

***In Vitro* Medicinal Properties of  
Novel Compounds from *Croton  
steenkampianus***

**By  
Adeboye Mutiu Adelekan**

**Submitted in partial fulfillment of the  
requirements for the degree of**

**DOCTOR OF PHILOSOPHIAE:  
PLANT SCIENCE  
Department of Plant Science**

**Faculty of Natural and Agricultural  
Sciences  
University of Pretoria**

**Promoter: Prof JJM Meyer**

**2009**



I declare that the thesis/dissertation, which I hereby submit for the degree PHD Plant Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:

Date: 26/01/2009

## ACKNOWLEDGEMENTS

Glory be to God almighty for His grace and mercy over me throughout the study.

I also wish to thank the following people for the role they have played in the course of the research:

Prof JJM Meyer for the assistance, guidance and support given to me.

Prof AI Louw for his contribution.

The National Research Foundation and University of Pretoria for their financial support.

Dr Ahmed Hussein for his help in the isolation and identification of the compounds and for arranging antiplasmodial bioassay.

Dr Adrian Basson for help with the cytotoxicity testing.

Dr Benedict Bapela for assistance with the TB assay.

Dr Emmanuel Tshikalange for help with anti-HIV assay.

Prof P Smith (University of Cape Town), Bruce Tshilamulele and Luis David (Smithsonian Tropical Research Institute, Panama) for help with the malaria bioassays.

Eric Palmer, Department of Chemistry, University of Pretoria for the assistance with the NMR.

Prof Peet van Roogen for assistance with x-ray crystallography.

My wife Tsholofelo D Adelekan for her support and encouragement.

All my friends and well-wishers.

## SUMMARY

### ***In Vitro* Medicinal Properties of Novel Compounds from *Croton steenkampianus***

by

**Adeboye Mutiu Adelekan**

**Promoter: Prof JJM Meyer**

**Department of Plant Science**

**Doctor of Philosophiae**

The effect of infectious diseases on the population in the developing countries is of utmost concern. Malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) are the three major infectious disease threats. They account for approximately half of the mortality caused by infectious diseases, which is almost half of the mortality in the developing countries. With no vaccine likely in the foreseeable future, drugs remain the best means of controlling infectious diseases. In the industrialized nations at the present time, some 50% of all prescribed drugs are derived or synthesized from natural products (animals, marine species, plants and micro-organisms). It has been estimated that plants are the most important source of medicine for more than 80% of the world's population. As previous work on the leaves of *Croton steenkampianus* gave promising results and revealed that it still contained bioactive compounds that could be isolated, it was chosen for further work.

The bioassay guided fractionation of the ethanol crude extract using silica and Sephadex column chromatography resulted in the isolation of six compounds: three flavonoids (quercetin, tamarixetin and eriodictyol), one new indane (**1**) (2,6-dimethyl-1-oxo-4 indanecarboxylic acid) and two new diterpenes (steenkrotin A (**2**) and steenkrotin B (**3**)) with novel skeletons. The structure of the compounds was determined using NMR, IR, UV, MS and X-ray crystallography.

Ethanol crude extract, quercetin, steenkrotin A, steenkrotin B and the indane were tested against four strains of *Plasmodium falciparum* (D6, D10, Dd2 and W2). Quercetin showed good antiplasmodial activity against the D10 and Dd2 strains. The antiplasmodial activity of steenkrotin A and crude extract were moderate. The antimalarial activity of steenkrotin A in particular is promising, as it showed more activity against resistant strains. The indane, and steenkrotin B were not active against the strains of *P. falciparum* used ( $IC_{50} > 10 \mu\text{g}/\text{m}$ ). The  $IC_{50}$  of the compounds improved when they were combined with chloroquine. However, the  $IC_{50}$  of chloroquine was still the lowest. The compounds showed moderate bioactivity against *Bacillus cereus* and *Escherichia coli*. The three new compounds (**1**, **2** and **3**) tested against *Mycobacterium* (H37Rv) were not active ( $IC_{50} > 10 \mu\text{g}/\text{ml}$ ). The indane (**1**) showed anti-HIV activity at  $50 \mu\text{g}/\text{ml}$  against reverse transcriptase. The antioxidant activity of the compounds tested ranged from weak to excellent ( $>280.00 \mu\text{g}/\text{ml}$  for compound **1** and **2** to  $0.05 \mu\text{g}/\text{ml}$  for quercetin).

The cytotoxicity of the compounds and extract were determined against Vero cells lines. Their  $IC_{50}$  values ranged from 34.0 to 305.9  $\mu\text{g}/\text{ml}$ , which is higher and better than that of chloroquine. The  $IC_{50}$  values obtained are: chloroquine (25.0), quercetin (33.6), steenkrotin A (35.0), ethanol extract (45.0), tamarixetin (53.8), indane (248.2) and steenkrotin B (305.9).



## CONTENTS

Summary.....	i
Acknowledgements.....	iii
List of figures.....	vii
List of tables.....	xi
List of abbreviations.....	xii

### Chapter 1: Introduction

1.1 Medicinal plants.....	3
1.2 Traditional medicine.....	4
1.2.1 African traditional medicine.....	5
1.2.2 American traditional medicine (North, Central and South).....	6
1.2.2.1 North America.....	6
1.2.2.2 Central and South America.....	7
1.2.3 Australian and Southeast Asian medicine.....	7
1.2.4 Ayurvedic medicine (Indian traditional medicine).....	8
1.2.5 Chinese traditional medicine.....	9
1.2.6 European medicine.....	10
1.2.7 Classical Arabic and North African traditional medicine.....	12
1.3 Drug discovery from medicinal plants.....	15
1.4 Synthesis and role of plant secondary metabolites.....	18
1.4.1 Terpenes.....	19
1.4.1.1 Monoterpenes.....	20
1.4.1.2 Sesquiterpenes.....	20
1.4.1.3 Diterpenes.....	21
1.4.1.4 Triterpenes.....	21
1.4.2 Phenolic compounds.....	21
1.4.2.1 Flavonoids.....	22
1.4.3 Nitrogen containing compounds.....	23
1.4.3.1 Alkaloids.....	24
1.4.3.2 Cyanogenic glycosides.....	25
1.5 Infectious diseases.....	25
1.5.1 Malaria.....	26
1.5.2 Human immunodeficiency virus.....	28



1.5.3 Tuberculosis.....	30
1.6 Antioxidant activity .....	31
1.7 <i>Croton steenkampianus</i> .....	32
1.8 Objectives.....	33
1.9 Scope of the thesis.....	33
1.10 Hypothesis.....	34
1.11 References.....	35

## **Chapter 2: Bioassay guided fractionation of the crude extract from *Croton steenkampianus***

2.1 Introduction.....	43
2.2 Materials and Methods.....	43
2.2.1 Collection of plant materials.....	43
2.2.2 Methods.....	44
2.2.2.1 Preparation of the crude extract.....	44
2.2.2.2 Bacterial culturing and antibacterial testing.....	44
2.2.2.3 Isolation and identification of compounds.....	45
2.2.2.4 Structure elucidation.....	46
2.3 Results and Discussion.....	48
2.4 References.....	75

## **Chapter 3: Antiplasmodial bioactivity of crude extract and isolated compounds**

3.1 Introduction.....	79
3.2 Methods.....	80
3.2.1 Culture medium and washed human erythrocytes.....	80
3.2.2 <i>In vitro</i> culturing of malaria parasites.....	80
3.2.3 Giemsa stained thin blood smear preparations.....	81
3.2.4 <i>In vitro</i> synchronisation of malaria parasites.....	81
3.2.5 Preparation of microculture plates.....	82
3.2.6 Determination of antiplasmodial activity with the Malstat method.....	82



3.2.7 Determination of antiplasmodial activity with the microfluorimetric method.....	83
3.2.7.1 Fluorimetric susceptibility test.....	83
3.2.7.2 Synergistic activity.....	84
3.3 Results and Discussion.....	85
3.4 References.....	87

#### **Chapter 4: Antibacterial and antioxidant activity of isolated compounds**

4.1 Introduction.....	91
4.2 Materials and Methods.....	92
4.2.1 Qualitative determination of antibacterial activity.....	92
4.2.2 Quantitative determination of antibacterial activity.....	92
4.2.3 Antimycobacterial testing.....	93
4.2.4 Antioxidant activity.....	94
4.2.4.1 Qualitative assay.....	94
4.2.4.2 Quantitative assay.....	94
4.3 Results and Discussion.....	95
4.4 References.....	100

#### **Chapter 5: Anti-HIV activity of the isolated compounds**

5.1 Introduction.....	104
5.1.1 HIV in South Africa.....	105
5.1.2 Anti-HIV compounds.....	106
5.1.3 Reverse transcriptase (RT).....	107
5.1.4 Replication of HIV.....	107
5.2 Materials and Method.....	110
5.2.1 Materials.....	110
5.2.2 Method.....	110
5.3 Results.....	111
5.4 Discussion.....	111
5.5 References.....	113





## Chapter 6: Cytotoxicity of the isolated compounds

6.1 Introduction.....	117
6.2 Materials and Method.....	118
6.2.1 Plant materials.....	118
6.2.2 Preparation of extract and isolation of the compounds.....	118
6.2.3 Cell culture.....	118
6.2.4 Toxicity screening (XTT viability assay).....	119
6.3 Results and Discussion.....	120
6.4 References.....	124

## Chapter 7: General discussion and conclusion

7.1 Introduction.....	127
7.2 Bioassay guided fractionation of the ethanol crude extract and isolated compounds.....	127
7.3 Biological evaluation of the isolated compounds .....	128
7.4 References.....	130

## Appendix

Appendix 1 Paper published from thesis.....	132
---	-----

## LIST OF FIGURES

### Chapter 1

Figure 1.1 The structure of artemisinin and arteether.....	16
Figure 1.2 Schematic representation of a typical medicinal plant drug discovery process and development.....	17
Figure 1.3 Main pathways leading to secondary metabolites .....	19
Figure 1.4 Monoterpenes commonly found in essential oils.....	20
Figure 1.5 The pathways of secondary metabolites derived from precursors in the shikimate pathway.....	22
Figure 1.6 Basic structures of some flavonoids.....	23
Figure 1.7 Structures of some alkaloids.....	24
Figure 1.8 Global malaria distribution.....	27
Figure 1.9 Distribution of malaria in Africa.....	27
Figure 1.10 <i>Croton steenkampianus</i> leaves.....	33

### Chapter 2

Figure 2.1 Schematic representation of the bioassay guided isolation of active compounds from <i>C. steenkampianus</i> .....	47
Figure 2.2 Typical results obtained from the pooled fractions from the silica column tested for antibacterial activity.....	48
Figure 2.3 TLC plates showing antibacterial activity of pure compounds.....	49
Figure 2.4 Structures of isolated compounds.....	50
Figure 2.5 <sup>1</sup> H-NMR spectrum of tamarixetin.....	52
Figure 2.6 <sup>1</sup> H-NMR spectrum of quercetin.....	53
Figure 2.7 <sup>13</sup> C-NMR spectrum of quercetin.....	53
Figure 2.8 <sup>1</sup> H-NMR spectrum of eriodictyol.....	54
Figure 2.9 <sup>1</sup> H-NMR spectrum of indane.....	55
Figure 2.10 <sup>13</sup> C-NMR spectrum of indane.....	55
Figure 2.11 COSY spectrum of indane.....	56
Figure 2.12 HMQC spectrum of indane.....	56



Figure 2.13 HMBC spectrum of indane.....	57
Figure 2.14 NOESY spectrum of indane.....	57
Figure 2.15 HMBC correlation of partial structure.....	58
Figure 2.16 <sup>1</sup> H-NMR spectrum of steenkrotin A.....	62
Figure 2.17 <sup>13</sup> C-NMR spectrum of steenkrotin A.....	63
Figure 2.18 COSY spectrum of steenkrotin A.....	63
Figure 2.19 NEOSY spectrum of steenkrotin A.....	64
Figure 2.20 DEPT 135 spectrum of steenkrotin A.....	64
Figure 2.21 MS data of spectrum of steenkrotin A.....	65
Figure 2.22 X-ray structure of steenkrotin A.....	65
Figure 2.23 <sup>1</sup> H-NMR spectrum of steenkrotin B.....	68
Figure 2.24 <sup>13</sup> C-NMR spectrum of steenkrotin B.....	69
Figure 2.25 DEPT 135 spectrum of steenkrotin B.....	69
Figure 2.26 COSY spectrum of steenkrotin B.....	70
Figure 2.27 HMQC spectrum of steenkrotin B.....	70
Figure 2.28 HMBC spectrum of steenkrotin B.....	71
Figure 2.29 <sup>1</sup> H-NMR spectrum of steenkrotin B acetate.....	71
Figure 2.30 <sup>13</sup> C-NMR spectrum of steenkrotin B acetate.....	72
Figure 2.31 COSY spectrum of steenkrotin B acetate.....	72
Figure 2.32 HSQC spectrum of steenkrotin B acetate.....	73
Figure 2.33 HMBC spectrum of steenkrotin B acetate.....	73
Figure 2.34 NEOSY spectrum of steenkrotin B acetate.....	74

## Chapter 4

Figure 4.1 Bioautogram of the indane in lanes 1-7.....	95
Figure 4.2 Qualitative antioxidant assay.....	97
Figure 4.3 Quantitative antioxidant assay.....	97
Figure 4.4 Antioxidant activities of the crude extract and compounds.....	99



## Chapter 5

Figure 5.1 Human immunodeficiency virus.....	105
Figure 5.2 The HIV replication cycle.....	108
Figure 5.3 The immature and mature forms of the HIV.....	109

## Chapter 6

Figure 6.1 The reduction of yellow tetrazolium salt MTT to purple formazan.	118
Figure 6.2 Sample plate design.....	119
Figure 6.3 Activity of the isolated compounds on the growth of Vero cells in $\mu\text{g/ml}$ .....	121



## LISTS OF TABLES

Table 1.1 Botanical drugs used in traditional medicine which led to useful modern drugs.....	14
Table 2.1 NMR spectroscopic data for compound <b>1</b> .....	51
Table 2.2 NMR spectroscopic data for compounds <b>2-4</b> .....	60
Table 2.3 Significant NOE data of compounds <b>2-4</b> .....	61
Table 3.1 Antiplasmodial activity of compounds and extract.....	85
Table 4.1 MIC of compounds against <i>B. cereus</i> and <i>E. coli</i> .....	96
Table 4.2 Quantitative antioxidant activities of the ethanol crude extract and the isolated compounds.....	97
Table 6.1 Cytotoxicity of the crude ethanol extract and compounds isolated from <i>C. steenkampianus</i> on Vero cells.....	120

## LIST OF ABBREVIATIONS

- $^{13}\text{C}$ -NMR: Carbon nuclear magnetic resonance  
 $^1\text{H}$ -NMR: Proton nuclear magnetic resonance  
AIDS: Acquired immune deficiency syndrome  
APAD: 3-Acetylpyrimidine adenine dinucleotide  
COSY: Correlated spectroscopy  
DEPT: Distortionless enhancement by polarization transfer  
DHFR: Dihydrofolate reductase  
DHODase: Dihydroorotate dehydrogenase  
DHPS: Dihydropteroate synthase  
DMSO: Dimethylsulfoxide  
DPP: Dimethylallyl pyrophosphate  
EDTA: Ethylenediaminetetra-acetic acid  
FPIX: Ferriprotoporphyrin IX  
HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid  
HIV: Human immunodeficiency virus  
HMBC: Heteronuclear multiple bond correlation  
HMQC: Heteronuclear multiple quantum correlation  
HSQC: Heteronuclear single quantum coherence  
IPP: Isopentenyl pyrophosphate  
IR: Infrared  
LD<sub>50</sub>: 50% Lethal dose  
MS: Mass spectroscopy  
MTCT: Mother-to-child transmission  
MTT: 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide  
NBT: Nitroblue tetrazolium  
NMR: Nuclear magnetic resonance  
NOESY: Nuclear overhauser effect spectroscopy  
NSP: National strategic plan  
PBS: Phosphate buffer saline  
PEP: Post-exposure prophylaxis  
PF: Potentiating factor



SP: Sulphadoxine-pyrimethamine

STD: Sexual transmitted disease

STI: Sexual transmitted infection

TLC: Thin layer chromatography

TMS: Tetramethylsilane

TRIS: N-tris (hydroxymethyl) aminomethane

UNAIDS: Joint United Nations programme on HIV/AIDS

UNGASS: United Nations general assembly session on HIV/AIDS

UNICEF: United Nations children's fund

USAID: United States agency for international development

UV: Ultraviolet

WHO: World health organization