Sokoine University of Agriculture

PhD Thesis

A new Ellagic Acid Rhamnoside and Other Biologically Active Compounds from Roots and Stems of *Synadenium glaucescens* Pax

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A New Ellagic Acid Rhamnoside and Other Biologically Active Compounds from Roots and Stems of *Synadenium glaucescens* Pax

The thesis is submitted in Fulfilment of the Requirements for Degree of Doctor of Philosophy in Phytochemistry of Sokoine University of Agriculture

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EXTENDED ABSTRACT

Medicinal plants have been used as a source of drugs and drug templates. The extracts of *Synadenium glaucescens* Pax (SG) from roots and stems contain bioactive secondary metabolites against bacterial and viral infections. However, majority of its pure compounds were uncharacterized and their phytochemical profiles in different phytogeographical regions were unknown. Therefore, this study aimed at characterization of more compounds which are promising drug leads. Furthermore, the study investigated the phytochemical profiles of SG from three regions of Tanzania.

Samples of SG were collected from Njombe, Morogoro and Tanga regions as representatives of three phytogeographical zones of Tanzania. All sample were dried in a cold and dark room between 15 to 18 ^{o}C at the Tanzania Tree Seed Agency-Morogoro. Samples were pulverized using a laboratory milling machine (Christy Hunt Engineering Ltd, England) to afford approximately 1 mm particles' size. The isolation of pure compounds involved root barks and stem barks of SG from Njombe. Phytochemical profiling involved five parts (root barks, root wood, stem barks, stem wood and leaves). Sample extraction was done by total maceration using either methanol or ethanol. Fractionation of the crude extracts was done through vacuum Liquid chromatography in solvent gradient systems from Hexane, ethyl acetate/ dichloromethane and finally methanol/ ethanol. Concentration of extracts was done using rotary evaporator at <60 ^{o}C. Isolation of pure compounds was done by column chromatography in a solvent gradient system. A single spot on the thin layer chromatography plate in both treatments was an indicator for a pure compound. Structures of compounds were elucidated through (1D and 2D) of a 600 MHz Bruker Avance III HD nuclear magnetic resonance (NMR) machine. Confirmation of structures involved

LC/GCMS, FTMS-ESI, NIST library and in comparison, to the literature. The SciFinder database was used for reference of a new compound. The processing NMR spectral data was performed using Bruker TopSpin 3.6.2 while compound structures were drawn in ChemDraw Professional 16.0.

Biological activities involved cytotoxicity and antibacterial tests of pure compounds. Cytotoxicity studies were done using Brine shrimp assay involving *Artemia salina c*ysts which were hatched and used within 24 hours of incubation. The varying concentrations (2400- 240 µg/ml) in 1mL of 20 % v/v DMSO were employed in duplicate. The compounds' concentration killing 50 % of the nauplii larvae was determined. An *In vitro* antibacterial efficacy of pure compounds was done by using microtitre plate assay. Four standard strains of bacteria were used (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC51559, *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 27853. The activities of the compounds were evaluated using the observed MIC values.

Determination of the phytochemical profiles on the thin layer chromatography plates used an optimal 4 µL volume of 5 mg/mL. The number of compounds were recorded at 254nm, 365nm of an ultraviolet lamp and after colour reaction. Data from the thin layer chromatography were analysed in JMP statistical software.

A total of fifteen (15) compounds were isolated and elucidated. A new ellagic acid rhamnoside 3',4'-di-*O*-methylellagic acid-4-α-*L*-rhamnopyranoside, 28 mg (**32**) was isolated as greyish powder. it showed high res. FTMS-ESI$^-$ of 476.0921 and melting point of 251.0~ 252.7 ^oC. Other known phenolics 3,4,3'-*tri*-*O*-methylellagic acid, 14 mg (**33**), hemicosanylferulate,65 mg (**34)** and octacosylferulate, 19 mg (**35)** were also isolated. The study also led to isolation of

two hydrocarbons from the root barks: 1-nonacosene, 11 mg (**38),** and hexacosane,173 mg (**39**) and one fatty acid from the stem barks; hexacosanoic acid, 26 mg (**40).** The isolated triterpenoids include Lupeol, 65 mg (**18**) from the stem barks, euphol, 1855.5 mg (**28**) from both roots and stem barks, epifriedelanol, 167 mg (**36**) from both roots and stem barks and a steroid, β-sitosterol 64 mg (**30**) from the root barks.

Cytotoxicity tests indicated that all the tested compounds were non-toxic (LC_{50} > 100 µg/mL) up to a maximum tested concentration of 2400 µg/mL. Octacosylferulate demonstrated strong activity against *S. aureus* (MIC= 0.125 mg/mL)

Phytochemical screening by TLC indicated a significant variation in concentration and distribution of compounds in SG among the three regions ($p < 0.0001$). Njombe registered the highest number of compounds (mean= 12.4000) while Morogoro had the least of all (mean= 9.7000). Euphol, a triterpenoid in root barks was detected to be at the highest concentration in Tanga and the lowest in Morogoro. Derivatives of ellagic acid were detected in root barks at all ages and locations. The results also indicated continuous disappearance of red fluorescing compounds in stems barks and leaves as the plant age increased. The number of compounds increased with age while the optimal age for a maximum number of phytochemicals was 3 to 4.5 years. Furthermore, the stem barks registered the highest number of phytochemicals (>15).

A new compound: 3',4'-di-*O*-methylellagic acid-4-α-*L*-rhamnopyranoside along with 14 other known compounds were isolated from root and stem barks of SG. The isolated compounds during this study represent six major groups of secondary metabolites of pharmacological importance which are Phenolics, triterpenoids, steroids, long chain hydrocarbons, long chain amine and Fatty acids. This new compound is suggested to serve as a marker for

standardization and quality control of any formulation from the root barks of *S. glaucescens* Pax.

IKISIRI KUU

Mimea mbali mbali ukiwamo mvunjakongwa hutumika kutibu magonjwa mbali mbali yatokanayo na vijidudu vya bakteria na virusi hapa Tanzania. Viduo vya mizizi na mashina vimekuwa na ufanisi mkubwa kulinganisha na sehemu nyingine za mmea huu. Hata hivyo kemikali zilizotambuliwa zilikuwa ni chache na kuathiriwa na umri na mazingira haukujulikana. Hivyo utafiti huu ulijikita katika kudukua na kutenganisha kemikali nyignine zaidi ya zilizofahamika awali ambazo zipatikana katika viziduo vya *ethyl acetate* na *dichloromethane* vya magome ya mizizi na ya mashina mtawalia. Pia mchango wa wa mazingira ya kijiografia na umri wa mmea huu wa mvunjakongwa juu ya upatikanaji na kiwango cha kemikali mbali mbali ulitafitiwa.

Sampuli za mmea dawa (magome ya mizizi (SG2), mtima wa mizizi (SG3), magome ya shina (SG5), mtima wa shina (SG6) pamoja na majani (SG7) zilikusanywa kutoka mikoa mitatu ya Tanzania ambayo ni Njombe, Morogoro na Tanga. Sampuli zilizotumika kudukua, kutenganisha na kuainisha maumbo ya kemikali-dawa zilikusanywa kutoka Njombe tu. Sampuli hizi zilikaushiwa kwenye chumba cha maabara yenye mwanga hafifu kwa joto la sentigredi kati ya 15 na 18 kwa wakala wa mbegu za miti Morogoro-Tanzania.*Udukuaji na utenganishaji wa kemikali*: Kemikali pombe aina za methanoli na ethanoli zilitumika kudukua mkusanyiko wa kemikali kutoka katika magome ya mizizi na magome ya mashina. Aidha viziduo hivyo vilitenganishwa Zaidi kwa njia ya *vacuum Liquid Chromatography* kwa kutumia *hexane*, *ethyl acetate* na hatimaye methanoli au ethanoli. Viziduo vyake vilitengwanishwa na ‘solvent’ hizo kwa kutumia *rotory evaporator* (nyuzi joto zisizozidi 60 ili kupata viduo vikavu ambavyo vilitunzwa kwenye jokofu la maabara sentigredi $^{-}$4 hadi muda wa matumizi. Utenganishaji wa kemikali ulitumia mbinu ya *column chromatograph* wakati usafishaji wake

ulitumia aidha methanoli au hexane kwa *vacuum suction*. Aidha kuonekana kwa doti moja ya kemikali husika kwenye karatasi ya 'thin layer chromatography' ulitumika kuhakiki usafi wa kemikali husika. Kwa upande wa sampuli zilizotumika kuainisha madhara ya mazingira na umri kwenye mgawanyiko wa kemikali katika mmea dawa wa mvunjakongwa methanoli ilitumika kupata viduo vyote vya sehemu tano za mti husika. Jumla ya mimea isiyopungua kumi na tano kutoka kila eneo ilichakatwa katika sehem zake tano na ilifanyiwa udukuzi. Viduo vilitenganishwa na methanoli kwa jotoridi la feni. Mbinu ya *thin layer chromatography* ilitumika ambapo ujazo wa 4 *µL* (5 *mg/ mL*) ya kila sampuli iliwekwa kwenye karatasi hiyo maalumu ya *TLC*. Idadi ya kemikali zilizokuwemo ilihesabiwa kwa kutumia mwanga wa taa maalumu (254 nm na 365 nm) na baada ya kutumia *vanillin reagent*.

Vifaa maalum vya *Nuclear Magnetic Resonance*, *Gas/Liquid Chromatography Mass spectrometry' na FTMS-ESI*$^{-}$ na rejea za machapisho mbali mbali vilitumika kuanishia tabia za kemikali na kutambua maumbo yake. Uchakataji wa taarifa za NMR ulitumia programu ya Bruker TopSpin 3.6.2. Nayo maumbo ya kemikali zote yalichorwa kwa kutumia programu ya ChemDraw Professional 16.0.

Jumla ya kemikali kumi na tano (15) zilitenganishwa kutoka viduo vya ethyl acetate na dichloromethane vitokanavyo na magome ya mizizi na mashina kwa mtiririko huo. Utafiti huu ulifanikisha kutenganishwa kwa kemikali mpya aina ya fenoliki ilijulikana kwa jina la *3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside, 28 mg (**32**)* pamoja na fenoliki nyingi *za 3,4,3'-tri-O-methylellagic acid, 14 mg (**33**),* vitohoo vya tindikali ya ferulic*; hemicosanylferulate, 65 (**34**) na octacosylferulate, 19 mg (**35**).* Aidha makundi mengine *ya* kemikali yalitenganishwa kama vile; *hydrokaboni mbili; 1-nonacosene, 11 mg (**38**),* na *hexacosane, 173 mg (**39**), fatty acid; hexacosanoic acid, 26 mg* (**40**). Kundi jingine ni

triterpenoids; *Lupeol*, 65 mg (**18**) na *euphol*, 1855.5 mg (**29**), na *epifriedelanol*, 167 mg (**36**) ziliyopatikana kwenye sehemu zote mbili (magome ya mizizi na mashina). Pia kemikali ya kundi la *steroid*; *β-sitosterol*, 64 (**30**) nayo pia ilipatikana kwenye magome ya mizizi na mashina mtawalia pamoja na '*long chain amine'*.
Matokeo ya utafiti huu yamebaini kuwa umri wa mmea-dawa, sehemu za mmea na mazingira ya kijiografia yanaathiri idadi na aina ya kemikali kiwango kikubwana ($p < 0.0001$). Aidha, ilibainika kuwa kemikali aina ya *Euphol* ilikuwamo kwa kiwango kikubwa kwenye sampuli za kutokea Tanga ikilinganishwa na maeneo mengine. Kemikali mtohoo za ellagic acid zilipatikana katika mimea ya umri wote na maeneo yote ya utafiti huu. Pia mvunjakongwa kutoka mkoa wa Njombe ilionekana kuwa na idadi kubwa ya kemikali ikifuatiwa na Tanga huku Morogoro ikionyesha kuwa nazo chache. Kemikali nyingine za magome ya mashina na majani zilionekana kupotea kadiri ya umri ulivoongezeka.
Matokeo ya majaribio ya kiwango cha sumu yalibainisha kuwa kemikali hizi hazina sumu kiwango cha juu cha 2400 *mg/mL* kilichotumika wakati wa majaribio.

Utafiti huu umewezesha kupatikana kwa kemikali mpya ya *3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside* pamoja na nyingine kumi na nne (14). makundi ya *Phenolics, triterpenoids, steroids, long chain hydrocarbons, long chain amine na Fatty acids* yalitenganishwa. Aidha kemikali nyingine kama vile *3,4,3'-tri-O-methylellagic acid, hemicosanyl ferulate, octacosyl ferulate na Lupeol* zimepatikana kwa mara ya kwanza katika mmea huu wa mvunjakongwa. Inapendekezwa kemikali hii mpya ya *3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside* kutumika kama kemikali rejea kwenye bidhaa zitokanazo na mizizi ya mvunjakongwa.

Maneno muhimu: *mimea dawa, traitapenoidi, ellagic acid euphorbiaceae, ramnosaidi, bakteria*

DECLARATION

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

Frank Rwegoshora — Date

(PhD candidate)

The above declaration is confirmed:

Prof. Faith Mabiki — Date

(Principal Supervisor)

Dr. Francis Machumi — Date

(Co-supervisor)

Dr. Musa Chacha — Date

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Prof. Claus Cornett — Date

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LIST OF PUBLISHED PAPERS AND SUBMITTED MANUSCRIPTS

1. F. Rwegoshora, F. Mabiki, F. Machumi, M. Chacha, B. Styrishave and C. Cornett. (2023). A new ellagic acid rhamnoside from *Synadenium glaucescens* Pax and cytotoxicity evaluation- Journal of Chemistry of natural compounds. Vol.4 **Accepted.**
2. Rwegoshora F, Mabiki F, Machumi F, Chacha M, Styrishave B, Cornett C. Isolation, and toxicity evaluation of feruloyl ester and other triterpenoids from *Synadenium glaucescens* Pax. J Phytopharmacol 2022; 11(5):347-352. doi: 10.31254/phyto.2022.11506.
3. Rwegoshora F, Mabiki F, Machumi F, Chacha M, Styrishave B, Cornett C. Isolation, and elucidation of antibacterial compounds from the root and stem barks of *Synadenium glaucescens* Pax. for publication East African Journal of Science, Technology, and Innovation. **Accepted**
4. Rwegoshora F, Mabiki F, Machumi F, Chacha M, Styrishave B, Cornett C. Phytochemical profiles of *Synadenium glaucescens* Pax from different phytogeographical regions of Tanzania**.** to the Journal of Medicinal Plants Research. **Submitted**

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My family (wife; Praxeda Kalikwendwa Henerco, our children, my mother, and siblings) are highly acknowledged for their moral and spiritual support.

Finally, I express my acknowledgement to residents of Mtulingala village (Njombe), Chakwale village (Morogoro) and Kibindu village (Tanga) for their cooperation during collection of plant material.

DEDICATION

This work is dedicated to my wife Praxeda Kalikwendwa Henerco, My children Baraka, Davis (Akiza), Joan (Wekisha) and Davina (Wokusingiza). My parents; father the Late Longino Thomas, Mother Lucy Longino, young brothers Anord and Edibert, my brother the Late Denis E. Zimbihya and the late Dr. Sijaona Msigala.

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THESIS ORGANIZATION

The thesis has been developed in ‘publishable papers format’ and comprises seven chapters. The first chapter consists of the introduction of the overall study. From chapter two to five is a description of the commonality of the concepts in the separate published papers or manuscripts submitted to the journals. The sixth chapter consists of general discussions while the seventh is the Conclusions and Recommendations. The arrangement of the papers follows the arrangement of the objectives.

LIST OF ABBREVIATIONS

SG	*Synadenium glaucescens*
EtOAc	Ethyl acetate
DCM	Dichloromethane
EtOH	Ethanol
DMSO	Dimethyl sulphoxide
MeOH	Methanol
Hex	Hexane
PE	Petroleum ether
ALU	Artemether-lumefantrine
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
COSY	Correlation Spectroscopy
HSQC	Heteronuclear Single Quantum Coherence Spectroscopy
ROESY	Rotating Overhauser Enhancement Spectroscopy
HMBC	Heteronuclear multiple bond correlation
NMR	Nuclear Magnetic Resonance
LC	Liquid Chromatography
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
MS	Mass spectrometry
FTMS	Fourier Transform Mass Spectrometry
ESI	Electrospray ionization
TLC	Thin Layer Chromatography
EA	Ellagic acid
FE	ethyl acetate fraction

FM	methanol fraction
FD	Dichloromethane fraction
VLC	Vacuum Liquid Chromatography
LC	Lethal Concentration
R_f	Retention factor
EPS	exopolysaccharides
MIC	Minimum Inhibitory concentration
TPC	Total Phenolic Content

1.0 CHAPTER ONE: INTRODUCTION

1.1 Plants as potential source of drugs

A plant is termed to be medicinal if , in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for the synthesis of useful drugs (Sofowora *et al*., 2013). The usage of medicinal plants as a key component of complementary and alternative medicine, has acquired renewed interest in developed countries (Sánchez, *et al.,* 2020). These chemical substances are commonly known as phytochemicals; a class of bioactive non-nutrient compounds which usually existing in fruits, vegetables, grains, and other food-based plants (Chen *et al.*, 2014) as well as other plant parts including roots, leaves (Aljubiri *et al*., 2021) and stems. On the other hand, these chemicals have served a medicinal function in both animal and human infections. The development of antimicrobial resistance is among the factors that has accelerated the initiatives for searching new sources and drug agents (Ginsburg and Deharo, 2011; Guo, 2016). The usage of these plants as a key component of complementary and alternative medicine, has acquired renewed interest in both developed and developing countries (Sánchez *et al*., 2020). However, the remarkable chemical diversity of plants and their impressive capability to synthesize highly complex novel compounds with 'drug-likeness' properties (Koehn and Carter, 2005, Harvey *et al*., 2015; Jia, *et al*., 2020;), provide substantial evidence that new drugs may still be discovered amongst the estimated 350,000 known vascular plant species (Howes *et al*., 2020). Medicinal plants are regarded as rich resources of traditional medicines and from these plants many of the modern medicines are produced. It is for this reason the use of traditional medicine for primary health care is estimated to be 80% of the population (Ginsburg and Deharo, 2011; Kayombo, 2013). Plants' extracts and formulations have

always proved to be effective against human and animal infections. The neem tree *Azadrachta indica* has always been a good source of quinine (Fig.1, **1**) drug against malaria before plasmodia started showing resistance against it. For decades, drug resistance has been the major obstacle in the fight against infections such as malaria, and the search for new drugs together with the combination therapy constitutes the major approach in responding to this situation (Zofou *et al*., 2012). For example, the antimalarial drug namely artemether/ lumefantrine (ALU) contains an active lead Artemisinin (Fig.1, **2**) (which was previously isolated from *Artemisia annua* (Guerriero *et al*., 2018). Its discovery revolutionized the approach to treating malaria, illustrating a change in approach from using quinoline-based drugs to which parasites were showing increasing resistance. The discovery of artemisinin paved the way for the development of new classes of drugs (Fitzgerald *et al*., 2019). Another notable natural compound Taxol/ Paclitaxol (**3**) is an antileukemia and antitumor agent which was originally isolated from the Pacific yew plant *Taxus brevifolia.* Vincristine (Fig.1, **4**) and vinblastine are another anticancer agent isolated from Madagascar periwinkle (*Vinca rosea* Linn), while Pilocarpine (Fig.1, **5**) isolated from *Pilocarpus jaborandi* Holmes *Rutaceae* is used to treat glaucoma (Thomas, 2007). Morphine (**6**), which is used as an analgesic drug, was first isolated from the *Opium poppy*. Although its isolation always led to a small amount, the natural one has served as precursor in a semi synthesis of the drug.

1.2. Biological activities of Plant Derived compounds

Plants are known for their range of biological activities such as cytotoxicity and anti-bacterial which is associated with biologically active compounds known as secondary metabolites. For example, Najmi *et al.,* (2022) reported a flavonoid, Epigallocatechin-3-O-gallate from *Camellia*

sinensis (green tea) as active principle for antibacterial potential, teixobactin from *Eleftheria terrace* is active against gram positive bacteria like vacomycin resistant enterococci and methicillin-resistant *S. aureus.* Plant volatile oils from plants are reported by (Dorman and Deans, 2000). Despite known potentials, the use of plants for medications in animals and humans may be constrained by toxicity status of their extracts or pure compounds (Kalala *et al.*, 2015). Brine shrimp lethality test (BSLT) invented by Meyer *et al.,* (1982) is among the key methods accepted for predicting toxicological properties of plant extracts and pure compounds. The extract or test compound is evaluated on the basis of concentration killing 50 % of nauplii (LC_{50}) according to Moshi *et al*., (2010) LC50 <1.0 µg/ml – highly toxic; LC50 1.0-10.0 µg/ml – toxic; LC50 10.0-30.0 µg/ml – moderately toxic; LC50 >30 <100 µg/ml – mildly toxic, while LC50 > 100 µg/ml as non-toxic. Although the toxic compounds may simply not be safe for consumption, they are good candidates for anti-cancer drugs (Kumar *et al*., 2018). It is important that a plant undergoes important evaluation tests for effectiveness and safety of the consumers.

1.3 Secondary metabolites of medicinal Plants

Natural products also termed as secondary metabolites are chemical compounds of limited distribution in either plants, microbes, or phytoplankton. They are synthesized on exposure of an organism to environmental stress (Liu *et al*., 2016). In turn these metabolites serve an important role through promotion of health and therapeutic activities (Blunder *et al*., 2017). Several studies have been focusing on natural products of medicinal plants as a substitute for some pharmaceutical drugs in certain diseases in developing countries (Elmarimi *et al*., 2019). Natural ingredient produced by plants are widely used for therapeutic treatment, because they are believed to have

fewer side effects and are cheaper than synthetic drugs (Dhaniaputri *et al.,* 2022) and relatively accessible easily. Pharmaceutically significant secondary metabolites (phytopharmaceuticals) include alkaloids, glycosides, flavonoids, volatile oils, tannins, resins, and terpenoids. Currently, most of these secondary metabolites are either isolated from wild or cultivated plants, as their chemical synthesis is either extremely difficult or economically infeasible (Jha and Bansal, 2018). These form the active component of a medicinal plant which is developed into drug or drug lead. Finding active ingredients from the plants is one of the important ways to develop new drugs which can be applied to the treatment of human diseases (Xu *et al.,* 2018) as well as animal infections. Before technological advancement (prior 1800s), the active constituents of most based medicines were generally plant-based medicines were mostly used as extract, decoction, oil, powder, the pure forms of compounds were unknown (Gurnani *et al.*, 2014). Thus, in the early 19th century, scientists began to extract and modify the active ingredients from the plants as in fast developing countries (Joe *et al*., 1998) as cited by (Elmarimi *et al.*, 2019).

Secondary metabolites from plants have become popular in pharmaceutical industries due to their ability to trigger a pharmacological or toxicological effect in humans and animals (Guerriero *et al*., 2018). In this view, natural products represent a source of potential new pharmacophores that are needed for fighting diseases and disease infections including killing the parasite (Wells, 2011). The analysis of medicinal plants has a long history, especially regarding assessing a plant's quality. There was development from the earliest techniques like organoleptic using the physical senses of taste, smell, and appearance. Then gradually these led on to more advanced instrumental techniques (Fitzgerald *et al.*, 2019). Discoveries and development in the field of natural products and antibiotics

takes its time back since the isolation of penicillin (Fig.1, **7**) by Alexander Fleming (Gaynes, 2017). Studies have been carried out globally to verify their efficacy and some of the findings have led to the production of plant-based medicines (Dar *et al*., 2017; Sofowora *et al*., 2013).

Different groups of plants' secondary metabolites like terpenes and phenolic compounds have pharmacological potential (Dorman and Deans, 2000). Phenolics like chalcones are reported to have anti-cancer, anti-inflammatory, anti-oxidant (Kumar *et al*., 2018),anti-tuberculosis (Qian *et al*., 2010) and anti-fungal (Manayi *et al*., 2013) profiles. compounds that contain at least one benzene rings, terpenes for are a group of organic compounds made of isoprene units of five (5) carbon atoms which are either aliphatic or cyclic. These units depending on the number of C-atoms, form monoterpenes (C-10), Sesquiterpenes (C-15), diterpenes (C-20), Sesterterpenes (C-25), triterpenes (C-30), tetraterpenes (C-40) and polyterpenes (>C-40). The chemical diversity and a vast array of biological functions of triterpenes continue to attract considerable research interest across a range of research disciplines (Ncube and Van Staden, 2015). Triterpenes constitute a large structurally diverse group of natural compounds biogenetically derived from active isoprene. Two C15 units build squalene or related acyclic 30-carbon precursors (Nazaruk and Borzym-Kluczyk, 2015). It is reported that 30% of 187 natural products recorded between 2012- 2020 from African medicinal plants anti- malaria/ plasmodial properties were terpenoids (Bekono *et al.*, 2020). Triterpenoids are also reported to have other pharmacological activities including; antiedemic, anti-inflammatory activity, promotion of healing and keratinization (Duke *et al.*, 2001). Lupenone, is a triterpene which is reported for various pharmacological activities including anti-inflammatory, anti-virus, anti-diabetes, anti-cancer, improving Chagas disease without major toxicity (Xu *et al.*,

2018). Artemisinin (Fig.1, 2) is a sesquiterpene which was previously isolated from *A. annua* is currently used and is a very effective antimalarial. It is further reported to be far superior over quinine and chloroquine (Guo, 2016; Gurnani et al., 2014). Further studies on Artemisinin, artemether and artesunate, as well as other artemisinin, have brought the global anti-malarial treatment to a new era, saving millions of lives all around the world for the past 40 years (Guo, 2016).

1.3 Biosynthetic pathways for secondary metabolites

The processes of biosynthesis and accumulation of secondary metabolites arise from highly regulated processes that require both genetic and environment-specific controls (Hadacek, 2002). There are mainly four pathways through which secondary metabolites are synthesized. These include; the Shikimate, Malonic-acid, Mevalonic-acid, and Methylerythritol-phosphate pathway (Dhaniaputri, Suwono, and Lukiati, 2022). Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: terpenoids, alkaloids (nitrogen containing compounds), and phenolics (Eljounaidi and Lichman, 2020). The saponins, tannins and glycosides are other groups of these metabolites. While phenolic and polyphenols are synthesized through Shikimate pathway in higher plants, phenolics in bacteria and fungi are synthesized through mevalonate pathway *(*Dhaniaputri *et al.*, 2022). Ellagic acids (EA) and garlic acids are known to be synthesized through the shikimate pathway and indirectly via hydroxycinnamic acids, the terpenoids (C-30) are synthesized through mevalonate pathway and a recently characterized 2C-methyl-D-erythritol-4-phosphate (MEP) pathway which is also known as the 1-deoxy-D-xylulose- 5-phosphate (DXP) pathway (Abdallah and Quax, 2017).

1.5 Medicinal value of Euphorbiaceous species

The family Euphorbiaceae is among the largest of the flowering plants families with a variety of chemical components (Lima and Medeiros, 2020). Its species are reported potential sources of terpenoids. The triterpene alcohols of the euphane, lanostane and cycloartane-type skeleton (Lima and Medeiros, 2020). They further assert that terpenoids including, euphol, obtusifoliol, lanosterol, cycloartenol and 24-methylene cycloartenol), diterpene alcohol ingenol and the ingenane diterpenoid esters have been isolated from *E. azorica*. Its classification and chemistry have of late been subjects of interest possibly because of the wide variety of chemical composition of its members (Mwine and Van Damme, 2011). Plant members of Euphorbiaceae are reported to contain several pharmacologically active compounds including anti-inflammatory, anticancer, anticarcinogenic (Bishayee *et al*., 2011), antidiabetic (Nazaruk and Borzym-Kluczyk, 2015; L. Wang et al., 2018) and antimicrobial activities (Nzogong *et al*., 2018). Another Euphorbiaceae, *Homalanthus nutans* is used by traditional healers in India for the treatment of viral hepatitis (Gurnani *et al.,* 2014). They further assert that a phorbol ester prostratin from its wood has indicated promising results against AIDS. The family is also reported among the families with potent hypoglycemic effect (Patel *et al*., 2012). In the same study by Patel *et al.,* (2012) the oral administration of ethanolic extract of *Ricinus communis* showed to have antidiabetic properties in mice model as it caused increase in insulin levels, improvement in lipid profiles and the animal body weight.

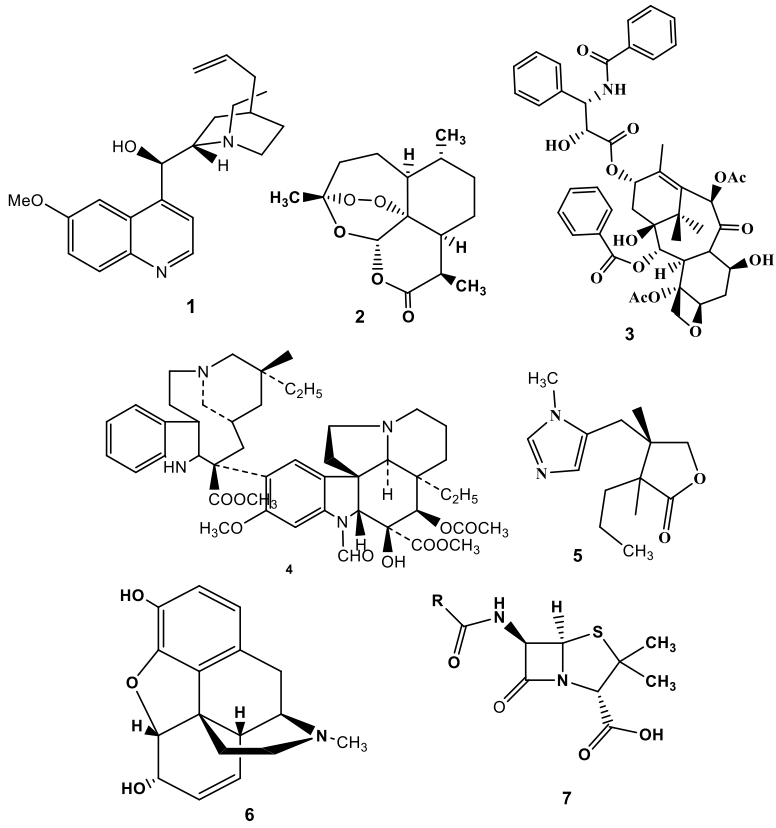

Figure 1: Structures of drugs in use from natural sources of plants and fungi

The tetracyclic triterpenes (Fig.2) with anti-inflammatory effect in the TPA-induced inflammation in mice were isolated from *Euphorbia maculate.* Among these include; lanostane-type triterpenoids named (3S,4S, 7S,9R)- 4-methyl-3,7-dihydroxy-7(8→9) a*beo*-lanost-24(28)-en-8-one (**8**) and 24-hydroperoxylanost-7,25-dien-3β-ol (**9**), cycloeucalenol (**10**), gramisterol (**11**),cycloart-23-en-3β,25-diol (**12**), Obtusifoliol (**13**), urs-12-ene-3β, 11α-diol (**14**), Neoilexonol (**15**), 12-Oleanene-3β,11β-diol (**16),** (3β,15α, 16α)-15,16-epoxy- Olean-12-en-3-ol (**17**), Lupeol (**18**), mutliforenol (**19**), 3-hydroxycycloart-25-ene-24-hydroperoxide (**20**), 3β-hydroxy-26-nor-9,19-cyclolanost-23-en-25-one (**21**) and cycloart-23en-3β,25-diol (**22**) just to mention a few (Sun *et al.,* 2018)

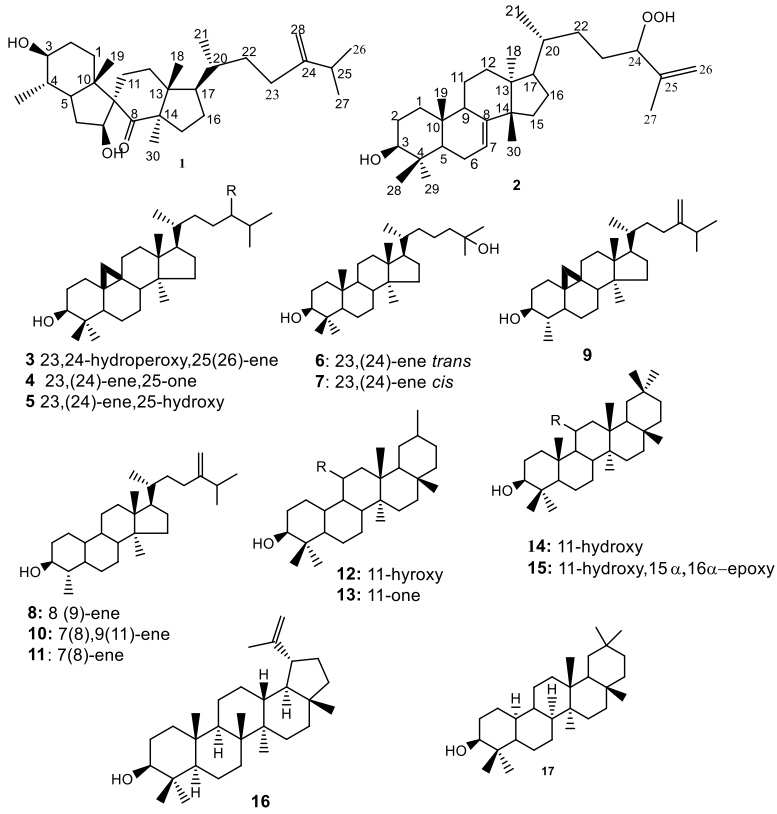

Figure 2: Structures of triterpenoids isolated from Euphorbia species.

Lupeol (Fig. 2, **18**) is a triterpenoid which has been isolated from different plant species like; the chloroform fraction of ethanolic extract of *Ziziphus xylopyrus*, (Retz) Willd (Gandagule, Duraiswamy *et al.,* 2018), ethyl acetate fraction of the roots of *Combretum Hypopolinum* (Diels.) Okafor (Muhammad et al., 2021), Lupeol acetate from latex of *Himatanthus drasticus* is known for anti-inflammatory effect (Lucetti et al., 2010). From *Euphorbia fischeriana* new triterpenoids, the tirucalane fisuphanins A (**23**) and B (**24)** (Fig.2) have been isolated (Huang *et al*., 2022). Although these tirucalane compounds were not tested, the dry roots of the *E. fischeriana* from which they were isolated have been used for the treatment of edema, abdominal distension, abdominal pain, chronic bronchitis, cough, asthma, tuberculosis, scabies and hemorrhoids and fistulas.

1.6 Synadenium species as potential source of dug leads.

The genus Synadenium is reported to contain about 24 species including *S. glaucescens* Pax. Synadenium species are in folk medicine for treatment of peptic ulcers and inflammatory diseases (Munhoz *et al.*, 2014). Despite a continuous use of crude extracts/ formulation in the traditional medicine therapy, several scholars have taken more steps of isolating, elucidating, and testing pharmacological properties of different groups of compounds. *S. grantii* as the most studied species and among is reported to contain compounds including tigliane, synadenol, phorbol type diterpenoids, triterpenoids, anthocyanins, unsaponifiable substances and coumarins (Costa *et a*l., 2012). Also the long chain hydrocarbons and fatty acids have been isolated from *Synadenium spp*. Phorbo esters (4-deoxyphorbol-12,13-ditiglate (**25)** and Query 3,4,12,13-tetraacetylforbol-20-phenylacetate (**26)** were isolated from the leaves of *S. grantii* Hook *f* (Hassan *et al*., 2012; Jesuíno *et al*., 2019). These compounds were

reported for anti-inflammatory and activity and prevent abdominal writhing (dysmenorrhea) in the mice model (Jesuíno et al., 2018). Diterpene phorbo compounds in *Synadenium spp* are held responsible for causing skin irritation (Nishizuka, 1984) as cited by (Beutler, 2009). These types of terpenes may be present in *S. glaucescens* Pax since this characteristic (skin irritation) is also present in its leaves and stem parts. The latex of *S. umbellatum* Pax is known for treatment diseases including *Diabetes mellitus*, Hansen´s disease, trypanosomiases, leukemia and several malignant tumors (Melo-Reis et al., 2010). This plant is also used in the folk medicine for gastric problems as well as analgesics and anti- inflammatory properties (Borges *et al*., 2013). Accurate mass measurement by Rodrigo et al., 2016) for compounds from the methanolic bark extract indicated 23 polyphenolic compounds including; ellagic and garlic acid derivatives and flavanols. The lectin (glycoproteins) from latex of *S. carinatum* has been reported to control *Toxoplasma gondi* infections (de Souza *et al.*, 2016). These compounds are from time to time reported from different member species of *Synadenium* which implies that these plants are likely to exhibit similar medicinal values.

Synadenium glaucescens Pax (Fig. **3**) commonly called mvunjakongwa (in Kiswahili), liyugi in Bena and igoole in Kaguru. It is another member plant which has drawn researchers' attention due to its pharmacological potentials. The crude extracts from this plant have demonstrated to be active against viral infections (Mabiki *et al*., 2013). A triterpenoid 3β-friedelanol isolated from its roots and leaves tested active against Newcastle Disease Virus (Credo *et al*., 2022) as well as against standard and Methicillin Resistant bacteria strains (Msengwa *et al*., 2023). Other triterpenoids like lanosterol (**28**), euphol (**29**),and a steroid β-sitosterol (**30**) are also reported from various extracts of this plant *(Credo et al., 2022; Nyigo et al.*, 2016; Rwegoshora *et al*., 2022). This study reports on fifteen (15) compounds that

have been isolated for the first time or re-isolated, elucidated and were evaluated for cytotoxicity and antibacterial efficacy.

Figure 3: Photograph of two years aged *Synadenium glaucescens Pax*

1.7 Marker compounds for plant- derived products

A marker compound (in this context) is a chemical constituents within a medicinal that can be used to verify its potency or identity (Chothani *et al*., 2012). A selection criteria for a compound to serve as a marker relies on the

amount present in each crude drug (Moon et al., 1999) as cited by (Weon *et al.*, 2016) or the bioactive compound in a mixture of several of them. However, it is argued that a chemical marker (in the medicinal sample in this case) does not necessary need to be the active component, but rather the specific analyte(s) detectable by the analytical methodology employed (Parker *et al.*, 2007). Since the marker compound is used as a unique identity for specific product, it seems necessary to determine them in *S. glaucescens* Pax for quality control of its herbal medicines and other formulations.

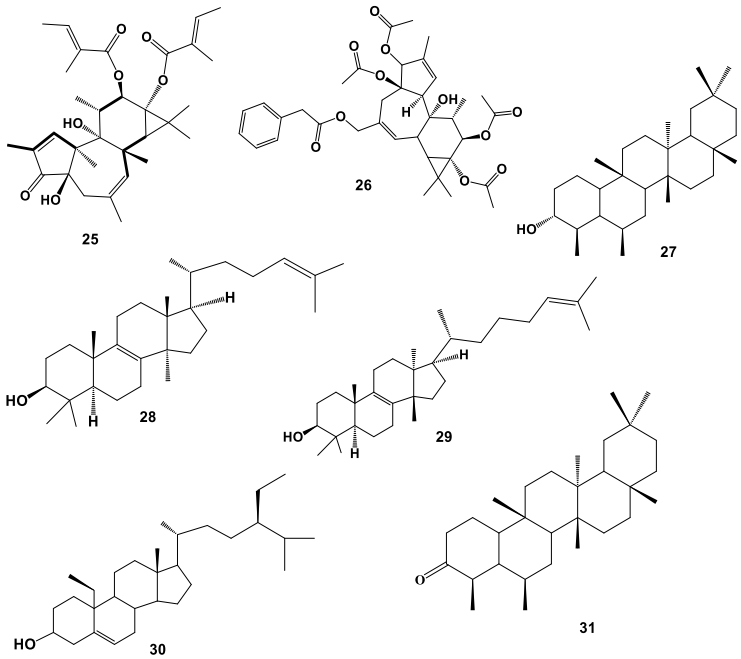

Figure 4: Some compounds isolated from *Synadenium spp.*

1.8 Intraspecies variations of Chemical profiles

Plants of the same species may display variations in their bioactive phytochemicals depending on how they interact with their environment in a development process (Peñuelas and Llusià 1997; Liu et al. 2016). Variations in the soil nature, season, their geographical location *(*Adegbaju *et al.,* 2021; Inbathamizh and Padmini, 2013; Muraina *et al.*, 2008). These weather related factors like water deficit, flooding, chilling, freezing, high temperature, salinity, light intensity are reported to affect its secondary metabolites (Dhaniaputri *et al*., 2022). In a study on the phytoconstituents of *Moringa olefera* seeds indicated a variation in amount of saponins, alkaloids, flavonoid contents among three senatorial zones (Ukwueze *et al*., 2019). Different aged leaves of *Moringa oleifera* indicated a variation in the total phenolic content and antioxidant activity (Kumar and Yadav, 2017). An assessment of *Lavandula pedunculata* (Mill.) Cav from different geographical origins on the total phenolic contents, antioxidant, anti-inflammatory potential and anti-proliferative capacity revealed a variation in concentration as well as the activity among the thirteen locations whose samples were assessed (Lopes et al., 2018). They further asserted that samples from Alentejo location had a higher bioactivity compared to Trás-os-Montes samples. In another study on Brazilian purple grapes for the chemical markers and *in vitro* antioxidant activity, the results indicated a significant variation in which Paraná state had the highest average values for bioactive compounds (Margraf *et al.,* 2016). They further assert that greater iron reduction capacity was observed in those samples. According to Vilanova et al. (2015) there were differences in levels of anthocyanins, flavonols, phenolic acids and resveratrol in the musts of Mencía (*Vitis vinifera*) grapes cultivated in different locations of north-western Spain. In this context, a change in geographical location eventually imposes differences in the

effectiveness of the plant be it on the medicinal or nutritional aspect.

Tanzania is endowed with over 10000 plant species of which 25 % are wild medicinal and distributed in five phytogegraphical regions. These regions include; the Afro–montane region including the Eastern Arc mountains, Lake-basin regions such as Lake Tanganyika and Lake Victoria, the Somali – Maasai region in central and northern part of Tanzania, Zambezi region, which is covered by the Miombo woodlands in the Western and Southern part of the country, and the Zanzibar – Inhambane region consisting of coastal, thickets, forests and woodlands (Nahashon, 2013). Although some plants serve for nutraceuticals. contain natural compounds which have a medicinal value. The bran part of the pigmented rice cultivars contains acetylated procyanidin (Oki *et al*.,2002), anthocyanins, and other phenolics that have significant free radical scavenging activity (Pitija *et al*., 2013 and Sriseadika et al., 2012). Other plants contain natural antioxidants, such as tocopherols, tocotrienols (Sookwong *et al*., 2007), oryzanols (Pereira-Caro *et al*., 2013), phytosterols (Afinisha *et al*., 2011), and phenolic compounds as they were cited by Suttiarporn et al., (2015). Traditionally, medicinal plants have been used as crude extracts.

In traditional practice, the harvesting of medicinal plants by practitioners has always paid less attention to the individual age of a plant. However, it is evident that there are fluctuations in behavioral distribution of phytochemicals with growth age among the useful plants. Biosynthesis of phytochemicals in plant has majorly dependence on their genetic make-up (Adegbaju *et al*., 2020). Other scholars argue that the concentration of many oxidative phytochemicals among others is significantly affected by other environmental stress conditions such as post-harvest processing and plant maturity (Adegbaju *et al*., 2020;

Nobossé *et al.*, , 2018; Wang *et al.*, 2016). As the plant approached maturity to flowering stage of *Celosia argentea* L, there was noticed an increase in the amount of proanthocyanidin (Adegbaju *et al*., 2020). On the other hand, the biological activity of medicinal plants is reported to change with the respect to the plant age (Mutalib, 2015). An assessment of the effect of age in coffee leaves showed variations on total phenolic content (TPC), anti-oxidant and anti-inflammatory activities of the extract (Chen *et al.*, 2017). The mature coffee leaves showed the highest TPC and anti-inflammatory activity was reported by Chen *et al.,* (2017) that the bound phenolics and flavonoids were dramatically elevated, and their antioxidant activity increased as the age increased. Regardless of the different ripening stages, higher contents of phenolics and flavonoids, stronger antioxidant and antiproliferative activities were found in the peels than in the flesh of *Citrus reticulate.*

1.10 Statement of the Problem and Significance of the Study

Before this study, there was limited information on the number of pure compounds isolated and elucidated from *S. glaucescens.* Likewise, the antibacterial efficacy and cytotoxicity status of its pure compounds was unknown. On the other hand, the status of phytochemical profiles of different parts of *S. glaucescens* Pax with respect to age and location were unknown. Lack of this information challenged the process of developing new drugs and good sustainable harvesting practice.

1.11 Significance of the Study

This study has led to characterization of a new ellagic acid derivative along with other fourteen (14) known biologically active compounds. A new ellagic acid derivative contributes to a global pool of drug templets. The cytotoxicity results indicated both compounds were nontoxic at the maximum

ested concentration. An octacosylferulate isolated from the stem bark extract indicated strong activity against *S. aureus*. *S. glaucescens* Pax is a promising source of new pharmacophores if further studies are conducted on anticancer, antioxidant and antidiabetic activities. The information on phytochemical profiles of studied locations is expected to guide the traditional medicine towards an eco-friendly harvesting of medicinal plants.

1.12 Research Objectives

1.12.1 Overall Objective

To enhance potential uses of *Synadenium glaucescens* through characterization of its phytochemical and biological properties

1.12.2 Specific Objectives

- To elucidate the structures of pure compounds isolated from the roots and stems of *Synadenium glaucescens* Pax.
- To determine the bioactivity of isolated compounds from roots and stems of *Synadenium glaucescens* Pax
- To determine the phytochemical profiles of *Synadenium glaucescens* Pax from different phytogeographical zones of Tanzania

2.0 CHAPTER TWO

A new ellagic acid rhamnoside from *Synadenium glaucescens* Pax and its cytotoxicity evaluation

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A NEW ELLAGIC ACID RHAMNOSIDE FROM *Synadenium glaucescens* AND ITS CYTOTOXICITY EVALUATION

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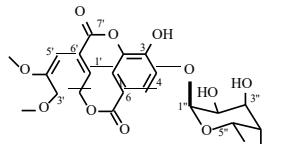
A phytochemical investigation of the ethyl acetate fraction of root bark extract of *Synadenium glaucescens* Pax (*Euphorbiaceae*) led to the isolation of two derivatives of ellagic acid. These included one new 3′,4′-di-*O*-methylellagic acid-4α, L-rhamnopyranoside (**1**) along with a known 3,4,3′-tri-*O*-methylellagic acid (**2**). Their structures were elucidated by 1D, 2D NMR, while a High res. MS (FTMS-ESI$^-$) was used for accurate mass measurement, which was compared with SciFinder data as well as with available literature. The BSLT results indicated that both **1** (LC_{50} = 2736.03 μg/mL) and **2** (LC_{50} = 531.19 μg/mL) were nontoxic at a maximum test concentration of 2400 ppm.

Keywords: ellagic acid, rhamnose, *Synadenium glaucescens*, *Euphorbiaceae*, antimicrobial, anticancer, antioxidant, phenolics, brine shrimp.

The use of higher plants as sources of medicine which can be traced back to over 1000s years [1, 2] is based on experience, the possibility for non or low toxicity, accessibility, and affordability [3, 4]. About 25% of the drugs prescribed worldwide today are reported to originate from plants and 121 such active compounds are in active use [5, 6]. Plants in the family *Euphorbiaceae* are known for their medicinal value against human and animal infections and some extracts are patented and registered as drugs and are available on the market [7]. Traditional practice and studies that involved crude extracts from *S. glaucescens* Pax reported the containment of bioactive compounds for antiviral [8], antifungal, antibacterial and acaricidal effect. Therefore, this plant is proving to be a candidate for providing compounds of medicinal value. Despite the observed potential of crude extracts, previous studies on pure compounds reported only the isolation of octacosanol and erythrinacinate c [9], euphol and β-sitosterol from root barks and leaves [10], and friedelanol from leaves [11], which created a need for the isolation and identification of more active compounds. This study thus reports on the isolation and structure assignment of two phenolic compounds—namely a new ellagic acid derivative 3′,4′-di-*O*-methylellagic acid-4α-L-rhamnopyranoside and a known 3,4,3′-tri-*O*-methylellagic acid isolated from the root bark of *S. glaucescens*.

Studies by other scholars on ellagic acid and its derivatives isolated from other plant species have reported pharmacological properties. Pure ellagic acid and its derivatives are also reported for their antioxidant and antimicrobial activity [12, 13], anticancer [14, 15], antidepressant-like activity [16], cardiovascular injuries prevention [17, 18] antidiabetic [19], neuroprotective, and anti-inflammatory actions [20]. 3,4,3′-tri-*O*-methylellagic acid isolated from*Combretum dolichopetalum* roots is known for its antidiabetic activity in mice models [21], as well as its antioxidant and cytotoxic effect [22]. Despite the limited information on pharmacological tests of compound **1**, the derivatives of ellagic acid with sugar moieties are known. The 3,3′-di-*O*-methyl ellagic acid 4′-*O*-β-D-xylopyranoside [23], ellagic acid-3,3,4-trimethoxy-4-*O*-α-L-rhamnopyranoside are reported for displaying antitumor activity [20]. The 3-*O*-methyl ellagic acid 4-*O*-β-D-glucopyranoside and 2,3,8-tri-*O*-methylellagic acid isolated from *Conocarpus lancifolius* demonstrated less toxicity in normal rat glioma cells and human embryonic kidney cells, significant antitumor properties against murine lymphocytic leukaemia, human colon cancer and human breast [22]. Since those compounds have close structure similarities to **1** and **2** there is a possibility for them to exhibit the same pharmacological effect.



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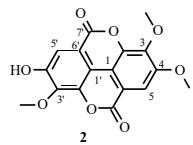

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TABLE 1. **^{13}C NMR Spectral Data of Compounds 1 and 2 (150 MHz, DMSO-d_6, δ, ppm)**

C atom	1	2	C atom	1	2
1	111.98	111.65	6′	117.64	111.90
2	141.56	140.95	7′	158.23	158.27
3	141.85	141.45	3-OCH_3		60.98
4	152.80	153.77	3′-OCH_3	61.59	56.70
5	111.63	107.45	4-OCH_3		61.27
6	112.68	113.34	4′-OCH^3	60.98	
7	158.39	158.45	1″	99.80	
1′	111.68	111.17	2″	70.03	
2′	141.05	140.80	3″	70.41	
3′	140.16	140.16	4″	71.52	
4′	150.24	152.59	5″	70.27	
5′	114.08	112.47	6″	17.89	

TABLE 2. **^{1}H NMR Spectral Data of Compounds 1 and 2 (600 MHz, DMSO-d_6, δ, ppm, J/Hz)**

C atom	1	2	C atom	1	2
5	7.79 (1H, s)	7.62 (1H, s)	3-OMe		4.04 (3H, s)
5′	7.52 (1H, s)	7.53 (1H, s)	4-OMe		4.06 (3H, s)
1″	5.58 (1H, d, J = 1.4)		3′-OMe	4.05 (3H, s)	4.00 (3H, s)
2″	3.96 (1H, br.s)		4′-OH		10.81(1H, s)
3″	3.72 (1H, d, J = 9.1)		4′-OMe	4.06 (3H, s)	
4″	3.36 (1H, d, J = 4.1)		2″-OH	4.84(1H, s)	
5″	3.52 (1H, dq, J = 9.2, 6.2)		3″-OH	4.97 (1H, s)	
6″	1.15 (3H, d, J = 6.1)		4″-OH	5.19 (1H, s)	
3-OH	10.84 (1H, s)				

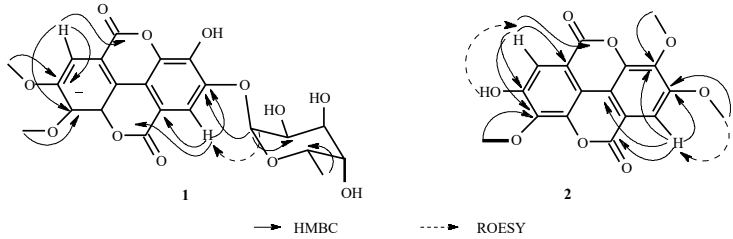

Fig. 1. Key ^{1}H–^{1}H correlations for compounds **1** and **2**.

Isolation of these derivatives of ellagic acid potentially contributes to the field of natural products, medicinal value of *S. glaucescens* and eventually to a future development of new medicines and vaccines.

Compound **1**, $C_{22}H_{20}O_{12}$, 28 mg, grayish powder, mp 251.0–252.7°C. HPLC-ESI$^-$ *m/z* 475.4119 [M – H]$^-$, FTMS-ESI *m/z* 476.0921. UV detection, 254 nm (blue spot) and 365 nm (purple fluorescence) of an ultraviolet lamp agrees with a highly conjugated nature of this polyphenolic compound. This conjugation nature was thought to contribute to the lowering of the chemical shifts of the carbonyl carbon signals from 167–180 ppm (theoretical values) to 158 ppm.

The ^{13}C NMR spectrum of compound **1** indicated twenty-two carbon signals (Table 1), whereas twenty protons were observed from its ^{1}H NMR (Table 1). The carbon signals corresponded to two methoxy groups (2 carbon atoms) at δ 60.98 and 61.59, two phenyls (12 carbon atoms) between δ 111.63–152.80, two carbonyl carbons at δ 158.39 and 158.23 and one rhamnose group (6 carbon atoms) at δ 17.89 and 70.03–99.80. The characteristic methyl group in the deoxyhexose, at C-6″ was observed at δ 17.89 and its anomeric proton signal appeared as a doublet at δ_H 5.58 attributed from a small coupling constant of 1.4 Hz to an α-configurated anomeric proton, which was in agreement with the values from [24]. A proton at δ 1.15 (3H, d, J = 6.2 Hz) in the ^{1}H NMR was a characteristic for the methyl group (C-6″) of the rhamnose sugar. The protons of the rhamnose sugar as indicated in Table 2 were compared and in agreement with [25–27]. The ^{1}H NMR signals (each 3H, s) at δ 4.06 and 4.05 were characteristic two methoxy attachments (3′-OCH_3 and 4′-OCH_3) while two aromatic protons were observed at δ 7.79 (C-5) and 7.52 (C-5′). The singlet protons at δ 4.84–5.19 were characteristic OH attachments of the sugar group at positions 2″, 3″, and 4″, while the δ_H 3.36–3.96 and at 5.58 were attributed to the rhamnose protons. The data from DEPT135 spectrum indicated the presence of only seven (C–H), three CH_3, twelve quaternary carbons and no CH_2. These spectral results together with C–H correlations in HSQC in H-6″, H-3″, H-4″, H-2″, H-5″, H-3′, H-4′, H-1″, H-5′, H-5, and the HMBC correlations for MeO-4′ to C-4′, MeO-3′ to C-3′, H-1″ to C-2″

and C-4, H-6″ to C-4″ and C-5″, H-5′ to C-3′, C-4′, and C-7′, H-5 to C-4, C-6, and C-7, established the crude structure of **1**. These spectral data were compared and closely related to ^{13}C NMR data by [25] but they varied in positions of the sugar and methoxy attachments. However, the position of the sugar moiety was confirmed from the ROESY data which indicated a correlation of the anomeric proton H-1″ with the phenolic proton H-5. Lack of H-H correlation between this anomeric proton and methoxy protons in ROESY data implied the rhamnose is not adjacent to any of the methoxy group (Fig. 1). The final structure **1** was confirmed by the high res. FTMS-ESI$^-$ *m/z* 475.0887 [M – H]$^-$ and fragment ions of FTMS-MS-ESI$^-$. The fragmentation pattern of the preceding molecular ion peak, giving *m/z* 328.0081 and 313.0291, suggested the ionization by loss of the rhamnose group ($C_6H_{11}O_4^-$, *m/z* 147.0806), followed by the methyl (CH_3^-, 14.979) group, respectively, at cone voltage of 25 and collision energy (CE) of 30. Thus, compound **1** was concluded to be 3′,4′-di-*O*-methylellagic acid-4α, L-rhamnopyranoside. Based on the spectral information and SciFinder database, this is a new derivative of ellagic acid and reported for the first time from *S. glaucescens* Pax. Others closely related to compound **1** are known to derive from the stem barks of *Dipentodon sinicus* [25] and *Euphorbia aphylla* [26].

Compounds **2**, $C_{17}H_{12}O_8$, 14 mg, pale-yellow solid. ESI-MS *m/z*: 343.2891 [M – H]$^-$ and *m/z* 344.3129 for a neutral molecule. The spectral data from ^{13}C and ^{1}H NMR (Table 1) indicated seventeen (17) carbon atoms and twelve (12) protons, respectively. The DEPT135 indicated two C-H signals at δ 107.2 and 111.4) and three CH_3 signals due to –OCH_3 groups at δ_C 56.4, 60.7, and 61.0. The ^{1}H NMR indicated three singlet proton signals (at δ 4.00, 4.04, and 4.06) due to OCH_3 substitution at carbon 3, 4, and 3′, respectively. The HSQC spectrum indicated C-H correlations of 3′-OCH_3/C-3′, 3-OCH_3/C-3, 4-OCH_3/C-4, H-5/C-5 and H-5′/C-5. The HMBC spectral data indicated correlations from H-5′ to C-3′, C-4′, C-6′, and C-7′, H-5 to C-1, C-3, C-4, C-5, and C-7, 4-OCH_3 to C-4, 3-OCH_3 to C-3 and 3′-OCH_3 to C-3′. A careful inspection of the ROESY spectrum indicated correlations of the methoxy protons, H-4 to the aromatic proton H-5 and the proton of the OH group, H-4′ correlated with the aromatic proton H-5′ (Fig. 1). These key correlations also confirmed the attachments of substituent groups. These NMR spectral data were in agreement with literature data [27–29] and it was assigned as 3,4,3′-tri-*O*-methylellagic acid. Its structure was finally confirmed by HPLC-MS/MS, for *m/z* 343.2891 [M – H]$^-$ and indicated fragment ions of *m/z* 328.0181, 312.7756, 297.9110, and 280.9110. This fragmentation pattern was related to consecutive loss of the three CH_3 of the methoxy groups at C-3, C-4, and C-3′ and finally an OH group at C-4′. Despite isolation of compound **2** for the first time from *S. glaucescens* Pax, it was previously isolated from *Euphorbia acaulis*, *Syzygium aromaticum* leaves, *S. guineense*, *S. cumini* seeds and roots of *Combretum dolicopetalum*.

Both compounds **1** and **2** demonstrated to be nontoxic (LC_{50} > 100 μg/mL) on the brine shrimp larvae (naupuli) at the maximum tested concentration of 2400 ppm. These LC_{50} values, 2736.03 μg/mL and LC_{50} 531.19 μg/mL for **1** and **2**, respectively, could support the traditional use of this plant extracts against both animal and human related ailments.

EXPERIMENTAL

General. The molecular masses of **1** and **2** were first determined by the HPLC-MS/MS. An Agilent 1100 liquid chromatography system (Agilent Technologies, Germany) coupled to a Quattro Micro API triple quadrupole Mass Spectrometer (Waters, Micromass Limited, UK) was used. The LC systems consisted of a degasser, G1379A; binary pump, G1312A; auto sampler G1313A; column oven, G1316A; and diode array detector (DAD), G1315B. The chromatography system was coupled to a Quattro Micro API triple quadrupole Mass Spectrometer (Waters, Micromass Limited, UK) equipped with an electrospray ionization (ESI) interface. The acquisition and treatment of data were done using Masslynx 4.1 software (Waters Corporation, USA). The nebulizing and collision gases were nitrogen and argon, respectively. High-resolution mass spectrometry (HR-MS) analysis was carried out on a freshly calibrated QExactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a custom-build DESI-MSI ion source. NMR spectroscopy experiments for compounds **1** and **2** were obtained using a 600 MHz Bruker Avance III HD equipped with a cryogenically cooled 5-mm dual probe optimized for ^{13}C and ^{1}H and TMS (as the internal standard).

Plant Material. The plants of *Synadenium glaucescens* Pax were identified by a botanist and assigned voucher specimen number HOS/FM 3672. The fresh root samples were collected from Mtulingala Village (Njombe region, Tanzania) in November of 2018. The samples were processed into bark and wood parts then air-dried in a dark room at 15°C (at Tanzania tree Seeds Agency, Morogoro) to prevent loss of labile and light sensitive compounds. Dry samples were pulverized using a laboratory milling machine (Christy Hunt Engineering Ltd, England) at the Department of Animal Science and Production (DASP) of Sokoine University of Agriculture to obtain approximately 1 mm particle size.

Extraction and Isolation. A total of 2.5 kg of powdered root barks were extracted by Maceration using methanol. For a maximum extraction, filtration was done three times consecutively after every 48 h until it showed a clear solution. The extract was air-dried at room temperature to obtain 300 g of solid brown residues. 70 g of the extract were pre-adsorbed on the silica gel 60 (70–230 mesh ASTM, Merck, KGaA, Darmstadt, Germany). The slurry was partitioned by vacuum liquid chromatography (VLC) in a solvent polarity gradient yielding hexane, ethyl acetate, and methanol extracts. The filtrates were dried using a rotary evaporator at 30°C and 55°C to afford three crude fractions: 204 mg He (oilish), 24 g EtOAc (yellow solid), and 32.33 g MeOH (brown solids). EtOAc fraction (22.21 g) was subjected to repeated column chromatography (CC) with solvent gradient systems of EtOAc–dichloromethane (DCM) ranging from 0:100–100:0 EtOAc–DCM then the solvent in vials was air evaporated. The vial components were eluted on TLC silica gel 60 F254 (made in

Germany, Merck KGaA, 64271 Darmstadt) affording fourteen fractions (F1–F14). Purification of Frs. F12 and F10 yielded compounds **1** and **2**, respectively. An ultraviolet lamp (254 and 365 nm) from Cole-Parmer (800)323-4340, Cat # 97620-41) was used for compound visualization on TLC. Both compounds **1** and **2** appeared as blue spots on the TLC at a UV light of λ 254 nm and purple fluorescence at 365 nm. Compound **1** indicated the R_f of 0.24 at 100% EtOAc and 0.69 at 10:90 MeOH–EtOAc. Compound 2 exhibited the R_f 0.16 in 100% DCM, 0.83 in 50% EtOAc–DCM and 0.91 in 100% EtOAc on the eluted TLC. These R_f values were a characteristic of polar compounds. The melting point (MP) was recorded using a Stuart SMP30 Cole-Parmer machine.

Cytotoxicity Evaluation. Compounds **1** and **2** were subjected to Brine Shrimp Lethality Test (BSLT). The eggs of Brine shrimps (*Artemia salina*) were used according to [30], whereas their toxicity status was interpreted according to [31] in which the concentration killie 50% of the naupuli, LC_{50} < 1.0 μg/mL – highly toxic; LC_{50} 1.0–10.0 μg/mL – toxic; LC_{50} 10.0–30.0 μg/mL – moderately toxic; LC_{50} > 30 < 100 μg/mL – mildly toxic, and > 100 μg/mL as non-toxic.

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3.0 CHAPTER THREE

Isolation and toxicity evaluation of feruloyl ester and other triterpenoids from *Synadenium glaucescens* Pax

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Isolation and toxicity evaluation of feruloyl ester and other triterpenoids from *Synadenium glaucescens* Pax

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ABSTRACT

The use of plants as sources of drug agents is attributed by factors among which are the easy accessibility to plants, less toxicity and little or no drug resistance. An improvement in both traditional medicine and drug discovery field necessitates investigation of pure compounds in any plant with medicinal value. *Synadenium glaucescens* Pax of the family Euphorbiaceae is among the medicinal plant in Tanzania which are proven to contain bioactive compounds against microbial infections. Analysis of ethanolic and methanolic extracts of root and stem barks respectively aided to isolated six pure compounds (**SG1- 6**). These compounds were analyzed by both 1D, 2D NMR and GC-MS while their spectral processing was achieved in the Bruker TopSpin 3.6.2. Among these compounds, one was a phenolic (hemicosanyl ferulate-**SG1),** three triterpenoids, (lupeol- **SG2**, epifriedelanol- **SG4** and euphol- **SG5**), one steroid (β-sitosterol- **SG6**) and a long chain alkene (1-nonacosene- **SG2**). Cytotoxicity evaluation by Brine shrimp lethality test (BLST) indicated the compounds under report were practically non-toxic.

Keywords: Triterpene, Lupeol, Friedelanol, NMR, Euphorbiaceae, Brine shrimp.

INTRODUCTION

Natural products isolated from various sources especially plants, have long been used in treatment of human ailments. Despite estimates varying depending on the definition of what is considered a natural product-derived drug, between 25 and 50% of currently marketed drugs owe their origins to natural products [1]. Among these, the triterpenoids form diverse structures of secondary metabolites and are widely distributed in both edible and ethnomedicinal plants [2, 3]. Plant members of Euphorbiaceae are reported to contain these triterpenes which are known for pharmacological activities including anti-inflammatory, anticancer, anticarcinogenic [4, 5], antidiabetic [6,7] and antimicrobial activities [8]. *Synadenium glaucescens* Pax is a medicinal plant of Euphorbiaceae which is used against both animal and human ailments. Phytochemical screening by Mabiki et al., 2013 [9] for dichloromethane extracts indicated presence of more than one hundred compounds including triterpenoids (euphol, lanosterol and lupeol backborn), steroids, long chain hydrocarbons and fatty alcohols while ethanolic extracts contained polyphenolic compounds as major constituents. Despite the large number of compounds in *S. glaucescens* Pax (SG) and the reported pharmacological potential of this plant, the isolations and structure assignment of pure forms have sorely reported only six of them namely euphol, β-sitosterol [10], erythrinacinate C and octacosanol [11], β and α-friedelanol [12] from leaves. Additionally, there is evidence from bioactivity studies of the crude extracts that the most bioactive compounds were in the SG root and stem barks [13]. However, the number of pure compounds isolated from the rootbark was limited to only two. This study aimed at isolating more pure compounds which would be associated with the previously observed bioactivities. Phytochemical investigations of the ethylacetate (EtOAc) and dichloromethane (DCM) extracts of the root and stem barks respectively resulted into isolation of one phenolic compound (hemicosanyl ferulate), three triterpenoids (Lupeol, epifriedelanol, Euphol), one long chain alkene (1-nonacosene) and a sterol (β-sitosterol). Their toxicity evaluation indicated they are none toxic at the tested concentrations.

MATERIALS AND METHODS

Plant collection and Processing

Plant authentication in Njombe region was done by a botanist and the voucher specimen (voucher no. 3672) was stored in the herbarium of the Department of Botany- University of Dar es Salaam (UDSM). Sample were collected from mtulingala village of Njombe district (08°34′ to 08°49′ S and 034°55′ to 035°10′ E), December 2018. The roots (NSG2) and stems (NSG5) parts were peeled to separate barks and wood parts. The root and stem barks of *S. glaucescens* Pax were air dried in a cold dark room at 15 ºC to retain the light and temperature sensitive compounds.

Chemicals and apparatus

All chemicals used in this study were of analytical grade. They were obtained from either Loba Chemie, Mumbai-India i.e. ethylacetate (EtOAc), Dichloromethane (DCM) and Petroleum ether (PE) or Finar Chemical, Gujarat-India i.e. Methanol (MeOH) and Ethanol (EtOH). Moreover, silica gel 60 (70- 230 mesh, 60 angstrom pore size) and Thin Layer Chromatography Aluminium sheets (TLC, silica gel 60 F254) were obtained from Merck KGaA group, Darmstadt, Germany. An ultraviolet lamp (wavelength λ; 254 and 365 nm) was used for illumination of the TLC sheets.

Extraction and isolation of pure compounds

Powdered 2500 g of root barks (SG2) and 1200 g of stem barks (SG5) were extracted by maceration using ethanol and methanol solvents respectively according to [14, 15]. Each sample was packed in opaque bottles in either 500 g or 600 g followed by 2 L solvent. Mixtures were shaken for 5 minutes before they were placed in a dark room. Filtration of the mixture was done thrice after every 72 hours' time until a clear solution was formed as a maximum extraction. All extracts were air dried to evaporate the solvents affording 300 g NSG2 brown residues and 185 g NSG5 dark green residues. Fractionation of 90 g for each sample by vacuum liquid chromatography (VLC) packed with 90 g of silica gel and eluted using 5 L of n-hexane, 10 L ethyl acetate and 5 L ethanol (from least to the most polar solvent) yielded 262 mg for hexane (He), 60 g for ethyl acetate (EtOAc) and finally 20.2 g for EtOH in root bark extract (SG2FE) while in the stem bark extract, only two solvents (12.5L of DCM and 7 L of MeOH) were used to afford 35.5g and 50 g respectively. The SG2FE column (58 g of EtOAc fraction) was eluted at a solvent gradient systems of petroleum ether (PE) to 50 % MeOH: DCM. Based on the TLC profiles of eluted vials, a total of 15 (*fr.* 1-15) sub fractions were collected from 155 vials. Purification of precipitates in **fr**.1, 5, 8 and 10 cleanup with MeOH until a single sport for each compound was observed on an eluted TLC plate afforded compound **SG1**, **SG3, SG4, SG5** and **SG6.** Isolation of pure compounds from the DCM fraction of the stem bark extract, (SG5FD), 32.5 g was achieved by column chromatography. The column was eluted at 30 % EtOAc/ PE along to 30 % MeOH/ EtOAc to afford 10 sub-fractions (*frb.* 1- 10). Repetitive cleanup of *frb.* 3 by vacuum suction on the filter paper, Whatman 1 afforded compound **SG2** and again **SG4**.

Spectroscopic analyses

Nuclear magnetic Resonance (NMR) spectroscopy experiments for compounds were obtained using a 600 MHz Bruker Avance III HD equipped with a cryogenically cooled 5 mm dual probe optimized for ^{13}C and ^{1}H, and TMS (as internal standard). The chemical shifts (□) were recorded in ppm. Both compounds were dissolved in deuterated solvents either dichloromethane, chlorofoam or acetone prior to analysis. Mass spectrometric data were acquired on a Gas Chromatograph (GC) machine (6890N, Agilent Technologies, Germany) coupled with an MS detector (5973, Agilent Technologies, Germany) using electron impact ionization. The obtained NMR spectral data were processed in Bruker TopSpin version 3.6.2.

Cytotoxicity evaluation of Pure compounds

The egges of brine shrimps (*Artemia salina*) were hatched and used. The brine shrimp lethality test was carried out using the standard procedure as described by [16], with slight modifications. The stock solution of the study compounds were prepared by dissolving 40 mg of each compound in 1ml of 20 % v/v DMSO. A serial dilution was made to afford final concentrations of 2400, 1200, 800, 400 and 240 μgml^{-1}. Each concentration was tested in duplicate making 10 vials per test compound and one set of vials was prepared using 20 % v/v DMSO as a negative control. Ten live naupuli were transferred into each vial containing test compound with sea salt solution using Pasteur pipettes, followed by immediate adjusting the volume of the sea salt solution to 5 mL mark. The vials were maintained at room temperature on a laboratory bench for 24 hours after which the survivors were counted. The concentration of the killing 50 % of the naupuli larvae (LC_{50}) was determined from the graph.

RESULTS AND DISCUSSION

Compound **SG1** ($C_{31}H_{52}O_4$, Fig. 1), 65 mg was isolated as white powder from the root bark extract. The ^{13}C NMR (150 MHz, CD30D, δppm) indicated benzylic signals at δ_C 127.6 (C-1), 111.4 (C-2), 150.2 (C-3), 148.9 (C-4), 116.1 (C-5), 124 (C-6), the olefinic carbons at 145.6 (C-**7),** 116.6 (C-8), carbonyl carbon (167.6, C-9), 56.4 (OMe), 64.8 (C-1'). Other aliphatic chain signals were 31.7 (C-19'), 30.4- 30.5 for the C-4' to C-18'), 23.4 (C-20'), 30.4 (C-2'**),** 26.8 (C-3'), and 14.4 (terminal CH_3, C-21'**).** The ^{1}H NMR (600 MHz, CD_3OD) afforded two trans-olefinic protons at δ_H 7.58 (1H, *d*, 16.0, H-7), 6.38 (1H, *d*, *J*=16.0, H-8) and three aromatic protons 7.14 (1H, *dd, J*=8.2, 2.2Hz, H-2), 6.90 (1H, *d, J*= 8.2, H-5) and 7.34 (1H, *d, J*= 1.9, H-6). It further exhibited a methoxy signal at 3.93 (3H, *s,* -OCH_3), and a methylene at δ 4.15 (2H, *t, J*= 6.6, C-1'), 1.65 (2H, *qui*, H-2'), 1.41 (2H, *m,* C-3') and 0.88 (3H, *t,* CH_3). These structural clues suggested a feruloyl ester moiety [17,18]. The presence of an aliphatic alcohol moiety was indicated by the triplet signal at δ 0.88 (terminal methyl), the broad singlet at δ 1.29 for CH_2 and the downfield triplet at δ 4.15 that corresponded to a methylene adjacent to an oxy-carbonyl function. The broad singlet in ^{1}H NMR (at 1.29 ppm, 34H) corresponded to a total of seventeen (17) CH_2 groups thus making 21 carbon atoms in the aliphatic chain. The larger coupling constant between H-7 and H-8 (*J*= 15.9) suggested a trans-geometry [17,18]. A key HMBC correlation was observed between the ferulic acid and the long chain alcohol at δ_C 167.8 (C-9) to δ_H 4.15 (H-1') Based on the 1D and 2D spectral data and in comparison to the available literature [18–21], the **SG1** was assigned as hemicosanyl ferulate. This compound was previously isolated from the stem bark of *Pavetta owariensis* of the family Rubiaceae and *Aristolochia kankauensis*.

Compound **SG2, (**$C_{30}H_{50}O$, 65 mg) was isolated as white powder from the stem bark extract**.** The ^{13}C-NMR (Table 1) indicated thirty (30) carbon signals while the ^{1}H NMR spectrum had many signals concentrated in the high field region which was a characteristic of triterpene skeleton. Two olefinic carbon signals in 13C-NMR spectrum were observed at □c□148.8 (C-20) and 109.8 (C-29) indicating the exocyclic double bond. The □c 78.7 was due to a carbon bearing OH group (C-3). The ^{1}H NMR spectrum indicated seven singlet methyls at □H 0.72, 0.74, 0.82, 0.97, 1.00, 1.18, and 1.74. These experimental data ^{1}H and ^{13}C NMR were compared and in agreement with the literature [22–26] and was finally concluded **SG2** to be Lupeol.

Compound **SG3**, ($C_{29}H_{58,}$ 11 mg), white waxy solid was isolated from the root bark extract. Its TLC profile indicated a retention factor (R_f) value of 0.96 in PE and reacted bright purple with vanillin reagent. This was an indicator for non-conjugated moiety. The ^{13}C NMR spectrum indicated fourteen (14) carbon signals one of them being the longest and broad at □c 29.9□which indicated the CH_2 chain stretch. Two peculiar olefinic carbons were observed at 114.3 (C-1) and 139.5 (C-2) due to a terminal π-bond while □c 14.3 corresponded to the terminal CH_3. The ^{1}H NMR indicated key signals at □H 5.79 (*m,* 1H) corresponding to a proton attached to an olefinic β-carbon, 4.94 (*dd,* 2H, *J*= 17.1 Hz and 10.2 Hz) for the two terminal hydrogen atoms at the chiral carbon rendering them different chemical environment. The other signals at □H 2.02 (*q,* 2H, *J* = 7.2) and 1.35 (*qui*, 2H) were due to γ and δ- CH_2 attachments while 0.86 (*t,* 3H, *J*= 6.5 Hz) was terminal CH_3. The final structure was confirmed by 2D NMR data (HSQC and HMBC) together with the GC_MS data indicated m/z 405.4 which was finally assigned as 1-nonacosene (Fig. 1). To the best of our knowledge, this long chain alkene is reported for the first time from *S. glaucescens* Pax but had been isolated from *Cissampelos mucronata* [27].

Compound **SG4**, ($C_{30}H_{52}O$), was isolated from bark extracts of both root (155 mg) and stem (12 mg) as white powder. The ^{13}C NMR spectrum indicated thirty (30) carbon signals. Comparative analysis of ^{13}C and DEPT135 NMR identified signals were eleven CH_2, six quaternary, five (C-H) and eight CH_3 signals were observed. The peculiar carbon signal at 73.1 represented a C-O stretch at C-3 of a triterpene skeleton. ^1H NMR spectrum indicated many proton signals in the high field region. Peculiar signals of ^1H NMR included a broad singlet at □H 3.70 representing a proton at C-3 bearing the-OH group in a trans-orientation [27]. Seven more proton signals for seven methyls resonated in the high field region as singlets at □ 0.95 (3H, s, C-24), 0.86 (3H, s, C-25), 1.00 (3H, s, C-27), 1.02 (3H, s, C-26), 1.00 (3H, s, C-28), 0.94 (3H, s, C-29) and 1.18 (3H, s, C-30). One methyl doublets resonated at δ 0.92 (3H, d, J = 7.3 Hz) and was assigned to C-23). Based on the experimental data (Table 1) and the available literature data [12,29,30], the **SG4** (Fig. 1) was concluded to be an epi-friedelanol (also known as β-friedelanol). Despite epifriedelanol being reported for the first time from the root and stem regions of *S. glaucescens* Pax, it was previously isolated from its leaves [12], stem barks of *S. grantii* [30], *Euphorbia neriifolia* [31] and leaves of *Pouteria ramiflora*.

Compound **SG5**, ($C_{30}H_{50}O$), R_f 0.6 in DCM, and [M^+], m/z 426.4 was isolated from bark extracts of both root (1834 mg) and stem (21.5 mg) as white powder. Their spectral data (^{13}C NMR, ^1H NMR and GC-MS) were compared and concluded to be the same compound. The ^{13}C NMR spectrum indicated thirty (30) carbon signals, four (4) of which resonated in olefinic regions at □c 134.7 (C-8), 134.1 (C-9), 125.8 (C-24) and 133.1 (C-25). The ^1H NMR indicated seven singlets at □H 0.77, 0.78, 0.88, 0.95, 0.98 and 1.26 (*s,* 6H) representing seven methyls. Comparison of these experimental data with the literature [10,33] and the GC-MS data, the compound **SG5** was confirmed to be euphol (Fig.1)

Compound **SG6**, ($C_{29}H_{50}O$, 62 mg) and the R_f 0.36 in DCM was isolated from the root bark extract as white shiny crystals. The ^{13}C NMR spectrum indicated a total of twenty nine (29) carbon signals (Table 1), which is a sterol characteristic. Two important olefinic carbon signals resonated at high field □C 141.0 (C-5) and 122.0 (C-6) while a signal at □C 72.1 was due to a proton attached to a carbon bearing the OH group at C-3. Its ^1H NMR spectrum indicated two main singlets at □H 0.66 (*s*, 3H) and 0.99 (*s*, 3H) assigned to two methyl groups at C-18 and C-19. Four more signals assigned to four secondary methyls appeared as doublets at □H 0.79, 0.82, 0.83 and 0.90 (*J*= 6.7, 6.8, 7.4 and 6.5) respectively. An olefinic proton at □H 5.33 (*br d,* 1H, *J*= 5.3) was assigned to H-6 while a proton at C-3 resonated as a multiplet at □H 3.50. These experimental data were in agreement with [10,34–36] together with the GC-MS data, m/z 414.3, and a melting point of 134.6-136.1 ºC helped to confirm **SG6** as β-sitosterol. This compound was earlier isolated from the SG leaves but it is for the first time isolated from the root barks of this plant.

Brine shrimp toxicity test

The degree of toxicity for each tested compound in the BLST was evaluated according to [33] in which the LC_{50} <1.0 µg/ml – highly toxic; LC_{50} 1.0-10.0 µg/ml – toxic; LC_{50} 10.0-30.0 µg/ml – moderately toxic; LC_{50} >30 <100 µg/ml – mildly toxic, while LC_{50} > 100 µg/ml as non-toxic.

The test results (Table 2) indicated that 100 % of the compounds had the LC_{50} values greater than 100 µg/ml suggesting them to be non-toxic. Despite their toxicity category, compound **SG1** (feruloyl ester) showed the least LC_{50} values (most toxic) of all whereas **SG2** (lupeol) was the least. Since the compounds were obtained from crude extract of the high polar solvents, these results (at maximum test concentration of 24000 µg/ml) support the safety in traditional use of extracts from root and stem regions of *S. glaucescens* Pax.

Table 1: ^{13}C NMR Experimental data for the isolated triterpenoids (**SG2, SG4,** and **SG5**) and a steroid (**SG6**) from the root and stem barks of Synadenium glaucescens Pax

Position	SG2 (150 MHz, CD_2Cl_2, δ ppm)	SG4 (150 MHz CD_2Cl_2, δ ppm	SG5 (150 MHz, CD_2Cl_2, δ ppm)	SG6 (150 MHz, $CDCl_3$, δ ppm)
1	39.3	16.4	35.8	36.7
2	27.9	35.8	28.4	37.5
3	79.3	73.1	79.3	72.1
4	42.3	49.8	39.4	42.5
5	55.6	37.7	51.5	141
6	19.0	42.3	19.2	122
7	33.9	18.1	28.2	32.1
8	42.6	53.7	134.7	31.9
9	55.4	38.9	134.1	50.4
10	37.7	61.9	37.8	36.4
11	22.2	35.9	22.1	21.3
12	24.5	30.5	31.5	40
13	39.2	38.4	44.7	42.6
14	45.3	40.2	50.6	57
15	25.3	32.9	30.3	26.3
16	34.3	36.7	28.7	28.5
17	47.1	30.3	50.3	56.3
18	50.9	43.4	15.9	34.2
19	50.1	36.1	20.5	19.3
20	149.4	28.6	36.5	32.2
21	28.4	33.4	19.5	24.5
22	42.4	39.8	36.0	46.1

23	28.1	12.0	25.3	23.3
24	16.2	16.8	125.8	12.2
25	17.0	18.6	131.3	29.4
26	16.5	19.0	17.9	20
27	15.7	20.5	26.0	19.6
28	17.0	31.2	28.6	19
29	110.4	35.3	15.9	12.1
30	21.6	32.1	24.7	

Reference for: **SG2-** *Lupeol* [22–26], **SG4-** *epifriedelanol* [12,18,19] , **SG5-** *Euphol* [10, 33], and **SG6-** *β-sitosterol* [10, 23-25]

Table 2: LC_{50} on Brine shrimps for the pure compounds isolated from the root and stem barks of *Synadenium glaucescens* Pax

Compound	Concentration (µgmLL^{-1})	% Mortality	LC_{50} (µg/mL)
SG1 (feruloylester)	2400	80	762.807
	1200	65	
	800	50	
	400	50	
	240	0	
SG2 (Lupeol)	2400	40	25813.42
	1200	20	
	800	10	
	400	0	
	240	0	
SG3 (1-nonacosene)	0	0	NT
	0	0	
	0	0	
	0	0	
	0	0	
SG4 (epifriedelanol)	2400	0	1088.48
	1200	0	
	800	0	
	400	0	
	240	0	
SG5 (Euphol)	2400	80	825.52
	1200	70	
	800	40	
	400	25	
	240	0	
SG6 (β-sitosterol)	2400	40	5688.20
	1200	25	
	800	0	
	400	0	
	240	0	

* NT= not tested

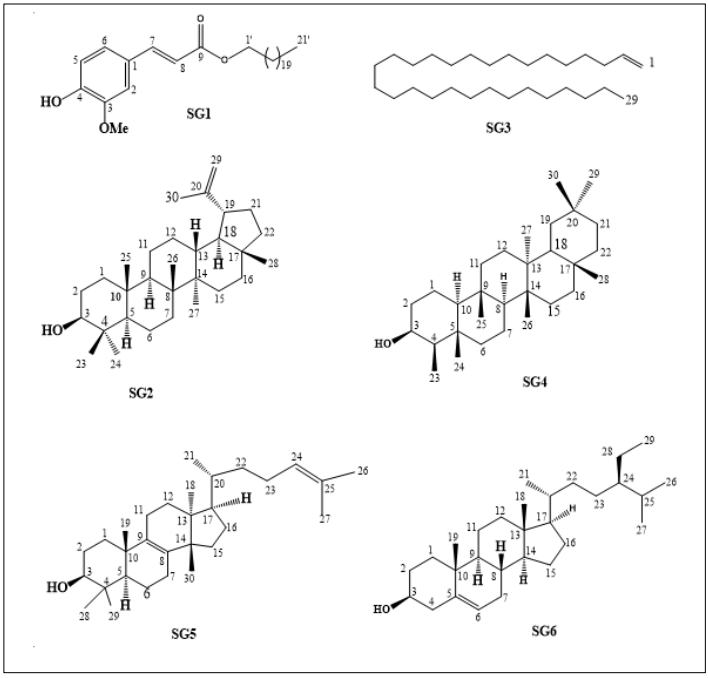

Figure 1: Structures of compounds isolated from the root barks (**SG1, SG3-6**) and stem barks (**SG2**) of *Synadenium glaucescens* Pax

Figure legend

Chemical structures of the compounds **SG1-6** were drawn using ChemDraw Professional 16.0 software.

CONCLUSION

This study (to the best of researchers' knowledge) reports for the first-time isolation of hemicosanyl ferulate (also known as eicosyl ferulate) from *S. glaucescens* Pax. Additionally, this is the first-time report on isolation of the triterpenoids, Lupeol, euphol (from the stem bark extracts) and epifriedelanol from both root and stem barks including the steroid β-sitosterol from the root regions of *S. glaucescens* Pax. These compounds demonstrated to be nontoxic to brine shrimps at the tested concentrations.

Limitation of the study

Cytotoxicity of 1-nonacosene was not done due to its insufficient weight.

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Conflict of Interest

None declared.

Supplementary information

Supplementary figures (Figure 1-23) can be downloaded by following the given link.

Link:
http://www.phytopharmajournal.com/Vol11_Issue5_06_SupFig.pdf

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4.0 CHAPTER FOUR

Isolation and antibacterial efficacy of fatty acid and phenolic compound from roots and stems of *Synadenium glaucescens* Pax

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Isolation and elucidation of antibacterial compounds from roots and stems of *Synadenium glaucescens* Pax

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ABSTRACT

Extracts of *Synadenium glaucescens* Pax are reported to contain biologically active compounds against bacterial and viral infections. This study aimed at isolating pure compounds from its bioactive extracts as well as evaluating their antibacterial efficacy. A phytochemical investigation of the root and stem barks involved total maceration in methanol (MeOH). The root barks extract was then partitioned by Vacuum Liquid Chromatography (VLC) in a solvent gradient system to afford three fractions of Hexane (Hex), Ethyl acetate (EtOAc) and finally MeOH for the least, moderate and most polar compounds respectively. The MeOH extract of stem barks was also partitioned in the same way by using two solvents; dichloromethane (DCM) and MeOH. All fractions were finally dried on a rotary evaporator at < 60 ^{O}C of the water bath. Isolation of pure compound from the EtOAc and DCM led to isolation of four compounds namely; hexacosane (**G1**)**,** β-sitosterol (**G2**), octacosyl ferulate (**G3**) and hexacosanoic acid (**G4**). Their structures were analyzed and confirmed through NMR, GC-MS and in comparison, with literature. Antibacterial assay for **G1, G3** and **G4** against *P. aeruginosa*, *E. coli, S. aureus*, and *E. faecalis* was achieved by broth serial microdilution. Compounds, **G3** demonstrated strong activity against *S. aureus* (MIC = 0.125 mg/mL) and weak activity against the rest strains (MIC = 2 mg/ mL). Also, the test results indicated **G1** had weak activity against all tested strains (MIC = 2 mg/mL or above). While **G4** demonstrated a moderate activity (1.0 mg/mL) against *E. coli, S. aureus, E. faecalis* and weak against *P. aeruginosa* (MIC > 2 mg/mL). These findings support traditional use and promise for antibacterial drug agents from *S. glaucescens* Pax.

Key words: *antimicrobial, NMR, phytochemistry, ferulic acid, Euphorbiaceae, drug, octacosylferulate*

Introduction

Antimicrobial resistance is a serious threat against humankind that has fueled research initiatives towards search for new therapeutics (Canli *et al.,* 2017; Nzogong *et al.*, 2018). It is the mission of the World health Organization (WHO) that searching for new medicines be among the interventions towards effective antimicrobial drugs for both preventive and curative measures that protect patients from potentially fatal diseases (WHO, 2015). Despite a limited number of these molecules being evaluated for their suitability for use as drugs (Fabricant and Farnsworth 2001), about 25 % to 50 % have served as the source and inspiration for a large fraction of the current pharmacopoeia (Kingston, 2011). Their diverse structures, forms and concentrations in different organism including plants make them serve a defense mechanism in live organisms (Ostria *et al.,* 2022). These compounds are used either as natural structures or derivative forms (Shelar and Shirote 2011). It is asserted that about 75 % of the drugs for infectious diseases come from natural products (Newman and Cragg 2016) including plants. Infections from resistant bacteria are now too common, and some pathogens have even become resistant to multiple types or classes of antibiotics (Frieden, 2013) thus necessitates a need for new drug agents and sources. *Escherichia coli* is a known versatile and frequently deadly pathogen which is associated with diarrhoeal diseases (Hunter, 2003), urinary tract infections (UTI) and meningitis (Kaper *et al.,* 2004). Other bacteria such as *P. aeruginosa* is reported to cause pulmonary infections, respiratory insufficiency, UTI and finally morbidity and mortality (Ochoa *et al.,* 2013) while *S. aureus* is known for causing illnesses ranging from mild skin and wound infections to fatal sepsis or multi-organ failure (Chen *et al.,* 2022). *Synadenium glaucescens* Pax (Euphorbiaceae) is a medicinal plant in Tanzania whose crude extracts and pure compounds have been reported for alleviating bacterial and viral infections (Mabiki *et al.,* 2013; Credo *et al*., 2022). Previous phytochemical screening and isolation experiments on *S.glaucescens* Pax revealed the

presence of phenolics, terpenoids, aliphatic hydrocarbons, fatty acids, steroids and more others (Mabiki *et al.,* 2013; Rwegoshora *et al.,* 2022). These groups of compounds are known for their medicinal value in both traditional and modern pharmaceutical industries. However, the number of potential compounds isolated and elucidated from these bioactive sections of this plant is still limited. This study aimed at isolating more pure compounds which are responsible for efficacy of root and stem extracts of this plant.

Materials and methods

Plant collection and Processing

Plant authentication in Njombe region was done by a botanist and the voucher specimen (HOS/FM 3672) was stored in the herbarium of the Department of Botany- University of Dar es Salaam (UDSM). Sample collection was done in Njombe district 1656 m above the see level, (08°34′ to 08°49′ S and 034°55′ to 035°10′ E), in December 2018. The roots (SG2) and stems (SG5) parts were peeled to separate barks and wood parts. The root and stem barks of *S. glaucescens* Pax were air dried in a cold dark room at 15 ^{o}C at the laboratory of Tanzania Tree Seed Agency (TTSA). The conditions were meant to retain the light and temperature sensitive compounds.

Extraction and compounds isolation

Two main samples of root barks and stem barks were involved in the isolation of pure compounds. 1.2 kg of powdered SG stem barks were extracted by total Maceration using methanol (MeOH). For a maximum extraction, filtration was done thrice consecutively after every 72 hours until it showed a clear solution and afforded 185 g of dry extract. 90 g of this extract were pre-adsorbed on silica gel 60 (70-230 mesh ASTM, Merck, KGaA, Darmstadt-Germany). The slurry was partitioned by vacuum liquid chromatography (VLC) in a solvent polarity gradient with yield of 35 g dichloromethane (DCM) and 50 g MeOH extracts. 32.5 g of the DCM fraction were subjected to a

column chromatography starting the solvent systems of 100 % PE to 30% MeOH/DCM. After TLC profiling of the eluted vials, a total eleven fractions (*Fs. 1- 11)* were afforded depending on the TLC results. A repetitive clean up *Fs.1, Fs.5* and *Fs.7* by using MeOH yielded compound **G1, G2** and **G3** respectively. The ultraviolet lamp (254 and 365 nm) from Cole-Parmer (800)323-4340, Cat # 97620-41) was used for compound visualization on TLC. Chemical treatment for visualization of non-conjugated was done by using vanillin (spraying) reagent. A single compound spot on the TLC plate under both treatments (UV and chemical treatment) was used to indicate a pure compound. The melting point (MP) was recorded using a Stuart SMP30 Cole-Parmer machine. The same extraction and partition procedure above) was adopted for the 1.65 kg of powdered root barks with some modification in the extraction solvents. Ethanol (EtOH) was used for the total maceration yielding 98 g dry brown residues while VLC involved a series of Hexane (He), ethyl acetate (EtOAc) and finally EtOH. 85 g of 98 g extract were subjected to VLC. The filtrates were dried using a rotary evaporator at 30 ^{o}C and 60 ^{o}C to afford three (3) crude fractions: 262 mg He (brown-waxy solid), 60 g EtOAc (marigold yellow solid) and 20.1 g EtOH (brown solids). A dry packing of a silica gel column performed using 58 g of EtOAc fraction after multiple elution for establishing suitable solvent systems. Solvent gradient systems of 100 % petroleum ether (PE) - 20 % MeOH/ DCM that afforded 155 vials. The vial components were air-dried and profiled on TLC silica gel 60 F_{254}, (Made in Germany, Merck KGaA, 64271 Darmstadt) and the plates treated with vanillin reagent. Basing on the TLC profiles eleven (*fr.1- fr.10*) fractions were afforded. Repetitive purification of *fr.2* yielded **G4**.

Anti-bacterial assay

In vitro antibacterial activity test for compounds G1, G3 and G4 was performed by two-fold serial microdilution method according to the previous described procedure CLSI, 2006

and (Begum *et al.,* 2014; Hiranrat, 2010) with some modifications. These compounds were dissolved in 80 % DMSO v/v to make a stock solution of 4 mg/mL and serially diluted in duplicates for every bacteria strain under test. A standard drug, Gentamicin (Gentakel 10) was used as a positive control while 80 % v/v DMSO served for as a negative control. The representative Gram-negative standard strains of bacteria; *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested while and *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 51559 reprented the Gram positive group. All standard bacteria strains were obtained from the Microbiology Laboratory, Department of Biosciences in the College of Natural and Applied sciences (SUA). They were tested against each compound in duplicate by using a sterile 96-well polystyrene microtitre plate. Two rows of the 96-well microtiter plate were labeled for the negative control, two for the positive control (Gentamicin), two rows for only broth and bacteria. Each well of the plate was loaded with 50 µL of Mueller Hinton broth (MBH) followed by addition of 50 µL DMSO in the negative control rows, 50 µL of Gentamicin, initial concentrations of 1.56 mg/mL for *Enterococcus faecalis* and 0.78 mg/ml for *P. aeruginosa, E. coli and S. aureus* were put in the positive control rows and serially diluted to the last well. 50 µL of 4 mg/ml for each compound in the rows making a total volume of 100 µL. Then 50 µL of the mixture were drawn from the first rows to the subsequent rows until the last ones. The 50 µL from the last wells were discarded. A volume of 50 µL bacteria suspension of 24-hour old culture was adjusted to a density of bacterial cell of approximately 1.5×10^8 CFU/ mL equivalent to 0.5 McFarland and inoculated into the wells to retain a volume of 100 µL. The plates were incubated at 37 ^{o}C for overnight. The MIC values were recorded visually from the well as the lowest concentration showing no growth. The compounds' efficacy criteria for antibacterial activity was according to previous description by (Mbunde *et al.,* 2019; Sartoratto *et al.*, 2004) as follows: MIC $\leq$ 0.5 mg/ml

(strong activity), MIC = 0.6-1.5 mg/ml (moderate activity) and MIC > 1.5 mg/ml (weak activity).

Results and discussion

Structure elucidation of isolated compounds

Compound **G1,** 173 mg, Melting point: 58- 59.5 ºC reacted purple with vanillin reagent (UV negative). It was isolated as white powder from DCM fraction of the stem bark. Based on both 1D and 2D spectra, its molecular formula was determined to be $C_{26}H_{54}$, hexacosane. The ^{1}H NMR (CD_2Cl_2, 600 MHz,) spectrum indicated two main signals; at δ 0.88 (6H, t*, J*= 6.8*)* which were characteristic two terminal CH_3 at H-1 and H-26, a second signal at δ 1.26 (48H, *br. s*) was equivalent to protons of twenty-four CH_2 stretch. The ^{13}C NMR (CD_2Cl_2, 150 MHz,) spectrum indicated signals in the shielded region (δ 14.5 – 32.5) which is signal characteristic for the carbon atoms of saturated hydrocarbon. The important signals at δ 32.5 (C-3 and CH_2, C-24), long CH_2 stretch was observed between 30.2 - 30.3 (C-5 to C-22). 29.9 (C-4 and C-23), 23.3 (C-2 and C-25) and the terminal methyls at 14.5 (CH_3, C-1 and C-26), These spectral data were in agreement with (Aljubiri, *et al*., 2021; Credo *et al*., 2022). The COSY spectrum indicated a correlation for the terminal methyls (6H, *t)* with the broad singlet representing $H_3C–CH_2$ connectivity. The HSQC spectral data confirmed the correlation for H-C (1), H-C (26), and the H atoms of the of the long CH_2 chain. The HMBC spectrum indicated C- H corrections for H-1 to C2, C3 or H-26 to C-25 and C-24. These spectral data and literature helped to conclude the structure of G1 to be hexacosane (Figure 1). To the best of researchers knowledge, isolation of **G1** (hexacosane) is a first time report in *S. glaucescens* Pax but it was previously reported in *Euphorbia balsamifera* (Aljubiri *et al.,* 2021).

Compound **G2,** 2 mg was isolated as white crystalline from the DCM fraction of stem barks. The ^{1}H NMR spectrum indicated most protons in the low field region which is a characteristic of tetracyclic skeleton. Two singlets each 3H at δ 0.69 and 1.01 were due to CH_3 for H-18 and H-19. The

olefinic proton, H-6 was observed at 5.35 (1H, br d, *J*=5.3) appealing to a characteristic of sterol while a signal at *δ* 3.5 (1H, m) was a proton H-3 of the skeleton. Other evident protons due to secondary methyls were evident at 0.82, 0.84, 0.85, 0.93 and both appeared as doublets *J*= 6.8, 7.5, 6.8 and 6.6 respectively. Its ^{13}C NMR indicated 29 carbon signals which is a characteristic of phytosterols. Two olefinic carbons at *δ* 141.6 (C, C-5) and 122.0 (CH, C-6) while 72.1 was a carbon bearing the OH group (CH, C-3), other carbon signals were 57.4 (C-14), 56.7 (C-17), 50.8 (CH, C-9), 46.5 (CH_2, C-22), 42.9 (C, C-13), 40.4 (CH_2, C-12), 37.9 (CH_2, C-2), 37.1 (CH_2, C-1), 36.7 (C, C-10), 34.5 (CH_3, C-18), 32.5 (CH_2, C-7 and CH, C-20), 32.3 (CH, C-8), 29.8 (CH, C-25), 28.8 (CH_2, C-16), 26.6 (CH_2, C-15), 24.8 (CH_3, C-21), 23.6 (CH_2, C-23), 21.7 (CH_2, C-11), 20.1 (CH_3, C-26), 19.8 (CH_3, C-27), 19.3 (CH_3, C-19), 19.1 (CH_2, C-28), 12.9 (CH, C-24), and 12.3 (CH_3, C-29) as per numbering of the sitosterol skeleton. Based on the DEPT 135 NMR, a total of six CH_3, eleven CH_2, one CH and three quaternary carbon signals were observed. These experimental data were compared and in correlation with (Edilu *et al.,* 2015; Lokadi and Munkombwe, 2015; Nyigo *et al.,* 2016; Ododo *et al.,* 2016; Rwegoshora *et al.*, 2022) together with GC-MS data at m/z 414, **G2** was concluded to be β-sitosterol, $C_{29}H_{50}O$ (Figure 1). Despite its earlier isolation from leaves (Ododo *et al.,* 2016) and root barks (Rwegoshora *et al.,* 2022), it is for the first time reported from the stem barks of *S. glaucescens* Pax.

Compound **G3** (19 mg), white powder was isolated from DCM fraction of SG stem barks. Its ^1H NMR (CD_2Cl_2, 600 MHz) afforded two trans-olefinic protons at *δ* 7.58 (1H, d, *J*= 15.9, H-7), 6.31 (1H, d, *J*=15.9, H-8) and three aromatic protons 6.69 (1H, d, *J*= 8.1, H-6), 7.14 (1H, d, *J*=7.8, H-2), and 6.90 (1H, d, *J*= 7.9, H-5). It further exhibited signal at δ 5.91 (1H, *s*, -OH), methoxy signal at 3.93 (3H, *s,* -OCH_3), and a methlene at δ 4.15 (2H, t, *J*= 6.7, H-1'), 1.68 (2H, m, H-2'), 1.39 (2H, m, H-3') and a terminal methyl at 0.88 (3H, t, *J*= 6.9, H-28'). A broad singlet (48H) in ^1H NMR

equivalent to twenty-four CH_2 stretch was observed at δ 1.26. The ^{13}C NMR (CD_2Cl_2, 150 MHz) indicated the signal for a carbonyl carbon at 167.6 (C-9), six benzylic carbon signals at 148.5 (C-3), 147.5 (C-4), δ 127.7 (C-1), 123.5 (C-6), 115.1 (C-5) and 110.0 (C-2). The olefinic carbons resonated at 144.8 (C-7) and 116.4 (C-8) while 56.6 represented the 3-OCH_3). The observed aliphatic chain signals included 65.1 (C -1'). 32.5 (C-19'), 30.2- 30.3 for the CH_2 of C-4' to C-18'), 23.3 (C-27'), 30.4 (C-2'**),** 26.8 (C-3'), and 14.4 for the terminal methyl (C-28'**).** Important HMBC correlations included H-1' to the carbonyl carbon (C-9), and C-2', 3-OCH_3 and OH to C-3, H-2 to C-3, H-7 and H-8 to C-1, H-28' to C-26' and C-27'. These spectral data were compared to and in agreement with (Baldé *et al.*, 1991; Evans *et al.*, 2016; Katagiri *et al.*, 1997; Rwegoshora *et al*., 2022) and it was assigned as octacosyl ferulate ($C_{38}H_{66}O_4$) which is also known as erythrinacinate b (Figure 1). Although this compound is reported for the first time from *S. glaucescens,* it was earlier isolated from *Pavetta owariensis* (Katagiri *et al.,* 1997) and *Erythrina caffra* (Baldé *et al.,* 1991). Other closely related compounds including hemicosanylferulate (eicosyferulate) from the root barks (Rwegoshora *et al.,* 2022) and erythrinacinate c from leaves (Nyigo *et al.*, 2022) of *S. glaucescens* have also been isolated.

Compound **G4**, 26 mg was isolated as white powder from the root bark extracts. It reacted purple with vanillin reagent when hot which was an indicator for non conjugation nature of the C- C skeleton. The ^1H NMR (CD_2Cl_2, 600 MHz) indicated four main signals on an aliphatic functionality; δ 2.33 (2H, t, *J*= 7.4) assigned to protons of C-2 which is α-methlene next to carboxylic acid group (O-CH_2), δ 1.61 (2H, m) due to protons of the β- methlene (C-3) and δ_H 0.88 (3H, t, *J*= 6.9) corresponding to terminal CH_3. A broad singlet observed at δ 1.22- 1.30 (46H) made an equivalence of twenty-three CH_2 chain. The ^{13}C NMR (CD_2Cl_2, 150 MHz,) indicated a single carbon signal in the high filed region at δ_C 175.9 (C, CO_2 C-1) due to carbonyl carbon (C=O) while the

rest carbons were in low field region (for aliphatic chain functionality). They included δ 33.8 (CH_2, C-2), 32.5 (CH_2, C-24), broad CH_2 stretch at δ 30.2- 30.4 (CH_2, C_4- C_{24}), 25.3 (CH_2, C-3), 23.3 (CH_2, C-25) while δ 14.4 was a characteristic signal for terminal CH_3. Other CH_2 signals in the ^{13}C NMR included δ 29.6, 29.8 and 29.9 which were also evident in the DEPT135 NMR spectrum. More confirmation of the structure was made using the HSQC spectrum C-H (26), C-H (2), C-H (3) and $(CH_2)_n$ and the HMBC; H-2 to C-1, C-3 and C-3, H-25 to C-24, H-26 to C-23 and C-25. GC-MS, m/z 396, calcd. 396.6899 equivalent to hexacosanoic acid ($C_{26}H_{52}O_2$). These spectral data were in agreement with (Credo *et al.,* 2022; Nyigo *et al.,* 2022; Rehan *et al.,* 2020; Yamamoto *et al.,* 2015). Based to these spectral and literature information, compound **G4** was assigned to be hexacosanoic acid (Figure 1) which is also known as cerotic acid.

Antibacterial efficacy

Compounds from stem and root barks of *S. glaucescens* demonstrated variable activities against bacteria standard strains. Hexacosane (**G1)** demonstrated weak activity (2 mg/mL) against all three test bacteria strains except on *E. faecalis* which was moderate (1.5 mg/mL). **G3,** octacosyl ferulate showed strong activity against *S. aureus* (MIC= 0.125 mg/mL), moderate activity against *E. faecalis* (MIC= 1.5 mg/mL), weak activity against *E. coli* (2 mg/mL) and *P. aeruginosa* (> 2 mg/mL). The Weak activity of phenolic compounds against Gram- negative was also reported (Canli *et al.*, 2017). *S. aureus* is arguably among the most problematic of all bacterial pathogens owing in large part to the persistent emergence of antibiotic resistant strains (Quave *et al.,* 2012). Therefore, the observed activity of this phenolic compound (Octacosyl ferulate) suggests the possibility of this plant to serve for source of drugs against infections associated with *S. aureus*. Compound **G4** (hexacosanoic acid demonstrated moderate activity (1 mg/ mL) against three of the tested strains except *P. aeruginosa*

on which it showed weak activity (MIC > 2 mg/mL. These results for hexacosanoic acid are supported by other literatures which indicated that the long chain fatty acid possess bactericidal activity. For example, palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid were found active against *S. pyogenes* and *S. aureus* through inhibition of FabI gene (Zheng *et al.*, 2005). However, it was noted that the standard drug (Gentamicin) was superior of all isolated compounds with MIC values; *S. aureus* (0.003 µg/mL), *E. faecalis* (0.003 µg/mL, *P. aeruginosa* (0.006 µg/mL) and *E. coli* (0.006 µg/mL). Similar result of less susceptibility of Gram negative over Gram positive bacteria to the natural compounds is also reported in EtOAc extracts of *Carpobrotus edulis* (Chokoe *et al.*, 2008). Since the activity of the any drug is associated with its molecular structure (Duque et al., 2018) including the pharmacophore (Duke *et al.,* 2001) and molecular weight (Solvents *et al.,* 2010), a systematic structural modification may improve their activity. Comparing the activities of compounds, there were maximum activities against Gram-positive bacteria (*S. aureus* and *E. faecalis*) due to its antibacterial action against cell wall synthesis (Yamamoto *et al.,* 2015). It is further explained that Gram-positive bacteria have a cell wall composed mostly of peptidoglycan with no protective outer membrane. This structural characteristic makes easy penetration of the toxic phytochemicals into the cells. As opposed to Gram- negative organisms (e.g. *E. coli and P. aeruginosa* in this case) have less peptidoglycan but contain an outer membrane composed of lipopolysaccharide and lipoproteins which make them less permeable (Chokoe *et al.,* 2008). The moderate and weak activities of the compounds against *P. aeruginosa* is associated bacterial biofilm formation favored by the presence of exopolysaccharides (EPS) embedded in an extracellular matrix and to the production of type IV pili, T4P (Ochoa *et al*., 2013). These results confer with findings that phenolic compounds are among the secondary metabolites in plants that show a wide

range of distinct biological activities (Chen *et al.,* 2014; Zhang, *et al.,* 2022).

Conclusion

Isolation of these biologically active compounds; hexacosane (**G1**), β-sitosterol (**G2),** Octacosylferulate (**G3**), (from the stem bark) and hexacosanoic (**G4**) acid from the root barks is reported for the first from *S. glaucescens* Pax. **G3** demonstrated strong antibacterial activity against *S. aureus* while **G1** demonstrated the weakest activities against all tested bacteria strains. The root and stem barks are the potential source of antibacterial agents.

Recommendation

Further studies on structure activity relationship for **G3** are suggested to enhance its efficacy.

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Declarations

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Conflict of interest

The authors declare no conflict of interest.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are included in the Supplementary material but

are also available from the corresponding author on reasonable request.

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FIGURES

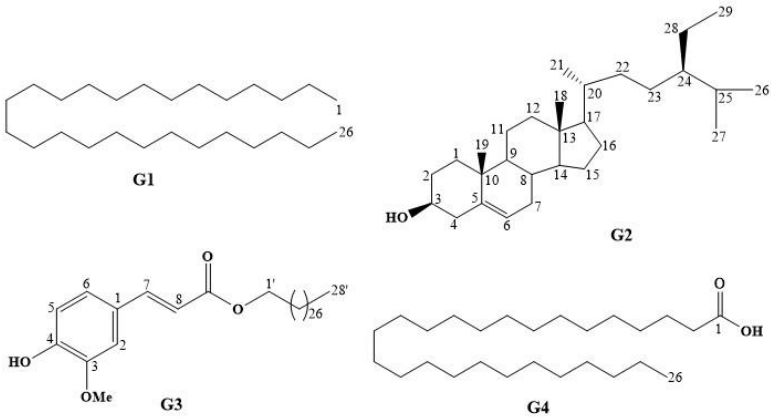

Figure 5: Structures of compounds isolated from *S. glaucescens* Pax

5.0 CHAPTER FIVE

Phytochemical profiles of *Synadenium glaucescens* Pax from different phytogeographical regions of Tanzania

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Phytochemical profiles of *Synadenium glaucescens* Pax from different phytogeographical regions of Tanzania

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ABSTRACT

Synadenium glaucescens Pax (SG) is a wild medicinal plant which grows nearly in all phytogeographical zones of Tanzania. An extensive exploitation of this plant in the traditional medicine has created an alert for evaluation of its phytochemical profile. Representative samples for phytochemical profiling were collected from Njombe, Morogoro and Tanga each representing one phytogeographical zone. The chemical profiles of root barks, root wood, stem barks, stem wood and leaves of SG at different ages were assessed by a thin layer chromatography method. The results indicated that location, age and plant part had significant influence on the concentration and

distribution of SG phytochemicals ($p< 0.0001$) among the three regions. While Njombe registered the highest number of compounds (mean= 12.4000), Morogoro had the least of all (mean= 9.7000). Euphol, a triterpenoid in root barks was detected to be at the highest concentration in Tanga and the lowest in Morogoro. Another conjugated compound (UV^{+}), with blue fluorescence (f_b) was detected at all ages among Tanga samples. Derivatives of ellagic acid were detected in root barks at all ages and locations. The results also indicated continuous disappearance of red fluorescing compounds in stems barks and leaves as the plant age increased. The number of compounds increased with age while the optimal age for a maximum number of phytochemicals was 3 to 4.5 years. Furthermore, the stem barks registered the highest number of phytochemicals (>15). The variations observed in this study need to be considered while harvesting of this plant for formulation. Further studies on full identification of the profiled phytochemicals and the bioassay guided approaches are suggested.

Key words: *Euphorbiaceae, antioxidant, fluorescence, phytochemicals, phytogeography, phenolics, medicinal plants, secondary metabolites*

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INTRODUCTION

Synadenium glaucescens Pax (SG) of family Euphorbiaceae is a common wild medicinal plant that grows in different parts of Tanzania. Studies on crude and pure compounds from this plant have demonstrated anti-viral (Mabiki et al. 2013; Nyigo et al. 2016; Credo et al. 2022), anti-bacterial (Msengwa et al., 2023) and anti-helminth properties. The plant is reported to contain different secondary metabolites including 3β and 3α-friedelanol (Credo et al., 2022), euphol and β-sitosterol, erythrinacinate c and Octacosanol (Nyigo et al. 2016; Nyigo et al. 2022). The isolation of hemicosanylferulate, lupeol, nonacosene were also reported from its roots and stems (Rwegoshora et al., 2022) while more others are yet to be isolated. SG is known to grows in all phytogeographical zones of Tanzania described by Nahashon (2013) including the Zambezi region (Njombe), Afro–montane region (Morogoro) and the Zanzibar – Inhambane region (Tanga). It is asserted that any variation in factors including individual age (Adegbaju and Afolayan, 2020; Nobossé and Mbofung, 2018; Wang and Liu, 2016) tends to affect chemical composition of plant. Other external factors including soil nature, season, their (Inbathamizh and Padmini 2013; Al-Hmadi et al. 2021) are also reported to have their significant effect. As a result, plants of the same species tend to display variations in their bioactive phytochemicals depending on how they interact with their environment in development process (Peñuelas and Llusià 1997; Liu et al. 2016). It is argued that secondary metabolites are synthesized for self-defense against environmental stress (Liu et al., 2016). However, these metabolites turn to be of medical use to both human and animal ailments. Therefore, these novel compounds which may be used as medicines to relieve or cure various diseases (Ahmad, and Kumar, 2015; Guerriero et al., 2018) are assessed for their effectiveness and safety (Raya et al., 2015; Soni and Gauttam, 2015). Medicinal plants as sources of the majority of medicines, either traditional or western are challenged with over

harvesting or exploitation (van Wyk and Prinsloo 2018; Papageorgiou et al. 2020). In order to obtain a high-quality efficacious herbal drug, the appropriate part of the medicinal plant must be harvested at the optimum stage of development (Pandey and Savita 2017; Boor and Lefebvre 2021) and their geographical location should be well selected (Obaineh and Shadrach, 2013). Therefore, this study aimed at assessing the chemical profiles of SG from different regions to ascertain the quality factors such as number of compounds and concentration which can affect its efficacy and dosage different ages and phytogeographical zones. The findings are expected to be a guide tool for sustainable harvesting and manufacturing practice of *Synadenium glaucescens* based products.

MATERIALS AND METHODS

Sampling area, procedure, and measurements

With the aid of a botanist from the University of Dar es Salaam the plant was identified and assigned a voucher specimen number of HOS/FM 3672, the fresh plants were collected from three phytogeographical zones in which the plant was readily available. Zone 1, Mtulingala vilage (Njombe) within 50 m diameter around an elevation 1656 m S08°47'08.5" E 34°53'22.2", zone 2, kibindu village (Tanga), 554 m S 05°34'00.9" E 038°9'57.4" to 567m S 05°34'04.9" E 038°09'54.4" and zone 3, Chakwale village (Morogoro), 1119 m S 06°00'08.5" E 036°58'09.9 to 1132 m S 06°00'05.5" E 036°58'09.9. To suppress the effect of seasonal variation, all samples were collected in November 2018. The voucher specimen number HOS/FM 3672 was deposited at the herbarium of botany department, College of Natural and Applied Sciences of the University of Dar es Salaam.

A selective random sampling of at least fifteen *S. glaucescens* Pax trees from each site led to a total of 50 samples. The stem's diameter at breast height (DBH) and corresponding annual rings were recorded for every sampled plant. A plot of DBH (in centimeters) against annual rings

afforded to obtain the linear regression equation (y = 1.0406x + 0.3533) which was used to calculate the number of rings for the uncut stems. Each plant was processed into five separate parts in small pieces of root barks (SG2), root woods (SG3), stem barks (SG5), stem woods (SG6) and leaves (SG7). All samples were immediately transferred to liquid nitrogen until drying time. Both samples were dried in cold and dark room (15 ºC) to protect the temperature and light sensitive compounds at the Tanzania Tree Seed agency (TTSA) laboratories.

Determination of the plant age

The total number of annual rings on stem discs of each plant were used to estimate the plant age (in years) according to the formula 1 below (Verheyden and Koedam, 2004). In this case, time lag of 2 years appeared between the actual age of the tree and the number of growth rings formed at 130 cm height was considered for the diameter at breast height (DBH).

$$Plant\ age\ (years) = \frac{Total\ number\ of\ annual\ rings\ at\ DBH}{2} \ldots (1)$$

Sample extraction and profiling

Dry samples were pulverized separately using a laboratory milling machine (Christy Hunt Engineering Ltd, England) to obtain approximately 1 mm particle size. Each sample was subjected to total maceration with methanol (MeOH) at room temperature in a dark to protect volatile and light sensitive compounds. For maximum extraction, the re-soaking and filtration was done after every 72 hours three consecutive times. Sample extracts were air-dried at room temperature to afford dry solid residues which were then stored at 4 ºC until further use. The pulverized samples (about 1 mm particle size) were subjected to total maceration using methanol. Extraction was done three times consecutively after every 72 hours to ensure maximum

phytoconstituents. All extract solutions were air dried at room temperature until solid residues were formed. Standardization of extracts led to a concentration of 5 mg/mL more best compound detection on the TLC. Three pure standard compounds were selected based on availability and polarity strength to suit the polarity range of compounds in crude extracts under study. These compounds: trimethoprim (TMP), Salphamethoxazole (SMX) and salphadoxine SDX) were used as the chromatographic constants. A concentration of 10 ppm for each standard was sufficient for being visualized on thin layer chromatography plate. In each case a volume 4.0 µL of the sample was spotted on the TLC paper and eluted at different solvent systems of 2 %, 10 % and 18 % Methanol/ dichloromethane. The selected combination of chromatographic constant was simultaneously spotted on the same TLC and eluted along with study samples. At each elution, the relative retention factors (R_f) for the observed compounds were recorded and compared. The strength of the spot on the TLC i.e., strong or faint considered to be equivalent to its concentration (amount) in a sample under analysis.

Determination of the relative retention factor (r_{ij}) of compounds

The formula for calculating the retention factor of the component phytochemicals relative to the selected standards was adopted from (Berezkin, 2006) as indicated in formula 2 below,

$$r_{ij} = \frac{l_{ri}}{l_{rj}} \times \frac{l_j}{l_i} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where: *lri= net retention distance for compound i, li= virtual distance for I*

lrj= net retention distance for standard j, and
lj= virtual distance for standard compound j

DATA ANALYSIS

The data were analysed with JPM Statistical software.

RESULTS AND DISCUSSION

Most of the plants used during the study were of the age between 0.5 and 2 years (Morogoro,72.22%; Njombe,66.11%; and Tanga, 66.67 %). The oldest plant was 8.5 years. The absence of higher aged plants in the sampled areas could be attributed to its over harvesting and bush clearing.

The results indicated that both location, plant part and individual age had a significant effect ($p< 0.0001$) on the profiles of phytoconstituents of SG. It was revealed that this plant has a big spectrum of compounds from highly polar, moderate to the least (nonpolar) compounds (Table 1). Many compounds of this plant were detected in Njombe (mean= 12.4000), Tanga (mean= 10.7000) and the least in Morogoro (mean= 9.4000). It is argued that the climatic variation like high altitude of Njombe favoured their phytoconstituent accumulation (Gololo, Nthai, and Mogale, 2019; Nayeem, 2017). In a study by (Muraina et al., 2008) it showed similar trend for the extract yields of *Anoigeissus leiocarpus* from colder against hot areas. The least number of compounds in Morogoro samples (Figure 1) could be due to abiotic factors like the nature of the soil in the respective area. These results are in line with variation with location which were recorded in *Morus nigra* (Kattak et al.2015). Euphol, a UV^{-} triterpenoid was at the highest concentration (strong spot) in Tanga followed by Njombe while it was the least (a faint spot) for Morogoro samples (Figure 2). In a study by Gololo et al., (2019) there was noticed a difference in antioxidant

capacity among the leaves of *Aloe greatheadii* var. *davyana* from different locations.

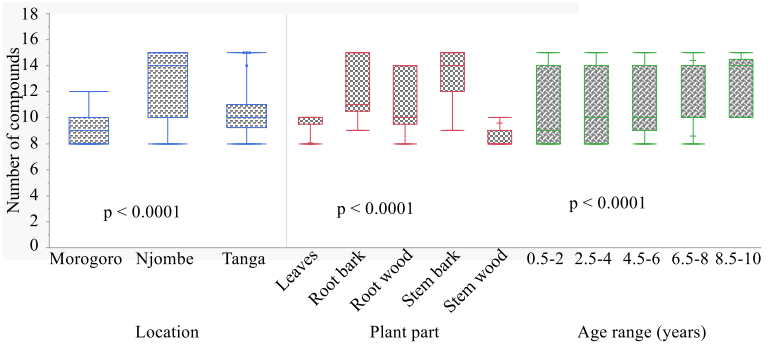

Figure 6: A graph of number of number of compounds in relation to location, plant part and age of SG Pax

It was observed that leaves and stem wood contained fewer compounds as compared to the root and stem barks (Figure 1 and Figure 3). However, the highest number of compounds were detected in stem barks. This could be attributed to maximum exposure of stem regions to stress (Liu et al., 2016). Similarly, wood parts of roots and stems are less exposed to stress thus tend to generate fewer secondary metabolites for self-defence. Among all samples, leaves did not show major difference in the profiles of compounds at all ages. This can be attributed to the fact that they develop seasonal wise thus tend to have closely similar ages before their senescence which don't impact chemical profiles. The leaf senescence occurs during summer and leaves the tree bare whose regeneration starts in the wet season. A nonconjugated triterpenoid was detected in all plant parts although it was at the highest concentration in root barks.

With respect to plant age, the two derivatives of ellagic 3′,4′-di *O*-methyl ellagic acid-4-α-*L*-rhamnopyranoside and 3,4,3′-tri-*O*-methyl ellagic acid (Figure 2d) were detected at all ages and locations in root back samples. It implied that

their synthesis in not affected by either location or developmental stage.

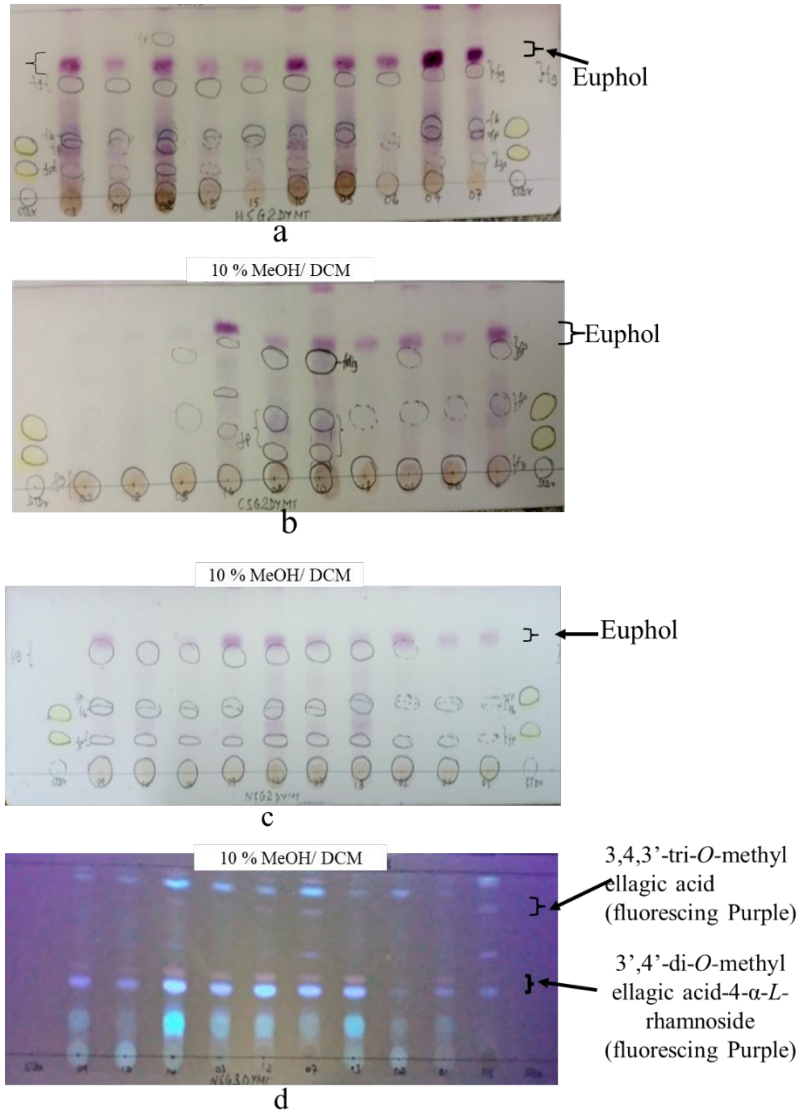

Figure 2: TLC chemical profiles for Njombe (a) and Morogoro (b) and Tanga (c) viewed after chemical treatment with vanillin reagent and at 365 nm UV lamp (d)

An Illumination of stem bark extracts with UV light at 365 nm indicated presence of many conjugated compounds (Fluorescing, Figure 3) than non-conjugated compounds (purple spots after chemical treatment of the TLC.

While euphol increased in concentration with age, some compound could not be detected in SG with less than 1 year age. The red fluorescing (f_R) compounds in leaves and stem barks decreased in concentration (faint spots) as the plant age increased. This was linked with a decrease in chlorophyll green pigment as the plant matures. According to (Nobossé et al., 2018), the increase in leaf age had a remarkable effect on the phenolic contents, flavanoids and the chlorophyll which in turn influenced a variation in antioxidant activity of the extracts.

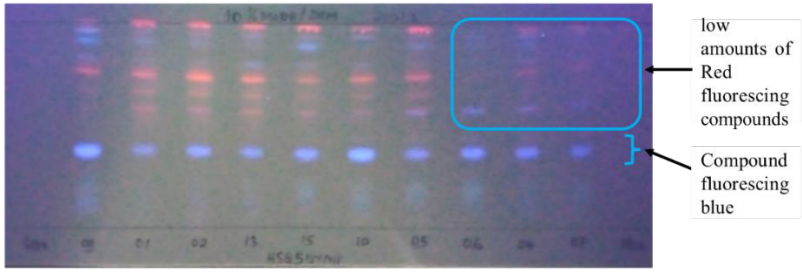

Figure 3: A TLC plate of compounds' profiles at increasing age (from left to right) viewed at 365 nm of an ultraviolet lamp.

Table 1: Relative retention factors of compounds' distribution in different parts of Synadenium glaucescens Pax

	Relative retention values (*rij*)				
S/N	**Root bark**	**Root wood**	**Stem bark**	**Stem wood**	**leaves**
1	139.46	0.96	26	16	
2	6.64	0.98	4.94	4.1	4.04
3	4.27	0.17	3.43	0.72	2.58
4	2.42	0.72	1.27	0.48	1.25
5	1.34	0.35	0.34	0.17	0.82
6	1.11	0.17	9.75	0	0.45
7	1.38	0.11	2.53	0.05	0.12
8	0.72	0.09	0.06	0.03	0.06
9	0.34	0.03	0.02	0.02	0.02
10	0.52	0.19	0.01	0.01	0
11	0.52	0.05	0.005	NE	
12	0.52	0.03	0.004		
13	0.52	0.02	0.004		
14	NE	0	0.001		
15			NE		

Where: NE- no elution at 18 % MeOH/DCM

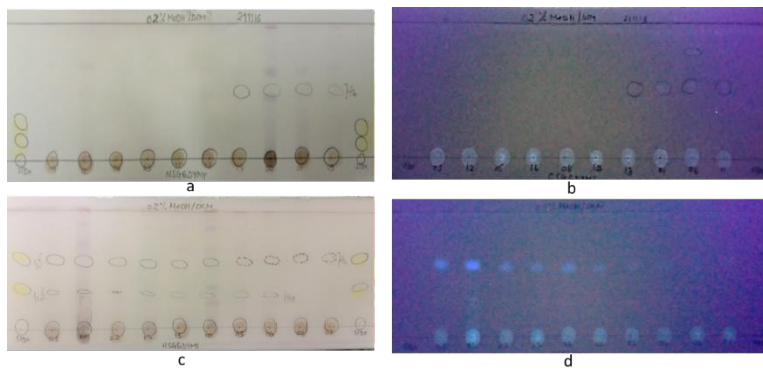

Figure 7: The TLC profiles of stem wood from Njombe (a), Morogoro (b), and Tanga (c) as viewed at UV 365 nm (b and d) and after chemical treatment (a, c)

CONCLUSION

The study revealed that there were significant variations in phytoconstituents of *Synadenium glaucescens* Pax due to location, age, and plant part. While derivatives of ellagic acid were not affected by age and location, euphol was the highest concentration in Tanga samples. It is recommended that any formulation that prefers euphol at higher concentration can consider collection of SG from Tanga region. However, Njombe region would be another preference for a big spectrum of phytochemicals from SG. For effective and safety of its extracts and formulations it recommended to conduct bioassay guided experiments to determine key plant ages that contain most pharmacologically active compounds.

Disclosure of conflict of interest

The authors confirm to have no conflict of interest.

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6.0 CHAPTER SIX: GENERAL DISCUSSION

6.1 Isolation and characterization of Pure compounds from the stem and root barks

Synadenium glaucescens Pax (Euphorbiaceae) is characterized with compounds of different types including phenolics, long chain compounds, terpenoids and steroids just to mention a few. Phytochemical analysis of moderately polar (DCM) and polar (EtOAc) extracts ended up on the isolation and full elucidation of fifteen compounds distributed in the array of groups as mentioned above. Previous studies on optimization of chemical constituents.

6.1.1 Phenolic compounds of *Synadenium glaucescens* Pax

Phenolic compounds are the secondary metabolites in plants, which show a wide range of distinct biological activities, (Zhang *et al.*, 2022). This study availed four phenolic compounds which included two derivatives of ellagic acid while the rest two were ferulic acid esters. A new derivative of ellagic acid 3',4'-*di*-*O*-methylellagic acid-4 α, *L*-rhamnopyranoside, $C_{22}H_{20}O_{12}$ (**32**), and the known 3,4,3'-*tri*-*O*-methylellagic acid (**33)** as well as hemicosanylferulate ($C_{31}H_{52}O_4$) were both isolated from the ethyl acetate fraction of root bark extract. The octacosyferulate (erythrinacinate b), $C_{38}H_{66}O_4$ was isolated from stem barks extracts. A new ellagic acid rhamnoside (Fig.5 **32**(a) and Fig**.**6,**32**)**,** 28 mg, grayish powder, High resolution FTMS-ESI$^-$, *m/z,* 476.0921 conformed the molecular formula of $C_{22}H_{20}O_{12}$ and a melting point of 251.0~ 252.7 ºC. Compound **33,** pale-yellow solid**,** molecular formula $C_{17}H_{12}O_8$,14 mg, ESI-MS, *m/z* 343.2891 $[M-H]^-$ and *m/z* 344.3129 for a neutral molecule. Their NMR spectral data (Table 1 and 2), the HMBC and ROESY (Fig.5) leading to full structures elucidation were agreement with literature data (Begum *et al*., 2014; Hiranrat, 2010; Tukiran *et al.*, 2018) as discussed in details at chapter two. Their UV light detection at 254 nm (blue spot) and 365 nm (purple

fluorescence) was an indicator for highly conjugated phenolics. Its structure was finally confirmed by HPLC-MS/MS, for m/z 343.2891 $[M-H]^-$ and indicated fragment ions of m/z; 328.0181, 312.7756 and 297.9110 and 280.9110. This fragmentation pattern was related to consecutive loss of the three -CH_3 of the methoxy groups at C-3, C-4 and C-3' and finally an OH group at C-4'. Despite isolation of compound **32** and **33** for the first time from *S. glaucescens* Pax, **33** it was previously isolated from *Euphorbia acaulis* (Bindra *et al.*, 1988), *Syzygium aromaticum* leaves (Begum *et al.*, 2014), *S. guinense, S. cumini* seeds (Quave *et al.*, 2012) and roots of *Combretum dolichopetalum* (Ríos *et al.*, 2018).

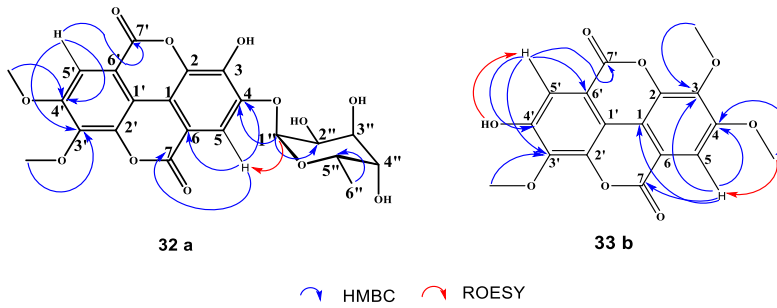

Figure 8: Key H-C and ^{1}H-^{1}H correlations for compound **32** and **33**

Table 2: ^{1}H NMR chemical shifts for compounds **32** and **33** (600 MHz, DMSO-d_6, δ, ppm, J/Hz)

Position (^{1}H)	**32**	**33**
5	7.79 (1H, s)	7.62 (1H, *s*)
5'	7.52 (1H, s)	7.53 (1H, *s*)
1"	5.58 (1H, d, *J*=1.4)	
2"	3.96 (1H, br, *s*)	
3"	3.72 (1H, d, *J* = 9.1)	
4"	3.36 (1H, d, *J* = 4.1)	
5"	3.52 (1H, dq, *J*=9.2, 6.2)	
6"	1.15 (3H, d, *J*=6.1)	
3-OH	10.84 (1H, s)	
3-OMe		4.04 (3H, s)
4-OMe		4.06 (3H, s)
3'-OMe	4.05 (3H, s)	4.00 (3H, s)
4'-OH		10.81(1H, s)
4'-OMe	4.06 (3H, s)	
2"-OH	4.84(1H, s)	
3"-OH	4.97 (1H, s)	
4"-OH	5.19 (1H, s)	

Phenolics including ellagic acids and its derivatives are reported to have potent antioxidant properties and prevention of cancer (Dai and Mumper, 2010). It is further asserted that the preventive effects of these secondary plant metabolites in terms of cardiovascular, neurodegenerative diseases and cancer have drawn attention of nutritionist for food supplement (Rassmusen *et al.,* 2005, Arts *et al.,* 1991, Hetog *et.,* 2005 and Cole *et al.,* 2005) as cited by (Dai and Mumper, 2010). The natural ellagic acid (EA) is reported to be a potential anti-Human rhinovirus (anti-HRV) through its ability to inhibit virus replication (Park *et al*., 2014), anticarcinogenic and antimutagenic (Maas *et al.*, 2019), antioxidant and anti-inflammatory in which it acts as a neuro protective agent (de Oliveira, 2016). The compound is also reported to inhibits cardiac arrhythmias (protects the heart from necrosis), hypertrophy and hyperlipidaemia (Kannan and Quine, 2013). Also, the derivatives of ellagic acid are in line with the parent compound (EA). The EA-peracetate (3,4,3′,4′-*tetra*-*O*-acetylellagic acid) is reported to have antitumor activity and help in boosting the immunity system in mice model (Ren *et al*., 2012). The natural derivatives of ellagic acid with sugar moiety are known from plants. Some example include 3,4′-dimethoxyellagic acid 3′-O-β-D-xylopyranoside and 1,6-di-O-ellagoyl-β-D-glucopyranose from *Punica granatum* are reported to have an antihypoglycemic activity (Olennikov *et al.,* 2019). According to this literature, the isolation of such compounds closely related to **32** suggest the possibility of its use as drug lead for the same disease. Additionally further investigation on the SG extracts may result to formulations of antidiabetic medicines. In a study by Zhang *et al*., (2014), the 3,3'-Di-O-methyl ellagic acid-4'-O-β-d-xylopyranoside isolated from *Euphorbia hylonoma* was reported for anticancer through inhibiting the proliferation of Human hepatoma (HepG2) cells.

Ferulic acid esters (compound **34** and **35**) are among the types of phenolic compounds present in *S. glaucescens* Pax. The hemicosanylferulate, $C_{31}H_{52}O_4$ (**34**), 65 mg was isolated as white powder from the EtOAc fraction while compound **35,** octacosylferulate, $C_{38}H_{66}O_4$,19 mg (Fig.6) was isolated as white powder from DCM fraction of SG stem bark' extracts. The ^{13}C NMR Table 2 and other spectral data as discussed at Chapter three and four together with literature confirmed the hemicosanylferulate and octacosylferulate (Amor *et al.*, 2020; Jordão *et al*., 2017; Prabha *et al*., 2019; Uzor and Osadebe, 2016; Kannan and Quine, 2013). Despite their first isolation in *S. glaucescens* Pax, compound **35** was previously isolated from the stem bark of *Pavetta owariensis* of the family Rubiaceae (Baldé *et al.*, 1991) and *Erythrina caffra* (Chukwujekwu *et al*., 2016) and from *Aristolochia kankauensis*. These spectral data for compound **35** were compared to and in agreement with (Baldé *et al*., 1991; Katagiri, *et al.*, 1997; Ododo *et al*., 2016; Rwegoshora e*t al*., 2022).

Table 3: ^{13}C-NMR (150 MHz) spectral data for the phenolic compounds isolated from root and stem barks of *Synadenium glaucescens* Pax

Position (C)	**32**	**33**	**34**	**35**
1	111.98	111.65	127.6	127.7
2	141.56	140.95	111.4	110.0
3	141.85	141.45	150.2	148.5
4	152.80	153.77	148.9	147.5
5	111.63	107.45	116.1	115.1
6	112.68	113.34	124.0	123.5
7	158.39	158.45	145.6	144.8
8			116.6	116.4
9			167.6	167.6
1'	111.68	111.17	64.8	65.1
2'	141.05	140.80	30.4	30.4
3'	140.16	140.16	28.6	26.8
4' (4'- 18')	150.24	152.59	30.4-30.5	30.2-30.3
5'	114.08	112.47		
6'	117.64	111.90		
7'	158.23	158.27		
3-OCH_3		60.98	56.4	
19'			31.7	32.5
20'			23.4	
21'			14.4	
27'				23.3
28'				14.4
3'-OCH_3	61.59	56.70		
4-OCH_3		61.27		
4'-OCH^3	60.98			
1"	99.80			
2"	70.03			
3"	70.41			
4"	71.52			
5"	70.27			
6"	17.89			

NMR solvent were DMSO-d_6 for compound **1** and **2**, Acetone-d_6 for **SG1** and CD_2Cl_2 for **G3**

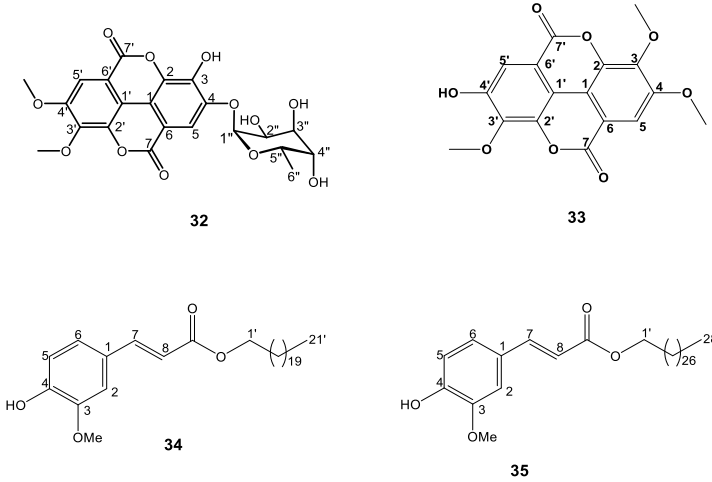

Figure 9: Structures of Phenolic compounds isolated from the root and stem barks of *S. glaucescens* Pax.

6.1.2 Triterpenoids and Steroid of *Synadenium glaucescens* Pax

The triterpenoids are a large group of natural products derived from C-30 precursors which are estimated to nearly 200 different triterpene skeletons from natural sources or enzymatic reactions (Xu *et al.*, 2004). These compounds are synthesized through Mevalonate and deoxyxylulose pathway in which diphosphate derivatives are used. The dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) are joined head to tail It is further asserted that they are structurally consistent with being cyclization products of squalene, oxidosqualene, or bis-oxidosqualene. Terpenoids are divided into monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, and triterpenes depending on its carbon. Abdallah and Quax, (2017) assert that many terpenoids have therapeutic properties such as antimicrobial, anti-inflammatory, immunomodulatory and chemotherapeutic properties that make them of great interest in the medical field, flavours and fragrances industries and as sources of biofuel.

The ^{1}H NMR spectrum is always characterized with several protons' signals in the shielded region of the spectrum (0- 2 ppm). The ^{13}C NMR spectra for such triterpenoids always consist of thirty (30) carbon signals. This study findings reported on structure analysis and elucidation of three triterpenoids that were isolated from the stem and root bark extracts of *S. glaucescens* Pax. They include Lupeol (**18**), euphol (**29**), epifriedelanol (**36**) and one steroid β-sitosterol (**30**).

Compound **18**, lupeol ($C_{30}H_{50}O$, 65 mg) was isolated as white powder from the stem bark extract. The NMR experimental data from ^{1}H and ^{13}C NMR were compared and in agreement with the literature (Adeosun *et al.*, 2019; Emaikwu *et al*., 2020; Gandagule *et al.*, 2018; Muhammad *et al*., 2021) for its confirmation. Compound **36**, epifriedelanol ($C_{30}H_{52}O$), was isolated from bark extracts of both root (155 mg) and stem (12 mg) as white powder. Its structure was experimentally, and literature confirmed. Peculiar signals of ^{1}H NMR included a broad singlet at δ_H 3.70 representing a proton at C-3 bearing the-OH group in a trans-orientation (Akande *et al.*, 2022). Based on the experimental data and the available literature data (Salazar et al. 2000; Rodrigues et al. 2017; Credo et al. 2022), was concluded to be epifriedelanol (also known as (3)β-friedelanol). Despite epifriedelanol being reported for the first time from the root and stem regions of *S. glaucescens* Pax (Rwegoshora et al., 2022), it was previously isolated from its leaves (Credo *et al*., 2022), stem barks of *S. grantii* (Munhoz *et al*., 2014)*, Euphorbia neriifolia* (Chang *et al*., 2012) and leaves of *Pouteria ramiflora* (Rodrigues *et al*., 2017). This compound was reported to exhibit anti-Newcastle activity when an *in ovo* assay in chicken embryos was done (Credo *et al*., 2022). It was also reported to demonstrated antitumor activity by preventing crown gall tumors formation in a potato disc

assay (Kundu *et al.*, 2000), reduces cellular senescence in human primary cells (Yang *et al.*, 2011). From this evidence, epifriredelanol could serve to produce anti-Newcastle drugs, dietary supplements or cosmetics that modulate tissue aging and age associated diseases.

Compound **29**, euphol ($C_{30}H_{50}O$), R_f 0.6 in DCM, and [M^+], m/z 426.4 was isolated from bark extracts of both root (1834 mg) and stem (21.5 mg) as white powder. Their spectral data (^{13}C NMR, ^1H NMR and GC-MS) were first compared and then concluded to be the same compound. The ^{13}C NMR spectrum indicated thirty (30) carbon signals, four (4) of which resonated in olefinic regions at δ_C 134.7 (C-8), 134.1 (C-9), 125.8 (C-24) and 133.1 (C-25). The ^1H NMR indicated seven singlets at δ_H 0.77, 0.78, 0.88, 0.95, 0.98 and 1.26 (*s,* 6H) representing seven methyls. Comparison of these experimental data with the literature (Abdel-Sattar et al., 2014; Nyigo et al., 2016), the GC-MS data and the NIST library, the compound **29** was confirmed to be euphol.

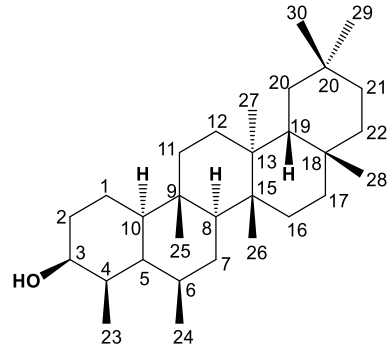

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Figure 10: Structure of epifriedelanol (β-friedelanol) isolated from the root and stem barks of *Synadenium glaucescens* Pax.

Compound **30**, β-sitosterol ($C_{29}H_{50}O$) and the R_f 0.36 in DCM was isolated from the root bark (62 mg) and stem bark (4 mg) extract as white shiny crystals. These experimental data were in agreement with (Lokadi and Munkombwe, 2015; Nyigo *et al*., 2016; Ododo *et al*., 2016) together with the GC-MS data, m/z 414.3, and a melting point of 134.6- 136.1 ^oC helped to confirm **30** as β-sitosterol (Fig.2, **30**). This compound was earlier isolated from the SG leaves, but it is for the first time isolated from the root and stem barks of this plant. it is reported to have antidiabetic property(Aurang , 2017; Babu and Jayaraman, 2020), analgesic (Villaseñor *et al.*, 2002) and antibacterial (Kurniawan *et al.*, 2021; Ododo et al., 2016). The identification of these compounds suggests great medicinal potential of *S. glaucescens* Pax.

6.1.3 Elucidation of Long chain compounds from *Synadenium glaucescens* Pax

Long chain hydrocarbons are among the natural compounds in medicinal plants. This study successfully led to isolation and elucidation of two long chain hydrocarbons (hexacosane, $C_{26}H_{54}$, **39** and 1-nonacosene, $C_{29}H_{58}$, **38**) were isolated from the root bark extracts while a long chain fatty acid, hexacosanoic acid was isolated from the stem barks of *S. glaucescens* Pax.

Compound **38**, 1-nonacosene ($C_{29}H_{58}$, 11 mg), white waxy solid was isolated from the root bark extract. Its TLC profile indicated a retention factor (R_f) value of 0.96 in PE and reacted bright purple with vanillin reagent which meant it to be a non conjugated whereas the larger R_f value was an indicator for a less polar compound. The structural details of it have been discussed in chapter three. The final structure was confirmed by 2D NMR data (HSQC and HMBC) together with the GC-MS data indicated m/z 405.4 which was finally assigned as 1-nonacosene (Fig. 9). To the best of our knowledge, this long chain alkene is reported for the first time from *S. glaucescens* Pax but had been isolated

from *Cissampelos mucronata* (Akande *et al*., 2022). Compound **39,** hexacosane**,** 173 mg, Melting point: 58- 59.5 ^{o}C reacted purple with vanillin reagent (UV negative) was isolated as white powder from DCM fraction of the stem bark. Based on both one and two dimensions ^{1}H and ^{13}C NMR, its molecular formula was determined to be $C_{26}H_{54}$, hexacosane. These spectral data were in agreement with (Aljubiri *et al*., 2021; *Credo et al*., 2022). More details on its elucidation were discussed in chapter four. To the best of researchers' knowledge, isolation of **39** (hexacosane) is a first-time report in *S*. *glaucescens* Pax but it was previously reported in *Euphorbia balsamifera* as a member of family Euphorbiaceae. Compound **40**, hexacosanoic acid (26 mg) was isolated as white powder from the root barks. It reacted purple with vanillin reagent when hot which was an indicator for non-conjugation nature of the C-C skeleton. Its structure was elucidated through NMR, GC-MS, m/z 396, *calcd*. 396.6899 and NIST library ascertained to be hexacosanoic acid $C_{26}H_{52}O_2$. Absence of the -OH proton signal in the ^{1}H NMR is associated with the long chain nature of the molecule which tends to weaken its intensity. It is also reported that after addition of the solvent, the OH tends to exchange for the deuterium which does not produce a peak in a typical NMR spectrum. The experimental spectral data were in agreement with (Yamamoto et al. 2015; Rehan et al. 2020; Credo et al. 2022). This acid is also known as cerotic acid. These compounds are a class of secondary metabolites synthesized through acetate biosynthetic pathway.

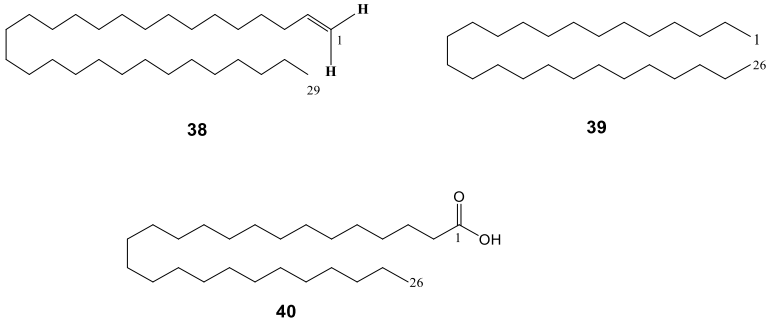

Figure 11: Long chain compounds isolated from the root bark (**37, 39**) and stem barks (**38**) of *S. glaucescens* Pax.

6.2 Cytotoxicity evaluation of pure compounds

The use of herbal medicine has been linked with the belief of being high safe or less toxic to users (Kingston, 2011). cultural acceptability, availability and lower costs (Merlin *et al*., 2019) It is argued that toxicity studies concerning herbal medicine should reflect their traditional use to allow for rational discussions regarding their safety for their beneficial use (Merlin et al., 2019). Some of the isolated compounds were subjected to brine shrimp (*A. salina*) lethality test and found to be nontoxic at the maximum test concentration of 2400 µg/mL. Compound **33** (3,4,3'-tri-O-methylellagic acid) demonstrated the highest LC_{50} value (531.19 µg/mL) while β-sitosterol demonstrated the least toxicity value LC_{50} as presented in Table 3.

Table 4: LC_{50} for the compounds isolated from the stem and root barks of *Synadenium glaucescens* Pax

Compound	LC_{50} (µg/mL)
hemicosanyl ferulate (**34**)	762.807
3',4'-di-*O*-methylellagic acid-4 α, *L*-rhamnopyranoside (**32**)	2736.03
3,4,3'-tri-*O*-methylellagic acid (**33**)	531.19
Lupeol (**18**)	25813.42
Epifriedelanol (**36**)	1088.48
Euphol (**29**)	825.52
β-sitosterol (**30**)	5688.20

6.3 Antibacterial evaluation of Pure compounds

Compounds from stem and root barks of *S. glaucescens* demonstrated variable activities against bacteria standard strains. Hexacosane (**39)** demonstrated weak activity (2 mg/mL) against all three test bacteria strains except on *E. faecalis* which was moderate (1.5 mg/mL). **35,** octacosylferulate showed strong activity against *S. aureus* (MIC= 0.125 mg/mL), moderate activity against *E. faecalis* (MIC= 1.5 mg/mL), weak activity against *E. coli* (2 mg/mL) and *P. aeruginosa* (> 2 mg/mL). The Weak activity of phenolic compounds and generally other compounds like the hexacosane above against Gram- negative is also reported by Canli *et al.,* (2017). *S. aureus* is arguably among the most problematic of all bacterial pathogens owing in

large part to the persistent emergence of antibiotic resistant strains (Quave et al., 2012). Therefore, the observed activity of this phenolic compound (Octacosyl ferulate) suggests the possibility of this plant to serve for source of drugs against infections associated with *S. aureus*.

Compound **40** (hexacosanoic) acid demonstrated moderate activity (1 mg/ mL) against three of the tested strains except *P. aeruginosa* on which it showed weak activity (MIC> 2 mg/mL. The observed activities are greatly in coherence with other scholars that have reported these compounds to be of medical importance including; antibacterial, antiprotozoan, anti-algal, antifungal and antiviral (Desbois and Smith, 2010). Essential fatty acids are known for medicinal value against cancer, insulin resistance, skin permeability, cardiovascular disease and depression (Melariri *et al.,* 2012). Linolenic ($C_{18}H_{30}O_2$) and linoleic acid ($C_{18}H_{32}O_2$) have been reported to be effective against *P. falciparum.* For example, palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid are reported active against *S. pyogenes* and *S. aureus* through inhibition of FabI gene (Zheng *et al*., 2005). However, it was noted that the standard drug (Gentamicin) was superior of all isolated compounds with MIC values; *S. aureus* (0.003 mg/mL), *E. faecalis* (0.003 mg/mL, *P. aeruginosa* (0.006 mg/mL) and *E. coli* (0.006 mg/mL). Similar result of less susceptibility of Gram negative over Gram positive bacteria to the natural compounds is also reported in EtOAc extracts of *Carpobrotus edulis* (Vázquez *et al.,* 2011). Since the activity of the any drug is associated with its molecular structure including the pharmacophore (Duque *et al.,* 2018) and molecular weight (Mohamed *et al.,* 2022), a systematic structural modification may improve their activity. Comparing the activities of compounds, there were maximum activities against Gram-positive bacteria (*S. aureus* and *E. faecalis*) due to its antibacterial action against cell wall synthesis (Rehan *et al*., 2020). It is further explained

that Gram-positive bacteria have a cell wall composed mostly of peptidoglycan with no protective outer membrane thus easy penetration of the toxic phytochemicals into the cells as opposed to Gram- negative organisms (e.g. *E. coli and P. aeruginosa* in this case) have less peptidoglycan but contain an outer membrane composed of lipopolysaccharide and lipoproteins which make them less permeable (Chokoe *et al.*, 2008). The moderate and weak activities of the compounds against *P. aeruginosa* is associated bacterial biofilm formation favoured by the presence of exopolysaccharides (EPS) embedded in an extracellular matrix and to the production of type IV pili, T4P (Ochoa *et al*., 2013). These results of octacosylferulate confer with findings that phenolic compounds are the most abundant secondary metabolites in plants that show a wide range of distinct biological activities (Chen *et al*., 2014; Zhang *et al.,* 2022).

The Thin layer chromatography (TLC) is a simple effective and quick method used to identify the phytochemical constituents in a sample (Karthika and Paulsamy, 2015). The TLC results indicated a variation in chemical composition and their concentration among the study sites. For example, euphol (UV negative, rij= 6.64) showed highest concentration in Tanga (Handeni) compared to Njombe and Morogoro samples. The observed variation in chemical composition among *S. glaucescens* Pax from Njombe, Morogoro and Tanga regions is supported by other scholars. It is asserted that the contents of bioactive compounds in traditional herbal medicines depend on plant origin, cultivation sites, harvest seasons (Weon *et al*., 2016), and age.

7.0 CHAPTER SEVEN: GENERAL CONCLUSION AND RECOMMENDATION

7.1 Conclusion

Synadenium glaucescens Pax is a promising source of drugs due a large spectrum of compounds of pharmacological importance which have been unveiled by this study. The fifteen (15) compounds isolated during this study represent six major groups of secondary metabolites of pharmacological importance which are Phenolics, triterpenoids, steroids, long chain hydrocarbons, long chain amine and Fatty acids. A new compound in nature which is a derivative of ellagic acid 3',4'-di-*O*-methylellagic acid-4-α-*L*-rhamnopyranoside (**32**) along with a known 3,4,3'-*tri*-*O*-methylellagic acid (**33**) were for the first time isolated. Octacosyl ferulate (**35**) from stem bark demonstrated the strongest activity (MIC 0.125 mg/mL) against *S. aureus* of compounds evaluated for antibacterial efficacy. The isolated triterpenoids are well known for the bioactivity. Cytotoxicity evaluation for the pure compounds indicate that they are safe. The age of *a S. glaucescens* Pax imparted less effect on secondary metabolite profiles compared to their geographical location. The variation of phytochemicals due to geographical location and age reported in this study is expected to guide traditional medicine practitioners during sample collection and formulations from SG.

7.2 Recommendation

Isolation of compounds with limited amount in the studied extracts by advanced methods like HPLC-MS and HPLC-NMR are suggested. The new compound 3',4'-di-*O*-methylellagic acid-4-α-*L*-rhamnopyranoside (**32**) is recommended as a marker compound for quality control of formulations from the root bark extracts of *S. glaucescens* Pax. Additionally, more studies on pharmacological activities including anti-cancer, anti-diabetic, and antioxidant are

recommended. For enhanced effectiveness and safety more toxicity studies on animal cell lines of the isolated compounds are suggested. It is recommended that any formulation that prefers euphol at higher concentration may consider collection of *S*. glaucescens from Tanga region. However, Njombe region may be another preference for a big spectrum of phytochemicals.

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APPEDINCES

Appendix I: Supplementary material for Paper one

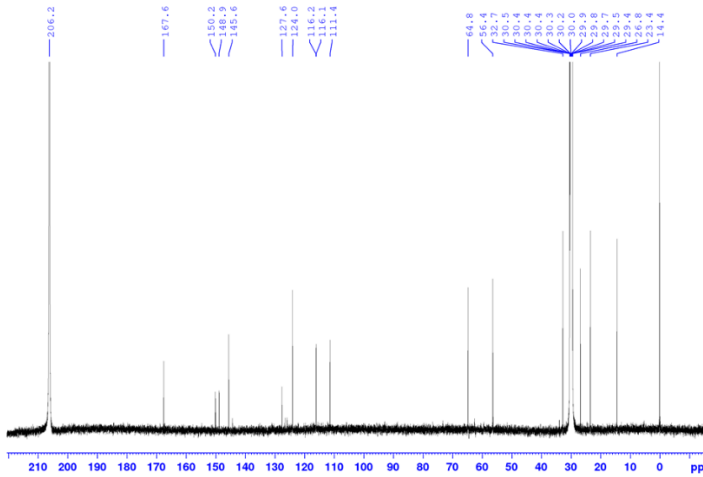

Figure S12: ^{13}C NMR (150 MHz, CD_3OD) spectrum of compound SG1 (Hemicosanyl ferulate)

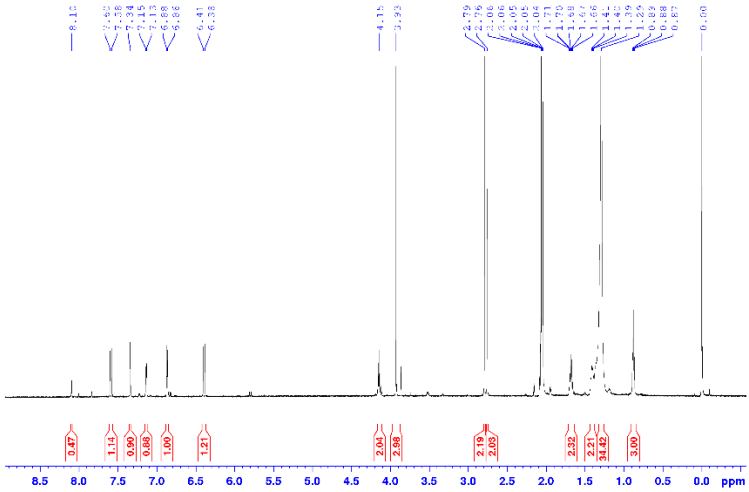

Figure S13: ^{1}H NMR (600 MHz, CD_3OD) spectrum of compound **SG1** (Hemicosanyl ferulate)

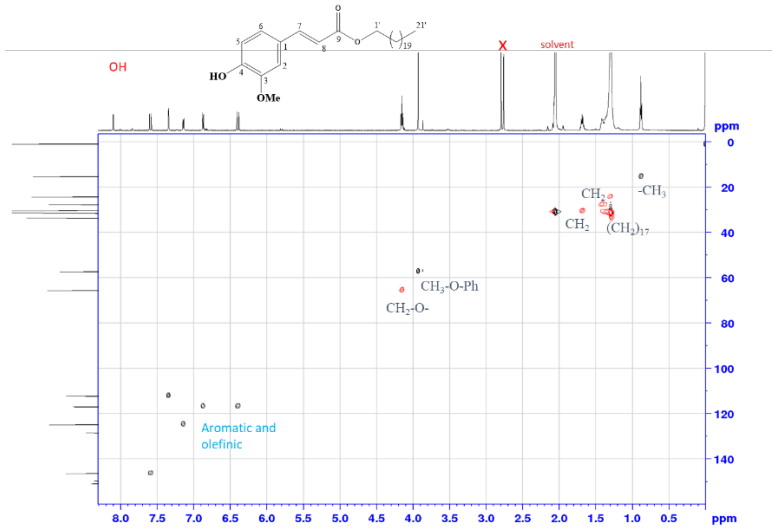

Figure S14: HSQC NMR (CD3OD) spectrum for compound SG1 (Hemicosanyl ferulate)

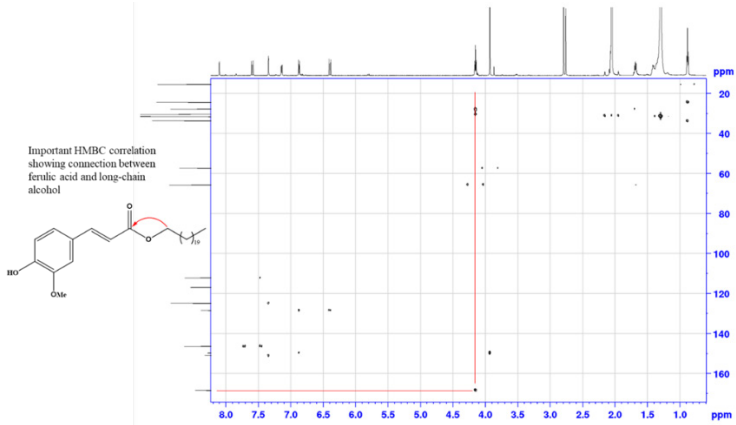

Figure S15: HMBC NMR (CD3OD) spectrum for compound SG1 (Hemicosanyl ferulate)

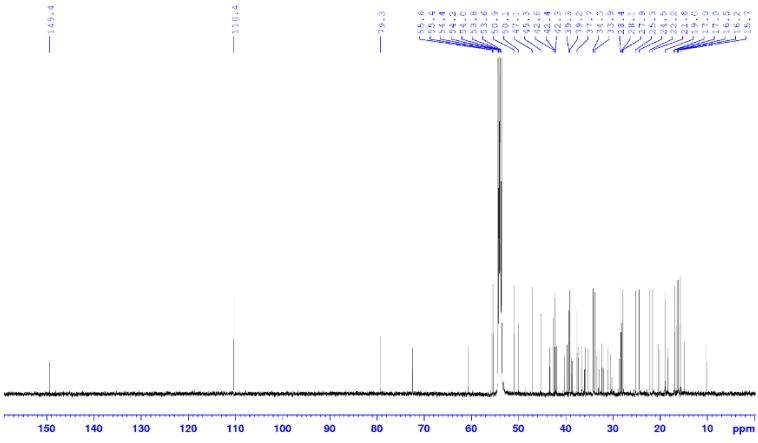

Figure S16: ^{13}C NMR (CD_2Cl_2, 150 MHz) spectrum of compound **SG2** (Lupeol)

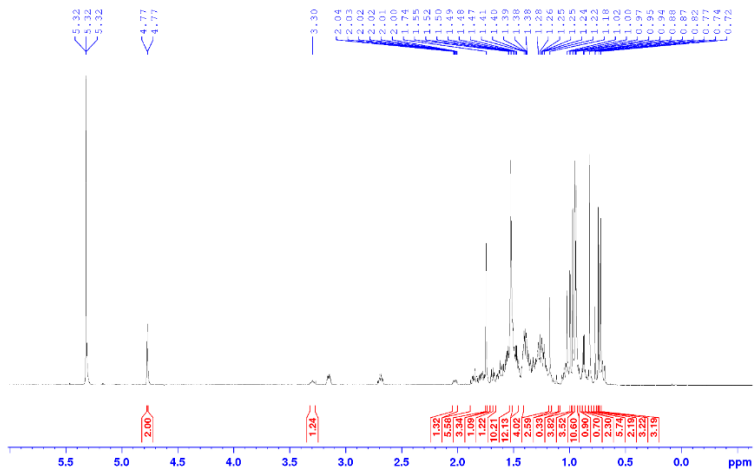

Figure S17: ^{1}H NMR (600 MHz, CD_2Cl_2) spectrum of compound **SG2** (Lupeol)

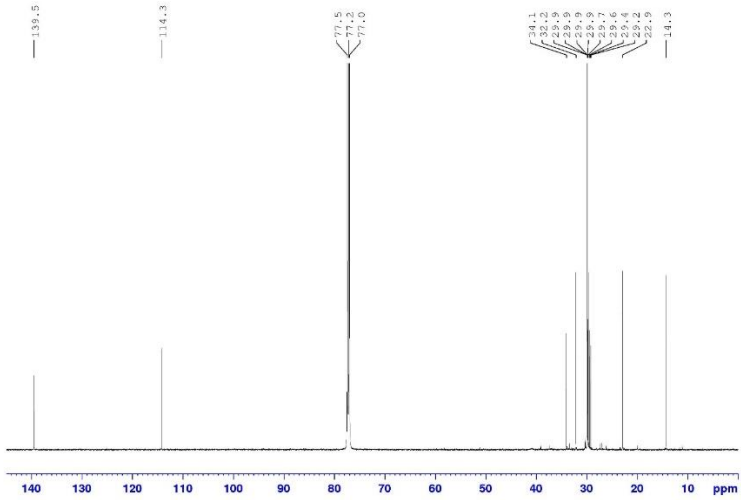

Figure S18: ^{13}C NMR (150 MHz, CDCl3) spectrum of compound **SG3** (1-nonacosene)

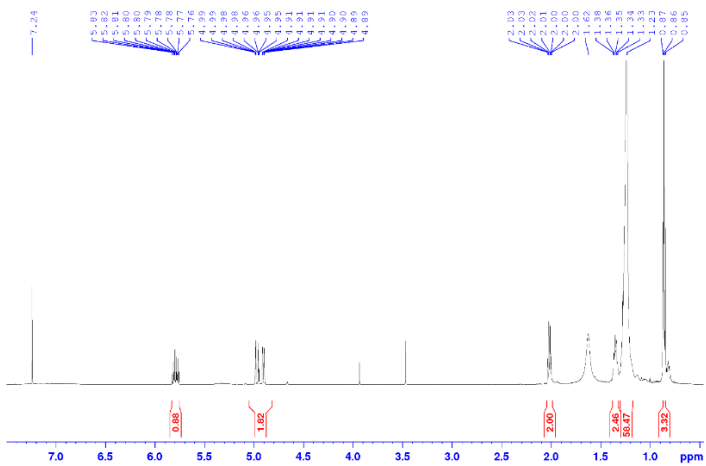

Figure S19: ^{1}H NMR (600 MHz, CDCl3) spectrum of compound **SG3** (1-nonacosene)

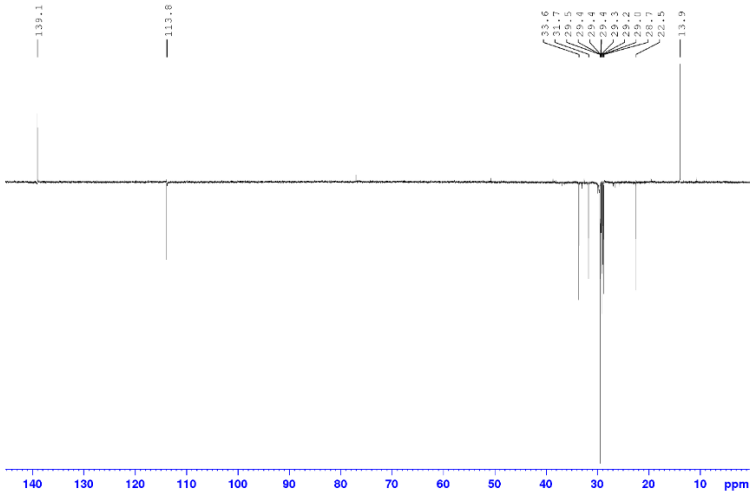

Figure S20: DEPT135 NMR spectrum (150 MHz, CDCl3) of compound **SG3** (1-nonacosene)

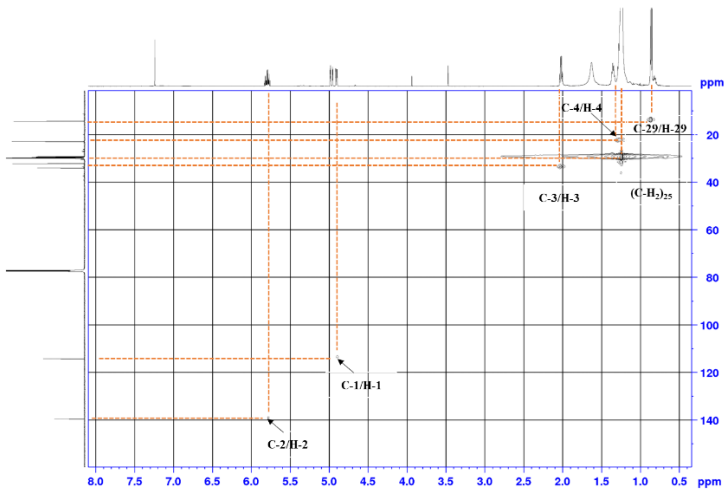

Figure S21: HSQC NMR spectrum of compound **SG3** (1-nonacosene)

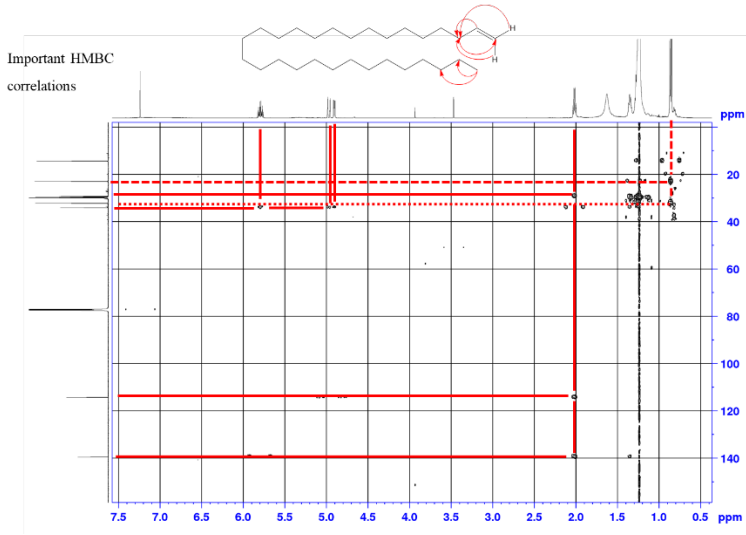

Figure S22: Important HMBC correlation spectrum of compound **SG3** (1-nonacosene)

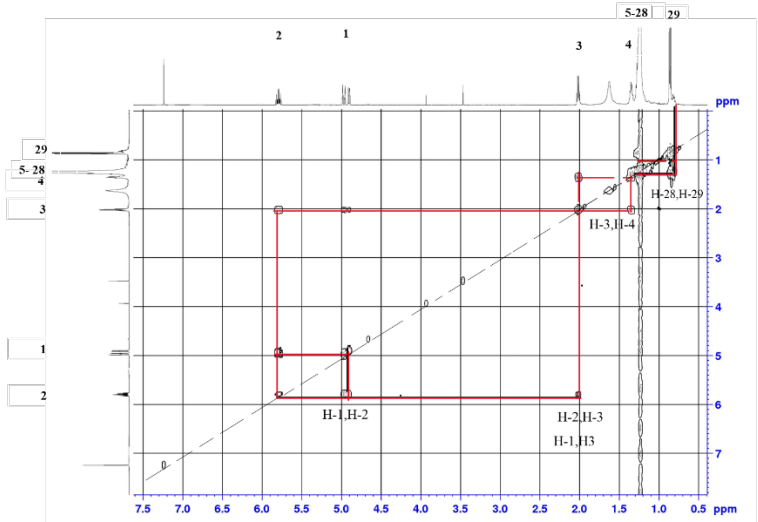

Figure S23: COSY (^{1}H-^{1}H correlation) NMR spectrum of compound **SG3** (1-nonacosene)

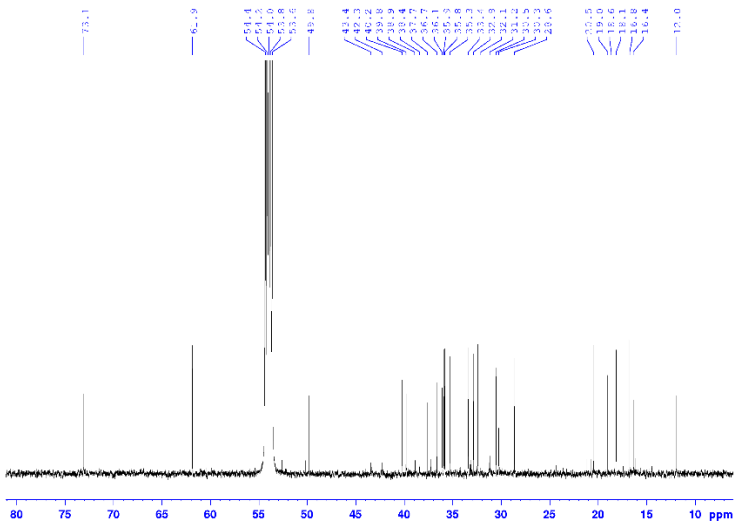

Figure S24: ^{13}C NMR (CD_2Cl_2, 150 MHz) spectrum for compound **SG4** (epifriedelanol)

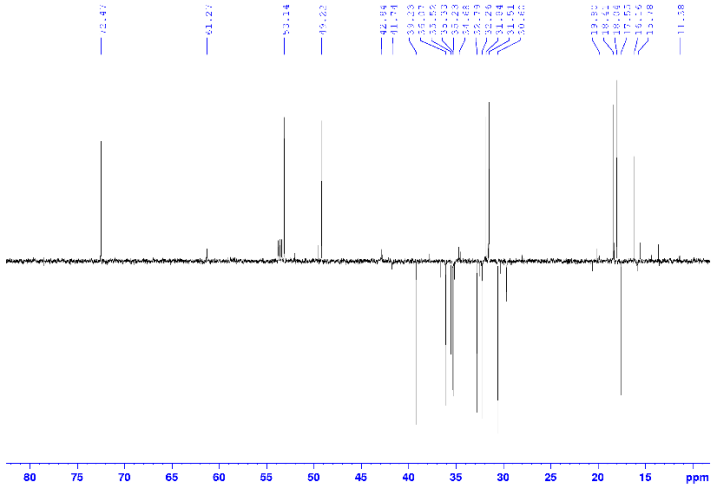

Figure S25: DEPT135 NMR (CD_2Cl_2, 150 MHz) spectrum for compound **SG4** (epifriedelanol)

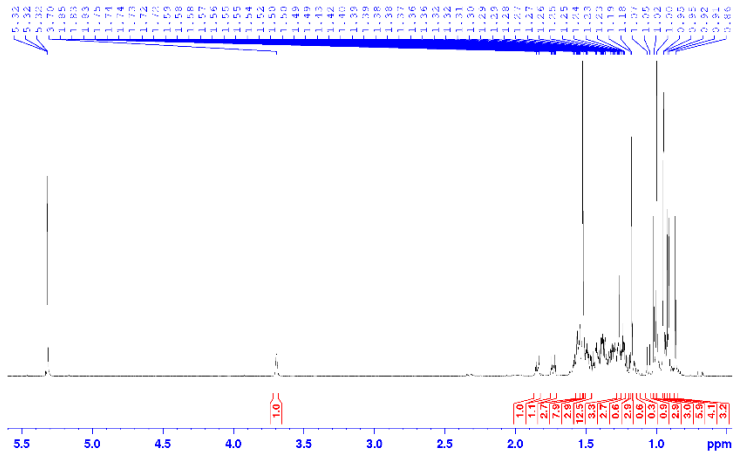

Figure S26: ^{1}H NMR (CH_2Cl_2, 600 MHz) spectrum for compound **SG4** (epifriedelanol)

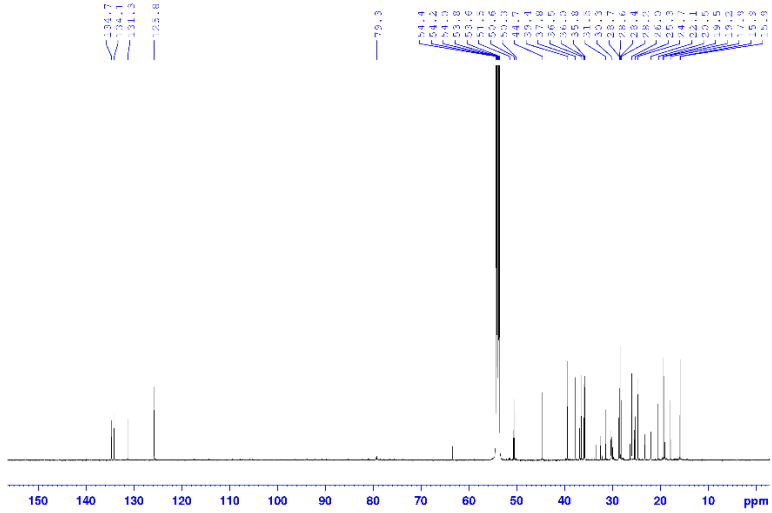

Figure S27: ^{13}C NMR (CH_2Cl_2, 150 MHz) spectrum for compound **SG5** (euphol)

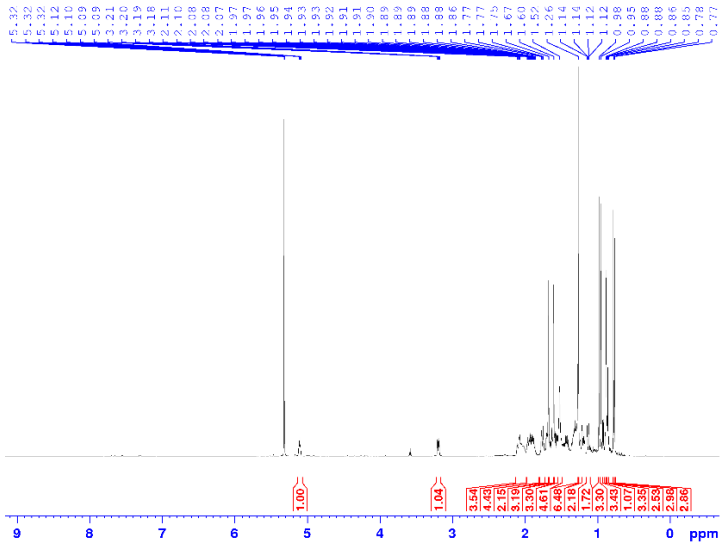

Figure S28: ^{1}H NMR (CH_2Cl_2, 600 MHz) spectrum for compound **SG5** (euphol)

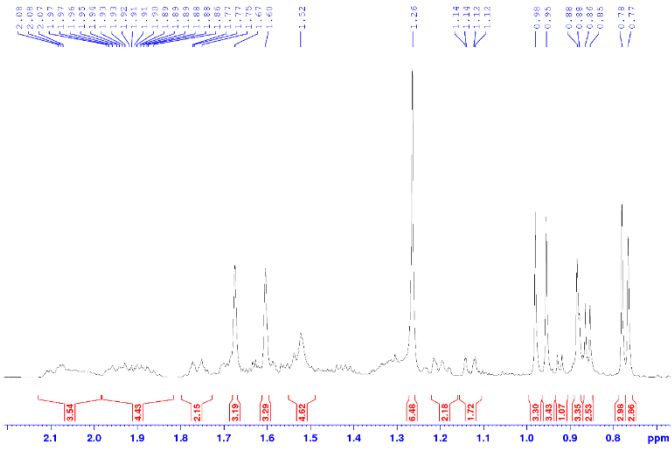

Figure S29 Extended high field region (0.70- 2.20 ppm) ^{1}H NMR (CH_2Cl_2, 600 MHz) spectrum of compound **SG5** (euphol)

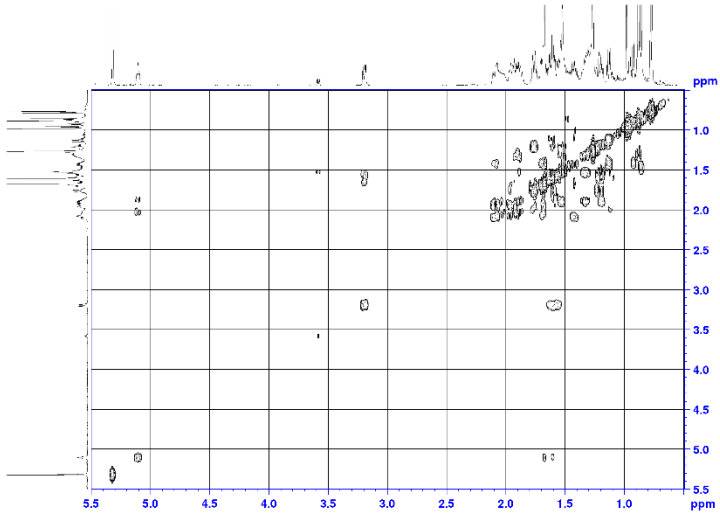

Figure S30: (^{1}H- ^{1}H) COSY NMR spectrum for compound **SG5** (euphol)

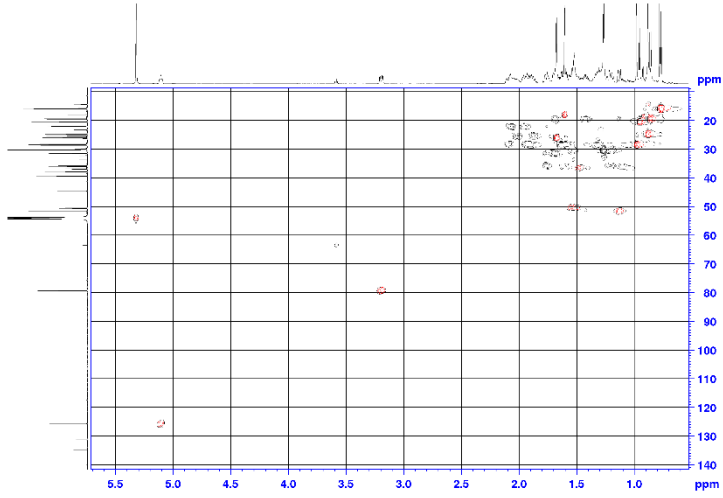

Figure S31: HSQC NMR spectrum for compound **SG5** (euphol)

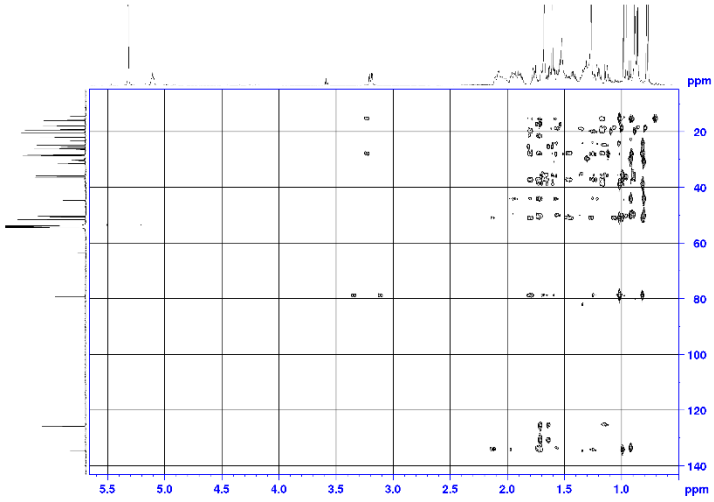

Figure S32: HSQC NMR spectrum for compound **SG5** (euphol)

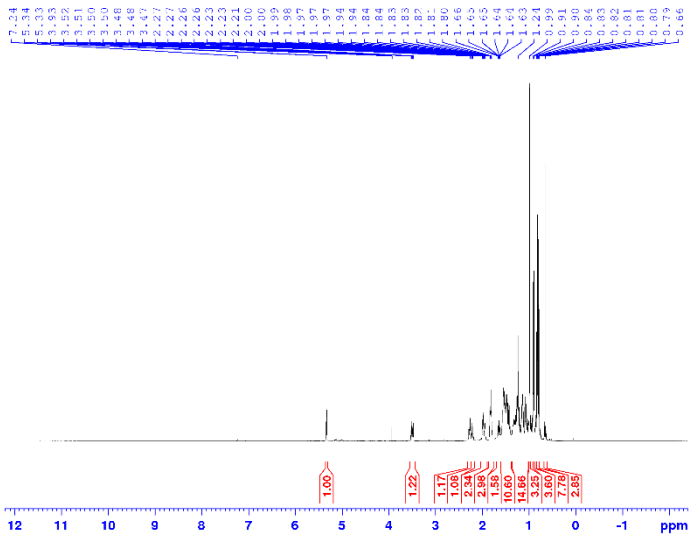

Figure S33: ^{1}H NMR (CDCl3, 600 MHz) spectrum of compound **SG6** (β-sitosterol)

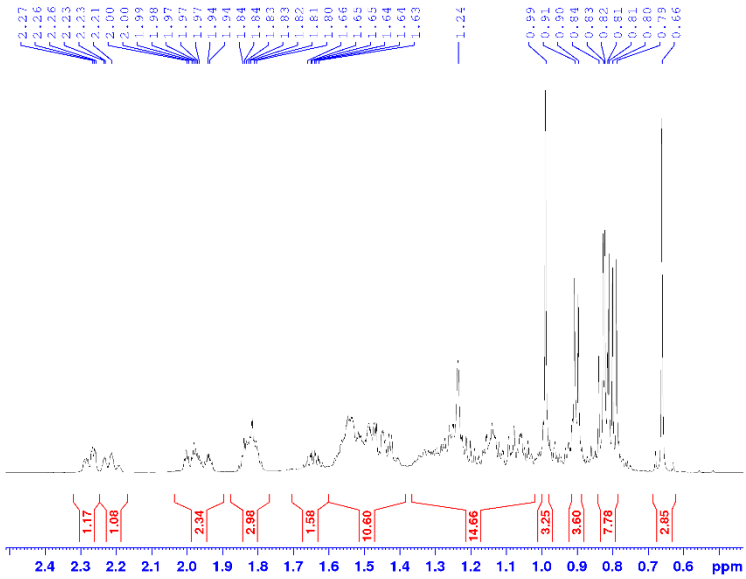

Figure S34: Extended region (0.50- 2.50 ppm) ^{1}H NMR ($CDCl_3$, 600 MHz) of compound **SG6**

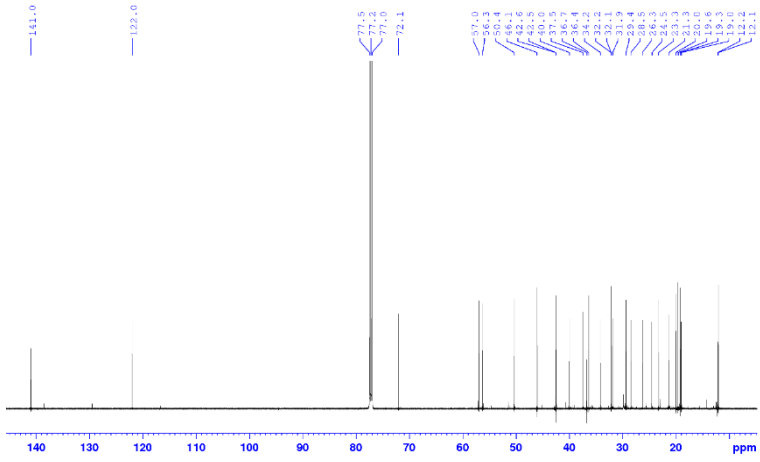

Figure S35: ^{13}C NMR (CDCl3, 150 MHz) spectrum of compound **SG6** (β-sitosterol)

Appendix II: Supplementary materials for Manuscript One

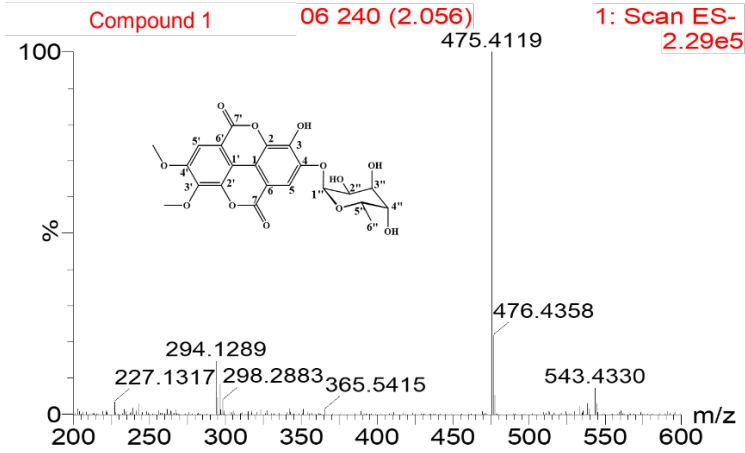

Figure S36: HPLCMS-ESI$^-$ spectrum of compound **1** (3', 4'-di-*O*-methylellagic acid 4-α-*L*-rhamnopyranoside

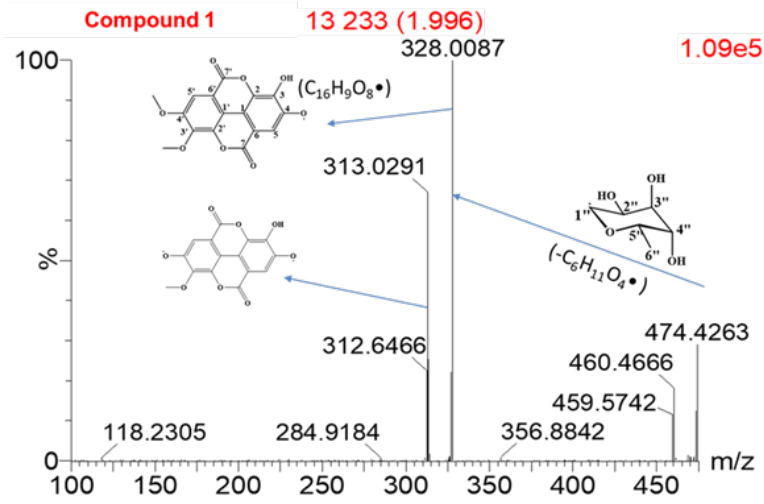

Figure S37: HPLCMS/MS-ESI$^-$ spectrum of daughter ions of compound 1 (3', 4'-di-*O*-methylellagic acid 4-α-*L*-rhamnopyranoside)

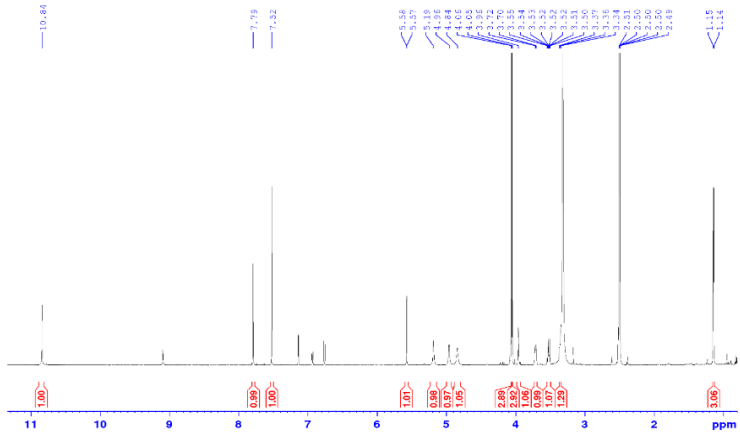

Figure S38:[1]H-NMR (600 MHz, DMSO-d_6) spectrum of compound 1 (3', 4'-di-O-methylellagic acid 4-α-L-rhamnopyranoside)

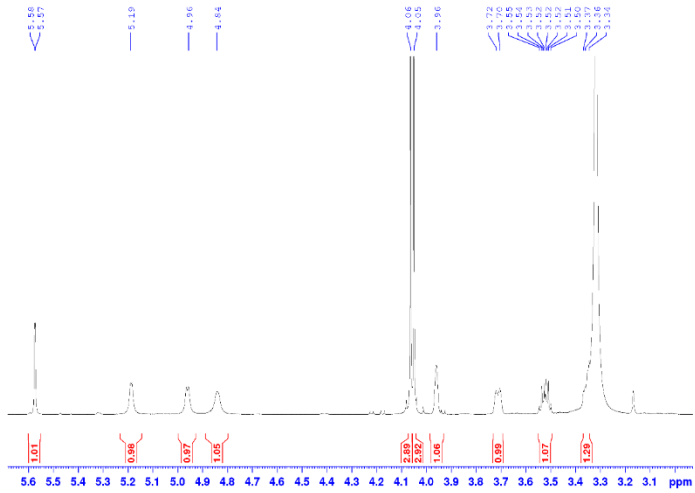

Figure S39: Extended region of 3.00- 5.70 ppm of [1]H-NMR (600 MHz, DMSO-d_6) spectrum of compound 1 (3',4'-di-*O*-methylellagic acid 4-α-*L*-rhamnopyranoside)

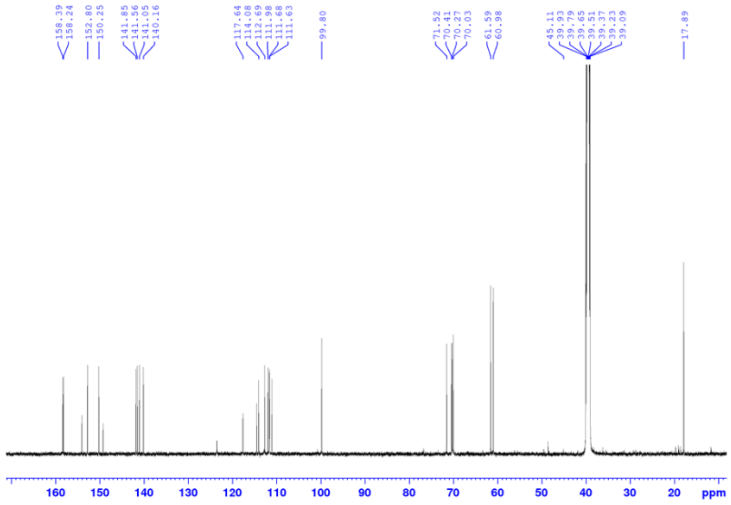

Figure S40: ^{13}C-NMR (150 MHz, DMSO-d_6) spectrum of compound 1 (3', 4'-di-*O*-methylellagic acid 4-α-*L*-rhamnopyranoside)

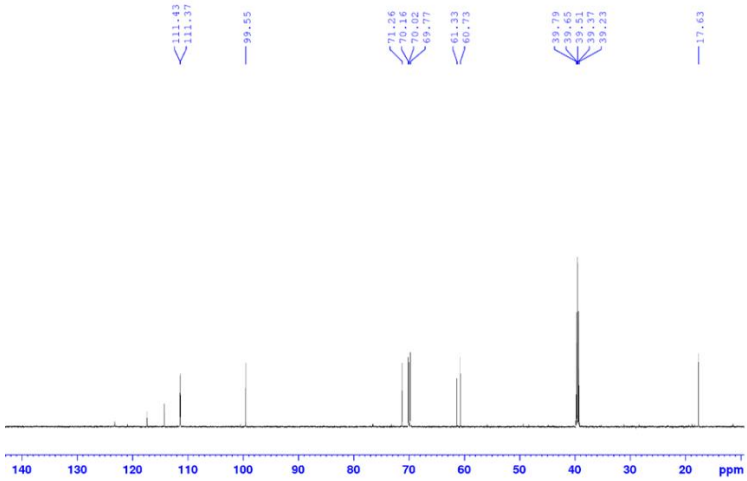

Figure S41: DEPT135 (150 MHz, DMSO-d_6, δ, ppm) spectrum of compound **1** (3',4'-di-*O*-methylellagic acid 4-*α*-*L*-rhamnopyranoside)

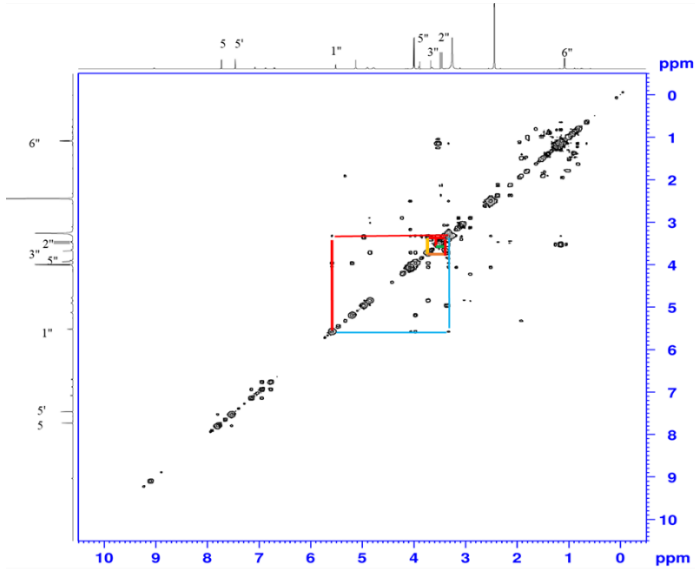

Figure S42: COSY Spectrum of compound **1** (3',4'-di-*O*-methylellagic acid 4-*α*-L-rhamnopyranoside)

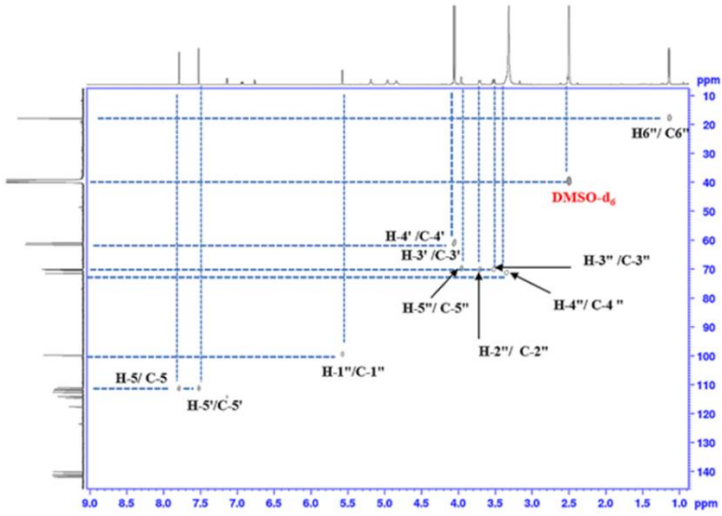

Figure 43: HSQC spectrum of compound **1** (3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside)

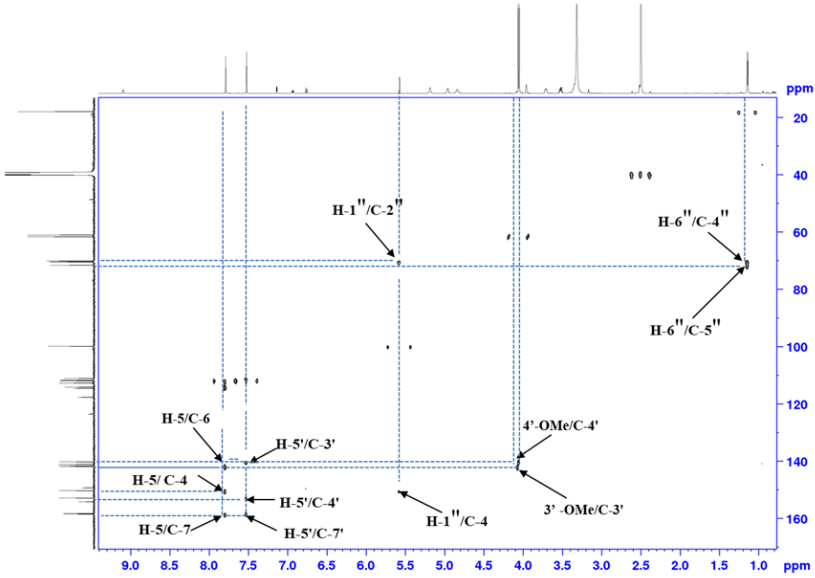

Figure 44: HMBC spectrum of compound 1 (3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside)

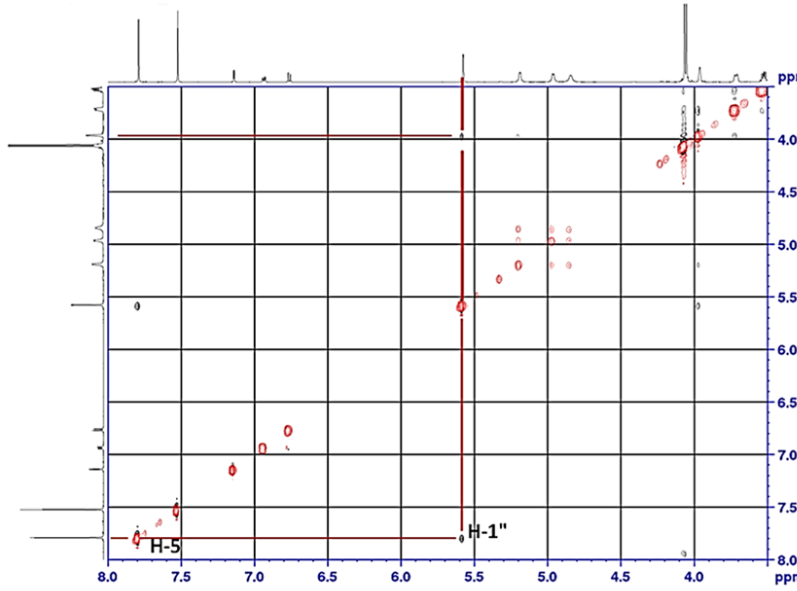

Figure 45: Key ROESY (1H-1H) correlation spectrum of compound 1 (3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside)

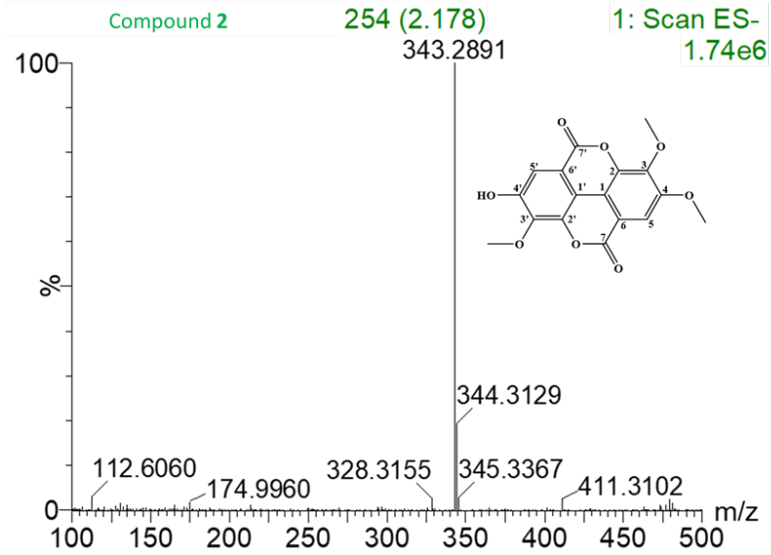

Figure 46: HPLCMS-ESI spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

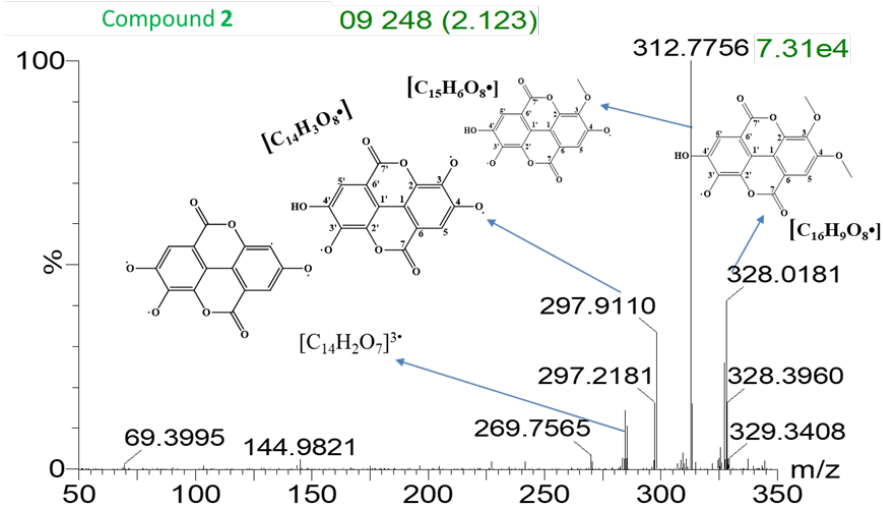

Figure 47: MS/MS-ESI$^-$ spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

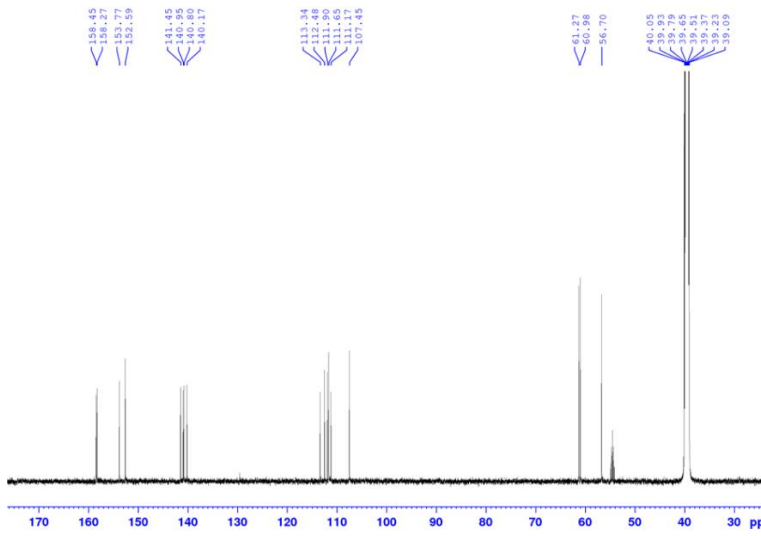

Figure 48: ^{13}C-NMR (150 MHz, DMSO-d_6, δ ppm) spectrum of compound **2** (3,4,3'-tri-*O*-methylellagic acid)

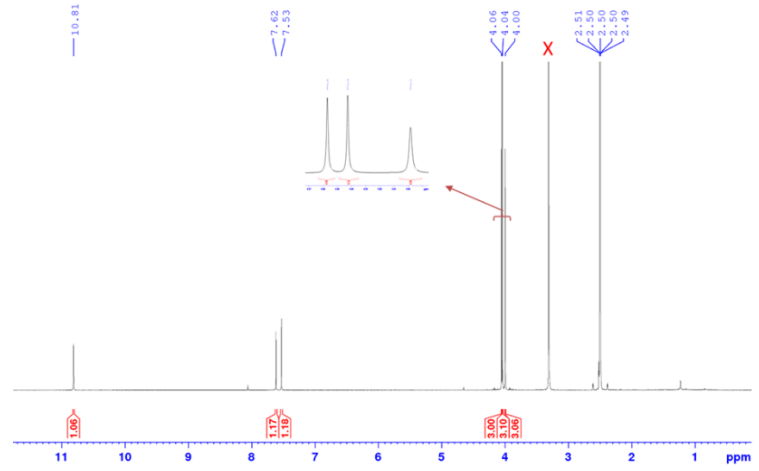

Figure 49: ^{1}H-NMR (600 MHz, DMSO-d_6, δ ppm) spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

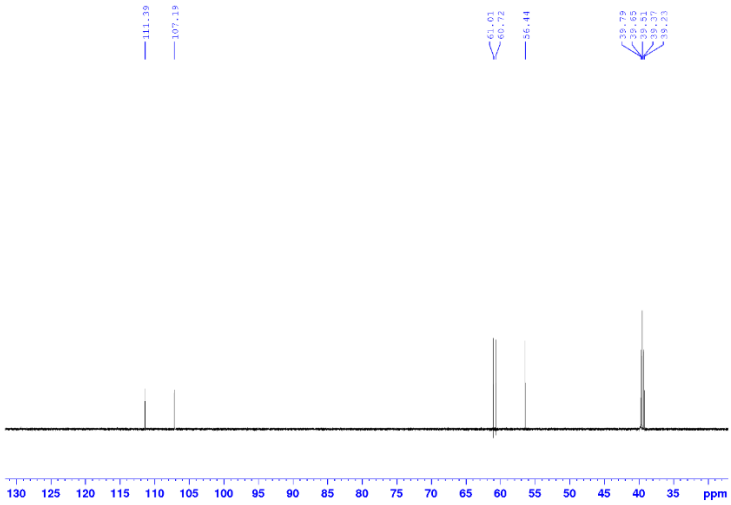

Figure 50: DEPT135 spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

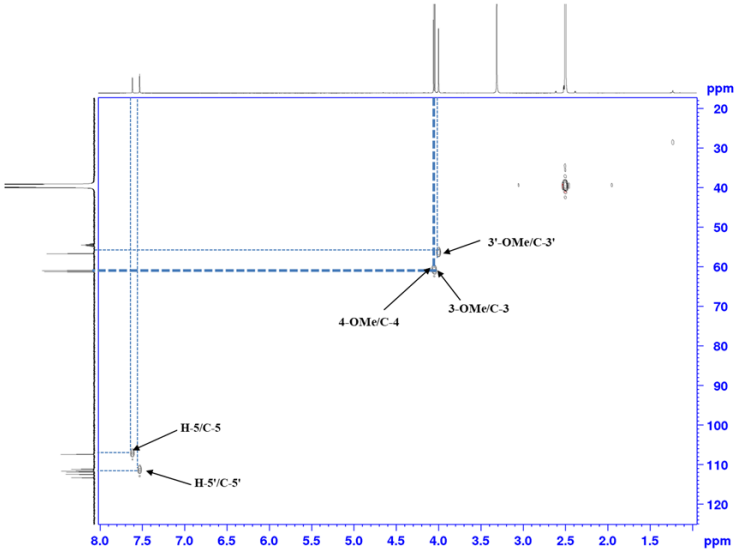

Figure 51: HSQC spectrum of compound **2** (3,4,3'-tri-O-methylellagic acid)

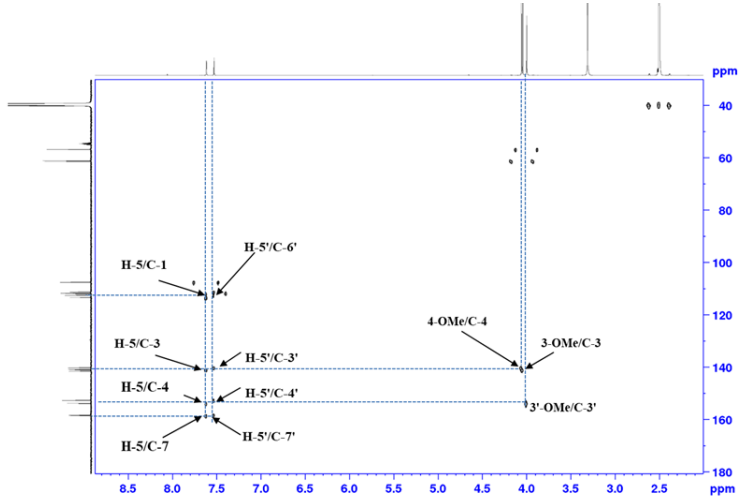

Figure 52: HMBC spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

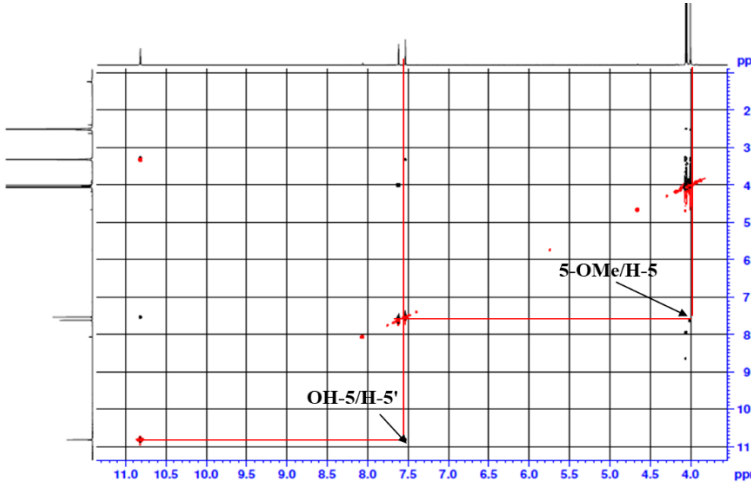

Figure 53: Key ROESY (^{1}H-^{1}H) correlation spectrum of compound **2** (3,4,3'-*tri*-*O*-methylellagic acid)

Appendix III: Supplementary material for Manuscript Two

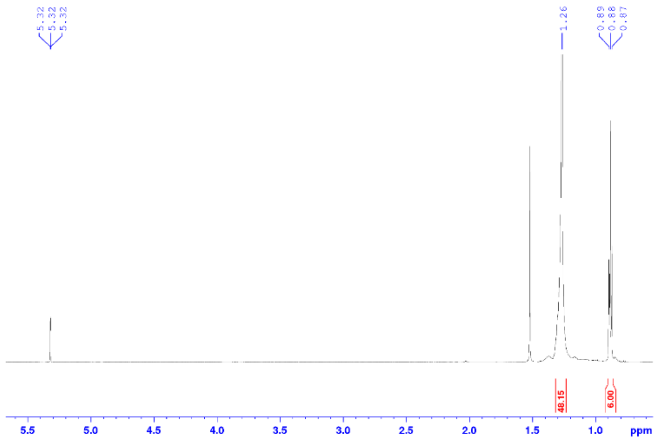

Figure S54: ^{1}H NMR (CD_2Cl_2, 600 MHz,) of compound G1 (Hexacosane)

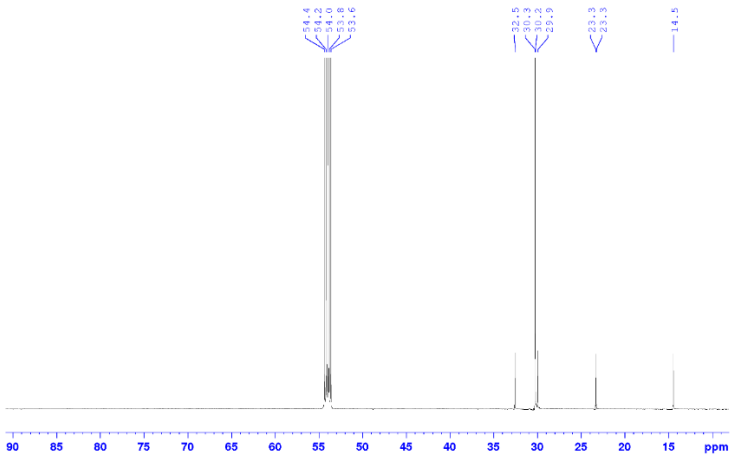

Figure S55: ^{13}C NMR (CD_2Cl_2, 150 MHz,) of compound **G1** (Hexacosane)

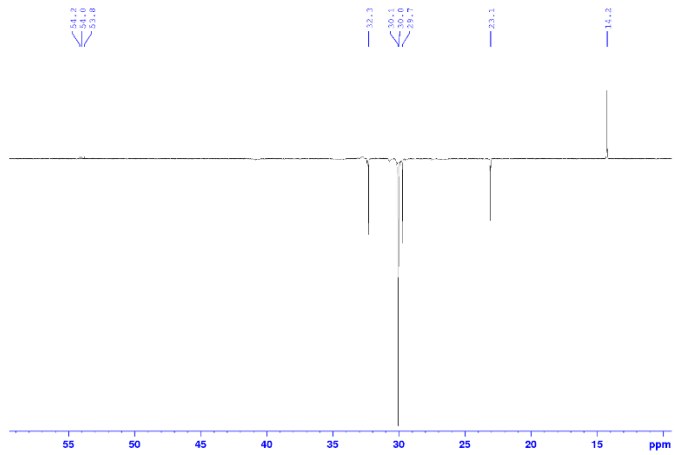

Figure S56: DEPT 135 NMR (CD_2Cl_2, 150 MHz,) of compound G1 (Hexacosane)

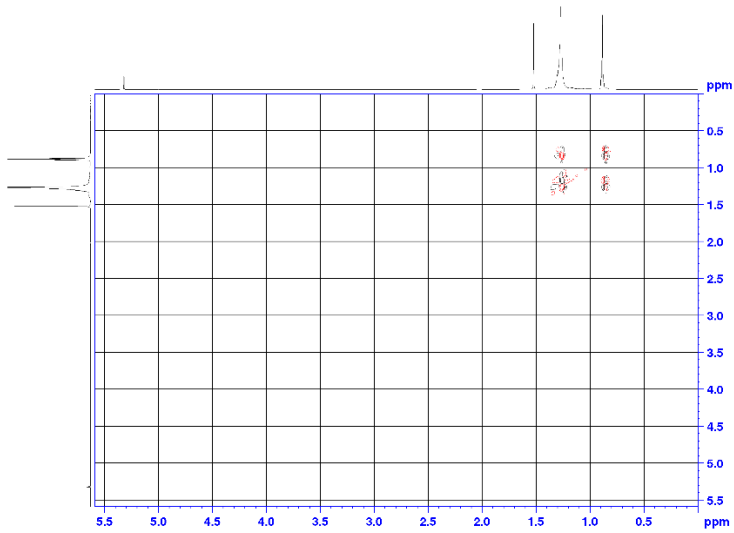

Figure S57: COSY (^1H: ^1H correlation) spectrum of compound **G1** (hexacosane)

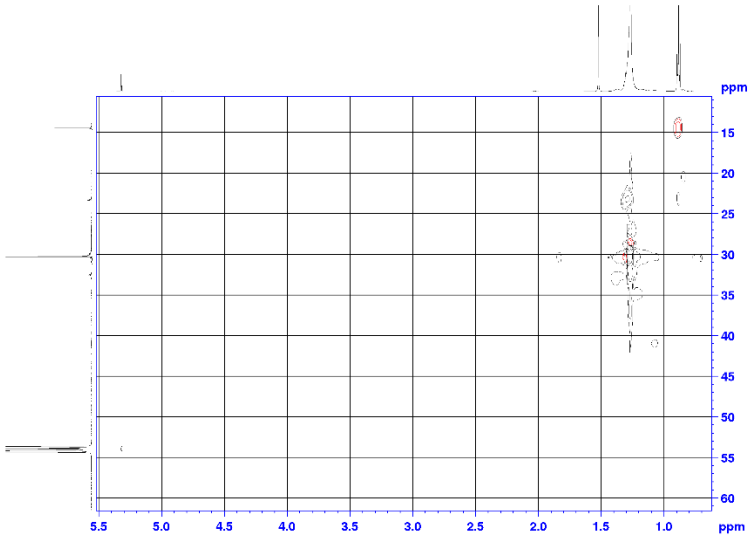

Figure S58: HSQC spectrum of compound **G1** (hexacosane)

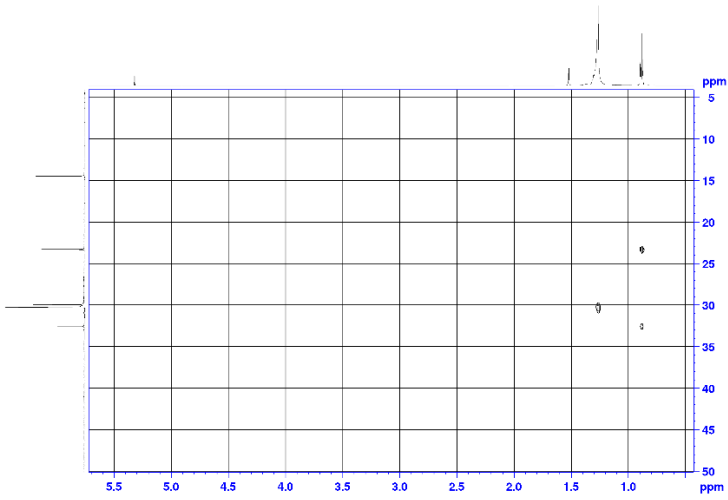

Figure S59: HMBC spectrum of compound **G1** (hexacosane)

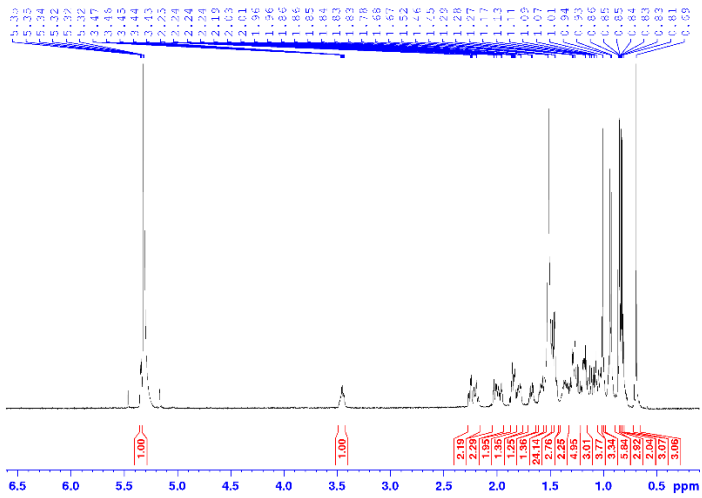

Figure S60: ^{1}H NMR (CD_2Cl_2, 600 MHz) of compound **G2** (β- sitosterol)

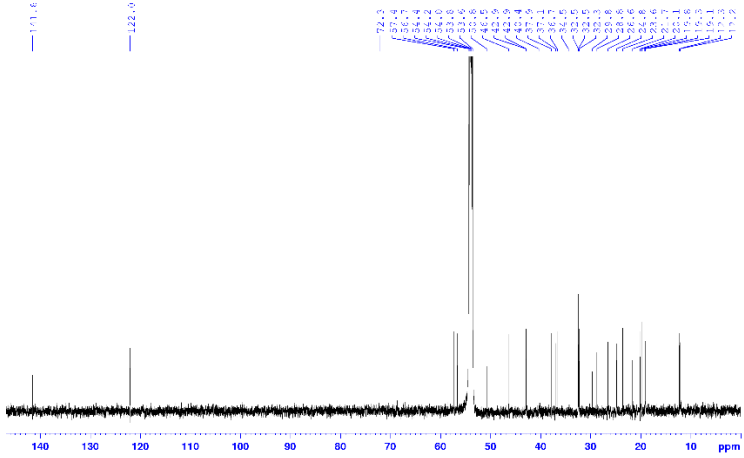

Figure S61: ^{13}C NMR (CD_2Cl_2, 150 MHz) of compound **G2** (β- sitosterol)

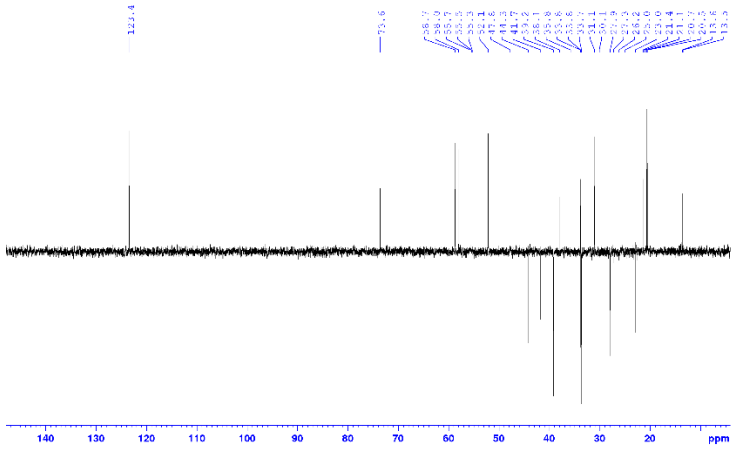

Figure S62: DEPT 135 NMR spectrum of compound **G2** (β-sitosterol)

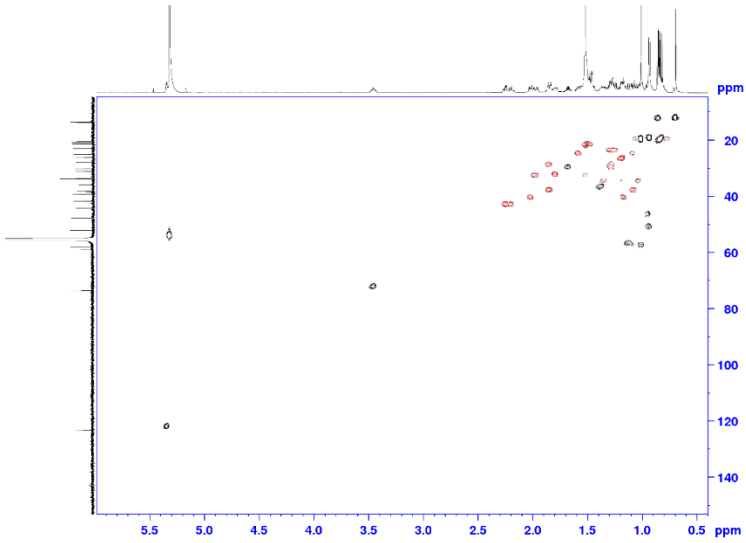

Figure S63: HSQC NMR spectrum of compound **G2** (β-sitosterol)

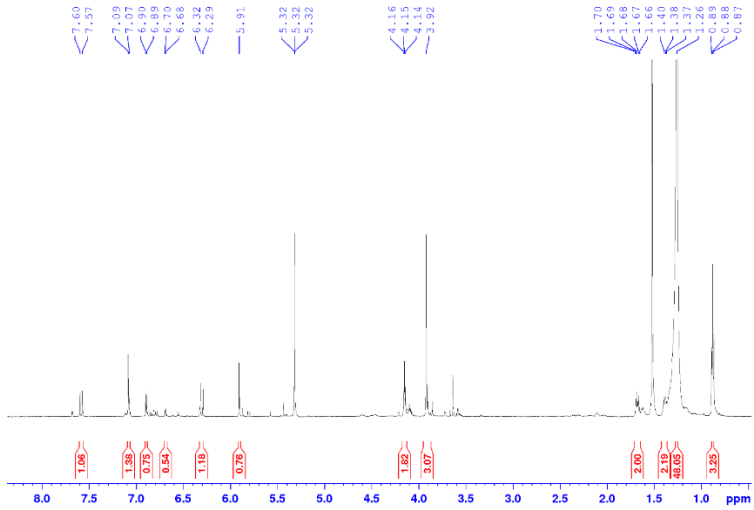

Figure S64: ^{1}H (CD_2Cl_2, 600 MHz) of compound **G3** (Octacosyl ferulate)

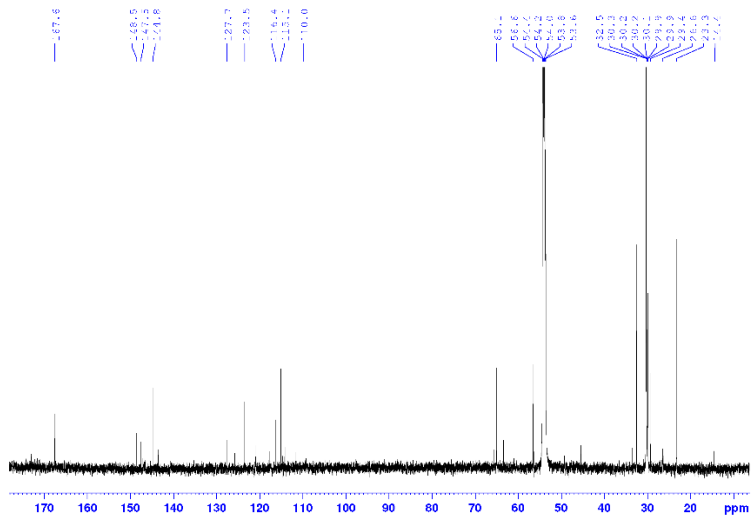

Figure S65: ^{13}C NMR (CD_2Cl_2, 150 MHz) of compound **G3** (Octacosyl ferulate)

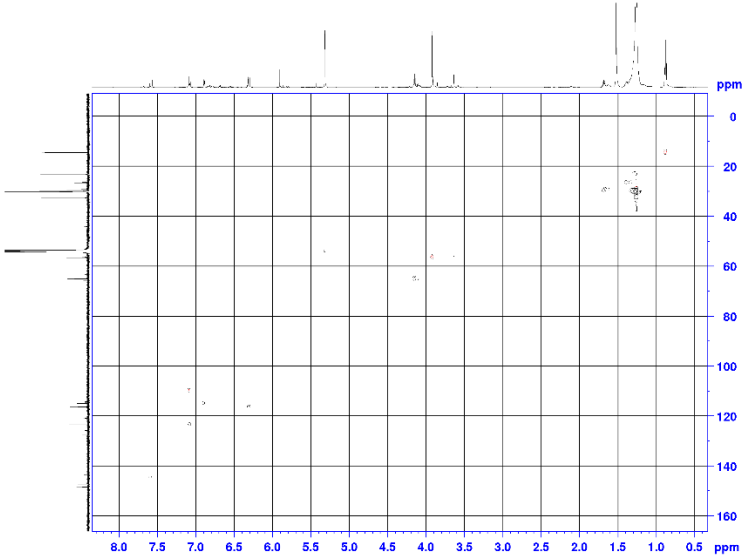

Figure S66: HSQC NMR spectrum of Compound **G3** (Octacosyl ferulate)

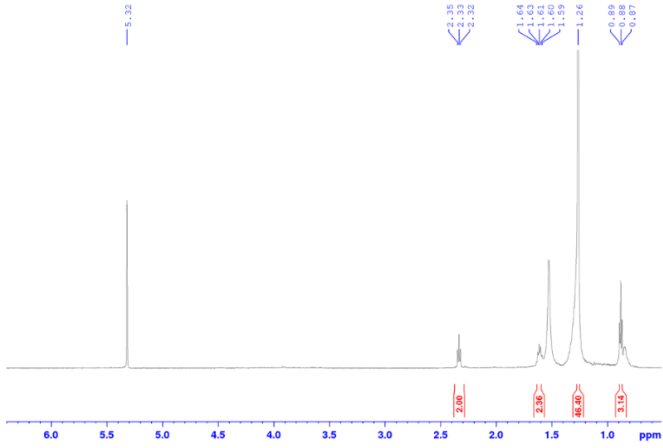

Figure S67: ^{1}H NMR (CD_2Cl_2, 600 MHz) of compound **G4** (hexacosanoic acid)

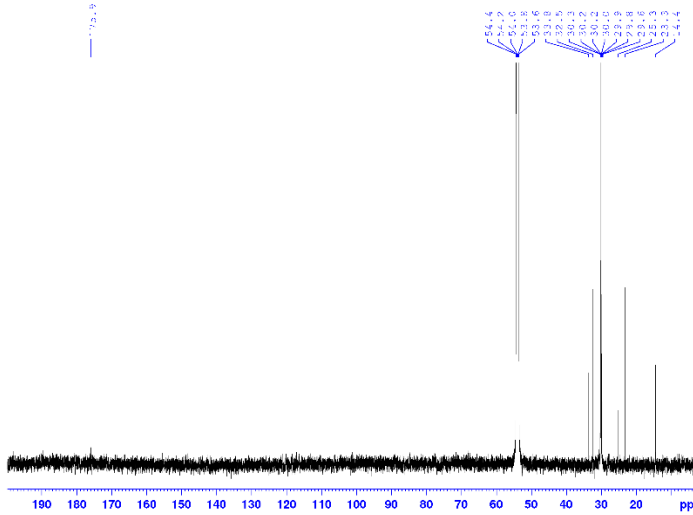

Figure S68*:* ^{13}C NMR (CD_2Cl_2, 150 MHz) of compound **G4** (hexacosanoic acid)

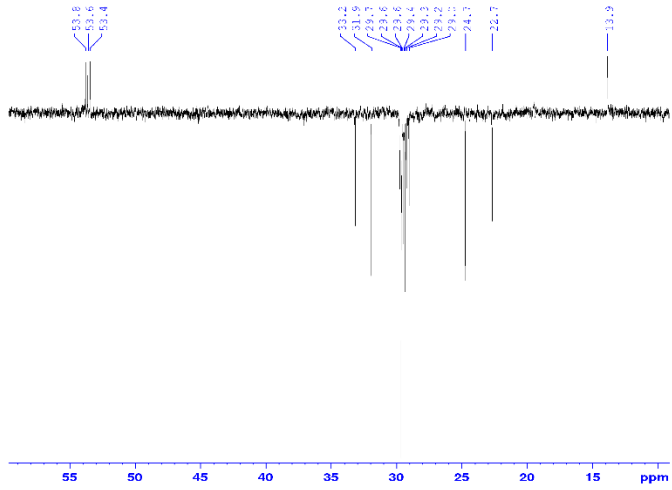

Figure S69: DEPT 135 NMR (CD_2Cl_2, 150 MHz) of compound **G4** (hexacosanoic acid)

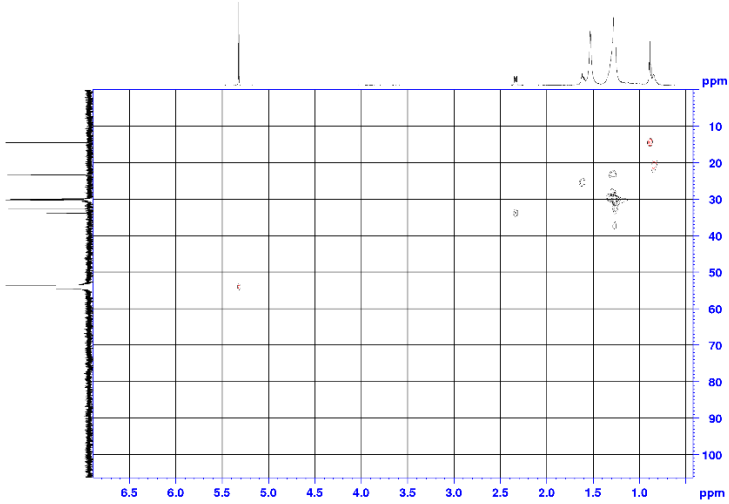

Figure S70: HSQC NMR spectrum of compound **G4** (hexacosanoic acid)

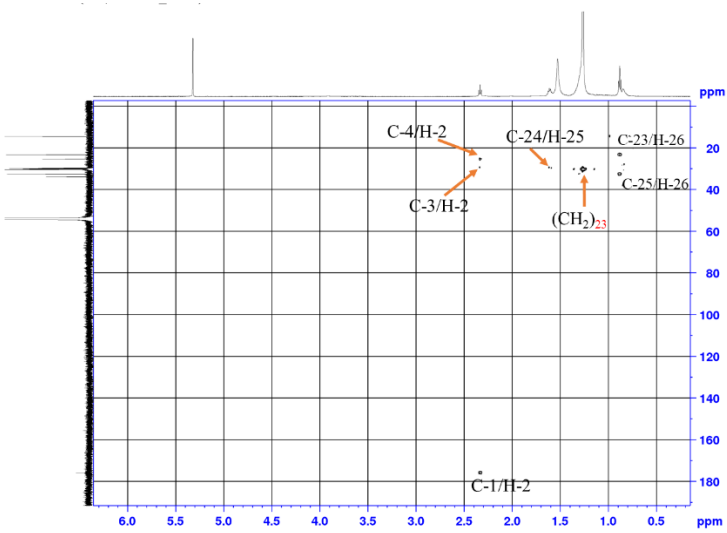

Figure S71: HMBC NMR spectrum of compound **G4** (hexacosanoic acid)

Kuhusu Tasnifu Hii

Mmeadawa wa mvunjakongwa umekuwa ukitumika kutibu magonjwa yanayosababishwa na bakteria na virusi katika sehemu mbali mbali za Tanzania. Utafiti huu ulijikita katika kudukua na kutenganisha kemikali muhimu ambazo zinaaminika kuwa ndizo huvifanya viduo vya mmea huu kuwa na uwezo wa kutibu magonjwa mbali mbali.

Utafiti huu amewezesha kupatikana kwa kemikali mpya 3',4'-di-*O*-methylellagic acid-4-α-*L*-rhamnopyranoside pamoja na nyingine kumi na nne (14). katika makundi ya *Phenolics, triterpenoids, steroids, long chain hydrocarbons, long chain amine na Fatty acids* yalitenganishwa. Aidha kemikali nyingine kama vile *3,4,3'-tri-O-methylellagic acid, hemicosanyl ferulate, octacosyl ferulate na Lupeol* zimepatikana kwa mara ya kwanza katika mmea huu wa mvunjakongwa. Inapendekezwa kemikali hii mpya ya *3',4'-di-O-methylellagic acid-4 α, L-rhamnopyranoside* kutumika kama kemikali rejea kwenye bidhaa zitokanazo na mizizi ya mvunjakongwa. Majaribio ya tabia za sumu za kemikali hizi kwa kutumia lava za *Artemia salina* yalionyesha kuwa kemikali hizi ni salama hadi kiwango cha juu kilichojaribiwa (2400 µg/ mL). Matokeo ya utafiti huu yamebaini kuwa umri wa mmea-dawa, sehemu za mmea na mazingira ya kijiografia yanaathiri idadi, kiwango na aina ya kemikali kwa kiwango kikubwa ($p < 0.0001$). Matumizi ya viziduo vya mmea huu hayanabudi kutumika kwa tahadhali na kuzingatia viwango hivi kwa ajili ya usalama wa afya ya mlaji.