

**BIOLOGICAL AND PHYTOCHEMICAL INVESTIGATIONS OF *SYNADENIUM*
GLAUDESCENS PAX (EUPHORBIACEAE)**

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**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF
AGRICULTURE. MOROGORO, TANZANIA.**

EXTENDED ABSTRACT

Synadenium glaucescens is a traditional medicinal plant used in Tanzania for management of various infections and diseases affecting animals including human. The diseases treated include HIV, TB and East Coast fever among others. Despite the recorded traditional utilization, limited scientific information exists regarding its biological and phytochemical importance. This current study, therefore aimed at investigating its toxicological, pharmacological and phytochemical properties. Toxicological studies involved the assessment of dermal toxicities using guidelines stipulated in the Organization for Economic Cooperation and Development (OECD). Acaricidal activity was evaluated using Larval and Adult immersion tests as described by Drummond. In pharmacological assays, an in vitro HIV-1 reverse transcriptase (RT) was used for screening of anti-HIV activity using a Roche HIV kit while chromatographic and spectroscopic techniques were used in phytochemical studies.

Irritation indices from dermal toxicity studies ranged between 3.2 and 0.05. According to Draize, these indices range are considered as mild to moderate irritancy since none of them could reach Primary Irritation Index (PII) of 5 or above which is classified as irritant. On the other hand, acute dermal toxicity tests showed no overt signs of toxicity. Similarly the extracts did not produce any sensitization reaction. Acaricidal studies showed low larvicidal (corrected mortality 37.5%) and adulticidal (corrected mortality 33.33%, LC₅₀ 666.91) activities respectively for methanol and ethanol extracts from leaves. Other extracts of this plant were inactive in these tests.

Anti HIV tests indicated aqueous root and leaves together with dichloromethane extracts from roots to have high activities with IC₅₀ values of 3.96 µg/mL, 6.04 µg/mL and 11.43

$\mu\text{g/mL}$ respectively. Ethyl acetate and methanol extracts showed low effectiveness in the inhibition of HIV-1 RT as indicated by their very high values of IC_{50} relative to positive control. From the phytochemical studies, four compounds namely euphol and erythrinacinate C from the root barks and β -sitosterol and octacosanol from the leaves were isolated. All compounds have been reported from other plant species but are isolated from this plant species for the first time.

The findings showed that dried extracts are dermally safe while possessing potential anti HIV activities. The extracts also shows to possess diversity of compounds that could be responsible for anti HIV activities. Further investigations are however, recommended for this plant species especially on anti HIV activities and phytochemistry for discovery of drug leads.

DECLARATION

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

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DEDICATION

My wife Kissa Ackson Kales and my children; Brian, Baraka, Beatrice and Belicia

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THESIS ORGANIZATION

The thesis has been developed in “published papers format” and comprises three chapters. The first chapter consists of the introduction of the overall study. The chapter describes the commonality of the concepts in the separate manuscripts. Chapter two contains original published papers and those intended for publication in different journals. The third chapter consists of general discussions and conclusion. The arrangement of the papers follows the arrangement of the objectives.

LIST OF PAPERS

- Paper I:** Assessment of Dermal Irritation and Acute Toxicity Potentials of Extracts from *Synadenium glaucescens* on Healthy Rabbits, Wistar Albino Rats and Albino Mice. Vitus A. Nyigo, Robinson H. Mdegela, Faith P. Mabiki and Hamisi M. Malebo. *European Journal of Medicinal Plants* 10(4): 1-11, 2015.
- Paper II:** Evaluation of acaricidal efficacy of *Synadenium glaucescens* (euphorbeaceae) against cattle ticks of *boophilus* species. Vitus A. Nyigo, Robinson H. Mdegela, Hamisi M. Malebo, Faith P. Mabiki and Gerda Fouche. *Journal of Medicinal Plant research* 10(21): 278-285, 3 June, 2016.
- Paper III:** Studies on anti-HIV activity of extracts from *Synadenium glaucescens* (pax). Vitus A. Nyigo, Rudzan Nthambelen, Robinson H. Mdegela, Faith P. Mabiki, Hamis M. Malebo and Gerda Fouche. Planned for submission to the *African Journal of biotechnology*.
- Paper IV:** Isolation and identification of euphol and β -sitosterol from the dichloromethane extracts of *Synadenium glaucescens*. Vitus A. Nyigo, Xolan Peter, Faith P. Mabiki, Hamis M. Malebo, Robinson H. Mdegela and Gerda Fouche. *Journal of Phytopharmacology*, 5 (3): 100-104, 2016.
- Paper V:** Isolation and identification of Long chain aliphatic compounds from the dichloromethane extracts of *Synadenium glaucescens*. Vitus A. Nyigo, Xolan Peter, Tlabo Leboho, Hamis M. Malebo, Faith P. Mabiki, Robinson H. Mdegela and Gerda Fouche. Planned for submission to the *Journal of Phytopharmacology*.

LIST OF ABBREVIATIONS

AFNET	African Natural Products Training Network
AIT	Adult Immersion Test
CC	Column Chromatography
COSY	Correlated Spectroscopy
CSIR	Council for Scientific and Industrial Research
DARAS	Department of Animal, Aquaculture and Range Sciences
DCM	Dichloromethane
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
dUTP	deoxyuridine Triphosphatase
ELISA	Enzyme Linked Immunosorbent Assay
EMR	Electro Magnetic Radiation
EtOH	Ethanol
GC	Gas Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
LIT	Larva Immersion Test
MeOH	Methanol
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
OECD	Organization for Economic Cooperation and Development
RF	Radio Frequency
RT	Reverse Transcriptase

SUA	Sokoine University of Agriculture
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

The use of plants in the treatment of animal diseases and control of agricultural pests dates back to time immemorial (Fang Ji *et al.*, 2009; Petrovska, 2012). In human, traditional herbal medicines are used for treatment of various ailments including different kind of fevers, epilepsy, asthma, malaria and skin disorders among others. In other animals such as cattle the common diseases treated with medicinal plants include mastitis, East Coast Fever (ECF), anaplasmosis and trypanosomiasis (Laudato and Capasso, 2013; Mabiki *et al.*, 2013). In chicken the common diseases include Newcastle disease, Infectious Bursal Disease, Fowl Pox and Fowl typhoid among others (Laudato and Capasso, 2013; Mabiki *et al.*, 2013).

It is from these varied medicinal potentials of plants that generations have accumulated different experiences and knowledge of their use. The use and efforts for its development however, in the past was discouraged by the modern science which regarded methods of traditional knowledge as primitive and backward (Abdullahi, 2011). Consequently many modern health practitioners continued turning away from their traditional counterparts despite their contribution to meeting the basic health needs of majority of the population, especially the rural people in developing countries. It is until recent years especially in the last decade when traditional medicines through the use of herbal drugs regained popularity with many countries worldwide returning back to the use of herbal medicines in well processed products.

It is also important to note that, despite the therapeutic values that traditional medicine have been providing for centuries, many inherent negative effects accompany the use of

traditional medicine. These include, toxicity of some of the plants, presence of toxic contaminants resulting from entire processing of medicinal plants (Chan, 2003) or deliberate adulterations with modern medicines by unfaithful practitioners (Ernst, 2002) or heavy metals (Lynch and Braithwaite, 2005). Worldwide, the adulteration of herbal medicines with synthetic drugs or heavy metals is a potentially serious problem which needs to be addressed by adequate regulatory measures. Thus, in their review of Asian traditional medicines, Lynch and Braithwaite (2005) reported a deliberate additions of heavy metals such as lead, mercury and arsenic probably on therapeutic purposes. On the other hand, Ernst, 2002 reported an adulteration of Chinese herbal medicines with synthetic drugs such as mefenamic acid, diazepam, prednisolone and indometacin. The clinical effects were in some case serious: somnolence, massive gastro-intestinal bleeding, Cushing's syndrome, diabetes, hypertension, arrhythmia.

In some cases, medicinal plants can be mistakenly confused with toxic plants. Vanherweghem (2008) reported that at least 100 cases of extensive interstitial fibrosis of the kidneys were observed in Belgium and France in women who followed a weight-loss regimen that included the use of Chinese herbs unintentionally taken as *Stephania tetrandra*. After investigations it was found that in fact the plant was not *Stephania tetrandra*, but inadvertently replaced by another Chinese herb, namely *Aristolochia fangchi*.

1.1 Role of Traditional Medicinal Plants in the Development of Human

Medicines

Despite the negative attitudes of the herbal treatment developed by modern science, the contribution of traditional medicines in the development of the modern medicines cannot be over emphasized. Thus development of science in drug discovery which

revolutionalised the control of human and agricultural diseases has in most cases relied on these accrued experiences and knowledge from traditional medicines. Many different chemicals and formulations have been developed from plants as pharmaceuticals and as pesticides (Table 1).

Quinine (**1**) is one of the most important anti-malarial drug isolated from *Cinchona* barks in 1820 (Achan *et al.*, 2011). This drug is still one among the current most popular anti-malarial drugs for complicated cases. Research efforts to obtain new anti-malarials still continue and in the advent, artemisinin (**2**) which is also an antimalarial agent from Chinese traditional herbal plant *Artemisia annua* was discovered in 1971 (Li and Wu, 1998; Liao, 2009; Tu, 2011). This drug is known for its rapid action as an anti-malarial and also for its low toxicity and efficacy against *Plasmodium falciparum*. Vincristine (**3**) and vinblastine (**4**) are indole alkaloids from plants and are very useful drugs for treatment of cancer. The compounds were isolated from *Catharanthus roseus* (L.) formerly known as *Vinca rosea* and Madagascan periwinkle. In folklore medicine, extracts of the leaves of this plant were reputed to be useful in the treatment of diabetes. In an attempt to verify the antidiabetic efficacy, serendipitously, the extracts led instead to the discovery and isolation of vinblastine and vincristine, which are used in the clinical treatment of a variety of cancers (Noble, 1990). Taxol (**5**) is another important natural product compound derived from plant for cancer medication that interferes with the growth of cancer cells and slows their growth and spread in the body. This compound was first isolated in 1969 from a pacific yew plant *Taxus brevifolia* through a bioassay guided approach and its structure was described in 1971 (Wall and Wani, 1995). This drug is most important for treatments of breast, lung and ovarian cancer.

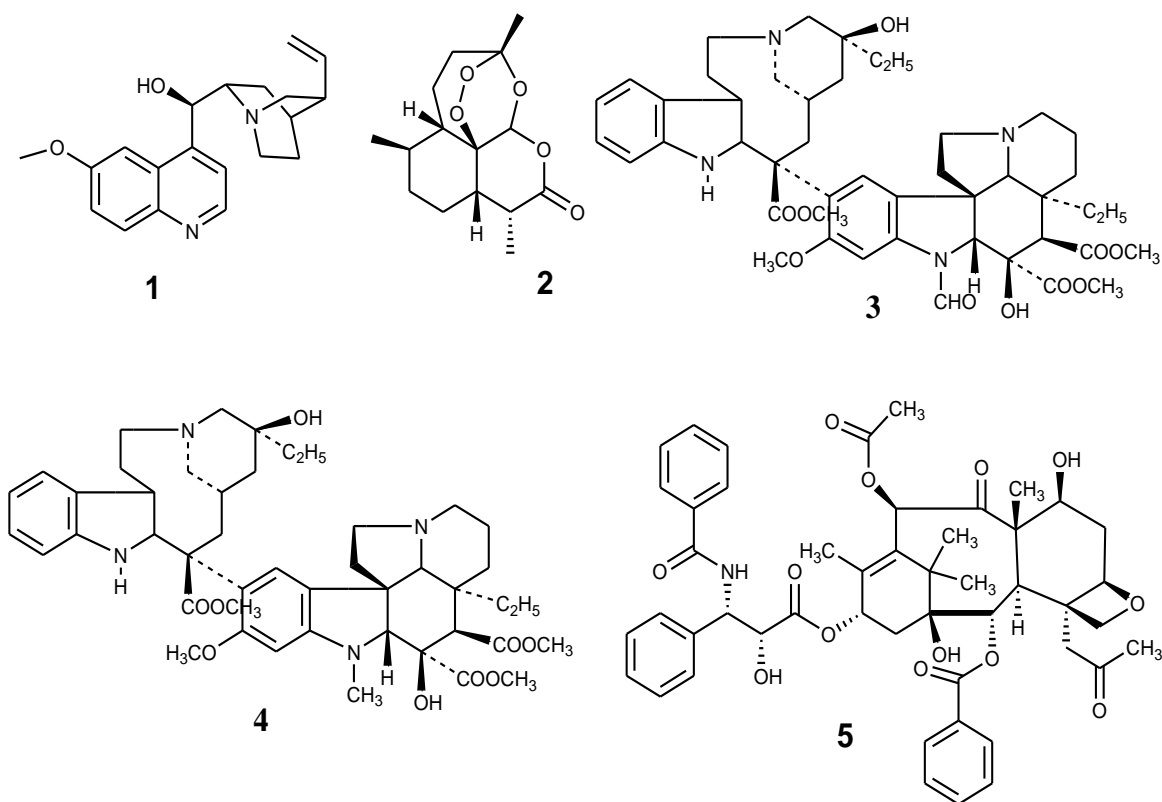
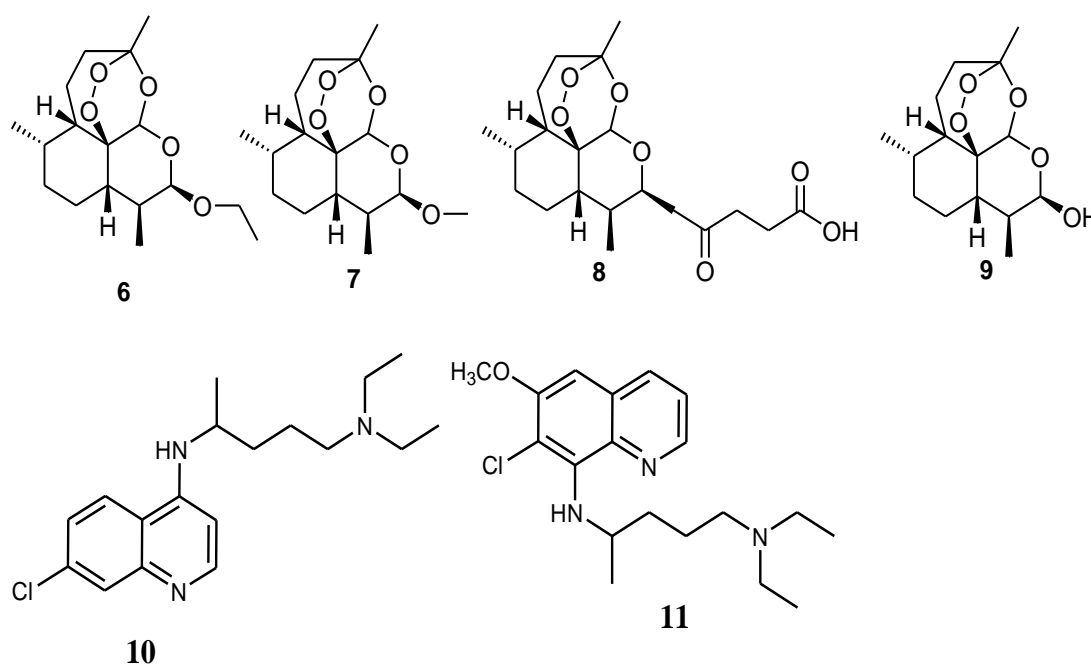


Table 1: Pharmaceuticals of Plant Origin

Drug/chemical	Action/Clinical Use	Plant species	References
Igoxin	Cardiotonic	<i>Digitalis purpurea</i> <i>Cephaelis</i>	<i>Fabricant, 2001</i>
Emetine	Amoebicide, emetic Sympathomimetic,	<i>ipeacacuanha</i>	<i>Fabricant, 2001</i>
Ephedrine	antihistamine	<i>Ephedra sinica</i>	<i>Fabricant, 2001</i>
Palmatine	Antipyretic, detoxicant	<i>Coptis japonica</i> <i>Cinchona</i>	<i>Fabricant, 2001</i>
Quinidine	Antiarrhythmic	<i>ledgeriana</i> <i>Cinchona</i>	<i>Fabricant, 2001</i>
Quinine	antimalarial, antipyretic	<i>ledgeriana</i>	<i>Fabricant, 2001</i>
<i>Berberine</i>	Anti-bacterial and protozoal	<i>Berberis vulgaris</i>	<i>Omulokoli, 1997</i>
Morphine	Analgesic	<i>Papaver somniferum</i>	<i>Fabricant, 2001</i>
Atropine	Anticholinergic	<i>Atropa belladonna</i>	<i>Fabricant, 2001</i>
L-Dopa	Anti-parkinsonism	<i>Mucuna sp</i>	<i>Misra and Wagner, 2007</i>
Taxol	Antitumor agent	<i>Taxus brevifolia</i>	<i>Wall and Wani, 1995</i>

1.2 Modification of Drugs

In the 19th century, scientists focused in the modifications of existing drugs in order to increase activity. Among the success of this strategy was the discovery of specific lactone reduction in artemisinin making it an important lead for synthesis of many derivatives that easily dissolve either in oil or water. Therefore, studies of the structure/activity relationship from this compound led to the discovery of arteether (**6**), artemether (**7**), artesunate (**8**) and dihydroartemisinin (**9**). Artemisinin and its derivatives are currently being used as effective antimalarial drugs in the fight against *P. falciparum* malaria in many malaria endemic countries around the world. Similarly, modification of quinine yielded other useful drugs such as chloroquine (**10**) and pamaquine (**11**) and both drugs are currently still in use while chloroquine is mainly being used for prophylaxis of *P. falciparum* malaria. Pamaquine is still used in preventing relapse of *Plasmodium vivax* malaria (Greenwood, 1995).



In the 1980s a need to synthesize many chemical compounds rapidly and at low costs produced a new branch of chemistry known as combinatorial chemistry. Two decades later, the world witnessed the advancement of this branch of chemistry which resulted in the production of significant amount of synthetic chemicals (Fang Ji *et al.*, 2009). This seemed to be industrially able to compete with natural product extracts and purified bioactive phytochemicals for High Throughput Screening resources and emerged as the preferred option. However, many of the resulting drug candidates produced through this system were not promising. Thus, the integration of combinatorial chemistry with other mechanisms for lead generation was then considered to be a viable strategy. On these bases, a combination of synthetic chemistry and natural product leads were found to be a good starting point for combinatorial chemistry for discovery of novel analogs (Topliss, 2002).

In general the synthetic and drugs of plant origins are acknowledged for significant contribution in the development of human health (Topliss *et al.*, 2002; Aktar *et al.*, 2009). However, diverse challenges still exists which include the emergency of multi drug-resistant micro-organisms, side effects of modern drugs, emerging diseases for which no medicine is available, inaccessibility and non-affordability (Mendis *et al.*, 2007; Aktar *et al.*, 2009). Consequently, traditional medicines has regained popularity with time, evidenced by diverse human efforts in search of new alternative chemical ingredients which are more efficacious, safe and affordable to poor communities. Plants still emerge as the major targets and have so far offered a significant contribution to mankind in terms of both human health and agricultural development.

1.3 Role of Medicinal Plants in the Control of Animal Diseases and Agricultural Pests

The value of medicinal plants utilizations is not only limited to human, but also in agriculture through management of livestock diseases and agricultural pests. Pests and diseases are major constraints of the agricultural production because they lower productivity. The major concern of the farmers is optimal production hence controlling the effects of pests and diseases are important step to meeting this desire.

Despite the fact that scientific investigations of veterinary traditional practices is not old, there is significant information regarding the use of plants for animal disease treatment which in most cases is based on self-medication common in animals such as chickens, sheep, butterfly and chimpanzee (Grade, 2009). The plants that are eaten by sick animals are likely to be rich in natural products mainly comprising of tannins and alkaloids which are known to have antiviral, antibacterial, antifungal and anthelmintic properties (Cowan, 1999; Hintz *et al.*, 2015). In the recent years, more efforts are being put in search of drugs for control of livestock diseases through management of parasites responsible for transmission of various diseases. The management of ticks in cattle for example is of vital importance. On the other hand, several compounds used for control of insects and agricultural pests have been developed from plants (Table 2).

Table 2: Pesticides of Plant Origin

Drug/chemical	Action/Clinical Use	Plant species	References
Rotenone	Insecticide	<i>Lonchocarpus nicou</i> <i>Chrysanthemum</i>	<i>Duke 1990</i>
Pyrethrins	Insecticide	<i>Cinerariaefolium</i>	<i>Duke 1990</i>
Nicotine	Insecticide	<i>Nicotiana tabacum</i>	<i>Duke 1990</i>
Sabdilla		<i>Schoenocaulon</i>	
Alkaloids	Insecticides	<i>officinale</i>	<i>Shivanandappa and Rajashekar (2014).</i>
Ryania	Insecticide	<i>Ryania speciosa</i>	<i>Shivanandappa and Rajashekar (2014).</i>
Azadirachtin	Insecticide	<i>Azadirachta indica</i>	<i>Shivanandappa and Rajashekar (2014).</i>

1.4 Family *Euphorbeacea*

This is a complex heterogeneous family consisting of approximately 322 genera and 8900 species around the world (Balakrishnan and Chakrabarty, 2007). Plants in this family are distributed in the temperate, sub-tropical and tropical regions all around the globe. The major characteristics of plants in this family including those of genus *Synadenium* is the frequent occurrence of plants with poisonous milky sap which may cause inflammation and a blistering rash in the incidences of skin contact.

For example, white mangrove or blind-your-eye mangrove latex causes blistering on contact and temporary blindness if in contact with the eyes (Balakrishnan and Chakrabarty, 2007). The latex of spurge has been used as a laxative. A number of plants of the spurge family are of considerable economic importance. Famous plants in this family include cassava (*Manihot esculenta*), castor plant (*Ricinus communis*), Barbados nut (*Jatropha curcas*) and the Para rubber tree (*Hevea brasiliensis*). Many are grown as ornamental plants, such as poinsettia (*Euphorbia pulcherrima*) while theefy spurge (*Euphorbia esula*) and Chinese tallow (*Triadica sebifera*) are invasive weeds in North America. In medicine, some species of *Euphorbiaceae* have proved to be effective against genital herpes' (Charles *et al.*, 2007). Plant species of the family euphorbiaceae are reported to contain a number of interesting biologically active compounds, some possess irritant or poisonous properties, antitumour as well as cytotoxic active constituents (Ahmad *et al.*, 2006). Euphorbiaceae species are also well known to contain tumor promoting constituents (Vogg *et al.*, 1999).

1.5 Genus *Synadenium*

1.5.1 Some Recorded Traditional Uses of Plants in the Genus *Synadenium*

Plants in this genus are recorded for various traditional utilizations by different communities in the world where root and leaves are plant parts that have found high utilization. In some incidences the use of latex alone has been witnessed. Table 3 indicates some of the ethno-medical utilization of some plants of the genus *Synadenium*.

Table 3: Ethnomedicinal Uses and Practices of some Species in the Genus *Synadenium*

Species	Plant Part	Ethnomedicinal use and Practice	References
<i>S. angolense</i>	Root	Infusion used in adult human for treatment of tuberculosis, stomach aches, dropsy urinal-genital systems and during child births.	Bossard, 1993
<i>S. cupulare</i>	Latex	Latex used to treat wounds by rubbing on external wounds.	Arnold and Gulumian, 1984
<i>S. compactum</i>	leaves	Dried and boiled leaves used to treat weakening bones and anaplasmosis. Boiled roots are used to treat theilerosis while soaked barks are used for ectoparasites.	Njoroge and Bussmann, 2006
<i>S. glaucescens</i>	Dried roots	Burned mixtures of dried roots, snake bones and python and baboon skin in petroleum jelly is used to treat paralysis by applying on incision made on paralyzed part.	Hedberg <i>et al.</i> , 1983
	dried latex from leaves and stem	Used as fish poison, thus used in fishing. It is also used for treatment of boils in human when taken orally. The latex is also used as a drastic purgative.	
	Dried root	Treatment HIV, TB and east coast fever.	
	Fresh leaves	Juice from fresh leaves is used for treatment of excessive menstruation.	
	Dried roots	Dried roots used for treatment of severe coughs.	
<i>S. umbellatum</i>	Latex	Treatment of diabetes mellitus, Hansen's disease, trypanosomiasis, leukemia and several malignant tumors.	Ortencio, (1997), Da Mota <i>et al.</i> , 2012
<i>S. grantii</i>	Latex	Neoplastic disease and gastric disorders such as peptic ulcers and gastritis. It is prepared by adding 18 drops of latex to 1 liter of water.	Costa <i>et al.</i> , 2012, Ortencio <i>et al.</i> , 1997

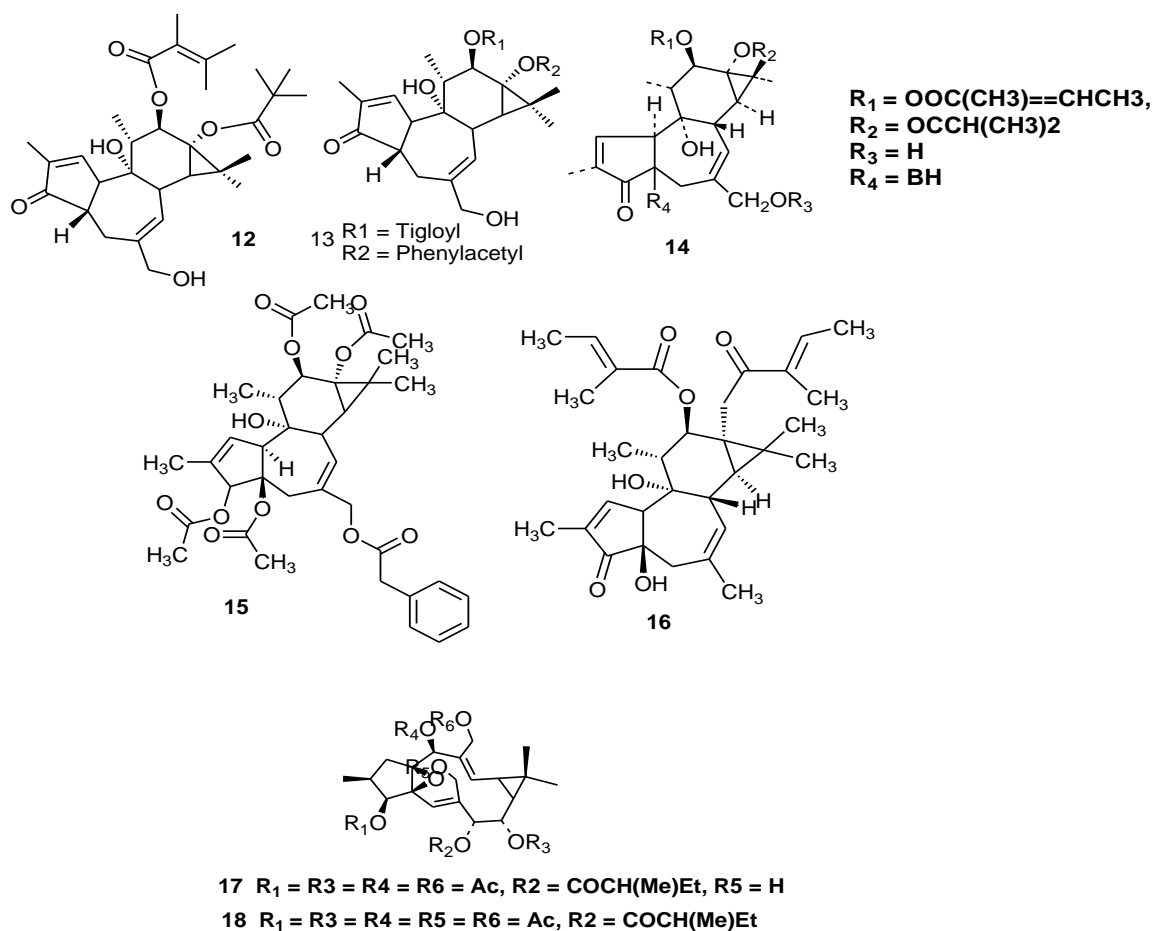
1.5.2 Phytochemistry of Plants in the Genus *Synadenium*

While many genera of the family Euphorbiaceae are well known for their diverse chemical ingredients and biological activities (Sabandar *et al.*, 2013), the genus *Synadenium* is not thoroughly investigated, thus bearing little phytochemical information. However, extensive studies have been done on the popular ornamental species such as *S. grantii*. Phytochemical screening of different morphological parts of the *S. grantii* has indicated dominance of the phorbol type diterpenoids in the latex showing a range of biological activities (Hassan *et al.*, 2012).

Few available natural chemicals (Table 4) however, have revealed potential medical benefits with some of them showing slight to significant harm when subjected to human body. These available chemicals represent few classes of compounds that have been isolated from this genus including anthocyanins, diterpenes and triterpenes (Olivier *et al.*, 1992; Andersen *et al.*, 2010; Hassan *et al.*, 2012; Munhoz *et al.*, 2013).

1.5.2.1 Diterpenes

Diterpenes are a group of compounds with twenty carbons (C₂₀) based on four isoprene units. These compounds are limited in occurrence and are considered as resins due to their higher boiling points. Investigations from the latex of *S. grantii* yielded novel diterpenes (**12-14**) which were also found to be responsible for skin irritation (Kinghorn, 1980; Neuwinger (1994). A recent investigation from the same species has yielded novel phorbol type diterpenes (**15-16**) which when tested for their biological activities exhibited anti-HIV activity in human (Hassan *et al.*, 2012). Olivier *et al.* (1992) reported the isolation of two esters of Synadenol (**17**) and (**18**) from the latex of *Synadenium compactum*.



1.5.2.2 Anthocyanins

Anthocyanins are water-soluble vacuolar pigments which vary in colors from red, purple or blue depending on pH. Anthocyanins belong to a parent class of flavonoids synthesized via the shikimic acid pathway. This class of compounds has been reported from *S. grantii* by 7 Andersen *et al.* (2010), who isolated six anthocynins having furanose sugar moieties (**17-19**) from the leaves of this species which were however not assessed for their biological activities.

1.5.2.3 Triterpenes

Triterpenes are compounds with thirty carbons (C_{30}) based on six isoprene units and which biosynthetically are derived from the mevalonic acid pathways. Triterpenes have

been reported only from *S. grantii* among the species of this genus. Thus, in the earlier studies, the *S. grantii* yielded several triterpenes namely, euphorbol (**20**), lanosterol (**21**), euphol (**22**) and tirucallol (**23**). More recently investigations of *S. grantii* (Munhoz *et al.*, 2013) resulted into the isolation of more triterpenoids namely, friederin (**24**) and 3 β -friedelinol (**25**). The different classes of chemical compounds from *Synadenium* are also listed in Table 4.

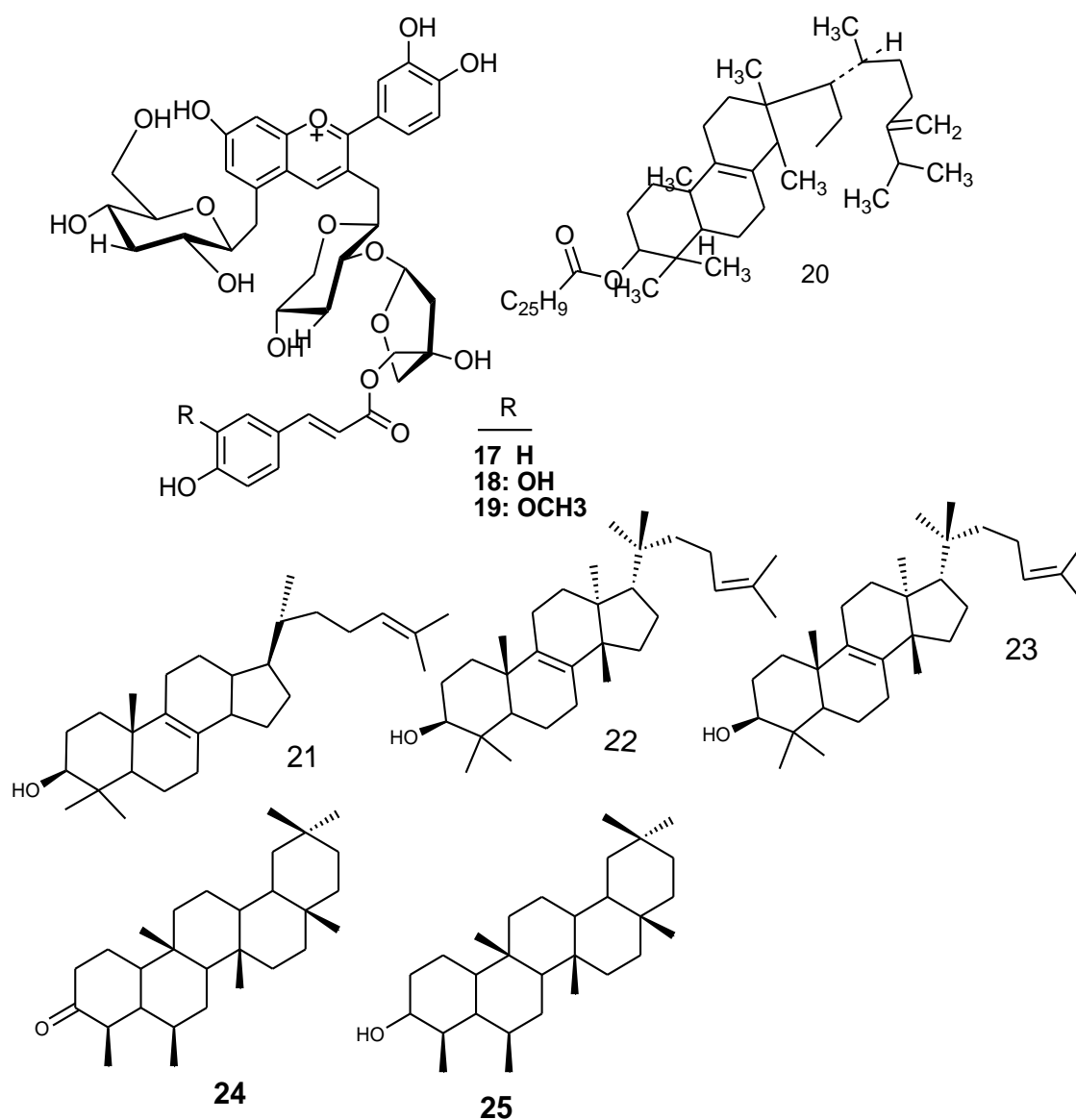


Table 4: Chemical Compounds Isolated from *Synadenium* Species

Structure no	Compound name	<i>Synadenium</i> spp	Reference
A. Diterpenoids			
12	Name un identified (Skin irritant) 12-tigloyl-4-deoxyphorbol-13-	<i>S. grantii</i>	<u>Kinghon</u> , 1980
13	isobutrate	<i>S. grantii</i>	Neuwinger, 1994
14	4-deoxyphorbol diterpene	<i>S. grantii</i>	Neuwinger, 1994
15	Synagrantol A	<i>S. grantii</i>	Hassan <i>et al.</i> , 2012
16	Synagrantol B	<i>S. grantii</i>	Hassan <i>et al.</i> , 2012
17	2-methylbutanoate tetraacetate	<i>S. grantii</i>	Olivier <i>et al.</i> , 1992
18	2-methylbutanoate pentaacetate	<i>S. grantii</i>	Olivier <i>et al.</i> , 1992
B. Anthocyanin			
19	Cyanidin 3-xyloside-5-glucoside	<i>S. grantii</i>	Andersen <i>et al.</i> , 2010
20	Cyanidin 3-xyloside	<i>S. grantii</i>	Andersen <i>et al.</i> , 2010
21	Unidentified	<i>S. grantii</i>	Andersen <i>et al.</i> , 2010
22	Unidentified	<i>S. grantii</i>	Andersen <i>et al.</i> , 2010
C. Terpenoids			
23	Euphorbol	<i>S. grantii</i>	Munhoz <i>et al.</i> , 2014
24	Lanosterol	<i>S. grantii</i>	Munhoz <i>et al.</i> , 2014
25	Euphols	<i>S. grantii</i>	Hassan <i>et al.</i> , 2012
26	Tirucallol	<i>S. grantii</i>	Hassan <i>et al.</i> , 2012
27	Friedelin	<i>S. grantii</i>	Munhoz <i>et al.</i> , 2014
28	3 β -friedelinol	<i>S. grantii</i>	Munhoz <i>et al.</i> , 2014

1.6 Pharmacological Effects

Table 5 lists the different pharmacological activities reported from plants of the genus *Synadenium*. These properties indicates the potency of plants from the genus as medicinal plants

1.6.1 Antiparasitic and Antiplasmodial Effects

Investigation of the Latex lectin from *S. carinatum* (ScLL) leaves was found to possess anti-leishmania effect. Evaluation of the latex against *Leishmania amazonensis* promastigotes/amastigotes (Afonso-Cardoso *et al.*, 2011) revealed that, pretreatment of

murine inflammatory peritoneal macrophages with ScLL reduced by 65.5% the association index of macrophages and *L. amazonensis* promastigotes. ScLL also showed the ability to reduce the growth of *L. amazonensis* amastigote intracellular forms, showing no *in vitro* cytotoxic effects in mammalian host cells.

In the study to assess anti plasmodial effects, Hassan *et al.* (2011) reported the effect of chloroform extract of *S. grantii* leaves against *Trypanosoma cruzi*, *Trypanosoma brucei* and *Plasmodium falciparum*. The extract was active against *Trypanosoma brucei* and *Plasmodium falciparum*, respectively with IC₅₀ values of 8.11 µg/mL and 23.70 µg/mL. On the other hand, the extract exhibited very high potency against *Trypanosoma cruzi* (IC₅₀ 2.21 µg/mL).

1.6.2 Antiulcer Activity

Anti-ulcer agents are class of drugs, exclusive of antibacterial drugs used to treat ulcers in the stomach and the upper part of the small intestine. In the test for anti-ulcer activity in rats, pure latex of *S. grantii* showed promising results. In the study two samples were prepared from leaf latex, one being pure latex and the other was diluted latex. In the findings, pure latex exhibited 90% gastric protection while the diluted latex had 6% protection (Costa *et al.*, 2012). However, the diluted form of extract is the common form of use in traditional utilization for ulcer protection (Ortencio, 1997).

1.6.3 Antitumoral and Antiangiogenic Effects

Angiogenesis is the process of creating new blood vessels from pre-existing blood vessels. The process is important for the growth and development of an organism, as well as in wound repair but also critical for tumor growth and metastasis. The body controls

angiogenesis by balancing stimulatory and inhibitory factors. Disease occurs when this delicate balance is disturbed. Thus antiangiogenic treatment is a highly promising therapeutic approach. Many activities have been going on for many years now aiming towards the discovery of angiogenesis inhibitors. Antitumoral and antiangiogenic effects were reported from a Brazilian plant *S. umbellatum* Pax by studying partitioned fractions using mice bearing Ehrlich Ascites Tumor (EAT) cells. These various fractions of crude plant extract possessed anti-tumoral and antiangiogenic activities (Nogueira *et al.*, 2008). Nogueira *et al.* (2008) further reported that after the mice were treated with extracts intraperitoneally for 10 days with *S. umbellatum*, the extracts showed a significant effect with 25mg/kg ethanolic extract producing the best results in prolonging life span. In these animals, the levels of vascular endothelial growth factor were markedly decreased after the treatment (Nogueira *et al.*, 2008).

1.6.4 Antiasthmatic Effect

Costa *et al.* (2012), reported that oral administration of *S. carinatum* latex lectin significantly inhibited neutrophil and eosinophil extravasations in models of acute and chronic inflammation. However, it reduced eosinophil and mononuclear blood counts during chronic inflammation. It also reduced IL (interleukin)-4 and IL-5 levels but increased interferon- γ and IL-10 in an asthma inflammatory model suggesting that it might induce a TH2 to TH1 shift in the adaptive immune response.

1.6.5 Antiviral Activity

This has been reported from *S. glaucescens* extracts against Newcastle Disease (ND) virus (Mabiki *et al.*, 2013). In this in ovo assay, embryonated chicken eggs were treated with the crude extract at a concentration of 0.2 mg/mL. The results indicated significant higher embryo survival and embryo weight increase in groups treated with *S. glaucescens*

extracts as compared to the positive control with root bark and ethanol extract demonstrating the highest activities (Mabiki *et al.*, 2013).

1.6.6 Antibacterial Activity

Antibacterial tests using extracts from *S. glaucescens* were carried out in mice using *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Findings indicated that mice infected with the two bacteria and treated with root extracts had significantly less ($p < 0.05$) severe skin lesions compared to mice from the untreated group which basically showed significant activity against tested bacteria (Max *et al.*, 2014).

1.6.7 Antinociceptive and Anti-inflammatory Activities

Antinociceptive effect is the action or process of blocking the detection of a painful or injurious stimulus by sensory neurons. These activities have been revealed from the investigation of *S. umbellatum* (Borges *et al.*, 2012) using ethanol (EtOH), hexane (HE), chloroform (CCl₃) and methanol/water (MeOH/H₂O) fractions. The test methodology involved the use of acetic acid-induced abdominal writhing test, formalin-induced paw licking test, tail flick test, croton oil-induced ear edema test and carrageenan-induced peritonitis test. Findings indicated that EtOH and MeOH/H₂O have antinociceptive activity involving the opioid system and anti-inflammatory activity. These results show that EtOH presented antinociceptive activity, probably involving the opioid system, anti-inflammatory activity in the croton oil-induced ear edema test and leukocyte migration into the intraperitoneal cavity. Methanol/water also presented anti-inflammatory activity in the croton oil-induced ear edema test (Borges *et al.*, 2012).

Table 5: Pharmacological and Toxicological Effects of some Species in the Genus *Synadenium*

Species	Activity	Description	Reference
<i>S. carinatum</i>		Evaluated the antiparasitic effects of the plant against <i>Leishmania amazonensis</i> in which pretreatment of murine inflammatory. peritoneal macrophages with <i>S. carinatum</i> latex lectin reduced by 65.5% the association index of macrophages and L. (L)	Alfonso-cardoso <i>et al.</i> , 2011
	Molluscidal	<i>amazonensis</i> promastigotes	
	anti-asthmatic	Oral administration of <i>S. carinatum</i> Latex Lectin significantly inhibited neutrophil and eosinophil while reducing eosinophil and mononuclear blood counts during chronic inflammation. It also reduced IL (interleukin)-4 and IL-5 levels but increased interferon-g and IL-10 in an asthma inflammatory model suggesting that it might induce a TH2 to TH1 shift in the adaptive immune response.	Rogério <i>et al.</i> , 2007
<i>S. grantii</i>	Anti-ulcer activity	Pure latex indicated 90% gastric protection in rats as compared to 6% of dilute extract which is a common form for traditional utilization	Costa <i>et al.</i> , 2012
	Irritant activity	External application of methanol extract of dried latex in mouse exhibited an irritant activity	Kinghorn, 1980
	Hypothermic activity	Ethanol (95%) extract of fresh latex from stem exhibited hypothermic activity and rabbits at doses of 5.0 and 10 mg/kg body weight	Unnikrishnan <i>et al.</i> , 1988
	Hypertensive activity	Ethanol (95%) extract of fresh leaf applied intravenously exhibited hypertensive activities in dog at a dose of 0.6mg/kg making it atropinized	Unnikrishnan <i>et al.</i> , 1988
	Hypotensive activity	Ethanol (95%) extract of fresh leaf exhibited hypotensive activity in dog at the dose of 0.6 mg/kg causing prolonged fatal hypotension, reversed by bilateral vagotomy.	Unnikrishnan <i>et al.</i> , 1988
	Pleteles secretory activity	ethanol (95%) extract of fresh leaf applied intravenously showed activity in platelet secretion in dog at dose of 0.6mg/kg	Unnikrishnan <i>et al.</i> , 1988
	Inotropic effects	Ethanol (95%) extract of fresh leaf showed inotropic effect when at dose levels between 0.08 and 2 mcg/ml	Unnikrishnan <i>et al.</i> , 1988

Table 5: *Continue*.....

Species	Activity	Description	Reference
<i>S. glaucescens</i>	Antiviral activity	Leaves, roots and stem extracts had antiviral actives against new castle virus, infectious bursal disease virus and fowl pox virus in ovo. The extracts increased embryo survival and weight significantly at 0.2mg/ml as compared to the positive control	Mabiki <i>et al.</i> , 2013
	Molluscidal activity	Water extract of oven dried leaf was active against <i>Biomphalaria Pfeifferi</i>	Kloos <i>et al.</i> , 1987
	Anti-bacterial activity	Ethanol root extracts exhibited significant activities (p<0.005) against staphylococcus aureus and pseudomonas aeruginosa	Max <i>et al.</i> , 2014
<i>S. umbellatum</i>	anti tumoral activity	Extract from this plant decreased the viability of Ehrlich ascites tumor (EAT) cells through apoptotic pathways	Da mota <i>et al</i> 2012, Nogueira <i>et al.</i> , 2008
	Anti angiogenic effects	In the test for anti tumoral activity in mice, the extract concurrently markedly decreased the levels of vascular endothelial growth factor after the treatment	Nogueira <i>et al.</i> , 2008
	Antinoceptive activity	when extracts from leaves were tested using the acetic acid-induced abdominal writhing test, formalin-induced paw licking test, tail flick test, croton oil-induced ear edema test, and carrageenan-induced peritonitis test, Ethanolic and methanol/water extracts significantly reduced the number of acetic acidinduced abdominal writhes while hexane, chloroform did not	Borges <i>et al.</i> , 2013
	anti inflammatory activity	Ethanol and methanol extracts from leaves had an anti-inflammatory activity in the croton oil-induced ear edema test, and leukocyte migration into the intraperitoneal cavity.	Borges <i>et al.</i> , 2013
	Mutagenicity and antimutagenic activities	Latex exhibited mutagenicity in higher doses and anti-mutagenicity in lower doses	melo-reis <i>et al.</i> , 2011
<i>S. pereskiaefolium</i>	Contractory inhibition	The aqueous extract from stems and leaves of this plant showed a contraction inhibition against of an ileum isolated from Guinea pig	Kerstin <i>et al.</i> , (1991)

1.7 Toxicological Effects

Toxicological studies for plants from the genus have pre clinically been conducted in oral and dermal route using test animals. The animals used include murines, rabbits and wistar

albino rats in most cases. The dried extracts have proven less toxic except pure latex which in some instances has proven toxic especially when used in a pure form. Thus, in the study to evaluate pre-clinical toxicity of the latex and of the ethanolic extract of the leaves (EEL) of *S.umbellatum*, administrated by oral route, in Wistar female rats, Azerado *et al.* (2009) revealed that neither animal death nor behavioral and physiological alterations were observed in the dose level of 2000 mg/kg. However, the latex caused congestion and leukocyte infiltration of the kidneys, liver and lungs.

During studies of anti-bacterial effects of *S. glaucescens*, Max *et al.* (2014) simultaneously conducted the histopathological examination of kidney and livers of the study mice. Findings indicated that tissues of these organs exhibited hydropic degenerations and desquamated tubular epithelium together with necrosis around central vein in livers of animals infected with *S. aureus* and treated with 50 mg/kg. From the observation, the study concluded that higher doses of the tested extract could be harmful to the mice. Studies on the skin application of methanol extract of *S. grantii* in mice revealed some irritant activity (Kinghorn, 1980).

1.8 *Synadenium Glaucescens*

Synadenium glaucescens (Fig. 1) endemic to the East African region and is found in Tanzania, Kenya, Democratic Republic of the Congo and Burundi. The specie is known as “Mvunjakongwa” in Swahili. It is endemic and grows in several regions in Tanzania (Mosha *et al.*, 2001). *Synadeium glaucescens* though reported poisonous and perceived as of no medicinal value, 23 communities in Tanzania have used it effectively in the treatment and control of human and animal diseases with no reported adverse effects (Mosha *et al.*, 2001). In the coastal, Morogoro and Kilimanjaro regions, the juice of fresh, crushed leaves is drunk to treat excessive menstruation and as a purgative (Chhabra *et al.*,

1984). A leaf decoction with lime juice, baking soda and honey added is drunk to treat asthma; the ashes of dried leaves are mixed with water and applied to treat leprosy (Schmelzer *et al.*, 2008). A root bark extract is taken with sugar to treat severe cough, tuberculosis and as ear drop to treat earache (Neuwinger, 2000; Newmark, 2002). In Tanga region it is used to prepare medicine to human and for control of poultry diseases mainly Newcastle disease (Wickaman *et al.*, 2006). The latex is also used as a fish poison (Neuwinger, 2004). Excessive use of its concoctions for purgative purposes, cause poisoning and even death. Its latex cause irritation and can causes blindness (Mosha *et al.*, 2001). The plant species is known for various medicinal properties as claimed by various traditional medicinal uses. Mabiki *et al.* (2013) reported various traditional medicinal utilization of this plant in Njombe district, Mtulingara village including anti HIV, wound healing, treatment of worms, anti TB and many others.



Figure 1: *Synadenium Glaucescens* Growing in Maize Field

Virtually, no any information exists regarding natural products that have so far been isolated from this plant. The only available information on natural product from this plant is the isolation of one pharmacologically active compound of which the establishment of chemical structure was not accomplished Rukunga *et al.*, 2012). Preliminary study of the isolated compound indicated it to be β -glycosides attached to alkyl group and was deduced to be an inhibitor of contraction of guinea pig ileum (Rukunga *et al.*, 2012).

1.9 Problem Statement and Justification

Despite the recorded traditional utilizations in Tanzania and elsewhere around the world (Table 5), limited information is available on biological and phytochemical characteristics of *S. glaucescens*. This is due to limited studies that have so far focused towards investigating this plant. This might be due to negative perceptions that this plant is poisonous and lacks medicinal value. Mabiki *et al.* (2013) documented the antiviral and antibacterial characteristic of this plant while Rukunga *et al.* (2012) document one and the only phytochemical isolate which however its structure was not established. Thus, many biological properties including toxicological, pharmacological and phytochemical characteristics of *S. glaucescens* remain undocumented. The consequence of this is an inadequate knowledge on the actual therapeutic values, safety indices and phytochemical contents of the plant extracts. This has created a gap for further scientific validation of extracts from this plant.

1.10 Significance of the Research

Despite its usefulness in combating various diseases, the utilization of plant based products in communities has many inherent problems. Most of these extracts lack scientifically proven information on their efficacies and levels of toxic effects. Some of

the commonly used plant extracts lack information of chemical components they possess. As a result, the immediate and long-term use has in some instances been accompanied with some health risks. The planned study is expected to reveal the dermal toxic effects and efficacies of *S. glaucescens* extracts as an acaricide and as anti HIV. The study reveals the phytochemical constituents of this plant species thus, contribute knowledge to science.

1.11 Research Objectives

1.11.1 Main Objective

The general objective of the study was to investigate for some biological and phytochemical characteristics of *S. glaucescens*.

1.11.2 Specific Objectives

- i. To assess the *in vivo* dermal toxicity of extracts of *S. glaucescens* using animal models,
- ii. To establish the ectoparasitic activity of *S. glaucescens* extracts against cattle ticks,
- iii. To determine the pharmacological effects of extracts against HIV virus,
- iv. To establish chemical structures of isolated chemical compounds *from S. glaucescens*.

1.12 Limitation of the Study

The major limitation to this study was the complexity of plant extracts for isolation of compounds. The Plant extracts from this plant appears to possess a lot fatty acids components that most of time run together with the intended compounds. As result

isolation is complicated and because of repeated trials of clean up to obtain pure compounds, the amounts of pure compounds obtained were also small, thus, limiting the bioassays.

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CHAPTER TWO

PAPER I

Assessment of Dermal Irritation and Acute Toxicity Potential of Extracts from *Synadenium glaucescens* on Healthy Rabbits, Wistar Albino Rats and Albino Mice

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Authors' contributions

This work was carried out in collaboration with all authors, they were all responsible in the design of study. Author VAN wrote the protocol and was responsible for data collection analysis and the first drafting of the manuscript. All authors contributed to critical review of the protocol and manuscript. They also read and approved the final manuscript. The corresponding author had the final responsibility to submit the manuscript for publication.

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ABSTRACT

Aim: This study was conceived for the purpose of evaluating dermal toxicity potentials of extracts from *S. glaucescens* which is known for many traditional application 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). This followed the scanty availability of information regarding

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dermal toxicity of this and many other plants in Tanzania despite the growing practice of utilizing plant products and extracts respectively to control and treat ectoparasites, and skin disorders.

Materials and Methods: The dried plant materials were subjected to sequential solvent extraction using organic and aqueous solvents. All test animals were obtained from Sokoine University of Agriculture (SUA), Tanzania. Thus, irritation, and acute dermal toxicity tests were respectively conducted in healthy rabbits and albino rats using the Organization for Economic Cooperation and Development (OECD) guidelines. Albino mice were used to test skin sensitization using method developed by Sailstad.

Results and Discussion: Irritation indices ranged from 3.2 and 0.05, thus according to Draize these are considered as mild and moderate irritants since none of them could reach PII of 5. On the other hand, findings from acute dermal toxicity tests showed no any overt signs of toxicity after two weeks of treatment. Similarly the extracts did not produce any sensitization reaction based on the mouse ear diameter taken by vernier calipers.

Conclusion: Findings from this study have shown that, extracts from dried plant of *S. glaucescens* exhibit neither sensitization nor acute dermal toxicity effects except for mild to moderate irritancy. The findings therefore suggests that extracts from dried plant parts of *S. glaucescens* under 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.

Keywords: Irritation; sensitization; acute; pathology.

1. INTRODUCTION

Treatment of various ailments through traditional medicines is a common practice in many countries around the world [1,2]. Skin disorders are among diseases that are managed by traditional medicines [3-5]. These disorders occur worldwide and they affect people of all ages from neonates to the elderly [1]. The common skin diseases afflicting people and which are also treated with traditional medicines include: *Tinea capitis*, *Tinea corporis*, scabies, acne, *Erythema multiforme*, leg ulcers, localized vitiligo, seborrheic dermatitis and all types of eczema [3,6,7].

Despite the enormous advantages in the treatment of these diseases, traditional medicines are also implicated for various side-effects [8] both internal and external to human and animals. Recently, a number of publications reporting dermal toxicity potential of various plant preparations used traditionally for skin applications have emerged. Ernest, 2000 [9] reported the potentials of many plants to cause dermal toxicity. In his review, he registered plants such as St John's Wort, *Piper methysticum* (kava), *Aloe vera*, *Eucalyptus* sp, *Cinnamomum camphora* (camphor), *Lawsonia inermis* (henna) and *Pausinystalia yohimbe* to have great potential of dermatological side effects. Other herbal treatments used for dermatological conditions such as Chinese oral herbal remedies for atopic eczema, have the potential to cause systemic adverse effects [9]. On the other hand

pure latex from the majority of plants has been found to exhibit severe skin irritations [4,5]. Many other organic plant extracts that have so far been studied revealed mild to moderate skin toxicity [4,5].

In Tanzania like many other countries, the use of traditional medicament for skin related problems in human and livestock are a common phenomenon. During the survey to determine the prevalence of skin disease in Tanzania, Satimia et al. [6] revealed the use of both traditional and modern medicine in the treatment of various skin disorders. Similarly Moshi et al. [10] reported the use of *Jatropha curcas*, *Ricinus communis*, *Zehrenia scabra* and *Tricalysia coriacea* medicinal plants in the treatment of various skin conditions in Kagera region in Tanzania.

Synadenium glaucescens is among of the Tanzanian traditional medicinal plants which is known for many traditional application 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). The plant is endemic in the East African Region and occurs in Tanzania, Kenya, Democratic Republic of the Congo and Burundi. *Synadenium glaucescens* has been proven to have strong antiviral and antibacterial activities [11]. Other species within the same genus were earlier reported to exhibit molluscicidal and insecticidal activities indicating their potential as biopesticides [12].

Despite the recorded utilization of plants through the dermal route in human and animals, there is scanty information regarding dermal toxicity of Tanzanian medicinal plants. This is in comparison with the amount of information that exists on cytotoxicity of different medicinal plants using the brine shrimp lethality test [13-14].

The aim of this study was to evaluate the toxicity potentials for of *S. glaucescens* commonly used for treatment of dermatological conditions in humans and livestock.

2. MATERIALS AND METHODS

2.1 Plant Materials

Plant materials (leaves and root barks) of *Synadenium glaucescens* (S.G) Pax were harvested from Mufindi District in Tanzania during May and August 2012. The World Health Organization (WHO) guideline on Good Agricultural and Collection Practices (GACP) for medicinal plants was used [15]. Thus, roots were dried under room temperature while some minor modifications were considered for leaves in which drying was effected in place with half day shade and half day sun due to the fact that leaves for this plant contain latex. The dried plant materials were pulverized and then subjected to solvent extraction with different polarities sequentially in ascending order starting with hexane, dichloromethane, ethyl acetate, methanol and ultimately water. After filtration, the

extracts were dried *in vacuo* and in a freeze dryer to obtain different organic and water extracts, respectively (Table 1).

2.2 Management of Experimental Animals

Healthy male white New Zealand rabbits (1.4-2.3 kg), healthy female adult wistar albino rats (71-89 g) and healthy female young adult albino mice (15-27 g) were used in the dermal toxicity for acute dermal irritations, acute dermal toxicity and skin sensitization studies, respectively. All animals were obtained from Soikoine University of Agriculture (SUA), Department of Animal Sciences and Production (DASP) for rabbits and in the Faculty of Veterinary Medicine for rats and mice. Rabbits were caged individually while rats and mice were caged in groups; all animals were supplied with conventional laboratory diets and water at *ad libitum* [16,17]. All female animals were nulliparus and non-pregnant. Housing was maintained at 22°C±3°C temperature and 40-65% relative humidity with a 12 hours light-dark cycle. Prior to the tests, animals were acclimatized in a laboratory condition for at least a week [16,17].

2.3 Laboratory Procedures

2.3.1 Acute dermal irritation tests

Acute dermal irritation tests were performed using the Organization for Economic Cooperation and Development (OECD) guidelines [16].

Table 1. Extract type and codes

Codes	Plant part	Extract type
GR-297-66201B	S.g (Root)	Dichloromethane (DCM) extract of S.g root prepared by extracting plant with DCM, after the plant materials extracted by Hexane
GR-297-66201C	S.g (Root)	Ethyl acetate (EOAc) extract of S.g root prepared after sequential extraction with Hexane and DCM and plant residue extracted with EtOAc
GR-297-66201D	S.g (Root)	Methanol (MeoH) extract of S.g prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with water (H ₂ O)
GR-297-66201E	S.g (Root)	Water extract of S.g root after sequential extraction with the above solvents
GR-297-66202B	S.g (leaves)	DCM extract of S.g leaves prepared by extracting plant with DCM, after the plant materials having been extracted by Hexane
GR-297-66202C	S.g (leaves)	EtOAc extract of S.g leaves prepared after sequential extraction with Hexane DCM, EtOAc; and plant residue extracted with EtOAc
GR-297-66202D	S.G (leaves)	MeoH extract of S.g leaves prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with H ₂ O
GR-297-66202E	S.g (leaves)	Water extract of S.g leaves after sequentially extracted with above solvents
GR-297-6603A	S.g (Root)	Ethanol Extract; fresh ground root barks extracted with ethanol

Twenty four hours before the test, fur from the backs of all rabbits was clipped using electric clipper exposing approximately 6cm² of skin [16]. Half a gram of each sample moistened with water and in some cases with a drop of sunflower cooking oil was evenly and gently applied using a small spatula in a test site while distilled water and in some case mixed with a drop of sunflower cooking oil was applied on the control side in this case the right side. Both sides were then covered using gauze of cotton cloth followed by a plastic sheath and then supported in place by a non irritating adhesive tape. After 4 hours the coverings were taken out and the test substance washed using distilled water. In cases where the substances were hard to get out especially for organic extracts, sunflower cooking oil was first used to soften and then water applied for cleansing. The test sites were then examined at 1, 24, 48 and 72 hours for dermal reaction using Draize scoring criteria (Table 1).

The Primary Irritation Indexes (PII) of test substances were then calculated using the formula below

$$(PII) = \frac{\text{sum of erythema/oedema}}{\text{No of test sites} \times \text{grading interval}}$$

And the long form of the formula is;

$$PII = \frac{\sum \text{erythema grades at 1, 24, 48 and 72 hr} + \sum \text{edema grade at 1, 24, 48 and 72 hr total}}{\text{number of observations (number of animal)} \times \text{observation intervals (4)} \times 2 \text{ (erythma and oedema)}}$$

The extract was then classified according to Draize method of classification using the PII scoring as mildly irritant if (PII < 2), moderately irritant (2 ≤ PII ≤ 5), and severe irritant (PII > 5)

2.3.2 Acute dermal toxicity tests

2.3.2.1 Selection of dosage

In acute dermal toxicity studies, only leaf extracts were tested. These extract included, water, methanol, ethyl acetate and dichloromethane respectively denoted as GR-297-6602E, GR-297-6602D, GR-297-6602C and GR-297-6602B. Selection of dosage was guided by procedures stipulated in OECD draft guideline no 434. Sighting study was conducted to all extracts tested. The limit dose of 2000 mg/kg was selected for the main study based on the fact that the 1000mg/kg as a start dose in the sighting study could not show any sign of toxicity when considering animal weight changes for two weeks.

2.3.2.2 Animals and preparation

The test in the albino rats were performed according to the OECD draft guideline number 434 [17]. A total of ten animals (all females) were divided into two groups of five animals each for a treatment and a control groups. Approximately 24 hours before the study, fur was removed from the dorsal area of the trunk of the animals by clipping to obtain at least 10% of the body surface area while taking care to avoid abrading the skin. Depending on the type of extract; the test substances were moistened with either sunflower cooking oil or distilled water then applied uniformly over a shaved area using a small spatula. Liquid test substances were used undiluted. The test substances were held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. At the end of the exposure period, residual test substance was removed using water or sunflower oil or both. Cage side observation was made daily, but weight measurement was taken weekly for 15 days. Observation included evaluation of skin and fur, eyes, respiratory effects, salivation, diarrhea, urination, and central nervous system effects (tremors and convulsion, gait and posture, reactivity to handling or sensory stimuli and altered strength). By the 15th day rats were humanely sacrificed and organs were carefully taken out for gross and histopathological examinations.

2.3.2.3 Gross and histopathological examination

Organs (Kidney, liver and lungs) were processed for histopathological examination using a standard procedure [18]. The organ tissues were first examined grossly for any observable effects and then sections (5 μM) were fixed in 10% buffered formalin embedded in paraffin, stained with haematoxylin-eosin (HE) and examined under light microscope. Tissue samples were then evaluated for degree of deformation and necrosis. The histopathological pictures of tissues from the different animal groups were evaluated by a pathologist and pictures taken using a digital camera.

2.3.3 Dermal sensitization studies

2.3.3.1 Selection of appropriate solvent

The criteria for selecting the right solvents were based on solubility of the test substances [4]. The procedure involved dissolving the extract in different trial solvents under both room and

raised temperatures using water bath. The solvents dissolved the largest part of the extract were selected as dissolution solvent for both sighting and actual tests. Thus, a range of organic solvents were tested. The extracts tested for solubility included dichloromethane (DCM) and ethyl acetate for both root barks and leaves. DCM extracts exhibited good solubility in chloroform and acetone while ethyl acetate extracts were soluble in ethanol. Therefore, acetone and ethanol were selected respectively as solvents for DCM and ethyl acetate extracts

2.3.3.2 Range-finding irritation test

Using the selected solvent, range-finding irritation test was performed and appropriate concentrations selected [4,16]. Sixteen female mice were randomized into eight groups; the groups were then randomly placed into different concentrations, two animals per each concentration. 100 μ l of the respective concentration of the test solution was applied to the backs and area was then immediately dried using an electric drier. Ten microlitres of the respective concentration of test solution was then applied to the dorsal and ventral regions of the left ear and dried immediately. The mice were returned to their cage and left undisturbed for overnight. On day 1, each mouse was anesthetized with diethyl ether and thicknesses of both ears were measured and results were recorded. On the same day immediately and on subsequent days (days 2 and 3) 100 μ l of the test solvent were again applied at the backs and on day 4, the skin of all animals was inspected for dermal irritation and scored as described above. The mildly irritant concentration on the belly regions (8 mg/ml) was chosen to be the dose for induction of the actual test and the highest non-irritant concentration to the ear (10 mg/ml) was chosen to be the challenge dose of the actual sensitization test [19,20].

2.3.3.3 Actual tests

The Mouse ear swelling test (MEST) method (C) by Sailstad et al. [21] was used to evaluate the sensitization potentials of some extracts from *S. glaucescens*. Thus, female mice were anesthetized using ether and the back of each mouse was shaved using small hair clipper [21]. Test chemicals or vehicle was applied: 100 μ l without Freund's Complete Adjuvant (FCA) for 2 consecutive days. Chemical challenge occurred on day 6.

2.4 Data Analysis

Analysis of data varied depending on the study. Data from irritation were presented as scores from observation based on the Draize scoring criteria (Table 2.) after which PII was then calculated. Data from acute dermal toxicity were analyzed using Statistical Analysis Systems (SAS) software version 9.3 and the results expressed as mean weight \pm standard error. The student *t*-test was used to perform statistical tests and if the statistical power (*p*) was less than 0.005, the change was considered significant and the drug was considered less toxic. In the sensitization study, only the ear diameter was taken before and after sensitization and challenge. The mean diameter were calculated using excel and then compared to control.

Table 2. Draize scoring criteria for erythema and oedema

End point measured	Grade
No erythema /oedema	0
Very slight erythema/ oedema (barely perceptible)	1
Well defined erythema/ oedema	2
Moderate to severe oedema	3
Severe erythema/ oedema (beef redness) to eschar formation preventing grading of erythema	4

Maximum value is 4

3. RESULTS

3.1 Dermal Irritation

The extracts tested for dermal irritations were ethyl acetate, dichloromathene and water preparations from roots barks and leaves. Additionally, methanol leaf extract was also tested for irritations. The highest primary irritation index (PII) recorded in all extracts was 2.00 from the ethyl acetate of leaves while the lowest was 0.03 from water extracts of leaves. Thus, all of them fall into mild ($PII < 2$) and moderate ($2 \leq PII \leq 5$) irritant categories. The individual irritation scores (Table 3a and b) shows that the effects were pronounced in the first days but faded away with time and on 8 day's time almost all signs had faded away (Table 3a and 3b). None of the extracts from *S. glaucescens* could therefore be classified as an irritant.

From the findings, ethyl acetate and dichloromethane extracts were relatively more irritating than water and methanol extracts.

Table 3a. Average Irritation scores for leaf extracts of *S. glaucescens* in rabbits

Time intervals (hrs)	Draize scores (sums of erythema & oedema)			
	Ethyl acetate	Dichloromethane	Methanol extract	Water extracts
1	3.4	3	1.2	0.2
24	3.2	2.2	0	0
48	3.2	3.2	0	0
72	3	3	0	0
8th day	0	0	0	0
16th	0	0	0	0
Pll	2.0	1.43	0.15	0.025

Table 3b. Average Irritation scores for root extracts of *S. glaucescens* in rabbits

Time intervals in hours	Draize scores (sums of erythema & oedema)		
	Ethyl acetate	Dichloromethane	Water extracts
1	3.4	4.4	2
24	1.8	3	0.2
48	3	3.4	0
72	2.8	1.6	0
8th day	0	0	0
16th	0	0	0
Pll	1.375	1.55	0.275

3.2 Dermal Sensitization

Non occluded sensitization was performed using chloroform and ethyl acetate crude extracts from root barks and leaves of *S. glaucescens*. Eight milligram per milliliter (8 mg/ml) was selected as induction dose (The mildly irritant concentration on the belly regions) and 10 mg/ml (highest non-irritant concentration to the ear) selected as a challenge dose of the actual sensitization test 1 [9,20]. Each test extract was validated using a negative control and the right ear which was the non challenged ear. The results indicate that no sensitization was observed in any of the extracts for both test and negative control after 24 and 48 hours of post challenge.

3.3 Acute Dermal Toxicity

3.3.1 Selection of dosage for acute dermal studies

A starting dose of 1000 mg/kg in the sighting study could not show any sign of toxicity when considering animal weight changes for two weeks (Fig. 1a). The few observable weight losses for some of the animals in the 2000 mg/kg dosage was taken as toxic signs from the plant extracts and therefore was the main criterion for its selection. The drop of weight was obvious in the first week but rose to normal in the second week (Fig. 1b).

3.3.2 Clinical observation and mortality

Few hours after application of extracts many animals from the test groups showed some signs of discomfort and restlessness manifested by movements around the cage and limited intake of food and water. However, the eating and drinking slowly resumed in the same day and after removal of occlusion and test drugs, in the next day all animals resumed to normal state. Despite the discomfort, no adverse clinical signs were evident from all the animals in the test and control groups (Table 3). Albeit no scoring was done, the observable irritations caused by extracts were ranked to be mild or moderate with DCM and ethyl acetate extracts ranging highest. None of the animals showed any signs of edema from any of the extracts and control groups. Observation indicated that none of animals was found neither in a moribund condition nor showing any severe pain and/or enduring signs of severe distress [17]. Further observation was conducted for appearances of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma [17] and none of these signs was observed to any single animal in all the tests and control groups (Table 3).

3.3.3 Body weight trends

The means of weights of individual animals in the test groups are shown in Table 3 while the trend in weight change in the two weeks is shown in

Fig. 2. The results show significant mean weight increase in week one ($P = 0.0003$) from the initial weight. However, the change in weight of in the second week was not significant.

3.3.4 Gross pathology

Gross pathological examination of organs revealed no difference that could be established between test and control groups thus, no evidence of organ toxicity was associated to the extracts.

3.3.5 Histopathological examination

Histopathological examinations of organs also revealed no lesion suspected from drug effects in any of the test and control groups. All organs (kidney, liver and lungs) were normal with none of them showing degenerative changes of parenchyma, only blood vessel congestion in both control and test groups which were not linked to any chemical effect was evident (Figs. 2 and 3)

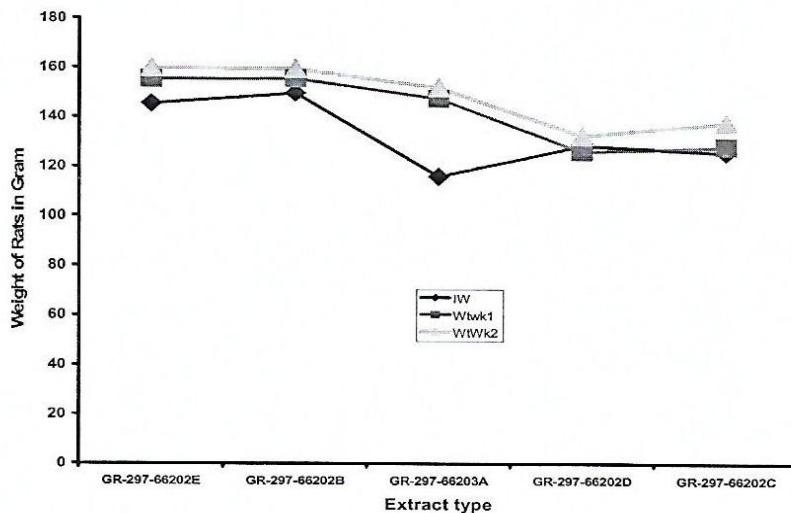


Fig. 1a. Weight change during sighting study for dermal toxicities for 1000mg/kg

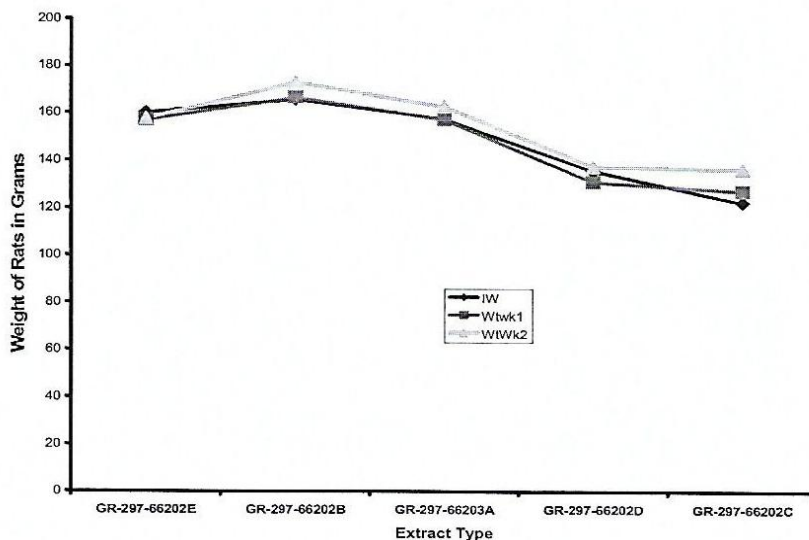


Fig. 1b. Weight changes during sighting study for dermal toxicities for 2000mg/kg

Table 4. Average Weight during the actual acute dermal toxicity tests

Extract code	Week 1			
	Means ± std dev (gm)	P=0.05	Mean Cwtw1 (gm)	p>0.05
GR-297-6602B	99.42±11.64		24.40±3.64	
GR-297-6602B	106.78±10.24		23.98±2.51	
GR-297-6602B	108.38±6.86		28.86±4.06	
GR-297-6602B	120.62±10.90		23.96±8.98	
Control (Dist H ₂ O)	94.80±10.58		11.18±2.29	
Overall	106.00±0.11	***	22.48±0.34	***
Week 2			Cwtw2	
GR-297-6602B	111.22±11.46		11.80±4.54	
GR-297-6602C	117.04±12.48		10.26±2.67	
GR-297-6602D	119.38±10.54		11.00±4.98	
GR-297-6602E	133.54±13.71		12.92±3.34	
Control (Dist H ₂ O)	109.80±8.52		15.00±6.66	
Overall	118.20±0.26	NS	14.82±0.70	NS

Cwtw1 = change of weight one week after treatment from initial weight, Cwtw2 = change of weight one week from weight in week one

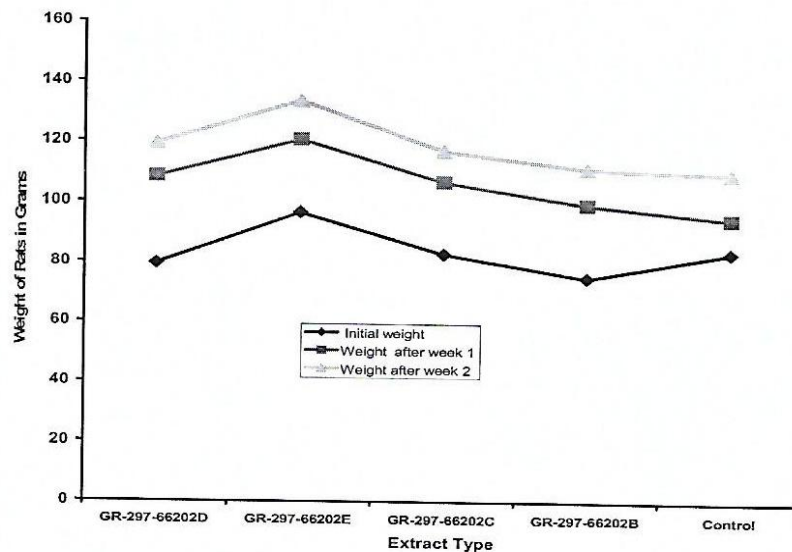


Fig. 2. Weight change during actual dermal toxicities tests

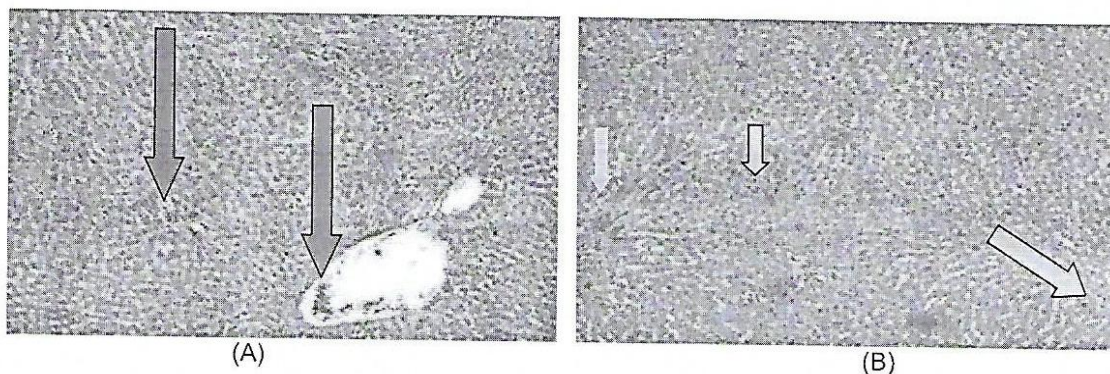


Fig. 3. (a) Photomicrograph of liver (a) from Methanol extracts (Blue arrows), (b) from distilled water ((yellow arrows) showing central vein and small blood vessel congestion H&E, X10 magnifications

4. DISCUSSION

4.1 Dermal Irritation

From this study, the calculated primary irritation indexes (PII) ranged from 0.05–3.2 for test substances and zero for control. Methanol extracts from leaves and water extracts from both leaves and roots exhibited PII of less than 2. According to Draize, [22] classification, these tree products were considered to have mild irritant effects. The other four extracts from dichloromethane and ethyl acetate extracts which are mid polar fractions from both plant parts were more irritant compared to polar fractions of water and methanol. These were concluded to have a moderate irritant effect since the PII was greater than 2 but less than 5. This could probably be due to fact that much of ingredients with irritant characteristics were contained in these portions of extracts. Elsewhere, similar findings have been obtained from other plant species of this family. Bigoniya et al. [5] reported a mild to moderate irritation in medium and polar fractions of *Euphorbia neriifolia* but very severe irritations from fresh and dried latex [5]. The dermal irritation tests for fresh latex was not studied due to ethical grounds as high level of toxicity in most plants of the family Euphorbiaceae is found in the fresh latex. The irritation of medium polar and polar fraction is associated with the presence of phorbol type diterpenes esters while the less polar fractions are generally reported to contain triterpenes which make them non irritant [23]. However, According to Draize, [22] classifications all extracts in this study are considered not severe irritants because the PII of less than 5.

4.2 Dermal Sensitization

Unlike in the irritation study, dermal sensitization revealed no effect that can be related to the test extract. The control group and the test groups had similar ear diameter and even the right ears of all groups appeared the same. These results indicate that although the extracts from this plant exhibit mild to moderate irritations (section 5.1), generally they are not sensitizers. The methodology used in this evaluation was shorter in terms of induction and challenge interval and had not been extensively utilized in dermal sensitization tests. However, it demonstrated a similar effect in the earlier study by Sailstad et al. [5].

4.3 Acute Dermal Toxicity Study

Acute dermal toxicity results indicate that all animals in the tests and control groups did not exhibit any overt clinical signs of toxicities. The discomfort Signs experienced initially could probably be due to mild irritations and experiences of foreign materials (extract, occlusion cloth and adhesive tape) in the body. The loss in body weight which is an important maker of gross toxicity as stated by Banerjee et al. [24] did not occur and this further indicates that the extract did not cause acute toxicity. The significant mean weight increase in week one ($P = 0.0003$) from the initial weight further confirms that no effects were caused by the test chemicals. The non significant weight change in week two likely emerged from normal growth retardation that occurs with time. Drastic toxicity or interference with absorption of nutrient is normally reflected in body weight reduction [24]. No gross and histopathological lesion were observed.

5. CONCLUSION

Findings from this study have shown that, extracts from dried plant of *S. glaucescens* exhibit neither sensitization nor acute dermal toxicity effects except for mild to moderate irritancy. The findings therefore suggests that extracts from dried plant parts of *S. glaucescens* under 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The research protocol was approved by the postgraduate studies committee of the Department of Veterinary Medicine and Public Health of the Sokoine University of Agriculture (SUA) in Tanzania.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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PAPER II**Evaluation of acaricidal efficacy of *Synadenium glaucescens* (Euphorbiaceae) against
Boophilus species**

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Full Length Research Paper

Evaluation of acaricidal efficacy of *Synadenium glaucescens* (Euphorbiaceae) against boophilus species

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Synadenium glaucescens is a traditional medicinal plant used by some communities in Tanzania for the management of various diseases in animals and human including the use for control of ticks in cattle. The aim of this study was to investigate the 'acaricidal effect' of extracts from this plant on *Boophilus decoloratus* and *B. microplus*. The methodology involved the use of larval and adult immersion tests. Results indicated low larvicidal (corrected mortality 37.5%) and adulticidal (corrected mortality 33.33%, LC₅₀ 666.91) activities respectively for methanol and ethanol extracts from leaves. Other extracts of this plant showed a non-significant activity of mortality. Thus, it is not recommended for field trials, rather additional research is needed to determine its potentials especially using fresh plant material

Key words: *Synadenium glaucescens*, Acaricidal activity, ticks, Tanzania.

INTRODUCTION

Records indicated that the number of people relying on agriculture has gone down as from 2001 to 2010, yet still it is the only sector that provides a livelihood for the majority of the communities and any other industry in the world (Upton, 2004; World Bank, 2008; Cervantes-Godoy and Dewbre, 2010). In the agricultural sector, livestock keeping is one among important activities that is practiced by many poor communities in developing world

(Randolph et al., 2007). In 2004, Upton reported that livestock keeping provided over half of the value of global agricultural output and was the main source of income in many countries. Literature indicates that the number of animals is further experiencing a remarkable increase especially in developing world (Randolph et al., 2007; Thornton, 2010). Despite this amazing increase, livestock keeping is constrained by diseases transmitted by ectoparasites

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(Njoroge et al., 2006). The harmful effects of ectoparasites on the productivity of livestock are well documented (Bagavan et al., 2009; Gazim et al., 2011). Ticks and tick-borne diseases are important causes of losses to the livestock industry, in particular, the production of cattle and small ruminants in tropical and subtropical areas. The diseases are associated with a reduction in productivity, fertility and in some instances may result in the death of an animal (Bagavan et al., 2009; Gazim et al., 2011). A worldwide loss due to diseases transmitted by ticks and the costs of tick control is very high (Minjauw and McLeod, 2003). The economic importance of ticks is principally due to the ability to transmit a wide spectrum of pathogenic microorganisms, such as protozoa, rickettsiae, spirochaetes, and viruses.

In Africa, tick-borne protozoan diseases (e.g. theileriosis and babesiosis) and rickettsial diseases e.g. anaplasmosis and heartwater (cowdriosis) are the main health and management problems of domestic ruminants. Tick-borne diseases that are reported to affect livestock productivity in the East African Region include East Coast Fever, anaplasmosis, babesiosis and cowdriosis (McCosker et al., 1993; Kagaruki et al., 1996). In Tanzania, tick-borne diseases contribute to over 72% of the annual cattle mortality (Mtei and Msami, 1996; Kivaria, 2007). Ticks from the genus *Boophilus* are important due to their ability to transmit pathogens in cattle such as *Anaplasma marginale*, *Babesia bigemina*, *Brucella ovis*, *Babesia traubmanni* and *Borrelia theileri*.

Control of ticks aims at either eradication or prevention and has for a long time depended much on chemical control mainly synthetic chemicals. Main methods of applications include regular dipping of animals and sprays. Despite these novel efforts of control means, ticks control experiences many challenges, which include a rampant development of resistance against common control chemicals such as synthetic pyrethroids, organophosphates, and amitraz. The building and maintenance of dipping tanks or sprays and the purchasing of acaricides for tick control and therapeutic agents hike farmer's production costs.

This situation is pressing for concerted efforts to search for novel effective and eco-friendly anti-tick natural products. Natural sources especially plants are believed to be arsenals of such control agents and due to their versatile application; they are currently the main target. A study from Korea for example with a detailed analysis of ethnoveterinary plants revealed 143 medicinal plants in use for treatment of cattle diseases (Song and Kim, 2010). While some laboratory tests results report moderate toxic effects of herbal plants on adult ticks and larvae (Bagavan et al., 2009). Some plants reveal significant activity against economically important tick species including species resistant to acaricides (Borges et al., 2003; Sunil et al., 2013; Ghosh et al., 2013; Nawaz et al., 2015).

This study was therefore conceived to assess the

activity of crude plant extracts from *S. glaucescens* against cattle ticks of the genus *Boophilus*. This plant has been reported to possess various pharmacological and insecticidal activities especially on its use as anti-ticks and in the post-harvest grain storage by local communities. However, there are no scientific reports regarding its acaricidal potentials against ticks. Nonetheless, other species of this genus have indicated good pesticidal activities against various ectoparasites (Bagavan et al., 2009; Hassan et al., 2012), thus building a base for investigating this plant species.

MATERIALS AND METHODS

Plant materials

Plant materials (leaves and root barks) of *S. glaucescens* Pax were harvested from Mufindi District in Tanzania during May and August 2012. The World Health Organization (WHO, 2003) guideline on Good Agricultural and Collection Practices (GACP) for medicinal plants was used. Thus, roots were dried at room temperature while some minor modifications were considered for leaves in which drying was effected in place with half day shade and half day sun because leaves of this plant contain a large amount of latex (Nyigo et al., 2015). The dried plant materials were pulverized and then subjected to extraction using solvents with different polarities sequentially in ascending order starting with hexane, dichloromethane, ethyl acetate, methanol and ultimately water. After filtration, the extracts were dried *in vacuum* and in a freeze dryer to obtain different organic and water extracts, respectively (Table 1).

Ticks collection for adulticidal testing

Tests of plant extracts against adult ticks were conducted at the Faculty of Veterinary Medicine, Department of Veterinary Medicines and Public Health of the Sokoine University of Agriculture (SUA). Engorged adult ticks (*Boophilus decoloratus*) were collected from naturally infested cattle pastured on local freelance grazing from different areas of Morogoro and Coast regions in Tanzania. During collection, the researchers first enquired information on the application of acaricides to ensure that none has been applied 45 days before tick collection (Rosado-Aguilar et al., 2010; Gazim et al., 2011). Ticks were then washed with water and dried with a paper towel and were subjected into different groups for testing and control.

Adult ticks for larva production

Test of extracts against larvae was conducted at the University of Free State, South Africa. Fully engorged female ticks *B. microplus* and *B. decoloratus* were received from Clinvet International on 7 July, 2014. They were washed with tap water, dried and distributed into 5 conical flasks containing 20 females each. The flasks were incubated at $26 \pm 2^\circ\text{C}$ at a Relative Humidity of $>70\%$ for oviposition and hatching, and the hatch date was determined to be the 26th of August 2014. Testing was performed between 17 and 25 days post hatching.

Sample preparation

Required weights of extract were prepared and dissolved using appropriate solvents. For organic extracts, the decision of solvent to

Table 1. Types of extracts of *S. glaucescens* and their codes.

Codes	Plant part	Extract type
RDCM	Root	Dichloromethane (DCM) extract of root prepared by extracting plant with DCM, after the plant materials extracted by Hexane
Rwater	Root	Water extract of the root after sequential extraction with Hexane, DCM, EtOAc, MeOH; and plant residue extracted with water (H ₂ O)
LDCM	Leaves	DCM extract of the leaves prepared by extracting plant with DCM, after the plant materials having been extracted by Hexane
LMeOH	Leaves	MeOH extract of leaves prepared after sequential extraction with DCM, EtOAc, and plant residue extracted with MeOH
Lwater	Leaves	Water extract of the leaves after sequentially extracted with hexane, DCM, ethyl acetate and MeOH
LEtoH	Leaf	Ethanol Extract; fresh ground dried leaves extracted with ethanol
REtoH	Root	Ethanol Extract; fresh ground root barks extracted with ethanol

use was reached after trials between DMSO and Tween 80. Since the solubility of extracts in DMSO was very low (Figure 1a), Tween 80 was chosen to be the dissolution solvent for organic extracts due its relatively better solubility (Figure 1b). Aqueous extracts were dissolved in distilled water while organic extracts were dissolved in 2% tween in distilled water. With aqueous extracts, the required amount of distilled water was measured and directly poured in the sample while for organic extract samples, the process involved first dissolving an extract in a known amount of tween 80 and diluted with distilled water to make the required volume of a solvent and in both cases, dissolutions were assisted by warming in a water bath. The controls composed of two solvents; 2% tween 80 in distilled water for organic extracts and 100% distilled water for aqueous extracts.

Larval immersion test (LIT)

Larvae obtained from the engorged female ticks of *B. microplus*, and *B. decoloratus* were rested unfed for 16 to 25 days after hatchability (Gazim et al., 2011). Approximately 200 larvae were placed between two round Whatman no 1 filter papers (diameter 120 mm) to form a larvae sandwich, placed in a pie plate. Ten milliliters of 1% solution from plant extracts was then poured over the larvae sandwich to expose them to the solution. Each run also included a positive control (300 ppm -Field concentration of Chlorfenvinphos- Supadip 30% m/v) and a negative control (diluent). After 30 min, excess solution was drained from the filter paper sandwich, then approximately 100 larvae were transferred to a clean filter paper (Whatman no 1, diameter 250 mm) envelope which was crimped closed as well as taped with masking tape over the crimped area to ensure that larvae cannot escape.

The envelopes were then placed in an incubator at a temperature of $26 \pm 2^\circ\text{C}$ and RH $\geq 70\%$ for 72 h. After 72 h each envelope was opened and turned over to allow dead larvae to fall onto a clean filter paper circle (Whatman no 1, diameter 250 mm). Live larvae still clinging to the filter paper envelope were counted by squashing each larva counted onto the filter paper envelope. Then the filter paper containing dead larvae was inspected for any possible live larvae, which were also counted as live and picked up with a masking tape strip. The remaining larvae were then considered dead. Both counts were documented on a datasheet and transferred to a spreadsheet. Efficacy of extract to kill the larvae was determined against a negative control (diluent) by calculating corrected mortality Abbott's formula (Abbott, 1925).

Adulticidal tests through adult immersion test

The adult immersion tests (AIT) as described by Drummond et al. (1976) and Holdsworth et al. (2006) was adopted with some modification for acaricidal activity tests of crude extracts of plant materials from *S. glaucescens* against *B. decoloratus* adult ticks. Ticks were grouped into four groups each with 12 engorged female ticks, three treated with different concentrations (triplicates) and one negative control. Both treatment and control groups were placed in perforated cloth specially made to be able to hold the ticks while allowing them to be in contact with solvents (Figure 2). The ticks were then immersed for five minutes in 20 ml of the diluted crude extract with tween 80 and the control group immersed in tween with distilled water (Rosado-Aguilar et al., 2010) and distilled water alone. The ticks were then transferred into Petri dishes and observed for mortality for a maximum of three days at the condition of temperature and humidity described previously. The criteria used to diagnose dead ticks included the lack of movement of legs and change of cuticle color (Pirali-Kheirabadi and Teixeira da Silva, 2011). Efficacy of extract to kill the adult ticks was determined against negative controls, that is, distilled water for aqueous extracts and 2% tween 80 in distilled water for organic extracts by calculating Corrected mortalities

Definition of test scores for crude extracts

Definition of test scores was adopted from those reported by Rosado-Aguilar et al. (2010) as follows. Activity of crude plant extracts were classified in mean % of mortality of adult ticks and larvae at 24, 48 and 72 h as; high mortality (86-100%); relatively high mortality (71-85%); moderate mortality (56-70%); low mortality 31-55%; and non-significant activity of mortality (0-30%) (Rosado-Aguilar et al., 2010).

Statistical analysis

All data were recorded in an excel sheet and used it to perform descriptive statistics such as arithmetic means of triplicate tests and percentage mortalities of adult ticks and larvae in test and control groups. Efficacy of extract to kill the adult ticks and larvae for all extracts concentrations was calculated using Abbott's formula (Abbott, 1925).

Table 2. Corrected Mortalities (%) of ticks from Root extracts of *S. glaucescens*.

Extract type	Corrected %age Mortalities in different concentrations (mg/ml)								
	24 h			48 h			72 h		
	100	200	300	100	200	300	100	200	300
REtoH	2.86	8.57	9.57	3.06	8.57	12.11	9.57	13.3	16.56
RDCM	5.56	5.56	8.57	5.56	8.57	12.11	8.57	9.97	16.56
Rwater	2.78	5.56	5.56	2.78	2.78	5.56	2.78	5.56	5.56
LEtoH	9.16	9.16	16.7	16.7	16.67	25	25	33.3	33.33
LDCM	0	0	8.33	8.33	16.67	18.21	16.67	18.2	18.21
Lwater	0	0	0	0	0	0	9.05	9.05	16.67

$$\text{Corrected mortality} = \frac{(\text{Mortality in test bottles [\%]} - \text{Mortality in control bottle [\%]})}{(100 - \text{mortality in control bottle [\%]})} \times 100$$

The corrected mortality results of adult ticks were then used to calculate lethal concentrations LC₅₀ and LC₉₀ for each extract using a graph pad Software version 5.0.

RESULTS

Adulticidal tests

Table 2 shows corrected percentage mortalities of adult ticks against different dried extracts of root barks and leaves of *S. glaucescens*. The minimum and highest mortalities in the last day of observation were 2.78 and 33.33%, respectively. These activities are regarded low especially when the highest mortality recorded below 50%, which appeared on the third day of the observation. However, among the extracts, the ethanol extracts from leaves was the most active (33.33%) while water extracts showed the least activity (2.78%).

Figure 3 shows the trend of mortality from day one to the third day of observation. It is evident that despite the low activity of extracts yet the tendency showed that the percentage mortality slightly increased with number of days and with increase in concentrations

Table 3 shows the lethal concentrations LC₅₀ and LC₉₀ of the different extracts. The high values are an indication of less effectiveness of the extracts. After 72 h, the LC₅₀ of almost all of extracts are in terms of thousands except for LEtoH (666.91). This further indicates that the activities of the extracts were low including the most active amongst them.

Larvicidal activities

The larvicidal activity was tested only using two extracts. Table 4 shows the larvicidal activity of methanol and water extracts from the leaves of *S. glaucescens*. Similar results are observed in the larvicidal test as indicated in

the adulticidal tests. Despite their higher susceptibility than adults (Williams et al., 2015), yet the activity of the extract against larvae was low with the highest and least mortality being 37.5 and 3.2% respectively (Table 4) with *B. decoloratus* larvae exhibiting higher resistance as compared to *B. microplus*.

DISCUSSION

Synadenium glaucescens is known for many traditional uses including use as pesticides agent in post harvests storage. Apart from traditional utilization, no any systematic study on acaricidal activity of the crude extracts from this plant had previously been reported. The existing reports are on pesticidal activities of other species in the genus (Afonso-Cardoso et al., 2011, Hassan et al., 2012). Thus, the evaluation of this plant on its effect in ticks is being reported for the first time and was based on these traditional values of the plant species and the existing pesticidal information in the genus. The study doses in this study are high and appear different from many studies that have been done on an acaricidal activity of various plant extracts (Bagavan et al., 2009; Rosado-Aguilar et al., 2010).

This is because during trials for an establishment of concentrations, the lower doses (25 and 50 mg/ml) could not perform well thus, necessitating trials of higher concentrations. Despite high test concentrations, yet extracts showed to exhibit very low activities on the adult ticks at 24, 48 and 72 h (Table 2). This is also indicated by high values of lethal concentrations (Table 3), which imply that the extracts exhibits low acaricidal effects. Therefore, most of the extracts have been grouped to bear non-significant activities while only one extract (LEtoH) exhibit low activity on adult ticks. Though only two extracts were tested for larvae efficacy, similar results have been observed where one extract exhibited low activity and the other exhibiting non-significant activity. This low activity against larvae further justifies the low effectiveness of the extracts as acaricide because larvae have relatively high susceptibility as compared to

Table 3. Lethal concentrations of Adult ticks after immersion in Root extract of *S. glaucescens*.

Extract type	LC ₅₀ and LC ₉₀ of Corrected %age Mortalities(CM) for Root and Leaf					
	24 h		48 h		72 h	
	LC ₅₀	LC ₉₀	LC ₅₀	LC ₉₀	LC ₅₀	LC ₉₀
REtoH	1481.67	2673.92	1130.09	2014.07	1264.26	2418.66
RDCM	3086.16	5741.96	1459.64	2681.02	1158.7	2159.95
Rwater	3463.79	6141.49	3530.46	6408.15	3463.79	6341.49
LEtoH	1220.95	2286.2	933.57	1893.93	666.91	1627.29
LDCM	-	-	920.58	1730.3	4395.24	9590.04
Lwater	-	-	-	-	1208.14	2258.01

Table 4. Corrected percentage mortalities of larvae against leaf extracts of methanol and water.

Extract type	Conc (%)	Total	Alive	Dead	Total	Alive	Dead	Mortality	CM	
										Larva species: <i>B. microplus</i>
LMeOH	1	139	76	63	127	64	63	47.4	37.5	
LWater	1	137	104	33	127	87	40	27.7	14.1	
Larva species: <i>B. decoloratus</i>										
LMeOH	1	54	41	13	95	86	9	14.8	5.1	
LWater	1	104	92	12	119	102	17	13	3.2	

Conc = Concentration; CM = Corrected mortality.

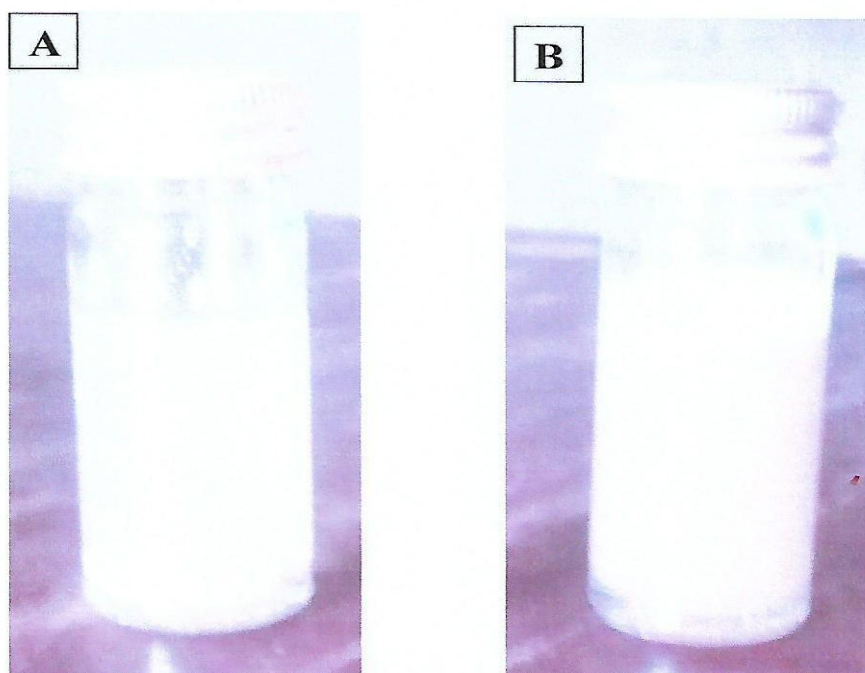
**Figure 1.** Extract dissolves in DMSO (a) and Tween 80 (b).



Figure 2. Adult tick immersion in a test and control solvents.

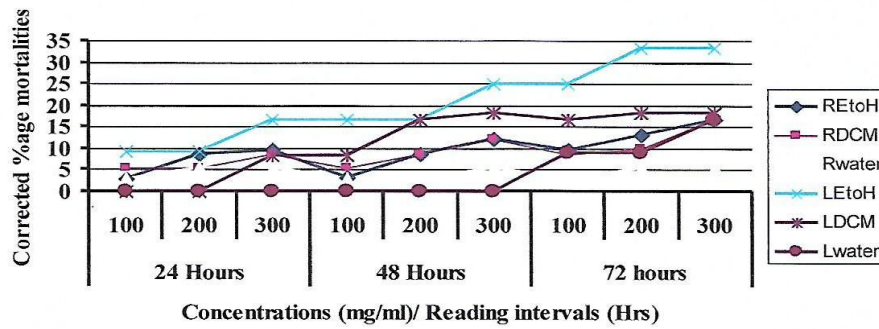


Figure 3. Mortality trends of adult ticks in the observation intervals.

adult ticks (Williams et al., 2015). These results are quite different from researchers' expectations and the claimed traditional efficacy on post-harvest storage protections. The reason for this difference is not well understood. However, it could probably be associated with conditions at which the test materials were used. In the traditional utilization, it is common that people use the fresh plant

materials, but in this case, plant materials were dried for the purpose of standardization. Some changes may have happened on the constituents during processing that resulted from operational conditions such as temperature and pH (Durairaj et al., 2009). Since the current results were observed within 72 h, the duration of observation could also have affected the results especially if the

product has a slow onset of acaricidal actions (Holdsworth et al., 2006). Maybe longer time observations, which have also been the case for some studies could have a different result from the current observation (Holdsworth et al., 2006; Righi et al., 2013). None of the tested extracts could kill even 50% of the test subjects despite the high dosages used. Thus, none of the plant extracts is considered effective against tested ticks species. We, therefore, suggest further research on the plant by using fresh plant materials especially leaves as the fresh leaf latex has also shown to have activities on pest (Afonso-Cardoso et al., 2011).

Conclusion

Since the activity of extracts in adults and larvae were less than 50%, the extracts are concluded to exhibit low to non-significant activity against ticks under the conditions of the test described. Thus, it is not recommended for field trials, rather additional research is needed to determine its potential using fresh plant material especially those with latex.

Conflict of Interests

The authors have not declared any conflict of interests.

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Paper III

Studies on Anti-RT activity of extracts from *Synadenium glaucescens* (Pax)

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Abstract

An *in vitro* HIV-1 reverse transcription (RT) assay was used for screening of anti-HIV activity of extracts from *Synadenium glaucescens*. The assay involved quantitative determination of retroviral RT activity by incorporation of digoxigenin (DIG) and biotin-labeled Complementary Deoxyribose Nucleic Acid (cDNA) strand from Ribose Nucleic Acid (RNA) polymerized by the action of HIV-1 RT. The DIG moieties were then detected by anti-DIG antibodies conjugated to horseradish peroxidase (HRP) enzymes and the absorbance read at 405 nm. Findings indicated that among seven plant extracts tested, three extracts namely, root and leaves together with dichloromethane extracts from roots to have high activities with IC₅₀ values of 3.96 µg/mL, 6.04 µg/mL and 11.43 µg/mL. The other extracts; methanol and ethyl acetate showed low effectiveness in the inhibition of HIV-RT indicated by their very high IC₅₀ values in the inhibition of HIV-1 RT compared to the positive control. The general result however, show that the activity of all extracts were far less than the activity of the positive control (BP 36, IC₅₀ 0.79) but far higher than the negative control (IC₅₀ 0). These findings have thus, demonstrated that aqueous extracts from roots and leaves of *S. glaucescens* together with DCM extracts of root possess potent anti-HIV-1 activity. The results further indicate that since anti HIV activity was exhibited by polar and mid polar fractions, the polar compounds are the probable cause of anti- HIV activities.

1.0 INTRODUCTION

Statistics indicate that Human Immunodeficiency Virus (HIV) continues to be a major global public health issue, having claimed more than 25 million lives over the past three decades. According to 2016 statistics, approximately 36.7 million people were living with HIV/AIDS worldwide (UNAIDS, 2017; WHO, 2017). Eastern and Southern Africa is the

most affected region, with nearly 19.4 million people living with HIV (UNAIDS, 2017) which accounts for approximately 43% of all people living with HIV globally (UNAIDS, 2017).

Since its first appearance in early 1980's, scientists have devoted significant efforts in drug discovery research for cure of HIV/AIDS (WHO, 2002). One among the most important outcome of these efforts is the availability of current highly active antiretroviral therapy (HAART) and no doubt that it has provided a significant impact on HIV/AIDS (Delaney, 2006; Lange and Ananworanich, 2014). These drugs have been vital in the prolongation of survival and to alleviate suffering of the victims. However, none of these drugs is claimed to be a complete cure for this disease. Incidences of resistance of some strains and side effects to patients under HAART have been reported (Boden *et al.*, 1999, WHO, 2017).

Several anti-HIV compounds have been developed. However, there are adverse effects and limitations associated with using chemotherapy for the treatment of HIV infection (Wang *et al.*, 2008). Thus, herbal medicines have frequently been used as an alternative medical therapy by HIV positive individuals and AIDS patients. Consequently, people especially in rural areas have been opting for alternative treatment and the easiest direction has been towards utilization of traditional medicines. The use of traditional medicinal plants by different communities for treatment of various ailments is not new, it has been in use since time immemorial.

In the recent years, the use of herbal drugs in treatment of HIV has become a common phenomenon all around the globe. In Africa, for example traditional herbal medicines are often used as primary treatment for HIV/AIDS and for HIV-related problems including

dermatological disorders, nausea, depression, insomnia and weakness (Hodgsons and Rachanis, 2002). Similarly in Tanzania, the use of plants for HIV treatment and management of opportunistic infection in people living with HIV/AIDS is a common practice and dates back just few years after HIV/AIDS was first reported in early 1980's.

On surveying information regarding the use of plants for traditional treatment of HIV in Zambia, Chinsembu and Hedimbi (2009) documented about 36 plant families containing 46 plant species with known anti-HIV active compounds and known modes of action. Similarly, use of traditional herbal medicine by AIDS patients after HIV diagnosis was noted in one study in Uganda (Langlois-Klassen *et al.*, 2007). Due to the current wide utilization of herbal drugs despite scarcity of evidence on effectiveness and the possibility of serious side effects, some African countries such as South Africa are currently promoting traditional medicines for the treatment of HIV and associated-symptoms (Manfred *et al.*, 2000). *Hypoxis hemerocallidea* (African potato-an immunostimulant) and *Sutherlandia* are among good examples of two plants used for HIV/AIDS treatment in sub-Saharan Africa.

To validate the claims of traditional healing through herbal drugs, scientists have been trying to conduct scientific studies and few of these plant remedies have been scientifically evaluated for efficacy, safety and quality. Many of these tested plants have showed promising results (Mode *et al.*, 2013; Lowe *et al.*, 2014). Two extracts, one from *Trigonostema xiphophylloides* (TXE) and one from *Vatica astrotricha* (VAD) inhibited HIV-1 replication and syncytia formation in CD4+ Jurkat cells, and had little adverse effects on host cell proliferation and survival (Park *et al.*, 2009). Several compounds naturally obtained from plants have shown to possess promising activities that could

assist in prevention and control of this disease. These compounds have been classified as alkaloids, coumarins, phenolics, proteins, sugars, flavonoids, terpenoids, tannins, proteins, alkaloids and inhibit various steps of the HIV life cycle (Chinsembu and Hedimbi, 2009; Helfer *et al.*, 2014). Helfer *et al.*, 2014 further reported *pelargonium sidoides* to have strong ant HIV activity which interferes directly with viral infectivity and blocks the attachment of HIV-1 particles to target cells, protecting them from virus entry. Analysis of the chemical footprint of anti-HIV activity indicates that HIV-1 inhibition is mediated by multiple polyphenolic compounds with low cytotoxicity and can be separated from other extract components with higher cytotoxicity. Although several of these reports are available, there is still a lack of scientific evidence to substantiate these claims and reports. The present research activity was conceived in order to scientifically proof the claims of anti HIV activity of *S. glaucescens* with the main objective being to investigate the anti HIV activity of extracts from this plant.

2.0 MATERIALS AND METHODS

2.1 Plant Materials

Plant materials were collected from Wanging'ombe district in January 2014 and drying was effected in the same month. Root barks were dried under complete shade in specially drying room while leaves were dried under half day sun and half day shade. Leaves needed a certain degree of sun to prevent it from rotting due to possession of large amount of latex. Grinding of dried plant materials was done in February 2014 at the Department of Animal, Aquaculture and Range Sciences, Sokoine University of Agriculture (SUA), Tanzania.

2.2 Methods

2.2.1 Extraction

The powdered root barks and leaves were extracted with different organic and aqueous solvents with increasing polarity. The extraction was done at room temperature by soaking for two days in solvents of increasing polarity starting from hexane, dichloromethane, ethyl acetate, methanol and water. Filtration was done through Whatman No1 filter paper. Solvents from the filtrates were removed from the organic extracts by rotary evaporator with at 40 °C water bath temperatures while water extracts were freeze dried. After complete drying, the extracts (Table 1) were kept in cold room for intended research including bioassays and phytochemistry.

Table 1: Extract Type and Codes

CODES	PLANT PART	EXTRACT TYPE
GR-297-66201B	Root	DCM extract prepared by extracting plant with DCM, after the plant materials extracted by Hexane
GR-297-66201C	Root	EtOAc extract prepared after sequential extraction with Hexane and DCM and plant residue extracted with EtOAc
GR-297-66201D	Root	MeoH extract prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with H ₂ O
GR-297-66201E	Root	Water extract after sequential extraction with the above solvents
GR-297-66202B	Leaves	DCM extract prepared by extracting plant with DCM, after the plant materials having been extracted by Hexane
GR-297-66202C	Leaves	EtOAc extract prepared after sequential extraction with Hexane DCM, EtOAc; and plant residue extracted with EtOAc
GR-297-66202D	Leaves	MeoH extract prepared after sequential extraction with DCM, EtOAc, MeOH; and plant residue extracted with H ₂ O
GR-297-66202E	Leaves	Water extract after sequentially extracted with above solvents

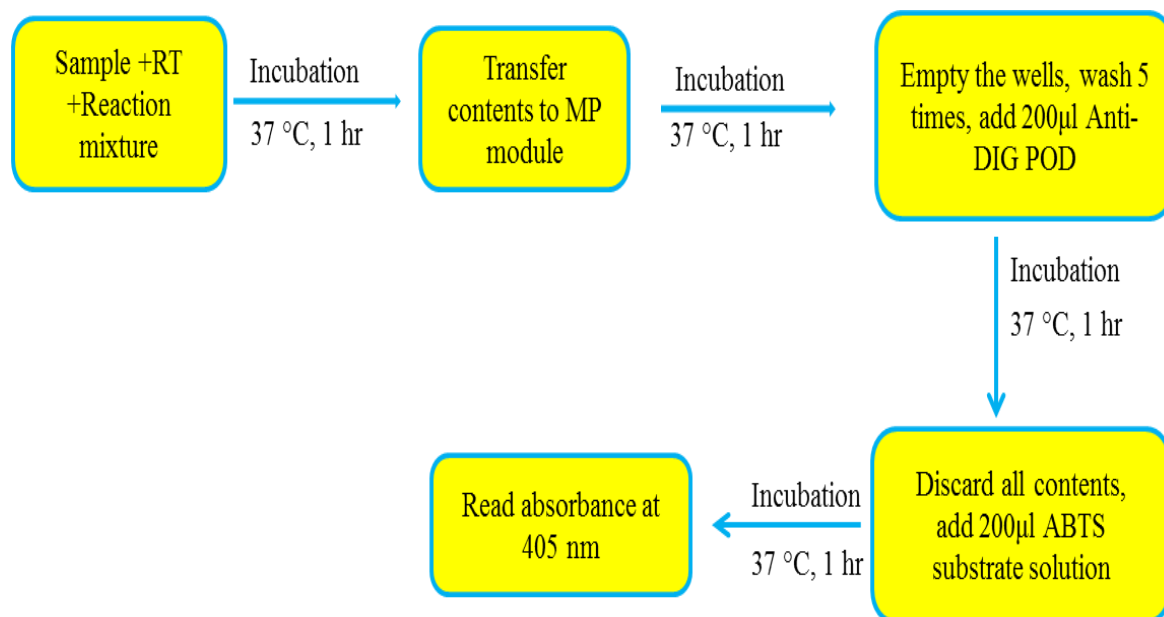
2.2.2 Anti HIV screening of crude extracts through Reverse transcriptase inhibition

The major steps involved in carrying out RT assay are outlined in **Scheme 1**. High through-put screening using six (10-fold) serial dilutions (100-0.001 mg/mL) for each sample in dimethyl sulfoxide (DMSO) was carried out for the crude extracts. The RT enzyme was diluted in lysis buffer to achieve 20 μ L diluted RT/well (=2 ng RT/well). Twenty microlitres of the following were added to wells in a 96-well round bottom plate: lysis buffer (positive control wells); pre-diluted RT (all wells, except blank control); test compounds (test compound wells); and a reaction mixture was added to all wells with samples. The plates were sealed with parafilm and incubated at 37 °C for 1 hour (Roche, 2010).

Ready to use microplate (MP) modules were placed in a strip frame 5 minutes before the end of incubation period. Samples (60 μ L) were transferred from the 96-well plates to the MP modules in the strip frame. The wells were then sealed using a self-adhesive cover foil (parafilm) and incubated at 37 °C for 1 hour. A concentration 200 μ L/well Anti-DIG-POD working dilution was prepared, 10 minutes before the end of incubation period, by diluting an appropriate volume of Anti-DIG-POD solution in conjugate dilution buffer (Roche, 2010).

After incubation, the self-adhesive cover was removed and the wells were emptied by quickly inverting the plate over the sink. No pipette is used at this stage because it will damage the DNA bound to the surface of the plate. The well were washed 5 times with 200 μ l washing buffer per well for about 30 seconds each using a multichannel pipette. The last washing buffer was removed from the wells and 200 μ l of Anti-DIG-POD working dilution was immediately added to each well using a multichannel pipette. The plates were sealed with parafilm and incubated at 37 °C for 1 hour (Roche, 2010).

ABTS (2, 2'-Azinobis [3-ethylbenzothiazole-6-sulfonic acid]-diammonium salt) substrate solution (200 μ L/well) was prepared 30 minutes before end of incubation period, by dissolving ABTS tablet (s) in substrate buffer (1 ABTS tablet is dissolved in 5ml of substrate buffer). The solution is then covered with aluminum foil and kept in the dark at room temperature until use. Following the incubation period, the wells were emptied and 200 μ L of ABTS substrate solution was added to each well using a multichannel pipette. The plates were then incubated in the dark at room temperature for about 2-5 minutes until color development occurs. Absorbance was read at wavelength of 405 nm using a Tecan Infinite F500 plate reader. Blank wells did not contain RT (lysis buffer was added instead of RT), RT activity in these wells is therefore 0%. Positive control wells contains RT, but no inhibitor was added (lysis buffer was added instead of the compound), RT activity in these wells is therefore 100%. Nevirapine, a commercially available drug that is currently used as non-nucleoside RT inhibitor, was used as a standard. RT in 10% DMSO was used as sample control (this indicates the level of RT inhibition due to the presence of the solvent used to dissolve the compounds). Dose-response curves with non-linear regression sigmoidal curves were used to determine the 50% inhibitory concentration (IC_{50}) of plant extracts using Graph Pad Prism, version 6 software (Graph Pad Prism User Guide, 1995-2014) (Appendix A). This was interpreted as the concentration of the extract that reduced RT activity by 50% when compared to controls.



Scheme 1: Outline of major steps in carrying out RT assay

2.2.3 Data analysis

The enzyme (RT) activity was calculated using the following equation (Roche, 2010).

Enzyme activity (%) = $[\text{Absorbance}_{\text{compound}} - \text{Absorbance}_{\text{Blank}}] / (\text{Absorbance}_{\text{positive}} - \text{Absorbance}_{\text{Blank}}) \times 100$. Analyses of concentration-response data were performed by the use of nonlinear curve-fitting program Prism (Graph Pad Software Inc., CA and USA) to determine IC_{50} values. The results were average of 3 independent experiments.

3.0 Results and Discussion

HIV-1 is a member of retrovirus family, virally encoded by reverse transcriptase (RT) enzyme that catalyzes the conversion of viral RNA to proviral DNA. This point is an important target where the test extract may act to inhibit HIV infection. In this study the Anti-RT activity was evaluated in presence and absence of extract. Results on inhibition of RT activity by *Synadenium glaucescens* plant extract fractions are presented in Table

2. Among the seven extract tested, three extracts namely; aqueous root and leaves together with dichloromethane from roots showed to have high retroviral inhibition activities with percentage inhibition of 100 and 98 and 85% at 100 mg/mL respectively. Among the three, water extracts from both root and leaves were the most active with IC_{50} values of 3.96 and 6.04 $\mu\text{g/mL}$ respectively followed by dichloromethane extract which exhibited an IC_{50} of 11.43 $\mu\text{g/mL}$. The methanol and ethyl acetate extracts showed low effectiveness in the inhibition of HIV-RT indicated by their high values of IC_{50} in the inhibition of HIV-RT compared to the positive control, the CSIR proven active plant extract against HIV (Table 2). The general result however, show that the activity of all extracts were far less than the activity of the positive control (BP 36, IC_{50} 0.79) but generally higher than the negative control (IC_{50} 0).

Based on the relatively higher activity shown by the two most active extracts, it suggests that the active compound(s) might be located in the polar portion of these plant extracts. It is therefore expected that the active compound(s) will have polar solubility characteristics. These findings are congruent to study reported by Wang *et al.*, 2008, which reported mid polar and polar compounds from plants as anti HIV. Similarly in his review on anti HIV compounds from natural sources, Singh *et al.*, 2005 reported several classes of anti HIV compounds which are polar and mid polar. These include various classes of compounds such as alkaloids, coumarins, terpenes, quinones, saponins, lignin and flavonoids.

Table 2: Anti-HIV activity of plant extracts from *S.glaucescens* evaluated by neutralization assay

Sample Number	Concentration/%age inhibition							IC ₅₀
	0.01	0.1	0.3	1.6	8	40	100	IC ₅₀
GR-297-66201D	0	17	34	13	43	76	98	11.43
GR-297-66201E	14	25	35	25	69	95	100	3.96
GR-297-66202A	18	28	29	16	17	31	10	0
GR-297-66202B	17	19	17	22	28	27	25	906
GR-297-66202C	0	12	0	0	3	19	12	1000
GR-297-66202D	0	21	7	20	23	17	6	453.1
GR-297-66202E	0	11	14	35	53	79	85	6.04
BP 36 (+VE control)	0	38	30	65	87	97	100	0.79
-VE Control	0	0	7	0	0	0	0	0

Figure 1a and b shows the trend in percentage inhibition of the different extracts with concentrations. The three more active extracts showed a clear increasing trend of inhibition while the other extracts exhibited an undefined trend.

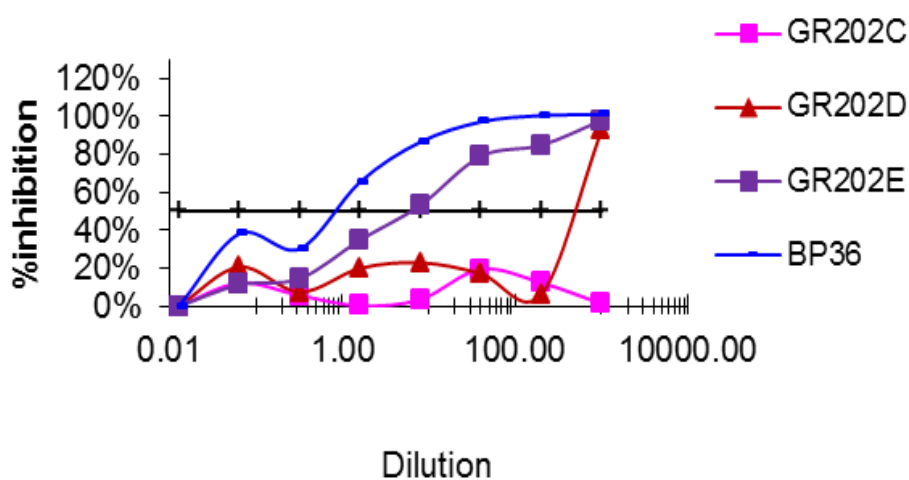


Figure 2a: Concentration versus percentage inhibition of RT of leaves extracts from *S. glaucescens*

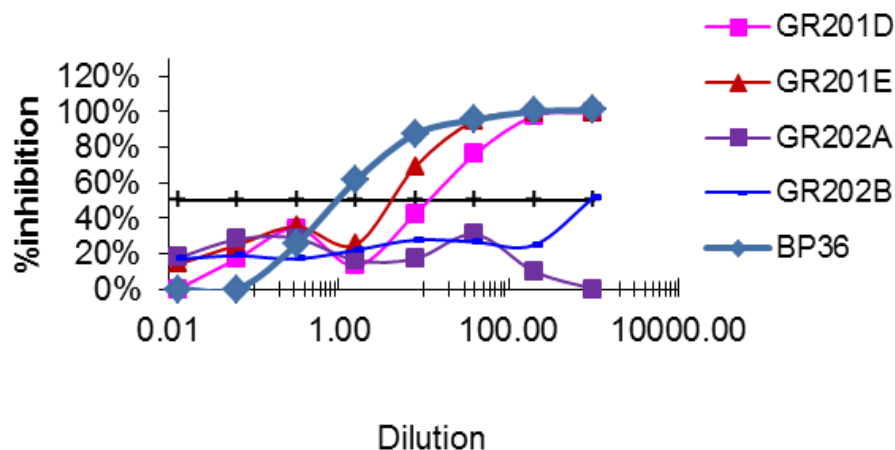


Figure 1b: Concentration versus percentage inhibition of RT of roots and leaves extracts from *S. glaucescens*

CONCLUSION AND RECOMMENDATION

These findings have demonstrated that aqueous extracts from roots and leaves of *S. glaucescens* together with DCM extracts of root possess potent anti-HIV-1 activity. The results further indicate that since anti HIV activity was exhibited by polar and mid polar fractions, the polar compounds are the probable cause of anti-HIV activities. The results therefore suggest that the observed anti HIV activity is attributable to the polar compounds in the crude extracts.

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Paper IV**Isolation and identification of Euphol and β -sitosterol from the Dichloromethane extracts of *Synadenium glaucescens* (Pax)**

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Isolation and identification of euphol and β -sitosterol from the dichloromethane extracts of *Synadenium glaucescens*

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ABSTRACT

Purification of dichloromethane extract from root barks and leaves of *Synadenium glaucescens* respectively resulted into the isolation of two compounds namely Euphol and β -sitosterol. Chemical structures were established mainly by using ^1H and ^{13}C NMR data and by comparing current NMR data with those reported in the literature. Both compounds are known and have been isolated from other plant species but are being reported from this plant species for the first time.

Keywords: *Synadenium glaucescens*, euphol, β -sitosterol

INTRODUCTION

Like many countries around the world, utilization of plants as traditional medicines is a common phenomenon in Tanzania. *Synadenium glaucescens* is a plant species which belongs to a small genus of about 15 species indigenous to eastern Africa [1]. Plants in this genus are shrubs or trees with sub-fleshy cylindrical branches and copious milky latex, monoecious [2]. *S. glaucescens* is among of Tanzanian plants that are traditionally claimed to be used for treatment of various ailments. These include HIV, TB, sores, wounds and worms [3]. Many plants from this genus are known for different biological activities including anti-ulcer activity [4], antioceptive, anti-inflammatory anti-plasmodial activity [5], anti-asthmatic effect [6], anti-tumoral and anti-angiogenic effects [7]. Albeit few studies have so far been conducted in *S. glaucescens*, the recent laboratory findings have shown that the plant exhibits anti-bacterial activities [8], strong anti-viral activities *in vitro* [9] and against infectious basal disease virus together with fowl pox virus [10]. We could however, hardly find any information regarding secondary metabolites isolated from this plant species.

Phytochemical screening of dichloromethane extracts indicated the presence of lanosterol and cycloartenol sterols as main compounds while ethanolic extracts showed to possess phenolic compounds [11]. The only available information on natural product from this plant is the isolation of one pharmacologically active compound of which the establishment of chemical structure was not accomplished. Preliminary study of the isolated compound indicated it to be β -glycosides attached to alkyl group and was deduced to be an inhibitor of contraction of guinea pig ileum [12]. Earlier Euphol was isolated from *Synadenium grantii* [13] and from some other genera in the family Euphorbiaceae [14, 15]. Different bioassay studies have shown great pharmacological potentials of euphol. Research has indicated the ability of this compound to reduce the severity of colitis in two models of chemically-induced mouse colitis. This suggests that this compound could be a potential molecule in the management of inflammatory bowel diseases [16]. Other pharmacological effects include antioceptive effects in inflammatory and neuropathic pains [17], inhibition of tumor artificially induced by 12-O-tetradecanoyl phorbol-13-Acetate [18] and selective inhibitions of human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis [19]. β -sitosterol (2) is among phytosterols which is found in many plants. Many phytosterols are well known for their anticancer effects against different types of cancer [20]. Although it has been isolated from different species [21, 22, 23], this compound is also being reported for the first time from this plant species. β -sitosterol is known to have many health benefits including control of heart diseases, lowering cholesterol content [24] and inhibition of colon cancer [25].

Research works in this paper describes the isolation and structural elucidation of euphol (1) and β -sitosterol (2) from *S. glaucescens* for the first time. The two compounds have however, been isolated earlier from other plant species [14, 21].

MATERIALS AND METHODS

General experimental Procedures

Thin layer Chromatography (TLC) and Column Chromatography (CC) were performed on silica gel 60 as stationary phase (particle size 0.04-0.036 mm, 230-400 mesh, ASTM E. Merck, Germany). Melting point measurements of compounds were done using the reichert thermogalen hot stage microscope (NCRL, Austria 1863) which is adapted to the requirements of thermal microscopy and provides optimum conditions to achieve fast and reliable results. Nuclear Magnetic Resonance (NMR) spectroscopy was used to determine the spatial disposition of the molecular frameworks of the isolated compounds within different chemical environments. NMR Spectral data i.e Proton and carbon spectra including the two dimensional spectra were collected in 600MHz Varian type Nuclear Magnetic Resonance (NMR) spectrophotometer at 30°C temperature in chloroform. Chemical shifts are given in δ (ppm), TMS was used as internal standard material and the coupling constants (J) are given in Hz. A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. [Optimisation of the chromatographic separation was done utilising a Waters BEH C18 column (150 mm x 2.1 mm, 1.7 μ m) and the column temperature controlled at 60 °C.

Plant materials

Plant materials were collected from Njombe region, Tanzania in January 2014 and were immediately subjected for drying. Root barks were dried under complete shade in specialised drying room while leaves were dried under half day sun and half day shade [26] to prevent it from rotting due to its high content of latex. Grinding of dried plant materials was effected at the Department of Animal Science and Production (DASP), Sokoine University of Agriculture (SUA), Tanzania.

Extraction and Isolation

Extraction and isolation were carried out using methods described by Harbone [27]. Thus, Dried root barks (1.18 kg) and leaves (1.8 kg) of *Synadenium glaucescens* were successively extracted by maceration at room temperature with n-hexane [2x3000 ml], Chloroform [2x3000 ml], Ethyl acetate [2x3000 ml], methanol [2x3000 ml] and water [2x3000 ml]. The process involved soaking plant materials for 24 hours (x2). Extracts were obtained by removing solvents from the filtrates through rotary evaporation at 40°C water bath temperature. Ten grams of extracts from root barks was dissolved in 500 ml distilled chloroform and pre adsorbed in silica gel. The sample was then partitioned in a vacuum liquid chromatography (VLC) column and eluted with hexane: ethyl acetate respectively starting with ratios of 10:90, 30:70, 40:60, 50:50, 60:40, 80:20 and 100:0 to obtain 7 different fractions. Fraction 2 and 3 were combined and subjected for repeated column chromatography using ethyl acetate: hexane solvent gradient system in increasing polarity from 10:90 ethyl acetate/hexane to 60:40 ethyl acetate/ hexane to obtain 32 fractions. Fractions 10-13 were combined and further purification in preparative TLC using 20: 80 ethyl acetate/Hexane to obtain compound 1. The dichloromethane leaf extract was partitioned into a silica gel column by first pre adsorbing 2.5g of extract into silica gel and fractionated into a column using hexane/ethyl acetate (5:95 - 0:100) which resulted into 15 fractions. Fraction 2 and 3 were then combined and then partitioned into repeated preparative thin layer chromatography (Prep TLC) using hexane/ethyl acetate (15:85) to obtain compound 2.

Euphol (1): White powder (5 mg); ^1H NMR (CDCl₃, 600 MHz): see Table 1; ^{13}C NMR (CDCl₃, 600 MHz): see Table 1

β -Sitosterol (2): White powder (4 mg); mp: 128-130°C; ^1H NMR (CDCl₃, 600 MHz): see Table 2; ^{13}C NMR (CDCl₃, 600 MHz): see Table 2; MS (m/z): 397 [M-OH]⁺

RESULTS AND DISCUSSION

In this paper we report the isolation of two compounds, a triterpene and a sterol namely respectively as euphol (1) and β -sitosterol (2) [Figure 1]. Both compounds have earlier been isolated from other plant species but being reported from *S. glaucescens* for the first time. Isolation of the compounds was effected through chromatographic technique and their structures established based on NMR spectroscopic data together comparison with other existing data (Table 1 & 2). Both compounds are soluble in chloroform and are white crystalline in nature.

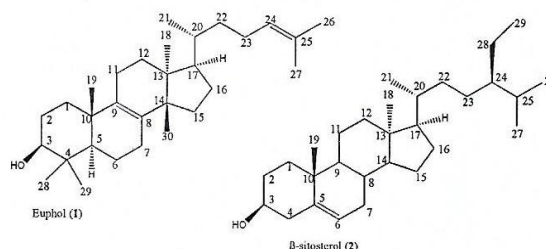


Figure 1: Structures of Euphol (1) and β sitosterol (2)

Compound 1 was obtained through Column Chromatography and pre TLC. The ^{13}C NMR spectra showed 30 carbon signals suggesting that the compound could possess a triterpenoid skeleton. The ^1H NMR spectra exhibited signals mostly concentrated in the high field region which are typically triterpene signals. The more peculiar signals in the proton NMR included an olefinic proton (δ_{H} 5.08, 1H, t) and an axial proton on an oxygen-bearing carbon (δ_{H} 3.21, dd, $J=11.4$, 4.2 Hz). It further exhibited seven singlets characteristic of tertiary methyl groups (δ_{H} 1.66, 1.58, 0.98, 0.93, 0.85, 0.78, 0.73) and a secondary methyl group (δ_{H} 0.84, d, $J=6.0$) which form the eighth methyl groups in the skeleton. The ^{13}C -NMR spectra exhibited four signals (δ_{C} 134.27, 133.78, 131.03 and 125.43) characteristic of olefinic carbons indicating that the compound is unsaturated and contains two double bonds. The carbon signal (δ_{C} 79.29) was characteristic of carbon bearing a hydroxyl group. These data were in agreement with the structure of Euphol (1). The NMR data of 1 (Table 1) is in agreement with the published values [14].

Compound 2 was identified using ^1H and ^{13}C spectra and confirmed using mass spectral data (m/z): 397 [M-OH]⁺. Signals in the ^1H NMR spectrum were observed mainly in the up field region. The spectra exhibited only two signals with high chemical shifts values; the first one resonated in the olefinic region and the other one was observed a little up field region. The olefinic signal at δ 5.3 (1H, br d, $J=4.8$ Hz) appeared to be characteristic of the sterols, and it was assigned to H-6 proton in the β -sitosterol (2) chemical skeleton. The ^1H -NMR spectra of compound 2 also exhibited a signal corresponding to the proton connected to C-3 hydroxyl group which appeared as a multiplet at δ 3.50 (1H, m). Six other proton signals were evident which include four secondary methyl groups (δ_{H} 0.91, 0.82, 0.81 and 0.79) all doublets with $J=6.6$, 7.2, 6.4 and 6.4 Hz respectively) and two tertiary methyl groups (δ_{H} 0.66 and 0.99). The ^{13}C NMR spectra exhibited 29 carbon signals, characteristic of phytosterols. These data were in agreement with the structure of β -sitosterol (2). The NMR data of 2 (Table 2) is in agreement with the published values [21].

Table 1: ^1H and ^{13}C NMR data for compound 1 and from the literature

Position	Experimental		Literature [14]	
	^{13}C	^1H	^{13}C	^1H
C-1	35.49		35.4	
C-2	27.9		27.7	
C-3	79.2	3.21 (dd, 1H, J=1.4, 4.2 HZ)	78.9	3.24, (1H, dd, J = 11.6, 4.8 HZ)
C-4	37.5		37.3	
C-5	51.2		50.9	
C-6	19.17		18.9	
C-7	28.16		27.9	
C-8	134.3		134	
C-9	133.8		133.5	
C-10	39.2		38.9	
C-11	21.7		21.5	
C-12	28.3		28.1	
C-13	44.4		44.1	
C-14	50.3		49.9	
C-15	31.1		30.9	
C-16	29.9		29.8	
C-17	49.9		49.6	
C-18	15.74	0.78 (3H, s)	15.6	0.80 (3H, s)
C-19	20.36	0.93 (3H, s)	20.1	0.95 (3H, s)
C-20	36.07		35.8	
C-21	19.12	0.83 (3H, d, J=6.0)	18.9	0.84 (3H, d, J=3.3HZ)
C-22	35.66		35.2	
C-23	24.74		24.7	
C-24	125.43	5.08 (t, 1H)	125.2	5.09 (1H, t, J=7.5HZ)
C-25	131.03		130.8	
C-26	17.88	1.66 (3H, s)	25.7	1.68 (3H, s)
C-27	25.93	1.58 (3H, s)	17.7	1.60 (3H, s)
C-28	24.68	0.85 (3H, s)	24.4	0.87 (3H, s)
C-29	28.27	0.98 (3H, s)	28	1.00 (3H, s)
C-30	15.85	0.73 (3H, s)	15.5	0.75 (3H, s)

Table 2: ^1H and ^{13}C NMR data for compound 2 and from the literature

Position	Experimental		Literature [21]	
	^{13}C	^1H	^{13}C	^1H
1	37.5		37.5	
2	40		31.9	
3	72.1	3.50 (m, 1H)	72	3.53 (tdd, 1H, J=4.5, 4.2, 3.8 HZ)
4	42.6		42.5	
5	141	5.33 (t, 1H)	140.9	35.36 (t, 1H, J = 6.4HZ)
6	121.9		121.9	

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7	32.2		32.1	
8	31.9		32.1	
9	50.4		50.3	
10	36.8		36.7	
1	21.3		21.3	
12	40.0		39.9	
13	42.6		42.6	
14	57.0		56.9	
15	26.4		26.3	
16	28.5		28.5	
17	56.3		56.3	
18	36.4		36.3	
19	19.3	0.91 (d, 3H, J=6.6)	19.2	0.93 (d, 3H, J=6.5 HZ)
20	34.2		34.2	
21	24.5		26.3	
22	46.1		46.1	
23	23.3		23.3	
24	12.2	0.82 (t, 3H, J=7.2HZ)	12.2	0.84 (t, 3H, J=7.2HZ)
25	29.4		29.4	
26	20.0	0.81 (d, 3H, J=6.4HZ)	20.1	0.83 (d, 3H, J=6.4HZ)
27	19.6	0.79 (d, 3H, J=6.4)	19.6	0.81 (d, 3H, J=6.4)
28	19.0	0.66 (s, 3H)	19.0	0.68 (s, 3H)
29	12.1	0.99 (s, 3H)	12.0	1.01 (s, 3H)

Limitation of the study

No biological assays were conducted for these compounds. The intended assays were anti HIV tests but the assay facilities were not available in the country. Samples needed to be sent abroad but time was limiting due to fact that the researcher was a student with a bound registration period

CONCLUSION

Two compounds, a triterpene and a sterol were isolated for the first time from *S. glaucescens*. Respectively, the compounds were isolated from root barks and leaves. The structures of the isolated compounds were identified as euphol (1) and β -sitosterol (2) on the basis of ^1H and ^{13}C NMR spectroscopic data and by comparing them to those reported in the literature.

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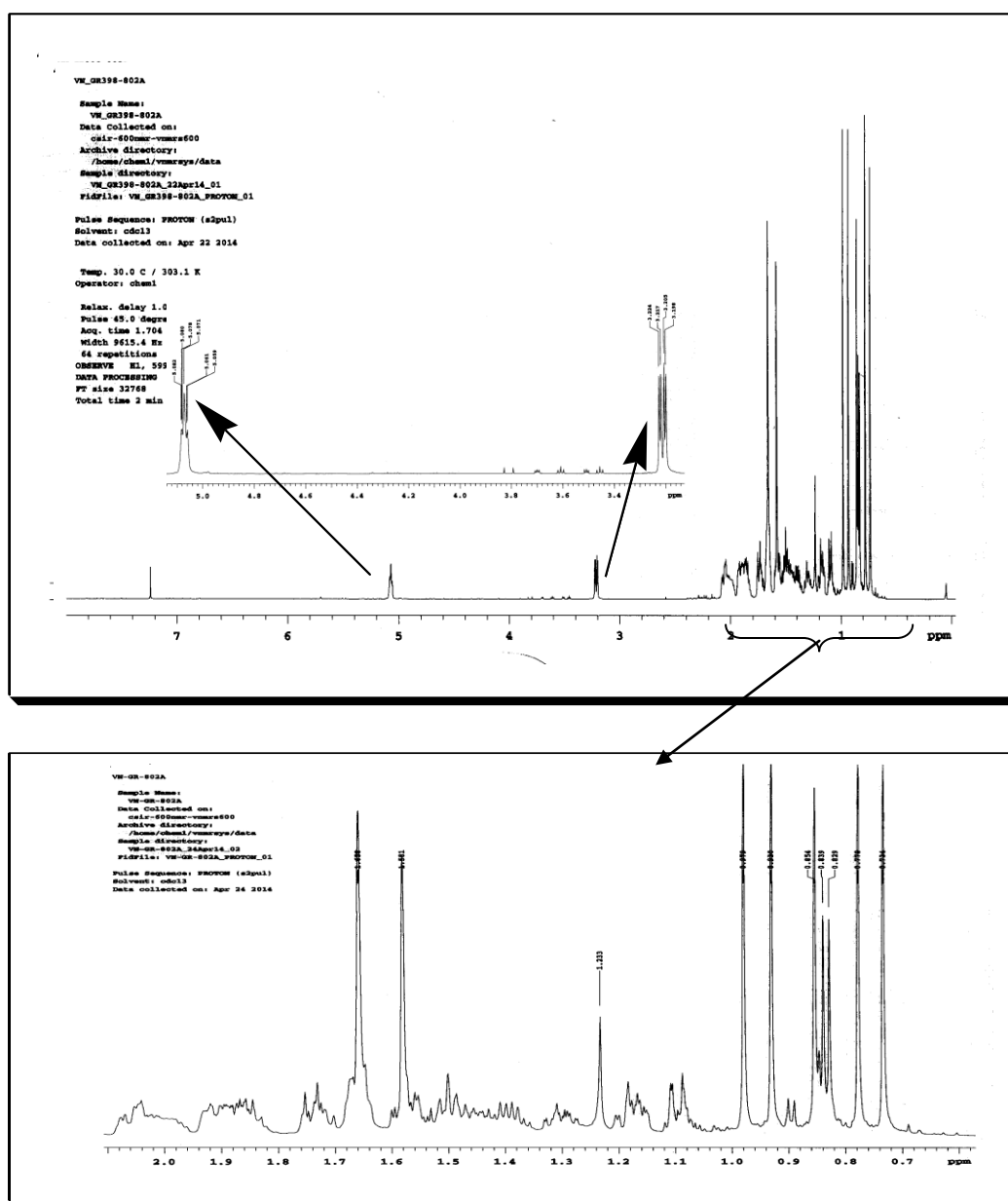
Nyigo VA, Peter X, Mabiki F, Malebo HM, Mdegela RH, Fouche G. Isolation and identification of euphol and β -sitosterol from the dichloromethane extracts of *Synadenium glaucescens*. The Journal of Phytopharmacology 2016;5(3):100-104.

SUPPLEMENT I

For paper IV: NMR spectra for Euphol (1) and β -sitosterol (2)

SUPPLEMENT I

Paper IV: NMR spectra for compound 1 and 2

Fig 1: ^1H - NMR spectrum for Euphol with expansions

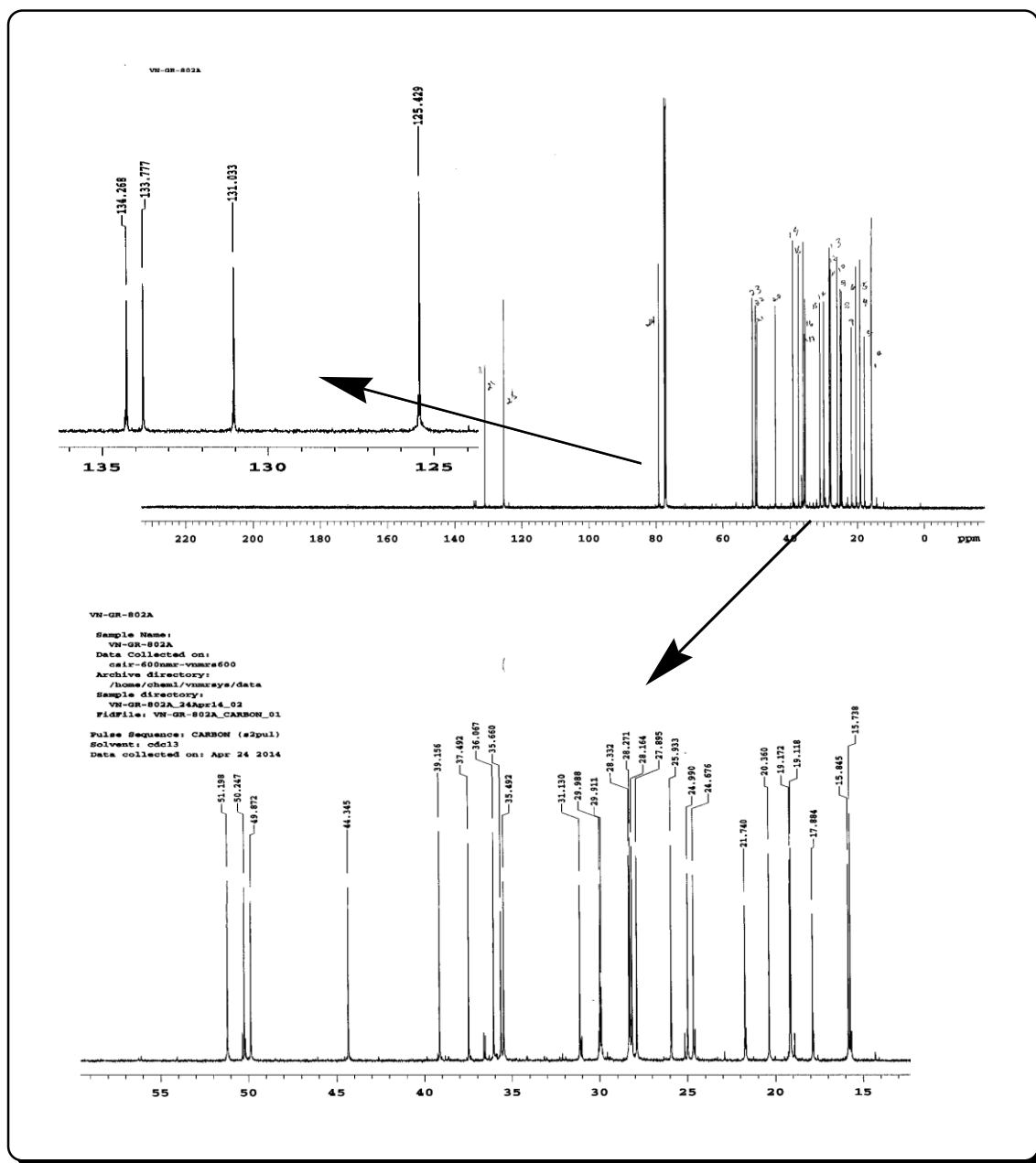


Fig 2: ^{13}C - NMR spectrum for Euphol with expansions

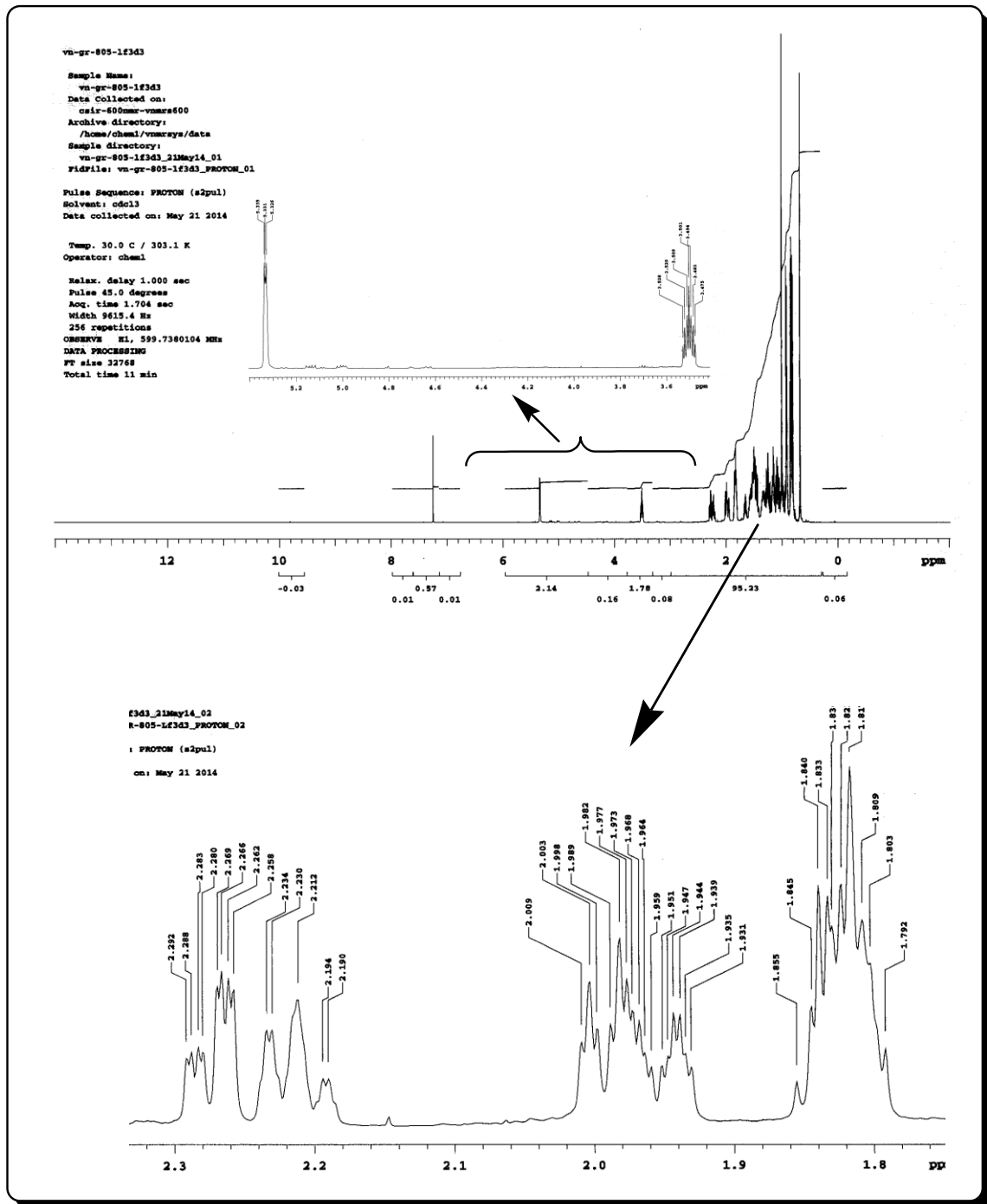


Fig 3: ^1H - NMR spectrum for β -sitosterol with expansions

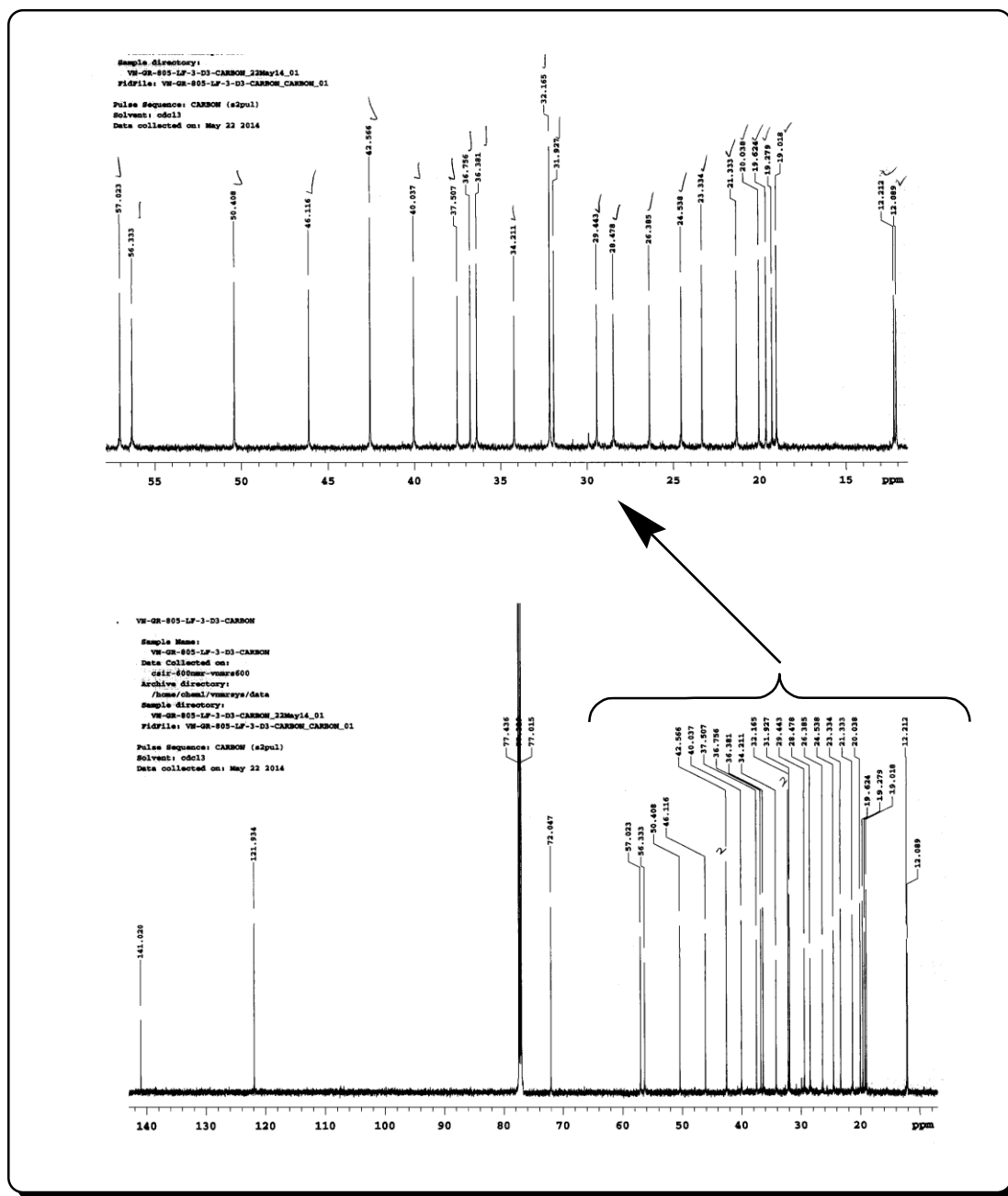


Fig 4: ^{13}C - NMR spectrum for β -sitosterol

PAPER V**Isolation and Identification of Natural Products with Long Carbon-Chain from
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**Isolation and Identification of Long -Chain Aliphatic Compounds from *Synadenium
Glaucescens***

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Morogoro, Tanzania

Abstract

Purification of dichloromethane extract from root barks and leaves of *Synadenium glaucescens* extracts through chromatographic techniques resulted into the isolation of two compounds, namely erythrinacinate C and 1- octacosanol. Chemical structures were established mainly using both one and two dimensional ¹H and ¹³C NMR data and by comparison of the current NMR data with those from literature. Mass spectrometry data were used for confirmation through molecular ion peak. Both compounds are known to have been isolated from other plant species but are being reported from this plant species for the first time.

1.0 INTRODUCTION

Synadenium glaucescens has many recorded traditional utilization including medicinal uses and others such as construction materials and firewood. Despite the recent biological investigation that has so far been conducted for this plant (Mabiki *et al.*, 2013; Nyigo *et al.*, 2015), very little is known regarding phytochemical investigation for this plant. This study was therefore deliberately conceived to investigate for chemical composition of this plant. Thus, phytochemical investigation of dichloromethane extracts of root barks and leaves respectively resulted into the isolation of erythrinacinate C (**1**) and 1-Octacosanol (**2**). erythrinacinate C (**1**) is an ester of ferulic acid and was first isolated from *Erythrina spp* with very limited bio assay data. The only available information regarding its bioassay information is the anti-microbial assay against various bacteria which indicated the compound to possess low potency as it possessed minimum inhibitory concentrations of above 100 µg/mL (Nkengfack *et al.*, 1997). 1-Octacosanol (**2**) is among long chain aliphatic alcohols collectively known as polyicosanols. Although it is being reported for the first time from this plant species, this compound has earlier been isolated from other plants of different species, including *Symplocos racemosa* (Gupta, 2015), *Tinospora cordifolia* (Thippeswamy *et al.*, 2008) and *Holoptelea integrifolia* (Ganie and Yadav, 2014). It has also been reported to be isolated from other sources especially waxes (Jackson and Eller, 2006). Information shows important uses of long-chain aliphatic alcohols such as treatment of various chronic diseases including diabetes and hypercholesterolemia (Damgé *et al.*, 1995; Yacilla, 2002; Tayler *et al.*, 2003, Jackson and Eller, 2006). Octacosanol has specifically been reported to possess antinoceptive and anti-inflammatory activities (De Oliveira *et al.*, 2012), which signify the relevance of the compound for the pharmacological control of pain and inflammatory processes.

2.0 MATERIALS AND METHODS

General methodology

Thin layer Chromatography (TLC) and Column Chromatography (CC) were performed on silica gel 60 as stationary phase (particle size 0.04-0.036 mm, 230-400 mesh, ASTM E. Merck, Germany). Melting point measurements of compounds were done using the reichert thermogalen hot stage microscope (NCRL, Austria, 1863) which is adapted to the requirements of thermal microscopy and provides optimum conditions to achieve fast and reliable results.

Nuclear Magnetic Resonance (NMR) spectroscopy was used to determine the spatial disposition of the molecular frameworks of the isolated compounds within different chemical environments. NMR Spectral data i.e. Proton and carbon spectra including the two dimensional spectra were recorded on 600 MHz Varian type Nuclear Magnetic Resonance (NMR) spectrophotometer at 30 °C temperature in chloroform. Chemical shifts are given in δ (ppm), TMS was used as internal standard material and the coupling constants (J) are given in Hz. A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data. Optimisation of the chromatographic separation was done utilising a Waters BEH C18 column (150 mm x 2.1 mm, 1.7 μ m) and the column temperature controlled at 60 °C.

2.1 Plant materials

Plant materials were collected from Mtulingara village in Njombe district and were immediately subjected to drying process. Root barks were air dried under the shade in special drying room while leaves were dried under half day sun and half day shade (Nyigo *et al.*, 2015). The need for some degree of sun heat for leaf drying was due to the

fact that it possesses large amount of latex, thus preventing it from rotting. Grinding of dried plant materials was effected at the Department of Animal Science and Production (DASP), Sokoine University of Agriculture (SUA), Tanzania.

EXTRACTION AND ISOLATION

Respectively, 800 g and 1.2 kg of dried root barks and leaves of *Synadenium glaucescens* were extracted at room temperature successively with n-hexane [2 x 2000 mL], dichloromethane [2 x 2000 mL], ethyl acetate [2 x 2000 mL], methanol [2 x 2000 mL] and water [2 x 2000 mL]. The filtrates from organic solvents were evaporated using a rotary evaporator with 40 °C water bath temperatures and the aqueous extracts were freeze dried. The process involved soaking plant materials for 24 hours (x2) for each extraction in solvents of increasing polarity. After complete drying the extracts were kept in cold room until required for bioassays and phytochemistry.

Thirteen grams of DCM extracts from root bark was dissolved in 300 mL of distilled chloroform and was pre adsorbed on silica gel. The sample was evaporated through rotary evaporator and then subjected to VLC column and eluting with gradient solvent mixtures of ethyl acetate/hexane (5:95-100:0) followed by chloroform/methanol (5:95-20:80) and a total of 13 VLC fractions were obtained. Fraction 3 and 4 were combined and subjected for repeated column chromatography. Initially gradient elution was applied using ethyl acetate: hexane in increasing polarity from 10: 90 to 20: 80 ethyl acetate/ hexane. Then isocratic elution was applied using 20:80 ethyl acetate/hexane mixture to obtain 25 fractions. Fractions 10-15 were combined and subjected for purification on prep TLC which yielding compound **1**. The pre adsorbed dichloromethane leaf extract (5 g) was subjected to VLC and eluted using a gradient ethyl acetate/hexane which resulted into 12

VLC fractions. Fraction 3 (20:80 ethyl acetate/hexane) was subject into column and was eluted using an isocratic (20:80 ethyl acetate/hexane) solvent system. Crystalline compound obtained in fractions 11 through 13 were washed using methanol to obtain compound **2**.

RESULTS AND DISCUSSION

Compound 1 was isolated as white crystals. The molecular formula was determined to be $C_{24}H_{38}O_4$ based on both one and two dimensional 1H and ^{13}C spectra and MS data. Thus MS analysis displayed a molecular ion peak at m/z 413 $[M + Na]^+$. The proton NMR exhibited signals at δ 7.60 (d, $J=15.6$ Hz, 1H) and δ 6.28 (d, $J=15.6$ Hz, 1H). The coupling constants of 15.6 Hz implied that the signal belong to olefinic protons in a trans-position and were assigned to carbon 1' and 2'. One 1H doublet of doublet at δ 7.15 ($J= 8.4$ 1.8 Hz) represented the aromatic H-2. The coupling constants are typical of meta- and ortho-splitting patterns between H-3 and H-2 and a long range coupling in a W patterns through conjugation to H-6. A 1H doublet at δ 6.90 ($J = 8.4$ Hz) was ascribed to another aromatic proton H-3. The ortho coupling constant is typically explaining the coupling with H 2. A proton doublet at δ 7.02 ($J = 1.8$ Hz) was assigned to H-6 while a 3H singlet at δ 3.91 was typical of a methoxy group and the presence of one hydroxyl group was shown by a broad singlet at δ 5.81. The broad singlet at δ 1.10 – 1.38, the presence of two proton triplets at δ 4.17 and the three proton triplets at δ 0.87 indicated the presence of a long chain aliphatic moiety. The signal δ 4.17 and δ 0.87 are typical of the methylene and methyl protons of the aliphatic chain attached respectively at the beginning and at the end of the aliphatic chain. The two-dimensional (2D) NMR spectra, mainly HMBC, HSQC and COSY were key in supporting the proposed structure. COSY revealed correlations between H2'-1', H2-3 and H-1''-2''. In the HMBC, the methoxy proton at carbon C-5

showed long range correlations to hydroxyl bearing carbon (C-4). Proton at C-6 showed long range correlation to C-4, C-2 and C-1. The carbonyl carbon (C-3') showed HMBC long range correlations with protons at C-1' and C-1". These data are also supported by the ^{13}C -NMR data. The signal at δ 169.90 indicated the presence of carbonyl carbon (C-3') of an ester group. The presence of eight unsaturated carbons in the low field region characteristic of an aromatic carbons and one extra double bond outside the aromatic region were in line with signals in ^1H -NMR. The signals at δ 144.6 and δ 109.3 were due to side chain C-C double bond and the signals were assigned to C-1' and C-2' respectively. Except for carbon 1" at 67.24 and the terminal methyl group at δ 14.1, the specific assignment of most carbon resonances from the side chain was tricky due to their structural similarity. Thus, the numbers of CH_2 groups between the two signals were determined through integration. The structure was then compared with data from literature (Volken, 1999; Singh *et al.*, 2005; Wang *et al.*, 2013). All these data together confirmed an ester of ferulic acid. The complete ^1H - and ^{13}C -NMR chemical shift of the compound and those from literature are shown in Table 1.

Compound 2: Was obtained as white crystals from column fractions (Ethyl acetate/hexane) and the structure were determined based on NMR and MS data. Thus, the Mass spectrum displayed a molecular ion peak at m/z values of 409 $[\text{M} - \text{H}]^-$. The proton NMR exhibited four signals, the broad singlet at δ 1.10 – 1.38, the presence of two proton triplets at 3.52, the quintet at δ 1.55 and the three proton triplets at δ 0.86. These signals are characteristic of a long chain aliphatic moiety and the compound was concluded to be a fatty alcohol Wang *et al.*, 2013. The molecular formula was determined as $\text{C}_{28}\text{H}_{58}\text{O}$ which corresponded to *n*-octacosanol. The ^{13}C NMR data of *n*-octacosanol showed only one methyl group (δ_{C} 14.10), all other signals were methylene groups, one of them (δ_{C} 63.11) attached to an hydroxyl functional group. These findings indicated the presence of

a fatty alcohol. The length of the methylene chain was determined by means of the integrals resulting from the ^1H NMR spectrum. Interpretation of these integrals indicated the presence of 25 methylene groups at δ_{H} 1.27, leading to the final formula $\text{CH}_3-(\text{CH}_2)_{27}-\text{OH}$ and was identified as 1-octacosanol. The melting point (80-82 $^{\circ}\text{C}$) is typical of 1-octacosanol.

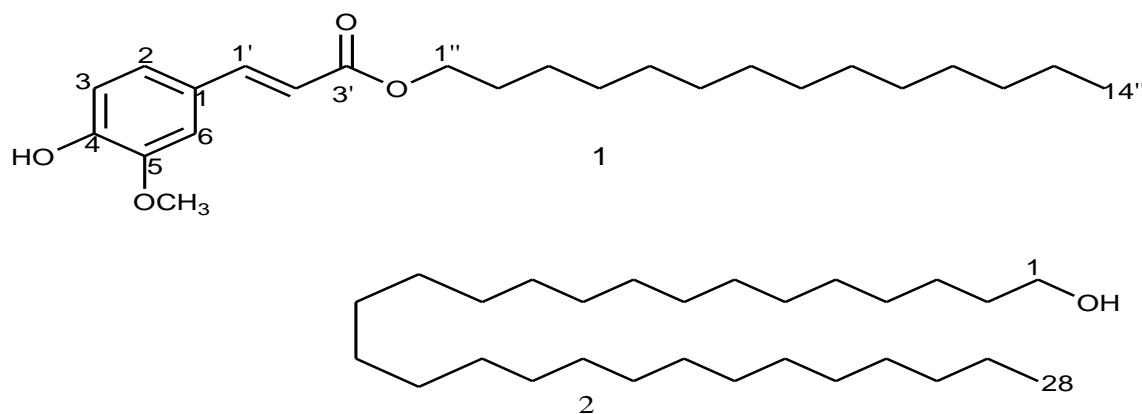


Fig 1: Structures of erythrinacinate C (1) and octacosanol (2)

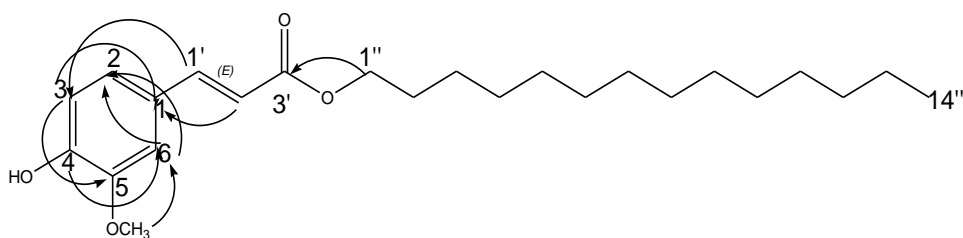


Fig 2: HMBC correlation of erythrinacinate C (1)

Identification of erythrinacinate C (1) and 1-octacosanol (2)

Erythrinacinate C (1): White crystals (10 mg); mp: 75-76 $^{\circ}\text{C}$ ^1H NMR (CDCl_3 , 600 MHz): see Table 1; ^{13}C NMR (CDCl_3 , 600 MHz): see Table 1; MS (m/z): 413 $[\text{M} + \text{Na}]^+$.

1-Octacosanol (2): White crystals (20 mg), mp: 80-82°C ^1H NMR (CDCl_3 , 600 MHz): see Table 4; ^{13}C NMR (CDCl_3 , 600 MHz): MS (m/z): 409 $[\text{M} - \text{H}]^-$.

Table 1: ^1H and ^{13}C NMR Data for compound 1

Carbon no	Experimental		Literature	
	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -NMR
C-3'	167.4		167.3	
C-3	147.9		147.9	
C-4	146.8		147.9	
C-1'	144.6	7.60 (d, 1H, J=15.6Hz)	144.6	7.60 (1H, d, J = 16.0, =CH ₂ , H-2)
C-1	127.1		127.1	
C-6	123.0	7.06 (dd, 1H, J=1.8Hz, 8.4Hz)	123.0	7.06 (dd, J = 8.6, 2.2 Hz, H-6)
C-2	115.8	7.02 (d, 1H, J= 1.8Hz)	115.7	7.02 (1H, d J=2.2)
C-5	114.7	6.90 (d, 1H, J=8.4Hz)	114.7	6.93 (1H, d J = 8.6, H-5)
C-2'	109.3	6.27 (d, 1H, J=15.6Hz)	109.3	6.26 (1H, J = 16.0 HZ, =CH ₂),
C-1''	64.6	4.17 (t, 2H, J = 6.6Hz)	64.6	4.15 (2H, t, J = 7.3 HZ, OCH ₂ R)
3-OMe	56.0	3.91 (s, 3H, -OCH ₃)	55.9	3.8 (3H, s, 3-OCH ₃)
C-12''	31.9		31.9	
C-10''	29.7		29.7	
C-6''	29.7		29.7	
C-8''	29.6		29.7	
C-9''	29.6	1.12-1.38 (24H, bs)	29.7	1.10 - 1.39 (24H, bs (CH ₂) ₁₂)
C-7''	29.6		29.7	
C-5''	29.6		29.7	
C-11''	29.4		29.3	
C-4''	29.3		29.3	
C-2''	28.8		28.8	
C-3''	26.0		26.0	
C-13''	22.7		22.7	
C-14''	14.1	0.87 (t, 3H, J = 7.2 Hz)	14.1	0.88 (3H, t, J = 7.3Hz, Me)

CONCLUSION

Two compounds, including an ester of ferulic acid and a fatty alcohol were isolated from *S. glaucescens*. The first compound was isolated from root bark while the second was isolated from leaves of *S. glaucescens*. The structures of the isolated compounds were identified as erythrinacinate C (**1**) and 1-Octacosanol (**2**) on the basis of spectroscopic data and by comparing with those reported in the literature. The complete ^1H and ^{13}C NMR spectral assignments of the isolated compounds were made based on COSY, HSQC, HMBC and spectroscopic data.

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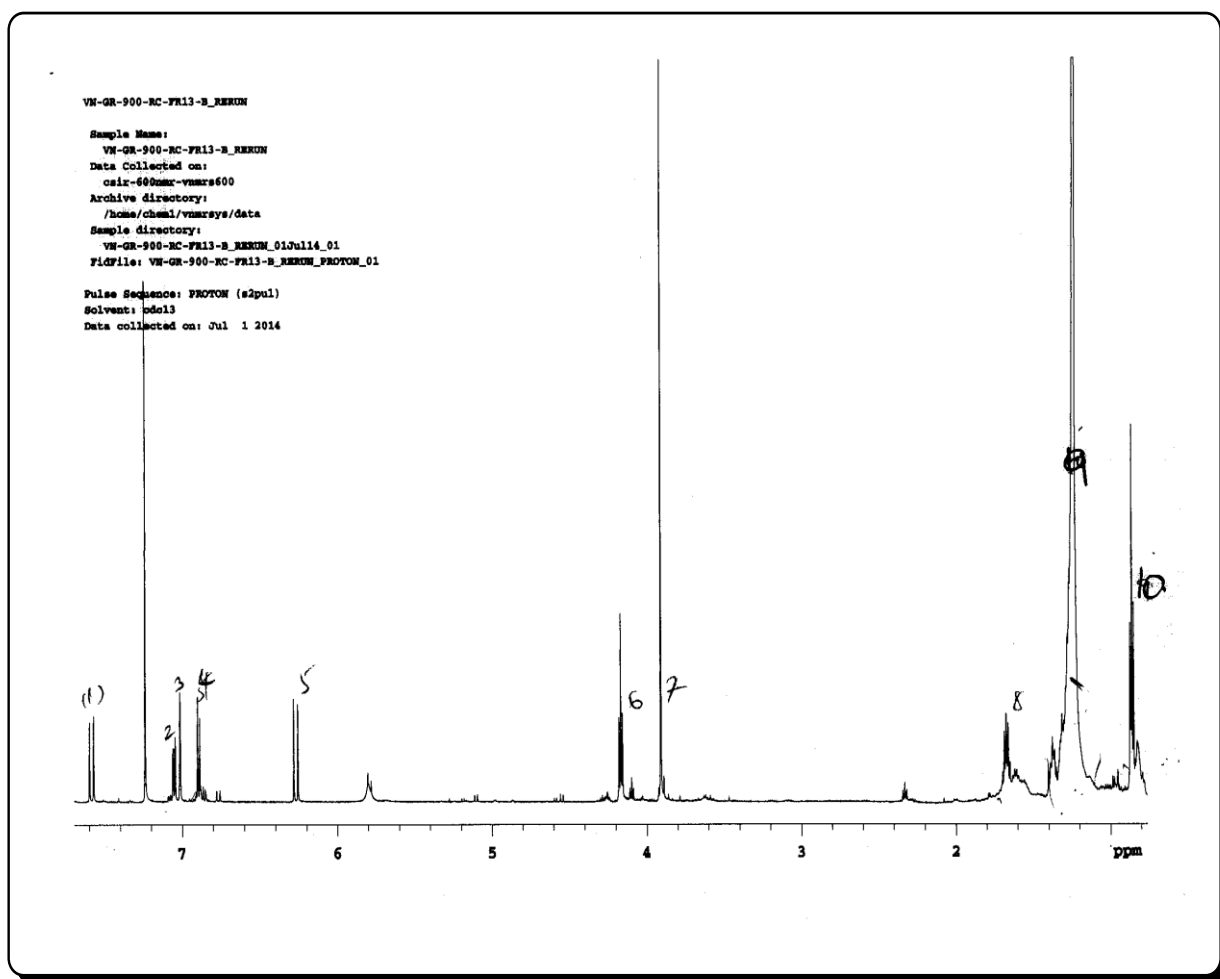
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SUPPLEMENT I

For paper V: NMR and MS spectra for Erythrinacinate (1) and 1-Octacosanol (2)

SUPPLEMENT I

Paper V: NMR and MS spectra for compound 1 and 2

Figure 1 (a): $^1\text{H-NMR}$ for Erythrinacinate C (whole spectrum)

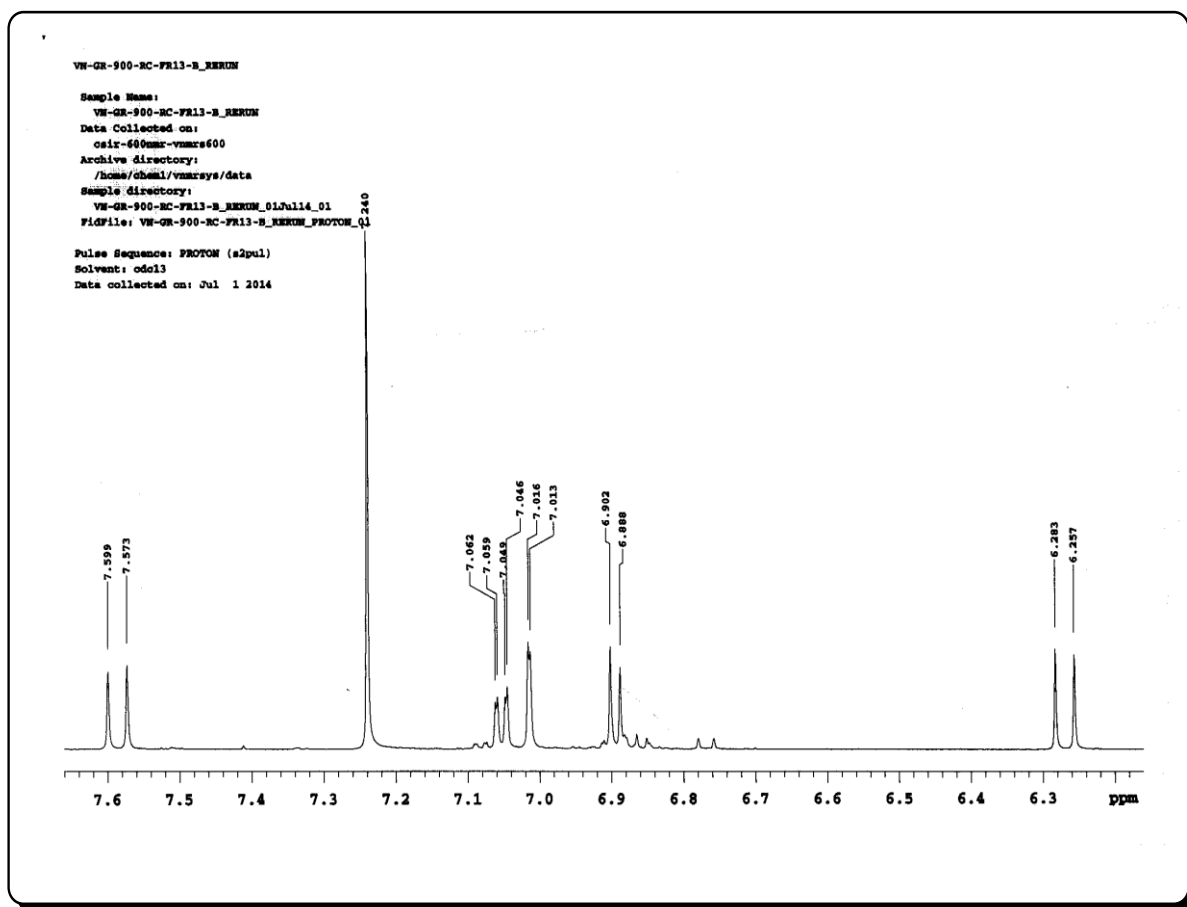


Figure 1 (b): ¹H-NMR for Erythrinacinate C (an expansion)

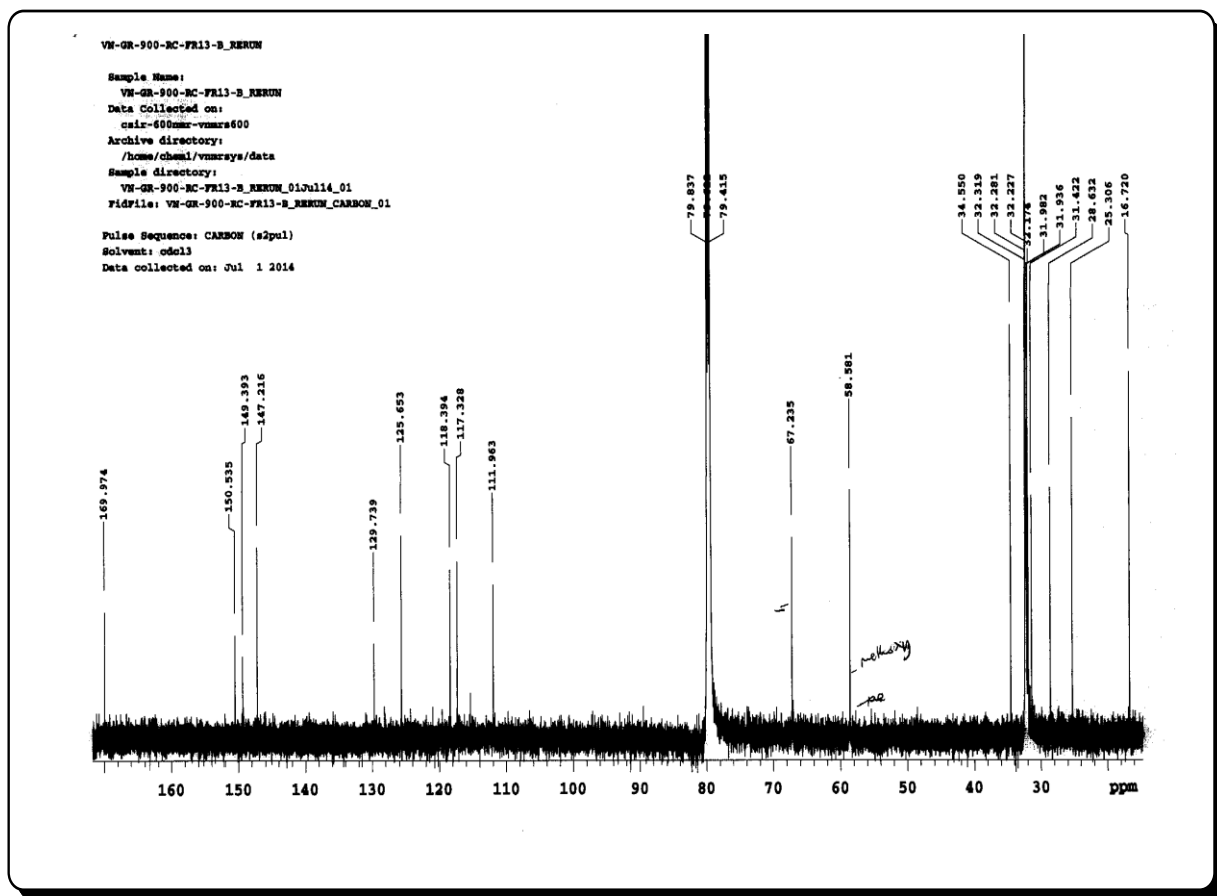


Figure 2: ^{13}C -NMR for Erythrinacinate C

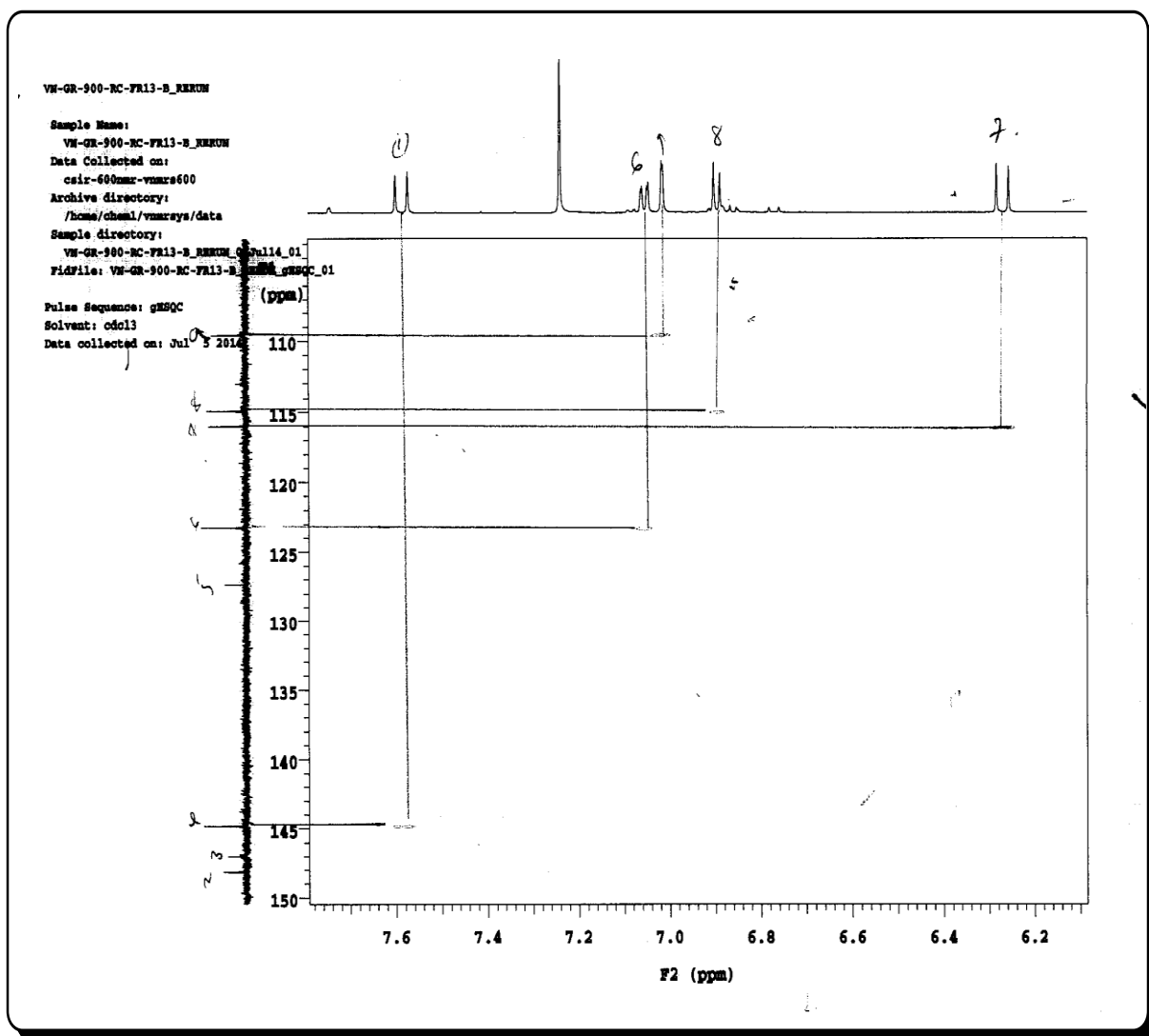


Figure 3: HSQC plot for Erythrinacinate C

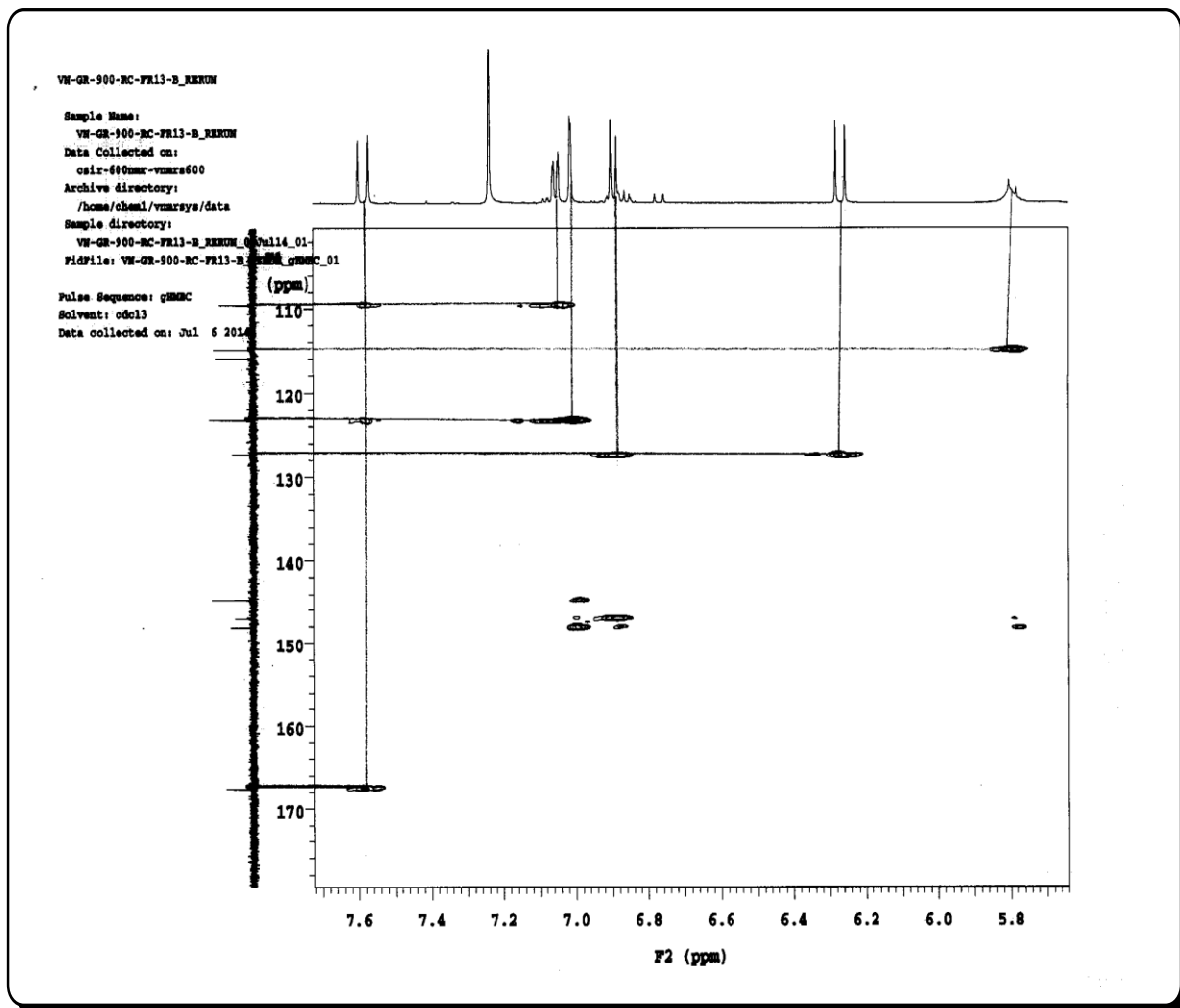


Figure 4: HMBC plot for Erythrinacinate C

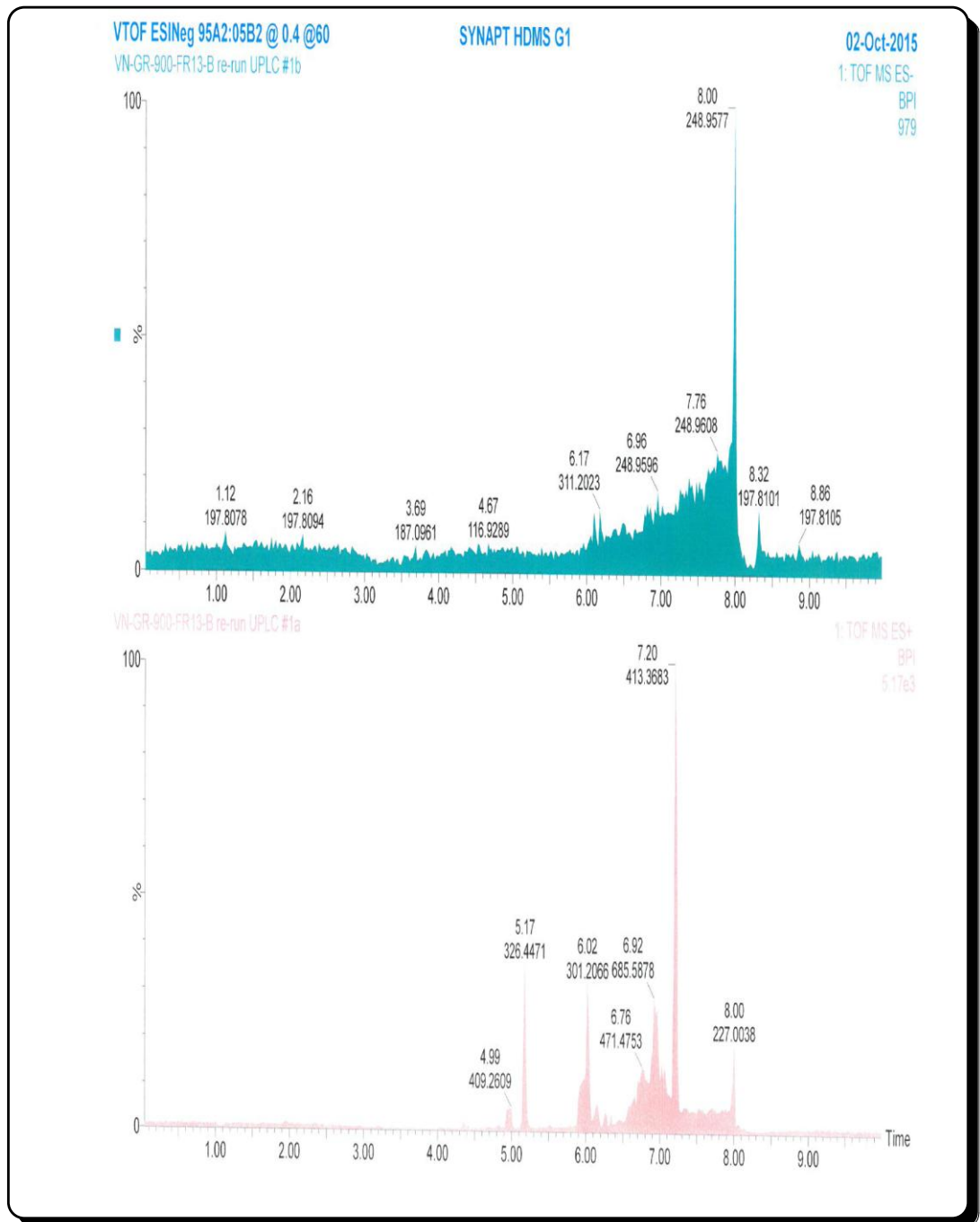
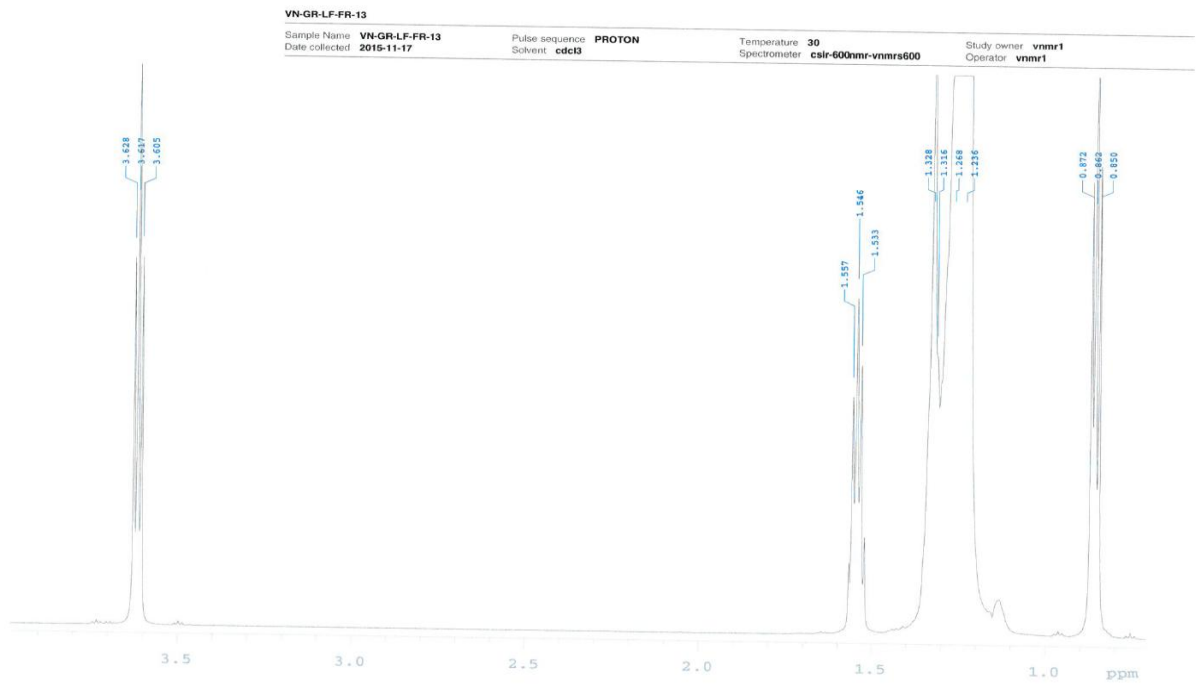
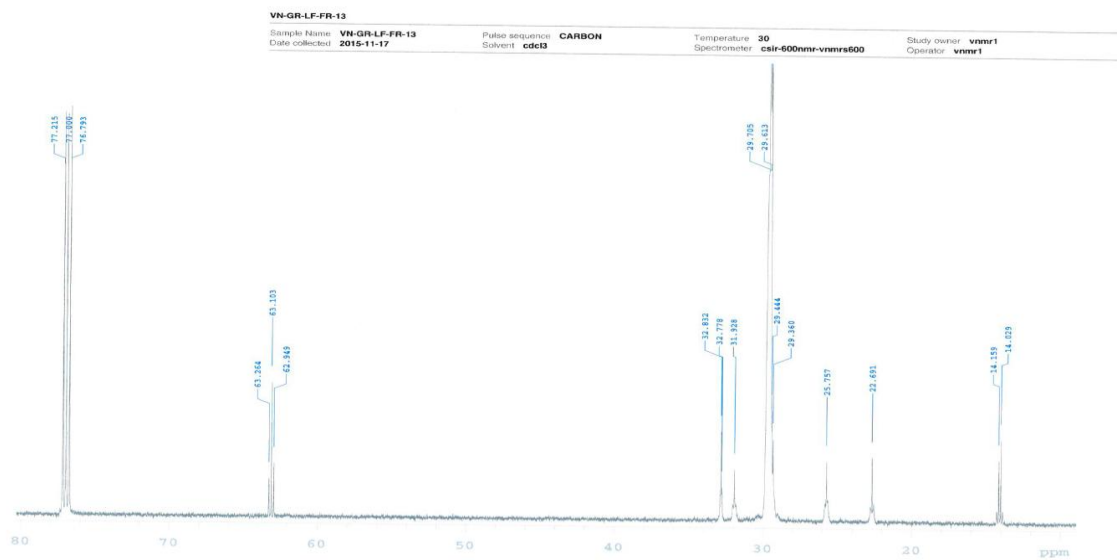


Figure 6: Ms Spectrum of Erythrinacinate C



(a)



(b)

Figure 7: (a) ^1H -NMR and (b) ^{13}C -NMR spectra for 1-Octacosanol

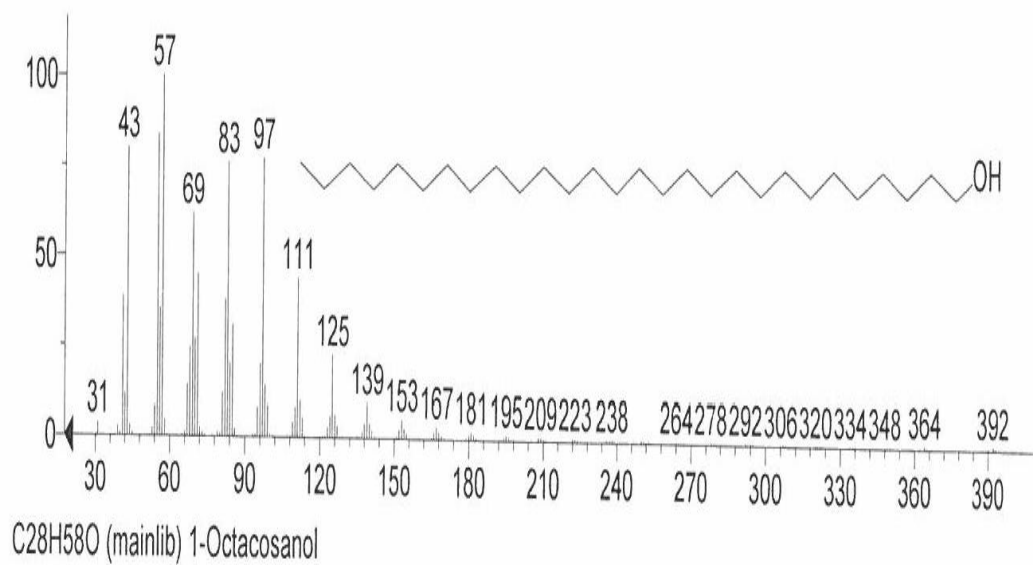


Figure 8: GC-MS spectrum for 1-Octacosanol

CHAPTER THREE

3.0 GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

The documentation of biological and chemical information of plants is an important step towards drug discovery from natural sources. This current research which reports biological and chemical investigation of *S. glaucescens* creates an important knowledge base as it registers some important toxicological, pharmacological and natural product information for this plant. The fact that it is the first time results from dermal toxicity, anti acaricidal, anti HIV and phytochemical analyses are reported from this plant, it adds value to science and creates an important avenue for further investigations on this plant species.

Studies on dermal toxicity is important for products that are used in skin care and skin treatment. For many years, researchers have given highest priority to addressing human and animal exposures associated with ingestion and inhalation. Despite many uses of plants through dermal routes, dermal exposure has been considered less important in many areas except some few fields such as cosmetics and drugs. Research of this plant species has documented the first ever study of this kind in Tanzania and has indicated the dried extracts of this plant to possess moderate to mild irritancy. Similar results have been found elsewhere from other species of plants (Teshome *et al.*, 2008; Bigoniya *et al.*, 2010).

Unlike in the irritation study, dermal sensitization studies revealed the dried plant extracts to have no effect that can be related to the test extract, thus indicating that dried extracts are not sensitizers. Acute dermal toxicity results showed that all animals in the tests and control groups did not exhibit any overt clinical signs of toxicities. Only the discomfort

signs were evident during the initial stages and were associated to mild irritations and experiences of foreign materials (extract, occlusion cloth and adhesive tape) in the body. It is therefore generally concluded that the plant can cause neither acute dermal toxicity nor sensitization except for mild to moderate irritations.

Toxicological investigations of the effects of plant extracts on ticks was based on the medical and economic importance of ticks that has long been recognized due to their ability to transmit diseases to humans and animals. Ticks cause great economic losses to livestock, and adversely affect livestock hosts in several ways. The availability of control agents for ticks is thus very important and that a continued search for new acaricide will ensure constant supply even when the current acaricide are rendered obsolete due various reasons including resistance. The evaluation of acaricidal effects of different extracts of *S. glaucescens* against cattle ticks as an effort to search for new traditional acaricide has revealed only ethanol leaf extracts to exhibit some activity (low) against adult ticks and the rest exhibited non-significant activities. Extracts tested against larvae showed similar results where one of the extract exhibited low activity and the other exhibiting non-significant activity.

This low activity against larvae further justifies the low effectiveness of the extracts as acaricides because larvae are believed to have relatively high susceptibility as compared to adult ticks (Williams *et al.*, 2015). It is witnessed that none of the tested extracts was able to kill at least 50% of the test subjects, thus concluded that none of the plant extracts is considered effective against the tested tick species. It is however, recommended that further studies needed to be carried out using different parameters such as testing the egg laying and hatchability inhibition and the use of fresh materials instead of dried one.

Assessment of Anti HIV activity was conducted through the use of HIV-RT using the Roche, 2010 Test Kit in presence and absence of extracts. Among the seven extract tested, three extracts; water fraction of root and leaves and dichloromethane fractions from root showed the highest retroviral inhibition activities with percentage inhibition of 100, 98 and 85 at 100 mg/ml respectively. Among the three, the water extracts from both root and leaves were the most active with IC_{50} values of 3.96 $\mu\text{g/mL}$ and 6.04 $\mu\text{g/mL}$ respectively followed by the dichloromethane extracts which exhibited the IC_{50} values of 11.43 $\mu\text{g/mL}$. The rest of extracts showed low effectiveness in the inhibition of HIV-RT, indicated by their very high IC_{50} values in the inhibition of HIV-RT compared to the positive control, the CSIR proven active plant extract against HIV (table 1) in chapter two, paper three. These findings have demonstrated that aqueous extracts from roots and leaves of *S. glaucescens* together with DCM extracts of root possess potent anti-HIV-1 activity.

The results further indicate that since anti HIV activity was exhibited by polar and mid polar fractions, the polar compounds are the probable cause of anti- HIV activities. These findings have thus, demonstrated that aqueous extracts from roots and leaves of *S. glaucescens* together with DCM extracts of root possess anti-HIV-1 activity. The results therefore suggest that since anti HIV activity was exhibited by polar and mid polar fractions, the polar compounds are the probable cause of anti- HIV activities. Further research on anti HIV activity is recommended especially using different methods and extracts.

Isolation of compounds from natural sources is an important step towards lead identification for development of new drugs. Different isolation approaches are known and bioassay-guided fractionation has been the state-of-the art method for identifying

bioactive natural products for many years. This approach involves repetitive preparative-scale fractionation and assessment of biological activity up to isolation of pure constituents with selected biological activity. This approach is resource intensive in terms of time, finances and human. Chemists sometimes isolate compounds without using bio assay guided approach and tests the compounds for their biological activities. In the current study, a non-bio assay guided isolation of chemical compounds from root bark and leaves of *S. glaucescens* was employed due to limited resources. This resulted into the isolation and identification of four compounds which include a sterol, fatty alcohol, triterpene, and an ester of ferulic acid. The first two compounds were isolated from leaves and the last two being isolated from the root barks. The structures of the isolated compounds were respectively identified as β -sitosterol, 1-octacosanol, euphol and erythrinacinate C on the basis of spectroscopic data and by comparing with those reported in literature. The complete ^1H and ^{13}C NMR spectral assignments of the isolated compounds were made based on COSY, HSQC, HMBC and spectroscopic data.

Further isolation is recommend especially of polar compounds and testing for anti HIV Activity. The bioassay approach is highly recommended for furthering this research. Despite the fact that the thesis is completed. Since all compounds were also not tested for their biological activities, it is recommended that when resources are available, all compounds have to be tested for biological activities especially anti HIV activity.

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