KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

COLLEGE OF HEALTH SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

DEPARTMENT OF PHARMACEUTICS

ANTIMICROBIAL AND TOXICITY STUDIES OF *ERYTHROPHLEUM IVORENSE* (LEGUMINOSEAE) AND *PARQUETINA NIGRESCENS* (ASCELPIADACEAE)

BY

LOUIS ADU-AMOAH

MARCH, 2014

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BY

LOUIS ADU-AMOAH

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DECLARATION

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no materials previously published by another person nor material which has been accepted for the award of another degree of the University, except where due acknowledgement.

LOUIS ADU-AMOAH .....

(PG 5981111) Signature Date

Certified by

DR. CHRISTIAN AGYARE .....

Supervisor Signature Date

Certified by

PROF. K. OFORI-KWAKYE ..... Date

Head of Department Signature

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DEDICATION

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ABSTRACT

Erythrophleum ivorense and *Parquetina nigrescens* are plants found in the tropical regions and they are used in African traditional medicine to treat various ailments including wounds, boils and anaemic conditions. Some species of plant in the *Erythrophleum* genus are also known to be poisonous and toxic to several livestock. However, there is no information on the toxicity of *E. ivorense* and *P. nigrescens* has been reported to possess cardiotoxicity. This study is to determine the antimicrobial, cytotoxicity and subchronic toxicity properties of methanol leaf extract (EIML) and methanol stem barks extract (EIMB) of *E. ivorense* and methanol leaf and other aerial part extract of *P. nigrescens* (PNML). Agar well diffusion and micro-dilution methods were used to assess the antimicrobial activity of the methanol extracts against two Gram-positive and Gram-negative bacteria and a fungus. In the subchronic toxicity studies, rats were administered with 100, 300 and 1000 mg/kg body weight of the extracts (EIML, EIMB and PNML) for 35 days. Concentrations from 0.1 to 100 µg/mL of the extracts were used to determine the influence of the extracts on viability, proliferation and release of lactate dehydrogenase (LDH) on HaCaT keratinocytes. The extracts (EIML, EIMB and PNML) showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* NTCC 10073, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853 and clinical strain of *Candida albicans* at concentrations of 12.5 to 100 mg/mL with mean zones of inhibition ranging from 10 to 23 mm. The MICs of EIML, EIMB and PNML extracts ranged between 2.0 to 6.0 mg/mL against Gram-positive bacteria, 3.0 to 8.0 mg/mL against the Gram-negative bacteria and 2.0 to 4.0 mg/mL against *C. albicans*. Phytochemical screening of EIML, EIMB and PNML extracts revealed the presence of alkaloids, tannins, flavonoids, sterols, cardiac glycosides and terpenoids. HPLC finger-printing of the extracts (EIML, EIMB and PNML) showed chromatograms with peaks appearing after different retention times from 1 to 13 min for EIML, 1 to 5 min for EIMB and 1 to 9 min for PNML extracts and these can be used for quality

control purposes. In the sub-chronic toxicity studies, tissues from the kidney and liver of the rats treated with lower doses (100 to 300 mg/kg body weight) of EIML extract showed highly vascularized kidneys with numerous glomerular tufts, healthy hepatocytes and sinusoids in liver. However, there were persistent renal tissue inflammation and glomerular degeneration in kidney, and increased inflammatory infiltrates with few vacuolations, scarrings in liver in rats treated with higher extract dose of 1000 mg/kg body weight of rat. The rats treated with EIMB extract showed persistent renal and hepatocyte inflammations with glomerular and hepatocyte necrosis at all administered doses (100, 300 and 1000 mg/kg body weight) which are indications of renal and hepatic toxicities. Though rats administered with 100 and 300 mg/kg of PNML extract showed renal haemorrhage and inflammation and hepatic inflammation, the rats administered with 1000 mg/kg body weight showed restoring glomerular tufts and improved vasculature and liver with reduced inflammatory infiltrates with healthy hepatocytes. All extracts decreased the viability and proliferation of the HaCaT keratinocytes significantly ($p<0.05$) within concentrations of 10 to 100 µg/mL. There was also release of LDH from the HaCaT keratinocytes within 0.1 to 100 µg/mL but not statistically significant ($p>0.05$). Methanol extracts of *E. ivorense* and *P. nigrescens* exhibited toxic effects on kidney and liver cells of rats treated with extract doses of 100, 300 and 1000 mg/kg body weight for 35 days.

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ABBREVIATIONS AND PREFIXES

%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microliter
µm	Micrometre
ALT	Alanine aminotransferase
AS	Apoptotic space
AnST	Antimicrobial Susceptibility Tests
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under curve
BP	British Pharmacopoeia
BUN	Blood urea nitrogen
CBC	Complete blood count
CDC	Centre for Disease Prevention and Control
cfu	Coliform forming units
cm	Centimetre
CO_2	Carbon dioxide
CoNS	Coagulase-negative *Staphylococcus*
CV	Congested vein
DAEC	Diffusely adhering *Escherichia coli*
DG	Degenerating glomeruli
DMSO	Dimethylsulfoxide
EAggEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EIMB	*Erythrophleum ivorense* methanol bark
EIML	*Erythrophleum ivorense* methanol leaf
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*

EUCAST	European Committee for Antimicrobial Susceptibility Testing
FCS	Foetal calf serum
g	Gram
G	Glomeruli
h	Hour
H	Haemorrhage
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
HGB	haemoglobin
HN	Hepatocyte necrosis
HP	Hepatocytes
HPLC	High performance liquid chromatography
HT	Hyalinized tissue
IC	Infiltrating cells
IC	Inflammatory cells
IH	Inflamed hepatocytes
IT	Inflamed tissue
KNUST	Kwame Nkrumah University of Science and Technology
LDH	Lactate dehydrogenase
MBC	Minimum bactericidal concentration
MCH	mean corpuscular haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	mean corpuscular volume
MDR	Multidrug resistance
MFC	Minimum fungicidal concentration
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
MRVP	Methyl Red-Voges Proskauer
MTT	Methylthiazolyl tetrazolium chloride

NG	Necrotic glomerulus
nm	Nanometres
NNIS	National Nosocomial Infections Surveillance
NTCC	National Typed Culture Collection
OT	Occluding tubules
PAUC	Percentage area under curve
PNML	*Parquetina nigrescens* methanol leaf
RBC	Red blood cell
RnT	Renal tissue
RT	Regenerating tissue
RV	Reduced vacuolation
S	Sinusoids
SEM	Standard error mean
SN	Satellite necrosis
ST	Scar tissue
TLC	Thin layer chromatography
TV	Tissue vasculature
UNESCO	United Nations Educational, Scientific, and Cultural Organization
USA	United States of America
UV	Ultra violet rays
V	Vacuolation
WHO	World Health Organization

CHAPTER ONE

1.0 Introduction

Throughout history, medicine and medical care have been of great concern to mankind. The inclusion of plants in traditional medicines dates back to several thousands of years (Abu-Rabia, 2005) and predates the introduction of antibiotics and other modern drugs (Haslam *et al.,* 1989) due to their great source of bioactive compounds which are exploited in traditional and recent systems of medicines, pharmaceutical intermediates and chemical agents for synthetic drugs (Nath *et al.,* 2011).

These compounds have been employed in the development and manufacturing of several medicines which are used to treat various ailments including bacterial, viral, fungal and parasitic infections. In most developing countries, the use of medicinal plants is on the increase because medications made from them represent as a relatively cheaper option of medication in terms of cost and complexity (WHO 2002; Zirihi *et al.,* 2005).

The World Health Organisation (WHO) defines herbal medicines as finished, labelled medicinal products that contain as active ingredients, aerial or underground part of plants or other plant materials, or combination thereof, whether in the crude state or as plant preparations. Plant materials include juices, gums, fatty oils, essential oils and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant materials combined with chemically defined active substances, including chemically defined isolated constituents of plants are not considered to be herbal medicines (WHO, 1998).

Plants, like all other organisms employ enzyme-mediated chemical reactions or pathways in synthesizing several molecules needed to build up their tissues and ensure overall proper

functioning. These reactions expressed through metabolic pathways are classified as either primary metabolism or secondary metabolism with their associated compounds termed primary metabolites and secondary metabolites respectively. Plant primary metabolites are the compounds present in all plants and used in building and maintaining the cells (e.g. chlorophyll, adenosine triphosphate) whereas secondary metabolites including flavonoids, alkaloids, saponins are for defense mechanisms of the plant and therapeutic purposes (Kauffman *et al.,* 1999; Wink, 1999).

Easy availability, easy biodegradability, easy to handle, low cost, safety for mankind and environmental usage, greater acceptance amongst the users and minimum side effects have been some of the factors that have accentuated the usage of medicinal plants (Akharaiyi, 2011).

Plants and their constituents have found usage in manufacturing food supplements, flavouring agents (Hammer *et al.,* 1999; Adetuyi *et al.,* 2004; Popoola *et al.,* 1994). In 1998, United Nations Educational, Scientific and Cultural Organization (UNESCO) realized the growing usage of medicinal plants in industrialized countries as a result of their ability to extract and develop several drugs and therapeutics from them, and other traditionally used herbal remedies (UNESCO, 1998).

However, the recurrent usage of these plants and their products and their associated misuse have led to the development of resistant strains in organisms whose infections were previously so easily managed and cured (Norrby *et al.*, 2005). There has also been the production of a plethora of antibiotics to treat microbial infections but the most are also becoming redundant due to the upsurge of multi-drug resistant strains (Bandow *et al.,* 2003). *Streptococcus pneumonia, Streptococcus pyogenes* and Staphylococci, the organisms that cause respiratory and cutaneous infections, as well as Pseudomonads and members of the Enterobacteriaceae which cause diarrhoea, urinary tract infections, and sepsis, are now resistant to virtually all of the antibiotics that were used to treat them (Kayser *et al.,* 2005). It is therefore needful that new or modified

and more potent antibiotics be developed to combat the emergence of the intractable problem of microbial resistance.

1.1 Phytomedicine

The exploitation of plants for medicinal benefits is not of a recent vintage with the earliest known records traced back to the Ayurveda health system around 1500 BC, which is still practiced in India, Sri Lanka and other countries (Chopra, 2002). In various communities all over the world, the general indigenous folks as well as their traditional herbal medicine practitioners have employed various parts of different plants for the treatment of different diseases. In most of these societies, their medical systems have a close association with their unique traditional systems. These characteristic traditional systems of medicine are usually based on the people's use of natural and local products, which stems from how they view the universe and life (Toledo *et al.,* 2009).

For the purposes of developing safer and yet cheaper medications, there has been marked rise in the practice of complementary and alternative medicine in developing countries in response to World Health Organization directives. The end result has been the albeit several pre-clinical and clinical studies done on many of the plants used in folk medicine to treat infections so as to provide the scientific basis for their efficacy (Vijaya and Ananthan, 1997; Dilhuydy, 2003).

Today it is estimated that about 80% of the world's population especially people from developing countries depend on traditional medicines of plant origin (Nath *et al.,* 2011) with medicines derived from plant substances accounting for about 25% of all prescribed medicines (Zheng *et al.,* 2001; Egwaikhide *et al.,* 2007). Beyond the traditional belief systems of many communities there has been studies and documentation of the scientific basis for the efficacy of plants in phytomedicine (Okwu and Morah, 2007a; 2007b; Okoli *et al.,* 2007). The screening of medicinal plants has led to the discovery of several compounds with therapeutic or medicinal

properties (Harborne and Baxter, 1995), with some exhibiting activities against microorganisms (Okigbo and Igwe, 2007). These bioactive constituents obtained as a result of phytochemical screening of medicinally used plants include essential oils, alkaloids, glycosides, flavonoids, saponins (Bourgaud *et al.*, 2001). Dietary polyphenolic constituents derived from plants have been shown to be more effective antioxidants in *in vitro* studies than α - tocopherol and ascorbic acid and thus might contribute significantly to their *in vivo* protective effects (du Toit *et al.*, 2001). These antioxidants may offer effective protection against peroxidative damages caused by free radicals in living systems (Diplock *et al.*, 1998).

Despite the existence of potent antimicrobial agents, resistant or multi-resistant strains keep resurfacing, thus necessitating the need for the search and development of new antibiotics (Silver, 1993). It is therefore very necessary that the search for newer antibiotic sources be a continuous process.

1.1.2 Economic benefits of medicinal plants

About 20% of the population of the United States are known to be users of herbal remedies representing over $5 billion annually (Bedi and Shenefelt, 2002; Basch *et al.*, 2005; Bent, 2008). The United States of America in 1996 reported a 37% increase in the sales of botanical products or preparations over that recorded in the preceding year (Klink, 1997).

Aside the fundamental therapeutic relevance and importance of most plants, other uses have also been found for them. Some plants and their derived phytoconstituents are used as flavouring agents while others act as food additives and preservatives (Adetuyi *et al.*, 2004, Popoola *et al.*, 1994; Okwu, 1999, 2001). Some plants have been used in the manufacture of adhesives, textiles, dyes, paints, plastic and rubber industries (Adetuyi *et al.*, 2004). Jambolão which is jambolan

plum obtained from *Eugenia jambolana* is consumed in several forms such as pies, drinks, juices and wine (Viegas *et al.*, 2007).

1.1.3 Therapeutic benefits of plants

It is known that over 35,000 to 70,000 extracts from plants have been screened for their medicinal purposes (Farnsworth and Soejarto, 1991), yet there still exist the opportunities of isolating a large reservoir and repertoire of substances with manifold chemotherapeutic activities from plants yet to be studied. This opportunity when pursued can help in finding new and better substances that could serve as precursors for the development of more potent and effective drugs to combat the diverse range of existing diseases and even the recently emerging diseases (Rahman *et al.*, 2009).

The biological studies done on plants in the *Ochna* genus revealed that *Ochna macrocalynx* has cytotoxic activity (Tang *et al.*, 2003), while *Ochna afzelii* thwarts activity of beta lactamase enzyme (Gangoué-Piéboji *et al.*, 2007). Herbalists in Eastern Nigeria treat gonorrhoea, asthma, cough, skin ulcers, burns and wounds with decoction of *Datura metel* L. (Dabur *et al.*, 2004). Withanolide-like steroids isolated from Tunisian *D. metel* were treated against human lung carcinoma cells (A549) and human colorectal adenocarcinoma cells (DLD-1) and they were found to be cytotoxic as they blocked the S-phase in the cell cycle and induced apoptosis (Bellila *et al.*, 2011). Extracts of *Asclepias tuberosa* and *Cynanchum paniculatum* have been found to increase the proliferation of human skin fibroblasts (Hatani *et al.*, 2004; Kikuchi *et al.*, 2008). Though *Petersianthus macrocarpus* has been shown to have toxic effects, it is also found to be very useful in the treatment of infestation with *Loa loa* (Mengome *et al.*, 2010).

Methanol leaf extracts of *Eugenia jambolana* have been reported to have hypoglycaemic (Sundaram *et al.*, 2009), hepatoprotective (Sisodia and Bhatnagar, 2009), anti-ulcer (Chaturvedi

et al., 2007), anti-nematodal activities (Meyer *et al.*, 2008). They are also known to possess phototoxic, (Coutinho *et al.*, 2009), antibacterial (Coutinho *et al.*, 2010), modulation of antibiotic activity (Coutinho *et al.*, 2010) and antioxidant activity (Santos *et al.*, 2010) with secondary metabolites such as alkaloids, tannins, phlobatannins, triterpenes, and saponins being present in the leaves (Kaneria *et al.*, 2009). These natural products have found further usage in the treatment of diarrhoea and also show anti-inflammatory, astringent, carminative activities (British Herbal Pharmacopoeia, 1983).

Relatively cheaper and less toxic alternative therapies for sickle cell disease management have been found in the phytoconstituents from seeds of *Cajanus cajan* (Ekeke and Shode, 1985) and roots of *Zanthoxyllum macrophylla* (Sofowora *et al.*, 1975).

1.1.4 Toxicity of medicinal plants

Aside the medicinal values of some components of plants, some are also known to be toxic and harmful to humans and animals. Some alkaloids such as pyrrolizidine alkaloids have been shown to irritate to the gastro-intestinal tract. They also inhibit the enzyme cholinesterase and subsequently lead to drowsiness, salivation, laboured breathing, trembling, loss of consciousness, coma and death due to paralysis (Ahmad *et al.*, 1994; Mattocks, 1986; Mattocks and Jukes, 1987; Roberts and Wink, 1998).

Leaves of *Galega officinalis* has been shown to cause sub-endocardial haemorrhage and accumulation of alveolar capillaries and eosinophilic protein-rich oedema fluid in the lungs of animals. Galegine, a major compound in the plant has been implicated in these disorders (Keeler *et al.*, 1992).

Rats treated with ethanol extract of aerial parts of *Echinophora platyloba* have shown to exhibit intra-alveolar haemorrhagia, congestion of capillaries and alveolar, increase in relative lung

weight and lactate dehydrogenase (LDH) release (Mirghazanfari *et al.*, 2012). Oils of *Eucalyptus torquata* stems, leaves and *E. sideroxylon* leaves have been shown to exhibit cytotoxic activities on human breast adenocarcinoma (MCF7) cell line (Ashour, 2008).

1.2 Search for new antimicrobials

Many plants have been studied for various biological activities and the treatment of diverse range of ailments. The management of bacterial, fungal and to lower extent viral infections have been aided by the novelty of compounds isolated from plants and developed into various medications such as strychnine, aspirin, taxol are of plant origin (Agyare *et al.*, 2006).

However, over several years, the wide use and misuse of these drugs have led to a significant adaptation of the pathogens to the once effective drugs, resulting in resistant strains. Most of these pathogens have achieved their desired resistance by several mechanisms. The presence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens (Oluwatuyi *et al.*, 2004).

There can be structural and chemical modification of antimicrobial agents to which resistance has developed in the bid to extending their lifespan and improving their efficacy. Successes in this area have been reported with antifungals such as the azoles (Jeu *et al.*, 2003), antiviral agents such as the non-nucleoside reverse transcriptase inhibitors (De Clercq, 2001) and various antibacterial agents including β-lactams and quinolones (Poole, 2001).

It is therefore needful to discover and develop new compounds that could block or arrest resistance mechanisms and improve the treatment of infections from resistant strains (Oluwatuyi *et al.*, 2004; Sibanda and Okoh, 2008). Combination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity and to obtain synergistic antimicrobial activity. It is not surprising then that many pharmaceutical

companies are concentrating significant component of their effort on improving on the prominent categories of antimicrobial agents (Taylor *et al.,* 2002).

1.3 Microbial infections

It is reported that 70% of the over 2 million annual cases of microbial infections that occur in USA hospitals are as a result of strains of microorganisms that are resistant to at least one antibiotic (Alexandria, 2005). Resistance of microorganisms to antimicrobials has now become a worldwide problem (El Astal *et al.,* 2005). The United Kingdom reported that level of methicillin-resistant *Staphylococcus aureus* (MRSA) infections was low a decade ago but now accounts for approximately 50% of all *Staphylococcus aureus* isolates (Adcock, 2002).

The National Nosocomial Infections Surveillance (NNIS) system in the USA reported that by the year 2000, the prevalence of resistant *Pseudomonas aeruginosa* had increased to 17.7% for imipenem, 27.3% for quinolones and 26.4% for third-generation cephalosporins (Fridkin *et al.,* 1999).

This problem of microbial resistance needs to be curtailed so as to preserve lives and reduce the cost of treating infections.

1.3.1 Socio-economic impact of infectious diseases

One of the greatest reasons for all the efforts being put into the fight and eradication of diseases is the evident impact on communities. Diseases that arise from microbial infections (bacteria, fungi viruses and parasites) result in disabilities, mortality and adverse socio-economic impacts in the lives of several people (WHO, 2010). Antibiotics are a scarce resource ascurrent use decreases their future value due to perisistent problem of development of resistance by the

pathogenic bacteria. Since resistance is therefore not wholly eradicable it must be managed to reduce its associated socio-economic burden.

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In the United States of America, it was estimated in 1995 that about $ 4 billion is spent annualy on the treatment of bacterial infections and combatting the development of resistance by the pathogenic bacteria to used antibiotics (John and Fishman, 1997). The Centre for Disease Prevention and Control (CDC) estimated that about $180,000 is spent in treating multi-drug resistant tuberculosis (Rajbhandary, 2005) though about $ 13,000 – 30,000 is spent in the standard treatment of tuberculosis (Wilton *et al.*, 2001).

There is a wide spread of parasitic worm infestation throughout tropical and subtropical regions, with prevalence rate in some communities as high as 90% (Haburchak, 2008). Ghana Health Service in 2003 reported that parasitic worm infestation was the major cause of anaemia in children in Ghana with an 80% prevalence rate of anaemia among rural pregnant women and children. The burden of cost in eradicating many of these infectious diseases especially in developing countries impacts adversely on their already challenged economies and their general welfare (Bachmann and Booysen, 2003). As a result quite a number of these poor people shy away from seeking medical attention (Kamolratanakul *et al.*, 1999), making the infections they suffer from more pervasive and persistent. As a result the gap between health conditions and general living status of people in developed and developing nations keep widening (Hall and Taylor, 2003).

1.3.2 Mode of transmission of microbial infections

The transmission of these pathogenic microorganisms must be established before infection can occur. Parasites have several host(s) within which they move to complete their life cycles before establishing infections. Bacteria, fungi and viruses also are transmitted from one host to the other

and they may cause infections. These pathogenic infections of humans are achieved either directly or indirectly. Hosts may become affected through the direct transmissions route are as a result of:

- **direct skin or mucosal contact** with contaminated hands, body fluids, animals or objects; this includes sexual transmission of infection.
- **inhalation** of contaminated aerosols (e.g. *Legionella pneumophilia*) or droplets generated by sneezing, coughing, talking.
- **ingestion** of food or drink contaminated with infective eggs or larvae in the soil (e.g. hookworms) (WHO, 2010).
- **inoculation, penetration or injection** of contaminated fluids (e.g. during surgery or drug administration).
- **vertical transmission** from mother to baby (e.g. transplacental).
- **vector:** bites from flies, mosquitoes that are carriers of the pathogens (Brooks *et al.*, 2005).

1.3.3 Some Bacterial Infections

1.3.3.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium that divides three-dimensionally to form clumps or clusters of cells. It is non-motile, non-sporulating and facultative anaerobes which thrives well on most microbial media. Like all other species of this genus, *S. aureus* produces the enzyme catalase which is used in the diagnostic laboratory for rapid identification. *S. aureus* is the most virulent member of the genus and produces the enzyme coagulase. Coagulase-negative *Staphylococcus* (CoNS) describes all the other species in this genus and they are weaker pathogens as compared to *S. aureus* (Brooks *et al.*, 2005).

Structurally, the *Staphylococci* produce carbohydrate capsules and slime. The slimes are used to create a biofilm that gives protection to the organism from host immune or antibiotic attacks. *S. aureus* establishes its pathogenicity by the production of toxins that are harmful to its host. Examples include coagulase that clots plasma, enterotoxins that cause vomiting and diarrhoea, and hyaluronidase that degrades hyaluronic acid in connective tissues (Brooks *et al.,* 2005).

S. aureus is a major cause of nosocomial infections especially in intensive care units of hospitals (Weber *et al.,* 1999). It is reported that 67 to 90% of vascular access site infections of patients on haemodialysis are as a result of *S. aureus* and these can lead to septicaemia (Chow *et al.,* 1989; Yu *et al.,* 1986; Quarles *et al.,* 1985) and may cause metastatic infections in bones (osteomylitis), joint (septic arthritis), internal lining of the heart (endocarditis) (Francioli and Masur, 1982; Mathews *et al.,* 1980; Moxley *et al.,* 1980). *S. aureus* carriers are known to have blood stream infection at a rate four times faster than those who are non-carriers (Yu *et al.,* 1986) and causes significant morbidity and mortality (Nsouli *et al.,* 1979).

S. aureus has been implicated in a number of infections of the skin and tissues (boils, carbuncles, abscesses, impetigo, blood (septicaemia and toxic shock syndrome), brain (brain abscess), lungs (pneumonia) and gut (enterocolitis and food-poisoning) (Brooks *et al.,* 2005).

1.3.3.2 *Bacillus subtilis*

The *Bacilli* are species that form tough and durable spores which aid the organisms in surviving harsh environmental and nutritional conditions. *Bacillus subtilis* is a rod-shaped Gram-positive, catalase-positive, lactose fermenter, and spore forming bacteria that are found in the soil and vegetation. *B. subtilis* hardly produces any toxins and it is largely believed not cause virulent infections in humans (Edberg, 1991). However, probiotic strains of *B. subtilis* isolated from immune-compromised people have been implicated in recurrent septicaemia (Oggioni *et al.,*

1998). *B. subtilis* has also been isolated from surgical wound-drainage sites from a subphrenic abscess, breast prosthesis and from two ventriculo-atrial shunt infections (Logan, 1988).

1.3.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, oxidase-positive, non-lactose fermenting, strictly aerobic, long and cylindrical, Gram-negative bacillus. *P. aeruginosa* thrives in minimal carbon and nitrogen sources, including simple media and moist surfaces.

It is an opportunistic pathogen. It expresses antigenically diverse capsules (K antigen), lipopolysaccharides (O antigen) and flagella (H antigen). It adheres to its hosts and takes up DNA by means of its pili (outer membrane proteins, some of which act as receptors for human molecules). This enables it to secrete exotoxins which help in establishing its pathogenicity. They are found not to be susceptible to antiseptic solutions and many antibiotics (Brooks *et al.*, 2005). It is pervasive and can be found in human and animal gut and soil (Hall *et al.*, 1998), sewage (Wheater *et al.*, 1980; Schwartz *et al.*, 2006), rivers (Baleux and Troussellier, 1989), lakes (Sherry, 1986), and moist surfaces. It may cause nosocomial infections (Brooks *et al.*, 2005).

In 2003, the NNIS reported that *Pseudomonas sp.* was responsible for 18.1% of pneumonia infections, 3.4% of bloodstream infections, 9.5% of surgical site infections and 16.3% of urinary tract infections (Gaynes and Edwards, 2005).

1.3.3.4 *Escherichia coli*

Escherichia coli is a Gram-negative rod, catalase-positive, oxidase-negative, indole-positive, ferments mannitol and also produces gas from glucose. It is a haemolytic bacterium. The strains that cause diseases in the intestine are markedly distinct from those that are implicated in

extraintestinal diseases (Brooks *et al.,* 2005; Johnson and Stell, 2000). Six categories of this organism exist and these are enterotoxigenic *Escherichia coli* (ETEC), enteroinvasive *Escherichia coli* (EIEC), enterohaemorrhagic *coli* (EHEC), enteropathogenic *Escherichia coli* (EPEC), enteroaggregative *Escherichia coli* (EAggEC), and diffusely adhering *Escherichia coli* (DAEC) (Prescott *et al.,* 2002).

In 1995, a report by the Advisory Committee on the Microbiological Safety of Food showed *E. coli* infections were lower than that of *Salmonella* and *Campylobacter* infections. Large outbreaks of *E. coli* infections were recorded in the USA, Japan, Scotland and Australia (WHO, 1997). There were 17 cases of *Escherichia coli* (0157:H7) infections in Washington and California, USA in 1994 (CDC, 1995).

1.3.4 Fungal Infections

Fungal infections are referred to as mycoses and are categorized into five (5) groups according to the tissue of the host that is affected:

- superficial (scalp, beard, moustache, trunk, neck, face, arms)
- cutaneous (beard hair, scalp hair, smooth or bare parts of skin, groin, buttocks, feet and nails)
- subcutaneous (legs, feet, puncture wounds)
- systemic (lungs, bones, viscera, central nervous system, within phagocytes)
- opportunistic (respiratory, mucous membrane and sometimes brain) mycoses (Prescott *et al.*, 2002).

Immunocompromised persons suffer from many infections including candidiasis caused by *Candida albicans* and related species, oesophagus and mouth cryptococcosis by *Cryptococcus neoformans* and aspergillosis by *Aspergillus* species.

1.3.4.1 *Candida albicans*

Candida albicans which causes candidiasis is one of the commonest fungus that affect humans. It is a pervasive diploid sexual fungus (Hull *et al*., 2000; Soll *et al*., 2003) and it is commonly found on the gastrointestinal tract and mucus membrane of oral and vaginal regions of humans as commensals (Basma *et al.,* 2011; Cristina *et al.,* 2001; Molero *et al.,* 2010). The virulence attributed to *C. albicans* arises from its ability to switch from yeast to filamentous form, a transformation induced by temperature, pH, nutrient concentration, cell density or human serum (Sudbury, 2011). Immuno-compromised patients tend to battle with infections from *C. albicans* (Fichtenbaum *et al.,* 2000).

1.4 Methods for determination of antimicrobial activity

1.4.1 Susceptibility testing

Antimicrobial Susceptibility Tests (AnST) are the various laboratory techniques employed to study the susceptibility or otherwise of microorganisms to antimicrobial agents. Several methods exist to aid in the determination of microbial susceptibility and may lead to different results (Lampinen, 2005). There is a need to subject all methods to several standardized factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preferences. These precautionary measures for AnST methodologies, used in epidemiological surveillance of antimicrobial drug resistance are critical if data are to be compared among national or international surveillance and monitoring programmes. Screening of plant extracts or compounds must be done through standardized *in vitro* tests, with determination of minimum inhibitory concentration (MIC) of natural products and compounds currently used as antibiotics (Devienne and Raddi, 2002).

Diffusion and dilution methods are commonly used to determine antimicrobial activity. The diffusion method comprises of the agar well diffusion, the agar disk diffusion and bioautography methods. The dilution method comprises of the agar dilution and broth dilution methods.

1.4.2 Diffusion methods

Agar well diffusion technique

Molten agar stabilized at 45°C is aseptically inoculated with specific inoculums size usually 10^5 colony forming units per mL of test microorganism. The seeded agar is poured into a sterile petri dish and allowed to set. Wells are made at equidistant from one another using graded sterile cork borer of a desired diameter. A standard volume of different concentrations of the antimicrobial agent is poured in the labelled wells and allowed to stand for about 30 min or an h and then incubated at 37°C for 24 hour (Mbata *et al.,* 2006). The zones of growth inhibition are then recorded.

Agar disk diffusion technique

A major problem in the 1960s was the pluralization of modified methods resulting in the lack of standardized method for the determination of susceptibility of bacteria to antimicrobial agents. A detailed and careful review by Kirby and Bauer, of the existing susceptibility tests (Bauer *et al.,* 1966) laid the foundation for the work by WHO committee in 1961 to standardize the methods leading to the development of a standardized procedure for single antimicrobial disk susceptibility testing (Jorgensen and Turnidge, 2007) now called the Kirby-Bauer disk diffusion test (Bauer *et al.,* 1966). In this method a 6-mm filter paper disk is earlier impregnated with a known concentration of an antimicrobial compound (Lourens *et al.,* 2004; Salie *et al.,* 1996) and dried for 2 h, under a lamina air flow chamber, and placed on a seeded agar plate (Nostro *et al.,* 2000; Baris *et al.,* 2006).

The disk quickly absorbs water from the agar commencing the diffusion of the antimicrobial agent into the surrounding agar. The antimicrobial agent diffuses at relatively slower rate through the agar than the rate at which antimicrobial diffuses out of the disk. The result is the establishment of a high concentration of antimicrobial at points closest to the disk and a logarithmic reduction in concentration for distances further away from the disk (Jorgensen and Turnidge, 2007) and this is measured as zones of growth inhibition. The concentrations that produce them are preliminary (Jorgensen and Turnidge, 2007) and so *in vivo* testing of blood and urine samples are required to calculate the obtainable level of a given antimicrobial that may be effective against a particular infection. This information is juxtaposed with zones of inhibition to interpret the result (Wikler, 2006).

Bioautography

This is an expedient way for studying the effect of plant extracts and pure substances on (Hostettmann, 1999) microbial agents, human and plant pathogens. It can be used in the target-directed isolation of active constituents (Hostettmann, 1999). Bioautographic assays include paper and thin-layer chromatography (TLC) methods. The TLC bioautographic methods include agar diffusion, direct TLC bioautographic detection and agar-overlay (Rios *et al.*, 1988). The direct bioautography is useful for microorganisms that can grow directly on the TLC plate, examples including *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. (Homans and Fuch, 1970) and also for some bacteria (Hamburger and Cordell, 1987), where well defined zones of inhibition can be seen from organisms that are susceptible to the antimicrobial agent. A developed TLC plate of the antimicrobial agent is placed on the seeded agar. The active compounds in the antimicrobial agent diffuse from the stationary phase to the layer of the seeded agar and then incubated. After incubation, the plate is sprayed with a tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) which is converted to a formazan

dye by the living microorganism. The zones of inhibition are observed as clear spots against a purple background (Zheng *et al.*, 2005).

Susceptibility of the fungus *Candida albicans* and bacteria such as *Bacillus subtilis* have also been studied using the agar-overlay procedure where the developed TLC plate is placed on part of seeded agar that is poured and allowed to set before the remaining volume of the seeded agar is poured over it (Rahalison *et al.*, 1991). If phenol red is incorporated into media containing 0.6% agar and the plates are sprayed with MTT, clearer results are obtained, with dark red coloured inhibition zones appearing against a blue background. This method works successfully with a range of microorganisms including *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Saxena *et al.*, 1995). The agar-overlay technique is a hybrid of the two other methods and is applicable to a broad spectrum of microorganisms.

1.4.3 Dilution methods

Agar well dilution technique

The agar dilution method is more adaptable than the broth dilution method and is devoid of difficulties like sample solution, contamination and determination of MIC breakpoints (Silva *et al.*, 2005). A stock solution of the extract is prepared in its extracting solvent and filter-sterilized. The stock is then incorporated in molten agar and cooled to 50°C in a water bath, to obtain different concentrations of the extract in the agar. Although Muller-Hinton agar has always been the agar of choice (European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2003) nutrient agar has also been used (Grierson and Afolayan, 1999; Afolayan and Meyer, 1995). Different researchers have suggested overnight inoculum preparation of culture dilutions of 1:100 (Afolayan and Meyer, 1995) or 1:10 (Meyer and Dilika, 1996) in broth. EUCAST (2003) recommends an inoculum density of about 10^7 colony forming units per millilitre (cfu/mL) and using replicator pins, micropipette or standard loop to transfer about 1 µL (10^4

cfu/mL) of the inoculum. In some reports, the seeded plates are left overnight before streak, to allow the solvent to evaporate (Grierson and Afolayan, 1999). The organisms are streaked in radial patterns on the agar plates and incubated at 37°C for 24 to 48 h. The MIC is defined as the lowest concentration of the extract inhibiting the visible growth of the test organisms on the agar plate (Nostro *et al.*, 2000; Hammer *et al.*, 1999).

Broth dilution methods

These methods were the earliest methods developed to study the susceptibility of microorganisms to antimicrobial agents although they were relatively tedious (Kirby *et al.*, 1957; Jorgensen and Turnidge, 2007). However, there is increased sensitivity for small quantities of the antimicrobial agent. It helps to distinguish between bacteriostatic and bactericidal effects and it can be used to determine the MIC of the antimicrobial agents (Langfield *et al.*, 2004). This method is adaptable to a wide range of microorganisms. It is not expensive to repeat and the results are reproducible. A stock solution of the antimicrobial agent is prepared in a suitable solvent, ideally that used in the extraction provided it does not exhibit antimicrobial activity (Grierson and Afolayan, 1999) or in dimethylsulfoxide (DMSO) (Salie *et al.*, 1996; Nostro *et al.*, 2000; Baris *et al.*, 2006). The wells of the micro-titre plate are filled with equal volumes of broth. Different volumes of stock antimicrobial agent are added to the wells and different volume of sterile water as diluents added to create different and graded concentrations of the antimicrobial agent. Lastly, an equal volume of the microbial culture is added to the wells and incubated at 37°C for 24 h (Lourens *et al.*, 2004) or 35–37°C for 16 to 20 h for non-fastidious organisms (EUCAST, 2003). The standard inoculum size for this method is usually 10^6 cfu/mL (Lourens *et al.*, 2004; Basri and Fan, 2005). Microbial culture with an optical density of 0.4 (log-phase) at 620 nm or a 12 h broth culture with extent of turbidity as a 0.5 McFarland turbidity standard may be used as inoculum size (Baris *et al.*, 2006). The set-up is not made to stand

beyond 30 min of standardization of inoculum otherwise there is the risk of changes in inoculum density (EUCAST, 2003).

Changes in turbidity are indicators of microbial growth with the lowest concentration of the well showing no growth recorded as the MIC of the antimicrobial agent. Indicators like tetrazolium salts or resazurin dye are added after the incubation period and left for about 6 h and changes in colour or absence of colour is noted (Umeh *et al.,* 2005). Other researchers also use spectrophotometry to measure absorbance at wavelength of 620 nm to assess presence of growth in wells of microtitre plates (Devienne and Raddi, 2002; Matsumoto *et al.,* 2001) with the concentration that shows sharp decline in the absorbance value (Devienne and Raddi, 2002) or the lowest concentration which gives a zero absorbance reading (Salie *et al.,* 1996) is deemed to be the MIC. The preparations that do not show growth are subcultured into broth. The broth which would show no growth is recorded as the minimum bactericidal concentration (MBC).

1.5 Toxicity

Toxicity is classified into four (4) levels mainly: acute, subacute, chronic and subchronic. Acute toxicity is the toxic effect realized on the administration of small quantities of the medicinal agent over a brief time. This shows the possible hazardous effects that would arise in a short exposure to the therapeutic agent and thus serves as a fundamental screening test for the possible behaviour of plants (Shetty *et al.,* 2007). This would help in the establishment of therapeutic index (the ratio between the lethal dose and the pharmacologically effective dose in the same strain (LD 50/ED 50). A greater index is indicative of a safer drug.

In the determination of chronic toxicity, small doses of the medicinal agent is administered to the laboratory animals over an extensive period of time, sometimes even up to a year or two years. This procedure determines the effect of long time exposure of the animals to the therapeutic agent. In subchronic toxicity studies, smaller doses are administered to the test animals over a

long period of time about 1 to 3 month and signs of toxicity studied and measured (Nsiah *et al.*, 2006).

Several blood parameters such as haemoglobin (HGB), packed cell volume (PCV), white blood cell (WBC), red blood cell (RBC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCH) are monitored. The general biochemistry is also studied where levels of lymphocytes, neutrophils, eosinophils, platelets, triglyceride and other parameters are studied for indication of toxicity or otherwise.

1.6 Cytotoxicity

The predictive value of *in vitro* cytotoxicity tests is based on the idea of 'basal' cytotoxicity, that the basic functions of cells are susceptible to toxic chemicals and that the toxicity can be measured by assessing cellular damage. The usefulness of medicinal plants for the treatment of diseases is a common knowledge. However, possible side effects and toxicity of their components must not be overlooked. The need to rapidly evaluate the potential toxicity of these compounds has led to the development of various cytotoxicity assays (Barile *et al.*, 1994; Davila *et al.*, 1990; Todd *et al.*, 1999). This has informed many pharmaceutical companies involved in research and development of drugs to screen compound libraries to remove potentially toxic compounds early in the drug discovery process. Studies that reveal toxic effects can help in the prioritization between chemical series, and identify structure-toxicity relationships (Todd *et al.*, 1999) and this will eventually reduce the cost in the research and development of medicines.

Three (3) basic parameters are considered in the study of cytotoxic effects of medicinal plants. The first assay is the measurement of cellular metabolic activity where a reduction in the overall activity could be indicative of cellular damage. This can be done by the measure of cellular ATP

levels or mitochondrial activity (via (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) metabolism).

The second parameter is the assessment of membrane integrity of cells. Cell membrane forms functional and protective barrier around cells and regulates movement of substance into and out of the cell via transporters, receptors and secretion pathways. When membrane integrity is compromised owing to cellular damage, the cells become 'leaky'. The result is a gradual increase in the release of lactate dehydrogenase (LDH) into the extracellular medium which is indicative of the cellular damage since this enzyme is normally present in the cytosol and cannot be measured extracellularly unless cell damage or apoptosis occurrs (Decker and Lohmann-Matthes, 1988). Other assays measure the uptake of fluorescent dye (ethidium homodimer) normally excluded from intact cells. Also the absorption of dissolved formazan in the visible region correlates with the number of intact living cells. Cytotoxic compounds are able to damage and destroy cells and thus decrease the reduction of MTT to formazan (Mossman, 1983).

Since dead cells normally detach from a culture plate, and are washed away in the medium, a direct measure of cell numbers through counting can be done. The measurement of total cell protein or DNA in a cell is proportional to the number of intact cells under study. These changes in metabolic activity serve as indicators of early cell injury while effects on membrane integrity are indicative of more serious injury, leading to cell death (Davila *et al.*, 1990).

1.7 Justification of study

The frequent and wide spread use and misuse of several drugs including antimicrobials agents is gradually creating an obdurate problem of combating diseases. Some of these microorganisms responsible for various infections have developed resistance to once effective antimicrobials,

thus there is a need to research into and develop new antimicrobials that would be potent against these emerging resistant pathogens (Chambers, 2006).

Antimicrobial agents are among the most commonly used and misused of all medications. The inevitable consequences of their wide spread use has been the emergence of antibiotic resistant pathogens, fuelling an ever increasing need for new antimicrobials (Chambers, 2006). Herbal remedies used in traditional folk medicine provide an interesting and a large source for the potential development of new drugs for chemotherapy which might help to overcome the growing problem of drug resistance and the severe side effects associated with some antibiotics (Al-wadh *et al.,* 2001).

In spite of the advantageous health properties of the biologically active compounds of most plants, some have been implicated as having other detrimental effects (Adeoye and Oyedapo, 2004). Several species of *Erythrophleum* which include *E. guineense, E. lasicanthum, E. chlorostachys* and *E. africanum* are known to be poisonous (Griffin *et al.,* 1971; Loder *et al.,* 1974). The leaves and roots of *Parquetina nigrescens* are used as poultice to treat wounds, boils, carbuncles, and worm infections in ethno-medicine (Agyare *et al.,* 2009).

Nigrescigenin and g-strophanthin are cardiac glycoside (cardenolides) that have been isolated from *P. nigrescens* by Berthold *et al.* (1965) and Brandt *et al.* (1966). Diverse concentrations of cardiac glycosides that are administered are known to elicit positive inotropic effects on isolated myocardial tissue (Coraboeuf *et al.,* 1953; Brauwald *et al.,* 1961; Akera, 1977; Bentfeld *et al.,* 1977). Contractile force of isolated atria of guinea-pigs was shown to increase upon the administration of *P. nigrescens* (Aworet-Samseny, 2011).

The aims of the study is to determine the antimicrobial activity and toxicity profile of the methanol leaf and stem bark extracts of *Erythrophleum ivorense* (A Chev.) and methanol leaf and other aerial parts extracts of *Parquetina nigrescens* (Afzel) Bullock.

1.8 Main aim

To determine the antimicrobial, cytotoxic and subchronically toxic (*in vivo*) activities of methanol leaf and stem bark extracts of *Erythrophleum ivorense* and methanol leaf and other aerial parts extract of *Parquetina nigrescens*.

1.8.1 Specific objectives

- To prepare methanol leaf and stem bark extracts of *E. ivorense*, and methanol leaf and other aerial part extracts of *P. nigrescens* using the cold maceration method.
- To perform preliminary phytochemical screening of the methanol extracts of *E. ivorense* and *P. nigrescens.*
- To determine antimicrobial activity of the methanol extracts against selected bacteria and fungus.
- To determine the minimum inhibitory and bactericidal concentrations of the extracts of *E. ivorense* and *P. nigrescens* against the test organisms.
- To determine the cytotoxicity of the methanol extracts of *E. ivorense* and *P. nigrescens* on HaCaT keratinocyte cells.
- To determine the *in vivo* sub-chronic toxicity of the *E. ivorense* and *P. nigrescens* extracts in Wistar rats.

1.9 Plant materials

1.9.1 *Erythrophleum ivorense* (A Chev.)

Description

Erythrophleum ivorense (A Chev.) is a large tree found growing in tropical regions in Africa including Cameroon, Central African Republic, Gabon, Ghana, Cote d'Ivoire, Liberia, Sierra Leone, Tanzania, Congo DR and Zambia. It is known as "Potrodom" in Asante-Twi in Ghana

and sasswood or ordeal tree in English. It is also described as the 'ugly' plant. It can grow up to 40 m tall, usually bole cylindrical, but it may occasionally be fluted at the base, with or without buttresses at old age. The diameter is usually 60 to 90 cm wide. The outer bark is predominantly scaly, often fissured and grey in appearance. The inner bark is reddish (thus its sap's name 'red-water') and granular. Its young twigs are brown and hairy. It possesses alternate, bipinnately compound leaves with 2 to 4 pairs of pinnae. The stipules are minute, petiole is 2 to 7 cm long, and the rachis often 5 to 15 cm long. The leaflets alternate, having 6 or 8 to 14 per pinna. These leaflets are elliptical to ovate in shape and up to 8.5 cm by 4 cm. Their bases are asymmetrical with shortly acuminate apex (Irvine, 1961; Burkill, 1995).

There has been reports of toxicity associated with the *Erythrophleum* species to livestock. Several of its species which include *E. ivorense, E. guineense, E. lasicanthum, E. chlorostachys, E. suaveolens* and *E. africanum* are believed to be poisonous (Watt and Bayer-Brnadwyle, 1962; Griffin *et al.,* 1971; Loder *et al.,* 1974).

Economic uses

The bark of *E. ivorense* is traded as 'sassy-bark' and 'casca bark'. They are employed in constructional works, used as fuel and lightings throughout the world. A bark decoction, when added to palm wine makes it a powerful alcoholic drink, a usual practice in Sierra Leone (Bosch, 2006).

Medicinal uses

In Sierra Leone, bark extract is given as emetic and laxatives. It is also used to treat pains (Loder *et al.;* 1974*).* Treatment of small pox is done in Cote d'Ivoire by rubbing crushed bark of young branches on infected skin. The barks is used as ordeal poisons in hunting in Liberia and Gabon, and as fishing poison in Sierra Leone (Dongmo *et al.,* 2001). Menstrual cycle complications and

pulmonary diseases are managed by decoctions from leaves and the bark of its twigs, respectively (Burkill, 1995).

Chemical constituents

Early investigations of *E. ivorense* led to the discovery of erythrophleine, an alkaloid (Bosch, 2006). The alkaloidal constituents are esters of tricyclic diterpene acids, and are of two (2) main types (i.e. dimethylaminoethylesters: made up of cassaine, cassaidine and erythrophleguine. Studies have shown that though the alkaloidal content in the seeds is less than that in the stem barks, there is a higher level of toxicity in the seeds than in the stem bark (Bosch, 2006).

Biological activity

Adeoye and Oyedapo (2004) reported that there was reduction in the activity of plasma alanine aminotransferase and liver aspartate aminotransferase and concentration of liver glycogen, liver protein, creatinine and haemoglobin in an *in vivo* administration of total alkaloidal fractions of the stem bark of *E. guineense* in Sprague-Dawley rats. The alkaloids such as cardenolides (digitoxine and ouabain) have a stimulant effect on the heart of animals. The dimethylaminoethylesters (cassaine) exhibited strong anaesthetic and diuretic effects, increased contractions of the intestine, uterus, and heart muscles while cassaidine caused depressive effects (Cronlund, 1976).

Fig. 1: Leaves and stem bark of *E. ivorense*

Source: http://www.help-congo-stories.org/index.php/la-foret-tropicale-de-conkouati/un-peu-de-botanique/les-arbres/251-erythrophleum-ivorense

1.9.2 *Parquetina nigrescens* (Afzel) Bullock

Description

Parquetina nigrescens (Afzel) Bullock belongs to the family Ascelpiadaceae. It is a slender, glaborous twining shrub that grows up to tops of forest trees. The leaf is glaborous, broadly oblong-elliptic, caldate-acuminate base rounded to subcordate, glossy and glaucous below. It is present in low bushes in savannah areas, farm clearings in forests and transition forests in West African countries including Ghana and Cote d'Ivoire (Irvine, 1961).

Medicinal uses

P. nigrescens has been extensively used in traditional medicine in West Africa to treat variety of disorders including rheumatism, boils and wounds (Angenot 1934; Watt and Breyer-Brandwijk 1962; Bouquet 1967, 1972; Ogundaini and Okafor, 1987). In Eastern region of Cote d'Ivoire, an aqueous extract of the leaves is used as an oral anti-asthma remedy. Poultice of leaves and roots are used to treat wounds, boils, carbuncles, worm infections and stomach problems in Ghana (Agyare *et al.,* 2009; Adeyemi, 1994).

The decoction of the leaves of *P. nigrescens* is used to treat intestinal worm infections (Agyare *et al.,* 2009). It is also used as poisons against the larvae of beetles in the Kalahari Desert in southern Africa (Neuwinger, 1995). The powdered root is applied to treat bites and rheumatism in Ghana Adeyemi (1994). Latex obtained from the plant is used for skin diseases (Dokosi, 1998; Irvine, 1961).

Chemical constituents

Nigrescigenin, periplogenin and g-strophanthin are some bioactive constituents that have been isolated from the leaves, seeds and roots (Schenker *et al.,* 1954; Mauli and Tamm 1957; Berthold

et al., 1965a, b; Brandt *et al*., 1966; Marks *et al.*, 1975). Other studies into the fractions of root extracts showed the existence of three cardenolides namely cymarin, strophanthidine and strophanthidine glycosides (Marks *et al.*, 1975).

Biological activity

The aqueous root extract is used to treat anaemia (Agbor and Odetola, 2001; Erah *et al.*, 2003). Aqueous leaf extract have analgesic, anti-inflammatory and antipyretic effects (Owoyele *et al.*, 2009). Hydro-methanol leaf extracts of the plant have oxytocin-like effects as it stimulates increased uterine contraction by mobilizing extracellular calcium (Datté *et al.*, 1996). The plant also has cardiotonic and catecholamine-like effects (Datté and Ziegler, 2001). *P. nigrescens* root extracts have been found to possess anti-sickling properties (Kade *et al.*, 2003; Imaga *et al.*, 2010). Pharmacological investigations indicated that leaf extract of *P. nigrescens* increases the contractile response in isolated myometrium smooth muscle of pregnant rat (Datté *et al.*, 1996). Moreover, the aqueous extract of the plant has a stimulant effect in guinea-pig isolated auricles (Datté and Ziegler 1998; Datté *et al.*, 1999).

Fig. 2: Leaves and tendrils of *P. nigrescens*

Source: http://www.medicinalplantsinnigeria.com/plantgalleryp.php

CHAPTER TWO

2.0 Materials and methods

2.1 Materials

2.1.1 Plant materials

Dried leaves and stem bark of *Erythrophleum ivorense*

Dried leaves and tendrils of *Parquetina nigrescens*

2.1.2 Animals used

Wistar rats (weighing 135 to 200 kg)

2.1.3 Chemicals

Name	Manufacturer/Source
Acetic anhydride 99.9% v/v	GPR, BDH, Poole, UK
Chloroform 99.9% v/v	GPR, BDH, Poole, UK
Ethanol 96% v/v	Scharlau Chemicals Ltd, Poole, England
Ethyl acetate 99.8% v/v	GPR, BDH, Poole, UK
Glycerol	GPR, BDH, Poole, UK
Methanol 99.9% v/v	GPR, BDH, Poole, UK
Petroleum ether	GPR, BDH, Poole, UK
Hydrogen peroxide	Ernest Chemist Ltd, Accra, Ghana
Acetone 99.8% v/v	BDH, Prolabo, France.
Fehling's solution	GPR, BDH, Poole, UK
Sulphuric acid 98.5% v/v	GPR, BDH, Poole, UK
Hydrochloric acid 36% v/v	GPR, BDH, Poole, UK
Sodium Hydroxide	GPR, BDH, Poole,UK
Aqueous Ammonia	Merck Chemicals Ltd, UK

Iron (II) Chloride	GPR, BDH, Poole,UK
Methylthiazolyl tetrazolium chloride (MTT) dye	Sigma-Aldrich, Michigan, USA
Dietary pellets	GAFCO, Tema, Ghana
Defribrinated horse blood	Oxoid Ltd, Basingstoke, UK.
Dragendorff	Merck Chemicals Ltd, UK

2.1.4 Reference compounds	**Manufacturer/Source**
Ciprofloxacin hydrochloric powder 99.8% w/w	Zhejiang Xin Ltd, China.
Ketoconazole powder 99.9% w/w	Aryton, Drug, Accra, Ghana.

2.1.5 Instrument and Equipment/Glass ware	**Manufacturer/Source**
Rotary evaporator (R-210)	Buch, Germany.
AutoFlow NU-8700 Dual Chamber Water Jacket CO_2 Incubator	Nuaire Corporation, Plymouth, Minnesota, USA
Hot air oven	Sanyo, OMT Oven, Weiss Technik, UK
Gallenkamp Plus II cooled incubator	Weiss Technik, UK
Laminar air flow cabinet Model T2 2472	Skan AG, Switzerland
Thermostatically controlled water bath R 76	New Brunswick, Edison New Jersey, USA
UV Spectrophotometer	Cecil, 2000 series, Basildon Ltd, UK
Electronic weighing balance	Ohaus Corporation, Pine Brook, NJ, USA.
Portable autoclave	Basildon Ltd.UK.
No. 5 cork borer	Gerber Instruments, AG, Holland
Test tubes	Fisher Scientific GmbH, Schwerte, Germany
1ml, 10ml dropping pipettes	Fisher Scientific GmbH, Schwerte, Germany

250ml, 500ml conical flask	Fisher Scientific GmbH, Schwerte, Germany
Beakers (50ml, 250ml,500ml, 600ml, 1L)	Fisher Scientific GmbH, Schwerte, Germany
Petri dish	Fisher Scientific GmbH, Schwerte, Germany
Frost free freezer	Mistral, UK
Laboratory Milling Machine	Christy and Norris Ltd, Chelmsford, UK.
Haematology autoanalyzer	Hitachi 7060, Japan
Refrigerator	Sharp Corporation, UK
HPLC pump	Spectra System, USA
HPLC column 250 x 4.6 mm (5 microns)	Spectra System, USA
UV 1000 Detector	Spectra System, USA
SCM 1100 Degasser	Spectra System,USA
Filter paper Whatmann 10	Sigma-Aldrich, Michigan, USA
ELISA cell proliferation test kit	Roche, Switzerland
CBC bottles containing EDTA-2K	Sewon Medical Co., Republic of Korea
Microtitre reader	Thermo Scientific, Asheville, NC, USA
Light Microscope	Diagnostic Instruments, London, UK
Incubator	Thermo Scientific, Asheville, NC, USA

2.1.6 Culture media

Nutrient agar	Oxoid Ltd, Basingstoke, UK.
Nutrient broth	Oxoid Ltd, Basingstoke, UK.
Sabouraud agar	Oxoid Ltd, Basingstoke, UK.
Sabouraud broth	Oxoid Ltd, Basingstoke, UK.
Koser's citrate	Oxoid Ltd, Basingstoke, UK.

Macconkey agar	Oxoid Ltd, Basingstoke, UK
Plate Count Agar	Oxoid Ltd, Basingstoke, UK.
Methyl Red Voges Proskauer (MRVP) broth	Oxoid Ltd, Basingstoke, UK.
Mannitol salt agar	Oxoid Ltd, Basingstoke, UK
Cetrimide agar	Oxoid Ltd, Basingstoke, UK
Potato dextrose agar	Oxoid Ltd, Basingstoke, UK
Tryptone broth	Oxoid Ltd, Basingstoke, UK
HaCaT keratinocytes medium (DMEM medium (high glucose) supplemented with 2mM L-glutamine and 10% foetal calf serum	Life Technologies Corporation, USA
Phosphate buffer solution	Sigma-Aldrich, Disendorf, Germany
Triton X-100	Sigma-Aldrich, Disendorf, Germany
Lysis buffer	Clontech Laboratories, Inc., USA

2.1.7 Skin cells

HaCaT keratinocytes were provided by Prof. Dr. Norbert E. Fusenig, Division of Differentiation and Carcinogenesis, German Cancer Research Centre (DKFZ), Heildelberg, Germany

2.2 Methods

2.2.1 Collection and identification of plant materials

The leaves and barks of *E. ivorense* were collected from the Botanic Garden, University of Ghana, in November 2011 by Mr. John Yaw Amponsah. The leaves and tendrils of *P. nigrescens* were collected by Mr. Eric Gyebi from Jachie in the Bosomtwi District of the Ashanti Region in December 2011. The plant materials were authenticated by Dr. Alex Asaase of the Department of Botany of University of Ghana, Legon. Voucher specimens of the plant materials have been kept in the Ghana Herbarium, University of Ghana, Legon, Accra.

2.2.2 Extraction of plant materials

The plant materials were washed with tap water, air dried at room temperature (25-30°C) for 6 days and then powdered using a laboratory milling machine. Eight hundred grams (800 g) of the powdered leaf of *E. ivorense,* and 650 g of powdered bark of *E. ivorense* and 700 g of powdered leaf and other aerial parts of *P. nigrescens* were weighed and each soaked in 2.5 L of 70% methanol in a stoppered container. These were shaken for about 5 min and left to extract by means of maceration for 72 h (shaking the mixture intermittently) (Parekh *et al.,* 2005). The mixtures were filtered using a fine mesh into a porcelain crucible. The filtrates were then lyophilized and placed in air-tight containers and labeled as *E. ivorense* methanol leaf (EIML), *E. ivorense* methanol bark (EIMB), and *Parquetina nigrescens* methanol leaf and other aerial parts (PNML) extracts. The extracts were stored in a dessicator.

2.2.3 Phytochemical screening of methanol extract

2.2.3.1 Test for alkaloids

Two hundred milligram of the methanol extracts (EIML, EIMB and PNML) was dissolved in 5 mL dilute HCl in a steam bath and filtered. To a 1 ml of this filtrate four drops of Mayer's reagent were added. A cream or pale yellow precipitate that is observed indicates the presence of alkaloids.

Four drops of Dragendoff's reagent were added to another 1 ml of filtrate. The formation of an orange precipitate indicates the presence of alkaloids (Akinyemi *et al.,* 2005).

2.2.3.2 Test for tannins

Five hundred milligram the methanol extracts (EIML, EIMB and PNML) was boiled in 20 mL of water in a test tube and then filtered. One millilter of 5% ferric chloride was added to 2 mL of

the aqueous filtrate and observed for colouration. A brownish green or a blue-black colouration indicated the presence of tannins (Parekh *et al*., 2007).

2.2.3.3 Test for saponins

Two hundred milligram of the methanol extracts (EIML, EIMB and PNML) was boiled with 20 mL of distilled water in a water bath and filtered. Ten (10) mL of the filtrate was transferred into a test tube and tightly corked and shaken vigorously for a stable persistent froth or foam. The persistence of the froth for 5 min gives an indication of the presence of saponins (Nsiah and Opoku, 2005).

2.2.3.4 Test for flavonoids

Five hundred milligram of the methanol extracts (EIML, EIMB and PNML) was dissolved in distilled water and filtered. Two milliliters of dilute ammonia solution was added to 5 mL of the aqueous filtrate and then followed by 1 mL (0.5M) concentrated H_2S0_4. The presence of a yellow colouration indicated the presence of flavonoids (Martinez, 2003).

2.2.3.5 Sterols

Two hundred milligram of the methanol extracts (EIML, EIMB and PNML) was dissolved in 5.0 mL of chloroform and filtered using a filter paper. One milliliter of ethanolic acetic anhydride was added 2.0 mL of the chloroform filtrate. Two milliliters of concentrated H_2S0_4 was then added down the side of the test tube till the formation of a basal layer. A bluish – green ring at the interface indicates the presence of steroids (Akinpelu *et al*., 2008).

2.2.3.6 Test for cardiac glycosides (Keller-Killani test)

To 2 mL of the methanol extracts (EIML, EIMB and PNML), 1 mL glacial acetic acid and 2 drops of $FeCl_3$ were added. One millilitre of concentrated H_2SO_4 (0.5M) also was gently added to the mixture. The appearance of brown ring at the interface indicated the presence of (deoxysugar characteristic of cardenolides) cardiac glycosides. A violet ring also appeared below the brown ring (Trease and Evans, 1989).

2.2.4 HPLC Finger-printing of methanol extracts

Modified methods of Srivastava *et al.* (2004) and Ding *et al.* (2011) was used to determine the HPLC finger-printing of the methanol extracts (EIML, EIMB and PNML) using a reverse phase Jupiter C_{18} 300R column (250 x 4.6 mm). Concentrations of 10 mg/mL of extracts were prepared and a volume of 10 µL injected into the columns with a mobile phase of methanol-water (3:7 v/v). The run time for the column of extracts was 10 min at 22°C under a pump pressure of 21MPa and flow rate of 1.0 mL/min. The resultant chromatograms were observed at a wavelength of 254 nm. The retention times and area under curve of the chromatograms were then determined.

2.2.5 Test organisms

2.2.5.1 Typed bacterial strains

Staphylococcus aureus ATCC 25923, *Bacillus subtilis* NTCC 10073, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 4853, were obtained from the Microbiology Research Laboratory, Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana.

2.2.5.2 Clinical strain

Clinical strain of *Candida albicans* was obtained from the Bacteriology Unit, Komfo Anokye Teaching Hospital, Kumasi, Ghana.

2.2.5.3 Standardization of microbial inoculum size

The inoculum size of the test organisms was determined by measurement of absorbance of suspensions of cultures using the ultraviolet spectrophotometer (Moosdeen *et al.*, 1988). The test organisms were subcultured in 10 mL nutrient broth and incubated at 37ºC (for bacteria) and 25ºC (for *Candida albicans*) for 24 h. Serial dilutions of 1 in 10, 1 in 10^2, 1 in 10^3 and 1 in 10^4 of this 24 h broth culture were prepared. The absorbances of these dilutions were determined at a wavelength of 420 nm. The various dilutions were cultured via the plate count method using plate count agar at 37ºC for bacteria and 25ºC for *Candida albicans* for 24 h. The viable counts (colony forming units/cfu) for the various dilutions of the test organisms were made using a colony counter. Graph of the log of cfu/mL against absorbance of the cultures were plotted (Appendix II).

2.2.5.4 Maintenance of microbial cultures

The test organisms were maintained in Eppendorf tubes containing 30% glycerol broth on agar slants. These were stored in a - 4ºC frost free freezer. Samples were taken from these master cultures and sub-cultured in broth and incubated at 37^{o}C for 24 h prior to usage in the work.

2.2.6 Antimicrobial susceptibility testing

The agar well diffusion technique described by Agyare *et al.* (2013) was used to determine the antimicrobial activities of the extracts. Distilled water was used as the solvent for the leaf extracts while 50% v/v methanol was used as the solvent for the bark extract. Concentrations of 12.5, 25, 50 and 100 mg/mL of the extracts were prepared. Twenty milliliters of sterilized nutrient agar was melted and stabilized at 45°C in a water bath. A suspension of *S. aureus* to 10^6 cfu/mL was prepared and used in the tests. One hundred microlitres (10^6 cfu/mL) of the test organism was added to the 20 mL stabilized nutrient agar and rolled in the palm to allow for even distribution of the organisms and poured into a sterile Petri dish. The agar-organism mixture was allowed to set and with a sterile cork borer of diameter 8 mm four wells equidistant to one another were made in the set agar and labelled with the concentrations they were to be filled with. Two hundred microlitres of the concentrations of each extract were put in the wells and allowed to stand for 1 h so the extract could diffuse into the agar. The Petri dishes were incubated at 37°C for 24 h. Ciprofloxacin (10 µg/mL) and ketoconazole (400 µg/mL) were used as reference antimicrobial agents for the bacteria and fungus respectively. The diameters of zones of growth inhibitions around the various wells were measured and recorded. The experiment was repeated three times for all the test organisms including *E. coli*, *P. aeruginosa*, *B. subtilis* and *C. albicans*.

2.2.7 Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the extracts was determined using the broth dilution technique as described by Agyare *et al.* (2012). Each well of the 96 well microlitre plate was filled with 100 µL double strength nutrient broth and 20 µL of the 10^6 cfu/mL of *S. aureus*. Stocks of 50 mg/mL of the extracts were prepared. Appropriate volumes of sterile water and the

stocks were added to each well to make up to 200 µL with concentrations being 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL. The plates were covered and incubated at 37°C for 24 h. After incubation, 20 µL of 1.25 mg/mL of 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) was added to each well and the appearance of blue to purple colour indicates microbial growth while the persistence of the yellow colour of the MTT shows no microbial growth. The MIC of the extract for a particular organism was recorded as the lowest concentration of the extracts where no growth was observed (Wiegand *et al.,* 2008). The experiment was repeated three times for all the test organisms including *E. coli*, *P. aeruginosa*, *B. subtilis* and *C. albicans*.

2.2.8 Determination of minimum bactericidal and fungicidal concentrations (MBC/ MFC)

The minimum bactericidal and fungicidal concentration of the extracts was determined using the broth dilution technique as described by Aibinu *et al.* (2007). Each well was filled with 100 µL double strength nutrient broth and 20 µL of 1.0 x 10^6 cfu/mL of *S. aureus*. Stocks of 50 mg/mL of the extracts were prepared. Appropriate volumes of sterile water and the stocks were added to each well to make up to 200 µL with concentrations from 1.0 to 10 mg/mL. The plates were covered and incubated at 37°C for 24 h. After incubation, aliquots were pipetted from each well and plated unto Petri dishes containing nutrient agar for bacteria and sabouraud agar for the fungus. These agar plates were incubated at 37°C (bacteria) and 28°C (fungus) for 24 h. The MBC and MFC were recorded as the lowest concentration that did not show any growth on the surface of agar respectively

2.2.9 Subchronic toxicity studies

2.2.9.1. Ethical approval for animal studies

Ethical clearance and approval for the subchronic toxicity studies in Wistar rats was given by the Ethical Committee on Animals of the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana in accordance with the Guide for Care and Use of Laboratory Animals, NIH, Department of Health Services Publication, USA, no. 83-23, revised 1985. (Garber *et al*., 2010).

2.2.9.2 Handling and preparation of test laboratory animals

Fifty (50) male Wistar rats were obtained from the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana and housed in stainless steel cages containing wood shavings for bedding. They were made to acclimatize to holding facilities for 2 weeks before experimental procedure was started. The rats were randomly divided into 110 groups (5 rats per group), which consisted of 9 treatment groups and 1 control group. They were fed on normal laboratory dietary pellets and given tap water *ad libitum* during the study. Environmental conditions were maintained at a temperature of 29±2°C and a relative humidity of 40±10% with 12 h light/dark cycle.

2.2.9.3 Administration of extracts to animals

The rats were fasted for 12 h prior to administration of doses. The extract were administered to the rats as aqueous suspensions of finely grounded powder of by oral gavage, using a curved, ball-tipped stainless steel feeding needle connected to a syringe. For thirty-five (35) days the groups were administered with specific concentrations of extracts as follow:

Group 1: 100 mg/kg of EIML

Group 2: 300 mg/kg of EIML

Group 3: 1000 mg/kg of EIML

Group 4: 100 mg/kg of EIMB

Group 5: 300 mg/kg of EIMB

Group 6: 1000 mg/kg of EIMB

Group 7: 100 mg/kg of PNML

Group 8: 300 mg/kg of PNML

Group 9: 1000mg/kg of PNML

Group 10: Distilled water

The weights of the individual animals in the respective groups are as follow:

Animal code	**Body weight of animals / g**									
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
A	120	110	110	115	135	95	130	105	120	145
B	110	120	110	105	135	110	110	130	140	115
C	130	135	130	120	125	115	100	115	125	115
D	135	120	135	125	130	110	105	110	120	120
E	150	125	135	150	130	135	115	100	140	100

Key/Legend

EIML – *Erythrophleum ivorensis* methanol leaf extract

EIMB – *Erythrophleum ivorensis* methanol bark extract

PNML – *Parquetina nigrescens* methanol leaf and other aerial part extract

2.2.9.4 Biochemical test for determination of toxicity

The animals were fasted overnight prior to necropsy and blood collection. At the end of the experimental period animals were anaethesized prior to euthanization and then decapitated after neck dislocation. Blood samples were taken from the animals in each group into complete blood count (CBC) bottles containing ethylenediaminetetraacetic acid (EDTA-2K). Haematological analyses which measured parameters such as red blood cell count, haemoglobin concentration, hematocrit, mean corpuscular cell volume, mean corpuscular cell haemoglobin, mean corpuscular cell haemogolobin concentration, platelet count, white blood cell count, and differential WBC count were determined using automatic haematology analyzer.

Portions of uncoagulated blood were centrifuged at 3000 rpm for 10 min and analyzed using an autoanalyzer. Serum biochemical indicators such as glucose, total cholesterol, blood urea nitrogen (BUN), creatinine, total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, creatine kinase, albumin/globulin ratio, triglycerides, phosphorus, calcium and chloride were measured.

2.2.9.5 Histopathological studies

Histopathological examinations were performed on the kidney and liver of both treated and untreated Wistar rats as described by Wasfi *et al.* (1994) and Guntupalli *et al.* (2006). The tissues were fixed in 10% formalin. They were then dehydrated sequentially in ethanol concentrations of 50 to 100%. The tissues are then rinsed in xylene to remove the dehydrant (ethanol) and finally embedded in paraffin for strengthening and easy dissection. Proir to sectioning the tissues, they are 'deparaffinized' by rinsing in xylene, followed by washing in decreasing concentration of ethanol (100% to 50%) before rehydrating the tissues with water. Tissue sections of thickness 6 µm were made and stained with hematoxylin-eosin (H-E) dye to impart contrast for

photomicroscopic viewing. The tissues were then observed under a light microscope at magnification of x 600.

2.2.10 *In vitro* cell proliferation test (ELISA BrdU-Assay)

The method described by Agyare *et al.* (2009) was used to determine the influence of the methanol extracts on cell proliferation using ELISA cell proliferation test kit developed by Roche, Switzerland. Each well of the 96-well plate was seeded with 10^5 HaCaT keratinocyte cells in HaCaT keratinocytes medium (HM medium). Concentrations of 0.10, 1.0, 10.0, 50.0 and 100.0 µg/mL of extracts were made in 100 µL HaCaT keratinocytes medium and plates incubated for at 35°C with 5% CO_2 for 24 h. After incubation each well was treated with a volume of 10 µL labelling solutions and plates re-incubated at 35ºC with 5% CO_2 for 24 h. The test medium was later removed and 100 µL fixation-denaturation solution added to the HaCaT keratinocytes and left to stand for 30 min at 20-25ºC. The fixation-denaturation solution was removed and a 50 µL antibody solution BrdU-POD (peroxidase-marked-mouse anti-BrdU-antibody) added to each well and incubated at 20-25ºC for 90 min with constant agitation of the plates. The antibody solution was then washed off three times and 50 µL substrate solution added to the wells. The plates were finally incubated at room 20-25 ºC to undergo a peroxidase reaction until the development of a clear blue solution. A 10 µL 1M H_2SO_4 solution was added to the wells to truncate peroxidase reaction. Upon halting the reaction there was a colour change of the solutions in the wells from blue to yellow. The absorbance of this resultant yellow solution was measured at wavelength of 450 nm against 690 nm.

2.2.11 Influence of extracts on cell viability

The influence of methanol extracts (EIML, EIMB and PNML) on viability of HaCaT keratinocytes was determined according to the method described by Agyare *et al*. (2011). Concentrations of 0.10, 1.0, 10.0, 50.0 and 100.0 µg/mL of the methanol extracts in HaCaT keratinocytes medium were made in the wells of 96-well micro titre plate and then 10^5cells added per well. The plates were incubated at incubated at 35ºC with 5% CO_2. HaCaT keratinocyte cells treated with 5% foetal calf serum (FCS) and untreated HaCaT keratinocyte cells served as positive and negative controls respectively. After incubation each well was washed with 100 µL of phosphate buffer solution (PBS) and then filled with 50 µL MTT solution and re-incubated at 35ºC with 5% CO_2 for 12 h and washed with 2 x 100 µL PBS. The soluble formazan crystals formed were dissolved in 50 µL DMSO. The intensity of the violet coloured formazan crystals was measured at 595 nm against 690nm. The absorbance recorded for the dissolved formazan at this wavelength would correspond with the number of viable HaCaT keratinocyte cells. Percentage viable cells (absorbance of test wells/absorbance of control wells × 100%) were determined. The experiments were repeated three times.

2.2.12 Cytotoxicity test

Cytoplasmic enzyme lactate dehydrogenase (LDH) released from the cytosol of HaCaT keratinocytes when damaged or under stress was determined according to the method described by Agyare *et al.* (2011). Concentrations (0.10, 1.0, 10.0, 50.0 and 100.0 µg/mL) of the extracts in 100 µL HaCaT keratinocytes medium were made in the wells of 96-well microtitre plates and each well seeded with 10^5 cells and then incubated for 48 h at 35ºC with 5% of CO_2. After incubation 25 µL of this supernatant was pipetted into 96-well microtitre plate and 25 µL lysis buffer added to both the supernatant and the adherent lysed cells in the wells. This was incubated

at 28°C for 1 h and frequently agitated. Afterwards 25 µL of substrate mix was added to both the untreated supernatant and lysed cell medium in the microtitre plate and incubated in dark at 20ºC for 30 min. The reactions were finally halted by the addition of 10 µL HCL-isopropanol solution to each well. The above procedure was repeated for untreated HaCaT keratinocytes and 10% Triton X-100 in FCS as negative and positive controls respectively. After incubation for 4 h measurement of LDH enzyme released into the 25 µL supernatant and the absorbance were determined at 450 nm against 690 nm.

2.3 Statisitcal Analyses

All statistical analyses were done using Graph Pad prism version 5.0 windows (Graph Pad Software, San Diego, CA, USA). A one-way analysis of variance (ANOVA) was done for the data followed by Newman-Keuls post test. The values of $p<0.05$ were considered to be significant. The graphs were plotted using Graph Pad prism version 5.0 windows and Microsoft Office Excel 2007(Microsoft Inc., California, USA)

CHAPTER THREE

3.0 Results

3.1 Extraction of plant material

The ground powder of the plant materials were soaked in 70% hydro-methanol to extract the active principles and then concentrated to dryness at 40ºC. The yields of the methanol extracts (EIML, EIMB and PNML) were expressed as percentages (Table 3.1.1).

Table 3.1 Yield of methanol extracts of *E. ivorense* and *P. nigrescens* (related to the dried material).

	Methanol extracts		
	EIML	EIMB	PNML
Yield (% w/w)	9.76	15.54	7.68

EIML - methanol leaf extract of *E. ivorense*; EIMB - methanol bark extract of *E. ivorense*; PNML - methanol leaf extract of *P. nigrescens*.

3.2 Phytochemical screening

The methanol extracts (EIML, EIMB and PNML) were screened for phytochemical constituents.

Table 3.2 Phytochemical constituents of methanol extracts of *E. ivorense* and *P. nigrescens*.

Phytochemical	**EIML**	**EIMB**	**PNML**
Saponins	+	+	+
Hydrolysable tannins	-	+	-
Condensed tannins	+	-	+
Alkaloids	+	+	+
Terpenoids	-	+	+
Sterols	+	-	+
Flavonoids	+	+	+

Cardiac glycosides	-	+	+

Key: (+) = presence of secondary metabolite; (-) = absence of secondary metabolites

3.3 HPLC analyses of methanol extracts

The HPLC finger printing of the methanol extracts were done using different solvent systems. These HPLC finger printings are used as identification for the plants. Different peaks in the chromatograms represent different compounds or constituents present in the plant extract (Figures 3.3.1 to 3.3.3).

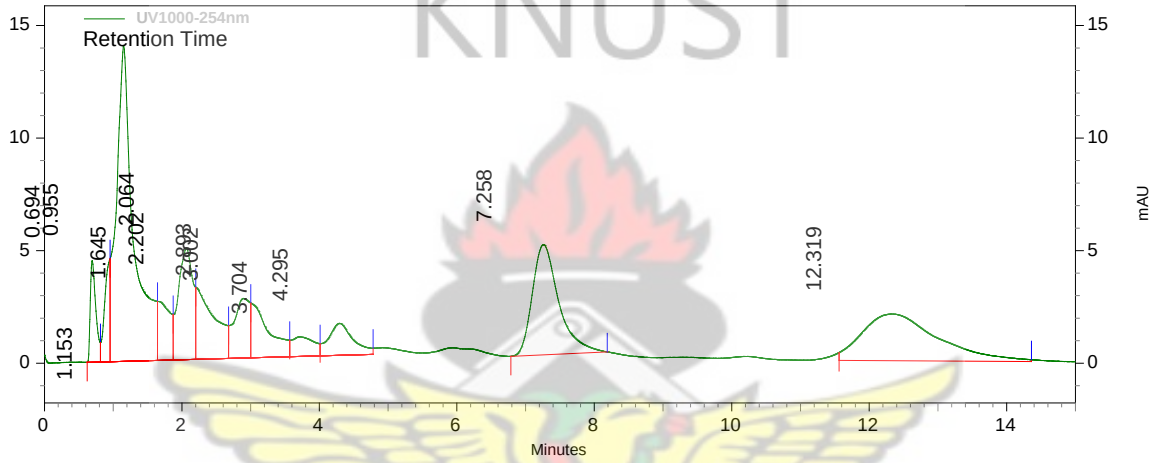

Fig. 3.3.1: HPLC chromatogram of methanol leaf extract of *E. ivorense* at λ 254 nm.

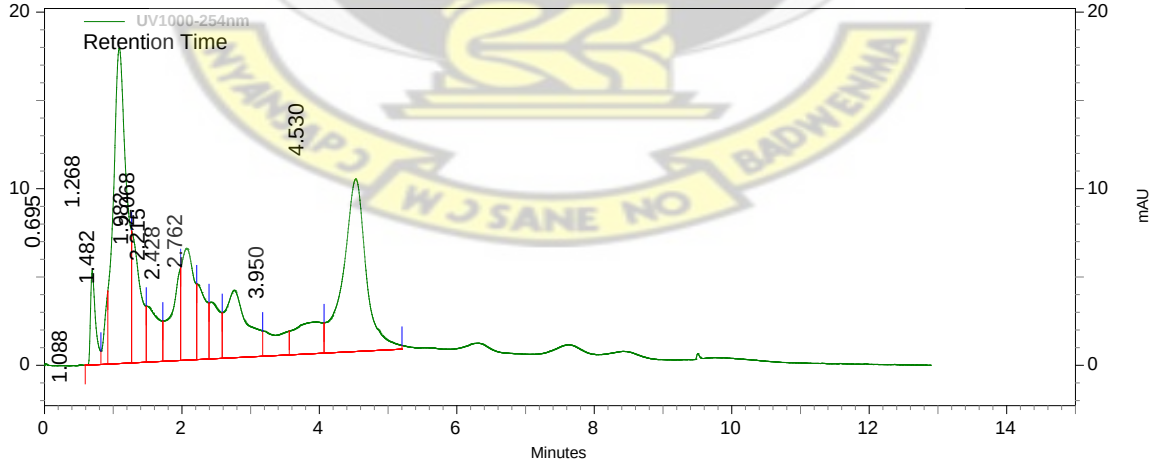

Fig. 3.3.2: HPLC chromatogram of methanol bark extract of *E. ivorense* at λ 254 nm.

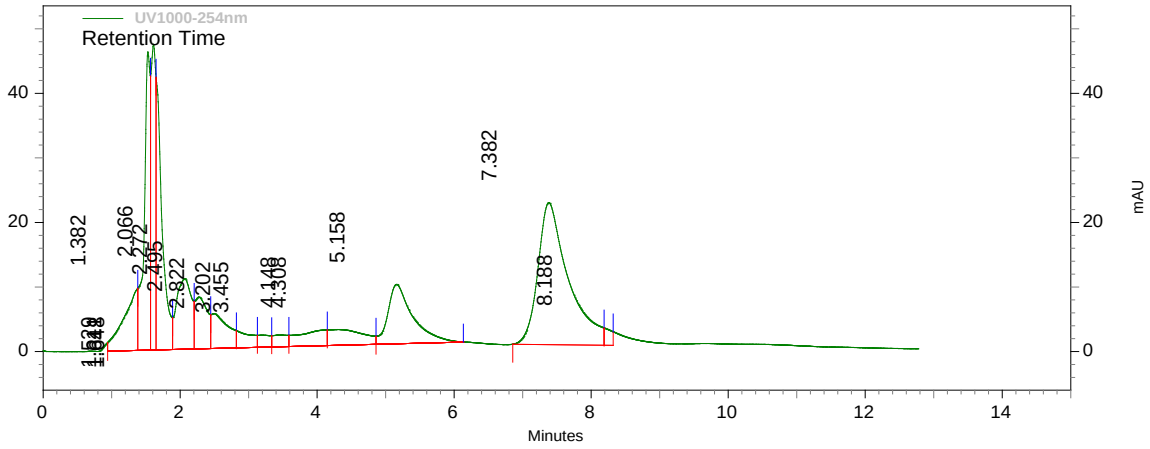

Fig. 3.3.3: HPLC chromatogram of methanol leaf extract of *P. nigrescens* at λ 254 nm

3.4 Antimicrobial activity

3.4.1 Agar diffusion method

The agar well diffusion method was used to determine the antimicrobial activity of the methanol extracts. The methanol leaf extract of *E. ivorense* showed narrow spectrum antimicrobial activity against the Gram-positive bacteria (*S. aureus* and *B. subtilis*) but not the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) at concentrations of 12.5 to 100 µg/mL (Table 3.4.1.1) in the agar well diffusion technique. The methanol bark extract of *E. ivorense* exhibited broad spectrum antimicrobial activity against the test organisms except *E. coli* at concentrations of 12.5 to 100 µg/mL (Table 3.4.1.2). The methanol leaf and other aerial part extract of *P. nigrescens* however exhibited broad spectrum antimicrobial activity against the *C. albicans*, *S. aureus* and *P. aeruginosa* (at concentrations of 12.5 to 100 µg/mL), and *B. subtilis* (at concentrations 50 to 100 µg/mL) but no activity against *E. coli* at any of the concentrations used (Table 3.4.1.3). The standard references ciprofloxacin showed broad spectrum antibacterial activity at 10 µg/mL while ketoconazole showed activity against *C. albicans* (fungus) at 400 µg/mL (Table 3.4.1.4).

Table 3.4.1.1: Mean zones of growth inhibition of *E. ivorense* leaf extract against test organisms

Concentration (mg/mL)	**Mean zones of inhibition (mm) + SEM**		
	B. subtilis	*S. aureus*	*C. albicans*
12.5	10.67±0.33	14.67±0.33	13.00±0.58
25	13.33±0.33	16.67±0.33	16.00±0.58
50	14.33±0.67	18.67±0.33	17.67±0.33
100	17.33±0.33	23.00±0.0	20.67±0.33

Diameter of well = 8mm; SEM = Standard Error Mean.

Table 3.4.1.2: Mean zones of growth inhibition of *E. ivorense* bark extract against test organisms

Concentration (mg/mL)	**Mean zones of inhibition (mm) + SEM**			
	B. subtilis	*S. aureus*	*P. aeruginosa*	*C. albicans*
12.5	13.67±0.33	10.67±0.67	10.33±0.33	12.67±0.88
25	14.67±0.33	12.33±0.33	10.67±0.33	14.33±0.33
50	16.33±0.67	14.00±0.57	12.00±0.57	16.67±0.33
100	18.00±0.0	16.33±0.33	14.00±0.57	18.33±0.33

Diameter of well = 8mm; SEM = Standard Error Mean.

Table 3.4.1.3: Zones of growth inhibition of *P. nigrescens* leaf and other aerial part extract against test organisms

Concentration of extract (mg/mL)	**Mean zones of inhibition (mm) + SEM**			
	B. subtilis	*S. aureus*	*P. aeruginosa*	*C. albicans*
12.5	0.0±0.0	14.67±0.33	10.67±0.33	11.00±0.0
25	0.0±0.0	17.33±0.33	11.33±0.33	11.33±0.33
50	11.33±0.33	18.00±0.33	13.67±0.33	12.67±0.33
100	12.67±0.33	19.00±0.57	15.67±0.33	14.33±0.33

Diameter of well = 8mm; SEM = Standard Error Mean.

Table 3.4.1.4: Zones of growth inhibition of reference drug against test organisms

Concentration of reference drug	**Mean zones of inhibition (mm) + SEM**				
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*
Ciprofloxacin(10 µg/mL)	29.33±0.67	20.67±0.67	26.67±2.4	22.33±1.45	nd
Ketoconazole(400 µg/mL)	nd	nd	nd	nd	13.67±0.88

nd = not determined; Diameter of well = 8mm; SEM = Standard Error Mean.

3.4.2 Minimum inhibitory concentrations of the extracts against test organism

The minimum concentrations of the extracts and standard reference that inhibited (MIC) the growth of the test organisms were determined using the broth dilution technique. There were

generally lower MICs for the Gram-positive bacteria as compared with the Gram-negative bacteria in all the extract. The leaf extract of *E. ivorense* showed higher MICs than its bark extract. The MIC of EIML extract ranged from 2.0 to 8.0 mg/mL, that for the EIMB extract ranged between 2 to 4 mg/mL while the MIC for PNML extract was between 3 to 8 mg/mL (Table 3.4.2.1).

Table 3.4.2.1: Minimum inhibitory concentrations of extracts and reference drug against test organisms

	MIC				
	Extract (mg/mL)			Reference drug (µg/mL)	
Test organism	**EIML**	**EIMB**	**PNML**	**Ciprofloxacin**	**Ketoconazole**
B. subtilis	4.0	2.0	6.0	0.030	nd
S. aureus	2.0	4.0	3.0	0.250	nd
E. coli	5.0	4.0	8.0	0.125	nd
P. aeruginosa	8.0	3.0	6.0	0.250	nd
C. albicans	4.0	2.0	4.0	nd	5.00

EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens*.

3.4.2 Minimum bactericidal and fungicidal concentrations (MBC/MFC) of the extracts against test organism

The methanol extracts (EIML, EIMB and PNML) exhibited bactericidal and fungicidal activities against the test organisms. The EIMB extract showed a better activity compared with the EIML and PNML extracts against the test organisms (Table 3.4.2.2).

Table 3.4.2.2: Minimum bactericidal and fungicidal concentrations of extracts antimicrobials against test organisms

Test organism	**Extracts** (mg/mL)		
	EIML	**EIMB**	**PNML**
B. subtilis	7.0	4.0	8.0
S. aureus	5.0	5.0	5.0
E. coli	8.0	7.0	10.0
P. aeruginosa	10.0	7.0	12.0
C. albicans	6.0	4.0	5.0

EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens*.

3.5 Sub-chronic toxicity studies

Biochemical tests were done on the blood samples taken from the rats. Several haematological and serum biochemistry indicators were studied for possible effects of end organ subchronic toxicity. The mean white blood cell counts measured in rats treated with the extracts were lower than the untreated rats (Fig. 3.5.1). However, red blood cells, haemoglobin and haematocrits concentrations of the animals in the treated groups were higher than those of the untreated groups (Table 3.5.1). The animals treated with EIMB extract showed increase in white blood cell concentration but a reduced concentration in those treated with EIML and PNML extracts (Table 3.5.1). In the serum biochemistry analysis, animals treated with the methanol extracts (EIML, EIMB and PNML) showed higher albumin, cholestrol and total bilirubin concentrations (Table 3.5.2).

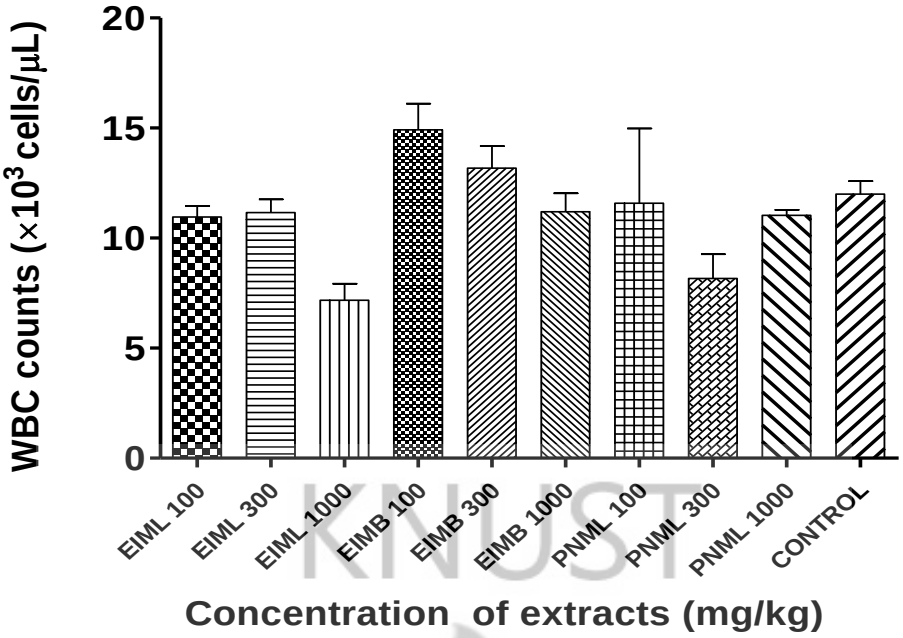

Fig. 3.5.1: White blood cells (WBC) counts in rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens. p>0.05.*

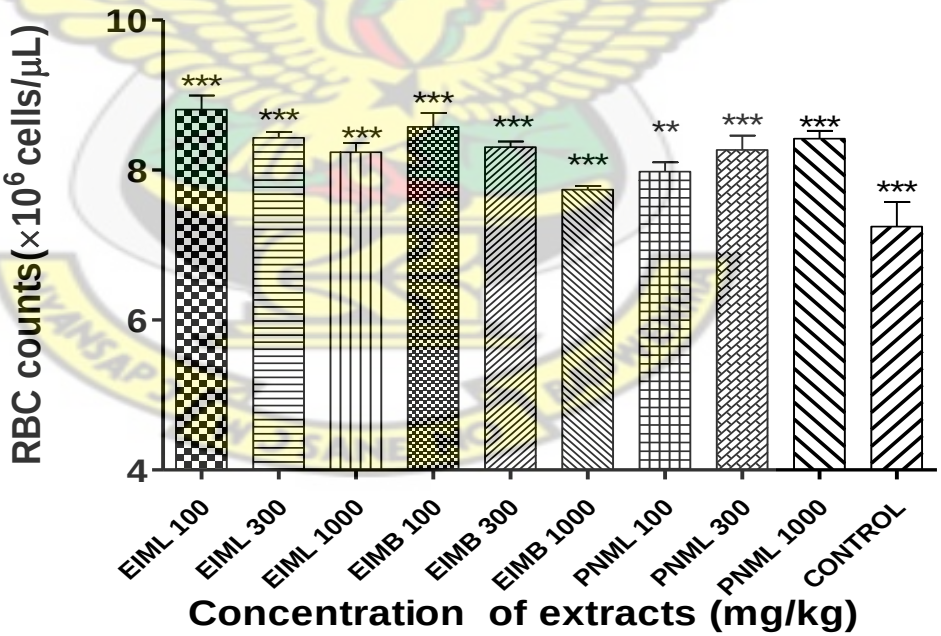

Fig. 3.5.2: Red blood cells (RBC) counts in rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* ***p<0.0001

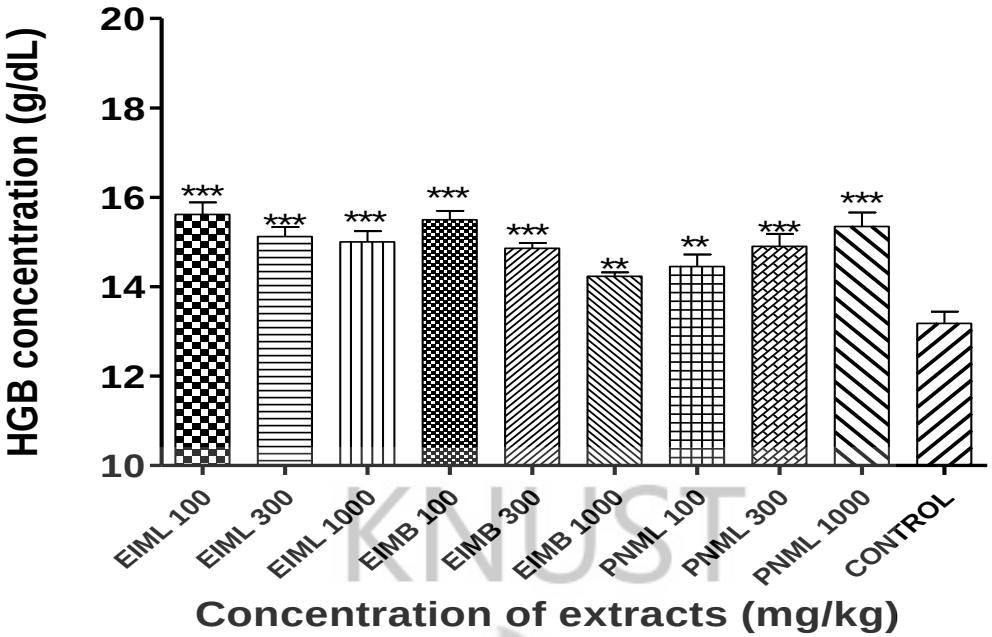

Fig. 3.5.3: Haemoglobin concentrations in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* *** *p<0.0001;* ***p<0.001*

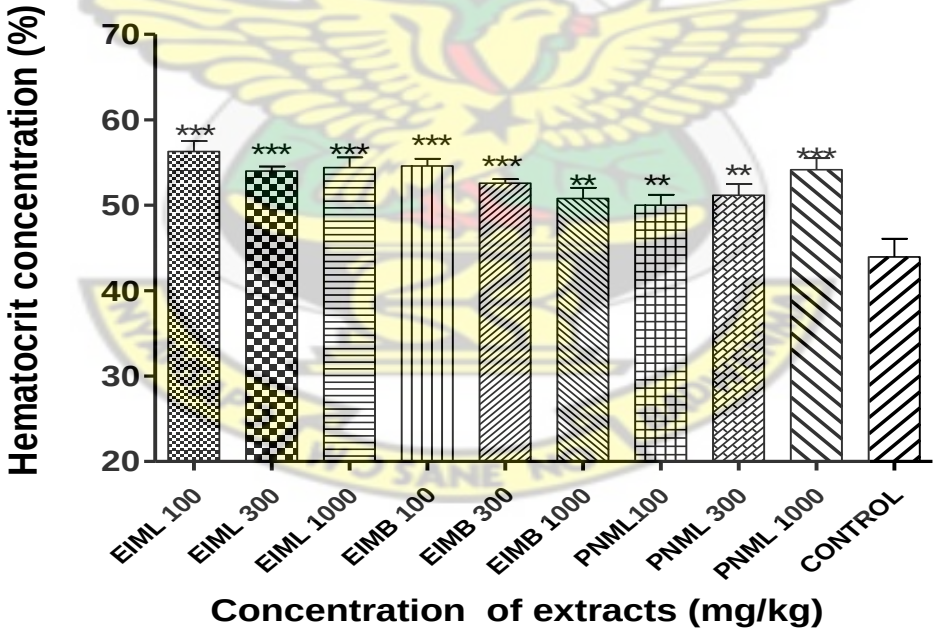

Fig. 3.5.4: Percentage haematocrits in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens*

*** p<0.0001; **p<0.01

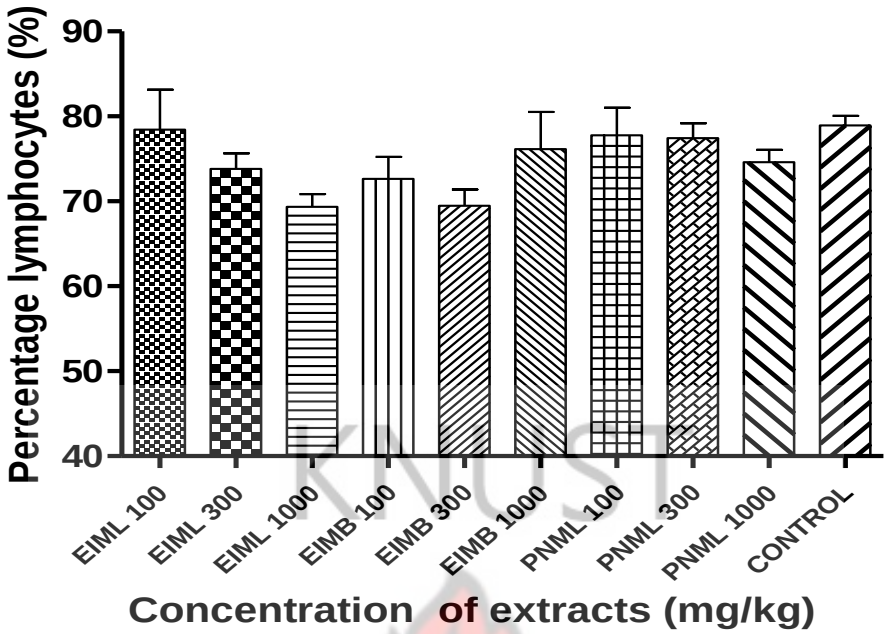

Fig. 3.5.5: Percentage lymphocytes in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* *p>0.05*

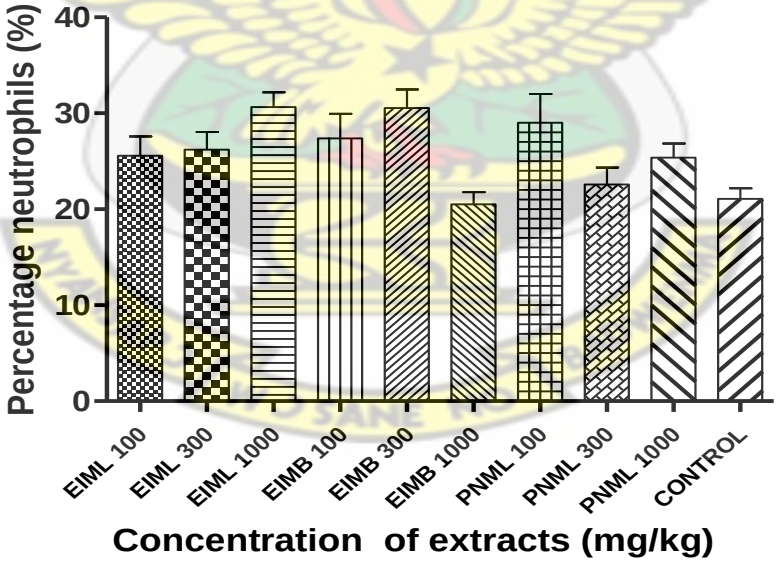

Fig. 3.5.6: Amount of neutrophils in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* *p>0.05*

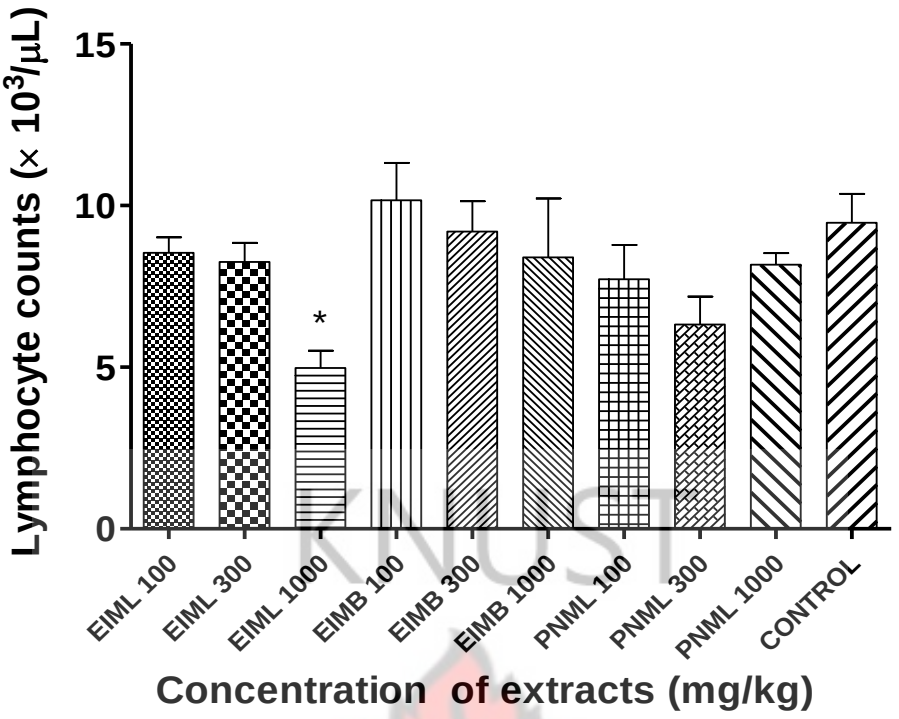

Fig. 3.5.7: Lymphocytes counts in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* *p<0.01

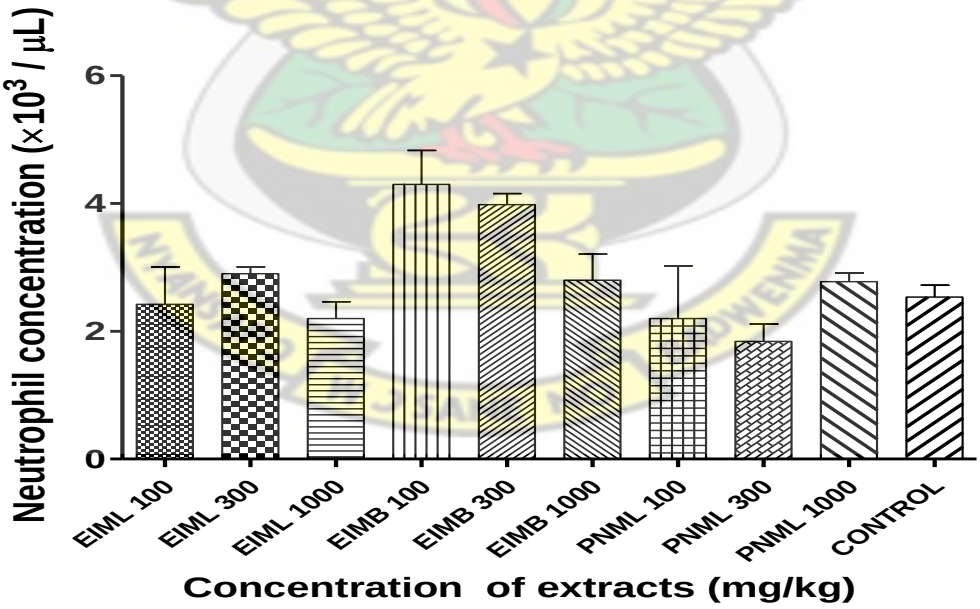

Fig. 3.5.8: Neutrophils counts in blood of rats treated with different concentrations of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens. p>0.05*

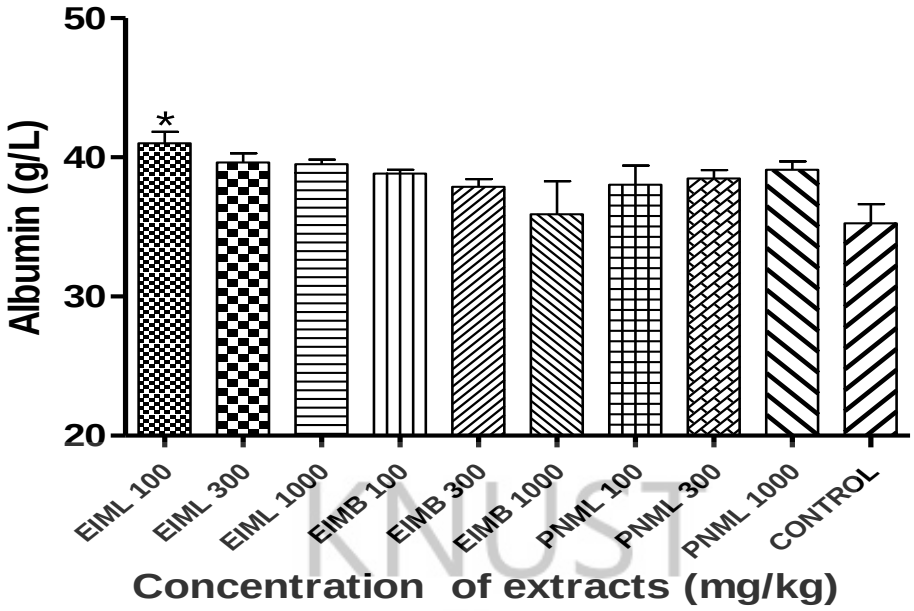

Fig. 3.5.9: Albumin concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens.* * *p<0.01*

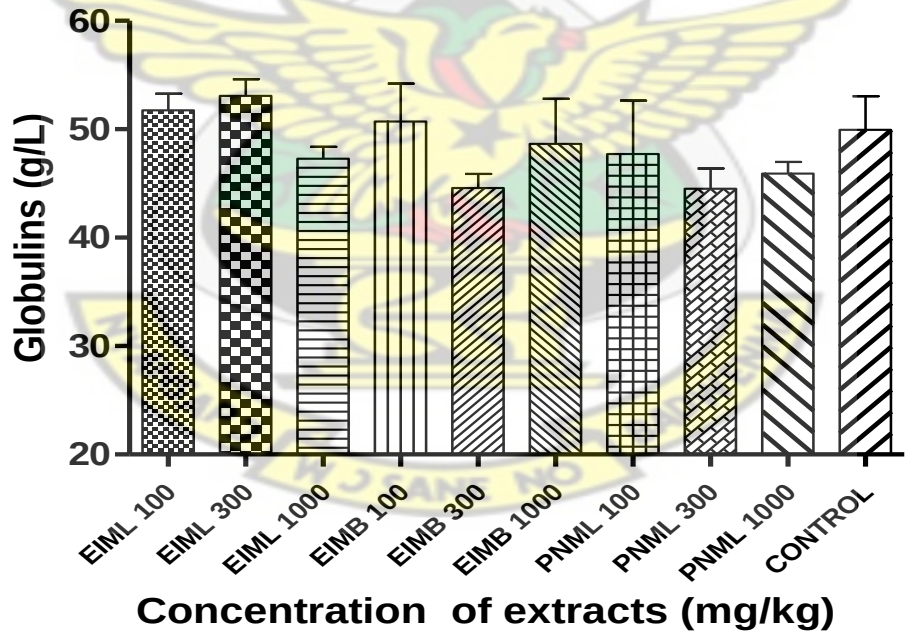

Fig. 3.5.10: Globulin concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens.* *p>0.05*

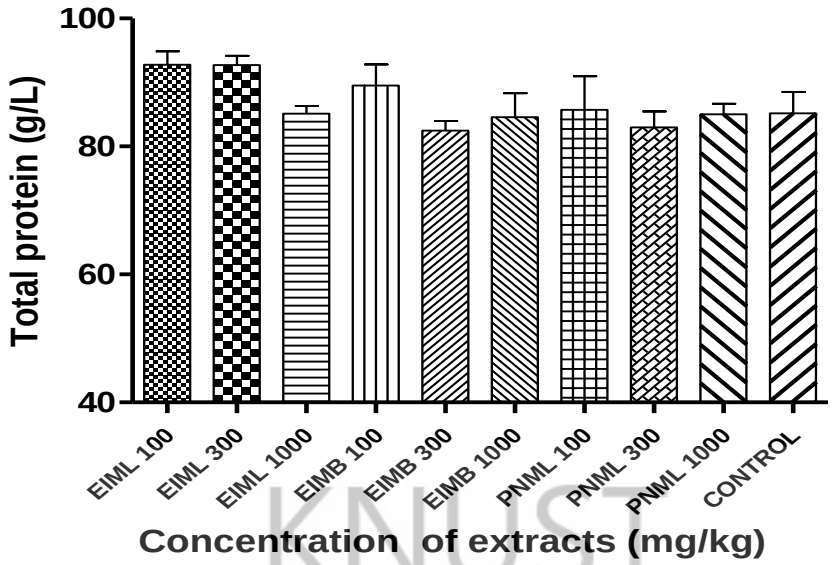

Fig. 3.5.11: Total protein concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens. p>0.05*

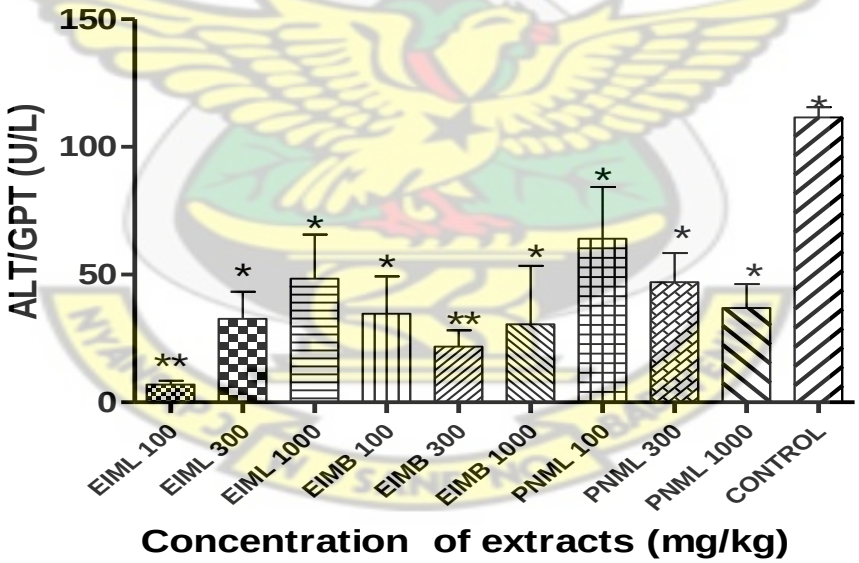

Fig. 3.5.12: Alanine aminotransferase concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens. **p<0.01*

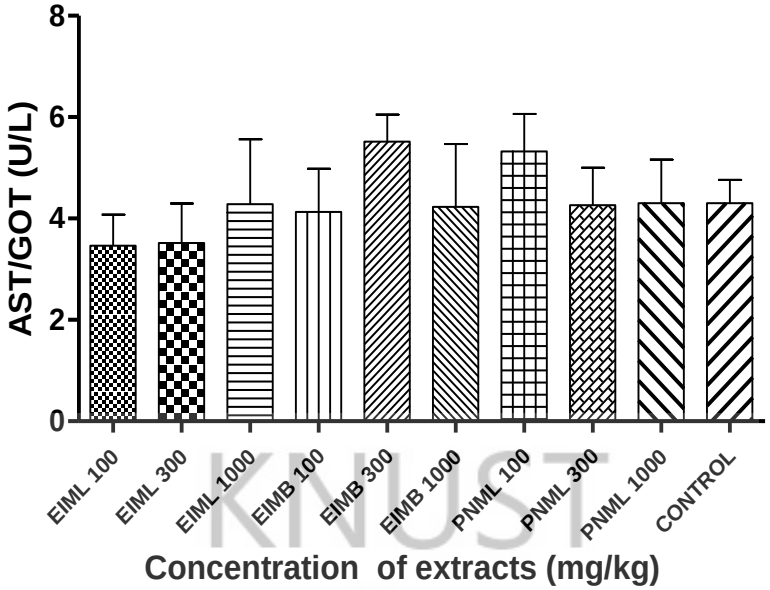

Fig. 3.5.13: Aspartate aminotransferase concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens p>0.05*

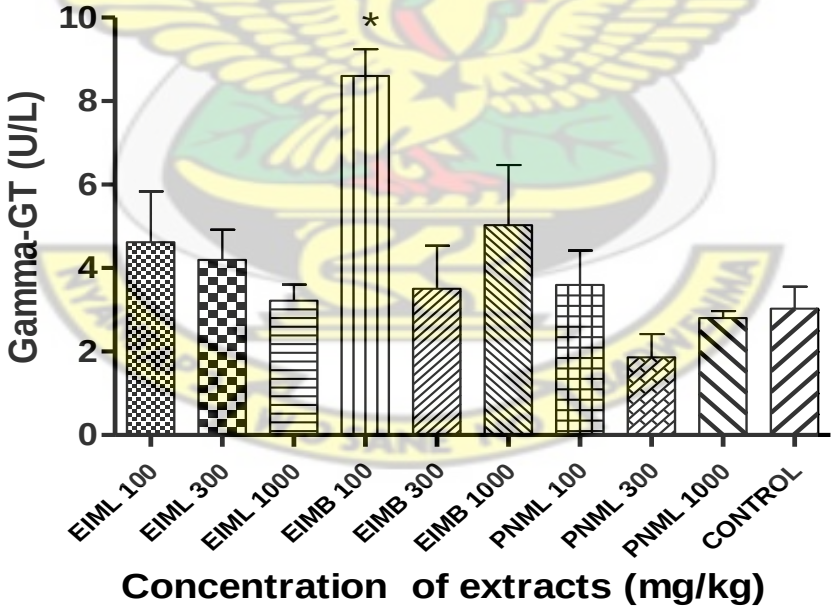

Fig. 3.5.14: Gamma glutamyl transferase concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens * p<0.01*

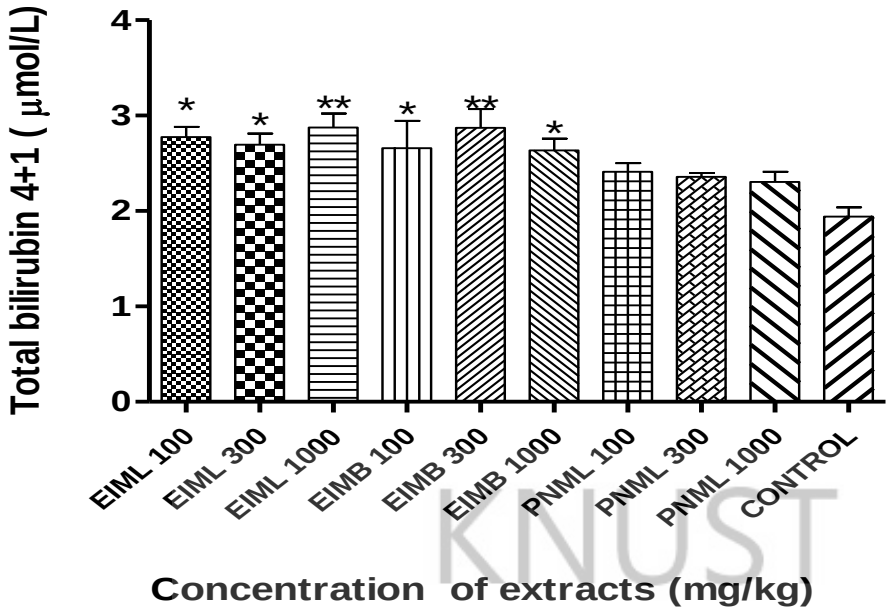

Fig. 3.5.15: Total bilirubin concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens.* *** p< 0.01; **p< 0.01*

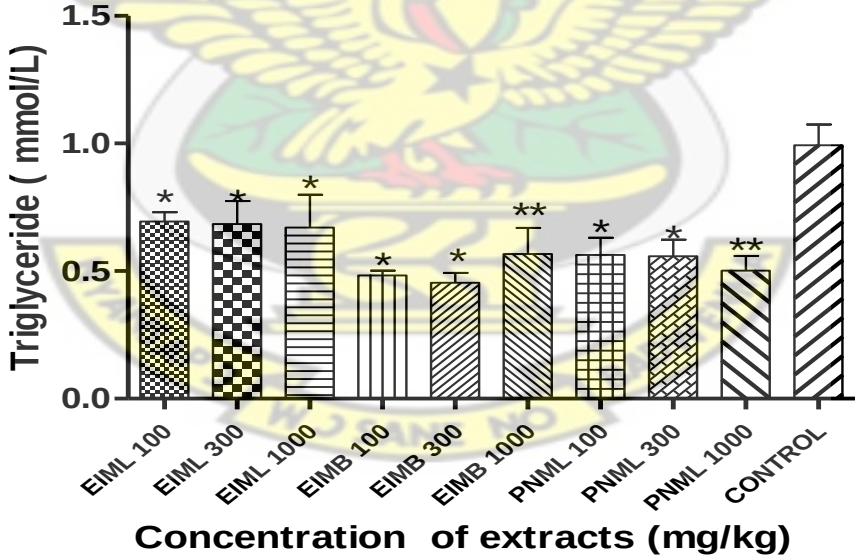

Fig. 3.5.16: Triglyceride concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* ** *p< 0.01*

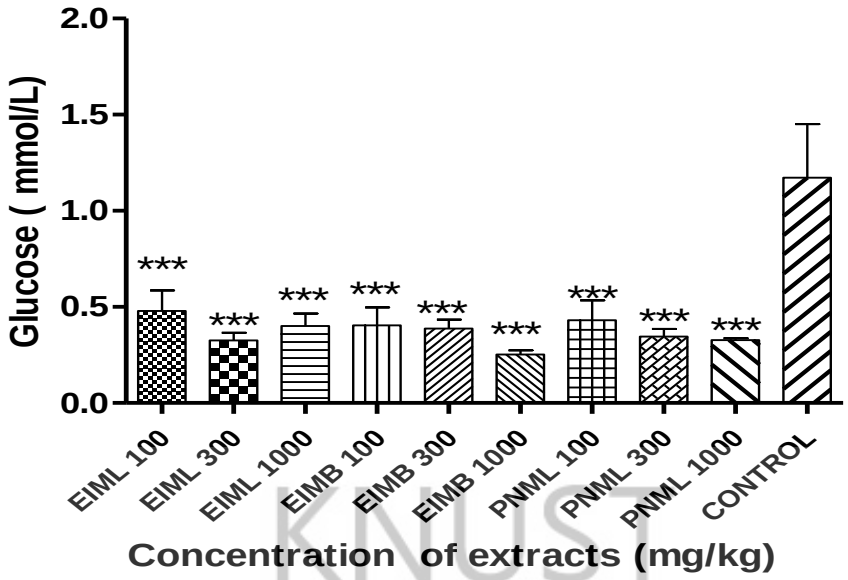

Fig. 3.5.17: Glucose concentration in blood of rats treated with different concentration of extracts. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens.* ****p< 0.0001*

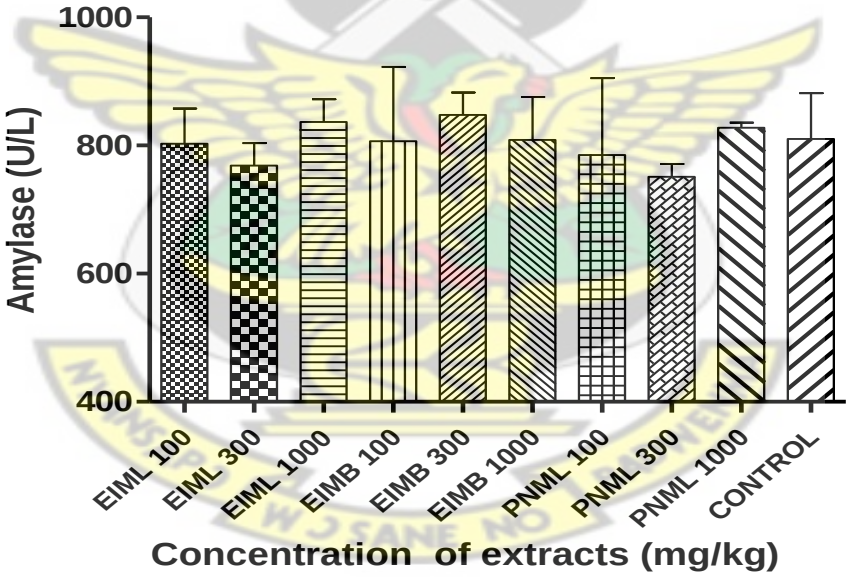

Fig. 3.5.18: Amylase concentration in blood of rats treated with different concentration of extract. EIML – methanol leaf extract of *Erythrophleum ivorense*; EIMB – methanol bark extract of *Erythrophleum ivorense*; PNML – methanol leaf and other aerial part extract of *Parquetina nigrescens* *p>0.05*

Table 3.5.1: Effects of methanol extracts on haematological parameters of Wistar rats in subchronic toxicity studies.

Parameters	Extracts and their concentration (mg/kg body weight)									
	EIML 100	EIML 300	EIML 1000	EIMB 100	EIMB 300	EIMB 1000	PNML 100	PNML 300	PNML 1000	CONTROL
WBC X 10^3/µL	11.0±0.50	11.2±0.60	7.18±0.75	16.2±0.12	13.2±1.00	12.1±0.74	8.20±0.47*	8.16±1.11*	11.0±0.25	12.0±0.58
RBC X 10^6/µL	8.81±0.19*	8.43±0.08*	8.24±0.12*	8.58±0.18 *	8.31±0.08*	7.74±0.05*	7.98±0.12*	8.27±0.19*	8.42±0.14*	7.25±0.33
HGB (g/dL)	15.6±0.27*	15.1±0.21*	15.0±0.24*	15.5±0.20 *	14.9±0.12*	14.2±0.09*	14.5±0.27*	14.9±0.28*	15.4±0.31*	13.2±0.26
HCT (%)	56.3±1.25*	54.0±0.54*	54.4±1.17*	54.6±0.83 *	52.6±0.46*	50.8±1.25*	50.0±1.25*	51.2±1.33*	54.2±1.35*	43.9±2.15
MCV (fL)	63.9±0.75	64.1±0.55	66.0±0.87*	63.7±0.70	63.3±0.71	65.6±1.64*	62.7±0.41	61.9±0.52	64.3±0.64	60.8±1.27
MCHC(g/dL)	27.8±0.21*	28.0±0.37*	27.6±0.37*	28.4±0.50	28.3±0.16	28.1±0.79	28.9±0.69	29.2±0.31	28.4±0.21	30.0±0.43
PLT X 10^3/µL	702±56.10	730±35.4	688±31.8	839±117	767±36.7	647±23.1	748±32.4	665±23.4	751±19.2	596±42.7
LYM (%)	78.4±4.74	73.8±1.85	69.3±1.52	72.6±2.60	69.5±1.96	72.8±7.69	72.8±11.1	77.4±1.78	74.6±1.46	78.9±1.12
NEUT (%)	21.6±4.74	26.2±1.85	30.7±1.52	27.4±2.60	30.5±1.96	27.2±7.69	27.3±11.1	22.6±1.78	25.4±1.46	21.1±1.12
LYM # X 10^3/µL	8.54±0.48	8.26±0.59	4.98±0.53*	10.2±1.16	9.20±0.94	8.40±1.82	7.73±1.06	6.32±0.87	8.18±0.35	9.47±0.90
NEUT# X 10^3/µL	2.42±0.58	2.90±0.11	2.20±0.26	3.97±0.85	3.98±0.17	2.80±0.40	4.70±3.20	1.84±0.27	2.78±0.14	2.53±0.19
RDW_SD(fL)	37.0±0.36	38.7±1.53	38.2±0.92	36.9±0.06	38.1±0.74	42.0±2.76*	36.0±1.63	34.3±0.96	37.4±0.93	35.2±0.38
RDW_CV(%)	15.9±0.39	15.7±0.95	14.9±0.29	15.1±0.41	16.3±0.36	17.3±0.84	14.9±1.19	14.3±0.61	15.1±0.35	15.0±0.72
PDW(fL)	8.80±0.18	8.64±0.21	9.16±0.25	8.97±0.47	8.24±0.20	8.50±0.21	9.38±0.51	8.78±0.24	8.60±0.08	9.35±0.45
MPV(fL)	7.16±0.05	7.08±0.10	7.32±0.09	7.23±0.29	6.82±0.07 *	6.97±0.07	7.45±0.21	7.12±0.12	7.13±0.10	7.60±0.00
P_LCR(%)	7.90±0.38	7.66±0.50	8.88±0.74	8.37±1.15	6.16±0.46	6.97±0.24	9.75±1.14	7.62±0.52	7.68±0.58	9.10±0.00

*$p<0.05$

Table 3.5.2: Effects of methanol extracts on general biochemical parameters of treated and untreated Wistar rats.

Parameters	Extracts and their concentration (mg/kg body weight)									
	EIML 100	EIML 300	EIML 1000	EIMB 100	EIMB 300	EIMB 1000	PNML 100	PNML 300	PNML 1000	CONTROL
Albumin(g/L)	41.0±0.82	39.6±0.63	39.5±0.33	38.8±0.26	37.9±0.55	35.9±2.37	38.0±1.40	38.5±0.62	39.1±0.60	35.3±1.38
Globulins(g/L)	51.8±1.51	53.1±1.53	47.3±1.09	50.7±3.52	44.6±1.29	48.7±4.14	47.7±4.94	44.5±1.89	45.9±1.06	49.9±3.12
Tot protein(g/L)	92.8±2.11	92.7±1.44	85.1±1.22	89.5±3.31	82.5±1.50	84.6±3.76	85.7±5.20	83.0±2.50	85.0±1.66	85.2±3.36
ALT/GPT(U/L)	6.94±1.46	32.6±10.6	48.5±17.2	34.7±14.6	21.8±6.33	30.5±22.9	64.0±20.3	47.1±11.4	36.9±9.42	112±4.02
AST/GOT(U/L)	3.46±0.61	3.52±0.78	4.28±1.28	4.13±0.85	5.52±0.53	4.23±1.24	5.33±0.74	4.26±0.74	4.30±0.86	4.30±0.46
Dir bil 4+1	0.002±0.07	-0.11±0.097	-0.15±0.11	2.26±0.13 *	2.24±0.05 *	0.74±0.76	2.27±0.28 *	2.14±0.08 *	1.99±0.04 *	-0.23±0.23
Tot bil 4+1	2.78±0.11 *	2.69±0.12 *	2.87±0.15 *	2.66±0.29 *	2.87±0.20 *	2.63±0.12 *	2.41±0.09	2.36±0.04	2.31±0.11	1.94±0.09
Indirect bil	2.77±0.08	2.80±0.17	3.03±0.09	0.40±0.31 *	0.64±0.16 *	1.89±0.88	0.08±0.21 *	0.22±0.04 *	0.32±0.11 *	2.41±0.04
Gamma-GT(U/L)	4.62±1.22	4.20±0.72	9.22±2.23	8.60±3.86	7.06±3.83	5.03±1.44	11.1±7.90	1.86±0.56	4.30±1.64	6.37±2.88
Creatinine(µmol/L)	81.0±4.58	72.5±3.94	75.5±5.46	73.9±5.15	76.2±1.70	75.8±6.05	72.1±7.73	71.3±2.91	73.8±1.92	59.7±3.20
Urea(mmol/L)	8.92±0.56	7.13±0.62	8.79±0.44	3.42±1.97 *	6.56±0.47 *	8.50±1.48	4.18±0.88 *	3.87±0.77 *	2.83±0.56 *	10.5±1.71
Cholestrol(mmol/L)	6.25±0.13	6.19±0.11	6.19±0.39	5.97±0.21	5.58±0.16	5.51±0.38	5.55±0.52	5.72±0.23	5.75±0.11	5.10±0.20
Triglyceride(mmol/L)	0.69±0.04 *	0.68±0.09 *	0.67±0.13 *	0.48±0.02 *	0.45±0.04 *	0.57±0.10 *	0.56±0.07 *	0.56±0.07 *	0.50±0.06 *	0.99±0.01
HDL chol(mmol/L)	1.34±0.08	1.39±0.06	1.40±0.08	1.23±0.10	1.35±0.00	1.54±0.13	1.27±0.19	1.32±0.04	1.33±0.10	1.06±0.09
LDL chol(mmol/L)	4.60±0.09 *	4.50±0.09	4.48±0.26	4.52±0.11	4.03±0.13	3.70±0.28	4.03±0.31	4.15±0.17	4.30±0.07	3.58±0.16
Coronary Risk	4.72±0.24	4.50±0.16	4.42±0.06	4.87±0.23	4.17±0.16	3.57±0.11 *	4.54±0.36	4.33±0.10	4.62±0.15	4.83±0.18
Glucose(mmol/L)	0.48±0.11 *	0.33±0.04	0.40±0.07 *	0.40±0.09 *	0.39±0.05 *	0.25±0.02 *	0.43±0.11 *	0.35±0.04 *	0.33±0.01 *	1.17±0.28
Uric acid(µmol/L)	112±8.49	104±13.70	157±14.9*	127±27.8	121±14.3	85.0±12.9	124±16.0	98.6±10.7	113±5.81	82.3±8.88
Amylase(U/L)	803±55.3	769±34.80	837±35.50	807±11.6	848±35.2	809±67.3	785±12.0	751±19.80	828±7.77	810±71.50

*$p<0.05$

3.6 Histopathological investigations of kidney and liver tissues from both treated and untreated rats

The histopathological examination of the sectioned kidney and liver tissues showed various extents of toxicity and injury after the thirt-five day treatment with the EIML, EIMB and PNML extracts. The renal tissues showed inflammation and glomerular degeneration in rats treated with 1000 mg/kg of EIML (Fig. 3.6.1, G3) extract while the renal tissues of rats treated with all dose levels of EIMB extract (100 to 1000 mg/kg) showed toxicity including tissue inflammation, renal haemorrhage, hyalination and hepatocyte degeneration (Fig. 3.6.1, G4 to G7). The PNML extract also showed renal haemorrhage and inflammation at treatment doses of 100 and 300 mg/kg (Fig. 3.6.1, G7-G8). The hepatic tissues showed marginal infiltration of inflammatory cells (Fig. 3.6.2, G2) in rats administered with EIML extract at 300 mg/kg but a more pronounced extent of inflammation, few vacuolation and scarring at treatment with 1000 mg/kg EIML extract (Fig. 3.6.2, G3). There was necrosis and inflammation in tissues of rats treated with EIMB extract concentration of 100 to 1000 mg/kg (Fig. 3.6.2, G4 to G6) and scarring, inflammation and occlusion or congestive veins in rats treated with PNML extracts at 100 to 300 mg/kg (Fig. 3.6.2, G7 to G8).

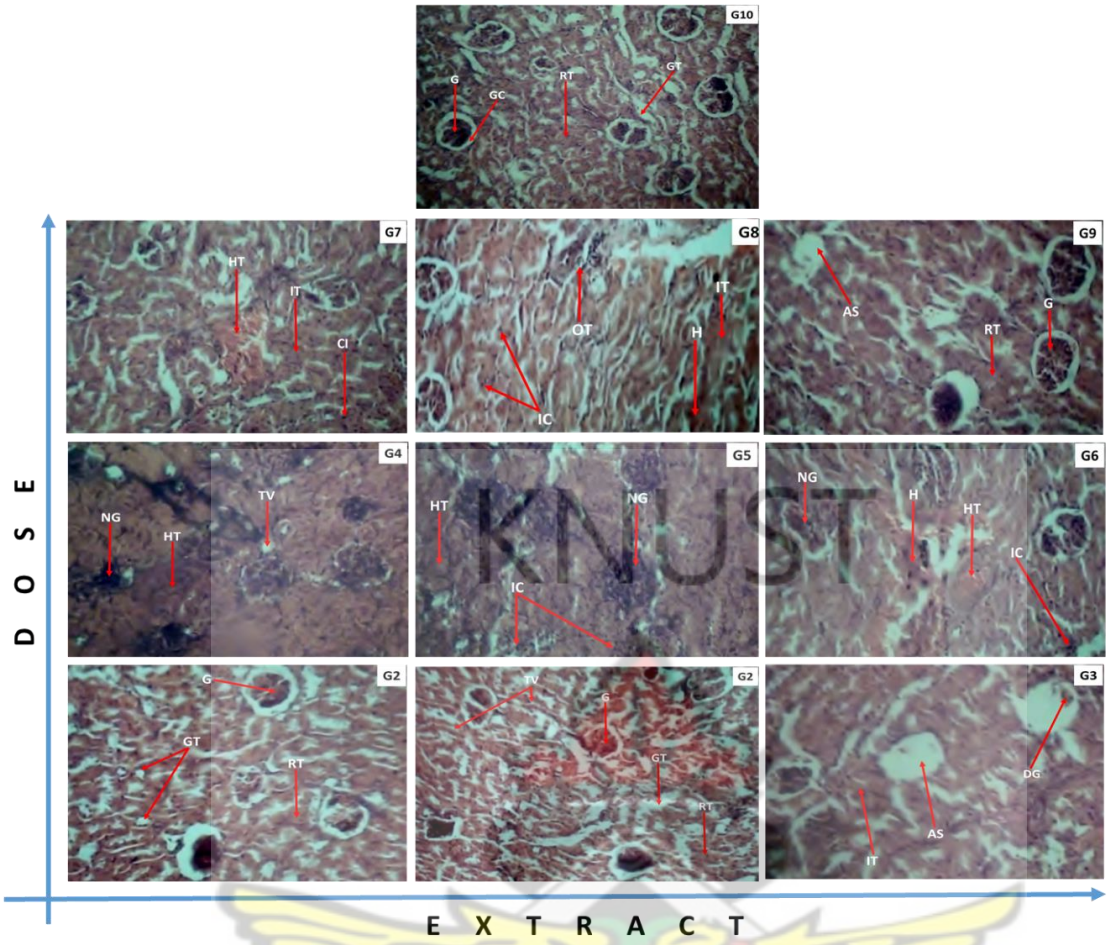

Fig 3.6.1: Effects of *E. ivorense* leaf and stem bark and *P. nigrescens* extracts on kidney tissue of treated and untreated Wistar rats. **(G1) EIML 100mg/kg:** Improved vasculature and a good number of glomerular tufts with no notable changes. (**G2) EIML 300 mg/kg:** Highly vascularized kidney with a good number of glomerular tufts. (**G3) EIML 1000 mg/kg:** Persistent renal tissue inflammation and glomerular degeneration, evident of reduced immunity. (**G4) EIMB 100 mg/kg:** Diseased kidney with profuse renal tissue inflammation, glomerular necrosis and vacuolation, evident of kidney degeneration. (**G5) EIMB 300 mg/kg:** Profuse glomerular degeneration with persistent renal tissue inflammation, indicative of persistent poor treatment response. (**G6) EIMB 1000 mg/kg:** Persistent renal toxicity with profuse haemorrhage, glomerular degeneration, reflective of treatment failure. (**G7) PNML 100 mg/kg:** Persistent hyalination and haemorrhage with focal inflammatory cells infiltration indicative of persistent kidney damage due to reduced immunity. (**G8) PNML 300 mg/kg:** Profuse haemorrhage and persistent renal tissue inflammation reflective of poor immune response. (**G9) PNML 1000 mg/kg:** A good number of restoring glomerular tufts with improved vasculature suggestive of recovery. (**G10) Control 000 mg/kg:** Normal kidney highly vascularized with a good number of glomerular tufts. **Legend: RnT**: Renal Tissue; **RV**: Reduced Vacuolation; **TV**: Tissue Vasculature.**AS**: Apoptotic Space; **DG**: Degenerating Glomeruli; **G**: Glomeruli; **GT**: Glomerular Tubules; **H**: Haemorrhage; **HT**: Hyalinized Tissue; **IC:** Infiltrating Cells; **IT**: Inflamed Tissue **NG**: Necrotic Glomerulus; **OT:** Occluding Tubules.

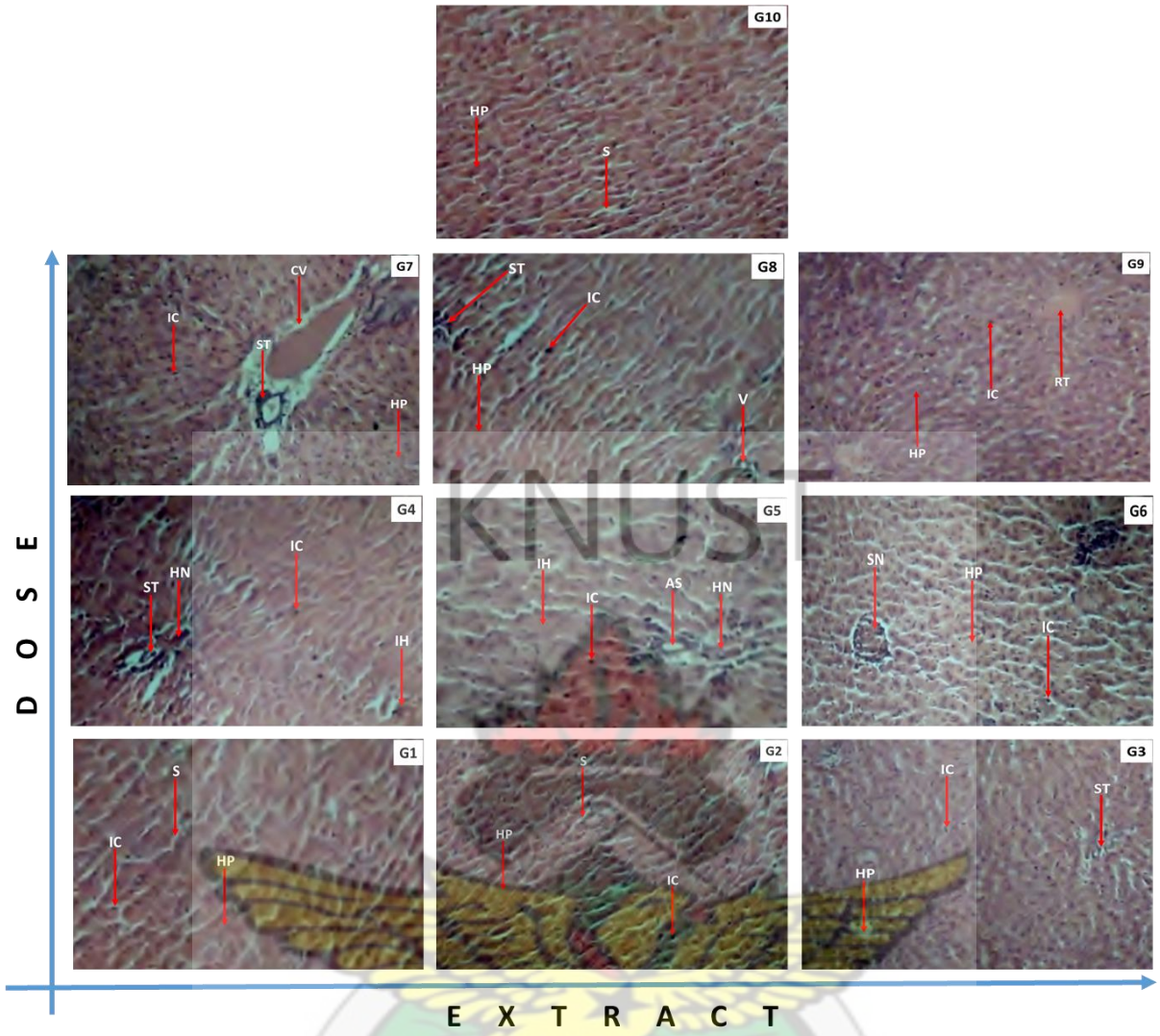

Fig 3.6.2: Effects of *E. ivorense* leaf and stem bark and *P. nigrescens* extracts on liver tissue of both treated and untreated Wistar rats. **(G1) EIML 100 mg/kg:** Healthy hepatocytes and sinusoids, indicative of effective treatment response. (**G2) EIML 300 mg/kg:** Marginal inflammatory infiltrates with healthy hepatocytes and sinusoids, indicative of recovery. (**G3) EIML 1000 mg/kg:** Increased inflammatory infiltrates with few vacuolations and scarrings, indicative of persistent disease. (**G4) EIMB 100 mg/kg:** Diseased liver with profuse inflammatory cell infiltration and hepatocyte necrosis. (**G5) EIMB 300 mg/kg:** Inflamed liver with hepatocyte necrosis, suggestive of sustained hepatotoxicity. (**G6) EIMB 1000 mg/kg:** Sustained inflammation with satellite hepatocyte necrosis, suggestive of impending hepatotoxicity. (**G7) PNML 100 mg/kg:** Increased inflammatory infiltrates with scarring and central venous congestion suggestive of poor immunity. (**G8) PNML 300 mg/kg:** Persistent inflammation with few scarrings and occluding vasculature, evident of sustained disease. (**G9) PNML 1000 mg/kg:** Reduced inflammatory infiltrates with healthy hepatocytes and tissue proliferation, indicative of liver restitution. (**G10) Control 000 mg/kg:** Normal liver with healthy hepatocytes and sinusoids. **Legend: AS**: Apoptotic Space; **CV**: Congested Vein; **HN**: Hepatocyte Necrosis; **HP**: Hepatocytes; **IC**: Inflammatory Cells; **IH**: Inflamed Hepatocytes; **RT**: Regenerating Tissue; **S**: Sinusoids; **SN**: Satellite Necrosis; **ST**: Scar Tissue; **V**: Vacuolation.

3.7 Influence of methanol extracts on viability, proliferation and cytotoxicity of HaCaT keratinocytes

3.7.1 Proliferation of HaCaT keratinocytes

The ELISA-BrdU assay was used to determine the influence of the methanol extracts on the proliferation of the HaCaT keratinocyte cells. Concentrations of 0.1, 1, 10, 50 and 100 µg/mL of methanol extracts (EIML, EIMB and PNML) were administered to HaCaT keratinocytes in the ELISA BrdU test, to study their effect on proliferation of the cells. EIML extract reduced the proliferation of the HaCaT keratinocytes at concentrations between 10.0 to 100 µg/mL (Fig. 3.7.1.1) while EIMB reduced the proliferation at 1.0 to 100 µg/mL (Fig. 3.7.1.2). The PNML extract however reduced proliferation at all used concentration (0.1 to 100 µg/mL) (Fig. 3.7.1.3).

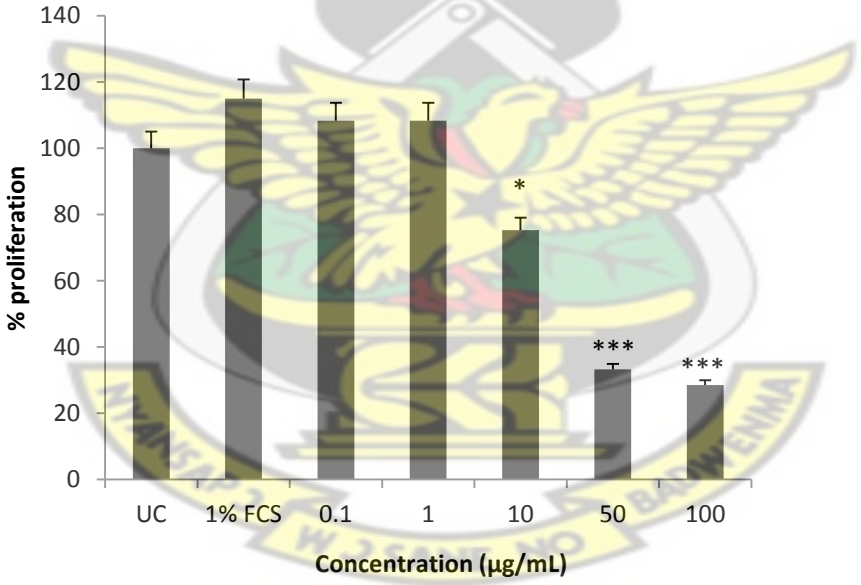

Fig. 3.7.1.1: Influence of methanol leaf extract of *E. ivorense* leaf extract on proliferation of HaCaT keratinocytes. ****p<0.0001, *p< 0.01*

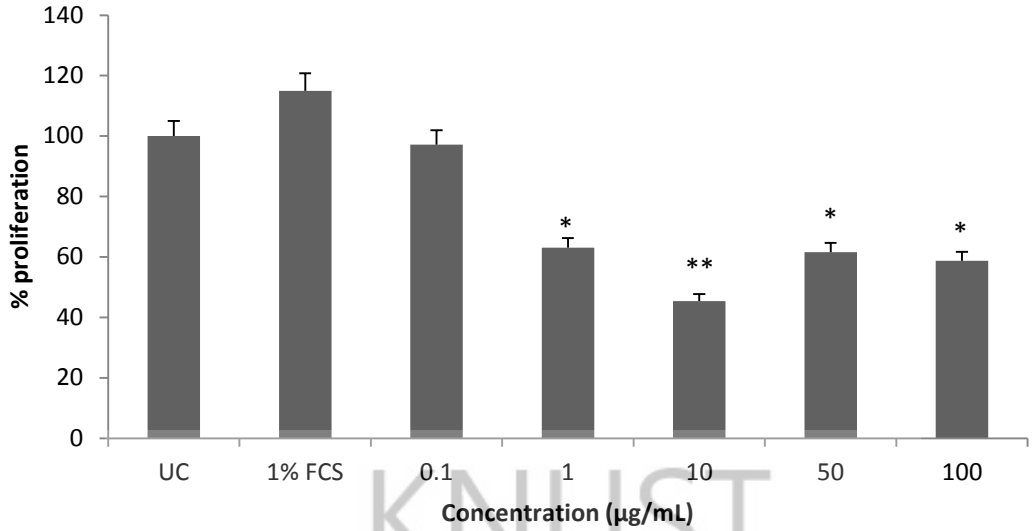

Fig. 3.7.1.2: Influence of methanol bark extract of *E. ivorense* on proliferation of HaCaT keratinocytes. ***p<0.001*

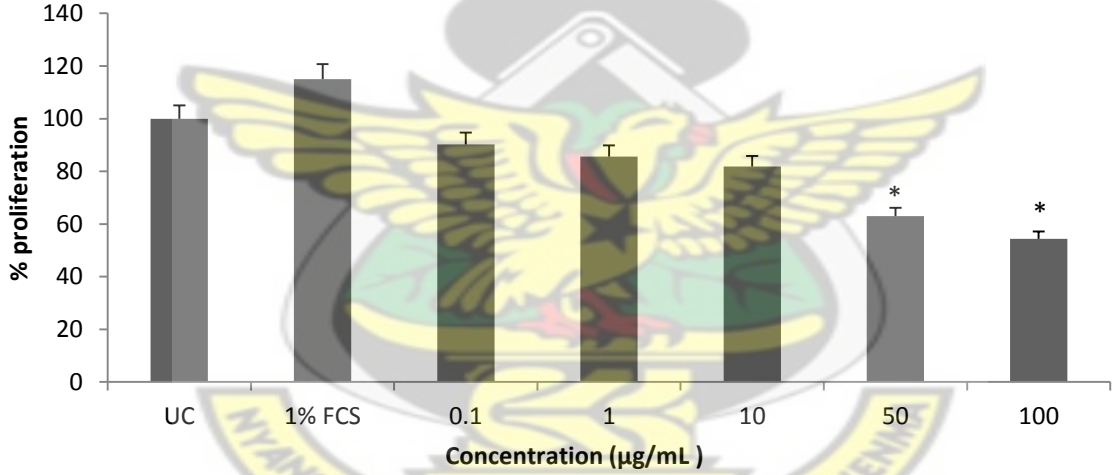

Fig. 3.7.1.3: Influence of methanol leaf and other aerial part extract of *P. nigrescens* on proliferation of HaCaT keratinocytes. **p<0.05*

3.7.2 Viability of HaCaT keratinocytes

The MTT reduction technique was used to study the effects of methanol extracts on the viability of the HaCaT keratinocyte cells. EIML and EIMB reduced the counts of viable cells at

concentrations 0.1 to 100 µg/mL (Fig. 3.7.2.1 and Fig. 3.7.2.2 respectively), while the PNML exhibited similar effect at higher concentrations 50 to 100 µg/mL (Fig. 3.7.2.3).

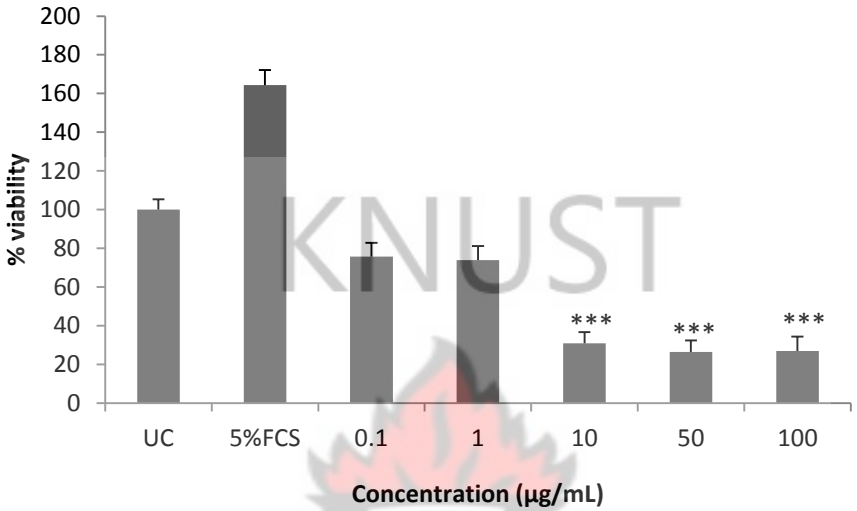

Fig. 3.7.2.1: Influence of methanol leaf extract of *E. ivorense* on viability of HaCaT keratinocytes cells. uc = untreated cells; FCS= foetal calf serum.*** *p<0.0001*

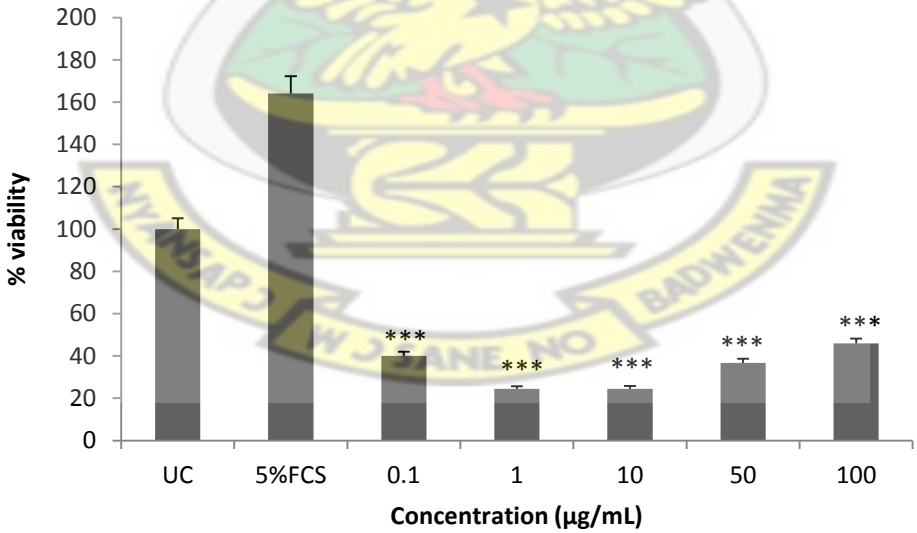

Fig. 3.7.2.2: Influence of methanol bark extract of *E. ivorense* on viability of HaCaT keratinocytes cells. uc = untreated cells; FCS= foetal calf serum; ***$p< 0.0001$

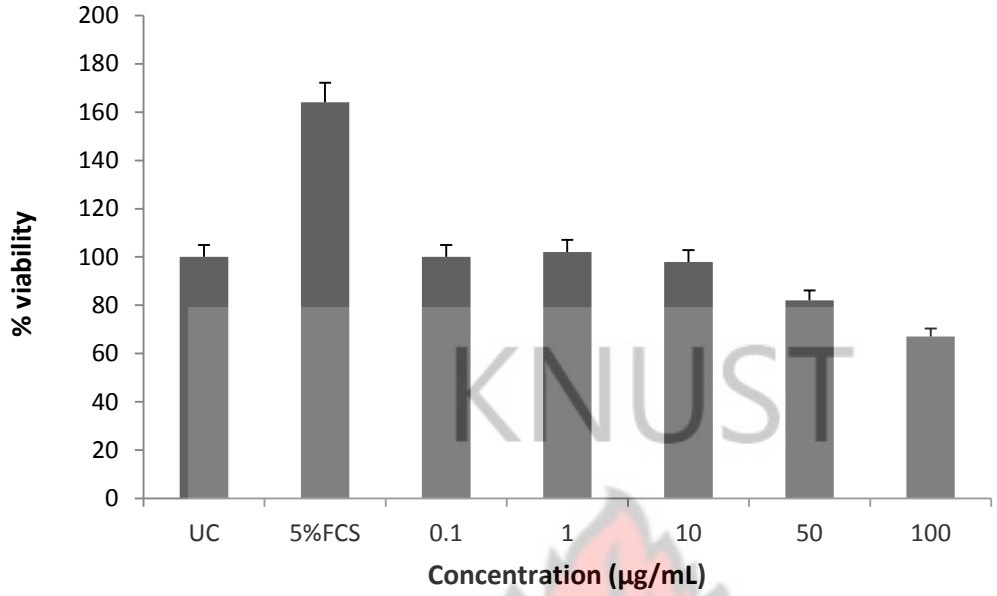

Fig. 3.7.2.3: Influence of methanol leaf and other aerial part extract of *P. nigrescens* on viability of HaCaT keratinocytes. uc = untreated cells; FCS= foetal calf serum; *$p<0.01$*

3.7.3 Release of lactate dehydrogenase (LDH) from HaCaT keratinocyte cells

The assay which measures the release of LDH into extracellular environments of damaged or stressed cells was used to study the cytotoxic activity of the methanol extracts on HaCaT keratinocytes. Concentrations of 0.1, 1, 10, 50 and 100 µg/mL of methanol extracts (EIML, EIMB and PNML) were administered to HaCaT keratinocytes to find out the level of cytotoxicity. There were smaller increase in amount of LDH released from HaCaT keratinocytes to which EIML and EIMB extracts were administered as compared to the PNML extract (Fig. 3.7.3.1 to Fig. 3.7.3.3).

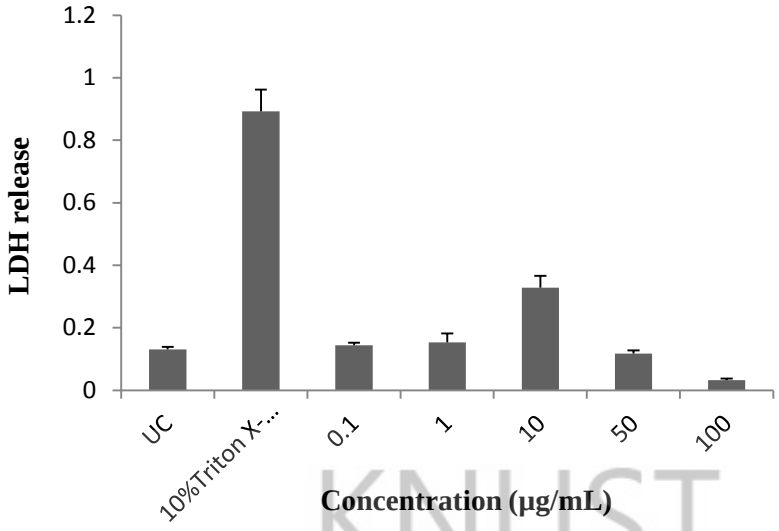

Fig. 3.7.3.1: Influence of methanol leaf extract of *E. ivorense* on the release of LDH from HaCaT keratinocyte cells. uc = untreated cells *p>0.05*

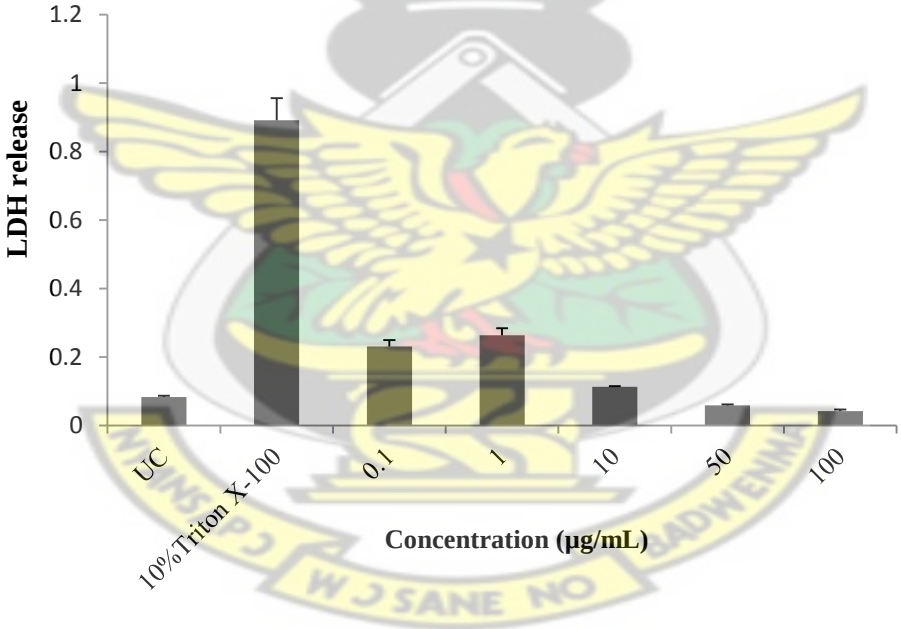

Fig. 3.7.3.2: Influence of methanol bark extract of *E. ivorense* on the release of LDH from HaCaT keratinocyte cells. uc = untreated cells **p<0.05*

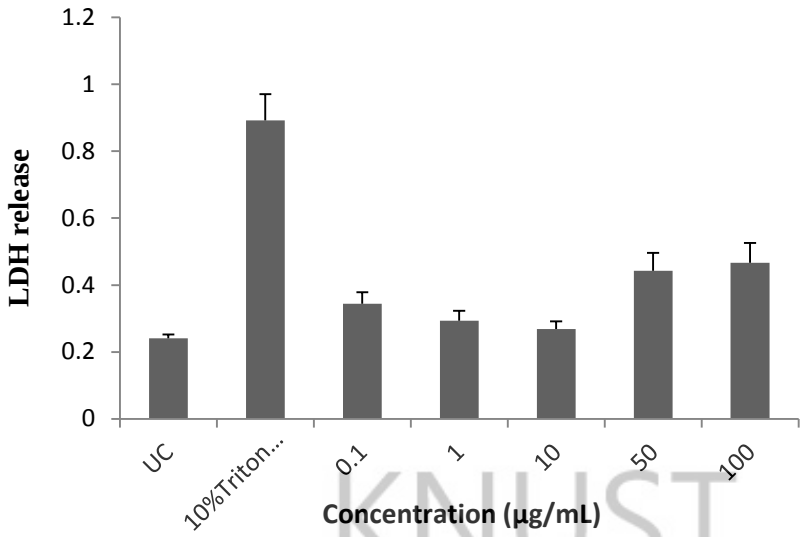

Fig. 3.7.3.3: Influence of methanol leaf and other aerial part extract of *P. nigrescens* on the release of LDH from HaCaT keratinocyte cells. uc = untreated cells *p>0.05*

CHAPTER FOUR

4.0 Discussion

Health practitioners and the inhabitants of various communities have used different parts of plants to treat various infections. Plants are a large reservoir of therapeutic compounds from which several medications have been developed (Olaleye, 2007). They have been used to treat diseases caused by bacterial, fungal, viral and parasitic organisms. These plants are prepared and used in various forms including decoctions, poultice, infusions, tinctures and macerations. These compounds, mainly, secondary metabolites, are usually responsible for the exertion of the medicinal properties of the plants (Oksman-Caldentey and Barz, 2002). However, some of these plants have also been shown to cause severe side effects and toxicities to their users and thus require the attention of researchers. Traditional medical practitioners use the leaves of *E. ivorense* to treat menstrual problems (Burkill, 1995). The stem-barks are used to treat pulmonary diseases (Burkill, 1995) and small pox (Dongmo *et al.,* 2001, Burkill, 1995). The leaves and tendrils of *P. nigrescens* are also used to treat wounds, carbuncle, worm infection and stomach problem (Agyare *et al.,* 2009).

Traditional healers normally use water as extractants in medicinal plant preparations for the treatment of ailments. Majority of the antimicrobial components of plants that have been identified are however not water soluble. Reports have shown that extracts made from organic solvents are more potent and usually exhibit better and consistent antimicrobial activity (Parekh *et al*., 2005; Parekh *et al*., 2006). Methanol thus may be a better extracting solvent (extractant) than water.

The methanol extracts were screened for their phytochemical constituents and the leaf extracts of *E. ivorense* (EIML) showed the presence of saponins, condensed tannins, sterols, and flavonoids, whereas the bark extract (EIMB) showed the presence of saponins, hydrolysable tannins,

flavonoids, alkaloids, terpenoids and cardiac glycosides. These phytochemicals were also found in aqueous leaf extract of *E. africanum* (Hassan *et al.,* 2007) and ethanol stem bark extract of *E. guineense* (Adeoye and Oyedapo, 2004) which belong to the genus *Erythrophleum*. The *P. nigrescens* extracts showed the presence of saponins, condensed tannins, alkaloids, terpenoids, sterols, flavonoids and cardiac glycosides and this is consistent with earlier report by Imaga *et al.* (2010).

Antimicrobial, oestrogenic, anti-inflammatory, enzyme-inhibition (Havsteen, 1993; Harborne and Baxter, 1999), antiallergic, antioxidant (Middleton and Chithan, 1993), cytotoxic, vascular and antitumour activity (Harborne and Williams, 2000) have been attributed to flavonoids. The presence of these therapeutic compounds may support the use of *E. ivorense* and *P. nigrescens* in the treatment of microbial infections.

Chromatographic fingerprinting of herbal extracts is used for both qualitative and quantitative determination of the possible constituents and quality control measures (Chang *et al.,* 2009; Chitlange, *et al.*, 2009; Negi and Singh, 2011). Several approaches are used to analyze patterns of chromatograms and these patterns consider signal intensity, retention time, peak area, and peak height of each independent peak without overlap. HPLC chromatograms are used to classify and discriminate different herbal materials (Zhang *et al.,* 2005; Wan, 2006).

The HPLC chromatogram of EIML extract showed peaks after different retention times between 1 to 13 min. The EIMB extract showed distinct peaks within 1 to 5 min, while the PNML extract showed peaks between 1 to 9 min. These individual peaks may be characteristic of various phytoconstituents or compounds present in the extracts. These unique fingerprints at the specific retention times are quality control and identification parameters that could help us distinguish between plant species (Negi and Singh, 2011).

Aside their usage to treat infectious disease due to their vast therapeutic properties, antimicrobials that are sourced from plant have a concomitant effect of alleviating some side effects often associated with synthetic antimicrobials (Iwu *et al.,* 1999). Antimicrobial activity screening of the extracts using the agar well diffusion method showed zones of growth inhibition at concentrations of 12.5, 25.0, 50.0 and 100 mg/mL against the test organisms. The zones of growth inhibition of methanol extracts of *E. ivorense* were relatively bigger than those for the *P. nigrescens* extracts (Tables 3.4.1.1 to 3.4.1.3). *E. ivorense* have a comparatively better antimicrobial activity than the *P. nigrescens.*

The zones of growth inhibition ranged between 11 and 23 mm for Gram-positive bacteria, between 10 to 16 mm for Gram-negative bacteria and between 11 to 21 mm for *C. albicans.* The methanol leaf extract of *E. ivorense* showed no activity against *P. aeruginosa.* None of the extracts at the various concentrations used showed activity against *E. coli*. The rate of diffusion of the antimicrobial agent through the agar is dependent on the diffusion and solubility properties of the agent in the agar (Bauer *et al.*, 1966) and the molecular weight of the antimicrobial compound where larger molecules will diffuse at a slower rate than lower molecular weight compounds. The inactivity of the extracts against some of the organisms may be attributed to a poor diffusion of the plant extract through the agar to exert their antimicrobial activity (Agyare *et al*., 2013; Eloff, 2007).

The MICs of EIML, EIMB and PNML extracts ranged from 2.0 to 6.0 mg/mL against Gram-positive bacteria, 3.0 to 8.0 mg/mL against the Gram-negative bacteria and 2.0 to 4.0 mg/mL against *C. albicans*. Generally, the MICs of the extracts for the test organisms were relatively lower in bark extract of *E. ivorense* compared to the leaf extract of *E. ivorense*, and the leaf and other aerial part extract of *P. nigrescens* (Table 3.4.2.1). Thus, the bark extract was more active against the test microorganisms than the other extracts used in this study since a lower MIC

value is indicative of the fact that antimicrobial activity against the test pathogenic organisms could be achieved at lower concentrations. Fabry *et al.* (1998) reported that extracts with low MICs (< 8 mg/mL) indicate a potent antimicrobial activity of the extracts.

Haas et al. (2010) reported that the type of antimicrobial action exhibited by an antimicrobial agent may be determined as either static or cidal by calculating the MBC to MIC (MBC:MIC) ratios. The ratio of MBC to MIC which is greater than 4 indicates bacteriostatic or fungistatic activity while MBC: MIC ratios <4 represent bacteriocidal or fungicidal activity. The ratio of MBC to MIC of methanol extracts (EIML, EIMB and PNML) were less than 4 and hence indicate that the extracts exhibit bacteriocidal and fungicidal actions against the test organisms.

Bacteria that colonize wounds include *S. aureus, Streptococcus spp.* and *P. aeruginosa* (Agyare *et al.*, 2013). The observed antimicrobial activity of the methanol extracts against these test organisms may support the folkloric use of *P. nigrescens* to treat wound infections and boils (Agyare *et al.*, 2009).

Secondary metabolites including alkaloids, terpenoids, and flavonoids etc. from plants have been found to possess antimicrobial activity (Dahanukar *et al.*, 2000; Adesokan *et al.*, 2008). Plants have been found to synthesize flavonoids in response to microbial infection and these flavonoids have shown *in vitro* antimicrobial activity against microorganisms (Bennet and Wallsgrove, 1994). The antimicrobial activity is believed to be due to their effectiveness in inactivating microbial adhesions and metabolism, and also impeding development and functioning of enzymes, cell envelope and proteins (Hugo and Russel, 1998; Prescott, 2002; Talaro and Talaro, 2001). These phytochemical constituents also form complexes with polysaccharides and proteins in the microbes rendering them inactive (Tsuchiya *et al.*, 1996). Tannins have also shown to be active against bacteria and filamentous fungi including *C. albicans* (Scalbert, 1991) and possess

astringent properties (Agyare *et al.,* 2013). Olowosulu and Ibrahim (2006) reported that flavonoids have been shown to have antimicrobial activity and this activity, according to Tsuchiya *et al.* (1996) may be attributed to their ability to complex with extracellular and soluble proteins, and cell walls of the bacteria. Therefore, the antimicrobial activity of the methanol extracts (EIML, EIMB and PNML) may be due to the physiological activities of the phytochemical constituents present in them.

Tribulus terrestris, Panicum coloratum and *Nolina texana* are plants with considerable amounts of saponins which active principles have shown to be poisonous to animals. These steroidal sapogenins isolated from these have been found to be hepatotoxic (Shumaik *et al.,* 1988; Miles *et al.,* 1993). They act by converting to glucuronide conjugates of epsimilagenin and these conjugates crystallize in bile and lead to biliary blockage and cholangitis (inflammation of bile ducts) in liver (Miles *et al.,* 1991; Miles *et al.,* 1993).

The methanol extracts (EIML, EIMB and PNML) were screened for their potential toxic effect on blood parameters and organs of rats. There were significant increase ($p<0.0001$), between the mean counts of red blood cells (RBC), haemoglobin (HGB) and hematocrit concentrations at all doses of administration of the extracts as compared to the control group. This is fairly consistent with other reports which also observed significant increases in these haematological parameters at 400, 800 and 1600 mg/kg dose levels of aqueous leaf extract of *P. nigrescens* (Agbor and Odetola, 2005) and at 50 and 100 mg/kg body weight of aqueous root extract of the same plant (Owoyele *et al.,* 2011). This may support the folkloric use of parts of this plant in Nigeria to treat anaemia (Owoyele *et al.,* 2011).

However, there was a decrease, though not significant ($p>0.05$) in the mean white blood cells (WBC) and lymphocytes counts at all doses of leaf extracts of *E. ivorense* and leaf and other

aerial part extract of *P. nigrescens* but rather an increase in the mean counts for the rats administered with the bark extract of *E. ivorense* (Table 3.5.1). The EIMB extract showed increase in WBCs and lymphocytes ($p>0.05$) at dose of 100 mg/kg. The reduction in the mean counts of WBCs in rats administered with aqueous leaf extract of *P. nigrescens* has also been reported in previous reports (Agbor and Odetola, 2001; Nsiah *et al.*, 2006). Owoyele *et al.* (2011) also reported decrease in mean counts of WBCs in rats treated with lower doses of 50 and 100 mg/kg body weight of aqueous root extracts of *P. nigrescens*. The active principles including phenols, cardiac glycosides, terpenoids, saponins, alkaloids, tannins, and steroids may be responsible for these effects (Owoyele *et al.*, 2011). There was no significant difference ($p>0.05$) in the mean counts and percentage concentration of neutrophils in the treated rats compared with untreated control group. Nonetheless, the results from the effects of the extracts on WBCs should prompt caution in administration of PNML extracts as they may affect the immune response of persons who use them.

The serum biochemical analyses of blood samples showed increase, though not significant ($p>0.05$), in albumin, creatinine and cholesterol concentrations of rats treated with the extracts (EIML, EIMB and PNML) compared to the untreated rats (Table 3.5.2). There were significant increases ($p<0.01$) in the total bilirubin but decreases in triglyceride concentrations in rats administered with 100, 300 and 1000 mg/kg body weight of EIML and EIMB extracts compared with the untreated animals (Table 3.5.2). The rats administered with PNML extract however showed no significant increase in total bilirubin but significant decrease ($p<0.01$) in triglyceride concentrations when compared with the untreated group. However, all the extracts did not show significant ($p>0.26$) increases in high-density lipoproteins (HDL) but a significant increase ($p<0.05$) in low-density lipoproteins (LDL) cholesterol concentration in all treated groups as compared to the untreated group. Increase in these lipid profiles have been reported for *P.*

nigrescens aqueous root extract at 100 and 150 mg/kg body weight by Owoyele *et al.* (2011). Similar effects of different plant extracts on lipid profiles have been reported (Lindequist *et al.*, 2005; Venkatesan *et al.*, 2003). These changes in lipid profile indicators may thus be useful in investigating the metabolism of lipids in the rats and how prone they may be to coronary diseases (Owoyele *et al.*, 2011).

The reduction in the blood glucose level was significant ($p<0.0001$) for all the extracts compared to the untreated (Table 3.5.2). Similar findings were reported with extracts of *P. nigrescens* which reduced significantly the glucose level in Wistar rats induced with diabetes (Saba *et al.*, 2010) and this may justify the folkloric use of *P. nigrescens* as an antidiabetic agent (Saba *et al.*, 2010). *E. ivorense* must be investigated for possible source of antidiabetic agent.

Histopathological investigations of kidney and liver tissues showed marked morphological variations in tissues from rats given doses of the various extracts as compared to the untreated group. The kidney tissues from rats administered with doses of 1000 mg/kg of EIML (Fig. 3.6.1, G3), 100 to 1000 mg/kg EIMB (Fig. 3.6.1, G4 to G6) and 100 to 300 mg/kg of PNML body weight extracts (Fig. 3.6.1, G7 to G8) showed persistent renal tissue inflammation and glomerular degeneration and necrosis. There were also apoptotic space and occluding tubules. Neutrophils are the predominant granulocytes that are seen in initial stages of acute inflammation (Alberts and Bruce, 2005). These neutrophils are packed with granules containing inflammatory factors like leukotrienes (Shen and Louie, 2005). These inflammatory factors could be involved in the evidence of inflammation shown in the micrograph of tissues. These inflammations could cause a reduced flow of blood through kidneys. The result is a surge in blood creatinine concentration (Table 3.5.2) and a decrease in the renal plasma clearance of creatinine (Fox, 2003). Cheesbrough (1991) showed increase in serum creatinine levels of rats treated with aqueous leaf extract of *Erythrophleum africanum* and the associated reduction in renal function.

Since kidney and liver are organs of metabolism and excretion respectively of xenobiotic molecules such as saponins, alkaloids and tannins, the presence of these secondary metabolites in *E. ivorense* may be responsible for the observed hepatorenal toxicities (Hassan *et al.*, 2007).

The PNML extract at 1000 mg/kg however showed good number of restoring glomerular tufts with improved vasculature which are suggestive of recovery from tissue damage (Fig. 3.6.1 G8). Tissue from kidney of the control group showed highly vascularized kidney with a good number of glomerular tufts (Fig. 3.6.1, G10) indicative of no end organ toxicity from the administration of only water.

In the examination of the liver tissues, the EIML extract showed increased inflammatory infiltrates with few vacuolations and scarrings (cirrhosis), indicative of persistent damage while the EIMB extract treated rats showed liver tissue with profuse inflammatory cell infiltration and hepatocyte necrosis. The PNML extract at 1000 mg/kg body weight showed reduced inflammatory infiltrates with healthy hepatocytes and tissue proliferation, indicative of liver restitution (Fig. 3.6.2 G9).

Alanine transaminase (ALT), aspartate transaminase (AST) and γ-glutamyl (GGT) are enzymes found in the cytoplasm of cells and they are involved in amino acid metabolism but only released into systemic circulation after cells have been damaged (Sallie *et al.*, 1991; Cheesbrough, 1991). Hassan *et al.* (2007) reported increases in serum levels of ALT and AST in treating rats with 2000 to 3000 mg/kg body weight of *E. africanum* and suggested that the extracts must have affected the permeability of liver cell membranes and made them leaky, thus the leakage of ALT and AST to raise their serum levels. An increase in the level of ALT and AST in blood serum of treated rats above normal ranges in the untreated rats may explain the liver damages by the methanol extracts (EIML, EIMB and PNML).

Spier et al. (1987) reported that hydrolysable tannins which are astringents bind to proteins in plasma and body organs resulting in coagulation and necrosis. Blood from all the animals treated with the extracts (EIML, EIMB and PNML) showed increase in serum total protein concentration. Hassan *et al.* (2007) has also reported increases in the concentration of serum total protein in rats administered with extract of *E. africanum*. The increases in serum total protein concentration have been attributed to liver injury and hepatic toxicity (Gatsing *et al.*, 2006; Emerson *et al.*, 1993). The methanol extracts EIML, EIMB and PNML) may therefore be toxic to animals due to the observed increase in serum total protein concentration. Therefore, methanol leaf and bark extracts of *E. ivorense* and leaf and other aerial part extract of *P. nigrescens* may be toxic to animals even at low concentrations.

All the methanol extracts at concentrations 10, 50 and 100 µg/mL caused a gradual reduction in the proliferation of the HaCaT keratinocytes as compared to the untreated cells (Figures 3.7.1.1 to 3.7.1.3) with significant reductions observed in treatments with EIML ($p<0.0001$) and EIMB ($p<0.001$) extracts. Hence these methanol extracts may be investigated for possible influence on cancerous cell lines or tissues.

All cells may lose their viability either through necrosis (usually as a result of infection with an agent exogenous or foreign to the affected cells) or by apoptosis. MTT reduction assay is performed to investigate the activity of mitochondrial and nonmitochondrial dehydrogenases of compounds as a potential indication of their cytotoxic effect (Döll-Boscardin *et al*., 2012). In the MTT assay, endonuclear dehydrogenases of the endoplasmic reticulum and mitochondrial dehydrogenases of the mitochondria of intact or viable HaCaT keratinocyte cells cleave the tetrazolium rings in the MTT molecules, yielding purple formazan crystals of MTT insoluble in aqueous solutions but soluble in dimethyl sulfoxide (Mosmann, 1983). Significant reduction in the viability of the HaCaT keratinocytes were observed from treatment with 10, 50 and 100

µg/mL of leaf extract of *E. ivorense*, 0.1 to 100 µg/mL of bark extracts of *E. ivorense* ($p<0.0001$) and 100 µg/mL leaf and other aerial parts extract of *P. nigrescens* ($p<0.01$) as compared with the untreated cells. The methanol extracts therefore showed the potential to alter the enzymatic activity of the mitochondria of HaCaT keratinocyte cells and initiate a primary and initial injury of the cells that eventually lead to the loss of viability in the HaCaT keratinocyte cells. This injury or loss of integrity could be as a result of a possible decrease in membrane potential of the HaCaT keratinocytes and a resultant depolarization or change in fluidity of the mitochondrial membrane thereby making the cells higly permeable to cytotoxic agents (Novgorodov and Gudz, 1996; Richter and Schlegel, 1993).

Lactate dehydrogenase (LDH) enzymes are found in the cytosol of cells but can be measured extracellularly when the cells are lysed or injured. There was an increase in the amount of LDH released from the cells treated with concentrations of 1 to 10 µg/mL of the methanol extracts (EIML, EIMB and PNML) compared with the untreated cells (negative control) though not statistically significant ($p>0.05$). In the study, cells that are in damage or under stress would release cytoplasmic LDH into the medium either as a result of cytoplasmic membrane disruption or necrosis (Korzeniewski and Callewaert, 1983). A similar report that has been given in cytotoxic studies of essential oils of *Eucalyptus benthamii* and its related terpenes on tumor cell lines (Döll-Boscardin *et al*., 2012). The release of LDH from HaCaT keratinocytes treated with low concentrations of the extracts may give indication that higher concentration may exhibit cytotoxicity. There is the need to isolate the bioactive compounds responsible for the biological activities observed for the extracts (EIML, EIMB and PNML) of *E. ivorense* and *P. nigrescens.*

CHAPTER FIVE

5.1 Conclusion

The methanol leaf and bark extracts of *E. ivorense* and the methanol leaf and other aerial part extract of *P. nigrescens* contained secondary metabolites including flavonoids, tannins, saponins, sterols, alkaloids and terpenoids.

The methanol leaf and bark extracts of *E. ivorense* and the methanol leaf and other aerial part extract of *P. nigrescens* exhibited broad spectrum antimicrobial activities against the test organisms. The MICs of the extracts against Gram positive bacteria ranged between 3.0 to 6.0 mg/mL while those for Gram negative bacteria ranged between 3.0 to 8.0 mg/mL. The MICs of the extracts against *C. albicans* was between 2.0 to 4.0 mg/mL.

The rats treated with the methanol extracts (EIML, EIMB and PNML) exhibited toxicity at 100 to 1000 mg/ kg body weight on kidney and liver tissues within the 35 days of administration. The kidney tissues from groups administered with the extracts showed diseased conditions including inflammation of cells, necrotic tissues and apoptotic spaces. The liver tissues from treated rats showed cirrhosis, inflammation and necrosis. All the extracts at concentrations of 0.1 to 100 mg/mL did not induce increase in HaCaT keratinocytes proliferation.

5.2 Recommendations

- Bioactivity-guided isolation and characterization of the active compounds from the two plants should be performed.
- Acute and chronic toxicity studies of the plants should be done.
- Methanol leaf and stem-bark extracts of *E. ivorense* must be investigated for the possible presence of antidiabetic agent or compounds.

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APPENDICES

APPENDIX I: PREPARATION OF CULTURE MEDIA

1. NUTRIENT AGAR (CM 0003, Oxoid)

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

Twenty grams (28g) of nutrient agar powder was weighed into a conical flask and dissolved in distilled water to 1 litre. This was then heated on a water bath to boil and poured into glass test tubes (20 mL per tube) and sterilised in an autoclave at 121^{o}C for 15min.

2. NUTRIENT BROTH (CM 0001, Oxoid)

Composition	Quantity (g)
'Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0

Thirteen grams (13g) of nutrient broth powder was weighed into a beaker and dissolved in distilled water to 1 litre. The mixture was then poured into glass tubes (10 mL per tube) and sterilized in an autoclave at 121^{o}C for 15 min.

3. PLATE COUNT AGAR (CM 0325, Oxoid)

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

Plate count agar (17.5 g) powder was weighed into a beaker and dissolved with in distilled water to 1 litre. The solution was heated on a water bath to boil and poured into glass test tubes (20 mL per tube). They were then sterilised in an autoclave at 121^{o}C for 15min.

4. MACCONKEY AGAR (CM 0007, Oxoid)

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

MacConkey agar powder (51.5 g) was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was boiled and poured into glass test tubes (20 mL per tube). They were sterilized in an autoclave at 121^{o}C for 15 min.

5. MANNITOL SALT AGAR (CM 0085, Oxoid)

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

One hundred and eleven grams (111g) mannitol salt agar powder was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was boiled over boiling water in a water bath and poured into glass test tubes (20 mL per tube). They were sterilized in an autoclave at 121^{o}C for 15 min.

6. CETRIMIDE AGAR (CM 0579, Oxoid)

Composition	Quantity (g)
Gelatin peptone	20.0
Magnesium chloride	1.40
Potassium sulphate	10.0
Cetrimide	0.3
Agar	13.6

Cetrimide agar powder (45.3g) was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was boiled over boiling water in a water bath and poured into glass test tubes (20 mL per tube). They were sterilized in an autoclave at 121^{o}C for 15 min.

7.0 POTATO DEXTROSE AGAR (CM 0139, Oxoid)

Composition	Quantity (g)
Potato extract	4.0
Dextrose	20.0
Agar	15.0

Thirty nine grams (39g) of potato dextrose agar powder was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was boiled over boiling water in a water bath and poured into glass test tubes (20 mL per tube). They were sterilized in an autoclave at 121^{o}C for 15 min.

8.0 KOSER'S CITRATE MEDIUM (CM 0155, Oxoid)

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

Koser's citrate powder (5.2g) was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was then distributed into glass test tubes (10mL per tube) and sterilized at 121^{o}C for 15 min in an autoclave.

9. TRYPTONE BROTH (CM 0073, Oxoid)

Composition	Quantity (g)
Tryptone	10.0
Sodium chloride	5.0
Bromocresol purple	0.04

Fifteen grams (15g) of tryptone broth powder was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was then distributed into glass test tubes (10mL per tube) and sterilized at 121^{o}C for 15 min in an autoclave.

10. MRVP MEDIUM (CM 0043, Oxoid)

Composition	Quantity (g)
Peptone	7.0
Glucose	5.0
Phosphate buffer	5.0

Seventeen grams (17g) of the powder was weighed into a beaker and dissolved in distilled water to 1 litre. The solution was then distributed into glass test tubes (10mL per tube) and sterilized at 121^{o}C for 15 min in an autoclave.

APPENDIX II: Standardisation of microbial suspensions

Table A1: Absorbance of 24 h broth culture

S. aureus		*B. subtilis*		*E. coli*		*P. aeruginosa*		*C. albicans*	
Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL	Mean Absorbance	Log cfu/mL	Mean Absorbanc	Log cfu/mL	Mean Absorbance	Log cfu/mL
0.639	6.693	0.329	1.505	0.497	8.255	0.494	7.303	0.336	1.079
0.662	7.622	0.331	2.283	0.4976	7.255	0.576	8.441	0.340	1.477
0.746	8.626	0.358	2.455	0.502	9.255	0.671	9.362	0.359	1.833
0.790	9.672	0.379	2.626	0.822	10.255	0.781	10.340	0.524	2.100

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

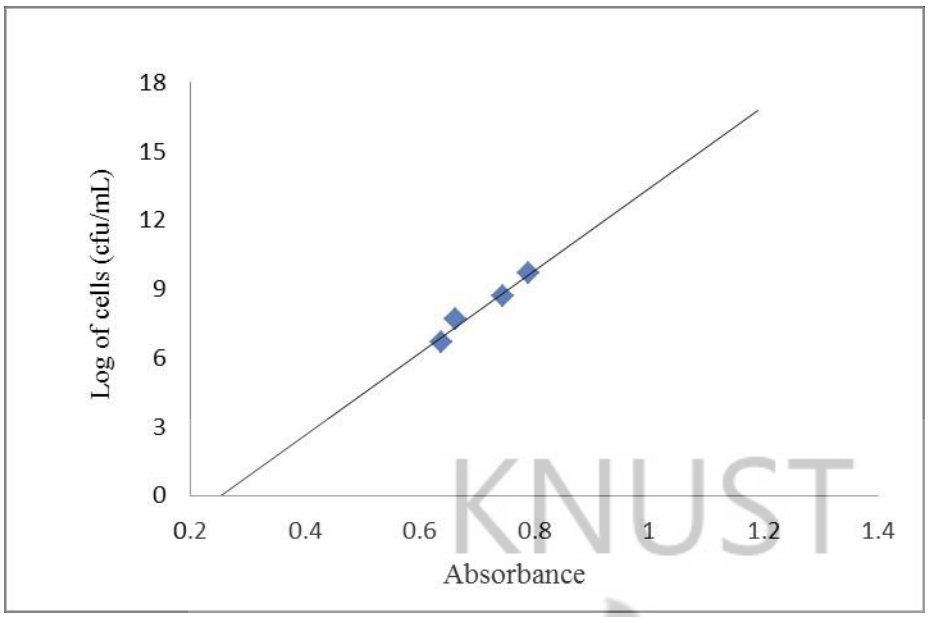

Fig. A1: Standardisation of *Staphylococcus aureus* suspension

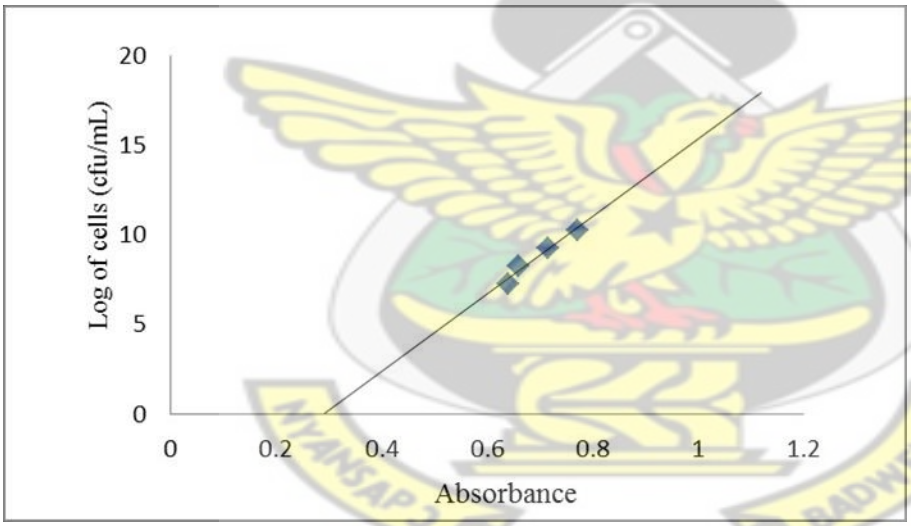

Fig. A2: Standardisation of *Bacillus subtilis* suspension

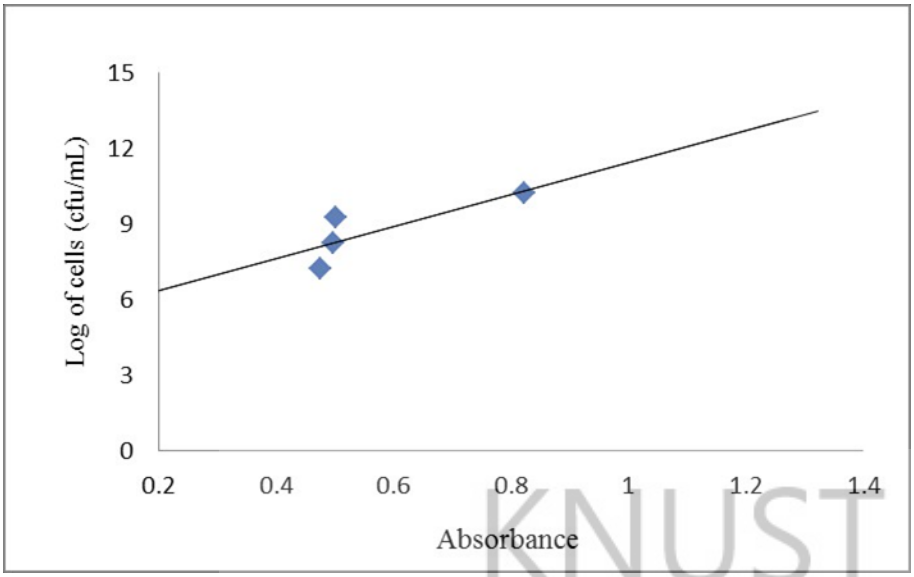

Fig A3: Standardisation of *Escherichia coli* suspension

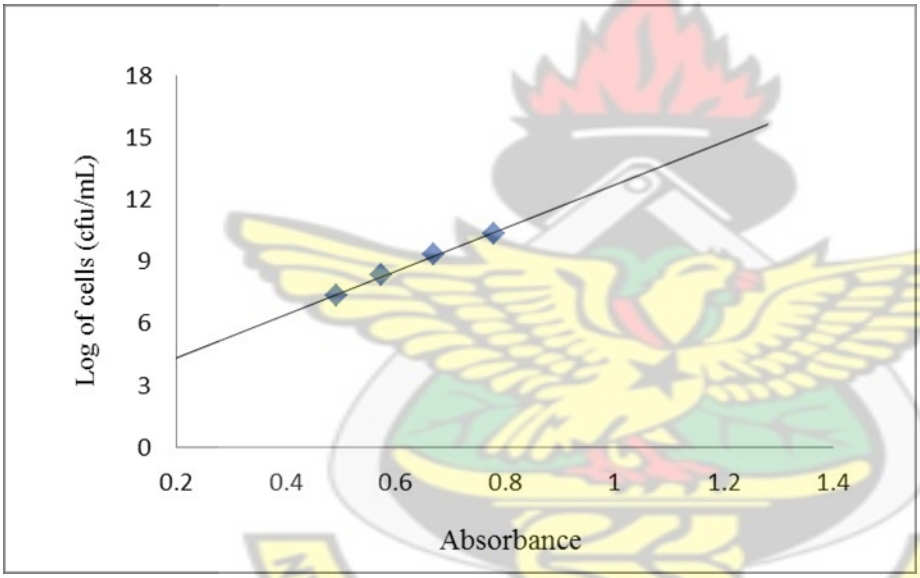

Fig. A4: Standardisation of *Pseudomonas aeruginosa* suspension

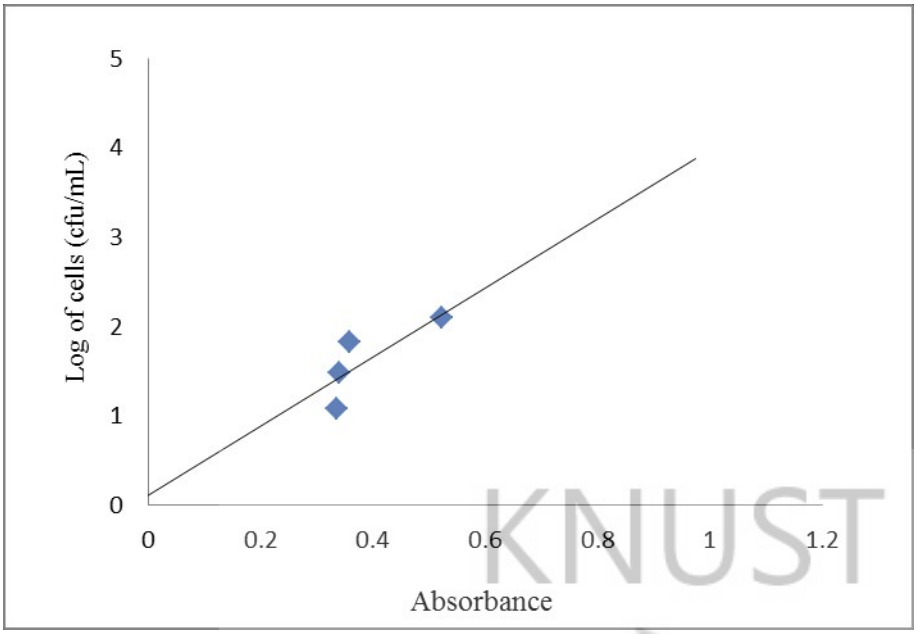

Fig. A5: Standardisation of *Candida albicans* suspension

APPENDIX III: CONFIRMATORY TESTS ON TEST ORGANISMS

1. *Staphylococcus aureus*

S. aureus was identified by culturing a suspension of organisms on mannitol salt agar folloed by coagulsae test. The appearance of yellow colonies on the surface of the agar after 24 h incubation at 37 ºC indicates the presence of *S. aureus.* Furthermore, the isolated organisms on mannitol salt agar are re-cultured in nutrient broth. A quantity of 0.1mL of a 24 h broth culture of the isolated colonies was inoculated into nutrient agar containing 10% $^{v}/_{v}$ rabbit blood plasma and incubated for 24 h at 37 ºC. The coagulation of the plasma (coagulase positive) confirms the suspension to be *S. aureus.*

2. *Bacillus subtilis*

Starch and casein hydrolysis was used to indentify *B. subtilis*. Nutrient agar containing 10% $^{w}/_{v}$ starch was prepared. A loopful of 24 h broth culture of the suspension of organism was streaked at the middle on the surface of nutrient agar containing 10% $^{w}/_{v}$ starch and incubated at 37 ºC for 24 h. After incubation the plate was then sprayed with iodine solution. Clear region due to the hydrolysis of starch were seen against a blue black surrounding. The clear zones were indicative of the presence of *B. subtilis.*

3. *Escherichia coli*

MacConkey agar was seeded with the organisms by streaking and the plate incubated at 37ºC for 24 h. The appearance of red-violet colonies on the surface of the agar after incubation indicates the presence of *E. coli*. The indole and Methy Red-Voges Proskauer (MRVP) tests were used as confirmatory test where this organism is known to be MR positive and VP negative. In the indole test 0.1 mL of the suspension of organism as inoculated in tryptone broth and incubated at 37 ºC for 24 h. After incubation Kovac's reagent was added to the tryptone broth. The appearance of a pink/red ring at the interface or meniscus of the broth indicated the presence of *E. coli.* The MRVP test was performed by inoculating a 24 h broth culture of the organisms in MRVP broth. After incubation the culture was divided into two for the distinct MR and VP tests. In the MR test methyl red was added to the culture and the appearance of pink/red colouration showed that the organism is MR positive. α-naphthol and KOH solutions were added to the second part of the broth culture for the VP test. The absence of a cherry red colouration indicates that the organism was VP negative.

4. *Pseudomonas aeruginosa*

Two loopfuls of a suspension of the microorganisms was streaked on cetrimide agar and incubated at 37ºC for 24 h incubation. The observation of greenish colonies on the surface of the agar after incubation indicates the presence of *P. aeruginosa.* Ten milliliters of Koser's citrate medium was inoculated with a loopful of the colonies. The conversion of the green Koser's citrate to blue colouration confirmed the identity of *P. aeruginosa.*

5. *Candida albicans*

A 24-h broth culture of the suspension of organisms was streaked on the surface of potato dextrose agar containing 1g chloramphenicol and incubated at 25°C for 72 h. Two loopfuls of colonies were streaked on the surface of eosin methylene blue agar. The appearance of white colonies indicated the presence of *C. albicans.*

APPENDIX IV: HPLC Retention Time and area under curve (AUC) of constituents of methanol extracts

Table A2:HPLC characterization of methanol leaf extract of *Erythrophleum ivorense*

Retention Time	AUC	PAUC
0.694	25524	2.859
0.955	23860	2.673
1.153	247167	27.690
1.645	31898	3.573
2.064	72952	8.173
2.202	60074	6.730
2.893	41712	4.673
3.002	44765	5.015
3.704	19638	2.200
4.295	35694	3.999
7.258	130679	14.640
12.319	158673	17.776

AUC = Area under curve; PAUC = Percentage area under curve

Table A3: HPLC characterization of methanol bark extract of *Erythrophleum ivorense*

Retention Time	AUC	PAUC
0.695	26837	2.894
1.088	233267	25.152
1.268	63689	6.867
1.482	38717	4.175
1.982	49306	5.316
2.068	77190	8.323
2.215	39714	4.282
2.428	33698	3.633
2.762	86522	9.329
3.950	50354	5.429
4.530	228148	24.600

AUC = Area under curve; PAUC = Percentage area under curve

Table A4: HPLC characterization of methanol leaf and other aerial part extract of *Parquetina nigrescens*

Retention Time	AUC	PAUC
1.382	132487	5.481
1.529	278279	11.513
1.611	218376	9.035
1.648	257334	10.647
2.066	165480	6.846
2.272	100644	4.164
2.495	91189	3.773
2.822	38775	1.604
3.202	22793	0.943
3.455	26792	1.108
4.148	68882	2.850
4.308	85240	3.527
5.158	231149	9.563
7.382	680546	28.156
8.188	19070	0.789

AUC = Area under curve; PAUC = Percentage area under curve