PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY INVESTIGATION OF *NICANDRA PHYSALOIDES* GAERTN (SOLANACEAE)

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A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in Pharmacognosy and Complementary Medicine

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This thesis is dedicated to my late sister Everline Imali for always being there for me and encouraging me that I am well able to make it in life.

To my dear husband Martin for the moral support, patience ,words of encouragement and understanding.

My lovely children Yovela and Brighton for being the source of motivation and joy in doing the work

To my parents Mr. Charles and Mrs. Irene Mukungu for bringing out the best in me

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND SYMBOLS

A. D.	*Anno Domini* (Year of our Lord)
AIDS	Acquired Immuno Deficiency Syndrome
B.C	Before Christ
^{o}C	degrees Celsius
CDC	Centre for Disease Prevention and Control
CLSI	Clinical Laboratory Standards Institute
DEA	Diethylamine
ESI-TIC	Electrospray Ionization Total Ion Current
g	gram
HIV	Human Immunodeficiency Virus
IR	Infra Red
KNH	Kenyatta National Hospital
m/z	mass/charge ratio
MALDI	Matrix Assisted Laser Desorption
mg	milligram
MIC	Minimum Inhibitory Concentration

MRSA	Methicillin Resistant *Staphylococcus aureus*
NCCLS	National Committee on Clinical Laboratory Standards
NCE	New Chemical Entity
NMR	Nuclear magnetic resonance
RF	Retention Factor
SARS	Severe Acute Respiratory Syndrome
SEA	South East Asia
TLC	Thin Layer chromatography
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
UV	Ultra Violet
WHO	World Health Organisation
WTO	World Trade Organization.
µ	Micron
µg	microgram
µl	microlitre

ABSTRACT

An enormous variety of medicinal plants are used worldwide by about 80 % of the world population, although in most cases no scientific studies have been done to prove the efficacy of these medicinal plants. Considering that most present-day western medicines are based on the traditional medicinal plants of European, Mediterranean and Arabic origin, the variety of plants in use around the world may very well represent an enormous treasure for drug development. The objectives of this study were to investigate the phytochemical properties of *Nicandra physaloides*, to isolate and characterize its components and to screen the crude extracts and isolated compounds for antimicrobial activity. This plant is used in traditional medicine as an analgesic, anti-inflammatory, anthelmintic, wound healing and as an insecticide.

The plant was collected from gardens surrounding Kenyatta National Hospital, Nairobi county, in Kenya in August 2009. It was air dried in the laboratory and the whole aerial parts which included the stems, fruits, leaves and flowers milled into powder for use. The extraction was done by cold maceration. The crude extract was evaporated to dryness *en vacuo*. Alkaloids were extracted from the extract and the remaining extract was subjected to column chromatography on normal silica gel. Thin layer chromatography using precoated aluminium plates was used to monitor the fractions. Two crystalline compounds were isolated and analyzed using spectroscopic methods. One of the compounds was identified as withanicandrin. The second compound was a mixture of phytosterols, two of which were identified as stigmasterol and β-sitosterol.

Some extracts of the plant and the isolated compound were prepared and screened for both antibacterial and antifungal activities using agar diffusion method. Petroleum ether extract,

ethylacetate, methanol extract and the alkaloid mixture, at a concentration of 50 mg/ml, showed activity against all the bacteria tested namely; *Staphylococcus aureus, Escherichia coli and Bacillus pumilus.* Withanicandrin lacked activity against any of the bacterial micro-organisms.

Petroleum ether extract, alkaloid mixture and withanicandrin, showed activity against the *S. cerevisiae* and *C. albicans*. Withanicandrin had the highest activity against these fungal micro-organisms.

From this study, *Nicandra physaloides* extracts showed both antibacterial and antifungal activities thus confirming scientific basis of the use in the folklore in management of wounds. The isolated compound, withanicandrin, showed antifungal activity. Further work should be done to improve on this activity.

CHAPTER ONE

INTRODUCTION

1.1 Background information

For thousands of years, natural products have played an important role throughout the world in treatment and prevention of human diseases. These products have come from various sources including terrestrial plants, terrestrial microorganisms, marine organisms and terrestrial vertebrates and invertebrates (Newman *et al*., 2000). Among these products used, herbal drugs constitute a major part in the traditional system of medical practice (Santos *et al*., 2007).

The use of plants as medicines is older than recorded history. Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years B.C (Solecki and Shanidar, 1975). The first known written record of herbal remedies was by a Sumerian physician around 2200 B.C. The records of King Hammurabi of Babylon of 1800 B.C include instructions for using medicinal plants. He prescribed use of mint in digestive disorders (Dee, 2009). The entire Middle East has a rich history of herbal healing. There are texts from the ancient cultures of Mesopotamia, Egypt and India that illustrate use of many medicinal plants (Aaboe and Asger, 1991). The first extensive record was made by Hippocrates in Greece in the fifth century B.C when he described about 400 herbs and their medicinal properties. In the first century A.D, Dioscorides expanded the list to around 600 herbs. Eventually, herbs were widely popularized by the writings of Nicholas Culpeper in the seventeenth century when he categorized all known herbs and their uses (McCarl, 1996).

Many of the plants used as medicines seem to have been developed initially through observation of their use by animals. Although their use appears to be much more prevalent among humans than animals, understanding the prominence in human use is bound to perspectives gleaned from studies of self-medication in animals and differences and similarities between humans and animals in the types of herbal medicine practiced (Benjamin, 2005). The development may also have arisen through trial and error.

The Chinese have for a long time taken full advantage of medicinal plants. The Emperor Shen Nung (c 2695 BC), the father of Chinese medicine, studied medicinal plants and verified their medicinal properties. He is said to have tasted hundreds of herbs to test their medicinal value. Over 11,000 herbal remedies were developed and used in China for thousands of years. These include *Ephedra sinica* (known as Ma Huang in Chinese), the source of the celebrated adrenergic drug ephedrine and *Artemisia annua*, the source of artemisinin, an antimalarial drug in current clinical use (Chen, 2000). Traditional Chinese medicine is still in common use in China today. More than half the population regularly use traditional remedies, with the highest prevalence of use in rural areas. Chinese herbs are most commonly used for infections, respiratory problems and rheumatological disorders (Wai *et al*., 1995). Herbal products account for approximately one fifth of the entire Chinese pharmaceutical market (Li, 2000).

In India, it is reported that traditional healers use up to 2500 plant species in total and 100 species of plants serve as regular sources of medicine (Pei, 2001). The majority of India's population still receive medical services from indigenous practitioners who treat their patients according to the principles of three ancient systems of Indian medicine namely; the Ayurveda, the Siddha medicine of South India, and the Unani or Graeco-Arabic medicine (Mel, 1987).

Africa is endowed with many plants that can be used for medicinal purposes. Indeed, out of the approximated 6400 plant species utilised in tropical Africa for various purposes, more than 4000 species are used as medicines (Stanley, 2004). Medicinal plants in Africa are used in the treatment of many diseases and illnesses. Other plants are also used for their symbolic and spiritual significance. In sub-Saharan Africa today, traditional healers far outnumber modern health practitioners and the majority of the population use herbal medicine. The majority of poor populations in East Africa have access to only traditional health care (Green, 1994).

Although practice of traditional medicine is widespread throughout the world, it is an integral part of individual cultures. Its practice is based mainly on traditional beliefs handed down from one generation to another for hundreds or even thousands of years. Unfortunately, much of this ancient knowledge and many valuable plants are being lost at an alarming rate. There is hardly any written record of these traditional African practices.

The Industrial Revolution and the development of organic chemistry resulted in a preference for synthetic drugs for pharmacological treatment of diseases. The reasons for this were that pure compounds were easily obtained in large quantities through synthesis, structural modifications to produce potentially more active and safer drugs could be easily performed and the economic power of the pharmaceutical companies was increasing. Furthermore, throughout the development of human culture, the use of natural products had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture. Drugs from natural resources were considered either an option for poorly educated or low income people or simply as religious superstition of no pharmacological value (Rates, 2001).

In recent years, there has been growing interest in alternative therapies including the use of natural products, especially those derived from plants (Goldfrank *et al.,* 1982; Levi, 2006; Gazzaneo *et al*., 2005). The use of conventional medicines results in side effects and other problems that are rarely seen with use of herbal drugs. Furthermore, about 30 percent of the world's population does not have access to essential medicines due to their unavailability and unaffordability (WHO, 2004). The World Health Organization recognizes the important role of traditional medicine in developing countries. It accepts that traditional systems will continue to play an important part in providing services to very large numbers of people, particularly in rural areas (WHO, 1976). These herbs are used in management of both acute and chronic diseases, including treating common cold, controlling blood pressure, cholesterol and several infectious diseases and other conditions. In some cases, traditional medicine tends to thrive in conjunction with westernization, modernization and urbanization, as in the case of countries such as Ghana and Nigeria. In some countries, such as China, the government has promoted a dual system in which paramedical personnel are trained in both traditional and modern orthodox diagnostic and treatment procedures (David *et al.,* 1988; Shuang and Mitchell, 2008).

The chemical constituents of plant medicines are usually part of the physiological activities of living plants hence they are believed to have a better compatibility with the human body. Since these secondary metabolites have been elaborated within living systems, they are often perceived as showing more drug-likeness and biological friendliness than totally synthetic molecules (Koehn and Carter, 2005), making them good candidates for further drug development (Balunas and Kinghorn, 2005). The large proportion of natural products in drug discovery has stemmed from these diverse chemical structures of natural products.

The potential use of higher plants as a source of new drugs is still under-exploited. Of the estimated 250,000–500,000 plant species, only around 21,000 plants have been used for medicinal purpose in the world (Rates, 2001). It is estimated that only 5000 species have been studied for medical use (Payne *et al*., 1991), 500 species of which have been thoroughly investigated as potential source of new drugs (Sara, 1992; Cathrine and Prabavathi, 2011). In most cases, only pharmacological screening or preliminary studies have been carried out.

1.2 Plants as sources of pharmaceuticals

A multitude of plants, often of unreliable quality, are readily available over-the-counter from herbal suppliers and general stores thus promoting self-medication with these substances. The herbs are available in different forms: teas, syrups, oils, liquid extracts, tinctures, and dry extracts (pills or capsules). Examples of these products are the herbal teas or other homemade remedies such as *Allium sativa* and *Zingiber officinale*. Some pharmaceutical preparations are available as crude extracts or standard enriched fractions such as tinctures, fluid extracts, powder, pills and capsules. Common formulations include St. John's Wort and milk thistle. When subjected to extraction and purification procedures, plants yield compounds of interest, which can themselves be active and used directly as drugs such as quinine, digoxin and ergotamine, or as precursors such as diosgenin in semisynthetic processes. With well-defined pharmacological activity or structure-activity relationship studies, these compounds can be used as prototypes for total synthesis of drugs as in the case of morphine.

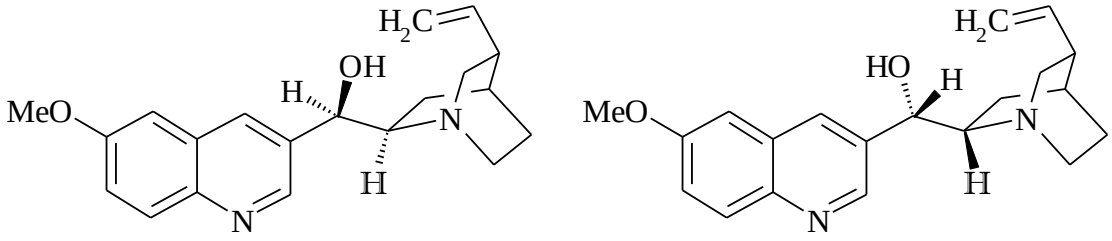
There are several approaches in drug discovery. One can simply screen everything that can be collected in large quantities (massor blind-screening approach). Such a strategy requires enormous investments of money and time and some luck. A second method is the

chemotaxonomy-oriented approach. In this method, plants are selected for screening based on their taxonomic relationships. For example, one can select plants that belong to certain families or genus that are likely to contain certain classes of compounds such as alkaloids, steroids, amino acids or glycosides. Lastly, one can choose simply to learn from the peoples who already use the flora as medicines, selecting for further study those plants already used as remedies according to the traditional knowledge.

Traditional knowledge on use of plants as medicines serves as powerful resource, which greatly facilitates intentional, focused and safe natural product drug discovery. It offers a more holistic approach to drug design and presents several possible targets for scientific analysis. Based on the folklore, many drugs have been developed and are in use in our conventional systems of medicine. This is well illustrated by the fact that 74 percent of chemical compounds derived from plants used as drugs today have the same or related use in Western medicine as they do in traditional medical systems (Farnsworth, 1988).

An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural product-derived drugs comprised twenty-eight percent of all new chemical entities (NCEs) launched onto the market (Newman *et al*., 2003). In addition, twenty-four percent of these NCEs were synthetic or mimic natural compounds, based on the study of pharmacophores related to natural products (Newman *et al*., 2000). The combined percentage (fifty-two percent of all NCEs) suggests that natural products are important sources for new drugs and are also suitable lead compounds in drug development. Of the 252 drugs considered as basic and essential by the WHO, 11 percent are exclusively of plant origin. At least 119 chemical substances from 90 plant species are important drugs used all over the world (Sara, 1992).

In Peru, the stem bark of *Cinchona ledgeriana*, a rainforest tree was used in management of fevers. The alkaloid quinine was isolated from the plant in 1820 A.D. For many years the alkaloid quinine (figure 1.1) was extracted only from the bark of this tree and processed into pills to treat malaria. Total synthesis of this alkaloid was successfully done by American chemists in 1944 (Woodward and Doering, 1944). Several quinine total syntheses have since been achieved to date but the process is very expensive. The isolation of the alkaloid from cultivation sources therefore remains the most viable option of obtaining quinine. Another alkaloid from the tree called quinidine (figure 1.1), an isomer of quinine, was found to be useful as antiarrythmic agent. The tree bark therefore remains the only source of production of quinine-based drugs.



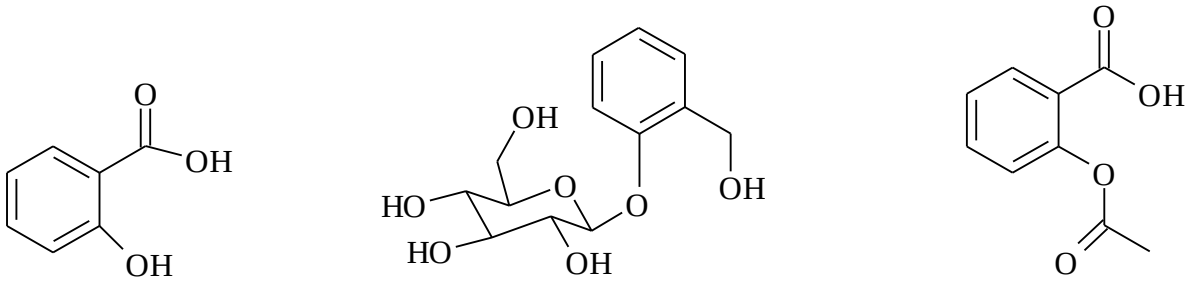
Quinine Quinidine

Figure 1.1 Chemical structures of quinine and quinidine

Khellin from *Ammi visnaga* (L.) Lamk. was used as a bronchodilator in the United States until it was shown to produce nausea and vomiting after prolonged use. In 1955, a group of chemists in England set about to synthesize khellin analogs as potential bronchodilators with fewer side effects. This eventually led to the discovery of chromolyn, used as sodium chromoglycate, which stabilizes cell membranes of mast cells in the lungs to prevent the allergen induced release of histamine (Sneader, 1985). Further studies have led to the synthesis of amiodarone, a useful antiarrythmic agent.

Galantamine hydrobromide, an amaryllidaceae alkaloid, obtained from *Galanthus nivalis* that has been used traditionally in Bulgaria and Turkey for neurological conditions (Howes *et al.*, 2003; Heinrich and Teoh, 2004). This drug was launched onto the market as a selective acetyl cholinesterase inhibitor for the treatment of Alzheimer's disease. It slows the process of neurological degeneration through inhibition of acetyl cholinesterase as well as binding to and modulating the nicotinic acetylcholine receptor.

Salicin (figure 1.2), a glycoside that is metabolized in the body to salicylic acid was originally derived from the white willow bark (*Salix alba*) and the meadowsweet plant. The salicylic acid (figure 1.2) is the precursor of acetylsalicylic acid (aspirin). The bark of white willow had long been used as a traditional remedy for pain, inflammation, and fever (Hedner and Everts, 1998).

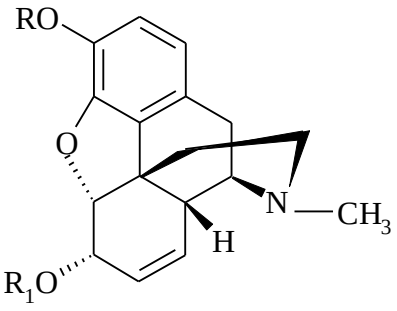


Salicylic acid Salicin Acetylsalicylic acid

Figure 1.2 Chemical structures of salicylate analogues

Catharanthus roseus (Apocyanaceae) also known as *Vinca rosea*, is native to the Caribbean basin and had historically been used to treat a wide range of diseases. European herbalists used the plant for conditions as varied as headache to a folk remedy for diabetes. It has more than 400 known alkaloids, some of which are approved as antineoplastic agents to manage leukemia, Hodgkin's disease, malignant lymphomas, neuroblastoma, rhabdomyosarcoma, Wilms' tumor, and other cancers (El-Sayed and Cordell, 1981).

The opium poppy (*Papaver somniferum*) yields morphine and codeine which are narcotic analgesics. Over the centuries the crude extract derived from poppies has been widely used as a sedative. Although pure morphine was isolated in 1803, it was not until 1833 that chemists were able to isolate and purify it on a commercial scale. Total synthesis of morphine was achieved later in 1952. The development of narcotic analgesics is a good example of the traditional approach in drug development. Other semisynthetic forms of morphine have been developed such as heroine. Figure 1.3 below shows chemical structure of morphine and related compounds.



	R	**R_1**
Morphine	H	H
Codeine	CH_3	H
Heroine	CH_3CO	CH_3CO

Figure 1.3 Chemical structures of morphine and related compounds

It is estimated that sixty percent of anti-tumour and anti-infectious drugs in the market or under clinical trials are of plant origin (Yue-Zhong, 1998). It is not economical to synthesize majority of these compounds therefore they are still obtained from plants. In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicines and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical tests (Williamson *et al*., 1996).

Traditional healers have long used plants to prevent or cure infectious conditions. Many of these plants continue to be used without sufficient scientific evidence on their pharmacological activities. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, which have been found to have antimicrobial properties in *in vitro*

studies. Passion flower for instance is widely employed by herbalists and natural health practitioners around the world today. Its traditional uses includes alcohol withdrawal, antibacterial, anti-seizure, anti-spasm, aphrodisiac, asthma, attention deficit hyperactivity disorder (ADHD), *Helicobacter pylori* infection, hemorrhoids, high blood pressure and menopausal symptoms (Dhawan et al., 2002). Phytochemical investigation of *Passiflora incarnata* and *Passiflora edulis* and occasional analysis of other species revealed that the members of this genus contain alkaloids, phenols, cyanogenic compounds and glycosyl flavonoids (Bradley, 1992; Newall *et al*., 1996; Birner and Nicolls, 1973) whereby the major active principle with antimicrobial activity was found to be a polyacetylenic compound, passicol. *Acacia concinna* has been used for hair care in India for centuries. Antidermatophytic activity of the plant was confirmed using crude extracts with various solvents (Todkar *et al*., 2010).

1.3 Challenges in the management of infectious diseases

Infectious diseases pose a major challenge to health systems in many resource limited and developing countries. These diseases are a major cause of death, disability and social and economic disruption for millions of people (WHO, 2002; Breman *et al*., 2004). It is estimated that infectious diseases are the number one cause of death accounting for approximately one-half of all deaths in tropical countries. Between 14 and 17 million people die each year due to infectious diseases majority of whom live in developing countries (WHO, 2002). In the recent years, mortality has been increasing in developed countries, such as the United States of America (U.S.A). It is estimated that infectious diseases are the underlying cause of death in 8 % of the

deaths occurring in the U.S.A. These statistics are alarming given that it was once believed that infectious diseases would be eliminated by the year 2000 in the developed countries.

Prior to Alexander Fleming's discovery of penicillin in 1928, many millions of people died prematurely because of infectious diseases. After the discovery of penicillins, many other antibiotics became available to effectively treat the growing number of infections (Iruka *et al*., 2005). Despite the existence the many antibiotics for the management of infectious diseases, many people lack access to the required preventive and treatment facilities. These diseases therefore still impact negatively on families, communities and economies. Infectious disease epidemics may last a few weeks or a few months and reduce productivity at work places. Institutions may experience reduced attendance at work due to infections, fear of infections or absenteeism of workers caring for their families. There is also lost productivity due to morbidity and high health care costs caused by infectious diseases. Broader economic problems may be caused by reduced work forces which may initiate economic downturn and further unemployment. Above all, millions of people are disabled every year by infectious diseases. Measles, for example, can result in blindness, deafness or brain damage.

The emergence of new infectious diseases such as Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS) has further complicated the situation. Other new or unrecognized diseases, such as Severe Acute Respiratory Syndrome (SARS), continue to emerge at a rapid pace and frequently with significant mortality and financial costs (WHO, 2005; Willow, 2011). In 2002 and 2003, SARS, believed to have emerged in China, spread around the world, from Asia to North and South America and Europe. Before it was finally contained, SARS infected 8,098 people, nearly 800 of them died (EPR, 2003).

Antibiotic resistance is an international problem that compromises the treatment of all infectious diseases. Much evidence shows that irrational use of antibiotics causes the development of resistant bacterial strains. Gastrointestinal, respiratory, sexually transmitted and nosocomial infections are leading causes of diseases and death in the developing world. The management of all these conditions has been critically compromised by the appearance and rapid spread of resistance (Iruka *et al.,* 2005). The European Centre for Disease Prevention and Control (CDC) reported the spread of antibiotic-resistant bacteria across Europe.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacteria of particular concern. Some MRSA strains are resistant to the newly introduced potent antibiotics daptomycin and oxazolidinones (such as Linezolid). Many European Union states have reported infections with *Klebsiella pneumonia* that are resistant to carbapenems. In the Middle East where antibiotics are used more extensively, reported resistance to antibiotics is very high. There are many reports of bacterial resistance in Iran, Lebanon, United Arab Emirates, Turkey, and other countries in the region. A more recent problem is the multidrug-resistant tuberculosis (TB) which is a major public health problem that threatens progress made in TB management and control worldwide. One of the reasons for development of resistance is due to indiscriminate use of antibiotics. For example, antibiotics are cheap in most countries in the Middle East and can be obtained without prescription in pharmacy outlets (Farrokh, 2012). To achieve the UN Millennium Development Goals, particularly goal 6, to control HIV/AIDS, tuberculosis, malaria, and other diseases (UN MDGs, 2007), more attention should be given to this global threat.

1.4 Plants as antimicrobial agents

It is estimated that twenty-five to fifty percent of current pharmaceuticals are derived from higher plants. However, none of these products are antimicrobials (Goldfrank *et al.*, 1982). Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It is reported that, on average, two to three antibiotics derived from microorganisms are launched each year (Clark, 1996). Though most of the clinically used antibiotics are produced by soil microorganisms, higher plants have also been a source of antibiotics. Examples of these are the bacteriostatic and antifungicidal properties of Lichens, the antibiotic action of allicin and other compounds in *Allium sativum* (garlic), or the antimicrobial activity of goldenseal (*Hydrastisca canadensis*) (Trease and Evans, 1972).

Much has been written about the antimicrobial effects of cranberry juice. Historically, women were told to drink the juice in order to prevent and even cure urinary tract infections. In the early 1990s, researchers found that the monosaccharide fructose present in cranberry and blueberry juices competitively inhibited the adsorption of pathogenic *E. coli* to urinary tract epithelial cells, acting as an analogue for mannose (Zafriri *et al*., 1989).

The chewing stick is widely used in African countries as an oral hygiene aid. The sticks are obtained from different species of plants, and within one stick the chemically active component may be heterogeneous. Crude extracts of one species used for this purpose, *Serindeia werneckei*, inhibited the periodontal pathogens *Porphyromonas gingivalis* and *Bacteroides melaninogenicus in vitro* (Rotimi *et al*., 1988).

The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha,* and related species, has been used for many years as amoebicidal drug as well as for the treatment of abscesses due to of *Entamoeba histolytica* infections.

Researchers have continued to test plants for antimicrobial activities. In a recent study carried out at the Department of Microbiology and Immunology, Botucatu Biosciences Institute, tests were performed utilizing extracts from *Allium sativum* (garlic bulbs), *Zingiber officinale* (ginger rhizomes), *Caryophyllus aromaticus* (clove flower buds), *Cymbopogon citratus* (lemon grass), *Psidium guajava* (guava leaves) and *Mikania glomerata* (guaco leaves) against *Enterococcus*, *Escherichia, Staphylococcus* and *Salmonella* species. The extracts from garlic and ginger presented the most intense activity against Gram-negative bacteria. Gram positive strains were more susceptible to guava and clove extracts (Silva and Fernandes, 2010).

Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis* which has antimicrobial activity and increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

1.5 Literature review on *Nicandra physaloides*

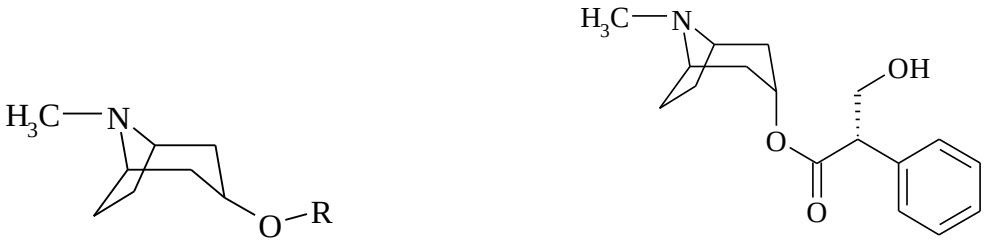
1.5.1 Family Solanaceae

The family Solanaceae is one of the most intriguing plant families in the world. It is one of the largest families in the plant kingdom with more than 3,000 species (D'Arcy, 1986). These include the edible species such as the potato (*Solanum tuberosum),* eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), capsicum (*Capsicum species*) and cape gooseberry (*Physalis peruviana*). This family also includes species that are grown as ornamentals such as those belonging to the genera *Browallia, Brunfelsia, Cestrum, Datura, Nicotiana, Salpiglossis, Solanum, Solandra* and *Nicandra* among others. Well known are the trumpet-like flowers of *Datura* species which are popular as ornamental plants and which can produce flowers of up to 30 cm. long in a variety of colours.

All of the Solanaceae family plants are toxic in some way. Their toxins range from mild to very high. Many of them, like potatoes and tomatoes are safe for humans, yet not safe for some animals. Jimson Weed (*Datura stramonium*) and mandrake (*Mandragora officinarum*) are highly toxic to humans, yet when used correctly can have substantial medical benefits. Tobacco is another member of the Solanaceae family that is mildly toxic but people usually smoke it. Humans have also learned to use the toxicity of peppers to make spray which is used as deterrent to both humans and animals.

Plants in the Solanaceae family are known for possessing a wide range of alkaloids. There are two main kinds of alkaloids. The first are the steroidal alkaloids such as the solanodine alkaloids, which are found in high concentrations in raw potatoes (Sandra, 2002). The second main alkaloid type is the tropane alkaloids and these come in many different varieties in the Solanaceae family.

For humans, these alkaloids can be desirable or toxic. The toxicity of these alkaloids has helped in evolution and survival of the species because of their anti-feedant properties (Griffin and Lin, 2000). Chemically, tropane alkaloids have a characteristic bicyclic chemical structure and include atropine, scopolamine and hyoscyamine. Figure 1.4 shows the general structure of tropane alkaloids and structure of hyoscyamine.



Common structure of tropane alkaloids Hyoscyamine

Figure 1.4 Common structure of tropane alkaloids and chemical structure of hyoscyamine

Pharmacologically, tropane alkaloids are powerful anticholinergics. They can reverse cholinergic poisoning caused by overexposure to pesticides and chemical warfare agents such as sarin. They can also halt many types of allergic reactions. Scopolamine, a commonly used ophthalmological agent, dilates the pupils and thus facilitates examination of the interior of the eye. They can also be used as antiemetics in people prone to motion sickness or those receiving chemotherapy. Atropine has a stimulant effect on the central nervous system whereas scopolamine has a sedative effect (Domino *et al*., 1967). The plants containing these alkaloids were eaten for their hallucinogenic properties by the native Americans (Cecilia and James, 2005). *Datura* is allegedly used as one of the ingredients in zombie powder in Haiti. The psychological experiences appear to be a dreamlike trance or stupor (Stephen *et al*., 2011). However the toxicity of these plants limits their use as recreational drugs.

The pharmacological activities of plants in Solanaceae family is well documented in the literature. These include *Capsicum annum* (Cichewicz and Thorpe, 1996), and *Withania* spp. (Ramzi *et al*., 2005) which have antimicrobial activities. *Solanum trilobatum* is one of the important medicinal plants, more commonly available in Southern India and has been used in herbal medicine to treat various diseases like respiratory problems, bronchial asthma and tuberculosis (Govindan *et al*., 2004). *In vitro* antimicrobial activity shows that *S. trilobatum* extract has activity against a number of bacteria. The plant extracts from leaves, flowers, stems and fruits revealed antimicrobial activity against both Gram positive bacteria and Gram negative bacteria (Swapna and Kannabiran, 2006). Other pharmacological activities have been observed in this family too. *Physalis minima*, a small herbaceous annual plant which grows as weed in crop fields has several medicinal applications. It is used as a tonic, a diuretic, a laxative, an anti-inflammatory agent, management of enlarged spleen and as a helpful remedy in ulceration of the bladder. The leaves are crushed and applied over snakebite site (Karthikeyani and Janardhanan, 2003). Fruits of this plant are used to cure spleen disorders (Anonymous, 1969).

1.5.2 *Nicandra physaloides* (L.) Gaertn

1.5.2.1 Description and distribution

Nicandra is a monotypic genus of flowering plants in the nightshade family containing the single species *Nicandra physaloides*. It is known by the common names Apple of Peru and shoo-fly plant. It is a coarse, erect annual plant reaching three to eight feet in height and about half as wide. It has large alternate leaves reaching up to one foot long and resembles *Datura* leaves. The leaves are ovate-cordate in shape. The young plants have dark colored ‘‘dots’’ that decorate the adaxial surfaces of the leaves. They are seen to be small cuticular spikes or trichomes. Their

margins are shallowly lobed, bluntly dentate, or undulate. The petiole of each leaf is long and slender, tilting at an upward angle; there are a few hairs near its base, otherwise it is hairless. The flowers are pale blue in colour with white throats and are bell-shaped. The flower becomes lantern-like towards the end of its bloom. The fruits appear prickly and are enclosed in papery inflated calyxes. Each fruit has a dry berry in it. The stems are angular and largely hairless.

Nicandra physaloides is native to Peru and it is recognized elsewhere as an exotic species. It is kept as an ornamental plant but has a tendency to be weedy and has consequently become a noxious weed in the tropics. The preference is full or partial sun, moist conditions and a loamy fertile well drained soil. Most vegetative growth occurs during the late spring and summer. This species is a summer annual. The size of a plant is variable, depending on soil fertility and availability of moisture (Huxley, 1992). All parts of the plant are mildly poisonous (Tsarong, 1994). Figure 1.5 is a mature flowering *N. physaloides* plant.

Figure 1.5 Mature flowering *Nicandra physaloides* plant (Photograph taken in september 2010 at KNH grounds).

1.5.2.2 Use in traditional medicine

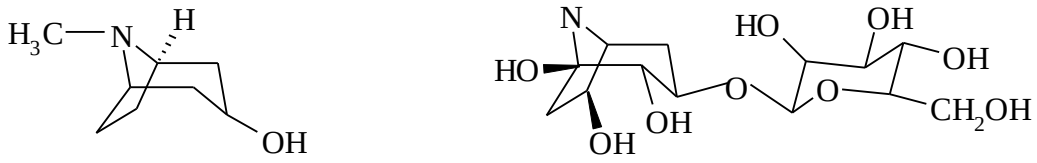
The plant possesses diuretic properties and an alkaloid-tropine with mydriatic action has been reported (Chopra *et al.,* 1986; William and David, 2000). The plant has also been used as an analgesic, anthelmintic, antibacterial, anti-inflammatory and as febrifuge. A decoction of the seeds is used in the treatment of fevers (Manandhar, 2002). The plant is commonly used as an insecticide and pediculicide. It is also used as an insect repellent administered by rubbing exposed skin with the tender stems and foliage. In some communities the plant's boiled extract is mixed with milk and set as fly poison (Chopra *et al*., 1986).

1.5.2.3 Previous work reported on *Nicandra physaloides*

Nicandra physaloides has been investigated for various biological activities. The methanolic and aqueous extract of the leaf, fruit, stem and root of *Nicandra physaloides* were active against *Bacillus subtilis*, *Mycobacterium phelei*, *Proteus mirabilis* and *Staphylococcus epidermidis*. Both methanolic and aqueous extract of leaves and roots were active against *Candida albicans* and *Aspergillus flavus* (Mann *et al*., 2008).

Aqueous and alcoholic extracts of *Nicandra physaloides* leaves have been tested for diuretic activity in rats. They both showed significant increase in excretion of sodium, potassium and chloride ions in the urine in a dose dependent manner. The obtained effect was comparable to that of furosemide. The study supported the presence of effective diuretic constituents in the aqueous and alcoholic extract of *Nicandra physaloides* (Devi *et al*., 2010).

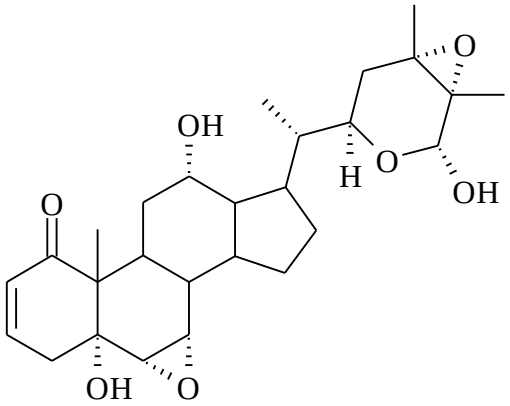
The alkaloid tropine and closely related compound calystegine β_1 glucopyranoside(3-O-β-D-glucopyranosyl-1α, 2β, 3α, 6α-tetrahydroxy-*nor*-tropane) (figure 1.6) were reported by William and David (2000) and Russel *et al.* (1996) respectively.



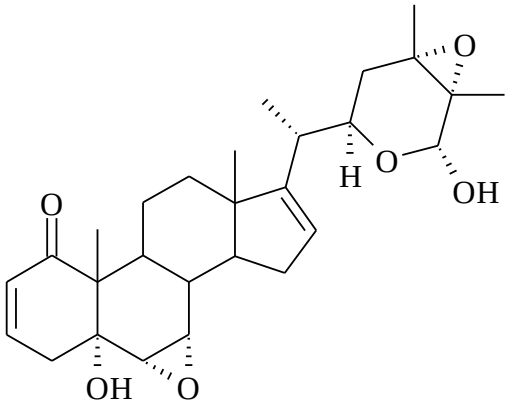
Tropine Calystegine β_1 glucopyranoside

Figure 1.6 Chemical structures of tropine and calystegine β_1 glucoside.

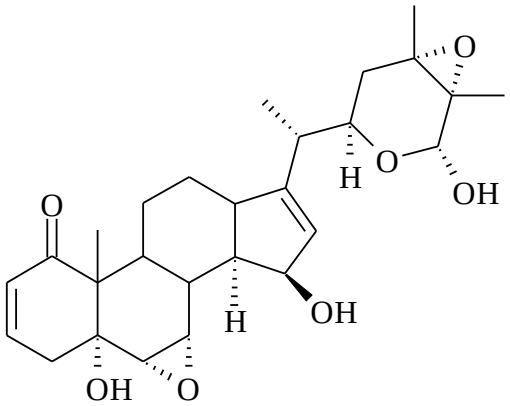
Olga *et al.* (1964) demonstrated the insecticidal properties of *Nicandra physaloides.* Kirson *et al*. (1972) elucidated the structure of nicandrenone, the insecticidal compound, identified in aqueous extracts as inhibiting the feeding of insect larvae. Other compounds related to nicandrenone (ergostane-related compounds, nicaphysalin A, B, C, D and E) were isolated from the methanolic extracts of fresh whole plants by Kazushi Shingu *et al*. (1994). Michael *et al.* (1976) had earlier isolated 17 distinct products, which were also mainly nicaphysalins and named them Nic 1-17 from the aqueous extract of the leaf of *N. physaloides* over several years of a study. One of the compounds (Nic-15), a terpene, was identified as loliolide. Nic 1 was the same as nicandrenone. Six of the products were non-crystalline. Another closely related compound, withanicandrin, which is the first natural occurring 12-oxowithanolide, was isolated by chromatographic fractionation of the extract of the leaves of *N. physaloides* (Kirkson *et al*., 1972). Some of these compounds are shown in figures 1.7 and 1.8.



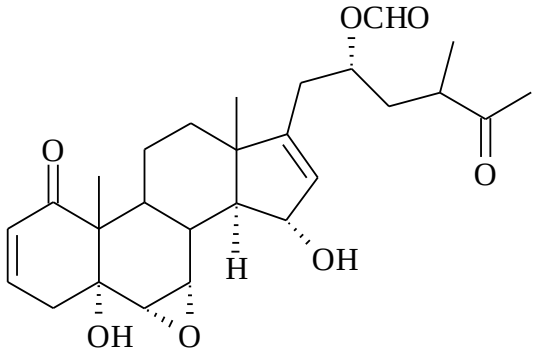
Nicaphysalin A



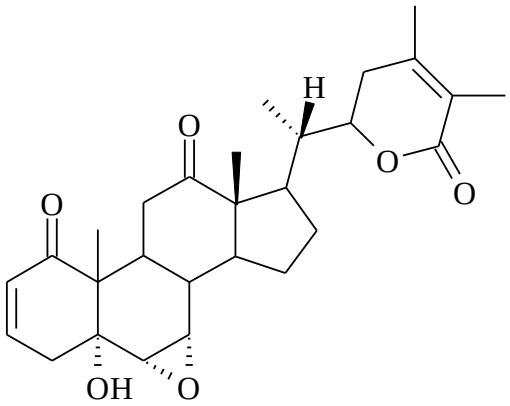
Nicaphysalin B



Nicaphysalin C

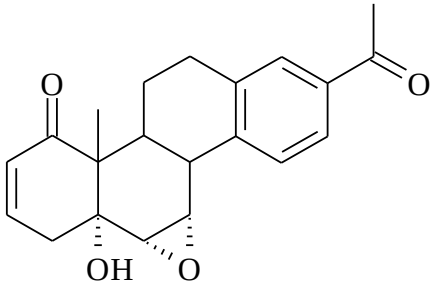


Nicaphysalin D

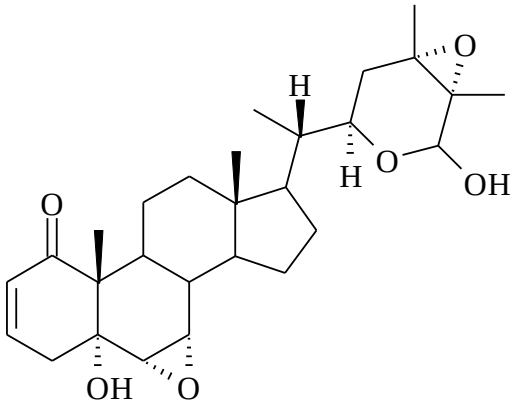


Withanicandrin

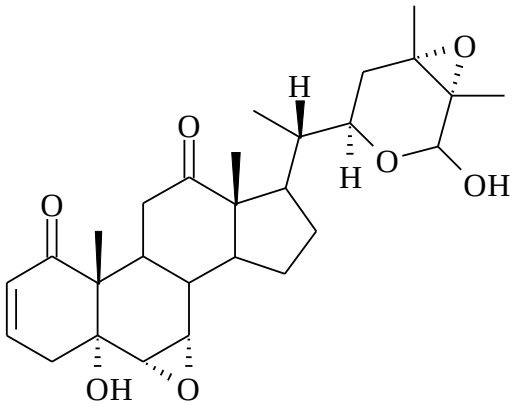
Figure 1.7 Chemical structures of selected nicaphysalins



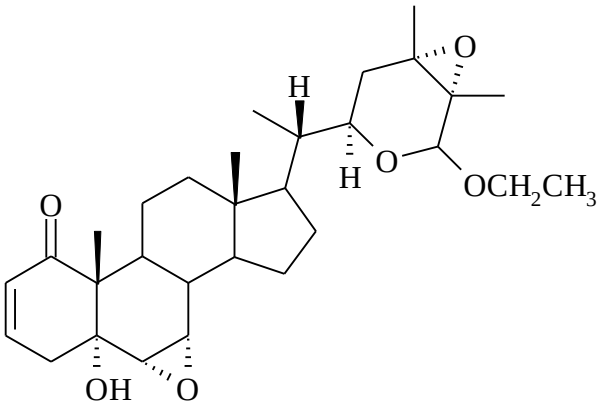
Nic-10



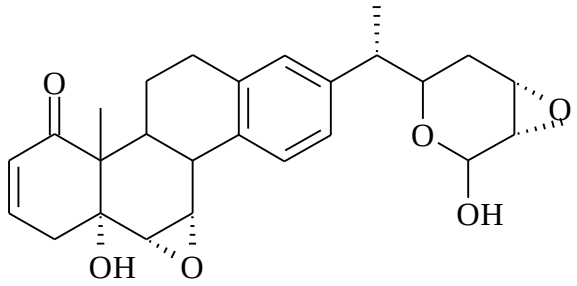
Nic-3



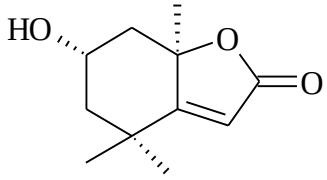
Nic-7



Nic-11



Nic-1 (nicandrenone)



Nic -15 (Loliolide)

Figure 1.8 Chemical structures of Nic compounds isolated from *N. physaloides*.

Through the work done by Dimetry and El-Gengaihi (2003), six compounds were isolated from the petroleum ether extract of the plant. They demonstrated ability to cause mortality in female two-spotted spider mites. However, these compounds did not affect the mite eggs with which they came in contact.

1.6 Study justification

The remarkable success of antimicrobial drugs generated a misconception in the late 1960s and early 1970s that infectious diseases had been conquered. The use of synthetic compounds led to a decline in the use of plants in modern medicine. However, 40 years later, infectious diseases remain the third-leading cause of death in the United States and the second-leading cause of death worldwide (WHO, 2002). These diseases present a great morbidity and mortality burden in developing nations. In these countries, many people lack access to preventive and curative care either due to the absence of such facilities or because they are unaffordable. The management of infectious diseases has also been affected by the emergence of resistance to the available antimicrobial agents making them less useful in treatment of many infectious diseases. The impact of antimicrobial resistance is particularly significant in developing countries, where the cost constraints prevent the widespread application of newer, more expensive agents. The situation is further complicated by the emergence of new infectious diseases. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics has raised the spectrum of untreatable bacterial infections and added to the urgency for the search for new infection fighting strategies (Sieradski *et al*., 1999).

A study carried out has shown that there has been a dramatic increase of infectious diseases among people of the 25-44 year old age group since the 1980s (Pinner *et al*., 1996). The spectre of bioterrorism, which gained widespread public attention after 11 September 2001, has magnified the problem, because genetic engineering of pathogens could render them resistant to currently available antimicrobials (Gilligan, 2002). These negative health trends call for a renewed interest in infectious disease in the medical and public health communities and alternative strategies on treatment and prevention. The development of new antimicrobials is therefore encouraged as one of the approaches to solving the problem (Fauci, 1998).

Although the need for new antimicrobials is increasing, development of such agents faces significant obstacles (Smolinski *et al*., 2003). Out of the several new pharmaceuticals from plants introduced onto the market very few are antimicrobials. A number of factors make antimicrobial agents less economically attractive targets for development than other drug classes (Projan, 2003). For example, the increasing therapeutic needs of the aging U.S.A population has shifted drug discovery efforts towards agents that treat chronic medical conditions that are more prevalent among elderly persons. The developing countries therefore need to develop homegrown solutions to infectious diseases since they are the most affected.

Plant based antimicrobials represent a vast untapped source for medicines especially to the developing world which is endowed with rich natural resources and folklore. Evaluating plants from the traditional African system of medicine, provides clues as to how these plants can be used in the treatment of diseases. Continued and further exploration of plant derived antimicrobials needs to be considered with much more interest. Plant based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic

antimicrobials (Aiyegoro and Okoh, 2009). In order to halt the trend of increased emerging and resistant infectious disease, it will require a multi-pronged approach that includes the development of new drugs. This approach is in line with the sixth millennium development goal which seeks to stop and reverse the spread of infectious diseases by 2015 (UN MDGs, 2007).

Traditional healers have long used plants to manage infectious diseases including *Nicandra physaloides*. Previous research on the plant showed that it has antimicrobial activity (Mann *et al.,* 2008) . However no active antimicrobial compounds were isolated. This present study therefore seeks to verify antimicrobial activity of the plant found in Kenya and the antimicrobial components.

1.7 Objectives

1.7.1 General objective

To investigate the phytochemical profile and antimicrobial activity of *Nicandra physaloides* found in Kenya.

1.7.2 Specific objectives

1. To carry out solvent extraction of *Nicandra physaloides* aerial parts.
2. To carry out isolation of phytochemical constituents of *Nicandra physaloides*.
3. To characterize isolated compounds by physical and spectroscopic techniques.
4. To screen the crude extracts and isolated compounds for antibacterial and antifungal activities.

CHAPTER TWO

EXPERIMENTAL

2.1 Introduction

Plants contain secondary metabolites which can be extracted with suitable solvents. The detection of these active principles in medicinal plants plays a strategic role in both qualitative and quantitative phytochemical investigation of crude plant extracts (Pascual *et al*., 2002). These bioactive phytocompounds can be used directly as therapeutic agents, as starting materials for the synthesis of drugs or as lead compounds for the development of pharmacologically active compounds.

2.2 Principles of phytochemical investigations

2.2.1 Plant preparation and extraction

Information on how the plant is used by communities is important since the preparation procedure may give an indication of the best extraction method. The formulation used provides information about pharmacological activity, mode of administration and the appropriate dosage levels to be tested. However, certain considerations must be taken into account when the ethnopharmacological approach of plant selection is chosen. For instance, each community has its own concepts of health or illness, as well as different health models (Elisabetsky and Posey, 1986). The signs and symptoms should be translated, interpreted and related to western biomedical concepts, thus allowing a focused study of a particular therapeutic property (Gottlieb and Kaplan, 1993; Souza, 1996).

Once the plant is identified, the next step is its collection and botanical identification, then it should be submitted to a stabilisation process. Stabilisation is usually done by drying the material at ambient temperature, but can also be carried out in an oven with controlled airflow and temperature. Stabilization can also be done by freezing, lyophilisation, or use of alcohol vapour. The dried or stabilised plant material should then be powdered and subjected to suitable extraction procedures.

Extraction is done to obtain extracts or compounds that are relatively pure at a high yield (Williamson *et al*., 1996). Water soluble compounds and proteins are extracted in buffers or water. Organic solvents are used to extract soluble organic compounds. Alcohol is a good all-purpose solvent for preliminary extraction. It is non toxic and reasonably selective. Several methods are used for extraction including organic solvent extraction, supercritical fluid extraction and steam distillation. Solvent extraction is commonly used and includes techniques such as maceration and percolation. Supercritical fluid extraction involves extraction of active ingredients using gases.

2.2.2 Fractionation and isolation of compounds

Chromatographic fractionation of plant extracts is largely achieved by use of liquid-solid column chromatography (LSC). In this procedure, the principle underlying the separation of compounds is adsorption at the solid-liquid interface. The mechanism of separation depends mainly on the nature of the stationary phase which results in the differences in migration rates among the sample components (Fried and Sherma, 1994). Increasing the polarity of the mobile phase increases the rate of movement of compounds down the column. There are two methods by which sample components are eluted: isocratic elution and gradient elution.

In isocratic elution, the mobile phase composition remains constant during the entire chromatographic run. This method is preferred for simple mixtures whose components have retention factors within a narrow range. To adequately handle narrow mixtures that have both weakly and strongly retained components like crude plant extracts, gradient elution is used. Gradient elution involves changing the mobile phase composition either stepwise or continuously as the elution proceeds. Initially, the mobile phase is composed entirely or mostly of the less polar solvent which facilitates separation of low-polarity components. The polarity is gradually increased enabling elution of the more polar components.

The eluents are collected as a series of fractions which are then examined appropriately for the presence of compounds. Thin layer chromatography (TLC) is commonly used to monitor the fractions collected. The homogeneous fractions based on the visualization of spots on TLC plate are then combined, evaporated. These fractions are usually contaminated with small amounts of other similar compounds and therefore need purification to yield pure compounds. The purification process is chosen depending on the structure, stability and quantity of isolated compounds. Several methods are available including freeze drying, chromatography, crystallization, sublimation and distillation. Aqueous solutions can be purified by freeze drying whereas crystalline compounds are usually purified by crystallization from suitable solvent or mixtures of solvents. The purity of the isolated compound can be ascertained by use of spectroscopic methods or determinations of its melting point or boiling point.

Each fraction and/or pure compound can then be subjected to bioassay and toxicity evaluation. This strategy is called bioactivity-guided fractionation. Bioassays can be performed using microorganisms, molluscs, insects, cellular systems such as enzymes and receptors, cell cultures

and isolated organs or *in vivo* tests in mammals, amphibians or birds (Hamburger and Hostettman, 1991; Souza, 1996).

2.2.3 Techniques in structure elucidation and identification

Elucidation of the molecular structure of unknown compounds is accomplished by use of a combination of the various spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and infrared (IR) spectroscopy (Verpoorte, 1989).

Nuclear Magnetic Resonance (NMR) spectroscopy is an important tool for determining the structure of organic compounds. Any nucleus with a spin has magnetic properties that can be used to yield chemical information. When irradiated with a radio frequency signal the nuclei spin in a molecule can change from being aligned with the magnetic field (lower energy) to being opposed to it (higher energy). The energy frequency at which this occurs is what is measured and displayed as an NMR signal (chemical shift). The position of the chemical shift in an NMR spectrum depend on its local structural environment of the proton producing the signal. Electrons around the nucleus shield the proton from the externally applied magnetic field causing the NMR signal to be generated at a higher magnetic field. Nuclei of vicinal protons influence each other's effective magnetic field. This result into spin-spin coupling that is shown as splitting of a signal to give multiple peaks. However equivalent protons do not couple each other. The area of the peak produced in an NMR spectrum is a measure of the number of protons it represents. This area is shown as an integration value. The most common nuclei utilized in NMR are ^{1}H and ^{13}C. Many functional groups can be identified by their ^{1}H and ^{13}C chemical shifts.

The utility of infrared spectroscopy in structure elucidation is based on the presence of characteristic absorption bands of specific functional groups in a molecule. The exact frequency

at which a given vibration occurs is determined by the strengths of the bonds involved and the mass of the component atom.

Mass spectrometry (MS) is an analytical technique that separates ionized particles such as atoms, and molecules. The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound. Several ionization methods have been developed and include chemical ionization, electron impact, Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption (MALDI) among others. Some of the ionization methods such electron impact result in fragmentation of the molecular ion to other secondary ions. However some methods such as ESI ionizes molecules by the addition or removal of a proton, with very little extra energy remaining to cause fragmentation of the sample ions (Shibdas and Shyamalava, 2012). The formed ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundances.

A mass spectrum will usually be presented as a vertical bar graph, in which each bar represents an ion having a specific mass-to-charge ratio (m/z) and the length of the bar indicates the relative abundance of the ion. The highest-mass ion in a spectrum is normally considered to be the molecular ion, and lower-mass ions are fragments from the molecular ion, with assumption that the sample is a single pure compound. Since a mass spectrometer separates and detects ions of slightly different masses, it easily distinguishes between different isotopes of a given element such as bromine and chlorine. The intensity ratios of isotope pattern depend on the natural abundance of the isotopes. Identification of the compounds is achieved by comparison of the molecular weights of the compound and the fragmented ions obtained by MS with those in a appropriate databases.

Mass spectrometry can be coupled to both gas-phase and liquid-phase separation techniques, enabling the structural analysis of complex mixtures after their chromatographic separation.

2.3 Principles of antimicrobial assays

Antimicrobial assays involve use of standard microorganisms to qualitatively or quantitatively determine certain chemical compounds. Some of the secondary metabolites found in plants affect microbial growth, the rate of inhibition of the growth being a function of the amount of compounds present. Screening of potential antimicrobial compounds from plants may be performed with pure compounds or crude extracts. The test substance is added to a liquid or gel medium, the medium is inoculated with the assay microorganism and the response measured as numerical counts, optical density, weight or area. The screens used to determine antimicrobial susceptibility are the broth dilution assay and the disc or agar well diffusion assay.

In the agar diffusion assays, a substance with biological activity is allowed to diffuse through an agar gel previously seeded with a test organism. For antibiotic assays, a clear zone of inhibition appears around the point of application. The growth of microorganisms is inhibited depending on concentration of drug and the concentration gradient. The diameter of the zone of inhibition is used to determine the activity of the compounds.

In the broth tube assay, liquid media is used; a small inoculum of microorganism is added to liquid nutrient media and kept at optimum conditions for growth. The turbidity is observed in the tube as an indicator of growth. It is measured as an estimate of the potency of drug used by measuring light scattering property or absorbance at 550- 650 nm. Most tube assays are for growth promoting substances such as vitamins.

The ideal microorganism should be sensitive to the substance being assayed, be easily cultivated and produce a response that is easily measurable. The microorganism should be suitable for the assay of several compounds. The incubation period for the assays varies from several hours to few days depending on the microorganism in use. The incubation temperature chosen should give good growth and the medium should contain factors necessary to support the growth of the microorganisms.

2.4 Solvents, materials, reagents and equipment

2.4.1 Solvents, materials and reagents

General purpose reagents; ethyl acetate, petroleum-ether, chloroform and methanol (Kobian Kenya Ltd, Kenya), were distilled and used for extraction, fractionation and isolation of compounds. Analytical grade methanol and acetone (Sigma-Aldrich GmbH, Seelze, Germany) were used for recrystallisation of compounds. Sulphuric acid (Loba Chemie, PVT Ltd, Mumbai, India), ammonia solution (Pharmacos Ltd, Essex, England) were used in extraction of alkaloids. Dimethyl sulfoxide (Fischer Scientific, Loughborough, United Kingdom) was used in preparation of aqueous suspensions for antimicrobial activity.

Filtrations to remove particulate matter from crude extracts and the solutions of isolated compounds were done using Whatmann filter paper No.1 (Whatmann International Ltd, Maidstone, England).

Column chromatography on normal phase silica gel of porosity 60Å and particle size 63-200 μm (Sigma–Aldrich GmbH, Seelze, Germany) was used for fractionation of the crude extracts. Thin Layer Chromatography was carried on pre-coated aluminium plates with 0.2 mm thick layer of normal phase silica gel 60 GF_{254} (Sigma-Aldrich GmbH & Co., Seelze, Germany).

Vanillin (1% w/v), prepared using vanillin powder (BDH Chemicals Ltd., Poole, England) in concentrated sulphuric acid (LobaChemie, PVT Ltd. Mumbai, India) and iodine resublimed general reagent (Merck, Damstadt, Germany) were used as visualizing agents.

2.4.2 Equipment

A Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) connected to a Laborota4000 cooler (Polyscience, Niles, USA), a WB2000® water bath (Heidolph Electro GmbH & Co. KG, Kelheim, Germany) and a diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany) was used to reduce to dryness extracted material for column chromatography and antimicrobial activity testing.

The extracts were fractionated using glass column whose dimensions were 2 cm internal diameter and 50 cm long equipped with a glass wool filter. The chromatographic fractions were collected using a SuperFrac™ automatic fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). Thin layer chromatograms were visualized using Min UVIS® ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany). Extracts and isolated compounds for recrystallisation were kept in Indesit refrigerator (Indesit, Italy) maintained at 4-6 °C.

The melting points of the isolated compounds were determined using SMP10 melting point apparatus (Barloworld Scientific Ltd., Stone, Staffordshire, ST15 O.S.A, U.K) in capillary tubes.

All the glassware used in the antimicrobial activity studies were sterilised in a Memmert® universal oven (Memmert GmbH & Co. KG, Schwabach, Germany) using dry heat at 150 ºC for 1 hour. A portable autoclave (Dixon's Surgical Instruments Ltd., Essex, UK) was used to sterilize the nutrient media and distilled water at 121ºC for 15 min. The micro-organisms were incubated in Freez I® incubator (Analis, Suarlee, Belgium). The bench work involving use of

micro-orhanisms was carried out in a Bioflow® laminar flow cabinet (Vermeulin L. J BVBA, Westmalle, Belgium) while the cork borer and wire loop were sterilized by flame from a Bunsen burner. The zones of inhibition diameters were measured using a hand-held electronic digital vernier calliper with a precision of 0.1 mm.

2.5 Methods

2.5.1 Collection of plant material

The aerial parts of *N. physaloides* were collected around Kenyatta National Hospital grounds, Nairobi in August 2009. The material included stems, leaves, flowers and fruits. The sample was verified by plant taxonomist and voucher specimens (SOP/NAM/2009/01) deposited at the School of Pharmacy, herbarium University of Nairobi, Kenya. The plant was air dried at ambient condition, ground to powder and kept in plastic containers until use.

2.5.2 Preparation of visualizing reagents

Vanillin (1 % w/v) was prepared by dissolving 1 g of vanillin in 100 ml of concentrated sulphuric acid with gentle swirling. This was kept in a dark cabinet away from light until use within one week.

Iodine vapour was prepared by placing 10 g of iodine resublimed general reagent in a chromatographic tank to saturation.

2.5.3 Solvent extraction

The dried and ground aerial parts of *N. physaloides* was extracted by wetting 500 g of the powdered material with 450 ml ammonium hydroxide (NH_4OH) (50 % v/v) and macerated using 2 litres of ethyl acetate overnight at room temperature with continous stirring. The material

was washed twice with 2 litres of ethyl acetate. The filtered extract was partitioned with equal volume of 2 % sulphuric acid in a separating funnel. The organic layer was reduced to dryness using rotary evaporator and used in the fractionation by column chromatography. The aqueous layer was used in extraction of alkaloids. The extraction is illustrated in figure 2.1.

The aqueous layer containing the alkaloids was alkalinized using ammonia solution and extracted using 3 x 500 ml portions of ethyl acetate. The aqueous layer was discarded whereas the organic layer extracts were combined and evaporated to dryness using rotary evaporator to obtain alkaloids mixture. The crude alkaloids mixture was desalted using 30 ml methanol, filtered and evaporated to dryness before storing in the refrigerator.

2.5.4. Column chromatography of ethylacetate extract

Thin layer chromatography was performed on the extracts to determine the suitable mobile phase for column chromatography. Fractionation by column chromatography was carried out using silica gel as the stationary phase. For this purpose, 10 g of the ethyl acetate extract was loaded onto a column packed with 120 g of silica gel. The column was eluted with chloroform-methanol mixture by gradient elution. The eluents were collected in 5 ml fractions using a fraction collector. Every other fourth tube of the fractions was profiled by thin-layer chromatography using 10 % methanol in chloroform as the mobile phase. The TLC profile is shown in appendix 1. The spots in the chromatograms were visualized under UV at 254 nm and 366 nm and by exposure to iodine as well as spraying with 1% vanillin. The chromatograms were analyzed and fractions showing similar profile were pooled to yield seven fractions coded F1 to F7. All the fractions were evaporated *in vacuo* to a volume of about 10 ml and dried in

open air on the laboratory bench. The extraction and fractionation processes are illustrated in figure 2.1.

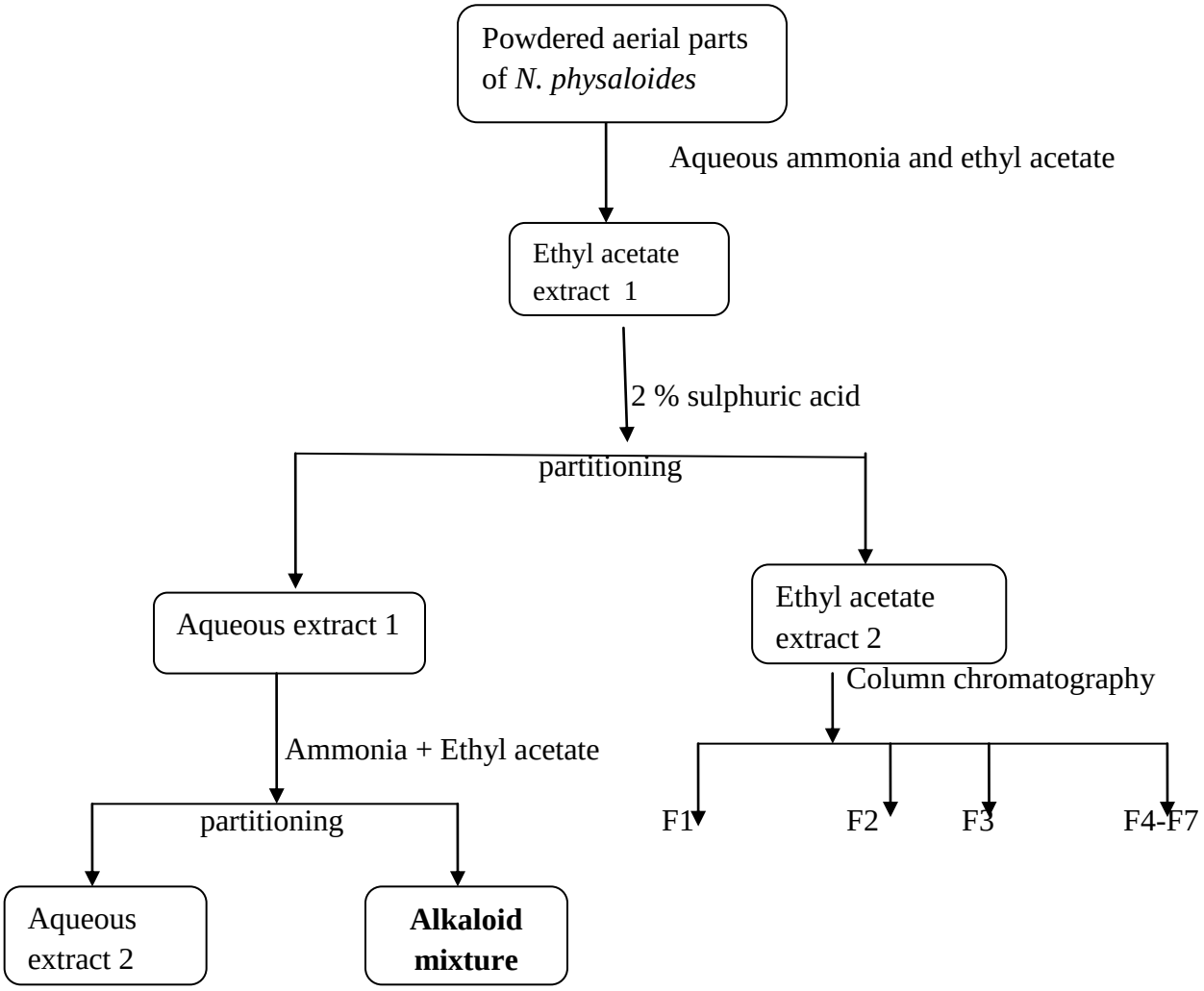


Figure 2.1. Organogram showing extraction and fractionation of *N. physaloides*

2.5.5 Antimicrobial activity testing

Bacterial test microorganisms: *Staphylococcus aureus* (NC 07447*)*, *Escherichia coli* (ATCC 25922) and *Bacillus pumillus* (NC 08241) were subcultured onto slants of Tryptone Soy Agar and incubated at 37 ºC for 24 hours while the fungal test microorganisms; *Candida albicans* (NCPF 3179) and *Sacchromyces cerevisiae* (ATCC 9763) were subcultured on Sabouraud dextrose agar at 25 ºC for 48 hours. One hundred grams each of the air-dried and coarsely powdered plant material was extracted with methanol, petroleum ether, and ethyl acetate separately overnight by cold maceration. Each of the extracts was filtered and evaporated under reduced pressure using rotary evaporator. The samples were weighed and dissolved in dimethyl-sulphoxide (DMSO) to make the final concentrations of about 50 mg/ml for the extracts and 30 µg/ml of the withanicandrin solution. Gentamicin and nystatin which were used as standards were prepared to concentrations of 30 µg/ml each.

The plate agar diffusion method was adopted according to Kavanagh, (1972) to assess the antibacterial activity of the prepared extracts. Nutrient agar was prepared according to manufacturer's instructions. They were sterilized in an autoclave and allowed to cool to about 50 ºC on the bench. Loopful of the standardized microorganism stock suspensions was mixed with 100 ml of the sterile nutrient agar to produce an inoculum of approximately 1 x 10^6 colony forming units per ml. After mixing, 20 ml of the inoculated nutrient agar was placed into each sterile petri dish to form uniform thickness of about 3 mm. The agar was left to set and in each of these plates 8 bores, 6 mm in diameter, were cut using a sterile cork borer and the agar discs were removed. Alternate bores were filled with 50µl of each extracts, isolated compound, DMSO and standards using microtiter-pipette and allowed to diffuse at room temperature for half an

hour in a laminar flow cabinet. The plates were then incubated at 37 ºC for 18 hours. Two replicates were carried out for each extract against each of the test organism. After incubation the diameters of the results and growth inhibition zones were measured, averaged and the mean values were tabulated.

Gentamicin was used as a positive control for bacterial microorganisms because it inhibits the growth of both Gram-positive and Gram-negative bacteria (Marshal & Williams, 2003) whereas nystatin was used for the fungal micro-organisms because it inhibits the growth of yeasts. The negative control, dimethylsulphoxide (DMSO), is a colourless liquid and an important aprotic solvent which dissolves both polar and non-polar compounds from a plant.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Isolation of compounds and structure elucidation

3.1.1 Instrumentation

3.1.1.1 Nuclear magnetic resonance apparatus

The nuclear magnetic resonance spectroscopic data was obtained using a Varian-Mercury 200 MHz spectrometer (Varian Inc. Palo Alto, Carlifornia, USA) with a magnet from Oxford Instruments (Oxford, UK). The data was acquired using an on-line computer (Sun Micro-Systems, California, USA) and analyzed using Varian software.

3.1.1.2 Mass spectrometer

Mass spectrometric data was obtained using an Agilent 6230 Accurate-Mass Time-of- Flight (TOF) LC/MS (Agilent Technologies, California, USA) operating at 175eV.

3.1.1.3 Infra-red spectrophotometer

A Fourier transform infrared spectrophotometer (IRPrestige-21, Shimadzu Corporation, Kyoto, Japan) was used for infrared spectroscopy. A hydraulic press machine (Perkin-Elmer GmbH, Germany) was used to prepare the sample IR discs and IR solution software was used in analysis and recording.

3.1.2 Isolation of compounds

3.1.2.1 Isolation of steroidal mixture, NK 01

The fraction F2 (Figure 2.1) from the chromatographic column formed a precipitate at ambient temperature after four days which was washed with methanol and dissolved in chloroform. The chloroform solution was left overnight to crystallize. Further recystallization in methanol-chloroform (10:90) under slow evaporation at ambient temperature produced the colourless crystals (NK 01) with a yield of 0.15 % w/w.

3.1.2.2 Isolation of withanicandrin

Fraction F3 (Figure 2.1) formed clear crystals on the sides of the wall of the test tube after three days at ambient temperature. The crystals were scrapped off and redissolved in ethyl acetate and allowed to recystallize. Further recrystallization was done using acetone under slow evaporation producing clear crystals which was identified as withanicandrin.

3.1.2.3 Alkaloid mixture

The alkaloid mixture was purified by addition of 5 % w/v lead acetate solution and sonicated to remove other plant components. This was then dissolved in methanol and filtered. The filtrate was evaporated to dryness. The dried extract was fractionation by column chromatography using silica gel as the stationary phase and ethylacetate as mobile phase. However, no pure compound was isolated. The TLC profile is shown in appendix 2.

3.1.3 Structure elucidation of isolated compounds

3.1.3.1 Steroidal mixture, NK 01

From the LC/MS data shown in appendices 3.a to 3.d, there are at least five compounds present in NK 01 with m/z values of 430, 428, 414, 413 and 402.

Appendix 3.e shows ^{1}H-NMR of NK 01 whose values are;

^{1}H-NMR (C_2D_6O, 200 MHz): δ 5.32, 5.30, 5.2, 5.23, 5.20, 5.16, 5.13, 5.08, 3.72, 3.70, 3.41, 3.38, 3.36, 2.89, 2.23, 2.20, 2.17, 2.16, 2.10, 2.07, 2.06, 2.05, 2.04, 2.03, 2.01, 2.00, 1.98, 1.94, 1.89, 1.87, 1.86, 1.80, 1.77, 1.74, 1.73, 1.72, 1.70, 1.68, 1.67, 1.65, 1.64, 1.64, 1.61, 1.57, 1.56, 1.54, 1.53, 1.51, 1.50, 1.48, 1.45, 1.44, 1.43, 1.43, 1.42, 1.41, 1.39, 1.36, 1.34, 1.33, 1.29, 1.28, 1.28, 1.26, 1.25, 1.24, 1.24, 1.21, 1.17, 1.13, 1.11, 1.07, 1.04, 1.02, 0.98, 0.97, 0.95, 0.94, 0.90, 0.88, 0.87, 0.86, 0.85, 0.84, 0.82, 0.80, 0.78, 0.74, 0.72

The intergration values for the ^{1}H-NMR of NK 01 were not provided, therefore only the ^{13}C-NMR data was useful in attempting to elucidate the structures of NK 01.

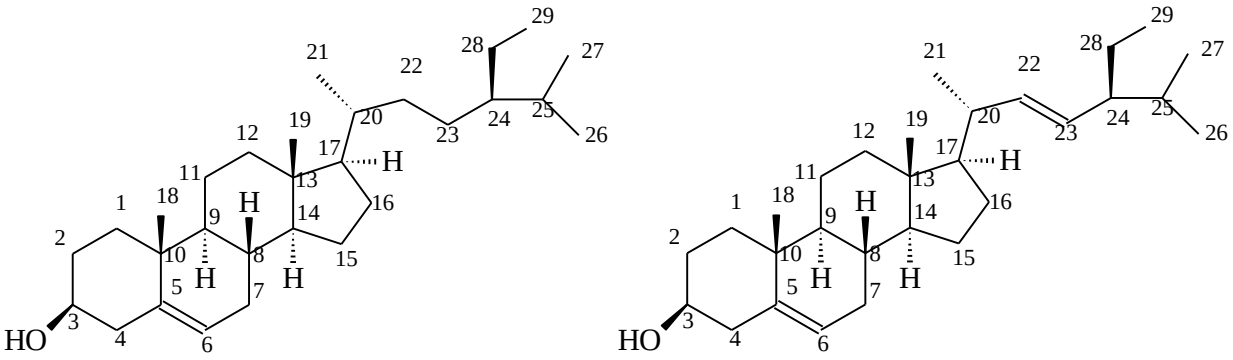
Appendix 3.f shows ^{13}C-NMR of NK 01. The values were compared to literature values of stigmasterol and β-sitosterol as shown in Table 3.1.

Table 3.1 Chemical shifts of ^{13}C-NMR of NK 01 and literature values of β-Sitosterol and stigmasterol (Saeidnia S. *et al.* 2011).

Carbon number	β-Sitosterol		Stigmasterol	
	Literature values	NK 01	Literature values	NK 01
1	37.3	37.6	37.3	37.6
2	31.7	31.8	31.7	31.8
3	71.8	71.0	71.8	71.0
4	42.3	42.4	42.2	42.3
5	140.8	141.7	140.8	141.7
6	121.7	120.9	121.7	120.9
7	31.9	31.9	31.9	31.9
8	31.9	32.0	31.9	32.0
9	50.2	50.5	50.2	50.5
10	36.4	36.6	36.4	36.6
11	21.1	21.1	21.1	21.1
12	39.8	39.9	39.7	39.9
13	42.3	42.3	42.2	42.3
14	56.8	56.9	56.9	56.9
15	24.3	24.3	24.4	24.3
16	28.3	28.4	28.9	28.8
17	56.1	56.3	56.0	56.1
18	11.9	11.7	12.0	11.9
19	19.8	19.8	19.4	19.4
20	36.2	36.2	40.5	40.7
21	18.8	18.7	21.2	21.2
22	34.4	34.0	138.3	138.7
23	26.1	26.1	129.3	129.4
24	45.8	46.0	51.6	51.5
25	29.2	29.2	31.9	31.9
26	19.0	19.1	19.0	19.1
27	19.4	19.4	21.1	21.0
28	23.1	23.1	25.4	25.4
29	12.0	11.6	12.0	11.6

Most of the ^{13}C-NMR data were in agreement with those of β-Sitosterol and Stigmasterol as shown in table 3.1 above. The ^{13}C NMR values at 71, 120, 129, 138 and 141 are characteristic of stigmasterol whereas 71, 120 and 141 are characteristic of β-Sitosterol with only one double bond. More ^{13}C-NMR values were unaccountanted for which implies presence of other compounds whose structures could not be elucidated from the available data.

From the LC/MS data, which indicates a mixture of compounds, the m/z values of 414 and 413 as shown in appendices 3.b and 3.c further confirm the presence of β-Sitosterol and stigmasterol respectively. The structures of the 2 compounds are shown in figure 3.1.



β-Sitosterol stigmasterol

Figure 3.1 Chemical structures of β-Sitosterol and stigmasterol

This is the first time for β-Sitosterol and stigmasterol to be isolated from *N. physaloides.*

β-Sitosterol and stigmasterol have severally been isolated together (Maria *et al*., 2001; Suparb B. and Aorn P., 1991) from different plants. Their separation is very difficult as they co-elute. Their separation through column chromatography is almost impossible. Repeated recrystallization has also proven difficult because both of them produce crystals in solution and produce dual mixture each time. In preparative-TLC, no separation occurs as they have equal Rf values.

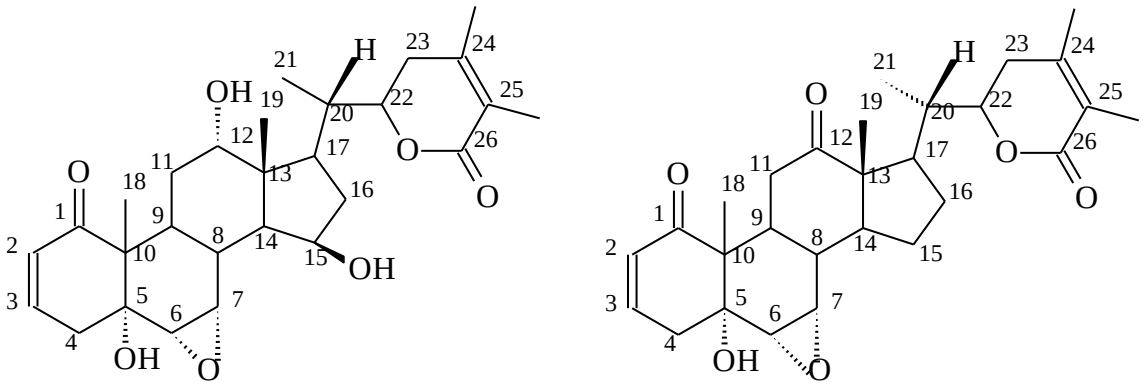
3.1.3.2 Withanicandrin

The melting point of withanicandrin is 269 °C (Kirkson et al., 1972) whereas the isolated compound was 269-273 °C which confirms the identity of the isolated compound.

Appendix 4.a shows the IR values of withanicandrin.

IR cm^{-1} 3477.66, 2968.45, 2945.30, 2370.51, 1765.93, 1650, 1684.79,1700, 1382.96, 1267.23

Appendix 4.b shows the ^{1}H-NMR ($CDCl_3$, 200 MHz) spectral data which was compared to the literature values of a closely related compound 15β-hydroxynicandrin (table 3.2) in $CDCl_3$ (Adriana *et al*., 1995).



15β-hydroxynicandrin withanicandrin

Figure 3.2 Chemical structures of 15 β-hydroxynicandrin and withanicandrin

Appendix 4.c shows the ^{13}C-NMR of withanicandrin. The values were compared to the literature values of withanicandrin (Kirkson *et al.*, 1972) as shown in table 3.3.

Table 3.2 Chemical shifts for ^{1}H-NMR of withanicandrin and literature values of 15 β-hydroxynicandrin (Adriana *et al*., 1995)

Hydrogen number	15β-hydroxynicandrin	withanicandrin
2	5.87 *dd*	6.59 (1H, dd)
3	6.61 *ddd*	5.83 (1H, m)
4 α	2.52 *dd*	2.51 (1H, m)
4 β	2.70 *ddd*	2.68 (1H, m)
5	-	3.41 (OH, br, s)
6	3.05 *d*	3.09 (1H, d)
7	3.72 *dd*	3.55 (1H, dd)
8	2.21 *dt*	2.22 (1H, m)
9	2.10 *dt*	2.00 (1H, m)
11 α	2.85 *dt*	2.68 (1H, m)
11 β	1.61 *ddd*	1.62 (1H, m)
12	3.96 *br*	-
14	1.87 *dd*	1.88 (1H, m)
15	4.45 *dt*	1.57 (2H, m)
16 α	2.32 *ddd*	1.49 (2H, m)
16 β	1.41 *ddd*	-
17	1.92 *ddd*	1.68 (1H, m)
18	1.06 *s*	1.12 (3H, s)
19	1.20 *s*	1.25 (3H, s)
20	2.12 *m*	2.17 (1H, m)
21	1.10 *d*	0.98 (3H, d)
22	4.35 *dt*	4.40 (1H, dt)
23 α	1.99 *dd*	1.96 (1H, m)
23 β	2.51 *dd*	2.48 (1H, m)
27	1.89 *s*	1.89 (3H, s)
28	1.94 *s*	1.95 (3H, s)

Table 3.3 ^{13}C-NMR chemical shifts of experimental and literature values of withanicandrin (Kirson *et al.*, 1972).

Carbon number	Literature values	Experimental values
1	202.1	202.7
2	128.8	129.0
3	139.8	140.2
4	36.7	36.8
5	73.2	73.4
6	56.2	56.4
7	56.8	57.1
8	35.7	35.7
9	37.7	37.8
10	51.5	51.6
11	38.4	38.5
12	212.0	212.5
13	57.7	57.8
14	52.9	53.0
15	23.6	23.8
16	27.7	27.3
17	42.8	42.8
18	11.5	11.6
19	14.7	14.9
20	39.5	39.9
21	13.1	13.7
22	76.3	76.6
23	29.1	30.1
24	149.3	149.8
25	121.8	122.0
26	166.1	167.3
27	12.5	12.7
28	20.5	20.0

The ^{1}H-NMR spectrum of withanicandrin was characteristic of the steroidal structure for the withanolide class of compounds (Glotter *et al.,* 1978). They were similar to those observed for other steroidal lactones having a 17 α-oriented side chain (Frolow *et al.,* 1981). However, the full ^{1}H-NMR for withanicandrin was not available in previous work therefore, the data was compared to that of 15β hydroxynicandrin. The broad signal at 3.96 in 15 β-hydroxynicandrin was lacking in withanicandrin as there is no proton in this position. At C-15, 15β-hydroxynicandrin showed only one signal downfield whereas withanicandrin has two signals much upfield due to the lack hydroxyl group which is seen in the former compound. There is a duplet at C-2 due to the coupling by the single proton at C-3. The proton at C-6 is coupled by single proton at C-7, thus producing a duplet whereas the C-7 proton is coupled by 2 protons (C-6 and C-7) making it a double duplet. Only the methyl group at C-21 was coupled by the single proton at C-20 to produce duplet, the rest were attached to quartenary carbons thus represented as singlets.

The data shown in table 3.2 compares the ^{13}C-NMR literature values of withanicandrin to those of the isolated compound. The isolated compound's values were in good agreement with the reported values (Kirkson *et al.*, 1972). Further confirmation using the distortionless enhancement by polarization transfer (DEPT) spectrum indicated that there were signals for five methyl, five methylene and ten methine, hence 8 quartenary carbons. This further confirmed that there were 28 carbons in the molecule.

The infrared (IR) spectrum of withanicandrin shown in appendix 3.a indicates the presence of hydroxyl groups (3477 cm^{-1}) (Glotter *et al.*, 1978). There was a broad band at 1700 which represents presence of α, β-unsaturated δ-lactone, ester and α, β-unsaturated ketone. A peak at 1650 cm^{-1} indicates presence of double bonds (Emma *et al.*, 2012).

Further support for the structure of the compound was obtained from mass spectroscopy based on ESI TIC scan fragm 175.0V, as shown in appendix 4.d, shows pseudomolecular ion peak at *m/z* 507.235 $[M+K]^+$ and a dimer at 975 [2M+K]. This indicates that the molecular ion has mass value of 468. Together with the NMR data, the molecular formula was proposed to be $C_{28}H_{36}O_6$.

3.2 Antimicrobial activities

3.2.1 Data analysis

Activity index was calculated by comparing the zone of inhibition of each test solution with that of the standard used.

$$\text{activity index} = \frac{\text{inhibition diameter of sample}}{\text{inhibition diameter of standard}}$$

3.2.2 Antibacterial activities

The *in vitro* antimicrobial activities of the aerial parts of the various crude extracts of *Nicandra physaloides* and withanicandrin are shown in Table 3.4. Sample results for *E. coli* on a petri dish are shown in appendix 5.

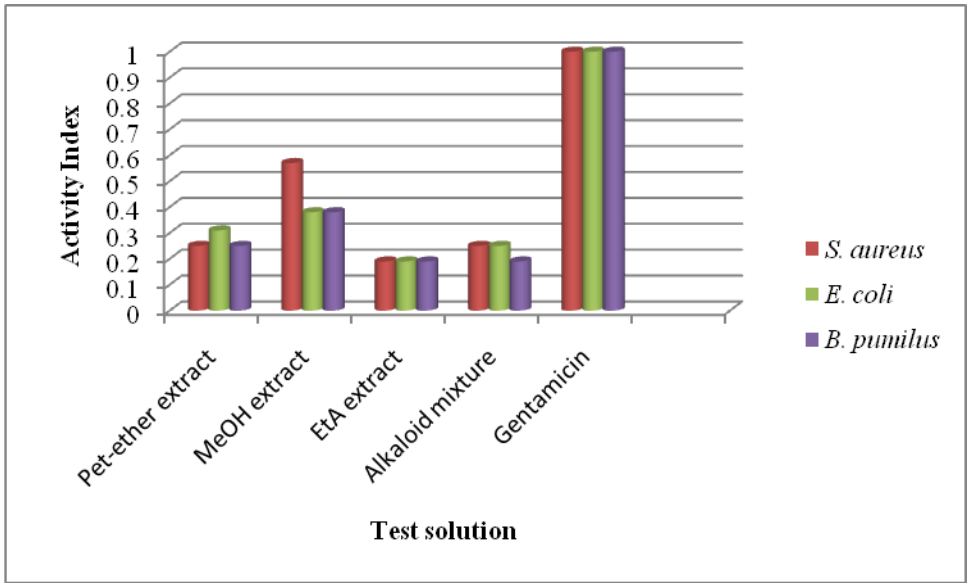
Table 3.4 Zones of inhibition (mm) of various test solutions against various bacteria micro-organisms

Test solution	Zones of inhibition (mm)		
	S. aureus	***E. coli***	***B. pumilus***
Petroleum-ether	4.0	5.0	4.0
extract[a]	9.0	6.0	6.0
Methanol extract[a]	3.0	3.0	3.0
Ethylacetate extract[a]	4.0	4.0	3.0
Alkaloid mixture[a]	0.0	0.0	0.0
Withanicandrin[b]	16.0	16.0	16.0
Gentamicin[b]			

[a] Concentration of 50 mg/ml; [b] Concentration of 30 ug/ml

Mean zone size from duplicate tests

The activity index of each sample was calculated and the results are shown in Figure 3.3



MeOH= Methanol; EtA=Ethylacetate 1; Pet ether= Petroleum ether

Figure 3.3 Activity indices of various extracts against bacterial micro-organisms

3.2.3 Antifungal activities

The various *N. physaloides* extracts were also tested against two fungal organisms; *S. cerevisiae* and *C. albicans.* The zones of inhibition were tabulated in table 3.5. Appendices 6 and 7 show the results of the test solutions against *C. albicans* and *S. cerevisiae* respectively as seen on petri dishes.

Table 3.5 Zones of inhibition (mm) of various test solutions against fungal micro-organisms

Test solution	**Zones of inhibition (mm)**	
	S. cerevisiae	***C. albicans***
Petroleum-ether extract[a]	10.0	6.0
Methanol extract[a]	0.0	0.0
Ethylacetate extract[a]	0.0	0.0
Alkaloid mixture[a]	10.0	8.0
Withanicandrin[b]	13.0	10.0
Nystatin[b]	18.0	18.0

[a] Concentration of 50 mg/ml; [b] Concentration of 30 ug/ml

Mean zone size from duplicate tests

The calculated activities of the various test solutions were compared and illustrated in the figure 3.

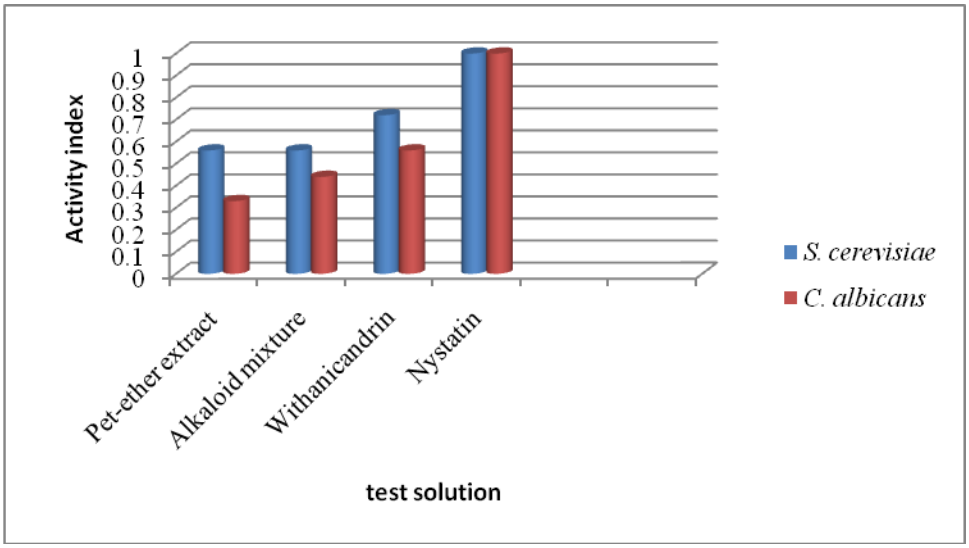


Figure 3.4 Activity indices of various extracts against fungal micro-organisms

In preliminary screening for antimicrobial activity, inhibition diameters higher than 1 mm are generally considered as positive results (Rosoanaivo and Ransimamanga, 1993). The petroleum ether, ethyl acetate and methanol extracts, each at a concentration of 50 mg/ml were found to be active against all the bacterial micro-organisms. The methanol extract had the highest activity. The petroleum ether extract, alkaloid mixture and withanicandrin were all active against the *S. cerevisiae* and *C. albicans*. Withanicandrin activity was the highest compared to all the other extracts against these fungal micro-organisms.

This is the first time that withanicandrin has been shown to have antifungal activity. Previous work on this compound was focused on antifeedant activity. Other withanolides have shown potent tumor-inhibitory activity, immune-modulator, antifungal, antihyperglycemic and mild inotropic and chronotropic effects on the heart (Maryam K. *et al*., 2012).

CHAPTER FOUR

CONCLUSIONS AND RECOMMENDATIONS

Chromatographic fractionation of the aerial of *Nicandra physaloides* yielded one pure crystalline compounds, withanicandrin and a mixture of phytosterols, two of which were identified as β-sitosterol and stigmasterol using the spectroscopic methods. Withanicandrin has been previously reported from *N. physaloides* (Kirson *et al*.,1972) It is the first time to report β-sitosterol and stigmasterol from the plant.

The petroleum ether extract, methanol extract, ethylacetate extract and alkaloid mixture at a concentration of 50 mg/ml showed activity against *Staphylococcus aureus, Bacillus pumilus* and *Escherichia coli.* The methanol extract had the highest activity whereas the isolated compound, withanicandrin, did not show any activity against all the bacterial organisms used in the study. The petroleum ether extract, the alkaloid mixture and withanicandrin showed activity against *S. cerevisiae* and *C. albicans*. The activity of withanicandrin against the fungus was the higher compared to all the other extracts. Literature search shows that *N. physaloides* possesses antibacterial activity against *Bacillus subtilis, Mycobacterium phelei*, *Proteus mirabilis* and *Staphylococcus epidermidis* (Mann *et al.,* 2008). This study confirms some of the antibacterial activity of the plant and reports newer antimicrobial activity thus supporting the folklore use of the plant in the management of wounds (Manandhar, 2002).

Many compounds especially withanolides have been isolated from *N. physaloides*. However, for the first time, two phytosterols have been isolated and identified from the plant. Three more

compounds were isolated but were not identified. More work should be carried out to isolate and identify other phytosterols present in this plant.

In previous work, a mixture of withanicandrin and daturalactone showed antifeedant activity in insects (Ascher *et al*., 1987). This is the first time the antifungal activity of withanicandrin is being demonstrated. In this study withanicandrin showed activity against two fungal micro-organisms. Antimicrobial activities should be carried out on many other compounds isolated from the plant previously. There is also need to optimize these activities and if possible, modifying the structure to give higher activity. Toxicological studies should also be carried out on the compound. There is need to isolate newer compounds and test them for activity.

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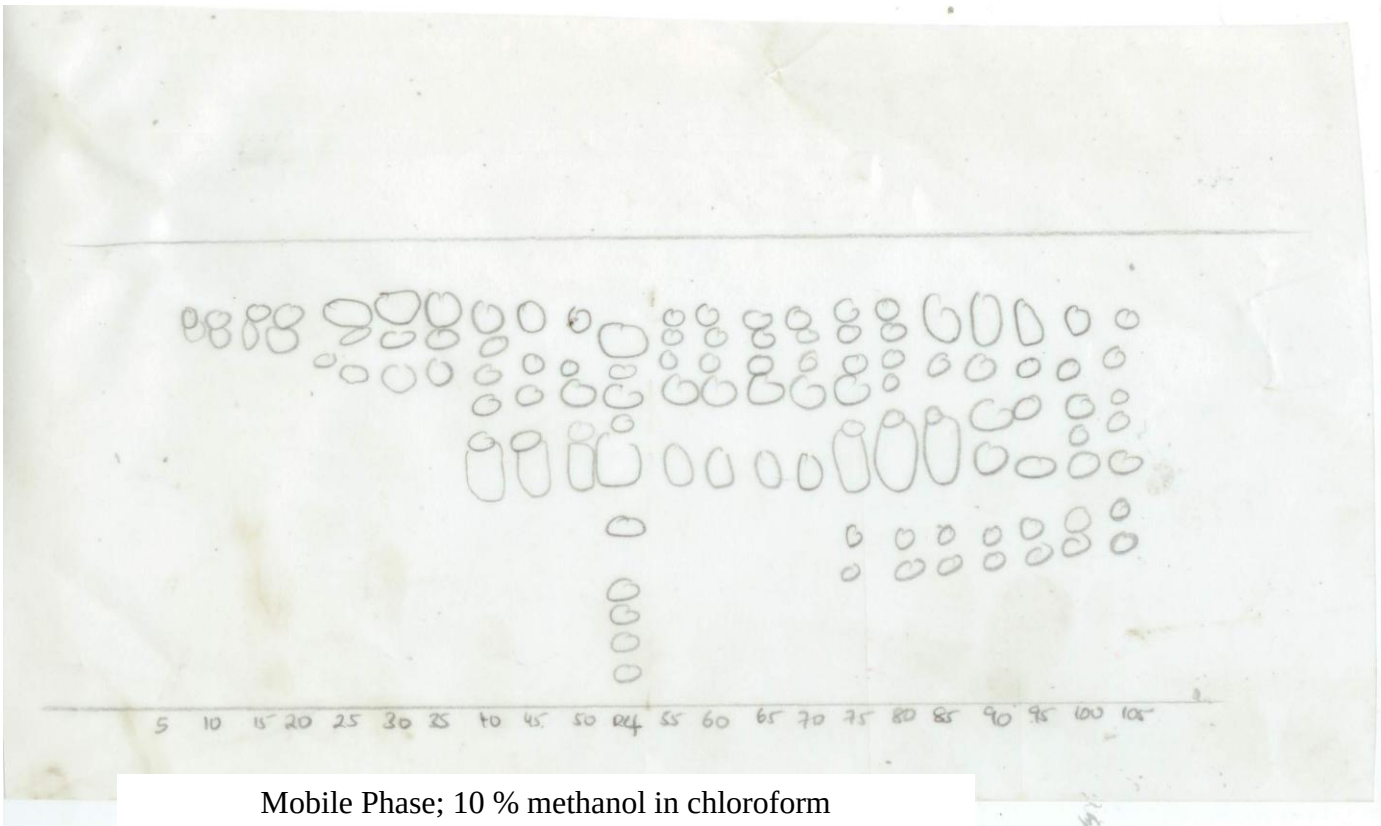
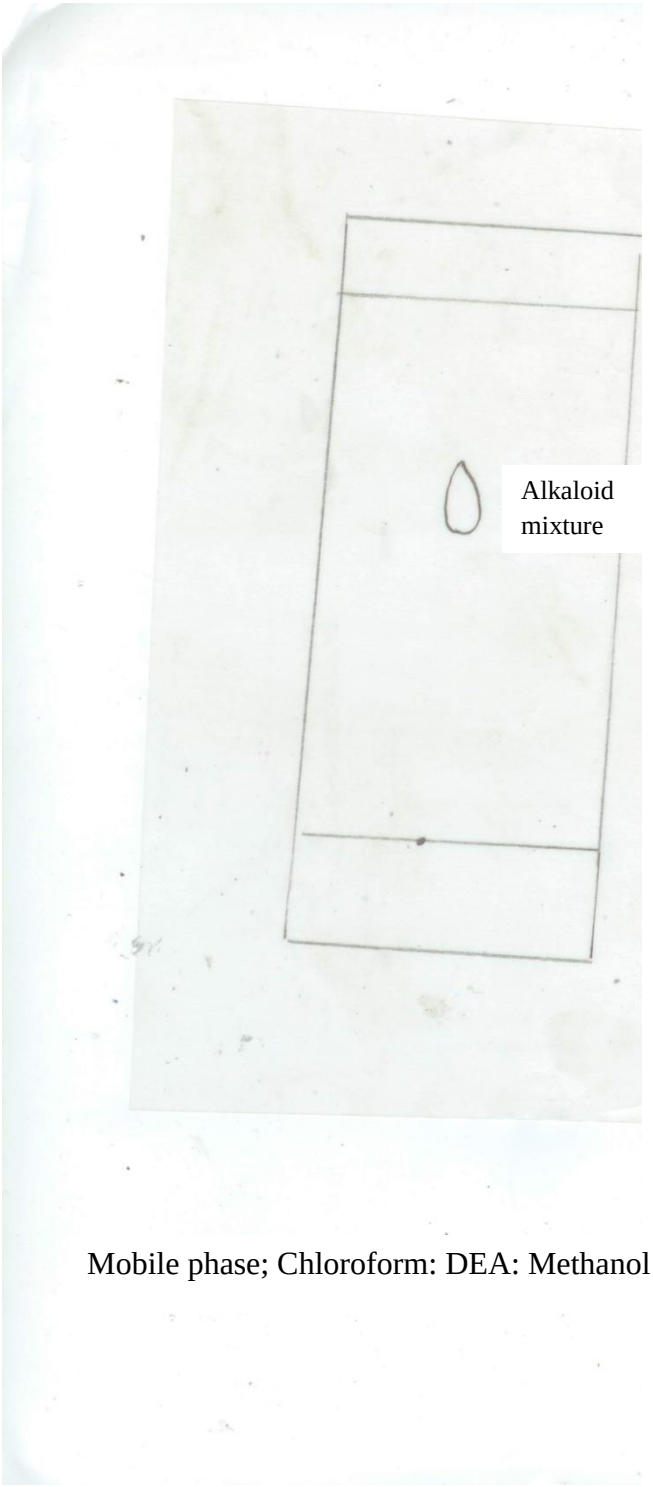
APPENDICES

Appendix 1 TLC of the ethylacetate extract from column chromatography



Mobile Phase; 10 % methanol in chloroform

Appendix 2 TLC Chromatogram of Alkaloid Mixture



Mobile phase; Chloroform: DEA: Methanol = 5:1:4