

Full Length Research Paper

Antifungal activity of epithelial secretions from selected frog species of South Africa

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Resistance to antibiotics has been acknowledged as a major global public health problem. The use of antimicrobial peptides to provide alternatives to combat multi-drug antibiotic resistance is beginning to attract increasing attention. The high diversity of amphibian skin peptides renders anurans an important potential source for the discovery of novel pharmacophores. This study aimed to investigate the antifungal activity of skin secretions from selected frogs (*Amietia fuscigula*, *Strongylopus grayi* and *Xenopus laevis*) and one toad (*Amietophrynus pantherinus*) of the south Western Cape Province of South Africa. Initially, different extraction techniques for the collection of skin secretions were tested and optimized, thereafter the extracts were tested against three fungal species of medical and agricultural importance that is, *Candida albicans*, *Fusarium verticillioides* and *Aspergillus flavus*. Chemical stimulation gave the best yield by mass, and secretions from *A. fuscigula* showed the best activity with an MIC of 40 µg / ml against *C. albicans* and 200 µg / ml against *A. flavus*. In general, *C. albicans* and *A. flavus* were the most sensitive while *F. verticillioides* was the most resistant. From this study it appears that bioprospecting of South African frog species has the potential to yield potential therapeutic lead agents.

Key words: Antifungal, African anurans, antimicrobial peptides (AMP), *Candida albicans*, *Aspergillus flavus*, bioprospecting, minimum inhibitory concentrations (MIC).

INTRODUCTION

Fungi are an important cause of human, animal and plant disease. Fungal infections in particular, have increased dramatically due to their opportunistic occurrence as a result of reduced immune status associated with the human immunodeficiency virus/ acquired immunodeficiency syndrome (HIV/AIDS) pandemic, cancer chemo-

therapy and transplantation surgery (Ascioglu et al., 2002). In particular, oral and genital candidosis due to *Candida albicans* and related species is a significant cause of morbidity and mortality (Jarvis, 1995). Treatment is limited by the narrow range of effective antifungal agents available, their toxicity (Feldmesser,

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Abbreviations: HIV/AIDS, Human immunodeficiency virus/acquired immunodeficiency syndrome; PPE, porcine pulmonary edema; NTDs, neural tube defects; HBV, hepatitis B virus; AMPs, antimicrobial peptides; EDTA, ethylene diaminetetraacetic acid; PDA, potato dextrose agar; PDB, potato dextrose broth; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MIC, minimum inhibitory concentration.

2003), emergence of resistance strains (Klepser, 2001) and relapse and re-infection (De Bruyne, 1997).

With all the research focus on human disease, very little attention has been given to fungi and the toxins they produce that pose a threat to agriculture, and thereby cause disease in humans. 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 and 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 fungi associated with corn / maize used for human and animal consumption throughout the world (Marasas et al., 1984). *F. verticillioides* and *Fusarium moniliforme* produce fumonisins which have been implicated as a risk factor in the development of oesophageal cancer in the Transkei region of South Africa as well as in the Cixian and Linxian counties of the People's Republic of China (Sydenham et al., 1996) and is a cause of leukoencephalomalacia in horses and porcine pulmonary edema (PPE) (Marasas et al., 1988). More importantly fumonisin exposure has been associated with neural tube defects (NTDs) (Marasas et al., 2004). Another important fungus is *Aspergillus flavus* which grows on crops left on the ground or stored in poor conditions and produces the aflatoxin mycotoxins. Aflatoxins are field and storage mycotoxins which are potent carcinogens, mutagens and immuno-suppressing agents (Katerere et al., 2008). They can act in synergy with the Hepatitis B virus (HBV) to increase the risk of hepatocellular carcinoma (Bhat and 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).

For centuries plants have been the major source of active compounds for pharmaceutical products (Springfield and Weitz, 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). The utilization of animal-based medicines also has a long history from ancient times (Weiss, 1947; Angeletti et al., 1992; Rosner, 1992) and scientists are increasingly exploring the use of metabolites from animals for antimicrobial activity.

Amphibians in particular offer an attractive source of novel antimicrobials because they exist in microorganism-rich environments, causing them to produce potent antimicrobial peptides (AMPs) as an innate form of defence (Govender et al., 2012). Given the respiratory and antimicrobial functions of the amphibian skin, it is likely that some of the molecules found in amphibian secretions may be of use in the treatment of skin and respiratory infections (Clarke, 1997). Extensive studies have been conducted on AMP of frogs belonging to the

genus *Rana* (Che et al., 2008; Simmaco et al., 1998a).

However there have been few studies on the antimicrobial activity of African frog species despite the large number of species and high endemicity which is typical of tropical West Africa, southern Africa and the Madagascari islands (Channing, 2001; Glaw and Vences, 2007; Poynton, 1999; Rödel, 2000). South Africa alone is home to over 100 anuran species (Minter et al., 2004) of which nearly half are found in the Western Cape Province and 27 species are endemic to the south Western Cape region (De Villiers, 2008). This large diversity and density may correlate to a great molecular diversity creating high potential for the discovery of novel therapeutic peptides.

This study aimed to extract and test secretions of frog and toad species found in the South Western Cape Province for antifungal activity. The first phase investigated the optimum extraction method. This method was then used to obtain secretions from three frog (*Amietia fuscigula*, *Strongylopus grayi* and *Xenopus laevis*) and one toad species (*Amietophrynus pantherinus*) which were then tested for activity against three fungal species using the microplate bioassay method.

MATERIALS AND METHODS

Ethical considerations

An application was made to and approved by the Cape Peninsula University of Technology (CPUT) Health and Applied Sciences Research Ethics committee. A permit from the Cape Nature Conservation of South Africa was obtained for the collection of the specimens used. Upon capture the species were identified by Dr Abeda Dawood, a qualified zoologist.

Field collection

A pond at the University of the Western Cape, Cape Town, South Africa, was identified for the collection of specimens of *X. laevis*. A total of 15 medium sized *X. laevis* frogs were collected, of which 12 weighing between 22 and 30 g were randomly selected for the testing of extraction techniques. These specimens of frog were collected using a home-made bucket trap with a narrowing entry hole during May 2008. This entry hole is large enough to let the frog in but not out. Ox liver was used as bait to attract the frogs into the bucket. The bucket with ox liver in hosiery material was submerged overnight and then collected in the morning. All frogs captured, besides the three frogs used in the skin harvesting technique, were released back where they were collected.

Four specimens from three species of frogs (*A. fuscigula*, *S. grayi* and *X. laevis*) and one toad species (*A. pantherinus*) were collected in the wild from the Oude Molen Village, Pinelands, Cape Town, South Africa during June 2008. The numbers collected were limited by the number of frogs available. Frogs and collecting sites are illustrated in Figures 1 and 2.

Evaluation of extraction techniques of frog and toad secretions

The four techniques tested to extract the frog skin secretions were swabbing, physical stimulation, tissue harvesting and chemical stimulation. Three *X. laevis* were used for each technique. The



Figure 1. A map of South Africa showing the location of the study site.

extracts obtained were freeze-dried and then weighed and the yield calculated per body mass. The extracts were in each case re-constituted into 100 μ l of sterile distilled water.

Swabbing - a novel approach

This was a new method developed for this study. The dorsal surface of three frogs was massaged gently with a cotton swab. The swabs were then placed in Eppendorf tubes, sealed and stored at -20°C . A 100 μ l of distilled water was used to wash off the secretions on the swab and this sample was used further in the analysis.

Physical stimulation - a novel approach

A frog was placed in a Ziploc[®] bag with 30 ml of double distilled water as illustrated in Figure 3 and was shaken in the bag, gently for 5 min. The distilled water containing the skin secretions was then poured from the bag into 50 ml centrifuge tubes and an additional 10 ml of double distilled water that was used to rinse the frog in the bag was added to the 50 ml centrifuge tube. The 40 ml samples were freeze-dried and then stored at -20°C for further analysis.

Tissue harvesting

The tissue harvesting technique of Goraya et al. (2000) was modified in this study. Frogs were individually euthanized by placing the frog in a bottle that contained tricaine methane sulfonate (MS 222) dissolved in distilled water. As much of the dorsal skin of the frogs was then removed and stored at -20°C . The tissue was homo-

genized in 10 ml of distilled water and freeze dried.

Chemical stimulation

This technique was adapted from Che et al. (2008). Cotton wool was soaked in anhydrous ether and placed into a tube. An individual frog was placed in a clean 5 l glass bottle. The plastic test tube with the cotton wool soaked in anhydrous ether was put into the bottle and the lid was tightened. The frog was left in the bottle for 2 min. The plastic test tube was removed from the bottle and the frog was rinsed with 15 ml of buffer (0.1 M phosphate buffer, containing 5 mM ethylene diaminetetraacetic acid (EDTA), pH 6.0) to wash off the skin secretions. The buffer containing the secretions was then freeze-dried.

Testing for anti-fungal activity

Three fungal species of medical and agricultural importance were used viz: *F. verticillioides* (MRC 826), *A. flavus* (MRC 3954), *C. albicans* (MRC 8907). These fungi were obtained from the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit of the South African Medical Research Council (MRC). The fungal isolates were grown on potato dextrose agar (PDA) for five days at 25°C and then stored 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) standardized to 0.5 McFarland. The microtitre plate method described in detail by Katerere and Eloff (2005) and Thembo and co-workers (2010) was used. The indicator p-INT was included at the beginning of the experiment and visual inspection of the plates was done every 24 h and MIC recorded for up to five days.



Figure 2. Specimens collected from the Oude Molen area. **A)** *Amietia fuscigula*, **B)** *Strongylopus grayi*, **C)** *Amietophrynus pantherinus* and **D)** *Xenopus laevis*.



Figure 3. The physical stimulation technique being applied to the *X. laevis*.

Table 1. Secretion yields of anurans collected from the Oude Molen area, Pinelands, Cape Town.

Catalogue number	Family	Species	Common name	Mass of species (g)	Absolute yield (mg)	Yield (mg/g)
AD 326	Ranidae	<i>Amietia fuscigula</i>	Cape River frog	80.08	140	1.75
AD 327	Bufoidea	<i>Amietophrynus pantherinus</i>	Western Leopard toad	6.06	130	21.45
AD 328	Ranidae	<i>Strongylopus grayi</i>	Clicking Stream frog	10.28	80	7.78
AD 330	Pipidae	<i>Xenopus laevis</i>	Platanna	2.01	90	44.78

RESULTS

Extraction technique selection

The amount of secretions obtained from the swabbing, skin harvesting and physical was so small that it was impossible to analyse using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the Bradford assays. In contrast, the chemical stimulation technique yielded a larger quantity of secretions which. A total of 50 mg was obtained which translated to a yield of 0.66 mg/g. Due to its superiority and ease of use, the chemical stimulation method was selected as the method of choice for subsequent use. The four species collected and the yield obtained are shown in Table 1.

A wide variation in the yield of secretions was observed. It would have been 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. Unfortunately, due to the limitations on our collecting permit we could not collect more than one specimen for each species. It might be worth investigating whether the yield is repeatable with a larger sample size.

In this study, the frogs were stimulated once and released back into their natural environment. Mangoni et al. (2001) stated that frogs that have been pharmacologically depleted of skin antimicrobial peptides will not easily recover unless the animals are gradually exposed to bacteria or fungi in their environment. In their depleted state these frogs will succumb to infection if suddenly exposed to otherwise innocuous microbes. This seems to indicate that AMPs are inducible rather than constitutive compounds.

Antifungal activity for the different frog and toad species was found to be between 0.04 and 12.5 mg/ml (Table 2). Good inhibition was shown by all frog secretions on *C. albicans* with minimum inhibitory concentration (MIC) values of between 0.04 and 0.19 mg/ml after 120 h. Apparent biofilm formation was evident with *C. albicans* after 72 h of exposure to the extract. This makes the potency of extracts from *A. fuscigula* (AD 326) all the more interesting. *Candida* species are well known for

biofilm formation which results in reduced sensitivity (d'Enfert, 2006). Similar results were obtained against *A. flavus*, where relatively low concentrations of 0.19 to 0.39 mg/ml were obtained after five days. *F. verticillioides* was generally resistant to all extracts.

In the case of isolates of *Aspergillus* spp. conidium formation was enhanced on plates after five days. Conidial formation may be an indication of stress and it might ironically result in increased biosynthesis of mycotoxin (Guzman-de-Peña and Ruiz-Herrera, 1997). 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, that is, the ability of a test extract to inhibit fungi for a certain time and lose activity thereafter (Thembo et al., 2010). It is evident that the extracts are particularly stable and maybe fungicidal against *C. albicans*, with MIC not exceeding 0.19 mg / ml after five days. AD 326 is particularly stable and potent.

DISCUSSION

Frog skin secretions were previously obtained by either electrical stimulation (Dourado et al., 2007; Kim et al., 2007; Nascimento et al., 2007), chemical stimulation using norepinephrine (Conlon et al., 2007; Nascimento et al., 2007; Rollins-Smith and Reinhart, 2005) or skin harvesting (Goraya et al., 2000; Roseghini et al., 1989). More recently the irritant chemical stimulation technique (Che et al., 2008) has been used to good effect. 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 the bioassays. 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 on their original collection sites.

Following the discovery in 1987 of the magainins in skin secretions of the African clawed frog *X. laevis* (Zaslouff, 1987), attention has been increasingly focused upon the skins of anurans as a potential source of

Table 2. Antifungal activity of anuran extracts against three fungal species.

Extract species	MIC (mg ml ⁻¹)											
	48 h			72 h			96 h			120 h		
	Fv ^a *826	Ca ^b *8907	Af ^c *3954	Fv 826	Ca 8907	Af 3954	Fv 826	Ca 8907	Af 3954	Fv 826	Ca 8907	Af 3954
<i>Amieta fuscigula</i> (AD 326)	0.39	0.04	0.02	1.56	0.04	0.19	1.56	0.04	0.19	12.5	0.04	0.19
<i>Amietophrynus pantherinus</i> [#] (AD 327)	0.39	0.04	0.02	1.56	0.19	0.19	1.56	0.19	0.19	1.56	0.19	0.39
<i>Strongylopus grayi</i> (AD 328)	0.39	0.04	0.02	3.12	0.09	0.09	3.12	0.19	0.09	12.5	0.64	0.39
<i>Xenopus laevis</i> (AD 330)	0.39	0.02	0.02	3.12	0.09	0.09	3.12	0.19	0.09	12.5	0.19	0.39
Amphotericin B*		0.52			1.17			6.38			21.0	

^a*Fusarium verticillioides* (MRC 826); ^b*Candida albicans* (MRC 8907); ^c, *Aspergillus flavus* (MRC 3954); *average MIC in µl/ml; #denotes toad species.

novel antibiotics (Nicolas and Mor, 1995; Simmaco et al., 1998b). Che et al. (2008) stated that the skin of amphibians, particularly those belonging to the families of Pipidae, Hylidae, Hyperoliidae, Pseudidae and Ranidae, synthesize and secrete a diverse array of antimicrobial peptides. Similar to the study of Zasloff (1987), the present study found that low concentrations of *X. laevis* secretions were able to inhibit growth of *C. albicans*. Skin secretions from the frog species *A. fuscigula*, (Pyxicephalidae), *A. pantherinus* (Bufonidae) and *S. grayi* (Pyxicephalidae) have not been previously tested for antifungal activity and this study is the first to do so. Peptides have been isolated from the skin secretions of toads belonging to the family *Bufonidae* (Clarke, 1997; Maciel et al., 2003, 2006) however, none of these studies focused on the species *A. pantherinus* or any other South African bufonid representatives.

Previous studies have reported that secretions from *Rana septentrionalis* (Bevier et al., 2004), *Rana areolata* (Ali et al., 2002), *Amolops loloensis* (Wang et al., 2008) showed no activity against *C. albicans*. In the present study, the frog skin secretions were active against not only *C. albicans* but also *A. flavus*. Strains of *Candida* spp have been used in previous similar studies (Ali et al., 2001; Basir et al., 2000; Wang et al., 2007) and the activity found ranged from 0.03 to >0.10 mg/ml. The results are similar to those obtained in this study. There is a need to isolate more secretions from the most active species, re-test them and isolate and elucidate the bioactive peptides for possible further research and development.

Conclusion

This study demonstrates the potential of finding novel antimicrobial peptides from anurans of the south Western Cape and South Africa. The use of peptides to provide alternative approaches to combating multi-drug resistant organisms is gaining momentum. This study presents a strong case for broadening research focus from biopros-

pecting for antimicrobials in medicinal plants to Africa's amphibian species, which are largely unexplored and may present a treasure trove for finding novel therapeutics.

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