KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICS

PHYTOCHEMICAL CHARACTERISATION, ANTIMICROBIAL AND WOUND HEALING PROPERTIES OF *CLAUSENA ANISATA* (RUTACEAE)

BY

NICHOLAS AGYEPONG

JULY, 2012

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By

NICHOLAS AGYEPONG

A THESIS SUBMITTED TO THE DEPARTMENT OF PHARMACEUTICS, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE AWARD OF

MASTER OF PHYLOSOPHY (PHARMACEUTICAL MICROBIOLOGY)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

COLLEGE OF HEALTH SCIENCES

JULY, 2012

DECLARATION

I, Nicholas Agyepong, do declare that this dissertation, Phytochemical characterization, antimicrobial and wound healing activity of *Clausena anisata* (Rutaceae) is the outcome of an original research work carried out by me, Nicholas Agyepong of the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science Technology, Kumasi, under the supervision of Dr. Christian Agyare and that neither in whole nor in part has been published or presented for another degree elsewhere except for the references to other works which have been duly cited.

NICHOLAS AGYEPONG ..... 18/10/2012 .....

Student (ID. No. 20137746) Signature Date

Certified by:

Dr. Christian Agyare ..... 19/10/2012 .....

(Supervisor) Signature Date

Certified by:

Prof. Ofori-Kwakye ..... 22/10/12 .....

(Head of Department) Signature Date

ACKNOWLEDGEMENTS

I am most grateful to the Almighty God for his mercy and protection throughout my study in the University. My sincere gratitude to my supervisor Dr. Christian Agyare of the Department of Pharmaceutics for his invaluable intellectual guidance, encouragement during my laboratory work and presentation of this thesis.

I am also grateful to Mr. S.Y Gbedema of the Department of Pharmaceutics for the conception of this project and initial guidance.

My sincere thanks also go to Dr. Paul Osei Sampane of the Department of Pathology for supervising the histopathological aspect of the research and Dr. Nicholas Tittiloye, Head of Department Pathology, Komfo Anokye Teaching Hospital, Kumasi, for permitting me to use their facility. I also express my sincere appreciation to all the technical staff especially Adwoa Konadu Ababio in the Department for their support in the histopathological studies.

I also wish to thank Mr. Martin Adarkwa-Yiadom of Ghana Standard Authority, Accra, for his assistance in the HPLC work.

I express my profound gratitude to all the lecturers including Mrs Vivian E. Boamah and the postgraduate students especially Yaw Duah Boakye and Kwame Apenteng of the Department of Pharmaceutics for their constructive criticisms and contributions in making this project a success.

My special thanks go to the Head, Rev. Prof. Charles Ansah of Department of Pharmacology and the technical staff especially Mr. Thomas Ansah for the expert manner they helped in the course of carrying out the wound healing aspect of the project.

I am also grateful to Miss Liticia Akawaboa Lincoln for her prayer support and encouragement throughout my studies.

Finally, I wish to sincerely thank Mr. Sixtus Bayaa-Martin and Mr. William Ofori-Appaw for their support and assistance in my statistically analysis.

DEDICATION

This work is dedicated to my mum, Madam Alice Frimpong and all those whose support and contributions have made my education this far possible.

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Oral presentation

Agyepong N, Agyare C, Gbedema SY, Adu F, Boamah VE, Ugboduma AO. Antimicrobial and antioxidants activities of ethanol extract of *Clausena anisata* Willd Hooke. 5th Western Africa Network of Natural Products Research Scientists (WANNPRES). AAPS Ethnoveterinary Symposium Ada, Osun State, Nigeria. 6 to 8th August, 2012.

Poster presentation

Agyepong N, Agyare C, Gbedema SY, Adu F, Boamah VE, Ugboduma AO. Antimicrobial activities of ethanol leaf extract and fractions of *Clausena anisata.* 5th Annual Convention of Ghana Biomedical Convention (GBC). Noguchi Memorial Institute of Medical Research. Legon, Accra, Ghana. 31st July, 2012 to 2nd August, 2012.

ABSTRACT

Clausena anisata Willd Hooke. belongs to the family Rutaceae. It is a shrub widely used in many parts of West Africa including Ghana for the treatment of bacterial and fungal infections on the skin including boils, ringworm and eczema. The leaf in the form of poultice is also used for the management of wounds. The study was to evaluate the antimicrobial and wound healing activities of ethanol leaf extract of *C. anisata.* The antimicrobial properties of the ethanol leaf extract of *C. anisata* was investigated using agar well diffusion and micro dilution techniques for determination of the minimum inhibitory concentrations (MIC) against Gram-positive bacteria (*Bacillus substilis* NCTC 10073, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 *Bacillus thuringiensis* ATCC 13838), Gram-negative bacteria (*Pseudomonas aeruginosa* TCC 4853, *Proteus vulgaris* ATCC 4175) and clinical isolate of *Candida albicans*. The antimicrobial activity of the ethanol extract by the agar well diffusion at the concentration of 20 mg/mL showed zones of growth inhibition range of 25.0 to 17.2 mm and 20.8 to 20.4 mm against the Gram-positive and Gram-negative organisms respectively. The MIC range of 4.5 to 0.5 mg/mL for Gram-positive bacteria, 2.5 to 1.0 mg/mL Gram negative bacteria and 5.5 mg/mL against *C. albicans* were determined using the ethanol extract. The MICs of the methanol fraction was 5.0 to 0.6mg/mL and 3.0 to 1.0 mg/mL for Gram-positive and Gram-negative respectively, the chloroform fraction, 7.5 to 3.0 mg/mL and 6.5 to 2.0 mg/mL for Gram-positive and Gram-negative organisms respectively and petroleum ether fraction, 8.0 to 4.5 mg/mL for Gram-positive and Gram-negative respectively. The ethanol extract exhibited static action against the test organisms within a concentration range of 22.0 to 0.5 mg/mL using time kill kinetics determination. The wound healing activity of ethanol leaf extract of the *C. anisata* was investigated using excision model. Concentrations of 4, 7 and 10% w/w aqueous cream of the

extract were tested with silver sulphadiazine (1% w/w) as the reference wound healing agent. The progression of wound healing was determined by the periodic assessment of the contraction of excision wounds and histopathlogical studies. The extract cream of the leaf (7% w/w) was found to significantly increase the rate of wound contraction ($p< 0.05$) at day 9 and day 13 ($p< 0.01$) compared to the untreated wound. Antioxidant activity was also determined using, 2 diphenyl-1 picrylhydrazyl (DPPH) radical scavenging method. The N-propyl gallate, used as the reference antioxidant agent, had IC_{50} of 4.19 µg/mL with the extract having the IC_{50} of 32.9 µg/mL. The phytochemical screening of *C. anisata* reveals the presence of tannins, flavonoids, steroids, saponin, glycosides and alkaloids. The HPLC finger-printing of the ethanol extract and fractions were determined for quality control purposes. These biological activities exhibited by the extract may justify the medicinal uses of leaves of *C. anisata*.

ABBREVIATION AND PREFICES

ANOVA	Analysis Of Variance
ATCC	American Typed Culture Collection
AUC	Area under curve
BSAC	British Society for Antimicrobial Chemotherapy
BP	British Pharmacopoeia
cfu	Colony forming unit
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
^0C	Degree Celsius
EUCAST	European Committee for Antimicrobial Susceptibility Testing
g	Gram
h	Hour
HPLC	High performance liquid chromatographic
MBC	Minimum Bactericidal Concentration
IC_{50}	Half-maximal Inhibitory Concentration
MFC	Minimum fungicidal concentration
MIC	Minimum Inhibitory Concentration
MTT	Methylthiazolyl tetrazolium chloride
min	Minutes
mg	Milligram
mL	Millilitre
NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standard
NCTC	National Collection of Type Cultures
NPG	N-Propylgallate
%	Percentage
R_f	Retardation factor
SD	Standard Deviation
Sec	Seconds
SEM	Standard Error Mean

Tween 20	Polyoxythylene (20)-Sorbitan monoaiurate
TLC	Thin Layer Chromatography
µg	Microgram
µL	Microlitre
ZOI	Zone of Inhibition

CHAPTER ONE

1.0 Introduction

Infectious diseases have remained a major cause of death and disability, throughout the history of mankind, accounting for about 22% of the global disease burden. It has been known to be the number one cause of death in tropical countries accounting for approximately half of all fatalities (Lopez *et al.*, 2006). In addition, its mortality rates are also increasing in developed countries (Pinner *et al.*, 1996). These negative health trends are thought to be driven largely by socio-economic, environmental and ecological factors (Morens *et al.*, 2004; Dassak *et al.*, 2000; Woolhouse, 2008). Jones *et al.* in 2008 reported the emergence of 335 infectious diseases between 1940 and 2004 in the global human population.

The discovery of penicillins in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases particularly in developed countries. However, despite this success, the increased use of antibiotics led to the development of resistance, to the effect that diseases that were thought to have been controlled by antibiotics later re-emerged as resistant infections (Norrby *et al.*, 2005). The clinical efficiency of many of these existing antibiotics for the treatment of microbial infections is being threatened by this emergence of multidrug resistant pathogens (Bandow *et al.*, 2003). This is as result of bacterial pathogens which have evolved numerous defence mechanisms against antimicrobial agents.

Apart from the narrow spectrum of antimicrobial agents, many of them have also been found to be neurotoxic, nephrotoxic or ototoxic and few others cause severe damage to the liver and cause bone marrow depression (Chong and Pagano, 1997). The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the

screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio, 1996; Scazzocchio *et al.*, 2001).

Medicinal plants play a central role in the healthcare systems of large proportions of the world's population, particularly in developing countries, where herbal medicine has a long and uninterrupted history of use (Koduru *et al.*, 2007). According to the World Health Organization (WHO), up to 80% of the population in Africa depends on traditional herbal medicine for primary health care, accounting for around 20% of the overall drug market (WHO, 2004). The popularity of such plants in these communities is largely due to their local availability and price affordability (Voravuthikunchai and Kitpipit, 2005) and also the effectiveness of these medicinal plants in the treatment of various diseases.

Medicinal plants have been found useful as antimalarial, antisickling, anti-helminthic, anti-microbial, anti-fungal, anti-hypertensive and anti-schistosomal agents (Prescott *et al.*, 2002). The medicinal actions of plants are unique to particular plant species or groups, consistent with the concept that the combination of secondary metabolites in a particular plant is taxonomically discrete (Parekh *et al.*, 2006). Plant-based drugs are gaining popularity because of several advantages such as milder side effects, better patient tolerance, less expensive and acceptance due to a long history of use (WHO, 2004). Herbal medicines provide rational means for the treatment of many diseases that are believed to be incurable in other systems of medicine (European Scientific Cooperative on Phytotherapy, ESCOP, 1999). Their bioactive principles appear as alternatives for the control of even resistant species of bacteria and human pathogens and their use have been shown to have a scientific basis (Mathias *et al.*, 2000; Ganguly *et al.*, 2001; Martino *et al.*, 2002).

Medicinal plants have also attracted the attention of the pharmaceutical and scientific communities. This involves the isolation and identification of secondary metabolites produced by plants and their use as active principles in medicinal preparations (Taylor *et al.*, 2001). Many of the plant secondary metabolites are constitutive, existing in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack (Osbourne, 1996).

The secondary metabolites produced by plants are overwhelming, with wide ranging chemical, physical and biological activities (Taylor *et al.*, 2001). These constitute a source of bioactive substances and currently the scientific interest has increased due to the search for new drugs of plant origin. A number of plant secondary metabolites have been used as anticancer, antidiabetic and antimicrobial agents. Phytochemical studies of the stem bark of *Clausena anisata* revealed the presence of coumarins, limonoids, reducing sugars, tannins, flavonoids, quinones and alkaloids (Cakrabirty *et al.*, 1995; Ito *et al.*, 2000). Antimicrobial (Hutchings, *et al.*, 1996), antidiabetic (Ojewole, 2002) antiparasitic and central nervous depressant (Makanju, 1983) properties of *C. anisata* leaves have also confirmed its therapeutic potentials in traditional medicine.

Many West African countries such as Ghana, Benin, Nigeria, and Togo, are very rich in herbal plants which are noted for natural therapies; however, infections from microorganisms are also rampant, with malaria being endemic as well. It is therefore imperative, to look for phytochemical agents that are capable of treating these infections and provide an excellent remedy for a whole host of diseases which afflict the majority of people in this region.

1.1 JUSTIFICATION OF THE STUDY

Bacteria and fungi resistance to antimicrobial drugs has continued to grow in the last decades. The increased prevalence of their resistance is due to extensive use, misuse and substandard brands of antimicrobials. This resistance has rendered the most current available antimicrobial agents ineffective to control microbial infections and has created a major public health problem. The situation has been worsened by high costs of primary health care, lack of access to medical health care and side effects of available drugs. All these have resulted in an increase in severity of microbial infections, compounded with high mortality rates in some cases. This development has led to resurgence in the search for new, broad spectrum and more potent antimicrobial agents.

Public interest has increased for natural therapies (mainly phytomedicine) all over the world due to the belief that phytomedicine is devoid of side effects since millions of people globally have been using phytomedicines for thousands of years for the treatment of certain diseases where conventional medicines have not been effective (Okigbo and Mmeka, 2006). Again there is an improvement in the quality, proof of efficacy and safety of phytomedicines which give consumers better preference over the use of high cost of synthetic drugs.

In Ghana, many infections have been known to be treated with herbal remedies by herbal practitioners and therefore natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity.

The quest for solutions to the global problem of microbial infections and antibiotic resistance in pathogenic microoganisms has often focused on the isolation and characterization of new antimicrobial compounds from a variety of sources including medicinal plants. The aim of this study therefore, is to evaluate the antimicrobial activity of leaf extracts of *Clausena anisata* (Willd) Hook. f. ex Benth and to determine its wound healing properties.

1.2 Main objective

To perform preliminary phytochemical screening and determine the antimicrobial and wound healing properties of ethanol leaf extract of *Clausena anisata.*

1.2.1 Specific objectives

- To prepare ethanol leaf extracts of *C. anisata.*
- To perform preliminary phytochemical screening of the extract.
- To determine the HPLC and TLC finger-printing of the extract and fractions.
- To determine the antimicrobial activity of the extract and fractions against both typed and clinical isolates of the test bacteria and fungus.
- To fractionate the crude extract and determine the antimicrobial activity of the various fractions
- To determine the minimum inhibitory concentrations of the ethanol extract and its fractions of *C. anisata* and minimum bactericidal concentration of the ethanol extract against the test organisms.
- To study the time-kill kinetics of the ethanol extract on test organisms.
- To determine the antioxidant properties of the ethanol leaf extract
- To determine the wound healing properties of ethanol leaf extract *in vivo.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Phytomedicines

The role of herbal medicine as the major remedy in traditional healing system has been in medical practice for thousands of years. However, the study of higher plants for the purpose of detecting antimicrobial agents in their tissues is of comparatively recent origin and the early investigation in this area focused on those plants that have found application in the age-old practice or their blind usage as therapeutics for human and animal diseases (Benjamin and Oguntimelun, 1983; Okigbo and Nmeka, 2005).

The use of plants has made great contribution to maintaining human health and thus, a majority of the world's population in developing countries still rely on herbal medicine to meet their needs (WHO, 2010). Ghana, Nigeria and Benin are among the West Africa countries, rich in rare and useful herbs, thus providing a vast area of medicinal plant research for novel drug development (Ghana Society for Development Dialogue, GSDD, 2008).

Traditional medicine is undoubtedly the oldest form of medicine and probably evolved simultaneously with the evolution of human beings (Wanzala *et al.*, 2005) or even much earlier. According to Akinyemi *et al.* (2005), herbal medicine has been shown to have genuine utility and about 80% of rural population depend on it as primary health care. The WHO states that 74% of these plants have modern indications that correlate with their traditional, cultural and sometimes ancient uses (Wanzala *et al.*, 2005), and advocate that, countries should interact with traditional medicine practitioners with a view of identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origin.

Medicinal plants serve as the main source of medicine to rural poor communities that do not have access to modern medical services, and in Africa majority of people seek medical attention from traditional medical practitioners than from medical practioners (WHO, 2005). In Ghana, the government recognises the role of traditional medical practitioners in the health delivery system, and has tried to galvanise their activities in a more formal way. It's among the first in developing a traditional medicine research plant, at the Centre for Research into Plant Medicine, and also in developing a code of ethics in traditional medicine (GSDD, 2008).

About 25% of conventional drugs are derived from medicinal plants (Spore, 1992). Investigation of some indigenous plants used locally by herbal practitioners for their antimicrobial properties has yielded positive results (Okigbo and Igwe, 2007). Many studies indicate that, in some plants there are many substances such as peptides, alkaloids, essential oils, phenols, coumarins and flavonoids which confer antimicrobial properties to them. These compounds have potentially significant therapeutic application against human pathogens including bacteria, fungi, helminthes and other parasites (Arora and Keur, 1999; Okigbo and Igwe, 2007). The search for natural products to cure diseases represents an area of great interest in which plants have been the most important source because of the prevalence of microbial resistance to existing synthetic drugs.

2.1.2 Economic benefits of phytomedicines

There has been a renewed interest in natural products globally. This interest is as a result of consumers' belief that natural products are superior over conventional medicines (Iwu *et al.*, 1999). This has therefore resulted in a dramatic increase in sales of plant-based products in the last decade. Sales of botanical products between 1995 and 1996 in the United States were approximately $ 3.2 billion (Gruenwald, 1997; Calixto, 2000).

The potential for developing plant based antimicrobials into medicines appear rewarding, from both the perspective of drug development and the perspective of phytomedicines. The immediate source of financial benefit from plant-based antimicrobials from the herbal products market offers many opportunities for those cultivating new crops, as many of the plants that are wild must be cultivated domestically today to match increasing demands (Iwu et al. 1999; Okigbo and Mmeka, 2006). For instance, *Hydrastis canadensis,* one of the top selling herbal preparations with antimicrobial properties in the US herbal market, has undergone domestication in order to supply the demand of the herbal products market (Iwu *et al.*, 1999).

2.1.3 Therapeutic benefits of phytomedicines

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources, and the discovery of penicillin led to later discoveries of antibiotics. Though soil microorganisms produce most of the clinically used antibiotics, higher plants have also been a source of antibiotics. Examples of these are the bacteriostatic and fungicidal properties of lichens, antibiotic action of allicine in *Allium sativum* (garlic) and the antimicrobial action of berberines in golden seal *Hydratis canadensis* (Trease and Evans, 2002).

Plant-based antimicrobials have enormous therapeutic potentials and they are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu, 1993). Effectiveness of plants as antimicrobial agents is hinged on their mode of action in the body. Generally, they have tropism for specific organs or systems in the body with resultant multiple effects on the body (Okigbo and Mmeka, 2006). Their actions are often beyond symptomatic treatment of the disease. For instance, *H. canadensis* not only has antimicrobial activity, but also increases blood supply to the

spleen, thus promoting optimal activity of the spleen to release mediating compounds (Murrary, 1998).

2.2 Plant products as antimicrobials

The search of biologically active compounds including antimicrobials from plants has always been of great scientistific interest. Approximately 25% of all prescriptions sold in the United States between 1999 and 2000 were from natural products (Cespedes *et al.*, 2006). A vast number of plant species have been screened for antimicrobial actions (Ogunlana and Ramstad, 1975; Leven *et al.*, 1979; Nair and Burke, 1990; Okigbo and Mmeka, 2006; Mensah *et al.*, 2006). Currently, the interest in this area has increased tremendously due partly to certain disadvantages that are associated with the use of many synthetic antimicrobial agents and to the rising incidences of multidrug resistance against these agents (Odama *et al.*, 1997). These shortcomings include their toxicity, the ability of organisms to develop resistance to the drugs previously known to be effective and loss of potency of the drug with time. On the other hand, the merits of herbal medicine over orthodox drugs have been speculated to have minimal or no side effects on the function of vital body organs and consistent potency (Nkere, 2003; Okigbo and Omodamiro, 2006).

Many plants are used as antimicrobials and their healing properties as claimed by local medicinal practitioners range from headache through skin diseases to gonorrhoea and syphilis (Akobundu, 1987; Burkill, 1997; Mensah *et al.*, 2003). Other ailments treated with these medicinal plants include asthma, cough, diarrhoea, malaria, diabetes, haemorrhage, wounds and tooth extraction. The plant parts extract mostly used as antimicrobials include the leaves, roots, barks and stems (Edeoga and Erita, 2001). Though a lot of the screened plants that have antimicrobial properties

have not been used in modern medicine, their usage in traditional medical practice is very high (Iwu *et al.*, 1999).

2.2.1 Need to explore new antimicrobials

World Health Organization (WHO) has shown great concern in documenting the use of medicinal plants from different parts of the world (Dev, 1997). Many developing countries have intensified their efforts in documenting the ethnomedical data on medicinal plants. However, an unexplored reservoir of phytochemical information is being rapidly destroyed by deforestation. About 10–20% of all plant species become extinct and above all, there is a continuing threat and pressure upon the existing plants due to fast-growing industrialization and commercial exploitation of pharmaceutical industry (Vogel, 1991).

Though a vast number of plants have not been studied for their antimicrobial properties these may become new sources of plants for antimicrobial activity. There is therefore a need for further collaborative biological screening of plant extracts in single or combination form. Phytochemicals derived from plant products are quite effective in controlling the growth of microorganism. The recent approaches of bioautography assay and high-throughput screening methods are the most suitable method to detect the antimicrobial component present in the extract (Martini and Eloff, 1998).

With the current trend on increasing awareness in traditional medicine, the plant-derived agents have been attracting much interest as natural alternatives to synthetic compounds because microbes have slowly developed resistance against antibiotics. Scientists are trying to tap the pharmaceutical and food values of these many unidentified plants. It is believed that the plants

will be a major source of new chemicals and raw materials for the pharmaceutical industry (Fauci, 1998).

2.2.2 Limitations of antimicrobials

Bacterial infections were the major cause of death in the 1940s. After the introduction of antibiotics, the prevalence of infectious diseases has gradually declined worldwide (Armstrong *et al.*, 1999). In the United States (US) antibiotics were the most frequently prescribed class of therapeutic drugs in 1981. The six most commonly prescribed drugs were erythromycin, amoxicillin, penicillin, tetracycline, ampicillin and sulfamethoxazole. However, these antibiotics have common side effects such as upset stomach, diarrhoea and vaginal candidiasis. Side effects are more often severe and may disrupt the function of the kidney, liver, bone marrow and other organs. Antibiotics can also cause mild allergic reactions such as an itchy rash or slight wheezes or some cases severe allergic reactions (anaphylaxis) that can be life threatening and usually include swelling of the throat, inability to breathe and low blood pressure (Merck, 2003).

Antimicrobial agents, including chlorhexidine, delmapinol chloride and triclosan are used to control dental plague for the prevention of dental caries and periodontal diseases. Amoxicillin and metronidazole are sometimes applied for treatment of periodontitis. Although these agents exhibit broad-spectrum of antimicrobial activity, the efficacy of some agents is now becoming unreliable due to resistance and quality of available antimicrobial products on the market (Guggenheim *et al.*, 2001). Moreover the side effects of these antimicrobial agents limit their therapeutic uses. Amongst the antimicrobials, chlorhexidine is regarded as the gold standard for plague control (Guggenheim *et al.*, 2001, Shapiro *et al.*, 2002). However, its clinical application

is limited because of its side effects, such as bitter taste, stains on teeth, hypersensitivity and generalised allergic reactions.

The evolution of resistant strains or new pathogens, the increasing number of immune compromised patients and developments of multi-drug resistant bacteria have created an urgent need for new therapeutic interventions. There has been almost complete lack of new drugs that will kill bacteria by novel mechanism of action (Cudic *et al.,* 2002). The need for the development of new antimicrobial agents is necessary and therefore phytomedicines can be considered as alternatives.

2.3 Microbial infections

An infectious disease is a clinically evident illness resulting from the presence of pathogenic biological agents, including pathogenic viruses, bacteria, fungi, protozoa, multicellular parasites, and aberrant proteins known as prions. These pathogens are able to cause disease in animals and/or plants. Infectious pathologies are also called communicable diseases or transmissible diseases due to their potential of transmission from one person or species to another by a replicating agent (Ryan and Ray 2004)

Globally, infectious disease is the number one cause of death accounting for approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries, such as the United States. Death from infectious disease, ranked fifth in 1981, became the third leading cause of death in 1992, an increase of 58%. It is estimated that infectious disease is the underlying cause of death in 8% of the deaths occurring in the US (Pinner *et al.*, 1996). The increases are attributed to increases in respiratory

tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosocomial and community acquired infections. Furthermore, the most dramatic increases of infections occur in the 25 to 44 year old group (Pinner *et al.,* 1996).

These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and renewed strategies on treatment and prevention. Proposed solutions are outlined by the US Centre for Disease Control as a multi-pronged approach that include prevention, (such as vaccination), improved monitoring and the development of new treatments. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998).

2.3.1 The socio-economic impact of infectious diseases

Diseases caused by bacteria, viruses, fungi and other parasites are major causes of death, disability and social and economic disruption for millions of people globally (WHO, 2010; UNAIDS, 2008). Despite the existence of safe and effective interventions, many people lack access to needed preventive and treatment care. The lost productivity, missed educational opportunities and high health-care costs caused by infectious diseases directly impact on family and communities.

Infections are prevalent in developing countries, where co-infections are common. The adverse impact of infectious diseases is most severe among the poorest people, who have the fewest material, physical and financial resources to draw from and limited or no access to integrated health care, preventive tools and medications (WHO, 2010). These diseases raise awareness of global vulnerability, the need for strong health care systems and the potentially broad and borderless impact of the disease (Jamison *et al.*, 2006).

Over 9.5 million people die each year due to infectious diseases and about 80% of them live in developing countries (WHO, 2010). Children are particularly vulnerable to infectious diseases. Pneumonia, diarrhoea and malaria are the leading cause of death among children under five years, with cerebral malaria which can cause permanent mental impairment. These infectious diseases are also destructive to the health of adults, causing disability, a diminished quality of life, decreased productivity or death (Jamison *et al.*, 2006; WHO, 2010).

2.3.2 Mode of transmission of microbial infections

Microorganisms and parasites may complete their life cycles by passing from one host to the next either directly or indirectly via one or more intermediate host species. Direct transmission may involve contact between hosts (e.g. venereal disease) or by specialized and unspecialized transmission stages of the organism that are picked up by inhalation (e.g. common cold), ingestion (e.g. pinworm) or penetration of the skin (hookworm). Indirect transmission can involve biting by the vector (flies, mosquito, tick) that serve as intermediate hosts or penetration by free-living transmission stages that are produced by molluscan and other intermediate hosts. In some cases the parasites is ingested when an infected intermediate host is eaten by predatory or scavenging primary host.

A special case of direct transmission arises when the infection is conveyed by a parent to its unborn offspring (egg or embryo) as can occur in syphilis and rubella and for many viral infections of arthropods, a phenomenon known as the "vertical transmission" (Fine 1975).

2.3.3 Bacteria infections

2.3.3.1 *Bacillus subtilis*

Bacillus subtilis is rod-shaped Gram-positive, catalase-positive, lactose fermenter and a spore forming bacteria that are naturally found in soil and vegetation and appears to have a low degree

of virulence to humans. It does not produce significant quantities of extracellular enzymes (subtilisin) or possess other virulence factors that would predispose it to cause infection (Edberg, 1991). However there are a number of reports where *B. subtilis* has been isolated from human infections. Infections include cases of endocarditis in drug abuse patients, pneumonia, bacteraemia, septicaemia and infection of a necrotic axillary tumor in some breast cancer patients. Isolation of *B. subtilis* has also been made from surgical wound-drainage site from a subphrenic abscess, from breast prosthesis and from two ventriculo-atrial shunt infections (Logan, 1988).

2.3.3.2 *Bacillus thuringiensis*

Bacillus thuringiensis is an aerobic, Gram-positive, rod shape and catalase positive belongs to the genus of bacillus as well as several other species that synthesize important antibiotics can cause an array of infections such as ear infections, meningitis, urinary tract infection and septicaemia. Most of these conditions occur as secondary infections in immuno-compromised hosts. They may exacerbate previous infection by producing tissue-damaging toxins or metabolites that interfere with treatment (Perreteven *et al.*, 2005).

2.3.3.3 *Proteus vulgaris*

Proteus vulgaris is Gram-negative bacilli, facultative anaerobe, oxidase negative, but catalase and nitrase positive. *P. vulgaris* is usually found to inhabit the intestinal tract of animals and also can be found in soil, water, faecal matter, and raw meat. It is considered to be an enteric pathogen, but can cause urinary tract infections, respiratory infections and wound infections (Murray, 1998; Shin *et al.*, 2005). All *Proteus* species, however, are highly motile with peritrichous flagella.

2.3.3.4 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive cocci, catalase-positive, coagulase positive, usually oxidase-negative and facultative anaerobe, which belongs to the family of Micrococcaceae and the group of staphylococci. *S. aureus* can be distinguished from other staphylococcal species on the basis of gold colony pigmentation, production of coagulase, fermentation of mannitol and trehalose, and production of heat stable thermonuclease (Ryan and Ray, 2004).

Most *S. aureus* isolates are enclosed in a polysaccharide capsule, which can be categorized into eleven different serotypes. Beneath the capsule there is a cell wall with a thick and highly cross-linked peptidoglycan layer and teichoic acid, which is typical of Gram-positive bacteria (van Wely *et al.*, 2001).

It is estimated that 20% of the human population are long-term carriers of *S. aureus* which may be found as a commensal on skin and less common in the throat but also occurs in the nose frequently (Whitt *et al.*, 2002). *S. aureus* is the most common species of staphylococcus to cause *Staphylococcus* infections and can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteraemia, and sepsis (Lowry, 1998). Their successful pathogenicity is a combination of bacterial immune-evasive strategies. One of these strategies is the production of carotenoid pigment staphyloxanthin, which is responsible for the characteristic golden colour of *S. aureus* colonies. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant which helps the microbe to evade the reactive oxygen species which the host immune system uses to kill pathogens (Liu *et al.*, 2005).

2.3.3.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, catalase positive, oxidase positive obligate aerobe, asporogenous and monoflagellated bacterium pathogen with versatile and opportunistic characteristics in terms of its genetics, metabolic potential and mechanisms of virulence. It is an obligate aerobe but can also respire anaerobically on nitrate or other alternative electron acceptors. It is a very ubiquitous microorganism, and can therefore be found in almost every part of the environments such as soil, water, humans, animals, plants, sewages and hospitals (Lederberg and Joshua, 2000).

P. aeruginosa is an opportunistic human pathogen and seldom infects healthy individuals. Instead, it often colonizes immuno-compromised patients, like those with cystic fibrosis, cancer or HIV/AIDS (Botzenhardt and Doring, 1993).

The pathogenesis of *Pseudomonas* infection is multifactorial due to a wide array of virulence determinants possessed by the bacterium. Due to its multiple and diverse determinants of virulence, it is able to cause a wide range of diseases such as bacteremic pneumonia, sepsis, burn wound infections, meningitis, septicemia, urinary tract infections, chronic lung infections, endocarditis, dermatitis and osteochondritis (Fine *et al.*,1996; Diekema *et al.*,1999).

2.3.3.6 *Enterococcus faecalis*

Enterococcus faecalis is Gram-positive cocci that form short chains or in pairs, facultative anaerobe, lactose fermenter, catalase negative and exhibit gamma-hemolysis on sheep's blood agar (Fisher and Philips, 2009). They are commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals (Ryan and Ray, 2004)

E. faecalis can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment, where there are naturally high levels of antibiotic resistance. These infections may be local or systematic and include urinary tract and abdominal infections, wound infections, bacteraemia, and endocarditis (Murray, 1998). *E. faecalis* is capable of surviving numerous environmental challenges including extremes of temperature and the presence of bile salts. Due to their acquired resistance to multiple antibiotics, these bacteria have become a major health problem.

2.3.3.7 *Streptococcus pyogenes*

Streptococcus pyogenes is a gram-positive bacterium that usually grows in pairs or chains. It has been classified as a beta-hemolytic streptococcus because when cultured on a blood agar plate the red blood cells are ruptured by the bacteria and are therefore known as flesh eating bacterium (Bessen, 2009).

S. pyogenes is a free-living organism, however, it has quite narrow ecological niche. Its known biological host is the human and like many other streptococci, lacks an environmental reservoir of importance. Thus, transmission is direct human-to-human, which mostly occurs through respiratory droplets or skin contact for its maintenance. Streptococcal organisms tend to reside extra-cellularly, but can also be found within the mammalian host cells of the respiratory tract (Osterlund *et al.*, 1997; Cleary *et al.*, 1998).

S. pyogenes causes a wide array of infections and is known to be responsible for a minimally estimated 616 million cases of throat infections (pharyngitis, tonsillitis) worldwide per year and 111 million cases of skin infections (primarily non-bullous impetigo) in children living in developed countries (Carapetis *et al.*, 2005).

2.4 Fungal infections

Fungal infections are diseases caused by the growth of fungi in or on the body. The incidence of fungal infections has increased significantly over the past decades and it is expected to continue to increase in the foreseeable future (Perlroth *et al.,* 2007). The increase in fungal infections is mainly due to the expanding population of immuno-suppressed persons. Nosocomial fungal infections are becoming more common and are also significant cause of morbidity and mortality (Ostrosky-Zeichner *et al.*, 2002).

In most healthy people fungal infections are mild, involving only the skin, hair, nails or other superficial sites and they clear up spontaneously. They include the ringworm caused by *Microsporum canis* and athlete's foot caused by *Trichophyton rubrum*. Fungi can also invade internal organs of the body, especially the lungs, where the infections resemble pneumonia or pulmonary tuberculosis. These infections usually occur in people whose immune system has been suppressed by diseases such as acquired immunodeficiency syndrome (AIDS), by anticancer drugs, or by radiation, patients being treated with steroidal hormones and people with diabetes and those being treated with antibiotic drugs for a long time (Ostrosky-Zeichner *et al.*, 2002). Two fungi often found in such cases are *Cryptococcus* and *Aspergillus,* which are opportunistic pathogens. Many drugs are available for treating fungal infections. These include both intravenous and oral drugs, and many agents are available for topical application including clotrimazole, ketoconazole, miconazole etc.

2.4.1 *Candida albicans*

Candida albicans is one of the predominant pathogens in fungal infection. It is a ubiquitous diploid asexual fungus (yeast) commonly found on the mucus membrane of mammals. In humans, they live in the mouth and gastrointestinal tract. Under normal circumstances, *C. albicans* lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis (Ellepola and Samaranayake, 2000). Candidiasis also known as "thrush" is a common condition that is usually easily cured in people who are immuno-competent. To infect host tissue, the usual unicellular yeast-like form of *C. albicans* reacts to environmental cues and switches into an invasive, multicellular filamentous form. In recent years, there has been a marked increase in treatment failures of candidiasis in patients receiving a long–term antifungal therapy. These effects are clearly observed in HIV–infected patients with oropharyngeal candidiasis infection (Fichtenbaum *et al.*, 2000).

2.5 Wounds

A wound is a disruption of the continuity of tissues produced by external force. It may also be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissues (Nalwaya *et al.*, 2009). The skin serves as a protective barrier against the outside world, and therefore any break in it must be rapidly and efficiently mended (Martin, 1996).

2.5.1 Classification and types of wounds

Wounds are generally classified by their level of chronicity as either acute or chronic. Acute wounds usually follow trauma or inflammation and are caused by external damage to intact skin and usually heal within six weeks (Bowler *et al.*, 2001). Acute wounds include surgical wounds, bites, burns, minor cuts and abrasions and more severe traumatic wounds such as lacerations,

crush or gunshot injuries. In contrast, chronic wounds, fail to heal after six weeks and have pathological characteristic underlying endogenous mechanisms associated with a predisposing condition that compromises the integrity of dermal and epidermal tissue that inhibit or delay healing (Bowler *et al*., 2001; Kumar and Leaper, 2008). Chronic wounds include pressure ulcers, venous leg ulcers and diabetic foot ulcers (De la Torre and Chambers, 2008) and these have negative economic impact.

2.5.1.1 Chronic wounds

These are wounds that have failed to progress through normal stages of healing and therefore enter a state of pathogenic inflammation (Menke *et al*, 2007). These wounds require prolonged time to heal, do not heal or recur frequently and are major cause of physical disability. This presents a significant burden to patients and the National Health Scheme (NHS) in both developed and developing countries. In the UK, 200,000 patients had chronic wounds between 2000 to 2001 and this had a negative impact on their quality of life (Franks and Morgan, 2003). Common symptoms include pain, exudate and odour, and these are frequently associated with poor sleep, loss of mobility and social isolation. The cost to the NHS of caring for patients with chronic wounds between 2005-2006 was estimated at $2.3 to 3.1 billion per year which is about 3% of the total estimated out-turn expenditure on health ($89.4 billion) for the same period in the US (Hostetler, 2006)

Venous leg ulcers

This is most commonly caused by venous hypertension resulting from valvular incompetence in the superficial, deep or perforating veins. Sustained venous hypertension causes swelling, restricted blood flow and damage to the skin and other tissues. The prevalence rates of venous

leg ulcers generally fall in the range of 1.2 to 3.2 per 1,000 people in Europe and North America and this increase with age (Graham, 2003). A study conducted between 2001 and 2002 in UK showed that the prevalence of venous ulceration in a local population was 0.3/1,000 in men and 0.5/1,000 in women (Moffatt, 2004).

Pressure ulcers

Pressure ulcer is an area of damage to the skin and underlying tissue that is caused by unrelieved pressure, friction and/or shear forces. A severe pressure ulcer is susceptible to infection and may be life-threatening. It is estimated that one in five hospital inpatients has a pressure ulcer (Clark, 2004). In the UK, about 400,000 individuals develop a new pressure ulcer annually and therefore increase the cost to the NHS because prolonged hospital treatment is needed in serious cases and those at risk must be protected (Bennett, 2004).

Foot ulcers

Foot ulceration is a common complication of diabetes. Gradual loss of sensation renders the foot susceptible to even minor trauma. Susceptibility to infection and peripheral vascular disease inhibit healing once injury has occurred and this may lead to gangrene and amputation. The age-adjusted rate of lower-limb amputation is estimated to be 15 times higher in individuals with diabetes than in the general population in Europe (Armstrong, 1997). In the UK it is estimated that there are about 64,000 individuals with active foot ulceration at any time and 2,600 amputations annually in patients with a foot ulcer (Gordois, 2003)

2.5.2 Wound infections

Wound infections are the invasion of tissues by one or more species of microorganisms, but usually the most common microorganisms are the *S. aureus*, *S. epidermidis*, *P. aeruginosa S.*

pyogenes, K. pneumonia and some species of *Candida* (White *et al.*, 2001; Ratliff *et al.*, 2008). These bacteria may affect all of the processes of wound healing (inflammation to remodelling) and they are always present in the surface and deep tissues of all wounds (Jones *et al.*, 2008). Through different stages of healing, these microbes seem to interact with acute and chronic wounds in similar but distinct ways. Their infection triggers the body's immune system; causes inflammation, tissue damage and slows the healing process (Kingsley, 2001). This infection may be localised or confined to wound site or may spread to other organs and/or into the blood and cause a systemic infection (septicaemia) (White *et al.*, 2001). The symptoms of wound infections are redness or discolouration, swelling, high temperature, pain, tenderness, pus drainage and itching (White *et al.*, 2001).

Wound infection may be due to many factors including the number of bacteria per gram of tissue, the pathogenicity and virulence of the organism and the ability of the host to counteract with an efficient immune system (Edwards & Harding, 2004). When the bacterial load exceeds 10^5 organisms per gram of tissue or the immune system becomes suppressed, infection develops (Robson *et al.*, 1999). In some cases, the beta-haemolytic streptococci cause infection at significantly lower levels which delayed healing response regardless of the bacterial count (Edwards and Harding, 2004). Thus, the number of organisms in a wound cannot always be used as an indication of invasiveness (McGuckin *et al.*, 2003).

Wound infection is a major cause of morbidity and mortality that is associated with subsequent high costs worldwide. The management of bacteria wounds infection is a problem which places a considerable drain on healthcare systems and major cause of increased length of hospital stay by an average of ten days due to the delay in recovery which results in doubling of hospital costs

(Plowman, 2000). Infections of surgical wounds are one of the most common causes of hospital acquired infection (HAI) in the United Kingdom. The cost of HAIs resulting from infected wounds is estimated to be £162 million per year (Plowman *et al.*, 1999). It is also estimated that, surgical site infections are the most common infections among post-operative patients in the United States, accounting for approximately 25% of all nosocomial infections (Patel *et al.*, 2008).

2.5.3 Wound healing process

Wound healing process or wound repair is a complex and dynamic process of restoring cellular structure and tissue layers. It is also an intricate process in which the skin (or another organ-tissue) repairs itself after injury (Nguyen *et al.*, 2009). In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) exists in steady-state equilibrium, forming a protective barrier against the external environment. Once the protective barrier is broken, the normal (physiologic) process of wound healing is immediately set in motion.

Wound healing may be affected by many factors and therefore can be categorized into extrinsic and intrinsic factors. The extrinsic factors impinge on the patient from the external environment and these include mechanical stress, temperature, debris, infections, chemical stress, medications and others such as smoking and alcohol abuse. Intrinsic factors directly affect the performance of healing by bodily functions through the patient's own physiology or condition. These include age of the individual, health and nutritional status (Gosain and Dipietro, 2004) The phases of normal wound healing include haemostasis, inflammation, proliferation, and remodelling. However, the classic model of wound healing is divided into three phases: the inflammation,

proliferation and remodelling phases (MacKey and Miller, 2003). Each phase of wound healing is distinct, although the process is continuous, with each phase overlapping the next.

2.5.3.1 Haemostasis

During haemostasis, injured tissue initiates a response that first clears the wound of devitalized tissue and foreign materials, setting the stage for subsequent tissue healing and regeneration. The initial vascular response involves a brief and transient period of vasoconstriction and haemostasis. The intense period of vasoconstriction last for 5 to10 minutes, followed by active vasodilation which is also accompanied by an increase in capillary permeability. Platelets aggregated within a fibrin clot secrete a variety of growth factors and cytokines that set the stage for an orderly series of events leading to tissue repair (MacKey and Miller, 2003; Singer and Clark, 1999).

2.5.3.2 Inflammatory phase

In this phase, bacteria and debris are phagocytized and removed, and growth factors such as platelet derived growth factors (PGF), fibroblast growth factors (FGF), keratinocyte growth factors (KGF), epithelial growth factors (EGF) and cytokines are released. These growth factors are responsible for the migration and division of cells involved in the proliferative phase. This presents itself as erythema, swelling, high body temperature and often associated with pain. The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. The neutrophils engulf debris and microorganisms, providing the first line of defence against infection. Neutrophil migration ceases after the first few days post-injury if the wound is not contaminated. If this acute inflammatory phase persists, due to wound hypoxia, infection, nutritional deficiencies, medication use, or other factors related

to the patient's immune response, it can lead to tissue damage (Stadelmann *et al.*, 1998; Houghton *et al.*, 2005).

In the late inflammatory phase, monocytes converted in the tissue to macrophages, which digest and kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Macrophages begin the transition from wound inflammation to wound repair by secreting a variety of chemotactic and growth factors that stimulate cell migration, proliferation and formation of the tissue matrix (Houghton *et al.*, 2005).

2.5.3.3 Proliferative phase

The proliferative phase is responsible for actually closing the lesion. The phase involves re-epithelialisation (wound recovery with new epithelium), which starts few hours after the lesion and includes the movement of epithelial cells from the margins and the epidermal appendices located in the lesion core, fibroplasia and angiogenesis, which comprise of the granulation tissue responsible for taking over the area of the damaged tissue for about 4 days after the lesion. Fibroblasts produce a new extracellular matrix required for cell growth, whereas new blood vessels carry oxygen and nutrients required for local cellular metabolism (Singer and Clark, 1999).

The duration of this phase is dependent on the size of the wound. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances essential to wound repair, including glycosaminoglycans (mainly hyaluronic acid, chondroitin-4-sulphate, dermatan sulphate, and

heparin sulphate) and collagen. These form an amorphous, gel-like connective tissue matrix necessary for cell migration (Stadelmann *et al.*, 1998).

2.5.3.4 Remodelling phase

In this phase of the healing process there is an attempt to recover the normal tissue structure. It is a phase marked by maturation of elements and affections to the extracellular matrix, leading to proteoglycan and collagen deposits. In a late phase, fibroblasts of granulation tissue are transformed into myofibroblasts and behave as contractile tissue that responds to agonists that stimulate the smooth muscles. At the same time, there is extracellular matrix reorganization, which transforms the transient matrix into a definitive one, whose phenotypic intensity, observed in scars, reflects the intensity of the phenomena that occurred and the level of balance between them (Gabbianni *et al.*, 1972).

As a result of maturation and remodelling processes, most vessels, fibroblasts and inflammatory cells disappear from the wound site through a process of migration, apoptosis or other unknown death cell mechanisms. Conversely, if there is persistent cellularity at the site, there will be formation of hypertrophic scars or keloids (Arnold and West, 1991).

2.5.4 Natural products used as wound healing agents

Medicinal plants that possess wound healing activity perform their action through their bioactive compounds agents they possess. Not every phytochemical has wound healing activity, however, the following compounds or secondary metabolites are believed to assist in the wound healing process (Araújo *et al.*, 2008).

Polyphenol compounds have the potential as natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelators (Nagulendran *et al.*, 2007). Most polyphenols including flavonoids have been found to increase the activity of catalase and glutathione peroxidase, which detoxify H_2O_2 by converting it to O_2 and H_2O (Oaka *et al.*, 2005). They are also known to stimulate wound healing of incision and dead space wounds and reduce the period of epithelialization in the excision wounds (Azeez *et al.*, 2007; Sasidharan *et al.*, 2010). And this can increased tensile strength and collagen content for better epithelialization. Flavonoids have been known to possess potent antioxidant and free radical scavenging effect, which is believed to be one of the most important components of wound healing (Shenoy *et al.*, 2009). The high mobility of the electrons in the benzenoid nucleus of flavonoids accounts for both their antioxidant and free-radical scavenging properties (Havsteen, 2002). Studies have shown that antimicrobial activities of plants can also be attributed to their flavonoid content (Owoyele *et al.*, 2005) and may be helpful in prevention of wound infection.

Tannins are also phenolic compounds that typically act as astringents and are found in a variety of herbal products used as wound healing agents. Their astringent and antimicrobial properties are responsible for wound contraction and increased rate of epithelialization (Panda and Tripathy, 2009). Medicinal plants that are known and or used for their wound healing or anti-inflammatory properties tend to have high tannin contents (Araújo *et al.*, 2008; Agyare *et al.*, 2011).

Alkaloids are known to promote wound healing process due to their antioxidant and antimicrobial activities (Sachin *et al.*, 2009). Aqueous extracts from *Symphytum*

asperum and *Symphytum caucasicum* contain allantoin, claimed to be a cell proliferation stimulating agent responsible for their wound-healing property (Barbakadze *et al.*, 2009).

Saponins are known to promote wound healing process due to their antioxidant and antimicrobial activities (Sachin *et al.*, 2009). For example, asiaticoside a saponin is thought to be one of its active constituents of *Centella asiatica*. And this can increased tensile strength and collagen content for better epithelialization (MacKay and Miller, 2003).

Topical application of these compounds or extract with antimicrobial and antioxidants properties on wounds have shown to improve significantly wound healing and protect tissues from oxidative damage (Thiem and Grosslinka, 2003). Antioxidants therefore enhance the healing of infected and noninfected wounds by reducing the damage caused by oxygen radicals (Martin, 1996).

2.5.5 Models for determination of wound healing agents

2.5.5.1 Excision wound model

Excision wounds are used to evaluate the rate of wound contraction and epithelialization for *in vivo* models (Nalwaya *et al.*, 2009). The excision wound is done by excising the full thickness of circular skin of an anaesthetized animal (Karodi *et al.*, 2009). The wound contraction is assessed by tracing the wound area first on transparent paper and subsequently transferring to a graph paper (Barua *et al.*, 2009). In excised wound, since the edges are not in contact with each other, contraction and epithelialization are necessary for the repairing process (Ghasemi *et al.*, 2010). Epithelialization and wound

contraction are the two parameters to be studied in case of excision wound (Malviya *et al.*, 2009).

2.5.5.2 Incision wound model

Incision wound model is an *in vivo* model used for the evaluation of wound breaking strength (Ghasemi *et al.*, 2010). In this model, two longitudinal paravertebral incisions are made through the skin and cutaneous muscles at a distance 1.0 cm from the midline on either side of the vertebral column of anaesthetized rat (Annan and Dickson, 2008). The parted skin is sutured and the skin breaking strength of the wound is measured after ten days of wound induction (Barua *et al.*, 2009).

2.5.5.3 Dead space analysis

Dead space is used for the assessment of *in vivo* models. This can be made by making a pouch through a small cut in the skin of the animal (Paschapur *et al.*, 2009). A polypropylene tube is implanted subcutaneously beneath the skin. The day of the wound creation is considered as day zero. On the 10^{th} day, the animals are sacrificed by overdose of anaesthesia, the polypropylene tube are carefully removed and dried in an oven at 60°C to a constant weight, and the weight is recorded. The level of increase (%) in the weight of granuloma tissue formed is calculated relative to the control (Okoli *et al.*, 2009; Azeez *et al.*, 2007). Dead space wound is important to evaluate the physical and mechanical changes in the granuloma tissue (Paschapur *et al.*, 2009). In dead space wound, granulation tissue dry weight, breaking strength and hydroxyproline content are the important parameters to consider (Malviya *et al.*, 2009).

2.5.5.4 Chorioallantoic membrane model

This is for *in vitro* model using embryonated chicken eggs. A nine day old embryonated eggs are selected and a small window is made in the shell (Barua *et al.*, 2009). Albumin is removed on the 4th day after fertilization to drop the embryo away from the shell and to allow the chorioallantic membrane to develop in a way that is accessible to treatment (Nalwaya *et al.*, 2009). Through the window, a sterile disc treated with the extract of interest is placed inside the egg at the junction of two blood vessels. The window is resealed and the egg is incubated at 37°C for three days. The window is then be opened and the growth of new capillary observed (Barua *et al.*, 2009).

2.6 Antioxidant assay (Free radical scavenging assay)

An antioxidant is an agent that inhibits oxidation of a susceptible substrate. They are metabolites found naturally in the body and in plants. Plants produce wide range of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid tocopherols and tocotrienols, which mobs up the free radicals as result of respiratory burst during the inflammation phase in wound healing process.

There is no single method which is adequate for determining the antioxidant capacity of compounds as different methods may give diverse results and therefore several methods based on different mechanisms must be used to determine the antioxidant activities (Wod *et al.*, 2006). Antioxidant capacity assays can be broadly classified as electron transfer (ET) and hydrogen atom transfer (HAT) based assays. The majority of HAT assays is kinetics based, and involves a competitive reaction scheme in which antioxidant and substrate compete for peroxyl radicals thermally generated through the decomposition of compounds.

2.6.1 Electron Transfer (ET) based assays

The ET−based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. ET assays is further grouped into Trolox equivalent antioxidant capacity (TEAC) assay, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu and Ferric reducing ability of plasma (FRAP) methods, *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) assay and the Cu(II) reduction capacity assay, each using different chromogenic redox reagents with different standard potentials (Resat *et al.*, 2007). The electron transfer reaction of these methods is as shown below.

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

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

2.6.2 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay

This assay is one of a few stable and commercially available organic nitrogen radicals and has a UV visible absorption range of 515 to 520 nm. Upon reduction, colour of the solution fades or bleaches (purple to yellowish), thus reaction progress of the mixture is monitored at 517nm for 30 minutes by a spectrophotometer until the absorbance is stable. The percentage of the DPPH remaining is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as inhibitory concentration $_{(50)}$ (IC_{50}). The time needed to reach the steady state with (IC_{50}) concentration is calculated from the

kinetic curve and defined as T_{IC50}. The DPPH assay is technically simple (Resat *et al.*, 2007; Agyare *et al.*, 2009).

2.7 Methods for determining antimicrobial activity

2.7.1 Susceptibility testing

The antimicrobial susceptibility test (AST) is an essential technique in modern biological science. It is used to determine resistance of certain microbial strains to different antimicrobials and in pharmacology research, to determine the efficacy of novel antimicrobials from biological extracts against different microorganisms. Though this could lead to variations in results obtained, yet the methods are widely employed nowadays to screen plant extracts for antimicrobial activity and to determine minimum inhibitory concentration (MIC) of antimicrobial agents.

Although current standard AST methods approved by various organizations like American National Committee for Clinical Laboratory Science (NCCLS), British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) exist, for guidelines of antimicrobial susceptibility testing of convenient drugs, these might not be exactly applicable to plant extracts and modifications have to be made (Hammer *et al.*, 1999). Antimicrobial susceptibility standard tests are broadly classified into diffusion and dilution methods for convenience. Diffusion tests include agar well diffusion, agar disk diffusion, poison food technique, and bio-autography, while dilution methods include agar dilution, broth micro-dilution and broth macro-dilution technique (Tenover *et al.*, 1995).

2.7.2 Diffusion methods

2.7.2.1 Agar disk diffusion assay

The agar disk diffusion method of antimicrobial test was developed in 1940 (Heatley, 1944). The procedure which was accepted by NCCLS and widely used nowadays, is a modification of that described by Bauer, Kirby, Sherris and Truck (commonly known as Kirby-Bauer test) (Bauer, *et al.*, 1959a, 1966b).

The agar disk diffusion technique has been widely used to assay plant extracts for antimicrobial activity (Freixa *et al.*, 1996; Salie *et al.*, 1996; Ergene *et al.*, 2006). In this method, sterilized filter papers disks (Whatmann No. 1) with diameter of 6 mm are saturated with filtered plant extract of desired concentration (Salie *et al.*, 1996). The impregnated discs are then placed onto the surface of a suitable solid agar medium like Mueller-Hinton, trypton soy agar (Lourens *et al.*, 2004) or nutrient agar (Doughari, 2006) pre-inoculated with test organisms. The standard inoculum size is 1 x 10^6 colony forming unit per millilitre (cfu/mL) of bacteria for inoculating diffusion plates (Baris *et al.*, 2006).

Some researchers dry the impregnated paper disk before putting on the inoculated plates (Lourens *et al.*, 2004; Salie *et al.*, 1996: Nostro *et al.*, 2000; Baris *et al.*, 2006). The drying time of impregnated paper disk varies among researchers from 2 h to overnight under a laminar flow cabinet (Basri and Fan, 2005). Plates are then incubated for 24 h at 37°C for bacteria and 48 h at 25°C for fungi (Salie *et al.*, 1996; Baris *et al.*, 2006). After incubation, the diameter of the zone of growth inhibition is measured to the nearest whole millimeter at the point wherein there is a prominent reduction of 80% growth.

2.7.2.2 Agar well diffusion assay

The principle of agar well diffusion is similar to that of agar disk diffusion assay. A standardized concentration of inoculum with fixed volume is spread evenly on the surface of gelled agar plate. A hole of about 6 to 12mm is aseptically punched in the gelled agar using a sterile cork borer. A fixed volume of plant extract is then introduced into the agar well and incubated at optimum temperature and duration depending upon the test microorganism (Mbata *et al.,* 2006; Norrel and Messely, 1997).

2.7.3 Dilution methods

2.7.3.1 Broth micro-dilution assay

The microliter plate or broth micro-dilution method has provided potentially useful technique for determining minimum inhibitory concentration (MIC) of a large number of test samples. Minimum inhibitory concentration is the lowest concentration of antimicrobials that will inhibit the visible growth of microorganisms after overnight incubation. It is important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. In the microliter plate method, a stock solution of the extract is first obtained in a suitable solvent (Grierson and Afolayan, 1999) or in DMSO (Salie *et al.*, 1996; Nostro *et al.*, 2000; Baris *et al.*, 2006). Mueller-Hinton broth or water is often used as a diluent in the wells of the microtitre plate before transferring an equal volume of stock solution to the plate. The EUCAST (2003) recommends certain supplemented Mueller-Hinton broth for non-fastidious microorganisms.

Two fold serial dilutions are made from the first well to obtain a concentration range. Minimum inhibitory concentration range of 5 to 8 serial dilutions can represent achievable concentrations for the used antimicrobials (Mendoza, 1998). The inoculum size for this procedure is usually

$1x10^6$cfu/mL (Lourens *et al.*, 2004; Basri and Fan, 2005). Some researchers have used microbial culture with an optical density of 0.4 at 620 nm or a 12 h broth culture adjusted to a 0.5 McFarland turbidity standard (Baris *et al.*, 2006). An equal volume of microbial culture is added to the wells and incubated at 37°C for 24 h (Lourens *et al.,* 2004). After incubation, plates are examined for changes in turbidity as an indicator of growth. The well with the least concentration in which there was no visible growth is recorded as the MIC of the extract. Indicators such as tetrazolium salts or resazurin dye (Umeh *et al.*, 2005) or spectophotometry are used to determine presence of growth (Devienne and Raddi, 2002). For the spectophotometric method, the absorbance is usually at 620 nm with a blank as negative control (Salie *et al.,* 1996). Concentration with sharp decline in absorbance value (Devienne and Raddi, 2002) or the lowest concentration which gives a zero absorbance reading is the MIC of the plant extract or the test phytochemical (Salie *et al.,* 1996).

2.7.3.2 Broth macro-dilution assay

The basic principle of this assay is the same as the broth micro-dilution assay. But the test is performed in a test tube. In macro-dilution assay, a set of test tubes with different concentrations of plant extract with the same volume are prepared. Tubes are inoculated with equal volumes of test microorganisms of standard inoculums size. After incubation, tubes are examined for changes in turbidity as an indicator of growth. MIC of the plant extract or the test phytochemical is determined using the various methods described above (Matsumoto *et al.*, 2001).

2.7.4 Bio-autography

Bio-autography is a very convenient way of testing plant extracts and pure phytochemical compounds for their effect on both human pathogenic and plant pathogenic microorganisms. It

can be employed in the target directed isolation of active constituents (Hostettmann, 1999). Bio-autography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components (Nostro *et al*., 2000; Schmourlo *et al*., 2004). Bio-autography overcomes the challenge of isolating antimicrobial compounds from crude extracts with complex chemical components by simplifying the process of their isolation and identification. It relatively uses very little amount of sample which is ideal for plant extracts and also allows the determination of the polarity of the active compounds (Runyoro *et al*., 2006; Silva *et al*., 2005) compared to different methods of AST bio-autography is a practical and reproducible test which is easy to perform.

Bio-autography localizes antimicrobial activity on a chromatogram usually have three approaches, which are direct bio-autography, where the microorganism grows directly on the thin layer chromatographic (TLC) plate, contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and agar overlay bio-autography, where a seeded agar medium is applied directly onto the TLC plate. These procedures are based on the agar diffusion technique, whereby the antimicrobial agent is diffusion from the thin layer or paper chromatogram to an inoculated agar plate. The zones of growth inhibition are then visualised by suitable growth indicator such as MTT (Hamburger and Cordell, 1987).

Paper chromatography followed by bio-autography was first used by Goodall and Len, (1946) to estimate the purity of penicillin. In this method, developed paper chromatogram was placed onto the inoculated agar layer enabling the diffusion of antibiotics from paper to agar containing microorganisms. Thin layer chromatography-bio-autography was introduced by Fisher and

Leutner. (1961). This method is usually grouped into three categories, agar diffusion or contact bio-autography, immersion or agar overlay bio-autography and direct bio-autography (Rios *et al.*, 1998).

2.8 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products (Cannell, 1998). This technique is currently used in various analytical techniques as the main choice for finger-printing studies for the quality control of herbal plants and products (Fan *et al.*, 2006). Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize the active compound. The bioactive compound is often present as minor component in the extract and the resolving power of HPLC is ideally suited to the rapid processing of such multicomponent samples on both an analytical and preparative scale. Many HPLC instruments now are modular in design and comprise a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer.

Chemical separations are done using HPLC by utilizing the compounds that have different migration rates given a particular column and mobile phase. The extent or degree of separation is usually determined by the choice of stationary phase and mobile phase, identification and separation of phytochemicals is often done using isocratic system.

2.9 Plant under study

2.9.1 Description

Clausena anisata (Willd) Hook. f. ex Benth also known as the Horsewood or Maggot killer belongs to the family Rutaceae. It is a forest undergrowth shrub or small tree that grows in the

Savannah region of West Africa. It is locally known as 'Sasadua' by the Akans, 'Ayida/Ayra' by the Ewes' and 'Samanyobli' by the Gas'of Ghana. The tree can grow up to about ten (10) meters high in or on the margins of evergreen forests. The leaves are pinnate, compound with 10 to 17 alternate and with a terminal one. They are densely dotted with glands and have a strong scent when crushed. It can be compared with anise seed. The branched inflorescences originate with an axillary spray. The small white flowers have orange-yellow stamens. Flowering time is normally from August to November (Iwu, 1993; Hutchings, 1996).

2.9.2 Chemical constituents

Phytochemical studies performed on the different parts of *C. anisata* revealed diverse bioactive compounds of which some have been isolated. And compounds such as coumarins, which include furanocoumarins, imperatorin, oxypeucedanine and chalepin have been isolated from the roots and stem bark (Chakraborty *et al.*, 1995; Mester *et al.*, 1997). Clausenol, a carbazole alkaloid has also been isolated from alcoholic extract of stem bark (Devienne and Raddi, 2002).

Studies on the leaf have revealed the presence of coumarins, limonoids, reducing sugars and alkaloids in the plant (Chakraborty *et al.*, 1995; Ito *et al.*, 2000). Characterization of leaf essential oils showed that the oils were of β-pinene and sabinene chemotypes (Senthikumar and Venkatesalu, 2009). The steam distillation of fresh leaves yields sweet smelling, brownish-yellow oil of which the major component is estragole (Okunade, 1987) and that the composition pattern of essential oils are affected by geographical and climatic conditions (Lahlou, 2004).

2.9.3 Medicinal uses

In West Africa countries such as Ghana and Nigeria, a mixture of *C. anisata*, *Afraegle paniculata* and *Azadirachtha indica* is taken against gut disturbances and also the concoction

used as antimalarial agent (Uwaifo, 1984). Decoction from the leaf is used as effective remedies against worm infections, respiratory ailments, heart disorders, hypertension, malaria fever, rheumatism, insanity, convulsion, arthritis and other inflammatory conditions, crushed leaves are used to treat wounds (Hutchings *et al.,* 1996). In Tanzania and other parts of West Africa, traditional healers use paste prepared from leaves for the treatment of oral candidiasis and fungal infections of the skin (Hamza *et al*., 2006) and management of epilepsy and convulsion (Moshi *et al.,* 2005). In South Africa, the leaves are used to control high blood pressure. In some parts of Africa and in the Philippines, the burning of fresh leaves is utilized to repel mosquitoes (Okunade, 1987). The pounded roots, with lime and Guinea grains, are applied to treat rheumatism and other pains in Nigeria, where also the leaves are considered anthelmintic (Okunade, 1987). In some parts of West Africa, the concoction of the stem bark and root is considered as a remedy for cough (Hutching *et al.,* 1996).

2.9.4 Biological activity

Various extracts and compounds from *C. anisata* have been found to possess varying biological activities. Pharmacological properties such as the antimicrobial activity of the stem bark (Chakraborty *et al.,* 1995), antidiabetics property of the root and stem bark (Ojewole, 2002), antiparasitic and central nervous depressant activity of the leaf and stem bark (Makanju, 1983) have been reported. Ethnopharmacological studies have revealed that the mechanism of antidiabetic action of the root bark extract appears to involve the stimulation of β-cells of the pancreas to secrete insulin (Marles and Farnsworth, 1995). Methanol root extract possesses hypoglycaemic property (Ojewole, 2002).

Fig. 2.1: Leaves and aerial parts of *C. anisata.* (Source: Nicholas Agyepong, October, 2011)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

The air dried leaves of *Clausena anisata* (KNUST/HML/2012/L061).

3.1.2 Animal used

Sprague Dawley rats (170-195g)

3.1.3 Chemicals

Name	Manufacturer/Company
Ethanol (96% v/v)	GPR, BDH, Poole, UK.
Methanol (99.9% v/v)	GPR, BDH, Poole, UK.
Chloroform (99.9% v/v)	AR, Merck, UK.
Petroleum ether (80% v/v)	Scharlau chemicals Ltd, Poole, UK.
Acetone (99.8% v/v)	BDH, Prolabo, France.
Ethyl acetate (99.8% v/v)	BDH, Prolabo, France
Tween (20)	Atlas chemicals industries Inc, UK.
Ketamine hydrochloride injection (500mg/mL)	Laboratorio Sanderson, Santiago, Chile.
Sodium chloride intravenous infusion (0.9% w/v)	Kabi Pvt Ltd Pune, India.
Methylthiazolyl tetrazolium chloride	Sigma-Aldrich, M5655, USA
Fehling's solution	GPR, BDH, Poole, UK
Sulphuric acid (98.5% v/v)	GPR, BDH, Poole, UK
Hydrochloric acid (36% v/v)	GPR, BDH, Poole, UK

Sodium hydroxide (96% v/v)	GPR, BDH, Poole, UK
Dragendorff (50% v/v)	AR, Merck, UK
Ammonia (30% v/v)	AR, Merck, UK
Iron (II) chloride (97% v/v)	GPR, BDH, Poole, UK
Anisaldeyde reagent (98% v/v)	Aldrich, Germany.

3.1.3 Reference Compounds

	Manufacturer/Company
Ciprofloxacin hydrochloride (99.9% w/w)	Zhejiang Xin Ltd, China.
Ketoconazole (99% w/w)	Aryton, Drug, Accra, Ghana.
2, 2-Diphenyl-1-picryhydrazyl (90% w/w)	Sigma Aldrich, Damstadt, Germany.
N-Propyl gallate (99% w/w)	Sigma, USA.
Silver sulphadiazine cream (1% w/w)	Arytons Drugs, Accra, Ghana

3.1.4 Instruments and Equipment/Glass wares

Name	**Manufacturer/ Company**
Rotary evaporator (R-210)	Buch, Germany.
Hot air oven	Sanyo, OMT Oven, Gallenkamp, UK.
Gallenkamp Plus II cooled incubator	Gallenkamp, UK.
Laminar air flow cabinet	Model T2 2472 Skan AG, Switzerland.
Thermostatically controlled water bath	R76 New Brunswick, Edison N.J., USA.
colony counter	Gerber Instruments, AG, Holland.
Digital Camera	Nikon CoolPix L11 L10, UK.
UV Spectrophotometer	Cecil, 2000 series, Basildon, Ltd.UK
Electronic weighing balance	Ohaus corporation, Pine Brook, NJ, USA.
No. 5 Cork borer	Gerber Instruments, AG, Holland.

Portable autoclave	Basildon, Ltd.UK.
Laboratory Milling Machine	Christy and Norris Limited, Chelmsford, UK.
TLC plate (silica gel 60 F_{254})	Merck, K GaA, Damstadt, Germany.
500mL separating funnel	GMBH, Werthiem, Germany.
Beakers (50, 250,500, 600 mL, 1L)	GMBH, Werthiem, Germany.
Test tubes	GMBH, Werthiem, Germany.
1mL, 10 mL dropping pipette	GMBH, Werthiem, Germany.
250 mL, 500 mL conical flask	GMBH, Werthiem, Germany.
Chroma tank	Matheson Inc. Cambridge, UK.
Capillary tube	GMBH, Werthiem, Germany.
Petri dish	GMBH, Werthiem, Germany.
HPLC Pump	Spectra system, USA.
Autosampler	Spectra system, AS3000, Germany.
UV Detector	Spectra system, UV1000, USA.
Degasser	Spectra system, SCM1100, USA.
Column	Hypersil Gold, C_{18} 150 x4.6mm, USA.

3.1.6 Cell cultures

3.1.6.1Typed cultures

The standard strain of *Staphylococcus aureus* NCTC 10788, *Bacillus subtilis* NCTC 10073, *Pseudomonas aeruginosa* ATCC 4853, *Proteus vulgaris* ATCC 4175, *Bacillus thuringiensis* ATCC 13838, *Enterococcus faecalis* ATCC 29212 were obtained from National Collection of Typed Cultures, U.K and American Typed Collection Cultures, USA.

3.1.6.2 Clinical cultures

The clinical cultures of *Staphylococcus aureus, Enterococcus faecalis* and *Candida albicans* were obtained from the Bacteriology Unit at Komfo Anokye Teaching Hospital (KATH), Kumasi. *Pseudomonas aeruginosa, Proteus vulgaris* and *Streptococcus pyogenes* were also obtained from the Microbiology Unit of Kumasi Centre for Collaborative Research (KCCR).

3.2 Methods

Solvent extractions, fractionation, phytochemical screening, thin layer chromatography, bio-autography, as well as infrared investigations, antioxidant assay, antimicrobial tests and excision wound healing methods were undertaken to achieve the set objectives.

3.2.1 Maintenance of cell cultures

The cultures were maintained on Muller-Hinton agar slants and kept in the refrigerator at 4°C as the master cultures. All necessary confirmatory tests were also performed on all the test organisms to ascertain their authenticity. During the experimental period, the stock were sub-cultured and incubated for 24 h at 37°C then tested biochemically for purity before use as described by Elgayyar *et al.* (2000). When needed, a sample of the master culture was aseptically transferred into sterile nutrient broth (test bacteria) or Sabouraud broth (*C. albicans*) and incubated at 37°C for 18 to 24 h.

3.3 Confirmatory test on clinical isolates

Morphological examination (Gram staining) and biochemical tests were performed on the clinical isolates to confirm their authenticity. The tests include cultivation on selective media, indole test, catalase, citrate utilization, oxidase test and nitrate reduction test (Appendix II B.2).

The various biochemical tests and staining methods were performed on the typed cultures and observations were then compared with that of clinical isolates to confirm their authenticity.

3.3.1 Selective/differential media

3.3.1.1 Cetrimide agar

This was used to identify *P. aeruginosa*. Three tubes of 20 mL cetrimide agar were melted at 100°C, stabilised at 45°C for 15 min and poured into different sterile petri dishes to set. Two loopful of *P. aeruginosa* were streaked on the agar and incubated inverted at 37°C for 24 h. Greenish colonies was identitied to be *P. aeruginosa*

3.3.1.2 Slanetz bartley agar

This was used to identify *E. faecalis*. Three tubes of 20 mL of the agar were melted at 100°C, stabilised at 45°C for 15 min and poured into different sterile petri dishes to set. Two loopful of *E. faecalis* were streaked on the agar and incubated inverted at 37°C for 24 h. The red or maroon colonies produces was identified to be *E. faecalis*.

3.3.1.3 Eosin Methylene Blue (EMB) agar

This was used to identify *P. vulgaris*. Three tubes of 20 mL EMB agar were melted at 100°C, stabilised at 45°C for 15 min and poured into different sterile petri dishes to set. Two loopful of *P. vulgaris* isolates were streaked on the agar and incubated inverted at 37°C for 24 h. *P. vulgaris* is non-lactose-fermenting showing pink colonies.

3.3.1.4 Mannitol salt agar

This was used for the identification of pathogenic staphylococci. Three tubes of 20 mL mannitol salt agar were melted at 100°C, stabilised at 45°C for 15 min and poured into different sterile petri dishes to set. Two loopful of *S. aureus* suspension was streaked on the agar and incubated

inverted at 37°C for 24 h. The pathogenic staphylococci ferment mannitol changing the colour of the medium from red to yellow thereby produced colonies surrounded by bright yellow zones.

3.3.1.5 Sabouraud dextrose agar

Sabouraud dextrose agar in 4% chloramphenicol was used to identify *C. albicans*. Three tubes of 20 mL sabouraud dextrose agar were melted at 100°C, stabilised at 45°C for 15 min and 4% chloramphenicol was added. The media was poured into different sterile petri dishes to set. Two loopful of broth culture of *C. albicans* was streaked on the agar and incubated inverted at 37°C for 24 h. White milky colonies were found to be *C. albicans*.

3.3.1.6 Blood agar base

This was used to identify *S. pyogenes*. *S. pyogenes* isolates was streaked on blood agar and incubated inverted for 24 h. The production of clear zones (beta-hemolysis) around colonies confirmed *S. pyogenes*.

3.3.2 Biochemical test

3.3.2.1 Indole test

The indole tests were performed by growing the isolates in sterile tryptone water and incubating for 24 h. After incubation, three drops of Kovacs' reagent was added to the broth culture, by the use of pipette. Presence of indole was detected by the appearance of red a red layer in the medium while its absence was shown by a yellow.

3.3.2.2 Oxidase test

The oxidase reagent was prepared dissolving 0.1g of tetramethyl-p-phenylenediamine in 10 mL of water. The isolates were streaked on nutrient agar and incubated at 37°C for 24h. Apiece of filter paper was placed in a petri dish and three drops of the freshly prepared oxidase reagent

added. The culture of the isolates was smeared across the impregnated paper with the platinum loop. A positive reaction was indicated by the appearance of dark purple colour on the paper within 10 sec.

3.3.2.3 Catalase test

A loopful of the isolate was transferred onto a glass slide and 2 drops of the 3% hydrogen peroxide was added. Catalase positive test was indicated by the formation air or gas bubbles. No gas bubble production indicates catalase negative.

3.3.2.4 Citrate utilisation

Using a straightened platinum wire, the isolates were inoculated into Koser's citrate medium and incubated at 37°C for 72 h. Citrate utilisation was detected by turbidity and colour change of the medium from light green to blue.

3.4 Collection and identification of plant material

The fresh leaves of *C. anisata* were collected from the Physique Garden of the Faculty of Pharmacy and Pharmaceutical Sciences between October and November, 2010. The plant material was authenticated by Mr. G.H. Sam of the Department of Herbal Medicine, Kwame Nkrumah University of Science and Technology, with the voucher specimen number, KNUST/HML/2012/L 061.

3.4.1 Extraction and fractionation procedures

The fresh leaves of *C. anisata* were thoroughly washed with tap water, to get rid of dirt and soil particles. This was air dried in the laboratory between the temperatures of 30 to 38°C for five days and milled into powder by means of a laboratory milling machine. Two hundred (200) grams of powdered leaves was weighed and one litre of 70% v/v ethanol added and extracted

using cold maceration method for 72 h. This was subsequently filtered through Whatman filter paper (number 10). The filtrates were concentrated using rotary evaporator at 45°C under vacuum. The concentrated extract was poured into porcelain crucibles and dried using a hot air oven (Sanyo OMT Oven) at 40°C for 72 h to a constant mass. The extract obtained was labelled and kept in a desiccator. The extraction procedure was also repeated using different solvents (aqueous, petroleum ether and ethyl acetate).

The ethanol crude extract was further fractionated with methanol, petroleum ether and chloroform using the separating funnel method. Twenty (20) grams of the crude extract was dissolved in 100 mL methanol, shaken and sequentially extracted in non-polar, intermediate and polar fractions with 200 mL of petroleum ether, 200 mL of Chloroform and 150 mL of methanol. The fractions obtained were concentrated under vacuum on rotary evaporator and dried 40°C. The fractions obtained were kept in the desiccator.

3.4.2 Preliminary phytochemical screening of the ethanol extract

3.4.2.1 Cardiac glycosides

One (1) mL of glacial acetic acid was added to 2 mL of the alcoholic filtrate and 2 drops of iron (II) chloride were added followed by 1 mL of concentrated sulphuric acid. Appearance of brown ring at the interface indicated presence of cardiac glycosides (Trease and Evans, 2002).

3.4.2.2 Tannins

Two (2) mL of 5% iron (II) chloride was added to 2 mL of aqueous filtrate. Formation of yellow brown/dark green precipitate indicated the presence of tannins (Jigna *et al.*, 2007).

3.4.2.3 Saponins

Ten (10) mL of aqueous filtrate was transferred into a test tube and tightly corked. This was then shaken vigorously to observe foamy appearance. The foam persisting for more than 5 min indicated the presence of saponins (Nsiah and Opoku, 2005).

3.4.2.4 Steriods

Two (2) mL of acetic anhydride was added to 4 mL of chloroform filtrate followed 2 mL of concentrated sulphuric acid. Bluish green ring indicated the presence of steroids (Akinpelu *et al.*, 2008).

3.4.2.5 Terpenoids

Two (2) mL acetic anhydride was added to 5 mL chloroform filtrate concentrated Sulphuric acid was then added carefully to form layer. Reddish brown coloration at the interface indicates the presence of terpenes (Akinyemi *et al.*, 2006). However there was no colour formation indicating the absence of terpenoids.

3.4.2.6 Alkaloids

One (1) % of 1.5 mL hydrochloric acid was added to 2 mL methanol filtrate. After heating the solution in water bath, 6 drops of Mayors' reagent was added. Formation of orange precipitate indicated the presence of alkaloids (Akinyemi *et al.*, 2006).

3.4.2.7 Flavonoids

Two (2) mL of dilute ammonia solution was added to 5 mL portion of the aqueous filtrate of extract followed by addition of 1 mL concentrated sulphuric acid. A yellow colouration observed in each extract indicated the presence of flavonoids (Martinez, 2003).

3.4.2.8 Phenolics

One (1) mL of 1% ferric chloride solution was added to 2 mL of aqueous filtrate. Bluish green colour indicated phenols (Martinez, 2003).

3.4.2.9 Anthraquinone glycosides

Twenty (20) mg of the ethanol extract was boiled with 10 mL of dilute sulphuric acid for 5 min and filtered while still hot. The filtrate was allowed to cool and shaken with an equal volume of chloroform. The chloroform layer was separated and shaken with half its volume of dilute ammonia. The presence of red colour indicated the presence of anthraqiunone glycosides (Banso and Adeyemo, 2006).

3.4.3 Thin layer chromatography (TLC) of ethanol extract and different fractions

The ethanol extract, methanol, petroleum ether and chloroform fractions were subjected to TLC analysis on 5.0 x 10.0 cm silica gel plate. The extract and fractions (10 mg/mL) were spotted on the TLC plate 2.0 cm above the plate at 0.5 cm intervals from each spot. After thorough drying, the plate was placed in the solvent system (30:70 acetone and petroleum ether) in the chromatank to develop. This was repeated three times and the plate was observed in the day light and under visible ultra violet light (wavelengths of 254 and 365nm). The plate was sprayed with anisaldehyde reagent and again observed under day light and UV light (wavelength 254 and 365 nm). The retardation factor (R_f) values of the separated spots/bands were determined.

3.4.4 High Performance Liquid Chromatographic (HPLC) analysis of ethanol extract and different fractions of *C. anisata*

The HPLC finger printing of the extract and different fractions analysis was performed on a Thermo Finnigan HPLC system using Hypersil Gold C_{18}, reversed-phase column (150 x4.6 mm),

ith 0.5auf particle size. The concentrations of extract/ fractions were 10 mg/mL. The optimum HPLC performance conditions for the analysis were: Injection volume: 10µL, Detection wavelength: 254 nm, Mobile phase: methanol: water/50:50 (isocratic condition, Temperature: 22°C, Pump pressure: 28MPa, Flow Rate: 1mL/min and Running time: 10 min. The wavelength and the retention time with their area under the curve (AUC) of the peaks were determined for the identification of various fractions and extract of *C. anisata.*

3.4.5 Bio-autography assay

Two tubes (20 mL) of Mueller-Hinton agar were melted at 100°C, stabilized at 45°C for 15 min and seeded with 0.1mL overnight broth culture (10^6cfu/mL) of *S. aureus* and *C albicans*. This was then spread thinly over the TLC plate such that the separate constituents were in contact with the seeded agar. The agar was allowed to set and was kept for 1 h to allow diffusion, after which it was incubated at 37°C for 24 h. After incubation, the plate was sprayed with MTT. Bacterial growth inhibition was observed as a clear yellow zone against purple background.

3.4.6 Determination of antioxidant activity

The determination of the free radical scavenging activity of the crude extract and solvent fractions were carried out using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Mensor *et al.* (2001). Four concentrations (0.1, 0.3, 1.0 and 3.0 mg/mL) of the ethanol extract were prepared. One millitre of a 0.3 mM DPPH in methanol was added to 2.0 mL of the extracts and to standard with concentrations (0.01, 0.03, 0.1 and 0.3 mg/mL), and allowed to stand at room temperature in a dark chamber for 30 min. The UV absorbance of the extract was initially determined and found to have a maximum range at 510 to 520 nm, hence the change in colour

from deep violet to light yellow was then measured at 517 nm. The decrease in absorbance was then converted to percentage antioxidant activity using the formula;

Percentage inhibition (%) = $(A_0 - A_1)/ A_0 x100\%$

A_0 is the absorbance of control; A_1 is the absorbance of the extract/sample at 517nm.

Blank = Methanol (1.0 mL) plus sample solution (2.0 mL); Negative control = DPPH solution (1.0 ml, 0.25 mM) plus methanol 2.0 mL and N-Propyl galllate used as reference compound.

3.5 Determination of antimicrobial activity

The agar-well diffusion method as described by Ver Poorte *et al.* (1988), was modified for the antimicrobial investigation. The inoculum of each test organism obtained was prepared by growing each pure isolate in nutrient broth overnight at 37°C. The overnight broth culture of each test organism was diluted with sterile normal saline to give inoculum size of 10^6cfu/mL. Molten nutrient agar, stabilised at 45°C for 15min was seeded with 0.1mL of the test organism and poured into sterile Petri dishes to a depth of 4 mm (20 mL per plate) and allowed to solidify. This was poured into the sterile Petri dishes. *C. albicans* was grown on sabouraud (4 %) dextrose agar. Four wells equidistant from each other were bored with cork borer (No.5) with diameter of 10.0 mm. The ethanol extract and fractions were reconstituted with sterile distilled water and stock concentration of 100 mg/mL prepared. The concentrations of 5.0, 10.0, 15.0 and 20.0 mg/mL of the extract and fractions were used. One hundred microliter (100 µL) of each concentration was put in the wells, left for 1h to allow diffusion. The nutrient agar plates were incubated at 37°C for 24 h while the sabouraud agar plates were incubated at 37°C for 72 h for *C. albicans*. The zones of inhibition were recorded. The procedure was repeated three times for each test organism.

3.5.1 Determination of minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The MIC, MBC and MFC of the ethanol extract and fractions were determined according to the modified method of Greenwood (1989). Concentrations of extract (50, 25, 12.5, 6.25, 3.20, 1.60, 0.80 and 0.40 mg/mL) were prepared from stock solution of 100 mg/mL. 0.1mL of test organism (containing 10^6cfu/mL) was pipette into each of the test tubes containing the mixture of the broth and different concentrations of the extracts. This was incubated at 37°C for 24 h after which 0.2 mL of Methylthiazolyl tetrazolium chloride (MTT) was added. Bacterial growth inhibition was indicated as yellow whiles the presence of purple colour indicated bacterial growth. The MIC was recorded as the least concentration of plant extract that completely inhibited the growth of the test organism. Bactericidal activity was determined when no growth occurred by streaking on nutrient agar (for bacterial) and sabouraud agar (*C. albicans*) and incubating for 24 h after MIC determination.

3.5.2 Determination of time kill-kinetics of ethanol extract

The method of Spangler *et al.* (1997) was modified and used for the time-kill assay against the test organisms. Viable counts of the test organisms were first determined from an 18 h culture suspension. Four (4) 20 mL test tubes containing 5.0 mL double strength nutrient broth were labelled C_1 to C_4. The time kill assays of the extract against the selected test organisms were determined using the factor of MIC concentrations of the test organisms (MIC, 2 × MIC, 3 × MIC and 4 × MIC). Specific volumes were drawn from freshly prepared stock concentration of 100 mg/mL of the extract into the test tubes and sterile water added to make up 10.0 mL of concentrations, 4.5, 9.0, 13.5 and 18.0 mg/mL (for *E. faecalis*) as shown in the Table 3.1. Hundred microlitre (100 uL) of 10^6 cfu/mL suspension of test organism was added to test tubes

containing the concentrations of the extracts. The test tubes were incubated at 37°C in a thermostatically controlled water bath with the shaker set at 40 shakes per min. The time kill kinetics were determined at 0, 1, 2, 3, 4, 5, 6, 12 and 24 h. Exactly 1.0 mL volume of the reaction mixture was withdrawn at the appropriate time and transferred to 9.0 mL of normal saline to produce a 10 fold dilution to neutralize the effects of the extracts carry-over from the test suspensions. This was further diluted serially in two other 10 folds in sterile normal saline. One (1.0) mL of the final dilution is inoculated into a stabilised molten count plate agar, poured into a petri dish, allowed to set and then incubated inverted at 37°C for 24 h. The Petri dishes were marked into segments and the colonies counted with the aid of a colony counter. Each experiment was done in triplicate. Plates yielding 10 to 50 colonies were selected for counting. Data were analysed by expressing growth as the log10 colony forming unit per millilitre (cfu/mL). A graph of Log10 of viable count per mL against time was plotted to determine the time-kill kinetics. A control consisting of nutrient broth and the test organism without extract was used as growth control in each experiment. The above procedure was repeated for the *C. albicans* but with the time intervals of 0, 6, 12, 24, 30, 36, 48, 54 and 72 h

Table 3.1 Composition of media used for the time-kill kinetics

Test Tube	C1	C2	C3	C4	Growth Control
Conc. of extract (mg/mL)	4.5	9.0	13.5	18.0	-
Volume of double Strength nutrient broth (mL)	5.0	5.0	5.0	5.0	5.0
Volume of Extract (mL)	0.45	0.90	1.35	1.80	-
Volume of sterile water (mL)	4.45	4.00	3.55	3.10	4.90
Inoculum size (mL)	0.10	0.10	0.10	0.10	0.10
Total volume of medium (mL)	10.0	10.0	10.0	10.0	10.0

The C1, C2, C3 and C4 are the concentrations of 4.5, 9.0, 13.5 and 18.0 mg/mL respectively. The control is the broth containing the test organism without the extract. The above concentrations were applicable for *E. faecalis*. The same procedure was the same for the other test organisms but at different concentrations based on their MICs.

3.6 Ethical clearance for *in vivo* studies

The protocol for the wound healing experiment was approved by the Animal Ethical Committee, Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, in accordance with the National Institutes of Health Guidelines for care and use of laboratory Animals (NIH, Department of Health Services Publication No. 83-23, revised 1985).

3.6.1 Determination wound healing activity of *C. anisata* using excision wound models

Thirty healthy Sprague-Dawley rats, of either sex, of comparable age and weighing 170 to 195g were used. They were housed in metal cages and maintained on normal commercial pellet diet. The animals were given water *ad libitum* and maintained under laboratory conditions (room temperature, 24 to 28°C, relative humidity of 60 to 70% and 12 h light to dark cycle).

3.6.2 Preparation of emulsifying ointment (BP, 2007)

Emulsifying ointment contains 30% w/w, emulsifying wax 50% w/w, white soft paraffin and 20% w/w liquid paraffin.

Table 3.2: Formula for preparation of emulsifying ointment (Scaled factor: x 0.1)

Composition	Master formula (g)	Scaled quantities used (g)
Emulsifying wax	300.0	30.0
White soft paraffin	500.0	50.0
Liquid paraffin	200.0	20.0

3.6.3 Preparation of aqueous cream (BP, 2007)

Aqueous cream contains 30% w/w emulsifying ointment, 1% w/w phenoxyethanol and 69% w/w purified water. The phenoxyethanol (preservative) was not added in the preparation of ethanol extract cream due to the additive antimicrobial activity that may be exhibited.

Table 3.3: Formula for preparing aqueous cream (Scaled factor x 0.25)

Composition	Master formula (g)	Scaled quantities used (g)
Emulsifying ointment	300.0	75.0
Phenoxyethanol	10.0	-
Purified distilled water*	690.0	175.0

* The mass of the purified distilled water converted to volume (density of water 1.0 g/mL).

3.6.4 Preparation of ethanol extract cream

The BP (2007) method for the preparation of aqueous cream was modified and used for the preparation of the ethanol extract cream. The emulsifying wax (30.0 g) was heated to melt and white soft paraffin (50.0 g) was then added. The mixture was stirred gently with glass rod until completely melted. The liquid paraffin (20.0g) was added with continuous stirring to obtain a homogenous mixture. The emulsifying ointment was then allowed to cool. A total of 250 g

aqueous cream was prepared for the wound healing. This was done by dissolving 75.0 g emulsifying ointment in 175. 0 mL purified distilled water. Concentrations doses of 4, 7 and 10 %w/w cream of the ethanol leaf extract of *C. anisata* were formulated (50.0 g) each and used in wound healing studies.

3.5.5 Excision wound model

The animals were anaesthetized with ketamine (500 mg/mL) at 0.2 mL per 150 g body weight. The dorsal fur of the animals were shaved to a circular diameter of 40 mm by means of razor blades and the anticipated area of the wound to be created was outlined on the shaved skin of the animals with ammonium oxalate crystal violet paint. The shaved area was cleaned with 70% v/v ethanol before excision wounds were created using slightly modified method as described by Bhakta *et al.* (1998). A full thickness of the skin with circular diameter of 20 mm and (an approximate area of 314 mm^2) were created along the markings using toothed forceps, surgical blades and pointed scissors. The entire wound was left open for 24 h and the animals divided into six groups of five animals each.

Group 1: Animals were topically treated with silver sulphadiazine (1% w/w) cream.

Group 2: Animals were treated with aqueous cream (control).

Group 3: Animals were untreated and allowed for natural wound healing to take place.

Group 4, 5 and 6: Animals were treated with concentrations of 4, 7 and 10% w/w of *C. anisata* ethanol extract cream respectively.

Wound treatment commenced on the second day of wound creation. The drugs were then topically applied to the wounds 24 hourly for 19 days. During the course of treatment, scaled photographs of the wound areas were taken (by means of a high resolution Digital Camera, alongside a millimetre scale every 48 h starting from the 1st day of wound treatment. The wound

areas were then determined with the aid of a computer programme (Graph pad prism, San Diego, CA, USA).

3.5.6 Parameters used to assess wound healing properties

The process of wound healing and the quality of the regenerated tissues at the wounded site were assessed by determining the following parameters:

3.5.6.1 Wound size/contraction

Wound size measurement can be used to monitor the progress of healing through changes in the area of the wound with time. The size of the wound was measured at regular interval of 72 h. An excision wound area was measured by vernier calipers and ruler and expressed in percentage of healed wound area (Rashed *et al.*, 2003).

3.5.6.2 Histopathological studies

Wound tissue specimens from untreated and treated animals were taken during healing process at day15. The cross sectional full thickness wound scar, of about 6 mm thick sections from each group were collected at the end of the experiment to evaluate the histopathological alterations (Sadaf *et al.*, 2006). Samples were fixed in 10% buffered formalin for 24 h and dehydrated with a sequence of ethanol-xylene series of solutions, processed and blocked with paraffin at 40-60°C and then sectioned into 5 to 6 µm thick sections. The sections were stained with hematoxylin and eosin stain (HE), Van Gieson's stain (VG) and toluidine blue stain (TB). Hematoxylin and Eosin stained sections and Van Gieson's stained sections were checked for collagen deposition. Toluidine blue stained sections were used to study mast cells (Jagetia and Rajanikant, 2005).

3.7 Statistical analysis of the data

Graph Pad prism version 5.0 windows (Graph Pad Software, San Diego, CA, USA) were used for all the statistical analysis. The data was analysed by two-way analysis of variance (ANOVA) followed by Bonferroni post-test. The values of $p<0.5$ were considered to be statistically significant. The graphs were plotted using Sigma plot for Windows Version 11.0 (Sytsat software Inc., Germany)

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction of *C. anisata* with different solvent

The yield of individual solvent extract was obtained from 200 grams of powdered leaves in 1000 mL of each solvent by cold maceration extraction and the yields expressed in percentages. The ethanol compared with other solvent had the highest percentage yield. The ethanol extract also exhibited significant antimicrobial activity against all the test organisms (Table 4.1) and this was basis for which it was selected for the studies.

Table 4.1: Percentage yields of the *C. anisata* extract with different solvent

Solvent for extraction	Ethanol	Aqueous	Pet ether	Ethyl acetate
Yields (%w/w)	8.51	7.76	6.45	7.55

4.2 Antimicrobial Activity of different solvent extracts of *C. anisata*

The different solvent extracts were found to exhibit antimicrobial activity against the test organisms using agar diffusion method (Fig 4.1 to 4.3). The ethanol extract showed relatively high degree of activity against all the test organisms, *E. faecalis* (E.f), *B. subtilis* (BS), *S. aureus* (SA) *P. aeruginosa* (PA), *P. vulgaris* (PV) *B. thurigiensis* (BT) and *C. albicans* (CA), exhibiting broad spectrum of action.

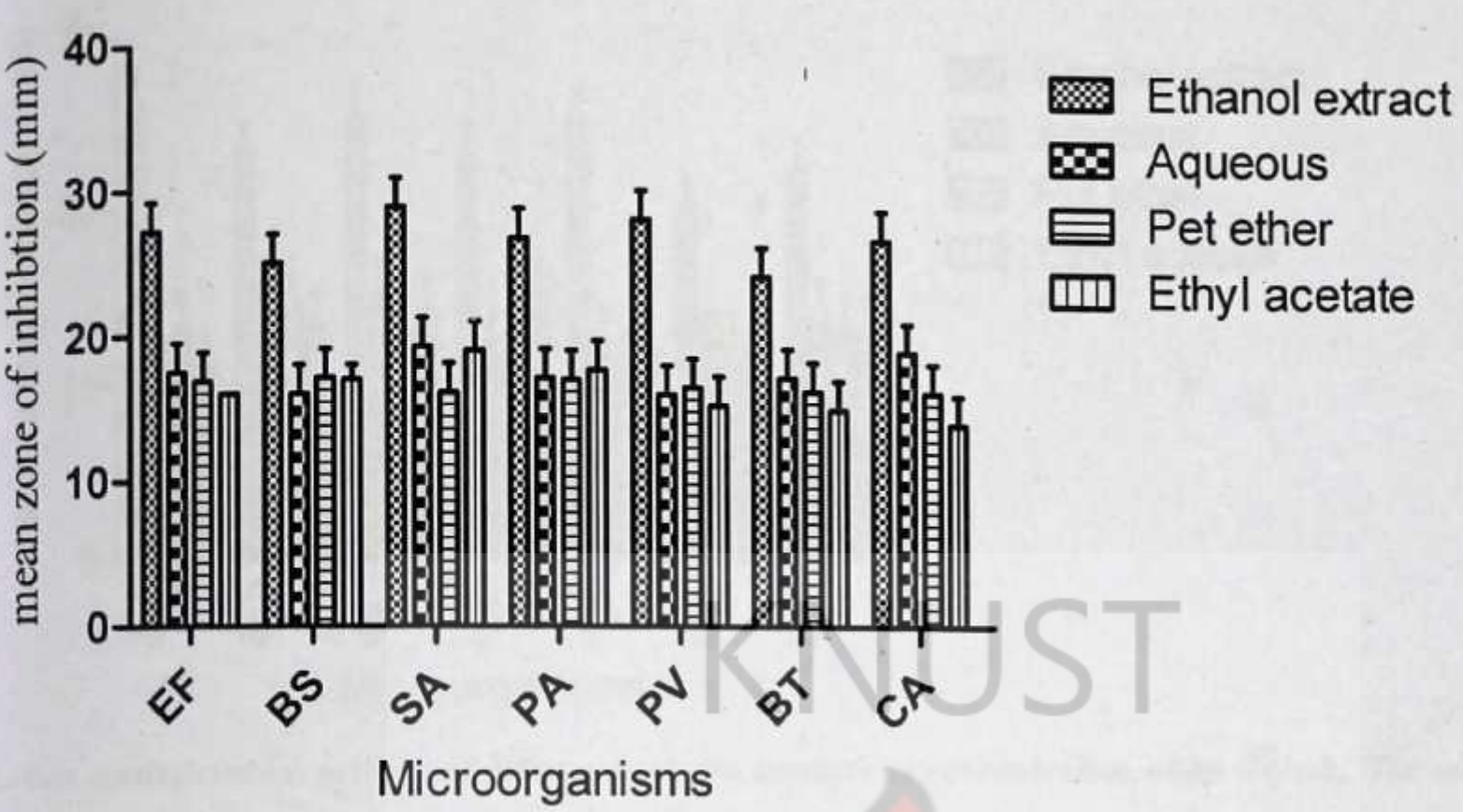

Fig. 4.1: Antimicrobial activity of different solvent extracts at concentration of 50 mg/mL. The comparison of antimicrobial activity of different solvents extract at concentration of 50 mg/ml against the test organisms by agar diffusion method showed varied levels of activity.

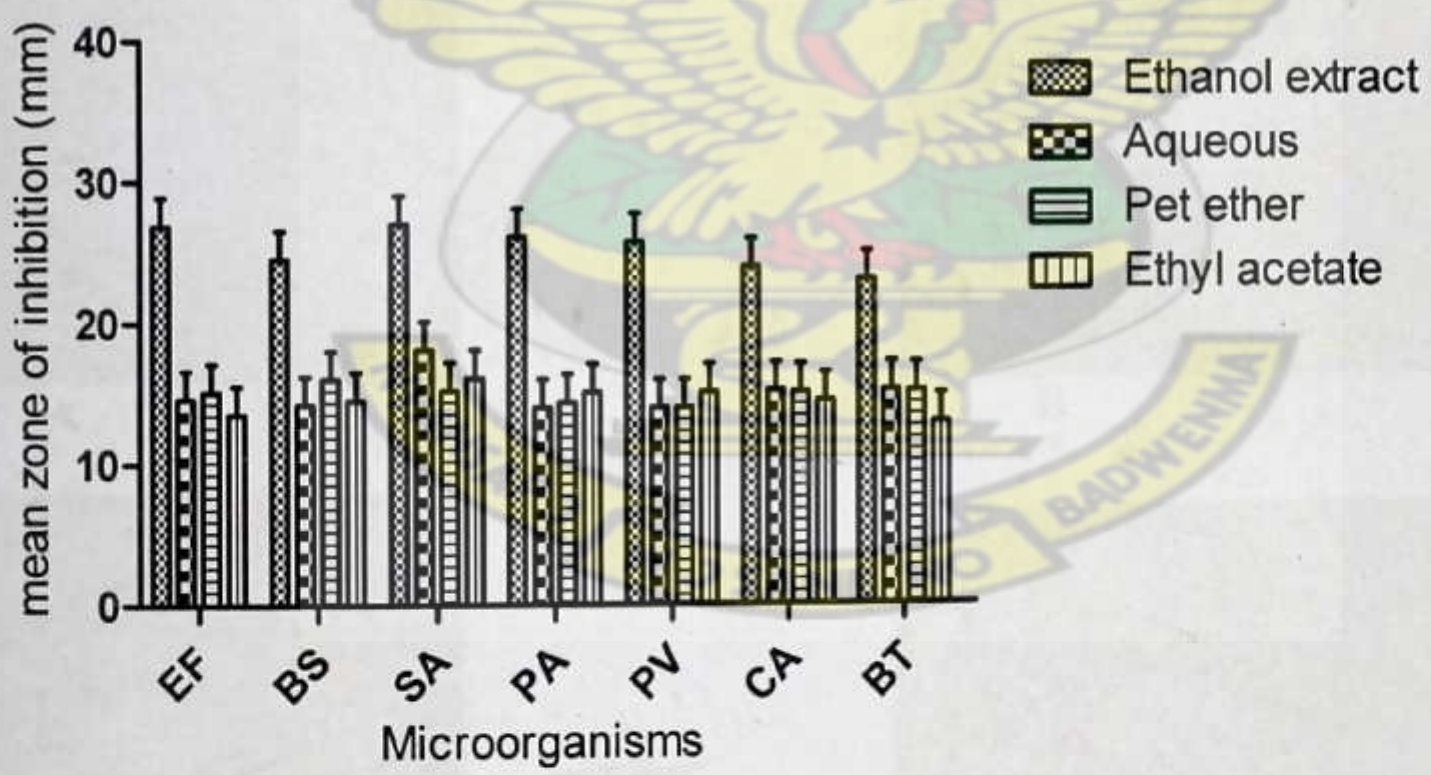

Fig. 4.2: Antimicrobial activity of different solvent extracts at concentration of 40 mg/mL. The comparison of antimicrobial activity of different solvents extract at concentration of 40 mg/ml against the test organisms by agar diffusion method showed varied levels of activity.

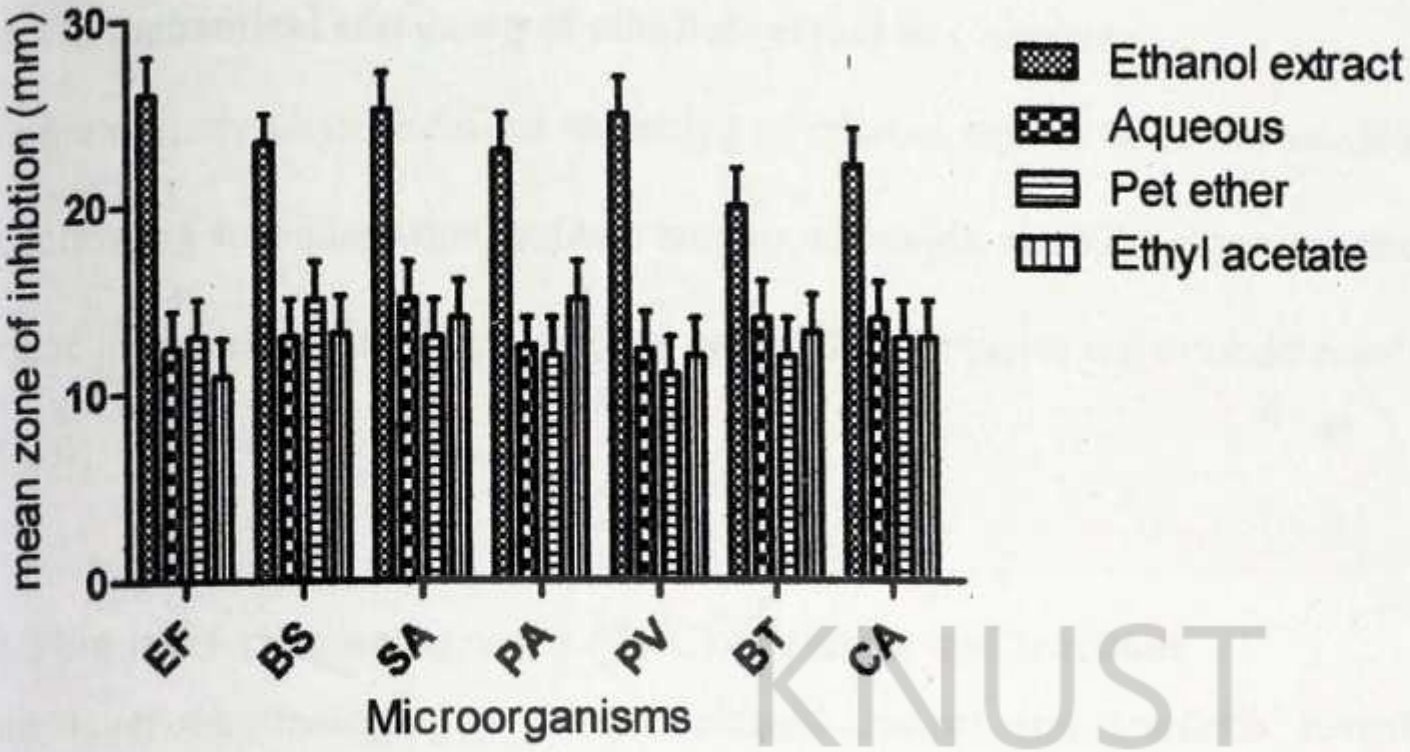

Fig. 4.3: Antimicrobial activity of different solvent extracts at concentration of 30 mg/mL. The comparison of antimicrobial activity of different solvents extract at concentration of 30 mg/ml against the test organisms by agar diffusion method showed varied levels of activity.

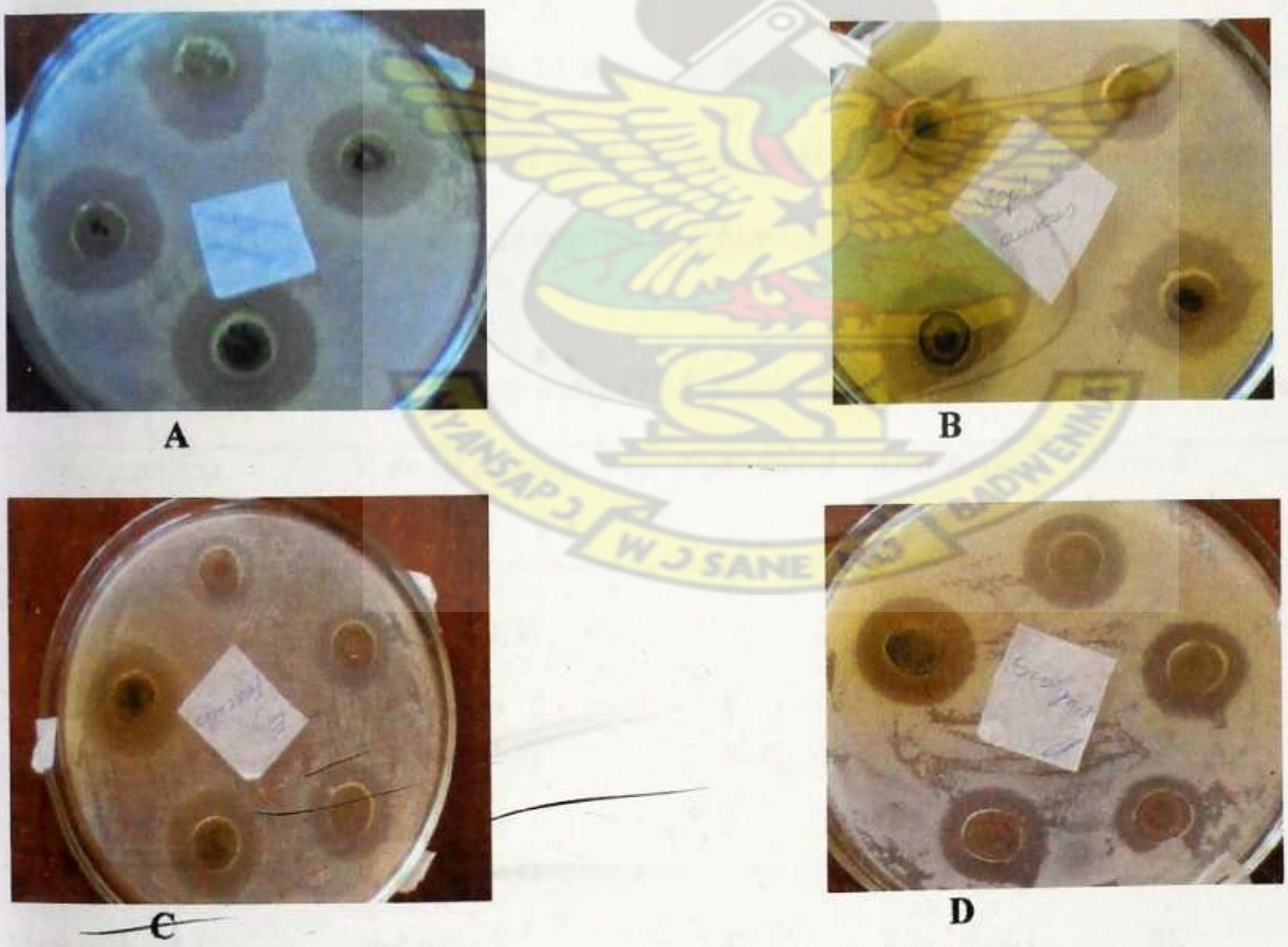

Fig. 4.4: The zones of growth inhibition of ethanol extract using agar diffusion method. Concentrations 20, 15, 10, 5 and 2.5 mg/mL) against *C. albicans* (**A**), S. *ureus* (**B**), *E. faecalis* (**C**) and *P. vulgaris* (**D**)

4.3 Phytochemical screening of ethanol extract of *C. anisata*

The preliminary phytochemical screening of ethanol extract of *C. anisata* was found to contain the following secondary metabolites: tannins, alkaloids, steroids, saponins, phenolics, flavonoids, cardiac glycosides and anthraquinones glycosides. Terpenes were not detected (Section 3.4.2.1 to 3.4.2.9).

4.4 Thin layer chromatography (TLC) of extract and fractions

Thin layer chromatography of the ethanol extract and fractions revealed various bands (indicating different compounds) under UV light (254 and 365nm). The pet ether, chloroform and methanol fractions revealed a number of characteristic bands which could be used in the identification of the extracts or the plant material (Fig. 4.5). The bands (compounds) showing characteristic blue fluorescence at 365nm with R_f of values 0.83 and 0.24) were found to be present in the ethanol extract and the various fractions could be used as preliminary identification of the *C. anisata* leaf extract and its fractions (Table 4.2).

Table 4.2: Thin layer chromatography (TLC) analysis ethanol leaf extract and fractions

Extract/fractions	Solvent system	No. of prominent spots	Distance of spot (cm)	Solvent front (cm)	Rf value
Ethanol crude extract	Pet ether + acetone (7:3)	6	7.2, 6.6, 6.2, 5.7, 2.8, 2.1	8.7	0.83, 0.76, 0.71, 0.66, 0.32, 0.24
Petroleum ether	Pet ether + acetone (7:3)	6	7.1, 6.5, 6.0, 5.5, 3.1, 2.0	8.7	0.83, 0.75, 0.69, 0.63, 0.36, 0.24
Chloroform	Pet ether + acetone (7:3)	5	7.3, 6.1, 5.8, 3.0, 2.0	8.7	0.84, 0.70, 0.67, 0.34, 0.24
Methanol	Pet ether + acetone (7:3)	3	7.2, 2.8, 2.1	8.7	0.83, 0.32, 0.24

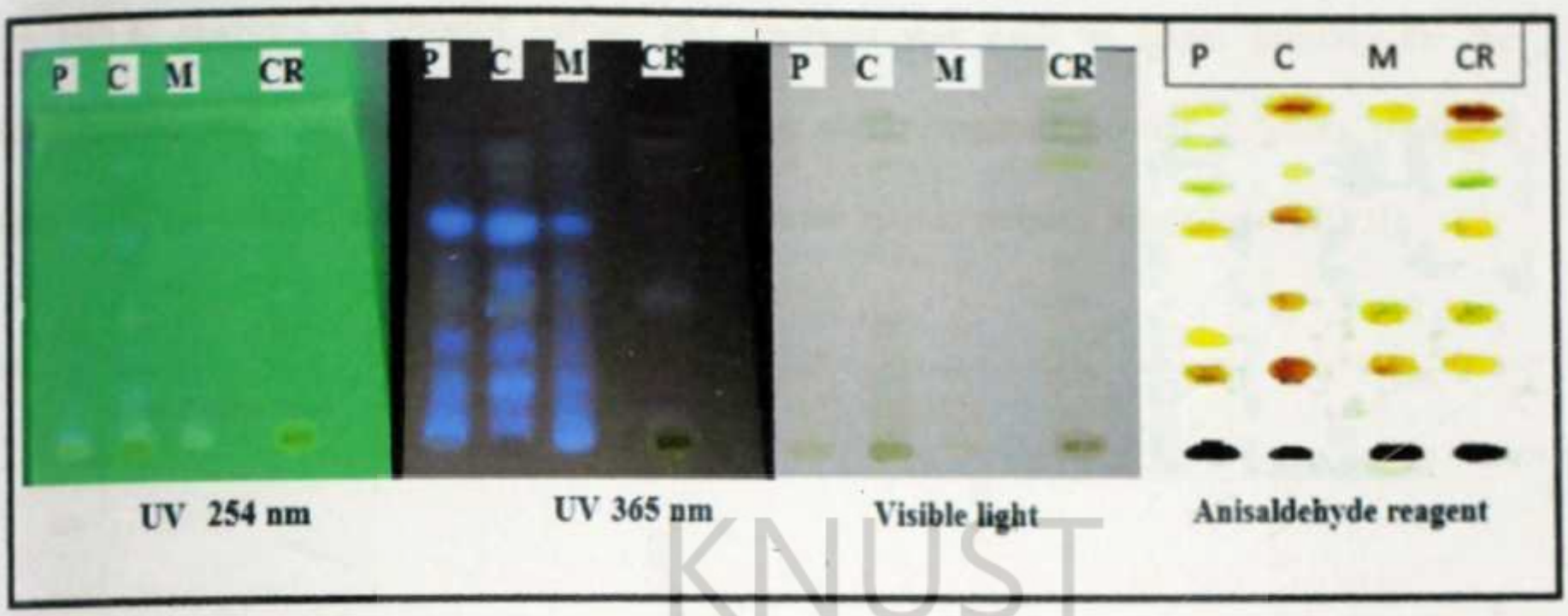

Fig.4.5: TLC chromatogram of ethanol extract and fractions. Thin layer chromatom of crude ethanol extract (**CR**), methanol (**M**), petroleum ether (**P**) and chloroform (**C**) fractions visualised under UV (254 and 365nm), day light and sprayed with anisaldehyde reagent. Six (6) prominent bands were revealed under the UV light of the ethanol crude extract. The petroleum ether, chloroform, and methanol fractions showed 6, 5 and 3 spots respectively under the same condition. This is due to the maximum absorbance of the compounds.

4.5 HPLC analysis of *C. anisata* ethanol leaf extract and fractions

The HPLC analysis of ethanol extract and fractions was used as finger printing for the identification and quality control purposes. The chromatogram showed different peaks of compounds for ethanol extract and fractions within the 10 min running time (Appendix II).

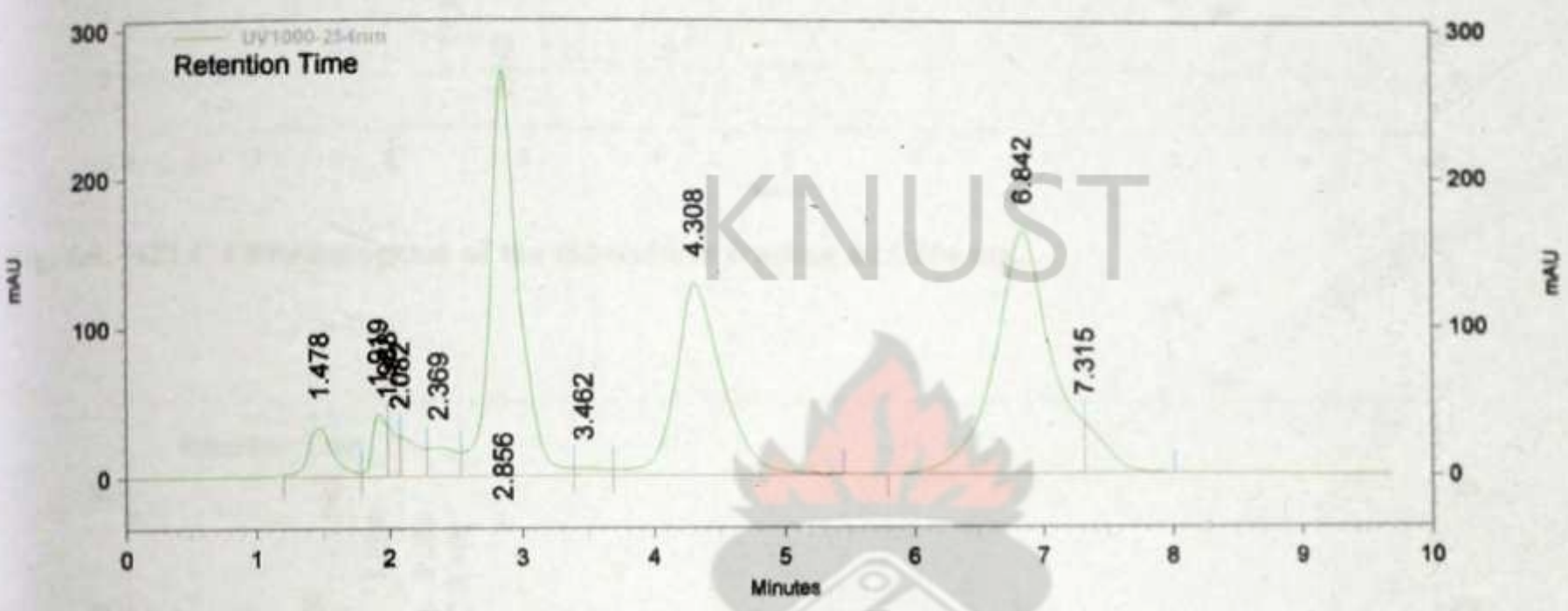

Fig. 4.6: HPLC Chromatogram of the ethanol extract at λ 254 nm.

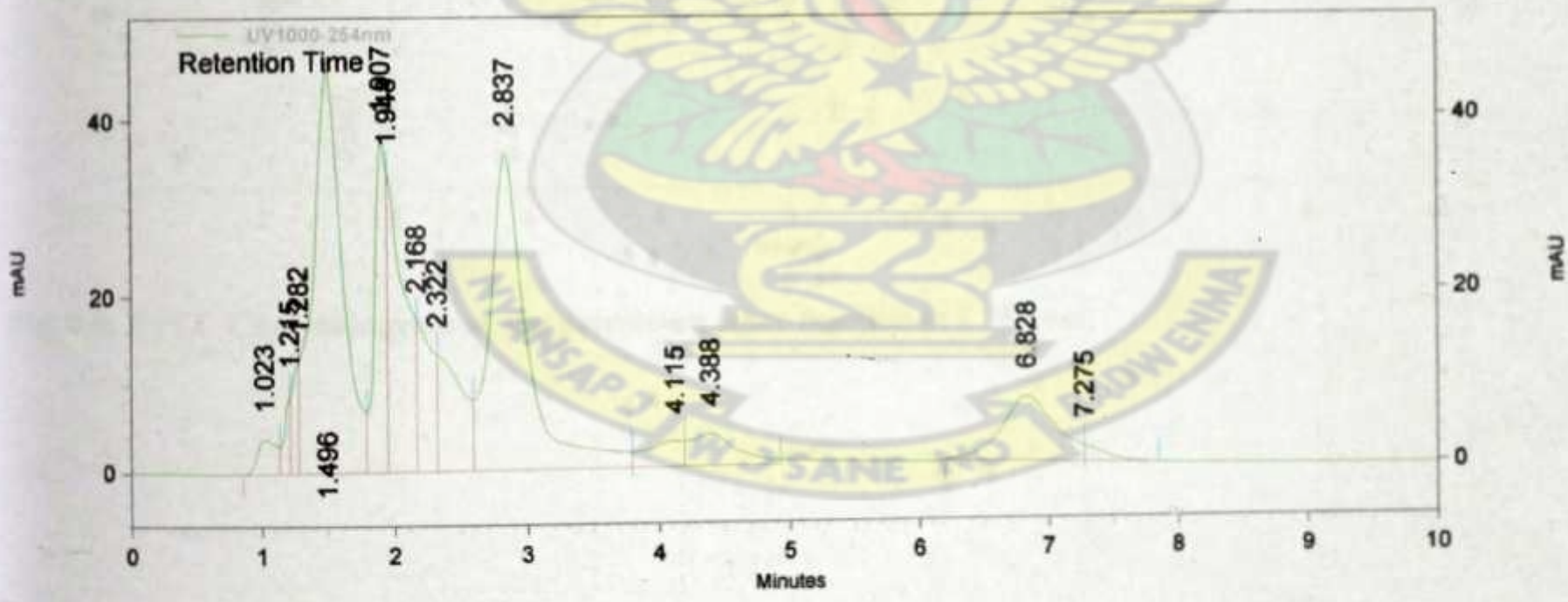

Fig. 4.7: HPLC Chromatogram of the methanol fraction at λ 254 nm.

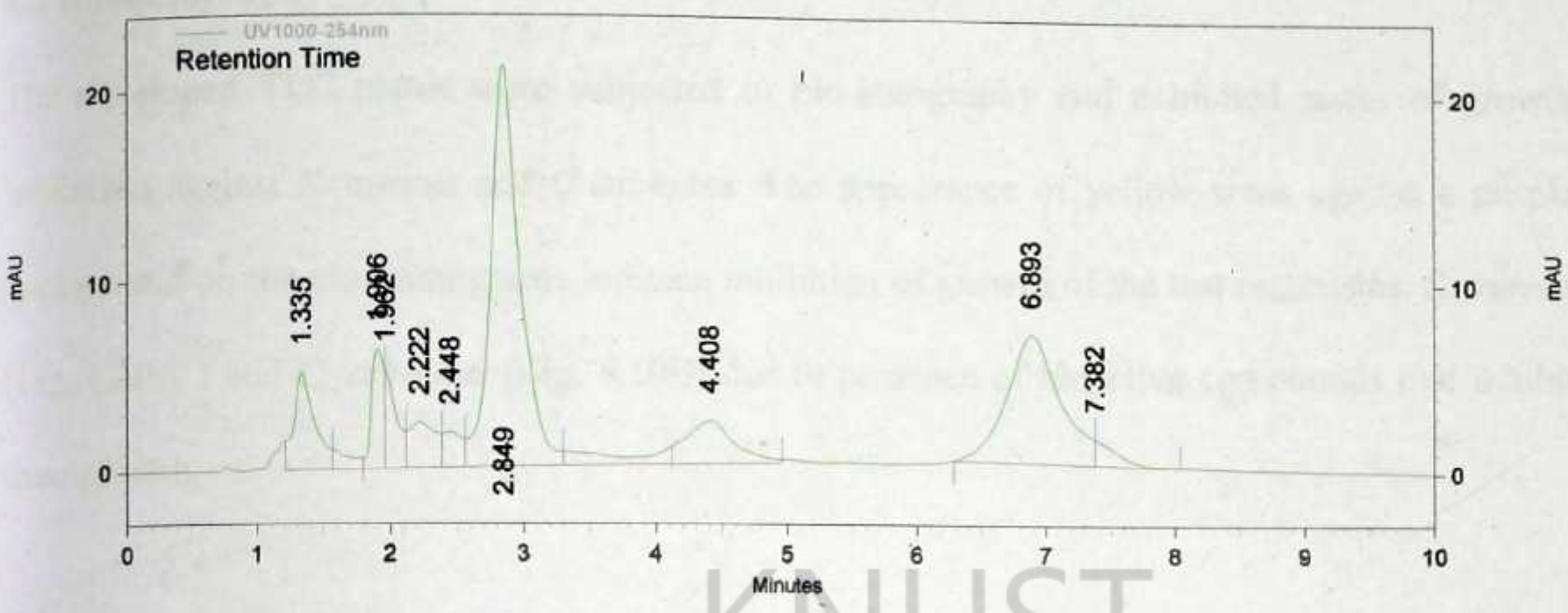

Fig. 4.8: HPLC Chromatogram of the chloroform fraction at ʎ 254 nm.

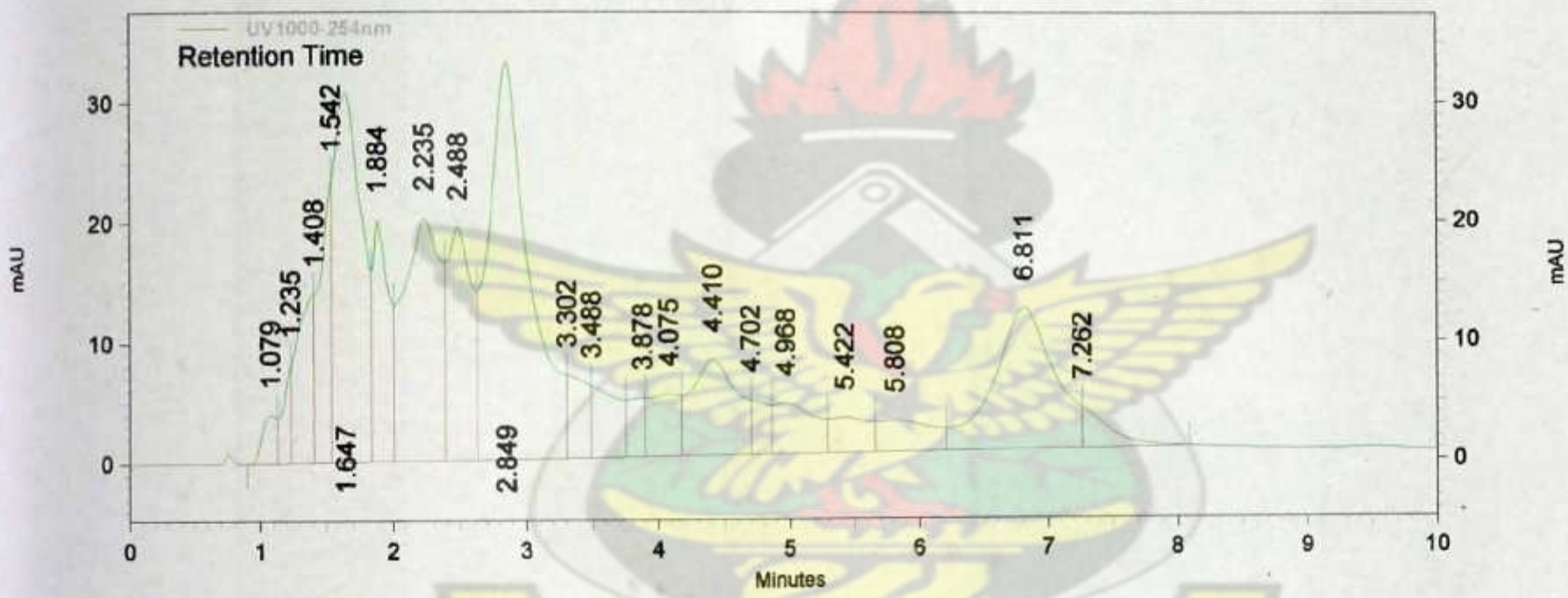

Fig. 4.9: HPLC Chromatogram of the petroleum ether fraction at ʎ 254 nm.

4.6 Bio-autography

The developed TLC plates were subjected to bio-autography and exhibited zones of growth inhibition against *S. aureus* and *C albicans.* The appearance of yellow areas against a purple background on the chromatograms indicate inhibition of growth of the test organisms, *S. aureus* (Fig. 4.10A) and *C. albicans* (Fig. 4.10B) due to presence of bioactive compounds that inhibit their growth.

Fig 4.10: Bio-autography of ethnol extract and fractions of *C. anisata*. The bio-autography showed zones of growth inhibition against *S. aureus* (**A**) and *C. albicans* (**B**). The zones of bacteria growth inhibition were visible as yellow clear areas against purple background after spraying with MTT. Crude ethanol extract (**CR**), methanol (**M**), petroleum ether (**P**) and chloroform (**C**) fractions

4.6.1 Antimicrobial activity of ethanol extract by agar diffusion method

The agar well diffusion method was used for the determination of antimicrobial activity against the test typed bacteria and *C. albicans* using ciprofloxacin and ketoconazole as reference antibacterial and antifungal agents respectively. The mean zones of growth inhibition was determined from three independent determinations (n=3) for all the test organisms. Though the ethanol extract exhibited a broad spectrum of activity, it was more active against the Gram-positive than Gram-negative for both typed and clinical test organisms at all the different concentrations.

Table 4.3: Mean zones of growth inhibition of ethanol extract against test organisms

Concentration. (mg/mL) Extract	Mean zones of inhibition (mm) + SEM						
	P. aeruginosa	*P. vulgaris*	*B. thuringiensis*	*S. aureus*	*E. faecalis*	*B. subtilis*	*C. albicans*
20.0	19.00 ±0.58	18.00 ±0.58	18.67 ±0.33	20.67 ±0.33	21.33 ±0.33	20.00 ±0.00	20.33 ±0.33
15.0	17.67 ±0.68	16.33 ±0.33	17.00 ±0.00	18.33 ±0.33	20.00 ±0.58	17.33 ±0.33	19.00 ±0.00
10.0	16.00 ±0.58	15.00 ±0.00	14.67 ±0.33	16.00 ±0.00	17.33 ±0.33	16.33 ±0.33	17.00 ±0.00
5.0	14.33 ±0.33	13.00 ±0.58	13.00 ±0.58	14.00 ±0.58	15.33 ±0.33	13.00 ±0.00	15.33 ±0.33
Ciprofloxacin (0.002)	20.50 ±0.67	21.80 ±0.68	24.50 ±0.33	23.50 ±0.33	25.60 ±0.58	19.10 ±0.11	nd
Ketoconazole (0.002)	nd	nd	nd	Nd	nd	nd	30.00 ±0.11

The mean zones of growth inhibition was determined from three independent result (n= 3) for all test organisms.

nd: not determined, SEM: standard error mean.

Table 4.4: Mean zones of growth inhibition of clinical and typed bacteria

Conc. (mg/mL) Extract	Mean zone of inhibition/mm + SEM (clinical strains)				Zone of inhibition (mm) + SEM (typed strains)			
	P. aeruginosa	*P. vulgaris*	*S. aureus*	*E. faecalis*	*P. aeruginosa*	*P. vulgaris*	*S. aureus*	*E. faecalis*
20.0	14.33 ±0.33	15.67 ±0.33	19.33 ±0.33	20.00 ±0.00	22.00 ±0.58	18.00 ±0.58	20.67 ±0.33	21.33 ±0.33
15.0	13.67 ±0.33	14.00 ±0.33	14.00 ±0.00	16.67 ±0.33	17.67 ±0.68	16.33 ±0.33	18.33 ±0.33	20.00 ±0.58
10.0	13.00 ±0.00	12.33 ±0.33	13.33 ±0.33	13.67 ±0.33	16.00 ±0.58	15.00 ±0.00	16.00 ±0.00	17.33 ±0.33
05.0	12.00 ±0.00	11.67 ±0.33	11.00 ±0.00	12.00 ±0.00	14.33 ±0.33	13.00 ±0.58	14.00 ±0.58	15.33 ±0.33
Cipro. (0.002)	19.30 ± 0.67	21.30 ±0.68	18.00 ±0.00	22.30 ±0.33	20.50 ±0.11	21.80 ±0.31	23.50 ±0.00	25.60 ±0.67

The mean zones of growth inhibition was determined from three independent result (n= 3) for all test organisms.

SEM: standard error mean; Conc: Concentration of extract; Cipro: ciprofloxacin.

4.6.2 Antimicrobial activity of the different fractions against test organisms

The different fractions showed comparatively varying degrees of activity against the test organisms by agar well diffusion method. The methanol and chloroform fractions showed significant activity compared to the petroleum ether fraction ($p< 0.05$) at a concentrations of 20 mg/mL against *E. faecalis* and *S. aureus*. The methanol fraction showed significant activity compared to chloroform and petroleum ether fractions ($p<0.05$) at 20 mg/mL against the test organisms except *C. albicans*.

Table 4.5: Antimicrobial activity of the different fractions

Test organisms	Concentration (mg/mL)	Mean zones of growth inhibition ± SEM		
		Petroleum ether	Chloroform	Methanol
E. faecalis	20.0	17.3 ± 0.33	21.0 ± 0.58	24.0 ± 0.58
	15.0	16.3 ± 0.67	19.0 ± 0.33	22.0 ± 0.88
	10.0	14.3 ± 0.34	17.3 ± 0.66	19.3 ± 0.33
	5.0	11.7 ± 0.33	14.3 ± 0.67	16.6 ± 0.67
B .subtilis	20.0	13.3 ± 0.33	14.0 ± 0.58	18.7 ± 0.33
	15.0	11.3 ± 0.33	13.3 ± 0.57	16.6 ± 0.33
	10.0	0.00 ± 0.00	11.3 ± 0.33	14.3 ± o.33
	5.0	0.00 ± 0.00	11.0 ± 0.00	11.3 ± 0.00
P. aeruginosa	20.0	16.7 ± 0.88	15.3± 0.33	18.3± 0.33
	15.0	12.7 ± 0.33	13.7 ± 0.33	16.3 ± 0.33
	10.0	11.3 ± 0.33	12.3 ± 0.33	13.3 ± 0.33
	5.0	0.00 ± 0.00	11.3 ± 0.33	11.7 ± 0.33
S. aureus	20.0	17.3 ± 0.33	20.3 ± 0.33	25.0 ± 0.57
	15.0	15.7 ± 0.33	18.7 ± 0.33	20.3 ± 0.33
	10.0	14.3 ± 0.33	16.3 ± 0.33	18.7 ± 0.33
	5.0	11.7 ± 0.33	12.7 ± 0.33	15.6 ± 0.34
P. vulgaris	20.0	18.6 ± 0.31	19.0 ± 0.57	22.6 ± 0.11
	15.0	16.3 ± 0.33	17.7 ± 0.33	19.3 ± 0.33
	10.0	13.3 ± 0.11	15.0 ± 0.57	17.3 ± 0.33
	5.0	12.7 ± 0.33	13.3 ± 0.33	13.1 ± 0.11
B. thuringiensis	20.0	12.7 ± 0.33	13.7 ± 0.67	16.0 ± 0.58
	15.0	11.0 ± 0.00	12.6 ± 0.67	14.7 ± 0.33
	10.0	0.00 ± 0.00	11.3 ± 0.33	12.3 ± 0.33
	5.0	0.00 ± 0.00	0.00 ± 0.00	11.0 ± 0.00
C. albicans	20.0	15.3 ± 0.33	15.1 ± 0.31	15.6 ± 0.33
	15.0	13.0 ± 0.58	12.7 ± 0.33	13.3 ± 0.33
	10.0	0.00 ± 0.00	11.3 ± 0.33	11.7 ± 0.33
	5.0	0.00 ± 0.00	11.0 ± 0.00	0.00 ± 0.00

The mean zones of growth inhibition was determined from three independent result (n=3) for all test organisms.

SEM: standard error mean, Conc: Concentration of the extract.

4.6.3 Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations (MICs/MBCs) of the ethanol extract and fractions

By broth dilution method, the extracts showed various degrees of MICs and MBCs against the test microorganisms. There were lower MICs for Gram-positive test organisms compared with the Gram–negative test organisms, indicating that the ethanol extract were more active against the Gram-positive test organisms (Table 4.6). The methanol fraction showed highest activity against the test organisms as compared with chloroform and petroleum fractions based on the MICs (Table 4.7).

Table 4.6 MICs and MBCs of the ethanol extract by broth macro-dilution technique

Test Organisms	Ethanol Extract		Ciprofloxacin	Ketoconazole
	MIC (mg/mL)	MBC (mg/mL)	MIC (µg/mL)	MIC (µg/mL)
E. faecalis	4.5	19.0	25.0	nd
B. subtilis	0.5	4.0	20.0	nd
S. aureus	0.9	15.0	20.0	nd
P. aeruginosa	2.5	11.0	40.0	nd
P. vulgaris	1.0	14.0	25.0	nd
B. thuringiensis	1.5	17.0	30.0	nd
C. albicans	5.5	27.5	nd	2.5

nd: not determined

Table 4.7: MIC of fractions of *C. anisata* leaf extract by broth macro-dilution technique

Test Organisms	MIC (mg/mL)		
	Methanol fraction	Chloroform fraction	Pet ether fraction
E. faecalis	2.0	6.5	8.0
B. subtilis	0.6	3.0	4.5
S. aureus	1.5	3.0	4.5
P. aeruginosa	2.0	4.5	4.0
P. vulgaris	1.0	2.0	2.5
B. thuringiensis	5.0	7.5	6.5
C. albicans	5.0	7.0	10.0

4.7 Time kill kinetics

The time-kill kinetics studies conducted showed static activity at the different concentrations of the ethanol extracts against all the test organisms and therefore the extract reduced the number of cells rather than acting bateriocidally.

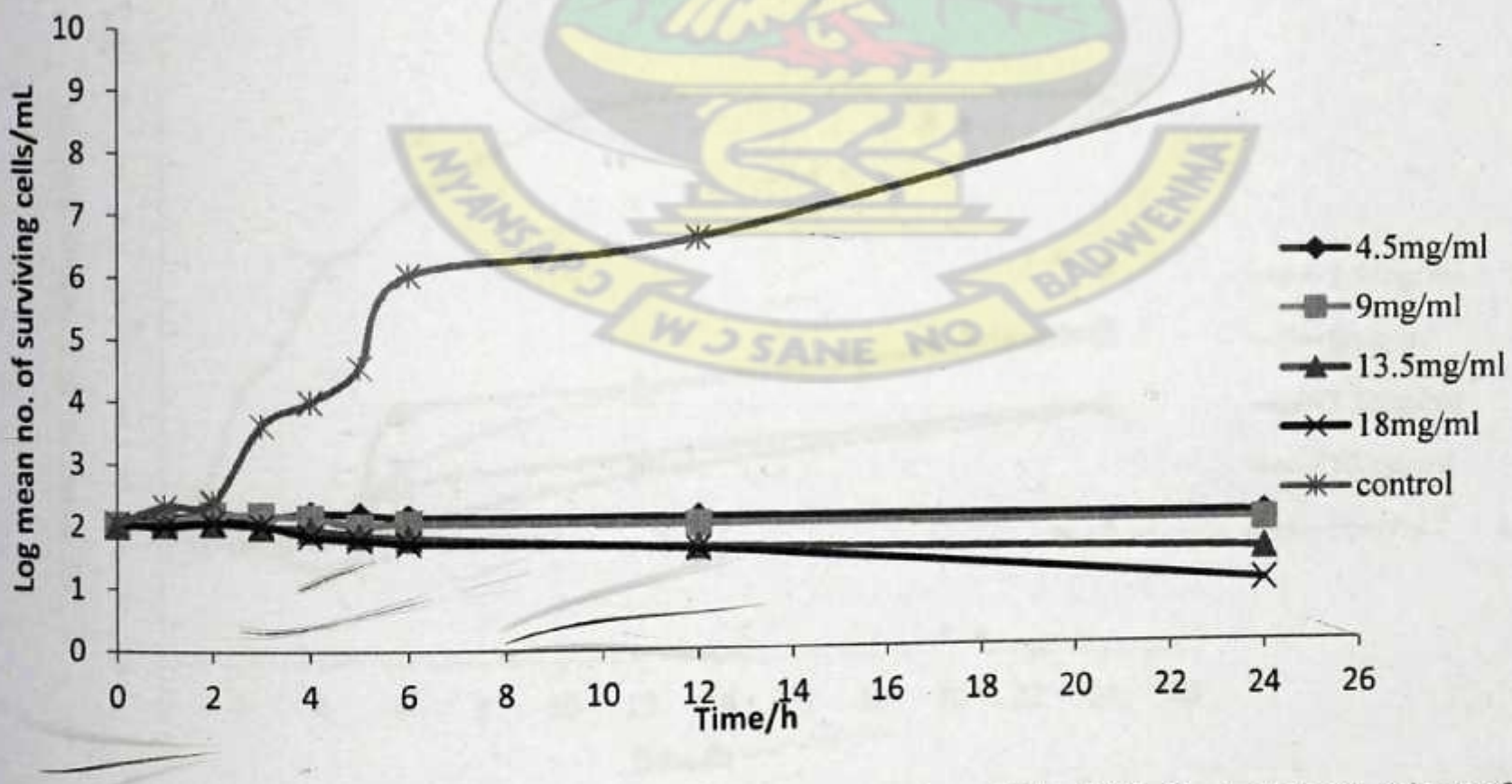

Fig 4.11: Survival of *E. faecalis* in extract at different concentration within 24 h. Control: Broth culture of *E. faecalis* without the extract.

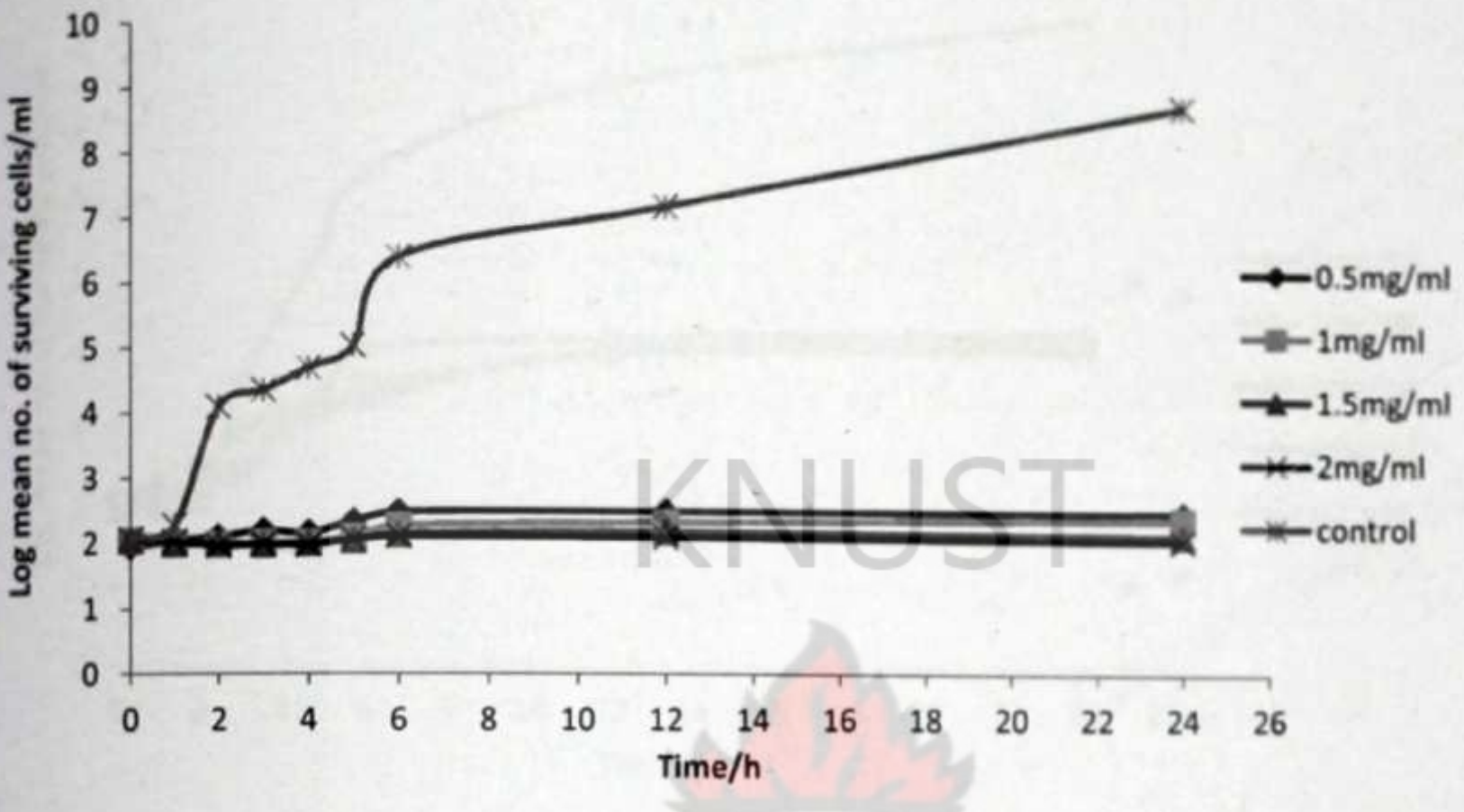

Fig. 4.12: Survival *B. subtilis* in extract different concentration within 24 h. Control: Broth culture of *B. subtilis* without the extract.

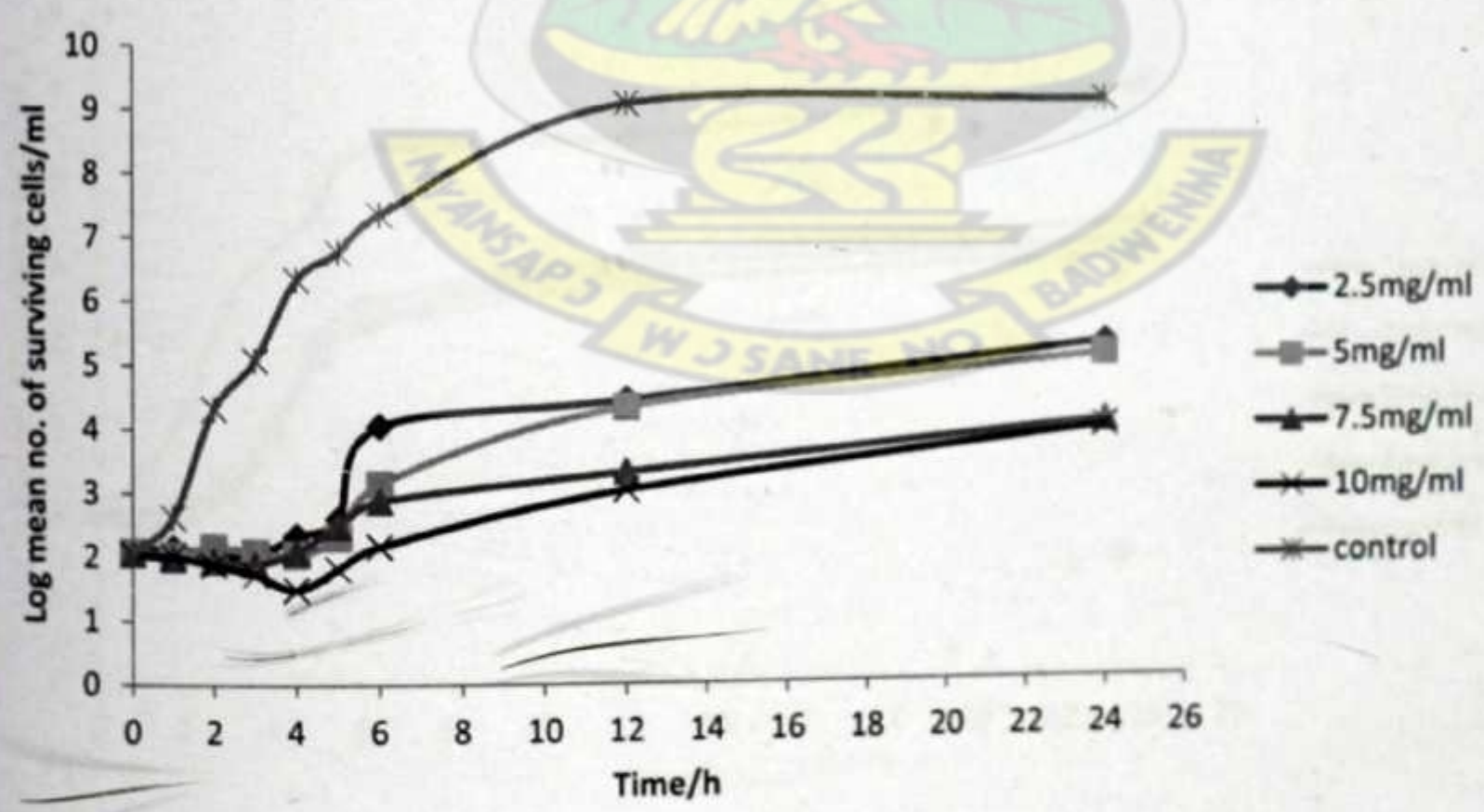

Fig. 4.13: Survival of *P. aeruginosa* in extract at different concentration within 24 h. Control: Broth culture of *P. aeruginosa* without the extract.

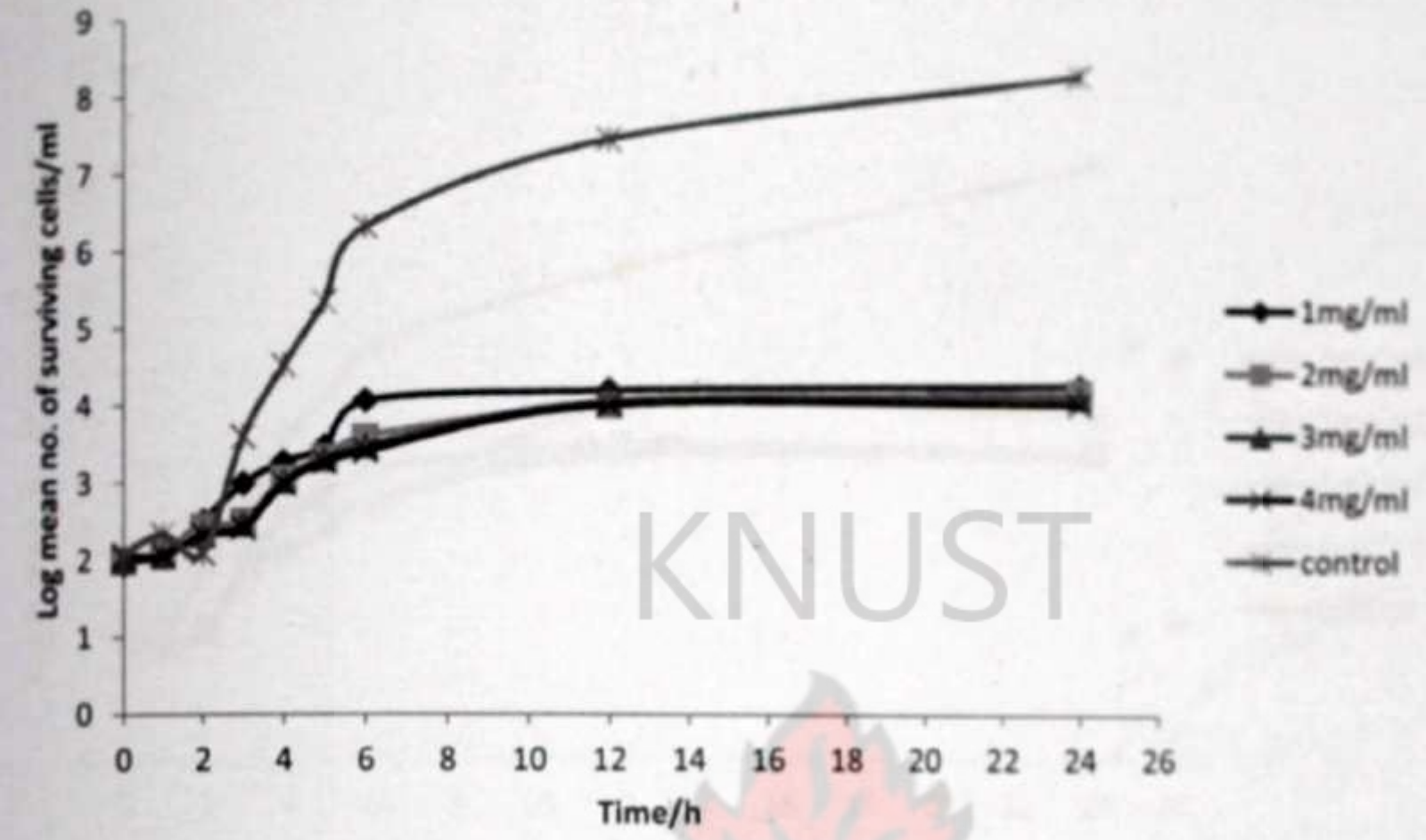

Fig. 4.14: Survival of *P. vulgaris* in extract at different concentration within 24 h. Control: Broth culture of *P. vulgaris* without the extract.

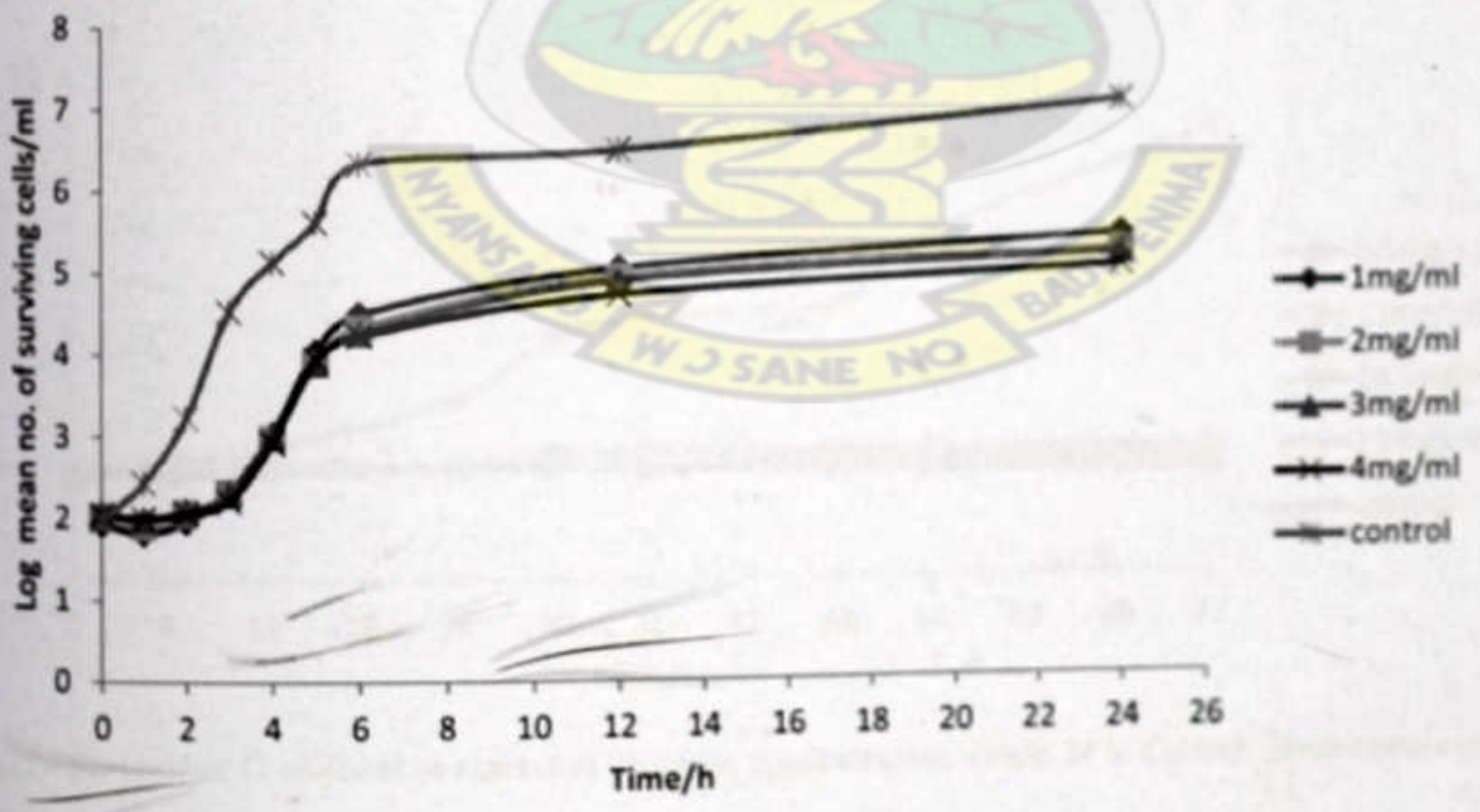

Fig. 4.15: Survival of *S. aureus* in extract at different concentration within 24 h. Control: Broth culture of *S. aureus* without the extract.

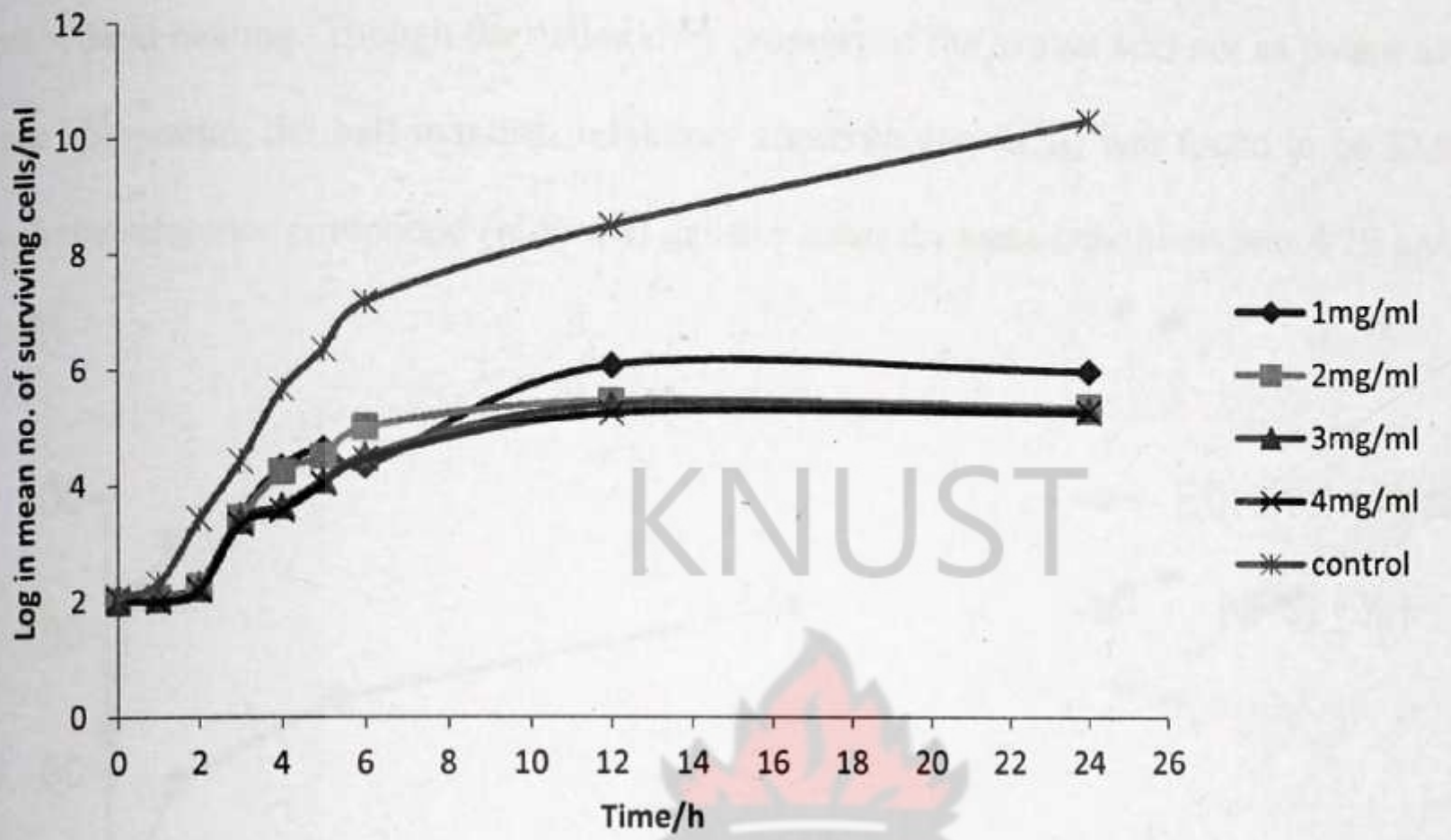

Fig. 4.16: Survival of *B. thuringiensis* in extract at different concentration within 24 h. Control: Broth culture of *B. thuringiensis* without the extract.

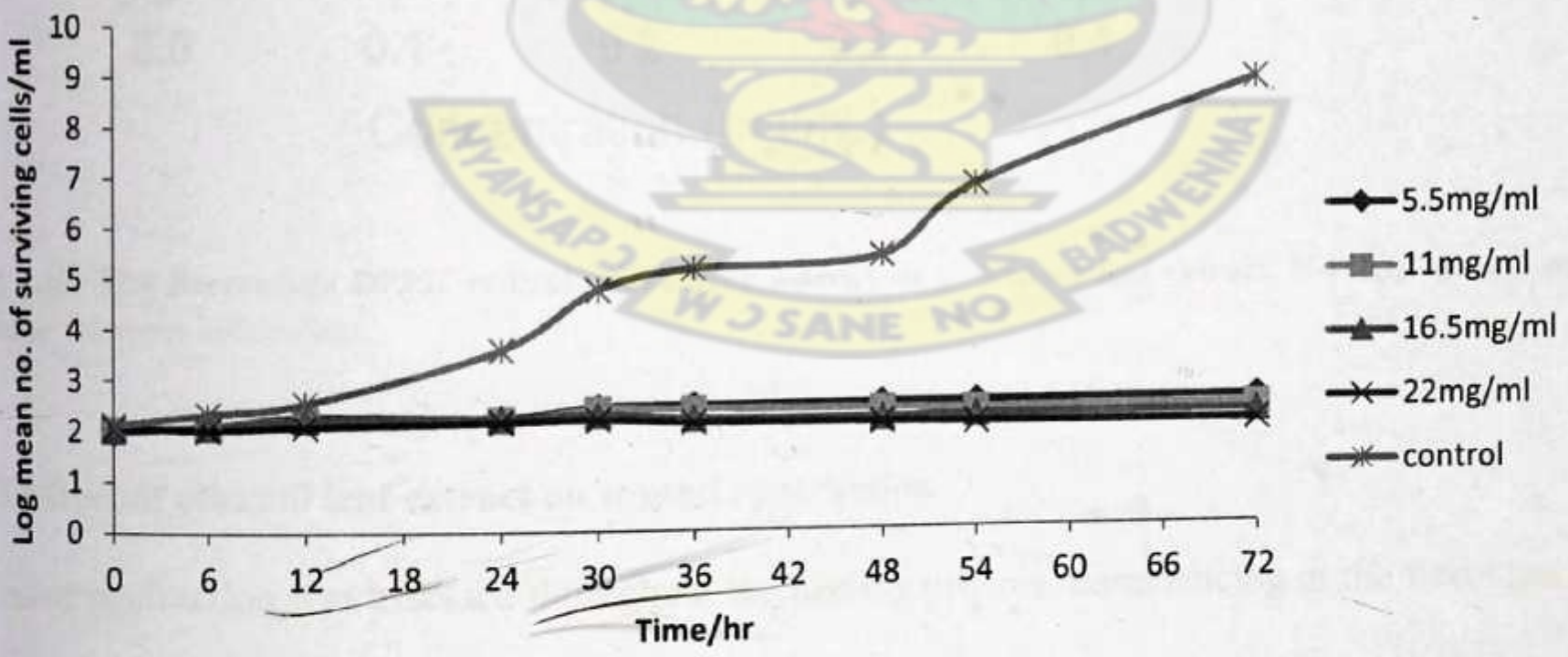

Fig. 4.17: Survival of *C. albicans* in extract at different concentration within 24 h. Control: Broth culture of *C. albicans* without the extract.

4.8 Antioxidant activity of the ethanol extract

The leaf extract of *C. anisata* has been found to exhibit antioxidant activity and therefore promote wound healing. Though the antioxidant property of the extract was not as potent as the reference compound, the half maximal inhibitory concentration (IC_{50}) was found to be 32.9 µg /mL with the reference compound (N-Propyl gallate) under the same conditions was 4.19 µg/mL. (Fig 4.18).

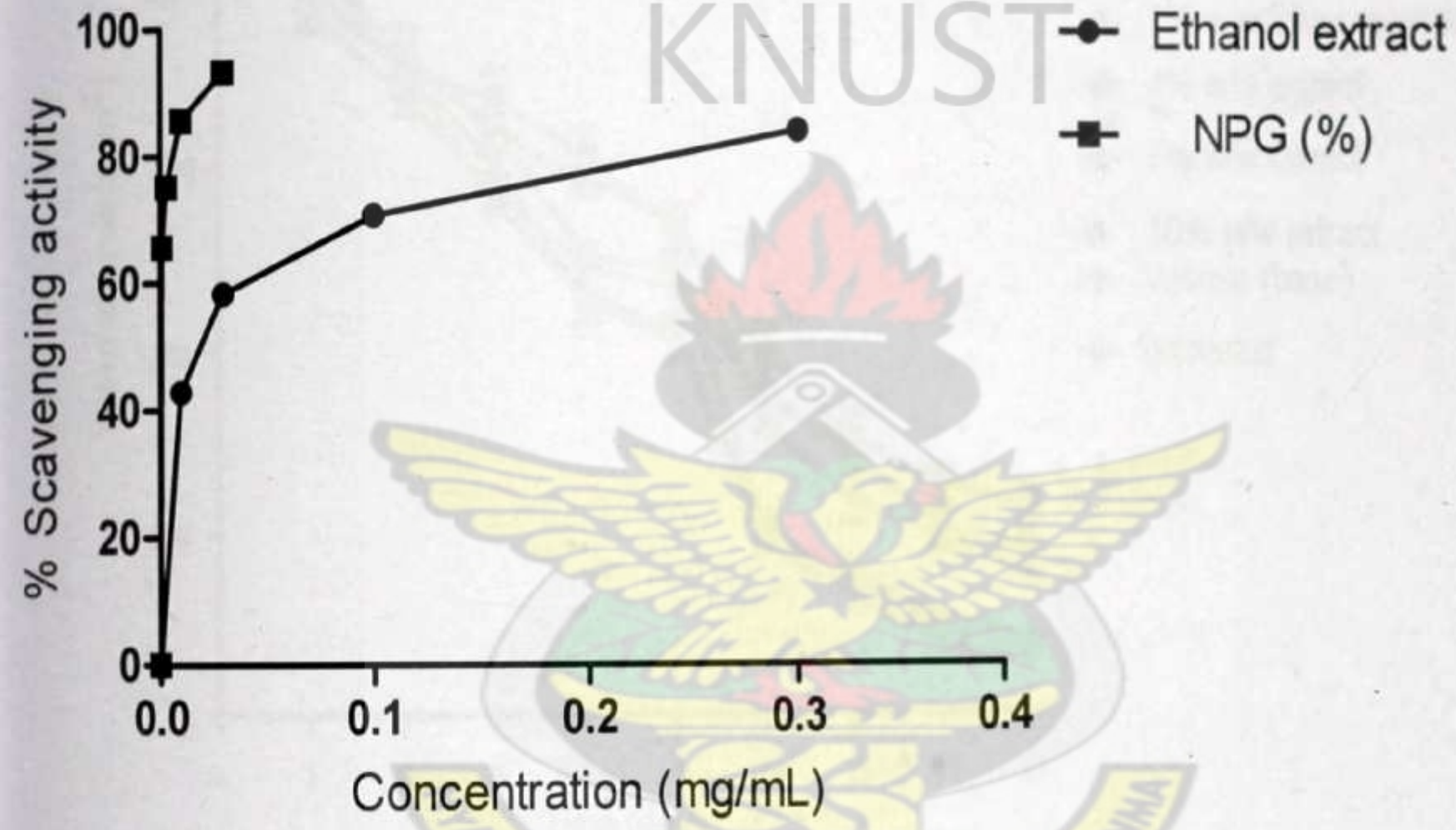

Fig 4.18: The Percentage DPPH radical Scavenging activity of *C. anisata* leaf extract. N-Propyl gallate was used as reference antioxidant.

4.9 Effect of ethanol leaf extract on wound contraction

Wound contraction was assessed throughout the healing process, commencing in the fibroblastic stage where the area of the wound undergoes shrinkage. The progression of wound healing was determined by the periodic assessment of the contraction of excision wounds. The ethanol cream extract at 7% w/w concentration was found to significantly increased the rate of wound

contraction ($p < 0.05$) at day 9 and day 13 ($p < 0.01$) compared to the untreated. There was no significant difference between the 7, 10% w/w ethanol extract and 1% w/w silver sulphadiazine used as reference wound healing agent. (Appendix II)

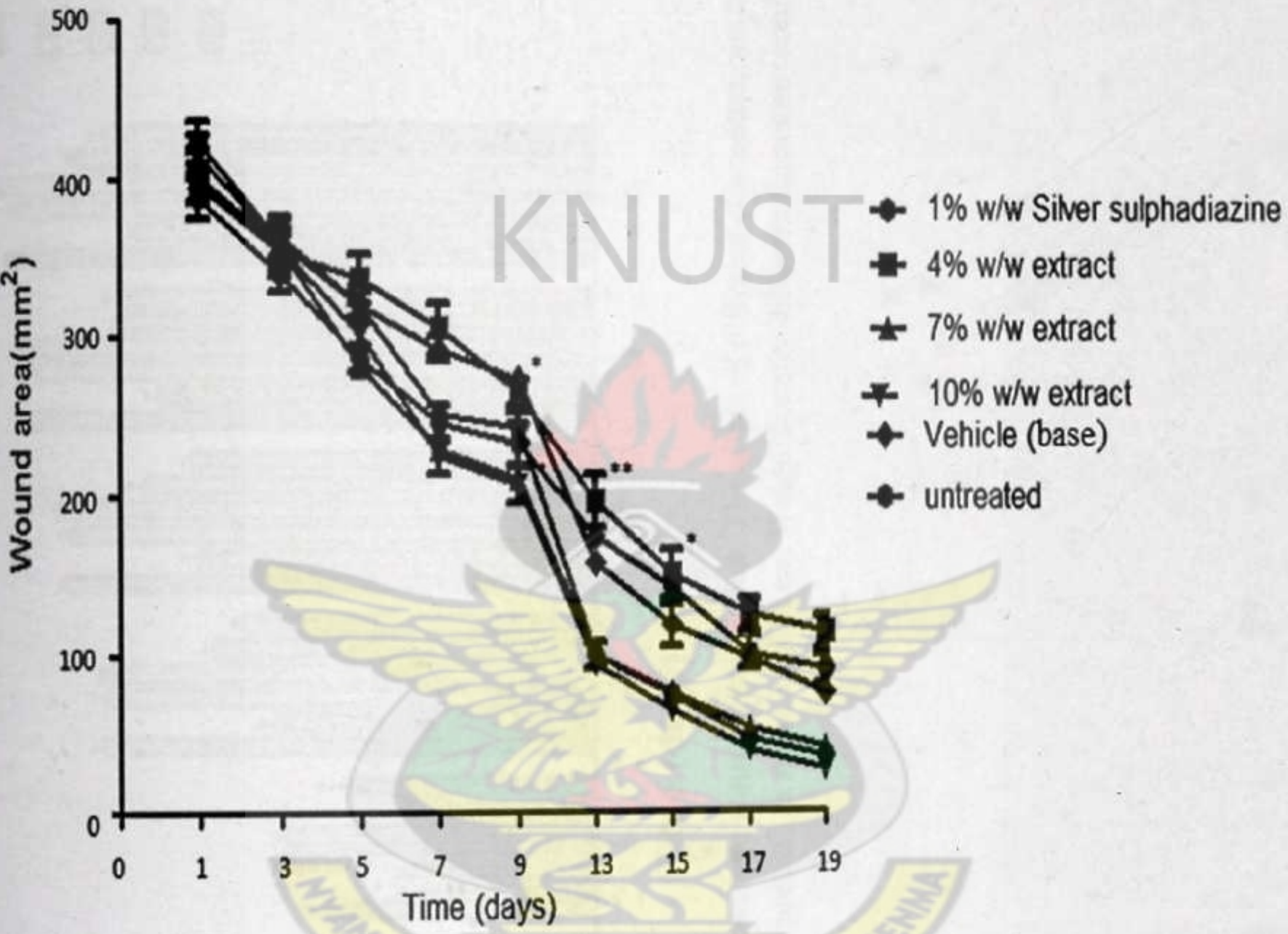

Fig 4.19: Effect of ethanol extract on wound contraction. The effect of silver sulphadiazine (1% w/w), vehicle and extracts (4, 7 and 10% w/w) on wound contraction. The values were expressed mean ± SEM (n=5) significantly different from untreated (*p< 0.05, **p<0.01 and ***p< 0.001).

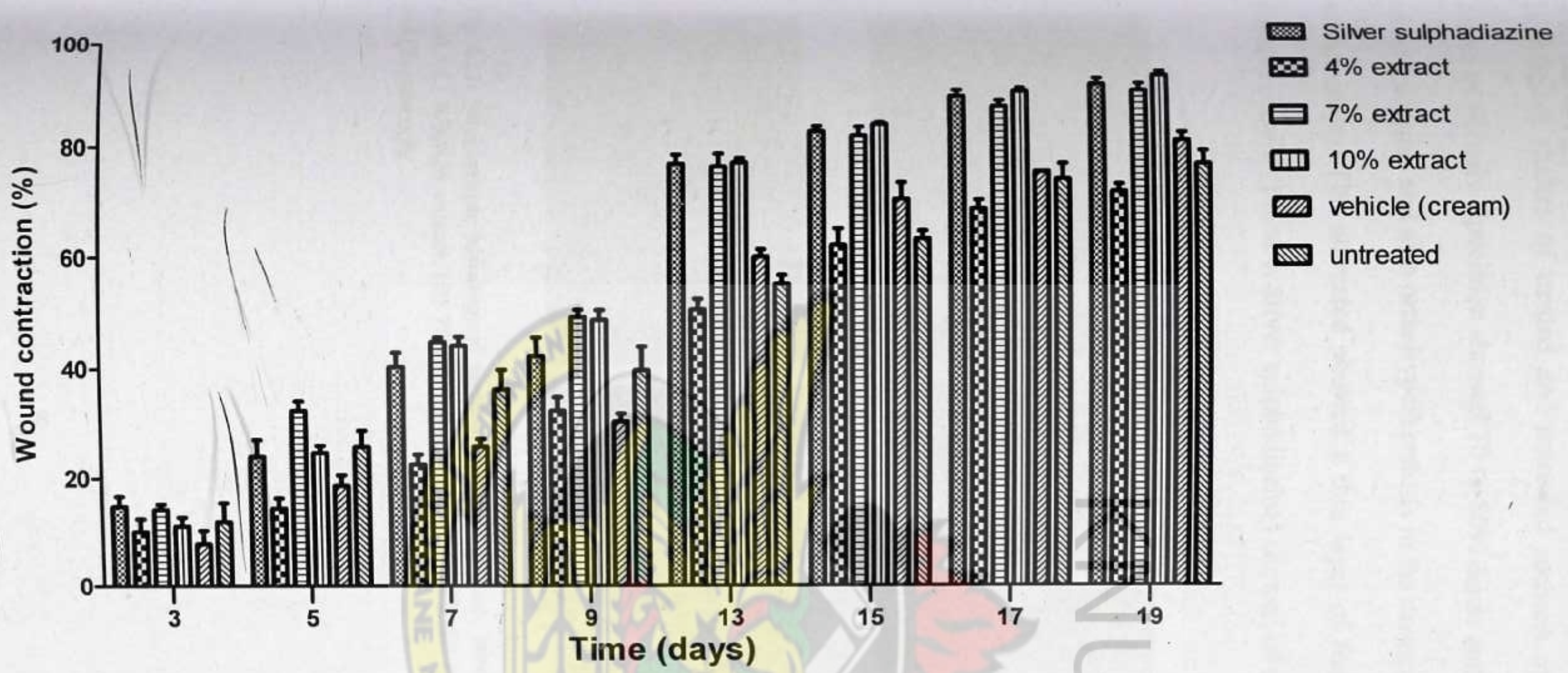

Fig. 4.19: Effect of ethanol extract on percentage wound contraction. The effect of different concentrations of ethanol extracts on rate of wound contraction the values were expressed as mean ± SEM (n=5) significantly different from untreated (*p< 0.05, **p<0.01 and ***p< 0.001).

4.9.1 Histological studies

Histological studies of treated and untreated sections of the wound tissues showed varying degree of fibrosis. Specimen showed 70 to 80% dense and thickened fibrosis, late stage atrophy of the epidermis and also orthohyperkeratosis in the dermoepidermal junction for 7 and 10% w/w extract cream. The untreated showed a thin layer of fibrosis of the epidermis. However, the positive control (1% w/w silver sulphadiazine) showed 60 to 70% fibrosis.

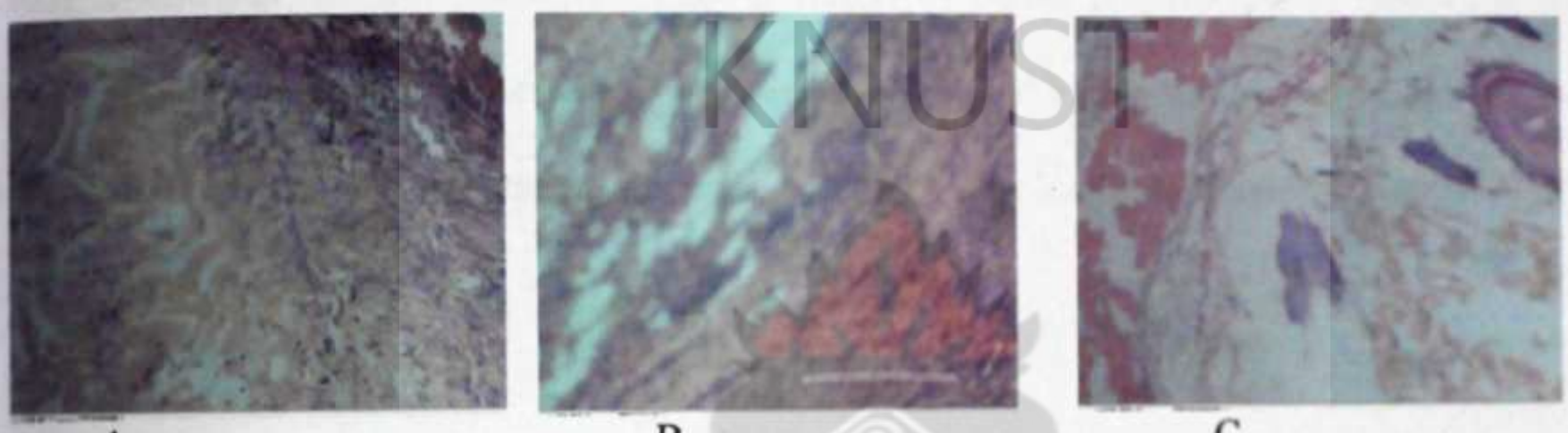

Fig. 4.21: Microscopic histological section of excised tissues. Histological section stained with hematoxylin and eosin, **(A)** 10%w/w extract, **(B)** 7%w/w extract, **(C)** untreated, **(D)** 1%w/w silver sulphadizine, **(E)** 4%w/w extract and **(F)** base only

CHAPTER FIVE

5.0 DISCUSSION

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and microbes (Erdogrul, 2002). Antimicrobial agents from plants have been shown to have genuine utility and about 80% of rural population depends on it as primary health care (Akinyemi *et al.*, 2005). Traditional medical practitioners use a variety of herbal preparations to treat different kinds of microbial diseases. The leaves of *C. anisata* are used by the traditional healers for the treatment of various skin infections, ulcers, sores and other microbial infections (Hamza *et al.*, 2006).

In this study, different solvents (ethanol, aqueous, petroleum ether and ethyl acetate) were used for the preliminary extraction and the ethanol extraction had the highest percentage yield of 8.51% w/w compared to the aqueous (7.76%), petroleum ether (6.45%)and ethyl acetate (7.55%) (Table 4.1). The preliminary *in vitro* antimicrobial activity of the different solvent extracts against the test organisms was determined using the agar well diffusion method. The solvents extracts showed varying degrees of antimicrobial activity, with ethanol extract exhibiting the highest activity at the same concentrations as the other solvent extracts, against all the test organisms. Though traditional healers use primarily aqueous extract, solvents extracts have been reported to give more consistent antimicrobial activity compared to water extract (Parekh *et al.*, 2005). Thus the ethanol extracted more of the constituents than aqueous, petroleum ether and ethyl acetate. Hence ethanol could be a better extracting solvent for most of the constituents of *C. anisata* leaves. The antimicrobial activity exhibited by the various extracts may give an

indication of the use of the plant for the treatment of infectious diseases by the traditional healers.

The result of the preliminary phytochemical screening of *C. anisata* ethanol leaf extract showed the presence of tannins, saponins, flavonoids, steroids, phenolics, anthraquinonnes, glycosides, cardiac glycosides and alkaloids. Studies have shown that phytochemical constituents such as flavonoids have been found *in vitro* to be effective against a wide range of microorganisms (Harborne, 1993; Owoyele *et al*., 2005). Tannins and saponins have been reported to prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for them (Sodipo *et al*., 1991; Panda and Tripathy, 2009). Studies have also shown that, growth of many fungi, bacteria and viruses are inhibited by tannins (Chung *et al*., 1998; Panda and Tripathy, 2009) and the presence of flavonoids, tannins and saponins in the ethanol leaf extract may therefore be responsible for the antimicrobial properties of the *C. anisata*.

The presence of flavonoids was confirmed on the TLC chromatogram by the characteristic yellow fluorescence bands at 365 nm in the pet ether, chloroform and methanol fractions on the thin layer chromatography. This suggests that *C. anisata* possibly contains non-polar and polar flavonoids (Fig. 4.5). The TLC of the three fractions also revealed a number of characteristic bands which could be used in the identification of the plant. For example, the compound showing characteristic blue fluorescence 365 nm (R_f: 0.83 and 0.24) could be used to identify the *C. anisata* leaf extract. The chromatogram further indicates the presence of similar compounds in the pet ether and chloroform fractions. This explains why the two fractions showed similar

antimicrobial activity (Table 4.5). The inhibition of growth on bio-autography TLC plates by the ethanol extract and fractions against the test bacterium and fungus indicates that antimicrobial agents are present in the non-polar and polar solvents (Fig 4.10). The bio-autography is not a quantitative measure of antimicrobial activity but only indicates the number of compounds that were separated with antimicrobial properties.

The HPLC finger-printing was used for the identification and quality control purposes. The ethanol extract and its fractions showed a characteristic peak under the chromatographic conditions. The peaks (compounds) and their retention times were obtained as preliminary identification measured by reversed-phase HPLC which was also used by Crozier *et al.* 1997, in identification and quantitative analysis of flavonoids in tomatoes, onions and lettuce.

Studies conducted by Gundidza *et al.* (1994) and Chakraborty *et al.* (1995) on isolation of bioactive compounds from the stem bark extracts of *C. anisata* revealed the presence of alkaloids (clausenol and clausenine), with antimicrobial activity.

The antimicrobial activity investigated using the different fractions, showed no significant difference between the activity of the petroleum ether and chloroform fraction. There was however significant difference between the activity of the methanol fraction and the petroleum ether, chloroform fractions ($p<0.05$) at the concentration of 20 mg/mL against the test organisms.

The ethanol extract showed maximum growth of inhibition at all the concentration levels (20.0, 15.0, 10.0 and 5.0 mg/mL) against *S. aureus, E. faecalis and P. vulgaris,* which are pathogenic agents of many infectious diseases and may justify the use of *C. anisata* leaves by the herbal

practitioners for the management of various microbial infections. The Gram-positive bacterium, *S. aureus* is known to cause serious diseases such as pneumonia, meningitis in hospital patients (Curran and Al-Salihi, 1980) and mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, esteomyelitis and food poisoning (Mylotte *et al.*, 1987). *P. vulgaris* and *E. faecalis* cause the urinary tract infections (UTI), pulmonary tract infections, burns, wounds, dysentery-like diarrhoea and other blood infections (Ryan and Ray, 2004). Antimicrobial activity of ethanol extract showed comparatively lower inhibition of growth against the clinical isolates of *P. aeruginosa, S. aureus, E. faecalis and P. vulgaris* and this may be due to the development of resistance as a result of misuse or abuse of available antibacterial agents.

The MIC of the ethanol extract indicates the level of antimicrobial activity against all test organisms (fungi, Gram-positive and Gram negative bacteria). The MIC values ranged between 5.5 and 0.5 mg/mL. Senthilkumar and Venkatesalu, (2009) reported that essential oil extracted from the leaves of *C. anisata* had MIC of 0.250 to 0.125 mg/mL against similar test organisms except *C. albicans* which was not included in the study.

Time-kill studies have been used to investigate numerous antimicrobial agents. They are often used as the basis for *in vitro* investigations into pharmacodynamic drug interactions. They also provide descriptive or qualitative information on the pharmacodynamics of antimicrobial agents. The time kill kinetics of the *C. anisata* ethanol extracts against test organisms gave variable effect among the test organisms (Fig. 4.11-4.16). The extracts exhibited static activity and therefore the time kill kinetics was observed as the reduction of the number of cells of the test organisms by the inhibitory effect of the extract. There was a high reduction for *E. faecalis*, between 12 to 24 h at the concentration of 18.0 mg/mL (Fig. 4.11).

Flavonoid, tannins and alkaloids detected in *C. anisata* leaf extract may be responsible for the wound healing activity. Studies have shown that phytochemical constituents such as flavonoids are known to promote the wound healing process mainly due to their astringent and antimicrobial properties which appear to be responsible for the wound healing and increased rate of epithelialization (Tsuchiya *et al.,* 1996). Thus, the enhanced wound healing property of *C. anisata* could be attributed to the free radical scavenging action and the antibacterial property of the phyto-constituents present in the plants (Leite *et al.*, 2002). Flavonoids have many therapeutic uses due to their antifungal, antioxidant and wound healing properties (Nayak *et al.*, 2009; Okuda, 2005) and hence the wound healing property of *C. anisata* could be a function of either the individual or the additive effects of the phytochemical constituents.

The ethanol extract of the leaf had antioxidant activity with the IC_{50} of 32.9 µg/mL and the N-Propyl gallate (reference antioxidant) under the same conditions was found to be 4.19 µg/mL (Fig 4.18). Though the antioxidant property of the extract was not as potent as the reference antioxidant, this may likely protect the cells against oxidative stress. Thus the DPPH free radical scavenging activity of the ethanol extract of *C. anisata* may suggest that external application of the extracts on the wounds entrapped free radicals liberated in the inflammation phase from the cells surrounding the wound and also have the ability to protect cells from microbial infections. The antioxidant activity of the extract may be due to phenolic compounds because of their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). The phenolic compounds have been found to be capable of regenerating endogenous tocopherol, in the phospholipid bilayer of lipoprotein particles, back to its active antioxidant form and are also

known to inhibit various types of oxidizing enzymes (Hall and Cuppett, 1997). These potential mechanisms of antioxidant action make the diverse group of phenolic compounds the target in the search for health beneficial phytochemicals (Halliwell and Gutteridge, 1989; Hall and Cuppett, 1997).

The 7% w/w leaf extract cream was found to significantly increase the rate of wound contraction ($p< 0.05$) at day 9 and day 13 ($p< 0.01$) compared to the untreated. However, there was no significant difference for 7 and 10% w/w which virtually had almost the same influence on the wound contraction.

Wound repair involves fibroblasts migration from the wound edges to the wound site, proliferation and subsequently production of collagen, the main component in the extracellular matrix. Stimulation of fibroblasts is one mechanism by which herbal extracts might enhance the wound repair process even though, keratinocytes also need to migrate from the wound edge to provide a provisional matrix for the fibroblasts to migrate on, this might be accelerated secondary to a mature dermal matrix (Agyare *et al.*,2011; Priya *et al.*, 2004). These migrations of fibroblasts may be as a result of phytoconstituents such as tannins and flavonoids in the herbal extract that may have a growth factor-like activity or have the ability to stimulate the early expression of growth factors (Priya *et al.*, 2004).

Histological observations of the sections of wound tissue treated with 7 and 10%w/w extract cream, showed 70 to 80% dense and thickened fibrosis compared with silver sulphadiazine (1%w/w) showing 60 to 70%, which was characterized by the accumulation of fibroblasts for the deposition of collagen to form a cross link with other collagen for the contraction of wound during healing. The high accumulation of fibroblasts may be due to tannins or flavonoids present

in the leaf extract of *C. anisata*, as reported by Priya *et al.* (2004). There is also orthohyperkeratosis in the dermo-epidermal junction which is further supported by an increased in the proliferation of fibroblasts, which are responsible for collagen synthesis and therefore better contraction of the wound. The 4% w/w extract showed 20 to 30% scanty inflammatory cells made of lymphocyte and plasma cells characterized by decrease in collagen content and further decrease in the proliferation of fibroblasts, which are responsible for collagen synthesis. The untreated tissues specimen showed a thin layer of fibrosis of the epidermis indicating a slow wound healing process.

The above result may confirm some of the uses of *C. anisata* leaves for the treatment of the microbial infections and various forms of wounds. The bioactive compounds in the ethanol extract and its fractions responsible for the various biological activities including antimicrobial, antioxidant and wound healing properties should be isolated and characterized.

Conclusion

The ethanol extract of *C. anisata* exhibited antimicrobial, antioxidant and wound healing properties. The extract showed broad spectrum antimicrobial inhibitory activity against the test organisms. The MIC range of 4.5 to 0.5 mg/mL and 2.5 to 1.0 mg/mL was determined against the Gram-positive and Gram-negative test organisms respectively. The MIC against *C. albicans* was 5.5 mg/mL. The ethanol extract exhibited static activity against all the test organisms.

The ethanol extract were found to contain tannins, alkaloids, steroids, saponins, phenolics, flavonoids, cardiac glycosides and anthraquinones glycosides. The presence of flavonoids was characteristic on the TLC chromatogram for all the different fractions and ethanol crude extract. The HPLC and TLC finger-printing of the ethanol and its fractions were developed.

The ethanol extract exhibited antioxidant activity with IC_{50} of 32.9µg/mL. A significant difference in wound size or contraction was observed on day 9^{th} ($p<0.05$) and day 13^{th} ($p<0.01$) for the 7% w/w ethanol extract compared to the untreated. The 7% w/w ethanol extract influenced the development of high levels of fibrous connective tissues which may enhance the proliferation of fibroblasts an important process in the wound healing.

The leaves of *C. anisata* extract therefore, have great potential as natural antimicrobial and wound healing agents. The ethanol extract stimulated wound contraction thus facilitating wound healing process and the inhibitory activity against the test organisms may confirm its uses in the traditional medicine.

Recommendations

- Further studies should be done on isolation and characterization of the bioactive compounds responsible for the antimicrobial, antioxidant and wound healing properties of leaf extract of *C. anisata*.
- The mechanisms of action responsible for the above biological properties of the *C. anisata* extract and its bioactive compounds should also be studied.
- Toxicological evaluation or studies especially on skin cells (fibroblast and keratinocytes) should be carried out on the leaf extract of *C. anisata* to determine its toxicity profile.

APPENDICES

APPENDIX I

PREPARATION OF CULTURE MEDIA

1. NUTRIENT AGAR (Oxoid Ltd. England)

Composition	Quantity (g)
Lab-lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water	1.0 L

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. This was distributed into glass tubes (20 mL per tube) and sterilized at 121°C for 15 min in an autoclave.

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

Composition	Quantity (g)
Beef extract	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Distilled water	1.0 L

Nutrient broth powder (13g) was weighed into a conical flask and dissolved in distilled water to 1 litre. This was distributed into glass tubes (10 mL per tube) and sterilized at 121°C for 15 min in an autoclave.

3. CETRIMIDE AGAR (Oxoid Ltd. England)

Composition	Quantity (g)
Gelatin peptone	20.0
Magnesium chloride	1.4
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.6
Distilled water	1.0 L

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

4. MANNITOL SALT AGAR (Oxoid Ltd. England)

Composition	Quantity (g)

Lab-lemco powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
Distilled water	1.0 L

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 was distributed into glass tubes and sterilized at 121°C for 15 min in an autoclave.

5. BLOOD AGAR BASE (Scharlau Chemie, Spain)

Composition	Quantity (g)
Casein peptone	12.0
Meat peptone	11.0
Sodium azide	0.4
Starch	1.5
Sodium chloride	5.0
Agar	15.00
Distilled water	1.0 L

Nutrient agar powder (44.5 g) was weighed into 950 mL of distilled water in conical flask and brought it to the boil. This was distributed into glass tubes and sterilize at 121°C for 15 min in an autoclave. The blood agar was cooled to 45-50°C and defibrinated horse blood was aseptically added (Oxoid Ltd England) at 5% proportion.

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

Composition	Quantity (g)
Peptone	10.0
Lactose	10.0
Dipotassium Hydrogen phosphate	2.0
Yellow Eosin	0.4
Methylene blue	0.06
Agar	15.0
Distilled water	1.0 L

Eosin methylene blue agar (37.5 g) was dissolved in 1 litre of distilled water. This was boiled and distributed into glass tubes and then sterilized at 121°C for 15 minutes in an autoclave.

7. MACCONKEY AGAR (Scharlau Chemie, Spain)

Composition	Quantity (g)
Peptone	20.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.0

Neutral red	0.03
Crystal violet	0.001
Agar	15.0
Distilled water	1.0 L

Maconkey agar powder (51.5 g) was weighed into 1 litre of distilled water in conical flask. By heating to melt in a boiling water bath, it was then distributed into glass tubes (20 mL per tube) and sterilized at 121°C for 15 minutes in an autoclave.

8. MUELLER HINTON AGAR (Oxoid Ltd. England)

Composition	Quantity (g)
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Distilled water	1.0 L

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 (20 mL per tube) and sterilized at 121°C for 15 min in an autoclave.

9. PLATE COUNT AGAR (Oxoid Ltd. England)

Composition	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 is distributed into glass tubes and sterilized at 121°C for 15 minutes in an autoclave.

10. SABOURAUD DEXTROSE AGAR (AR Merck, UK)

Composition	Quantity (g)
Peptone	10.0
D (+) Glucose	40.0
Agar	15.0
Distilled water	1.0 L

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)

Composition	Quantity (g)

Casein peptone	5.0
Meat peptone	5.0
D (+) Glucose	20.0
Distilled water	1.0 L

Thirty (30) g of powder was dissolved in 1 litre of distilled water. This was dispensed into glass tubes (10 mL per tube) and sterilized at 121°C for 15 minutes in an autoclave.

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

Composition	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
Distilled water	1.0 L

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 petri dish immediately. This was not autoclaved.

13. TRYPTONE WATER (Oxoid Ltd. England)

Composition	Quantity (g)
Tryptone	10.0
Sodium chloride	5.0
Distilled water	1.0 L

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

14. KOSER CITRATE MEDIUM (Oxoid Ltd. England)

Composition	Quantity (g)
Sodium Ammonium phosphate	1.5
Potassium Dihydrogen phosphate	1.0
Magnesium sulphate	0.2
Sodium citrate	2.5
Bromothymol blue	0.016
Distilled water	1.0 L

Koser citrate powder (5.2 g) was weighed into a conical flask and dissolved in 1L distilled water. The mixture was distributed into glass tubes in portions of 10 mL and

sterilized at 121°C for 15 minutes in an autoclave.

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

16. Rabbit blood 5% v/v proportion.

Standardization of test microorganisms

Nutrient broth (10 mL) was aseptically inoculated with *S. aureus* from the stock culture and incubated at 37°C for 18 to 24h. 1mL of the resultant suspension was serially diluted 10^3, 10^4, 10^5 and 10^6 times in test tubes containing 9.0 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 (A1 and A2) and Graphs (figure 1to 7) shows results of the Log10 cfu/mL plotted against the absorbance.

Table A.1: Absorbance of 24 h broth culture

S. aureus		*E. faecalis*		*B.thuringiensis*	
Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL
0.639	6.693	0.648	7.107	0.337	1.644
0.662	7.622	0.718	8.233	0.344	2.125
0.746	8.626	0.752	9.152	0.356	2.571
0.790	9.672	0.831	10.220	0.455	2.921

The mean absorbance was determined from three independent result (n=3)

Table A. 2: Absorbance of 24 h broth culture

B. subtilis		*P. vulgaris*		*P. aeruginosa*		*C. albicans*	
Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL
0.329	1.505	0.456	7.246	0.494	7.303	0.336	1.079
0.331	2.283	0.490	8.196	0.576	8.441	0.340	1.477
0.358	2.455	0.502	9.210	0.671	9.362	0.359	1.833
0.379	2.626	0.822	10.255	0.781	10.340	0.524	2.100

The mean absorbance was determined from three independent result (n=3)

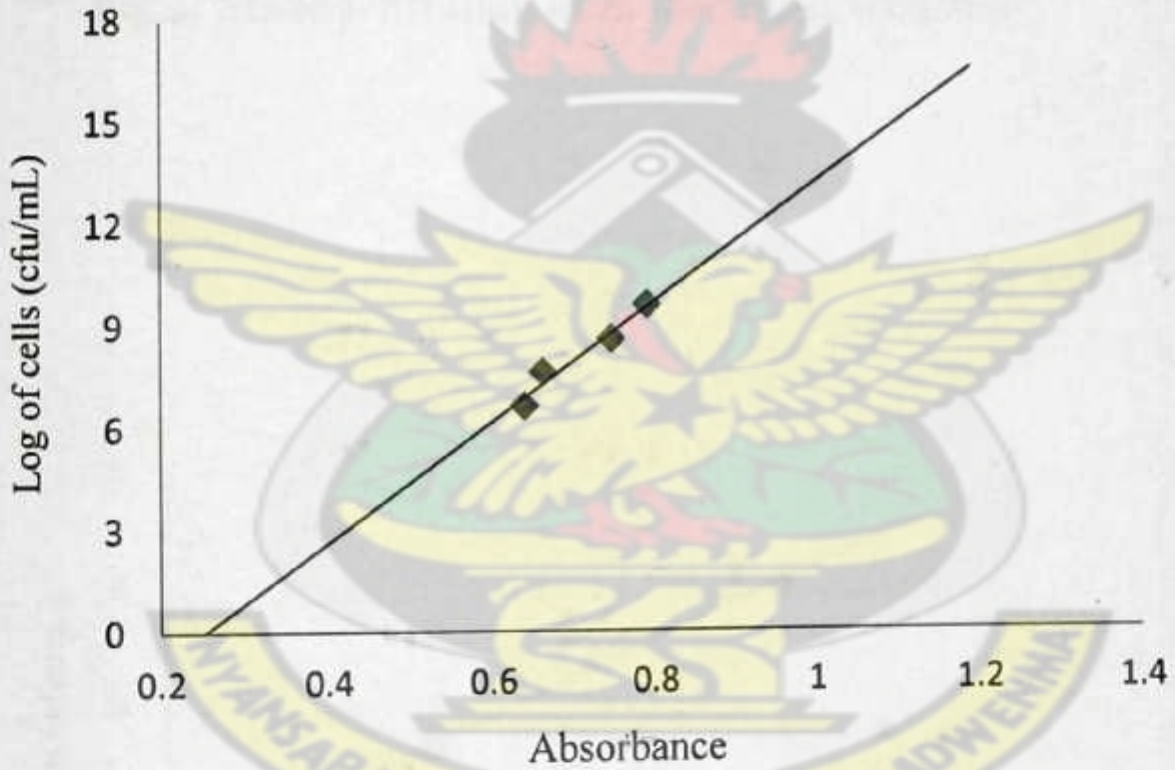

Fig.1: Standardization of *S. aureus* suspension

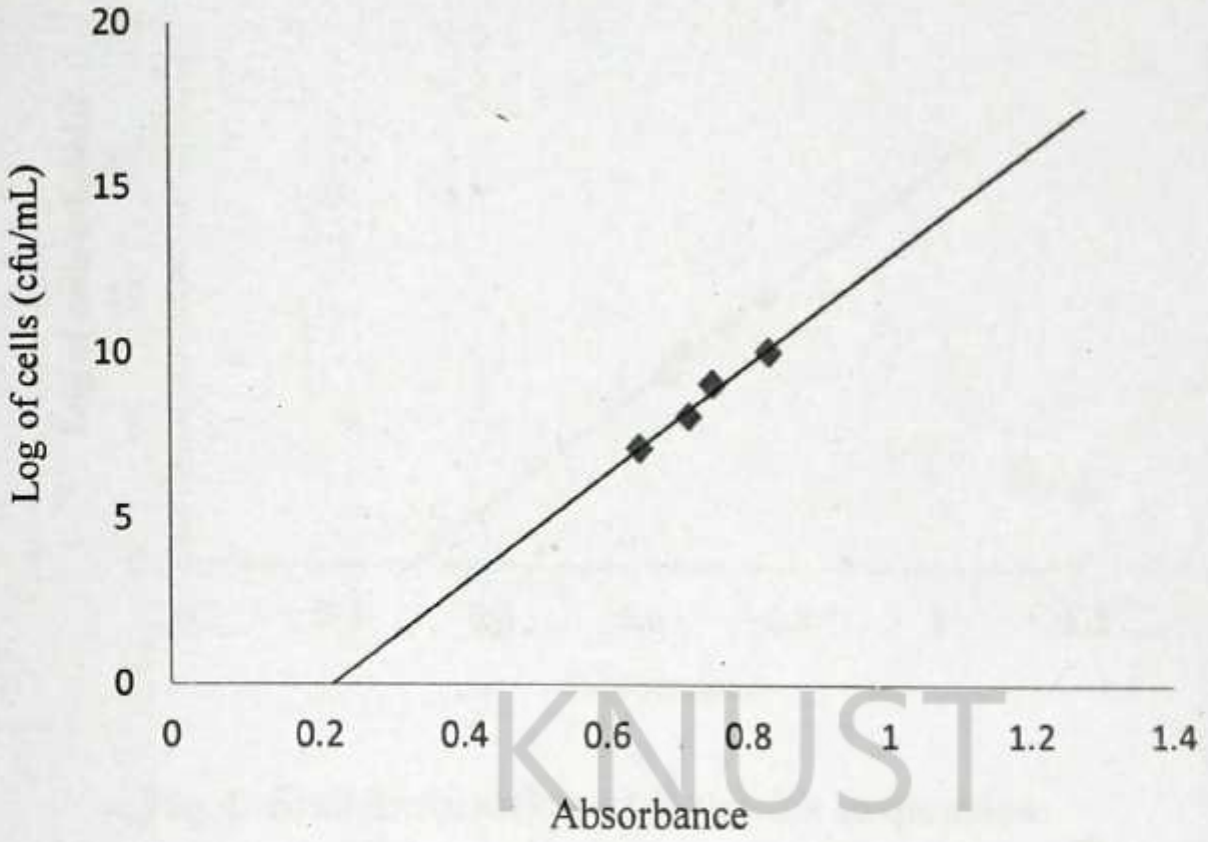

Fig.2: Standardization of *E. faecalis* suspension

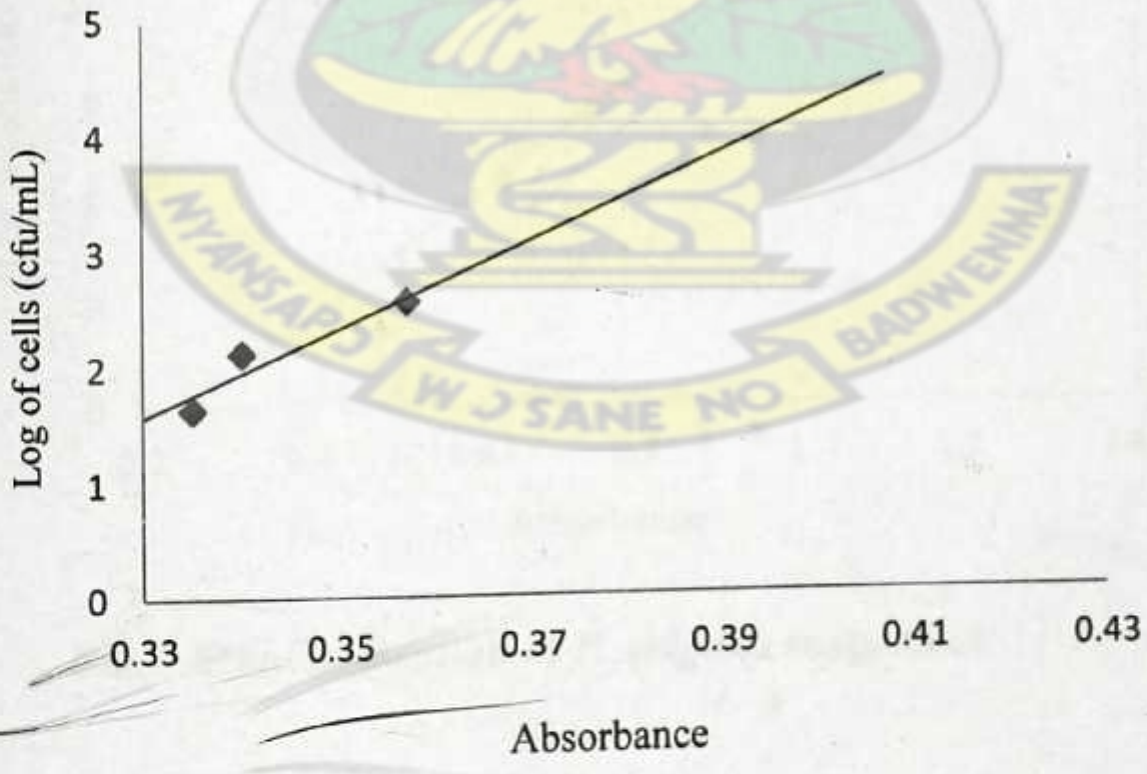

Fig.3: Standardization of *B. thuringiensis* suspension

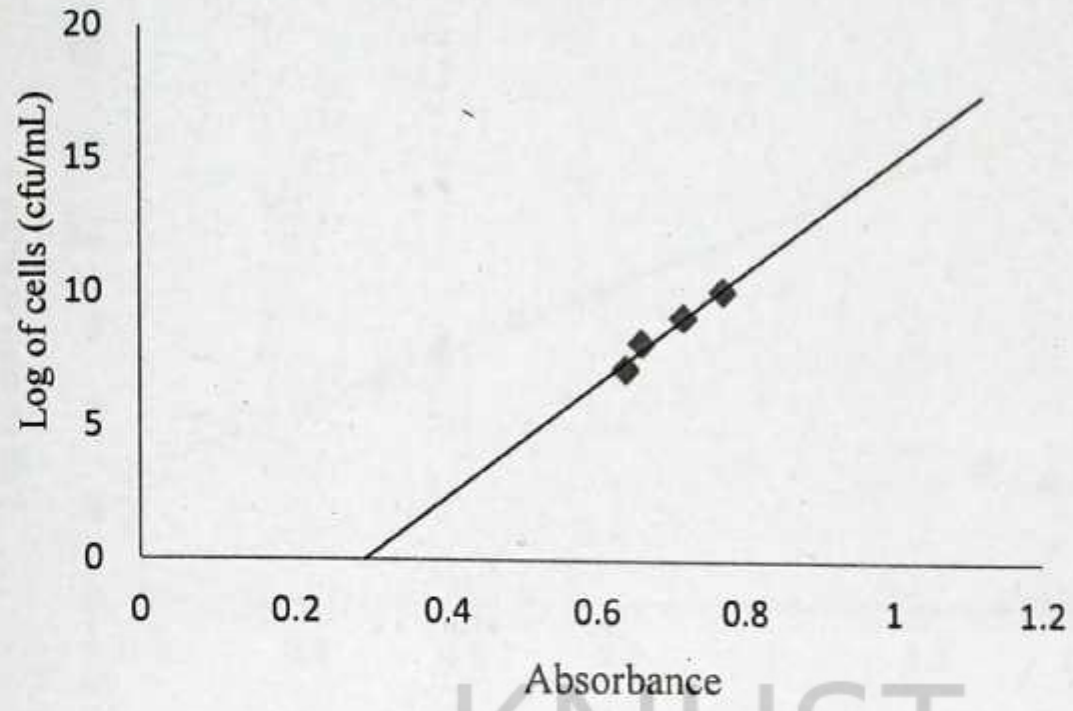

Fig.4: Standardization of *B.subtilis* suspension

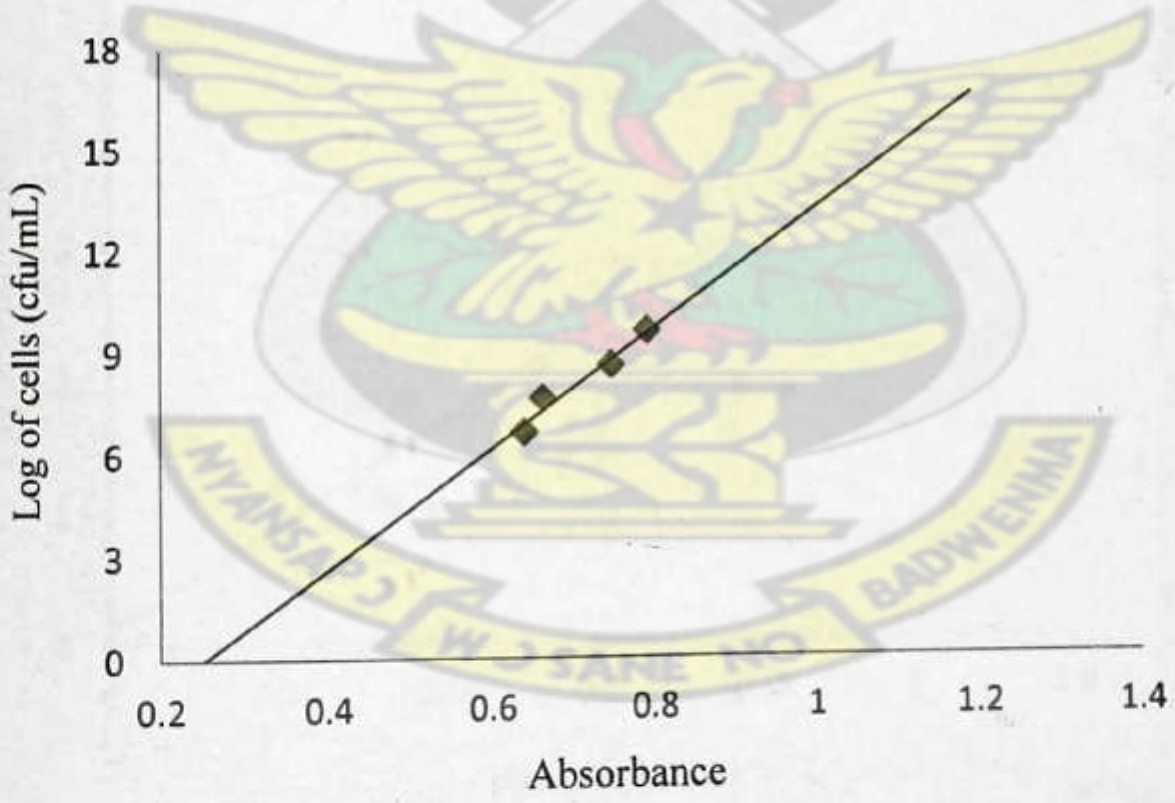

Fig.5: Standardization of *P. vulgaris* suspension

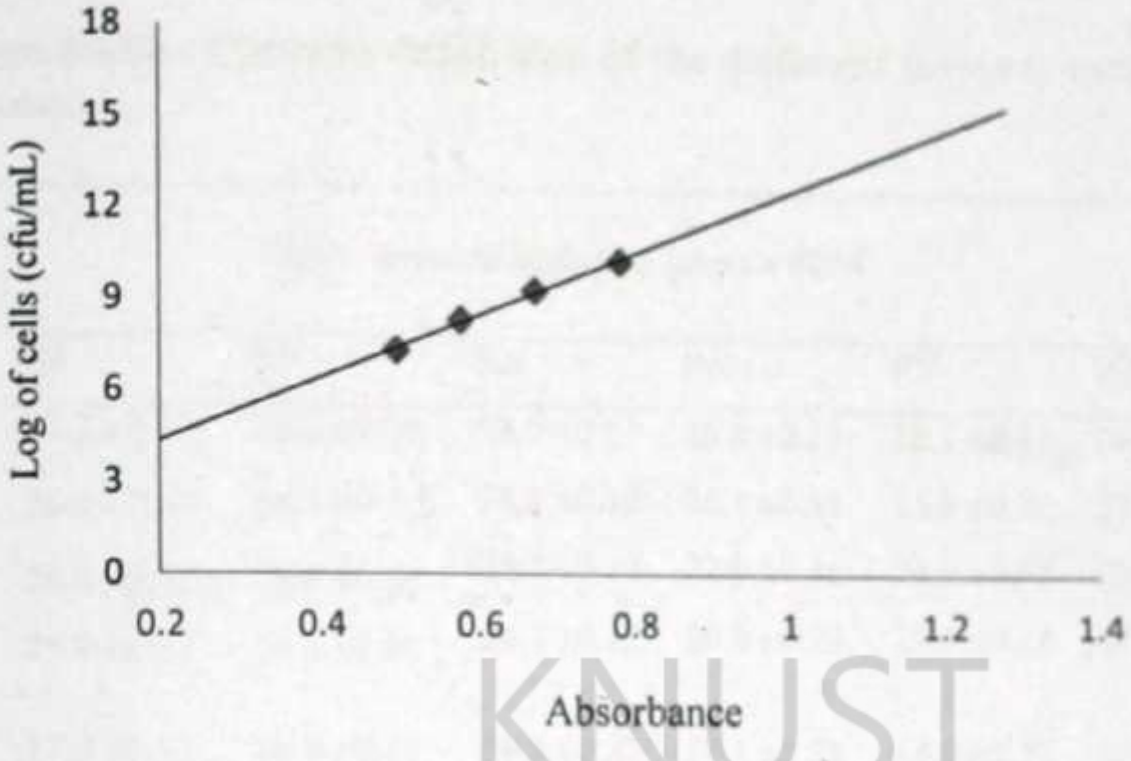

Fig.6: Standardization of *P. aeruginosa* suspension

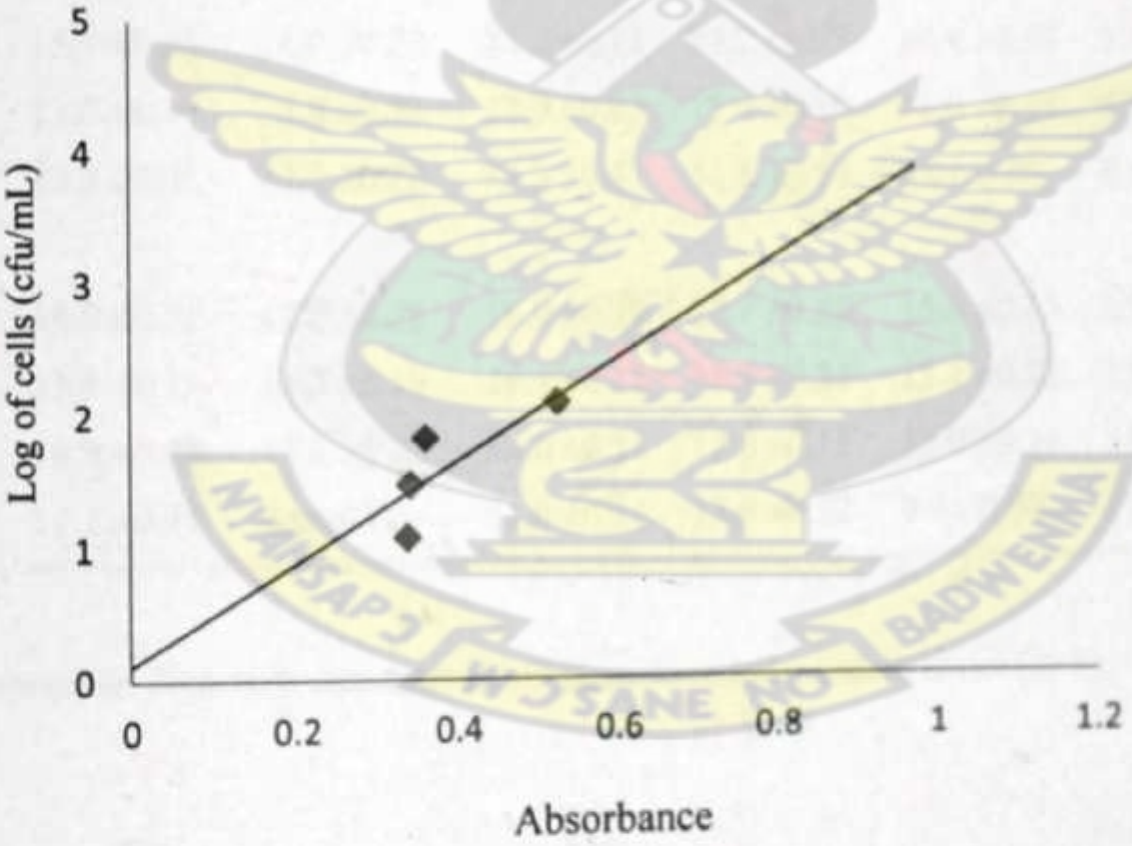

Fig.7: Standardization of *C. albicans* suspension

APPENDIX II

Table B. 1 Average zones of growth–inhibition of the different solvents extracts against test organisms.

Solvents	Conc. mg/mL	Mean zones of inhibition (mm) ± SEM						
		EF	BS	SA	PA	PV	BT	CA
Ethanol Extract	50	27.2 ±0.13	25.0 ±0.10	28.9 ±0.11	26.8 ±0.21	28.1 ±0.33	24.2±0.23	26.8±0.57
	40	26.9 ±0.22	24.5 ±0.33	27.0 ±0.10	26.1 ±0.11	25.8 ±0.22	23.1±0.21	24.0±0.16
	30	26.0 ±0.21	23.9 ±0.16	25.1 ±0.33	22.9 ±0.16	24.9 ±0.57	20.0±0.10	22.2±0.11
	20	25.0 ±0.11	23.3 ±0.28	24.3 ±0.22	20.8 ±0.23	20.4 ±0.11	17.2±0.33	19.7±0.23
Aqueous Extract	50	17.5 ±0.31	16.0 ±0.11	19.3 ±0.22	17.1 ±0.21	16.0 ±0.57	17.2±0.23	19.0±0.11
	40	14.6 ±0.41	14.2 ±0.22	18.1 ±0.16	15.2 ±0.33	14.0 ±0.11	15.3±0.12	15.2±0.31
	30	12.0 ± 0.23	13.0 ±0.11	15.0 ±0.33	13.0 ±0.12	12.3 ±0.22	14.0±0.11	14.0±0.13
	20	0.0 ±0.00	12.3 ±0.21	13.0 ±0.13	11.0 ±0.16	12.0 ±0.12	0.0 ±0.00	12.0±0.11
Petroleum ether extract	50	16.9 ±0.22	17.1 ±0.14	16.1 ±0.33	17.0 ±0.21	16.5 ±0.33	16.3±0.28	16.2±0.24
	40	15.1 ±0.43	16.0 ±0.22	15.2 ±0.11	13.2 ±0.33	14.4 ±0.57	15.2±0.31	15.1±0.33
	30	13.0 ±0.11	15.0 ±0.33	13.0 ±0.23	12.0 ±0.10	11.0 ±0.11	12.0±0.33	13.0±0.16
	20	12.2 ±0.41	12.1 ±0.22	13.3 ±0.41	11.8 ±0.57	0.0± 0.00	0.0±0.00	11.9±0.23
Ethyl acetate Extract	50	16.0 ±0.22	17.0 ±0.32	19.0 ±0.10	17.7 ±0.58	15.3 ±0.13	15.0±0.12	14.0±0.32
	40	13.5 ±0.11	14.5 ±0.16	16.1 ±0.12	15.8 ±0.31	15.1 ±0.22	13.0±0.11	14.5±0.22
	30	10.9 ±0.58	13.2 ±0.22	14.0 ±0.11	15.0 ±0.11	12.0 ±0.16	13.3±0.31	13.0±0.11
	20	11.1 ±0.11	0.0± 0.00	11.0 ±0.11	13.0 ±0.22	0.0 ±0.00	12.0±0.16	11.0±0.21

The mean zones of inhibition of growth was determined from three independent result (n=3)

B.2 Confirmatory test on clinical isolates

Test organisms	Identification	Indole	Oxidase	Catalase	citrate
S. aureus	Bright yellow zones around the colonies	–	–	+	–
P. aeruginosa	Greenish colonies	–	+	+	+
P. vulgaris	Pink colonies	+	–	+	+
E. faecalis	Maroon/ red colonies	–	–	–	–
S. pyogenes	Clear zones around colonies	–	–	–	+
C. albicans	White milky colonies	nd	nd	nd	nd

nd= not detected

B. 3 HPLC Retention time and percentage area of ethanol extract

Peak	Retention time for peak	AUC	Percentage AUC
1	1.478	448419	3.202
2	1.919	296616	2.118
3	1.988	164314	1.173
4	2.082	263095	1.879
5	2.369	273731	1.955
6	2.856	4018205	28.696
7	3.462	77939	0.557
8	4.308	3194893	22.816
9	6.842	4886522	34.897
10	7.315	379023	2.707

B.4 HPLC Retention time and percentage area of methanol fraction

Peak	Retention time for peak	AUC	Percentage Area
1	1.023	38771	1.412
2	1.215	27132	0.988
3	1.282	44360	1.615
4	1.496	710326	25.862
5	1.907	227860	8.296
6	1.948	308059	11.216
7	2.168	137019	4.989
8	2.322	170694	6.215
10	2.837	701500	25.540

B.5 HPLC Retention time and percentage area of petroleum ether fraction

Peak	Retention time for peaks	AUC	Percentage AUC
1	1.079	29929	0.870
2	1.235	33739	0.981
3	1.408	121619	3.534
4	1.542	141298	4.106
5	1.647	443837	12.899
6	1.884	164020	4.767
7	2.235	382843	11.126
8	2.488	244258	7.099
9	2.849	703277	20.438
10	3.302	72554	2.109

B.6 HPLC Retention time and percentage area of petroleum ether fraction

Peak	Retention time for peaks	AUC	Percentage AUC
1	1.335	55623	7.268
2	1.906	39554	5.168
3	1.962	29953	3.914
4	2.222	33172	4.335
5	2.448	18022	2.355
6	2.849	319913	41.802
7	4.408	58057	7.586
8	6.893	196017	25.613
9	7.382	14986	1.958
10	7.657	3456	2.946

B. 7 MICs of the ethanol extract (mg/mL) by Broth dilution technique.

Test Organisms	Concentrations (mg/ml)										
	200	100	50	25	12.5	6.25	3.20	1.60	0.80	0.40	MIC
E. faecalis	–	–	–	–	–	–	+	+	+	+	4.5
B. subtilis	–	–	–	–	–	–	–	–	–	+	0.5
S. aureus	–	–	–	–	–	–	–	–	+	+	0.9
P. auringinosa	–	–	–	–	–	–	–	+	+	+	2.5
Pr. vulgaris	–	–	–	–	–	–	–	–	+	+	1.0
B. thuringiensis	–	–	–	–	–	–	–	+	+	+	1.5
C. albicans	–	–	–	–	–	–	+	+	+	+	5.5

B.8 MBCs of the ethanol extract (mg/mL) by Broth dilution technique.

Test Organisms	Concentrations (mg/ml)										
	200	100	50	25	12.5	6.25	3.20	1.60	0.80	0.40	MBC
E. faecalis	–	–	–	–	+	+	+	+	+	+	19.0
B. subtilis	–	–	–	–	–	–	+	+	+	+	4.0
S. aureus	–	–	–	–	+	+	+	+	+	+	15.0
P. auringinosa	–	–	–	–	–	+	+	+	+	+	11.0
Pr. vulgaris	–	–	–	–	+	+	+	+	+	+	14.0
B. thuringiensis	–	–	–	–	+	+	+	+	+	+	17.0
C. albicans	–	–	–	+	+	+	+	+	+	+	27.5

B.9 Result for percentage DPPH radical Scavenging activity (Extract) and (N- Propyl gallate)

Concentration of solution		Absorbance at 517 nm								Mean Scavenging Activity (%)	
Extract (mg/ml)	NPG (ug/mL	DPPH + Extract				DPPH+ NPG					
		1	2	3	Average	1	2	3	Average	Extract	NPG
3.0	300.0	0.311	0.311	0.312	0.3113	0.133	0.134	0.134	0.1336	84.31	93.13
1.0	100.0	0.574	0.574	0.574	0.5740	0.282	0.281	0.282	0.2817	70.56	85.55
0.3	30.0	0.814	0.814	0.815	0.8143	0.485	0.485	0.485	0.4850	58.24	75.12
0.1	10.0	1.116	1.116	1.117	1.1163	0.670	0.669	0.669	0.6693	42.76	65.64

$A_{control\ (DPPH)} = 1.950$

B.10 The time-kill kinetics of ethanol extract against *E. faecalis*

	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per mL				
	Concentration (mg/mL)									
Time(h)	4.5	9	13.5	18	control	4.5	9	13.5	18	control
0	108	109	103	111	115	2.03342	2.03743	2.01284	2.04532	2.0607
1	137	121	109	101	210	2.13672	2.08279	2.03743	2.00432	2.32222
2	152	160	113	117	249	2.18184	2.20412	2.05308	2.06819	2.3962
3	144	152	102	105	$42x10^2$	2.15836	2.18184	2.0086	2.02119	3.62325
4	160	141	90	70	$98x10^2$	2.20412	2.14922	1.95424	1.8451	3.99123
5	155	102	73	61	$352x10^2$	2.19033	2.0086	1.86332	1.78533	4.54654
6	140	111	64	53	$105x10^4$	2.14613	2.04532	1.80618	1.72428	6.02119
12	135	105	45	41	$420x10^4$	2.13033	2.02119	1.65321	1.61278	6.62325
24	125	98	33	10	$110x10^7$	2.09691	1.99123	1.51851	1	9.04139

B.11 The time-kill kinetics of ethanol extract against *B. subtilis*

	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per Ml				
	Concentration (mg/mL)									
Time(h)	0.5	1.0	1.5	2.0	control	0.5	1.0	1.5	2.0	control
0	88	111	116	118	122	1.944483	2.045323	2.064458	2.071882	2.08636
1	109	102	98	107	187	2.037426	2.0086	1.991226	2.029384	2.271842
2	125	95	102	91	$123x10^2$	2.09691	1.977724	2.0086	1.959041	4.089905
3	156	101	94	97	$22x10^3$	2.193125	2.004321	1.973128	1.986772	4.342423
4	143	103	100	94	$48x10^3$	2.155336	2.012837	2	1.973128	4.681241
5	215	125	111	115	$105x10^3$	2.332438	2.09691	2.045323	2.060698	5.021189
6	298	183	136	128	$242x10^4$	2.474216	2.262451	2.133539	2.10721	6.383815
12	311	213	151	126	$156x10^5$	2.49276	2.32838	2.178977	2.100371	7.193125
24	321	254	144	120	$71x10^7$	2.506505	2.404834	2.158362	2.079181	8.851258

B.12 The time-kill kinetics of ethanol extract against *P. aeruginosa*

	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per mL				
	Concentration (mg/mL)									
Time(h)	2.5	5.0	7.5	10.0	Control	2.5	5.0	7.5	10.0	control
0	126	120	112	110	118	2.100371	2.079181	2.049218	2.041393	2.071882
1	137	109	96	102	411	2.136721	2.037426	1.982271	2.0086	2.613842
2	117	144	88	76	$205x10^2$	2.068186	2.158362	1.944483	1.880814	4.311754
3	111	122	79	54	$117x10^3$	2.045323	2.08636	1.897627	1.732394	5.068186
4	215	110	121	31	$212x10^4$	2.332438	2.041393	2.082785	1.491362	6.326336
5	401	208	310	65	$56x\ 10^5$	2.603144	2.318063	2.491362	1.812913	6.748188
6	$110x10^2$	$141x10^1$	$75x10^1$	147	$217x10^5$	4.041393	3.149219	2.875061	2.167317	7.33646
12	$278x10^2$	$217x10^2$	$210x10^1$	$109x10^1$	$118x10^7$	4.444045	4.33646	3.322219	3.037426	9.071882
24	$198x10^3$	$128x10^3$	$116x10^2$	$95x10^2$	$128x10^7$	5.296665	5.10721	4.064458	3.977724	9.10721

B.13 The time-kill kinetics of ethanol extract against *P. vulgaris*

Time (h)	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per mL				
	Concentration (mg/mL)									
	1.0	2.0	3.0	4.0	control	1.0	2.0	3.0	4.0	control
0	88	101	97	111	121	1.944483	2.004321	1.986772	2.045323	2.082785
1	124	117	110	115	213	2.093422	2.068186	2.041393	2.060698	2.32838
2	311	271	210	204	119	2.49276	2.432969	2.322219	2.30963	2.075547
3	96 x10^1	317	281	254	385 x10^1	2.982271	2.501059	2.448706	2.404834	3.585461
4	181 x10^1	112 x10^1	101 x10^1	94 x10^1	318 x10^2	3.257679	3.049218	3.004321	2.973128	4.502427
5	282 x10^1	205 x10^1	198 x10^1	177 x10^1	211 x10^3	3.450249	3.311754	3.296665	3.247973	5.324282
6	110 x10^2	386 x10^1	287 x10^1	248 x10^1	196 x10^4	4.041393	3.586587	3.457882	3.394452	6.292256
12	156 x10^2	98 x10^2	111 x10^2	101 x10^2	281 x10^5	4.193125	3.991226	4.045323	4.004321	7.448706
24	211 x10^2	175 x10^2	147 x10^2	120 x10^2	246 x10^6	4.324282	4.243038	4.167317	4.079181	8.390935

B.14 The time-kill kinetics of ethanol extract against *S. aureus*

Time (h)	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per mL				
	Concentration (mg/mL)									
	1.0	2.0	3.0	4.0	control	1.0	2.0	3.0	4.0	control
0	85	108	117	112	97	1.9294189	2.0334238	2.0681859	2.049218	1.9867717
1	66	81	98	101	271	1.8195439	1.908485	1.9912261	2.0043214	2.4329693
2	92	117	125	116	168 x10^1	1.9637878	2.0681859	2.09691	2.064458	3.2253093
3	217	210	198	165	338 x10^2	2.3364597	2.3222193	2.2966652	2.2174839	4.5289167
4	107 x10^1	96 x10^1	101 x10^1	85 x10^1	136 x10^3	3.0293838	2.9822712	3.0043214	2.9294189	5.1335389
5	111 x10^2	78 x10^2	82 x10^2	90 x10^2	410 x10^3	4.045323	3.8920946	3.9138139	3.9542425	5.6127839
6	318 x10^2	217 x10^2	186 x10^2	172 x10^2	217 x10^4	4.5024271	4.3364597	4.2695129	4.2355284	6.3364597
12	114 x10^3	88 x10^3	80 x10^3	54 x10^3	318 x10^4	5.0569049	4.9444827	4.90309	4.7323938	6.5024271
24	280 x10^3	181 x10^3	165 x10^3	114 x10^3	126 x10^5	5.447158	5.2576786	5.2174839	5.0569049	7.1003705

B.15 The time-kill kinetics of ethanol extract against *B. thuringiensis*

Time (h)	Mean number of surviving cells per ml (x 10^3)					Log mean number of surviving cellls per m				
	Concentration (mg/mL)									
	1.5	3.0	4.5	6.0	control	1.5	3.0	4.5	6.0	control
0	114	103	97	110	119	2.0569049	2.0128372	1.9867717	2.0413927	2.075547
1	126	118	107	98	203	2.1003705	2.071882	2.0293838	1.9912261	2.307496
2	195	178	167	154	$260x10^1$	2.2900346	2.25042	2.2227165	2.1875207	3.4149733
3	$303x10^1$	$271x10^1$	$242x10^1$	$209x10^1$	$260x10^2$	3.4814426	3.4329693	3.3838154	3.3201463	4.4149733
4	$203x10^2$	$171x10^2$	$438x10^1$	$361x10^1$	$441x10^3$	4.307496	4.2329961	3.6414741	3.5575072	5.6444386
5	$414x10^2$	$311x10^2$	$117x10^2$	$103x10^2$	$216x10^4$	4.6170003	4.4927604	4.0681859	4.0128372	6.3344538
6	$211x10^3$	$98x10^3$	$316x10^2$	$277x10^2$	$142x10^5$	4.3242825	4.9912261	4.4996871	4.4424798	7.1522883
12	$121x10^4$	$291x10^3$	$248x10^3$	$178x10^3$	$312x10^6$	6.0827854	5.463893	5.3944517	5.25042	8.4941546
24	$113 x10^4$	$268 x10^3$	$231 x10^3$	$216 x10^3$	$279 x10^6$	6.0530784	5.4281348	5.363612	5.3344538	10.445604

B.16 The time-kill kinetics of ethanol extract against *C. albicans*

	Mean number of surviving cells per mL (x 10^3)					Log mean number of surviving cells per mL				
	Concentration (mg/mL)									
Time (h)	5.5	11.0	16.5	22.0	control	5.5	11.0	16.5	22.0	control
0	97	106	103	117	132	1.986772	2.025306	2.012837	2.068186	2.120574
1	123	125	118	107	215	2.089905	2.09691	2.071882	2.029384	2.332438
2	190	170	167	121	351	2.278754	2.230449	2.222716	2.082785	2.545307
3	179	162	173	146	$39x10^2$	2.252853	2.209515	2.238046	2.164353	3.591065
4	281	254	198	157	$58x10^3$	2.448706	2.404834	2.296665	2.1959	4.763428
5	301	249	163	137	$152x10^3$	2.478566	2.396199	2.212188	2.136721	5.181844
6	312	238	154	128	$258x10^3$	2.494155	2.376577	2.187521	2.10721	5.41162
12	319	225	166	119	$65x10^5$	2.503791	2.352183	2.220108	2.075547	6.812913
24	342	233	173	114	$78x10^7$	2.534026	2.367356	2.238046	2.056905	8.892095

B.17 Effect of treatment of wound with time (days)

Days	Wound area (mm^2)					
	Untreated	Silverzine (1% w/w)	Vehicle (base)	Concentration 4% w/w	7% w/w	10% w/w
1	387.3 ±0.21	422.9 ±0.33	394.1 ±0.11	395.0 ±0.10	415.6 ±0.10	401.8 ±0.10
3	339.9 ±0.18	359.8 ±0.17	363.2 ±0.15	353.3 ±0.15	356.5 ±0.11	357.1 ±0.11
5	286.6 ±0.14	320.7±0.15	320.5 ±0.22	337.0 ±0.44	280.5 ±0.22	301.9 ±0.31
7	246.4 ±0.11	251.6 ±0.33	292.6 ±0.11	305.5 ±0.33	229.7 ±0.15	224.7 ±0.21
9	232.9 ±0.33	243.6 ±0.33	274.6 ±0.21	266.0 ±0.11	211.3 ±0.22	206.6 ±0.11
13	174.6 ±0.15	98.8 ±0.57	158.5 ±0.33	197.4 ±0.33	99.2 ±0.24	93.6 ±0.33
15	143.2 ±0.22	74.1 ±0.16	118.4 ±0.16	150.9 ±0.16	75.8 ±0.33	65.1 ±0.22
17	101.3 ±0.10	45.8 ±0.17	98.5 ±0.22	125.4 ±0.17	52.9 ±0.58	39.7 ±0.18
19	68.9 ±0.16	35.7 ±0.22	76.1 ±0.17	104.8 ±0.41	40.2 ±0.11	29.6 ±0.33

B.18 Percentage contraction of wound area with time (days)

Days	Wound area contraction (%)					
	Untreated	Vehicle (base)	Concentrations			
			Silverzine (1% w/w)	4% w/w	7% w/w	10% w/w
3	12.1 ±0.14	8.0 ±0.10	14.8 ±0.11	10.1 ±0.14	12.2 ±0.22	11.2 ±0.26
5	25.7 ±0.22	18.6 ±0.12	23.9 ±0.56	14.5 ±0.33	32.4 ±0.16	24.7 ±0.16
7	36.1 ±0.10	25.7 ±0.12	40.2 ±0.22	22.5 ±0.41	44.7 ±0.10	44.0 ±0.22
9	39.5 ±0.18	30.3 ±0.18	42.1 ±0.12	32.2 ±0.22	49.1 ±0.11	48.5 ±0.44
13	54.9 ±0.11	59.8 ±0.16	76.5 ±0.33	50.5 ±0.44	75.9 ±0.57	76.7 ±0.21
15	63.0 ±0.10	70.0 ±0.26	82.4 ±0.41	61.8 ±0.22	81.6 ±0.22	83.8 ±0.10
17	73.7 ±0.16	75.0 ±0.33	89.1 ±0.34	68.3 ±0.21	87.2 ±0.16	90.0 ±0.14
19	76.4 ±0.22	80.8 ±0.11	91.5 ±0.61	70.9 ±0.13	90.2 ±0.14	93.3 ±0.10

B.19 Antimicrobial activity of formulated cream against test microorganisms

Concentration (mg/mL)	Zones of inhibition ± (SEM)			
	S. aureus	*P. aeruginosa*	*S. pyogenes*	*C. albicans*
1.0	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ± 0.00
3.0	12.1 ±0.11	11.3 ±0.33	12.0 ±0.00	12.6 ±0.56
4.0	15.3 ±0.33	13.1 ±0.33	14.6 ±0.67	13.6 ±0.66
5.0	16.0 ±0.00	14.4 ±0.11	15.8 ±0.33	14.8 ±0.33
7.0	20.1 ±0.33	18.8 ±0.67	19.3 ±0.33	19.0 ±0.00
10.0	22.3 ±0.57	20.1 ±0.33	21.0 ±0.00	21.6 ±0.67
Vehicle (base)	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00	0.0 ±0.00
Silver sulphadiazine	21.3 ±0.33	17.5±0.11	21.6 ±0.57	nd
Ketoconazole	nd	nd	nd	17.6 ±0.66

nd= not detected

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