ANTIMICROBIAL ACTIVITY STUDY AND CHEMICAL CHARACTERIZATION OF PURE COMPOUNDS FROM *SYNADENIUM GLAUCESCENS* PAX

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN NATURAL PRODUCTS' TECHNOLOGY AND VALUE ADDITION OF SOKOINE UNIVERSITY OF AGRICULTURE.

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ABSTRACT

The modern medicine particularly drugs' discovery and their development is a result of improvement of the local knowledge of the health practitioners on the medicinal plants and animal products. This study aimed at isolating and testing the bioactive compounds from *Synadenium glaucescens* collected from Njombe Region; Tanzania. Extraction was done using Soxhlet method while the isolation involved a series of chromatographic techniques. Nuclear magnetic resonance (NMR) technique was used for compound identification. Colour reaction was done using Vanillin reagent. Microdilution and agar well diffusion methods were used to test for antimicrobial activity of the isolates and/or compounds. The crude extracts obtained were 1.99 % and 6.25 % by weight of the pulverized stem wood and the stem bark respectively. The percentage composition of the pure compounds from crude extracts ranged between 0.5 % – 8.25 %. Out of nine isolates (5 from stem bark and 4 from stem wood (coded C_1 – C_9), two compounds C_6 and C_9 were identified. All compound isolates were conjugated except C_2. The majority of fractional extracts reacted bluish purple with vanillin reagent which implies the characteristics of terpenoids. The NMR data were compared to literature, and indicated compound C_6 to be a fatty acid and compound C_9 showed to have lupeol acetate backbone based on the ^{1}H and ^{13}C NMR spectroscopic data. All tested isolates were bioactive against the tested standard bacterial and fungal strains at different efficacies. Isolate C_7 demonstrated the highest activity of MIC (0.01 mg/ml) against *A. baumannii* and *S. enterica,* and the largest zone of inhibition (27 mm) against *C. tropicalis.* These results are recorded for the first time for this plant and present a good direction towards drugs discovery. Further studies on identification and bioassays of the compounds from stem of *S. glaucescens* are recommended.

DECLARATION

I FRANK RWEGOSHORA do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work, done within the registration period and that has neither been submitted nor being concurrently submitted in any other institution.

Signature _____ _____

Frank Rwegoshora Date
(MSc candidate)

The above declaration is confirmed by:

_____ _____

Dr. Faith P. Mabiki Date
(Supervisor)

_____ _____

Prof. Robinson H. Mdegela Date
(Supervisor)

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Last but not least, I express my sincere thanks to my family, mother and young brothers as well as Dr. Cliffson Maro my fellow candidate for their tireless prayers and advice throughout the period of this study. Their spiritual and social support helped me to remain strong in all difficult situations.

DEDICATION

This work is dedicated to my beloved wife Praxeda Henerico, my son Akiza, my parents (the late Longino Thomas and my mother Lucy Longino) and young brothers Anord and Edibert.

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LIST OF ABREVIATIONS

$CDCl_3$	Deuterated chloroform
DCM	Dichloromethane
EtOAc	Ethyl acetate
INT	Iodonitrotetrazoleum violet
MeOH	Methanol
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
NP	Natural Products
PE	Petroleum ether
SDA	Sabouraud Dextrose Agar
SM-CoSE	Solomon Mahlangu college of Science and Education
SUA	Sokoine University of Agriculture
TLC	Thin layer chromatograph
WHO	World Health Organization

CHAPTER ONE

1.0. INTRODUCTION

Natural products (NP) are chemical compounds or substances of natural origin from living organism. They are sourced from tissues of plants, marine organism or microorganism and many others which possess biological effect on other organisms. Natural products may be of medical, nutritional or pesticidal value. They are more specifically referred to as secondary metabolites; the small organic molecules of limited distribution having molecular weight of approximately less than 2000 amu (Dewick, 2002, Sarker *et al.,* 2006., Sarker *et al*., 2007). Secondary metabolites are produced as a result of external stimuli, abiotic stress, biotic stress, injury, heat and cold (Lade *et al*., 2014). They include; carotenoids, phytosterines, saponins, phenolic compounds, alkaloids, glycosinates, terpenes, proteins, sugars, amino acids, lignin, cellulose and many others (Demirezer, 2013, Liang and Fang, 2006). Plants so far provide the largest source of NP, producing more than 200,000 different bio-active natural products (Field, 2010). Generally, Natural products have always been exploited to promote health and served as a valuable source for the discovery of new drugs (Waltenberger *et al.,* 2016). They help to protect the human and animal body against diseases and infections. Natural products from plants constitute higher in this field than other organisms. Ahmad *et al.,* (2006) asserted that, the curative properties of plants must have sprung from instinct. They further stated that the primitive peoples first used plants as food and, as result of this ingestion; the link with some plant properties would have been learnt.

The use of these products for medicinal purpose includes the traditional and advanced technological levels. However, the traditional level of health therapies occupies a large group and unelucidated natural products (Maroyi, 2013).

1.1 Diversity of Natural Products in Health Therapies

Natural products and herbal remedies use in traditional folklore medicine have been the source of many medically beneficial drugs because they elicit fewer side effects, relatively cheap, affordable and claimed to be effective (Wal *et al.,* 2015)

It is asserted that, in early civilizations, illness was usually believed to be due to divine punishment (Williams *et al.,* 2006). They continually argued that with time of exploration, there was renewed interest in the usefulness of plants in the treatment of diseases. The demand for health therapies based on medicinal plants is increasing as the population increases. Natural products have always been exploited to promote health and served as a valuable source for the discovery of new drugs (Waltenberger *et al.,* 2016). According to the WHO report of 2013; about 80% of the people especially in rural areas where health facilities are limited, use traditional medicine as their primary health care. According to Borges *et al*., (2013), approximately 49% of the drugs developed between 1981 and 2002 were obtained from natural products, or similar semi-synthetic or synthetic compounds based on natural products' structures. While plants have continued serving a large component, animals and microorganism also still make an appreciable contribution to the field of natural products. Some fungi and bacteria continue to serve pharmacological function in the past and the present life. For example, some have been isolated from filamentous fungi (*Penicillium sp*), like penicillin which was first isolated from *Penicillium notatum* by Alexander

Fleming, and its other derivatives (isolated from fungi) have largely been used in treatment of various diseases. The other antifungal drug is Griseofulvin a tricyclic spirodiketone, first isolated from *Penicillium griseofulvum* (Francois, (2005) as cited by Spampinato and Leonardi, (2013)).

1.2 State of Use of Traditional Medicine in Tanzania

It is estimated that 60% of the urban and 80% of the rural population in Tanzania depend on traditional medicines, most of which are derived from plants (Mabiki, 2013a, b). According to Stanifer *et al.,* (2015), about 70 % of the Tanzanian population frequently access healthcare through traditional healers or vendors. From time to time, there is an increasing number of Tanzanians who opt for the traditional medication, and the increased number of traditional health practitioners. It was stated to be attributed to poverty which is an issue of serious concern in developing countries (Mabiki *et al.,* 2013a) and thus fail to cover conventional medicinal drugs, facilities and medical personnel (Kayombo *et al.,* 2012). This is irrespective of their technical and advanced knowledge on the biochemical knowledge. Lack of scientific knowledge is among the factors that may put the users at risks. Plant extracts may contain more than one compound, some of which are curative while others are toxic. The Julocrotine isolated from *Croton sylvaticus* Hoechst ex Krauss (Forest fever-berry) tested to be toxic against brine shrimp while traditional healers claim to be using the leave extracts for treating cancer (Kapingu *et al.,* 2012).

Decoction of the leaves and root bark of is used traditionally for the treatment of tuberculosis, inflammation, as a purgative, as a wash for body swelling caused by kwashiorkor or by tuberculosis and for the treatment of malaria (Beentje, 1994 and Kokwaro, 1976) as sited by Kapingu *et al*., (2012).

1.3 Traditional Use of *Synadenium* species

In Tanzanian communities there is wide use of Euphorbiaceae and in particular, the *S. glaucescens* (Fig.1). *Synadenium* is a genus in the complex and heterogeneous Euphorbiaceae family of approximately 300 genera and 7500 species that have been identified worldwide (Bittner *et al*., 2001 and Rahmann and Akter, 2013). The plant is commonly used in Tanzania and other East African communities despite being reported and documented as a poisonous plant (Mosha *et al*., 2002). The plant is used as an insect repellant when planted surrounding the buildings. Decoction of its various parts like root stem and leaves, either free or in a combination with other plant and animal products are used to treat various human and animal diseases as well as for controlling pests. Some of the human diseases that are controlled by *S. glaucescens* are fungal infections on the skin, a leaf decoction with lime juice and honey treats asthma, a root bark extract is mixed with sugar to treat severe cough or tuberculosis, an ear drop to treat an ear ache (Mabiki *et al.,* 2013b)

Figure 1: *Synadenium glaucescens* Shoot

1.4 Problem Statement and Justification

The development of bacterial and fungal resistance to presently available antibiotics has necessitated the search of new antibacterial agents especially of natural origin including those from *Synadenium glaucescens*. The medicinal values of *S. glaucescens* from both traditional knowledge and advanced studies have sorely based on the crude extracts from its root, stem and leaves. There is no any pure compound as well as the corresponding bioactivity reported from its stem. This poses a number of effects like its unnecessary and overharvesting contributing to its extinction, unknowns on dose estimation and their mode of action. This created a gap of knowledge which this study focused on.

1.5 Significance of the Study

The results of this study will contribute towards understanding of the process of drug discovery from natural origins; especially medicinal plants, and on the chemistry of bioactive compounds from crude extracts which is of vital importance in drug discovery. Furthermore, the finding of this study contributes to natural product database and their biological activity will knowledge in the field of medicine and phamrmacology.

1.6 RESEARCH OBJECTIVES

1.6.1 Overall Objective

To study the bioactivity and to characterize compounds from *Synadenium glaucescens* Pax stem.

1.6.2 Specific Objectives

i. To determine the structures of isolated compounds from stem bark and wood of *S. glaucescens*.

ii. To assess the antibacterial and antifungal properties of isolated compounds of the dichloromethane extracts of the stem bark and wood of *S. glaucescens*

iii. To establish the structural-activity relationship of isolated compounds from stem wood and those from stem bark.

CHAPTER TWO

2.0 LITERATURE REVIEW

Natural products have served several important purposes in human and animal life including medicinal function (Field, 2010). Various secondary metabolites have served a great medicinal function in treatment of a wide range of diseases and infections. According to Nkunya (2005), Tanzania is endowed with a great abundance of floral diversity, which is estimated to constitute about 10,000 vascular plant species, of which at least 25 % are considered to be indigenous to the country. Medicinal plants were the main source of products used to sustain health until the nineteenth century, when the German chemist Friedrich Wohler in 1828, attempting to prepare ammonium cyanate from silver cyanide and ammonium chloride, accidentally synthesized urea (Ahmad *et al.,* 2006). In most rural areas particularly, from emerging economy countries including Tanzania, the uses of decoctions or infusions of the crude extracts from various parts of the plants have been the common approach. For example, Kisangu *et al.,* (2007) documented 75 plant species from 44 families including Euphorbiaceae which were being used by the Haya in Bukoba rural district (in Tanzania) for management of HIV/AIDS opportunistic infections.

2.1 Pure Secondary Metabolites in the Modern Health Practices

Plants and their derivatives are known to be important in pharmacological research due to their great potential as a source for a variety of biologically active ingredients used in drug development (Leite *et al.,* 2014). Despite some plants being reported to possess poisonous properties, in an extensive screening program of plants used in

traditional medicine, researchers have provided scientific evidence for their rational use in treating infectious diseases, inflammation, including disorders of the central nervous system (Ahmad, *et al.*, 2006).

Since the development of the germ theory there have been several initiatives for looking and development of new drugs. The work of Alexander Fleming discovered the antibacterial penicillin from the molds which became wide spread in the mid of the 20th century. Other developments include artemisinin from *Artemisia annua*. Artemisinin and its derivatives are the current gold standard medicine for malaria treatment. Malaria remains as one of the most significant disease responsible for over 250 million cases and over 800,000 deaths annually (Wells, 2011). Various drugs of plant origin have been documented, such as; Quinine (an alkaloid) has an anti-malaria, analgesic, and antipyretic properties. This drug was originally isolated from the Cinchona tree. Paclitaxel (taxol), a diterpenoid originally obtained from the *Taxus brevifolia*, the Pacific yew plant is a source of drug used to treat ovarian, breast, lung, pancreatic and other cancers (Priyadarshini and Aparajitha, 2012). According to Block, (2015), *Cytisus triflorus* from the family Fabaceae is a source of naturally occurring *2, 6-Bis(1,1-di-tert-methyl)-4-methyl phenol* which is used traditionally to treat abdominal pain, wound healing, as a haemostatic (a substance that stops bleeding), as an antifungal, and also as a hypotensor (a substance that lowers blood pressure) in Algeria.

2.2 Medicinal Value of Family Euphorbiaceae

The family Euphorbiaceae has approximately 300 genera and over 8000 species that have been identified worldwide (Bittner *et al*., 2001). A survey of medicinal plants in

Tanzania conducted between 1979 and 1989 in Tabora region in Tanzania documented a total of 127 medicinal plants which were indigenously used to treat about 66 human diseases of which 9 plants were from the family Euphorbiaceae (Ruffo, 1991).

2.2.1 Euphorbiaceae: Ethno-medicinal Uses.

The plants in the family of Euphorbiaceae have made a remarkable contribution in the list of natural products. According to Hassan *et al.*, (2012), this family is widely known for its diverse medicinal uses which often are associated with phorbol-type diterpenoid polyols. They continually assert that, its ester derivatives are known for their ability to inhibit HIV-induced cytopathic effect. Euphol, a triterpenoid from *Euphorbia kansui* is reported to possess the molecules which can be used in the management of inflammatory and neuropathic pain states (Dutra *et al.*, 2012 and Yasukawa, 2000). The prominence of euphol prominent in *Euphorbia tirucalli* is reported to be associated with anti- cancer activity against gastric and breast cancer according to Sadeghi-Aliabadi *et al*., (2009); and Zhang *et al*., (2012) as cited by Vuong *et al.*, (2014). Euphol displayed a significant activity against *Staphylococcus aureus* at MIC values of 1mg/ml and 0.2mg/ml for the ATCC 6538P and clinical isolate respectively (Upadhyay *et al*., 2010). It was therefore suggested that the activity of their chemical composition may be due to the presence of tannins exhibiting toxic effects on microorganisms tested. The study by Okeleye *et al.*, (2011) on the extracts of the Stem Bark of *Bridelia micrantha* (Hochst., Baill) have shown to be active against the growth of *Helicobacter pylori*

2.2.2 *Synadenium* Species in Health Therapies

The genus *Synadenium* contains about 24 species and poses a wide range of chemical compounds including tigliane, senadenol, phorbol type diterpenoids, triterpenoids, and anthocyanins (Hassan *et al.,* 2012). The isolated compounds from members of this genus are reported to have immunoregulatory and antitumor properties. Compounds from *S. grantii* are reported to have ability to inhibit HIV (Evans, 1986) as cited by Hassan *et al.,* (2012). In folk medicine, people use the latex from *S. grantii* Hook to treat neoplasic diseases and gastric disorders such as peptic ulcers and gastritis (Costa *et al.*, 2012). Among the compounds isolated from the leaves of the *S. grantii* include diterpene esters (3, 4, 12, 13 - tetraacetylphorbol-20 -phenylacetate and 4 - deoxyphorbol-12 ,13 -ditiglate), have been active against *Typanosoma cruzi*, *T. brucei* and *Plasmodium fulciparum* (Hassan *et al.,* (2012). The studies on *S. umbellatum*, the extrats have demonstrated good anti-inflammatory activity of both in America and Africa (Borges *et al.* 2013). This plant as reported by (Melo-Reis *et al.* (2009). latex is used to treat *Diabetes mellitus*, Hansen´s disease, tripanosomiases, leukemia and several malignant tumors. Latex of *S. carinatum* in has been widely used to treat a great number of inflammatory disorders. In Afonso- Cardoso and his colleague's experiment (2007 and 2011), the Lectin from *S. carinatum* was found to have a protective effect on *Leishmania amazonensis* infection in BALB/c mice; and the extract treats allergic disease.

2.2.3 Ethno medicinal Value of *Synadenium glaucescens*

Despite *S. glaucescens* being reported and documented as a poisonous plant (Mosha *et al.* 2002), ethnic communities in Tanzania and Kenya use it effectively in treatment and control of various human and animal diseases as well as pests (Wickama *et al.*,

2006 as cited by Mabiki *et al.*, 2013a). In Tanzania, it is planted surrounding the buildings to serve as repellant for the ants. The latex is used as a fish poison and water extracts of the leaves have demonstrated antimolluscidal activity against *Biomphalaria pfeifferi* (Ouma, and Butterworth, 1987; Neuwinger, 2004 as cited by Mabiki *et al.*, 2013b). Antimicrobial studies done have shown a remarkable effect on Newcastle disease virus, infectious bursal disease virus and fowl pox virus, bacterial and fungal infections (Mabiki *et al*., 2013b). This plant has been proved to serve as future drug component. The dermal toxicity study by Nyigo *et al.*, (2015) using the extracts from dried leaves and root barks of *S. glaucescens* suggested that the short term use of different extracts from dried leaves and root barks applied on skin of animals do not cause any adverse effects both externally and internally. This plant has widely been useful for many traditional applications in human and animals including healing wounds, boils, HIV, worms and application on the swollen lymph nodes of cattle suffering from East Coast Fever (ECF), (Mabiki *et al.* 2013a).

2.2.4 Compounds Isolated from *Synadenium glaucescens*

Preliminary characterization of the compounds from *S. glaucescens* by Rukunga *et al.* (1990) revealed the presence of β-glucoside attached to groups which could be methane, methylene. The current research work by Nyigo, *et al.*, (2015), reported isolation of a tetracyclic triterpene; Euphol from the root bark and a sterol; β-sitosterol was isolated from its leaves. The former is known for its wide range of pharmacological properties including anti-inflamatory and nueopathic pain (Dutra *et al.*, 2012) while the later is reported to enhance cardiovascular health.

However, there are no other investigations that have been conducted from other parts of this plant thus building the interest of this study.

2.3 Antibacterial and Antifungal Resistance

Over the last three decades, an increasing number of antibiotic resistance mechanisms have been emerging in members' bacteria and other microorganisms (Findlay, 2011, McConnell *et al.* 2012). Drug resistance is one of the most serious health threats the world is facing today. The rapid emergence of bacterial infections that are resistance to many drugs is creating the need for new drug development (Lopez *et al.*, 2001), and some pathogens have even become resistant to multiple types or classes of antibiotics (Frieden, 2013 and Radji *et al.*, 2013). However, Gram negative bacteria are reported to be less susceptible to the antibiotics than Gram positive bacteria. According to Rosell (1987) as cited by El Shoubaky and Salem (2014), this difference is attributed by a more complex structure and composition of Gram negative bacterial cell wall. Courvalin (1994) asserts that spread of "old" resistance genes into "new" bacterial hosts (i.e. genera or species that were previously uniformly susceptible) is a widely known mechanism since the early findings of antibacterial resistance. Furthermore, Findlay (2011) asserts that, they resist by disabling the antibiotics or development of impermeable protein membrane. Spampinato and Leonardi, (2013), state that the mechanism of resistance is different depending on the mode of action of antifungal compounds.

Gram-negative bacteria account for approximately 69% of the cases of bacterial food-borne disease (Mendonca *et al;* 1994), and account for the mortality rate ranging 30 to 70% (Tamma *et al.*, 2010). Among the common resistant bacteria species include;

Staphylococcus aureus which is responsible for healthcare associated infections as well as community acquired ones (Kurlenda and Grinholc, 2012). Plant derived medicines have been part of traditional health care system in most parts of the world for thousands of years and nowadays there is increasing interest in plants as sources of agents to fight microbial diseases (Natarajan *et al*. 2005).

2.3. 1Antibacterial and Antifungal Activity of the Plant-based Extracts and Their Application

Various plants' extracts have served the antibacterial and antifungal value, either as crude, pure or in a combined therapy. The Indonesian water soluble green tea extract, *Camelia sinensis* has demonstrated inhibition of the methicin resistant *S.aureus* and the multidrug resistant *P. aeruginosa* (Radji et al., 2013). Cocaine from and the coca plant has for so long been known as a painkiller, the resinous gum from the plant Pinus edulis which was used for the treatment of abscesses. This plant has pinene oils that are effective in killing Staphylococci bacteria (Steiner R, 1986) as cited by Williams et al. (2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection and Preparation

Synadenium glaucescens stem samples were collected from Mtulingala village, Njombe district in Njombe region which is in the Southern Highlands of Tanzania. The samples were further processed and tested in the Natural Products and Ecotoxicology Laboratory at the Faculty of Veterinary Medicine, and the Chemistry Laboratory - Solomon Mahlangu Campus (SMC) of Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

3.2 Materials

Equipments and reagents were obtained from Technonet Scientific Ltd, who is the SUA supplier. Bacteria and fungus standard strains; American Type Culture (ATCC) were obtained from the microbiology laboratory of SUA and Muhimbili University of Health and Allied Sciences in Dar es Salaam, Tanzania.

3.2.1 Test Microorganisms

The bacterial strains included gram negative strains: Eschelichia coli (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883), Enterobacter cloaca (ATCC 23355), *Proteus mirabilis* (ATCC 12453), *Shigella sonnei* (ATCC 25931), Enterobacter cloaca (ATCC 23355), *Salmonella enterica sub spp Enterica serovar enteritidis* (ATCC 13076), *Salmonella para typhi* (ATCC 9150), *Pseudomonas aeruginosa* (ATCC 27853) and gram positive strains included *Staphylococcus aureus* (ATCC 25923), Enterococcus faecalis (ATCC 51299) and *Acinetobacter baumannii* (ATCC 19606). In this study a single standard culture of fungus was used *Candida tropicalis* (ATCC 13803).

3.2.2 Chemicals and Reagents

Mueller- Hinton broth (MHB) from OXOID LTD, Basingstoke, Hemisphere, England was used and had the following properties: pH 7.3 ± 0.1 at 25 ^{o}C, beef, dehydrated infusion from 300, Casein hydrolysate 17.5, starch 1.5, agar 17.0 which meets the CLSI standard M6-A.The Sabouraud Dextrose Agar (SDA) produced by the OXOID LTD, Basingstoke, Hemisphere, England had the following specifications: pH 5.6 ± 0.2 at 25^{o}C, nutrient contents; mycological peptone 10.0, glucose 40.0, agar 15.0.

The reagents included; Dichloromethane, ethyl acetate, petroleum ether, chloroform, sulphuric acid and vanillin. They were both analytical grade, and were obtained from SUA supplier.

3.3 Plant Material Pretreatment and Processing

The shoot region of *S. glaucescens* was carefully harvested, the stem was cleaned using distilled water to remove dust and debris and then pilled to obtain the stem bark and stem wood separately, and then air- dried at room temperature (25- 30 ^{o}C) and pulverized to 1 mm particle size as indicated in Mabiki *et al.,* (2011). The sample was then stored at $^{-}$4 ^{o}C in the chemistry laboratories at SM-CoSE.

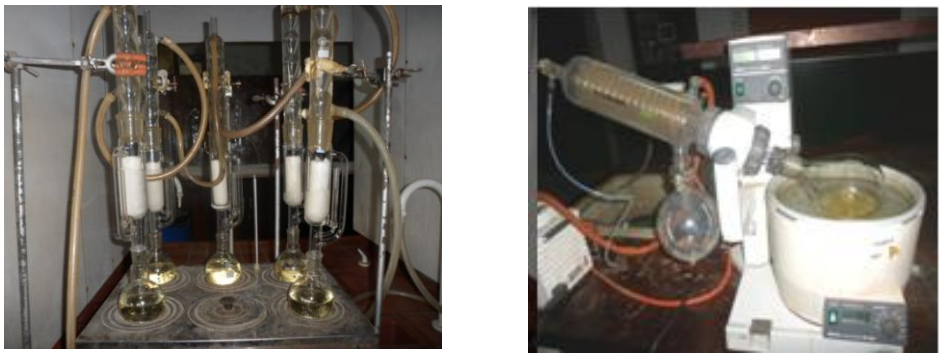
a b

Figure 2*: Synadenium glaucescens* stem wood (a) stem and back (b)

3.4 Extraction, Isolation and Purification

3.4.1: Extraction and Isolation

Soxhlet technique was used for extraction of less polar compounds by using dichloromethane (DCM) solvent. The samples of 278.34 g and 84 g of pulverized stem wood and stem bark respectively were subjected into different thimbles and placed in the 250 mL Soxhlet flasks followed by 200 mL of DCM in each of the flask (Fig. 3a) at 30 ^{o}C for a maximum of four (4) hours. The extract-solvent solution was separated using a rotary evaporator (Fig. 3b).



a b

Figure 3: Soxhlet apparatus (a), Rotary evaporator (b)

The isolation (Fig. 5 and 6) was conducted by column chromatography technique in which the sample were dissolved in a little DCM and adsorbed on silica gel (60 – 120 mesh) for the preparation of slurry (Fig. 4a). The extracts were dried in air and chromatographed over silica gel column packed in petroleum ether. The column was eluted with ethyl acetate-petroleum ether mixture in the order of increasing polarity from 10: 90 to 80:20. The fractions (Fig. 5) were obtained from the TLC, whereas the UV lamp of λ 254 nm (for non fluorescing compounds) and 365 nm (for fluorescing compounds) as well as chemical treatment. The treatment (colour reaction) test involved the vanillin reagent prepared by mixing 0.2 g/L (vanillin/Ethanol), and 0.1

mol/L (H_2SO_4/ Ethanol) in a ratio of 1:1. Eluted compounds were visualized on the TLC sheets after spraying with vanillin reagent.

The separation was conducted by using a column chromatography technique in a dry packing system of the sample (Fig 4). Stationary phase for separation according to polarity involved Silica gel while separation and purification according to particle size was achieved using Sephadex. The mobile phase included; Petroleum ether (PE) and ethyl acetate (EtOAc) in varying polarities depending on the separation behaviour of the compounds. The polarity ranged from 15 % to 40 % EtOAc/PE. For purification by Sephadex, mobile phase was 50:50 of $CHCl_3$: MeOH was used. At room temperature, the collected sample–solvent vials were exposed allowing the solvent to evaporate thereby leaving the extracts concentrated. Nature of the compounds from the chromatograms was observed first under UV lamp of wavelength 254 nm and 365 nm, followed by colour reaction test (chemical treatment)using the vanillin reagent.

a b

Figure 4: (a) compound slurry (b) Slurry parking in a Column Chromatograph

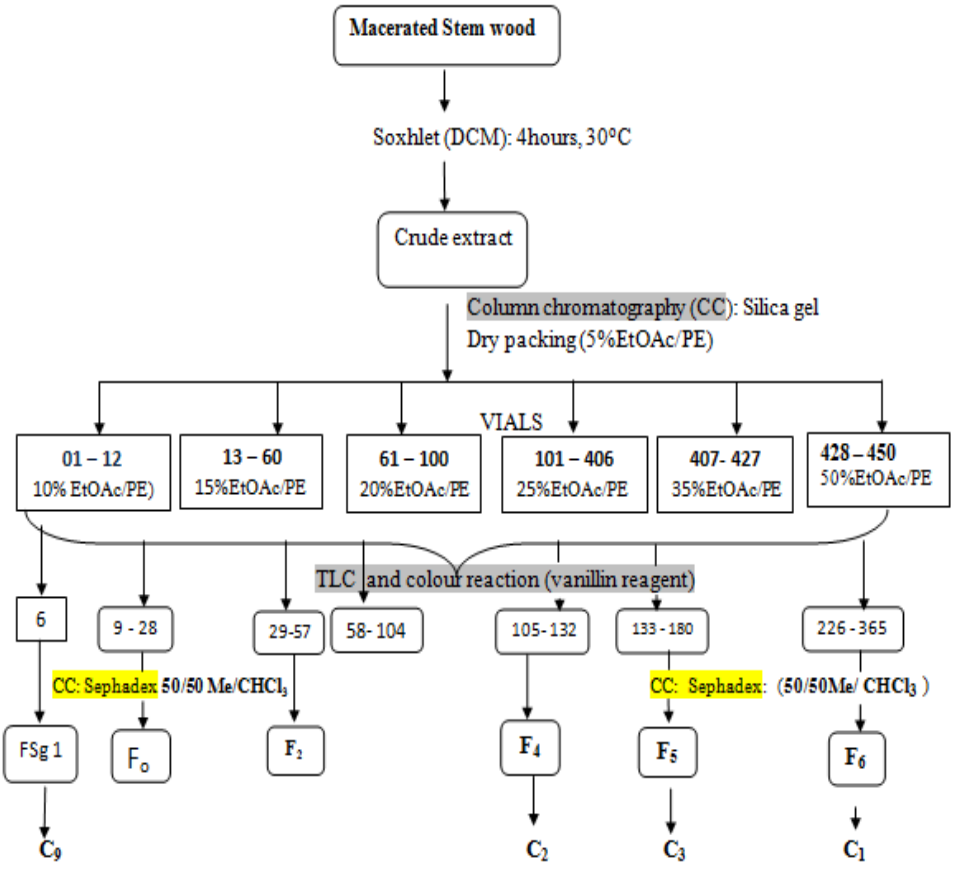


Figure 5: Scheme for the Isolation of compounds from *Synadenium glaucescens* stem wood

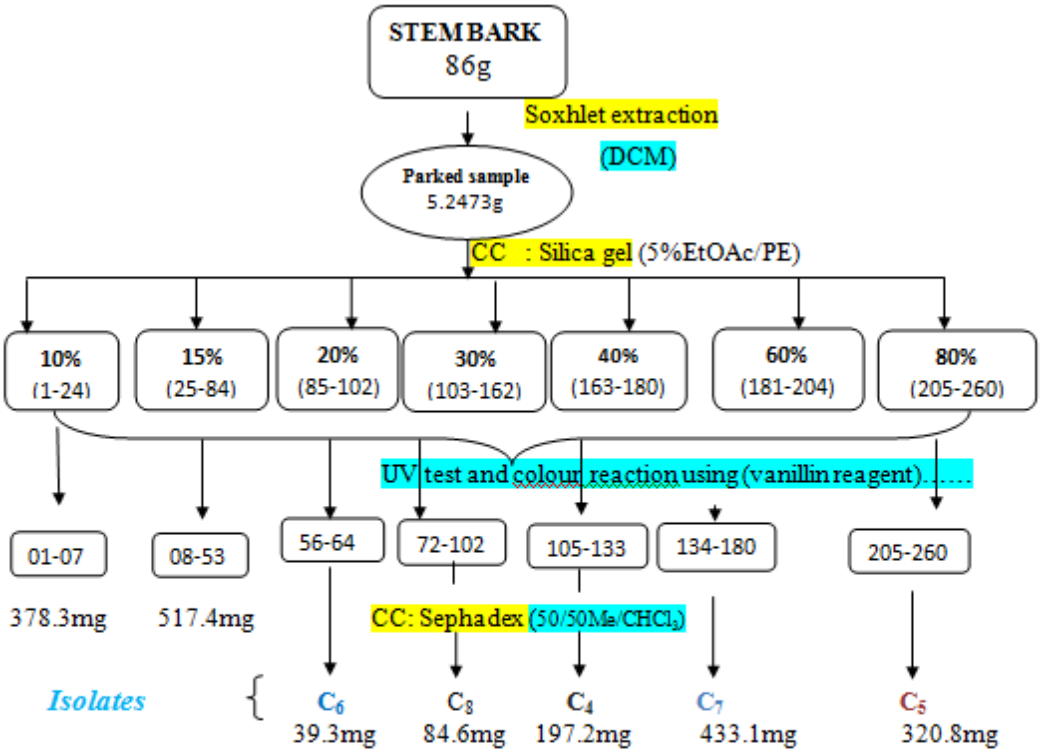


Figure 6: Scheme Isolation of compounds from *Synadenium glaucescens* stem bark

3.4.2 Chemical Treatment with Vanillin Reagent

The reagent was prepared by mixing 0.2 g/l (vanillin/Ethanol), and 0.1 mol/L (H_2SO_4/ Ethanol) in a ration of 1:1. Colour reaction was used to trace any non – conjugated compounds meaning those which were UV negative. This was done by spraying the vanillin reagent on the TLC sheet which contained eluted compounds. The wet TLC was immediately heated in an oven at a temperature 110^{o}C for 5minutes. The colour developments were observed and recorded as they were numbered from the lowest to the highest polarity, i.e 100% PE, 10% EtOAc/ PE and 40% EtOAc/ PE (see Fig. 7).

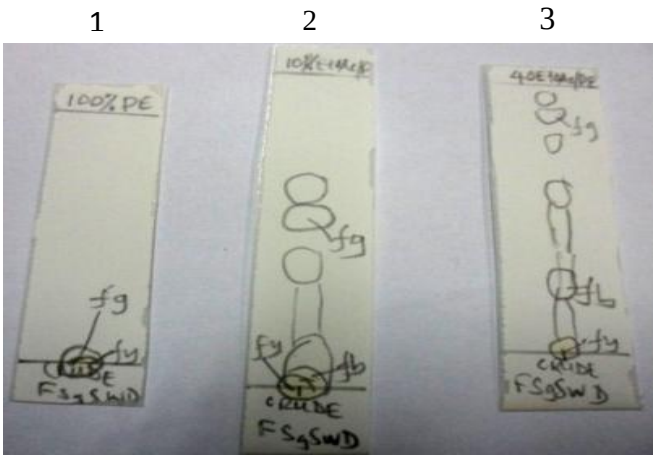


Figure 7: TLC Finger print of compounds from *Synadenium glaucescens* Crude Extract stem

3.4.3 Purification of Isolates

Purification of the isolates involved Sephadex® LH-20 (Pharmacia) as thestationary phase that separates compounds by particle size. A constant ratio of mobile phase 1:1 of chloroform: methanol was used. A single spot under both tests was a criterion for pure compound isolated. The isolated compounds were crystallized and weighed. The physical and chemical properties were also studied and recorded. Each crude extract was profiled for the compounds present by chromatographic technique in which Thin Layer chromatograph (TLC) was the stationary phase. A single spot under both Ultra Violet test and a colour reaction was a criterion for pure compound (Fig. 8). The pure compounds and the fractions were then weighed and recorded. The physical and chemical properties of the crude extracts, isolates and pure compounds were also studied and recorded.

3.5 Determination of the Extraction Yield

The yields of the crude and pure extracts were calculated by using the equation 1 and 2 shown below, respectively. The percentage yields were used to compare the content of the respective compounds in the plant parts studied.

$$\%Yield = \frac{Amount\ (g)\ of\ the\ crude\ extract\ obtained}{Amount\ (g)\ of\ the\ dry\ sample} \times 100 \qquad \ldots\ldots 1$$

$$\%Yield = \frac{Amount\ (mg)\ of\ the\ Isolate}{Amount\ (mg)\ of\ the\ crude\ extract} \times 100 \qquad \ldots\ldots 2$$

3.6 Determination of Antimicrobial Activity of the Isolates

Both antibacterial and antifungal activity tests of the isolated compounds were conducted in an *in vitro* assay.

3.6.1 Antibacterial Activity Test

Nine isolates C_1—C_8 were screened for the *in vitro* antimicrobial efficacy using microdilution method according to Clinical and Laboratory Standards Institute (CLSI) of 2015 with some few modifications. Briefly, the bacterial growth culture was prepared by dissolving 38 g of MHB in 1liter of distilled water then boiled to dissolve. The medium was then sterilized in the autoclave at temperature of 121 ^{o}C and pressure of 1034.214 mbar for 15 minutes. Bacterial standard strains were mixed with the broth using a sterile wire loop. Gentamicin of concentration 0.1 mg/ml was used as a positive control for standard drug while sterile distilled water was used as negative control against the bacterial strains tested.

3.6.1.1 Determination of Minimum Inhibitory Concentration

A volume of 100 microlitre of sterile distilled water was poured into the twelve (12) wells of microtitre plates. The same volume of each 10 mg/ml of compound isolates C_1 to C_8 dissolved in acetone (that is; 10 mg of the isolate in 1 ml of acetone) was added in the wells at serial dilution to the 10^{th} well. Well number 11 was not treated with any isolate and considered as the positive control for bacterial growth. One hundred μL of the bacteria were inoculated into the wells number 1 to 11. Plates were then incubated overnight at a 37 ^{o}C. After which, 100 μL of 0.2 mg/ml of

Iodonitrotetrazolium violet (INT) were added in all twelve wells of microtitre plates so as to reveal the status of bacterial inhibition.

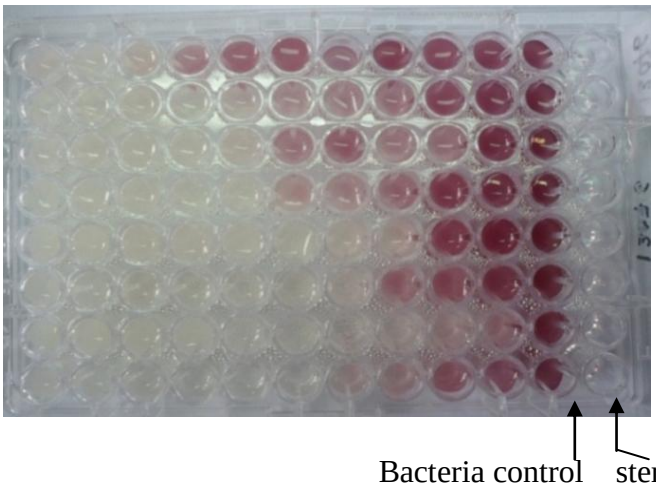


Figure 8: Microtitre plate determining MIC

Inhibition mechanism a Positive control: Gentamicin is a bactericidal antibiotic that works by irreversibly binding the 30S subunit of the bacterial ribosome, interrupting protein synthesis (Milanesi and Ciferri, 1996).

3.6.2 Antifungal Activity Test

This test involved the isolates C_1, C_4, C_5, C_7 and C_8 against a standard strain of *Candida tropicalis* (ATCC 13803). Isolates C_2, C_3, C_6 and C_9 were not tested for antifungal activity since were isolated in very small quantity and got finished at antibacterial activity test. The agar well diffusion method with reference to Perez, *et al.,* (1990) was adopted in which the Sabouraud Dextrose Agar (SDA) was used. A 65 g of the agar were suspended into 1litre of distilled water and brought to boil to dissolve completely. The solution was then sterilized by autoclave at 121^{o}C for 15minutes. A standard drug, Ketoconazole at 20mg/ml in sterile water was used as positive control for the antifungal activity of the isolated compounds.

3.7 Sample Analysis

The chemical structures for the isolates were studied and separated using chromatographic techniques and analyzed by nuclear magnetic resonance (NMR) technique. The ^{1}H and ^{13}C NMR spectra were recorded using deuterated chloroform ($CDCl_3$) on a Bruker 400 MHz ultra shield machine at Stockholm University (Sweden).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Phytochemistry Results of Isolates

A total of 1.99% and 6.25% of crude extract by weight were obtained from stem wood and stem bark respectively. The percentage yields of the pure compounds (C_1 to C_8) are summarized in Table 1 below;

Table 1: Percentage Yield by Mass of Isolated compounds

Compounds	%composition
C_1	2.23
C_2	0.58
C_3	0.75
C_4	3.76
C_5	6.11
C_6	0.75
C_7	8.25
C_8	1.61

The characteristics of isolated compounds are as shown in Table 2. The stem wood of *S. glaucescens* contains a total of six (6) less polar compounds of which one was Ultra violet (UV) negative and the rest five being UV positive. The UV Positive test indicates a conjugation system in their moiety whereas the UV negative do not. Two stem wood compounds were brightly fluorescing green and blue (labeled f_g and f_b respectively). Their fluorescence can be explained with their behaviour in energy absorption and emission. During their stabilization they emit energy in the visible region whose wavelength is equivalent to that of green and blue light respectively.

Table 2: Quantification and Characteristic Features of Isolates

	Other features			Solubility			
Isolate	**Mass (mg)**	**UV Test**	**Appearance**	DCM	MeOH	50%$CHCl_3$/ MeOH	$CHCl_3$
C_1	18.8	+ve, F_b	Brown-waxy	✓	⁎⁎white ppt	✓	✓⁎
C_2	14.1	-ve	Oilish yellow	✓	⁎⁎	✓⁎	✓⁎
C_3	19.6	+ve	Brown solid, colourless (in liquid)	✓	✓⁎	✓⁎	✓⁎
C_4	19.0	+ve	Dirt brownish	✓	✻ milky	✓⁎	✓⁎
C_5	18.8	+ve	Red-brown, oily	✓	⁎⁎	✓⁎	✓⁎
C_6	16.8	+ve	Dirt brownsolid	✓	⁎⁎	✓⁎	✓⁎
C_7	16.7	+ve	Dirt brownsolid	✓	⁎⁎	✓	✓⁎
C_8	15.6	+ve	Yellowish crystalline	✓	⁎⁎	✓⁎	✓⁎
C_9		+ve	Colourless crystalline	✓			

Key

✓ Highly soluble ✓⁎ Soluble ✻ Slightly insoluble ⁎⁎ Insoluble

F_b- Fluorescing blue

4.1.2 An Attempted NMR structure elucidation of compounds C_6 and C_9

Among the compounds that were isolated, partial spectroscopic data were obtained for only two compounds (C_6 and C_9). However, the complete structures could not be confirmed due to insufficient spectroscopic data that were available at the time of writing this thesis, in particular the lack of MS and 2D spectral data.

4.1.2.1 Compound C_9 with a Lupeol Acetate backbone

^{1}H and ^{13}C NMR (Appendix 3 and 4) established the isolated compound to have a lupeol acetate backbone. The ^{1}H NMR indicated a *doublet at* 5.25 ppm which is due to C_3, *singlet* signals at 4.75 and 4.58 due to 2H attached to terminal double bond protons at C_{29}. The *dd* at 4.167 (J 1.72 and 1.54) is due to proton at C-3 which attached at the chiral centre, this carbon renders protons at C_2 unequivalent therefore rendering them at different chemical shifts (δ). Most protons showed signals in the shielded region between $^{\delta}$ 0.5 and 1.75. When the NMR data for C_9 (Table 3) were compared with literature (Sobrinho *et al.,* 1991 and Jamal *et al*., 2008), it appeared to have several correlation features with lupeol acetate, thus indicating the compound to possess a lupeol backbone. The presence of the compounds with lupeol functionality in dichloromethane extracts was first reported by Mabiki (2011). The lupeol compound was reported in euphorbeaceae by Kapingu *et al.,* (2012) from the forest fever berry (*Croton sylvaticus* Hoechst) and in many other plant species by Wal *et al.,* (2015).

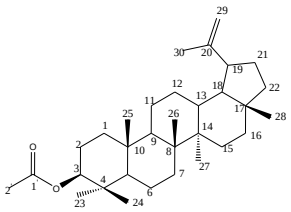


Figure 9: Lupeol acetate

Table 3: ^{13}C NMR Data for Isolate C_9

Experimental		Literature (Sobrinho *et al.*, 1991and Jamal *et al*., 2008)
Carbon #	δppm	δ ppm
2`	173.0	171.0
20	150.0	150.39
3	130	__
29	109.17	109.27
4	61.67	__
5	55.0	55.25
9	50.0	50.26
18	48.33	48.21
19	43.0	42.73
8	41.0	40.77
12	40.0	__
1	39.8	38.56
4	38.0	37.68
10	37.0	36.98
16	36.0	35.5
7	34.0	34.17
18	33.9	__
19	32.0	__
21	29.9	29.75
23	27.8	28.0
15	27	27.38
23	25	__
2	23	23.61
11	21.5	20.61
30	20	19.24
6	18	17.97
25	16	16.14
27	14.0	14.46

4.1.2.2 Isolate C_6; a Fatty Acid Derivative

^{1}H and ^{13}C NMR (Appendix 1 and 2) established the isolated compound to have an aliphatic fatty acid moiety. The ^{1}H NMR spectrum indicated a weak *singlet* 9.75ppm. This signal is a characteristic of acidic proton. It is asserted that in most cases, acid proton chemical shifts for fatty acid usually don't appear and if they appear, it is expected at around 10-13ppm. However, the chemical shift value lowers as the carbon chain increases, 2-*triplets* at 2.33ppm which indicate protons due to α-C, and 0.88 ppm is a characteristic of the ^{1}H of terminal C, while the literature range is 0.8- 0.9 ppm while the signal of the methylene (CH_2) protons at 1.2–1.4 ppm (Knothe and Kenar, 2004). The ^{13}C NMR displayed signals at 179 ppm, indicating the presence of the terminal CH_3-group.The literatures suggest it as the characteristic of an aliphatic chain of fatty acid. Most C-signals appeared in the shielded region between $^{\delta}$ 0- 30 ppm. These included; 34, 32, 30, 24, 22, 14 and 1 ppm.

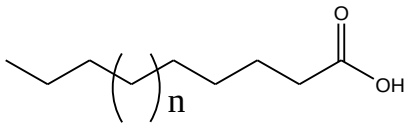


Figure 10: Aliphatic fatty acid

n= number of -CH_2 in the chain

Therefore, from the spectra data compound C_6 suggested to be an aliphatic fatty acid. However, the degree of carbon chain could not be confirmed due to lack of important data from the mass spectrometry.

4.2 Bioactivity Results of isolates

4.2.1 Antibacterial Efficacy

All eight (8) isolates used in this study inhibited the growth of both Gram positive and Gram negative standard bacterial strains at various MIC levels that ranged between 5.0 mg/ml and 0.01 mg/ml. Isolate C_7 demonstrated the highest activity of 0.01mg/ml against *A. baumannii* while the same concentration was demonstrated by both C_7 and C_8 against *S. enterica* (Table 4). The least activity was observed in isolate C_1 against *E. faecalis.* Thus, *S. glaucescens* pax demonstrated its potential against both Gram positive and Gram positive bacteria as well as the fungal infection (Table 4, Table 5 and Table 6 respectively).

Table 4: MIC for Isolates C1 to C8 against Gram Negative Standard Bacterial Strains

		MIC (mg/ml) On Gram Negative Bacteria Standard Strains								
STRAIN NAME	CODE	C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	Positive Control
E. coli	ATCC 25922	0.625	0.156	0.313	0.313	0.156	0.313	0.078	0.313	0.001
A. baumannii	ATCC 19606	0.625	0.625	0.625	0.313	0.156	0.078	0.010	0.020	0.001
S. enterica	ATCC 13076	0.156	0.039	0.020	0.039	0.039	0.039	0.010	0.010	0.001
P. aeruginosa	ATCC 27853	2.500	2.500	1.250	0.625	0.625	0.156	0.625	1.250	0.002
S. paratyphi	ATCC 9150	1.250	2.500	0.313	0.078	0.625	0.078	0.313	0.313	0.025
E. faecalis	ATCC 51299	5.000	ND	ND	0.625	ND	0.078	0.039	0.625	0.025
P. mirabilis	ATCC 12453	1.250	ND	ND	0.313	ND	0.156	0.039	0.078	0.003

Key ATCC- American Type Culture Collection ND- not tested

MIC – minimum inhibitory concentration

Table 5: MIC for Isolates C_1 to C_8 against Gram Positive Standard Strain

STRAIN NAME	CODE	C2	C3	C5	C_1	C_4	C_6	C_7	C_8	Positive control
S. aureus	ATCC 25923	2.5	0.625	0.156	0.625	0.156	0.156	0.078	0.313	0.005
K. pneumoniae	ATCC 13883	ND	1.25	0.156	0.625	0.313	0.313	0.156	0.156	0.001
E. cloaca	ATCC 23355	ND	ND	ND	0.625	0.625	0.313	0.078	0.020	0.016
S. sonnei	ATCC 25931	ND	ND	ND	0.625	0.156	0.078	0.078	0.156	0.003

Key ATCC- American Type culture collection ND- not tested

MIC – Minimum Inhibitory Concentration

From Table 4 above, the variations in the MIC values could be explained by the chemical nature (structure) of the compounds themselves as well as the morphological (cell wall) adaptation of the strain itself. Though, it was difficult to really tell that there were structural differences if elucidation was incomplete.

The extent the drug penetrates the pathogen's cell accounts for the effectiveness of it (Matsumoto *et al.,* 2001). Some bacteria like *Salmonella spp* are highly affected by the alkaline pH, which suggests for alkaline functionality in the isolated compounds (Mendonca, 1994). The previous studies on crude extracts by Mabiki (2011, demonstrated the lowest sensitivity of compounds from *S. glaucescens* against *S. typhimurium* demonstrated the highest resistance of all tested microorganisms. It was suggested that the resistance is attributed by the presence of resistant genes like PSE and CARB-type. Therefore, this information may imply the absence of such genes in the *S. enterica* (ATCC 13076). The possible activity mechanism is as discussed on chapter four (section, 4.3).

4.2.2 Antifungal Activity on *Candida tropicalis*

Candida tropicalis is among the fungi species that cause more than 90% of invasive fungal infections. However, the relative prevalence of the species depends on the geographical location, patient population, and clinical settings (MacCallum, 2012) as cited by Spampinato and Leonardi, (2013). Candidiasis infections due to *C. tropicalis* have increased dramatically on a global scale. It is thus proclaiming this organism to be emerging pathogenic yeast (Spampinato and Leonardi, 2013).

The isolates were inhibited the growth of the *C. tropicalis* at different levels (Table 6) in the Agar well diffusion method. The inhibition from some compounds was higher than that of standard drug as shown in Table 6 below. This can be explained by their respective chemical structure relationship.

Table 6: Inhibition Zones against *Candida tropicalis*

Isolate		Standard
C_1	13	14
C_4	15	14
C_5	19.5	14
C_7	27	14
C_8	13.5	14

The fungal cell wall is a unique organelle that fulfills the criteria for selective toxicity. Among the feature put into account when fighting fungi/fungal infections involves several considerations that include inhibition of the cell wall formation and cell membrane disruption. It involves inhibiting cell division by targeting the microtubule responsible for forming the mitotic spindle or by inhibiting DNA transcription.

According to Spampinato and Leonardi (2013), most antifungal agents targeted to ergosterol binding or biosynthesis does not cross-react with host cells. Despite all of the isolated compounds being active against *C. tropicalis,* again Isolate C_7 (27 mm) was found to be the most active followed by C_5 while C_1 (13 mm) was the least active. Isolates C_4, C_5, C_7 and C_8 were more active than the standard drug (Ketoconazole). This suggests that they possess significant antifungal properties and thus a systematic structural elucidation can suggest a new antifungal lead.

4.3 Structure - Activity Relationship for Isolate C_6 and C_9

Biological activities of compound are said to be related to their molecules structures and shape (Nitiema *et al.,* 2012, Desbois and Smith, 2010). Understanding the chemistry of the bioactive compounds is of vital importance in the field of natural products and drug discovery (Tiang and Shuo, 2006). In order to make their remedies acceptable, a scientific evaluation of these bioactive natural products and identifying the active principles as well as their mechanism of action is important (Wal *et al.,* 2015). For instance, the lupeol acetate has bacteriastatic and fungastatic properties such that it is a component in the drugs used to control bacterial and fungal infections. The lupeol, a triterpene as a parent chain, as well as the lupeol acetate (a derivative) have served in the treatment of various bacterial infections from *E. coli*, *Bacillus* and *Staphylococcus* species (Wal *et al.*, 2015). Fatty acids on the other hand are known to possess antibacterial, antimalarial and antifungal activities (Pohl *et al.,* 2011). The essential fatty acids are reported to have shown diverse medicinal properties and potential beneficial effects on diseases. Melariri *et al., (*2012) have reported such these diseases are such as cancer, insulin resistance, skin permeability, cardiovascular

disease and depression. It is suggested that their antimicrobial activities is attributed to long-chain unsaturated fatty acid (C16 - C20). Examples include palmitoleic, oleic and linolenic acids, etc (El Shoubaky and Salem, 2014). It is further explain that the mechanism of antibacterial activity of fatty acids is a function of the length of the carbon chain and the presence, number, position and orientation of double bonds. In addition to this, the -OH group on the carboxyl group seems to be important for the antibacterial activity thus suggesting for the MIC values in both antibacterial and antifungal efficacies for C_6 and C_9 isolates studied here.

Antibacterial and antifungal structure – activity relationship of other isolates (C1, C2, C3, C4, C5, C7 and C8 could not be explained as their chemical structures were not established.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The study aimed at studying the bioactivity and structural properties of the pure compounds isolated from the stem of *S. glaucescens* and therefore to advance the biochemical knowledge and value on the plant itself. It also focused on modifying the traditional knowledge of the utilization of the plant extract against various infections in Tanzanian communities .The isolated compounds though not fully characterized, showed to be active against tested bacterial and fungal standard strains similar to reported activities in crude extracts. This study has shown that *S. glaucescens* stem contains a good number of compounds including those with Lupeol acetate moiety and the aliphatic fatty acids. However, structures of isolated compounds could not be confirmed at the molecular structure level due to lack of some spectroscopic data like MS, IR and melting points.

5.2 RECOMMENDATION

Further characterization, structural elucidation of unidentified compounds and an *in ovo* as well as *in vivo* assay studies are recommended. This will generate baseline information for comparing the displayed bioactivity of these compounds and thus adding value to the science of natural products and in the new drug discovery.

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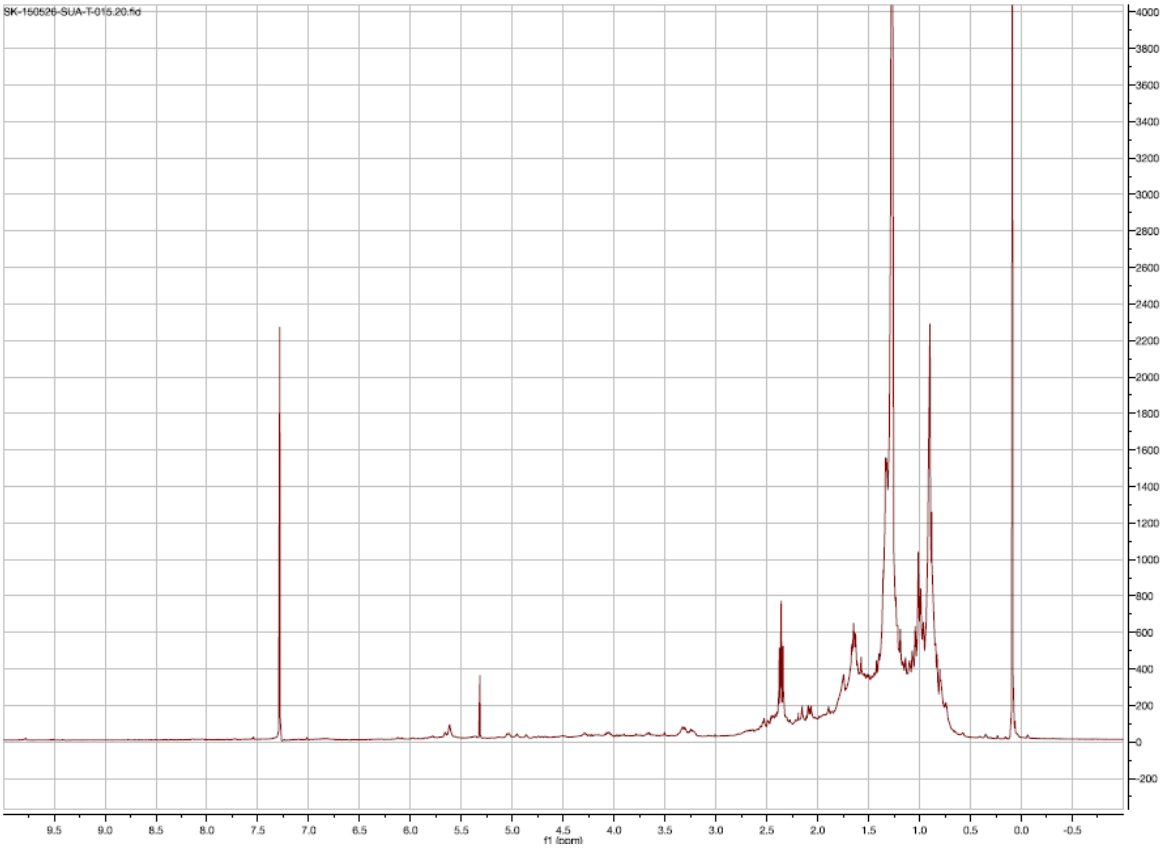
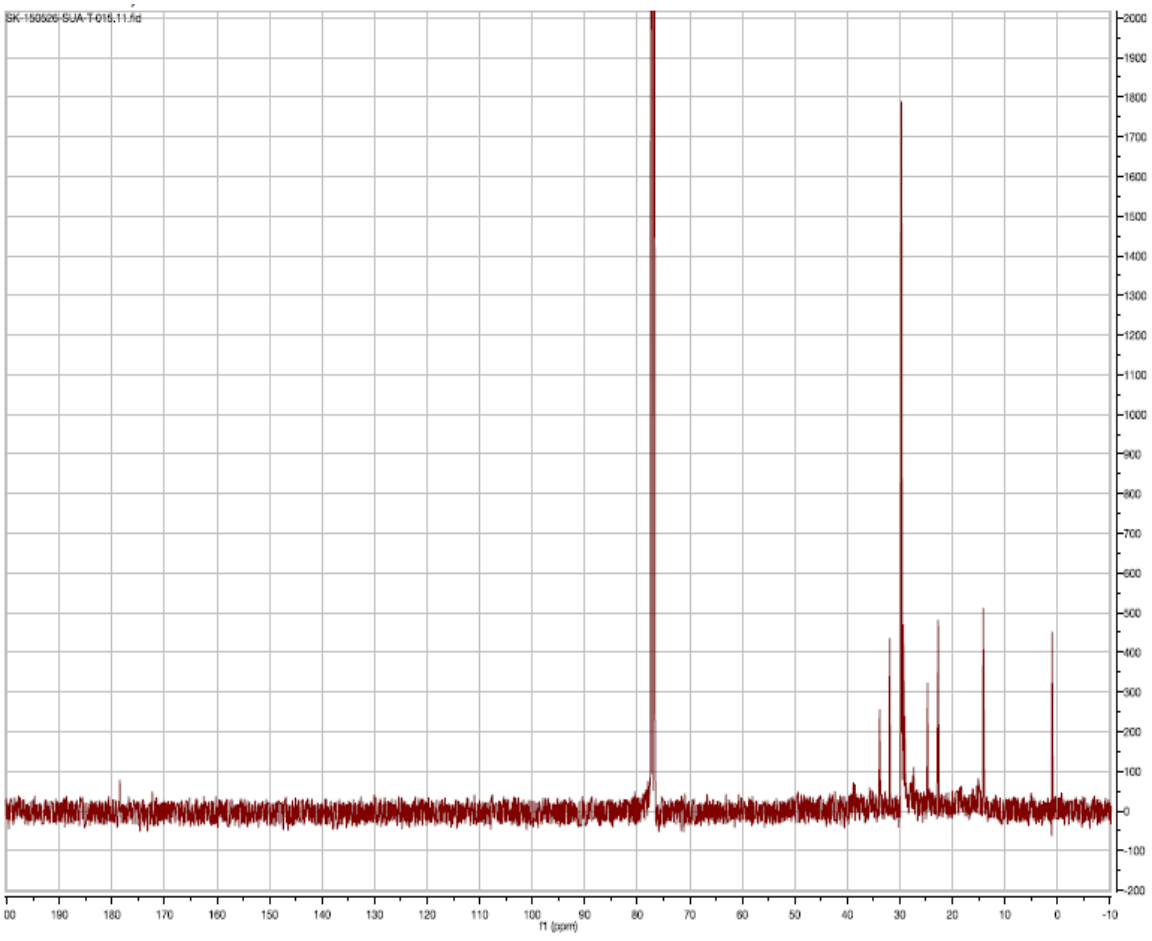
APPENDICES

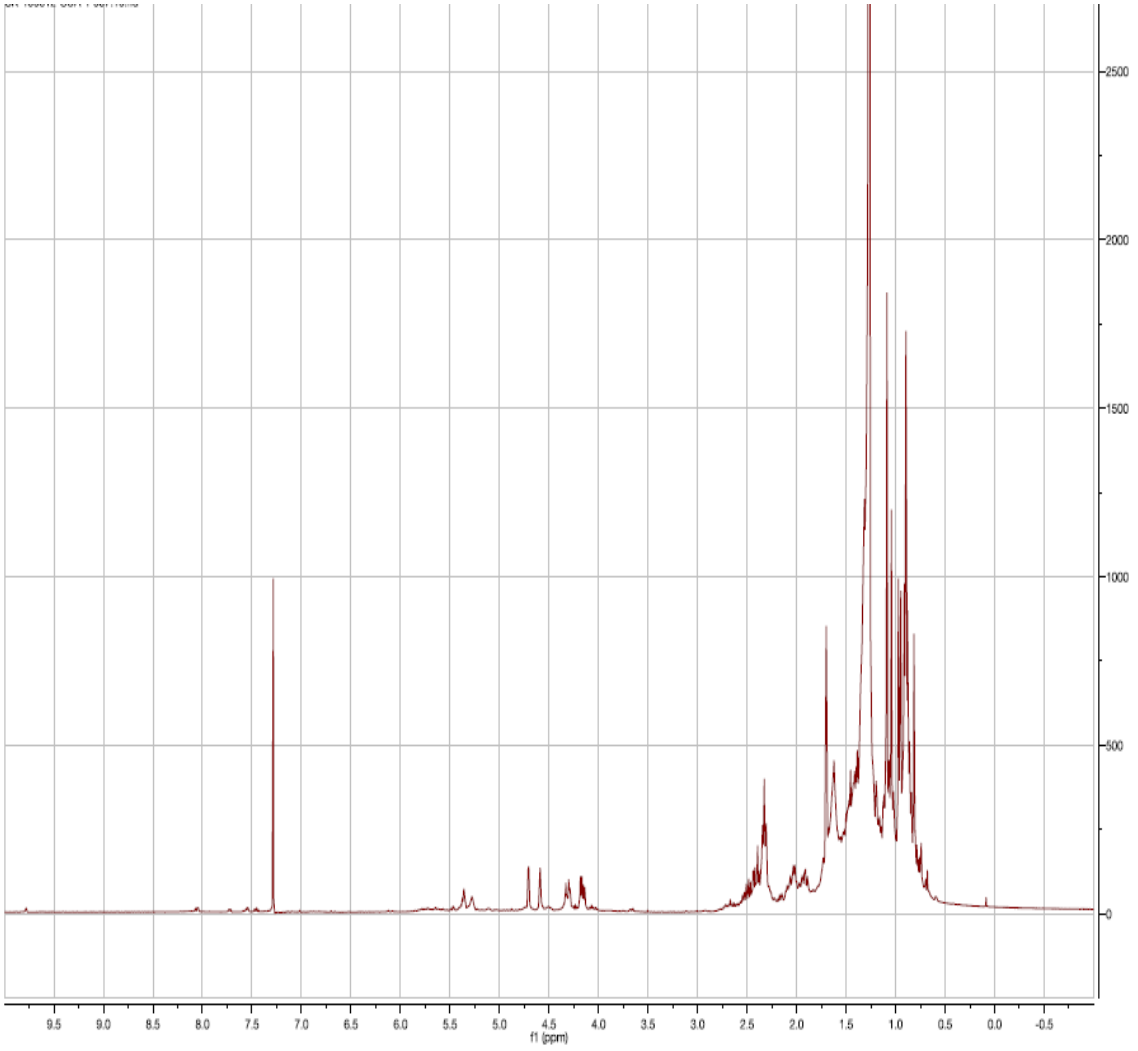
Appendix 1: ^{1}H NMR Spectrum for Isolate C_6



Appendix 2: ^{13}C NMR for Isolate C_6



Appendix 3: ^{1}H NMR for Isolate C_9



Appendix 4: ^{13}C NMR for Isolate C_9