KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL

DEPARTMENT OF PHARMACOGNOSY

PHARMACOGNOSTIC STUDIES AND ANTI-INFECTIVE PROPERTIES OF *HILLERIA LATIFOLIA* (LAM.) H. WALT. (PHYTOLACCACEAE)

By

ANTONIA OTOO

SEPTEMBER, 2014

PHARMACOGNOSTIC STUDIES AND ANTI-INFECTIVE PROPERTIES OF *HILLERIA LATIFOLIA* (LAM.) H. WALT. (PHYTOLACCACEAE)

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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In the

Department of Pharmacognosy,

Faculty of Pharmacy and Pharmaceutical Sciences

By

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KWAME NKRUMAH UNIVERSITY OF SCIENCE & TECHNOLOGY,

KUMASI

SEPTEMBER, 2014

DECLARATION

I hereby declare that this experimental work described in this thesis is my own work towards the award of an MPhil and that to the best of my knowledge, it contains no material previously published by another person for the award of any degree of the University, except where due acknowledgment has been made in the text.

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Antonia Adwoa Otoo Date

Certified by

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Prof. A.Y. Mensah Date

(Supervisor)

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Prof. M.L.K Mensah Date

(Supervisor)

…… ……..……

Prof. A.Y. Mensah Date

(Head of Department)

DEDICATION

This thesis is dedicated to my late mother Mrs. Agatha Otoo. Your years spent with me were very memorable and will be forever cherished.

ACKNOWLEDGEMENT

All glory and honour to my Lord and Master Jesus Christ who has brought me this far. Dear Lord I am forever grateful for the successful completion of this study.

I would also like to express gratitude to Prof. A.Y. Mensah for his support and direction throughout my study. God richly bless you. Special thanks also go to Prof. M.L.K Mensah for his direction and great sense of correction. This work wouldn't be possible without you. Thank you.

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ABSTRACT

The study sought to investigate the Pharmacognostic properties and anti-infective activities of *Hilleria latifolia* (Lam.) H. Walt. which has immense medicinal use in the African sub-region, especially Ghana. The Pharmacognostic properties of the plant as well as the antimicrobial and antiplasmodial activities were assessed. Pharmacognostic studies carried out on the plant revealed the presence of abundant calcium oxalate crystals in all plant parts. Tetragonal crystals were found beneath the epidermal cells and were not embedded in the palisade cells. Different types of prismatic crystals and rosette crystals were found in the palisade cells of the leaf. The leaves had anomocytic stomatal arrangement and possessed abundant prismatic crystals (3.9-110.5 µm in length) and multicellular clothing trichomes (94.7-347.2 µm in length). Vein islet and veinlet termination numbers averaged 18 and 32 respectively. The physical parameters revealed the highest extractive value for alcohol in all plant parts; leaf (24.048 ± 0.208 $\%^{w}/_{w}$), stem (10.012 ± 1.652 $\%^{w}/_{w}$) and root (12.036 ± 0.564 $\%^{w}/_{w}$). The leaf gave the highest values for total ash (16.667 ± 0.286 $\%^{w}/_{w}$) and water soluble ash (16.028 ± 0.434 $\%^{w}/_{w}$). Phytochemical screening revealed the presence of alkaloids, triterpenoids, tannins and glycosides in all parts of the plant. Flavonoids, phytosterols and saponins were present only in the roots and anthraquinones were absent in all parts of the plant. Pharmacopoeial standards for the plant have also been established through the pharmacognostic studies. The methanolic, ethyl acetate and petroleum ether extracts of the leaves, stem and roots were tested against six micro-organisms; *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *C. albicans* using the agar dilution method. The ethyl acetate extract of the leaves was the most active, inhibiting *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* at MIC's between 250 µg/ml and 500 µg/ml; the positive controls used were Ciprofloxacin

and Ketoconazole for bacteria and fungus respectively. The methanol extract of the whole plant, tested against *Mycobacterium smegmatis*, recorded an MIC above 500 µg/ml with the positive control Rifampicin recording an MIC of 0.01 µg/ml. The antiplasmodial activities of the extracts were investigated in multidrug resistant *P. falciparum* (K1) parasites using the Parasite Lactate Dehydrogenase Assay. The petroleum ether extract of the leaf, gave the highest antiplasmodial activity with IC_{50} value of 28.18 µg/ml. The petroleum and ethyl acetate root extracts showed moderate antiplasmodial activities with IC_{50} values of 100 µg/ml each. Similarly, that of the stem was 89.25 µg/ml. The positive controls used, Artemether and Chloroquine, gave IC_{50} values of 19.3 ng/ml and 412.01 ng/ml respectively. The present work has demonstrated that *Hilleria latifolia* possesses some level of anti-infective activity as suggested by folklore medicine, thus giving scientific credence to its use.

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ABBREVIATIONS

ADB	Albumin Dextrose Catalase
APAD	3-acetylpyridine adenine dinucleotide
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLF	Centre for a Livable Future
CNS	Central Nervous System
DAPI	4′, 6-diamidino-2- phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
IC_{50}	Half maximal inhibitory concentration
MB	Middlebrook
MIC	Minimum Inhibitory Concentration
MTT	Diphenyl tetrazolium bromide
PABA	Para amino benzoic acid
TLC	Thin Layer Chromatography
UV	Ultraviolet
WHO	World Health Organisation
µg/ml	microgram per millilitre
p.o	per os (by mouth)

DETAILS OF PUBLICATIONS

Published papers

Amponsah, I. K., Mensah, A. Y., Otoo, A., Mensah, M. L. K., Jonathan, J. (2014). Pharmacognostic studies on *Hilleria latifolia* (Lam.) H. Walt., *Asian Pacific Journal of Tropical Biomedicine*, 4(1):930-935

Published abstracts

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Poster presentations

Otoo, A. A., Mensah, A.Y., Mensah, M. L. K., Gbedema, S. (2013). Investigation of the antimicrobial and antiplasmodial activities of *Hilleria latifolia* (Lam.) H. Walt. Presentation made at the College of Health Sciences 5th Annual Scientific Conference, KNUST from 14th to 15th November, 2013.

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Plants have since prehistoric times played a major role in the lives of man; as medicines, foods and many more. Ancient records which date back to 60,000 years ago give evidence of the use of plants such as *Alcae rosea* (Malvaceae) commonly known as hollyhock by the Neanderthals in the treatment and management of diseases. (Cowan, 1999). Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still used as part of the habitual treatment of various maladies. Plants such as bearberries, cranberries, garlic and tea tree among others have been reported in various ancient manuals of phytotherapy as potent drugs in the treatment of urinary tract infections (Heinrich *et al.*, 2004).Over the years, many conventional medicines have been obtained from plants and have achieved great success in the treatment of diseases. Artemisinin derivatives, which are very effective antimalarials, were derived from *Artemisia annua* of the family Asteraceae (Griffith *et al.*, 2007). Quinine used for the treatment of severe malaria as well as some heart diseases was obtained from the bark of *Cinchona succuribra* (Rubiaceae). Colchicine for the treatment of gout came from *Colchicum autumnale* of the family Colchicaceae. Many other compounds such as taxol from *Taxus brevifolia* of the family Taxaceae have been useful in treating various forms of cancer including ovarian, breast, lung, prostate, oesophageal as well as Kaposi's sarcoma (Saville, *et al.*, 1995; Hoffman 2003). Vinblastine and Vincristine from *Catharanthus roseus* of the family Apocynaceae have been used as anticancer agents (Hoffman 2003; Mitchell and Ahmad, 2006). Morphine,

an alkaloid obtained from *Papaver somniferum* (Papaveraceae), is a very potent narcotic analgesic used in the management of intense pain caused by cancer and surgery (Rossi *et al*., 2005).

Although conventional medicines have been useful in the treatment of diseases, factors such as drug resistance by microbes, undesirable side effects and adverse drug reactions have caused a great resurgence in the use of herbal medicines. This has in turn led to the commercialization of herbal medicines as they are considered to be safe and have no or relatively few side effects. The World Health Organisation (WHO) estimates that about 60% of the world's population use herbal medicine for treating their sicknesses and up to 80% of the population living in the African sub-region depends on traditional medicine for some aspects of primary health care (WHO, 2002), owing to their availability, affordability and safety among other reasons (Prakash *et al*., 2013).

In Ghana, the integration of herbal medicine practice into mainstream health care by the Ghana Health Service brings to bear the contribution of herbal medicine to the health of the population. The increased interest in the use of herbal medicines however has not been without challenges. In the past decades, some useful medicinal plants such as *Cosmos atrosanguineus* (Asteraceae) have become extinct (Lewis and Elvin-Lewis, 1995) and this has led to substitution and in many cases adulteration of herbal drugs with substandard or inferior drugs. In many cases, the adulterants have been spurious drugs, superficially similar but inferior drugs, substandard commercial varieties etc. Similarities in the names in traditional systems of medicine, where the vernacular names of plants are used interchangeably have often led to the unintentional substitution and adulteration of drugs (Prakash *et al*., 2013). This burning problem in the herbal industry has thus necessitated the need for standardisation of medicinal plants.

1.2 JUSTICATION

Infections and infectious diseases have been a major cause of death worldwide for years, and although various conventional medicines have been used successfully in the treatment and management of these diseases, they continue to be major killers in low-resource settings and in the developing world (WHO, 2011). The WHO reported that people predominantly die of infectious diseases such as lower respiratory tract infections, AIDS, diarrhoeal related diseases, malaria and tuberculosis and these collectively account for almost one third of all deaths in low income countries (WHO, 2011). Apart from the death toll from infectious diseases, the economies of countries are affected as they affect the productivity of the nation.

Various anti-infective agents have been useful in treatment and management of infectious diseases. However, there have been some setbacks; the emergence of resistant strains of common pathogens has been a bane in the treatment. An example is the emergence of multidrug-resistant bacteria like Methicillin resistant *Staphylococcus aureus* and *Mycobacterium tuberculosis.* This has created a situation in which there are few or no treatment options for infections with certain microorganisms (Gupta and Bhakta, 2012; Aires de Sousa and de Lencastre, 2004). There has also been the rapid emergence of new infections, and the resurgence of several infections that appeared to have been controlled leading to the subsequent failure of the available antimicrobial agents to treat these infections and infectious diseases (Morse, 1995)**.** Another setback is the unwanted side effects and adverse drug reactions such as pruritis, anaphylaxis, Stevens- Johnson syndrome where the surfaces of the eye, lips and mouth may be eroded, toxic epidermal necrolysis and many others have been associated with the use of conventional medicines particularly antibiotics (Rang, 2005)

One disease of public health importance especially in sub - Saharan Africa is malaria. It is caused by the *Plasmodium* parasite. The World Health Organization estimated that in 2010, there were 219 million documented cases of malaria. That year, the disease killed between 660,000 and 1.2 million people many of whom were children in Africa (Nayyar *et al*., 2012; WHO, 2012). The disease has a heavy burden in some countries, where it may be responsible for 30 to 50 percent of hospital admissions, up to 50 percent of outpatient visits, and up to 40 percent of public health spending (WHO, 2003). The discovery of quinine and the artemisinin in the 17th and 20th centuries respectively (Jacoby and Youngson, 2004) have been extremely helpful in the treatment of malaria. However, some synthetic antimalarials such as chloroquinine, a derivative of quinine become ineffective over the years. The devastating impact of resistance to existing antimalarials such as and other antimicrobial agents calls for research to develop new anti-infectives (Kaur *et al*., 2009; Mutabingwa *et al*., 2001). These reasons have thus created the necessity for studies directed towards the development of new anti-infective agents to combat diseases. Natural products have provided lead compounds in the past and remain a good source for compounds of medicinal importance (Olga, 2014; Verpoorte, 2002).

The present study aims to investigate the anti- microbial and antiplasmodial potentials of *Hilleria latifolia*, a plant indigenous to most sub-Saharan African countries including Ghana. Various parts of the plant have been used traditionally over the years for the treatment of diseases. The leaves or roots in the form of poultices are applied to boils or applied topically to painful areas and to treat persistent headache. A leaf decoction is taken to treat bloody cough. In Nigeria, the leaves are eaten in soup to treat gonorrhoea and in Congo, the leaves are used to treat gynaecological disorders in which purging is

considered necessary (Dokosi, 1998; Iwu, 1993). Its wide use in the treatment and management of infections and infectious diseases serve as a reasonable basis for the investigation of possible anti-infective properties of *Hilleria latifolia* (Lam.) H. Walt. .The pharmacognostic properties of the plant will also be investigated to aid in proper identification of the plant because of its numerous ethnopharmacological uses.

1.3 AIM AND OBJECTIVES

1.3.1 Aim

- To investigate the Pharmacognostic and anti-infective properties of *Hilleria latifolia* (Lam.) H. Walt. of the family Phytolaccaceae.

1.3.2 Objectives

The objectives of this research are to:

- Carry out pharmacognostic studies on the plant; including quantitative microscopy, physicochemical properties, thin layer chromatography, phytochemical screening and fluorescence analysis.
- Determine the antimicrobial activity of the whole plant, leaf, stem and root of *Hilleria latifolia* against some selected micro-organisms using the agar dilution and the Spot Culture Growth Inhibition Assays.
- Investigate the antiplasmodial potential of extracts of the leaf, stem and root of the plants against multi drug resistant *Plasmodium falciparum* parasites using the Parasite Lactate Dehydrogenase Assay.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANTIMICROBIAL AGENTS

Since their discovery decades ago, antimicrobial agents have substantially reduced the threat posed by infectious diseases. Over the years, antimicrobials have saved the lives and eased the suffering of millions of people, especially in Africa where the menace of endemic diseases is greatly felt (WHO, 2002).

Resistance to antimicrobial agents is recognized at present as a major global public health problem. Infectious diseases account for approximately one-half of all deaths in countries in tropical regions. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics, drug resistant microorganisms and the emergence of unknown disease-causing microbes, pose enormous public health concerns (Iwu, 1991).

2.2 CONVENTIONAL ANTI-INFECTIVES

Antibiotics are used to treat and prevent infections and to promote growth in animals. Their mechanism of action is to either kill (bactericidal) or inhibit the growth (bacteriostatic) of pathogen .These actions may be based on the drug concentration, the phase of growth of the pathogen and the species (Puttaswamy *et al*., 2012).

2.2.1 Mechanisms of Antimicrobial Agents

The ideal antimicrobial agent is selective, that is, it targets the microorganism but not the mammalian cells. The mechanism of action of different antimicrobial agents is ultimately

aimed at inhibition of cell wall synthesis, inhibition of protein synthesis, interference with the conversion of para-aminobenzoic acid (PABA) to folate , inhibition of nucleic acid synthesis and inhibition of DNA gyrase (Nguyen and Thompson, 2006). The table 2.1 below summarises the common antibacterial agents and their mechanisms of action.

Table 2.1 Classes of antibiotics and their mechanisms of action

Class of drug	Example	Mechanism of action
Aminoglycosides	Gentamycin	Inhibit protein synthesis
Cephalosporins	Cephradine	Inhibit cell wall synthesis
Chloramphenicol	Chloramphenicol	Inhibit protein synthesis
Macrolides	Erythromycin	Inhibit protein synthesis
Monobactams	Aztreonam	Inhibit cell wall synthesis
Nitroimidazoles	Metronidazole	Inhibit nucleic acid synthesis
Penicillins	Benzylpenicillin	Inhibit cell wall synthesis
Quinolones	Ciprofloxacin	Inhibit DNA gyrase
Sulphonamides	Sulphamethaxazole	Compete with PABA
Tetracyclines	Oxytetracycline	Inhibit protein synthesis

2.2.2 Development of Resistance

A drug is said to be ineffective when a pathogen or test organism is not inhibited by the usually achievable concentrations of the drug or anti-infective agent with normal dosage schedules and/or demonstrates minimal inhibitory concentrations that fall in the range in which specific microbial resistance mechanisms (eg, β lactamases) are likely and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies

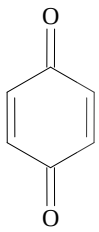
(Franklin *et al*., 2012). Antimicrobial resistance and drug resistance as a whole is an increasingly serious threat to global public health that requires action across all government sectors and society (WHO, 2014). In 1998, the Institute of Medicine estimated that antimicrobial resistance costs the United States between $ 4 billion and $ 5 billion annually. These costs are higher now as the problem of resistance has grown and intensified worldwide (CLF, 2009).

2.3 ANTIMICROBIAL AGENTS FROM PLANTS

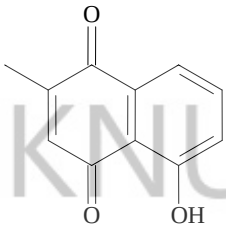
Plants have a great ability to synthesize secondary metabolites (Cowan, 1999). At least 12,000 of these secondary metabolites have been isolated, a number estimated to be less than 10% of the total (Mallikharjuna *et al*., 2007). In many cases, these substances are thought to serve as plant defense mechanism against predation by microorganisms, insects and herbivores. Useful antimicrobial phytochemicals can be grouped into such categories as phenolics and polyphenols, terpenoids and essential oils, alkaloids, lectins and polypeptides.

Phenols and phenolic acids, as exemplified by cinnamic and caffeic acids, are common representatives of phenyl-propane derived compounds which have been found to be active against viruses , bacteria and fungi. Quinones, e.g. 1,4-benzoquinone **[1]** are very common in nature and are characteristically very reactive, as well as naphthoquinones such as plumbagin **[2]** and anthraquinones [**3**]. In addition to providing free radicals, quinones and naphthoquinones are known to form a reversible complex with nucleophilic amino acids in proteins often leading to inactivation of protein and loss of function; hence the range of quinone antimicrobials is huge. Probable targets in the microbial cell are the surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes

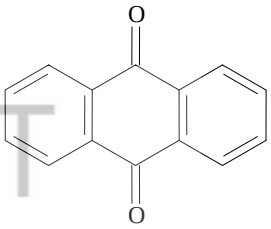
(Cowan, 1999). Methyljuglone [**4**] and diospyrin [**5**], which are naphthoquinone derivatives and were isolated from *Euclea natelensis*, have been found by Adeniyi *et al*. (2000) to be particularly effective against *Mycobacterium tuberculosis*. The two naphthoquinone compounds were also found to inhibit several antibiotic resistant as well as antibiotic susceptible strains of *M. tuberculosis*.



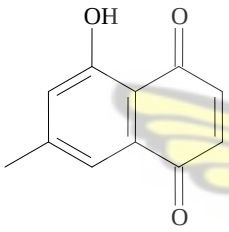
1,4-benzoquinone [1]



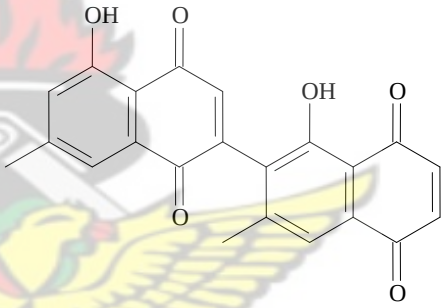
Plumbagin [2]



Anthraquinone [3]

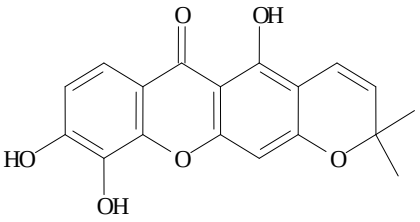


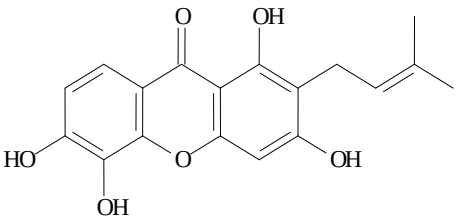
Methyljuglone [4]



Diospyrin [5]

Flavonoids are phenolic compounds (Fessenden and Fessenden, 1982). They have been found *in vitro* to be effective antimicrobial substances against a wide range of micro organisms. Their activity is thought to be due to their ability to form a complex with extracellular and soluble proteins and to complex with bacterial cell wall (Cowan, 1999). Coumarins are also phenolic substances several of which have been found to have antimicrobial properties (Cowan, 1999). Xanthones, jacareubin [**6]** and 1, 3, 5, 6-tetrahydroxy-2-(3, 3-dimethylallyl) xanthone [**7**], isolated from *Calophyllum basiliense* were also reported to be active against *Escherichia coli* by Yasunaka *et al*. (2005).



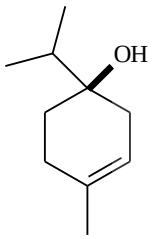


Jacareubin [6] **1, 3, 5, 6-tetrahydroxy-2-(3, 3-dimethylallyl) xanthone [7]**

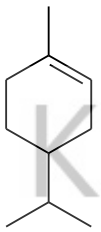
The antibacterial properties of essential oils have been extensively established against a variety of bacterial strains including *Clostridium sporogens*, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. The essential oils of *Piper nigrum*, *Thymus vulgaris* and *Ocimum gratissimum* have been shown to be active against both gram positive and gram negative bacteria (Dorman and Deans, 2002). Important constituents of essential oils that have been shown to have antibacterial action include terpinen-4-ol [**8**], terpinolene [**9**], menthone [**10**] and pinene-(-) [**11**]. A number of terpenoids with various degrees of antibacterial activities have been isolated from different plants. These include 6β-hydroxyisopimaric acid [**12**], ferruginol [**13**], 1-oxoferruginol [**14**], horminone [**15**] and 7-acetylhorminone [**16**] isolated from some *Salvia* species (Ulubelen, 2003).

Alkaloids have been found in many plants with demonstrable antibacterial actions. Paulo *et al*. (1994) demonstrated that cryptolepine [**17**] and cryptoheptine [**18**], isolated from the root of *Cryptolepis sanguinolenta* were active against several bacterial species including *E. coli*, *Staph. aureus*, *Vibrio cholerae* and *Shigella dysenteriae* with MIC ranging between 1.5-50 µg/ml. Two quinolinone alkaloids, waltherione-A [**19**] and O-methylwaltherione-A [**20**] were also isolated from *Waltheria dourahinha* (Sterculiaceae) by Hoelzel *et al*. (2005) and tested for antibacterial activity, but only O-

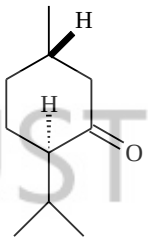
methylwaltherione-A had a moderate activity against both gram positive and gram negative bacteria. Baumgartner *et al.* (1990) assessed the effect of two indolizidine alkaloidal compounds, ficuseptine and antofine isolated from *Ficus septica* (Moraceae) on different strains of bacteria including *E. coli* and *B. subtilis* with a low MIC ranging between 1 and 10 µg/ml against *B. subtilis* and *E. coli* respectively.



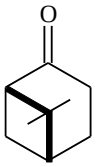
Terpinen-4-ol [8]



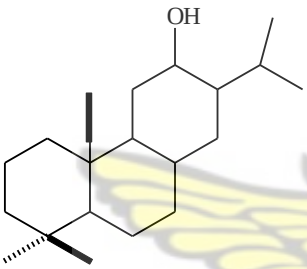
Terpinolene [9]



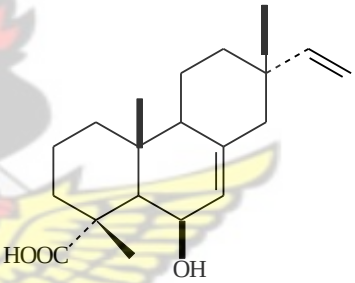
Methone [10]



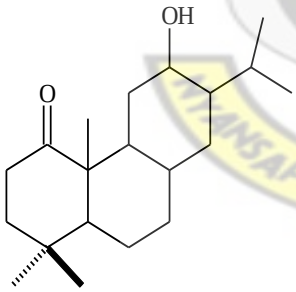
Pienene-(-)[11]



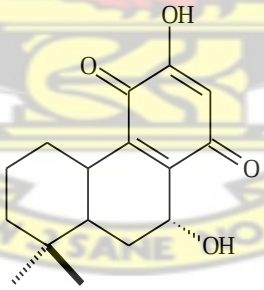
Ferruginol [13]



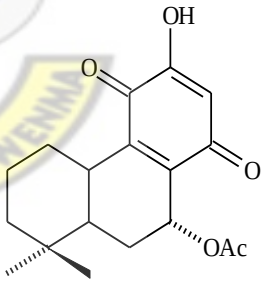
6-beta-hydroxyisopimaric acid [12]



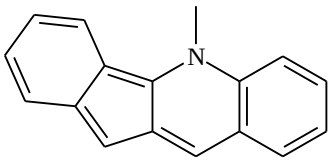
1-oxoferruginol [14]



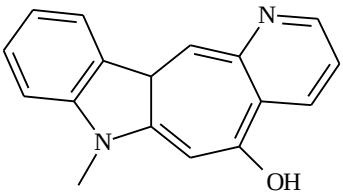
Horminone [15]



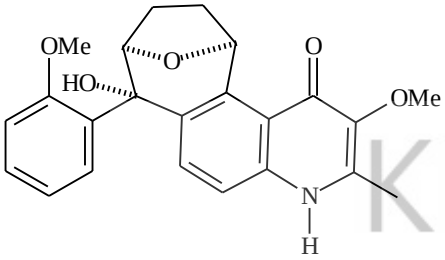
7-acetylhorminone [16]



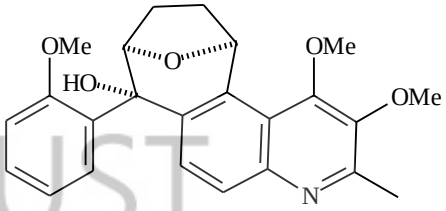
Cryptolepine [17]



Cryptoheptine [18]



Waltherione-A [19]



O-methylwaltherione-A [20]

2.4 ANTIMICROBIAL ASSAYS

Antimicrobial susceptibility tests have over the years been used to test and determine the efficacy of potential antimicrobials from biological extracts against a number of different microbial species. The tests have been very useful in screening plant extracts for antimicrobial activity and also useful in determining the minimum inhibitory concentration (the lowest concentration at which organisms or pathogens are inhibited) of the drug under study (Das *et al*., 2010)

Antimicrobial susceptibility tests are broadly classified into diffusion and dilution methods. The diffusion tests include agar disc diffusion, agar well diffusion and bioautography. The dilution tests include agar dilution, broth microdilution and broth macrodilution methods (Tenover *et al*., 1995).

2.4.1Diffusion Methods

2.4.1.1 Agar disc diffusion assay

In this assay, antibiotic impregnated discs are used to test the susceptibility of a particular microorganism, particularly bacteria (Mohanty *et al*., 2010). Discs are saturated with antimicrobial agents at desired concentration and placed on seeded agar and incubated at 37 ^{o}C for 24hours for bacteria. The discs may be impregnated with the drug after or before being placed on the inoculated plate (Lourens *et al*., 2004; Salie *et al*., 1996). Refrigeration of the plates containing the seeded agar and disc is sometimes done for about an hour to allow pre-diffusion of the drug from the disc into the agar before incubation (Schmourlo *et al*., 2004). The antibiotic diffuses radially outwards, becoming less concentrated as it does so. A clear zone of inhibition appears where growth has been inhibited. The larger this is, the more susceptible the organism. Zones of inhibition are then measured from the circumference of the disc to the circumference of the inhibition zone (Salie *et al*., 1996).

The agar disc diffusion assay is usually qualitative as the amount of extracts that adhere to the disc is not quantitatively determined (Dilika *et al*., 2000; Leite *et al*., 2006). This method is not suitable for quantification of the bioactivity of the extract. Another limitation of this method is its inability to distinguish between bactericidal and bacteriostatic effects (Parekh *et al*., 2006; Silva *et al*., 2005; Tepe *et al*., 2004).

2.4.1.2 Agar well diffusion assay

In this assay, a standardized concentration of inoculums with fixed volume is spread evenly on the surface of gelled agar plate. Wells which range between 6 mm- 8 mm in diameter are punched aseptically on the agar with the aid of a sterile cork borer. A fixed

volume of the test substance is then introduced into the bored medium and incubated at optimum temperature and duration depending on the test organism (Mbata *et al*., 2006; Norrel and Messely, 1997).

2.4.1.3 Bioautography

Bio-autography localizes antimicrobial activity on a chromatogram using three approaches: direct bio-autography; where the micro-organism grows directly on the thin-layer chromatography (TLC) plate, contact bio-autography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact and agar overlay bio-autography; where a seeded agar medium is applied directly onto the TLC plate (Hamburger and Cordell, 1987; Rahalison *et al*., 1991). Bioautography is also employed as a preliminary phytochemical screening technique, by bioassay guided fractionation, to detect active components (Nostro *et al*., 2000; Schmourlo *et al*., 2004) and overcomes the challenge of isolating antimicrobial compounds from crude extracts. Despite the high sensitivity of this method, its applicability is limited to micro-organisms that easily grow on TLC plates. Other limitations to this method are the need for complete removal of residual low volatile solvents, such as *n*-butanol, trifluoroacetic acid and ammonia and the transfer of the active compounds from the stationary phase into the agar layer by diffusion (Cos *et al*., 2006).

One advantage of this method is the fact that the assay allows localizing antimicrobial activities of an extract on the chromatogram and thus supports a quick search for new antimicrobial agents through bioassay-guided isolation (Cos *et al*., 2006).

2.4.2Dilution Methods

2.4.2.1 Agar dilution method

Agar dilution susceptibility testing is regarded as the golden standard for all other susceptibility testing methods (Hendrisksen, 2003). This assay involves the incorporation of different concentrations of the test substance into a nutrient agar medium followed by the application of a standardized number of cells to the surface of the agar plate (Wiegand *et al*., 2008). In this method, a stock solution of the extract is prepared in its extracting solvent, filter-sterilized (0.22 μm) and then incorporated in molten agar, cooled to 50 ºC in a water bath to obtain different concentrations of the extract in the agar (Silva *et al*., 2005). The bacterial inoculum is standardized according to McFarland standard and inoculated on the surface of agar and incubated at 37 ºC for 24 hours in bacteria. The lowest concentration of antibiotic that inhibits visible growth on surface of agar is taken as the MIC (Sridhar, 2006).

The agar dilution method has also been proven and accepted to be an equally good and optimal technique as compared to broth microdilution. However, the agar dilution method allows the determination of susceptibility and minimum inhibitory concentrations (MIC) whereas the broth microdilution method can only be used for the determination of MIC. The agar dilution method has been recommended as an alternative to broth microdilution (Barry *et al*., 2001; Fuchs *et al*., 2001; Kohner *et al*., 1994).

2.4.2.2 Broth microdilution method

The micro-titre plate or broth microdilution method has provided a potentially useful technique for determining MIC values of large numbers of test samples. Its advantages over diffusion techniques include increased sensitivity for small quantities of extract;

ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC (Langfield *et al*., 2004). This method can also be used for a wide variety of microorganisms. It is not expensive and it produces reproducible results. In the micro-titre plate method, a stock solution of the extract is first obtained in solvent, usually the solvent used for extraction (Grierson and Afolayan, 1999) or in DMSO (Salie *et al*., 1996; Nostro *et al*., 2000; Baris *et al*., 2006).

2.4.2.3 Broth macrodilution method

The basic principle of this assay is the same as the broth microdilution assay. But the test is performed in a test tube. In the macrodilution assay, a set of test tubes with different concentrations of plant extract with the same volume are prepared. Tubes are inoculated with test microorganisms of standard concentrations as discussed above. After incubation, tubes are examined for changes in turbidity as an indicator of growth. MIC of the plant extract or the test phytochemical can be determined using the above discussed methods (Das *et al*., 2010).

2.5 MALARIA

Malaria is a parasitic disease transmitted by mosquitoes. It is transmitted through the bite of an infected female *Anopheles* mosquito. The disease is caused by the *Plasmodium* parasite. There are many species of the *Plasmodium* parasite recorded, four of which are infectious to humans. These include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. The most common types are the *P. falciparum* and *P. vivax* species while the most deadly and prevalent in Africa is the *P. falciparum* species (Akande and Musa, 2005). The *P. falciparum* species is responsible

for about 75% of infections whereas the *P. vivax* is responsible for about 20% of malaria infection (Nadjm and Behrens, 2012). Another species, *P. knowlesi* has also been found to cause severe infections in humans although it also causes infections in monkeys (Cox-Singh *et al*., 2008).

2.5.1 Burden of Malaria

Malaria is a great cause of poverty and a major hindrance to the economic development of many countries (Gollin and Zimmermann, 2007; Worrall *et al*., 2005). The economic impact of the disease is estimated to cost Africa $12 billion every year which includes the costs of health care, days lost to education or school by students, working days lost to the disease, loss of investment and tourism (Greenwood *et al*., 2005).

2.5.2 Antiplasmodial Assays

The *Plasmodium* life cycle consists primarily of two phases; the sexual phase (sporogony) which occurs in the mosquito vector and the asexual phase (schizogony) which occurs in the host. Antimalarial assays are carried out primarily by two approaches: the *in vivo* approach and the *in vitro* approach (Nogueira and Estólio do Rosario, 2010).

In vitro screening of compounds for antimalarial activity is based on the ability to culture *P. falciparum in vitro* in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% haematocrit at 37°C under reduced oxygen (typically 3–5% O_2, 5% CO_2, 90–92% N_2) in tissue culture (RPMI 1640) media containing either human serum or Albumax (a lipid-rich bovine serum albumin) (Noedl *et al.*, 2003).

2.5.2.1 Isotopic assays

In this assay parasite growth was assessed by measuring the incorporation of ^{3}H-hypoxanthine, which is taken up by the parasite for purine salvage and DNA synthesis (Desjardins *et al.*, 1979). The parasites are cultured in the presence of different concentrations of the drug to be tested. The test drug is incorporated into media containing reduced concentrations of hypoxanthine. Radiolabelled ^{3}H-hypoxanthine is added for an additional incubation period before the cells are harvested and radioactive counts are measured. IC_{50} values can be determined by linear regression analyses on the linear segments of the dose–response curves (Fidock *et al.*, 2004).

A limitation of this method is the high cost of the necessary equipment, such as liquid scintillation counters and harvesting machines. The method is also quite complex. Ethanolamine is an alternative to radiolabeled hypoxanthine. Its major advantage is that the culture medium can be supplemented with hypoxanthine to improve parasite growth. Several other precursors such as palmitate, inositol, serine, choline, and isoleucine have also been suggested for use in isotopic assays (Nogueira and Estólio do Rosário, 2010).

2.5.2.2 WHO Schizont maturation assay

This assay is based on the maturation (growth) of *P. falciparum* in a 24 to 36 hours microculture. This assay is a low-cost alternative for testing small numbers of compounds. It involves the incubation of parasites with test compounds for 48 or 72 hours after which the parasitaemias of the treated and untreated parasites are compared. The numbers of parasites that mature into schizonts are counted in GIEMSA stained

thick films. This assay is laborious and requires experienced microscopists. However, it is economical (Fidock *et al.,* 2004; Nogueira and Estólio do Rosario, 2010).

2.5.2.3 Flow cytometry

Flow cytometry has also been used to test candidate antimalarial compounds, and takes advantage of the fact that human erythrocytes lack DNA. In the simplest use of this technology, parasites are fixed after the appropriate period of incubation with test compounds, then either the parasitized cells are stained with hydroethidine (which is metabolized to ethidium) or the parasite nuclei are stained with DAPI (4′,6-diamidino-2- phenylindole) (Heyde *et al.,* 1995). Counts of treated and control cultures are then obtained by flow cytometry.

2.5.2.4 Parasite lactate dehydrogenase assay

This is also a colorimetric assay method and 3-acetylpyridine adenine dinucleotide (APAD) is utilised as a substrate for malaria parasite lactate dehydrogenase.

The basis of the Parasite lactate dehydrogenase (pLDH) assay is the observation that the lactate dehydrogenase (LDH) enzyme has the ability to rapidly use 3-acetyl pyridine NAD (APAD) as acoenzyme in the reaction, leading to the formation of pyruvate from lactate (Makler and Hinrichs, 1993). In contrast, human red blood cell LDH carries out this reaction at a slow rate in the presence of APAD. The development of APADH is measured, and there is a correlation between levels of parasitemia and the activity of parasite LDH (Nogueira and Estólio do Rosário, 2010). An advantage of this method is that radiolabelled substrates are not required. Being a colorimetric method,

parasite growth is shown clearly by the production of a dark blue colour and so results can be observed without the aid of a plate reader.

2.6 PHARMACOGNOSTIC STUDIES

The use of herbal medicines is on the increase globally. To meet the growing demand, due to commercialization, the natural drug is easily adulterated with low grade material and in some cases completely substituted. Thus it is important to establish standards for authentication of efficacious medicinal plants used in the treatment of diseases. Pharmacognostic studies will ensure plant identity and lay down standardization parameters which will help prevent adulteration (Chanda, 2014). Such study does not only help in authentication but also ensures reproducible quality of herbal products. It involves the establishment of standardization parameters such as organoleptic features, microscopic parameters, physicochemical parameters and the chemical profile.

2.6.1 Organoleptic features

Organoleptic evaluation of a drug or plant refers to the evaluation by colour, odour, size, shape, taste and texture etc. Organoleptic evaluation can be done by means of sensory organs. The evaluation procedure provides the simplest and quickest means to establish the identity and purity of a drug and hence the quality of a particular drug. The colour of the drug usually gives information on the origin of a drug. e.g. Materials derived from aerial parts are usually green (Mukherjee, 2002).

2.6.2 Microscopic Parameters

Every plant possesses characteristic tissue structure that can be identified through the study of tissue arrangement, cell wall structure and cell contents. Microscopic features can be qualitative or quantitative. Qualitatively, the presence and type of starch grains, calcium oxalate crystals, stomata and epidermal cells are of diagnostic importance. Quantitatively the estimation of leaf constants such as stomatal index, vein-islet and veinlet termination numbers are used for identification, especially of closely related species (Mukherjee, 2002).

2.6.3 Physical parameters

The physical evaluation of crude drugs is done by the determination of various physical characteristic using physicochemical techniques. Parameters determined include moisture content, foreign matter, ash value and extractive values.

2.6.3.1 Moisture content

Moisture content is the quantity of water contained in a material or plant. Every plant has a quantity of water or moisture. However, in dried plant materials high moisture content can be a disadvantage. High moisture content of crude drugs is responsible for its decomposition due to microbial attack or chemical changes. Presence of moisture in a crude drug is uneconomical. The moisture content of crude drug, according to the WHO, should not exceed 10 $\%^{w}/_{w}$ (Mukherjee, 2002).

2.6.3.2 Ash values

Ash values are used to determine the purity and quality of crude drugs. The determination of ash values focuses on three areas; total ash, water soluble ash and acid insoluble ash. The World Health Organisation recommends an acid insoluble ash value of not more than 2% for sample with no specified values (Modi *et al.*, 2010; Mukherjee, 2002). Water soluble ash constitutes the part of total ash content which is soluble in water. It is a good indicator of either previous extraction of the water soluble salts in the drug or incorrect preparation (Mukherjee, 2002). Generally, high ash values are indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing (Mukherjee, 2002).

2.6.3.3 Extractive values

Extractive values are useful for the evaluation of crude drugs. They give an idea about the nature of the chemical constituents in a crude drug. They are also useful for the estimation of chemical constituents soluble in a particular solvent. The extractive values can be used to indicate adulteration with foreign matter, poor quality and incorrect processing of the plant during drying, storage etc. (Mukherjee, 2002).

2.7 THE FAMILY PHYTOLACCACEAE

The family Phytolaccaceae is also known as the Pokeweed family and belongs to the order Caryophyllales. It is a family of 18 genera and contain between 75-135 species of plants (Steinman, 2010). It is widely distributed in temperate, subtropical and tropical North America, Africa, West Indies, Central America, South America, Asia, Africa, Pacific Islands, Australia etc (Schmelzer, 2007).

2.8 THE GENUS *HILLERIA*

Hilleria comprises 3 South American species and belongs to the tribe Rivineae. The genus *Hilleria* falls in the major group of Angiosperms. Three species so far have been identified in this genus; *Hilleria latifolia*, *Hilleria longifolia* and *Hilleria secunda* (Ruiz& Pav.) H. Walt. (http: //www. gbif.org).

2.9 *HILLERIA LATIFOLIA*

Hilleria latifolia is plant from the Phytolaccaceae (http://www.theplantlist.org). It is commonly known as forest spinach or pepper herb. Some common local names include Avegboma (Ewe), Kukluigbe (Ewe), Akople (Ewe), Aka ato (Igbo), Vanfraudaka (Baoule), Anafranaku (Akan), Nyabatoo kplai (Ga) (Woode and Abotsi, 2011).

Figure 2.1The aerial part of *H. latifolia*

2.9.1 Botanical Description

Hilleria latifolia is a perennial plant which grows as a shrub, herb or tree to about 2 metres tall (Kokwaro, 2009; Dequan and Larsen, 2003). It bears simple, alternate leaves which have entire margins. It is exstipulate and petiolate. The leaves are ovately shaped

or elliptical to broadly lanceolate. The leaf base is rounded to cuneate. It has a long acuminate apex (Kokwaro, 2009; Schmelzer, 2007).

It bears head shaped bisexual flowers which are zygomorphic. It possesses a pedicle usually 1-2 mm long. The sepals are usually green to white, turning yellow to red. Petals are usually absent. It has free stamens, a superior ovary and is almost sessile (Iwu, 1993; Kokwaro, 2009; Schmelzer, 2007).

The fruits are yellow to dark red to purple berries or utricles. It has a thin pericarp, reticulately wrinkled which adheres to the seeds (Steinman, 2010).

2.9.2 Geographical Distribution

The plant *Hilleria latifolia* occurs in tropical Africa from Guinea east to Ethiopia, and south to Angola, Mozambique and northern South Africa. It also occurs in Madagascar and in Sri Lanka (Schmelzer, 2007).

2.9.3 Ethnomedicinal uses

In La Cote d'Ivoire, a decoction of the leaves is used to treat food poisoning (Odugbemi, 2008; Schmelzer, 2007). A decoction of the whole plant is taken orally for the treatment of urethral discharges. It may be taken alone or boiled in palm nut soup for the same purpose (Iwu, 1993). In Nigeria, the leaves are boiled in soup to treat gonorrhoea. In Congo, the leaves are used to treat gynaecological disorders in which purging is necessary and in combination with the stem sap of *Costus afer* (Schmelzer, 2007). The leaf decoction is taken orally to treat coughing of blood (haemotypsis) and a paste of the flowers in orange juice is used to treat asthma (Schmelzer, 2007).

In East Africa, a decoction of the whole plant is used for bathing to treat skin diseases. A quantity is also drunk for the same purpose (Kokwaro, 2009). Similarly, the leaves or whole plant are applied as poultices or lotions to treat various skin infections (Odugbemi, 2008; Schmelzer, 2007). Leprosy is treated by the application of a poultice of *Hilleria latifolia* (Lam.) H. Walt. and *Piper guineense* (Schmach and Thonn). The leaves or whole plant is used in the treatment of headaches (Odugbemi, 2008). The pulped leaves are applied topically to painful areas for relief. The leaves together with *Piper guineense* (Schmach and Thonn) are used topically to treat swellings. The plant in combination with *Althernanthera pungens* (Kunth) or *Capsicum annum* L. is used topically in the treatment of rheumatism (Schmelzer, 2007).

In Ghana, decoction of the leaves and twigs are used as a steam bath or taken orally to treat jaundice and guinea worm infestations (Iwu, 1993). The leaves or whole plant are utilized in the treatment of breast cancers. The plant together with *Momordica charantia* (Curcubitaceae) is used in the treatment of breast cancer (Abbiw, 1990; Odugbemi, 2008). The leaf decoction is administered as an enema to treat ascites. The leaf sap and that from the plant is used as a haemostatic and applied as eardrops for ear infections respectively.

2.9.4 Non medicinal uses

In Ghana, the presence of the plant is an indication that the soil is suitable for cocoa cultivation. The leaves are eaten as vegetables and the dried fruits as a relish in Ghana, Nigeria and Cameroon. The Masai people of Kenya used the blackened stems for cosmetic purposes; for drawing eyebrows (Schmelzer, 2007).

2.9.5 Biological activities

The antimicrobial activity of the methanolic, ethyl acetate and hexane fractions of *Hilleria latifolia* were investigated by Assob *et al.* (2011) using the agar well diffusion and broth dilution methods. The results indicated that the ethyl acetate and the methanol fractions were active against *Pseudomonas aeruginosa* and *Salmonella typhi* with MIC's of 0.62mg/ml for both. The hexane fraction was also found to be weakly active.

The ethanol extract of *Hilleria latifolia* was tested for antinociceptive activity using acetic acid induced abdominal writhing, glutamate, formalin, capsaicin and the tail immersion test. The plant extract, with morphine and diclofenac as reference drugs, showed significant anti-nociceptive activity with the extract producing dose-related anti-nociception in the models used (Woode and Abotsi, 2011).

A study conducted by Abotsi *et al*. (2012) to assess the anti-inflammatory potential of the ethanolic extract of *Hilleria latifolia* revealed that the extract (10-300 mg/ kg, *p.o*.) significantly inhibited carrageenan-induced foot oedema in 7-day old chicks. Further investigations using the Freund's adjuvant induced-arthritis model in rats revealed that the extract exhibited significant anti-arthritic properties when applied to established adjuvant arthritis.

Abotsi *et al.* (2012) also investigated the *in vitro* antioxidant potential of the ethanolic extract of the plant. The extract (0.03-1 mg/ ml) exhibited Fe^{3+} reducing activity, scavenged DPPH and prevented lipid peroxidation. These results were comparable to that of *N-propyl*. The findings revealed that the extract has antioxidant properties which may partly account for its anti-inflammatory activity.

Neurobehavioural properties of the ethanolic extract of the plant were evaluated. The extract (10-300 mg/ kg, *p.o.*) exhibited anxiolytic activity similar to diazepam in all the

anxiety models used. The results from the study suggested that the extract had anxiolytic and antidepressant effects (Woode *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 PLANT COLLECTION AND PROCESSING

A quantity of *Hilleria latifolia* shoot was collected from the KNUST Community Centre in February, 2012. The plant sample was authenticated at the Department Of Pharmacognosy, KNUST and voucher specimen KNUST/HM1/2012/WP005 was deposited at the Department of Herbal Medicine Herbarium, KNUST. The collected plants were screened for foreign matter after which the leaves, stems and roots were separated. The plant parts were then air-dried for about two weeks. The dried plant materials were milled and stored in paper bags until ready for use.

3.2 PHARMACOGNOSTIC STUDIES

The fresh plant parts and the dried powdered materials were used for the standardization of *H. latifolia*.

3.2.1 Organoleptic evaluation

Organoleptic evaluation was done by observing the leaves and taking note of the colour, size, odour and other parameters.

3.2.2 Macroscopic evaluation

Different macroscopic parameters of the leaves, stem and roots were noted. Evaluation of the leaves included the type of leaf, shape, arrangement, apex, margin, venation, base, texture etc.

3.2.3 Microscopic and histological techniques

3.2.3.1 Transverse sections

A qualitative microscopic analysis was conducted on the transverse section of the midrib and petiole of the leaf and a free hand illustration of the transverse section of the petiole was made. Lignified, cellulosic and other identifying features were studied staining with toluidine blue, phloroglucinol in concentrated HCl and N/50 iodine (Shah *et al*., 2013). Microscopic evaluation of the tissues was supplemented with photomicrographs of different magnifications taken with Leica light microscope DM 1000 LED (Wetzlar, Germany).

3.2.3.2 Powder microscopy

The finely powdered leaves, stems and roots of *H. latifolia* were studied under the microscope. Small amounts of the different plant parts were placed separately on a slide and mounted using chloral hydrate, phloroglucinol in conc. HCl and iodine solution. Photomicrographs of the different cellular structures and inclusions were taken.

3.2.3.3 Quantitative microscopy

A quantitative microscopy was conducted on a leaf sample. This included stomatal number, stomatal index, palisade ratio, vein islet and veinlet termination numbers. They were evaluated according to the method described by Kumar *et al*., (2012).

3.2.3 Physicochemical parameters

Physicochemical analysis such as total ash, water soluble ash, acid insoluble ash, pet-ether, alcohol and water soluble extractives as well as loss on drying of various plant

parts were determined according to standard methods (Khandelwal, 2008). The pHs of the various solvent extractives were determined.

3.2.4 Fluorescence analysis

The water, petroleum ether and ethanol extractives were observed for characteristic fluorescent colours in visible light and under UV short and long wavelengths (Najafi and Deokule, 2010).

3.2.5 Phytochemical screening

The presence of secondary metabolites such as tannins, alkaloids, glycosides, terpenoids, sterols etc were determined according to standard methods (Kokate, 1999).

3.2.6 Thin layer chromatography profile

Five (5) grams of the powdered plant materials were cold-macerated with 30 ml of chloroform for 24 hours. The filterate was concentrated under vacuum to 5 ml and used for the experiment. Analytical TLC on silica gel G60 F_{254}, 0.25 mm layer developed with pet-ether: chloroform (1:4). Separated compounds were observed for characteristic fluorescence under UV 365 nm. They were then by sprayed with anisaldehyde/H_2SO_4 (Figure 4.10).

3.3 EXTRACTION

One kilogram each of the powdered plant materials were sohxlet extracted with petroleum ether, ethyl acetate and 70% methanol successively. The liquid extract was then concentrated to a syrupy mass using the rotary evaporator at 40˚C. It was then stored

in a desiccator until required for further use. The percentage yields of the various extracts are presented in Table 3.1

Table 3.1: Percentage yield of plant extracts

EXTRACT	ABBREVIATION CODE	% YIELD
Methanol extract of leaves	HLM	15.3
Ethyl acetate extract of leaves	HLEA	13.0
Petroleum ether extract of leaves	HLP	5.2
Methanol extract of stem	HSM	11.5
Ethyl acetate extract of stem	HSEA	10.9
Petroleum extract of stem	HSP	6.7
Methanol extract of roots	HRM	10.2
Ethyl acetate extract of roots	HREA	9.2
Petroleum ether extract of root	HRP	5.9

3.4 ANTI-INFECTIVE ASSAYS

3.4.1 Antimicrobial assay

3.4.1.1 Microorganisms used

Five bacterial species; three gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NTCC 100073 and clinical strains of *Streptococcus pyogenes*) and two gram negative bacteria (*Pseudomonas aeruginosa* ATCC 4853 and *Escherichia coli* ATCC 25922) and one fungal isolate *Candida albicans* were used for the antimicrobial assay. *Mycobacterium smegmatis* mc^2155 (ATCC 700084) was also used.

3.4.1.2 Standardisation of microorganisms

The microorganisms used in the antimicrobial assay with the exception of *M. smegmatis* were standardized using the MacFarland's standards. Twenty four hour broth cultures of all the organisms to be used were made and diluted serially with sterile water after which comparison with the MacFarland's standards were made to obtain organism concentration of 10^5 CFU/ ml.

The organism *Mycobacterium smegmatis* mc^2155 (ATCC 700084) was grown at 37 ˚C in an incubator in 100 ml of Middlebrook (MB)7H9 broth medium enriched with 10%$^{v}/_{v}$ albumin dextrose catalase (ADB; BD Biosciences) and in MB7H10 with 10%$^{v/}_{v}$ Oleic acid albumin dextrose catalase (OADC) for solid agar growth at 37 ^{o}C. Incubation continued until mid-exponential phase was obtained (OD_{600} is around 1). The quality control of the organisms was checked by staining *Mycobacterium smegmatis* with a modified Ziehl-Neelsen acid fast staining protocol using a TB colour kit according to the manufacturer's procedure (Gupta and Bhakta, 2012).

3.4.1.3 Agar dilution and Spot culture growth inhibition assays

The agar dilution method as described by Vanden-Berghe and Vlientinck (1991) and Vlientinck (1991) was used in testing the antimicrobial properties of the plant extracts. Two millimetres of pre-warmed (60 ˚C) solubilised plant extract and its four fold dilutions were mixed with equal volumes (2.0 ml) of double strength nutrient agar (Oxoid). The wells of a 96 microtitre plate were filled with 300 µl of the mixture. After solidification at room temperature, all wells were inoculated with 5 µl of 24 hour broth cultures of concentration 10^5CFU/ml.

After incubation at 37 ˚C for 24 hours, 5 µl of Diphenyl tetrazolium bromide (MTT) was added to the various wells. Observation of a blue colour change indicated growth at that particular concentration. Inhibition was observed where there was no colour change and the Minimum Inhibitory Concentration (MIC) was taken at that concentration. Ciprofloxacin was used as the positive control. The negative control used was the double strength agar without any incorporated extract. Concentrations of the extracts used for the assay ranged between 32.5 µg/ml and 1000 µg/ml. Nine extracts of *H. latifolia* were employed in this assay (the methanol, ethyl acetate and petroleum ether extracts of the leaf, stem and root).

The methanol extract of the whole plant was tested against *Mycobacterium smegmatis*. The extract was dissolved in sterile DMSO (the final concentration of DMSO was 0.1%) to make a stock solution from which, different working stock concentrations were prepared using serial dilutions. In this assay, 2 µl of 10^3 cells of *Mycobacterium smegmatis* were placed with 2 ml of MB7H10/OADC (Oleic acid-albumin-dextrose catalase) containing different concentrations of the extracts in each well of a 24-well Costar plate (Appleton Woods) previously filled with boiled and cooled Middlebrook 7H10 agar medium. The Middlebrook 7H10 agar medium used had been cooled down to 55 ℃ in a prewarmed water bath. Incubation of *Mycobacterium smegmatis* was done for 2 days. The positive control used was Rifampicin and the negative control used was DMSO (Dimethyl sulphoxide) and water. The whole extract of the plant was tested at a concentration range of 3.75-500 µg/ml (Gupta and Bhakta, 2012).

3.4.2 Antiplasmodial assay

3.4.2.1 Washing of Human (A+ erythrocyte) blood for Parasite culture

About 300 ml of fresh human A^+ non-sickling blood which had previously tested negative for HIV and Hepatitis B antibodies was transferred into 50 ml centrifuge tubes. The blood sample was then centrifuged at 2500 rotations per minute (rpm) for 10 minutes. Three layers; a yellow layer consisting of plasma (on top), a supernatant buff layer consisting of white blood cells (middle) and a layer of packed red blood cells (bottom) resulted after centrifugation. The plasma layer on top was transferred into a sterile tube for further treatment and the supernatant buff layer was discarded in bleach. An equal volume of phosphate buffer saline (PBS) was added to the layer of red blood cells and thoroughly mixed. The mixture was then centrifuged for another 10 minutes. A resulting supernatant layer formed on top of the red blood cells was removed and discarded. Washing was repeated three times until the blood sample was ready for parasite culturing (Makler and Hinrichs, 1993).

3.4.2.2 Preparation of plates

The first and second columns of a 96 well microtitre plate were filled with 50 µl of the solubilized extract. Two rows were allotted for each extract. A twofold serial dilution was done from the second column to the twelfth column in each row. A volume of 50 µl of parasitised red blood cells (1.5% parasitemia and 5% haematocrit) was then added to each well. All wells of the plates except wells in the first column were previously filled with complete medium. Two rows were left for the control and blank. The wells for the control contained no extracts and the wells for the blank contained only uninfected red blood cells. The starting concentration of the extracts was 500 µg/ml which was serially

diluted to the lowest concentration of 0.244 µg/ml. The concentrations of the surfactants used were below 1%. The extracts were then made up to solution in complete medium. The plates were covered, placed in a modular incubator chamber (Billups – Rothenberg) and flushed with gas (3 % O_2, 4 % CO_2 and 93 % N_2) for 5 mins. The chamber was then sealed and placed in an incubator at 37 ^{o}C for 48 hours. After incubation, 50µl of a mixture of nitroblue tetrazolium (1000ug/ml) and Phenazine ethosulphate (500 ug/ml) (20:1) and 50 µl of APAD (3- acetylpyridine –adenine dinuceotide) reagents were added to the contents in the wells and thoroughly mixed. The plates were then covered and kept in a dark, cool place for 15 minutes. The optical densities were then measured on a plate reader (MRX Dynatech Laboratories). The percentage inhibitions of the extracts were calculated and log dose response curves were plotted. The IC_{50} values of the extracts were determined by regression analysis to the linear section of the curves. Tests were carried out in duplicates. The positive controls employed were Artemether and Chloroquine phosphate. Concentrations of the positive control were from 5 µg/ml and below (Makler and Hinrichs, 1993).

CHAPTER FOUR

RESULTS

4.1 PHARMACOGNOSTIC STUDIES

4.1.1 Organoleptic features of leaves

The organoleptic characters of the leaf are displayed in table 4.1.

Table 4.1 Organoleptic characters of *H. latifolia*

Parameter	Description (Fresh mature leaves)
Colour	Green on adaxial and light green on abaxial surfaces
Odour	Characteristic pungent
Shape	Ovate to lanceolate
Leaf base	Oblique
Margin	Entire
Apex	Acute, short to long acuminate
Surface	Glabrous
Texture	Leathery
Venation	Reticulate

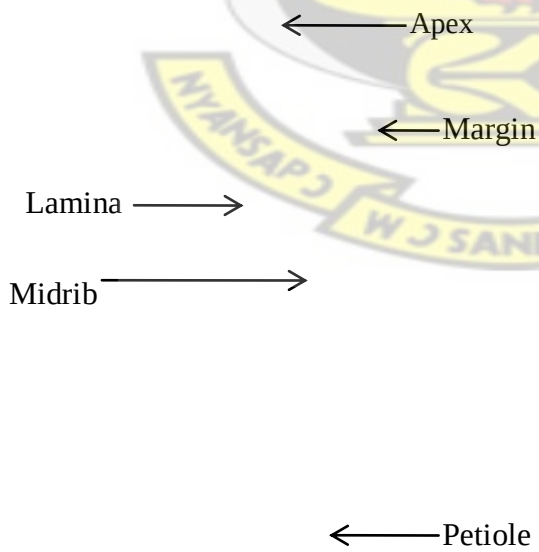


Figure 4.1 Macromorphological features of the leaf of *H. latifolia*

4.1.2 Leaf microscopy

It is hypostomatic with anomocytic type of stomata on the abaxial surface (Figure 4.2). Leaf microscopy showed the presence of abundant tetragonal crystals (3.9-110.5 µm in length) which are situated between the epidermal cells and palisade cells and multicellular clothing trichomes (94.7-347.2 µm in length) (Figure 4.2).

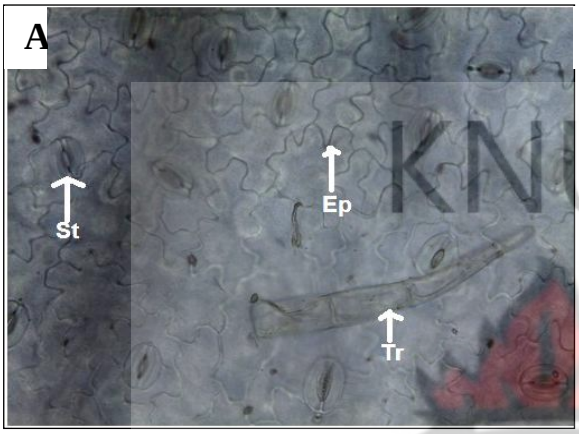


Figure 4.2 Microscopic features of the leaves stomata, epidermal cells and trichomes (A) and tetragonal calcium oxalate crystals situated below the epidermis (B)

Cr: Tetragonal crystals; St: Stomata; Ep: Epidermal cell; Tr: Trichome

A very important diagnostic feature of the leaf is the presence of rhombic, cuboidal and rosette calcium oxalate crystals in the palisade mesophyll cells (Figure 4.3). The leaf measurements are shown in table 4.2.

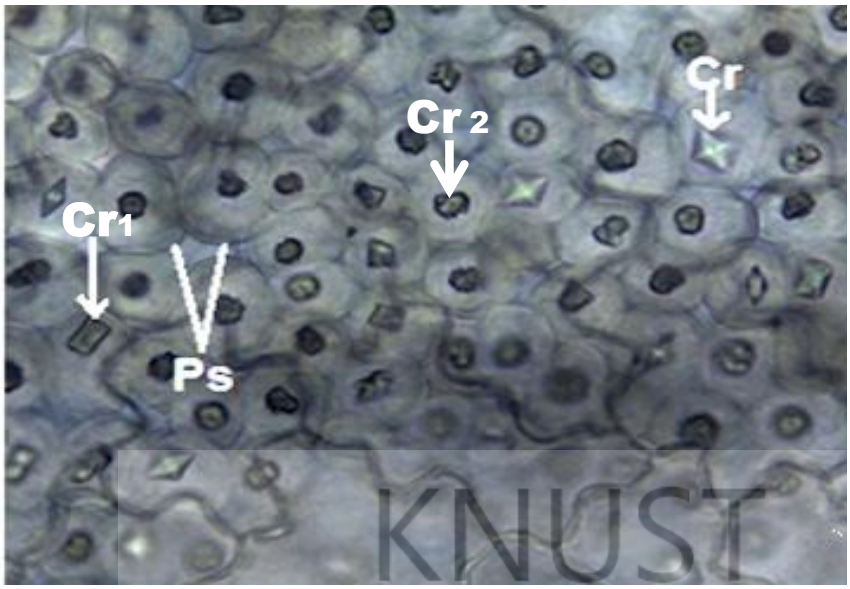


Figure 4.3 Microscopic features of leaf surface showing palisade cells and calcium oxalate crystals (embedded in palisade cells)

Cr: Rhombic calcium oxalate crystal; Cr $_1$: Cuboidal calcium oxalate crystal;
Cr_2 : Rosette crystals ; Ps: Palisade cells

Table 4.2 Leaf constants of *H. latifolia*

Parameter	Value	Average
Stomatal number (Lower epidermis)	170 -218	194
Stomatal index (Lower epidermis)	21-27	24
Vein islet number	10-31	18
Veinlet termination number	28-40	34
Palisade ratio (Upper epidermis)	4.5 -8.3	-

4.1.3 Transverse section of midrib and petiole

Transverse of the midrib shows a depression on the ventral side and a concave dorsal surface (Figure 4.4). Both surfaces are lined with elongated epidermal cells with no conspicuous cuticle. Large number of uniseriate covering trichomes (3- 8 celled) arises from both epidermal layers with very few glandular trichomes (unicellular head and unicellular stalk) on the abaxial surface. Cortical parenchyma cells contain a large number of prismatic crystals. The vascular bundle is collateral with lignified xylem elements towards the adaxial surface and phloem towards the abaxial side. There is a clear vascular cambium between the xylem and phloem.

In transverse section, the petiole is not circular but depresses towards the ventral surface with a depression on both upper lateral sides (Figure 4.5). All other features were similar to that of the midrib (Figure 4.4).

Figure 4.4 TS of the midrib of *H. latifolia* (free hand sketch)

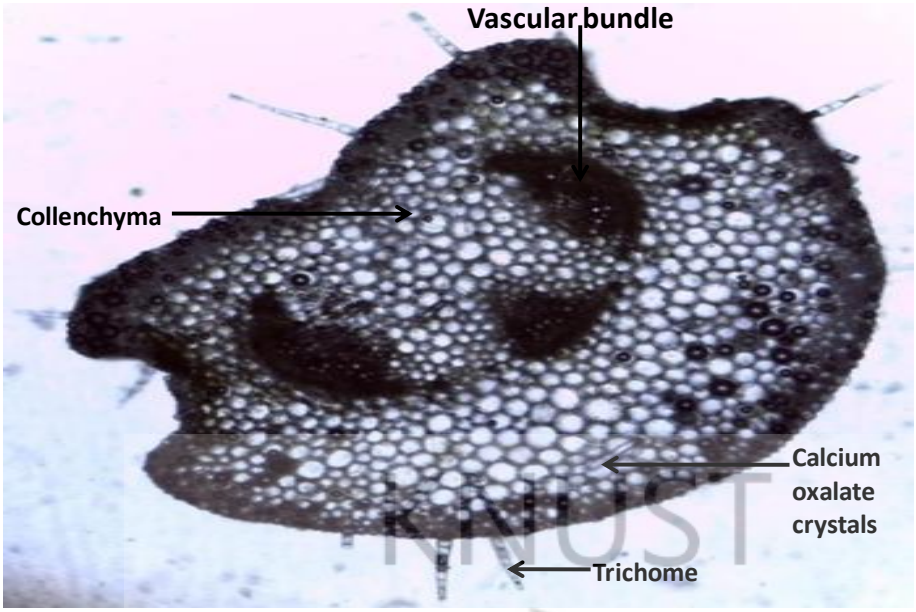


Figure 4.5 TS of the petiole of *H. latifolia*

4.1.4 Powder microscopy

The powder of *H. latifolia* displayed the presence of abundant calcium oxalate crystals in all parts of the plant (Figure 4.6a, Figure 4.7b, Figure 4.8b). A large number of broken pieces of multicellular clothing trichomes occurred in the leaf powder (Figure 4.6b) and very few in the stems. The leaf powder showed the presence of stomata (Figure 4.6a) with the root, the only conspicuous starch grain bearing part of *H. latifolia* (Figure 4.8b). Also present in the powdered stem are lignified vessels with annular and scalariform thickening (Figure 4.7). Pieces of cork cells (Figure 4.8a) and epidermal cells (Figure 4.7d) occurred in the roots and stem powders respectively.

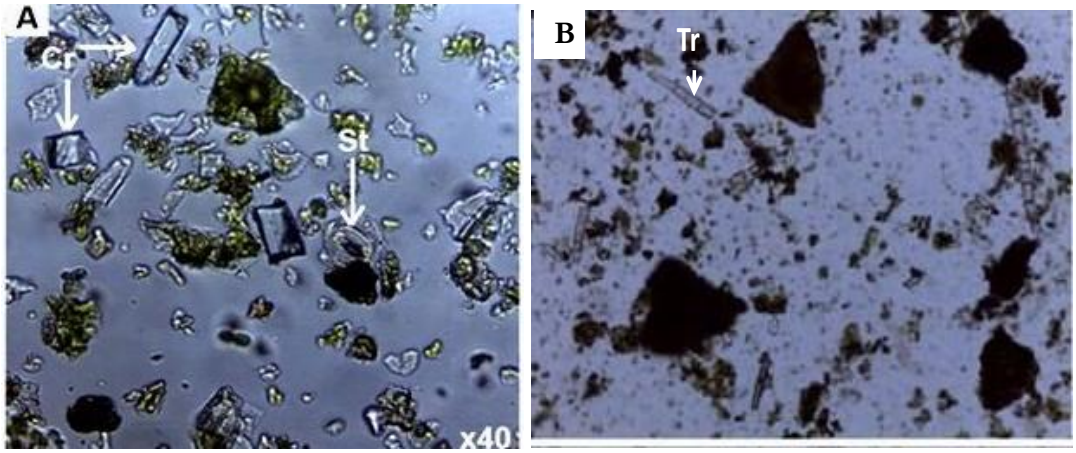


Figure 4.6 Microscopic features of the powdered leaves

Cr: Broken crystals; St: Stomata; Tr: Broken trichome

Figure 4.7 Microscopic features of the powdered stem

Av: Annular vessels; Sc: Scalariform vessels Cr; Broken crystals; Ep: Epidermal cells

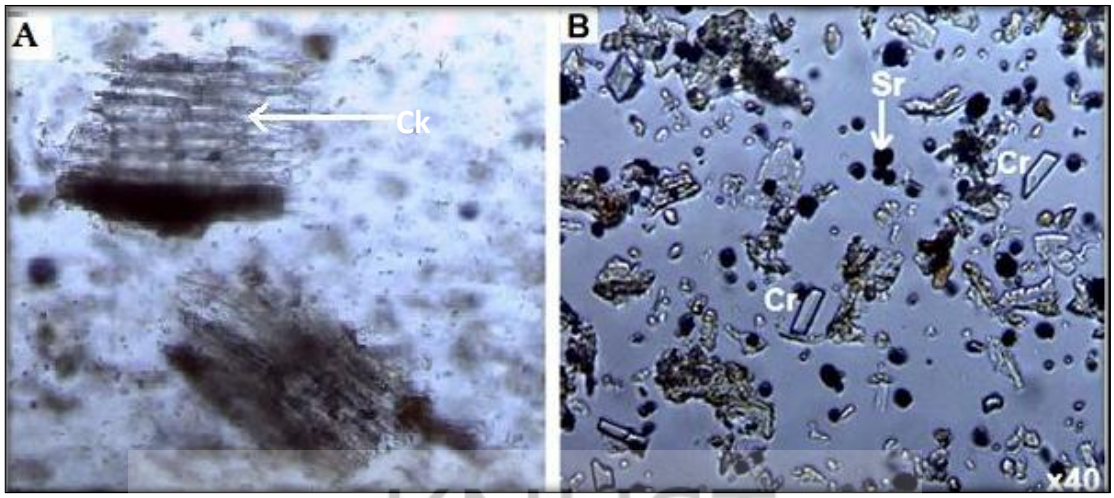


Figure 4.8 Microscopic features of the powdered roots

Ck: Cork cells; Sr: Starch grains; Cr: Broken crystals

4.1.5 Physicochemical parameters of *H. latifolia*

The extractive and ash values of the leaves, stems and roots of *H. latifolia* are shown in table 4.3. The pH values of the petroleum ether, ethanol and water soluble extractives are shown in Figure 4.9. The pet-ether soluble extract of all plant parts was basic (pH > 7) whereas the ethanol and water soluble extracts were weakly acidic to neutral (Figure 4.9)

Table 4.3 Physicochemical values

Physical parameter ($\%^{w}/_{w}$)	Leaves	Stems	Roots
Pet-ether soluble extractive	3.092 ± 0.428	5.579 ± 0.888	4.156 ± 0.157
Alcohol soluble extractive	24.048 ± 0.208	10.012 ± 1.652	12.036 ± 0.564
Water soluble extractive	27.060 ± 1.060	10.170 ± 0.670	15.8 ± 0.871
Total Ash	16.667 ± 0.286	7.389 ± 0.246	5.424 ± 0.200
Acid-insoluble Ash	0.490 ± 0.002	0.985 ± 0.003	0.493 ± 0.004
Water soluble Ash	16.028 ± 0.434	6.404 ± 0.246	4.907 ± 0.204
Moisture content	80.650 ± 1.190	74.61 ± 0.790	66.14 ± 0.860

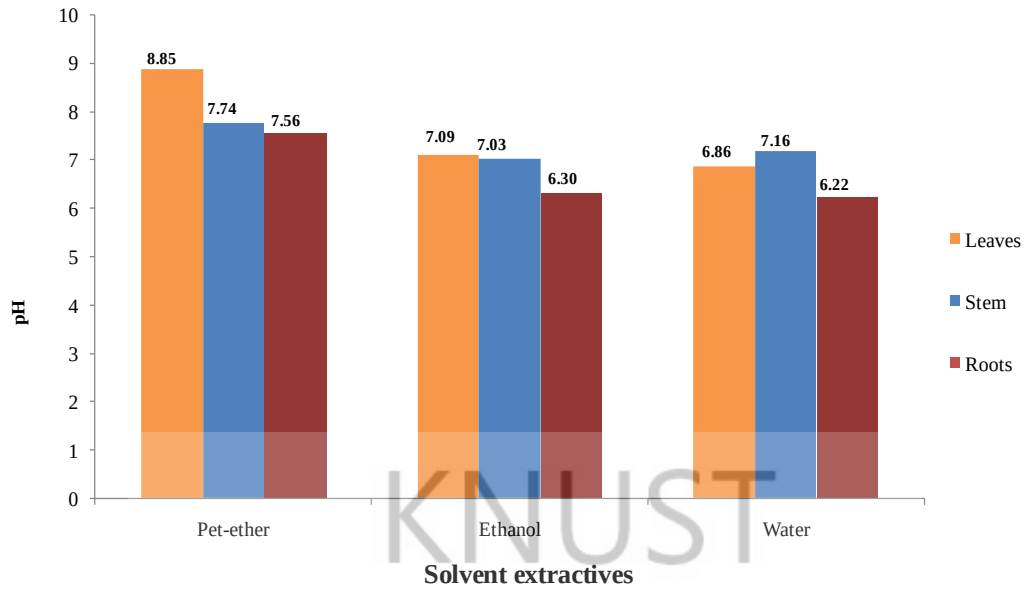


Figure 4.9 pH of solvent extractives

4.1.6 Fluorescence analysis of extractives

The pet-ether, ethanol and water soluble extractives were observed in visible light and under ultraviolet light at 254 and 365nm wavelengths for their characteristic fluorescence colours. The leaf, stem and root extract showed characteristic fluorescence colours which are displayed in Table 4.4.

Table 4.4 Fluorescence characters of plant extractives

Plant extractives	Visible light	Long wavelength	Short wavelength
(Leaf) Pet-ether	Green	Red	Yellow
(Leaf) Ethanol	Dark green	Red	Black
(Leaf) Water	Olive yellow	Light green	Dark brown
(Stem) Pet- ether	Light yellow	Pink	Light yellow
(Stem) Ethanol	Light green	Rose-pink	Dark brown
(Stem) Water	Straw	Light green	Dark brown
(Root) Pet-ether	Pale yellow	Red	Pale yellow
(Root) Ethanol	Pale yellow	Light blue	Pale yellow
(Root) Water	Straw	Light green	Pale yellow

4.1.7 Phytochemical screening of plant parts

Phytochemical analysis showed the presence of triterpenoids, tannins and reducing sugars in all the plant parts. Alkaloids were absent from all plant parts (Table 4.5).

Table 4.5 Chemical constituents of plant parts

Phytochemical	Leaves	Stems	Roots
Triterpenoids	+	+	+
Phytosterol	-	-	+
Alkaloids	+	+	+
Flavonoids	-	-	+
Saponins	-	-	+
Glycoside (reducing sugars)	+	+	+
Tannins	+	+	+
Anthraquinones	-	-	-

+: present - : absent

4.1.8 Thin Layer Chromatography (TLC)

The TLC chromatogram showed two prominent pink spots (L_6, L_7, S_3, S_4, R_3, R_4) in all three extracts with R_f values of 0.7 and 0.6 respectively. The leaf extract showed three additional pink spots with R_f values of 0.93, 0.88 and 0.83. The stem and root extracts contained two compounds with characteristic blue and light blue florescence under UV 365 nm (Figure 4.10).

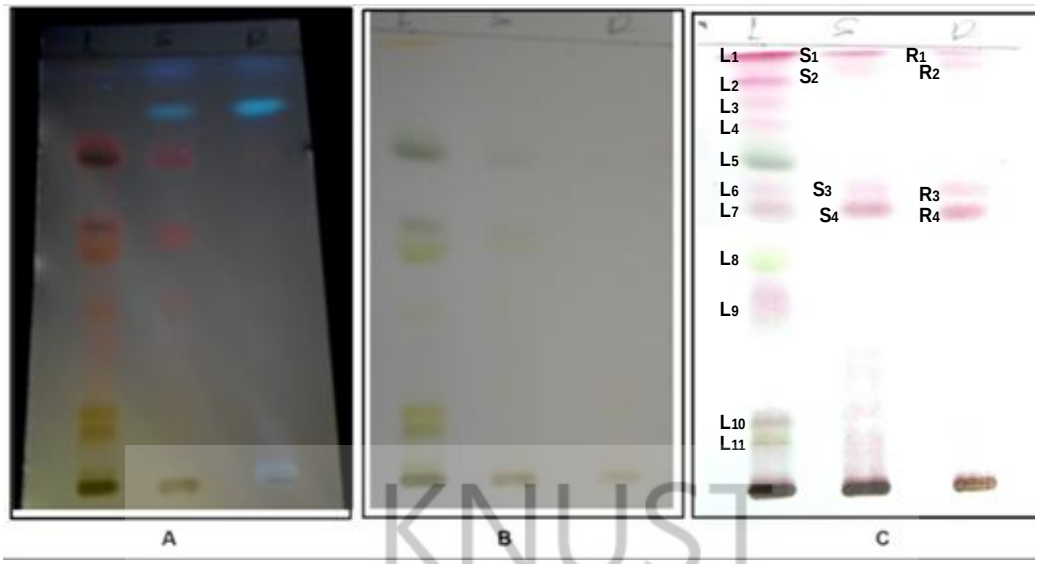


Figure4.10 TLC chromatogram of the leaf (L), stem (S) and root(R)

A (under uv 365nm), B (visible light), C (sprayed with anisaldehyde/conc H_2SO_4)

Table 4.6 R_f values of spots

Spot	L_1	L_2	L_3	L_4	L_5	L_6	L_7	L_8	L_9	L_{10}
R_f value	0.96	0.92	0.85	0.81	0.74	0.7	0.6	0.54	0.41	0.17

Table 4.6 cont.

Spot	L_{11}	S_1	S_2	S_3	S_4	R_1	R_2	R_3	R_4
R_f value	0.12	0.97	0.94	0.7	0.6	0.98	0.95	0.7	0.6

4.2 ANTIMICROBIAL ASSAY

The antibacterial and antifungal activities of the plant were investigated using the agar dilution method as described by Vanden-Berghe and Vlientinck (1991) .The plant extract were tested against six micro-organisms; *B. subtilis*, *S. aureus*, *S. pyogenes*, *P. aeruginosa*, *E. coli* and *C. albicans*. Their susceptibility, as indicated by the MICs, is shown in Table 4.9

The whole extract of the plant was tested against *M. smegmatis* .The MIC of the extract was above 500 µg/ml with the positive control Rifampicin recording an MIC of 0.01 µg/ml.

Table 4.7 MIC values of plant extracts (µg/ml)

ORGANISM	HLM	HLEA	HLP	HSM	HSEA	HSP	HRM	HREA	HRP	CONTROL
B. subtilis	>1000	250	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1
S. aureus	>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	2
S. pyogenes	>1000	1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	<1
P. aeruginosa	>1000	500	>1000	>1000	>1000	>1000	>1000	>1000	500	<1
E. coli	>1000	250	>1000	>1000	>1000	>1000	>1000	1000	1000	<1
C. albicans	>1000	500	>1000	>1000	>1000	>1000	>1000	>1000	1000	5

Key:

HLM ; methanol extract of leaves, HLEA; ethyl acetate extract of leaves, HLP; petroleum ether extract of the leaves, HSM; methanol extract of stem, HSEA; ethyl acetate extract of the stem, HSP; petroleum ether extract of the stem, HRM; methanol extract of roots, HRP; petroleum ether extract of the roots, HREA ethyl acetate extract of the root

4.3 ANTIPLASMODIAL ASSAY

The antiplasmodial activity of the plant was assessed by the Parasite Lactate Dehydogenase Assay. The optical densities of the plates were measured and the percentage inhibitions of the extracts were calculated as shown in Table 4.2. The results of the percentage inhibition of the extracts are shown in Table 4.9. Log dose response curves were plotted and the IC_{50} values of the extracts were determined by regression analysis to the linear section of the curves as shown in Figure 4.11- 4.19. The IC_{50} values are presented in Table 4.4. The antiplasmodial activities of the extracts were graded as very active $IC_{50} \leq 25\mu g/ml$; active $IC_{50} > 25 \leq 50$ µg/ml; moderately active $> 50 \leq 100$ µg/ml and weakly active $IC_{50} > 100 \leq 1000$ µg/ml (Gessler *et al*., 1994; Ramazani *et al*., 2010).

Table 4.8 Calculation of percentage inhibition and IC_{50} values

Concentration of sample	Mean test absorbance	Corrected absorbance (Growth)	% Growth	% Inhibition of growth
µg/ml	A_{test}	$G = A_{test} - A_{un}$	$\%G = (G/Tg) \times 100$	100 - % G

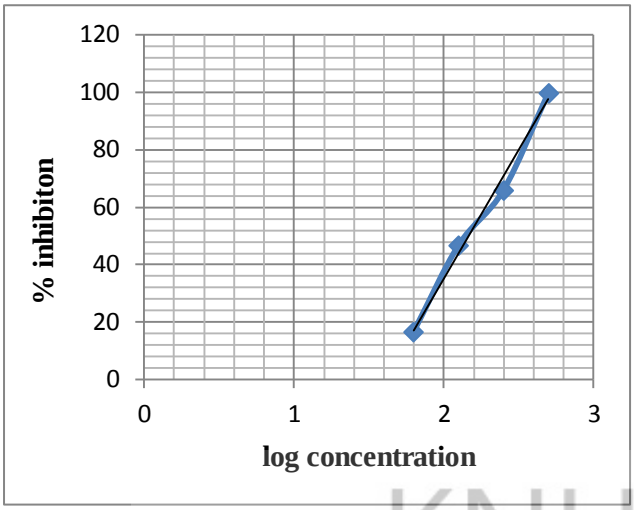
A_{test} = Absorbance of the test A_{un} = The average absorbance of the blank/uninfected Tg = Total growth

Table 4.9: Percentage inhibition of the plant extracts

Conc. (µg/ml)	HLM	HLEA	HLP	HSM	HSEA	HSP	HRM	HREA	HRP
500.00	99.6594	89.1802	116.3279	97.5432	90.6866	110.4334	99.7234	103.3926	98.8079
250.00	65.8639	69.4007	108.5340	63.7345	79.0284	81.1243	85.2367	94.3543	91.0795
125.00	46.7066	45.5605	108.9270	42.7802	58.9542	55.9742	42.0123	60.9846	50.7346
62.50	16.6444	14.4176	72.7409	15.2345	43.8903	17.5941	28.9878	19.8209	25.9447
31.25	11.4703	13.3697	41.4015	10.6780	20.6396	9.1125	31.0750	13.9919	13.3042
15.63	3.6436	0.5981	33.4439	5.3780	15.7275	-0.4498	9.1502	7.9008	4.1676
7.81	15.1053	-2.0216	28.4008	12.9086	15.3673	6.9839	-1.7890	-0.8099	-2.9713
3.91	35.3432	-3.2987	37.7338	30.5688	-96518	24.6021	-7.2201	-0.1222	-5.6238
1.95	17.6923	-1.0392	13.7299	16.1235	6.0670	15.8913	-1.5520	-3.2987	-2.4801
0.98	50.4726	-2.4473	45.9207	42.9085	5.9360	35.3105	2.3832	-4.8051	-4.5431
0.49	7.2459	4.0694	39.5349	5.9867	5.4448	34.2299	-7.7893	1.0893	-2.1526
0.24	72.4789	19.8537	77.6203	7.6004	18.6420	70.2849	3.5888	13.4679	7.9991

Key:

HLM; methanol extract of leaves, HLEA; ethylacetate extract of leaves, HLP; petroleum ether extract of the leaves, HSM; methanol extract of stem, HSEA; ethylacetate extract of the stem, HSP; petroleum ether extract of the stem, HRM; methanol extract of roots, HRP; petroleum ether extract of the roots, HREA ethyl acetate extract of the roots

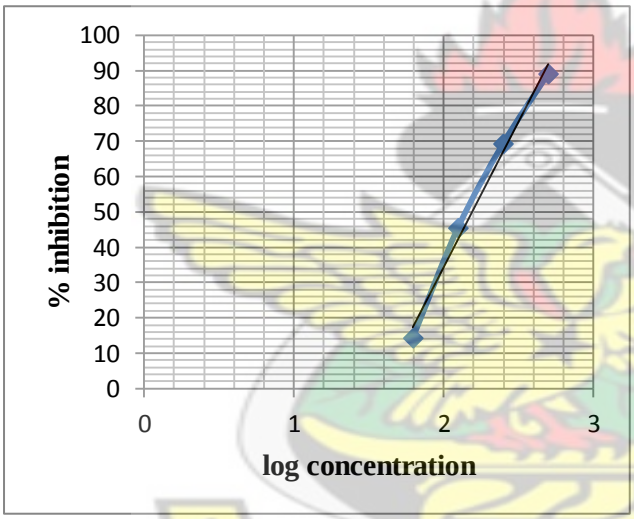


Log concentration at 50% inhibition = 2.17

IC_{50}= Antilog of 2.17

IC_{50}=151.36 µg/ml

Figure 4.11 Percentage inhibition against log concentration of methanol leaf extract

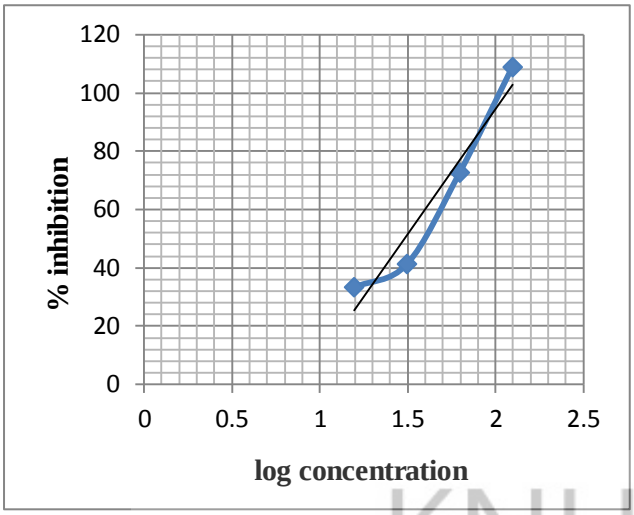


Log concentration at 50% inhibition = 2.17

IC_{50}= Antilog of 2.17

IC_{50}=147.91µg/ml

Figure 4.12 Percentage inhibition against log concentration of ethyl acetate leaf extract

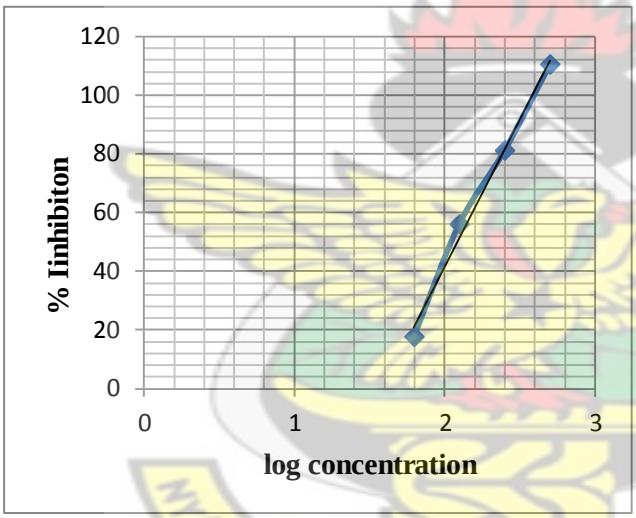


Log concentration at 50% inhibition = 1.45

IC_{50}= Antilog of 1.45

IC_{50}=28.18µg/ml

Figure 4.13 Percentage inhibition against log concentration of pet-ether leaf extract

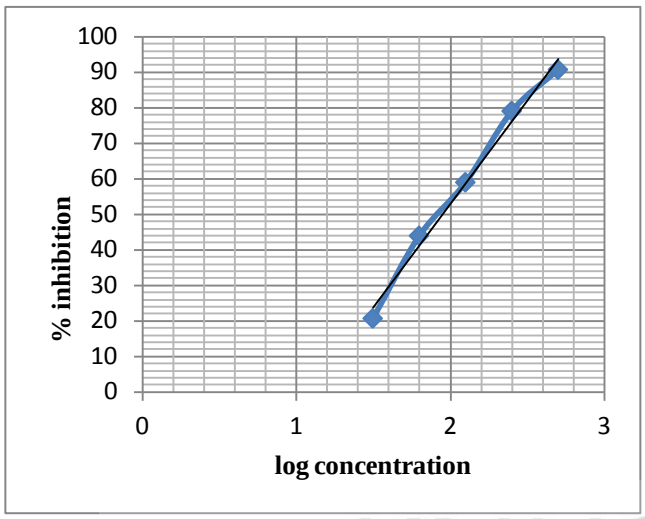


Log concentration at 50% inhibition = 2.05

IC_{50}= Antilog of 2.05

IC_{50}=112.20 µg/ml

Figure 4.14 Percentage inhibition against log concentration of methanol stem extract

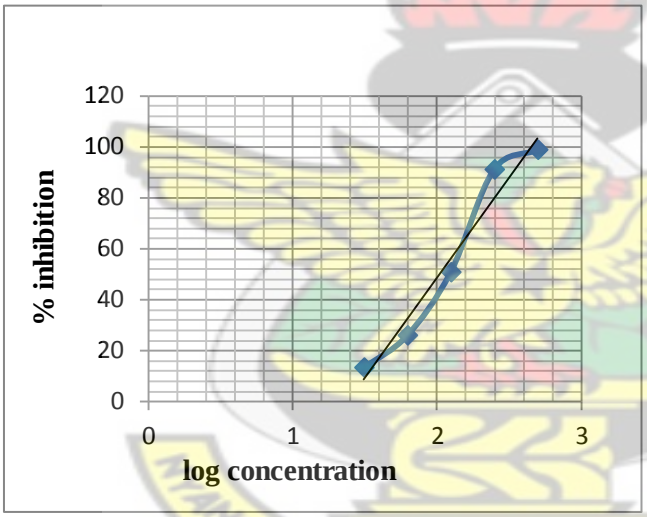


Log concentration at 50% inhibition = 1.95

IC_{50}= Antilog of 1.95

IC_{50}=89.25 µg/ml

Figure 4.15 Percentage inhibition against log concentration of ethyl acetate stem extract

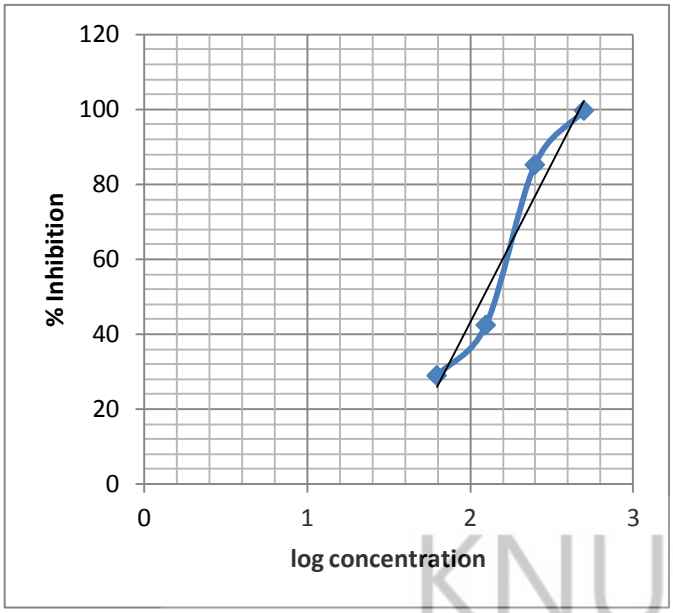


Log concentration at 50% inhibition = 2.00

IC_{50}= Antilog of 2.00

IC_{50}=100 µg/ml

Figure 4.16 Percentage inhibition against log concentration of pet-ether stem extract

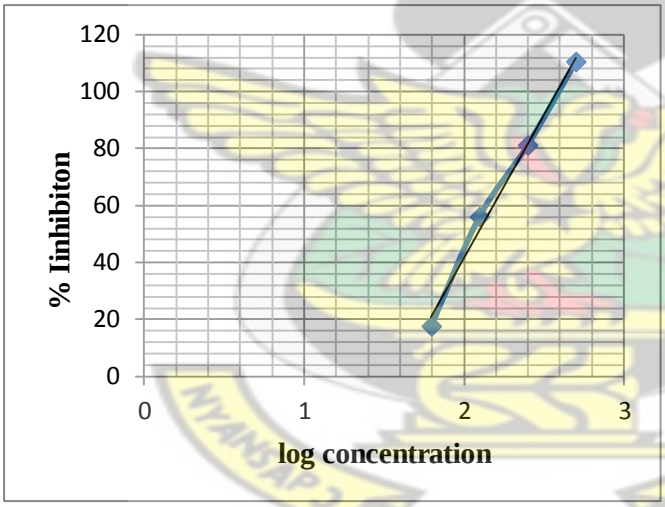


Log concentration at 50% inhibition = 2.08

IC_{50}= Antilog of 2.08

IC_{50}=120.23 µg/ml

Figure 4.17 Percentage inhibition against log concentration of methanol root extract

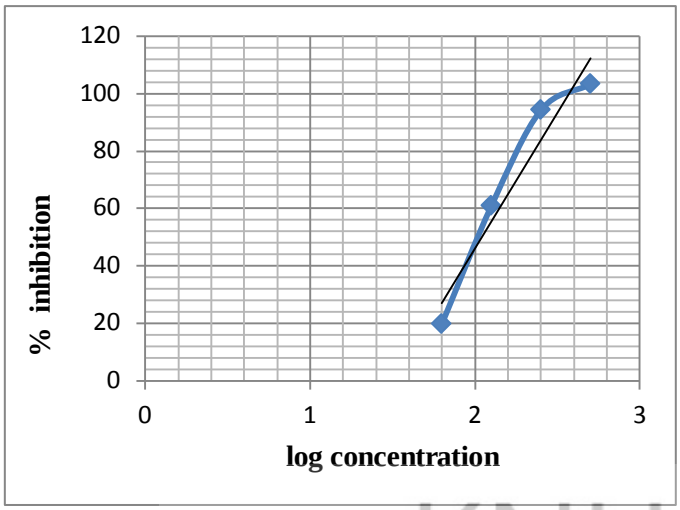


Log concentration at 50% inhibition = 2.03

IC_{50}= Antilog of 2.03

IC_{50}=107.15 µg/ml

Figure 4.18 Percentage inhibition against log concentration of pet-ether root extract



Log concentration at 50% inhibition = 2.00

IC_{50}= Antilog of 2.00

IC_{50}=100 µg/ml

Figure 4.19 Percentage inhibition against log concentration of ethyl acetate root extract

Table 4.10 Antiplasmodial activity of extracts expressed as IC_{50}

EXTRACT	IC_{50}	ACTIVITY
HLM	151.36 µg/ml	Weakly active
HLEA	147.91 µg/ml	Weakly active
HLP	28.18 µg/ml	Active
HSM	112.20 µg/ml	Weakly active
HSEA	89.25 µg/ml	Moderately active
HSP	107.15 µg/ml	Weakly active
HRM	120.23 µg/ml	Weakly active
HREA	100 µg/ml	Moderately active
HRP	100 µg/ml	Moderately active
Artemether	19.3 ng/ml	Very active
Chloroquine	412.0 1ng/ml	Very active

Key:

HLM; methanol extract of leaves, HLEA; ethyl acetate extract of leaves, HLP; petroleum ether extract of the leaves, HSM; methanol extract of stem, HSEA; ethyl acetate extract of the stem, HSP; petroleum ether extract of the stem, HRM; methanol extract of root, HRP; petroleum ether extract of the roots, HREA ethyl acetate extract of the roots

CHAPTER FIVE

DISCUSSION

5.1 PHARMACOGNOSTIC STUDIES

*Hilleria latifolia i*s used extensively in traditional medicine for the treatment of diseases especially as an anti-infective, anti-inflammatory and analgesic agent (Schmelzer *et al.*, 2008). The anti-nociceptive, anti-inflammatory and antioxidant activity of the ethanol extracts of the aerial parts of the plant have been established (Woode *et al.*, 2011; Abotsi *et al*., 2012). However there are no documented pharmacopoeial standards for the correct identification and standardisation of this plant thus the pharmacognostic features of *Hilleria latifolia* are being reported for the first time.

Hilleria latifolia, as a natural drug, can be described as a shrubby herb up to 2 m tall, with some weak bristly hairs on young branches. The leaves are alternate, simple and entire with no stipules. The petiole is (1–7) cm long. The blade is ovate or elliptical to broadly lanceolate (Schmelzer *et al*., 2008). There are no petals; the sepals and stamens are four each with one very short style. It produces smooth reddish matured fruits, about 2 mm in diameter (Iwu, 2014). The plant has a disagreeable odour with the effect being strongest in the roots.

In the histological examination and powder microscopy, all parts of the plant contain prismatic crystals with the occurrence of rosette and rhombic crystals in the palisade cells (Figure 4.3), a very important diagnostic feature. Pharmacognostic studies carried on *Petriveria alliacea* of the same family revealed the presence of large prismatic calcium oxalate crystals in the leaves and stem, a feature similar in *H. latifolia*. However, *Phytolacca decandra* and *Phytolacca americana* also of Phytolaccaceae were reported to

contain acicular calcium oxalate crystals (Evans, 2009; www.henriettes- herb.com) which were absent in *H. latifolia*. According to Kubitzki *et al*. (1993) one important systematic character in leaf anatomy in the Phytolaccaceae is the different kinds of calcium oxalate crystals in the leaves. In the present study, various kinds of crystals; rhombic, cuboidal, and rosette crystals were observed in the leaf and this supports the findings by Kubitzki *et al*. (1993). Uniseriate clothing trichomes occur on both the leaf and stem. This agrees with reports by Dalin *et al*. (2008) which indicated that uniseriate trichomes occur in the Phytolaccaceae.

The ash values established in this study (Table 4.7) may be useful in the determination of the purity and quality of *H. latifolia*. The extractive values give an idea about the nature of the chemical constituents present in the plant and is useful for the estimation of specific constituents soluble in that particular solvent used for the extraction as well as the determination of exhausted materials. The water, ethanol and pet-ether soluble extractives gave characteristic fluorescence in visible light and under ultraviolet light, which may be useful in the detection of adulteration (Table 4.4).

Phytochemical evaluation and chemical profiling are useful for the quality assessment of plant materials. Preliminary phytochemical analysis of the plant ascertained the presence of triterpenoids, phytosterols, reducing sugars and tannins (Table 4.5) which is consistent with a report by Abotsi *et al*. (2012) The TLC chromatogram (Figure 4.10), developed for *H. latifolia*, may be useful in the identification of the various parts of the plant and the detection of adulterants.

5.2 ANTIMICROBIAL ASSAY

Hilleria latifolia finds use in a number of folklore medicines as an anti-infective. Therefore in the present studies, its antimicrobial activity was evaluated against six micro

organisms using the agar dilution method described by Vanden-Berghe and Vlientinck (1991). Methanol, ethyl acetate and pet-ether extracts of the leaf, stem and root were used. The ethyl acetate extract of the leaf and the pet-ether extract of the root were the most active (Table 4.7). The most susceptible organisms were the gram negative bacteria *P. aeruginosa* and *E. coli*. All other extracts showed weak activity against the organisms giving MIC's of above 1000 µg/ml. Previous studies have shown that plants of the family Phytolaccaceae are active against a number of gram negative bacteria especially *Pseudomonas aeruginosa* (Tadeg *et al*., 2005; Pacheco *et al*., 2013). The antimicrobial activity of *H. latifolia* however was more active as compared to the plant investigated by Pacheco *et al*. (2013) which recorded MIC's above 50 mg/ml. The ethyl acetate extract was also active against the fungus *C. albicans*, giving an MIC of 500 µg/ml. The antimicrobial compounds in the leaves of *H. latifolia*, may be medium polar in nature, since the highest activity resided in the ethyl acetate extract. Thus the present studies have shown that *Hilleria latifolia* exhibits some level of antimicrobial activity and therefore supports to an extent its use in folklore medicine.

In some parts of Africa, the boiled leaves of *Hilleria latifolia* are used to treat haemotypsis, a condition suggestive of possible tuberculosis. The mycobacterial potential of the plant was thus investigated using the spot culture growth inhibition assay. An MIC higher than 500 µg/ml was obtained for the whole extract of *Hilleria latifolia*. Thus whole plant extract of *H. latifolia* showed weak anti-mycobacterium activity. Previous work on some members of the family Phytolaccaceae gave MICs a little over 100 µg/ml (weak activity) against *Mycobacterium bovis* (Tosun *et al*., 2005).

5.3 ANTIPLASMODIAL ASSAY

In this study, the antiplasmodial activity of *H. latifolia* was investigated. The methanolic, ethyl acetate and petroleum ether extracts of the plant were tested against multidrug resistant *P. falciparum* parasites using the Parasite lactate dehydrogenase assay. The petroleum ether extract of the leaves, showed the highest activity (IC_{50} = 28.18 µg/ml). The pet-ether extract of the roots, the ethyl acetate extract of the stem and root, showed moderate antiplasmodial activity giving IC_{50} values between 89.25 µg/ml and 100 µg/ml. All other extracts showed weak antiplasmodial activity (Table 4.10). Thus, the antiplasmodial constituents of *H. latifolia* reside in the non-polar pet-ether extract of the leaves.

5.4 CONCLUSION

Hilleria latifolia can be said to possess some level of antimicrobial activity and may be particularly useful in managing infections caused by *P. aeruginosa*. The ethyl acetate extract of the leaves showed the strongest antimicrobial and antifungal activities inhibiting all microorganisms tested. The petroleum ether extract of the leaves showed considerable antiplasmodial activity with an IC_{50} value of 28.18 µg/ml. The plant exhibits some level of antiplasmodial activity but weak antimycobacterial activity. The plant contains abundant calcium oxalate crystals amongst other significant pharmacognostic properties. The study thus supports to some extent the folkloric use of the plant in the treatment of various infections. Standardisation parameters for the plant have been determined for its proper identification.

5.5 RECOMMENDATION

It is recommended that future studies on the plant should focus on isolating and identifying the chemical principles in the ethyl acetate and petroleum extracts of the leaves as they showed the highest antimicrobial and antiplasmodial activities respectively.

This study has revealed that the plant is a rich source of various types of calcium oxalate crystals. This plant can be very useful as a teaching material in Pharmacognosy practical lessons.

REFERENCES

Abbiw, D. K. (1990).Useful Plants of Ghana West African Uses of Wild and Cultivated Plants. Intermediate Technology Publications Ltd., London, UK.

Abotsi, W. K., Ainooson, G. K. and Woode, E. (2012). Anti-inflammatory and antioxidant effects of an ethanolic extract of the aerial parts of *Hilleria latifolia* (Lam.) H. Walt. (Phytolaccaceae). *African Journal of Traditional, Complementary and Alternative Medicines*, 9(1): 138-52.

Adeniyi, B. A., Fong, H. H. S., Pezzuto, J. M., Luyengi, L. and Odelola, H. A. (2000). Antibacterial activity of diospyrin, isodiospyrin and bisi-sodiospyrin from the root of *Diospyros piscatoria* (Gurke) (Ebenaceae). *Phytotherapy Research*, 14:112–117.

Aires de Sousa, M. and de Lencastre, H. (2004). Bridges from hospitals to laboratory: genetic portraits of Methicillin resistant *Staphylococcus aureus* clones. *FEMS Immunology Medical Microbiology*, 40: 101-111.

Akande, T. M. and Musa, I. O. (2005). Epidemiology of malaria in Africa. *African Journal of Clinical and Experimental Microbiology*, 6(2):107-111.

Assob, J. C. N., Kamga, H. L. F., Dickson, S. N., Njunda, A. L., Nde, P. F., Asongalem, E. A., Njouendou A. J., Bertrand Sandjon, B. and Penlap, V. B. (2011). Antimicrobial and toxicological activities of five medicinal plant species from Cameroon Traditional Medicine. *BMC Complementary and Alternative Medicine*, 11(70):1-11.

Baris, O., Gulluce, M., Sahin, F., Ozer, H., Kilic, H., Ozkan, H. Sokmen M., and Ozbek, T. (2006). Biological activities of the essential oil and methanol extract of *Achillea Biebersteinii* Afan. (Asteraceae). *Turkish Journal of Biology*, 30: 65-73.

Barry, A. L., Fuchs, P. C. and Brown, S. D. (2001). Identification of β-lactamase-negative, ampicillin-resistant strains of *Haemophilus influenzae* with four methods and eight media. *Antimicrobial Agents of Chemotherapy*, 45*:* 1585-1588.

Baumgartner, B., Erdelmeier, C. A. J. and Otto, S. (1990). An antimicrobial alkaloid from *Ficus septica*. *Phytochemistry*, 29(10): 3327-3330.

Centre for a Livable future, (2009). Centre for a Livable future Congressional Briefing. Antibiotic Resistance: A multibillion Dollar Health Care Crisis.

Chanda, S. (2014). Importance of Pharmacognostic study of medicinal plants: An overview. *Journal of Pharmacognosy and Phytochemistry*, 2(5): 69-73.

Cos, P., Vlietinck, A. J., Vanden Berghe, D. and Maesa. L. (2006). Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology*, 106: 290–302.

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12(4): 564–582.

Cox-Singh, J., Davis, T. M., Lee, K. S., Shamsul, S. S., Matusop, A., Ratman, S. *et al*., (2008). *Plasmodium knowlesi* malaria in humans in widely distributed and potentially life threatening. *Clinical Infectious Diseases: Oxford Journals*, 46(2): 165-167.

Dalin, P., A˚gren, J., Bjo¨rkman, C., Huttunen, P. and K¨arkk¨ainen, K. (2008). Induced Plant Resistance to Herbivory; Leaf Trichome Formation and Plant Resistance to Herbivory. Springer Science Business Media B.V. pp 80-105.

Das K., Tiwari, R. K. S. and Shrivastava, D. K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*, Vol. 4(2):104-111.

Dequan, L. and Larsen, K. (2003). Phytolaccaceae. *Flora of China*, 5, 435-436.

Desjardins, R. E., Canfield, C. J., Haynes, J. D. and Chulay, J. D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semi-automated microdilution technique. *Antimicrobial Agents and Chemotherapy*, 16: 710–718.

Dilika, F., Bremner, P. D. and Meyer, J. J. M. (2000). Antibacterial activity of Linoleic and oleic acids isolated from *Helichrysum pedunculatum*: a plant used during circumcision rites. *Fitoterapia,* 450-452.

Dokosi, O. B. (1998). Herbs of Ghana. Ghana Universities Press; Accra: pp 746.

Dorman, H. J. and Deans, S. G. (2002). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, 88: 308-316.

Evans, W. C. (2002). Trease and Evans' Pharmacognosy. 15th edition. Saunders Limited. pp 537.

Gessler, M., Nkunya, M., Mwasumbi, L., Heinrich, M. and Tanner, M. (1994). Screening Tanzanian medicinal plants for antimalarial activity. *Acta Tropica*, 56(1): 65-77.

Fessenden, R. J. and Fessenden, J. S. (1982). Organic Chemistry, 2nd edition, Willard Grant Press, Boston.

Fidock, D. A., Rosenthal, P. J., Croft, S. L., Brun, R. and Nwaka, S. (2004). Antimalarial drug discovery: efficacy models for compound screening. *Nature Reviews*, 3: 509-518.

Franklin, R. C., Wilker, M. A., Alder, J., Dudley, M. N., Hindler, J. A., Eliopolus, G. M. *et al.*, (2012). Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically. Vol. (32): 2.

Fuchs, P. C., Barry, A. L. and Brown, S. D. (2001). Influence of variations in test methods on susceptibility of *Haemophilus influenza* to ampicillin, azithromycin, clarithromycin, and telithromycin. *Journal of Clinical Microbiology,* 39: 43-46.

Gollin, D. and Zimmermann, C. (2007). Malaria: Disease Impacts and Long-Run Income Differences. University of Connecticut and IZA Discussion Paper No. 2997.

Greenwood, B. M., Bojang, K., Whitty, C. J. and Targett, G. A. (2005)."Malaria". *Lancet*, 365(9469): 1487–1498.

Grierson, D. S. and Afolayan, A. J. (1999). Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. *Journal of Ethnopharmacology*, 66: 103-106.

Griffith, K. S, Lewis, L. S., Mali, S. and Parise, M. E. (2007). "Treatment of Malaria in the United States". *Journal of the American Medical Association*, 297: 2264-2277.

Gupta, A. and Bhakta, S. (2012). An integrated surrogate model for screening of drugs against *Mycobacterium tuberculosis*. *Journal of Antimicrobial Chemotherapy*, 56:1-12.

Hamburger, M. O. and Cordell, G. A. (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. *Journal of Natural Product*, 50: 19-20.

Heinrich, M., Barnes, J., Gibbons, S. and Williamson, E. M. (2004). Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone, Edinburgh, pp. 245–252.

Hendriksen, R. S. (2003). MIC susceptibililty testing of *Salmonella* and *Campylobacter*. Laboratory protocols, Level 2 Training Course. 4th Edition, pp 2-28

Heyde, H. C. V., d. Elloso, M. M., Waa, J. V., Schell, K. and Weidanz, W. P. (1995). Use of hydroethidine and flow cytometry to assess the effects of leucocytes on the malaria parasite *Plasmodium falciparum*. *Clinical Diagnostics Laboratory Immnunology*, 2: 417-425.

Hoelzel, S. C. S. M., Vieira, E. R., Morel, A. F. (2005). An unusual quinolinone from *Waltheria douradinha*. *Phytochemistry*, 66(10): 1163-1167.

Hoffman, D. (2003). Medical Herbalism: The Science and Practices of Herbal Medicine, Healing Arts Press, Rochester.

Iwu, M. M. (2014). *Hilleria latifolia*, Handbook of African medicinal plants. 2nd edition, CRC Press Taylor and Francis. pp 236.

Iwu, M. (1993). Handbook of African Medicinal Plants. CRC Press, Boca Raton, Florida USA.

Iwu, M. M., Unaeze, N. C., Okunji, C. O., Corley, D. G., Sanson, D. R., Tempesta, M. S. (1991). Antibacterial aromatic isothiocyanates from the essential oil of *Hippocratea welwitschii* roots. *International Journal of Pharmacognosy*, 29:154 -158.

Jacoby, D. B. and Youngson, R. M. (2004). Health and Fitness. Marshall Cavendish Corporation.

Kaur, K., Jain, M., Kaur, T. and Jain, R. (2009). Antimalarials from nature. *Bioorganic and Medicinal Chemistry*, 17: 3229-3256.

Khandelwal, K. R. (2008). Practical Pharmacognosy. 19th Edition, Nirali Prakashan, Pune. pp 157-159.

Kubitzki, K. Rohwer, J. G. and Bittrich, V. (1993) Flowering Plants-Dicotyledons: Magnoliid, Hamamelid and Caryophyllid Families. pp 507

Kohner, P. C., Rosenblatt, J. E. and Cockerill, F. R. (1994). Comparison of agar dilution, broth dilution and disk diffusion testing of ampicillin against *Haemophilus* species by using in-house and commercially prepared media. *Journal of Clinical Microbiology*, 32: 1594-1596.

Kokate, C. K. (1999). Practical Pharmacognosy. Vallabh Prakashan, India.

Kokwaro, J. O. (2009). Medicinal plants of East Africa. East African Literature bureau, Nairobi, Kenya. pp 224.

Kumar, D., Kumar, K., Kumar, S., Kumar, T., Kumar, A. and Prakash, O. (2012). Pharmacognostic evaluation of leaf and root bark of *Holoptelea integrifolia* Roxb. *Asian Pacific Journal of Tropical Biomedicine*, 169-175.

Langfield, R. D., Scarano, F. J., Heitzman, M. E., Kondo, M., Hammond, G. B. and Neto, C. C. (2004). Use of a modified microplate bioassay method to investigate

antibacterial activity in the Peruvian medicinal plant *Peperomia galiodes*. *Journal of Ethnopharmacology*, 94(2-3): 279-281.

Leite, S. P., Vieira, J. R. C., de Madeiros, P. L., Leite, R. M. P., Lima, V. L., Xavier, H. S. and Lima (2006). Antimicrobial activity of *Indigofera suffruticosa*. *Evidence Based Complementary Alternative Medicine*, 2(3): 261-265.

Lewis, W. H. and Elvin-Lewis M. P. (1995). Medicinal plants as sources of new therapeutics. *Annals of Missouri Botanic Garden*, 82:16–24.

Lourens, A. C. U., Reddy, D., Baser, K. H. C., Viljoen, A. M. and Van Vuuren, S. F. (2004). *In vitro* biological activity and essential oil composition of four indigenous South African *Helichrysum* species. *Journal of Ethnopharmacology*, 9: 253-258.

Makler, M. T. and Hinrichs, D. J. (1993). Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitaemia. *American Journal of Tropical Medicine and Hygiene*, 48, 205–210.

Mallikharjuna, P. B., Rajanna, L. N., Seetharam, Y. N. and Sharanabasappa, G. K. (2007). Phytochemical Studies of *Strychnos potatorum* L.f. - A Medicinal Plant. *E-Journal of Chemistry*, 4(4): 510-518.

Mbata, T. I., Debiao, L. and Saikia, A. (2006). Antibacterial activity of the crude extract of Chinese Green Tea (*Camellia sinensis*) on *Listeria minocytogenes*. *Internet Journal of Microbiology*, 2(2).

Mitchell, S. M. and Ahmad, M. H. (2006). A review of medicinal plant research at the University of the West Indies, Jamaica, 1948-2001. *West Indian Medical Journal*, 55(4): 243-269.

Modi, D. C., Patel, J. K., Shah, B. N. and Nayak, B. S. (2010). Pharmacognostic Studies of the seed of *Syzygium*. *An International Journal of Pharmaceutical Sciences*, 1(1): 20-26.

Mohanty, A., Das, C., Dash, S. and Sahoo, D. C. (2010). Physicochemical and antimicrobial study of polyherbal formulation. *Pharmacie Globale International Journal of Comprehensive Pharmacy*, 4(4): 1-3.

Morse, S. S. (1995). Factors in the Emergence of Infectious Diseases. *Perspectives*, 1(1): 7-15.

Mukherjee, P. K. (2002). Quality Control of Herbal Drugs- An Approach to evaluation of Botanicals. Business Horizons Pharmaceutical Publishers.

Mutabingwa, T., Nzila, A., Mberu, E., Nduati, E., Winstanley, P., Hills, E. and Watkins, W. (2001). Chlorproguanil-dapsone treatment of drug-resistant *falciparum* malaria in Tanzania. *Lancet*, 358: 1218-1223.

Nadjm, B. and Behrens, R. H. (2012)." Malaria: An update for physicians". *Infectious Disease Clinics of North America*, 26(2): 24-259.

Najafi, S. and Deokule, S.S. (2010). Pharmacognostic study of *Tylophora dalzelli* HOOK f. *Journal of Medicinal Plant Resources*, 4(5): 403-406.

Nayyar, G. M. L., Breman, J. G., Newton, P. N. and Herrington, J. (2012). "Poor-quality antimalarial drugs in Southeast Asia and sub-Saharan Africa". *Lancet Infectious Diseases,* 12 (6): 488–96.

Nguyen, L. and Thompson, C. J. (2006). Foundations of antibiotic resistance in bacterial physiology: the mycobacterial paradigm. *Trends in Microbiology*, 14: 304-312.

Noedl, H., Wongsrichanalai, C. and Wernsdorfer, W. H. (2003). Malaria drug-sensitivity testing: new assays, new perspectives. *Trends Parasitology*.19: 175–181.

Nogueira, F. and Estólio do Rosário, V. (2010). Methods for assessment of antimalarial activity in the different phases of the *Plasmodium* life cycle. *Revista Pan-Amazônica de Saúde,* 1: 109-124.

Norrel, S. A. and Messely K. E. (1997). Microbiology Laboratory Manual Principles and Applications: Prentice Hall. Upper Saddle River, New Jersey.

Nostro, A., Germano, M. P., D'Angelo, V., Marino, A. and Cannatelli, M. A. (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letter of Microbiology*, 30(1): 379-384.

Odugbemi, T. (2008). Outline and pictures of medicinal plants from Nigeria.Vol 10 University of Lagos Press, Lagos, Nigeria, pp 95.

Olga, G. (2014). Novel Approaches to Solving the Anti-Infective Crisis. BIO International Conference – The Global event for Biotechnology, San Diego-CA

Pacheco, A. O., Moran, J. M., Giro, Z. G. and Rodriguez, A. H. (2013). *In vitro* antimicrobial activity of the total extracts of the leaves of *Petiveria alliacea* L (Anamu). *Brazilian Journal of Pharmaceutical Sciences*, 49(2): 241-250.

Parekh, J., Karathia, N. and Chanda, S. (2006). Screening of some traditionally used medicinal plants for potential antibacterial activity. *Indian Journal of Pharmaceutical Science*, 68(6): 832-834.

Paulo, A., Pimentel, M. and Gomez, E. T. (1994). *Cryptolepis sanguinolenta* activity against diarrhoeal bacteria. *Journal of Ethnopharmacology*, 44(2): 73-77.

Prakash, O., Jyoti, Kumar, A., Kumar, P. and Manna, N. K. (2013). Adulteration and substitution in Indian medicinal plants. *Journal of Medicinal Plants Studies*, 1(4):127.

Puttaswamy, S., Lee, B., Amighi, B., Chakraborty, S. and Sengupta, S. B. (2012). Novel Electrical Method for the Rapid Determination of Minimum Inhibitory Concentration (MIC) and Assay of Bactericidal/Bacteriostatic Activity. *Biosensors and Bioelectronics*.

Rahalison, L., Hamburger, M., Hostettmann, K., Monod, M. and Frenk, E. (1991). A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical Analysis*, 2:199–203.

Ramazani, A., Zakeri, S., Sardari, S., Khodakarim, N. and Djadidt, N. (2010). *In vitro* and *in vivo* anti-malarial activity of *Boerhavia elegans* and *Solanum surattense Malaria Journal*. 9:124

Rang, H. P. (2005) Pharmacology. Edinburgh: Churchill Livingstone. pp 146

Rossi, G. C., Brown, G. P., Leventhal, L., Yang K., and Pasternal, G. W. (2005). Novel Receptor Mechanisms for Heroin and morphine-6-glucuronide analgesia. *Neuroscience Letters*, 216(1):1-4.

Salie, F., Eagles, P. F. K. and Lens, H. M. J. (1996). Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology*, 52(1): 27-33.

Saville, M. W., Lietzau, J., Pluda, J. M., Wilson, W. H., Humphrey, R. W., Fiegel, E., Steinberg, S. M., Broder, S. *et al*., (1995). "Treatment of HIV-associated Kaposi's sarcoma with Paclitaxel''. *The Lancet*, 346(8966): 26-8.

Schmelzer, G. H., Gurib-Fakim, A., Arroo, R., Bosch, C. H., de Ruijter, A., Simmonds, M. S. J., eds. (2008). Plant Resources of Tropical Africa 11(1) – Medicinal Plants 1. Backhuys Publishers, Wageningen, Netherlands. pp 237

Schmelzer, G. H., (2007). *Hilleria latifolia* (Lam.) H.Walt. In: Schmelzer, G. H. and Gurib-Fakim, A. (Editors). Prota 11(1): Medicinal plants/Plantes médicinales 1. PROTA, Wageningen, Netherlands.

Schmourlo, G., Mendonca-Filho, R. R., Alviano, C. S., Costa, S. S. (2004).Screening of antifungal agents using ethanol precipitation and bioautography of medicinal food plants. *Journal of Ethnopharmacology*, 96(3): 563-568.

Shah, G., Chawla, A., Baghel, S. U., Raha, S., Singh, S. P. and Dhawan, K. R. (2013). Pharmacognostic standardization of the leaves of *Melaleuca lecadendron*. *Pharmacognosy Journal*, 5: 143-148.

Silva, M. T. G., Simas, S. M., Batista, T. G. F. M., Cardarelli, P., Tomassini, T. C. B. (2005). Studies on antimicrobial activity, *in-vitro*, of *Physalis angulata* L.

(Solanaceae) fraction and physalin B bringing out the importance of assay determination. *Memorias do InstitutoOswaldoCruz*, 100(7): 779-782.

Sridhar, R. P. N. (2006). Antibiotic susceptibility testing, Department of Microbiology Jagadguru Jayadeva MurugEndra Medical College, Davangere (http://www.microrae.com).

Steinman, V. W. (2010). Neotropical Phytolaccaceae. In: Milliken, W., Klitgard, B. and Baracat, A. (2009 onwards). Neotropikey- Interactive key and information resources for flowering plants of the Neotropics.

Tadeg, H., Mohammed, E., Asres, K. and Gebre-Mariam, T. (2005). Antimicrobial activities of some selected traditional Ethiopian medicinal plants used in the treatment of skin disorders. *Journal of Ethnopharmacology*, 100: 168–175.

Tenover, F. C., Swenson, J. M., O'Hara, C. M. and Stocker, S. A. (1995).Ability of Commercial and Reference Antimicrobial Susceptibility Testing methods to detect vancomycin resistance in *Enterococci*. *Journal of Clinical Microbiology*, 33(6): 1524-1527.

Tepe, B., Donmez, E., Unlu, G., Polissiou, M. and Sokmen, A. (2004). Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbretet Aucher ex Bench.) and *Salvia multicaulis* (Vahl).*Journal of Food Chemistry*, 84(4): 519-525.

Tosun, F., Akyu¨z Kizilay, C., S¸ener, B. and Vural, M. (2005). The Evaluation of Plants from Turkey for *in vitro* antimycobacterial activity. *Pharmaceutical Biology*, 43(1): 58–63.

Ulubelen, A., (2003). Cardioactive and antibacterial terpenoids from some *Salvia* species. *Phytochemistry*, 64: 395-399.

Vanden-Berghe, D. A. and Vlientinck, A. J. (1991). Screening Methods for Antibacterial and Antiviral Agents from Higher Plants. In: Hostettmann, K. (Editor). Methods in Plant Biochemistry VI. Assays for Bioactivity, Academic Press: London, 47-69.

Verpoorte, R. (2000). Pharmacognosy in the new millennium: lead finding and biotechnology. *The Journal of Pharmacy and Pharmacology*, 52(3): 253-262.

Wiegand, I., Hilpert, K. and Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, 3(2): 163-75.

Woode, E. and Abotsi W. K. (2011).Antinociceptive effect of an ethanolic extract of the aerial parts of *Hilleria latifolia* (Lam.) H. (Phytolaccaceae). *Journal of Pharmacy and Bioallied Sciences*, 3(3): 384-396.

Woode, E., Abotsi, W. K. M. and Mensah, A. Y. (2011). Anxiolytic and antidepressant-like effects of an ethanolic extract of the aerial parts of *Hilleria latifolia* (Lam.) H. Walt. in mice. *Journal of Natural Pharmaceuticals*, 2(2): 62-71.

World Health Organisation, (2011). Fact sheet on antimicrobial resistance or Drug resistance.

World Health Organisation, (2000). Document on General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. Geneva.

World Health Organisation, (2002). WHO Traditional Medicine Strategy 2002-2005. Geneva.

World Health Organisation, (2014). Antimicrobial Resistance, Fact Sheet N°194.

World Health Organisation, (2003). Africa Malaria Report, 2003.

World Malaria Report, (2012). Fact Sheet –World Health Organisation.

Worrall, E., Basu, S. and Hanson, K. (2005). "Is malaria a disease of poverty? A review of the literature". *Tropical Health and Medicine*, 10(10): 1047–59.

Yasunaka, K., Abe, F., Nagayama, A., Okabe, H., Lozada-Pérez, L., López-Villafranco, E., Muñiz, E. E., Aguilar, A. and Reyes-Chilpa, R. (2005). Antibacterial activity of crude extracts from Mexican medicinal plants and purified coumarins and xanthones. *Journal of Ethnopharmacology*, 97(2): 283-299.

http://www.theplantlist.org/tpl/record/kew.html accessed 7th June 2014.

http://www.gbif.org/species/hilleria.html accessed 23rd July 2014.

http://www.henriettes-herb.com/ec;ectic/bpc1911/phytolacca.html accessed 3rd March, 2013.