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# CHAPTER 1

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## INTRODUCTION

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### 1.1 Background

#### 1.1.1 Phytomedicines: the greening of modern medicine

Naturally occurring substances of plant, animal and mineral origin have provided a continuing source of medicines since the earliest times known to man, but it is the plant kingdom, in particular, which has proved to be of the most use for treating most of our ailments. During the course of history, experimentation has succeeded in distinguishing those plants, which have beneficial effects from those, which are toxic or merely non-effective. Through trial and error, human beings have discovered ways of relieving pain and sickness, and of living in harmony with nature. This process has gradually evolved throughout the whole world over a period of thousands of years and it is estimated that some 20,000 plant species are used medicinally (Penso 1982).

Scientifically based medicine has accumulated a great amount of knowledge about the causes of sickness and their cures. Against this background, traditional healers and their practices appear as a mainstay of the grossest darkness, which holds the human mind. Yet contrary to this frequently held assumption, some of the medicines used by the traditional healers are surprisingly effective. Many of our medicinal plants, which have continued to be used clinically, are now given in the form of isolated compounds or in standardised preparation. More and more people in developing countries utilise traditional medicine for their major primary health care needs. During the last decades, extensive investigations have been done on

medicinal plants. The obtained results often justified the use of the plants in folk medicine and constituted a serious basis for the improvement of the efficacy, the safety and the quality of herbal remedies used all over the world. Moreover plants are known to offer excellent perspectives for the discovery of new therapeutic products including anti-infectious agents (Cox & Balick 1994). Scientists have shown a keen interest in the knowledge of traditional healers all over the world. Higher plants, in particular, contribute to Western medicine in being ingredients of approximately one quarter of prescriptions dispensed (Phillipson & Anderson 1989). Although available in the United States for little more than 10 years, ethnomedicines represent an important class of natural therapeutics that are broadly used and accepted world-wide (Israelsen 1995). Plants and plant products are present in 14 of the 15 therapeutic categories of pharmaceutical preparations, which are currently recommended to medical practitioners in the United Kingdom. Numerous examples from medicine impressively demonstrate the innovative potential of natural compounds and their impact on progress in drug discovery and development (Phillipson & Anderson 1989).

### 1.1.2 Ethnopharmacology and the utilization of herbal drugs

The ability to correlate ethnobotanical reports with corresponding scientific studies could lead to improved selection of plants for further study in the health care system of a country. Ethnopharmacology provides scientists with an alternative approach for the discovery of antimicrobial agents, namely the study of medicinal plants with a history of traditional use as a potential source of substances with significant pharmacological and biological properties. The combination of analysing ethnomedical information and scientific studies on plant extracts may reduce the number of plants that need to be screened for drug discovery attempts, resulting in a corresponding greater success rate than by random selection and mass bioscreening (Vlietinck *et al.* 1995).

Drug development based on ethnomedical leads has followed the identification of plant species with biologically active compounds and the characterisation and

standardisation of traditional recipes for reformulation as medicines. During the 1800s, the active principles of a number of plant drugs were isolated and it was realised that the clinical effects of drugs such as opium, cinchona and ipecacuanha could be attributed to the chemical compounds morphine, quinine and emetine, respectively (Phillipson & Anderson 1989). The recognition of many African plants as medicines led to the isolation of several biologically active molecules; examples range from the well known physostigmine (from *Physostigma venenosum*) used for the treatment of glaucoma to the recently identified antiviral agents from *Ancistrocladus abbreviatus* (Iwu 1994). Some recent work done in drug development in India, relates to species of *Commiphora* (used as a hypolipidaemic agent), *Picrorhiza* (which is hepatoprotective), *Bacopa* (used as a brain tonic), *Curcuma* (anti-inflammatory) and *Asclepias* (cariotonic) (Jain 1994).

The National Cancer Institute (NCI), Bethesda-Maryland, screened 21,881 extracts derived from over 10,500 samples for activity against the human immunodeficiency virus (HIV); 2320 of these extracts were of medicinal plant origin. Approximately 18% of the total number of extracts showed significant anti-HIV activity of which four plant-derived compounds are in preclinical development at the NCI (Cragg *et al.* 1994). In the Amazonia, ethnobotanical studies have identified plants documented by early travellers; these include *Paullinia yoco* and *Ilex guayusa*, which are used as stimulants (Schultes 1994). Numerous drugs have entered the international pharmacopoeia *via* the study of ethnobotany and traditional medicine. This process has recently resulted in clinical trials of 'Provir' an oral product from a medicinal plant for the treatment of respiratory viral infections and 'Virand' a topical antiviral product for the treatment of herpes in Shamam pharmaceuticals, a development-stage company (king & Tempesta 1994).

### 1.1.3 South African traditional medical practice

In the traditional African worldview the natural environment is a living entity whose components are intrinsically bound to mankind. The wealth of information on folk medicine coupled with the enormous diversity of the South African flora (about 24000 species) focussed our attention on the use of folk medicine as the starting point for our research. The traditional doctors in this country known as inyanga or ngaka have been practising for centuries. They play a significant part in health care in so much that they treat about 60-70% of patients (Matthe 1989). The detailed analysis of the pharmacological properties of medicines used traditionally brought to light that there are innumerable acids, alkaloids, flavonoids, terpenoids, oils, gums, resins, fats etc. present in plants. Some of these ingredients do have a specific pharmacological reaction towards sickness, which are recognised by pharmacologists (Watt & Breyer-Brandwijk 1962; Hutchings 1966; Van Wyk *et al.* 1997).

Natural product research continues to provide a tremendous variety of lead structures, which are used as templates for the development of new drugs by the pharmaceutical industry. The field of ethnobotanical research has expanded greatly in recent years as the value of this type of research has come to be more widely recognised. The traditional use of medicinal plants and the pharmacological activity of extracts previously investigated shows that a viable approach to pharmaceutical research in the areas of various diseases such as arthritis, tuberculosis (TB), cancer, diabetes, bacterial and viral infections etc. is needed. Medicinal plants possess many potentially valuable therapeutic agents which needs further research to investigate their effectiveness.

## 1.2 Tuberculosis

### 1.2.1 Epidemiology

Tuberculosis remains a serious health problem in many regions of the world, especially in developing nations. It is a contagious disease and is becoming an epidemic in some parts of the world. TB is considered to have the fifth highest fatality rate in the world (Kochi 1997). It is estimated that 30-60% of adults in developing countries are infected with *Mycobacterium tuberculosis*. Approximately 8-10 million individuals develop clinical TB and 3 million die of TB each year (WHO/IUATLD 1989). The WHO suggests that the number of new cases world-wide will rise from the current 7 million a year to 10 million by 2015. It is estimated that between 2000 and 2020, nearly 1-billion more people will be newly infected, 200-million people will get sick and 70 million will die from TB if control of the disease is not strengthened (New Scientist 1998). Within the black population of South Africa TB is endemic. The prevalence of TB in South Africa increased from 0.9% in 1975 to 3.9% in 1991 among black gold miners (Murray *et al.* 1996). A program audit of 2473 TB patients in KwaZulu-Natal, South Africa, was conducted between 1991 and 1994. It was found that monthly admissions increased from 34 per month in 1991 to 66 in 1994 (Wilkinson *et al.* 1996). In South Africa, over 3 in every thousand people die of TB, the highest rate in the world. One out of every 200 people suffers from active tuberculosis. TB is the most commonly notified disease in South Africa and the fifth largest cause of death among the black population (South African Tuberculosis Association 1998). In the United States, the number of TB cases steadily decreased until 1986 when an increase was noted; TB cases has continued to rise since. Ten million individuals are infected in the USA, with approximately 26000 new cases of active disease each year (National Jewish Medical and Research Center 1994).

TB has already been recognised as one of the most frequent opportunistic infections in persons with Human Immunodeficiency Virus (HIV) infection in developing countries. HIV infection results in an impairment of the immune system and

entails a substantial risk of TB in those individuals who are or become infected with the tubercle bacillus (Grange & Davey 1990). It is said that there is an exchange of “evil services” between the two diseases, tuberculosis and HIV- infections, warranting the term of cursed duet (Chretien 1990). The diagnosis of infection caused by *M. tuberculosis* is of increased public health concern following increases in the number of cases in developing countries and major increases in developing countries is associated with the spread of HIV infection (Wilkinson *et al.* 1996). The TB epidemic in Africa reached its peak with the advent of HIV infection. The problem became serious enough that, in 1991, Stamford raised the question ‘“Is Africa Lost?”’ (Stamford *et al.* 1991). TB accounts for almost one-third of AIDS death worldwide, which kills 2-million people each year and infects 8-million more almost all of them in developing world. It causes about 40% of AIDS death in Africa and Asia. (WHO/TB/98.258). Control of the TB epidemic linked with HIV infection will depend largely on the adequate treatment of TB, and possibly of effective chemoprophylaxis, not just for HIV-infected persons but for the community as well (WHO/IUATLD 1989).

### 1.2.2 TB in animals

Studies on TB have been directed not only on humans, but also towards marine creatures and domestic animals. *Mycobacterium* causes TB in many animal species including humans. Generally, *Mycobacterium bovis* infects cattle and cervids, but it has the potential to infect virtually all species of mammals (Stead 1997). Bovine TB is well-established in cattle in Australia and New Zealand and causes a serious public health problem, especially in children through ingestion of TB-infected milk (Tweddle & Livingstone 1994).

There is sufficient evidence to suggest that TB infection in animals is widely distributed and is found at significantly high prevalence in some population of animals. Diverse pathological conditions causing the strandings and/or deaths of several species of sea lions and seals on the Northern Coast of the province of Buenos Aires was reported. Bacterial isolates from the diseased sea lions and seals showed characteristics consistent with *M. bovis*, whereas some demonstrated the properties of *M. tuberculosis*.



More recently, cases of TB have been detected in an Australian sea lion, and in a New Zealand fur seal (Bernardelli *et al.* 1996). Ferrets were recognised as significant wild life vectors of TB in New Zealand. Ten out of eighty animals captured from the wild were severely infected (Cross *et al.* 1999). TB was diagnosed on a game ranch in Zambia outside a national park in free ranging animals like kaffie lechwes and bushbucks (Zieger *et al.* 1998). In some buffalo herds the prevalence of TB is as high as 70% in the Kruger National Park of South Africa. A number of lions, cheetahs and baboons contracted the disease and died recently by consuming infected buffalo meat and carcasses (Keet *et al.* 1996).

The zoonotic transmission of TB infection in humans is likely to occur by aerosolization of infected particles produced from the cough of live animals, or by the housing of infected material in the rendering plant or postmortem laboratory (Fanning *et al.* 1991). The epidemiology of *M. bovis* infections in man were reported in 1974 and 1975 in Sweden in areas where cattle TB had previously been common (Sjogren & Hillerdal 1978). A case-control study of TB in cattle done between December 1993-1995 in Northern Ireland suggested that a TB outbreak among people staying close to the animal farm was associated with bovine TB in cattle. Infected cattle played a significant role in the transmission of TB (Denny & Wilesmith 1999). There were 34,959 human deaths from all forms of TB in England and Wales in 1931, of which an estimated 2147 deaths (6.1%) were attributed to TB infection from cattle (Minor 1999).

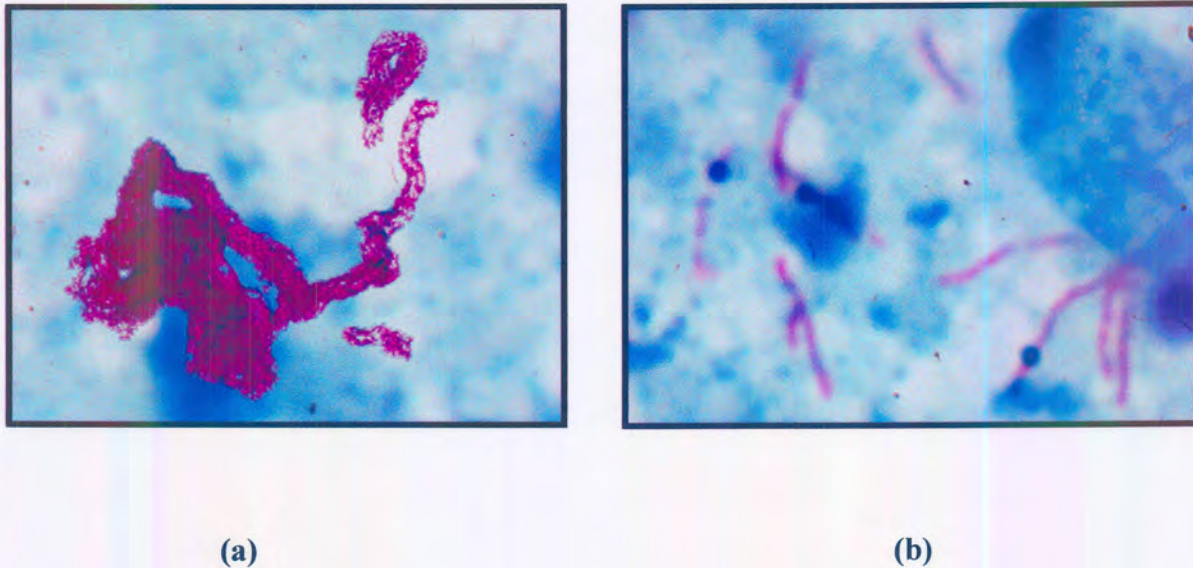
TB infection in a fully susceptible population in any game park, leads to the establishment of the disease in a particular species which then become maintenance host of the disease. The presence of a large number of maintenance hosts in the diverse ecosystem of game park may be expected to infect the disease further to other species in the park and neighbouring areas. Additionally the close association between humans and animals especially baboons in the parks particularly in some of the tourist camps and local dwellings, should be a cause for concern, as the infection, which is known as zoonosis, may also be contracted by man (Keet *et al.* 1996).



### 1.2.3 *Mycobacterium tuberculosis*; the causative agent

Robert Koch first isolated the causative agent of tuberculosis, *Mycobacterium tuberculosis* in 1882. Koch found the bacillus constantly associated with the clinical disease, isolated it in pure culture, reproduced the disease in guinea pigs and rabbits, and recovered the bacillus in pure culture, from the experimentally infected animals. The shape of *M. tuberculosis* is slender, straight, or a slightly curved rod with rounded ends. The organisms vary in width from 0.2 to 0.5  $\mu\text{m}$  and in length from 1 to 4  $\mu\text{m}$ . The most unique structure of the mycobacterial cell is its cell wall, a multilayered structure approximately 20 nm thick. The bacilli are non-motile, non-sporogenous, and nonencapsulated. They are difficult to stain with the gram stain but are usually considered to be gram positive. The Ziehl-Neelsen stain is useful in staining organisms and once stained they are resistant to decolourization with acidic alcohol. For this reason they are often referred to as acid-fast bacilli. The Ziehl-Neelsen stain is generally used for the visualisation of the organism. With this stain, the bacilli appear as brilliantly stained red rods against a deep sky-blue background. Organisms often have a beaded appearance because of their polyphosphate content and unstained vacuoles (Joklik *et al.* 1968) (Figure 1.1).

TB most commonly occurs in communities who live in poor socio-economic conditions. This is because people living in poor conditions have little immunity to the TB infection as a result of malnutrition, physical stress, poor access to health services, overcrowded conditions etc. This bacterium primarily affects the lungs and may infect anyone at any age. If someone who is actively infected by tuberculosis sneezes or coughs, it is easy for people nearby to inhale the tuberculosis bacteria. However, inhaling the organism does not usually mean that one can develop the active disease. A person's natural body defences are usually able to control the infection so that it does not cause disease. In this case, the person would be infected but not have the active disease. Only about 10% of those infected will actually develop TB in their



**Figure 1.1.** *Mycobacterium tuberculosis* stained uniformly by the Ziehl-Neelsen method: (a) Colonies x 400 (b) Straight and curved rods x 1000 of *M. tuberculosis* (Courtesy: WHO/TB/98.258).

lifetimes. The active disease can occur in an infected person when the body's resistance is low or if there is a large or prolonged exposure to the bacteria that overcome the body's natural defences. The body's response to active TB infection produces inflammation, which can eventually damage the lungs. Mycobacterium attacks mitochondrial membranes, causing functional damage to membrane-associated respiration and oxidative phosphorylation (National Jewish Medical and Research Center 1994).

The amount of damage caused by TB may be quite extensive, yet the symptoms may be minimal. The usual symptoms of TB are

- Fever
- Coughs
- Weight loss
- Loss of appetite
- Weakness
- Night sweats
- Dyspnoea
- Shortness of breath
- Chest pain, signs of chest disease etc. (South African Tuberculosis Association 1998).

#### **1.2.4 Multidrug-resistant TB**

TB therapy has been revolutionised and the present treatment regimes for TB are based on multidrug therapy with usually 3 or 4 antituberculosis drugs. However, the problem of multidrug-resistant tubercle bacilli is emerging for various drugs for example; isoniazid, ethambutol, rifampin, streptomycin etc. (Girling 1989; Grange & Davey 1990). Risk factors for the spread of multidrug-resistant tuberculosis (MDR TB) include poor compliance, convergence of immunosuppressed patients, delayed diagnosis or treatment and poor or inadequate ventilation facilities. MDR TB is very difficult to treat and requires a longer period of treatment. Sometimes, surgery is needed to remove areas of destroyed lungs that are heavily infected by mycobacterium and inaccessible to drugs (National Jewish Medical and Research Center 1994).

A recent WHO report states that, globally, 2% of all cases of tuberculosis are multidrug resistant-by definition, resistance to rifampin plus isoniazid (plus/minus other resistances). Such cases can be treated in the USA and other high resource regions but at a great cost (> US\$ 250,000 per case!) and using very long courses of rather toxic drugs, thereby raising serious problems of compliance (WHO 1997). South Africa

is witnessing an explosion in the number of cases of drug-resistant tuberculosis. In some parts of South Africa, 1 in 10 cases of TB is resistant to treatment. A research group funded by the British Pharmaceuticals Company Glaxo Wellcome, says that the number of cases of MDR TB in the KwaZulu-Natal region has risen by 300 percent in just one year. (New Scientist March 1997). An estimated 2000 South Africans contract multidrug resistant TB each year and more than half of these patients die within a period of two years(WHO/TB/98.258). It is essential to have new antituberculosis agents due to the increasing resistance of mycobacterium to these classic antituberculosis drugs, preferably those that can readily and simply be produced from some local source.

### 1.3 Other Bacterial infections

Man is host to a variety of pathogenic bacteria, protozoa and viruses. Persons who are deficient in the production of circulating antibodies are highly susceptible to respiratory infections by Gram-positive bacteria; persons who are deficient in T cell functions, however, tend to succumb to infections by fungi and viruses, as well as to bacteria which grow predominantly intracellularly (Stanier *et al.* 1958).

Toxins produced by pathogenic bacteria, could result in serious complications. The pathogenicity of some of the Gram-positive bacteria has sparked an acute awareness among people recently. *Staphylococcus aureus*, a Gram-positive bacterium is associated with the ability of the organism to produce a number of different toxins. Of these, the enterotoxin is responsible for a common type of food poisoning. On the other hand, the exotoxin causes necrosis of the skin and lyses red blood cells during the development of boils or other local abscesses. From these local inflammations, the organisms frequently spread by way of the lymphatics and the blood. Hence, Staphylococcal infections often develop into more serious diseases such as pneumonia, meningitis, endocarditis, osteomyelitis and many others. Most *Bacillus* organisms are usually straight rods with parallel sides and may be arranged in varying configuration. Some outbreaks of food poisoning have been attributed to *B. cereus* and *B. subtilis*



which occasionally cause human eye infections (Delaat 1979).

Similar to Gram-positive bacteria Gram-negative bacteria also cause impaired function in the human body which may be acute, manifest at short notice or can be relatively long in duration. *Escherichia coli* are short plump rods and they uniformly stain Gram-negative. *E. coli* is the most frequent cause of urinary tract infections, which may take the form of cystitis, pyelitis, pyelonephritis, appendicitis, peritonitis, postoperative wound infection, infantile diarrhoea etc. They are also associated with the secondary infection of the lungs (Delaat 1979). *Pseudomonas aeruginosa* typically causes infections, not frequently fatal, in victims of severe burns and in cancer patients who have been treated with immuno-suppressive drugs. *Klebsiella pneumoniae* are short to fairly long bacilli, they all produce capsules and are nonmotile. *K. pneumoniae* can cause severe enteritis in children, as well as pneumonia and upper respiratory tract infections in man generally. *Enterobacter* species are frequently isolated from patients suffering from urinary tract infections or septicemia (Stanier *et al.* 1958).

The pathogenicity of some of the bacterial species is quite significant because of their resistance to known antibiotics. The emergence of methicillin-resistant *S. aureus*, vancomycin-resistant enterococci and multiresistant Gram-negative bacteria has become a serious issue recently (Rao 1998). In a study done earlier it was found that 36 strains of *B. cereus* were highly resistant to lincomycin, polymyxin B and penicillin G-cephalosporin (Arribas *et al.* 1988). Fifty methicillin-resistant strains of *S. aureus* had been isolated at a hospital in Osaka between 1986 and 1990 of which a few were also resistant to streptomycin and kanamycin (Kondo *et al.* 1991).

## 1.4 Viral infections

Viruses damage their hosts either by destroying the cells in which they multiply or by triggering hypersensitivity reactions. Serious viral infections are mumps, measles, influenza and skin infections caused by various medically important viruses such as

adenovirus, herpesvirus, papovavirus, paramyxovirus etc. Among these viruses, herpes simplex virus (HSV) is one of the most widespread viruses in the human population. HSV infections in man are seen either as primary or recurrent infections. Acute gingivo-stomatitis, cervicitis and eczema are examples of primary infections, whereas cold sores, kerato-conjunctivitis are most common among the recurrent infections (Timbury 1986). Primary infections are caused by the herpes simplex virus type II (HSV II). HSV I is associated with the recurrent infections such as, infections of the facial area, such as cold sores. It produces skin infections but the major target organs in humans are oral mucous membranes (Stanier *et al.* 1958).

## 1.5 Medicinal plants with antibacterial and antiviral activity

Over the past decade there has been a proliferation of literature on the antibacterial and antiviral properties of plant extracts. There are several reports on *in vitro* inhibition of mycobacterium by medicinal plants. Ten of 408 ethanolic extracts of plants such as *Actaea spicata*, *Angustura vera*, *Cinnamomum camphora*, *Piper cubeba* (*Cubeba officinalis*), *Guaiacum officinale*, *Ipomea purga*, *Rhamnus cathartica* etc. inhibited growth of *Mycobacterium tuberculosis* H37Rv at dilutions of 1 in 160 to 1280 and a high proportion of the other extracts inhibited growth at lower dilutions (Grange & Davey 1990). It was found that *M. tuberculosis* was also sensitive towards the Rwandese medicinal plants, *Pentas longifolia*, *Tetradenia riparia* and *Bidens pilosa*. The active compound isolated from the leaves of *T. riparia* was tested against *M. tuberculosis* and showed activity at 100 µg/ml (Van Puyvelde *et al.* 1994). *Hydrocotyle asiaticum* inhibited growth of *M. tuberculosis* at a dilution of 1:20 (Grange & Davey 1990). The pharmacognostical studies on the Chinese traditional drug A-ji-ba-mo used as a remedy for tuberculosis was derived from the roots of *Dipsacus asperoides* (Zhou *et al.* 1994). From the root extracts of *Salvia hypargeia*, a new compound, hypargenin, was isolated which showed significant activity against *M. tuberculosis* (Ulubelen *et al.* 1988).

A number of plants have been cited in the literature as being used for medication against various bacterial and viral infections or as containing biologically active compounds. Research conducted by Noristan, Pretoria, suggests that from a total of about 300 plants screened, at least 31% show marked analgesic, anti-inflammatory and anti-infective properties. (Theunis *et al.* 1992). Out of 100 medicinal plants of Rwanda, 30% of the plants tested showed activity against *B. subtilis* and *S. aureus* (Boily & Van Puyvelde 1986). A significant antibacterial activity was displayed by a novel diterpene diol isolated from *Iboza riparia* (De Kimpe *et al.* 1982). The zones of inhibition produced by water and methanolic extracts of *Bridelia ferruginea* ranged from 4 to 20 mm when tested against *S. aureus*, *E. coli*, *Klebsiella* sp., *Streptococcus pyrogenes* etc. (Irobi *et al.* 1994). Organic extracts of *Helichrysum crispum* inhibited the growth of *M. smegmatis* and *P. aeruginosa* (Salie *et al.* 1996). The active principle 3-O-methylquercetin isolated from the flowers of *H. odoratissimum* displayed antimicrobial activity (Van Puyvelde *et al.* 1989).

Acute and recurrent herpes simplex virus infections are world-wide in distribution and several antiviral compounds have been introduced into therapeutic use (Meyers *et al.* 1982; Palmieri *et al.* 1987). Their conflicting efficacy in recurrent infection and in immunodeficient patients, as well as the problem of prohibitive costs in developing countries, has necessitated the search for alternative drugs. The minimum inhibition dose for herpes simplex virus was shown to be 0.1 mg/ml and 1.0 mg/ml for the ethanol extract of *Rheum officinale* and of *Annona muricata* respectively (Wang *et al.* 1996; Padma *et al.* 1998). In an ethnopharmacological screening of some of the selected medicinal plants used in Argentina such as *Polygonum punctatum*, *Lithraea molleoides*, *Sebastiania brasiliensis* etc. showed *in vitro* antiherpetic activity with the 50% effective dose (ED<sub>50</sub>) ranging from 0.039 to 0.169 mg/ml (Kott *et al.* 1999).

Plants have been endowed with therapeutic virtues both in legend and in scientific literature and are being used in treating various ailments such as coughs, colds, other pathogenic bacterial and viral infections etc. The use of antimicrobials from natural vegetation has a great impact in human health care of undeveloped





countries. Herbal medicine has been used for centuries in rural areas by local healers and has been improved in industrialised countries. A number of substances used in modern medicine for the treatment of serious diseases have originated from research on medicinal plants (Irobi *et al.* 1994). Indeed, over half the world's 25 best selling pharmaceuticals for 1991 owe their origin to one of a range of natural source materials (Table 1.1) (Sneader 1985; Phillips & Drew 1992).

**Table 1.1** The World's 25 Best Selling Pharmaceuticals (Sneader 1985; Phillips & Drew 1992)

Position 1991	Product	Therapeutic Class	Sales \$m
1	Ranitidine	H <sub>2</sub> antagonist	3,032
2	<sup>a</sup> Enalapril	ACE inhibitor	1,745
3	<sup>a</sup> Captopril	ACE inhibitor	1,580
4	<sup>a</sup> Diclofenac	NSAID	1,185
5	Atenolol	β-antagonist	1,180
6	Nifedipine	Ca <sup>2+</sup> antagonist	1,120
7	Cimetidine	H <sub>2</sub> antagonist	1,097
8	<sup>a</sup> Mevinolin	HMGCoA-R inhibitor	1,090
9	<sup>a</sup> Naproxen	NSAID	954
10	<sup>a</sup> Cefaclor	β-lactam antibiotic	935
11	Diltiazem	Ca <sup>2+</sup> antagonist	912
12	Fluoxetine	5HT reuptake inhibitor	910
13	Ciprofloxacin	Quinolone	904
14	Amlodipine	Ca <sup>2+</sup> antagonist	896
15	<sup>a</sup> Amoxicillin/ clavulanic acid	β-lactam antibiotic	892
16	Acyclovir	Anti-herpetic	887
17	<sup>a</sup> Ceftriaxone	β-lactam antibiotic	870
18	Omeprazole	H <sup>+</sup> pump inhibitor	775
19	Terfenadine	Anti-histamine	768
20	<sup>a</sup> Salbutamol	β <sub>2</sub> -agonist	757
21	<sup>a</sup> Cyclosporin	Immunosuppressive	695
22	<sup>a</sup> Piroxicam	NSAID	680
23	Famotidine	H <sub>2</sub> antagonist	595
24	Alprazolam	Benzodiazepine	595
25	<sup>a</sup> Oestrogens	HRT	569

<sup>a</sup>Natural product derived

Overall, the world TB situation is thought to be improving very little despite the existence of effective therapy and a partially effective vaccine. The objective of the research activities should be to curb the anticipated increase in TB in developing countries where *M. tuberculosis* infection is highly prevalent. Exploration of new directions, in academic research and in tuberculosis control policy is required. Ideally, any new drug would be capable of acting in synergy with the existing drugs and showing efficiency against the MDR TB. As part of the screening programme for the detection of potentially useful antimycobacterial agents in South African medicinal plants, plant extracts have been examined for their inhibitory activity against a reference strain (H37Rv) and multidrug resistant strains of the human tubercle bacillus *M. tuberculosis* in this project. Compounds isolated from the plant possessing the highest antimycobacterial activity have also been investigated for their inhibitory action against other bacterial species and herpes simplex virus.

## 1.6 Scope of the thesis

### 1.6.1 Antimycobacterial activity of plant extracts

The traditional use of some medicinal plants such as, *Acacia nilotica*, *Cassine papillosa*, *Chenopodium ambrosioides*, *Combretum molle*, *Croton sylvaticus*, *Cryptocarya latifolia*, *Ekebergia capensis*, *Protasparagus africanus*, *Rapanea melanophloeos*, etc. by South Africans in curing TB related symptoms such as fever, cough, chest disease, night sweats etc. has been reported (Watt & Breyer-Brandwijk 1962; Pujol 1990; Hutchings 1996). However, no attention has previously been given to the laboratory evaluation and detection of antituberculosis activity in South African medicinal plants. Scientific basis for the utilisation of such plants in South African folk medicine is required and therefore, the present study was undertaken.

The antimycobacterium activity of 20 local medicinal plants that have been used in the treatment of TB symptoms was investigated in this study. Plant extracts were

screened for activity against a drug-sensitive and a few drug-resistant strains of *M. tuberculosis*.

### **1.6.2 Comparative study of the different methods for susceptibility testing of *M. tuberculosis***

Standardisation of *in vitro* susceptibility testing is greatly needed to achieve uniformity among the test methods used to evaluate tuberculosis therapeutics. At present, two methods are being employed; a conventional and a rapid radiometric method for isolation and susceptibility testing of mycobacterium from extrapulmonary specimens and for drug susceptibility testing. The technique employed in the conventional method for drugs/plant extracts susceptibility testing permits the most economical utilisation of equipment. It is possible in a routine bacteriology laboratory to provide data which are satisfactory within 5-6 weeks. The so-called critical concentrations of the antituberculosis drugs using conventional methods were developed originally for testing in Lowenstein-Jensen egg medium; later, equivalents were found for 7H10 agar, 7H11 agar and 7H12 broth media (Siddiqi *et al.* 1981).

Recent studies have suggested that the BACTEC radiometric method is superior to conventional methods in detection time and recovery rate of mycobacterium. In 1977, Middlebrook *et al.* showed that a new liquid medium, 7H12, used with the semi-automated BACTEC system for the detection of mycobacterial growth could have clinical laboratory usefulness. The first evaluation of rapid radiometric drug susceptibility testing of *M. tuberculosis* was reported by Snider *et al.* in 1981. BACTEC is a rapid test and the results are generally reported in 4 to 5 days, while the conventional results are reported in 3 to 4 weeks (Siddiqi *et al.* 1985). However, it has been suggested that improved detection of mycobacterium is possible if radiometric and conventional methods are used together (Takahashi & Foster 1983). Previous studies have shown that the conventional method is comparable to the radiometric method for testing susceptibility of *M. tuberculosis* strains to drugs (Laszlo *et al.* 1983; Fadda & Roe 1984).

In the present study, the conventional method (7H11 agar medium) was used for susceptibility testing for the preliminary screening of 20 plant extracts against *M. tuberculosis*. Results were later confirmed and compared by rapid radiometric method by testing the plant extracts with good activity, against the sensitive and the multidrug-resistant strains.

### 1.6.3 Cytotoxicity assay of plant extracts

Cytotoxicity evaluation of the plant extracts and their active principle is required for its effective therapeutic use. After establishing the antimycobacterial activity of 14 plant extracts against *M. tuberculosis*, the next step was to isolate the active

compound(s) from one of the most active and the least toxic plants. Cytotoxicity assay of the plant samples were carried out on monkey kidney cells in collaboration with the Virology department of the University of Pretoria with the intention to choose a plant for the isolation of the active compound(s) with anti-TB activity and low toxicity.

### 1.6.4 Antibacterial activity of *Euclea natalensis*

Out of 14 plants the acetone extract of *Euclea natalensis* A.DC. was found to be one of the most active extracts we investigated for activity against *M. tuberculosis* and comparatively less toxic than the other plants.

*Euclea natalensis* is a tree of the Ebenaceae family with a somewhat spreading crown (Figure 1.2). It occurs in a variety of habitats including coastal and inland forest as well as bushveld. It is widely distributed in tropical and sub-tropical Africa and is common on the east coast of South Africa (Van Wyk & Van Wyk 1997) (Figure 1.3). It has been reported in literature that the roots of *E. natalensis* are used by indigenous people of South Africa for various pathological bacterial infections. Powdered root bark of this species is used as an ingredient in medicines to treat urinary tract infections, venereal diseases, and dysmenorrhoea. Root bark infusions are applied in sores and wounds (Pujol 1990). The custom of cleaning teeth and the gums with a chewed



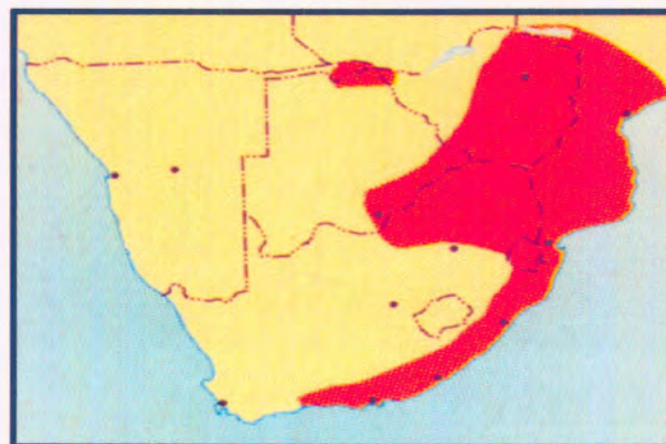


(a)



(b)

**Figure 1.2** *Euclea natalensis*: (a) Tree (b) Fruit.



**Figure 1.3** Distribution of *Euclea natalensis* in Southern Africa (Van Wyk & Van Wyk 1997).

root of *E. natalensis*, in the belief that it benefits oral health, is practised in South Africa by married women of an African Zanzibar community (Stander & Van Wyk 1991). According to one investigational report of the Herbal Medicine Trade in KwaZulu-Natal, root bark of *E. natalensis* are used to prepare an Imbhiza (blood purifying decoction taken for glandular swellings and sores) and are widely used for curing TB-related symptoms, various chest diseases such as bronchitis, pleurisy, chronic asthma etc. by the Zulus, a tribe of South Africa (personal communication; Bryant 1966).

It was therefore, decided to investigate the antibacterial activity of acetone and water extracts of *E. natalensis* against some of the common bacterial species. Five Gram-positive bacteria: *Bacillus cereus*, *B. pumilus*, *B. subtilis*, *Micrococcus kristinaea*, *Staphylococcus aureus* and six Gram-negative bacteria: *Enterobacter aerogenes*, *E. cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens* were tested for susceptibility in this study.

### 1.6.5 Isolation, purification and identification of the active compound(s) from *E. natalensis*

Out of 14 plants tested for toxicity and activity it was found that *E. natalensis* possesses high antimycobacterial activity and comparatively less toxicity than the other plants investigated.

Our objective was to isolate the active compound(s) and evaluate the minimal inhibitory concentration (MIC) of the isolated compound(s) for *M. tuberculosis*. Through the activity-guided fractionation of the acetone extract of *E. natalensis* two closely related bioactive compounds (diospyrin and 7-methyljuglone) were isolated and identified. The MICs of the isolated compounds were determined for drug sensitive as well as drug-resistant strains of *M. tuberculosis*. Synergistic inhibitory action of the compounds was also investigated against H37Rv, a drug-sensitive and a drug-resistant strain, resistant to two drugs isoniazid and rifampin of *M. tuberculosis*.



The MICs of the two isolated compounds were determined for eleven bacterial species and the synergistic effect of the compounds was also evaluated against the bacterial species.

### 1.6.6 Antiviral activity of *E. natalensis*

It has been reported that extracts of the root of *E. natalensis* are believed to be used as a natural remedy for various skin infections by South Africans. The charred and powdered root is used as an application to the skin lesions in leprosy and internally for ancylostomiasis by the local people in South Africa (Watt & Breyer-Brandwijk 1962; Bryant 1966; Khan *et al.* 1978) It was therefore, decided to investigate the inhibitory activity of acetone and water extracts of *E. natalensis* and an isolated compound, diospyrin against HSV I *in vitro*.

## 1.7 Structure of the thesis

The preliminary screening of 20 South African medicinal plants against a sensitive strain of *M. tuberculosis* by using the more economical conventional agar plate method is described in **chapter 2**. The antimycobacterial activities of the acetone and water extracts of the plants are reported.

**Chapter 3** reports on the antimycobacterial results of 14 selected plant samples by the BACTEC radiometric method. The effectiveness of agar plate method and BACTEC radiometric method in susceptibility testing of *M. tuberculosis in vitro* is compared and discussed in this chapter.

The cytotoxicity evaluation of 14 plant samples is dealt with in **chapter 4**. ID<sub>50</sub> values of the acetone extracts against monkey kidney cells are reported.

**Chapter 5** describes the antibacterial activity of the crude acetone and water extracts of the most active and least toxic plant, *E. natalensis*. MICs against 11 bacterial species have been determined.

In **chapter 6** the isolation and identification of the first active compound from *E. natalensis* has been described. The MICs of the isolated compound for *M. tuberculosis* and eleven pathogenic bacterial species have been analysed.

**Chapter 7** deals with the antiherpes activity of the acetone and water extracts of *E. natalensis*. Inhibitory activity of the isolated compound diospyrin has been investigated as well.

In **chapter 8** the isolation and identification of the second active compound from *E. natalensis* is described. The MICs of the second isolated compound against *M. tuberculosis* and eleven pathogenic bacterial species are also reported. Synergistic inhibitory actions of the two active compounds isolated from *E. natalensis*, have been evaluated against *M. tuberculosis* and the bacterial species.

In the general discussion and conclusion in **chapter 9** an attempt is made to synthesize a more coherent picture of the results of this study.

Finally **chapter 10** summarises the motives of the entire project, the importance of medicinal plant's folkloric use and entails the recommendations from the findings of this study.

## 1.8 References

- ARRIBAS, G.M.L., PLAZA, C.J., ROSA, M.C. & MOSSO, M.A. 1988. Characterization of *Bacillus cereus* strains isolated from drugs and evaluation of their toxins. *J. Appl. Bacteriol.* 64(3): 257-264.
- BERNARDELLI, A., BASTIDA, R., LOUREIRO, J., MICHELIS, H., ROMANO, M.I., CATALDI, A. & COSTA, E. 1996. Tuberculosis in sea lions and fur seals from the south-western Atlantic coast. *Rev. Sci. Tech. Off. Int. Epiz.* 15(3): 985-1005.
- BOILY, Y. & VAN PUYVELDE, L. 1986. Screening of medicinal plants of Rwanda (Central Africa) for antimicrobial activity. *J. Ethnopharmacol.* 16: 1-16.
- BRYANT, A.T. 1966. Treatment of Diseases. In: Zulu Medicine and Medicine-Men, Ch.9, pp. 44-50. C. Struik, Cape Town.
- CHRETIEN, J. 1990. The cursed duet, tuberculosis and HIV. *Bull. Int. Union. Tuberc.* 65: 25-28.
- COX, P.A. & BALICK, M.J. 1994. The ethnobotanical approach to drug discovery. *Sci. Amer.* 270: 60-65.
- CRAGG, G.M., BOYD, M.R., CARDELLINA, J.H., NEWMAN, D.J., SNADER, K.M. & MCCLOUD, T.G. 1994. Ethnobotany and drug discovery: the experience of the US National Cancer Institute. *Ciba Found Symp.* 185: 178-190.
- CROSS, M.L., SWALE, E., YOUNG, G. & MACKINTOSH, C. 1999. Effect of field capture on the measurement of cellular immune responses in wild ferrets (*Mustela furo*), vectors of bovine tuberculosis in New Zealand. *Vet. Res.* 30(4): 401-410.

- DE KIMPE, N., SCHAMP, N., VAN PUYVELDE, L., DUBE, S., CHAGNON-DUBE, M., BORREMANS, F., ANTEUNIS, M.J.O., DECLERQ, J.P., GERMAIN, J.P. & VAN MEERSCHE, M. 1982. Isolation and structural identification of 8(14), 15-sandaracopimaradiene-7 $\alpha$ , 18-diol from *Iboza riparia*. *J. Org. Chem.* 47: 3628-3630.
- DELAAT, A.N.C. 1979. Gram-Negative facultatively anaerobic rods. In: *Microbiology for the Allied Health Professions*, 2<sup>nd</sup> edn, Ch. 15, pp. 201-207. Henry Kimpton Publishers, London.
- DENNY, G.O. & WILESMITH, J.W. 1999. Bovine tuberculosis in Northern Ireland: a case-control study of herd risk factors. *Vet. Rec.* 144(12): 305-310.
- FADDA, G. & ROE, S.L. 1984. Recovery and susceptibility testing of *Mycobacterium tuberculosis* from extrapulmonary specimens by the BACTEC radiometric method. *J. Clin. Microbiol.* 19: 720-721.
- FANNING, A., EDWARDS, S. & HAUER, G. 1991. *Mycobacterium bovis* infection in humans exposed to elk in Alberta. *Can. Dis. Wkly. Rep.* 17(44): 239-243.
- GIRLING, D.J. 1989. The chemotherapy of tuberculosis. In: *The Biology of the Mycobacteria*, eds. C. Ratledge, J.L. Stanford & Grange, J.M. Vol. 3, pp. 43-47. Academic Press, London.
- GRANGE, J.M. & DAVEY, R.W. 1990. Detection of antituberculosis activity in plant extracts. *J. App. Bacteriol.* 68: 587-591.
- HUTCHINGS, A. 1996. *Zulu Medicinal Plants*. University of Natal Press, Pietermaritzburg.

- IROBI, O.N., MOO-YOUNG, M., ANDERSON W.A., & DARAMOLA, S.O.1994. Antimicrobial activity of bark extracts of *Bridelia ferruginea*. *J. Ethnopharmacol.* 43: 185-190.
- ISRAELSEN, L.D. 1995. Phytomedicines:the greening of modern medicine. *J. Altern. Complement Med.* 1(3): 245-8.
- IWU, M.M. 1994. African medicinal plants in the search for new drugs based on ethnobotanical leads. *Ciba Found Symp.* 185: 116-126.
- JAIN, S.K. 1994. Ethnobotany and research on medicinal plants in India. *Ciba Found Symp.* 185: 153-164.
- JOKLIK, W.K., WILLETT, H. P. & AMOS, D.B. 1968. Mycobacteriaceae. In: Zinsser Microbiology, 17<sup>th</sup> edn, Ch. 34, pp. 651-664. Appleton-Century-Crofts/ New York.
- KEET, D.F., KRIEK, N.P.J., PENRITH, M.L., MICHEL, A. & HUCHZERMEYER, H. 1996. Tuberculosis in buffaloes (*Syncerus caffer*) in the Kruger National Park: Spread of the disease to other species. *Onderstepoort J. Vet. Res.* 63: 239-244.
- KHAN, M.R., MUTASA, S.L., NDAALIO, G. & WEVERS, H. 1978. Antibiotic action and constituents of root bark of *Euclea natalensis* A. DC. *Pakistan J. Sci. Ind. Res.* 21: 197-198.
- KING, S.R. & TEMPESTA, M.S. 1994. From shaman to human clinical trials: the role of industry in ethnobotany, conservation and community reciprocity. *Ciba Found Symp.* 185:197-206.

- KOCHI, A. 1997. Tuberculosis control – is DOTS the health breakthrough of the 1990s? *Wld. Hlth. F.* 18 (3/4): 225.
- KONDO, S., IKEDA, Y., HATTORI, S., HAMADA, M. & TAKEUCHI, T. 1991. Susceptibility of methicillin-resistant *Staphylococcus aureus* to various antibiotics. Classification by aminoglycoside-modifying enzymes and antibiotics active against MRSA. *Jpn. J. Antibiot.* 44(11): 1211-1215.
- KOTT, V., BARBINI, L., CRUANES, M., MUNOZ, J.D., VIVOT, E., CRUANES, J., MARTINO, V., FERRARO, G., CAVALLARO, L. & CAMPOS, R. 1999. Antiviral activity in Argentina medicinal plants. *J. Ethnopharmacol.* 64(1): 79-84.
- LASZLO, A., GILL, P., HANDZEL, V., HODGKIN, M.M. & HELBECQUE, D.M. 1983. Conventional and radiometric drug susceptibility testing of *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* 18: 1335-1339.
- MATTHE, D.S. 1989. Ethnomedical science and African medical practice. *Med. Law.* 7(5):17-21.
- MEYERS, J.D., WADE, J.C. & MITCHELL, C.D. 1982. Multicellular collaborative trial of intravenous acyclovir for treatment of mucocutaneous Herpes simplex virus in the immunocompromised host. *Am. J. Med.* 73: 229-235.
- MIDDLEBROOK, G., REGGIARDS, Z. & TIGERTT, W.D. 1977. Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *Am. Rev. Respir. Dis.* 115: 1067-1069.
- MINOR, R. 1999. Bovine tuberculosis. *Vet. Rec.* 144 (21): 596.

- MURRAY, J., KIELKOWSKI, D. & REID, P. 1996. Occupational -disease trends in Black South- African gold miners-An autopsy-based study. *Amer. J. Resp. Med.* 153(2): 706-710.
- NATIONAL JEWISH MEDICAL AND RESEARCH CENTRE. 1994. Medfacts from the National Jewish Centre for Immunology and Respiratory Medicine. Colorado.
- PADMA, P., PRAMOD, N.P., THYAGARAJAN, S.P. & KHOSA R.L. 1998. Effect of the extract of *Annona muricata* and *Petunia nyctaginiflora* on Herpes simplex virus. *J. Ethnopharmacol.* 61(1): 81-83.
- PALMIERI, G., AMBROSI, G., FERRARO, G., AGRATI, A.M. & PALAZZINI, E. 1987. Clinical and immunological evaluation of oral ribavirin administration in recurrent Herpes simplex infections. *J. Int. Med. Res.* 15: 264-275.
- PENSO, G. 1982. Index Plantarum Medicinalium Totius Mundi Eorumque Synonymorum. Organizzazione Editoriale Medico Farmaceutica, Milan.
- PHILLIPS & DREW. 1992. Global Pharmaceutical Review.
- PHILLIPSON, J.D. & ANDERSON, L.A. 1989. Ethnopharmacology and Western medicine. *J. Ethnopharmacol.* 25: 61-72.
- PUJOL, J. 1990 Natur Africa. The herbalist handbook, pp. 40-57. Jean Pujol Natural Healers Foundation, Durban.
- RAO, G.G. 1998. Risk factors for the spread of antibiotic-resistant bacteria. *Drugs* 55(3): 323-330.
- SALIE, F., EAGLES, P.F. & LENG, H.M. 1996. Preliminary antimicrobial screening of four South African Asteraceae species. *J. Ethnopharmacol.* 52(1): 27-33.



- SCHULTES, R.E. 1994. Amazonian ethnobotany and the search for new drugs. *Ciba Found Symp.* 185: 106-112.
- SIDDIQI, S.H., LIBONATI, J.P. & MIDDLEBROOK, G. 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 13: 908-913.
- SIDDIQI, S.H., HAWKINS, J.E. & LASZLO, A. 1985. Interlaboratory Drug Susceptibility Testing of *Mycobacterium tuberculosis* by a Radiometric Procedure and Two Conventional Methods. *Am. Soc. Microbiol.* 22 (6): 919-923.
- SNEADER, W. 1985. Drug Discovery: The Evolution of Modern Medicines. John Wiley & Sons: Chichester, U.K.
- SNIDER, S.H., LIBONATI, J.P. & MIDDLEBROOK, G. 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 18: 689-696.
- SOUTH AFRICAN TUBERCULOSIS ASSOCIATION. 1998. Tuberculosis. Sunsite Southern Africa.
- STAMFORD, J.L., GRANGE, J.M. & POZNIAK, A. 1991. Is Africa Lost? *Lancet* 2: 557-558.
- STANDER, I. & VAN WYK, C.W. 1991. Toothbrushing with the root of *Euclea natlensis*. *J. Biol. Buccale.* 19: 167-172.
- STANIER, R.Y., ADELBERG, E.A. & INGRAHAM, J.H. 1958. Important Groups of Unicellular Eubacteria. In: General Microbiology, Ch. 18, pp. 407-415. The Macmillan Press, London.

- STEAD, W.W. 1997. The origin and erratic global spread of tuberculosis. How the past explains the present and is the key to the future. *Clin. Chest Med.* 18(1): 65-77.
- SJOGREN, I. & HILLERDAL, O. 1978. Bovine tuberculosis in man-reinfection or endogenous exacerbation. *Scand. J. Respir. Dis.* 59(3): 167-170.
- TAKAHASHI, H. & FOSTER, V. 1983. Detection and Recovery of Mycobacteria by a Radiometric Procedure. *J. Clin. Microbiol.* 17(2): 380-381.
- THEUNIS, F.G., ISABEL, I. & SNYCKERS, F. O. 1992. Folk medicine: a viable starting point for pharmaceutical research. *S. Afr. J. Sci.* 88: 190-192.
- TIMBURY, M.C. 1986. Herpes virus diseases. In: Notes on Medical Virology, 8<sup>th</sup> edn, Ch. 1, pp. 72-80. Churchill Livingstone, Edinburgh and London.
- TWEDDLE, N.E. & LIVINGSTONE, P. 1994. Bovine tuberculosis control and eradication programs in Australia and New Zealand. *Vet. Microbiol.* 40: 23-39.
- ULUBELEN, A.E., TUZLACI, E. & JOHANSON, C. 1988. Diterpenoids from the roots of *Salvia hypergeia*. *J. Nat. Prod.* 51 (6): 1178-83.
- VAN PUYVELDE, L., DE KIMPE, N., COSTA, J., MUNYJABO, V., NYIRANKULIZA, S., HAKIZAMUNGU, E. & SCHAMP, N. 1989. Isolation of flavonoids and a chalcone from *Helichrysum odoratissimum* and a synthesis of helichrysetin. *J. Nat. Prod.* 52(3): 629-633.
- VAN PUYVELDE, L., NTAWUKILIYAYO, J.D. & PORTAELS, F. 1994. *In vitro* inhibition of mycobacteria by Rwandese medicinal plants. *Phytother. Res.* 8: 65-69.

- VAN WYK, B. & VAN WYK, P. 1997. Field guide to trees of Southern Africa, pp. 184-185. McKenzie Street, Cape Town.
- VAN WYK, B.E., VAN OUDTSHOORN, B. & GERICKE, N. 1997. Medicinal Plants of South Africa. Briza Publications, Pretoria.
- VLIETINCK, A.J., VAN HOOF, L., TOTTE, J., LASURE, A., VANDEN BERGHE, D., RWANGABO, P.C. & MVUKIYUMWAMI, J. 1995. Screening of a hundred Rwandese medicinal plants for anti-microbial and antiviral properties. *J. Ethnopharmacol.* 46: 31-47.
- WANG, Z., WANG, G., XU, H. & WANG, P. 1996. Anti-herpes virus action of ethanol-extract from the root and rhizome of *Rheum officinale* Baill. *Chung. Kuo. Chung Yao Tsa Chih.* 21(6): 364-366.
- WATT, J.M. & BREYER-BRANDWIJK, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2<sup>nd</sup> edn. E & S Livingstone, Edinburgh.
- WHO/IUATLD working group. 1989. Tuberculosis and AIDS. *Bull. Int. Union Tuberc.* 64: 8-11.
- WHO/TB/98.258. 1998. Laboratory services in tuberculosis control Part II Microscopy, Geneva, Switzerland.
- WILKINSON, D., DAVIES, G.R., CONNOLLY, C. 1996. Directly Observed Therapy for Tuberculosis in Rural South-Africa, 1991 through 1994. *Am. J. Pub. Hlth.* 86(8): 1094-1097.
- WORLD HEALTH ORGANIZATION., 1997. Anti-tuberculosis Drug Resistance in the World. The WHO/IUATLD Project on Anti-tuberculosis Drug Resistance Surveillance. Geneva: World Health Organization Global Tuberculosis Programme.



ZHOU, G. C., KOMATSU, K., YAMAJI, S., NAMBA, T. 1994. Pharmacognostical studies on the traditional drugs used by chinese minority race, called "Yi" (I) on "A-ji-ba-mo" derived from *Dipsacus* plants. *Natur. Med.* 48 (2): 131-140.

ZIEGER, U., PANDEY, G.S., KRIEK, N.P. & CAULDWELL, A.E. 1998. Tuberculosis in Kafue Lechwe (*Kobus leche kafuensis*) and in a bushbuck (*Tragelaphus scriptus*) on a game ranch in central province, Zambia. *J. S. Afr. Vet. Assoc.* 69(3): 98-101.

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# CHAPTER 2

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## **SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* USING THE AGAR PLATE METHOD**

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## Chapter 2

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# SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* USING THE AGAR PLATE METHOD

### Abstract

Twenty South African medicinal plants used to treat pulmonary diseases were screened for activity against *Mycobacterium tuberculosis* by the conventional agar plate method. Acetone and water extracts of the plants were investigated for their inhibitory action against H37Rv, a drug-sensitive strain of *M. tuberculosis*. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml. Six water extracts showed activity at concentrations ranging from 0.5 to 5.0 mg/ml. The activity against *M. tuberculosis* was the highest in water extracts of *Euclea natalensis* (0.5 mg/ml) and *Helichrysum melanacme* (1.0 mg/ml). In this study we were able to establish evidence of the inhibitory nature of plant extracts and determine the concentration required to inhibit *M. tuberculosis*. Susceptibility testing of *M. tuberculosis* by the agar plate method is reliable, economical, reproducible and are readily applicable to large scale screening of plants for their inhibitory activity against *M. tuberculosis*.



## 2.1 Introduction

Susceptibility testing of *Mycobacterium tuberculosis* by the agar plate method is one of the most commonly used testing procedures to evaluate drugs/plant extracts for antituberculosis activity. The agar plate method is a modified version of the proportion method of Canetti and Co-workers (1963). The modification, including the preparation and concentration of drugs, was described by Vestal (1975). The principle of the proportion method is based on determining the proportion of resistant tubercle bacilli present in the bacterial population (Canetti *et al.* 1963).

Every culture of tubercle bacilli contains some mutants resistant to antibacterial drugs. The difference between a resistant strain and a susceptible strain is that the proportion of resistant bacteria among the total number of bacterial strain is much higher in a resistant strain than in a susceptible one (Canetti *et al.* 1963). When 1% or more of microorganisms tested are resistant to the drug, the population is considered resistant to that chemotherapeutic agent. The inoculum size of the mycobacteria in the testing procedure is such that it is certain to show 1% of resistant microorganisms to the drugs (Middlebrook & Cohn 1958). Resistance on the part of the microorganisms is certainly significant when at least 1% of the total bacterial population develops at the so-called critical concentrations, that is, the weakest concentration at which susceptible bacilli are unable to grow in the presence of the drug. Any smaller proportion of resistant microorganisms have no clinical significance. The critical concentration of antituberculosis drugs was originally developed for testing in Lowenstein-Jensen egg medium, later equivalents were found for 7H10 agar, 7H11 agar and 7H12 broth media (Lee & Heifets 1987).

The use of some medicinal plants such as, *Acacia nilotica*, *Cassine papillosa*, *Chenopodium ambrosioides*, *Combretum molle*, *Croton sylvaticus*, *Cryptocarya latifolia*, *Ekebergia capensis*, *Euclea natalensis*, *Protasparagus africanus*, *Rapanea melanophloeos*, etc. by South Africans in treating TB related symptoms such as fever,

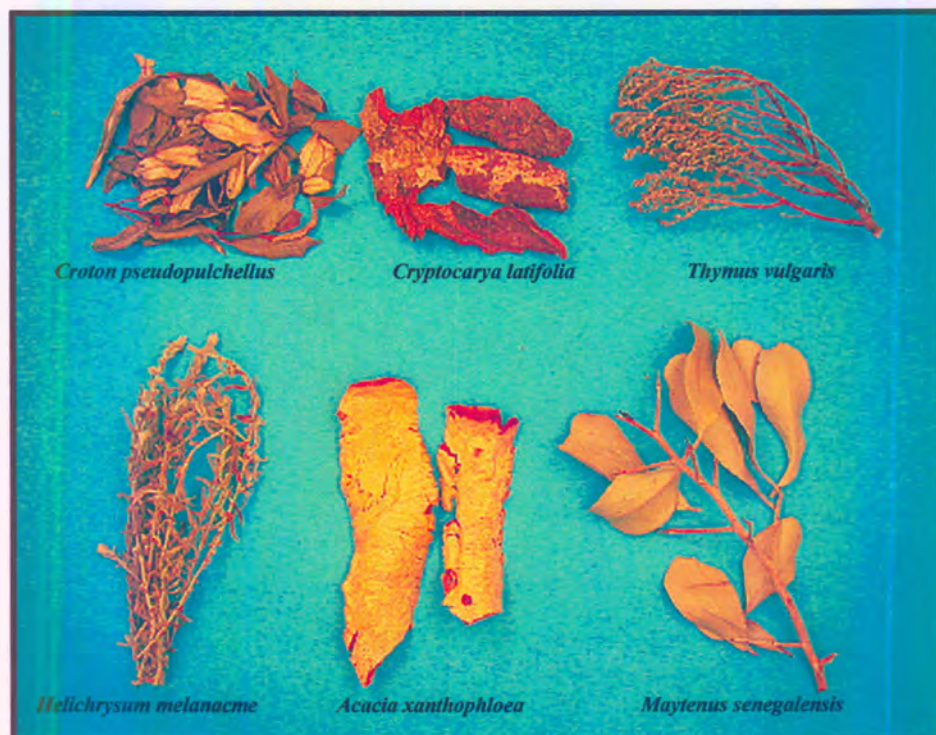


cough, chest disease, night sweats etc. have been reported (Watt & Breyer-Brandwijk 1962; Pujol 1990; Hutchings 1996). A scientific basis for the utilisation of such plants in folk medicine is required because one of the prerequisites for the success of primary health care is the availability and use of suitable drugs. Plants that are used in traditional medicine have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. A combination of information indicating that a specific plant has been used in a local health care system for centuries, together with efficacy and inhibitory activity data, can help scientists to establish whether it should or should not be considered acceptable for medicinal use (Akerele 1984). It was therefore decided to investigate the acetone and water extracts of 20 ethnobotanically selected South African medicinal plants, *in vitro* against *M. tuberculosis* by the conventional agar plate method.

## **2.1 Materials and methods**

### **2.1.1 Plant material**

All plants were collected from the south and central parts of South Africa (Lady Grey, Aliwal North, Elliot, Barkley East, Durban, Umlazi). Different parts of the plants for example, stem, root, bark, leaves etc. were collected as used by our indigenous people to treat TB symptoms such as, fever, blood in the sputum, cough etc. (Figure 2.1). The selection of plants was based on the actual experience of traditional healers as well as information culled from published sources. The plants were identified at the HGWJ Schweickerdt herbarium of the University of Pretoria and also at the herbarium of the National Botanical Institute, Pretoria. Herbarium voucher specimens are preserved in the herbarium of the University of Pretoria (Table 2.1)



**Figure 2.1** Examples of ethnobotanically selected plant parts

### 2.2.2 Preparation of plant extracts

Acetone and water extracts of each plant sample were prepared. About 40 g of plant material was homogenized and three times extracted with acetone. The extract was filtered and concentrated to dryness at reduced pressure. The acetonc residue was later dissolved in dimethyl sulphoxide (DMSO) to obtain a concentration of 500 mg/ml because of the toxicity of acetone towards bacteria.

Water extract was prepared by boiling about 20 g of non-homogenised plant material in 500 ml of distilled water for 30 min under reflux. The extract was filtered and concentrated to dryness at reduced pressure. The residue was later dissolved in distilled water to a concentration of 500 mg/ml.

### 2.2.3 Determination of antimycobacterial activity

#### 2.2.3.1 Preparation of bacterial media

1 litre of Middlebrook 7H11 agar (Difco laboratories) containing 0.5% glycerol, was prepared and sterilized by autoclaving at 121<sup>0</sup>C for 15 min. After cooling to 55<sup>0</sup>C, 100 ml of Middlebrook OADC enrichment fluid (Difco) was added to the medium.

The acetone and water plant extracts (500 mg/ml) were sterilised by filtering through 0.22µm syringe fitted filters and then incorporated in the medium before solidification, to obtain final concentrations of 5.0, 1.0 and 0.5 mg/ml. Control experiments showed that the final amount of DMSO (1%) in the media had no effect on the growth of *M. tuberculosis*. The mixture (10 ml) of plant extract and medium was poured in glass bottles and solidified in slants. All tests were done in triplicate.

#### 2.2.3.2 Interpretation of results

Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. These cultures were routinely tested for susceptibility to the primary drugs streptomycin (SM), isoniazid (INH), ethambutol (EB) and rifampin (RIF). A sensitive strain of *M. tuberculosis*, H37Rv reference strain (a strain frequently used for drug susceptibility testing), was used in the screening procedure.

Standard inoculum was prepared for the sensitive strain in Middlebrook-Dubos 7H9 broth containing 0.5% Tween 80 to obtain a concentration of 1 mg/ml (wet mass). The bacterial cultures which were used to prepare the standard inoculum, were maintained on Lowenstein-Jensen medium (Figure 2.2). A representative amount of growth was picked from the cultures by using a sterile applicator stick. This sample was transferred to a sterile screw-capped tube containing six to eight glass beads and 3-4 ml of Middlebrook-Dubos 7H9 broth.





**Figure 2.2** Colonies of *M. tuberculosis* on Lowenstein-Jensen medium

A homogenous suspension was obtained by placing the tube on a Vortex mixer for 5 min. After the large particles had settled, more broth was added and adjusted to McFarland no.1 turbidity standard, approximately. The H37Rv sensitive strain suspension was divided into two portions. One portion was saved for the rapid radiometric susceptibility test (chapter 3) and the other was used for the agar plate susceptibility testing.

The suspension was diluted to  $1 \times 10^{-2}$  mg/ml and  $1 \times 10^{-4}$  mg/ml. To each bottle containing plant extract, 0.2 ml of the  $1 \times 10^{-2}$  mg/ml of bacteria was added. For the control tubes (medium + 1% DMSO), 0.2 ml of the two dilutions ( $1 \times 10^{-2}$  and  $1 \times 10^{-4}$  mg/ml) of the inoculum were used. The antimicrobial activity was evaluated after 6 weeks of incubation at  $37^{\circ}\text{C}$ . The number of colonies growing on the medium with plant extracts,  $N^{-2}$  for the dilution  $1 \times 10^{-2}$  mg/ml, was compared with the growth on the control series,  $NO^{-2}$  for  $1 \times 10^{-2}$  mg/ml and  $NO^{-4}$  for  $1 \times 10^{-4}$  mg/ml (Canetti *et al.* 1963).



The following criteria were used for the interpretation of the results:

- $N^{-2} \geq NO^{-2}$  : the strain is considered as resistant;  
 $NO^{-4} \leq N^{-2} \leq NO^{-2}$  : the strain is considered as partially susceptible;  
 $N^{-2} \leq NO^{-4}$  : the strain is considered as sensitive (< 1% growth).

All procedures involving transfer of cultures were carried out in a biological safety cabinet.

### **2.3 Results and Discussion**

Good growth of *M. tuberculosis* (H37Rv) was evident in the bottles containing only Middlebrook medium, within 5 to 6 weeks. All the results were recorded after 6 weeks. Fourteen acetone and 6 water extracts out of 20 inhibited growth of the organism at a concentration ranging from 0.5 to 5.0 mg/ml (Table 2.1). Acetone extracts of *Combretum molle*, *Croton pseudopulchellus*, *Ekebergia capensis* and *Helichrysum odoratissimum* inhibited growth of the H37Rv strain at a concentration of 0.5 mg/ml. It has been reported that the roots of *E. capensis* growing in the KwaZulu-Natal province of South Africa is used in a decoction to treat gastritis, hyperacidity and coughing (Pujol 1990). *C. molle* and *H. odoratissimum* are widely used in the Eastern cape region of South Africa against fever, coughs and cold (Hutchings & Johnson 1986; Pooley 1993). Bark of *Cassine papillosa* is used to clean the digestive tract and for chest congestion by the Zulus, a tribe of the KwaZulu-Natal province of South Africa (Pujol 1990). Ground bark of *Cryptocarya latifolia* is mixed with crocodile fat to treat chest ailments (Gerstner 1941). In the present study it was found that *Cryptocarya latifolia* and *Cassine papillosa* showed activity at 0.5 and 1.0 mg/ml respectively against H37Rv strain. Only 6 water extracts showed activity at concentrations ranging from 0.5 to 5.0 mg/ml (Table 2.1). The activity against *M. tuberculosis* was the highest in extracts of *Euclea natalensis* (0.5 mg/ml) and *Helichrysum melanacme* (1.0 mg/ml). Inhibitory effect of *Croton pseudopulchellus* on the growth of *M. tuberculosis* is shown in Figure 2.3.





**Table 2.1** Antimycobacterial activity of plant extracts on the H37Rv strain of *Mycobacterium tuberculosis* as determined by the agar plate method

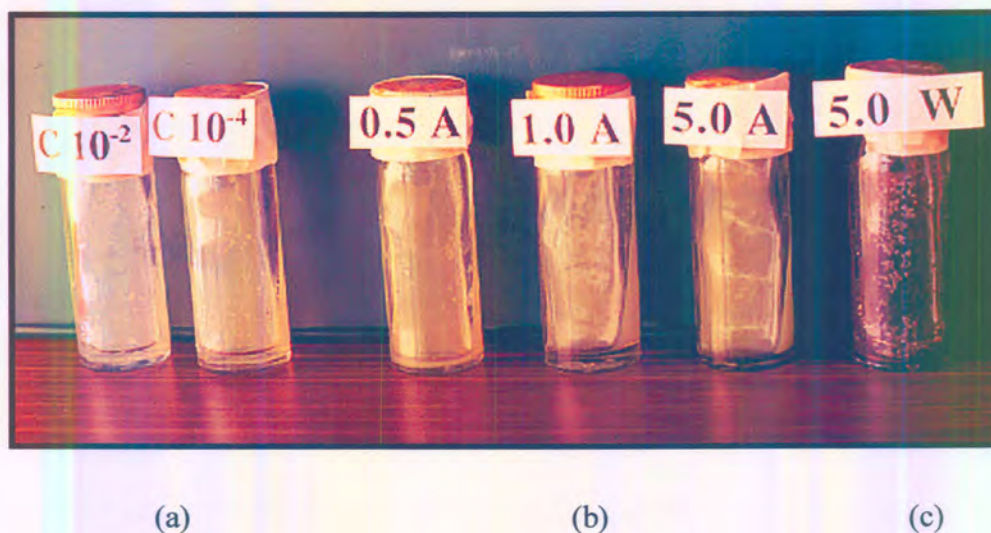
Plant species	Plant part used <sup>a</sup>	MIC <sup>b</sup> of plant extracts (mg/ml)		Voucher specimen no.
		Acetone	Water	
<i>Acacia xanthophloea</i> Benth	B	0.5	na <sup>c</sup>	E.P. 96
<i>Cassine papillosa</i> (Hochst.) Kuntze	B	1.0	na	R.A.L. 595
<i>Chenopodium ambrosioides</i> L.	A	0.5	na	N.L. 9
<i>Combretum molle</i> R. Br. Ex G. Don	B	0.5 <sup>d</sup>	na	E.P.81
<i>Croton pseudopulchellus</i> Pax	A	0.5	na	N.L.30
<i>Cryptocarya latifolia</i> Sond.	B	0.5 <sup>d</sup>	5.0	A.E.V.W.1031
<i>Ekebergia capensis</i> Sparrm	B	0.5	na	E.A.P.60
<i>Euclea natalensis</i> A. DC.	R	0.5	0.5	N.L. 22
<i>Gunnera perpensa</i> L.	R	na	na	N.L. 24
<i>Helichrysum melanacme</i> DC.	W	0.5	1.0	N.L. 16
<i>Helichrysum odoratissimum</i> (L.) Sweet	W	0.5	na	N.L. 18
<i>Maytenus senegalensis</i> (Lam.) Excell	A	0.5 <sup>d</sup>	na	N.L. 31
	R	na	na	
<i>Nidorella anomala</i> Steetz.	W	0.5	5.0	N.L. 28
<i>Nidorella auriculata</i> DC.	W	0.5	na	N.L. 29
<i>Polygala myrtifolia</i> L.	A	0.5	na	N.L. 20
<i>Rapanea melanophloeos</i> (L.) Mez	B	5.0 <sup>d</sup>	na	N.L. 32
<i>Rumex crispus</i> L.	A	na	na	Afol. 14
<i>Senecio serratuloides</i> DC. Var. <i>serratuloides</i>	A	na	5.0	N.L. 8
<i>Tetradenia riparia</i> (Hochst.) Codd	R	na	na	N.L. 23
<i>Thymus vulgaris</i> L.	A	0.5	5.0	N.L. 5

<sup>a</sup>plant part used: W, whole plant; A, aerial parts; R, root; B, bark.

<sup>b</sup>minimal inhibitory concentration.

<sup>c</sup>not active at the highest concentration (5.0 mg/ml) tested.

<sup>d</sup>partially susceptible.



**Figure 2.3** Inhibitory effect of acetone and water extracts of *Croton pseudopulchellus* on the growth of *M. tuberculosis*. (a) Control - colonies of *M. tuberculosis*. (b) Acetone extract (0.5, 1.0 and 5.0 mg/ml) - total inhibition of *M. tuberculosis*. (c) Water extract (5.0 mg/ml) - colonies of *M. tuberculosis*.

The results of the present study show a good correlation between traditional medicinal use of the plants and antibiotic activity as has previously been reported in other ethnopharmacological studies as well. In a British Columbian study 95% of the plants designated as potentially antibiotic, based on ethnobotanical data, exhibited antibiotic activity (McCutcheon *et al.* 1992). Similarly in Rwanda 50% of the plants used in native medicine to treat infectious diseases had an antimicrobial activity (Boily & Van Puyvelde 1986).

The MIC values for the extracts of *Rapanea melanophloeos* and *Senecio serratuloides* were found to be 5.0 mg/ml. These extracts do not have a good potency level based on their high MIC values, implying the active compounds would probably not be pharmaceutically useful (Rios *et al.* 1988). Reasons for the relatively high MIC

values could be that the extracts tested are still in an impure form, or that the active compound(s) are present in very low concentrations. Some of the plant species for example, *Gunnera perpensa*, *Rumex crispus*, and *Tetradenia riparia* did not show activity in this screening test even at a concentration of 5.0 mg/ml although these plants are also reported as being used to treat symptoms similar to that caused by TB (Watt & Breyer-Brandjwijk 1962; Hutchings 1996). It is possible that these plants are effective against colds, coughs or chest pains caused by diseases other than TB. Many traditional healers prefer to use a combination of several plants to treat a disease. This combination could have an antimicrobial activity, where separate constituents have not.

## 2.4 Conclusion

In this study we were able to analyse the antimycobacterial properties of South African medicinal plants and determine the concentrations required to inhibit *M. tuberculosis in vitro*. Fourteen of the 20 acetone extracts showed inhibitory activity at a concentration of 0.5 mg/ml. Susceptibility testing of *M. tuberculosis* by the agar plate method is reliable, economical, reproducible and are readily applicable to large-scale screening of plants for their inhibitory activity against *M. tuberculosis*.

## 2.5 References

- AKERELE, O. 1984. WHO'S traditional medicine programme: progress and perspective. *WHO Chronicle* 38(2): 76-81.
- BOILY, Y. & VAN PUYVELDE, L. 1986. Screening of medicinal plants of Rwanda (central Africa) for antimicrobial activity. *J. Ethnopharmacol.* 16: 1-13.
- CANETTI, G., FROMAN, J., GROSSET, J., HAUDEROY, P., LANGEROVA, H., MAHLER, G., NEISSNER, G., MITCHISON, A. & SULA, L. 1963. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull. WHO.* 29: 565-578.
- GERSTNER, J. 1941. A preliminary checklist of Zulu names of plants with short notes. *Bantu Studies* 13 (1): 49-64.
- HUTCHINGS, A. & JOHNSON, C.T. 1986. Glimpses of a Xhosa herbal. *Veld and Flora* 72: 59-62.
- HUTCHINGS, A. 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg.
- LEE, C.N. & HEIFETS, L.B. 1987. Determination of minimal inhibitory concentrations of antituberculosis drugs by radiometric and conventional methods. *Am. Rev. Respir. Dis.* 136: 349-352.
- MCCUTCHEON, A.R., ELLIS, S.M., HANCOCK, R.E.W. & TOWERS, G. H.N. 1992. Antibiotic screening of medicinal plants of the British Columbian native peoples. *J. Ethnopharmacol.* 44: 157-169.



- MIDDLEBROOK, G. & COHN, M.L. 1958. Bacteriology of tuberculosis: Laboratory methods. *Amer. J. Pub. Hlth.* 48: 844-853.
- POOLEY, E. 1993. The Complete Field Guide to Trees of Natal, Zululand and Transkei, pp. 20-25. Natal Flora Publications Trust, Natal Herbarium, Durban.
- PUJOL, J. 1990. Natur Africa. The herbalist handbook, pp. 40-57. Jean Pujol Natural Healers Foundation, Durban.
- RIOS, J.L., RECIO, M.C. & VILLAR, A. 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. *J. Ethnopharmacol* 23: 127-149.
- VESTAL, A.L. 1975. Procedures for the isolation and identification of mycobacteria. U.S. Department of Health, Education, and Welfare publication no. (CDC) 76-8230. Centres for Disease Control, Atlanta.
- WATT, J.M. & BREYER-BRANDWIJK, K.M. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2nd edn. E & S Livingstone, Edinburgh.

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# CHAPTER 3

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## **SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM* *TUBERCULOSIS* USING THE BACTEC RADIOMETRIC ASSAY**

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## Chapter 3

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# SUSCEPTIBILITY TESTING OF *MYCOBACTERIUM TUBERCULOSIS* USING THE BACTEC RADIOMETRIC ASSAY

### Abstract

Fourteen South African medicinal plants that showed good activity against *Mycobacterium tuberculosis*, using the conventional agar method were screened to confirm their inhibitory activity employing the BACTEC radiometric method. Acetone plant extracts were screened against the H37Rv strain as well as a strain resistant to the drugs isoniazid and rifampin. The minimal inhibitory concentration (MIC) of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala*, and *Polygala myrtifolia* was found to be 0.1 mg/ml against the H37Rv strain. Extracts of *Chenopodium ambrosioides*, *E. capensis*, *E. natalensis*, *Helichrysum melanacme*, *Nidorella anomala* and *Polygala myrtifolia* were active against resistant strain at 0.1 mg/ml. Eight plants showed activity against both the strains at a concentration of 1.0 mg/ml.



### 3.1 Introduction

The introduction of radiometric techniques in the field of mycobacteriology is a relatively recent development. Radiometric respiratory with the BACTEC TB-460 system is a well-documented technique for susceptibility testing of *Mycobacterium tuberculosis*. An automated radiometric detection of mycobacterium growth has opened new opportunities for determining the susceptibility of mycobacteria quantitatively, on the basis of the minimal inhibitory concentration (MIC) of drugs. The BACTEC procedure for drug susceptibility testing for mycobacteria is based on the same basic principle employed in the conventional method, however there are some differences. In the BACTEC radiometric assay liquid medium is used, the growth of *M. tuberculosis* is monitored radiometrically and the results are available within 5 to 6 days. On the other hand, in conventional method the growth of *M. tuberculosis* is monitored on solid agar medium, mycobacterial colonies are counted only after about 3 weeks and the results are available within 3 to 4 weeks. Several published studies have reported that results obtained by the BACTEC method compared well with the conventional method (employing 7H10/ 7H11 media). The accuracy and reproducibility of the BACTEC method has also been evaluated with excellent results (Siddiqi *et al.* 1981; Snider *et al.* 1981) The drugs or the plant extracts are incorporated in a 7H12 Middlebrook TB medium, and the critical proportion of resistance of *M. tuberculosis* is evaluated at the 1% level (Middlebrook *et al.* 1977).

Given the activity of 14 acetone plant extracts at 0.5 mg/ml against the H37Rv strain by the agar plate method, it was decided to compare these results with the radiometric method and examine the activity of these plant extracts on a drug-resistant strain of *M. tuberculosis* as well. The value of such comparisons in evaluating the usefulness of new techniques is also discussed.

## 3.2 Materials and methods

### 3.2.1 Plant material

Acetone extracts of fourteen plants which showed good activity against *M. tuberculosis* using conventional agar plate method were prepared as mentioned in chapter 2 (section 2.2.2). The acetonic residue was later dissolved in DMSO to obtain a concentration of 500 mg/ml because of the toxicity of acetone towards *M. tuberculosis*.

### 3.2.2 Determination of antimycobacterial activity

The 14 plant extracts were tested against a strain (MRC strain no. CCKO28469V), resistant to two drugs, isoniazid (INH) and rifampin (RIF) and the previously studied sensitive H37Rv strain. Bacterial cultures utilised in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria.

The 7H12 Middlebrook TB medium (Middlebrook *et al.* 1977) which is an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and  $^{14}\text{C}$ -labelled substrate (palmitic acid) as a source of carbon, was used for these studies. Growth of the organism leads to the consumption of the substrate, with subsequent release of  $^{14}\text{CO}_2$  into the atmosphere above the medium in the sealed vial, and the BACTEC TB-460 instrument (Johnston Laboratories, Towson, MD) detects the amount of  $^{14}\text{CO}_2$  and records it as a growth index (GI) on a scale of 0 to 999 (Figure 3.1).



**Figure 3.1** BACTEC TB-460 instrument

In preparing sensitive and resistant strains of *M. tuberculosis* for MIC determination, a vial containing 7H12 Middlebrook TB medium was inoculated with homogenized cultures prepared as described in chapter 2 (section 2.2.3.1) in a special diluting fluid, Middlebrook-Dubos 7H9 broth having the no.1 McFarland standard optical density. When growth in this vial reached a GI reading of 400 to 500, the 7H12 broth culture was used undiluted to inoculate a set of vials, 0.1 ml per vial, yielding  $1 \times 10^4$  to  $1 \times 10^5$  colony forming units/ml (CFU/ml) (Heifets *et al.* 1985).



Plant extracts were analysed for activity at concentrations of 1.0, 0.5 and 0.1 mg/ml. Extracts were added into the vials to obtain final concentrations of 1.0, 0.5 and 0.1 mg/ml together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. Each vial of PANTA is formulated with polymyxin, amphotericin, nalidixic acid, trimethoprim and azlocillin. PANTA supplement was added to suppress the rapidly growing contaminants that might otherwise overgrow the slowly growing mycobacteria. Two plant extract-free vials were used as controls: 1 vial was inoculated in the same way as the vials containing plant extracts, and the other was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population ( $1 \times 10^2$  to  $1 \times 10^3$  CFU/ml) found in the extract-containing vials (Figure 3.2). All tests were done in triplicate.



(a)

(b)

**Figure 3.2** BACTEC vials used for susceptibility testing by the radiometric method:  
(a) Control (b) Vials with plant extracts.

All procedures involving transfer of cultures were carried out in a biological safety cabinet and the bottle tops were wiped with gauze pads soaked with 5% phenol before removal from the hood. An ultraviolet light located under the hood of the BACTEC instrument could be turned on in case of an accident during operation. In addition, a constant-volume air pump exhausted the chamber through absolute filters at a flow rate of 1 ft<sup>3</sup>/min to protect the environment from aerosols that might be produced during collection of the gas sample from the inoculated bottles.

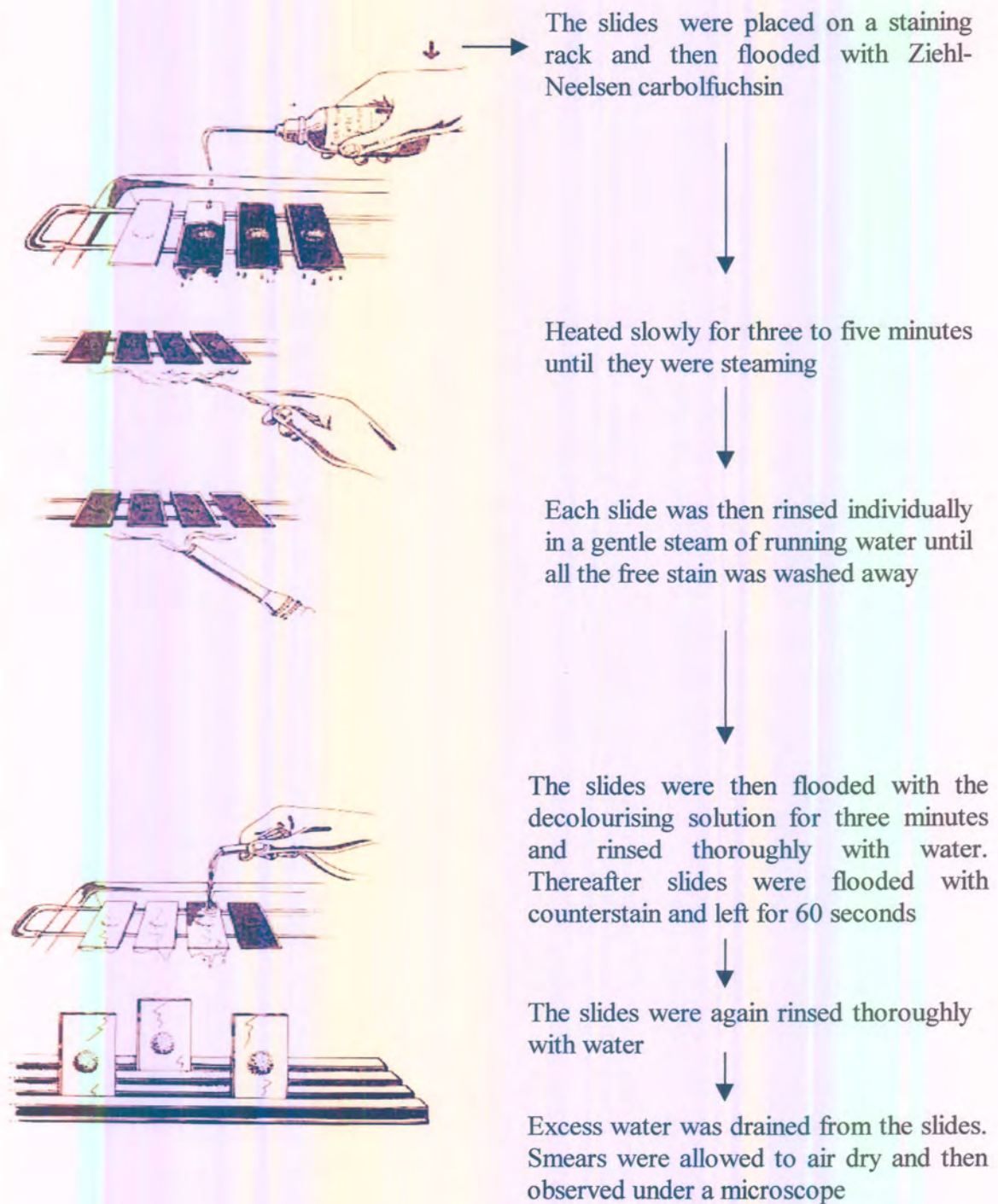
The principles of the radiometric proportion method (Siddiqi *et al.* 1981) state that the concentration of the plant extract that produce a daily GI increase and final GI reading lower than in the 1:100 control can be considered the concentration inhibiting more than 99% of the bacterial population. The MIC was defined as the lowest concentration of drug that inhibited more than 99% of the bacterial population.

Inoculated bottles were incubated at 38<sup>0</sup>C. Each bottle was assayed everyday at about the same hour until cumulative results were interpretable. A BACTEC instrument was used to measure the <sup>14</sup>CO<sub>2</sub> produced from radiolabeled palmitate contained in culture medium, as described previously. The difference in the GI values of the last two days is designated as ΔGI. The GI reading of the vials containing the plant extract was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing 100 times lower dilution of the inoculum, than the vials with plant extract, reached a GI of 30 or more. If the ΔGI value of the vial containing the plant extract was less than the control, the population was reported to be susceptible to the extract. The test was read for 1 or 2 additional days if the ΔGI of the control vial was close to the ΔGI of the extract-containing vial. In such cases, depending upon the increasing or decreasing pattern of the GI reading, the culture was reported as borderline susceptible (0.8 to 1% resistant population).

Whenever results suggested contamination (e.g., large, rapid increases in GI), bottles were inspected grossly and the organisms were stained by Ziehl-Neelsen stain to determine whether the visible microbial growth was mycobacterial (Kleeberg *et al.* 1980; WHO/TB/98.258) (Figure 3.3).

With this stain, the bacilli appear as brilliantly stained red rods against a deep sky-blue background (Figure 1.1). Organisms often have a beaded appearance because of their polyphosphate content and unstained vacuoles (Joklik *et al.* 1968).





**Figure 3.3** Ziehl-Neelsen staining ( Kleeberg *et al.* 1980; WHO/TB/98.258)

The basic reagents of Ziehl-Neelsen stain, and the staining procedures are as follows:

- **Ziehl-Neelsen carbolfuchin**

**Fuchin**

Basic fuchin 3.0 g

95% ethanol 100 ml

Basic fuchin was dissolved in ethanol .....Solution 1

**Phenol**

Phenol crystals 5.0 g

Distilled water 100 ml

Phenol crystals were dissolved in distilled water.....Solution 2

*Working solution*

10 ml of solution 1 was combined with 90 ml of solution 2.

- **Decolourising agent: 3% acid-alcohol**

Concentrated hydrochloric acid 3 ml

95% ethanol 97 ml

Concentrated hydrochloric acid was carefully added to 95% ethanol.

- **Counterstain: Methylene blue**

Methylene blue chloride 0.3 g

Distilled water 100 ml

Methylene blue chloride was dissolved in distilled water.



## 3.3 Results and Discussion

### 3.3.1 Inhibitory activity of plant extracts

Results were interpreted on day 6 or 7 when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 3.1).

Of the 14 plant extracts, 12 were observed to be active on the H37Rv strain at a concentration of 0.5 mg/ml. Our laboratory evaluation of *Croton pseudopulchellus*, *Ekebergia capensis*, *Euclea natalensis*, *Nidorella anomala*, and *Polygala myrtifolia* showed inhibition of growth of the organism at a concentration of 0.1 mg/ml which verified the ethnobotanical reports of these plants. In East Africa, leaf decoctions of *Croton sp.* are used to cure TB and various respiratory ailments (Kokwaro 1976). Green leaf tinctures of *Chenopodium ambrosioides* and one of the *Polygala sp.* are used as cough suppressants by the Xhosa, a tribe of the Eastern Cape region of South Africa (Watt and Breyer-Brandwijk 1962) and for chest pain in Zimbabwe (Gelfand *et al.* 1985). Decoctions made from the chopped bark of *E. capensis* and from the roots of *E. natalensis* are taken as emetics for heart burn, respiratory chest problems and coughs (Bryant 1966).

The drug resistant strain (CCKO28469V) was inhibited by all 14 plant extracts at a concentration of 1.0 mg/ml. The MIC of a few plants, such as, *C. ambrosioides*, *E. capensis*, *E. natalensis*, *Helichrysum melanacme*, *N. anomala* and *P. myrtifolia*, was found to be 0.1 mg/ml. In Zimbabwe, roots and leaves of *Maytenus senegalensis* are used for various respiratory ailments including pneumonia and TB (Gelfand *et al.* 1985). In our investigation it was found that the extract of *M. senegalensis* was active at 1.0 mg/ml only against the resistant strain of *M. tuberculosis*.

**Table 3.1** Effect of plant extracts on the growth of the sensitive strain (H37Rv) and resistant strain (CCKO28469V) of *Mycobacterium tuberculosis* as determined by the radiometric method.  $\Delta GI^a$  values of the control vials were  $29 \pm 4.04$  and  $24 \pm 4.04$  for the sensitive and resistant strains respectively

Plant species	Sensitive strain		Resistant strain	
	MIC <sup>a</sup> (mg/ml)	$\Delta GI^b$ values of plant extracts (mg/ml)	MIC (mg/ml)	$\Delta GI$ values of plant extracts (mg/ml)
<i>Acacia xanthophloea</i> Benth	0.5	$4 \pm 2.1$ (S <sup>c</sup> )	0.5	$-1 \pm 1.0$ (S)
<i>Chenopodium ambrosioides</i> L.	0.5	$2 \pm 0.5$ (S)	0.1	$2 \pm 2.1$ (S)
<i>Combretum molle</i> R. Br. Ex G. Don	1.0	$22 \pm 2.1$ (S)	1.0	$0 \pm 1.0$ (S)
<i>Croton pseudopulchellus</i> Pax	0.1	$2 \pm 0.5$ (S)	0.5	$1 \pm 1.1$ (S)
<i>Cryptocarya latifolia</i> Sond.	0.5	$20 \pm 2.4$ (S)	1.0	$7 \pm 1.7$ (S)
<i>Ekebergia capensis</i> Sparrm	0.1	$3 \pm 1.4$ (S)	0.1	$2 \pm 1.0$ (S)
<i>Euclea natalensis</i> A. DC.	0.1	$-1 \pm 1.0$ (S)	0.1	$2 \pm 1.0$ (S)
<i>Helichrysum melanacme</i> DC.	0.5	$-1 \pm 1.1$ (S)	0.1	$-6 \pm 2.5$ (S)
<i>Helichrysum odoratissimum</i> (L.) Sweet	0.5	$0.1 \pm 0.5$ (S)	0.5	$0 \pm 1.7$ (S)
<i>Maytenus senegalensis</i> (Lam.) Excell	na <sup>e</sup>	$76 \pm 10.5$ (R <sup>d</sup> )	1.0	$11 \pm 6.0$ (S)
<i>Nidorella anomala</i> Steetz.	0.1	$-2 \pm 2.0$ (S)	0.1	$5 \pm 2.0$ (S)
<i>Nidorella auriculata</i> DC.	0.5	$-2 \pm 0.5$ (S)	0.5	$0 \pm 0.5$ (S)
<i>Polygala myrtifolia</i> L.	0.1	$-2 \pm 0.5$ (S)	0.1	$0 \pm 1.0$ (S)
<i>Thymus vulgaris</i> L.	0.5	$4 \pm 3.4$ (S)	0.5	$3 \pm 3.0$ (S)

<sup>a</sup>minimal inhibitory concentration.

<sup>b</sup> $\Delta GI$  values are means  $\pm$  standard deviation.

<sup>c</sup>susceptible.

<sup>d</sup>resistant.

<sup>e</sup>not active at the highest concentration (1.0 mg/ml) tested.

### 3.3.2 Comparison of the two susceptibility testing methods

The results obtained from the radiometric method were satisfactory and comparable with that obtained from the agar plate method. Good correlation between susceptibility test results of the radiometric assay and conventional methods for *M. tuberculosis* has been reported by other investigators. The first evaluation of rapid radiometric drug susceptibility testing of *M. tuberculosis* was reported by Snider *et al.* in 1981. According to them the overall agreement of radiometric results of susceptibility testing of *M. tuberculosis* with those obtained by a conventional method was 98% with specificity and sensitivity (Snider *et al.* 1981). Susceptibility testing of *M. tuberculosis* strains, resistant to INH, RIF and ethambutol was done by Laszlo (1983) using the two methods. The statistical analyses of the radiometric versus conventional methodology showed no significant differences between the two methods. Another report states that in comparing the susceptibility results of the two methods, there were 95% agreement with strains resistant to streptomycin and INH and 100% agreement with strains resistant to ethambutol and RIF (Fadda & Roe 1984).

In the present study, overall, the BACTEC results for *M. tuberculosis* were in high agreement with the results of the conventional method. The minor discrepancies between BACTEC and conventional results were among those plant extracts, which had low activity for e.g., *Combretum molle*, *Cryptocarya latifolia* and *M. senegalensis*. *M. tuberculosis* cultures were reported to be partially susceptible when agar medium containing these plant extracts at a concentration of 0.5mg/ml were tested for activity. The MICs of these plant extracts were found to be 1.0 mg/ml by the radiometric method. The differing results using the  $^{14}\text{CO}_2$  and the conventional agar plate methods are not really surprising when one considers what each measures. The  $^{14}\text{CO}_2$  method measures the metabolism of palmitic acid whereas the agar plate method uses bacterial growth as an end point. It is therefore, expected that plant extracts would effect these processes in different ways and to different extents.

The agar plate method for plant extract susceptibility testing is well standardized and widely used, but the main disadvantage of this method is the long waiting period before the results can be obtained. The time period required by the radiometric method for susceptibility testing was significantly fast, only 6 to 7 days. In liquid medium there are more cells to drug contact and due to the shorter incubation time, also less likelihood of heat related breakdown of testing compounds. Another advantage in using the BACTEC radiometric assay is that 7H12 broth does not contain Tween-80, which probably could effect the results by changing the surface-active properties of bacteria (Youmans & Youmans 1948). The indication of resistance of organism to the plant extract could be observed much earlier than susceptibility; since the release of labelled CO<sub>2</sub> occurred more rapidly if there was no inhibition by the plant.

### 3.4 Conclusion

This study has indicated that some plants might be of value in the continuing struggle to control tuberculosis. Our results seem to verify the traditional use of most of the plants investigated being used as folk medicine against TB. Six plants showed significant activity against the drug-resistant strain at a concentration of 0.1 mg/ml. Conventional and BACTEC methods for the susceptibility testing of *M. tuberculosis*, are standardised and reliable. Our experience indicates that due to the agar plate method being more cost effective than the radiometric assay, for preliminary screening of plant extracts, tests by the agar plate method is desirable but for improved specificity of the results the radiometric assay is recommended. 7H12 liquid medium in conjunction with conventional media appears to maximise the accuracy of the activity of plant extracts.

### 3.5 References

- BRYANT, A.T. 1966. Treatment of Diseases. In: Zulu Medicine and Medicine-Men, Ch.9, pp. 44-50. C. Struik, Cape Town.
- FADDA, G. & ROE, S.L. 1984. Recovery and susceptibility testing of *Mycobacterium tuberculosis* from extrapulmonary specimens by the BACTEC Radiometric Method. *J. Clin. Microbiol.* 19(5): 720-721.
- GELFAND, M., MAVI, S., DRUMMOND, R.R.& NDEMERA, B. 1985. Special complaints and medicines best known by N'anga. In: The Traditional Medical Practitioner in Zimbabwe, Ch. 4, pp. 35-37. Mambo Press, Geveru, Zimbabwe.
- HEIFETS, L.B., ISEMAN, M.D., COOK, J.L., LINDHOLM-LEVY, P.J. & DRUPA, I. 1985. Determination of *in vitro* susceptibility of *M. tuberculosis* to cephalosporins by radiometric and conventional methods. *Antimicrob. Agents Chemother.* 27: 11-15.
- JOKLIK, W.K., WILLET, H. P. & AMOS, D.B. 1968. Mycobacteriaceae. In: Zinsser Microbiology, 17<sup>th</sup> edn, Ch. 34, pp. 651-664. Appleton-Century-Crofts/ New York.
- KLEEBOG, H.H., KOORNHOF, H.J. & PALMHERT, H. 1980. Laboratory manual of tuberculosis methods. Tuberculosis Research Institute, MRC, Pretoria, South Africa.
- KOKWARO, J.O. 1976. Medicinal plants of East Africa, pp. 45-47. East African literature Bureau, Kampala, Nairobi, Dar-es-salaam.





- LASZLO, A., GILL, P., HANDZEL, V., HODGKIN, M.M. & HELBECQUE, D.M. 1983. Conventional and radiometric drugs susceptibility testing of *Mycobacterium tuberculosis* complex. *J. Clin. Microbiol.* 18: 1335-1339.
- MIDDLEBROOK, G., REGGIARDS, Z. & TIGERTT, W.D. 1977. Automable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. *Am. Rev. Resp. Dis.* 115: 1067-1069.
- SIDDIQI, S.H., LIBONATI, J.P. & MIDDLEBROOK, G. 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 13: 908-913.
- SNIDER, S.H., LIBONATI, J.P. & MIDDLEBROOK, G. 1981. Evaluation of a rapid radiometric method for drug susceptibility testing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 18: 689-696.
- WATT, J.M. & BREYER-BRANDWIJK, K.M. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2nd edn. E & S Livingstone, Edinburgh.
- WHO/TB/98.258. 1998. Laboratory services in tuberculosis control Part II Microscopy, Geneva, Switzerland.
- YOUMANS, A.S. & YOUMANS, G.P. 1948. The effect of "Tween 80" *in vitro* on the bacteriostatic activity of twenty compounds for *Mycobacterium tuberculosis*. *J. Bacteriol.* 56: 245-252.

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# CHAPTER 4

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## CYTOTOXICITY ASSAY OF PLANT EXTRACTS

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# CYTOTOXICITY ASSAY OF PLANT EXTRACTS

### Abstract

Fourteen South African medicinal plants which exhibited significant antimycobacterial activity *in vitro* was evaluated for their cytotoxic properties against primary vervet monkey kidney cells (VK). *Acacia xanthophloea*, *Chenopodium ambrosiodes* and *Ekebergia capensis* showed significant toxicity against VK cells exhibiting an ID<sub>50</sub> between 0.7 to 6.0 µg/ml whereas the remaining plant extracts exhibited moderate cytotoxicity. The crude acetone extract of *Euclea natalensis* was found to have the least cytotoxicity. At a concentration of 0.1 mg/ml the VK cells did not exhibit altered morphology or growth characteristics indicative of cytotoxic effect. *E. natalensis* was therefore selected for the isolation of its active principle because of its antimycobacterial properties and low cytotoxicity.

## 4.1 Introduction

Several thousands of chemicals that are used for therapeutic purposes, have been tested for toxicity using different types of cell lines *in vitro* but even now the battle to get regulatory agencies to accept the evidence of these types of tests continues. A useful test should be able to provide information on the dose-effect relationship including the dose range for potential human exposure. Human cell lines such as, prostate, stomach, liver, colon, as well as animal cells such as monkey kidney cells, rat prostate cells are used for laboratory evaluation of toxicity of compounds these days (Yoo *et al.* 1998; Ren & Tang 1999). The various cellular and intracellular barriers are surprisingly constant amongst the vertebrates and in addition recent evidence suggests that certain receptors show only small variations throughout the mammalian species (Martin 1981). According to Enzmann *et al.* (1988), any chemical that is toxic not only in rodents but also in fish, appears to be likely to have the ability to affect basic cell mechanisms that are similar in all vertebrates. Cell culture toxicity testing is a valuable and an inexpensive approach for short term toxicity testing.

Cytotoxicity evaluation of the plant extracts, which show activity against human-pathogens, *in vitro*, is essential before they could be considered for their impact on progress in drug discovery. Studies on the cytotoxicity of the extracts are useful to evaluate the toxicological risks. According to one earlier report, a low molecular weight fraction obtained from Aloe vera (*Aloe barbadensis*) gel was found to be of similar potency to toxic substances sodium dodecyl sulphate, aloe-emodin and aloin (an anthraquinone and its precursor present in Aloe vera cortex) on a weight to weight basis. It has been suggested that every effort must be made to limit the amount of this toxin in the commercially prepared Aloe vera gel products (Aviala *et al.* 1997).

During our investigation it was found that plants selected for our research based on ethnobotanical data and on the actual experience of traditional healers showed promising activity in antimycobacterial screening and it was therefore,

decided to evaluate their cytotoxic properties. The aim was to choose one plant out of the 14, which had a low toxicity, for the isolation of its active compound(s). The plant extracts were screened for cytotoxicity against primary vervet monkey kidney (VK) cells.

## 4.2 Materials and methods

### 4.2.1 Plant material

The plant extracts were prepared as described in chapter 2 (section 2.2.2). The acetonetic residue was later dissolved in DMSO to obtain a concentration of 500 mg/ml from which aliquots were diluted with Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) Ltd., Kelvin, South Africa), to obtain the desired concentrations.

### 4.2.2 Cell culture

Plant extracts were evaluated for toxicity against VK cells according to standard cell culture techniques, as outlined by Girst *et al.* (1979). Monolayers of VK cells were prepared by seeding 25-cm<sup>3</sup> flasks or 96-well microtitre trays with 10<sup>5</sup> cells/ml. Eagle's MEM, supplemented with 10% heat inactivated (56<sup>0</sup>C for 30 min) fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) and containing 100 U/ml penicillin and 100 µg/ml streptomycin was used for the propagation of the cells. Cell cultures were incubated in a humidified CO<sub>2</sub> atmosphere (4%CO<sub>2</sub>/96% filtered air) at 37<sup>0</sup>C.

Multilayer cells present in the tissue culture plates were first rinsed three times with 10% phosphate buffer saline (PBS), and then 3 ml of 0.1% trypsin EDTA were added onto them. This helped to loosen the cells adhered to the bottom surface of the plates. The plates were incubated for 5 minutes at 37<sup>0</sup>C. Fresh maintenance medium



(MM) was poured in the tissue culture plates and the content was transferred to a test tube. The test tube was then centrifuged for 5 minutes at 3000 rpm. MM was essentially the same as the propagation medium except that it contained only 1-2% FCS.

After centrifugation the cells settled at the bottom of the test tubes and the supernatant was discarded. Fresh MEM was mixed thoroughly with the cells. 100  $\mu$ l of these freshly mixed cells in MEM was transferred to microtitre plates and incubated at 37<sup>0</sup>C for 24 hours. The monolayer cells were then formed in the microtitre plates.

### **4.2.3 Cytotoxicity assay**

Plant extracts were dissolved in DMSO with a maximum of 0.1% and doubling dilutions of the extracts from a concentration of 100  $\mu$ g/ml to 0.1  $\mu$ g/ml were prepared in MM. The extracts were tested for cytotoxicity by exposing the monolayer of the cell cultures to 200  $\mu$ l of dilutions of the extracts at 37<sup>0</sup>C. Cells were incubated for 6 days at 37<sup>0</sup>C and were monitored and observed on the first, third and sixth day. Monolayers of cells exposed to MM, without the addition of plant extracts were used as controls. Results were expressed as the dose that inhibits 50% cell growth after the incubation period (ID<sub>50</sub>). All the testing procedures were carried out in a safety cabinet.

## **4.3 Results and Discussion**

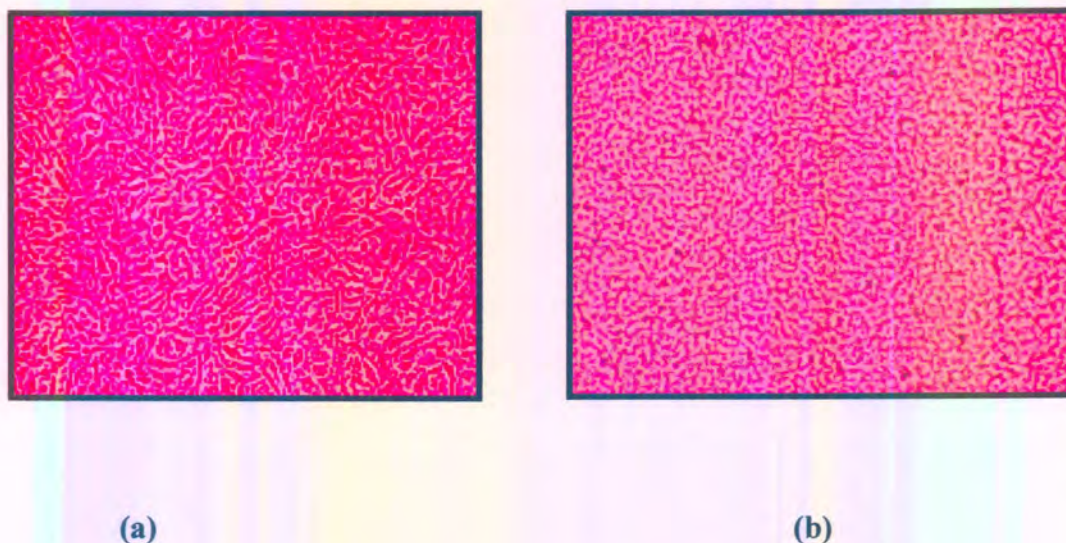
The concentrations of the plant extracts at which 75% of the vervet monkey kidney cells were alive until the sixth day was considered to be the highest concentration which is non toxic to the cells. The ID<sub>50</sub> values of the plant extracts are given in the Table 4.1.

**Table 4.1** ID<sub>50</sub> (µg/ml) values of 14 plant extracts on monkey kidney cells

Plants used for cytotoxicity assay	ID <sub>50</sub> <sup>a</sup> (µg/ml)
<i>Acacia xanthophloea</i> Benth	3.1
<i>Chenopodium ambrosiodes</i> L.	0.7
<i>Combretum molle</i> R. Br. Ex G. Don	10.0
<i>Croton pseudopulchellus</i> Pax.	10.0
<i>Cryptocarya latifolia</i> Sond.	10.0
<i>Ekebergia capensis</i> Sparrm	6.0
<i>Euclea natalensis</i> A. Dc.	100.0
<i>Helichrysum melanacme</i> DC.	10.0
<i>Helichrysum odoratissimum</i> (L.) Sweet	10.0
<i>Maytenus senegalensis</i> (Lam.) Excell	10.0
<i>Nidorella anomala</i> Steetz	10.0
<i>Nidorella auriculata</i> DC.	20.0
<i>Polygala myrtifolia</i> L.	20.0
<i>Thymus vulgaris</i> L.	50.0

<sup>a</sup>Dose that inhibits 50% cell growth

The non-toxic concentration of the plant extracts tested against the VK cell culture was found to be at concentrations ranging from 100.0 to 0.7 µg/ml. *Acacia xanthophloea*, *Chenopodium ambrosiodes*, and *Ekebergia capensis* showed significant toxicity against VK cells exhibiting ID<sub>50</sub> values between 0.7 to 6.0 µg/ml. The other plant extracts exhibited moderate cytotoxicity (Figure 4.1). ID<sub>50</sub> values of the plants tested in this study are comparable to those reported previously for various plant extracts against different cell lines. The compounds isolated from the liverwort *Plagiochila ovalifolia* showed significant cytotoxicity against P-388 murine leukemia tumour cells exhibiting an ID<sub>50</sub> of 0.05 µg/ml (Toyota *et al.* 1998). 8-hydroxyisodiospyrin isolated from *Diospyros maritima* showed cytotoxicity against hepatoma (ID<sub>50</sub>= 1.72 µg/ml),



**Figure 4.1** Cytotoxic effect of acetone extract of *Acacia xanthophloea* against primary vervet monkey kidney cells: (a) Control. (b) Cytopathic effect of the acetone extract.

colon carcinoma ( $ID_{50}=2.24 \mu\text{g/ml}$ ), nasopharynx carcinoma ( $ID_{50}= 1.85 \mu\text{g/ml}$ ) and cervical carcinoma ( $ID_{50}= 1.92 \mu\text{g/ml}$ ) (Kuo *et al.* 1997).

Cytotoxicity testing on cell cultures has been a controversial issue. The major criticism of this type of study has been that metabolism of foreign compounds (toxification/detoxification) differs from one cell to another. There could be some interesting differences, e.g. the pH in the stomach differs from that in cell culture and could lead to different absorption rates of partially ionised compounds. Toxicity testing, especially when animal cell cultures are used, may not to be a reliable tool to evaluate toxicological risks for humans. Previously, tests on experimental cell cultures cannot provide irrefutable proof of the safety or toxicity of a substance for the human

species (Schramm & Teichmann 1979). However, most of the research in the last few years on comparative effects of *in vitro* and *in vivo* cytotoxicity assays seem to point to strong similarities in early phases of response to the tested compounds (Kuo *et al.* 1997). The combination of ethnomedical information, *in vitro* and *in vivo* cytotoxicity assay results and extensive use of the plants by indigenous people for curing ailments seem to be an appropriate criteria to evaluate the plant samples for their toxicity.

#### 4.4 Conclusion

The crude acetone extract of *E. natalensis* was found to have the lowest cytotoxicity amongst the 14 plants analysed by the cytotoxicity assay. At a concentration of 100 µg/ml the VK cells did not exhibit altered morphology or growth characteristics indicative of cytotoxic effect. *E. natalensis* was therefore, selected for the isolation of its active compound(s), because of its antimycobacterial properties and low cytotoxicity. *A. xanthophloea*, *C. ambrosiodes*, and *E. capensis*, showed significant toxicity against VK cells whereas the remaining plant extracts exhibited moderate cytotoxicity.

## 4.5 References

- AVIALA, H., RIVERO, J., HERRERA, F. & FRAILE, G. 1997. Cytotoxicity of a low molecular weight fraction from *Aloe vera* (*Aloe barbadensis* Miller) gel. *Toxicol* 35(9): 1423-1430.
- ENZMANN, H., IATROPOULOS, M., BRUNNEMANN, K.D., BOMHARD, E., AHR, H.J., SCHLUETER, G. & WILLIAMS, G.M. 1998. Short-and intermediate-term carcinogenicity testing-A review Part 2: Available experimental models. *Food Chem. Toxicol.* 36: 997-1013.
- GIRST, N.R., BELL, E.J., FOLLETTE, E.A.C. & URQUHART, G.E.D. 1979. Diagnostic Methods in Clinical Virology, 3rd edn., pp. 60-79. Blackwell Scientific Publications, Oxford.
- KUO, Y.H., CHANG, C.I., LI, S.Y., CHOU, C.J., CHEN, C.F., KUO, Y.H. & LEE, K.H. 1997. Cytotoxic constituents from the stems of *Diospyros maritima*. *Planta Med.* 63(4): 363-365.
- MARTIN, P. 1981. Using animal tests to detect human carcinogens. *Occup. Hlth.* 33(10): 529-532.
- REN, W & TANG D.G. 1999. Extracts of *Solanum muricatum* (Pepino/CSG) inhibit tumour growth by inducing apoptosis. *Anticancer Res.* 19(1A): 403-408.
- SCHRAMM, T. & TEICHMANN, B. 1979. Testing of chemicals for carcinogenic activities and some problems related to the assessment of carcinogenesis risks to men. *Pol. J. Pharmacol. Pharm.* 31(6): 639-645.





TOYOTA, M., TANIMURA, K. & ASAKAWA, Y. 1998. Cytotoxic 2,3-secoaromadendrane-type sesquiterpenoids from the liverwort *Plagiochila ovalifolia*. *Planta Med.* 64(5): 462-464.

YOO, I.D., YUN, B.S., LEE, I.K., RYOO, I.J., CHOUNG, D.H. & HAN, K.H. 1998. Three naphthalenes from root bark of *Hibiscus syriacus*. *Phytochemistry* 47(5): 799-802.

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