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ANTIFUNGAL ACTIVITY OF EPITHELIA FROM SELECTED FROG SPECIES OF THE SOUTH WESTERN CAPE OF SOUTH AFRICA

by

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DECLARATION

I, Thashlin Govender, declare that the contents of this thesis represent my own unaided work, and that thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed

19-01-2009

Date

ABSTRACT

Resistance to antibiotics has been acknowledged as a major global public health problem. The use of peptides to provide alternatives to combat multi drug resistant organisms is of current relevance to overcome antibiotic resistance. The high diversity of amphibian skin peptides render these animals a potential source for the discovery of novel drugs. This qualitative study analyses the skin secretions of the frog species *Amietia fuscigula, Amietophrynus pantherinus, Strongylopus grayi* and *Xenopus laevis* found in the south Western Cape of South Africa for antifungal activity. Various techniques for the extraction of frog skin secretions were tested in a preliminary study. Results of the preliminary study indicated that chemical stimulation of the frog was a successful technique for the extraction of skin secretions. Extracts were bioassayed using the microtitre plate technique to determine their potency against *Candida albicans*, *Fusarium verticillioides* and *Aspergillus flavus,* which are fungi of medical and agricultural importance. Further analyses by biological and chemical fingerprinting were undertaken to compare the constituents of the frog secretions. This allowed for the purification and elucidation of the most active extract.

The results showed that the extracts have antifungal properties. The total activity of secretions against fungi from the frog *Amietia fuscigula* was most potent. Results from the MS-MS spectra after purifying all the extracts, indicated the presence of a single compound of interest with a molecular weight of 1968 from the frog *Amietia fuscigula*. The MS-MS resolution below 700 amu (indicated by 'R' in the sequence below) was poor and did not allow for the elucidation of the peptide sequence. The proposed sequence assigned to the peptide was 'R'-Ser-Gly-Met-Ser-Pro-Ser-Ile-Pro-Ala-Ile-Glu-Ala-Met.

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Above all, salutations and prostrations to God for having blessed me with "happy endings."

I am – You are – We are one.

Jai Hind!

जय राम रमारमनं समनं
भवतापभयाकुल पाहि जनं

From the chorus of 'We all stand together':

Win or lose, sink or swim
One thing is certain, we'll never give in
Side by side, hand in hand we all stand together.

'We All Stand Together' was performed by Paul McCartney and the Frog Chorus for the children's series - Rupert the Bear. The Frog Chorus was an ensemble of the St Paul's Choir together with the King's Singers.

DEDICATION

I dedicate, this my contribution to science, to my great friend and mentor whose treasured camaraderie and support makes me fortunate in so many ways

for Soshan Soobramoney

- ever full of *joie de vivre.*

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GLOSSARY

Abbreviations

AMP	Antimicrobial Peptides
BSA	Bovine Serum Albumin
C-18	Carbon 18
CAL	Central Analytical Laboratory
CPGR	Centre for Proteomics and Genomics
GPS	Global Positioning System
IIDMM	Institute of Infectious Disease and Molecular Medicine
INT	p-iodontrotrotetrazolium
LC-MS	Liquid Chromotography – Mass Spectrometry
MALDI TOF	Matrix Assister Laser Desorption Ionisation – Time Of Flight
MIC	Minimum Inhibitory Concentration
MRC	Medical Research Council
MS	Mass Spectrometry
PDA	Potato Dextrose Agar
PROMEC	Programme on Mycotoxins and Experimental Carcinogenisis
Q-TOF	Quadrupole – Time Of Flight
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
TLC	Thin Layer Chromatography
TFA	Trifluoroacetic Acid

CHAPTER ONE

Background and Literature Review

1.1 Antibiotics and antibiotic resistance

Antibiotics have been called the single most significant discovery in medicine (Silver & Bostian, 1993) and can be described as chemical substances derived from microbial sources; used to treat microbial infections (Katzung, 1998). The 1900s saw the development of dyes to treat bacterial infections, the first being pyocyanase, a blue pigment produced by *Pseudomonas aeruginosa* that reduced the growth of other bacteria *in vitro* (Edwards, 1980). An important event in the history of antibiotics was the discovery of penicillin by Alexander Fleming in 1929. The real potential for its use was only recognised with the advent of the Second World War, which commercialised the use of the medication for the treatment of septic wounds (Levy, 1997). The golden era of antibiotic discovery and development in the 1940s and 1950s (Chicarelli - Robinson *et al.*, 1997) allowed for the effective management of bacteriological pathogens.

The term "resistance" is used to describe the relative insensitivity of a microbe to an antimicrobial drug that has been tested and compared with other isolates of the same species (Rex *et al.*, 1997). In contrast, clinical failure is described as the lack of success of an appropriate therapy for a certain indication to result in a clinical response. An explanation for clinical failure may be, for example, antifungal resistance. Other causes such as an impaired immune function, poor bioavailability of the drug given, or accelerated metabolism of the drug, are possible causes of treatment failure (Rex *et al.*, 1997). Primary resistance occurs in organisms never exposed in that host to a drug of interest. In contrast, secondary resistance, also defined as acquired resistance, arises after exposure of the organism to the drug. Intrinsic resistance is defined as resistance of all or almost all isolates of one species to a certain drug, for example, the resistance of *Candida krusei* to fluconazole (Rex *et al.*, 1997). The term "clinical resistance" can be used to describe failure of therapy or relapse of

infection with an organism not associated with resistance, for example, changes in the host's immune system (Rex *et al.*, 1997).

The gains made in improving human health using antibiotics have been partly lost due to the emergence of antibiotic resistant organisms and the increased incidence of newly described pathogenic fungi and bacteria (Torres - Viera *et al.,* 2000). Penicillins are reported to be the most widely used antibiotics and therefore account for some of the drastic consequences of antibiotic misuse (Jawetz *et al.*, 1998). *Pneumococci* have been known to be susceptible to penicillins; however in Papua New Guinea and South Africa, outbreaks of meningitis and pneumonia due to penicillin-resistant *pneumococci* have been observed (Jawetz *et al.*, 1998).

Resistance to antimicrobial agents has been acknowledged at present as a major global public health problem (WHO, 2000). For example, certain hospital acquired nosocomial pathogens are already resistant to available antibiotics and therefore untreatable (Weinstein & Hayden, 1998; Norrby *et al.*, 2005). Furthermore, an increase in mucosal and systemic fungal infections and the concomitant increased usage of antifungal agents for prophylaxis is most likely the main cause of the development of antifungal drug resistance (Helmorhorst *et al.*, 1999). Medical advances have also led to increased numbers of immune-compromised patients living longer, which has in turn led to an increase in the incidence of fungal infections that have not always been amenable to treatment (Torres-Viera *et al.,* 2000). The increase in resistant strains to current regimens of antimicrobials has led to increased efforts in the search for new antimicrobial agents.

Microbial resistance to antibiotics results in increased human morbidity, mortality, and therefore increased costs in health care (Silver & Bostian, 1993). The cost of drugs is a sizable proportion of total health care expenditure in developing countries (Sofowara, 1984). There is a wide variation in the prescribing of antimicrobials and other drugs. In primary health care 30-60% of patients receive antibiotics perhaps twice what is clinically needed (Holloway, 2000). Misuse of antibiotics is common and may take the form of incorrect dosage

or inappropriate prescription (Holloway, 2000). Generally, the treatment of patients with an infection is managed by the identification and isolation of the bacteria and fungi involved. Having done this the sensitivity of the pathogen is determined. However due to the lag phase of the patient presenting themselves to a medical practitioner, the initial treatment of the infection is based on the clinical impression. This is deduced from the history of the patient, physical examination, signs and symptoms, microscopic examination, epidemiology and rapid laboratory tests (Katzung, 1998).

According to Weiner and Pepper (1985), antimicrobial treatment should be managed with agents that treat the suspected microorganism. Treatment with antimicrobial agents should be based on thorough clinical findings and it is imperative that clinical specimen culture and sensitivity tests be used to establish the rationale of the antibiotic use. The etiological factors for the patient's illness have to then be established and individuals exposed to the initial patient should be protected in order to prevent secondary or nosocomial infections where necessary. On the other hand, antimicrobial agents are usually used before the causative pathogen for a particular illness or susceptibility to a particular antimicrobial agent is known. This use of antimicrobial therapy is termed empirical (presumptive) therapy and is based on clinical experience (Katzung, 1998). The justification for empirical therapy is that infections are best treated early and to withhold treatment until the results of clinical specimens are available may expose the patient to risks of morbidity or death. Initiation of empirical therapy to a certain extent should however conform to a well - defined protocol (Serage, 2003).

1.2 Traditional medicine in drug discovery

The medicinal interaction between humans and animals has been shown throughout the world (Gudger, 1925; Branch & Da Silva, 1983). Animal - based medicines have been utilised since ancient times (Weiss, 1947; Angeletti *et al.*, 1992; Rosner, 1992). Popular remedies were obtained from parts of an animal's body, from products of its metabolism (corporal secretions and excrements), or from non - animal materials (nests and cocoons) (Hancock & Patrzykat, 2002).

Historically antibiotics have been developed from secondary metabolites of fungi, for example penicillin from *Penicilium notatum* (Springfield, 2006). For centuries, plants too have been a major source of active compounds for products (Springfield, 2006). In recent years, the search for new pharmaceuticals of natural origin has intensified and been extended to include sources other than plant material (Clarke, 1997).

According to Hancock and Patrzykat (2002), antimicrobial peptides represent an important natural response for overcoming microbial infections. The peptides are produced by bacteria, fungi, plants, insects, amphibians, crustaceans, fish, birds and mammals, including man, either constitutively or in response to the presence of a microbe (Mor & Nicholas 1994; Hancock & Patrzykat, 2002).

The Chinese have traditionally administered frog skin and secretions of toad parotid glands to regulate internal corporal functions and fertility or as a treatment for dog bites (Costa-Neto, 1998). Peptides extracted from the scraped skin secretions of *Phyllomedusa bicolor* are used in Chinese folk medicine for the treatment of depression, stroke, seizures and cognitive loss in ailments such as Alzheimer's disease (Amato, 1992). Traditional healers in Nagaland, India use the dorsal skin of frogs to cover wounds of patients (Purna Sai *et al.*, 1995). In Vietnam, the lack of adequate medical supplies to treat napalm burns during the Vietnam war in the 1960s, led surgeons to investigate traditional Vietnamese remedies for burns. They found that the use of amphibian skins from the genus *Rana* as temporary grafts for patients with severe skin loss was a successful means of treatment (Le, 1992). When testing these grafts, experimentally wounded Wistar rats dressed with frog skin, as opposed to controls dressed with cotton gauze showed that healing was faster with the group treated with the frog skins. Biochemical estimations of wound granulation were carried out every two days until complete healing was achieved. These showed that the frog skin treated group produced higher levels of the amino acid hydroxyproline than the controls (Purna Sai *et al.*, 1995).

1.3 Pharmacological investigation of frogs

Amphibians exist in microorganism - rich environments, causing them to produce potent antimicrobial peptides as a defence. Biologically active compounds such as amphibian antimicrobial peptides are secreted by non - lymphoid cells on the mucosal surfaces of the respiratory and gastrointestinal tracts, and by the granular glands of the skin (Barra & Simmaco, 1995; Nicolas & Mor, 1995; Woodhams *et al.*, 2005; Pal *et al.*, 2006). These peptides are released in skin secretions, often at very high concentrations.

Given the respiratory and antimicrobial functions of the amphibian skin, it is likely that some of the novel molecules found in amphibian granular gland secretions may be of use in the treatment of skin and respiratory infections (Clarke, 1997). For example, an antibiotic effective against *Staphylococcus aureus* (which often causes abscesses and boils) and against viruses that are rarely affected by antibiotics was discovered from a frog species of the genus *Rana* (Channing, 2001).

Bactericidal and fungicidal peptides synthesized in the skins of certain frogs represent a promising source of potential therapeutic agents (Nicolas & Mor, 1995). For example, the skin secretions of the African clawed frog, *Xenopus laevis*, contained high concentrations of a diverse array of biologically active components that included thyrotropin - releasing hormones, peptide glycine - leucine amide (PGL^a) and the myotropic peptides caerulein, xenopsin and levitide (Lazarus & Attila, 1998). Their helical, amphiphilic structures have an affinity for microbial membranes causing dissipation of ion gradients (Zasloff, 1987; Chen *et al.*, 1988). These peptides are water soluble and nonhemolytic. These peptides inhibit *Candida albicans* growth (Zasloff, 1987). According to Zasloff (1987) the peptides identified from *X. laevis* appear to represent a previously unrecognised class of vertebrate antimicrobial peptides.

Extensive studies have been conducted on antimicrobial peptides of frogs belonging to the genus *Rana* (Simmaco *et al.*, 1998; Conlon *et al.*, 2004; Che *et al.*, 2008). This genus

comprises of more than 250 species and are distributed worldwide, except for the polar regions, southern South America and most of Australia (Pal *et al.*, 2006). Frogs of this genus have proved to be a rich source of peptides with antibacterial and antifungal activity (Pal *et al.*, 2006). About 160 antimicrobial peptides have been identified from more than 20 ranid amphibians (Clark *et al.*, 1994; Gabay, 1994; Barra & Simmaco, 1995; Conlon *et al.*, 2004).

Peptides have been isolated from the secretions of *Rana ornativentris* (Kim *et al.*, 2001), *Rana japonica* (Isaacson *et al.*, 2002), *Rana tagoi* (Conlon *et al.*, 2003); *Rana pirica* (Conlon *et al.*, 2004), *Rana okinavana* (Conlon *et al.*, 2005) and *Odorrana graham* (Che *et al.*, 2008). These studies resulted in the discovery of broad - spectrum antibacterial and antifungal activities of certain peptides, such as esculentin-1, ranalexin-1 and ranatuerin, (Conlon *et al.*, 2004). The dermaseptins, produced by the South American Arboreal frog, *Phyllomedusa sauvagii* are lytic, linear, cationic, lysine - rich peptides (Mor, *et al.,* 1991). Another South American tree frog, *Phyllomedusa bicolor*, produces Skin-PYY (SPYY) which is an antifungal compound closely related to NPY, as well as a neuropeptide, and gastrointestinal tract peptide (PYY) (Vouldoukis *et al.*, 1996). SPYY permeates phospholipid membranes and inhibited *C. neoformans, C. albicans,* and *A. fumigatus* growth (Vouldoukis *et al.*, 1996). A study conducted by Basir *et al.* (2000) on the pickerel frog *Rana palustris* were able to isolate as many as 22 peptides with different growth – inhibitory activity towards bacteria and fungi from skin secretions, showing the diverse peptides found in secretions of frogs.

The antimicrobial peptides found in the Ranidae consist of between 10 and 46 amino acid residues and on the basis of structural similarity can be grouped into multiple families (Goraya *et al.*, 2000; Conlon *et al.*, 2004). These families of peptides are believed to be related evolutionarily and are proposed to have risen as a result of multiple gene duplication events (Duda *et al.*, 2002). There are no conserved regions of amino acid sequence in the ranid families of peptides that are associated with antimicrobial activity but the peptides are almost invariably cationic, relatively hydrophobic, and have the propensity to form an

amphipathic α-helix in the environment of a phospholipid vesicle or in a membrane - mimetic solvent such as trifluoroethanol (Tossi *et al.*, 2000).

1.4 Taxonomy of amphibians

Amphibians are widespread throughout the world except for high latitudes in the Arctic, Antarctic, some oceanic islands and extremely dry deserts (Underhill, 1988). Despite adopting a life on land, amphibians remain dependant on moist or aquatic habitats, particularly for breeding. The class Amphibia which comprises of more than 5000 species are represented in South Africa by the orders Anura and Gymnophiona (Underhill, 1988). The south Western Cape of South Africa is a unique biogeographic region. According to Minter *et al.* (2004), overall amphibian species richness is high in the south Western Cape, where 21 - 30 species are recorded per grid cell (676 km^2).

1.4.1 Anatomy of amphibians

Like many of the higher vertebrates, amphibians possess a vertebral column that protects the central nervous system. Frogs and toads have limbs which bear fingers and toes, external eardrums, eyelids, skin glands, a tongue, voice box, and sternum (Underhill, 1988). Amphibians have a three - chambered heart, and most have paired lungs. Frogs and toads are characterised as cold blooded. The ectoderms of the animal are warmed by the external environment (Underhill, 1988). There are few physical differences between frogs and toads. Frogs have a smooth, moist skin with few warts and live near or in water; whilst toads have a rough, drier skin with warts and live on land and breed in water only (Passmore & Carruthers, 1979). Toads have large parotid glands found behind the eyes (Underhill, 1988). Frogs have a narrower body and waist; their hind legs are longer for hopping and webbed feet for swimming. In contrast, toads have broader, flatter bodies, shorter hind legs and walk rather than hop.

1.4.2 Secretory glands of frogs

The amphibian skin is a morphologically, biochemically and physiologically complex organ which fulfils a wide range of functions necessary for the organism's survival (Clarke, 1997). The skin of the frog is a thin, flexible integument that aids in respiration and water absorption (Wager, 1986). The skin is highly vascular which makes respiration possible, but at the same time excluding disease organisms (Wager, 1986). The integument consists of two major layers: the epidermis and dermis. The epidermis is made up of germinative layers which in turn are made up of basal cells. These cells produce a non - keratinised layer, which are frequently shed during summer months (Minkoff, 1975).

The dermis contains connective tissue and the layer beneath the germinative layer contains the mucous and the pigment cells known as chromatophores (Wager, 1986). The chromatophores enable the frogs to alter their colour for protective purposes and to aid in regulating body temperature (Underhill, 1988). The skin of frogs and toads contain poisonous glands which produce toxins that are toxic to other animals. These glands are either scattered throughout the skin or concentrated in specific areas (Underhill, 1988). Figure 1.1 illustrates the gland of a frog's skin.

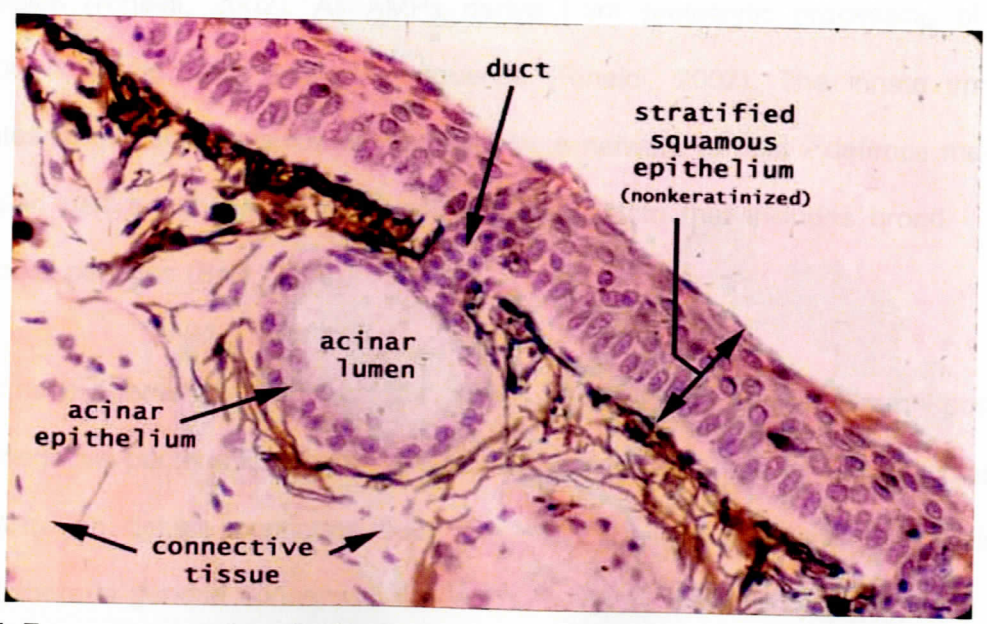

Figure 1.1: Transverse section of a frog's skin (nonkeratinised, stratified squamous epithelium) invaginating to form a simple gland, with one single secretory unit connected to the surface by a short duct (King, 2002)

The skin of frogs provides a potential avenue of entry for bacteria, fungi, and other invaders (Channing, 2001). The compounds secreted by the glands play various roles, either in the regulation of physiological functions of the skin or in defence against predators or microorganisms (Barra & Simmaco, 1995). The skin glands produce a range of substances that make a mammal predator very ill, without killing it. The cytoplasm of the skin gland cells are rich in granules and the lumen is reduced into a small empty cavity. Contraction of myocytes surrounding the glands causes a synchronous discharge of their content with a holocrine mechanism (Goraya *et al.*, 2000). These discharges contain peptides which have the ability to inhibit growth of numerous pathogenic microorganisms, including enveloped viruses, Gram - positive and Gram - negative bacteria, protozoa, and fungi (Nicolas & Mor, 1995).

1.5 Antimicrobial peptides

Defence against invading microbes is a problem faced by all multi - cellular organisms. One key component of the host - resistance apparatus is innate immunity (Kimbrell, 2001). Among the main effector molecules of the innate immunity are antimicrobial peptides (AMPs). These are gene - encoded, ribosome - synthesised peptides comprising of ~10 - 50 amino acids (Rinaldi, 2002). All AMPs derive from proteolytic processing of a larger precursor, which includes a signal sequence (Rinaldi, 2002). The innate immunity of vertebrates to microbial invasion is mediated by a network of host - defence mechanisms involving in part a nonspecific chemical defense system that includes broad - spectrum antimicrobial peptides (Bals, 2000).

Peptides have been shown to inhibit the growth of enveloped viruses, Gram - positive and Gram - negative bacteria, protozoa, fungi and even cancer cells *in vitro* (Nicolas & Mor, 1995). Although debate continues over the specific killing mechanism of antimicrobial peptides, there is general consensus that microbial death initiated by the cationic peptides involves cell membrane disruption and subsequent unregulated ion exchange with the environment (Sitaram & Naggaraj, 2002). This explanation is validated by the observation

that antimicrobial peptides work rapidly, apparently far too quickly for any process that involves translocation of the peptide across the cell membrane for binding to an intracellular target molecule (Sitaram & Naggaraj, 2002). They are relatively small molecules, their action is fast and lethal to a large spectrum of pathogens, and they seem to escape many of the drug resistance mechanisms (Mor & Nicholas, 1994).

Peptide antibiotics, which are believed to interact with the microbial membrane leading to the disruption of cellular integrity and cell death, may be a promising new class of antifungal or antimicrobial agents (Mor & Nicolas, 1994; Helmorhorst *et al.*, 1999). The antifungal properties of peptides have been studied for nearly forty years (Georgopapadakou & Walsh, 1996). During the past 10 - 15 years, interest in their antifungal nature has expanded due to increased resistance of fungal pathogens to currently used antifungal drugs and the toxicity or adverse host reactions of other anti - infective drugs (Georgopapadakou & Walsh, 1996). The mechanism of bacterial cell lysis by peptides generally involves non - specific interaction with the membrane (phospho) lipids rather than binding to specific receptors on the cell membrane. Therefore microorganisms develop resistance to antimicrobial peptides at rates that are less than those observed for conventional antibiotics (Yeaman & Yount, 2003). On the negative side, the toxicities of many of the peptides and their rapid rate of clearance from the circulation mean that *in vitro* applications may be more appropriate than systemic administration (Conlon, 2004).

Peptides have been isolated from plants and animals. Table 1.1 provides an overview of these novel peptides. Diverse applications have been demonstrated for antimicrobial peptides as anti - infective agents (Zasloff, 2002). These applications are listed below:

- The broad spectrum of antimicrobial peptides positions them for consideration as 'chemical condoms' to limit the spread of sexually transmitted diseases, including Neisseria, Chlamydia, HIV/AIDS and Herpes simplex virus (HSV) (Yasin *et al.*, 2000).

- Microbial colonisation and growth on the surfaces of synthetic polymeric materials is a problem that complicates the use of medical devices such as intravenous catheters. One solution is the use of magainin peptides, which when covalently bound to insoluble polymeric beads, retains antimicrobial activity (Haynie, 1995).
- Introduction of antimicrobial genes into plants and animals has been successful in transferring some benefit against disease. Agricultural uses have progressed, as demonstrated in tobacco (De Gray *et al.*, 2001) and potatoes (Osusky *et al.*, 2000).
- The possibility of alleviating the pulmonary bacterial infections associated with cystic fibrosis by transferring a genetic construct capable of expressing super physiological levels of cathelicidin (LL37) has been demonstrated in an animal model (Bals *et al.*, 1999).
- The re - engineering of human macrophages to express β-defensins to enhance their efficacy against *Mycobacterium tuberculosis* has been proposed (Kisich *et al.*, 2001).
- The discovery that the essential amino acid isoleucine can pharmacologically stimulate expression of β-defensin genes in isolated enteric cells suggests that new classes of therapeutics could be developed on the basis of their ability to turn on endogenous antimicrobial peptides (Fehlbaum *et al.*, 2000).

Table 1.1: Overview of antimicrobial peptides that have been discovered from animals and plants. Table adapted from Zasloff (2002)

Representative peptides	Origin	Tissue	Reference
α-helical			
Cecropin A	Silk moth	Epithelial tissue, Blood Cell, Haemolymph	(Steiner *et al.*, 1981)
Magainin 2	*Pipidae* frog	Epithelial tissue	(Zasloff, 1987)
Pexiganan	Synthetic		
Dermasepin 1	*Rana* Frog	Epithelial tissue	(Simmaco *et al.*, 1998)
LL-37	Human	Epithelial tissue, Blood Cell	(Yang, 2000)
Buforin II	Vertebrate	Epithelial tissue	(Kim, 2000)
One disulphide bond			
Bactenecin 1	Cow	Blood Cell	(Selsted, 1992)
Thanatin	Insect	Blood Cell	(Ulvatne & Vorland, 2001)
Brevnin 1T	*Rana* frog	Epithelial tissue	(Mangoni *et al.*, 2000)
Ranalexin	*Rana* frog	Epithelial tissue	(Simmaco *et al.*, 1998)
Ranateurin 1	*Rana* frog	Epithelial tissue	(Romeo *et al.*, 1988)
Esculentin 1	*Rana* frog	Epithelial tissue	(Selsted *et al.*, 1985)
Two disuphide bonds			
Tachyplesin	Horseshoe crab	Blood Cell	(Matsuzaki, 1999)
Androctonin	Scorpion	Haemolymph	(Brey, 1993)
Protegrin 1	Pig	Blood Cell	(Yang *et al.*, 2000)
Three disuphide bonds			
α-defensin (HNP3)	Human	Blood Cell, Epithelial tissue	(Tang, 1999)
β-defensin (TAP)	Cow	Epithelial tissue, Blood Cell	(Diamond, 1994)
Θ-defensin	Monkey	Blood Cell	(Tang, 1999)
Defensin (sapecinA)	Insect	Epithelial tissue, Blood Cell, Haemolymph	(Stoven *et al.*, 2000)
Thionin (crambin)	Plant	Epithelial tissue	(Dangl & Jones, 2001)
Four disulphide bonds			
Defensin	Radish	Seeds, Epithelial tissue	(Thevissen, 2000)
Drosomycin	*Drosophila*	Haemolymph	(Imler & Hoffmann, 2001)
Hepcidin	Human	Liver	(O'Neil, 1999)
Linear, not α-helical			
Bac 5	Cow	Blood Cell	(Selsted, 1992)
PR-39	Pig	Blood Cell	(Agerberth, 1991)
Indolicidin	Cow	Blood Cell	(Selsted, 1992)
Apidaecin	Honeybee	Haemolymph	(Brey, 1993)
Pyrrhocoricin	Insect	Haemolymph	(Engstrom, 1993)
Histatin 5	Human	Saliva	(Kimbrell & Beutler, 2001)

1.6 Commercial drug development from antimicrobial peptides

Zasloff (2002) recently named at least seven companies exploring the use of AMPs as drugs. If antimicrobial peptides become available on the market, Boman (1991) explains that resistance of mutants will not occur provided that antimicrobial peptides are administered as the following regimen in clinical medicine:

- As microbial membranes being the targets. Experiments carried out indicate that it is difficult to isolate mutants with an altered membrane composition. It has been done, but the changes introduced (altered charge) decreased the viability of the mutants to such an extent that they could hardly survive in nature.
- Should the peptide structure (without any modifications) come from an animal, make certain it does not involve a new selection pressure. This means that the resistance mutations that could occur have already happened. If a natural peptide structure is altered (for patent reasons), the argument above is still valid.
- If one is to mimic nature one should always work with a combination of peptides. A resistant mutant would then have to be altered in such a way that several peptides made from different genes are simultaneously inactivated. This is however unlikely to happen.

1.7 Bioprospecting of South African frogs

Frog species from a limited number of families and locations have been studied for antimicrobial activity (Barra & Simmaco, 1995). In sub - Saharan Africa, amphibians are represented by a large number of frog families, genera and species of which a number of families and genera are endemic. South Africa is home to 114 frog species (Minter, 2004). The Western Cape Province has 51 frog species of which 27 are endemic to the south Western Cape (De Villiers, 2008). Figure 1.2 lists the frog and toad species found in South Africa.

Class: *Amphibia*	
Order: *Anura*	
Arthroleptidae	• *Arthroleptis* • *Leptopelis*
Bufonidae	• *Amietophrynus* • *Vandijkophrynus* • *Poyntonophrynus* • *Capensibufo* • *Schismaderma*
Brevicipitidae	• *Breviceps*
Heleophrynidae	• *Helephryne*
Hemisotidae	• *Hemisus*
Hyperoliidae	• *Afrixalus* • *Hyperolius* • *Kassina* • *Semnodactylus*
Microhylidae	• *Phrynomantis*
Petropedetidae	• *Microbatrachella* • *Poyntonia*
Pipidae	• *Xenopus*
Phrynobatrachidae	• *Phrynobatrachus*
Ptychadenidae	• *Ptychadena*
Pyxicephalidae	• *Tomopterna* • *Pyxicephalus* • *Arthroleptella* • *Natalobatrachus* • *Amietia* • *Strongylopus* • *Cacosternum* • *Anhydrophryne*
Rhacophoridae	• *Chiromantis*

Figure 1.2: List of amphibian families and genera found in South Africa. Adapted from Minter *et al.*, 2004

The Cape Floristic Region of South Africa has been recognised as biologically distinctive and rich in endemic frog and toad species (Pressey, Cowling & Rouget, 2003). Due to the region's high endemism and threats to its biodiversity, it has also been identified as a global biodiversity hotspot (Mittermeier *et al.*, 1998). The high species diversity may reflect a high molecular diversity from frog secretions and a potential for novel peptides to be discovered

from South African frog skin secretions. This study aims to analyse secretions of frog species found in the south Western Cape Province for antifungal activity.

Literature searches show that studies have been done on the frog species, *Xenopus laevis* which are found in South Africa. The phylogenetics and antibiotics of African Ranine frogs has also been studied. In the Du Preez (1997) study nucleotide sequence comparisons were used to investigate the genetic relationships within the subfamily Raninae. Results from this study showed that two species *Amietia vertebralis* and *Amnirana galamensis* generated a product after Polymerase Chain Reaction (PCR) cycling with two specified Ranalexin primers (Clark *et al.*, 1994). The sizes of the product that were identified were inconsistent with the size of Ranalexin cDNA, therefore warranting further investigation.

1.8 Epidemiology of fungal disease

The living world is divided into the five kingdoms of Planta, Animalia, Fungi, Protista and Monera. Fungi are eukaryotic, heterotrophic, unicellular to filamentous, rigid cell walled, spore - bearing organisms that usually reproduce by both sexual and asexual means and are insensitive to antimicrobial antibiotics (Rippon, 1988). Exposure to certain fungi can cause human illness through three specific mechanisms: generation of a harmful immune response (e.g. allergy or hypersensitivity pneumonitis), direct infection by the organism, and toxic - irritant effects from mould by - products (Bush *et al.*, 2006).

Alleviating human ill - health in the 21st century will have to be done simultaneously on two areas, namely infectious disease and chronic non-communicable diseases (WHO, 2000). With the increasing burden of disease causing a strain on public health and the economics of a country, drug resistance has become an important problem in a variety of communicable diseases including HIV/AIDS, tuberculosis, and other bacterial and fungal infections which have profound effects on human health (White *et al.*, 1998). At the same time, there have been dramatic increases in the incidence of opportunistic fungal infections, most commonly

as a result of reduced immune status associated with the HIV/AIDS epidemic, cancer chemotherapy and organ and bone marrow transplantation (White *et al.*, 1998).

The range of human infections caused by the yeast *Candida albicans* and several related species is significant (McCullough, 1995). The infections range from oral and genital thrush to systemic infections in patients who are already seriously ill with other diseases (McCullough, 1995). In sub Saharan Africa where 70% of the world cases of HIV/AIDS are reported (UNAIDS, 2004), oral candidiasis including oropharyngeal and oesophageal candidiasis, are common, causing significant morbidity among patients. Oral candidiasis is usually treated by tropical antifungal agents, which include nystatin, miconzole, fluconazole, itraconazole and amphotericin B. The treatment of *Candida* infections faces a number of challenges including: limited number of effective antifungal agents, toxicity of the available antifungal agents (Feldmesser, 2003), resistance of *Candida* to commonly used antifungals (Klepser, 2001), relapse of *Candida* infections (Debruyne, 1997) and the high cost of antifungal agents (Feldmesser, 2003). When relapses occur, the infections tend to be increasingly refractory to treatment. The challenges associated with the management of *Candida* infections necessitate the discovery of new antifungal agents, in order to widen the spectrum of activity against *Candida* and combat strains expressing resistance to the available antifungal agents (Runyoro *et al.*, 2006).

With all the focus on human disease, very little attention has been given to fungi that pose a threat to agriculture, and thereby cause disease in humans. In Africa and the rest of the developing world subsistence farming is common with home - grown crops which are the major source of food for many households, irrespective of quality conditions (Shepard, 2003). Subsistence farming is widespread in rural Africa and is a strategy by poor rural households to reduce expenditure on food and ensure food security (Watkinson & Makgetla, 2002). Food production and storage therefore play an important role in stabilising seasonal food production (Bankole *et al.*, 2006). The fungus, *Fusarium verticillioides,* is one of the most common seed - borne fungus associated with corn used for human and animal consumption

throughout the world (Marasas, 1984). *Fusarium verticillioides* was the predominant fungus isolated from mouldy corn implicated in a field outbreak of equine leukoencephalomalacia in South Africa in 1970. Fumonisins, mycotoxins produced by *F. verticillioides,* were found in fungus contaminated corn worldwide (Marasas, 2001). Fumonisins cause field outbreaks of mycotoxicosis in animals, are carcinogenic in rats, and disrupt sphingolipid metabolism (Marasas, 2001). Fumonisins have also been associated with a higher risk of oesophageal cancer in humans (Gelderblom *et al.*, 1988; Sydenham *et al.*, 1996) as well as neural tube defects in infants (Van Waes *et al.*, 2005; Missmer *et al.*, 2006).

Another fungus that has negative effects on agriculture and consequently on human health is *Aspergillus flavus. A. flavus* produces a toxin called aflatoxins on agricultural products such as nuts, grains, and cotton (Geiser *et al.*, 1998). In many countries, strict controls are placed on aflatoxin levels in food and feed products. Yet the cost of lost crops and animals, reduced yield and monitoring aflatoxin levels is steadily increasing (Dvorackova, 1992). Without these strict controls aflatoxins would remain a significant public health problem (Wogan, 1992). Aflatoxins are potent carcinogens and can act in synergy with the Hepatitis B virus (HBV) to cause hepatocellular carcinoma (Bhat & Vasanthi, 2003). Perinatal exposure to aflatoxins has been shown to stunt growth (low height for age) and may contribute to infant mortality as a result of protein energy malnutrition (Gong *et al.*, 2002). The ability to reduce or eliminate aflatoxin contamination via biological control methods requires an understanding of the life history of the fungus, which is currently rudimentary (Wogan, 1992).

The incidence of fungal infections stemming from agricultural contamination and human pathogens has increased the need for novel antifungal agents (White *et al.*, 1998). Hence research into the development of novel pharmaceutical products of natural origins, such as frog secretions of South African frogs, is important. This may result in the discovery of novel antimicrobial peptides for the development of antibiotics. This will assist in providing alternatives to the public health dilemma of antibiotic resistance.

1.9 Research question

Do secretions from the epithelia of selected frogs of the south Western Cape have antifungal activity?

1.10 Aim and objectives of the study

The aim of the study is to qualitatively establish if skin secretions from selected frogs of the south Western Cape Province of South Africa have antifungal properties.

1. To test different extraction techniques for collecting frog skin secretions. This will form part of a preliminary study.
2. To optimize the extraction technique.
3. To determine the extracts efficacy or potency against three fungal species of medical and agricultural importance.
4. To determine and compare the biological and chemical constituents of the frog secretions.
5. To purify and elucidate the chemical structures of the most active extract.

CHAPTER TWO

Materials and Methods

An application (HASREC 4.3) was made to the Cape Peninsula University of Technology Health and Applied Sciences Research Ethics committee, for ethics approval prior to any of the following experiments being carried out by the investigator. All experiments followed the committees' ethical guidelines. A permit from the Cape Nature Conservation of South Africa was obtained for the collection of the 12 frogs used in the preliminary study and the four frogs used in the final study.

2.1 Preliminary study: Techniques for collecting skin secretions

Four techniques intended to extract skin secretions were tested on one frog species i.e. *Xenopus laevis* (Figure 2.1). Two of the four techniques were designed by the author for the preliminary study.

Figure 2.1: Dorsal view of *Xenopus laevis* collected from a pond at the University of the Western Cape

The other two techniques were previously described and modified for the purposes of the preliminary study. The optimal technique was selected for the final study on the basis of the amount of secretions obtained, the ease of applying the method as well as ethical considerations.

2.1.1 Collection of *Xenopus laevis*

A pond at the University of the Western Cape, Bellville, South Africa, was identified for the collection of frogs in the preliminary study. The pond has a high density of *Xenopus laevis.* The study aimed to test the extraction techniques on the *X. laevis* species, hence a pond like the one identified at the University supplied an ample amount of specimens from a single location. Frogs were collected using a home - made bucket trap (Figure 2.2).

Figure 2.2: Home - made bucket trap. The exterior of the trap is shown in A, whilst B depicts the interior

The trap consisted of a bucket with a hole large enough to let frogs in but not out. Ox liver was used as bait. The ox liver was placed in hosiery material, so as to restrict the frogs from eating all of the bait. The trap was submerged into the University pond and left overnight. The frogs were then transported to a laboratory at the Cape Peninsula University of Technology (CPUT) for testing of the various extraction techniques. At this stage the frogs

were identified as *Xenopus laevis* using the classification key from Passmore & Carruthers (1995) and Channing (2006).

2.1.2 Extraction of frog secretions

The four techniques tested were swabbing, physical stimulation, tissue harvesting and chemical stimulation. As previously mentioned these techniques were evaluated using yield, ease of application of method and ethical considerations.

2.1.2.1 The swabbing technique

The swabbing technique was designed by the author for the preliminary study. Cotton buds were used as swabs. The dorsal surfaces of three frogs were each gently swabbed. The swabs were then placed into eppendorf tubes and stored at -20 °C. A 100 µl of distilled water was used to wash off the secretion from the swab. The secretions from the swab were then biologically analysed for proteins using SDS - PAGE.

2.1.2.2 Physical stimulation technique

This was the second technique designed by the author for the preliminary study. Three frogs were used for this technique. A frog was placed in a Ziploc bag with 30 ml of double distilled water. The frog was shaken gently for five minutes, as illustrated in Figure 2.3.

Figure 2.3: The physical stimulation technique being applied to a *X. laevis*

The distilled water containing the skin secretions were then poured from the bag into 50 ml blue cap tubes. An additional 10 ml of double distilled water was used to rinse of the frog. The 10 ml of double distilled water was added to the 30 ml in the blue cap tube. The samples were then stored at -20 °C. One hundred microlitres of extract from the 40 ml was analysed for proteins using SDS - PAGE.

2.1.2.3 Tissue harvesting technique

The tissue harvesting technique of Goraya (2000) was modified and applied in the preliminary study. Three frogs were individually euthanased by placing the frog in a bottle that contained tricaine methane sulfonate (MS 222) dissolved in distilled water. The dorsal skin of the frogs were then removed (Figure 2.4).

Figure 2.4: The removal of skin from a *X. laevis*

The skin tissue was then stored at -20 °C. The tissue was macerated and dissolved in 10 ml of distilled water. One hundred microlitres of the extract was then analysed for proteins using SDS - PAGE.

2.1.2.4 Chemical stimulation technique

This technique was adapted from Che *et al.* (2008). The technique was applied on three frogs of *X. laevis*. Cotton wool was soaked in anhydrous ether and placed into a blue cap tube. An individual *X. laevis* frog was placed in a clean five litre glass bottle. The blue cap tube with the cotton wool soaked in anhydrous ether was put into the bottle that contained the frog and the lid was fastened. A frog was left in the bottle for two minutes (Figure 2.5).

Figure 2.5: The chemical stimulation technique being applied to a frog

The blue cap tube was removed from the bottle and the frog was rinsed with 15 ml of buffer (0.1 M phosphate buffer, containing 5 mM EDTA, pH 6.0) to wash off the skin secretions. The buffer containing the secretions were then poured into a round bottom flask and placed on ice. The solution was quickly centrifuged at 4 °C at 4000 rpm for 5 minutes and the supernatant was freeze - dried. The samples were then weighed and 5 mg of the dried supernatant fraction of the frog secretions were then dissolved in 10 ml of double distilled water to form a stock solution (0.5 mg/ml). One hundred microlitres was analysed from the stock solution using SDS - PAGE.

2.1.3 Protein electrophoresis using SDS - PAGE

SDS - PAGE was used to fractionate protein molecules in the extract. SDS - PAGE separates proteins according to their molecular weights and charge (Laemmli, 1970). The frog secretions from the four techniques were analysed on a 10% SDS - PAGE using the Mini Protean III system (Bio - Rad) with 1 mm thick spacers. Sample extracts (100 µl) were then mixed with an equal volume (100 µl) of 2X Laemmli sample buffer (Laemmli, 1970). Gels were made from a 40% pre - mix acrylamide: bisacrylamide (37.5:1) (Biorad). The separating gel consisted of 10% acrylamide: bisacrylamide (0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05%, ammonium persulphate and 0.1% TEMED). The stacking gel consisted of 4 % acrylamide: bisacrylamide (62.5 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulphate and 0.1 % TEMED). The protein molecular weight marker used was Page Ruler Unstained Protein Ladder (Fermentas). After loading the samples on the gel, the gel was electrophoresed at 110 V through the stacking gel and then at 120 V through the resolving gel. After electrophoresis was complete the gels were stained with Coomassie staining solution for at least 30 minutes while shaking on a belly dancer shaker at room temperature. The gels were then destained and the images captured with a digital camera (Olympus Optical Co).

2.1.4 Bradford protein assay

Extracts obtained from the chemical stimulation technique were analysed using the Bradford assay. The assay is colorimetric and is used to measure total protein concentration (Bradford, 1976). The Bradford assay is based on the proportional binding of the Coomassie stain to proteins. The colour of the dye reagent before adding the extracts is brown. As the protein concentration increases, the colour changes to a darker blue. The Bradford assay uses 5 mg/ml Bovine Serum Albumin (BSA) stock solution as a standard. The different concentrations of BSA used to produce a standard curve were 0, 125, 250, 500, 750 and 1000 µg/ml. The samples were diluted 50 times (2 µl sample) using 98 µl PBS (Phosphate Buffered Saline). These were prepared in duplicate.

The Bradford dye reagent was added last and incubated at room temperature for 5 minutes. The absorbance was read at 595 nm with an Ultraspec 1000 spectrophotometer (Pharmacia Biotech). Dependent on the results, the absorbance of the standards would then be used to plot a standard curve: absorbance vs. BSA concentration. The equation generated by the linear regression (y = mx + c) was used to calculate the concentration of the protein sample using its measured absorbance (Bradford, 1976).

2.2 Final study

2.2.1 Collection sites

The Oude Molen area is located in the south Western Cape of South Africa (Figure 2.6) and is rich in frog species. The GPS coordinates for the site are 33° 56' 25.3" S; 18° 22' 11.1" E. The Oude Molen consists of three water bodies and was identified as our collection site for the final study. Four species of frogs and toads were collected from the Oude Molen.

Figure 2.6: A map of South Africa showing the location of the study site (adapted from the South African Tourism Services, 2008)

Once the frogs and toad had been captured with the use of nets, they were placed into clean plastic containers with holes in the lids, for the supply of air. These frogs were stored in

cooler boxes which contained ice. The frogs were taken to the laboratory where the extraction technique described in Section 2.1.2.4 was specifically used to obtain skin secretions.

Che *et al.* (2008) used 30 frogs of the same species which each weighed approximately 30 to 40 g, while in this study one frog of each species were used. Forty millilitres of buffer solution was used to wash of each specimen, whilst Che, *et al.* (2008) used 500 ml of buffer (total volume). The solution was quickly centrifuged at 4 °C at 4000 rpm for 5 minutes and the supernatant was freeze - dried. The samples were then weighed and 5 mg of the dried supernatant fraction of the frog secretions were then dissolved in 10 ml of double distilled water to form a stock solution. The stock solution was then stored at -20 °C.

2.2.2 Microtitre plate bioassay

This method is a colorimetric assay and the change in colour of the indicator from colourless to pink is used to demonstrate fungal growth. Three fungal species were used viz: *Fusarium verticillioides* (MRC 826), *Aspergillus flavus* (MRC 3954), *Candida albicans* (MRC 8907). These fungi were obtained from the Programme on Mycotoxins and Experimental Carcinogenisis (PROMEC) Unit of the South African Medical Research Council (MRC). The fungal species were used in both the preliminary and final study.

The fungal isolates were grown on Potato Dextrose Agar (PDA) for five days, in an incubator at 4 °C. Fungal suspensions were prepared by suspending the spores in a solution of 0.05 % Tween 20. Prior to use, the suspensions were diluted in Potato Dextrose Broth (PDB) until the turbidity was the same as that of the 0.5 McFarland standard (0.05 ml $BaCl_2.2H_2O$, 9.95 ml H_2SO_4). The McFarland standard was used as a reference to approximate the number of fungi in a liquid suspension (Eloff, 1998). The suspensions were then mixed with PDB and were ready for use in the microtitre plate bioassay.

In brief, 100 µl of water was added to all wells of the microplate. In each of the first row of wells, 100 µl of the test extract at a concentration of 50 mg/ml was added to the 100 µl of

water previously added and mixed. After all the extracts were added; a serial dilution was done in the following manner: 100 µl from each of the first wells were pipetted into the wells of the next row. This was done all the way to the last row of wells on the plate. The extract concentrations were therefore reduced by 50 % from one row to the next. Forty microlitres of a 0.2 mg/ml p - iodonitrotetrazolium (INT) solution was added to each well before incubation. One microlitre of test fungi viz: *F. verticillioides* (MRC 826), *Aspergillus flavus* (MRC 3954), *Candida albicans* (MRC 8907) were pippetted into each well. The microtiterplate was covered and incubated in an incubator at 25 °C with 100 % humidity for five days. The lowest concentration at which a decrease in turbidity (pink colour) is apparent compared to the next dilution is taken as the Minimum Inhibitory Concentration. Fungal growth is indicated by the dark pink colour of the INT reduced to formazan (Eloff, 2000). The MIC was recorded as the lowest concentration of the extract that inhibited fungal growth every 24 hours for five days, and stability graphs were constructed.

2.3 Chemical fingerprinting

Frog secretions were further analysed using chemical fingerprinting to indicate the presence of proteins, based on specialised techniques such as Thin Layer Chromatography (TLC) and Liquid Chromotography - Mass Spectrometry (LCMS). The two techniques were applied on all four extracts.

2.3.1 Thin Layer Chromatography (TLC) method

Thin Layer Chromatography was carried out, as described by Masoko and Eloff (2005), using TLC F254 MERCK plates with the following solvents used as a mobile phase:

- Chloroform/ethyl acetate/formic acid, (CEF) [5:4:1],
- Ethyl acetate/methanol/water, (EMW), [40: 5.4: 4.0]

The 20 x 20 cm aluminum plates were cut in half and prepared in duplicate for each of the solvent systems described. To each plate, 5 µl of a 50 mg/ml solution was loaded on a line (1

cm apart). The plates were placed into saturated tanks and allowed to develop. The plates were then sprayed with a ninhydrin reagent and heated until dry. The separated components were visualised under UV light at 240 nm and 360 nm.

2.3.2 LC - MS analysis of extracts

Mass spectrometry measures the mass - to - charge ratio of ions, which assists in determining the composition of a sample by generating a mass spectrum. Extracts were initially sent to the University of Stellenbosch Central Analytical (CAL) facility for LC - MS analysis. The extracts were analysed with the Q-Tof Ultima Mass Spectrometer (Waters) using the method of Rautenbach *et al.* (2007). In brief, electrospray ionisation mass spectrometry (ESMS) was performed on a Waters Q - Tof Ultima mass spectrometer (Millipore-Waters, La Jolla, USA) fitted with a Z-spray electrospray ionisation source. Ten microlitres of the sample solution (100 ng frog secretions in 50 % acetonitrile) was introduced into the ESMS via Waters Acquity UPLC™. The carrier solvent was 50% acetonitrile in 0.1% formic acid, delivered at a flow rate of 300 µL / min during each analysis. A capillary voltage of 3.5 kV was applied, with the source temperature set at 100 °C and cone voltage at 35 V. Data acquisition was in the positive mode. UPLC-MS analysis was done on a Waters Acquity Ultra Performance Liquid Chromatograph connected to the above Waters Q - TOF Ultima mass spectrometer. Separation was achieved on a Waters UPLC BEH C_{18} column (2.1 x 50 mm, 1.7 µm spherical particles, Millipore-Waters), using a 0.1% of trifluoroacetic acid. The analyte was then eluted onto a multi - source plate.

Additional analysis of the extracts were done at the University of Cape Town's Institute of Infectious Disease & Molecular Medicine (IIDMM), Centre for Proteomic and Genomic Research (CPGR). The difference between the analysis done at the CAL facility and the CPGR was that the frog extracts underwent purification. In brief, 15 mg of freeze dried extracts were dissolved in 300 µl of analytical grey water from a Milli Q filtration system (Millipore-Waters, La Jolla, USA). Of this, 100 µl of the solution was aliquoted into a 1.5 ml eppendorf tube and centrifuged at 1200 xg for 10 minutes to remove particulates. The

supernatant (40 µl) was loaded on an in - house manufactured C-18 micro - spe column. The column was then washed with 0.1 % Trifluoroacetic acid (TFA) (Sigma Aldrich, St Louis, USA) and eluted. The eluate (70% acetonitrile; 0.1% TFA containing 5 mg/ml Alpha – cyano – 4 - hydroxy cinnamic acid [α – CHCA]) was then eluted onto a multi - source plate. Matrix Assisted Laser Desorption Ionisation – Time of Flight (MALDI - TOF TOF) using Applied Biosystems 4800 (ABI 4800) Tof was used to acquire MS/MS spectra. Parent ion spectra were recorded in reflector mode with grid voltage set to 16 kv. Spectra were acquired in the positive ion mode (1 kv) with a total of 300 shots of sub - spectrum laser intensity set to 3300 arbitrary units. The acquisition was internally calibrated using pep - mix 4 (Laser Biolabs, France).

CHAPTER THREE

Results and Discussion of Results

This chapter describes and discusses the results obtained from the preliminary and final studies. All experiments followed the ethical guidelines of the Cape Peninsula University of Technology Research Ethics committee.

3.1 Preliminary study

3.1.1 Collection of *X. laevis*

Frogs collected with the bucket trap from the university pond were identified as *Xenopus laevis* using the catalogue described in Passmore & Carruthers (1995) and Channing (2006). A total of 15 medium sized *X. laevis* frogs were collected, of which 12 frogs were randomly selected for the testing of extraction techniques. The frogs used in the preliminary study weighed between 22 - 30 g. All frogs captured, besides the three frogs used in the skin harvesting technique, were released back into the university pond. The secretions obtained from the swabbing, skin harvesting and physical stimulation techniques yielded minute secretions and could not be quantified. In contrast, the chemical stimulation technique yielded a larger quantity of secretions and were freeze dried and weighed (Table 3.1).

Table 3.1: Quantity of freeze - dried secretions collected from the three *Xenopus laevis* specimens using the chemical stimulation technique

Frog	Weight of frog (g)	Quantity of secretions (mg)	Yield (mg/g)
1	24.4	20	0.82
2	28.4	20	0.70
3	23.4	10	0.43

In this study, yield is defined as the amount of dry secretion (mg) per weight (g) of the frog. The yield, which serves to measure the effectiveness of the stimulation technique, is calculated by dividing the amount of the secretions obtained in milligrams by the weight of the frog in grams. There is a difference in the yield of secretions obtained from the frogs. The

weight of frog one and three are similar. However the quantity of secretions obtained from frog three was half of that of frog one. Frog two weighed the most, but had the same quantity of secretions as frog one. Hence frog one established a greater yield of secretions. An experimental error may have occurred in the application of the chemical stimulation technique on frog three.

3.1.2 Protein visualisation using electrophoresis

The secretions obtained from the *X. laevis* were electrophoresed using a 10% SDS - PAGE. Extracts from the skin harvesting, swabbing and the physical stimulation techniques were electrophoresed on Gel 1. Extracts from the chemical stimulation technique were electrophoresed on Gel 2. Figure 3.1 shows the result of the SDS – PAGE.

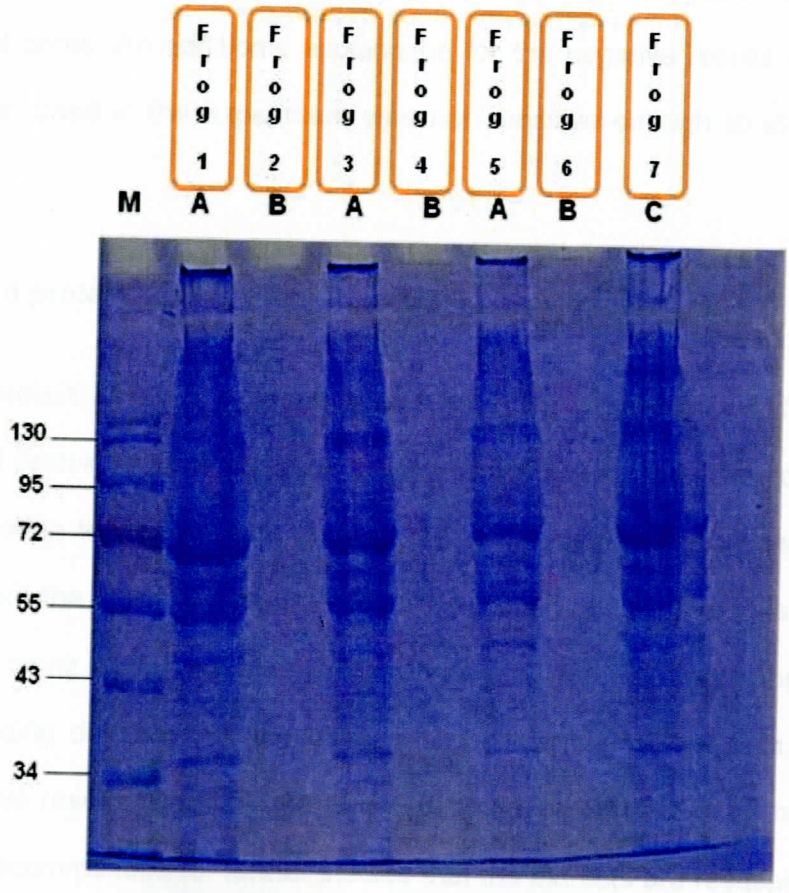

Figure 3.1: SDS - PAGE of *Xenopus laevis* secretions obtained via the skin harvesting technique (Lane A), swabbing technique (Lane B) and physical stimulation technique (Lane C). The lane M indicates the protein molecular weight marker (KDa)

Several bands were found to be present on the gel (Figure 3.1). The bands seen on the gel indicate proteins present in the extract at the molecular weight indicated. These bands do not

necessarily mean that the band is made up of one protein but rather that there are several proteins present with the same molecular weight. The marker used to facilitate band sizing indicated consistent molecular weights at 55 KDa, 72 KDa and 130 KDa for the secretions obtained using the skin harvesting technique. Similar molecular weights were attained from the extracts of the skin harvesting (Lane A) and physical stimulation technique (Lane C).

Electrophoreses of the extracts from the chemical stimulation technique was done on a separate gel. The extracts from the chemical stimulation technique showed no protein bands present at the molecular weights used in this experiment. No protein bands present on the gel does not signify that there are no proteins present in the extract. The extracts may have been too dilute for protein molecules to be detected. Alternatively, the extracts consisted of extremely small or large molecules of proteins which may have been unable to migrate through the gel pores. An additional explanation for the negative results could be that the coomassie stain used in the experiment was not sensitive enough to identify the protein bands.

3.1.3 Bradford protein assay

The Bradford protein assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. The assay is colorimetric. Secretions obtained from the chemical stimulation technique were used in this experiment. Protein concentrations were not obtained from the extracts using the Bradford protein assay. This was indicated by no colour change being observed after adding the dye. For all the sample extracts the absorbance reading detected was negative, which confirmed the no colour change of the dye. The negative results obtained from the experiment could be due to the extracts being too dilute. It is recommended for further studies that the extracts are resuspended in smaller amounts of buffer. A standard curve could not be constructed due to the negative results obtained from the assay.

Even though extracts from the chemical stimulation technique attained negative results from both the SDS - PAGE and Bradford assay, the extracts from the technique could be applied and used in various experiments once it had been freeze dried. These secretions were in a powder form and stock concentration solutions could be formulated when dissolved in water. Secretions from the frog were weighed, unlike in the other techniques tested in the preliminary study. The technique seemed to be the most humane because it was non - destructive, quick, non - invasive, caused minimal stress and provided an acceptable amount of secretions from the frog. For these reasons this technique was selected for use in further studies.

3.1.4 Microtitre assay

The microtitre assay was used to quantify the level of activity of these extracts by determining their Minimum Inhibitory Concentration (MIC) values. The microtiter plate has the advantage of rapidly analysing adhesion of multiple fungal strains or growth conditions within each experiment. The method is robust, inexpensive, provides reproducible results and is 30 times more sensitive than other methods described in literature (Eloff, 1998). Most authors use agar diffusion assays to determine the antimicrobial activity of extracts (Hewitt & Vincent, 1989). The technique works well with defined inhibitors (Hewitt & Vincent, 1989), but when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998).

In microbial assays, serial dilution of the extract in a number of test tubes followed by the addition of the test organism to determine the MIC for the test organism using turbidity is used as an indication of growth (Eloff, 1998). This technique requires relatively large quantities of extracts and is therefore not useful in bioassay guided isolation of antimicrobial compounds. However, the microtitre plate method requires small quantities of the sample, can be used for a large number of samples and leaves a permanent record (Eloff, 1998).

In this study, the microtitre plate technique allows for triplicate bioassays to be carried out on the secretions obtained from each frog. The antifungal activity of the *Xenopus laevis* secretions were assayed against isolates of three fungal species. *Candida albicans* (MRC 8907) and *Aspergillus flavus* (MRC 3954) are fungal strains that pose negative effects on human health and agriculture. The procedure followed is explained in Section 2.2.2 of Chapter 2. The MIC values of the frog secretions against the test fungi are shown in Table 3.2.

Table 3.2: Minimum Inhibitory Concentrations of secretions from a serial dilutions of the *Xenopus laevis* secretions against the test fungi. Results were recorded every 24 hours for 7 days

Fungal Species	Strain	MIC (mg/ml)						
		24hrs	48hrs	72hrs	96hrs	120hrs	144hrs	168hrs
Candida albicans	MRC 8907	0.02	0.02	0.02	0.02	0.02	0.09	0.09
Aspergillus flavus	MRC 3954	NG	0.02	0.04	0.04	0.09	0.09	0.09
Fusarium verticillioides	MRC 826	0.04	0.09	0.39	0.78	1.56	1.56	1.56
		MIC (µl/ml)						
Amphotericin B		6.25	6.25	12.5	12.5	25	50	50

NG: No Growth

The MIC results were similar for the triplicate assays; therefore the standard deviation is equal to zero. This finding is common with MIC's. The technique worked well with all of the test fungi. *Candida albicans* and *Aspergillus flavus* were more sensitive to the extracts than *Fusarium verticillioides.* The extracts after 7 days, inhibited the growth of *C. albicans* and *A. flavus* at relatively low concentrations of 0.09 mg/ml. *F. verticillioides* followed with an average MIC of 1.56 mg/ml. The MIC exhibited that the extract activity was superior to the control.

In the case of isolates of the *Aspergillus* spp. tested, growth inhibition was observed, but conidium formation was enhanced on plates after five days. Conidium formation is known to occur when fungi are under stress due to fungicides, nitrogen fertilisers (Edward, 2004; Jouany, 2007) and environmental factors such as temperature, water availability or co - inoculation, which causes competition between species for resources (Xu *et al*, 2007). It is

likely that the enhanced conidium information observed in this study was due to stress induced by the added frog extracts. The impact of sporulation on the extracts merits further investigation. In the *Candida alibicans* microtitre plate, biofilm developed at the bottom of the plate by the third day of incubation (Figure 3.2).

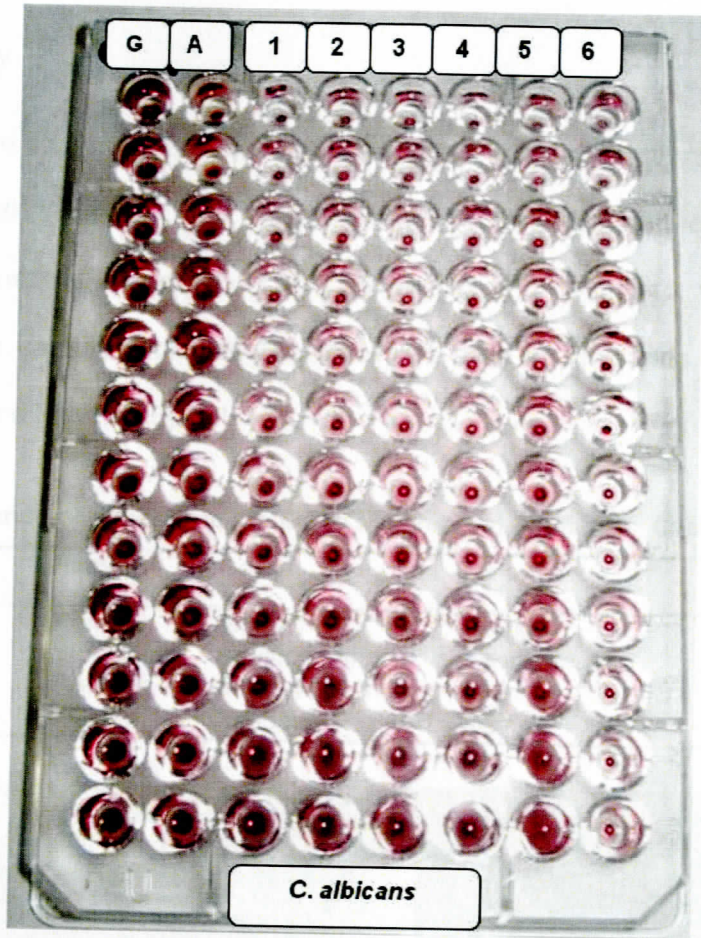

Figure 3.2: Biofilm formation after seven days of incubation on the *C. albicans* microtitre plate. Row G is the growth control (negative control), row A contains Amphotericin B (positive control) and rows 1 to 6 contain the *X. laevis* frog secretions

Biofilms are multicellular matrices of bacteria surrounded by extracellular polysaccharides called a glycocalyx. The glycocalyx acts as a physical barrier and is strongly anionic thereby protecting the fungal microcolony from external agents (Jeyasekaran, *et al.*, 2000). The biofilm progressed with time to wells of higher concentrations of extracts. Biofilm formation may be indicative of the fungi being under stress, as once biofilms are established the fungi appear to have greater resistance to exogenous deleterious agents such as antibiotics, detergents or biocides than their planktonic counterparts (Lewis, 2001). In this study, the extracts were not able to inhibit biofilm growth.

3.1.6 Thin Layer Chromatography (TLC) analysis

TLC plates with the frog secretions were sprayed with a ninhydrin spray reagent. No chromophoric compounds were detected. A possible reason for this was that the concentrations of amino acids in the extracts used were too low to be detected.

3.2 Final study

This is a qualitative study and the aim is to investigate if frog secretions, from selected frog species of the south Western Cape, have antifungal activity. Subsequent to the preliminary study where the method of extraction was developed and optimised; three frogs and one toad species were captured from the Oude Molen area in Pinelands, Cape Town, South Africa (Table 3.3 and Figure 3.3).

Table 3.3: Amphibians collected from the Oude Molen area, Pinelands, Cape Town

Catalogue Number	Family	Genus	Species	Common name
AD 326	Ranidae	*Amietia*	*fuscigula*	Cape River frog
AD 327	Bufonidae	*Amietophrynus*	*pantherinus*	Western Leopard toad
AD 328	Ranidae	*Strongylopus*	*grayi*	Clicking Stream frog
AD 330	Pipidae	*Xenopus*	*laevis*	Platanna

Figure 3.3: Specimens collected from the Oude Molen area. A: *Amietia fuscigula*, B: *Strongylopus grayi*, C: *Amietophrynus pantherinus* and D: *Xenopus laevis*

3.2.1 Collection of frog secretions

Frog secretions were acquired using the chemical stimulation technique, as explained in Section 2.1.2.4 of Chapter 2. The quantities of frog secretions from the various species are shown in Table 3.4.

Table 3.4: The mass of the frog specimens, dry mass of secretions obtained after freeze drying and the yields of secretions obtained

Catalogue Number	Species	Mass of species (g)	Mass of secretion after freeze drying (mg)	Yield (mg/g)
AD 326	*Amietia fuscigula*	80.08	140	1.75
AD327	**Amietophrynus pantherinus*	6.06	130	21.45
AD329	*Strongylopus grayi*	10.28	80	7.78
AD330	*Xenopus laevis*	2.01	90	44.78

** Denotes a toad*

There is a variation in the yield of secretions obtained from the frogs. One would have expected that the larger the frog the greater the skin surface area and number of secretory glands therefore more secretions would be obtained. The results indicate that this was not the case. In this study, the frogs were stimulated once and released back into its natural environment. Mangoni *et al.* (2001) state that frogs that have been pharmacologically depleted of skin antimicrobial peptides will not re - accumulate skin antimicrobial peptides unless the animals are gradually exposed to bacteria or fungi in their environment. In their depleted state these frogs will succumb to overwhelming infection if suddenly exposed to otherwise innocuous microbes. In the past, acquisition of skin secretions necessitated the sacrifice of the frog (Mangoni *et al.*, 2001)

3.2.2 Microtitre plate bioassay

The MIC of the fungi was determined as described in Section 2.2.3 of Chapter 2. All the extracts had substantial antifungal activity with MIC values between 0.04 and 12.5 mg/ml. Good inhibition was shown by all frog secretions on *Candida albicans* with MIC values of between 0.04 and 0.19 mg/ml after 120 hours (Table 3.5).

Table 3.5: MIC values of frog secretions against *Candida albicans* (MRC 8907). Results were recorded every 24 hours for five days

Extract species	MIC (mg/ml)				
	24hrs	48hrs	72hrs	96hrs	120hrs
Amietia fuscigula (AD 326)	0.02	0.04	0.04	0.04	0.04
**Amietophrynus pantherinus* (AD 327)	0.02	0.04	0.19	0.19	0.19
Strongylopus grayi (AD 328)	0.02	0.04	0.09	0.19	0.19
Xenopus laevis (AD 330)	0.02	0.02	0.09	0.19	0.19
	MIC (µl/ml)				
Amphotericin B	0.04	0.39	0.39	0.39	0.78

* *Denotes a toad*

Biofilm formation was evident with *C. albicans* after 72 hours of exposure to the extract. Similar results were obtained against *Aspergillus flavus,* where relatively low concentrations of 0.19 to 0.39 mg/ml were obtained (Table 3.6) after five days.

Table 3.6 MIC values of frog secretions against *Aspergillus flavus* (MRC 3954). Results were recorded every 24 hours for five days

Extract species	MIC (mg/ml)				
	24hrs	48hrs	72hrs	96hrs	120hrs
Amietia fuscigula (AD 326)	NG	0.02	0.19	0.19	0.19
**Amietophrynus pantherinus* (AD 327)	NG	0.02	0.19	0.19	0.39
Strongylopus grayi (AD 328)	NG	0.02	0.09	0.09	0.39
Xenopus laevis (AD 330)	NG	0.02	0.09	0.09	0.39
	MIC (µl/ml)				
Amphotericin B	NG	0.39	1.56	12.5	50

* *Denotes a toad*
NG: No Growth

In the case of isolates of *Aspergillus* spp. conidium formation was enhanced on plates after five days. The conidium growth had similarly occurred with the fungal strain during the preliminary study. Fungi grew freely in the control after five days at 50 mg/ml. Extracts inhibited *Fusarium verticillioides* at 3.12 to 12.5 mg/ml after five days (Table 3.7). Extracts from AD 326, AD 328 and AD 330 against *Fusarium verticillioides* showed MIC's of 0.04 mg/ml after 24 hours which rose to no more than 12.5 mg/ml after 120 hours, while the extract from AD 327 rose from 0.04 mg/ml after 24 hours to 3.12 mg/ml after 120 hours.

Table 3.7: MIC values of frog secretions against *Fusarium verticillioides* (MRC 826). Results were recorded every 24 hours for five days

Extract species	MIC (mg/ml)				
	24hrs	48hrs	72hrs	96hrs	120hrs
Amietia fuscigula (AD 326)	0.04	0.39	1.56	1.56	12.5
**Amietophrynus pantherinus* (AD 327)	0.04	0.39	1.56	1.56	3.12
Strongylopus grayi (AD 328)	0.04	0.39	3.12	3.12	12.5
Xenopus laevis (AD 330)	0.04	0.39	3.12	3.12	12.5
	MIC (µl/ml)				
Amphotericin B	0.78	0.78	1.56	6.25	12.5

* *Denotes a toad*

Candida albicans and *Aspergillus flavus* were most sensitive compared to *Fusarium verticillioides.* The extract from *A. fuscigula* was most active against test fungi compared to the other extracts, particularly against *C. albicans* and *A. flavus* (Figure 3.4). If the MIC values of the frog secretions are compared to that of the control, in most cases the values obtained are two to five times lower than Amphotericin B. *F. verticillioides* was most sensitive to AD 327 with an MIC of 3.12 mg/ml at 120 hours. In general, *F. verticillioides* was the most resistant to all of the extracts.

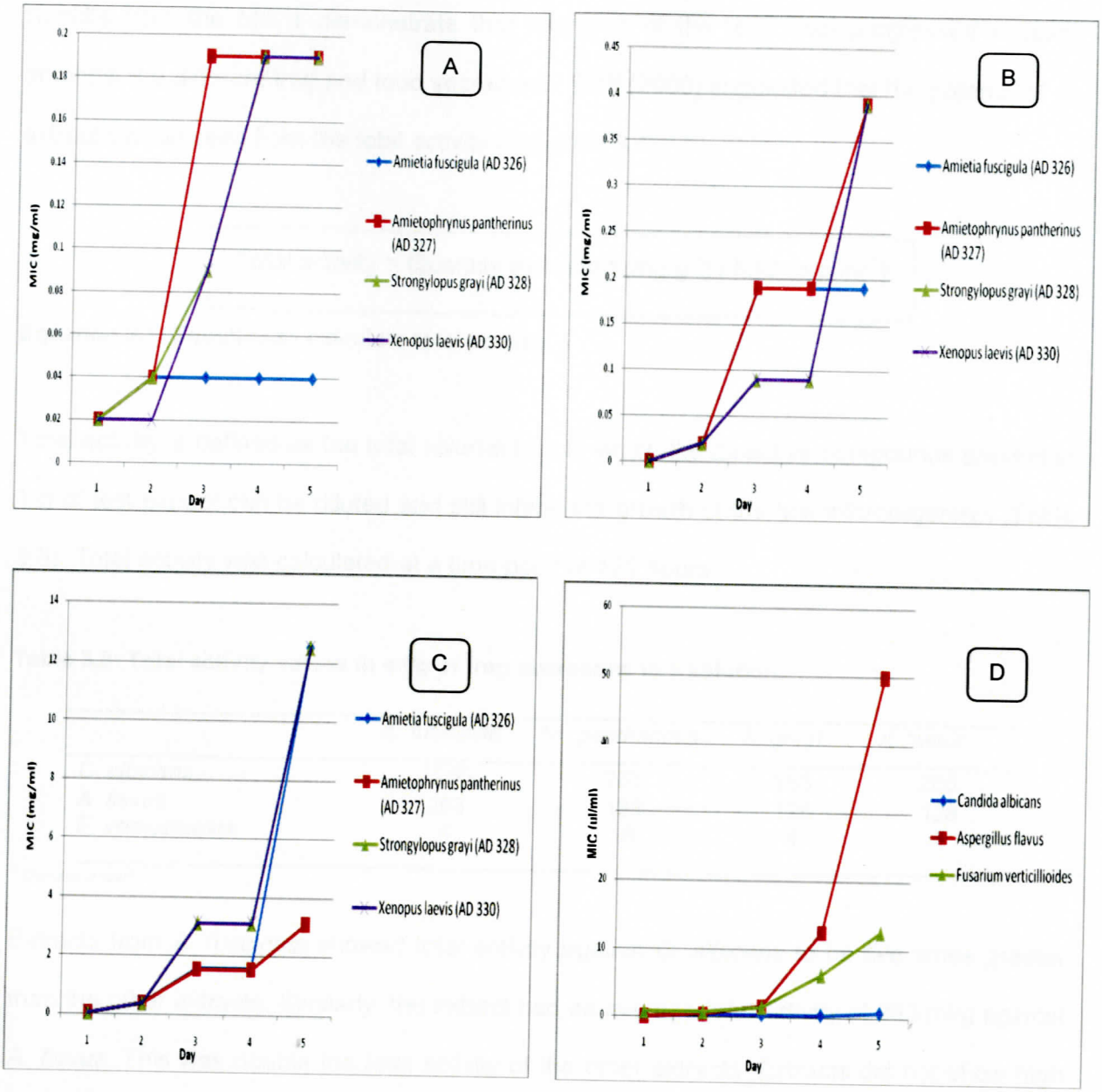

Figure 3.4: Stability graphs of the frog secretions over a five day period. (A) = frog secretions vs *Candida albicans,* (B) = frog secretions vs *Aspergillus flavus,* (C) = frog secretions vs *Fusarium verticillioides*, (D) = Amphotericin B vs the test fungi

In stability graphs, the lower and more horizontal (flatter) the curve, the better the activity of the test extract. A more horizontal line depicts fungicidal activity as opposed to fungistatic activity, i.e. the ability of a test extract to inhibit fungi for a certain time and lose activity thereafter. It is evident that the extracts in general do inhibit the growth of the test fungal species. Extracts from AD 326 in Figure 3.4 (A) is shown to have a horizontal curve against *Candida albicans* indicating good activity of the extract. *Aspergillus flavus* was not sensitive to Amphotericin B (Graph D) and after five days grew freely.

Results from the MIC's demonstrate that inhibition of the test fungi progressed similarly amongst the different frog and toad secretions. Eloff (2000) suggested that the potency of an extract can be seen from the total activity (Equation 3.1).

$$\text{Total activity} = \text{Quantity extracted } (mg.g^{-1}) \,/\, \text{MIC } (mg.ml^{-1})$$

Equation 3.1: Equation to calculate total activity

Total activity is defined as the total volume (ml) to which the bioactive compounds present in 1 g of test extract can be diluted and still inhibit the growth of the test microorganism (Table 3.8). Total activity was calculated at a time point of 120 hours.

Table 3.8: Total activity values in ml/g of frog secretions in a solution

	A. fuscigula	**A. pantherinus*	*S. grayi*	*X. laevis*
C. albicans	1250	263	263	263
A. flavus	263	128	128	128
F. verticillioides	4	16	4	4

* *Denotes a toad*

Extracts from *A. fuscigula* showed total activity against *C. albicans* to be five times greater than the other extracts. Similarly, the extract had an average total activity of 263 ml/g against *A. flavus.* This was double the total activity of the other extracts. Extracts did not show high total activity against *F. verticillioides.*

3.3 Protein visualisation using gel electrophoresis

The secretions obtained from the *X. laevis* were electrophoresed using a 10% SDS - PAGE as described in Section 2.1.3 of Chapter 2. The extracts showed no protein bands present at the molecular weights used in this experiment. As stated in the preliminary study, negative results do not indicate that there are no proteins present in the extract. The extracts may have been too dilute, consisted of extremely small (<10 kDa) or large molecules (>130 kDa) of proteins which may have been unable to migrate through the gel pores or the commassie stain used in the experiment was not sensitive enough to identify the protein bands.

3.4 Chemical fingerprinting

3.4.1 TLC analysis

As with the earlier analysis, no compounds were detected using this method. This shows that no free amino acids were present in the extracts. A possible reason for the negative results may have been due to the extracts being too diluted for the purpose of this experiment. This warrants future research into the use of solvents and reagent sprays to obtain a chemical fingerprint for the frog extracts.

3.4.2 LC - MS analysis

The supernatant of the frog secretions were analysed as described in Section 2.3.2 of Chapter 2. The MS - MS spectra (Figures 3.5 to 3.8) show that there is a difference in the chemical constitution of the different extracts. Analysis of the spectra showed high levels of background activity present. A significant change was seen in the chromotographs after the extracts were purified (Figures 3.5 to 3.8). Each y - peak represents a peptide fragment. Background activity was not entirely removed from the spectra; however the MS - MS spectra indicated the presence of a single compound (peak of interest shown in Figure 3.5) with a molecular weight of 1968 from the extract of *A. fuscigula*. The peak of interest was most abundant in comparison to spectra from the other three extracts and rendered sufficient information to attempt to assign a sequence to the peptide.

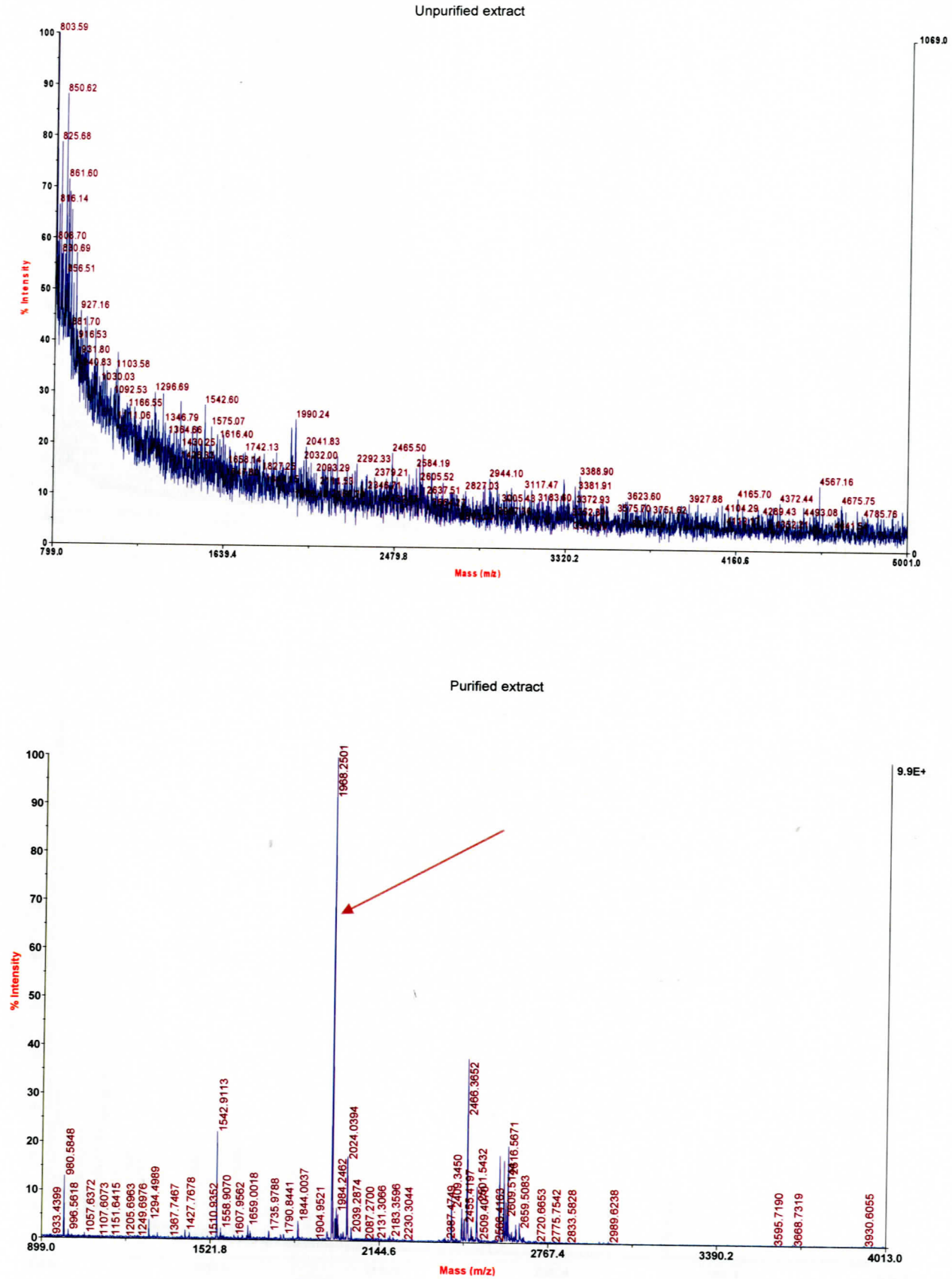

Figure 3.5: MS - MS spectra from the extract of *A. fusigula* prior to purification and after purification. The 'peak of interest' (1968 ion), is marked by an arrow

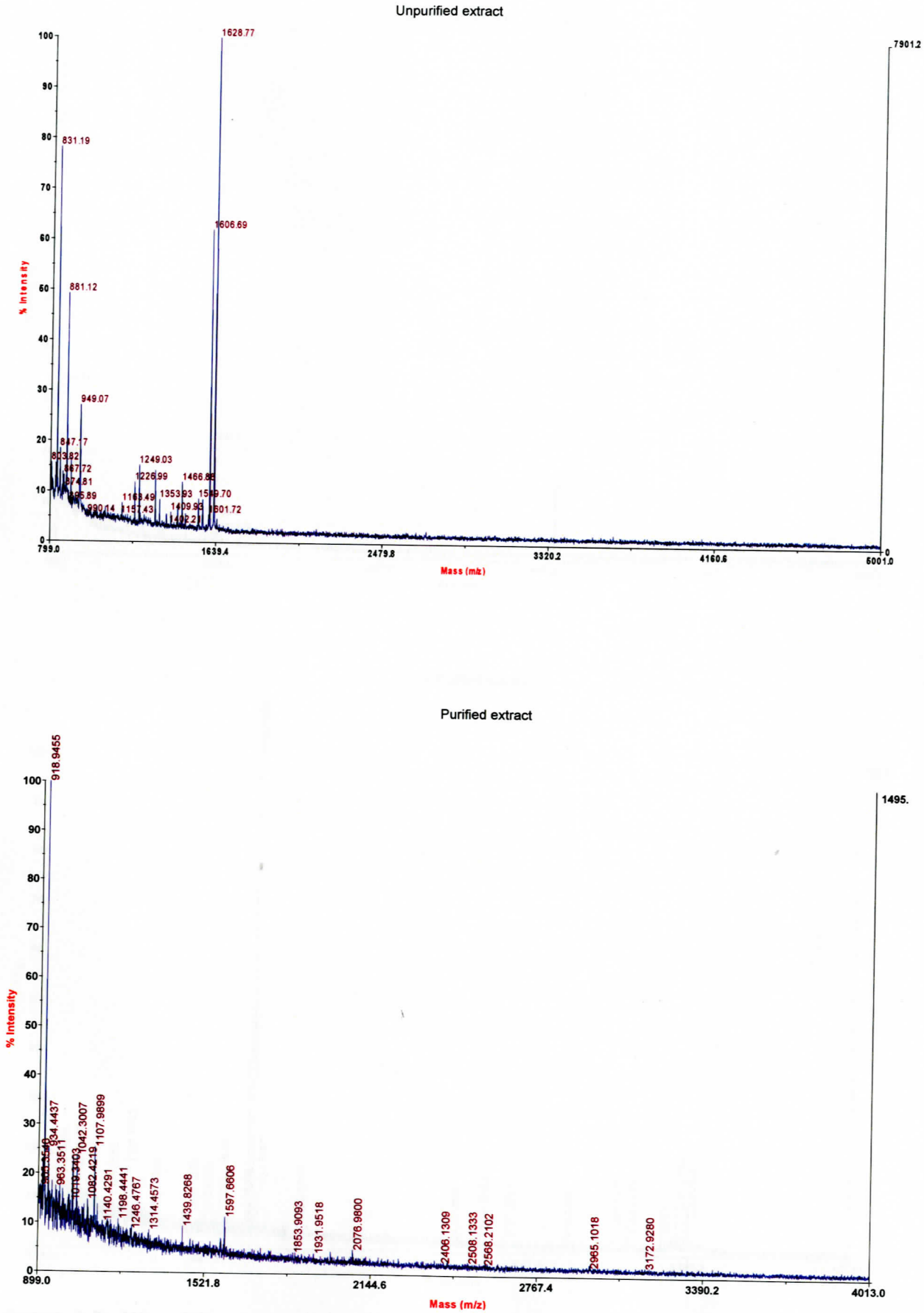

Figure 3.6: MS - MS spectra from the extract of *A. pantherinus* prior to purification and after purification

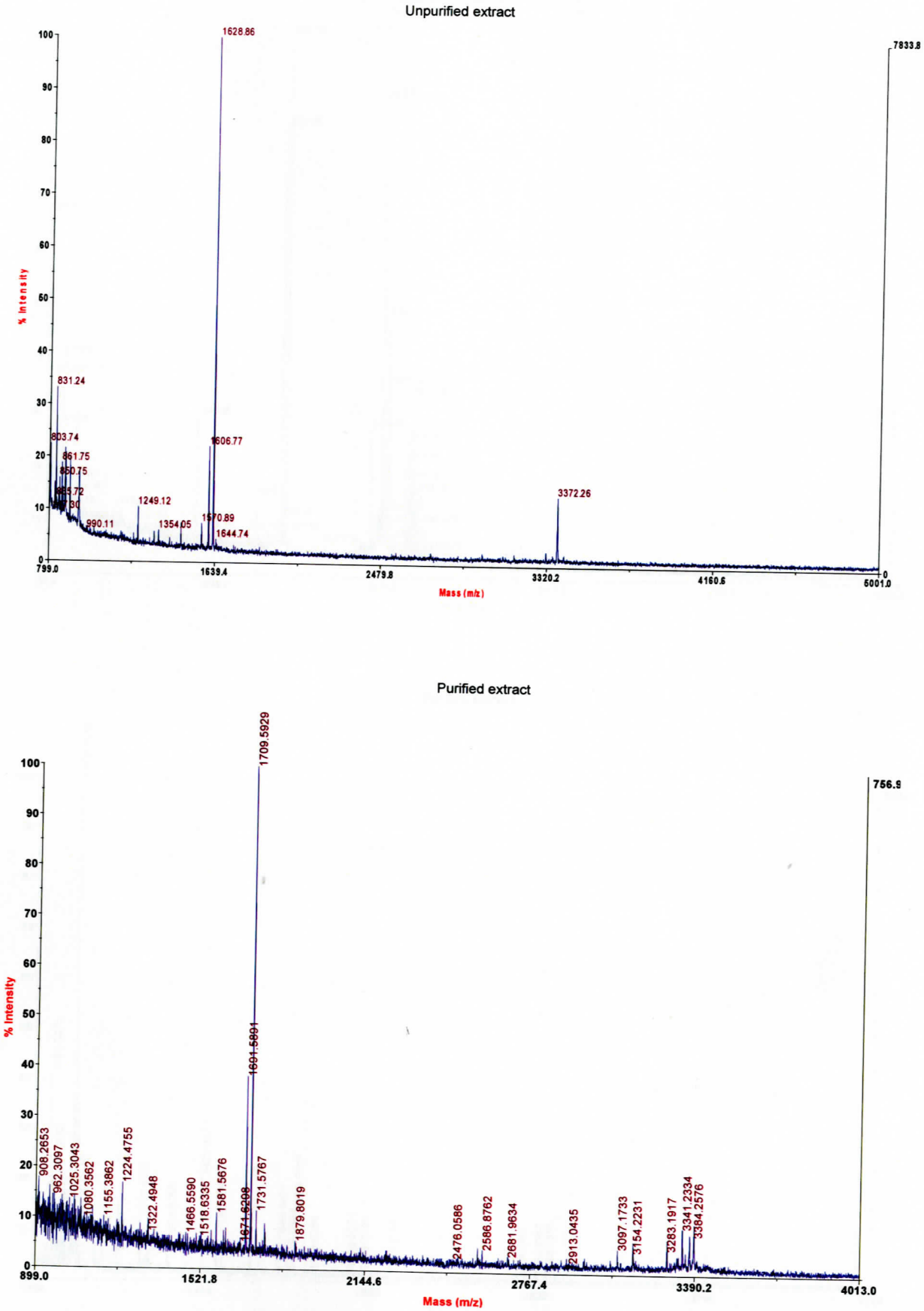

Figure 3.7: MS - MS spectra from the extract of *S. grayi* prior to purification and after purification

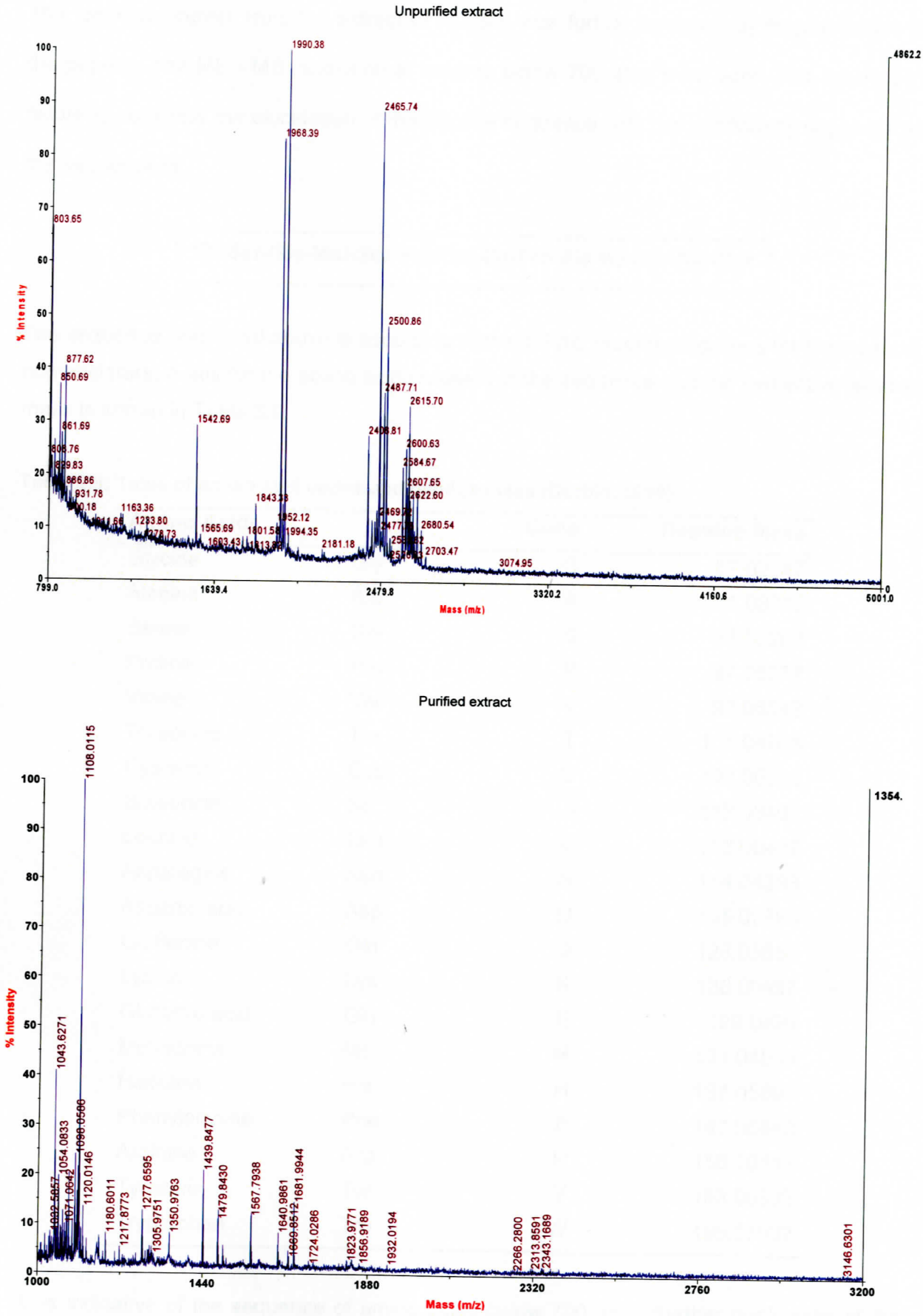

Figure 3.8: MS - MS spectra from the extract of *X. laevis* prior to purification and after purification

The 'peak of interest' from the extract of AD 326 was further analysed by fragmentation of the peptide. The MS - MS resolution at masses below 700 amu were poor. This resulted in failure to complete the elucidation of the peptide sequence. We can confidently suggest that the sequence is:

'R'-Ser-Gly-Met-Ser-Pro-Ser-Ile-Pro-Ala-Ile-Glu-Ala-Met

The sequence presented above is according to the IUPAC recommendations for biochemical nomenclature. A key for the amino acids present in the sequence and the respective residue mass is shown in Table 3.9.

Table 3.9: Table of amino acid codes and residue mass (Durbin, 1998)

Amino Acids		Code	Residue Mass
Glycine	Gly	G	57.02147
Alanine	Ala	A	71.03712
Serine	Ser	S	87.03203
Proline	Pro	P	97.05277
Valine	Val	V	99.06842
Threonine	Thr	T	101.04768
Cysteine	Cys	C	103.00919
Isoleucine	Ile	I	113.08407
Leucine	Leu	L	113.08407
Asparagine	Asn	N	114.04293
Aspartic acid	Asp	D	115.02695
Glutamine	Gln	Q	128.05858
Lysine	Lys	K	128.09497
Glutamic acid	Glu	E	129.0426
Methionine	Met	M	131.04049
Histidine	His	H	137.05891
Phenylalanine	Phe	F	147.06842
Arginine	Arg	R	156.10112
Tyrosine	Tyr	Y	163.06333
Tryptophan	Trp	W	186.07932

"R" is indicative of the sequence of amino acids below 700 amu. Further purification of the extract will have to be done in future to complete the sequence. This can be done by LC - MS or alternatively by automated Edman degradation.

CHAPTER FOUR

General Discussion and Conclusion

In this chapter the following will be discussed: extraction techniques of skin secretions from frogs, the antifungal activity of the frog secretions; the peptide structure identified from the frog *A. fuscigula* and future research in the field.

Frog skin secretions were previously obtained by either electrical stimulation (Dourado *et al.*, 2007; Kim *et al.*, 2007; Nascimento *et al.*, 2007), or hormonal stimulation using norepinephrine (Rollin-Smith *et al.*, 2005; Conlon *et al.*, 2007) or skin harvesting (Roseghini *et al.*, 1989; Daly, 1998; Goraya *et al.*, 2000). More recently the chemical stimulation technique (Che *et al.*, 2008) has been used to obtain frog skin secretions. In the Che *et al* (2008) study, 30 frogs of the same species were used, whereas in the present study a single frog provided sufficient yield for determining antifungal activity. Furthermore, the skin secretions could be obtained in the field and the animals could be released at their site of capture. This reduced the amount of stress imposed on the frogs and ensured that the frogs were released back into their original collection sites.

Following the discovery in 1987 of the magainins in skin secretions of the African clawed frog *Xenopus laevis* (Zasloff, 1987); attention has been increasingly focused upon the skins of frogs and toads as a source of antimicrobial peptides (Nicholas & Mor, 1995; Simmaco *et al.*, 1998). Che *et al.* (2008) state that the skin of anuran amphibians, particularly those belonging to the families Pipidae, Hylidae, Hyperoliidae, Pseudidae and Ranidae, synthesise and secrete a diverse array of antimicrobial peptides. As described by Zasloff (1987), the *X. laevis* frog skin secretions, once freeze dried, were soluble in water. Similar to Zasloff (1987), the present study found that low concentrations of *X. laevis* secretions were able to inhibit growth of *Candida albicans*. Skin secretions from the frog species *Amietia fuscigula, Amietophrynus pantherinus* and *Strongylopus grayi* have not been previously tested for

antifungal activity. *Amietia fuscigula* and *Strongylopus grayi* belong to the family *Pyxicephalidae* and *Amietophrynus pantherinus* is from the family *Bufonidae*. The present study is the first to investigate antifungal activity of skin secretions of frogs from the family *Pyxicephalidae* and the toad species *Amietophrynus pantherinus*. Peptides have been isolated from the skin secretions of toads belonging to the family *Bufonidae* (Clarke, 1997; Maciel *et al.*, 2003; Maciel *et al.*, 2006). However, none of these studies focussed on the species *Amietophrynus pantherinus* or any other South African bufonid representatives.

Frog skin secretions have been tested against the fungus *C. albicans* and it was found that *Rana septentrionalis* (Bevier *et al.*, 2004), *Rana areolata* (Ali *et al.*, 2002), *Amolops loloensis* (Wang *et al.*, 2008) had no activity detected against *C. albicans.* In the present study, the frog skin secretions were tested against three species of fungi; *C. albicans*, A. *flavus* and *F. verticillioides,* which are fungi of medical and agricultural importance. Extracts from all specimens were active against all three species of fungi. The extracts had antifungal activity with MIC values between 0.04 and 12.5 mg/ml. Minimum Inhibitory Concentrations from the present study showed that *Candida albicans* and *Aspergillus flavus* were most sensitive to the extracts as compared to *Fusarium verticillioides.* Fungal strains of *Candida* have been used in previous studies to test the antimicrobial properties of frog secretions (Basir *et al.*, 2000; Ali *et al.*, 2001 & Wang *et al.*, 2007). The present study provides a novel approach in treating infections caused by *A. flavus* and *F. verticillioides,* which are fungal strains of medical and agricultural importance.

In a comparative study investigating the antimicrobial properties of indigenous South African aromatic plants with commercially available essential oils, the MIC values against pathogens ranged between 4 – 16 mg/ml (Van Vuuren, 2006). Antifungal activity of cecropins (silkworms) were found to be 0.03 mg/ml (Moore, *et al.*, 1996). In another study where resistance of animal fungal pathogens were tested using various solvents using bioassays, the average MIC obtained from all the solvents tested was 253 mg/ml (Eloff, 2007). In studies where frog secretions were tested against fungal strains MIC's were recorded at 0.03

mg/ml (Wang *et al.*, 2007), 0.04 mg/ml (Ali *et al.*, 2001), 0.15 mg/ml (Basir *et al.*, 2000) and ranged between 0.03 to >0.10 mg/ml. When comparing results of the present study, the MIC obtained from the bioassays indicate that small concentrations (0.04 to 12.5 mg/ml) of frog skin secretions are potent enough to inhibit fungal strains.

The chromatographs presented in this study indicate that the chemical fingerprint is highly specific for each species of frog. MS - MS spectra indicated the presence of a single compound with a molecular weight of 1968 from the extract of *A. fuscigula*. The peptide was partially elucidated, and could be identified as a putative protein structure. Further research is warranted into the peptides found in the skin secretions of this species.

As described in the present qualitative study, skin secretions have antifungal properties and therefore have therapeutic potential in the treatment of fungal infections in both man and agriculture. The results obtained in the present study demonstrated the potential of novel antimicrobial peptides being isolated from frog species in the south Western Cape and South Africa. The use of peptides to provide alternative approaches to multi - drug resistant organisms is of current relevance to the pharmaceutical industry and for public health (Daly, 1998).

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