PHYTOCHEMICAL AND ANTI-DREPANOCYTOSIS
STUDIES OF CAJANUS CAJAN, CALLISTEMON
VIMINALIS, MELALEUCA BRACTEATA VAR.
REVOLUTION GOLD AND SYZYGIUM GUINEENSE

By

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Submitted in fulfilment of the academic requirements for the degree of
Master of Science in the School of Chemistry, University of KwaZulu-
Natal, Westville, Durban, South Africa

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DECLARATION

The experimental work described in this thesis was carried out in the School of Chemistry, Faculty of Science and Agriculture, University of KwaZulu Natal, Durban, South Africa under the supervision and the co-supervision of Professor Francis Oluwole Shode and Dr Neil Koorbanally, respectively.

This study represents original work by the author and has not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Signed:

_______________________________________
Damien S. Tshibangu

We hereby certify that the above statement is correct.

Signed:

_________________    ______________________
Dr. Neil Koorbanally     Professor Francis O. Shode
Ph.D. (Natal)           Ph.D. (Sheffield)
DEDICATION

To Jesus-Christ, my God, the one who helped me, when performing this research.

To my wife, Thérèse Ngomba Muamb and our boys, Moïse Kasombo, Emmanuel Kazadi, Aaron Mwamba, Joseph Musasa and Elie de Dieu Tshibangu, for your patience and all your love.
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To all our students at the Université de Kinshasa, who worked for a long time with me during the collection of plant materials and also in remembrance of one of them who passed away after successfully completing her Honours degree, Frida Mandiangu; may her soul rest in peace.
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Finally, I would like to express my deepest thanks and profound gratitude to Third World of Academy and Science (TWAS), for 2008 Research and Advanced Training fellowship awarded to me.
ABSTRACT

All over Africa, traditional healers use medicinal plants to prepare medicines to treat a wide range of illnesses. One of these illnesses is sickle cell anaemia or drepanocytosis or sicklemia. This disease is particularly common among sub-Saharan Africans with a clear predominance in equatorial Africa. However, it also exists in North Africa, Greece, Turkey, Saudi Arabia and India. An estimated 50 million people are affected worldwide. A literature review on sickle cell anaemia revealed that a number of plants have anti-drepanocytosic activity. The availability and frequency of ethnobotanic use of plants were taken into account when selecting the plants investigated in this study. *Cajanus cajan, Callistemon viminalis, Melaleuca bracteata var. Revolution Gold* and *Syzygium guineense* (from DRC and South Africa) were selected for study. The selected plants were subjected to modern phytochemical analysis. A total of 8 compounds were isolated from the plants’ extracts and their structures determined by modern spectroscopic techniques (1D and 2D NMR, FT-IR and MS). *S. guineense* from DRC (Democratic Republic of Congo) yielded flavanonoid glycoside (A) as its major chemical constituent. The South African *S. guineense* afforded 4 compounds namely betulinic acid (B), sitosterol (C), friedelan-3-one (D) and a betulinic acid derivative (E). *Cajanus cajan* showed the presence of fatty acids, one of them was characterized as an unsaturated fatty acid (I). *Callistemon viminalis* afforded one compound, betulic acid (F) and *Melaleuca bracteata* afforded two compounds which were characterized as betulinic acid acetate (G) and ursolic acid acetate (H).
\[ \text{CH}_3-(\text{CH}_2)_n-\text{CH}_2-\text{CH}:=\text{CH}-\text{CH}_2-\text{CH}:=\text{CH}-(\text{CH}_2)_n-\text{CH}_2-\text{CH}_2-\text{COOH} \]

I

The investigation of the anti-drepanocytosis activities of the extractives and their crude extracts showed in vitro antisickling activity.

Ethyl acetate crude extracts of *Callistemon viminalis* and *Melaleuca bracteata*; hexane, dichloromethane and ethyl acetate crude extracts of *Syzygium guineense* of DRC, betulinic acid, betulinic acid acetate and maslinic acid showed a high antisickling activity, more than 70% of normalization. The compound BF$_4$, a fatty acid, from *Melaleuca bracteata* was found to have a medium activity, between 50 and 70% of normalization and oleanolic acid showed the weakest activity, between 10 and 50 % of normalization.

Maslinic acid and oleanolic acid which were used for anti-sickling bioassay were isolated and characterized from *Syzygium cordatum* by my supervisor, Professor Shode.

Others crude extracts and pure isolated compounds were found to be non-active anti-sickling agents. These included crude hexane and methanol extracts of *Cajanus cajan*; crude dichloromethane extract of *Callistemon viminalis*; crude dichloromethane, methanol and 80% aqueous methanol extracts of *Melaleuca bracteata*; crude hexane, dichloromethane, ethyl acetate, and methanol extracts of *Syzygium guineense* (South Africa); ursolic acid from *Melaleuca bracteata* and flavanone glycoside from *Syzygium guineense* of DRC.

This is the first report of the in vitro anti-sickling activity of betunilic acid, betulinic acid acetate, oleanolic acid, and maslinic acid.
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<th>Description</th>
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<tr>
<td>AIDS</td>
<td>Acquired Immuno Deficiency Syndrome</td>
</tr>
<tr>
<td>Aq. MeOH</td>
<td>Aqueous Methanol</td>
</tr>
<tr>
<td>BC</td>
<td>Before Jesus Christ</td>
</tr>
<tr>
<td>br s</td>
<td>broad singlet</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>$^{13}$C-N.M.R.</td>
<td>Carbon 13 Nuclear Magnetic Resonance spectroscopy</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COSY</td>
<td>COrelation SpectroscopY</td>
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<tr>
<td>COX</td>
<td>CycloOXygenase</td>
</tr>
<tr>
<td>Cpd</td>
<td>Compound</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarization Transfer</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRC</td>
<td>Democratic Republic of Congo</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl Acetate</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transformed InfraRed spectroscopy</td>
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<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrometry</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Coherence</td>
</tr>
<tr>
<td>$^1$H- NMR</td>
<td>Proton Nuclear Magnetic Resonance spectroscopy</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>INERA</td>
<td>Institut National d’ Etudes et Recherches Agronomiques</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MCN</td>
<td>Minimal concentration of normalization</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>mp</td>
<td>Melting point</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser Enhancement Spectroscopy</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
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<td>Q</td>
<td>Quartet</td>
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<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
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<td>Reference</td>
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<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Ratio-to-front</td>
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<td>Singlet</td>
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<td>SA</td>
<td>South Africa</td>
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<td>t</td>
<td>Triplet</td>
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<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>Tetramethylsilane</td>
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<td>United States of America</td>
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CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Medicinal Plants in African Traditional Medicine - an Overview

1.1.1 Historical perspectives

Traditional medicine is defined by the World Health Organization (WHO) as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses. Recourse to folk medicines, which are mainly based on plants, enjoys a respectable position nowadays. Plants have always been considered a healthy source of life for all people. Several well-known plant species which extracts were listed as part of folk medicine as far back as 2600 BC, are still being used today as treatments for inflammation, influenza, coughing and parasitic infestation.

Chinese herb guides document the use of herbaceous plants as far back in time as 2000 BC.

In developing countries, where the availability of modern health services is limited, traditional medicine is popular among the people of both urban and rural areas. Most of the people who patronize the traditional healers believe that traditional medicine is safe, effective, and inexpensive. The World Health Organization estimates that, even in many developed countries, a great portion of the population uses of traditional health remedies, especially medicinal plants. Although easy access to modern medicine is available in these countries, the use of medicinal herbs has kept its popularity for historical and cultural reasons. On the other hand, in the developing countries in Africa and other parts of the world, 65-80% of the population depend largely on medicinal plants for basic healthcare.

1.1.2 Medicinal Plants in Traditional African Medicine
Traditional African Medicine (TAM) is defined as a holistic discipline involving extensive use of indigenous herbalism combined with aspects of African spirituality. Practitioners of traditional African medicine claim to be able to cure a wide range of conditions, including cancers, acquired immune deficiency syndrome (AIDS), psychiatric disorders, high blood pressure, cholera, infertility, and most venereal diseases. Other conditions include epilepsy, asthma, eczema, hayfever, anxiety, depression, benign prostatic hypertrophy, urinary tract infections, gout, and healing of wounds and burns.

Despite numerous attempts at government interference, ATM system of healing continues to thrive in Africa and practitioners can be found in many other parts of the world. Under colonial rule, many nations considered traditional diviner-healers to be practitioners of witchcraft and outlawed them for that reason. In some areas of colonial Africa, attempts were also made to control the sale of traditional herbal medicines. After Mozambique obtained independence in 1975, diviner healers were sent to re-education camps. Opposition to traditional medicine has been particularly vehement during times of conflict, when people have been more likely to call on the supernatural realm. More recently, interest has been expressed in integrating traditional African medicine with the continent's national health care systems. In Kwa-Mhlanga, 65 kilometers from Pretoria, South Africa, a 48-bed hospital combines traditional African medicine with homeopathy, iridology, and other Western healing methods, as well as traditional Asian medicine. Founded by a traditional African healer, the hospital is said to be the first of its kind in South Africa.

WHO has encouraged some African countries to enhance development in Traditional African Medicine. At present, WHO is supporting clinical studies on antimalarials in three African countries; the studies are revealing good potential for herbal antimalarials. In contrast, African concepts of health and medicine have been portrayed as nothing more than witchcraft by Hollywood and the Western media. Nevertheless, some of these herbal medicines have indeed been proven effective against AIDS-related opportunistic infections. Africa is endowed with an enormous wealth of plant resources, it has been estimated that between 450 000 - 500 000 plant species are known to occur in the forest regions alone, and many of them have been used for several centuries in traditional medicines for the
prevention and treatment of diseases. Although a large number of research publications is available on the constituents and biological activities of medicinal plants from Africa, the development of therapeutic agents from African medicinal plants has remained a somewhat neglected subject.\textsuperscript{13} Several scientists have documented African medicinal plants and their uses.\textsuperscript{14-16} For example, Hutchings et al.\textsuperscript{14} have published several medicinal plants used by the Zulus. Some of these Zulu medicinal plants are listed in Table 1.1.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Asparagus africanus</td>
<td>Emetics for nausea and colic</td>
</tr>
<tr>
<td>2  Alepidea amatymbica</td>
<td>Colds, coughs, influenza</td>
</tr>
<tr>
<td>3  Bulbine latifolia</td>
<td>Eczema, syphilis</td>
</tr>
<tr>
<td>4  Cissampelos fasciculata</td>
<td>Charm against lightening</td>
</tr>
<tr>
<td>5  Clausena anisata</td>
<td>Tapeworm remedy</td>
</tr>
<tr>
<td>6  Curtisia dentate</td>
<td>Aphrodisiac, diarrhoea</td>
</tr>
<tr>
<td>7  Drimia elata</td>
<td>Stomach ailments, hypertension</td>
</tr>
<tr>
<td>8  Dioscorea sylvatica</td>
<td>Blood problems</td>
</tr>
<tr>
<td>9  Gasteria cruocheri</td>
<td>Charms</td>
</tr>
<tr>
<td>10 Gunnera perpensa</td>
<td>Cystisis, easy child birth</td>
</tr>
<tr>
<td>11 Haworthia fasciata</td>
<td>Protective charms</td>
</tr>
<tr>
<td>12 Helichrysum odoratissimum</td>
<td>Coughs and colds</td>
</tr>
<tr>
<td>13 Hydnora africana</td>
<td>Diarrhoea</td>
</tr>
</tbody>
</table>
Table 1.1 Continued.

<table>
<thead>
<tr>
<th></th>
<th>Plant Name</th>
<th>Medical Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td><em>Protorhus longifolia</em></td>
<td>Heartburn, bleeding of the stomach</td>
</tr>
<tr>
<td>17</td>
<td><em>Rhoicissus digitata</em></td>
<td>Pregnancy delivery, cattle diseases</td>
</tr>
<tr>
<td>18</td>
<td><em>Rubia cordifolia</em></td>
<td>Impotence, control of menses</td>
</tr>
<tr>
<td>19</td>
<td><em>Strychnos henningsii</em></td>
<td>Stomach complaints</td>
</tr>
<tr>
<td>20</td>
<td><em>Strychnos decussata</em></td>
<td>Cramps, protective charms</td>
</tr>
</tbody>
</table>

African medicinal plants have advanced with publications arising from the following research areas: antimicrobial (16%), molluscicidal (11%), antimalarial (7%), plant toxicology (7%), anti-tumour-related studies (4%) and others (54%). This continent has a long and impressive list of medicinal plants based on local knowledge. Countries such as Burkina Faso, Madagascar, Mali and Tanzania have made partnership arrangements with traditional health practitioners and the private sector as equal. For instance, *Securidaca longepedunculata* is a tropical plant found almost everywhere in Africa. The dried bark and root are used in Tanzania as a purgative for nervous system disorders.

Throughout East Africa, the plant’s dried leaves are used for wounds and sores, coughs, venereal disease, and snakebite. In Malawi, the extract of the leaves are used for wounds, coughs, bilharzias, venereal disease, and snakebite as well as headache. The extract of the dried leaves are used in Nigeria for skin diseases. According to one pharmaceutical researcher, the root is used in Botswana and Zimbabwe for malaria. Meanwhile, in Angola, the dried root is used as both a fish poison and as an aphrodisiac. The same dried roots have religious significance in Guinea-Bissau and are understood to have a psychotropic effect. The root bark is used for epilepsy in Ghana.\textsuperscript{15,16}
Phytolacca dodecandra, Tetrapleura tetraptera, Urginea epigea and Swartzia madagascariensis had become an international research interest for the control of schistosomiasis. The root of Cryptolepis sanguinolenta, used traditionally for treating urinary infections, is strongly antimicrobial and the active principle is cryptolepine 1.1. The common chewing sticks used by the Africans in various communities for traditional dental care have been reported to possess activity against oral microbial flora and to contain various minerals which can hinder plaque formation. Ancistrocladus abbreviatus, a Cameroonian plant species, was studied in the laboratory of the National Cancer Institute in the United States of America (USA), it showed a strong anti-HIV activity. This activity was attributed to the alkaloids, Michellamines A 1.2 and B 1.3 isolated from the plant.

![Structure of Cryptolepine 1.1](image)

![Structure of Michellamine A 1.2](image)
Some plant drugs, even in the crude form, from Africa, are well known in the international markets today. For instance *Rauwolfia vomitoria* which is a major source of reserpine, a major tranquilizer and an antihypertensive; ginger (*Zingiber officinale*), which contains gingerol used as spice, carminative and important medicinal product, produced also in Nigeria. *Capsicum annum* produces capsain and capsacin, used as spice and medicine; *Physostigma venenosum* also known as Calabar bean, produces physostigmine or eserine used in ophthalmia; *Syzygium aromaticum* is a dental remedy and also *Chrysanthemum cinerariifolium*, called pyrethrum flower, produces the natural pyrethrins, a class of insecticides. Others include *Catharanthus roseus*, also called the Vinca or Madagascar Rose periwinkle, used in the management of leukaemia and Hodgkin’s disease; *Agave sisalana*, exported by Tanzania, is rich in hecogenin and is employed for the partial synthesis of steroidal drugs such as corticosteroids and oral contraceptives; also *Cinchona succirubra*, which yields quinine which is a key antimalarial drug of long history. Practitioners of traditional African medicine claim to be able to cure a wide range of conditions, including cancers, acquired immunodeficiency syndrome (AIDS), psychiatric disorders, high blood pressure, cholera, infertility, and most venereal diseases. Others include epilepsy, asthma, eczema, hay fever, anxiety, depression, benign prostatic
hypertrophy, urinary tract infections, gout, and healing of wounds and burns.\textsuperscript{14,17} \textit{Zanthoxylum zanthoxyloides} (Lam.) Waterm, an antisickling and anticancer plant, was found to contain alkaloids: berberine, fagaronine, chelerythrine, canthin-6-one and benzoic acid derivatives as the main active ingredients.

### 1.2 Drepanocytosis

#### 1.2.1 Introduction

Drepanocytosis (from the Greek drepnos, sickle), also known as Sickle Cell Disease (SCD), also known as haemoglobinose S or sicklemia or sickle cell anemia. It is a hereditary disease characterized by the alteration of haemoglobin, a protein of the transport of oxygen in the blood. This autosomal recessive genetic disease results from a genetic defect which induces the substitution of the sixth amino acid (glutamic acid) of the $\beta$ chain of haemoglobin with another amino acid (valine).\textsuperscript{18} This is illustrated in Figures 1a and 1b.

![Structure of haemoglobin showing points of mutation in drepanocytes (sickle cells).](image)

Figures 1a and 1b Structure of haemoglobin showing points of mutation in drepanocytes (sickle cells).
SCD remains one of the diseases treated traditionally by the use of medicinal plants in Africa. This genetic disease affects Africans for several centuries and nowadays it is widespread all over the world. The African subtropical region is the most affected with 25-30% of heterozygotes HbAS or HbCS. Unfortunately, up-to-date, there is no efficient therapy. Proposed therapies remain very expensive for most of the population who have recourse to medicinal plants, in order to treat drepanocytosis.

The Blood

The blood is a red colored liquid that delivers necessary substances to the body's cells such as nutrients and oxygen and has an important role to transport waste products away from those same cells. It is slightly denser and approximately 3-4 times more viscous than water. Blood consists of cells which are suspended in a liquid.

In human body, blood performs many important functions within the body including:

- supply of oxygen to tissues (bound to haemoglobin, which is carried in red cells);
- supply of nutrients such as glucose, amino acids and fatty acids (dissolved in the blood or bound to plasma proteins (for example, bloods lipids);
- removal of waste such as carbon dioxide, urea, and lactic acid;
- immunological functions, including circulation of white blood cells and detection of foreign material by antibodies;
- coagulation which is one part of the body's self-repair mechanism;
- messenger functions, including the transport of hormones and the signalling of tissue damage;
- regulation of body pH (the normal pH of blood is in the range of 7.35 - 7.45) (covering only 0.1 pH unit);
- regulation of core body temperature;
- hydraulic functions.
History
In 1904, James B. Herrick (1861-1954), a physician (cardiologist) in Chicago, was the first medical scientist to give a medical description of sickle cell disease. Forty-five years later, James Neel showed that the transmission of this disease was Mendelian. The same year Linus Pauling showed that it was due to an abnormal structure of haemoglobin, characterized by low solubility. This was the first time to discover the molecular origin of a genetic disease. In 1956, the British Vernon Ingram showed that it was due to the replacement of an amino acid in the abnormal haemoglobin. This demonstrated for the first time that genes determine the nature of each amino acid in a protein. In 1978, Tom Maniatis isolated the gene for beta globin.

1.2.2 Geographical distribution
Sickle cell anaemia is particularly common in populations of Africans in sub-Saharan Africa, with a clear predominance in Equatorial Africa (reaching in some populations the frequency of 30%), but it also exists in North Africa, Greece, Turkey, Saudi Arabia, Italy (Sicily), Anatolia and India. An estimated of 50 million people are affected in the world. In Africa, about 300,000 infants are born with this genetic abnormality and the majority are HbSS.

In rural villages, as few as 2% of sickle cell disease patients survive beyond the age of 5 years. This distribution overlaps quite well with that of another disease, malaria, which has an infectious origin: *Plasmodium falciparum*. In the United States of America (USA), sickle-cell anaemia primarily affects African Americans where the distribution of the S gene is about 8% of the black population. The disease occurs in approximately 1 in every 500 African-American births. Figure 2 shows the geographical distribution of sickle anaemia worldwide which seems to overlap with area where the malaria parasite is endemic, especially in tropical Africa, the Middle East, and Asia.
1.2.3 Characteristics of Drepanocytosis

Red cells from homozygous blood, which contain almost exclusively of HbS, acquire the property to polymerize in low oxygen tension. This explains why the sickling of red blood cells is triggered by lack of oxygen in the blood (hypoxia). Figure 3 shows the normal and sickle-shaped red blood cells.
The disease is reported in infants, but is not usually evident at birth because the red cells of newborns still contain 50-90% of foetal haemoglobin. The symptoms of this disease can appear at six months of age.\textsuperscript{18}

The usual acute manifestations of sickle cell disease are of three kinds:

- **Vaso-occlusive crises**: clots clog an artery, causing sudden and severe pain in one part of the body (often the hands, feet, hips, and abdomen). These crises can be very painful;
- **Haemolytic anaemia**: red blood cells of sickle cell disease are abnormal. Under low oxygen tension, the red blood cells assume a sickle shape, and are consequently entrapped by the spleen, where they are destroyed. This destruction leads to a decrease in the number of erythrocytes and thus a regeneration of anaemia;
- **Infections**: Infections are more common in sickle cell disease, especially pneumonia in young children.\textsuperscript{18}

Chronic manifestations of sickle cell disease include (i) stroke, which can result from a progressive narrowing of blood vessels; (ii) cholelithiasis (gallstones) which may result from excessive bilirubin production and precipitation due to prolonged haemolysis; (iii) jaundice, yellowing of the skin, may occur due to the inability of the liver to effectively remove bilirubin from the filtering of damaged red blood cells out of the blood supply as well as blocks in the organ’s blood supply, and so on.\textsuperscript{18}

### 1.2.4 Identification tests

- A simple blood test, by the microscopic observation of fresh blood stored between glass slide and cover slide, with or without the addition of a reducing agent (potassium metabisulfite, or ascorbic acid), can determine if an individual carries at least one haemoglobin S gene.\textsuperscript{27} Test by electrophoresis: haemoglobin samples are identified by the way they behave in an electric field.
In USA, most newborn infants are tested for sickle-cell anemia before they leave the hospital. If it is diagnosed early, some of the complications, particularly severe infections, can be prevented with antibiotics and vaccinations.27

Today, tests can detect carriers (people who have the heterozygous allele but are not sick).29

1.3 Past and Present Research and Development of Anti-drepanocytosis Drugs - An overview

A. Chemotherapy

Treatment of sickle-cell anemia is geared towards preventing infection, reducing organ damage, and minimizing pain and discomfort.31 Several agents were developed by rational drug design to inhibit the sickling process in vitro. These agents interfere with the mechanism and/or kinetics of the sickling process.

Unfortunately, most of the agents couldn’t show promising success in terms of clinical use.32 If sickle cell anemia is diagnosed early, some of the complications, particularly severe infections, can be prevented with antibiotics and vaccinations. Daily penicillin prophylaxis is the most commonly used treatment during childhood. Also, the daily treatment with the cancer drug hydroxyurea has recently been shown to reduce the number of pain episodes and the severity of the anemia.32-34 Regular blood transfusions treat anemia by replenishing red blood cells and preventing other complications, such as stroke.29 Bone marrow transplantation cures sickle-cell anemia in a small number of children who are able to find an acceptable, related bone marrow donor.35, 36 Milder crises of the vaso-occlusive crisis which is caused by sickle-shaped red blood cells that obstruct capillaries and restrict blood flow to an organ can be treated using diclofenac or naproxen. For more severe crises, most patients are treated by the use of intravenous opioid or associated with diphenylamine.37, 25
B. Phytotherapy

As alluded to in section 1.2.1 previously, drepanocytosis remains one of the diseases treated traditionally by the use of medicinal plants. In developing countries, the use of medicinal plants to treat drepanocytosis is gaining popularity among the people of both urban and rural areas. Ethnic groups on indigenous traditional medicine, as source of information have played a vital role in the discovery of novel products from plants as chemotherapeutic agents. Some previous studies carried out on plants used by traditional healers to treat drepanocytosis revealed their interesting antisickling activities.

Some examples of medicinal plants used traditionally are elaborated below:

(i) *Fagara zanthoxyloides.* In 1973, the root extracts of *F. zanthoxyloides* was shown to possess antisickling properties.

(ii) *Adansonia digitata.* In 1988, Adesanya and co-workers showed that *A. digitata* which is used traditionally to treat sickle cell anaemia in Nigeria has a little inhibitory activity against sickle cells.

(iii) *Cajanus cajan.* Ekeke and Shode, in 1985 showed that the water-soluble extract of the seeds of *C. cajan* reversed sickled cells to normalcy and also, inhibited sickling.

(iv) *Stephania cepharantia.* An investigation of the leaves of *S. cepharantia* showed that its aqueous and ethanolic extracts had interesting antisickling activity.

(v) Furthermore, Egunyomi and co-workers showed the *in vitro* antisickling activity of the methanol extract of the parts of two plants used in SCD management in Nigeria.

(vi) From 2007 up-to-date, several works of Mpiana and co-workers showed promising antisickling activities of some crude extracts of plants and fractions of some plants used traditionally against sickle cell anaemia. Some of these plants are: *Syzygium guineense, Cajanus cajan, Alchornea cordifolia, Hymenocardia acida, Annona senegalensis, Jatropha curcas,* and *Ocimum basilicum.*
1.4 Aim and Objectives of Research

The main aim of this project was to isolate and characterize compounds with possible anti-drepanocytosis activity from selected medicinal plants. The selected plants were *Cajanus cajan*, *Callistemon viminalis*, *Melaleuca bracteata* var. *revolution gold*, *Syzygium guineense* (Congolese origin), and *Syzygium guineense* (South African origin).

Specific objectives:

- To identify, collect, and prepare the leaves or seeds of the selected plants for extraction;
- To extract the powdered plant materials sequentially using different organic solvents;
- To isolate, purify, and characterize compounds from the plants extracts with promising bioactivities using chromatographic methods;
- To determine the antisickling activities of crude extracts and purified compounds.

This work is subdivided into 5 chapters. The first chapter is a general introduction and some literature review while the second to the fourth chapters present the literature review of studied plants, the description of the extractives, and results obtained from the phytochemical examination of the study plants. The last chapter presents the results of the biological tests performed on the crude extracts and the isolated compounds. A conclusion and some suggestions on the project end this thesis.
1.5 References


CHAPTER TWO

PHYTOCHEMICAL EXAMINATION OF SYZYGIUM GUINEENSE

2.1 Introduction

_Syzygium_ is a genus of flowering plants that belongs to the Myrtle family or Myrtaceae. The genus comprises about 1100 species, and has a native range that extends from Africa and Madagascar through southern Asia. Its highest levels of diversity occur from Malaysia to northeastern Australia, where many species are very poorly known and many more have not been described taxonomically. Most species are evergreen trees and shrubs. Several species are grown as ornamental plants for their attractive glossy foliage, and a few produce edible fruits that are eaten fresh or used in jams and jellies.¹ ²

2.2 _Syzygium guineense_ (Willd.) DC.

_S. guineense_ is one of the most widespread African tree species. It certainly occurs in a greater range of vegetation types and shows a larger variety of growth forms than any other African plant. It is widely distributed in the rain forests of the Guineo-Congolian Region and the montane forests of the Afromontane Region, as well as in riverine forests and woodland in the Sudanian Region. It ranges from a lofty forest tree 30 m or more tall. The flowers are essentially uniform and the fruits show only slight difference in shape. Variation in leaf-shape and size at first sight appears to be continuous, but in the field much of this variation is seen to be closely correlated with ecology and habit.¹ ²

Scientific classification

- Domain: **Eukaryota**
- Kingdom: **Plantae**
- Subkingdom: **Viridaeplantae**
- Phylum: **Tracheophyta**
Subphylum: **Euphyllophytina**
Infraphylum: **Radiatopses**
Class: **Magnoliopsida**
Subclass: **Rosidae**
Superorder: **Myrtanae**
Order: **Myrtales**
Suborder: **Myrtineae**
Family: **Myrtaceae**
Subfamily: **Myrtoideae**
Tribe: **Syzygieae**
Genus: **Syzygium**
Specific epithet: guineense
Botanical name: **Syzygium guineense** (Wild.) DC.²

**General uses**
As medicines, *Syzygium guineense* is used as a febrifuge and anti-aborifiacient. It’s also used for menstrual cycle, malnutrition, debility, naso-pharyngeal affections, pain-killers, pulmonary troubles, laxatives, heart, skeletal structure, stomach troubles, pregnancy, diarrhoea, dysentery, arthritis, rheumatism, venereal diseases and anaemia. The fruits of this plant are eaten as meal (sauces, condiments, spices, flavourings), while its barks and woods have different uses, such as building materials for carpentry and related applications; farming, forestry, hunting and fishing apparatus; fuel and lighting; pastimes-carving, musical instruments, games, toys. This plant is also used as ornamental plant.²
2.3 Previous work done on S. guineense

A. Phytochemistry

The essential oil from dried leaves of S. guineense from Benin was analyzed by gas phase chromatography coupled to mass spectrometry and it showed the presence of caryophyllene oxide (7%), δ-cadinene (7.5%), viridiflorol (7.5%), epi-α-cadinol (9.8%), α-cadinol (12.7%), cis-calamen-10-ol (14%), citronellyl pentanoate (15.2%), β-caryophyllene (20.1%) and α-humulene (39.5%). Djoukeng et al. isolated ten triterpenes from the leaf extracts of S. guineense namely betulinic acid 2.1, oleanolic acid 2.2, a mixture of 2α-hydroxyoleanolic acid 2.3, 2α-hydroxyursolic acid 2.4, arjunolic acid 2.5 and asiatic acid 2.6, a mixture of terminolic acid 2.7, and 6-hydroxyasiatic acid 2.8, and a mixture of arjunolic acid 28-β-glucopyranosyl ester 2.9 and asiatic acid 28-β-glucopyranosyl ester 2.10.

![Chemical structures of 2.1 and 2.2](image1)

![Chemical structures of 2.3 and 2.4](image2)
Furthermore, the complex extract of *S. guineense* afforded six ellagic acid conjugates; the known 3-0-methylellagic acid-4′-O-α-rhamnopyranoside, two new ellagic acid rhamnopyranosides, 3-O-Me ellagic acid-4′-O-α-2″-O-acetylrhamnopyranoside and 3-O-Me ellagic acid-4′-O-α-3″-O-acetylrhamnopyranoside.\(^5\)

Phytochemical study of the leaves of *S. guineense* from Mali and from Nigeria was carried out and showed the presence of some secondary metabolites such as alkaloids, tannins, sterols, triterpenes, anthocyanins, leucoanthocyanins, saponins, and flavonoids.\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)

In 1987, Eyélé Mvé-Mba found large amounts of *cis*-guaiene (30%) and *β*-caryophyllene (15.7 %) in essential oil from the leaves of *S. guineense* from Gabon.\(^9\)

### B. Pharmacology

Triterpenes isolated and characterised from the plant are biologically active against bacteria (*Escherichia coli*, *Bacillus subtilis* and *Shigella sonnei*).\(^4\) Tsakala in 1996 showed the activity against strains of *Salmonella E.*, *Shigella D.*, *Shigella F.*, *E. coli* and *Enterobacter A.* of a dried aqueous decoction on *S. guineense* collected in the Democratic Republic of Congo (DRC).\(^10\) Malele reported that a methanol extract of *S. guineense* bark inhibited intrinsic contractions of rabbit isolated ileum.\(^11\) Amadou Diallo in 2005 showed antioxidant and anti-inflammatory activities of an aqueous decoction of the leaves of *S. guineense* from Mali.\(^6\) Oyewale in 2007 showed the antimicrobial effects of secondary metabolites identified from *S. guineense* from Nigeria, and cytotoxicity tests carried out on plant extract showed activities against the following pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Candida albican*, *Salmonella typhi*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.\(^7\) In 2008, Mpiana established the antisickling activity, *in vitro*, of the leaves of *S. guineense* collected in DRC.\(^8\)
2.4 Extractives from *S. guineense* (Congolese origin)

A. Foreword to experimental

1. General extraction protocol and chromatographic techniques

The general extraction protocol of the plant materials followed in this research is summarised in Scheme 2.1, page 27.

The extracts were subjected to column chromatography over silica gel 60 (0.040 – 0.63 mm [230-400 mesh]). The column was eluted with different eluents as specified below. Collected fractions were monitored on TLC plate (silica gel 60 F<sub>254</sub> aluminium barking from Merck, Germany). Fractions of different volumes as specified below were collected and similar fractions according to the TLC analysis were pooled to give combined fractions and labelled.

2. Nuclear Magnetic Resonance (NMR) spectroscopy

The $^1$H, $^{13}$C and all 2D NMR spectroscopy were recorded using a 400 MHz Bruker spectrometer at the University of KwaZulu-Natal, Westville Campus. All the spectra were recorded at room temperature using deuteriochloroform (CDCl<sub>3</sub>) or a mixture of deuteriochloroform and deuteriomethyl sulfoxide (DMSO) as specified on each spectrum. All spectra were referenced according to the central line of deuterioated chloroform at $\delta_H$ 7.24 for $^1$H-NMR spectra and $\delta_C$ 77.20 for $^{13}$C -NMR spectra.

3. Infrared spectroscopy (IR)

All spectra were recorded using a Perkin-Elmer Spectrum FTIR spectrophotometer. Samples were calibrated against an air background and crystalline samples were directly placed on the window and then analysed in KBr.
4. **Gas chromatography-mass spectrometry (GC-MS)**

Mass spectrometry of compound was recorded using an Agilent 6890 series gas chromatography system. The compound was dissolved in HPLC grade methanol (1:100) and helium (at 0.8 mL/minute). A gas carrier programmed to 300°C (5°C/minute) was first maintained at 200 °C for 4 minutes.

5. **Liquid chromatography-mass spectrometry (LC-MS).**

The liquid chromatography was recorded using an Agilent 1100 series LC/MSD Trap system. Compounds were dissolved in HPLC grade methanol and the following parameters were observed while running the instrument: Injection: 6 µl; flow: 0.3 ml/min; time: 1.5 minutes; maximum pressure: 150 bar, minimum pressure: 0 bar, temperature 24.4 - 26.8 °C. Mobile phases used were acetonitrile 95% and Millipore water 5%.

**2.4.1 Extraction and isolation**

The leaves of *Syzygium guineense* were collected in Kinshasa, DRC, and authenticated at the Institut National d’ Etudes et Recherches Agronomiques (INERA), Faculty of Science, Université de Kinshasa. A voucher specimen is deposited at the INERA Herbarium no. Carlier 53; Errard 6303/6558; Breyne no. 36.199. The leaves were dried at room temperature and powdered using a laboratory blender.

*S. guineense* leaves (powder, 281.7g) were extracted sequentially, after 24 hours, with n-hexane, dichloromethane, ethyl acetate, methanol, and 80% aqueous methanol as shown in Scheme 2.1. Each extraction was optimized by repeating the maceration twice. Each solvent extract was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give hexane-solubles (DS/2/A) (6.11g, 2.2%), dichloromethane-solubles (DS/2/B) (3.01g, 1.1%), ethyl acetate-solubles (DS/2/C) (1.69g, 0.6%), methanol-solubles (DS/2/D) (72.27g, 25.7%), and aqueous methanol-solubles (DS/2/E) (2.10g, 0.7%), respectively.
The ethyl acetate extract was triturated with ethyl acetate during which a white solid was obtained. This solid was filtered, weighed (0.63 g), coded as DS/2/C/2, and subjected to column chromatography over silica gel 60 (0.040 – 0.63 mm [230-400 mesh]). The column was eluted with a mixture of hexane - ethyl acetate (1:1 to 3:7) and ethyl acetate - methanol (9:1). Collected fractions were monitored on TLC plate (silica gel 60 F254 aluminium backing from Merck, Germany). A total of 185 fractions of 7 mL volumes each were collected. Similar fractions according to the TLC analysis were pooled to give 4 (SA, SB, SC, SD) combined fractions. Combined fraction SA eluted with hexane - ethyl acetate (1:1) afforded one spot; greenish oily liquid, in small amounts. Combined fractions SB eluted with hexane-ethyl acetate 1:1 and SC and SD eluted with ethyl acetate – methanol 9:1 afforded two spots each. Combined fraction SC (280 mg) contained the major compound, whitish solid, contaminated by SD; while the combined fraction SB was in small quantities.
Scheme 2.1 General extraction protocol adopted for the isolation of plant material
Acetylation of SC:
Further purification of SC was achieved by acetylation using standard method to give an acetylated mixture. This mixture was purified by preparative TLC (silica gel 60 F_{254} Merck 5715) to give acetylated compound A'.

2.4.2 Results and Discussion
A. Structural characterization of Acetylated Compound A’:

Physical data:
Name: Flavanone glycoside peracetate
Yield: 0.280g (0.071 %)
Experimental melting point: 40-41°C (Literature: not found)
Molecular formula: C_{43}H_{48}O_{22}
Molecular mass (LC-MS): m/z: 916.83 g mol\(^{-1}\) [M\(^+\)]; 408.9, 331.8, 304.1, 272.7, 212.7 and 152.7 as base peak. (Figure 7.9)
FT-IR: \(\nu \text{ cm}\(^{-1}\) (KBr): 2991.82 (C-H); 1748.99 (Ester); 1259.93 (methyl groups); 1226.21 (Aromatic ethers); 787.43 (meta substituted aromatic ring). (Figure 7.8)

\(^{1}\)H-NMR (CDCl\(_3\)): see Table 2.1 (Figure 7.1).\(^{12}\)

The COSY spectrum (Spectrum A.4) and NOESY spectrum (Figure 7.6) confirm these assignments of H-5' and H-6' as they are correlating, as well as HMBC (Figure 7.7), where H-5' is correlating to H-6' is correlating to H-2' (\(^3\)J). The HSQC (Figure 7.5) correlated the aromatic protons described above to signals at \(\delta_{C} 122.1\ (d, \ C-6')\); \(\delta_{C} 127.43\ (d, \ C-5')\); \(\delta_{C} 106.0\ (s, \ C-6)\); \(\delta_{C} 102.4\ (s, \ C-8)\). C-7 is HMBC correlating with C-1'' (\(^3\)J). This is confirming the glycoside bond to aglycone of these two carbons. There is a \(^2\)J correlation between C-4 and H-5' which is a C→H correlation.
$^{13}$C-NMR (Figure 7.2). A literature search revealed compound A to be a flavanone glycoside. The fully assigned NMR data of compound A acetate is shown in Table 2.1.\textsuperscript{12, 13}

**Proposition of structure of Compound A peracetate**

![Structure of acetylated flavanone glycoside A.](image)

It is therefore proposed tentatively that the structure of compound A is 7-[[2-O-(6-deoxy-$\alpha$-L-mannopyranosyl)-$\beta$-D-glucopyranosyl] oxy]-2,3-dihydro-5-hydroxy 2-(4-hydroxyphényl)-4H-1-benzopyran-4-one as shown in Figure 2.12. Further chemical experimentation and semi-synthesis are necessary to confirm the proposed structure.

![Proposed structure of compound A: Flavanone glycoside 7-[[2-O-(6-deoxy-$\alpha$-L-mannopyranosyl)-$\beta$-D-glucopyranosyl] oxy]-2,3-dihydro-5-hydroxy 2-(4-hydroxyphényl)-4H-1-benzopyran-4-one](image)
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<th>$\delta_H$</th>
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<th>NOESY</th>
<th>HMBC C$\rightarrow$H</th>
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<td>Hb-3; Ha-3; H-2</td>
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<td>-</td>
<td>-</td>
<td>Ha-3 $^3$J; Hb-3 $^3$J</td>
</tr>
<tr>
<td>5</td>
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<td>-</td>
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<td>H-2 $^3$J; H-5 $^3$J</td>
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<td>-</td>
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<td>7.45 <em>d</em>; J:1.72</td>
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<td>H-2$^<em>$; H-6$^</em>$; H-2</td>
<td>H-3$^3$J; H-6 $^4$J</td>
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<td>7.15 (br d)</td>
<td>H-5$^*$</td>
<td>H-3$^<em>$; H-5$^</em>$</td>
<td>H-2 $^3$J</td>
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<td>5.13 <em>d</em></td>
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<td>2$^{**}$</td>
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<td>5.03 <em>dd</em></td>
<td>H-1$^{<strong>}$; H-3$^{</strong>}$</td>
<td>H-5$^{<strong>}$; H-3$^{</strong>}$</td>
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<td>H-3''; H-5''; H-6''</td>
<td>H-5''  J; H-2''  J</td>
</tr>
<tr>
<td>5''</td>
<td>74.1(CH)</td>
<td>5.33 ddd</td>
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</tr>
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<td>H-3''  J</td>
</tr>
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<td>5.09 dd</td>
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<td>H-1''  J; H-3''  J</td>
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<td>H-2''  J</td>
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2.5 Extractives from *Syzygium guineense* of South African (S.A.) origin

2.5.1 Extraction and isolation

The leaves of *Syzygium guineense* were collected on the campus of the University of KwaZulu Natal in South Africa. The fresh plant materials were blended using a laboratory blinder (Russell Hobbs). The homogenate materials were used for further studies.

*S. guineense* (756 g) of fresh homogenated leaves were extracted sequentially in n-hexane, dichloromethane, ethyl acetate, methanol and aqueous methanol 80% as shown in scheme 2.1. Each extraction was optimised by repeating the maceration twice. Each solvent extraction was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give hexane-solubles (DS/8/A) (3.0g, 0.39%), dichloromethane-solubles (DS/8/B) (7.90g, 1.05%), ethyl acetate-solubles (DS/8/C) (0.60g, 0.08%), methanol-solubles (DS/8/D) (5.00g, 0.66%), and aqueous methanol-solubles (DS/8/E) (2.10g, 0.28%), respectively.

The dichloromethane extract was subjected to column chromatography using the mixture of hexane - ethyl acetate (9:1 to 6:4). A total of 90 fractions of 40 ml each were collected and 10 similar fractions (FA, FB, FC, FD, FE, FF, FG, FH, FI and FJ) were combined. Combined fraction FA eluted with hexane 100% afforded one spot; a greenish oily liquid, in small amounts. Combined fractions FC (brownish oily) and FF (whitish solid) eluted with hexane-ethyl acetate 8:2 and 7:3 respectively, afforded two spots each. The combined fraction FF (110 mg) contained the major compound. Combined fraction FD (brownish oily) and FE (brownish oily) afforded three spots each, in small amounts; while the combined fraction FB afforded four spots, but in small quantities.
Preparative TLC
For further purification, 20 mg of the combined fraction FF were submitted to a preparative TLC analysis. Two compounds were obtained chromatographically pure in hexane-ethyl acetate 7:3.

The methanol extract was subjected to column chromatography using the mixture of hexane-ethyl acetate (9:1 to 3:7). A total of 120 fractions of 40 ml each were collected and 13 similar fractions labelled SD1, SD2, SD3, SD4, SD5, SD6, SD7, SD8, SD9, SD10, SD11, SD12 and SD13 were combined. Combined fraction SD1 eluted with hexane-ethyl acetate 9:1 afforded 6 spots of greenish oily liquid, in traces. Combined fraction SD3 eluted with hexane-ethyl acetate 9:1 afforded one spot of yellowish oily liquid, in traces. Combined fractions SD2, SD4, SD5, SD8 and SD9 eluted with hexane-ethyl acetate 9:1 for SD2, SD4, hexane-ethyl acetate 8.5-1.5 for SD5 and hexane-ethyl acetate 8:2 for SD8 and SD9 afforded two spots each. The combined fractions SD6, SD7, SD12 and SD13 afforded three spots each, while the combined fractions SD10 and SD11 afforded four spots each. The combined fractions SD1, SD5 and SD9 gave the products chromatographically pure, after precipitating them in hexane for the SD1 and SD5 and in dichloromethane for SD9.

2.5.2 Results and discussion
A. Structure elucidation of compound B from dichloromethane extract

Physical data:
Name: 3-Hydroxy-20(29)-lupen-28-oic acid (Betulinic acid)
Yield: 0.110 g (0.015 %)
Experimental melting point: 315-316 °C (Literature: 316-318 °C)\textsuperscript{15,16}
Molecular formula: C\textsubscript{30}H\textsubscript{48}O\textsubscript{3}
Molecular mass (LC-MS): m/z: 455.2 (M-H)\textsuperscript{+} (Figure 7.14)
FT-IR: $\nu_{\text{cm}^{-1}}$ (KBr): 2920.25 and 2851.42 (C-H stretching); 1724.37 (carboxylic acid) (Figure 7.13)
The $^1$H-NMR (CDCl$_3$+DMSO) spectrum (Figure 7.10) showed a deshielded characteristic proton at $\delta$ 3.18 (1H, $dd$, H-3) assigned to the proton of C-3 which is bonded to the hydroxyl group. This compound shows also two one proton broad singlets at $\delta$ 4.58 (1H, br $s$, H-29) and $\delta$ 4.71(1H, br $s$, H-29), characteristic of the vinyl methylene group in a lup-20(29)-ene skeleton. The other protons are assigned as follows: $\delta$ 3.1 (1H, $t$, H-19); $\delta$ 1.67 (3H, br $s$, Me-30); $\delta$ 0.67 (3H, br $s$, Me-23); $\delta$ 0.79 (3H, br $s$, Me-24); $\delta$ 0.84 (3H, br $s$, Me-25); $\delta$ 0.86 (3H, br $s$, Me-26); and $\delta$ 0.88 (3H, br $s$, Me-27). The remaining protons, CH and CH$_2$ groups, 25H appear, between $\delta$ 0.99-2.26 ppm. The assigned values agreed with the literature values. The minimal differences are due to the different solvents used.

$^{13}$C-NMR: see Table 2.2 (Figure 7.11). A literature search revealed compound B to be betulinic acid.

**Structure of Compound B: Betulinic acid:**

![Structure of Compound B: Betulinic acid](image-url)
Table 2.2: $^{13}$C-NMR (100.6 MHz) spectral data for compound B

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<th>$\delta_C$ Lit.$^{(18)}$</th>
<th>$\delta_C$ Cpd B</th>
<th>Carbon Position</th>
<th>$\delta_C$ Lit.$^{(18)}$</th>
<th>$\delta_C$ Cpd B</th>
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<td>32.2(CH$_2$)</td>
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<tr>
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<td>27.4</td>
<td>27.4(CH$_2$)</td>
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<td>56.3</td>
<td>56.2(C)</td>
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Structure elucidation of compound C from methanol extract

**Physical data**

Name: 22, 23-dihydrostigmasterol or Stigmast-5-en-3-ol (β-Sitosterol)

Yield: 0.021 g (0.0028 %)

Physical description: white crystals

Literature melting point: 136-140 °C

Experimental melting point: 136-139 °C (Literature: 136-140 °C)$^{19,20}$

Molecular formula: C$_{29}$H$_{50}$O
Molecular mass (LC-MS): m/z: 413.0 (M-H)$^+$, 392.9, 374.9, 358.9, 331.9, and 313.7. (Figure 7.19)

FT-IR: $\nu_{\text{cm}^{-1}}$ (KBr): 3387.81 (Hydroxyl group), 2932.67 (-C-H stretching); 1686.51 (double bond of sp$^2$ carbons) (Figure 7.18)

The $^1$H-NMR (CDCl$_3$) spectrum (Figure 7.15) shows a characteristic proton at $\delta_H$ 3.58 (1H, m, H-3), which is assigned at C-3, bonded to hydroxyl group. The proton $\delta_H$ 5.33 (1H, m, H-6) is assigned to C-6, characteristic of the double bond occurring between C-5 and C-6. Two singlets appearing at 0.65 and 1.00 were assigned for the group of C-18 and C-19 respectively. The doublets for methyl groups of C-21, C-26 and C-27 appear at respectively $\delta$ 0.92 ($d, J = 8.0$ Hz); $\delta$ 0.82 ($d, J = 7.2$ Hz), $\delta$ 0.84 ($d, J = 7.7$ Hz) and $\delta$ 0.82 ($d, J = 7.2$ Hz). This is correlating to the literature value. The signals at $\delta$ 1.84, $\delta$ 2.02 and $\delta$ 2.24 were assigned to the multiplets of three CH$_2$ groups. The remaining protons appeared as multiplets at $\delta$ 1.08 to $\delta$1.79. This is correlating to the literature value.$^{21}$

$^{13}$C- NMR: see Table 2.3. (Figure 7.16). A literature search revealed compound C to be sitosterol.$^{22}$

**Structure of Compound C: Sitosterol**

![Figure 2.14 Structure of Compound C: Sitosterol](image-url)
Table 2.3: $^{13}$C-NMR (100.6 MHz) spectral data for compound C

<table>
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<tr>
<th>Carbon Position</th>
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<th>$\delta^{}_{C}$ Cpd C</th>
<th>Carbon Position</th>
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<td>9</td>
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<td>25</td>
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<td>29.15(CH)</td>
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<td>24.29</td>
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</tbody>
</table>

C. Structure elucidation of compound D from methanol extract

**Physical data**

Name: Friedelan-3-one

Yield: 0.019 g (0.0025 %)

Experimental melting point: 261-262 °C (Literature 261-262 °C)$^{23}$

Molecular formula: C$_{30}$H$_{50}$O

Molecular mass (GC-MS): m/z: 426 (M$^+$), 411, 341, 302, 287, 273, 146, 231, 218, 205, 191, 179, 163, 149, 137, 123, 109, 95, 81, 69 and 55 (Figure 7.24)
FT-IR: $\nu_{\text{cm}^{-1}}$ (KBr): 2927.14 (C-H stretching), 1715.37 (carbonyl group), 1379.43 (methyl groups) (Figure 7.23)

The $^1$H-NMR (400 MHz, CDCl$_3$) $\delta_H$ (ppm): 0.76 (3H, s, H-24), 0.88 (3H, s, H-25), 0.90 (3H, d, H-23), 0.96 (3H, s, H-30), 1.03 (3H, s, H-26), 1.1 (3H, s, H-27), 1.1 (3H, s, H-28), 1.19 (3H, s, H-29), 1.26 (3H, s, H-30), 1.97 (1H, m, H-1a), 1.69 (1H, m, H-1b), 2.28 (2H, m, H-2b, H-4), 2.40 (1H, m, H-2a), 2.00 (1H, m, H-2b), the remaining protons were assigned at $\delta$1.30-1.60 (m) (Figure 7.20) $^{24}$

$^{13}$C- NMR: see Table 2.4 (Figure 7.21). A literature search revealed compound C to be friedelan-3-one.$^{18, 24}$

**Structure of Compound D: Friedelan-3-one**

![Structure of Compound D: Friedelan-3-one](image)

Figure 2.15 Structure of Compound D: Friedelan-3-one
Table 2.4: $^{13}$C-NMR (100.6 MHz) spectral data for compound D

<table>
<thead>
<tr>
<th>Carbon Position</th>
<th>$\delta_C$ Lit$^{[18,21]}$</th>
<th>$\delta_C$ Cpd D (DEPT)</th>
<th>Carbon Position</th>
<th>$\delta_C$ Lit$^{[18,21]}$</th>
<th>$\delta_C$ Cpd D (DEPT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.3</td>
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<td>36.0(CH$_2$)</td>
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<tr>
<td>2</td>
<td>41.5</td>
<td>41.5(CH$_2$)</td>
<td>17</td>
<td>30.0</td>
<td>29.9(C)</td>
</tr>
<tr>
<td>3</td>
<td>213.2</td>
<td>213.7(C)</td>
<td>18</td>
<td>42.8</td>
<td>42.8(CH)</td>
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<tr>
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<td>19</td>
<td>35.3</td>
<td>35.3(CH$_2$)</td>
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<tr>
<td>5</td>
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<td>42.2(C)</td>
<td>20</td>
<td>28.1</td>
<td>28.2(C)</td>
</tr>
<tr>
<td>6</td>
<td>41.3</td>
<td>41.3(CH$_2$)</td>
<td>21</td>
<td>32.7</td>
<td>32.8(CH$_2$)</td>
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<td>18.2(CH$_2$)</td>
<td>22</td>
<td>39.2</td>
<td>39.3(CH$_2$)</td>
</tr>
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<td>53.1(CH)</td>
<td>23</td>
<td>6.8</td>
<td>6.8(CH$_3$)</td>
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<td>37.4</td>
<td>37.4(C)</td>
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<td>14.7(CH$_3$)</td>
</tr>
<tr>
<td>10</td>
<td>59.4</td>
<td>59.5(CH)</td>
<td>25</td>
<td>17.9</td>
<td>17.9(CH$_3$)</td>
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<td>35.6</td>
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<td>26</td>
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<td>20.3(CH$_3$)</td>
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<tr>
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<td>27</td>
<td>18.6</td>
<td>18.7(CH$_3$)</td>
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<td>39.7(C)</td>
<td>28</td>
<td>32.1</td>
<td>32.1(CH$_3$)</td>
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<td>38.3(C)</td>
<td>29</td>
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<td>15</td>
<td>32.4</td>
<td>32.4(CH$_2$)</td>
<td>30</td>
<td>31.8</td>
<td>31.8(CH$_3$)</td>
</tr>
</tbody>
</table>

D. Characterization of compound E from methanol extract

Physical data
Name: Derivative of betulinic acid
Yield: 0.020 g (0.026 %)
Experimental melting point: 258-260 °C (Literature 259-260 °C)$^{25}$
Molecular formula: C$_{32}$H$_{50}$O$_4$
Molecular mass: the mass spectrum showed a molecular ion peak at m/z: 497.3 (M-H)$^+$. (Figure 7.33)
FT-IR: $v_{cm}^{-1}$ (KBr): 3330.12 (alcohol group), 2951.12, 2933.78, and 2876.61 (-C-H stretching), 1240.41 (ester stretching) (Figure 7.32)

The $^1$H-NMR (CDCl$_3$+DMSO) spectrum (Figure 7.25) showed a characteristic deshielded proton at $\delta_H$ 4.41 assigned to the proton of C-3 which is bonded to the hydroxyl group. The compound showed also two one proton broad singlet at 4.55 (H-29) and 4.69 (H-29), characteristic of the vinyl methylene group in a lup-20(29)-ene skeleton. HSQC confirmed the proton of C-31 at $\delta_H$ 1.98. The others remaining protons are assigned in Table 2.5 below.

NOESY (Figure 7.30) showed a correlation between the protons of C-30 and the two of C-29; this confirms the presence of the double bond.

$^{13}$C- NMR (Figure 7.26). A literature search conducted according to the information presented above indicated that compound E is a derivative of betulinic acid. This structure was not completely elucidated. The fully assigned NMR data of this compound is shown in Table 2.5 below.
Table 2.5 $^{13}$C-NMR (100.6 MHz) spectral data for compound E

<table>
<thead>
<tr>
<th>Carbon Position</th>
<th>δ_C Lit (18,22)</th>
<th>δ_C (DEPT)</th>
<th>δ_H</th>
<th>COSY</th>
<th>NOESY</th>
<th>HMBC C→H</th>
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<td>38.8 (CH₂)</td>
<td>1.61; 0.89</td>
<td>H-2</td>
<td>H_a-5</td>
<td>H-25 $^3$J; H-2 $^2$J</td>
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<td>27.4</td>
<td>27.4 (CH₂)</td>
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<td>H-3; H-1</td>
<td>H-5; H_a-3; H-23</td>
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<tr>
<td>3</td>
<td>78.9</td>
<td>78.9 (CH)</td>
<td>4.41</td>
<td>H-2</td>
<td>H-23 H-2; H_a-5</td>
<td>H-23 $^3$J; H-1 $^3$J</td>
</tr>
<tr>
<td>4</td>
<td>38.8</td>
<td>38.7 (C)</td>
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<td>-</td>
<td>-</td>
<td>H-2 $^3$J; H-23 $^2$J</td>
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<td>55.5 (CH)</td>
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<td>H-6</td>
<td>H-6; H-2; H_a-3; H_a-1; H_a-9</td>
<td>H-23 $^3$J</td>
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<tr>
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<td>18.3 (CH₂)</td>
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<td>H-24; H-5; H_a-2</td>
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<td>H-13; H-12; H-11; H-25, H_a-5</td>
<td>H-26 $^3$J; H-25 $^3$J</td>
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<td>H-16; H_β-7</td>
<td>H-27 J</td>
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<td>H-13; H-15; H_α-22</td>
<td>H-18 J</td>
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<td>H-18 J</td>
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<td>H-19</td>
<td>H-22 J</td>
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<td>H-30 J; H-18 J</td>
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<td>H-30 J; H-18 J</td>
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<td>H-2 J</td>
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<td>H-5 J; H-1 J; H-9 J</td>
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<td>28</td>
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<td>-</td>
<td>-</td>
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<td>H-18 J; H-22 J</td>
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Table 2.5 Continued.
Table 2.5 Continued.

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<td>H₆ 4.55</td>
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</tr>
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<td>21.3(CH₃)</td>
<td>1.98</td>
<td>-</td>
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2.6 Conclusion

- During the extraction, methanol extract of *S. guineense* of DRC (leaves) gave the highest crude extract while the ethyl acetate extract gave the lowest yield.
- In *S. guineense* of South Africa, the crude extract in dichloromethane was the highest; while ethyl acetate contained the lowest.
- Column chromatography of the EtOAc extract of *S. guineense* of DRC gave a flavanone glycoside, which was identified as a flavanone glycoside.
- Column chromatography of the dichloromethane and methanol extracts of *S. guineense* of South Africa gave betulinic acid and sitosterol, friedelan-3-one and a derivative of betulinic acid, respectively.
2.7 References


CHAPTER THREE

PHYTOCHEMICAL EXAMINATION OF CALLISTEMON VIMINALIS AND MELALEUCA BRACATEATA var. Revolution Gold

3.1 Introduction

3.1.1 Callistemon viminalis

*Callistemon* is a genus of around 34 species in the Myrtle family Myrtaceae. All except four species are endemic to Australia, the others occurring in New Caledonia. *Callistemons* are commonly known as "bottlebrushes" because of the cylindrical, brush-like shape of the flower spike. They are very popular for gardens and landscaping both in Australia and overseas and numerous cultivars have been brought into cultivation. In nature, *Callistemons* are often found along watercourses or along the edges of swamps. They are generally plants of open forest or woodland in relatively high rainfall areas and are found in South Florida and are also popular in Southern California.\(^1\), \(^2\), \(^3\), \(^4\)

**Name meaning: Callistemon viminalis**

*Callistemon*: from the Greek, *kallistos*, most beautiful and *stema*, a stamen;

*viminalis*: from the Latin, *vimen*, a long flexible shoot or osier.\(^5\)

**Classification**

The problem with the classification of *Callistemon* and *Melaleuca* on the basis of the arrangement of the stamens is that this supposed difference is not clear cut and *Callistemon* tends to merge into *Melaleuca* rather than being unambiguously distinct.

The well known *Callistemon viminalis* is one that has often been discussed as not easily fitting the accepted definition of *Callistemon*.\(^1\)
Description:

*Callistemon viminalis* is probably the most widely cultivated of all bottlebrushes. Only *C. citrinus* would challenge that status. The species and its cultivars are widely planted in Australia and overseas.

The weeping bottlebrush is typically a small tree with pendulous foliage although some forms are more pendulous than others. It’s an attractive tree even when not in bloom and reaches a height of about 10 meters in its natural habitat, forming a wide rounded crown if the lower branches are pruned off. It is usually smaller in cultivation, particularly in temperate areas where it is an attractive and reliable small tree for street planting. The brushes are usually about 70 mm long by 50 mm diameter, bright red in colour and are usually seen in spring and sometimes in autumn. The plant performs best in medium to heavy soils and can tolerate less than perfect drainage but may be damaged by moderate to heavy frost. It responds to annual fertilizing after flowering. Although the plant will respond to pruning, this can have the effect of destroying the weeping habit. Weeping bottlebrush flowers mature into woody capsules that are distinctive of this genus. Leaves are narrow and lance shaped growing up to 10 cm in length. The leaves are a very attractive bronze-green when they emerge in spring, gradually turning dull green as they mature.¹ ⁷

Usage

In traditional medicine, *Callistemon viminalis* has a large use against several diseases. It’s also has insecticidal properties. Bottlebrushes make excellent garden plants. Plants are all woody shrubs which range from 0.5 m to 10 m tall. The flowers can be spectacular and are irresistible to nectar-feeding birds and insects. Most species are frost tolerant.⁶ The brittle wood of this plant may make it unsuitable for windy areas.

One of the best uses is for lawn specimens, or screens on large properties, with a regular maintenance program. Drought tolerance and relative freedom from pests and disease only add to this bottlebrush's attractiveness.⁸
Scientific classification

Kingdom : Plantae - Plants
Subkingdom : Tracheobionta- Vascular plants
Superdivision : Spermatophyta- Seed plants
Division : Magnoliophyta- Flowering plants
Class : Magnoliopsida- Dicotyledons
Subclass : Rosidae
Order : Myrtales
Family : Myrtaceae - Myrtle family
Genus : Callistemon R. Br. – bottlebrush
Species : Callistemon viminalis (Sol. ex Gaertn.) Cheel–Weeping bottlebrush.

Some Callistemon species:
Common Callistemon species include Callistemon citrinus, Callistemon pallidus, Callistemon viminalis, Callistemon acuminatus Cheel, Callistemon brachyandrus Lindl., Callistemon chisholmii Cheel, Callistemon citrinus (Curtis) Skeels - Crimson Bottlebrush, and Callistemon coccineus F.Muell.

3.1.2 Melaleuca bracteata
Melaleuca is a genus of plants in the Myrtle family Myrtaceae. There are well over 250 recognized species, most of which are endemic to Australia. A few species occur in Malaysia and 7 species are endemic to New Caledonia and Papua New Guinea. It’s also cultivated in Indonesia (Java), in China and in United States (California). The genus Melaleuca is native to Australia (New South Wales, Northern Territory, Queensland, South Australia, Western Australia and it was named in this country, in 1858, by Ferdinand J.H. von Mueller, Government Botanist of Victoria. The genus name comes from the Greek melas = black, and leukos = white; its origin is obscure, but may refer to the black trunk and white branches of some species; the species name is from the Latin bractea = bract, referring to the conspicuous bracts, or leaf-like structures of the flower.
Description:
The species are shrubs and trees growing (depending on species) to 2–30 m tall, often with flaky, exfoliating bark. The leaves are evergreen, alternately arranged, ovate to lanceolate, 1.5–3 mm wide, 5–11-veined, 1-25 cm long and 0.5-7 cm broad, apex acute to acuminate, glabrous or occasionally pubescent; sessile, with an entire margin, dark green to grey-green in colour. Flowers solitary or in threes within each bract, white. Petals ± circular, 1.5–2 mm long. Stamens 16–25 per bundle; claw 3–4 mm long. Fruit subglobose, 2–3 mm diam., orifice c. 2 mm diam.; sepals persistent. These flowers are produced in dense clusters along the stems, each flower with fine small petals and a tight bundle of stamens; flower colour varies from white to pink, red, pale yellow or greenish. The fruit is a small capsule containing numerous minute seeds.17

Scientifique classification

Classification : Melaleuca bracteata F. Muell.
Kingdom : Plantae
Plants Subkingdom : Tracheobionta
Super division : Spermatophyta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Rosidae
Order : Myrtales
Family : Myrtaceae
Genus : Melaleuca L.
Species : Melaleuca bracteata F. Muell.18, 19
Some *Melaleuca* species

This is a list of some plants in the genus *Melaleuca*, which is in the myrtle family, Myrtaceae.

*Melaleuca acacioides, Melaleuca acerosa, Melaleuca acuminata, Melaleuca adenostyla, Melaleuca adnata, Melaleuca agathosmoides, Melaleuca alsophila, Melaleuca alternifolia* (Tea tree), *Melaleuca amydra, Melaleuca bracteata*. 20

**Synonyms:**

- *Melaleuca genistofolia* Auct.
- *Melaleuca monticola* J. M. Black. 11

**Common names:** *Melaleuca bracteata* is commonly known as:

- river tea tree;
- revolution gold;
- revolution green;
- paper barks;
- honey myrtles;
- punk trees;
- black tea-tree;
- black ti-tree. 11, 12, 13

**Economic importance:**

*Melaleuca* species are used as environmental (ornamental) plants. In Australia, *Melaleuca* species are sometimes used as food plants. Both in Australia and other tropical areas worldwide, they are used as popular garden plants. *Melaleuca* populations have nearly quadrupled in southern Florida over the past decade. 11-13
3.2 Previous work on *Callistemon viminalis* and *Melaleuca bracteata*

### 3.2.1 *Callistemon viminalis*

**a) Phytochemical studies**

El Dib reported the isolation of nine phenolic compounds from the aerial parts of *Callistemon viminalis*. These are: (i) gallic acid, (ii) Me gallate, (iii) quercetin 3-O-α-L-arabinofuranoside (avicularin), (iv) quercetin 3-O-β-D-galactopyranoside (hyperin), (v) quercetin 3-O-α-L-rhamnopyranoside (quercitrin), (vi) quercetin 3-O-β-D-glucuronopyranoside, (vii) quercetin, (viii) 1-O-galloyl-β-D-glucopyranose (glucogallin), and (ix) 2,3,5-(S)-flavogallonoyl-4,6-(S)-hexahydroxydiphenoyl-D-glucopyranose (castalagin).\(^{21}\)

In 2007, twenty-nine compounds were identified in *C. viminalis* and the majority of them were found to be primarily monoterpenes. Cineole was found to be the major compound (78.3%). Other results of analyse indicated, however the presence of linalool and myrcene as relatively prominent compounds.\(^{22}\)

1, 8-cineole (47.94 %), linalool (13.03 %), limonene (10.90 %) were found as major components in *C. viminalis* by Mahmoud, Ibrahim when comparing *Callistemon lanceolatus* DC. and *Callistemon viminalis*, two Egyptian plants.\(^{23}\) Several works carried out on essential oil from *Callistemon viminalis* leaves resulted in the identification of 1, 8-Cineole, α-pinene and menthol acetate as major components in the oil.\(^{24-27}\)

Two novel epimeric compounds, viminadione A (1) and viminadione B (2), have been isolated from *C. viminalis* by Bhupinder and his collaborators.\(^{28,29,30}\)

**b) Pharmacological studies**

Aqueous extracts of *Conocarpus erectus*, *Callistemon viminalis* and *Bucida buceras* showed anti-viral effects against *Pseudomonas aeruginosa*.\(^{31}\) *C. viminalis* and two others plants, *Conocarpus erectus* and *Bucida buceras*, were found to cause a significant
inhibition of Las A protease, Las B elastase, pyoverdin production, and biofilm formation, when their aqueous extracts were examined their effects on *Pseudomonas aeruginosa* virulence factors and the QS system.\textsuperscript{32}

1,8-cineole which is one of the major compounds in *Callistemon viminalis* was found to have potent fumigant toxicity against 3 major stored-grain insects: *Sitophilus oryzae*, *Tribolium castaneum* and *Rhyzopertha dominica*, while Tetradecahydroxanthenediones exhibited interesting insecticidal activity.\textsuperscript{28, 29, 30, 31}
3.2.2 *Melaleuca bracteata*

a) Phytochemical studies

Many *Melaleuca* species have been subjected to phytochemical studies in the past. Li, Sijiao and his collaborators using the optimum extraction methods and gas chromatography conditions of essential oil in the leaves and branches of *Melaleuca bracteata* explored the extraction processing feasibility. The results showed that the highest yield of essential oil was 0.91% after 9 hours of steam distillation extraction. By means of gas-chromatography method to analyze the content of major component of the extracted essential oil, the result was about 94% (terpinene, eucalyptol, terpilenol, and so on). \(^3\)

In 2009, Adesanwo and Shode identified betulinic and oleanolic acids, two triterpenes from this plant. \(^3\) Naidu in his investigation showed the stress tolerance of some *Melaleuca* species, attributable to their ability to accumulate large quantities of organic compounds known as osmoprotectants or proline (betaine) and analogues, which can be easily extracted from these plants and used in seed treatment and foliage application to increase the stress tolerance of economic crops. \(^3\), \(^3\)

Aboutabl et al. investigated the essential oils from three *Melaleuca* species growing in Egypt. It was reported that eugenol is the main component of *M. bracteata* (97.7%); 1, 8-cineole constitutes 57.2% of the oil of *M. quinquenervia* and 33.7% of *M. armillaris*, and Terpinen-4-ol amounts to 24.8% in *M. armillaris*. \(^3\) Early in 1955, Penfold and Willis confirmed the presence of citronellal in *Eucalyptus citriodora* and of phenyl ethers in *Melaleuca bracteata*. \(^3\)

\[ \text{3.1} \]

\[ \text{3.2} \]
Methyleugenol, elemicin and evodionol were identified in *Melaleuca bracteata* in significant concentration up to 60-90% for the first one.\(^{39, 41}\)

**Traditional Aboriginal uses**

Aborigines used the leaves traditionally for many medicinal purposes, including chewing the young leaves to alleviate headache and for other ailments. The softness and flexibility of the paper bark itself made it an extremely useful tree to aboriginal people.

It was used as a bandage, as a sleeping mat, and as material for building humpies. It was also used for wrapping food for cooking (in the same way aluminum foil is today), as a disposable raincoat, and for tamping holes in canoes.\(^{11, 12, 13}\)

Its wood is close-grained, heavy, hard and durable and could be useful for posts and poles. The leaves contain essential oils and it is an excellent ornamental, a good shelter tree and has potential for erosion control on stream banks.

Although it produces large amounts of pollen it is of minor importance for honey production due to poor flavour and low density.\(^{11-13}\)

**(b) Pharmacological studies**

**Modern uses**

Scientific studies have shown that tea tree oil made from *Melaleuca alternifolia* is a highly effective topical antibacterial and antifungal, although it may be toxic when ingested internally in large doses or by children. In rare cases, topical products can be absorbed by the skin and result in toxicity.\(^{44-47}\) The oils of *Melaleuca* can be found in organic solutions of medication that claims to eliminate warts, including the Human papilloma virus. *Melaleuca* oils are the active ingredient in “Burn-aid”, a popular minor burn first aid treatment and this oil is also used in many pet fish remedies (such as Melafix and Bettafix) to treat bacterial and fungal infections. Bettafix is a lighter dilution of tea tree oil while Melafix is a stronger dilution. The remedies are often associated with Betta fish, but are also used with other fish.\(^{42, 43, 45}\) An essential oil obtained from the leaves is germicidal and is used in insecticides to increase their potency. The oil is heavier than water.\(^{46}\)
Known Pharmacological activities

Melaleuca bracteata is one of the most studied medicinal plants and shows a large number of pharmacological activities namely: Acaricide, adrenergic, aldose-reductase-inhibitor, allelochemic, allergenic, analgesic, anaesthetic, antiaggregant, antiarachidonate, antibacterial, anticonvulsant, antidote, antiedemic, antienterococcic, antiescherichic, antiestrogenic, antifeedant, antigenotoxic, antiherpetic, antiinflammatory, antikeratotic, antileukemic, antimitotic, antimutagenic, antinitrosating, antioxidant, antipneumonic, antiprostaglandin, antipseudomonic, antipyretic, antiradicular, antisalmonella, antiseptic, antispasmodic, antistaphylococcic, antithromboxane, antitumor, antiallergic, antiulcer, antiulcerase, antiviral, apifuge, CNS-depressant, CNS-stimulant, COX-1-inhibitor, COX-2-inhibitor, calcium-antagonist, cancer-preventive, candidicide, carcinogenic, carcinogenic, carminative, choleric, chronotropic, circulatory-stimulant, cytochrome-P450-inhibitor, cytotoxic, dermal, dermatitigenic, emetic, enterorelaxant, flavor, fungicide, fungistat, hepatoprotective, herbicide, histaminic, hypoglycemic, hypotensive, hypothermic, inotropic, insecticide, insectifuge, insectiphile, irritant, juvabional, larvicide, laxative, lipoxygenase-inhibitor, monoaminergic, motor-depressant, mutagenic, myorelaxant, narcotic, nematicide, neurotoxic, perfumery, pesticide, prostaglandin-synthesis-inhibitor, sedative, sprout-inhibitor, teratogenic, termiticide, tranquilizer, trichomonicide, trichomonistat, trypsin-enhancer, tyrosinase-inhibitor, ulcerogenic, varroacide, vasodialator, vermifuge, vibriocide.

3.3 Extractives from Callistemon viminalis

3.3.1 Extraction and isolation

The leaves of Callistemon viminalis were collected in Durban city, in South Africa. Callistemon viminalis fresh leaves (powder 221.7g) were extracted sequentially in dichloromethane and in ethyl acetate as showed in scheme 2.1. Each extraction was optimised by repeating the maceration twice. Each solvent extraction was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give...
dichloromethane-solubles (DS/12/B) (10.26 g, 4.63 %) and ethyl acetate-solubles (DS/12/C) (6.10 g, 2.75 %), respectively.

The ethyl acetate extract was subjected to column chromatography using the mixture of hexane - ethyl acetate (9.5:0.5 to 6.5:3.5) A total of 95 fractions of 60 ml volumes each were collected and 7 similar fractions labelled successively :VC1, VC2, VC3, VC4, VC5, VC6, and VC7 were combined. Combined fraction VC1 eluted with hexane - ethyl acetate 9.5:0.5 afforded four spots of greenish oily liquid, in small amounts. Combined fractions VC2 and VC3, whitish solid, eluted with hexane-ethyl acetate 9:1 and 8:2 respectively, afforded one spot each. VC2 was in traces, while CC3 contained the major compound (0.595 g). The combined fractions VC4, VC5 and VC7 (whitish solid each) afforded two spots each. The combined fraction VC6 (whitish solid) afforded three spots, in small quantities.

### 3.3.2 Structure elucidation of compound F from VC2

**Physical data**

- **Name:** 3-Hydroxy-20(29)-lupen-28-oic acid
- **Yield:** 0.595 g (0.27 %)
- **Experimental melting point:** 315-317 °C (Literature 316-318 °C) (Lit. see compound B)
- **Molecular formula:** C$_{30}$H$_{48}$O$_3$
- **Molecular mass:** m/z: 456.3 (M$^+$), 413.0; 364.8 and 313.8 as base peak. (Figure 7.38)
- **FT-IR:** v$_{cm^{-1}}$(KBr): 3457.55 (alcohol group), 2940.34 (-C-H stretching), 1240.17 (Figure 7.37)
- **The $^1$H-NMR (CDCl$_3$+DMSO) spectrum** (Figure 7.34) $^{55}$
- **The $^{13}$C- NMR spectrum** (Figure 7.35).$^{56}$

This compound was identified as betulinic acid which has already been identified in this study (See Compound B) (Section 2.5.2). The spectral data of compound F were identical with those of compound B.
3.4. Extractives from *Melaleuca bracteata*

3.4.1 Extraction and isolation

The leaves of *Melaleuca bracteata* were collected in Durban city, South Africa. *Melaleuca bracteata* fresh leaves (powder 400 g) were extracted sequentially in dichloromethane, ethyl acetate and methanol as showed in scheme 2.1. Each extraction was optimised by repeating the maceration twice. Each solvent extraction was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give dichloromethane-solubles (DS/10/B) (37.32 g, 9.33 %); ethyl acetate-solubles (DS/10/C) (3.21 g, 0.80 %) an methanol-solubles (17.56 g, 4.39 %), respectively.

**Acetylation of DS/10/B**

Further purification of dichloromethane extract was achieved by acetylation using standard method to give an acetylated mixture. This mixture was subjected to column chromatography with the mixture of hexane - ethyl acetate (9:1 to 7:3). A total of 65 fractions of 40 ml volumes each were collected and 4 similar fractions were combined and pooled to give M3A, M3B, M3C and M3D. Combined fractions M3B and M3D afforded one compound of whitish solid each. Combined fractions M3A and M3C afforded two spots each of whitish solid.

3.4.2 Structure elucidation of Compound G from M3B

**Physical data**

Name: 3-acetyloxy -20(29)-lupen-28-oic acid (Betulinic acid acetate)

Yield: 0.420 g  (0.11 %)

Experimental melting point: 258-259 °C (Literature 259-260 °C) \(^{57}\)

Molecular formula: C\(_{32}\)H\(_{50}\)O\(_4\)

Molecular mass: m/z: 498.7 (M\(^+\)). (Figure 7.43)
FT-IR: $\nu_{\text{cm}^{-1}}$ (KBr): 2919.38 and 2851.06 (-C-H stretching), 1729.37, 1691.91 and 1641.71 characteristic of carboxylic acid. 1240.17 (Figure 7.42).

The $^1$H- NMR spectrum (Figure 7.39) showed two deshielded characteristic protons at $\delta_H$ 4.44 ($dd$, 1H) and $\delta_H$ 2.97 (t, 1H) assigned to the proton of C-3 and C-19 which are bonded to the hydroxyl group and the double bond on positions C-20 and C-29 respectively. This compound showed also two one proton broad singlet at $\delta_H$ 4.58 (s, 1H, H$\alpha$-29) and $\delta_H$ 4.70 (s, 1H, H$\beta$-29), characteristic of the vinyl methylene group in a lup-20(29)-ene skeleton. The remaining protons were assigned at $\delta_H$ 2.14 (s, 3H, Me-Ac), 1.62 (s, 3H, 30-Me) and the complex CH-, CH$_2$ 25H which were assigned at $\delta_H$ 1.03-2.25.$^{58}$

$^{13}$C- NMR (Figure 7.40). A literature search conducted according to these information revealed compound G to be known betulinic acid acetate. The fully assigned $^{13}$C-NMR data of this compound is shown in Table 3.1 compared to literature value.$^{56}$

**Structure of Compound G: Betulinic acid acetate.**

![Structure of betulinic acid acetate](image-url)
Table 3.1: $^{13}$C-NMR (100.6 MHz) spectral data for compound G

<table>
<thead>
<tr>
<th>Carbon Position</th>
<th>$\delta_C$ Lit$^{3b,38}$</th>
<th>$\delta_C$ Cpdl G</th>
<th>Carbon Position</th>
<th>$\delta_C$ Lit$^{3b,38}$</th>
<th>$\delta_C$ Cpdl G</th>
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<td>- (CH$_2$)</td>
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<td>47.00</td>
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<tr>
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<td>19</td>
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<td>171.1(C)</td>
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<td>32</td>
<td>-</td>
<td>23.7(CH$_3$)</td>
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</table>
3.4.3 Structure elucidation of Compound H from M3D

**Physical data**

Name: 3β-(Acetyloxy)-urs-12-en-28-oic acid

Yield: 0.0121 g (0.003 %)

Literature melting point: 286-287°C

Experimental melting point: 285-287°C (Literature: 286-287°C)\(^{59}\)

Molecular formula: \(\text{C}_{32}\text{H}_{50}\text{O}_{4}\)

Molecular mass: 456 (479-23) (M\(^+\)) (Figure 7.47). This corresponds to the molecular formula of \(\text{C}_{30}\text{H}_{48}\text{O}_{3}\). This compound didn’t give the mass of ursolic acid acetate. The obtained mass was the one of ursolic acid. This can be due to the decomposition of the compound, the functional group acetate could be reduced in hydroxyl, as this analysis was performed more than 3 months after the isolation of the compound. The peak observed at 439.0 corresponds to the loss of hydroxyl group, characteristic of acetic acid. The other intense peaks are 393, 338.8 and 290.9 as base peak.

**FT-IR:** \(\nu_{\text{cm}^{-1}}\) (KBr): 2940.24 and 2866.13 (-C-H stretching), the strong peak observed at 1243.75 corresponds to an ester stretching. (Figure 7.46)

The \(^1\text{H}-\text{NMR}\) spectrum (CDCl\(_3\)) (Figure 7.44) showed two deshielded characteristic protons at \(\delta_H\) 4.49 (1H, m) assigned to the proton of C-3 which is bonded to the hydroxyl group and \(\delta_H\) 5.22 (1H, br s, olefinic-H) assigned to C-12 characteristic of the olefinic-H. The proton at \(\delta_H\) 2.16 (1H, d, J=11.3 Hz) is assigned to C-18, while the one at \(\delta_H\) 2.14 (3H, s) is characteristic to the protons of the acetate group (-COOCH\(_3\)). These values are correlating with the literature review.\(^{60}\)

\(^{13}\text{C}-\text{NMR}\) (Figure 7.45). A literature search revealed compound G to be known Ursolic acid acetate. The fully assigned \(^{13}\text{C}-\text{NMR}\) data of this compound is shown in Table 3.2 compared to literature value.\(^{56}\)
Structure of Compound H: Ursolic acid acetate

Figure 3.3 Structure of Compound H: Ursolic acid acetate

Table 3.2: $^{13}$C-NMR (100.6 MHz) spectral data for compound H

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<th>$\delta_C$ Cpd H</th>
<th>Carbon Position</th>
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</table>
3.5 Conclusion

- During the extraction, dichloromethane extract of *C. viminalis* (leaves) gave the highest crude extract while the ethyl acetate extract gave the lowest yield.
- In *M. bracteata* (leaves), the crude extract in dichloromethane was the highest; while ethyl acetate contained the lowest.
- Column chromatography of the EtOAc extract of *Callistemon viminalis* gave one compound identified as betulinic acid.
- Column chromatography of the acetylated dichloromethane extract of *M. bracteata* gave two compounds, which were identified as betulinic acid acetate and ursolic acid acetate.
3.6 References


CHAPTER FOUR

PHYTOCHEMICAL EXAMINATION OF CAJANUS CAJAN
(FABACEAE)

4.1 Introduction

*Cajanus cajan (L.) Millsp.* is a leguminous shrub, perennial member of the family Fabaceae, recognized as having 32 species. Commonly known as Pigeon pea, it probably evolved in South Asia and appeared about 2000 BC in West Africa, which is considered a second major centre of origin.

Scientific names: *Cajanus bicolour* DC., *Cajanus indicus* Spreng, *Cajanus flavus* DC., *Cytisus cajan* (L.) (Basionym)

Common names:
Congo pea, pigeon pea, red gram, yellow dahl (English); ambrévade, pois d'Angole (French); straucherbse (German); arhar, tuver, dahl (India); feijoa-guandu, guandú, guisante-de-Angola (Portuguese); cachito, gandul (Spanish); kachang (Asia); Guandul, poroto guandul, poroto paraguayo, sachacafé, falso café, arveja (Argentina); guando (Brazil); frijol de árbol (Mexico); Cumandái (Paraguay); Angola pea (United Kingdom); Puerto Rican bean (Hawaii).

Scientific classification

- **Kingdom**: Plantae
- **Subkingdom**: Tracheobionta
- **Division**: Magnoliophyta
- **Class**: Magnoliopsida
- **Subclass**: Rosidae
- **Order**: Fabales
4.2 Description

*Cajanus cajan* is an annual, or more usually short-term perennial shrub that may reach 4-5 m in height, but usually 1-2 m only, woody at the base, with a variable habitat, but usually erect. Deep and quick growing tap root. Angular stem resulting from three ribs starting from the base of each petiole. Leaves trifoliate, alternate set in a spiral along the stem. Leaflets oblong, lanceolate 5-10 cm long x 2-4 cm wide, pubescent likewise the stem. Lateral petioles, 2-3 mm the terminal one reaching 10-20 mm. Stipules linear 2-3 mm long, stipules filiform 1-2 mm long. Flowers usually yellow but they may also be striated with purple streaks or plain red. Corolla 20-25 mm, with the flag 18-20 mm wide. Calyx 10-12 mm long, with 5 linear teeth. Inflorescence composed of racemes having 5-10 flowers on top of an axillary, little divided peduncle. Pods flat, with an acuminate tip, pubescent and of variable colour, 5-9 cm long x 12-13 mm wide, containing 2-9 seeds in shades of brown, red or black. Husks bearing deep, oblique furrows underlining the septa between the seeds. Life span, up to 5 years. Pigeon pea is extremely variable from the genetic viewpoint hence there are many cultivars. *Cajanus cajan* is very heat-tolerant. It prefers hot moist conditions. It will grow at temperatures above 35°C under adequate soil conditions of moisture and fertility. It does not tolerate frost, but will grow in temperatures to just above frost level.\(^5\)
Distribution

It is believed to originate from tropical India and today it is widely distributed in most tropical countries throughout the world especially South America. It is also found in Afghanistan, Bangladesh, Bhutan, Sri Lanka. In Africa, the main countries growing Pigeon pea are Kenya, Malawi, Uganda, Tanzania, and Nigeria, but it is also grown in southern Africa in warm regions. It is grown mainly as a subsistence crop. It has become naturalized in the northerly parts of southern Africa.

Usage:

a. Medicinal uses

It is useful in the treatment of internal organ swelling. Some herbal practitioners and researchers are of the opinion that it diminishes the swelling of internal organs like stomach, liver and intestines. In case of wound or cancer of these organs it is helpful in reducing them. Green leaves of pigeon peas ground in water and added to half boiled water should be applied externally on the affected body part.

In Peru, Brazilian and Argentina the leaves are prepared in an infusion for anaemia, coughs hepatitis, diabetes, urinary infections, and yellow fever. The flowers are prepared in an infusion for dysentery, bronchitis, pneumonia and menstrual disorders; while the seeds are prepared in a tea for inflammation and blood disorders.

b. Common uses

Pigeon peas are nutritionally important, as they contain high levels of protein (20–32% crude protein), and the important amino acids methionine, lysine, and tryptophan. In combination with cereals, pigeon peas make a well-balanced human food. In Dominican Republic and Hawaii; pigeon peas are grown for canning. In Ethiopia, not only the pods but the young shoots and leaves are cooked and eaten. In Malagasy, the leaves are used as food for the silkworm.
Pigeon peas are in some areas an important crop for green manure. Nitrogen fixing ability for a density of 7,000-10,000 plants ha is of the order of 100-120 kg N₂ / ha / year. The crop has long been used as a windbreak and shade for young coffee trees, forest seedling nurseries and vegetable beds, and is an important honey-producing plant. The woody stems of pigeon peas are used as firewood, fencing and thatch. Its hay is an effective substitute for more expensive industrial concentrates.⁷,¹⁵
4.3 Previous work on *Cajanus cajan* Pigeon pea

Chemical analysis of this plant revealed the presence of: 2'-O-methylcajanone, 2'-hydroxygenistein, 5,7,2'-trihydroxyisoflavone, alpha-amyrin, beta-amyrin, beta-sitosterol, cajafлаванone, cajaisoflavone, cajanin, cajanone, cajaquinone, concajanin, ferreirin, genistein, isogenistein-7-O-glucoside, lupeol, phenylalanine, and stigmasterol.\(^4,6,9\)

In 2009, the analysis of the oil of *Cajanus cajan* revealed the presence of sesquiterpenes in the leaves, stem and seeds. The major compounds identified were α-himachalene, β-himachalene, γ-himachalene, α-humulene and α-copaene.\(^16\)

4.4 Pharmacological review

In several clinical studies, scientists have reported that seed extracts of pigeon pea inhibit red blood sickling and may be beneficial for people with sickle cell anaemia. Laboratory studies with animals report that the seeds have some anti-nutritional qualities and reported to contain trypsin inhibitors and chymotrypsin inhibitors which reduce or inhibit pancreatic amylase and lipase.\(^4\)

A medicine containing longistyline a carboxylic acid and pinostrobin from breaking branch and leaves of *Cajanus cajan* was prepared for treating herpesvirus. These two compounds showed an interesting activity.\(^13\)

In 2009, *Cajanus cajan* crude extracts and its active ingredients (longistyline A, lactone, pinostrobin, vitexin and isovitexin) were used to produce a medicine for resisting gram-positive bacteria, and treating children pneumonia caused by *Staphylococcus aureus*, upper respiratory tract infection, and food poisoning. Antioxidant activity of *Cajanus cajan* was shown on the crude extracts and the isolated compounds such as cajaninstilbene acid, pinostrobin, vitexin and orientin.\(^18-22\)
4.5. Extractives from *Cajanus cajan*

4.5.1 Extraction and isolation

The seeds of *Cajanus cajan* were purchased by Professor Shode, from a local market in Lagos, Nigeria in 2007. The seeds were ground using a laboratory blender (Russell Hobbs). The powdered seeds were used for further studies.

*Cajanus cajan* seeds (powder, 1654.5 g) were extracted sequentially with n-hexane, dichloromethane, ethyl acetate, methanol, and aqueous methanol 80% as shown in Scheme 2.1. Each extraction was optimised by repeating the maceration twice. Each solvent extraction was concentrated under reduced pressure and allowed to dry at room temperature and weighed to give hexane-solubles (DS/6/A) (7.33 g, 0.44 %), dichloromethane-solubles (DS/6/B) (9.23 g, 0.56 %), ethyl acetate-solubles (DS/6/C) (52.24 g, 3.16 %), methanol-solubles (DS/6/D) (75.15g, 4.54 %), and aqueous methanol-solubles (DS/6/E) (41.72 g, 2.72 %), respectively.

The ethyl acetate extract was subjected to column chromatography over silica gel 60 (0.040 – 0.63mm [230-400 mesh]). The column was eluted with the mixture of hexane - ethyl acetate (9:1 to 3:7). Collected fractions were monitored on TLC plate (silica gel 60 \( F_{254} \) aluminium barking from Merck, Germany). A total of 185 fractions of 7 ml volumes each were collected. Similar fractions according to the TLC analysis were pooled to give 4 (SA, SB, SC, SD) combined fractions. Combined fraction SA eluted with hexane - ethyl acetate (1:1) afforded one spot; greenish oily liquid, in small amounts. Combined fractions SB eluted with hexane-ethyl acetate 1:1 and SC and SD eluted with ethyl acetate – methanol 9:1 afforded two spots each. Combined fraction SC (280 mg) contained the major compound, whitish solid, contaminated by SD; while the combined fraction SB was in traces. A total of 55 fractions of 40 ml volumes each were collected. Similar fractions according to the TLC analysis were pooled to give 13 (CC1, CC2, CC3, CC4, CC5, CC6, CC7, CC8, CC9, CC10, CC11, CC12 and CC13) combined fractions.
Combined fractions CC1, CC4 and CC5 eluted with hexane - ethyl acetate (9:1) afforded one spot each of orange oily liquid, in traces. Combined fractions CC2, CC6, CC8, CC9 and CC10 eluted with hexane-ethyl acetate 9:1 for CC2 and hexane – ethyl acetate 8:2 for the others afforded three spots each of orange oily liquid. Combined fractions CC3, CC5, CC7, CC12 and CC13 eluted with hexane – ethyl acetate 9:1 for CC3 and CC5; hexane-ethyl acetate 8:2 for CC7 and CC12 and hexane – ethyl acetate 7:3 for CC13 afforded two compounds each of orange oily liquid.

**Liquid-liquid extraction of DS/6/D**

The methanol extract (40g) was dissolved in water and added into a separatory funnel and extracted with butanol. The butanol extract was dried with sodium sulphate anhydrous before evaporation under reduced pressure. Four grammes of crude butanol extract were obtained. The BuOH extract was subjected to column chromatography with the mixture of hexane - ethyl acetate (9:1 to 3:7). A total of 64 fractions of 40 ml volumes each were collected and 11 similar fractions labelled respectively CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD9, CD10 and CD11 were combined. Combined fractions CD1, CD2, CD9, CD10 and CD11 eluted with hexane 100% and hexane-ethyl acetate 9:1 for CD2, hexane-ethyl acetate 8:2 for CD9, hexane-ethyl acetate 7.5:2.5 for CD10 and hexane – ethyl acetate 7:3 for CD11 afforded one spot of orange oily liquid each. Combined fractions CD5, CD7 and CD8 eluted with hexane – ethyl acetate 9:1 for CD5 and CD7; hexane-ethyl acetate 8:2 for CD8 afforded two compounds each of orange oily liquid. Combined fraction CD3 eluted with hexane –ethyl acetate 9:1 afforded a mixture of four compounds in traces. Combined fractions CD4 and CD6 eluted with hexane-ethyl acetate 9:1 afforded a mixture of three compounds, in small quantities.

**4.5.2 Characterisation of isolate from *Cajanus cajan***

Pure compounds from the chromatographic separation in section 4.5.1 were submitted to $^1$H and $^{13}$C-NMR analysis.

**Characterisation of Compound H: fraction CC4**
Physical data

Yield: 0.0009 g (0.00005 %)

Molecular mass (LC-MS): m/z: 383.9 (M⁺); the other intense peaks were observed at 350.9, 313.0, 278.9, 226.7, 179.7, and 119.7 as base peak. (Figure 7.51)

FT-IR: $\nu_{\text{cm}^{-1}}$ (KBr): a broad band at 3454.97 and a medium peak at 1160.64 which are characteristic of hydroxyl group. The sharp peaks at 2923.21 cm⁻¹ and 2853.81 cm⁻¹ are typical of C-H stretching. A strong peak at 1739.96 cm⁻¹ is characteristic of carbonyl group, which attests the presence of carboxylic acid. (Figure 7.50)

The $^1$H- NMR spectrum (Figure 7.48) showed some sets of protons, characteristic of fatty acids. The peak at $\delta_H$ 0.87 ppm is characteristic of the terminal methyl group. The strong peak at $\delta_H$ 1.25 ppm represents a long chain of CH₂ groups. The peak at $\delta_H$ 5.3 ppm belongs to the allylic protons. The peak at $\delta_H$ 2.0 ppm belongs to the proton of CH₂ bonded to the allylic carbons and the one appearing at $\delta_H$ 2.7 ppm can be attributed to the protons of CH₂ which is separating two allylic groups.²⁴

The $^{13}$C- NMR spectrum (Figure 7.49) revealed 34 signals of carbons ($\delta_C$ ppm).

173.30, 173.27, 172.84, 130.213, 129.99, 128.08, 127.89, 68.89, 62.09, 34.19, 34.06, 34.03, 31.93, 31.53, 29.70, 29.66, 29.62, 29.48, 29.36, 29.35, 29.27, 29.20, 29.18, 29.12, 29.09, 29.05, 27.20, 25.63, 24.87, 22.69, 22.57, 14.11, 14.06.

According to the literature, the signals appearing at 14.1 ppm are characteristic to the methyl groups. The one at $\delta_C$ 29.4 ppm to 29.9 ppm are characteristic to methylene group. Glycerol appears at 68.9 and 62.1 ppm, while the olefinic and allylic appear at 127 to 130 ppm and 27.3 to 25.6, respectively. The peaks at $\delta_C$ (ppm) 173.3 and 34.2 are identified as C1 and C2, respectively.²⁴
Proposed class structure of compound I

\[
\text{CH}_3-(\text{CH}_2)_{n}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_{n}-\text{CH}_2-\text{CH}_2-\text{COOH}
\]

Figure 4.1 Class structure of compound I

4.6. Conclusion

- During the extraction, methanol extract of *Cajanus cajan* (seeds) gave the highest crude extract while the hexane extract gave the lowest yield.
- Column chromatography on the ethyl acetate and on methanol extracts of *Cajanus cajan* showed the presence of fatty acids.
4.7. References


16 Fu, Yujie; Zu, Yuangang; Liu, Xiaolei; Liu, Wei; Kong, Yu; Gu, Chengbo; Wu, Nan (2009) Application of Cajanus cajan extract in drugs for resisting gram positive bacteria. *Faming Zhuanli Shengqing Gongkai. Shuomingshu*, CN 101485714 A 20090722.

17 Kong, Yu; Fu, Yu-Jie; Zu, Yuan-Gang; Liu, Wei; Wang, Wei; Hua, Xin; Yang, Mei (2009) Ethanol modified supercritical fluid extraction and antioxidant activity of cajaninstitilbene acid and pinostrobin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves. *Food Chemistry*, 117(1), 152-159.


20 Kong, Yu; Fu, Yu-Jie; Zu, Yuan-Gang; Liu, Wei; Wang, Wei; Hua, Xin; Yang, Mei (2009). Ethanol modified supercritical fluid extraction and antioxidant activity of cajanin-stilbene acid and pinostrobin from pigeon pea \([Cajanus cajan\ (L.)\ Millsp.]\) leaves. \textit{Food Chemistry}, \textbf{117}(1), 152-159.


SUB-CHAPTER FOUR

MASLINIC ACID AND OLEANOLIC ACID

These two compounds, maslinic acid 4.1 and 4.2 were isolated and fully characterized by my supervisor, Professor Shode from the leaves of Syzygium cordatum for the purpose of including them as blind control in the anti-sickling assay.

4.1

4.2
5.0 Introduction

One of the main objectives of this project was to determine whether the crude extracts or any of their pure isolated chemical constituents had anti-sickling activity. There are different ways of doing this and some of these methods are described in the *Journal of Laboratory and Medicine*. In this study, the Emmel test was used as technique of performing anti-sickling study.

5.1 Biological materials

Blood samples used to perform the anti-sickling activity of the crude extracts and the pure isolated compounds in this study were collected from a known drepanocitary center named “Centre de Médecine Mixte et d’Anémie SS” located in Kinshasa area, Democratic Republic of Congo. The blood samples were first characterized by Hb electrophoresis on cellulose acetate gel, in order to confirm their SS nature, as previously reported by Mpiana. They were found to be SS blood and were then stored in a refrigerator at ± 4°C for not more than seven days. These SS blood samples were so used to perform biological tests.

5.2 Emmel test

* A drop of physiologic solution is put on a glass slide and a drop of blood is added on the physiologic solution. Two drops, one of sodium metabisulfite (Na$_2$S$_2$O$_5$) solution 2% and another of the extract are also successively added, and then the slide glass is covered using a cover glass which is hermetically covered ; within 2 hours in order to accelerate the sickling shape, with melts paraffin.
Assay is observed under optical microscope and the number of observed erythrocytes is determined using Thomas` cell.³

5.3 Biological activity

Blood samples were put in contact with diluted plant crude extracts and pure isolated compounds at different concentrations, using physiologic solution (NaCl 0.9 %) as dilution solvent; according to Emmel’s test procedure which was performed in this study as reported above.

5.4 Data analysis.

A microscopic pictures obtained were taken using an optical microscope (Motic). In order to convert the photonic micrograph image into digital image, a Canon digital camera (Zoom 6 X) was used, which allowed to digitize micrographs that were treated using Motic Images 2000 version 1.3 Software, on Windows XP. Thomas` cell was used to observe the number of erythrocytes.

5.5 Results and discussion

In the aim to verify the plants antisickling activity, an in vitro bioassay has been performed on different crude extracts of Syzygium guineense of D.R. Congo, Syzygium guineense of South Africa, Melaleuca bracteata, Callistemon viminalis and Cajanus cajan. These tests were also performed on the different pure isolated compounds from these plants and for this purpose; the Emmel test has been used. Minimal concentrations of normalization (MCN) which correspond to the maximal of normalization rate were calculated for the active crude extracts and the pure compounds. The obtained results are summarized in Table 5.1 below.
**Table 5.1**: In vitro antisickling activity evaluation of crude extracts and isolates using Emmel test.

<table>
<thead>
<tr>
<th>N°</th>
<th>Product</th>
<th>Sample Code</th>
<th>MCN</th>
<th>Degree of normalization</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atropin (1mg/mL)</td>
<td>Reference</td>
<td>0.81µg/mL</td>
<td>69%</td>
<td>+++</td>
</tr>
<tr>
<td>1</td>
<td>Crude hexane extract</td>
<td>DS/6/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Crude methanol extract</td>
<td>DS/6/D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Callistemon viminalis</td>
<td>DS/12/B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Crude dichloromethane extract</td>
<td>DS/12/C</td>
<td>22.23 mg/mL</td>
<td>74%</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>Crude ethyl acetate extract</td>
<td>DS/12/C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Melaleuca bracteata</td>
<td>DS/10/B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Crude dichloromethane extract</td>
<td>DS/10/D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Crude ethyl acetate extract</td>
<td>DS/10/C</td>
<td>10.57 mg/mL</td>
<td>82%</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>Crude methanol extract</td>
<td>DS/10/E aq</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Crude aqueous methanol extract</td>
<td>DS/10/EAq</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Compound B (Betulinic acid)</td>
<td>DS/10/C/A</td>
<td>0.34 µg/mL</td>
<td>78%</td>
<td>+++</td>
</tr>
<tr>
<td>10</td>
<td>Compound G (Betulinic acid acetate)</td>
<td>DS/10/C/Ab</td>
<td>0.71 µg/mL</td>
<td>87%</td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>Compound H (Ursolic acid)</td>
<td>DS/10/C/Ac</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Compound BF4 (Fatty acid)</td>
<td>DS/10/B/F4</td>
<td>1.70 µg/mL</td>
<td>62%</td>
<td>++</td>
</tr>
<tr>
<td>13</td>
<td>Syzygium cordatum</td>
<td>OA</td>
<td>12.63 µg/mL</td>
<td>49%</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Compound (Oleanolic acid)</td>
<td>MA</td>
<td>0.42 µg/mL</td>
<td>89%</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>----------</td>
</tr>
<tr>
<td>15</td>
<td>Crude hexane extract</td>
<td>DS/2/A</td>
<td>12.07 mg/mL</td>
<td>76%</td>
<td>+++</td>
</tr>
<tr>
<td>16</td>
<td>Crude dichloromethane extract</td>
<td>DS/2/B</td>
<td>5.78 mg/mL</td>
<td>84%</td>
<td>+++</td>
</tr>
<tr>
<td>17</td>
<td>Crude ethyl acetate extract</td>
<td>DS/2/C</td>
<td>0.12 mg/mL</td>
<td>71%</td>
<td>+++</td>
</tr>
<tr>
<td>18</td>
<td>Crude methanol extract</td>
<td>DS/2/D</td>
<td>17.87 mg/mL</td>
<td>73%</td>
<td>+++</td>
</tr>
<tr>
<td>19</td>
<td>Compound A (Flavanone glycoside)</td>
<td>DS/2/C/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Syzygium guineense (Leaves from South Africa)**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Crude hexane extract</td>
<td>DS/8/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Crude dichloromethane extract</td>
<td>DS/8/B</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Crude ethyl acetate extract</td>
<td>DS/8/C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Crude methanol extract</td>
<td>DS/8/D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>Compound D (3-oxo-friedelin)</td>
<td>DS/8/D/SD1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>Compound C (Sitosterol)</td>
<td>DS/8/D/SD5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Legend:**

+++ : > 70% of normalization
++ : 50 to 70% of normalization
+ : 10 to 50% of normalization
- : negative test.

MCN : Minimal concentration of normalization.

Atropin: Positive control
Table 5.2 Grouping of test samples according to degree of anti-sickling activity.

<table>
<thead>
<tr>
<th>High Activity</th>
<th>MCN</th>
<th>Medium Activity</th>
<th>Low Activity</th>
<th>Zero Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine (69%)</td>
<td>0.81 µg/mL</td>
<td>DS/10/B/F4 (62%) (MCN = 1.70 µg/mL)</td>
<td>Oleanolic acid (49%) (MCN = 12.63 µg/mL)</td>
<td>DS/6/A</td>
</tr>
<tr>
<td>DS/12/C (74%)</td>
<td>22.23 mg/mL</td>
<td></td>
<td></td>
<td>DS/6/D</td>
</tr>
<tr>
<td>DS/10/C (82%)</td>
<td>10.57 mg/mL</td>
<td></td>
<td></td>
<td>DS/12/B</td>
</tr>
<tr>
<td>DS/10/C/A (78%)</td>
<td>0.34 µg/mL</td>
<td></td>
<td></td>
<td>DS/10/D</td>
</tr>
<tr>
<td>DS/10/C/Ab (87%)</td>
<td>0.71 µg/mL</td>
<td></td>
<td></td>
<td>DS/10/Eaq</td>
</tr>
<tr>
<td>Maslinic acid (89%)</td>
<td>0.42 µg/mL</td>
<td></td>
<td></td>
<td>DS/10/C/Ac</td>
</tr>
<tr>
<td>DS/2/A (76%)</td>
<td>12.07 µg/mL</td>
<td></td>
<td></td>
<td>DS/2/C/2</td>
</tr>
<tr>
<td>DS/2/B (84%)</td>
<td>5.78 mg/mL</td>
<td></td>
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<td>DS/8/A</td>
</tr>
<tr>
<td>DS/2/C (71%)</td>
<td>0.12 mg/mL</td>
<td></td>
<td></td>
<td>DS/8/B</td>
</tr>
<tr>
<td>DS/2/D (73%)</td>
<td>17.87 mg/mL</td>
<td></td>
<td></td>
<td>DS/8/C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DS/8/D</td>
</tr>
<tr>
<td></td>
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<td>DS/8/SD1</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>DS/8/SD5</td>
</tr>
</tbody>
</table>

Key:

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS/12/C</td>
<td>Ethyl acetate extract of <em>C. viminalis</em></td>
</tr>
<tr>
<td>DS/10/C</td>
<td>Ethyl acetate extract of <em>M. bracteata var. revolution gold</em></td>
</tr>
<tr>
<td>DS/10/C/A</td>
<td>Compound B (Betulinic acid)</td>
</tr>
<tr>
<td>DS/10/C/Ab</td>
<td>Compound G (Betulinic acid acetate)</td>
</tr>
<tr>
<td>DS/2/A</td>
<td>Hexane extract of <em>S. guineense</em> (DRC; leaves)</td>
</tr>
<tr>
<td>DS/2/B</td>
<td>DCM extract of <em>S. guineense</em> (DRC; leaves)</td>
</tr>
<tr>
<td>DS/2/C</td>
<td>Ethyl acetate extract of <em>S. guineense</em> (DRC; leaves)</td>
</tr>
<tr>
<td>DS/2/D</td>
<td>Methanol extract of <em>S. guineense</em> (DRC; leaves)</td>
</tr>
<tr>
<td>DS/10/B/F4</td>
<td>Fatty acid from <em>M. bracteata var. revolution gold</em></td>
</tr>
<tr>
<td>DS/6/A</td>
<td>Hexane extract from <em>Cajanus cajan</em> (seeds)</td>
</tr>
</tbody>
</table>
The results of the Emmel test show that maslinic acid, betulinic acid, its acetate have the highest activities. Comparatively, these three compounds have higher activity than the reference (Atropin), which is one of the most taken medicines by sicklers”.

According to Tables 5.1 and 5.2, the highest activities, more than 70% of normalization, were found in crude ethyl acetate extracts of *Callistemon viminalis* and *Melaleuca bracteata*, and in hexane, dichloromethane and ethyl acetate extracts of *Syzygium guineense* of DRC, and on the other hand, in the isolated pure compounds, betulinic acid, betulinic acid acetate and maslinic acid showed high activity.

The compound BF$_4$, a fatty acid from *Melaleuca bracteata* was found to have a medium activity, between 50% to 70% of normalization. Oleanolic acid showed the weakest activity, between 10% to 50% of normalization.

Some crude extracts and pure isolated compounds were found to be non-active. These were: crude hexane and methanol extracts of *Cajanus cajan*; crude dichloromethane extract of *Callistemon viminalis*; crude dichloromethane, methanol and 80% aqueous methanol extracts of *Melaleuca bracteata*; crude hexane, dichloromethane, ethyl acetate and methanol extracts of *Syzygium guineense* of South Africa; ursolic acid from *Melaleuca bracteata* and flavanone glycoside from *Syzygium guineense* of DRC (Table 5.2).

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS/6/D</td>
<td>Methanol extract from <em>Cajanus cajan</em> (seeds)</td>
</tr>
<tr>
<td>DS/12/B</td>
<td>DCM extract from <em>C. viminalis</em></td>
</tr>
<tr>
<td>DS/10/D</td>
<td>Methanol extract from <em>M. bracteata var. revolution gold</em></td>
</tr>
<tr>
<td>DS/10/Eaq</td>
<td>Aqueous methanol extract of <em>M. bracteata var. revolution gold</em></td>
</tr>
<tr>
<td>DS/10/C/Ac</td>
<td>Compound H (Ursolic acid)</td>
</tr>
<tr>
<td>DS/2/C/2</td>
<td>Flavononoid glycoside</td>
</tr>
<tr>
<td>DS/8/A</td>
<td>Hexane extract from <em>S.guineense</em> (SA; leaves)</td>
</tr>
<tr>
<td>DS/8/B</td>
<td>DCM extract from <em>S.guineense</em> (SA; leaves)</td>
</tr>
<tr>
<td>DS/8/C</td>
<td>Ethyl acetate extract from <em>S.guineense</em> (SA; leaves)</td>
</tr>
<tr>
<td>DS/8/D</td>
<td>Methanol extract from <em>S.guineense</em> (SA; leaves)</td>
</tr>
<tr>
<td>DS/8/SD1</td>
<td>Compound D (3-oxo-friedelin)</td>
</tr>
<tr>
<td>DS/8/SD5</td>
<td>Sitosterol</td>
</tr>
</tbody>
</table>
Figures 5.1 to 5.8 show some morphologies which illustrate the modification of drepanocytes by crude extracts of plants and the isolated pure compounds. These micrographs were taken in hypoxic conditions, where the SS blood erythrocytes are assumed to change morphology. Figure 5.1 shows the drepanocyte morphology of untreated SS blood (standard), where the majority of erythrocytes are sickled, confirming the SS nature of the blood. Figure 5.2 shows the morphology of the SS blood treated with the methanol extract of *S. guineense* of DRC. It showed significant activity. It can be seen in this figure that a high number of erythrocytes recovered to normal form comparatively to the standard, indicating the antisickling activity of the extract. Figures 5.3, 5.4 and 5.5 show morphologies of SS blood treated with betulinic acid, betulinic acid acetate and maslinic acid. The population of non-sickled cells is high in all of them. This is a confirmation of the antisickling activity of these compounds. Compound BF₄ showed a relatively low to medium antisickling activity, while Figures 5.7 and 5.8 show morphologies of SS blood in the presence of compound C (Sitosterol) and one crude extract (Hexane extract from *S. guineense* of South Africa) which were found to be non-active since most of the cells remained sickled upon treat with these extractives.

![Figure 5.1 Morphology of drepanocytes of untreated SS blood (standard) (X500)](image-url)
Figure 5.2 Morphology of SS blood treated with MeOH crude extract of *S. guineense* of DRC (MCN: 17.87 mg/mL; 73%) (X500)

Figure 5.3 Morphology of SS blood treated with Betulinic acid (MCN: 0.34µg/mL; 78%) (X500)
Figure 5.4 Morphology of SS blood treated with Betulinic acid acetate (MCN: 0.71µg/mL; 87%) (X500)

Figure 5.5 Morphology of SS Blood treated with Maslinic acid (MCN: 0.41µg/mL; 89%) (X500)
Figure 5.6 Morphology of SS blood treated with compound DS/10/BF4 (MCN: 1.70µg/mL; 62%) (X500)

Figure 5.7 Morphology of SS blood treated with sitosterol (X500)

Figure 5.8 Morphology of SS blood treated with crude hexane extract of *S. guineense* (SA) (X500)
Figures 5.9 and 5.10 show the normalization rates of drepanocytes versus the concentration of betulinic acid and betulinic acid acetate and maslinic acid, respectively. These curves show that the normalization rate of erythrocytes increases with the concentration of compounds and reaches maximal normalization at a specific concentration and remains constant.
The minimum concentration corresponding to the maximal normalization rate is called minimal concentration of normalization (MCN). The MCN values are 0.34 µg/mL (78%), 0.71 µg/mL (87%) and 0.42 µg/mL (89%) for betulinic acid, betulinic acid acetate and maslinic acid, respectively. A low value of the MCN is a good indication of the antisickling activity.

5.6 Conclusion

In conclusion, the high anti-sickling activity of the plants’ extracts and the pure isolates listed in Table 5.2 is very encouraging. This is the first report of the anti-sickling activity of oleanolic acid, maslinic acid, betulinic acid, and betulinic acid acetate. This discovery is novel and it offers research opportunities to use these already known bioactive triterpenes as lead compounds in the development of new, potent, and affordable anti-sickling drugs. Furthermore, the active crude extracts from the leaves of S. guineense (DRC) offer opportunity to discover known or unknown anti-sickling agents after fractionation, purification, and bioassay of the chemical constituents.

5.7 References


General Conclusion

The main aim of this project was to isolate and characterize anti-drepanocytosic compounds from selected medicinal plants. To achieve these goals, five medicinal plants were studied. These included *Syzygium guineense* (from DRC and SA), *Cajanus cajan*, *Callistemon viminalis*, and *Melaleuca bracteata* var. *revolution gold*. Chromatographic methods were used to isolate and purify compounds. The *in vitro* antisickling activities of crude extracts and purified compounds were performed using the Emmel test. Elucidation of structures of isolated compounds was performed using spectroscopic techniques.

The leaves of *S. guineense* were collected in Kinshasa area, in D R Congo and on Westville campus of the University of KwaZulu Natal in South Africa. The seeds of *Cajanus cajan* were purchased by Professor Shode, from a local market in Lagos, Nigeria in 2007, while the leaves of *Callistemon viminalis* and *Melaleuca bracteata* were collected on Durban city, in South Africa. Several extractions were performed on different plant materials using organic solvents: hexane, dichloromethane, ethyl acetate, methanol and aqueous methanol 80%. Methanol extract from the leaves of *S. guineense* of D R Congo gave the highest extract (25.65%), while ethyl acetate extract contained the lowest (0.59%). Dichloromethane extract from the leaves of *S. guineense* of South Africa gave the highest extract (1.05 %), while ethyl acetate extract contained the lowest (0.08 %). Dichloromethane extract from the leaves of *C. viminalis* gave the highest extract (4.63%), while ethyl acetate extract contained the lowest (2.75%). Dichloromethane extract of *M. bracteata* gave the highest extract (9.33%), while ethyl acetate extract contained the lowest (0.80%). In the seeds of *C. cajan*, methanol extract gave the highest extract (4.54%), while hexane extract contained the lowest (0.44%).

The n-hexane crude extract of *S. guineense* of D.R. Congo in hexane - ethyl acetate (9:1) showed 10 compounds. The dichloromethane crude extract in hexane - ethyl acetate (8:2) presented 3 compounds. The ethyl acetate crude extract gave a precipitate when concentrated under reduced pressure. TLC analysis of this solid in the solvent system
hexane – ethyl acetate (7:3) revealed the presence of 3 compounds. The column chromatography performed on the above solid gave one pure compound which was identified as known flavanone glycoside. The methanol and aqueous methanol crude extracts could not give interesting separation. These extracts were conserved for further studies.

In *S. guineense*, SA., the column chromatography performed on this extract led to the isolation of one pure compound identified as betulinic acid. The ethyl acetate extract afforded three pure compounds after chromatographic separation; these were identified after spectroscopic analysis as Sitosterol, 3-oxo-friedelin and a derivative of betulinic acid.

The two species of *Syzygium guineense*, one from South Africa and the other one from DRC, gave different results. This could be due to the influence of the geographical and climatic regions, the season and the time of collection which can affect their secondary metabolism.

Betulinic acid was obtained from *C. viminalis*.

Acetylated ethyl acetate extract of *M. bracteata* afforded two pure compounds, which were identified as betulinic acid acetate and ursolic acid acetate.

Fatty acids were obtained from *Cajanus cajan*.

*In vitro* anti-sickling activity studies carried out on different crude extracts and pure isolated compounds gave some positive results. Some crude extracts and pure isolated compounds showed high antisickling activities, more than 70% of normalization; these were ethyl acetate extracts of *Callistemon viminalis* and *Melaleuca bracteata*; hexane, dichloromethane and ethyl acetate extracts of *Syzygium guineense* of DRC, betulinic acid, betulinic acid acetate and maslinic acid. A fatty acid from *Melaleuca bracteata*, coded BF₄,
was found to have low activity, between 50 and 70% of normalization and oleanolic acid showed the weakest activity, between 10 and 50% of normalization.

However, some crude extracts and pure isolated compounds were found to have no anti-sickling activity; these were: crude hexane and methanol extracts of *Cajanus cajan*; crude dichloromethane extract of *Callistemon viminalis*; crude dichloromethane, methanol and aqueous methanol (80%) extracts of *Melaleuca bracteata*; crude hexane, dichloromethane, ethyl acetate and methanol extracts of *Syzygium guineense* of South Africa; ursolic acid from *Melaleuca bracteata* and flavanone glycoside from *Syzygium guineense* of DRC.

According to these results, it can be seen that the activity of ethyl acetate extract from *Melaleuca bracteata* in particular must arise from the betulinic acid and its acetate, two compounds which were found in this extract. Betulinic acid and maslinic acid were found to have the highest activities.

I wish to recommend further investigations on the hexane, dichloromethane, ethyl acetate, and methanol extracts of *S. guineense* from DRC, in order to identify the active principles. Furthermore, different derivatives of betulinic acid and maslinic acid should be synthesized, in order to compare their anti-sickling activities with the starting material.
APPENDIX 1
SPECTRA OF COMPOUNDS
Figure 7.1: $^1$H-NMR spectrum of Compound A.
Figure 7.2: $^{13}$C-NMR spectrum of Compound A.
Figure 7.3: DEPT spectrum Compound A
Figure 7.4: COSY spectrum Compound A
Figure 7.5: HSQC spectrum of Compound A
Figure 7.6: NOESY spectrum of Compound A.
Figure 7.7: HMBC spectrum of Compound A
Figure 7.8: FT-IR spectrum of Compound A.
Figure 7.9: LC-MS spectrum of Compound A.
Figure 7.10: $^1$H-NMR spectrum of Compound B.
Figure 7.11: $^{13}$C spectrum of Compound B.
Figure 7.12: DEPT spectrum of Compound B.
Figure 7.13: FT-IR spectrum of Compound B.
Figure 7.14: LC-MS spectrum of Compound B.
Figure 7.15: $^1$H-NMR spectrum of Compound C.
Figure 7.16: $^{13}$C-NMR spectrum of Compound C.
Figure 7.17: DEPT spectrum of Compound C.
Figure 7.18: FT-IR spectrum of Compound C.
Figure 7.19: LC-MS spectrum of Compound C.
Figure 7.20: $^1$H-NMR spectrum of Compound D.
Figure 2.1: $^{13}$C-NMR spectrum of Compound D.
Figure 7.22: DEPT spectrum of Compound D.
Figure 7.24: GC-MS spectrum of Compound D.
Figure 7.23: FT-IR spectrum of Compound D.
Figure 7.25: $^1$H-NMR spectrum of Compound E.
Figure 7.26: $^{13}$C-NMR spectrum of Compound E.
Figure 7.27: DEPT spectrum of Compound E.
Figure 7.33: LC-MS spectrum of Compound E.
Figure 7.32: FT-IR spectrum of Compound E.
Figure 7.28: COSY spectrum of Compound E.
Figure 7.29: HSQC spectrum of Compound E.
Figure 7.30: NOESY spectrum of Compound E.
Figure 7.31: HMBC spectrum of Compound E.
Figure 7.34: $^1$H-NMR spectrum of Compound F.
Figure 7.35: $^{13}$C-NMR spectrum of Compound F.
Figure 7.36: DEPT spectrum of Compound F.
Figure 7.37: FT-IR spectrum of Compound F.
Figure 7.38: LC-MS spectrum of Compound F.
Figure 7.39: $^1$H-NMR spectrum of Compound G.
Figure 7.40: $^{13}$C-NMR spectrum of Compound G.
Figure 7.41: DEPT spectrum of Compound G.
Figure 7.42: FT-IR spectrum of Compound G.
Figure 7.43: LC-MS spectrum of Compound G.
Figure 7.44: $^1$H-NMR spectrum of Compound H.
Figure 7.45: $^{13}$C-NMR spectrum of Compound H.
Figure 7.46: FT-IR spectrum of Compound H.
Figure 7.47: LC-MS spectrum of Compound H.
Figure 7.48: $^1$H-NMR spectrum of Compound I.
Figure 7.49 $^{13}$C-NMR spectrum of Compound I.
Figure 7.50: FT-IR spectrum of Compound I.
Figure 7.51: LC-MS spectrum of Compound I.