Isolation and Characterisation of Bioactive Compounds from *Commelina benghalensis* Linn: Biological activity analysis of extracts against Wil-2 NS lymphoma cancer cell lines and selected pathogenic microorganisms

By

MATLOU P. MOKGOTHO

RESEARCH THESIS

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Supervisor: **Prof Mampuru L.J.**
Department of Biochemistry, Microbiology and Biotechnology, University of Limpopo

Co-Supervisor: **Prof Eloff J.N.**
Department of Paraclinical Sciences, University of Pretoria

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DECLARATION

I declare that the thesis hereby submitted to the **University of Limpopo** for the degree of **PHILOSOPHIAE DOCTOR (PhD)** has not been previously submitted by me for the 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.

_____

M.P Mokgotho (Mr) 25 September 2009

DEDICATION

I dedicate this work to all the people who always believed in me and positively contributed to my success.

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LIST OF ABBREVIATIONS

A

ACE	acetone
AGC	accelerated gradient chromatography
AIDS	acquired immunodeficiency syndrome
AO/EB	acridine orange/ethidium bromide
ATCC	American type culture collection

B

BEA	benzene: ethanol: ammonium hydroxide

C

CAAX	cysteinyl-leucyl-isoleucyl/valinyl-methionine
$CHCl_3$	trichloromethane
CI	compound I
CII	compound II
^{o}C	degree Celsius
CEF	chloroform: ethyl acetate: formic acid
^{13}C-NMR	carbon-13 nuclear magnetic resonance
CoA	coenzyme A
CO_2	carbon dioxide
COSY	correlation spectroscopy

D

DEPT	distortionless enhancement by polarisation transfer
DCM	dichloromethane
D-MEM	Dulbecco minimum Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribose nucleic acid
1D-NMR	one dimensional nuclear magnetic resonance
2D-NMR	two dimensional nuclear magnetic resonance
dH_2O	distilled water
DPPH	2,2-diphenyl-1-picrylhydrazyl

E

EBRT	external beam radiotherapy
EGCG	epigallocatechin gallate
EMW	ethyl acetate: methanol: water
EMR	electromagnetic radiation
EtOAc	ethyl acetate

F

F2	fraction 2
FBS	fetal bovine serum
FTase	farnesyl pyrophosphate transferase

G

g	gram

GAP	GTPase activating protein
GGTase	geranylgeranyl pyrophosphate transferase
GTP	guanosine triphosphate
GDP	guanosine diphosphate

H

H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
Hex	hexane
^{1}H-NMR	proton nuclear magnetic resonance
h	hour
HMBC	heteronuclear multiple bond correlation
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMQC	heteronuclear multiple quantum coherence
HPLC	high performance liquid chromatography

I

INT	*p*-iodonitrotetrazolium violet
IR	infrared

K

$K_3Fe(CN)_6$	potassium ferricyanide
kDa	kilodalton

L

LC/MS	liquid chromatography/mass spectroscopy
LC/DAD/UV	liquid chromatography/diode array detection/ultraviolet
LC/UV/MS	liquid chromatography/ultraviolet/mass spectroscopy

M

MeOH	methanol
mg	milligram
MH2	isolated compound
MHz	megaHertz
MIC	minimum inhibitory concentration
min	minutes
ml	millilitre
MS	mass spectroscopy
MTT	3-(4,5-dimethylthiozyl-2-yl)-2,5-diphenyl tetrazolium bromide

N

NaCl	sodium chloride
NCI	national cancer institute
nm	nanometer
NMR	nuclear magnetic resonance

O

OH	hydroxyl radicals

P

PBS	phosphate buffered saline
PI	propidium iodide
PP_i	inorganic pyrophosphate
ppm	parts per million
PSN	penicillin, streptomycin, neomycin

R

R_f	retardation factor
RF	radio frequency
RPMI-1640	Roswell Park Memorial Institute 1640
ROS	reactive oxygen species

S

SEM	standard error of the mean
SO_4^-	superoxide ion
SOD	superoxide dismutase
SRB	sulforhodamine B

T

TLC	thin layer chromatography

U

µl	microliter
U	units

UV	ultraviolet

V

VLC	vacuum liquid chromatography
v/v	volume to volume
v/w	volume to weight

W

WHO	World Health Organization

ABSTRACT

Traditional medicine based on herbal remedies has always played a key role in the health systems of many countries. *Commelina benghalesis* Linn is frequently used in traditional medicine as an anti-inflammatory, anticancer and anti-diarrheal agent. It is used as coarse food for livestock and in other countries like Ghana the leaves are cooked and eaten as a vegetable. Several other medicinal benefits of this plant have been reported viz., as a heating pad for sore feet, treatment for sore throat, burns, eye complaints and leprosy. Despite its several uses, the plant is not well investigated and the biologically active compounds are not yet fully elucidated. The current study investigated the anti-oxidative, anti-bacterial, anti-fungal and anti-proliferative (anti-cancer) activity of the organic solvents-extracted crude extracts from *C. benghalensis*. The study also reports on the isolation, purification and structural elucidation of the bioactive entities inherent to *C. benghalensis*.

For experimental purposes, the fleshy stem samples of the plant were collected from the north-western parts of Mpumalanga Province under the guidance of a traditional healer and the materials were subsequently dried and milled to a fine powder. Different crude extracts were prepared by using *n*-hexane, dichloromethane (DCM), acetone and methanol. The chemical profiles of the resultant crude extracts were analysed by thin layer chromatography (TLC); the TLC plates were developed in benzene/ethanol/ammonia (90:10:1) [BEA], chloroform/ethyl acetate/formic acid (5:4:1) [CEF] and ethyl acetate/methanol/water (40:5.4:4) [EMW].

Consequently, methanol extracted a greater quantity of the plant material than the other solvents used. For the detection of the chemical entities extracted, vanillin-sulphuric acid and *p*-anisaldehyde-sulphuric acid reagents were sprayed on the chromatograms and heated at 110^{o}C for optimal colour development. Most of the separated compounds reacted with vanillin-sulphuric acid; however, the chemical profiles of the different extracts displayed little differences.

The stable free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), was used to evaluate the antioxidant activity of the four crude extracts. The TLC-DPPH method exposed few compounds that possess antioxidant activity in the methanol extract developed in BEA; the other extracts displayed little or no antioxidant activity. Four bacterial strains *viz*., *Escherichia coli* ATCC 27853, *Enterococcus faecalis* ATCC 21212, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 25922, were used as test organisms for the evaluation of the extracts' antibacterial activity. The results showed compounds on the chromatograms that demonstrated antibacterial activity against all the bacteria tested. The *n*-hexane and DCM extracts had 2 compounds with the most active antibacterial activity, followed by acetone extract with 4 compounds having less activity. Methanol extract had showed 2 compounds with less antibacterial activity. Furthermore, the serial micro-dilution assay was used to determine the minimum inhibitory concentration (MIC) values of the individual extracts. The MIC values from the *n*-hexane and DCM extracts ranged between 0.15-0.62 mg/ml; however, the methanol

extract had low activity, with a high MIC value of 2.5 mg/ml. The observed MIC values corresponded and complemented the bioautography results.

The compounds that displayed the most growth inhibitory activity of the test bacterial strains were then targeted for further isolation and purification. This was accomplished by using the bioassay-guided isolation of the target antibacterial compounds from the *n*-hexane extract, since this extract contained most of the target compounds. The compounds were successfully isolated and their chemical structures were elucidated by means of nuclear magnetic resonance (NMR) and the masses and structure of the pure compounds were confirmed by mass spectroscopy (MS). The purified compounds were found to be two of the well known plant sterols *viz*., a mixture of *β*-sitosterol/stigmasterol and a long carbon chain compound identified as pentatriacontane. These purified plant sterols, together with the *n*-hexane and DCM extracts, were further evaluated for their cytotoxicity on the normal Monkey kidney Vero cells using 3-[4,5-dimethylthiazol-2-yl]-2-5 diphenyltetrazolium bromide (MTT) assay. There was no observable inhibitory activity that was exerted on the normal Monkey kidney Vero cells at the experimental concentrations (0 – 250 µg/ml) used. However, the *n*-hexane and DCM extracts demonstrated a concentration-dependent inhibitory activity against Wil-2 NS cancerous cells. The purified compounds were not tested for their efficacy against the Wil-2 NS cells since there was no sufficient material left to complete these studies. The DCM extract was the most toxic at 15.6 µg/ml (7%), as compared to the *n*-hexane extract when used at the same concentration. However, higher concentrations (62.5 µg/ml) of the *n*-hexane

extract had a higher inhibitory activity (>55%) than the DCM extract. The *n*-hexane extract was further fractionated and the Hex70 and Hex30 fractions had more antiproliferative activity against Wil-2 NS cells, with the Hex30 fraction displaying the greatest inhibitory effect at lower concentrations (39 µg/ml, 28%) than the Hex70 fraction under similar concentrations (16%).

In conclusion, the *C. benghalensis* extracts contained antibacterial compounds that were non-polar based on the quantity present in the extract. A mixture of plant sterols was isolated together with a long hydrocarbon chain compound, pentatriacontane. The isolated sterols have antibacterial activity and it could be speculated that these sterols are the source of the protective effect for the plant against undesirable plant pathogens. The antiproliferative activity against cancerous lymphoma cells (Wil-2 NS) was due, in part, to these isolated sterols. Furthermore, the Hex30 fraction had a higher antiproliferative activity against the Wil-2 NS cells. However, the DCM extract had the highest antibacterial and anticancer activity. This suggests that the DCM extract may possess compounds that elicit anticancerous activity, and when combined they may act in a synergistic manner. Future endeavour will be focused towards further purification and characterisation of the bioactive entities of the DCM extract.

The overall outcome of this study is the validation of the ethnobotanical claims in the use of *C. benghalensis* as a medicine for treatment of various skin ailments that are related to bacterial infections and skin tumours. The study thus corroborates the assertion by the practitioners of indigenous medicine

that extracts of *C. benghalensis* proffer beneficial effects toward alleviation of skin outgrowths.