BIOACTIVITY POTENTIAL OF EXTRACTS FROM *SYNADENIUM GLAUCESCENS* PAX (EUPHORBIACEAE)

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FAITH PHILEMON MABIKI

FOR REFERENCE ONLY

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

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EXTENDED ABSTRACT

The bioactivity of *Synadenium glaucescens* (Pax) extracts was studied in order to advance the knowledge that would enhance the utilization and commercialization of the plant. Participatory and questionnaire survey methods were used to establish ethnobotanical uses of the plant. Extracts from leaves, roots and stem samples were obtained using cold and hot extraction techniques. Brine shrimp test was involved for cytotoxicity studies. Using an *in ovo* method, extracts were tested against three viruses of veterinary importance. The agar well diffusion method and minimum inhibitory concentration were used to determine antibacterial and antifungal activity. Soxhlet extraction technique was used for optimization studies and GC-MS and HPLC for phytochemical screening.

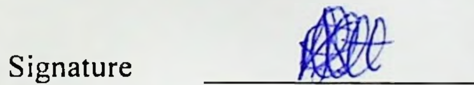
A total of 220 respondents were interviewed and majorities (94%) were aware of ethnomedical value of *S. glaucescens.* Twenty six uses were documented. Leaves and roots were the parts mostly used for ethnomedical purposes and grandparents were responsible for knowledge transfer. The cytoxicity test indicated LC_{50} values less than 30µg/ml for all extracts. More than 50% of the extracts prevented deaths, deformation and formation of pox lesions in embryos challenged with Infectious bursal disease virus and Fowl Pox virus at 0.2 mg/ml, without affecting the host cells. The extracts inhibited multiplication of Newcastle Disease virus at lowest concentration of 0.1 mg/ml. Treatment with ethanolic extracts from the root bark resulted into higher antiviral activity against the three viral particles. Extracts from hot extraction showed higher antibacterial and antifungal activity compared to the extracts from cold extraction. Gram positive bacteria were more sensitive to extracts

than the Gram negative bacteria. *Streptococcus pyogenes* and *Candida albicans* were the most sensitive bacteria and fungus respectively. Ethanol extracts demonstrated higher antibacterial and antifungal activity than other solvent extracts. Higher extraction yields were obtained within 4 hours of extraction at 30°C for dichloromethane and 75°C for ethanol and particles size of 1 mm. Dichloromethane and ethanolic extracts were composed of triterpenoids and polyphenolic compounds respectively.

These findings demonstrate the potential and the feasibility of using *S. glaucescens* extracts for treatment of viral, bacterial and fungal diseases. Furthermore, it validates the ethnobotanical uses at community level.

DECLARATION

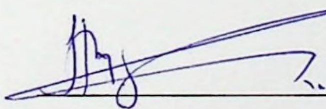
I, FAITH PHILEMON MABIKI, do hereby declare to the Senate of Sokoine University of Agriculture that, this thesis is my own original work, done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

Signature  08/11/2013

Faith Philemon Mabiki Date

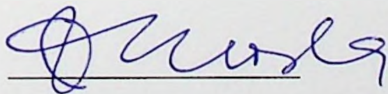
(PhD Candidate)

The above declaration is confirmed:

 11-11-2013

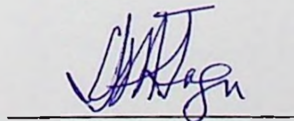
Prof. R. H. Mdegela Date

(Supervisor)

 11/11/2013

Prof. R. D. Mosha Date

(Supervisor)

 09-11-2013

Dr. J. J. Magadula Date

(Supervisor)

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Last but not the least my mother Elestina Mabiki, my husband Omath, my sons Jair, Jean-Abdon, Jed and my Brothers Albert and Baraka, sisters; Leah, Ellyjalia and Clara for your encouragement and prayers throughout the study period.

DEDICATION

This work is dedicated to my late father Mr. Philemon G. Mabiki, my mother Elestina L. Mwipopo and my beloved husband Omath S. Sanga, my sons Jair, Jean-Abdon and Jed.

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PAPER II: *In Ovo* antiviral activity of *S. glaucescens* (Pax) crude extracts on Newcastle disease virus. **Mabiki,** F. P[1,2]., Mdegela, R.H.[2], Mosha, R.D.[2] and Magadula, J.J.[3]. Published in the *Journal of Medicinal Plant Research 7 (14), 2013 863-870.*

PAPER III: Antiviral activity of crude extracts of *S. glaucescens* (Pax) against infectious bursal disease and Fowlpox virus. **Mabiki,** F. P[1,2]., Mdegela, R.H.[2], Mosha, R.D.[2] and Magadula, J.J.[3]. Published in the *Journal of Medicinal Plant Research 7(14), 2013: 871-876.*

PAPER IV: Bioactive crude extracts of *S. glaucescens* (Pax) against selected bacteria and fungi of health importance **Mabiki,** F. P[1,2]., Mdegela, R.H.[2], Mosha, R.D.[2] and Magadula, J.J.[3] Manuscript intended for submission to the *Journal of Medicinal Plant Research 2013.*

PAPER V: Optimization of extraction conditions and phytochemical screening of root extracts of *Synadenium glaucescens* Pax. **Mabiki,** F. P[1,2]., Magadula, J.J. [3], Mdegela, R.H.[2], Mosha, R.D.[2] and. Published in the *Journal of International Chemistry* 5 (4), 2013: 103-112.

[1]Faculty of Science, Sokoine University of Agriculture.

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture.

[3]Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences.

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DECLARATION OF THE PAPERS

I, **Faith Philemon Mabiki**, do hereby declare to the Senate of Sokoine University of Agriculture that the listed papers and supplements listed above that makes this thesis, summarises my independent efforts. It is my original work and will not be part of another thesis in published papers format in any other University.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
DMSO	Dimethyl Sulphoxide
EtOH	Ethanol
GC-MS	Gas Chromatography – Mass Spectrum
HPLC	High performance liquid Chromatography
INT	Iodonitrotetrazolium violet
ITM	Institute of Traditional Medicine
MHA	Muller-Hilton agar
MIC	Minimum Inhibitory concentration
MUHAS	Muhimbili University of Health and Allied Sciences
SDA	Saborouds dextrose agar
SUA	Sokoine University of Agriculture
TM	Traditional Medicine
WHO	World Health Organization

CHAPER ONE

1.0 INTRODUCTION

Natural products are chemical compounds produced by living organisms that exert a biological effect on others (Colegate and Molyneux, 1993). These compounds are known as secondary metabolites and are usually small molecules unique to a particular species or group of organisms. Natural products often have an ecological role in regulating the interactions between plants, microorganisms, insects and animals. They can be defensive substances, antifeedants, attractants and pheromones. Natural products are diverse and found in all types of environment ranging from aquatic to terrestrial. They are sourced from microorganism, animal and plants. In such high biodiversity pool, only about 10% have been studied for their natural chemical diversity and bioactivity (Cragg and Newman, 2005). It has been estimated that, of the world biodiversity of about 250,000 species of higher plant, only about 6-15% of them have either been investigated for bioactive compounds or phytochemicaly studied (Farnsworth, 1988; Balandrin *et al.,* 1993; Fabricant and Farnsworth, 2001). Marine organisms have provided over 3,000 new compounds over the past thirty years which is less compared to the available potential (da Rocha *et al.,* 2001; Mayer *et al.,* 2010). It is estimated that microbial world encompasses secondary metabolites close to 50,000 known compounds with diverse array of chemical structures (Gunatilaka and Wijeratne, 2011).

Humans have always relied on natural products and have continued to explore their application potential in various aspects in life. Natural products have provided man with useful substances such as medicines, flavouring agents, cosmetics and pest

control agents. The use of extracts from tobacco (*Nicotiana tabacum*) and pyrethrum flowers (*Chrysanthemum cinerarifolium*) was a breakthrough in controlling pests in agricultural products from production to market as well as insects of human and animal health importance. Isolation of pyrethrin, the insecticidal constituents of pyrethrum flowers led to synthesis of their analogues such as bioresmethrin, cypermethrin and deltamethrine which are used as active ingredients in many pesticides. In Tanzania for instance, over 50% of insecticides in the market contain synthetic pyrethrins (TPRI, 2013). Other plant species mostly used include *Tephrosia vogelii, Tagetes minuta, Azadirachta indica, Nicotiana tabacum, Neorautanenia mitis* and most of the *Euphorbia* species.

1.1 Natural products as medicine

The use of natural products as medicines has been described throughout the history of man in the form of traditional medicines, remedies, potions and oils with many of their bioactive natural products still being unidentified. In spite of the advent of the modern high throughput drug discovery and screening techniques, traditional knowledge of medicinal plants has always guided the search for new cures by providing clues to the discovery of valuable drugs (Tyler, 1986; Fabricant and Farnsworth, 2001). In more recent times, natural products have continued to be significant sources of drugs and leads for formulation. Their dominant role is evident in the approximately 60% of anticancer compounds and 75% of drugs for infectious diseases that are either natural products or natural product derivatives (Newman *et al.*, 2003; Crag *et al.*, 2005). In 1985, it was estimated that around 80 % of the world's population relied on medicinal plants as their primary health care source

(Farnsworth *et al.*, 1985). To date still majority of population in Africa, Asia and Latin America use traditional medicines for their primary health care needs (WHO, 2008). In industrialized countries such as USA, plant-based traditional medicines or phyto-therapeuticals are often termed complementary or alternative medicine (CAM). The use of herbal products formulations specifically products made of combinations of herbs over those based on single herbs in USA has increased steadily over the last 10 years (Blumenthal *et al.,* 2006).

In Tanzania traditional medicines depend heavily on plants and are the most common form of primary healthcare (Stangeland *et al.,* 2008). According to Tanzania Traditional Medicine Act 2002, a traditional practitioner (denoted traditional healer/herbalist) is defined as a person who is recognized by the community that he/she lives as competent to provide health care using plants, animals, minerals substances and other methods based on social, cultural and religious backgrounds as well as knowledge, altitude and beliefs that are prevalent in the community regarding physical, mental and social well being and cause of disease and disability. It is estimated that over 60% of health seeking population have a traditional healer as the first point of contact (Mhame, 2000). About 80% of Tanzania population comprising rural and urban depend on traditional medicine for their primary health care (Stangeland *et al.,* 2008). In 2000, the estimated ratio of traditional health practitioner to the human population was 1:400, while that of doctors to patients' was 1:20,000 (Mhame, 2000). Over the centuries, people have developed a wide variety of technologies for exploitation of nature and the ecosystems. Exploration of medicinal properties of plants, microorganisms, extracts of animals and marine life has been

through careful observations, trial and error, together with a vast heritage of knowledge and expertise in different ethnic cultures and civilizations. Most of such indigenous knowledge was handled down, through the ages, by oral tradition while the practices in general had to meet the need of the local communities.

1.2 Plant secondary metabolite: Classification and drug sourced

Since time immemorial, man has been sourcing natural products from plants for different purposes. During 18th to 20th centuries, a number of scientific studies were carried out to explore the vast potential of plants and other organisms as sources of useful chemical substances (Tyler, 1986). These efforts revealed a great potential of chemical substances from plants, some of which are useful in crude and others in pure form. However, the potential of plants as sources of new drugs is largely unexploited the state which prompts more searches for bioactive compounds from plants. Higher plants are vascular plants with developed and specialized tissues for conducting water, minerals, and photosynthetic products. They produce economically important organic compounds known as secondary metabolites, such as oils, resins, tannins, natural rubber, gums, waxes, dyes, flavours, fragrances, and pesticides (Hadacek, 2002). These chemicals often have an ecological role representing chemical adaptations to environmental stresses, or serve as chemical defensive agents against micro-organisms, insects and higher predators, and even other plants. These chemicals have played a role in commercial development of new drugs since are considered more environmentally friendly and safe to human. Chemists have been able to isolate, identify and synthesize the active constituent of many medicinal plants in order to study the physiological activities. Through

pharmacognosy it has been possible to identify active principles which act as lead compounds for total synthesis, semi synthesis or as template for development of new drugs with improved biomedical properties over those found naturally. Based on their biosynthetic pathways, secondary metabolites are divided into polyketides and fatty acids, terpenoids and steroids, phenylpropanoids, alkaloids, specialized carbohydrates and amino acids and peptides (Hanson, 2003).

1.2.1 Polyketides and fatty acids

Polyketides are natural products that are formed by the stepwise condensation of acetate (ethanoate) units. Fatty acids are also biosynthesised through a similar process. Polyketides are an important group of natural products as they results to bioactive compound of medical importance to human (Staunton and Weissman, 2001). These compounds include most of the oxygenated compounds such as phenolic compounds: flavonoids, phenolic acids, polyphenols, stilbenes, tannins, coumarins, lignans and lignins (Surveswaran *et al.,* 2006). Phenolic compounds play an important role in preventing human cells and the organs from damage of unsaturated fatty acids by lipid peroxides and by absorbing and neutralizing free radicals (Simic *et al.,* 2007). Fatty acids mainly unsaturated are present in plants and animal fats. The most fatty acids available in plants are unsaturated fatty acids which in different forms are used for medicinal purposes. The most common are the linolenic acid derivatives such as α-linolenates, for example α linoleic and eicosapentaeinoic acids, which are referred as ‘essential fatty acids’ (EFAs). Since these fatty acids all have a double bond three carbons from the methyl end of the chain, they are grouped together under the term ω-3 fatty acids (omega- 3 fatty

acids). These acids are responsible for reduction of many human disorders and prevention of diseases such as heart attack and atherosclerosis (Dewick, 2002).

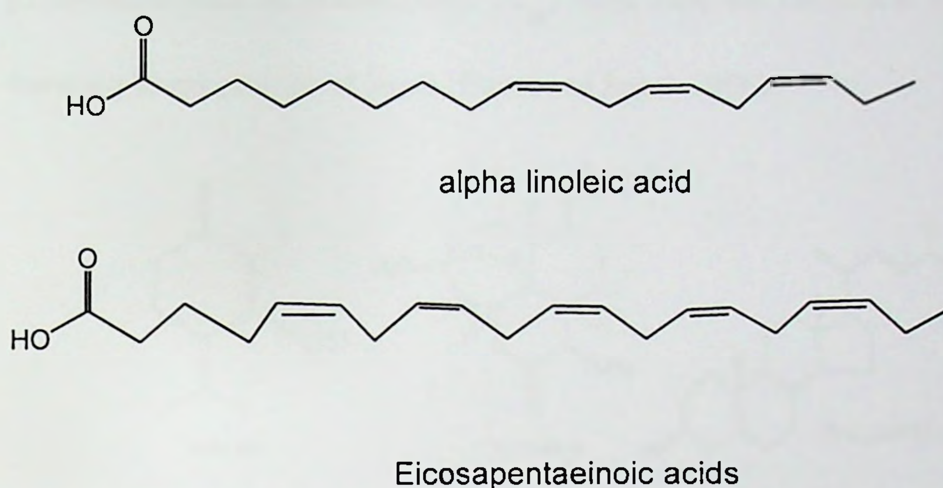


Figure 1: Some omega–3 fatty acids

1.2.2 Terpenoids and steroids

Terpenoids and steroids are derived biosynthetically from isopentenyl diphosphate. Terpenoids have an immense variety of unrelated structures occurring as sesquiterpenoids (C_{15}), diterpenoids (C_{20}), triterpenoids (C_{30}) and tetraterpenoids (C_{40}). Steroids have a common tetracyclic carbon skeleton resulting from modified terpenoids biosynthesized from the triterpene lanosterol. Menthol is a monoterpenoid found in the essential oil of the field mint, *Mentha arvensis,* and possesses useful physiological properties including local anaesthetic and refreshing effects. It is used to flavour sweets, tobacco and toothpaste. Artemisinin obtained from *Artemisia annua* are sesquiterpenoid lactones which have recently been recommended by the WHO for treatment of resistant strains of malaria. This compound contains unusual peroxide which is associated with its biological activity. Taxol (or paclitaxel),

originally obtained from the bark of the Pacific yew, *Taxus brevifolia* is a diterpenoids possessing antitumour activity. Steroids from plants known as phytosterols such as brassicasterol (C_{28}) from rape oil (*Brassica rapa*) helps in lowering blood cholesterol levels (Pascal and Segal, 2006).

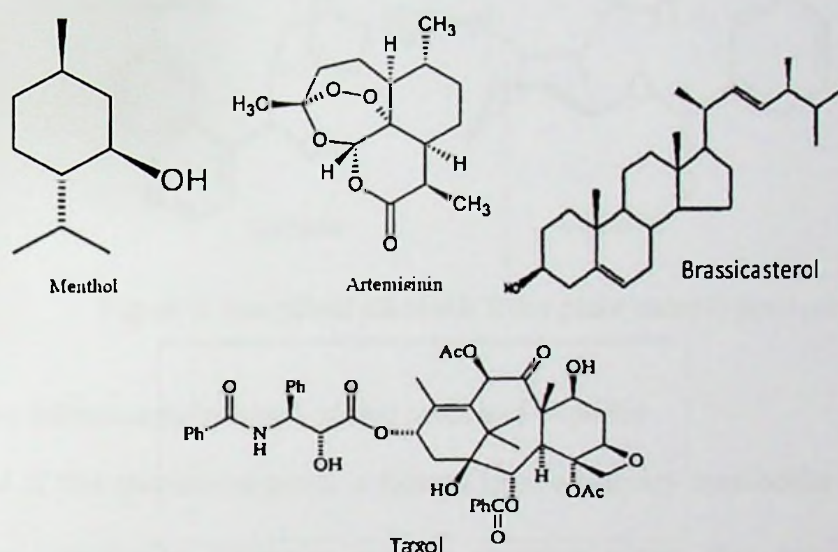


Figure 2: Medicinal terpenoids from plants natural products

1.2.3 Alkaloids

Alkaloids are derived biosynthetically from amino acids. Some of the first natural products to be isolated from medicinal plants were alkaloids. Since many of the initially discovered alkaloids originated from plants, early definitions of an alkaloid included these three characteristics (nitrogen-containing, basicity, and plant origin) (Cordell *et al.*, 2001). Many alkaloids have neuroactive properties and interact with the receptors at nerve endings. Common known alkaloid containing plants include the coca plant, *Erythroxylon coca.* The plant produces cocaine which has a paralysing effect on sensory nerve endings and produces a sense of excitement.

Cinchona ledgerianu bark reserves quinine the alkaloid containing the quinoline ring system. It is used as an antimalarial drug and as a bitter substance in tonic waters (Hanson, 2003).

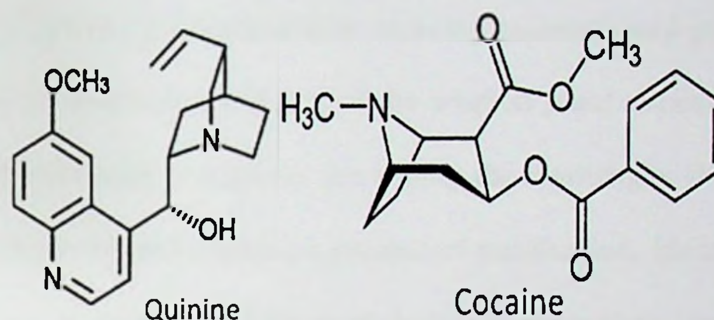


Figure 3: Medicinal alkaloids from plant natural products

1.2.4 Specialized carbohydrates, amino acids and peptides

Compound of this specialized group is formed from a primary metabolite attached to a secondary metabolite. Examples are less common sugars that are attached to natural products as part of a glycoside. The non-sugar portion is known as the aglycone, and it may be a terpenoid, alkaloid or polyketide. For instance, antibiotics such as the penicillin are formed from small peptides and the nitrogenous portion of the alkaloids are derived from amino acids such as lysine and tyrosine (Hanson, 2003)

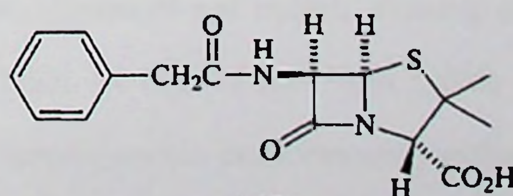


Figure 4: Structure of Penicillin

1.3 The perception that limits interest in natural products from plants

Despite the opportunity that the natural products (especially the plant derived products) have in pharmaceutical industry, several challenges have been experienced towards their development. Difficulties with sourcing authenticated plant materials due to inadequate documentation and loss of the original plant collections together with lack of simple bioassay procedures are among the challenges (McChesney *et al.*, 2007). The complexity and expensive process of purification, identification and structure elucidation of natural products contributed to the wrong perspective that natural products were a difficulty drug source (Tyler, 1986; McChesney *et al.*, 2007). These challenges have been addressed by the recent advance in capability and sophisticated technology in bioassays and analytical techniques (Butler, 2004; Dias *et al.*, 2012). Concern over the availability of large quantity of a chemical entity required for development and market needs, is the most limiting factor in pharmaceutical industries with interest in natural products (McChesney *et al.*, 2007). Secondary metabolites are found in low quantities in plants and usually isolated in low amounts. Plant-cell culture techniques and semi and total synthetic approaches have been employed to meet the demand challenge (Tyler, 1986). Application of developed production system incorporating agronomic systems for biomass production ensures the sustainable and reliable sourcing of bioactive compounds (McChesney *et al.*, 2007). In view of that Khan (2006) recommended that the mechanization of the harvest process, extraction and purification systems should be economical.

1.4 Toxicity and safety of plant crude drugs

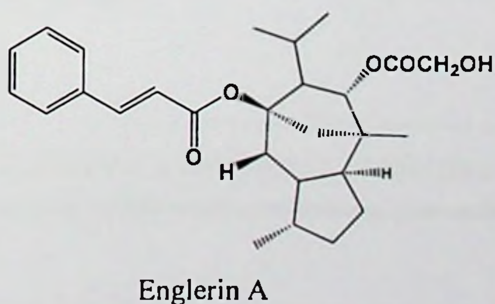
The reliance on plants as source of medicines warrants scientific validation of their safety, efficacy, quality and the appropriate dosage of the plant material. With the increasing acceptance of herbal medicine as an alternative form of health care, the screening of the medicinal plants for active compounds is increasingly becoming important (Shai *et al.*, 2008). Despite the difficulties in collection of traditional medicinal plant due to their seasonal unavailability, the possibility of infective or harmful treatments, uncertain dosages and lack of standardisation have been the major drawbacks of the traditional plant remedies (McGau *et al.*, 2005). Plants commonly used in traditional medicine are perceived to be safe. The safety is based on their long usage in the treatment of diseases according to the knowledge accumulated over century's (Fennel *et al.*, 2004). However, recent scientific research has shown that many plants used as food or in traditional medicine are potentially toxic, mutagenic and carcinogenic (Fennel *et al.*, 2004). Furthermore, it is well known that poisoning from traditional medicines is usually a consequence of misidentification, incorrect preparation or inappropriate administration and dosage (Stewart and Steenkamp, 2000); frequently due to self-administration (Popat *et al.*, 2001) rather than innate risks of using traditional healthcare. Traditional healers possess considerable knowledge of medicinal plants and how to avoid acute poisoning. Thus, toxicity of plants should not be a limiting factor for ethnomedical and ethnoverterinary utilization but rather a stepping stone towards further studies on proper identification, safer formulations, dose standardization and searching for diversity of uses of species.

1.5 Comparison between conventional and traditional drug discovery approach

Ethnomedical search presents the most promising way in selecting the candidates for drug discovery programs. This is done through conducting ethnobotanical surveys, use of databases such as NAPRALERT, following up information of plants used in organized traditional medical systems such as herbalism, folklore and shamanism, field work, notes placed on voucher herbarium specimens by the botanist at the time of collection and information from books and reviews on medical botany and herbs (Fabricant and Farnsworth, 2001). A study by Farnsworth (1988) indicates that of 119 known useful plant-derived drugs, 74% of the chemical compounds used as drugs have the same or related use as the plants from which they were derived. Another study identified 122 compounds from only 94 species of plants, and of these 80% were used for the same (or related) ethnomedical purposes (WHO, 1985). Other approaches taken in plant drug discovery include; random selection followed by chemical screening, random selection followed by one or more biological assays and follow-up of biological activity reports (Fabricant and Farnsworth, 2001).

The conventional drug discovery approaches are well known and have been blamed of being time consuming and expensive. An average of 12 years and cost of between $400 – 650 millions is needed for new drug to penetrate the market from the laboratory using the conventional approach. This has lead to many drugs not reaching the market (Gupta *et al.,* 2012). For example, in paclitaxel (Taxol); its chemical structure was reported and identified as the cytotoxic active constituent of extracts of *Taxus brevifolia* in 1971. It was approved for marketing as a cancer chemotherapeutic agent at the end of 1992, 20 years later. Similarly, several

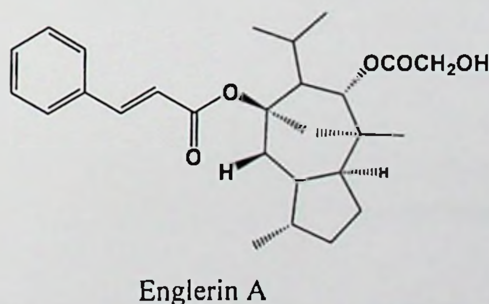
bioactive compounds have been isolated between 1988 and 2008 by natural products chemists in Tanzania. Although these compounds have shown interesting anticancer, antimalarial, antileishmanial, antimicrobial and Anti-HIV bioactivity (Magadula and Erasto 2009), they have not been exploited for commercialization. The most interesting was the elucidation of a novel guaiane sesquiterpenes Englerin A from the stem bark of *Phyllanthus engleri* (Euphorbiaceae) which exhibited 1000-fold selectivity relative to taxol against six renal cancer cell lines (Ratnayake *et al.*, 2009). So far none of the isolated compounds have penetrated the market. The time frame and cost call for initiative in reducing the time and improve efficiency of the drug discovery process. On the other hand the situation is prompting for used crude drugs in most of the developing countries like Tanzania to reach the market. Recognizing the potential of each approach the model that combines both approaches was adopted while setting the structure of the current work (Figure 6).



Englerin A

Figure 5: A guaiane sesquiterpenes from *Phyllanthus engleri*

bioactive compounds have been isolated between 1988 and 2008 by natural products chemists in Tanzania. Although these compounds have shown interesting anticancer, antimalarial, antileishmanial, antimicrobial and Anti-HIV bioactivity (Magadula and Erasto 2009), they have not been exploited for commercialization. The most interesting was the elucidation of a novel guaiane sesquiterpenes Englerin A from the stem bark of *Phyllanthus engleri* (Euphorbiaceae) which exhibited 1000-fold selectivity relative to taxol against six renal cancer cell lines (Ratnayake *et al.*, 2009). So far none of the isolated compounds have penetrated the market. The time frame and cost call for initiative in reducing the time and improve efficiency of the drug discovery process. On the other hand the situation is prompting for used crude drugs in most of the developing countries like Tanzania to reach the market. Recognizing the potential of each approach the model that combines both approaches was adopted while setting the structure of the current work (Figure 6).



Englerin A

Figure 5: A guaiane sesquiterpenes from *Phyllanthus engleri*

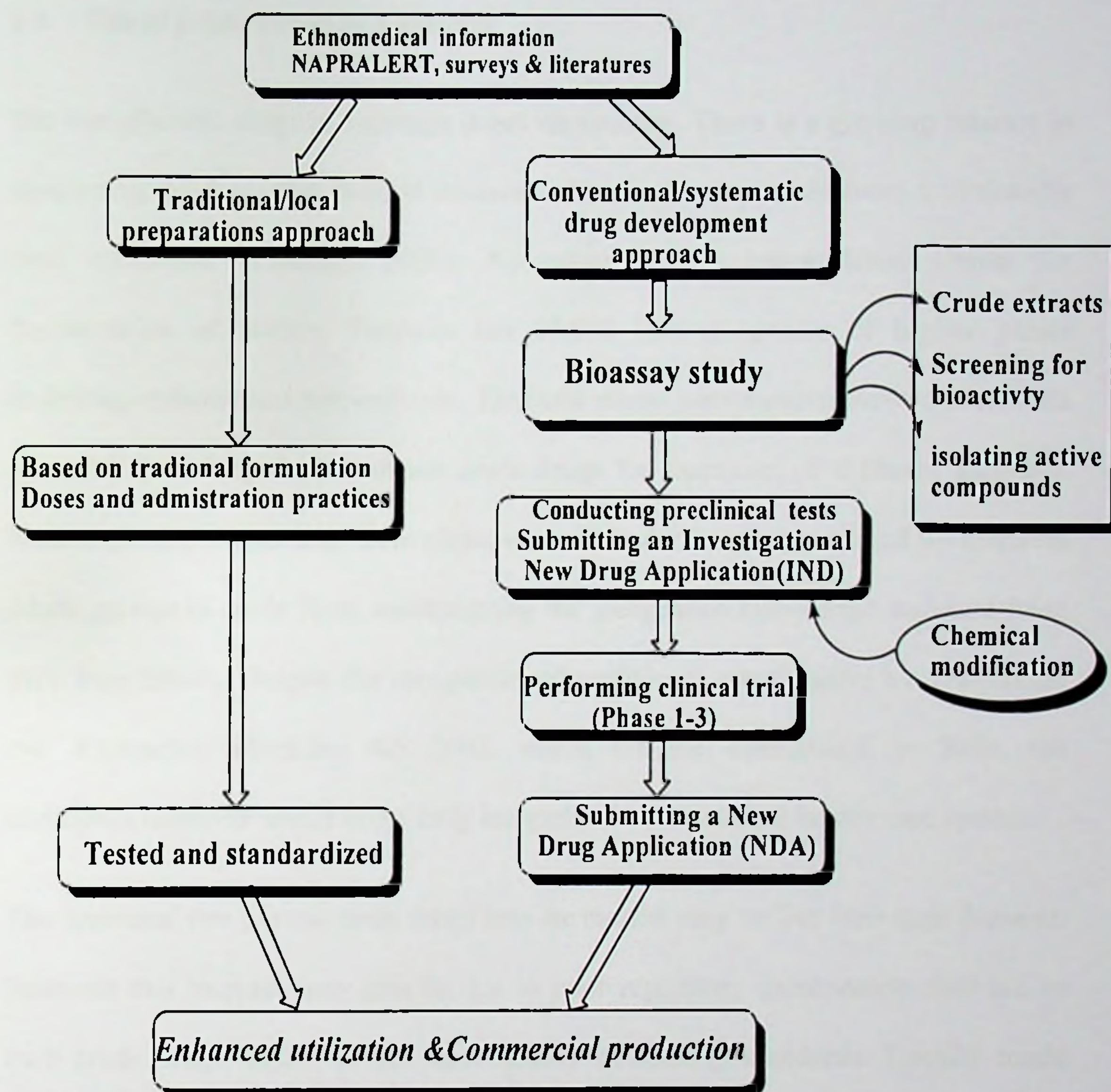


Figure 6: Drug discovery approaches adopted during the study[1]

[1] NAPRALERT is an acronym for Natural Products Alert Data base

1.6 Use of crude drugs in Tanzania

The use of crude drugs in Tanzania is not uncommon. There is a growing interest in developing products that contain mixtures of natural compounds from traditionally used medicines (Charlish, 2008). According to the International Union for Conservation of Nature, Tanzania has 10,008 known species of higher plants including endemic and non endemic. Tanzania ethnic communities have utilized this potential for a long time to obtain crude drugs for treatment of different ailments. Natural products especially from plants are well understood and utilized by different ethnic groups in crude form, incorporating the indigenous knowledge acquired from their fore fathers. Despite the recognition of traditional practitioners by Traditional and Alternative Medicine Act 2002, which became operational in 2005, the traditional medicine sector is not fully integrated in the national health care system.

The increased rate of new crude drugs into the market may reflect their high demand. However this increase may also be due to poor regulatory mechanisms that fail to curb crude drugs which do not meet quality and safety standards. Locally made traditional formulations are existing in the market in very crude and affordable price. These include detergents, soaps shampoo, antimalarial drugs and drugs which control opportunistic infections especially for people affected with HIV/AIDS. This traditional approach is characterized by use of traditional experiences during preparation and dosage. These may or may not pass through the clinical trial before entering into the market. The responsible parts for preparation of traditional product include herbal clinics, herbal companies and individual herbalists. Such individual herbalists include the Maasai who prepare and sell these products in street where the

prescription depend on the explanation during the purchasing. Herbal clinics follow a normal hospital routine, laboratory tests and prescribe their herbal formulation. Herbal clinics which operate throughout the country, not only prepare the crude drugs, but also move a step further by providing free public lessons on how to use the herbs as food supplements, for personal control and treatment of ailments. Some formulated crude drugs are indicated in table 1. The responsible bioactive herbs are always a secret especially when they have to enter the market. Some of these preparations have gained international market such as Ngetwa 3 and fiterawa which are also used in European countries.

Table 1: Some herbal formulations found in the Tanzanian market

Trade name ®	Purpose	Formulating agent
Ngentwa 3	Malaria and diabetes	Ngetwa3 herbal medicine, Mbeya
Fiterawa	Treatment of ulcers	Rahabu Ulcers Clinic Centre, Dar Es Salaam
Antipa SOSOA	Antimalarial, typhoid treatment	Antipa Natural therapies, Dar Es Salaam
Ngoka 11	Antimalarial and antiulcer	Ngoka Herbal Products Co. Ltd., Dar Es Salaam
Morizela Juice	Diabetes and nutritional supplement	Institute of traditional medicine (ITM), MUHAS, Dar Es Salaam
Ravo cream	Skin fungal infections	
Prucan capsules	Benign prostate hypertrophy	
Pumu syrup	Asthmatic condition	
TMS 2001	Malaria treatment	National Institute for Medical Research (NIMR), Dar Es Salaam
Warburgistat	Treatment of opportunistic infections	
Persivin	Treatment of Prostate hypertrophy in men	
Persican	Treatment of diabetes	
Mundex	Treatment of Erectile dysfunction in men	
NIMREX	Herbal chest, cold and cough remedy	
NIMREVIT	Health drink	
Fukuza Mbu	Herbal mosquito repellent	
Kiluwicide	Mosquito Larvicide	
Liwa herbal soap; Eden garden hair jelly; food jelly; Pen herbal soap; Fruit herbal soap; Woman wash herbal soap; Mwali herbal soap; Alovala herbal soap; Habalt soda herbal soap	Skin care, sanitation and antiseptic, antifungal	Ivan products herbal cosmetic making, Mbeya
Natural herbal soap; herbal soap; Neem herbal; Moringa soap; Baobab herbal soap; Aloe Vera herbal soap; Turmalic herbal soap	Skin care, sanitation and antiseptic, antifungal	Seaweed centre women today, Zanzibar
Mwarobaini Soap (Neem tree soap)	Skin care, sanitation and antiseptic, antifungal	University of Dar es Salaam, Dar Es Salaam

1.7 Common medicinal plant families used in Tanzania

Several studies have been done to document traditional uses and treatments of medicinal plants in Tanzania (Kitula, 2007; Maregesi *et al.*, 2007; Augustino *et al.*, 2011; Amri and Kisangau, 2012; Moshi *et al.* 2012). The most common families used for crude drug preparation include Fabaceae (Papilionaceae), Euphorbiaceae, Verbenaceae, Compositae (Asteraceae), Combretaceae, Annonaceae, Asteraceae and Solanaceae.

1.8 The family Euphorbiaceae

Synadenium glaucescens which focused in this study belongs to the family euphorbiaceae. The family euphorbiaceae is among the largest families of flowering plants composed of over 300 genera and 8,000 species. The family is heterogeneous and complex, distributed all over the world with all sorts of plants ranging from large woody trees through climbing lianas to simple weeds that grow prostrate to the ground (Bruyn *et al.*, 2006). Euphorbiaceous species have developed a wide range of adaptation capabilities, due to their exposure under different stimuli that lead to production of a diverse range of secondary metabolites (Mwine and van Damme, 2011). The fact that they produce diverse secondary metabolites have made the family of pharmaceutical importance and therefore, a good starting point for a search of phytomedicine of human and veterinary importance. Plant species of the family euphorbiaceae are reported to contain a number of interesting biologically active compounds. Furthermore, a number of Euphorbiaceae species have shown to contain alkaloids, anthraquinones, diterpenoids, triterpenoids, phenols, flavonoids, saponins,

tannins, essential oils, esters, epoxides and fatty acids (Mwine and van Damme 2011). These compounds have demonstrated diverse medicinal properties as anti-bacterial, antifungal, antiprotozoa and pesticidal. Others contained irritant or poisonous properties, antitumour as well as cytotoxic active constituents (Ahmad *et al.*, 2006; Spiridon and Kintzios *et al.*, 2006). Euphorbiaceae species are also well known to contain tumor promoting constituents (Vogg *et al.*, 1999). Between 1998 and 2008, eight (8) US patents were granted for medicinal Euphorbiaceae extracts (Mwine and van Damme 2011). In 2010 two (2) Tanzania patents were granted to Sokoine University of Agriculture for the antiviral and antibacterial activity of the euphorbiaceae species (*Synadenium glaucescens* Pax) extracts.

1.8.1 The genus *Synadenium:* Ethnobotanical and ethnomedical uses

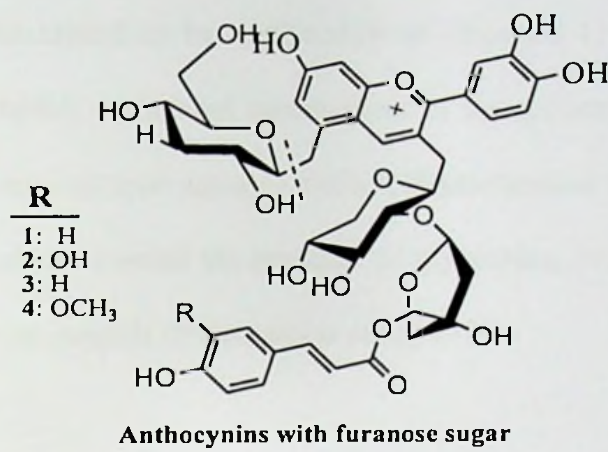
The genus *Synadenium* is small consisting about 18 species which are distributed in tropical and subtropical regions of Africa and the Americans, as shrubs or trees. The species within the genus produce toxic sap like many of the members of the family Euphorbiaceae (Neuwinger, 2004). Despite production of toxic sap, the species are used by different ethnic groups as medicinal plants in different parts of world (Dey and De, 2011; Mwine and van Damme, 2011). *Synadenium umbellatum* is a medicinal plant used to treat several diseases in Midwest Brazil (Nogueira *et al.*, 2008). Ethnoveterinary use of *Synadenium compactum* is reported among the Kikuyu of Kenya, and is the most utilised plant species in traditional management of East Coast fever (ECF) in cattle by the tribe. Treatment involves direct application of plant extracts especially latex on the lymph nodes or the plant liquid preparations that are given orally to cattle (Njoroge *et al.*, 2006). *Synadenium grantii* (African milk

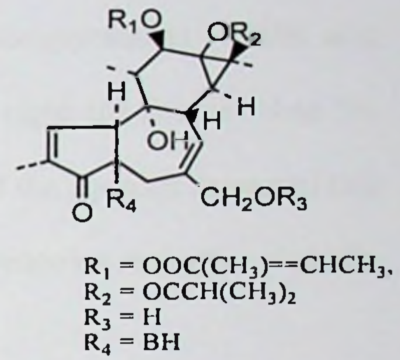
bush), is used in ethnomedical treatment of wound and as antitheilerial drug (Kinabo *et al.*, 2002; Premaratna *et al.*, 1981). *Synadenium pereskiifolium* is also used in the preparation of an anti- asthmatic drug regimen by traditional doctors in Kenya (Hermansson et al., 1991). *Synadenium volkensii* is used for treatment of Newcastle diseases in chickens and ECF in cattle in northern Tanzania (Minja, 1994). *Synadenium glaucescens* is used by different ethnic group in Tanzania for human and animal treatments (Schmelzer *et al.*, 2008). However the plant lacks documentation, scientific validated doses and formulation. This study was conceived to cover this gap.

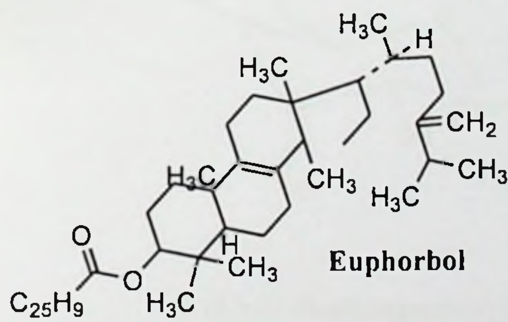
1.8.2 The genus *Synadenium:* Phytochemistry and Bioactivity

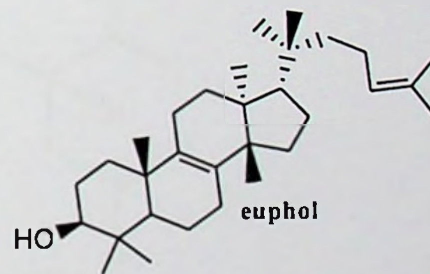
Phytochemical studies of the members of genus *Synadenium* are still limited. Several tigliane, senadenol, phorbol type diterpenoids, triterpenoids and anthocyanins have been isolated (Costa *et al.*, 2012). Extensive studies have been done on the popular ornamental species such as *S.grantii*. Phytochemical screening of different parts of the *S. grantii* has indicated dominance of the phorbol type diterpenoids in the latex showing a range of biological activities. Recently, studies on the latex and barks of the same species have lead to a novel compound found to be responsible for skin irritation, with the anti ulcer activity using rat model (Costa *et al.*, 2012). In another study, two new Phorbol-type diterpene esters (3, 4 , 12, 13 - tetraacetylphorbol-20-phenylacetate and 4-deoxyphorbol-12,13 -ditiglate) were isolated from the dichloromethane extract of the leaves from the same species. The extracts had marginal cytotoxicity on *Trypanosoma brucei* and *Plasmodium falciparum* but showed high potency against *Trypanosoma cruzi*. Another study reported the

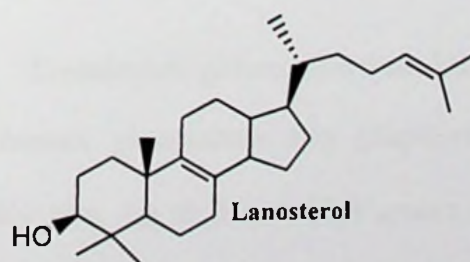
isolation of Anthocynins with furanose sugar from leaves of same species. However, none of the compounds were assessed for biological activity (Andersen *et al.*, 2010). Earlier, several other compounds namely euphorbol, lanosterol, euphols and tirucallol were isolated from the same species but no bioactivity test was done on them. Phytochemical results showed presence of tannins, terpenes, unsaponificable substances, coumarins and anthraquinones in the crude bark extract (Costa *et al.*, 2012). The Brazilian plant *S. umbellatum*, has been studied and various fractions of crude plant extract indicated to possess antitumoral and anti angiogenic activities (Nogueira *et al.*, 2008). A d-galactose-binding lectin isolated from the latex of *S. carinatum* is used to treat a number of inflammatory disorders, since it has anti-asthmatic and immunoregulatory activity (Rogerio *et al.*, 2007).











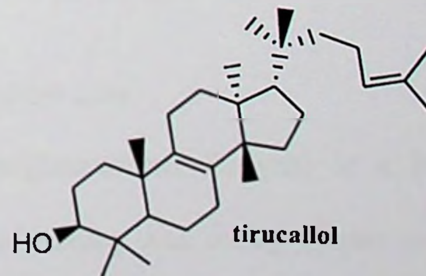


Figure 7: Bioactive compounds from *S. grantii*

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In another study, the pharmacologically active compounds were isolated from the aqueous extracts of leaves and stems of *S. pereskiifolium*. The compound was identified to be a glycoside of structure 1: 2-O-β-D-glucopyranosyl-1-malic acid which stimulated contractions of the guinea pig ileum eight times more than the original total aqueous extract. Phytochemical screening of the aqueous extract of this plant revealed the presence of glycosides, terpenoids, flavonoids and other phenolic compounds (Hermansson *et al.*, 1991).

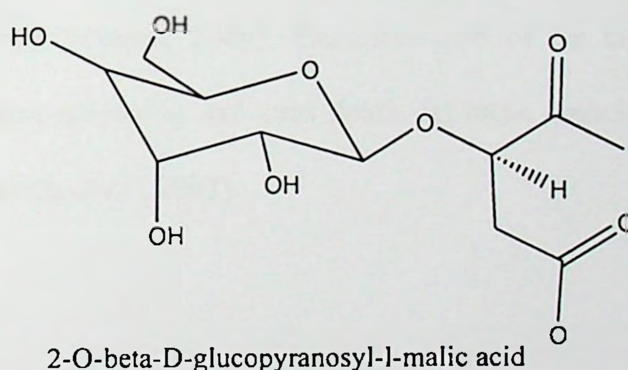

Figure 8: A glucoside from *S. pereskiifolium*

1.8.3 *Synadenium glaucescens* Pax: Ethnomedical uses

Synadenium glaucescens Pax (*Euphorbia neoglaucescens* Bruyns) is a bush or shrubby tree, up to 9 m which grows in sandy stony and rocky slopes with dry deciduous woodland, altitude 300-1800 m above sea level (Bruyn *et al.*, 2006). *Synadenium glaucescens* is known as "*Mvunjakongwa*" in kiswahili is endemic and grows in several regions in Tanzania (Mosha *et al.*, 2001). *Synadenium glaucescens* though reported to be poisonous and perceived as of no medicinal value, ethnic communities in Tanzania have used it effectively in the treatment and control of

human and animal diseases with no reported adverse effects (Mosha *et al.*, 2001). In the coastal, Morogoro and Kilimanjaro regions, the juice of fresh, crushed leaves is drunk to treat excessive menstruation and as a purgative (Chhabra *et al.*, 1984). A leaf decoction with lime juice, baking soda and honey added is drunk to treat asthma; the ashes of dried leaves are mixed with water and applied to treat leprosy (Schmelzer *et al.*, 2008). A root bark extract is taken with sugar to treat severe cough, tuberculosis and as ear drop to treat earache (Neuwinger, 2000; Newmark, 2002). In Tanga region it is used to prepare medicine for human and for control of poultry diseases mainly Newcastle disease (Wickama *et al.*, 2006). The latex is also used as a fish poison (Neuwinger, 2004). Excessive use of its concoctions for purgative purposes, cause poisoning and even death. Its latex causes irritation and can cause blindness (Mosha *et al.*, 2001).

Fig 9: Photograph of *S. glaucescens* tree

1.8.4 Phytochemistry of *S. glaucescens*

Phytochemical information of the *S. glaucescens* is limited. Phytochemical screening of the aqueous extract of the leaves of *S. glaucescens* is reported to have positive reaction for tannins, triterpenoids, coumarins and polyases while the methanol extract showed the presence of steroids, triterpenes and anthocyanins and the ether extract contained carotenoids, steroids, triterpenoids and volatile oils (Hutchings *et al.,* 1996). Chemical characterization of pharmacologically active compounds is

reported from the leaves and stems of *S. glaucescens*. Preliminary analysis of the compound revealed to be β-glycosides attached to alkyl group and was deduced as an inhibitor of contraction of guinea pig ileum. Its structural elucidation however was not concluded (Rukunga *et al.*, 1990).

1.9 Originality of the study idea

The reported study was first conceived after witnessing the treatment of child infected with tape worm using the root extract of *S. glaucescens.* This was witnessed in 2002 at a village named Idenyimembe, Njombe district in the former Iringa region which is the current Wanging'ombe district in Njombe region. After a through ethnobotanical literature search it was found that *S. glaucescens* is exploited among the Hehe and Bena communities in Njombe and Iringa region, as well as other regions in Tanzania (Schmelzer *et al.*, 2008). Despite that scientific information for *S. glaucescens* was limited and thus create a gap of knowledge.

1.10 Problem statement and Justification

Synadenium glaucescens grows in Iringa and Njombe regions (former Iringa region) where it is used by ethnic groups for treatments of diseases and for other domestic uses. The major ethnic groups found in Njombe and Iringa are Bena and Hehe respectively. The use of plants for medication among the two ethnic groups dates back to their fathers (Erdtsieck, 2003). *Synadenium glaucescens* is named *"Liyugi"* in Hehe/Bena languages. Despite its high use among the Bena and Hehe the uses are not documented. Studies by Mathias (1982) who documented some medicinal plants of the Hehe, did not list the use of *S. glaucescens*. Although recent studies by Shangali *et al.* (2008) and Kitula (2007) documented the medicinal plants of the

Hehe ethnic groups of the Udzungwa mountains but there was no mention of the use of *S. glaucescens*. This has created not only an ethnobotanical gap of knowledge, but also a gap in scientific validation of traditional uses and commercialization of products from this plant. Despite of bioassays and phytochemical studies reported Rukunga *et al.* (1990) and Hutchings *et al.* (1996) a detailed bioassay guided and phytochemical screening focusing on drug discovery and commercialization is limited. This creates a gap in phytochemical evidence of the extracts from *S. glaucescens*.

Most of the traditional treatment use crude and scientifically unproven doses. They are also associated with traditional rituals which have resulted into social problems and even deaths. Despite being neglected by natural product scholars for the reasons of being regarded to be poisonous, *S. glaucescens* has for decades been deployed to control and treat diseases within different parts in Tanzania. It is expected that, the output from this study will provide the scientific evidence on the alleged activity of this plant. It is further expected that the detailed phytochemical analyses reported in this study will contribute new knowledge in natural product chemistry as far as isolation and characterization of extracts and compounds of *S. glaucescens* are concerned. The generated knowledge will contribute to development of new drugs/formulations of natural origin specifically by enhancing the utilization and commercialization of products from *S. glaucescens*.

1.11 Objectives of the Study

1.11.1 General Objective

The main objective of this study was to advance knowledge towards utilization and commercialization of *S. glaucescens* through bioactivity studies of its extracts and pure compounds in Tanzania.

1.11.2 Specific Objectives

i. To establish the ethnobotanical importance of *S. glaucescens* growing in Njombe and Iringa region in the Southern Highlands of Tanzania.

ii. To study the antiviral potential of crude extracts from different morphological parts of *S. glaucescens.*

iii. To study the antibacterial and antifungal activity of crude extracts from different extraction techniques.

iv. To optimize the extraction conditions for higher yields of crude extracts.

1.12 Choice of Methods

1.12.1 Study area

A detail study area description is given in Paper I. To reveal the ethnobotanical importance of *S. glaucescens,* a purposive ethnobotanical survey was conducted in the Southern Highland zone of Tanzania covering one district from Iringa and another from Njombe regions (Fig. 10).

1.12.2 Ethnobotanical Survey

Indigenous knowledge plays an important role in guiding plant candidates for natural products. This is independent of the drug discovery route (Figure 6). Interviews using semi-structured questionnaires were conducted in urban and rural setups of both Njombe and Iringa region as explained in Paper I. Four focused group discussion were conducted for follow-ups targeting traditional healers, traditional believers and elderly people. Cross tabulation and basic descriptive analysis using SPSS 16.0 version were used to analyse coded data obtained during the survey.

1.12.3 Sample collection

Growth and maturation of plant tissues involve a series of complex reactions, which leads to differences in the phytochemistry of the plants (Mahmood *et al.*, 2012). In addition to that different parameters such as season, variety, stages of maturity and climatic conditions influence the phytochemical composition of a plant Gull *et al.*, 2012). Climatic factors are very important because they can interfere in the chemical biosynthesis particularly in the final concentration of bioactive compounds. With this regard the effect of geographical location was considered in order to minimize the possible error during bioactivity studies. Therefore all samples for bioactivity studies were collected from the same location thought the study and only male plants were used. The samples collected were divided into 5 parts and coded as shown in Table 1 of paper II.

1.12.4 Choice of extraction methods

Crude samples were extracted using cold and hot extraction methods depending on type of crude extract needed. Cold extraction technique is usually employed to avoid

heat decomposition for labile compounds in plants and is one of the most extensive techniques employed for the extraction and separation of chemical constituents in medicinal plant. However most of ethnomedical preparation involves heat at some stage. Local extraction methods were adopted based on the information provided during the survey and was used to prepare local crude extract (Paper II). Cold and hot sequential extraction using three solvents were done using Hexane (H) or Petroleum ether (PET), Dichloromethane and ethanol (E) respectively. For each of the extraction 15 extracts were obtained and coded as shown in Paper II and III. The yields were calculated using equation 1 (Paper V).

1.12.5 Supercritical carbon dioxide extraction

Supercritical fluid is a rapidly developing method using supercritical fluid (SCF) to produce bioactive compounds under mild conditions (Simandi *et al.*, 2002). A SCF is defined as a substance above its critical temperature (T_C) and critical pressure (P_C). The critical point represents the highest temperature and pressure at which the substance can exist as a vapour and liquid in equilibrium (Brunner 2010). The phenomenon can be easily explained with reference to the phase diagram for pure carbon dioxide (Figure 1). Supercritical fluids commonly used include supercritical carbon dioxide ($ScCO_2$) and supercritical water (ScH_2O). Supercritical carbon dioxide extraction was used for extracting the root barks and leaves of *S. glaucescens*.

1.12.6 Choice of derivatisation of crude extracts for GC-MS screening

Derivatization that prepares non-reactive derivatives of fatty acids such as the methyl esters which are also more volatile than the free acid components is

necessary before Gas Chromatography (GC) analysis. It renders the thermal stable compounds easily adsorbed in the injector and hindered functional groups easily detectable. This enhances the sensitivity for Electron Capture Detection (ECD) during the analysis. During experiments (Paper V), Silylation was used as method of derivatisation. Silylation produces trimethylsilyl derivatives that are more volatile and less stable. Silylation agents *N*-trimethylsilylimidazole (TMSI) and derivatization with Trimethylchlorosilane (TMCS) in Bistrimethylsilyltrifluoroacetamide (BSTFA) were used and during derivatization for fatty acids analysis fresh Sodium hydroxide was used.

1.12.7 Choice of test microorganisms

The choice of test microorganisms used during bioscreening was guided by the findings of the ethnobotanical survey. On the other hand the choice considered the availability of testisolates and strains and technical capacity of the laboratories. In both cases the microorganism were of human and veterinary importance. Antiviral studies of the extract were tested against three viruses of veterinary importance. These were Newcastle disease virus (paper II), infectious bursal disease virus and fowl pox virus (paper II) and. Eight standard strains and 7 local (L) Gram positive and negative bacterial and fungal strains and isolates were tested (Paper IV). For cytotoxic studies shrimp larvae (*Artemia salina* Leach) were used. *Artemia salina* eggs are cheap and available; the larvae have high sensitivity to toxins thus qualifies for this purpose.

1.12.8 Choice of bioactivity methods

Bioactivity testing methods were chosen according to their expected reliability and

usefulness of the data to be produced.

1.12.8.1 Cytotoxicity extracts

Brine Shrimp Lethality Test (BST) was chosen for cytotoxicity studies considering several advantages over other screening methods. *Artemia salina* cysts are commercially available and can be maintained indefinitely in the laboratory in their cyst form. The *A. salina* cysts are easily induced to hatch and the assay is quick. The assay is simple and can be performed at low cost. It requires small sample volume and can be performed with high sample throughput (Ruebhart *et al.*, 2008). The brine shrimp eggs were obtained from the institute of traditional medicine at MUHAS and bioassay conducted as detailed in Paper I.

Figure 11: Brine Shrimp Lethality Test Setup

1.12.8.2 Antiviral methods

In ovo method was used for testing the efficacy of the extracts against the viral strains. *In ovo* method involves the use of embryos which are the most delicate stage of development. This was considered as an important measure for effectiveness and

toxicity of the plant extracts (Papers II and III).

Figure 12: Inoculation during an *In Ovo* Assay

1.12.8.3 Antibacterial and antifungal studies

For antibacterial and antifungal tests, the agar well diffusion method was considered for screening. However, agar well diffusion method is limited to diffusivity of components thus could be used for quantification purposes. Hence standard two-fold microdilution techniques were adopted for determination of Minimum Inhibitory Concentartion (MIC). Microdilution increases sensitivity for small quantities of extract, distinguish between bacteriostatic and bactericidal effects and avail quantitative determination and this was chosen for MIC.

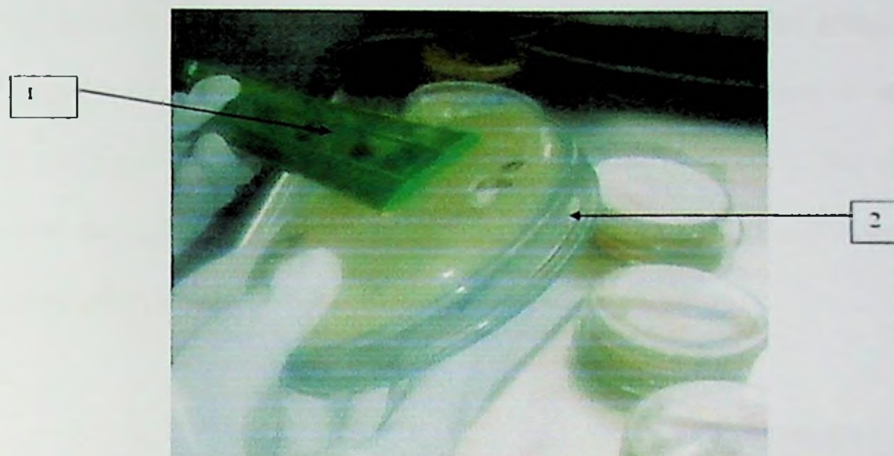

Figure 13: Measurement of Zone of Inhibition (1= Ruler, 2 = Plate)

1.12.9 Gas Chromatography – Mass Spectrometer (GCMS)

The extracts were derivatised to enhance the sensitivity before analysing with GC-MS. Gas chromatography coupled with mass spectrometry is versatile and high technique for separation and identification of organic compound in plant extracts. During the study a Perkin-Elmer Clarus 560 GC-MS with an auto-sampler was used for qualitative and quantitative phytochemical studies.

Figure 14: GC Machine

1.12.10 High performance liquid chromatography (HPLC)

The polyphenolic components are detected at a wavelength of 254 nm using a diode array detector (DAD). Thus the presence of polyphenolic compounds in ethanolic extract was determined by a HPLC fitted DAD detector (Paper V). The effect of temperature on ethanolic extracts was analysed by HPLC fitted with a UV detector at 254 nm (detailed method in paper V).

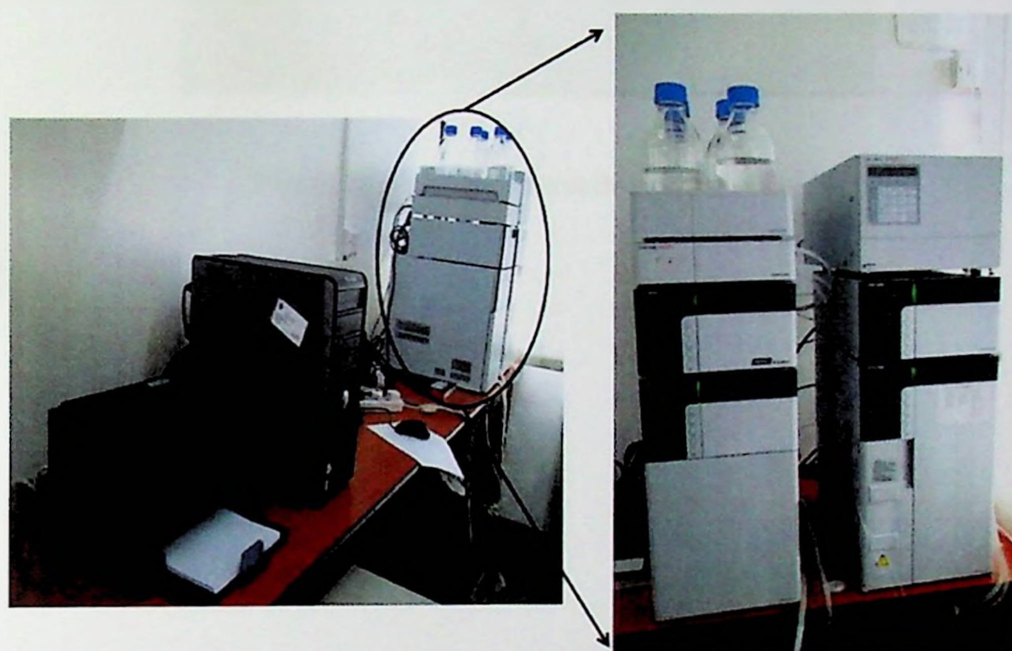

Figure 15: **HPLC Machine**

1.12.11 Optimization of extraction condition

The root bark samples were used due to its higher bioactivity as demonstrated on the bioactivity screening test. Soxhlet method was selected not only for its superiority but also considering that most of the ethnomedical preparations involve heat (Paper V).

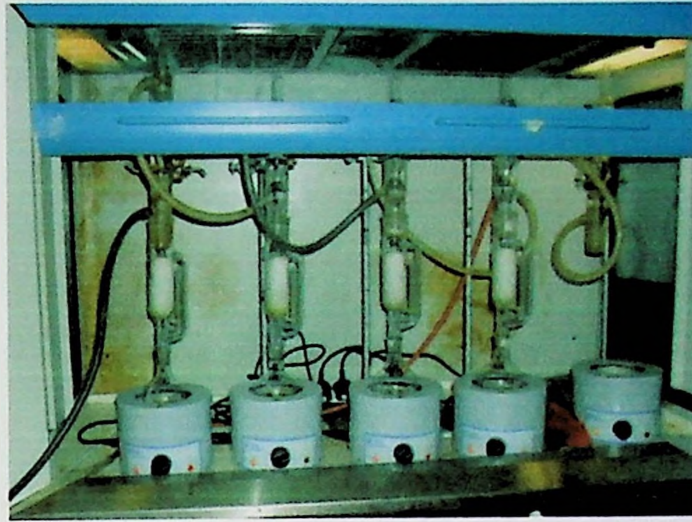

Figure 16: Soxhlet Extraction Apparatus

1.13 Limitation of the study

In the execution of this study several limitations were encountered which include:

i. Failure to describe the exact mode/mechanism of action of the extracts against the virus/pathogens

The structure–activity relationship (SAR) is the relationship between the chemical or 3D structure of a molecule and its biological activity. The mechanism to which the drug or group bioactive molecules work in treating the disease depends on SAR. Despite of bioactivity reported in this study, no SAR could be developed due lack of structure of the responsible compounds. Several pure compounds were isolated but their structure could not be elucidated due to lack of important analytical information such as spectral data of the compounds.

ii. Worked on extracts more than on pure compound

Few pure compounds were tested for bioactivity due to small amounts isolated and stability of the compounds.

iii. Time limitation

The research reported was scheduled for less than four years. Due to time limitations further analysis of samples which needed equipment from abroad were limited. It also limited performance of *in vivo* experiments.

iv. Decomposition of compounds

Lack of some analytical equipments such NMR machine demanded sample to be sent abroad. Some of the pure compounds isolated were decomposing during transit such that spectra data could not be obtained for structure elucidation.

v. Limited cytotoxicity studies

In plant bioactivity studies, it is important to screen the isolated compounds for cytotoxicity against various cell lines to gain an indication of potential toxicity. Further investigation of the compounds prior to potential drug development, including acute and chronic toxicity and other *in vivo* toxicity trials, need to be undertaken. During the study the cytotoxicity studies were limited to brine shrimp lethality test. However brine shrimp test cannot validate the toxicity in other cells as we have seen in viral and bacterial cells.

1.14 Thesis organization

The thesis has been developed in "published papers format" comprising of three chapters. The first chapter consisting of the extended abstract and introduction of the overall theme studies. The chapter offers a description of the commonality of concepts presented in separate papers. Chapter Two contains a series of originality published papers in different journals and three supplements. The arrangement of the papers follows the arrangement of the objectives and the lists of publication. Traditional knowledge and ethnobotanical uses of a neglected toxic species of *S. glaucescens* Pax (Euphorbiaceae) in Tanzania (Paper I). *In Ovo* antiviral activity of *S. glaucescens* (Pax) crude extracts on Newcastle disease virus (paper II), Antiviral activity of crude extracts of *S. glaucescens* (Pax) against infectious bursal disease and Fowlpox virus (paper III), Bioactive crude extracts of *S. glaucescens* (Pax) against selected bacteria and fungi of health importance (Paper IV) and Optimization of extraction conditions and phytochemical screening of root extracts of *Synadenium glaucescens* Pax (Paper V) respectively.

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CHAPTER TWO

PAPER I

Traditional Knowledge and Ethnobotanical Uses of *Synadenium glaucescens* (Pax) Euphorbiaceae a Neglected plant Species of in Tanzania

[1]Faith PM, [2]Mdegela RH, [2]Mosha RD and [3]Magadula JJ,

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel/Fax: +255 23 260 4647/260 3511- 4

[3]Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith PM, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, P. O. Box 3038, Mazimbu Campus, Morogoro, Tanzania (EA). Tel/Fax.: +255-23-2603-404 Emails: fmabiki@yahoo.com, fmabiki@suanet.ac.tz

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[1]Faith PM, [2]Mdegela RH, [2]Mosha RD and [3]Magadula JJ,

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel: +255 23 260 4647/260 3511- 4, Fax: +255 23 260 4647

[3]Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith PM, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, P. O. Box 3038, Mazimbu Campus, Morogoro, Tanzania (EA). Tel/Fax.: +255-23-2603-404 Emails: fmabiki@yahoo.com, fmabiki@suanet.ac.tz

Abstract

Background: *Synadenium glaucescens* is reported as poisonous plant and perceived as of no value despite ethnobotanical uses within communities in Tanzania. The uses, safety and efficacy of the plant extracts used are not documented. This study reports the ethnomedical and ethnoveterinary uses of different parts used, diseases treated, methods of preparations, doses and administration routes. The study reports domestic and field uses and cytotoxicity of the extracts.

Method: Interviews using semi-structured questionnaires were conducted in selected urban and rural setups in Mufindi district, Iringa region and Makambako town in Njombe region. Focused group discussions were conducted for follow-ups targeting traditional healers, traditional believers and elderly people (above 50 years). Brine shrimp lethality test was used to test the cytotoxicity of extracts from *S. glaucescens*.

Results: A total of 220 people were interviewed. It was found that 94% of the respondents were aware of the medicinal value of the plant, among which 78% had personally used it for medicinal purposes. Twenty six (26) ethnobotanical uses including ethnomedical, ethnoveterinary and other domestic uses were recorded. The most frequently used parts for ethno medicine were leaves (47%) and roots (37%) while root decoction was the most dominant extract used orally and fresh leaves for external use only. Ninety six percent (96%) of the respondents argued that elders had more knowledge on the traditional uses of the plant and majority (59%) acquired the knowledge from their grandfathers. The informants (78.9%) reported the plant as highly toxic and cytoxicity test using brine toxicity test that showed LC_{50} of less than

30μg/ml in all the extracts. Domestication of the plant species is taken as adaptation strategy towards the species disappearance in the wild.

Conclusions: Indigenous knowledge plays an important role in the primary health care system of Tanzania. This study has revealed the richness of indigenous knowledge among the Hehe and Bena community on the ethnobotanical uses of *S. glaucescens*. The brine shrimp lethality results confirm the ethnomedical potential of the *S. glaucescens.* In addition the results support the traditional usage of the studied plants and serves as a guide for further studies.

Key words: *Ethnoverterinary, Ethnomedical, Indigenous knowledge, S. glaucescens, crude extracts*

Introduction

The use of plants as the cheap and accessible source of medicines, food and many other life purposes have for long been practiced in many poor communities around the world. Medicinal plants are essential natural resource which constitutes one of the potential sources of new products and bioactive compounds for drug discovery and development. Despite of the advent of the modern high throughput of drug discovery and screening techniques, traditional knowledge of medicinal plants has always guided the search for new cures by providing clues to the discovery of valuable drugs [1, 2]. In Tanzania, the majority of people have for long been utilizing the indigenous knowledge (IK) and experience to treat and control both human and animal diseases using plants. Traditional medicines have been the most common form of primary healthcare in Tanzania [3]. It is estimated that 60% of the urban and 80% of the rural population in Tanzania depend on traditional medicines, mostly drugs derived from plants for their primary health care [4]. Different ethnic groups in the country have over centuries, developed a wide variety of technologies with due regard to the nature and the ecosystem. These ethnic groups have explored the medicinal properties of plant extracts through careful observation, trial and errors by the indigenous communities, collection of a vast heritage of knowledge and expertise which was verbally passed over from one generation to another. Verbal transfer of the traditional practices from one generation to another has resulted into lack of written documents on traditional medical practices that has made its promotion difficult. Such situation has prompted the need for documentation of traditional

practices in the recent years. Several studies have been done to document traditional uses and treatments of medicinal plants in Tanzania [5-12].

Among important medicinal plants with value in traditional rituals is *Synadenium glaucescens* that belong to the family Euphorbiaceae. The genus *Synadenium* consists of about fifteen species that are reported to produce toxic sap like many other members of the family Euphorbiaceae [13]. Despite production of toxic sap, the species are exploited by different ethnic communities as medicinal plants [14, 15] in different parts of the world. *Synadenium grantii* (African milk bush), is used ethnomedically for wound treatment [16], *Synadenium pereskiifolium* is a key recipe in traditional anti- asthmatic preparation [17]. *Synadenium umbellatum* is a medicinal plant used to treat several diseases in Midwest Brazil [18]. *Synadenium compactum* is the most utilised plant species in traditional management of East coast fever in cattle by Kikuyu in Kenya [19]. *Synadenium glaucescens Pax* (*Euphorbia neoglaucescens* Bruyns), in Swahili "*Mvunjakongwa*" and in Hehe/Bena "*Liyugi*" is endemic and grows in several regions in Tanzania [20]. *Synadenium glaucescens* though reported poisonous and perceived as of no medicinal value [21, 22], ethnic communities in Tanzania have used it effectively in the treatment and control of human and animal diseases with no reported adverse effects. In the Costal, Morogoro and Kilimanjaro region, the juice of fresh, crushed leaves is drunk to treat excessive menstruation and as a purgative [10]. A leaf decoction with lime juice, baking soda and honey added is drunk to treat asthma; the ashes of dried leaves are mixed with water and applied to treat leprosy [23]. A root bark extract is taken with sugar to treat

severe cough, tuberculosis and as ear drops to treat earache [24-25]. The latex is also used as a fish poison [26]. In Tanga region the plant in named "*Muuwi*" in Shambaa and is used to prepare medicine for human disease and for control of poultry diseases mainly Newcastle Disease [27]. The use of plants for medication among the *Hehe* and *Bena* ethnic groups dates back to their fathers [28]. Despite the use of S. *glaucescens* among the *Hehe* and *Bena* ethnic groups in Iringa and Njombe regions the uses are not documented. Studies by Mathias (1982), who documented some medicinal plants of the *Hehe* did not list the use of *S. glaucescens* despite its use among the community [29]. Recent studies by Shangali *et al.*, (2008) documented the medicinal plants of the Hehe ethnic group of the Udzungwa Mountains but also did not mention the use of *S. glaucescens*. The aim of this study was to establish the IK and ethnobotanical uses of *S. glaucescens* species among the *Hehe* and *Bena* ethnic groups of Mufindi and Njombe districts. The information documented included diseases treated, preparations, doses and route of administration and other domestic uses.

Materials and methods

Study area

The purposive filed survey was done between 2010 and 2011, in the former Iringa region in Mufindi and Njombe districts in Iringa and Njombe region respectively. Iringa region is situated in the southern higherlands of Tanzania (Figure 1). It is characterised by a series of mountain ranges and volcanoes covered in a mosaic

forest, grassland and Miombo Woodland with about 2,000 species of vascular plants. The former Iringa region was divided in two regions Njombe region and Iringa region. In the current status, the surveyed Njombe district fall under Wanging'ombe and Njombe districts in Njombe region while Mufindi district falls under Iringa region. Mufindi and Njombe districts covered 20% of the former Iringa region with Hehe and Bena ethic communities making more than half of the population of the region. Njombe district dominated by the Bena ethnic group lay between latitudes 8.8° 5′ and 19.8° 30′ S of the Equator and between longitudes 34.5° and 35.8° longitudes, within an altitude of 1000 to 2000m above sea level and Mufindi district which is dominated by Hehe ethinic groups lies between latitude 8° 00′- 9° 15′S and longitude 34° 35′– 35° 55′E within an altitude between 1700-2000m above sea level. The districts receive up to 1600mm of unimodel pattern of rainfall between November and April. Crop cultivation, livestock husbandry and forestry that provide food and cash are common practises holding the economy of the districts. Common grown food crops in the districts include maize, beans, wheat, tea and common cash crops is tea. Cattle, goats, pigs and chicken are common livestock kept of which chicken is the most common source of income and animal derived protein [30].

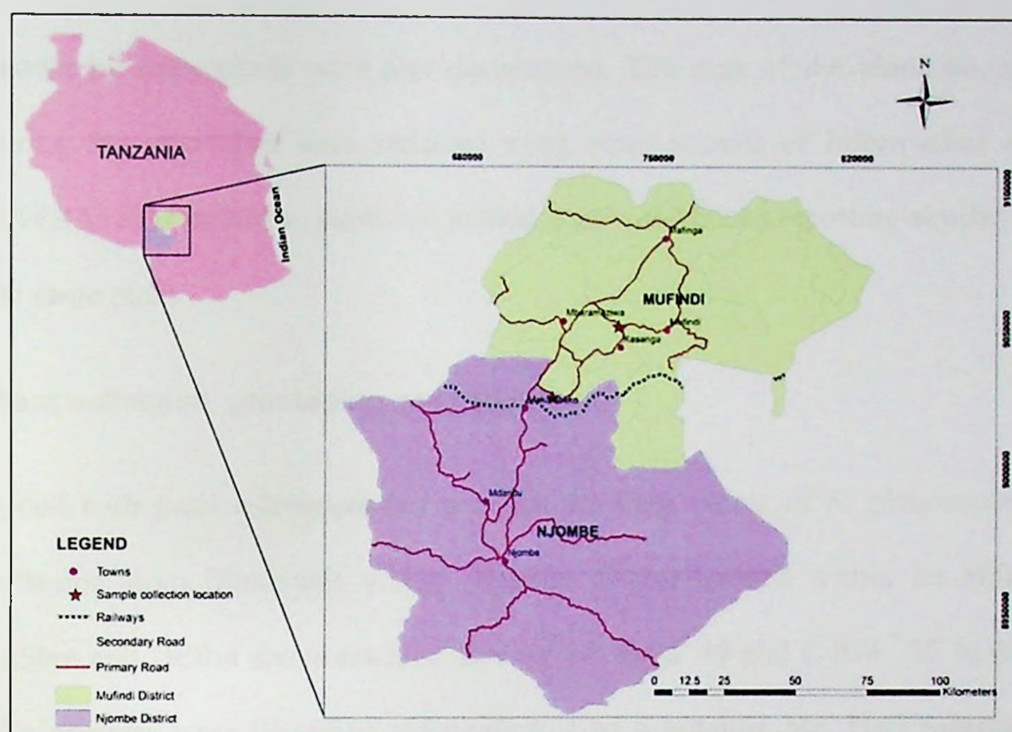

Figure 1: Map of Tanzania showing location of Mufindi and Njombe region and sampling sites

Data collection and analysis

Interviews using semi-structured questionnaires were conducted in urban and rural setups that included Makambako main market and Mtulingala village in Njombe district, Igowole town and Ibatu village in Mufindi district. Focused group discussion was conducted for follow-ups targeting traditional healers, traditional believers and elderly people (above 50 years). The middle (31-50 years) and young ages (14-30 years) were also included to help understand the trans-generation knowledge of the plant in the community. Information collected included general awareness on the use of *S. glaucescens* knowledge, morphological parts used, the diseases treated, methods of preparation, dosage, formulations, and route of administration, side effects and any other use apart from ethnomedicine. Different names of the plant

known by respondents were also documented. The uses of the plant documented during the interviews were validated using other sources of information such as NAPRALERT database, published journal papers and books reporting similar uses of the same plant.

Plant collection, processing and extraction

Aided with local informants and botanist, the flesh plants of *S. glaucescens* were collected from Mtulingala village, Njombe district located within an altitude of 1650m and 1950m above sea level at S 08° 34' to 08° 49' and E 034 ° 55' to 035 ° 10'. The samples were identified and confirmed by a botanist Mr. Haji Selemani and voucher specimen was stored in the herbarium at the Botany Department of botany, College of Natural and Applied Sciences of the University of Dar es Salaam (UDSM) Tanzania, where voucher specimen with number 3672 was deposited. The roots, stems and leaves of *S. glaucescens* were cleaned and mechanically separated to get five parts; the root bark, root wood, stems bark, stem wood and leaves, the parts were denoted 1,2,3,4 and 5 respectively. The samples 1-5 were air dried, pulverized to a particle size of 1 mm for use during extraction. During extraction the samples were soaked consecutively in petroleum ether (P), dichloromethane (D) and ethanol (E) (2 x 48h) at room temperature (*ca.* 30 °C). The extracts were concentrated using a rotary evaporator at reduced pressure. After drying in desiccators the samples were used for cytotoxicity test.

Cytotoxicity test

Brine Shrimp lethality Test (BST) was used as a simple and rapid general bioassay

for assessing cytotoxicity of the crude extracts (Meyer *et al.*, 1982) [31]. Ten *Artemia salina napulii* were subjected into solutions of 240,120, 80, 40, 24, 16, 8 and 4 µg/ml concentrations of the plant extracts in DMSO, and then diluted with sea water to make 10 ml at room temperature. The numbers of survived larvae were recorded after 24 hours and using the log Probit method the lethal concentration (LC_{50}) were calculated for each extract.

Results and discussions

Awareness and use of *S. glaucescens*

A total of 220 people were interviewed. Sixty eight percent (68%) of the respondents were male and 32% female. Majority of the respondents were peasants (78.1%) and businessmen (13.2%). The age groups were divided into 14 -30 years (33%), 31-50 years (49.8%) and above 50 years (16.6%). It was found that 95% of the respondents were aware of the ethnobotanical uses and other value of the plant, among which 78% had used it for medical purposes and the frequent parts used during medication were leaves (47%) and roots (37%), Figure 2. During the follow-ups discussions it was revealed that the leaves were the mostly used due to their high content of sap. Leaves and sap were boiled or warmed before use. If used fresh it was strictly for external use only to avoid toxic effects. The root extracts were used for oral route and most of the time in boiled form. The majority of the respondents (71%) had used for animal treatments, 15% for human treatments and 14% for both animal and human treatment. The respondents were able to mention other names of the plant

from other tribes as shown in Table 1.

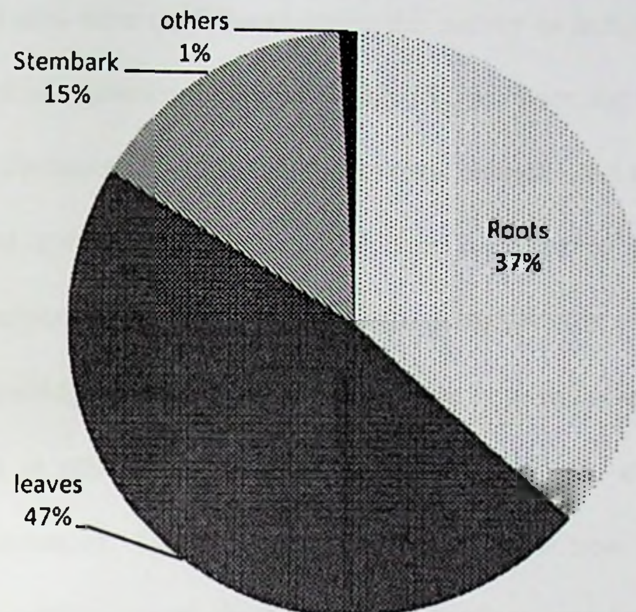

Figure 2: Most parts used during treatments

Table 1: Tribal names of *Synadenium glaucescens* obtained during the survey

Name	Tribe
Ohjoke	Kinga
Mvunjinjoki	Haya
Munua	Shambaa
Igole	Kaguru
Munyula, Liyuge, livelevele, liyugi,liyanganyanga	Bena/Hehe
Lilangali	Pangwa
Mvunjakongwa	Swahili
Mwasa, Kinyunyu, Mnua, Muswoswo, Tiha, Tula, Tupa	NAPRALERT
Omoitanjoka	Haya
Ikigili	unknown tribe

Ethnomedical and others uses

Twenty six (26) uses were established during this survey as indicated in Table 2. The uses included 19 ethnomedical uses, of which 15 uses were for treatment of human ailments. Root decoction was the most dominant extract used orally. This implies that the bioactive compounds are water soluble and heat stable [32]. The respondents explained the purpose of involving heat was for detoxification purposes. Indigenous people have knowledge and they own it. The bridge between science and indigenous knowledge such as ethnobotany is of vital importance in the current world and it gains more acceptances. Though scientists for long time now have assumed that indigenous communities must change to meet modern standards, it is a current feeling that scientists and society must begin to respect the consecrated knowledge that indigenous people have known for generations. The knowledge has been learnt from direct observation of the land over thousands of years and gathered lots of practical experiences on continued use of the land and vegetation in particular [33].

Table 2: The traditional uses of the plant *Synadenium glaucescens* as obtained during the survey

	Ethnobotanical Use	Plant Part used	Type of extract	Method of Preparations/Doses	Route of administration
	(a)Ethnomedical/Ethnoveterinary uses / Disease treated				
1	Ear ache	Leaves		Leaf warmed in warm ash, and then squeezed to produce a juice. Two drops of it is dropped inside the ear three times a day	External
2	Gastrointestinal worms (for animal and Human)	Stems/roots	Decoction	2 to 3 pieces (3-4cm) of stems are boiled with meat in about 1.75 a litre till remains like a quarter the amount. Two food spoons for adults or 1 tea spoon for children, till it finishes.	Oral
3	Swollen and pain legs	Leaves		The Leaves are warmed and gently rubbed on the legs	External
4	Tumour control	Sap/Root	Decoction	The drop of sap is applied on the tumour growth till it diminishes or the root is boiled in water the extract is drunk	External/Oral
5	East Cost fever in cattle	Sap/leaves/ste m	Decoction	Flesh leaves/stem with sap is rubbed on the swollen lymph node and left till it burst or the root is boiled and the extract is given to the animal is given one full cup (200ml) for 7 days (It works at very early stage of the disease)	External/oral
6	Ring worms	Roots/sap		Roots are washed then boiled small amount of sap used to wash the affected part or 1 drop of the sap dropped on the ring worm for human being once a day	External
7	Skin disease in cattle	Leaves	Decoction	The leaves are boiled in water; one cup of the extract is given to the cow.	Oral
8	Boils	Leaves/sap		Fresh leaves are squeezed to produce juice used to put on the boil or a drop of sap is applied on it or the leaves are used to rub the boil once a day, till it heals	External

9	Teeth ache	Roots	Decoction	Roots boiled with water and the extract used for tooth brushing for few days. Or A drop of the sap in applied direct on the tooth or brush the tooth with the root stick	Oral
10	Sexual transmitted diseases (STIs) and Human immunodeficiency Virus (HIV) symptoms	Sap/ Root	Decoction	Boiled root/sap mixed with other medicinal plants (not mentioned). The extract is drunk for several days	Oral
11	Infection leading to swelling of lymph nodes in human	Roots	Decoction	Roots are boiled and extract drunk	Oral
12	Severe Coughing/blood cough/tuberculosis	Roots	Decoctions	Few pieces of the root is boiled in about 5liters of water, till it remains about 350 mL. One table spoon to half a cup x 3 a day. The dose depend on age of a patient	Oral
13	Stomach ache	Roots/leaf	Decoction	Pieces of root or leaf are boiled in water mixed with honey. Then 1 cup (200ml) drunk 3 times a day for adults or 1 teaspoon x3 a day for children	Oral
14	Backbone/neck aches	Root	Decoction	Pieces of Roots boiled and the extract is drunk	Oral
15	Phlegmon/Cellulites	Roots		A piece of root is dried then ground to powder, the sick person leaks the powder (lamba)	Oral
16	Wounds (human and animal)	Leaves/root	Decoction	Pieces root boiled and the extract used to wash the wound. Juice from warmed leaves is dropped in the wound	External
17	Eye pain	Leaves	Decoction	A leaf is boiled in water (not concentrated) and the extract is used to wash the face specifically eye parts	External
18	Newcastle disease	leaves		About 200g of leaves, cut into pieces are soaked in about 2litres of water. The mixture is left for sometime then given to chicken to drink in early stages of the diseases.	Oral
19	Induction of abortion	Leaves/Root	Decoction	Leaves/roots Boiled and juice drunk	Oral

b) Non medical uses

20	Sticker/glue	Sap	An amount of sap is warmed for sometime then used as glue	
21	Pesticide: Killing pest animal like rats, guinea pigs and pigs	Stem/leaves	A good amount crushed leaves or stems mixed with meat is left around for an animal to eat	Oral
22	Illegal fishing/Fish poison	Leaves/Sap	Fresh leaves with sap crushed and thrown in water. After some time the fish floats and they picked with hands	
23	Insect repellent	Whole Plant	The plant is grown near each pillar of the grain storage structures (Kihenge in Hehe/Bena) to stop them from being destroyed by Ants	
24	Food storage (Grain storage)	Leaves	Leaves are dried, powdered then mixed with dry grains before storage	
25	Farm Protection (super natural belief)	Whole Plant	Grown in the border or near the entrance of the farm to harm people with bad intension. Believed to cause swelling of the bell/cause irritation put on a bad person faeces.	
26	Grave marker	Whole plant	Planted in the grave to mark the position of the graves	

Awareness on toxicity and side effects

Indigenous people have accumulated knowledge with experience on how to handle the plant due to its toxicity and side effect if happens due to mishandling and overdose. Thus, 78.9% of the respondents were aware on the toxicity of the plant. The sap produced by the plant was reported to be responsible for corrosiveness and toxicity. Despite acknowledging that the plant is toxic, they argued that, it does not stop them from exploiting for medicinal and other uses as they have learnt through years on how to handle the plant. The toxicity of the plant was mentioned to be severe in human than in animals and so lower doses were recommended for human treatment. The side effects such as eye blindness would occur if the sap gets in contact, and corrosive to skin, where as stomach aches, bloody diarrhea, pains even death were reported as the main side effects due to overdose. Most of the species from Euphorbiaceae family and genus *Synadenium* in particular are reported to contain irritant diterpene and phorbol esters which are toxic to human and animals [34].

Adaption to disappearance of the plant in the wild

The respondents have demonstrated a high knowledge in observing the trend towards disappearance of the plant in the wild. Ninety six percent (96%) agreed that the plant is available, of them 80% agreed that the abundance of the plant is less compared to few years back. This can be attributed by many factors including deforestation for farming, drought and over harvesting of the plant. As an adaptation to disappearance of the plant in the wild, 69% of respondents have attempted to grow the plant, of them 99% domesticated the plant successfully. To protect children from contacting the plant is grown in farms far from residential houses. The respondents also reported that the plant was easy to grow and it does not need much water for germination during propagation even for growing.

Flow of knowledge and continuous use of the plant

Majority of the respondents (96%) argued that elders had more knowledge on the traditional uses of the plant. About 59% acquired the knowledge on the plant from their grandfathers (Figure 3). This could indicate that men took role of traditional healing and transfer of the knowledge to the new generations than women. This is attributed by gender influences on local knowledge distribution due to culturally assigned roles between men and women or division of labour among the community [35]. Like in many other traditions, the knowledge of the use of *S. glaucescens* among the Hehe and Bena is passed over verbally. This kind of knowledge transfer is affected by change of culture, behaviour, division of labour and gender roles within the community. Despite the willingness shown by the majority (80%) of the respondents that they will continue using the plant for their benefit, majority (86%) agreed that the plant is in less use compared to the past times. The reason for less use was mentioned to be due to the availability of medical officers and dispensaries around the area. Further 85% of the respondents argued that poor knowledge transfer from elders to younger generation is the main reason for having less use of traditional methods of treatments.

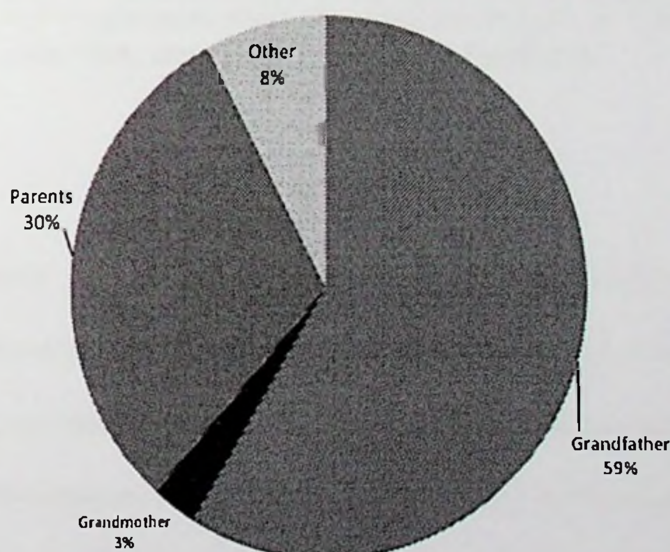

Figure 3: Source of knowledge of ethnobotanical uses of *S. glaucescens*

Result of the brine shrimp lethality test

The results in Table 3 indicates that the extracts from *S. glaucescens* could be both bioactive and anticancer as they were all demonstrating the LC_{50} below 30 μg/ml lower to 0.65 μg/ml and below by SD3 and SD2 respectively. The Dichloromethane extracts were the most toxic followed by petroleum ether and ethanol extracts respectively. This validates the experience as reported by the local people that the plant is very corrosive and toxic to human being and sometime used for fishing. The latex from the plant is reported to cause blindness and locally used to control tumor growth. Studies have shown that other species from this genus have antitumour properties [18]. The LC_{50} values have been reported for many toxins and plant extracts [36, 37]. According to Meyer et al. (1982) and Parra et al. (2001), LC_{50} value lower than 1000 μg/ml is considered bioactive in cytotoxicity evaluation of plant extracts by BST bioassay. Other scholar consider the cytotoxicity below 100 μg/ml and consider LC_{50} of <20 μg/ml very toxic and suggestive to anti-cancer activity.

Table 3: Showing brine shrimp lethality results

Sample code	SP1	SP2	SP3	SP4	SP5	SD1	SD2	SD3	SD4	SD5	SE1	SE2	SE3	SE4	SE5
LC50 in ug/ml	9.8	3.6	6	3.6	8.2	3.4	0	0.65	5.6	27.7	10	1.81	12	15.7	25.3

Where S = *Synadenium*, P = petroleum ether, D = dichloromethane, E = ethanol, No. 1, 2, 3, 4, 5 = rootbark, root wood, stem bark, stem wood and leaves respectively

Conclusion

This ethnobotanical study presents the first documentation on S. *glaucescens*, a plant species considered toxic and therefore its ethnomedical, ethnoveterinary and domestic importance were neglected. In addition, the study has revealed the richness of indigenous knowledge among the Bena and the Hehe communities in Iringa and Njombe region. The brine shrimp lethality results confirm the ethnomedical potential of the *S. glaucescens.* In addition the results support the traditional usage of the studied plants and serves as guide for further development studies.

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Authors' contributions

FPM, RHM, JJM and RDM identified the research area and designed the study which implemented as part of natural product network in Tanzania, Kenya and Uganda. FPM and RHM involved in preparation and implementation of the interviews, compiling the information, analysis and structured the manuscript. JJM and RDM participated in refining data analysis and drafting as well as enrichment of the manuscript. All authors read, revised and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

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PAPER II

In Ovo Antiviral Activity of *Synadenium glaucescens* (Pax) Crude Extracts on Newcastle Disease Virus

Faith Philemon Mabiki[1,2], Robinson H. Mdegela[2], Resto D. Mosha[2], and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel/Fax: +255 23 260 4647/260 3511- 4

[3]Instute of Traditional Medicine, Muhimbili University of Health and Applied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith Philemon Mabiki, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania (EA). Tel/Fax.: +255-23-2603-404 Emails: fmabiki@yahoo.com , fmabiki@suanet.ac.tz

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Full Length Research Paper

In ovo antiviral activity of *Synadenium glaucescens* (pax) crude extracts on Newcastle disease virus

Faith Philemon Mabiki[1,2*], Robinson H. Mdegela[2], Resto D. Mosha[2] and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Mazimbu campus, Morogoro, Tanzania.
[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania.
[3]Institute of Traditional Medicine, Department of Natural Product Development and Formulation, Muhimbili University of Health and Applied Sciences, P.O. Box 65001 Dar Es salaam, Tanzania.

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Investigation on the effect of root bark and wood, stem bark and wood, leaves and sap of *Synadenium glaucescens* extracts against Newcastle disease (ND) virus was done using an *in ovo* assay. Viable 9 days embryonated chicken eggs were arranged into 25 treatment groups (n = 5). Groups 1 to 21 were challenged with a 13C/SUA virulent strain of ND virus treated with extract at concentration of 0.2 mg/ml. Un-inoculated group saved as negative control and groups inoculated with virus and diluent saved as positive controls. Haemagglutination test was used to quantify the amount for ND virus units. Embryo survival and embryo weight were significantly higher ($P \leq 0.05$) in groups treated with *S. glaucescens* extracts than the positive control. The root bark demonstrated significantly higher antiviral activities ($P \leq 0.05$). Furthermore, treatments with ethanolic extract SE1 resulted into 100% embryo survival, 91.2% mean embryo weight and reduced viral load by 99.2%. The minimum dose of SE1 with the highest efficacy was 0.2 mg/ml. The percent mean embryo weight and haemagglutination test demonstrated negative correlation ($R^2 = 0.94$). These findings validate the ethnoveterinary potential of *S. glaucescens* and the feasibility of its use for treatment and control of ND.

Key words: Ethnoveterinary, Euphorbiaceae, poultry viral diseases, Newcastle disease, *Synadenium glaucescens*.

INTRODUCTION

The use of plant extracts for control of Newcastle disease (ND) in rural Tanzania is not uncommon (Buza and Mwamuhehe, 2001). *Synadenium glaucescens* (euphorbiaceae) has been deployed by communities in Tanzania for ethnomedical (Chhabra et al., 1984) and ethnoveterinary purposes (Wickama et al., 2006; Mabiki et al., 2011). The water extract of the leaves and stems of *S. glaucescens* have demonstrated antimolluscicidal activity (Kloos et al., 1987) and weak inhibition of electrically induced contractions of the guinea-pig ileum (Rukunga et al., 1990). Newcastle, which is one of the diseases claimed to be treated and controlled by this plant (Wickama et al., 2006; Mabiki et al., 2011), is among the serious challenges for development of poultry industry in Tanzania under rural setting (Yongolo et al., 2002; Komba et al., 2012). Though studies on efficacy of plant extracts against viruses is growing and *S. glaucescens* is increasingly being reported for its ethnoveterinary potential, there are limited controlled laboratory studies of the extracts on ND virus (NDV). Therefore, the aim of this study was to assess the effectiveness of different extracts of *S. glaucescens* on NDV. The findings of this study provide valuable information on the usefulness of the plant against ND.

*Corresponding author. E-mail: fmabiki@gmail.com or fmabiki@suanet.ac.tz. Tel/Fax: +255-23-2603-404.

Table 1. The coding of extracts.

Plant part (PP)	PP Code	Extract codes			
		Laboratory sequential extraction			Traditional extraction (T)
		Petroleum ether (P)	Dichloromethane (D)	Ethanol (E)	
Root Bark	1	SP1	SD1	SE1	ST1
Root wood	2	SP2	SD2	SE2	ST2
Stem Bark	3	SP3	SD3	SE3	ST3
Stem Wood	4	SP4	SD4	SE4	ST4
Leaves	5	SP5	SD5	SE5	ST5
Sap	6	na	na	na	ST6

S: *Synadenium*; na: not applicable

Table 2. Grouping and treatment allocation for the *in ovo* assay.

Group (G): n = 5	Treatment
G1 to G15	NDV + extract + DMSO ; 15 different extracts (SP/SD/SE 1-5)
G16 to G21	NDV + extract + DMSO; 6 different extracts (ST 1-6)
G22	NDV alone (V+)
G23	NDV + DMSO (V+)
G24	Egg + DMSO + Sample (VS+)
G25	Untreated embryonated chicken egg, ECE (V-)

MATERIALS AND METHODS

Plant collection and processing

Aided by local informants and botanist, the fresh plants were collected in Njombe region, Njombe district in Southern Highlands of Tanzania. The samples were identified and confirmed by a botanist and voucher specimen was stored in the herbarium at the Botany Department, College of Natural and Applied Sciences of the University of Dar es Salaam (UDSM) in Tanzania with specimen's number HOS/FM 3672. The roots, stems and leaves of *S. glaucescens* were cleaned and mechanically separated to get five parts; the root bark, root wood, stem bark, stem wood, and leaves. The parts were used as fresh or air dried and pulverized to a particle size of 1 mm for use. The sap was used as fresh.

Extraction of samples

In the laboratory sequential extraction (LSE), the dry samples were soaked sequentially in petroleum spirit, dichloromethane and then ethanol twice each for 72 h for each solvent. After filtration, the extracts were dried using rotary evaporator to obtain 15 crude extracts. Extracts for use were dissolved in dimethyl sulphoxide (DMSO) to make a concentration of 0.2 mg/ml. Traditional method of extraction (TE) was adopted from traditional practitioners and users of the plant. 100 g of fresh samples were mixed with 2 L of water and was boiled while allowing evaporation, till the volume of the solution was reduced to 100 ml. The sap was used after four fold dilution. The extracts from both methods were coded as indicated in Table 1. The extracts were stored at 4°C before being used for antiviral tests.

Antiviral screening

The test organism 13C/SUA NDV strain, supplied by the Bacteriology and Mycology Laboratory, Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture was used. *In ovo* assay following the procedure by Sally (1998) and Senne (2002) with slight modification was used to test the antiviral potential of *S. glaucescens* extracts. Embryonated chicken eggs (ECE) of 9 days old were checked for viability by candling before being used. The ECE were randomized into 25 groups (n = 5), allocated as shown in Table 2. A hole was made through egg shell just above the air sac to allow vertical inoculation of 0.1 ml of the inoculum. The inoculum was prepared by mixing 0.1 ml virus suspension and 0.9 ml crude extract (0.2 mg/ml). After inoculation, the inoculated site was sealed with paraffin wax, and then the eggs were kept at 4°C (refrigerated) for 1 h. The eggs were then incubated at 37°C for 5 days with the air sac uppermost. Un-inoculated eggs (V-), eggs with virus suspension only (V+) and eggs Injected with DMSO and virus (VS+) served as controls for all experiments.

The challenged ECE were observed daily for death of embryo for 4 days. After 5 days, the eggs were chilled and growing embryos were observed for growth and weight change. The assessments of antiviral activity were based on survival of the embryo; percentage mean embryo weight (%MEW) (Equation 1) and the viral load in the allantoic fluid during harvesting.

$$\text{MEW}(\%) = \frac{\text{MEW of the embryo harvested from treated ECE}}{\text{MEW of embryos from untreated ECE (V-)}} \times 100$$

Dose dependent study

The dose dependent study was done using the ethanolic extract of the root bark which demonstrated the highest antiviral activity against NDV. The extract concentration ranging from 0.05 to 0.3 mg/ml was used. The assessments of antiviral activity were based on the weights of the embryo, their survival and physical observation.

Haemagglutination (HA) test of the allantoic fluid collected from ECE challenged with the inoculums

HA test was used to quantify the amount of NDV using two fold dilutions in a V-shaped 96 well micro titre plates. One percent chicken red blood cells were two fold diluted, and then titrated with allantoic fluid collected from NDV challenged ECE with NDV. After allowing it to stand for 45 min at room temperature (28°C), the end point was recorded for interpretation and calculation of the HA titre. Micro wells with a sharp button of red blood cells at the bottom were considered HA negative, while the ones with a hazy film of red blood cells or no button at the bottom of the V-bottom well were considered HA positive. The endpoints for HA were used in calculation of the HA titre of the test samples and the reduction of viral load due to extracts exposure (Equation 2).

$$\text{Reduced} \quad \text{HA} \quad (\%) \;=\; \frac{C - E}{C} \times 100$$

where C= the base two logarithmic HA titre of the virus control and E= the base two logarithmic HA titre of the Challenged ECE.

Haemagglutination inhibition (HI) test against NDV antibodies in hatched chicks Ten eggs challenged with the inoculums of NDV with SE1 and 10 eggs EV were left to hatch 12 days post treatment. Serological testing protocol for the HI test as previously described by Allan and Gough (1974) with slight modification was used to detect the presence of antibodies against NDV in chickens on days 0 and 7 post hatching. Sera prepared from hatched chicks blood samples were made into two fold serial dilution with PBS in micro titre plate up to the 11th well, while the 12th wells column were left with neat sera that served as control. The 4 HA units NDV antigen were added up to the 11th well and kept at 28°C for 25 to 30 min. A 1% volume of chicken red blood cells (RBCs) suspension in phosphate buffered saline (PBS) prepared from centrifuged and washed chicken blood was added into each well. The samples forming a button shaped settling of RBCs were recorded as positive and the maximum dilution of each sample causing HI was considered as the end point.

Statistical analysis

Data collected were analyzed using CoStat Version 6.400, (CoHort Software, USA). The weights for different groups were reported in %MEW ± Standard deviation at 95% confidence interval. The differences of MEW were further analyzed by one-way analysis of variance (ANOVA) and significance was reported at $P \leq 0.05$. Comparison of means was performed by Tukey-Kramer test.

RESULTS

Antiviral activity of extracts obtained by sequential laboratory extraction method

The results on the antiviral activities of the extracts against the NDV in ECE showed that there was a significant difference in activity between extracts ($P \leq 0.05$). The %MEW of treated ECE ranged between 22 and 92% as compared to the unchallenged ECE (Figures 1 to 3). Generally, the embryos treated with ethanolic extracts demonstrated higher %MEW well above 50%, implying more activity from extracts of this solvent as compared to P and D solvents. The results further indicated that %MEW for SE1 were significantly higher ($P \leq 0.05$) as compared to other ethanolic extracts (Figure 1). There was no significant difference (P> 0.05) between the %MEW of the extracts SP3 (18.2%), SP5 (19.1%), SD1 (17.2%), SD4 (22.8%), SE2 (22.7%) and the two positive controls EV+ (12.8%) and EVS+ (18.6). All embryos in two positive control groups died within 30 h, while those treated with the extracts that had similar level of activity demonstrated up to 70% death of embryos in five days of observation. The %MEW of the SE1C had no significant difference with the EV-.

Antiviral activity of extracts obtained by the traditional extraction method

The results from extracts obtained by local extraction method against the ECE challenged with NDV are as shown in Figure 2. The results show that there was a significant difference in activity between extracts ($P \leq 0.05$). Extract ST2 of the root wood demonstrated higher MEW of 91.6% implying higher activity than other extracts. There were no deaths of embryos observed from these extracts. There was no significant difference ($P > 0.05$) among %MEW of ST1 (88.5%), ST3 (84.05%) and ST2 (91.6%) extracts. No significant difference ($P > 0.05$) of %MEW between ST4 (49.8%) and ST5 (49%) was observed. In these groups, 20 to 100% death of embryos was observed from days 2 to 5 and the difference was significant ($P > 0.05$). ST6 demonstrated the least activity with %MEW of 28.4% which was lower and not significant ($P < 0.05$) when compared with the positive control, with 100% death of embryo within 16 h.

HA test results for ECE challenged with the inoculum

The HA results for ECE challenged with the inoculum are as shown in Figure 3. The viral load in eggs challenged with NDV mixed with extracts was reduced to 99.6%, which was the highest. The lowest HA tires were recorded in allantoic fluids from ECE treated with ST1, ST2 and ST3 extracts prepared using traditional extraction method, each showing the 2 HA units equivalent to 99.6% reduction of the viral load. SP2, SP4, SE1, SE3 and SE5 demonstrated similar HA titres of 3 HA units equivalent to 99.2% reduction of virus units as compared to the EVS+. The highest viral load was on ECE treated with SP5, SD1, SD2 and SD4 extracts with512 HA units. The mean antibody titres against NDV in hatched chicks at concentration of 0.2 mg/ml were all negative.

Relationship between percent mean weight of embryo and HA titres

The HA titres correlated well with the %MEW with R^2 = 0.94 for extracts extracted using laboratory method and R^2 = 0.97 for extracts extracted using the traditional

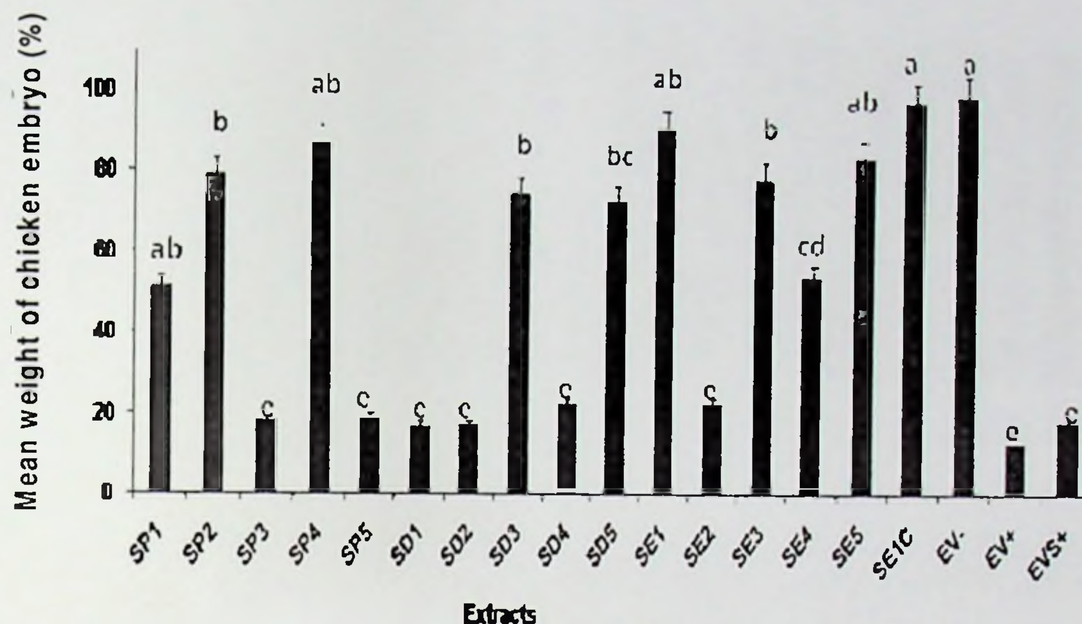

Figure 1. Percentage of embryo weights in eggs infected with NDV and treated with extracts in comparison to the negative control. Where, S= *Synadenium*, P= Petroleum ether, D= Dichloromethane, E= Ethanol, No. 1, 2, 3, 4, 5 = root bark, root wood, stem bark, stem wood and leaves, respectively, EV+ = Positive control group of eggs inoculated with a NDV treated with extracts; EVS+ Positive control group of eggs inoculated with a NDV and DMSO, EV- Negative control group of un-inoculated eggs and SE1C – Egg with Extract SE1 alone. Extracts with the same letter were not significant different (P > 0.05).

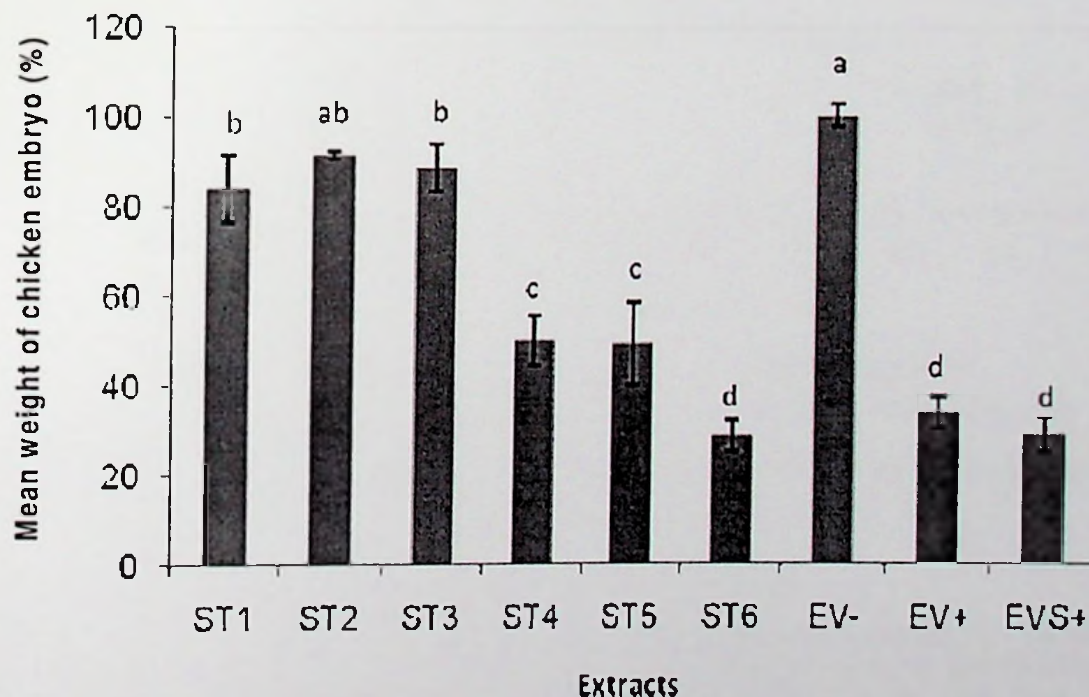

Figure 2. Percentage of embryo weights in eggs infected with NDV treated with extracts extracted using traditional method. Extracts with the same letter were not significant different (P > 0.05). Where, S= *Synadenium*, T= Traditional extraction, No. 1, 2, 3, 4, 5, 6= root bark, root wood, stem bark, stem wood, leaves and Sap, respectively. EV+= Positive control group of eggs inoculated with a NDV treated with extracts; EVS+ Positive control group of eggs inoculated with a NDV and DMSO, EV- Negative control group of un-inoculated eggs. Extracts with the same letter were not significant different (P > 0.05)

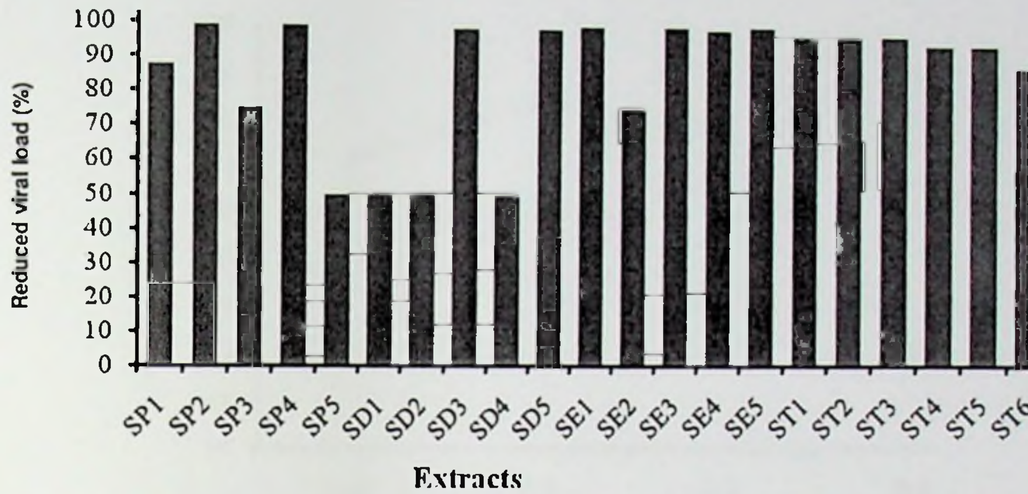

Figure 3. Percentage Reduced Viral Load due to HA test. Where, S= *Synadenium*, T= Traditional extraction, P= Petroleum ether, D Dichloromethane, E= Ethanol; No. 1, 2, 3, 4, 5, 6 = root bark, root wood, stem bark, stem wood, leaves and Sap, respectively

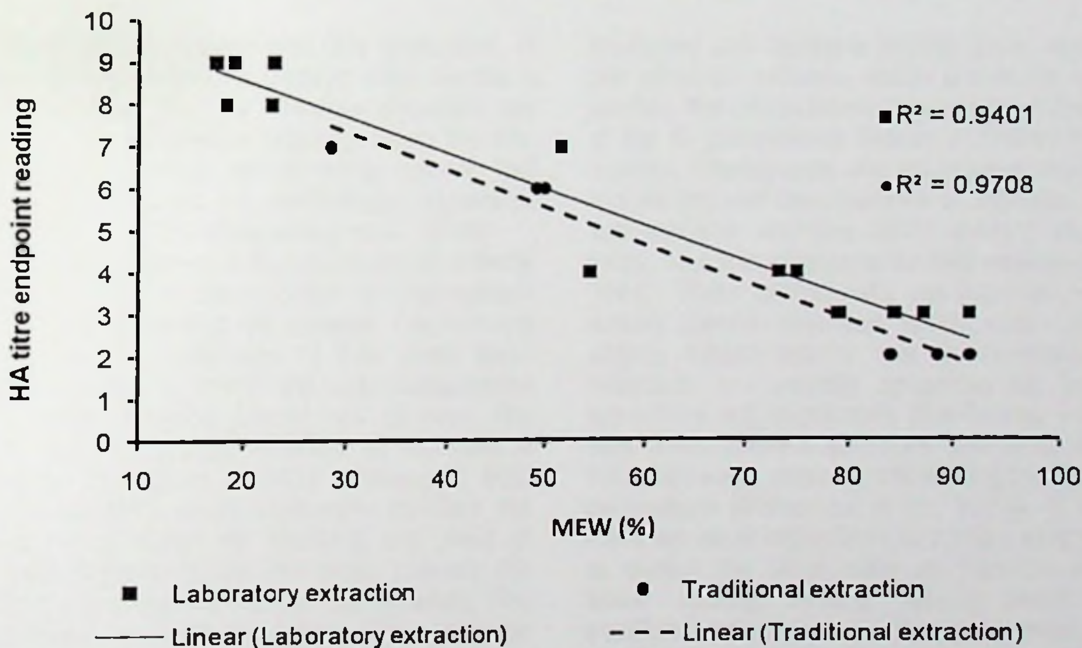

Figure 4. Graph of HA titre endpoint reading against %MEW. Whereas MEW is the mean embryo weight.

technique (Figure 4).

Dose dependent study

The results from the dose dependent study done using SE1 at concentrations ranging from 0.05 to 0.3 mg/ml are indicated in Figure 5. The highest %MEW was observed at concentration of 0.1 mg/ml implying higher activity. Deaths of embryos at 20% were observed after 72 h at 0.3 mg/ml.

DISCUSSION

This study has demonstrated for the first time the activity of S. *glaucescens* extracts against NDV while relating the embryo weight and the viral load. The extract was considered active if it inhibited virus replication in the embryo cells thus allowing embryo growth, also if the extract reduced or decreased the viral load in ECE and thus preventing the death of embryo. The invasion of the NDV into the embryo cell is enabled by the functioning of the fringe of glycoprotein spikes present on the virus

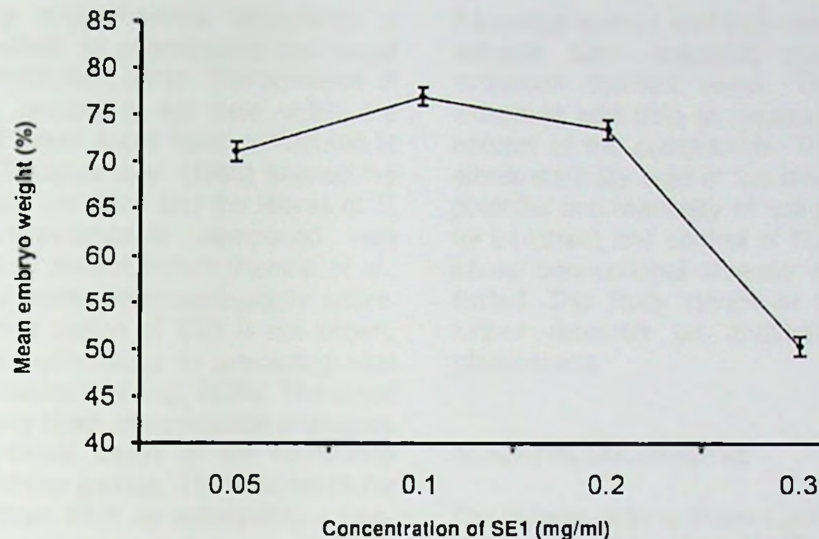

Figure 5. A dose dependent study using the ethanolic extract (SE1). Concentration of extracts with the same letter were not significant different ($P > 0.05$).

envelope (Young et al., 2002) and the replication of viruses inside the cytoplasm of embryo cells causes a disease. Their mechanisms of causing diseases are always based on the replication capacity using the embryo cell metabolic pathways, which mostly leads to cell deaths or suffering to death for multicellular organisms (Hightower and Brant, 1974; Wakamatsu et al., 2006).

The goal of antiviral search is the discovery of antiviral agents that are specific for the inhibition of viral multiplication without affecting normal cell division. The extracts of *S. glaucescens* as presented in this study have demonstrated the ability to inhibit the viral multiplication without significantly affecting normal cell division. The continuation of embryo growth unveiled by increase in weight and organ formation in NDV challenged ECE implies that the extracts could potentially interfere the viral replication cycle either by blocking one point of propagation mechanisms inside the cells, prevent the invasion mechanism or kill the virus in the inoculate. The ability demonstrated by more than 80% of the extracts reducing the HA titre by more than 50% (Figure 3) shows that the extracts can inhibit the replication of virus. The fact that HA titre correlated strongly and negatively with the %MEW (Figure 4) with R^2 of 0.94 and 0.97, indicate that the extracts were highly responsible with inhibiting the multiplication of virus in the embryo without affecting the growing embryo cells. The significant difference in %MEW for different extracts could be attributed to the diversity of compounds in the extracts. Each extract had a different degree of inhibitory activity and specificity against the virus and/or its essential enzymes. The ethanolic extracts prepared by the LSE (SE1, SE3, SE4, SE5) and water extracts from TE method (ST1, ST2 and ST3), were more active as compared to other extracts. Water is a common extraction solvent in traditional treatment and contains mainly polar compounds unlike the ethanolic extracts which are more refined. In other studies, the phytochemical analysis of the water extracts of the *S. glaucescens* leaves indicated the presence for tannins, triterpenoids and coumarins while the methanol extract showed the presence of steroids, triterpenes and anthocyanins and the ether extract contained carotenoids, steroids, triterpenoids and volatile oils (Neuwinger, 1994). These compounds are reported to have antiviral activity (Jassim and Naji, 2003), and could have shown slightly higher activity due to synergistic effect. Viral infections are usually accompanied by a variety of symptoms not necessarily due to the virus directly, but associated immune functions and other important metabolic pathways, thereby, influencing multiple physiological parameters (Mohamed et al., 2010). It is possible that there are other ingredients in a plant preparation that help to control the virus such as immune modulation and tissue healing among others which justifies why traditional treatments involves preparation of concoction of different plants to attain the maximum beneficial effect of a medicinal plant preparations (Gessler et al., 1994). However, the water extract is hard to handle in isolating pharmacologically active compounds for drug discovery research, because it needs more sophisticated instrumentation not available in many developing laboratories (Rukunga et al., 1990).

The ethanolic extracts from the LSE method are more purified with specific concentration; thus, present a better candidate for further research on drug discovery as perceived from this study. The root barks extract SE1 have shown activity at minimum concentration of 0.1 mg/ml. The extract was denoted as ST1 in water extraction and presented higher activity (88.9% and reduced HA titre of 99.6%) despite the different methods of

preparation. Preliminary phytochemical screenings of SE1 indicated the presence of polyphenolic and sugar moiety compounds (unpublished data). The presence of compounds with sugar moiety is not new within the *Synadenium* genus and these could have contributed to activity of the extracts. Rukunga et al. (1990) isolated the glucoside compound from the stem and the leaves of *S. glaucescens* and glucopyranoside compound was isolated from *Synadenium pereskiifolium* (Kerstin et al., 1991), both compounds were pharmacologically active. Though the mechanism of action of SE1 is not known, the mechanisms of the nucleosides in preventing viral replication are known (Jassim and Naji, 2003). The sugar moiety is the main building block of nucleoside analogues which are used as antiviral drugs of the nucleoside reverse transcriptase inhibitor groups. These interrupt the formation of viral DNA from RNA by substituting a look-alike analogy that resembles nucleosides (building blocks) used by the virus to synthesize DNA thus blocking the viral replication. The polyphenols act principally by binding to the virus and/or the protein of the host cell membrane and thus arrest absorption of the virus (Van den Berghe et al., 1986). The high activity of the ether extracts SP4 and SP2 could have been attributed by the presence other antiviral active compounds such as steroids, triterpenes and anthocyanins carotenoids, triterpenoids which have been reported present in this plant species (Neuwinger, 1994; Jassim and Naji, 2003).

Antiviral drug specificity to the pathogen is very vital in searching for antiviral drugs. The selectivity of the extracts SP3, SP5, SD1, SD2, SD4 and SE2 was poor and thus they demonstrated toxicity towards both the virus and embryo. The %MEW due to their treatment was not significant to the positive control ($P > 0.05$) (Figure 1) and could reduce the viral by 50% (Figure 3). Higher death rate of embryo up to 70% in four days of observation were contributed with mainly the D extracts which imply being more toxic than other extracts. The toxicity of these extracts could be attributed by the presence of phorbol esters reported in the family Euphorbiaceae and the genus *Synadenium* (Gunjan et al., 2007). The fact that these were toxic to the virus they can be used in biosecurity measure as natural disinfectants for decontamination purposes. With the LSE method, these toxic components were separated from the safer and active extracts, unlike the water extraction which is common among traditional treatments which gives a credit to the method especially when considering safety issues.

Conclusion

This study has demonstrated for the first time the antiviral potential of the extracts of *S. glaucescens*. The results indicate clearly that the plant contains antiviral chemical constituents against ND virus which was shown by the strong negative correlations between the %MEW of the harvested embryo and their HA titres. The polar root bark extracts have indicated more activity despite the extraction method used. The laboratory sequential extraction was able to separate the toxic and the safer content of the compounds. These findings validate the ethnoveterinary uses of the plant and demonstrate a high potential and feasibility of using *S. glaucescens* extracts for treatment and control of ND especially in rural areas where conventional disease management options are limited. This study stands as a stepping stone towards further research on antiviral drug search from *S. glaucescens*.

ACKNOWLEDGEMENTS

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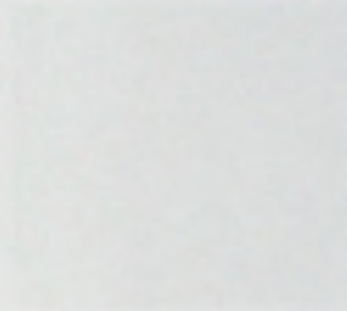
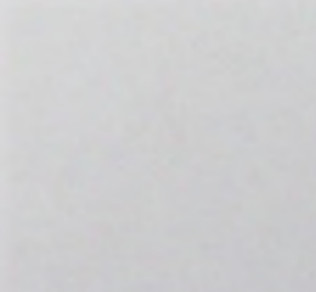
SUPPLEMENT I:

FOR PAPER II: Effect of Ethanolic Extract (ES1) on Embryo Growth: in Photos





SUPPLEMENT I:

FOR PAPERS I

The effect of ethanolic extract of the root on Embryo growth: in Photos

Figure. 1: An embryo which neither challenged with Newcastle Disease Virus nor treated with ES1. Incubated for 5 day, harvested live

Figure 2: An embryo challenged with Newcastle Disease Virus then treated with ES1, incubated for 5 day, harvested live

Figure 3: An embryo challenged with Newcastle Disease Virus, dies within 24 hours of incubation

PAPER III

Antiviral activity of crude extracts of *Synadenium glaucescens* (Pax) against Infectious Bursal Disease and Fowlpox virus

Faith Philemon Mabiki[1, 2], Robinson H. Mdegela[2], Resto D. Mosha[2], and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel: +255 23 260 4647/260 3511- 4, Fax: +255 23 260 4647

[3]Instute of Traditional Medicine, Muhimbili University of Health and Applied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith Philemon Mabiki, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, fmabiki@yahoo.com , fmabiki@suanet.ac.tz

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Journal of Medicinal Plant Research

Full Length Research Paper

Antiviral activity of crude extracts of *Synadenium glaucescens* (Pax) against infectious bursal disease and fowlpox virus

Faith Philemon Mabiki[1]*, Robinson H. Mdegela[2], Resto D. Mosha[2] and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania.
[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania.
[3]Instute of Traditional medicine Department of Natural Product Development and Formulation, Muhimbili University of Health and Applied Sciences, P.O. Box 65001 Dar Es salaam Tanzania.

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The effect of crude extracts from different morphological parts of *Synadenium glaucescens* against infectious bursal disease virus (IBDV) and fowlpox (FP) virus using an *in ovo* assay were investigated. Viable 9 days embryonated chicken eggs were challenged with viral strains then treated with *S. glaucescens* extracts at concentration of 0.2 mg/ml. Un-inoculated group were saved as negative control and groups inoculated with virus and diluent saved as positive controls. The treatments were observed daily and embryo weights were measured 5 days post-inoculation. Embryo survival and mean embryo weight were significantly higher ($P \leq 0.001$) in groups treated with *S. glaucescens* extracts than the positive control. More than 50% of the extract prevented death and deformation of embryo and formation of pock lesions in embryos. Furthermore, the treatments with ethanolic extract of the root bark demonstrated significantly higher mean embryo weight compared to other extract for both viruses ($P \leq 0.001$). The mean embryo weights from eggs challenged with infectious bursal disease virus and fowlpox virus treated with the extract were 6.3 ± 2 and 5.9 ± 0.5 g, respectively. These findings demonstrate potential and feasibility of using *S. glaucescens* extracts for treatment of the viral diseases. Furthermore, it validates the ethnoveterinary exploitation at community level.

Key words: Chicken, gumboro, Fowlpox disease, liyugi, mvunjakongwa and viral infection

INTRODUCTION

Controlling diseases in tropical and sub-tropical countries like Tanzania is a continuing battle. In developing countries like Tanzania where poverty is an issue of serious concern, farmers have opted on traditional treatment and control of both human and animal diseases. This has been taken as a means of buffering up the lack of access and financial resource to afford buying the commercial vaccines and drugs. Traditional plants preparations for decades have been deployed and are increasingly been reported locally as effective to control and treat different kind of diseases including viral diseases affecting chicken. The use of plant extracts for control of viral diseases in rural Tanzania is not uncommon (Buza and Mwamuhehe, 2001).

*Corresponding author. E-mail: fmabiki@yahoo.com. Tel/Fax: +255-23-2603-404.

Table 1. Grouping and treatment allocation for the in ovo assay.

Group (G): n = 5	Treatment
G1 to G15	IBDV+extract+DMSO; 15 different extracts (SP/SD/SE 1-5)
G16 to G30	FPDV+Extract+DMSO; 15 different extracts (SP/SD/SE 1-5)
G31	IBDV alone (V-)
G32	FPVD alone (V-)
G33	IBDV+DMSO (VS+)
G34	FPDV+DMSO (VS+)
G35	Untreated embryonated chicken egg ECE (V-)

Synadenium glaucescens (euphorbiaceae) is been deployed by communities in Tanzania for ethnomedical (Chhabra et al., 1984) and ethnoveterinary purposes (Mabiki et al., 2011; Wickama et al., 2006; Wickama et al., 2004). The plant is known as Liyugi in Bena/Hehe language and Mvunjakongwa in Swahili language. The water extract of the leaves and stems of *S. glaucescens* have demonstrated antimolluscicidal activity (Kloos et al., 1987) and weak inhibition of electrically induced contractions of the guinea-pig ileum (Rukunga et al., 1990). The viral diseases IBD and FP are among serious challenges for development of poultry industry in Tanzania (Yongolo et al., 2002). Despite the reports on the use of the plant in controlling various diseases, there is no report on the use of *S. glaucescens* in controlling both infectious bursal disease (IBD) and fowlpox disease (FPD). The aim of the study was to investigate the effectiveness of extracts from different morphological parts of *S. glaucescens* against infectious bursal disease virus (IBDV) and fowlpox disease virus (FPDV). Findings from this research provide valuable information on the usefulness of the plant against IBD and FPD in chicken.

MATERIALS AND METHODS

Plant collection and processing

Aided with local informants and botanist, the fresh plants were collected in Njombe region, Njombe district in southern higherlands of Tanzania. The samples were identified and confirmed by a botanist and voucher specimen was stored in the herbarium at the Botany Department, College of Natural and Applied Sciences of the University of Dar es Salaam (UDSM) in Tanzania, with specimen's number HOS/FM 3672. The roots, stems and leaves of *S. glaucescens* were cleaned and mechanically separated to get five parts; (1) the root bark, (2) root wood, (3) stem bark, (4) stem wood and (5) leaves. The samples 1 to 5 were air dried and pulverized to a particle size of 1 mm for use during extraction.

Extraction of crude extracts

The five dried and pulverized samples 1 to 5 each were soaked sequentially in solvents with increasing polarities [that is, petroleum spirit (P), dichloromethane (D) and ethanol (E)] twice each for 72 h for each solvent. After filtration, the extract was dried using rotary evaporator to obtain 15 crude extracts. The extracts were stored at 4°C before being used for antiviral tests. Extracts for use were dissolved in dimethyl sulphuroxide (DMSO) to make a concentration of 200 µg/ml. The extracts were coded with two letters, S denoting *Synadenium* followed by letter (P, D or E) denoting solvent type used and number 1 to 5 denoting the plant part.

Antiviral screening

Test organisms

The local strains of IBDV and FPDV were supplied by the Bacteriology and Mycology Laboratory, Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture. *In ovo* assay following the procedure by Sally (2002) and Senne (1998), with slight modification, was used to test the antiviral potential of *S. glaucescens* extracts. Embryonated chicken eggs (ECE) which were 9 days old, were checked for viability by candling before being used. The ECE were randomized into 35 groups (n = 5) and allocated as shown in Table 1. During inoculation, a hole was made through the egg shell just above the air sac to allow vertical inoculation of 0.1 ml of the inoculum into the chorialallantoic fluid. The first 15 groups were treated with 15 inocula made by mixing 0.9 ml of 0.2 mg/ml of different extracts and 0.1 ml of IBDV, and the second 15 groups were treated with 15 inoculum made by mixing 0.9 ml of 0.2 mg/ml different extracts and 0.1ml of FPV.

A group of un-inoculated ECE (V-) served as negative control, two groups of ECE inoculated with 0.1 ml virus suspension only (V+) served as a positive control, and a group of ECE inoculated with 0.9 ml diluents (dimethylsulphoxide) and virus (VS+) served as positive control to study the effect of solvent. After inoculation, the inoculated site was sealed with paraffin wax then the eggs were kept at 4°C (refrigerated) for one hour. The eggs were then incubated at 37°C with the air sac uppermost. Embryos survival was monitored daily by checking the embryo movements, blood vessels and time of embryo death through candling of eggs. Five days post-inoculation, the eggs were chilled and growing embryos were observed for growth and weight change. The assessments of antiviral activity were based on survival of the embryo, mean embryo weight (MEW), formation of pock lesions on eggs and embryo for the eggs infected with FPV and deformation of embryos observed in chicken embryonated eggs infected with IBDV.

Statistical analysis

Data collected were analyzed using CoStat Version 6.400 (CoHort Software, USA). The weights for different groups were reported in

MEW (%) ± standard deviation at 95% confidence interval. The differences of MEW were further analyzed by one-way analysis of variance (ANOVA) and significance was reported at P ≤ 0.05. Comparison of means was performed by Tukey-Kramer test.

RESULTS

Observed time of embryo death, embryo deformation and Pox lesions formation

Death time and embryo conditions during incubation and during harvest are as shown in Table 2. More than 50% of the extracts prevented death, prevented deformation of embryo and also prevented formation of pock lesions. A hundred percent death was recorded in the two positive controls of IBDV (EVS+ and EV+) as well as in embryos treated with *Synadenium* dichloromethane 1 and 2 (SD1, SD2) and *Synadenium* ethanol 2 (SE2) extracts within five days of incubation in IBDV treatment group. In all dead embryos, there was deformation of embryo and pox lesion formation except for SD1 and SD2 groups.

Mean embryo weights of embryonated chicken eggs challenged with IBDV treated with extracts

There was a significant difference between MEW treated with different extracts (P ≤ 0.001) in ECE challenged with IBDV, as indicated in Figure 1. The MEW in the positive control was significantly lower (P < 0.001) than that of the negative control and most of the extracts. There was no significant difference between the positive controls and the four extracts; SE2, SD1, SD4 (P > 0.001). The MEW of treated ECE ranged between 1.3 to 6.3.2 g. Generally, the embryos treated with petroleum ether extracts demonstrated higher MEW well above 4 g, followed by ethanolic extract and dichloromethane extracts which were the lowest. The results further indicated that MEW of the ECE treated with ethanolic extract SE1 were significantly higher (P ≤ 0.001) 6.3 ± 0.2 g compared to all treatment groups.

Mean embryo weights of embryonated chicken eggs challenged with FPDV treated with extracts

Figure 1 shows the data about the effect of different extracts on the weights of FPDV infected embryos. The data shows that there was a significant difference between MEW of different extracts (P ≤ 0.001) in ECE. The MEW in the positive control was significantly lower (P ≤ 0.001) than that of the negative control and majority of the extracts except SP3, SP5, SE2, SD2 and SD1. The MEW of treated ECE ranged between 1.3 ± 0.1 to 6.3 ± 0.2 g. Generally, the embryos treated with ethanolic extract demonstrated higher MEW well above 4 g, followed by and dichloromethane extracts that recorded the lowest MEW. The results further indicate that MEW of the ECE treated with ethanolic extract SE1 were significantly higher (P ≤ 0.001). The extract SE1 recorded MEW of 5.98 ± 0.5 g compared to all treatment groups.

DISCUSSION

This study is the first to report the activity of S. *glaucescens* extracts against IBDV and FPV using chicken embryo model. A hundred percent death of embryo within three days post inoculation implies that the viruses were virulent. It is clear from Table 2 that treatment with extracts prevented death of embryo or prolonged survival of embryo compared to positive control. Furthermore, it prevented embryo deformation in ECE challenged with IBDV and formation of lesions in ECE challenged with FPDV. Treatment with extract SD1, SD2 and SE2 indicated toxicity to the embryo and possibly viral strains. This is indicated by early mortality of the embryos with neither deformation nor pox lesion. The fact that these were toxic to the virus, they can be used in biosecurity measure as natural disinfectants for decontamination purposes.

The mean embryo data in Figures 1 and 2 records higher MEW in most of the ECE treated with extracts which indicates the ability of extract to prevent effects of viral strains on the growing embryo. The effects of the extract is then linked to increase of MEW of infected embryos and thus extract differed significantly in their effect on the viral strains by demonstrating different MEW of the treated embryo for each treatment. The continuation of embryo growth unveiled by increase in weight and organ formation in ECE challenged FPDV implies that the extracts could potentially interfere the viral replication cycle either by blocking one point of propagation mechanisms inside the cells, prevent the invasion mechanism or kill the virus in the Inoculum.

The significant difference in MEW for different extracts could be attributed to the diversity of compounds in the extracts. Each extract had a different degree of inhibitory activity and specificity against the virus and/or its essential enzymes. A diversity of antiviral agents with diversified mechanism is reported in plants (Jasmin et al., 2003; Mohamed et al., 2010). Different researchers have reported the attempts to use plant to inhibit the effects of virus on cells with positive and negative results.

Simon et al. (2007) and Esimone et al. (2007) reported negative results in *in vitro* screening of antiviral activity of more than 9 plant species from the Brazilian flora and Nigeria against IBDV and none of them were active. However, several species are reported to have the potential to inhibit the effect of IBDV and FPDV on cells

Table 2. Embryo deaths as observed from day 1 to day 5 post inoculation with IBDV and FPDV.

Inoculum		Time of embryo death (in days)					Other observations[a,b]
Extract in DMSO	Virus	Day 1	Day 2	Day 3	Day 4	Day 5	
SP1	IBDV						No embryo deformation
	FPV	0	0	0	0	1	Pock lesions
SP2	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SP3	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	1	Pock lesions
SP4	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SP5	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	Pock lesions
SD1	IBDV	3	2	0	0	0	No embryo deformation
	FPV	4	1	0	0	0	No pock lesions
SD2	IBDV	3	2	0	0	0	No embryo deformation
	FPV	3	1	1	0	0	No pock lesions
SD3	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SD4	IBDV	0	0	0	0	1	No embryo deformation
	FPV	0	0	1	2	0	Pock lesions
SD5	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SE1	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SE2	IBDV	1	3	1	0	0	Embryo deformation
	FPV	1	2	2	0	0	Pock lesions
SE3	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SE4	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
SE5	IBDV	0	0	0	0	0	No embryo deformation
	FPV	0	0	0	0	0	No pock lesions
EV+	IBDV	0	0	2	2	1	Embryo deformation
	FPV	0	0	0	0	2	Pock lesions

EVS+	IBDV	0	0	3	2	0	Embryo deformation
	FPV	0	0	0	0	2	Pock lesions

[a]Embryo deformation = included any observed haemorrhage from ruptured blood vessels, perforation in membrane/skin and deformed shape of the embryo. [b]Spot like dots formed on chorio-allantoic membranes/egg shells due to infection with FPV.

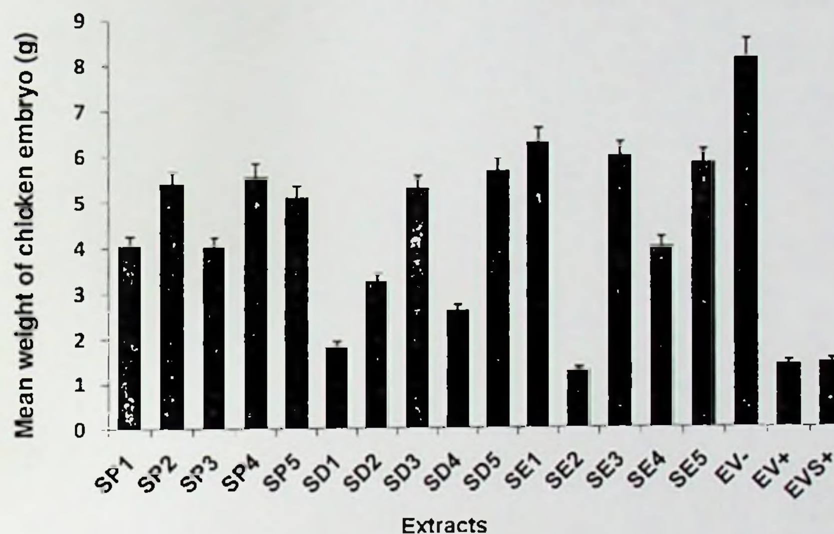

Figure 1. Mean embryo weights following inoculation of embryonated chicken eggs (ECE) with IBDV treated with different concentrations of crude extracts of *S. glaucescens*. Where S = synadenium, P = petroleum ether, D = dichloromethane, E = ethanol, No. 1, 2, 3, 4, 5 = rootbark, root wood, stem bark, stem wood and leaves, respectively. EV+ = positive control group of eggs inoculated with; EVS+ = positive control group of eggs inoculated with IBDV and DMSO, EV- = negative control group of un-inoculated eggs.

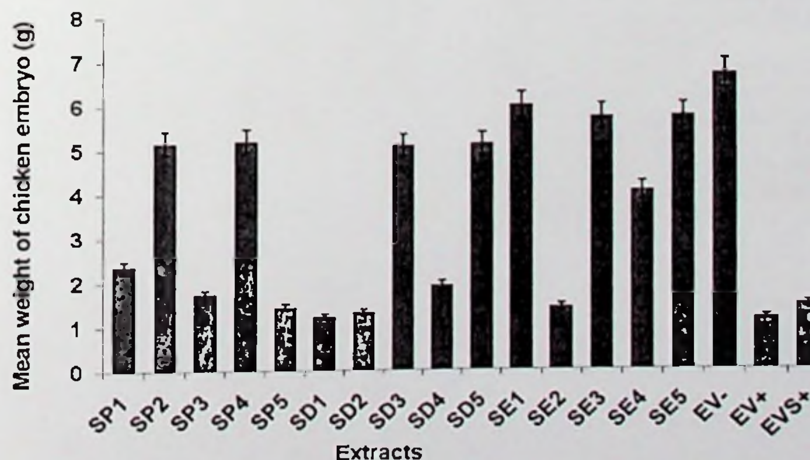

Figure 2. Mean embryo weights following inoculation of embryonated chicken eggs (ECE) with FPDV treated with different concentrations of crude extracts of *S. glaucescens*. Where S = synadenium, P = petroleum ether, D = dichloromethane, E = ethanol, No. 1, 2, 3, 4, 5 = rootbark, root wood, stem bark, stem wood and leaves, respectively, EV+ = positive control group of eggs inoculated with FPDV; EVS+ = positive control group of eggs inoculated with FPDV and DMSO, EV- = negative control group of un-inoculated eggs.

as reported by Meenakshi et al. (2009) on the *in vitro* activity of extracts from *Moringa oleifera*, *Holarrhena antidysenterica*, *Synzium aromaticum*, *Allium sativum*, *Piper nigrum* and *Azadirachta indica* against IBDV. Extracts from leaves of *Acacia arabica* and *Eugenia jambolana* are reported to have inhibited the replication of goat pox virus *in vitro* (Bhanuprakash et al., 2008). Mechanisms of the different compound responsible for the reaction and their mechanism is well explained by Jasmin et al. (2003).

The polar extract ethanolic SE1 recorded higher MEW in treating both ECE challenged with IBDV and pox, it prevented death, deformation and formation of pox lesions in ECE challenged with both IBDV and FPDV. In other studies, the polar extracts of *S. glaucescens* have demonstrated molluscicidal activity (Kloos et al., 1987). The activity of this extract could be attributed by the antiviral agents such as those with sugar moiety and other group of compounds reported to have antiviral activity which were previously reported in various extracts of *S. glaucescens* (Rukanga et al., 1990; Neuwinger, 1994; Jasmin et al., 2003).

Conclusion

These studies have demonstrated for the first time the antiviral potential of the extracts of *S. glaucescens.* The results indicate clearly that the plant extracts from S. *glaucescens* contains antiviral chemical constituents which can act against both IBDV and FPDV. These findings validate the ethnoveterinary uses of the plant and demonstrate a high potential and feasibility of using *S. glaucescens* extracts for treatment and control of IBD and FPD, especially in rural areas where conventional disease management options are limited. This study stands as a stepping stone towards further research on antiviral drug search from *S. glaucescens.*

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PAPER IV

Bioactive Crude Extracts of *Synadenium glaucescens* (Pax) against Selected Bacteria and Fungi of Health Importance

Faith Philemon Mabiki[1], Robinson H. Mdegela[2], Resto D. Mosha[2], and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel: +255 23 260 4647/260 3511- 4, Fax: +255 23 260 4647

[3]Instute of Traditional medicine, Muhimbili University of Health and Allied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith Philemon Mabiki, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania (East Africa). Tel/Fax.: +255-23-2603-404
Emails: fmabiki@yahoo.com, fmabiki@suanet.ac.tz

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BIOACTIVE CRUDE EXTRACTS OF SYNADENIUM GLAUCESCENS (PAX) AGAINST SELECTED BACTERIA AND FUNGI OF HEALTH IMPORTANCE

Faith Philemon Mabiki[1], Robinson H. Mdegela[2], Resto D. Mosha[2], and Joseph J. Magadula[3]

[1]Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania. Tel/Fax.: +255-23-2603-404

[2]Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania. Tel: +255 23 260 4647/260 3511- 4, Fax: +255 23 260 4647

[3]Instute of Traditional Medicine, Muhimbili University of Health and Allied Sciences, P.O. Box 65001 Dar Es salaam Tanzania

Corresponding author: Faith Philemon Mabiki, Faculty of Science, Department of Physical Sciences Sokoine University of Agriculture, P. O. Box 3038, Solomon Mahlangu Campus, Morogoro, Tanzania (East Africa). Tel/Fax.: +255-23-2603-404 Emails:

fmabiki@yahoo.com, fmabiki@suanet.ac.tz

Abstract

The study aimed at investigating the effect of crude extracts from different morphological parts of *Synadenium glaucescens* against selected bacteria and fungi of health importance using an *in vitro method.* Ten bacterial and two fungal strains and isolates were tested using the agar well diffusion method. Thirty crude extracts from root barks, root wood, stem barks, stem wood and leaves of *Synadenium glaucescens* were obtained using cold and hot sequential extraction methods with increasing polarity. Extract (10mg/ml) was used to test the potential to inhibit the growth of the microbes. Activities of extracts were based on the diameter of inhibition zone around the wells. Minimum inhibitory concentration was done using dilution method. All extracts at different strength inhibited the growth of test microbes. Extracts from hot extraction showed more activity compared to those extracts from cold extraction method Gram positive bacteria were more sensitive to extract than the Gram negative. *Salmonella typhimurium* demonstrated the highest resistance towards the extracts. Ethanol extracts demonstrated higher antimicrobial activity as compared to hexane, petroleum ether and dichloromethane extracts. The highest inhibition was demonstrated by Dichloromethane extract of the stem wood (32mm) against *S. pyogenes.* Eighty seven (87%) and 62.7% of extracts demonstrated moderate to strong growth inhibition against *C. albicans* (ATCC 90028) and *A. niger* (AZN 8240) respectively. The study validates the use of *S. glaucescens* for traditional medication as reported by different ethnic groups in Tanzania against bacteria and fungal infections.

Key words: *Antibacterial, antifungal, crude extracts,* Euphorbiaceae, *Soxhlet,*

Introduction

The vast increase of infectious diseases and occurence of microbial resistance have accelerated the search for newer and alternative compounds (Cowan, 1999). Medicinal plants are essential natural resources which constitute one of the potential sources of new products and bioactive compounds for drug discovery and development (Fabricant and Farnsworth 2001). Use of plants as the cheap and accessible source of medicines has been practiced in poor communities around the world. In Tanzania, the majority poor have for long been utilizing the indigenous knowledge (IK) and experience in treating and controlling both human and animal diseases using plants as a copying strategy for the ever increasing cost of the conventional drugs. Traditional medicines have been the most common form of primary healthcare in Tanzania (Stangeland et al 2008). It is estimated that 60% of the urban and 80% of the rural population in Tanzania depend on traditional medicines, mostly drugs derived from plants for their primary health care (Mbwambo et al 2007). Several plant species are documented being used by different ethnic systems in the country, *Synadenium* being among them (Moshi et al 2012, Kitula 2007, Maregesi 2007, Augustino et al 2011 and Amri and Kisangau 2012 and Mabiki, 2013ab).

The genus *Synadenium* (Euphorbiaceae) consists of about 15 species which are distributed in tropical and subtropical regions of Africa and the Americans. The species of the genus *Synadenium* are used in different ethinic systems in the world for traditional treatments of diseases. *Synadenium grantii* (African milk bush), is reported to be used for wound treatment and with potential antitheilerial activity, (Kinabo *et al.,* 2002). *Synadenium pereskiifolium* is a key recipe in traditional anti-asthmatic preparation Kerstin *et al.,* (1991) and *S. umbellatum* used to treat several diseases in Midwest Brazil (Nogueira *et al.*, 2008). A d-galactose-binding lectin from *S. carinatum,* latex have showed anti-asthmatic and immunoregulatory potential (Rogerio, *et al.*, 2007). *Synadenium compactum*

is the most utilised plant species in traditional management of East Cost Fever (ECF) in cattle by Kikuyu in Kenya. Treatment involves direct application of plant extracts especially latex on the lymph nodes or the plant liquid preparations that are given orally to the cattle (Njoroge *et al*., 2006). *Synadenium volkensii* is used for treatment of Newcastle diseases in chicken and East coast fever in cattle in Tanzania (Minja 1994).

Synadenium glaucescens Pax (*Euphorbia neoglaucescens* Bruyns), in Swahili "*Mvunjakongwa*" and in hehe/bena *"Liyugi"* is endemic and grows in several regions in Tanzania (Bruyn 2006). *Synadenium glaucescens* though reported poisonous and perceived as of no medicinal value, ethnic communities in Tanzania have used it effectively in the treatment and control of human and animal diseases. In the costal, Morogoro and Kilimanjaro region, the juice of fresh, crushed leaves is drunk to treat excessive menstruation and as a purgative (Chhabra et al., 1984). A leaf decoction with lime juice, baking soda and honey added is drunk to treat asthma; the ashes of dried leaves are mixed with water and applied to treat leprosy (Schmelzer et al., 2008). A root bark extract is taken with sugar to treat severe cough, tuberculosis and as ear drops to treat earache (Newmark 2002, Neuwinger 2000, Schmelzer et al., 2008). In Tanga region the plant in named "*Muuwi*" in Shambaa and is used to prepare medicine to human and for control of poultry diseases mainly Newcastle Disease (Wickama et al., 2006). Despite the reported use of the *S. glaucescens* among the community in the country, there is limited scientific publication on its antibacterial and antifungal activity. This study aimed at investigating the antibacterial and antifungal effect of extracts of *S. glaucescens* against bacteria and fungi of health importance, to validate its use among the community.

Materials and methods

Plant collection and processing

Aided with local informants and botanist, the fresh plants were collected in Njombe region, southern higherlands of Tanzania. The samples were

identified and confirmed by a botanist and voucher specimen was stored in the herbarium at the Botany Department, College of Natural and Applied Sciences of the University of Dar es Salaam (UDSM) in Tanzania, with specimen's number 3672. The roots, stems and leaves of *S. glaucescens* were cleaned and mechanically separated to get five parts; (1) the root bark, (2) root wood, (3) stem bark, (4) stem wood and (5) leaves. The samples 1 to 5 were air dried, to avoid any chemical decomposition; dried plant materials were stored in cool dry place and pulverized into smaller particle sizes of 1mm diameter just before the extraction

Extraction of crude samples

Cold Sequential extraction

Cold sequential extraction (CSE) method was done targeting labile compounds. The dry samples were soaked sequentially in petroleum spirit, dichloromethane and then ethanol twice each for 72 h for each solvent. After filtration with filter paper watman no.1, the extracts were dried using rotary evaporator to obtain 15 dry crude extracts which were coded as indicated in Table 1. The extracts were stored at 4°C before being used for antibacterial or antifungal tests. Extracts for use were dissolved in dimethyl sulphoxide (DMSO) to make a specific concentration.

Hot Sequential Extraction (Soxhlet extraction)

Soxhlet extraction technique is a common technique employed for the extraction and separation of heat stable compounds in medicinal plant. Ten grams (10g) of the dry ground samples (1mm diameter) was placed into thimbles (33mm diameter, 80mm length) in the extraction chamber and extracted using a common Soxhlet apparatus consisting of a condenser, a Soxhlet chamber, and an extraction flask. Sequential extraction with 200ml of each solvent (hexane, dichloromethane the ethanol), extraction time of 4hours at a temperature of 30°C for DCM and 70°C for Ethanol was employed. Thimbles containing the samples were air-dried at room

temperature for 24hours before adding the next solvent. Liquid extracts obtained after filtering with filter paper watman no.1, were evaporated using the rotary evaporator to obtain the dry crude extracts. The samples were coded as shown in table 1. The extracts were stored at 4°C before being used for antibacterial or antifungal tests. Extracts for use were dissolved in dimethyl sulphoxide (DMSO) to make a specific concentration.

Table 1 Codes of Extracts used during the study

Plant Part (PP)	PP Code	Cold / Soxhlet (S) extraction		
		Petroleum ether (P) / Hexane (H)	Dichloromethane (D)	Ethanol (E)
Root Bark	1	P1/SH1	D1/SD1	E1/SE1
Root wood	2	P2/SH2	D2/SD2	E2/SE2
Stem Bark	3	P3/SH3	D3/SD3	E3/SE3
Stem Wood	4	P4/SH3	D4/SD4	E4/SE4
Leaves	5	P5/SH4	D5/SD5	E5/SE5

Antimicrobial screening

Test microorganisms

Eight standard strains and 7 local (L) isolates were tested. Bacterial strains included Gram positive *Staphylococcus aureus* (ATCC 29213 and ATCC 259230), *Enterococcus faecalis* (ATCC 29212). Gram negative; *Escherichia coli* (ATCC 25922 and ATCC 259523), *Pseudomonas aeruginosa* (ATCC 27853). Four local isolates were *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Gram negative) and *Bacillus subtilis* and *Streptococcus pyogenes* (Gram positive). Two Standard fungal strains were *Aspegillus niger (AZN 8240), Candida albicans* (ATCC 90028). Bacterial cultures cells were maintained at 37°C on Muller-Hilton (MH) agar on slants until needed. Fungal cultures were maintained at 37°C on Saborouds dextrose agar SDA on slants until needed. All microbial species used were supplied by the Bacteriology and Mycology Laboratory, Department of Veterinary Microbiology and Parasitology, Sokoine University of Agriculture or Faculty of Veterinary Medicine, Department of Paraclinical Science, Pretoria University.

Antibacterial and antifungal testing

The antimicrobial assay was done by using the agar well diffusion method with reference to Perez, *et al.*, (1990). Muller- Hinton (MH) or Saboroud dextrose agar (SDA) plates were prepared by pouring 20 ml of warm molten into the sterile Petri dishes plates then incubated at 37^0C for 6hours. The bacterial and fungal isolates were serially diluted into inoculum of $1X10^{-2}$cfu streaked into MH plates, and left for about 30 minutes to dry. After allowing the plates to dry, wells with 6 mm diameter were punched with sterile cork borer on each plate and get labeled in accordance to the test extract bacterial. A test extract 100 µl of a 10mg/ml were introduced into the well, left to defuse for 30 minutes then incubated at 37°C for 24 hrs. Gentamycin 10 µg/ml and Ketoconazole (200 µg/ml) were used as positive control for bacteria and fungi respectively and DMSO as a negative control. The assessment of antimicrobial activity was based on measurements of the diameter (in mm) of the zone of inhibition formed around the wells. The experiments were done in duplicate. Inhibition zone data were analysed descriptively using Microsoft excel statistical package (2007).

Minimum inhibitory concentration (MIC)

Minimal inhibitory concentrations (MIC) are regarded as the lowest concentration of extract that inhibits growth of test organisms. Standard two-fold dilution techniques by Eloff (1998) and Obi *et al.* 2007 were adopted at different times. The assay was initiated by pouring sterile water aliquots (100 µl) into wells of microtitre plates. Exactly 100 µl of 10 mg/ml extract prepared in acetone or 50 mg/ml in DMSO was added in row A and mixed using a micropipette. The two-fold dilution technique was performed to row H and the additional 100µl in row H was discarded. Two columns were used as sterility control (no cultures were added) and negative growth control (the extracts were replaced with 100 µl of acetone, DMSO or 50% DMSO/Acetone). A 0.5 McFarland standard suspension of test bacteria was made in nutrient broth,

from which 100 μl of the final inoculums containing approximately 1 x10^{8}cfu/mL was added to the appropriate wells to make a final volume of 200 μl in each well except the sterility controls. The microtitre plates were sealed in a plastic bag with a plastic film sealer before incubating at 37°C in humidified incubator for 18 hours for bacteria and 28 °C for 48hours for fungi. After incubation 40 μl of 0.2 mg/ml INT was added to each well and plated were incubated for a further 2 hours before observation in antibacterial activity assays. The development of red colour, resulting from the formation of the red/purple formazan, was indicative of growth (positive indicator of cell viability). MIC values were regarded as the lowest concentrations of the extracts that inhibit the growth of the test organisms (decrease in the intensity of the red formazan colour). Gentamycin and Ketoconazole were used as a positive control in the antibacterial and antifungal tests respectively. The experiments were performed in duplicate.

Results

Antibacterial activity of extracts obtained by the cold and Hot extraction method

The antibacterial results are as shown in figures 1 and 2. The results show that extracts were able to inhibit the growth of both Gram positive and Gram negative bacteria despite the method and solvent of extraction. Generally, extracts from Soxhlet extraction showed more activity compared to the extracts from cold extraction and the Gram positive were more sensitive than the Gram negative. The highest inhibition was demonstrated by D4 (32mm) against *S. pyogenes*. High sensitivity of *S. pyogenes* to the crude extracts was also seen with D2, E1, E2, E4, SE1 and SE3 with zone of inhibition (ZI) of 30mm and P2 with ZI of 25mm. *Streptococcus pyogenes* was sensitive to all the extracts with lowest ZI of 8mm demonstrated by D1. The same sensitivity was observed with *B. Subtilis (L)* where 96% of the extract were inhibited the growth of *S. aureus* (ATCC 259230).

Pseudomonas aeruginosa (L) isolate were the most susceptible Gram negative bacteria followed by *E. coli* (ATCC 259523) and *S. typhimurium (L)* being the least sensitive. All extract except SD1 inhibited the growth of *E. coli* (ATCC 259523). Only 13.3% of the extracts that is D2, E1, E4 and E5 inhibited the growth of *S. typhimurium (L)* with ZI of 10.5 mm, 15 mm, 20 mm and 16 mm respectively. *Salmonella. typhimurium (L)* is considered the most resistant of test microbes used in this study. The ethanolic extract despite the method of extraction demonstrated higher inhibition potential compared to petroleum ether, hexane and dichloromethane.

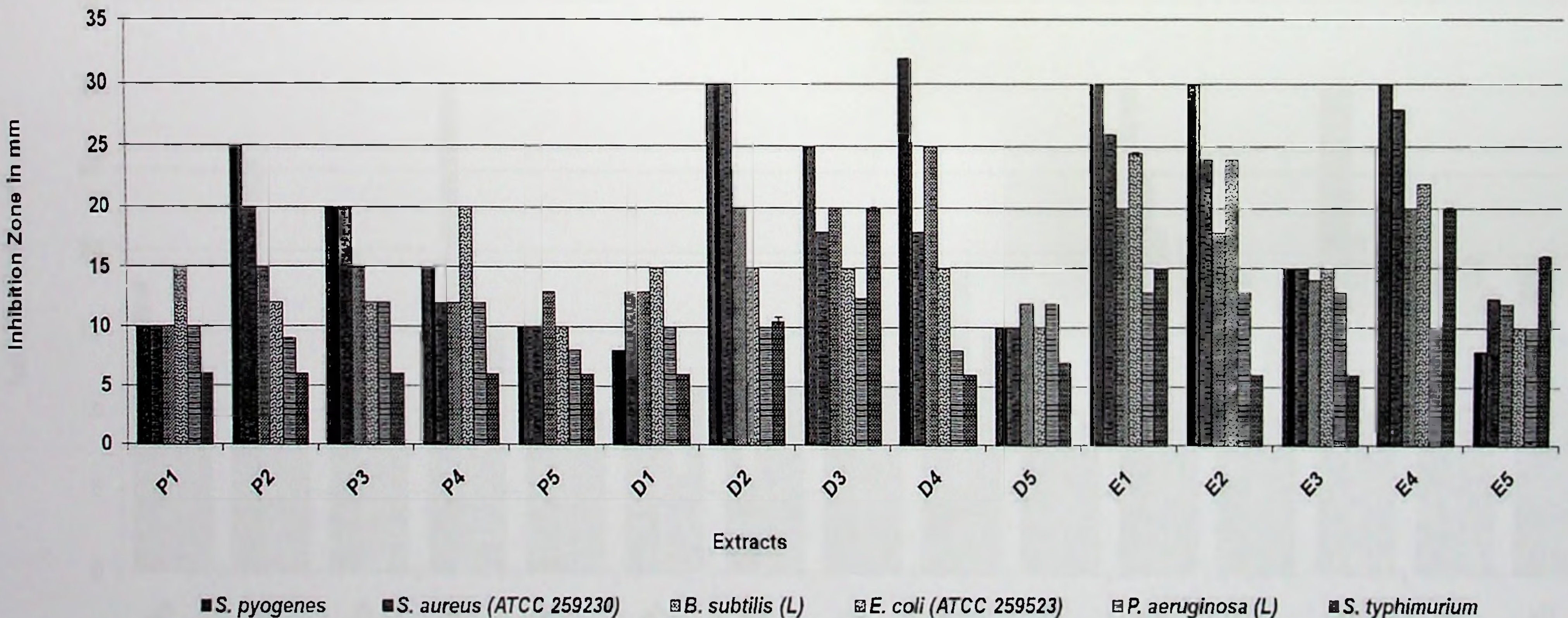

Figure 1: Antibacterial Zone of inhibition demonstrated by *S. glaucescens* extracts obtained using cold extraction method. Diameter of the well 0.6 mm (included). P = Petroleum ether, D = dichloromethane, E = Ethanol. No. 1, 2, 3, 4, 5, 6 = rootbark, root wood, stem bark, stem wood and leaves. Zone of inhibition of 6mm indicated no activity.

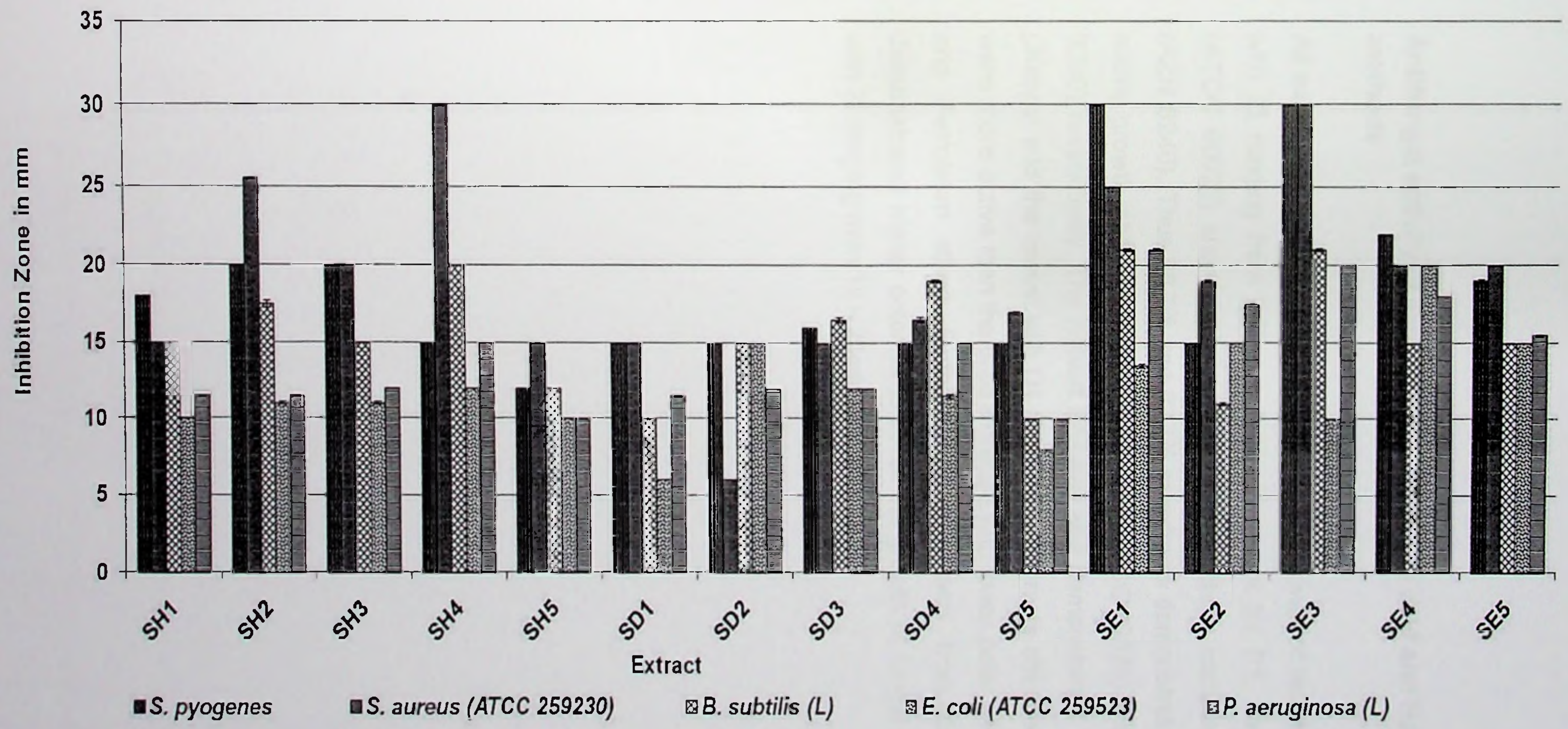

Figure 2: Antibacterial Zone of inhibition demonstrated by *S. glaucescens* extracts obtained using hot extraction method. Diameter of the well 0.6 mm (included). P = Petroleum ether, D = dichloromethane, E = Ethanol. No. 1, 2, 3, 4, 5, 6 = rootbark, root wood, stem bark, stem wood and leaves; S = Soxhlet. Zone of inhibition of >6 mm indicated no activity.

Antifungal activity of extracts obtained by the cold and Hot extraction methods

All extracts at different strength inhibited the growth of test fungal microbes with ZI ranging from 7-30mm (Table 2) except for P1. *Candida albicans* (ATCC 90028) showed slightly higher susceptibility compared to *A. niger* (AZN 8240). Thus 86.7% and 62.7% of extracts demonstrated moderate to strong growth inhibition against *C. albicans* (ATCC 90028) and *A. niger* (AZN 8240) respectively. The highest inhibition was demonstrated with extract P4 (30mm) and the lowest with D1 (7±0.8mm). Extracts obtained from Ethanol were more active than the once extracted with lower polar solvents (Hexane and Petroleum ether). Furthermore the extracts from Soxhlet method demonstrated higher potential to inhibit the growth of fungal species in test with ZI ranging from 12 – 25mm.

Table 2: Growth inhibition zones demonstrated with *S. glaucescens* extracts on growth of test fungi

Extract	Zone of inhibition in mm ± SD	
	C. albicans (ATCC 90028)	*A. niger (AZN 8240)*
P1	10	NA
P2	14.5±0.05	10
P3	12	10
P4	30	10
P5	10	10
D1	12	7±0.08
D2	20	14
D3	15	14
D4	25	13
D5	20	10
E1	12	15
E2	10	14.5±0.05
E3	16	8
E4	20	20
E5	12	10
SH1	12	14
SH2	16	20
SH3	14	21.5±0.05
SH4	10	10
SH5	12	15
SD 1	12	20
SD 2	12	15
SD 3	12.5±0.05	27
SD 4	13.5±0.05	25
SD5	12	22
SE1	14.5±0.05	25
SE 2	14	12.5±0.05
SE 3	15	16
SE 4	14	16
SE 5	18	15
Control (+)	1.8	1.8
Control (-)	0	0

Diameter of disk = 6.0 mm (inclusive); NA=no inhibition, 7-10mm= Weak sensitivity; 11-14mm= moderate sensitivity; >14 = strong sensitivity. P = Petroleum ether, D = dichloromethane, E = Ethanol. No. 1, 2, 3, 4, 5, 6 = rootbark, root wood, stem bark, stem wood and leaves; S = Soxhlet. Zone of inhibition of >6 mm indicated no activity.

Minimum inhibitory concentrations of some selected active extracts

Minimum Inhibitory Concentrations for some of selected the active extracts are indicated in table 3. Gram positive bacteria were inhibited more thus lower MIC values compared to Gram negative bacteria. E1 and SE1 demonstrated the lowest MIC values (0.08 mg/ml) for both *S. aureus* strains. The lowest MIC value for Gram negative strains were 0.125mg/ml demonstrated with SE1 to *E. coli* (ATCC 25922).

Table 3: Minimum inhibition concentration (mg/ml) of some selected extracts

	Minimum inhibition concentration in mg/ml					
Plant extracts	***S. aureus (ATCC 259230)***	***S. aureus (ATCC 29213)***	***E. coli (ATCC 259523)***	***E. coli (ATCC 25922)***	***P.aeruginosa (ATCC 27853)***	***E. faecalis (ATCC 29212***
E1	0.08	-	0.63	-	-	-
E3	0.31	-	1.25	-	-	-
SD1	0.125	0.06	6.25	NI	1.000	0.06
SE1	0.08	0.125	0.63	0.125	0.250	0.25

E1 = ethanolic extract of the root obtained by cold extraction, E3 = ethanolic extract of the stem bark obtained by cold extraction, SD1 = dichloromethane extract of the root bark obtained by Soxhlet extraction, SE1 = ethanolic extract of the root obtained by cold extraction, NA= No inhibition.

Discussions

This study reports for the first time the *in vitro* antibacterial and antifungal potentials of the extracts of *S. glaucescens,* and so it saves as scientific proof of the ethnomedical uses as mentioned by the community. The results

Discussions

This study reports for the first time the *in vitro* antibacterial and antifungal potentials of the extracts of *S. glaucescens,* and so it saves as scientific proof of the ethnomedical uses as mentioned by the community. The results indicate that, extracts are active against both bacteria and fungi and therefore the plant has the potential for treatment of bacterial and fungal diseases. The Gram positive bacteria were generally more susceptible compared to Gram negative bacterial isolates. This is in agreement with previous reports that plant extracts are more active against Gram positive bacteria than Gram negative bacteria (Vlietinck et al, 1995; Rabe and Van Staden, 1997). The resistance of against most Gram negative bacterial strain antibiotics is associated with the presence of double membrane layer and trans-membrane efflux which together lead to combined exclusion effect to antimicrobial compounds (Parekh and Chanda (2007). Furthermore, the highest resistance to the crude extracts shown is also reported in common used antibiotics. Studies by Parekh and Chanda (2007) who tested twelve species of Indian medicinal plants, found that *S. typhimurium* were resistant to all tested plants. The resistance of *S. typhimurium* is attributed to its genetic makeup as explained by Brisabois et al., (1997). *S. typhimurium* is build up with resistant genes, PSE and CARB-type, located on an integron, a new family of genetic components into which many resistance agents can fit (Brisabois et al., 1997). These genes are responsible with resistance in both the human and animals.

The ethanol extracts were found to be the most effective antimicrobial agent as compared to other extract. This possibly, were attributed by type of compounds in the extract, their intrinsic bioactivity, their ability to dissolve or diffuse in different media used in the assay and their ability to penetrate to the microbes (Eloff,1998, Costa et al., 2012). Ethanolic extract contains polar compounds such as phenolic compounds such hydrolysable and condensed tannins, coumarins and anthraquinones, anthocyanins, flavonoids and

glucosides which are usually stable, highly polar and of low molecular weight, These properties are responsible for the bioactivity of many polar extracts of *Synadenium* genus (Costa et al., 2012). In another study a pharmacologically active glucoside was isolated from aqueous extract of the leaves of S. *glaucescens* and was responsible for the inhibition of the electrically induced contractions of a quinea pig ileum (Rukanga et al., 1990). Similar studies indicated higher antibiotic activity of *S. grantii* methanol extracts against the ATCC strains of *Escherichia coli, Pseudomonas aeruginosa*, *Enterococcus faecalis, Staphylococcus aureus* and *Candida albicans* (Chougale et. al., 2011*)*. The aqueous extract of the leaves and stems of *S. glaucescens* is reported to have given positive reaction for tannins, triterpenoids and coumarins while the methanol extract showed the presence of steroids, triterpenes and anthocyanins and the ether extract contained carotenoids, steroids, triterpenoids, volatile oils and glucosides (Neuwinger 1994, Rukunga *et al.*, 1990). The activity shown by less polar solvent is attributed by the presence of mainly triterpenes and phorbal ester reported in this genus (*Larissa* et. al., 2012, Chougale *et. al., 2011,* Mwine and Damme, 2011*)*.The root bark extract were more active compared to other morphological parts of the plant. Dichloromethane extract inhibited both gram negative and gram negative bacteria and fungi. It showed the lowest MIC value of 0.06mg/ml *with S. aureus* (ATCC 29213) and *E. Faecalis* (ATCC 29212). The ethanol extract of the root back (E1 and SE1) despite the method of extraction have shown the broad nature of activity. The ethanol extract of the root inhibited all test micrograms with the lowest MIC value of 0.08mg/ml against ATCC 259230. The same trend was observed with antifungal activity. Despite that less polar compound been reported to be of high antifungal activity, polar fractions of *S. glaucescens* have shown higher activity compared to less polar fraction from Petroleum, hexane and dichloromethane solvents. Extracts from less polar fraction contains terpenoids which responsible for most antibacterial and fungal activity (Masoko and Ellof 2005).

Majority of the extract from soxhlet extraction demonstrated higher activity compared to cold extracts. This could be attributed by the purity nature of the compound and nature of the compounds. Cold extraction technique is usually employed to avoid heat decomposition for labile compounds in plants and is an extensively technique employed for the extraction and separation of chemical constituents in medicinal plant. However most of ethnomedical preparation involves heat at some stage. Soxhlet extraction method is a hot extraction and considered to be superior to cold extraction in minimizing time, solvent use and give higher yield however during the process the labile compound may decompose.

Novel use of microtitre plates

Agar well diffusion method is limited to diffusivity of components thus could be used for quantification purposes. Standard two-fold microdilution techniques were adopted for Minimum inhibitory concentration (MIC). Microdilution advantage over diffusion techniques include increased sensitivity for small quantities of extract, ability to distinguish between bacteriostatic and bactericidal effects and ability to avail quantitative determination of the MIC. It is as well a cheap and presents reproducible results (Klančnik *et. al.* 2010).

Conclusion

This study discloses the ability possessed by extracts prepared from different morphological parts of *S. glaucescens*, obtained using different extraction methods possessing the ability to inhibit the growth of standard and local isolates of bacteria, and fungi of clinical importance *in vitro*. This implies that *Synadenium glaucescens* extracts possess compounds with antimicrobial properties that can be used as antimicrobial and antifungal agents. The study further validates the use of S. glaucescens for traditional medication as reported by different ethnic groups in Tanzania. The ethanol extract have demonstrated more potency than other and this is useful information in attempts towards development of new drugs. Further studies are needed for the ethanol extracts of the root bark for isolation of

responsible compounds and for further pharmacological evaluations.

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SUPPLEMENT II:

For Papers I-IV: Sample extraction Methods and the Crude Extracts Yield

SUPPLEMENT II:

FOR PAPERS I – IV

Sample extraction Methods and the Crude Extracts Yields

1.0 Introductions

Growth and maturation of plant tissues involve a series of complex reactions, which leads to differences in the phytochemistry of the plants (Mahmood *et al.*, 2012). In addition to that different parameters such as season, variety, stages of maturity and climatic conditions influence the phytochemical composition of a plant Gull e*t al.*, 2012). Climatic factors are very important because they can interfere in the chemical biosynthesis particularly in the final concentration of bioactive compounds. With this regard the effect of geographical location was considered in order to minimize the possible error during bioactivity studies. Therefore all samples for bioactivity studies were collected from the same location throught the study and only male plants were used.

1.1 Plant Sample Collection, processing and Extraction

The samples collected were divided into 5 parts and coded as indicated in in papers II to IV.

1.2 Extraction Methods

1.2.1 Hot and cold extraction

Crude samples were extracted using cold and hot extraction methods depending on type of crude extract needed. Cold extraction technique is usually employed to avoid heat decomposition for labile compounds in plants and is one of the most extensive

techniques employed for the extraction and separation of chemical constituents in medicinal plant. However most of ethnomedical preparation involves heat at some stage. Local extraction methods were adopted based on the information provided during the survey and was used to prepare local crude extract when needed. Conventional solvent extraction method used included cold soaking and soxhlet extraction method. Soxhlet extraction method is a hot extraction and considered to be superior to cold extraction in minimizing time, solvent used and giving higher yield. However during the process the labile compounds may decompose. Five solvents including hexane, petroleum ether, dichloromethane, ethanol and water were used for extraction. Sequential extraction using three solvents were done using Hexane (H) or Petroleum ether (PET), Dichloromethane and ethanol (E) respectively. The yields were calculated using equation 1. in Paper V.

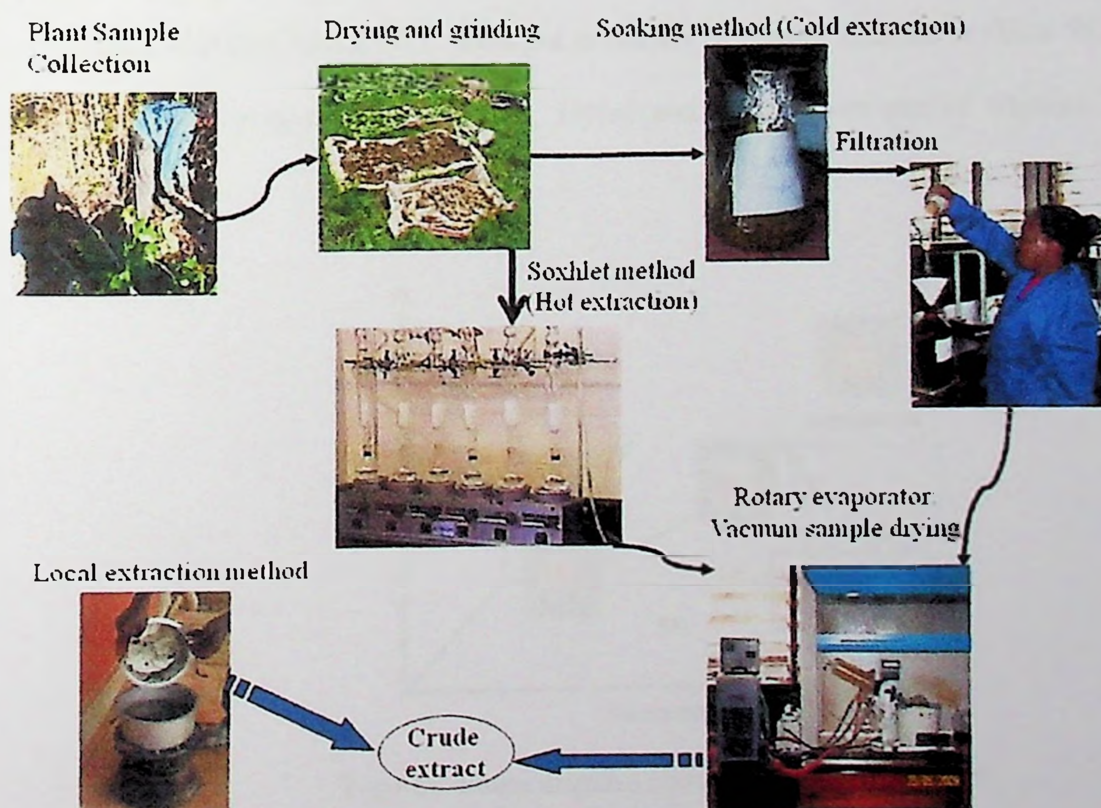

Fig. 1: Different extraction methods for obtaining crude extract

1.3 Supercritical Carbon dioxide Extraction

Supercritical fluid is a rapidly developing method using supercritical fluid (SCF) to produce bioactive compounds under mild conditions (Simandi *et al.*, 2002). A SCF is defined as a substance above its critical temperature (T_C) and critical pressure (P_C). The critical point represents the highest temperature and pressure at which the substance can exist as a vapour and liquid in equilibrium (Brunner 2010). The phenomenon can be easily explained with reference to the phase diagram for pure carbon dioxide. Supercritical fluids commonly used include supercritical carbon dioxide ($ScCO_2$) and supercritical water (ScH_2O). Supercritical carbon dioxide extraction was done for the *S. glaucescens* rootbark (coded as $SgRCO_2$) and the *S. glaucescens* leaves (coded as $SgLCO_2$). During the study 134g and 106g of milled SgRb and SgL respectively were put in $ScCO_2$ extraction equipment (Thar SCF500 CO_2), and then extracted at 50°C, 350bar and $ScCO_2$ flow rate of 40g/min for 4 hours.

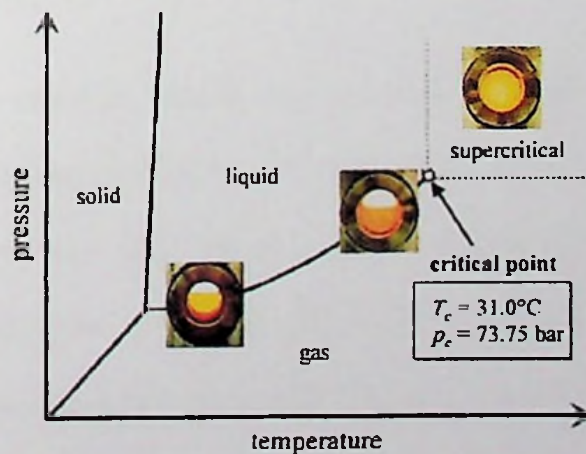

Figure1: Phase diagram showing $ScCO_2$ conditions

1.4 Extraction yields from different extraction techniques

1.4.1 Comparison between cold and hot extractions

Extraction techniques used during extraction yielded different amount depending on the methods and the extracting solvent. Generally the yields for soxhlet extraction were higher compared to the cold extraction technique and SCO_2 extraction method. The yields lowered towards ethanol extracts except for the ethanol extract of the root bark (SE1) with the highest yield of 14%. This indicates that, SE1 is composed more of polar components than less polar (More Phytochemical work of SE1 is reported in Paper V. Soxhlet technique uses the Soxhlet apparatus, at elevated temperatures and has reported to yield more than other methods such as cold extraction (Ntonifor *et al.*, 2002). Although this method and other methods involving heat are reported to be prone to contamination from co-extracted lipids, artefacts formation, or losses of labile or volatile compounds, the method was adopted considering that, most of the traditional preparation involve boiling and thus the labile/volatile components were not of interest for this purpose.

Cold extraction of the root bark yielded much lower which signify that the plant accumulates little secondary metabolites and much water or primary metabolites. Higher yield from the root bark indicated more accumulation of secondary metabolite in the root bark; this may explain why the root is reported more frequently used as traditional medicine practices than other parts of the plant (Kitula, 2007).

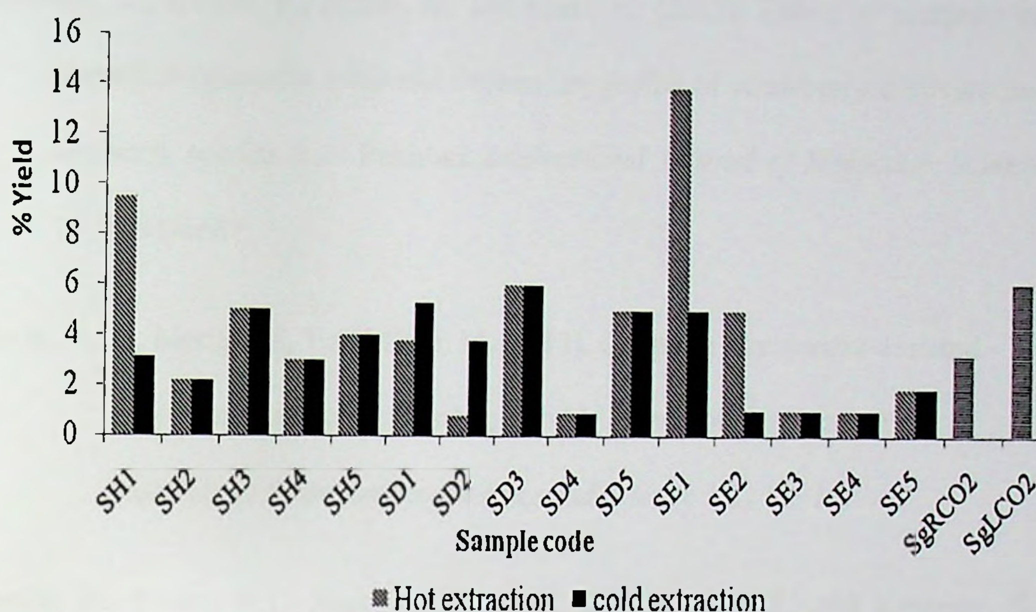

Figure 20: Percent yields of crude extracts

D=Dichloromethane, P= hexane, E= Ethanol, l=leaves, W= wood, S= stem, b=bark, R = Root

1.4.2 SCO_2 extraction

Supercritical carbon dioxide extraction yielded higher for the leaves than roots. This can be explained by considering the solvent extraction condition set such as solvent properties. Solvent properties for SCO_2 are tuneable and thus yield can be increased by tuning the operation parameters of the SCO_2 extraction such as temperature, pressure and mixing of other solvent during the process (Quitain, *et al.*, 2013).

1.5 References

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PAPER V

Optimization of Extraction Conditions and Phytochemical Screening of Root Extract of *Synadenium glaucescens* Pax

Faith P. Mabiki [1,3], Joseph J. Magadula[2], Robinson H. Mdegela[3] & Resto D. Mosha[3]

[1]Faculty of Science, Sokoine University of Agriculture, Morogoro, Tanzania

[2]Institute of Traditional medicine, University of Health and Allied Sciences, Dar es Salaam Tanzania

[3]Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania

Correspondence: Faith P. Mabiki, Department of Physical sciences, Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Morogoro, Tanzania, Tel/Fax: +255-23-2603-404. Email: fmabiki@gmail.com, fmabiki@suanet.ac.tz

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Faith P. Mabiki[1,3], Joseph J. Magadula[2], Robinson H. Mdegela[3] & Resto D. Mosha[3]

[1] Faculty of Science, Sokoine University of Agriculture, Morogoro, Tanzania

[2] Institute of Traditional Medicine, University of Health and Allied Sciences, Dar es salaam, Tanzania

[3] Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro, Tanzania

Correspondence: Faith P. Mabiki, Department of Physical Sciences, Faculty of Science, Sokoine University of Agriculture, P. O. Box 3038, Morogoro, Tanzania. Tel: 255-23-260-3404. E-mail: fmabiki@gmail.com, fmabiki@suanet.ac.tz

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Abstract

Optimization of extraction conditions and phytochemical screening of the root bark of *Synadenium glaucescens* were carried out in a stepwise manner in order to obtain the highest yields and the constituents of the extracts. Sequential extraction using Soxhlet method was performed using dichloromethane, hexane and petroleum ether, respectively, each followed by ethanol. Extraction conditions included: running time of 2 to 6 hours, temperature at 25 °C to 95 °C and particle size ranging from 0.4mm to >3mm diameter. Phytochemical screening was done using derivatisation techniques, gas chromatography-mass spectrometry and high performance liquid chromatography. Extraction with dichloromethane followed by ethanol resulted in a higher yield by 25%, within 4 hrs of extraction, particle size of 1mm, at temperatures of 30 °C for dichloromethane and 75 °C for ethanol. Fatty acid analysis indicated absence of free fatty acids in both Dichloromethane and ethanolic extracts. Silylation and Thin Layer Chromatography indicated the presence of non hindered and hindered functionality and the presence of triterpenoids in the dichloromethane extract. Phytochemical screening of the dichloromethane extracts indicated that it is composed of two main triterpenoids that best matched with Lanosterol (42%) and Cycloartenol (31%). Other minor compounds identified through chromatographic analysis were phytol, ergostadiol, hentriacontane, sitastirol aceate, lupeol and hopenone. The ethanolic extracts indicated the presence of polyphenolic compounds.

Keywords: HPLC, GCMS, soxhlet extraction, phytochemical screening

1. Introduction

The members of the Euphorbiaceae family are widely utilized for different purposes in the world (Mwine & Damme, 2011). The genus *Synadenium* is indigenous to East Africa (Dev & Koul, 1997). The species *Synadenium glaucescens* (*Mvunjakongwa* in Swahili, *Liyugi* in *Bena* language) are found growing in several regions in Tanzania and indigenous people have been using them for treatment of both animal and human illnesses. A juice made from freshly-crushed leaves is usually ingested for treatment of excessive menstruation (Chhabra, Uiso & Mshiu, 1984). A leaf decoction with lime juice, baking soda and honey added is ingested to treat asthma; the ashes of dried leaves are mixed with water and applied to the skin to treat leprosy (Schmelzer, Gurib-Fakim, Arroo, Bosch, de Ruijter, & Simmonds, 2008). A root bark extract is taken with sugar to treat a severe cough or tuberculosis or taken as an ear drop to treat an ear ache (Schmelzer et al., 2008). The same species is also used to control poultry diseases such as Newcastle disease (Wickaman, Mbaga, Madadi, & Byamungu, 2006). The latex is used as a fish poison and water extracts of the leaves have demonstrated antimolluscidal activity against *Biomphalaria pfeifferi* (Kloos, Thiongo, Ouma, & Butterworth, 1987; Neuwinger, 2004).

Water is a common traditional solvent used during ethnomedical extractions. However, technical difficulties exist in the isolation of pharmacologically active compounds from aqueous extracts. The aqueous extract of the leaves and stems of *S. glaucescens* is reported to have a positive reaction for tannins, triterpenoids and coumarins while the methanol extract has steroids, triterpenes and anthocyanins and the petroleum ether extract contains carotenoids, steroids, triterpenoids, volatile oils and glucosides (Neuwinger 1994; Rukunga, Gunnar, & Kofi-Tsekpo, 1990). The extracts prepared from the roots of *S. glaucescens* are used in oral administration for

animal and human treatments. However, the yield during preparation and dosing and the chemical constituents are usually unknown. The work reported here aimed to stepwise optimize the extraction conditions to obtain higher yields and to screen the chemical constituents of the root bark of *S. glaucescens*. The work involved sequential extraction of the root bark of *S. glaucescens* using three solvents as a less polar option followed by ethanol as a highly polar solvent for each. The phytochemical screening was done using gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC-UV). Identification of compounds was done by comparing the Kovat's index and computer data base of the National Institute for Standard and Technology (NIST) library.

2. Methodology

2.1 Plant Collection and Pre-Treatment

With the help of a botanist and local informants, fresh root bark was collected from the field at altitudes between 1650m and 1950m above sea level located between 08°34′ to 08°49′ S and 034°55′ to 035°10′ E in Njombe district, Njombe region in the southern highlands of Tanzania. The collected plant parts were air dried in an open space (28-30 °C) for 5 days. To avoid any chemical decomposition, dried plant materials were stored in a cool, dry place and pulverized into smaller particles prior to extraction.

2.2 Chemicals

All chemicals used in this study were purchased from Sigma or Fisher Scientific through Sokoine University of Agriculture, Tanzania and the University of York, UK. The chemicals included Hexane (H) Petroleum Ether (PE), Dichloromethane (DCM), Ethanol (Et), *N*-trimethylsilylimidazole (TMSI), Trimethylchlorosilane (TMCS) and Bistrimethylsilyltrifluoroacetamide (BSTFA).

2.3 Extraction Methods

Soxhlet method was used, in which the dry ground samples were placed into thimbles (33 mm diameter, 80 mm length) and extracted using a common Soxhlet apparatus consisting of a condenser, a Soxhlet chamber, and an extraction flask. Thimbles containing the samples were air-dried at room temperature for 24 hours before adding the next solvent, while the filtrate was evaporated using the rotary evaporator to obtain the dry crude extracts that were measured for yield and phytochemical analysis.

2.4 Determination of Extraction Yield

The yields of the crude extracts were calculated using Equation 1. The percentage yield obtained was used to compare the efficiency of different extraction methods.

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

2.5 Optimization of Extraction Conditions

The effects of four main factors, namely solvent, running time, temperature, and particle size of the sample, were investigated to optimize the extraction process for high yields of crude extracts. The process involved sequential extraction of 10 g of pulverized sample with less polar solvent (Hexane, Petroleum ether or Dichloromethane) at similar boiling points followed by ethanol at 60 °C. The extraction temperature varied from 25 °C for less polar solvents to 95 °C for ethanol. Reaction time varied between 2-6 hours while the particle size was fixed between 0.4 mm and particles greater than 3mm size. These parameters were varied at the time maintaining the optimized factors, while the solvent to sample ratio was kept constant in all experiment. Each experiment was repeated at least twice, and the calculated yields were compared.

2.6 Sample Analysis with High Performance Liquid Chromatography (HPLC)

The effect of temperature on ethanolic extracts was studied by dissolving 5 mg of dry ethanolic extract in 1ml of HPLC grade methanol and then filtering with a microfilter with 0.2 µm pore size. The HPLC (Shimadzu 20AD) fitted with an auto sampler and a SPA UV detector at 254 nm was used for analysis. A reversed-phase supelco C-18 column (150 x 4.60 mm and particle size of 5 µm) was used for separation with the column temperature set at 40 °C. The sample injection volume was 1µL and flow rate of 1mL/min; mobile phase: Solvent A: Water; Solvent B: Methanol. The following low gradient elution system was used: 0–8 min, 5% B; 8–20 min, 10%–90% B; 20–27 min, 90%–3% B; 27–30 min 97% B. The detection of phenolic contents was done under the same conditions using an HPLC Model Hewlett Packard 1090 liquid chromatography series II, fitted with the Diode array detector (DAD).

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2.7 Derivatisation with N-Trimethylsilylimidazole (TMSI)

A 50 g sample was mixed with 1ml pyridine and 0.25 ml of TMSI in a reaction vial. The mixture was then heated at 70 °C for 20 min in a heating block. The resulting liquid was analysed in the GC-MS.

2.8 Derivatization with Trimethylchlorosilane (TMCS) in Bistrimethylsilyltrifluoroacetamide (BSTFA)

A 200 µL of TMCS in 1% BSTFA was mixed with 3mg of plant sample in 100 µl Toluene in a closed reaction vial and left to react at 75 °C for 30 min.

2.9 Fatty Acids Derivatisations

Fresh Sodium methoxide (1N) was prepared by dissolving 40 mg of Sodium Hydroxide in 1 ml methanol. 15 µL of the solution was then added to 5 mg of the crude plant sample into a reaction vial diluted with 1 ml hexane. The mixture was incubated at room temperature while stirring at 300 r/min for 20 min. The resulting solution was analyzed using GC-MS.

2.10 Sample Analysis with Gas Chromatography-Mass Spectrometry (GCMS)

A Perkin-Elmer Clarus 560 GC-MS with an auto-sampler was used. A high temperature, non-polar DB5HT capillary column (30 m × 0.25 mm x 0.25 µm) was used. The auto sampler injection volume was 0.5 µl with a split ratio of 25:1. The oven temperature programme was 60 °C for 1 min, ramped up at 8 °C/min to 360 °C and then held for 10 minutes.

2.11 Kovat's Index Calculations

In order to identify the composition of the crude extracts, two methods were employed: calculation of the Kovat's Index (KI) and the NIST library. KI is commonly used as a method which links retention times on all different columns in order to identify the chemical components of a complex mixture in GC. KI was calculated using the Equation 2, KI of an even C12–C60 alkane standard ASTM® 5442 (Aldrich) as shown in Figure 2, was then used in calculating the KI of the unknown GC traces for identification of the components.

$$I = 100\times\left(n+(N-n)\left(\frac{t_{unknown}-t_n}{t_N-t_n}\right)\right) \quad (2)$$

I = Kovats Index, n = The number of carbons in the smaller linear alkane (standard); N = The number of carbons in the larger linear alkane (standard); t = Retention time.

The NIST library is a database containing a fully-evaluated collection of mass spectra, KI and GC data. The NIST mass spectra search database software as installed in the GCMS equipment was used to identify the best match chemical structures of the different components in the unknown crude samples.

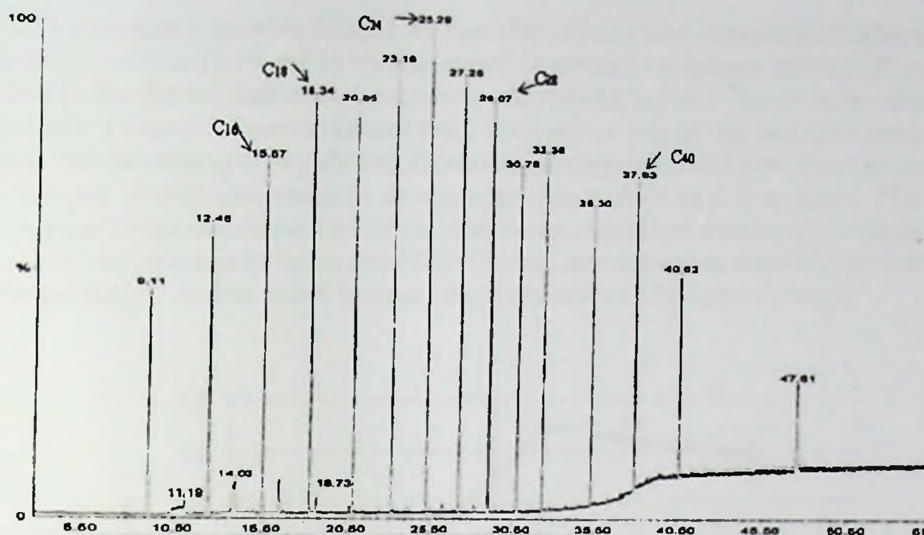

Figure 1. GC traces for a C12-C60 Standard

3. Results and Discussions

3.1 Solvent Optimization

Figure 2 shows the capabilities of each sequential extraction system by percentage. Based on the finding, extraction starting with DCM-ethanol is a better solvent system to attain the highest total yield. The yield obtained by extraction with DCM increased by 6.4% and 4.4% compared to extraction by hexane and petroleum ether, respectively. The total yield of DCM-ethanol extraction was 3.7% higher than the hexane and petroleum ether extraction system. GC traces of hexane and DCM were identical implying that they have similar content. Following the high yields of DCM-Ethanol extraction system, this method was chosen for further optimization studies.

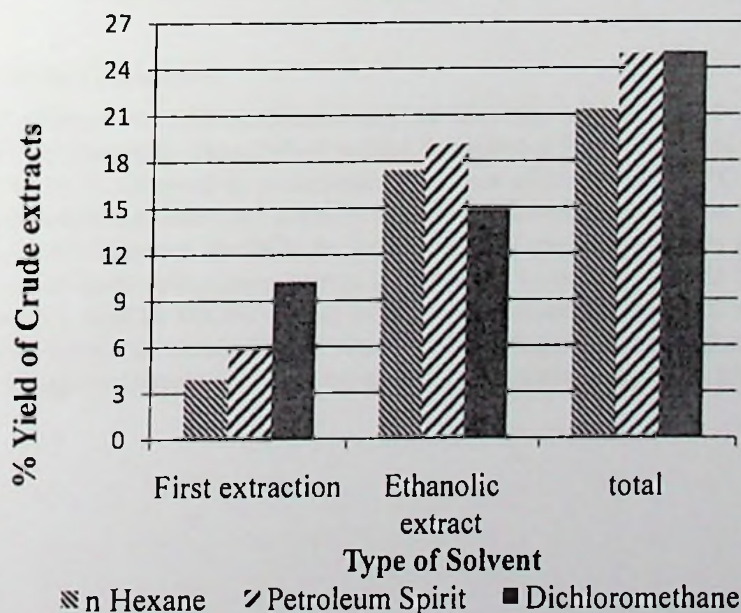

Figure 2. Yields of the three solvent systems at near boiling point with particle size 1.5 mm and running time of 4 hrs

3.2 Effect of Extraction Time

The effect of extraction time is shown in Figure 3. An extraction time of 4 hours demonstrated higher yield for both DCM and ethanol extraction, 10.5% and 14.5% respectively. Obtaining the maximum quantity of extracts at 4 hrs extraction indicates that the root bark of *S. glaucescens* would yield the highest at this extraction duration. Lower yields obtained after 2 hours of extraction indicate that 2 hours was not enough time to extract maximum content from the sample. The decrease of 0.6% yield with 6hrs extraction compared to 4hrs extraction can be explained by either a loss of more volatile components or decomposition due to the long time exposure. This trend could increase with increased exposure time and the nature of the extract composition contrary to the results obtained by Ahmad et al. (2010) during the optimization extraction of herbal Leonuri, showing that extraction with a less polar solvent hexane increased over time from 6 hours to a maximum yield of 7.25% after 12 hours.

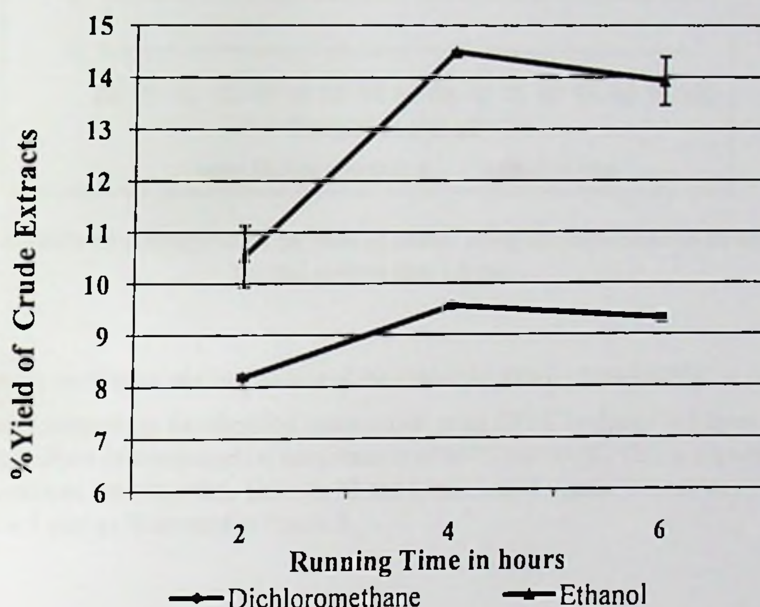

Figure 3. The effect of reaction time on yield of crude extract using dichloromethane- ethanol solvents with the temperature near the boiling point and a particle size of 1.5 mm

3.3 Effects of Extraction Temperature

Figure 4 shows the effect of extraction temperature on yield. Extraction with DCM at 30 °C yielded the highest by 10%, followed by extraction with ethanol which yielded the highest at 75 °C. The yield with DCM increased by 1.1% from 25 °C to 30 °C followed by an inconsistent decrease of 0.1-0.3% to 45 °C. Increasing temperature enhances both the diffusion coefficient and solubility of extracts to the solvent and, hence, improves extraction rate (Richardson et al., 2002). However, for DCM the increase in temperature gave slightly lower yields due to high solvent volatility, reducing the solvent turnover rate. Increasing the temperature from 55 °C to 75 °C with ethanol extraction increased the yield by 9%, after which an increase in temperature by 10 °C resulted in a decrease in yield by 3% which doubled after another 10°C increase. The decreasing yield could be due to the decomposition of some compounds at high temperatures or the evaporation of some volatile compounds from the crude extracts.

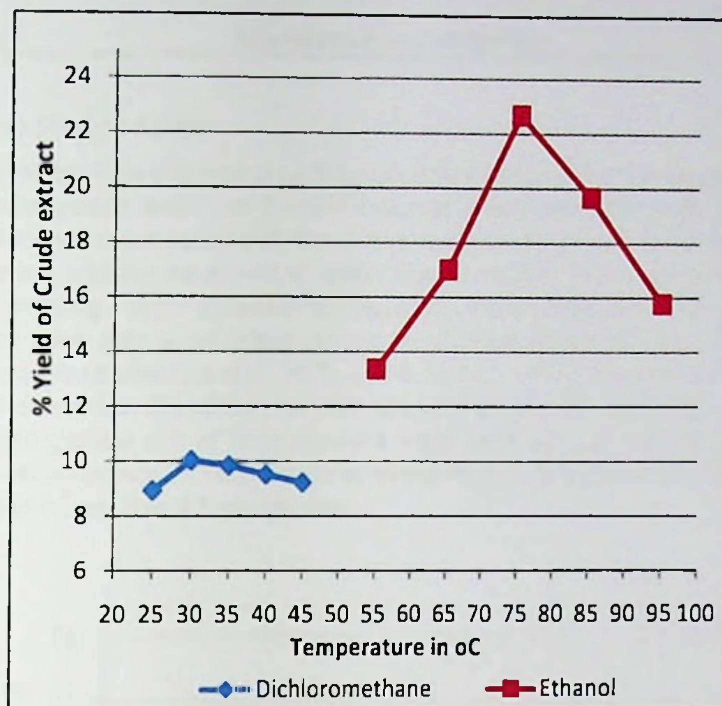

Figure 4. The effect of extraction temperature on yield of extract using dichloromethane-ethanol, running time 4 hrs and particle size 1.5 mm

3.4 Effect of Temperature on Chemical Composition of the Ethanolic Extract Using HPLC

The effect of higher temperature on the chemical composition using HPLC is shown in Figure 5. HPLC analysis indicates some decomposition of compounds at temperatures of 85 °C and 95 °C. This is expressed by a reduction of intensity of some peaks at retention time between 12 and 14min and increased intensities of peaks at retention time between 5.5 and 6.5 min as illustrated in Figure 5.

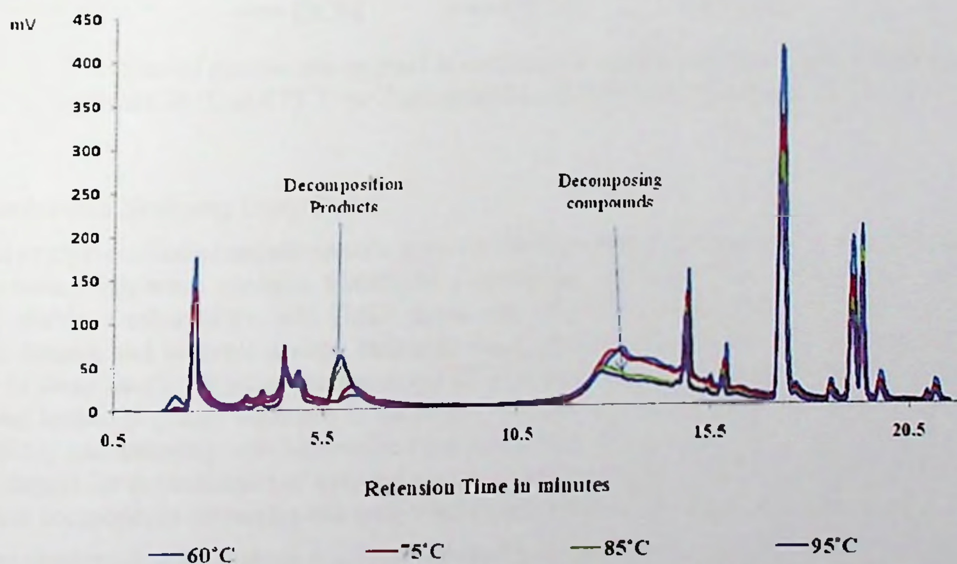

Figure 5. HPLC chromatogram showing the effect of temperature on chemical composition

3.5 Effects of Particle Size of a Sample

Low yields of crude extract were observed in particles larger than 1mm diameter compared to those less than 1mm (Figure 6). For particles greater than 1 mm the yield decreased as the particle size increased; this may possibly be due to the decreased solvent entrance and diffusion of the extract from the particle to the solvent. A smaller particle size offers more surface area for the solvent to diffuse into the sample. This improves interaction between the sample and solvent implying a larger extraction rate and, hence, higher yields. However, when the sample particle is too small agglomeration may occur, which reduces the effective surface area available for diffusion of the solvent in the solid sample (Richardson et al., 2002; Tzia & Liadakis, 2003). The principle holds for a particle size of 0.4mm that yielded less than 2% of the total yield compared to particles with 1mm size. Generally, it can be concluded that a small particle size of 1mm provide a higher yield of crude extracts from the root bark of *S. glaucescens* using Soxhlet extraction. These results are similar to those by Sayyar et al. (2009) who found less than 7% yield with particles of less than 0.5 mm diameter.

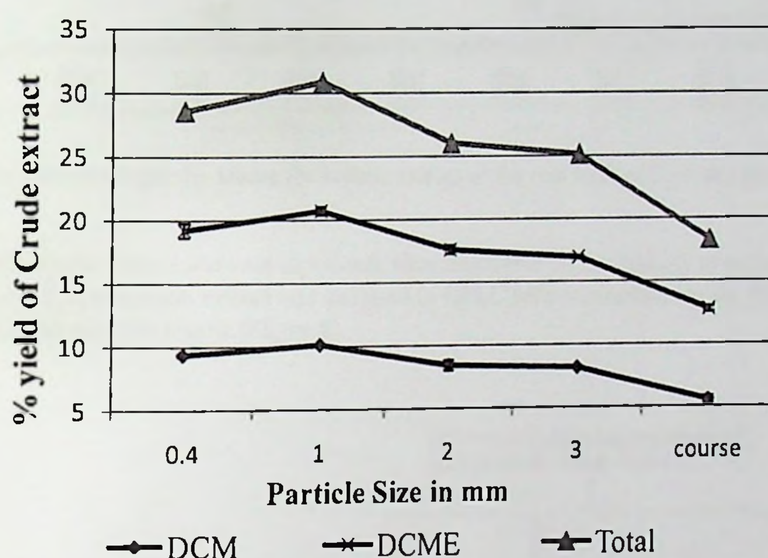

Figure 6. The effect of particle size on yield of extract using dichloromethane - ethanol, running time 4hrs temperature 30 °C and 75 °C for Dichloromethane (DCM) and ethanol (DCME), respectively

3.6 Phytochemical Screening Using GCMS

Fatty acid analysis indicated negative results implying that there were no free fatty acids in the root bark extracts of *S. glaucescens*. Silylation produces trimethylsilyl derivatives which are more volatile, less stable, and more thermally stable. Derivatisation with TMSC mixed with BSA was used for detecting the presence of alcohols, alkaloids, amines and biogenic amines, carboxylic acids, phenols, and steroids. The two reagents indicated the presence of these functional groups in hexane and DCM extracts. Derivatisation with TMSI revealed the presence of hindered hydroxyl groups especially in the ethanol extract of the root. TMSI is a derivatisation reagent which reacts quickly and smoothly with hindered and unhindered hydroxyl and carboxyl groups. TMSI is considered to be the strongest for derivatisation of hydroxyl groups. The GC/MS analysis of hexane and DCM extracts showed an identical composition containing two main triterpenoids of tirucallol or euphol and cycloeuphornol backbone.

Thin layer chromatography analysis at 254 nm indicated a UV negative compound which reacted greenish to deep purple after a colour reaction with vanillin reagent spray and heating. This is an indication of the presence of unconjugated terpenoids and hydroxyl functionality. DCM is reported to have higher potentials of extracting terpenoids and is used specially for this purpose (Tiwari et al., 2011). The dichloromethane extracts were composed of mainly two triterpenoids, see Figure 7. The major compound appeared at RT 32.92 min with calculated KI of 3,273 constituting 42% of the total chemical contents, and it matched best with lanosterol in the NIST library. The second compound appeared at RT 33.3 with calculated KI of 3,326 constituting 31% of the total chemical contents and matched best with cycloartenol in the NIST computer library (Figure 7). The family Euphorbiaceae is characterized with different types of triterpenoids, among which are those with tirucallol, euphol,

cycloeuphornol and euphorbol backbone (Rizk, 1987). Other minor compounds identified during GCMS analysis include phytol, ergostadiol, hentriacontane, sitastirol aceate, lupeol and hopenone.

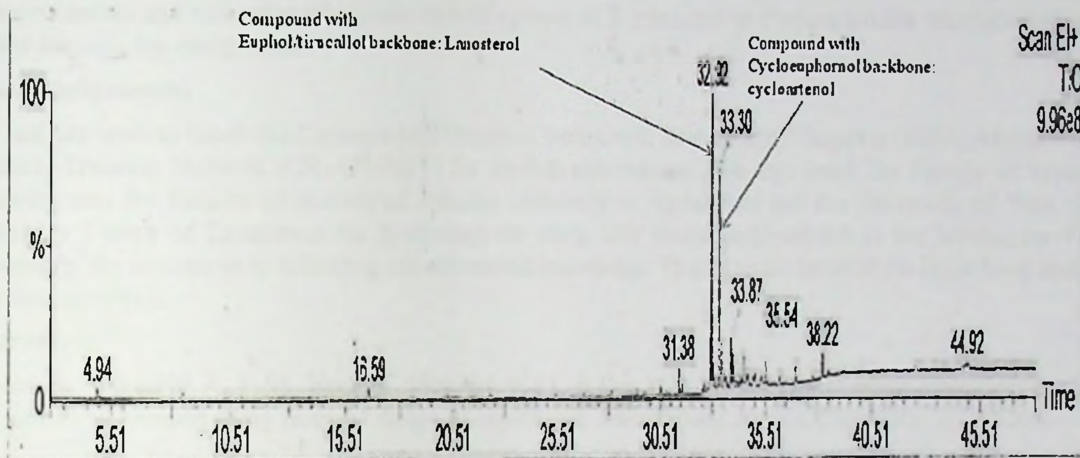

Figure 7. Gas Chromatography traces for hexane extract of the root bark of *Synadenium glaucescens*

The GC traces for ethanolic extract were not significant, thus considered either artefacts or decomposition products of the major product. The ethanolic extract was analysed in HPLC with parameters set for detection of phenolic compounds and showed positive results (Figure 8).

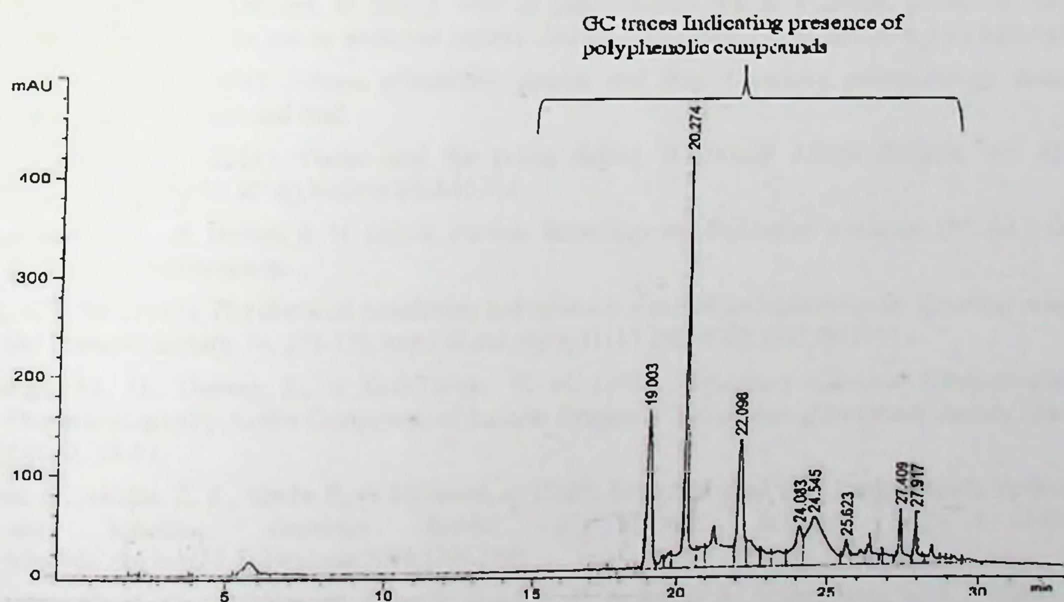

Figure 8. HPLC traces acquired with a reversed-phase gramin C-18 column 150 x 4.60 mm; particle size, 5 µm; temperature, 40 °C; injection volume, 10 µL, DAD λ, 254 nm and gradient system; specifically set for detection of polyphenolic content in the ethanol extract of the root bark of *Synadenium glaucescens*

4. Conclusion

Four factors affecting the yield from Soxhlet extraction of the root bark of *S. glaucescens* were studied. DCM–ethanol extraction system presented higher extract yields. The optimum condition for extraction was obtained at a running time of 4 hours with a temperature of 30 °C for DCM and 75 °C for ethanol and a particle size

of 1mm. This study shows that extraction with hexane yields low amounts of extracts. Extraction with petroleum ether yields almost the same amount of extracts with DCM but the mass separation is poor in petroleum ether. Phytochemical screening revealed that the less polar extracts are mainly characterized by triterpenoids while the polar component is composed of polyphenolic compounds. This work should be considered the first information on optimization and screening of organic solvent extracts of *S. glaucescens*. Further studies are recommended to further identify the compounds.

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SUPPLEMENT III:

FOR PAPER V: Phytochemical Screening of Extracts

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FOR PAPER V

Phytochemical Screening of Extracts

1.0 Introductions

The family euphorbiaceae is characterized by different types of triterpenoids, among which are those with tirucallol, cycloeuphornol and Euphorbol backbone. All extracts were analysed to study the phytochemistry of the extracts. Thin layer chromatography (TLC) and gas chromatography as explained in Paper V were used. The results are presented in Figure 1 and Table 1.

1.1 Thin layer Chromatography

Thin layer Chromatography analysis (Figure 1) support this observation; UV analysis of the TLC at shorter wavelength does not give many sports however after colour reaction with vanillin and heating the compounds react greenish to deep purple which is an indication of the presence of unconjugated terpenoids and alcohol functionality.

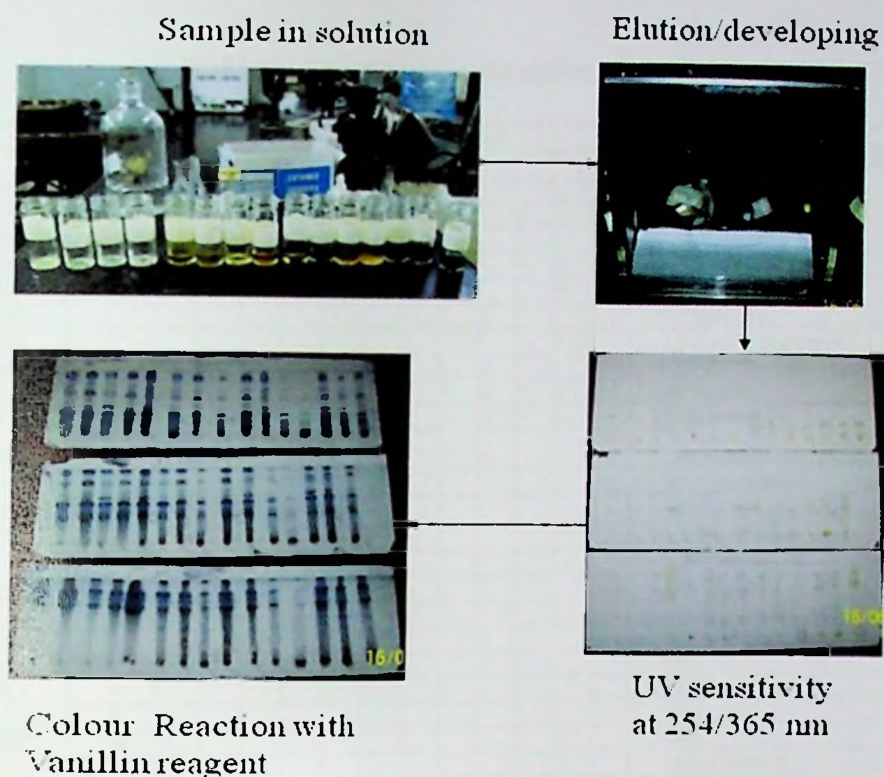

Figure 1: Thin layer chromatography results

1.2 Phytochemical composition of extracts

The content of the extract, there retention time, KI indices, abundance and their best much are as shown in the table 1. Most of the compounds are extracted with hexane and dichloromethane, while few very polar compounds are extracted with ethanol. The most abundant constituents extracted from the hexane and dichloromethane extracts are steroids with best match of the tirucallol backbones. DCM is reported to have higher potentials of extracting terpenoids and is used specially for this purpose (Tiwari *et al.*, 2011). Most of the compounds are also common to all parts of the plant studied.

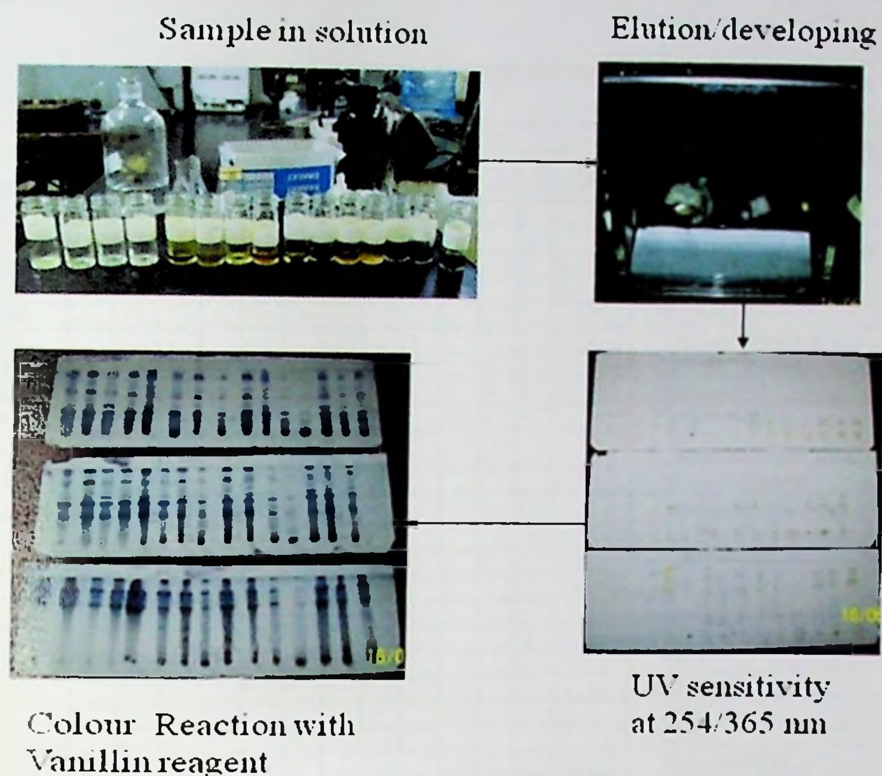

Figure 1: Thin layer chromatography results

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Table 1: Chemical Composition of extracts

Peak RT	Sample Abundance In %															KI	Best match structure from KI and NIST
	SH1	SH2	SH3	SH4	SH5	SD1	SD2	SD3	SD4	SD5	SE1	SE2	SE3	SE4	SE5		
3.24													V		•		
3.88											•						
4.92													•	•	V		
5.17														x			
9.44												x			x	1,219	piperitol
10.97														x		1,310	aminoacetophenone
11.96												•	•			1,369	Benzene triol
12.83											V	V	•	x		1,423	Ionone
13.61											x	•	x	•		1,473	decalactone
16.37						x		•	x		x	•				1,658	Octanoic acid heptyl ester
17.40														V		1,718	dodecalactone
18.69								x		x						1,828	Diethyl 2-hydroxyglutarate, ucinol
20.17		•	"	x		•	•	•	V	x				x		1,946	Hexadecanoic acid
21.37			"	x												2,126	Kaurene
22.02		•	"	•		"			•	•						2,093	phytol
22.28										•						2,114	octadecanoic acid
23.91						"										2,270	
24.36						"										2,313	
24.84			"			"										2,358	
25.40																2,411	
26.07			•	•												2,852	
26.08																2,853	octacosane
26.41							•									2,514	
26.77			•	•		•										2,913	
27.53		V						V	•	•						2,631	Hexacosane
27.96			"		"	x										2,678	
29.35			"	•	•	x										2,831	
30.50				"	"	•										2,967	
30.78						"										3,000	
31.34	•	•	•	•	V		x									3,070	Hentriacontane
31.37																3,074	sitastirol acetate,
31.58							x									3,100	
32.24					"											3,183	
32.48					"		x									3,214	ethylybutyl-
32.81		"	•	•	"											3,259	Pentadecanone, heptadecane, 2,6,10,15-tetramethyl-
32.87	"	"	"	x	•											3,267	phytol
32.91	V				"	•										3,273	octacosyl acetate
32.99						x										3,284	heptytrifloro acetate
33.22		V	V	•	"	V				V					•	3,315	stigma-5,22-diene-3-ol,acetate,(3b)-, cyclonostanol acetate
33.42						x										3,342	lupeol
33.55				x		x										3,360	ergostadinol
33.87	•	•		•		•										3,404	
33.90				•	"											3,408	
34.25				V	"											3,456	lanostediene- ol, acetate
34.44			x			x										3,482	
34.72			•		"											3,521	
34.90				•	"											3,545	
35.36				•	"	x										3,609	hopenone
36.19						x										3,735	
36.93	"															3,848	
36.99						x										3,857	
38.22	"															4,044	

100	√	Most Abundant peak
61-99%	*	Large abandance
31-60%	x	Medium Abundance
11-232%	•	Low Abundance
less than 5%	"	traces

Quitain, A. T., Moriyoshi, T.and Goto, M. (2013). Coupling microwave-assisted drying and supercritical carbon dioxide extraction for coconut oil processing. *Chemical engineering and science* 1:1, 12–16.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. and Kaur, H., (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia* 1: 1, 98 – 106.

CHAPTER THREE

3.0 CONCLUSION

3.1 Major Findings

The aim of this study was to advance knowledge towards utilization and commercialization of *S. glaucescens* by studying the biological activities of its extracts. The model that combined traditional and conventional drug discovery approaches was adopted while setting the structure of the current work. Ethnobotanical survey to document uses of the plant in ethnomedicine and ethnoveterinary practices was important.

Synadenium glaucescens is highly utilized within the community. Great awareness on the ethnomedical importance of *S. glaucescens* was shown by the respondents. Since the plant is poisonous, it is utilized with great care to avoid toxic effects. Indigenous people trust their knowledge and practice it while transfer the knowledge is verbally.

The extracts from *S. glaucescens* demonstrate a broad spectrum of activity against virus, bacteria and fungi of both human and animal importance. Findings in this study validate the ethnoveterinary and ethnomedical exploitation among the community in the Iringa and Njombe region in particular and Tanzania in general. Furthermore, it demonstrates a high potential and feasibility of using *S. glaucescens* extracts for treatment and control of viral diseases especially in rural areas where conventional disease management options are limited. This study stands as a stepping stone towards further research on antiviral drug search from *S. glaucescens*.

The ethanol extract have demonstrated more potency than other and this is useful information in attempts towards development of new drugs.

Synadenium glaucescens constitutes less polar bioactive compounds which are more toxic than polar compounds. Both have demonstrated a very narrow safety margin. Hot extractions are more effective than cold extractions with maximum yield at 30˚C dichloromethane then ethanol at 70˚C keeping the particle size at 1 mm and 4 hours extraction time with soxhlet extraction method.

3.2 Relevance of the Study

The bridge between science and indigenous knowledge such as ethnobotany, are of vital importance in the current world and it gains more acceptance. it is a current feeling that scientists and society must begin to respect the consecrated knowledge that indigenous people have known for generations. In view of the findings, publications and patents obtained through this study, the plant has the potential to contribute in improving and enhancing human and animal health and contribute in poverty reduction. Furthermore, It contribute to the development of new drugs/formulations of natural origin which will further create employment opportunities.

3.3 Recommended Future Studies

Based on findings from this study, future studies should focus on isolation of responsible active compounds of ethanolic extract and their pharmacological evaluations. Extraction of pure compounds, their chemical modification and associated bioativity is also recommended. *In vivo* (semi field and field) antibacterial and antiviral studies towards pure and crude drug formulation are

recommended. Wide screening of the cytotoxicity using different cell line is important to validate the toxicity. Furthermore, investigation of anticancer properties, formulation of disinfectants/antiseptics from the toxic extracts and formulation of products extracts against ecto-parasites and protozoa are recommended. Preliminary screening against pathogens of human importance such HIV virus and bacteria causing tuberculosis have shown promising results. It is therefore recommended that further studies be done on these pathogens of human importance.

APPENDICES

Appendix 1: Questionnaire

Ethnobotanical survey of *Synadenium glaucescens*

Section 1: Personal information of the respondent

Please put a tick or write to provide the needed information

1. Sex: i) Male ____ ii) Female ____
2. Age: ____________
3. Tribe: ____________
4. Origin: Region_________,
   District_________,Ward___________Village_________
5. Religion: Christian [ ] Muslim [ ] traditional religion [ ]
   Others; mention___________
6. Occupation: Peasant [ ] Businessman [ ] Traditional healer [ ]
   Others; mention___________

Section 2: Information about the use of Synadenium glaucescens

Please put a tick or write to provide the needed information

7. Have you ever heard about *Synadenium glaucescens*:
   a) Yes
   b) No
8. Mention the other name of this plant which you know______________
9. Who informed you about *Synadenium glaucescens* for the first time?
   a) Grandfather b) Grandmother c) Mother d) Others mention....................

10. What are the uses of *Synadenium glaucescens*? (Describe how it used)

11. Which part the plant is used most (roots, leaves or stems) _____

12. Have you ever used this plant in person?

    a) Yes

    b) No

13. If your answer is yes in question 12,

    a. To whom did you use for? Human being or Animals________

    b. Which disease did you treat? Mention____________

    c. Which part did you use; Roots, Leaves, Stem bark

    d. How much and by what means is it used? Explain

14. Do you know any undesirable effects from this plant?

    a) Yes b) No

15. If your answer is yes in the question 14 what are those effects?

    a. Mention the effects you have heard

    b. Mention the effect you have seen

16. Have you ever propagated this plant?

    a) Yes b) No

17. If your answer is yes for question 16, did it grow?

    a) Yes b) No

18. For each of the statements below, please tick the opinion that mostly applies to you.

Statement	Strongly agree	Agree	Disagree	Strongly disagree
This plant is very poisonous to human beings				
This plant is very poisonous to animals				
This plant is available in our location				
Nowadays the plant is not readily available as it was in the past				
Most people use the plant in our area				
Nowadays the extent of using this plant has declined compared to the past				
Old people are more informed than youth on the uses of the plant				
Youths are not informed about the plant because old people have not disseminated the knowledge				
Presence of veterinarian, dispensaries lead to decline in extent of using the plant				
The detailed information about the plant are obtained from Traditional herbalists				
In person I use the plant and will continue using it because is so helpful				

Section 3: Opinion of the respondent

Give any other information on this plant you wish to be known

Appendix 2: Focused group discussion guide questions

1. What are the importances of S. *glaucescens* in your community?

2. Which diseases are treated or controlled using the extracts of *S. glaucescens?*

3. How do you prepare different formulations for treatments?

4. How you know and administer the doses for the specific treatment?

5. How is the information on the use of *S. glaucescens* transferred from one generation to another?

6. What do you comment on the availability of *S. glaucescens* in the recent years compared to past years?

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