ANTIMICROBIAL PROPERTIES OF *PHYLLANTHUS* SPECIES

BY

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DECLARATION

This is my original work and has not been presented for a degree in any other University or Institution.

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DEDICATION

To my mother Margaret, wife Caren and progeny Wendy, Darlene and Cheryl. They are a great inspiration in my life.

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ABBREVIATIONS AND SYMBOLS

ATCC:	American Type Culture Collection
Cfu:	Colony forming units
CH_2Cl_2:	Dichloromethane
HIV/AIDS:	Human Immunodeficiency Virus/Acquired Immune Deficiency
MeOH:	Methanol
Ml:	Milliliter
MHA:	Mueller-Hinton Agar
NCPF:	National Collection of Pathogenic Fungi
NCTC:	The National collection of Type Cultures
S.T.D:	Sexually Transmitted Disease
S.T.I:	Sexually Transmitted Infections
SDA:	Sabouraud Dextrose Agar
T.B:	Tuberculosis
TLC:	Thin Layer Chromatography
TSA:	Trypson Soya Agar
UV:	Ultra Violet
WHO:	World Health Organisation
CA:	*Candida albicans*
EC:	*Escherichia coli*
SA:	*Staphyloccocus aureas*
BS:	*Bacillus subtilis*
BP:	*Bacillus pumillus*

ABSTRACT

The antimicrobial activities of two species, *Phyllanthus amarus* and *Phyllanthus odontodenius* traditionally used for treatment of microbial diseases were investigated in comparison to *Phyllanthus niruri*. Aqueous (hot and cold water), methanol and dichloromethane: methanol (1:1) crude extracts of the two plants were evaluated for *in vitro* activity against the test organisms, *Candida albicans, Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli* and *Klebsiella pneumoniae*. The organisms were also exposed to two standard antibiotics, 0.32mg/ml *Gentamycin* (for bacteria) and 0.30mg/ml *Nystatin* (for fungus). Disk diffusion method was employed to screen the antimicrobial activities of both the extracts and for the standard antibiotics. *In vitro* antimicrobial susceptibility activity was screened by using Nutrient Agar (NA). The methanol extracts of *P. odontodenius* showed the strongest activity against all the organisms both at 100mg/µl and 50mg/µl followed by dichloromethane: methanol (1:1), hot water and cold water extracts. The solvents in comparison to antibiotics showed 80% activity for methanol, 48% for CH_2CL_2: MeOH 1:1, 43% in hot water and 28% for cold water.

Crude extract profiling carried out on *P. amarus, P. odontodenius* and *P. niruri*using thin layer chromatography (TLC) indicated that the compounds had similar Rf values The species possess significant antimicrobial activity and confirms the justification by herbalists for the use of the extracts for treatment of measles, diarrhoea, pneumonia, malaria, common cold and other microbial diseases. Therefore, bioassay guided fractionation, isolation and characterization studies of compounds from the extracts will yield information on the active components and their mechanism of action.

CHAPTER ONE

1.0 INTRODUCTION

Plants continue to be a major source of medicines, as they have been throughout human history (Hobbs, 1994).They have been used as traditional treatments for numerous human diseases for thousands of years. Their medicinal value lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic components (Hill, 1952). The presence of these constituents is believed to account for the antimicrobial potency. In developing countries, drugs are not often affordable thus approximately 60–80% of the world's population still relies on traditional medicines as remedies for the treatment of common illnesses (Owolabi *et al.*, 2007).

According to World Health Organization (WHO), medicinal plants are the best source to obtain a variety of drugs to combat serious diseases (Nascimento *et al.*, 2000). WHO advocates that countries should interact with other aspects of traditional medicine with a view to identifying and exploiting safe and effective remedies for ailments of both microbial and non-microbial origins. The Kenyan government development plan of 1989 strongly recommended that medicinal plants should be urgently studied, preserved and developed as alternative medicines and possible elucidation of new therapeutic preparations (Republic of Kenya). This study aimed at fulfilling some of the recommendations of the WHO and the national policy on traditional drug research.

Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic value (Nostro *et al.*, 2000; Tanaka, 2002). Traditional medicine is an important part of African cultures and local medicinal system vary between different cultural groups and regions (Makhubu, 2006). Herbs are now very popular in developing countries on account of improved knowledge about the safety, efficacy and quality assurance of ethno- medicine. In recent years, secondary plant metabolites (phytochemicals) have been extensively investigated as a source of medicinal agents. Thus, it is anticipated that phytochemicals with good antibacterial activity will be used for the treatment of bacterial infections. This is because, according to Arora and Keur (1999), the success story of chemotherapy lies in the continuous search of new

drugs to counter the challenges posed by resistant strains of micro organisms. Studies by Okigbo et al on 'Effects of plants and medicinal plant combinations as anti-infectives' indicate that in some plants there are many substances such as peptides, tannins, alkaloids, essential oils, phenols, and flavonoids among others which could serve as sources for antimicrobial production. These substances or compounds have potentially significant therapeutic application against human pathogens including bacteria, fungi and viruses (Arora and Keur, 1999; Okigbo and Omodamiro, 2006).

Phyllanthus niruri is a herb belonging to the family Phyllanthaceae and is indigenous to S. America, India and China. Closely related species are widely distributed in many tropical countries where it is considered a weed. In Kenya they are common at the Coast, Nairobi, Central, Eastern, Nyanza and Western provinces where they are used by locals as remedy for stomach problems (Kokwaro, 1993). A root decoction of *Phyllanthus delpyanus* Hutch is employed against Sexually Transmitted Diseases by the Digo while *Phyllanthus fischeri* Pax fruit decoction is employed against roundworm by the Nandi (Beentje, 1994).

Phyllanthus contains about 25 Species and 33 accepted taxa overall, found in tropical and subtropical regions worldwide, with about 13 species native to Kenya. Within Kenya, these plants are found in all provinces (Beentje, 1994). The Kenyan species are herbs, shrubs or trees. Several trees are in the Shimba Hills National Park with examples of *Phyllanthus delpyanus and Phyllanthus sacleuxii* (Beentje, 1994). In many parts of the world *Phyllanthus niruri* is used to control several health conditions including malaria (Ajaiyeoba, *et al.,* 2004), hepatitis B (Wang, 1995) and various ailments including: liver conditions, kidney gall stones and HIV/AIDS (Ogata, 1992).

This study therefore was focused on screening the two *Phyllanthus* species (*P. odontodenius* and *P. amarus)* for their antimicrobial activity against *Candida albicans,* a fungus, *Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis,* gram standard bacteria and *Escherichia coli* and *Klebsiella pneumoniae* which are gram negative bacteria and compare their activity with that of *P.niruri.* Thereafter, thin layer chromatography was done to confirm the presence of the phytochemicals in these species.

1.1 LITERATURE REVIEW

1.1.1 *Phyllanthus niruri*

Phyllanthus niruri has been used in wide number of traditional ailments such as jaundice, gonorrhoea, frequent menstruation, and diabetes and topically as a poultice for skin ulcers, sores, swelling, and itchiness (Bharatiya, 1992). The plant has a role in liver disorders due to its febrifuge, antiseptic, astringent, stomachic, deobstruent and diuretic actions (Nadkarni, 1993). It corrects GIT troubles like dyspepsia, colic, diarrhoea and dysentery and tones the GIT tract back to function (Thyagarajan *et al.*, 1988). The young shoots of the plant are administered in the form of an infusion for the treatment of chronic dysentery (Meixa, 1995). *Phyllanthus niruri* primarily contains lignans (e.g., phyllanthine and hypophyllanthine), alkaloids, and bioflavonoids (e.g., quercetin) (Caring Ambassadors Program inc. 2008)

This plant is distributed in all the tropical regions of the Planet and there are no paleobotanic studies accounting for its geographic origin with accuracy. Some people say that it is native to India because Linnaeus (1770 - 1778) probably reported a first specimen from that country. It also grows very abundantly in other tropical zones.

1.1.2 Description

Phyllanthus niruri belongs to Phyllanthaceae (Phyllanthoideae pro parte, Euphorbiaceae sensu lato) family (Wurdack *et al.*, 2004). The generic name *Phyllanthus* has more than 300 species, and it means "leaf and flower" because the flowers, as well as the fruit, seem to become one with the leaf. In fact, even though it appears to be so, it is not a compound leaf, but has little thin and symmetric branches that make the leaf look like a plumose leaf. Each little leaf of that branch carries in the angle a flower and the fruit. The specific name *niruri* may come from a Hindu term, and was adopted by Linneaus (1770 - 1778).

This is a plant that grows well in moist and shady places, and it spreads quickly by the invasive capacity of its large root, consuming with greed the nutrients within the ground,

in such a way that it could damage the surrounding plants. *Phyllanthus herbs* can be found in all the world's tropical places: on roads, valleys, on riverbanks and near lakes.

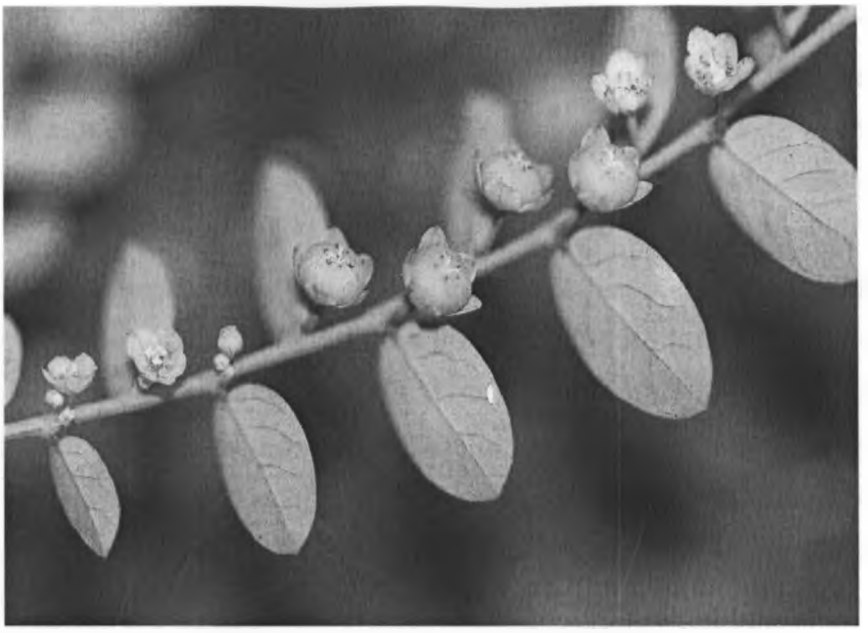

Plate 1 An image of *Phyllanthus niruri*

It is a small weed recognized by the small stonelike berries along the stalk under the leaves.

Stems: Erect 30 to 60 cm tall and 1 to 2.5 mm wide, a few horizontal branches, from 5 to 10 cm long and almost filiform.

Leaves: Whole, hairless and pale on the underside, elliptic shaped, short petiole, obtuse 7 to 12 mm apex, disposed alternately one over the other on each side of the stem, so that they resemble the folioles of a compound leaf.

Flowers: Chanca piedra Spanish name for *Phyllanthus niruri* has small, single, monoic flowers that grow in the angle of each leaf, with whitish or yellowish sepals and a green longitudinal stripe. Male flowers are very small, they have three sessile stamens, they are less abundant and we can find them near the base of the branch.

Fruits: They are eschizocarpic, capsules, globular and flattened, with 2 to 3 millimetres

Root: It is large and somewhat branched.

1.1.3 Worldwide traditional medicinal uses of *Phyllanthus niruri*

Phyllanthus niruri is a herb found in many parts of the world. It is known for a variety of uses such as hepatoprotective action, lipid lowering action, antidiabetic action, antifungal action to name a few. For example in Benin the hot water extract of the entire plant is administered orally, to reduce fevers, and as a laxative (Halberstein and Saunders, 1978).

In the Dominican Republic, the hot water extract of leaves is administered orally as a popular fever remedy (Ricardo, 1944). The decoction of dried leaves and roots is taken orally for fever, and for good health in Fiji. Also, dried entire plant, ground in buttermilk is administered orally for jaundice. Fresh leaf juice is used externally for cuts and bruises. For eye diseases the juice is mixed with castor oil and applied to the eye. Infusion of dried leaves is administered orally for dysentery and diarrhea. Infusion of green root is taken orally to treat heavy menstrual periods (Singh, 1986)

The hot water extract of leaves is administered orally as a cholagogue in French Guyana (Duke, 1975) while in Haiti the decoction of dried leaves is taken orally for or used in bath for fever, and orally for indigestion (Weninger *et al.,* 1986). Hot water extract of dried entire plant is administered orally as a spasmolytic and is also against fever (Weninger *et al,* 1982). In India, the Fresh plant juice is taken orally for genito-urinary disorders (Sahu, 1984). Also, the fruit is used externally for tubercular ulcers, scabies and ringworm (Chauhan, *et al* 1977). Hot water extract of dried entire plant is administered orally for diabetes (Jain and Sharma, 1967) and for asthma in ayurvedic medicine (Sircar, 1984).

Fresh leaf juice or fresh root juice are taken orally for venereal diseases in Papua-New Guinea. Decoction of dried entire plant is also administered orally to treat venereal diseases (Holdsworth *et al.,* 1989). A cupful of the decoction of dried leaf when taken orally daily is a treatment for diarrhoea (Holdsworth and Balun, 1992). In Philippines, the decoction of dried entire plant is used as a bath for newborns because it is believed to remove disease-causing elements from the skin. Orally the decoction is used for coughs in infants (Velazco, 1980). Hot water extract of leaf and stem is taken orally for fevers in Puerto Rico (Loustalot and Pagan, 1949) whereas in Tanzania hot water extract of fresh

entire plant is administered orally for gonorrhea (Khan *et al.*, 1978). In Thailand, hot water extract of commercial sample of the entire plant, is administered orally as an antipyretic (Mokkhasmit *et al.*, 1971) while the hot water extract of dried aerial parts administered orally is used as a diuretic, as an antipyretic, and for malaria (Kitisin, 1952). Hot water extract of dried entire plant is administered orally as an anti-inflammatory agent (Wasuwat, 1967). In Virgin Islands hot water extract of the plant is taken orally to increase the appetite (Oakes and Morris, 1958)

The hot water extract of roots together with that of *Citrus aurantifolia* roots is taken orally to increase appetite in West Indies and hot water extract of entire plant administered orally, is taken for malarial fever. The plant is boiled and the tea taken. Water extract of the leaves and roots is taken orally for diabetes, and as a diuretic (Asprey and Thornton, 1955).

A number of the *Phyllanthus* species have been reported to have extensive history in medicine systems (Mdlolo, 2008). Substantial amount of the genus are used widely in traditional medicine for the treatment of flu, dropsy, diabetes, jaundice, gall and bladder calculus, and liver disease (Unander *et al.*, 1995; Calixto *et al.*, 1998; Dhiman and Chawla, 2005).

1.1.4 Pharmacological uses of *Phyllanthus niruri*

The bioactivity of plants used as herbs appears to be derived from 'secondary metabolites', such as the polyphenols (Huffman, 2003). Polyphenols, the most numerous and widely distributed class of phytochemicals, include classes of chromones, coumarins, lignans, stilbenes, xanthones and the ubiquitous flavonoids (Hertog *et al.*, 1994; Kromhout *et al.*, 1996). Within the past decade, many polyphenols, particularly the flavonoids, have been found to possess relatively potent antioxidant, antiatherosclerotic, antiinflammatory, antimutagenic, antitumor and antiviral activities (Nijveldt *et al.*, 2001).

Observational studies have repeatedly shown that diets high in plant-based foods and beverages are associated with a lower risk of chronic diseases, such as cardiovascular disease and some forms of cancer (Hertog *et al.*, 1996; Hertog *et al.*, 1993; Hertog *et al.*,

1995; Hollman *et al.*, 1999; Hu, 2003; Riboli and Norat, 2003; Rimm *et al.*, 1996) and suggest this correlation may be attributable to the phytochemical constituents as well as to the macro- and or micronutrient content of these foods. Further research is thus necessary to better understand and quantify the contributions of phytochemicals to health promotion and disease prevention.

Most of these species have pharmacological properties for example *Phyllanthus niruri* has demonstrated *in vitro* antibacterial actions against *Staphylococcus*, *Micrococcus* and *Pasteurella* bacteria as well as *in vivo* and *in vitro* anti-malaria properties, which validates other traditional uses of the genus (Veeramuthu *et al.*, 2006). Extracts of *Phyllanthus* had been used as antiviral source to treat hepatatis B (Venkateswaran *et al.*, 1987; Thyagarajan et al., 1988; Blumberg *et al.*, 1989; Lam *et al.*, 2006). Powis and Moore (1985) studied the aqueous extracts of *Phyllanthus amarus* and found it to inhibit viral DNA replication *in vitro*. In addition, they eliminated detecTable virus from the sera of woodchucks (*Marmota monax*) acutely or chronically infected with the woodchuck hepatitis virus (WHV). The methanol extracts of five *Phyllanthus* species from India was reported to have strong antioxidant activity (Kumaran and Karunakaran, 2007).

P. niruri has antifungal activity on ringworm, ulcers, scabies and jaundice. Its ethanol extracts have extensive antibacterial and antiviral actions and are antiprotozoal against *Amoeba berghei* and anthelmintic to *Hyme-nollepis nana.* Aqueous extracts of the leaves produced an oral hypoglycemic effect comparable to that of toluene butanamide (Unander, 1996). According to Pettit *et al.* (1990), the root of *Phyllanthus acuminatus* inhibited the growth of murine P-388 lymphocytic leukemia and B-16 melanoma cell lines.

The *Phyllanthus* genus is a source of plant chemicals. Extracts of *Phyllanthus* have secondary compounds like alkaloid, flavonoid, lignin, phenol, tannin and terpene. Many of the "active" constituents are attributed to biologically active lignin, glycosides, flavonoids, alkaloids, ellagilannins and phenyl propanoids that are found in the leaf, stem and roots of the plant. Common lipids such as sterols and flavonols also occur in the plant (Unander *et al.*, 1990, 1991).

A number of the *Phyllanthus* species have been reported to have extensive history in medicine systems (Unander *et al.*, 1990, 1991). Researches and review on *Phyllanthus* species indigenous to some countries are known for their numerous antimicrobial and antiviral activities.

Some of these activities are hepatoprotective effect; Hepatitis B is one of the major diseases inflicting human population. Conventional treatment with interferon – alpha is very expensive and has many serious side effects. Alternative herbal medicine using extracts of *Phyllanthus niruri* and *Phyllanthus urinaria* have been reported to be effective against Hepatitis B and other viral infections. A study reports quantitative determination of the anti viral effect of these herbs in well-defined in vitro systems (Meixa, *et al.*, 1995)

Phyllanthus niruri has been reported to exhibit marked antihepatitis B virus surface antigen activity in *in-vivo* and *in-vitro* studies. It was postulated that *Phyllanthus niruri* might inhibit proliferation of the virus by inhibiting replication of the genetic material of the virus (Thyagarajan et *al.*, 1988)

Hepatoprotective effect of an ayurvedic medicine; herbal preparation HPN – 12 orally administered to male albino rats at 1ml/100g body weight was found to be effective against liver damage (Latha and Rajesh, 1999). Research in Japan and India in the 1980's demonstrated the liver -healing properties of *Phyllanthus niruri*. The primary compounds responsible are *phyllanthin, hypophyllanthin* and *triacontanal*. Glycosides found in *Phyllanthus niruri* demonstrated Aldose reductase (AR) inhibitory activity in studies conducted by Japanese research group in 1988 and 1989 (Shimizu, 1989).

HIV Replican Inhibition; Aqueous extract of *Phyllanthus niruri* is reported to have inhibitory effect on human immunodeficiency virus. The alkaloidal extract of *Phyllanthus niruri* was thus found to exhibit sensitive inhibitory response on cytopathic effects induced by both the strains of human immunodeficiency virus on human MT-4 cells in the test concentrations (Naik and Juvekar, 2003). Extracts of five medicinal plants: *Aristolia indica, Cassia occidentalis, Phyllanthus niruri*, *Withania somnifera and Tinospora cordifolia* increased CD4 count in HIV standard patients (Natarraj, 2000).

Lipid Lowering Activity; Lipid lowering activity of *Phyllanthus niruri* alcohol extracts in triton induced hyperlipidaemia was examined in rats. It was observed that administration of *Phyllanthus niruri* at the dose of 200mg/kg simultaneously with triton lowered the level of total cholesterol, phospholipid and triglyceride by 27, 25 and 24 percent respectively. In an experiment with cholesterol fed rats, *Phyllanthus niruri* at a dose of 100 mg/kg lowered the elevated level of low-density lipoprotein lipids in hyperlipidemic and drug fed animals (Chandra, 2000).

Anti-diabetic Activity; alcohol extract of *Phyllanthus niruri* was found to reduce significantly the blood sugar in normal rats and in alloxan diabetes rats (Raphael *et al.*, 2000).

Anti-malarial Activity; the ethanolic, dichloromethane and lyophilized aqueous extracts of *Cassia occidentalis* root bark, *Morinda morindoides* leaves and whole plants of *Phyllanthus niruri* were evaluated for their antimalarial activity in vivo. Each lyophilized aqueous extract was less active than the corresponding ethanolic extract (Neraliya and Gaur, 2004).

Activity against Filarial Mosquito (*Culex quinquefasciatus*); 18 plants were evaluated for juvenile hormone analogue activity against *Culex quinquefasciatus*. Of these acetone extracts of 8 plants namely *Commelina benghalensis, Ageratum conyzoides , Achyranthus aspera, Sida acuta, Euphorbia pulcherrina, Rivinia humilis, Ruellia tuberosa* and *Phyllanthus niruri* possessed significant juvenile hormone activity. The LC_{50} values of 5 most active plants namely *Phyllanthus niruri, Amaranthus spinosus, Antegonon leptopus, Corchorus aestuans, Corchorus benghalensis* were determined to be 13,16,17,17,14ppm respectively (Calixto, 1984).

Anti-spasmodic activity; research done in Brazil at the Federal University of Santa Catarina in 1984 on *Phyllanthus niruri* revealed an alkaloid (phyllanthoside) in the leaves and stem with strong antispasmodic activity (Grewal, nd). It served as a relaxing agent for smooth muscles and they concluded that its spasmolytic action probably accounted for the efficacy of *Phyllanthus niruri* in expelling stones (Grewal, nd). Also methanol extract of dried callus tissue showed analgesic activity (Santos, 1994)

Analgesic activity; methanol extract of dried callus tissue showed activity (Santos, 1994). Chromosome Aberration Inhibition; water extract of dried fruit and leaves was active versus chromosome damage induced by lead nitrate and aluminium sulphate in bone marrow chromosomes (Holdsworth and Wamoi, 1982).

In Vitro study of effects of an aqueous extract of *Phyllanthus niruri* on the model of calcium oxalate crystals endocytosis by Madin-Darby canine kidney cells; the extract exhibited a potent and effective non concentration dependent inhibitory effect on the calcium oxalate crystals internalization (Campos and Schor, 1999).

1.1.5 Plant compounds with antimicrobial activity

Mainstream medicine is increasingly receptive of the use of antimicrobial and other drugs derived from plants, as traditional antibiotics become ineffective and because of the rapid rate of plant species extinction. There is a feeling among natural-products chemists and microbiologists alike that the multitude of potentially useful phytochemical structures which could be synthesized chemically is at risk of being lost irretrievably (Cowan, 1999).

Many of the earliest isolated pure compounds with biological activity were alkaloids. Naturally occurring alkaloids are nitrogenous compounds that constitute the pharmacogenically active basic principles of flowering plants. A benzylisoquinoline alkaloid, papaverine was shown to have inhibitory effect on several viruses and indoquinoline alkaloids from *Cryptolepsis sanguinolenta* displayed activity against a number of gram negative bacteria and yeast (Silva *et al.*, 1996).

The phenolics and polyphenols are another group of PSM that have exhibited antimicrobial activity. Important subclasses in this group of compounds which have been found to have antimicrobial activity include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins. This group includes metabolites derived from the condensation of acetate units (tepernoids), those produced by the modification of aromatic amino acids (phenylpropanoids and coumarins), flavonoids, isoflavonoids

and tannins. Flavones, flavonoids and flavonols have been known to be synthesized by plants in response to microbial infection so it is not surprising that they have been found, *in vitro*, to be effective antimicrobial substances against a wide array of microorganisms.

Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions have been assigned to tannins. These are soluble in water, alcohol and acetone and gives precipitates with proteins (Basri and Fan, 2005). Tannins have been traditionally used for protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, hemorrhoids and diarrhea (Ogunleye and Ibitoye, 2003). As a group, coumarins have been found to stimulate macrophages, which could have an indirect negative effect on infections (Cowan, 1999).

External plant surfaces are often protected by biopolymers for example, waxes fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid alkaloids and other compounds which inhibit the development of fungi and bacteria. Cell walls of at least some monocotyledons also contain antimicrobial proteins, referred to as thionins (Angeh, 2006).

1.1.6 Practical clinical application of plant antimicrobial compounds

Bacteria have evolved numerous defences against antimicrobial agents and drug-resistant pathogens are on the rise. This resistance is conferred by multidrug resistance pumps (MDRs), membrane translocases that extrude structurally unrelated toxins from the cell. These protect microbial cells from both synthetic and natural antimicrobials (Stermitz *et al.*, 2000). Secondary metabolites resemble endogenous metabolites, ligands, hormones, signal transduction molecules or neurotransmitters and thus have beneficial medicinal effects on humans due to their recognition in potential target sites (Parekh *et al.*, 2005).

The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments and could help curb the problem of these multi-drug resistant organisms. In a study done with *Pseudomonas aeruginosa*, which is resistant to different antibiotics, its

growth was inhibited by extracts from clove, jambolan, pomegranate and thyme (Nascimento *et al.*, 2000).

Moreover, the synergistic effects of extracts with antimicrobial activity in association with antibiotics can provide effective therapy against drug resistant bacteria. These synergistic combinations represent a largely untapped source of new pharmaceutical products with novel and multiple mechanisms of action that can overcome microbial resistance. Recent developments in plant biotechnology have created the tools to produce botanical mixtures at a level comparable to that of pure drug compounds (Gibbons, 2003), and through biosynthesis and bioengineering dependence on large amount of plant material is reduced, limiting depletion of biogenetic resources in forests. These compounds, however, should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on beneficial normal microbiota (Iwu *et al.*, 1999). It would be beneficial to standardize methods of extraction and *in vitro* testing so that the search for new antimicrobial drugs from plants could be more systematic and to facilitate proper interpretation of results (Cowan, 1999).

1.1.7 Experimental approaches

There are multiple factors that may affect the outcome of susceptibility tests and standardized methods are more likely to be reproducible than unstandardized methods. Standardization is required for intra- and inter-laboratory reproducibility as results may be significantly influenced by the method used (EUCAST, 2003). Standard criteria for evaluation of plant antimicrobial activity are lacking and results greatly differ between authors. Sometimes it is difficult to compare results obtained, when dealing with plant extracts, with published results in the literature because several variables influence the results, such as the environmental and climatic conditions under which the plant grew, choice of plant extracts, choice of extraction method, antimicrobial test method and test microorganisms (Nostro *et al.*, 2000; Hammer *et al.*, 1999). The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant although it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of plants are unique to a particular plant species or

group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh *et al.*, 2005). They also vary between tissues (higher concentrations occur in bark, heartwood, roots, branch bases and wound tissues), among species from tree to tree and from season to season (Gottlieb, 1990). In their work, Mitscher *et al.* (1972) found that extracts are generally richest in antibacterial agents after the flowering (sexual) stage of their growth is complete, and that plants taken from stressful environments were particularly active.

1.2 Research Problem

Despite the extensive use of antibiotics and vaccination programmes, microbial diseases continue to be a leading cause of morbidity and mortality worldwide. Widespread antibiotic resistance, the emergence of new pathogens in addition to the resurgence of old ones, and the lack of effective new therapeutics exacerbate the problems. Keeping in view the great impact microbial diseases have on health, it was therefore important to conduct a research with the aim of screening crude extracts of two *Phyllanthus* species (*F. odontodenius* and *P. amarus)* for their antimicrobial activity against *Candida albicans,* a fungus, *Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis,* gram standard bacteria and *Escherichia coli* and *Klebsiella pneumoniae* which are gram negative bacteria and compare their activity with that of *P.niruri.*

1.3 Research Justifications

Antibiotic resistance has become a global concern as the clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug-resistant pathogens. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for the development of novel drugs because of the great diversity in their chemical structure. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. This study is therefore expected to

contribute towards finding more effective, less toxic and less costly drugs for treatment of microbial diseases.

1.4 Research Questions

1. Do the Kenyan *Phyllanthus species* (*P. odontodenius* and *P. amarus)* extract have antimicrobial activities?
2. Are the antimicrobial activities comparable to those of *P. niruri*?

1.5 Hypothesis

The two Phyllanthus species (*P. odontodenius* and *P. amarus)* possess important phytochemicals with antimicrobial activity.

1.6 Objectives of the study

1.6.1 General objective

To screen two Kenyan *Phyllanthus* herbs (*P. odontodenius* and *P. amarus)* in comparison to *Phyllanthus niruri* for their antimicrobial activity.

1.6.2 Specific Objectives

1. To identify *Phyllanthus* species collected from two ecological regions in Kenya, Nairobi area and Siaya district, Nyanza province.
2. To investigate the *in vitro* activity of extracts of *Phyllanthus amarus, Phyllanthus odontadenius and Phyllanthus niruri* on *Candida albicans, Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Klebsiella pneumonia, Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli.*
3. To correlate the effects of commercial extracts (*P. niruri*) in the Kenyan market vs. the two *Phyllanthus* species found in Kenya.
4. To compare the effects of Nutrient Agar, Muller- Hinton Agar and Trypson Soy Agar on susceptibility testing.

5. To profile crude extracts of *P. amarus, P. odontodenius* and *P. niruri*using thin layer chromatography (TLC).

1.7 Expected outcome

1. Herbal remedy - It is hoped that this study will generate more information on *Phyllanthus* species found in Kenya which could lead to a new remedy to many microbial pathogens.
2. Capacity building - The outcome of this undertaking will offer considerable contribution to research in herbal medicine. Standard findings of the study may get rid of cost of importation hence lower product cost. This may also lead to commercialization of the product.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Plant collection and interviews with local community

Field collections of *Phyllanthus* species was carried out in Langata forest Nairobi area and Ligingo village, Siaya district, Nyanza province. In the latter, this was conducted in collaboration with the communities in these regions in order to seek traditional uses.

The specimens were divided into two portions; one set was deposited in the University of Nairobi herbarium and the second set was used for extractions for biological assays and TLC screening.

Interaction with the communities in the two regions during collection provided an insight on the traditional uses of the plant.

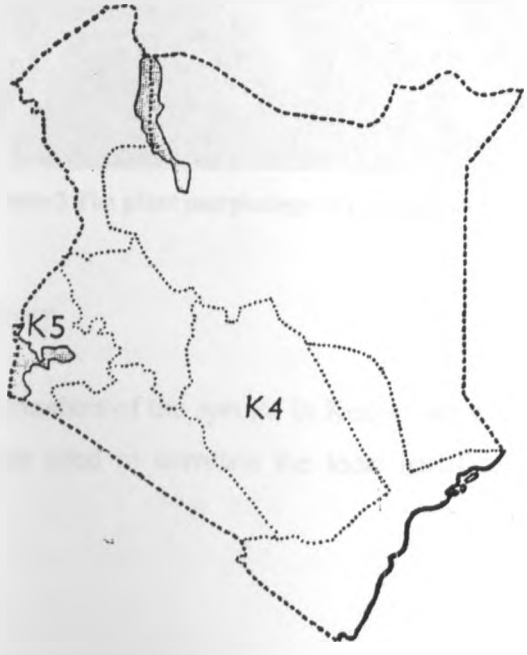

Figure 1 : Collection sites K4 and K5 adopted from floral regions of Kenya (Beentje, 1994)

2.2 Identification of the plants

Preliminary identification of the plants was done in the field by Dr. Beatrice Anyango, School of Biological Sciences, University of Nairobi. Herbarium specimens were prepared and photographs taken to aid in the confirmation of the identity of the plants. Voucher specimens were deposited in the Herbarium of the School of Biological Studies, University of Nairobi, Kenya, where identity of the plants was confirmed by comparison with available voucher specimens. The identification was further confirmed at the National Museums of Kenya Herbarium.

Plate 2 The plant morphology of *Phyllanthus* herb

2.3 Ethnobotanical survey

The ethnobotanical information of the species in Kenya was collated from literature and local herbalist. This was used to correlate the local herbs' ethnobotany with that of *Phyllanthus niruri*.

2.4 Processing of the plant materials

Whole plant materials were sorted and chopped into smaller pieces, where necessary, and dried under the shade. The dried plant material was ground to various degrees of fineness depending on their botanical structures using an electric grinder.

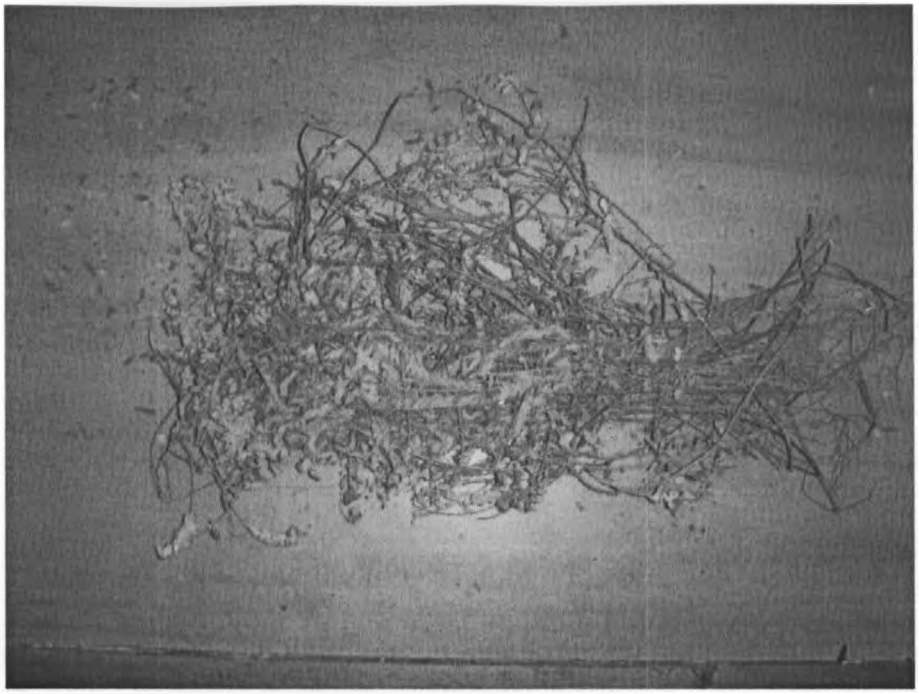

Plate 3 Drying of *Phyllanthus niruri* in the shade

2.4.1 Preparation of crude extracts

Plant extracts were prepared by soaking 20g of dried powder in cold water, hot water methanol or dichloromethane: methanol 1: 1 according to standard extraction method (Harbourne, 1998). These were set for 8 hours. The plant extracts were decanted then filtered using filter funnels fitted with Whatman No 1 filter papers into Erlenmeyer flask. The filtrate was concentrated using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) before drying in a freeze drier (Chemlab instruments ltd). Dry powder extract was reconstituted with sterile distilled water as the solvent. These were then used for susceptibility tests.

2.5 Preparation of disk and Wells

Paper disks (diameter 6mm prepared by punching Whatman No. 1 filter paper) were impregnated with 2µl of the final extract, the equivalent of 2 mg/ml of dried plant extract.

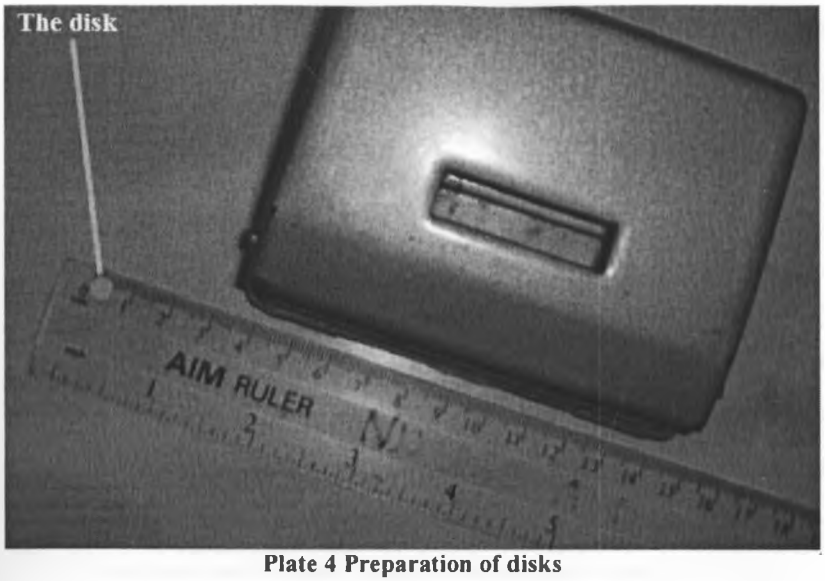

Plate 4 Preparation of disks

Petri dishes with 15-20ml of 1ml inoculum in 100ml media per plate; 6million CFU/ml equivalent to 0.5MacFalant was used for the bioassays. Wells were used in subsequent trials where the media was drilled with 8.1 mm punch.

Plate 5 Wells preparation

2.5.1 Antimicrobial screening

Different solvent extracts of *Phyllanthus niruri, P. odontadenius* and *P. amarus* were screened against a total of six bacterial strains and one fungus. The test organisms, *Candida albicans, Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli* and *Klebsiella pneumoniae* were obtained from the Microbiology laboratory of the University of Nairobi.

2.5.2 Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments was prepared by aseptically transferring a loopful of cells from the stock cultures (provided by School of Biological Studies and School of Pharmacy University of Nairobi) to test tubes of Nutrient agar for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 hrs at and 37°C and 25°C respectively. The cultures was diluted with fresh nutrient agar and Sabouraud dextrose broth to achieve optical densities corresponding to 2.0×10^6 colony forming units (CFU/ml) for bacteria and 2.0×10^5 spore/ml for fungal strains.

2.5.3 Antimicrobial susceptibility test

The Kirby-Bauer disc diffusion (Bauer et al, 1966) and Well methods (Cooper 1955) were used to screen the antimicrobial activity. The tests were carried out at the School of Biological Sciences, University of Nairobi where the disk diffusion method was used. The infectious organisms, used for the tests could only be obtained and handled at the School of Pharmacy, University of Nairobi. This introduced the Well diffusion method as the normal procedure at the School of Pharmacy.

In vitro antimicrobial activity was screened by using Nutrient agar (NA). The NA plates were prepared by pouring 15 ml of molten media into sterile Petri plates. The plates were allowed to solidify for 15 minutes and 100µl of inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. 2µl of 1mg/100µl/disk

extracts of cold water, hot water, methanol and dichloromethane: methanol 1: 1 was loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 minutes. The plates were kept for incubation at 37°C and the zone of inhibition measured in mm after 24h growth. A control experiment was set up by using drops of sterile distilled water in place of different solvent systems. A standard control 0.32mg/ml Gentamycin for bacteria and 0.30mg/ml Nystatin for fungi were used. At the end of incubation, inhibition zones formed around the disc were measured with a Vanier caliper in millimetre. Further tests were carried out using Mueller-Hinton agar (MHA) and Trypson Soya agar (TSA). The change of media was to compare the effects on susceptibility testing. There were five replicas.

2.6 Thin layer chromatography

The study was carried out to compare the migration patterns of compounds in crude extracts of *P. amarus, P. odontadenius and P. niruri*. Each extract was reconstituted into aqueous solution, as all were soluble, before spotting 25µl on a chromatography sheet and the chromatography plates with silica, as the adsorbent. The plates were placed in chromatographic tanks containing solvent systems of varying polarities (water, hexane: methanol 9:1, CH_2CL_2: Acetone 9:1, CH_2CL_2: Hexane 8:2). The developed plates, after air-drying, were examined under the UV lamp, for clarity of chromatograms, at wavelengths of 254nm and 366nm.

2.7 Type and source of test strains used

Table 1 The Bacterial and Fungal Cultures

Ref. Number	Product Description	Format
NCPF3179	*Candida Albicans*	Standard
NCTC08241	*Bacillus Pumilus*	Standard
NCTC07447	*Staphylococcus Aureus*	Standard
NCTC10400	*Bacillus Subtilis*	Standard
NCTC07743	*Micrococcus Luteus*	Standard
ATCC25922	*Escherichia Coli*	Standard
Clinical Isolate	*Klebsiella Pneumoniae*	

2.8 Sampling design

Purposive Sampling Non-probability Sampling was employed i.e. Subjects were selected for a good reason tied to purposes of research.

2.9 Data collection and analysis

2.9.1 Data collection technique and tools

Data was obtained by measuring inhibition zone in millimetres using transparent rulers and Vanier calipers.

2.9.2 Research design and data entry

Nested design was used and data entered in a Table.

2.9.3 Data analysis

All results were as mean S.E. The statistical analysis of the data was performed using the Bonferroni type multiple *t*-test. A value of $p \leq 0.05$ was considered statistically significant. The antimicrobial activity of *Phyllanthus* extracts was calculated using ANOVA test to test for significant difference ($P>0.05$) between the media used and significant difference ($P<0.05$) between extraction solvent.

CHAPTER THREE

3.0 RESULTS

3.1 Species Identification

Phyllanthus collected from Nairobi's Langata forest matched voucher specimen of *P. odontadenius*.

Plate 6 A voucher specimen of *P. odontodenius*

The herb collected from Ligingo, Siaya district was identified as *Phyllanthus amarus*.

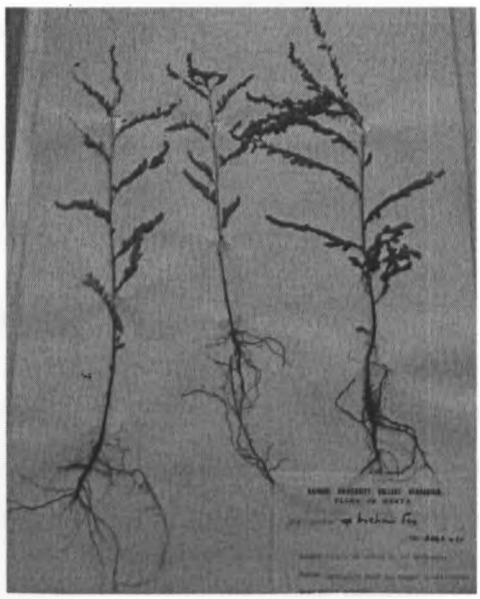

Plate 7 Voucher specimen of *P. amarus*

3.2 Ethnomedicine

The ethnomedicinal data of *Phyllanthus amarus* collected from Siaya, Nyanza province. A myriad of names were given to the herb from awour awour to anyidhra. Being considered a weed by diverse populace the name was given as oboke.

Table 2: Ethnomedicine of *P. amarus* as collected from Siaya district.

Vernacular name	Parts used	Preparation method	Application	Condition treated
Anyidhra*	Whole plant	Pounded plant mixed with oil	Massage	Muscle aches and hunch back tendency in children.
		Decoction	Oral	Measles, malaria stomach ailments, measles and sexually transmitted diseases particularly gonorrhoea.

* Kokwaro 1993

Ethnomedicinal information on *P. amarus* as collated from literature

The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic. It is useful in dropsy, jaundice, diarrhoea, dysentery, intermittent fevers, and diseases of urino-genital system, scabies ulcers and wounds (Thyagarajan, 1982; Thyagarajan, 1988; Thyagarajan and Venkateswaran, 1987).

There was no ethnomedicinal information on *P. odontadenius* or any collated from literature.

3.3 Antimicrobial activity

At a concentration of 50µl of 1mg/100µl/well, *C. albicans* showed no susceptibility to any of the extracts. *Escherichia coli, Staphyloccocus aureas, Bacillus subtilis* and *Bacillus pumillus* were susceptible. The results are shown in Table 2 below.

Table 3: Antimicrobial activity of three *Phyllanthus* species extracts of different solvents

		Microorganism				
		Candida albicans	*Escherichia coli**	*Staphyloccocus aureas***	*Bacillus subtilis***	*Bacillus pumillus***
P. odontadenius	CH_2CL_2:meoh	-	+	++	++	++
	meoh	-	+	++	++	++
	cold water	-	+	++	++	++
	hot water	-	+	++	++	++
P. amarus	CH_2CL_2:meoh	-	+	++	++	++
	meoh	-	+	++	++	++
	cold water	-	-	-	-	-
	hot water	-	+	++	++	++
P. niruri	meoh	-	+	++	++	++

- ...No inhibition, + ...active, ++ ... very active, ** gram standard * gram negative bacteria

Preliminary results showed no action on *Candida albicans.* Gram standard bacteria were more susceptible than the gram negative. Cold water was the least active solvent.

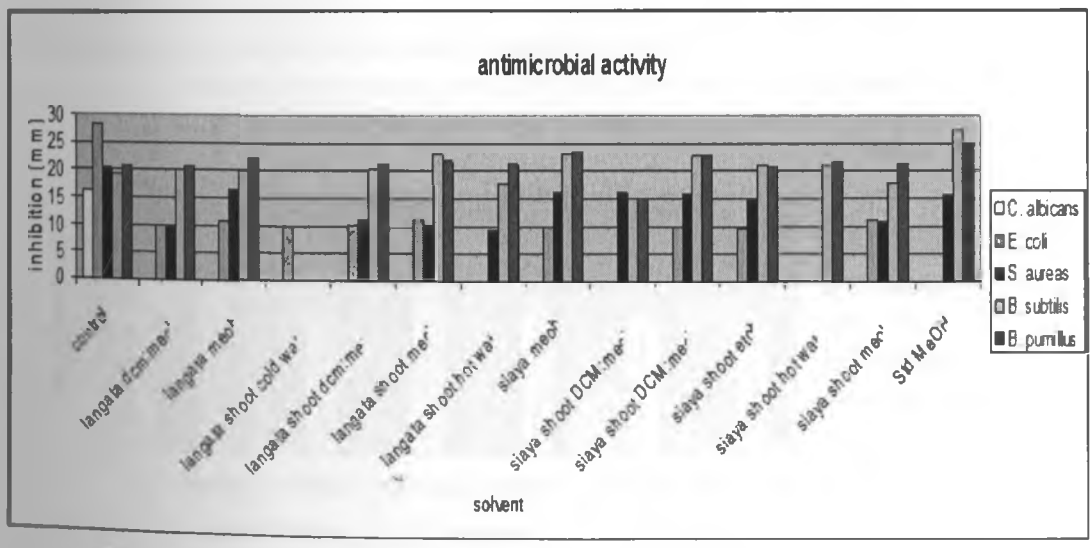

Figure 2 Antimicrobial activity of *Phyllanthus* species extracts with different solvents

At a concentration of above 50mg/µl the activity of the extracts was standard on all the test organisms with *P. odontadenius* extracts showing the greatest activity as shown in Table 3.

Table 4: Antimicrobial activities of methanol extracts of *Phyllanthus* species at a concentration of 100mg/µl and 50mg/µl.

	100mg/µl			50mg/µl		
	P.odontadenius	*P. amarus*	*P. niruri*	*P.odontadenius*	*P. amarus*	*P. niruri*
Staphyloccocus aureas	+++	++	+	+	+	-
Bacillus subtilis	++++	+++	+	++	+	-
Eschelichia coli	++++	++++	+	++	++	+
Bacillus pumilus	+++	++	+	+	+	+
Klebsiella pneumoniae	+++	+++	+	++	+	-
Candida albicans	+++	++	+	++	+	+

Inhibition < 40% -, Inhibition > 40% +, Inhibition >50% ++, Inhibition >60% +++, Inhibition >70% ++++

The antimicrobial activity of methanol extracts is shown in Figure 3

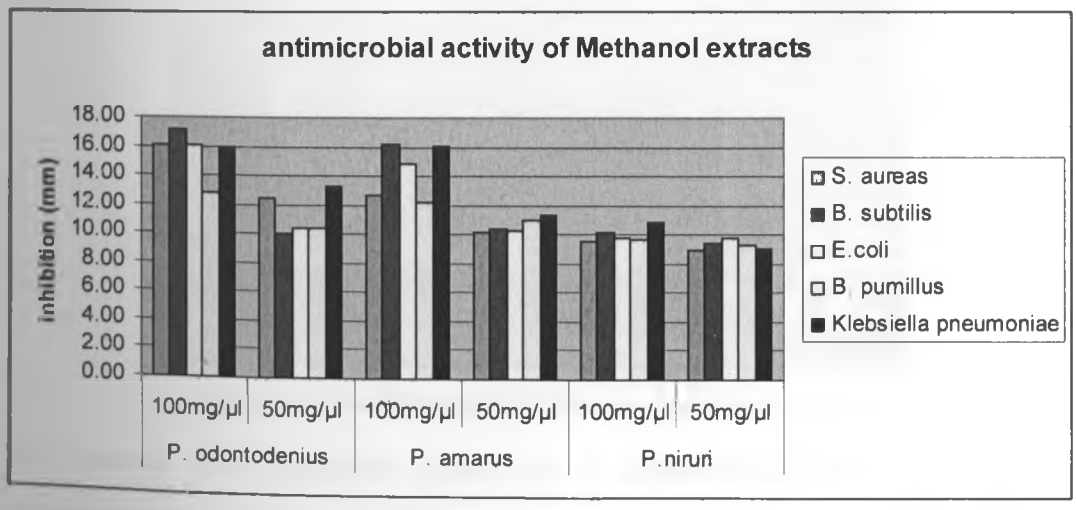

Figure 3 Antimicrobial activity of methanol extracts at different concentrations

Susceptibility of the test microbes to the extracts showed high activity with *P. odontadenius* and *P. amarus*. At a concentration of 100mg/µl *P. niruri* matched activity of *P. amarus* at 50mg/µl concentration.

Plates 7, 8, 9 and 10 show the standard and negative results of the susceptibility tests

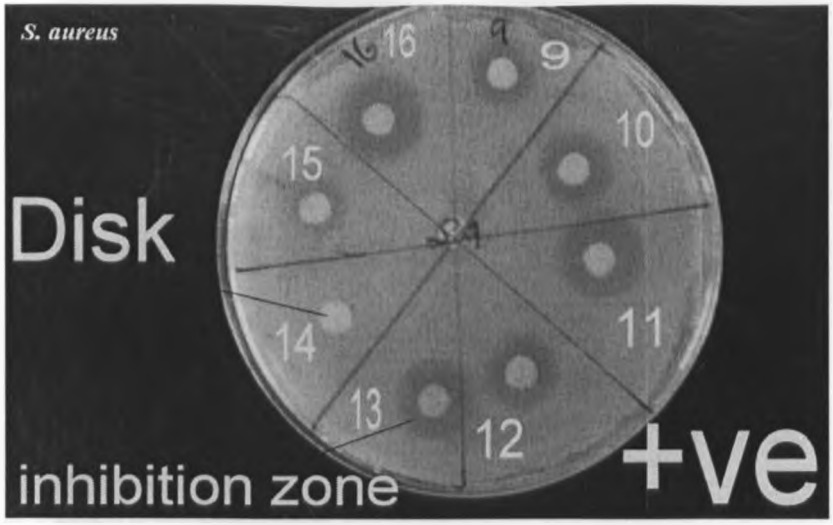

Plate 8 Disk standard results of *Staphylococcus aureas* showing inhibition zones

9- *P. amarus* ethanol extract, 10- *P. amarus* methanol extract, 11- *P. odontadenius*, 12- *P. odontadenius* methanol extract, 13- *P. amarus* MeOH extract, 14- *P. amarus* cold water extract 15- *P. amarus* root cold water extract, 16-*P. odontadenius* CH_2CL_2:MeOH extract. SA- negative control.

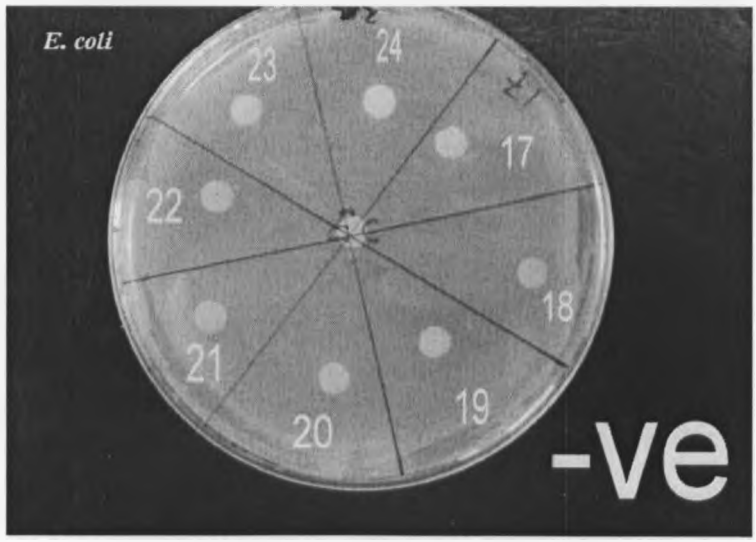

Plate 9 Results of *Escherichia coli* showing no inhibition zones

18-*P. amarus* CH_2CL_2:MeOH extract, 19- *P. odontadenius* MeOH extract 21- *P. odontadenius* hot water extract, 22-*P. amarus* shoot hot water extract 23 and 24 blank.

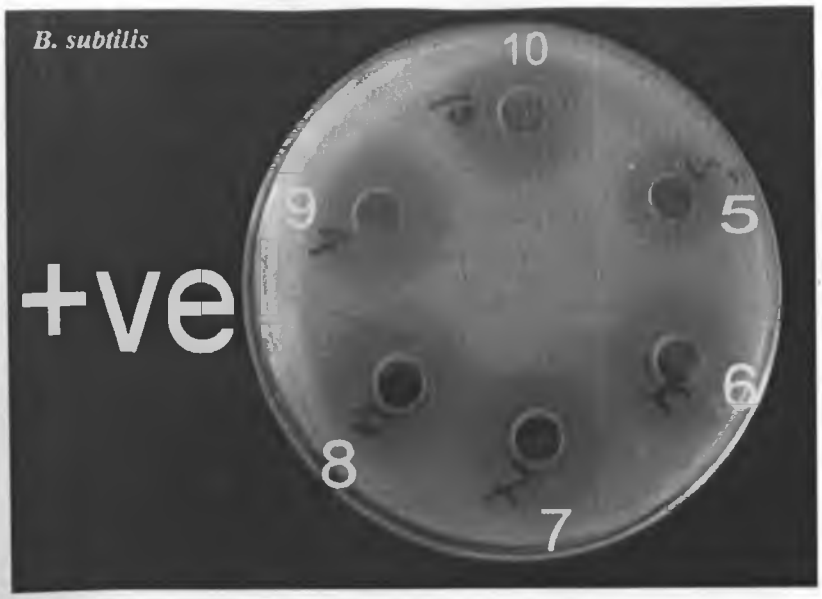

Plate 10 Results of *Bacillus subtilis* showing inhibition zones

5- *P. amarus* methanol extract, 6- *P. odontadenius*, 7- *P. odontadenius* methanol extract, 8- *P. amarus* MeOH extract, 9- *P. amarus* root cold water extract, 10-*P. odontadenius* CH_2CL_2:MeOH extract.

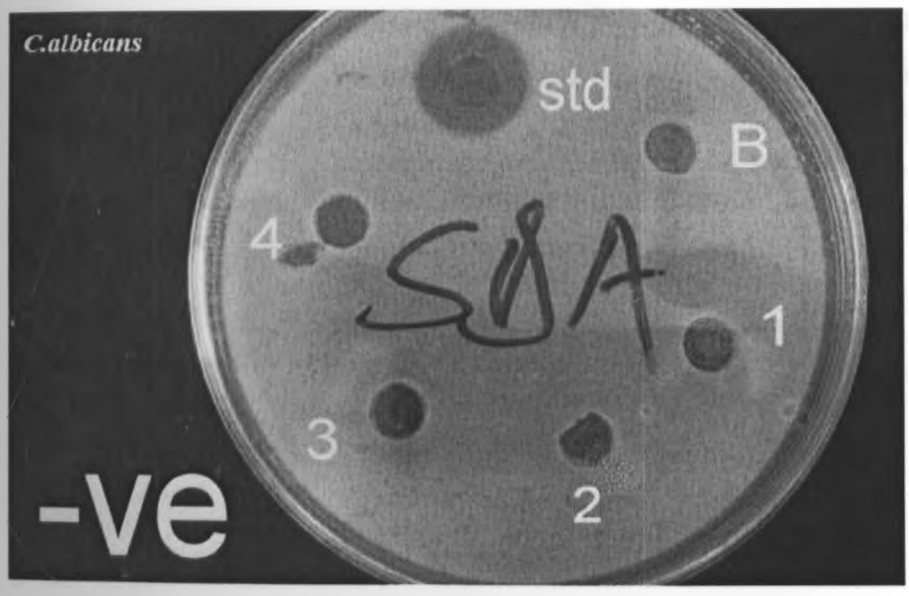

Plate 11 Wells negative results of *Candida albicans* showing inhibition zones

Std- standard control, B- blank negative control, 1- *P. odontadenius* cold water extract, 2- *P. niruri* MeOH extract, 3- *P. amarus* CH_2Cl_2: MeOH extract, 4-*P. niruri* cold water extract.

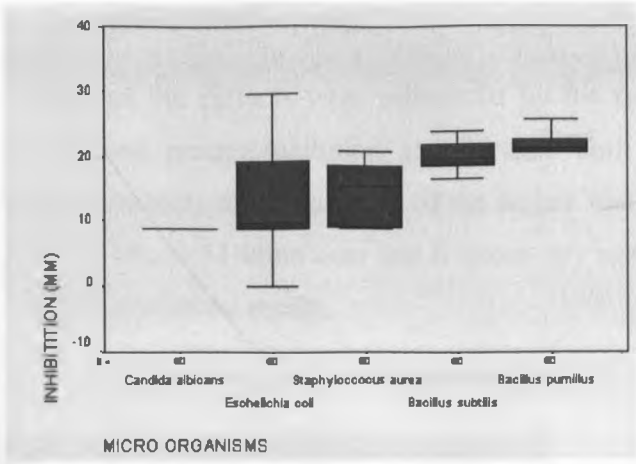

Figure 4 Comparison of inhibition between test microorganisms

Most extracts were active against the bacteria. Cold water extract showed least activity with minimal activity on *E. coli.* The solvents in comparison to antibiotics, standard control, showed 80% activity for methanol, 48% for CH_2CL_2: MeOH 1:1, 43% in hot water and 28% for cold water. The most active extract was methanol with the effects being highest in *Bacillus pumillus.* At 2mg/ml, all crude extracts showed no activity on *Candida albicans. Escherichia coli* showed the least activity for bacteria, Figure 4 shows *Bacillus pumillus* being the most susceptible bacteria with median close to 20mm. sterile deionised water was used as negative control.

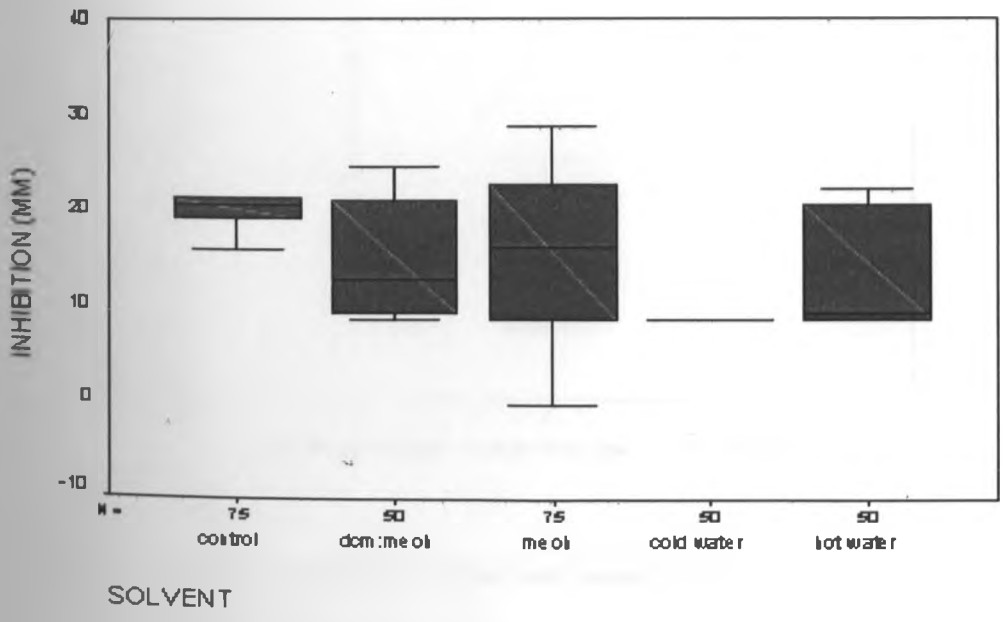

Figure 5 Comparisons between the extraction solvent used

3.4 Effects of media on antimicrobial activity

The antimicrobial effects of the extracts were influenced by the type of media used. Mueller-Hinton agar showed greater inhibition activity than both nutrient agar and Tryptone Soya agar. Susceptibility testing on each of the isolate was first performed on nutrient agar, and then on Mueller-Hinton agar and tryptone soy agars. Figure 6 below gives a graphical representation of the results.

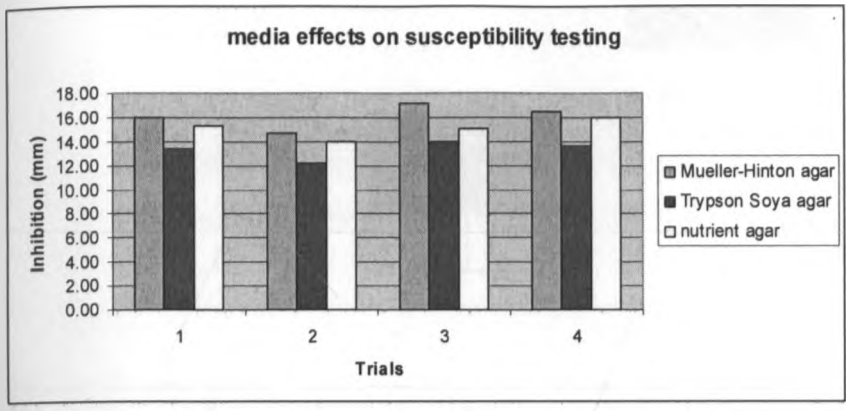

Figure 6 Media influence on susceptibility testing

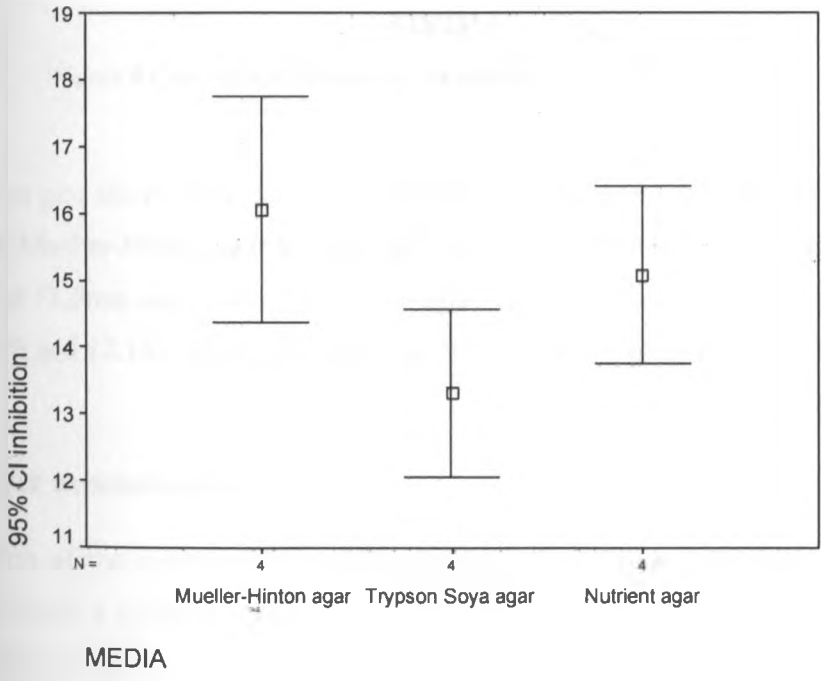

Figure 7 A comparison between media on susceptibility testing

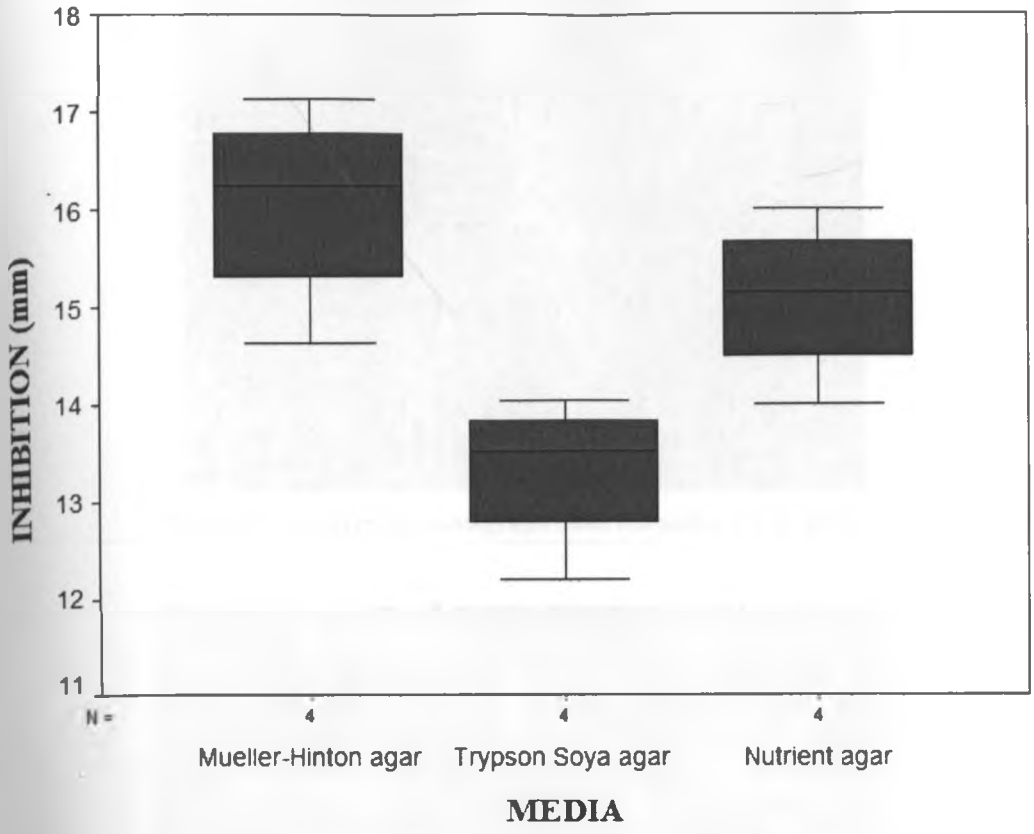

Figure 8 Comparison between media effects on susceptibility tests

From the box plot above, the mean and confidence interval shows that 50% of the inhibition in Mueller-Hinton agar was above 15mm compared to trypson soy agar whose median lay at 13.5mm and 15mm for nutrient agar. The deviation from Mueller-Hinton agar is 6.12% and 17.13% for nutrient agar and trypson agar respectively.

3.5 Thin layer chromatography (TLC)

A comparison of the crude extracts of the three species, *P.niruri, P.odontadenius* and *P.amarus* chemical profile on TLC using CH_2CL_2 Hexane, CH_2CL_2 Acetone and CH_2CL_2 Methanol, as solvent systems, showed similarities in compounds polarity.

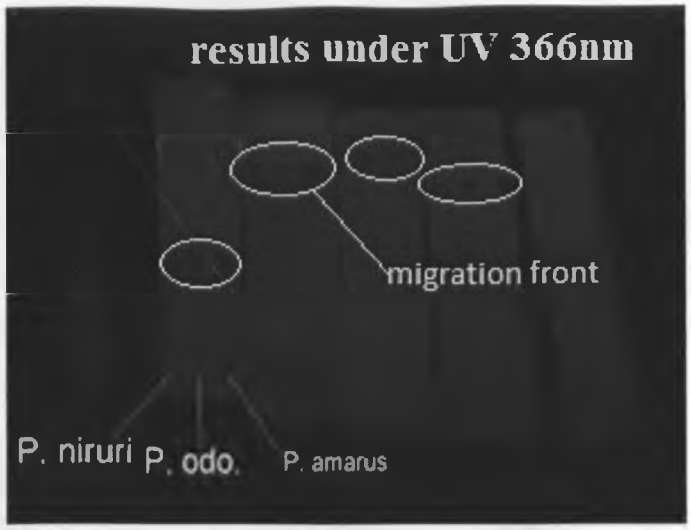

Plate 12 Thin layer chromatograph observed under UV of 366 nm

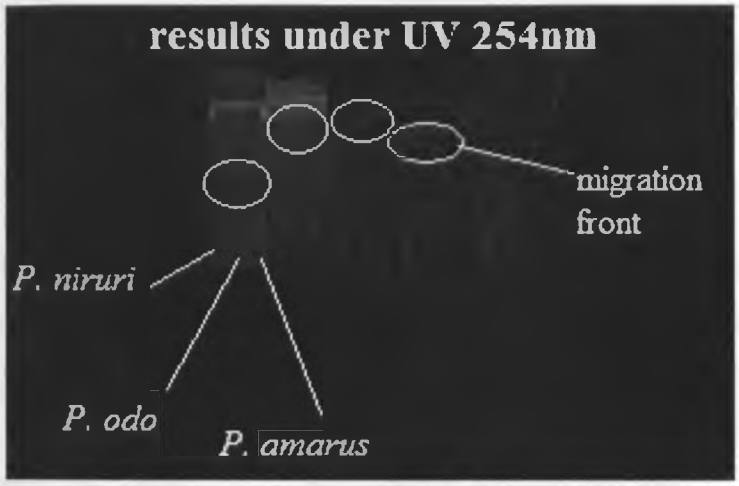

Plate 13 UV visualization of TLC under UV of 254 nm

Plates 11 and 12 show similarity in compounds based on migration and Rf values for the three *Phyllanthus* species.

CHAPTER FOUR

4.0 DISCUSSION

In this study, the antimicrobial effects of crude aqueous, dichloromethane: methanol and methanolic extracts of three different *Phyllanthus* species, *P.odontadenius P.amarus, P.niruri,* on *Candida albicans, Bacillus pumilus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Klebsiella pneumonia, Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* were investigated.

The antimicrobial properties of the *Phyllanthus* extracts were determined by antimicrobial susceptibility test using Kirby- Bauer disc diffusion and well methods. Disc diffusion methods are used extensively to investigate the antibacterial activity of natural substances and plant extracts (Bartner *et al.*, 1994). These assays are based on the use of discs as reservoirs containing solutions of the substances to be examined. In case the activity is low, higher concentrated solutions are used. Because of the limited capacity of discs, holes or wells are preferably used (Bartner *et al.*, 1994).

The three plants had at least one extract active against the test microbial organisms. The methanol extracts were the most active exhibiting high antimicrobial activity followed by dichloromethane: methanol extracts and aqueous extracts in that order as shown in Figure 5. Cold water was the least active solvent while all the extracts were less active against *Candida albicans.* All extracts of *P. odontadenius* also showed activity against all the tested bacteria as compared to *P.amarus* where cold water extract did not show any activity.

The antimicrobial activity of the *Phyllanthus* species extracts with different solvent systems showed that at a concentration of above 50mg/µl the activity of the extracts was standard on all the test organisms with *P. odontadenius* extracts showing the greatest activity as shown in Table 3. Figure 3 shows the antimicrobial activity of methanol extracts at different concentrations which showed a broad spectrum of activity against all the microorganisms employed. Susceptibility of the test microbes to the extracts showed high activity with *P. odontadenius* and *P. amarus.*

At a concentration of 100mg/µl *P. niruri* matched activity of *P. amarus* at 50mg/µl concentration. This means that *P. odontadenius* and *P. amarus* contain high concentrations of phytochemicals responsible for antimicrobial activity than *P. niruri.*

However, the present results revealed that the methanolic extracts were more effective than the aqueous extract in inhibiting the growth of the test microbes (Parekh and Chanda, 2007). It is possible that the active chemical constituents were not soluble in methanol, dichloromethane: methanol or water. The drying process may have caused changes to occur in some of the chemical constituents found in these plants (Parekh and Chanda, 2007). Thus future research should centre on the effects of different solvents and drying methods on the efficacy of the plant extracts as microbial agents.

Plates 11, 12, 13 and 14 show the standard and negative results of the susceptibility tests. The tested plant extracts were most active against gram-standard microorganisms than gram-negative microorganisms. This is in agreement with previous reports by the several authors (Buwa and Staden, 2006; Perumalsamy, 1999 and Valsaraj *et al.*, 1997).

The aqueous, methanol, and methanol: dichlomethane (1:1) extracts of *Phyllanthus* species demonstrated varying levels of antimicrobial activity against the test organisms. The methanol extracts of all plants showed the highest inhibitory activity with effects highest in *Bacillus pumillus* and at 2mg/ml there was no activity in *Candida albicans* while it showed the least activity in *Escherichia coli.* In general, among the tested microbial strains, bacteria were found to be more sensitive to many of the test agents than fungi.

The higher activity of the methanol extracts may be due to higher solubility of the active compounds in these solvents. The polarity of methanol was better able to extract the active antibacterial compounds in the plant which exhibited higher activity with higher zones of inhibition. The aqueous extracts had little activity against the test organisms. Cold water extract showed least activity with minimal activity in *E. coli.*

This is supported by Fawole *et al.*, (2008), Parekh and Chanda, (2007) and Boer *et al.*, (2005). They found out that water extracts showed no / poor antimicrobial activity than those made using organic solvents. Among reasons reported could be that some active substances were present in water extracts but at concentrations at which bioactivity was no longer detectable.

Another reason could be that the active substances were soluble in organic solvents and basically not present in water extracts. This finding is also in agreement with that of Clarkson *et al.*, (2004) who reported that the inactivity of water extracts may have been because extract were not prepared according to the traditional methods, which in some cases involved mixed with other plants/herbs and boiling for several hours.

It is therefore worth noting that the traditional practitioners use water because that's all they have at their disposal, and success may be due to administration of the concoctions/decoctions in large quantities i.e (or in combination with other herbs) in a basin, cups, water glasses and in all or most cases, the treatment involves using the extracts for a long period of time (Yineger *et al.*, 2008; Lukelal *et al.*, 2008; Erasto *et al.*, 2005). The aqueous extracts may possibly be active against bacterial strains which were not tested in the current study as reported by Shale *et al.* (1999).

Concentration of 0.32mg/ml of Gentamycin and 0.30mg/ml of Nystatin were used as standard controls for bacteria and fungi respectively. Gentamycin, a standard antibiotic, had a better antibacterial activity than methanol extract of *Phyllanthus* species. The solvents in comparison to antibiotics showed 80% activity for methanol, 48% for CH_2CL_2: MeOH (1:1), 43% in hot water and 28% for cold water. The antibacterial activity of various plant species has been compared to that of standard antibiotics (Parekh and Chanda, 2008).

The antimicrobial activity of the extracts of *Phyllanthus* species could be to the presence of lignans; phyllanthin and hypophylanthin, flavonoids, triterpenoids, glycosides and tannins, present in the plant extract (Rajesh *et al.*, 2002). Phytochemical constituents like flavonoids (Tsuchiya *et al.*, 1996) and triterpenoids (Scortichini and Rossi, 1991) are known to prevent gastric ulcer due to the astringent and antimicrobial properties, which

appear to be responsible for gastro-protective activity. P-cymene, a monoterpenoid has been tested for antimicrobial properties using the disc diffusion method, in which it revealed a good antimicrobial activity (Medeiros *et al.*, 2003).

More importantly, there have been no side effects or toxicity reports for many years on this plant (Rajeshwar *et al.*, 2008). Although there has been extensive research on this plant, there is still a lot of scope for further research, especially towards the mechanism of biological activity of phytochemicals from *phyllanthus* species.

There was higher activity (large inhibition zones) in *B.pumilus* and *S.aureus* as compared to other bacteria and fungi. It is common observation that Gram-negative bacteria are more resistant to many compounds than Gram-standard ones (Gerald McDonnell and A. Denver Russell, 1999). This is generally ascribed to the morphological differences between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components of the thick layer. This makes the cell wall impermeable to lipophillic solutes with an exclusion limit of 600 Da (Nikaido and Varra, 1985). Particularly *E. coli* is incriminated in several infections for their insensitivity to antibiotics (Morse *et al.*, 1986).

The mechanisms by which microorganisms generally survive the action of antimicrobial agents are poorly understood and remain debatable. But one of the proposed mechanisms to the resistance is genetic factor for *E. coli (*Wolfrey and Enright, 1990). It was also observed that, after exposure to a given antibiotic agent, *E. coli* was found to decline in number for the first two hour then rapidly increase almost at the same rate as the control.

Hence, this is in congruency with the observation in this experiment that almost all of the extracts have shown lesser activity against *E. coli* than that of *S. aureus*. The Gram-standard bacteria should be more susceptible having an outer thin peptidoglycan layer which is not an effective permeability barrier.

The small inhibition zones (10 mm) of the extract in almost all the strains tested could be associated with diffusion problem of the active constituent(s). If the active constituents were macromolecules, there could be diffusion problem on the agar media as these

molecules move slowly on such a matrix system. Even though it was prepared at the same concentration as those of other extracts, it has thicker consistency which might be responsible for the reduced-size of the coloured zone that appeared on the dish containing 15% agar.

Moreover, the plant materials were extracted without prior deflating and hence, the high amount of fatty material and pigment might also inhibit the process of diffusion of the active principle(s). In comparison, the extracts of *Phyllanthus niruri, P. amarus and P. odontadeneus* did not show much difference in activity though *P. amarus* showed slightly higher inhibitory activity. The results of antimicrobial activity also support the claims by the traditional medicine practitioners to treat microbial diseases.

The outcome of susceptibility testing is known to be influenced by several factors, some of which include the medium used for bacterial culture, type of drug tested, and the type of organism (Bauer, 1996, Baron, 1994). These factors were also observed to influence susceptibility testing results in this study.

The standard medium for the Kirby Bauer method of susceptibility testing is Mueller-Hinton agar. In Kenya, because nutrient and tryptone soy agars are sometimes used as substitutes for Mueller-Hinton, this prompted the evaluation of the quantitative effect of the two media on the quality of susceptibility testing. Previous studies on the subject have compared susceptibility testing on Mueller-Hinton agar with other standard susceptibility testing media such as Oxoid sensitivity test medium and Iso-Sensitest agar. Because nutrient and tryptone soy agars are general purpose media rather than standard susceptibility testing media, there is hardly any data comparing these media with Mueller-Hinton in susceptibility testing. In this study, using nutrient and tryptone soy agars in susceptibility testing introduced a deviation from the correct results in 6.12% and 17.13% cases respectively.

The high discrepancy of susceptibility results observed between Mueller-Hinton agar and each of nutrient and tryptone soy agars for majority of the drugs tested raises doubts about the reliability of the nutrient and tryptone soy agars for microbial susceptibility testing. While similarity of susceptibility results have been reported between Mueller-

Hinton agar and certain media including Oxoid sensitivity test medium and Iso-Sensitest agar, high discrepancy have been reported for other media such as Wilkins-Chalgren agar (Carol, 1972; Traub, 1998).

The suitability of culture media for susceptibility testing is often associated with the composition which could affect growth of the test organism or drug activity in various ways (Baron, 1994). For media of poor suitability such as nutrient and tryptone soy agars, there is usually the presence of antagonistic substances or unsuitable pH that inhibits drug activity (Baron, 1994). In comparison between the media used it was noticed that it influenced the results of inhibition but formed similar distributions.

The findings of the study discourage the use of nutrient and tryptone soy agars in the Kirby Bauer method as practiced by some laboratories in Kenya, due to the considerable error margin these media may introduce into microbial susceptibility results.

Traditionally, thin layer chromatography (TLC) has been widely used for chemical analysis of medicinal plants and it is included as a method for identification in monographs of herbal drugs in most Pharmacopoeias throughout the world. For example, in the European Pharmacopoeia, TLC is mentioned as a primary tool for identification as part of monographs on all medicinal plants, most extracts and synthetic drugs. A Comparison of the three species, *P. niruri, P. odontadenius and P. amarus* on TLC using CH_2CL_2: Hexane, CH_2Cl_2 Acetone and CH_2Cl_2 Methanol, showed similarities in compounds polarity (Plates 15 and 16). This showed similarity in crude extracts run on the plates.

Data are displayed as chromatograms with respect to spots observed visually under UV at both at 254 and 366 nm. These spots could either be made of pure compound or mixtures of similar compounds such as lignans (phyllanthin and hypophyl-anthin), flavonoids, triterpenoids, glycosides and tannins, which have been shown to be present in the extracts of *P. niruri* (Rajesh *et al.*, 2002).

But extracts from natural products are commonly mixtures of large number of components. Therefore, the bands were most likely from mixtures of relatively similar

components. These bands together with the photographs of the successive fractions are assumed to characterize the plant material better and hence, can serve as a starting point for complete quantitative standardization.

The compounds found in the three species of *Phyllanthus* are similar as is indicated in the TLC plates. The R_f values of 0.55 and 0.63 respectively compare with those of lignans; phyllanthin and hypophyllanthin compounds as indicated by Kamlesh, *et al* 2006. These two compounds have been indicated for hepatoprotective activity (Sayyada, et al, 2006).

The potential for developing antimicrobials from higher plants appears rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials (Iwu *et al.*, 1999). Continued further exploration of plant- derived antimicrobials is needed today.

Further research is necessary to determine the identity of the antimicrobial compounds from within these plants and also to determine their full spectrum of efficacy. However, the present study of in vitro antimicrobial evaluation of these three plants forms a primary platform for further phytochemical and pharmacological studies.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

The results of the antimicrobial screening show that all extracts exhibited appreciable antibacterial properties inhibiting growth of all bacteria. Thin layer chromatography results also indicate that the three plants studied may contain similar types of phytochemicals such as lignans (phyllanthine and hypophyllanthine), alkaloids, terpenoids, glycosides, tannins and flavonoids known for their therapeutic effects.

This study therefore has provided a basis to the folkloric use of this plant as a remedy for urinary tract infection, skin disease and other infections caused by the pathogens studied as practiced ethnomedically all over the world. It also justifies the folklore medicinal uses and claims about the therapeutic values of this plant as curative agent. Therefore, further purification and characterization of the phytochemicals should be done to determine their efficacy as antimicrobial agents.

This would be important in view to obtaining useful chemotherapeutic agent. Compounds with high activity should then be subjected to animal and human studies to determine their effectiveness in whole-organism systems, in particular toxicity studies as well as an examination of their effects on beneficial normal microbiota. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated.

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APPENDIX

Post Hoc Tests

Multiple Comparisons

Dependent Variable: inhibition

	(I) MEDIA	(J) MEDIA	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Mueller-Hinton agar	Trypson Soya agar	2.7500(*)	.63667	.005	.9724	4.5276
		Nutrient agar	.9825	.63667	.317	-.7951	2.7601
	Trypson Soya agar	Mueller-Hinton agar	-2.7500(*)	.63667	.005	-4.5276	-.9724
		Nutrient agar	-1.7675	.63667	.051	-3.5451	.0101
	Nutrient agar	Mueller-Hinton agar	-.9825	.63667	.317	-2.7601	.7951
		Trypson Soya agar	1.7675	.63667	.051	-.0101	3.5451
LSD	Mueller-Hinton agar	Trypson Soya agar	2.7500(*)	.63667	.002	1.3098	4.1902
		Nutrient agar	.9825	.63667	.157	-.4577	2.4227
	Trypson Soya agar	Mueller-Hinton agar	-2.7500(*)	.63667	.002	-4.1902	-1.3098
		Nutrient agar	-1.7675(*)	.63667	.022	-3.2077	-.3273
	Nutrient agar	Mueller-Hinton agar	-.9825	.63667	.157	-2.4227	.4577
		Trypson Soya agar	1.7675(*)	.63667	.022	.3273	3.2077
Bonferroni	Mueller-Hinton agar	Trypson Soya agar	2.7500(*)	.63667	.006	.8824	4.6176
		Nutrient agar	.9825	.63667	.472	-.8851	2.8501
	Trypson Soya agar	Mueller-Hinton agar	-2.7500(*)	.63667	.006	-4.6176	-.8824
		Nutrient agar	-1.7675	.63667	.065	-3.6351	.1001
	Nutrient agar	Mueller-Hinton agar	-.9825	.63667	.472	-2.8501	.8851
		Trypson Soya agar	1.7675	.63667	.065	-.1001	3.6351
Sidak	Mueller-Hinton agar	Trypson Soya agar	2.7500(*)	.63667	.006	.8891	4.6109
		Nutrient agar	.9825	.63667	.401	-.8784	2.8434
	Trypson Soya agar	Mueller-Hinton agar	-2.7500(*)	.63667	.006	-4.6109	-.8891
		Nutrient agar	-1.7675	.63667	.063	-3.6284	.0934
	Nutrient agar	Mueller-Hinton agar	-.9825	.63667	.401	-2.8434	.8784
		Trypson Soya agar	1.7675	.63667	.063	-.0934	3.6284

* The mean difference is significant at the .05 level.