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ANTIMICROBIAL, ANTIBIOTIC MODULATION AND WOUND HEALING ACTIVITIES OF ENTANDROPHRAGMA ANGOLENSE

BY

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By

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DECLARATION

I hereby declare that this thesis is my own work towards 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 acknowledgment has been made in the text.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

Oral presentations

1. Agyare C., Ugboduma A.O. Adu F., Gbedema S.Y., Annan, K., Boamah V.E., Agyepong N. Antimicrobial activities of methanol stem bark extract of *Entandrophragma angolense* (Welw.) C.DC. 5th Western Africa Network of Natural Products Research Scientists (WANNPRES). AAPS Ethnoveterinary Symposium Ada, Osun State, Nigeria. 6 to 8th August, 2012.
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ABSTRACT

Entandrophragma angolense (Welw.) C.DC. (Meliaceae) is a deciduous plant commonly found on the West African Coast. This study was undertaken to evaluate the antimicrobial, antioxidant and wound healing activities of the methanol stem bark extract of *E. angolense*. Phytochemical screening for secondary metabolites was carried out on the crude extract of the plant. The crude extract was investigated for its antimicrobial activity; using the agar well diffusion method, minimum inhibitory concentration (MIC) using broth dilution technique; minimum bactericidal concentration (MBC), resistance modulation and time kill kinetics. The antimicrobial activity of the crude extract and various solvent fractions were evaluated against five bacteria species and a fungus; (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* NCTC 10073, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and a clinical strain of *Candida albicans*). For the time kill kinetics MIC, 2×MIC, 3×MIC and 4×MIC of the crude extract were used against the test organisms. Bacterial and fungal resistance modulation studies were carried out by testing the activity of the reference antibiotics in the presence of sub-inhibitory concentrations of the crude extract. The antioxidant activity was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method and N-propyl gallate was used as reference antioxidant. The excision wound model was used for the determination of wound healing activities of the crude extract using formulated creams (1, 4 and 16%) w/w. The wound size and histopathological studies were the parameters used to evaluate the wound healing process. Phytochemical screening of the crude extract revealed the presence of tannins, flavonoids, alkaloids, terpenoids, saponins and cardiac glycosides. The MICs of the crude extract against the test organisms were: *S. aureus* (11.0 mg/mL), *E. faecalis* (9.0 mg/mL), *B. subtilis* (13.0 mg/mL) *E. coli* (17.0 mg/mL), *P. aeruginosa* (19 mg/mL) and *C. albicans* (15.0

mg/mL). The various solvent fractions (pet ether, ethyl acetate and aqueous) exhibited varying antimicrobial activities, with the highest activity exhibited by the aqueous fraction. The crude extract exhibited bacteriostatic and fungistatic activity against all test bacteria and fungus respectively. The crude extract enhanced the antimicrobial effects of some reference antibiotics whilst reducing the activity of others against the test bacteria and fungus. Time kill studies showed that the extract exhibited an inhibition of bacterial and fungal growth. The 4% w/w formulated cream exhibited significant wound healing activity similar to 1% w/w silver sulphadiazine (reference agent). The results of histopathological examination also supported the outcome of the extract cream in facilitating the formation of granulation tissue and re-epitheliasation in the wound healing process. The crude extract exhibited radical scavenging activity with IC_{50} of 18.1µg/mL. The enhanced wound healing activity may be ascribed to the free radical scavenging activity and the different phenolic compounds present which are responsible for the antimicrobial and antioxidant activities of the crude extract.

CHAPTER ONE

1.0 INTRODUCTION

Nature has been the source of active ingredients present in medicinal plants and has formed the basis of sophisticated traditional medicine systems that has been in existence from ancient times. The widespread use of herbal remedies and healthcare preparations can be traced to the occurrence of medicinal properties in these herbs. For thousands of years people have used these plant products to flavour and preserve foods and as well as in the treatment of diverse ailments. Popular observations on the use and efficacy of medicinal plants have significantly contributed to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not completely known (Robbers *et al.*, 1996).

WHO defines traditional medicine as comprising therapeutic practices that have been in existence for hundreds of years, before the development and spread of modern scientific medicine and are still in use today (WHO, 2008).

Research carried out by various scientists have revealed that the mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) have become ineffective with their over prescription and misuse (Borris, 1996). It therefore becomes necessary that new sources especially plant sources be investigated. In recent time's research have been directed towards the use of natural ~~products~~ for the treatment and control of infections.

A large number of plants in different locations around the world have been extracted and semi purified to investigate their chemical constituents due to their safety profile, ease of availability, historical, economic and cultural reasons (Srivastava *et al.*, 1996).

It is considered that because of the structural and biological diversity of their constituents, medicinal plants offer a unique and renewable resource for the discovery of potential new drugs and biological entities (Balandrin *et al.*, 1985). It is estimated that only 5,000 plant species have been studied exhaustively for medical application out of the total of 250,000 to 300,000 species (Abelson, 1990).

1.1 Medicinal plants

For centuries, humankind has used plants for their healing abilities, without any explanation for their effectiveness. Today, however, the fields of organic chemistry and pharmacology have quantitatively determined which chemical factors in given plant are responsible for its therapeutic effects (Wink, 1999). These distinctions regarding these chemical constituents are due to the combinations of some secondary metabolites present. In the physiology of plants there are those compounds which occur in all cells and play a central role in the metabolic and reproductive processes of plant cells. They are known as primary metabolites and include carbohydrates, lipids, proteins, chlorophyll and nucleic acids (Kaufman *et al.*, 1999). There are those responsible for the cellular structuring of the plant cells; they are made up of some high molecular weight polymeric materials such as celluloses, lignins and proteins. The last group are those compounds that are characteristic of a limited range of species mostly produced as by-products from primary metabolites and are called the secondary metabolites (Kaufman *et al.*, 1999; Wink, 1999).

Although secondary metabolites have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has shown important role of these chemicals in plants (Wink, 1999).

These secondary metabolites do induce adaptive features important for their ecological fitness and survival by providing a defensive role, inter-plant competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Kaufman *et al.*, 1999; Wink and Schimmer, 1999).

Medicinal plants have been used for the management of all types of diseases throughout the history of humankind. The scientific basis of this activity has been established for many plants. Some examples of pharmacologically active compounds that have been gotten from medicinal plants include digoxin, extracted from foxglove (*Digitalis purpurea*) leaves, and recognized for its positive cardiovascular effects long before the active constituent was identified (Pervaiz *et al.*, 2006). Similarly, etoposide and teniposide currently used in cancer chemotherapy were extracted from *Podophyllum peltatum*, a plant traditionally used by Native Americans to treat cancer. Morphine and codeine from the latex of the opium poppy used as analgesic to relief pain continue to be in clinical use (Imbert, 1998). Many plants have also been found to have potent antimicrobial activity, examples of which include the methanolic extract of *Psidium guajava* which has been shown to possess antibacterial effect on *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Abdelrahim *et al.*, 2002). Furofuran lignans also isolated from the *Propolis sp.* has shown antibacterial activity against *S. aureus* and *E. coli* (Bankova *et al.*, 1999).

1.1.1 Medicinal plants as source of resistance modulatory agents

Managing infections using antimicrobial compounds has brought about the problem of antibiotic resistance. Resistance to two or more classes of antimicrobial agents has been a common finding reported in human and veterinary medicine which has been limiting the available therapeutic options.

The presence of efflux pumps and multidrug resistance (MDR) proteins have contributed to the intrinsic and acquired antibiotic resistance in these microorganisms (Li *et al.*, 1994). Therefore the discovery and development of new compounds that would either block or circumvent these resistance mechanisms could improve the containment, treatment and eradication of these strains (Oluwatuyi *et al.*, 2004).

The antimicrobial properties of medicinal plants have been investigated by a number of studies worldwide and have now been evaluated for MDR Pump or Efflux Pump inhibitors against various multidrug resistant bacteria (Adriana *et al.*, 2007).

The screenings of some medicinal plants have revealed their antimicrobial, antioxidant, antiulcer, antimalarial and anti-inflammatory properties amongst others. These properties have been used in treating chronic diseases such as typhoid fever, gastroenteritis, dysentery, hepatitic disorders, osteoporosis and diabetic wounds which are diseases typical of tropical countries (Sofowora, 1993; Nick *et al.*, 1995).

Research carried out have also shown that several combinations of natural and synthetic chemical compounds such as antibiotics, have presented a direct activity against some microorganisms. In this association, the medicinal plants or their sub-products can inhibit or increase the therapeutic effect of conventional drugs (Nascimento *et al.*, 2000). The effects of these combinations that aid in changing the microbial susceptibility to antibiotics for inhibition of efflux of pumps, do characterise these compounds (natural products of plant origin; extracts and phytoconstituents) as modifiers of antibiotic activity (Wolfart *et al.*, 2006; Molnar *et al.*, 2004).

Research work carried out by Adu *et al.* (2009) has showed the potentiation of antimicrobial effects of some antibiotics by *Corynanthe pachyceras* (Rubiaceae) extracts, thus modulating the bacterial resistance of some microorganisms to antibiotics.

1.1.2 Chemical constituents present in medicinal plants

Plants contain a large and heterogeneous group of biologically active compounds, which include three broad categories of plant secondary metabolites as natural products; terpenes and terpenoids (~25,000 types), alkaloids (~12,000 types), and phenolic compounds (~8,000 types) (Croteau *et al.*, 2000). These plants have limitless ability to synthesize some of these aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963).

Phenolics are secondary metabolites synthesized by plants that are ubiquitous throughout the plant kingdom. They are present in significant amounts in many commonly consumed fruits, vegetables, grains and beverages. Over 8000 phenolic compounds have been isolated from different natural products. These include quinones, flavonoids (flavonols, flavones), phenolic acids, coumarins, and tannins. Each group of this phytochemicals is further divided into subgroups on the basis of its chemical structure (Edeoga *et al.*, 2005).

Flavonols are the most widespread flavonoids in foods, and some examples include; quercetin, kaempferol and myrecetin (Beecher, 2003). Since they are known to be synthesized by plants in response to microbial infection it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Tsuchiya *et al.*, 1996). Flavonoids found in varying amounts in foods and medicinal plants have been shown to exert potent antioxidant activity against the superoxide radical (Hertog *et al.*, 1993).

Many biological activities and antibacterial effects have been reported for plant tannins and flavanoids (Scalbert, 1991; Chung *et al*., 1998). The effects of some terpenes on microorganisms have been seriously studied since (at least) the 1980's (Andrews *et al*., 1980). A number of studies have also shown saponins to have inhibitory effects on protozoa (Iwu *et al*., 1999). Chewonarin *et al*. (1999) isolated an alkaloid from *Hibiscus sabdariffa* and demonstrated its ability to interfere with cell division and prevent mutagenesis hence used as therapeutic agents in the management of cancer.

1.2 Antioxidants

Oxygen is an element obligatory for life; living systems have evolved to survive in the presence of molecular oxygen and for most biological systems. Oxidative properties of oxygen play a vital role in diverse biological phenomena. Oxygen has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events (Shinde *et al*., 2006). Oxidation is one of the most important processes, which produce free radicals in food, chemicals and even in living systems. The causes of the poisonous properties of oxygen were obscure prior to the publication of Gershman's free radical theory of oxygen toxicity in 1954, which states that the toxicity of oxygen is due to partially reduced forms of oxygen (Gerschman *et al*., 1954). In the normal metabolic process of the body energy obtained by the oxidation of carbohydrates, fats and proteins through both aerobic and anaerobic processes often leads to the generation of free radicals. Also the production of various substances such as toxins and wastes as by-products can play a role in the overproduction of these free radicals which are responsible for various tissue injuries (Valko *et al*., 2006).

1.2.1 Reactive oxygen species (ROS)

Free radicals are atoms or groups of atomic fragments with an odd (unpaired) number of electrons in its outermost atomic or molecular orbital. This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally achieve stable configuration. They are capable of attacking the healthy cells of the body, causing them to lose their structure and function.

Free radicals are formed from molecules by the haemolytic cleavage of a chemical bond and via redox reactions, once formed these highly reactive radicals can start a chain reaction (Bahorun *et al.*, 2006; Valko *et al.*, 2006). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) describe free radicals and other non-radical reactive derivatives. These reactive species at low or moderate concentrations play vital roles in defence against infectious agents and in the function of a number of cellular signalling systems. However when there is an overproduction on one end and a deficiency of enzymatic and non-enzymatic antioxidants on the other, the harmful effects of free radicals is escalated causing potential biological damage termed as oxidative or nitrosative stress (Kovacic and Jacintho, 2001; Ridnour *et al.*, 2005).

The toxicity of lead, pesticides, cadmium, ionizing radiation, alcohol, cigarette smoke, UV light and many other pollutants may all be due to their free radical initiating capability (Langseth, 1996). Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immuno-competency decline, liver diseases, diabetes mellitus, inflammation, renal failure, brain dysfunction and stress amongst others (Gupta *et al.*, 2008). To prevent free radical damage the body hence has a defence system of antioxidants.

1.2.2 Functions of antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). To protect the cells and organ systems of the body against reactive species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize these free radicals.

The human body naturally produces antioxidants but the process is not 100 percent effective in case of overwhelming production of free radicals and that effectiveness also declines with age (Sies, 1991; Goldfarb, 1993).

Antioxidants are substances that delay or inhibit free radicals by safely interacting with single free radicals and neutralizing them by donating one of their own electrons, ending the carbon reaction to those target molecules and hence, preventing any oxidative damage to cells concerned. They are thus absolutely critical for maintaining optimal cellular and systemic health and well-being (Ozsoy *et al.*, 2008). Naturally, there is a dynamic balance between the amount of free radicals produced in the body and antioxidants to scavenge or quench them and protect the body against deleterious effects.

The amount of antioxidant principles present under normal physiological conditions may be insufficient to neutralize free radicals generated. The body cannot manufacture these micronutrients so they must be supplied in the diet. The principal micronutrient antioxidants are vitamin E, beta-carotene, and vitamin C. Additionally, selenium a trace metal that is required for proper function of one of the body's antioxidant enzyme systems, is sometimes included in this category (Sies, 1991).

There is an increasing interest in the study of antioxidant substances mainly due to the findings of the therapeutic effects of these free radical scavengers. A great number of medicinal plants worldwide have been known to possess strong antioxidant properties (Baratto *et al.*, 2003; Katalynic *et al.*, 2006).

1.2.3 The role of antioxidants in wound healing

Wound healing processes are well organized biochemical and cellular events leading to the growth and regeneration of wounded tissue in a special manner. Healing of wounds involves the activity of an intricate net work of blood cells, cytokines and growth factors which ultimately leads to the restoration to normal condition of the injured skin or tissue (Clark, 1991).

It is desired that at the site of wound closure a flexible and fine scar with high tensile strength is desired. To promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient, it becomes important to explore factors that have influence on wound healing outcome (Menke *et al.*, 2007)..

Antioxidants present in medicinal plants aid to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. They counter the excess proteases and ROS often formed by neutrophil accumulation in the wounded area at the inflammatory stage of wound healing and protect protease inhibitors from oxidative damage. As such some fibroblasts and other cells may be destroyed by excess ROS and skin lipids will be made less flexible hence these antioxidant substances alleviate the possibility of adverse events occurring by free radicals (Houghton *et al.*, 2005).

Studies have also shown that phytochemical constituents like flavonoids, tannins and triterpenoids are known to promote the wound healing process mainly due to their astringent, anti-inflammatory, antioxidant and antimicrobial properties which appear to be responsible for the wound healing and increased rate of epithelialisation (Tsuchiya *et al.*, 1996; Scortichini and Pia, 1991).

The process of wound healing could be a function of either the individual or the additive effects of the phytochemical constituents so that the overall antioxidant effects appear to be important in the successful treatment of wounds (Krishnan, 2006)..

1.2.4 Methods for determination of antioxidant properties

The chemical principle of antioxidant capacity assays of various products gives varying results depending on the reactions involved. The end result is the same, regardless of mechanism, but kinetics and potential for side reactions differ. The assays can be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET). The majority of the *HAT-based assays* apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds.

These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays. The *ET-based assays* measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change is correlated with the sample's antioxidant concentrations. The ET-based assays include the total phenols assay by Folin–Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion

reducing antioxidant power (FRAP), 'total antioxidant potential' assay using a Cu (II) complex as an oxidant and DPPH (Huang *et al.*, 2005).

It must be appreciated at the outset that no single assay will accurately reflect all radical sources or antioxidants in a mixed or complex system because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved.

Some of the methods available for the in vitro measurement of antioxidant capacity in medicinal plants are described below:

1.2.4.1 Ferric ion reducing antioxidant power (FRAP)

This method is one of the most rapid test and very useful for routine analysis. It measures antioxidant power with the help of an oxidant, i.e. ferric (Fe^{3+}) is reduced to ferrous (Fe^{2+}) at low pH causing the formation of a coloured ferrous–tripyridyltriazine complex. In the FRAP assay, reductants (antioxidants) in the sample reduce the Fe (III) tripyridyltriazine complex, present in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593nm. The change in absorbance is proportional to the combined (total) ferric reducing/antioxidant power of the antioxidants in the sample. FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues.

However, since it cannot detect compounds that act by radical quenching (H transfer), it cannot measure the antioxidant capacity of certain antioxidants accurately (particularly thiols and proteins), which can react with iron (II) and SH group-containing antioxidants. This causes a serious underestimation in serum (Ou *et al.*, 2002).

1.2.4.2 Folin-Ciocalteu reagent (FCR)

The FCR assay has for many years been used as a measure of total phenolics in natural products, and the basic mechanism is an oxidation/reduction reaction. This assay measures the change in colour when metal oxides are reduced by polyphenolic antioxidants such as gallic acid and catechin, resulting in a blue solution with maximal absorption at 765 nm. The standard curve is prepared using gallic acid, and results are reported as gallic acid equivalents.

The method is simple, sensitive and precise. However, the reaction is slow at acid pH and it lacks specificity (Wolfe, 2007).

1.2.4.3 Trolox equivalence antioxidant capacity (TEAC)

The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation (2, 2'-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid) $ABTS^{\cdot+}$. When ABTS is incubated in the presence of a peroxidase and hydrogen peroxide or in the presence of hydroxyl, peroxyl, alkoxyl and inorganic radicals,. $ABTS^{\cdot+}$ radical cation is generated. A diode-array spectrophotometer is used to measure the loss of colour when the antioxidant is added to the blue-green chromophore ABTS 2, 2'-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid).

As the radical cation $ABTS^{\cdot+}$ begins to form, the absorbance increases which has a characteristic long wavelength absorption spectrum showing maxima at 660, 734 and 820 nm. Results of test compounds are expressed relative to Trolox (McAnalley *et al.*, 2003). This assay is operationally simple and has been used in many research laboratories for studying antioxidant capacity (AOC). ABTS radical can be used over a wide pH range and can be used to study effects of pH on antioxidant mechanisms. $ABTS^{\cdot+}$ is a stable radical not found in the human body and thus represents a "nonphysiological" radical source (Berg *et al.*, 1999).

1.2.4.4 Total antioxidant potential assay using Cu (II) as an Oxidant

The method is based on reduction of Cu (II) to Cu (I) by reductants (antioxidants) present in a sample. A chromogenic reagent, bathocuprione (2, 9-dimethyl-4, 7-diphenyl- 1, 10 phenathroline) forms a 2:1 complex with Cu (I), which has a maximum absorbance at 490 nm. It has been found that 1 mol of R-tocopherol can reduce 2 mol of Cu (II) to Cu (I) (Yamashita *et al*., 1998).

1.2.4.5 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay

This is the most widely reported method for screening of antioxidant activity of many plants. The DPPH is one of the few stable organic nitrogen radicals, which bears a deep purple colour. This assay is based on the measurement of the reducing ability of methanol solution of antioxidants towards DPPH. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. Antioxidant assays are based on measurement of the loss of DPPH colour (turns yellow) at 517 nm after reaction with test compounds and the reaction is monitored by a spectrophotometer. The percentage of the remaining DPPH· ($DPPH^{\cdot}_{REM}$) is proportional to the antioxidant concentration and the concentration that causes a decrease in the initial DPPH· concentration by 50% is defined as IC_{50} (Noruma *et al*., 1997).

It is a rapid, simple and inexpensive method which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. DPPH is stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility. The DPPH radical is not commonly found in biological systems (Noruma *et al*., 1997).

1.3 Wounds

A wound is defined as the disruption in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue (i.e. muscle, bone, nerves) following injury to the skin or underlying tissues/ organs (Hutchinson, 1992). This is normally caused by surgery, a blow, a cut, chemicals, heat or cold, friction or shear force, pressure or as a result of disease, such as leg ulcers or carcinomas (Enoch and Price, 2004; Leaper and Gottrup, 1998).

Medicinal plants have been used for many years as topical and internal preparations and have been shown to be very beneficial in wound care, promoting the rate of wound healing with minimal pain, discomfort, and scarring to the patient (Mackay and Miller, 2003).

Two types of wound do occur; acute and chronic. In the acute wounds there is an orderly process that results in sustained restoration of anatomic and functional integrity within the expected time frame. They are usually caused by cuts or surgical incisions (Lazarus *et al.*, 1994). However, various physiological and mechanical factors may impair the stepwise progression of healing process and results in the development of chronic wound that takes much longer time to heal.

Chronic wounds on the other hand are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation (Menke *et al.*, 2007). These wounds require a prolonged time to heal, do not heal, or recur frequently and are a major cause of physical disability. Some common examples of chronic wounds are diabetic foot ulcer, pressure ulcer and the venous leg ulcer.

Frequent causes of chronic wounds are local infection, hypoxia, trauma, foreign bodies and systemic problems such as diabetes mellitus, malnutrition, immunodeficiency or medications (Krishnan, 2006).

The size, location and depth of wounds are major determinant in types of wounds that are been formed as it may lead to a complete perforation of an organ or the body, with the additional complication of entrance and exit wounds. Large wounds are more serious than small ones, they usually involve more severe bleeding, more damage to the underlying organs or tissues and a greater degree of shock. On the other hand small wounds are sometimes more dangerous than large ones; they may become infected more readily due to neglect.

Two classes of wounds are explained below:

1.3.1 Mechanical wounds

1.3.1.1 Abrasions

Abrasions are made when the skin is rubbed or scraped against something rough. It usually involves the superficial layers of the epidermis only. Rope burns, floor burns, and skinned knees or elbows are common examples of abrasions. This kind of wound is often contaminated by dirt or grit that gets embedded in the tissues. When they heal there is no permanent scar (Young, 2002).

1.3.1.2 Lacerations

Lacerations are wounds in which the tissues are torn rather than cut. They have ragged, irregular edges and masses of torn tissue underneath. These wounds are usually made by blunt, rather than sharp objects. A wound made by a dull knife, for instance, is more likely to be a laceration than an incision. Many of the wounds caused by accidents with machinery are lacerations; they are often complicated by crushing of the tissues and are frequently contaminated with dirt, grease, or other materials which are normally embedded in the tissues (Hollander and Singer, 1999).

1.3.1.3 Incisions

Incisions (cuts, slashes or slices) are wounds that tend to have smooth edges and are made by sharp cutting instruments such as knives, razors, scalpels and broken glass. Incisions tend to bleed freely because the blood vessels are cut clean with a shallow end as compared to lacerations that are deep with ragged edges. There is little damage to the surrounding tissues.

Of all classes of wounds, incisions are the least likely to become infected, since the free flow of blood washes out many of the microorganisms (germs) that cause infection (Sorensen *et al.*, 2003).

1.3.1.4 Punctures

Punctures are usually, narrow wounds in the skin and usually from a pointed object (nails, needles, wire, and bullets) that penetrate into the tissues while leaving a small surface opening. Some punctures could also be exerted into the underlying organs i.e. entering the cavity of the body e.g. stab wounds from knives and are called penetrating wounds (Mendez and Black,

1999). Small puncture wounds do not bleed freely however; large puncture wounds may cause severe internal bleeding. The possibility of infection is great in all punctures especially if the wound is deep and difficult to clean or if the penetrating objects was contaminated (Mendez and Black, 1999). To prevent anaerobic infections, primary closures are not made in the case of puncture wounds.

1.3.1.5 Avulsions

An avulsion is the tearing away of tissue from a body part. The piece of skin could be hanging from the body or completely removed. Bleeding is usually heavy in this type of wound and most often involves ears, fingers and hands. In certain situations, the torn tissue may be surgically reattached (Nayduch, 1999).

1.3.1.6 Amputations

Amputation involves the cutting or tearing off a body part such as a finger, toe, hand, foot, arm or leg. Bleeding is heavy and requires a tourniquet to stop the blood flow to the affected area. Shock is certain to develop in these cases. If medical protocols are followed appropriately, the affected body parts can often be successfully reattached (Bosse *et al.*, 2002).

1.3.1.7 Contusions

A contusion or bruise is an effusion of blood into the tissues due to rupture of subcutaneous vessels usually capillaries. It is caused by an object striking the body with enough force to crush the tissues beneath the skin such as a fist, stone, stick, whip and boots. If the blow is over a bony area, the bone also may be contused.

This results in blood and waste products leaking into the area around the cells. These blood and waste products gradually travel to the skin, producing the characteristic "black and blue mark" of a contusion. Usually there is no loss of continuity of skin but they may be associated with abrasions or lacerations (Eaglstein, 1998).

1.3.1.8 Thermal wounds

Due to cold

I. Frost bites

This is most common cold injury which occurs in mountaineers. It is caused by the exposure to temperatures (risk exist below -2°C) low enough to cause crystal formation in the tissues. Frost bite develops after exposure for 1 hour. They are developed based on factors such as wind chill, altitude, duration, wetness and prior exposure. Fingers, toes and ears are most commonly involved (Hassi and Makinen, 2000).

II. Trench foot

Trench foot also known as immersion foot is a non-freezing cold injury which occurs when the feet are wet for long periods of time. Symptoms range from swelling, cold and blotchy skin, numbness, a prickling or heavy feeling in the foot to the formation of red and painful blisters which in severe cases is followed by skin and tissue falling off due to peripheral nerve damage and tissue necrosis. Trench foot can involve the toes, heels or the entire foot and can be prevented and treated (Marcus, 1979).

Due to heat

I. Burns

Burns are wounds that are caused by application of heat or chemical substances to the external or internal surface of the body which causes destruction of tissues. Exposure to temperatures above 65°C for two seconds is sufficient to produce burns (Monafo and Bessey, 2002).

1.3.2 Wound healing cascade

In humans and more widely in all mammalian species, the wound healing process is a dynamic one which can be divided in three consecutive and overlapping phases: haemostasis/inflammatory, proliferation (new tissue formation) and maturation (remodelling). The transition from one stage to another depends on the maturation and differentiation of the main cell populations involved, among which are the keratinocytes, the fibroblasts and the macrophages (Gurtner *et al.*, 2008). It is critical to remember that wound healing is not linear and often wounds can progress both forward and backward through the phases depending upon intrinsic and extrinsic forces at work within the patient. The phases are explained below:

1.3.2.1 Haemostasis/Inflammation

This phase is the body's natural response to injury. The first event occurring after injury is haemostasis a process by which the the body prevents blood loss (coagulation) within the first 5-10 minutes. This involves the formation of a cellular system comprised of cells called platelets that circulate in the blood and serve to form a platelet plug over damaged vessels and, a second system based upon the actions of multiple proteins (called clotting factors) that act in concert to produce a fibrin clot.

These two systems work in concert to form a clot; adverse changes in either system can yield disorders that cause either too much or too little clotting (Leibovich and Ross, 1975). Once haemostasis has been achieved, blood vessels then dilate to allow essential cells, antibodies, white blood cells, growth factors, enzymes and nutrients to reach the wounded area. This leads to a rise in exudates level so the surrounding skin needs to be monitored for signs of maceration (Leaper and Gottrup, 1998).

The predominant cells secreted to work are the phagocytic cells neutrophils and macrophages; mounting a host response and autolysing any devitalized necrotic or sloughed tissue. Neutrophils are the first cells to be formed. They massively infiltrate the wound during the first 24hr after injury. They are attracted by the numerous inflammatory cytokines produced by the activated platelets, endothelial cells, as well as by the degradation products from pathogens (Ross and Odland, 1968).

The release of these cytokines during this apoptotic process is an important component in macrophage recruitment. Macrophages infiltrate the wound massively 2 days post injury and exacerbate at this stage an intense phagocytic activity (Ross and Odland, 1968; Leibovich and Ross, 1975). It is normally at this stage that the characteristic signs of inflammation can be seen; erythema, heat, oedema, pain and functional disturbance.

1.3.2.2 Proliferation

The proliferation process begins few hours after the wound formation. Here the wound is rebuilt with new granulation tissue which is comprised of collagen and extracellular matrix and into which a new network of blood vessels develop, a process known as angiogenesis.

A massive angiogenesis is dependent upon the fibroblast receiving sufficient levels of oxygen and nutrients supplied by the blood vessels for the healing process of the rebuilding tissue (Chung, 2010).

Later, some of the fibroblasts differentiate into myofibroblasts. These contractile cells will help bridge the gap between the wound edges (Singer and Clark, 1999). During the same time, growth factors produced by the granulation tissue will favour proliferation and differentiation of epithelial cells, which finally resurface at the wound site, restoring the epithelial barrier integrity in a process known as epithelialisation (Gabbiani, 2003).

1.3.2.3 Maturation

Maturation is the final phase and it entails a gradual involution of the granulation tissue and dermal regeneration. This step is associated with the apoptosis of myofibroblasts, endothelial cells and macrophages. Cellular activity is reduced and the number of blood vessels in the wounded area regress and decrease. The remaining tissue is therefore composed mostly of extracellular matrix proteins, essentially collagen type III. This would be remodelled by the metalloproteinase produced by the epidermal cells, endothelial cells, fibroblasts and the macrophages remaining in the scar and be replaced by collagen type I (Hutchinson, 1992).

1.3.2.4 Infections of wounds

When the initial inflictions on a wound are extensive, infections may develop as organisms that previously colonized the skin and gut may now invade tissues. Some important organisms causing wound infections are *Staphylococcus aureus*, *Streptococcus pyogenes*, *Corynebacterium* sp., *Escherichia coli* and *Pseudomonas aeruginosa* (Kumar *et al*., 2007).

In addition, such an environment may allow secondary invaders to cause infections. These organisms or their liberated toxins may overwhelm the local protective environment with resultant systemic sepsis resulting in chronic wounds (Altemeier *et al.*, 1984; Thomson, 1984). Wounds do heal by primary intention or secondary intention depending upon whether the wound may be closed with sutures or left to repair, whereby damaged tissue is restored by the formation of connective tissue and regrowth of epithelium (Cooper, 2005). Understanding the healing process and nutritional influences on wound outcome is critical to successful management of wound patients.

1.3.3 Assessment parameters used for wound healing

A thorough wound assessment provides baseline data about the status of the wound and valuable information that can assist in identifying short- and long-term goals of care, enabling the effects of treatment to be monitored at each stage of healing (Keast *et al.*, 2004). The purpose of any wound measurement is to monitor the progress of healing through changes in the length, width or area of a wound. This can be done using the following techniques:

1.3.3.1 Linear measurement

This is the simplest method of determining wound area. This technique quantifies the surface area by measuring the two maximal perpendicular dimensions directly from the wound site with the aid of a tape measure or ruler. The greatest length is then multiplied by the perpendicular greatest width. This type of calculation has been shown to overestimate wound area by 10% to 44%, with accuracy decreasing as wound size increases (Majeske, 1992).

This method is quick, inexpensive and easy to perform and most reliable when measuring near-circular wounds however, this ruler-based technique is considered less reliable than other measurement techniques particularly for wounds which are large, irregular or cavitous such as ulcers (Wild *et al.*, 2008; Goldman and Salcido, 2002).

1.3.3.2 Wound tracing

This is another two dimensional wound measurement tool that aids in sequential comparisons of tracings to monitor healing progression. A transparent film is placed over the wound surface and the wound perimeter traced with an indelible marker. The film usually consists of two sheets: one sheet in contact with the wound (sterile), which is later discarded to minimize contamination, and the other on which the tracing is performed (Plassmann, 1995). This technique allows more accurate area calculation when the wound is irregular but it is also limited in deciding where the boundary of the wound lies, which affects the reliability and accuracy of this technique (Charles, 1998).

1.3.3.3 Scaled photographs

Photography is a non-contact objective method of recording wound measurements, which provides a systematic, evidence-based approach. This method of assessment uses a photograph that has been processed by a special scanner so that a scaled ruler is incorporated at the edge of the photograph. The ruler is used to calculate length and width, which are expressed in simple measurements (Berg *et al.*, 1990). However, the use of photography has its limitations as there is the potential for magnification errors.

Wound images, if not properly corrected to accommodate natural body curvature, may result in distortion of the wound perimeter and subsequent inaccuracies in measurement computations this is particularly problematic for larger wounds.

1.3.3.4 Tensile strength

The force required to open the healing action is known as tensile strength. It is used to measure the degree of healing. It also indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue (Kuwano *et al.,* 1994). Wounding strips of (varying width and length) healed tissues along with normal skin at both ends is excised out and tensile strength is measured with the help of a tensiometer (Tremayne and Srebrolow, 2007).

The total breaking load is measured in Newtons (N) and the breaking strength is calculated as mass in kg by the equation:

Tensile strength = Total breaking load ÷ Cross sectional area of the wound

1.3.3.5 Histopathological study

This tool is used to assess and monitor the reformation and healing of cells within wounds. In wound models thin sections of tissue specimens from the samples are stripped out from the healed wounds and fixed in solutions used to preserve tissues for routine histological studies. The tissue sections are then stained routinely with primary or specific stains and qualitatively observed under the microscope for histological changes (Bancroft and Gamble, 2008).

1.4 Plant under study

Entandrophragma angolense (Welwitsch) C.DC. belongs to a genus of eleven species of deciduous trees belonging to the mahogany family of Meliaceae, abundantly found in the rain forests, mangrove swamps to semi-deserts of the tropical regions. The geographical distribution of this plant cuts across Equatorial Guinea to Sudan, Uganda and Kenya, Democratic Republic of Congo and Angola (Muteeba, 2000). In West Africa *E. angolense* is most common in moist semi-deciduous forest, particularly in regions with an annual rainfall of 1600 to 1800 mm. However, it can also be found in evergreen forest, but its abundance strongly declines in regions with an annual rainfall of more than 2300 mm (Muteeba, 2000). In East Africa it occurs in lowland and mid-altitude rainforest, but sometimes also in gallery forest and thickets, up to 1800 m altitude.

It strongly prefers well-drained localities with good water-holding capacity. *E. angolense* trees are commonly planted at the roadsides and occasionally used as shades in banana, coffee and tea plantations (Aubreville, 1950). Some common trade names given to this plant include the Mountain Mahogany (English), Tiama (Cote d'Ivoire), Edinam (Ghana), Kilula (Congo) and Gedu Nohor (Nigeria).

1.4.1 Taxonomy

Kingdom: Plantae
Phyllum : Trachiophyta
Division : Magnoliophyta
Class : Magnoliopsida
Order : Sapendales
Family : Meliaceae
Genus : Entandrophragma
species : angolense

1.4.2 Morphology

E. angolense is a huge emergent tree with height of 50 to 60 m tall and a strong bole at 30 to 40 m often with blunt buttresses up to 6 m high which is slightly curved and elliptical in cross-section often extending into surface roots. The leaves are pinnate with 5 to 9 pairs of leaflets; each leaflet is 8 to 10 cm long with a flat petiole of 8 to 18 cm long, often slightly winged at base, and clustered at the ends of the branches (Muteeba, 2000).

The bark surface of this plant is pale greyish brown to orange-brown, smooth but becoming scaly with irregular scales up to 20 cm in diameter leaving a concave crown dome-shaped marked with large leaf scars and lenticels (Muteeba, 2000).

A

B

Figure 1.1: A: *E. angolense* tree; B: base of the bole of the plant of *E. angolense* . Source: http:// database.prota. org/search. hmtl

The flowers are yellowish and the petals are about 2 mm long. The fruit is a five valved capsule containing numerous winged seeds. The fruits have a pendulous cylindrical capsule of 3 to 5 cm, with brown to black coloration, and many small lenticels, dehiscing from the base with 5 woody valves. The seeds are 6 to 9.5 cm long and most of them are attached to the upper part of the central column along the large apical wing (Muteeba, 2000).

C

Figure 1.2: C: leaves, fruits and seeds *of E. angolense* plant. Source : http:// database.prota. org/search. hmtl

The heartwood is pale pinkish brown to pale reddish brown, slightly darkening upon exposure to deep reddish brown. The wood is medium-weight, with a density of 510–735 kg/m³ at 12% moisture content. It air dries slowly and is liable to warping and distortion. Most of the woods are not suitable as timber and thus used as firewood and for charcoal production (Oteng-Amoako, 2006).

1.4.3 General uses of *E. angolense*

The wood of this plant is highly valued and forms a great source in the carpentry work, building materials, veneer and plywood; it is also used in flooring, interior trim and panelling, stairs and ship building. Other by-products that are obtained from this plant include: dyes, stains, inks, tattoos, mordants, exudations (gums and resins) (Oteng-Amoako, 2006).

1.4.4 Medicinal uses of *E. angolense*

The aqueous decoctions of this plant is used in african folk medicine for the treatment of fevers and malaria and other gastrointestinal disorders including diarrhoea and peptic ulcers in humans predominantly in Cameroon and Ivory Coast (Irvine, 1961; Obih *et al.*, 1986; Bray *et al.*, 1990). It is also used in external applications as an anodyne against stomach and ear aches, rheumatic or arthritic pains as well as to treat eye and ear infections and swellings. Some other species belonging to this family such as *Azadirachta indica, Entandrophragma candollei, Entandrophragma utile* and *Khaya grandifoliola* are also widely used as antimalarials or antipyretics in traditional medicine (Irvine, 1961; Obih *et al.*, 1986; *Bray et al.*, 1990; Weenen *et al.*, 1990).

1.4.5 Biological activities of *E. angolense*

Research carried out on this plant revealed it as one of the most promising source of compounds with antiplasmodial properties. Limonoids isolated from the root bark methanol extract of *E. angolense* have been shown to exhibit moderate *in vitro* antimalarial activities against *Plasmodium falciparum* (Bickii *et al.*, 2007).

There has been a report on the antiulcer activities of the methanol stem bark extract of this plant (Njar *et al.*, 1994) as well as its effects on the gastrointestinal smooth muscle and transit time in mice have recently been reported (Orisadipe *et al.*, 2001). Structural studies of tetranortriterpenoids from the Congolese species of *E. angolense* against malaria, have also displayed considerable antifeedant activity against Spodotera insects (Okorie and Taylor, 1977).

1.4.6 Phytochemical constituents of *E. angolense*

Research carried out on the seeds this plant indicated a total fat content of 45 to 62% and high gross energy levels of 30.9 kJ/g. Seed oils gotten from three species of this genus contained up to 50% of their acids as 11-18:1, which is the greatest concentration of this acid ever found in nature. These species also are relatively abundant in 16:1 and 16: 2 (Hilditch, 1964). Two of the isolated compounds 7α-obacunylacetate and 24-methylenecycloartenol have exhibited pronounced activity against chloroquine resistant strains of *P. falciparum* (Bray *et al.*, 1990). Gedunin and β-sitosterol have been isolated from the bark and leaves of the plant. Entandrolide has also been isolated from the seeds (Akisanya *et al.*, 1960). The structural elucidation performed by spectroscopic methods: mass spectrometry, 1D and 2D NMR have isolated sixteen compounds and found five compounds to be new rings namely: methyl angolensante, methyl 6-acetoxyangolensante, secomahoganin, 3β-hydroxy-3-deoxycarapin and xyloccensin (Nkunya *et al.*, 1991; Bickii *et al.*, 2000).

1.5 General objective

The active principles of many drugs found in plants have exhibited antimicrobial and wound healing properties. A number of secondary metabolites or active compounds isolated from plants have been demonstrated in animal models as active principles responsible for direct effect on the wound healing processes due to their anti-inflammatory and antimicrobial properties (Cowan, 1999). Therefore this study is generally aimed at determining the antimicrobial and wound healing properties of the stem bark of *E. angolense*. This is to ascertain the folkloric uses of this plant as an agent used for the treatment of microbial infections and wounds.

1.5.1 Specific objectives

The specific objectives of this study are:

- To extract bioactive compounds in crude form from the stem bark of the *E. angolense* using methanol (70%).
- To determine the class of secondary metabolites present in the methanol extract.
- To determine the antimicrobial activity of the crude extract and the various solvent fractions against both typed and clinical isolates of bacteria and fungi
- To determine the minimum inhibitory and bacteriocidal concentration of the methanol extract.
- To determine the resistance modulatory effects of the methanol extract on the antimicrobial activity of some reference antibiotics against the test organisms.
- To determine the time kill kinetics of the methanol extract against the test organisms.
- To determine the antioxidant activity of the methanol extract.
- To determine the wound healing activity of the methanol extract

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Plant collection and preparation

The *E. angolense* plant was identified and stem bark collected from the forest at the bottom of mountain Ejuanoma near Nkawkaw in the Eastern region of Ghana. It was authenticated by Mr. G.H. Sam of the Department of Herbal Medicine, (FPPS, KNUST), where a voucher specimen, (number P'cog/He/0036), is deposited at the herbarium. The stem bark was cleaned of unwanted foreign materials, cut into small pieces and air dried between temperature of 33 °C to 40 °C on a blotter for 1 week. The dried plant material was then ground into coarse powder using a lab mill machine (Type 8, Christy and Norris Ltd. England). The powdered sample was kept in a brown paper bag and stored for further use.

2.2 Extraction process

An amount of 400 g of the powdered sample was extracted with 2.5 L of methanol 70% (GPR, BDH Ltd. Poole, UK) using the cold maceration method for 72h with constant stirring at intervals using a glass rod. At the end of extraction, the first supernatant was extracted by filtering using a cheese cloth and then followed by a filter paper (No.10 125mm Whatmann, England). The filtrate obtained was concentrated under vacuum on a rotary evaporator (Buch, Germany) at 40°C and finally evaporated to dryness using a hot air oven (Sanyo OMT Oven, Gallenkamp, UK) at 40°C. The weight and yield of the dried methanol extract was taken and recorded and then kept in an airtight sample bottle and stored at room temperature for further studies.

2.3 Test organisms

A total of six microorganisms were used for the antimicrobial, time kill and resistance modulation studies. Typed Gram-positive bacterial strains used included: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NCTC 10073, and *Enterococcus faecalis* ATCC 29212. Typed Gram-negative bacterial strains used included: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and a clinical isolate of fungus *Candida albicans*. However a clinical isolate of Gram positive *Streptococcus pyogenes* was also included and used only for the in vitro antimicrobial activity of the formulated cream in the wound healing assay (Table 3.10). All test organisms used were obtained from Kumasi Centre for Collaborative Research in Tropical medicine (KCCR) and Komfo Anokye Teaching Hospital (KATH).

2.3.1 Maintenance of test organisms

The bacterial cultures were maintained on Mueller-Hinton (MH) agar (Oxoid ltd. England) slants and stored at 4°C. The yeast was also maintained on the Sabouraud Dextrose Agar (SDA) (AR Merck, UK) slant and stored at 4°C. Cultures were reactivated before every test. Activation of the bacterial cultures was carried out by streaking culture from the slants on to a Muller Hinton (MH) agar plate and then incubating them overnight at 37°C. A single colony was then aseptically transferred from this plate into sterile nutrient broth (Licfi Chem, Italy) and incubated for 18-24 h at 37°C prior to the test. In the case of fungi, Sabouraud broth (SB) was used for the activation and incubated at 25°C for 48 h.

Confirmatory tests were carried out on the clinical isolates using selective media and biochemical tests. The results on the biochemical tests are recorded in appendix II.

2.4 Qualitative phytochemical tests

The crude extract was screened for the presence of various phytochemical constituents using modified procedures as described by Sofowara (1993), Trease and Evans (2002) and Harborne (1998).

2.4.1 Flavonoids

About 0.2 g of the crude extract was dissolved in 10 mL distilled water in a test tube. 5 mL of dilute ammonia solution (NH_3) (GPR, BDH Chemicals, Poole UK) was added to a portion of the aqueous filtrate followed by an addition of 1mL of concentrated sulphuric acid (H_2S0_4) (GPR, BDH Chemicals, Poole UK). The formation of an intense yellow colour which becomes colorless on addition of dilute acid indicates the presence of flavonoids (Sofowara, 1993).

2.4.2 Terpenoids

The crude extract (0.2 g) was dissolved in 10mL of methanol and 5mL was placed in a test tube. Next 2 mL of chloroform (AR, Merck, UK) was added to the solution. 3 mL of H_2S0_4 was then carefully added to form a layer. A reddish brown colouration formed at the interface gives a positive result for the presence of terpenoids (Odebiyi and Sofowora, 1978).

2.4.3 Tannins and Phlobatannins

About 0.2 g of the crude extract was stirred with 10 mL of distilled water in a test tube and then filtered. Four drops of ferric III chloride ($FeCl_3$) 1% (GPR, BDH Chemicals, Poole UK) was added to 2 mL of the filtrate, an occurrence of a blue- black or dark-green precipitate indicates the presence of tannins.

0.3 g of the extract was boiled with aqueous hydrochloric acid (HCl) 1% (GPR, BDH Chemicals, Poole UK). The deposition of a red precipitate at the bottom of the test tube was taken as evidence for the presence of phlobatannins (Trease and Evan, 2002).

2.4.4 Alkaloids

Crude extract (0.2 g) is dissolved in 5mL HCl on a water bath and then filtered. Of the filtrate, 1mL is taken into a test tube and 1mL of Mayer's reagent (potassium mercuric iodide) is added. The appearance of a turbid buff-coloured precipitate is an indicator for the presence of alkaloids (Sofowora, 1993).

2.4.5 Steroids

About 0.2 g of the crude extract was dissolved in 4mL of chloroform and filtered. 2 mL of acetic anhydride (CH_3CO) 2O) (GPR, BDH Chemicals, Poole UK) was added to the filtrate followed by 2 mL of H_2S0_4. A colour change from reddish violet to blue or green indicates the presence of steroids (Trease and Evans, 2002).

2.4.6 Anthraquinones

About 0.2 g of the crude extract was shaken with 10mL of benzene (GPR BDH Chemicals, Poole, UK) and filtered. 5mL of NH_3 10% was then added to the filtrate and shaken. The appearance of a pink or violet colour in the ammoniacal (lower) phase was taken as the presence of free anthraquinones (Sofowora, 1993).

2.4.7 Cardiac glycosides

To 0.5 g of the crude extract 1mL of glacial acetic acid (CH_3COOH) (AR, Merck, UK) was added followed by 1 to 2 drops of $FeCl_3$ 1% and then 1 mL of H_2S0_4. Appearance of a brown ring at the interface indicates presence of cardiac glycosides. A violet ring may also appear below the brown ring (Trease and Evans, 2002).

2.4.8 Saponins

An aqueous crude extract of 0.5 g was made and shaken vigorously for a stable froth. Frothing persistence of more than 5 minutes indicates presence of saponins. 3 drops of olive oil was then added to the frothing and shaken vigorously, then observed for the formation of an emulsion. Formation of emulsion also indicates the presence of saponins (Sofowora, 1993).

All phytochemical screenings were performed in triplicate.

2.5 Antimicrobial screening

The antimicrobial activity of the crude extract was determined using a modification of the agar diffusion method as described by Jussi-Pekka *et al.* (2002). Several concentrations of the crude extract (64, 32, 16, 8, 4 and 2 mg/mL) were prepared in methanol. 20 millilitres Mueller-Hinton agar was melted and stabilized at 45 °C for 15 minutes and innoculated with 0.1 mL of an 18 to 24 h broth culture of *S. aureus* containing approximately 10^5 cfu / mL. A cork borer No.5 with a diameter of 10 mm was used to bore 4 wells per plate (equidistant from each other in the plate). The wells were labelled and filled with 0.1mL of the above concentrations. The plates were allowed to stand for 1 h and then incubated at 37°C for 24 h, (Gallenkamp plus II cooled incubator, UK). The same procedure was repeated for *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa*.

Sabouraud dextrose agar was used for *C. albicans* and was incubated at 25°C for 48 h. Reference antibiotics, 0.01mg/mL of ciprofloxacin (Zhejiang Xin Ltd. China) and 1mg/mL of ketoconazole (Sigma-Aldrich, Co. llc, USA) were used as positive controls. Methanol was used as negative control. The formation of clear inhibition zones around the wells was regarded as positive results. The zone sizes were then measured and the results recorded. The screening was performed in triplicate.

2.6 Minimum Inhibitory Concentration (MIC) by Broth Dilution Method

The MIC of the crude extract was determined using the broth dilution method as described by (Akinpelu and Kolawale, 2004). Concentrations of 30, 20, 16, 8, 4, 2 and 1mg/mL were prepared in broth in 2mL quantities. A control test tube containing methanol and water (20:80) was also prepared. 0.1mL of 18 to 24 h of *S. aureus* broth culture containing 10^5cfu/mL was added to each tube of nutrient broth. The same procedure was repeated for *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa*. Sabouraud broth was used for the *C. albicans*.

Concentrations of the reference antibiotics ciprofloxacin (0.005 to 0.01mg/mL) and ketoconazole (0.00125 to 0.01mg/mL) were also prepared in broth. The test tubes were then incubated at 37°C for 24 h (bacteria) and 25°C for 48 h (fungi) and observed for growth. The lowest concentration of the crude extract which inhibited the visible growth of the organisms was recorded as the MIC value. Absence of growth was confirmed using 0.1mL of 1.25mg/mL methyl thiazolyl tetrazolium chloride (MTT) solution (Sigma-Aldrich, Co. llc, USA).

Bacterial growth inhibition was indicated as a yellow colour whilst the presence of a purple colour indicated bacterial growth. The tests were performed in triplicate.

2.7 Determination of minimum bactericidal and fungicidal concentration (MBC and MFC)

The MBC and MFC of the crude extract were determined using a modification of the method described by Aibinu *et al.* (2007). Test tubes that showed no visible growth in the previous test (section 2.6) after 24 to 48 h incubation were streaked onto Mueller-Hinton agar and incubated at 37°C for 24 h (bacteria) and Sabouraud agar at 25°C for 48 h (fungus). The MBC and MFC were determined as the lowest concentration that prevented bacterial and fungal growth respectively after incubation. Plain agar plates were also incubated serving as the control. All tests were performed in triplicates to ensure consistency.

2.8 Solvent fractionation of the crude extract

An amount of 10 g of the crude extract was sequentially extracted into non-polar, intermediate and polar fractions with 100 mL of petroleum ether (Scharlau Chemicals, Ltd. UK), 100 mL of ethyl acetate (BDH Chemicals, UK) and 150 mL of methanol/water (7:3). The fractions obtained were concentrated under vacuum on a rotary evaporator and dried at 40°C. The antimicrobial activities of the various solvent fractions were tested using the agar diffusion method as described in section 2.4.

2.9 Time kill kinetics

This was carried out using a modified method outlined by Adeniyi *et al*, (2000). Various concentrations of crude extract (MIC, 2×MIC, 3×MIC and 4×MIC) were prepared in 5mL nutrient broth. 0.1 mL of *S. aureus* containing approximately 10^5 cfu / mL was added to each test tube and kept at 37°C. Aliquots (0.1mL quantities) were withdrawn at intervals of 0, 1, 2, 3, 4, 5, 6, 12 and 24 h and inoculated into stabilized 20 millilitre plate count agar.

The plates were incubated inverted at 37°C for 24 h (bacteria) and 25°C for 48 h (fungi). Viable counts were determined by counting the colonies that formed using a colony counter (Gerber Instruments, L. Schneider &Co. AG Holland). The same procedure was repeated for *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa* and *C. albicans.* A test tube containing only broth and test organisms was used as the control. The procedure was performed in triplicate to ensure consistency. Data were analyzed using XML spread sheet. A graph of Log10 viable count per mL against time (h) was plotted to determine the time-kill kinetics.

2.10 Resistance modulatory activities

2.10.1 Minimum Inhibitory Concentration (MIC) of reference antibiotics

The MICs of the antibiotics were determined using the broth dilution method (Akinpelu and Kolawale, 2004). The 96-well microtitre plates were prepared by dispensing 50µL of the inoculated double strength nutrient broth plus a calculated volume aliquot of the antibiotics (ciprofloxacin, amoxicillin, tetracycline, erythromycin and ketoconazole) in the appropriately labelled wells. An adequate volume of sterile water and 10µL was added to each well to achieve a total volume of 100 µL.

Concentrations ranging from 1.25 to 10240 µg/mL were used for the antibiotics. The same procedure was repeated for *B. subtilis*, *Ent. faecalis*, *E. coli* and *P. aeruginosa*. Sabouraud broth was used for the *C. albicans*. A control was performed for the nutrient broth and the organisms. The plates were incubated for 24 h at 37°C (bacteria) and 25°C for 48 h (fungi). Microbial growth was determined after incubation period by the addition of 50 µL of MTT solution. The experiments were performed in triplicate.

2.10. 2 Method for antibiotics modulation by methanol extract

The modulation assay was performed by testing the activity of the reference antibiotics (ciprofloxacin, amoxicillin, tetracycline, erythromycin and ketoconazole) in the presence of sub-inhibitory concentrations of the crude extract using the method of Oluwatuyi *et al.* (2004). Serial dilutions (1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120 and 10240 µg/mL) of each of the antibiotics were prepared using sub-inhibitory concentrations (5000 and 10000 µg/mL) of the crude extract as vehicle. The 96-well plates were prepared by dispensing 50µL of the nutrient broth into each well.

A calculated volume of the various antibiotic solutions were added to the appropriate labelled wells followed by 10 µL of *S. aureus* broth culture containing 10^5cells.

Enough vehicle was added when necessary to achieve a final volume of 100 µL. The same procedure was repeated for *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa*. Sabouraud broth was used for the *C. albicans.*

A control was performed for the nutrient broth. The plates were incubated at 37°C for 24 h (bacteria) and 25°C for 48 h (fungus). After the incubation period, 20µL of MTT solution was added to each well. Inhibition of bacterial growth was visible as a yellow colouration while presence of growth was detected by the appearance of a purple colour in the well. The experiments were performed in triplicate.

2.11 Antioxidant activity (free radical scavenging activity)

The free radical scavenging activity of the extract was determined by a modified method described by Braca *et al*, (2001). Different concentrations of 100, 50, 25 and 12.5 µg/mL of the crude extract and 30, 10, 3 and 1 µg/mL of the reference agent N-propyl gallate (Sigma- Aldrich, USA) were used for the test. 0.002% w/v of DPPH (Sigma-Aldrich Damstadt, Germany) solution was prepared in methanol. 1 mL each of the different concentrations of the crude extract and the reference agent was added to 3 mL of the prepared DPPH solution. All mixtures were shaken and kept in a dark chamber for 30 minutes and change in colour from purple to yellow noted. The absorbance of different concentrations of the extract were measured at 517 nm using a UV-VIS-1 spectrophotometer (Cecil, 2000 series).

Standardization was carried out by running absorbance of methanol as a blank. The absorbance of the DPPH + methanol solution (4mL) as control (A_{DPPH}) was also determined.

The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Percent inhibition} = \frac{(A_{DPPH} - A_{sample})}{A_{DPPH}} \times 100$$

Where A_{DPPH} = the absorbance of DPPH solution used as control

A_{sample} = the absorbance of the extract/reference antioxidant.

The results were analysed using Graph Pad Prism Version 5.0 for Windows (Graph Pad Software Inc, San Diego, CA, USA) statistical package programme. The readings for all the concentrations and control were performed in triplicate.

2.12 Determination of wound healing activity of extract creams of *E. angolense*

2.12.1 Ethical clearance for animal work

The study was conducted upon approval by the Animal Ethical Committee, Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana and in accordance with International guidelines for the Care and Use of Laboratory Animals (Smith *et al.*, 2007).

2.12.2 Animals

The wound healing activity was done using the excision wound model in rats. The experiments were carried out with 30 healthy Sprague Dawley rats weighing between 150 to 200g from the animal house, Department of Pharmacology, KNUST, Ghana. The animals were housed in metal cages, kept at standard environmental conditions of temperature (31±1°C) with 12:12 light/dark cycle and humidity (60± 0.2%) and fed with a standard pellet diet (AGFCO, Tema) and given water (*ad libitum*).

2.12.3 Formulation of crude extract creams

Different concentrations (1%, 2%, 4%, 8%, and 16% w/w) of aqueous cream of methanol extract of *E. angolense* were prepared. Silver sulphadiazine 1% w/w (Ayrton Drugs, Accra, Ghana) was used as the reference wound healing agent and the aqueous cream as the vehicle. The preparation of aqueous cream is recorded in appendix VI. The antimicrobial activities of the various creams and reference agent were tested using the agar diffusion method as described in section 2.4.

2.12.4 Excision wound model

Excision wounds were subsequently inflicted as described by Morton and Malone, (1972). The dorsal fur of the rats was shaved using razor blades; the shaved area was sterilized with 70% ethanol (GPR BDH Chemicals Poole, UK) and then anaesthetized intraperitoneally with 500 mg/kg body weight ketamine hydrochloride injection (Laboratorio Saunderson, Santiago, Chile). Wounds margins were made using a sterile round seal from a predetermined area on the back of each animal and excision wound area of 314.2 mm^2 was created with a pair of sharp scissors. Prior to treatment, the wounds of all experimental animals were left untreated for 24 h post wounding.

2.12.5 Experimental design

The rats were divided into 6 groups to give 5 rats per group

Group 1: untreated (negative control).

Group 2: treated with silver sulphadiazine 1% w/w (positive control).

Group 3: treated with aqueous cream (vehicle).

Groups 4, 5 and 6 were treated with 1, 4 and 16% w/w of methanol extract creams respectively.

The extract creams (1, 4 and 16%) w/w was chosen for wound healing. The creams were administered topically once a day for 21 days and for those not completely healed after the study treatment continued till wounds were healed.

2.12.6 Assessment parameters for wound healing

2.12.6.1 Wound size

The changes in wound size were measured in mm at 3 day intervals for 15 days using a millimetre rule. Graph Pad Prism Version 5.0 for Windows (Graph Pad Software Inc, San Diego, CA, USA) statistical package programme was used for all statistical analyses. Values were expressed as mean ± SEM (n=5) and analyzed using the two way analysis of variance (ANOVA), followed by Bonferroni post test. $P < 0.05$ was considered statistically significant in all analyses. The percentage of wound size was calculated, as a percentage of the corresponding original wound size on day 1 post treatment (Saha *et al.*, 1997). Scaled photographs of wound areas were taken with the aid of a high resolution digital camera (Cannon, PC 1355, Cannon Inc. China) during the course of treatment.

2.12.6.2 Histopathological studies of wounds

Whole wound scars were collected on the 16th day of treatment from wound area and surrounding margins from 2 rats per group and immediately fixed in Bouin's solution for histological assessment. The fixed tissues were dehydrated through increasing grades of ethanol and embedded in paraffin wax. The tissues were then cut to 5 µm sections with a rotary microtome, deparaffinised, mounted on clean glass slides and stained with haematoxylin and eosin (H&E). The glass slides were then observed under the microscope for histopathological changes. The sections were observed for the degree of cell repair; granulation tissue replacement and re-epithelisation (Chung, 2010).

CHAPTER THREE

3.0 RESULTS

The extraction of the *E. angolense* stem bark using methanol 70 % gave a percentage yield of 10.7 % w/w (weight of methanol extract = 42.9 g; weight of dried plant material= 400 g).

3.1 Phytochemical screening

The phytochemical screening of the methanol extract showed the presence of the flavonoids, saponins, alkaloids, tannins, cardiac glycosides and terpenoids. Steriods and anthraquinones were absent. Observations and inferences made in the phytochemical tests are summarised in table 3.1.

Table 3.1 Phytochemical screening of the *E angolense* methanol extract

Chemical constituents	Observation	Inference
Flavonoids	Yellow colouration	+
Terpenoids	Red ppt. at the interface of test tube	+
Tannins and Phlobatannins	Darkish green colour/ red ppt. at the bottom of test tube	+
Alkaloids	Turbid buff- colour	+
Steroids	Absence of steroidal ring and no change in colour	-
Anthraquinones	Absence of a pink/violet colour	-
Cardiac glycosides	Violet ring formed at the interface of test tube	+
Saponins	Frothing and formation of emulsion	+

+ = present; - = absent; ppt. = precipitate

3.2 Antimicrobial activity of the methanol extract

The methanol extract exhibited a broad spectrum of activity against the test bacteria as well as antifungal activity (table 3.2). The methanol stem bark extract exhibited a broad spectrum of activity against the test organisms (*S. aureus*, *B. Subtilis*, *E. faecalis*, *E. coli*, *P. aeruginosa* and *C. albicans*) using the agar diffusion method as shown in tables (4.1 and 4.1.1).

Gram-positive bacteria were found to be more susceptible to the methanol extract than the Gram-negative bacteria. The extract exerted the highest zone of inhibition against *E. faecalis* which was followed by *S. aureus, and B. subtilis*. *E. coli* had a low zone of inhibition and the least activity was exhibited against *P. aeruginosa.* A moderate zone of inhibition was exerted by *C. albicans*.

Table 3.2 Antimicrobial activity of methanol extract against test organisms

oncentration (mg/mL)	Mean zones of inhibition (mm)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
64	23.33± 0.33	19.00±0.33	24.33±0.33	18.67±0.33	18.00±0.58	20.33 ±0.33
32	21.00±0.58	16.33±0.33	21.67±0.88	16.00±0.33	16.00±0.58	18.00±0.58
16	18.00±0.58	15.33±0.33	18.67±0.33	14.67±0.33	14.25±0.25	15.75±0.25
8	15.33±0.33	13.33±0.33	16.67±0.33	13.33±0.33	12.50±0.50	14.50±0.50
4	13.33± 0.33	12.33±0.33	13.67±0.33	11.67±0.33	0.00	12.75±0.25
2	12.00± 0.00	11.33±0.33	11.67±0.33	0.00	0.00	0.00

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.

S.A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

The solvent fractions of the methanol extract exhibited varying antimicrobial activities as shown in tables 3.3, 3.4 and 3.5 below. The activity of the reference antibiotics against the test organisms is recorded in table 3.6. Pictures showing zones of inhibition of aqueous fractions against some test organisms is shown in figure 3.1 below.

Table 3.3 Antimicrobial activity of pet ether fraction against test organisms

Concentration (mg/mL)	Mean zones of inhibition(mm)	
	S. A	**C. A**
100	17.67±0.33	16.00±0.33
25	15.00±0.58	14.33±0.33

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.

S. A: *Staphylococcus aureus*; C.A: *Candida albicans*.

Pet. ether fraction showed no antimicrobial activity against *B. subtilis*, *E. faecalis*, *E. coli* and *P. aeruginosa*.

Table 3.4 Antimicrobial activity of ethyl acetate fraction against test organisms

oncentration (mg/mL)	Mean zones of inhibition(mm)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
100	16.67±0.88	16.67±0.33	19.67±0.88	18.33±0.33	16.00±0.58	17.00±0.58
50	15.33±0.68	14.67±0.33	18.33±0.33	17.33±0.33	15.67±0.33	16.33±0.33
25	14.67±0.33	12.67±0.33	17.00±0.58	15.67±0.33	14.33±0.33	15.33±0.33
12.5	13.33±0.33	0.00	15.33±0.33	13.00±0.58	12.00±0.58	0.00
6.25	11.33±0.33	0.00	13.33±0.33	0.00	0.00	0.00
3.125	0.00	0.00	12.00±0.58	0.00	0.00	0.00

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.

Table 3.5 Antimicrobial activity of aqueous fraction against test organisms

Concentration (mg/mL)	Mean zones of inhibition(mm)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
100	22.18±0.73	19.67±0.73	23.83±0.92	18.33±0.93	17.50±0.76	19.17±0.60
50	20.33±0.33	18.33±0.33	21.33±0.73	17.50±0.29	17.50±0.50	18.33±0.33
25	19.18±0.60	17.33±0.60	19.83±0.18	17.50±0.50	15.33±0.33	16.33±0.33
12.5	16.50±0.50	13.50±0.29	18.50±0.29	15.50±0.29	13.33±0.33	15.17±0.44
6.25	15.33±0.88	13.33±0.33	15.50±0.87	11.71±0.44	11.71±0.71	13.33±0.33
3.125	13.33±0.33	11.17±0.44	13.18±0.44	0.00	0.00	11.17±0.44

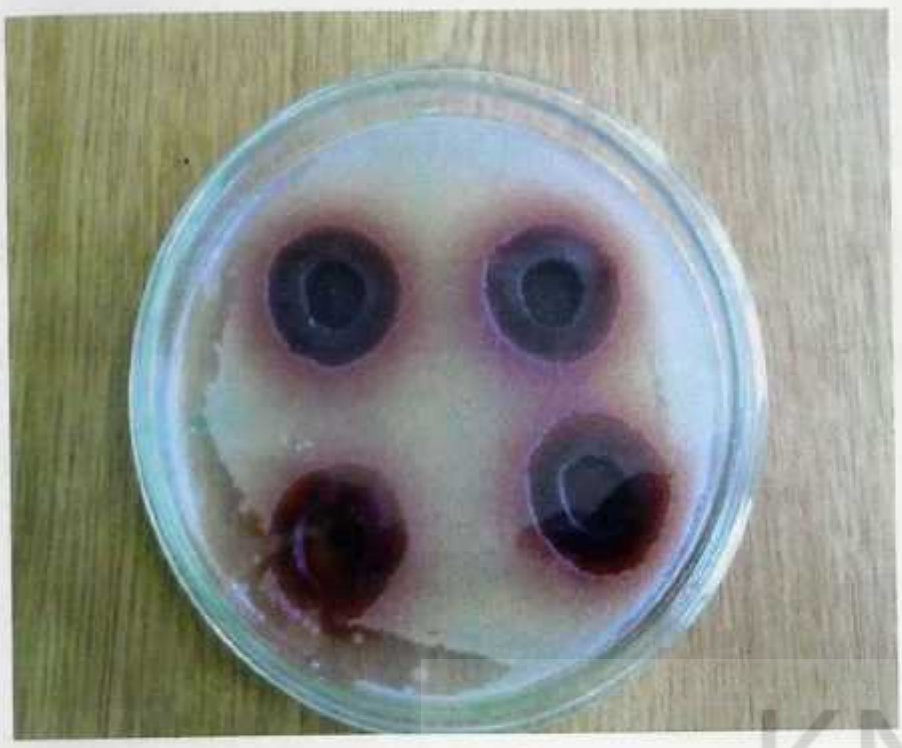
Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.

Table 3.6 Antimicrobial activity of reference antibiotics against test organisms

Concentration (mg/mL)	Mean zones of inhibition(mm)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
Ciprofloxacin (0.01)	28.67±0.58	21.67±0.88	23.67±0.58	34.00±0.58	29.68±0.88	nd
Ketoconazole (1)	nd	Nd	nd	nd	nd	28.33±0.33

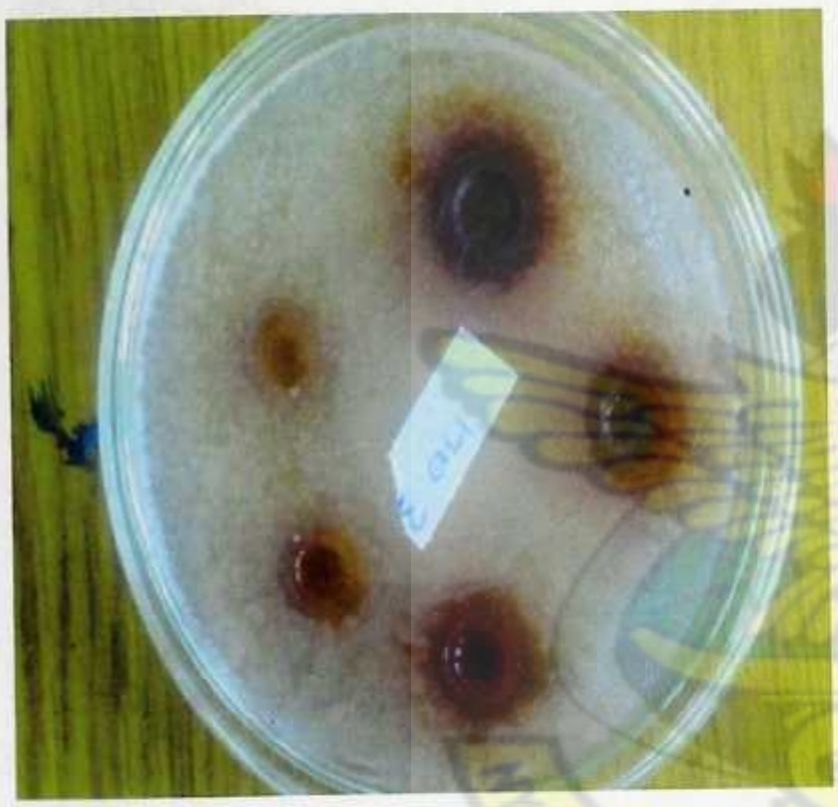
Diameter of well = 10 mm; nd = not determined; values for the zone of inhibition are the mean of three independent determination.

S.A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; E.F: *Enterococcus faecalis*; E.C: *Escherichia coli;* P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.



A

B



C

D

Figure 3.1: Antimicrobial activities of aqueous fractions (100, 50, 25, 12.5 and 6.25 mg/mL) of crude extract using the agar diffusion method of against A: *Enterococcus faecalis*; B: *Staphylococcus aureus*; C: *Escherichia coli*; D: *Candida albicans*.

3.3 Minimum Inhibitory Concentration (MIC) of methanol extract

The MIC exhibited by the methanol extract and reference antibiotics against the test organisms using the broth dilution method are recorded in table 3.7. No Minimum bactericidal or fungicidal activity could be established even at concentrations as high as (150mg/mL) indicating its bacteriostatic activity.

Table 3.7 MIC of the methanol extract and reference antibiotics against test organisms

Antimicrobial agent	Minimum inhibitory concentration (mg/mL)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
Methanol extract	11	13	9	17	19	15
Ciprofloxacin	0.04	0.02	0.03	0.0025	0.08	nd
Ketoconazole	nd	Nd	nd	nd	nd	0.0025

nd= Not determined

3.4 Time kill kinetic studies of methanol extract

Graphs showing the effects of the methanol extract against the test organisms are recorded in figure 3.2 to 3.7 below. The extract showed growth inhibition of the test organisms at all concentrations as compared to the control. The control showed the distinct growth curve patterns for all the test organisms. Tables on the time kill studies are recorded in Appendix III.

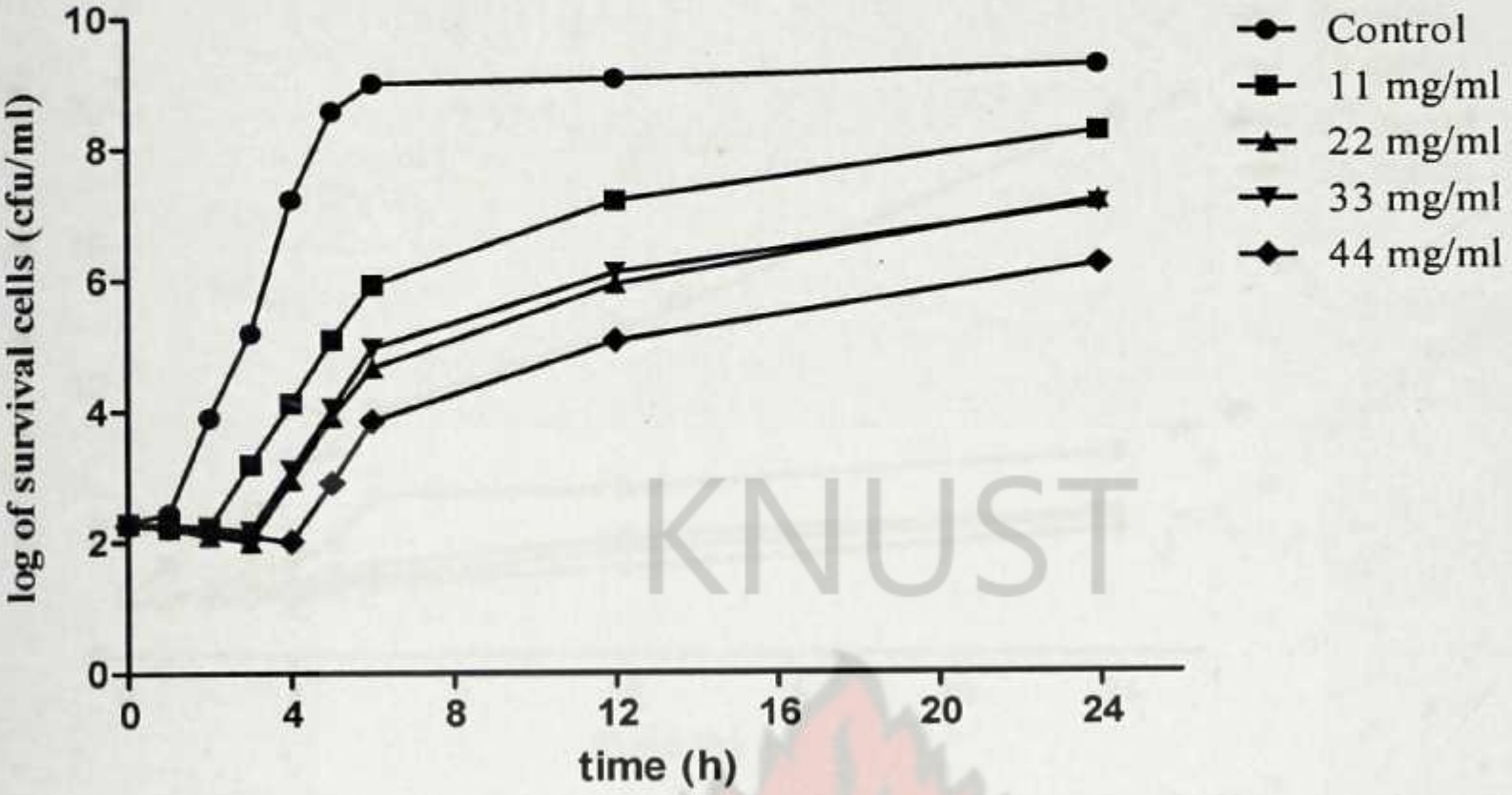


Figure 3.2: Time kills kinetics of methanol extract against *S. aureus*

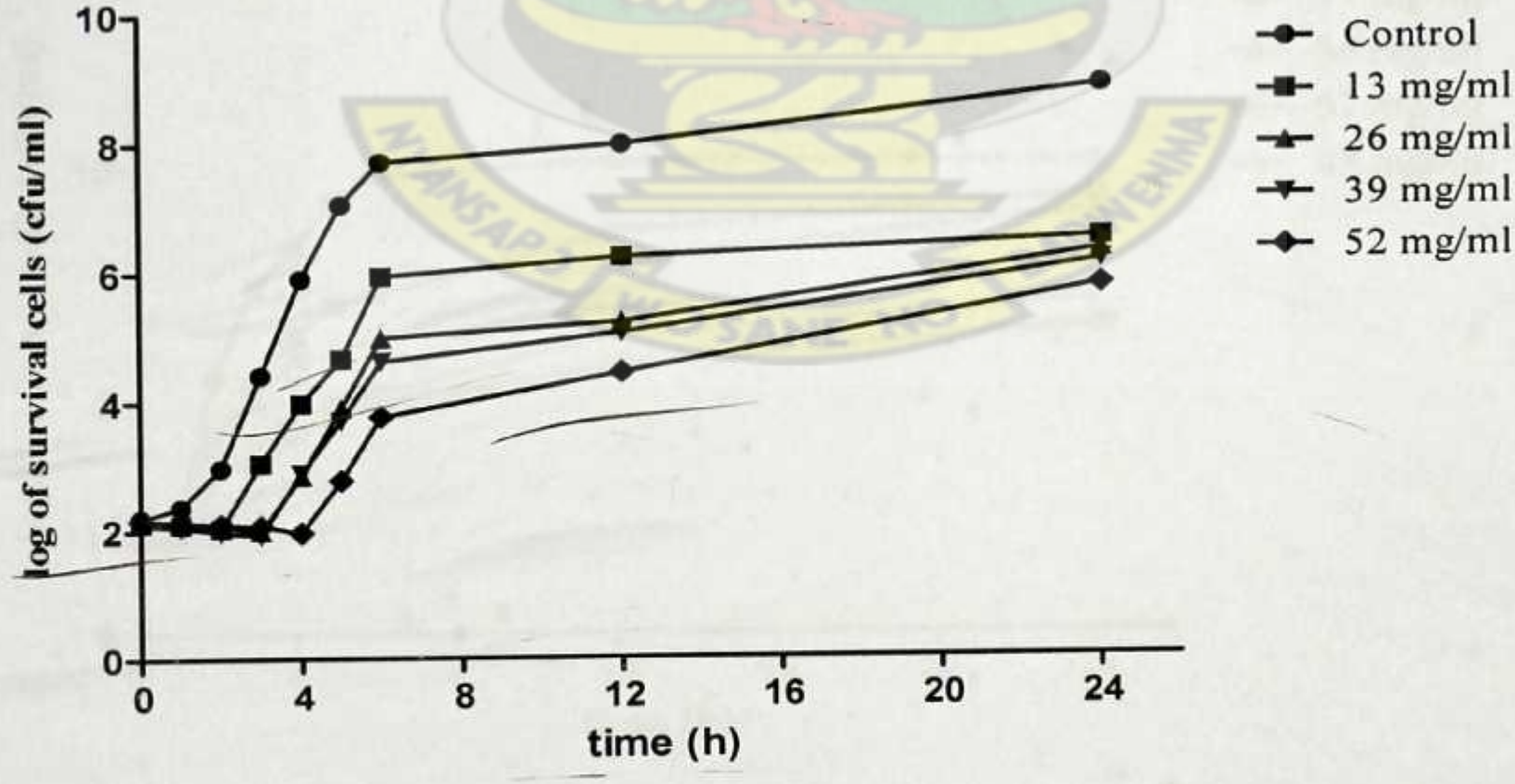


Figure 3.3: Time kill kinetics of methanol extract against *B. subtilis*

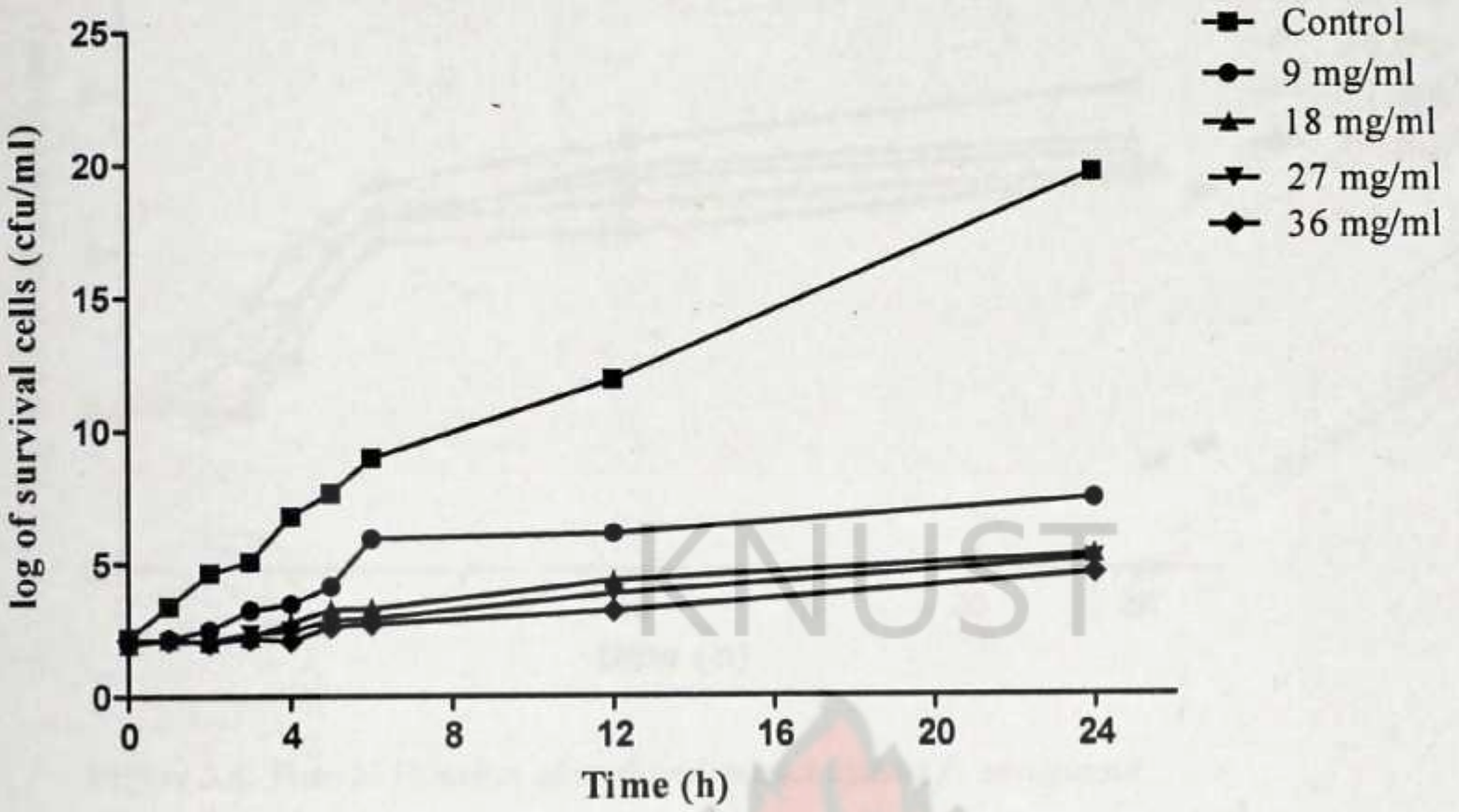


Figure 3.4: Time kill kinetics of methanol extract against *E. faecalis*

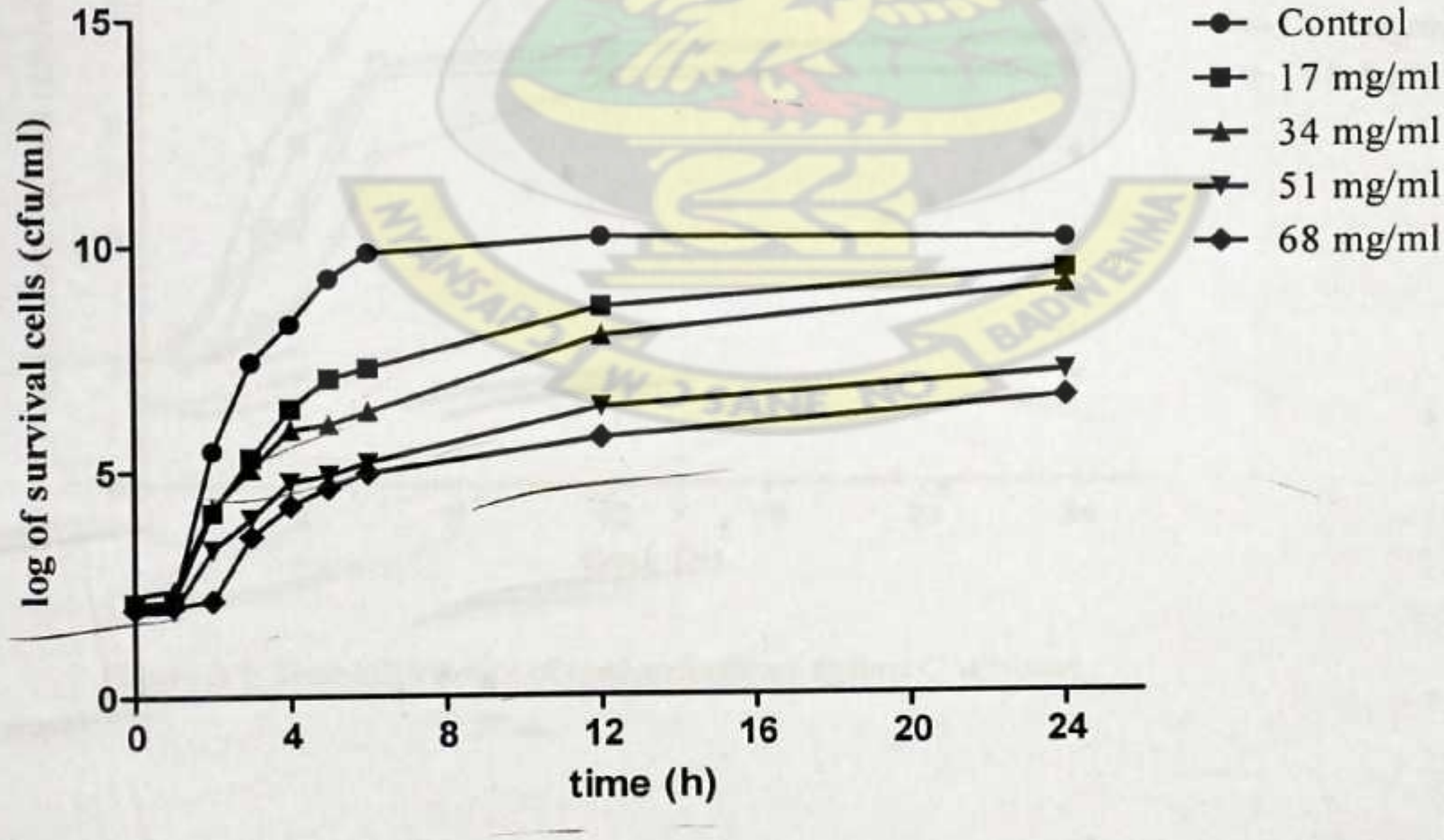


Figure 3.5: Time kill kinetics of methanol extract against *E. coli*

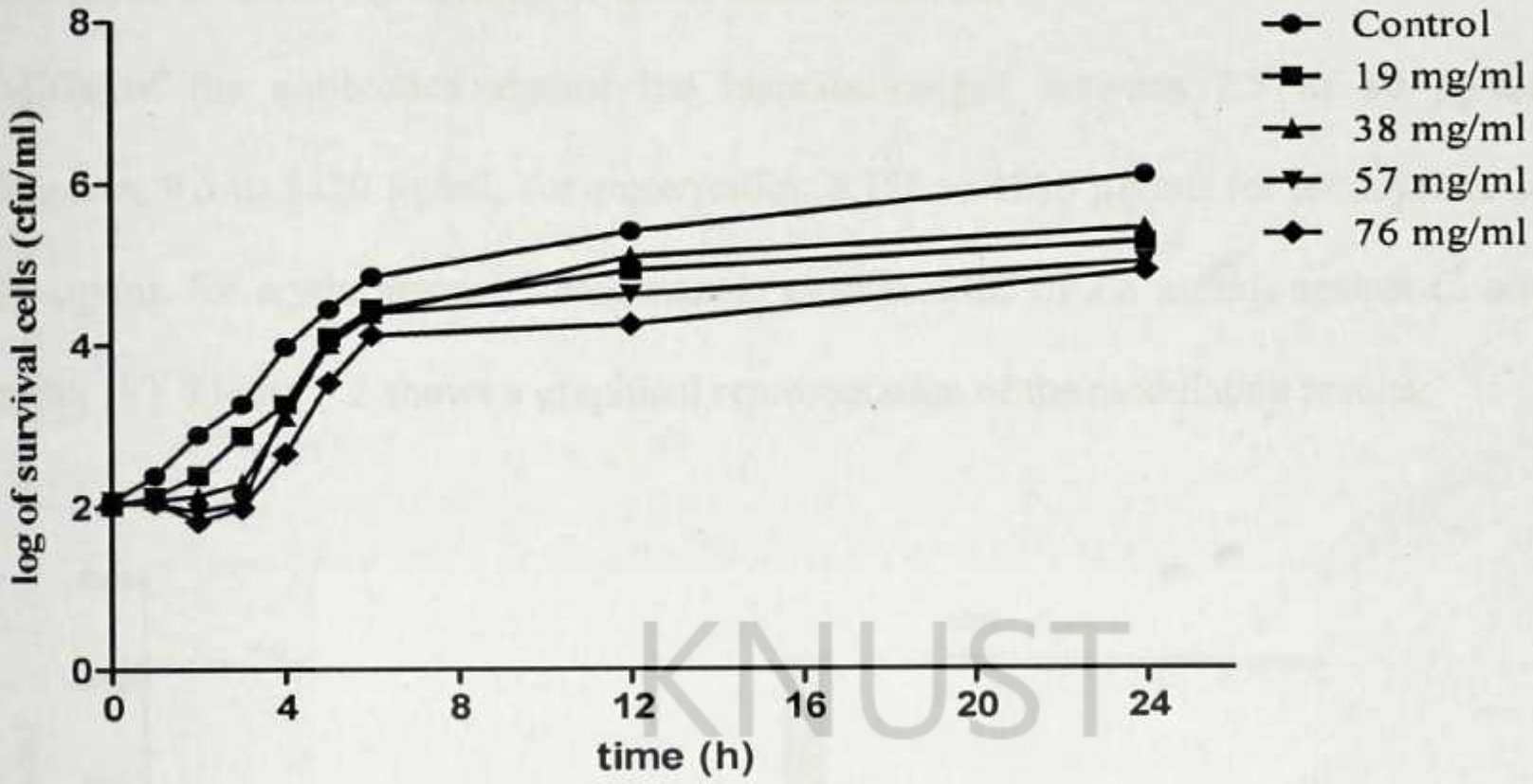


Figure 3.6: Time kill kinetics of methanol extract against *P. aeruginosa*

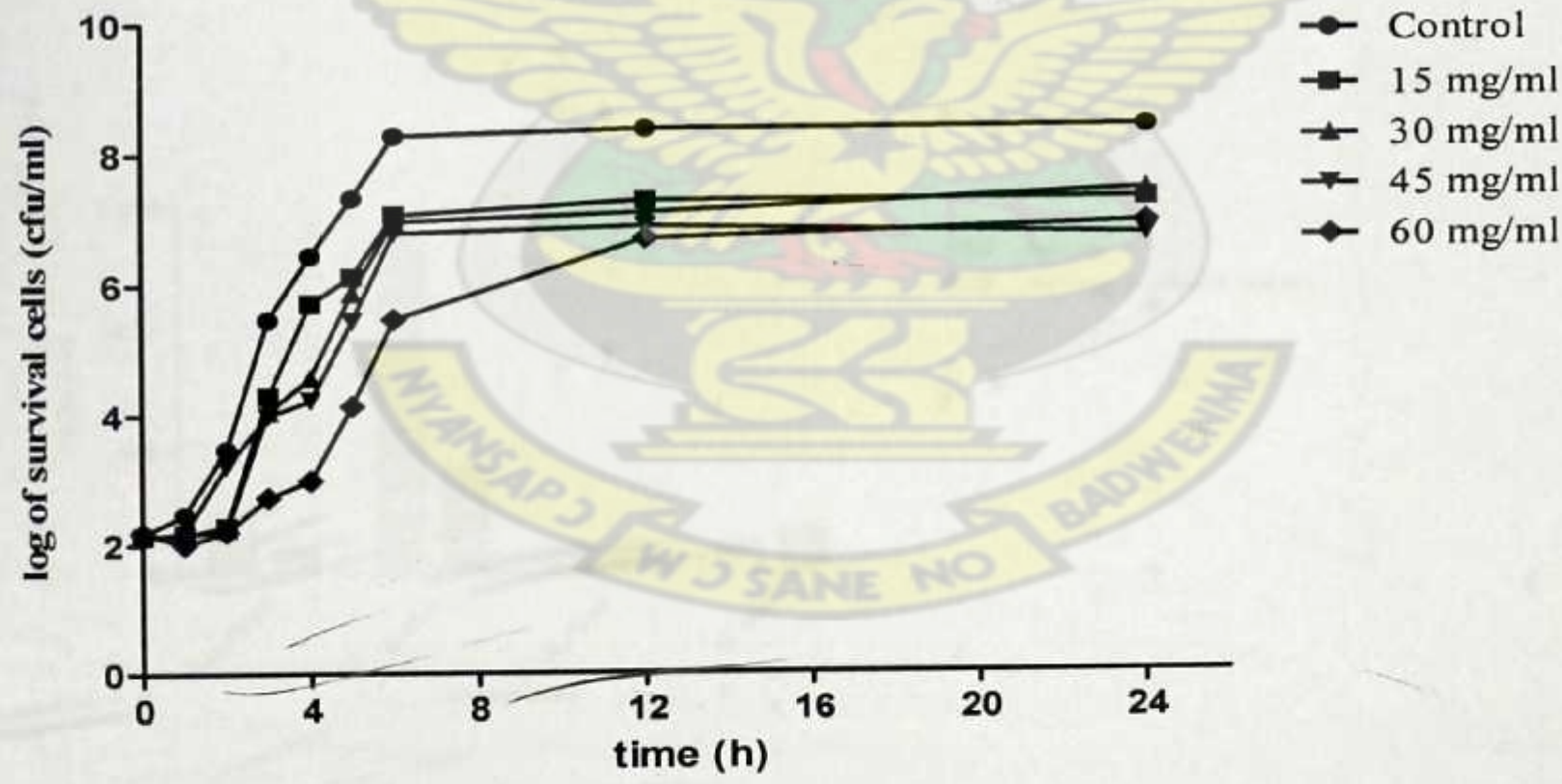
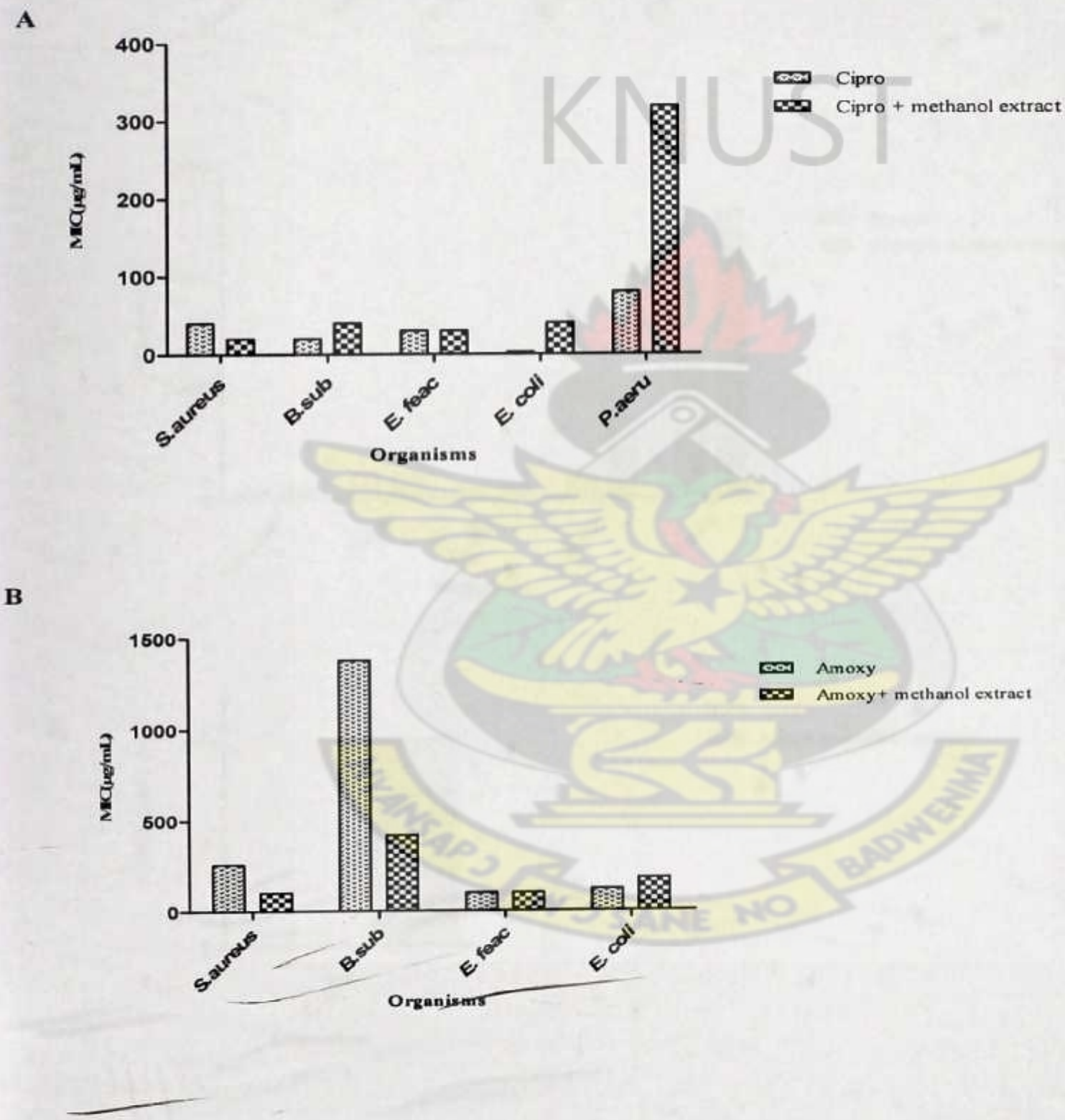


Figure 3.7: Time kill kinetics of methanol extract against *C. albicans*

3.5 Resistance modulatory activity of the methanol extract

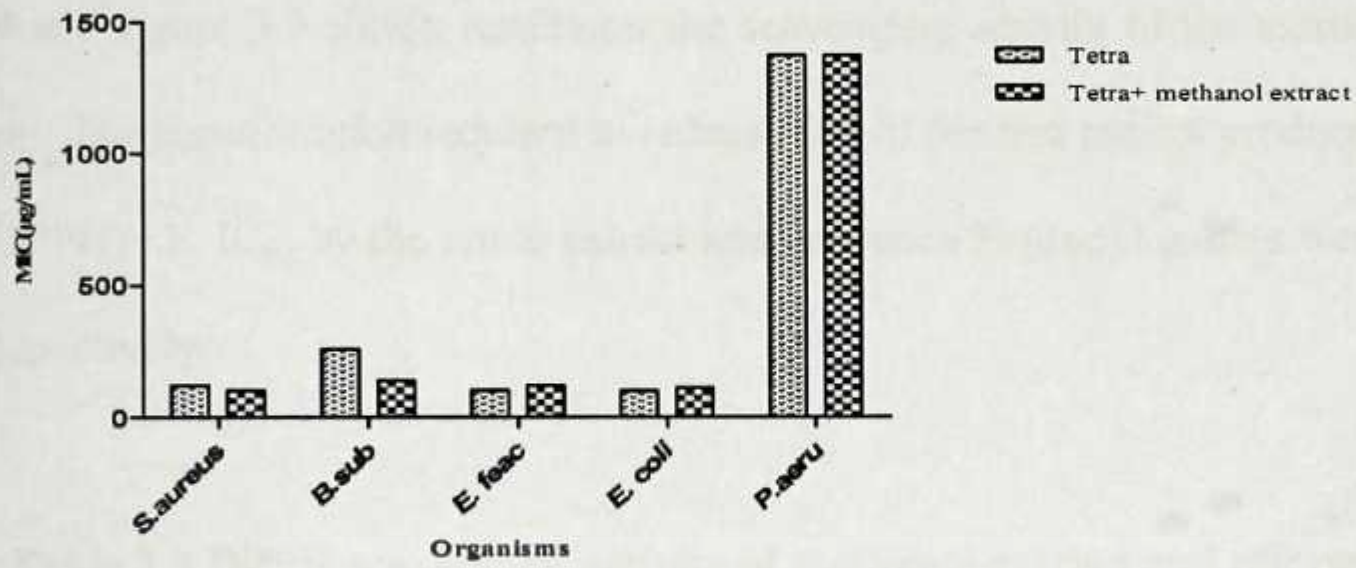
The MICs of the antibiotics against the bacteria ranged between 2.5 to 80 µg/mL for ciprofloxacin, 0.5 to 5120 µg/mL for amoxycillin, 0.156 to 1280 µg/mL for tetracycline and 20 to 2560 µg/mL for erythromycin. Ketoconazole gave an MIC of 2.5 µg/mL against *C. albicans* (Appendix IV). Figure 3.2 shows a graphical representation of the modulation results.



There was antimicrobial activity of amoxycillin against *Pseudomonas aeruginosa*

Figure 3.8: MICs of reference antibiotics and testing of the reference antibiotics in the presence of sub-inhibitory concentrations of the crude extract. A: Ciprofloxacin; B: Amoxycillin. Analyses and graphs were expressed using Graph Pad Prism Version 5.0 statistical package programme.

C



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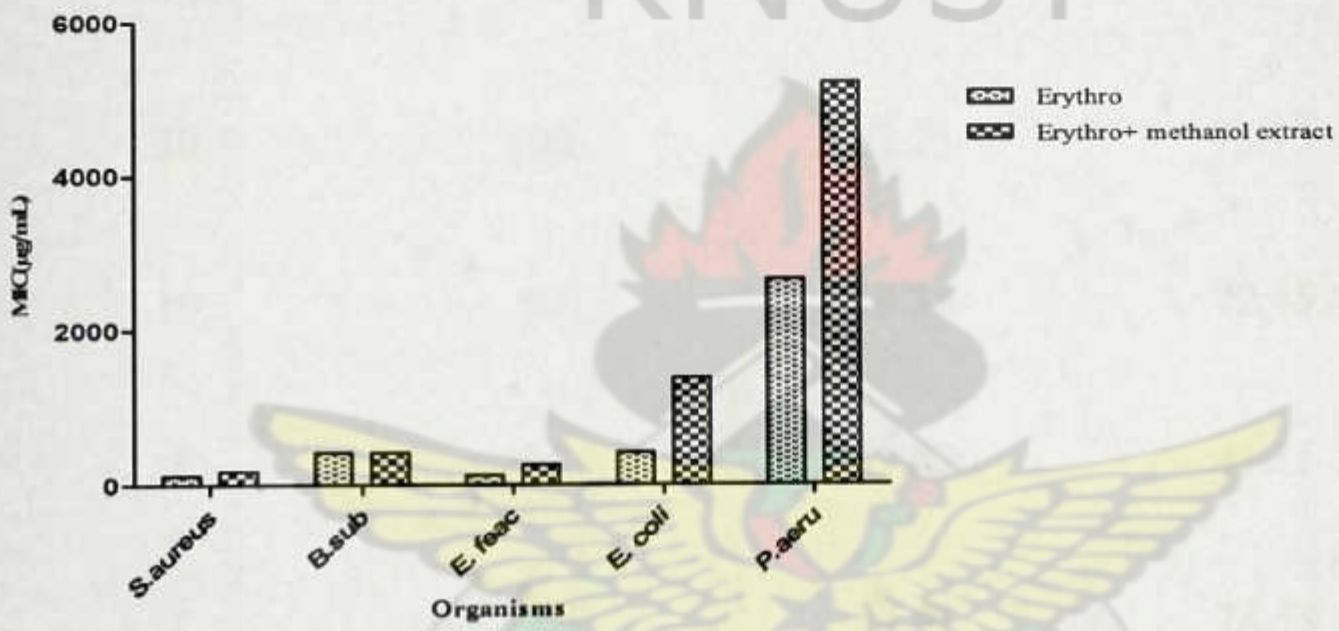

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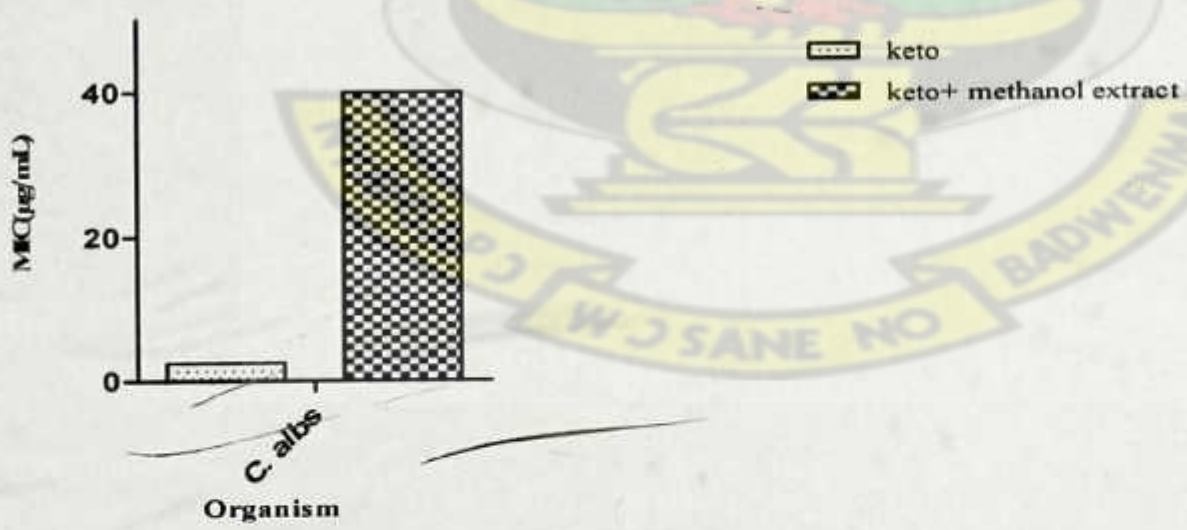

Figure 3.9: MICs of reference antibiotics and testing of the reference antibiotics in the presence of sub-inhibitory concentrations of the crude extract. C: Tetracycline; D: Erythromycin; E: Ketoconazole. Analyses and graphs were expressed using Graph Pad Prism Version 5.0 statistical package programme.

3.6 Antioxidant activity

Table 3.9 and figure 3.9 shows results on the scavenging activity of the extract and reference antioxidant. The concentration required to reduce 50% of the free radical produced by the radical system (DPPH) i.e. IC_{50} by the crude extract and reference N-propyl gallate were 18.1 and 3.01 µg/mL respectively.

Table 3.8 DPPH scavenging activity of methanol extract and reference (NPG)

Concentration (µg/mL)		Mean Scavenging activity (%)	
NPG	ME	NPG	ME
30	100	62.73	75.13
10	50	49.35	72.45
3	25	41.82	41.11
1	12.5	24.91	28.58

NPG = N- propyl gallate; ME = Methanol extract

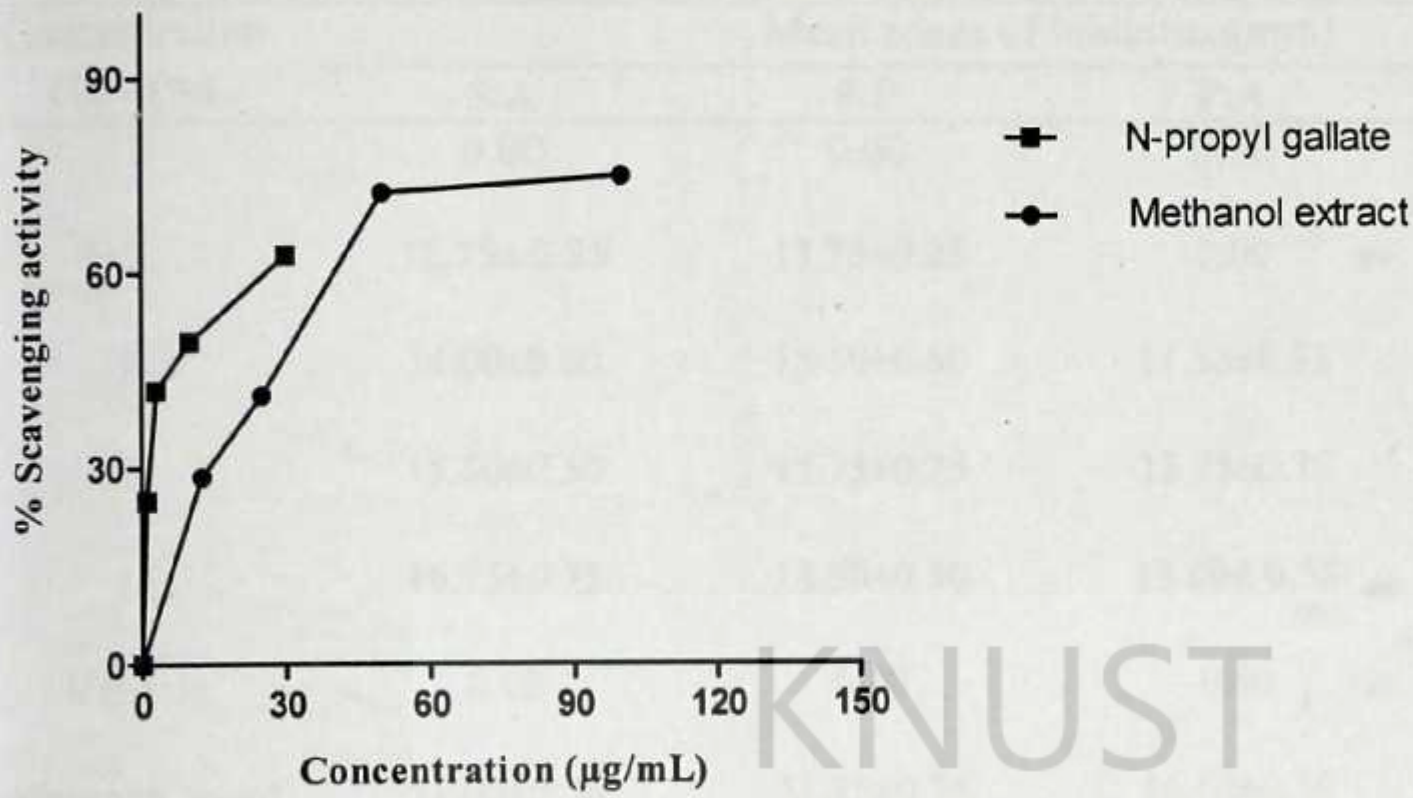

Figure 3.10: Free scavenging activity of methanol extract and N-propyl gallate

3.7 Wound healing activity of the crude extract creams

Results obtained from the treatment of excision wounds of the rats showed that all treatment groups showed better decrease in wound sizes during the 15 days of the wound healing process as compared to the untreated group. Graphical interpretation of wound diameter and wound contraction was used to illustrate the wound healing process of the study. The antimicrobial activity of the extract creams, vehicle and reference drug are recorded in table 3.10. Graphs on results of the wound diameter and contraction is summarised in figure 3.10 and 3.11. Tables on the wound diameter and contraction results are recorded in appendix VI.

Table 3.9 Antimicrobial activity of formulated creams against some test organisms

Concentration (% w/w)	Mean zones of inhibition(mm)			
	S.A	**S.P**	**P.A**	**C.A**
1	0.00	0.00	0.00	0.00
2	12.75± 0.25	13.75±0.25	0.00	0.00
4	14.00±0.00	15.50±0.50	11.33±0.33	12.33±0.00
8	15.50±0.50	15.75±0.25	12.75±0.75	14.33±0.33
16	16.75±0.75	18.50±0.50	13.00± 0.58	15.33±0.33
Vehicle	0.00	0.00	0.00	0.00
reference agent	21.00±0.50	21.25±0.75	16.50±0.50	17.75±0.75

Diameter of well = 10 mm; values for the zone of inhibition are the mean of three independent determination.

S. A: *Staphylococcus aureus*; S. P: *Streptococcus pyogenes*; P. A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

3.7.1 Wound diameter

Results obtained from the treatment of excision wounds of the rats showed that all treatment groups showed decreased wound sizes during the 15 days of the wound healing process. The untreated showed a distinct normal wound healing in absence of any medicaments (figure 3.10).

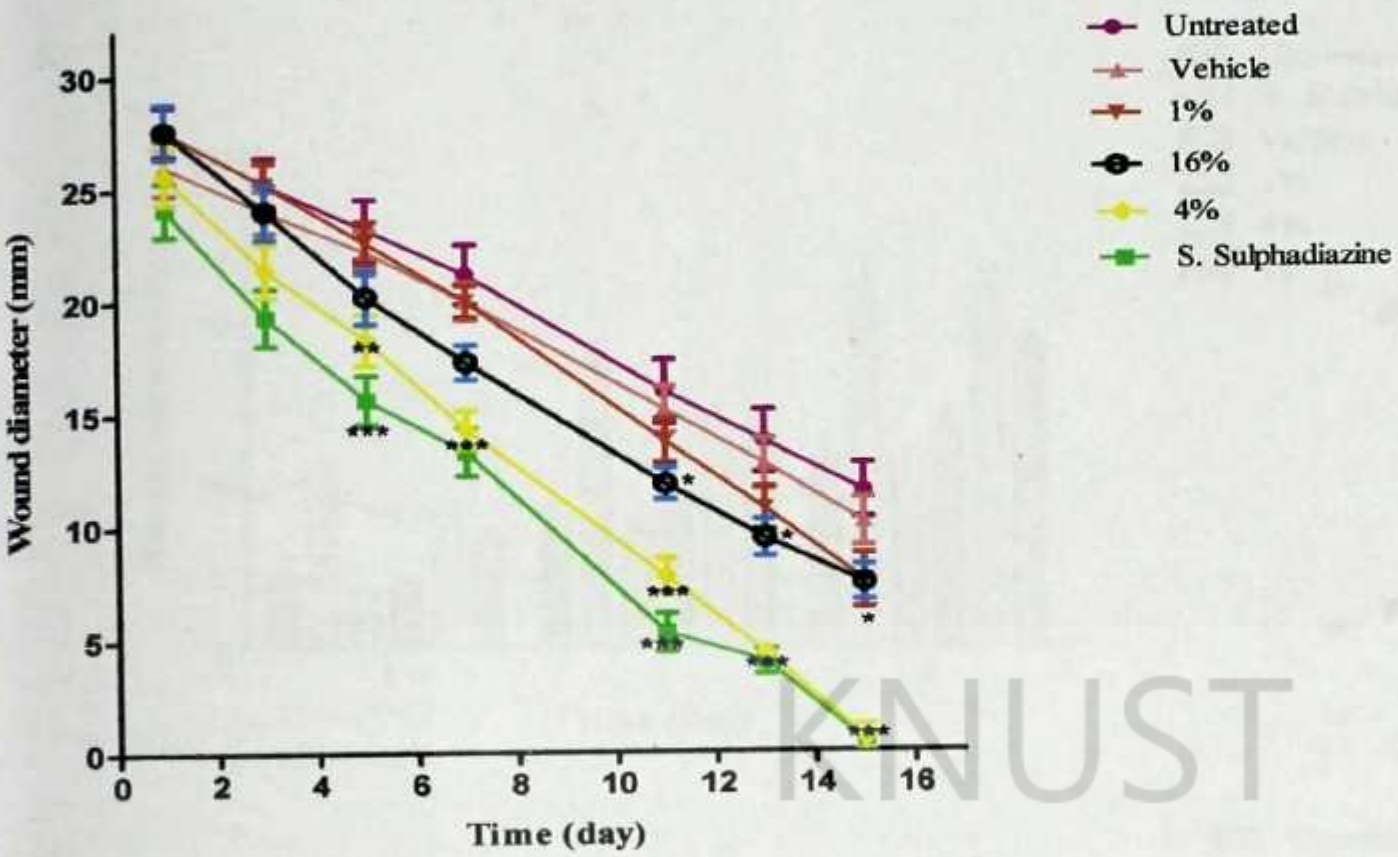

Figure 3.11: Effect of crude extract creams of *E. angolense* plant on wound diameter. Values are expressed as mean ± S.E.M. (n=5) for days 1, 3, 5, 7, 11, 13 and 15. Significance was determined using two way analysis of variance (ANOVA) followed by Bonferroni's post-tests with p values of (*p<0.05, **p< 0.01, ***p<0.001). Analyses and graphs are expressed as treatment groups' compared to the untreated group.

3.7.2 Wound contraction

Figure 3.11 was used to show the percentage wound contraction on the 15 days of treatment. All treatment groups exhibited varying wound contractions in the wound healing process (figure 3.11). The 4% w/w cream and reference drug was found to exert an increased wound contraction as compared to the other treatment groups during the 15 days of study.

The rate of wound contraction for day 5, 7, 11, 13 and 15 were (28.52, 44.14, 69.53, 82. 81 and 97.66 %) was 4% extract cream, (35.2, 44.81, 78.01, 83.40 and 97.66 %) for the reference agent and (15.94, 23.19, 42.03, 50 and 57.97 %) for the untreated group.

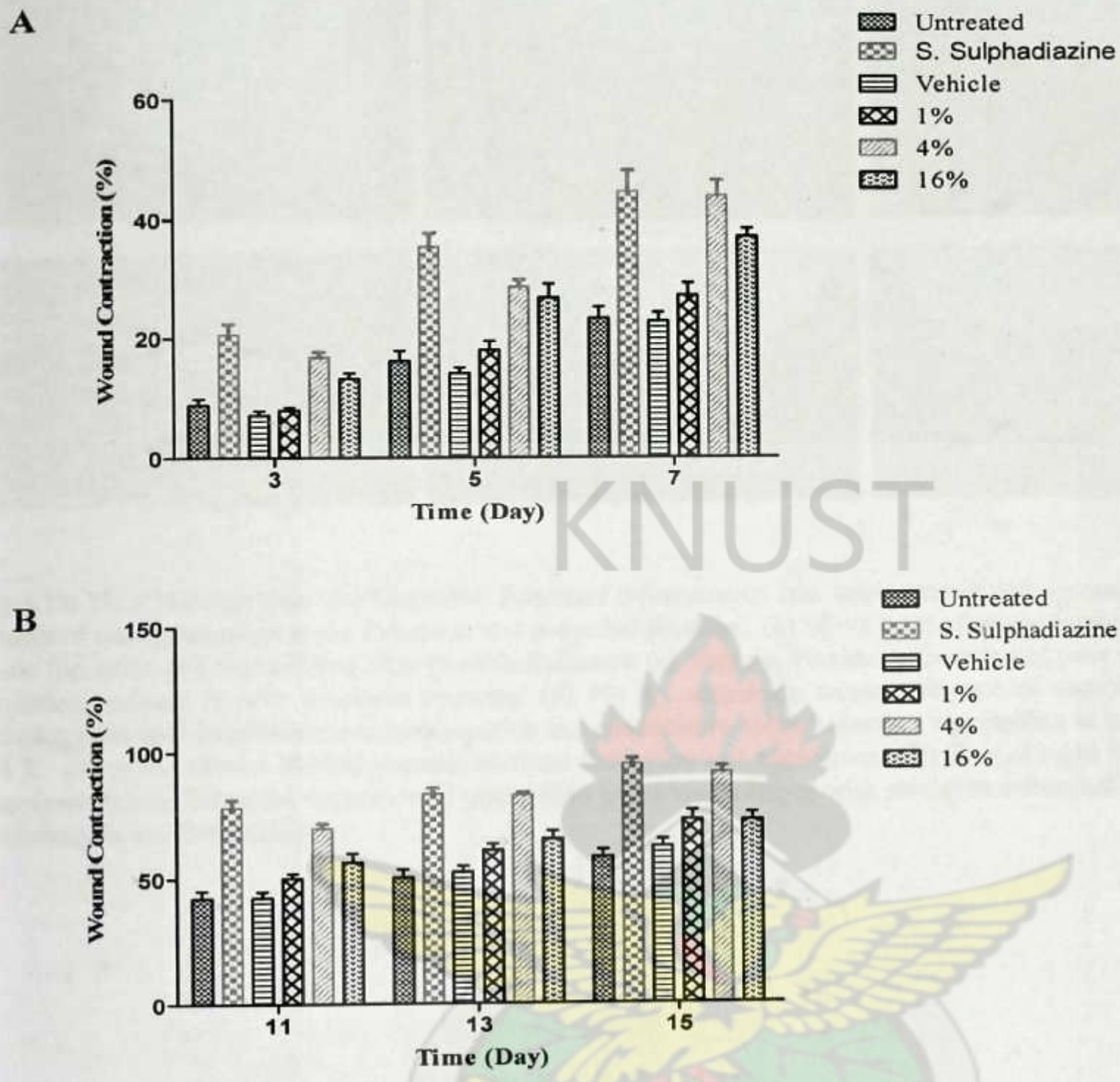

Figure 3.12: Effect of crude extract creams on wound contraction in figureA and B. The wound contraction of each time point was measured as the percentage reduction of original wound size. Values are expressed as mean ± S.E.M. (n=5) for days 3, 5, 7, 11, 13 and 15.

3.7.3 Histopathological evaluation

Histological assessment of the excised tissue samples from all treatment groups exhibited a generalised regeneration of tissues during the wound healing process. A closer examination of granulation tissue sections revealed that tissue regeneration was facilitated in all treatment groups at varying degree.

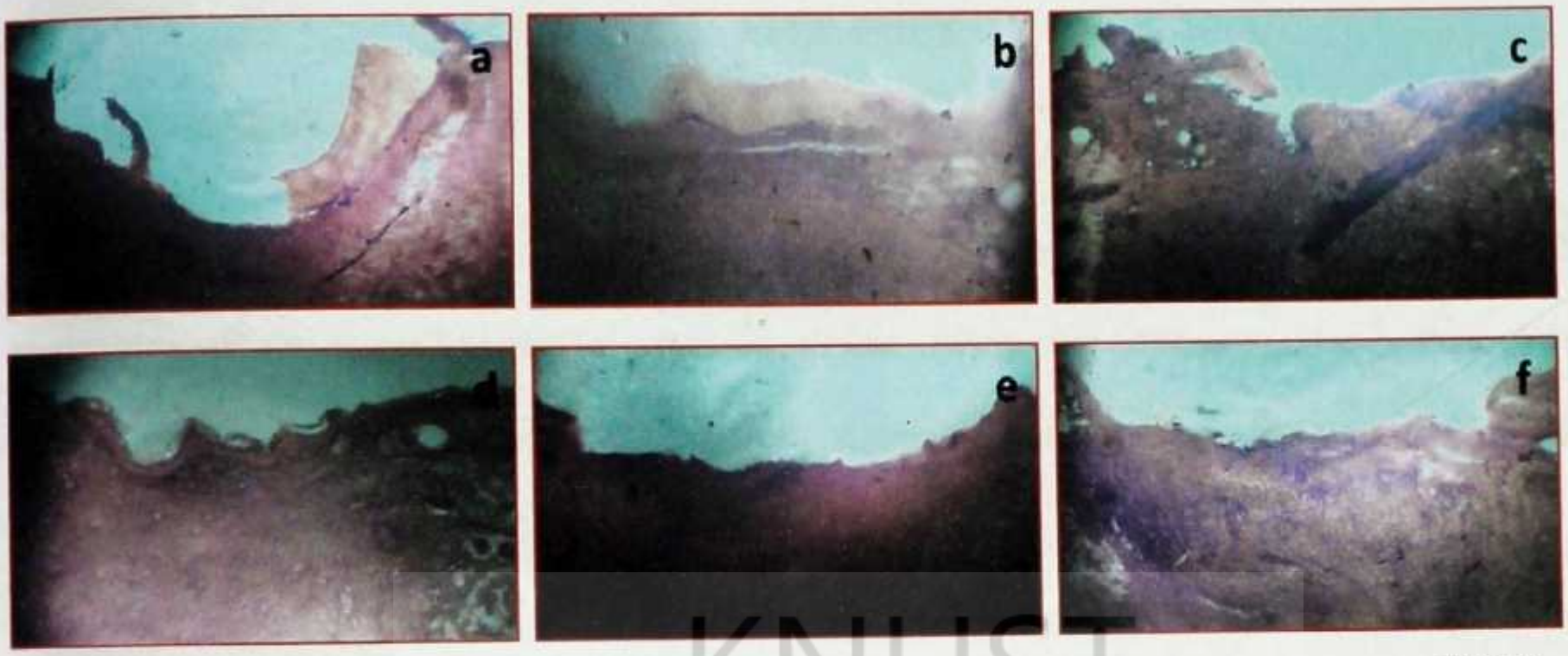

Fig 3.13: Skin Micrographs. (a) Untreated: Persistent inflammation and angiogenesis with incomplete wound area, evident of poor granulation tissue formation and re-epithelialisation. **(b)** Silver sulphadiazine: Appreciable granulation tissue formation and tissue filling with re-epithelialisation **(c)** Vehicle; Persistent necrosis and poor granulation tissue formation, evident of poor treatment response. **(d)** 4% *E. angolense* cream: Presence of vacuolation as obvious pathology but with improvement in collagenation and enhanced re-epithelialisation, manifesting as uneven scaring. **(e)** 1% *E. angolense* cream: Marked granulation tissue formation and angiogenesis, evident of rapid healing **(f)** 16% *E. angolense* cream: Enhanced deposition of granulation tissue and collagen with persistent inflammation. **Note:** Stain = Hematoxylin and Eosin (HE).

CHAPTER FOUR

4.0 DISCUSSION

The phytochemical screening of the crude extract showed the presence of tannins, flavonoids, terpenoids, alkaloids, cardiac glycosides and saponins. Plants that contain tannins as their major components are astringent in nature and are used in treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003).

The crude extract exhibited a broad spectrum of activity against the test bacteria used. The antimicrobial activities against the test organisms may be ascribed to the presence of phytoconstituents found in the crude extract. Gram positive bacteria (*Ent. faecalis*, *Staph. aureus* and *B. subtilis*) were found to be more susceptible to the crude extract with MICs of 9, 11 and 13 mg/mL than the Gram negative bacteria (*E. coli* and *P. aeruginosa*) which gave MICs of 17 and 19 mg/mL respectively. Ciprofloxacin was active against all the test bacteria with MICs ranging from 0.0025 mg/mL for *E. coli* to 0.08 mg/mL for *P. aeruginosa* (Table 3.7) respectively as expected.

The difference in sensitivity patterns could be as a result of the morphological differences between these microorganisms (Ahmadu *et al*., 2006). The Gram positive bacteria have only an outer peptidoglycan layer which is not an effective barrier against antimicrobial agents. *Ent. faecalis*, *Staph. aureus* and *B. subtilis* were the most susceptible bacteria, an observation that may be attributed to the presence of single membrane of the organisms which made it more accessible to permeation by the principles of the crude extract.

Gram negative bacteria on the other hand do possess an outer phospholipidic membrane which carries the structural lipopolysaccharide components, hence making the cell wall complex and impermeable to antimicrobial chemical substances (Ahmadu *et al*., 2006). *E. coli* gave a low

sensitivity whilst *Ps. aeruginosa* exhibited the least sensitivity. Generally *P. aeruginosa* exhibits low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes. They could easily evolve specific resistance either by mutation in chromosomally-encoded genes or by the horizontal gene transfer of antibiotic resistance determinants making them one of the most worrisome organisms mostly in chronic infections (Poole, 2004). This property could have contributed to making it impossible for the bioactive constituents to diffuse through their cell walls.

The crude extract also exhibited a higher activity against the clinical isolate of *C. albicans* with an MIC of 15 mg/mL than the Gram negative bacteria, although it was far lower in activity compared to the ketoconazole which had an MIC of 0.0025 mg/mL.

The solvent fractions of the crude extract exhibited varying antimicrobial activities; the aqueous fraction gave the highest zone of inhibition (Table 3.3) followed by the ethyl acetate fraction (Table 3.4) whilst the pet ether fraction gave the least activity (Table 3.5). In comparing the antimicrobial activities of the fractions to the crude extract (Table 3.2) it was observed that the crude extract exhibited a higher activity against all test organisms as compared to the individual fractions. This goes to explain the synergistic effect of the bioactive compounds present in the crude extract.

Based on this result it can be said that the activities exerted by the polar fractions (aqueous and ethyl acetate) could be due to the presence of a high proportion of active ingredients in these fractions.

Research carried out by Fabry *et al*, (1998) has shown that extracts having MIC values below 8mg/mL possess some high antimicrobial activity. The MIC of the crude extract found in the range of 9 mg/mL to 19 mg/mL, explains its bacteriostatic activity against the test organisms. Low antimicrobial activity could be associated with either the presence of either small amounts of potent active compounds, or large amounts of less potent compounds in the crude extract of *E. angolense*.

From the time kill studies, although there were inhibitory effects exerted by the crude extract against the test organisms as compared to the control, it was also observed that from the 3rd to the 24th h there was a gradual increase in the level of surviving organisms as seen in figures 3.2 to 3.7.

In some parts of the African continent, herbal medicines are sometimes administered concomitantly with antibiotics and the resulting effects could either be beneficial or deleterious. Both crude extracts and pure compounds of some plants have been reported to potentiate the activity of antibiotics (Esimone *et al*., 2006). The results on the antimicrobial activities of the reference antibiotics in the presence of sub-inhibitory concentrations of the crude extract showed varying levels of activity. Some of the antibiotics were enhanced while others exhibited resistance or showed no change in activity against the test organisms.

The activity of amoxycillin activity was increased in folds of 3, 128 and 4 against only the gram positive bacteria (*Ent. faecalis*, *Staph aureus* and *B. subtilis*) respectively. For *E. coli*, the antimicrobial effect of the antibiotic was reduced as resistance against the organism increased whilst no effects were shown with *P. aeruginosa* (Table C2 in appendix IV). Generally bacteria that are resistant to amoxycillin do so by producing enzymes that inactivate or cleave off their binding sites (Scherrer and Gerhardt, 1971).

The enhancement of the amoxycillin activity by the crude extract could be attributed to the structural modification of this antibiotic which prevented enzymatic cleavage or inactivation of the enzyme by binding to the active site in the cell membrane hence inhibiting some steps in cell wall (peptidoglycan) synthesis.

Ciprofloxacin activity was enhanced two folds against only *S. aureus*. The resistance modulatory effect of ciprofloxacin by the crude extract could have been due to its ability to inhibit the enzymes involved in the DNA replication process of the microorganisms (Scherrer and Gerhardt, 1971). In other test organisms the formation of a complex (antibiotic/extract) could have posed some antagonistic reactions that reduced the potency of the antibiotic and could also be responsible for the resistance.

The antimicrobial activity of tetracycline was enhanced against only *Staph aureus* and *B. subtilis* (8 and 4 folds) respectively. Tetracycline showed a reduced activity (0.03 and 0.02 folds) against *Ent. faecalis* and *E. coli* respectively in the presence of the crude extract whilst no change in activity was exhibited by *P. aeruginosa*. Tetracyclines do normally exert their activity against microorganisms by binding to the 30S ribosomal subunits hence inhibiting the protein synthesis (Ahmadu *et al.*, 2006). In the presence of the crude extract there could have been an antagonistic reaction between the bioactive constituents of the extract and antibiotic hence reducing the antibacterial effects of the antibiotic against those organisms.

Also there could have been an increased activity of the organisms' efflux pumps which expelled tetracycline out from their cells or inhibition of the permeability of the drug into the organism.

In the case of erythromycin there was reduction in antibacterial activity against all test bacteria (*Staph aureus*, *Ent. faecalis*, *E. coli* and *Ps. aeruginosa*) except *B. subtilis* which exhibited no change in activity. Erythromycin also normally exerts its activity against microorganisms by binding to the 50S ribosomal subunits hence inhibiting the protein synthesis (Ahmadu *et al.*, 2006). Its reduced activity could be due to the reasons already stated above.

Ketoconazole belongs to the azoles family which do exert their activity by inhibiting enzymes involved in synthesis of ergosterol which is an important component of fungal cell membranes (Ertuk *et al.*, 2006). This antifungal agent showed a lower MIC of 2.5µg/mL against *C. albicans* compared to the combination with the crude extract which gave an MIC of 40 µg/mL (16 times less potent). This could be due to the inability of ketoconazole to diffuse through cell wall in the presence of the extract and inhibit the enzymes responsible for the synthesis of the cell membrane of this organism.

Natural antioxidants that are present in herbs and spices are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. Spices and herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds (Aquil *et al.*, 2006). In the antioxidant study there was a reduction of methanol DPPH solution in the presence of crude extract and N-propyl gallate resulting in the formation of non radical DPPH-H reaction. The crude extract was able to reduce the stable DPPH indicating its antioxidant activity. The concentration required to reduce 50% of the free radicals produced by the radical system (DPPH) i.e. IC_{50} by the crude extract and reference antioxidant was 18.1 and 3.01 µg/mL respectively (figure 3.9). The reference antioxidant was found to be 6 times more potent than the extract. Flavonoids and tannins are major group of phenolic compounds that are known to possess potent antioxidants activity.

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Polterait, 1997). The presence of these bioactive compounds in the crude extract is likely to be responsible for the free radical scavenging effects observed.

Wound healing or repair is a natural process initiated in response to an injury, and proper healing is essential for the restoration of disrupted anatomical continuity and functional status of the skin (Begum and Nath, 2000). For a pure compound or a plant extract to be a good wound healing agent it should possess two or more of the following properties: antimicrobial, antioxidant, anti-inflammatory, stimulate collagen formation, fibroblast and keratinocytes proliferation and diffrenciation (Houghton *et al*., 2005).

The formulated creams 4 and 16% w/w exhibited antimicrobial activities against *Staph aureus*, *S. pyogenes*, *P. aeruginosa and C. albicans* with the exception of the 1% w/w (Table 3.10). In comparing the antimicrobial activity (using the agar diffusion method) of the crude extract (table 3.2) to the formulated cream it was observed that the zones of inhibition were slightly higher for the crude extract than the formulated cream although no significance ($p > 0.05$) was recorded. Differences in activity for the formulated cream could be due to the delayed release of bioactive compound from the cream base before exertion of activity against the organisms. This could also have played a role in the healing process of the wounds using the extract creams.

In the wounding process all the excised wounds were enlarged 24 h post wounding, an indication of progressing pathology due to acute inflammation. It was observed that, 12 h into wounding on the 1st day, the wounds had soon been covered by a thin scab, which became hardened within the first 2 days of repair in accordance with Paddock's accession (2003).

It was observed that in the topical application of the creams on the wounds as at day 3, all treatment groups except those receiving the vehicle did not show any increase in wound size. In all these cases inflammation had also been reduced. This is an indication of effective drug intervention against inflammatory responses and subsequently facilitation of wound healing. However for the vehicle some exudates were observed at the wounds for the first 7 days of treatment and were reduced as treatment progressed.

Wound size was generally reduced compared to their original sizes as well as wound contraction had also increased at varying degree (as seen in figure 3.11) in all treatment groups by the end of 15 days. All the test animals were able to cope well with the injuries and no mortality was recorded.

In all the treatment groups the formulated extract creams (1, 4 and 16%), vehicle and reference drug (silver sulphadiazine) enhanced the wound healing activity at varying degrees in the course of the 15 days of treatment compared to the untreated (figure 3.10).

The 4 % cream exhibited significant value ($p<0.01$) on the 5^{th} day of treatment. The reference drug and the 4 % cream exhibited similar significance $p<0.001$ on the, 7, 11, 13 and 15^{th} day of treatment as compared to the untreated group. The 16% cream had a significant value ($p<0.05$) enhancing the wound healing activity on the 11, 13 and 15 day of treatment as compared to the untreated.

The reference agent had a better enhancement of wound healing than the 1% cream and vehicle, but there was no significant difference ($p>0.05$) between the 1% cream and vehicle on all days of treatment. The reference drug showed a rapid facilitation of healing process whilst the untreated showed the normal healing process of wounds (figure 3.10).

Wounds treated with the 4 % cream and reference agent was completely healed on day 15 of treatment. However wound healing with the 16% cream stagnated after day 7 which was showed by a decrease in the rate of wound closure (figure 3.10). This could probably be attributed to the toxicity of the extract to the cells at high concentrations. Wounds treated with 16% cream were fully healed on the 17th day. The 1% cream also exhibited an enhanced reduction in wound size as compared to the vehicle and the untreated which exhibited a minimal reduction in wound size. Wounds treated with 1% were fully healed on 19th day whilst those treated with the vehicle and the untreated were fully healed on the 21st day.

From the histopathological studies (figure 3.12) the reference agent exhibited good granulation tissue formation and re-epithelialisation as expected. The 4% cream showed an increased formation of granulation tissue, with improvement in collagen formation and enhanced re-epithelialisation. The antioxidant activity (IC_{50} 18.1µg/mL) of the crude extract contributed to the enhance tissue formation as antioxidants play a role in reducing oxidative stress and products at the wound sites.

Wounds treated with the vehicle showed persistent necrosis and poor granulation tissue formation (figure 3.12). Insufficient levels of oxygen and nutrients supplied by the blood vessels probably could have contributed to the necrosis of the underlying tissue cells.

The 16% extract cream showed an enhanced deposition of granulation tissue and collagen with persistent inflammation. The inflammation could be due to toxicity exerted by the extract and correlates with declined reduction of wound sizes after day 7 of treatment (figure 3.10). The untreated group showed an incomplete wound healing process, evident of poor granulation tissue formation and re-epithelialisation. In the absence of medicaments (cream or reference agent) a minimal reformation of the tissues was exhibited (figure 3.10).

The promotion of wound closure of groups treated with the creams compared to the untreated group may be due to a rapid process of angiogenic potential. The phytoconstituents present in the crude extract may have possessed proangiogenic activity facilitating the production of growth factors which in turn, stimulated the angiogenesis process. Angiogenesis is one of the most essential components of the repair process, as it enhances the nutrient supply to sustain cell metabolism, creates an intact delivery system, and facilitates the clearance of debris (Giordano and Johnson, 2001). Studies carried on geraniin and furosin isolated from *Phyllanthus muellerianus* (Kuntze) Exell used in traditional medicine indicated a significant induction of cellular proliferation of skin fibroblast and keratinocytes (Agyare *et al*., 2011).

Examination of the tissue samples showed varying degrees of formation of granulation tissue, which could have resulted from an increased synthesis of collagen and therefore an increase in the wound contraction of treated groups and reference agent as compared to the untreated group. Collagen is a major protein of the extracellular matrix which is a constituent of growing cells that ultimately, not only contributes to wound strength and integrity, but has been known to also play a vital role in homeostasis and in epithelialisation at the latter phase of healing (Gupta *et al*., 2009).

The wound healing action could be due to the presence of tannins, flavonoids and alkaloid found in the crude extract which is responsible for the free radical scavenging activity and is believed to be one of the most important components of wound healing. Reactive oxygen species (ROS) when produced in excessive amounts can results in oxidative stress thereby causing cytotoxicity and delayed wound healing; hence the elimination of ROS could be an important strategy in healing of wounds (Dissemond *et al*., 2002).

Therefore the antioxidant potential exhibited by the crude extract (IC_{50} 18.1µg/mL) makes it a good therapeutic agent. The individual or synergistic effects of the antimicrobial and antioxidant constituents of the crude extract could be responsible for enhancing the formation of underlying tissues in the wound healing process

5.0 CONCLUSION

The study showed that the methanol stem bark extract of *E. angolense* contained tannins, flavonoids, alkaloids, saponins, terpenoids and cardiac glycosides. The crude extract and various solvent fractions exhibited a broad spectrum activity against both bacteria and fungus with the MICs ranging from 9 to 19 mg/mL, and that the extract had bacteriostatic and fungistatic activity against the test organisms used. The crude extract enhanced the antimicrobial effects of some antibiotics against the test organisms whilst reducing the potency of most other antibiotics used. An inhibition profile was established against the test organisms in the time kill studies. The crude extract exhibited an antioxidant activity with an IC_{50} of 18.1 µg/mL. The creams (1, 4 and 16% w/w) formulated from the crude extract facilitated the wound healing process with 4% cream being the best and was similar to the reference wound healing agent and the histopathological studies supported this potential activity with a better formation of tissues. This study therefore offers a scientific support to the folkloric use of this plant for the management of infections hence providing a rationale for its use in wound healing medicaments and possible treatment of wounds and skin infections.

6.0 RECOMMENDATIONS

It is recommended that

1. Further investigation into the bioactivities of *E angolense* and isolation of beneficial compound(s) that could be used as prominent drugs in treatment of microbial infections and wound healing therapy.

2. Toxicological studies should be carried out on the extract to determine its safety profile. This would aid in the formulation of products that will be efficacious with low toxicity and profound therapeutic output.

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APPENDICES

Appendix I: Preparation of culture media

1. BLOOD AGAR BASE (Scharlau Chemie, Spain)

Formula	Quantity (g)
Casein peptone	12.0
Meat peptone	11.0
Sodium azide	0.4
Starch	1.5
Sodium chloride	5.0
Agar	15.00

An amount of 44.5 g of powder was weighed, added to 950 mL of distilled water and brought to boil. It was distributed into glass tubes and sterilize at 121°C for 15 minutes in an autoclave. To obtain Blood Agar cool it was cooled to 45-50°C and defibrinated horse blood (Oxoid Ltd England) at 5% proportion was aseptically added to the agar base .

2. CETRIMIDE AGAR (Oxoid Ltd. England)

Formula	Quantity (g)
Gelatin peptone	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.6

Cetrimide agar powder (45.3 g) was weighed into a conical flask and dissolved in about 900mL distilled water by heating in a boiling water bath. 10 mL of glycerol was added and made up to 1 litre. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

3. EOSIN METHYLENE BLUE AGAR (Scharlau Chemie, Spain)

Formula	Quantity (g)
Peptone	10.0
Lactose	10.0
Dipotassium Hydrogen phosphate	2.0
Yellow Eosin	0.4
Methylene blue	0.06

Agar	15.0

An amount of 37.5 g powder was weighed and added to 1 litre of distilled water. It was brought to boil and distributed in glass tubes. It was sterilized at 121°C for 15 minutes in an autoclave.

4. MACCONKEY AGAR (Scharlau Chemie, Spain)

Formula	Quantity (g)
Peptone	20.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.00
Neutral red	0.03
Crystal violet	0.001
Agar	15.0

An amount of 51.5 g of powder was weighed and added in 1 litre of distilled water. It was brought to boil and sterilized at 121°C for 15 minutes in an autoclave.

5. MANNITOL SALT AGAR (Oxoid Ltd. England)

Formula	Quantity (g)
Lab-lemco powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol Red	0.025
Agar	15.0

Mannitol salt agar powder (111 g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture is distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

6. MUELLER HINTON AGAR (Oxoid Ltd. England)

Formula	Quantity (g)
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Mueller Hinton agar powder (38 g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

7. NUTRIENT AGAR (Oxoid Ltd. England)

Formula	Quantity (g)
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.00

Nutrient agar powder (28 g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

8. NUTRIENT BROTH (Licfi/chem. Bacteriology pdts, Italy)

Formula	Quantity (g)
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

Nutrient broth powder (13g) was weighed into a conical flask and dissolved in distilled water to 1 litre. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

9. PLATE COUNT AGAR (Oxoid Ltd. England)

Formula	Quantity (g)
Tryptone	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	9.0

Plate count agar powder (17.5 g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

10. SABOURAUD DEXTROSE AGAR (AR Merck, UK)

Formula	Quantity (g)
Peptone	10.0
D(+) Glucose	40.0

Agar	15.0

Sabouraud dextrose agar powder (65g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture was distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

11. SABOURAUD BROTH (Scharlau Chemie, Spain)

Formula	Quantity (g)
Casein peptone	5.0
Meat peptone	5.0
D(+) Glucose	20.0

An amount of 30 g of powder was dissolved in 1 litre of distilled water. It was dispensed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

12. SLANETZ BARTLEY AGAR (Lab M Ltd. UK)

Formula	Quantity (g)
Trytose	20.0
Yeast extract	5.0
Dextrose	2.0
Potassium phosphate	4.0
Sodium azide	0.4
TTC	0.1
Agar	12.0

Slanetz bartley agar powder (43.5 g) was weighed into a conical flask and dissolved in distilled water to 1 litre by heating in a boiling water bath. The mixture was brought to cool at 50° C and distributed into plates immediately.It was not autoclaved.

AppendixII: Reagents used for confirmatory test on organisms

1. KOVAC'S (Oxoid Ltd. England)

Formula	Quantity
p-dimethylaminobenzaldehyde	5 g
Amyl alcohol	75 mL
Conc.HCl	25 mL

The aldehyde was dissolved in the alcohol by gently warming in a water bath (50 to

55°C). It was then cooled and the acid added to it.

2. KOSER CITRATE MEDIUM (Oxoid Ltd. England)

Formula	Quantity (g)
Sodium Ammonium Phosphate	1.5
Potassium Dihydrogen Phosphate	1.0
Magnesium sulphate	0.2
Sodium citrate	2.5
Bromothymol blue	0.016

Koser citrate powder (5.2 g) was weighed into a conical flask and dissolved in distilled water to 1 litre. The mixture was distributed into glass tubes in portions of 10 mL and sterilized at 121°C for 15 minutes in an autoclave.

3. TRYPTONE BROTH

Formula	Quantity (g)
Tryptone	10.0
Sodium chloride	5.0

Tryptone water powder (15 g) was weighed into a conical flask and dissolved in distilled water to 1 litre. The mixture was distributed into glass tubes in portions of 10 mL, and sterilized at 121°C for 15 minutes in an autoclave.

4. Hydrogen peroxide 6% w/v of H_2O_2 (Bellson and Co. Ltd. Southport, England).

5. Rabbit blood 5% v/v proportion.

6. Confirmatory test on organisms using selective media

The test organisms were steaked on the different selective media to observe their growth and appearance of colonies. Selective media used include: Cetrmide agar, MacConkey agar, Mannitol salt agar, Slanetz Bartley, Sabouraud Dextrose Agar and Blood base agar.

6.1 Cetrimide agar

This medium is used to confirm *Pseudomonas aeruginosa*. Two tubes of 20 mL of Cetrimide agar were melted and stabilized at 45^0 C for 15 minutes then poured into sterile petri dishes and allowed to set. A loopful of 24 h broth culture of test

bacteria was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. The presence of greenish colonies was a confirmation for the presence of *P. aeruginosa*.

6.2 MacConkey agar

This medium is used to confirm *Escherichia coli*. Two tubes of 20 mL of MacConkey agar were melted and stabilized at 45^0 C for 15 minutes then poured into sterile petri dishes and allowed to set. A loopful of 24 h broth culture of test bacteria was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. The presence of red violet colonies was a confirmation for the presence of *E. coli*.

6.3 Mannitol salt agar

This medium is used to confirm *Staphylococcus aureus*. Two tubes of 20 mL of Mannitol salt agar were melted and stabilized at 45^0 C for 15 minutes then poured into sterile petri dishes and allowed to set. A loopful of 24 h broth culture of test bacteria was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. The presence of bright yellow colonies was a confirmation for the presence of *S. aureus*.

6.4 Slanetz Bartley agar

This medium is used to confirm *Enterococcus faecalis*. Two tubes of 20 mL of Slanetz Bartley agar were heated in a boiling water bath, brought to cool at 50^o C and distributed into plates immediately. A loopful of 24 h broth culture of test bacteria was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. The presence of maroon colonies was a confirmation for the presence of *E. faecalis*.

6.5 Sabouraud dextrose agar

This medium is used to isolate and identify *Candida albicans*. Two tubes of 20 mL of Sabouraud dextrose agar were melted and stabilized at 45^0 C for 15 minutes and 4%

chloramphenicol was aseptically added to the agar, poured into sterile petri dishes and allowed to set. A loopful of 24 h broth culture of isolate was streaked on the surface of the agar and incubated inverted at 25^0 C for 48 h. Confirmatory test was carried out by streaking the isolates on Eosin Methylene Blue agar. The presence of white colonies was a confirmation for the presence of *C. albicans*.

6.6 Blood agar base

Blood agar was used to isolate and identify *Streptococcus pyogenes*. Two tubes of 20 mL of Blood agar base were melted and stabilized at 45^0 C for 15 minutes and 5 % defibrinated horse blood was aseptically added to the agar, poured into sterile petri dishes and allowed to set. A loopful of 24 h broth culture of isolate was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. The presence of clear zones (beta-heamolysis) around colonies was a confirmation for the presence of *S. pyogenes*

6.7 Nutrient agar

This medium is used to confirm *Bacillus subtilis*. Two tubes of 20 mL of Nutrient agar agar were melted and stabilized at 45^0 C for 15 minutes and 1% fresh prepared starch was aseptically added to the agar, poured into sterile petri dishes and allowed to set. A loopful of 24 h test bacteria was streaked on the surface of the agar and incubated inverted at 37^0 C for 24 h. After incubation the surface of the agar was sprayed with iodine and observed. The presence of clear zones around a blue-black ground was a confirmation for the presence of *S. pyogenes*

7. Biochemical tests

7.1 Catalase test

This test was performed by placing 2 drop of fresh 3 % hydrogen peroxide (H_2O_2) on a clean glass slide and aseptically transfering a loopful of the bacteria on to slide with a brief mixing of the cells in the peroxide.

The presence of catalase was confirmed by the formation of air bubbles in the emulsion.

7.2 Indole test

This test was performed by inoculating test bacteria into sterile tryptone broth and incubating it for 24 h. 5 drops of Kovac's reagent was added to the fresh overnight culture broths and positive results were shown by the presence of a red or red-violet colour in the surface alcohol layer of the broth. A negative result appears yellow.

7.3 Citrate test

For the identification of citrate utilization the test bacteria were inoculated on Koser Citrate medium and the results were observed after 24 hours of incubation. The appearance of a colour change in the medium from green to blue was recorded as a positive result.

7.4 Coagulase test

Three test tubes were taken and labelled "test", "negative control" and "positive control". Each tube was filled with 0.5 mL of 1 in 10 diluted rabbit plasma. To the tube labelled test, 0.1 ml of overnight broth culture of test bacteria was added. To the tube labelled positive control, 0.1 ml of overnight broth culture of known *S. aureus* was added and to the tube labelled negative control, 0.1 ml of sterile broth is added. All the tubes are incubated at 37°C and observed up to 4 h. A positive result was indicated by gelling of the plasma, which remains in place even after inverting the tube.

Table A.1 Results on confirmatory tests of organisms

		BIOCHEMICAL TESTS			
Organisms	**Culture media**	**Catalase**	**Indole**	**Coagulase**	**Citrate**
S. aureus	bright yellow colonies	+	-	+	-
B. subtilis	Clear zones around the bacterial growth	+	-	-	+
E. faecalis	Maroon colonies	-	-	-	-
E. coli	Red violet colonies	+	+	-	-
P. aeruginosa	Greenish colonies	+	-	-	+
S. pyogenes	Clear transparent yellow zones around colonies	-	-	-	+
C. albicans	White / cotton-like colonies	nd	nd	nd	nd

nd= not determined

Appendix III: Tables on time kill studies of crude extract

Tables B.1 to B.6 shows results on crude extract (MIC, 2×MIC, 3×MIC and 4×MIC) mg/mL against the test organisms.

Table B.1 Time kill kinetics of methanol extract against *Staphylococcus aureus*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	11	22	33	44	Control	11	22	33	44
0	204	192	187	196	181	2.31	2.28	2.27	2.29	2.26
1	275	187	165	178	169	2.44	2.27	2.22	2.25	2.23
2	8×10^{3}	172	124	165	153	3.90	2.24	2.09	2.22	2.19
3	155×10^{3}	156	102	149	121	5.19	3.19	2.01	2.17	2.08
4	172×10^{5}	132×10^{2}	890	127×10^{1}	103	7.24	4.12	2.95	3.10	2.01
5	38×10^{7}	124×10^{3}	82×10^{2}	111×10^{2}	800	8.58	5.09	3.91	4.05	2.90
6	102×10^{7}	85×10^{4}	46×10^{3}	92×10^{3}	71×10^{2}	9.01	5.93	4.66	4.96	3.85
12	127×10^{8}	16×10^{5}	9×10^{5}	13×10^{4}	122×10^{3}	9.10	7.23	5.95	6.13	5.09
24	218×10^{10}	207×10^{6}	181×10^{5}	157×10^{5}	189×10^{4}	9.34	8.31	7.26	7.20	6.28

Table B.2 Time kill kinetics of methanol extract against *Bacillus subtilis*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	13	26	39	52	Control	13	26	39	52
0	157	132	146	122	149	2.20	2.12	2.16	2.09	2.17
1	210	125	133	114	136	2.36	2.20	2.12	2.06	2.13
2	950	109	117	95	128	2.98	2.04	2.07	1.98	2.11
3	263×10^{2}	112×10^{1}	98	86	119	4.42	3.05	1.99	1.93	2.08
4	82×10^{4}	96×10^{2}	72×10^{1}	79×10^{1}	93	5.91	3.98	2.86	2.90	1.97
5	116×10^{5}	47×10^{3}	81×10^{2}	52×10^{2}	61×10^{1}	7.06	4.67	3.91	3.72	2.79
6	54×10^{6}	89×10^{4}	99×10^{3}	43×10^{3}	59×10^{2}	7.73	5.95	5.00	4.63	3.77
12	101×10^{5}	177×10^{4}	173×10^{3}	119×10^{3}	286×10^{2}	8.00	6.25	5.24	5.08	4.46
24	82×10^{7}	34×10^{5}	218×10^{4}	147×10^{4}	63×10^{4}	8.91	6.53	6.34	6.17	5.80

Table B.3 Time kill kinetics of methanol extract against *Enterococcus faecalis*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	9	18	27	36	Control	9	18	27	36
0	142	98	92	119	130	2.18	1.99	1.96	2.08	2.11
1	2355	139	132	109	124	3.37	2.14	2.12	2.04	2.09
2	408×10^{1}	294	109	113	105	4.61	2.47	2.04	2.05	2.02
3	114×10^{2}	168×10^{1}	160	204	143	5.06	3.23	2.20	2.34	2.16
4	571×10^{3}	287×10^{1}	540	281	118	6.76	3.46	2.73	2.45	2.07
5	43×10^{5}	135×10^{2}	1780	940	390	7.64	4.13	3.25	2.98	2.59
6	9×10^{7}	82×10^{3}	192	12×10^{2}	510	8.95	5.91	3.28	2.91	2.70
12	86×10^{10}	133×10^{4}	214×10^{2}	72×10^{2}	1550	11.93	6.12	4.33	3.86	3.19
24	61×10^{18}	28×10^{6}	205×10^{3}	144×10^{3}	41×10^{3}	19.8	7.45	5.31	5.16	4.61

Table B.4 Time kill kinetics of methanol extract against *Escherichia coli*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	17	34	51	68	Control	17	34	51	68
0	98	125	177	78	91	1.99	2.10	2.25	1.89	1.96
1	133	137	238	82	107	2.12	2.14	2.38	1.91	2.03
2	293×10^{8}	124×10^{2}	156×10^{2}	187×10^{1}	138	5.46	4.09	4.19	3.27	2.14
3	27×10^{5}	198×10^{3}	111×10^{3}	91×10^{2}	36×10^{2}	7.43	5.30	5.05	3.96	3.56
4	189×10^{6}	252×10^{4}	84×10^{4}	59×10^{3}	175×10^{2}	8.28	6.40	5.92	4.77	4.24
5	197×10^{7}	116×10^{5}	109×10^{4}	83×10^{3}	48×10^{3}	9.29	7.06	6.04	4.92	4.63
6	72×10^{8}	198×10^{6}	213×10^{4}	157×10^{4}	92×10^{3}	9.86	7.30	6.33	5.20	4.96
12	159×10^{8}	44×10^{7}	101×10^{6}	26×10^{5}	55× 104	10.20	8.64	8.00	6.42	5.74
24	130×10^{88}	27×10^{7}	120×10^{8}	143×10^{5}	39×10^{5}	10.11	9.43	9.08	7.16	6.59

Table B.5 Time kill kinetics of methanol extract against *Pseudomonas aeruginosa*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	19	38	57	76	Control	19	38	57	76
0	119	104	117	109	120	2.08	2.02	2.07	2.08	2.04
1	247	137	123	115	109	2.39	2.14	2.09	2.04	2.06
2	780	246	138	92	67	2.89	2.39	2.14	1.95	1.83
3	185×10^1	750	195	112	98	3.27	2.88	2.29	2.05	1.99
4	94×10^2	185×10^1	129×10^1	169×10^1	450	3.97	3.27	3.11	3.23	2.65
5	28×10^3	124×10^2	102×10^2	98×10^2	34×10^2	4.45	4.09	4.01	3.99	3.53
6	69×10^3	281×10^2	239×10^2	247×10^2	132×10^2	4.84	4.45	4.38	4.39	4.12
12	262×10^3	85×10^3	127×10^3	44×10^3	182×10^2	5.42	4.93	5.10	4.63	4.26
24	136×10^4	198×10^3	294×10^3	112×10^3	87×10^3	6.13	5.30	5.47	5.05	4.94

Table B.6 Time kill kinetics of methanol extract against *Candida albicans*

Time (hr)	Mean number of surviving cells per/ ml (mg/ml)					Log of surviving cells per/ ml (mg/ml)				
	Control	15	30	45	60	Control	15	30	45	60
0	146	135	143	132	155	2.16	2.13	2.16	2.12	2.19
1	295	141	138	153	149	2.47	2.15	2.14	2.19	2.17
2	1330	195	176	1650	158	3.47	2.29	2.25	3.22	2.20
3	297×10^3	198×10^2	113×10^2	98×10^2	540	5.47	4.30	4.05	3.99	2.73
4	282×10^4	52×10^4	38×10^3	17×10^3	980	6.45	5.72	4.58	4.23	2.99
5	212×10^5	135×10^4	76×10^4	28×10^4	139×10^2	7.33	6.13	5.88	5.45	4.14
6	194×10^6	117×10^5	94×10^5	62×10^5	298×10^3	8.29	7.07	6.97	6.79	5.47
12	246×10^6	195×10^5	129×10^5	79×10^5	51×10^5	8.39	7.29	7.11	6.90	6.71
24	275×10^8	211×10^6	285×10^6	58×10^5	89×10^5	8.44	7.32	7.46	6.76	6.95

Appendix IV: Tables on the resistance modulatory effects of crude extract on reference antibiotics

Results obtained from testing the reference antibiotics in the presence of sub-inhibitory concentrations of the crude extract is shown in Table C.1 to C4.

Table C.1 Minimum Inhibitory Concentration (MIC) of reference antibiotics

Reference (µg/mL)	Minimum inhibitory concentration(µg/mL)					
	S. A	**B. S**	**E. F**	**E. C**	**P. A**	**C. A**
Ciprofloxacin	40	20	30	2.5	80	nd
Amoxycillin	160	1280	0.5	20	>5120	nd
Tetracycline	20	160	0.5	0.156	1280	nd
Erythromycin	20	320	20	320	2560	nd
Ketoconazole	nd	nd	nd	nd	nd	2.5

nd= not determined

Table C.2 on Minimum Inhibitory Concentration (MIC) of antibiotics + methanol extract

Organisms	**Cipro only (µg/mL)**	**Cipro+ extract (µg/mL)**	**Potency**	**Amoxy only (µg/mL)**	**Amoxy+ extract (µg/mL)**	**Potency**
S. A	40	20	2	160	1.25	128
B. S	20	40	0.5	1280	320	4
Ent. F	30	30	1	0.5	0.156	3.2
E. C	2.5	40	0.06	20	80	4
P. A	80	320	0.25	>5120	>5120	-

Table C.3 Minimum Inhibitory Concentration (MIC) of antibiotics + methanol extract

Organisms	Tetra only (µg/mL)	Tetra+ extract (µg/mL)	Potency	Erythro only (µg/mL)	Erythro + extract (µg/mL)	Potency
S. A	20	2.5	8	20	80	0.25
B.S	160	40	4	320	320	1
E. F	0.5	20	0.03	20	160	0.13
Esc. C	0.156	10	0.02	320	1280	0.25
P. A	1280	1280	1	2560	5120	0.25

S.A: *Staphylococcus aureus*; B.S: *Bacillus subtilis*; Ent.F: *Enterococcus faecalis*; E.C: *Escherichia coli*; P.A: *Pseudomonas aeruginosa*; C.A: *Candida albicans*.

Table C.4 Minimum Inhibitory Concentration (MIC) of the antibiotic + methanol extract

Organism	Keto only (µg/mL)	Keto+ extract (µg/mL)	Potency
C. A	2.5	40	16

Appendix V: A table on antioxidant activity of methanol extract

Table D.1 DPPH scavenging activity of crude extract and reference (N-propyl gallate)

Conc. (µg/mL)		Absorbance (517 nm)								Mean Scavenging activity (%)	
NPG	ME	DPPH+N-propyl gallate			Mean	DPPH+ crude extract			Mean	NPG	ME
30	100	0.133	0.134	0.134	0.134	0.287	0.288	0.289	0.288	62.73	75.13
10	50	0.182	0.181	0.182	0.182	0.318	0.319	0.320	0.319	49.35	72.45
3	25	0.209	0.208	0.209	0.209	0.681	0.682	0.683	0.682	41.82	41.11
1	12.5	0.270	0.269	0.268	0.269	0.836	0.835	0.834	0.835	24.91	28.58

Appendix VI: Tables on wound healing activity of *E. angolense* methanol extract

A table on the preparation of emulsifying ointment and formulated creams is shown in E.1 and E2. Tables on the percentage wound contraction of the crude extract creams; vehicle and reference wound healing agent on the excised wounds of the rats is shown in Table E.3 and E.4.

Table E.1 Preparation of emulsifying ointment (BP, 2007)

EMULSIFYING OINTMENT(master formula × 0.1)		
Ingredients	Master formula (g) w/w	Scaled quantity (g) w/w
Emulsifying wax	300	30
White soft paraffin	500	50
Liquid paraffin	200	20
Total	1000	100

Table E.2 Preparation of aqueous cream (BP, 2007)

AQUEOUS CREAM (master formula × 0.2)		
Ingredients	Master formula (g) w/w	Scaled quantity (g) w/w
Emulsifying ointment	300	60
Phenoxyethanol	10	-
Distilled water	690	140
Total	1000	200

Mass of water was converted to volume (density of water = 1g/mL): - = not used

The preparation of the aqueous cream was determined using a modification as described by the BP (2007). Emulsifying ointment (100 g) was prepared using emulsifying wax, white soft paraffin and liquid paraffin (30: 50: 20 g w/w). They were melted and stirred under low heat. Aqueous cream (200 g) was prepared by dissolving 60 g of the emulsifying ointment in 140 mL of distilled water and stirring constantly under low heat until a homogenous mixture was obtained. Crude extract creams (1, 4 and 16 % w/w) for the wound healing studies were prepared by incorporating (1, 4 and 16 g) of the methanol extract into the aqueous cream to achieve a total volume of 20 g each for all three concentrations. Phenoxyethanol was not added to the preparation of the extract cream due to the additive antimicrobial activity that may be exhibited by the preservative.

Table E.3 Results on wound diameter with time for all treatment groups

Days	Untreated	Reference	Vehicle	1%	4%	16%
1	27.60±1.08	24.10±1.20	26.00±1.23	27.60±1.21	25.60±1.25	27.60± 1.21
3	25.20±1.20	19.30±1.27	24.00±0.95	25.20± 0.97	21.40±1.19	24.00±1.23
5	23.20±1.32	15.62±1.09	22.20±0.97	22.60± 0.93	18.30±1.13	20.20±1.20
7	21.20±1.32	13.30±1.06	20.00±0.84	20.00±0.71	14.30±0.85	17.30± 0.77
11	16.00±1.41	5.30±0.85	15.20±1.02	13.80±0.97	7.80±0.78	11.90±0.70
13	13.80±1.39	4.00± 0.55	12.80±1.11	10.80± 0.92	4.40±0.25	9.50± 0.82
15	11.80±1.21	0.40±0.40	1.020±1.07	7.60±1.21	0.60± 0.60	7.50± 0.79

Table E.4 Results on wound contraction with time for all treatment groups

Days	Untreated	Reference	Vehicle	1%	4%	16%
3	8.80±1.06	20.55±1.87	7.56±0.92	7.95±0.50	16.50±1.05	13.13±0.98
5	8.07±1.25	18.91±3.27	7.56±0.91	10.33±0.92	14.52±1.62	15.84±2.33
7	8.72±0.45	14.79±2.98	9.82±1.54	11.38±1.43	21.57±3.22	14.01±1.85
11	14.10±1.29	47.01±4.06	13.78±1.51	19.18±2.85	32.83±3.25	15.48±3.14
13	13.92±2.54	43.00±14.32	16.04±2.78	22.12±2.50	42.02±4.92	20.66±2.86
15	16.06±0.65	48.33±17.2	20.70±2.30	31.41±5.71	52.00±13.2	21.37±3.71

Appendix VII: Standardization of test microorganisms.

Sterile nutrient broth (10mL) was aseptically innoculated with *Staph. aureus* from the stock culture and incubated at 37°C for 18 to 24 h. 1mL of the resultant suspension was serially diluted to 10^3, 10^4, 10^5 and 10^6 times in test tubes containing 9 mL sterile nutrient broth. Plain nutrient broth was used as a blank to zero the spectrophotometer and absorbance of the suspensions of organisms were then determined at 480 nm. After absorbance readings were taken the number of viable cells per milliliter of the broths was determined by means of the pour plate method with the aid a colony counter. Tables (F1 and F2) and Graphs (figure F1 to F7) shows results of the Log10 cfu/mL plotted against the absorbances.

Table F1: Number of surviving cells and their absorbances at 480 nm

S. aureus		*E. faecalis*		*S. pyogenes*	
Absorbance	Log cfu/mL	Absorbance	Log cfu/mL	Absorbance	Log cfu/mL
0.639	6.6928	0.648	7.1072	0.335	1.6435
0.662	7.6222	0.718	8.2330	0.340	2.1271
0.746	8.6263	0.752	9.1523	0.357	2.5705
0.790	9.6721	0.831	10.2201	0.456	2.9205

Table F2: Number of surviving cells and their absorbances at 480 nm

B. subtilis		*E. coli*		*P. aeruginosa*		*C. albicans*	
Absorbance	Log cfu/mL	Absorbance	Log cfu/mL	Absorbance	Log cfu/mL	Absorbance	Log cfu/mL
0.329	1.5052	0.476	7.2455	0.494	7.3032	0.336	1.0792
0.331	2.2833	0.497	8.1959	0.576	8.4409	0.340	1.4771
0.358	2.4548	0.502	9.2095	0.671	9.3617	0.359	1.8325
0.379	2.6263	0.822	10.2552	0.781	10.3404	0.524	2.1003

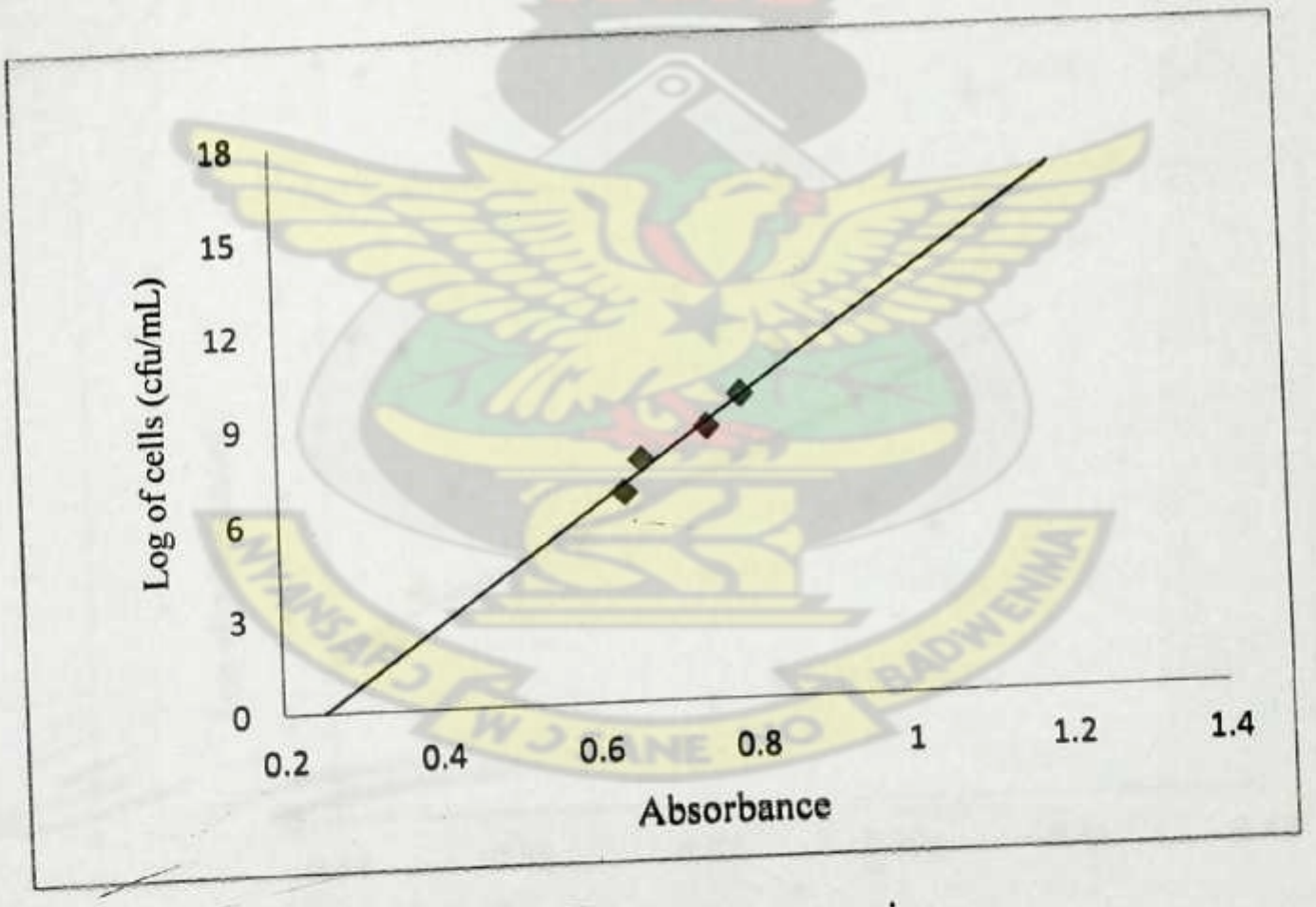

Figure F1: Standardization of *S. aureus* suspension

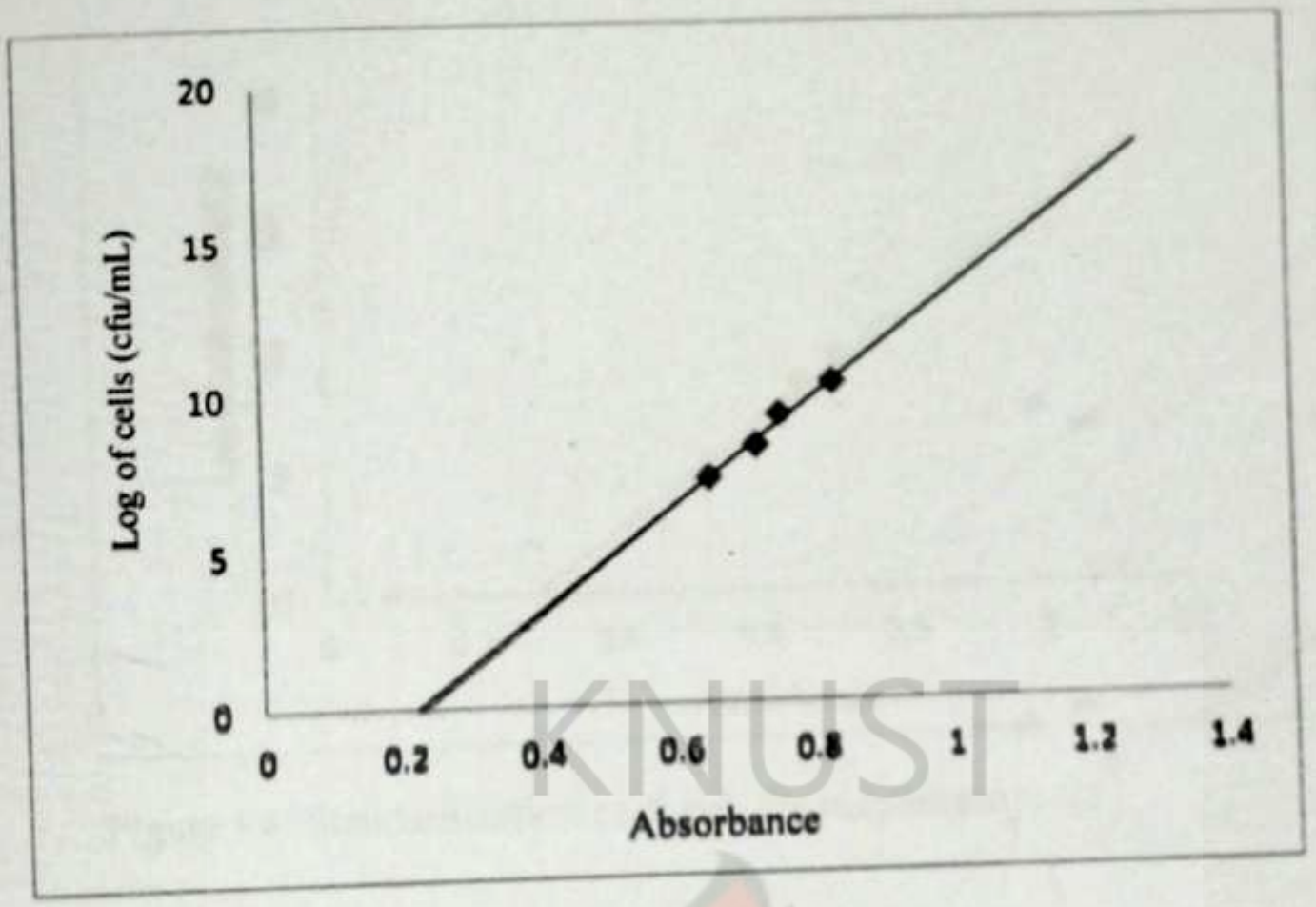

Figure F2: Standardization of *E. faecalis* suspension

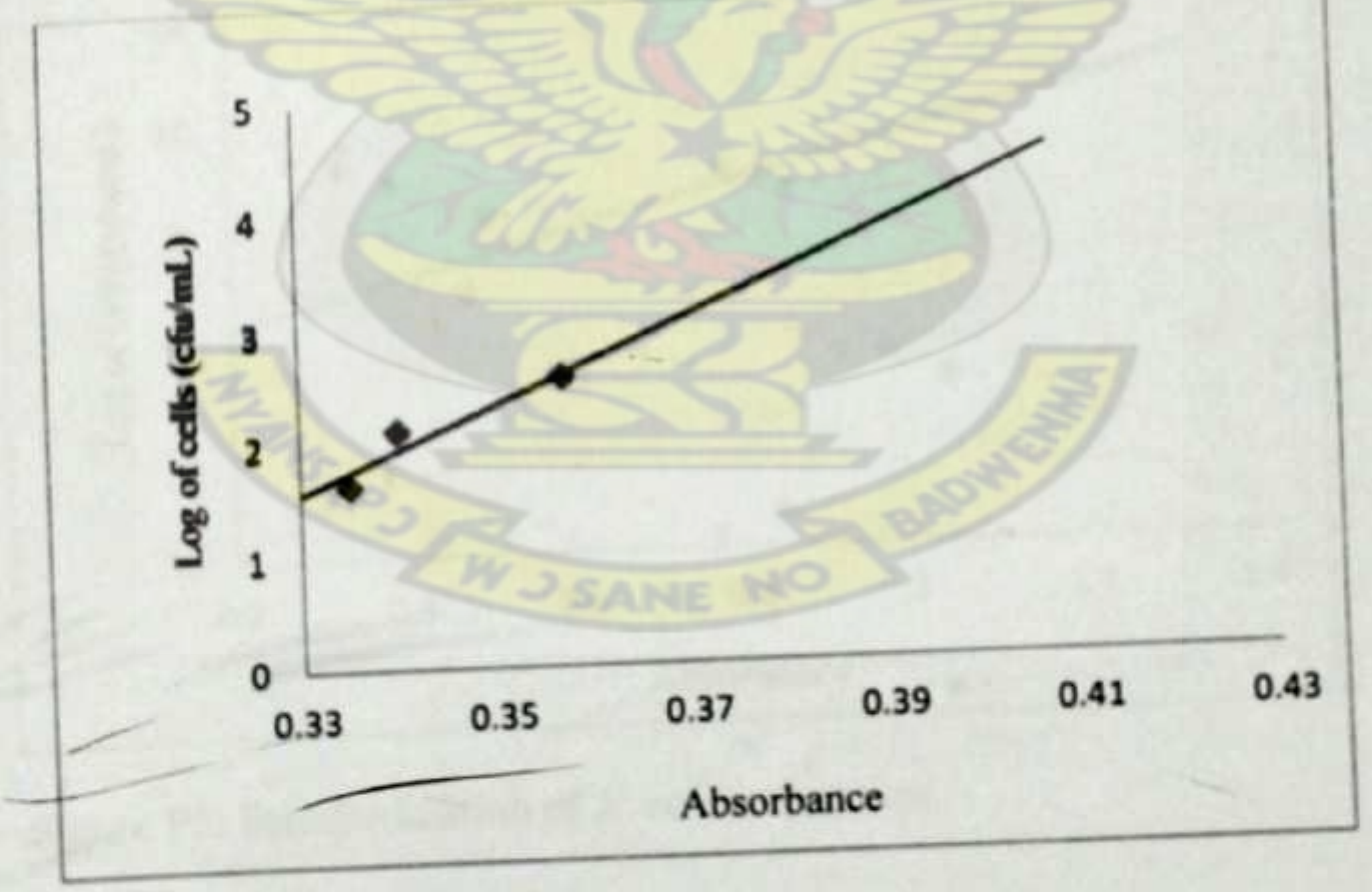

Figure F3: Standardization of *S. pyogenes* suspension

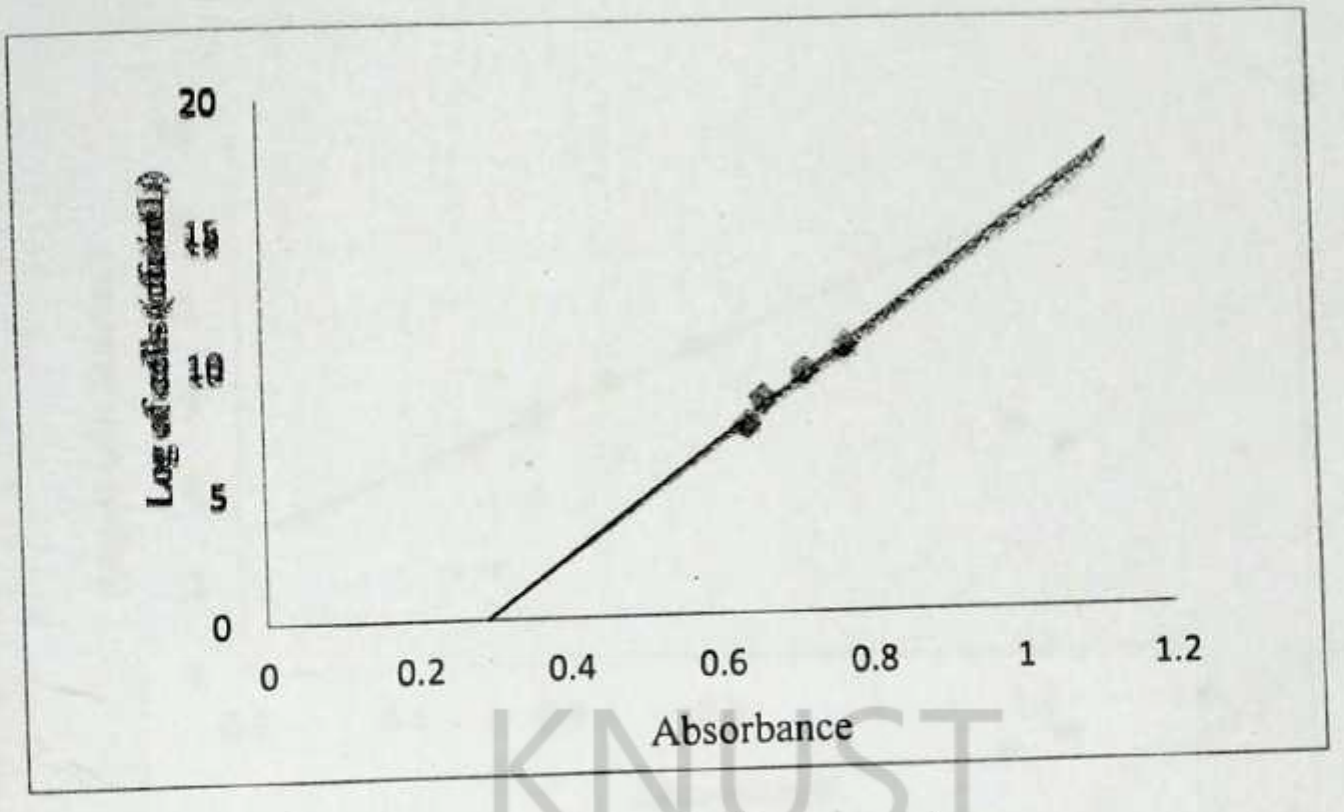

Figure F4: Standardization of *B.subtilis* suspension

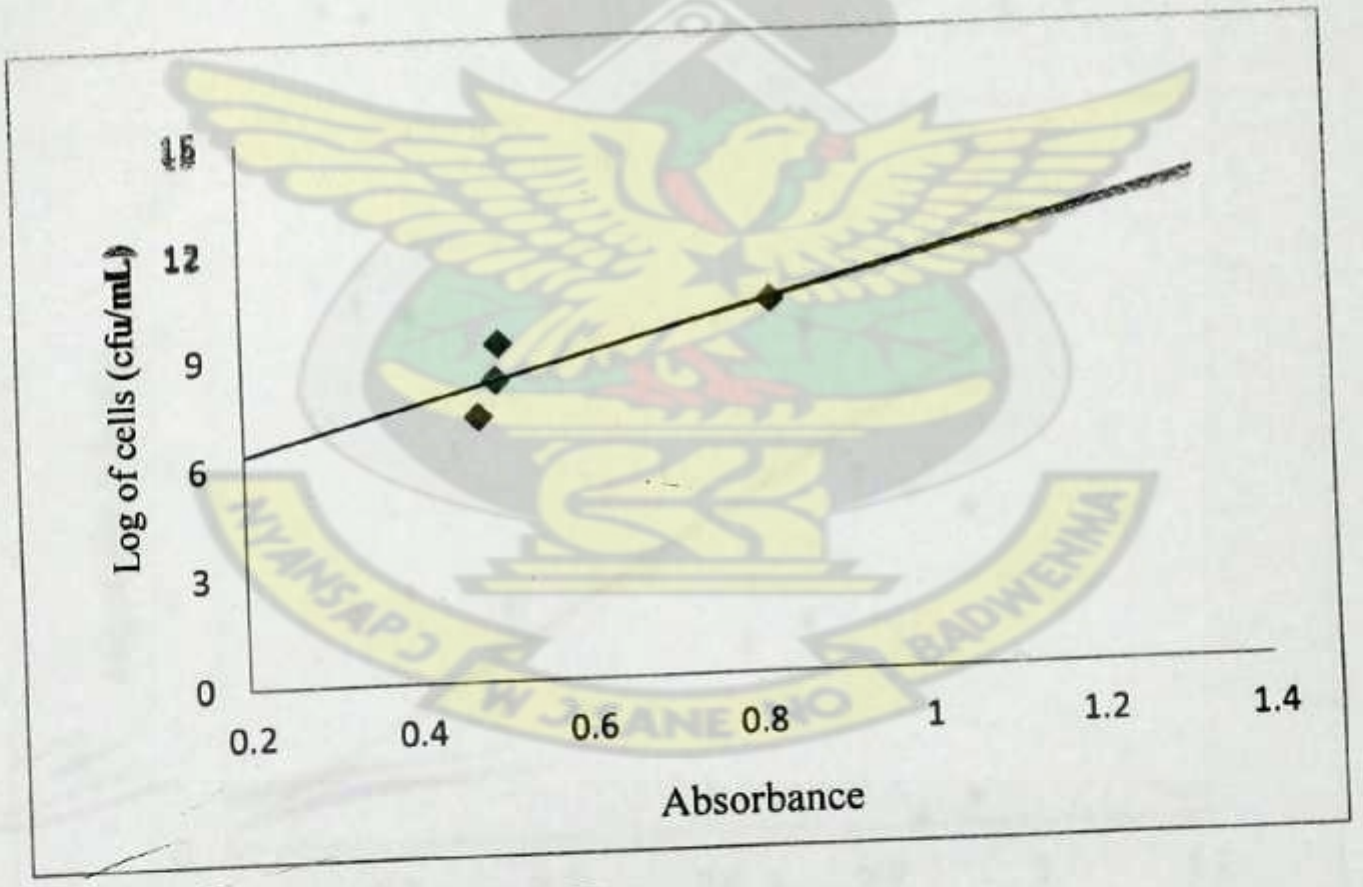

Figure F5: Standardization of *E. coli* suspension

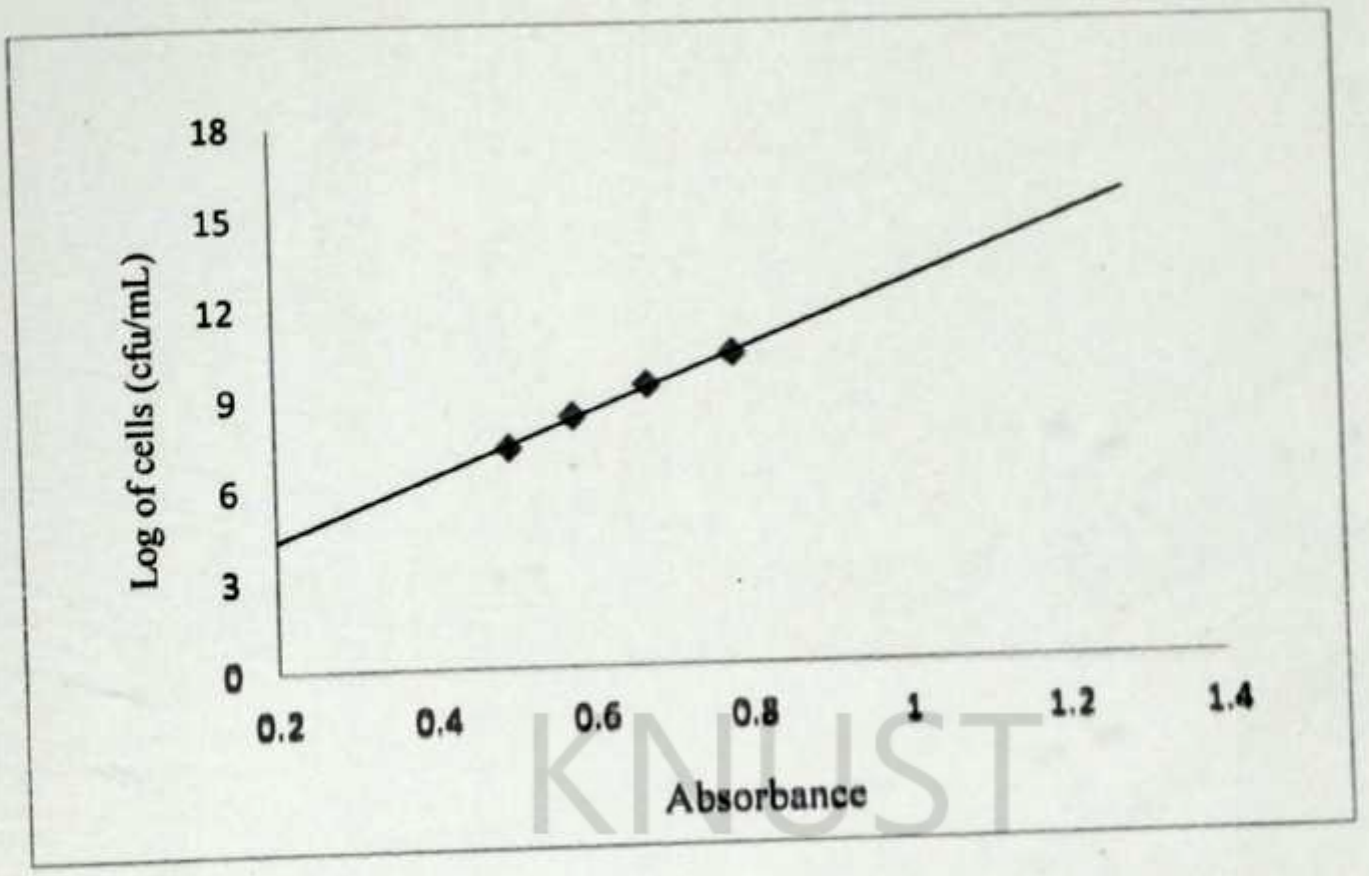

Figure F6: Standardization of *P. aeruginosa* suspension

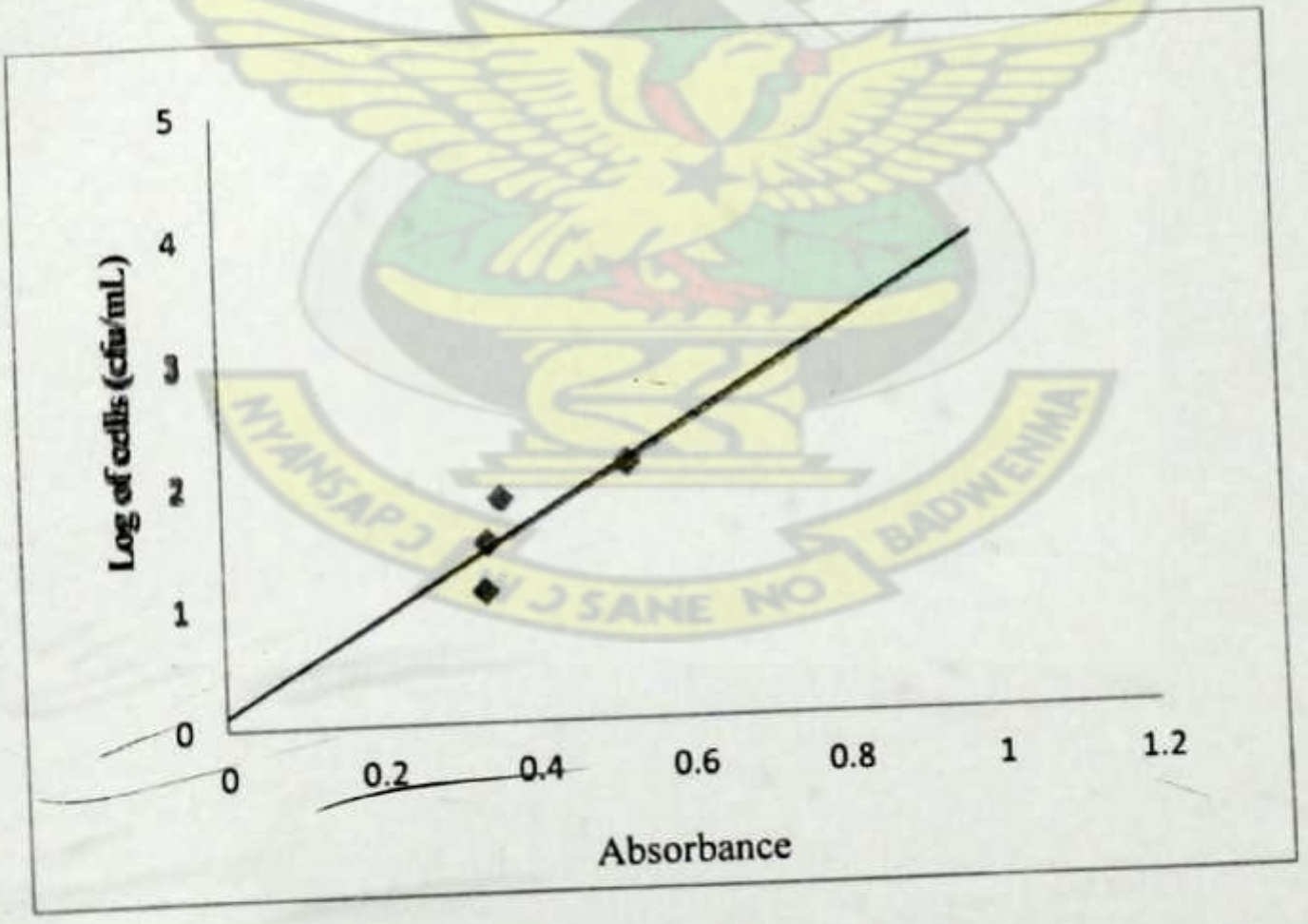

Figure F7: Standardization of *C. albicans* suspension