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Antimicrobial and wound healing activities of *Clerodendron Splendens* G. Don

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Antimicrobial and Wound Healing Activities of *Clerodendron splendens* G. Don

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DECLARATION

I hereby declare that this submission is my own work toward the M. Phil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Clerodendron splendens G. Don (Fam. Verbanaceae) is a well known plant in Ghanaian folklore medicine for treating various skin infections and in wound healing. Based on its traditional use, this plant was evaluated for its antimicrobial, wound healing and antioxidant properties. Juice expressed from the fresh leaves and ethanol, acetone and chloroform extracts of the dried leaves, stem and root, all exhibited various degrees of antimicrobial activity against the test microorganisms: *Pseudomonas aeruginosa (NCIMB1042), Escherichia coli (NCTC9002), Staphylococcus aureus(NCTC4163), Bacillus subtilis (NCTC10073), Proteus mirabilis (NCTC13376), Klebsiella pneumonia (NCTC5055)* and *Candida albicans (NCTC3255),* with minimum inhibitory concentrations ranging from 0.15 to 7mg/ml. The time-kill kinetics studies conducted suggested microbistatic activity of the various extracts against these test microorganisms employed.

The extracts (fresh juice, 3, 30, 300 and 1000 mg/ml) were topically applied twelve hourly to excision wounds created on the back of Sprague Dawley rats in groups of seven. Two more groups serving as the controls were also treated with 1%$^{w}/_{w}$ Silver sulphurdiazine cream and the vehicle (normal saline + tween80). The extracts showed faster rate of wound contraction in the first 5 days than the controls. Higher breaking strengths were recorded, especially in the 300mg/ml treated group, suggesting the possibility of higher collagen content of the repaired tissues than in the control groups. *C. splendens* also exhibited antioxidant activity in the DPPH (1-diphenyl-2- picrylhydrazyl) radical scavenging assay with IC_{50} value of 5.61± 0.04 mg/ml. Thus this study provides a scientific rationale for the traditional use of this plant in the management of wounds and other infectious conditions.

CHAPTER ONE

1.1 INTRODUCTION

The use of herbs and other natural products for the restoration of health dates back into ancient times. The earliest known medical document is a 4000-year–old Sumerian clay tablet that recorded plant remedies for various illnesses. By the time of the ancient Egyptian civilization, a great wealth of information had already been gathered about existing medicinal plants. Among the many remedies prescribed was mandrake (*Mandragora afficinarum)* for pain relief, and garlic (*Allium sativum*) for the treatment of heart and circulatory disorders. This information, along with hundreds of other remedies, was preserved in the Ebers papyrus about 3500 years ago (Loriaux, 2006).

Ancient China is also a source of information about the early medicinal uses of plants. The 'Pun-tsao', a Chinese pharmacopoeia published around 1600, contained thousands of herbal cures that are attributed to the works of Shen-nung, China's legendary emperor who lived over 4500 years ago (Levetin and McMahon, 1999).

Medicinal plants have been used in virtually all cultures as a source of medicine.

The widespread use of herbal remedies as those described in ancient texts such as the Bible can be traced to the occurrence of bioactive principles in these plants (Hoareau and DaSilva, 1999). In the Bible (Numbers 11:4-6 and Luke 11:42) onions (*Allium cepa*) and garlic which are well known herbs and rue (*Ruta graveolens*) which is a spice and an aromatic stimulant, among other garden herbs are recorded.

William Withering in 1779 was the first in the medical field to scientifically investigate a folk remedy; his studies of foxglove (*Digitalis purpurea*) as a

treatment for dropsy (congestive heart failure) set the standard for pharmaceutical chemistry (Levetin and McMahon, 1999).

Herbal medical practice has been the main form of treatment especially among indigenous populations around the world particularly in developing countries, and the World Health Organization has also estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare (WHO, 2002). Currently even the industrialized nations are developing interest in the use of herbs. In the United States, increasing public dissatisfaction with conventional medicine and its increased cost, combined with an interest in returning to natural or organic remedies with the mistaken impression that natural products are safe, has led to an increase in the use of herbal medicines (Mathews *et al*, 1999).

In Germany it is estimated that roughly 600 to 700 plant-based medicines are available and are prescribed by approximately 70% of German physicians (Hart and Shumaka, 2008). In Germany, herbal medicines are actually prescribed more often than conventional medicines. These plant based medicines have been studied and the claims of a number of compounds in them shown to be valid while others have been ineffective or more toxic than prescription alternatives (Hart and Shumaka, 2008).

Scientific investigation into some folk remedies has provided leads to bioactive compounds and many of these compounds have been developed into drugs. Examples of these folk remedies are as follows: willow (*Salix purpurea*), the plant from which the famous analgesic, aspirin was first obtained (Hedner and Everts, 1998), quinine (an antimalarial) from *Cinchona succirubra*, artemisinin (qinghaosu) which is the antimalarial from a Chinese medicinal herb (*Artemisia*

annua) (Klayman, 1985), Forskolin, which is the antihypertensive agent from *Coleus forskohlii* Briq, (Kinghorn, 1987) and the ginkgo tree (*Ginkgo biloba*), mentioned in Chinese medicinal books used in antiasthmatic and antitussive preparations (Hamburger *et al,* 1991).

Folklore medicine seems to have a stock of valuable lead compounds which when scientifically investigated can provide very useful drugs especially in the management of infectious conditions. As the development of bacterial resistance to antibiotic persists, man turned to his prehistory and found literally thousands of phytochemicals, which inhibit all types of microorganisms, broadly effective without inducing microbial resistance. New compounds inhibiting microorganisms such as benzoin from *Styrax benzoin* and emetine have been isolated from plants (Cox, 1994). The new plant-based antibiotics may help in the treatment of infections caused by antibiotic-resistant microorganisms being encountered in chemotherapy.

1.2 THE SEARCH FOR BIOLOGICALLY ACTIVE METABOLITES

The detection of biologically active metabolites in a material is one of the strategic approaches in the search for potentially new drugs and plants have been useful in this kind of approach.

1. Clues to the presence of bioactive metabolites in plant come from the folklore uses of such plants. Quinine and aspirin for example were discovered when the folkloric use of the plants *Cinchona succirubra* and *Salix purpurea* (Hedner and Everts, 1998) were investigated.

2. Other clues to useful metabolites come from observations by scientists in the field. It was this method that led to the discovery of the principal antibiotics, penicillin by Alexander Fleming. In his laboratory, he observed that a contaminating colony of microorganism prevented the growth of bacteria form the region where it was growing (Harbarth *et al*, 2003).

3. Bioactive metabolites can also be identified in plants by searching for a particular type of compound. Castanospermine for example was discovered when the search for alkaloids with glycosidase-inhibiting activity was initiated (Waterman, 1990).

4. Serendipitous discoveries have also been reported (Waterman, 1990); the Madagascan periwinkle (*Catharanthus roseus*) from which very potent anticancer agents (vincristine and vinblastine) were developed was indigenously employed for the management of diabetic mellitus (Murata, 2003).

1.3 BIOASSAY TECHNIQUES

1.3.1 Antimicrobial bioassays

All antimicrobial bioassays aim at the fast and reliable detection of antimicrobial activity. These tests are simple but require specialized skills to enable them be carried out.

1.3.1.1 Agar Diffusion methods

The agar diffusion method relates the varying concentrations of antimicrobial agent between the point of application and the area of inhibition. Generally, an area of no growth which is a clear zone can be related to the concentration of the agent.

The diffusion methods rest on the principle that the antimicrobial agent diffuses through the agar in which bacterial cells are capable of growth. It is desirable that the organisms are uniformly distributed on the agar in petri dishes or large plates. The samples under test are applied into wells or cylinders or disks placed on the seeded agar plates. The zone of inhibition of growth is measured and it is expected that zone length will be proportional to the concentration of the antibiotic (Olaniyi and Ogunlana, 1998).

1.3.1.2 Serial dilution method

A standardized microbial inoculum is added to tubes containing serial dilutions of an antibiotic, and the growth of the microorganisms is monitored as a change in turbidity. In this way, the minimum concentration of the antibiotic that prevents growth of the microorganisms in vitro can be determined. The MIC indicates the minimal concentration of the antibiotic that must be achieved at the site of infection to inhibit the growth of the microorganisms tested. The method is usually not sensitive enough for desired accuracy but it has an advantage of ease of performing the tests with many microorganisms. Reports show that the use of this method for antibiotics can still be reproducible if special attention is paid to various details that include the following: sensitivity of the test microorganism, constancy of composition of medium, inoculums size, temperature of incubation and pH of the medium (Olaniyi and Ogunlana, 1998).

1.3.2 Bioautography

This is a technique that has been used to screen for antimicrobial activity. The extract is developed on a thin-layer chromatographic plate (TLC) and allowed to

dry. The developed TLC plate is overlaid with a seeded agar. Compounds diffuse from the chromatoghraphic layer into the agar and after incubation, zones of inhibition are made visible with the appropriate stains such as p-iodonitrotetrazolium chloride (Hamburger and Cordell, 1987).

1.3.3 Antiviral activity

A bioautoghraphic assay to screen extracts for antiviral agents involves overlaying *Herpes simplex* virus (HSV)–infected CV-1(monkey kidney) cells with a developed thin layer chromatographic plate. Time is allowed for diffusion of the antimicrobials from the plate into the tissue culture. After incubation, areas of viral growth inhibition caused by the extracts are looked out for. This assay also gives indication of cytotoxicity by showing inhibition of the CV-1 cells (Tan *et al*, 1991).

1.3.4 Brine shrimp lethality test

The brine shrimp lethality test (BSLT) is a technique capable of detecting a broad spectrum of bioactivity, including cytotoxicity and pesticidal activity (Evieyer, *et al*, 1982). The eggs of *Artemis salina* (crustacean) when placed in brine solution hatch within 48hours into shrimps. Compounds and extracts are tested in vials containing 5ml of brine solution and 10 brine shrimps in 3 replicates initially at 10, 100 and 1000 ppm. Survivors are counted after 24h with the aid of a stereoscopic microscope, and LC_{50} values at the 95% confidence limit calculated. The LC_{50} is the concentration that is lethal to 50% of the shrimps. It has been used in the

detection of active antitumor agents and pesticides produced by plants (Alkofahi *et al*, 1989).

1.3.5 Crown–Gall Tumour Bioassay

This bioassay is fairly accurate in predicting *in vivo* murine antileukin activity. Crown–gall is a neoplastic disease induced by *Agrobacterium tumefaciens*, a bacterium known to infect crop plants and is due to the transfer of tumor–inducing plasmid from the bacterium to the plant genome. In this assay, potato tubers are surface sterilized, and a core of the tissue is extracted from each tuber. A 2cm piece is cut from each end, and the remainder is sectioned into 0.5cm thick disks that are then placed onto petri dishes containing 1.5% water agar. The 1.5% water agar is to provide a substrate that is sterile and contains enough moisture for optimal growth of the bacterium. A solution of the extract or compound dissolved in a suitable solvent is spread over a disk, and the solvent is allowed to evaporate. The disks are then inoculated with 0.1ml of the bacterial suspension and the plates are incubated at 27^0C. The assay measures the inhibition of tumors induced by the bacterium on potato disc by various plant extracts (Galski *et al*, 1980).

1.4 WOUND HEALING PROPERTIES OF COMPOUNDS

Wound healing is the body's natural process of regenerating dermal and epidermal tissues in a wounded area. When an individual is wounded, a set of complex biochemical events takes place. These events may be categorized into the following stages: the inflammatory, proliferative, and remodeling phases (MacKay and Miller, 2003). Compounds which are known to promote these stages can be

therapeutically employed to accelerate the wound-healing process (Reddy *et al*, 2007).

1.4.1 Inflammatory phase

Immediately after a blood vessel is breached, blood comes in contact with collagen, triggering blood platelets to begin secreting inflammatory factors. Ruptured cell membranes also release other inflammatory factors like thromboxanes and prostaglandins thus initiating the clotting cascade. This causes the vessel to spasm to obtain hemostasis hence prevent blood loss and to collect inflammatory cells in the area. This vasoconstriction lasts five to ten minutes and is followed by vasodilatation (Stadelmann *et al*, 1998).

Within an hour of wounding, polymorphonuclear neutrophils (PMNs) arrive at the wound site and become the predominant cells in the wound. Neutrophils and macrophages phagocytise debris and also kill bacteria by releasing free radicals called a 'respiratory burst'(Greenhalgh, 1998). Within a short time there is pain, reddening and edema of the surrounding tissue which are all classical symptoms of inflammation caused by release of eicodanoids, prostaglandins and leukotriens (Houghton *et al*, 2005).

Because inflammation plays roles in fighting infection and inducing the proliferation phase, it is a necessary part of healing. However, inflammation can lead to tissue damage if it lasts too long; hence reduction of inflammation is frequently a goal in therapeutic settings. Some plant extracts such as *Calendula*

succus promote wound healing by inhibiting further eicosanoid synthesis and free radicals (MacKay and Miller, 2003; Houghton *et al*, 2005).

1.4.2 Proliferative phase

About three days after the wound occurs, fibroblasts begin to enter the wound site, marking the onset of the proliferative phase. Stem cells originating from parts of uninjured blood vessels develop pseudopodia and push through the wound site. This leads to angiogenesis where new blood vessels form to provide oxygen and nutrients to the healing tissue (Deodhar and Rana, 1997). *Symphytum officinale* has been reported to promote this phase by enhancing cell division and the growth of connective tissues throughout the wounds (MacKay and Miller, 2003).

Vitamin A on the other hand does not only promote epithelial cell differentiation but is also an antioxidant and therefore reduces damage to cells by oxygen radicals. Substantial evidence supports the use of vitamin A as a perioperative nutritional supplement.

By the third post-wounding day, Fibroblasts begin secreting appreciable collagen. Collagen production continues rapidly for two to four weeks, after which its destruction matches its production and so its growth levels off. Collagen deposition is important because it increases the strength of the wound (Bulbuller *et al*, 2003).

Vitamin C (ascorbic acid), a common wound healing agent is an essential cofactor for the synthesis of collagen, proteoglycans, and other organic components of the intracellular matrix of soft tissues such as bones, skin, capillary walls, and other connective tissues. Ascorbic acid deficiency causes abnormal collagen fibers and

alterations of the intracellular matrix that manifests as cutaneous lesions, poor adhesion of endothelium cells, and decreased tensile strength of fibrous tissue (Porto *et al*, 2002).

A*loe vera* is also known to increase collagen content and the degree of collagen cross-linkage within the wound. C*entella asiatica* extract is also known to stimulate type-1 collagen production (MacKay and Miller, 2003).

1.4.3 Epithelialization

The formation of granulation tissue in an open wound allows the reepithelialization phase to take place, as epithelial cells migrate across the new tissue to form a barrier between the wound and the environment. Basal keratinocytes from the wound edges and dermal appendages such as hair follicles, sweat glands and sebacious glands which are the main cells responsible for epithelialization advance in a sheet across the wound site and proliferate at its edges, ceasing movement when they meet in the middle (DiPietro and Burns, 2003).

About seven days after wounding takes place, fibroblasts differentiate into myofibroblasts and the wound begins to contract. Contraction peaks at 5 to 15 days post wounding and this occurs in order to reduce the size of the wound. A large wound can become 40 to 80% smaller after contraction. Wounds can contract at a speed of up to 0.75 mm per day, depending on how loose the tissue in the wounded area is. As the actin in myofibroblasts contracts, the wound edges are pulled together. Methanol leaf extract of *Leucas hirta* possesses wound healing potency,

evident by the increased rate of wound contraction which may be attributed to the phytoconstituents like flavonoids, alkaloids, tannins (Manjunatha et al, 2006). Haruan (*Channa striatus*) treated wounds have also shown an increased rate of wound contraction, leading to quicker healing as confirmed by the increased healed area when compared to controls (Saringat and Sheikh, 2000).

When the levels of collagen production and degradation equalize, the maturation phase of tissue repair is said to have begun. During Maturation, type III collagen, which is prevalent during proliferation, is gradually degraded and the stronger type I collagen is laid down in its place. As the phase progresses, the tensile strength of the wound increases approaching 50% that of normal tissue and ultimately becoming as much as 80% as strong as normal tissue, the wound is said to have matured (Lorenz and Longaker, 2003). Wound strength is acquired from remodeling of collagen, brought about by the inter and intra molecular protein cross-linking (Chithra *et al*, 1998), polypeptide formation by the combination of glycine with aspartic and glutamic acid in the presence of leucine, methionine, alanine and arginine (Mat Jais *et al*, 1994). Wounds treated with *Channa striatus* cream have shown greater tensile strength, and it may be inferred that it increases the amount of collagen.

1.5 Methods for assessing wound healing properties of compounds

The progress of wound healing and the quality of the regenerated tissues at the wounded site may be assessed by determining the following parameters:

1.5.1 Wound size

Wound size measurement can be used to monitor the progress of healing through changes in the length, width, area or volume of the wound with time. The size of the wound is measured at regular intervals. The size of wound may be assessed by various methods including the following:

1.5.1.1 Simple measurement: the wound surface area is determined by measuring its linear dimensions with a tape measure or ruler. An alternative method of calculating wound surface area is based on the formula for an ellipse (that is length x width x 0.785).

1.5.1.2 Wound tracing: In this assessment, a pen is used to trace the outline of the wound directly onto sterile transparent film at certain time intervals (Plassmann, 1995). The tracings can be entered into a data processing system using a simple scanner (Langemo *et al*, 1998).

1.5.1.3 Scaled photographs: Scaled photographs of the wound area are taken at certain time intervals and these are processed by a special scanner that calculates the area (Resch *et al*, 1988 and Berg et al, 1990).

1.5.2 Measurement of Tensile strength

Tensile strength is the resistance to breaking under tension. It indicates how effectively the repaired tissue resists breaking and may indicate in part the quality of the repaired tissue. The instrument used for measurement is the tensiometer according to Vaisberg *et al*, 1989. For the quantitation, one of the edges of the

wound is fixed while applying a measurable force to the other one (Fig. 1). The load in grams required to disrupt the wound is determined after complete healing of the wound (Rashed *et al*, 2003).

1.5.3 Wound bed assessment

The wound bed is filled with granulation tissue, the quality of which can be a means of assessing healing progression. The tissues may be observed visually or microscopically for various characteristics. Healthy granulation tissue appears pink in colour and is an indicator of healing while unhealthy granulation tissue is dark red in colour, often bleeds on contact, and may indicate the presence of wound infection. Excess granulation or overgranulation may also be associated with infection or non-healing wounds. Chronic wounds may be covered by white or yellow shiny fibrinous tissue, and healing will proceed only when it is removed. It is of extreme importance that this tissue be assessed during the healing of the wound because the type of tissue at the base of the wound will provide useful information relating to expectation of total healing time and the risk of complications, for example, bone at the base may suggest osteomyelitis and therefore a delayed or non-healing wound. Wound bed that is covered with necrotic tissue, slough or eschar impedes healing (Izadi and Ganchi, 2005).

1.5.4 Microbiological examination of wounds

Exposure of subcutaneous tissue following wounding provides a moist, warm, and nutritionally rich environment that favors microbial colonization and proliferation. Since colonization most frequently occurs by a group of potentially pathogenic microbes, any wound is at some risk to become infected (Bowler, 1998). Infected

wounds heal less rapidly and also often result in the formation of unpleasant exudates with concomitant killing of regenerating cells. Antibacterial and antifungal compounds in traditional remedy may prevent this occurring and may underlie its use in treating wounds (Houghton *et al*, 2005). Topical antimicrobial agents, including both antiseptics (Iodine, Ethanol) and antibiotics, are currently helpful in reducing the risk of infection. Microbial resistance to antibiotics has however become a worldwide medical, economical, and public health problem (Milatovic and Braveny, 1987). Antiseptic agents are therefore gaining renewed interest and usage because they have a lower propensity to induce bacterial resistance than antibiotics. Molecular iodine and silver are potent broad-spectrum antimicrobial agents that promote healing in microbially compromised wounds (Bowler et al, 2001).

Widespread opinion among wound care practitioners is that aerobic or facultative pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and hemolytic streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds (Daltrey *et al*, 1981).

Triphala which is a traditional ayurvedic herbal formulation consisting of three medicinal plants, *Terminalia chebula*, *Terminalia bellirica*, and *Phyllanthus emblica*, have been reported to possess numerous biological and pharmacological activities when applied to wounds (Wohlmuth, 2002). In particular *Terminalia chebula* has antibacterial activity against the main primary organisms in wound infections, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and haemolytic streptococci (Ahmad *et al*, 1998).

In assessing the antimicrobial activity of these extracts, various antimicrobial susceptibility methods may be employed. The extract is applied to the wounds and periodic swabs taken to determine the antimicrobial profiles of these wounds. This includes the numbers and types of organisms present.

1.6 ANTIOXIDANTS

1.6.1 The role of antioxidants in wound healing

An antioxidant can be defined as an agent that inhibits oxidation of a susceptible substrate. They are metabolites found naturally in the body and in plants. Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E) {Hollman, 2001}.

Reactive oxygen species (ROS) play a vital role in wound healing by serving as cellular messengers that drive numerous aspects of molecular and cell biology. In the inflammation phase of healing, neutrophils and macrophages are attracted into the injured tissue. They phagocytize, kill, and digest microorganisms and eliminate wound debris through their characteristic “respiratory burst” activity and phagocytosis (Clark and Moon, 1999) that results in the generation of free radicals such as superoxides. Superoxide is rapidly converted to membrane permeable form, H_2O_2, by superoxide dismutase. Release of H_2O_2 may promote formation of other oxidants that are more stable including hypochlorous acid, chloramines, and aldehydes, together this suggests that the wound site is rich in oxidants (Khanna *et al*, 2001). At high concentrations, these ROS can induce severe tissue damage and

even lead to neoplastic transformation which further impedes the healing process by causing damage to cellular membranes, DNA, proteins and lipids as well (Martin, 1996).

Cells have developed mechanisms to detoxify ROS. There are two major strategies which convey partial resistance against oxidative stress to most cell types: small antioxidant molecules like ascorbate, polyunsaturated fatty acids or sugars (mainly mannitol), and ROS-scavenging enzymes, such as superoxide dismutases (SOD), catalase, and various peroxidases (Heike Steiling *et al*, 1999). Topical applications of compounds with free-radical-scavenging properties in patients have shown to improve significantly wound healing and protect tissues from oxidative damage (Thiem and Grosslinka, 2003). Antioxidants therefore enhance the healing of infected and noninfected wounds by reducing the damage caused by oxygen radicals (Martin, 1996).

The combined effect of ascorbic acid (vitamin C) on collagen synthesis, antioxidant status, and immunomodulation makes it an appropriate supplement for wound repair protocols (MacKay and Miller, 2007).

Vitamin E is also used in skin care to prevent scaring. It also functions as the major lipophilic antioxidant thereby preventing peroxidation of lipids, resulting in more stable cell membranes. Prophylactic administration of vitamin E has also been shown to increase breaking strength and normalize healing of wounds exposed to preoperative irradiation (Chandan *et al,* 2002). Anecdotal reports also claim that topical vitamin E is valuable for speeding wound healing and improving cosmetic

outcome of burns and other wounds, including surgical scars (MacKay and Miller, 2007).

1.6.2 Antioxidant assay

Antioxidant capacity assays may be broadly classified as electron transfer (ET) and hydrogen atom transfer (HAT) based assays. The majority of HAT assays is kinetics based, and involves a competitive reaction scheme in which antioxidant and substrate compete for peroxyl radicals thermally generated through the decomposition of azo compounds.

ET−based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. ET assays include the ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), CUPRAC (Cupric ion reducing antioxidant capacity), DPPH (2, 2-Diphenyl-1-picrylhydrazyl), Folin-Ciocalteu and FRAP (Ferric reducing ability of plasma) methods, each using different chromogenic redox reagents with different standard potentials (Reşat *et al*, 2007).

1.6.2.1 ET-Based Assays

Grouped into this category are the total phenols assay, Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay, the *N*, *N*-dimethyl-*p*-phenylenediamine (DMPD) assay, and the Cu (II) reduction capacity assay. These methods involve two components in the reaction mixture, antioxidants and oxidant (also the probe). They are based on the following electron-transfer reaction:

Probe (oxidant) + e (from antioxidant) → reduced probe + oxidized antioxidant

The probe itself is an oxidant that abstracts an electron from the antioxidant, causing color changes of the probe. The degree of the color change is proportional to the antioxidant concentrations. The reaction end point is reached when color change stops. The change of absorbance is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which is expressed as Trolox equivalence (TE) or gallic acid equivalent (GAE) {Reşat *et al*, 2007}.

1.6.2.2 2, 2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay

DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 517 nm. Upon reduction, the solution color fades; the reaction progress is conveniently monitored by a spectrophotometer until the absorbance is stable. Upon reduction, the color of the solution fades. The percentage of the DPPH remaining is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as T_{EC50}. The DPPH assay is technically simple. (Reşat *et al*, 2007).

1.6.2.3 Total Antioxidant Potential Assay Using Cu (II) as an Oxidant.

The method is based on reduction of Cu (II) to Cu (I) by antioxidants present in a sample. A chromogenic reagent, bathocuproine (2, 9-dimethyl-4, 7-diphenyl-1, 10-phenanthroline), forms a 2:1 complex with Cu (I), which has a maximum

absorbance at 490 nm. It was found that 1 mol of α-tocopherol can reduce 2 mol of Cu (II) to Cu (I) (Reşat *et al*, 2007).

1.7 PLANT UNDER STUDY

1.7.1 Description

Clerodendron splendens G. Don known as the flaming glorybower belongs to the family Verbenaceae. It originates from West Africa. It is a climbing shrub with branchlets that have finely pubescent leaves about 15.2 by 8.9 cm. The leaves are avate or narrowly lanceolate, fairly glandular below and rounded at base. This plant flowers from November to April. It has one long, white or deep red conspicuous corolla which is nearly 1inch in length, with pink stamens, in showy corymbose cymes. The stamens are long and protruding. Calyx is red when in fruit. Fruiting is from November to January. The seeds are black, enclosed in an orange substance as observed in Fig 1.1 and 1.2. *Clerodendron splendens* is mostly seen growing on cultivated land between food crops, especially cassava and maize plants, and therefore considered a noxious weed among many in Ghana (Irvine, 1961).

Fig. 1.1 Picture of *Clerodendron splendens* when flowering

Fig. 1.2 Picture of *Clerodendron splendens* when in fruit

1.7.2 Folkloric Uses of *Clerodendron splendens* and other species

The genus Clerodendron consists of about 400 herbs, vines, shrubs, and trees of the tropics, many of which are grown as garden plants (Irvine, 1961).

The leaves of *Clerodendron splendens* are formulated into lotion and applied to bruises and sores, the dry powdered leaves are also applied to blisters caused by burns (Irvine, 1961). Some of the indigens use the leaves for various skin diseases such as *Tinea capitis* and boils. It is also known to enhance wound healing. The volatile oil of *Clerodendron splendens*, prepared by extraction of the fresh flowers with n-hexane was found to be active against S*taphylococcus aureus* and *Candida albicans* (El Deeb and Kadriya, 2003). Anti pyretic and anti inflammatory effects of extracts have also been revealed (Shehata *et al*, 2001).

In European countries, it is a decorative shrub mainly used for ornamental purposes. It is suitable for covering all kinds of fences and arches, in full sun. Some of the other species are used for medicinal purposes while many more such as *Clerodendron buchholzii, Clerodendron fallax, Clerodendron fragrans* and *Clerodendron sinuatum* are grown for ornamental purposes.

Other species of *Clerodendron* that have been reported to be of medicinal use are listed below;

1.Clerodendron capitatum; A decoction of the root is taken hot for severe stomach pains and also for the treatment of orchitis and elephantiasis of the scrotum, the leaf-pulp with *Capsicum pepper* being used as an enema for the same purpose. A decoction of the stem is considered febrifugal and a decoction of leaves and inflorescence is used as a gargle for tooth ache.

2.Clerodendron polycephalum; the leaf infusion is applied to snake bites in Nigeria.

3.Clerodendron umbellatum; the leaf-pulp is used on snake bites and also as an enema to hasten expulsion of the placenta at childbirth, and for gonorrhea (Irvine, 1961).

1.7.3 Phytochemical constituents of *Clerodendron splendens*

Clerodendron splendens leaves and flowers have been reported to contain carbohydrates, glycosides, unsaturated sterols, triterpinoids, flavonoids and volatile oils. The major sterol detected was 24ß-etylcholesta-5, 22E, 25(27)-trien-3ß-ol (also known as 25(27)-dehydroporiferasterol). Trace of its 22-dihydro derivative, clerosterol (also known as 25(27)-dehydroclinonasterol) was also found. The dominant n-alkane present was C29 (n-nonacosane) and the dominant n-alkanol was C28 (n-octacosanol) {Pinto *et al*, 1985}.

1.8 AIM OF STUDY

Antimicrobial agents from plant sources are gaining renewed interest and usage because they have a lower propensity to induce bacterial resistance than antibiotics and highly capable of enhancing wound healing (Bowler et al, 2001). Therefore this study seeks to investigate the antimicrobial and wound healing properties of *Clerodendron splendens (Fam.Verbenaceae)* which may justify the traditional use of this plant. This investigation was done by carrying out the following tests:

- Screening of leaves, stem and roots of *Clerodendron splendens* for antimicrobial activity against some test microorganisms.
- Determining the time-kill kinetics of the extracts on test organisms.
- Determining the wound healing properties of *Clerodendron splendens* in vivo.
- Assaying leaf extracts for antioxidant properties.
- Screening the plant phytochemically to establish the classes of compounds present.

It is hoped that results of this tests will aid in the proper use of this plant and probably promote further investigations leading to the detection and isolation of compound(s) that will be invaluable in wound healing.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Test organisms

The following test organisms were obtained from the National Type Culture Collection, U.K., and maintained on agar slants for use in the bioassay. Whenever needed, a sample of the stock test microorganism was aseptically transferred into sterile nutrient broth (for bacterial) or Sabouraud broth (for fungus) and incubated 18 to 24 hours.

1. Escherichia coli (NCTC 9002)

2. Pseudomonas aeruginosa (NCIMB 1042)

3. Proteus mirabilis (NCTC 13376)

4. Klebsiella pneumonia (NCTC 5055)

5. Staphylococcus aureus (NCTC 4163)

6. *Bacillus subtilis* (NCTC 10073)

7. Candida albicans (NCTC 3255)

2.2 Collection of Plant Materials

The leaves, stems and roots of the *Clerodendron splendens* were collected from Asokore Mampong in the Ashanti Region between April and July 2007. The plant was authenticated by Mr Ntim of The Forestry Herbarium Resource Management Support Centre, Forestry Commission, Kumasi on April 10th 2007. A herbarium specimen (number FP 08/08/1) is being kept in the Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi.

2.3 Extraction of *Clerodendron splendens*

2.3.1 Extraction procedure

2.3.1.1 Fresh leaf extract

The fresh leaves of *Clerodendron splendens* were thoroughly washed with tap water, to get rid of dirt and soil particles. The washed leaves were weighed (112.8g) and by means of porcelain mortar and pestle triturated into a fine pulp. The pulp was then strained with white calico and 27.4ml of fresh leaves extract of Density 1.067g/ml was obtained. This was kept in an amber coloured bottle for antimicrobial testing. Ten milliliter of this extract was dried at 40^0C and the dried weight in the extract determined to be 1.9g.

2.3.1.2 Dried material extracts

The stem, roots and remaining leaves were separately, air dried for 7 days and milled into powders by means of a Lab Mill Machine (Type 8, Christy and Norris Limited). Three hundred (300) grams of powdered leaves was weighed and 1.5L of 70% Ethanol added. The mixture was shaken and left to stand at room temperature with intermittent shaking for 72 hours. The mixture was subsequently filtered through Whatman filter papers (number 10) and poured into porcelain crucibles. The filtrate was evaporated to dryness using a Hot Air Oven (Sanyo OMT Oven, Gallenkamp) at 37^oC to a constant mass. The extract obtained was labeled and kept in a desicator for use. A similar procedure was used to obtain extracts from the stem and roots. Extraction was also done with Acetone and Chloroform using the same procedure and the yields are as recorded in Table 3.1.

2.3.2 Screening *C. splendens* for antimicrobial activity using the Cup-Plate bioassay technique

Twenty (20) ml of Nutrient agar (Oxoid) was melted in a boiling water bath and stabilized at 45^{o}C for 15 minutes in a thermostatically controlled water bath (Model R76 New Brunswick Scientific, Edison N.J., USA). The molten agar was aseptically inoculated with 0.1ml of an 18hour suspension of *E. coli* (of absorbance 0.04 at 480nm wavelength filter) containing approximately10^{6}cfu/ml and rolled in the palm to mix thoroughly. The seeded agar was then transferred into a sterile petri dish under a laminar air flow cabinet, (Model T2 2472 Skan AG, Switzerland). The agar was allowed to set and Cork borer No 7 (diameter 11.5mm) was flamed and used to bore four (4) wells equidistant from each other in the agar. The wells were labeled and filled with 0.2ml of the fresh leaves extract. The plates were allowed to stand for 1 hour to enable the extract diffuse into the medium and then incubated at 37^{o}C for 24 hours. The zones of inhibition were then measured. This experiment was repeated using *Pseudomonas aeruginosa* suspension (of absorbance 0.06), *Klebsiella pneumonia* suspension (of absorbance 0.05), *Proteus mirabilis* suspension (of absorbance 0.03), *Staphylococcus aureus* suspension (of absorbance 0.4), *Bacillus subtilis* suspension (of absorbance 0.05) and *Candida albicans* suspension (of absorbance 0.07), but in the case of *C. albicans*, Sabouraud agar was used and it was incubated at 25^{o}C for five (5) days. The results are as recorded in Table 3.1. All the experiments were performed in triplicates.

In determining the activity of 70% ethanol leaves extract on *E. coli*, a 10mg/ml solution was made by weighing 100mg of the ethanol extract into a sterile beaker and about 5ml 50% Methanol and 0.1ml Tween 80 added and stirred to dissolve. The solution was made to 10 ml volume with 50%$^{v}/_{v}$ methanol. Five (5) ml of this solution was pipetted into a 10ml volumetric flask, diluted to 10ml and mixed thoroughly to obtain a solution of 5mg/ml. Further two fold dilutions were made to obtain 2.5 and 1.25mg/ml solutions. Molten and stabilized agar was inoculated with 0.1ml of an 18h suspension of *E.coli*, rolled in the palm to evenly distribute the organisms. This was transferred into sterile petri dishes under a laminar air flow cabinet, (Model T2 2472 Skan AG, Switzerland). The agar was allowed to set and Cork borer No 7 (diameter 11.5mm) was used to bore four wells, equidistant from each other in the agar. The wells were labeled C_1 to C_4 and filled with 0.2ml of the 10, 5, 2.5 and1.25 mg/ml solutions respectively. It was preincubated at room temperature (at about 27^{o}C) for 1 hour and incubated at 37^{o}C for 24h in an incubator (Model Gallenkamp Pluss II). The zones of growth inhibition were measured. This experiment was repeated for *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* but in the case of *C. albicans*, Sabouraud agar was used and it was incubated at 25^{o}C for five (5) days. The results are as recorded in Table 3.4 All the experiments were performed in triplicates.

The vehicle, 50% Methanol and Tween 80 was the negative control while Neomycin sulphate and Clotrimazole (Sigma-Aldrich, UK) were used as the standards for bacteria and fungi respectively. The activities of the stem and root

extracts were also determined on the above organisms using the Cup-plate method (Banty, 1976). Results are recorded in Tables 3.1.1 to 3.2.1.

2.3.3 Minimum inhibitory concentration determination of the extracts by the Broth Dilution method

Nine tubes of 5ml double strength Nutrient Broth (Oxoid) were labeled T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9 and T_{10}. Four ml of the fresh leaves extract obtained from the extraction in Section 2.2.1 was added to the first test tube (by means of a sterile pipette). To T_2 was added 3.5ml and 3.0ml, 2.5ml, 2ml, 1.5ml, 1ml, 0.5ml added to T_3, T_4, T_5, T_6, T_7 and T_8 respectively. The appropriate volumes of water as in Table 2.0 were added to each tube and shaken to form a uniform mixture and inoculated with 1ml of an 18h culture of *E. coli* (absorbance of 0.04 at 480nm). To T_9 was added 1ml extract to determine the sterility of the extract and the medium, while T_{10} contained the vehicle (50% Methanol and Tween 80) and 1ml *E.coli* only, to determine whether the vehicle or medium used, had any inhibitory effect on the organism. The tubes were incubated at 37^{o}C for 24h and examined for turbidity (growth). The experiment was done in triplicates (Results- Tables 3.1.7-3.1.9).

Table 2.1 Determination of MIC of fresh leaves extract

Test tube	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
Volume of double strength nutrient broth(ml)	5	5	5	5	5	5	5	5	5	5
Volume 0f 100% extract(ml)	4.0	3.5	3.0	2.5	2.0	1.5	1	0.5	1.0	0
Inoculum size(ml)	1	1	1	1	1	1	1	1	0	1
Volume of sterile water(ml)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4
Total volume of medium(ml)	10	10	10	10	10	10	10	10	10	10

Concentration(mg/ml)	76	66.5	57	47.5	38	28.5	19	9.5	19	-

For the determination of the minimum inhibitory concentrations of 70% Ethanol leaves extract, nine (9) tubes of 5ml of double strength nutrient broth (Oxoid) were labeled T_1,T_2,T_3,T_4,T_5,T_6,T_7,T_8 and T_9. The ethanol extract of *Clerodendron splendens* leaves was weighed (0.5 g) into a 25ml beaker and dissolved in 50% aqueous-methanol with two drops (0.1ml) of tween 80. The solution was transferred into a 10ml volumetric flask and made up to the graduated mark with 50% aqueous-methanol and mixed thoroughly to make a 50mg/ml solution of 10 ml, labeled L_1. Quantities of 4 and 0.4 ml of L_1 were respectively transferred into T_1 and T_2. One (1) millilitre was diluted 25 folds resulting in 2mg/ml solution (L_2), of which 1 and 0.1 ml quantities were also pipetted into T_3 and T_4 respectively. L_2 was also diluted 250 folds to obtain 0.008mg/ml solution (L_3) and 2.5, 0.25, 0.025 ml of it were transferred into T_5, T_6 and T_7 respectively. Calculated amounts of sterile distilled water were added to the tubes such that when 1 ml quantities of the suspension of test organism ($1x10^6$cfu/ml) were introduced, the final volumes were 10 ml in each case (Results recorded in Table 3.8). To T_8 was added 1ml extract but no inoculum, to ascertain the sterility of the extract and the medium while T_9 contained the vehicle (50% aqueous-methanol and Tween 80) and the test organism, to ascertain whether the vehicle used, had any inhibitory effect on the organism. The experiment was done in triplicates and the tubes incubated at 37^oC for 24 hours. The whole exercise was repeated for the acetone and chloroform extracts. Observation was then made for signs of growth and the results are as recorded in Table 3.7.

The experiments were performed in triplicates. Results of can be found in Table 2.1.The range of concentrations was then narrowed to obtain a more accurate MIC.

Table 2.2 Determination of MIC of ethanol leaves extract

Test tube	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9
Volume of double strength nutrient broth(ml)	5	5	5	5	5	5	5	5	5
Volume 0f extract(ml) **(**L_1=50mg/ml; L_2=2mg/ml; L_3=0.008mg/ml)	4 (L_1)	0.4 (L_1)	1 (L_2)	0.1 (L_2)	2.5 (L_3)	0.25 (L_3)	0.025 (L_3)	1.0	0
Inoculum size(ml)	1	1	1	1	1	1	1	0	1
Volume of sterile water(ml)	0.0	3.6	3.0	3.9	1.5	3.75	3.97	4.0	4.0
Total volume of medium(ml)	10	10	10	10	10	10	10	10	10
Concentration(mg/ml)	20	2.0	0.2	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	0.2	-

2.3.4 Killing kinetics of the ethanol leaves extract

Four (4) 100ml bottles containing 10ml double strength nutrient broth were labeled T_1 to T_4. A 100mg/ml solution of the Ethanol leave extract was prepared by weighing 2g of extract into a beaker and dissolved in about 6ml of sterile distilled water and 0.2ml of polysorbate 80. The resulting solution was made up to 20ml in a graduated volumetric flask. Quantities of this solution were added to the bottles as shown in Table 2.2: Two (2) ml of the stock solution was added to the 10ml double strength nutrient broth in T_1 to produce a solution of concentration

10mg/ml when diluted to 20ml. Seven milliliter of water was added and the bottle was shaken to ensure thorough mixing. To T_2 was added 1.2 ml extract, 7.8ml water and shaken and to mix the contents. To T_4 was added 0.2ml Tween 80, 9.6ml sterile distilled water and no extract, to serve as the control. An 18h suspension of *E. coli* was obtained shaken and 1ml quantities were added to each of the bottles. The bottles were kept at a constant temperature of 37^0C in a thermostatically controlled water bath (Model R76 New Brunswick Scientific, Edison N.J., USA) with the shaker set at 40 shakes per minute. Aliquots of 1ml were withdrawn from the bottles at time intervals of 0min., 30min, 1h, 2h, 3h, 4h, 6h, 12h and 24h and the activity of the extract was neutralized by dilution in 9ml, sterile normal saline to produce a 10 fold dilution. Two other ten fold dilutions were made and 1ml samples were pipetted from each, into sterile petri dishes. Twenty milliliter molten agar, stabilized at 45^oC was added to each of the petri dishes and mixed thoroughly with the 1ml samples. The set agars were then incubated inverted at 37^oC for 48h. The petri dishes were marked into segments and the colonies counted with the aid of a colony counter (Type 95.06.17, Gerber Instruments, L.Schneider &co.AG). The viable count per ml at each given time was calculated. The experiment was repeated in triplicates and the averages taken (Appendix C). A graph of Log_{10} of viable count per ml against time was plotted to determine the time-kill kinetics (Fig 3.1).

The procedure was repeated using the 10mg/ml, 6mg/ml and 3mg/ml of the extracts in the medium on all the other organisms: *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Baccillus subtilis and Staphylococcus*

aureus. In the case of *Candida albicans*, Sabouraud Agar was used and incubation done at room temperature (about 27^0C) for three days. The results are recorded in Appendix C and the graphs obtained are Fig 3.2 - 3.7.

Table 2.3 Determination of Time – kill kinetics of extracts

Test tube	T_1	T_2	T_3	T_4
Volume of double strength nutrient broth(ml)	10	10	10	10
Volume 0f 100mg/ml extract(ml)	2	1.2	0.3	0
Volume of sterile water(ml)	7	7.8	8.7	9
Inoculum size(ml)	1	1	1	1
Total volume of medium(ml)	20	20	20	20
Concentration of extract(mg/ml)	10	6	3	0

2.4 Wound-healing activity of *Clerodendron splendens* in Excision wound model in rats

Forty nine healthy male Sprague Dawley rats, weighing 190–195 g obtained from the animal house of the Kwame Nkrumah University of Science and Technology were used for the study. They were housed in metal cages and maintained on Normal Commercial Pellet Diet (GAFCO, Tema). The animals were given water (ad libitum) and maintained under laboratory conditions (room temperature, 24-28^{o}C, relative humidity of 60-70% and 12 hour light–dark cycle).

2.4.1 Preparation of the plant extracts

The Fresh leaves extract of *Clerodendron splendens* as well as the ethanol leaf-extract (as obtained in section 2.2.1) were employed in the wound healing study at concentrations of 190mg/ml (fresh leaves extract) and 3, 30,300 and 1000mg/ml (ethanol extract).

2.4.2 Excision wound model

The animals were anaesthetized with diethyl ether prior to and during creation of the wounds. The dorsal fur of the animals were shaved to a circular diameter of about 45mm by means of razor blades and the anticipated area of the wound to be created was outlined on the shaved skin of the animals with Ammonium oxalate crystal violet paint (with a cork of diameter 25mm, hence an approximate area of 490mm^2). The area was cleaned with ethanol before the rats were inflicted with excision wounds. A full thickness of the skin with circular diameter of 25 mm and an approximate area of 490mm^2 was created along the markings using toothed forceps, surgical blades and pointed scissors. The entire wound was left open and the animals divided into seven groups of seven animals each.

Group 1 animals were topically treated with 1%$^w/_w$ Silver Sulphadiazine Ointment (Aytons Drugs, Ghana).

Group 2 were treated with vehicle (Tween 80 and Normal saline) only, as the control.

Group 3 Animals were treated with the fresh leaves extract of concentration 190mg/ml.

Groups 4, 5, 6 and 7 were topically treated with 3, 30,300 and 1000mg/ml concentrations of *Clerodendron splendens* ethanol extracts respectively.

Wound treatment was commenced on the 2nd day of wound creation. The drugs were then topically applied to the wounds 12 hourly for 19 days. During the course of treatment, scaled photographs of the wound areas were taken (by means of a high resolution Digital Camera, *Nikon* CoolPix L11 L10) alongside a millimeter scale (Fig 2.1) every 48h starting from the 1st day of wound treatment. The wound areas were then determined with the aid of a computer programme (Sigma Pro Scan 5).

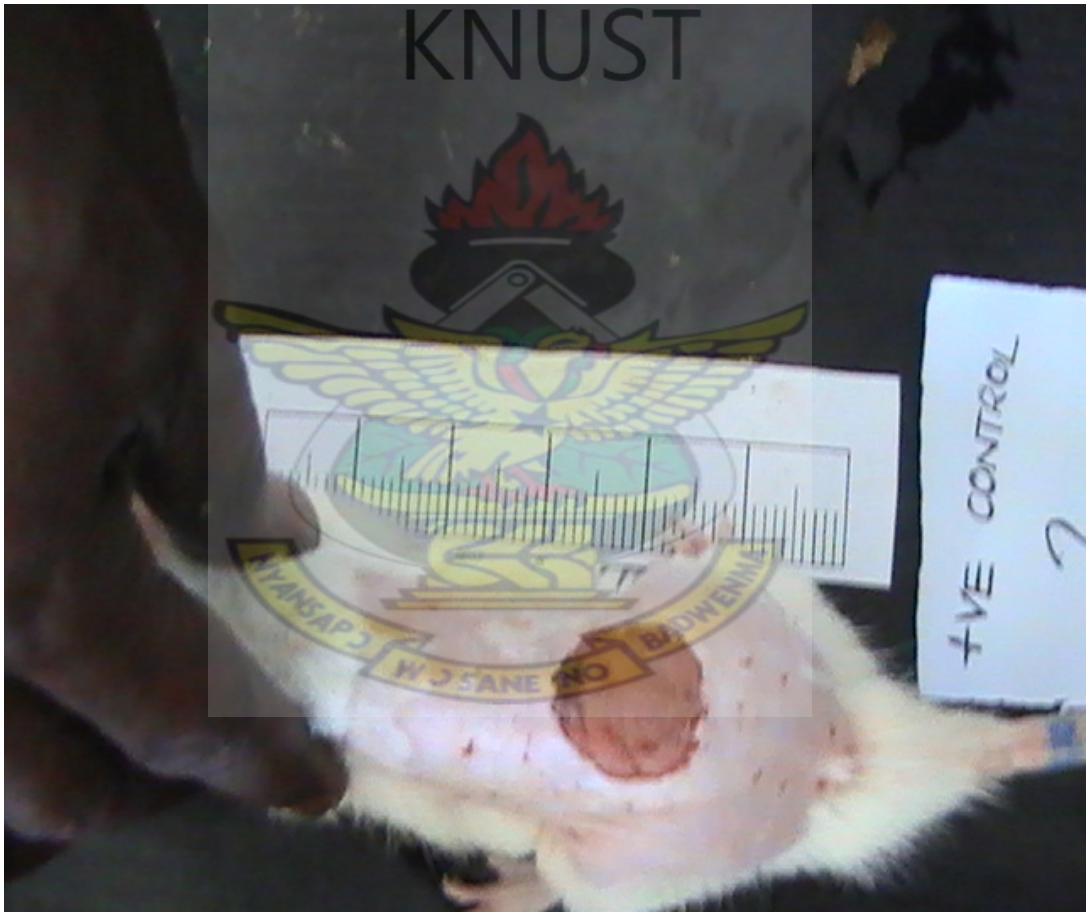

Fig. 2.1 Measurement of wound area

2.4.3 Determination of Tensile strength

On day twenty of treatment, the animals were anaesthetized with diethyl ether. The regrown dorsal fur in and around the healed tissue was shaved and a rectangular outline of 8mm width and 20mm length was marked with the healing scar in the middle. Healing tissue along with normal skin at two ends was excised. The strips of 8mm width and 20mm length were cut out from all the groups of animals and loaded between the upper and lower holder of the apparatus in such a way that the effective load bearing size was 8mm×8mm with the wound scar remaining in the centre. Weights were then introduced gradually to the tissue until it was disrupted. The total breaking load required to disrupt the tissue was measured in grams.

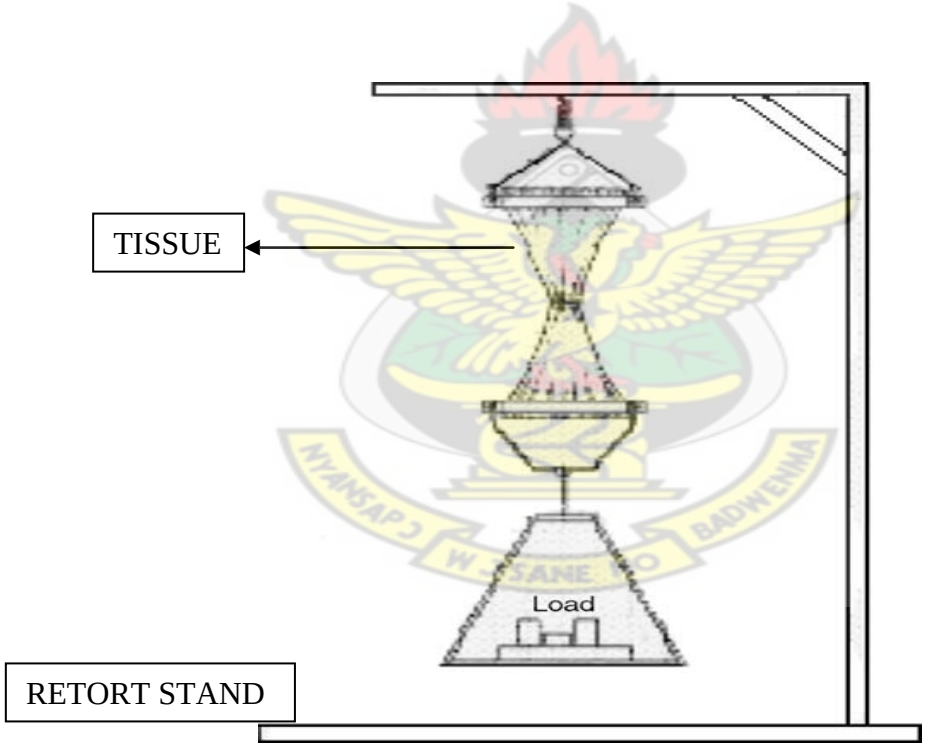

Fig. 2.2 Tensiometer, designed according to the method of Vaisberg *et al*., 1989 to measure tensile (breaking) strength.

2.5 Antioxidant assay of *Clerodendron splendens* extract using the DPPH

method

Concentrations of 40, 20, 10, 5, 2.5 and 1.25mg/ml of ethanol extract of the leaves were prepared. Four hundred milligrams (400mg) of the extract was weighed into a beaker and dissolved in 10ml methanol (of analytical grade) to obtain a solution of 40mg/ml. This solution was filtered through a microfilter into a test tube (T_1). Five ml of this solution was pipetted into tube 2 (T_2) and diluted with 5ml methanol to obtain a solution of 20mg/ml. Further two fold serial dilutions were made to obtain concentrations of 10, 5, 2.5 and 1.25mg/ml solutions in T_4, T_5 and T_6 respectively. Two hundred milligrams (200 mg) of DPPH was also weighed into a 500 ml volumetric flask and dissolved in methanol to produce 0.04 mg/ml solution. A quantity (0.1 ml) of the 40mg/ml extract solution was mixed with 10ml freshly prepared DPPH solution. This mixture was shaken and left to stand in the dark for 30min at room temperature (about 25^0C) after which its absorbance ($A_{Ext\text{-}DPPH}$) was determined at 517nm wavelength using a previously standardized UV Spectrophotometer (Cecil, 2000 series) (by running the absorbance of methanol). To another test tube ($A_{Ext\text{-}meth}$) was added 0.1 ml of the 40mg/ml extract and 10 ml of methanol and treated in similar manner as above. The absorbance of the pure DPPH 0.04 mg/ml solution ($A_{control}$) was also determined. The whole procedure was repeated for the other extract concentrations (20, 10, 5 and 2.5 mg/ml). L-ascobic acid (the standard) was employed at concentrations of 1000,500, 250, 125, 67.5 and 33.75µg/ml. For each extract concentration, three replicate trials were conducted. The results are recorded in Table 4.0.

2.6 PHYTOCHEMICAL SCREENING OF PLANT MATERIAL

2.6.1 Test for the presence of saponins

For each of the plant material (leaves, stem and roots), 2g was weighed and shaken with 5 ml of water in a test tube. Foaming, which did not persist on warming for more than five minute, was considered as the absence of saponins (Banso and Adeyemo, 2006). The results are recorded in Table 5.1.

2.6.2 Test for the presence of tannins

About 2 g of the powdered plant material was boiled with 25mls of water for 5 minutes; it was then cooled and filtered. To 1ml aliquot of the aqueous extract was added 5 drops of 1% ferric chloride. Blue–green colouration was taken as an indication of the presence of condensed tannins (Evans, 2002). The results are recorded in Table 5.1.

2.6.3 Test for the presence of alkaloids

A quantity (about 4g) of the powdered sample was extracted with 30ml of ammoniacal alcohol (ammonia/alcohol 1:9). The filtrate was then evaporated to dryness and the residue extracted with about 2ml 1% H_2SO_4. This was then filtered and the filtrate rendered alkaline with dilute ammonia solution. The alkaline solution of the extract was then put in a separating funnel and partitioned with chloroform. The chloroformic layer was then separated into a crucible and allowed to evaporate to dryness. About 1 ml 1% H_2SO_4 was added to the residue followed by three drops of Dragendorff's reagent. Observation was made for the

presence of orange precipitate which was taken as an indication for the presence of alkaloids (Evans, 2002). The results are recorded in Table 5.1.

2.6.4 Test for the presence of glycosides

The powdered plant material (2g) was introduced into two different test tubes. To one of the test tubes (T_1) was added 5ml dilute Sulphuric acid while 5ml water was added to the other tube (T_2). The two tubes were heated for three minutes and the contents filtered. The filtrates were made alkaline with 0.5ml 20% NaOH and Fehling's solution A and B added and boiled for three minutes. The presence of more reddish brown precipitate in the filtrate of T_1 than T_2 was taken as positive test for the presence of glycosides (Banso and Adeyemo, 2006). The results are recorded in Table 5.1.

2.6.5 Test for the presence of anthraquinone glycosides

2g of the sample was boiled with dilute H_2SO_4 for 5 min and filtered while still hot. The filtrate was allowed to cool and shaken with an equal volume of chloroform. The chloroform layer was separated and shaken with half its volume of dilute NH_3 solution. The absence of red colour was taken as the absence of anthraqiunone glycosides (Banso and Adeyemo, 2006). The results are recorded in Table 5.1.

2.6.6 Test for the presence of cyanogenetic glycosides

2g of the powdered drug was weighed in a conical flask and moistened with few drops of water. A strip of sodium picrate paper was suspended by means of a cork in the neck of the flask. The tube was warmed gently on a water bath and the paper observed for a reddish–purple colour change (Evans, 2002). The results are recorded in Table 5.1.

2.6.7 Test for the presence of phytosterols (Lieberman's test)

About 2g of the powdered leaves of *Clerodendron splendens* was extracted with chloroform by warming for 30minutes, and filtered. Two ml of acetic anhydride was added to the chloroform layer followed by 4 drops of concentrated H_2SO_4 added along the sides of the test tube. A blue-green colouration indicated the presence of steroids (Evans, 2002). The test was repeated for the stem and root. The results are recorded in Table 5.1.

2.6.8 Test for the presence of terpenoids (Salkowski test)

The leaves, stem and roots (2g) were extracted with 10ml ethanol. Five ml of each extract was mixed with 2ml of chloroform and concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids in the leaves (Odebiyi and Sofowora, 1978). The results are recorded in Table 5.1.

2.6.9 Test for the presence of flavonoids

The powdered leaves of f *Clerodendron splendens* (about 2g) was extracted with 15mls of ethanol (96%). To the ethanolic extract was added a small piece of piece of magnesium ribbon, this was followed by dropwise addition of concentrated

hydrochloric acid. An orange colour indicated the presence of flavonoids, (Odebiyi and Sofowora, 1978). Results are recorded in Table 5.1.

CHAPTER THREE

3. RESULTS

Table 3.1 Percentage yield of extracts

These are the yields obtained from extracting 300g of each plant material

Solvent for extraction	Percentage Yield of Extract		
	Leaves	Stem	Root
70% Ethanol	6.6	5.3	5.3
Acetone	4.4	0.006	1.73
Chloroform	3.0	0.260	0.135

3.1 Antimicrobial activity of the extracts of *Clerodendron splendens*

The various extracts (fresh leaves, ethanol, chloroform and acetone) were found to exhibit antimicrobial activity against the test microorganisms in the agar diffusion method employed (Tables 3.1 to 3.4).The fresh leaves extract was active against four of the test organisms namely *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus mirabilis* and, *Candida albicans*. The other extracts also showed various degrees of activity with the ethanol extract of the leaves exhibiting a broad spectrum of activity. The activities of various concentrations of Neomycin sulphate against the bacterial strains is also recorded in Table 3.5. The activity of Clotrimazole (an

antifungal) was also determined on *Candida albicans* and the MIC as determined by the broth dilution method was 0.086 µgm/ml.

Table 3.2.1 Zones of growth–inhibition of fresh leaves extract against test organisms

Organism	***Zone of inhibition(mm)**
Escherichia coli	-
Pseudomonas aeruginosa	-
Proteus mirabilis	22.0
Klebsiella pneumoniae	-
Staphylococcus aureus	27.0
Bacillus subtilis	30.0
Candida albicans	16.0

*the readings are the mean of three replicate trials.

Table 3.2.2 Zones of growth–inhibition of chloroform extracts against test organisms

		Zones of growth-inhibition (mm)						
Extract	**Conc.(mg/ml)**	***E.coli***	***P.aeru***	***P.mira***	***K.pneu***	***S.aur***	***B.sub***	***C.alb***
Leaves	1.25	13.0	18.5	17.0	-	12.5	12.8	13.1
	2.5	14.5	20.5	15.0	-	13.5	13.0	14.1
	5.0	16.0	21.3	16.0	-	14.0	14.0	15.0
	10.0	17.0	22.0	17.0	13.5	15.5	15.1	16.1

Stem	1.25	18.5	18.5	13.0	12.5	12.0	13.5	13.0
	2.5	19.5	19.3	13.5	15.5	14.0	13.0	14.0
	5.0	20.5	20.5	14.3	15.5	14.5	14.5	16.5
	10.0	22.0	21.6	16.3	17.1	15.0	16.0	17.3
Root	1.25	-	-	-	-	-	16.0	-
	2.5	-	-	-	-	-	18.8	-
	5.0	-	-	-	-	-	20.6	-
	10.0	-	-	-	-	-	21.6	-

Table 3.2.3 Zones of growth–inhibition of acetone extracts against test organisms

		Zones of growth-inhibition (mm)						
Extract	**Conc.(mg/ml)**	***E.coli***	***P.aeru***	***P.mira***	***K.pneu***	***S.aur***	***B.sub***	***C.alb***
Leaves	1.25	12.5	-	-	13.0	13.0	-	12.5
	2.5	13.0	-	-	14.5	13.5	-	12.5
	5.0	16.0	-	-	15.0	14.0	-	12.5
	10.0	17.5	-	16.0	16.0	16.5	-	13.0
Stem	1.25	18.0	14.6	13.0	14.0	12.0	13.0	14.5
	2.5	19.5	16.6	13.3	14.0	13.7	13.0	14.8
	5.0	21.0	16.8	15.0	15.5	14.2	14.0	16.0
	10.0	22.5	19.7	17.5	17.1	14.9	16.3	16.8
Root	1.25	-	-	-	22.5	-	15.5	26.7
	2.5	-	-	-	23.3	-	16.5	28.0
	5.0	-	-	-	25.5	-	17.5	30.0
	10.0	18.0	15.0	14.0	25.8	12.0	21.5	31.7

Table 3.2.4 Zones of growth–inhibition of ethanol extracts against test organisms

		Zones of growth-inhibition (mm)						
Extract	**Conc.(m g/ml)**	***E. coli***	***P. aeru***	***P.mira***	***K.pneu***	***S.aur***	***B.sub***	***C.alb***

Leaves	1.25	13.5	13.6	14.5	13.0	11.6	-	12.5
	2.5	15.0	15.5	14.6	13.5	12.5	13.0	13.0
	5.0	15.5	15.5	15.0	14.4	13.0	13.5	13.5
	10.0	17.0	16.6	15.5	16.0	13.1	14.0	13.5
Stem	1.25	21.0	18.5	14.0	-	15.0	15.6	13.5
	2.5	23.0	18.5	16.0	-	15.3	16.3	14.5
	5.0	24.0	21.0	17.5	-	17.5	17.5	15.0
	10.0	25.0	19.0	19.5	16.5	18.8	19.0	16.0
Root	1.25	-	-	16.5	24.0	18.5	16.0	24.0
	2.5	-	-	18.0	25.0	19.0	18.0	27.5
	5.0	-	-	19.5	27.0	19.0	19.5	30.1
	10.0	-	-	23.0	26.0	22.0	22.5	30.5

Table 3.2.5 Zones of growth–inhibition of standard drugs against test organisms

	Zones of growth-inhibition (mm)						
Conc. (µg/ml)	***E. coli***	***P. aeru***	***P.mira***	***K.pneu***	***S.aur***	***B.sub***	****C.alb***
0.0625	-	17	21	21	23	22	20
0.125	-	23	23	23	24	23	21
0.25	-	25	24	24	26	26	22
0.5	17	29	27	26	31	43	23

*Clotimazole (µg/ml)

3.2 Minimum Inhibitory Concentrations of the extracts of *C. splendens* against the test organisms.

In the broth dilution technique, the extracts exhibited various MICs against the test microorganisms as shown in the tables bellow:

Table 3.3 MIC of fresh leaves extract (mg/ml) - broth dilution method

Organism	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	MIC
E. coli	+	+	+	+	+	+	+	+	+	+	-
P. aeruginosa	+	+	+	+	+	+	+	+	+	+	-
P. mirabilis	-	-	-	-	-	-	+	+	+	+	28.5
K. pneumoniae	+	+	+	+	+	+	+	+	+	+	-
S. aureus	-	-	-	-	-	-	+	+	+	+	28.5
B. subtilis	-	-	-	-	-	-	+	+	+	+	28.5
C. albicans	-	-	-	-	-	-	+	+	+	+	28.5
Vol. of extract	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	1.0	0.0	
Conc extract (mg/ml)	76	66.5	57	47.5	38	28.5	19	9.5	19	0.0	

Where – represents no growth; + represents growth

For *Proteus mirabilis* the MIC is the concentration of extract in T_6

Dry mass per ml of fresh leaves extract is 0.19g (190mg)

Mass of extract in T_6 = 285mg Therefore conc. of extract = 285/10

= 28.5mg/ml

Table 3.4 MIC of extracts (mg/ml)

The minimum inhibitory concentrations against the various organisms ranged from 0.15 to 7mg/ml.

	Plant material	*E.coli*	*P.aeru*	*P.mira*	*K.pneu*	*S.aur*	*B.sub*	*C.alb*
Chloroform	**Leaves**	1.5	4.0	1.5	1.5	1.5	1.5	1.5
	Stem	0.3	1.5	0.15	3.0	2.6	0.15	3.0
	Root	-	-	-	-	-	0.5	-
Acetone	**Leaves**	3.0	-	4.0	2.0	1.5	-	1.5
	Stem	0.4	0.15	1.5	3.0	1.5	1.5	1.5
	Root	7.0	6.0	3.0	3.0	0.6	0.3	0.5
Ethanol	**Leaves**	3.0	1.5	1.5	2.0	1.5	1.5	0.15
	Stem	0.3	1.5	4.0	3.0	1.5	1.5	1.5

	Root	-	-	1.5	2.0	1.5	1.5	0.15
Standard drugs	**Neomycin (µg/ml)**	65	8.4	2	49	15	2.5	*0.086

*Clotrimazole (mg/ml), *E. coli (Escherichia coli), P. aeru (Pseudomonas aeruginosa), P. mira (Proteus mirabilis), K. pneu (Klebsiella pneumonia), S. aur (Staphylococcus aureus), B. sub (Bacillus subtilis), C. alb (Candida albicans)*

3.2 Time-kill kinetic studies of ethanol leaves extract

The graphs (Figures 3.1-3.7) show the behavior of the cells in the presence of 10, 6 and 3mg/ml extracts.

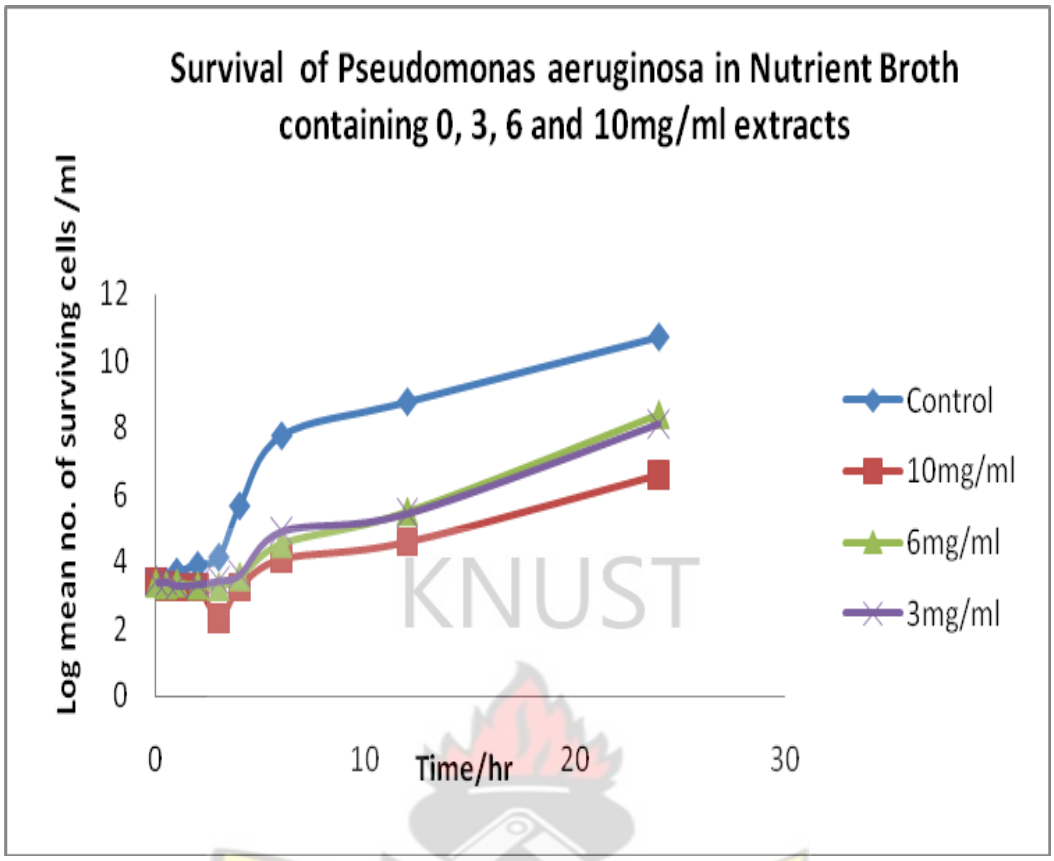
Survival of Pseudomonas aeruginosa in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells /ml
12
10
8
6
4
2
0
0
10
20
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.1

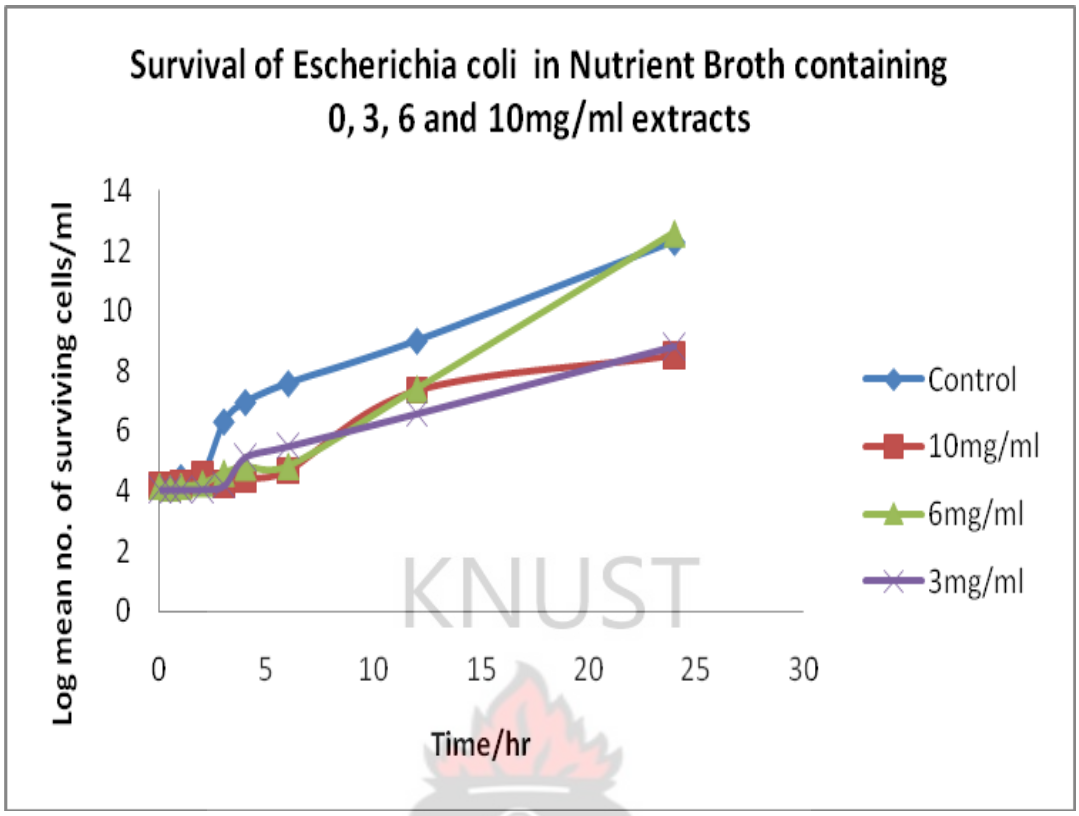
Survival of Escherichia coli in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells/ml
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.2

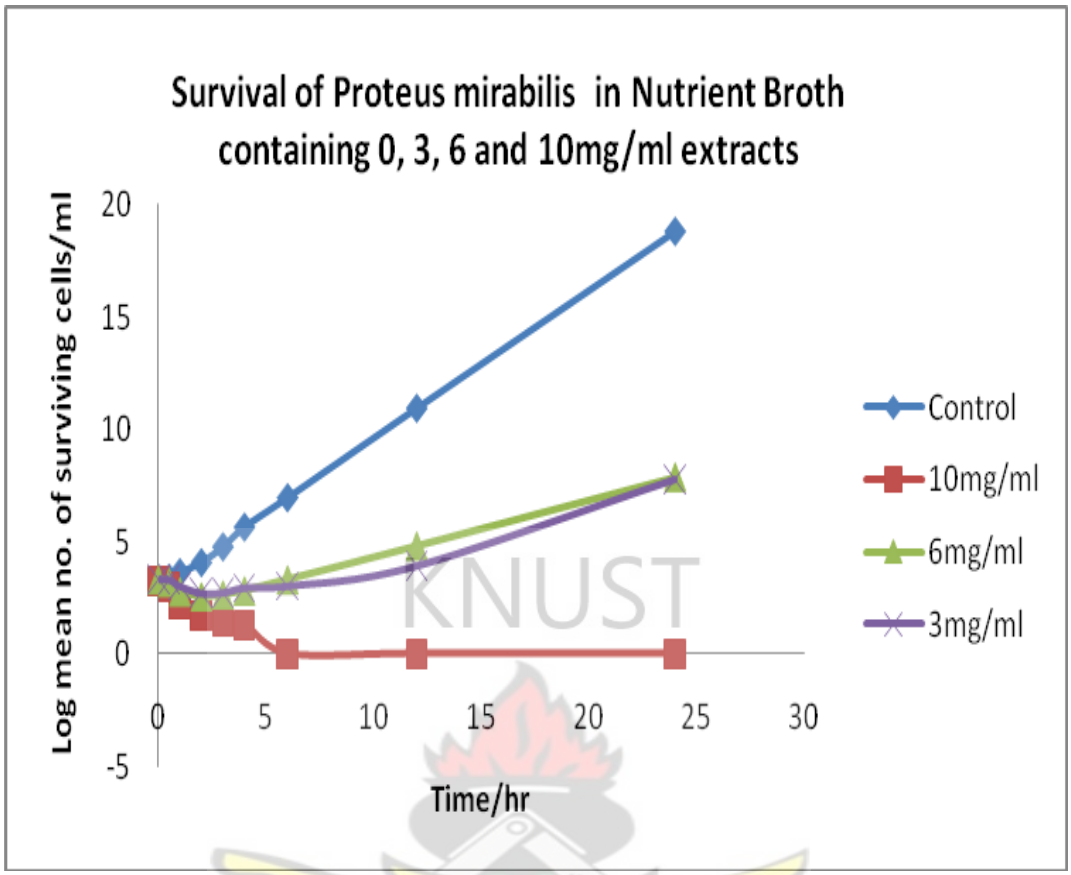
Survival of Proteus mirabilis in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells/ml
20
15
10
5
0
-5
0
5
10
15
20
25
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.3

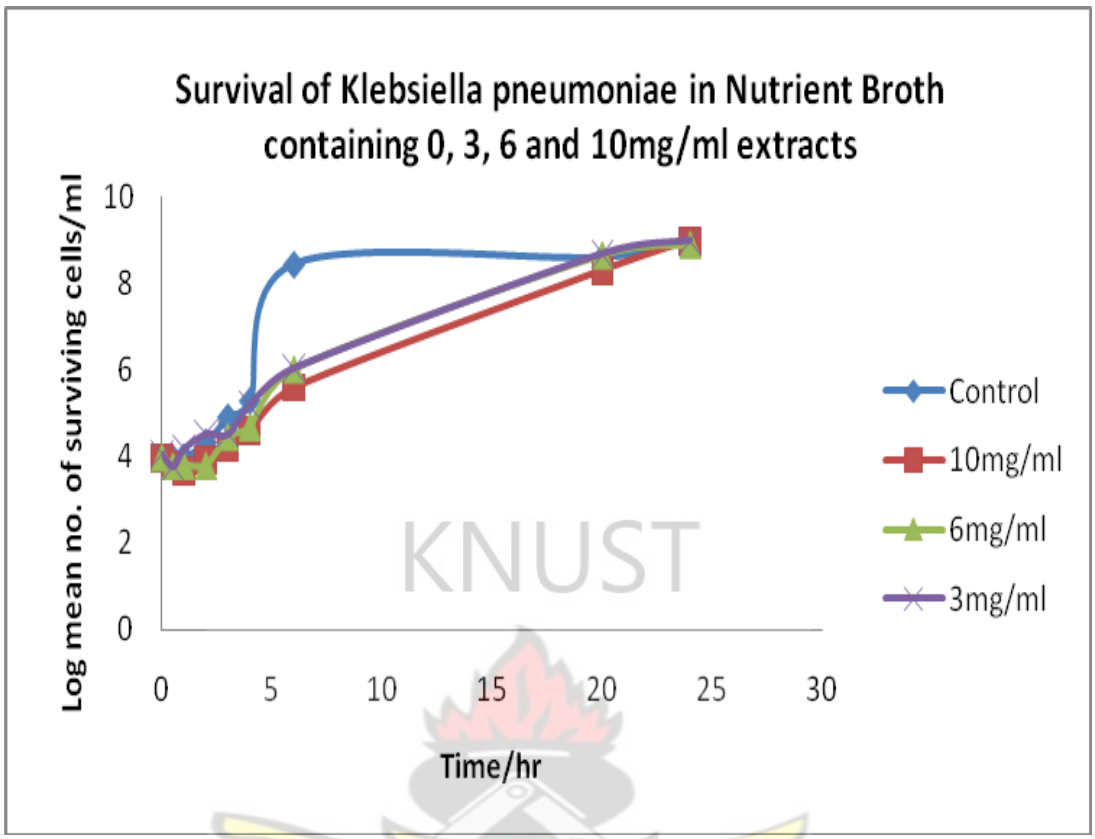
Survival of Klebsiella pneumoniae in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells/ml
10
8
6
4
2
0
0
5
10
15
20
25
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.4

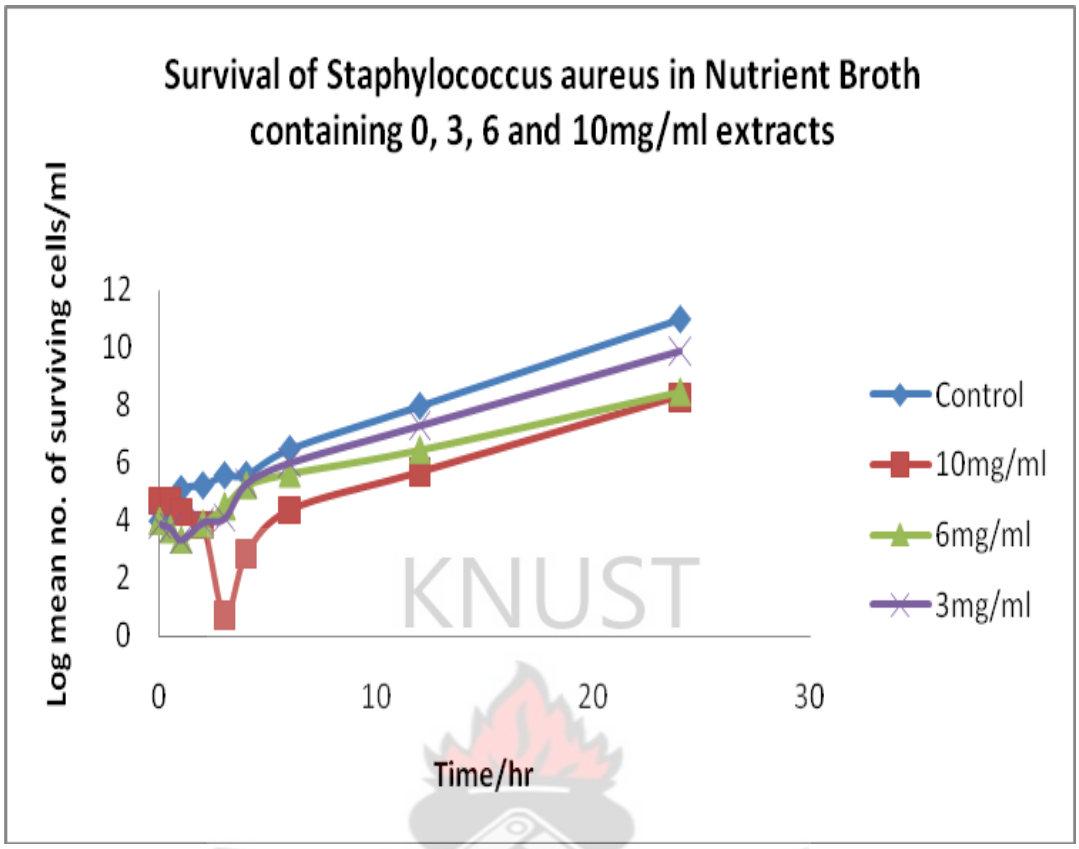
Survival of Staphylococcus aureus in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells/ml
12
10
8
6
4
2
0
0
10
20
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.5

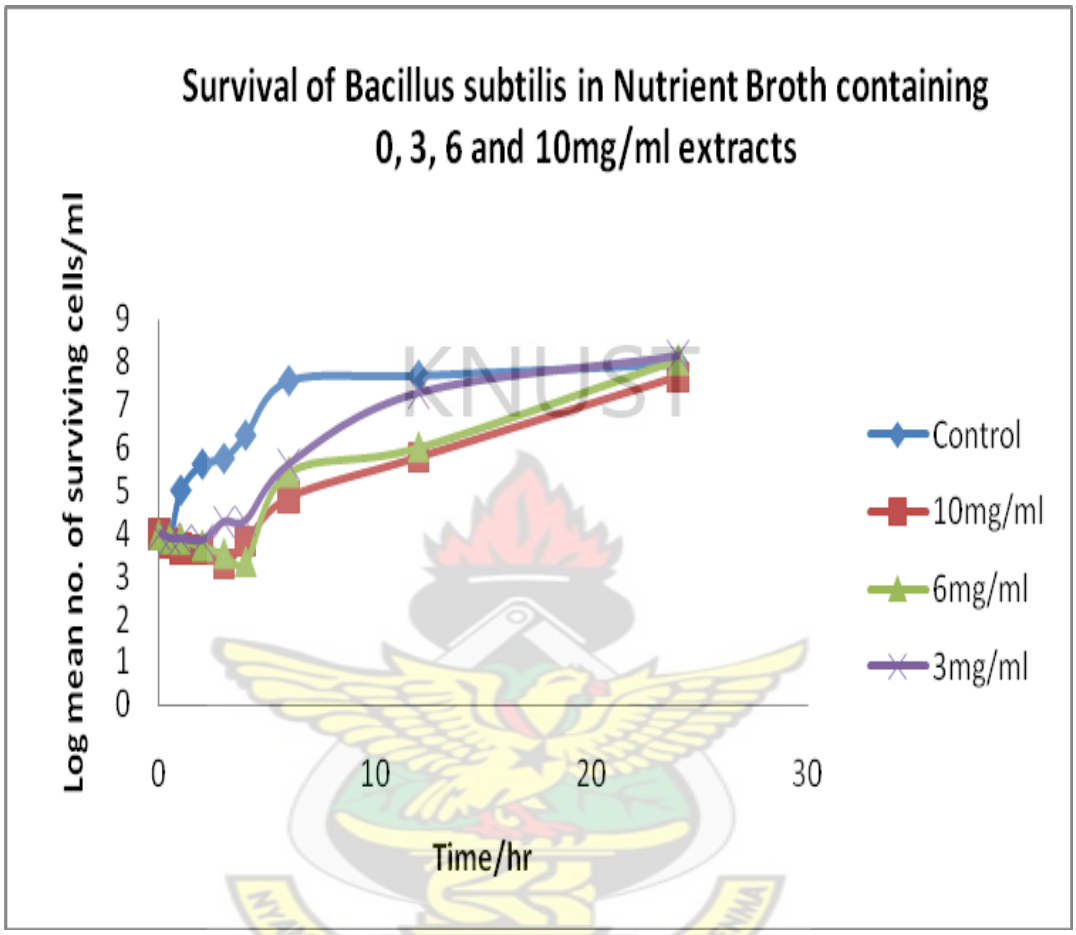
Survival of Bacillus subtilis in Nutrient Broth containing 0, 3, 6 and 10mg/ml extracts
Log mean no. of surviving cells/ml
0
1
2
3
4
5
6
7
8
9
0
10
20
30
Time/hr
Control
10mg/ml
6mg/ml
3mg/ml

Fig 3.6

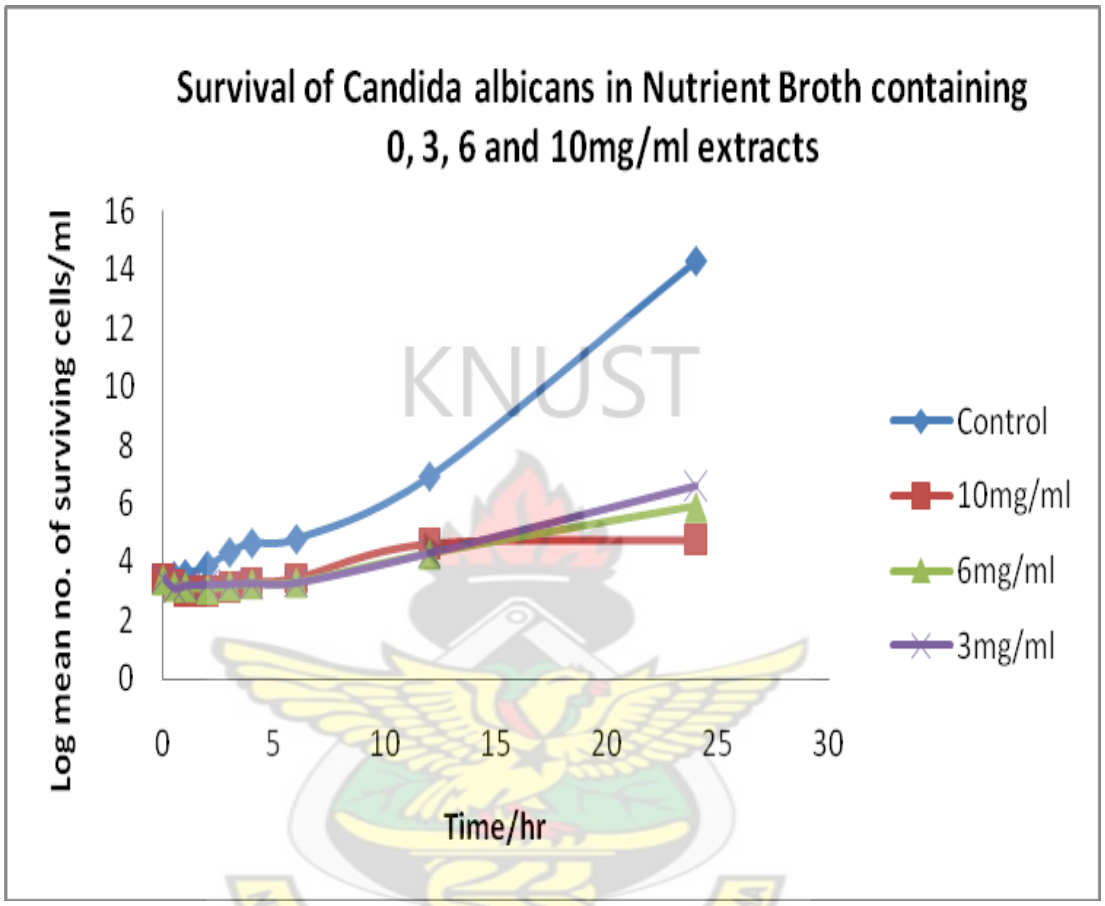

Fig 3.7

3.3 Results for the Wound healing activity

The effect of *C. splendens* extracts on wounds inflicted on rats was monitored by measuring the size of the wound with time and these are recorded in the tables below.

Table 3.5.1 Effect of *C. splendens* fresh leaves extract on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	218.28	94.09	109.16	75.73	28.67	11.48	3.2	1.58	0.56	0.02
2	227.72	109.49	104.9	76.46	24.47	13.85	5.18	3.38	1.35	1.42
3	200.5	107.17	127.99	65.68	35.87	20.19	14.64	11.09	2.97	2.3
4	230.72	127.37	161.5	78.84	47.99	33.55	18.41	15.35	3.12	10.56.
5	197.87	124.96	162.32	83.39	29.81	20.73	14.18	5.63	4.77	0.45
6	207.76	100.69	145.53	80.89	36.75	19.45	8.83	4.37	1.69	1.44
7	198.36	129.91	149.33	85.55	46.05	31.4	22.53	7.39	4.92	5.83

Table 3.5.2 Effect of 3mg/ml extract on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	294.86	193.59	207.91	86.07	46.13	19.11	13.25	7.58	4.15	2.16
2	211.2	107.23	110.97	40.26	20.8	9.79	5	3.4	0.78	0.49
3	202.59	141.25	154.01	66.26	36.15	20.63	8.07	3.63	0.59	0.09
4	238.35	124.23	122.27	66.68	33.05	18.57	8.34	5.99	2.31	1.59
5	194.98	124.67	137.92	70.41	48.65	19.77	14.18	10.78	4.42	2.14
6	194.68	120.57	117.83	54.54	29.22	10.5	11.15	7.38	4.18	1.03
7	212.97	146.68	119.36	49.5	23.54	13.45	4.7	2.1	0.84	0.19

Table 3.5.3 Effect of 30mg/ml extract on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	184.19	121.99	134.13	89.41	44.43	27.69	9.27	6.34	4.18	0.32
2	190.55	133.72	193.14	80.17	42.47	34.23	20.09	10.54	3.39	0.41
3	260.52	150.43	193.16	98.25	50.88	22.33	20.56	4.33	3.01	1.09
4	281.86	160.53	164.36	86.38	45.24	32.71	20.1	9.58	4.84	5.03
5	195.43	149.09	153.12	71.43	40.66	24.35	12.97	10.31	3.78	1.68
6	200.88	132.25	151.01	74.22	35.42	19.96	8.99	3.22	3.09	1.86
7	179.03	109.42	122.12	79.18	37.82	14.84	11.57	3.45	2.24	0.51

Table 3.5.4 Effect of 300mg/ml extract on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	185.87	120.74	105.46	54.93	23.04	15.82	8.82	6.98	4.59	0.87
2	155.31	91.45	152.74	44.06	13.82	10.45	8	1.16	0.42	0.03
3	186.62	130.53	150.4	58.91	37.84	25.34	10.23	8.66	7.62	4.07
4	209.18	128.21	120.36	60.76	29.1	19.16	10.69	11.06	5.17	2.44
5	187.07	132.48	140.85	65.41	31.02	13.6	7.42	0.77	0.68	1.68
6	274.98	145.22	141.55	78.92	41.15	29.38	22.26	5.02	7.42	1.86
7	197.68	107.04	111.32	62.41	30.47	15.59	8.85	4.42	0.82	0.51

Table 3.5.5 Effect of 1000mg/ml extract on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	153.44	127.48	114.08	92.65	50.77	35.21	8.82	2.65	0.34	0.65
2	240.77	176.5	239.63	60.08	30.6	17.12	8	1.26	0.15	0.42
3	161.93	142.34	171.45	77.51	30.44	11.33	10.23	2.75	0.8	0.17
4	186.17	154.48	150.54	54.79	25.6	17.5	10.69	5.55	2.58	0.83
5	229.56	154.92	77.95	79.01	45.19	24.46	18.54	8.69	6.81	0.72
6	181.67	133.33	160.79	71.95	42.7	29.76	24.58	8.8	8.46	7.13
7	262.73	194.42	218.8	0.000	54.69	33.54	25.79	17.16	3.9	0.11

Table 3.5.6 Effect of vehicle on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	200.35	98.43	112.66	67.28	43.61	19.56	12.58	4.28	2.4	1.8
2	213.94	133.23	156.19	77.78	44.65	23.29	21.48	9.79	3.16	1.06
3	139.03	111.05	128.71	67.52	46.22	25.72	14.31	7.98	5.24	2.01
4	159.87	109.78	127.43	69.05	33.08	22.82	9.11	6.38	1.95	0.35
5	168.66	91.07	98.59	45.68	24.84	6.78	4.98	2.41	0.52	0.94
6	181.99	117.42	118.08	57.98	33.2	20.12	13.95	3.79	2.27	1.61
7	222.55	126.57	121.74	64.86	43.47	24.2	11.9	5.25	2.11	0.84

Table 3.5.7 Effect of standard drug on wound size with time

	Time (days)									
Rats	**1**	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**	**19**
1	201.85	166.92	178.72	74.48	32.94	22.21	13.13	14.47	6.12	2
2	314.33	209	173.58	62.88	28.71	14.37	5.54	1.25	0.56	0.29
3	195.28	139.58	122.3	46.39	25.6	14.21	5.4	2.51	0.75	0.26
4	132.78	112.77	74.73	37.02	20	8.39	2.54	1.7	0.39	0.07
5	172.69	144.2	133.72	45.89	24.49	17.1	2.5	2.2	0.00	0.00
6	186.28	136.83	163.57	48.86	28.81	16.41	7.51	4.94	1.15	0.28
7	198.89	155.82	127.61	50.24	27.16	17.8	6.53	4.1	1.79	0.13

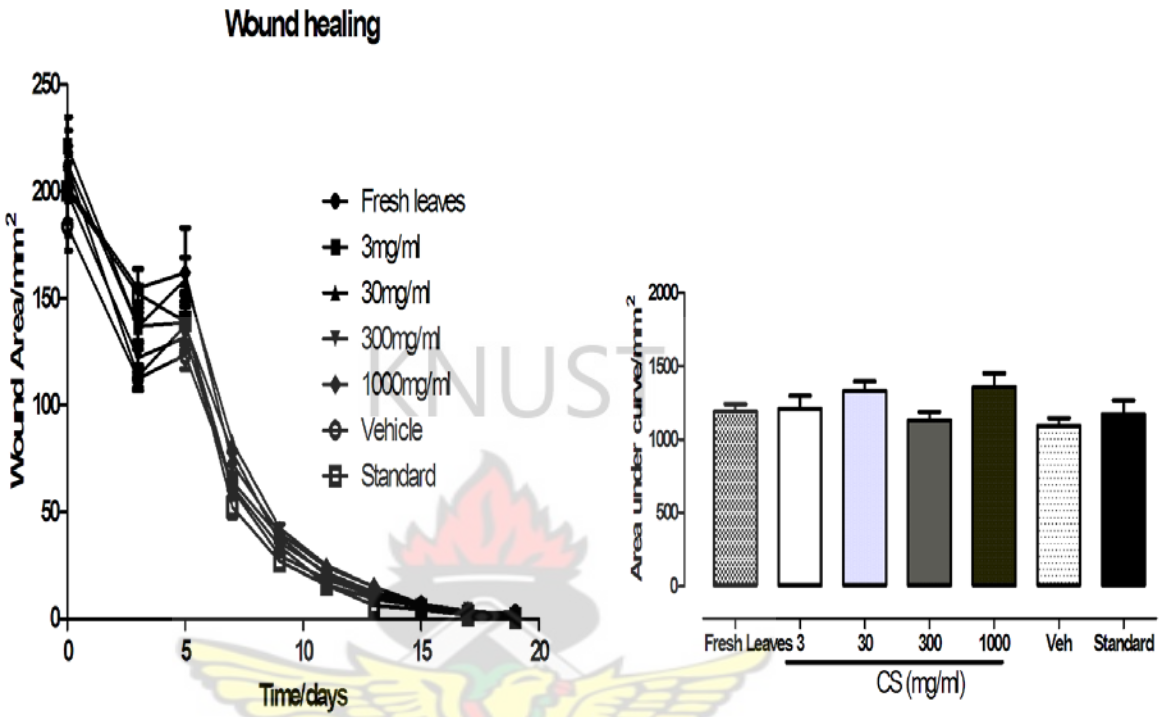

Fig 3.8.1 Decrease of wound area with time and area under curves

3.4 Rate of wound contraction

The fresh leaves and the ethanol extracts (especially the 3mg/ml) showed more percentage of wound healing than the control group during the first few days (Days3-5). By the 7th day the control had contracted to the same extent as the fresh leaves. Complete healing was recorded at about day 15 for almost all the groups.

The degree of wound contraction was calculated from the formula below:

Degree of wound contraction =

[1-(wound area on corresponding day/wound area on day zero)]× 100%.

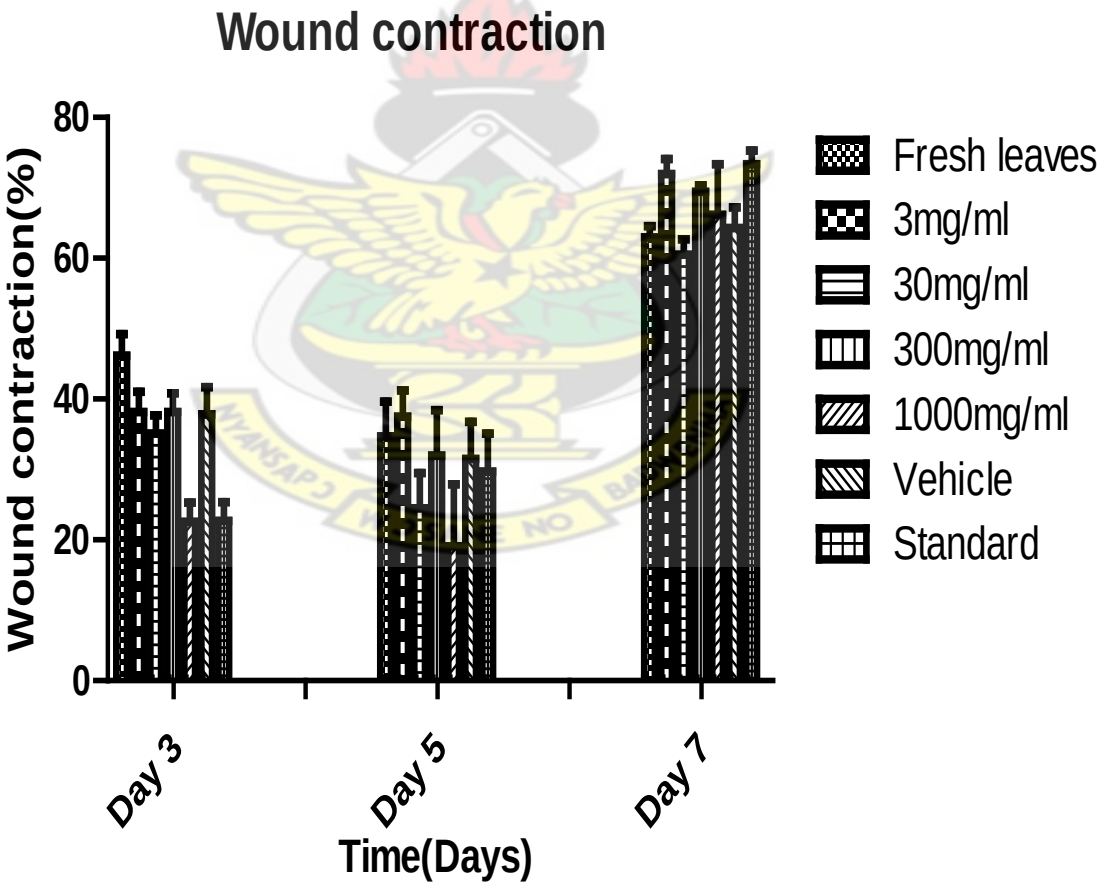

Fig 3.8.2 Percentage contraction of wound

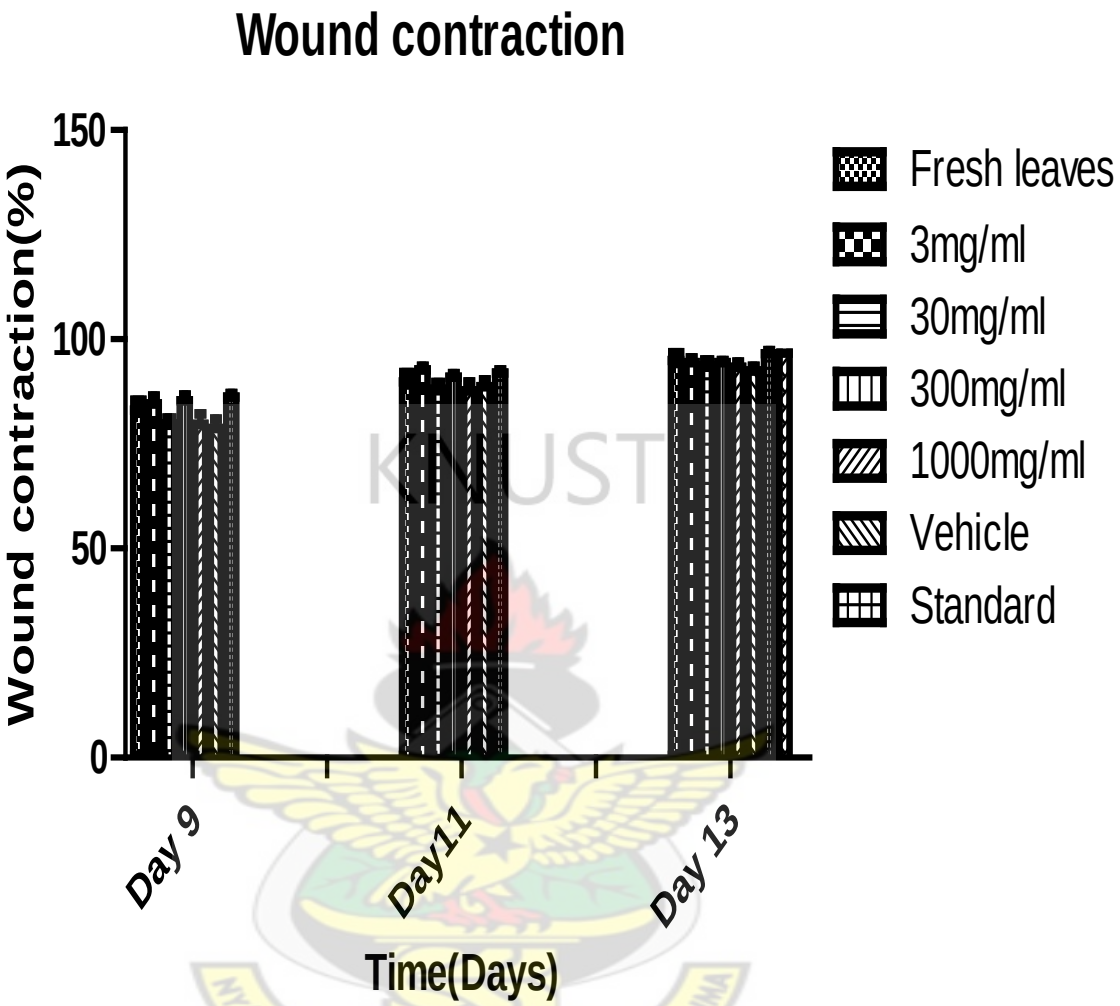

Fig 3.8.3 Percentage contraction of wound

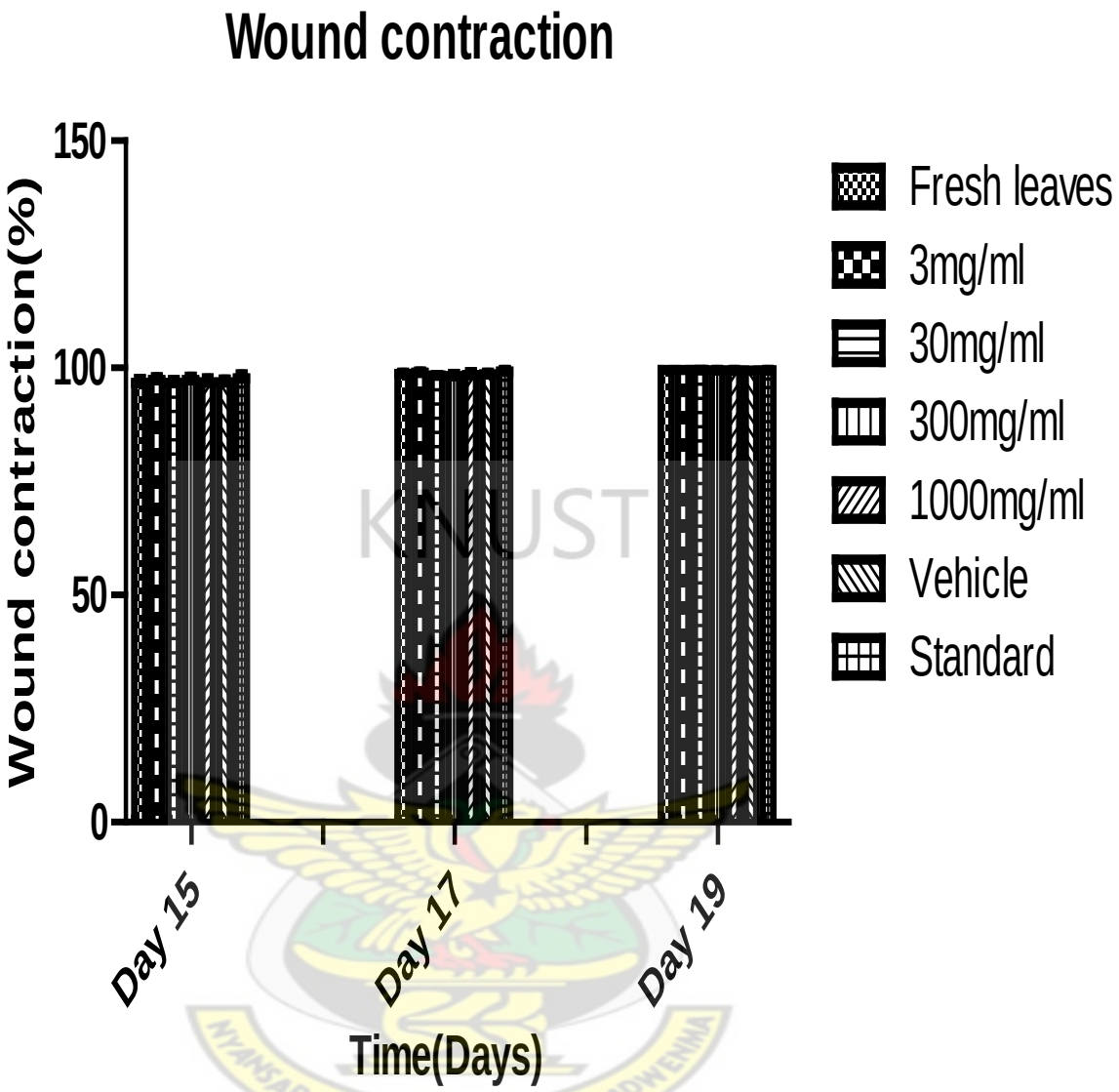

Fig 3.8.4 Percentage contraction of wound

Table 3.6.1 Results for tensile strength measurement (grams)

Rats	Fresh leaves	3mg/ml	30mg/ml	300mg/ml	1000mg/ml	Vehicle	Standard
1	314.14	416.14	461.14	451.14	501.14	351.14	551.14
2	281.14	401.14	311.14	476.14	621.14	431.14	421.14
3	397.00	301.14	426.14	471.14	481.14	441.14	546.14
4	466.14	296.14	566.14	576.14	401.14	401.14	461.14
5	421.14	326.14	451.14	461.14	341.14	459.6	411.14
6	501.14	316.14	321.14	581.14	441.14	541.14	461.14
7	393.81	421.14	321.14	331.14	464.49	591.14	391.14

3.5 Mean tensile strength determination

An increase in concentration of the extract from 3mg/ml, 30mg/ml and 300mg/ml, showed an increase in the breaking strength of the wound. The 300mg/ml group exhibited a slightly higher breaking strength than the standard drug.

Table 3.6.2 Effect of topical treatment on tensile strength of wounds

Animal groups	Fresh leaves	3mg/ml	30mg/ml	300mg/ml	1000mg/ml	Vehicle	Standard
Tensile strength(g)	396.3 ±34.88	354.0 ±21.24	408.3 ±36.02	478.3 ±31.96	464.5 ±39.13	459.5 ±36.64	463.3 ±24.05

Values are mean ± S.E. (standard error), *n* = 7 mice (in each group).

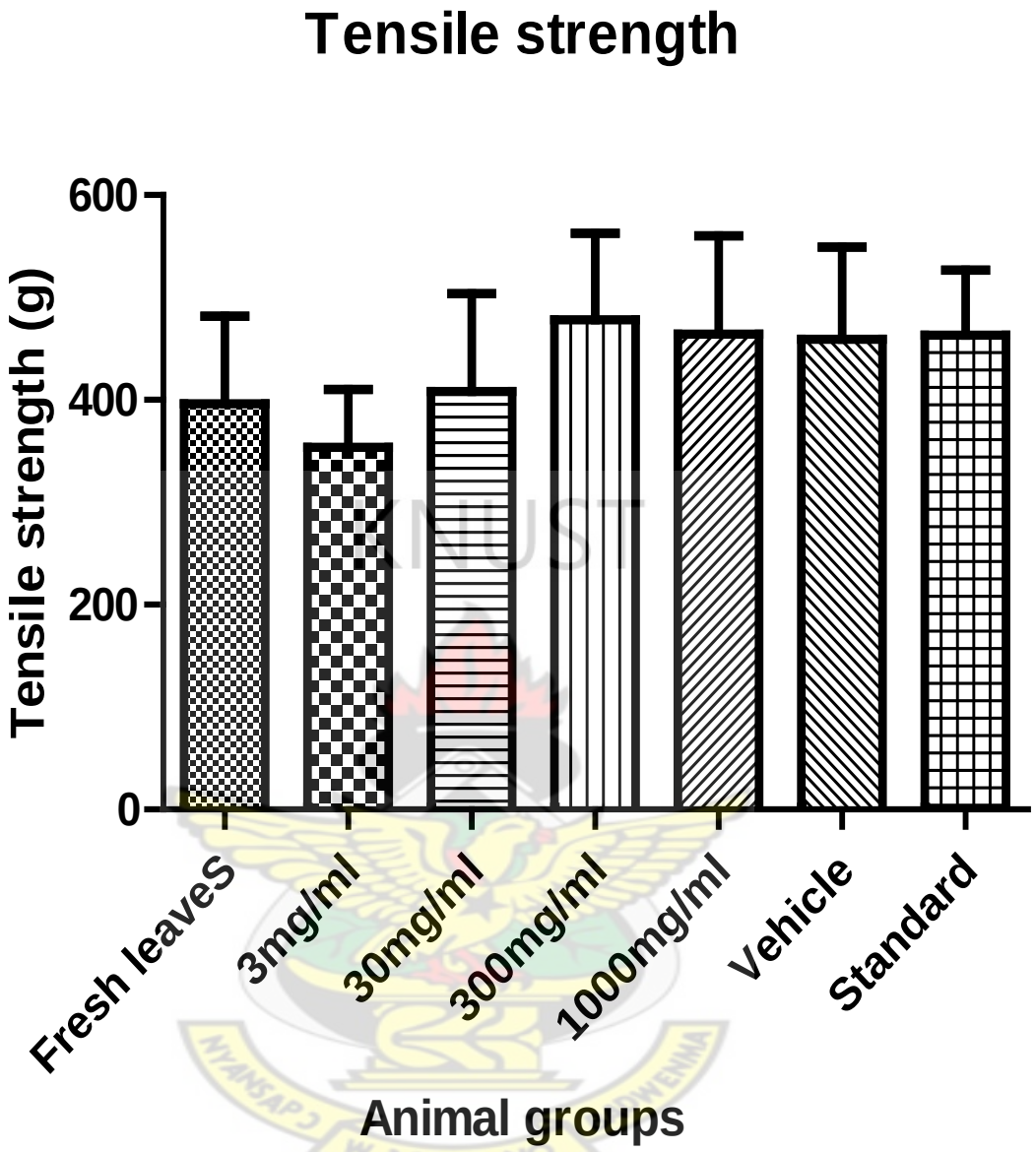

Fig 3.9 Tensile strength of wounds

3.6 Antioxidant activity

The $IC_{50,}$ which is the half maximal inhibitory concentration (a measure of the effectiveness of a compound in inhibiting biochemical function), was determined to be 5.61±0.040 mg/ml .That of L- ascorbic under the same conditions was 0.4873 ±0.0 122 mg/ml. Hence ascorbic acid is about 11 times stronger than the extract as an antioxidant.

Table 3.7.1 Results for DPPH radical scavenging activity

Concentrations of solutions		Absorbance (nm)											
		$A_{Ext\text{-}DPPH}$			$A_{Ext\text{-}Meth}$			$A_{Asc\ acid\text{-}DPPH}$			$A_{Asc\ acid\text{-}Meth}$		
Ext (mg/ml)	**Asc. acid (µg/ml)**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
40	1000	0.153	0.154	0.153	0.112	0.115	0.114	1.481	1.480	1.482	0.028	0.028	0.028
20	500	0.133	0.133	0.132	0.050	0.050	0.050	1.415	1.415	1.415	0.028	0.028	0.028
10	250	0.388	0.389	0.389	0.035	0.039	0.037	1.337	1.337	1.336	0.031	0.031	0.031
5	125	0.952	0.957	0.957	0.045	0.044	0.044	1.151	1.151	1.151	0.031	0.031	0.031
2.5	67.5	1.128	1.128	1.128	0.014	0.013	0.013	0.765	0.766	0.766	0.031	0.031	0.031
1.25	33.75	1.344	1.344	1.345	0.008	0.008	0.008	0.224	0.224	0.224	0.031	0.031	0.031

$\mathbf{A}_{control}$ (DPPH) = 1.585

DPPH radical scavenging activity (%) = $(A_{Control} - A_{Extract}) / A_{Control} \times 100$

Radical scavenging activity of 40mg/ml extract

=1.585 - (0.1533-0.1136) / 1.585 ×100%

=97.5%

Table 3.7.2 Results for Percentage DPPH radical scavenging activity

Extract conc. (mg/ml)	Mean Scavenging activity (%)	Ascorbic acid conc. (mg/ml)	Mean Scavenging activity (%)
40	97.50	1.0000	87.82
20	94.81	0.5000	53.60
10	77.80	0.2500	29.33
5	42.50	0.1250	17.39
2.5	29.67	0.0625	12.50
1.25	15.70	0.03375	8.30

3.7 Phytochemical screening

Phytochemcal tests revealed the presence of phytosterols, reducing sugars, tannins, terpenoids and flavonoids.

Table 3.8 Phytochemical tests

Test	Leaves	Stem	Roots
Phytosterols	+++	++	-
Condensed tannins	++	++	++
Terpenoids	+	+	+++
Alkaloids	+	+	++
Glycosides			
General tests for glycosides	+	+	+
Saponins	-	-	-
Anthraquinone glycosides	-	-	-
Cyanogenetic glycosides	-	-	-
Flavonoids	+	+	-

+ (Present in traces), ++ (present in moderate quantities), +++ (present in abundant quantities), - (absent)

CHAPTER FOUR

4 Discussions

4.1 Antimicrobial activity and time-kill kinetics studies

The leaves of *Clerodendron splendens* is used in folklore medicine for the treatment of wound and various skin infections (Irvine, 1961). In this study the various plant parts (leaf, stem and root) yielded different quantities of extracts using ethanol, acetone and chloroform. The leaf extracts had higher yield for all the solvents. Chloroform, 70% ethanol, and acetone yielded leaf extracts of 6.6, 4.4 and 3% respectively. Of the solvents used, the ethanol extracted more of the constituents than chloroform and acetone. Hence ethanol could be a better extractant for most of the constituents of *Clerodendron splendens* leaves.

The extracts exhibited antimicrobial activity against all the test organisms (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans)* as seen in Table 2.1-2.4. Hence this study appears to lend evidence to the effectiveness of this plant in folklore treatment of wound and skin infections. The minimum inhibitory concentrations of *Clerodendron splendens* extracts ranged between 0.15 and 7mg/ml. Neomycin sulphate (standard antibacterial agent) had MIC ranging from 1.5 to 65µg/ml and Clotrimazole (standard antifungal agent) had MIC of 0.086µg/ml against *Candida albicans* (Results recorded in Table 2.5*)*. The standard drugs gave MICs which are far lower than that of the extracts.

The time-kill kinetics studies showed that the extracts inhibited the growth of the various test organisms, while against some organisms such as *Proteus mirabilis,* the action was bactericidal for the 10mg/ml extract and the live cells was reduced to almost zero within a three hour period (Fig 3.3). For the 3 and 6mg/ml extracts

the action was bacteriostatic, inferring that the antimicrobial activity is concentration dependent.

Against *Pseudomonas aeruginosa*, the various concentration of extracts significantly reduced the log phase, but their extent of inhibition did not vary much from each other (Fig 3.1). For *Klebsiella pneumonia,* inhibition from the normal curve was observed about five to eleven hours (Fig 3.4).

For *Staphylococcus aureus*, the 10 mg/ml extract inhibited the multiplication of the organism to a higher extent than the 6mg/ml and 3mg/ml within the first three hours (Fig 3.5). The action of the extracts seems to be concentration dependent.

The various concentrations of extracts were capable of inhibiting *Candida albicans* to similar extents (Fig 3.7).

The controls showed the typical growth curves in nutrient broth for all the microorganisms.

Antimicrobial activity of these extracts infer that application of these extracts on wounds prevented microbes from invading the wound resulting in the protection of the wounds against infections.

4.2 Antioxidant and wound healing activity

Wound healing involves a chain of well orchestrated biochemical and cellular events leading to the growth and regeneration of wounded tissue in a specific manner (Clark, 1991). Results from this study suggests that treatment of Sprague Dawley rats with the fresh and ethanol extracts of the leaves of *Clerodendron Splendens* accelerated the wound healing process. The fresh juice and the ethanol extracts of leaves showed more percentage of wound healing than the control

group during the first five days (Fig 3.8.2). By day seven the control group had contracted to the same extent as those treated with the extracts. Complete healing was recorded at about day 16 for all the groups.

The breaking strength of the formed tissue, which may be due to the increase in collagen concentration (Udupa, 1995), increased with increase in concentration of the extract. The 300mg/ml extract had breaking strength higher than the standard as well as the vehicle treated wounds (Fig 3.9).

The ethanol extract of the leaves, used in the wound healing study also had antioxidant activity and very likely protected the cells against oxidative stress. The IC_{50} of the extracts was estimated to be 5.61±0.040 mg/ml. That of L- ascorbic under the same conditions was 0.4873 ± 0.0122 mg/ml (Table 3.72). Thus the antioxidant activity of the ascorbic acid is about 11.5 times that of the extract. This DPPH radical scavenging activity of the extract suggests that external application of the extracts on the wounds entrapped free radicals liberated from the wound surrounding cells which have the ability to protect cells from microbes.

The preliminary phytochemical analysis of the plant showed the presence of tannins, phytosterols, terpenoids, flavonoids and traces of alkaloids. Any one of the observed phytochemical constituents present could be responsible for the wound healing activity. Recent studies have however shown that phytochemical constituents like flavanoids and terpenoids promote the wound-healing process due to their astringent and antimicrobial properties, which appear to be responsible for

wound contraction and increased rate of epithelialisation (Scortichini and Rossi, 1991).

Some plant extracts also promote wound healing due to their antioxidant effect by altering the redox environment of the wound and hence reducing the concentration of oxygen radicals, thereby reducing their damage to cell membranes. This antioxidant effect could be due to the phenolic constituents, like flavonoids. Flavonoids are also known to reduce lipid peroxidation, prevent the onset of cell necrosis and also improve vascularity, hence enhancing the supply of blood to the area. Any drug that inhibits lipid peroxidation like flavonoids is also believed to increase the viability of collagen fibres and promote DNA synthesis (Nayak *et al*, 2006), these factors all enhance wound healing.

Hence, synergistic effect of antimicrobial and antioxidant activity as well as being able to increase the rate of wound contraction accelerated the wound-healing process.

5.0 CONCLUSION

In conclusion, this study confirms the promising wound healing activity, of *Clerodendron splendens.* Results indicated that the fresh juice from the leaves as well as the ethanol extract stimulated wound contraction, increased the breaking strength of the repaired tissue and possibly reduced the incidence of wound infection. The extract's inhibitory activity against the various organisms confirms its effectiveness in the folkloric use and also provides a rationale for the use of its preparation in skin and wound healing medicaments for the possible treatment of wounds, burns and skin infections. The minimum inhibitory concentrations against the various organisms ranged from 0.15 to 7mg/ml.

6.0 RECOMMENDATIONS

1. The various principles in the extracts may be isolated and their specific activities as well as their mechanism of action determined.

2. Stability studies should be conducted on the active principles of this plant.

3. Short and long term toxicity studies should be performed on the extracts before its use may be widely recommended.

4. The extracts should be formulated into an appropriate dosage form (cream or lotion) to make for convenient use.

5. People living within the area that this plant grows should be educated on the benefits of this plant when applied to wounds.

APPENDIX

A. CULTURE MEDIA

1. NUTRIENT AGAR (MERK)

Formula (in g/l)

Peptone from meat	3.45
Peptone from casein	3.45
Sodium chloride	5.10
Agar- agar	13.00

Nutrient agar powder (22.5g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

2. NUTRIENT BROTH (OXOID)

Formula (in g/l)

Lab lemco Powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar no 3	15.5

Nutrient broth powder (25g) was weighed into a conical flask and dissolved in distilled water to 1L. The mixture was distributed into glass tubes in portions of 10ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30mmin in an autoclave (Arnold and sons Ltd, Basildon).

3. EOSIN METHYLENE BLUE AGAR (SCHARLAU)

Formula (in g/l)

Peptone	10000
Lactose	10000
Dipotassium hydrogen phosphate	2000
Yellowish eosin	400
Methylene blue	65
Agar	15000

Final pH 7.1

Eosin methylene blue agar powder (37.5g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

4. MACKONKEY AGAR NO 2 (OXOID)

Formula (in g/l)

Peptone	20.0
Lactose	10.0
Bile salts No 2	1.50
Sodium chloride	5.00
Neutral red	0.05
Crystal violet	0.001
Agar	5.00

MaCkonkey agar powder (51.5g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

5. MANNITOL SALT AGAR (OXOID)

Formula (in g/l)

Lab –lemco Powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol Red	0.025
Agar	15.0
Water to	1000ml

Mannitol salt agar powder (111g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

6. CETRIMIDE AGAR (OXOID)

Formula (in g/l)

Gelatin peptone	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.6

Cetrimide agar powder (45.3g) was weighed into a conical flask and dissolved in about 900ml distilled water. Ten (10) ml of glycerol was added and made up to 1L. The mixture was heated in a boiling water bath to dissolve, distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

7. SABOURAUD 4% DEXTROSE AGAR (MERK)

Formula (in g/l)

Peptone	10.0
D (+) Glucose	40.0
Agar –Agar	15.0

Sabouraud dextrose agar powder (65g) was weighed into a conical flask and dissolved in distilled water to 1L by heating in a boiling water bath. The mixture was distributed into glass tubes in portions of 20ml, plugged firmly with cotton and sterilized by autoclaving at 115^{o}C for 30min in an autoclave (Arnold and sons Ltd, Basildon).

B. Standardization of test organisms

Ten (10) ml sterile nutrient broth was inoculated with *E.coli* from the stock cultures by means of a platinum loop. These were incubated at 37^0C for 18h. One milliliter of the resultant suspension was serially diluted 10, 10^2, 10^3,10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, $10^{11}$and 10^{12} times in several 10ml Nutrient Broths. Plain nutrient broth was used as a blank to zero a Digital Photocolorimeter (Model 312E) at 480nm (wavelength of maximum absorbance). The absorbances of the resultant suspensions of organisms were then determined. The number of viable cells per milliliter of the broths were determined by means of the pour plate method, with the aid a Colony counter (Type 95.06.17, Gerber Instruments, L.Schneider &co.AG). Results recorded in Tables C1 to C4. Graphs of Log_{10} cfu/ml was plotted against Absorbances (Fig C1 to C7). From the graphs the range of absorbances of broths containing 10^5 - 10^7 viable cells per ml were read and tabulated in Table C5.

Table B.1 The Absorbances of Colony forming units of *Bacillus subtilis and Proteus mirabilis* cells in Nutrient broth

Bacillus subtilis			***Proteus vulgaris***		
Absorbance	Cfu/ml	Log cfu	Absorbance	Cfu/ml	Log cfu
1.28	1.09×10^9	9.0294	1.70	1.95×10^{10}	10.29073
0.41	2.14×10^8	8.3324	0.73	3.91×10^9	9.59176
0.10	4.28×10^7	7.6334	0.20	7.81×10^8	8.89279
0.06	8.59×10^6	6.9345	0.05	1.56×10^8	8.19382
0.04	4.3×10^6	6.6335	0.01	3.125×10^7	7.49485
0.01	2.14×10^6	6.3324	0.01	6.25×10^6	6.79588
0.00	3.44×10^5	5.5366	0.01	5.0×10^4	6.09691

Table B.2 The Absorbances of Colony forming units of *Escherichia coli and Pseudomonas aeruginosa* cells in Nutrient broth

Escherichia coli			*Pseudomonas aeruginosa*		
Absorbance	**Cfu/ml**	**Log cfu**	**Absorbance**	**Cfu/ml**	**Log cfu**
0.33	2.8×10^{8}	8.4597	0.27	6.62×10^{8}	8.8210
0.19	9224×10^{4}	7.9649	0.08	2.649×10^{7}	7.4231
0.06	5765×10^{4}	7.7608	0.06	1.324×10^{7}	7.1221
0.05	2306×10^{4}	7.3628	0.05	3.162×10^{6}	6.5000
0.03	1153×10^{4}	7.0618	0.02	5.29868×10^{5}	5.72416
0.02	230.6×10^{4}	6.3629	0.01	1.0597×10^{5}	5.0251
0.01	4612×10^{2}	5.6639	0.00	2.1195×10^{4}	4.3262
0.00	922.4×10^{2}	4.9649			

Table B.3 The Absorbances of Colony forming units of *Klebsiella pneumonia* and *Staphylococcus aureus* cells in Nutrient broth

Klebsiella pneumonia			*Staphylococcus aureus*		
Absorbance	**Cfu/ml**	**Log cfu×10^2**	**Absorbance**	**Cfu/ml**	**Log cfu**
0.85	7.8659	7.3×10^{7}	1.85	4.8×10^{8}	8.6816
0.27	7.1669	1.510^{7}	0.88	9.60×10^{7}	7.9826
0.04	6.4679	29375×10^{2}	0.28	1.92×10^{7}	7.2837
0.03	5.0700	1175×10^{2}	0.07	3.84×10^{6}	6.5847
0.02	4.3710	2350×10^{2}	0.03	7.68×10^{5}	5.8857
0.02	3.6720	47×10^{2}	0.01	1.537×10^{5}	5.1868
0.01	2.9731	940	0.01	3.075×10^{3}	4.4878

Table B.4 The Absorbances of Colony forming units of *Candida albicans* cells in Nutrient broth

Candida albicans		
Absorbance	**Cfu/ml**	**Log cfu**
0.38	1.875×10^{7}	7.2730
0.34	1.5625×10^{7}	7.1938
0.09	3.125×10^{6}	6.4948
0.06	9.375×10^{5}	5.9719
0.05	6.456×10^{5}	5.8100
0.03	6.25×10^{5}	5.7959
0.02	1.25×10^{5}	5.0969
0.01	2.50×10^{4}	4.3979
0.00	5.00×10^{3}	3.6989

Table B.5 Standardized Absorbances of suspension of organisms containing 10^{5}-10^{7}colony forming units per ml.

Organisms	Absorbances
Staphylococcus aureus	0.02-0.28
Bacillus subtilis	0.01-0.09
Escherichia coli	0.02-0.09
Pseudomonas aeruginosa	0.05-0.09
Proteus mirabilis	0.01-0.63
Klebsiella pneumoniae	0.04-0.06
Candida albicans	0.06-0.36

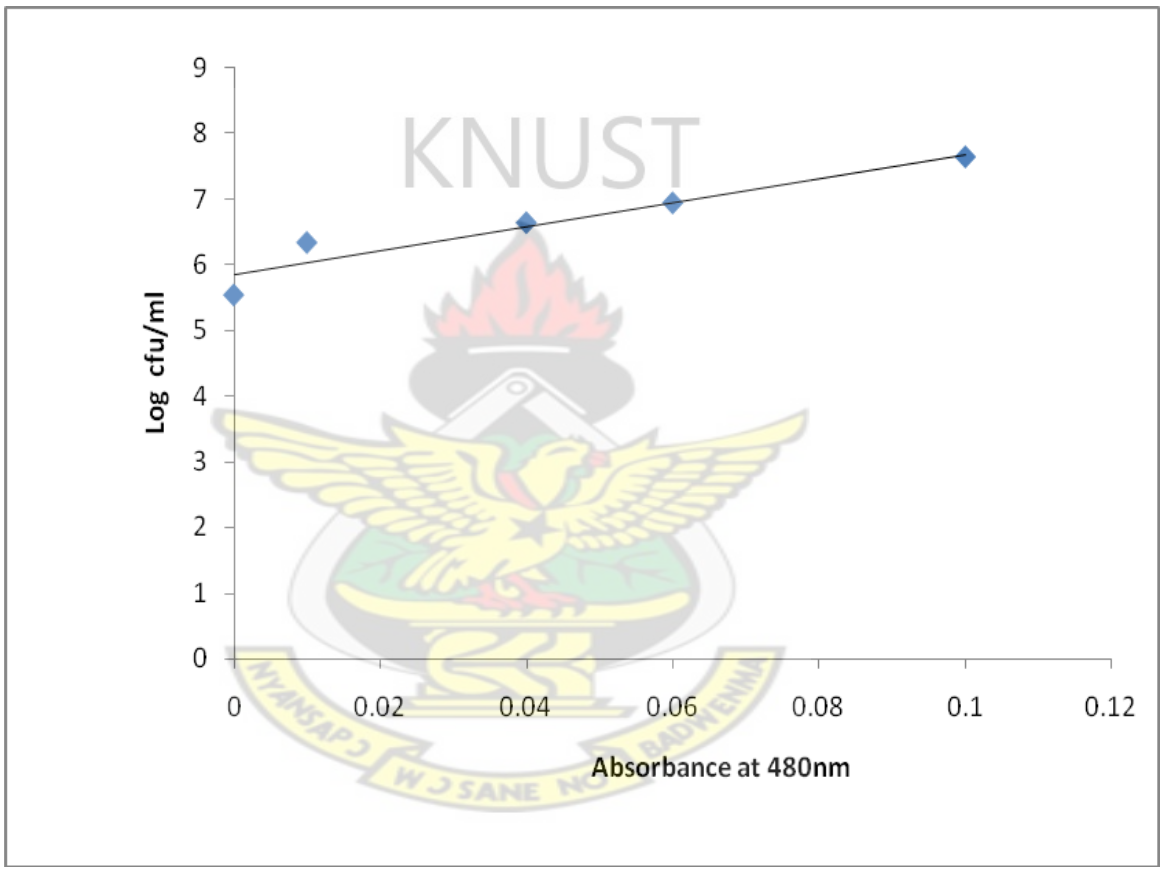

Fig. B.1 Standardization of *Bacillus subtilis* suspension

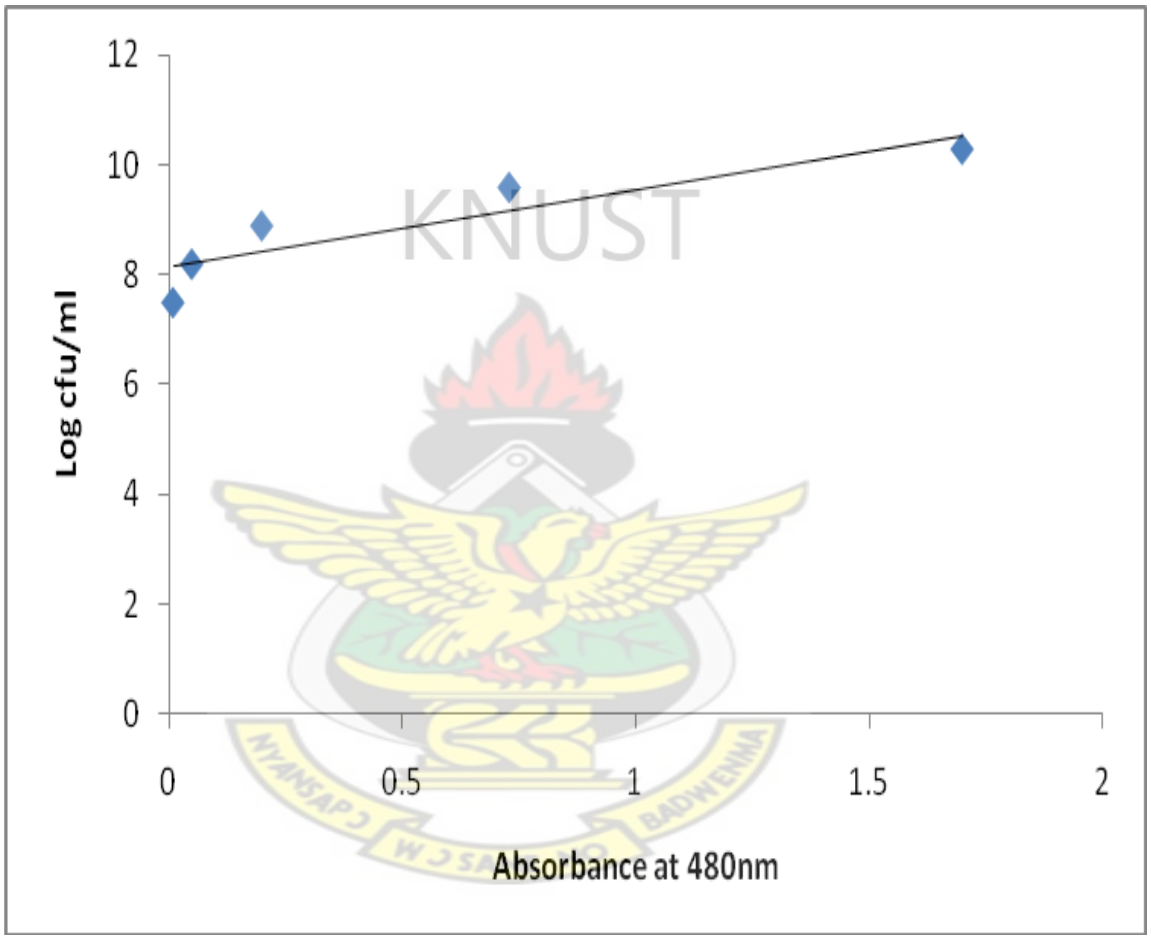

Fig. B.2 Standardization of *Proteus vulgaris* suspension

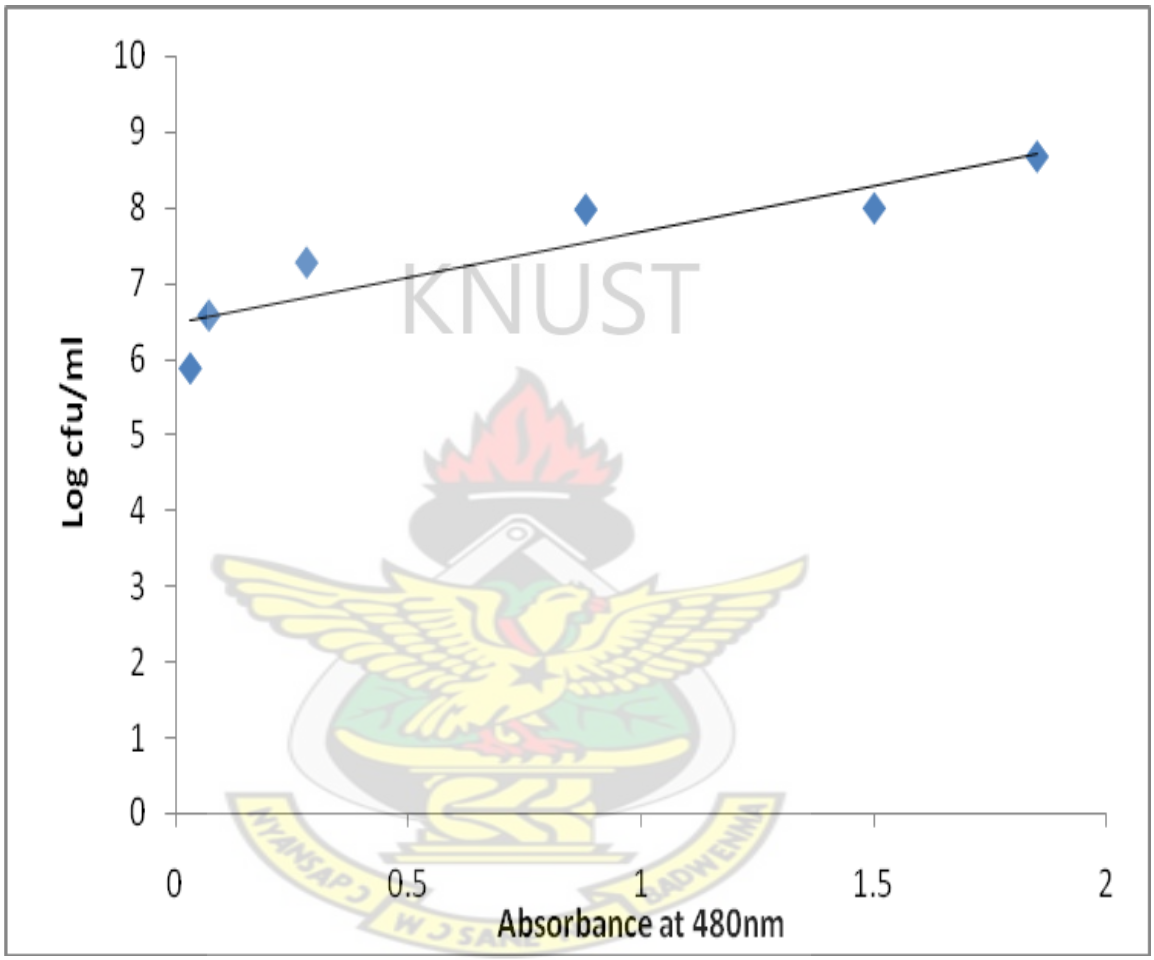

Fig. B.3 Standardization of *Staphylococcus aureus* suspension

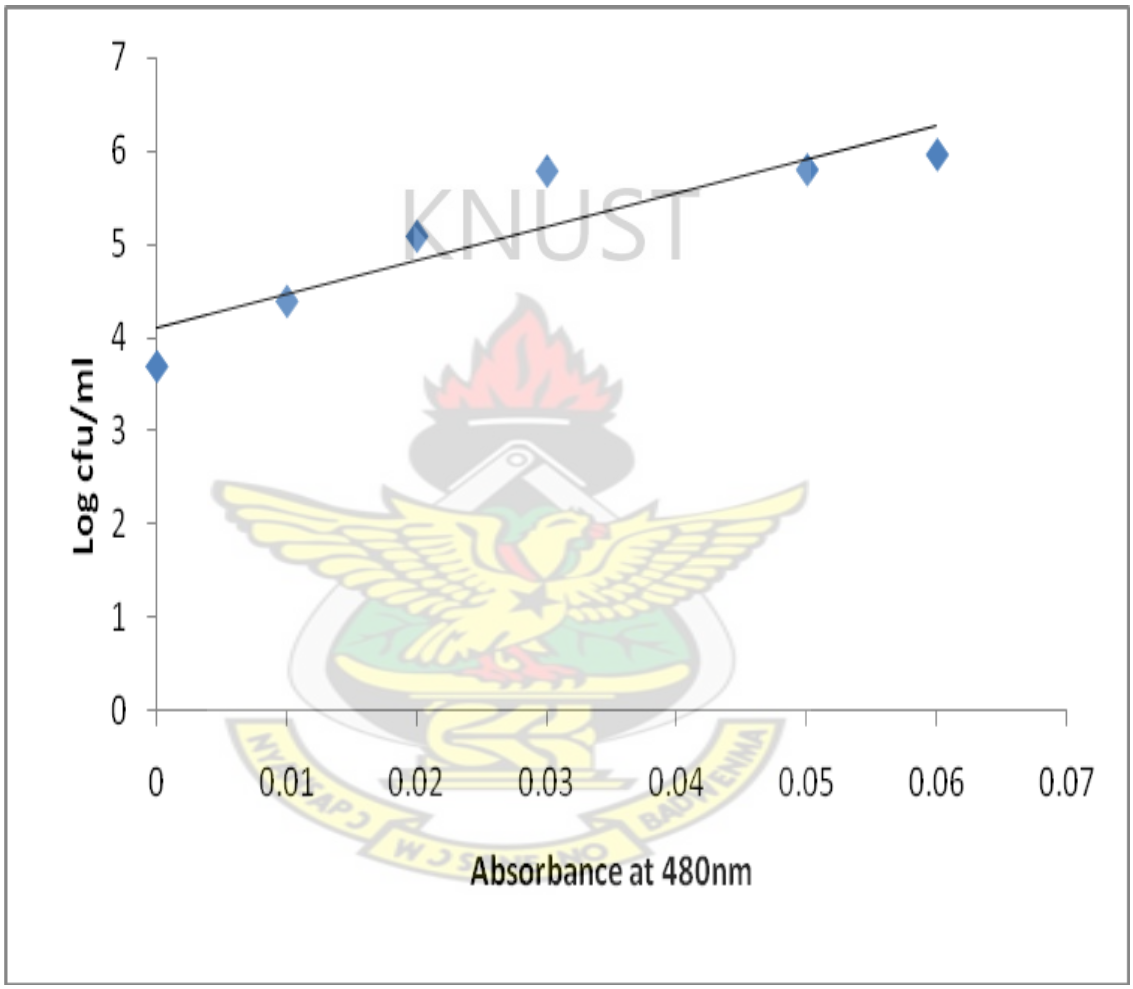

Fig. B.4 Standardization of *Candida albicans* suspension

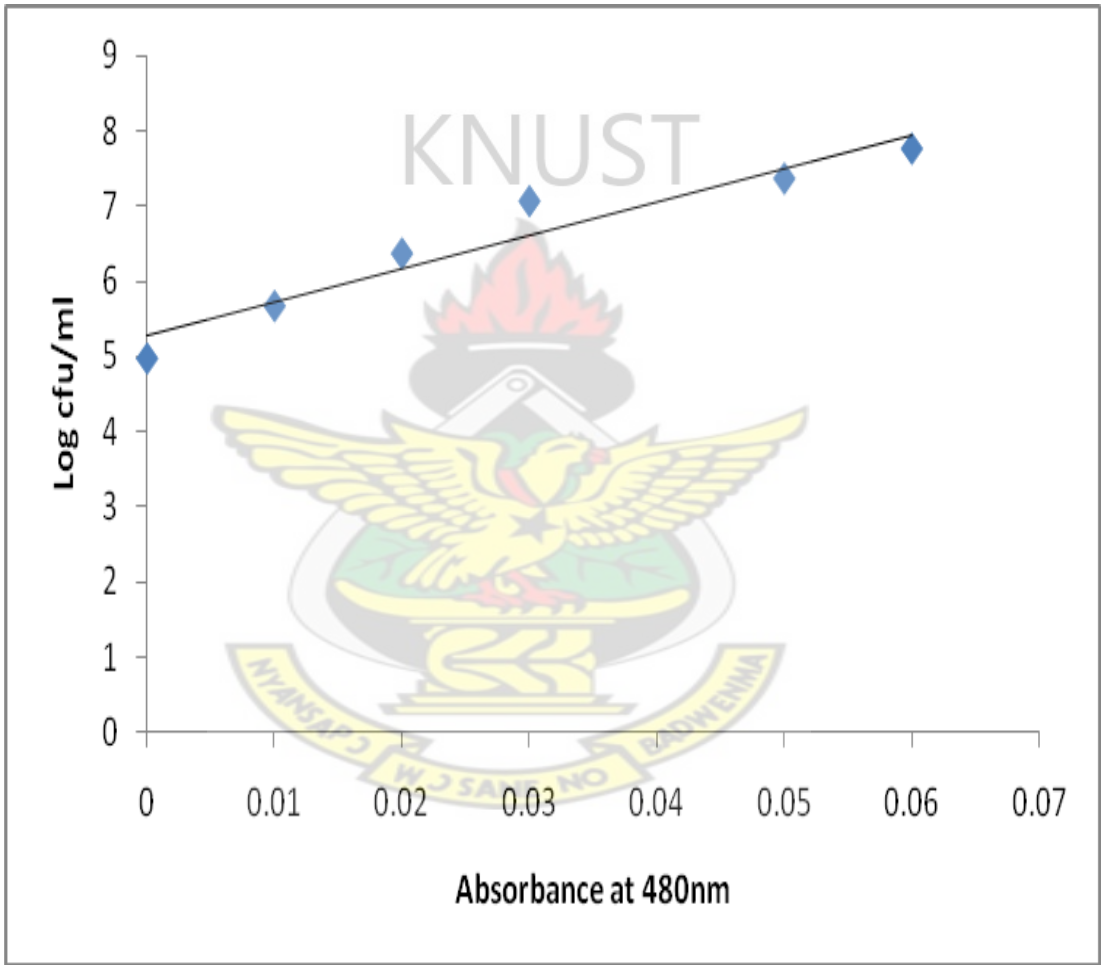

Fig. B.5 Standardization of *Escherichia coli* suspension

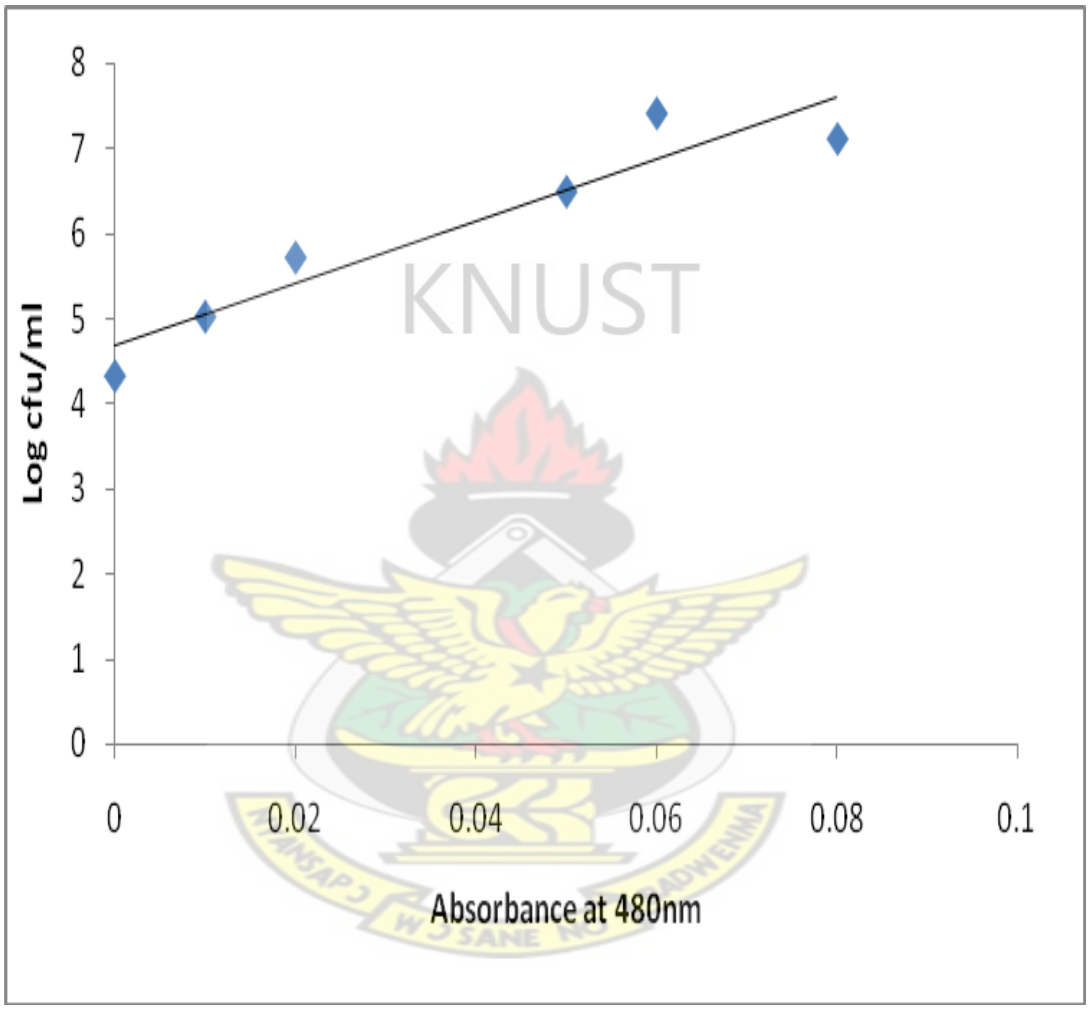

Fig. B.6 Standardization of *Pseudomonas aeruginosa* suspension

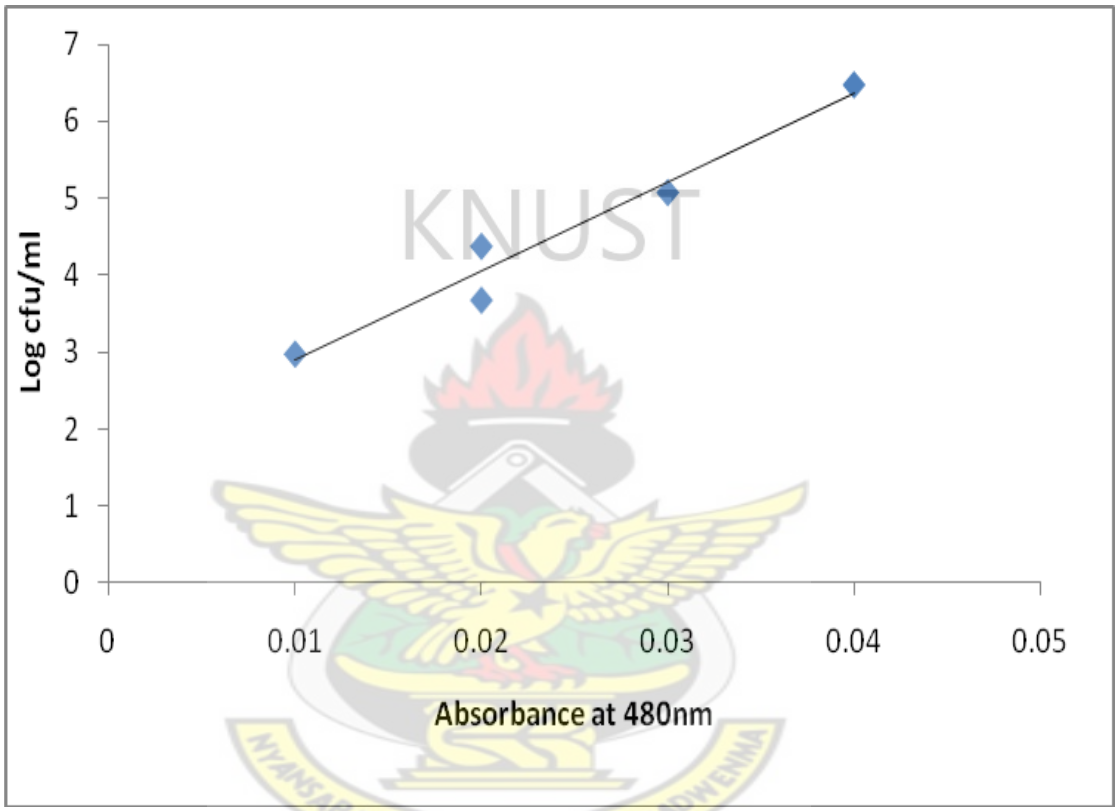

Fig. B.7 Standardization of *Klebsiella pneumoniae* suspension

C. Time-kill kinetics of ethanol leaves extract of C*lerodendron splendens*

When the test organisms were exposed to different concentrations of the Ethanol leaves extract of *Clerodendron splendens*, in nutrient broth and aliquots taken at time interval and viable counts performed on them using the pour plate method, colonies of surviving cells were observed on the media. These were countered and recorded below in Table C.1 to C.7.

Table C.1 Surviving *Escherichia coli* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				**Log Mean number of surviving cells per ml**			
Time (h)	Control	3mg/ml of extract	6mg/ml of extract	10mg/ml of extract	Control	3mg/ml	6mg/ml	10mg/ml
0	14000	14400	11600	16000	4.15	4.16	4.06	4.20
0.5	15990	12666	11466	13600	4.20	4.1	4.05	4.13
1	27537	14666	11600	18800	4.44	4.16	4.06	4.27
2	35175	17866	11733	37800	4.55	4.25	4.07	4.57
3	21×0^{6}	40400	16000	17733	6.30	4.60	4.20	4.25
4	9×10^{6}	63918	143000	26400	6.95	4.80	5.15	4.42
6	38×10^{6}	67635	326960	6×10^{6}	7.58	4.83	5.51	6.77
20	1×10^{6}	26×10^{6}	4×10^{6}	23×10^{6}	9.00	7.41	6.60	7.36
24	2×10^{12}	4×10^{6}	694×10^{6}	339×10^{6}	12.3	12.60	8.84	8.53

Table C.2 Surviving *Pseudomonas aeruginosa* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				**Log Mean number of surviving cells per ml**			
Time (h)	Control	3mg/ml	6mg/ml	10mg/ml	Control	3mg/ml	6mg/ml	10mg/ml
0	2080	2560	2460	2660	3.32	3.4	3.39	3.42
0.5	2636	2650	2150	1820	3.42	3.42	3.33	3.26
1	4983	2050	2440	1780	3.7	3.31	3.39	3.25
2	8035	2240	2090	1720	3.9	3.35	3.32	3.23
3	14030	2840	1920	200	4.14	3.45	3.28	2.3
4	465365	4335	3825	1785	5.67	3.64	3.58	3.25
6	$58E6\times10^{6}$	84420	36582	12060	7.76	4.93	4.56	4.08
20	$586E6\times10^{6}$	$287E3\times10^{6}$	323E3	$39E3\times10^{6}$	8.77	5.46	5.51	4.59
24	$5E10\times10^{6}$	$136E6\times10^{6}$	$252E6\times10^{6}$	$4E6\times10^{6}$	10.7	8.13	8.4	6.6

Table C.3 Surviving *Klebsiella pneumoniae* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				Log Mean number of surviving cells per ml			
Time (h)	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**
0	9030	11160	10180	9030	9.95	3.95	4.00	4.05
0.5	9240	5830	6700	6530	3.96	3.81	3.80	3.77
1	9890	7760	6470	4360	3.99	3.64	3.81	4.18
2	21507	15240	9460	7900	4.33	3.90	3.81	4.52
3	83013	33330	29920	16170	4.91	4.20	4.475	4.52
4	194970	161304	49720	37840	5.29	4.60	4.70	5.20
6	284×10^{6}	113×10^{4}	106×10^{4}	368852	8.45	5.60	6.03	6.05
20	470×10^{6}	491×10^{6}	471×10^{6}	217×10^{6}	8.60	8.30	8.67	8.69
24	1×10^{9}	1×10^{9}	824×10^{6}	1×10^{9}	9.00	9.00	8.91	9.00

Table C.4 Surviving *Proteus mirabilis* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				Log Mean number of surviving cells per ml			
Time (h)	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**
0	2020	2040	2010	2011	3.30	3.30	3.30	3.30
0.5	2355	1900	1500	990	3.37	3.28	3.18	3.00
1	4080	980	595	160	3.61	2.99	2.77	2.20
2	11440	465	350	55	4.06	2.67	2.54	1.74
3	57120	510	425	30	4.76	2.7	2.63	1.47
4	434520	838	648	20	5.64	2.92	2.81	1.30
6	9E6	1020	2040	0	6.95	3.00	3.30	0.00
20	8.6×10^{10}	8158	63712	0	10.93	3.91	4.80	0.00
24	6.1×10^{18}	60×10^{6}	73×10^{6}	0	18.80	7.78	7.86	0.00

Table C.5 Surviving *Baccilus subtilis* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				Log Mean number of surviving cells per ml			
Time (h)	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**
0	8705	9330	9220	9408	3.94	4	3.96	3.97
0.5	8844	7600	7640	5520	3.95	3.88	3.88	3.74
1	105×10^{3}	7420	7040	4400	5.02	3.87	3.85	3.64
2	424×10^{3}	6840	5080	3990	5.63	3.84	3.7	3.6
3	575×10^{3}	18425	3240	1920	5.76	4.27	3.51	3.28
4	2×10^{6}	19796	2140	6280	6.3	4.3	3.33	3.8
6	37×10^{6}	420×10^{3}	$266E3\times10^{3}$	67602	7.57	5.62	5.42	4.83
20	49×10^{6}	19×10^{6}	1×10^{6}	590×10^{3}	7.69	7.28	6	5.77
24	91×10^{6}	137×10^{6}	112×10^{6}	$45E6\times10^{6}$	7.96	8.14	8.04	7.65

Table C.6 Surviving *Staphylococcus aureus* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				Log Mean number of surviving cells per ml			
Time (h)	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**
0	9620	7480	10000	48000	3.98	3.87	4	4.68
0.5	13090	5230	5360	45200	4.12	3.72	3.72	4.66
1	122360	2070	2260	20000	5.09	3.32	3.35	4.30
2	165624	8250	8075	6940	5.22	3.92	3.91	3.84
3	368235	11648	32400	5	5.57	4.07	4.51	0.70
4	384384	192156	175200	740	5.58	5.28	5.24	2.86
6	3×10^{6}	$1E6\times10^{6}$	$449E3\times10^{6}$	23400	6.48	6.00	5.65	4.37
20	96×10^{6}	$20E6\times10^{6}$	$3E6\times10^{6}$	478421	7.98	7.30	6.47	5.68
24	$1E\times10^{11}$	8×10^{9}	3×10^{8}	2×10^{8}	11.00	9.90	8.48	8.30

Table C.7 Surviving *Candida albicans* cells in the presence of 0, 3, 6 and 10mg/ml Ethanol leaves extract of *Clerodendron splendens*.

	Mean number of surviving cells per ml				Log Mean number of surviving cells per ml			
Time/h	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**	**Control**	**3mg/ml**	**6mg/ml**	**10mg/ml**
0	3040	2720	2651	2570	3.48	3.43	3.42	3.40
0.5	3460	1245	1621	1690	3.53	3.10	3.20	3.22
1	4040	1520	1520	1025	3.6	3.18	3.18	3.01
2	7930	1685	1221	980	3.89	3.22	3.09	2.99
3	22440	1710	1655	1350	4.35	3.23	3.21	3.13
4	45900	1802	1890	1950	4.66	3.26	3.28	3.29
6	63628	1899	2160	2730	4.80	3.28	3.33	3.44
20	8.9×10^{6}	20000	20000	45000	6.94	4.30	4.30	4.65
24	206×10^{12}	4×10^{6}	800800	60060	14.31	6.60	5.90	4.78

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