EFFECT OF CRUDE ROOT EXTRACT FROM *SYNADENIUM GLAUCESCENS* ON SELECTED BACTERIAL INFECTIONS IN ALBINO MICE (*MUS MUSCULUS*)

BY

CHRISTER MWAGENI

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ABSTRACT

The plant, *Synadenium glaucescens*, is widely used in Tanzania to treat various diseases including skin conditions, sores and wounds. However, systematic information about the effectiveness of traditionally prepared *S. glaucescens* extracts against microbial agents is scanty. *In vivo* studies using mice were carried out to investigate the effect of root extract from the plant against two pathogenic species of bacteria. A total of 120 mice were used in two experiments involving two bacterial species, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In each experiment, 60 mice were randomly divided into 6 groups with or without bacterial infection and with or without root extract. Clinical signs and survival rate were monitored and skin, kidneys and livers were examined for gross lesions, histopathological changes and bacterial counts. Results indicated that mice infected with the two bacteria and treated with the root extract from S. *glaucescens* had significantly less ($P < 0.05$) severe skin lesions compared to untreated control groups. Histopathological study of liver and kidney tissues showed hydropic degeneration and inflammatory reaction in uninfected groups receiving the extracts. Protein in tubular lumens, desquamated tubular epithelium and necrosis around central veins were observed in livers of mice infected with *S. aureus* following infection and treatment with the extract. It is concluded that root extract from S. *glaucescens* had significant bacterial activity against the two tested bacteria. Histopathological changes in the kidneys and livers of mice which received the extract alone suggest that high doses of prolonged exposure of the tested extract could be harmful to the mice. Further studies are needed to find out optimum dosage and whether the extract is harmful to other organs.

DECLARATION

I, Christer Mwageni, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and it has neither being submitted nor being concurrently submitted for a degree award in any other institution.

Christer Mwageni
(MSc. Applied Microbiology)

16/11/2011
Date

The declaration above is confirmed by

Robert A. Max, (PhD)
(Supervisor)

17/11/2011
Date

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DEDICATION

This work is dedicated to my Husband Majige. J. Mabula and my beloved children Filbert, Sabina and Faraja for their encouragement during the entire period of study. You are always loved and valued simply for being you.

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LIST OF ABBREVIATIONS AND SYMBOLS

^0C	degree Celsius
spp	Species
mm	Millimeter
mg	Milligram
g	gram
kg	Kilogram
ml	Milliliter
cfu	Colony Forming Unity
WHO	World Health Organization
HE	Hemotoxylin-eosin
h	Hour
µL	microliter
I P	intra-peritoneal
SUA	Sokoine University of Agriculture
FVM	Falcult of Veterinary Medicine

CHAPTER ONE

1.0 INTRODUCTION

Many plants possess medicinal properties and have been used by humans worldwide as natural remedies against various diseases since time immemorial (Amara *et al.*, 2008). Recently there has been a growing interest in the use of plants and plant products to treat diseases or improve health in humans and animals mainly due to inaccessibility of modern medical system, economic and cultural factors easy accessibility, efficacy on treatment and affordable cost in getting traditional health services and the assumed lack of side effects are main reasons in preferring traditional medicine to modern medicine (Yirga, 2010).

According to assessment of World Health Organization (WHO) about 80% of world population depends on medicinal plants for their health care needs (Gulfraz *et al.*, 2006). In some developing countries, traditional medicine is preferred because of its good availability and affordability (Bussmann and Sharon, 2006). Ethno-pharmacological information together with phytochemical studies of medicinal plants have enabled pharmaceutical industries to develop potent drugs from plants.

Numerous studies (Valgas, *et al* 2007) have been carried out to extract various natural products for screening antimicrobial activity but attention has not been focused intensively on studying *in vivo* anti microbial activity of these medicinal plants (Abu-Shanab *et al.*, 2004). For instance, antibacterial properties of Euphorbiaceae were found to corroborate its use in traditional medicine (Chika *et al.*, 2007). Extracts from the plants were used in sore and wound healing and in the ear and treatment of small ear abscesses (boils). Species of Euphorbiaceae have been used for treatment of many other ailments including malaria,

diarrhea, heart diseases, diabetes, hemorrhages, hepatitis, jaundice and scabies (Mwine and Van Damme, 2011). Euphorbiaceae are also known to cure urinol-genital infections, (e.g., gonorrhea) and are used as astringent and diuretic (Kumar and Chaturvedi, 2010). *Synadenium* species are among Euphorbiaceae genus which is found in the eastern and southern Africa and widely used in the treatment of different infectious diseases including malaria, stomach-ache, urinal-genital problems, excessive menstruation, tuberculosis and cardiac palpitations (Melo-Reis *et al.*, 2010).

Although a number of scientific investigations have been undertaken to study bioactivities of this Euphorbiaceae family plants, there are no reports on *in vivo* antibacterial activity of the plant, *S. glaucescens*. This study was therefore aimed at investigating the effect of root extract from *S. glaucescens* on healing of experimentally induced bacterial abscesses using mice model. Results were expected to act as a template for further studies to validate the use of this plant in treating various bacterial infections.

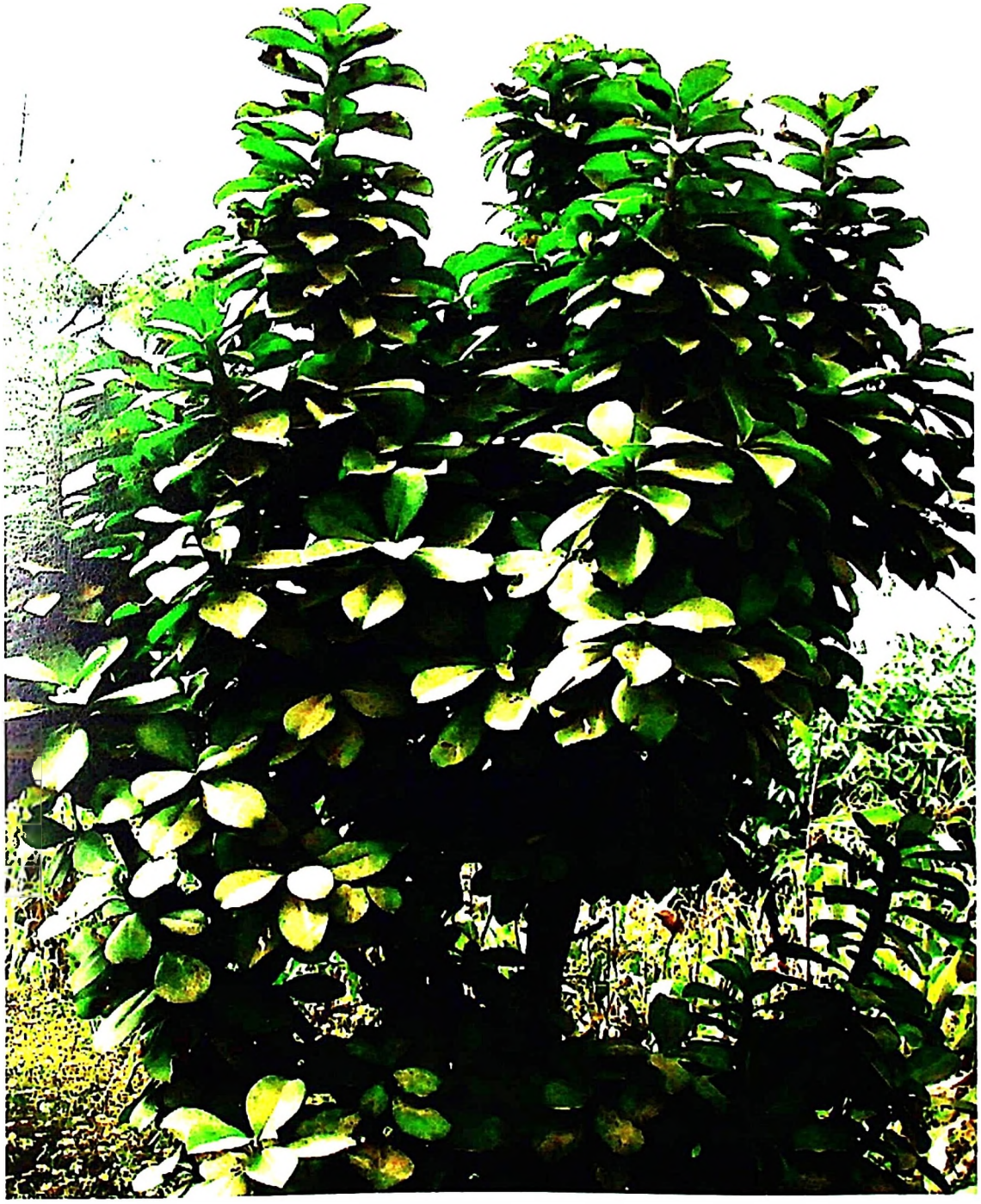

Figure 1: A full grown test plant (about 3m high), *Synadenium glaucescens*

1.1 Problem statement and justification

The plant *S. glaucescens* is widely used in Tanzania to treat different diseases, either applied topically or taken orally using traditional methods of preparation. However the information about the effectiveness of traditionally prepared *S. glaucescens* extracts against microbial agents is not available.

1.2 Objectives

1.2.1 General objective

To investigate the effect of crude root extract of *S. glaucescens* against selected pathogenic bacteria of public health importance in albino mice.

1.2.2 Specific objectives

i) To study the effectiveness of ethanolic extract from roots of *S. glaucescens* on healing of experimentally induced bacterial abscess in mice.

ii) To determine the minimum effective dose of the test extract on healing of the experimentally induced bacterial abscess in mice.

iii) To determine, using histological examination, if the test extract has any effect on two important organs (liver and kidney) of the treated mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Medicinal uses of Euphorbiaceae

Plants of Euphorbiaceae family have been widely used in America and tropical Africa for treatment of malaria, diarrhea, diabetes, heart diseases, hemorrhages, hepatitis, jaundice, and scabies (Kumar and Chaturvedi, 2010). Euphorbiaceae are also known to cure urinal-genital infections (Kumar and Chaturvedi, 2010). Some species are used in folk medicine to treat skin diseases, severe headache, intestinal parasites and warts (Rojas *et al.*, 2009). Also several macrocyclic diterpenoids with antibacterial, anticancer, PGE2-inhibitory, prolylendopeptidase inhibitory, anti-HIV, and analgesic activity have recently been isolated from different *Euphorbia* species (Rojas *et al.*, 2009). Aqueous extract of *Euphorbia hirta* has been used in traditional medicine for the treatment of dysentery, colic, ulcers, asthma and chronic bronchial infections in humans (Sudhakara *et al.*, 2006) The extract also showed activity against *E. coli*, *Proteus vulgaris*, *P. aeruginosa* and *S. aureus* (Rojas *et al.*, 2009).

The latex of *Euphorbia pseudograntii* is applied to warts and leaf sap to treat cardiac problems. Several drops of latex from warmed leaves are taken to expel intestinal parasites and sometimes tapeworm (Rojas *et al.*, 2008). In Tanzania and Uganda the latex is applied to abscesses to mature them (Nicholson, 2008). Leaf preparations are externally applied to different skin infections (Kumar and Chaturvedi, 2010). A decoction of the stem bark or the latex is taken to expel a retained placenta. Crushed leaves are rubbed into scarifications to treat backache, leaf ash is taken in water to treat a sore throat, a root extract or sap from the crushed stem is used as ear drops to treat earache. The latex is an

ingredient of arrow poison also used as a fish poison and can be fatal if ingested (Schmelzer and Gurib-Fakim, 2008).

Plant leaves of *Euphorbia pseudograntii* were ground and small balls are inserted into the reproductive tract to treat female sterility, excessive menstruation and painful menstruation in humans (Nicholson, 2008). Ground leaves in water are administered against difficult child birth, a plant decoction is taken for its astringent, vulnerary and anthelmintic properties and crushed plants are used as a poultice for broken arms (Schmelzer and Gurib-Fakim, 2008). In some tribes crushed leaves are eaten to treat amoebic dysentery, a leaf extract is applied as an enema to treat inflammations (Mwine and Van Damme, 2011). Headache is treated by rubbing leaf powder mixed with palm oil on the head. An infusion or decoction of the leaves is also taken orally to treat fungal infection (Schmelzer and Gurib-Fakim, 2008). The crushed whole plant is eaten with bread as a treatment against kidney stones (Kumar and Chaturvedi, 2010).

2.2 Methods of preparation of medicinal plant extracts

Medicinal plants can be prepared in many different ways depending on the part of the plant being used, the illness being treated and the major constituents of the plant. Some methods used in preparing medicinal plants for use include: Tinctures, capsules and tablets, decoctions, infusions, juices and poultices.

2.2.1 Tinctures

Herbal tincture preparations are made with alcohol or distilled water using dried or fresh herbs. Dried or fresh herbal liquids of tinctures are consumed in teas, water or juices. One teaspoon taken up to three times daily is often the dosage amounts for herbal tinctures. Herbs medicinal ingredients extracted in alcohol or vinegar as tincture will remain efficacious for up to two years and is one of the best methods of long time storage and

effective extracting. Alcohol is also a very effective natural preservative. Since a tincture is easily assimilated in the body, it is an effective way to administer herbal compounds (Chevallier, 2001).

2.2.2 Capsules and tablets

Capsules and tablets contain a ground or powdered form of raw herbs. In general there are little differences between the two in terms of clinical results. Because finely milled herbs degrade quickly, it is important that herbs be freshly ground and then promptly encapsulated or put in tablet form within twenty four hours of being powdered (Chevallier, 2001). With the exception of certain herbal concentrates both capsules and tablets tend to be much less strong and potent than tinctures and extracts (Chevallier, 2001).

2.2.3 Decoctions

Decoction is the methods of extracting herbs in a more vigorous way. Herbal decoctions are usually used for roots, barks, and twigs, where by herbs are placed in a pan of cold water and simmered for up to an hour, strained and drunk hot or cold (Chevallier, 2001). The usual dose is one cupful three times daily or hourly for acute conditions. The decoction can be stored in a refrigerator for up to one day (Chevallier, 2001).

2.2.4 Infusions

Herbal infusions are made from leaves and flowers of the plants. These parts of plants are more fragile. Due to fragility, these parts of plants are steeped in boiling water rather than simmered because they release their active ingredients more easily than roots and barks. Infusions are made by boiling one quart of water, pouring one table spoonful of herb in one quart of boiling water and then left to steep for 10 minutes before straining. The amount of herbs used is then modified according to taste (Chevallier, 2001).

2.2.5 Juices

The fresh plant materials are pounded or chopped and then filtered through a fine piece of cloth or the plant parts are just squeezed to extract the juice and then a small amount of water is added and then presses once again. Some herbs need to be cooked for about 30 minutes in order to extract their juice; the juices extracted from many herbs can be taken internally or applied externally. This method is excellent for getting minerals and vitamins from the plant (Chevallier, 2001).

2.2.6 Poultices

Herbal remedies are sometimes applied directly to the skin as poultices, usually on rashes, wounds and as topical pain relieving remedies (Chevallier, 2001). Poultices are prepared in various ways. It is made by grinding, crushing or pounding herbs or the fresh plant material with a little warm water, molasses, natural oils, or honey to make a paste. Paste can be applied directly to the skin and covered with a piece of clean cloth. If the herb used is potent such as onion, garlic, ginger, or mustard, a layer of thin cloth (gauze) between the skin and the herb may be used. The cloth can then be covered with plastic wrap to hold in the moisture. The poultice can be changed every 3 to 4 h or whenever it dries up. These poultice ingredients also have properties to draw out infections and reduce inflammation. These poultices can even be used to treat chest congestion, hemorrhoids and earaches (Chevallier, 2001).

2.3 Part of the plant used

Roots were used because traditional healers in the area where the test plants were collected prefer the same part of the plant for treatment of various ailments.

2.4 Choice of solvents

Quality of plant extract depends on plant material, choice of solvents and the extraction methods Successful determination of biologically active compound from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions include low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to dissociate. Initial screening of plants for possible antimicrobial activities typically begins by using the crude or ethanol extractions and can be followed by various organic solvent extraction methods.

2.5 Traditional preparations

Before the introduction of modern medicines, disease treatment was entirely managed by herbal remedies (Samy and Gopalakrishnakone, 2007). It is estimated that about 80% of the world population residing in rural areas of the developing countries still rely mainly on medicinal plants (Gulfraz *et al.*, 2006). Medicinal plants are the only affordable and accessible source of primary health care for them, especially in the absence and lack of access to modern medical facilities. Studies reveal that there are more traditional medicine providers than the modern medicine providers especially in the rural areas (Gulfraz *et al.*, 2006). The use of traditional medicine has increased in developed countries also, mainly due to the failure of modern medicine, to provide effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites (Hoareau, 1999). The adverse effects of chemical drugs, questioning of the approaches and assumptions of modern medicine, their increasing costs and greater public access to information on traditional medicine has also led to an increase in interest in alternative treatments (WHO, 2002).*Synadenium* (Euphorbiaceae) was used as latex collected from cut branch and directly applied to wounds till they healed (Kuvar and Bapat, 2010).

2.6 Systematic preparations

Many of the traditional medicines originating from plants are prepared by either chemical methods or traditional methods. In traditional medicine, Euphorbiaceae plants are used for treatment of various microbial diseases such as skin infections, diarrhea, dysentery and gonorrhea (Uduak *et al.*, 2010). The ethanol and water extracts of the whole plant showed significant antifungal activity against the dermatophytes, trichophyton, mentagrophyles and microsporum *in vitro* and *in vivo* in goats and rabbits. Water extract inhibit growth of spore formations and enterotoxin production of *Clostridium perfringens* type A. Ethanolic extracts from aerial parts showed significant anti bacterial activity against *E. coli* and *B. subtilis*. An aqueous ethanol extract showed significant antibacterial activity *in vivo* against *Shigella dysenteriae* in tests with rats (Schmelzer and Gurib-Fakim, 2008).

Ground dry leaves of *Synadenium* spp. have been used for treatment of headache, catarrh, and flu, while dried leaves are eaten to treat asthma. Latex is placed in a hollow tooth to treat tooth ache, latex may also be rubbed on infected wound. *S. glaucescens* is a succulent tree endemic to Tanzania and morphologically similar to *Euphorbia pereskiifolis* which are used for treatment of diseases in Kenya. The juice of fresh crushed leaves is drunk to treat excessive menstruation in women and as a purgative. Traditional doctors use a leaf decoction, mixed with lemon juice, baking soda, and honey, for effective treatment of asthma (Schmelzer and Gurib-Fakim, 2008). The ash of dried leaves are mixed with water and applied to treat leprosy. A root bark extract is taken with sugar to treat severe cough and tuberculosis. A root extract is used as ear drops to treat ear-ache (Schmelzer and Gurib-Fakim, 2008).

2.7 Why medicinal plants?

Consequent on the current emergence and re-emergence of resistant strains of most microorganisms, together with side effects of most conventional drugs, interest in the use of plant and plant products in the management of diseases is increasing. Indeed, nature has remained a veritable source of medicinal agents since ancient times (Valgas *et al.*, 2007). Although *in vitro* and *in vivo* antimicrobial properties of some medicinal plants have been widely reported, only a few have received significant scientific backing (Ayogu, 2009).

A number of plants which are used as natural remedies showed promising biological activity (Das *et al.*, 2010). This may indicate that the use of these plants in traditional medicine is of value, especially in terms of topical application where bioavailability and biotransformation are less important (McGaw *et al.*, 2007). The lack of data correlating activity in the assays with activity in *in vivo*, restrict the value of these plant remedies (McGaw *et al.*, 2007).

2.8 *In vitro* and *in vivo* test

In vitro testing determines the inherent susceptibilities of microorganisms to chemical agents whereas *in vivo* testing gives a more realistic insight of the test agent because it takes into account a variety of host factors such as metabolic processes and anti-infective defense mechanisms (Zak and O'Reilly, 1991). The testing of potential antimicrobial agents using animal models with infectious disease is a long-established practice and is acknowledged as an essential prerequisite of anti-infective therapy. Animal models of infectious disease bridge the gap between the *in vitro* characterization and the clinical evaluation of antimicrobial agents. However, the ultimate utility of such experiments in the development of antimicrobial agents is still debated. The majority of agents active *in*

vitro are devoid of significant activity *in vivo*. While some may also exert demonstrable effects *in vivo*, the correlation is generally considered poor (Zak and O'Reilly, 1991).

Promising biological activity displayed by a number of plant extracts supports the use of these plants as natural remedies, but systematic *in vivo* tests are required to fully ascertain their medicinal properties and potential toxicity. Drawbacks of traditional plant remedies include seasonal unavailability of plants, the possibility of uncertain dosages, ineffective or harmful treatments and lack of standardization (McGaw *et al.*, 2007).

2.9 S. aureus and *P. aeruginosa*

One of the most common human pathogenic bacteria are staphylococcus, particularly *Staphylococcus aureus.* The *staphylococci* are responsible for a variety of diseases in humans and animals. The *staphylococci* are the most common cause of hospital acquired infection and antibiotic resistant strains (MRSA) have become endemic in hospitals in most countries causing major public health concern. When it gets an opportunity, it causes superficial and systemic infections. The examples of pathogenic diseases caused by this bacterium include boils, impetigo and folliculitis. It can also cause serious infections of pneumonia, and infections of wounds and bones. Methicillin-resistant *S. aureus* (MRSA) are endemic in most hospitals in many industrialized countries and they are considered the most serious hospital-acquired pathogen as they can cause large outbreaks that are frequently difficult to treat using antibiotics (Lindsay, 2008).

P. aeruginosa is a gram-negative, aerobic, rod-shaped bacterium with unipolar motility and opportunistic human pathogen, *P. aeruginosa* typically infects the pulmonary tract, urinary tract, burns, wounds, and also causes other blood infections *P. aeruginosa* is naturally resistant to a large range of antibiotics and may demonstrate additional resistance

after unsuccessful treatment, in particular, through modification of a porin (Delden and Iglewski, 1998).

2.10 Mice model

An animal model is a living, non-human animal used during the research and investigation of human disease, for the purpose of better understanding the disease without the added risk of causing harm to an actual human being during the process (Perrett, 2010). The animal chosen will usually meet a determined taxonomic equivalency to humans so as to react to disease or its treatment in a way that resembles human physiology as needed. Many drug treatments and cures for human diseases have been developed with the use of animal models (Perrett, 2010). Animal models serving in research may have an existing, inbred or induced disease or injury that is similar to a human condition. When scientist, employ animal models in the study of human disease, the animals are frequently selected because of their similarity to humans in terms of physiology anatomy, and genetics. Also, animal models are often preferable for experimental disease research because of their unlimited supply and ease of manipulation (Simmons, 2008). Therefore, scientists cannot conduct research on just one animal or human, and it is easier for scientists to use sufficiently large number of animals to attain significant results. Among these animals, the majority of studies, especially those involving disease, have employed mice, not only because their genomes are similar to that of humans, but also because of their availability, ease of handling, high reproductive rates, and relatively low cost (Simmons, 2008).

Mouse models that mimic human disease play a vital role in understanding the cause and origin of diseases. Results of mouse model studies lend evidence toward the next step in biomedical research that leads to early detection of diseases, discovery of, new drugs, new combinations of treatments, or new methods. Additionally, scientists use mouse models

for investigating diseases in humans caused by diet, drugs, and environmental agents (Simmons, 2008).

2.11 Histopathology tests

According to Zak and Reilly (1991), studies with animals to determine toxicity of medicinal plants must be performed to ensure their safety. Guidelines that describe relevant parameters and interpretation of the results obtained are available. The toxicity testing in animals can give clear indications of possible short-term or long-term toxic effects and the maximum tolerable doses; it is thus, a prerequisite to clinical trials.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Test plant, laboratory animals and study site

Roots from mature trees of *Synadenium glaucescens* (ranging from 3 - 5 m in height) were collected from Makambako in Iringa Region where people grow the plant in their home gardens for medicinal use. Roots were selected due to the fact that they are commonly used by traditional healers. Moreover, studies by Das *et al.*, 2010, using various medicinal plants indicated that high concentration of bioactive compounds can occur in roots. Albino Swiss mice kept at the Laboratory Animal Unit were used to carry out *in vivo* studies. All the laboratory works were carried out at the Lab Animal Unit and Department of Microbiology and Parasitology respectively in the Faculty of Veterinary Medicine (FVM), Sokoine University of Agriculture (SUA) Morogoro.

3 2 Collection and handling of plant materials

Five kilograms (5 kg) of *S. glaucescens* roots were collected in clean bags and then washed using sterile water to remove any associated debris. The clean fresh roots were then air-dried on a laboratory bench for a week before being ground and filtered through 1.5 mm sieve.

3.3 Preparation of crude roots extract

Three portions of the air-dried sample, each weighing 30 g, were packed in thimble where Soxhlet extraction method was performed for 4 h, using ethanol 99% and then residues dried using rotary evaporator (BUCHI, Switzerland). The resultant powder was then measured 100 mg then dissolved in water for injection 2 ml (50 mg/ml) and passed through filter membrane .0.2 µL for bacteriological sterilization before inducement of the

extract to the mice. The extract was injected to the mice at the dose of 25 and 50 mg/mL based on the mean body weight of the mice which was 24 gm.

3.4 Experimental animals and their management

A total of one hundred and twenty (120) mice ranging between four and six weeks of age were selected randomly and kept in an experimental cages and left for a week to acclimatize with the experimental conditions (Kumari *et al.*, 2009). The mice were randomly put in groups of 10 animals each, kept in cages and fed on a basal feed (broiler finisher) with *ad libitum* access to drinking water. All mice received an oral dose of a broad-spectrum antibiotic (Erythromycin) to clear them of any infections prior to the experiment before being screened for bacterial infection one week before commencement of experiment. On the day of experiment, mice in each cage were marked by number and weighed.

3.5 Preparation of bacteria broth

Pure cultures of gram positive bacteria (*S. aureus* ATCC25923 and gram negative *P. aeruginosa* locally isolated strains) were cultivated in nutrient broth media and then serially diluted before incubation, overnight, in solid plate count agar. The resulting numbers of bacteria were 1.02×10^{9} cfu/mL and 2. 60 x 10^{10} cfu/mL respectively. The bacteria broths were kept in universal bottles at 4^{0}C until used.

3.6 Grouping and treatment allocation

Two separate experiments were carried out using 60 mice each and one bacterial species at a time (Table 1). Induction of bacterial abscesses was carried out using a method described by Dale *et al.*, (2004) with some modifications that the mice were shaved on the abdomen rather than the dorsal area. Exactly 100 µL of *S. aureus* and *P. aeruginosa* broth

were then injected subcutaneously under the shaved area. The test drug was administered to the mice through intra-peritoneal (i.p.) route (Chika *et al.*, 2007) once the induced abscesses were apparent.

In the first experiment (*S. aureus*), the 60 mice were randomly divided into six groups (n = 10). The first group (G1) served as a negative control i.e., the mice were neither infected with selected bacteria nor receive the test extract. Mice in the second and third groups (G2 and G3) were not infected but received two different doses of the test extract (25 and 50 mg) with no infection. The fourth group (G4) served as a positive control i.e., the mice were infected subcutaneously with 100 µL *S. aureus* broth into the previously shaved abdominal skin (Dale *et al.*, 2004) but not treated with the test extract. The mice in the last two groups (G5 and G6) were also infected with *S. aureus* and then treated with two different doses (25 and 50 mg/ml/kg body weight). The two doses were selected following a pilot experiment using four different doses whereby higher doses showed signs of toxicity to mice.

The second experiment was similar to the first experiment with the only exception that *P. aeruginosa* was used as a test bacterium.

Table 1: Grouping and treatment allocation

	Bacteria	Group	Infection	Extract (mg)
Experiment 1	*S. aureus*	G1	No	0
		G2	No	25
		G3	No	50
		G4	Yes	0
		G5	Yes	25
		G6	Yes	50
Experiment 2	*P. aeruginosa*	G1	No	0
		G2	No	25
		G3	No	50
		G4	Yes	0
		G5	Yes	25
		G6	Yes	50

3.7 Visual observation

Following the bacterial infections, mice from all the groups were monitored twice per day for twenty four hours for development of skin abscesses and any other lesions characteristic of each bacterium including swelling and redness.

After administration of the test extract, observations on clearance of the abscesses were monitored to the end of the experiments. Five mice from each group (control and test groups) were sacrificed and organs (skin, kidney and liver) were taken and preserved for bacterial count and histological examination.

3.8 Assessment of mice survival

Deaths of mice were recorded for up to 7 days after commencement of the treatments as an index of survival. Mean survival rate of mice that died was calculated by dividing the number of remaining live mice by the original total number of mice in the group.

3.9 Bacterial counts in tissues

Assessment of number of bacteria present in the sampled tissues was done using miles and misra method, where nutrient agar and Baird Parker media were used for *P. aeruginosa* and *S. aureus* respectively. A total of 144 samples were collected.

Mice were sacrificed and thoracic cavities were opened to remove kidney and liver, each organ was weighed and then 1 g was homogenized in 2 mL sterile normal saline. Skin lesions were sampled by first disinfecting the skin with 70% ethanol followed by removal of the infection site and underlying tissue using a sterile scalpel blade. One gram of the removed skin lesions was homogenized in 2 mL sterile normal saline. The homogenates (1 mL) of each tissue were serially diluted in tenfold in normal saline before pouring on plates and incubating at 37^0C for 24 h. Bacterial counts were expressed as numbers of *S. aureus* and *P. aeruginosa* colony forming units per gram per mL (cfu $g^{-1}mL^{-1}$) of tissue.

3.10 Histopathological examination of tissue samples

The remaining portions of kidney and liver were processed for histopathological examination using a standard procedure. Initially, tissues were grossly examined and then five micrometer thick sections were fixed in 10% buffered formalin, embedded in paraffin, stained with hemotoxylin-eosin (HE) and examined in a light microscopy. Tissue samples were evaluated for degree of deformation, infiltration of cells, protein formation in tubules, and necrosis. The histopathological pictures of tissues from the different animal groups were assessed blindly by a pathologist Pictures of the kidney and liver cells were taken using a still camera (Paxcam^{TM}-5).

3.11 Statistical analysis

All data obtained from the study were subjected to one way analyses of variance (ANOVA). A value of $P < 0.05$ was considered significant using the MS EXCEL (2007) package.

CHAPTER FOUR

4.0 RESULTS

4.1 Development of lesions following induced bacterial infections

Following the inducement of bacterial infections in mice, appearance of small skin lesions and abscesses occurred within 24 - 48 h of infection in G4 (infected + no extract) of both experiments. Infection due to *P. aeruginosa* was different from that of *S. aureus* in that the former had burnt appearance skin lesions whereby visible skin and kidney abscesses were the features seen in *S. aureus* infected mice. Following treatment, some clinical signs such as rough coat, hunched back, ocular discharge, moribund state and some mice finally died. Close gross/visual observation of the skin lesions of mice from G5 and G6 (infected + extract) showed disappearance of pus discharges from the skin within seven days of infection. All mice treated with the extract showed varying degrees of improvement on the skin lesions as compared to those mice in G4.

4.2 Mice survival assay

Results of the mice survival assay are shown in Table 2. In the first experiment all mice infected with *S. aureus* in a group which was not infected and not treated G1 and in group which was infected and not treated G4 the mice survived to the end of observation period. Two mice in each group (G2 and G3) (not infected and treated) died and the rest eight mice in each of these groups survived to the end of observation period. Seven mice in G5 (Infected and treated) survived and 3 died before the end of observation period while only 6 mice survived in G6 (Infected and Treated).

Figure 2: Skin lesion caused by *P. aeruginosa* (the arrow shows a burnt appearance skin lesion)

Figure 3: Skin abscesses caused by *S. aureus* infection

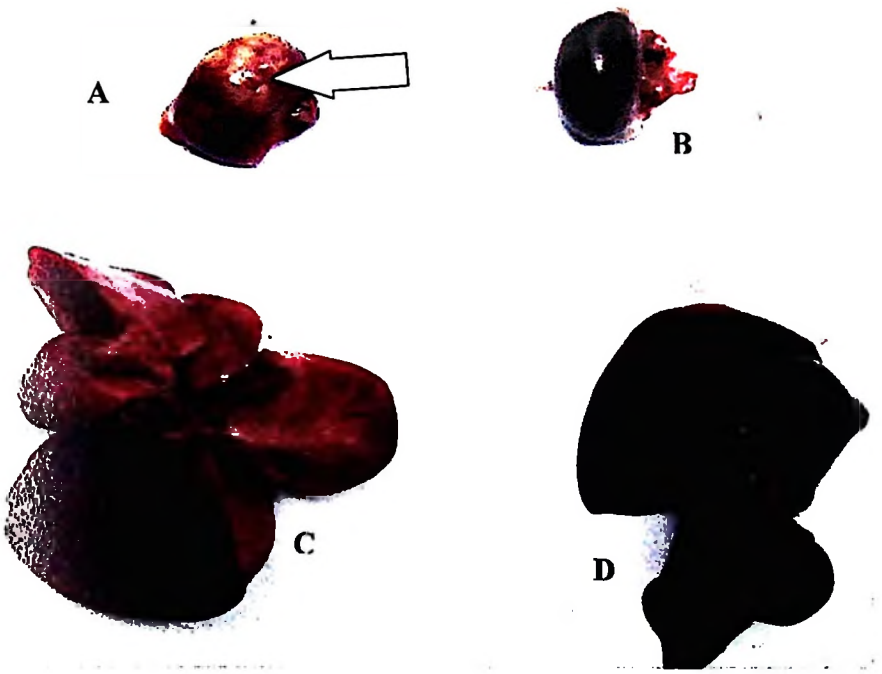

Figure 4: Abscesses in kidney due to *S. aureus* infections (A); normal kidney (B), liver infected by *P. aeruginosa* (C) and normal liver (D).

In the second experiment all mice in G1 (not infected and not treated) survived, 2 mice in each G2 and G3 (Not infected and treated) died. One mouse, five and seven mice died in G4 (infected and not treated), G5 (Infected and treated) and G6 (infected and treated) respectively.

Table 2: Effect of different doses of *S. glaucescens* extract on the survival of mice infected with *S. aureus* or *P. aeruginosa*

	Group	Infection	Extract (mg)	Deaths
Experiment 1	G1	No	0	0
(*S. aureus*)	G2	No	25	2
	G3	No	50	2
	G4	Yes	0	0
	G5	Yes	25	3
	G6	Yes	50	4
Experiment 2	G1	No	0	0
(*P. aeruginosa*)	G2	No	25	2
	G3	No	50	2
	G4	Yes	0	1
	G5	Yes	25	5
	G6	Yes	50	7

4.3 Tissue bacterial counts

Bacterial counts (cfu/mL) in different organs of mice following treatment with two different levels of test extract are presented in Table 3.

Table 3: Mean bacteria counts from different mice organs following induced infections and treatment with different doses of *S. glaucescens* crude root extract.

	Group	Infection	Extract dose (mg)	Mean Cfu/mL		
				skin	kidney	liver
Experiment 1	G1	No	0	3.0	2.6	4.0
(*S. aureus*)	G2	No	25	4.8	0.0	0.0
	G3	No	50	2.4	0.0	0.0
	G4	Yes	0	59.0	92.0	37.0
	G5	Yes	25	21.0	6.0	5.2
	G6	Yes	50	22.8	2.0	8.6
Experiment 2	G1	No	0	2.2	2.4	2.2
(*P. aeruginosa*)	G2	No	25	4.8	0.0	0.0
	G3	No	50	2.4	0.0	0.0
	G4	Yes	0	104.0	28.2	79.2
	G5	Yes	25	2.2	0.4	0.0
	G6	Yes	50	27.7	2.3	3.7

In both experiments, cfu/mL of organs from mice in G1 – G3 (no infection) were far less regardless of extract administration. Conversely, cfu/mL of G4 (infection without treatment) groups were significantly higher than those treated with either 25 or 50 mg extract per kg body weight. Regression between the dose of extract and cfu was found to be negative ($R^2 = 0.68$; $P = 0.37$) i.e., the cfu counts decreased with increasing dose of the extract.

Table 4: Summary of histopathological findings from the examined organ (experiment 1)

Bacteria	Group	Infection	Kidney			Liver	
			Histopathological change	Extract (mg)	Severity	Histopathological change	Severity
Experiment 1 (*S. aureus*)	G1 – G3	No	Desquamation of tubular epithelium	0	NO	Vacuolar or hydropic degeneration	NO
				25	+		+
				50	+		+
			Protein deposition in tubular lumen	0	NO	Necrosis	NO
				25	+		NO
				50	+		NO
			Inflammatory reaction (mononuclear cells in interstitial space)	0	NO	Inflammatory reaction (mononuclear cells)	NO
				25	+		+
				50	+		+
			Glomerular tufts	0	NO		
				25	+		
				50	+		
	G4 – G6	Yes	Desquamation of tubular epithelium	0	NO	Vacuolar or hydropic degeneration	NO
				25	+		+
				50	++		+++
			Protein deposition in tubular lumen	0	NO	Necrosis	NO
				25	+		NO
				50	+++		NO
			Inflammatory reaction (mononuclear cells in interstitial space)	0	NO	Inflammatory reaction (mononuclear cells)	NO
				25	NO		+
				50	NO		++
			Glomerular tufts	0	NO		
				25	NO		
				50	NO		

NO = not observed.

Table 5: Summary of histopathological findings from the examined organ (experiment 2)

Bacteria	Group	Infection	Kidney			Liver	
			Histopathological change	Extract (mg)	Severity	Histopathological change	Severity
Experiment 2 (*P. aeruginosa*)	G1 – G3	No	Desquamation of tubular epithelium	0	NO	Vacuolar or hydropic degeneration	NO
				25	+		+
				50	+		+
			Protein deposition in tubular lumen	0	NO	Necrosis around central vein	NO
				25	+		NO
				50	+		+++
			Inflammatory reaction (mononuclear cells in interstitial space)	0	NO	Inflammatory reaction (mononuclear cells)	NO
				25	+		+
				50	+		+
			Glomerular tufts	0	NO		
				25	+		
				50	+		
	G4 – G6	Yes	Desquamation of tubular epithelium	0	NO	Vacuolar or hydropic degeneration	NO
				25	NO		NO
				50	NO		+++
			Protein deposition in tubular lumen	0	NO	Necrosis around central vein	NO
				25	NO		NO
				50	NO		+++
			Inflammatory reaction (mononuclear cells in interstitial space)	0	NO	Inflammatory reaction (mononuclear cells)	NO
				25	NO		+
				50	NO		+++
			Glomerular tufts	0	NO		
				25	NO		
				50	NO		

NO = not observed.

4.4 Histopathological findings

Histological evaluation was carried out using samples from treated and untreated groups. Kidney and liver cells from those groups receiving extract and those infected with selected bacteria and then treated with 25 and 50 mg test extract per kg body weight showed some reactions.

In both experiments, kidneys and livers from mice in the control groups (G1, no infection, no extract) were normal. In G2 (no infection, 25 mg extract) and G3 (no infection, 50 mg extract) of both experiments, their liver cells had hydropic degeneration and inflammatory reactions. In the kidneys there was protein deposition in tubular lumen (Fig. 5), glomerular tuft (Fig. 6) and desquamation of the tubular epithelium.

In the group of mice which were infected with *S. aureus* then treated with the extract at a dose of 50 mg/kg body weight there were protein deposition in the tubular lumen and inflammatory reaction in kidney (Fig. 5). The livers showed the presence of inflammatory reaction and vacuolar or hydropic degeneration (Fig. 7). Tissue sections from groups of mice which were treated with the extract at 25 mg/kg body weight, showed mild desquamated tubular epithelium and protein in the tubular lumen of the kidneys. Liver tissue showed mild hydropic degeneration and inflammatory reaction. Groups infected with *P. aeruginosa* then treated with test extract at dose of 50 mg/kg body weight (G6) their liver underwent hydropic degeneration, necrosis formation (Fig. 7 and 8) and mononuclear cells inflammation. However, kidneys were normal. For those infected and treated with, 25 mg extract (G5), appearance of early stages of vacuolation and mononuclear cells infiltration in liver were observed while their kidneys were normal.

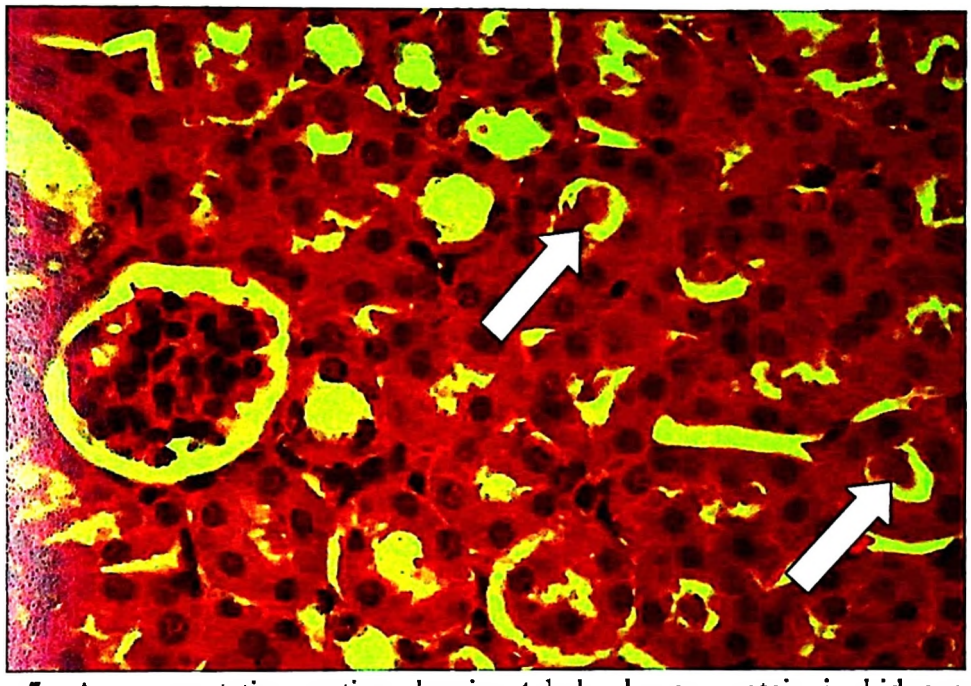

Figure 5: A representative section showing tubular lumen protein in kidneys from bacteria-infected-groups which received 50 mg root extract/kg body weight (200X)

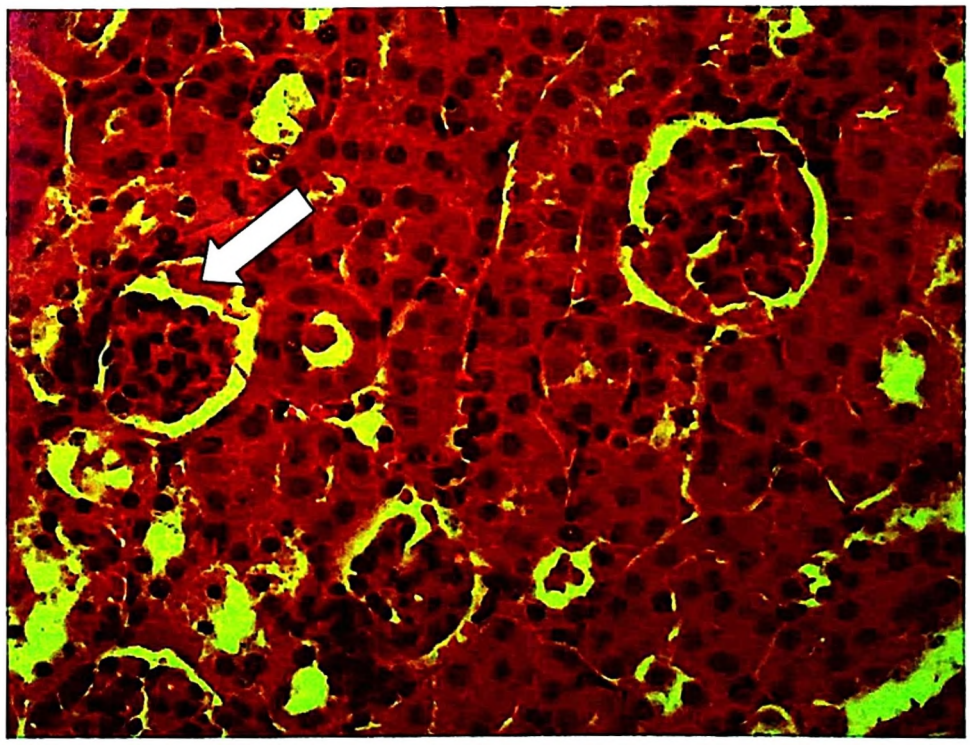

Figure 6: A representative section showing protein in glomerular tuft from bacteria-infected-groups which received 50 mg root extract/kg body weight (200X)

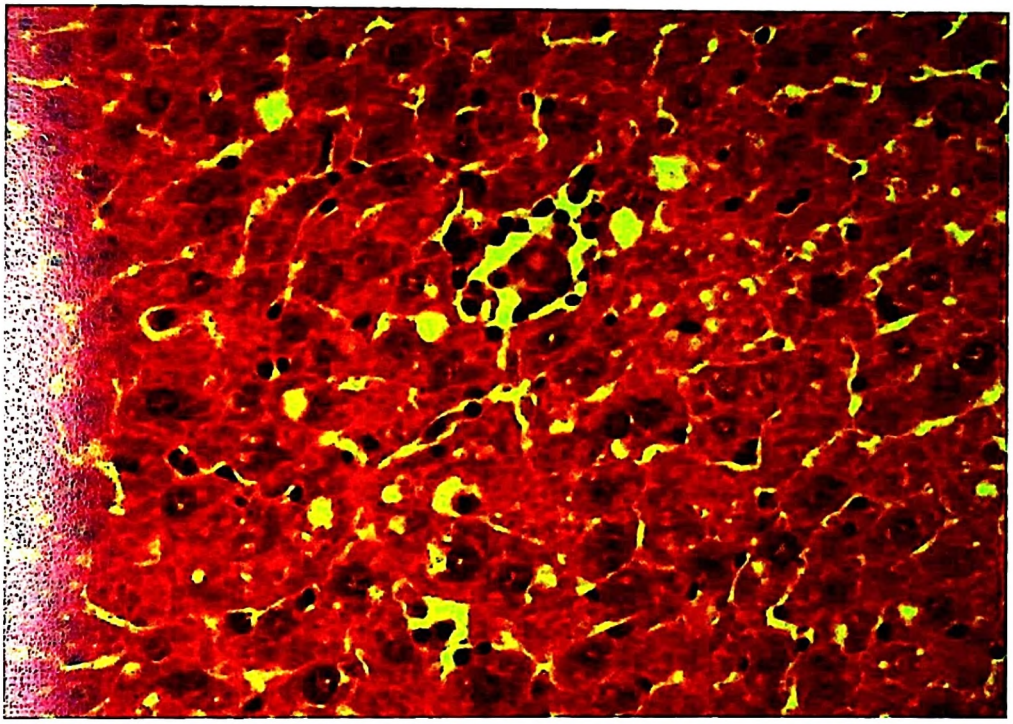

Figure 7 Hydropic degeneration of the hepatocytes from the *P. aeruginosa* infected group which received 50 mg extract/kg body weight (100X)

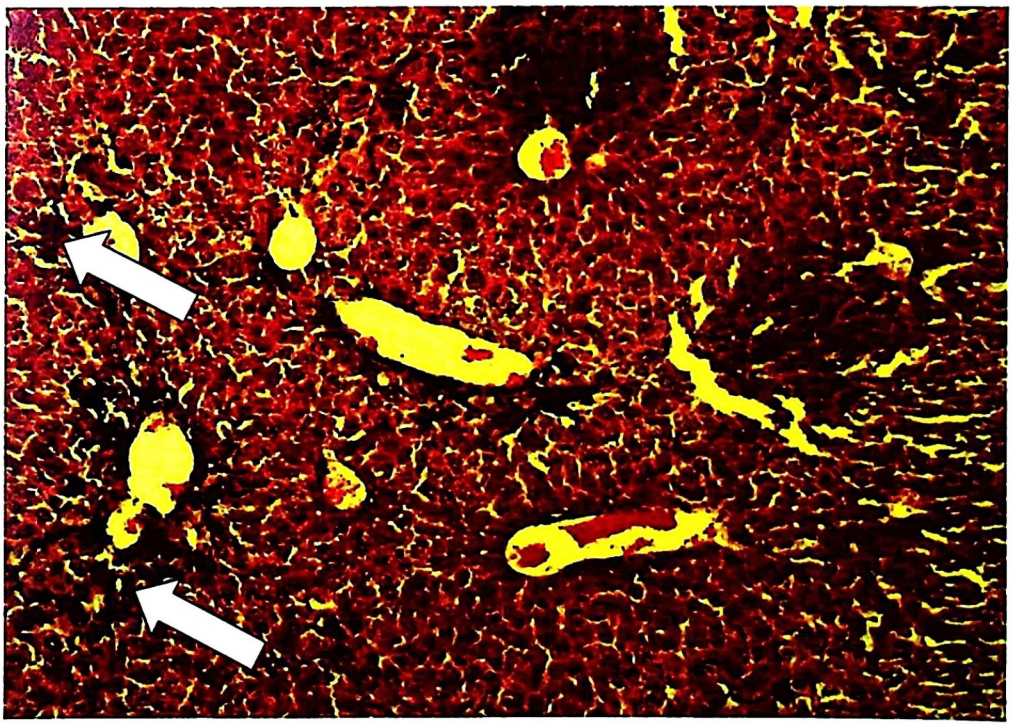

Figure 8: Liver necrosis around central vein from the *P. aeruginosa*-infected-group which received 50 mg extract/kg body weight (50X)

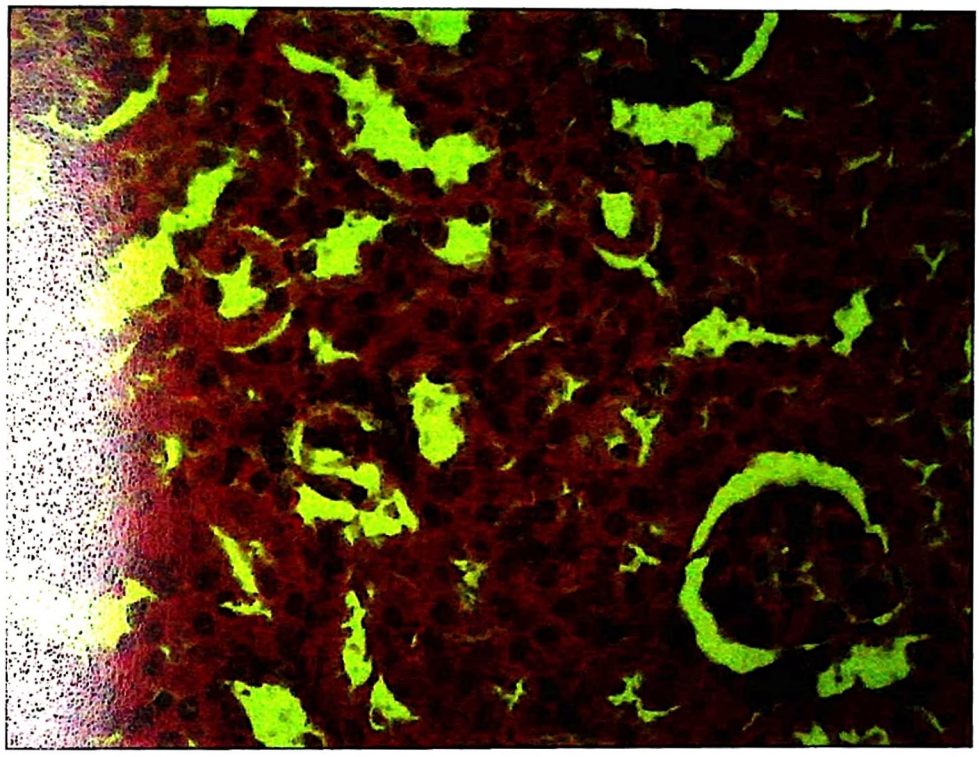

Figure 9: Histological section of a normal mouse kidney (200X)

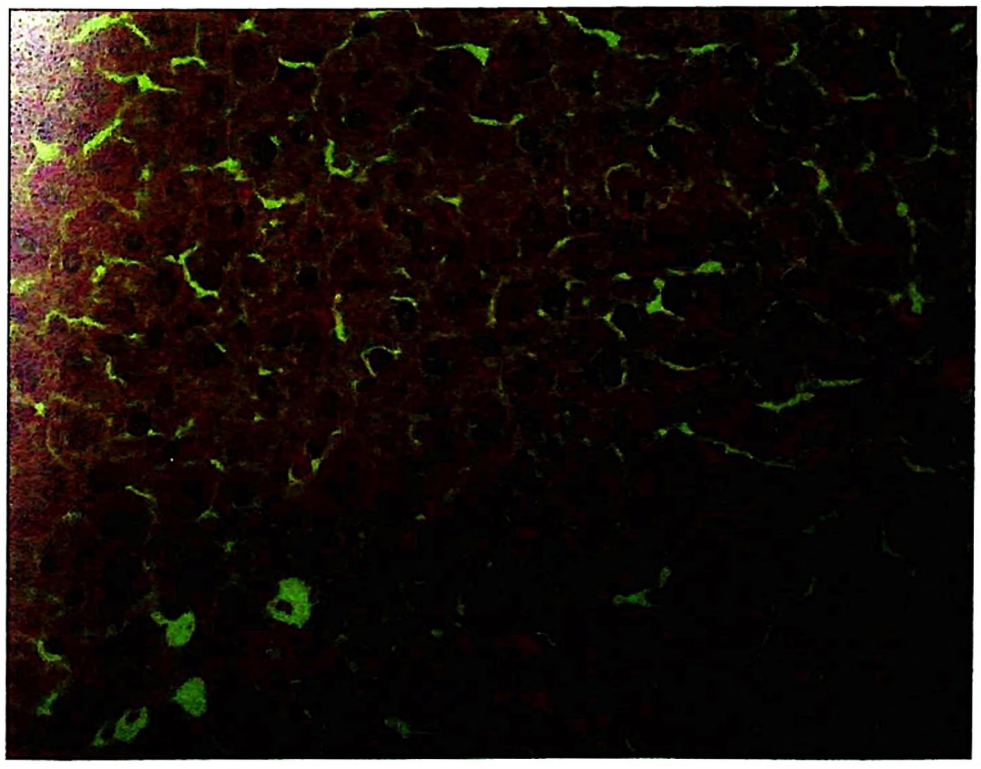

Figure 10: Histological section of a normal mouse liver (200X)

CHAPTER FIVE

5.1 DISCUSSION

The aim of the current study was to investigate the effect of crude ethanolic extract from roots of *S. glaucescens* against selected pathogenic bacteria using an *in vivo* assay. The assay involved experimental induction of bacterial infections to mice followed by treatment using the crude extract at different doses. Results indicated that induction of bacterial infections to mice lead to development of clinical signs and lesions characteristic of the test bacteria. On treatment with two different doses of the extract, it was clearly observed that the severity of clinical signs and lesions were significantly reduced. For instance, mice in groups receiving the extract showed disappearance of pus discharges from the abscesses as compared to those in the positive control groups. This was a clear indication that the extract had a negative impact on the development of bacterial infections (Abu-Al-Basal, 2009). Further antibacterial activity was demonstrated from the results of tissues bacterial counts and histopathological examinations of kidney and livers. In both experiments, mice infected with bacteria without treatment with the extract had significantly higher tissue bacterial counts than their treated counterparts (Table 3). This was an indication that the extract had a direct effect on the survival of the test bacteria in their hosts (Abu-Al-Basal, 2009). The presence of higher bacterial counts in skin than the internal organs (kidneys and lives) can be explained by the fact that skin of animal harbors large number of bacteria as normal flora (Kumari, *et al.*, 2009).

Histopathological examination of kidney and liver tissues indicated changes attributed to both extract and test bacteria. Administration of the extract induced a degree of desquamation of tubular epithelium, protein deposition, inflammatory reaction and formation of glomerular tufts in the kidney. In the liver, necrosis around central veins and

vacuolar degeneration were features associated with the extract administration. These findings suggest that the *S. glaucescens* extract could have some pathological effects on the mice's internal organs (Melo-Reis, *et al.*, 2010).

Two treatment doses were used to determine the optimum dose and whether the extract effect was dose-dependent or not. The current findings indicate a weak dose dependent effect as deduced from the regressions coefficient (R^2 = 0.68; P = 0.37); that is, the antibacterial activity increased with increasing dose of the extract. Since more mice deaths occurred when the dose was increased from 25 to 50 mg/kg bodyweight, especially with *P. aeruginosa* infection, it is suggested that high doses of the extract could be lethal.

In the current study, it was also observed that more mice deaths occurred when the extract was administered to *P. aeruginosa* infected groups than to those infected with *S. aureus* suggesting that the former bacterium became more virulent on administering the extract. Explanation behind this observation is not clear. However, it is known that *P. aeruginosa* tend to produce virulent factors as a defense mechanism when confronted by stressful agents like drugs (Adonizio. *et al.*, 2008). These factors, which include LasA protease, LasB elastase, pyoverdin, pyocyanin, alginate as well as toxins, are known to worsen the host animal clinically and may eventually lead to death.

5.2. Conclusion

In this research, the *in vivo* anti bacterial activity of *S. glaucescens* root extract was evaluated using mice. The results have demonstrated significant *in vivo* antibacterial activity against both test bacteria. However, the plant extract should be used with care since high doses were associated with some pathological kidney and liver changes.

5.3. Recommendation

Further studies are needed, to find out the most optimal dose of root extract in treating infections caused by the tested bacteria. Other studies should be done using other parts of plants such as leaves and stem bark. Phytochemical studies to isolate active compounds in the plant are also recommended.

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