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Bioactivity of the alkaloidal fraction of *Tabernaemontana elegans* (Stapf.)

by

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Declaration

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FACULTY OF HEALTH SCIENCES

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"Always bear in mind that your own resolution to succeed is more important than any one thing"

Abraham Lincoln

Abstract

Bacterial infections remain a significant threat to human health. Due to the emergence of widespread antibiotic resistance, development of novel antibiotics is required in order to ensure that effective treatment remains available. The aim of this study was to isolate and identify the fraction responsible for the antimicrobial activity in *Tabernaemontana elegans* (Stapf.) root extracts.

The active fraction was characterised by thin layer chromatography (TLC) and gas chromatography – mass spectrometry (GC-MS). Antibacterial activity was determined using the broth micro-dilution assay and antimycobacterial activity using the BACTEC radiometric assay. Cytotoxicity of the crude extract and fractions was assessed against primary cell cultures; lymphocytes and fibroblasts; as well as a hepatocarcinoma (HepG2) and macrophage (THP-1) cell line using the Neutral Red uptake and MTT assays. The crude root extracts were found to contain a high concentration of alkaloids (1.2% w/w).

GC-MS analysis identified the indole alkaloids, voacangine and dregamine, as major components. Antibacterial activity was limited to the Gram-positive bacteria and *Mycobacterium* species, with MIC values in the range of 64 – 256 µg/ml. When combined with antibiotics, additive antibacterial effects were observed. Marked cytotoxicity to all cell lines tested was evident in the MTT and Neutral Red uptake assays, with IC_{50} values ranging between 1.11 – 9.81 µg/ml.

This study confirms the antibacterial activity of *T. elegans* and supports its potential for being investigated further for the development of a novel antibacterial compound.

Keywords: Antibacterial natural products; Indole alkaloids; Methicillin-resistant *Staphylococcus aureus* (MRSA); *Tabernaemontana elegans*; Tuberculosis.

List of abbreviations

aDNA	Ancient DNA
AF	Alkaloidal fraction
ATCC	American Type Culture Collection
BF	Basic fraction
CFU	Colony forming units
DMEM-F12	Dulbecco's Minimum Essential Medium – F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed therapy, short course
EMB	Ethambutol
EMEM	Eagle's Minimum Essential Medium
FCPA	Ferric chloride-perchloric acid
FCS	Foetal calf serum
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
GC	Gas chromatography
GI	Growth index
HTS	High-throughput screening
INH	Isoniazid
LLE	Liquid-liquid extraction
MBC	Minimum bactericidal concentration
MDR-TB	Multi-drug resistant tuberculosis

MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistance *Staphylococcus aureus*
MS	Mass spectrometry
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Neutral Red	3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride
NIST	National Institute of Standards and Technology
PBS	Phosphate-buffered saline
PHA	Phytohaemaglutinin
PMA	Phorbol 12-myristate 13-acetate
PZA	Pyrazinamide
RMP	Rifampicin
RPMI-1640	Rosewell Park Memorial Institute medium 1640
TB	Tuberculosis
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organisation
XDR-TB	Extensively drug -resistant tuberculosis

Table of contents

List of Figures

Chapter 1

Chapter 2

Chapter 4

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

1 Literature Review

The introduction of antibiotics in the middle of the previous century, and the associated reduction in mortality attributable to bacterial infections was one of the significant medical advances of the 21st century. Rapid advances in the diagnosis and treatment of these infections generated widespread optimism and it was widely thought that the so-called 'war on microbes' had been won. Due to this presumed victory, research and development in the treatment of bacterial infections was de-prioritized. It has become apparent that the declaration of victory was premature, and that globally, a crisis of antibiotic resistance is looming. If future generations are still to have access to the life-saving antibiotics, a major paradigm shift will be required in the treatment of bacterial infections.

1.1 A history of antibiotics

Bacteria, the most ubiquitous living organisms on Earth, have always had an intricate relationship with humans[1]. These unicellular micro-organisms are able to colonize virtually any environment on the planet, and the human body is no exception. It is estimated that the bacteria present on the skin and in the gastrointestinal tract of a human outnumber the cells of the same body 10 to 1[2]. The vast majority of these bacteria present in the human are considered to be normal flora of the organs and are responsible for important physiological functions.

Certain bacterial species, however, upon gaining entry into the human body, are able to rapidly colonise tissues and are responsible for disease. Bacterial infections have been responsible for millions of human deaths throughout the ages and were the leading cause of death before the introduction of antibiotics[3]. Infection with the causative agents of diseases such as tuberculosis, the bubonic plague, meningitis, diphtheria and pneumonia often represented a death sentence, as no curative treatments were available.

In the early twentieth century, the search for antimicrobial compounds had yielded the compounds arsphenamine (Salvarsan) and sulphanilamide (Prontosil). Arsphenamine was limited in its efficacy, had a torturous administration regimen and associated with severe side effects[4]. Sulphanilamide represented a major step forward in the chemotherapy

of infections[5], but it was only in 1945, when the antibacterial properties of pencillin was discovered, that a major turning point in the treatment of bacterial infections was reached. Penicillin, isolated from the *Penicillium* moulds, and the isolation of streptomycin from the bacterium *Streptomyces griseus* a few years later, prompted the realization that certain bacteria and fungi produced antibacterial compounds as secondary metabolites. This finding provided the much needed impetus to start the highly successful search for other antibiotics in other micro-organisms.

As a result, many pharmaceutical companies began vast screening programs for the identification of new antibiotics, predominantly in the actinomycetes and fungi[6]. During this golden era of antibiotic discovery (1930 – 1960), ten novel classes of antibacterial drugs were introduced; in chronological order, these were the sulfonamides, beta-lactams, aminoglycosides, chloramphenicol, tetracycline, macrolides, glycopeptides, rifamycins, quinolones and trimethoprim[7]. Many of the prototype molecules for these classes were the natural products isolated directly from micro-organisms, but advances in synthetic chemistry allowed semi-synthetic derivatives with wider spectrums of activity and better pharmacokinetic properties to be developed.

These early successes in the development of new antibiotics, and their efficacy at treating infections prompted complacency in treatment of bacterial infections. It was assumed that the emergence of antibiotic resistance would not be so significant as to impact the efficacy of antibiotics on a large scale, and that the pace of antibiotic discovery would remain rapid. Fifty years onwards from the golden era of antibiotics, it is clear that both of these assumptions were false.

1.2 Antibiotic resistance

In each decade after the introduction of antibiotics, numerous newly-resistant species of bacteria were described. At present, resistance mechanisms have been identified for all classes of antibiotics available[8] and in certain bacterial strains, multiple resistance mechanisms have been acquired[9]. Infections caused by such strains are difficult and more costly to treat, and are associated with higher incidences of mortality and morbidity, clearly

demonstrating the clinical impact of antibiotic resistance[10]. A brief review of antibiotic resistance in selected bacterial pathogens follows.

1.2.1 Tuberculosis

The global epidemic of tuberculosis (TB) is a prime example of the antibiotic resistance crisis. An ancient disease, it was successfully brought under control due to advances in medical science and improvement in living conditions. It has recently re-emerged as a major cause of mortality, especially in the developing world, with a rising number of cases resistant to one or more of the antibiotics used to treat TB. As no major advances in new anti-tuberculosis agents have been introduced, the TB crisis threatens to spiral dangerously out of control.

TB has affected mankind since prehistory, as evidenced by bones dating to the Neolithic period (ca. 5000 BCE) demonstrating morphological changes characteristic of TB infection[11]. The morphological diagnosis of TB in these bones has been confirmed by ancient DNA (aDNA) studies[12]. From antiquity onwards, numerous authors have described a chronic lung disease with symptoms characteristic of TB[13], but it was only in 1882 that Robert Koch isolated and identified the bacterial pathogen responsible for TB.

Tuberculosis is caused by infection with the aerobic bacilli of the *Mycobacterium* genus, primarily *M. tuberculosis*. Once the bacilli have entered the lungs, they are taken up by alveolar macrophages. The macrophage is unable to destroy the bacillus, as the bacterium is able to prevent the phagosome-lysosome fusion, and prevent the subsequent acidification of the phagosome[14]. Consequently, the bacteria are able to multiply within the macrophage. Extracellularly, a complex interaction between macrophages, lymphocytes and neutrophils occurs, resulting in the sequestration of the infected macrophages within granulomas[15]. This cellular-based immune reaction to infection is able to prevent progression of infection to active TB disease.

In up to ten percent of infections, however, the immunological control of the infection fails and active TB develops[16]. Factors which suppress the immune system, such as co-infection with HIV, treatment with cytotoxic drugs, illicit drug use or clinical conditions such as silicosis, diabetes mellitus and chronic renal failure greatly increase the probability of active infection developing[17].

The symptoms of TB are predominantly pulmonary, with patients experiencing a persistent cough with blood in the sputum and chest pains, as well as symptoms characteristic of chronic immune activation, such as night sweats, fever and weight loss. The mycobacteria are transmitted between people through the respiratory aerosols of patients with active TB disease. It is thought that one person with untreated TB disease will transmit the infection to 10 – 15 persons annually[18].

The World Health Organisation (WHO) estimates that one third of the world's population is infected with *M. tuberculosis*, with a new infection occurring every second[18]. In 2009, it was estimated that there were 11 million cases of TB globally, resulting in 1.3 million deaths annually. Prevalence was highest in South-East Asia, with 4.9 milllion cases of active TB infection[19]. In Africa, the the prevalence figures are similar, with 3.9 million cases[19]. Together, these two regions account for 85% of the TB burden of the world[19]. Even though the incidence of new TB infections is stable or falling in all six WHO regions, corresponding increases in the global population have resulted in no substantial reduction in the number of reported TB cases[18].

The treatment of TB requires a regimen of a combination at least three antibiotics, taken for a minimum of six months. The first-line treatment options all consist of regimens combining isoniazid (INH), rifampicin (RMP), pyrinzamide (PZA) and ethambutol (EMB)[20]. The long course of antibiotic therapy is due to the slow doubling time of *M. tuberculosis* (16 – 20 hours). The rationale for the use of multiple anti-tuberculosis agents is to increase efficacy, prevent resistance from developing, and shorten the length of treatment[20]. Due to its duration, patient compliance to the treatment regimen is a major obstacle to successful treatment. The Directly Observed Therapy, Short course (DOTS) programme was implemented by the WHO in 1995 to address these compliance issues. One of the major components of DOTS is the supervised administration of the anti-tuberculosis agents by a healthcare professional. To date, 184 countries have implemented DOTS programmes, treating 41 million people with a success rate of 86%[19]. The implementation of the programme in high-burden, resource-poor settings, however, has been slow. As of 2000, it was estimated that less than 25% of all TB cases were treated under such a programme[21]. Despite the successes of the DOTS programme, there are still numerous medical, social,

economic and legal challenges which face patients and may result in non-completion of full treatment programme[22].

In circumstances where TB treatment is inadequately or erratically followed, drug resistance may occur. Cases of multidrug resistant tuberculosis (MDR-TB), defined as *M. tuberculosis* infections that are resistant to treatment with isoniazid and rifampicin, are rising, with approximately half a million cases reported in 2008[23]. Data collected in 27 high-burden MDR-TB countries, revealed that the majority of MDR-TB cases that are identified occur in patients that have previously received TB treatment[23]. A significant proportion of MDR-TB cases, however, are diagnosed in those who have never been treated for TB, indicating the ability of MDR-TB to be spread in a community setting[24;25]. The geographic distribution of MDR-TB cases in new TB cases, and previously treated TB cases, are shown in Figure 1.1 and Figure 1.2, respectively.

The treatment of MDR-TB requires the use of second-line anti-tuberculosis agents such as aminoglycosides (kanamycin and amikacin), polypeptides (capreomycin, viomycin and enviomycin), fluroquinolones (ofloxacin, ciprofloxacin and gatifloxacin), D-cycloserin or thionamides (ethionamide and prothionamide)[26]. Treatment with these agents is 10 times more expensive due to the longer duration of treatment required[27], and furthermore, is associated a less favourable adverse effect profile[28]. A sub-set of these MDR-TB infections have acquired resistance mechanisms to these second-line agents, and are termed extensively-drug resistant TB (XDR-TB).

The implications of the M/XDR-TB for public health are not fully known, but as the treatment of TB intensifies globally, the proportion of *M. tuberculosis* strains that are drug-resistant is bound to rise. In high-burden, low-resource settings, where the majority of M/XDR-TB cases are found, the additional challenges to effectively treat these cases will prove to be very demanding. However, with a mortality rate of up to 98% in those co-infected with M/XDR-TB and HIV[24], it is clear that these challenges will need to be addressed promptly.

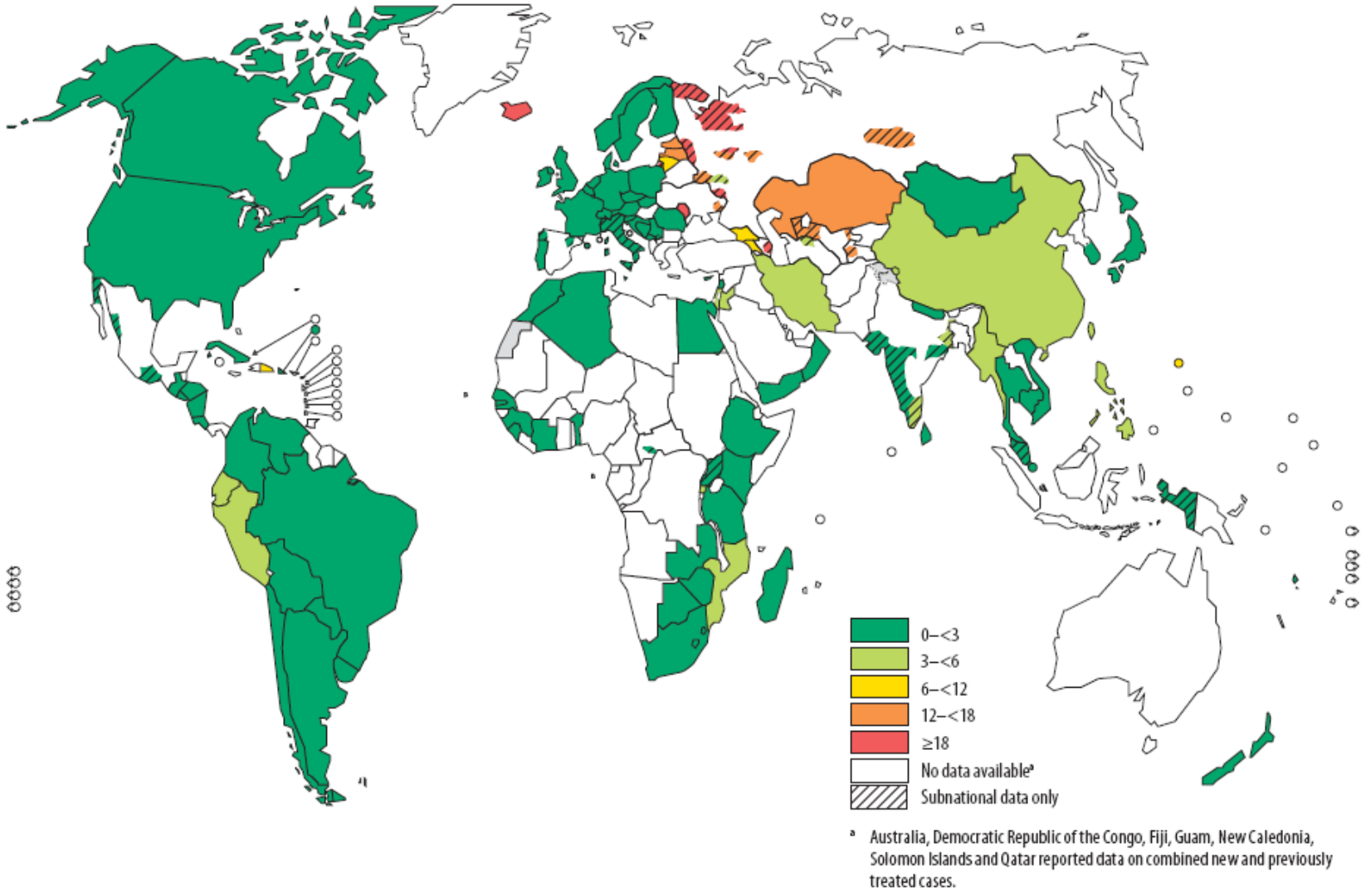

Figure 1.1: Geographic distribution of proportion of MDR-TB cases among new cases of TB, 1994 – 2009[23]

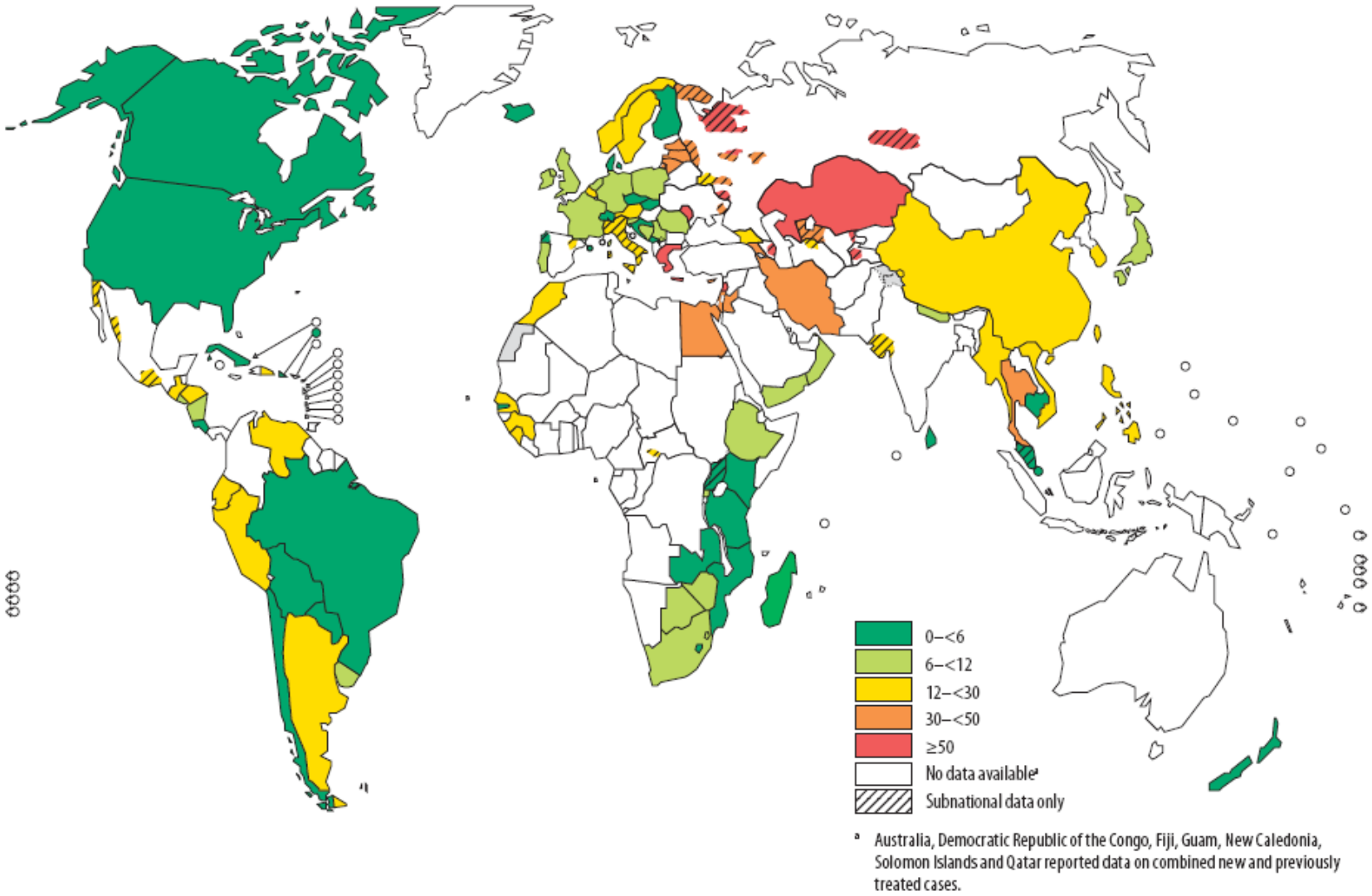

Figure 1.2: Geographic distribution of proportion of MDR-TB cases in previously treated TB cases, 1994 - 2004[23]

1.2.2 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive cocci that is found as part of the normal bacterial flora of the skin in approximately 30% of the population[29]. In certain cases, *S. aureus* causes infections ranging from skin and soft tissue infections to pneumonia, meningitis, osteomyelitis, endocarditis, sepsis and bacteraemia.

Empirical treatment for *S. aureus* infections has been with the β-lactam antibiotics, however, within the first five years after the introduction of penicillin, resistance to this antibiotic was found in up to 50% of *S. aureus* strains[30]. This resistance is conferred by the presence of genes encoding for the production of β-lactamases, enzymes which are able to destroy the β-lactam ring responsible for the antibacterial activity of the penicillins. Newer derivatives of the β-lactams were developed, such as methicillin, that are resistant to the effects of the β-lactamases. The ferocious pace of bacterial evolution, however, has ensured that strains of *S. aureus* have developed resistance mechanisms against these antibiotics as well.

Previously, the strains of methicillin-resistant *Staphylococcus aureus* (MRSA) were overwhelmingly associated with nosocomial settings[31]. In these strains, the high selective pressure associated with long periods of treatment with numerous antibiotics promoted the emergence of strains with multiple resistance mechanisms. Termed healthcare-associated MRSA (HCA-MRSA), these infections frequently target hospital patients undergoing invasive medical procedures, the immunocompromised and the elderly[32]. Treatment options for HCA-MRSA are limited due to the widespread resistance to available antibiotics, with only vancomycin available in the first-line setting[33]. Mortality of patients with HCA-MRSA infections has been shown to be higher than those patients infected with methicillin-sensitive *S. aureus*, even when co-morbid factors are controlled for[34].

In the late 1990s, a potentially more threatening form of MRSA emerged as the cause of disease outbreaks in the community[35]. This community-acquired MRSA (CA-MRSA) has been defined by the Centres for Disease Control (CDC) as identification of MRSA in a patient with signs and symptoms of infection, either in an outpatient setting or with 48 hours of admission to hospital, with no history of MRSA infection or colonisation, no history of admission to a hospital or nursing home in the previous year and the absence of dialysis,

surgery, permanent indwelling catheters or medical devices that pass through the skin to the body[36]. Infections caused by CA-MRSA ranged from epidemics of skin and soft-tissue infections[37], such as carbuncles and furuncles, to necrotising pneumonia and bacteraemia[38]. The population at risk for CA-MRSA is substantially different from those affected by HCA-MRSA, with healthy children and adolescents, as well as adults that spend a large portion of their time in close proximity with others (sports teams, soldiers, prison inmates) most at risk[39].

Of concern, is the fact that CA-MRSA is almost always associated with the expression of numerous virulence factors, such as Panton-Valentine leukocidin (PVL), which is responsible for tissue necrosis[40]. In case studies of patients with invasive (i.e. non skin and soft-tissue) CA-MRSA infections, mortality rates have been found to be as high as 35%[41]. A further development in the epidemiology of CA-MRSA is epidemics of CA-MRSA in hospital settings, where the natural virulence of the strain is potentiated by the high levels of co-morbidities of the patients infected.

It has been shown that levels of antibiotic-resistance in CA-MRSA is substantially less than that of HCA-MRSA, presumably due to the lower levels of antibiotic use in the general population. Most strains of CA-MRSA only exhibit complete resistance to the β-lactam antibiotics, with variable resistance to other agents[29]. Despite this fact, vancomycin remains the agent of choice in serious CA-MRSA infections[33]. This reliance on vancomycin for the treatment of serious MRSA infections is not a sustainable situation as vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* (VISA and VRSA, respectively) strains have been reported in the literature[42]. These infections, without the development of new antibiotics, will represent a growing source of mortality in the years to come.

1.3 Development of novel antibiotics

The development of novel antibiotics, either new molecules in existing classes or new classes of antibiotics, has lagged significantly behind the emergence of antibiotic resistance. The decline in research efficiency in this field is multi-factorial, and may be ascribed to economic, medical and priority changes within the drug development arena. As the impact

of antibiotic resistance increases, it is likely that drug development programs will return to the most promising source of antibacterial agents – natural products.

A relatively untapped area of natural product research is that of antibacterial compounds of plant origin. While the initial research in this area has not been immediately successful, it does not mean that this vast area of chemical space should be left unexplored. Research in this area will lead to a deeper understanding of the mechanisms employed by plants to protect themselves from bacterial invasion. This information may be vital in the future for the development of new antibiotics, or antibiotic combinations, which could be used to either treat resistant bacterial infections, or reverse resistance to existing antibiotics.

1.3.1 Current state of antibiotic development

All of the classes of antibiotics that have been approved for clinical use, as well as their year of introduction are listed in Table 1.1[43]. The de-prioritization of antibiotic development is clearly visible in the scarcity of novel antibiotic classes being approved since the mid 1980s. In the 20 year period 1983 – 2003, only three new classes were introduced, and a total of 49 new antibiotics were approved by the US Food and Drug Administration. The antibiotic pipeline is similarly poor, with a mere six compounds in clinical development[44]. The decline in approval of antibiotics is shown graphically in Figure 1.3[45].

In the past, research in the antibiotic field was predominantly driven by the pharmaceutical industry. The current dearth of new antimicrobials may in part be explained by the significant changes in the pharmaceutical industry over the past decades. These factors include a substantial increase in the costs of drug development, prioritizing research in fields which will yield the greatest return on investment, and a shift away from natural product research in favour of combinatory chemistry coupled with high through-put screening (HTS).

The cost of drug development was estimated to be USD $1.7 billion in 2003[46], up from USD $231 million in 1991[47], representing an increase of 735%. These costs can be attributed to, in part, the large amounts of data that are required by regulatory agencies for marketing approval. Pharmaceutical companies are under mounting pressure from their shareholders to develop new medicines that provide a substantial return on investment in

order to recoup these costs. As antibiotics are given only for short periods, their return on investment is generally less than that for chronic drugs. In a pharmacoeconomic analysis based on 2004 prices, ten days treatment with antibiotics cost a mean of US$ 85, in comparison to US$ 848 for antineoplastic drugs and US$ 301 for respiratory tract drugs, the most expensive therapeutic classes[48].

The HIV epidemic has also negatively impacted the development of new antibiotics. Research funding in pharmaceutical companies is split by therapeutic area, and as anti-retroviral drugs and antibiotics fall under the umbrella of infectious diseases, these two areas of research have been in direct competition for funding. With the HIV epidemic affecting an estimated 33.3 million people in 2009[49], and the chronic nature of treatment, the development of new anti-retroviral drugs has been at the expense of new antibiotics[45].

As a means to lower the costs involved in drug development, many pharmaceutical companies have shifted to a research platform based on the combination of combinatorial chemistry coupled to HTS[50]. It had been hypothesized that due to the advances in understanding the molecular basis of diseases, rational drug disease could guide the creation of synthetic molecules with the desired biological activity. Once a template molecule was identified, combinatorial chemistry could create a number of variations of that molecule, and HTS could be used to identify the molecules which were the most effective.

Due to the expected success of this platform, many natural product development programmes that were responsible for novel antibiotics were closed[51]. The return from the combinatorial chemistry/HTS, however, has failed to materialize. There are numerous reports in the literature of HTS methodologies for the discovery of new antibiotics, but none of these have been successful in indentifying new molecules with significant antibacterial activity[52].

Table 1.1: Antibiotic class with approximate year of clinical introduction, lead derivation, example of drug and mechanism of action[43]

Antibiotic class	Introduction	Derivation	Example	Mechanism
Sulphonamide	1935	Synthetic	Sulfapyridine	Anti-folate
β-lactam	1941	NP-derived	Penicillin	Bacterial cell wall
Bacterial peptide	1942	NP-derived	Bacitracin	Bacterial cell wall
			Polymixin	Bacterial cell membrane
Aminoglycoside	1944	NP-derived	Streptomycin	Protein synthesis
Cephalosporin	1945	NP-derived	Cephalosporin	Bacterial cell wall
Nitrofuran	1947	Synthetic	Nitrofurantoin	Various
Hexamine	1947	Synthetic	Methenamine mandelate	Release of formaldehyde
Chloramphenicol	1949	NP-derived	Chloramphenicol	Protein synthesis
Tetracycline	1950	NP-derived	Chlortetracycline	Protein synthesis
Isoniazid	1951	Synthetic	Isoniazid	Fatty acid biosynthesis
Viomycin	1951	NP-derived	Viomycin	Protein synthesis
Macrolide	1952	NP-derived	Erythromycin	Protein synthesis
Lincosamide	1952	NP-derived	Lincomycin	Protein synthesis
Streptogramin	1952	NP-derived	Virginiamycin	Protein synthesis
Cycloserine	1955	NP-derived	Cycloserine	Bacterial cell wall
Glycopeptide	1956	NP-derived	Vancomycin	Bacterial cell wall
Novobiocin	1956	NP-derived	Novobiocin	DNA synthesis

Antibiotic class	Introduction	Derivation	Example	Mechanism
Ansamycin	1957	NP-derived	Rifamycin	RNA synthesis
Nitroimidazole	1959	Synthetic	Tinidazole	DNA synthesis
Ethambutol	1962	Synthetic	Ethambutol	Bacterial cell wall
Quinolone	1962	Synthetic	Nalidixic acid	DNA synthesis
Fusidane	1963	NP-derived	Fusidic acid	Protein synthesis
Diaminopyrimidine	1968	Synthetic	Trimethoprim	Antifolate
Phosphonate	1969	NP-derived	Fosfomycin	Bacterial cell wall
Pseduomonic acid	1985	NP-derived	Mupirocin	Protein synthesis
Oxazolidinone	2000	Synthetic	Linezolid	Protein synthesis
Lipopeptides	2003	NP-derived	Daptomycin	Bacterial cell wall

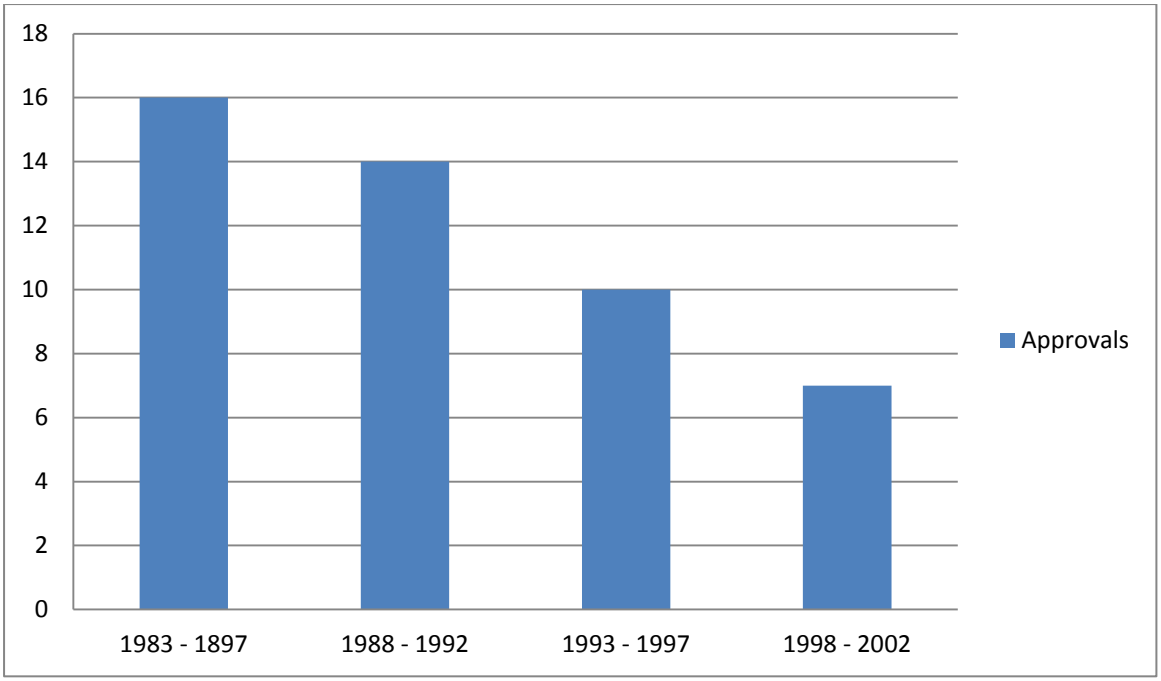

Figure 1.3: New antibacterial agents approved in the US, 1983 – 2002, per 5-year period[45].

1.3.2 Natural products as a source of novel antibiotics

Any molecule that is produced within a living organism and is able to exert an effect on a biological system is termed a natural product. These molecules often serve no immediate physiological function in the producing organism. The organism, however, is conferred a reproductive benefit through the ability of the molecule to affect the biological systems of other organisms. In higher organisms, such as plants, these molecules may provide a protective effect against infection by micro-organisms or predation by herbivores[53]. Evolution has thus acted as a screening process for these molecules, selecting for those which provide the most benefit to the organism.

Prior to the development of chemistry, natural products were the only source of biologically active molecules that could be utilized by mankind for the treatment of diseases. Numerous cultures have long histories and extensive pharmacopoeias detailing the use of plant and other natural substances for their ability to cure disease or ameliorate symptoms. As modern science developed, natural products served as the basis of pharmacology.

Natural products are estimated to be the source of 80% of all drugs approved in the pre-genomic era[54]. Despite the industry-wide move away from natural product drug discovery, natural products were the source of approximately 50% of drugs introduced in the period 1981 – 2002[55]. In the field of antibiotics, this figure is higher, with 75% of all antibiotics approved during 1981 – 2006 being natural products, or derivates of a natural product[56].

Despite the previous successes in developing natural products into antibiotics, the chemical space represented by natural products remains relatively unexplored. Methods to culture an estimated 99% of bacterial species found in soil do not exist[57], which hamper the effort to screen these organisms for biological activity. Furthermore, approximately 4% of the estimated 250,000 plant species have been assessed for significant biological activity[58]. The chemical diversity found among natural products is unsurpassed in comparison to that which can be created combinatorially, due to the presence of novel carbon skeletons and numerous chrial centres[50]. These chemical entities are also approximately 100-fold more likely to exert a biological effect than their synthetically produced counterparts[50], due to the

evolutionary screening process. All the while, the advantages of using natural products for drug discovery remain.

1.3.3 Antimicrobial secondary metabolites of plants

Due to the dire need for novel antibiotics, there has been an upsurge in screening of plants for antibacterial compounds. This field of research is multidisciplinary, utilizing aspects of pharmacognosy, medicinal and analytical chemistry, pharmacology and botany. Based on a PubMed search, 115 articles describing antimicrobial medicinal plants were published during the period 1966 – 1994; this figure doubled in the following ten years[59]. In the past, assessing terrestrial plants for antibacterial products has been relatively unpopular in comparison to the research conducted in micro-organisms. Various logistical, scientific and regulatory factors, some of which are common to all plant-derived natural products, can be ascribed to the reticent of the pharmaceutical industry to investigate this field.

The logistical factors include difficulty in obtaining a sustainable source of plant material which is needed for screening and development of a plant-derived antibiotic. The source of plant material is of even greater importance should a product be indentified that cannot be manufactured synthetically. The inherent variability in the chemical composition of various batches of plant material, even those collected from the same geographic region under similar climatic conditions, further add to these challenges[60].

When a crude plant extract is identified as having significant biological activity, the complexity of these extracts, especially in terms of closely-related chemical compounds and stereoisomers, make the process of isolating the responsible compounds a complex task. Furthermore, care has to be taken that the compound responsible for the activity has not previously been identified and investigated for its therapeutic usefulness, and that the compound is selective in its biological activity.

Through advances in various fields such as genomics, medicinal chemistry and chromatography, these challenges have become easier to surmount. Strategies have been developed for crude plant extracts to be screened for biological activities in time periods comparable to that of synthetic chemicals[54]. Developments in chromatography have allowed for rapid isolation of the responsible compound, and dereplication assays have been developed to ensure that the compound isolated has not been previously identified

and analysed[61]. New methodologies in chemistry have also allowed for total synthetic synthesis of natural products with the identified biological activity. Should the active natural product not be able to be fully synthesized, chemical analogues which retain the pharmacologically functional group are often a viable alternative[54]. As the challenges posed by the logistical and scientific factors lessen, it is highly likely that the rate at which biologically active natural products from plant sources are approved will increase.

The final hurdle which exists, and pertains exclusively to antibacterial plant compounds, is our understanding of how plants effectively prevent bacterial infections and how to effectively use this understanding to develop new antibiotics. It is well established that plants produce a number of low molecular weight compounds, termed phytoalexins, which are produced upon pathogenic infection of the plant[62]. When these compounds are assessed for antibacterial activity, however, only weak activity is found, and this activity is predominantly limited to Gram-positive bacteria[63].

In addition to the antibacterial phytoalexins, plants simultaneously produce compounds which are able to modulate bacterial physiology, especially in terms of the uptake and efflux of xenobiotics into the bacterial cell[64]. By virtue of altering the permeability of the bacterial cell, the phytoalexins are then able to exert their antibacterial effects. This is even in Gram-negative bacteria, which are morphologically better adapted to avoid the effects of xenobiotics. *In vitro* studies have shown that addition of compounds known to inhibit the efflux pumps found in bacteria significantly increase the potency of plant-derived antibacterial products[65].

Should it be the case that the vast majority of plant-derived antibacterial compounds require this type of synergism to be effective, it will require a major paradigm shift for drug discovery in this area. Robust methodologies for the identification of synergistic antibacterial compounds will need to be developed, and the scientific and regulatory communities worldwide will have to adjust their criteria for what constitutes a valid pharmacological entity. Without these changes, a significant number of potential new antibiotics will be lost, something which cannot be afforded given the current state of affairs.

1.4 Tabernaemontana elegans Stapf.

Tabernaemontana elegans Stapf (syn. *Conopharyngia elegans* (Stapf)), is a member of the *Apocynaceae* family. It is a small tree found in evergreen river fringes at low altitudes and in coastal scrub forest[66]. It is known in English as the toad tree due to the brown, wart-like skin of its fruit (Figure 1.4).

There are several reports of the ethnomedical use of *T. elegans*, indicating that *T. elegans* may contain compounds which are biologically active *in vivo*. Pertaining to antibacterial activity, a root decoction is applied as a wash to wounds, and drunk for pulmonary diseases and chest pains by the VhaVenda[67] and Zulu[68] people of South Africa. Other ethnomedical usages include treatment of heart diseases with the seeds, stem-bark and roots, the root-bark and fruits for cancer treatment, and a root decoction is said to have aphrodisiac properties[69].

There are only three reports of biological activity ascribed to extracts of *T. elegans*. In a previous study conducted in this laboratory, *T. elegans* was identified as having antibacterial activity against *S. aureus* and antimycobacterial activity against *M. smegmatis*[70], as well as anti-fungal activity against *C. albicans*[71]. An earlier study identified *T. elegans* as one of 8 *Tabernaemontana* species possessing antibacterial activity against Gram-positive bacteria[72]. In these studies, the compound(s) responsible for the reported biological activity was not identified.

A number of other members of the approximately 100 species of *Tabernaemontana* genus[73] have been assessed for biological activity. There have been reports of anti-amoebic[72], anti-cancer[74], anti-fertility[75], anti-inflammatory[76;77], anti-microbial[72;78], anti-oxidant[78], anti-ophidian[79], anti-protozoal[80-82], and anti-viral[72] properties, as well as acetylcholinesterase inhibition[83-85], depression of the central nervous system[86], and cardiovascular effects[87] for various *Tabernaemontana* species.

Figure 1.4: *Tabernaemontana elegans* (Stapf.)[66]

Previous phytochemical research has shown *T. elegans* to be particularly rich in monoterpenoid indole alkaloids, of which 24 have been isolated (Table 1.2)[88-91]. These alkaloids, which are structurally diverse, are commonly found in many members of the genus and are considered chemotaxonomically important[92]. To date, over 300 of these alkaloids have been isolated[73].

The biosynthetic pathway for the production of indole alkaloids requires tryptamine or tryptophan for the production of the indole nucleus, as well a C_9- or C_{10}- monoterpene moiety, which is derived from secologanin. The biosynthetic pathway for these alkaloids is shown diagrammatically in Figure 1.6. Viewed in terms of structural types and biogenetic origin, these indole alkaloids can be divided in ten classes: aspidospermatan, corynanthean, eburnan, heynean, ibogan, plumeran, strychnan, tacaman, vallesiachotaman and vincosan[92]. Of most chemotaxonomic importance is the corynanthean (C) and heynean (H) classes, which are rarely located outside the *Tabernaemontana* genus[92]. Adding to the chemical diversity of the alkaloids is the production of bisindolic alkaloids, which are heterodimeric indoles formed from the combination of two indole alkaloids from any of the classes. As shown in Table 1.2, the alkaloids of *T.elegans* are mainly of the aspidospermatan (A), corynanthean (C), and ibogan (I) classes, as well a number of bisindole alkaloids consisting of dimers of corynanthean-ibogan moeities. The generalized chemical structure for the aspidospermatan, corynanthean and ibogan classes are shown in Figures 1.7 – 1.9, respectively.

The majority of the literature for the species of *Tabernaemontana* has been focused on the isolation and characterisation of novel indole alkaloid structures. The biological activity of *T. elegans*, especially in terms of antibacterial activity, which has thus far been reported for crude extracts, remains less thoroughly investigated. Due to the clear need for new agents which may be used for the treatment of tuberculosis and other bacterial infections, further investigation of the bacterial properties of this plant was warranted.

Table 1.2: Alkaloids previously isolated from *Tabernaemontana elegans,* listed by indole alkaloid class

Alkaloid	Biosynthetic Class[b]	Plant part	Abundance in whole plant extract[c]	Reference
Apparicine	A2	WP	+++	88
16-*S*-OH-16,22-dihydro-apparacine	A2	WP	+	88
Tubotaiwine	A3	WP	+	88
Vobasine	C5	WP	++++	88
Vobasinol	C5	WP	+	88
Dregamine	C5	WP, RB	++++	88;89
Dregaminol	C5	WP	++	88
Tabernaemontanine	C5	WP, RB	++++	88;89
Tabernaemontaninol	C5	WP	++	88
Dregaminol-methylester	C5	WP	+	88
Isovoacangine	I1	WP	++	88
Conopharyngine	I1	RB	N/A	88
3-*R/S*-hydroxyconodurine	I1	WP	++	88
Tabernaelegantine A	C-I	WP, RB	+++	88-90
Tabernaelegantine B	C-I	WP, RB	+++	88;89 90
Tabernaelegantine C	C-I	WP, RB	++	88-90
Tabernaelegantine D	C-I	WP, RB	++	88-90
Tabernaelegantinine A	C-I	RB	N/A	90

Tabernaelegantinine B	C-I	RB	N/A	[90]
Tabernaelegantinine C	C-I	RB	N/A	[91]
Tabernaelegantinine D	C-I	RB	N/A	[91]
3-*R/S*-hydroxy-tabernaelegantine B	C-I	WP	++	[88]
3-*R/S*-methoxy-tabernaelegantine C	C-I	WP	++	[88]
Conoduramine	C-I	RB	N/A	[89]

a: WP – Whole plant extract; RB – Root bark extract

b: Vincosan (D), Corynanthean (C), Vallesiachotaman (V), Strychnan (S), Aspidospermatan (A), Plumeran (P), Eburnan (E), Ibogan (I), Tacaman (T), Bisindole (B).

c: ++++: Main component; +++: major component; ++: minor component; +: trace component. N/A: results not available.

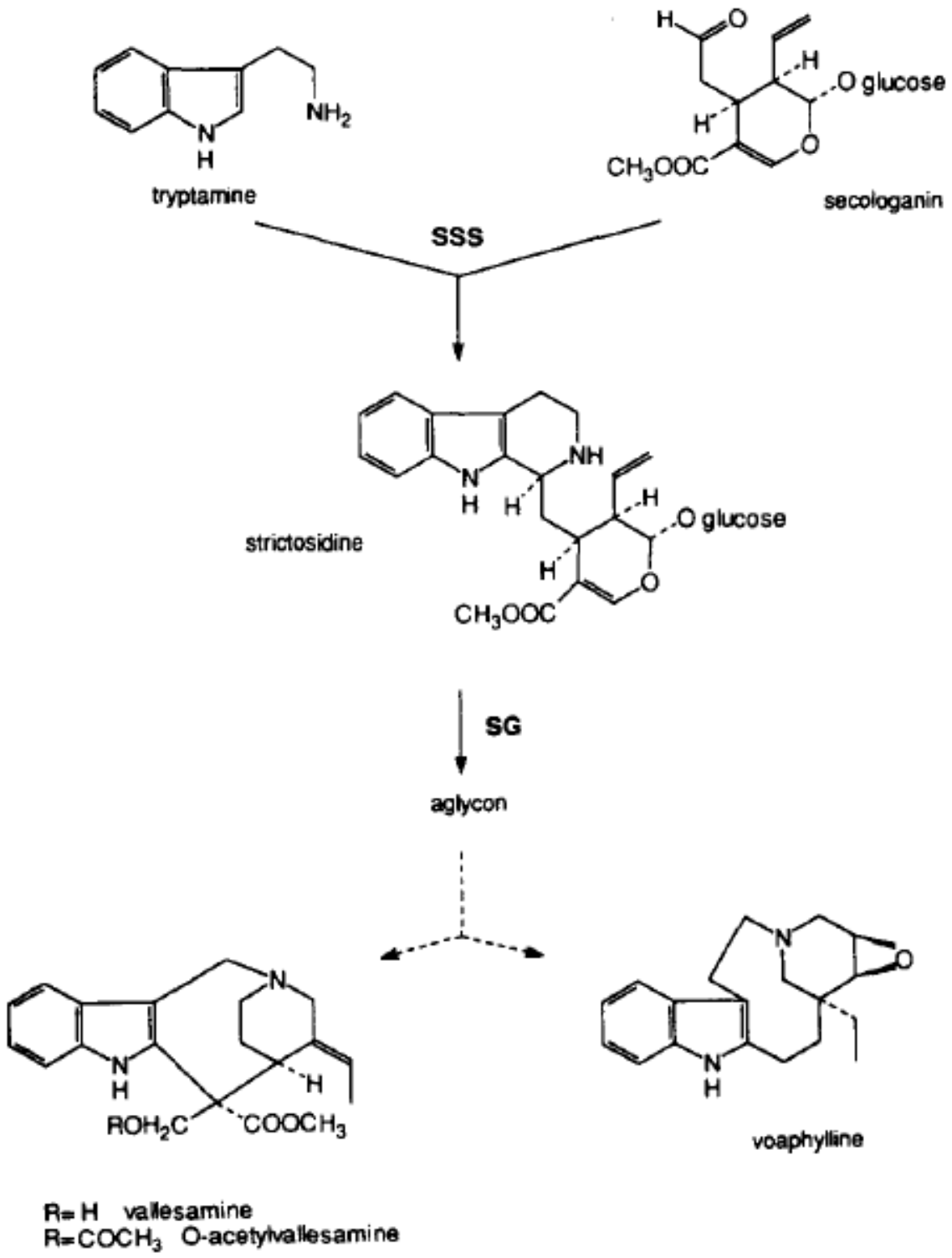

Figure 1.5: Biosynthesis of the main alkaloids of *Tabernaemontana* species[93]

SSS: strictosidine synthase; SG: strictosidine glucosidase.

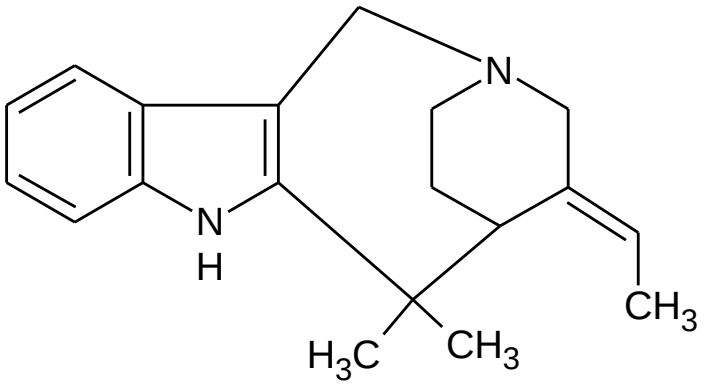

Figure 1.6: Generalized chemical structure of the aspidospermatan indole alkaloid class

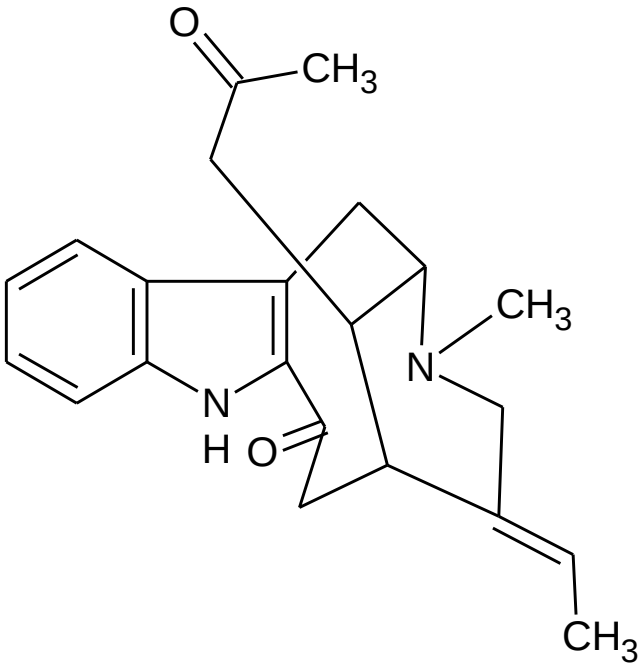

Figure 1.7: Generalized chemical structure of the corynanthean (C5) indole alkaloid class[94]

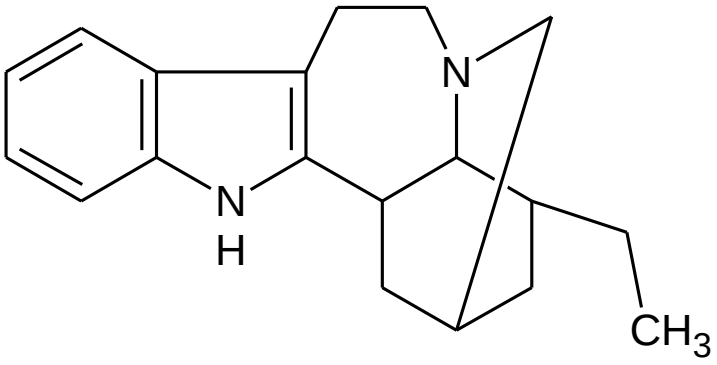

Figure 1.8: Generalized chemical structure of the ibogan (I1) indole alkaloid class[94]

1.5 Aim and objectives

1.5.1 Aim

The primary aim of this study was to isolate an antibacterial fraction from *Tabernaemontana elegans* and assess the spectrum of antibacterial activity, synergism of antibiotic effects, as well as the *in vitro* cytotoxicity against mammalian cells.

1.5.2 Objectives

The objectives of this study were to:

- Identify major phytochemical constituents of an ethanolic crude extract of *T. elegans* root.
- Isolate the alkaloidal fraction (AF) from the crude extract.
- Chemically characterise the major components of the AF.
- Investigate the spectrum of antibacterial activity possessed by the crude extract and AF.
- Investigate the synergistic antibacterial properties of the AF when combined with antibiotics.
- Determine the *in vitro* cytotoxicity of the crude extract and AF against mammalian cells.

2 Extraction and chemical characterisation

2.1 Introduction

The primary aim of the extraction process should be the creation of a reproducible, biologically-active, enriched extract that is compatible with biological assay systems. There are, however, very few methods that allow the extraction process to be monitored directly, especially if the end-point of the procedure is to assess its biological activity. While this may mean that much of the extraction process is performed by trial and error, careful consideration can greatly reduce the amount of error.

Plant material should be carefully selected, with care being taken to ensure that the identity of the plant is verified by an experienced botanist and that a voucher specimen is deposited for future reference. The collected plant material should be inspected for any visible signs of contamination. Where possible, all experimental procedures should be performed on the plant material that was collected at the same time and location. This is due to the chemical variability in extracts associated with differing geographic and climatic conditions[60].

The choice of initial extraction solvent and subsequent fractionation procedure is an important consideration. The secondary metabolites produced by plants can belong to a wide variety of phytochemical classes with specific physicochemical properties. Solvent selection should be based on literature and reported ethnomedical preparations[95], and whether a specific class of compounds in that particular plant are of interest. If there is no compelling evidence that a specific class of compounds is responsible for previously reported biological activity, or if biological activity has not previously been described, a wide range of solvents with differing polarity should be employed in order to extract as many classes of secondary metabolites from the plant.

Chemical characterisation of the active extract is needed to obtain a chemical fingerprint. This information may aid in further fractionation steps if a non-specific extraction methodology was employed, and can confirm for variability in biological activity of different batches of plant material. The recent advances in coupled chromatographic techniques, particularly gas and liquid chromatography-mass spectrometry, when combined

with mass spectra fragment libraries, allows for identification of known compounds in fractions. These techniques can provide extensive information using very little sample by separating and characterising multiple compounds based on available databases of well characterised compounds, both in terms of physicochemical and biological properties. This process is termed dereplication[61].

In the literature, numerous reports exist of the biological activity associated with the alkaloids of *Taberaemontana* species. In a pilot study performed in our laboratory, it was demonstrated that the antibacterial activity of the crude extract was limited to the alkaloidal fraction of *T. elegans*. As alkaloids can be extracted from plant material, with relative ease and in high yield, by an acid-base extraction methodology, an alkaloidal fraction was selected as the primary fraction of interest.

Due to documented methodology for alkaloid extraction, ethanol was selected as the initial extraction solvent. An acid-base partitioning step was employed to obtain an alkaloid-rich subfraction, a method which has been well described for the isolation of alkaloids. This method employs changes in pH in order to alter the solubility of alkaloids in aqueous solutions and liquid-liquid extraction as a means of separation of compounds. Thin layer chromatography was utilized to follow the fractionation process, to identify phytochemical groups present in the crude extract, and to confirm the selectivity of the alkaloidal extraction process by evaluating the enrichment of alkaloidal compounds within the subfraction. Gas chromatography-mass spectrometry was used to identify the major alkaloids present in the alkaloidal subfraction.

2.2 Materials and methods

2.2.1 Plant material

Roots of *Tabernaemontana elegans* Stapf. were collected from the Venda region of Limpopo, South Africa in February 2009. The plant material was authenticated and a voucher specimen (NH 1920) deposited at the Soutpanbergensis Herbarium (Makhado, Limpopo).

2.2.2 Extraction procedure

The collected roots were inspected for any signs of microbial and fungal contamination. The roots air-dried at room temperature and milled with a Wiley mill (Arthur Tomas Co., Philadelphia, USA). The plant material was then finely ground with an Ika Analytical Mill (Staufen, Germany). The powdered plant material was kept in a closed container, out of direct sunlight, at room temperature, until use. The powdered root was extracted by maceration in ethanol (Merck Chemical Co.) at a ratio of one hundred grams of plant material to one litre of ethanol. The flask was placed in an ultrasonic bath for an hour then left to stand overnight at ambient temperature. The extract was centrifuged (1000*g*, 5 minutes) and vacuum-filtered (0.44 µm, Millipore). The plant material was then further macerated under similar conditions two more times and the three extracts combined.

2.2.2.1 Preparation of the crude extract

An aliquot of the ethanol extract was concentrated to dryness with a rotary evaporator (Buchi, Switzerland) at a temperature of 40˚C under reduced pressure. Liquid-liquid extraction (LLE) was performed using distilled water and hexane as the two phases. The hexane-fraction contained the more lipophilic constituents of the extract and was not used any further after initial screening showed no significant activity in this subfraction. The aqueous fraction represented the water-soluble crude extract which was used in all further bioassays. The water phase was lyophilized and stored at 4˚C in a dessicator until use.

2.2.2.2 Preparation of the alkaloid fraction

An alkaloid fraction (AF) was obtained by an acid-base extraction method. An aliquot of the extract was taken to dryness at 40˚C with a rotary evaporator. The dried extract was partitioned between 2% acetic acid and hexane, with the hexane fraction containing the

lipophilic constituents being discarded. The aqueous layer was adjusted to pH=10 with ammonia in order to precipitate the alkaloids present. Using LLE, the precipitated alkaloids were collected into chloroform and dried using sodium sulphate, producing the alkaloidal fraction (AF). The remaining aqueous phase was termed the basic fraction (BF) and contained the balance of the non-alkaloidal constituents of the crude ethanolic extract of *T. elegans*. The extract procedure is shown diagrammatically in Figure 2.1.

The AF was taken to dryness at 40˚C using a rotatory evaporator, reconstituted in absolute ethanol at concentration of 100mg/mL and stored at -18˚C until use. The pH of the BF was adjusted to 7 with 2% acetic acid and lyophilized. The powered BF was stored at 4˚C in a dessicator until use.

2.3.3 Chemical characterisation

2.3.3.1 Phytochemical screening of the crude extract

Screening was performed on the crude extract to identify the major phytochemical groups present using thin layer chromatography (TLC). The alkaloidal fraction was also included in the phytochemical analysis in order to confirm the selectivity of the alkaloidal extraction process.

Stock solutions of 4mg/ml of the crude extract and the alkaloidal fraction were prepared in ethanol. A volume of 10 µl of the samples were spotted onto aluminium-backed silica TLC plates (Si60 F_{254}; Macherey-Nagel Alugram) and developed in an equilibrated TLC tank. Separation of the compounds was achieved with a mobile phase of 17:2:1 ethyl acetate:2-propanol:ammonia. Developed TLC plates were assessed for the presence of various phytochemical groups, by utilization of visible and ultraviolet (UV) light (254nm and 266nm (Camag Universal UV lamp, TL-600)), in addition to various spray reagents, which were selected to visualise specific classes of compounds according to methods published in literature[96;97]. The selected spray reagents for the phytochemical groups are listed in Table 2.1.

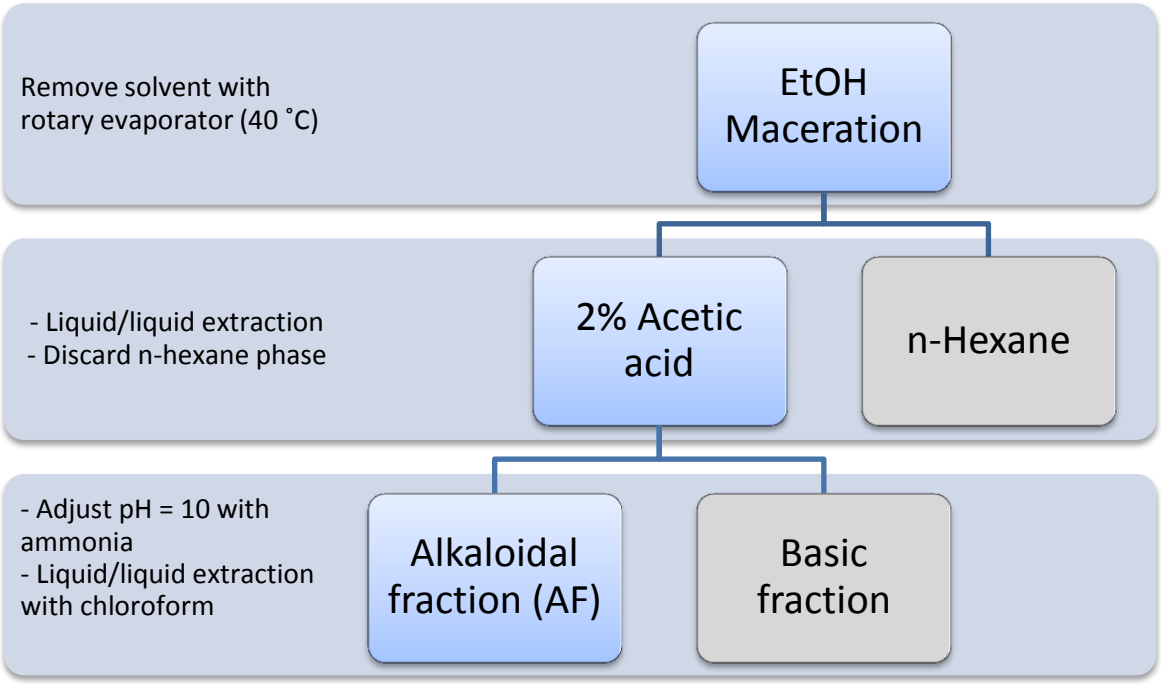

Figure 2.1: Extraction procedure utilized to produce alkaloidal and basic fractions.

Table 2.1. Visualisation agents selected for the phytochemical screening of the crude extract of *T. elegans*

Phytochemical group	Spray reagent and preparation	Positive reaction
Essential oils	Diphenylpicrylhdrazyl (DPPH): DPPH (0.06g) was dissolved in 100 ml of chloroform. The plate was heated at 110 °C for ten minutes.	Yellow bands on a violet background appear.
Flavonoids	Aluminium chloride: A 1% (w/v) solution of aluminium chloride was prepared in ethanol.	Yellow bands which florescence in long-wave UV light.
	Antimony(III)chloride: A 10% (w/v) solution of antimony(III) chloride was prepared in chloroform.	Bands which fluorescence in long-wave UV light.
Alkaloids **Nitrogen-containing compounds**	Dragendorff's reagent: Solution A: 1.7g basic bismuth nitrate was prepared in a 100ml of a 4:1 mixture of water and acetic acid (4:1) Solution B: 40g of potassium iodide was prepared in 100ml of water. A mixture of solution A (5 ml), solution B (5 ml), 20 ml acetic acid and 70 ml water was prepared.	Orange spots develop
Coumarins **Anthraquinone glycosides**	Potassium hydroxide: A 5% (w/v) solution of potassium hydroxide was prepared in methanol.	Visualisation of bands in daylight and long-wave UV.

Phytochemical group	Spray reagent and preparation	Positive reaction
Higher alcohols **Sterols / Steroids** **Essential oils**	Vanillin: Vanillin (1g) was dissolved in 100 ml concentrated sulphuric acid. The plate was heated at 120 °C until maximum colour development was achieved.	Blue to green spots
Phenols	Folin-Ciocalteu reagent: Solution A - A 20% (w/v) of sodium carbonate in water. Solution B - Folin-Ciocalteu (Sigma-Alrich (St Louis, MI)) reagent was diluted in a 1:3 ratio with water. Plates were sprayed with Solution A, allowed to dry briefly, and sprayed with Solution B.	Grey to black bands develop upon spraying with Solution B.
Amino acids **Amines**	Ninhydrin: Ninhydrin (0.2g) was dissolved in 100 ml ethanol. The plate was heated at 110 °C until maximum colour development was achieved.	Purple spots for primary amines; Yellow spots for secondary amines.
Reducing compounds **Lipids** **Sterols / Steroids**	Molybdophosphoric acid: A 5% (w/v) of molybdophosphoric acid was dissolved in ethanol.	Blue bands appear 1-2 minutes after spraying
Organic substances	Chromic acid: Potassium dichromate (5g) was dissolved in 100 ml of 40% sulphuric acid. The plate was heated at 150 °C until organic components charred.	Various coloured spots appear after brief heating. Extended heating produces black charred spots for most organic compounds with a high mass.

2.3.3.2 Thin layer chromatography of the alkaloidal fraction

As extensive data of the chromatographic properties of the *Tabernaemontana* alkaloids is available[94], TLC of the alkaloidal fraction was performed with the aim of identifying the major alkaloids present in the extract. Three mobile phases with varying polarities were used to determine multiple R*f* values, and in combination with UV- and chromogenic-properties was compared to the literature in order to tentatively identify the alkaloids. The crude extract was utilized as a control in these experiments.

Stock solutions of 4mg/ml of the alkaloidal fraction and crude extract were prepared in ethanol. A volume of 10 µl of the samples was spotted onto the silica TLC plate (Si60 F_{254}; Macherey-Nagel Alugram) and was developed using one of 3 mobile phases: (S1) toluene:absolute ethanol containing 1.74% ammonia (19:1), (S2) chloroform:methanol (9:1), (S3) ethyl acetate:2-propanol:ammonia (17:2:1). Prior to the equilibration of the TLC tank with S1, the atmosphere was saturated with ammonia for twenty minutes. After development, the plates were allowed to dry and visualized under short- and long-wave UV (254 and 360 nm, respectively).

Dried plates were sprayed with ferric chloride-perchloric acid (FCPA) reagent, which was prepared by dissolving 3.25% (w/v) iron(III) chloride in 35% perchloric acid. Immediate chromogenic reactions were noted. The plates were then heated with a hair dryer for a period of 3 minutes, and plates were assessed for any further development of colour or colour changes to existing bands.

The hR*f* values were calculated for all bands using the following formula:

$$hRf = \frac{\text{distance of band from point of sample application (mm)}}{\text{distance of solvent front from point of sample application (mm)}} \times 100$$

The hR*f* values, UV-data and chromogenic reactions were tabulated and compared with the available data in order to identify the alkaloids present.

2.3.3.3 Gas chromatography mass spectrometry (GC-MS) analysis of the alkaloidal fraction

GC-MS analysis of the crude extract and alkaloidal fraction was performed using an Agilent 7890 GC-MS equipped with a DB5-MS column (30m x 0.32 µm i.d., film thickness 0.25 mm, J&W Scientific). Helium was used as the carrier gas at a constant flow, and the injection split ratio was 50:1. The injectior temperature was 280°C. Column temperature was programmed to rise from 80°C to 310°C at 10°C/min for 23 minutes, then maintained at 310°C for 7 minutes.

The acquisition mode selected for mass spectrometry was scan mode, with a scan range of 35 - 550 Daltons and a threshold of 100 counts per second (cps). Solvent delay was 4.0 minutes. Quadrupole temperature was 150°C , with the transfer line temperature set to 280°C. GC and mass spectrometry parameters, data collection and analysis was performed by Agilent Chemstation.

2.3 Results and discussion

One kilogram of powdered *T. elegans* root was thrice macerated with ethanol and after acid-based extraction yielded approximately 12g of alkaloidal fraction, representing a yield of 1.2% (dry weight/weight). This is in agreement with other yields from *Tabernaemontana* species reported in the literature[87;98].

Phytochemical screening of the crude extract and alkaloidal fraction confirmed the selectivity of the acid-base extraction for alkaloids. Positive reactions were observed in the crude extract for the following spray reagents: diphenylpicrylhydrazyl (essential oils), Dragendorff (alkaloids and nitrogen-containing compounds), Folin-Ciocalteu reagent (phenols), molybdophosphoric acid (reducing compounds, steroids, sterols, lipids), ninhydrin (amines and amino acids), and vanillin (higher alcohols, sterols, steroids and essential oils). The alkaloidal fraction, however, did not show the same complexity of the crude extract, confirming that the acid base extraction procedure does enrich the alkaloids and results in the removal of most of the compounds of other classes. Previous phytochemical screening of various extracts from *Tabernaemontana* species have identified the presence of triterpenes/triterpenoids[73;84;98-100], steroids[73;84], phytosterols[99], flavonoids[84], phenyl propanoids[84] and phenolic acids[84] in these extracts.

Due to the substantial amount of TLC data available for the *Tabernaemontana* alkaloids, an attempt was made to identify major alkaloids present in the AF by hR*f* values in 3 different solvents systems, in combination with UV and chromatogenic reactions following exposure to ferric chloride-perchloric acid spray reagent. This approach was hampered by limited resolving power and selectivity, due to high prevalence of stereoisomers and that alkaloids of the same chemotaxonomic group to demonstrate the same colour changes upon exposure to the spray reagent. These limitations prevented definitive identification of the specific alkaloids present in the alkaloidal fraction by means of TLC.

In order to better characterise the major components of the alkaloidal fraction, GC-MS analysis of the alkaloidal fraction was performed. This is a well-documented technique that has been used by various research groups for identification of *Tabernaemontana* alkaloids in alkaloidal fractions[101-103].

GC-MS analysis of the crude extract and alkaloidal fraction indicated the presence of two major compounds that together accounted for more than 75% of all the detected compounds. These compounds were identified with a greater than 90% certainty by comparison to the NIST database to be the indole alkaloids, dregamine [20α, 19,20-dihydro-3-oxo Vobasan-17-oic acid methyl ester] and voacangine (12-methoxyibogamine-18-carboxylic acid methyl ester). A typical total ion chromatogram (TIC) for the alkaloidal fraction is shown in Figure 2.2. Two compounds eluting closely but at different times were identified as being the same compound, which could be due to minor isomeric differences in the structure or due to on-column degredation of a labile derivative, that then gives the expected product fragmentation in the mass spectrometer. The possibility of the latter is fairly large due to the compound being the most abundant in the chromatogram. Compounds identified in the alkaloidal fraction representing >5% of the total integrated area of the total ion chromatogram and with significant fragmentation pattern based compound match (>90%) to the NIST library are listed in Table 2.1.

Dregamine [20α, 19,20-dihydro-3-oxo Vobasan-17-oic acid methyl ester] is a corynthenean class indole alkaloid with an empirical formula of $C_{21}H_{26}N_2O_3$. This compound has been reported in other species of the *Tabernaemontana* genus, and has been previously isolated from *T. elegans* (Table 1.2; Chapter 1). The chemical structure for the compound is shown in Figure 2.2. The expected mass fragmentation pattern for dregamine from the NIST library is provided in Figure 2.3, while the observed fragmentation pattern during the GC-MS analysis is depicted in Figure 2.4. The physicochemical properties for the compound are: melting point (186-205 °C), $[\alpha]_D$: -93.1°, UV: 239 (4.18), 316 (4.27) nm, IR (KBr): 1653, 1730, 1245[104]. H NMR (C_6D_6) and ^{12}C NMR data have been described[104;105]. A method for the total chemical synthesis of dregamine has been reported in the literature[106].

Voacangine [12-methoxy- Ibogamine-18-carboxylic acid methyl ester] is an ibogan class indole alkaloidal with an empirical formula of $C_{22}H_{28}N_2O_3$. This class of alkaloid has also been reported in other species in the genus which again makes this a highly probable alkaloid to find in *T. elegans.* The chemical structure for the molecule is shown in Figure 2.5. The expected mass fragmentation pattern for voacangine from the NIST library is given in Figure 2.6, while the observed fragmentation pattern during the GC-MS analysis is provided in Figure 2.7. The physicochemical properties for the molecule are: melting point (136-137

°C)[107], $[\alpha]_D$: -34°[107], UV: 224 (log ε 4.23) 285 (3.85) 299 (3.85) nm[108]. ^{13}C NMR data has been described[109].

Analysis of the alkaloidal fraction by GC-MS is limited by the fact that it is expected that the more polar alkaloids that are present in the fraction are unlikely to be separated by gas chromatography[101;103], unless the alkaloids are derivatized to enhance their volatility. This fact is confirmed by the fact that the GC-MS TIC shows very few alkaloids while the TLC separation of the same fraction appears to have in excess of 30 compounds that show alkaloidal characteristics. The bisindole alkaloids have infrequently been detected by GC-MS, partially due to their high mass (>700 Daltons) and low volatility[110]. For the more polar indole alkaloids, as well as the high mass bisindoles, liquid chromatography-mass chromatography would be a more applicable technique for separation. This technique, however, lacks the predictable fragmentation patterns of gas phase ionisation as seen in GC-MS and the libraries to search. The use of LC-Q-TOF would, however, provide accurate mass information which would provide empirical formulas for the separated compounds. In future studies, it would be worthwhile to use both of these hyphenated techniques in parallel, in order to fully characterise the chemical composition of an alkaloidal fraction of the *Tabernaemontana* genus.

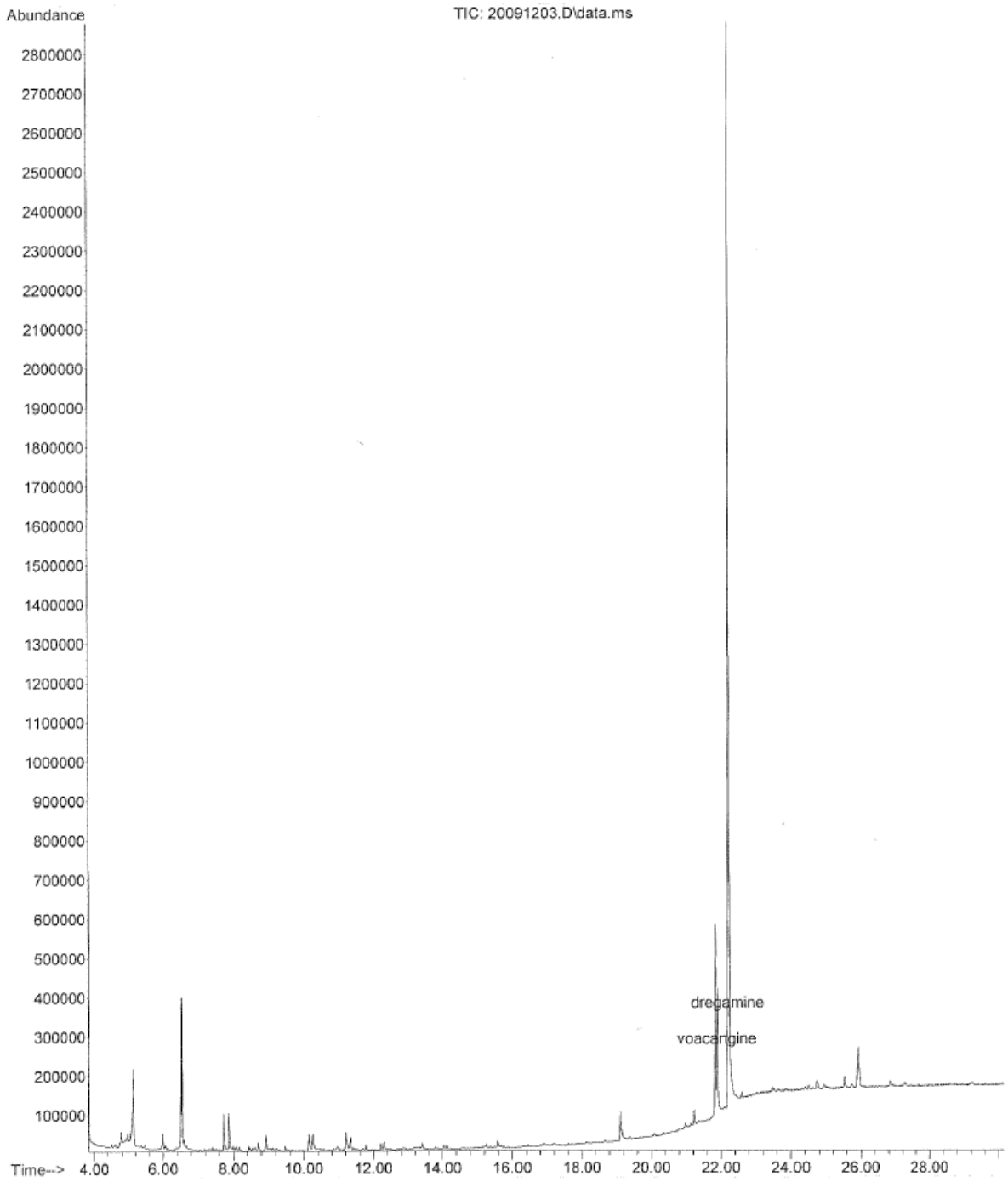

Figure 2.2: GC-MS total ion chromatogram of the alkaloidal fraction

Table 2.2 NIST library search report for GC-MS analysis of the alkaloidal fraction, listing all significant matches (>90%) for all peaks that were >5% of the total integrated area

Peak	Retention time	Total of integrated area (%)	Library match	Quality of match (%)	CAS
1	5.125	5.42	Decane	97	000124-18-5
2	6.536	7.43	Undecane	95	001120-21-4
7	21.842	8.23	Dregamine [Vobasan-17-oic acid, 19,20-dihydro-3-oxo, methyl ester (20.alpha)]	90	0002299-26-5
8	21.900	6.72	Voacangine [Ibogamine-18-carboxylic acid, 12-methoxy-, methyl ester]	93	000512-22-5
9	22.215	62.16	Dregamine [Vobasan-17-oic acid, 19,20-dihydro-3-oxo, methyl ester, (20.alpha)]	95	002299-26-5

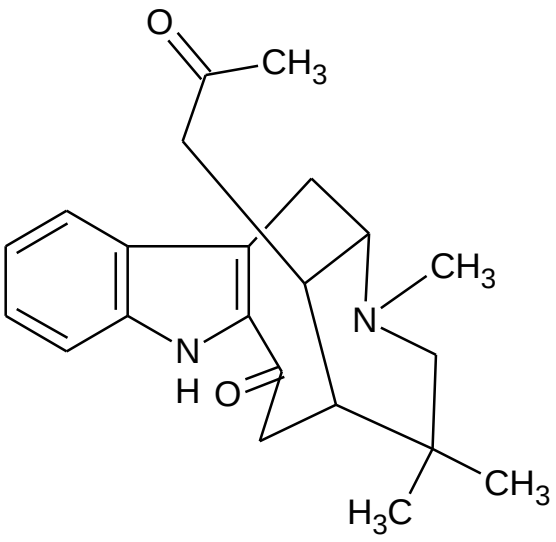

Figure 2.3: The chemical structure of dregamine[90]

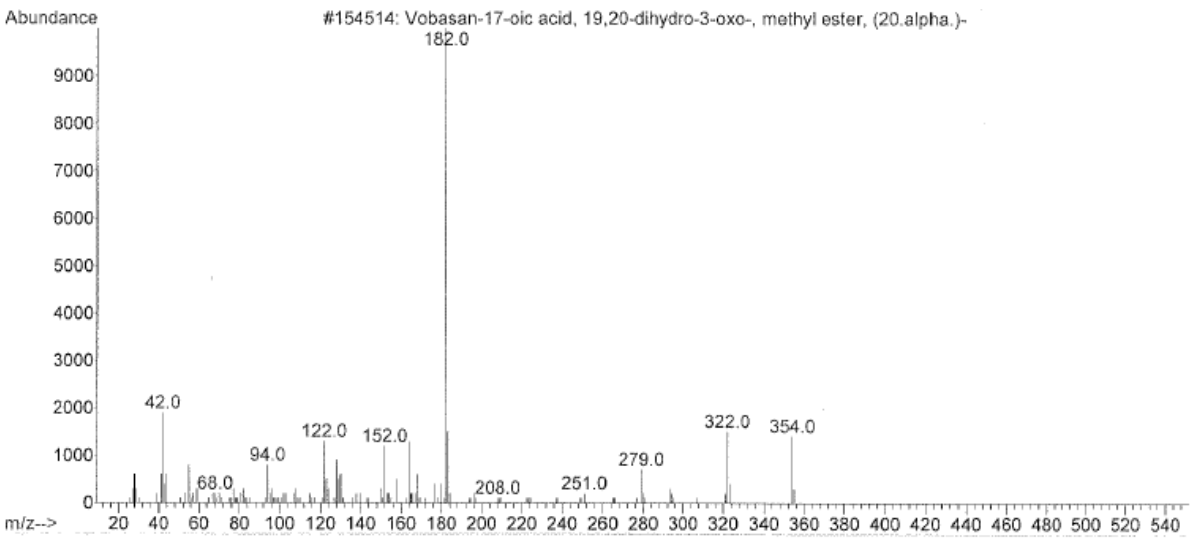

Figure 2.4: Expected mass fragmentation pattern of dregamine in the NIST library

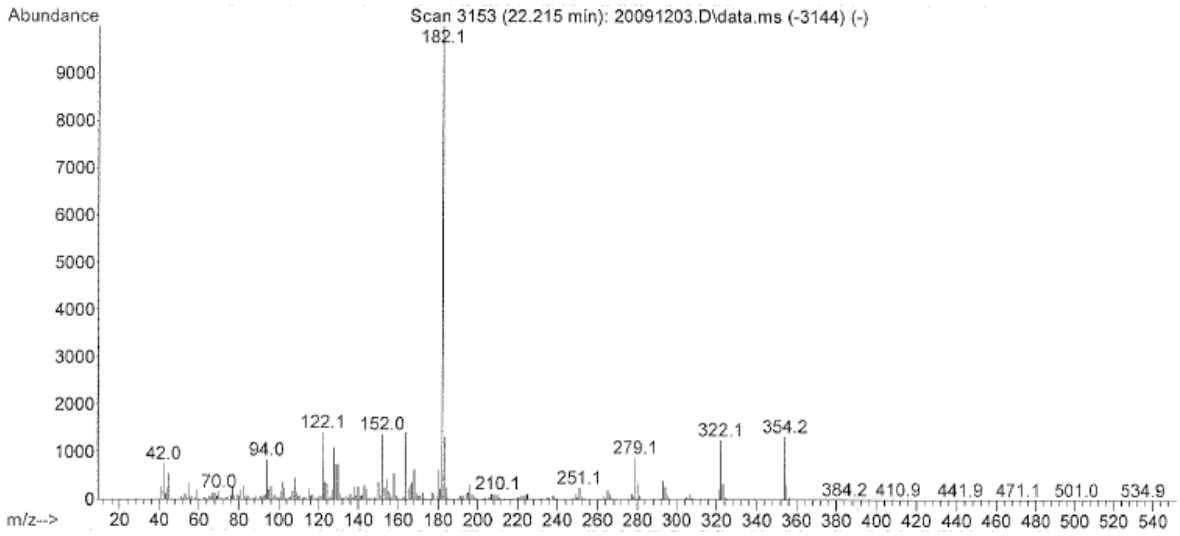

Figure 2.5: Observed mass fragmentation pattern of dregamine in the GC-MS analysis

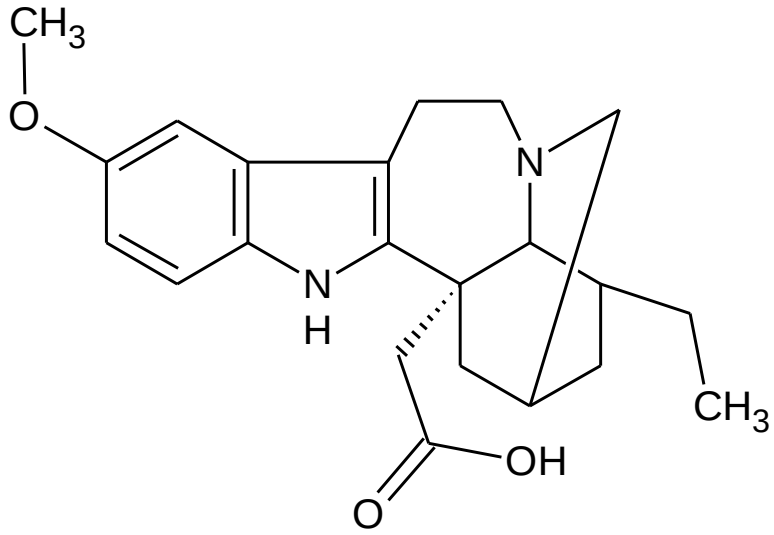

Figure 2.6: The chemical structure of voacangine[90]

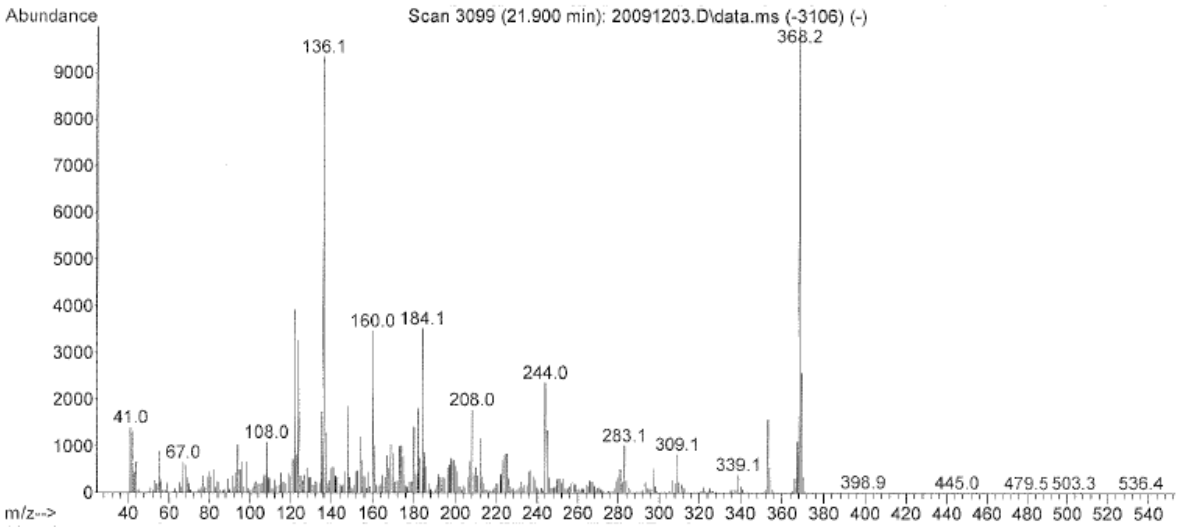

Figure 2.7: Expected mass fragmentation pattern of voacangine in the NIST library

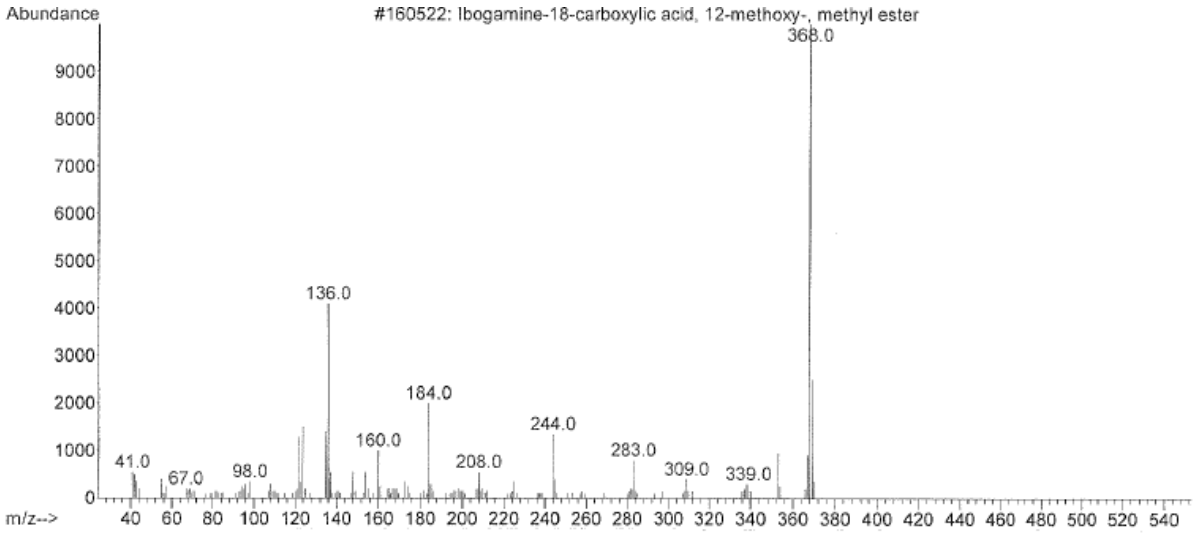

Figure 2.8: Observed mass fragmentation pattern of voacangine in the GC-MS analysis

3 Antibacterial assays

3.1 Introduction

3.1.1 Assessment of antibacterial activity of plant-derived natural products

The assays employed in screening plants for antibacterial activity have primarily been adaptations of those used in clinical microbiology, such as the diffusion and dilution inhibition assays. In general, the selection of which assay to use appears arbitrary, without consideration for the expected physicochemical properties of the active constituent, the inappropriate selection of micro-organisms, and the disagreement on a standard definition of significant activity.

Two commonly employed assays for the determination of antibacterial activity of plant extracts are the agar diffusion assays and the broth dilution assays. In the agar diffusion assay, the diameter of the zone of inhibition of bacterial growth is assumed to represent the antibacterial efficacy of the compound/extract, but the zone is also dependent on the physicochemical properties of the compound, which effects the diffusion rate through the agar. The results from the agar diffusion assay, therefore, cannot be viewed as quantitative when assessing antibacterial compounds with unknown characteristics. Non-polar compounds, due to their lipophilicity, and molecules with a high molecular weight, are known to diffuse poorly through agar and may be falsely thought to be inactive[95]. It is recommended that results from the diffusion assays be augmented with more quantitative data.

The broth dilution assay, however, is able to provide such quantitative data, and is a suitable method for the identification of antibacterial activity in natural products. Provided the sample is soluble in the liquid medium, and factors such as bacterial innoculum size and growth conditions are controlled for[95], the MIC for the various compounds (including known standards), plant extracts and fractions thereof are directly comparable.

The selection of bacteria is another important factor in the design of an antibacterial study. They should represent a wide spectrum of the bacterial kingdom, with particular emphasis on the species responsible for disease and where there is an unmet medical

need[59;95]. At a minimum, one Gram-positive and one Gram-negative species of bacteria should be included in the assay, but it is recommended that Gram-positive cocci, spore-forming Gram-positive rods, encapsulated and non-encapsulated members of the Gram-negative family *Enterobacteriaceae* as well as acid-fast bacteria are used[95]. Bacteria selected should be of reference strains, such as ATCC strains, and if clinical isolates are used, the identity and antibiotic-resistance profiles obtained and reported.

The concentration range of the plant extract or compound utilized in the assays should be carefully selected. A definition of what is deemed as true antibacterial activity should be considered in the methodology of the study, in order to ensure that only pharmacologically-significant antibacterial results are reported. A consensus regarding this issue indicates that any activity ascribed to a plant extract at a concentration less than 1 mg/ml, or 10 µg/ml for a pure compound can be described as significant, and worthy of further study[59;95;111].

The assessment of the antimycobacterial activity of the crude extract and alkaloidal fraction of *T. elegans* was initially assessed for activity using *M. smegmatis*, a rapidly growing, non-pathogenic mycobacterium. *M. smegmatis* serves well as a model for anti-tuberculosis activity, due to its similar antibiotic sensitivity profile to *M. tuberculosis*[112]. This allowed for safe, effective and rapid assessment of the antimycobacterial activity of *T. elegans,* prior to assays using the pathogenic *M. tuberculosis*, which are costly, time-consuming and carry the inherent risks of working with a highly pathogenic micro-organism. Due to the specialised growth requirements of *M. tuberculosis*, the BACTEC radiometeric assay was employed to assess antibiotic sensitivity to *T. elegans.* This method has been shown to have high levels of accuracy and reproducibility[113].

3.1.2 Assessment of synergistic antibacterial activity

While numerous methods for determining *in vitro* synergism have been described, three assays are currently favoured: the chequerboard, time-kill and Epsilometer (Etest) assays. Each of these assays measure different end-points in determining synergistic activity, and as a result correlation between the assays has been poor. There is little consensus on superiority of any one of the assays[114].

The chequerboard assay was selected due to compatibility with the experimental methodology utilized for determination of the antibacterial activity of the *T. elegans* extracts. Furthermore, as no literature is available on the synergistic effects of the *T. elegans* alkaloids in combination with antibiotics, this objective of the study was deemed as exploratory and the limited data provided by the chequerboard assay sufficient.

In this chapter, the antibacterial and synergistic properties of the ethanolic root extract of *T. elegans* and the enriched alkaloidal fraction were evaluated. The MIC and MBC of the extract and alkaloidal fraction were determined for three Gram-positive (*Bacillus subtilis, Enterococcus faecalis* and *Staphylococcus aureus*), three Gram-negative (*Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa*) and two mycobacteria (*Mycobacterium smegmatis* and *Mycobacterium tuberculosis*), as well as two clinical isolates of clinically-important pathogens (Isoniazid-resistant *Mycobacterium tuberculosis* and Methicillin-resistant *Staphylococcus aureus*). The MIC was determined using the broth micro-dilution assay and the MBC by viable colony counts. The synergistic combination of the alkaloidal fraction in combination with a control antibiotic was also assessed using the chequerboard assay for all bacteria that demonstrated susceptibility to the alkaloidal fraction. Fractional inhibitory concentrations were determined as per literature and categorized according to standard definitions of synergy.

3.2 Materials and methods

3.2.1 Plant material

The crude ethanolic extract, alkaloidal and basic fractions of *T. elegans* root were prepared as stated in Sections 2.2.2.1 - 2.2.2.2, respectively.

3.2.2 Micro-organisms

The crude extract and alkaloidal fraction were tested for antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* (ATCC 6633), *Enteroccocus faecalis* (ATCC 29212) and *Straphylococcus aures* (ATCC 12600), the mycobacteria *M. tuberculosis* $H_{37}R_V$ (ATCC 25177) and *M. smegmatis* (ATCC 14468), and the Gram-negative bacteria *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 9027). Samples were also tested against a clinical isolate of methicillin-resistant *S. aureus* (NHLS 363), obtained from the National Health Laboratory Service, Pretoria, and a clinical isolate of *M. tuberculosis* displaying resistance to isoniazid (MRC 3366), obtained from the Tuberculosis Unit, Medical Research Council, Pretoria.

Microbial cultures were maintained on Lowenstein Jensen medium (all strains of *M. tuberculosis*), Mannitol Salt Agar (all strains of *S. aureus*), Meuller-Hinton agar supplemented with 5% sheep blood (*E. faecalis*, *P. aeruginosa*) and Nutrient agar (*B. subtilis*, *E. coli*, *K. pneumoniae*, *M. smegmatis*).

3.2.2 Bacterial inocula

Bacterial inocula for all micro-organisms apart from *M. tuberculosis* were prepared by transferring colonies from a 24 hour, freshly prepared subcultures (72 hour subcultures in the case of *M. smegmatis*) to an aliquot of Mueller Hinton broth. A spectrophotometric optical density (OD), equivalent to a 0.5 McFarland turbidity standard (Sherwood Colorimeter; OD 0.8 at λ 550nm), was obtained by diluting the inoculum with additional broth.

For *M. smegmatis*, the aliquot of liquid media contained 0.02% (v/v) Tween 80 and 4-5 sterile 0.5mm glass beads. A homogeneous suspension was obtained by vortex mixing for 3 min and left to stand for 5 - 10 min in order for the particles to settle. After the large particles settled, the supernatant was transferred into a separate sterile test tube and

adjusted to a 0.5 McFarland turbidity standard. Before use in the assays, all inocula were diluted 100-fold with liquid media to obtain a concentration of 5 x 10^5 colony forming units (CFU/mL).

Inocula of *M. tuberculosis* were prepared by transferring colonies from a LJ medium slant to screw-capped round tube containing six to eight glass beads (1 – 2 mm) and 3 – 4 ml of diluting fluid (0.1% Tween 20). A homogeneous suspension was obtained by vortex mixing for 3 min and leaving the solution to stand for 5 - 10 min in order for large particles to settle. The supernatant was transferred to a separate sterile test tube and ≈ 2 - 5 ml of the diluting fluid added to adjust the tube to a 1 McFarland turbidity standard. One hundred microlitres of the adjusted suspension was added to a BACTEC vial, incubated at 37˚C and readings taken daily until GI reached 400 -500. Once this GI was obtained, contamination of the inoculum was assessed by removing 100 µL, plating on a blood agar plate and incubation for 24 h at 37˚C.

3.2.3 Anti-microbial assays

3.2.3.1 Broth micro-dilution assay

Determination of the minimum inhibitory concentration (MIC) for the all the selected bacterial species, apart from the strains of *M. tuberculosis,* was performed using the broth micro-dilution assay[115]. The MIC is defined as the lowest concentration of a compound or extract that is able to inhibit the growth of the bacteria. The minimum bactericidal concentration (MBC) of the crude extract and alkaloidal fraction was determined following the broth micro-dilution assay for all susceptible bacteria. The MBC is defined as the concentration of a compound or extract that results in the >99.9% killing of bacterium relative to the concentration of the bacterium present in the inoculum[115].

Stock solutions of the test samples and antibiotic standards were prepared in distilled water. The concentrations of the stock solutions were 4.096 mg/mL for the crude extract, 2.048 mg/mL for the alkaloidal and basic fraction, 32 µg/mL for ampicillin and 2 µg/mL for ciprofloxacin. In order to increase the solubility of the alkaloidal fraction in distilled water, the stock solution was acidified to ≈ pH 6.5 with acetic acid. All stock solutions were kept at -18˚C until use.

Using Mueller Hinton broth as the liquid media, serial two-fold dilutions of the test samples were made in the medium and 50 µL of the dilution transferred to the wells of a 96-well microtitre plate. The antibiotics, ampicillin (for the Gram-positive bacteria) and ciprofloxacin (for the Gram-negative bacteria and *M. smegmatis*) (Sigma-Alrich), were serially diluted in the media and served as the positive control. Wells containing 50 µL of media without plant extracts or antibiotics were used as growth controls. Wells containing 100 µl of medium served as the sterility control. Fifty microlitres of inocula was added to the all wells except the sterility controls to give a final volume of 100 µL before incubating at 37˚C in an ambient atmosphere for 24 h (72 h in the case of *M. smegmatis*).

The MIC was defined as the lowest concentration of a sample that visibly prevented the turbidity associated with microbial growth in a liquid medium. Each assay was performed in triplicate and on at least three different occasions.

The MBC of the samples for susceptible bacterial strains was determined following the MIC assay[116]. From all wells that demonstrated no apparent bacterial growth, the entire well contents (100 µL) were streaked in a straight line on a Mueller Hinton agar plate. After allowing the broth to dry, which prevented antibiotic carry-over, the plate was streaked to form a lawn of bacterial growth and incubated for 24 hours at 37˚C (72 hours in the case of *M. smegmatis*). A 100-fold dilution of the starting inoculum, spread on a Mueller Hinton agar plate, was used to determine the starting colony count.

After incubation, the plates were examined to determine at which concentration of the sample a 99.9% killing of the starting inoculum was achieved. For this, the formula:

$$n + 2\sqrt{n}$$

was used, where n = 0.1% of the initial test's colony count and $n + 2\sqrt{n}$ is the cutoff point for corrected MBC colony count[117]. This quantitative endpoint includes the 95% confidence limits for 99.9% bacterial killing. The lowest dilution of the sample that produces equal or fewer colonies than this value was deemed to be the MBC[115].

3.2.3.2 BACTEC radiometric assay

The BACTEC radiometric broth dilution assay was used to determine the MIC of the samples for the two strains of *M. tuberculosis*[113].

Stock solutions of the samples were prepared in ethanol, with a concentration of 43.13 mg/ml for the crude extract and 21.57 mg/ml for the alkaloidal fraction. The basic fraction was not assayed due to lack of antimycobacterial activity against *M. smegmatis* in the broth micro-dilution assay. Powdered isoniazid (Becton Dickinson, New Jersey, USA) was dissolved in sterile, distilled water at a concentration of 4.1 µg/ml. Serial dilutions of the stock solutions of the samples were made in sterile, double distilled water and 100 µl was transferred to BACTEC vials. This provided starting concentrations of 1.052 mg/ml, 512 µg/ml and 0.1 µg/ml for the crude extract, alkaloidal fraction and isoniazid, respectively. Three vials were used as controls, two containing only medium and the vehicle, representing solvent controls, while the third contained only medium and was the growth control. From the BACTEC vial containing the inoculum of *M. tuberculosis*, 100 µL was transferred to the vials containing the samples and the two solvent control vials. In the growth control vial, a 1:100 dilution of the inoculum was added, representing 1 x 10^3 – 1 x 10^2 CFU/mL, or 1% of the initial mycobacterial population.

Inoculated vials were incubated at 37˚C, with each vial assayed at 24 h intervals until the growth control vial reached a GI of greater than 30. The following day, final readings were taken and the ΔGI determined for all samples. The MIC was defined as the lowest concentration of a sample for which the ΔGI was less than the ΔGI of the growth control. If the GI of the sample was greater than 100, *M. tuberculosis* was deemed to be resistant to the sample, even if the ΔGI was less than that of the growth control.

The MBC for the strains of *M. tuberculosis* were not determined due to the long growth periods required for this assay and the poor reproducibility associated with this assay for the mycobacteria.

3.2.4 Synergistic antimicrobial activity assays

3.2.4.1 Chequerboard synergy assay

Assessment of the syngergistic activity between the alkaloidal fraction and the antibiotics that were utilized as controls for the broth micro-dilution assay was performed using a chequerboard synergy testing protocol[118]. This was performed for all bacterial strains apart from *M. tuberculosis*, for which the BACTEC radiometric synergy assay was used (Section

3.2.4.2) and the Gram-negative bacteria, which had proved to be unsusceptible to the alkaloidal fraction.

A dilution series of the alkaloidal fraction and of the antibiotics were made up in Mueller Hinton broth, with the concentration range representing 1/8 x MIC – 1 x MIC for the sample against the specific bacteria, as previously determined. The MIC values used were obtained in the broth micro-dilution assay as described in section 3.2.3.1. The concentration range for ampicillin used for the ampicillin-resistant strain of *S. aureus* (NHLS 363) that was used in the assay was the same as that was for the ampicillin-sensitive strain of *S. aureus* (0.008 µg/ml – 0.06 µg/ml). This was in order to observe whether there was anny effect by the alkaloidal fraction on antibiotic resistance.

In a four-by-four grid on a 96 well microtitre plate, 25 µl of the dilution series of the alkaloidal fraction was added along the x-axis of the grid, while 25 µl of the antibiotic dilution series was added to the y-axis of the grid. Wells containing 50 µl of sample-free medium served as growth controls and wells containing 100 µl of medium served as sterility controls. Each well containing 50 µl of the alkaloidal fraction/antibiotic mixture was inoculated with 50 µl of a bacterial inoculum, prepared as described in section 3.2.2. The microtitre plates were incubated at 37˚C in an ambient atmosphere for 24 hours (72 hours in the case of *M. smegmatis*).

The mean fractional inhibitory concentration (FIC) was used to interpret the results of the chequerboard synergy assay. The FIC was defined as:

$$FIC = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

The FIC was calculated for each non-turbid well along the growth/inhibition interface of the microtitre plate and the mean FIC calculated from these values. Synergism between the samples was defined as a mean FIC of ≤ 0.5, additivity as a value of > 0.5 to 4 and antagonism as a value of > 4[118]. Each four-by-four grid was repeated in triplicate on at least three different days and the mean of these FIC values is reported.

3.2.4.2 Radiometric synergy assay

For both strains of *M. tuberculosis*, the BACTEC radiometric method was used to determine synergy between the alkaloidal fraction of *T. elegans* and isoniazid.

A dilution series, with the concentration range 1/8 x MIC – 1 MIC, was made for the alkaloidal fraction and for isoniazid, based on the MIC values obtained in the assay described in section 3.2.3.2. For the isoniazid-resistant strain of *M. tuberculosis* (MRC 3366), the same concentration range for isoniazid was used as for the drug-sensitive strain of *M. tuberculosis* (0.01 µg/ml – 0.1 µg/ml). This was in order to observe if the alkaloidal fraction was able to reverse the isoniazid-resistance of the strain.

BACTEC vials were arranged in a four-by-four grid, and 50 µl of the alkaloidal fraction was added along the x-axis of the grid, while 50 µl of the antibiotic dilution series was added to the y-axis of the grid. The remainder of the BACTEC radiometric assay was performed as described in section 3.2.3.2.

The mean fractional inhibitory concentration (FIC) was used to interpret the results of the assay. The FIC was defined as stated in section 3.2.4.1. With the vials arranged in the four-by-four matrix, the FIC was calculated for each vial along the growth/inhibition interface and the mean FIC calculated from these values. Synergism between the samples was defined as a mean FIC of ≤ 0.5, additivity as a value of > 0.5 to 4 and antagonism as a value of > 4[118]. The assay was repeated in duplicate and the mean of the FIC values is reported.

3.3 Results and discussion

3.3.1 Assessment of antimicrobial activity by the broth micro-dilution assay and BACTEC radiometric assay

The MICs and MBCs obtained in the broth micro-dilution assay for the crude ethanolic extract and alkaloidal fraction of *T. elegans*, as well as the antibiotic controls, are reported in Table 3.1. The basic fraction did not possess antimicrobial activity against any of the selected bacteria (results not shown).

The antimicrobial activity of the crude ethanolic extract was significant, but limited to Gram-positive bacteria and the mycobacteria. The growth of the Gram-negative bacteria was unaffected at the concentrations tested. The MIC of the crude extract ranged from 64 µg/ml – 256 µg/ml. *B. subtilis* (ATCC 6633) demonstrated the most susceptibility to the antimicrobial-effects of the crude extract.

These results confirm earlier findings of the antibacterial properties of a whole-plant ethanolic extract of *T. elegans*, which showed antibacterial activity against *B. subtilis* and *S. aureus*; of the 18 *Tabernaemontana* species assayed, 8 species were active against the Gram-positive bacteria and only 3 species were active against the Gram-negative bacteria[72]. Antibacterial acitivty against Gram-positive, but not Gram-negative bacteria, has also been described for a methanolic whole-plant extract of *T. stapfiana*[119] and an organic solvent stem extract of *T. angulata*[120]. The only report of antimycobacterial activity in a crude extract was the screening study which served as the basis of this study; *T. elegans* possessed activity against *M. smegmatis* at a concentration of 1 mg/ml[70].

A screening study of ethnomedical plants used in India, however, failed to find antibacterial activity in sequential hexane- and methanol- extracts of the leaves and stems of *T. heyneana*[121]. The lack of activity in this study might be attributed to a combination of selection of plant parts, the method of extraction, as well as assay and concentrations selected.

Table 3.1 MIC and MBC values obtained in the broth micro-dilution assay

Micro-organism	Crude extract (µg/ml)		Alkaloidal fraction (µg/ml)		Antibiotic control (µg/ml)[a]	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-positive						
B. subtilis (ATCC 6633)	64	128	32	64	0.06	0.125
E. faecalis (ATCC 29212)	128	512	64	256	0.5	1
S. aureus (ATCC 12600)	128	128	32	64	0.06	0.06
S. aureus (NHLS 363)[b]	128	128	32	64	>2	NA
Gram-negative						
E. coli (ATCC 35218)	>1024	NA	512	512	0.125	0.125
K. pneumonia (ATCC 13883)	>1024	NA	>512	NA	0.125	0.125
P. aeruginosa (ATCC 9027)	>1024	NA	>512	NA	0.5	0.5
Mycobacteria						
M. smegmatis (ATCC 14468)	256	1024	32	128	0.5	0.5
M. tuberculosis (ATCC 25177)	128	ND	32	ND	0.1	ND
M. tuberculosis (MRC 3366)[c]	128	ND	32	ND	ND	ND

Key:
a: Antibiotic for Gram-positive bacteria – ampicillin; Antibiotic for Gram-negative bacteria- ciprofloxacin; Antibiotic for *M. smegmatis* – ciprofloxacin; Antibiotic for *M. tuberculosis* – isoniazid.

b: Clinical isolate of *S. aureus* – resistant to ampicillin, erythromycin, clindamycin and oxacillin

c: Clinical isolate of *M. tuberculosis* – resistant to isoniazid

NA: Not determined due to lack of activity; ND: Not determined

The alkaloidal fraction demonstrated the antibacterial activity against the same organisms as that of the crude extract, but at a significantly lower concentration, with the MICs ranging from 32 – 64 µg/ml (Table 3.1). These results support the hypothesis that the alkaloids, enriched in the alkaloid subfraction using the method described in 2.2.2.2, are primarily responsible for the antimicrobial activity of *T. elegans*.

The presence of drug resistance in the bacteria from clinical isolates tested in this study did not have an effect on the susceptibility of the bacteria to the crude extract or the alkaloidal fraction. This effect has been reported for another species of *Tabernaemontana*[119].

These finding confirm previous studies ascribing antibacterial activity to the alkaloidal fraction and/or isolated alkaloids from various *Tabernaemontana* species. The alkaloidal fraction of *T. catharinensis* was antimycobacterial against 3 species of mycobacteria, as well as Gram-positive and Gram-negative bacteria [78]. Of the alkaloids isolated from various *Tabernaemontana* species, conoduramine[122], conodurine[122], voacamine[122] and particularly the dimeric alkaloids of the voacamine-type[123], possess strong antibacterial activity against Gram-positive bacteria. The alkaloids ibogamine and voacangine have been reported to have proven antimycobacterial activity[124]. None of these compounds, however, have been previously identified in *T. elegans* (Table 1.2; Chapter 1), suggesting that the pharmacophore responsible for the antibacterial activity, may be common to a number of alkaloids in this genus.

Minimum bacteriocidal concentrations (MBC) were determined for the bacteria that demonstrated susceptibility to the crude extract and alkaloidal fraction, apart from the strains of *M. tuberculosis*. This was due to the long period required for growth on solid media and the associated problems of maintaining selective sterility. The MBCs for the bacteria tested ranged from 64 µg/ml – 1024 µg/ml. All values obtained were within two serial dilutions of the respective MICs obtained for the bacterium, indicating that the antibacterial alkaloids of *T. elegans* are bacteriocidal. The MBCs reported for an antibacterial methanolic extract of *T. stapfiana* are in agreement with the findings of the present study[119].

3.3.2 Assessment of synergy by the broth micro-dilution assay and BACTEC radiometric assay

The combination of the alkaloidal fraction of *T. elegans* and an antibiotic were tested for synergistic activity in the micro-organisms that displayed sensitivity to both of these agents. The mean FIC values from the chequerboard and BACTEC radiometric synergy assays, and the interpretation thereof, are provided in Table 3.2.

The combination of the alkaloidal fraction and either ampicillin for Gram-positive bacteria, ciprofloxacin for *M. smegmatis* or isoniazid for *M. tuberculosis*, reduced the required concentration of both agents to inhibit the growth of all bacteria assayed. The mean FIC ranged from 0.76 – 1.26, representing additive activity between the alkaloidal fraction and the antibiotics. This finding may indicate that the mechanism(s) of action responsible for the antibacterial activity of the alkaloidal fraction differs from those employed by antibiotics used in the assay, but mechanistic studies are required to support this hypothesis.

As the clinical isolates of *S. aureus* and *M. tuberculosis* were resistant to ampicillin and isoniazid respectively, MIC could not be determined and mean FIC cannot be reported. The synergy assays were performed, however, in order to assess whether the alkaloidal fraction, at a sub-MIC concentration, was able to restore the antibacterial activity of ampicillin and isoniazid. In these assays, the highest concentration of ampicillin and isoniazid tested was equal to the MIC obtained for the drug-sensitive strains in the antibacterial assays. In this manner, should the growth of the isolate be inhibited by the combination of a sub-MIC of the alkaloidal fraction, and a concentration of the antibiotic that would be inhibitory if the bacteria were susceptible to it, it would indicate that some of the resistance to the antibiotic has been reversed.

The combination of 16 µg/ml (1/2 x MIC) of the alkaloidal fraction and 0.06 µg/ml ampicillin produced an inhibitory effect on growth of the methicillin-resistant strain of *S. aureus*. The controls for the experiment, which consisted of either the alkaloidal fraction or

Table 3.2 Mean FIC and interpretation of FIC for synergy assays

Micro-organism	Alkaloidal Fraction MIC (µg/mL)[a]	Antibiotic MIC (µg/mL)[a, b]	Mean FIC	Interpretation of FIC
Gram-positive				
B. subilis (ATCC 6633)	32	0.06	0.76 ± 0.03	Additive
E. faecalis (ATCC 29212)	64	0.05	1.25 ± 0.00	Additive
S. aureus (ATCC 12600)	32	0.06	0.77 ± 0.04	Additive
Mycobacteria				
M. smegmatis (ATCC 14468)	32	0.5	1.26 ± 0.05	Additive
M. tuberculosis (ATCC 25177)	32	0.1	0.81 ± 0.00	Additive

Key

a: MIC values obtained in the broth micro-dilution assay

b: Antibiotic for Gram-positive bacteria – ampicillin; Antibiotic for *M. smegmatis* – ciprofloxacin; Antibiotic for *M. tuberculosis* – isoniazid

ampicillin at the above concentrations alone, did not produce the same effect. This effect was not seen, however, in isoniazid-resistant *M.* tuberculosis, where a mixture of 16 µg/ml of the alkaloidal fraction and 0.1 µg/ml of isoniazid failed to inhibit the growth of the mycobacteria.

The results obtained in this study indicate that the alkaloids present in *T. elegans* possess additive activity when combined with ampicillin or isoniazid, and that these alkaloids may be able to reverse resistance acquired against ampicillin.

Due to the limitations of the checkerboard assay, as well as the experimental methodology employed, no further conclusions can be made regarding the extent of the synergistic properties of the *T. elegans* alkaloids. No reports could be found in the literature of other studies assessing the synergistic antibacterial effects of extracts or alkaloids of the *Tabernaemontana* genus in combination with antibiotics.

In future studies, it would be worthwhile to utilize both the checkerboard and time-kill assays, so that results may be correlated. Furthermore, a number of alkaloidal fraction-antibiotic combinations should be tested, in order to assess if there are any differences in synergistic activity based on the mechanism of action of the antibiotic.

4 Cytotoxicity assays

4.1 Introduction

One of the fundamental *in vitro* toxicological assays performed is the assessment of the direct effects of a compound on the viability of a human cell line. Data obtained in these assays are not only useful in selecting the most promising candidate for further drug development, but also provide vital data for future pre-clinical studies. Furthermore, when assessing a compound or natural product extract with antimicrobial activity, these assays are imperative to ascertain the selectivity of these antimicrobial effects. As any 'hit' in these screening programs may be ascribed to non-specific toxicity rather than a selective action against the specific micro-organism, the possibility of toxic false positives needs to be investigated[95].

Numerous assays have been employed for the determination of the toxic cellular effects of xenobiotics on cells, assessing functions of cellular physiology such as membrane integrity, mitochondrial function or protein synthesis as a surrogate for cell viability[125]. While this approach to determining cell viability has been shown to be accurate and reproducible, each assay has been associated with certain limitations. In order to overcome these limitations and improve the reliability of the *in vitro* data, a number of cell viability assays should be run in parallel, providing a more comprehensive picture of the potential cellular toxicity via different mechanisms.

Two commonly used assays are the MTT and neutral red uptake assays. There are several factors known to limit the robustness of the MTT assay: certain reducing agents and antioxidants present in plant extracts affect formazan production[126], as do parameters of the medium, such as pH[127] and glucose concentration[128]. The netural red assay has been found to have good linearity with cell number[129], although this correlation is lower for weakly basic substances[130] and agents which cause lysosomal swelling by osmosis, such as mannitol[131].

Another important consideration for *in vitro* toxicology assays is the selection of cell types to be utilized. Ideally, these should consist of a mixture of primary cell cultures and

transformed cell lines, and be derived from a variety of organ systems. This approach, when assessing a fraction or compound for which the cytotoxic mechanism is unknown, is beneficial as it may indicate the organ systems most vulnerable to potential toxic effects, when the compound is given *in vivo*.

In this chapter, the effects of the crude ethanolic extract, the alkaloid fraction, and the aqueous fraction of *T. elegans* on the cell viability of human fibroblasts, heptatocytes, lymphocytes and macrophages using the MTT and neutral red uptake assays was assessed.

4.2 Materials and methods

4.2.1 Plant material & reagents

The crude ethanolic extract of *T. elegans*, as well as the alkaloidal and basic aqueous fraction was prepared as stated in Sections 2.2.2.1 and 2.2.2.2, respectively. All reagents were obtained either from Sigma-Aldrich or Merck Chemical Co., unless otherwise stated.

Stock solutions of the test reagents were prepared in distilled water. The concentrations of the stock solutions were 410 µg/ml for the crude extract and the basic aqueous fraction, 205 µg/ml for the alkaloidal fraction, and 1% (w/v) for saponin. In order to increase the solubility of the alkaloidal fraction in distilled water, the stock solution was acidified to pH 6.5 with acetic acid. All stock solutions were kept at -18˚C until use.

4.2.2 Cell lines

4.2.2.1 Normal human dermal fibroblasts

Normal human dermal fibroblasts, isolated from human foreskin tissue, were purchased from Southern Medical, South Africa. For use in the cytotoxicity assays, the isolated fibroblasts were trypsin-treated for 10 minutes, centrifuged (200*g*, 10 min) and the pellet resuspended in 1 ml of DMEM-F12 supplemented with 10% FCS. Cells were enumerated by crystal violet staining and diluted to a concentration of 5 x 10^4 cells/ml. To each well of a microtitre plate, 100 µl of the cell suspension was added, in addition to 80 µl of FCS-supplemented medium. The microtitre plates were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2 to allow fibroblast adhesion, after which cytotoxicity assays were performed.

4.2.2.2 Human lymphocytes

Human lymphocytes were isolated from whole blood following the procedure of Anderson[132]. From healthy volunteers who had given informed consent (Blanket Ethics Approval, University of Pretoria Ethics Committee), 300 ml of blood was drawn into a bag containing 30 µl of heparin solution (30 mg/ml). The blood was layered onto Histopaque 1077 and centrifuged (650*g*, 25 min). The top plasma layer was discarded and the lymphocyte layer, near the top of the Histopaque harvested and washed with RMPI-1640 medium. Following centrifugation (200*g*, 15 min), the supernatant was discarded and the

pellet resuspended in sterile, ice-cold ammonium chloride to lyse any contaminating erythrocytes. After a 10 minute incubation period, the suspension was centrifuged (200*g*, 10 min). The supernatant was discarded and the pellet resuspended in 1 ml of RPMI-1640 medium supplemented with 10% FCS.

The isolated lymphocytes were enumerated using crystal violet staining and a haemocytometer, and diluted to a concentration of 2 x 10^6 cells/mL in FCS-supplemented RPMI-1640 medium. To each well of a 96 well microtitre plate, 100 µl of the cell suspension was added, together with 60µL of medium. Following an hour incubation at 37˚C in a humidified atmosphere of 5% CO_2, 20 µl of a 5 µg/ml solution of phytohaematoglutinin (PHA) in medium was added to selected microtitre plates to produce populations of stimulated lymphocytes, while other microtitre plates received a further 20 µl of medium and represented resting lymphocytes. The PHA-stimulated and resting lymphocytes were used in the cytotoxicity assays.

4.2.2.3 HepG2 hepatocyte cell line

HepG2 cells (ATCC HB 8065), a human heptocelluar carcinoma cell line, were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FCS at 37˚C in a humidified atmosphere of 5% CO_2. For use in the cytotoxicity assay, HepG2 cells were trypsin-treated for 10 minutes, decanted from culture flasks and centrifuged (200*g,* 10 min). The pellet was resuspended in 1ml of FCS-supplemented EMEM medium and the cells enumerated by crystal violet staining. The HepG2 cells were diluted to a concentration of 5 x 10^5 cells/ml in EMEM medium, and 100 µl of the cell suspension plated in each of the wells of a 96 well microtitre plate, as well as 80 µl of EMEM medium. Following overnight incubation at 37 °C in a humidified atmosphere of 5% CO_2 to allow for cellular reattachment, cytotoxicity assays were carried out.

4.2.2.3 THP-1 monocyte cell line

THP-1 cells (ATCC TIB 2.2), a free-floating human myelomonocytic cell line, were cultured in THP medium at 37˚C in a humidified atmosphere of 5% CO_2. For use in the cytotoxicity assays, THP-1 cells were decanted from the culture flasks and centrifuged (650*g*, 10 min). The pellet was resuspended in 1 ml of THP medium and cells enumerated by crystal violet staining. The THP-1 cells were diluted to a concentration of 1 x 10^5 cells/ml in THP medium,

and 100 µl of the cell suspension was plated into the wells of a 96 well microtitre plate, as well as 80 µl of THP medium. The microtitre plates were incubated for an hour at 37 °C in a humidified atmosphere of 5% CO_2. Differentiation of the THP-1 monocytes into macrophages was induced by the addition of 20 µl of a 0.1 µg/ml solution of phorbol 12-myristate 13-acetate (PMA) in THP medium, followed by 48 hours of incubation at 37 °C in a humidified atmosphere of 5% CO_2. The 96 wells microtitre plates, containing the differentiated macrophages were used in the cytotoxicity assays.

4.2.3 Cytotoxicity assays

Two-fold serial dilutions of the crude extract (1.6 – 204.8 µg/ml), alkaloidal fraction (0.1 – 12.8 µg/ml), basic aqueous fraction (3.2 – 410 µg/ml) and saponin (0.0008 – 0.1% w/v), which served as the positive control, were prepared in the appropriate medium. To each well of the microtitre plate containing cells, 20 µl of the sample was added, and for the growth controls, 20 µl of medium. The cells were incubated for 3 days at 37˚C in a humidified atmosphere of 5% CO_2 before the cytotoxicity assays were conducted. All assays were conducted in triplicate, on three different days.

4.2.3.1 MTT assay

The MTT assay was performed as described by Mossman[133]. After exposure to the samples, 20 µl of MTT (5 mg/ml in PBS) was added to the wells of the 96 well microtitre plate and incubated for 4 hours at 37˚C in a humidified 5% CO_2 atmosphere. The plates were centrifuged (650 *g,* 10 min) and the medium removed without disturbing the pellet. The wells were washed once with 150 µl of phosphate-buffered saline (PBS), followed by centrifugation (650 *g,* 10 min). After removing the supernatant, the plates were dried overnight. Dimethyl sulphoxide (DMSO) (100 µl) was added to each well, and the plate gently agitated on a mechanical shaker for 60 minutes. The optical density was measured using a spectrophotometer (EL_x800 Universal Microplate Reader (Bio-tek Instruments, Inc.)) at λ 540 nm with a reference λ of 630 nm.

4.2.3.2 Neutral red uptake assay

The Neutral red uptake assay was performed as described by Borenfreund and Puerner[134]. Neutral red medium was prepared by dissolving Neutral Red in the appropriate medium for the cell line at a concentration of 100 µg/ml. The pH of the medium was adjusted to 6.4 with

1 M potassium dihydrophosphate (BDH Chemicals, England) and filter sterilized (0.22 µm, Millipore). For the assay, microtitre plates were centrifuged (200*g*, 5 min), the medium was removed and 200 µl of Neutral Red medium added to the wells. After incubation for 2 hours at 37˚C in a humidified 5% CO_2 atmosphere, the microtitre plates were centrifuged (650*g*, 10 min), the medium discarded, and the cells washed with 200 µl of PBS. The microtitre plates were centrifuged (1200*g*, 10 min), the PBS removed and 100 µl of the elution buffer was added, which consisted of ethanol: distilled water: acetic acid in a 50:49:1 ratio. Following gentle agitation on a mechanical shaker for 30 minutes, the microtitre plates were read using a spectrophotometer (EL_x800 Universal Microplate Reader (Bio-tek Instruments, Inc.)) at λ 540 nm with a reference λ of 630 nm.

4.2.4 Statistical analysis

The concentration of sample that prevented the survival of 50% of the cell population (IC_{50}) was determined by non-linear regression from optical density measurements. Sample optical density values were converted to a percentage of the average growth of control wells. Non-linear regression, using a sigmoidal dose-response curve model with a bottom constraint of 0, was performed using GraphPad 4 software.

Correlation between the IC_{50} values obtained in the MTT assay and Neutral Red uptake assay was determined by two-tailed Pearson correlation calculation performed using GraphPad 4 software.

4.3 Results and discussion

The crude ethanolic extract and the alkaloidal fraction of the roots of T*abernaemontana elegans* had significant effects on cell viability for all of the cell lines and primary cultures tested (Table 4.1). The IC_{50} values for the crude ethanolic extract in both assays ranged from 1.93 µg/ml – 19.27 µg/ml and showed dose-dependent response curves (Figures 4.1 – 4.5). Smaller IC_{50} values were obtained for the alkaloidal fraction, ranging from 1.11 µg/ml – 9.81 µg/ml (Figures 4.1 – 4.5). The basic fraction demonstrated no effects on cell viability at the highest concentration tested (410 µg/ml; results not shown). The lower IC_{50} values obtained for the alkaloidal fraction, combined with the non-toxicity of the basic fraction, demonstrate it is primarily the indole alkaloids of *T. elegans* that are responsible for the cytotoxicity observed in the crude extract.

Analysis of the IC_{50} values obtained in the MTT and Neutral Red Uptake assays showed a significant correlation of values between assays for the alkaloidal fraction (Pearson r = 0.84), however, no significant correlation was found for the crude ethanolic extract. This data may indicate that the constituents present in the crude extract were able to adversely affect the reliability of one of the assay systems. This highlights the importance for performing multiple cytotoxicity assays in parallel, especially when the constituents of an extract are unknown.

Previous research on crude extracts of *Tabernaemontana* species has shown conflicting evidence of cytotoxicity. An aqueous extract of *T. elegans* demonstrated weak toxicity to human lymphocytes, with IC_{50} concentrations ranging between 90 - 160 µg/ml[70]. The earliest investigation of the cytotoxic effects of *Tabernaemontana* species showed that an aqueous methanolic extract of *T. arborea* possessed an IC_{50} of 8 µg/ml against the P-388 cell line (murine lymphocytic leukaemia)[74]. For *T. divaricata,* methanol, dichloromethane and water root extracts were tested for cytotoxicity against the COR L23 (human non-small cell lung cancer) and MCF-7 (human breast adenocarinoma) cell lines using the sulforhodamine B assay. The methanolic and dichloromethane extract were found to be cytotoxic, however the aqueous extract did not produce an effect even at the highest concentration tested (> 25 µg/ml) [135]. Another study on *T. divaricata* confirmed these results[136]. On the contrary, a methanol extract of *T. divaricata* had no effect on resting human mesangial cells[137]. Furthermore, the viability of the human mesangial cells were not

Table 4.1 IC_{50} values obtained in the MTT and Neutral Red uptake cytotoxicity assays.

Cell line	IC_{50}					
	MTT assay			**Neutral red uptake assay**		
	Crude extract (µg/mL ± SD)	**Alkaloidal fraction (µg/mL ± SD)**	**Saponin (%)**	**Crude extract (µg/mL ± SD)**	**Alkaloidal fraction (µg/mL ± SD)**	**Saponin (%)**
Primary cell cultures						
Resting lymphocytes	4.52 ± 3.20	1.75 ± 1.35	0.006	19.27 ± 8.43	4.35 ± 0.58	0.004
PHA-stimulated lymphocytes	11.77 ± 4.20	1.67 ± 1.96	0.006	9.05 ± 5.10	2.30 ± 1.96	0.005
Normal human dermal fibroblasts	10.91 ± 2.54	5.91 ± 0.24	0.018	13.38 ± 2.59	9.81 ± 1.19	0.015
Cell lines						
HepG2 hepatocytes	5.81 ± 4.85	1.11 ± 0.49	0.005	1.93 ± 0.29	1.27 ± 0.26	0.003
THP-1 macrophages	16.77 ± 9.56	9.73 ± 4.10	0.010	8.10 ± 2.92	8.23 ± 0.88	0.012

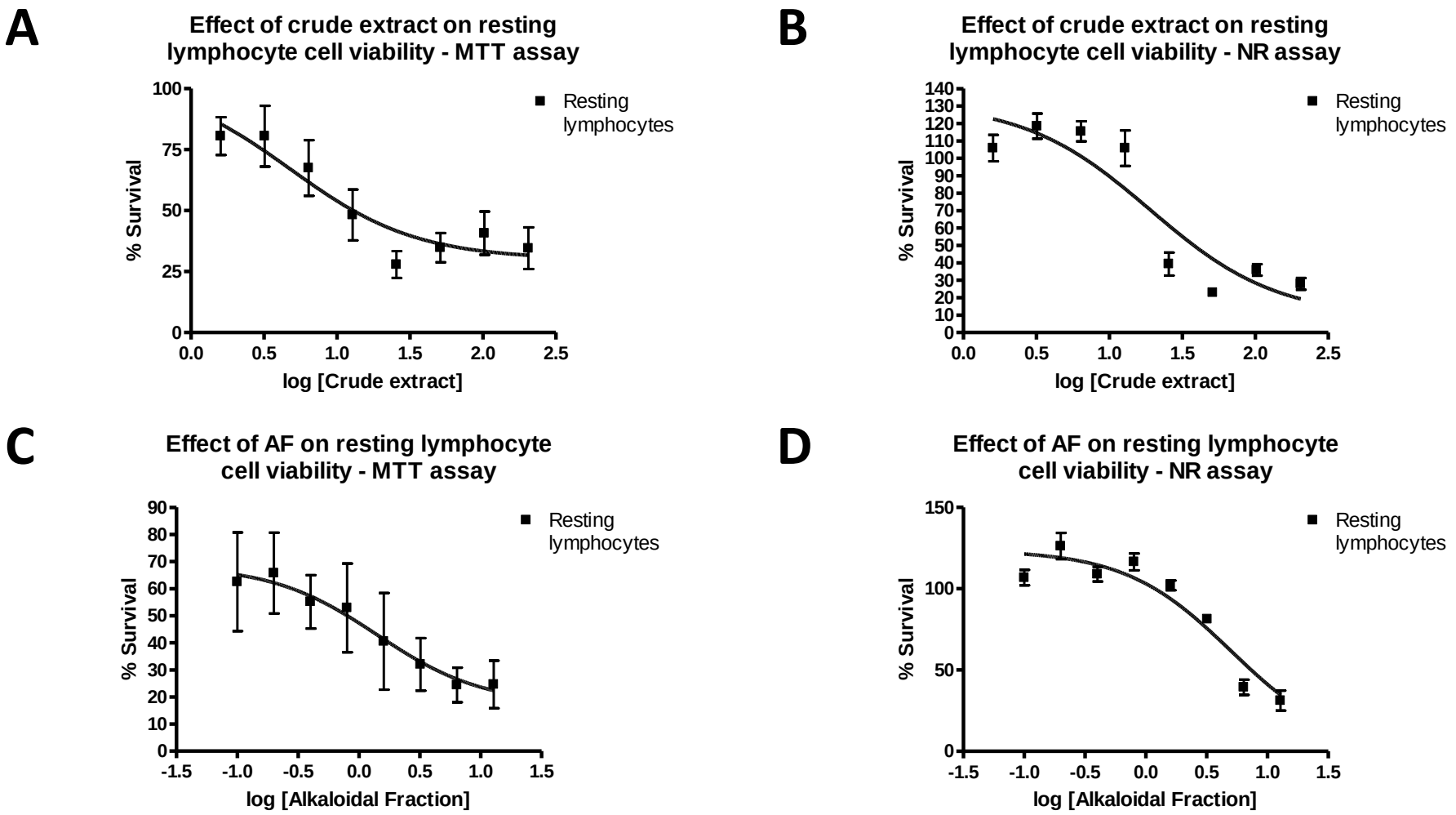

Figure 4.1: Effect of the crude ethanolic extract of *T. elegans* (A-B) and alkaloidal fraction (C-D) on the growth of resting lymphocytes, as measured by the MTT (A, C) and Neutral Red Uptake (B,D) assays after 72h of incubation.
Each point represents the mean of three different experiments ± standard error.

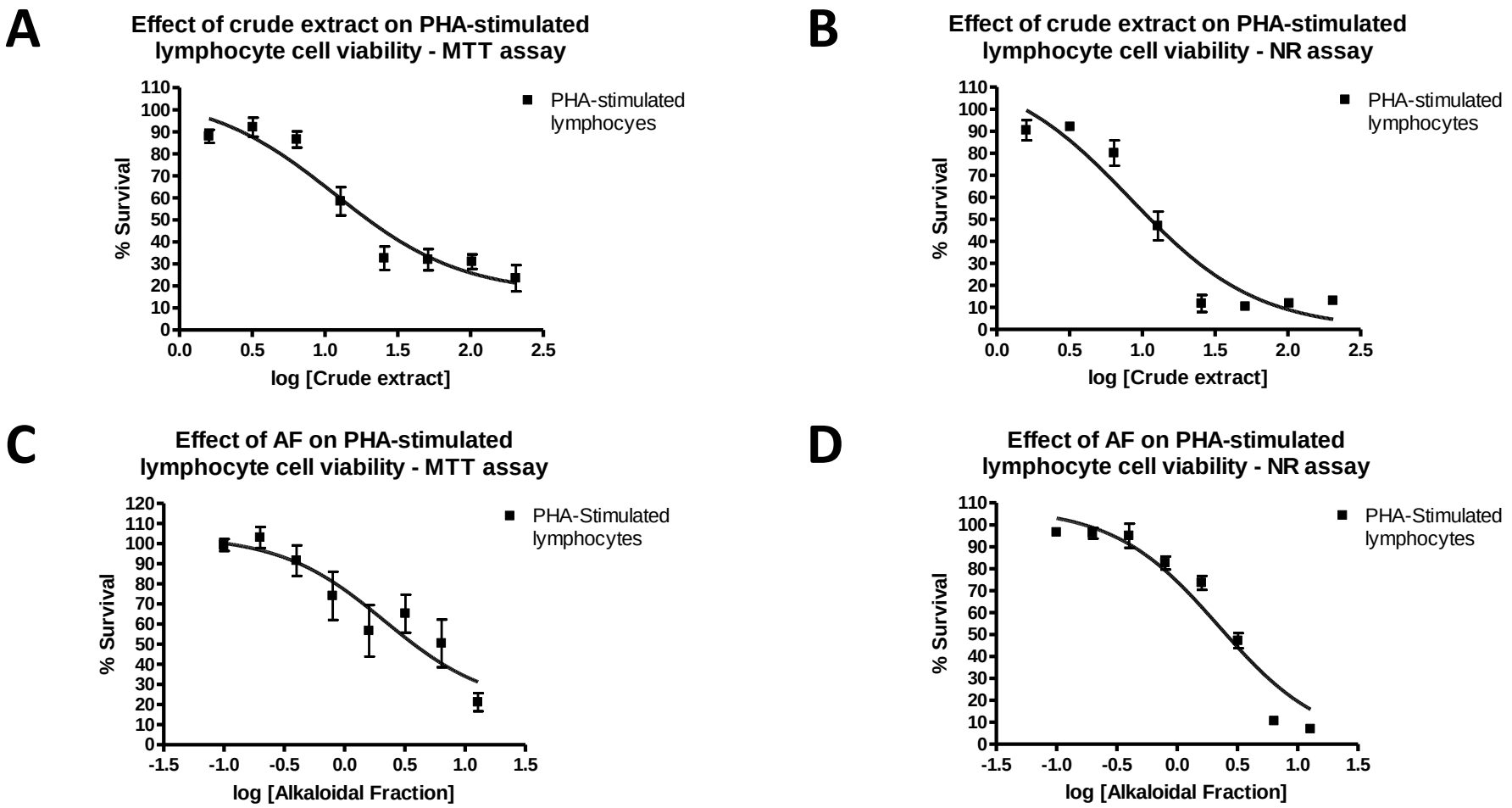

Figure 4.2: Effect of the crude ethanolic extract of *T. elegans* (A-B) and alkaloidal fraction (C-D) on the growth of PHA-stimulated lymphocytes, as measured by the MTT (A, C) and Neutral Red Uptake (B,D) assays after 72h of incubation.
Each point represents the mean of three different experiments ± standard error.

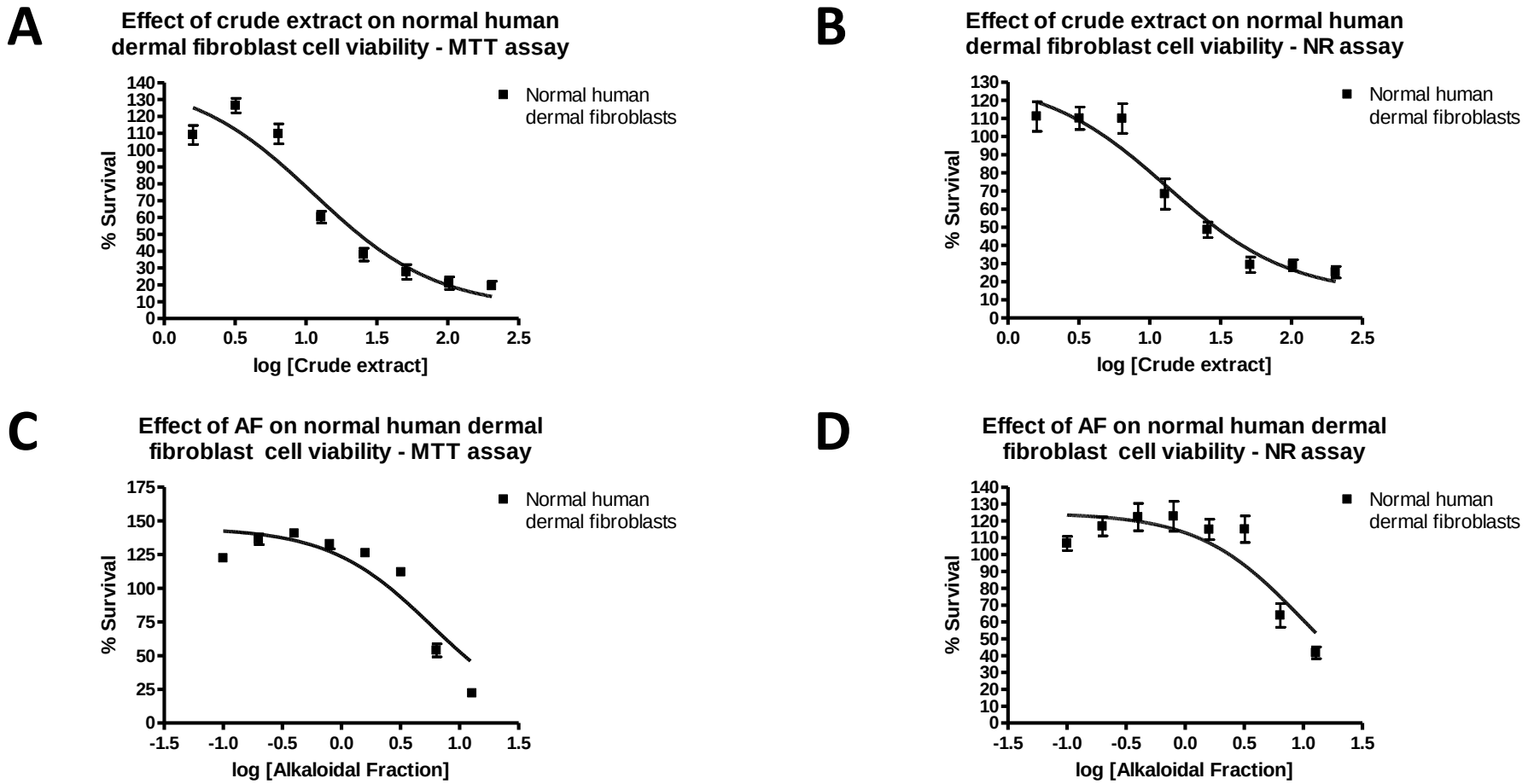

Figure 4.3: Effect of the crude ethanolic extract of *T. elegans* (A-B) and alkaloidal fraction (C-D) on the growth of normal human dermal fibroblasts, as measured by the MTT (A, C) and Neutral Red Uptake (B,D) assays after 72h of incubation.
Each point represents the mean of three different experiments ± standard error.

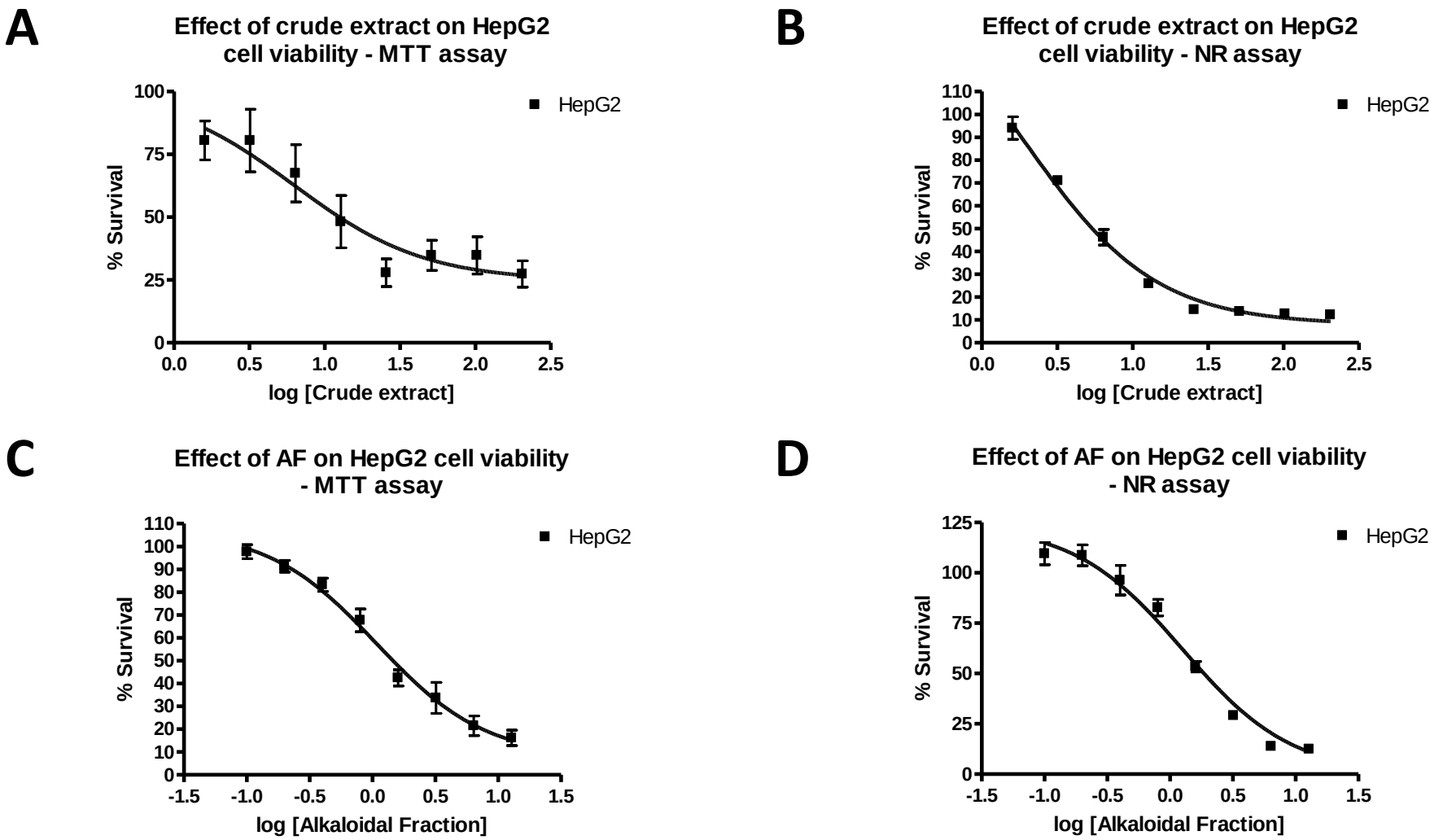

Figure 4.4: Effect of the crude ethanolic extract of *T. elegans* (A-B) and alkaloidal fraction (C-D) on the growth of HepG2 hepatocytes, as measured by the MTT (A, C) and Neutral Red Uptake (B,D) assays after 72h of incubation.
Each point represents the mean of three different experiments ± standard error.

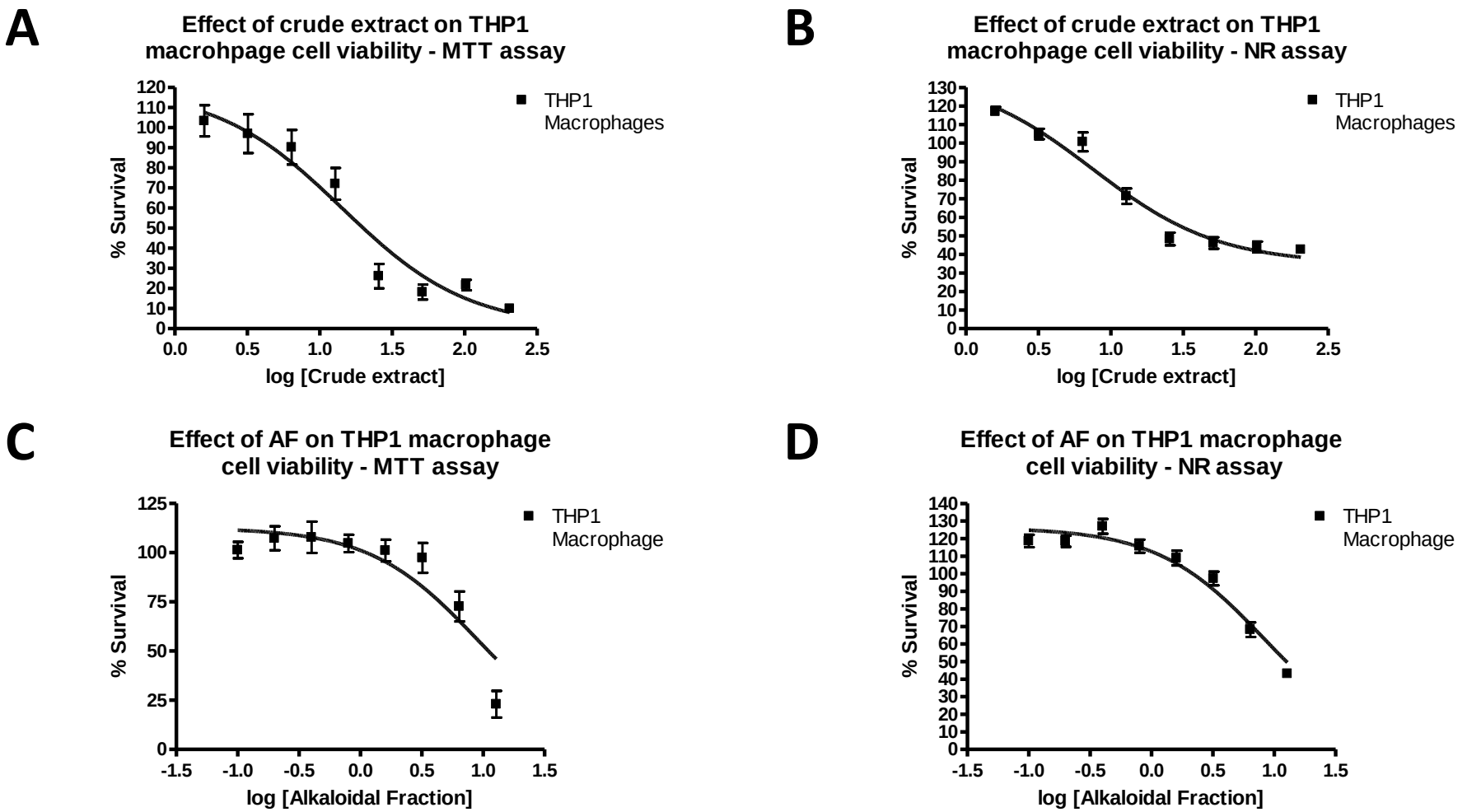

Figure 4.5: Effect of the crude ethanolic extract of *T. elegans* (A-B) and alkaloidal fraction (C-D) on the growth of THP1 macrophages, as measured by the MTT (A, C) and Neutral Red Uptake (B,D) assays after 72h of incubation.
Each point represents the mean of three different experiments ± standard error.

significantly decreased after 4 days of treatment with the extract[137]. An ethyl acetate extract of the stem bark of *T. laeta*, and leaf extracts of *T. amydalifolia* exhibited no cytotoxicity (IC_{50} > 20 µg/ml) in a KB human oral epidermoid carcinoma cell line [138;139]. Although a methanol extract of the leaves of *T. obliqua* was reported to have cytotoxic effects against the promonocytic U937 cell line by the authors, the IC_{50} value obtained (231.6 µg/ml ± 2.7 µg/ml) are substantially higher than what most would deem to be physiologically significant[140].

Several possible explanations exist for the lack of cytotoxicity activity reported in some of the crude extracts. It may be related to factors influencing the concentration of the alkaloids in the plant, such as pre-cursor availability[93;141], microbial attack[142], or other environmental stressors, and factors influencing the concentration of alkaloids in the crude extract, such as choice of plant part, the method of extraction and solvent system selected for extraction[101].

From the literature, more than 40 isolated *Tabernaemontana* alkaloids have been assayed for cytotoxicity (Table 4.2). The majority of these alkaloids have IC_{50} values below 20 µg/ml, indicating significant cytotoxic effects regardless of the type of cell or the cytotoxicity assay employed. Of the two alkaloids identified by GC-MS analysis of the alkaloidal fraction isolated in this study, cytotoxicity has been reported for voacangine by two independent research groups (Table 4.2.). Furthermore, two alkaloids previously isolated from *T. elegans*, conoduramine and isovoacangine, have also been associated with cytotoxic activity (Table 4.2). The cytotoxicity of the alkaloidal fraction of *T. elegans* may potentially be ascribed to the presence of these alkaloids.

The HepG2 cell line was particularly vulnerable to the cytotoxic effects of the crude extract and alkaloidal fraction, with IC_{50} values for the crude extract of 9.70 µg/ml and 6.49 µg/ml, and for the alkaloidal fraction of 2.55 µg/ml and 1.92 µg/ml, for the MTT and neutral red uptake assays, respectively. As the HepG2 cell line maintains some of the metabolic functions of hepatocytes, such as some of the cytochrome P450 subfamily enzymes[143], these data may indicate the constituents of the samples are converted to more toxic metabolites.

Table 4.2. IC_{50} values for indole alkaloids isolated from various *Tabernaemontana* species

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
Bicarpamontamine A	*T. sphaerocarpa*	S	HL60	MTT	0.91	[144]
			RPMI8226	MTT	0.37	[144]
			NCI-H226	MTT	1.27	[144]
			HCT116	MTT	1.05	[144]
			MCF7	MTT	1.89	[144]
Bicarpamontamine B	*T. sphaerocarpa*	S	HL60	MTT	0.37	[144]
			RPMI8226	MTT	1.42	[144]
			NCI-H226	MTT	1.8	[144]
			HCT116	MTT	0.6	[144]
			MCF7	MTT	1.35	[144]
Conodiparine A	*T. corymbosa*	L	KB	MTT	19.2	[145]
			KBV^-	MTT	13.5	[145]
19-oxo-conodiparine A	*T. corymbosa*	L	KB	MTT	21.4	[145]
			KBV^-	MTT	15	[145]
Conodiparine B	*T. corymbosa*	L	KB	MTT	20.8	[145]
			KBV^-	MTT	17	[145]
19-oxo-conodiparine B	*T. corymbosa*	L	KB	MTT	18.6	[145]
			KBV^-	MTT	13.6	[145]

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
Conoduramine	*T. johnstonii*	SB	P-388	N/S	20	[74]
			KB	N/S	19	[74]
	T. laeta	SB	A431	SRB	3.6	[138]
			BC1	SRB	0.8	[138]
			Col2	SRB	1.1	[138]
			HT	SRB	2	[138]
			KB	SRB	8.8	[138]
			KBV^-	SRB	0.6	[138]
			KBV^+	SRB	11.7	[138]
			LNCaP	SRB	11.1	[138]
			Lu1	SRB	5.3	[138]
			Mel2	SRB	1.7	[138]
			P-388	SRB	2.6	[138]
			U373	SRB	2.6	[138]
			ZR-75-1	SRB	1.3	[138]
Conodurine	*T. johnstonii*	SB	P-388	N/S	26	[74]
			KB	N/S	31	[74]
19-(2-Oxopropyl)-conodurine	*T. holstii*	R	P-388	N/S	2.4	[146]

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
Coronaridine	*T. calcarea*	WP	A2780	AB	9.9	[147]
	T. laeta	SB	A431	SRB	19.1	[138]
			BC1	SRB	7.5	[138]
			Col2	SRB	>20	[138]
			HT	SRB	>20	[138]
			KB	SRB	13.6	[138]
			KBV^-	SRB	1.9	[138]
			KBV^+	SRB	11.2	[138]
			LNCaP	SRB	10.7	[138]
			Lu1	SRB	10.9	[138]
			Mel2	SRB	>20	[138]
			P-388	SRB	3.8	[138]
			U373	SRB	12	[138]
			ZR-75-1	SRB	>20	[138]
(3S)-3-cyanocoronairdine	*T. divaricata*	SB	KB	MTT	2.2	[148]
11-hydroxycoronaridine	*T. calcarea*	WP	A2780	AB	4.8	[147]
Epivoacorine	*T. arborea*	S	P-388	N/S	1.7	[149]
Gabunamine	*T. johnstonii*	SB	P-388	N/S	1.3	[74]
			KB	N/S	5.8	[74]

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
Gabunine	*T. holstii*	R	P-388	N/S	3.2	[146]
	T. johnstonii	SB	P-388	N/S	3.2	[74]
Heyneanine	*T. calcarea*	WP	A2780	AB	10.7	[147]
19-*epi*-heyneanine	*T. calcarea*	WP	A2780	AB	8.9	[147]
Ibogamine	*T. calcarea*	WP	A2780	AB	3.5	[147]
10-Methoxyibogamine	*T. calcarea*	WP	A2780	AB	10.2	[147]
11-Methoxyibogamine	*T. calcarea*	WP	A2780	AB	4.9	[147]
Isovoacangine	*T. calcarea*	WP	A2780	AB	9.4	[147]
	T. johnstonii	SB	P-388	N/S	18	[74]
			KB	N/S	59	[74]
(3S)-3-cyanoisovoacangine	*T. divaricata*	SB	KB	MTT	1.9	[148]
Isovoacristine	*T. calcarea*	WP	A2780	AB	9.6	[147]
Jerantinine A	*T. corymbosa*	L	KB	MTT	0.76	[150]
Jerantinine A acetate	*T. corymbosa*	L	KB	MTT	0.44	[150]
10-O-methyljerantinine A	*T. corymbosa*	L	KB	MTT	4.77	[150]
Jerantinine B	*T. corymbosa*	L	KB	MTT	0.44	[150]
Jerantinine B acetate	*T. corymbosa*	L	KB	MTT	0.3	[150]
10-O-methljerantinine B	*T. corymbosa*	L	KB	MTT	2.93	[150]
Jerantinine C	*T. corymbosa*	L	KB	MTT	0.32	[150]

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
Jerantinine D	*T. corymbosa*	L	KB	MTT	0.28	[150]
Jerantinine E	*T. corymbosa*	L	KB	MTT	0.98	[150]
Jerantinine F	*T. corymbosa*	L	KB	MTT	5.1	[150]
Pericyclivine	*T. johnstonii*	SB	P-388	N/S	13	[74]
			KB	N/S	>100	[74]
Perivine	*T. johnstonii*	SB	P-388	N/S	20	[74]
			KB	N/S	70	[74]
Tabernamine	*T. johnstonii*	SB	P-388	N/S	2.1	[74]
3R/3S-hydroxytabernanthine	*T. calcarea*	WP	A2780	AB	7.9	[147]
Voacamine	*T. arborea*	S	P-388	N/S	2.6	[149]
	T. laeta	SB	A431	SRB	5	[138]
			BC1	SRB	2.9	[138]
			Col2	SRB	1.5	[138]
			HT	SRB	6.6	[138]
			KB	SRB	9.6	[138]
			KBV^-	SRB	2	[138]
			KBV^+	SRB	15.3	[138]
			LNCaP	SRB	12.6	[138]

Alkaloid	*Tabernaemontana* species	Plant part[a]	Cell line[b]	Assay[c]	IC_{50} (µg/ml)	Reference
			Lu1	SRB	11.2	[138]
			Mel2	SRB	3.8	[138]
			P-388	SRB	3	[138]
			U373	SRB	1.3	[138]
			ZR-75-1	SRB	2.8	[138]
Voacangine	*T. arborea*	S	P-388	N/S	6.8	[149]
	T. calcarea	WP	A2780	AB	10.4	[147]
(3S)-3-cyanovoacangine	*T.divaricata*	SB	KB	MTT	9.4	[148]
Voacristine	*T. calcarea*	WP	A2780	AB	11	[147]
19-*epi*-voacristine	*T. calcarea*	WP	A2780	AB	4	[147]
19-*epi*-voacristine hydroxyindolenine	*T. calcarea*	WP	A2780	AB	10.8	[147]
3-oxo-19-*epi*-voacristine	*T. calcarea*	WP	A2780	AB	6.8	[147]
Vobtusine	*T. sphaerocarpa*	S	HL60	MTT	11.64	[144]
			RPMI8226	MTT	28.52	[144]
			NCI-H226	MTT	14.89	[144]
			HCT116	MTT	27.56	[144]
			MCF7	MTT	29.6	[144]

Notes:

[a] : L – leaves; R – roots; S – stems; SB – stem-bark; WP – whole plant

[b]: A2780 – ovarian cancer; A431 – human epidermoid carcinoma; BC1 – human breast cancer; Col2 – human colon cancer; HCT116 – human colon cancer; HL60 – human blood premyelocytic leukaemia; HT – human fibrosarcoma; KB – human oral epidermoid cancer; KBV^- – vinblastine-resistant KB assessed in the absence of vinblastine; KBV^+ – vinblastine-resistant KB assessed in the presence of vinblastine (1 µg/ml); LNCaP – human prostate cancer; Lu1 – human lung canncer; MCF7 – human breast adenocarcinoma; Mel2 – human melanoma; NCI-H226 – non-small cell lung carcinoma; P-388 – murine lymphocytic leukaemia; RPMI8226 – multiple myeloma; U373 – human glioma; ZE-75-1 – hormone-dependant human breast cancer

[c]: AB – Alamar Blue assay; MTT – MTT assay; N/S – not stated; SRB – sulforhodamine B assay

The other cell types utilized in this study seem to have been equally affected by the cytotoxicity of the crude ethanolic and alkaloidal fraction, with the IC_{50} values obtained in the same range.

As no mechanistic studies have been conducted on the cytotoxicity of the these alkaloids, it can only be postulated that the alkaloids of the *Tabernaemontana* species are cytotoxic in a non-specific manner at concentrations below 20 µg/ml, based on the data obtained from this study as well as the literature.

5 Conclusions

The primary aim of this study was to isolate an antibacterial fraction from *Tabernaemontana elegans* (Stapf.) and assess the spectrum of antibacterial activity, synergism of antibiotic effects, as well as the *in vitro* cytotoxicity against mammalian cells. Through this aim, the study evaluated the potential of the antibacterial constituents of *T. elegans* for future pharmacological development.

The constituents of the crude ethanolic extract of the roots of *Tabernaemontana elegans* (Stapf.) were identified by thin layer chromatography as alkaloids, amines, phenols, steroids, sterols and lipids. The acid-base fractionation methodology employed in producing the alkaloidal subfraction was successful in separating the alkaloids from the other constituents of the crude ethanolic extract, in high yield. Based on the literature, previous studies using other *Tabernaemontana* species, and initial studies conducted in the laboratory, this alkaloidal fraction was selected as the fraction of interest, due to its potentiated antibacterial action.

Chemical characterisation of the alkaloidal fraction was performed by TLC and GC-MS. Due to the complexity of the alkaloidal fraction, it was not possible to conclusively identify any known alkaloids by TLC, despite substantial literature available on the chromatographic and chromogenic properties of the indole alkaloids of the *Tabernaemontana* genus. GC-MS identified two major components of the alkaloidal subfraction as dregamine and voacangine. Dregamine has been previously isolated from *T. elegans*. Voacangine has not been previously identified in *T. elegans*, but has been identified in numerous other *Tabernaemontana* species. When considering the number of alkaloids previously isolated from *T. elegans* and the complexity of the alkaloidal subfraction during TLC analysis, it is likely that a number of compounds present in the alkaloidal subfraction were not detected by GC-MS.

The broth micro-dilution assay and BACTEC radiometric assay were used to assess the antibacterial activity of the crude ethanolic extract and subfractions against 3 Gram-positive and 3 Gram-negative, as well as 2 mycobacteria. Two clinical isolates of bacteria possessing antibiotic resistance were also included in the panel of bacteria tested. The crude

extract and the alkaloidal subfraction possessed significant antibacterial activity (MIC ≤ 256 µg/ml) against the Gram-positive bacteria and mycobacteria, but did not influence the viability of the Gram-negative bacteria. Based on the MIC values obtained, it was shown that the acid-base fractionation step successfully enriched the antibacterial constituents of *T. elegans*. Furthermore, due to the lack of the activity in the basic fraction, it could be concluded that the compound(s) responsible for the antibacterial activity of the crude extract were alkaloidal in nature. Antibacterial activity has previously been ascribed to indole alkaloids isolated from various *Tabernaemontana* species, further substantiating the claim that these alkaloids are responsible for the antibacterial activity of *T. elegans*.

The presence of antibiotic resistance in the clinical isolates did not influence the susceptibility of the bacteria to either the crude extract or alkaloidal subfraction. This indicates that the bacterial compound(s) in *T. elegans* are able to circumvent the resistance mechanisms acquired by these bacteria.

The synergistic effects of the alkaloidal subfraction in combination with currently prescribed antibiotics in bacteria susceptible to the alkaloidal subfraction were assessed using the checkerboard synergy assay. FICs indicated that the alkaloidal fraction had additive effects in combination with ampicillin and isoniazid against Gram-positive and mycobacteria, respectively. The ability of the alkaloidal subfraction to reverse antibiotic resistance in isoniazid-resistance *M. tuberculosis* and methicillin-resistant *S. aureus* was also assessed. Results indicated that the alkaloidal subfraction was able to restore the antibiotic effect of ampicillin in MRSA. This effect was not seen with isoniazid in antibiotic-resistant *M. tuberculosis*.

The *in vitro* cytotoxicity of the crude extract and alkaloidal subfraction was assessed by the MTT and Neutral Red uptake assay in various mammalian primary cultures and cell lines. Significant cytotoxicity for both the crude extract and alkaloidal subfraction was observed in all cells, with IC_{50} values <20 µg/ml. As in the antibacterial assays, the acid-base fractionation step enriched the cytotoxic constituents of *T. elegans*. The cytotoxic properties of numerous indole alkaloids isolated from various *Tabernaemontana* species, including voacangine, have been previously described.

While the results obtained in this study confirm the antibacterial activity of the alkaloids of *T. elegans*, several limitations hamper the ability of the study to conclusively evaluate the pharmacological potential of these compounds. The primary limitation of this study was the use of a mixture of alkaloids in all of the assays. As each of the alkaloids may cause various biological actions, the testing of a mixture may cause inaccurate conclusions to be drawn about the components. This is particularly valid should the components have synergistic or antagonists activities.

Should there be no synergistic or antagonistic antibacterial actions between the alkaloids in terms of antibacterial action, the true magnitude of antibacterial activity of the active alkaloid would have been masked by the presence of the other alkaloids. In terms of the cytotoxicity, there is a possibility that the observed cytotoxicity is due to the other components in the alkaloids fraction and not caused by antibacterial alkaloid. However, due to the high number of cytotoxic alkaloids identified in *Tabernaemontana* species, it is likely that this is a class effect of these alkaloids. Unfortunately, these questions cannot be answered until bioassay guided fractionation of the alkaloidal fraction has been performed and the antibacterial alkaloid(s) is identified.

A further limitation of this study is the reliance on *in vitro* assays for determination of biological activity, particularly for the cytotoxicity assays. Due to the diverse factors that influence the absorption, distribution, metabolism and excretion of any molecule, there is the possibility that the data obtained *in vitro* may not correspond to the actual *in vivo* effects. Due to the dire need for new antibacterial agents, any compound with promising activity should be thoroughly assessed in an *in vitro* and *in vivo* setting, as far as possible.

This study confirms the antibacterial activity of *T. elegans.* The compounds responsible for this activity are highly likely to be the indole alkaloids, for which the genus is well known. Despite the promising *in vitro* antibacterial activity against myco- and Gram-positive bacteria, the significant cytotoxicity identified in this study may limit the usefulness of these compounds in the development of novel antibiotics. This is particularly evident when considering the number of previously identified cytotoxic alkaloids in this genus. While chemical modification of these alkaloids may produce less-toxic molecules that still retain an antibiotic effect, it is unlikely that the alkaloids present in *T. elegans* could be used

directly as antibiotics for human use, and thus have little potential for further pharmacological development.

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