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ANTIMICROBIAL AND CHEMICAL ANALYSES OF SELECTED *BULBINE* SPECIES

BY

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To
my children,
Dipika, Jivesh and Samika

*For Shri Vishnu
for the guidance and blessings*

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SUMMARY

The use of plant materials for the treatment of various diseases is very common in African countries. As traditional medicine used by the rural people does not always have a proper scientific basis, research programmes have to be undertaken to evaluate their therapeutic efficacy and safety. In traditional African medicine various *Bulbine* species are used to treat a number of conditions including sexually transmitted diseases, wound infections, dysentery and urinary tract infections.

The *Bulbine* species belong to the family Asphodelaceae. There are over fifty South African *Bulbine* species and they are mostly herbs. Their leaves are evergreen and succulent in appearance. *Bulbine* species have thick fleshy tuberous roots, are easy to grow, are able to withstand drought and heat and are able to grow in poor soil.

There is very little documented information on the antimicrobial activity and chemical properties of the *Bulbine* species. Therefore research programmes of this nature have to be undertaken.

Various *Bulbine* species, viz., *B. natalensis* Bak, *B. frutescens* Willd (yellow flowers), *B. narcissifolia* Salm Dyck, *B. abyssinica* A Rich and *B. frutescens* Willd (orange flowers) were collected. The plants were washed with tap water, air dried and separated into the different components. Each component was cut into small pieces and immersed in methanol: dichloromethane (1:1, v/v) for extraction. The organic solvent was decanted from the plant material and evaporated under reduced pressure. The resultant crude extracts were stored in glass vials in the freezer. In addition, the roots, stems and leaves of *B. natalensis* and *B. frutescens* (yellow flowers) were extracted aqueously.

The crude organic and aqueous were subjected to various tests to evaluate their antimicrobial and cytotoxic potential. To evaluate their antibacterial activities, the Disk Diffusion and Bore Well Methods were employed. The crude extracts were tested against various pathogens implicated in wound and urinary tract infections and dysentery. In these experiments the Disk Diffusion Method produced better results

than the Bore Well Method. The crude organic and aqueous extracts were found to be effective against many of the bacteria used in this study including *K. pneumoniae*, *S. aureus*, *S. typhi* and *S. flexneri* which are considered to be troublesome pathogens.

The TLC bioassay was employed to evaluate the antifungal potential of the various crude extracts against *Aspergillus* and *Penicillium* and the Disk Diffusion and Bore Well methods were used to evaluate the antifungal potential of *C. albicans*. The *Bulbine* species displayed no antifungal activity against *Penicillium* and limited antifungal activity against *Aspergillus*. The two method used to evaluate the antifungal activity of *C. albicans* was chosen because *C. albicans* grows in a similar manner to bacteria on solid and liquid culture media. Only the root extracts of the two *B. frutescens* varieties were inhibitory to *C. albicans*.

The Brine Shrimp Bioassay was used to ascertain the cytotoxic potential of the crude extracts. The majority of the extracts were cytotoxic at the most concentrated dilution (i.e., dilution 1) but not cytotoxic at the lower dilutions. The only extracts that were not cytotoxic at the most concentrated dilution were the organic extract of the root of *B. frutescens* (yellow flowers), the organic extract of the root of *B. narcissifolia* and the organic extract of the leaf of *B. abyssinica*.

TLC and column chromatography was carried out to evaluate the chemical composition of the *Bulbine* species. The TLC indicate that this technique could be a valuable tool in identifying the different species in the genus *Bulbine*. Column chromatogram was carried out on the extract which displayed a significant amount of antibacterial activity against the bacteria used in this study. The stem extract of *B. natalensis* was chosen for further analysis. The stem extract was fractionated into different fractions but unfortunately none of the chemical component could be identified.

According to the results obtained in this study, there is considerable scope for further studies of this genus.

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GLOSSARY

Acaulescent: Becoming stem-less.

Aerobic Bacteria: Bacteria requiring oxygen for growth.

Agar: A dried polysaccharide extract of red algae used as a solidifying agent in various microbiological media.

Aminoglycosides: Broad spectrum antibiotics containing an amino-sugar, an amino- or guanido-inositol ring and residues of other sugars that inhibit protein synthesis, e.g., kanamycin, neomycin and streptomycin.

Antibacterial agents: Agents that kill or inhibit the growth of bacteria, e.g., antibiotics, antiseptics and disinfectants.

Antibiotic: Substance of microbial origin that in very small amounts have antimicrobial activity, current usage of the term extends to synthetic and semi-synthetic substances that are closely related to naturally occurring antibiotics and that have antimicrobial activity.

Antibiotic Resistance: The natural or acquired ability of a microorganism to overcome the inhibitory effects of an antibiotic.

Antifungal agents: Agents that kill or inhibit the growth and reproduction of fungi, may be fungicidal or fungistatic.

Antimicrobial agents: Chemical or biological agents that kill or inhibit the growth of microorganisms.

Antiseptics: Chemical agents used to treat human or animal tissues usually the skin, to kill or inactivate microorganisms capable of causing infection. Usually not considered safe for internal consumption.

Aqueous: Of, relating to, or resembling water; made from, with, or by water; solutions in which water is the solvent.

Artifact: Appearance of something in an image or micrograph of a specimen that is due to causes within the optical system or preparation of the specimen and is not a true representation of the features of the specimen.

Aseptic techniques: Precautionary measures taken in microbiological work and clinical practice to prevent the contamination of cultures, sterile media, etc. and/or infection of persons, animals or plants by extraneous microorganisms.

Autoclave: Apparatus in which objects or materials may be sterilized by air free saturated steam under pressure at temperatures greater than 100°C.

Ayurveda: "Science of life"- Indian philosophy that forms the basis of Ayurvedic medicine.

Ayurvedic Medicine: Traditional system of medicine of India that has been practiced there for several thousand years.

Bacitracin: Antibiotic that inhibits bacterial cell wall synthesis.

Bacteraemia: Condition in which viable bacteria are present in blood.

Bacteria: Members of a group of diverse and ubiquitous prokaryotic, single celled organisms lacking a nucleus.

Candidiasis: Fungal infection caused by *Candida* species infecting the skin, nails or mucous membranes.

Capsule: A mucoid envelope composed of polypeptides and/or carbohydrates surrounding certain microorganisms.

Caulescent: Becoming stalked, where the stalk is apparent.

Cauline: Belonging to the stem or arising from it.

Cephalosporins: A heterogeneous group of natural and semi-synthetic antibiotics that act against a range of Gram positive and Gram negative bacteria by inhibiting the formation of cross links in the peptidoglycan.

Conidia: Thin walled asexually derived spores borne singly or in groups or clusters in specialized hyphae.

Conidiophores: Branches of mycelia bearing conidia.

Corm: Underground reproductive part of certain plants similar in appearance to a bulb from which the new stalk grows each year.

Dimorphic: In mycology, the tendency of some pathogens to alter their growth forms from mould to yeast in response to rising temperature.

Eczema: Skin disease causing redness, severe itching and scaling of the skin.

Emetic: Causing vomiting.

Endospores: Thick walled spores formed within the parent cells in bacteria.

Enterobacteriaceae: Family of Gram negative, facultative anaerobic rods.

Facultative anaerobes: Microorganisms capable of growth under aerobic or anaerobic conditions.

Freeze-dried cultures: Cultures that are frozen at a temperature of -20°C or lower to preclude microbial growth entirely. Freezing does not kill most microorganisms.

Fungi: A group of diverse, widespread unicellular and multicellular eukaryotic organisms lacking chlorophyll and usually bearing spores and often filaments.

Gram stain: Differential staining procedure in which bacteria are classified as Gram negative or Gram positive depending on whether they retain or lose the primary stain when subject to treatment with a decolourizing agent.

Herbalist: Person who grows, sells or specializes in herbs for medicinal use.

Herbal medicine: Use of plants and plant remedies in the treatment and prevention of disease.

Impetigo: An acute inflammatory skin disease caused by bacteria characterized by small blisters, weeping fluid and crusts.

Indigenous: Native to a particular habitat.

Indigenous/Traditional healers: One who uses medicinal plants in treating ailments.

Inflorescence: Arrangement of a plant's flowers on the stem.

Inhibition: Prevention of growth or multiplication of microorganisms.

Inoculate: To introduce microorganisms into an environment that will support growth.

Inyanga: Specializes in the use of herbal medicine. Possesses an extensive knowledge of curative herbs, natural treatments and medicinal mixtures of animal origin.

Isangoma: Traditional diviner and diagnostician who not only defines illnesses but also divines the circumstances of the illness.

Microorganisms: Microscopic organisms including algae, bacteria, fungi, protozoa and viruses.

Modern/Western/Orthodox medicine: Refers to medicine practiced by western-trained practitioners. Functions within an empirical scientific framework.

Penicillinase: A beta lactamase that hydrolyzes the beta lactam linkage of many penicillins rendering it ineffective as an antibiotic.

Penicillins: A group of natural and semi-synthetic antibiotics with a beta lactam ring that are active against Gram positive bacteria inhibiting the formation of cross links in the peptidoglycan of growing bacteria.

Peptidoglycan: The rigid component of the cell wall in most bacteria.

Perennial: Plants living for more than two years.

Periplasmic space: In Gram negative bacterial cells, the area between the outer cell wall membrane and the plasma membrane.

Petri plate: A round shallow flat bottomed dish with a vertical edge and a similar slightly larger structure that forms a loosely fitting lid, made of glass or plastic, widely used receptacles for various types of solid media.

Plasmid: Extrachromosomal genetic structure that can replicate independently within a bacterial cell.

Prophylaxis: Preventive treatment against disease.

Quinolone: A new class of synthetic antimicrobial drugs with broad-spectrum effects.

Rhizomatous: Having the character of a rhizome.

Rhizome: Root-like stem of some plants, growing along or under the ground and sending out both roots and shoots.

Sciatica: Pain in or near the sciatic nerve.

Sciatic nerve: The nerve that goes from the pelvis to the thigh.

Scrofula: Disease causing swelling of the glands, probably a form of tuberculosis.

Septicaemia: A condition in which an infectious agent is distributed throughout the body via the bloodstream.

Spore: An asexual reproductive or resting body that is resistant to unfavourable environmental conditions, capable of generating viable vegetative cells when conditions are favourable.

Stock culture: A culture that is maintained as a source of authentic subcultures; a culture whose purity is ensured and from which working cultures are derived.

Streptomycin: An aminoglycoside antibiotic produced by *Streptomyces griseus*, affecting protein synthesis by inhibiting polypeptide chain initiation.

Subterete: Somewhat terete.

Sub-terranean: Underground.

Susceptibility: The likelihood that an individual will acquire a disease if exposed to the causative agent.

Terete: Circular in transverse section, cylindrical and usually tapering.

Traditional medicine: Therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine, and are still in use today.

Tranquilizer: Drug for making an anxious person feel calm, sedative.

Umthandazi/umprofethi: Faith healer who is usually a professed Christian who belongs to one of the missions of African independent churches. They heal mostly through prayer, by laying hands on patients or providing holy water and ashes.

Vancomycin: Antibiotic that inhibits cell wall synthesis.

CHAPTER ONE

**INTRODUCTION
AND
LITERATURE
REVIEW**

1.1. INTRODUCTION

1.1.1. Background and Motivation for the study

Ancient civilizations used parts of different plants to concoct various potions, which were used to eliminate pain, to control suffering, and to counteract disease¹. Records from ancient Egypt, Assyria, China and India show that the use of medicinal plants extend back to earliest history². The *Rig-Veda* composed as early as 3000BC mentions the use of *Soma Rasha* (the juice of the Soma plant) as an Elixir of Life³. The Charka Samhita (900BC) is the first recorded treatise on *Ayurveda* and describes three hundred and forty-one plants that were used in medicine⁴. The earliest known pharmacopoeia resulted from the studies of native flora by the legendary Chinese emperor, Shen Nung. The emperor was familiar with many plants including ephedra and rhubarb. These plants are still in medicinal use today⁵.

The ancient Babylonians, Egyptians and Greeks were also aware of the medicinal values of a number of plants. Ancient Egyptians possessed an understanding of the human anatomy as well as knowledge of the medicinal uses of plants and animals. The Greek physician, Dioscorides, described six hundred plants of known medicinal value in his *De Materia Medica* (78AD). Of these plants, a surprisingly large number are still important in modern medicine. Aloe, belladonna, colchicum, hyoscyamus and opium are a few that were used in much the same manner as they are used today¹.

African traditional medicine has been used for more than one thousand years. The use of plant materials for the treatment of various diseases is very common in Third World countries⁶. In traditional African medicine, various *Bulbine* species are used to treat a number of conditions including sexually transmitted diseases (STDs), wound infections, dysentery and urinary tract infections (UTIs)⁷. The *Bulbine* species belong to the family Asphodelaceae and there are over fifty South African *Bulbine* species. These plants are mostly herbs with leaves that are evergreen and succulent in appearance. *Bulbine* species have thick fleshy tuberous roots and are easy to grow⁸. There is however, a lack of information on the chemical constituents, the long-term toxic potential and the

antimicrobial activity of the *Bulbine* species. Although herbal remedies are popular, reliable scientific information is scarce. Comprehensive, controlled and standardized studies on both the safety and efficacy of herbal remedies are thus imperative.

There is also increasing interest in traditional systems of medicine in developing countries. Modern medicines are expensive whilst traditional therapies are usually far less so⁹. The World Health Organization (WHO) is promoting traditional medicine as a form primary health care in developing countries and advocates the co-operation and mutual respect between traditional and modern health practitioners¹⁰. Further, with the advent of bacterial resistance to antimicrobial agents, there is need to find alternative therapies. The great diversity of antimicrobial agents might lead one to believe that, even in the light of bacterial resistance, at least one drug will work. However, this is not the case. Every year many patients die from infections for which there are no effective drugs. Drug resistance in pathogens like the staphylococci, *Pseudomonas* spp, *Salmonella* spp and the gonococci has been a serious problem for decades. Now, microorganisms that were never resistant are developing resistance to one or more drugs. Progressive drug resistance means that in many cases, microbial sensitivity or susceptibility to drugs cannot be assumed and infectious agents must be tested for possible resistance to prevent failure in therapy. This also increases the cost of medication¹¹.

It is clear that medical science, in a race with the microorganisms, is rapidly losing ground. The demand for plant based raw materials for pharmaceutical development has increased. Plant medicines may just solve the resistance problems.

The traditional African healers use the different *Bulbine* species to treat various diseases. Table 1 illustrates the myriad uses of these plants.

Table 1: Traditional uses of the *Bulbine* species

PLANT NAME	USED BY	PART USED	AILMENT/ TREATMENT
<i>Bulbine alooides</i>	Xhosa	Tubers	Rheumatism, syphilis, wound, rashes ¹²
	Zulu	Root	Diarrhoea, UTI, venereal disease (VD) ¹³
<i>Bulbine asphodeloides</i>	Zulu	Leaf Juice	Wounds, gravel rash, ringworm ¹²
	South Sotho South Sotho Xhosa	Crushed Leaf Leaf Juice Infusion of bulb and root	Dressing for burns ⁷ Cracked lips ⁷ Scrofula ⁷
	South Sotho Xhosa, Mfengu	Root Root	VD ⁷ Upset stomach, UTI ⁷
<i>Bulbine latifolia</i>	Mfengu, Hottentot	Decoction of dried root	Diarrhoea, dysentery, abdominal complaints, UTI, syphilis, lumbago ⁷
	Shona Zulu Xhosa	Leaf Leaf sap Tubers	Septic wounds ⁷ Eczema ¹² UTI, dysentery, diarrhoea ¹²
<i>Bulbine narcissifolia</i>	Sotho, Griquas	Leaf sap	Warts, corns, ringworm, gravel rash, diarrhoea, UTI ⁷
	Zulu Zulu	Decoction of root Cold infusion of leaf	Rheumatism, VD ⁷ Purgative ⁷
<i>Bulbine natalensis</i>	Zulu	Leaf juice	Itches, eczema ⁷
	Zulu	Root	Vomiting, VD, UTI, diarrhoea, blood disorders ¹²
<i>Bulbine rostrata</i>	Sotho	Leaf	Rheumatism, sciatica ⁷
<i>Bulbine tortifolia</i>	Zulu	Leaf juice	Wounds, sores ⁷
<i>Bulbine frutescens</i>	Zulu	Root and leaf infusions	Treatment of patients who are going mad as a result of being bewitched ⁷

Although the *Bulbine* species are extensively used for a variety of infectious conditions, there is very little documented information on their cytotoxic potential, antimicrobial activity and chemical compositions. Comprehensive, controlled and standardized studies on both the safety and efficacy of the *Bulbine* species were thus imperative.

1.1.2. Aims

The aims of this study were to investigate the antimicrobial activity and chemical composition of selected *Bulbine* species. The objectives were as follows:

-carry out the extraction of the leaves, roots and stems of the *Bulbine* species, viz.:

-*Bulbine natalensis* Bak

-*Bulbine frutescens* Willd (both yellow and orange flowers)

-*Bulbine narcissifolia* Salm Dyck

-*Bulbine abyssinica* A Rich,

using a combination of organic solvents (methanol: dichloromethane, 1:1, v/v) and water

-ascertain the antimicrobial activity of the crude organic solvent extracts of the leaves, roots and stems against a range of pathogens implicated in the diseases/ailments treated by the traditional healers employing the Disk Diffusion¹⁴ and Bore Well¹⁵ Methods

-ascertain the antifungal activity of the crude organic solvent extracts of the leaves, roots and stems using the Thin Layer Chromatography Bioassay¹⁶

-determine the cytotoxicity of the crude organic solvent extracts of the leaves, roots and stems employing the Brine Shrimp Bioassay¹⁷

-perform a preliminary chemical analysis of the crude organic solvent extracts of the leaves, roots and stems using thin layer chromatography (TLC) and column chromatography.

1.2. Literature Review

As a number of indications for the *Bulbine* species relate to infection and infectious conditions, it is pertinent to include a brief review of bacteriology and mycology in an attempt to understand the antimicrobial actions of the plant extracts.

1.2.1. Bacteriology

1.2.1.1. Size and Shape of Bacteria

The majority of bacteria range from 0,20 to 2,0 micrometres (μm) in diameter and from 2 to $8\mu\text{m}$ in length. They are unicellular structures which may occur as cylindrical (rod shaped), spherical (coccoid) or spiral forms^{11, 20, 21}.

Coccoid cells have a tendency to grow in aggregates. There exist assemblies of pairs (diplococci), groups of four (sarcinae), unorganized arrays like a bunch of grapes (staphylococci) and in chains like a string of beads (streptococci). Rod shaped bacteria occasionally occurs in chains while spiral shaped bacteria normally occur singly^{11, 20}.

1.2.1.2. Structure of Bacteria

Figure 1 illustrates the structure of a typical bacterial cell.

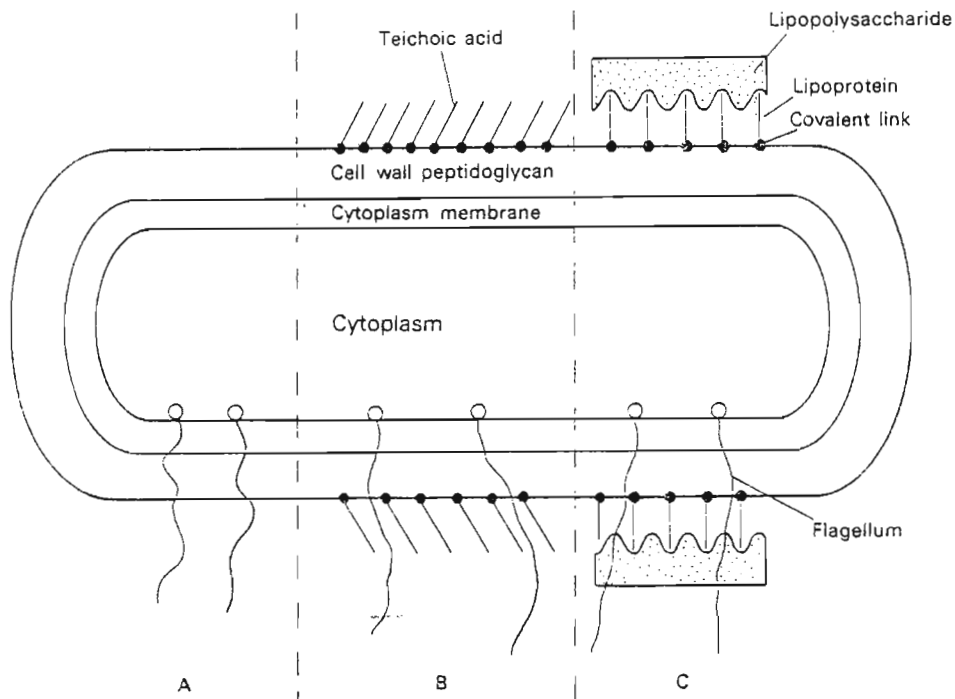


Figure 1: Structure of a Bacterial Cell²¹

The principal structures of the bacterial cell are shown in Figure 1. The protoplasm is bound peripherally by a very thin, elastic and semi-permeable cytoplasmic membrane. Outside, and closely covering this, lies the rigid supporting cell wall that is porous and relatively permeable. The cytoplasm consists of a watery sap packed with large numbers of small granules called ribosomes and a few membranous bodies called mesosomes. The cell also contains nuclear material^{11,22}.

Outside the cell wall there may be a protective gelatinous covering called a capsule. Some bacteria bear one or more kinds of filamentous appendages, viz., flagella (organs of locomotion), fimbriae (organs of adhesion) and pili (involved in the transfer of genetic material) protruding outward from the cell wall^{22,23}.

1.2.1.3. The Bacterial Cell Wall

The cell wall determines the shape of a bacterium and it provides the strong structural support necessary to keep a bacterium from bursting or collapsing due to changes in osmotic pressure. The cell walls of most bacteria gain this relatively rigid quality from a unique macromolecule called peptidoglycan. This compound is composed of a repeating framework of long glycan chains cross-linked by short peptide fragments to provide a strong but flexible support framework¹¹. Depending on their reaction to the Gram stain bacteria can be classified as either Gram positive (retain the primary stain and appear purple in colour) or Gram negative (stains with the counterstain and appears red in colour)^{11,24}.

Table 2 highlights the differences between the Gram positive and Gram negative cells walls.

Table 2: Comparison of Gram positive and Gram negative cell walls¹¹.

CHARACTERISTIC	GRAM POSITIVE	GRAM NEGATIVE
Number of major layers	1	2
Chemical make-up	Peptidoglycan Teichoic Acid Lipoteichoic Acid	Lipopolysaccharide Lipoprotein Peptidoglycan
Overall Thickness	Thicker (20-80 nanometres)	Thinner (8-11 nanometres)
Outer Membrane	No	Yes
Periplasmic Space	Absent	Present in all
Porin Proteins	No	Yes
Permeability to molecules	More permeable	Less permeable

Figure 2 is a schematic representation of the differences between the Gram positive and Gram negative cell walls.

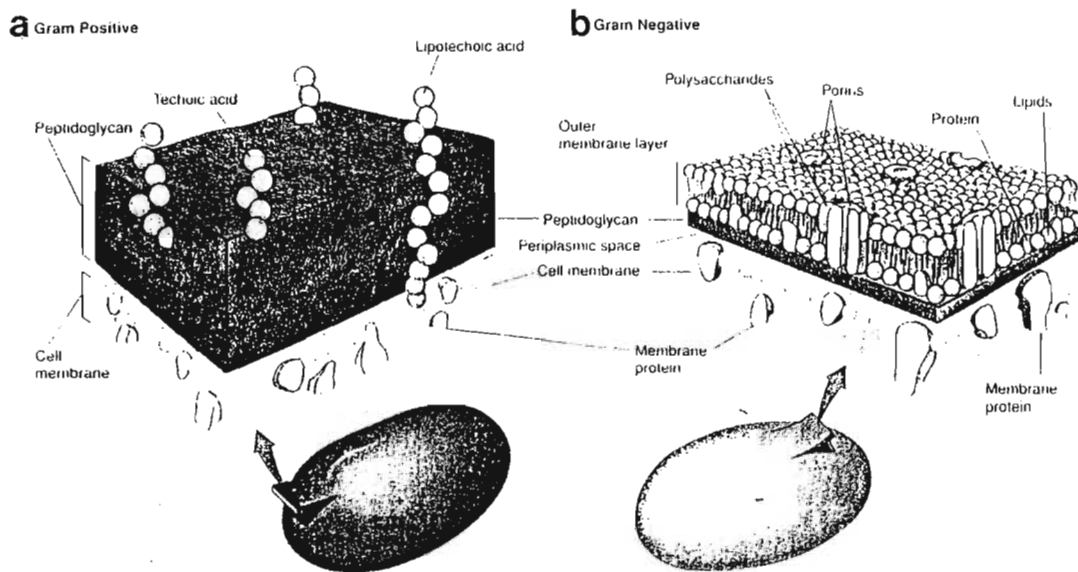


Figure 2: Bacterial cell walls, viz., (a) Gram positive cell wall and (b) Gram negative cell walls¹¹

The outer membrane of the Gram negative cell wall contributes an extra barrier and this makes Gram negative bacteria impervious to some antimicrobial agents. Generally, Gram negative bacteria are more difficult to inhibit or kill than are Gram positive bacteria. The exception is for alcohol based compounds, which can dissolve lipids in the outer membrane and disturb its integrity. Treating infections caused by Gram negative bacteria often requires drugs that act on a different target than in Gram positive bacteria and they commonly require more aggressive therapy than do Gram positive bacteria¹¹.

1.2.2. Mycology

Fungi can be divided into two categories, viz., macroscopic fungi (e.g., mushrooms, puffballs, gill fungi) and microscopic fungi (e.g., yeasts and moulds). Yeasts are unicellular organisms and moulds are multicellular filamentous organisms. The thallus (body) of a mould consists of long filaments of cells joined together. These filaments are called hyphae. When environmental conditions are suitable, the hyphae grow, intertwine and form a filamentous mass that is visible to the naked eye. Yeasts are non-filamentous, unicellular fungi that are typically spherical or oval²⁰.

Some fungi, mainly pathogens, exhibit dimorphism, i.e., two forms of growth. Such fungi can grow either as a mould or as a yeast. Dimorphism in fungi is temperature dependent. The fungus is yeast-like at 37°C and it is mould-like at 25°C²⁰.

Figure 3 illustrates the morphology of the yeasts whilst Figure 4 illustrates the morphology of the moulds.

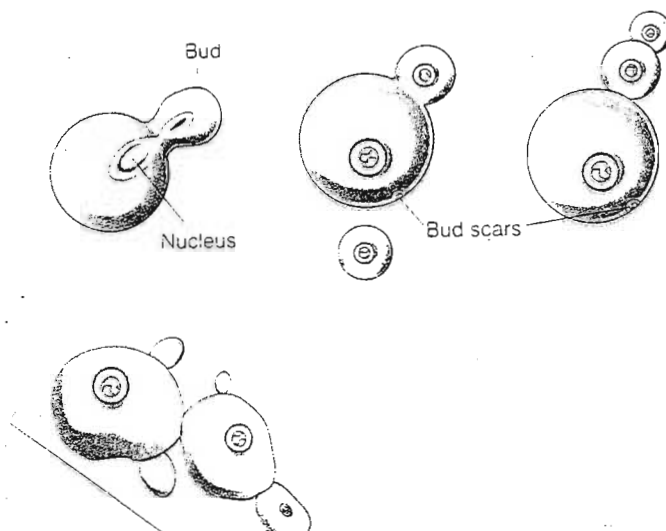


Figure 3: Microscopic morphology of yeasts¹¹

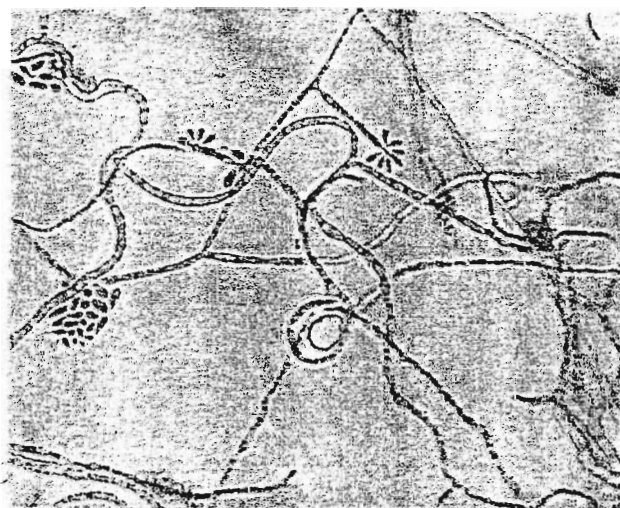


Figure 4: Micrograph of a mould showing long filamentous filaments of intertwined hyphae²⁵

1.2.3. Traditional Medicine in South Africa

1.2.3.1. Traditional Healers and reasons for consultation

There are broadly three types of traditional healers available to South Africans, viz.,

- ◆ The *isangoma* who is the diagnostician and augur of society. She consults the spirits to identify the source of affliction²⁶.
- ◆ The traditional doctor or *inyanga* who claims no affiliation to the spirits. He functions as an apothecary, preparing and dispensing various herbal remedies^{27,28}.
- ◆ The *umprofethi* or *umthandazi* (faith healer) who integrates Christian and traditional practices^{29,30}.

Despite phenomenal advances, modern medicine has failed to reach the masses. Between seventy to ninety percent of the population of rural African areas are not covered by public health services. People therefore resort to traditional methods for satisfying their medical needs³¹. A large percentage of the population cannot afford the products of the western pharmaceutical industry³² and for most Africans, traditional medicine in the principal and often the only form of health care^{9,33,34}.

There is an increasing interest in traditional medicines in developing countries. Modern medicines are expensive whilst traditional systems are usually less expensive⁹. Traditional medicine is accessible to the rural and underprivileged communities. The practitioner speaks the language of the people³³ and understands their psychology and feelings³⁵, whereas, there often exists a communication gap between the “givers” and “takers” of modern medicine. Very few doctors have a command of African languages and there is need for an interpreter posing a risk of wrong translation or inappropriate phraseology²⁶.

Despite the westernization of the Africans through urbanization and education, their belief in traditional remedies and healers remain firm³⁶. To the many people that use traditional healers, they are more easily available, accessible, acceptable and adaptable than western doctors are. For the many people that have grown up with this tradition, it is difficult for them to adapt to other systems of medicine³⁷.

1.2.3.2. The Integration of Traditional Healing Systems with Western Medicine

The WHO emphasized the utilization of traditional systems of medicines based on locally available raw materials. Because of the serious shortage of trained professional personnel in the field of health, it was recommended that existing traditional health care delivery systems be utilized³⁸.

The WHO advocates the co-operation and mutual respect between the traditional and modern health practitioners. Collaboration between the medical doctor and diviner is recommended since their spheres of competence, the physical and social, neither meet nor overlap but are independent and complementary. Although the herbalist and medical doctor share the same sphere of competence, i.e., body and mind, the means they employ differ so radically that they must be ranked as opposite rather than complementary and this rules out collaboration²⁷.

The WHO has suggested the retraining of traditional practitioners to serve as primary health care workers³⁹. In remote areas, where western medicine because of expense and distance has not made any impact on the local rural people, the training of certain traditional healers should be attempted to fill the gap and carry the gospel of health care supervision³⁰.

Several developing countries in Africa, Asia and Latin America have experimented with the integration of traditional and western health care systems. China has more than one thousand one hundred traditional medicine hospitals as well as traditional medicine departments in ninety percent of all hospitals³⁴. Government and independently initiated programmes for the integration of traditional and western medicines are found in several countries in Africa, viz., Ghana³⁴, Nigeria⁴⁰, Kenya³⁴, Tanzania³⁴, Zambia³⁴, Swaziland⁴¹ and Zimbabwe³⁴. In rural Nigeria, a doctor operated a highly successful programme combining the strengths of traditional and western psychiatric practices into a hybrid system of healing. In Zimbabwe, attempts to structure and improve the utilization of traditional medical practitioners have occurred within the framework of comprehensive and

promotive legislation. In Ghana, the government supports traditional practitioners. Here, there is the primary health training for indigenous healers programme which concentrates on traditional practitioners and improving co-operation and co-ordination with the western based health services. The programme is sensitive to the needs of both the practitioner and the demands of the traditional health care market³⁴.

The programmes in Zimbabwe and Ghana indicate that the potential for developing traditional medical systems have been recognized in Africa. The examples of these two countries illustrate that the feasibility of linking traditional medicine to national health care coverage relies, in part, on favourable government policies and supportive national legislation. The lack of established contact between traditional healers and health planners have been identified as further constraints between traditional and western medical practitioners in integrating health care⁴².

In South Africa, the actual provision of health care by traditional medical systems has not been sufficiently investigated, nor has the potential of the health care resources been adequately explored³⁴.

Nevertheless, the Department of Health recognizes that traditional healers have a role to play in health care and that it could be a possible supplement to conventional medicine.

1.2.3.3. Advantages and Disadvantages of consulting traditional healers

➤ Advantages

Traditional healers are reported to treat the whole person. They simply do not dispense medicines for particular symptoms³⁵. Some traditional healers seem to be effective in treating certain illnesses, e.g., controlling diarrhoea, sedating a patient, easing headaches and other pains, and reducing swelling²⁹.

Sometimes, a patient will remain with the traditional healer for a period of days, weeks or months. This may remove a patient from a stressful situation in his/her family or community and help him/her recover. Healers may help ease the pressure on overburdened

clinics by treating minor self-limiting psychosomatic conditions in which traditional therapies are appropriate and effective⁴¹.

Most traditional healers refer patients to clinics if they cannot treat a problem and in many cases, these diseases are diagnosed as cholera, tuberculosis, polio or bilharzia⁴¹.

African traditional medicine begins at the household level, where family members possess a great deal of information on the diagnosis and treatment of common symptoms³¹. The therapy employed by the traditional healers is not always limited to drugs or herbs. It frequently involves spiritual rituals and the regulation of diet and behaviour.

➤ **Disadvantages**

Many traditional healers carry out harmful practices and these include:

- ◆ giving medicinal enemas to treat and prevent childhood diarrhoeas
- ◆ inducing vomiting for patients with cardiac problems and tuberculosis
- ◆ traditional vaccinations done with unclean razors posing the risk of tetanus, hepatitis and acquired immunodeficiency syndrome (AIDS)
- ◆ medicines are given which are dangerous by themselves or if taken at the same time as modern medicines⁴¹.

There is evidence of unhealthy and dangerous practices by traditional healers, e.g., a woman collected menstrual secretions, debris from under the arms, vomit and dirt from under the nails and took it to a traditional healer who mixed it with certain herbs. This concoction was mixed with the husband's food in order to improve the marriage relationship⁴³.

1.2.4. Useful Medicinal Plants

In spite of the tremendous advances in medicine, there are a number of diseases for which modern medicine has no cure⁴⁴. There is a growing demand for herbal medicine and for support leading to the development of medicines from herbal sources. This growing demand is not only in the countries where these plants are traditionally used but also in countries such as the United States of America, United Kingdom, France and Germany¹⁰. In addition, the WHO has emphasized the utilization of indigenous systems of medicines based on the locally available raw material, i.e., medicinal plants.

In diseases for which modern medicine has no cure, symptomatic treatment is advocated. These diseases include arthritic diseases, liver disorders, cancer and AIDS. Recent data has shown that plant drugs may be the answer to such diseases. A number of formulations based on *Ayurvedic* medicines have been used for liver disorders and have been found to be effective. Artemisinin isolated from the plant *Artemisia annua* is effective against resistant strains of *Plasmodium falciparum* (causes malaria). Saponins from the Indian plant *Commifera mukul* have been approved as a hypolipidaemic agent. Taxol, isolated from the bark of the yew tree is useful for ovarian cancer whilst swansonins, isolated from *Swansonia* species, inhibits the replication of the AIDS virus by causing changes in the glycoproteins on the surface of the virus^{44,45}.

In recent years people all over the world are beginning to realize that the comforts provided to us by science and modern technology do not necessarily make life easier⁴⁶. Several of the very important and useful medicines we use today come from plants. Many of these have now been synthesized and the synthetic drugs are now being used⁴⁶. In other instances, it is easier to obtain the medicines from plants even if they can be synthesized. Some of these medicines are ephedrine, morphine, quinine, emetine, reserpine, digitalis, ergot and vincristine¹⁰.

Table 3 lists only a few examples of useful herbal remedies. There is considerable scope to screen these and other plants for active constituents that may be used in the future for the treatment of incurable diseases.

Table 3: Some useful herbal remedies.

COMMON NAME	SOURCE	USES
Echinaceae	<i>Echinaceae</i> species	Immunostimulant ⁴⁷
Garlic	<i>Allium sativum</i>	Lowers blood lipids ^{47,48}
Ginger	<i>Zingibar officinale</i>	Anti-emetic ⁴⁷
Valerian	<i>Valeriana officinalis</i>	Minor tranquillizer ⁴⁷
Feverfew	<i>Tanacetum parthenium</i>	Migraine prophylaxis ^{47,49}
Horse Chestnut	<i>Aesculus hippocastanum</i>	Topical varicose vein treatment ⁴⁹
Hawthorn	<i>Crategus oxyacantha</i>	Congestive heart failure (early stages) ⁴⁹
Artichoke	<i>Cynara scolymus</i>	Lipid lowering activity ⁴⁹
Peppermint	<i>Mentha piperita</i>	Irritable bowel syndrome ⁴⁹

1.2.5. Adverse effects of plant used medicinally

Although herbal preparations are widely used and generally appear to be safe, there may be the misconception that because herbs are “natural” they are devoid of the adverse effects of conventional medicines^{49,50,51}. Some plants are toxic to humans; others are associated with adverse side effects and most will cause problems when overdosed.

Many unsafe and ineffective herbs are on the market. Many do not conform to any official standards of quality; the identity and purity of many herbal preparations may not be those specified on the label⁵⁰. There are many causes of herbal poisoning such as misidentification of the plant species⁵² and unknown or ignored toxicity of a correctly identified plant⁵³. Medical professionals and the public should be alerted to the toxicity of herbal remedies. The users at particular risk are long term users⁵⁴, consumers of large amounts⁵⁴, babies⁵⁴, foetuses⁵⁴ and geriatrics⁵⁵. For example, comfrey has been traditionally used as a wound-healing agent. It contains highly carcinogenic compounds and should not be taken internally. It also contains pyrrolizidine alkaloids that can cause liver damage^{47,56}. In Southern Africa, acute renal failure is common amongst the Black population. The

commonest nephrotoxin in the black patients is herbal medicine⁵⁷ and *Callilepis laureola* was identified as the causative agent^{58,59}. The toxic action of *C. laureola* is also the cause of fatal liver necrosis⁶⁰.

Other examples of unsafe herbals include borage, calamus, chaparral, coltsfoot, liquorice and sassafras⁴⁷. Herbs used in teas could contain coumarins that could lead to abnormal menstrual bleeding⁵⁶. Some herbal medicines are mixtures with other ingredients of non-herbal origin, e.g., lead^{61,62} or undeclared western drugs, e.g., prednisolone, non-steroidal inflammatory agents, anti-rheumatic agents and paracetamol^{63,64}.

Toxic reactions related to the use of traditional remedies are due to a number of factors including difficulty in identifying the herbal⁶⁵, remedies may be mixtures⁶⁶, lack of demonstration of the safety and efficacy of the herb⁵⁴, variability in chemical constituents of the herb^{67,68,69}, adulteration^{70,71} and the synergistic effect of using a herbal medication in combination with another pharmacological product⁷².

Despite the above-mentioned, the use of herbal medicines is a persistent aspect of present day health care. Twenty-five percent of all prescriptions written are for plant products and another twenty-five percent are for agents based on plant products⁵⁴. Many people use traditional medicines in combination with orthodox medicines.

Generally, little is known about the consequences of such combinations. This is complicated by the lack of knowledge about what many of the herbal medicines contain⁶³. The herbal/traditional remedies should be viewed as one would view any manufactured drug or chemical in assessing health risk or safety⁵⁴.

Despite protests by herbalists, adverse reactions do occur with herbal remedies⁷³ and more studies are needed to ascertain the toxic potential of all herbal remedies.

1.2.6. The *Bulbine* species

1.2.6.1. The Asphodelaceae

The *Bulbine* species belong to the family Asphodelaceae. This family has a predominantly Old World distribution. It consists of four varied genera, viz.,

- ❖ *Bulbine* (yellow flowers)
- ❖ *Bulbinella* (yellow flowers)
- ❖ *Kniphofia* (yellow and red flowers)
- ❖ *Trachyandra* (white flowers)

These plants are rhizomatous and have thickened storage roots. The taxa are herbaceous with mesomorphic or occasionally succulent leaves. The species have central branched or unbranched pedunculate inflorescences⁷⁴.

1.2.6.2. Botany of the *Bulbine* species

The following is a brief botanical description of the *Bulbine* species with particular emphasis on the plants used in this study.

Figures 5-8 are a photographic representation of the different *Bulbine* species used in these experiments.

❖ *B. natalensis* Bak

Zulu name: **ibhucu**¹³

Afrikaans name: **rooi wortel**¹³



Figure 5: Photograph of *B. natalensis* Bak

B. natalensis is a perennial, acaulescent herb or subshrub with a cormose base⁷⁵. The plant has an aloe-like appearance with a rosette of fleshy thornless yellowish green leaves¹³. The leaves are firm to papery when dry. The yellow flowers are borne in elongated clusters on long flowering stems that are curved-erect to erect and 2-3 times as long as the leaves. The flowers are numerous⁷⁵. *B. natalensis* is known to many people as *Bulbine latifolia* but the latter is a distinct species from the Eastern Cape with dark green firm textured leaves and densely grouped flowers¹³. *B. natalensis* generally flowers in July and August. It is normally found in rocky areas with poor soil⁷⁶.

Distribution of *B. natalensis*: Central and Eastern South Africa, Zimbabwe, Mozambique, Gauteng, KwaZulu-Natal and the Free State⁷⁵.

❖ *B. frutescens* Willd

Synonyms: *Bulbine caulescens*, *Bulbine rostrata*¹²

Zulu names: **ibhucu, ithethe elimpofu**¹²

Common names: **burn jelly plant, cat's tail, rankkopieva, yellow and orange garlic**¹²

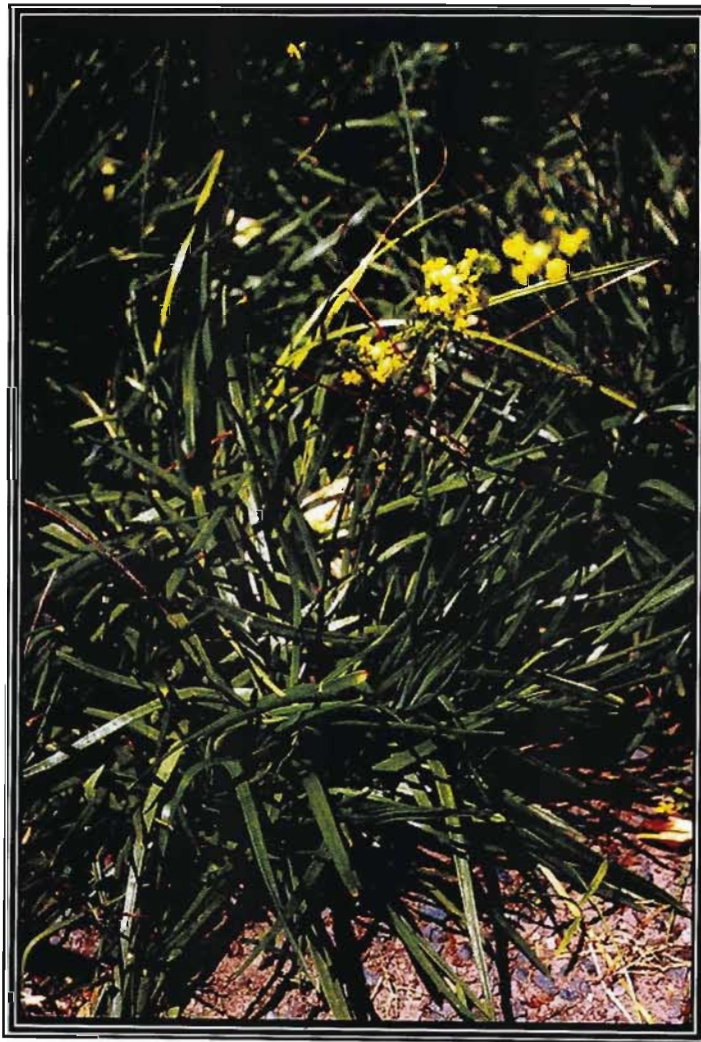


Figure 6: Photograph of *B. frutescens* Willd (yellow flowers)

B. frutescens is a perennial, acaulescent or almost caulescent subshrub with a woody base. The roots are frequently wiry, mainly subterranean and cauline. The stems are abbreviated or up to seventy centimetres long. The onion-like leaves that are terete, subterete or occasionally furrowed adaxially are generally green to glabrous green. The inflorescence is erect or slightly curved⁷⁵. These plants thrive in sandy soil. Flowering specimens are found throughout the year⁷⁷.

Distribution of *B. frutescens*: South Africa mainly the Eastern Cape⁷⁵.

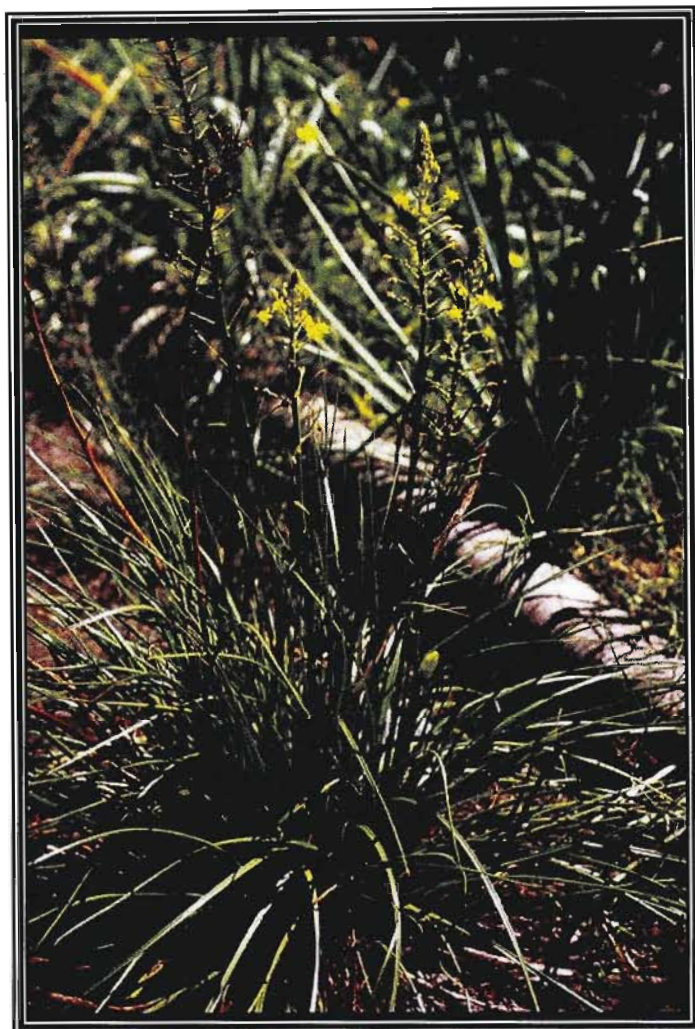
❖ *B. abyssinica* A Rich

Figure 7: Photograph of *B. abyssinica* A Rich

B. abyssinica is a perennial acaulescent herb with a rhizomatous base. The rhizome is compact and overtopped by persistent dry leaf bases. The roots are many, radiating; slender or swollen. The leaves are ten to many in a basal rosette, glabrous green, erect to slightly spreading. This robust plant has the flowering stem arising from a short rootstock. The inflorescence is generally erect⁷⁵. *B. abyssinica* is a widespread species and flowering specimens are recorded in all months except July⁷⁸.

Distribution of *B. abyssinica*: Central and Eastern Africa from Northern Somaliland to the Western Cape and Eastern Cape and Angola⁷⁵.

❖ *B. narcissifolia* Salm Dyck

Griqua name: **tloruthloru**

South Sotho name: **khomo-ea-basemane**

Common name: **kopiva, snake flower, wildekopiva**

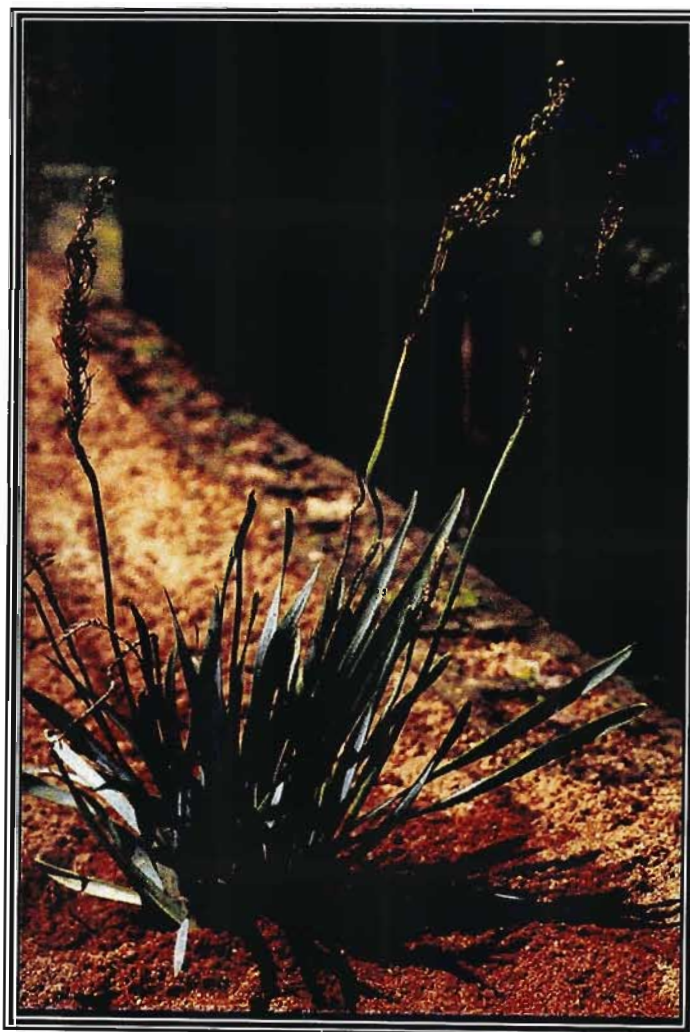


Figure 8: Photograph of *B. narcissifolia* Salm Dyck

B. narcissifolia is a perennial acaulescent herb with a rhizomatous base. The leaves are erect and strap shaped. The inflorescence is generally erect. Flowering and fruiting tend to occur twice a year, viz., February to April and September to November⁷⁵.

Distribution of *B. narcissifolia*: Southern Africa, Lesotho, Gauteng, Free State, KwaZulu-Natal, Western Cape and Eastern Cape⁷⁵.

CHAPTER TWO

MATERIALS AND METHODS

2.1. PREPARATION OF THE CRUDE EXTRACTS

The chemistry of the *Bulbine* species is largely unknown and not many documented studies have been carried out to ascertain their antimicrobial activities. A solvent system extracting both the polar and non-polar components was thus chosen. The combination of methanol and dichloromethane (1:1, v/v) was used to extract the polar and non-polar components respectively.

Recent literature shows a swing away from the traditional method of differential solvent extraction as well as the use of the Soxhlet apparatus in the extraction procedure because of the possibility of labile compounds being destroyed by heating. Robin *et al.*⁷⁹, Ariaswa *et al.*⁸⁰, Kumar *et al.*⁸¹, Roche *et al.*⁸² and Osawa *et al.*⁸³ employed methanol or dichloromethane as the extracting solvent.

The use of dry powdered material has also been superceded by the use of fresh material. Milling exposes labile constituents of the plant causing artifact formation. Powdered material has to be completely dry or remaining moisture may facilitate decomposition and oxidation. The *Bulbine* species are succulents by nature and it is difficult to dry these plants. In addition, drying could affect the components found in the aqueous fraction of the fleshy leaves. During the evaporation process labile compounds could be destroyed. Therefore, in these experiments the evaporation of solvents was carried at a temperature of 30°C.

The aqueous evaporation was carried out to see if there were any differences in the antimicrobial activities of the extracts utilizing the two different extracting solvents. The aqueous extract also mimicked the way that these plants were used by the traditional healers.

2.1.1. Collection of the Plant Material

The plants used were collected with the assistance of the curator of the herbarium at the University of Durban-Westville (UDW).

Table 4 gives an indication of the sources of the plants used in the study. Voucher specimens were deposited in the Ward Herbarium in the Department of Botany at UDW.

Table 4: Collection of the Plant Material

PLANT NAME	SOURCE
<i>B. natalensis</i>	UDW gardens
<i>B. frutescens</i> (yellow flowers)	UDW gardens
<i>B. frutescens</i> (orange flowers)	Nursery, Eastern Cape
<i>B. abyssinica</i>	Silverglen Nature Reserve Nursery, Durban
<i>B. narcissifolia</i>	Bergville

2.1.2. Organic Extraction

The plants were washed with tap water to remove sand and dirt and air-dried overnight at room temperature (25°C). The leaves were cut into small pieces and immersed in methanol: dichloromethane (1:1, v/v) for seventy-two hours at room temperature. The methanol: dichloromethane solution was decanted from the leaves and concentrated under reduced pressure at 30°C using a rotary vacuum evaporator. The crude extract was massed into glass vials and placed in the freezer (-20°C). The procedure was repeated for the roots and stems.

NB: The quantity of plant material used in the organic extraction is not given in the methodology because the amount of plant material used varied considerably for the different plants and also for the different plant components. However, all the quantities are stipulated in the results section in Table 7.

2.1.3. Aqueous Extraction

The extraction procedure was carried out for *B. natalensis* and *B. frutescens* (yellow flowers). Fresh leaves (approximately 20g) were cut and immersed in cold water (100ml) for twenty-four hours at room temperature. The aqueous solution was decanted from the leaves and used the same day. The procedure was repeated for the roots and stems. In addition, fresh leaves (approximately 20g) were cut and immersed in boiling water (100ml). The aqueous solution was decanted from the leaves for immediate use.

2.2. ANTIBACTERIAL ACTIVITIES

2.2.1. Bacteriology

This study investigated the activity of the *Bulbine* species against some of the pathogens implicated in the conditions mentioned in Table 1, viz.,

◆ **urinary tract infections**

*Escherichia coli*⁸⁴, *Klebsiella oxytoca*⁸⁴, *Klebsiella pneumoniae*⁸⁴, *Proteus mirabilis*⁸⁴, *Enterobacter aerogenes*⁸⁴, *Pseudomonas aeruginosa*⁸⁴, *Staphylococcus aureus*⁸⁴, *Citrobacter freundii*⁸⁴, *Enterococcus faecalis*⁸⁵

◆ **wound infections**

*E. aerogenes*⁸⁶, *Morganella morganii*⁸⁶, *P. mirabilis*⁸⁶, *S. aureus*⁸⁶, *Staphylococcus epidermidis*⁸⁶, *Bacillus* species⁸⁶

◆ **dysentery**

*Salmonella typhi*⁸⁴, *Shigella flexneri*⁸⁴

Table 5 shows the different bacteria used in this study with emphasis on their Gram stains, the diseases that they cause and the drug therapy used to treat resulting infections.

Table 5: Bacteria used in this study

BACTERIUM	GRAM STAIN	DISEASE CAUSED	DRUG THERAPY
<i>E. coli</i>	-ve	UTI ²² , gastroenteritis ¹¹ , septicaemia ¹¹	Aminoglycoside ¹¹ , ampicillin ¹¹ , quinolone ⁸⁷
<i>E. aerogenes</i>	-ve	UTI ²² , sepsis ¹¹ , septicaemia ¹¹	Cephmandole ¹¹ , aminoglycoside ²²
<i>S. typhi</i>	-ve	Typhoid fever ²² , salmonellosis ¹¹	Chloramphenicol ²² , ampicillin ¹¹
<i>S. flexneri</i>	-ve	Dysentery ¹¹	ampicillin ¹¹
<i>M. morgani</i>	-ve	Opportunistic or secondary infection ¹¹	aminoglycoside ²² , cephalosporin ²²
<i>C. freundii</i>	-ve	Opportunistic or secondary infection ¹¹ , UTI ²² , sepsis ²²	aminoglycoside ²² , chloramphenicol ²²
<i>K. pneumoniae</i>	-ve	Bronchopneumonia ²² , UTI ²²	cephalosporin ²² , gentamycin ¹¹
<i>K. oxytoca</i>	-ve	Hospital acquired infection ¹¹	cephalosporins ²²
<i>P. aeruginosa</i>	-ve	Infections of wounds and burns ¹¹	aminoglycosides ¹¹ , tetracycline ¹¹ , carbenicillin ¹¹
<i>P. mirabilis</i>	-ve	UTI ²² , septicaemia ¹¹	ampicillin ²²
<i>S. aureus</i>	+ve	Endocarditis ⁸⁷ , septicaemia ¹¹ , wound infections ²⁵ , impetigo ²⁵	cloxacillin ⁸⁷ , penicillinase resistant penicillins ¹¹ , vancomycin ¹¹ , erythromycin ¹¹
<i>S. epidermidis</i>	+ve	UTI ¹¹ , bacteraemia ¹¹	-
<i>E. faecalis</i>	+ve	UTI ¹¹ , wound infections ¹¹	ampicillin ⁸⁷ , penicillin ¹¹ , cephalosporin ¹¹
<i>M. luteus</i>	+ve	Wound infections ¹¹	-
<i>B. subtilis</i>	+ve	Wound and burn infections ⁸⁵	-

2.2.2. Preparation of the Bacterial Cultures

All the bacterial cultures used in this study were freeze-dried cultures purchased from the Council for Scientific and Industrial Research (CSIR). Stock cultures were inoculated onto Nutrient Agar (Oxoid) slants and stored at 5°C. Cultures for the experiments were overnight cultures prepared by inoculating Nutrient Broth (Oxoid) (5ml) with a single bacterial colony and incubating at 37°C for 18-24 hours. The bacterial cultures were adjusted with sterile Nutrient Broth using McFarland's Standard to yield approximately $1,0 \times 10^8$ colony forming units per millilitre (cfu/ml).

2.2.3. Preparation of the agar plates

Mueller Hinton Agar (Oxoid) was used as the standard test medium. The agar plates were prepared by pouring melted sterile agar (25ml) that had been autoclaved at 121°C for fifteen minutes into sterile 90mm plastic petri plates and allowing them to stand at room temperature until they had solidified.

2.2.4. Preparation of the crude extracts

The crude extract (50mg) was dissolved in distilled water (2ml). The concentration of crude extract per ml of water was as follows:

$$\begin{aligned}\text{Concentration} &= 0,05\text{g extract in } 2\text{ml water} \\ &= 0,025\text{g/ml.}\end{aligned}$$

Each disk was impregnated with crude extract solution (30 μ l). Therefore the amount of crude extract on each disk was 0,75mg. Each well was inoculated with 50 μ l of crude extract. Therefore the amount of crude extract in each well was 1,25mg.

2.2.5. Disk Diffusion Method¹⁴

The Mueller Hinton Agar plates were inoculated with the bacterial suspension using a sterile swab to achieve a lawn growth. Sterile paper disks (6mm in diameter) (Schleicher & Schuell, Dassel, Germany) were placed on the inoculated agar surface and impregnated with 30µl of crude extract solution. The plates were incubated aerobically at 37°C for 24 hours. After incubation, the zones of inhibition were measured.

2.2.6. Bore Well Method¹⁵

The Mueller Hinton Agar plates were inoculated with the bacterial suspension using a sterile swab to achieve a lawn growth. Wells were punched into the agar with a sterile stainless cork borer (diameter of well = 6mm). The wells were inoculated with crude extract solution (50µl) and incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured.

2.3. Mycology

2.3.1. Fungi used in this study

The following fungi were used in this study, viz.,

- ◆ *Candida albicans*
- ◆ *Aspergillus species*
- ◆ *Penicillium species*

All the fungi that were used were purchased from CSIR in Pretoria.

Table 6 shows the different fungi used in this study with emphasis on their medical importance.

Table 6: Fungi used in this study

FUNGUS	MEDICAL IMPORTANCE
<i>C. albicans</i>	Causes vaginitis, candidiasis, oral thrush ²⁰
<i>Aspergillus</i> species	Causes aspergillosis ²⁰
<i>Penicillium</i> species	Used for the mass production of the antibiotic penicillin ^{20,25}

2.3.2. Preparation of the fungal spores

Aspergillus and *Penicillium* were cultured on Potato Dextrose Agar (Oxoid) plates and incubated at room temperature for seven days. The spores were harvested by rinsing the plates with sterile Nutrient Broth. This solution was diluted with sterile Nutrient Broth to give a final concentration of 10^6 spores /ml. The number of fungal spores were evaluated using the plate count technique. To evaluate the number of spores using this technique, serial dilutions of the harvested spores were made with distilled water. Each dilution (0,1ml) was added to melted Potato Dextrose Agar (25ml), mixed well, poured into sterile 90mm petri plates and allowed to solidify. The plates were incubated at room temperature for 5-7 days. The number of spores were enumerated by counting the number of fungal colonies.

2.3.3. Preparation of *C. albicans*

The *C. albicans* culture used in the experiments was an overnight culture prepared by inoculating Nutrient Broth (5ml) with a single *C. albicans* colony and incubating at 37°C for 18-24 hours. The culture was adjusted with sterile Nutrient Broth using McFarland's Standard to yield approximately $1,0 \times 10^8$ cfu/ml.

2.3.4. Antifungal activity using the TLC Bioassay¹⁶

The TLC antifungal bioassay method was used to determine the antifungal activities of *Penicillium* and *Aspergillus*. The crude extract solutions were prepared by dissolving crude extract (50mg) in methanol: dichloromethane (1:1, v/v) (1ml). These crude extract solutions were applied to silica gel GF₂₅₄ aluminium backed plates (Merck) in bands 1cm wide (3 to 4 applications) and allowed to develop for 9cm.

The solvent system used for the separation of constituents was hexane: ethyl acetate: chloroform: formic acid (8:7:5:1, v/v).

Thereafter, the plates were sprayed with the fungal spores and incubated in a moist chamber in a dark cupboard at room temperature for fourteen days. After incubation, the zones of inhibition were measured.

2.3.5. Antifungal activity of *C. albicans*

The antifungal activity of *C. albicans* was assessed using the Disk Diffusion¹⁴ and Bore Well¹⁵ Methods. The crude extracts used were prepared as per method in Section 2.2.4.

2.4. Cytotoxic Activity

2.4.1. The Brine Shrimp Method

In the method by Meyer *et al.*¹⁷ the brine shrimp, *Artemia salina* Leach is used as a simple bioassay for natural products. The method is rapid (results are obtained twenty-four hours following the introduction of the shrimps), reliable, inexpensive, convenient as an in-house general bioassay tool and simple. Since most active plant principles are toxic at elevated doses, an approach to develop an effective general bioassay was needed to screen for substances that are toxic to zoologic systems. The tiny crustacean, the brine shrimp, is thus ideal. The eggs of the brine shrimp are readily available at low

cost in pet shops as a food for tropical fish and they remain viable for years in the dry state. In a brine solution, the eggs hatch within 48 hours, providing a large number of larvae (nauplii). The brine shrimp is a convenient probe for pharmacologic activities in plant extracts which may be manifested as toxicity towards the newly hatched nauplii¹⁷.

The brine shrimp belongs to the phylum Arthropoda⁸⁸, subphylum Crustacea, class Phyllopoda, subclass Sarsotraca and order Anostraca⁸⁹. The order Anostraca with one hundred and eighty-eight species including the brine shrimp⁸⁹, *Artemia salina* Leach⁹⁰. This group is characterized by the lack of a head shield and carapace⁸⁹ and the bodies are relatively slender⁹⁰. Brine shrimps are very small. They inhabit ephemeral ponds and hypersaline lakes and marine lagoons⁹¹. Under unfavourable conditions such as the evaporation of the pool and unfavourable temperatures, they produce resistant eggs which can restart the population when suitable conditions return⁸⁹. Brine shrimps are permanently planktonic and swim upside down⁹⁰.

Figure 9 is a diagrammatic representation of the brine shrimp.

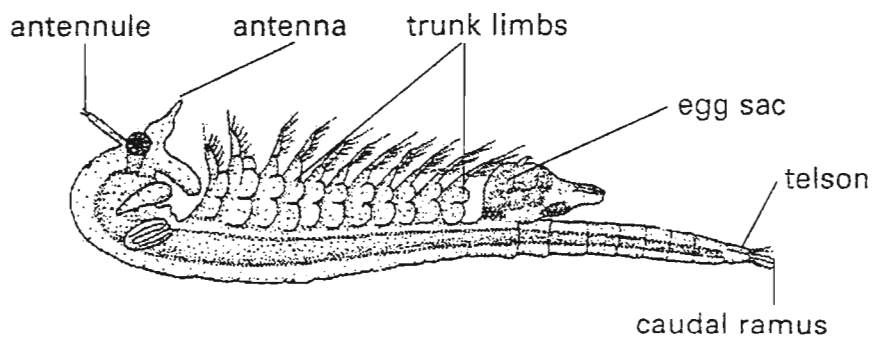


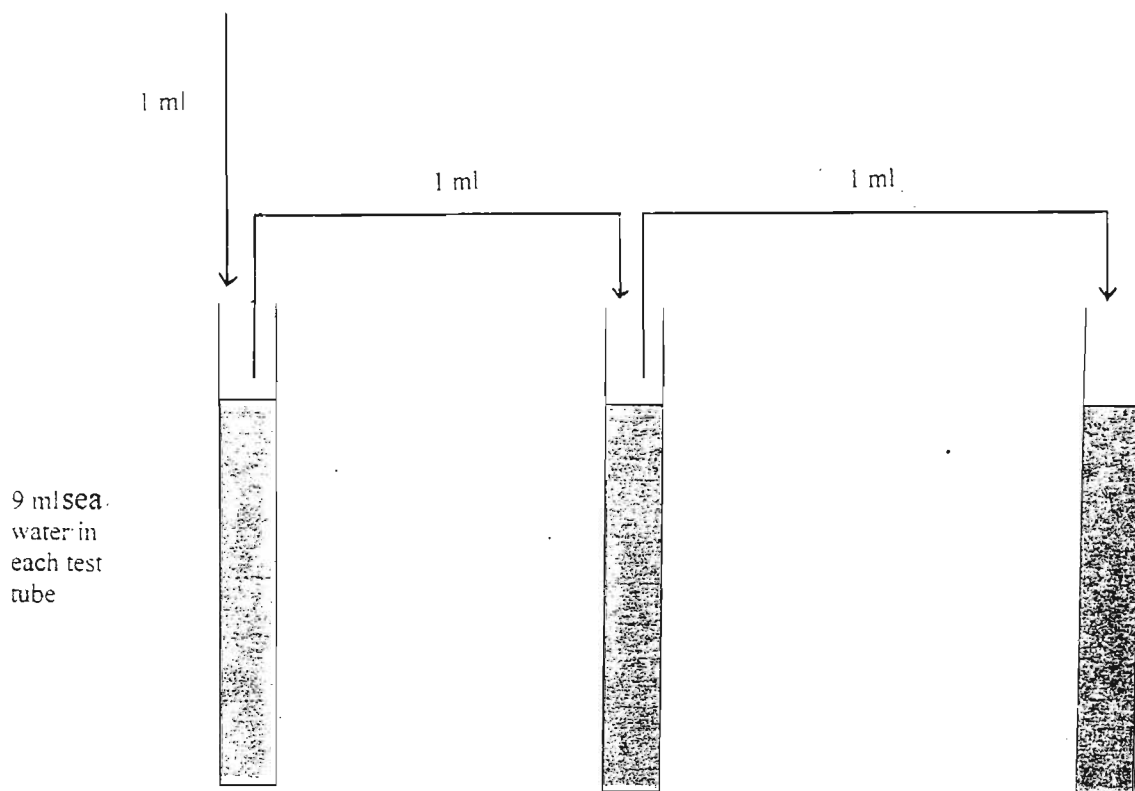
Figure 9: *Artemia salina*, an Anostracan brine shrimp⁹¹

2.4.2. Preparation of the crude extracts

The crude extract (50mg) was dissolved in distilled water (1ml). Thereafter three 1:10 serial dilutions were carried out using sea-water as the diluent.

Figure 10 illustrates the preparation of the extracts for the brine shrimp bioassay using the serial dilution method.

50mg extract + 1ml distilled water = 0,05g/ml



	test tube 1	test tube 2	test tube 3
Dilution:	1:10	1:100	1:1000
Concentration of extracts:	5mg/ml	0,5mg/ml	0,05mg/ml

Figure 10: Preparation of the crude extracts for cytotoxicity studies by the serial dilution method

2.4.3. Preparation of the brine shrimps

Dehydrated brine shrimp eggs were purchased from a pet shop. The brine shrimp eggs (2,5ml) were added to a beaker containing sea-water (500ml). A source of air was supplied. The beaker was covered with aluminium foil and left to incubate overnight at room temperature.

2.4.4. Methodology

The Brine Shrimp Bioassay of Meyer *et al.*¹⁷ was used to ascertain the cytotoxicity of the crude organic extracts. Brine shrimps (10) were added to each of the three 1:10 serial dilutions that were prepared. Brine shrimps (10) were also added to sea-water (10ml) to serve as a control. The procedure was carried out in triplicate. The test tubes were placed uncovered in a water bath set at a temperature of 30°C for 24 hours. After incubation the surviving brine shrimps were counted.

2.5. Chemical Analysis

2.5.1. Chemotaxonomy

Chemical data in taxonomy are an important adjunct to morphological evidence as it reflects the relationship of plants at another level of structural organization. TLC fingerprinting is a simple technique which can be used to resolve taxonomic problems. Apart from R_f comparisons, the most diagnostically important information in this technique is provided by variations or similarities in the colour of the bands that provide the fingerprint for the particular species¹⁸.

> Procedure

The crude extract (50mg) was dissolved in methanol: dichloromethane (1:1, v/v) (1ml). The TLC analyses were carried out on Merck silica gel GF₂₅₄ aluminium backed plates using three different solvent systems. The crude extract solutions were applied to the plates in bands 1cm wide (four applications) and allowed to develop for 9cm. In these experiments the crude extract solutions were applied onto the TLC plates as bands as per method described by Rogers *et al.*¹⁸ and not in the as the usual spots because it was found that the when the plates were developed finer details could be visualised on the TLC.

The three solvent systems used were:

◆ Solvent System 1:

Hexane: ethyl acetate (8:3, v/v) for the separation of non-polar constituents.

◆ Solvent system 2:

Chloroform: ethyl acetate: formic acid (5:4:1, v/v) for the separation of polar constituents

◆ Solvent system 3:

Hexane: ethyl acetate: chloroform: formic acid (8:7:5:1, v/v) for the separation of constituents of intermediate polarities.

A spray reagent consisting of p-anisaldehyde (5ml), concentrated sulphuric acid (5ml) and ethanol (90ml) was used to visualize colourless constituents. These appeared as coloured bands after the sprayed plates had been heated at 110°C for 2-5 minutes. The thin layer chromatograms were recorded as colour photocopies.

2.5.2. Column Chromatography

For liquid column adsorption chromatography, the stationary phase is a surface active solid (e.g., alumina, silica gel or charcoal) packed into a column and the mobile phase is a solvent composed of one or more organic liquids. The separation of a mixture results from the differential adsorption of the components onto the surface of the solid. Weakly

adsorbed solutes travel more quickly whilst strongly adsorbed solutes are retarded. The molecular interactions involved in adsorption can be of several types depending upon the nature (polarity) of the surface, the adsorbed solutes and the solvent¹⁹.

2.5.2.1. Preparation of the column

The glass column (internal diameter of column = 25mm) was wet packed with a slurry of silica gel 60 (Merck, 230-400 mesh) and ethyl acetate (the first solution that was used for chromatography). The column was tapped gently to promote uniform settling. After each portion had settled, the excess liquid was drained out and more slurry was added until the desired height was reached. The column was washed with additional amounts of ethyl acetate after which the liquid level was drained to the top of the slurry.

2.5.2.2. Methodology

The crude extract of the stem of *B. natalensis* (1g) was dissolved in ethyl acetate (5ml). This solution was applied to the top of the column (height of silica gel 60 column was 27cm) with a pasteur pipette as a narrow band. After the sample had just percolated into the column, ethyl acetate (50ml) was introduced. The eluate was collected in 10ml volume fractions (average elution rate was 0,667ml/minute). The column was gradient eluted with a solvent system consisting of ethyl acetate, ethyl acetate:chloroform and finally ethyl acetate:chloroform:methanol.

The eluted fractions were evaporated until dry, spotted onto TLC plates and developed in solvent system 3 (Refer to Section 2.5.1.). The fractions with similar TLC bands were combined and elution was stopped when all the bands (chemical components) from the original stem extract had been eluted from the column.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Yield of Crude Extract

Table 7 illustrates the amount of fresh plant material used and the mass and percent yield of the crude organic extract after the extraction and evaporation processes.

Table 7: Yields of crude organic extracts from the fresh plant material

PLANT PART	MASS OF FRESH MATERIAL (KG)	MASS OF CRUDE ORGANIC EXTRACT (G)	%YIELD
<i>B. natalensis</i>			
Leaves	9,0	29,0	0,32
Roots	0,5	28,29	5,6
Stems	1,8	76,0	4,2
<i>B. frutescens</i> (yellow flowers)			
Leaves	4,0	38,0	0,95
Roots	0,2	1,7	0,85
Stems	0,4	19,0	4,75
<i>B. narcissifolia</i>			
Leaves	5,0	35,0	0,7
Roots	0,5	17,0	3,4
Stems	0,3	5,9	2,0
<i>B. abyssinica</i>			
Leaves	2,0	6,0	0,3
Roots	0,1	2,1	2,1
<i>B. frutescens</i> (orange flowers)			
Leaves	0,3	3,0	1,0
Roots	0,075	2,2	2,0

3.2. Antibacterial Activity

3.2.1. Results

◆ Disk Diffusion Method

Tables 8 and 9 indicate the antibacterial activity of the crude organic solvent and aqueous extracts of the leaves, roots and stems of *B. natalensis*, *B. frutescens* (yellow flowers), *B. abyssinica*, *B. narcissifolia* and *B. frutescens* (orange flowers) against the different pathogens used in this study. Figures 11 – 20 illustrate the antibacterial activity of some of the crude organic solvent and aqueous extracts of the leaves, roots and stems of the *Bulbine* species against *S. epidermidis*, *S. aureus*, *S. typhi*, *M. morgani*, *K. pneumoniae* and *E. coli*.

NB: Many different concentrations of the crude organic extracts were used but the concentration prepared as per section 2.2.4 was found to be most suitable and the results in the 4 tables that follow are the zones of inhibition achieved when using these concentrations.

Table 8: Zones of inhibition (in mm) achieved with the Disk Diffusion Method using the crude organic solvent and aqueous extracts of the leaves, roots and stems of *B. natalensis* and *B. frutescens* (yellow flowers)

BACTERIUM	1	1a	1b	2	2a	3	3a	4	4a	4b	5	5a	6	6a
<i>B. subtilis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. freundii</i>	-	8,5	-	-	-	-	8,5	-	9,5	8,5	-	-	-	-
<i>E. coli</i>	-	-	-	-	-	-	-	-	8,5	8,0	9,0	8,0	-	9,5
<i>E. aerogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. faecalis</i>	-	-	-	-	-	11,0	-	9,5	-	-	11,5	-	10,0	-
<i>K. oxytoca</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	10,0	8,5	10,5	12,5	14,0	-	8,0	10,5	-	11,0	12,5	9,5	12,0	-
<i>M. luteus</i>	-	-	-	14,0	-	10,0	-	-	-	11,5	-	13,0	-	-
<i>M. morgani</i>	-	-	-	11,0	-	8,0	10,0	-	-	-	13,0	-	9,5	-
<i>P. mirabilis</i>	-	-	-	-	-	-	-	-	-	-	-	8,0	-	-
<i>P. aeruginosa</i>	-	-	-	11,0	-	10,5	-	-	-	-	-	-	-	-
<i>S. aureus</i>	-	-	-	10,5	-	11,0	-	-	8,5	7,5	-	-	11,0	-
<i>S. epidermidis</i>	-	-	-	12,5	-	12,5	-	-	9,5	-	11,5	-	11,0	-
<i>S. typhi</i>	-	-	-	13,0	-	10,5	-	-	11,5	-	-	-	9,5	-
<i>S. flexneri</i>	-	-	-	12,0	12,0	10,0	-	-	-	-	11,0	-	10,0	-

KEY:

- | | |
|--|--|
| 1. <i>B. natalensis</i> -organic solvent extr. of leaf | 4a. <i>B. frutescens</i> -aqueous extr. of leaf (cold water) |
| 1a. <i>B. natalensis</i> -aqueous extr. of leaf (cold water) | 4b. <i>B. frutescens</i> -aqueous extr. of leaf (hot water) |
| 1b. <i>B. natalensis</i> -aqueous extr. of leaf (hot water) | 5. <i>B. frutescens</i> -organic solvent extr. of stem |
| 2. <i>B. natalensis</i> -organic solvent extr. of stem | 5a. <i>B. frutescens</i> -aqueous extr. of stem |
| 2a. <i>B. natalensis</i> -aqueous extr. of stem | 6. <i>B. frutescens</i> -organic solvent extr. of root |
| 3. <i>B. natalensis</i> -organic solvent extr. of root | 6a. <i>B. frutescens</i> -aqueous extr. of root |
| 3a. <i>B. natalensis</i> -aqueous extract of root | (-) - no inhibition of growth |
| 4. <i>B. frutescens</i> -organic solvent extr. of leaf | |

Table 9: Zones of inhibition (in mm) achieved with the Disk Diffusion Method using the crude organic solvent extracts of the roots, leaves and stems of *B. narcissifolia* and the crude organic solvent extracts of the roots and leaves of *B. abyssinica* and *B. frutescens* (orange flowers)

BACTERIUM	7	8	9	10	11	12	13
<i>B. subtilis</i>	-	-	-	7,5	13,0	-	-
<i>C. freundii</i>	-	-	-	10,5	-	-	-
<i>E. coli</i>	10,0	-	-	-	-	-	-
<i>E. aerogenes</i>	9,0	-	-	-	10,0	-	-
<i>E. faecalis</i>	9,0	-	-	-	-	-	10,5
<i>K. oxytoca</i>	-	-	9,0	-	-	-	-
<i>K. pneumoniae</i>	11,5	8,0	9,5	-	-	-	11,0
<i>M. luteus</i>	-	-	-	11,0	9,0	-	-
<i>M. morgani</i>	9,5	-	8,0	11,0	10,0	11,0	10,0
<i>P. mirabilis</i>	-	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	10,5	-	-	-	-
<i>S. aureus</i>	-	8,0	-	9,0	11,5	-	-
<i>S. epidermidis</i>	-	-	9,0	11,5	-	-	-
<i>S. typhi</i>	11,0	8,5	10,0	-	-	-	-
<i>S. flexneri</i>	9,5	10,0	9,5	-	-	-	9,5

KEY:

7. *B. abyssinica*-organic solvent extr. of leaf
 8. *B. abyssinica*-organic solvent extr. of root
 9. *B. narcissifolia*-organic solvent extr. of leaf
 10. *B. narcissifolia*-organic solvent extr. of stem
 11. *B. narcissifolia*-organic solvent extr. of root

12. *B. frutescens* (orange flowers)-organic solvent extr. of leaf
 13. *B. frutescens* (orange flowers)-organic solvent extr. of roots
 (-) - no inhibition of growth

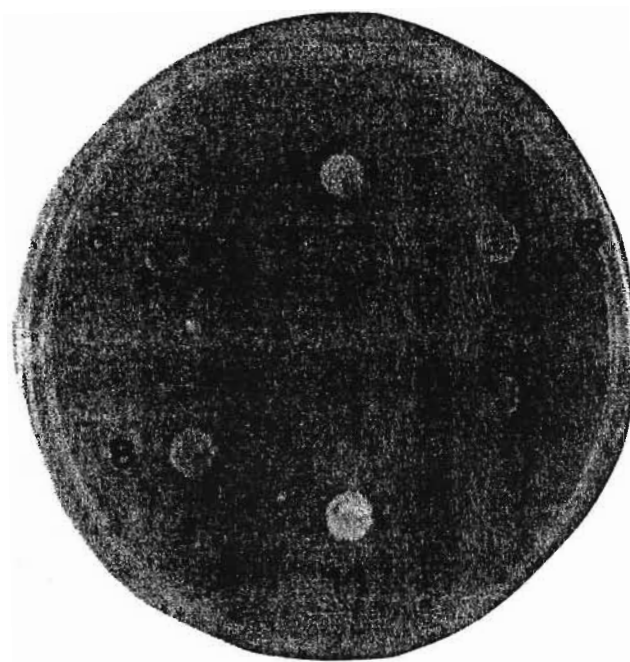


Figure 11: Activity of the crude organic solvent extracts of the roots of the *Bulbine* species against *S. typhi*

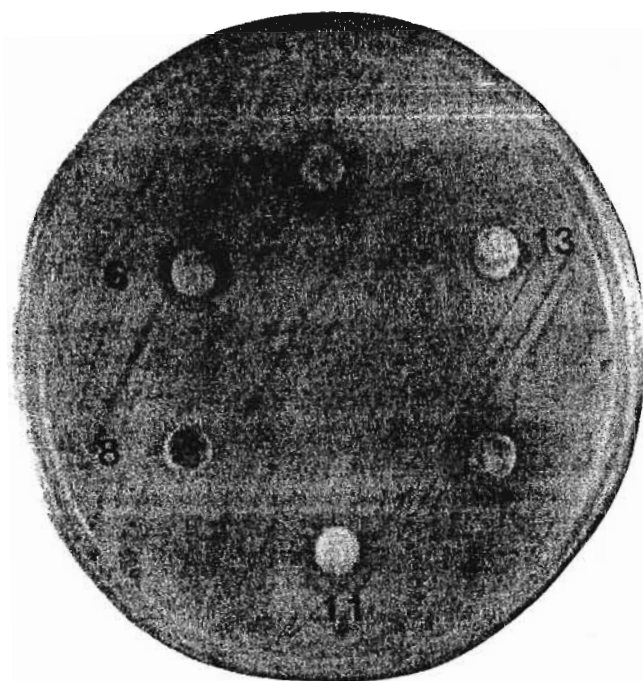


Figure 12: Activity of the crude organic solvent extracts of the roots of the *Bulbine* species against *S. epidermidis*

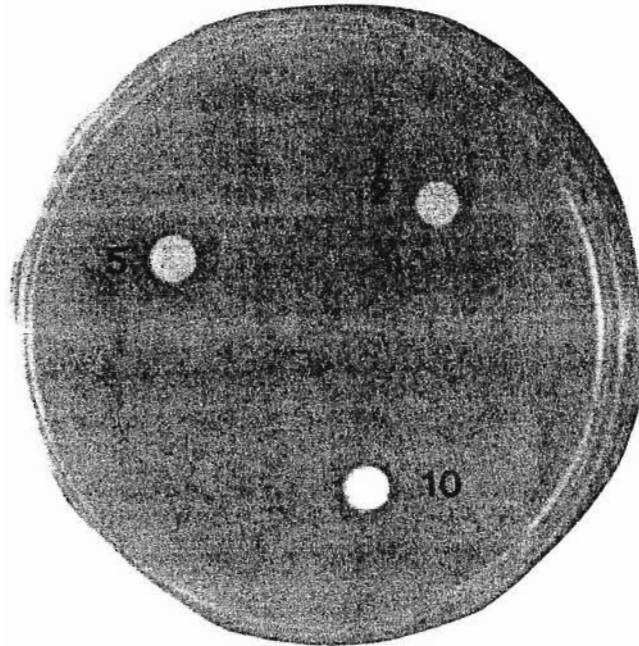


Figure 13: Activity of the crude organic stem solvent extracts of the stems of the *Bulbine* species against *M. morganii*

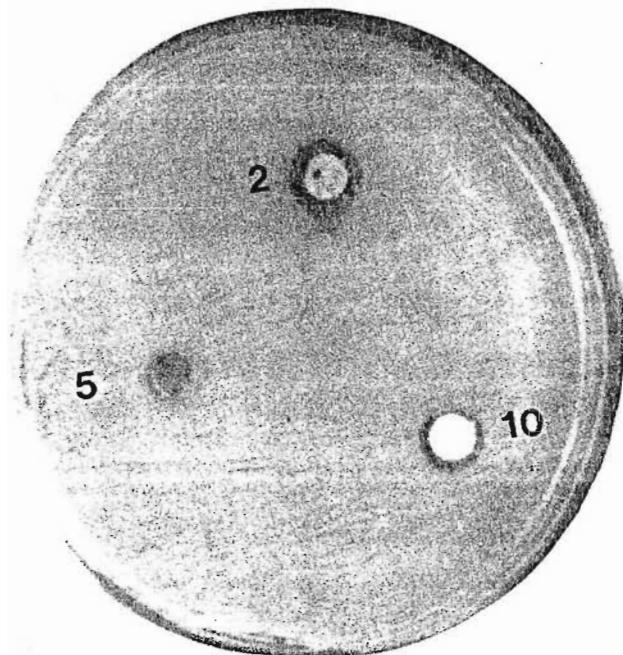


Figure 14: Activity of the crude organic solvent extracts of the stems of the *Bulbine* species against *S. aureus*

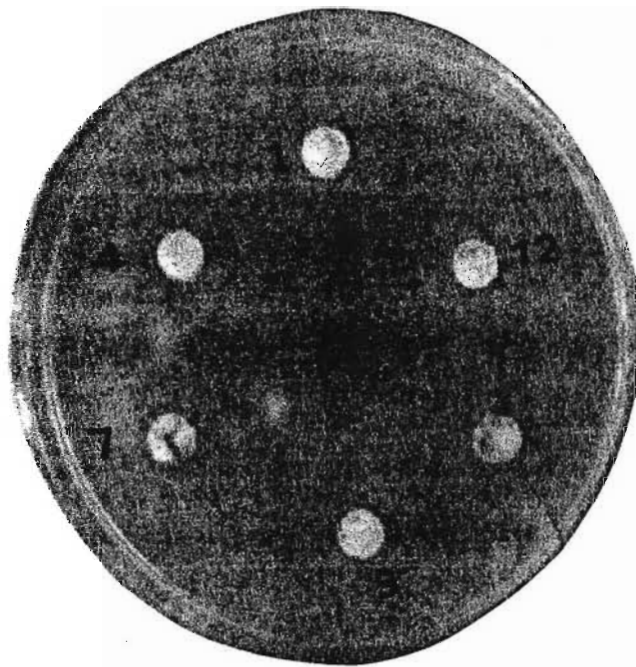


Figure 15: Activity of the crude organic solvent extracts of the leaves of the *Bulbine* species against *S. typhi*

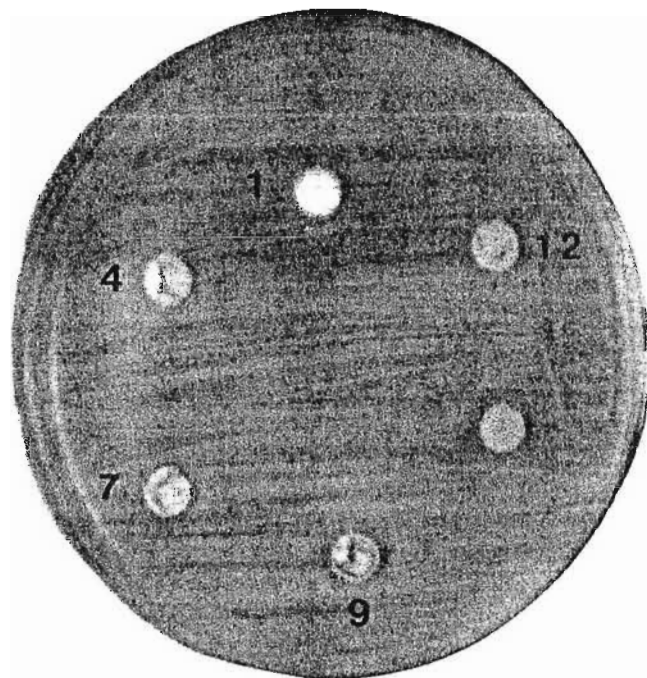


Figure 16: Activity of the crude organic solvent extracts of the leaves of the *Bulbine* species extracts against *S. epidermidis*

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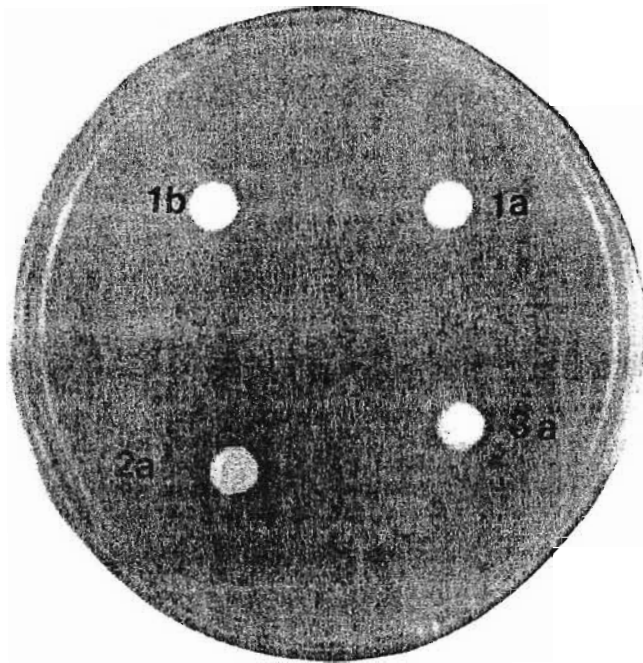


Figure 17: Activity of the crude aqueous extracts of the roots, stems and leaves of *B. natalensis* against *M. morgani*

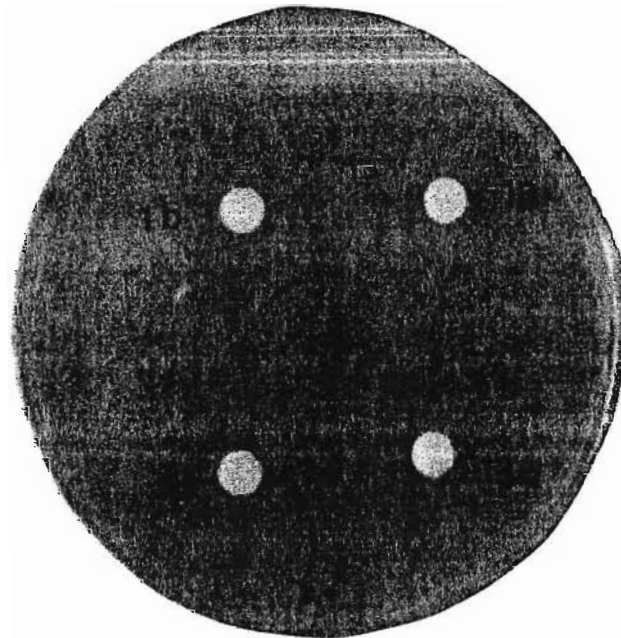


Figure 18: Activity of the crude aqueous extracts of the roots, stems and leaves of *B. natalensis* against *K. pneumoniae*

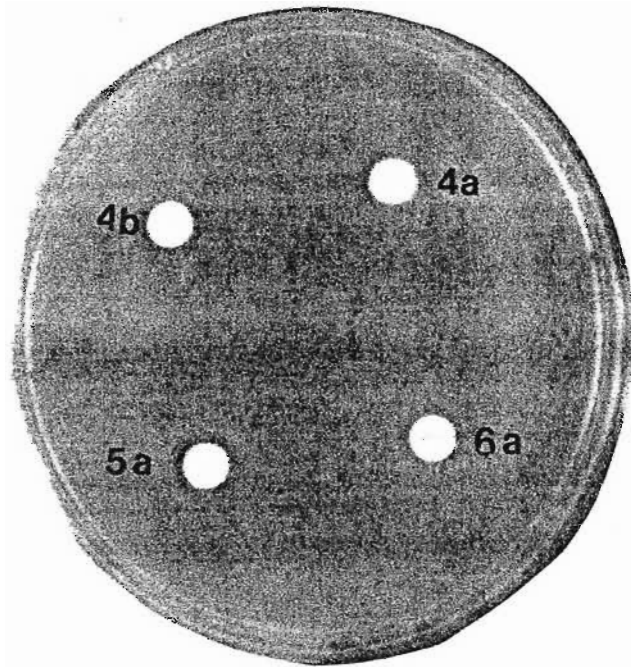


Figure 19: Activity of the crude aqueous extracts of the roots, stems and leaves of *B. frutescens* (yellow flowers) against *E. coli*

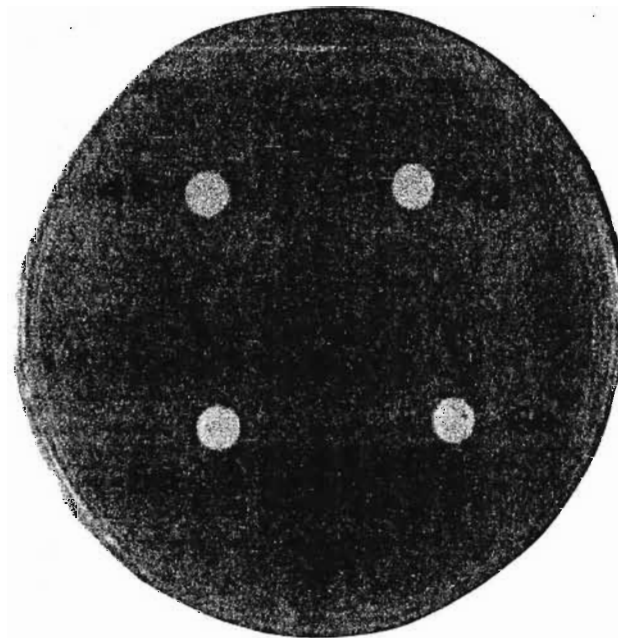


Figure 20: Activity of the crude aqueous extracts of the roots, stems and leaves of *B. frutescens* (yellow flowers) against *K. pneumoniae*

◆ Bore Well Method

Tables 10 and 11 indicate the antibacterial activity of the crude organic and aqueous extracts of the leaves, stems and roots of *B. natalensis*, *B. frutescens* (yellow flowers), *B. narcissifolia*, *B. abyssinica* and *B. frutescens* (orange flowers) against a range of pathogens. Figures 21-27 illustrate the antibacterial activity of some of the crude organic and aqueous extracts of the roots, stems and leaves of the *Bulbine* species against *P. mirabilis*, *S. aureus* and *S. epidermidis*.

Table 10: Zones of inhibition (in mm) achieved with the bore well method using the crude organic and aqueous extracts of the roots, stems and leaves of *B. natalensis* and *B. frutescens* (yellow flowers)

BACTERIUM	1	1a	1b	2	2a	3	3a	4	4a	4b	5	5a	6	6a
<i>B. subtilis</i>	-	-	-	10,0	-	-	-	-	-	-	-	-	-	-
<i>C. freundii</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. aerogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. faecalis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. oxytoca</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>M. luteus</i>	-	-	-	11,5	-	8,5	-	-	-	-	-	-	-	-
<i>M. morgani</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. mirabilis</i>	-	-	-	8,0	-	10,0	-	8,0	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. aureus</i>	-	-	-	11,5	9,5	9,0	-	10,5	-	-	-	-	-	-
<i>S. epidermidis</i>	-	-	-	12,5	-	-	-	-	-	-	-	-	-	-
<i>S. typhi</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. flexneri</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-

KEY:

1. *B. natalensis*-organic solvent extr. of leaf

1a. *B. natalensis*-aqueous extr. of leaf (cold water)

1b. *B. natalensis*-aqueous extr. of leaf (hot water)

2. *B. natalensis*-organic solvent extr. of stem

2a. *B. natalensis*-aqueous extr. of stem

3. *B. natalensis*-organic solvent extr. of root

3a. *B. natalensis*-aqueous extr. of root

4. *B. frutescens*-organic extr. of leaf

4a. *B. frutescens*-aqueous extr. of leaf (cold water)

4b. *B. frutescens*-aqueous extr. of leaf (hot water)

5. *B. frutescens*-organic solvent extr. of stem

5a. *B. frutescens*-aqueous extr. of stem

6. *B. frutescens*-organic solvent extr. of root

6a. *B. frutescens*-aqueous extr. of root

(-) - no inhibition of bacterial growth

Table 11: Zones of inhibition (in mm) achieved with the Bore Well Method using the crude organic extracts of the roots, stems and leaves of *B. narcissifolia* and the crude organic extracts of the roots and leaves of *B. abyssinica*, and *B. frutescens* (orange flowers)

BACTERIUM	7	8	9	10	11	12	13
<i>B. subtilis</i>	-	-	13,0	-	-	-	-
<i>C. freundii</i>	-	-	-	-	-	-	-
<i>E. coli</i>	-	-	-	-	-	-	-
<i>E. aerogenes</i>	-	-	-	-	-	-	-
<i>E. faecalis</i>	-	-	-	-	-	-	-
<i>K. oxytoca</i>	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	-	-	-	-
<i>M. luteus</i>	-	-	-	-	-	-	-
<i>M. morgani</i>	-	-	-	-	-	-	-
<i>P. mirabilis</i>	-	-	9,0	9,0	8,0	-	8,0
<i>P. aeruginosa</i>	10,0	-	10,0	-	-	-	-
<i>S. aureus</i>	-	-	8,0	-	8,5	-	-
<i>S. epidermidis</i>	-	-	-	-	6,5	-	-
<i>S. typhi</i>	-	-	-	-	-	-	-
<i>S. flexneri</i>	-	-	-	-	-	-	-

KEY:

7. *B. abyssinica*-organic solvent extr. of leaf
 8. *B. abyssinica* -organic solvent extr. of root
 9. *B. narcissifolia*-organic solvent extr. of leaf
 10. *B. narcissifolia*-organic solvent extr. of stem
 11. *B. narcissifolia*-organic solvent extr. of root
 12. *B. frutescens* (orange flowers)-organic solvent extr. of leaf
 13. *B. frutescens* (orange flowers)-organic solvent extr. of root
- (-) - no inhibition of bacterial growth

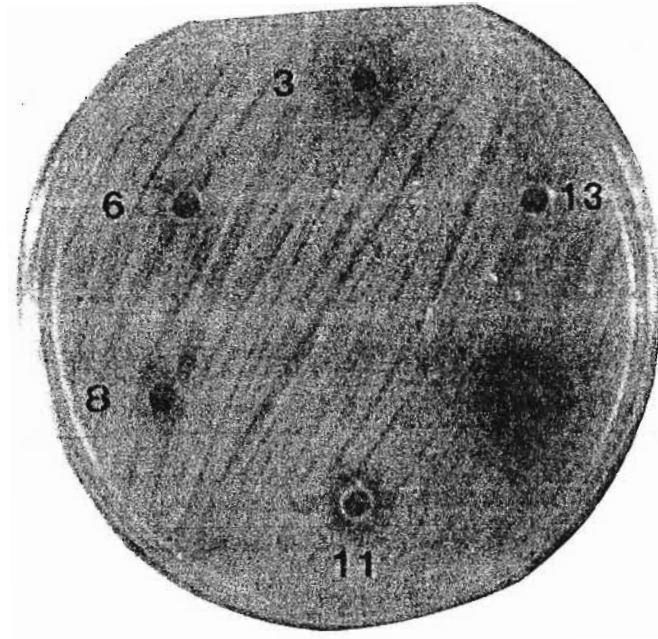


Figure 21: Activity of the organic solvent extract of the roots of the *Bulbine* species against *S. epidermidis*

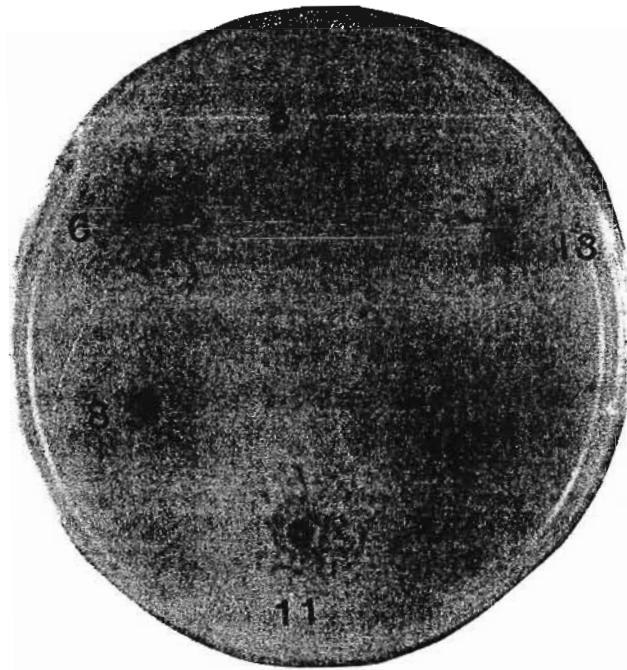


Figure 22: Activity of the organic solvent extract of the roots of the *Bulbine* species against *P. mirabilis*

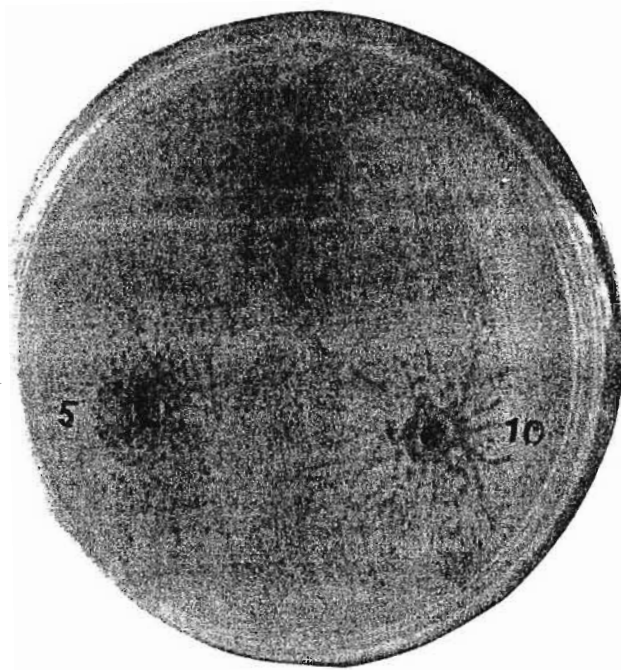


Figure 23: Activity of the organic solvent extract of the stems of the *Bulbine* species against *P. mirabilis*

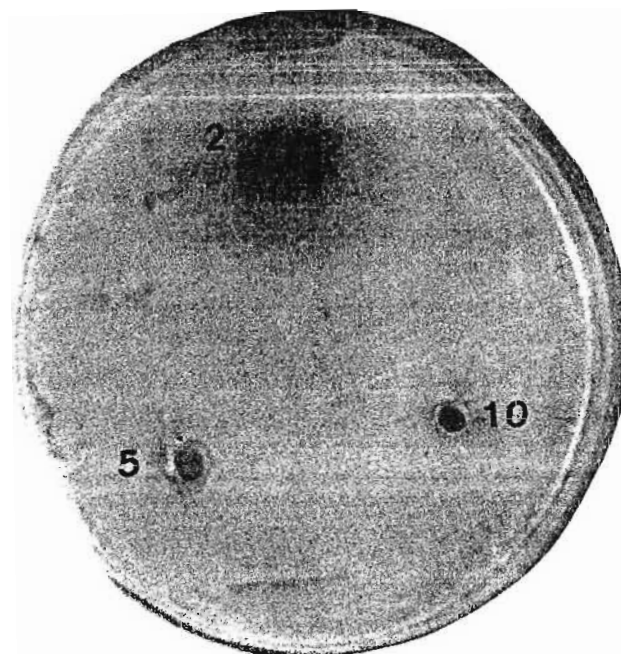


Figure 24: Activity of the organic solvent extract of the stems of the *Bulbine* species against *S. aureus*

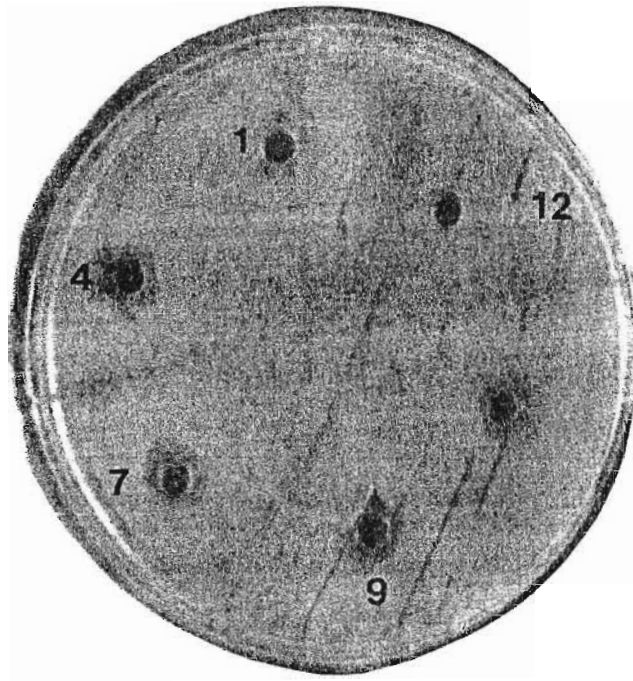


Figure 25: Activity of the organic solvent extract of the leaves of the *Bulbine* species against *S. aureus*

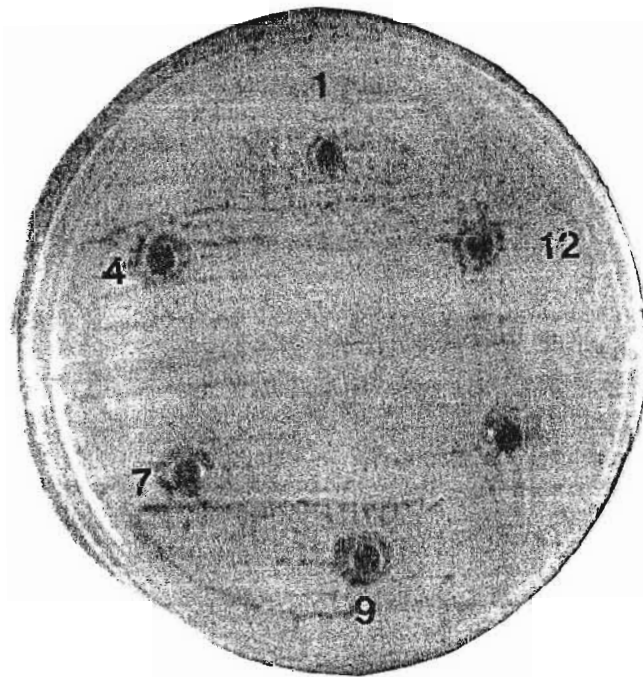


Figure 26: Activity of the organic solvent extract of the leaves of the *Bulbine* species against *P. mirabilis*

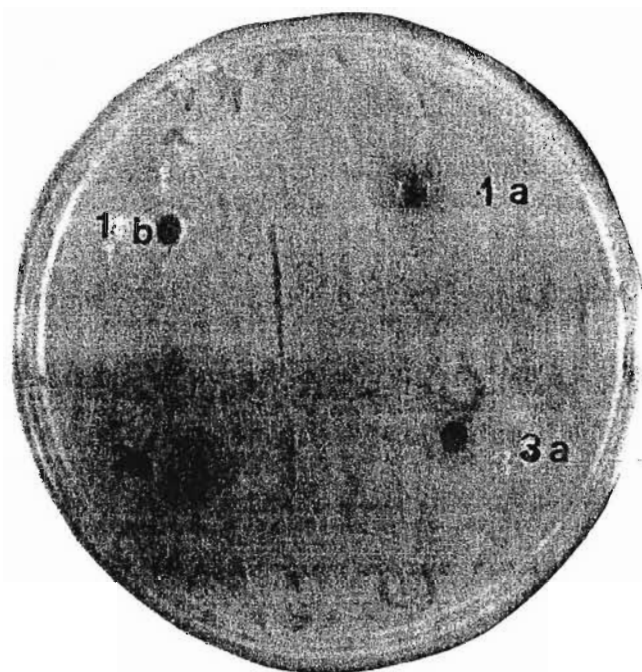


Figure 27: Activity of the aqueous extract of the leaves, stems and roots of *B. natalensis* against *S. aureus*.

3.2.2. Discussion

The concentrations used to ascertain the antibacterial activity of the different bacteria was prepared as per section 2.2.4 i.e., a solution containing 0,75mg of crude extract was inoculated onto each the disk whilst a solution containing 1,25mg of crude extract was placed in each well. Other concentrations were also used but this concentration was found to be the most effective dose. The other dosages that were used are as follows:

Crude organic (100mg) was dissolved in water (2ml) to give a concentration of 50mg/ml. Each crude organic solution (20 μ l) was inoculated on each disk. Therefore the amount of crude extract inoculated onto each disk was 1,0mg. Also, the crude organic extracts were prepared as per section 2.2.4 and crude extract solution (20 μ l) was inoculated onto each disk instead of 30 μ l. This implied that the amount of crude organic extract per disk was 0,5mg. (Refer to Appendix Two for results as per Tables 14 and 15).

It is evident from the results that the Disk Diffusion Method was more easily interpretable than the Bore Well Method (Tables 9-12). The only instance where the bore well method was more effective than the disk diffusion method was for the bacterium *P. mirabilis* (Tables 10 and 11 & Figures 22, 23 and 26). In the disk diffusion method the bacteria were inoculated on the surface of the agar to achieve a lawn growth. The disks, impregnated with the crude extracts, were placed on the agar surface and could therefore interact directly with the bacteria. In the bore well method, the extracts were placed into the wells. It was more difficult for the extracts to diffuse within the agar than onto the agar surface. Also, a larger volume needed to be placed in the wells to interact with the bacteria (50µl with the bore well method as opposed to 30µl with the disk diffusion method). Problems encountered in the bore well method may be overcome by incorporating the bacterial suspension with the melted agar and then pouring it into sterile petri plates and allowing them to solidify. The problem with this technique was that the agar had to be at the correct temperature when the bacterial suspension was added. If the agar was too hot the bacteria died and if it was too cold, the agar solidified. Also, to be homogenous, the bacterial suspension and the agar had to be mixed thoroughly to achieve uniform growth. The inoculation of the agar surface with the bacterial suspension was a practical and easier way of inoculation. Further, all the cultures used in this study were either aerobic or facultative anaerobic bacteria and thus more amenable to the disk diffusion method.

Crude organic solvent extracts for the disk diffusion and bore well methods were reconstituted in distilled water. All residual methanol and dichloromethane was removed during the evaporation process. Distilled water was chosen as the diluent because it would not interfere with the antimicrobial properties of the extracts. Other commonly used diluents such as the alcohols⁸⁵ and dimethyl sulphoxide (DMSO) have been shown to inhibit bacterial growth. Alcohol can also interfere with the integrity of the outer membrane of Gram negative bacteria¹¹ and thus make them more susceptible. This may have yielded false positive results.

According to Salie *et al.*⁹² and Battinelli⁹³ the crude extracts were first filter sterilized with a 0,45 micrometre (µm) membrane filter. In these experiments the crude extracts

were not filtered as it was thought that larger molecules (which could be antimicrobial) may be retained by the filter yielding false negative results. In other articles by Osawa *et al.*⁹⁴ and Tomas-Barberan *et al.*⁹⁵ no mention was made of filter sterilizing the crude extracts.

The antibacterial activity of the *Bulbine* species were evident from the results. With the crude organic solvent extracts, the leaf extracts of *B. natalensis* had less activity than the roots and stems extracts (Table 8). This was also the case for *B. frutescens* (yellow flowers) and *B. frutescens* (orange flowers) where the underground parts displayed more antibacterial activity than the aerial parts (Tables 8 and 9). However, with *B. abyssinica*, the leaf extracts showed more activity than the roots extracts (Table 9). With *B. narcissifolia*, the three components showed similar amounts of activity (Table 9). *B. frutescens* (yellow flowers) and *B. frutescens* (orange flowers) looked identical with the exception that their flowers were a different colour. However, their antimicrobial activity, whilst similar for the organic solvent extracts of the roots, were different for the leaf extracts (Tables 8 and 9). *B. abyssinica* looked similar to *B. frutescens* in terms of their leaf appearance. However, the leaf extracts of *B. abyssinica* showed more antibacterial activity than the leaf extracts of either *B. frutescens* varieties (Tables 8 and 9). The root extracts of *B. abyssinica*, though, displayed similar activities to the root extracts of the two *B. frutescens* varieties (Tables 8 and 9). It would be interesting to see the differences, if any, in the chemistry of these from the TLC. From Tables 8 and 9, the most effective crude extract was the organic solvent extract of the roots of *B. natalensis* which displayed activity against many of the pathogens used in the study.

With the aqueous extracts, it was interesting to note that the aqueous extract of the leaf of *B. natalensis* had more activity than the organic solvent extracts of the leaf (Table 8). This plant had thick fleshy leaves and it seemed that important antibacterial compounds were found in the aqueous component of the leaves. The hot water extract of the leaves also displayed antibacterial activity. This implied that the active component/s were not destroyed by heating (Table 8). In fact, heating seemed to enhance the activity of the leaf extracts. The aqueous extracts of the stems had less activity than the organic solvent extracts of the stem (Table 8). The aqueous extract of the stem had activity against *S. flexneri*. The aqueous extract of the root displayed antibacterial activity against *C.*

freundii and *K. pneumoniae*. The organic solvent extract of the root did not display any antibacterial activity against these two bacteria (Table 8).

The aqueous extract of the leaves of *B. frutescens* showed greater activity than the corresponding organic extract (Table 8). Whilst the hot and cold water extracts showed similar activity against *C. freundii*, *E. coli* and *S. aureus*, the cold water extracts showed more activity against *S. epidermidis* and *S. typhi*. It is interesting to note the activity of the aqueous extracts of the leaves against *S. typhi*, a notorious pathogen. The aqueous and organic extracts of the stems of *B. frutescens* showed similar activities whilst the organic extract of the roots showed more activity than the aqueous extract.

With the organic extracts, the amount of extract used in the diluent was known and therefore the concentration of the crude extracts was known. With the aqueous extract, a leaf for example, was placed in a volume of water and allowed to stand. The resultant extract was not concentrated but used as a dilute solution. More work needs to be done with known concentrations of crude aqueous extracts to ascertain the activity of these extracts against the bacteria.

Bacteria are classified as either Gram positive or Gram negative on the basis of the structure of their cell walls (Refer to Table 2 and Figure 2). The Gram negative bacteria have an extra layer called the outer membrane. This makes Gram negative bacteria more difficult to kill or inhibit than Gram positive ones. Therefore, treating infections by Gram negative bacteria requires different drugs and more aggressive therapy than treating Gram positive bacteria¹¹. The outer membrane can also render Gram negative bacteria resistant to antibiotics²⁵. Nevertheless, from Tables 8 to 11 and Figures 11, 13, 15, 17, 18, 19, 20, 22, 23, 25 and 26 it was evident that a number of Gram negative bacteria were susceptible to a number of the extracts. The most susceptible bacteria were *K. pneumoniae*, *M. morgani*, *P. mirabilis*, *S. typhi* and *S. flexneri* (organic extracts) and *C. freundii*, *K. pneumoniae* and *E. coli* (aqueous extracts).

A wide variety of the crude extracts inhibited the growth of *S. aureus* (Tables 8-11 and Figures 14, 24, 25 and 27). *S. aureus* is considered to be the most resistant of all non

spore forming pathogens²⁰ with well developed capabilities to withstand high salt concentrations, extremes in pH and high temperatures. It also resists the effects of many disinfectants and antibiotics¹¹. Most strains of *S. aureus* have acquired genes for penicillinase which makes them resistant to penicillin and ampicillin. Various other strains demonstrate tolerance to erythromycin, tetracyclines, chloramphenicol, aminoglycosides, cephalosporins and even methicillin and oxacillin that are usually unaffected by penicillinases. Furthermore, *S. aureus* has shown to have multiple resistance to antibiotics especially penicillin, ampicillin and methicillin¹¹.

Tables 8-11 and Figures 12, 16 and 21 showed that a number of the extracts inhibited the growth of *S. epidermidis*. This bacterium is often more resistant to drugs than *S. aureus*²³ and multiresistant strains are becoming increasingly prevalent²².

The organic solvent extracts of the roots, stems and leaves of *B. narcissifolia* and the organic solvent extracts of the stems of *B. natalensis* appeared to inhibit the growth of the sporing bacterium, *B. subtilis* (Table 9, 10 and 11). An old culture known to have endospores was used. Endospores can withstand hostile conditions to facilitate survival. They are capable of withstanding extremes in heat, drying, freezing, radiation and chemicals¹¹.

Bacteria which produce capsules are generally more resistant than non capsule forming bacteria¹¹. *Klebsiella* species generally produce capsules²⁰. They are generally more resistant to a wider range of antibiotics than most *E. coli* strains. However, in this study *K. pneumoniae* was susceptible to nine of the crude organic extracts and *K. oxytoca* was susceptible to just one of the extracts (Tables 8 and 9). With the crude aqueous extracts, *K. pneumoniae* was inhibited by six of the extracts (Table 8 and Figures 18 and 20). This illustrated the effectiveness of the *Bulbine* species against an encapsulated bacterium.

P. aeruginosa is extremely resistant to antimicrobial agents. Its outer membrane is one hundred times less permeable than that of *E. coli*²³. *P. aeruginosa* is resistant to soaps, dyes, quaternary ammonium disinfectants, drugs, drying and temperature extremes. It is

a chronic nosocomial pathogen that is very difficult to control¹¹. Generally *Pseudomonas* infections cannot be treated with a single drug therapy because the success rate is low with such therapy and because the bacteria can rapidly develop resistance when single drugs are used²³. Also, *P. aeruginosa* is normally intrinsically resistant to most commonly employed antimicrobial agents. It is also resistant to and may multiply in many disinfectants and antiseptics commonly used in hospitals²². From Tables 8, 9 and 11 it can be seen that *P. aeruginosa* was susceptible to many of the crude organic extracts. It is interesting to note that a bacterium as resistant as *P. aeruginosa* was actually susceptible to such small doses of crude extract.

From Tables 8 and 9 and Figures 11, 13, 15, 17, 18, 19, 20, 22, 23 and 26 it was evident that the crude organic extracts had been successful against the Enterobacteriaceae including *S. typhi*, *S. flexneri* (both reported to be problematic multiresistant bacteria¹¹), *M. morgani* and *K. pneumoniae* (resistant because of the presence of capsules) and successful to a limited extent against *E. coli*, *E. aerogenes*, *K. oxytoca* and *C. freundii*. However, from Table 9 it was evident that the aqueous extracts were more successful against *C. freundii* and *E. coli* than the organic extracts. From Tables 10 and 11 and Figures 22, 23 and 26 it was evident that the organic extracts inhibited *P. mirabilis*. Tables 9 and 10 illustrated the susceptibility of *S. typhi* to many of the extracts. *S. typhi* is the causative agent of typhoid fever, a serious problem killing approximately 25 000 people annually.

In conclusion, it can be seen from the results that many of the crude organic and aqueous extracts of the different *Bulbine* species are indeed antibacterial.

3.3. Antifungal Activity

3.3.1. Results

◆ *Penicillium* and *Aspergillus*

The *Bulbine* species used in this study did not display any antifungal activity against *Penicillium*. Figures 28-30 illustrate the antifungal activity of the root, leaf and stem extracts of *B. natalensis*, *B. frutescens* (yellow flowers), *B. frutescens* (orange flowers), *B. abyssinica* and *B. narcissifolia* against *Aspergillus*. The zones of inhibition are indicated by the clear zones



Figure 28: Antifungal activity of the root extracts of the *Bulbine* species against *Aspergillus*.



Figure 29: Antifungal activity of the stem extracts of the *Bulbine* species against *Aspergillus*

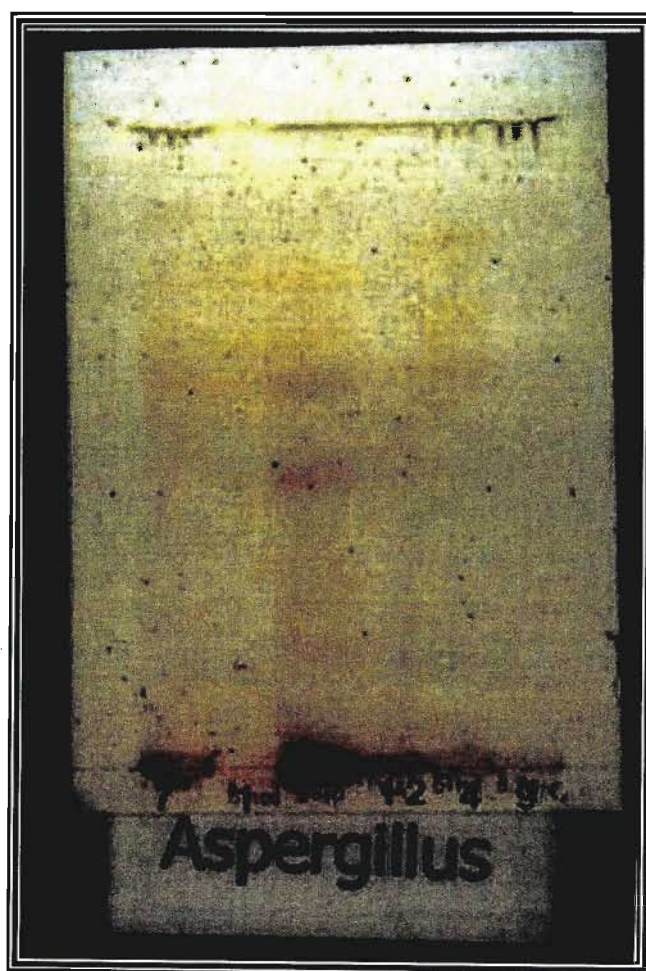


Figure 30: Antifungal activity of the leaf extracts of the *Bulbine* species against *Aspergillus*

♦ *C. albicans***Disk Diffusion Method:**

The organic solvent extracts of the roots of *B. frutescens* (yellow and orange flowers) were the only two extracts that inhibited the growth of *C. albicans*. The zones of inhibition were each 10,5mm in diameter.

Bore Well Method:

None of the extracts inhibited the growth of *C. albicans* and therefore no zones of inhibition were seen.

Figure 31 illustrates the activity of the root extracts of the two *B. frutescens* varieties against *C. albicans*. Figure 32 illustrates the inactivity of the *Bulbine* stem extracts against *C. albicans*.

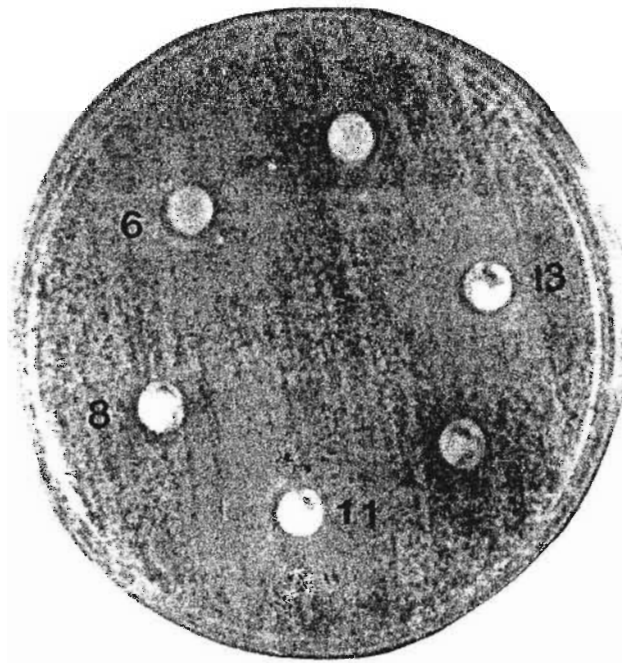


Figure 31: Antifungal activity of the root extracts of the *Bulbine* species against *C. albicans*

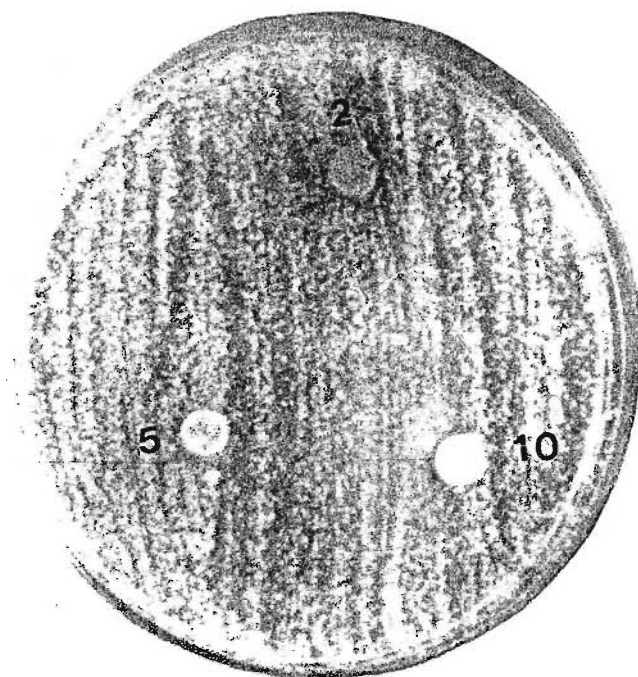


Figure 32: The inactivity of the stem extracts of the *Bulbine* species against *C. albicans*

3.3.2. Discussion

The organic solvent extracts of the roots of both the orange and yellow flowered *B. frutescens* species inhibited the growth of *C. albicans*. These two plants displayed similar activities with both extracts displaying zones of 10,5mm each (Figure 31). *C. albicans* is an oval budding yeast that produces a pseudomycelium both in culture and in tissues and exudates. It is a member of the normal flora of the mucous membranes of the respiratory, gastrointestinal and female genital tracts²³. Superficial *Candida* infections are common throughout the world²². Fungal overgrowth of the vaginal tract by *Candida* sometimes occurs as a result of population shifts among the microorganisms that compose the normal vaginal tract microbiota. This excessive growth of *Candida* causes vaginitis²⁵. Women taking antibiotics sometimes develop vaginitis due to an overgrowth of *Candida* which is normally held in check by the indigenous bacteria of the vaginal tract. Excessive douching or deodorants can also

cause *Candida* to flourish leading to vaginitis²⁰. Systemic forms of candidiasis may be localized e.g., in the urinary tract, heart valves, meninges or the peritoneal cavity or the infections may be widely disseminated and associated with septicaemia²². It is interesting to note that two of the crude organic extracts had activity against *C. albicans*.

With the TLC bioassay, one of the components of the root extract of *B. narcissifolia* seemed to be antifungal against *Aspergillus*. The clear zone is indicated by a black circle (Figure 28). Some of the components of the stem extract of *B. frutescens* (yellow flowers) and *B. narcissifolia* seemed to be antifungal against *Aspergillus* (Figure 29). *Aspergillus* is a fungus with septate hyphae and distinctive sporing structures. The spore bearing hypha (conidiophore) terminates in a swollen cell (vesicle) which is surrounded by 1 or 2 rows of cells (sterigmata) from which chains of asexual conidia are produced. *Aspergillus* grows in nature and in culture as mycelial fungi²². Immunosuppressed hosts or individuals exposed to high numbers of *Aspergillus* spores may develop a respiratory disease called aspergillosis²⁰. *Aspergillus* is commonly found growing in decaying vegetable and compost heaps and is a common source of infection for farmers and gardeners. In some cases an allergic response to the inhalation of *Aspergillus* spores may occur with symptoms of asthma that include difficulty in breathing. Within the body *Aspergillus* may form a hyphal mass called an aspergilloma. Invasive infections of pulmonary aspergillosis may be fatal²⁴. With the potential antifungal action of some of the crude extracts of the *Bulbine* species against the spores of *Aspergillus*, other studies need to be carried out using methods such as the disk diffusion and bore well techniques to verify the antifungal activity.

None of the components of the leaf, stem and root extracts of the *Bulbine* species inhibited the growth of *Penicillium*. *Penicillium* is a saprophytic fungus that usually grows on rotten vegetables, fruits, meats and other moist and dead organic substrates⁹⁶. *Penicillium* strains do not cause diseases and are used for the mass production of the antibiotic penicillin^{20,25}.

From the results, it was evident that the root and stem extracts displayed some antifungal activities against *C. albicans* and *Aspergillus* whilst the leaf extracts did not

display any. It is recommended that an aqueous extract of the leaves, stems and roots be tested against these fungi using the TLC bioassay.

Results obtained with the TLC bioassay need to be verified using other methods of antifungal testing. The fungal spores were added to the solvent and mixed well to produce a homogenous solution. This solution was then sprayed onto the TLC plates which were then incubated. If the fungal spores and the solvent are not mixed properly, the spore solution that was sprayed would have clumps of spores and therefore the results may be false positive or misleading. If the plates were not sprayed evenly, the growth of the fungus would be patchy, thereby giving false positive results. Some fungi, e.g., *Penicillium*, grow in clumps on the TLC plates and therefore the results are difficult to interpret. When the plates are incubated in a moist chamber, moisture condenses on the lid of the chamber. If this moisture falls onto the TLC plates, growth can be retarded/hindered and this could give false results. To overcome this problem, the lid of the incubation chamber was lined with filter paper to absorb the excess moisture and it was changed regularly as the TLC plates had to be incubated for fourteen days.

It is recommended that a standard antifungal agent which is known to be active against the fungi used in the study should be included in the TLC bioassay to preclude any false negative results.

3.4. Cytotoxic Activity

3.4.1. Results

Table 12 illustrates the number of surviving brine shrimps at each of the different dilutions of the crude organic extracts.

Table 12: Number of surviving brine shrimps

CRUDE EXTRACT	DILUTION 1	DILUTION 2	DILUTION 3
<i>B. natalensis</i> (leaves)	5	10	10
<i>B. natalensis</i> (stems)	7	10	10
<i>B. natalensis</i> (roots)	3	9	10
<i>B. frutescens</i> -yellow flowers (leaves)	1	10	10
<i>B. frutescens</i> -yellow flowers (stems)	1	10	10
<i>B. frutescens</i> -yellow flowers (roots)	10	10	10
<i>B. narcissifolia</i> (leaves)	5	10	10
<i>B. narcissifolia</i> (stems)	6	10	10
<i>B. narcissifolia</i> (roots)	10	10	10
<i>B. abyssinica</i> (leaves)	10	10	10
<i>B. abyssinica</i> (roots)	4	8	10
<i>B. frutescens</i> -orange flowers (leaves)	3	10	10
<i>B. frutescens</i> -orange flowers (roots)	0	10	10

Control: Number of survivors = 10

3.4.2. Discussion

In this experiment a slight modification of the Meyer *et al*¹⁷. method was made. Instead of using artificial sea-water, real sea water was used. It is difficult to obtain the correct salinity of sea-water and the brine shrimps need to thrive under ideal conditions. No food supplement had to be added because the sea-water contained all the nutrients that the brine shrimps needed. According to Meyer *et al.*, the brine shrimps took up to 48

hours to hatch. In these experiments, the brine shrimps hatched after 24 hours. The tank used to hatch the brine shrimps that was mentioned in the article by Meyer *et al.* was very elaborate. In these experiments only a beaker which was covered with aluminium foil was used. In this way this already very simple and convenient method was simplified even further.

With the exception of the root extract of *B. frutescens* (yellow flowers) and *B. narcissifolia* and the leaf extract of *B. abyssinica*, all the other extracts showed some degree of cytotoxicity at dilution 1 which had a concentration of 5mg/ml. Most of the crude organic extracts were not cytotoxic at dilution 2 which had a concentration of 0,5mg/ml and none of the crude organic extracts were cytotoxic at dilution 3 which had a concentration of 0,05mg/ml.

Further cytotoxicity tests were carried out with the 3 crude organic extracts which were not cytotoxic at dilution 1, i.e., the root extracts of *B. frutescens* (yellow flowers) and *B. narcissifolia* and the leaf extract of *B. abyssinica*. The concentration of the extracts used was 25mg/ml. After incubating for 24 hours the number of surviving brine shrimps in each of the tests was 10. This implied that these extracts were not cytotoxic at this relatively high concentration.

3.5. Chemical Analysis

3.5.1. TLC Fingerprinting

3.5.1.1. Results

Figures 33, 34 and 35 represent the TLC fingerprints of the stem, leaf and root extracts of the five different *Bulbine* species used in this study using three different solvent systems.

KEY:

Bn- *Bulbine natalensis*

Bnf- *Bulbine narcissifolia*

Bf- *Bulbine frutescens* (yellow flowers)

Ba- *Bulbine abyssinica*

Bfo- *Bulbine frutescens* (orange flowers)

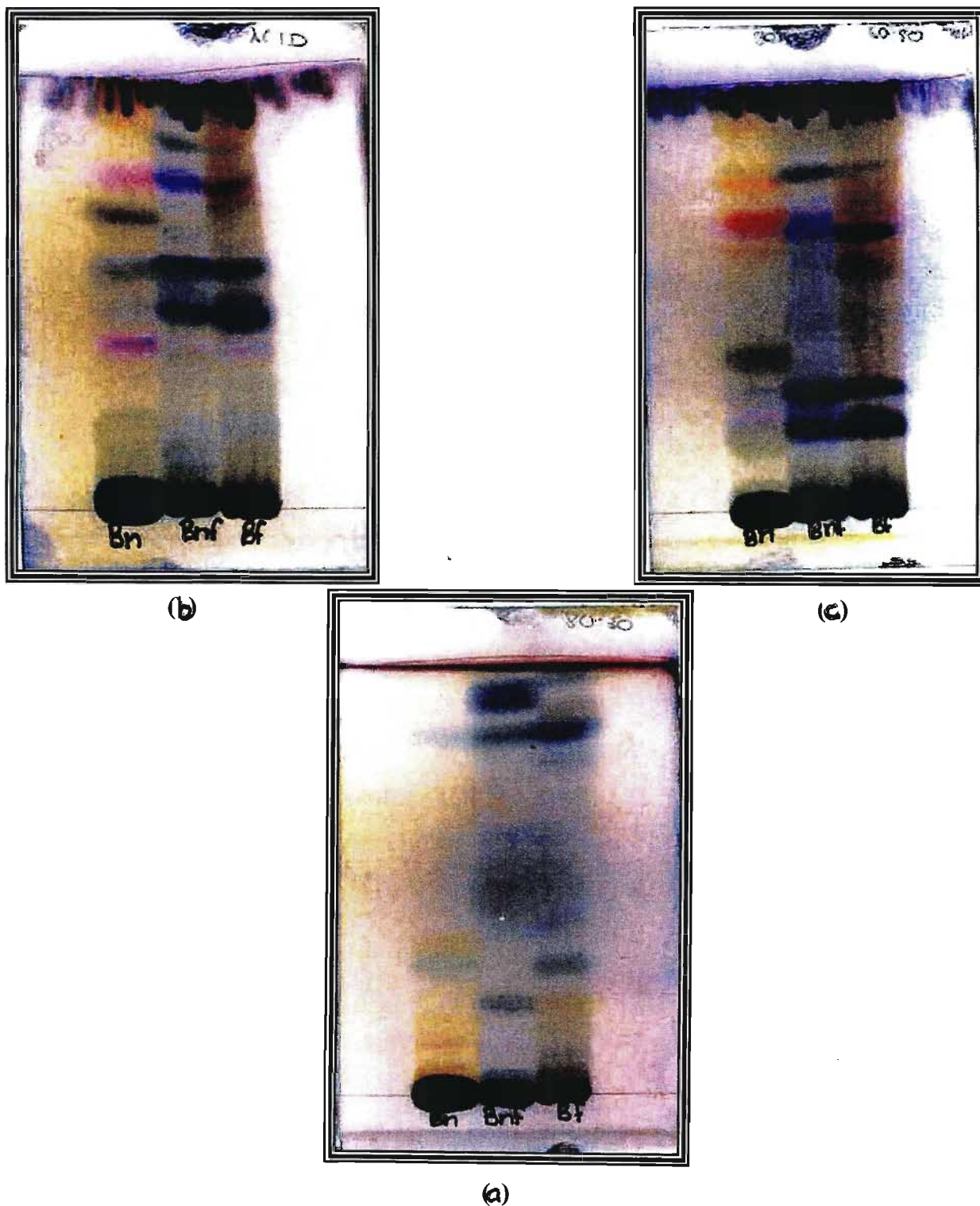


Figure 33: Representative TLC fingerprints of the stem extracts of the different *Bulbine* species using the three different solvent systems.

- a. Solvent system 1
- b. Solvent system 2
- c. Solvent system 3

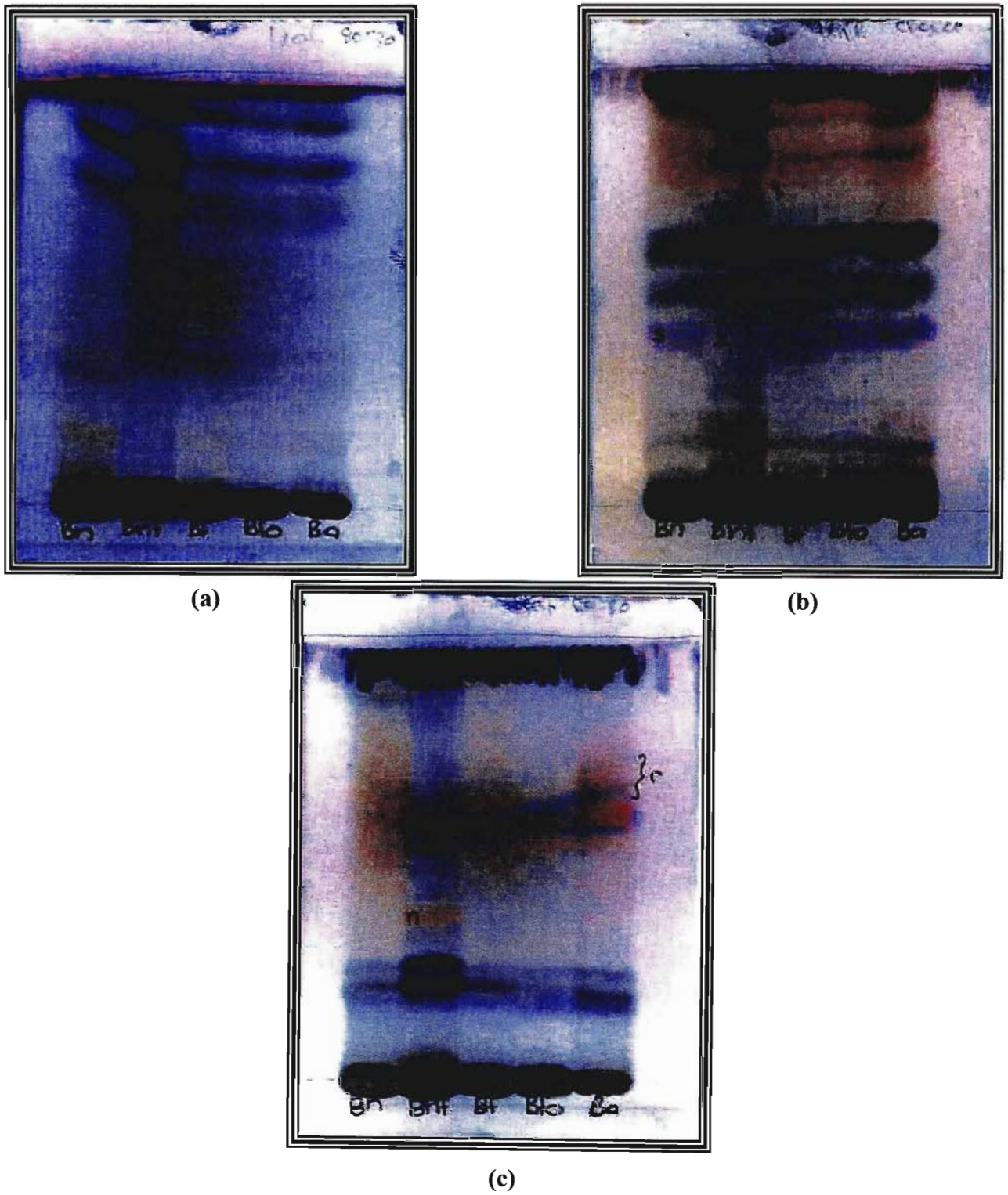


Figure 34: Representative TLC fingerprints of the leaf extracts of the different *Bulbine* species using three different solvent systems.

- a. Solvent system 1
- b. Solvent system 2
- c. Solvent system 3

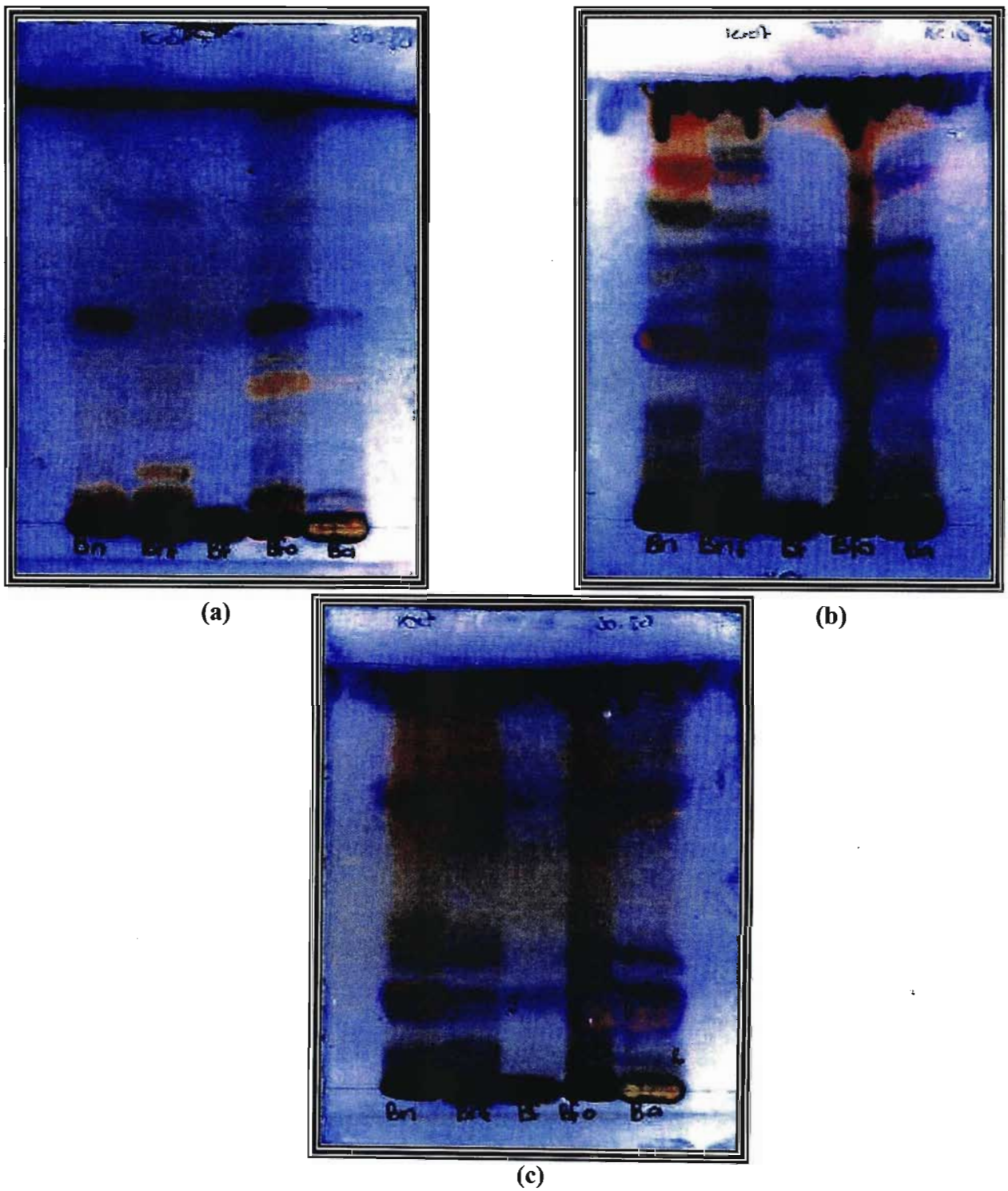


Figure 35: Representative TLC fingerprints of the root extracts of the different *Bulbine* species using three different solvent systems.

- a. Solvent system 1
- b. Solvent system 2
- c. Solvent system 3

3.5.1.2. Discussion

❖ Leaf Fingerprints

The close relationship between the five species was evident from the chromatograms (Figure 34). They all shared many major constituents. From Figure 34(c) it was evident that although *B. natalensis* showed many similarities to the other species, it also had certain differences. Visually, these leaves looked very different from the others. These were broad, fleshy leaves. The leaves of *B. narcissifolia* too, had many differences. They were different in appearance from the other leaves in that they were strap-shaped. The chromatograms of the leaf extracts of the two *B. frutescens* varieties and that of the leaf extract of *B. abyssinica* were very similar. Their leaves were almost identical in appearance and *B. frutescens* and *B. abyssinica* looked very similar. The chromatograms of the leaf extracts (Figure 34c) showed that the leaf TLC fingerprinting could be a useful tool in identifying the different species. The TLC showed that *B. natalensis* and *B. narcissifolia* although showing similarities were not closely related to the other three species. The chromatograms of *B. frutescens* (yellow flowers), *B. frutescens* (orange flowers) and *B. abyssinica* showed that these plants were closely related. The chromatograms using solvent system 2 (Figure 34b) and solvent system 1 (Figure 34a) showed many similarities between the five plants. The chromatograms using solvent system 1 cannot be used to conclusively identify the different species. Solvent system 2 showed many similarities between the plants and from Figure 34b it could be seen that *B. narcissifolia* was distinctive from the others.

❖ Stem Fingerprints

From Figures 33a, 33b and 33c it was evident that the TLC fingerprints of the stem extract of *B. natalensis* was different from that of *B. frutescens* and *B. narcissifolia*. The chromatogram obtained using solvent system 3 (Figure 33c) showed that the stem fingerprints had distinctly different components from the other two stems. These included the green band indicated by (a) and the orange band indicated by (b). The stem fingerprints of *B. narcissifolia* and *B. frutescens* showed many similar components including the blue bands indicated by (c) and the pink bands indicated by (d). This pink band was also evident in the *B. natalensis* stem extract chromatogram. The stem extract

of *B. natalensis* and *B. frutescens* also have two similar yellow bands indicated by (e) and (f). From these chromatograms it was evident that whilst all three species were related, they also had distinctive differences and that these stem fingerprints could be an aid in identifying the different species.

❖ Root Fingerprints

The chromatograms obtained using solvent system 3 (Figure 35c) showed that each of the root extracts had a distinctive fingerprint. The root extracts however, shared many components and these included the pink bands indicated by (g) and (h). The root extract fingerprints of *B. frutescens* (orange flowers) and *B. abyssinica* shared many similar components indicated by (i), (j), (k) and (l). These two species were very similar in appearance and even their roots looked similar. *B. natalensis* had a distinctive orange band (m) which the other roots did not have. The TLC using solvent system 1 did not show the differences between the species as clearly as the other two solvent systems (Figure 35a). The chromatograms obtained using solvent system 2 (Figure 35b) also highlighted the fact that whilst the root extracts shared many similarities, there were many differences making each root extract fingerprint unique.

Chemical studies on the *Bulbine* species by van Staden and Drewes⁹⁷ showed that a substance called knipholone was isolated from *B. latifolia* and *B. frutescens*. The major constituent isolated from the room temperature ethanol extract of *B. latifolia* was identified as biaryl anthraquinone knipholone 1 by nuclear magnetic resonance (NMR) spectroscopy. This compound was also identified in *B. frutescens*. However, this compound showed no activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*, *M. luteus* and *C. albicans*. The article by van Staden and Drewes did not mention the R_f value of any reference standard and therefore the R_f value obtained by these researchers when running a TLC of knipholone could not be used as a comparison.

Studies by Berzabin, Motlhagodi and Abegaz⁹⁸ indicated that *B. frutescens* and *B. abyssinica* contained knipholone anthrone, islandicin, aloe-emodin and chrysophanol. These studies, however, did not indicate whether the orange or yellow flowered *B.*

frutescens was used and further studies need to be carried out to see if there are any differences between the two varieties. This study seemed to indicate that the two *B. frutescens* varieties are slightly different in terms of their chemical composition. This article did not mention any Rf values and also the use of any reference standards. Also, no antimicrobial assays were conducted using these chemical compounds.

3.5.2. Column Chromatography

3.5.2.1. Results

Table 13 shows the different fractions that were obtained when the eluted samples with similar TLC were pooled. Figures 36 and 37 illustrate the TLC of each of the different fractions, i.e., 1-14. The sample numbers, e.g., 4, 5, 6 represent the order in which the fractions were eluted. In column chromatography the bands (chemical components) that appeared at the top of a TLC were eluted first. In the chromatogram of the stem of *B. natalensis* (Figure 33c) the yellow bands were at the top of the chromatogram. When eluting the samples, these bands (chemical components) were eluted first.

Table 13: Fractions that were obtained by combining the eluted samples with similar TLC.

FRACTION	SAMPLE COMBINATION
1	4,5,6
2	7,8,9,10,11
3	12,13,14,15,16
4	17,18,19
5	20,21,22
6	23,24,25,26,27,28,29,30
7	31,32,33,34,
8	35,36,37,38,39,40,41
9	42,43,44,45,46
10	47,48,49,50,51
11	52,53,54,55,56,57
12	58,59,60,61,62
13	63,64,65,66
14	67,68,69,70

Based on the results obtained with the TLC, fractions 11, 12, 13 and 14 were combined for further evaluation. This fraction was evaluated using nuclear magnetic resonance (NMR) spectroscopy but unfortunately due to solvent interference and time constraints on the NMR spectrometer, the chemical component could not be identified.

CHAPTER FOUR

Conclusions, Limitations and Recommendations

4.1. Conclusions

- ◆ The extraction procedure using the combination of organic solvents (methanol:dichloromethane, 1:1, v/v) was successful. The crude organic extracts displayed activity against a variety of bacteria that are reported to be pathogens. The organic solvent extract of the stem of *B. natalensis* each inhibited the growth of *K. pneumoniae*, *M. luteus*, *M. morgani*, *P. aeruginosa*, *S. aureus*, *S. epidermidis* and *S. flexneri*, the organic solvent extract of the root inhibited the growth of *E. faecalis*, *M. luteus*, *M. morgani*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *S. typhi* and *S. flexneri* whilst the organic solvent extract of the leaf was inhibitory to only *K. pneumoniae*. The organic solvent extract of the leaf of *B. frutescens* (yellow flowers) inhibited the growth of *E. faecalis* and *K. pneumoniae*, the organic solvent extract of the stem inhibited the growth of *E. faecalis*, *K. pneumoniae*, *M. morgani*, *S. aureus*, *S. epidermidis*, *S. typhi* and *S. flexneri* whilst the organic solvent extract of the root was inhibitory to *E. coli*, *E. faecalis*, *K. pneumoniae*, *M. morgani*, *S. epidermidis*, and *S. flexneri*. With *B. abyssinica* the organic solvent extract of the leaf was more bactericidal than the organic solvent extract of the root inhibiting the growth of *E. coli*, *E. aerogenes*, *E. faecalis*, *K. pneumoniae*, *M. morgani*, *S. typhi* and *S. flexneri* whilst the root extract inhibited the growth *K. pneumoniae*, *S. aureus*, *S. typhi* and *S. flexneri*. The organic solvent extract of the leaf of *B. narcissifolia* inhibited the growth of *K. oxytoca*, *K. pneumoniae*, *M. morgani*, *P. aeruginosa*, *S. epidermidis*, *S. typhi* and *S. flexneri*, the organic solvent extract of the stem inhibited the growth of *B. subtilis*, *C. freundii*, *M. luteus* *M. morgani*, *S. aureus* and *S. epidermidis* whilst the organic solvent extract of the root inhibited the growth of *B. subtilis*, *E. aerogenes*, *M. luteus*, *M. morgani* and *S. aureus*. With *B. frutescens* (orange flowers) the organic solvent extract of the root extract was more bactericidal than the leaf extract inhibiting the growth of *E. faecalis*, *K. pneumoniae*, *M. morgani* and *S. flexneri* whilst the organic solvent extract of the leaf inhibited the growth of only *M. morgani*.
- ◆ The disk diffusion technique produced better results than the bore well technique. From Tables 9-12, it was evident that when employing the Disk Diffusion Technique the growth of more bacteria was inhibited than when using the Bore Well

Technique. All the bacteria used in this study were either aerobic or facultative anaerobic bacteria and thus more amenable to the disk diffusion technique.

- ◆ The aqueous extracts displayed a limited amount of antibacterial activity. The aqueous extract of the leaf (cold water extraction) of *B. natalensis* inhibited the growth of *C. freundii* and *K. pneumoniae* as compared with the organic extract which inhibited the growth of only *K. pneumoniae*. The aqueous stem and root extracts of *B. natalensis* were less successful than the organic extract of the stem inhibiting the growth of *K. pneumoniae* and *S. flexneri* (stem extract) and *C. freundii*, *K. pneumoniae* and *M. morgani* (root extract). The aqueous extract of the leaf (cold water extraction) of *B. frutescens* (yellow flowers) was more inhibitory than the organic solvent extract of the leaf inhibiting the growth of *C. freundii*, *E. coli*, *S. aureus*, *S. epidermidis* and *S. typhi* whilst the organic solvent extract of the leaf was inhibitory to *E. faecalis* and *K. pneumoniae*. The aqueous extracts of the stem and root of *B. frutescens* (yellow flowers) were less successful than the organic extracts. The aqueous extract of the stem inhibited the growth of *E. coli*, *K. pneumoniae*, *M. luteus* and *P. mirabilis* whilst the aqueous extract of the root inhibited the growth of only *E. coli*.
- ◆ Some of the components in the aqueous extraction were not heat labile. The cold water extract of the leaf of *B. natalensis* inhibited the growth of *K. pneumoniae* (zone of inhibition = 8,5mm) whilst the hot water extract of the leaf also inhibited the growth of *K. pneumoniae* but with a slightly larger zone, i.e., 10,5mm. The hot water extract of the leaf of *B. frutescens* (yellow flowers) was inhibitory to the growth of *K. pneumoniae* and *M. luteus* whilst the cold water extract was not.
- ◆ A few of the crude organic solvent extracts had limited antifungal activity. None of the extracts inhibited the growth of *Penicillium*. The only extracts that inhibited the growth of *Aspergillus* were the root extract of *B. narcissifolia* and the stem extracts of *B. natalensis* and *B. narcissifolia*. The organic solvent extracts of the root of *B. frutescens* (both varieties) inhibited the growth of *C. albicans* when the Disk Diffusion Technique was employed.
- ◆ Most of the extracts were reported to be cytotoxic at the more concentrated dilutions and non cytotoxic at the lower concentrations. A few of the crude organic extracts were not cytotoxic at the most concentrated dilution. The root extracts of *B.*

frutescens (yellow flowers) and *B. narcissifolia* and the leaf extract of *B. abyssinica* were not cytotoxic at the most concentrated dilution. The root extract of *B. frutescens* (orange flowers) was cytotoxic at this dilution and killed all the brine shrimps.

- ◆ Figures 33-35 showed that the TLC fingerprints from the leaf, stem and root extracts could be used as a valuable chemotaxonomic tool in this genus.
- ◆ The orange and yellow flowered *B. frutescens* varieties displayed varying antibacterial and cytotoxic activities. The organic solvent extract of the leaf of the yellowed flowered variety inhibited the growth of *E. faecalis* and *K. pneumoniae* whilst the leaf extract of the orange flowered variety inhibited the growth of only *M. morganii*. The organic solvent extract of the root of the yellow flowered variety inhibited the growth of *E. faecalis*, *K. pneumoniae*, *M. morganii*, *S. aureus*, *S. epidermidis*, *S. typhi* and *S. flexneri* whilst the organic solvent extract of the root of the orange flowered variety was inhibitory to *E. faecalis*, *K. pneumoniae*, *M. morganii* and *S. flexneri*. The root extract of the yellow flowered variety was not inhibitory to the brine shrimps at dilution 1 whilst the root extract of the orange flowered variety killed all the brine shrimps at that concentration.

4.2. Recommendations and Limitations

4.2.1. Limitations

- ◆ The organic extraction procedure was carried out only for the stems of *B. natalensis*, *B. narcissifolia* and *B. frutescens* (yellow flowers). The other 2 plants were purchased from the nursery and a sufficient quantity of plants was not available to get an adequate yield to perform the antimicrobial tests.
- ◆ The aqueous extraction was not carried out concurrently with the organic extraction. Therefore the aqueous extraction was carried out only with the roots, stems and leaves of *B. natalensis* and *B. frutescens* (yellow flowers). These two plants were easily available as they were growing in the gardens at UDW. *B. narcissifolia* was

obtained from the Bergville area, *B. abyssinica* was purchased from a nursery whilst *B. frutescens* was obtained from Eastern Cape and therefore not available at the time of the aqueous extraction

4.2.2. Recommendations

- ◆ The extraction procedure should be carried out using both an organic solvent and water. The aqueous extraction should be carried out using both hot and cold water treatment to ascertain the differences, if any, in their activities. The aqueous extract will have to be concentrated to achieve a higher concentration of crude extract and the quantity of crude aqueous extract will have to be quantified to evaluate the possible antimicrobial concentrations.
- ◆ The experiments should be carried out using the stem extracts of *B. abyssinica* and *B. frutescens* (orange flowers).
- ◆ The method for the Brine Shrimp Bioassay should be amended. Instead of making dilutions of the crude extracts with sea water, the crude extracts should first be inoculated onto filter paper disks, dried and then added to the sea water. In this way, the liquid won't be dark and turbid and higher concentrations of crude extracts can be tested. It is also recommended that tests be carried out using the aqueous extracts in the same way as the crude organic extracts.
- ◆ The crude organic and aqueous extracts should be tested against the bacteria causing sexually transmitted diseases, i.e., *Neisseria gonorrhoea* and *Treponema pallidum* to ascertain any antibacterial activity. *N. gonorrhoea* is an anaerobe and needs specialized incubation conditions which were not available to the researcher. *T. pallidum* cannot be cultured in the laboratory on culture media. It has to be grown on the scrotum of a rabbit and these facilities were also not available to the researcher.
- ◆ The stem and root chromatograms had many common components. Therefore antimicrobial activity studies should be carried out with the different fractions of the roots and stems to ascertain similarities, if any, in their activities. It is also recommended that all the other crude organic extracts be fractionated and antimicrobial studies be carried out.

- ◆ Further chemical analysis of this family is needed. It is also recommended that further studies be carried out on the relationship between other members within the family Asphodelaceae to ascertain similarities and differences within the family.
- ◆ Further studies, preferably doctoral studies, should be carried out using the results already obtained to further elucidate the antimicrobial activity, cytotoxicity and chemical composition of this genus.

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APPENDICES

APPENDIX ONE

MATERIALS FOR ANTIMICROBIAL TESTING

1.1. Nutrient Agar (Oxoid)

Lab Lemco Powder	1,0g
Bacteriological Peptone	3,0g
Yeast Extract	2,0g
NaCl	5,0g
Agar No. 3	15,0g
Distilled Water	to 1000ml

Suspend 28g in 1000ml distilled water. Boil until dissolved. Autoclave at 121°C for 15 minutes.

1.2. Nutrient Broth (Oxoid)

Lab Lemco Powder	1,0g
Bacteriological Peptone	5,0g
Yeast Extract	2,0g
NaCl	5,0g
Distilled Water	to 1000ml

Suspend 13g in 1000ml distilled water. Autoclave at 121°C for 15 minutes.

1.3. Mueller Hinton Agar (Biolab)

Meat Infusion	5,0g
Casein Hydrolysate	17,5g
Soluble Starch	1,5g
Agar	14,0g
Distilled Water	to 1000ml

Suspend 38g in 1000ml distilled water. Autoclave at 121°C for 15 minutes.

1.4. Potato Dextrose Agar (Oxoid)

Potato Extract	4,0g
Glucose	20,0g
Agar	15,0g
Distilled Water	to 1000ml

Suspend 39g in 1000ml distilled water. Autoclave at 121°C for 15 minutes.

1.4.1. 0,5 M McFarland's Standard

1.4.2. 0,048M Barium Chloride

Barium Chloride	17,625g
Sterile Distilled Water	to 1000ml

1.4.3. 0,36N Sulphuric Acid

1N Sulphuric Acid	36ml
Sterile Distilled Water	to 1000ml

The Barium Chloride solution (0,5ml) was added to the sulphuric acid solution (99,5ml), stirred with a magnetic stirrer and dispensed into Bijou bottles in volumes used to grow cultures. The bottles were sealed well to prevent loss by evaporation and stored in the dark at room temperature. The solution was agitated vigorously on a vortex mixer immediately prior to use⁹⁹.

APPENDIX TWO

Table 14: Zones of inhibition (in mm) achieved with the Disk Diffusion Method using the crude organic solvent extracts of *B. natalensis*, *B. frutescens* (yellow flowers), *B. abyssinica*, *B. narcissifolia* and *B. frutescens* (orange flowers). The amount of crude extract impregnated on each disk was 1,0mg

BACTERIUM	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>B. subtilis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. freundii</i>	-	-	-	-	-	-	-	-	-	9,5	-	-	-
<i>E. coli</i>	-	-	-	-	10,0	-	9,5	-	-	-	-	-	-
<i>E. aerogenes</i>	10,0	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. faecalis</i>	-	-	11,0	9,0	10,0	9,5	9,0	-	-	-	-	-	-
<i>K. oxytoca</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	8,5	12,5	9,5	10,0	11,0	11,5	12,0	8,5	-	-	-	-	-
<i>M. luteus</i>	-	11,0	10,5	-	-	-	-	-	-	-	-	-	-
<i>M. morgani</i>	-	9,5	8,5	-	11,5	-	10,5	-	-	12,5	9,5	10,5	10,0
<i>P. mirabilis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	10,0	-	-	-	-	-	-	-	-	-	-
<i>S. aureus</i>	-	8,0	-	-	-	11,5	-	-	-	-	10,5	-	-
<i>S. epidermidis</i>	-	11,5	-	-	11,5	-	-	-	-	10,0	-	-	-
<i>S. typhi</i>	-	7,0	8,0	-	-	7,0	7,0	10,0	9,5	8,0	-	-	-
<i>S. flexneri</i>	-	10,5	-	-	-	-	-	-	7,0	8,0	-	-	7,5

KEY:

1. *B. natalensis*- organic solvent extr. of leaf
2. *B. natalensis*- organic solvent extr. of stem
3. *B. natalensis*- organic solvent extr. of root
4. *B. frutescens*- organic solvent extr. of leaf
5. *B. frutescens*- organic solvent extr. of stem
6. *B. frutescens*- organic solvent extr. of root
7. *B. abyssinica*- organic solvent extr. of leaf
8. *B. abyssinica*- organic solvent extr. of root

9. *B. narcissifolia*- organic solvent extr. of leaf
 10. *B. narcissifolia*- organic solvent extr. of stem
 11. *B. narcissifolia*- organic solvent extr. of root
 12. *B. frutescens* (orange flowers)- organic solvent extr. of leaf
 13. *B. frutescens* (orange flowers)- organic solvent extr. of roots
- (-) – No inhibition of growth

Table 15: Zones of inhibition (in mm) achieved with the Disk Diffusion Method using the crude organic solvent extracts of *B. natalensis*, *B. frutescens* (yellow flowers), *B. abyssinica*, *B. narcissifolia* and *B. frutescens* (orange flowers). The amount of crude extract impregnated onto each disk was 0,5mg.

BACTERIUM	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>B. subtilis</i>	-	-	-	-	-	-	-	-	-	-	9,5	-	-
<i>C. freundii</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. coli</i>	-	-	-	-	7,5	-	-	-	-	-	-	-	-
<i>E. aerogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. faecalis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. oxytoca</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. pneumoniae</i>	7,5	12,0	-	-	9,5	-	8,0	-	-	-	-	-	7,5
<i>M. luteus</i>	-	10,0	7,5	-	-	-	-	-	-	7,5	-	-	-
<i>M. morgani</i>	-	7,5	-	-	-	-	-	-	-	7,0	-	-	-
<i>P. mirabilis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i>	-	-	-	-	-	-	-	-	7,5	-	-	-	-
<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-	7,5	-	-
<i>S. epidermidis</i>	-	9,5	-	-	7,5	-	-	-	-	-	-	-	-
<i>S. typhi</i>	-	7,5	-	-	-	-	7,5	-	-	-	-	-	-
<i>S. flexneri</i>	-	-	-	-	-	-	-	-	-	-	-	-	-

KEY: Refer to Table 14.