

**ISOLATION AND CHARACTERIZATION OF ANTIMALARIAL
COMPOUNDS FROM ROOT BARK OF CALLICHILIA STENOPETALA
STAMP. (FAMILY APOCYNACEAE)**

**A THESIS SUBMITTED TO
THE SCHOOL OF POSTGRADUATE STUDIES OF THE UNIVERSITY
OF LAGOS, NIGERIA, AS PART OF THE REQUIREMENT FOR THE
AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) DEGREE IN
PHARMACOGNOSY**

BY

**ORABUEZE, IFEOMA CELESTINA
MARICULATION NUMBER: 039095021**

SEPTEMBER 2015

DECLARATION

The study, titled “**ISOLATION AND CHARACTERIZATION OF ANTIMALARIAL COMPOUNDS FROM ROOT BARK OF CALLICHILIA STENOPETALA STAPF. (APOCYNACEAE)**”, submitted to the School of Postgraduate Studies, University of Lagos, Lagos, Nigeria for the award of Doctor of Philosophy (Ph.D) Degree in Pharmacognosy, Pharmacy, is an original research carried out by **ORABUEZE, IFEOMA CELESTINA**, in the Department of Pharmacognosy, Faculty of Pharmacy of the University of Lagos under the supervision of **Dr. S. A. Adesegun, Prof. H.A.B. Coker** and **Dr. S. Ogbonnia**. It is hereby declared that this study has not been submitted previously (in whole or in part) to any institution for the purpose of awarding any academic degree.

Dr. S. A. Adesegun

Department of Pharmacognosy, Faculty of Pharmacy of the University of Lagos

Prof. H.A.B. Coker

Department of Pharmaceutical Chemistry, Faculty of Pharmacy of the University of Lagos

Dr. S. Ogbonnia

Department of Pharmacognosy, Faculty of Pharmacy of the University of Lagos

Orabueze, Ifeoma Celestina (Candidate)

DEDICATION

This research work is dedicated to God the almighty, the alpha and finisher of my faith and in lovely memory of a rare gem, late Mrs. Chinyelu Okoli (my late mother). She lives on in the lives she touched while on Earth.

ACKNOWLEDGEMENTS

I would like to convey my gratitude to my supervisors, Dr. Adeleke Adesegun, Professor Herbert Coker and Dr. Sunday Ogbonnia for their guidance, concern, understanding and their support throughout the development of this Ph.D. programme. I thank you all for giving me so much of your precious time and constructive comments towards completion of this dissertation.

I greatly appreciate Professor Andrew Marston (late) and Professor J.H van der Westhuizen for their supervisory role while doing part of the bench work under their supervision at the Natural Product Chemistry laboratory, Department of Chemistry, University of the Free State, South Africa. Prof. Marston was a strong name to be reckoned with in natural product isolation and left behind teams of powerful natural product researchers, working as one big family. Research under him was fun and great experience.

I respectfully appreciate Professor Odukoya (former Dean, Faculty of Pharmacy, University of Lagos) for her constant support both in administrative and academic matters, ensuring that there was always a step forward towards completion of the study. Thank you Ma, I really appreciate your support and “energy drive”. I would like to thank the current Dean, Faculty of Pharmacy, University of Lagos, Prof Silva, a great researcher and always ready to listen.

I am grateful for the support and mentorship of Dr. Ajayi, Dr. Olagbende-Dada, Dr. Sofidiya, Dr. Sowemimo, Dr. Oreagba, Dr. Azubike. Your contributions towards the completion of this study are immeasurable. I really appreciate your care and words of encouragement.

My appreciation extends to Professor Bezuidenhout and his team (Physical Chemistry department, University of the Free States, UFS) for GC-MS measurements; Professor John Davies (University of Cambridge) for the X-crystallography, Dr. Susan Bonnet (UFS) for useful suggestion in the column chromatography work and during NMR spectra recording .

My profound appreciation goes to Professor FAMILONI for the fatherly and professional advice received since the time of my MSc. and current Ph.D programme. I appreciate Dr. Aina of NIRM for his very useful suggestions and provision of resourceful materials.

My deepest appreciation is also dedicated to Dr. Kurma, Dr. Achilonu, Matthew, Dr. Anwar, Dr. Rossy, Du for NMR measurements. Without their help, this research work would not have been completed. I also wish to extend my thanks to my friends and colleagues in the Natural product and Phytochemistry laboratories, UFS; Khaya, Talkmore, Du, Kagee, Dr. Agbor, Dr. Le' Deu and Kaycee for those times we had to share each other's joy or stress/frustration in the course of purification and isolation. I would like to thank all my colleagues of the University of Lagos (the present and those already graduated) for the peaceful and friendly academic environment; Dr. Odimegwu, Dr. Alaribe, Dr. Illomuanya, Dr. Oludare, Nkemehule, Dr. (Mrs.) Isreal, Ini Okoko and others.

My kind regards goes to the technical staff that had to work some late evenings and even weekends towards completion of the work, Mr. Duncan, Mr. Adeleke, Mrs. Olorunyomi, Mr. Julius, Mrs. Olarinoye, Miss Umoh, etc. I really appreciate all your help and the extra miles taken on my behalf.

The financial support from University of Lagos in the form of staff development tuition free sponsorship, doctoral assistance grant, study leave and the free use of various laboratories within the University premises is gratefully acknowledged and appreciated.

Finally I would like to express my special appreciation to my family, nuclear and extended. Mum, when you predicted that one day I will have my Ph.D, little did I believe you, but then you have always been right in so many things. Regretfully she died at the beginning of the study; I pray that her gentle soul continues to rest in the bosom of God. I miss her greatly. I lovely appreciate my dad, Dr. G. L. Okoli, thank you for the educational foundation you and mum laid for your children. My siblings, Kenny (KK), Onyi, UK, Emmy (CC) and Nnamdi (Nndy), great are the parts you played in my life, my friends and love. I really appreciate you all. My beloved husband, you have been wonderful and so understanding. You covered so well, hours, weeks and months of my not being in the house. I really appreciate you with a lovely heart. My sweethearts; Ogo, Naza, Che-Che and Ify, thanks for your love and understanding and not giving mummy any trouble while she worked towards this goal.

I bow my head in humility, thankful and in gratitude to God for all His blessings.

TABLE OF CONTENTS

| | Page |
|---|------|
| Title page | i |
| Declaration | ii |
| Dedication | iii |
| Acknowledgement | iv |
| Table of Contents | v |
| List of Tables | vi |
| List of Figures | vii |
| Abstract | 1 |
| CHAPTER ONE | |
| 1.0. INTRODUCTION | 2 |
| 1.1. Background of the study | 2 |
| 1.2. Statement of the problem | 4 |
| 1.3. Aim and objectives | 5 |
| 1.4. Specific objectives of study | 5 |
| 1.5. Significance of study | 5 |
| 1.6. Definition of operational terms | 6 |
| 1.7. List of abbreviations and acronyms | 8 |

CHAPTER TWO

| | | |
|----------|--|----|
| 2.0 | LITERATURE REVIEW | 10 |
| 2.1. | Medicinal plants and biodiversity | 10 |
| 2.2. | Ethnobotany and Traditional Medicine | 13 |
| 2.3. | Plants in drug development | 16 |
| 2.3.1. | Ethnobotanical leads in natural product discovery | 16 |
| 2.3.2. | Secondary plant metabolites and drug discovery | 30 |
| 2.3.2.1. | Terpenes | 31 |
| 2.3.2.2. | Alkaloids | 36 |
| 2.4. | Malaria | 45 |
| 2.4.1. | Introduction | 45 |
| 2.4.2. | Causative agent life cycle | 48 |
| 2.4.3. | Transmission | 51 |
| 2.4.4. | Clinical symptoms | 52 |
| 2.4.5. | Diagnosis | 53 |
| 2.4.6. | Treatment/ Management of Malaria | 55 |
| 2.4.7. | Mechanism of action | 59 |
| 2.4.8. | Resistance | 61 |
| 2.4.9. | The health and economic burden of Malaria in Africa | 63 |
| 2.5. | Antioxidants | 64 |
| 2.5.1. | Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity | 66 |
| 2.6. | Purification, isolation and characterization of bioactive compounds from plant extracts | 68 |

| | |
|--|----|
| 2.6.1. Purification and Isolation of Natural products | 68 |
| 2.6.2. Solvent- Solvent Partition/Fractionation | 70 |
| 2.6.3. Chromatographic Methods | 70 |
| 2.6.3.1. Thin Layer Chromatography (TLC) | 71 |
| 2.6.3.2. Column Chromatography (CC) | 72 |
| 2.6.3.3. Vacuum liquid chromatography (VLC) | 74 |
| 2.6.3.4. High Pressure Liquid Chromatography (HPLC) | 75 |
| 2.6.3.5. Gel permeation chromatography (Size Exclusion Chromatography) | 77 |
| 2.6.3.6. Gas Chromatography (GC) | 78 |
| 2.6.3.7. Counter-current chromatography | 80 |
| 2.6.4. Fractional Crystallization | 80 |
| 2.6.5. Other separation methods | 82 |
| 2.7. Characterization and structure elucidation of bioactive compounds | 84 |
| 2.7.1. Spectroscopic Techniques | 84 |
| 2.7.1.1. Nuclear Magnetic Resonance Spectroscopy (NMR) | 84 |
| 2.7.1.2. One Dimensional NMR | 86 |
| 2.7.1.3. Two dimensional NMR (2D-NMR) | 86 |
| 2.7.2. Other Spectroscopic methods | 88 |
| 2.7.2.1. Gas Chromatography/Mass Spectrometry (GC/MS) | 88 |
| 2.7.2.2. Mass Spectrometry (MS) | 89 |
| 2.7.2.3. X-ray crystallography | 89 |
| 2.8. Nigerian medicinal plants used for malaria treatment | 90 |

| | |
|---|-----|
| 2.8.1. Isolated compounds characterized as antimalarials from Nigerian medicinal plants | 92 |
| 2.9. Nigerian medicinal plants having antioxidant activities | 95 |
| 2.10. Taxonomy and botanical description of Apocynaceae | 97 |
| 2.11. Medical/economic uses of Apocynaceae | 99 |
| 2.12. Phytochemistry: alkaloids of Apocynaceae family (Dogbane) | 101 |
| 2.13. Genus: <i>Callichilia</i> | 107 |
| 2.13.1. <i>Callichilia stenopetala</i> Stapf | 107 |

CHAPTER THREE

| | |
|--|-----|
| 3.0. MATERIALS/ METHOD | 117 |
| 3.1. Solvents, Reagents and Equipment | 117 |
| 3.1.1. Solvents | 117 |
| 3.1.2. Chemicals | 117 |
| 3.1.3. Equipment | 117 |
| 3.1.4. Reagents | 118 |
| 3.1.5. Parasite and animals | 119 |
| 3.2. Search and collection of plants | 121 |
| 3.2.1. Collection and taxonomical identification of study plant | 122 |
| 3.2.2. Pre-extraction preparation of <i>C. stenopetala</i> | 122 |
| 3.3. Extraction and Fractionation of <i>C. stenopetala</i> root bark | 122 |
| 3.4. Phytochemical screening of <i>C. stenopetala</i> | 124 |
| 3.4.1. Test for Anthraquinones | 124 |

| | |
|--|-----|
| 3.4.2. Test for phenols and tannins | 125 |
| 3.4.3. Test for saponins | 125 |
| 3.4.4. Test for alkaloids | 125 |
| 3.4.5. Test for flavonoids | 125 |
| 3.4.6. Test for steroids and triterpenes | 126 |
| 3.5. Pharmacological screening | 126 |
| 3.5.1. Acute toxicity test in mice | 127 |
| 3.5.2. <i>In vivo</i> anti-plasmodial assay of crude extract, fractions and the isolates | 127 |
| 3.5.3. Evaluation of antioxidant activity of crude extract, fractions and the isolates | 128 |
| 3.5.4. Determination of the Total Phenolic Content of the crude extract (TPC) | 129 |
| 3.6. Purification, isolation and spectroscopic characterization of compounds | 130 |
| 3.6.1. Chromatographic materials | 130 |
| 3.6.1.1. Thin layer chromatography (TLC) plates | 130 |
| 3.6.1.2. Preparation of the TLC development chamber | 130 |
| 3.6.1.3. Developing the plates | 131 |
| 3.6.1.4. Preparation of open column chromatography (CC) | 131 |
| 3.6.2. Separation of chemical constituents of hexane fraction of <i>C. stenopetala</i> (Hex) | 132 |
| 3.6.3. Separation of chemical constituents of chloroform fraction of <i>C. stenopetala</i> (CHCl ₃) | 134 |
| 3.6.3.1. Recrystallization | 134 |
| 3.6.4. Isolation of chemical constituents of EtOAc fraction | 135 |
| 3.6.5. GC-MS Studies | 137 |

| | |
|---|-----|
| 3.6.6. Spectroscopic analysis | 137 |
| 3.6.6.1. Nuclear Magnetic Resonance spectra (NMR) | 137 |
| 3.6.6.2. Infrared Spectroscopy (IR) | 138 |
| 3.6.6.3. X-ray diffraction (crystallography) | 138 |
| 3.7. Melting point determination | 138 |

CHAPTER FOUR

| | |
|---|-----|
| 4.0. RESULTS | 139 |
| 4.1. Plant search and determination of plant for study | 139 |
| 4.2. Extraction of <i>C. stenopetala</i> root bark | 145 |
| 4.2.1. Fractionation of <i>C. stenopetala</i> root bark | 145 |
| 4.3. Preliminary phytochemical analysis of <i>C. stenopetala</i> root bark | 146 |
| 4.4. Pharmacological activity | 150 |
| 4.4.1. Acute toxicity | 150 |
| 4.4.2. Anti-malarial activity | 150 |
| 4.4.2.1. Anti-malarial activity of crude extract of <i>C. stenopetala</i> | 150 |
| 4.4.2.2. Anti-malarial activity of the fraction | 152 |
| 4.4.3. Antioxidant activity | 154 |
| 4.4.3.1. Anti-oxidant activity of the crude extract and the fractions | 154 |
| 4.4.4. Determination of total phenolic content (TPC) | 158 |
| 4.4.5. Antimalarial and antioxidant activities of the isolates | 160 |
| 4.5. Purification, isolation and characterization of antimalarial compounds from active fractions of the crude extract | 164 |

| | |
|--|-----|
| 4.5.1. Hexane fraction: Isolation and spectroscopic results for characterization of compounds 1 and 2 | 164 |
| 4.5.1.1. Isolation of compound 1 | 164 |
| 4.5.1.2. Spectroscopic results for characterization and elucidation of compound 1 | 168 |
| 4.5.1.3. Isolation of compound 2 | 184 |
| 4.5.1.4. Spectroscopic results for characterization and elucidation of compound 2 | 185 |
| 4.5.2. Chloroform fraction: Isolation and spectroscopic results for characterization of compound 3 | 197 |
| 4.5.2.1. Isolation of compound 2 | 199 |
| 4.5.2.2. Spectroscopic results for characterization and elucidation of compound 3 | 194 |

CHAPTER FIVE

| | |
|----------------------------|-----|
| 5.0. DISCUSSION | 220 |
| SUMMARY OF FINDINGS | 235 |
| CONCLUSION | 237 |
| CONTRIBUTIONS TO KNOWLEDGE | 238 |
| REFERENCES | 239 |
| APPENDIX | 299 |

LIST OF TABLES

| | |
|---|-----|
| Table 1: Herbal drugs used in traditional medicine and which have given useful modern drugs | 27 |
| Table 2: Groups of alkaloid | 37 |
| Table 3: Some physiological import alkaloids of Apocynaceae family | 105 |
| Table 4: Macromorphological comparison of <i>Callichilia Spp</i> | 111 |
| Table 5: Phytochemical screening | 124 |
| Table 6: List of some medicinal plants used in treatment of malaria fever | 141 |
| Table 7: Suppressive activity of some antimalarial medicinal plants crude extracts at 500 mg kg ⁻¹ | 142 |
| Table 8: DPPH free radical scavenging activity of some antimalarial medicinal plants crude extracts | 143 |
| Table 9: Bio-activities summary of some antimalarial medicinal plants crude extracts | 144 |
| Table 10: Liquid – Liquid fractionation of crude extract of <i>C. stenopetala</i> root bark | 145 |
| Table 11: Phytochemical screening of the crude extract of <i>C. stenopetala</i> root bark | 146 |
| Table 12: Alkaloidal content of the fractions | 148 |
| Table 13: TLC results of alkaloidal content of chloroform (CHCl ₃) fraction | 149 |
| Table 14: Suppressive activity of crude extract of <i>C. stenopetala</i> root bark on parasitaemia in mice | 151 |
| Table 15: Anti-malarial activity of fractions <i>C. stenopetala</i> on parasitaemia in mice | 153 |
| Table 16: DPPH Free radical scavenging activity of the crude extract and Vit C | 155 |
| Table 17: Calibration data for gallic acid | 157 |
| Table 18: Antimalarial activity of the isolates on parasitaemia in rats | 161 |
| Table 19: Summary of bioactivity profile of the isolates | 162 |

| | |
|--|-----|
| Table 20: ^{13}C NMR and ^1H -NMR main signals for compound 1 | 182 |
| Table 21: ^{13}C NMR and ^1H -NMR main signals for compound 2 | 196 |
| Table 22: TLC results of Chloroform fraction | 199 |
| Table 23: TLC results of test tube/fraction 34 from chloroform fraction (Figure 47) | 200 |
| Table 24: ^{13}C NMR and ^1H -NMR main signals for compound 3 | 218 |

LIST OF FIGURES

| | |
|--|-----|
| Figure 1: Estimated incidence of malaria per 1000 population in the year 2000 | 47 |
| Figure 2: Causative agent life cycle | 49 |
| Figure 3a: Ring stage of " <i>Plasmodium falciparum</i> " in human red blood cells | 54 |
| Figure 3b: Stained thin blood film showing <i>Plasmodium falciparum</i> infection | 54 |
| Figure 4: Equipment for measuring Nuclear Magnetic Resonance (NMR) | 85 |
| Figure 5a: <i>Callichilia</i> spp microscopy (a) | 109 |
| Figure 5b: <i>Callichilia</i> spp microscopy (b) | 110 |
| Figure 6: <i>Callichilia stenopetala</i> plant showing leaf arrangement | 114 |
| Figure 7: <i>Callichilia stenopetala</i> plant showing its fruits | 115 |
| Figure 8: <i>Callichilia stenopetala</i> showing its flower | 116 |
| Figure 9: Schematic representation of extraction and partitioning of crude extract | 123 |
| Figure 10: Isolation scheme of compounds 1 and 2 | 133 |
| Figure 11: Isolation scheme of compound 3 | 136 |
| Figure 12: TLC chromatograms showing the alkaloidal content of various fractions of <i>C. stenopetala</i> | 147 |

| | |
|--|-----|
| Figure 13: TLC chromatograms of the CHCl ₃ fraction of <i>C. stenopetala</i> showing alkaloidal content | 149 |
| Figure 14: DPPH Free radical scavenging activity of crude extract and Vit C | 156 |
| Figure 15: DPPH Free radical scavenging activity of fractions of <i>C. stenopetala</i> root bark | 157 |
| Figure 16: Calibration curve for Gallic acid | 158 |
| Figure 17: DPPH free radical scavenging activity of the isolates | 163 |
| Figure 18: TLC Chromatogram of hexane fraction (1) | 164 |
| Figure 19: TLC Chromatogram of hexane fraction (2) | 165 |
| Figure 20: TLC of re-chromatogram of C ₂ of hexane fraction | 166 |
| Figure 21: TL Chromatogram, Illustration of the level of purity of compound 1 after recrystallization | 167 |
| Figure 22: ¹ H NMR spectrum of compound 1 | 170 |
| Figure 23: ¹³ C NMR spectrum of compound 1 | 171 |
| Figure 24: APT spectrum of compound 1 | 172 |
| Figure 25: GC-MS spectrum of compound 1 | 173 |
| Figure 26: IR spectrum of compound 1 | 174 |
| Figure 27: NOESY spectrum of compound 1 | 175 |
| Figure 28: COSY spectrum of compound 1 | 176 |
| Figure 29: HMBC spectrum of compound 1 | 177 |
| Figure 30: HSQC spectrum of compound 1 | 178 |
| Figure 31: 3 Dimensional ball and stick minimized energy mode for compound 1 | 179 |
| Figure 32: 3 Dimensional ball and stick minimized energy mode for compound 1 | 180 |

| | |
|---|-----|
| Figure 33: 3 Dimensional ball and stick minimized energy mode for compound 1 | 181 |
| Figure 34: The structure of compound 1 | 183 |
| Figure 35: Illustration of the level of purity of compound 2 after recrystallization | 184 |
| Figure 36: ^{13}C NMR spectrum of compound 2 | 187 |
| Figure 37: ^1H NMR spectrum of compound 2 | 188 |
| Figure 38: ATP spectrum of compound 2 | 189 |
| Figure 39: GC-Mass spectrum of compound 2 | 190 |
| Figure 40: IR spectrum of compound 2 | 191 |
| Figure 41: NOESY spectrum of compound 2 | 192 |
| Figure 42: COSY spectrum of compound 2 | 193 |
| Figure 43: HMBC spectrum of compound 2 | 194 |
| Figure 44: HSQC spectrum of compound 2 | 195 |
| Figure 45: Structure of compound 2 | 197 |
| Figure 46: TLC chromatogram of chloroform fraction | 199 |
| Figure 47: TLC chromatogram of open column chromatography of CHCl_3 fraction | 200 |
| Figure 48a and b: TLC chromatogram of open column chromatography of EtOAc fraction | 201 |
| Figure 49: TLC chromatogram of compound 3 | 202 |
| Figure 50: ^1H NMR spectrum of compound 3 | 205 |
| Figure 51: ^{13}C NMR spectrum of the compound 3 | 206 |
| Figure 52: APT spectrum of compound 3 | 207 |
| Figure 53: Expanded APT spectrum of compound 3 | 208 |
| Figure 54: IR spectrum of compound 3 | 209 |

| | |
|---|-----|
| Figure 55: GC Mass spectrum of compound 3 | 210 |
| Figure 56: NOESY spectrum of compound 3 | 211 |
| Figure 57: COSY spectrum of compound 3 | 212 |
| Figure 58: HMBC spectrum of compound 3 | 213 |
| Figure 59: HSQC spectrum of compound 3 | 214 |
| Figure 60: 3 Dimensional Ball and Stick minimized energy mode for compound 2 | 215 |
| Figure 61: 3 Dimensional Ball and Stick minimized energy mode for compound 2 | 216 |
| Figure 62: 3 Dimensional ball and stick minimized energy mode for compound 2 | 217 |
| Figure 63: Structure of compound 3 | 219 |