Isolation and characterization of compounds from *Podocarpus henkelii* (Podocarpaceae) with activity against bacterial, fungal and viral pathogens

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Declaration

I declare that the thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor has not previously been submitted by me for a degree at this or any other University. That it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

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Victor P. Bagla

Acknowledgement

This project is dedicated to my mum, late Princess Mamie Bagla, may your soul rest in perfect peace.

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List of abbreviations

Ace	Acetone
AIDS	Acquired immune deficiency syndrome
Amp-B	Amphotericin-B
Ann.s	*Annona senegalensis*
AS	*Acokanthera schimperi*
ATCC	American type culture collection
BEA	Benzene/ethanol/ammonium hydroxide
BERB	Berberine chloride
BD	Bovine dermis cells
CC_{50}	Cytotoxicity (50% cell death)
CE	*Carissa edulis*
CEF	Chloroform/ethyl acetate/formic acid
CDV	Canine distemper virus
CHB	Chronic hepatitis B
CMV	Cytomegalovirus
CPE	Cytopathic effect
CPIV	Canine Parainfluenza virus
CRFK	Crandell feline kidney cells
DCM	Dichloromethane
DMEN	Dulbecos minimun essential medium

DMSO	Dimethyl sulfoxide
DNA	Dioxiribonucliec acid
DPPH	1-1-diphenyl-2-picryl-hydrazyl
EC	*Ekebergia capensis*
EC_{50}	Effective concentration 50
ELIZA	Enzyme linked immunosorbent assay
EMW	Ethyl acetate/methanol/water
EsbL	Extended spectrum b-lactamase
FCS	Fetal calf serum
FHV	Feline herpes virus
FMDV	Foot and Mouth Disease Virus
FMD	Foot and Mouth Disease
GI	Gastrointestinal
HAART	Highly active antiretroviral therapy
HCMV	Human cytomegalovirus
Hep G2	Human hepatoma cell line
Hex	Hexane
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive care unit
INT	*p*-iodonitrotetrazolium violet,

LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MDR	Multidrug resistance
MEM	Minimum essential medium
Met	Methanol
MH	Müller-Hinton
MIC	Minimum inhibitory concentration
MPs	Medicinal plants
MRSA	Methicillin- resistant *Staphylococcus aureus*
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NCCLS	National Committee for Clinical Laboratory Standards
NCE	New chemical entities
N/D	Not done
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
OIE	Office International des Epizooties
OPC	Oropharyngeal candidosis
PBS	Phosphate buffered saline
PH	*Podocarpus henkelii*
PPR	Peste des petits ruminants

Pz	*Plumbago zeylanica*
REACH	Registration, Evaluation and Authorisation of Chemicals
R_f	Retardation factor
RNA	Ribonucliec acid,
ROS	Reactive oxygen species
RP	Rinderpest
RS	Reactive species
RSV	Respiratory syncytial virus
RVF	Rift Valley fever
Sca	*Schrebera alata,*
SD	Sabrouraud dextrose broth,
SI	Selectivity index,
SV	Simian virus
$TCID_{50}$	Tissue culture infective dose 50
TLC	Thin layer chromatography
UK	United Kingdom
UV	Ultraviolet
VREF	Vancomycin resistant *Enterococcus faecalis*
VZV	Varicella-zoster virus
WHO	World Health Organisation
4-NQO	Nitroquinoline-1-oxide

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Bagla, V.P., McGaw, L.J. and Eloff, J.N. Comparative cytotoxicity studies of extracts of selected medicinal plants on different cell types.

Abstract

Diseases caused by bacteria, fungi and viruses pose a significant threat especially to poor rural communities. Viral infections are frequently complicated by secondary bacterial and fungal infections which remain a major challenge globally and in particular, in sub Sahara Africa amongst humans and animals alike. The main aim of this study was to develop a low toxicity plant extract or isolated compound active against viral, bacteria and fungal pathogens from selected plant species.

Seven tree species that were investigated were *Acokanthera schimperi, Carissa edulis, Ekebergia capensis, Podocarpus henkellii, Plumbago zeylanica, Annona senegalensis* and *Schrebera alata* traditionally used in the treatments of various ailments were selected and extracted using solvents of varying polarity. Extracts of selected plants were tested for activity against two Gram positive and two Gram negative bacterial namely *Enterococcus faecalis* and *Staphylococcus aureus* and two Gram-negative species, *Pseudomonas aeruginosa* and *Escherichia coli* respectively, three fungal pathogens: *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigates* and four enveloped animal viruses: feline herpes virus–1 (FHV-1, dsDNA), canine distemper virus (CDV, ssRNA), canine parainfluenza virus-2 (CPIV-2, ssRNA) and lumpy skin disease virus strain V248/93 (LSDV, dsDNA). The presence of antioxidant constituents in the different extracts and cytotoxicity against three cell types CRFK, bovine dermis and Vero cells were determined. Bioautography and the serial microplate dilution methods were used to determine the number of antimicrobial compounds and antimicrobial activity of extracts against bacterial and fungal pathogens. Virucidal and attachments assays were used to determine the activity against viral pathogens. Qualitative antioxidant activities of extracts were tested using the DPPH reagent and cytotoxicity using the MTT assay.

Biological activity was observed in all the extracts against one or more organisms on bioautography. The intermediately polar system (CEF) separated more active constituents. Some extracts had compounds with similar R_f values active against one or more organisms. In both the antibacterial and antifungal assays, acetone extracts had the highest activity followed by DCM against one or more pathogens. Hexanes extracts were the least active. *P. henkellii* extracts had more active compounds against the bacteria and *Annona senegalensis* against the fungi. In the microdilution assay, *S. aureus* was the most susceptible bacterial organism to extracts of the different plant, followed by *P. aeruginosa* and *Escherichia coli,* and *E. faecalis* the least. *C. neoformans* on the other hand was the most susceptible fungal pathogen. In the antiviral assay, although activity was observed with hexane extracts of some plants in the virucidal assay, the most potent inhibition was observed with the acetone and methanol extracts of *Podocarpus henkelii* against CDV and LSDV in the virucidal assay and acetone extracts in the attachment assay.

In general the hexane was the least toxic while the intermediate polarity extracts were generally the most toxic indicating that highly polar compounds were possibly poorly or highly absorbed through membranes in the former and later respectively. Of the three cell types used CRFK was the most sensitive followed by bovine dermis and Vero cells the least. Cytotoxicity studies of extracts of the different plants revealed *A. senegalensis and A. schimperi* extracts were the most toxic plants in the cellular assay. These plants are toxic to animals and the cytoxicity is in line with the *in vivo* toxicity. The protective effects of antioxidant constituents in some extracts varied and appear to be influenced by the metabolism of the type of cell in culture. It also appears to suggest that metabolism in kidney-derived cells can be influenced by species variation in the origin of cells.

P. henkellii was selected for isolation of bioactive compound. Three compounds were isolated and their structure elucidated using ^{13}C and ^{1}H NMR and mass spectrometric data. The antibacterial, antifungal and antiviral activity of the isolated compounds 7', 4', 7'', 4''', tetramethoxy amentoflavone (C1), isoginkgetin (C2) and Podocarpusflavone–A (C3) were determined. Compound C2 was the most active against *E. coli* and *S. aureus* (MIC = 60 µg/mℓ) and a selectivity index (SI) value of 16.67. The compound was also active against *A. fumigatus* and *C. neoformans* (SI = 33.33) suggesting both antibacterial and antifungal activity with relative safety. Compound C3 had a broad spectrum of activity against *E. faecalis* and *P. aeruginosa* with SI values of 4. A less potent activity of the compounds was obtained in both the virucidal and attachment assays against test pathogens, indicating the lower activity of the compounds against tested viral pathogens. The studies further suggest structural activity relationship in the antimicrobial activity of biflavonoids. The compounds C1 and C2 had no toxic effect on the three cell types and mutagenicity studies indicated no activity of these compounds.

Podocarpusflavone-A occurs in every species of *Podocarpus* so far investigated, except *P. latifolius.* These studies represent the first isolation of bioactive compounds from *P. henkellii.* Although a different extractant was used than that used by traditional healers, the presence of antiviral compounds in *Podocarpus henkelii* against two unrelated viruses may justify on a chemotaxonomic basis the traditional use of related species *Podocarpus latifolius* and *Podocarpus falcatus* in the traditional treatment of canine distemper infection in dogs.