/mL well, mixing the well contents by rinsing the pipette tip, and adding 100 µL of the 1000 pg/mL standard to the 500 pg/mL well. Continue these dilutions to the 31.3 pg/mL standard well, out of which the extra 100 µL should be discarded.

Working Detector Preparation

(Note: One-step incubation of Biotin/Streptavidin reagents.) Add required volume of Detection Antibody to Assay Diluent. Within 15 minutes prior to use, add required quantity of Enzyme Reagent, vortex or mix well. For recommended dilutions, see lot-specific Instruction/Analysis Certificate. For a full 96-well plate, prepare 12 mL of Working Detector. Discard any remaining Working Detector after use.

Warnings and Precautions

1. Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
2. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
3. Capture Antibody contains < 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
4. Detection Antibody contains BSA and ProClin®-150 as a preservative.
5. Enzyme Reagent contains BSA and ProClin®-150 as a preservative.
6. Source of all serum proteins is from USDA inspected abattoirs located in the United States.

Recommended Assay Procedure

1. Coat microwells with 100 µL per well of Capture Antibody diluted in Coating Buffer. For recommended antibody coating dilution, see lot-specific Instruction/Analysis Certificate. Seal plate and incubate overnight at 4° C.
2. Aspirate wells and wash 3 times with ≥ 300 µL/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.
3. Block plates with ≥ 200 µL/well Assay Diluent. Incubate at RT for 1 hour.
4. Aspirate/wash as in step 2.
5. Prepare standard and sample dilutions in Assay Diluent. See "Standards Preparation and Handling".
6. Pipette 100 µL of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hours at RT.
7. Aspirate/ wash as in step 2, but with 5 total washes.
8. Add 100 µL of Working Detector (Detection Antibody + SAv-HRP reagent) to each well. Seal plate and incubate for 1 hour at RT.
9. Aspirate/ wash as in step 2, but with 7 total washes.
10. Add 100 µL of Substrate Solution to each well. Incubate plate (without plate sealer) for 30 minutes at room temperature in the dark.
11. Add 50 µL of Stop Solution to each well.
12. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract absorbance at 570 nm from absorbance 450 nm.

Assay Procedure Summary

1. Add 100 µL diluted Capture Ab to each well. Incubate overnight at 4° C
2. Aspirate and wash 3 times.
3. Block plates: 200 µL Assay Diluent to each well. Incubate 1 hr RT
4. Aspirate and wash 3 times.
5. Add 100 µL standard or sample to each well. Incubate 2 hr RT.
6. Aspirate and wash 5 times.
7. Add 100 µL Working Detector (Detection Ab + SAv-HRP) to each well. Incubate 1 hr RT
8. Aspirate and wash 7 times
9. Add 100 µL Substrate Solution to each well. Incubate 30 min RT in dark
10. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 min with λ correction 570 nm.

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Appendix 5

Cytometric bead array protocol used during this study

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3

Assay preparation

This section covers the following topics:

- Preparation of Human Th1/Th2 Cytokine Standards (page 18)
- Mixing Human Th1/Th2 Cytokine Capture Beads (page 20)
- Diluting samples (page 22)

Preparation of Human Th1/Th2 Cytokine Standards

Purpose of this procedure

The Human Th1/Th2 Cytokine Standards are lyophilized and must be reconstituted and serially diluted immediately before mixing with the Capture Beads and the PE Detection Reagent.

You must prepare fresh cytokine standards to run with each experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Human Th1/Th2 Standards. Transfer the standard spheres to a 15-mL conical, polypropylene tube. Label the tube "Top Standard."
2. Reconstitute the standards with 2.0 mL of Assay Diluent.
   a. Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.
   b. Gently mix reconstituted protein by pipet only. Do not vortex or mix vigorously.
3. Label eight 12 × 75-mm tubes and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
4. Pipette 300 µL of Assay Diluent to each of the remaining tubes.

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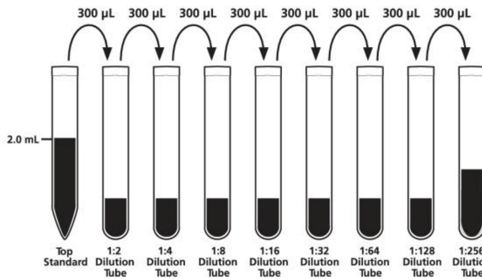
5. Perform a serial dilution:
   a. Transfer 300 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipet only.
   b. Continue making serial dilutions by transferring 300 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube.
   c. Mix thoroughly by pipet only. Do not vortex.



6. Prepare one 12 x 75-mm tube containing only Assay Diluent to serve as the 0-pg/mL negative control.

Concentration of standards

See the table in Performing the Human Th1/Th2 Cytokine II Assay (page 24) for a listing of the concentrations (pg/mL) of all six recombinant proteins in each standard dilution.

Next step

Proceed to Mixing Human Th1/Th2 Cytokine Capture Beads (page 20).

Mixing Human Th1/Th2 Cytokine Capture Beads

Purpose of this procedure

The Capture Beads are bottled individually (A1–A6). You must pool all six bead reagents immediately before mixing them in the assay.

Follow the procedure to mix the Capture Beads for all sample types. Then perform additional steps to incubate the beads in Serum Enhancement Buffer, if analyzing serum and/or plasma samples, to reduce the chances of false-positive results due to the effects of serum or plasma proteins.

Procedure

To mix the Capture Beads when testing any sample type:

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (eg, 8 unknowns, 9 cytokine standard dilutions, and 1 negative control = 18 assay tubes).
2. Vigorously vortex each Capture Bead suspension for a few seconds before mixing.
3. Add a 10-µL aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "mixed Capture Beads" (eg, 10 µL of IL-2 Capture Beads × 18 assay tubes = 180 µL of IL-2 Capture Beads required).
4. Vortex the bead mixture thoroughly.
5. If you are testing serum or plasma samples, you will need to incubate the Capture Beads in Serum Enhancement Buffer. Proceed to Additional steps when testing serum and plasma samples (page 21).

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Next step

The mixed Capture Beads are now ready to be transferred to the assay tubes. Discard excess mixed Capture Beads. Do not store after mixing.

To begin the assay, proceed to Performing the Human Th1/Th2 Cytokine II Assay (page 24). If you need to dilute samples having high cytokine concentration, proceed to Diluting samples (page 22).

Additional steps when testing serum and plasma samples

Note: These steps can also be performed when testing cell culture supernatants.

When testing serum and/or plasma samples:

1. Perform the procedure to mix the beads in Procedure (page 20).
2. Centrifuge the mixed Capture Beads at 200*g* for 5 minutes.
3. Carefully aspirate and discard the supernatant.
4. Resuspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal volume to amount removed in step 3) and vortex thoroughly.
5. Incubate the mixed Capture Beads for 30 minutes at room temperature, protected from light.

Next step

To begin the assay, proceed to Performing the Human Th1/Th2 Cytokine II Assay (page 24). If you need to dilute samples having a high cytokine concentration, proceed to Diluting samples (page 22).

Diluting samples

Purpose of this procedure

The standard curve for each cytokine covers a defined set of concentrations from 20 to 5,000 pg/mL. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the range of the generated cytokine standard curve. For best results, dilute samples that are known or assumed to contain high levels of a given cytokine.

Procedure

To dilute samples with a known high cytokine concentration:

1. Dilute the sample by the desired dilution factor (ie, 1:2, 1:10, or 1:100) using the appropriate volume of Assay Diluent.
2. Mix sample dilutions thoroughly.

Next step

Perform instrument setup using the Cytometer Setup Beads. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

Or, if you wish to begin staining your samples for the assay, proceed to Performing the Human Th1/Th2 Cytokine II Assay (page 24), and you can perform instrument setup during the 3-hour staining incubation.

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4

Assay procedure

- Performing the Human Th1/Th2 Cytokine II Assay (page 24)
- Data analysis (page 28)

Performing the Human Th1/Th2 Cytokine II Assay

Before you begin

- Prepare the standards as described in Preparation of Human Th1/Th2 Cytokine Standards (page 18).
- Mix the Capture Beads as described in Mixing Human Th1/Th2 Cytokine Capture Beads (page 20).
- If necessary, dilute the unknown samples. See Diluting samples (page 22).

Procedure for tubes

Follow these steps regardless of whether you are testing cell culture supernatants or serum/plasma samples. If testing serum and/or plasma samples, be sure to use the Capture Beads that you prepared specifically for the serum/plasma samples. See Additional steps when testing serum and plasma samples (page 21).

To perform the assay:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.
2. Add 50 µL of the Human Th1/Th2 Cytokine Standard dilutions to the control assay tubes as listed in the following table.

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8

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Tube label	Concentration (pg/mL)	Cytokine Standard dilution
8	1,250	1:4
9	2,500	1:2
10	5,000	Top Standard

3. Add 50 µL of each unknown sample to the appropriately labeled sample tubes.
4. Add 50 µL of the Human Th1/Th2 - II PE Detection Reagent to all assay tubes.
5. Incubate the assay tubes for 3 hours at room temperature, protected from light.

   Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.

6. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300 µL of Wash Buffer to each assay tube to resuspend the bead pellet.

Procedure for filter plates

To perform the assay:

1. Wet the plate by adding 100 µL of Wash Buffer to each well.
2. Place the plate on the vacuum manifold.
3. Aspirate for 2 to 10 seconds until the wells are drained.
4. Remove the plate from the manifold, then blot the bottom of the plate on paper towels.

5. Add 50 µL of each of the following to the wells in the filter plate:
   - Capture Beads (vortex before adding)
   - Standard or sample (add standards from the lowest concentration to the highest, followed by samples)
   - Human Th1/Th2 - II PE Detection Reagent
6. Cover the plate and shake it for 5 minutes at 1,100 rpm on a plate shaker.
7. Incubate the plate for 3 hours at room temperature on a non-absorbent, dry surface.

   Note: Place the plate on a non-absorbent, dry surface during incubation. Absorbent or wet surfaces can cause the contents of the wells to leak.

8. Remove the cover from the plate and apply the plate to the vacuum manifold.
9. Vacuum aspirate for 2 to 10 seconds until the wells are drained.
10. Remove the plate from the manifold, then blot the bottom of the plate on paper towels after aspiration.
11. Add 120 µL of Wash Buffer to each well to resuspend the beads.
12. Cover the plate and shake it for 2 minutes at 1,100 rpm before you begin sample acquisition.

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Next step

Acquire the samples on the flow cytometer. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

CBA samples must be acquired on the same day they are prepared. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

To facilitate the analysis of samples using the FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 pg/mL) to highest (Top Standard) concentration, followed by the test samples.
- If running sample dilutions, acquire sequentially starting with the most concentrated sample.
- Store all FCS files (standards and samples) in a single folder.

When you are finished acquiring samples, proceed to Data analysis (page 28).

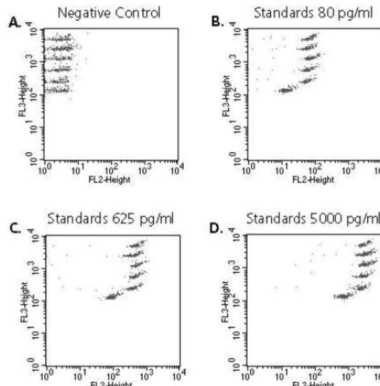
Data analysis

How to analyze

Analyze BD CBA Human Th1/Th2 Cytokine Kit II data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup and see the *Guide to Analyzing Data from BD CBA Kits Using FCAP Array Software*.

Typical Data

The following data, acquired using BD CellQuest™ software, shows standards and detectors alone.



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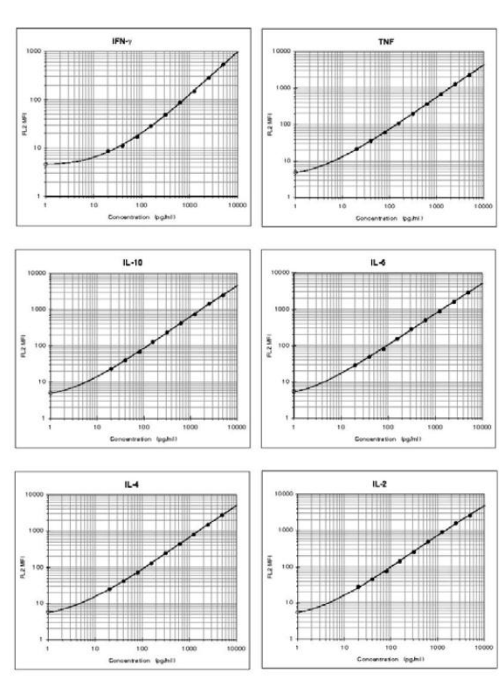
Standard curve examples

The following graphs represent standard curves from the BD CBA Human Th1/Th2 Cytokine Kit II.



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Appendix 6

Interleukin 12 ELISA standard curve prepared and used in this study

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Five Parameter Logistics - None / R-square=1.0000 RMSE=0.0115

a=0.047 b=-1.36472338598488 c=3297.09344733521 d=7.35769925167559 e=0.678783371724784

Response Values: 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5, 0, -0.5

Independent Values: 100, 1000

◆ Curve Points (click to hide)

● Unknown Points (click to hide)

ReaderFit.com

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Appendix 7

Th1/Th2 Cytometric bead array results

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Full printout

Experiment Name:	CBA 06_05_13 CO
Comments:	-
Operator:	FCAP Array Administrator
Creation Date:	5/7/2013
Date of analysis:	5/7/2013
Release Authorization:	Not finalized
Finalization time:	Not finalized
Instrument name:	BD FACS ARRAY
Instrument serial number:	00
Date of acquisition:	5/7/2013
Conditions:	-

Plex: Plex 1 (4F95-40E8)

Sample File Assignment

Folder: C:\BDExport\Carel 2012\CBA 06_05_13 CO\Plate_001\filtered\

Sample ID	Plate position	File
Std01	A1	filtered_Standard_01_Std01.fcs
Std02	A2	filtered_Standard_02_Std02.fcs
Std03	A3	filtered_Standard_03_Std03.fcs
Std04	A4	filtered_Standard_04_Std04.fcs
Std05	A5	filtered_Standard_05_Std05.fcs
Std06	A6	filtered_Standard_06_Std06.fcs
Std07	A7	filtered_Standard_07_Std07.fcs
Std08	A8	filtered_Standard_08_Std08.fcs
Std09	A9	filtered_Standard_09_Std09.fcs
Std10	A10	filtered_Standard_10_Std10.fcs
S0001	A11	filtered_S001_S001_001.fcs
S0002	A12	filtered_S002_S002_001.fcs
S0003	B1	filtered_S003_S003_001.fcs
S0004	B2	filtered_S004_S004_001.fcs
S0005	B3	filtered_S005_S005_001.fcs
S0006	B4	filtered_S006_S006_001.fcs
S0007	B5	filtered_S007_S007_001.fcs
S0008	B6	filtered_S008_S008_001.fcs
S0009	B7	filtered_S009_S009_001.fcs
S0010	B8	filtered_S010_S010_001.fcs
S0011	B9	filtered_S011_S011_001.fcs
S0012	B10	filtered_S012_S012_001.fcs
S0013	B11	filtered_S013_S013_001.fcs
S0014	B12	filtered_S014_S014_001.fcs
S0015	C1	filtered_S015_S015_001.fcs
S0016	C2	filtered_S016_S016_001.fcs
S0017	C3	filtered_S017_S017_001.fcs
S0018	C4	filtered_S018_S018_001.fcs
S0019	C5	filtered_S019_S019_001.fcs
S0020	C6	filtered_S020_S020_001.fcs
S0021	C7	filtered_S021_S021_001.fcs
S0022	C8	filtered_S022_S022_001.fcs
S0023	C9	filtered_S023_S023_001.fcs
S0024	C10	filtered_S024_S024_001.fcs
S0025	C11	filtered_S025_S025_001.fcs
S0026	C12	filtered_S026_S026_001.fcs

Sample ID	Plate position	File
S0027	D1	filtered_S027_S027_001.fcs
S0028	D2	filtered_S028_S028_001.fcs
S0029	D3	filtered_S029_S029_001.fcs
S0030	D4	* not acquired *
S0031	D5	* not acquired *
S0032	D6	* not acquired *
S0033	D7	* not acquired *
S0034	D8	* not acquired *
S0035	D9	* not acquired *
S0036	D10	* not acquired *
S0037	D11	* not acquired *
S0038	D12	* not acquired *
S0039	E1	* not acquired *

Analyte: IL-2

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	562	5.49	70.98%	0.00	0.00	-	0.00
Std02	20.00	532	10.24	41.57%	1.30	1.32	1.31%	20.80
Std03	40.00	436	15.77	34.83%	1.60	1.60	0.36%	39.47
Std04	80.00	447	27.90	36.19%	1.90	1.89	0.92%	76.83
Std05	156.00	491	57.82	45.72%	2.19	2.20	0.38%	159.02
Std06	312.50	507	123.09	501.03%	2.49	2.51	0.41%	320.02
Std07	625.00	501	251.55	45.88%	2.80	2.79	0.20%	617.17
Std08	1250.00	469	531.80	83.70%	3.10	3.10	0.19%	1266.99
Std09	2500.00	416	981.17	71.19%	3.40	3.38	0.52%	2400.46
Std10	5000.00	462	1875.88	42.94%	3.70	3.71	0.30%	5127.27

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.74, B=3.02, C=3.18, D=4.86
Fitting accuracy: R square = 99.99%

Analyte: IL-4

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	524	4.42	84.27%	0.00	0.00	-	0.00
Std02	20.00	587	10.14	41.29%	1.30	1.29	0.98%	19.42
Std03	40.00	459	16.31	29.65%	1.60	1.61	0.65%	40.97
Std04	80.00	505	25.18	30.41%	1.90	1.87	1.82%	73.87
Std05	156.00	572	49.48	30.82%	2.19	2.23	1.52%	168.46
Std06	312.50	507	89.58	25.60%	2.49	2.52	0.96%	330.25
Std07	625.00	483	147.55	37.24%	2.80	2.76	1.42%	570.38
Std08	1250.00	492	326.81	25.15%	3.10	3.13	1.00%	1342.63
Std09	2500.00	420	536.61	33.12%	3.40	3.36	1.12%	2290.97
Std10	5000.00	454	1138.32	25.95%	3.70	3.72	0.52%	5225.06

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.64, B=2.15, C=4.88, D=7.40
Fitting accuracy: R square = 99.90%

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Analyte: IL-6

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	450	3.27	106.46%	0.00	0.00	-	0.00
Std02	20.00	429	12.46	202.92%	1.30	1.28	1.37%	19.19
Std03	40.00	385	22.50	34.39%	1.60	1.61	0.40%	40.60
Std04	80.00	356	41.17	34.88%	1.90	1.92	0.69%	82.45
Std05	156.00	403	71.99	40.05%	2.19	2.19	0.16%	154.72
Std06	312.50	404	138.49	36.40%	2.49	2.50	0.36%	318.96
Std07	625.00	404	251.55	43.81%	2.80	2.79	0.21%	616.64
Std08	1250.00	387	464.55	37.20%	3.10	3.09	0.29%	1224.58
Std09	2500.00	366	857.21	41.08%	3.40	3.39	0.15%	2470.66
Std10	5000.00	380	1581.04	32.42%	3.70	3.71	0.25%	5105.59

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.51, B=1.79, C=5.10, D=7.95
Fitting accuracy: R square = 99.99%

Analyte: IL-10

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	495	3.07	106.66%	0.00	0.00	-	0.00
Std02	20.00	453	11.30	42.18%	1.30	1.30	0.04%	20.02
Std03	40.00	444	19.91	36.32%	1.60	1.61	0.41%	40.61
Std04	80.00	401	34.87	36.55%	1.90	1.89	0.81%	77.21
Std05	156.00	504	66.93	40.93%	2.19	2.20	0.15%	157.16
Std06	312.50	448	131.76	32.34%	2.49	2.51	0.56%	322.72
Std07	625.00	443	240.44	46.27%	2.80	2.79	0.38%	609.82
Std08	1250.00	493	477.29	67.97%	3.10	3.10	0.23%	1270.99
Std09	2500.00	395	864.96	70.05%	3.40	3.39	0.30%	2442.42
Std10	5000.00	429	1639.00	32.75%	3.70	3.70	0.13%	5057.14

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.49, B=1.88, C=4.80, D=7.66
Fitting accuracy: R square = 99.99%

Analyte: TNF

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	431	2.55	123.17%	0.00	0.00	-	0.00
Std02	20.00	449	10.24	48.42%	1.30	1.27	2.47%	18.57
Std03	40.00	369	19.72	52.72%	1.60	1.62	1.16%	41.75
Std04	80.00	383	35.85	36.07%	1.90	1.92	0.72%	82.58
Std05	156.00	459	64.52	42.88%	2.19	2.20	0.12%	156.94
Std06	312.50	449	125.35	41.14%	2.49	2.50	0.32%	318.24
Std07	625.00	401	225.71	49.76%	2.80	2.77	0.86%	591.20
Std08	1250.00	405	473.00	34.36%	3.10	3.11	0.44%	1289.74
Std09	2500.00	376	841.91	44.31%	3.40	3.38	0.60%	2384.76
Std10	5000.00	381	1714.44	29.42%	3.70	3.71	0.38%	5166.65

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.40, B=1.71, C=6.04, D=9.73
Fitting accuracy: R square = 99.97%

Analyte: IFN

Table of Standards

Sample ID	Nominal CC pg/ml	Event #	MFI	CV% (MFI)	Log CC	Fitted Log CC	Delta % (Log CC)	Fitted CC pg/ml
Std01	0.00	470	2.40	130.78%	0.00	0.00	-	0.00
Std02	20.00	527	6.23	59.26%	1.30	1.30	0.45%	19.73
Std03	40.00	394	10.86	45.78%	1.60	1.62	1.13%	41.70
Std04	80.00	420	18.90	39.16%	1.90	1.90	0.23%	79.21
Std05	156.00	482	34.87	877.22%	2.19	2.18	0.62%	151.21
Std06	312.50	499	75.35	39.86%	2.49	2.51	0.67%	324.80
Std07	625.00	421	138.49	69.59%	2.80	2.77	1.06%	583.68
Std08	1250.00	451	323.88	203.14%	3.10	3.12	0.75%	1318.71
Std09	2500.00	400	631.09	41.29%	3.40	3.40	0.15%	2529.30
Std10	5000.00	445	1217.81	67.08%	3.70	3.69	0.22%	4906.59

Fitting Equation

4 parameter logistic: log I = D + (A - D) / (1 + (log CC / C) ^ B)
Parameters: A=0.38, B=2.23, C=4.39, D=7.07
Fitting accuracy: R square = 99.97%

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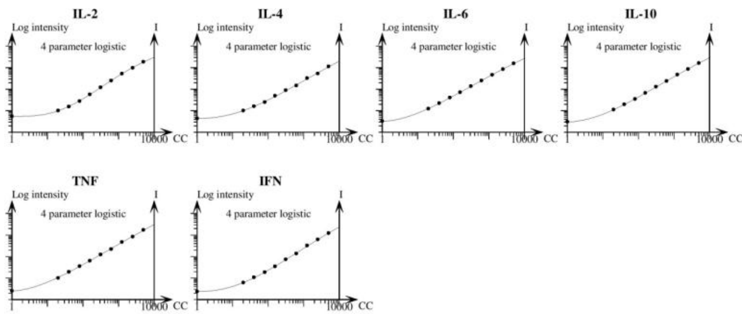
Grouped Fitting Plots



Colored Plate View

Color / Pattern Codes

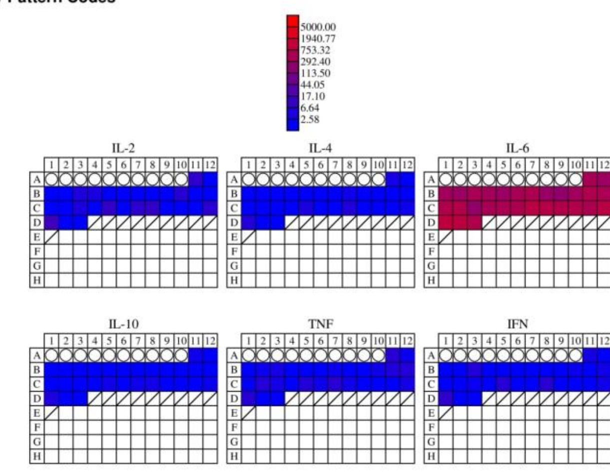


Table of Samples

Analyte: IL-2

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	481	5.98	65.12%	4.57	1.00	4.57	
S0002	516	5.49	67.55%	0.00	1.00	0.00	
S0003	502	4.88	67.83%	0.00	1.00	0.00	
S0004	476	5.24	84.07%	0.00	1.00	0.00	
S0005	448	5.67	64.71%	2.92	1.00	2.92	
S0006	508	5.61	70.74%	2.50	1.00	2.50	
S0007	492	5.21	74.79%	0.00	1.00	0.00	
S0008	486	4.88	72.89%	0.00	1.00	0.00	
S0009	524	5.04	67.22%	0.00	1.00	0.00	
S0010	463	5.49	67.72%	0.00	1.00	0.00	
S0011	546	5.04	73.45%	0.00	1.00	0.00	
S0012	495	5.85	83.94%	3.95	1.00	3.95	
S0013	481	5.38	66.05%	0.00	1.00	0.00	
S0014	500	5.26	74.39%	0.00	1.00	0.00	
S0015	558	4.65	123.12%	0.00	1.00	0.00	
S0016	469	4.99	81.41%	0.00	1.00	0.00	
S0017	454	5.64	65.00%	2.72	1.00	2.72	
S0018	451	4.83	76.07%	0.00	1.00	0.00	

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Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0019	465	6.23	62.74%	5.73	1.00	5.73	
S0020	490	5.10	69.41%	0.00	1.00	0.00	
S0021	451	6.04	65.50%	4.87	1.00	4.87	
S0022	444	6.23	71.93%	5.73	1.00	5.73	
S0023	460	5.10	72.89%	0.00	1.00	0.00	
S0024	477	5.49	60.88%	0.00	1.00	0.00	
S0025	506	5.32	71.42%	0.00	1.00	0.00	
S0026	491	5.92	61.41%	4.27	1.00	4.27	
S0027	449	7.06	61.65%	9.11	1.00	9.11	
S0028	427	5.26	70.34%	0.00	1.00	0.00	
S0029	137	4.94	73.33%	0.00	1.00	0.00	
S0030	-	-	-	-	1.00	-	-
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

Analyte: IL-4

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	485	4.62	75.47%	2.19	1.00	2.19	
S0002	446	3.87	84.99%	0.00	1.00	0.00	
S0003	448	3.39	200.14%	0.00	1.00	0.00	
S0004	500	3.63	84.54%	0.00	1.00	0.00	
S0005	461	4.23	75.54%	0.00	1.00	0.00	
S0006	503	3.96	77.27%	0.00	1.00	0.00	
S0007	515	3.57	92.63%	0.00	1.00	0.00	
S0008	549	3.91	79.94%	0.00	1.00	0.00	
S0009	515	3.83	87.80%	0.00	1.00	0.00	
S0010	467	4.52	76.54%	1.82	1.00	1.82	
S0011	517	3.70	88.44%	0.00	1.00	0.00	
S0012	548	4.05	6854.21%	0.00	1.00	0.00	
S0013	479	4.28	81.34%	0.00	1.00	0.00	
S0014	490	4.28	75.11%	0.00	1.00	0.00	
S0015	461	3.41	238.91%	0.00	1.00	0.00	
S0016	477	3.66	85.43%	0.00	1.00	0.00	
S0017	495	4.23	81.33%	0.00	1.00	0.00	
S0018	429	3.66	2455.01%	0.00	1.00	0.00	
S0019	504	4.75	76.71%	2.61	1.00	2.61	
S0020	497	3.70	90.04%	0.00	1.00	0.00	
S0021	476	4.21	85.63%	0.00	1.00	0.00	
S0022	509	4.88	71.70%	3.02	1.00	3.02	
S0023	509	3.57	89.79%	0.00	1.00	0.00	
S0024	502	3.85	81.89%	0.00	1.00	0.00	
S0025	501	3.78	129.11%	0.00	1.00	0.00	
S0026	502	4.52	434.99%	1.82	1.00	1.82	
S0027	438	5.10	68.81%	3.66	1.00	3.66	
S0028	460	3.57	93.33%	0.00	1.00	0.00	
S0029	163	3.45	312.82%	0.00	1.00	0.00	
S0030	-	-	-	-	1.00	-	-
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

Analyte: IL-6

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	371	124.21	29.50%	282.93	1.00	282.93	
S0002	388	87.17	26.64%	191.32	1.00	191.32	
S0003	394	105.50	24.20%	236.28	1.00	236.28	
S0004	358	82.54	27.46%	180.09	1.00	180.09	
S0005	391	128.80	25.58%	294.47	1.00	294.47	
S0006	426	107.43	34.24%	241.06	1.00	241.06	
S0007	446	85.99	26.81%	188.46	1.00	188.46	
S0008	417	125.35	25.59%	285.77	1.00	285.77	
S0009	414	147.55	23.44%	342.05	1.00	342.05	
S0010	432	76.74	35.22%	166.10	1.00	166.10	
S0011	429	60.53	28.79%	127.47	1.00	127.47	
S0012	415	100.82	31.97%	224.72	1.00	224.72	
S0013	391	161.53	33.02%	377.95	1.00	377.95	
S0014	442	133.56	24.29%	306.47	1.00	306.47	
S0015	397	164.48	27.22%	385.56	1.00	385.56	
S0016	344	173.66	25.92%	409.35	1.00	409.35	
S0017	375	66.32	29.30%	141.19	1.00	141.19	
S0018	374	131.16	27.12%	300.41	1.00	300.41	
S0019	380	132.35	28.41%	303.43	1.00	303.43	
S0020	407	141.02	34.10%	325.40	1.00	325.40	
S0021	425	217.70	30.64%	525.43	1.00	525.43	
S0022	401	183.34	29.84%	434.61	1.00	434.61	
S0023	365	170.54	25.40%	401.27	1.00	401.27	
S0024	407	176.83	29.90%	417.61	1.00	417.61	
S0025	454	141.02	30.51%	325.40	1.00	325.40	
S0026	396	214.77	32.12%	517.61	1.00	517.61	
S0027	387	251.55	32.56%	616.64	1.00	616.64	
S0028	395	227.76	30.88%	552.36	1.00	552.36	
S0029	130	165.98	28.79%	389.43	1.00	389.43	
S0030	-	-	-	-	1.00	-	-
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

Analyte: IL-10

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	448	3.26	92.03%	1.74	1.00	1.74	
S0002	423	2.75	103.58%	0.00	1.00	0.00	

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Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0003	433	2.11	103.05%	0.00	1.00	0.00	
S0004	416	2.54	104.26%	0.00	1.00	0.00	
S0005	384	3.09	84.75%	1.18	1.00	1.18	
S0006	456	2.48	127.18%	0.00	1.00	0.00	
S0007	470	2.35	119.12%	0.00	1.00	0.00	
S0008	441	2.13	112.32%	0.00	1.00	0.00	
S0009	446	2.46	112.77%	0.00	1.00	0.00	
S0010	454	2.77	101.28%	0.00	1.00	0.00	
S0011	471	2.19	109.52%	0.00	1.00	0.00	
S0012	501	2.89	102.98%	0.00	1.00	0.00	
S0013	422	2.40	114.72%	0.00	1.00	0.00	
S0014	458	3.00	106.41%	0.00	1.00	0.00	
S0015	468	2.38	295.45%	0.00	1.00	0.00	
S0016	420	2.35	110.48%	0.00	1.00	0.00	
S0017	493	2.46	140.19%	0.00	1.00	0.00	
S0018	434	2.29	110.02%	0.00	1.00	0.00	
S0019	455	3.33	115.88%	1.94	1.00	1.94	
S0020	471	2.43	143.52%	0.00	1.00	0.00	
S0021	447	3.26	1646.41%	1.74	1.00	1.74	
S0022	464	3.35	118.54%	1.99	1.00	1.99	
S0023	458	2.41	115.49%	0.00	1.00	0.00	
S0024	456	2.84	107.95%	0.00	1.00	0.00	
S0025	482	2.64	235.51%	0.00	1.00	0.00	
S0026	486	3.22	11785.16%	1.64	1.00	1.64	
S0027	378	3.72	95.74%	2.84	1.00	2.84	
S0028	455	2.46	341.97%	0.00	1.00	0.00	
S0029	147	2.05	130.71%	0.00	1.00	0.00	
S0030	-	-	-	-	1.00	-	-
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

Analyte: TNF

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	406	3.78	84.78%	3.92	1.00	3.92	
S0002	378	2.72	111.51%	1.62	1.00	1.62	
S0003	415	2.28	121.07%	0.00	1.00	0.00	
S0004	383	2.72	121.11%	1.62	1.00	1.62	
S0005	377	2.96	98.51%	2.17	1.00	2.17	
S0006	429	2.55	112.34%	0.00	1.00	0.00	
S0007	457	2.22	119.28%	0.00	1.00	0.00	
S0008	438	1.85	144.18%	0.00	1.00	0.00	
S0009	396	2.72	102.22%	1.62	1.00	1.62	
S0010	417	2.49	111.54%	0.00	1.00	0.00	
S0011	425	2.05	128.30%	0.00	1.00	0.00	
S0012	430	2.82	134.64%	1.86	1.00	1.86	
S0013	379	3.37	95.01%	3.05	1.00	3.05	
S0014	411	3.53	125.49%	3.39	1.00	3.39	
S0015	412	2.60	114.28%	1.31	1.00	1.31	
S0016	398	3.66	87.22%	3.65	1.00	3.65	

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0017	361	2.89	108.40%	2.02	1.00	2.02	
S0018	382	2.46	113.12%	0.00	1.00	0.00	
S0019	410	3.55	88.19%	3.43	1.00	3.43	
S0020	454	2.75	91.57%	1.70	1.00	1.70	
S0021	407	3.91	90.92%	4.20	1.00	4.20	
S0022	390	2.82	112.73%	1.86	1.00	1.86	
S0023	429	2.46	117.56%	0.00	1.00	0.00	
S0024	447	2.16	173.39%	0.00	1.00	0.00	
S0025	429	2.25	122.98%	0.00	1.00	0.00	
S0026	404	2.98	85.41%	2.21	1.00	2.21	
S0027	386	3.78	84.09%	3.92	1.00	3.92	
S0028	356	2.31	109.16%	0.00	1.00	0.00	
S0029	138	0.89	288.18%	0.00	1.00	0.00	
S0030	-	-	-	-	1.00	-	-
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

Analyte: IFN

Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0001	484	2.54	114.14%	2.23	1.00	2.23	
S0002	440	2.02	129.84%	0.00	1.00	0.00	
S0003	414	1.74	130.25%	0.00	1.00	0.00	
S0004	451	1.76	154.36%	0.00	1.00	0.00	
S0005	389	2.72	108.11%	3.19	1.00	3.19	
S0006	444	1.84	141.34%	0.00	1.00	0.00	
S0007	450	2.01	136.87%	0.00	1.00	0.00	
S0008	453	1.74	144.18%	0.00	1.00	0.00	
S0009	455	1.84	131.30%	0.00	1.00	0.00	
S0010	452	2.34	110.03%	0.00	1.00	0.00	
S0011	474	1.84	190.52%	0.00	1.00	0.00	
S0012	484	2.44	116.14%	1.61	1.00	1.61	
S0013	441	2.28	118.57%	0.00	1.00	0.00	
S0014	499	2.49	108.33%	1.94	1.00	1.94	
S0015	480	1.92	130.96%	0.00	1.00	0.00	
S0016	428	1.97	299.82%	0.00	1.00	0.00	
S0017	443	2.43	103.14%	1.47	1.00	1.47	
S0018	387	1.64	151.15%	0.00	1.00	0.00	
S0019	446	2.84	105.80%	3.77	1.00	3.77	
S0020	460	1.74	146.69%	0.00	1.00	0.00	
S0021	408	2.32	116.77%	0.00	1.00	0.00	
S0022	424	2.89	96.00%	4.02	1.00	4.02	
S0023	445	1.94	128.00%	0.00	1.00	0.00	
S0024	458	2.12	129.48%	0.00	1.00	0.00	
S0025	481	1.86	132.60%	0.00	1.00	0.00	
S0026	434	2.48	123.32%	1.84	1.00	1.84	
S0027	434	3.14	100.05%	5.21	1.00	5.21	
S0028	436	1.88	142.37%	0.00	1.00	0.00	
S0029	164	1.40	161.33%	0.00	1.00	0.00	
S0030	-	-	-	-	1.00	-	-

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Sample ID	Event #	MFI	CV% (MFI)	Calculated CC pg/ml	Dilution	Final CC pg/ml	Message
S0031	-	-	-	-	1.00	-	-
S0032	-	-	-	-	1.00	-	-
S0033	-	-	-	-	1.00	-	-
S0034	-	-	-	-	1.00	-	-
S0035	-	-	-	-	1.00	-	-
S0036	-	-	-	-	1.00	-	-
S0037	-	-	-	-	1.00	-	-
S0038	-	-	-	-	1.00	-	-
S0039	-	-	-	-	1.00	-	-

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Printed by FCAP Array Administrator

11 / 11

Experiment Name: CBA 06_05_13 CO
Experiment ID: DB08-6372

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Appendix 8

Enzyme kinetic graphs depicting the Vmax and Km-values

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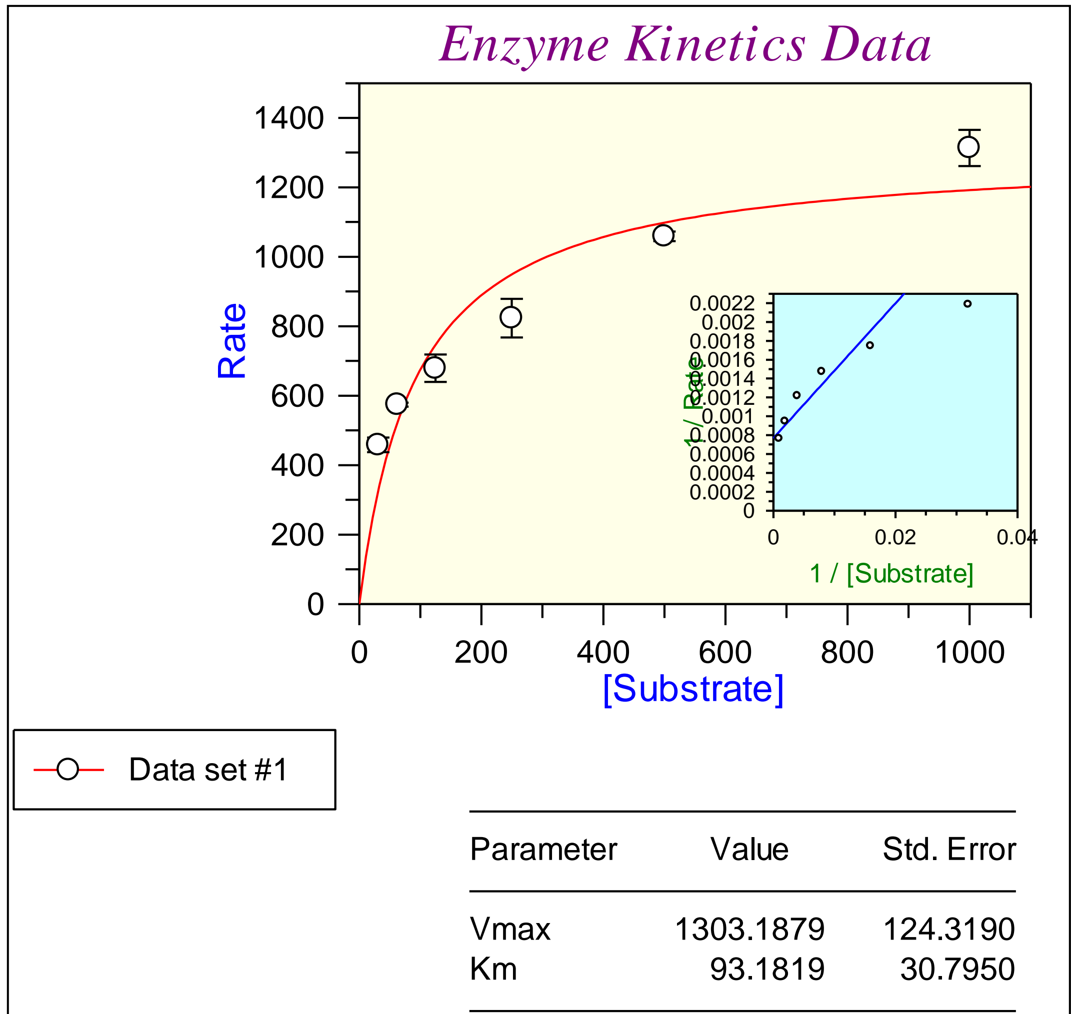
AQ2



Parameter	Value	Std. Error
Vmax	1303.1879	124.3190
Km	93.1819	30.7950

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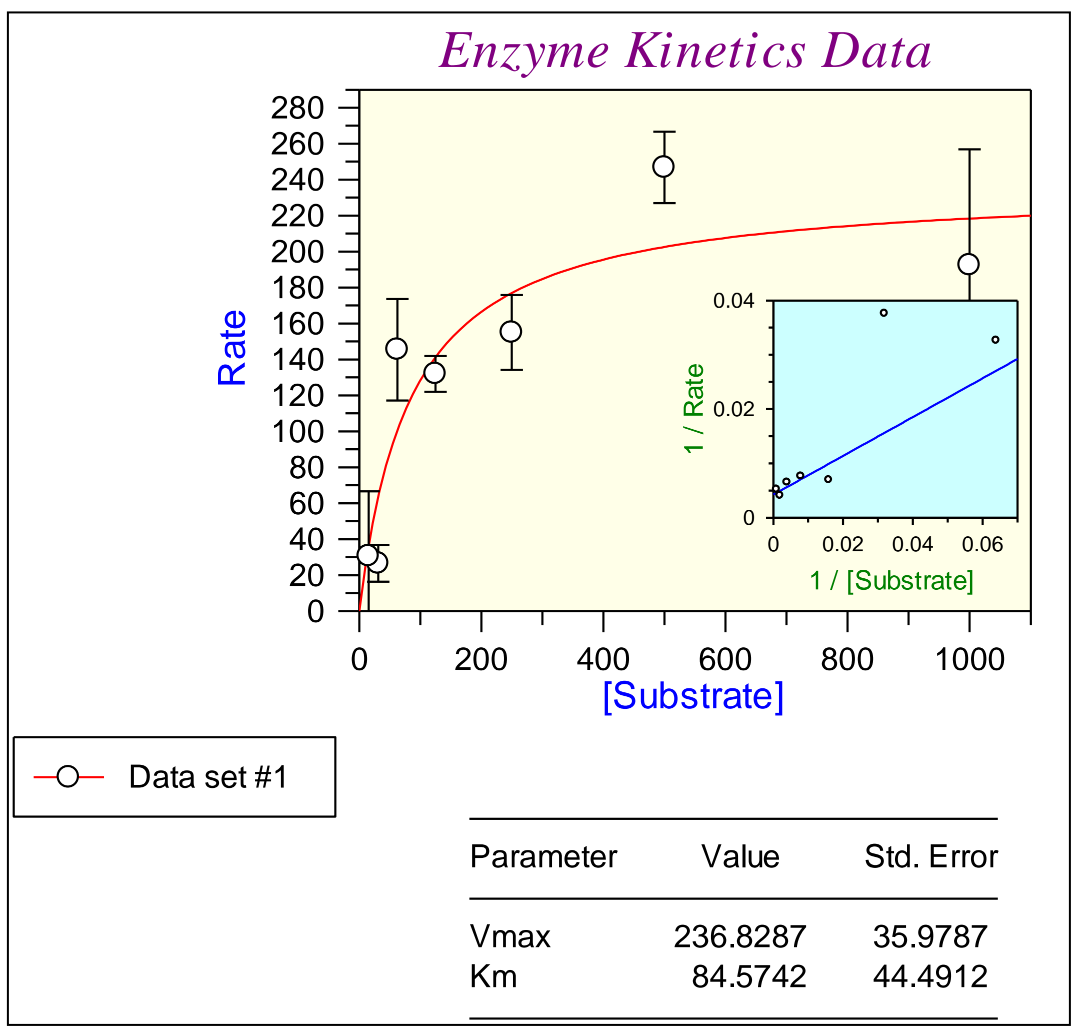
CB5



Parameter	Value	Std. Error
Vmax	236.8287	35.9787
Km	84.5742	44.4912

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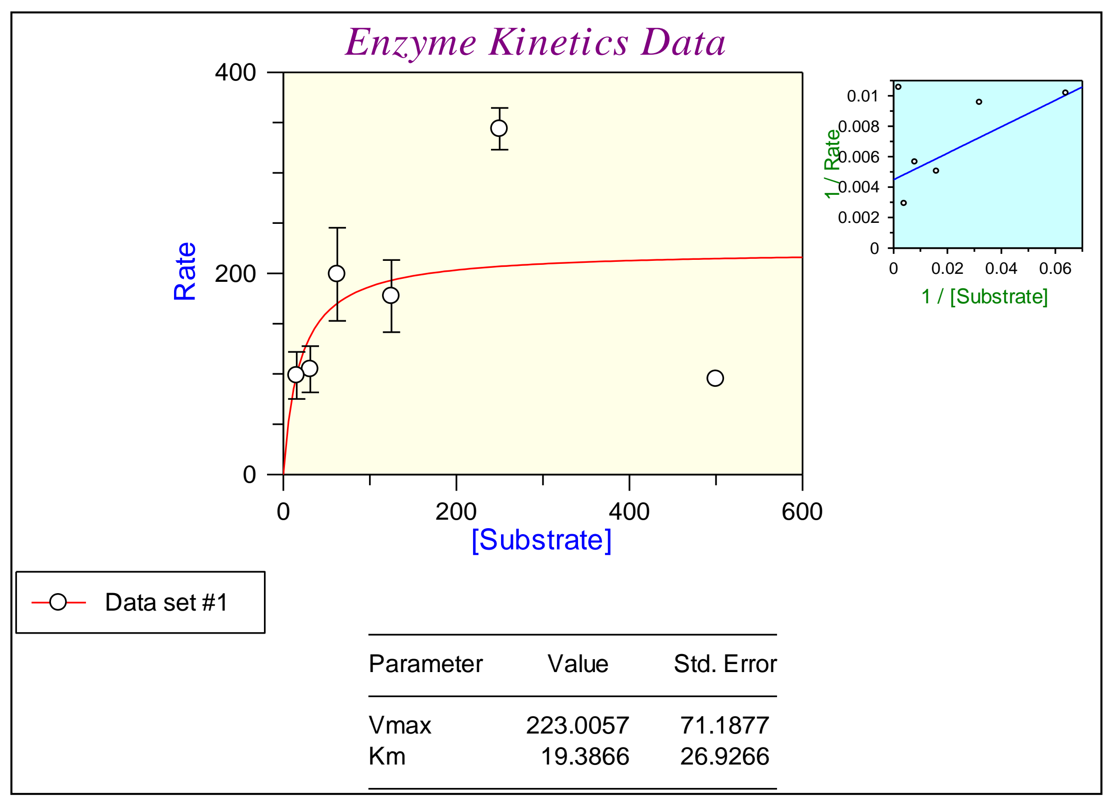
IL107



Parameter	Value	Std. Error
Vmax	223.0057	71.1877
Km	19.3866	26.9266

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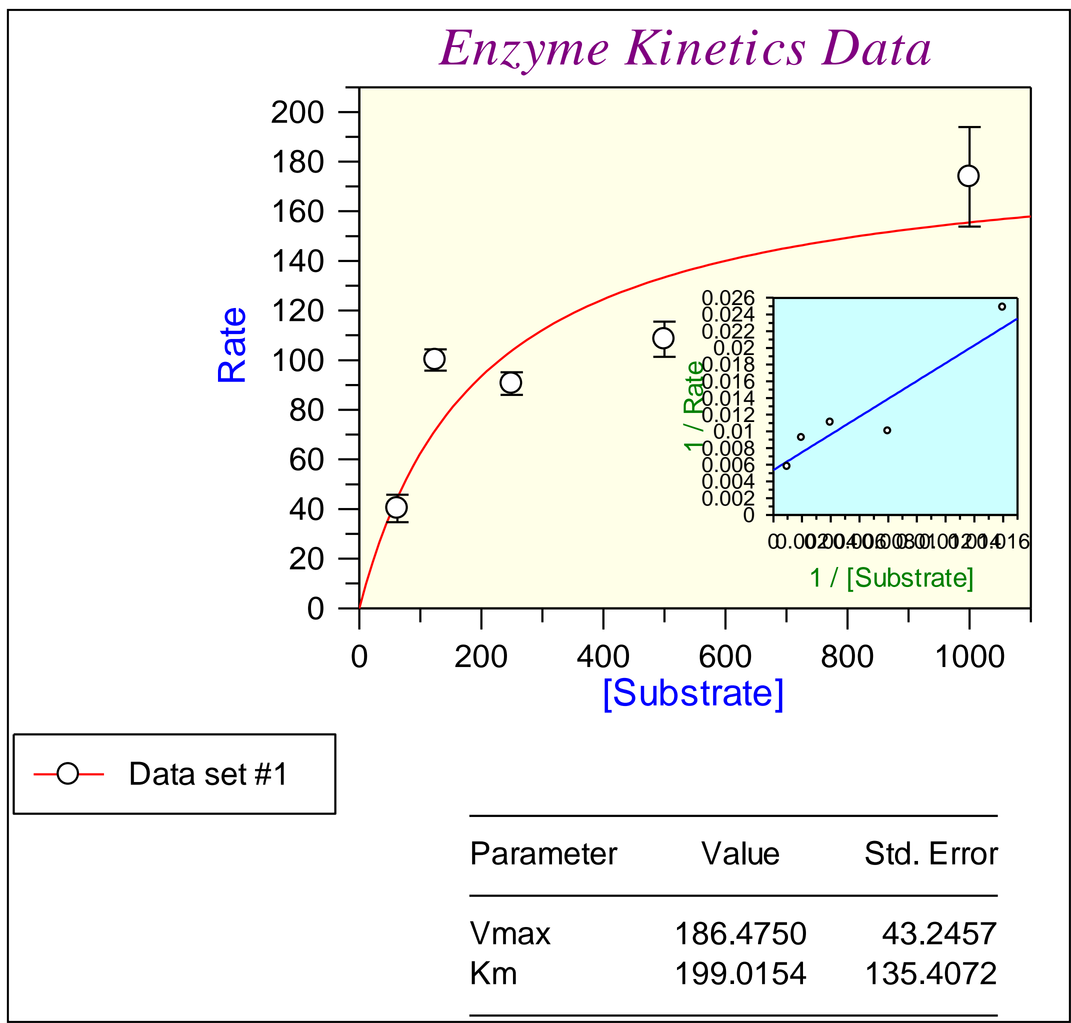
IL113



Parameter	Value	Std. Error
Vmax	186.4750	43.2457
Km	199.0154	135.4072

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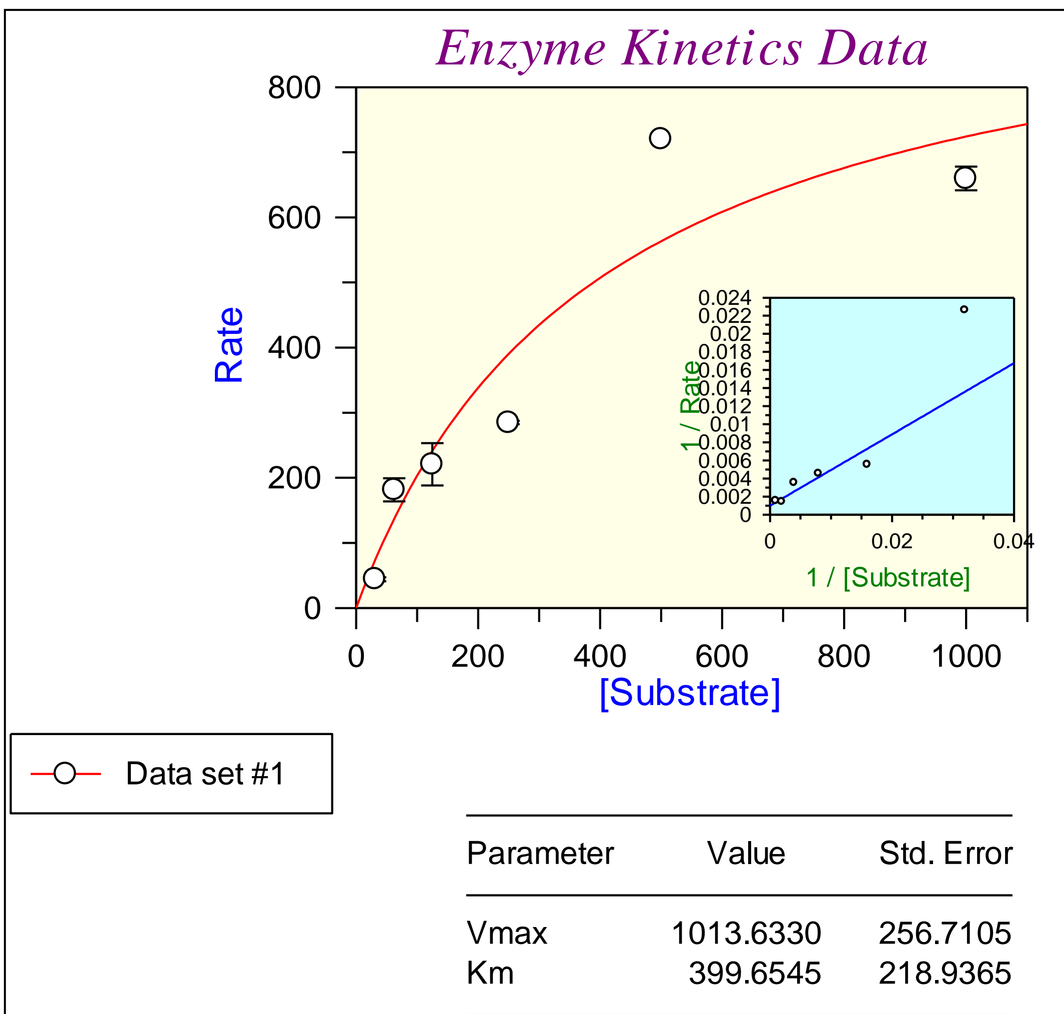
IL135



Parameter	Value	Std. Error
Vmax	1013.6330	256.7105
Km	399.6545	218.9365

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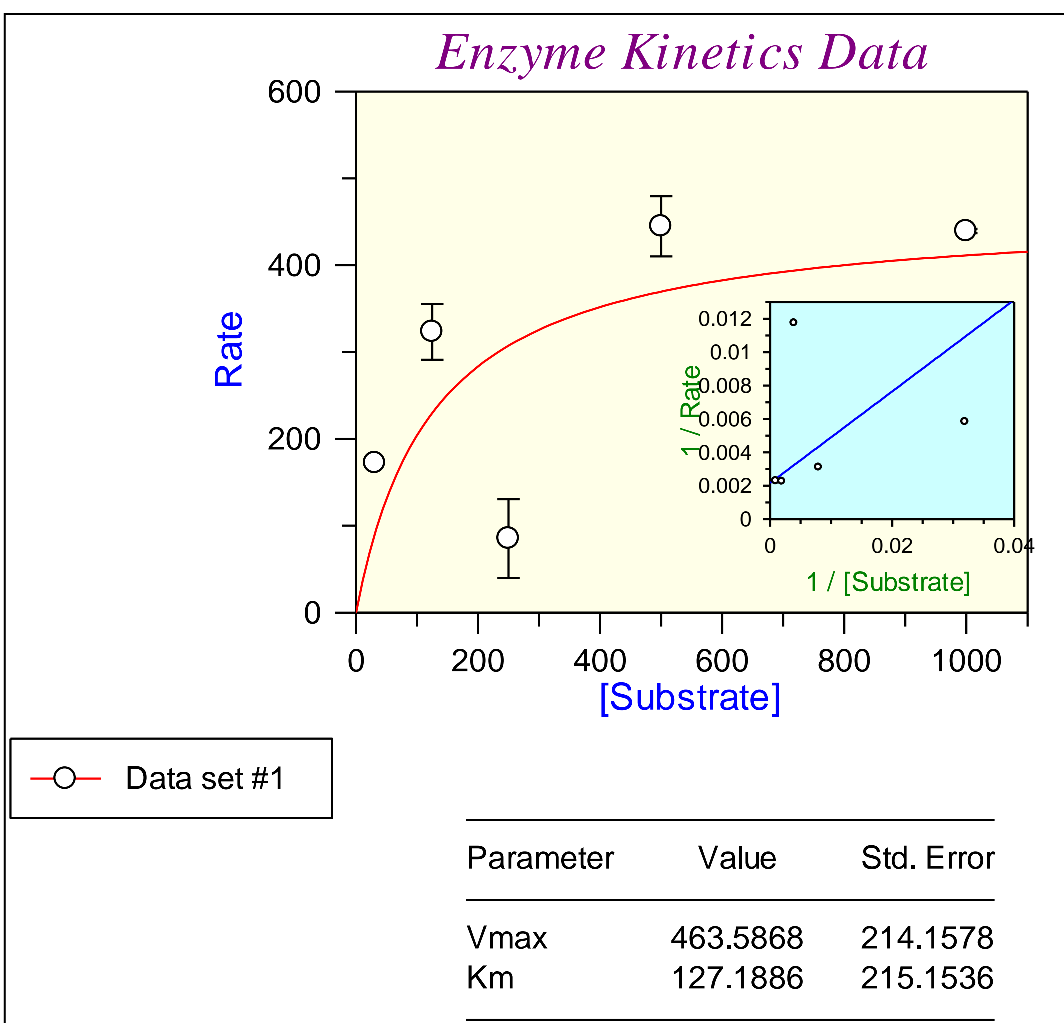
IL142



Parameter	Value	Std. Error
Vmax	463.5868	214.1578
Km	127.1886	215.1536

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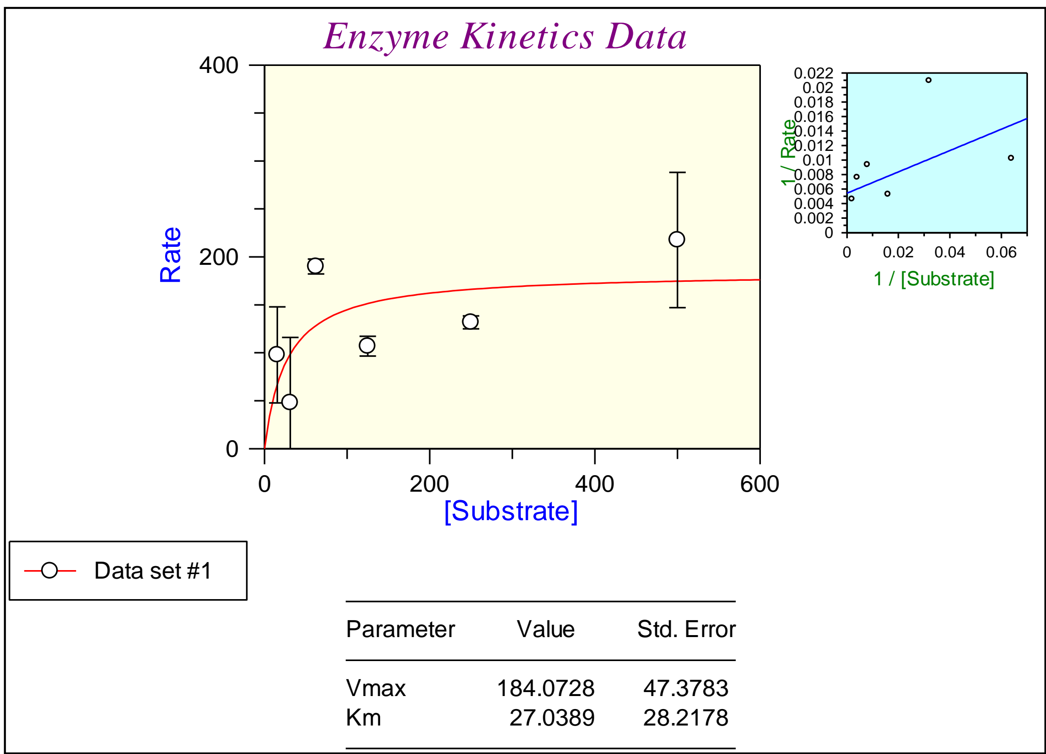
IL143



Parameter	Value	Std. Error
Vmax	184.0728	47.3783
Km	27.0389	28.2178

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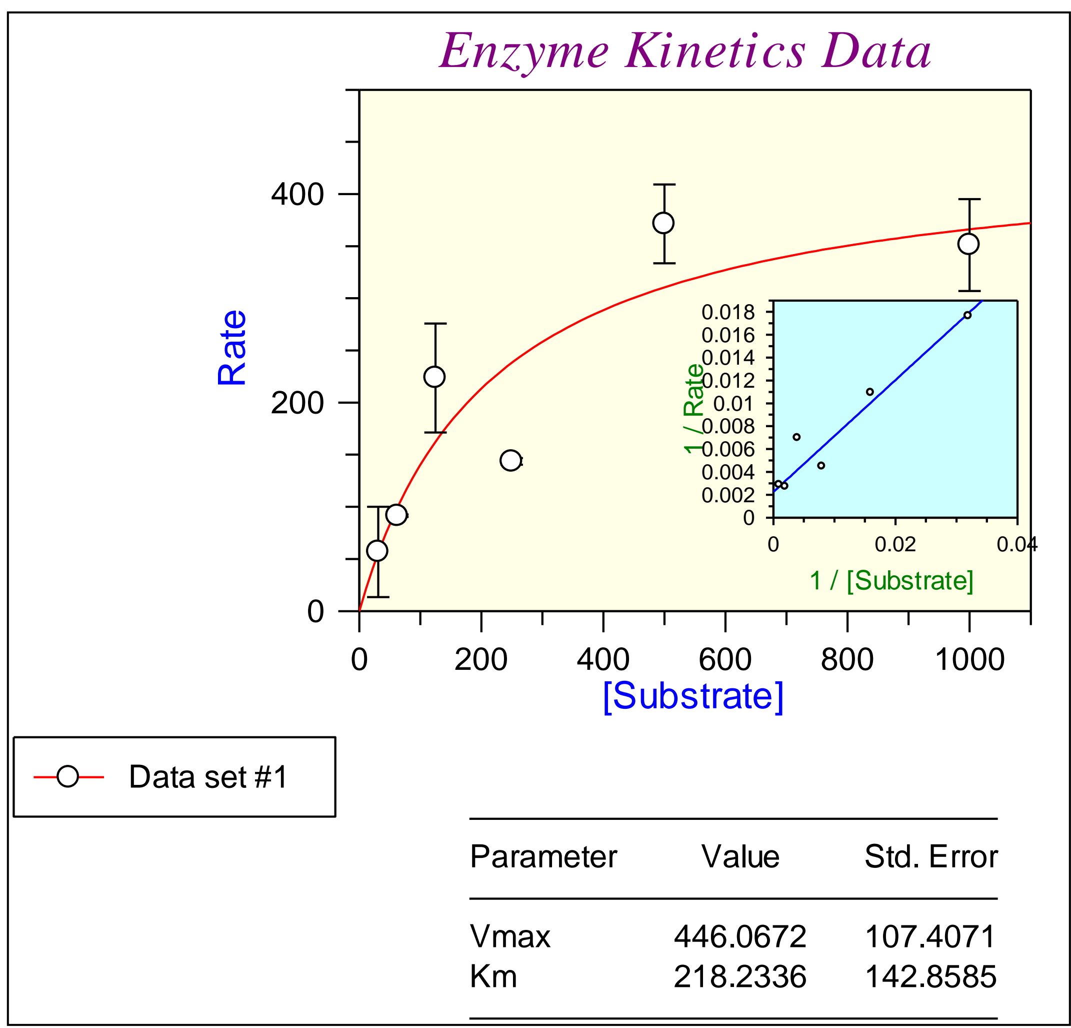
KM41



Parameter	Value	Std. Error
Vmax	446.0672	107.4071
Km	218.2336	142.8585

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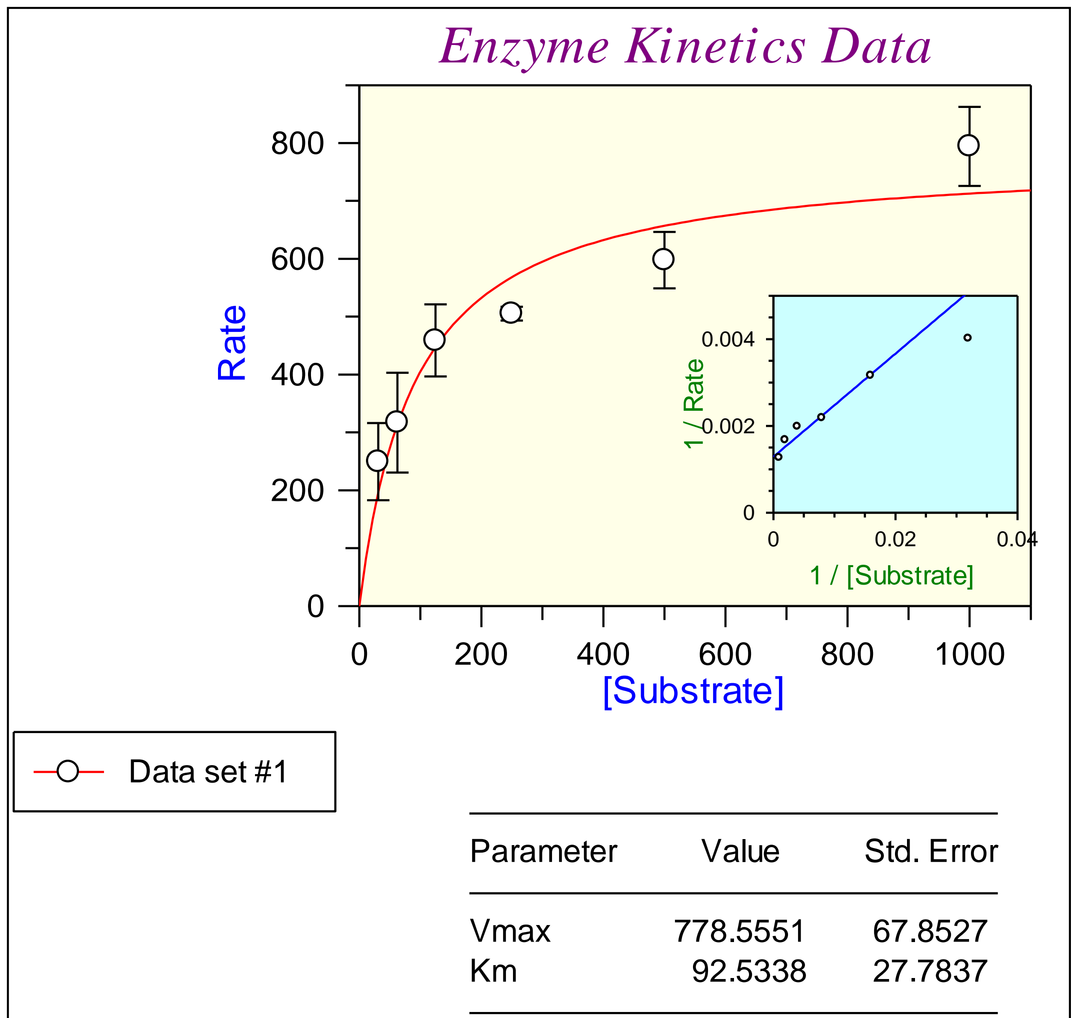
KM46



Parameter	Value	Std. Error
Vmax	778.5551	67.8527
Km	92.5338	27.7837

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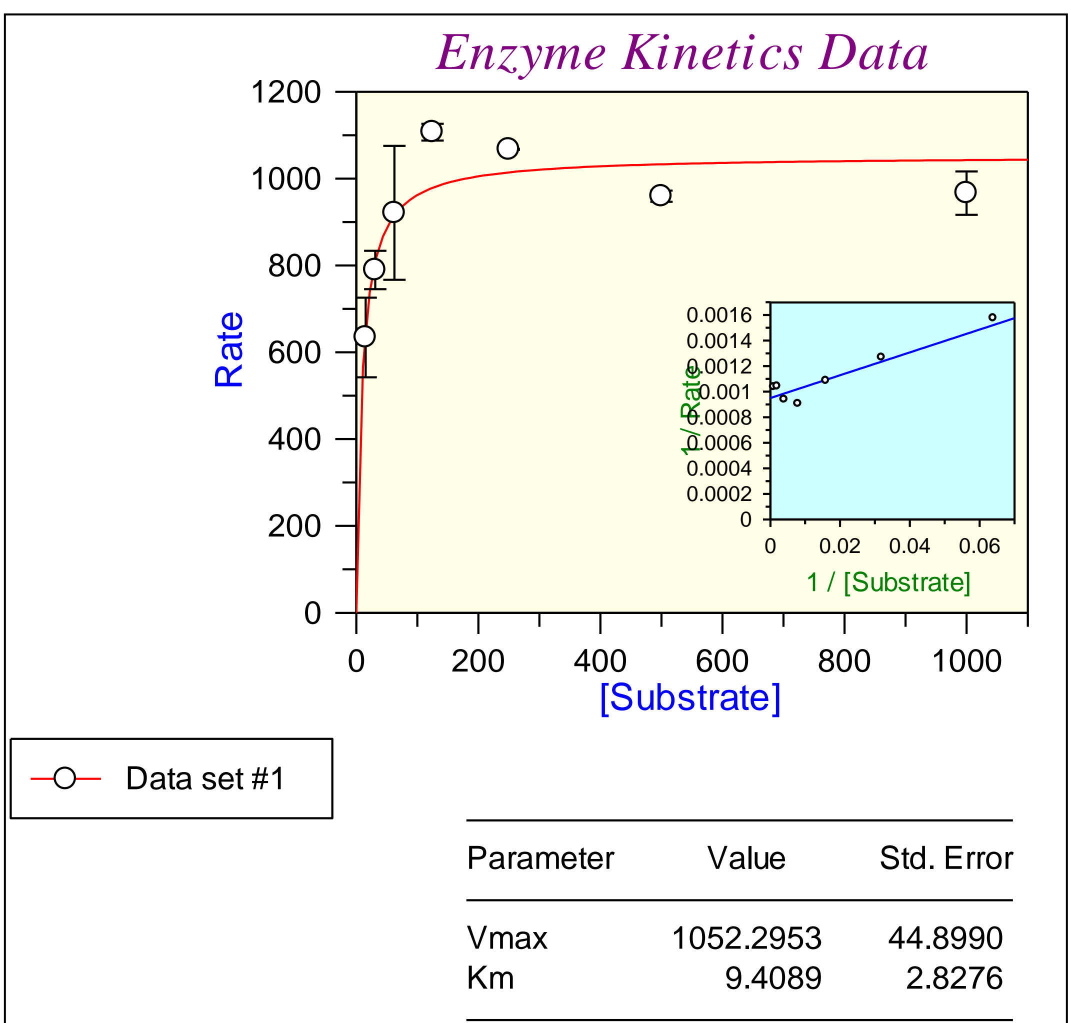
KM80



Parameter	Value	Std. Error
Vmax	1052.2953	44.8990
Km	9.4089	2.8276

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KM86

Enzyme Kinetics Data

Rate

120
100
80
60
40
20
0

0 200 400 600 800 1000

[Substrate]

1 / Rate

0.028
0.026
0.024
0.022
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0

1 / [Substrate]

Data set #1

Parameter	Value	Std. Error
Vmax	95.2374	14.6525
Km	73.0759	46.2051

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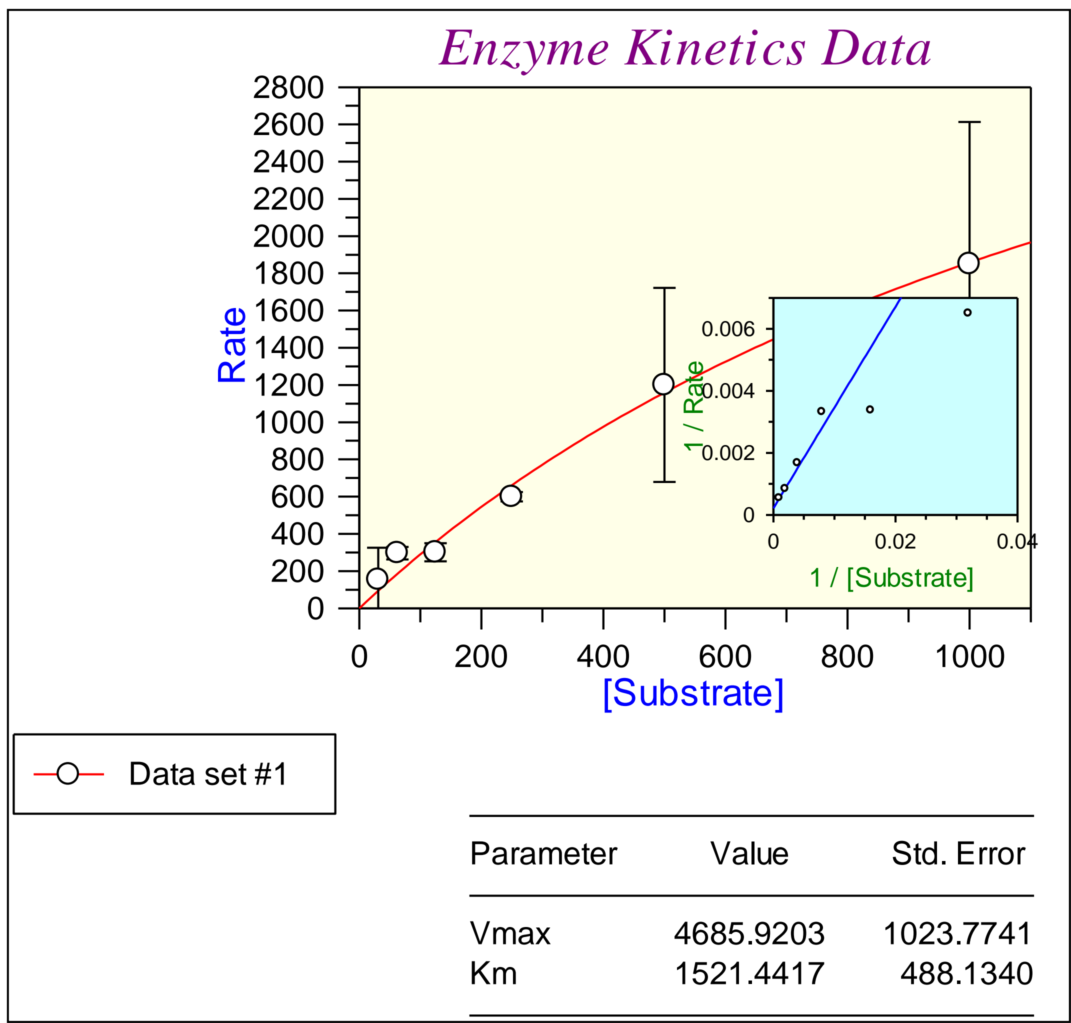
KM108



Parameter	Value	Std. Error
Vmax	4685.9203	1023.7741
Km	1521.4417	488.1340

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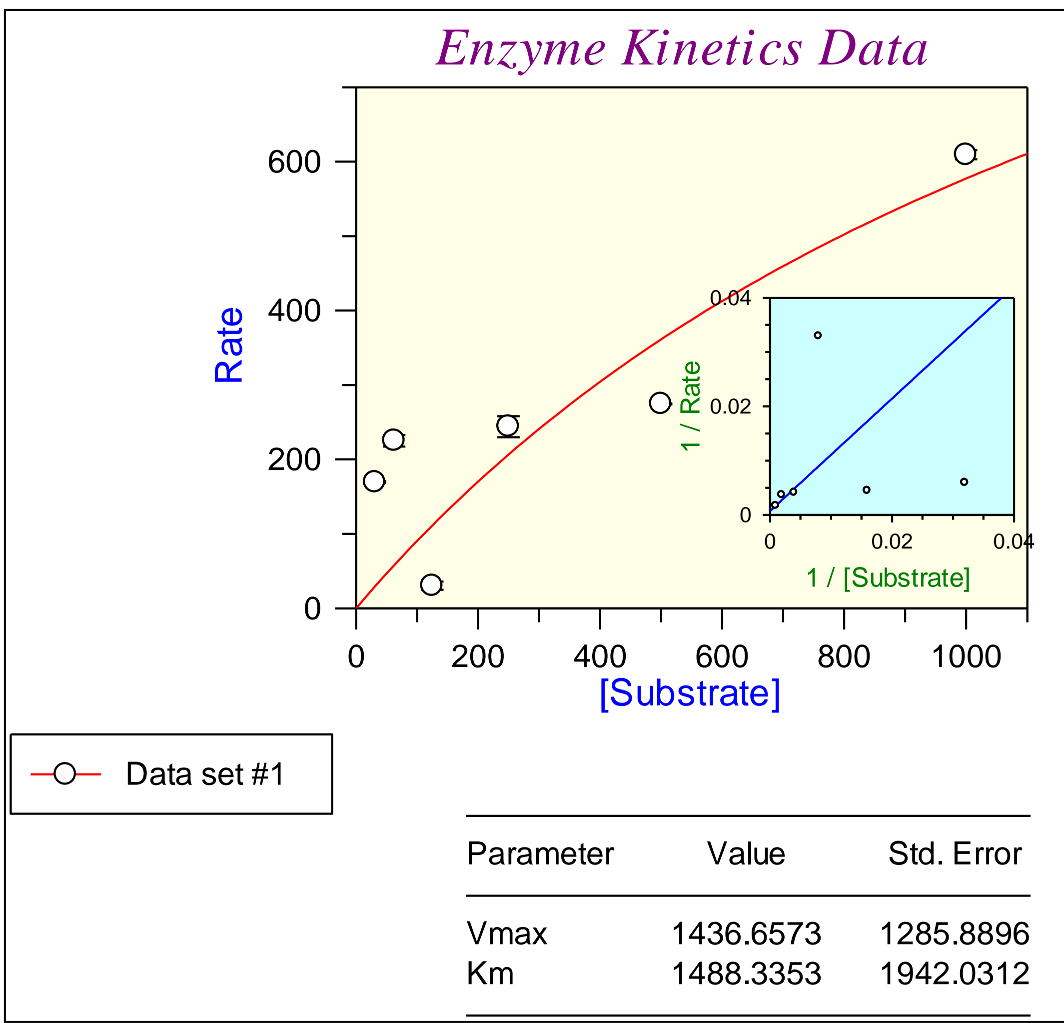
KM117



Parameter	Value	Std. Error
Vmax	1436.6573	1285.8896
Km	1488.3353	1942.0312

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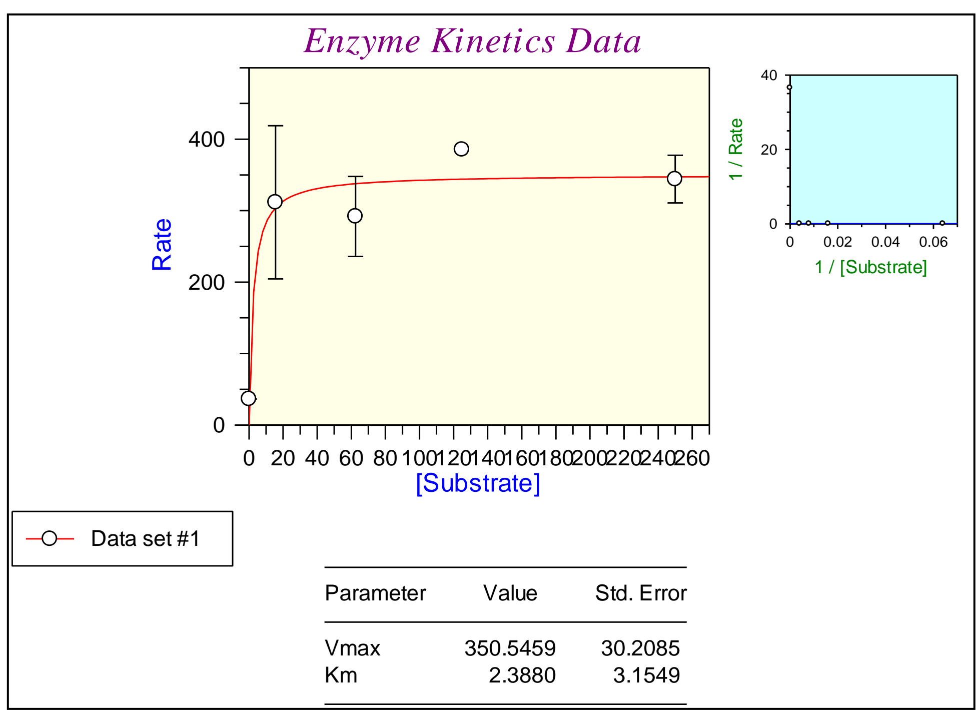
KM139



Parameter	Value	Std. Error
Vmax	350.5459	30.2085
Km	2.3880	3.1549

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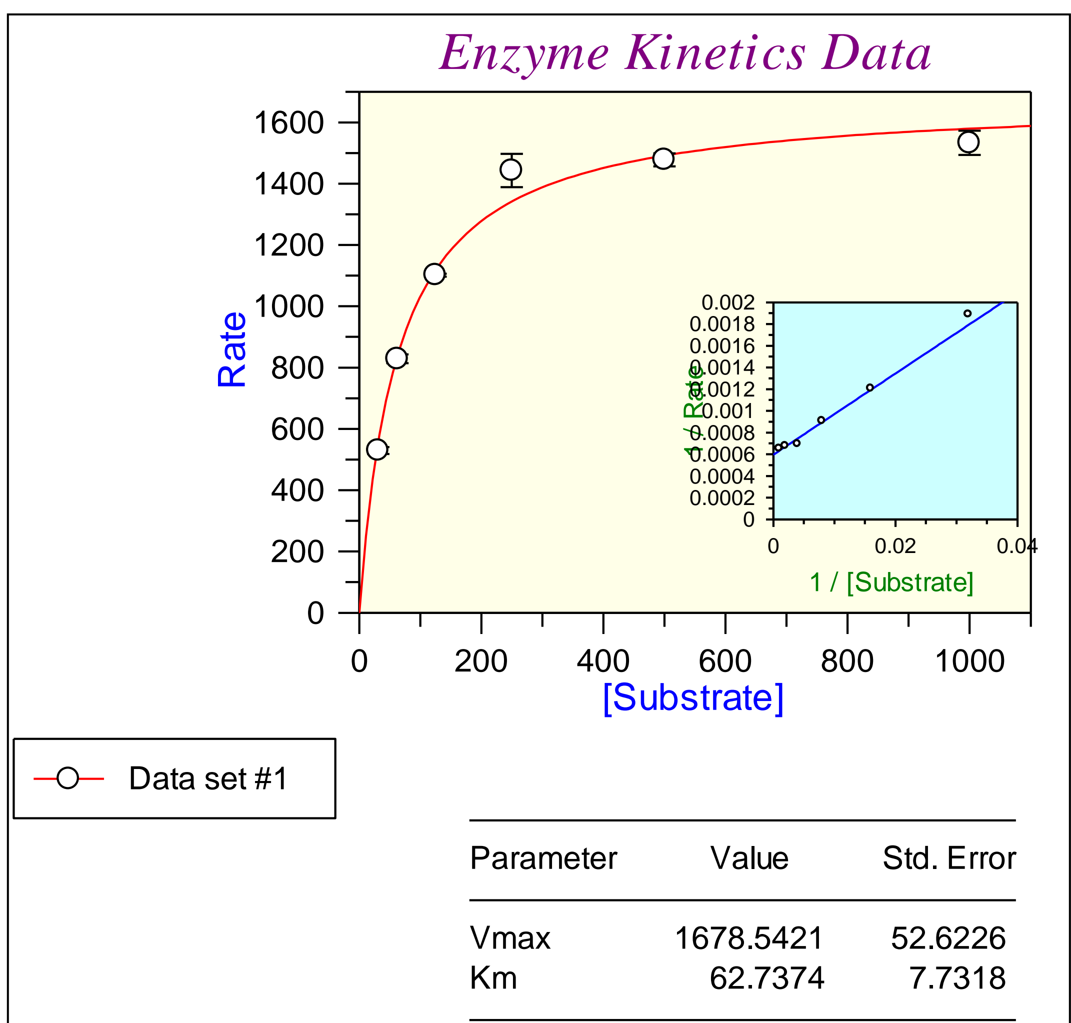
KM140



Parameter	Value	Std. Error
Vmax	1678.5421	52.6226
Km	62.7374	7.7318

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