PHARMACOGNOSTIC STUDY OF 5 MEDICINAL PLANT SPECIES FROM WESTERN CAPE PROVINCE (SOUTH AFRICA) FOR ANTI-TUBERCULAR ACTIVITY

BY

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To my dear wife Solange Musau Mpoyi and my sons Chançard Bamuamba Kapinga, Samuel Mpoyi Kapinga and Prince Ntumba Kapinga

Declaration

I declare that 'Pharmacognostic study of 5 medicinal plant species from Western Cape province (South Africa) for anti-tubercular activities' is my own work and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references.

KAPINGA BENOIT BAMUAMBA

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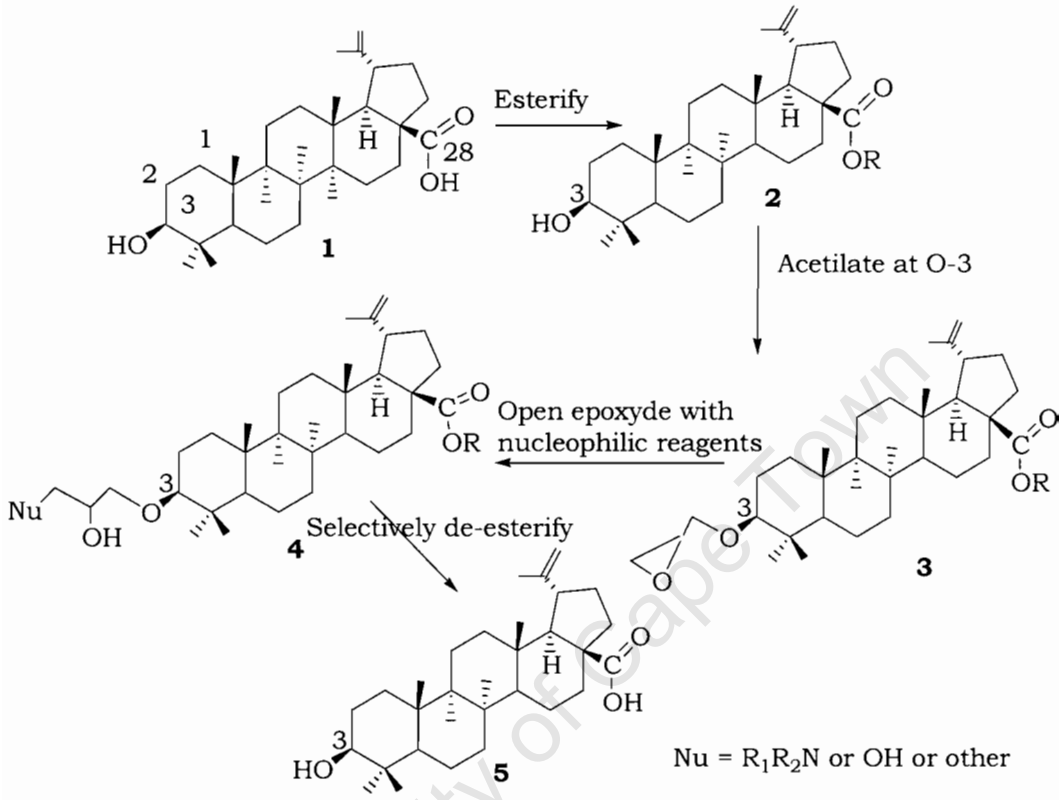
ABSTRACT

In our search for new anti-tuberculosis lead molecules, five medicinal plant species, *Olea capensis* (L.), *Tulbaghia alliacea* (L.), *Inula graveolens* (L.), *Leyssera gnaphaloides* (L.), and *Buddleja saligna* (L.) were collected in Cape Town and surrounding area and investigated for antimycobacterial activity following report of their therapeutic use in traditional medicine to treat infectious diseases such as tuberculosis. A bioassay guided fractionation of the acetone/water (4:1) crude extracts of *O. capensis* (leaves) and *T. alliacea* (rhizomes) showed no activity against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 252923, and M*ycobacterium aurum* A+. In contrast, the organic fractions (hexane, dichloromethane) of the acetone/water (4:1) crude extracts of *I. graveolens*, *L. gnaphaloides*, and *B. saligna* exhibited significant activity against *M. tuberculosis* H37Rv, *M. avium* 25291, *M. microti* ATCC 19422, and *M. scrofulaceum* ATCC 19987. The isolation and structure determination of the bioactive led to the identification of pentacyclic triterpenoids, ursolic acid (UA) and oleanolic acid as major antitubercular constituents of *B. saligna*, *L. gnaphaloides*, and *I. graveolens*. The in vitro cytotoxicity assays of the isolated bioactive constituents showed no cytotoxicity against Chinese Hamster Ovarian (CHO) cells line.

Subsequently, given the pharmaceutical value of the above finding, a survey on structure activity of pentacyclic triterpenoids was conducted. It was was found, for instance that selective substitutions at C-3 and/or C-28 and the double bond at UA, OA and betulinic and (**1**) BA) were made in order to improve anti-tumour and anti-HIV activity. However, thought a great number of modified bioactive pentacyclic triterpenoids is reported, none was tested against *Mtb*. Therefore, this study also explored a new synthetic route (**scheme 1**) toward a generation of (**5**), which may allow improving antitubercular, anti-HIV or anti-tumour activity, and/or specificity.



Scheme 1: Synthetic route to (**5**)

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CHAPTER I

INTRODUCTION

1.1 TRADITIONAL MEDICINE - PLANT NATURAL PRODUCTS - THE SEARCH FOR NEW DRUGS

1.1.1 Traditional medicine: A global perspective

Traditional medicine (TM) is defined as the totality of all knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating physical, mental or societal imbalances (WHO/OMS, 2003; Wilder, 2001). This knowledge and practice are mainly passed on verbally from generation to generation since long before the modern science and technology era (WHO/EDM, 2002).

According to recent reports, about 80% of the world's population relies chiefly on various traditional medicinal systems to satisfy their primary health care. There is an upsurge in the use of traditional and complementary medicine (CM), in both industrialised and developing countries (WHO.INT, 1998). In 2002, the WHO's acting coordinator of traditional medicine, Dr Xiaorui Zhang reported the following percentages of the population that have used complementary and/or alternative medicine at least once: Australia- 48 %, Canada - 70 %, USA - 42 %, Belgium - 40%, and France - 75 % (Zhang, 2002). This percentage stands at 80% in Africa, and 65% in India (Zhang, 2002). It was found in the Northern Province of South Africa that 9 out of 10 outpatients first consult a traditional medicine practitioner before going to a modern hospital (Rajendra, 1995).

Herbal medicines are the most widely used form of therapy, both in traditional and complementary medicine. Herbal medicines, as defined by several WHO guidelines, include crude plant materials such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered (WHO/EDM, 2002) The Secretariat of the Convention on Biological Diversity (CBD) reported that world herbal medicine sales amounted to US$60 billion in 2000

(Zhang, 2002). Herbal therapy is well established in many countries' cultures and traditions. Almost 80% of the people in rural areas of Asia, Latin America and Africa rely on traditional medicine, and herbal remedies constitute their principal source of medicine (Banquar, 1993). One of the reasons why traditional medicine is so popular in developing countries, is that it uses only herbal medicines, which are cheaper compared to modern drugs, and readily available even in remote rural areas (Banquar, 1993; Farnsworth, 1994a; Srivastava et al., 1996).

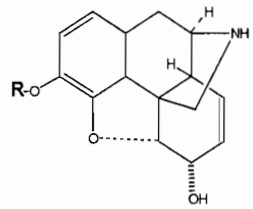
1.1.2 Plant natural products and new drug discovery

Plants have long provided humankind with a source of medicinal agents. Natural products at one point in history served as the source of all drugs (Balandrin et al., 1993). The initial development of the science of pharmacology used natural products to elucidate physiological processes (Williamson et al., 1996). Nowadays, at least 25% of all prescription drugs dispensed in the world contain active compounds derived from higher plants (Farnsworth, 1994b), of which 75% originated from traditional remedies (Gericke, 1993). However, the potential of higher plants as a source of new drugs is still largely under-explored. Of 370 000 to 500 000 species of plants that grow on the earth (Williamson et al., 1996; Hostettman, et al., 1996), only 5-10% have been scientifically investigated, fewer have been screened for biological activity (Hostettman et al, 1996).

Nowadays, many standard drugs in modern medicine are of plant origin; they are so popular that even some medical professionals are unaware of their plant origin (Williamson et al., 1996). Some examples of such drugs include (Bird, 1991; Cox, 1994):

Alkaloids:

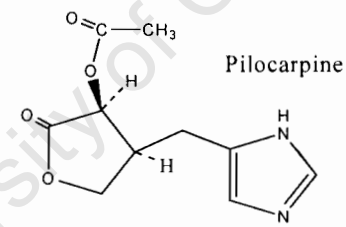
a) Codeine is a common ingredient of syrups used to suppress irritating coughs and/or as an analgesic to relieve aches and pains.



R = H: Codeine
R = -CH_3: Codeine

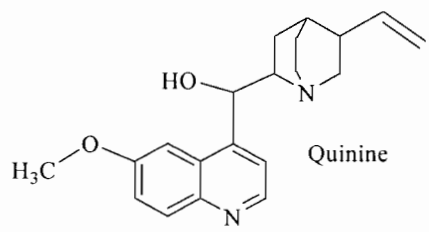
b) Morphine is an analgesic more potent than codeine. Both codeine and morphine are extracted from the opium poppy, *Papaver somniferum.*

c) Pilocarpine is an alkaloid extracted from *Pilocarpine jaborandi.* It is listed on the WHO essential drugs list for the treatment of chronic simple and acute angle glaucoma through inclusion in eye-drop formulations.



Pilocarpine

d) Quinine: an alkaloid isolated from an extract of the bark of the cinchona tree; it is a WHO essential drug used for resistant *Plasmodium falciparum* malaria and for the treatment of malaria due to any type of *Plasmodium* parasite.

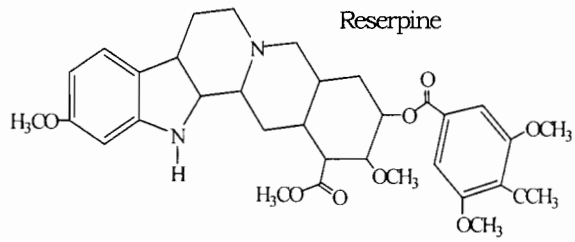


Quinine

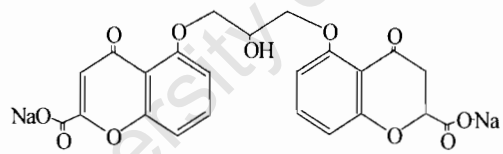
2) <u>Steroids:</u>

a) Diosgenin: was discovered in wild yams from Mexico and Guatemala and later became the starting material for the synthesis of the first contraceptive pill.

b) Reserpine is a common anti-hypertensive drug extracted from a shrub historically known for its tranquilizer properties.

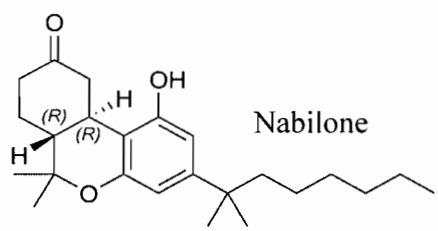


c) Sodium cromoglycate is an anti-asthmatic drug (active ingredient of Intal) derived from khellin, extracted from a Mediterranean medicinal plant used in the treatment of colic.



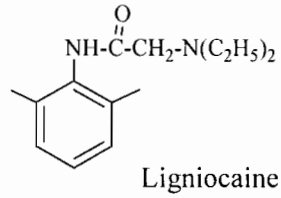
Sodium cromoglycate

d) Nabilone is an anti-emetic derived from *Cannabis sativa* (marijuana).



Plant constituents have also served as templates for the development of many drugs. The local anaesthetic, lignocaine, neuromuscular blockers from tubocurarine, carbenoxolone from glycyrrhetinic acid; chloroquin

from quinine, and bromocriptin from ergot alkaloids are a few examples of drugs of this category (Williamson et al., 1996).



Ligniocaine

1.1.3 The resurgence of interest in medicinal phytochemistry

There is a global resurgence of interest in plants as sources of medicines and of novel molecules of pharmacological interest. The following factors may justify this situation:

1. Many drugs of modern medicine are scarce and expensive for a large proportion of the population in developing countries, thus they chiefly rely on traditional herbal remedies, some of which have been used for generations by their ancestors (Williamson et al., 1996; Farnsworth, 1994[a]).

2. The fact that many important drugs used in modern medicine are of plant origin has lead to the expectation that the cure for some devastating diseases such as malaria, tuberculosis, cancers and HIV/ AIDS will come from the plant kingdom (Farnsworth, 1994[b]).

3. There is a perception in the global green revolution movement that herbal remedies are safer and less damaging to the body than synthetic drugs (Williamson et al, 1996).

Because of the abundance of the plant biodiversity they observe, it is a believe among scientists that this implies a great diversity of plant bio-active constituents as well (Farnsworth, 1994[b]).

The screening of plants can be channelled in two directions, one leading to drug discovery and commercialisation of the product, the other ending just with the adoption of a crude extract as a medicine (Houghton, 1995). It is common that the investigation into traditional medicine remedies reveals an unexpected pharmacological activity for illnesses for which the biochemical mechanisms have yet to be elucidated (Cox, 1994). A previously non-active natural product can also be modified to give a semi-synthetic drug with the desired biological activity. The following section will list some criteria commonly used by scientists for the selection of plants for biological screening that may bear therapeutic potential.

1.1.4 Principal steps in the process of drug discovery from plant material

In their book, `Human Medicinal Agents from Plants', Kinghorn and Balandrin (1995) divide the general strategy for drug discovery from plant material:

Step 1. Identification of the need for conducting the research.
Step 2. Selection of plants likely to possess the desired activity.
Step 3. Collection of plant material.
Step 4. Identification and preparation of voucher specimens.
Step 5. Preparation of plant extracts.
Step 6. Evaluation of the antimicrobial activity of the extracts.
Step 7. Fractionation of extracts, and isolation of compound.
Step 8. Structure determination of the active principles.
Step 9. Toxicity studies of the active principles.
Other steps, which are purely commercially oriented, include:
Step 10. Determination of structure-activity relationships.
Step 11. Evaluation of status for commercial development.

Step 12. Conduct clinical trials.

Step 13. Commercialisation.

Step 14. Conduct agronomic studies.

An individual or a small group of collaborators in the laboratory can conduct the first nine steps in this process. Steps 10 to 14 are expensive operations, which generally are conducted with the support of the pharmaceutical industry in prospect of commercialisation of the product. Only steps 1- 9 will be discussed.

Step 1: Identification of the need for the investigation

Here, the therapeutic need for new drugs must be determined. In the case where the drug is destined for commercial purposes, a preliminary economic assessment must be conducted. This includes the market potential of the kind of product, and the possibility for commercialization of the product. The community's need for a new drug can consist of a need for a cheap drug, or a drug that is culturally more acceptable, or the need for a drug to treat a disease that is yet to find a proper cure (Williamson et al., 1996).

Step 2: How to select plants for medicinal research

The selection of plant materials that are likely to contain the needed biological activity is a determinant step for any ethno-pharmacological research. It is true that not all plants in the flora contain biologically active compounds with potential therapeutic utility. The selection of plants is determinant in ethno-pharmacological research and is based on criteria that in practice guide the screening for biological activity:

(1) Researchers show high interest in investigating plants used in various traditional medicines (Houghton, 2000). A focus on traditional medicinal plants, some of which have been in use as medicines for centuries, is more likely to yield pharmacological activity, rather than a random screening of plants. Furthermore, with careful attention to the cultural environment, the traditional medicine methods can be a helpful guide for designing an appropriate research methodology (Cox, 1994).

(2) The need to address a particular community's demand (e.g. anti-cancer drug) can also be the driving force in choosing plant materials for investigation (Williamson et al., 1996).

(3) Considerations related to production costs are taken into account when engaging in phyto-pharmaceutical research. Operational costs such as cost of plant collection and replacement, and cost of lab operations are also concerned.

(4) The selection of plants can also simply be based on predictable chemical constituents of the plant according to available chemotaxonomic data. The plants that are likely to contain the desired phytochemicals will be automatically selected, whether they are traditionally used as medicines or not.

(5) Computerized databanks that can guide the selection of plant species based on plant taxonomy are available (Farnsworth, 1994b).

(6) A combination of criteria is usually used in order to optimize the chance of selecting desirable plant species for biological investigation. After the plant materials have been identified, they must be collected and handled in a manner to ensure that they preserve their original quality. Therefore, how to handle plant materials for scientific screening for bioactive compounds becomes an important issue.

Step 3: Collection of plant material

The ethno-pharmacological study of a plant must start with the collection of the correct plant species and preparation of herbarium voucher specimens (Cotton, 1996). Depending on the usefulness of the parts of the plant (leaves, stems, roots or bark), attention is given to factors such as the age of the plant, the habitat, and the season.

Step 4: Identification of plants

Collection of plant material

Scientific investigation of plant material commences with an accurate botanical identification of the plant. An experienced plant taxonomist who is involved in the identification, nomenclature and classification of plant species should authenticate the plant material to be investigated (Cotton, 1996). Recently, some computer software has been used in the identification of plant material, whereby the plant specimen is analyzed using a computer image (Tsichristzis et al., 1998).

Preparation of voucher specimens

A correct voucher specimen is an integral part of the study, because it allows permanent preservation of representative plant samples. Thus, a

voucher specimen of the plant must be conserved at a known herbarium for reference purposes.

Storage of raw plant material

Good storage that preserves the quality of plant material requires that:

- Plant material destined for storage must firstly be dried, because the storage of damp plant material can lead to spoilage by fungal and bacterial growth.
- Heat drying of plant samples should be avoided if possible, because heat drying can affect the quality of the active compound and/or modify the phytochemical composition of the plant (King's College, 1993). The choice of storage method of raw plant material largely depends on the nature of plant, the type of investigation and methods that are to be followed. Different studies require different modes of preservation of raw plant materials; the advantages and disadvantages of each method should be weighted accordingly in order to determine the way to proceed (King's College, 1993). Usually, raw plant materials are stored in a dried powdered form, which has the advantage of storage in tightly closed containers such as bottles.

Step 5: Preparation of plant extracts

Once the plant material has been properly prepared, the extraction of the plant's constituents must follow in order to proceed with the bioassay. Several methods of extraction can be used for this purpose but solvent extraction is the most common of them all; it includes maceration, percolation, soxhlet extraction, infusion, and distillation (Williamson et al., 1996). A concentrate of the crude extract can be obtained using a rotary vacuum evaporator, or freeze drier (in the case of aqueous extracts). Then a bioassay-guided fractionation of the extract helps to determine which fraction contains the active principle so that the

attention is given to the active fraction only, which allows one to save valuable time and minimizes the operational cost.

Step 6: Evaluation of the antimicrobial activity of the extracts

Agar plate bioassays

* Disk diffusion method: sterile hard paper disks (such as filter paper) are impregnated with the sample (in various concentrations) and deposited on an agar plate inoculated with a suspension of the microbial culture. The plate is then incubated overnight at 37 °C. Clear inhibition zones appear around the disks containing bioactive compound(s).

* Agar overlay spotting technique: based on a similar principle to the disk diffusion method. Instead of using impregnated disks, samples are directly spotted onto the surface of the inoculated plate.

Bioautography on TLC plates

The antimicrobial properties of compounds are tested on a TLC plate that serves as support for the culture and the test samples.

Step 7: Extraction, isolation, and purification of plant bioactive

In addition to classic liquid/liquid methods of separation, there are techniques for isolation and purification of phytochemicals, such as lyophilization, chromatographic techniques, filtration through selective membrane, etc..., some of which are used for high through-put in industry. In general, the use of one or other method of extraction and separation is dictated by the structure, stability, and the quantity of the compound to be isolated. The usefulness of the liquid/liquid method of separation lies in the fact that it allows a bioassay-guided fractionation of plant crude extracts before proceeding with further investigation; thus the investigator finds out in which fraction the activity lies, which can

help to simplify the purification process by starting the extraction with selective solvents if possible. However, if the desired compounds are unknown, it is preferable to perform several exhaustive extractions using different solvent systems with sequentially increasing polarity. A list of commonly used solvents is given in Table **1.1.**

Table 1.1: List of solvents (in order of increasing polarity).

Polarity	Solvent	Extractable
Low polarity	Light petroleum Hexane	Waxes Fats, fixed oils
	Cyclohexane Toluene Diethyl ether	Volatile oils and steroids
Medium polarity	Chloroform Ethyl acetate Dichloromethane	Alkaloids, free aglycone (from glycosides)
High polarity	Acetone n – Propanol Ethanol	Glycosides, Phenolics
	Methanol	Carbohydrate
	Aqueous alkali	Water-soluble compounds and acids

Modified from Harbone (1998)

Step 8: Structure determination of active compounds

Once the active compound has been isolated and purified, it comes down to the determination of its structure. Nowadays, structure elucidation can be carried out quickly and accurately using a combination of spectroscopic methods, suchnas X-Ray, MS, NMR IR and UV techniques. Spectroscopic techniques have recorded a spectacular development, thus the analysis of the MS and NMR data alone can lead to a complete elucidation of a complex structure. The coupling of the spectroscopic techniques with highly developed software and robots has rendered the task of structure elucidation even faster than ever.

Step 9: Toxicity studies of the active principles.

The in vitro cytotoxicity test is aimed at measuring the extent of the damage an active agent causes to normal living cells. Cytotoxic drugs may disturb fundamental mechanisms involving cell growth, differentiation, and activity of the chromosomes at some stage preceding their duplication (The New Encyclopedia Britannica Vol. 3). Cytotoxicity tests are important as they allow one to determine whether the agent can be used for therapeutic purposes in animals or humans, and what precautions must be taken in order to prevent harmful effects. Various types of cells exhibit different susceptibilities to toxic compounds. Cells that are most susceptible to inhibition and damage by cytotoxic agents appear to be rapidly proliferating cells, such as blood cells, lymph-forming systems, reproductive and epithelial tissues (e.g. intestinal mucosa), and neoplastic (cancerous) tissues. Cytotoxicity is measured in terms of the percentage inhibition of cell growth or percentage of normal cells killed following incubation with the drug for a defined time. IC_{50} is therefore defined as the sample concentration that causes death of 50% of the test cell population. In vitro cytotoxicity testing is performed on mammalian cell lines other than human cells (e.g. Chinese Hamster Ovarian (CHO) cells) because of restrictions which regulate the use of cells from human tissues in laboratory experiments. It is also advantageous to use fast growing and easy to handle cells, such as CHO cells.

I.2 TUBERCULOSIS AND DRUG-RESISTANT TB

1.2.1 TB DR-TB, A GLOBAL THREAT

Tuberculosis is defined as a chronic, granulomatous disease caused by *Mycobacterium tuberculosis* (*Mtb*). *Mtb* is a rod-shaped, non-spore forming, non-motile, aerobic bacterium (Xtra: Health, 2001). The causative microbe of TB has infected humankind since as early as 2000 to 4000 B.C. (Bloom, 1992). Pulmonary TB was already well described during Hippocrates' era (Cummins, 1949; Bloom, 1992). The TB pandemic was thought to have been eradicated in the early second half of the past century. However, during the last two decades this old disease has resurged with an alarming speed, both in developing and industrialized countries. Today TB is still the leading cause of death in the world from a single infectious disease (Bloom, 1992; Xtra: Health, 2001). There are approximately 10 million patients treated for active TB worldwide every year (Stop TB, 2000). The WHO reported 8 million newly infected TB patients in 1999. This number is expected to be 10.2 million new TB patients per year by 2005 (Lall and Meyer, 2001). Furthermore, a survey conducted in 1998 estimated that 1 in 10 new TB patients would be infected with multi-drug resistant *Mtb* (Li and Brainard, 2001). TB kills at least 2 to 3 million people per year (Bannenberg, 2001). The association of TB with HIV aggravates these trends. The vast majority of TB patients live in developing countries of the southern hemisphere, especially in poor and less educated communities. 1n 1993 the WHO listed TB as a global emergency (Steyn, 2003), because of its speed of expansion in the world, and the worsening conditions considered to be the co-factors of TB expansion, such as deepening poverty (poor housing, overcrowding and malnutrition) and lack of education in developing countries, the mass population displacement resulting from wars and natural catastrophes, tourism industry and association with HIV/AIDS.

1.2.2 MODERN ANTI-TB THERAPY

The anti-TB therapeutic strategies include early diagnosis and drug therapy, chemoprophylaxis, and BCG vaccination.

1.2.2.1 Anti-TB chemotherapy

Tuberculosis is a curable disease. Several drug regimens have shown high cure rates of more than 95% when the drugs are used correctly (Telenti and Iseman, 2000). Poor patient compliance is the single most important factor that compromises TB therapy.

TB chemotherapy started with streptomycin (SM), which appeared in the late 1940s; it was then followed by p-amino salicylic acid (PAS), which is no longer used. Isoniazid (INH) and rifampicin (RIF), which form the key anti-TB drugs today, were introduced into chemotherapy in the late 1960s (Petrini and Hoffner, 1999).

Rifabutin and rifapentine are analogues of RIF with slightly different characteristics. Pyrazinamide (PZA), another powerful anti-TB drug was already known in the 1950s. Ethambutol (EMB) and ethionamide (ETHN) are used as adjunct drugs to prevent the development of resistance (Petrini and Hoffner, 1999). INH, RIF, PZA, and EMB are classified as first-line TB chemotherapy drugs. The second line anti-TB drugs include quinolones (ciprofloxacin, ofloxacin, and sparfloxacin), aminoglycosides (kanamycin), capreomycin, ethionamide (ETH), thiacetazone and cycloserine (Petrini and Hoffner, 1999). The current short course anti-TB drug regimen consists of an initial intensive drug regime comprising 6 months RIF and INH, plus PZA for the first 2 months. If drug resistance is suspected, EMB can be added (Petrini and Hoffner, 1999). Both INH and PZA need to be activated by mycobacterial enzymes, catalase-peroxidase (KatG) and pyrazinamidase, respectively. Thus, a mutation in the activating enzyme can lead to resistance to these drugs. When RIF is

included in the regimen, the therapy may not need more than 6 months to eradicate the disease; otherwise, the treatment can take 12 - 18 months. INH and RIF are the most potent anti-TB drugs. They kill more than 99% of *Mtb* cells within 2 months of starting drug therapy (Rattan et al., 1998). PZA shows a high sterilizing effect when it is added to INH and RIF. PZA acts on semi-dormant bacilli that are not affected by any other anti-tubercular drug (Rattan et al., 1998). Streptomycin (SM) is also a bactericidal anti-tuberculosis drug. Its use is limited by the fact that it is available in injectable form only. SM is not recommended for pregnant patients because of its potential fetal ototoxicity. Terizidone and thiacetazone are listed as WHO essential drugs with tuberculostatic action. The choice of treatment regimen for TB should take into account factors such as:

-The cost of the medication and proximity of the healthcare centre (distance travel from and to the healthcare center).

-The potential toxicity of the prescribed anti-TB drugs. The choice of drug regimen must also consider whether the bacilli being treated are drug resistant or not and avoid the possibility of drug resistance arising during the treatment.

1.2.2.2 Anti-TB chemoprophylaxis

Anti-tuberculosis chemoprophylaxis consists of INH (4 to 5 mg/kg/day) administered to people who are in contact (e.g. staying in the same house) with active TB patients. The treatment must be continued for at least 3 months. However, if the skin test shows a positive result, or if there is risky contact the TB patient, the drug regimen should be continued for at least 12 months (SAMF, 2000).

1.2.2.3 Anti-TB vaccination

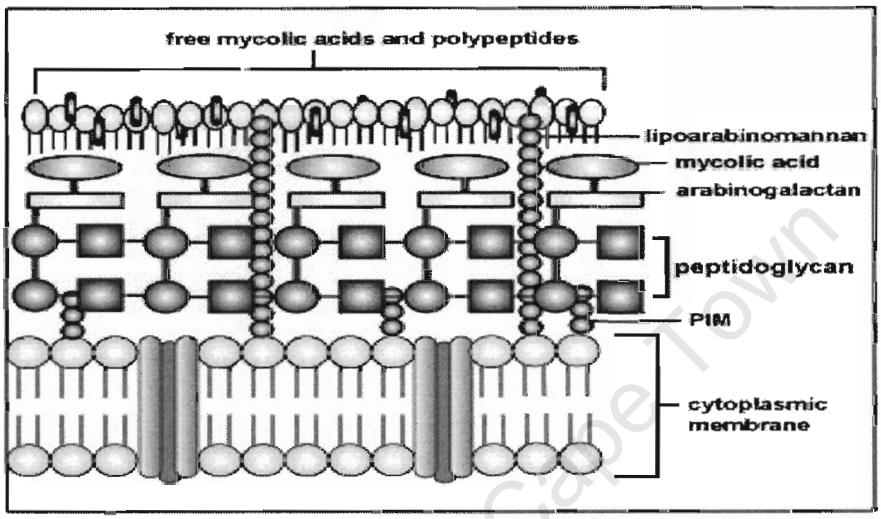
Anti-TB vaccination is given in the form of a live, attenuated *M. bovis* strain - Bacillus Calmette-Guerin (BCG). In South Africa, BCG is given to all children at birth, when starting school and when leaving school (SAMF, 2000). BCG's protective property is doubtful, but its value lies in protecting against disseminated disease such as TB meningitis (Steyn, 2003). BCG vaccination is prohibited for a person with active TB. The development of novel anti-TB vaccine continues, and includes investigation of attenuated *M. tuberculosis* strains and subunit vaccines (Steyn, 2003).

1.2.3 THE MYCOBACTERIAL CELL WALL

Mycobacteria are characterized by a highly hydrophobic cell wall, which plays an important role in selective permeability and constitutes a barrier to many drugs (Telenti and Iseman, 2000). It is also important to note the presence of a well-developed drug-efflux system that characterizes the mycobacteria (Telenti and Iseman, 2000). The mycobacterial cell wall is made up of peptidoglycan and a large amount of glycolipids, especially *mycolic acids*. The peptidoglycan layer is linked to arabinogalactan (D-arabinose and D-galactose) which is then linked to high-molecular weight mycolic acids. Mycolic acids account for up to 60% of the dry weight of the organism. The type of mycolic acid can be used as the basis for the differentiation of mycobacteria (Steyn, 2003). The arabinogalactan/mycolic acid layer is overlaid with a layer of polypeptides and mycolic acids consisting of free lipids, glycolipids, and peptidoglycolipids. There are also other glycolipids such as lipoarabinomannan and phosphatidylinositol mannosides (PIM). Because of its unique cell wall, when it is stained by the acid-fast procedure, it resists decolorization with acid-alcohol and remains red. With the

exception of a very few other acid-fast bacteria such as *Nocardia*, all other bacteria will be decolorized and stain blue, the color of the methylene blue counter stain.

Figure 1.2.1: Mycobacterial cell wall



(Extracted from: http://www.gsbs.utmb.edu/microbook/ch033.htm)

The bacilli's cell wall is a determinant factor in the pathogenicity and virulence of the micro-organism. All pathogenic mycobacteria are intracellular pathogens. It is the cell wall that helps the mycobacteria to survive within the macrophage by resisting oxidation (Steyn, 2003). Because of their characteristic hydrophobicity and their need for oxygen, mycobacteria are found at the water air interfaces.

1.2.4 MECHANISMS OF DRUG-RESISTANCE IN TB

The rising rate of drug-resistant TB (DR-TB) and multidrug-resistant TB (MDR-TB) constitutes a serious challenge to global control of TB. The term "Multidrug-resistance" refers to simultaneous resistance of *Mtb* to RIF and INH (Vareldzis et al., 1994). The natural factors that explain the

ability of *Mtb* to resist the antibiotics include its highly hydrophobic cell wall, its well-developed drug efflux systems, and the ability to produce drug-modifying enzymes such as lactamases, and aminoglycoside acetyl transferases (Telenti and Iseman, 2000). Resistance to antibiotics can be classified as primary when the patient has been infected through contact with a person who has MDR-TB, or acquired, when the bacilli were previously sensitive to treatment and have developed resistance during the course of medication. Primary resistance is predominant in rich countries and is usually found in places such as prisons, shelters for the homeless, hospitals and HIV/AIDS patients (Coimbra and Araujo, 2001). The acquired resistance usually results from patient non-compliance, bad prescription of anti-TB drugs, incorrect use of the drugs by the patient, or simply from too many modifications to the patient's therapy (Coimbra and Araujo, 2001).

Previous genetic and molecular analyses of anti-TB drug resistance indicated that *M. tuberculosis* acquires resistance through mutations that alter the drug target (Spratt, 1994). Another mechanism consists of titration of the drug through overproduction of the target (Davis, 1994). The selective permeability of the mycobacterial cell wall also contributes to drug resistance (Rattan et al., 1998). The mutations that confer resistance occur on the chromosome of individual bacilli, which means that multidrug-resistance comes about as a result of serial individual mutations in several independent genes. A summary of molecular mechanisms of resistance is presented in **Table 1.2**.

In MDR *Mtb*, over 95% of Rif-resistant strains possess a mutation in the *rpoB* gene encoding the beta subunit of DNA-dependent RNA polymerase, and 90% of INH resistant strains have mutations in *inhA*, *katG,* and *ahpG* (Taniguchi, 2000). The principal factor leading to INH resistance is mutation in the catalase-peroxidase enzyme, which activates INH to inhibit mycolic acid synthesis. Resistance to INH is 100 times more

frequent than resistance to RIF (Petrini and Hoffner, 1999). A better understanding of the mechanisms of action of anti-TB drug resistance at the molecular level can help in identification of new drug targets to override resistance and develop more efficient treatment regimens for MDR-TB patients.

Table 1.2: Mechanisms of drug resistance in *Mtb*

Drug	**Mechanism of action**	**Gene**(s) **involved**	**Product (location)**	**Role**	**Frequency** (%) **in resistant** *Mtb*
RIF	Inhibition of transcription	*rpoB*	Subunit of RNA polymerase	Drug target	95 - 100
	INH	Inh of mycolic acid biosynt.	*katG*	Catalase-peroxidase	60 - 70
		oxyR-ahpC	Alkyl hydro-reductase	Marker of resistance	20
		kasA		Drug target	Unknown
INH; ETH	Inh of mycolic acid biosynt.	*InhA*	Enoyl-ACP reductase	Drug target	10
PZA	Inhibit. of fatty acid synth.	*PcnA; fasA*	Amidase	Conversion of prodrug	72 - 97
EMB	Inhib. arabinogalactan syn.	*embCAB*	EmbCAB	Drug target	47 - 65
SM	Inhib. of protein synthesis	*rpsL*	Ribosomal protein S12	Drug target	52 - 59
	Inhib. of protein synthesis	*rrs* (16SRNA)	16s rRNA	Drug target	10
Kanam;Amika	Inhib. of protein synthesis	*rrs* (16SRNA)	16s rRNA	Drug target	76
Fluoroquinolone	Inhibition of DNA gyrase	*gyrA*	DNA gyrase	Drug target	75 - 94
	Inhibition of DNA gyrase	*gyrB*	DNA gyrase	Drug target (indirect)	In vitro
	Inhibition of DNA gyrase	*IfrA*	DNA gyrase	Transport	Not in *Mtb*
Clarithromycin	Inhib. peptitransfer function	*rrs (23sRNA)*	ibosome	Drug target	

1.2.5 THE NEED FOR NEW ANTI-TB DRUGS

The need for new anti-TB drugs is justified by:
(1) Urgent need for an improvement of the current TB treatment by shortening the duration of therapy and/or by providing a widely spaced intermittent drug regimen,
(2) The need for a simpler and more efficient treatment for MDR-TB,
(3) The need for improvement of the treatment of latent tuberculosis infection (LTB) (O'Brien and Nunn, 2001).

Although modern anti-TB chemotherapy is efficient enough to cure TB and DR-TB patients, the minimum 6-month treatment of TB poses a major obstacle to control of TB in the world. TB is most common in poor communities, among the less educated and in crowded and hopeless places such as prisons and refugee camps. These people are more likely to show non-compliance with a complicated drug regimen that is to last a minimum of 6 months. There is therefore a need for new anti-TB drugs that will allow reducing both the length of treatment and the frequency of the daily drug administration; new anti-TB drugs must also be cheap and available in order to be convenient even to lower-income communities.

1.2.6 Limitations of the existing drugs discovery approaches

Pharmaceutical discovery often begins with the hypothesis that a target protein in the body is involved in a certain disease and that compounds that block or inhibit the action of that target will provide therapeutic benefit. The search for these compounds typically starts by screening a collection of molecules to find "hits" that inhibit the target function. These hits are then improved through medicinal chemistry to create more advanced molecules that are tested in animal models of the disease

to determine whether they provide therapeutic benefit. Molecules that test positively in animal models are optimized to have the necessary properties to become drugs and are ultimately tested in human clinical trials.

Combinatorial chemistry has expanded the size of compound collections, and advances in automated high-throughput screening (HTS) have enabled the screening of million-compound libraries. Despite these advances, HTS is limited by the number of complex, fully formed compounds that can practically be made and stored in a collection. Even very large collections represent only a small fraction of the compounds that could be made. Thus, new approaches to searching the vast potential diversity of chemical compounds are highly desired. Another challenge with HTS is that it typically identifies compounds that bind to the main binding site of kinases. It can be difficult to find molecules that bind to the variable region of kinases using HTS; kinase inhibitors that bind to the variable region are believed to possess significant potential therapeutic benefit.

Fragment-based drug discovery offers an alternative strategy for identifying drug-like compounds that bind to proteins. In contrast with HTS where compounds are identified by measuring activity, fragment-based discovery involves the identification of multiple drug fragments by detecting the binding event itself. Individual fragments that bind to nearby sites on the protein are identified and combined to form a drug-like compound. By first determining which fragments interact with the protein in the area of interest and then combining them, there is higher probability of identifying novel compounds that productively bind to the protein. However, a key challenge to fragment-based drug

discovery is identifying the typically weakly binding fragments that are generally difficult to detect using conventional methods.

1.2.7 Progress in the development of new anti-TB drugs

Despite the need for new drugs to combat MDR-TB, there is so far no promising drug that is even in clinical trials (Telenti and Iseman, 2000). New drugs such as the rifamycins [rifabutin, rifapentin and rifalazil (KRM-1648)] and the quinolones have added to the list of anti–TB drugs, but all these drugs have failed to present the desired breakthrough in TB therapy. Most MDR-TB strains are susceptible to fluoroquinolone antibiotics. These include levofloxacin, ofloxacin, sparfloxacin, and moxifloxacin, which present a minimum inhibitory concentration (MIC) of less than 0.5 mg/L. There are also some reports supporting the efficacy of imipenem or amoxicillin/clavulanic acid in the treatment of MDR–TB. A novel class of antibiotic comprising oxazolidinone compounds (e.g. linozolid) appears to be promising. Their mechanism of action lies in the inhibition of an early step in protein synthesis (Telenti and Iseman, 2000).

The hope is that better understanding of the synthesis of the mycobacterial cell wall with mycolic acid synthesis inhibitors (i.e., thiolactomycin or thiourea isoxyl) may lead to the development of new specific anti-mycobacterial agents (Phetsuksiri, et al., 1999).

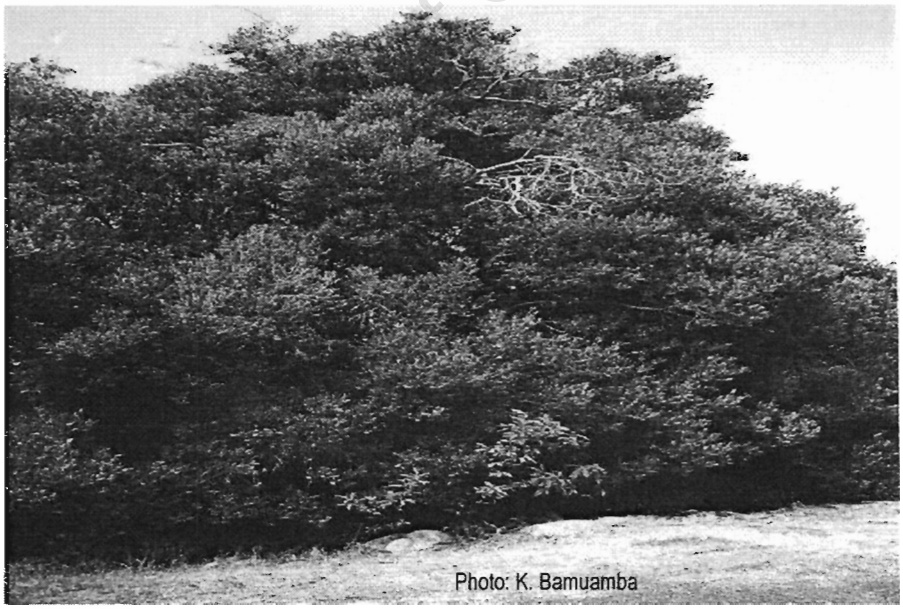
CHAPTER II

INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF OLEA CAPENSIS (L.), AND TULBAGHIA ALLIACEA (L.)

2.1 INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF *OLEA CAPENSIS* (L.)

BACKGROUND

Olea capensis (L.), also called Bastard black ironwood, is a shrub reaching the size of a medium tree of 15 to 20 m (**Figure 2.1**) (Goldblatt and Manning, 2000). An infusion of leaves of *O. capensis* is reportedly used by traditional medicine practitioners as to treat higher respiratory tract infections, or drunk as tea to treat a sore throat, bad back and kidney pains (Montagu Museum, 1998). The plant is distributed in South Africa from the Cape Peninsula eastward up to Kwazulu Natal province. *O. capensis* subsp. m*acrocarpa*, the true black ironwood, is often confused with the other bastard ironwood, *O. laurifolia*. The former is a smaller, small-fruited tree growing mostly in drier areas, whereas the latter is a tall, large-leaved, large-fruited tree of the forests (Goldblatt and Manning, 2000).



Picture 2.1: *Olea capensis (L.)* growing in Kirstenbosch Garden, Cape Town, South Africa

2.1.2. EXPERIMENTAL

2.1.2.1 Collection of plant material

O. capensis (leaves) were collected on 15 March 2001 from Kirstenbosch Garden in Cape Town. The species was authenticated by Mr H. Trinder-Smith, the curator at the Bolus Herbarium, Dept. Botany, University of Cape Town. A voucher specimen [K. Bamuamba, sn. (BOL)] was deposited in the Bolus Herbarium.

2.1.2.2 Preparation of crude extracts

The collected plant material (leaves and stems) was air-dried at 37 °C for 72 hours and then ground into fine powder before being solvent extracted. The extraction of the crude extract was carried out following two methods:

A) Since there were reports of the therapeutic use of aqueous infusions of the leaves of *O. capensis*, the first effort of this investigation was to verify the antimicrobial activity in *O. capensis*'s (leaves) aqueous extract. The protocol of this extraction was (Montagu, 1998):

100g of dried powdered plant material (leaves) was infused with 1 litre of boiling distilled water in an Erlenmeyer flask and shaken for ten minutes. The infusion was then left to cool at room temperature for 4 hours, and then filtered through filter paper (Whatman No 3) in a large funnel. The resultant filtrate was freeze-dried (Labotec Dry-o-vac freeze-drier). The residue was then re-suspended and bio-tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Mycobacterium aurum* A+.

B) The second mode of extraction was a maceration of the powdered plant material with a mixture of acetone/water (4:1). This method of extraction was aimed at extracting both lipophilic and hydrophilic compounds. The protocol followed was:

100g of dried powdered plant were extracted with 1000ml of acetone/water (4:1, v/v) at room temperature in a 2.5 litre Erlenmeyer flask. The mixture was shaken for one hour, and then allowed to stand overnight at room temperature. The resultant supernatant was filtered through filter paper (Whatman No 3, 18.5cm) into a separate flask. The remaining residue was retained in the original flask, and extracted with a further two portions of 1000ml acetone/water (4:1, v/v), as previously, for 6 hours. The three acetone/water extracts were combined, and the acetone was evaporated at 37 °C using a rotary evaporator (Buchi Rotovapo-R). The obtained aqueous mixture was transferred into a separating funnel where it was successively extracted (gently without vigorous shaking) with hexane, chloroform, and ethyl acetate (3x 100ml of each). The remaining aqueous solution was finally freeze-dried, and the residue dissolved in 100% methanol. Four extract fractions were so obtained: hexane fraction, chloroform fraction, ethyl acetate fraction, and aqueous fraction.

2.1.2.3 Antibacterial susceptibility test

The aqueous and organic extracts from *O. capensis* leaves were tested for anti-microbial activity against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *M. aurum* A+ using a modified Mosman's Microtiter MTT colorimetric assay (Mosman, 1983) and bioautography on TLC (Vladimir, 1973). Both the aqueous and the acetone/water (4:1) crude extracts of *O. capensis* leafs were found to be inactive against the test bacteria at 100

µg/spot (bioautography). Therefore, the investigation for the antibacterial activity of *O. capensis* was discontinued.

2.2 INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF TULBAGHIA ALLIACEA (L.)

2.2.1 Introduction

Tulbaghia alliacea (L.), one of ten species that make up the *Tulbaghia* genus, is a southern African herb that grows in a variety of habitats, from swampy lowland to barren ground, 0 – 2000m (Burbidge, 1978). *T. alliacea* is characterized by a rhizomatous rootstock with rhizomes often up to 10 cm long, and swollen. The tubercle and even the aerial parts have a strong and persistent smell that is typical of this species. The plant is distributed in southern Africa, extending northwards in the east up to Zimbabwe (Burbidge, 1978).

2.2.2 Antibacterial susceptibility assays

T. alliacea tubercles were ground and infused with boiling water as described in section 2.1.2.2 (A), and the obtained extracts were tested for antibacterial activity as described in section 2.1.2.3. Another extraction *T. alliacea* tubercle material was carried out using acetone/water as solvent as describe in section 2.1.2.2. Both aqueous and acetone/ water crude extracts of *T. alliacea* tubercles showed no antibacterial activity against the test bacteria at 100 µg/spot (bioautography), therefore, the investigation of this plant material was abandoned.

CHAPTER III

INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF BUDDLEJA SALIGNA (L.)

3.1 INTRODUCTION

Buddleja saligna Willd is commonly called: False Olive (English); Witolien (Afrikaans); unGqeba (Xhosa); and iGqeba-elimhlope (Zulu) (Aubrey, 2002). The plant belongs to the Buddlejaceae family, which comprise about 100 species distributed in the sub-tropical regions of Africa, America and Asia (Arciniegas et al., 1997). The genus was named in honour of an amateur botanist, Rev Adam Buddle (1660-1715). *Saligna* is a reference to the leaves being willow like. *B. saligna* grows as a shrub or small tree that can reach 5 to 10m tall. The leaves are long and narrow - somewhat similar to the olive from which it gets its common name - but more textured. The leaf upper surface is hairless and dark green, the underside is whitish with prominently raised venation. The flowers, which appear from spring to summer (August-January) are tiny, creamy white and borne in dense sprays usually at the ends of branches. The seeds are minute, forming in small, hairy capsules, which develop in the dried out flowers (October - March) (Aubrey, 2002).

The false olive is widespread in South Africa, from the Western Cape province through to Zimbabwe, extending inland from the coast to central South Africa and the Kalahari thornveld in the northwest. The habitats are very varied; it is found growing on dry hillsides, in mixed scrub, wooded valleys, and forest margins, along streams and in coastal bush (Aubrey, 2002, Van Wyk, B and Van Wyk P, 1997).

The plant was mentioned for the first time in 1962 by Watt and Breyers for its use in traditional medicine to treat against cough, cold and chest pain; its leaves were used as soap (Van Wyk, B and Van Wyk P, 1997); the roots are used as a purgative. It is important to note that there is no record of any phytochemical or biological investigation on *B. saligna*.

Picture **3.1:** *Buddleja saligna* tree

3.2 PREVIOUS INVESTIGATION ON OTHER *BUDDLEJA* SPECISES

3.2.1 Phytochemical studies

Previous studies on *Buddleja* species have reported the isolatation of:
- Saponins and glycosides from *Buddleja scordioides* (Avila and Romo de Vivar, 2002)
- Sesquiterpenoids from *Buddleja daviddii* (Yoshida et al., 1976)
- Diterpenoids from *Buddleja albiflora* (Houghton et al., 1996)
- Flavonoids from *Buddleja parviflora* (Arciniegas et al., 1997)
- Flavonoids and phenylethanoids from the leaves of *B. globosa* were found to possess wound-healing properties (Houghton, 1984a,b; Houghton et al., 2001).
- Terpozan, an active compound isolated from *B. parviflora* is used in traditional medicine as an anti-rheumatic agent (Arciniegas et al., 1997).
- Irridoids (Houghton, 1984b; Harborne, 1967).
- Phenylpropanoid: e.g.verbascoside from *B. davidii* (Houghton, 1985)

Worldwide, many *Buddleja* species are used in folk medicine (Arciniegas et al., 1997); the infusion of leaves of *Buddleja madagascariensis*, for example, is drunk to treat asthma, coughs and bronchitis. Therapeutic properties of these herbal remedies were attributed to its constituent triterpenoid saponin, Mimengoside B (Emam et al., 1997).

3.2.2 Biological studies

So far, the therapeutic properties of *B. saligna* are known only in traditional medicine: The infusion of leaves and stems is used by traditional medicine practitioners in the Cape region to treat cough, cold, tuberculosis and chest pain (Montagu Museum, 1998). Therefore, the present study set out to investigate the scientific basis of these claimed therapeutic properties of *B. saligna*.

3.3 RESULTS AND DISCUSSION

3.3.1 Extraction and purification of bioactive compounds

As for the investigation of *O. capensis* (see Chapter 2), it was important to make sure that the method of extraction of *B. saligna* plant material was as close as possible to the one used in traditional medicine, the aqueous infusion of leaves and stems. Thus, before proceeding to the extract with organic solvent systems, an aqueous infusion of *B. saligna* (leaves and stems) was prepared as described in section 2.1.2.2 (A), and tested against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *M. aurum* A+. However, no antibacterial activity was found in *B. saligna*'s aqueous extracts. Following this negative result, an extraction was carried out using a mixture of solvents, acetone/water (4:1, v/v). This solvent system was purposely designed to elute hydrophilic as well as lipophilic constituents from the plant material. A bioactive crude extract was obtained, and then divided using the liquid/liquid separation method described in Figure **3.6.** The hexane fraction (see Table **3.2**), which was found to be the most active, afforded six bioactive fractions by column chromatography: **Fr-BS1** and **Fr-BS2**, which produced the bioactive compounds **BS1** and **BS2**, and **Fr-BS3, Fr-BS4, Fr-BS5** and **Fr-BS6,** corresponding to bioactive mixtures **BS3, BS4, BS5** and **BS6** respectively (**Table 3.2**).

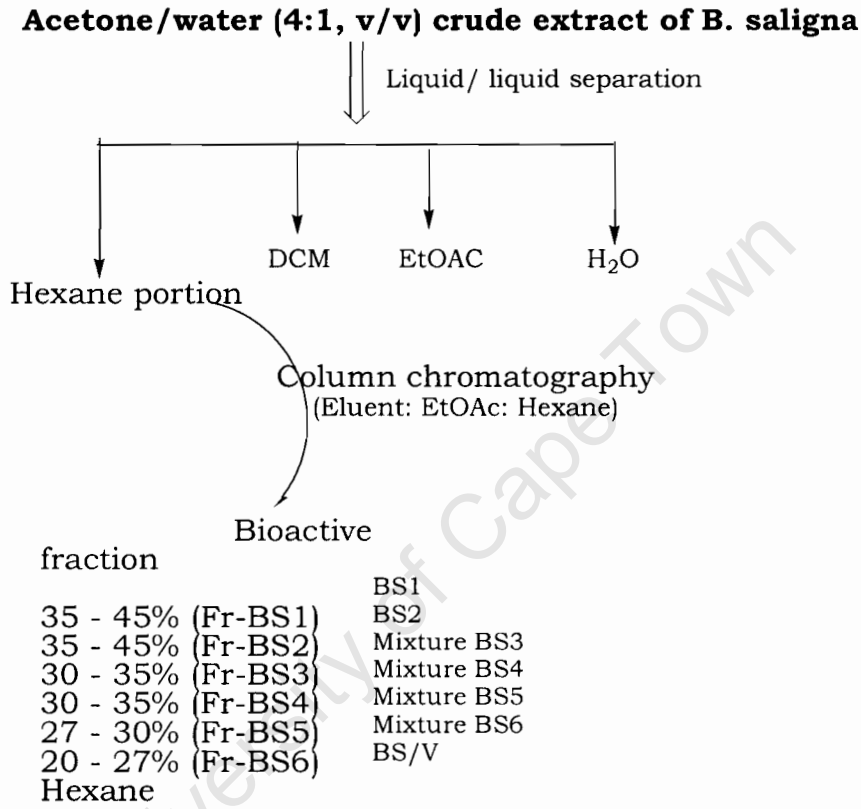
Table 3.1: Bioautography: *B. saligna* bioactive crude extracts

Antibacterial activity of *B. saligna* crude extract (SS = Acetone/water (4:1); (yield: 23.47%)				
Bacteria		*M. aurum* A+	*S. aureus* ATCC 5923	*E. coli* ATCC 25922
Growth inhibtn		+ + +	+ + +	+ + +
Extract fractionation (liquid/liquid separation)				
	Yield	*M. aurum* A+	*S. aureus* ATCC 5923	*E. coli* ATCC 25922
Hexane	2.7%	+ + +	+ + +	+ + +
CHCl2	3.66%	+ +	+ +	+ +
EthAc	2.4%	+	+	+

Legend: + Inhibition of bacteria

The acetone/water (4:1) crude extract was liquid/ liquid divided into hexane, dichloromethane, and ethyl acetate portions. The hexane portion was then column chromatographed on silica using the mixture ethyl acetate/ hexane as eluent (Table **3.2**)

Table 3.2: Bioactive constituents of chromatographed extract fractions



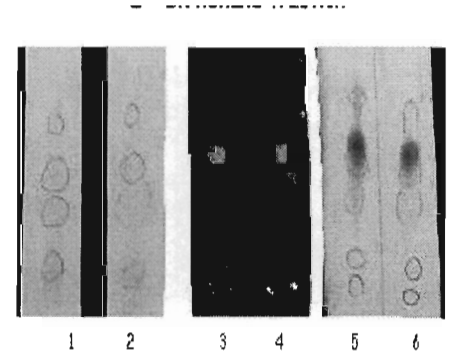
All the bioactive constituents resulted from the fractions ranging from 20% to 40% ethyl acetate in hexane (v/v). In contrast, compound **BS/V** was isolated from hexane fraction.

3.3.2 Antibacterial susceptibility testing of the isolated compounds

The evaluation of the antimicrobial properties of *B. saligna* crude extracts was initially carried out using the colorimetric MTT micro-titre plate method. However, because of the inconveniences (described in section **3.4.4**) the colorimetric micro-titre MTT assay method was abandoned. The assay was then carried out using bioautography on

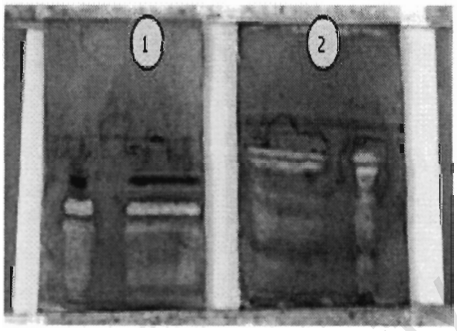
TLC plate method (Picture **3.2**). The results of this assay are detailed in Table **3.3 - 3.5**.

Picture 3.2: Revelation of bioactive compounds spots on TLC plate
(Photo by K. Bamuamba)



TLC monitoring:

1, 2: Spots under UV light
3, 4: Bioactive spots on bioautograph
5, 6: Spots revealed by cerium ammonium sulphate



Bioautography on TLC plates:
Legend

1: TLC of BS, hexane fraction
2: TLC of BS, dichloromethane fraction

Test bacteria

M, aurum A+ (indicator of *Mtb*)

Table 3.3: Bioautography: Anti- *M. aurum* A⁺ activity of *B. saligna*'s constituents

Sample	**Amount (µg/spot)**				
Dosage (µg/spot)	**10**	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *M. aurum* A+				
Rifampicin	-	-	-	-	+
BS6	-	-	-	-	+
BS5	-	-	-	-	+
BS2	-	-	-	-	+
BS3	-	+	+	+	+
BS4	-	-	+	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

Table 3.4: Bioautography: Anti-*S. aureus* ATCC 25923 activity of *B. saligna*'s constituents

Sample	**Activity against *S. aureus* ATCC 25923**				
Dosage (µg/spot)	**10**	**5**	**2.5**	**1.25**	**0.625**
Rifampicin	-	-	-	-	-
BS6	-	-	-	+	+
BS5	-	-	-	-	+
BS2	-	-	-	-	+
BS3	-	-	+	+	+
BS4	-	-	-	-	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

Table 3.5: Bioautography: Anti-*E. coli* ATCC 25922 of *B. saligna*

Sample	**Activity against *E. coli* ATCC 25922**				
Dosage (µg/spot)	**10**	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *E. coli* ATCC 25922				
Rifampicin	-	-	-	-	-
BS1	-	-	-	+	+
BS2	-	-	-	+	+
BS3	-	-	-	+	+
BS4	-	-	-	+	+
BS5	-	-	-	+	+
BS6	-	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

All the test samples showed significant activity against *M. aurum A+* (TB indicator mycobacterium): compounds **BS1, BS2 and BS3** recorded anti-*M. aurum* MIC = 1.25µg/spot, comparable to that of rifampicin. Sample **BS4** was the weakest, anti-*M. aurum* MIC = 10µg/spot, 8 times weaker then rifampicin. BS5 was the second weakest with an anti-*M. aurum* MIC = 5µg/spot. It may be not a surprise that samples **BS4** and **BS5** showed weaker activity since they were mixtures, hence much less pure than **BS1** and **BS2**. The presence of impurities in these samples may be a contributing factor to their weaker anti-*M. aurum* activity.

As for *M. aurum*, all the test samples showed also a significant activity against *S. aureus*. The recorded anti- *S. aureus* MIC was 1.25µg/spot for sample **BS2**, **BS3** and **BS5.** Once again, sample BS4 was the weakest, anti-*S. aureus* MIC = 5µg/spot; BS1 was the second weakest with an MIC = 2.5µg/spot. In contrast with rifampicin showed no activity against *S. aureus* and *E. coli.* All the test samples recorded an anti-*E. coli* MIC of 2.5µg/spot.

3.3.3 Evaluation of the anti-mycobacterial properties of the isolated compounds

The isolated compounds/mixtures were tested against *Mtb* H37Rv (using both disk diffusion and bioautography on TLC plates) and against non-tuberculosis mycobacteria (NTM), *M. microti* ATCC 19422, *M.avium* ATCC 25291, and *M.scrofulaceum* ATCC 19981, using the bioautographic method only. The results of these assays are detailed in Table **3.6 – 3.9**

Table 3.6: Disk diffusion: Anti-*Mtb* activity of *B. saligna*'s constituents

Sample	**Activity against *Mtb* H37Rv**					
Dosage (µg/disk)	**20**	**10**	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *Mtb* H37Rv					
Rifampicin	-	-	-	-	+	+
BS6	-	-	-	+	+	+
BS5	-	-	-	+	+	+
BS2	-	-	-	+	+	+
BS3	-	-	-	+	+	+
BS4	-	-	-	+	+	+

Legend: **+**: blue colour spots, indication of bacteria survival

-: clear zone of bacterial grow inhibition all around the impregnated disk (indication of Significant antibacterial activity)

All the test samples, **BS1**, **BS2**, **BS3**, **BS4**, **BS5**, and **BS6** exhibited significant anti-*Mtb* activity. An anti-*Mtb* MIC of 5µg/disk was observed on the agar plate for all the test samples, and 2.5 µg/ disk for rifampicin.

Bioautography on TLC plate

Purified compounds **BS1, BS2**, and mixtures **BS3, BS4, BS5,** and **BS6** were tested against the test mycobacterial species using bioautography on TLC plates as described in section **3.4.5.1**. The results of these assays are summarized in **Tables 3.7 – 3.10**:

Table 3.7: Bioautography: Anti-*Mtb* H37Rv activity of *B. saligna constit.*

Sample	**Activity against Mtb H37Rv**				
Dosage (µg/Spot)	**10**	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *Mtb* H37Rv				
BS1	-	-	-	+	+
BS2	-	-	-	+	+
BS3	-	-	+	+	+
BS4	-	-	-	+	+
BS5	-	-	-	+	+
BS6	-	-	-	+	+
Rifampicin	-	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

Table 3.8: Bioautography: Antimicrobial activity of *B. saligna* constituents against *M. microti* ATCC 19422

Sample	**Activity against *M. microti* ATCC 19422**			
Dosage (µg/spot)	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *M. microti* ATCC 19422			
BS1	-	-	+	+
BS2	-	-	+	+
BS3	-	-	+	+
BS4	-	-	+	+
BS5	-	-	+	+
BS6	-	-	+	+
Rifampicin	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

Table 3.9: Bioautography: Antimicrobial activity of *B. saligna* constituents against *M avium* ATCC 25291.

Sample	**Activity against *M. avium* ATCC 25291**			
Dosage (µg/spot)	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *M. avium* ATCC 25291			
BS1	-	-	-	+
BS2	-	-	-	+
BS3	-	-	-	+
BS4	-	-	-	+
BS5	-	-	-	+
BS6	-	-	-	+
Rifampicin	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

Table 3.10: Bioautography: Antimicrobial activity of *B. saligna* constituents against *M. scrofulaceum* ATCC 19981

Sample	**Activity against *M. scrofulaceum* ATCC 19981**			
Amount (µg/spot)	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *M. scrofulaceum* ATCC 19981			
BS1	-	-	-	+
BS2	-	-	-	+
BS3	-	-	-	+
BS4	-	-	-	+
BS5	-	-	-	+
BS6	-	-	-	+
Rifampicin	-	-	-	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

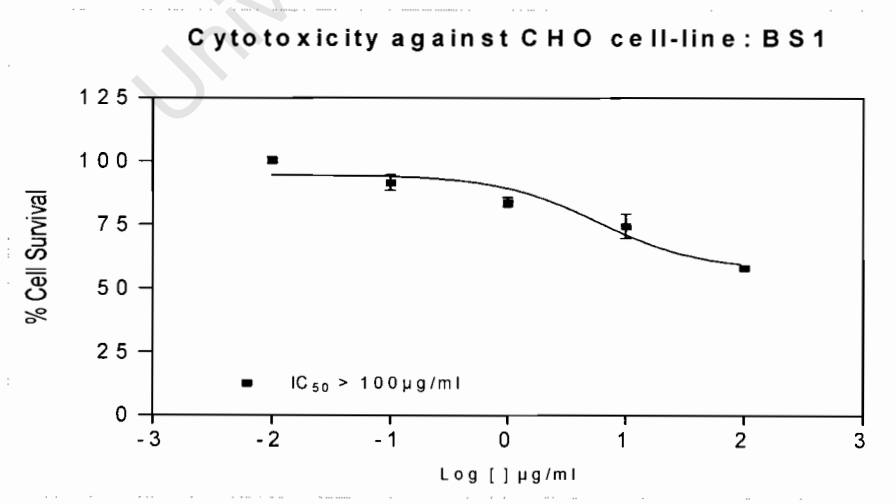
All the test samples showed significant antimycobacterial activity against the test mycobacteria: MIC = 2.5µg/spot against *Mtb* H37Rv (except **BS2**: MICs = 5µg/spot) and *M. microti* ATCC 19422, 1.25µg/spot against *M. avium* ATCC 25291 and *M. scrofulaceum* ATCC 19981. All the test samples were twice more active against *M. avium* ATCC 25291 than rifampicin.

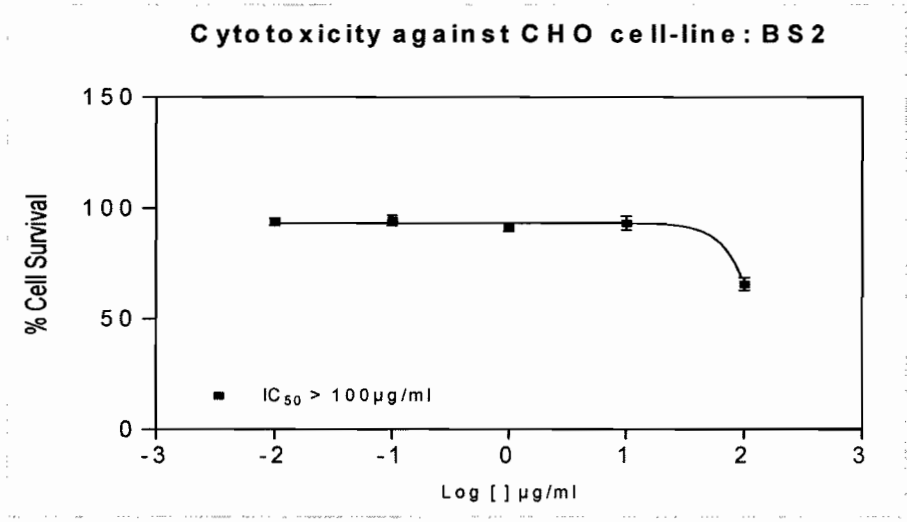
3.3.4 In vitro cytotoxicity testing

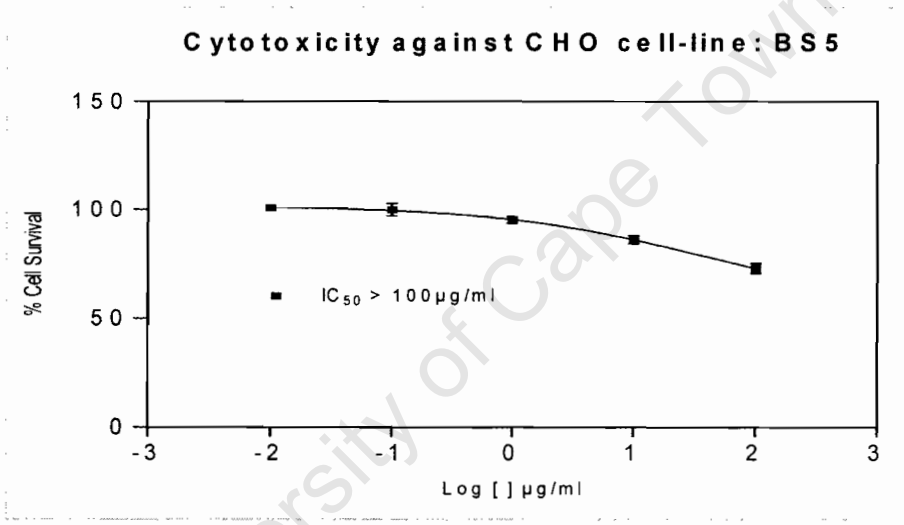
The aim of the in vitro cytotoxicity test was to measure the damage the antibacterial compounds cause to normal living cells. It allows one to determine whether the agent could be used for therapeutic purposes without excessive damage to host cells; thus the inhibitory concentration of the bioactive agent is determined (IC_{50}), an IC_{50} value greater than 100mg/ml was considered non toxic to normal cells.

As indicated by the dose-response graphs (Figure **3.1**), the IC_{50} values of the isolated compounds (BS1, BS2, BS3 and BS4) could not be determined since more than 50% cell survival was observed up to a concentration of 100 µg/ml. All the isolated compounds/mixtures killed less than 50% of CHO cells even at a concentration as high as 100µg/ml, with the exception of mixture **BS3**, which killed about 50% of the cells at a concentration of 41.4µg/ml. According to these results, the tested bioactive compounds/ mixtures can be considered no-toxic to CHO cells.

Figure 3.1: Dose response graphs: In vitro cytotoxicity against CHO cell







CHO IC_{50} = sample quantity ($\mu g ml^{-1}$) that kills 50% of CHO cells.
IC_{50}s(50% inhibitory concentration) were obtained using non-linear dose-response curve fitting analyses (Graph Pad Prism v.2.01 software)
IC_{50} – value is the average of the means of three experimental results.

Table 3.11: Antimycobacterial activity and CHO IC_{50}

Compound	**Anti-mycobacterial activity: MIC** (µg/spot) (Bioautography on TLC plates)				**CHO IC_{50}** (µg/ml)
	Mtb H37R**v**	***M. avium*** ATCC 25291	***M. scrofulaceum*** ATCC 19981	***M. microti*** ATCC 19422	
BS1	2.5	1.25	1.25	2.5	> 100
BS2	2.5	1.25	1.25	2.5	> 100
BS3	5	1.25	1.25	2.5	> 41.4
BS4	2.5	1.25	1.25	2.5	> 100
BS5	2.5	1.25	1.25	2.5	> 100
BS6	2.5	1.25	1.25	2.5	> 100
Emetine					0.07

3.3.5 Structure elucidation of the isolated compounds

Complete elucidation of the molecular structures of the isolated compounds was determined from analysis of NMR data (one-dimensional, ^{1}H, ^{13}C, and DEPT experiments, and two-dimensional, COSY, HSQC, and HMBC), and MS data (HREIMS and FABMS), and compared with data from the literature. Physicochemical properties such as solubility of the compounds, melting points, colours and smells were also important features.

3.3.5.1 Elucidation of the molecular structure of compound BS-V

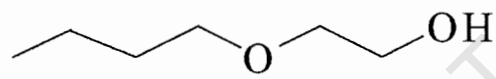
Compound **BS-V** was isolated from hexane fractions of the acetone/ water (4:1, v/v) of the crude extract of *B. saligna* (leaves and stems) as a colourless liquid at room temperature, soft smelling like an essential oil, Rf = 0.755. The isolation was carried out by preparative TLC (silica gel 60 on glass plates) eluted with hexane/ethyl acetate (7:5, v/v). **^{1}H NMR** data: the integration of the ^{1}H NMR peaks of **BS-V** showed a total of 14 protons. These included a broad singlet at δ3.63, which disappeared on D_2O-wash spectrum suggesting the presence of the hydroxyl group in the molecule. The presence of this hydroxyl group was seen as a deshielding of the shift of carbon down to ~δ71.9. **^{13}C NMR** showed a total of six peaks, which corresponded to six carbons of **BS-V** molecule. DEPT ^{13}C NMR data indicated 6 all protonated carbons, of which, five CH_2-, one CH_3, and zero CH. At this point, a complete assignment was easily achieved on the basis of HSQC, HMBC, Cosy, and compound **BS-V** was identified as 2-butoxyethanol. Comparison of ^{1}H and ^{13}C **NMR** data of **BS-V** with data from commercial available 2-butoxyethanol (99.98% ethylene glycol mono-butyl ether, Sigma, catalogue No: E-0883; Table **3.11**), confirmed that **BS-V** was 2-butoxyethanol.

Table 3.12: ^{13}C NMR spectrum assignment of BK-V

		2-butoxyethanol (Sigma, cat. No: E-0883)		**BS-V**
	Carbon	^{1}**H** (ppm)	^{13}**C δ**(ppm)	^{13}**C δ**(ppm)
1	$HO\text{-}CH_2\text{-}$	3.4 - 3.63 (m, 3H)	71.7	71.7
2	$\text{-}CH_2O\text{-}$	3.70 (m, 2H)	71.9	71.0
3	$\text{-}CH_2\text{-}$	3.80 (m, 2H)	61.8	61.8
4	$\text{-}CH_2\text{-}$	1.58 (m, 2H)	31.5	31.6
5	$\text{-}CH_2\text{-}$	1.37 (m, 2H)	19.0	19.2
6	$\text{-}CH_3$	0.87 (s, 3H)	13.6	13.8

[1]

^{13}C NMR data were measured at 75 MHz with the samples dissolved in CDC.. 2-butoxyethanol (Sigma) ^{13}C NMR data are listed here for comparison.



2-butoxyethanol

Butoxyethanol (BE) is a known hazardous and toxic synthetic agent, which does not build up, in biological material (ATSDR, 1998; WHO/CICADS, 1998; WHO/IPCS/ILO, 1993; U.S/ EPA, 2002; NJ/DHSS, 2001). It is miscible with water and soluble in most organic solvents (ATSDR, 1998) and can be easily absorbed through the skin to cause harmful effects. The isolation of 2-butoxyethanol from plant material has not been reported in the literature. The isolation of compound BS-V (2-butoxyethanol) from *B. saligna* plant material came as a surprise to the investigator, given the fact it is a synthetic agent that doesn't build up in biological material. However, some one can speculate that BS-V may be an impurity from the solvent system or from the plant material, which might have been soiled by chemical in use in the laboratory.

3.3.5.2 Structure Elucidation of compound BS1

Compound **BS1** was isolated as a whitish amorphous powder; mp. 280–282 °C, soluble in chloroform, precipitates from methanol. Compound **BS1** was one of seven bioactive constituents that were isolated from the extracts of *B. saligna* (leaves and stems). The five others, **BS2, BS3, BS4, BS5,** and **BS6** were found to be mixtures on TLC.

Preliminary analysis of NMR spectra (^{1}H and ^{13}C NMR spectrum: 30 carbon's peaks), MS spectra (EI MS and FAB), and physicochemical proprieties such as m.p. and solubility of the compound readily suggested that **BS1** was a hydroxylated pentacyclic triterpenoid of the urs/oleanolic acid type. Full details of the structure elucidation of **BS1** are given below to provide the basis for analysis of the other structures.

Figure 3.2: Fragmentation of pseudo molecular ion $[M+1]^{+}$ into A, B & C

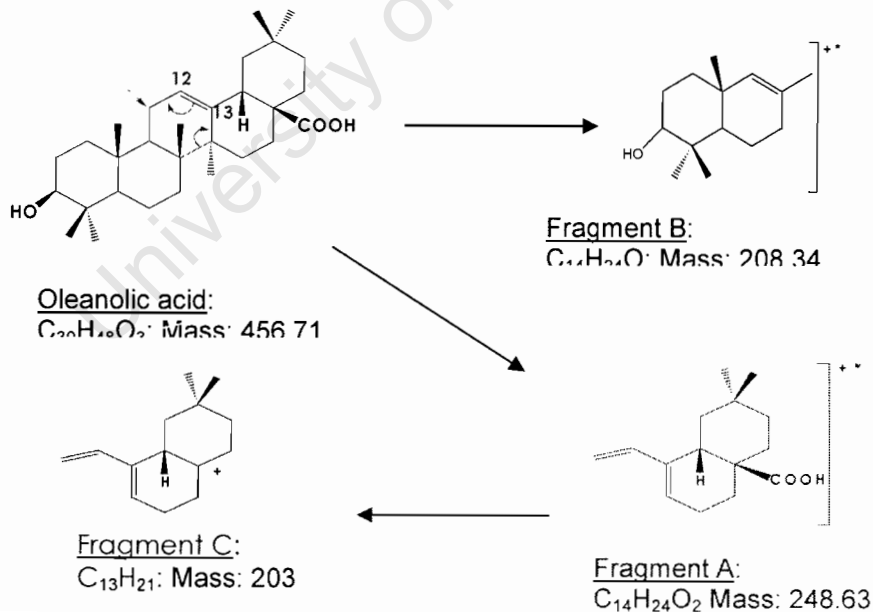
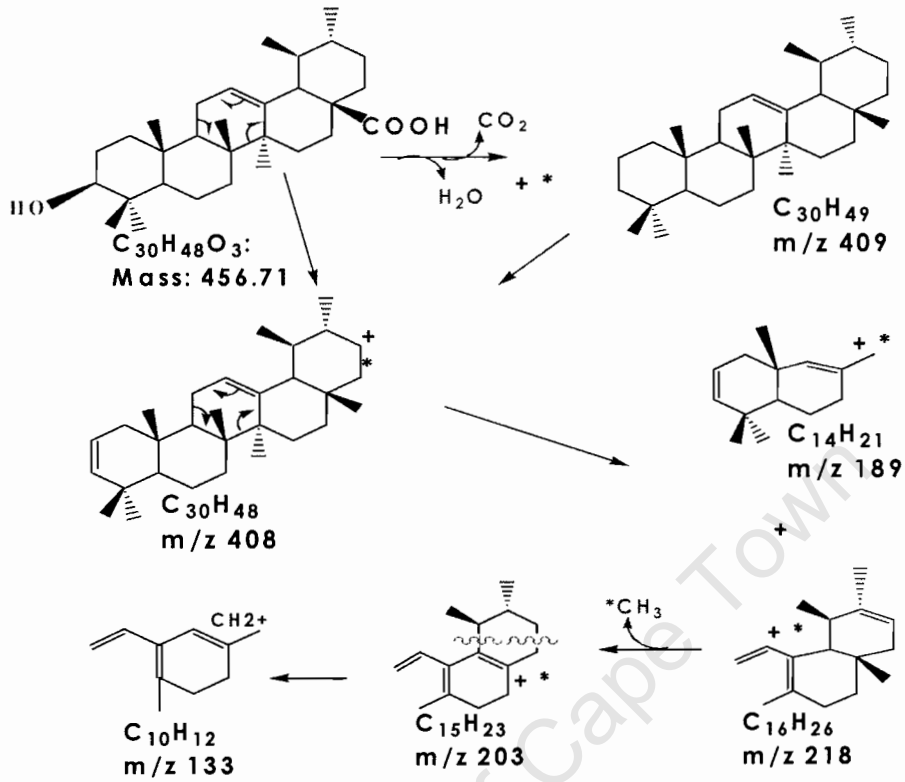


Figure 3.3: Mechanism of fragmentation of Urs/Oleane-12-en (Budzikiewicz et al., 1963)

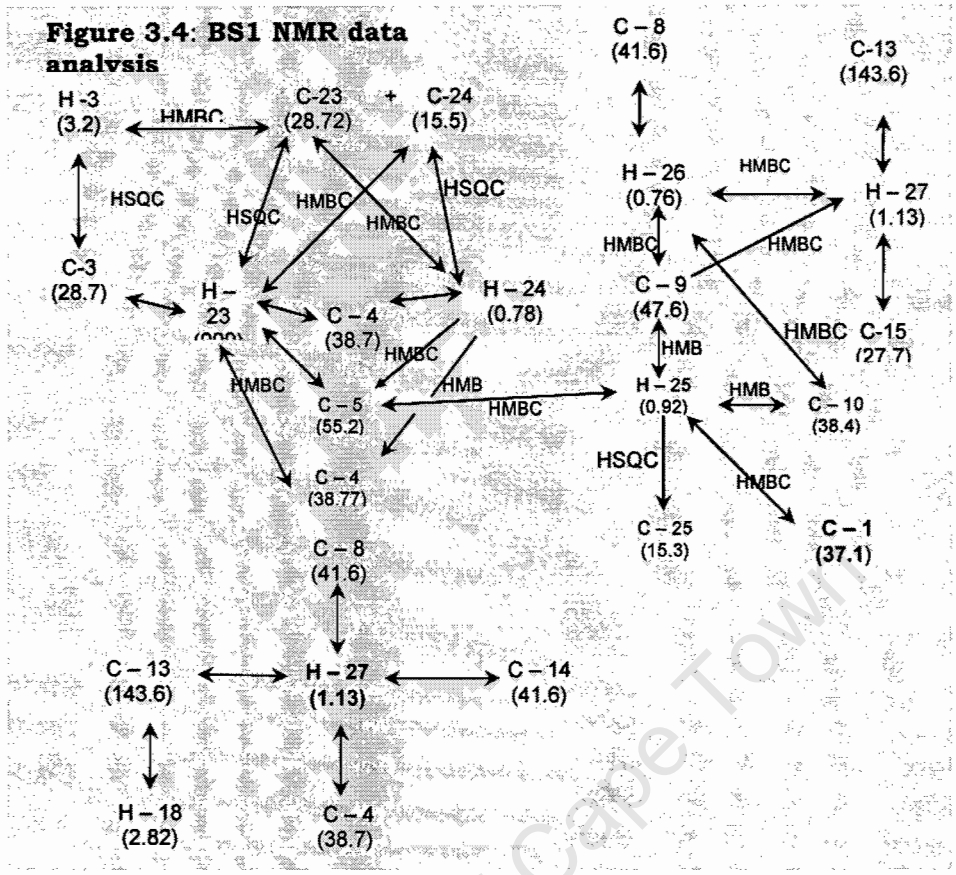


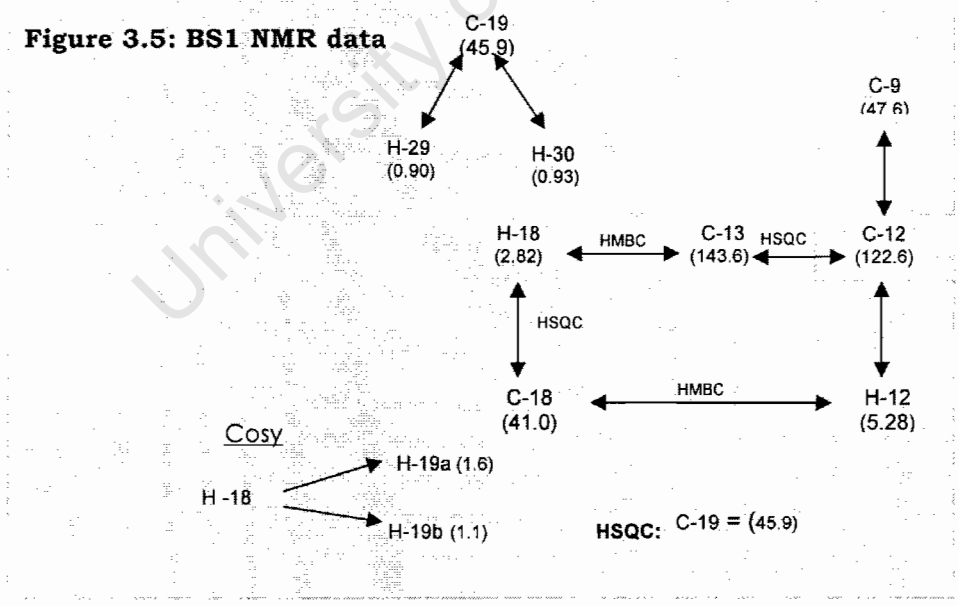
The MS spectrum of compound **BS1** showed a mass fragmentation pattern similar to the one that Budzikiewicz et al., (1963) described as characteristic of a triterpenoid pentacyclic molecule that contains an ethylenic function at C12 –C13 (see Figure **3.2 – 3.3**). The MS spectrum ($DCINH_3$ + isobutene negative mode) showed a major peak at m/z 455 [100%) that could correspond to the molecular ion fragment $[M - H]^+$ (i.e.: $C_{30}H_{47}O_3$), and a peak at m/z 458 that could correspond to $[M + 2H]^+$ (i.e.: $C_{30}H_{48}O_3^+$). The fragment at m/z: 440 ($C_{30}H_{48}O_2$) could be attributed to a loss of H_2O from the fragment at m/z 458, thereby suggesting the presence of an hydroxyl group in the molecule. Other meaningful peaks were observed at m/z 207, 203, 189, and at m/z 133. These fragments considered as fingerprints of an urs/oleane skeleton (Budzikiewicz et al., 1963). A complete assignment of the ^1H and ^{13}C was achieved by analysis of 1D and 2D NMR data. **^{1}H NMR** spectrum showed seven singlets (each integrating for three protons), which

corresponded to seven methyl group signals. A signal at δ 3.2 (dd, J = 6.4, 13.6 Hz, 1H) suggested the presence of a -OH group based on the chemical shift and its coupling patterns, which were typical of an α-axial proton adjacent to a methylene group in a six-membered ring in the chair conformation. It meant that the concerned proton was attached to C-3 that is adjacent to the quaternary carbon C-4 with its attached geminal dimethyl group. On the basis of HSQC C-3 was easily identified at δ 79,0 which is consistent with downshielded shift due to the hydroxyl group. These assignments were further confirmed by the correlations observed on HMBC spectrum between H-3 and two methyl carbons, C-23 (δ 28.7) and C-24 (15.5), as well as quaternary carbon C-4 (δ15.5). From HSQC the chemical shifts of H-23's at δ0.99 ppm and H-24's at δ 0.78 ppm could then be established, and HMBC showed that both these sets of methyl protons were long range coupled to C-4 (δ 38.4) and C-5 (δ 41.6). C-5 is coupled to H-25 (δ0.92), (with C-25 (δ 15.3) identifiable from HSQC, and H-25 is long-range coupled to C-10 (δ 38.4), C-9(δ 47.6), and C-1(δ 37.1).

The HMBC also revealed that C-9 and C-10 were both correlated to H-26 (δ 0.76), which correlated with C27 (δ 25.9), and C-8 (δ 41.6). The latter is long-range coupled with H-27 (δ 1.13).

HMBC established also the connectivity between H-27 and olefinic carbon C-13 (δ 43.6), as well as C-14 (δ 41.6) and C-15 (δ 27.7), and HSQC confirmed the assignment of C-27 (δ 25.9).





From the ethylenic proton H-12, the carbon C-18 (δ 41.0) was easily identified in the HMBC spectrum and hence, by HSQC, the characteristic dd for H-18 at δ 2.82 was identified. A summary of the

connectivities observed on the HSQC, HMBC and COSY spectra is givene in Figures **3.4** and **3.5**, and discussed below. In addition, the HMBC established correlation between H-18 to C-12 and C-13. HSQC allowed establishment of the correlation between C-18 and H-18, and by COSY revealed the latter coupling to H-19a (δ 1.6) and H-19b (δ 1.1). The presence of two distinct protons – and absence of a methyl group – on this carbon rules out the presence of the ursane skeleton. After identifying C-19 at δ 45.9 by HSQC, this was shown (HMBC) to correlate to H-29 (δ 0.90) and H-30 (δ 0.93), providing strong evidence for the geminal dimethyl group at C-20 in the oleane skeleton. Although it was impossible to unambiguously establish all the connectivities, enough evidences were gathered from NMR and MS data analysis for concluding that **BS1** was **oleanolic acid.** This was later confirmed by comparing the experimental ^{13}C NMR data of **BS1** with the literature (see **Table 3.13**). Another confirmation was obtained by comparing **BS1**'s melting point (282 - 284°C) with AO m.p (280 – 282 °C).

Table 3.12: Connectivity observed on BS1 HSQC and HMBC spectra

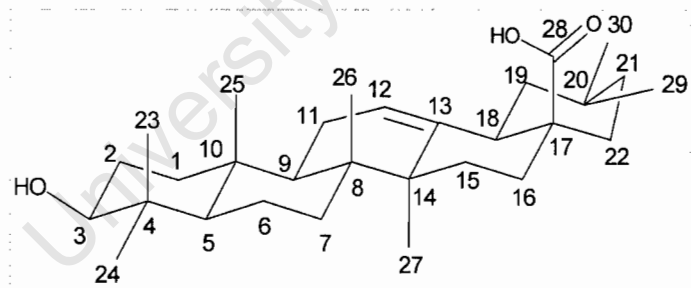
Proton	Connectivities observed	Proton	Connectivities observed
H-1	C-2, C-3	H-22	C-17, C-18
H-2	C-4	Me-23	C-3, C-4, C-5, C-24
H-3β	C-2, C-5	Me-24	C-3, C-4, C-5, C-23
H-5	C-4	Me-25	C-1, C-5, C-9, C-10
H-6	C-5	Me-26	C-7, C-8, C-9, C-14
H-9	C-8, C-10, C-25, C-26	Me-27	C-8, C-13, C-14, C-15
H-16	C-28	Me-28	C-16, C-17
H-18	C-13, C-19, C-20, C-28	Me-29	C-19, C-28
H-19	C-18, C-20, C-21,	Me-30	C-17, C-18, C-22
H-21	C-17, C-18, C-19, C-20		

^{13}C NMR assignment of **BS1** (in $CDCl_3$), comparison with methyl oleanolate, Takeoka et al (2000) BS1 ^{13}C data were measured at 75 MHz and ^{1}H at 300 MHz).

Table 3.13: ^{13}C NMR assignment of **BS1** in $CDCl_3$
(comparison with methyl oleanolate's data)

Methyl oleanolate			Compound BS1	
Carbon	**^{13}C: δ(ppm)**	**Carbon**	**^{13}C: δ(ppm)**	**Carbon**
16	CH2	22.97		22.96
17	C	46.56		46.56
18	CH	41.05	2.82 (dd; *J* = 4.8, 7.2 Hz)	41.04
19	CH	45.94		45.93
20	CH	30.67		30.67
21	CH2	33.85		33.84
22	CH2	32.48		32.48
23	CH3	28.12	0.99(s)	28.11
24	CH3	15.53	0.78(s)	15.53
25	CH3	15.33	0.92(s)	15.32
26	CH3	17.17	0.76(s)	17.16
27	CH3	25.94	1.13(s)	25.93
28	C=O	183.19		183.35
29	CH3	33.05	0.90(s)	33.05
30	CH3	23.59	0.93(s)	23.58
31	CH3	51.25		

BS1 ^{13}C NMR assignment (in $CDCl_3$), comparison with methyl oleanolate, Takeoka et al (2000). ^{3}C data were measured at 75 MHz and ^{1}H at 300 MHz).



Oleanolic acid

Oleanolic acid (OA) and its homologue, ursolic acid (UA), are extensively distributed in plants. They are known for a variety of biological activities including anti–inflammatory activity, antifungal, antiviral, and anti-tumour activity (Li and Wei-Jian, 2002). The anti-tumour activity of OA and UA is based on a mechanism of action that involves the inhibition of the DNA polymerase and DNA topoisomerases (Li et al., 2002). It is possible that the same mechanism of action is involved the anti-*Mtb* activity of the compounds.

3.3.5.3 Structures elucidation of BS2, BS3, BS4, BS5, BS6

Significant antimycobacterial activity were also recorded for the samples **BS2, BS3, BS4, BS5,** and **BS6**, which resulted from chromatographed fractions of the acetone/dichloromethane (9:1) crude extract of *B. saligna* (leaves and stems), **Fr-BS2, Fr-BS3, Fr-BS4**, **Fr-BS5**, and **Fr-BS6** respectively. In vitro cytotoxicity testing founded **BS2, BS3, BS4, BS5,** and **BS6** to be no toxic against CHO cells line. Although the NMR data of **BS2, BS3, BS4, BS5,** and **BS6** presented similar patterns than that of compound **BS1**, the TLC revealed that were rather mixtures containing **BS1** and related structures, with the **BS1** being the most preponderant constituent.

3.3.6 Discussion

All the isolated bioactive compounds from *B. saligna* (leaves and stems) were pentacyclic triterpenoids, type ursolic or oleanolic acid and their alkylated derivatives. Thus, the hydrophobic nature of these compounds lead to their poor diffusion through hydrophilic agar media. In contrast, the methanolic rifampicin solution diffused more easily in hydrophilic bacterial culture medium. As a result: two things were observed:

1) The growth inhibition zone around the disk impregnated with rifampicin was greater (anti-*Mtb* MIC: 2.5 µg/spot) than the test samples (anti-*Mtb* MIC: 5 µg/spot) on disk diffusion plates.

2) The anti-*Mtb* MICs measured using the diffusion technique were found to be greater than the those observed using bioautography on TLC plates, both for test samples and the positive control, rifampicin.

The hydrophilic or hydrophobic properties of the sample do not affect the results on bioautographic plates. Furthermore, antibacterial effects of the samples are more easily measurable (white spots on a blue background) on bioautographic plates than agar plates, especially in the

case of the slow-growing *Mtb*. Another important advantage of bioautography is that the duration of the experiment is considerable reduced, maximum 48 hours for anti-*Mtb* assay using bioautography, compared to three to six weeks when using the disk diffusion method. The anti-*Mycobacterium* activity of bioactive compounds isolated from *B. saligna* was comparable to rifampicin. These results show that both OA and UA possess anti-*Mycobacterium* properties. Indeed UA and its derivatives have been reported to have anti-microbial activity against *S. aureus*, Gram-negative bacteria and the fungus, *Microsporium lenosum* (Zaletova et al., 1986). UA also inhibits some food-associated bacteria and yeasts (Collins and Charles, 1987); it is also active against herpes simplex virus (Poehland et al., 1987). Oleanolic acid-type saponins also showed a broad-spectrum antifungal activity (Favel et al., 1994). However, UA and OA are lipophilic compounds, their correspondent glycosides or saponins are soluble in hydrophilic solvents (Avilla and Romo de Vivar, 2002). These hydrophilic derivatives may act as pro-drugs, with the active principles being their aglycon moieties; this may explain the therapeutic effects of *B. saligna* aqueous extracts.

3.3.7 Conclusion

B. saligna (leave and stems) contains pentacyclic triterpenoids, oleanolic acid and its alkylated derivatives, which possess significant activity against *Mtb* and the NTM, *M. avium* ATCC 25291, *M. microti* ATCC 19420 and *M. scrofulaceum* ATCC 19981. The fact that *B. saligna* bioactive constituents showed no toxicity against CHO cells should encourage further investigations that would assess their potential for development as anti-tuberculosis drugs. The finding of this investigation has given a scientific explanation for the alleged therapeutic effects of *B. saligna* plant-remedies that used in traditional medicine for the treatment of infectious diseases such as bronchitis and pulmonary tuberculosis.

3.4 EXPERIMENTAL

3.4.1 General

B. saligna (leaves and stems) (Picture **3.2**) were collected on the 15 March 2001 from Kirstenbosch National Botanical Garden in Cape Town with the help of Mr. Ivan Manuel, the Garden's curator. This plant species was authenticated by Mr H. Trinder Smith, the curator at the Bolus Herbarium, Department of Botany, University of Cape Town. A voucher specimen [K. Bamuamba, sn. (BOL)] was deposited at the Bolus Herbarium.

Picture 3.2: Leaves and stems of *B. saligna*

3.4.2 Preparation of Crude Extracts

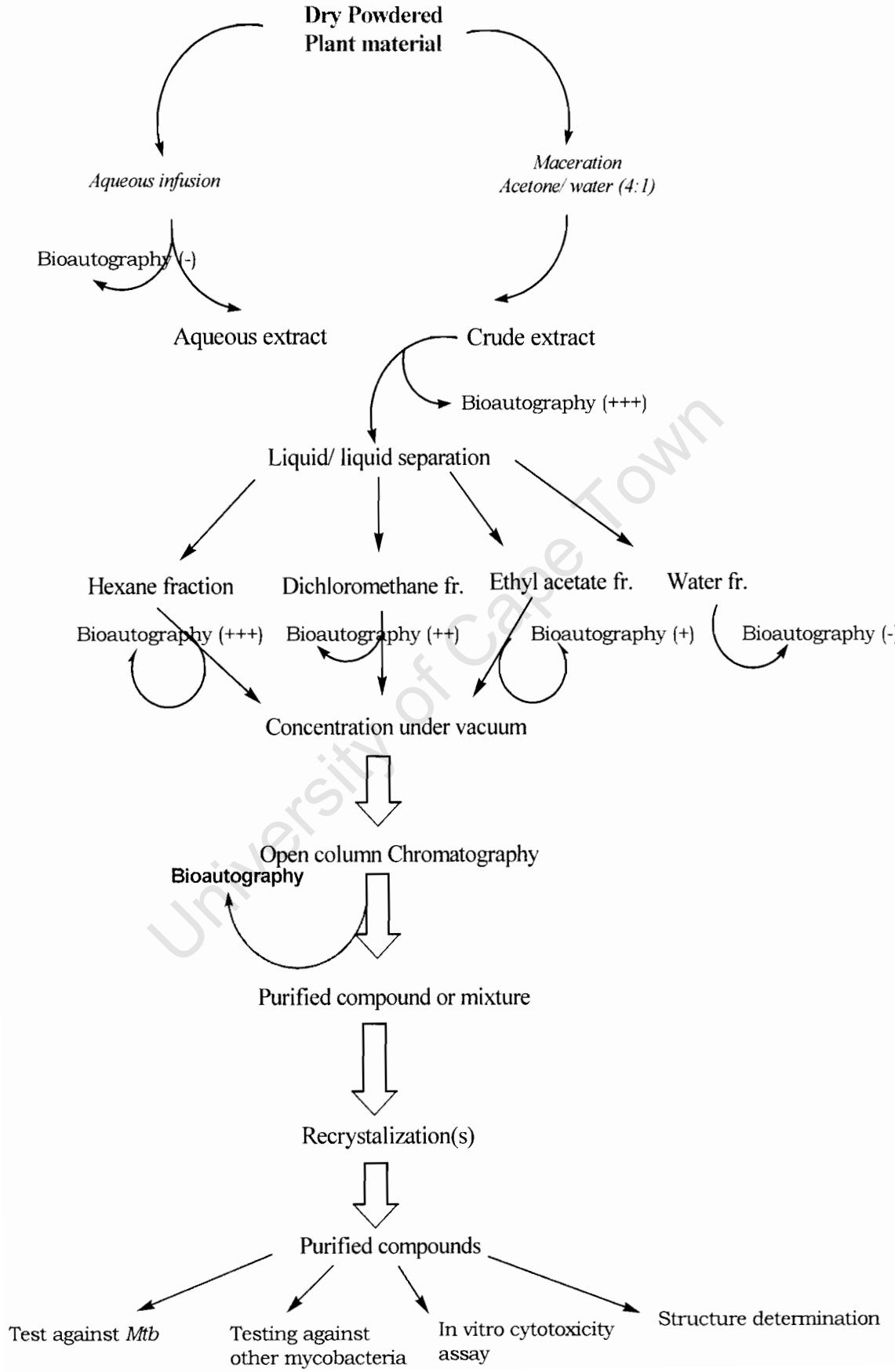
The collected plant material was air-dried at 37 °C for 72 hours, ground and sieved through an 850-micron mesh-sieve. Solvent extraction was carried out on dry powdered plant materials. Three different methods of extraction were used in this investigation in an attempt to improve the yield of bioactive compounds (Figure **3.6**):

A) The aqueous infusion of *B. saligna* (leaves and stems) is reportedly used by traditional medicine practitioners in the treatment of cough, cold and higher respiratory track infectious diseases and TB (Montague Museum, 1998). The same method of extraction was followed in order to verify the alleged therapeutic properties of the aqueous extracts of *B. saligna* (leaves and stems).

B) The second method of extraction, maceration with a mixture of acetone/water (4:1) was intended to extract lipophilic and hydrophilic compounds. The protocol was:

Dry leaf and stem material of *B. saligna* was extracted at room temperature with acetone/water (4:1 (v/v), 1000ml) in a 2.5L flask. The mixture was shaken for 15 to 20 minutes and allowed to stand overnight. The resultant supernatant was filtered through filter paper (Whatman No 1) and the residue was retained in the flask and extracted with a further two portions of acetone/water (4:1 (v/v), 1000ml) for 6 hours each. The combined three acetone/water extracts were evaporated under vacuum to obtain a concentrated aqueous suspension, which was then transferred into a separating funnel and divided into 4 extract fractions by sequentially extracting (liquid/ liquid separation) with hexane, dichloromethane, and ethyl acetate (3x100ml of each). The remaining aqueous solution was freeze-dried, and the residue re-suspended in methanol.

Figure 3.6: Scheme of the extraction and purification of bioactives



3.4.3 Bioassay-guided fractionation of plant extracts: Isolation and Purification of plant bioactive compounds

Preliminary antibacterial susceptibility tests were conducted on crude extracts using bioautography on TLC plates to determine whether the plant materials possessed bacterial activity. This step was followed by the isolation and purification of the bioactive constituents and the determination of their respective MICs against test bacteria. The hexane extract fraction (most active of all) that resulted from liquid: liquid fractionation of the acetone/water (4:1) crude extract of *B. saligna* (leaves and stems, 13g) was open-column chromatographed in silica gel 60. The column was washed with n-hexane (500 ml) to elute unwanted fatty substances, and then with a mixed solvent system made up of ethyl acetate in hexane (1:5). The ratio of ethyl acetate was sequentially increased by 2.5% every 500ml. Chromatographic fractions were collected in small quantities of 25ml each. The progress was monitored by TLC on Merck silica gel 60 F_{245}. Visualisation was accomplished under UV light (254 nm and 365 nm) and/or by spaying with anisaldehyde/sulphuric acid (1:1) or cerium ammonium sulphate reagent followed by heat (100 °C). Ceric ammonium sulphate is a powerful oxidant; it reacts non-specifically with most phytochemicals resulting in dark brown spots on the plate following heating.

After column chromatography, similar eluted fractions were combined and bio-tested against *M. aurum*. Inactive extract fractions were discarded to retain only bioactive fractions for further purification by column chromatography and/ or by recrystalization.

3.4.4 Cultivation of bacteria

Test bacteria, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *M. aurum* were grown overnight in 2YT broth medium at 37 °C with agitation (Sambrook et al, 1989).

Cultivation of Mtb H37Rv

Mtb H37Rv was grown at 37°C as 10ml standing culture (Middlebrook 7H9 Broth culture medium) for 14 days with intermittent agitation (Chung et al., 1995).

All the assays on *Mtb* H37Rv were carried out in a Biosafety Level 3 laboratory in the Department of Medical Microbiology, University of Cape Town. The purity of the *Mtb* H37Rv culture was checked by a Ziehl-Neelsen stain priory to bioautography.

The MTT colorimetric assay

The MTT colorimetric assay is based on the reduction of MTT (3-4, 5 dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide; also called thiazolyl blue) by metabolically active cells. Only living cells have the ability to reduce thiazolyl blue to its formazan derivative. The formation of the formazan precipitate is measured by reading the absorbance at 620 nm. This method was sensitive to colours other than that of formazan, including the colour of the extract and media. Thus, the final absorbance measured did not reflect the formation of formazan alone, but a mixture of components. Another inconvenience resulted from the insolubility of the lipophilic bioactive compounds in the hydrophilic microbial culture medium and the aqueous MTT solution, which required the addition of DMSO to an amount that is toxic to the bacteria. For these reasons, the micro-titre –MTT method was found to be inappropriate for this assay and was therefore abandoned.

Advantages of using bioautography on TLC plate method

In contrast, the bioautography on TLC plates was a more appropriate method because of its simplicity, sensitivity and compatibility with lipophilic molecules. It allows determination of the antibacterial activity

of an agent within a short time of 6 hours of contact between the agent and the test bacterium. In the case of the slow-growing *Mtb,* a longer period of incubation was needed after the MTT solution was applied to the bioautographic plates in order to allow the blue colour to develop. Thus, anti-*Mtb* bioautography was conducted over 48 hours: 24 hrs for the contact between sample and *Mtb* plus 24 hrs to allow the formazan colour to develop after the application of the MTT solution on TLC plates. The MICs measured on agar plates (disk diffusion method) were greater than those measured on the bioautograms.

3.4.5 Evaluation of anti-*Mtb* properties of the isolated compound

The isolated compounds/mixtures were tested against *Mtb* H37Rv (using both disk diffusion and bioautography on TLC plates) and against ntm, *M. microti* ATCC 19422, *M. avium* ATCC 25291, and *M. scrofulaceum* ATCC 19981, using the bioautographic method only.

3.4.5.1 Bioautography of isolated compounds against *Mtb* H37Rv

The bioautographic assays was carried out on two separate occasions for the evaluation of the anti-*Mtb* properties of the test samples in order to verify the reproducibility of the results. The protocol of this experiment was:

10µl of sample solutions (dissolved in dichloromethane at different concentrations: 2mg/ml; 1mg/ml; 0.5mg/ml; 0.25mg/ml; 0.125mg/ml; and 0.0625mg/ml) were spotted in five replicates each on a TLC plate (silica gel 60 on aluminium sheet). Rifampicin (Sigma, catalogue No: R3501) was used as a positive control for antimycobacterial activity. The spotted plates were left to dry completely. A 10 – 14 days-old *Mtb* H37Rv culture was spread on the spotted TLC plates (bacterial culture applied using sterile non-absorbent cotton wool). The inoculated TLC plates were placed in sealed plastic boxes containing damp paper towel, which

ensured sufficient humidity to prevent the plates drying out. The boxes were then incubated for 24 hrs at 37°C, after which an MTT aqueous solution (0.25% in sterile water) was spread on the plates using sterile cotton wool, as for the bacterial culture. The plates were incubated again at 37°C for 24 hrs in order to allow the formazan blue colour to develop. A clear spot on a blue background is indicative of the anti-*Mtb* activity of the compound present in that spot.

3.4.5.2 Disk diffusion method

Sterile filter-paper disks (Whitman 3 MM; 6 mm diameter) were each impregnated with 10µl of a sample solution (sample dissolved in dichloromethane at different concentrations: 2mg/ml; 1mg/ml; 0.5mg/ml; 0.25mg/ml 0.125mg/ml; and 0.0625mg/ml). The impregnated disks were left to dry completely. A 10 – 14 days-old *Mtb* H37Rv culture suspension (100µl) was spread on the surface of Middlebrook 7H9 agar plates (containing 0.2% glucose) using a sterile glass spreader. After fifteen minutes, the impregnated disks were placed on the agar. The culture plates were sealed in plastic bags and incubated at 37°C for three weeks, after which the culture-growth was clearly visible on the agar. A clear zone visible all around the impregnated-disk indicated the sample's inhibitory effects.

3.4.6 In Vitro Cytotoxicity Testing

The assay for in vitro cytotoxicity was performed on Chinese Hamster Ovarian (CHO) cells using the MTT colorimetric assay (Mosman, T., 1983). CHO cells are mammalian cells, they are easy to grow and handle. Emetine dihydrochloride (Sigma, catalogue No: E 2375) was used as a positive control, its solution was made up in sterile water and serially diluted in the cell culture medium to the required concentrations.

3.4.7 Structure determination: NMR and MS experiments

The NMR experiments were conducted with samples dissolved in $CDCl_3$ at 30 °C on a Variant Mercury-300BB spectrometer. The ^{1}H NMR spectra were recorded at the frequency 300.07567 MHz and the ^{13}C NMR at 75.4537 MHz.

The MS experiments were carried out using FAB in both negative and positive mode.

CHAPTER IV

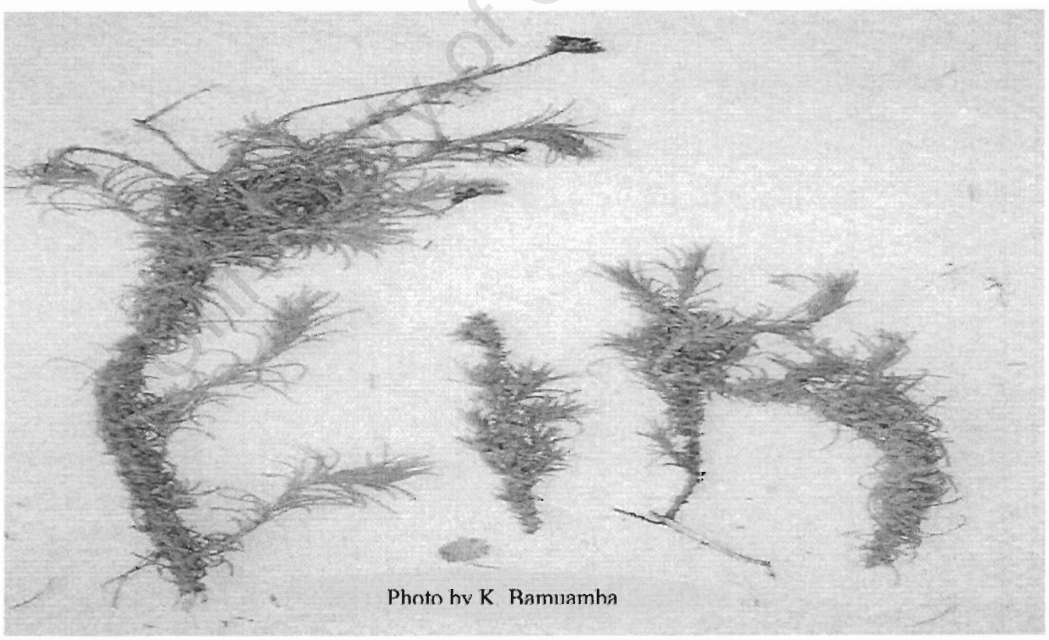
INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF *LEYSSERA GNAPHALOIDES* (L.) AND *INULA GRAVEOLENS* (L)

4.1 INVESTIGATION OF THE ANTIBACTERIAL PROPERTIES OF *LEYSSERA GNAPHALOIDES* (L.)

4.1.1 PLANT DESCRIPTION AND OCCURENCE

4.1.1 Plant description and occurrence

Leyssera gnaphaloides (**Picture 4.1**) belongs to the *Asteraceae* family (Daisy family). It is a tomentose-glabrescent shrublet that reaches 0.2 to 0.5m high; leaves are densely set on short crowded lateral branchlets. *L. gnaphaloides* is common on flats and lower mountain slopes of the Southern Cape region of the Western Cape province, South Africa (Bremer, 1978). It also occurs in a wide arc extending from Southern Namibia to the Eastern Cape.



Picture 4.1: *L. gnaphaloides* plant

4.1.2 PREVIOUS INVESTIGATIONS OF *L. GNAPAHLOIDES*

4.1.2.1 Phytochemical studies

Previous studies on *L. gnaphaloides* have reported the isolation of pentaynene and polyacetylene compounds, which are also common to the *Inuleae* family (Bremer, 1978; Harborne, 1977). Bohlmann and Zdero isolated a benzofurane derivative named Leysseral-angelicate from the leaves of *L. gnaphaloides*. The Leysseral-angelicate was found to be a constituent of many other *Leyssera* species (Bohlmann and Zdero, 1972). In addition, Tsichristzis and Jakupovic (1991) reported the isolation of kauren-18-oic acid and its corresponding alcohols, diterpenoid labdanes, 13-epimanoloide derivatives and an akur-15-ene derivative (Tsichristzis and Jakupovic, 1991; Gonzales et al., 1973).

4.1.2.2 Pharmacological properties

L. gnaphaloides was reportedly used in traditional-medicine in the Cape region (South Africa) in the treatment of bronchitis, cough, cold, and pulmonary tuberculosis (Montagu Museum, 1998).

The objectives of the present investigation were to find out if *L. gnaphaloides* contains some constituents which possess antimycobacterial properties, to isolate and purify the active principles, and determine their molecular structures.

4.1.3 RESULTS AND DISCUSSION

The investigation of *L. gnaphaloides* was carried out following a five-step bio-assay guided method:

(i) Preparation of the extracts and bioassay guided fractionation.
(ii) Isolation and purification of the antibacterial constituents.

(iii) Evaluation of the anti-mycobacterial properties of the isolates.
(iv) In vitro cytotoxicity testing of the bioactives against CHO cells.
(V) Elucidation of the molecular structures of the isolated compounds.

Preparation, and bioassay-guided fractionation of crude extracts of *L. gnaphaloides* plant material were conducted in the manner as described in sections **3.4.2 – 3.4.5**.

4.1.3.1 Antibacterial susceptibility testing of *L. gnaphaloides* constituents

The evaluation of the antibacterial properties of the isolated compounds from *L. gnaphaloides* was performed following the same procedure as described in sections **3.4.3 – 3.4.5**.

Aqueous extracts of *L. gnaphaloides* (entire plant) were found to be inactive against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *M. aurum* A+. *M. aurum* A+, a non-pathogenic, fast-growing *Mycobacterium* was used as an indicator of *Mtb*'s susceptibility because of its antibiotic susceptibility profile similarity to *Mtb* (Chung et al., 1995).

In contrast, the acetone/water (4:1) extract of ***L. gnaphaloides*** exhibited significant antibacterial activity against the test bacteria. After a sequential liquid/liquid separation of the acetone/water crude extract with hexane, dichloromethane, and ethyl acetate, the hexane fraction showed more potency than the other fractions. Ultimately, the hexane fraction extract was concentrated under vacuum and column chromatographed on silica gel using a mixture of hexane/ethyl acetate. Chromatography afforded the isolation of four bioactive compounds/mixtures designated **LG1, LG2**, **LG3,** and **LG4** (see Table 4.1)

Table 4.1: Summary table: Antimicrobial activity of *L. gnaphaloides* constituents

Extraction	**Aqueous** infusion	**Maceration** [acetone/water (4:1)]			
Isolated compound	-	LG1	L2	LG3	LG4
Antimicrobial activity	-	+	+	+	+

The results of the antimicrobial activity assays of these bioactive constituents are detailed in **Tables 4.2 - 4.5**.

Table 4.2: Bioautography: Anti-*M. aurum* activity of *L. gnaphaloides* bioactive constituents

Sample	**Amount** (µg/spot)				
	10	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *M. aurum* A+				
Rifampicin	-	-	-	-	+
LG1	-	-	+	+	+
LG2	-	-	+	+	+
LG3	-	-	+	+	+
LG4	-	-	+	+	+

Legend: **+**: Blue spot = Bacterial survival = No antibacterial activity

-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.3: Bioautography: Anti-*S. aureus* ATCC 25923 activity of *L. gnaphaloides* bioactive constituents

Sample	**Amount** (µg/spot)				
	10	**5**	**2.5**	**1.25**	**0.625**
	MTT reduced by *S. aureus* ATCC 25923				
Rifampicin	-	-	-	-	+
LG1	-	-	+	+	+
LG2	-	-	+	+	+
LG3	-	-	+	+	+
LG4	-	-	+	+	+

Legend: **+**: Blue spot = Bacterial survival = No antibacterial activity

-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.4: Bioautography: Anti-*E. coli* ATCC 25922 activity of *L. gnaphaloides* bioactive constituents

Sample	Amount (µg/spot)				
	10	5	2.5	1.25	0.625
	MTT reduced by *E. coli* ATCC 25922				
Rifampicin	-	-	-	-	+
LG1	-	-	+	+	+
LG2	-	-	+	+	+
LG3	-	-	+	+	+
LG4	-	-	+	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity

-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.5: Bioautography: The MICs of semi-purified L. *gnaphaloide*s bioactive constituents

Sample	MICs of the isolated compounds (µg/spot)		
	M. aurum A+	*E. coli* ATCC 25922	*S. aureus* ATCC 25923
Rifampicin	1.25	1.25	1.25
LG1	5	5	5
LG2	5	5	5
LG3	5	5	5
LG4	5	5	5

4.1.3.2 Evaluation of the antimycobacterial properties of the isolated compounds

The recent global resurgence of TB and MDR-TB has a strong correlation with the HIV/AIDS pandemic. It is estimated that more than half HIV/AIDS patients die of TB and it is also among HIV/AIDS patients that the highest rate of non-tuberculosis mycobacteria (NTM) infections occur. NTM cause opportunistic infections; they rarely infect humans other than HIV/AIDS patients (Wilson, 1997). The most common NTM species include *M. avium*, *M. kansasii*, *M. scrofulaceum*, and *M. microti.* A brief description will be given of each of these atypical *Mycobacterium* species.

M. avium is as a slow growing M*ycobacterium*, and it is the most common NTM (Wilson, 1997). *M. avium* grows and produces colonies in the lung like *Mtb*. Similarly, *M. scrofulaceum* is a slow-growing NTM, but it is much less common than *M. avium*. This bacterium causes opportunistic chronic infections such as scrofula (cervical adenitis) and chronic abscesses in humans suffering from HIV/AIDS. Infections due to *M. scrofulaceum* do not respond very well to drugs. Another type of atypical mycobacterium is *M. microti*, which is closely related to *Mtb* and is the least common infection due to atypical mycobacteria. It occurs almost exclusively in patients suffering from HIV/AIDS as an opportunistic infection.

In a separate set of assays, the purified bioactive compounds were tested against *Mtb* H37Rv, and the non-tuberculous mycobacteria, *M. avium* ATCC 25291, *M. scrofulaceum* ATCC 19981 and *M. microti* ATCC 19422. The evaluation of the anti-mycobacterial properties of the isolated compounds was carried out using the two previously described methods, namely, the disk diffusion method and bioautography on TLC plates. The results of these assays are given in Tables **4.6 – 4.10** below.

Table 4.6 Disk diffusion: Anti-*Mtb* activity of *L. gnaphaloides* isolated bioactive constituents

Sample	**Activity against *Mtb* H37Rv**					
Dosage (µg/disk)	**20**	**10**	**5**	**2.5**	**1.25**	**0.625**
Rifampicin	-	-	-	-	+	+
LG1	-	-	-	+	+	+
LG2	-	-	+	+	+	+
LG3	-	-	-	+	+	+
LG4	-	-	-	+	+	+

Legend: +: Bacterial growth

-: Clear zone all around the impregnated disk meaning the inhibition bacterial grow = Significant antibacterial activity

All the test samples showed significant anti *Mtb* H37Rv activity, with a zone of growth inhibition appearing around each disk on the agar. The observed anti-*Mtb* MICs were: 10 μg/disk for compound **LG2**, 5μg/disk for compounds **LG1, LG3, LG4**, and 2.5 μg/disk for rifampicin.

Table 4.7: Bioautography on TLC plates: Anti-*Mtb* activity

Sample	**MTT reduced by *Mtb* H37Rv**			
Dosage (μg/spot)	**5**	**2.5**	**1.25**	**0.625**
Rifampicin	-	-	+	+
LG1	-	-	+	+
LG2	-	-	+	+
LG3	-	-	+	+
LG4	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity

-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.8: Bioautography: Anti-*M. avium* ATCC 25291 activity

Sample	**MTT reduced by *M. avium* ATCC 25291**			
Dosage (μg/spot)	**5**	**2.5**	**1.25**	**0.625**
LG1	-	-	-	+
LG2	-	-	-	+
LG3	-	-	-	+
LG4	-	-	-	+
Rifampicin	-	-	-	+

Legend: **+**: Blue spot = Bacterial survival = No antibacterial activity

-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.9: Bioautography: Anti- *M. scrofulaceum* ATCC 19981 activity

Sample	**MTT reduced by *M. scrofulaceum* ATCC 19981**			
Dosage (µg/spot)	**5**	**2.5**	**1.25**	**0.625**
LG1	-	-	-	+
LG2	-	-	-	+
LG3	-	-	-	+
LG4	-	-	-	+
Rifampicin	-	-	-	+

Legend: +: Blue spot = Bacterial survival = No antibacterial active
-: White spot = No bacterial survival = Significant antibacterial activity

Table 4.10: Bioautography: Anti-*M. microti* ATCC 19422 activity

Sample	**MTT reduced by *M. microti* ATCC 19422**			
Dosage (µg/spot)	**5**	**2.5**	**1.25**	**0.625**
LG1	-	-	+	+
LG2	-	-	+	+
LG3	-	-	+	+
LG4	-	-	+	+
Rifampicin	-	-	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

4.1.3.3 The advantages of using the modified bioautography on TLC plates method

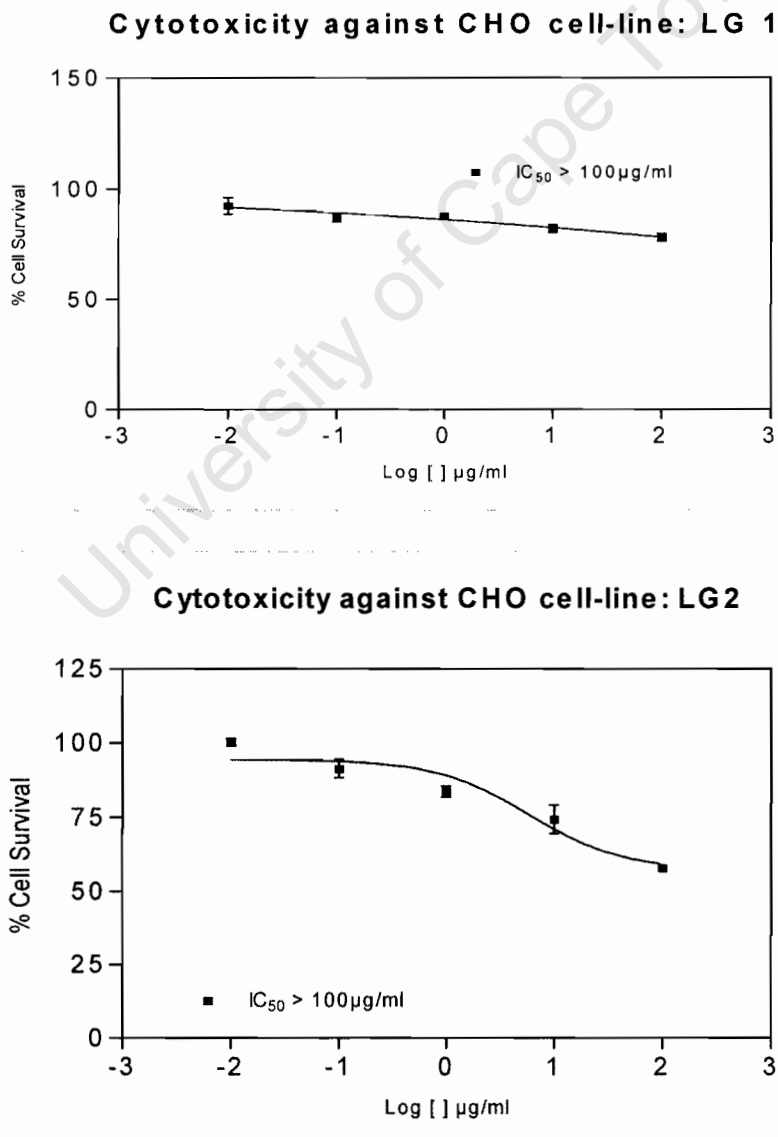
The results of the bioautography on TLC plates confirmed the anti-*Mtb* properties of the samples as observed using the agar disk-diffusion method. However, the MICs on the bioautograms were found to be lower (2.5 µg/disk) than observed on agar plates (5 µg/disk). In addition, the rifampicin anti-*Mtb* MIC on agar plates was lower (2.5 µg/disk) than those of the isolated compounds (5 µg/disk). This situation can be explained by the fact that the methanol solution of rifampicin easily diffused into the hydrophilic agar medium compared to the hydrophobic bioactive compounds. This highlights several important advantages of using

bioautography on TLC plates: it is a method that is applicable to both hydrophilic and hydrophobic compounds, is more sensitive than disk diffusion and requires only small amounts of samples.

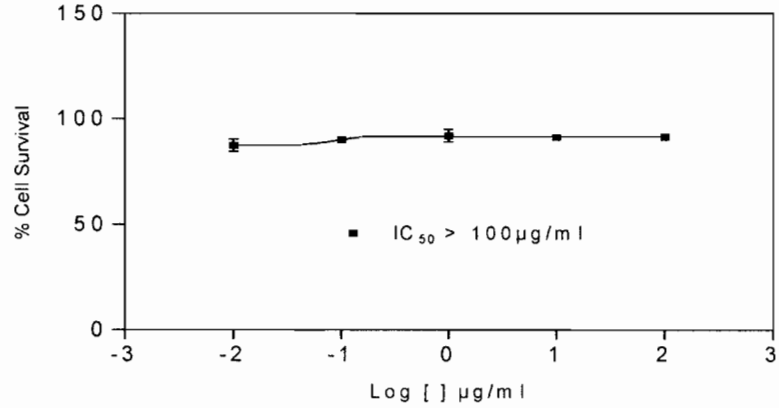
4.1.3.4 In vitro cytotoxicity assay of constituents of *L. gnaphaloides*

Cytotoxicity assays were performed on Chinese Hamster Ovarian (CHO) cells. Emetine dihydrochloride was used as a positive control. The results are presented in the form of dose-response graphs (Figure **4.1**)

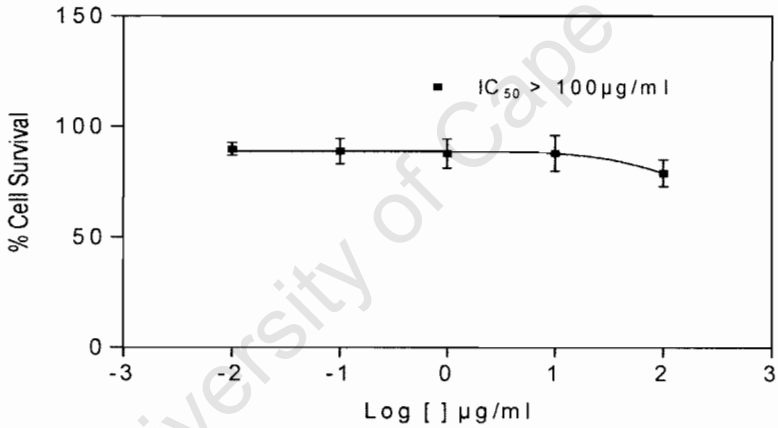
Figure 4.3: In vitro cytotoxicity against CHO cells



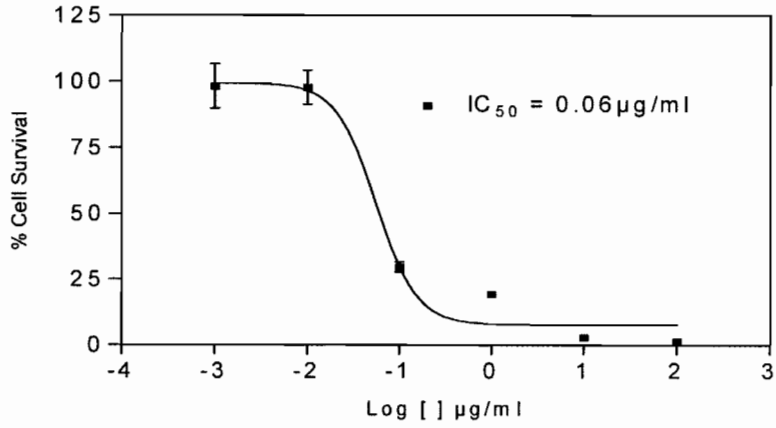
Cytotxicity against CHO cell-line: LG 3



Cytotoxicity against CHO cell-line: LG 4



Cytotoxicity against CHO cell-line: Emetine



After the isolated bioactive constituents from *L. saligna*, **LG1**, **LG2**, **LG3**, and **LG4** were tested and found to be significantly active against the test mycobacteria, it was interesting to assess whether these bioactive constituents were cytotoxic to normal cells. An in vitro cytotoxicity test was then performed on CHO cells line as described in section ***3.4.6.***

The result of this assay showed in vitro cytotoxicity profiles of the bioactive constituents of *L. saligna*, **LG1**, **LG2**, **LG3**, and **LG4** to be similar to that of compounds **BS1** and **BS2**, with 50% cell survival at a concentration of the bioactive agent as high as 100 µg/ml.

It is important to note that the cytotoxicity of an agent can also expressed in term of its selective index (SI). Practically, SI represents the ratio of the percentage of survival of normal cells to the percentage of survival of pathogenic bacteria; it is a numerical figure of the selectivity of the bioactive agent.. A summary of the results of the antibacterial susceptibility assay and the test for the in vitro cytotoxicity of bioactive constituents of *L. gnaphaloides* is give in Table **4.11** below.

Table 4.11: Summary table: Anti *Mycobacterium* activity and CHO in vitro cytotoxicity of *L. gnaphaloides* bioactive constituents

Compound	**Anti-mycobacterial MIC** (µg/spot) (Bioautography on TLC plates)				**CHO IC_{50}** (µg/ml)
	Mtb	***M. avium***	***M. scrofulaceum***	***M. microti***	
LG1	2.5	1.25	1.25	2.5	> 100
LG2	2.5	1.25	1.25	2.5	> 100
LG3	2.5	1.25	1.25	2.5	> 100
LG4	2.5	1.25	1.25	2.5	> 100
Emetine					0.06

4.1.3.5 Structure elucidation

Complete structure elucidation of the isolated compounds **LG1** and **LG2** was established by analysing data from ^{1}H and ^{13}C NMR spectra, including

DEPT, HSQC, and HMBC spectra. The NMR data were analysed together with data obtained from High Resolution Electron Impact Mass Spectra (HREIMS) and Fast Atom Bombardment Mass Spectra (FAB-MS). The experimental data were finally compared with data from the literature.

4.1.3.5.1 Structure elucidation of compound LG1

Compound **LG1** was isolated as a whitish amorphous powder, m.p 279 – 280 °C. **LG1** was one of the four related structures encountered in this investigation of *L. gnaphaloides*; the other three are **LG2, LG3** and **LG4**. Full details of the structural elucidation of **LG1** are given below to provide the basis for the elucidation of other structures.

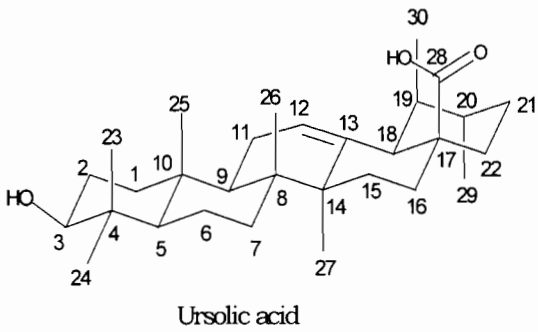
Preliminary analysis of HREIMS and NMR spectra suggested **LG1** to be a hydroxylated pentacyclic triterpenoid. This was possible since the EI-MS spectrometer was linked to a data bank which, by matching the experimental data to the data in the bank was able to suggest what type of compound the sample was most likely to be, a pentacyclic triterpenoid of the ursolic/oleanolic acid type. From this point on, these suggestions gave some sort of direction to the rest of data analysis process. Thus, when analysing the EI-MS spectrum, it could easily be assumed that the peak at m/z 455 represents $[M - H]^-$, which eventually corresponds to $C_{30}H_{47}O_2$, as previously described in Figure **3.2**. The presence of this fragment supported the assumption that **LG1** was a triterpenoid. In addition, other meaningful fragments such as fragments at m/z = 248 and m/z 208 also supported the assumption. The former is suggestive of the presence of a carboxyl group and the latter is indicative of the presence of a hydroxyl group in the molecule. Other meaningful peaks included fragments at m/z 203 (50%) [$C_{13}H_{21}$], m/z 189 (40%) and m/z 133 (65%). The presence of these fragments is practically considered as a fingerprint of 12-en-ursane or 12-en-oleane molecule types (Budzikiewicz et al., 1963). The mechanism leading to these fragments has already been elucidated (Figure **3.2 – 3.3**)

(Katerere et al., 2002). From this point, it became obvious that compound **LG1** is an ursolic or oleanolic type of skeleton (Figure **4.2**).

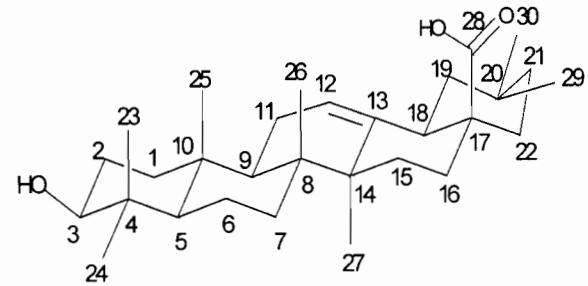
The ^{1}H NMR spectrum showed the presence of one doublet of doublets (dd) at δ 3.2, characteristic of a hydroxyl group, which was easily assigned to C-3 on the basis of COSY and HSQC spectra. The presence of this hydroxyl group was confirmed by the disappearance of its signal in the ^{1}H NMR spectrum of the D_2O-washed sample. There were seven methyl-group singlets (integrating three protons each) at δ 1.05 (H-23), δ 0.85 (H-24), δ 0.51 (H-25), δ 0.79 (H-26), δ 1.14 (H-27), δ 0.92 (H-29), and δ 0.90 (H-30). The ^{13}C NMR spectrum of compound **LG1** revealed the presence of 30 signals corresponding to 30 carbons that make up the presumed molecule. The complete assignment of all the carbon signals, together with all resolved proton signals was possible with the aid of the COSY, HMBC and HSQC spectra, and with reference to the literature, in particular the extensive review of ^{13}C data on triterpenoids by Mahato and Kundu (1994). The correlations observed in the HMBC and HSQC spectra are summarised in Tables **4.12**.

As a starting point, the olefinic carbons C-12 and C-13 as well as H-12 could readily be identified and their respective protons assigned based on their characteristic chemical shifts on the ^{13}C and ^{1}H NMR spectra, together with the HSQC spectrum. The assignment of H-18 (δ 2.82) followed from the HMBC and HSQC spectra. From the HMBC spectrum, H-18 was shown to be long-range coupled to C-20, C-29 and C-30. On the HSQC spectrum, H-19 showed a correlation with H-28.

Figure 4.2: Molecular structure of ursolic acid and oleanolic acid



Ursolic acid



Oleanolic acid

The HMBC spectrum revealed that H-19 was long range coupled to C-20, C-29, and C-30. The carbon signal at δ 52.78 was assigned to C-18, as it was the closest methine carbon to H-19. After assigning C-18, H-18 could be easily identified on the HSQC spectrum at δ 2.82, because of its correlation with C-13, and C-12 on HMBC. The methyl groups were then assigned as follows: firstly, the methylic proton signal at δ 1.09 was the only one that did not show correlation to any of the other methyl groups, suggesting that it was the one at H-27 on the α-face of the molecule. The geminal dimethyl groups, typical at C-4 in triterpenoids, were recognized from the common HMBC correlations observed for the signals at δ 0.87 and 0.99, suggesting these were Me-23 and Me-24. Of these two methyl signals, only the signal at δ 0.87 showed connectivity to the methyl signal at δ 0.92, suggesting that these were Me-24 (the β-methyl group at C-4) and Me-25 respectively. At this point, the signal at δ 0.99 was left only to be assigned as Me-23. Me-25 was in turn correlated to the signal at δ 1.09 corresponding to Me-26. After assigning C-3, C-4, C-18, C-19, C-20, C-28, C-29 and all the methyl groups, the HMQC, HMBC and HSQC spectra were used for the assignment of the methine carbons: C-5, identified from the connectivities shown in the HMBC spectrum for Me-23 and Me-24 (C-3, C-4 and C-5); C-13 could be identified from the connectivities shown for Me-27 (C-8, C-13, C-14 and C-15), which also led to the identification of the methylene carbon C-15; C-9 could be identified from the connectivities observed for Me-25 (C-1, C-5, C-9 and C-10), which also led to the

identification of the methylene carbon C-1 and the quaternary carbon C-10. C-2 was identified from the connectivities shown for H-1 (C-2 and C-3) and H-3β (C-2 and C-5). The analysis of the COSY, HSQC and HMBC spectra (Table **4.12**) confirmed these assignments as Me-26 showed a correlation to H-13, Me-27 showed a correlation to H-9, H-5 was shown to be correlated to H-1α, and Me-25 and Me-24 were both shown to be correlated to H-2β. The rest of the carbons were assigned using a combination of the various 2D NMR spectra and the reported literature data. It is important to note that the -COOH group's signal at C28 was not clearly observed on the initial ^{13}C NMR spectrum of **LG1**. However, the deshielding of the H-18 signal down to δ 2.827 ppm indicated the effects of a –COOH group (i.e. at C-28). This was confirmed after several days of ^{13}C NMR scans accumulation, which finally revealed the carboxyl group signal (C-28) at δ 178.3 ppm. A complete assignment of the ^{13}C NMR spectrum of **LG1** is given in the **Table 4.13**. The mass fragmentation pattern observed on the MS spectrum of compound **LG1** followed a pattern already described (Budzikiewicz et al., 1963) in section **3.3.5.2.**

Table 4.12: HMBC results of **LG1**

Proton	Connectivities	Proton	Connectivities
H-1	C-2, C-3	H-22	C-17, C-18
H-2	C-4	Me-23	C-3, C-4, C-5, C-24
H-3β	C-2, C-5	Me-24	C-3, C-4, C-5, C-23
H-5	C-4	Me-25	C-1, C-5, C-9, C-10
H-6	C-5	Me-26	C-7, C-8, C-9, C-14
H-9	C-8, C-10, C-25, C-26	Me-27	C-8, C-13, C-14, C-15
H-16	C-28	Me-28	C-16, C-17
H-18	C-13, C-19, C-20, C-28	Me-29	C-19, C-28
H-19	C-18, C-20, C-21,	Me-30	C-17, C-18, C-22
H-21	C-17, C-18, C-19, C-20		

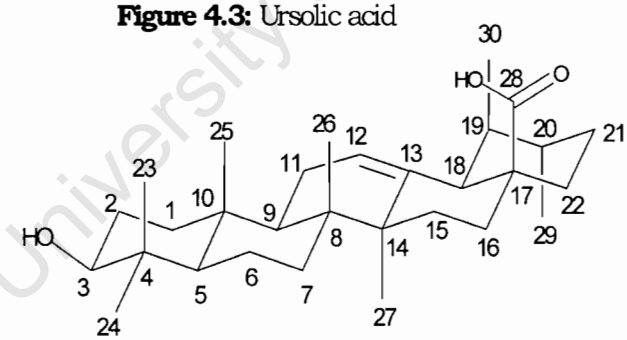
Table 4.13: 13C chemical shifts for **LG1**

Methyl ursolate			LG1	Methyl ursolate			LG1
Carbon		^{13}C	^{13}C	Carbon		^{13}C	^{13}C
1	CH_2	38.6	38.7	16	CH_2	24.2	24.2
2	CH_2	27.2	27.3	17	C	48.1	48.0
3	CH	79.0	79.1	18	CH	52.9	52.7
4	C	38.7	38.8	19	CH	39.0	39.1
5	CH	55.2	55.3	20	CH	38.9	38.9
6	CH_2	18.3	18.3	21	CH_2	30.6	30.6
7	CH_2	33.0	33.0	22	CH_2	36.6	36.7
8	C	39.5	39.6	23	CH_3	28.1	28.1
9	CH	47.6	47.6	24	CH_3	15.4	15.4
10	C	37.0	37.0	25	CH_3	15.6	15.5
11	CH_2	23.3	23.3	26	CH_3	16.9	17.1
12	CH	125.6	125.9	27	CH_3	23.6	23.6
13	C	138.1	138.0	28	C=O	178.0	178.0
14	C	42.0	42.0	29	CH_3	16.9	16.9
15	CH_2	28.0	28.1	30	CH_3	21.1	21.1
				31	CH_3	51.4	24.2

Compound **LG1** ($CDCl_3$; ^{13}C data were observed at 75 MHz and ^{1}H at 300MHz). ^{13}C chemical shift of methyl ursolate, Takeoka et al (2000) are listed for comparison

Compound **LG1** was thus concluded to be the known compound **ursolic acid** (figure **4.5**).

Figure 4.3: Ursolic acid



Indeed, ursolic acid and its derivative metabolite, 3β-ursane, are in fact constituents of numerous plants, including *Epigaea asiatica* (leaves), *Erica arborea* (leaves and stems), *Arbutus andrachne, Nerium oleander, Phillyrea latifolia, Rhododendron hymenathes*, and many varieties of apple and pear (Glasby, 1982). UA exists in two separate forms, which differ in their solubility in ethanol and their melting points (m.p.). The less soluble form has a m.p. of 291°C, its acetate derivative has a m.p. = 289 – 292 °C, and the more soluble form has a m.p. = 284 – 285°C, its acetate derivative has

a m.p = 286 °C. These two forms of metabolites are often found together in a plant sample (Glasby, 1982).

4.1.3.5.2 Structure elucidation of compound LG2

Compound **LG2** was isolated as a whitish amorphous powder, m.p. 278 °C, soluble in chloroform, precipitating from methanol. The MS spectrum (EI and FAB) of **LG2** presented features resembling to compound **BS1** (section **3.3.5.2**), the molecular peaks at m/z 456 (75%) and at m/z 455 (100%) corresponding to [M: $C_{30}H_{48}O_3$], and [M – H: $_{30}H_{47}O_3]^-$, and a fragment at m/z 470, (M + 15: $C_{31}H_{50}O_3]^*$. Based on MS fragments and the physicochemical properties, it was readily assumed that **LG2** was urs/oleane-12-en skeleton.

There were also similarities in ^1H and ^{13}C NMR spectra of compounds **LG2** and **LG1**; the ^{13}C spectra of these two compounds were virtually superposable. The only difference was the shifts for the ethylenic carbons peaks, δ 122.6 (C-12) and 143.6 (C-13) for **LG2** against δ 122.6 (C-12) and 143.6 (C-13) for **LG1.** According to Takeoka et al. (2000) and Mahato and Kundu (1994), shifts for the ethylenic carbon's peaks $\pm\delta$ 121.8 (C-12) and ±145.1 (C-13) are characteristic of an oleane skeleton whereas δ124.3 (C-12) and ±139.3 (C-13) are indicative of ursane skeleton (see **Table 4.14**). ^{13}C NMR data of **LG2** were found be in agreement with the literature (Table **4.15**). At this point, compound **LG2** was identified as **oleanolic acid** (Figure **4.4**).

Table 4.14: Differential ^{13}C shifts: 12-Ursene and 12-Oleanene skeleton

12-en-Urs		**12-en-Olean**	
Carbon	**^{13}C: δ(ppm)**	**Carbon**	**^{13}C: δ(ppm)**
12	124.3	12	121.8
13	139.3	13	145.1
3β	78.8	3β	78.8
3α	76.4	3α	76.4

Table 4.15: ^{13}C chemical shifts for **LG2**

Methyl oleanolate			**Compound LG 2**		
Carbon		**δ** (ppm.)	**^{1}H: δ** (ppm.)	**^{13}C: δ** (ppm.)	**Dept**
1	CH2	38.4		38.4	38.4
2	CH2	27.2		27.2	27.1
3	CH	79.0	3.23 (dd, *j* =7.4 Hz, 8.2Hz)	79.0	78.9
4	C	38.7	-	38.7	----
5	CH	55.2		55.2	55.2
6	CH2	18.3		18.3	18.3
7	CH2	32.7		32.6	32,6
8	C	39.3	-	39.3	----
9	CH	47.6		47.6	47.6
10	C	37.0	-	37.1	37.2
11	CH2	23.4		23.4	23.4
12	CH	122.4	5.28 (broad t, *j* = 3.5 Hz,)	122.6	
13	C	143.8	-	143.6	----
14	C	41.6	-	41.6	----
15	CH2	27.74		27.7	27.7

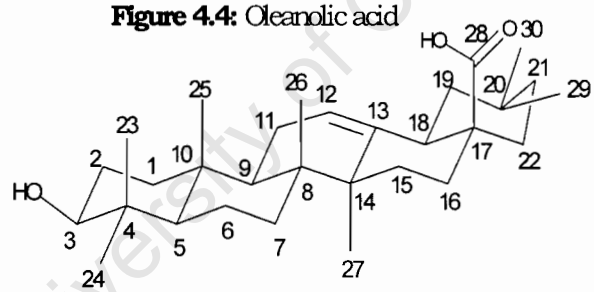
Methyl oleanolate			**Compound LG 2**		
16	CH2	23.1		22.9	
17	C	46.7	-	46.5	----
18	CH	41.3	2.85(broad dd, *J* = 13.5; 2.9 Hz)	41.0	
19	CH2	45.9		45.9	45.9
20	C	30.7	-	30.6	----
21	CH2	33.9		33.8	33.8
22	CH2	32.4		32.4	32.4
23	CH3	28.1	0.99(s)	15.5	28.1
24	CH3	15.5	0.78(s)	28.1	15.5
25	CH3	15.3	0.92(s)	15.3	15.3
26	CH3	16.8	0.76(s)	17.1	17.1
27	CH3	25.9	1.13(s)	25.9	25.9
28	C=O	178.2		178.2	
29	CH3	33.1	0.90s)	33.0	33.0
30	CH3	23.6	0.93(s)	23.5	23.5

Compound LG2 in $CDCl_3$; ^{13}C data measured at 75 MHz and ^{1}H at 300M.

^{13}C chemical shifts of methyl oleanolate, Takeoka et al. (2000).

In addition to compounds **LG1** and **LG2, s**ignificant anti-*Mtb* activity was also recorded with the samples **LG3** and **LG4**. However, TLC revealed the latter to be mixtures of constituents, which were structurally related to **LG1** and **LG2,** Rf values were too close that they could not be separated. This assumption was later supported by MS and NMR data of **LG3** and **LG4**, which presented features similar to those of **LG1** and **LG2**. It was noted, for instance, that the ^{13}C spectrum of sample **LG3** (Table **4.17**) was virtually superposable on the spectrum of **LG1,** presented 2 additional signals at δ41.36 and 39.69 ppm that were not in the ^{13}C NMR spectrum of **LG1**. However, the HSQC and HMBC spectra showed no connectivity between these peaks and the core molecular structure. This suggested that these additional peaks may have been from an impurity present in the sample. This assumption was supported by the presence of the peaks at m/z 471, and (M + 29) m/z 485 in the MS spectrum, corresponding possibly to fragments [5%, $C_{31}H_{51}O_3$]* and [$C_{32}H_{55}O_3$]* respectively.

Figure 4.4: Oleanolic acid



In addition to NMR and MS data, a colorimetric assay was carried out in order to compare sample **LG3** with the commercial reference samples of ursolic acid, oleanolic acid and β-amyrin by spraying with ethanolic sulphuric acid and heating. Oleanolic acid and β-amyrin gave orange-pinkish and yellow-orange colours respectively (Table **4.16**), whereas sample LG3 and ursolic acid revealed a pink colour.

Table 4.16: Colorimetric identification of compounds

Sample	Rf[a]	Colour[b]
LG3 (yellowish)	0.51	Pink
Ursolic acid (white)	0.51	Pink
Oleanolic acid (white)	0.531	Orange-pinkish
β-amyrin (white)	0.671	Yellow-orange

a = Rf on TLC (Polygram SiL G/UV_{254}, Macherey-Nagel); eluent: toluene/ethyl acetate (8:2). b = the colour of the spot on the TLC plate after spraying with sulphuric acid in ethanol, and heating.

Table 4.17: ^{13}C chemical shifts of **LG3**

LG3		
Carbon		**^{13}C: δ (ppm)**
1	CH2	38.6
2	CH2	27.2
3	CH	79.0
4	C	38.7
5	CH	55.2
6	CH2	18.3
7	CH2	33.6
8	C	39.5
9	CH	47.6
10	C	37.0
11	CH2	23.5
12	CH	125.9
13	C	137.9
14	C	42.0
15	CH2	28.0
16	CH2	25.9

LG3		
Carbon		**^{13}C: δ (ppm)**
17	C	47.9
18	CH	52.7
19	CH2	39.0
20	C	32.8
21	CH2	33.8
22	CH2	32.4
23	CH3	28.1
24	CH3	15.5
25	CH3	15.4
26	CH3	17.0
27	CH3	23.4
28	C=O	125.7
29	CH3	16.9
30	CH3	21.1
	$\underline{CH_2}$	41.3
	CH3	29.6

LG3 in $CDCl_3$; ^{13}C NMR data were measured at 75MHz and ^{1}H at 300MHz.

4.1.3.6 Discussion a conclusion

Aqueous extracts from *L. gnaphaloides* (entire plant) were found to be inactive against the test bacteria. In contrast, the organic-solvent extracts (acetone/water; 4:1, v/v) of the plant material exhibited significant activity against the test bacteria, which suggested that the antibacterial constituents of *L. gnaphaloides* were of a lipophilic nature. This assumption was then confirmed by further steps in this investigation which led to the isolation and structure elucidation of the bioactive compounds, four pentacyclic triterpenoids designated LG1, LG2, LG3, and LG4, and identified as ursolic and oleanolic acid, and their mixtures with their alkylated derivatives. Though these isolated bioactive compounds are soluble in organic solvents, their corresponding glycosides and saponins are soluble in polar solvents such as water (Avilla and Romo de Vivar, 2002). It is possible that these glycosides and saponins could act as pro-drugs, the aglycon moieties being the active principles. Indeed, the bioactivity of many saponins and glycosides derived from triterpenoids is well documented (Favel et al., 1994). Therefore, the therapeutic effects of *L. gnaphaloides* aqueous extracts may be explained by the fact that they contain glycoside and saponin derivatives of triterpenes.

4.1.5 EXPERIMENTAL

4.1.5.1 Collection of plant material

L. gnaphaloides (L.) (entire plant) was collected in March 2001 from Blaauwberg farm in the Atlantis area, about 50 km outside Cape Town. At the time of the collection, the plant bore pinkish flowers, some fresh, some already dying. The collection was carried out with the help of the botanist, Dr Gillian Scott. Mr T.H. Trinder-Smith, the curator at the Bolus Herbarium, University of Cape Town, authenticated the species. A voucher specimen was deposited at the Bolus Herbarium.

4.1.5.2 Preparation of plant crude extracts

L. gnaphaloides (entire plants) were air-dried at 37°C and ground into a powder. Dry ground plant material (100g) was extracted at room temperature with acetone/water (4:1v/v, 1000ml) by maceration overnight in a 2.5 litre flask. The extraction was then carried out following the same procedure as described in section **3.4.3.**

4.1.5.3 Bioassay-guided fractionation of plant extracts, isolation and purification of plant bioactive compounds

The experiments, bioassay-guided fractionation of plant extracts, isolation and purification of plant bioactive compound were conducted following the same procedures as described in Sections **3.4.3 - 3.4.7**

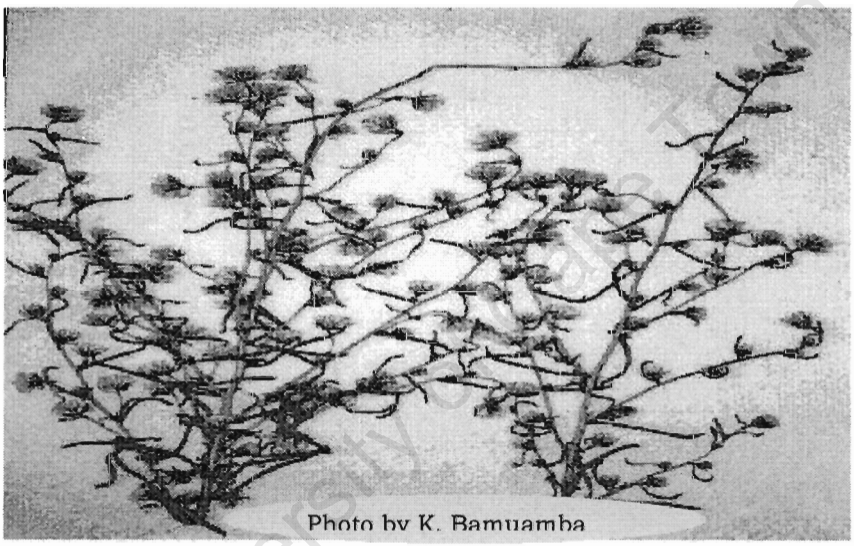
4.1.5.4 NMR Spectroscopic Experiments

The NMR spectra were obtained from samples dissolved in $CDCl_3$; the ^1H spectra were recorded on a Variant Mercury Spectrometer at a frequency of 300.0 MHz, and for ^{13}C at 75.0 MHz.

4.2 INVESTIGATION OF ANTIBACTERIAL PROPERTIES OF *INULA GRAVEOLENS* (L.)

4.2.1 INTRODUCTION

Inula graveolens (L.) (**Picture 4.2**), Asteraceae family, is commonly known as Kaapse Kakiebos or Cape khakiweed in the Western Cape Province, South Africa. It is also called *Camphor inula*, or *kanfer-inula*.. It is an annual herb, about 1m tall, leaves alternate, generally sessile, margins dentate or denticulate, hairy and glandular. There are about 100 *Inula* species worldwide (Europe, Africa, Asia), of which only two are typical southern African species: *Inula glomerata*, and *Inula paniculata* (Leistner, 2000).



Picture 4.2: *Inula graveolens* (L.)

It is believed that *I. graveolens* was originally from the Mediterranean region of Europe; it was introduced in the Cape region about three centuries ago (Bromilow, 1995). The plant has a very distinctive smell of essential oil. It is entirely covered with glandular hairs that make it sticky to the touch. *I. graveolens* is often found growing on gravel roadsides and waste places; the plant is also common amongst crops (Leistner, 2000).

4.2.2 PHARMACOLOGICAL PROPERTIES

Previous investigations on *I. graveolens* have reported the isolation of mono and sesquiterpenes from flowers and leaves (Rustaiyan et al., 1987; D'Alcontres et al., 1973), lipids and flavonoids from leaves and stems (Soueles and Philianos, 1979), carbohydrate compounds, benzenoid and phenylpropanoids from the aerial parts (Soueles and Philianos, 1979). A report on the biological activity of *I. graveolens* compiled by the NAPRALERT database (University of Illinois, May 15, 2002) indicated that in Australia, a feeding study (40 days) of the aerial parts of *I. graveolens* in undiluted ration showed no general toxicity to guinea pigs (Seddon and Carner, 1927). The same study also reported no toxic effects (unspecified dose) in guinea pig from aqueous extract of *I. graveolens* (leaves and stems) (Seddon and Carner, 1927).

In Iran, a biological activity study of the ethanolic extracts (ethanol 80%) of dry leaves and stems of *I. graveolens* at a concentration of 100 μg/ml showed antibacterial activity against *Bacillus anthracis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, but no activity against *Escherichia coli, Proteus vulgaris, Salmonella paratyphi, Shigella sonnei, Staphylococcus aureus,* and *Vibrio cholera* (Aynehchi et al., 1982). In Greece, *I. graveolens*'s aqueous extracts were found to be inactive against *Streptococcus faecium, Staphylococcus aureus,* and *Plasmodium berghei* in mice (Caldes et al., 1975); the same study also reported no in vitro cytotoxicity for *I. graveolens* against CA-9KB cells in cell culture.

The objectives of the present investigation were to find out if *I. graveolens*'s constituents possess antimycobacterial properties, to isolate and purify the active principles, and determine their molecular structures.

4.2.3 RESULTS AND DISCUSSION

The investigation of *I. graveolens* was conducted following the same procedure and methods as for the investigation of *L. gnaphaloides* and *B. saligna* described in section **4.1.3** and **3.4**, respectively.

4.2.2.1 Antibacterial Susceptibility Testing of *L. gnaphaloides* constituents

The aqueous extracts of *I. graveolens* (entire plant) were found to be inactive against the test bacteria, *M. aurum* A+ , *S. aureus* ATCC 25923, and *E. coli* ATCC 25922.

In contrast, the acetone/water crude extract of *I. graveolens* (entire plant) showed stronger antimicrobial activity than any other crude extract of plant materials that were tested in this investigation. The acetone/water crude extract of *I. graveolens* was liquid/liquid divided into hexane, dichloromethane, ethyl acetate, and water extract fractions. The hexane extract fraction was the most active of all, followed by dichloromethane and ethyl acetate extract fractions; the water extract fraction was inactive.

Column chromatography of the hexane extract fraction led to the isolation of the bioactive constituent **IG1**, which was later identified as oleanolic acid (**BS1**, previously isolated during this investigation from *B. saligna* plant material: section **3.3.5.2**) by comparing the NMR spectroscopy data of **IG1** and **BS1.**

In addition, a bioactive mixture, **IG2** was also isolated from the hexane extract fraction following column chromatography. **IG2** was found to be a mixture seemingly made up of **IG1** plus some impurities, which were too closely related to be separated using column chromatography. **IG2** had moderate antimicrobial activity compared to **IG1**, most probably because of the presence of these impurities in the sample.

It is important also to note the presence of other constituents of *I. graveolens* such as volatile oils, which were remarkable because of the special smell they confer to the *I. graveolens* plant and the oily moisture the plant leaves to the touch. It was also assumed that I graveolens contains unstable phytochemicals such as oxidable tannins. The observation that lead to this assumption was that concentred of acetone/water crude extract of *I. graveolens* or their ethyl acetate and water extract fractions were found to be unstable, they changed the colour to dark brown and becoming sticky to the touch when exposed to air, suggesting the presence of some unstable constituents such as oxidisable tannins. Indeed, since tannins are water soluble, this may explain the easy diffusion of *I. graveolens'* crude extract through the agar medium that created a wider zone of growth inhibition than were observed around the impregnated disks and around the sample's spot on the bioautograph.

4.2.2.2 Evaluation of the antimycobacterial properties

Compounds **IG1** and **IG2** were tested against *Mtb* H37Rv using the bioautography on TLC plates method as described in section **3.4.5.1**. Betulinic acid was used as positive control. The results of these experiments are given in Table **4.17** below:

Table **4.17:** Ant-Mtb activities of bioactive compounds isolated from *I. graveolens*

MTT reduced by *Mtb* H37Rv					
Sample		**Dosage** (µg/spot)			
		10	**5**	**2.5**	**1.25**
1	Betulinic acid	–	–	–	–
2	IG1	–	–	–	+
3	IG2	–	–	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity
-: White spot = No bacterial survival = Significant antibacterial activity

The anti-*Mtb* MICs of **IG1** and **IG2** were 2.5 and 5µg/spot, respectively, whereas BA was active at concentration below 1.25µg/spot. **IG2** was half s potent as **IG1**, this may be explained by the fact that **IG2** was a mixture of **IG1** plus a trace of structurally closely related impurities.

4.2.2.3 Elucidation of structures

4.2.2.3.1 Elucidation of the structure of compound IG**1**

The elucidation of the structure of compound **IG1** was realised by analysing the ^{1}H and ^{13}C NMR spectra. A comparison of **IG1**'s NMR data with those of **LG2** (OA, Figure **4.4**) showed that these compounds were identical. Their physico-chemical properties, m.p. (280 – 282 °C), and Rf. Were identical. Spots of these two compounds gave similar colour on TLC plate (yellow-pinkish) after spraying with ethanolic sulphuric acid and heating. The above elements lead to the conclusion that **IG1** was **oleanolic acid.**

4.2.2.3.2 Elucidation of the structure of compound IG2

Sample **IG2** was identified by TLC as a mixture containing predominantly the compound **IG1**, and a trace of others constituents that were structurally very close to **IG1** (Rf very close to each other) difficult to separated by column chromatography. Resemblance of these constituents was further demonstrated by the ^{1}H and ^{13}C NMR spectra of **IG2**, which show many similarities with that of **IG1.**

4.2.4 CONCLUSION

The therapeutic use of *I. graveolens* in traditional medicine for the treatment of infectious diseases such as TB is scientifically supported by its bioactive constituents such as oleanolic acid and its derivatives.

It is also assumed that *I. graveolens* may contain other constituents such as essential oils and tannins, which may be playing a synergistic role for the anti-microbial activities of the aqueous extracts of *I. graveolens* (entire plant).

4.2.5 EXPERIMENTAL

4.2.5.1 Collection of plant material

I. graveolens (entire plant) was collected in Kirstenbosch Garden (Cape Town, South Africa) on the 15 March 2001. The plant was wildly growing on gavel stony road-sides in the garden. The collection was carried out with the help of Mr. Ivan Manuel, the curator of Kirstenbosch Garden. A specimen of the plant was deposited in the Bolus Herbarium of the Dept. of Botany, University of Cape Town, South Africa.

The rest of the experimental work of on *I. graveolens* was carried out following the same procedure and methods as for the investigation of *L. gnaphaloides* and described in section **3.4.**

CHAPTER V

STRUCTURE ANTI-TB ACTIVITY RELATIONSHIP

5.1 INTRODUCTION

The resurgence of TB and DR-TB over the last two decades has led many scientists to search for new anti-TB drugs as a matter of urgency in order to overcome the global threat the pandemic is posing. Thus, a large number of naturally occurring and synthetic compounds have been reported for their antimycobacterial properties in these last two decades. A recent review of naturally occurring antimycobacterial compounds by Okunade et al. (2004) highlighted the structural diversity that characterises these naturally occurring bioactive compounds. They include terpenoids, saponins and glycosides, steroids, flavonoids and polyphenolic compounds. In this study, attention was particularly focused on pentacyclic triterpenoids because this is the class of compounds to which the bioactive constituents, ursolic acid, oleanolic acid, and their derivatives belong. These are the common active principles responsible for the anti-tuberculosis properties of *B. saligna, Inula graveolens* and *L. gnaphaloides*. UA, OA, and their homologue, betulinic acid (BA), are believed to be among the most likely antitubercular lead candidates for new anti-TB drugs. In their study, Wachter et al. (1999) found betulinic acid to be more potent against *Mtb* than ursolic and oleanolic acid.

Further, it is also important to note that numerous synthetic and semi-synthetic pentacyclic triterpenoid derivatives were produced in an attempt to improve the biological activity and selectivity of these molecules, mostly with regard to their anti-HIV activity. For instance, Baglin et al. (2003) published a review of natural and modified betulinic, ursolic and echinocystic acid derivatives as potential antitumor and anti-HIV agents.

This chapter will discuss the most potent antitubercular pentacyclic triterpenoids and their structure-activity relationships with particular attention to substitutions on C-3 and C-28.

5.2 NATURALLY OCCURING PENTACYCLIC TRITERPENOIDS: STRUCTURE-ANTITUBERCULAR ACTIVITY CORRELATION

5.2.1 URSOLIC, OLEANOLIC AND BETULINIC ACID

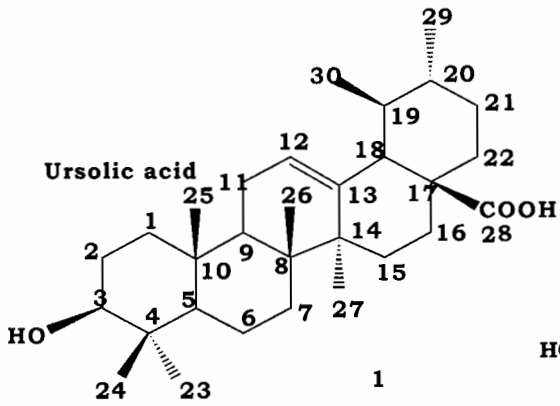
Pentacyclic triterpenoids are widely distributed in higher plants, and they are known to possess diverse biological activities. Compounds such as OA and UA, for instance, have been reported for several biological activities including anti-diabetic, anti-inflammatory, diuretic, anti-spasmodic, anti-atherosclerotic, anti-tumour, antimicrobial, anti-fungal, and anti-HIV (Li and Wei-Jian, 2002; Takeoka et al., 2000).

Jing-Zen and collaborators proposed that the antimicrobial mechanism of action of UA and OA and their derivatives is based on the inhibition of DNA polymerase (Jing-Zen et al., 2000). The same mechanism seems to explain the anti-tumour properties of these compounds. In the case of *Mtb*, drugs must pass through the cell wall, which is a highly hydrophobic structure made up mostly of mycolic acids. The *Mtb* cell wall is characterised by a high selective permeability and thus acts as a barrier to many drugs (Telenti et al., 2000). This important factor affects the antimicrobial agent's bioavailability to the microorganism. An example of this was reported by Cantrell and his collaborators who noticed a correlation between bioavailability and the polarity of some semi-synthetic anti-*Mtb* compounds (Cantrell et al., 2001).

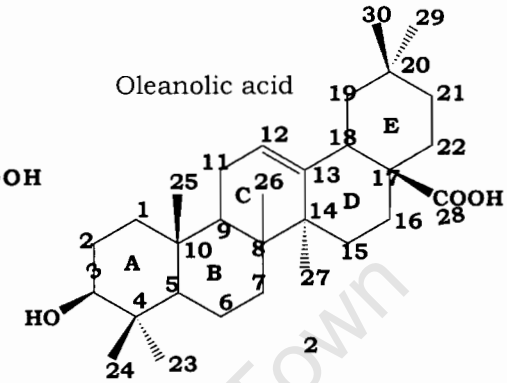
Comparing the anti-TB effects of some triterpenes, Wachter et al. (1999) found constituents containing the ursolic skeleton to be more potent than those containing the oleanolic skeleton are. Ursolic acid (**1**), for instance, is twice as active (MIC = 32 μM) as oleanolic acid (**2:** MIC = 64 μM) against *Mtb* H37Rv using the BACTEC 460 system (Cantrell et al., 2001). The only difference between OA and UA is the geminal methyl group at C-20 (OA), and C-19 and C-20 (UA) in ring E. It suggests that ring E is important for the

antitubercular activity of triterpenoid pentacyclic molecules such as OA, UA and betulin.

The saturation of the double bond at C12 – C13 decreases the activity of oleanolic acid. For example, erythodiol (**3**) is twice as active as friedelinol (**4**) [Wachter et al., 1999).



1



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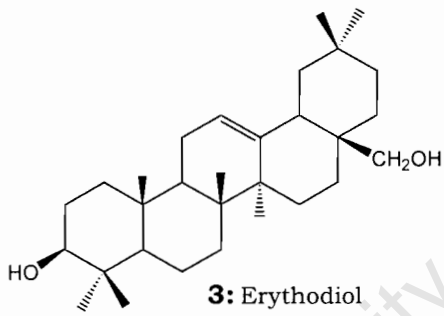
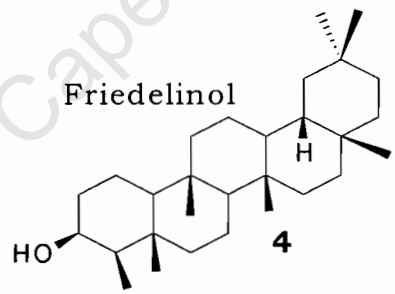


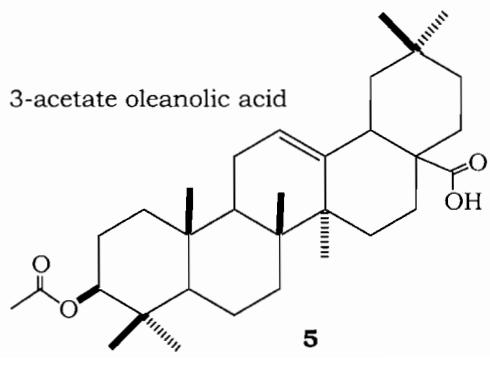
3: Erythodiol



4

3-O-acetyl oleanolic acid (**5**), isolated from *Prismatomeris fragrans* exhibited antimalarial activity against *Plasmodium falciparum*, antimycobacterial activity against *Mycobacterium tuberculosis,* and anticancer activity against human lung cancer cells, NCI-H187 (Kanokmedhakul et al., 2005). In contrast, compound (**6**), was found to be inactive against to malaria, tuberculosis and human lung cancer cells (Kanokmedhakul et al., 2005).

Sericic acid (**7**) from *Terminalia sericea* (Combretacea) and its homologue ajunolic acid (**8**) from *Syzygium cordatum* (Myrtaceae) are both OA derivatives, which were reported to have antimicrobial properties.



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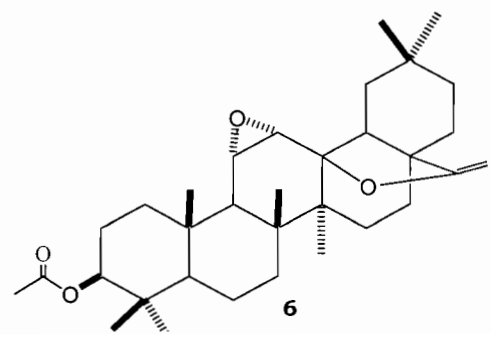
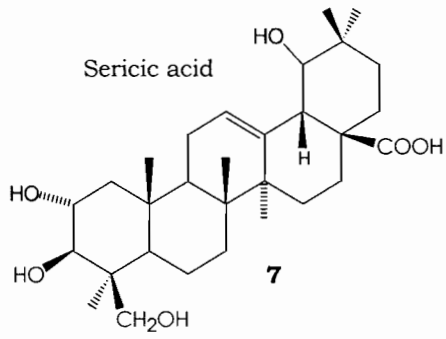


6

The decoction of bark leaves and roots from *S. cordatum* and *T. sericea* are reportedly used in traditional medicine for the treatment of tuberculosis, stomach complaints and diarrhoea (Wyk et al., 1997).



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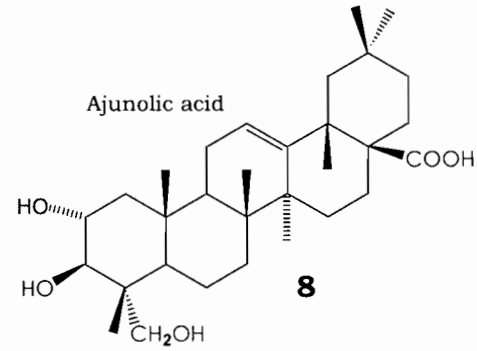
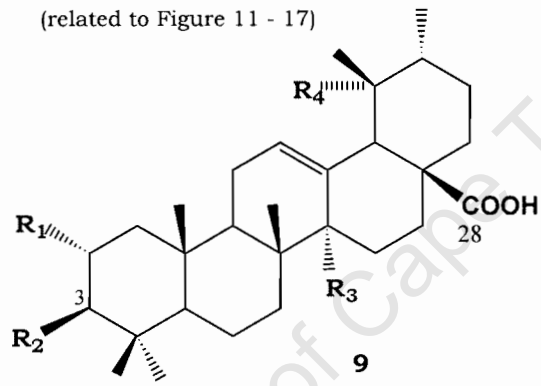


8

Ursolic acid skeleton
(related to Figure 11 - 17)



9

Figure	Compound	R	R_1	R_2	R_3	R_4
10	Uvaol	ß-OH	COOH	H	Me	H
11	Pomolic acid	ß-OH	COOH	OH	Me	H
12	Tormentic acid	ß-OH	COOH	OH	Me	σ-OH
13	Pomolic acetate	ß-OAc	COOH	OH	Me	H
14	Epi-tormentic acid	ß-OH	COOH	OH	Me	ß-OH
15	Euscaphic acid	σ-OH	COOH	OH	Me	σ-OH

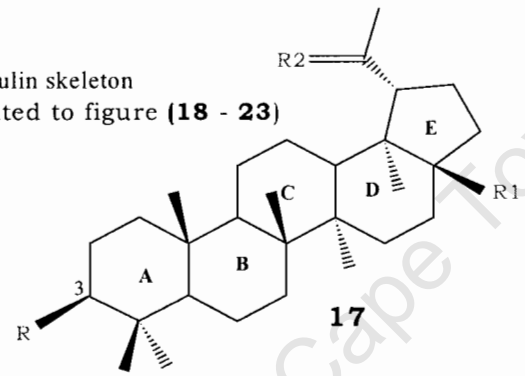
Some **UA** derivatives such as uvaol (**10**), pomolic acetate (**13**), and tormentic acid (**12**) were found to exhibit antitubercular activity as potent as **BA** and **UA**. Since pomolic acetate and tormentic acid are as active as **UA** is, it can be assumed that the presence of an -OH group at C-19 or an ester group at C-3 has no impact on the antitubercular activity (Wachter et al., 1999).

In contrast, the addition of two chemical groups on ring A, a $-CH_3$ at C-23 and an -OH at C-2 decreases the antitubercular activity. For example, epi-

tormentic acid (**14**), euscaphic acid (**15**), and niga-ichigoside (**16**) possess an antitubercular activity half of that of UA.

Figure	Compound	R	R_1	R_2
18	Betulinic acid	ß-OH	COOH	CH_2
19	Betulin	ß-OH	CH_2OH	CH_2
20	Lupeol	ß-OH	Me	CH_2
21	Lupenone	=O	Me	CH_2
22	Lupeol acetate	ß-OAc	Me	CH_2
23	3-OH-nor-lupen-20-one	ß-OH	Me	=O
24	3-Acetoxy-nor-lupen-20-one	ß-OAc	Me	=O

Betulin skeleton
related to figure **(18 - 23)**



17

In addition to OA and UA, betulinic acid (BA) and its derivatives (**18 – 24**) constitute a distinct class of antitubercular pentacyclic triterpenoids (Wachter et al., 1999). An analysis of betulinic acid and its analogues (**19** – **24**) shows that BA and betulin (**19**), with oxygen functionalities at C-28 have significant antitubercular activity, while those where C-28 is a methyl group have little or no activity, despite modifications at other positions (Wachter et al., 1999).

ZEORIN DERIVATIVES

Zeorin (**25**) is the most potent naturally occurring antitubercular terpenoid. Zeorin was found to be 400% more active than UA, but it also showed cytotoxicity against cultured P-388 cells (Wong et al., 1986). Because of its toxicity, zeorin is unlikely to be a suitable candidate as a new anti-TB drug. In contrast, BA, another potent antitubercular and antitumor agent (causing

apoptosis in melanoma cells), is reportedly being tested for use in humans (Pisha et al., 1995).

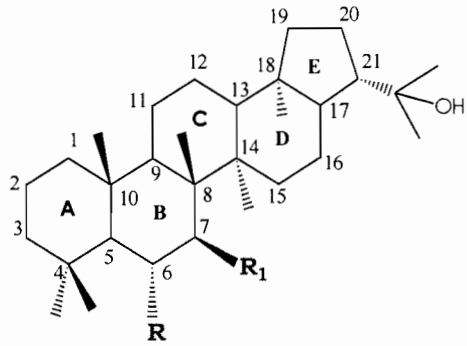
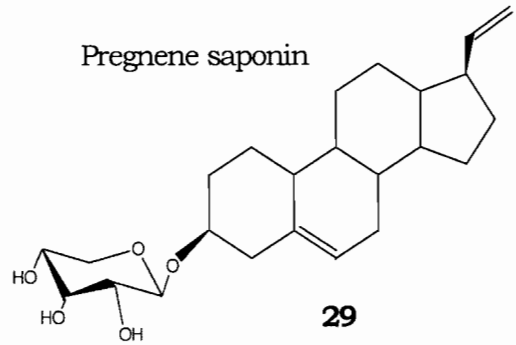


Figure	Compound	R	R_1
25	Zeorin	OH	H
26	7ß-acetyl-22-hydroxylhopane	H	OAc
27	7ß,22-dihydroxyhopane	H	OH

Although zeorin has strong anti-TB activity, its modified C-7 (**26** and **27)** derivatives, which have a acetal and a hydroxyl attached at C-7, respectively, have no significant activity (Pisha et al., 1995).

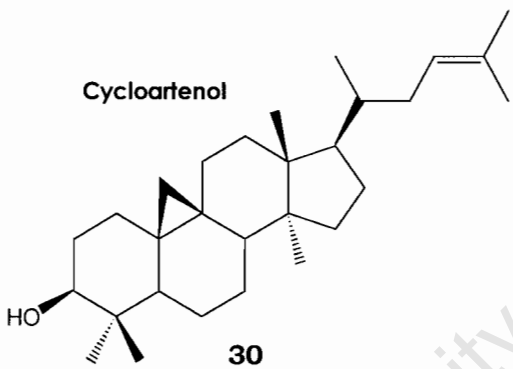
5.2.2 SAPONINS

The addition of a hydrophilic moiety to the above sterol skeleton (**28** – **29**) reduces the lipophilicity of the molecule and hence affects its bioavailability to *Mtb*. The saponin jugubogenin (**28**) extracted from the plant, *Colubrina retusa* (Rhamnaceae) is an example in this category of compounds; its anti-*Mtb* MIC is 10µg/ml (Elsohly et al., 1999) whereas its corresponding bioactive aglycon moiety epidioxysterol (**33**)has an anti-*Mtb* MIC ≤ 2.5 µg/ml (Saludes et al., 2002). Pregnene (**29**), a saponin from the gorgonian octocoral, *Eunicea pinta*, is another example in this class of compounds; its anti-*Mtb* MIC was found to be higher (6.25 µg/ml), than to its corresponding aglycon moieties stigmasta-4-en-3-one (**31**) and stigmasta-4, 22-dien-3-one (**33**) (anti-*Mtb* MIC < 2 µg/ml) (Saludes et al., 2002).



29

Although saponin derivatives of bioactive aglycon moieties such as Figures **30 - 34** have weak anti-*Mtb* activity, they may be acting as pro-drugs. It is also possible that their emulsifying physicochemical nature allows them to interpose in the TB cell membrane and disrupt the intra-extra cell exchanges with lethal effects for the bacilli.



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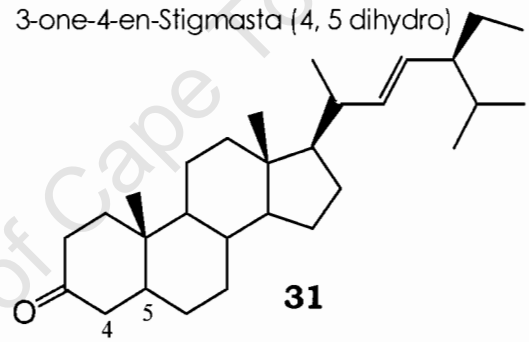
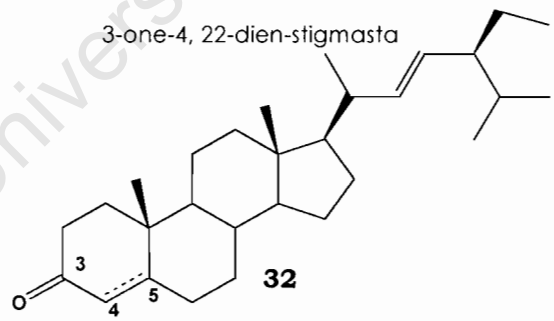


31



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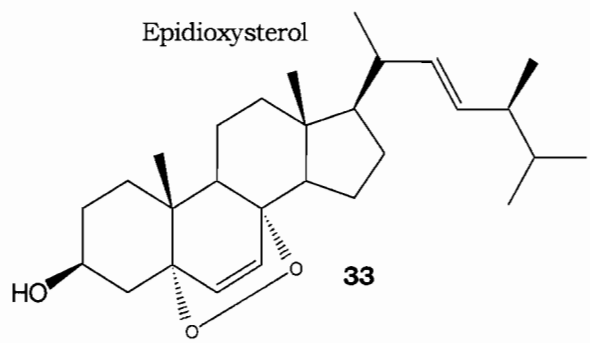
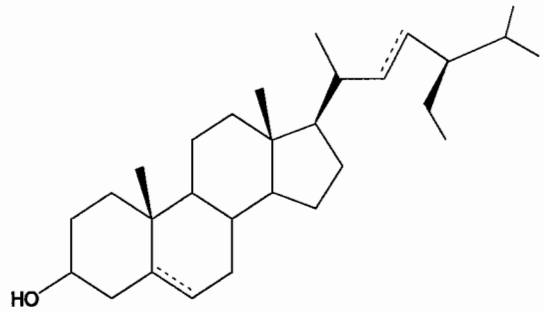


33



34: 22,23-dihydrositosterol
35: 22, 23- dehydro-sitosterol (stigmasterol)

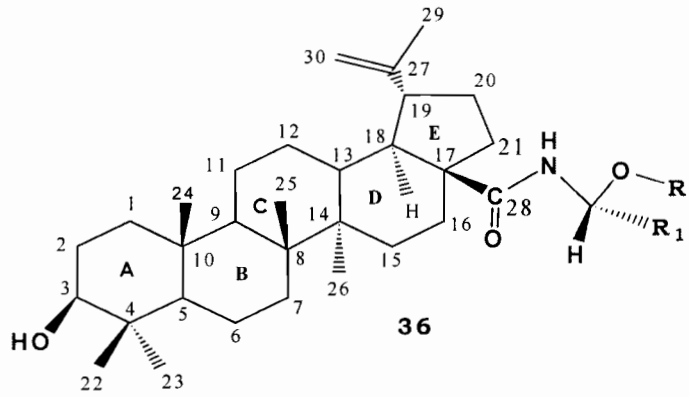
The 3-one-4-en-stigmasta (4, 5 dihydro, **31**), (3-one-4, 22-dien-stigmasta (**32**), and epidioxysterol (4, 5 deshydro, **33**) were reported to be potent anti-*Mtb* constituents of *Morinda citrifolia* (MICs ≤ 2.5 µg/ml; Saludes et al., 2002). Other bioactive compounds such as 22, 23- dihydro-β-sitosterol (**34**) isolated from *M. citrifolia*, and 22, 23- dehydro-β-sitosterol (stigmasterol, **35**) were also reported to have anti-*Mtb* activity with a MIC of 32 µg/ml and 125 µg/ml, respectively (Saludes et al., 2002).

5.3 MODIFIED BIOACTIVE PENTACYCLIC TRITERPENOIDS

Many naturally occurring and modified triterpenoid structures (UA, OA, and BA) have been reported for their anti-cancer and anti-HIV activities. It appears however, that only a few modified triterpenoid structures were particularly designed for anti-tuberculosis activity. Of all naturally occurring anti-tubercular pentacyclic triterpenoids (UA, OA, BA, and EA) BA was found to be the most potent; it has in addition a proven antitumor and anti-HIV activity. Based on a recent literature review of bioactive triterpenoids, it is apparent that structure modifications of UA, OA, and BA for anti-tumour and/or antiviral activity targeted particularly the –OH group at C-3, and/or the carboxyl group at C-28 and the double bond. These three functions are all determinant for Antitumor, anti-HIV, and anti-TB activity of triterpenoids compounds.

5.3.1 C-28 modified betulinic acid

36 is a general representation of the C-28 modified BA structures that possess anti-HIV and/or anticancer activities (Baglin et al, 2003).

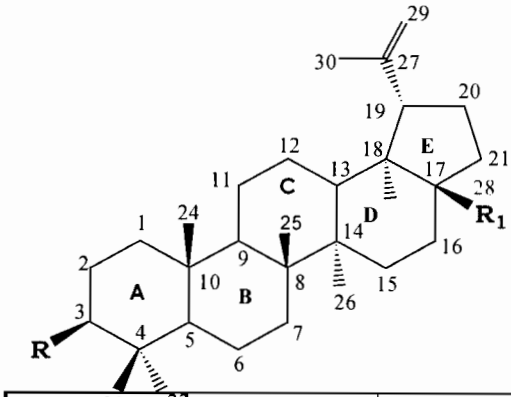


36

Figure	R	R_1
37	-	COOH
38	H_2N	COOH
39	HO-N=	COOH
40	HO-N=	CHNOH
41	CH_3O-N=	COOH
42	OH	H-CON$(CH_2)_{10}$-COO
43	OCO-$(CH_2)_3$-COOH	-COOH

5.3.2 C-3 alkylated betulinic and ursolic acid derivatives

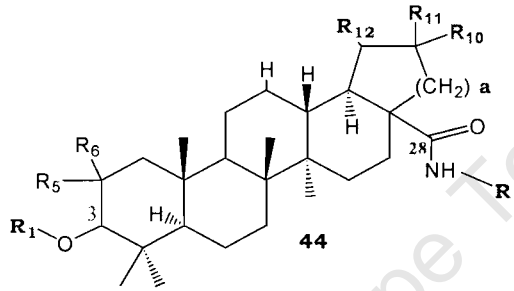
Baglin et al. (2003) also reported a number of betulinic and ursolic acid derivatives with substituents at C-3 (**40 – 43**) that possess anti-HIV activity.



42	CH_3	OCH_3
43	$CH(CH_3)_2$	OCH_3
Figure	**R**	**R_1**
40	H	OH
41	CH_3	OH

Acylation at C-3 was performed using various acylating (reagents) such as diclohexylcarbodiimide (DCC) and a catalyst, 4-dimethylaminopyridine (DMAP) (Baglin et al, 2003).

Other betulinic acid derivatives which have been reported for anti-HIV activity include the invention by Chin Ho Chen and Li Hang (patent application publication No.: US 2004/0204389 A1), which provides compounds of general structure shown in (**44**). BA is substituted at C-3 and C-28.



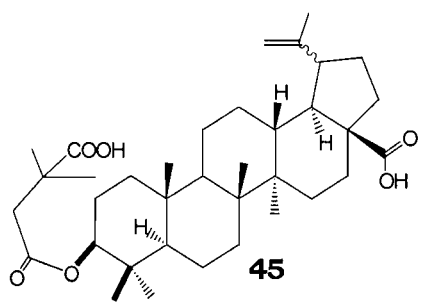
44

a	1 or 2
R10 and R11	H, $-CH_3$ or $-CH_2-CH_3$
R	$-(CH)_n-X-(CH)_m-Y-C-COOR'$
X	$-NH-CH_2-$; -NH-CO-; $-CHNH_2-$
Y	-Bn-

(Chen et al, 2004: Patent application publication No: US 2004/0204389 A1)

However, it is important to note that many of these compounds exhibited severe toxicities and side effects, required complicated dosing schedules or could lead to drug resistance (Chen et al, 2004).

Two of these compounds (**45 – 46**) were reported to be suitable candidates for the treatment of HIV (Chen et al, 2004: Patent application publication No: US 2004/0204389 A1)



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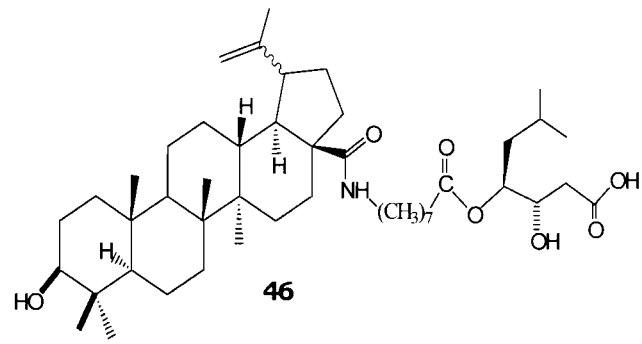


46

ANTI-TUMOUR EFFECTS

A number of investigations have been done regarding the relationship between structure and anti-tumour effects of pentacyclic triterpenoids, UA, OA, and BA. The most important observations in this regard can be summarised as follows:

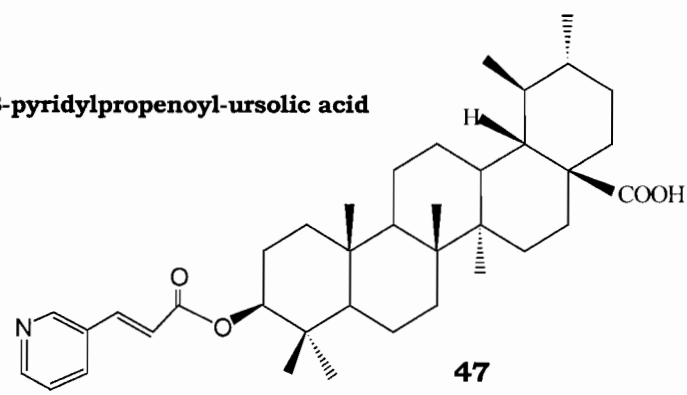
1. Lacaille-Dubois et al. (2000) found UA, OA, and BA to be effective cytotoxic agents against human colon adenocarcinoma cell lines (HT 29 cells) (Lacaille-Dubois et al., 2000).

2. The potassium salt of these acids, though more soluble, did not provide more potency than the corresponding original molecules, except for the potassium salt of BA, which exhibited greater cytotoxic activity than UA (Baglin et al., 2003). The hydrogen bonding capacity at C-28 and/or acidity is essential in the expression of the cytotoxic effects of the molecule (Baglin et al., 2003; Kim et al., 1998).

3. The acid function at C-28 and the hydroxyl group at C-3 are key to the anti-tumour activity of the pentacyclic triterpenoids, UA, OA, and BA (Baglin et al., 2003; Martin-Cordero et al., 2001).

4. The size limitation and the electronic density of the substituents are determinant factors for the activity (Baglin et al., 2003). For example, Lacaille et al. (2000) observed the loss of cytotoxic potency against HT29 cell lines when any cinnamoyl moiety was substituted at C-3. However, the use of a pyridylpropenoyl moiety for the C-3 substitution on ursolic acid provided a compound (**47**), which exhibited significant cytotoxicity.

3-pyridylpropenoyl-ursolic acid



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5.4 CONCLUSION

The phytochemical group of pentacyclic triterpenoids to which the bioactive compounds, UA, OA, BA, and their derivatives belong is pharmacologically important because of significant biological activities that characterise these constituents. The biological activities of pentacyclic triterpenoids include antitubercular, anti-tumour, and anti-HIV activities. The isolation of UA, OA and their derivatives from the traditional medicinal plants of the Cape province (South Africa), *B. saligna*, *L. gnaphaloides*, and *I. graveolens* supports the therapeutic use of these plant materials in the treatment of diseases such as TB.

In addition, this investigation has found the isolated bioactive constituents, UA, OA, and their derivatives to be non toxic against normal cells (the CHO cell line) (section **3.3.4** and **4.1.3.4**). This finding adds value to the pharmaceutical prospects of these compounds.

As part of the present investigation, a literature survey regarding structure activity relationships of pentacyclic triterpenoids revealed that there are very few reports concerning the modification of pentacyclic triterpenoids as potential anti-TB agents. In contrast, a long list of modified pentacyclic triterpenoid molecules with anti-tumour and or anti-HIV-1 potency is presented. This list of bioactive modified terpenoid guided the investigator to pin point the chemical functionalities of pentacyclic triterpenoids, which are important for anti-tumour and or anti-HIV activity and may be test for anttuberculosis activity as well. For example, the carboxyl at C-28, the hydroxyl at C-3 and ring E are key to anti-HIV and anti-tumour potency of UA, OA, BA and echinocystic acid. Thus substitutions at these key functional groups can provide molecular modifications likely to induce more potency and/or specificity. A considerable amount of structure modification work has been done with regard to the anti-tumour and anti-HIV activity of the triterpenoids, OA, UA, BA, and echinocystic acid (Baglin et al., 2001). For instance, concerning the modification of BA, the size limitation of the

substitutes at C-3, the importance of free carboxyl group at C-28 in comparison with other substituents such as –CHO, -$COOCH_3$, -CH_2-CH_3, and the importance of amino-acids substituents at C-28 were highlighted as necessary to obtain a good cytotoxic effects against melanoma cells (Lacaille-Dubois et al., 2000). On the contrary, the modification at C-20 reduces the anti-tumour activity.

Among the naturally occurring triterpenoids, BA was found to be a more potent anti-TB agent than UA and OA are. Furthermore, it is commercially available and cheaper than UA and OA. Its apoptosis-inducing ability and its favourable therapeutic index make BA a promising new lead worth pursuing.

These considerations prompted the present investigation directed at synthesis of derivatives of BA which would contribute to the study of structure/activity relationships with regard to antitubercular activity.

CHAPTER V

STRUCTURE ANTI-TB ACTIVITY RELATIONSHIP

5.1 INTRODUCTION

The resurgence of TB and DR-TB over the last two decades has led many scientists to search for new anti-TB drugs as a matter of urgency in order to overcome the global threat the pandemic is posing. Thus, a large number of naturally occurring and synthetic compounds have been reported for their antimycobacterial properties in these last two decades. A recent review of naturally occurring antimycobacterial compounds by Okunade et al. (2004) highlighted the structural diversity that characterises these naturally occurring bioactive compounds. They include terpenoids, saponins and glycosides, steroids, flavonoids and polyphenolic compounds. In this study, attention was particularly focused on pentacyclic triterpenoids because this is the class of compounds to which the bioactive constituents, ursolic acid, oleanolic acid, and their derivatives belong. These are the common active principles responsible for the anti-tuberculosis properties of *B. saligna, Inula graveolens* and *L. gnaphaloides*. UA, OA, and their homologue, betulinic acid (BA), are believed to be among the most likely antitubercular lead candidates for new anti-TB drugs. In their study, Wachter et al. (1999) found betulinic acid to be more potent against *Mtb* than ursolic and oleanolic acid.

Further, it is also important to note that numerous synthetic and semi-synthetic pentacyclic triterpenoid derivatives were produced in an attempt to improve the biological activity and selectivity of these molecules, mostly with regard to their anti-HIV activity. For instance, Baglin et al. (2003) published a review of natural and modified betulinic, ursolic and echinocystic acid derivatives as potential antitumor and anti-HIV agents.

This chapter will discuss the most potent antitubercular pentacyclic triterpenoids and their structure-activity relationships with particular attention to substitutions on C-3 and C-28.

5.2 NATURALLY OCCURING PENTACYCLIC TRITERPENOIDS: STRUCTURE-ANTITUBERCULAR ACTIVITY CORRELATION

5.2.1 URSOLIC, OLEANOLIC AND BETULINIC ACID

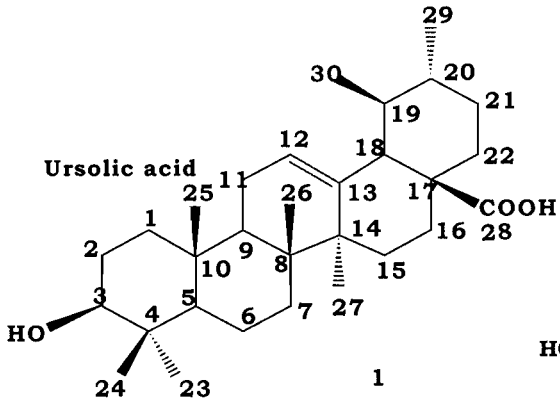
Pentacyclic triterpenoids are widely distributed in higher plants, and they are known to possess diverse biological activities. Compounds such as OA and UA, for instance, have been reported for several biological activities including anti-diabetic, anti-inflammatory, diuretic, anti-spasmodic, anti-atherosclerotic, anti-tumour, antimicrobial, anti-fungal, and anti-HIV (Li and Wei-Jian, 2002; Takeoka et al., 2000).

Jing-Zen and collaborators proposed that the antimicrobial mechanism of action of UA and OA and their derivatives is based on the inhibition of DNA polymerase (Jing-Zen et al., 2000). The same mechanism seems to explain the anti-tumour properties of these compounds. In the case of *Mtb*, drugs must pass through the cell wall, which is a highly hydrophobic structure made up mostly of mycolic acids. The *Mtb* cell wall is characterised by a high selective permeability and thus acts as a barrier to many drugs (Telenti et al., 2000). This important factor affects the antimicrobial agent's bioavailability to the microorganism. An example of this was reported by Cantrell and his collaborators who noticed a correlation between bioavailability and the polarity of some semi-synthetic anti-*Mtb* compounds (Cantrell et al., 2001).

Comparing the anti-TB effects of some triterpenes, Wachter et al. (1999) found constituents containing the ursolic skeleton to be more potent than those containing the oleanolic skeleton are. Ursolic acid (**1**), for instance, is twice as active (MIC = 32 μM) as oleanolic acid (**2:** MIC = 64 μM) against *Mtb* H37Rv using the BACTEC 460 system (Cantrell et al., 2001). The only difference between OA and UA is the geminal methyl group at C-20 (OA), and C-19 and C-20 (UA) in ring E. It suggests that ring E is important for the

antitubercular activity of triterpenoid pentacyclic molecules such as OA, UA and betulin.

The saturation of the double bond at C12 – C13 decreases the activity of oleanolic acid. For example, erythodiol (**3**) is twice as active as friedelinol (**4**) [Wachter et al., 1999).



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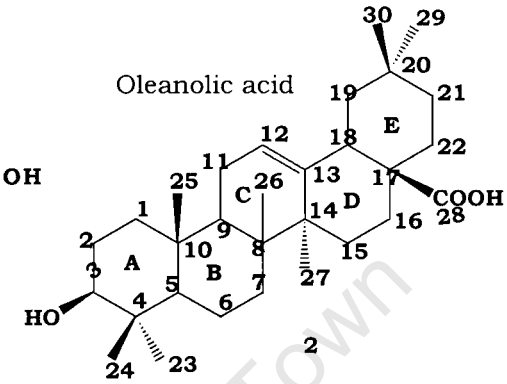

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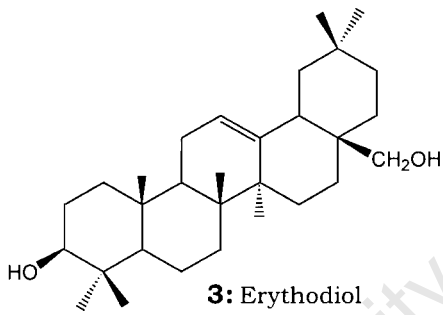
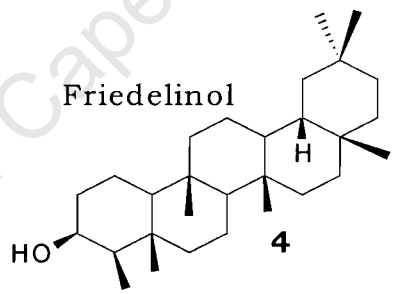


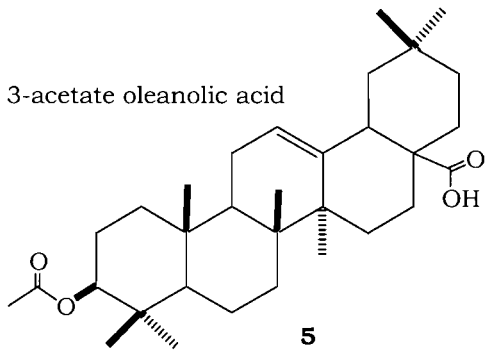
3: Erythodiol



4

3-O-acetyl oleanolic acid (**5**), isolated from *Prismatomeris fragrans* exhibited antimalarial activity against *Plasmodium falciparum*, antimycobacterial activity against *Mycobacterium tuberculosis,* and anticancer activity against human lung cancer cells, NCI-H187 (Kanokmedhakul et al., 2005). In contrast, compound (**6**), was found to be inactive against to malaria, tuberculosis and human lung cancer cells (Kanokmedhakul et al., 2005).

Sericic acid (**7**) from *Terminalia sericea* (Combretacea) and its homologue ajunolic acid (**8**) from *Syzygium cordatum* (Myrtaceae) are both OA derivatives, which were reported to have antimicrobial properties.



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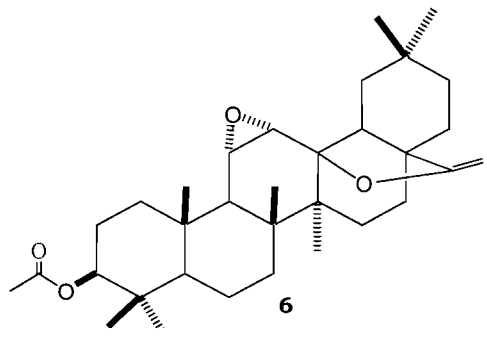

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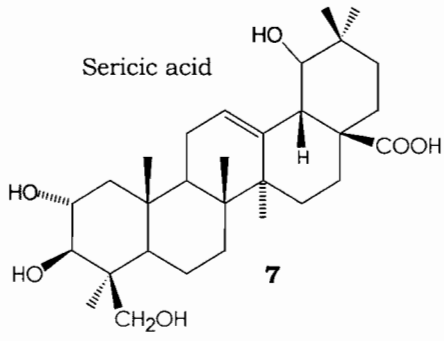
The decoction of bark leaves and roots from *S. cordatum* and *T. sericea* are reportedly used in traditional medicine for the treatment of tuberculosis, stomach complaints and diarrhoea (Wyk et al., 1997).



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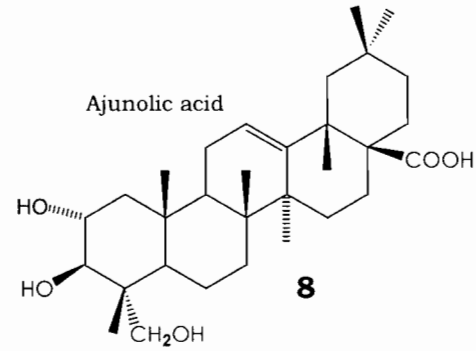

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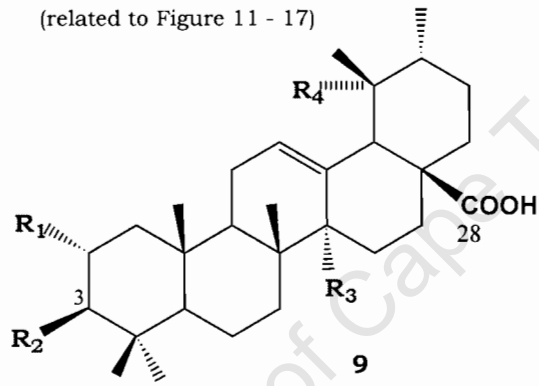
Ursolic acid skeleton
(related to Figure 11 - 17)



9

Figure	Compound	R	R_1	R_2	R_3	R_4
10	Uvaol	ß-OH	COOH	H	Me	H
11	Pomolic acid	ß-OH	COOH	OH	Me	H
12	Tormentic acid	ß-OH	COOH	OH	Me	σ-OH
13	Pomolic acetate	ß-OAc	COOH	OH	Me	H
14	Epi-tormentic acid	ß-OH	COOH	OH	Me	ß-OH
15	Euscaphic acid	σ-OH	COOH	OH	Me	σ-OH

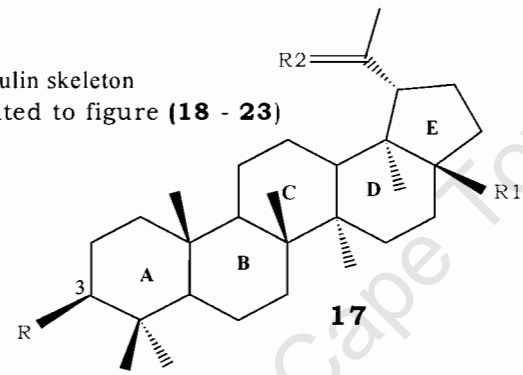
Some **UA** derivatives such as uvaol (**10**), pomolic acetate (**13**), and tormentic acid (**12**) were found to exhibit antitubercular activity as potent as **BA** and **UA**. Since pomolic acetate and tormentic acid are as active as **UA** is, it can be assumed that the presence of an -OH group at C-19 or an ester group at C-3 has no impact on the antitubercular activity (Wachter et al., 1999).

In contrast, the addition of two chemical groups on ring A, a $-CH_3$ at C-23 and an -OH at C-2 decreases the antitubercular activity. For example, epi-

tormentic acid (**14**), euscaphic acid (**15**), and niga-ichigoside (**16**) possess an antitubercular activity half of that of UA.

Figure	Compound	R	R_1	R_2
18	Betulinic acid	ß-OH	COOH	CH_2
19	Betulin	ß-OH	CH_2OH	CH_2
20	Lupeol	ß-OH	Me	CH_2
21	Lupenone	=O	Me	CH_2
22	Lupeol acetate	ß-OAc	Me	CH_2
23	3-OH-nor-lupen-20-one	ß-OH	Me	=O
24	3-Acetoxy-nor-lupen-20-one	ß-OAc	Me	=O

Betulin skeleton
related to figure **(18 - 23)**



17

In addition to OA and UA, betulinic acid (BA) and its derivatives (**18 – 24**) constitute a distinct class of antitubercular pentacyclic triterpenoids (Wachter et al., 1999). An analysis of betulinic acid and its analogues (**19** – **24**) shows that BA and betulin (**19**), with oxygen functionalities at C-28 have significant antitubercular activity, while those where C-28 is a methyl group have little or no activity, despite modifications at other positions (Wachter et al., 1999).

ZEORIN DERIVATIVES

Zeorin (**25**) is the most potent naturally occurring antitubercular terpenoid. Zeorin was found to be 400% more active than UA, but it also showed cytotoxicity against cultured P-388 cells (Wong et al., 1986). Because of its toxicity, zeorin is unlikely to be a suitable candidate as a new anti-TB drug. In contrast, BA, another potent antitubercular and antitumor agent (causing

apoptosis in melanoma cells), is reportedly being tested for use in humans (Pisha et al., 1995).

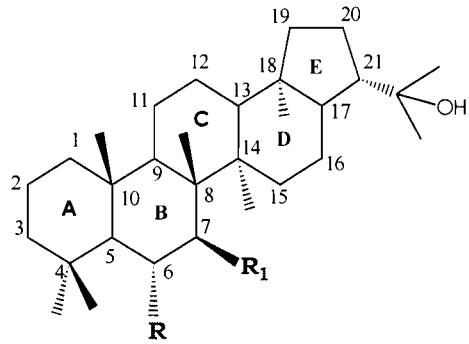
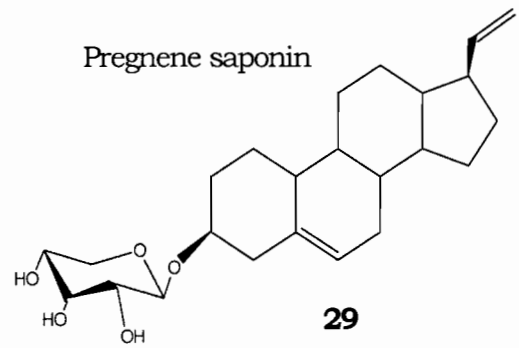


Figure	Compound	R	R_1
25	Zeorin	OH	H
26	7ß-acetyl-22-hydroxylhopane	H	OAc
27	7ß,22-dihydroxyhopane	H	OH

Although zeorin has strong anti-TB activity, its modified C-7 (**26** and **27)** derivatives, which have a acetal and a hydroxyl attached at C-7, respectively, have no significant activity (Pisha et al., 1995).

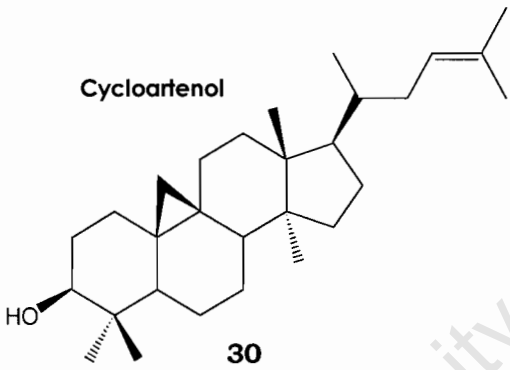
5.2.2 SAPONINS

The addition of a hydrophilic moiety to the above sterol skeleton (**28** – **29**) reduces the lipophilicity of the molecule and hence affects its bioavailability to *Mtb*. The saponin jugubogenin (**28**) extracted from the plant, *Colubrina retusa* (Rhamnaceae) is an example in this category of compounds; its anti-*Mtb* MIC is 10µg/ml (Elsohly et al., 1999) whereas its corresponding bioactive aglycon moiety epidioxysterol (**33**)has an anti-*Mtb* MIC ≤ 2.5 µg/ml (Saludes et al., 2002). Pregnene (**29**), a saponin from the gorgonian octocoral, *Eunicea pinta*, is another example in this class of compounds; its anti-*Mtb* MIC was found to be higher (6.25 µg/ml), than to its corresponding aglycon moieties stigmasta-4-en-3-one (**31**) and stigmasta-4, 22-dien-3-one (**33**) (anti-*Mtb* MIC < 2 µg/ml) (Saludes et al., 2002).



29

Although saponin derivatives of bioactive aglycon moieties such as Figures **30 - 34** have weak anti-*Mtb* activity, they may be acting as pro-drugs. It is also possible that their emulsifying physicochemical nature allows them to interpose in the TB cell membrane and disrupt the intra-extra cell exchanges with lethal effects for the bacilli.



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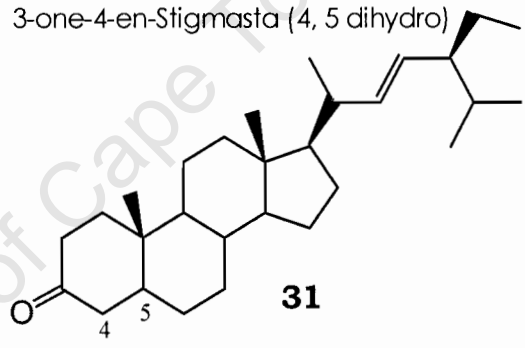

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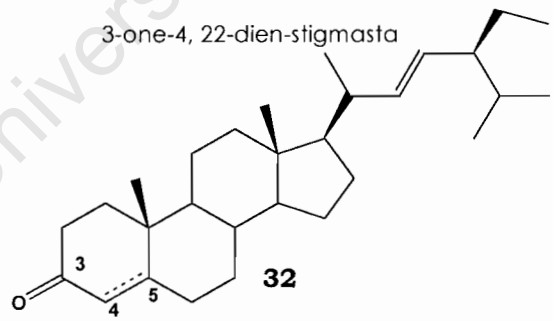

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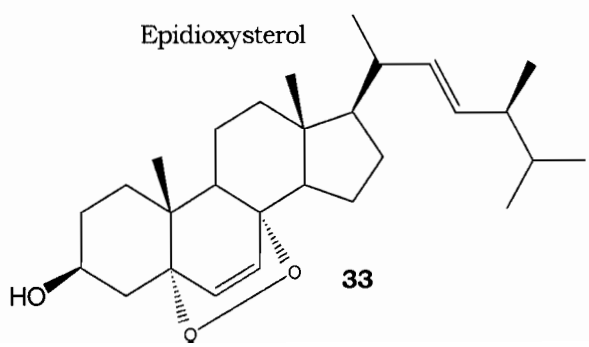

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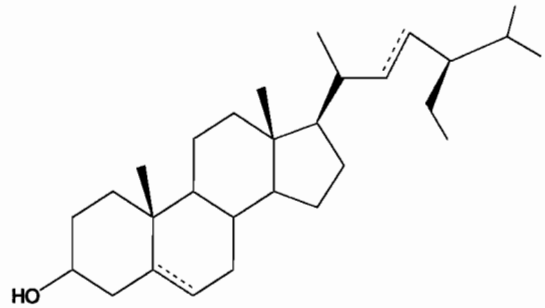


34: 22,23-dihydrositosterol
35: 22, 23- dehydro-sitosterol (stigmasterol)

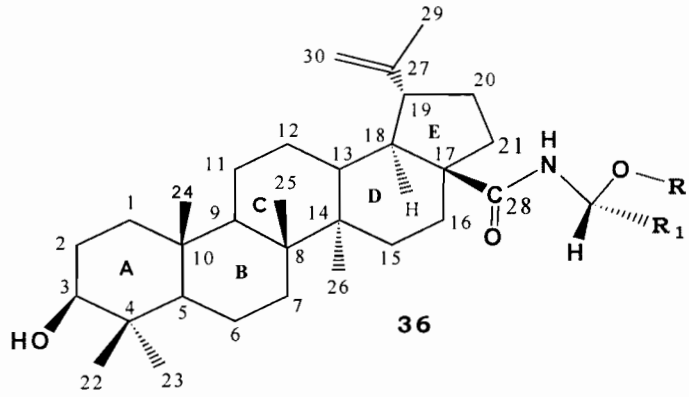
The 3-one-4-en-stigmasta (4, 5 dihydro, **31**), (3-one-4, 22-dien-stigmasta (**32**), and epidioxysterol (4, 5 deshydro, **33**) were reported to be potent anti-*Mtb* constituents of *Morinda citrifolia* (MICs ≤ 2.5 µg/ml; Saludes et al., 2002). Other bioactive compounds such as 22, 23- dihydro-β-sitosterol (**34**) isolated from *M. citrifolia*, and 22, 23- dehydro-β-sitosterol (stigmasterol, **35**) were also reported to have anti-*Mtb* activity with a MIC of 32 µg/ml and 125 µg/ml, respectively (Saludes et al., 2002).

5.3 MODIFIED BIOACTIVE PENTACYCLIC TRITERPENOIDS

Many naturally occurring and modified triterpenoid structures (UA, OA, and BA) have been reported for their anti-cancer and anti-HIV activities. It appears however, that only a few modified triterpenoid structures were particularly designed for anti-tuberculosis activity. Of all naturally occurring anti-tubercular pentacyclic triterpenoids (UA, OA, BA, and EA) BA was found to be the most potent; it has in addition a proven antitumor and anti-HIV activity. Based on a recent literature review of bioactive triterpenoids, it is apparent that structure modifications of UA, OA, and BA for anti-tumour and/or antiviral activity targeted particularly the –OH group at C-3, and/or the carboxyl group at C-28 and the double bond. These three functions are all determinant for Antitumor, anti-HIV, and anti-TB activity of triterpenoids compounds.

5.3.1 C-28 modified betulinic acid

36 is a general representation of the C-28 modified BA structures that possess anti-HIV and/or anticancer activities (Baglin et al, 2003).

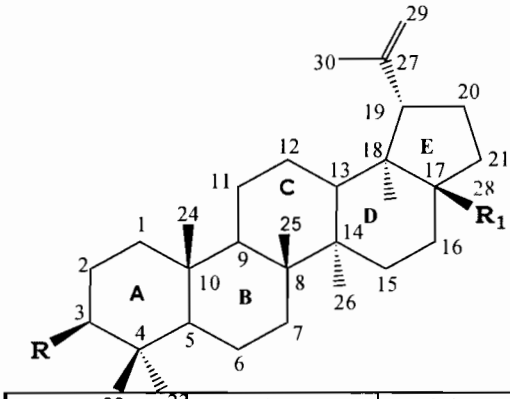


36

Figure	R	R_1
37	-	COOH
38	H_2N	COOH
39	HO-N=	COOH
40	HO-N=	CHNOH
41	CH_3O-N=	COOH
42	OH	H-CON$(CH_2)_{10}$-COO
43	OCO-$(CH_2)_3$-COOH	-COOH

5.3.2 C-3 alkylated betulinic and ursolic acid derivatives

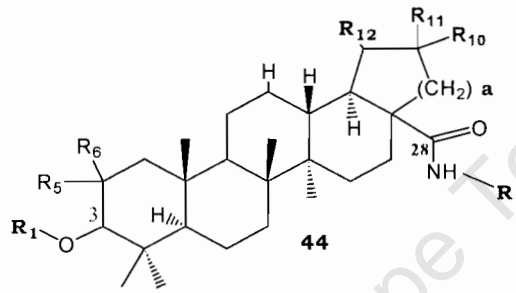
Baglin et al. (2003) also reported a number of betulinic and ursolic acid derivatives with substituents at C-3 (**40 – 43**) that possess anti-HIV activity.



42	CH_3	OCH_3
43	$CH(CH_3)_2$	OCH_3
Figure	**R**	**R_1**
40	H	OH
41	CH_3	OH

Acylation at C-3 was performed using various acylating (reagents) such as diclohexylcarbodiimide (DCC) and a catalyst, 4-dimethylaminopyridine (DMAP) (Baglin et al, 2003).

Other betulinic acid derivatives which have been reported for anti-HIV activity include the invention by Chin Ho Chen and Li Hang (patent application publication No.: US 2004/0204389 A1), which provides compounds of general structure shown in (**44**). BA is substituted at C-3 and C-28.



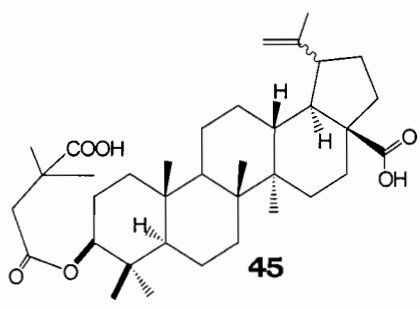
44

a	1 or 2
R10 and R11	H, $-CH_3$ or $-CH_2-CH_3$
R	$-(CH)_n-X-(CH)_m-Y-C-COOR'$
X	$-NH-CH_2-$; -NH-CO-; $-CHNH_2-$
Y	-Bn-

(Chen et al, 2004: Patent application publication No: US 2004/0204389 A1)

However, it is important to note that many of these compounds exhibited severe toxicities and side effects, required complicated dosing schedules or could lead to drug resistance (Chen et al, 2004).

Two of these compounds (**45 – 46**) were reported to be suitable candidates for the treatment of HIV (Chen et al, 2004: Patent application publication No: US 2004/0204389 A1)



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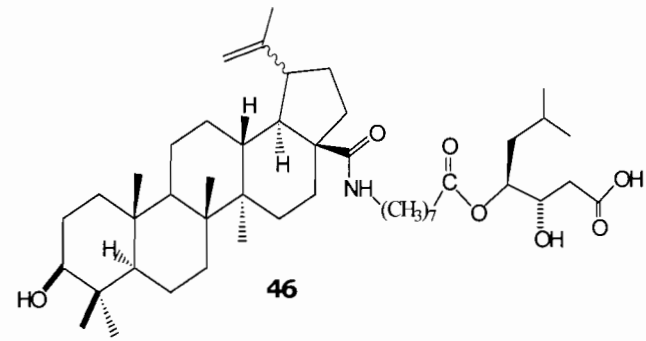

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ANTI-TUMOUR EFFECTS

A number of investigations have been done regarding the relationship between structure and anti-tumour effects of pentacyclic triterpenoids, UA, OA, and BA. The most important observations in this regard can be summarised as follows:

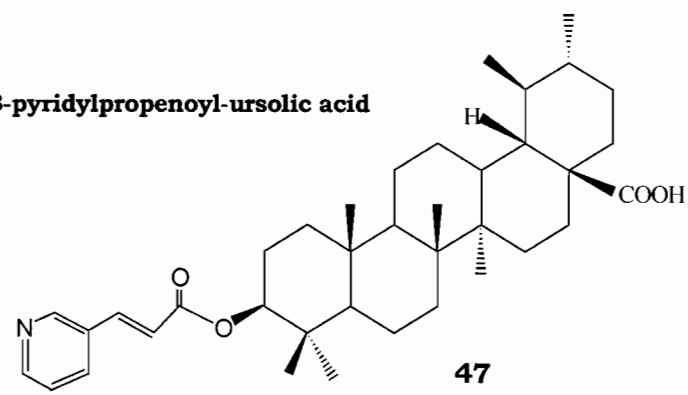
1. Lacaille-Dubois et al. (2000) found UA, OA, and BA to be effective cytotoxic agents against human colon adenocarcinoma cell lines (HT 29 cells) (Lacaille-Dubois et al., 2000).

2. The potassium salt of these acids, though more soluble, did not provide more potency than the corresponding original molecules, except for the potassium salt of BA, which exhibited greater cytotoxic activity than UA (Baglin et al., 2003). The hydrogen bonding capacity at C-28 and/or acidity is essential in the expression of the cytotoxic effects of the molecule (Baglin et al., 2003; Kim et al., 1998).

3. The acid function at C-28 and the hydroxyl group at C-3 are key to the anti-tumour activity of the pentacyclic triterpenoids, UA, OA, and BA (Baglin et al., 2003; Martin-Cordero et al., 2001).

4. The size limitation and the electronic density of the substituents are determinant factors for the activity (Baglin et al., 2003). For example, Lacaille et al. (2000) observed the loss of cytotoxic potency against HT29 cell lines when any cinnamoyl moiety was substituted at C-3. However, the use of a pyridylpropenoyl moiety for the C-3 substitution on ursolic acid provided a compound (**47**), which exhibited significant cytotoxicity.

3-pyridylpropenoyl-ursolic acid



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5.4 CONCLUSION

The phytochemical group of pentacyclic triterpenoids to which the bioactive compounds, UA, OA, BA, and their derivatives belong is pharmacologically important because of significant biological activities that characterise these constituents. The biological activities of pentacyclic triterpenoids include antitubercular, anti-tumour, and anti-HIV activities. The isolation of UA, OA and their derivatives from the traditional medicinal plants of the Cape province (South Africa), *B. saligna*, *L. gnaphaloides*, and *I. graveolens* supports the therapeutic use of these plant materials in the treatment of diseases such as TB.

In addition, this investigation has found the isolated bioactive constituents, UA, OA, and their derivatives to be non toxic against normal cells (the CHO cell line) (section **3.3.4** and **4.1.3.4**). This finding adds value to the pharmaceutical prospects of these compounds.

As part of the present investigation, a literature survey regarding structure activity relationships of pentacyclic triterpenoids revealed that there are very few reports concerning the modification of pentacyclic triterpenoids as potential anti-TB agents. In contrast, a long list of modified pentacyclic triterpenoid molecules with anti-tumour and or anti-HIV-1 potency is presented. This list of bioactive modified terpenoid guided the investigator to pin point the chemical functionalities of pentacyclic triterpenoids, which are important for anti-tumour and or anti-HIV activity and may be test for anttuberculosis activity as well. For example, the carboxyl at C-28, the hydroxyl at C-3 and ring E are key to anti-HIV and anti-tumour potency of UA, OA, BA and echinocystic acid. Thus substitutions at these key functional groups can provide molecular modifications likely to induce more potency and/or specificity. A considerable amount of structure modification work has been done with regard to the anti-tumour and anti-HIV activity of the triterpenoids, OA, UA, BA, and echinocystic acid (Baglin et al., 2001). For instance, concerning the modification of BA, the size limitation of the

substitutes at C-3, the importance of free carboxyl group at C-28 in comparison with other substituents such as –CHO, -$COOCH_3$, -CH_2-CH_3, and the importance of amino-acids substituents at C-28 were highlighted as necessary to obtain a good cytotoxic effects against melanoma cells (Lacaille-Dubois et al., 2000). On the contrary, the modification at C-20 reduces the anti-tumour activity.

Among the naturally occurring triterpenoids, BA was found to be a more potent anti-TB agent than UA and OA are. Furthermore, it is commercially available and cheaper than UA and OA. Its apoptosis-inducing ability and its favourable therapeutic index make BA a promising new lead worth pursuing.

These considerations prompted the present investigation directed at synthesis of derivatives of BA which would contribute to the study of structure/activity relationships with regard to antitubercular activity.

CHAPTER VI

SYNTHESIS OF 3-EPOXYPROPYLOXY AND SUBSTITUTED 3-(2'-HYDROXYPROPYL) - DERIVATIVES OF BETULINIC ACID

6.1 INTRODUCTION

6.1.1 General

Recent reviews of natural and modified BA have described a wide range of BA derivatives that possess anti-tumour and/or anti-HIV properties, which are mainly C-3 and/or C-28 substituted molecules. Previous studies established that selective substitutions at C-3, the double bond at C-29, and the carboxyl group at C-28 could leads to enhanced activity. Baglin et al. (2003) for example, reported a number of BA derivatives selectively substituted at C-3, and/or C-28, which showed an improved selective cytotoxicity against epidermoid carcinoma of the mouth.

A literature survey revealed that most C-3 modified BA molecules were substituted esters or amides, which were prepared to target the HIV and/or tumours. There is no report of such structural modification work specifically targeting antitubercular activity.

The objectives of the present work were:
- To prepare a C-3 epoxypropyl derivative (**3,** scheme 1) which would allow for generation of a variety of analogues (**5**) derived from nucleophilic opening of the epoxide. These could include alcohols and amino alcohols.
- To evaluate the in vitro anti-*Mtb* activity of **1- 5** against *Mtb* 3H 7Rv.

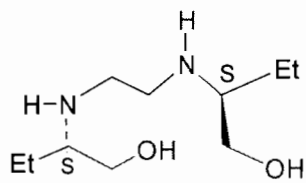
6.1.2 The rationale for focusing on alkylation at O-3

The reason choosing BA as starting material were already highlighted in section **5.4** (c). The focus on alkylation at O-3 was inspired by reports in the literature that various C-3 modified BA derivatives showed improved anti-HIV and/or anti-tumour activity (Baglin et al. 2003; Chen et al, 2004; Lacaille-Dubois et al., 2003; Kim et al., 1998; Martin-Cordero's et al., 2001). These also highlighted the importance of a free carboxyl group at C-30 in comparison with other functional groups such as –CHO, -$COOCH_3$, -CH_2-

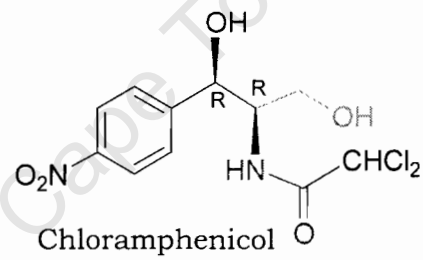
CH_3, although amino-acid substituents at C-30 also give good anti-tumour activity (Lacaille-Dubois et al., 2000).

Preparation of O-3 alkylated BA derivatives could also allow generation of products with increased polarity, which could in turn enhance the bioavailability of the agents.

The intention of this work was therefore to explore the idea of preparing BA derivatives substituted at O-3 with amino-alcohols and other known pharmacophores, which would enhance the desired biological activity of the compounds. Vicinal amino-alcohols are found as, for example pharmacophores in antibiotics such as ethambutol and chloramphenicol, but there are no reports to date of antitubercular BA derivatives having this structural feature.



Ethambutol



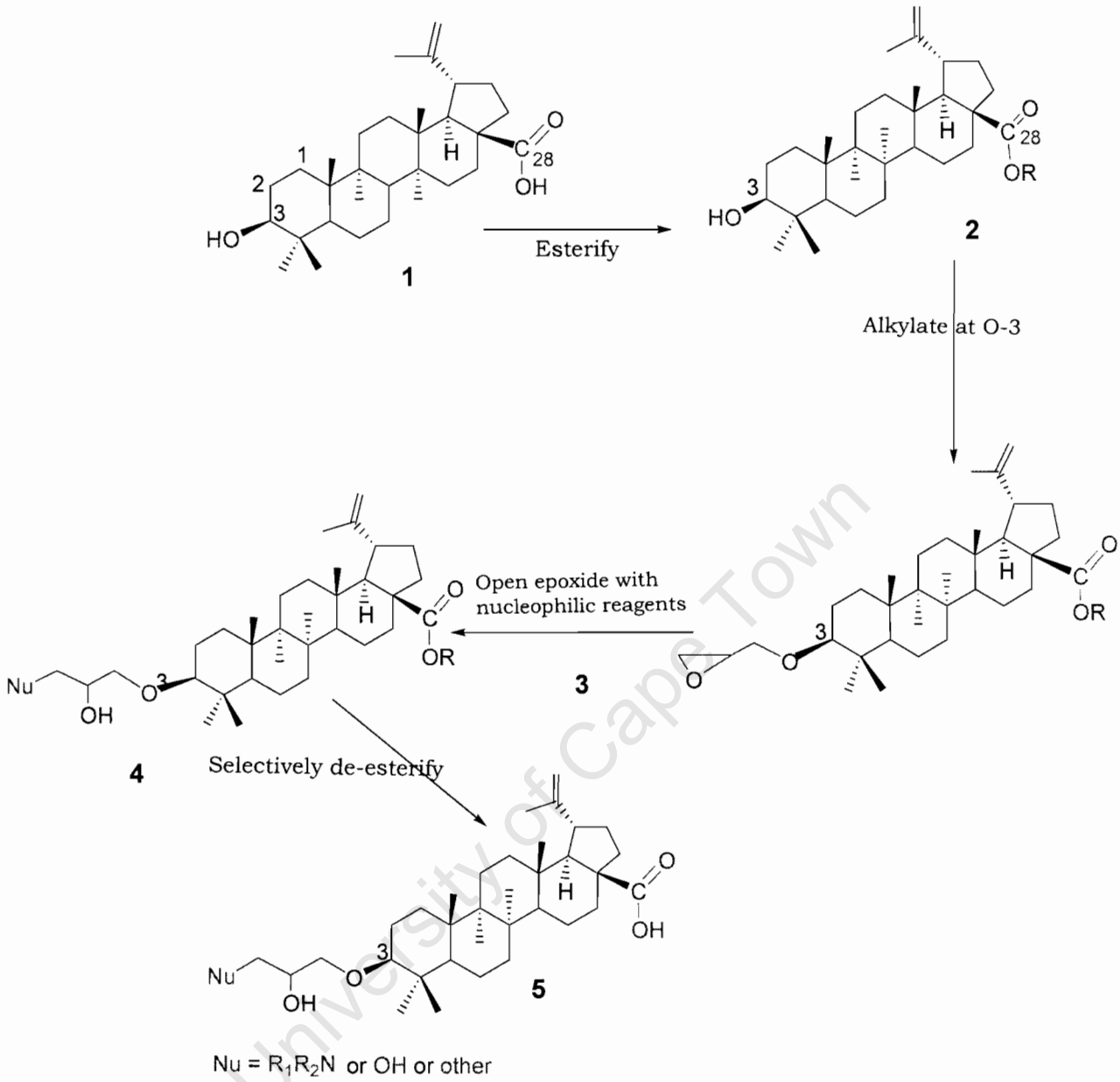
Chloramphenicol

The need was to develop a synthetic protocol that is simple, efficient, and allows for the generation of a range of related structures from a common core.

The objective as shown in scheme 1, was to prepare a C-3 epoxypropyl derivative (**3**) which would allow for the generation of a variety of analogues (**4**) derived from nucleophilic opening of the epoxide. These could include aminoalcohols, amino acids, etc...

To achieve this, it is necessary to first protect the carboxyl group to form (**2**), using a protection group which can be introduced selectively and then subsequently removed selectively in the presence of the double bond at C-29 and the alkyl substituent at O-3.

6.1.3 Proposed synthetic route

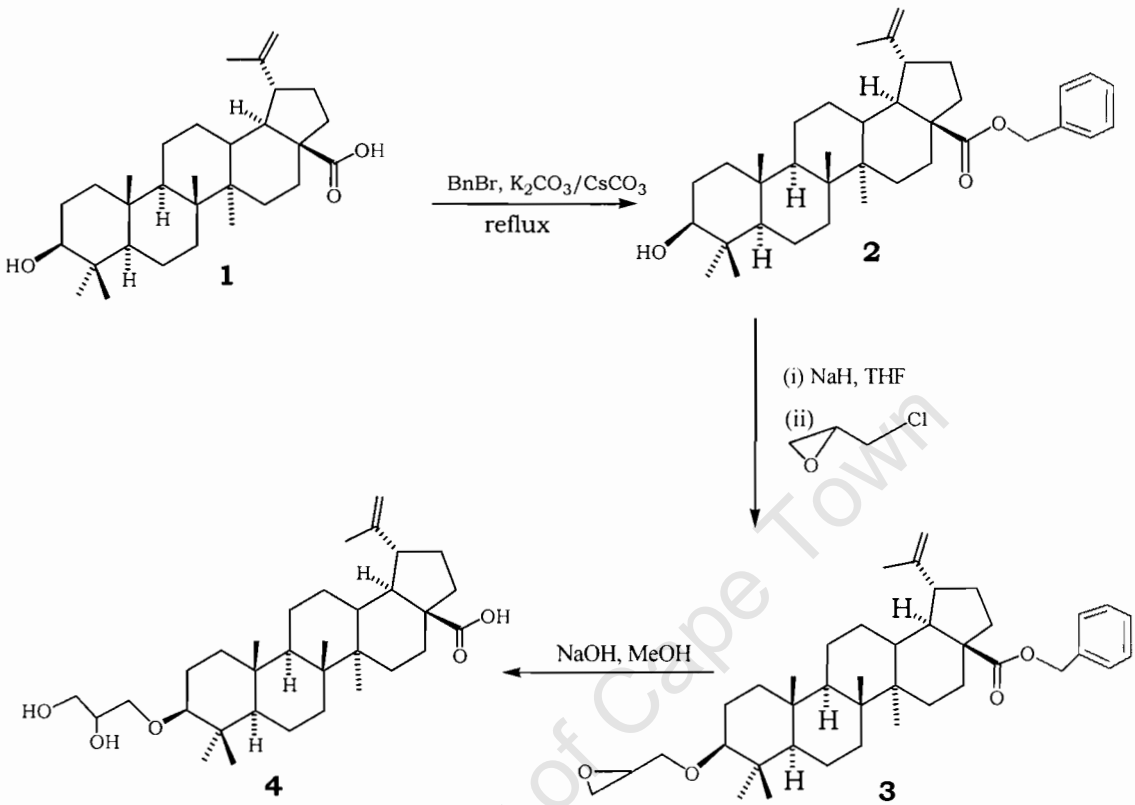


Scheme 1: Proposed synthetic route

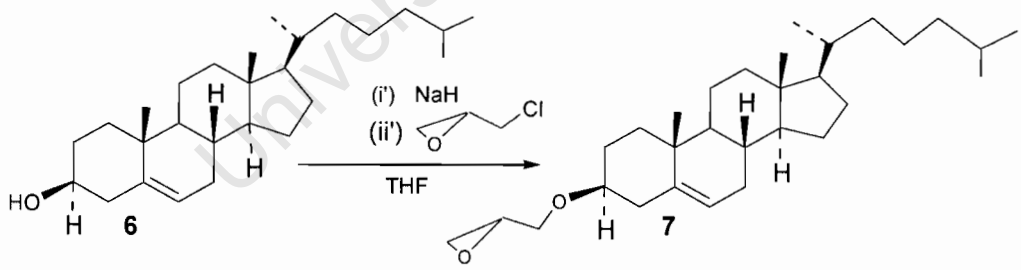
Alkylation at O-3 would follow to give epoxypropyl ether (**3**). Although it would be desirable to have chirally-pure derivatives, the racemic epoxypropyl derivative is readily available using epichlorohydrin. It was thought that this would provide suitable derivatives for preliminary testing. The epoxypropyl ether then represents a versatile building block for generating a variety of derivatives (**4**) by nucleophilic opening. Final deprotection at C-28 would give the target 0-3-substituted BA (**5**).

6.2 RESULTS AND DISCUSSION

6.2.1 Chemistry



Scheme 2: synthesis of 4



The proposed synthetic route to the desired derivatives is summarized in Scheme 2. In order to establish conditions for the crucial alkylation step, readily available cholesterol (**6**) was chosen as model compound. This was treated with NaH and epichlorohydrin (1-chloro-2-3-epoxypropane) in THF to give epoxypropyl derivative (**7**) in good yield (60 – 70%). The presence of the epoxypropyl group of (**7**) was confirmed by the ^{1}H NMR spectrum which showed additional peaks - compared against ^{1}H NMR spectrum of the starting material (**6**) at δ3.12 (m, 1H), 3.01 (dt, 1H, *J*=4.4Hz, *J*=10.8Hz), 2.81

(m, 2H), corresponding to H-1', H-2' and H-3' respectively. Before the same alkylation reaction could be carried out on betulinic acid (**1**) it was necessary to protect the carboxyl group at C-28. We chose the simple otion of forming the benzyl ester, since this was a known compound[ref?] and the benzyl ester is in principle easily removed by hydrolysis or hydrogenolysis. Benzylation was achieved by reacting (**1**) with benzyl bromide in the presence of K_2CO_3 and $CsCO_3$ in THF to give crystalline benzyl BA ester (**2**) in 97% yield. The analytical data of (**2**) corresponded with those in the literature, and C-13 assignments (Table) were carried out based on a detailed 2D NMR analysis of betulinic acid (D. Chen, MSc thesis, University of Cape Town, 1997). The presence of the benzyl ester was clear from both the ^1H and ^{13}C NMR spectra: a distinctive AB quartet for the benzylic methylene group was evident at δ5.1 in the ^1H NMR spectrum, and the corresponding carbon signal for the benzylic carbon appeared at δ65.7 in the ^{13}C NMR spectrum, while signals for the protons and carbons in the aromatic ring were also apparent.

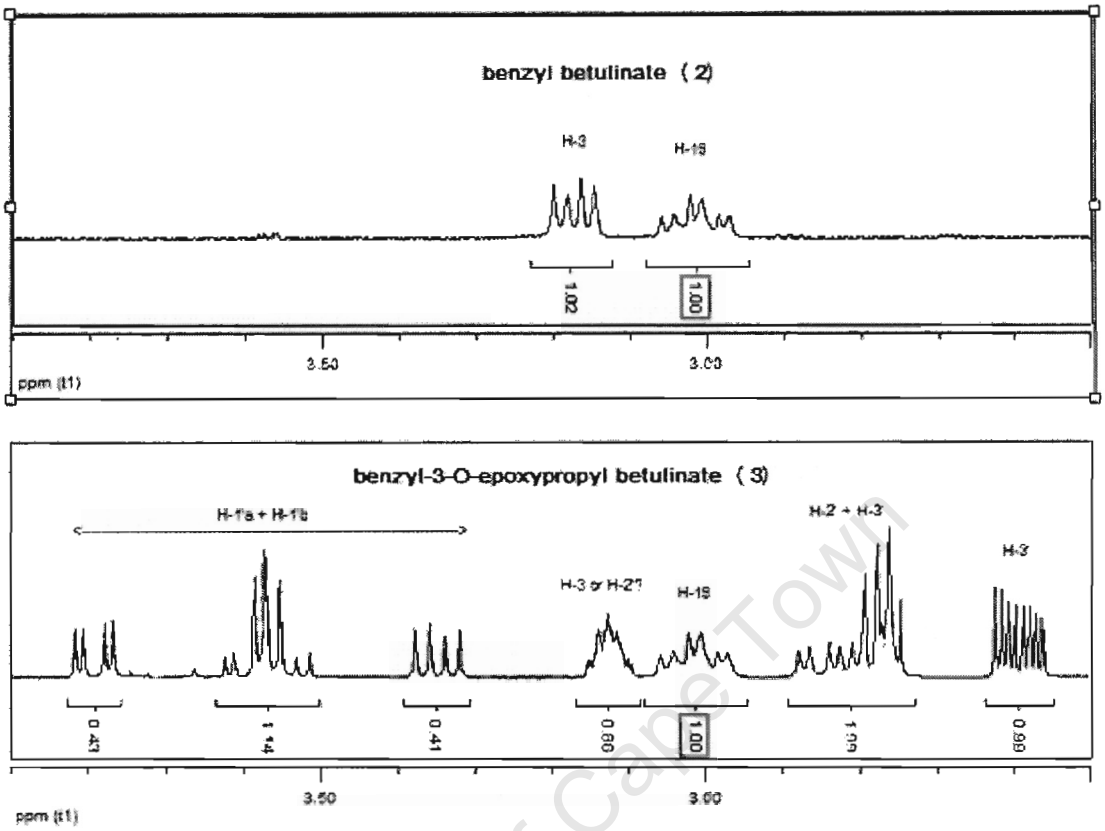
Carbon	**δ**(ppm) **BA**[ref?]	**Lit.**[ref?]	**δ** (ppm) **BZBA**	**Carbon**	**δ**(ppm) **BA**	**Lit.**	**δ** (ppm) **BZBA**
C-1	38.7	38.7	38.7	C-16	32.2	32.1	32.1
C-2	27.4	27.4	27.4	C-17	56.2	56.3	56.5
C-3	79.0	78.9	78.9	C-18	49.3	46.8	49.4
C-4	38.9	38.8	38.8	C-19	46.9	49.2	46.9
C-5	55.4	55.3	55.3	C-20	150.4	150.3	150.5
C-6	18.3	18.3	18.3	C-21	30.6	29.7	30.6
C-7	34.4	34.3	34.3	C-22	37.0	37.0	36.9
C-8	40.7	40.7	40.6	C-23	28.0	27.9	27.9
C-9	50.6	50.5	50.5	C-24	15.3	15.3	15.3
C-10	37.2	37.2	37.1	C-25	16.0	16.0	15.8
C-11	20.9	20.8	20.8	C-26	16.1	16.1	16.1
C-12	25.5	25.5	25.5	C-27	14.7	14.7	14.6
C-13	38.4	38.4	38.2	C-28	179.8	180.5	175.7
C-14	42.5	42.4	42.4	C-29	109.7	109.6	109.5
C-15	29.7	30.5	29.5	C-30	19.4	19.4	19.3
				C-1'			65.7
				Ar.			128.0, 128.2, 128.4, 136.4

Table 6.2: Comparison of ^{13}C NMR data of benzyl BA against BA values

The epoxypropyl ether (**3**) was then prepared by reacting (**2**) with epichlorohydrin in THF in the presence of NaH under conditions described above. The desired product was obtained in yields ranging between 55 – 60 %, together with as-yet unidentified by-products in minor proportions. The structure of (**3**) was confirmed by an analysis of the ^{1}H and ^{13}C NMR: a portion of the ^{1}H NMR spectrum of **3** is compared with that of **2** in figure 1 below. Key evidence for the presence of the epoxypropyl moiety in (**3**) included:

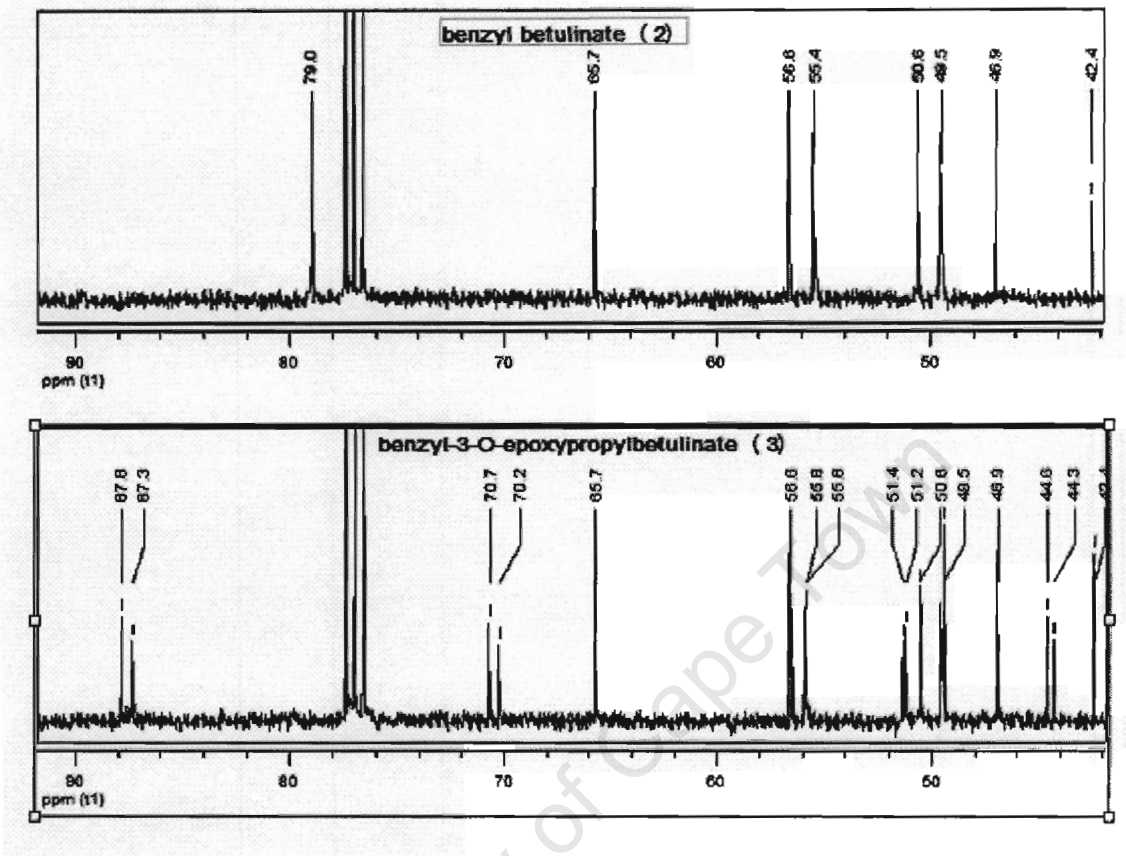
- The spectrum of **3** showed signals together integrating for 7 protons in the region δ2.6 – 3.8ppm, accounting for protons H-19, H-3, and the protons in the epoxypropyl group (two H-1's, H-2' and two H-3's)
- For these protons (except H-19) the spectrum showed two sets of signals consistent with the presence of two diastereomers as was expected
- As result of substitution at O-3, the clear dd typical for H-3 could no longer be observed: this peak appeared to have shifted upfield to ~δ2.8, where it was now overlapping with H-2' (methine proton in the epoxy group).
- The terminal methylene protons in the epoxypropyl group were represented by two clear sets of dd's at δ2.6, giving evidence of their coupling with vicinal proton H-2'.
- The sets of signals in the region δ3.35 - 3.8, integrating for 2 protons are assignable to the H-1' protons in the two diastereomers, given their coupling with each other and with H-2'.
- As expected there was no change in the signal for H-19 at δ3.0

Figure 1: Comparison of a region of the ^{1}H NMR spectra of **2** and **3**



Further confirmation of the structure of (**3**) was obtained from the ^{13}C NMR spectrum (Figure 2 and Appendix 6.2.1.3). The signal for C-3 had shifted downfield to 87ppm (from 78.9), consistent with alkylation of the alcohol at C-3, and the signal had split into two signals due to the two diastereomers present. New pairs of signals appeared for C-1' (δ70.2 and 70.7), the terminal C-3' methylene carbon in the epoxy group (δ51.2 and 51.4), and the methine C-2' in the epoxy group (δ44.3 and 44.6). It was noted that some other signals for carbons in the BA skeleton were also split due to the diastereomers present.

Figure 2: Comparison of region of ^{13}C NMR spectra of **2** and **3**

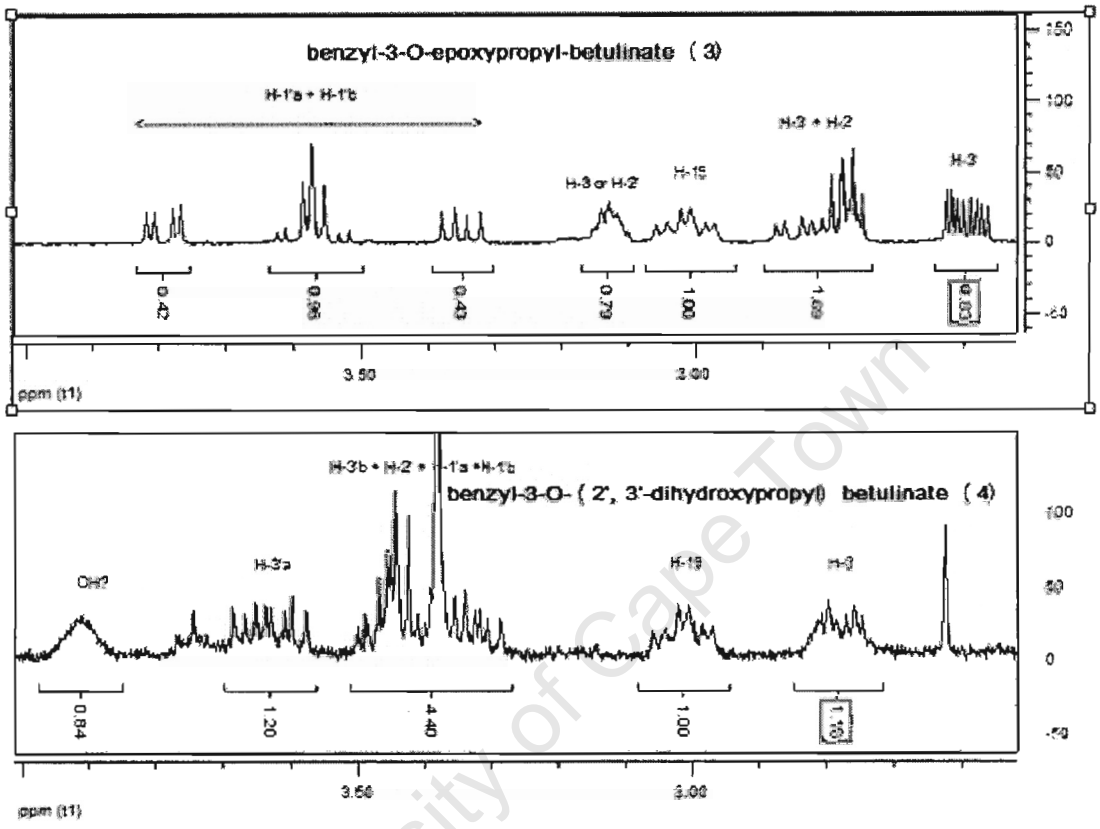


With the successful preparation of **3** it was in principle now possible to attempt nucleophilic opening of the epoxide to generate a range of substituted derivatives, which could then be converted to the final betulinic acid analogues by removal of the benzyl protecting group on the carboxylic acid. In order to confirm these possibilites we first attempted a direct conversion to the dihydroxypropyl derivative under conditions which were considered to be adequate for simultaneous hydrolysis of the benzyl ester.[ref] Epoxypropyl ether (**3**) was therefore treated with 4N NaOH in methanol/THF to give (**4**) as a whitish residue, recrystalizable from hexane and methanol. Analysis of the NMR data for compound (**4**) confirmed this to be benzyl-3-(2', 3'-dihydroxy-propyloxy)-betulinate, and that the benzyl ester had somewhat surprisingly not been hydrolysed under these conditions.

Assignment of the structure of **4** was based on analysis of the ^{1}H and ^{13}C NMR spectra. In the proton spectrum (Figure 3) a new set of signals in the

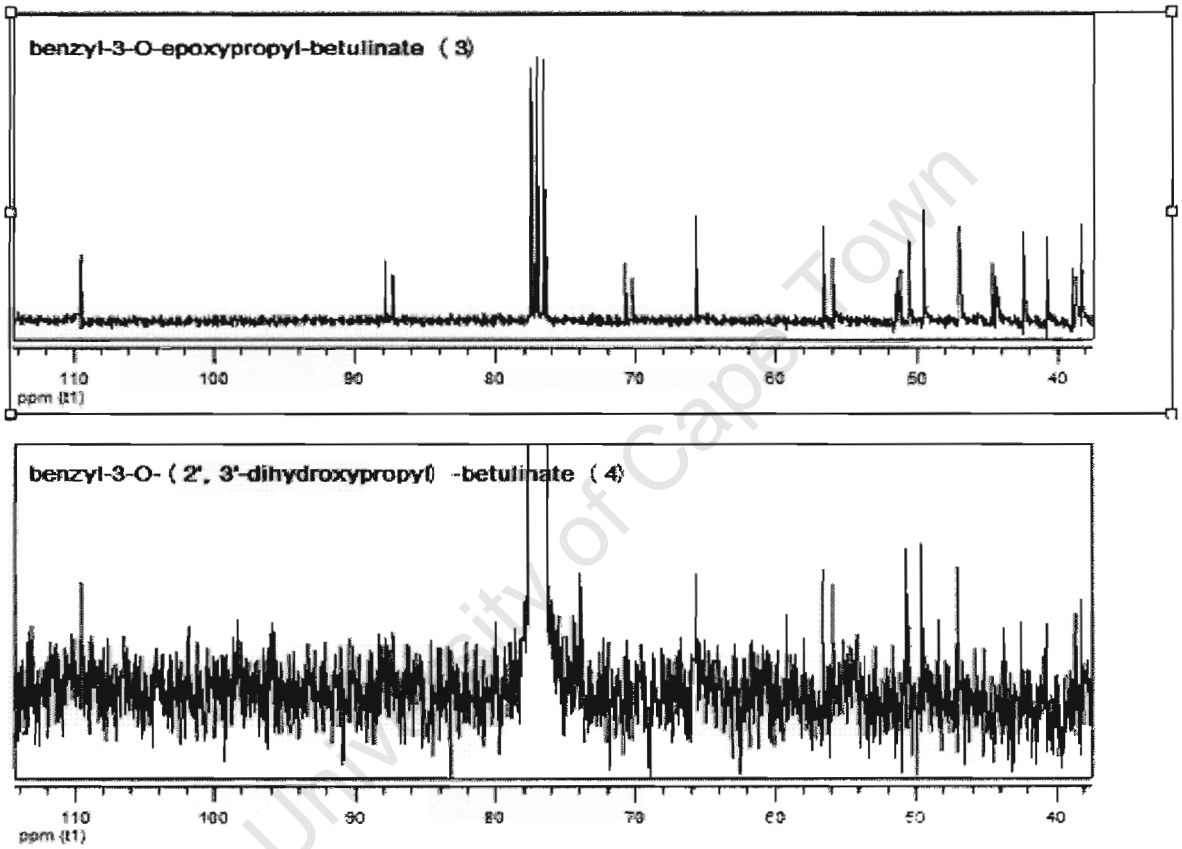
region δ2.6-3.9ppm integrated together for the 7 protons expected. Further key observations are summarized as follows:

Figure 3 Comparison of region of the ^{1}H NMR spectra of **3** and **4**



- The signals at δ2.6 for H-3' in the epoxypropyl group had disappeared and shifted to the region δ3.3-3.7, where hydroxylated methylene and methine protons are expected. (These signals together integrate for ~5 protons as expected.)
- No change was observed for the signal at δ3.0 for H-19 as expected.

The formation of 2',3'-dihydroxypropyl group was also confirmed by some change that were observed in the ^{13}C spectrum of compound (**4**) (Figure 4). The most significant was that signals at 44.5 and 51.5 due to C-2' and C-3' of the epoxyproplyl group have disappeared in the new spectrum, and a new signal appears at 74 which would be consistent with formation of a new secondary hydroxyl group at C-2'.



It was thus confirmed that the benzyl ester had not been hydrolysed under these conditions, and further experiments confirmed that the benzyl ester resisted hydrolysis at higher temperatures. The benzyl group was chosen as protecting group on the assumption that it would be readily removable by hydrolysis, while also in the awareness that it) could not be removed by hydrogenolysis because this would result in saturation of the double bond as well. In ongoing work it will clearly now be necessary to consider another strategy for selective protection and deprotection of the carboxyl group.

6.2.2 Evaluation of the anti-Mtb properties of compounds (1 - 4)

Compounds (**1**), (**2**), (**3**) and (**4**) were tested for antitubercular activity against *Mtb* H37Rv using bioautography on TLC plates as described in section **3.4.5.1**. The results of these assays are presented in Table **6.2.2** below:

Compounds (**1**), (**2**) and (**3**) were found to be inactive against *Mtb* H37Rv even at 100 µg/spot. These results are in line with the previous reports, which highlighted the importance of a free carboxyl group at C-28 for the anti-HIV and/or anti-tumour properties of pentacyclic triterpenoids and their derivatives (Baglin et al., 2003; Kim et al., 1998).

Table 6.1: Bioautography: Antitubercular activity of compounds 1- 4

MTT reduced by *Mtb* H37Rv

	Sample	Dosage (µg/ spot)					
		100	20	10	5	2.5	1.25
1	Betulinic acid	-	-	-		-	-
2	28-Benzyl-betulinic acid ester	+	+	+	+	+	+
3	28-Benzyl-3-epoxy-betulinic acid ester	+	+	+	+	+	+
4	3-(2',3'-dihydroxypropanol)-28-benzyl-BA	+	+	+	+	+	+

Legend: +: Blue spot = Bacterial survival = No antibacterial activity.
-: White spot = No bacterial survival = Significant antibacterial activity

6.3 EXPERIMENTAL

6.3.1 General procedures

All solvents were freshly distilled. Dichloromethane was distilled over phosphorus pentoxide. Diethyl ether and tetrahydrofuran were dried over sodium wire with benzophenone and distilled under nitrogen. Other solvents were purified according to standard procedures (Perrin and Armarego, 1988). Reactions were monitored by thin layer chromatography (TLC) using aluminium-backed precoated Merck silica gel 60 F254 plates and components visualized under a UV lamp or by immersing in either acidified anisaldehyde or ceric ammonium sulphate solutions followed by heating at 150 °C. The work-up typically involved threefold extraction with an organic solvent. All reagents were purchased from Aldrich or Merck.

Melting points were determined using a Reichert-Jung Thermovar hot-plate microscope and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR spectrometer in chloroform. Nuclear Magnetic Resonance spectra were recorded on a Varian Mercury 300 MHz (75 MHz for ^{13}C) or Varian Unity 400 MHz (100 MHz for ^{13}C) and were carried out in d-chloroform unless otherwise stated. Chemical shifts (δ) were recorded using residual chloroform (δ 7.24 in ^{1}H NMR and δ 77.00 in ^{13}C NMR) or tetramethylsilane (δ 0.00) as internal standard. For D_2O, dioxane (δ 3.75 in ^{1}H NMR and δ 67.19 in ^{13}C NMR) was used as the internal standard. For CD_3OD, the residual methanol peak (δ3.31 in ^{1}H NMR and δ49.00 in ^{13}C NMR) was used as internal standard. All chemical shifts are reported in ppm.

3-(2', 3'-epoxpropyloxy) - cholestane

An oven–dried, 50ml three-necked round-bottomed flask containing a magnetic stirring bar and fitted with reflux condenser and serum caps was charged with NaH coated in oil (435 mg, 10.9 mmol), and flushed with nitrogen. The NaH was then washed with hexane to remove the oil and suspended in THF (15ml). A solution of cholesterol (2.9 mmol) in THF (15ml)

was then added with vigorous stirring, via syringe over 2 minutes (mild effervescence), before being, the mixture was heated at reflux for 2 hours under nitrogen, and then allowed to cool under continuous stirring. When the gas evolution had ceased, epi-chlorohydrin (2ml, 10.9 mmol) was added to the solution via syringe and the reaction was heated at reflux again for 24 hours. After the reaction was complete (TLC), 2-propan-2-ol (5ml) was cautiously added to the cooled mixture and the latter was then concentrated under vacuum. The yellowish pasty residue was partitioned between water and ether and the water fraction extracted with ether (3 xs). The combined ether fractions were washed with brine, concentrated and then chromatographed on silica gel, eluting with a mixture of hexane/ethyl acetate (4:1) to give (**7**)) after removal of solvents, as a whitish amorphous powder (66%). **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 5.35 ppm (dd, 1H, *J*=2.4Hz, *J*=4.5Hz), 3.52 (m, 1H), 2.27 (m, 1H), 1.98 (m, 1H), 1.68 (s, 1H), 1.52 (m, 1H), 1.37 (s, 1H), 1.26 (m, 1H), 1.11 (m, 1H), 1.01 (m, 1H), 1.01 (m, 1H), 0.92 (d, 2H, *J*=6.6Hz), 0.87 (dd, 2H, *J*=1.7Hz, *J*=6.6Hz), 0.68 (s, 3H); 13C-NMR ($CDCl_3$) δ140.7, 121.6, 76.6, 71.7, 56.7, 56.1, 50.1, 39.7, 39.5, 37.2, 36.4, 36.1, 35.7, 31.9, 31.6, 28.2, 27.9, 24.2, 23.8, 22.7, 19.3, 18.7, 11.7.

28-benzyl-betulinate (betulinic acid benzyl ester)

A mixture containing BA (350mg, 0.767 mmol), BnBr (0.70ml; 0.055 mmol), $CsCO_3$ (210mg, 0.648 mmol), and K_2CO_3 (210mg, 1.519 mmol) in dry acetone (70ml) was placed in a 100 ml two necked round bottom flask and heated at reflux for 9hr. When the reaction was complete (TLC), the solvent was evaporated under vacuum, and the residue was extracted with dichloromethane (3x), concentrated and purified by column chromatography on silica gel using a mixture of hexane/ethyl acetate (4:1) as eluent to give (**2**) (402 mg, 97%) as colourless crystals, m.p. 202 °c, soluble in dichloromethane. The NMR spectra of (**2**) (appendix **6.2.1**) were obtained in $CDCl_3$. NMR data as well as the m.p. (202°C) of (**2**) were in agreement with the data from the literature (Bringman et al., 1997); ^{1}H-NMR (300 MHz) δ 5.12 (ABq, 2H, benzylic CH_2), 4.72 (d, 1H, *J* =2.3Hz, H-29), 4.59 (qd, 1H, *J*

=1.3Hz, *J* =2.7Hz, H-29), 3.17 (dd, 1H, *J* =5.3Hz, *J* =10.8Hz, H-3), 3.01 (dt, 1H, *J* =4.5Hz, *J* =10.9Hz, H-19), 2.28 (m, 1H), 2.19 (dt, 1H, *J* =3.7Hz, *J* =12.7Hz, H-13), 1.89 (m, 2H), 1.67 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H, 30-CH_3), 0.80 (s, 3H, -CH_3), 0.77 (s, 3H, -CH_3), 0.75 (s, 3H, -CH_3), 0.66 (dd, 1H, *J* =3.1Hz, *J* =8.3Hz, H-5). **^{13}C-NMR** δ175.7, 150.5, 136.4, 128.4, 128.2, 128.0, 109.5, 78.9, 65.7, 56.5, 55.3, 50.5, 49.4, 46.9, 42.4, 40.6, 38.8, 38.7, 38.2, 37.1, 36.9, 34.3, 32.1, 30.6, 29.5, 27.9, 27.4, 25.5, 20.8, 19.3, 18.3, 16.1, 15.8, 15.3, 14.6

3-(2', 3'-epoxypropyloxy)-28-benzyl-betulinate

An oven–dried, 50ml three-necked round-bottomed flask containing a magnetic stirring bar and serum caps was charged with NaH coated in fatty material (3.22 mmol), and connected to a nitrogen-flushed reflux condenser, the top of which was attached to a nitrogen line via a tap. The NaH was then washed (hexane) free of oil under nitrogen, and suspended in 10ml of THF. While the NaH suspension was strongly stirred, a solution of the benzyl ester of betulinic acid (**2**) (0.448mmol) in THF (10ml) was added via syringe over 2 minutes (mild effervescence), and the mixture was heated at reflux for 2 hours under nitrogen, after which it was allowed to cool below the refluxing temperature. When the gas evolution had ceased and the mixture was still warm and under stirring, a solution of epi-chlorohydrin (0.251ml, 3.22 mmol) was added to the solution via syringe and the reaction was heated at reflux again for 24 hours. After the reaction was completed, 2-propan-2-ol (5ml) was cautiously added to the cooled mixture and the latter was then concentrated under vacuum. The yellowish pasty residue was partitioned between water and ether. The water fraction was extracted with ether (2 xs). The combined ether fractions were washed with brine, concentrated, and then column chromatographed on silica gel and eluted with a mixture of hexane/ethyl acetate (4:1) to give (**3**) (178mg, 66%), a whitish amorphous powder, soluble in dichloromethane; **^{1}H-NMR** (300 MHz) δ 5.12 (ABq, 2H, benzylic CH_2), 4.72 (d, 1H, *J* =2.1Hz, H-29), 4.59 (dd, 1H, *J* =1.4Hz, *J* =2.2Hz, H-29), 3.79 (dd, 1H, *J* =3.3Hz, *J* =11.6Hz), 3.57 (m, 1H), 3.35 (dd,

1H, *J* =5.7Hz, *J* =11.6Hz), 3.13 (m, 1H), 3.01 (dt, 1H, *J* =4.4Hz, *J* =10.8Hz, H-19), 2.81 (m, 1H), 2.60 (ddd, 1H, *J* =2.7Hz, *J* =5.1Hz, *J* =11.0Hz), 2.28 (m, 1H), 2.18 (dt, 1H, *J* =3.6Hz, *J* =12.7Hz), 1.89 (m, 1H), 1.68 (s, 3H)

3-(2', 3'-dihydroxypropyloxy)-28-benzyl betulinate

A solution of compound (**3**) (75mg, mmol), THF (1ml), MeOH (1ml) was treated with NaOH 4N (1ml) at reflux. After the reaction was completed, the mixture was acidified with HCl to PH 4 and extracted with $CHCl_3$ (3 x 20ml). After the solvent was evaporated under vacuum, the residue was recrystalized from n-hexane/methanol (3x) to give (**4**), as a white crystal powder, m.p.> 320 °C, relatively soluble in CH_2Cl_2, sparsely soluble in MeOH; ^{1}H-NMR (300 MHz) δ 7.34 (m, 5H), 5.12 (ABq, 2H, benzylic CH_2), 4.72 (d, 1H, *J*=2.1Hz, H-29), 4.59 (dd, 1H, *J*=1.4Hz, *J*=2.2Hz, H-29), 3.91 (m, 1H), 3.63 (m, 1H), 3.39 (m, 2H), 3.01 (dt, 1H, *J*=4.7Hz, *J*=10.6Hz, H-19), 2.78 (m, 1H), 2.22 (m, 2H), 1.68 (s, 3H), 1.27 (s, 3H), 1.26 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.76 (s, 3H), 0.74 (m, 6H); **^{13}C NMR**: δ128.4, 128.2, 109.5, 73.9, 65.7, 59.2, 56.5, 55.8, 55.0, 50.5, 49.5, 46.9, 42.3, 40.6, 38.2, 36.9, 32.1, 30.6, 29.6, 29.5, 26.7, 25.5, 22.8, 20.9, 19.3

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

7.1 GENERAL DISCUSSION

Throughout history, the plant kingdom has provided humankind with many useful drugs which brought remedies to medical problems facing the world (Farnsworth, 1994). Traditional medicine, which plays an important role in the medical systems of most of developing countries, relies on herbal medicine. The fact that three out of five traditional medicine plant species in this investigation, *B. saligna, L. gnaphaloides I. graveolens* were found to possess significant anti-TB activities highlights the value of scientifically assessing the herbal medicines used by traditional medicine practitioners, and the plant kingdom in general.

Furthermore, this investigation has found pentacyclic triterpenoids, UA, OA, and their derivatives to be the common antitubercular constituents of the three bioactive plant species; they are believed to be widely distributed in higher plants. This fact gives to this group of compounds (UA, OA and BA) a considerable pharmaceutical value since they have been shown to have low in vitro toxicity against CHO cells line.

It would appear contradictory that all the medicinal plant material that were investigated was reportedly used by traditional medicine practitioners in the form of aqueous extracts whereas the isolated anti-TB constituents are all of lipophilic nature. The explanation to this may lie in water soluble constituents, glycosides and/or saponins, which may be acting as pro-drugs. Indeed, Emam et al. (1997), for instance, explained that the therapeutic properties of the infusion of *B. madagascariensis* (leaves), a *B. saligna* related species, in the treatment of asthma, coughs and bronchitis, is due to its constituent Mimengoside B. Mimengoside B is a saponin consisting of a sugar moiety linked to an aglycon derivative of UA or OA (Emam et al., 1997).

Therefore, it appears appropriate that a total phytochemical screening approach (instead of bioassay-guided fractionation) be applied for the

investigation of these plant materials in order to find what hydrophilic compounds are responsible for the biological activities of the aqueous extracts. Indeed, glycosides and saponins can be extracted in water and hydrolysed using a base or an acid to free their bioactive aglycon moieties. The latter will then be tested for anti-*Mtb* properties.

In this investigation, I modified the bioautography on TLC plate method to be suitable for the assay for the evaluation of the anti-*Mtb* properties of compounds. This modified method has the advantage of being simpler and faster than the conventional microtiter plate colorimetric method or the disk diffusion method. It allows completion of the assay in three days, whereas three weeks minimum are required when using the disk diffusion method or the microtiter plate colorimetric method.

The synthesis of BA derivatives was a step toward the investigation of the structure-antitubercular activity, which may provide molecules that have an improved specificity and potency.

7.2 CONCLUSION

The plant kingdom is still an important source of remedies capable of combating and eradicating devastating diseases and pandemics such as cancer, tuberculosis and HIV/AIDS. Many naturally occurring compounds have been reported for anti-*Mtb* activity, some more potent than the current standard first line therapeutic drugs such as rifampicin, and isoniazid. There is a genuine need to intensify the phytochemical exploration to find remedies to pandemics such as TB and HIV.

It is worth investigating the herbal remedies used by traditional medicine practitioners as they have a significant probability of containing bioactive constituents. This study has found three out of five investigated traditional medicinal plant species, *B. saligna, L. gnaphaloides I. graveolens* to possess

significant anti-TB activity. Interestingly, the isolation of similar antitubercular constituents, UA, OA and their derivatives from the three different plant materials not only supports their use in traditional medicine, but also highlights the pharmaceutical prospects of the isolated bioactive constituents. In addition, the pharmaceutical prospects of the isolated compounds, UA, OA, and their derivatives look even more promising since they showed no in vitro cytotoxicity against CHO cells (IC_{50}s ≥ 100 µg/ml).

Furthermore, a literature survey on structure activity relationship conducted during this study has revealed that structure modification at C-3, C-28 and the double bond of the isolated triterpenoids is likely to generate molecules with an improved activity and/or specificity. This finding acted as a motivating factor and inspired the development of a new synthetic route that allows the preparation of a variety of O-3-alkylated BA derivatives resulting from 3-epoxypropyl-BA (see Chap. VI).

In conclusion, the findings of this investigation are encouraging enough to propose further studies in order to assess the potential of the isolated bioactive constituents, UA, OA, BA and their derivatives to be developed as new anti-TB drugs. These studies would include structure anti-TB activity relationship investigations, the evaluation of the in vitro anti-TB activity of the derivatives, and the evaluation of the in vitro general cytotoxicity of the derivatives.

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APPENDIX