BIOCHEMICAL STUDIES OF SECONDARY METABOLITE OF *PENICILLIUM CHRYSOGENUM* GROWN ON SELECTED AGRO-WASTES

BY

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B.Sc. Med. Lab. Science, 1992 (RSUST); M.Sc. Biochemistry, 2005(UNILAG)

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A Thesis submitted to the School of Postgraduate Studies, University of Lagos in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy (Ph.D.) in Biochemistry.

UNIVERSITY OF LAGOS
SCHOOL OF POSTGRADUATE STUDIES

CERTIFICATION

This is to certify that the thesis:

"Biochemical Studies of Secondary Metabolite of Penicillium Chrysogenum Grown on Selected Agro Wastes."

Submitted to the School of Postgraduate Studies, University of Lagos

for the award of the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

is a record of original research work carried out

By

ONYEGEME-OKERENTA, Blessing Minaopunye
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DECLARATION

The research work contained in this thesis was undertaken entirely by me. No part of this study has been presented either in part or as a whole to any other institution or organization for the purpose of obtaining any degree or qualification.

ONYEGEME-OKERENTA, BLESSING MINAOPUNYE

DEDICATION

Affectionately dedicated to my husband Engr. Emenike Onyegeme-Okerenta. Thank you for believing in me and supporting me all the way.

ACKNOWLEDGEMENT

My gratitude goes first to GOD Almighty, the ONE who decrees a thing and it comes to pass. WHO upholds me with HIS right arm of righteousness, WHO strengthens me when I am weary, and rules over the affairs of men, to HIM be all the glory.

The success of any research is never limited to the individual who is undertaking the work. Most often, it is the collective efforts of the people that reflect in the success. It is impossible to thank everybody separately who are responsible for this day as it is dependent on several yesterdays. However, I take this opportunity to express my profound gratitude and thanks to some of the personalities involved with me in the process of completing this thesis.

First and foremost, I would like to express my sense of gratitude to my research supervisors Professor (Mrs.) Veronica Ihuaku Okochi and Professor Osaretin A. T. Ebuehi for their guidance, encouragement, the freedom of work and care they provided throughout my research work. Professor Okochi's sustained interest, perceptive comments, tireless enthusiasm and inspiring guidance throughout the course of this study will always remain a source of inspiration to me and it has contributed immensely to the content of research work presented here. I also acknowledge the professional input of Dr. S. Ogbonnaya of Pharmacognosy Department, University of Lagos.

My special thanks go to Messrs Uzoma Anthony Okafor, Ojomo, Akinrindoye, Stephen Apata, and Sunday Adelekan for sharing their expertise and useful discussion during biochemical studies. It is my pleasure to offer thanks to Mr. Ojobo of Lagos University Teaching Hospital Research Centre for assisting me in HPLC analysis of my culture extracts and autoanalysis of blood samples.

It was enriching to work with my senior collaborating colleagues in Biochemistry department. To Dr. Ngozi Awa Imaga, Dr. (Mrs.) Marian Igwo-Ezeikpe and Dr. (Mrs.) Rasheedat Bakare; your timely help and friendly discussion during the course of this research need to be gratefully acknowledged for the result is evident in this research.

I am grateful to Professor G. O. Gbenle and the postgraduate coordinator, Dr. (Mrs.) O. S. Odesanmi for their support and encouragement in my trying moments.

To my friends, Pastor & Mrs. Moses Ogianyo, Dr. Chinedu Nwodo, Venerable & Mrs. Sunday Opara, Rev. Canon & Mrs. Charles Obi Mordi, Rev. Canon & Mrs. Ikechukwu Anene, Rev. & Mrs. Reginald Chinweze, Rev. & Mrs. Charles Ibeh, Evang. & Mrs. Innocent Henry, Dr. (Mrs.) Asodike, Dr. (Mrs.) Onyeike, Mr. & Mrs. Sesun Daramola, Mrs. Rose Ajoku, Mrs. Margaret Wagwu, Mrs. Nwachinemere Nnaji, Mrs. Ebun Ohiosimuan, Mrs. Edith Dibia, Mrs. Ebele Chukwubuikem and others, I say thank you for upholding me in prayers.

Finally, words would not fail me in expressing my heartfelt thanks to my family especially to my mother Madam Augusta Igoni Benstowe who believe so much in child education. My appreciation also goes to my siblings Jenny, Opuaya, Hellen, Charles and Edna and also to my in-laws Mrs. Sabinah Okerenta, Mr. Sam O. Jumbo, Victor Hums, Darlington John Okechukwu and Ms. Christiana Nwakama.

I am indebted mostly to my husband, Emenike, and my children, Chigozie Tamunotonye, Tochi Nengi, Oluchi Eziuche, Ugochi Chidindu and Kelechi James for their unending support and encouragement during this programme. I am very grateful for their love, continual friendship and patience.

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LIST OF ABBREVIATIONS

ALT	–	Alanine aminotransferase
ALP	–	Alkaline phosphatase
AST	–	Aspartate aminotransferase
BCG	–	Bromocresol green
BUN	–	Blood urea nitrogen
CAT	–	Catalase
DNPH	–	Dinitrophenylhydrazine
DTI	–	Direct thrombin inhibitors
DSA	–	Diazotised sulphanilic acid
ESBL	–	Extended-spectrum β-lactamases
GFR	–	Glomerular filtration rate
GLUT	–	Glutathione
HLE	–	Human leucocytes elastase
HPLC	–	High performance liquid chromatography
LFT	–	Liver Function Test
MIC	–	Minimum inhibitory concentration
PCV	–	Packed Cell Volume
PMN	–	polymorphonuclear leucocytes
SEM	–	Standard error of mean
SOD	–	Superoxide dismutase
TBR	–	Total bilirubin reagent
TFPI	–	Thrombin activatable fibrinolysis inhibitor

TCT	–	Thrombin Clotting Time
TLC	–	Thin layer chromatography
TNR	–	Tri-Nitrite reagent
TT	–	Thrombin Time
UV	–	Ultra violet
VTE	–	Venous thromboembolism
WBC	–	White Blood Cell Count

OPERATIONAL DEFINITION OF TERMS

Agro-wastes: These are waste produced at agricultural premises as a result of agricultural activity. They include those liquid or solid wastes that result from agricultural practices, such as crop residue (for example, corn stalks, sugar cane pulp, cassava peels, and saw dust) and fertilizers.

Antibiotics: are chemical substances produced by living organisms, generally a microorganism that is harmful to other microorganisms. These compounds are formed naturally by fungi and bacteria, and are then released into the soil as part of nature's production chain.

Bioconversion: The conversion of organic materials, such as plant or animal waste, into usable products or energy sources by biological processes or agents, such as certain microorganisms.

Chromatography: Any of various techniques for the separation of complex mixtures that rely on the differential affinities of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass, such as paper, gelatin, or magnesia.

Coagulation: The process by which blood forms clots. It is an important part of homeostasis (the cessation of blood loss from a damaged vessel), wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel.

Histopathology: The microscopic examination of tissue in order to study the manifestations of disease.

Inflammation: A basic way in which the body reacts to infection, irritation or other injury, the key feature being redness, warmth, swelling and pain. Inflammation is now recognized as a type of nonspecific immune response.

Morphology: The branch of biology that deals with the form and structure of organisms without consideration of function.

Mortality: A fatal outcome or, in one word, death.

Oedema: The presence of abnormally large amounts of fluid in the intercellular tissue spaces of the body usually used to describe demonstrable accumulation of excessive fluid in subcutaneous tissues.

Papillary necrosis (also called **Necrotizing papillitis):** This occurs when the renal papillae become infracted; it is the localized death of cells or tissues through injury or disease.

Pathology: The study of disease. Pathology has been defined as "that branch of medicine which treats the essential nature of disease.

Potency: The relationship between the therapeutic effect of a drug and the dose necessary to achieve that effect.

Saccharification: The hydrolysis of soluble polysaccharides to form simple sugars. Hydrolysis is a chemical reaction during which one or more water molecules are split into hydrogen and hydroxide ions in the process of a chemical.

Thin Layer Chromatography: The chromatographic technique for separating and analysing mixtures of substances, using a thin layer of stationary phase attached to a glass plate and using the passage of liquid up the plate by capillary action as the mobile phase.

Thrombin: – a serine protease is the normal activation product of prothrombin in the coagulation cascade. It converts fibrinogen to fibrin.

Thrombosis: The formation or presence of a blood clot in a blood vessel. The vessel may be any vein or artery.

Toxicology: The study of the adverse effects of chemicals on living organisms. It is the study of symptoms, mechanisms, treatments and detection of poisoning, especially the poisoning of people.

ABSTRACT

Selected agro-wastes found in Lagos, Nigeria (cassava peels, corncob, sawdust, and sugarcane pulp) were compared with glucose and lactose as microbial substrates for cultivating *P. chrysogenum* (wild strain). This study was designed to give added value to agro waste as substrates to cultivate *P. chrysogenum* and subsequent production of secondary metabolite with antibiotic and anticoagulant properties. In the growth studies, corn cob and cassava peels significantly ($p < 0.05$) produced the highest amount of mycelia weight. Corn cob yielded a mycelia weight of 0.15 ± 0.02 and 0.92 ± 0.04mg/ml on the third and ninth day respectively while cassava peels yielded a mycelia weight of 0.13 ± 0.07 and 0.12 ± 0.02mg/ml on the third and twelfth day respectively. Mycelia weight of the organism, in media containing glucose, sugar cane and lactose was 0.12 ± 0.02, 0.068 ± 0.05 and 0.055 ± 0.03mg/ml respectively, was highest on the ninth day. Sawdust gave the least growth with a mycelia weight of 0.07 ± 0.01mg/ml on the third day. Cassava media has the highest carbohydrate content. Changes in extracellular protein secreted into the different media (every 3days for 21days) shows that culture media containing cassava peels gave the highest protein peak of 0.38 ± 0.08 mg/ml on the sixth day, while corncob gave an early peak of 0.30 ± 0.03mg/ml on the third day. Sawdust gave two protein peaks, 0.15 ± 0.03 on the third day and 0.25 ± 0.01mg/ml on the twelfth day. A total protein yield of 0.2 ± 0.05, 0.08 ± 0.02, 0.06 ± 0.02 mg/ml respectively was obtained with glucose, sugarcane pulp and lactose containing media on the third day. The results suggest that cassava peels, corncob and sugarcane pulp could serve as cheap fermentation substrates for the growth of the fungus. Optimum pH and temperature of growth and antibiotic production was 6.5 and 25^{O}C respectively. UV

modification of parent strain produced two mutant strains with 70% increase in penicillin production. *In vitro* antibacterial activity of the culture extracts was tested against some clinical bacterial isolates, namely, *B. subtilis, E. coli, P. mirabilis* and *P. aeruginosa*. Commercial Benzyl Penicillin was used as reference drug. The culture extracts and standard drug inhibited the growth of *B. subtilis* and *E. coli*. Zone of inhibition varied with the carbon source. Culture extracts and reference drug were not effective against the isolates of *P. aeruginosa* and *P. mirabilis* because they produce β-lactamase enzymes which hydrolyse the β-lactam present in the extract and reference drug. Antibacterial activity of extracts from cultures containing cassava peels and sugarcane pulp compared positively with that of the standard drug. The Minimum inhibitory concentration (MIC) of the reference drug against the susceptible organisms was 0.2 - 0.4mg/ml. For the culture extracts, the MIC ranged from 0.4 to 2.0mg/ml. It was 0.4 - 0.8mg/ml for cassava peels and sugarcane pulp, 0.6 - 0.8mg/ml for glucose and lactose, 0.8 - 1.0mg/ml for corncob and 1.0 - 2.0mg/ml for sawdust. Toxicity study showed that the extract is safe for use as there were no visible changes or recorded deaths 48 - 72hours after administration of the extracts. Haematological evaluation showed a significant decrease ($p<0.05$) in platelet count for both the extract and reference drug in the sub acute toxicity study as well as in infection and inflammatory conditions. The extract was shown to have a potent antithrombic and anticoagulant activities against thrombin and whole blood respectively. Higher concentrations of the extract and reference drug caused an increase in whole blood clotting time. At 6mg/ml of the extract and 5mg/ml of the reference drug, clot formation was not observed. There was complete inhibition of thrombin coagulation at concentrations above 10 and 20mg/ml of the reference drug and extract respectively.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Biomass in the form of crop residues and agro-industrial wastes are renewable carbon sources available in very large quantities both in industrialized and developing countries. They accumulate every year, particularly in major cities and settlements, causing enormous environmental pollution with adverse health, economic and social consequences. In Nigeria, *Mitragyna ciliata* ("Abora" in Yoruba, "Uburu" in Igbo, and "Guleya" in Hausa) is one of the woods used as raw materials for building and furniture making and saw-dust comes off as waste from this wood during processing. In a research involving the use of the leaves of this plant, mixed colonies of micro fungi were seen growing on the body of the plant (Abu *et al*., 2000). The growing population of the country imposes great demand on food production which in turn increases the output of agricultural waste. Although some of the waste is utilized as compost, or other functions that yield little economic return, much of it is simply left in fields to be plowed under or deposited in landfills where it contributes to air pollution, water contamination and public health issues. These challenges require that measures be put in place for recycling processes which are environmental friendly, thus minimizing agro-waste, adding economic value to our agricultural products and protecting the environment through biodegradation. In Nigeria, agro waste constitutes a major environmental pollutant (Solomon *et al*., 1999; Howard *et al*., 2003). These wastes can be converted into useful products thereby, turning wastes into wealth.

Numerous filamentous fungi naturally thrive on plant wastes because they can penetrate the dead plant matter and utilize the cell wall components as growth substrates (Grant and Long, 1981). Among them is *Penicillium* species, a common contaminant which colonizes a wide range of materials including wood, fabrics and leather objects. While some species produce

poisonous toxins or cause food spoilage, others such as *Penicillium chrysogenum* are beneficial to man. *P. chrysogenum* is the source of penicillin, the first antibiotics to be discovered (Volk, 2003). A strain of *P. chrysogenum* (PCL501) was among the micro fungi isolated from wood-waste dump in Lagos, Nigeria (Nwodo-Chinedu *et al*., 2005). The organism produces extracellular enzymes such as cellulases (Nwodo-Chinedu *et al*., 2007b) and xylanases (Chinedu *et al*., 2008) in media containing agro-wastes as sole carbon sources.

P.chrysogenum is a micro fungus of great medicinal importance used industrially for the production of several β-lactam antibiotics, particularly the penicillins (de Hoog *et al.,* 2000). The chance discovery of *P. notatum* by Alexander Fleming and the production of the revolutionary drug, Penicillin, is perhaps the most important finding in the history of therapeutic medicine (Volk, 2003). There is no gainsaying the fact that penicillin antibiotics has been of immense benefit to humanity because it is used worldwide in the treatment of many diseases. In spite of the long term use of this fungus in antibacterial production, efforts are still being made towards developing cheap and improved strains of the organism for greater yield of the penicillin antibiotics.

The penicillins and related β-lactam antibiotics exert their antibacterial activity by acylation of serine residues at the active site of enzymes (penicillin-binding proteins) involved in the biosynthesis of bacterial cell walls. All members of the extended family of penicillin and cephalosporin antibiotics contain an 'activated' β-lactam ring, as well as other structural features required for recognition by their target enzymes. β - Lactam derivatives have also been shown to inhibit a range of other enzymes with nucleophilic serine residues, including mammalian serine proteases such as elastase, and prostate specific antigen (Konaklieva, 2002 Stitzinger, 2007). Some β -lactams such as N-benzoazetidinone or cephalotin have also been reported to be inhibitors of mammalian serine proteases such as α-chymotripsin. Recently, series of neutral derivatives of cephalosporin and monocyclic β -lactams have been developed

as mechanism-based inhibitors of human leukocyte elastase (HLE) (Walselman *et al*., 1991). Leukocyte Elastase (LE) is a serine protease, expressed by polymorphonuclear (PMN) leukocytes, mainly neutrophils, that acts both intracellularly to kill engulfed pathogens and extracellularly as mediator of coagulation, immune responses, and wound debridement. Because LE has the potential to degrade some structural proteins of the extracellular matrix (ECM), such as elastin, fibronectin, and collagens, excess of LE activity has been involved in a number of inflammatory pathological conditions leading to impairment of ECM organization, these include rheumatoid arthritis, emphysema, cystic fibrosis, and tumour progression. Similarly, thrombin is the normal activation product of prothrombin in the coagulation cascade and is most commonly known for its ability to convert fibrinogen into fibrin. It is involved in normal recovery from injury through its roles in blood clotting, subsequent clot lyses, and tissue repair. However, it is instrumental in the initiation and propagation of pathological events such as oedema, inflammation, cell recruitment, and release of cellular products, mitogenesis, and angiogenesis (Hongbao *et al*., 2008). Thrombin is also a powerful stimulus for platelet activation and aggregation and is degraded by proteinase inhibitors such as antithrombin III, heparin cofactor II, and alpha-2 macroglobulin. Besides its central role in the coagulation cascade, thrombin is a potent platelet agonist and thus constitutes an interesting target for drugs that would prevent the formation of fibrin- and platelet-rich thrombi induced by thrombin (Cazenave *et al*., 2008). Penicillin G and related antibiotics may be inhibitory because they coat the platelet surface. Their effects on platelet functions are probably responsible for excessive bleeding and increased bleeding times observed in patients and volunteers receiving high doses of these antibiotics.

In all of these processes, thrombin is tightly regulated in terms of its formation and destruction. Congenital diseases associated with the absence or reduced production of thrombin (haemophilias) represents important clinical problems that fortunately are rarely

encountered. However, the unregulated production of thrombin in an inappropriate location leading to a thrombotic occlusion is a frequently encountered problem. For the past century, thrombosis in the venous circulation and the embolization of venous clots has been recognized to be important contributors to pathology. More recently, the significance of clot formation in the arterial circulation has led to the development of both biochemical and mechanical interventions to disrupt clots that cause myocardial infarction and stroke (Mann, 2003).

Epidemiologic studies have identified a wide variety of risk factors for clinical thrombosis, but no study provides sufficiently strong incentives to invoke anticoagulant prophylaxis in the absence of a clinically defined thrombotic syndrome. Methods for clinical intervention following the display of clinical thrombosis have been largely unchanged since the development of heparin in the 1920s and warfarin in the 1940s. The development of low-molecular-weight heparins has represented a major advance in therapy for venous thrombosis; however, these agents are still only available by subcutaneous injection (Mann, 2003).

The wider scope of this project is aimed at exploring the benefits of biotechnology to give value to large volumes of agro-wastes that litter and pollute our environment and transforming these wastes into wealth. This can be achieved by the use of micro organisms in biotransformation of the wastes to useful products like enzymes (for industrial application) and simple sugars (for production of biofuels). However, this particular research is designed to (i) isolate and modify indigenous *P. chrysogenum* grown on wood wastes in our own environment, (ii) use the micro fungus to ferment the wood wastes for natural antibiotic production, and (iii) characterize the antibiotic extract from the fermentation medium in comparison to the industrially synthesized commercial penicillin. The strategy is to use cost-effective and sustainable materials in the methods of the experiment while deriving environmentally friendly benefits from the entire exercise.

1.2 Statement of problem

All over the world today, use of natural products in healthcare management has gained prominence. Considering the current trends of thought and the extensive opportunity offered by biotechnology, it becomes reasonable to exploit Nigeria's rich tropical flora for microorganisms which can be used to biodegrade dead plant matter for antibiotic production. Nigeria is replete with agro-wastes which constitute the bulk of the solid waste generated all over the country. Biodegradation of these wastes can transform them into wealth.

Production of natural penicillin from agro-wastes is of interest because it belongs to the β-lactam family of antibiotics. Currently, there is increased interest in studies with β-lactam antibiotics because of the realization that besides their antibacterial effect, β-lactam derivatives are also inhibitors of serine proteases (e.g. elastase, and prostate specific antigen) involved in inflammatory diseases such as rheumatoid arthritis, emphysema, cystic fibrosis, and tumor progression (Walselman *et al*., 1991; Hongbao *et al*., 2008).

Epidemiologic studies have identified a wide variety of risk factors for clinical thrombosis, but no study provides sufficiently strong incentives to attract interest in studies involving anticoagulant prophylaxis in certain clinical conditions that may require such intervention. Methods for clinical intervention following the display of clinical thrombosis have been largely unchanged since the development of heparin in the 1920s and warfarin in the 1940s (Mann, 2003).

These considerations emphasize the need to investigate natural products that are cost effective and beneficial. Since biomass in the form of agro-wastes is renewable and sustainable raw materials, they are considered veritable substrates for this research work.

1.3 Overall aim of study

The focus of this study is aimed at exploring the benefits of proceses which give value to large volumes of agro-wastes that litter and pollute our environment. This can be achieved by the use of micro organisms in biotransformation of these wastes to wealth.

1.4 Objectives of study

Based on the overall aim, the objectives of this study are as follows:

1. Isolation of *P. chrysogenum* from wood wastes, determine appropriate conditions for optimum growth and production of natural penicillin and characterization of the antibiotic extract.
2. Investigation of potency of the natural antibiotic extract and determine its properties as a β-lactam compound *in vitro,* then modify the wild strain to obtain higher yield of the antibiotic produced by the micro fungus.
3. Determination *in vivo* how the natural antibiotics extracted influence the functions of serine proteases and oxidative stress enzymes and the toxic effect of the extract on the liver and kidney.
4. Determination of the antithrombic and anticoagulant properties of the culture extract *in vitro*.

1.5 Significance of study

This research is designed to add value to agro-wastes that litter and pollute our environment. Agro-wastes have been chosen as the raw materials because of the following:

- They are rich sources of microorganisms, such as, fungi and bacteria. The organisms utilize them as substrates for growth by producing substances, such as enzymes (cellulase) which degrade these wastes. Consequently, useful microorganisms can be isolated from agro-wastes.

- Agro-wastes (e.g. cassava peels, corncob, sawdust and sugarcane pulp) when processed, can serve as industrial low-cost microbial substrates for growth of microorganisms (e.g. *P. chrysogenum*) and subsequently generate metabolites which include:

    i. Antibiotics (e.g. penicillin). These are used in the medical industry for treatment of infectious diseases caused by pathogenic organisms e.g. gastroenteritis caused by *E. coli*.

    ii. Anticoagulants and antiproteases (e.g. β-lactam derivatives) which have been shown to be inhibitors of serine proteases (e.g. elastase, and prostate specific antigen) involved in inflammation such as rheumatoid arthritis, emphysema, cystic fibrosis etc.

  The importance of penicillin, an antibiotic, to humanity cannot be over emphasized. Penicillin-derived antibiotics are still the world's most significant weapon against infectious diseases. *P. chrysogenum* – a fungus is used for the production of antibiotics which has been beneficial to humanity. This is important, because penicillin-derived antibiotics are likely to remain one of the most potent defences against infectious diseases for several decades to come.

CHAPTER TWO

LITERATURE REVIEW

Industrialization and urbanization have led to increase in generation of waste into the environment from various sources. Waste generation is, therefore, a necessary outcome of consumption, due to insufficient bioconversion processes, general ignorance, wasteful habits and social attitudes. Biomass in the form of agricultural and forest wastes and residues accumulates every year in large quantities both in industrialized and developing countries. This results in a deterioration of the environment and a loss of potentially valuable resources (Dubey, 2003). In Nigeria, agro-industrial wastes abound in the form of wood-wastes and crop residues such as cereal straws, cassava peels, corncobs and sugarcane pulps (Abu *et al*., 2000). Numerous filamentous fungi naturally thrive on plant wastes because they can penetrate the dead plant matter and utilize the cell wall components as growth substrates and energy sources for fermentation, thereby reducing pollution-load and subsequently secrete value-added products such as enzymes (e.g. cellulose, pectinase, and xylanase) and antibiotics (e.g. Penicillin and Cephalosporin) into the fermentation media (Grant and Long, 1981; Solomon *et al*., 1999; Howard *et al*., 2003). Among these filamentous fungi is the *Penicillium* species. While some species produce poisonous toxins or cause food spoilage, others such as *P. chrysogenum* are beneficial to man. *Penicillium chrysogenum* is the source of penicillin, the first antibiotics to be discovered (Volk, 2003). *P. chrysogenum* (formerly, *Penicillium notatum*) is an important industrial organism due to its ability to produce several β-lactam antibiotics, particularly Penicillins used for the treatment of some bacterial infections (de Hoog *et al*., 2000). Most bacterial infections are usually accompanied by inflammation and oxidative stress.

2.1 Agro-wastes

Large quantities of agricultural wastes (agro-wastes) are generated in Nigeria. The environmental pollution problems associated with conventional disposal methods in Nigeria (Belewu and Banjo, 2000) necessitate the search for alternative, environmentally friendly methods of handling agro-wastes.

Agro waste contains vast amounts of nutrients (nitrogen, phosphorous and potassium), which on decomposition would be released to influence growth of microorganisms and subsequent production of useful primary and secondary products like enzymes (Ellouz *et al*., 2001; Chipeta *et al*., 2002; Vasquez-Alvarez *et al*., 2004), energy production (Oliveira, 2001; Sun and Cheng, 2002), biomass production (Duru and Uma, 2003; Tripodo *et al*., 2004) and organic acids like lactic acids, butanol, methanol, ethanol and antibiotics.

Cellulosic biomass is biodegradable and serves as carbon and energy source to an assortment of soil organisms, which utilize plant cell-wall materials (Grant and Long, 1981). By breaking down detritus materials, *P. chrysogenum* and other fungi convert complex carbon structures into more simple forms. This makes them the decomposers in an ecosystem. Fungi produce carbon dioxide through respiration, and they also leave behind nutrients such as nitrogen and phosphorus in the soil. These nutrients and gases are then absorbed by plants which are the producers of nutrients (food) in an ecosystem. The plants are eaten by primary consumers, the herbivores and omnivores which in turn are eaten by secondary consumers. Within all of these groups, organisms die and become the detritus materials which are broken down by fungi, such as *P. chrysogenum.*

2.2 Fungi

Fungi are eukaryotic, heterotrophic organisms that have cell walls made of polysaccharides. Their classification is based on their mode and appearance of asexual and sexual reproduction leading to spore production. Fungi are distinguished by their characteristic thallus (body; pl. = thalli). Typically, a filamentous fungus produces a haploid mycelium that is a branched network of walled tubes called hyphae (sing. = hypha). Vegetative growth occurs by apical extension of the hypha in the filamentous forms, or by budding or cell fission in yeast forms. Most fungi spend their lives in the haploid condition (Benson, 2005).

Sexual reproduction generally leads to the transient formation of diploid cells that quickly resolve into haploid spores. Most fungi are heterothallic, that is, they require the presence of an opposite mating type to reproduce sexually. Some are homothallic where both mating types are carried within the same hyphae. Except for the important exceptions of some yeast, all fungi are obligate aerobes.

Nutritionally, fungi are primary decomposers that will break down a variety of macromolecules using extracellular enzymes as a way to gain nutrients that are absorbed by the cells. Thus, many fungi are involved in the decomposition of cellulose, starch, pectin, proteins, fats, etc. Being soil organisms, fungi also compete with other microorganisms for food. This competition manifests itself by the production of a variety of antibiotics and acids that are useful in industry (Benson, 2005).

The Fungi kingdom is a diverse clad of heterotrophic organisms. Its lineage include mushrooms, rusts, smuts, puffball, truffles, movels, molds and yeasts as well as many less well known organisms. More than 70,000 species of fungi have been described; however, some estimates of total number suggest that 1.5 million species may exist. Fungi are often found when examining material under a microscope. While there may be little difficulty in

distinguishing protozoa from algae, fungi sometimes present a difficulty. They are non-photosynthetic eukaryotes, and therefore are never green. However, unlike protozoa, fungi are never motile, and are much less varied in cell shape. There are two principal groups of microscopic fungi, yeasts and molds (water molds and common molds). Yeasts are unicellular and roughly spherical in shape, most of them are in the sac fungi or club fungi. They reproduce by a process called "budding", whereby new cells pinch off an existing cell. The filaments of molds can be distinguished from those of algae and cyanobacteria because molds are not photosynthetic, and thus are not green. A specimen of a mold typically will contain a mixture of hyphae and spores (Benson, 2005).

2.2.1 *Penicillium chrysogenum*

P. chrysogenum is a mould that is widely distributed in nature, and is often found living on foods and in indoor environments. It was previously known as *Penicillium notatum* (Samson *et al*., 1977). It has rarely been reported as a cause of human disease. It is the source of several β-lactam antibiotics, most significantly penicillins and cephalosporins. These are converted into active pharmaceutical ingredients like amoxicillin, ampicillin, cephalexin and cefadroxil. (Volk, 2003). Other secondary metabolites of *P. chrysogenum* include various different penicillins, roquefortine C, meleagrin, chrysogine, xanthocillins, secalonic acids, sorrentanone, sorbicillin, and PR-toxin (de Hoog *et al*., 2000). Like the many other species of the genus *Penicillium*, *P. chrysogenum* reproduces by forming dry chains of spores (or conidia) from brush-shaped conidiophores. The conidia are typically carried by air currents to new colonization sites. In *P. chrysogenum* the conidia are blue to blue-green, and the mould sometimes exudes a yellow pigment. However, *P. chrysogenum* cannot be identified based on colour alone. Observations of morphology and microscopic features are needed to confirm its identity. The airborne spores of *P. chrysogenum* are important human allergens. Vacuolar

and alkaline serine proteases have been implicated as the major allergenic proteins. *P. chrysogenum* has been used industrially to produce penicillin and xanthocillin X, to treat pulp mill waste, to produce the enzymes polyamine oxidase, phosphogluconate dehydrogenase, and glucose oxidase (de Hoog *et al*., 2000).

The ability to produce penicillin appears to have evolved over thousands of years, and is shared with several other related fungi. It is believed to confer a selective advantage during competition with bacteria for food sources. However, some bacteria have developed the ability to survive penicillin exposure by producing penicillinases, enzymes that degrade penicillin. Penicillinase production is one mechanism by which bacteria can become penicillin resistant. The principal genes responsible for producing penicillin, pcbAB, pcbC and penDE are closely linked, forming a cluster on chromosome I. Some high-producing *Penicillium chrysogenum* strains used for the industrial production of penicillin have been shown to have multiple tandem copies of the penicillin gene cluster (Ligon, 2004).

The ability of *P. chrysogenum,* PCL501, newly isolated from wood-waste dump in Lagos, to grow in medium containing sugarcane pulps as sole carbon source has been studied (Nwodo-Chinedu *et al*., 2006). It was reported that the modified media formulated with sugarcane pulp as carbon sources supported the growth of the micro fungi as efficiently as the commercial media which were unmodified. Since, sugarcane pulp contains other cell-wall polysaccharides such as xylan in addition to cellulose, more simple sugars would be released in the media containing the complex cellulosic materials. This could account for the better growth of the organisms on the modified media containing sugarcane pulp (Nwodo-Chinedu *et al*., 2006). *P. chrysogenum* is an important industrial organism due to its capacity to produce penicillin, which is still one of the main commercial antibiotics. Penicillin yields have been increased through development of better production strains by classical mutagenesis procedures and optimization of the growth (Veenhuis, 2002; Chuan-Bao *et al*., 2002).

2.3 Penicillin – a classical β-lactam antibiotic

Penicillins are β-lactam antibiotics which have broad clinical utility. Penicillin was discovered in 1928 when Alexander Fleming's lab assistant left a window open overnight and had mold spores cover his *Staphylococcus* bacterial specimens in a Petri dish. At first he was very irritated at the contamination but as he was about to throw the specimens away, he noticed something interesting. He looked under the microscope at the bacteria surrounding the blue-green mold and noticed that many were dead or dying due to the mold preventing the bacteria from making new cell walls and reproducing. He identified the mold as *Penicillium notatum*, which releases the antibiotic penicillin G into the medium. After this he did some testing on humans and animals and discovered that not only did it kill bacteria, but that it was suitable for use in humans and animals. However, the discovery did not attract much attention until the 1940s when Howard Florey and Ernst Chain developed methods for mass production and application in humans, incited by the urgent war-time need for antibacterial agents. Army pilots sent back soil from around the world to be tested for the right kind of mold. Even the people of Peoria, Illinois were told to bring in any molds that they found around their homes. It has also been said that the scientists working on this project kept an eye out for similar looking molds while grocery shopping or when they were cleaning around the kitchen especially their refrigerators. The discovery of penicillin ushered in a new age of antibiotics derived from microorganisms (Diggins, 1999; Ligon, 2004).

The first clinical trials with penicillin were undertaken in 1941. In parallel with efforts to provide penicillin in large amounts, its structure was elucidated in 1945, when Hodgkin and Low showed by x-ray crystallography analysis that it is composed of a β-lactam structure.

The modern era of antimicrobial chemotherapy began following Fleming's discovery in 1929 of the powerful bactericidal substance penicillin, and Domagk's discovery in 1935 of

synthetic chemicals (sulphonamides) with broad antimicrobial activity. In the early 1940's, spurred partially by the need for antibacterial agents in World War II, penicillin was isolated, purified and injected into experimental animals, where it was found to not only cure infections but also to possess incredibly low toxicity for the animals. This fact ushered in the age of antibiotic chemotherapy and an intense search for similar antimicrobial agents of low toxicity to animals that might prove useful in the treatment of infectious disease. The rapid isolation of streptomycin, chloramphenicol and tetracycline soon followed, and by the 1950's, these and several other antibiotics were in clinical usage.

2.3.1 Chemical nature of penicillin

Structurally β-lactam antibiotics contain a 4-membered beta lactam ring (Figure 2.1A). The term Penam is used to describe the core skeleton of a member of a penicillin antibiotic. This skeleton has a molecular formula R-$C_9H_{11}N_2O_4S$, (Figure 2.1B) where R is a variable side chain (Pichichero, 2006). They are the products of two groups of fungi, *Penicillium* and *Cephalosporium* moulds, and are correspondingly represented by the penicillins (Figure 2.2) and cephalosporins. The beta lactam antibiotics inhibit the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains, mediated by bacterial carboxypeptidase and transpeptidase enzymes.

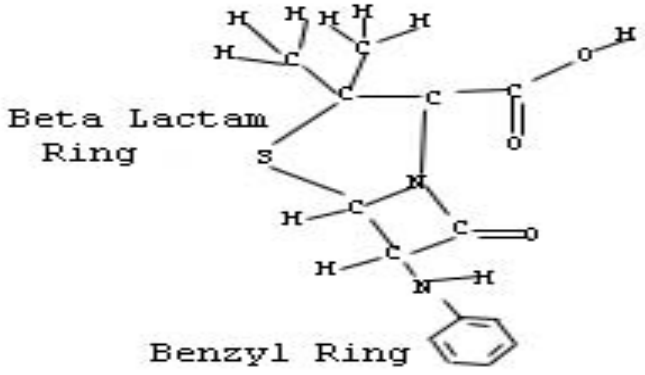


Figure 2.1A: Penicillin G

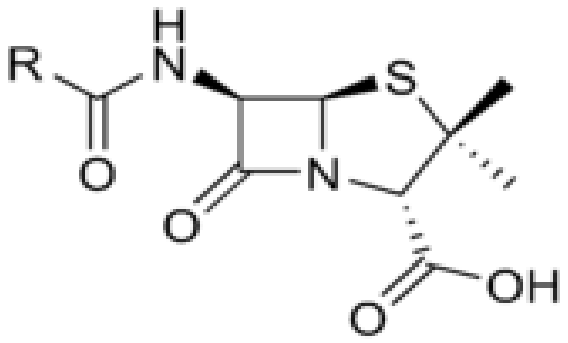


Figure 2.1B: Penicillin core structure. "R" is variable group

2.3.2 Biological function of Penicillin (β-lactam) antibiotics

β-lactam antibiotics are normally bactericidal and require that cells are actively growing in order to exert their toxicity. β-lactam antibiotics work by inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall. The β-lactam moiety (functional group) of penicillin binds to the enzyme (DD-transpeptidase) that links the peptidoglycan molecules in bacteria, which weakens the cell wall of the bacterium, thus causing cytolysis due to osmotic pressure. The result is cell death. In addition, the build-up of peptidoglycan precursors triggers the activation of bacterial cell wall hydrolases and autolysins, which further digest the bacteria's existing peptidoglycan (Veenhuis, 2002).

The discovery of antibiotics for clinical use is perhaps the most important discovery in the history of therapeutic medicine. The application of antibiotics to the therapy of infectious diseases may conceivably have saved more lives than any other medical development. The exact number of people using penicillin around the world every year is almost impossible to quantify but is more than a billion. Antibiotics account for about 8% of the world pharmaceuticals market. The most important property of a clinically-useful antimicrobial agent, especially from the patient's point of view, is its selective toxicity, i.e., the agent acts in some way that inhibits or kills bacterial pathogens but has little or no toxic effect on the animal taking the drug. This implies that the biochemical processes in the bacteria are in some

way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy (Veenhuis, 2002).

2.3.3 Biosynthesis of Penicillin

Penicillin biosynthesis is regulated by environmental factors such as the phosphate, carbon, nitrogen and oxygen content of the medium. The overall rate of penicillin synthesis is severely reduced under conditions of low oxygen. Reduction of oxygen supply leads to accumulation of isopenicillin N, a precursor of penicillin (Figure 2.2). The mechanism of oxygen control over penicillin synthesis is not well understood. Possibly, low oxygen levels directly affect the biosynthetic pathway of penicillin, which includes several oxidation reactions. It is also possible that a more efficient overall metabolism provided by higher oxygen levels indirectly results in higher penicillin yields. Regardless of the mechanism, technologies that improve aerobic metabolism in these organisms should have a positive effect on penicillin production (Veenhuis, 2002).

Biotechnological processes are in general very complex, by virtue of the great number of variables involved. The process of penicillin production by the *P. chrysogenum* filamentous fungus is a typical example of this complexity. The process basically comprises two steps: the rapid growth stage of the microorganism and the antibiotic production stage, in this case, a secondary metabolite. Both stages are interdependent, in such a way that abundant and quick cell growth is desirable for obtaining high productivity in the antibiotic biosynthesis. The choice of substrate, usually a carbohydrate, is important in this process, as this is a high cost raw material. It represents about 11% of the total cost of the antibiotic (Veenhuis, 2002).

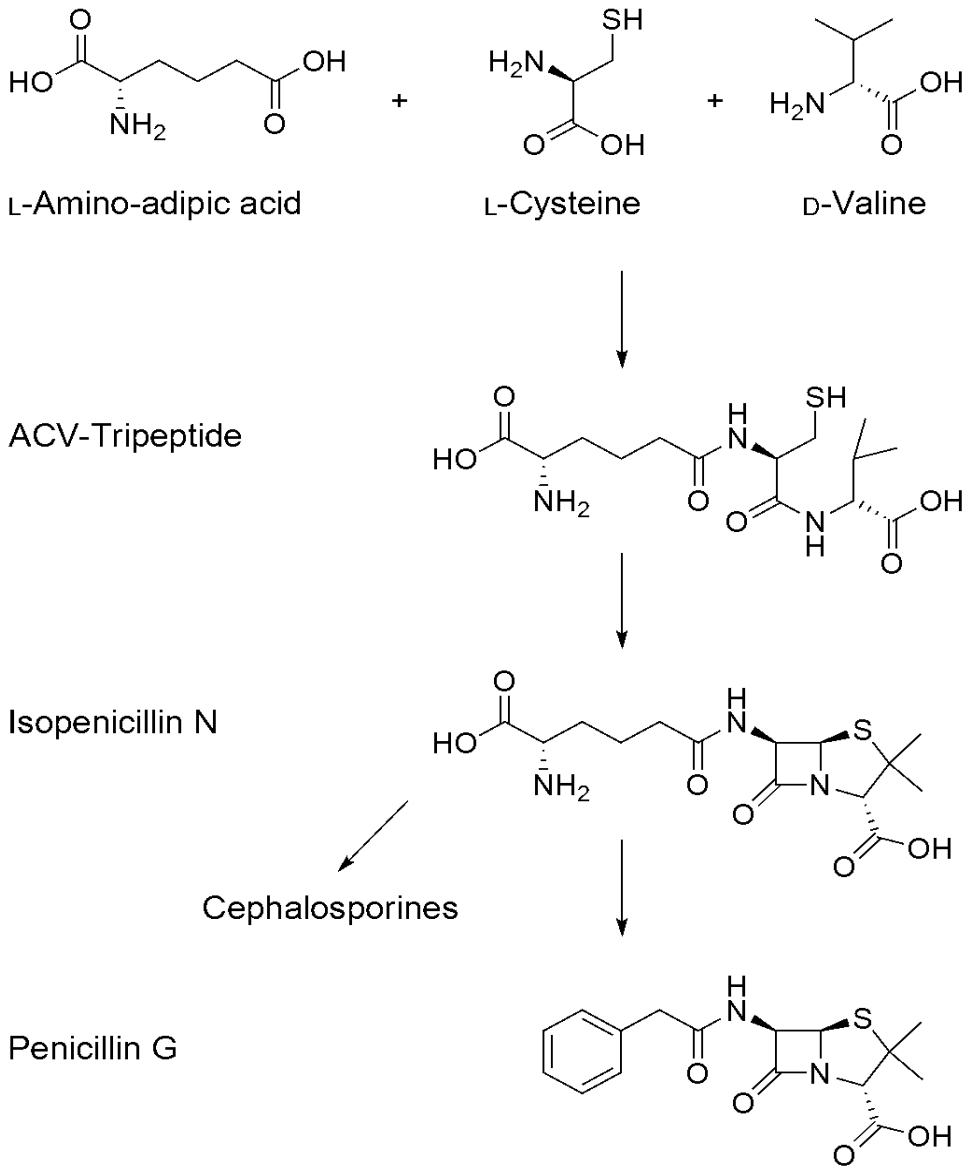
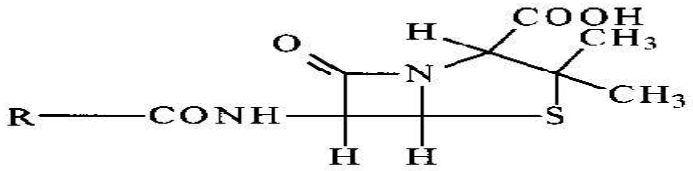


Figure 2.2: Biosynthesis of penicillin G and cephalosporins (Veenhuis, 2002).

2.3.4 Derivatives of Penicillin

There are other derivatives of penicillin which could treat a wider range of infections. The first real step forward was in the form of ampicillin (Figure 2.3). Ampicillin offered a broader spectrum of activity than either of the original penicillins and allowed doctors to treat a broader range of both Gram-positive and Gram-negative infections. Further developments led to amoxicillin, with improved duration-of-action. Further development also gave

flucloxacillin, important even now for its resistance to beta-lactamases produced by bacteria such as *Staphylococcus* species. The last in the line of true penicillins were the antipseudomonal penicillins, such as ticarcillin, useful for their activity against Gram-negative bacteria (McGrane *et al*., 1998).



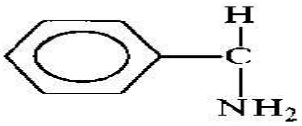
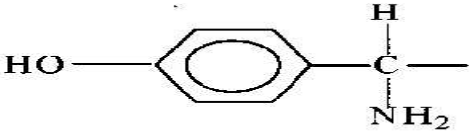
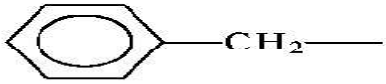
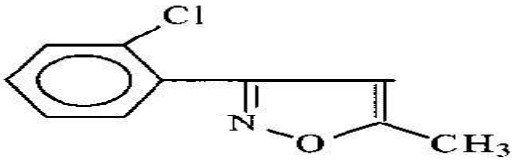
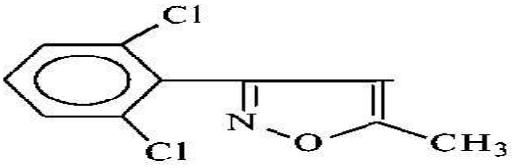
Penicillin	R
Ampicillin	
Amoxicillin	
Penicillin G	
Cloxacillin	
Dicloxacillin	

Figure 2.3: Structures of various derivatives of Penicillins. (McGrane, 1998).

2.4 β-lactamases and Penicillin function

Resistance to penicillin is now common amongst many hospital acquired bacteria. The resistance to penicillin has been partly due to the rise of beta-lactamase producing bacteria which secrete an enzyme that breaks down the beta-lactam ring of penicillin, rendering it harmless to the bacteria. These bacteria may remain sensitive to other beta-lactam antibiotics. Resistance also arises through modifications to the bacterial cell wall; this resistance usually extends to other beta-lactam antibiotics (McGrane *et al*., 1998). β-lactamase catalyses the opening and hydrolysis of the β-lactam ring of β-lactam antibiotics such as penicillins and cephalosporins. There are four groups, classed A, B, C and D according to sequence, substrate specificity, and kinetic behaviour: class A (penicillinase-type) is the most common. The genes for class A β-lactamases are widely distributed in bacteria, frequently located on transmissible plasmids in Gram-negative organisms, although an equivalent chromosomal gene has been found in a few species (Ambler, 1980; Philippon *et al*., 2002).

Class A, C and D β-lactamases are serine-utilising hydrolases - class B enzymes utilise a catalytic zinc centre instead. The three classes of serine β-lactamase are evolutionarily related and belong to a superfamily that also includes DD-peptidases and other penicillin-binding proteins. All these proteins contain an S-x-x-K motif, the Ser being the active site residue. Although clearly related, however, the sequences of the three classes of serine β-lactamases vary considerably outside the active site. The β-lactamases are enzymes produced by bacteria to hydrolyze β-lactam antibiotics such as penicillins, cephalosporins and carbapenems. These enzymes catalyze the hydrolysis of the β-lactam ring to effectively destroy the antibiotic's activity and enable bacteria to survive in the presence of these drugs. This is the major mechanism of resistance to β-lactam antibiotics in gram-negative bacteria (Philippon *et al*., 2002; Farkosh, 2008).

2.4.1 Penicillinase

Penicillinase is a specific type of β-lactamase, showing specificity for penicillins, again by hydrolysing the β-lactam ring. Molecular weights of the various penicillinases tend to cluster around 50,000. "Penicillinase" was discovered in 1940 and re-named β-lactamase when the structure of the β-lactam ring was finally elucidated. Penicillinase was the first β-lactamase to be identified: it was first isolated by Abraham and Chain in 1940 from *E. coli* even before penicillin entered clinical use but penicillinase production quickly spread to bacteria that previously did not produce it or only produced it rarely (Philippon *et al*., 2002; Farkosh, 2008)..

2.4.2 Extended-spectrum beta-lactamase (ESBL)

Extended-spectrum β-lactamases (ESBLs) are plasmid-mediated β-lactamases of predominantly Bush class A, so far described only in gram negative bacilli. ESBLs are capable of efficiently hydrolyzing penicillins, narrow spectrum cephalosporins, many extended-spectrum cephalosporins, the oxyimino group containing cephalosporins (cefotaxime, ceftazidime), and monobactams (aztreonam). β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) generally inhibit ESBL producing strains. Most ESBLs are mutants of TEM-1, TEM-2 and SHV-1; to date none has been described that are able to hydrolyze cephamycin or carbapenems (imipenem, meropenem). Over 25 different TEM and SHV ESBL variants have been claimed to date. ESBL producing isolates are most commonly *Klebsiella* species, predominantly *Klebsiella pneumoniae,* and *Escherichia coli* (*E. coli*). Most ESBL producing organisms are in the family Enterobacteriaeae and have been described in almost all members (Philippon *et al*., 2002; Farkosh, 2008).

Bush Class B β-lactamases include metaloproteases, which are capable of hydrolyzing carbapenems such as imipenim and meropenam. This class of β-lactamases have recently been reported in Japan among *Pseudomonas aeruginosa* and *Serratia marcescens*. In addition

to carbapenams, the isolates were resistant to other β-lactams and β-lactamase inhibitors with the exception of aztreonam (Naumovski *et al*., 1996; Hernandez *et al*., 2005; Farkosh, 2008).

2.5 Penicillin in enzyme function, inflammation and coagulation

The Penicillins and related β-lactam antibiotics have been shown to inhibit a range of enzymes with nucleophilic serine residues involved in inflammatory processes. These include mammalian serine proteases such as thrombin, elastase and prostate specific antigen (Konaklieva, 2002).

2.5.1 Inflammation

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Inflammation is not a synonym for infection: infection is caused by an exogenous pathogen, while inflammation is the response of the organism to the pathogen. In the absence of inflammation, wounds and infections would never heal and progressive destruction of the tissue would compromise the survival of the organism. However, inflammation which runs unchecked can also lead to a host of diseases, such as hay fever, atherosclerosis, and rheumatoid arthritis. It is for this reason that inflammation is normally tightly regulated by the body. Inflammation can be classified as either acute or chronic (Stitzinger, 2007).

2.5.1.1 Acute inflammation

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. It is

a short-term process which is characterized by the classic signs of inflammation - swelling, redness, pain, heat, and loss of function - due to the infiltration of the tissues by plasma and leukocytes and the slowing of blood flow, which are induced by the actions of various inflammatory mediators. It occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed, broken down, or walled off by scarring (fibrosis). Vasodilation occurs first at the arteriole level, progressing to the capillary level, and brings about a net increase in the amount of blood present, causing the redness and heat of inflammation. Increased permeability of the vessels results in the movement of plasma into the tissues, with resultant stasis due to the increase in the concentration of the cells within blood - a condition characterised by enlarged vessels packed with cells. Stasis allows leukocytes to marginate along the endothelium, a process critical to their recruitment into the tissues. Normal flowing blood prevents this, as the shearing force along the periphery of the vessels moves cells in the blood into the middle of the vessel (Stitzinger, 2007).

2.5.1.2 Chronic inflammation

Chronic inflammation is prolonged inflammation which leads to a progressive shift in the type of cells which are present at the site of inflammation and is characterised by simultaneous destruction and healing of the tissue from the inflammatory process. It is a pathological condition characterised by concurrent active inflammation, tissue destruction, and attempts at repair. Chronic inflammation is not characterised by the classic signs of acute inflammation listed above. Instead, chronically inflamed tissue is characterised by the infiltration of mononuclear immune cells (monocytes, macrophages, lymphocytes, and plasma cells), tissue destruction, and attempts at healing, which include angiogenesis and fibrosis.

Endogenous causes include persistent acute inflammation. Exogenous causes are varied and include bacterial infection, especially by *Mycobacterium tuberculosis*, prolonged exposure to

chemical agents such as silica, or autoimmune reactions such as rheumatoid arthritis (Serhan and Savill, 2005; Stitzinger, 2007).

2.5.2 Penicillin antibiotics and enzyme function

Serine proteases or serine endopeptidases (newer name) are a class of peptidases (enzymes that cleave peptide bonds in proteins) that are characterised by the presence of a serine residue in the active centre of the enzyme. The major groups found in humans include the chymotrypsin-like, the subtilisin-like, the alpha/beta hydrolase, and signal peptidase groups. Serine proteases participate in a wide range of functions in the body, including blood clotting (coagulation), immunity, and inflammation, as well as contributing to digestive enzymes in both prokaryotes and eukaryotes (Walselman *et al*., 1991; David and Michael, 2006). All three enzymes are synthesized by the pancreatic acinar cells, secreted in the small intestine and are responsible for catalyzing the hydrolysis of peptide bonds (Konaklieva, 2002).

Serine proteases are inhibited by serine protease inhibitors ("serpins"), a diverse group of enzymes that form a covalent bond with the serine protease, inhibiting its function. The best-studied serpins are antithrombin and alpha1-antitrypsin, studied for their role in coagulation/ thrombosis and emphysema/A1AT respectively.

Recently, a number of β-lactams, compounds widely used as antimicrobial drugs, have been identified as inhibitors of serine enzymes, in particular leucocyte elastase (LE). The first LE inhibitor β-lactams were naturally occurring bicyclic compounds, such as clavams and cephalosporins, but more recently synthetic monocyclic β-lactams have been developed. Because the latter perform with extremely good safety profiles and infrequent side effects, they could represent a good model base for designing powerful drugs able to inhibit LE and restore the altered protease/antiprotease ratio at the inflammatory sites (Konaklieva, 2002). Human leucocytes elastase [HLE], a serine protease released by the azurophilic granules of

human polymorphonuclear leucocytes [PMN] degrades elastin and other connective tissue components. It has been implicated in the pathogenesis of pulmonary emphysema and other inflammatory diseases such as rheumatoid arthritis and cystic fibrosis.

Mutations may lead to decreased or increased activity of enzymes. This may have different consequences, depending on the normal function of the serine protease. For example, mutations in protein C, when leading to insufficient protein levels or activity, predispose to thrombosis. Determination of serine protease levels may be useful in the context of particular diseases.

- Coagulation factor levels may be required in the diagnosis of haemorrhagic or thrombotic conditions.
- Faecal elastase is employed to determine the exocrine activity of the pancreas, e.g. in cystic fibrosis or chronic pancreatitis.
- Prostate specific antigen is used to determine prostate cancer risk.

2.6 Thrombin

Thrombin – a serine protease is the normal activation product of prothrombin in the coagulation cascade. It is evident that thrombin is a molecule with an extremely wide range of biological roles (blood coagulation, platelet activation, chemoattractant for neutrophils and monocytes, feedback inhibition, thromboembolism in cancer patients etc) and an extremely high level of regulation associated with it is due to its potency as a pro-coagulant and pro-inflammatory mediator. Thrombin is most widely recognized for its role in blood coagulation but the diversity of this protein's functions in the body is far more widespread. Indeed, it has been noted by one group of workers that almost every cell type tested (except erythrocytes) was responsive to thrombin (Hongbao *et al*., 2008).

2.6.1 Physiological Functions of Thrombin

2.6.1.1 Blood Coagulation

The blood coagulation system comprises a cascade of proteases that cleave precursor enzymes to form active enzymes, forming an extremely effective amplification system that culminates in the formation of thrombin. Thrombin, once formed, then catalyses the conversion of fibrinogen to fibrin to form a clot and, importantly, regulates the system by providing stimulatory and inhibitory feedback. The centrality of thrombin to this system makes it an extremely powerful enzyme in coagulation and makes it the most suitable enzyme to be a target for anticoagulant drug therapy, as to control the action of thrombin would be to control the entire coagulation system (Hongbao *et al*., 2008).

The main role of thrombin in coagulation is the cleavage of soluble fibrinogen to form insoluble fibrin, the basis of the haemostatic clot. Fibrinogen cleavage is an orderly process comprising of two distinct steps, both catalysed by thrombin. Once the coagulation cascade is initiated and thrombin is generated, the initial stimulus is often "turned off", and the cascade is maintained by the feedback effects of thrombin. This stimulatory feedback comes in the form of activation of factors V, VIII and XI. Factor V, when activated, associates with activated factor X and cleaves prothrombin to thrombin. Factor VIII, when activated, associates with activated factor IX and activates factor X. Factor XI, when activated, activates factor IX. In this way, the cascade is up-regulated, and a very large amount of product (i.e. fibrin) can be formed from a relatively small initial stimulus. To complement its role in fibrin clot formation, thrombin also plays a part in inhibition of lyses of that clot (Dahlback, 2000; Hongbao *et al*., 2008). This action is carried out by a plasma carboxypeptidase enzyme, which circulates in the blood as an inactive proenzyme and is activated by thrombin. This enzyme, termed "thrombin activatable fibrinolysis inhibitor" (TFPI) inhibits fibrinolysis by cleavage of carboxy-terminal lysine residues on the fibrin polymers. These residues are important in

assembling components of the fibrinolytic system, so their removal inhibits fibrinolysis (Hongbao *et al*., 2008).

2.6.1.2 Thrombin and Platelets

Supplementary to its role in coagulation, thrombin also plays a vital role in primary haemostasis, as an extremely potent activator of platelets. This thrombin-induced platelet activation is critical for adequate haemostasis (Hung *et al*., 1992). This activation is initiated by interaction of the thrombin molecule with a receptor on the surface of the platelet, with subsequent activation of various secondary messenger systems, of which the inositol triphosphate/calcium system is probably the most important. The interaction of these two molecules is interesting because the kinetics of the reaction suggest that what actually occurs is not simple ligand-receptor interaction, but something more akin to an enzyme-substrate interaction where thrombin enzymatically cleaves the receptor/substrate. The amino terminal extracellular domain of the receptor contains a cleavage site for thrombin, which is structurally similar to the anticoagulant hirudin and is therefore able to bind thrombin. Thrombin cleaves this site between Arg 41 and Ser 42 exposing a new amino terminal domain, which acts as a ligand for the receptor itself - termed a "tethered ligand" (Hongbao, *et al*., 2008). The role of thrombin in platelet activation is not an isolated one, but is closely associated with thrombin's other roles in coagulation, particularly fibrin formation. As platelet aggregation and coagulation go hand-in-hand during bleeding, it is fitting that the molecule that is central to and regulates one system also regulates the other. Whenever fibrin formation is required to achieve haemostasis, platelet aggregation will also be required, and vice versa (Cazenave et al., 2008; Hongbao, *et al*., 2008).

2.6.1.3 Thrombin and Inflammation

Thrombin has various actions in inflammation. It is a chemoattractant for neutrophils (Esmon, 2000) and monocytes (Becker, *et al*., 1998) that is, it induces the cells to move down a chemical gradient to where the thrombin is most concentrated, i.e. the site of injury. This allows the neutrophils and monocytes to carry out their phagocytic role if there is invading bacteria present. Thrombin stimulates the production of the cell-anchoring protein P-selectin from Weibel-Paladi bodies in endothelial cells, which is then expressed on their membrane. This molecule is important in the process of leucocyte "rolling", in which leucocytes are loosely bound to the vessel wall and therefore begin to slow down their flow rate, and roll along the endothelium, eventually stopping where they are required. Thrombin also stimulates endothelial cells to produce platelet activating factor (PAF). Although not implied in its name, PAF is a potent activator of neutrophils especially those bound to P-selectin (Esmon, 2000).

2.6.1.4 Thrombin and Cancer

Since the recognition of the association between cancer and thrombosis 140 years ago, a few significant strides have been made both in understanding of this complex relationship and in management of venous thromboembolism (VTE) in patients with cancer (Lee. 2006). It has been noted that multiple factors determine the risk of thrombosis in cancer patients. The most important cancer-related factors are the histology and the extent of the malignancy. Patients with adenocarcinomas and metastatic disease appear to have the highest risk. The exact mechanisms remain unclear but may include enhanced expression of tissue factor by tumour cells and host monocytes, unregulated release of cytokines (such as tumour necrosis factor) that indirectly activate coagulation, and suppression of natural anticoagulant pathways. - Chemotherapeutic agents also appear to activate coagulation through similar yet undefined mechanisms. Other extrinsic factors or conditions that also predispose patients with cancer to develop VTE include surgery and reduced mobility. Consequently, prophylaxis is highly

recommended to prevent VTE in patients undergoing surgery for malignancy, although the use of primary thromboprophylaxis in other clinical settings is not routine practice (Lee, 2006; Bick, 2006).

2.6.2 Thrombin – Beta-lactam interaction

β-lactam antibiotics (βLAs) may contribute to bleeding episodes in patients by depleting vitamin K-dependent coagulation factors, interfering with fibrin polymerization, or causing defects in platelet function. Despite well-documented platelet abnormalities in both normal and patient groups receiving βLAs**,** clinical bleeding primarily occurs in chronically ill and/or malnourished patients. Although platelet defects have been described at therapeutic concentrations for many, some antibiotics produce defective platelet function *in vitro* only at concentrations greatly in excess of those used therapeutically (Jean-Pierre, *et al*., 2008).

Pastakia, *et al.,* (1993) examined the effects of the β-lactam antibiotic penicillin G on platelet function and on specific membrane glycoproteins *in vitro*. Platelet concentrates exposed to 3 to 10 mmol/L penicillin for 48 hours showed irreversible inhibition of aggregation by thrombin in washed platelets after removal of the antibiotic. Although a brief 15-minute exposure to similar doses of penicillin also inhibited thrombin aggregation, the inhibition was reversed on removal of the penicillin by washing. Aggregation activity was also restored to normal levels by stimulation with high thrombin concentrations (>0.4 U/ml) (Pastakia, *et al.,* 1993).

2.6.3 Thrombin Regulation

Thrombin regulates its own production by being part of an inhibitory system. This is achieved via binding to a vascular endothelial cell protein called thrombomodulin. This leads to activation of protein C (causing inactivation of coagulation factors V and VIII and thus down-regulation of thrombin generation), and inhibition of thrombin's ability to form fibrin and activate factor XIII, platelets and coagulation feedback stimulatory proteins (Esmon, 2000). The protein C system of thrombin inhibition is extremely powerful under normal circumstances, and so must be confined to the site of injury. This confinement is achieved by the necessity of binding to thrombomodulin, which is expressed on damaged endothelial cell walls.

Thrombin is a powerful stimulus for platelet activation and aggregation and is degraded by proteinase inhibitors such as antithrombin III, heparin cofactor II, and alpha-2 macroglobulin. Besides its central role in the coagulation cascade, thrombin is a potent platelet agonist and thus constitutes an interesting target for drugs that would prevent the formation of fibrin- and platelet-rich thrombi induced by thrombin ((Konaklieva, 2002). Aside from mortality, significant additional morbidity occurs from both arterial or venous thrombotic events, including, but not limited to paralysis (non-fatal thrombotic stroke), cardiac disability (repeated coronary events), loss of vision (retinal vascular thrombosis) and foetal wastage syndrome (placental vascular thrombosis), stasis ulcers and other manifestations of post-phlebitic syndrome, (recurrent deep venous thrombosis [DVT]), etc. (Lee, 2006; Bick, 2006).

2.6.4 Thrombin clotting time test

The Thrombin Time (TT), also known as the Thrombin Clotting Time (TCT), is a test of the time it takes for a clot to form measuring the conversion of fibrinogen to fibrin. It is a coagulation assay which is usually performed in order to detect the therapeutic level of the

anticoagulant. It is also sensitive in detecting the presence of a fibrinogen abnormality. The thrombin time is used to diagnose bleeding disorders and to assess the effectiveness of fibrinolytic therapy. Reference values for thrombin time are 10 to 15 seconds or within 5 seconds of the control (Flanders *et al.,* 2003).

The thrombin time involves only the addition of bovine or human thrombin to platelet poor plasma. It, therefore, reflects the conversion of fibrinogen to fibrin but is also sensitive to the presence of inhibitors e.g. heparin. Thrombin cleaves fibrinogen; releasing fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from fibrinogen and converting fibrinogen into a fibrin clot (Flanders *et al.,* 2003).

There are however a new class of anticoagulants known as Direct thrombin inhibitors (DTIs) that binds directly to thrombin and block its interaction with its substrates. Some DTIs — such as recombinant hirudins, bivalirudin, and ximelagatran, either alone or in combination with melagatran acts as DTIs (Marcello *et al.,* 2005).

2.7 Role of biological markers in physiological and disease condition

2.7.1 Haematology

Haematology is the branch of science that is concerned with the study of blood, the blood-forming organs, and blood diseases. Haematology includes the study of aetiology, diagnosis, treatment, prognosis, and prevention of blood diseases. The most commonly performed test in haematology laboratory is the complete blood count (CBC) also called full blood count (FBC), which includes; white blood cell count, platelet count, haemoglobin level and several parameters of red blood cells. Coagulation is a sub-speciality of haematology; basic general coagulation tests are the thrombin time, prothrombin time (PT) and partial thromboplastin time (PTT) (Alternative Technologies, 2008).

2.7.1.1 Blood Cells

Blood consists of a fluid (plasma) which contains three main types of cells - red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes). The numbers of each type of blood cell vary between people and at different ages but these values are quite stable for healthy individuals. Each type of blood cell has different functions (Alternative Technologies, 2008).

2.7.1.2 Red cells

Although blood appears to be a red liquid (Plate 2.1A), it is actually composed of a yellowish liquid called plasma and billions of cells. The vast majority of these cells are red cells and these give blood its red colour. Besides the red cells, the blood also contains several types of infection-fighting white cells and tiny cell fragments called platelets which are essential for clotting (Alternative Technologies, 2008).

2.7.1.3 Platelets

Platelets are fragments of a much larger cell, the megakaryocyte (Plate 2.1B below) that stays in the bone marrow after it differentiates and matures from the stem cell. Under normal conditions platelets circulate in the blood for approximately 10 days as disk shaped, formed elements, and do not adhere to other cellular elements, the vascular endothelium, or to themselves. But within a few seconds after injury to a vessel, platelets adhere to the exposed collagen surface. Such platelets become “activated” and release their internal constituents, resulting in a growing aggregate of platelets, and the simultaneous occurrence of clot formation. This growing mass literally plugs the hole in the damaged vessel wall. Thus, platelet function can be described in terms of the following reactions: 1) attachment at the site of the injury (adhesion); 2) aggregation of platelets to each other; and 3) release of substance(s) that facilitate blood coagulation. Adhesion to a cut vessel wall is a complicated

process that has been shown to require plasma factors as well as key platelet membrane molecules. Aggregation of platelets to each other is mediated by numerous exogenous platelet agonists such as thrombin and adenosine diphosphate (ADP). Each of these agonists has one or more receptors on the platelet surface. Binding of the agonist unmasks specific sites for plasma fibrinogen on the platelet membrane, which is thought to somehow serve as the "bridge" between platelets (Alternative Technologies, 2008).

2.7.1.4 White Blood Cells

There are five distinctly different kinds of white blood cells, neutrophils, monocytes, lymphocytes, eosinophils and basophils. Some have the ability to change with needs and situations in the body (Plate 2.1C). So, for example, there are different monocytes found in different tissues, and different types of lymphocytes with different roles in fighting infections. These cells can leave the bloodstream, sliding out through the vessel walls and attacking invaders at the site of an infection.

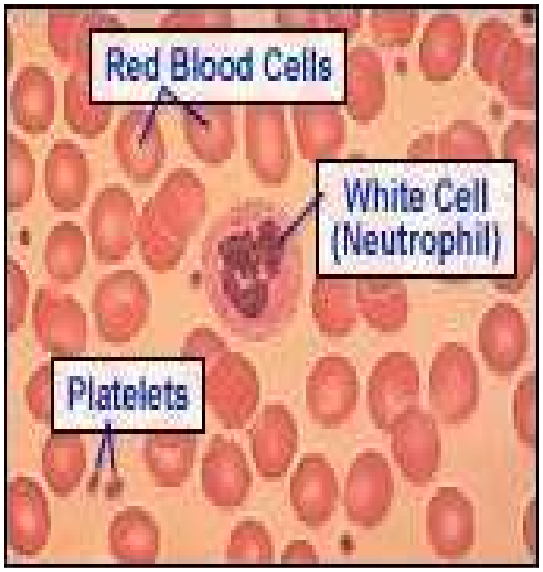


Plate 2.1A: Red blood cells

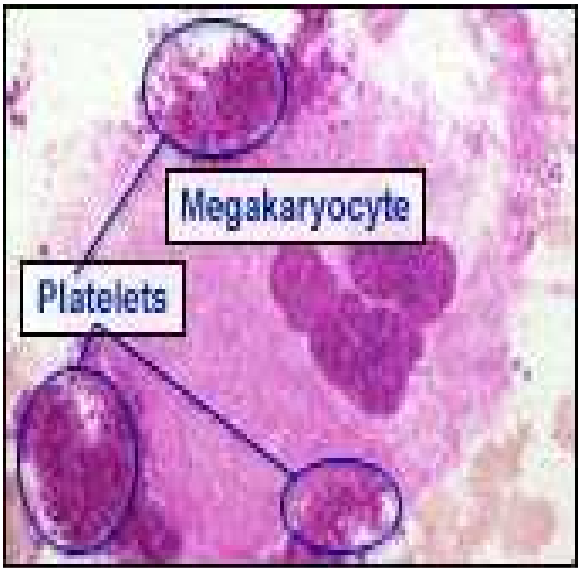
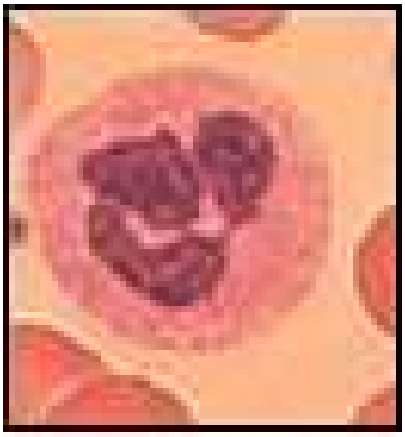
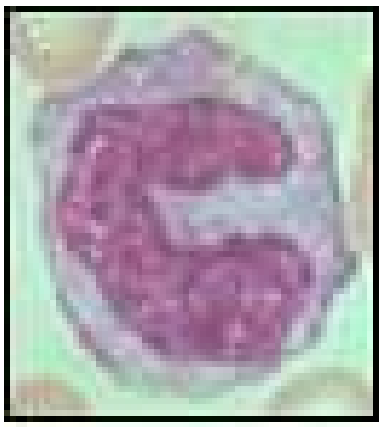


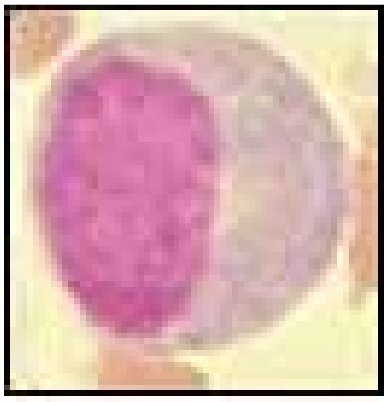
Plate 2.1B: Platelets



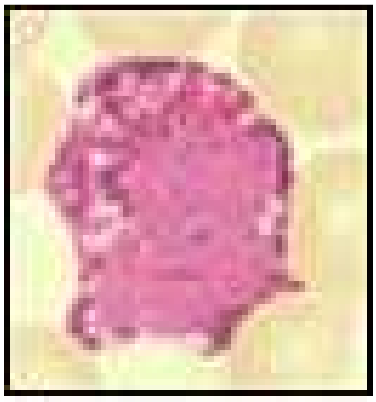
Neutrophil



Monocyte



Lymphocyte



Basophil

Plate 2.1C: White blood cells

2.8 Enzymes of oxidative stress: their interaction with penicillin antibiotics

The ability of organisms to use molecular oxygen was a major evolutionary breakthrough that enabled the production of significantly more energy from the breakdown of foods, amongst many other advantages. However, these advantages came at a cost: toxic by-products known as reactive oxygen species (ROS) are produced, which if left unchecked would seriously affect an organism's viability. These ROS include hydrogen peroxide, superoxide anion radicals, singlet oxygen, hydroxyl radicals and nitric oxide. ROS serve as normal signaling molecules, but unchecked they can damage a wide variety of molecules within cells, leading to oxidative stress. In order to limit the crippling effects of oxidative stress, a cell can respond by committing suicide, whereby the ROS produced by a cell's mitochondria can act as a trigger for apoptotic cell death through the activation of caspases. This is effective in the short-term, but high levels of oxidative stress can lead to serious tissue damage through excessive cell death and oxidative damage. Just how harmful these ROS can be is evidenced by the diseases they are involved in when their levels become too high, which include inflammatory joint disease (destruction of cartilage), insulin-dependent diabetes mellitus (destruction of pancreatic beta cells), asthma, cardiovascular disease, and many neurodegenerative diseases (destruction of nerve cells) including Alzheimer's disease and amyotrophic lateral sclerosis (ALS). To help protect against the destructive effects of ROS, aerobic organisms produce protective antioxidant enzymes such as catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), and glutathione peroxidase (EC 1.11.1.9). It was the evolution of these enzymes that made oxidative cellular metabolism possible (Ramazan and Namik, 2002; Devasagayam *et al*., 2004; Usor *et al*., 2005).

2.8.1 Antioxidants to the Rescue

Cells make a variety of antioxidant enzymes to fight the dangerous side-effects of life with oxygen. Two important players are superoxide dismutase, which converts superoxide radicals into hydrogen peroxide, and catalase, which converts hydrogen peroxide into water and oxygen gas. The importance of these enzymes is demonstrated by their prevalence, ranging from about 0.1% of the protein in an *Escherichia coli* cell to upwards of a quarter of the protein in susceptible cell types. These many catalase molecules patrol the cell, counteracting the steady production of hydrogen peroxide and keeping it at a safe level (Boon *et al*., 2007).

2.8.1.1 Catalase

Catalases are produced by aerobic organisms ranging from bacteria to man. Catalases (EC 1.11.1.6) are haem-containing proteins that catalyze the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen, thereby protecting cells from the toxic effects of hydrogen peroxide: Each molecule of catalase has four polypeptide chains, each composed of more than 500 amino acids, and nested within this tetrad are four porphyrin haeme groups - very much like the familiar haemoglobin, cytochromes, chlorophylls and nitrogen-fixing enzymes in legumes (Catalase may also take part in some of the many oxidatic reactions that occur in all cells.) Some haem-containing catalases are bifunctional, acting as a catalase and a peroxidase (EC 1.11.1.7). In these bifunctional catalase-peroxidases, a variety of organic substances can be used as a hydrogen donor, for example alcohol, which can be oxidized in the liver. These bifunctional catalases are closely related to plant peroxidases. There are also non-haem manganese-containing catalases, which occur in bacteria. Catalases are some of the most efficient enzymes found in cells. Each catalase molecule can decompose millions of hydrogen peroxide molecules every second. The cow catalase and the human catalases use Fe^{3+} to assist in this speedy reaction. The enzyme is composed of four identical subunits, each with its own active site buried deep inside (Brioukhanov *et al*., 2006; Boon *et al*., 2007).

2.8.1.1.1 Mode of action

Most catalases exist as tetramers of 60 or 75 kDa, each subunit containing an active site haem group buried deep within the structure, but which is accessible from the surface through hydrophobic channels. The very rigid, stable structure of catalases is resistant to unfolding, which makes them uniquely stable enzymes that are more resistant to pH, thermal denaturation and proteolysis than most other enzymes. Their stability and resistance to proteolysis is an evolutionary advantage, especially since they are produced during the stationary phase of cell growth when levels of proteases are high and there is a rapid rate of protein turnover. Haem-containing catalases break down hydrogen peroxide by a two-stage mechanism in which hydrogen peroxide alternately oxidizes and reduces the haem iron at the active site. In the first step, one hydrogen peroxide molecule oxidizes the haem to an oxyferryl species. In the second step, a second hydrogen peroxide molecule is used as a reductant to regenerate the enzyme, producing water and oxygen. Some catalases contain NADPH as a cofactor, which functions to prevent the formation of an inactive compound (Boon *et al*., 2007).

2.8.1.1.2 Applications of Catalase

Catalase is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene - some lens-cleaning systems disinfect the lenses by soaking them in a hydrogen peroxide solution, and catalase is used to decompose the peroxide before reinserting the lenses in the eye. Recently, catalase has begun to be used in the aesthetics industry in mask treatments combining the enzyme with hydrogen peroxide on the face to increase cellular oxygenation of cells in the upper layers of the epidermis (Brioukhanov *et al*., 2006; Boon *et al*., 2007).

2.8.1.2 Superoxide Dismutase

Superoxide dismutases (SODs) are antioxidant metalloenzymes catalyzing the redox disproportion in the (dismutation) of superoxide radical, O_2. It is generally accepted that in all SODs the metal ion (M) catalyzes dismutation of the superoxide radical through a cyclic oxidation reduction mechanism:

$$M^{3+} + O_2^{*-} \text{ --> } M^{2+} + O_2$$

$$M^{2+} + O_2^{*-} + 2H^{+} \text{ ---> } M^{3+} + H_2O_2$$

The four classes of SODs are known, distinguished by the metal prosthetic groups: Cu/Zn, Fe, Mn and Ni. Fe^{-} and Mn^{-} SODs constitute a structural family (Parker *et al.,* 1987; Parker *et al.,* 1988). Fe- and Mn- SODs are unequally distributed throughout the kingdoms of living organisms and are located in different cellular compartments (Liberman and Babal 2004; Valko *et al*., 2005). In particular, Fe-SOD is found in obligate anaerobes and aerobic diazotrophs (exclusively), facultative aerobes (exclusively or together with Mn-SOD).

Aerobic organisms have developed numerous mechanisms which protect the cell from the physiological generation of activated oxygen. However, when the generation of such species over whelms the cell's ability to detoxify them, cell injury can result. The superoxide dismutases dispose of superoxide anions. These metal-containing enzymes catalyze the dismutation of two molecules of the superoxide anion to give hydrogen peroxide and dioxygen.

$$O^{-}_2 + O_2\text{-} + 2H^{+} \text{ -----> } H_2O_2 + O_2$$

2.8.1.3 Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Usor *et al*., 2005; Boon *et al*., 2007; Soliman 2008)..

The enzyme glutathione peroxidase also catalyzes the reduction of hydrogen peroxide to water. Reduced glutathione is used as the source of reducing equivalents necessary to drive this reaction. In this process, two molecules of glutathione are oxidized to yield one molecule of glutathione disulfide (GSSG). Glutathione disulfide is efficiently reduced to glutathione by glutathione reductase. NADPH serves as the source of the reducing equivalents. The glutathione peroxidase-reductase system seems to be the first line of defence against hydrogen peroxide, and catalase is a secondary system. Glutathione peroxidase is found in the cytosol of most cells as well as within the mitochondria. Glutathione peroxidase also shows considerable activity toward organic hydroperoxides, converting them to their corresponding alcohols. By contrast, catalases will only catabolise hydrogen peroxide. The cytosolic and mitochondrial forms of glutathione peroxidase are dependent upon the metal selenium for activity. The importance of glutathione reductase is emphasized by the observation that inhibition of this enzyme also potentiates the cytotoxicity of oxidative stress. With the inhibition of glutathione reductase, oxidized glutathione is not converted to reduced glutathione, a result that limits the effectiveness of the peroxidase by limiting the supply of glutathione (Usor *et al*., 2005; Soliman 2008).

2.8.1.4 Lipid peroxidation (malondialdehyde)

Polyunsaturated fatty acids (PUFAs) are abundant in cellular membranes and in low-density lipoproteins (LDL) (Dekkers, *et al*., 1996). The PUFAs allow for fluidity of cellular membranes. A free radical prefers to steal electrons from the lipid membrane of a cell, initiating a free radical attack on the cell known through a process known as lipid peroxidation. As with any radical reaction, the reaction consists of three major steps: initiation, propagation and termination. Initiation is the step whereby a fatty acid radical is produced. The initiators in living cells are most notably reactive oxygen species (ROS), such as $OH\cdot$, which combines with a hydrogen atom to make water and a fatty acid radical (Acworth and Bailey 1997; Kaczmarski, *et al*., 1999; Brioukhanov *et al*., 2006; Boon *et al*., 2007; Soliman 2008).

Reactive oxygen species target the carbon-carbon double bond of polyunsaturated fatty acids. The double bond on the carbon weakens the carbon-hydrogen bond allowing for easy dissociation of the hydrogen by a free radical. A free radical will steal the single electron from the hydrogen associated with the carbon at the double bond. In turn this leaves the carbon with an unpaired electron and hence becomes a free radical. In an effort to stabilize the carbon-centred free radical molecular rearrangement occurs. The newly arranged molecule is called a conjugated diene (CD). The CD then very easily reacts with oxygen to form a peroxy radical. The peroxy radical steals an electron from another lipid molecule in a process called propagation. This process then continues in a chain reaction (Acworth and Bailey 1997; Ramazan and Namik, 2002).

The administration of antibiotics such as ampicillin, tetracycline, chloramphenicol and streptomycin bring about profound alteration in lipid peroxidation levels of different tissues of rat along with decrease in SOD and catalase activity and increase in the levels of reduced glutathione especially in the kidney. However some antibiotics used in the *in vitro* studies of

pancreatic cells did not produce any oxidative stress as evidenced by low levels of free radical measured in terms of MDA comparable to those of control. This difference could be attributed to the different tissues (liver, kidney, heart and not pancreas) used (Yogita *et al*., 2001). The mechanism by which antibiotics exert their toxic effect may be different in each case. Different tissues of animals respond differently to oxidative stress depending on the status of their own antioxidant defence system and nature of damaging agents.

However, among the antibiotics used (gentamycin, penicillin, streptomycin, tetracycline, neomycin erythromycin and chloramphenicol) none of the antibiotics led to development of oxidative stress except tetracycline. This was obvious by the increase in lipid peroxidation and moderate SOD and catalase activities (Yogita *et al*; 2001).

2.9 Liver and its enzymes

The liver is the largest gland, and the largest solid organ in the body. It is located on the right side of the upper abdomen below the diaphragm anatomy. It lies below the diaphragm in the thoracic region. The adult human liver normally weighs between 1.4 - 1.6kg. It holds approximately 13% (about 0.57 litres) of the total blood supply at any given moment and is estimated to have over 500 functions. The liver is dark reddish brown and is divided into two main lobes (the much larger right and the smaller left) which are further subdivided into approximately 100,000 small lobes, or lobules. About 60% of the liver is made up of liver cells called hepatocytes which absorb nutrients and detoxify and remove harmful substances from the blood. A hepatocyte has an average lifespan of 150 days. It receives its blood supply via the hepatic artery and portal vein (which transports nutrients from the intestine, or gut) (Bramstedt, 2006).

The liver plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, that

are essential for blood to clot (clotting factors); and detoxification. It produces bile, an alkaline compound which aids in digestion, via the emulsification of lipids. It also performs and regulates a wide variety of high-volume biochemical reactions requiring very specialized tissues (Eugene *et al*., 2003).

2.9.1 Liver Function Tests

As the liver performs its various functions, it makes a number of chemicals that pass into the bloodstream and bile. Various liver disorders alter the blood level of these chemicals. Some of these chemicals can be measured in a blood sample. Some tests that are commonly done on a blood sample are called liver function tests (LFTs). LFTs are sometimes referred to as a liver panel and they typically measure the following: alanine aminotransferase (ALT), aspartate aminotransferase e (AST), alkaline phosphatase (ALP), total protein and albumin, and total and direct bilirubin.

Among the most sensitive and widely used of these liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These enzymes are normally contained within liver cells. If the liver is injured, the liver cells spill the enzymes into blood, raising the enzyme levels in the blood and signalling the liver damage. The aminotransferases catalyze the conversion of one amino acid to the corresponding keto acid with the simultaneous conversion of another keto acid to an amino acid, hence the names "aminotransferases". Transamination reactions occur in many tissues (Jenifer 2008).

2.9.1.1 Alanine aminotransferase (ALT)

ALT is an aminotransferase enzyme (EC 2.6.1.2) found in serum and in various bodily tissues, large amounts of ALT occur in liver cells. The serum concentration is elevated, especially when there is acute damage to liver cells, as in viral or toxic hepatitis, and

obstructive jaundice. Significant elevation of the serum levels of ALT is a specific indicator of liver damage only in small animals and primates (Sarko *et al.,* 1979; AGA 2002; Chernecky and Berger, 2008; Jenifer 2008). It catalyzes the transfer of an α-amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate.

2.9.1.2 Aspartate aminotransferase (AST)

AST is normally found in a diversity of tissues including liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged. Following injury or death of physiologically active cells, the enzyme is released into the circulation. Elevated values may be found 8hours after injury and should peak in 24 - 36hours if the original episode is not repeated. It usually falls to normal in 4 - 6days. The amount of serum AST is directly proportional to the number of cells damaged and the interval of time between tissue injury and the test (AGA 2002; Chernecky and Berger, 2008; Jenifer 2008).

2.9.1.3 Alkaline phosphatase (ALP)

The ALP of the liver is produced by the cells lining the small bile ducts (ductoles) in the liver. Its origin differs from that of other enzymes called aminotransferases. If the liver disease is primarily of an obstructive nature (cholestatic), i.e. involving the biliary drainage system, the ALP will be the first and foremost enzyme whose activity will be elevated. If, on the other hand, the disease is primarily of the liver cells (hepatocytes), the aminotransferases will rise prominently. Thus, these enzymes are very useful in distinguishing the type of liver disease-cholestatic or hepatocellular (Sarko *et al.,* 1979; Pagana and Pagana, 2006).

2.9.2 Bilirubin

The majority of bilirubin (80%) is produced from the degradation of haemoglobin from erythrocytes undergoing normal (removal of aged cells) or abnormal destruction (i.e.

intravascular or extravascular haemolysis) within mononuclear phagocytes (principally splenic, hepatic and bone marrow macrophages). A small percentage (20%) is derived from the catabolism of various hepatic hemoproteins (myoglobin, cytochrome P450) as well as from the over-production of heme from ineffective erythropoiesis in the bone marrow. Within macrophages, a free heme group (iron + porphyrin ring) is oxidized by microsomal heme oxygenase into biliverdin and the iron is released (the iron is then stored as ferritin or released into plasma, where it is bound to the transport protein, transferrin). Biliverdin reductase then reduces the green water-soluble biliverdin into unconjugated bilirubin. Heme oxygenase is also located in renal and hepatic parenchyma, enabling these tissues to take up heme and convert it to bilirubin.

Unconjugated or free bilirubin is then released into plasma where it binds to albumin. Once within the hepatocyte, unconjugated bilirubin is transported with ligand (Y protein) or other proteins (e.g. Z protein) and the majority is conjugated to glucuronic acid by UDP-glucuronyl transferase. The remainder is conjugated to a variety of neutral glycosides (glucose, xylose). Bilirubin must be conjugated before it can be excreted into bile (conjugation makes bilirubin water soluble). Excretion into biliary canaliculi is the rate-limiting step of the entire bilirubin metabolism pathway and occurs via specific transporters, which are energy (ATP) dependent. Transfer into the canaliculi is facilitated by bile salt-dependent and bile salt-independent biliary flow (the latter of which is generated by a basolateral (sinusoidal or blood-side) Na/K ATPase pump (Sarko *et al.,* 1979; Baranano *et al*., 2002; Liu *et al*., 2008).

2.9.3 Total Protein and Albumin

Total protein measures albumin and all other proteins in blood while albumin is the main protein made by the liver, and it circulates in the bloodstream. The levels of albumin (a type of protein the body makes) and total protein can help tell how well the liver is functioning. The ability to make albumin (and other proteins) is affected in some types of liver disorder. A

low level of blood albumin occurs in some liver disorders. Serum protein determination is important because albumin is synthesized in the liver, and because serum globulins are produced by the Kupffer cells. Therefore, in typical chronic liver disease, the albumin/globulin ratio is reversed with diminution of albumin and elevation of globulin, which is a broad gamma type of elevation (Sarko *et al.,* 1979; Baron, 1985)

When the plasma albumin level falls below 20-25g/l, oedema is likely to develop. In a patient suffering from severe malnutrition of slow onset of the plasma albumin level may be low without there being oedema: conversely, a 'famine oedema' can present with a normal plasma albumin level though there may be excess fluid intake. In hypoalbuminaemia, there is increase synthesis, and probably catabolism, of lipids.

In chronic or severe acute liver disease, synthesis of albumin is impaired. The fall in plasma albumin that occurs after trauma, in malignancy and other long continued wasting disease, or in acute or chronic infection and other systemic illnesses, is partly due to liver damage, partly to impaired intake, and partly to an unexplained toxic destruction of protein (Sarko, *et al.,* 1979; Baron, 1985; Eugene *et al*., 2003; Bramstedt, 2006).

2.10 Kidney and its metabolites

The kidneys are complicated organs that have numerous biological roles. Their primary role is to maintain the homeostatic balance of bodily fluids by filtering and secreting metabolites (such as urea) and minerals from the blood and excreting them, along with water, as urine. Because the kidneys are poised to sense plasma concentrations of ions such as sodium, potassium, hydrogen, oxygen, and compounds such as amino acids, creatinine, bicarbonate, and glucose, they are important regulators of blood pressure, glucose metabolism, and erythropoiesis (the process by which red blood cells (erythrocytes) are produced) (Baron, 1985).

2.10.1 Kidney Function Tests

2.10.1.1 Urea

Blood urea nitrogen (BUN) measures the amount of urea nitrogen, a waste product of protein metabolism, in the blood. Urea is formed by the liver and carried by the blood to the kidneys for excretion. Because urea is cleared from the bloodstream by the kidneys, a test measuring how much urea nitrogen remains in the blood can be used as a test of renal function. However, there are many factors besides renal disease that can cause BUN alterations, including protein breakdown, hydration status, and liver failure (Baron, 1985).

An increase in urea level is known as azotaemia and may be caused by: impaired renal function, congestive heart failure as a result of poor renal perfusion, dehydration, shock, haemorrhage into the gastrointestinal tract, acute myocardial infarction, stress, excessive protein intake or protein catabolism.

A decreased urea may be seen in: liver failure, malnutrition, anabolic steroid use, over hydration, which can result from prolonged intravenous fluids, pregnancy (due to increased plasma volume), impaired nutrient absorption, Syndrome of inappropriate anti-diuretic secretion (Sarko. *et al.,* 1979; Baron, 1985).

2.10.1.2 Creatinine

Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). Creatinine is mainly filtered by the kidney, though a small amount is actively secreted. There is little-to-no tubular reabsorption of creatinine. If the filtering of the kidney is deficient, blood levels rise. As a result, creatinine levels in blood and urine may be used to calculate the creatinine clearance (ClCr), which reflects the glomerular filtration rate (GFR). The GFR is clinically important because it is a measurement of renal function. Ketoacids, cimetidine and trimethoprim reduce

creatinine tubular secretion and therefore increase the accuracy of the GFR estimate, particularly in severe renal dysfunction. BUN-to-creatinine ratio; ratio of urea to creatinine can indicate other problems besides those intrinsic to the kidney for example, a urea level raised out of proportion to the creatinine may indicate a pre-renal problem such as dehydration. Men tend to have higher levels of creatinine because they have more skeletal muscle than women. Vegetarians tend to have lower creatinine levels, because vegetables contain no creatine. Measuring serum creatinine is the most commonly used indicator of renal function. A rise in blood creatinine levels is observed only with marked damage to functioning nephrons. Generally a rising level of creatinine signifies an increasing problem with poorly performing kidneys (Gross, *et al*., 2009).

2.11 Electrolyte balance as markers in health and disease

The electrolyte panel is used to detect, evaluate and monitor electrolyte imbalances. It may be ordered as part of a routine examination or to help in the diagnosis of a chronic or acute illness that occurs as a result of inflammation. It may be requested at intervals to help monitor conditions such as renal disease and hypertension and also to monitor the effectiveness of treatment for known imbalances. Electrolytes are electrically charged minerals that are found in body tissues and blood in the form of dissolved salts. They help move nutrients into and waste out of the body's cells, maintain a healthy water balance and help stabilize the body's pH level. The electrolyte panel measures the main electrolytes in the body: Sodium (Na^+), Potassium (K^+), Chloride (Cl^-) and total Carbon dioxide (CO_2) (Lockless *et al*., 2008).

2.11.1 Electrolyte shift (imbalance)

Physiological functions in the body are regulated by electrolytes. A variety of symptoms have been associated with electrolyte imbalance and these include loss of body fluids through prolonged vomiting, diarrhoea, sweating or high fever. The most serious forms of electrolyte

imbalance in cancer patients include high blood calcium levels called hypercalcaemia or a disorder called tumour lyses syndrome, which results in electrolyte imbalance from the killing of cancer cells. Both of these conditions can be life threatening if not managed appropriately. The kidneys play a crucial role in regulating electrolytes. They control the levels of chloride in the blood and flush out potassium, magnesium as well as sodium. Therefore a shift in physiological levels of these electrolytes may be related to kidney function (Lockless *et al*., 2008).

2.11.2 Sodium/Potassium

Sodium is the primary cation (positive ion) in extracellular fluids in animals and humans. These fluids, such as blood plasma and extracellular fluids in other tissues bath cells and carry out transport functions for nutrients and wastes. Sodium is also the principal cation in seawater, although the concentration there is about 3.8 times what it is normally in extracellular body fluids (Eggeman, 2007).

Potassium cations are important in neuron (brain and nerve) function, and in influencing osmotic balance between cells and the interstitial fluid. Potassium is found in intracellular fluids, it is important in allowing muscle contraction and the sending of all nerve impulses in animals through action potentials. By nature of their electrostatic and chemical properties, K^+ ions are larger than Na^+ ions, and ion channels and pumps in cell membranes can distinguish between the two types of ions, actively pumping or passively allowing one of the two ions to pass, while blocking the other (Lockless *et al*., 2008). A shortage of potassium in body fluids may cause a potentially fatal condition known as hypokalaemia, typically resulting from diarrhoea, increased diuresis and vomiting. Deficiency symptoms include muscle weakness, paralytic ileus, ECG abnormalities, decreased reflex response and in severe cases respiratory paralysis, alkalosis and cardiac arrhythmia (Noskov *et al*., 2004; Shi *et al*., 2006; Lockless *et al*., 2008).

2.11.3 Chloride

Chloride is a chemical the human body needs for metabolism (the process of turning food into energy). It also helps keep the body's acid-base balance. The amount of chloride in the blood is carefully controlled by the kidneys. Chloride ions have important physiological roles. For instance, in the central nervous system, the inhibitory action of glycine and some of the action of gamma amino butyric acid (GABA) relies on the entry of Cl^- into specific neurons. Also, the chloride-bicarbonate exchanger biological transport protein relies on the chloride ion to increase the blood's capacity of carbon dioxide, in the form of the bicarbonate ion. The normal blood reference range of chloride for adults in most laboratories is 95 to 105 milliequivalents (mEq) per litre. The normal range may vary slightly from one laboratory to another (Baron, 1985).

2.12 Toxicology

Toxicology is “the science of poison” i.e. “the study of the adverse effects of chemicals or physical agents on living organism”. These adverse effects may occur in many forms, ranging from immediate death to subtle changes not realized until months or years later. They may occur at various levels within the body, such as an organ, a type of cell, or a specific biochemical levels. It is now known that various observable changes in anatomy or body functions actually result from previously unrecognised changes in specific biochemical functions in the body (Toxicology Tutor 2005). The historical development of toxicology began with early cage dwellers that recognised poisonous plants and animals and used their extracts for hunting or in warfare. Paracelsus 1500AD determined that specific chemicals were actually responsible for toxicity of a plant or animal poison. His studies revealed that small doses of a substance might be harmless or beneficial whereas larger doses could be toxic. This is now known as the dose response relationship, i.e. “All substances are poisons;

there is none which is not poison. The right dose differentiates a poison and a remedy." In 1800AD Orfila was the first to prepare a systematic correlation between the chemical poisons on specific organs by analyzing autopsy materials for poisons and their associated tissue damage (Ping *et al*., 1989; Sofowora, 1993).

Xenobiotic is the general term that is used for a foreign substance taken into the body. Thus, antibiotics such as benzyl penicillin and the extract from *P. chrysogenum* constitute xenobiotics to the rats. A xenobiotic in small amounts may be non-toxic and beneficial but when the dose is increased, toxic and lethal effects may result. Xenobiotics cause many types of toxicity by a variety of mechanisms. Some chemicals are themselves toxic. Others must be metabolized (chemically changed within the body) before they cause toxicity (Toxicology Tutor 2005).

2.12.1 Acute Toxicity

Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24hours. Toxicity may manifest itself by effects directed at different target organs and measurement of toxicity is death. The ability of a compound to cause death and do so in half of the animals when certain dose is administered defines the toxicity of the compound. The purposes of acute toxicity testing are to obtain information on the biologic activity of a chemical and gain insight into its mechanism of action. The information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling, and use of chemicals. The LD_{50} value, defined as the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period, is currently the basis for toxicological classification of chemicals (Kent and Gallo, 1998; Walum, 1998).

The cage side observation included any changes in the skin, fur, eyes, mucus membranes, circulatory system, autonomic and central nervous system, somato-motor activities, behaviour, etc. Other pharmacotoxic signs such as tremor, convulsion, salivation, diarrhoea, lethargy, sleepiness, morbidity, fasciculation, mydriasis, miosis, droppings, discharge, or hypotonia should be recorded (Ping *et al*., 1989).

2.12.2 Sub-acute Toxicity

The objective of the sub-acute toxicity tests is to generally evaluate and characterize effects of compound when they are administered to the experimental animals repeatedly, usually on daily basis over a period and pharmacological effects are particularly evaluated. The route of administration of a test compound is usually limited to the oral route whenever a compound is given on a daily basis for several weeks because repeated administration of the compound does not induce harmful effects in the animals and mice were used because blood samples can be obtained at intervals for chemistry conveniently (Kent and Gallo, 1998; Walum, 1998).

When new drugs are subjected to the prolonged toxicity tests, it is not uncommon for the animals to show, either during the test or at autopsy at the end of the test, some altered or even abnormal function of certain organs, blood chemistry and haematology. In these situations it is of considerable value to determine the degree of reversibility and of these effects (Walum, 1998; Toxicology Tutor, 2005).

The distribution of toxicants and toxic metabolites throughout the body ultimately determines the sites where toxicity occurs. Many ingested chemicals, when absorbed from the intestine, distribute first to the liver and may be immediately detoxified. The liver is particularly susceptible to xenobiotics due to a large blood supply and its role in metabolism causing hepatotoxicity (i.e. toxicity to the liver, bile duct and gall bladder). The site and rate of excretion is another major factor affecting the toxicity of a xenobiotic. The kidney is the

primary excretory organ, followed by the gastrointestinal tract, and the lungs (for gases). The kidney is highly susceptible to toxicants because high volume of blood flows through it and it filters large amounts of toxins, which can concentrate in the kidney tubules causing nephrotoxicity (i.e. toxicity to the kidneys). Confirmation of toxicity is enhanced by enzyme assay (Burkitt *et al*., 2002).

2.13 Histology/Histopathology

Histology is the study of the microscopic anatomy of cells and tissues of plants and animals. It is performed by examining a thin slice of tissue under a light microscope. The ability to visualize or differentially identify microscopic structures is frequently enhanced through the use of histological stains. Histology is an essential tool of biology and medicine. Histopathology refers to the microscopic examination of tissue in order to study the manifestations of disease (viral or not). Specifically, in clinical medicine, histopathology refers to the examination of a biopsy or surgical specimen by a pathologist, after the specimen has been processed and histological sections have been placed onto glass slides. This is the most important tool of the anatomical pathologist in routine clinical diagnosis of cancer and other diseases. Histological examination of tissues starts with surgery, biopsy, or autopsy (Merck Source, 2002).

2.14 Techniques for identification and quantitative analysis of biochemical compounds

2.14.1 Chromatography

This is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be

isolated. Chromatography may be preparative or analytical. Preparative chromatography seeks to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography normally operates with smaller amounts of material and seeks to measure the relative proportions of analytes in a mixture. The two are not mutually exclusive (Jonathan *et al*., 2007).

2.14.1.1 Thin Layer Chromatography (TLC)

TLC is a chromatography technique used to separate mixtures. It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the experimental solution based on the polarity of the components of the compound in question. Spots of colourless compounds on a TLC plate can be visualized by a technique often used for compounds containing aromatic rings, or other systems that absorb UV radiation at 254nm. The silica or alumina is impregnated with a fluorescent insoluble compound that absorbs UV light and emits it as visible light. When placed under a UV lamp, the plate emits a bright white light except where a UV absorbing compound is situated. Here, a dark spot is observed.

Its wide range of uses include: assaying radiochemical purity of radiopharmaceuticals, determination of the pigments a plant contains, detection of pesticides or insecticides in food, analysing the dye composition of fibres in forensics, or identifying compounds present in a given substance, monitoring organic reactions (Jonathan *et al*., 2007; Fair and Kormos, 2008).

2.14.2 High Performance Liquid Chromatography (HPLC)

HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. It utilizes a column that holds

chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

Sample to be analyzed is introduced in small volume to the stream of mobile phase. The analyte's motion through the column is slowed by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent (Lloyd and John, 2006; Xiang *et al*., 2006; Brandao and Roberto 2006).

2.15 Mutation

Mutations are changes to the nucleotide sequence of the genetic material of an organism. Mutations can be caused by copying errors in the genetic material during cell division, by exposure to ultraviolet or ionizing radiation, chemical mutagens, or viruses, or can occur deliberately under cellular control during processes such as hyper mutation. In multicellular organisms, mutations can be subdivided into germ line mutations, which can be passed on to

descendants, and somatic mutations, which are not transmitted to descendants in animals (Maki, 2002; Taggart and Starr, 2006).

2.15.1 Ultraviolet (UV) light

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than x-rays, in the range 400nm to 10nm. It is so named because the spectrum consists of electromagnetic waves with frequencies higher than those that humans identify as the colour violet. UV light is found in sunlight and is emitted by electric arcs and specialized lights such as black lights. As an ionizing radiation it can cause chemical reactions, and causes many substances to glow or fluoresce. Most people are aware of the effects of UV through the painful condition of sunburn, but the UV spectrum has many other effects, both beneficial and damaging, to human health.

UV light causes thymine base pairs next to each other in genetic sequences to bond together into thymine dimers, a disruption in the strand which reproductive enzymes cannot copy. This leads to frame shifting during genetic replication and protein synthesis, usually killing the organism. As early prokaryotes began to approach the surface of the ancient oceans, before the protective ozone layer had formed, blocking out most wavelengths of UV light, they almost invariably died out. The few that survived had developed enzymes which verified the genetic material and broke up thymine dimer bonds, known as excision repair enzymes. Many enzymes and proteins involved in modern mitosis and meiosis are extremely similar to excision repair enzymes, and are believed to be evolved modifications of the enzymes originally used to overcome UV light *(*Ellis *et al*., 2001: Hockberger, 2002*;* Maki, 2002; Taggart and Starr, 2006).

2.15.2 Chemical mutagens

Some chemical mutagens, such as nitrous acid and nitrosoguanidine, work by causing chemical modifications of purine and pyrimidine bases that alter their hydrogen-bonding properties. For example, nitrous acid converts cytosine to uracil which then forms hydrogen bonds with adenine rather than guanine. Other chemical mutagens function as base analogs. They are compounds that chemically resemble a nucleotide base closely enough that during DNA replication, they can be incorporated into the DNA in place of the natural base. Examples include 2-amino purine, a compound that resembles adenine and 5-bromouracil, a compound that resembles thymine. The base analogs, however, do not have the hydrogen-bonding properties of the natural base. Still other chemical mutagens function as intercalating agents. Intercalating agents are planar three-ringed molecules that are about the same size as a nucleotide base pair. During DNA replication, these compounds can insert or intercalate between adjacent base pairs thus pushing the nucleotides far enough apart that an extra nucleotide is often added to the growing chain during DNA replication. An example is ethidium bromide (Maki, 2002; Taggart and Starr, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

General laboratory glassware, Petri dishes (glass and sterile plastic), Inoculating loop and glass spreaders, Cork borer, Incubator, Oven, Autoclave, Weighing balance (Mettler Toledo AB204, Switzerland), Light Microscope, (Carl Zeiss, Germany, Swift instruments International, South Africa), Haematocrit reader, Neubauer counting chamber, Water baths (Gallenkemp, England), Centrifuges - Uniscope SM112 (Surgifriend Medicals, England), Ultracentrifuge-Superspeed RC 2-B (Sorvall Inc, Newtown Connecticut, USA) Spectrophotometers-Thermospectronic Genesys 20 (4001/1, USA) and Spectronic GenesysTM8, (USA), Freeze dryer (Thermoservant, Micro duodulyo, USA), Grounding machine-Malex Excella Mixer (Mumbai, India), Colorimeter-Biomedical colorimeter (USA), Distillation (Kjeldah system 1002 Distilling unit), Agilent 8453 UV Spectrometer, HPLC Analyzer (Agilent 1100 series G1316A COLCOM), DESAGA HP-UVIS Heidelberg Low Pressure Hg-Lamp, Haematology analyzer (Coulter A^{c}. T.diff., Beckman coulter, Miami, USA.), Silica gel GF pre-coated (250 microns) fluorescence alumina plates (10x20).

3.1.1 Reagents

All chemical reagents used for this study were of analytical grade.

Benzyl Penicillin (Retarpen, Sandox Austria) as a Reference drug, Potato Dextrose Agar (PDA) Nutrient agar were obtained from Merck, Darmstadt, (Germany), HemoStat thrombin time reagent was supplied by Human Geselischaft fur Biochemica und Diagnostica mbH – Germany.

The agro-waste substrates used for culture media preparation in this work are sawdust, sugarcane pulps, cassava peels and corncob. They are readily available and were purchased from the local markets and farmlands.

3.2 METHODS

3.2.1 Isolation of micro fungus (*P. Chrysogenum*)

Freshly prepared stock culture of the decomposing wood waste which was collected from sites at Okobaba sawmills at Ebuta-Metta, Lagos, was inoculated on three different media containing agar namely: Czepek-Dox Agar (CDA), Potato Dextrose Agar (PDA) and Sabouraud's Agar (SA) to determine the types of micro fungi present in the waste. The culture media were prepared with Ampicillin – an antibiotic to inhibit the growth of bacteria. A sterile glass spreader (hockey stick) was used to spread the inoculum on the surface of the respective agar medium. The cultures were incubated at $30^{O}C$ for 48 – 72 hours. Colonial characteristics of viable micro fungi were observed. Each colony that appeared on the plates was sub cultured on PDA and incubated at $30^{O}C$ for 48 – 72 hours. Further sub culturing was done until pure isolates of the three isolated micro fungi were obtained per plate and maintained on slant and stored at $4^{O}C$.

3.2.1.1 Identification of micro fungi

Isolates were identified based on their colonial and cultural morphologies and microscopic features of their sporulating structures (de Hoog *et al*., 2000). Preliminary identification of the fungal isolates was done with the assistance of Dr. A.A. Adekunle, a mycologist in the Department of Botany, University of Lagos.

3.2.1.2 Colonial and cultural morphologies

Each isolate was cultured on PDA and observed for the following characteristics:

1. Hyphae – The characteristics of the vegetative mycelia were observed for the presence of cross-walls. The Hyphae may be loosely interwoven or may form stomata from which the spores arise.
2. Pigmentation: The pigments may be diffusible or coloured. The colour of the colonies was observed for a period of 1 – 7 days.

3.2.1.3 Morphology of sporulating structures

Slide preparations of the sporulating isolates were stained with lactophenol-cotton blue and covered with cover slips. The microscopic features of the sporulating micro fungi were studied using a light microscope (Carl, Zeiss, Germany) and by the description given by Deacon (1980) and Talbot (1972).

Three filamentous micro fungi were isolated from the stock of decomposing wood waste collected from sites at Okobaba sawmills at Ebuta-Metta, Lagos. The identified fungi include: *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum*. Preliminary investigations show that culture extract of *A. flavus*, and *A. niger* did not produce visible inhibition against clinical isolates of *Escherichia coli* and *Bacillus subtilis* while that of *P. chrysogenum* did. Based on literatures as an antibacterial producing fungus, *P. chrysogenum* was identified and used throughout the course of this research.

3.2.2 Preparations of agro-waste materials

The agro-wastes used as fermentation substrates were prepared as follow:

Sawdust of gmelina Abora wood was collected from sawmills at Ikorodu, Lagos, Nigeria. While mature sugarcane (*Saccharum offinarum*) was purchased from Ire-Akari in Isolo,

Lagos. The peeled sugarcane was crushed and washed thoroughly in water to obtain a sugar-free fibrous pulp. The pulp was cut into small pieces and sun-dried for 3-5 days to remove moisture and make it easier to dry.

Fresh maize (Zea mays) was purchased from Mushin market, Lagos, Nigeria. Corncobs were obtained by removing the maize grains. Cassava peels were obtained from boundary market, Ajegunle, Lagos. The materials were cut into small pieces and sun-dried for 3-5 days to reduce the moisture content and make easier to grind. Grinding was done using Marlex, Excella Mixer Grinder (Mumbai, India). Fine powder obtained by passing the ground materials through a fine mesh sieve of about 0.5mm pore size was used as microbial substrates.

3.2.3 Growth media

A modification of the Mineral salt medium described by Kastner *et al*., (1994) was prepared for growth studies. This Basal media known as Mineral Salt Broth contained per litre: Ammonium acetate, 6.0g; $NaSO_3$, 0.5g; $ZnSO_4.7H_2O$, 0.02g; $MgSO_4.7H_2O$, 0.25g; KH_2PO_4, 6.0g; $FeSO_4.7H_2O$, 0.01g; distilled water 1000mL. Four different media were prepared each containing 10.0g Agro-waste material as carbon source: Sawdust (SD), Cassava peels (CAS), and Sugarcane pulp (SC), Corncob (CC). 10g of Glucose (GLU), and Lactose (LAC) respectively were used as controls. Duplicate flask of 50 ml of each medium were used for the growth studies. The pH was adjusted to 6.5 before autoclaving at 121^oC for 15 minutes, cooled and inoculated with 5ml spores suspension.

3.2.4 Strain

Pure isolates of *P. chrysogenum* (wild strain) used for this study were obtained from a wood-waste dump in Lagos, Nigeria as described previously (Nwodo-Chinedu *et al*, 2005; Nwodo-Chinedu *et al*, 2007b) and maintained on PDA slant at 4^oC. The organism was sub cultured on

PDA plates and incubated at 30^{o}C for 3-5 days to obtain the spore suspension used for growth and antibiotic production. The spores were washed with 0.1% Tween 80 in 0.1M potassium phosphate buffer (54 g. of KH_2PO_4/105 g. of K_2HPO_4) at pH 7.0 (Gordon G. Carter,1977). The density of this spore suspension was adjusted so that a 1 in 10 dilution had an optical density of 0.48 measured at 530nm in the spectrophotometer. 5ml of the undiluted suspension was then used to inoculate each growth flask.

3.2.5 Growth Measurements

3.2.5.1 Mycelia weight measurement

Growth was measured by determining the mycelia weight, protein production and carbohydrate utilization every 3 days for 21 days. The dry weight of the mycelia was measured by filtering the mycelia unto a previously weighed filter paper, washed with distilled water and dried at 70^{o}C to a constant weight (Meletiadis *et al*., 2001; Kim *et al*., 2002; Park *et al*., 2002). Protein production was assayed using the Folin-Lowry method (1951) and measurement of extinction coefficient of a cell-free media at OD 280 nm. Carbohydrate utilization was assayed using the Anthrone method as described by Morris (1948) and the absorbance read at 620 nm. Quantitative determination of Carbohydrate on 5ml of the blank media was carried out before the cells were inoculated. Turbidity of the media was also measured at OD 530 nm every day for 7 days against a cell-free media to monitor growth.

3.2.5.2 Turbidometric method

The growth of the organism was assessed by a modification of the turbidometric method described by Meletiadis *et al.,* (2001). Mineral salt media containing the various waste materials were prepared as described above. 50 ml portions of the different media were dispensed into 160ml sterile media bottles and autoclaved at 121^{o}C for 15 minutes. The fungal

isolate was sub-cultured on PDA plates and incubated at 25^{o}C for 5-7 days to obtain enough spores. The conidia were carefully rubbed with a sterile cotton swab and transferred into a sterile test tube. The resulting suspensions were vigorously homogenized by shaking manually for 10 seconds. One ml of the spore suspension was used to inoculate the sterile 50 ml of the media and the bottle was covered with sterile cotton wool. Tween 80 was used in order to prevent the growth of fungi on the surface of the media inside the bottles and also necessary to release the carbon complex in the medium. The turbidity of the supernatant was measured by Optical Density measurement taken every 24 hours for 7 days using Biomedical Colorimeter. Non-inoculated media and inoculated media at zero hours served as control. Actual growth of the organism was calculated thus:

OD_{530} at time, t, – OD_{530} control (time, zero) = OD of organism (due to growth)

3.2.5.3 Protein Determination

Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins. 5 ml of the alkaline solution was added to 1 ml of the cell-free culture supernatant, mixed thoroughly and allowed to stand at room temperature for 30 minutes. 0.5 ml of diluted Folin-Ciocalteau reagent was added rapidly to the test with immediate mixing. This was left to stand for 30 minutes and the extinction against a reagent blank read at 750 nm in a spectrophotometer. A protein standard curve was prepared by making a serial dilution of a 1.0 mg/ml protein – BSA (Bovine Serum Albumin) to get varying concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml. The protein content was quantified as above according to the method of Lowry *et al*., (1951).

The extinction of a 0.0024mg/ml BSA protein was read at A_{280} and this was used as a standard for other protein assays.

3.2.5.4 Quantitative determination of carbohydrate by Anthrone method

The Anthrone reaction is the basis of a rapid and convenient method for the determination of glucose, lactose, hexose, aldopentose and hexuronic acids, either free or present in polysaccharides. The blue-green solution shows an absorption maximum at 620 nm. A calibration curve was prepared by placing 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of standard sugar in different boiling tubes, the volume in each tube was made up to1 ml with distilled water and placed in ice-bath. 6ml of Anthrone reagent was added to each tube from a running burette (Anthrone reagent is corrosive, contains conc. Sulphuric acid). This was placed in boiling water-bath for exactly 10 minutes with a marble covering the tubes to prevent evaporation. Absorbance was read at 620 nm against a reagent blank upon cooling the tubes. The same procedure was carried out for the test samples and the values were read from the calibration curve (Morris 1948).

3.2.6 Determination of optimum conditions for growth of *P. Chrysogenum* and antibiotic production

3.2.6.1 Optimum pH of growth by turbidity and mycelia weight measurement

Cell free culture media containing cassava peel as carbon and energy source was prepared as in growth studies. 50 ml was dispensed into each bottle. The pH was adjusted in triplicates for each pH value. The pH range was from 3.5 to 8.5. This was autoclaved at $121^{O}C$ for 15 minutes, cooled and inoculated with 0.5ml of spore suspension of the organism. This was left to stand for 7 days with intermittent shaking. Optical density of each bottle was measured every day for 7 days at 530 nm. At the end of the 7^{th} day, the dry weight of the mycelia was measured by filtering the mycelia unto a previously weighed filter paper, washed with

distilled water and dried at 70^{o}C to a constant weight (Meletiadis *et al*., 2001; Kim *et al*., 2002; Park *et al*., 2002). Results were expressed as mean triplicate value of pH measured.

3.2.6.2 Optimum temperature of growth by turbidity and mycelia measurement

Culture media was prepared as above and pH adjusted to 6.5 (optimum pH). This was autoclaved at 121^{O}C for 15 minutes, cooled and inoculated with 0.5ml of spore suspension (organism). This was left to stand in the incubator for 7 days with intermittent shaking at different temperatures ranging from 20 - 50^{O}C. Optical density (turbidity) of each bottle was measured every day for 7 days at 530 nm. At the end of the 7th day, the dry weight of the mycelia was measured by filtering the mycelia unto a previously weighed filter paper, washed with distilled water and dried at 70^{o}C to a constant weight (Meletiadis *et al*., 2001; Kim *et al*., 2002; Park *et al*., 2002). Results were expressed as mean triplicate value of temperature measured.

3.2.6.3 Optimum pH of antibiotic production

In studies of optimun pH of production of antibacterial extract, 50 ml undiluted spore suspension was inoculated into a production media as stated earlier. Four different media were prepared each containing 10.0g agro waste material as carbon and energy sources: corncob (CC), sawdust (SD), cassava peels (CAS), and sugarcane pulp (SC). The flasks were incubated with intermittent shaking for 7days at various pHs at 25^{O}C, the contents were sieved through cotton wool to remove the large mycelia that has accumulated, and then centrifuged, and the filtrate was extracted with chloroform-phosphate buffer and assayed for antibiotic activity (Gordon, 1977; Oruc and Sonal 2005).

3.2.6.4 Extraction and partial purification of extract

After 7 days of fermentation, crude extract was obtained by centrifuging the culture filtrates at 4000g for 5 minutes to remove cells. 0.5ml of the crude extract was used to assay for antibacterial activity using a modified method of Grau and Halliday (1957), Oruc and Sonal (2005) and Laich *et al*., (2002) with *Bacillus subtilis*, *Escherichia coli* as test organism and the zone of inhibition noted. Partial purification and extraction of penicillin was done by adjusting the pH of the cell free crude extract to 2.5. 20 ml of chloroform and 1 ml of 10% phosphate buffer pH 2.5 was added to 9 ml of the culture filtrate, this was mixed and the layers allowed separating. The chloroform layer was drawn into a second separating funnel. A second 20 ml portion of chloroform was added to re-extract, the extracts were combined and washed with one or more 10 ml portions of 1% phosphate buffer, pH 2.5, the buffer washes were discarded. The penicillin was extracted from the chloroform with 10 ml of 1% phosphate buffer, pH 6.0. Antibacterial activity of the extract after partial purification was determined.

For determination of optimum pH, each batch of extract for pH was freeze-dried to a constant weight at $4^{O}C$ and the quantity of antibiotics produced was weighed and recorded against each pH value.

3.3 PHYSICAL PROPERTIES OF THE CRUDE AND PARTIALLY PURIFIED EXTRACT

TLC plates are made by mixing silica gel- an adsorbent, with a small amount of calcium sulphate (gypsum) - an inert binder and water. This mixture was spread as a thick slurry on a glass plate measured 15 cm x 5 cm to a depth of approximately 2mm, and the resultant plate was dried and activated by heating in an oven for 30 minutes at 110 °C (Peter, *et al.,* 2003; Sherma and Fried, 2003; Fair and Kormos, 2008).

Using a capillary tube, a small spot of sample solution and standard reference drug were applied respectively to the different spots on the plates, one centimetre from the base. The plates were then placed in a tank containing a solvent mixture of toluene:ethylacetate :acetic acid (2:2:1 v/v/v), and the tank sealed with paraffin wax (Sherma and Fried, 2003). Chromatogram was prepared in Triplicates. These were allowed to stand for 1 hr while the solvent (mobile phase) percolates through the silica gel (stationary phase). The plates were removed when the solvent front approaches the top of the plate and the position of the solvent front recorded before it was dried and placed in iodine chamber for one minute before it was viewed under a UV-scan at 325 nm (McGrane *et al.,* 1998; Peter *et al.,* 2003; Sherma and Fried, 2003). The different spots were quickly marked and the R_f (retention ratio or retardation factor) values calculated using.

$$R_f = \frac{\text{distance moved by solute}}{\text{distance moved by solvent}}$$

HPLC analysis of the crude extract, partially purified extract and reference drug was carried out with a μBondapak C_{18} column after a careful validation of the method of Laich *et al*., (2002). The extract was filtered through Whatman No1 filter paper. The suspension was left to settle for 1 hour at 4 ^{o}C and filtered again through a Whatman No 1 followed by a 0.45 micron and then a 0.22 micron filter. This crude extract was divided into 50 ml aliquots and stored in the dark at 4^{o}C until use. Benzyl Penicillin G at a concentration of 0.1 mg/ml was used as the control sample. A C_{18} gravity column was wetted with 100 ml of HPLC grade methanol and then washed with 50 ml of Milli-Q water. A 50 ml aliquot of crude extract was added to the column and allowed to adsorb. Buffer A was 50 mM sodium acetate, pH 4.5, and buffer B was acetonitrile. The flow rate was 1.0 ml/min, and the running conditions were as follows: min 1 to 3, buffer A; min 3 to 15, gradient buffer B, 0 to 60%; min 15 to 18, gradient buffer B, 60 to 80%; min 18 to 20, buffer B, 80%; min 20 to 22, gradient buffer B, 80 to 0%;

and min 22 to 25, buffer A (Laich *et al*., 2002; Oruc and Sonal , 2005; Brandao and Roberto, 2006). Eluting peaks were scanned between 200 nm and 300 nm with 1 nm intervals to determine absorbance maxima and minima. Penicillin eluted at approximately 2.35 minutes.

3.4 MODIFICATION BY GENETIC MUTATION FOR HYPER PRODUCTION OF ANTIBIOTIC

3.4.1 Transformation of *P. chrysogenum* using Ultra Violet irradiation

Further work on improving the yield was done by transformation and selection. High yielding natural selectant was subjected to UV irradiation. A modified method of Karanam and Medicherla (2008) was adopted. A completely sporulated slant of *P. chrysogenum* (wild strain) (5 days old slant) was taken. The spores were scrapped off into 5 ml of sterile water. The spore suspension was serially diluted up to 10^{-5} dilution. A 0.1-ml quantity of spore suspension was poured asceptically on a PDA medium contained in Petri dishes. The suspension was uniformly distributed using a sterile spreader.

The spore suspension was exposed to UV light. This was carried out in a UV Illuminator fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A°. The exposure was carried out in the dark at a distance of 16 cm away from the centre of the Germicidal lamp (UV light source) with occasional shaking. The exposure times were 5, 10, 15, 20, 25, and 30 minutes. Each UV exposed spore suspension was sealed with a foil paper and stored overnight in the dark to avoid photo reactivation. The plates were incubated for 3 days at 28°C. Selection was based on a 1% survival rate and changes in morphology, size and shape.

The best two transformed strains with 1% survival rate were isolated. The two transformed isolates were streaked on PDA Slants and incubated for 3 days at 28°C. The best two UV

transformed t strains were inoculated into a growth and production media containing cassava peel as agro-wastes substrate and antibacterial production was monitored and quantified using HPLC analytical method over a period of 5 days (Laich *et al*., 2002).

3.4.2 Mutation by exposure of organism to sodium nitrate ($NaNO_3$)

A modified method of Karanam and Medicherla (2008) was adopted. 9 ml of 10^{-6} dilution of *P. chrysogenum* (wild strain) was added to 1 ml of sterile stock solution of 0.01 M $NaNO_3$. One-ml aliquots of samples were withdrawn at intervals of 10 min up to 60 min. 0.5 ml of phosphate buffer; pH 7 was added to each sample and was neutralized with 0.5 ml of 0.1 M NaOH. 0.1 ml of this exposed suspension was placed on PDA medium and the suspension was uniformly distributed using a sterile spreader. Each $NaNO_3$-exposed spore suspension was stored overnight to avoid photo reactivation. The plates were incubated for 3 days at 28°C. After 3 days, some colonies were obtained and among them, four isolates were selected from the plates on the basis of their morphology, size and shape. These isolates were streaked on PDA slants and incubated for 3 days at 28°C.

These best isolates were inoculated into a growth and production media containing cassava peel as agro waste substrate and antibacterial production was monitored and quantified using HPLC analytical method over a period of 5 days (Laich *et al*., 2002).

3.4.3 Quantitative Analysis of modified strain extracts using HPLC

P. chrysogenum (wild strain), best UV, as well as best $NaNO_3$ mutated strains were fermented in cassava peel medium under the conditions described earlier. Samples from the supernatant were taken between 24 hr - 96 hr of fermentation. Fifty micro litres of the unprocessed supernatant of *P. chrysogenum* (wild strain), best UV- as well as best $NaNO_3$-transformed strains were analyzed usin HPLC method as described earlier.

3.5. BIOCHEMICAL PROPERTIES OF FUNGAL ANTIBACTERIAL EXTRACT *IN VITRO*

Test organisms for antibacterial studies were supplied by the Department of Microbiology, University of Lagos, Nigeria. Individual bacterial strains were tested against the different antibacterial extract, of *P. chrysogenum* grown on various agro-waste media by growing the bacteria as "lawns" on nutrient agar in the presence of different concentrations of standard commercial penicillin (Reference standard). *P. chrysogenum* (wild strain) was tested for its ability to produce antibacterial activity by bioassays on solid medium with *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* as test organisms.

3.5.1 Determination of the Standard Dose Response

The bioassay on solid medium was performed on nutrient agar plates. Agar plugs (6-mm diameter) were bore on the nutrient agar plates, 0.4 ml of various concentrations of the reference drug and antibacterial extract were allowed to diffuse in the plates in the cold (2 h at 5°C), the test organisms were overlaid on the agar plates and finally the cultures were incubated at 30°C for 24 h. The agar plates were checked after 24 hours for zone of inhibition. Inhibition zones were measured in millimetre using metre rule. The mean response for each standard dose level was determined by using the logarithmic scale for the standard solutions (doses) and the arithmetic scale for the mean zone diameters. The regression coefficient, b, was calculated as:

$$\underline{b} = \frac{n\sum XY(\sum X)(\sum Y)}{n\sum X^2-(\sum X)^2}$$

X = concentrations of reference drug

Y = mean zone of diameter for each concentration

n = number of samples (5)

b = Regression coefficient

Determination of Potency of the extract was calculated using the formula below

$$\text{Concentration of unknown} = \frac{Yu - Ys}{b}$$

Where Yu = mean response of unknown sample

Ys = mean response of reference standard drug

Antilog M' = potency of the assay solution relative to the standard; and (antilog M') x 100 = potency of the assay solution as a percentage of the reference dose of standard. The concentration of antibiotics per unit or sample size tested was calculated by multiplying the concentration per milliliter of solution by the appropriate dilution factor (Gordon, 1977; Oruc and Sonal 2005).

3.5.2 Tests for β–Lactamase activity

The β-lactamase production of *Pseudomonas aeruginosa* was determined by use of β-lactamase identification sticks (Oxoid, Wesel, Germany) with nitrocefin as the substrate (Hakim *et al.,* 2007). Cells from a 24-hour old culture of *Pseudomonas aeruginosa* were collected into a test tube, sonicated to lyse the cells before centrifuging at 4000g for 15 minutes. β-lactamase identification sticks with nitrocefin as the substrate were inserted into test tubes containing the enzyme and allowed to stand for 30 minutes and observed for colour change. Presence of β-lactamase caused a colour change from yellow to red. The supernatant containing the β–lactamase enzymes was used to assay for lactamase activity against the antibacterial extract. 500 µL of lactamase enzyme extract was incubated with 500 µL of the *P. chrysogenum* extract from agro waste and allowed to stand for 30 minutes at room temperature; this was introduced into wells on agar plates and streaked with *E. Coli*. The

controls were 500 µL of β–lactamase and 500 µL of *P.chrysogenum* extract. The plates were observed for inhibition of growth of test organism after 24 hrs of incubation.

3.5.3 Determination of the presence of β-lactam ring in the antibacterial extract

A modified method of Pitout *et al*., (1997) was adopted for β-lactamase studies**.** Overnight cultures of *Pseudomonas aeruginosa* in nutrient broth were diluted with 95 ml of fresh broth and were incubated with shaking for 90 min at 37^{O}C. Both cultures were incubated for a further 2 hr. Cells were harvested by centrifugation at 4^{O}C, washed with 1 M potassium phosphate buffer (pH 7.0), suspended and sonicated. After sonication, crude extracts were obtained by centrifugation at 5,000 *g* for 1 h. The β-lactamases in the sonic extracts were used for hydrolysis of crude extract and benzyl penicillin by monitoring the rate of hydrolysis on a UV spectrophotometer for 30 minutes.

1000 µL of the *P. chrysogenum* extract (10 mg/mL 10mM Sodium Phosphate buffer pH 7.0) was pipetted into 2 test tubes. Similarly, 1000 µL of the reference standard drug (10 mg/mL 10mM Sodium Phosphate buffer pH 7.0) was set up in duplicate tubes. The blank contains 1000 µL of 10 mM Sodium Phosphate buffer pH 7.0 and 1000 µL of normal saline. The reaction was started with the addition of 1000 µL of lactamase enzyme extract to the tubes containing the extract and reference standard drug and their extinction monitored at 265nm for 30 minutes with readings taken at 5 minutes interval. Changes in extinction were recorded. Extinction coefficient: $E^{mM} = 0.18$ at 264.4 nm (Reynolds, 1982).

3.5.4 Determination of Minimum Inhibitory Concentration (MIC)

The extract of *P.chrysogenum* showed good activity against two strains (*E. coli* and *B. subtilis*, in the solid agar well diffusion test. Those concentrations giving an inhibitory zone of 10.00 mm diameter were chosen to assay for the MIC with the agar dilution method according to NCCLS (1997) guideline (Olukoya *et al.,* 1993; Enwuru, *et al*., 2008). The inoculums were

prepared from an overnight broth cultures and adjusted to turbidity equivalent to 0.5 McFarland standards. A stock solution 5 mg/ ml was made and the serial dilutions of 2.0, 1.0, 0.8, 0.6, 0.4, and 0.2 mg/ml were prepared from it. A 1-ml portion of each dilution was introduced into the appropriate agar medium in each of the six petri dishes. Each petri dish was divided into two sections. A loopful of the diluted culture of each test organisms was inoculated by streaking on the surface of each section of the petri dish. The petri dishes were incubated at 37°C for 24hrs. A control which contained only nutrient agar and the test organism was also set up. The MIC was defined as the lowest concentration of the extract inhibiting the visible growth of each organism.

3.6 DETERMINATIONS OF BIOCHEMICAL CHANGES IN PLASMA ANALYTES FOLLOWING ACUTE AND SUB-ACUTE TOXICITY STUDIES, *E. COLI* INFECTION AND EGG ALBUMIN-INDUCED INFLAMMATION

3.6.1 Acute Toxicity

Thirty normal Wistar albino mice weighing approximately 20 grams each were obtained from the animal house at College of Medicine, Idi-Araba for acute toxicity test. Based on regulatory guidelines, five mice per group were used for sound scientific judgment as well as practical and economical factors (Toxicology Tutor 2005). They were fasted prior to administration of oral dose to prevent contamination with other substances in the gastrointestinal tract, which may complicate absorption of the test substance. It was noted that mice has a higher metabolic rate, thus with holding feed for 3-4 hr was considered adequate for this research work (Kent and Gallo 1989). The animals were grouped into six; five cages containing five albino mice were administered with five dosage ranges 500 mg/Kg to 1500 mg/Kg body weight while the sixth cage containing five mice which served as the control group was administere with water. Dosage was based on the body weight of the mice (expressed as weight of the test substance – *P. chrysogenum* extracts per kilogram body

weight of animal). It was administered to the different groups of mice by gavages with a feeding needle cannular.

3.6.2 Sub-acute toxicity studies to evaluate *in vivo*, the effect of the extract on some biochemical analytes under normal condition

Fifteen Wistar albino rats weighing approximately 200 grams were used for the sub-acute toxicity study carried out over a period of 7days. They were divided into three groups of five rats each:

Group 1 rats were administered intramuscularly with distilled water and served as the control while group 2 rats were administered intramuscularly with Benzyl Penicillin as Reference drug (2500mg/kg body weight). Rats i the third group were administered intramuscularly with extracts of *P. chrysogenum* from agro waste (2500mg/kg body weight).

In order to evaluate the biochemical changes occurring in the plasma following the administration of the extract, blood samples were collected after 7 days and certain chemical analytes were quantified which include liver function tests (AST, ALT, Alkaline phosphatase, bilirubin – total and direct, total protein and albumin), haematological indices (PCV, WBC count, WBC Differentials and platelet count), renal function test (urea, creatinine, electrolyte balance - Na^+, K^+, Cl^-), enzymes of oxidative stress (glutathione transferase, catalase, superoxide dismutase) and oxidation product (MDA). The animals were sacrificed after 7 days and vital organs aseptically removed and sent for histology studies.

3.6.3 *In vivo*, study to determine, the effect of extract following *E.coli* infection and egg albumin induced inflammation

A total of thirty-five Wistar albino rats weighing approximately 200 grams were used for this study. The rats were separated into seven groups (of five rats each). *E. coli* infection was done by injecting 0.5ml cell suspension of overnight broth culture into the sub cutaneous layer of the

right thigh of the rats. Acute inflammation was induced by injecting 0.5 ml of fresh egg albumin into the right thigh of the rats (Uche and Aprioku 2008). These animals were observed after 6 – 12hrs.

Group A: Infected with *E. coli*.

B: Egg albumin-induced inflammation

C: Infected with *E. coli*

D: Egg albumin-induced inflammation

E: Negative control (*E. coli* infected).

F: Negative control (Egg albumin-induced inflammation)

G: Positive control.

Groups A and B were administered with Extracts of *P.chrysogenum* from agro waste (1500mg/kg body weight in divided doses of 750mg/kg body weight twice daily) for 7 days while groups C & D: were administered with commercial Benzyl penicillin (1500mg/kg body weight in divided doses of 750mg/kg body weight twice daily) for 7 days. Groups E & F were given no treatment (Negative control). Group G is the positive control – they are normal rats that were neither infected nor treated.

Clinical signs were then observed at specific intervals shortly after dosing, frequently over the next 4 hr, and at least once daily thereafter. The cage side observation included any changes in the size, skin, fur, eyes, mucus membranes, behaviour, etc. (Kent and Gallo, 1989).

Blood samples were taken 24hrs before infecting the rats, this served as the control. For *in vivo* biochemical analyses of infected albino rats, blood was collected 24 hrs after bacterial infection with *E.coli* and injection of egg albumin to induce inflammation. Extract and reference drug were administered for 7 days. Blood was subsequently collected 4 days, 8 days

and 14 days after commencement of administration of extract and reference drug to respective groups. Haematological and biochemical analyses as stated in sub-acute toxicity study were also carried out.

3.7 COLLECTION OF BLOOD AND SAMPLE ANALYSIS

The blood of the mice and rats were collected from the corner of the eyes by capillary action into EDTA bottles for haematological analysis, lithium heparinised bottles for plasma and plain tubes for the serum were separated after centrifugation at 4000g for biochemical analyses. Plasma for thrombin assay was collected into bottles containing citrate (9ml of blood to 1ml of citrate) as anticoagulant.

3.7.1 Haematology

3.7.1.1 Haematocrit or Packed Cell Volume (PCV)

Heparinised capillary tubes were used to collect the sample from the bottle. It was immediately sealed at one end with plasticine. The capillary tubes were placed in the radial grooves of the micro-haematocrit centrifuge and centrifuged for 5 minutes. The percentages of the red blood cells were determined using haematocrit reader. The PCV became established as a reliable indication of anaemia. It is also the volume of erythrocytes expressed as a fraction of volume of whole blood in a sample.

3.7.1.2 Total White Blood Cell (WBC) Counts

Whole blood was diluted 1 in 20 with WBC diluting fluid (an acid reagent) which haemolyses the Red Blood cells, leaving the WBCs to be counted. The leucocytes (white cells) were then counted in a counting chamber under the microscope, and the number of cells in one litre of blood was counted.

WBC diluting fluid (0.38 ml) was measured and dispensed into well labelled test tubes using automatic micropipettes. 20 µl of anticoagulated whole blood was added into the tubes and mixed properly. The Neubauer counting chamber was assembled by moistening the chamber surface on each side of the grid areas and sliding a cover glass into position. Each side was pressed down until rainbow colours were seen. The diluted blood sample was re-mixed by inversion to ensure homogeneity. The micropipette was used to aspirate the diluted blood sample to fill one of the grids of the chamber, taking care not to over fill the area. The chamber was left for 2 minutes for the WBCs to settle.

The microscope objective (10x) was used to focus the rulings of the chamber and WBCs, with the condenser sufficiently closed to give a good contrast. The cells appeared as small black dots. Cells in the four large corner squares (total areas of 4 mm^2) were counted, including the cells lying on the lines of two sides of each large squares. The number of WBCs counted was reported as cells per micro litre of blood.

3.7.1.3 Differential white blood cell count

The anticoagulated whole blood sample was used to make a thin blood smear on a clean grease free labelled microscope slides. This was allowed to air-dry and quickly fixed using absolute methanol and stain with leishman stain. A thin blood film was prepared and fixed in methanol for 2 – 3 minutes. A 1:3 dilution of the leishman stain was prepared using one part of stain to two parts of water. The slides were covered with the diluted stain for 10 minutes. The stain was washed off in a stream of buffered water without tapping the stain off as this would have left a deposit of stain on the film. Differentiation was done by placing the slide in clean water for 3 minutes. The water was tipped off and the slides were left on the draining rack to dry.

3.7.1.4 Platelet count

Platelet count was done by the use of the new improved Neubauer Haemocytometer. Twenty microlitre of blood was taken from each experimental animal and put in the tube with 0.38 mL of diluting fluid (1% ammonium oxalate in distilled water). The contents were then mixed. With the use of a clean Pasteur pipette, 0.02 ml of the diluted blood was filled into the counting chamber of the Haemocytometer. The chamber was then kept humid by placing it in a petridish spread in wet cotton wool for 10 – 20 minutes to allow the platelets to settle for easy counting.

3.7.2 Liver Function Tests

These tests are used to analyze the serum for specific enzymes (AST, ALT and ALP) which are indicative of the functional state (metabolic state) of the liver.

3.7.2.1 Aspartate aminotransferase

Aspartate aminotransferase was measured by monitoring the conversion of oxaloacetate hydrazone formed with 2, 4-Dinitrophenylhydrazine (DNPH). 500 µl solutions (each) of phosphate buffer, L-Aspartate, α-oxoglutarate and DNPH were mixed with the serum sample and the absorbance read against a reagent blank after 5 mins at 546 nm in a spectrophotometer.

3.7.2.2 Alanine aminotransferase

Alanine aminotransferase was measured by monitoring the concentration of glutamic-pyruvic transaminase catalyzes reaction product, pyruvate hydrazone formed with 2, 4-Dinitrophenylhydrazine (DNPH). 500µl bromocresolgreen (BCG) reagent and the serum sample were mixed and incubated for 5 mins at 20-25 ^{O}C and the absorbance read against a reagent blank at 546 nm.

3.7.2.3 Total and Direct (Conjugated) Bilirubin

Bilirubin reacts with diazotised sulphanilic acid (DSA) to form a red azo dye. The absorbance of this dye at 546 nm is directly proportional to the bilirubin concentration in the sample. Water-soluble bilirubin glucoronides react directly with DSA whereas the albumin conjugated indirect bilirubin will only react with DSA in the presence of an accelerator: Total bilirubin = direct + indirect bilirubin.

Total bilirubin reagent (TBR) - Sulphanilic acid 14mmol/L, Hydrochloric acid 300mmol/L, Caffeine (accelerator) 200mmol/L, Sodium benzoate 420mmol/L.

T-Nitrite reagent (TNR):- Sodium nitrite 390mmol/L.

Direct bilirubin (DBR): - Sulphanilic acid 14mmol/L, Hydrochloric acid 300mmol/L, T-Nitrite reagent (DNR):- Sodium nitrite 390mmol/L.

Total Bilirubin: 1000µl of TBR were added into well labelled test tubes for samples and sample blank. 40µl of TNR was added, mixed and incubated for 5minutes at room temperature. 100µl of plasma was added to both the sample tubes and the blank. This was mixed and incubated for 30minutes at room temperature. The absorbance of the sample and blank was measured at 546nm.

Direct bilirubin: 1000µl of DBR were added into well labelled test tubes for samples and sample blank. 40µl of DNR was added, mixed thoroughly and 100µl of plasma sample was added to both the sample tubes and the blank within 2minutes. This was mixed and incubated for exactly 5minutes at room temperature. The absorbance of the sample and blank was measured at 546 nm.

3.7.2.4 Alkaline Phosphatase

Alkaline Phosphatase acts upon the AMP-buffered sodium thymolphthalein monophosphate (substrate). The addition of alkaline reagent stops the enzyme activity developing a blue

chromogen, whose intensity is proportional to the concentration of the alkaline phosphatase present.

Alkaline phosphatase (0.5mL) substrate was pipette into labelled test tubes and equilibrated to $37^{O}C$ for 3minutes. At time intervals, 0.05mLs of each standard, control and test samples (plasma) were added to the respective test tubes and they were mixed gently. Distilled water was used as sample for reagent blank. This was gently mixed and incubated for exactly 10 minutes at $37^{O}C$. 2.5mL alkaline phosphate colour developer was added at time interval and mixed. The absorbance of the sample and blank was measured at 590nm on a spectrophotometer against a reagent blank.

3.7.2.5 Determination of Total Protein

Cupric ions react with protein in alkaline solution to form a purple complex. The absorbance of this complex is proportional to the protein concentration in the sample.

1000µl of the reagent mixture (Sodium hydroxide 200mmol, Potassium sodium tartrate 32 mmol, Copper sulphate 12mmol, and Potassium iodide 30mmol per Litre of distilled water) was added, to 20µl plasma and standard, mixed and incubated for 10minutes at $20 – 25^{O}C$. The absorbance of the sample and standard was measured at 580nm against the reagent blank within 30minutes (Lowry *et al*., 1951).

3.7.2.6 Determination of Albumin

At a certain pH value, albumin is specifically combined with bromocresol green, to produce a coloured complex, which is photometrically measured (Doumas *et al.,* 1971). 10µl of plasma and standard were added in well labelled test tubes. 2.5ml of the reagent mixture (0.75g/L Bromocresol green in 50mM Succinate buffer pH 4.2) was added. This was well mixed and allowed to stand for 5minutes at room temperature and read at 630nm against a reagent blank.

3.7.3 Renal Function Test

3.7.3.1 Urea Determination

Urea is hydrolysed in the presence of water and urease to produce Ammonia and carbon dioxide. In a modified Berthelot reaction the ammonium ions react with hypochlorite and salicylate to form a green dye. The absorbance increase at 578 nm is proportional to the urea concentration in the sample (Tobacco, A. *et al*., 1979). Reagent 1 made up of 120mmol/L Phosphate buffer (pH 7.0), 60mmol/L Sodium salicylate, 5mmol/L Sodium nitruprusside and 1 mmol/L EDTA was constituted before the test. 10 µl of plasma and standard were added in well labelled test tubes. 1000µl of reagent 1a (1ml of Urease enzyme to 100ml of reagent 1 was added). This was well mixed and incubated for 3minutes at 37^{O}C. 1000µl of reagent 2 (Phosphate buffer(pH < 13) 120mmol/L, Hypochlorite 0.6g/l Cl. Urease enzyme, urea standard, sodium azide 0.095%) was added, mixed and incubated at 37^{O}C. Absorbance was measured within 60minutes at 578nm against a reagent blank.

3.7.3.2 Creatinine Determination

Creatinine in an alkaline solution forms an orange-red coloured complex with picric acid. The absorbance of this complex is proportional to the creatinine concentration in the sample. 1000µl of plasma, creatinine standard and distilled water were added in well labelled test tubes. 1000µl of 10% Trichloroacetic acid was added to deproteinize the plasma. This was carefully mixed centrifuged at 4000g for 5 – 10minutes. 1000µl of the deproteinized serum supernatant, creatine standard and water was carefully collected into clean labelled respective test tubes and 1000µl Reagent 1 (equal volume of Picric acid 26mmol/l and Sodium hydroxide 1.6mmol/l) was added. This was well mixed and incubated for 20minutes at 25^{O}C. Absorbance was measured at 546nm against a reagent blank.

3.7.4 Electrolytes

3.7.4.1 Sodium (Na^+)/Potassium (K^+)

A 1:5 dilution of the sample (plasma) was made with distilled water and introduced into the flame photometer. The electrical signals of the photo detector was amplified and displayed on the digital readout. The absorbance of sample is directly proportional to the concentration of electrolyte present; this was multiplied by the dilution factor to get the actual concentration of the test sample.

3.7.4.2 Chloride (Cl^-)

20µl of plasma, standard and distilled water were added in well labelled test tubes. 2000µl of test reagent was added to all the tubes, mixed and incubated for 5minutes at 15-25^{O}C. Absorbance was measured at 450nm against a cyanate reagent blank within one hour.

3.7.5 Enzymes of oxidative stress and inflammation

3.7.5.1 Catalase Enzyme Activity

Serum catalase activity was determined by measuring the decrease in absorbance at 240nm in a UV recording spectrophotometer by monitoring the decomposition of H_2O_2 for 1, 2, and 3 minutes as described by Usoh *et al*., (2005). The reaction mixture (3 ml) contained 0.1 ml of suitably diluted serum in phosphate buffer (50 mM, pH 7.0) and 2.9ml of 30mM H_2O_2 in phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240nm of 40.0 M-1 cm-1 was used for the calculation. The specific activity of catalase has been expressed as moles of H_2O_2 reduced per minute per mg protein.

3.7.5.2 Superoxide Dismutase (SOD) Enzyme Activity

Superoxide dismutase activity was assayed by its ability to inhibit the auto oxidation of adrenaline, and determined by the increase in absorbance at 480nm. Whole blood superoxide

dismutase was assayed utilizing the technique of Fridovich as described by Usoh *et al*., (2005). 1ml of whole blood was diluted with 9ml of distilled water to make a one in ten dilution of the whole blood. An aliquot of 2.0ml of the diluted blood was added to 2. 5ml of 0.05M carbonate buffer (pH 10.2) and left to equilibrate in the spectrophotometer. The reaction was started by the addition of 0.3ml freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480 nm was monitored for 1, 2, and 3minutes. A single unit of enzyme is defined as the quantity of SOD required to produce 50% inhibition of autoxidation (Usoh *et al*., 2005).

3.7.5.3 Determination of Malondialdehyde by Thiobabituric acid reactions (TBARS)

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of lipid peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red species absorbing at 535nm. 1.0ml of the sample was added to 2.0ml of TCA-TBA-HCL (15 % $^{w}/_{v}$ Trichloroacetic acid, 0.375 % $^{w}/_{v}$ Thiobarbituric acid and 0.25 N Hydrochloric acid) reagent and mixed thoroughly. The solution was heated for 15mins in a boiling water bath and allowed to cool. The flocculent precipitate formed was removed by centrifugation at 1000g for 10minutes. The absorbance of the sample was measured at 535nm against a blank containing all the reagents minus the lipid. Malondialdehyde concentration of the sample was calculated using an extinction coefficient of $1.56x10^{5}M^{-1}Cm^{-1}$ (Usoh *et al*., 2005)

3.7.5.4 Determination of reduced glutathione (GSH)

Glutathione peroxidise (GSH- reduced) was measured in a coupled enzyme system by measuring the decrease of DTNB (5,5-Dithio-bis 2-Nitrobenzoic acid). 0.1ml of sample was added into a test tube containing 1ml of 0.2M Tris EDTA buffer and 0.9ml of 20mM EDTA.

0.1 ml of 10nM DTNB was added and incubated for 30 mins at room temperature. The mixture was centrifuged and absorbance of the supernatant read against distilled water blank at 412nm. The specific activity of GST is expressed as μmol of GSH- DTNB conjugate formed/min/mg protein using an extinction coefficient of $13,600M^{-1}Cm^{-1}$ (Usoh *et al*., (2005).

3.7.6 Determination of Thrombin Time

The Thrombin Time is a simple test to screen for conditions that can interfere with conversion of fibrinogen to fibrin. A low potency thrombin is added to undiluted plasma and clot formation is timed. Blood samples of normal laboratory staff who volunteered for study were used. Blood samples with PCV of 36 – 40 %, Hb of 12 – 14 g/L and platelet count of above 150 – 400 x 10^9 were considered fit as these are established normal blood cell values. Blood was obtained by clean venipuncture using a sterile needle and syringe and collected into citrated bottles. An exact ratio of 9volumes of blood to 1volume of anticoagulant (32g/L citrate) was maintained. The anticoagulated specimen was then centrifuged at 3000*g* for 20 min. Plasma samples were then aliquoted and kept frozen at -6 to -8°C. Ten samples were collected in the same manner from healthy donors for reference interval studies. 0.2ml of citrated plasma was pipetted into pre-warmed test tubes at 37^OC, and incubated for 3 minutes. 0.1ml of the reconstituted Bovine thrombin reagent was added while a stop clock was simultaneously started. The timer was stopped at the appearance of a fine mesh of insoluble fibrin polymers that are recognized as the end point in thrombin clotting assays. The mean time of triplicate Thrombin Time determinations for each plasma sample were calculated and reported to the nearest 0.1seconds (Flanders *et al.,* 2003).

Effect of increasing "HemoStat thrombin" on the initial rate of clot formation on citrated plasma at room temperature was monitored at 400nm at intervals of 3 seconds for 15 seconds. A serial dilution of neat plasma was made by adding 0.2, 0.4, 0.6, 0.8 and 1ml plasma into

different text tubes and making up the volume to 1 ml with distilled water. 0.5ml of HemoStat thrombin was added to 1ml of the diluted plasma at room temperature. The absorbance was monitored every 3seconds for 15seconds at 400nm on a spectrophotometer against distilled water blank.

A modified method of Flanders *et al*., (2003) was adopted in the kinetic study where varying concentrations of the culture extract (1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 mg) as well as the reference standard were incubated with the test samples for 3 minutes, the Bovine thrombin reagent added and monitored visually for clot formation. The mixture after 5minutes was mixed thoroughly on a mixer and the absorbance read on a spectrophotometer at 400nm against individual blank. Normal individuals will generally exhibit a Thrombin Time of 14 - 16 seconds.

3.7.7 Whole Blood Clotting Time

In other to study patients thought to be suffering from blood clotting defects, various tests may be carried out and this include whole blood clotting time. Blood samples were aseptically collected into four plain test tubes with an internal diameter of 8 mm placed in $37^{O}C$ water bath and the stop clock simultaneously started. This was examined at intervals of 30seconds for clotting by gently tilting the tubes. The timer was stopped at the appearance of a clot. The clotting time was reported as the average of the times given by the four tubes. This method was modified and adopted in the kinetic study where varying concentrations of the culture extract (1, 2, 3, 4, 5, 10, 20, 30, 40 and 50mg) as well as the reference standard were placed in the test tubes in a $37^{O}C$ water bath and the whole blood added and observed for clotting.

3.8 HISTOLOGY

Three animals each from the sub-acute toxicity study and the positive control group were sacrificed using chloroform anaesthesia. The organs (liver and kidney) excised from the

animals were immersed in 10% phosphate buffered formaldehyde and left for 24 hours for fixation of the organs after which histology analysis was carried out.

3.9 STERILIZATION

Sterilization was very crucial to avoid contamination by microorganisms in the environment. Glass wares were washed thoroughly with detergents and sterilized by placing in an oven at 170^{o}C for a minimum of 60 minutes. Cotton wool and other dry materials were also sterilized by heating in the oven for at least an hour. Media in glass containers were sterilized by autoclaving at 121^{o}C for 15 minutes. Inoculation loop, cork borer and glass spreader used for transferring micro organisms were sterilized by flaming using Bunsen burner or spirit lamps.

3.10 STATISTICAL ANALYSIS

Results of this study were reported as means ± SEM (Standard error of mean) from 3 repeated determinations (n=5). The data were analyzed with Student's independent‘t’ test and one-way analysis of variance (ANOVA) test. All statistical analysis were performed with the program Statistical Package for the Social Science (SPSS 8.0 windows, version 13) and p value of < 0.05 was accepted as statistically significant.

CHAPTER FOUR

RESULTS

4.1 ISOLATION OF FUNGUS

Colonies of different types of fungi appeared on the Potato dextrose agar (PDA) and Czapek-Dox agar (CDA) media inoculated with stock culture of decomposing wood-waste. After series of sub culturing, three filamentous micro fungi were isolated from the stock of decomposing wood-waste on the PDA and CDA media. The identified fungi include: *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum*.

The number of colonies and size increased as incubation period increased. Growth of fungi was visible after 48 hours of incubation. By the 5^{th} day, growth of the organism has covered the entire surface of the Petri dish. Plate 4.1 is a 7-day pure culture of *P. chrysogenum* obtained after series of sub culturing and incubation. Table 4.1 is a Summary of the colonial and cultural characteristics, and microscopic features of the three identified fungal isolates - *Aspergillus flavus*, *Aspergillus niger* and *Penicillium chrysogenum*.

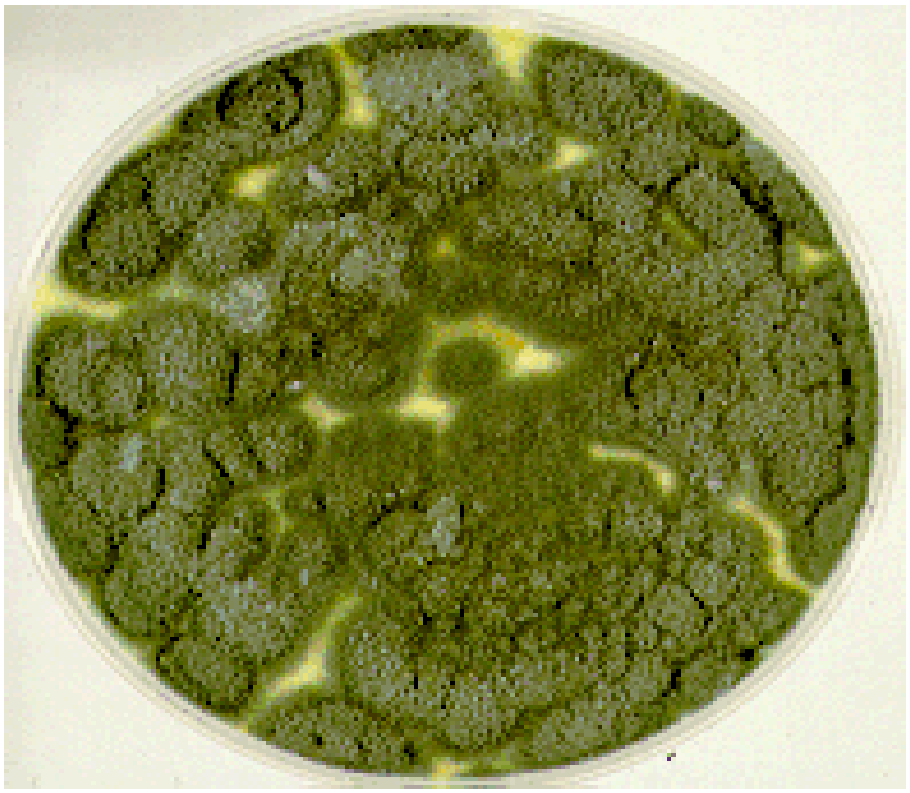


Plate 4.1: A 7-day old pure culture isolate of *P. chrysogenum* isolated on potato dextrose agar

4.2 SUMMARY OF THE COLONIAL AND CULTURAL CHARACTERISTICS AND MICROSCOPIC FEATURES OF THE THREE IDENTIFIED FUNGAL ISOLATES

Morphological features: Hyphae are septate and hyaline. Conidiophores are non-septate. Conidial heads are radial to loosely columnar.

Colonial characteristics: Yellow or olive green to lemon-green colour; dusty or flaky colonies

Fungal isolate: *Aspergillus flavus*

Morphological features: Hyphae are septate and hyaline. Cornidiophores are non-septate. The conidial heads are mop-like.

Colonial characteristics: Initially white, spores turn to thick black colour under 24 hours; dusty colonies

Fungal isolate: *Aspergillus niger*

Morphological features: Hyphae are septate and hyaline. Cornidiophores are branched. Cornidial heads are brush-like.

Colonial characteristics: White to blue-green (ash) colour; flaky colonies.

Fungal isolate: *Penicillium chrysogenum*

4.3 GROWTH MEASUREMENT OF *P. CHRYSOGENUM*

4.3.1 Turbidity Measurement

Turbidity of culture media monitored against a cell-free blank for 7 days at OD 530nm showed remarkable increase in the turbidity of the different agro-wastes media. A two day lag phase was observed in media containing sawdust and corncob (Figure 4.1A). Culture media enriched with CAS, SC, CC and SD supported the growth of *P. chrysogenum* as shown by remarkable changes in daily turbidity measurement (optical density) and when compared to a synthetic media made up of GLU, and LAC. Highest growth of the organism was obtained on the 2^{nd}, 4^{th} and 6^{th} day in media containing SC, GLU, CAS, while in media containing CC and SD, highest growth was obtained on the 3^{rd} day with additional peak on the 6^{th} day for CC. For LAC containing media, highest change in growth as compared to the agro-wastes media was recorded on the 2^{nd} day (Figure 4.1B).

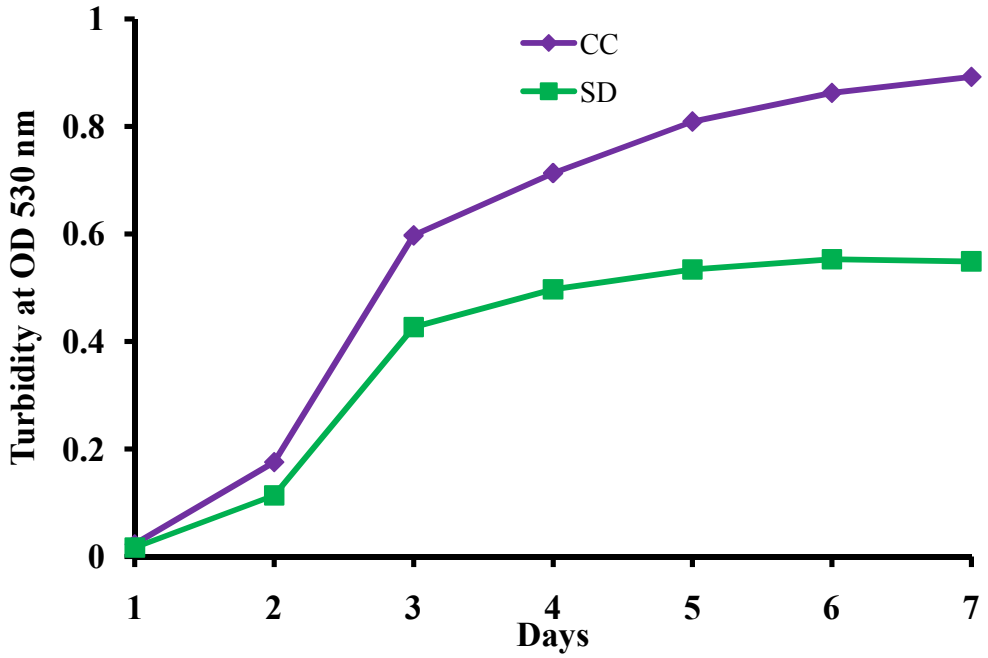


Figure 4.1A: Turbidity measurements of growth of *P. chrysogenum* showing lag phase after two days of growth on media enriched with SD (sawdust) and CC (corncob) for 7 days

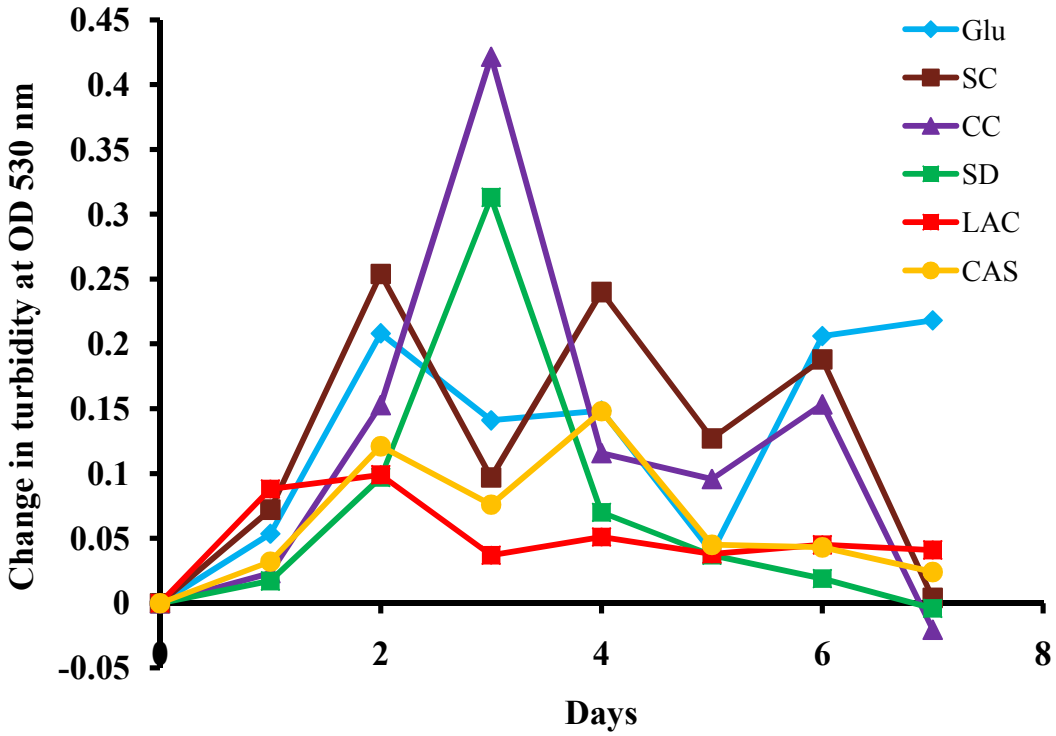


Figure 4.1B: Daily changes in turbidity measurements of growth of *P. chrysogenum* cultivated on various agro waste substituted media for 7 days. Mineral Salt Broth enriched with SD (sawdust), SC (sugarcane), CC (corncob), CAS (cassava peels), GLU (glucose) and LAC (Lactose).

4.3.2 Protein Yield

The protein yield of *P. chrysogenum* cultivated on the different substrates is shown in Figure 4.2. Changes in extracellular protein secreted into the different media (every 3 days for 21 days) shows that culture media containing cassava peels gave the highest protein peak of 0.38mg/ml on the 6th day, while corncob gave an early peak of 0.30mg/ml on the 3rd day. Sawdust gave two protein peaks, 0.15mg/ml on the 3rd day and 0.25mg/ml on the 12th day. A total protein yield of 0.2, 0.08, 0.06mg/ml respectively was obtained with glucose, sugarcane pulp and lactose containing media on the 3rd day. The downward slope obtained after the 15th day shows that the organism has stopped growing and are beginning to breakdown proteins for normal metabolism in the face of growth stoppage.

4.3.3 Mycelia Weight Measurement

Figure 4.3 shows the change in mycelia weight (every 3 days for 21 days) of *P. chrysogenum* cultivated in media containing the different substrates. There was a general increase in mycelia weight as incubation progressed until it attained a maximum value after which it began to decline. The period for highest mycelia weight varied with the different substrates. Best growth was obtained with CC and CAS. CC yielded a maximum growth of 0.15 and 0.92mg on the 3rd and 9th day respectively while CAS yielded a maximum mycelia weight of 0.13 and 0.12mg on the 3rd and 12th day respectively. Mycelia weight of the organism, in media containing GLU, SC and LAC was 0.12, 0.068 and 0.055mg respectively, was highest on the 9th day. However, sawdust gave the least growth with a maximum mycelia growth of 0.07mg on the 3rd day. The decline in values obtained after the 15th day shows a slow down in metabolic activities in the organism hence no increase in production of biomass.

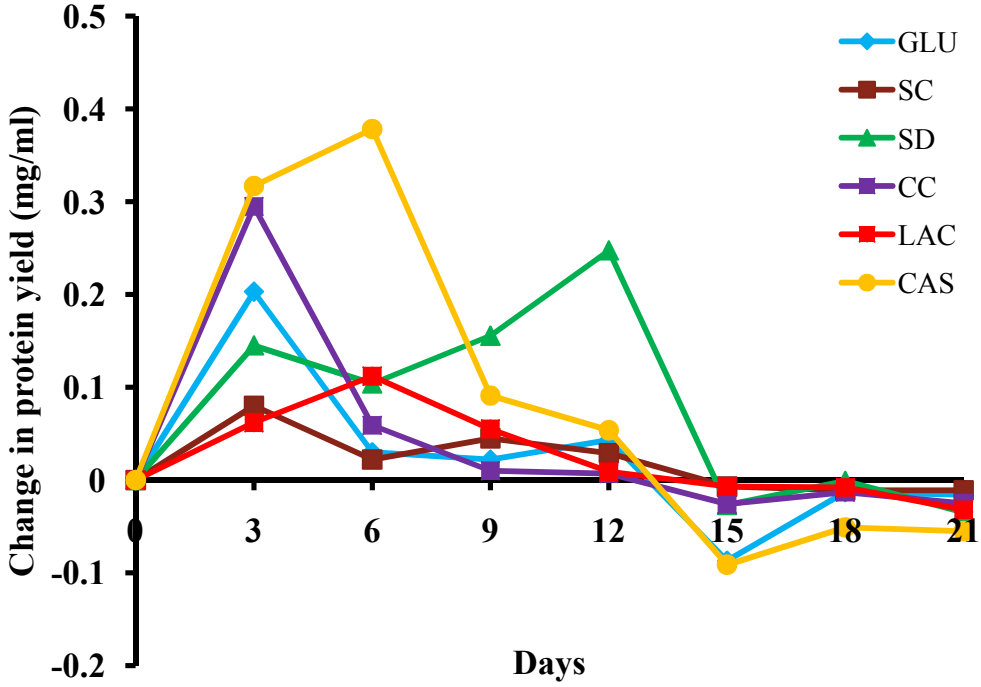


Figure 4.2: Changes in protein yield of *P. chrysogenum* cultivated on various agro waste substituted media for 21 days. Mineral Salt Broth enriched with SD (sawdust), SC (sugarcane), CC (corncob), CAS (cassava peels), GLU (glucose) and LAC (Lactose).

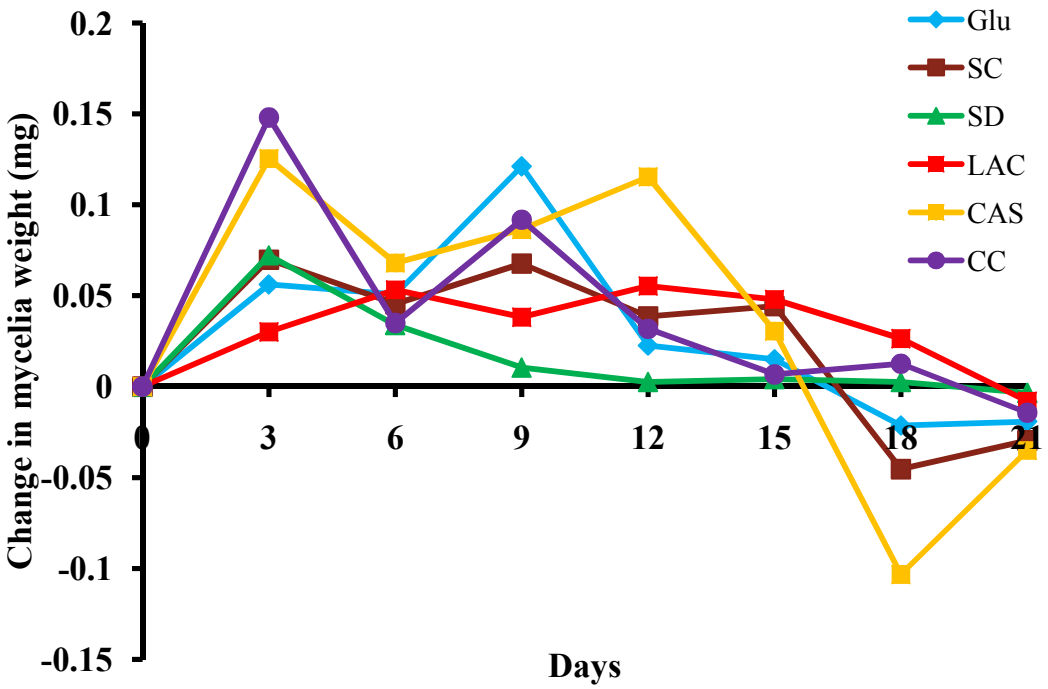


Figure 4.3: Changes in mycelia weight of *P. chrysogenum* cultivated on various agro waste substituted media for 21 days. Mineral Salt Broth enriched with SD (sawdust), SC (sugarcane), CC (corncob), CAS (cassava peels), GLU (glucose) and LAC (Lactose).

4.3.4 Carbohydrate utilization

To establish that *P. chrysogenum* utilizes carbohydrates present in agro waste for growth, quantitative determinations of carbohydrate present in a cell-free media used as blank was carried out. Figure 4.4. showed that cell-free media containing cassava peels gave the highest total sugar content of 4.25mg/ml before the incubation whereas that of corncob gave the least value of 2.98mg/ml. Cell-free media containing glucose, lactose and sugarcane pulp contained about the same amount of total sugar (3.82mg/ml) while that of sawdust contained 3.58mg/ml. Sawdust showed the most rapid decline, coming down to 0.045mg/ml on day 12 and 15; there was no detectable sugar in the media afterwards. At the end of 21-day incubation period, the residual sugar content was 1.98, 0.71, 0.62, 0.59 and 0.56mg/ml respectively for CAS, GLU, SC, LAC and CC.

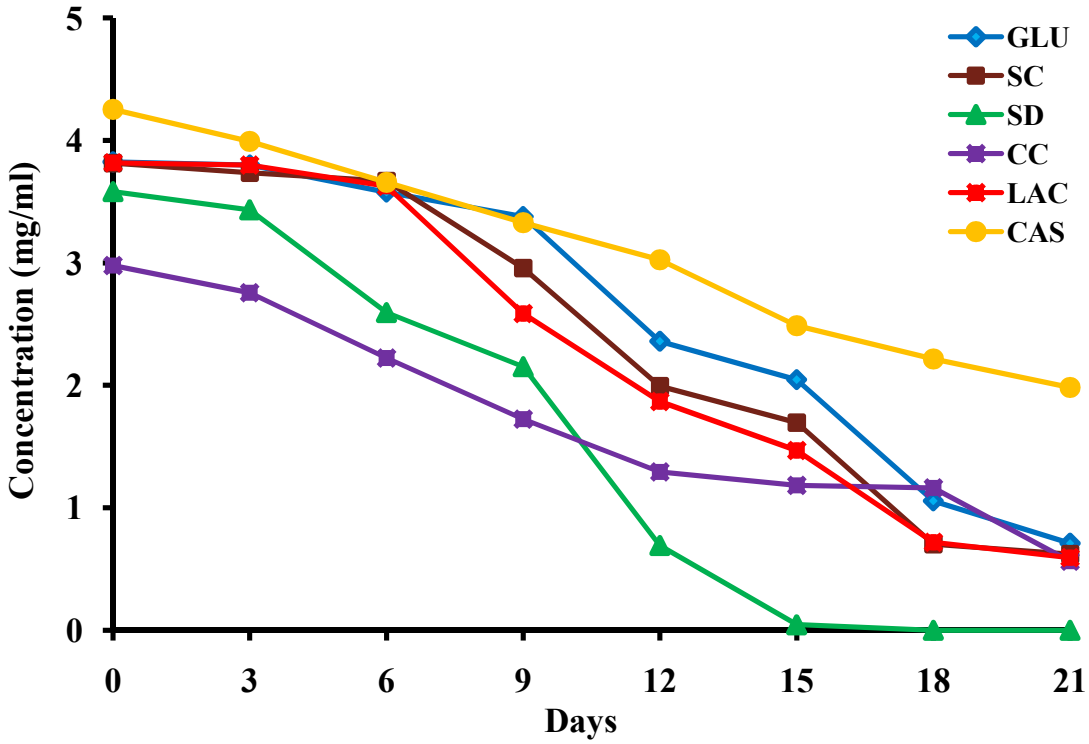


Figure 4.4: Carbohydrate utilization of *P. chrysogenum* cultivated on various agro-wastes substituted media for 21 days. SD (saw-dust), SC (sugar cane), CC (corn- cob), CAS (cassava peels), GLU (glucose) and LAC (Lactose).

4.4 OPTIMUM CONDITIONS FOR GROWTH OF *P. CHRYSOGENUM* AND ANTIBIOTIC PRODUCTION

4.4.1 Optimum pH of growth by mycelia weight measurement

The average mycelia weight of the organisms was measured as an indication of growth and was used to monitor the optimum pH of growth of the organism. The organism was cultivated in media of varying pH and growth was monitored by increase in the weight of mycelia produced. Figure 4.5 shows the increase in mycelia weight at various pH. Maximum weight of mycelia was measured at pH 6.5 and is therefore, the Optimum pH of growth of the organism.

4.4.2 Optimum pH of growth by turbidity measurement

P. chrysogenum grown on agro-waste substrate showed optimum growth at pH 6.5. Figure 4.6 shows the measurement of turbidity as an indication of growth of the organism. The change in turbidity of the medium was monitored for 7 days. Turbidity increased from pH of 3.5 and maximum values were recorded at pH 6.5 above which the values gradually decreased. There was no recorded change in turbidity at pH lower than 3.5.

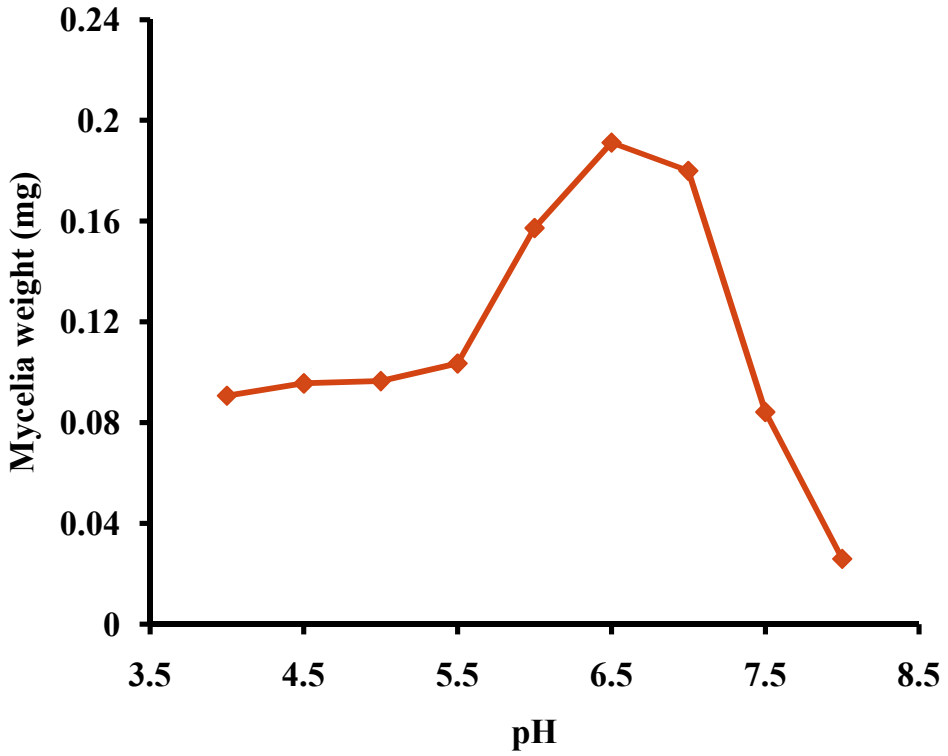


Figure 4.5: Optimum pH of growth of *P. chrysogenum* by mycelia weight measurement

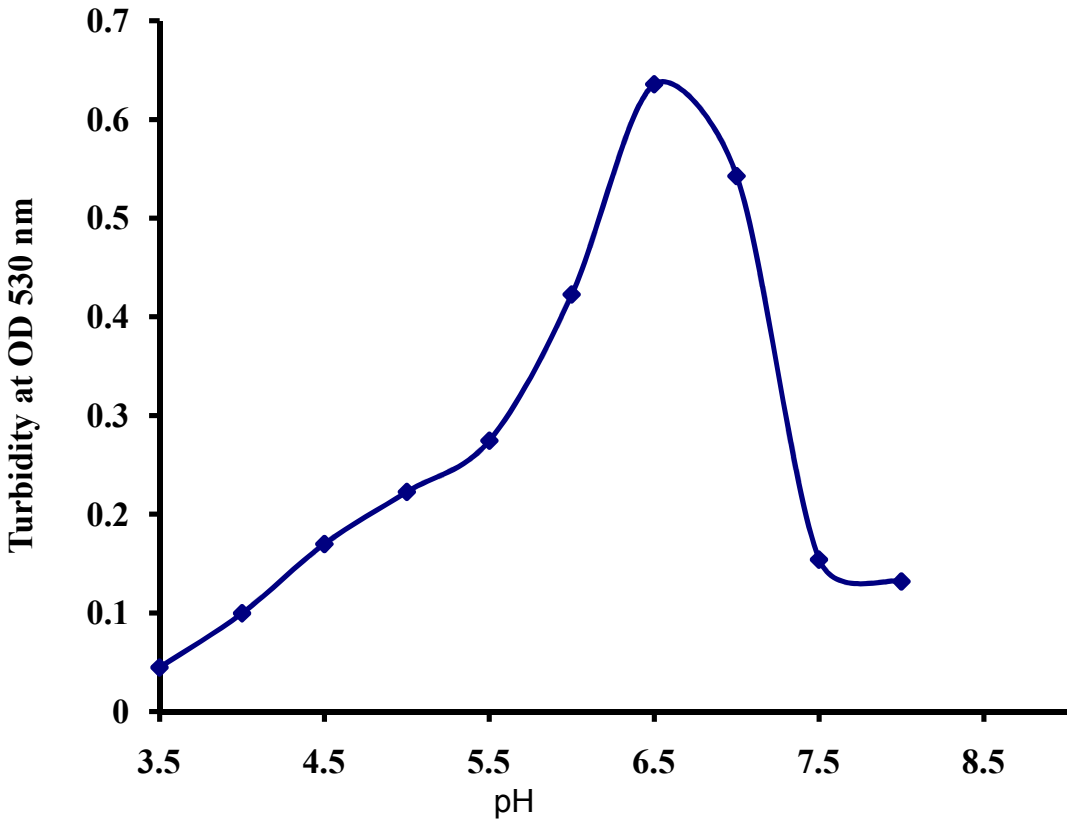


Figure 4.6: Optimum pH of growth of *P. chrysogenum* by turbidity measurement

4.4.3 Optimum temperature of growth by mycelia weight measurement

A change in mycelia weight and turbidity of the culture media was used to establish the optimum temperature of growth of the organism. The mycelia weight was highest at 25^0C (Figure 4.7).

4.4.4 Optimum temperature of growth by turbidity measurement

Figure 4.8 shows the optimum temperature for the turbidity of the medium. Turbidity reduces as the temperature increases. Therefore the observed optimum temperature for growth of *P. chrysogenum* was 25^0C.

4.4.5 Optimum pH of production of extract with antibacterial activity

The growth of *P. chrysogenum* is directly proportional to the amount of penicillin produced. Optimum production of extract of *P. chrysogenum* with antibacterial activity was recorded after 7 days of fermentation at pH 6.5 (Figure 4.9).

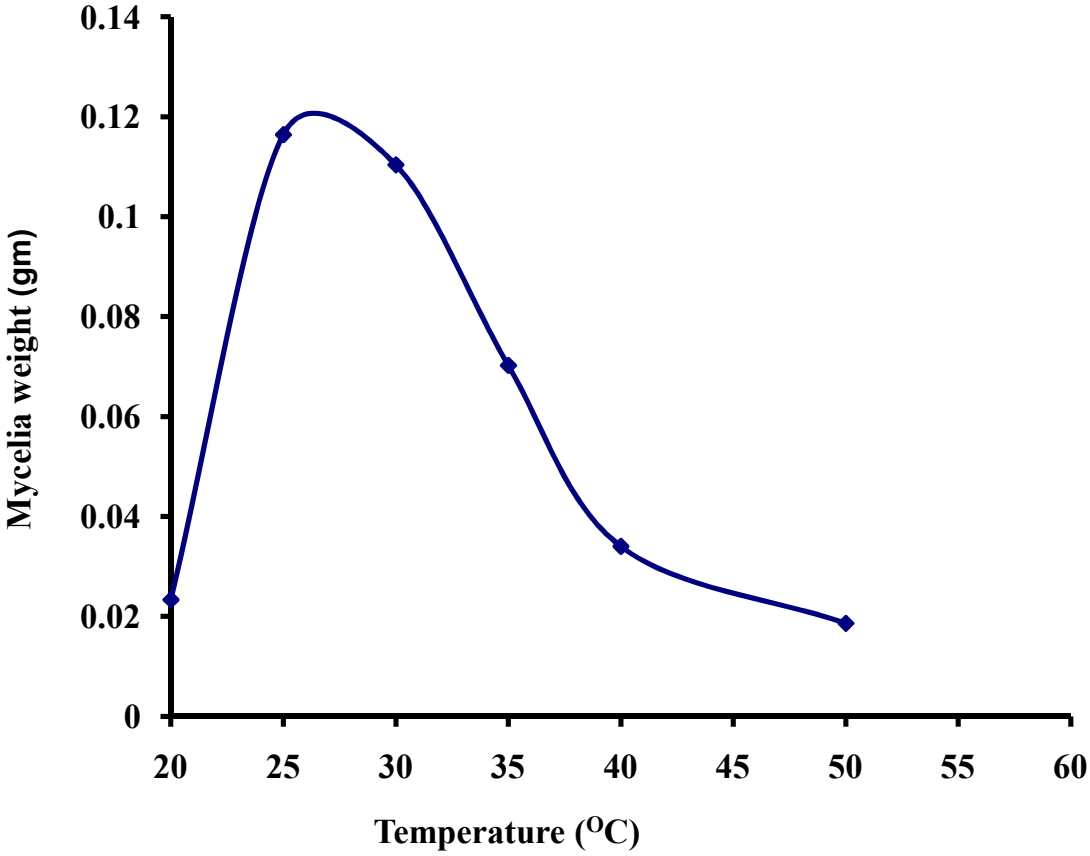


Figure 4.7: Optimum temperature of growth of *P. chrysogenum* by mycelia weight measurement

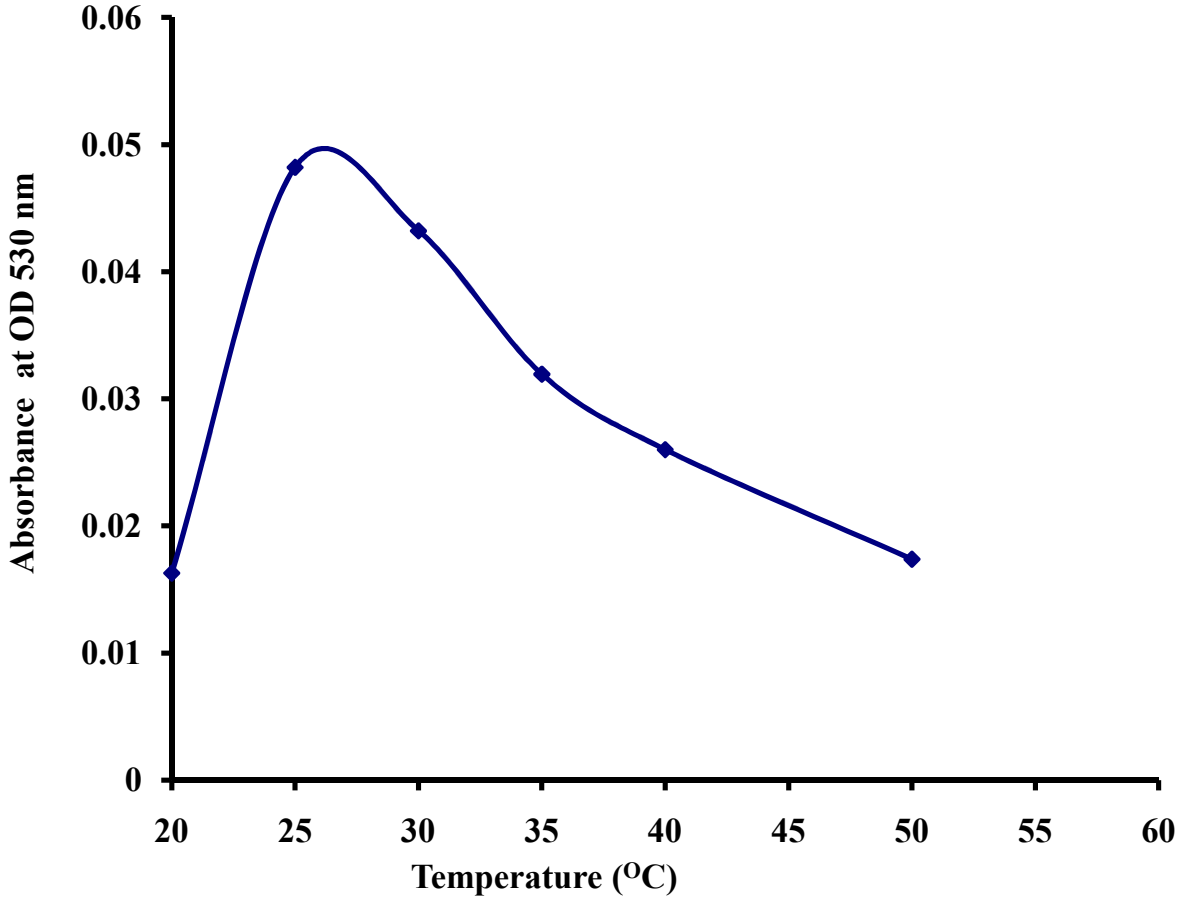


Figure 4.8: Optimum temperature of growth of *P. chrysogenum* by turbidity measurement

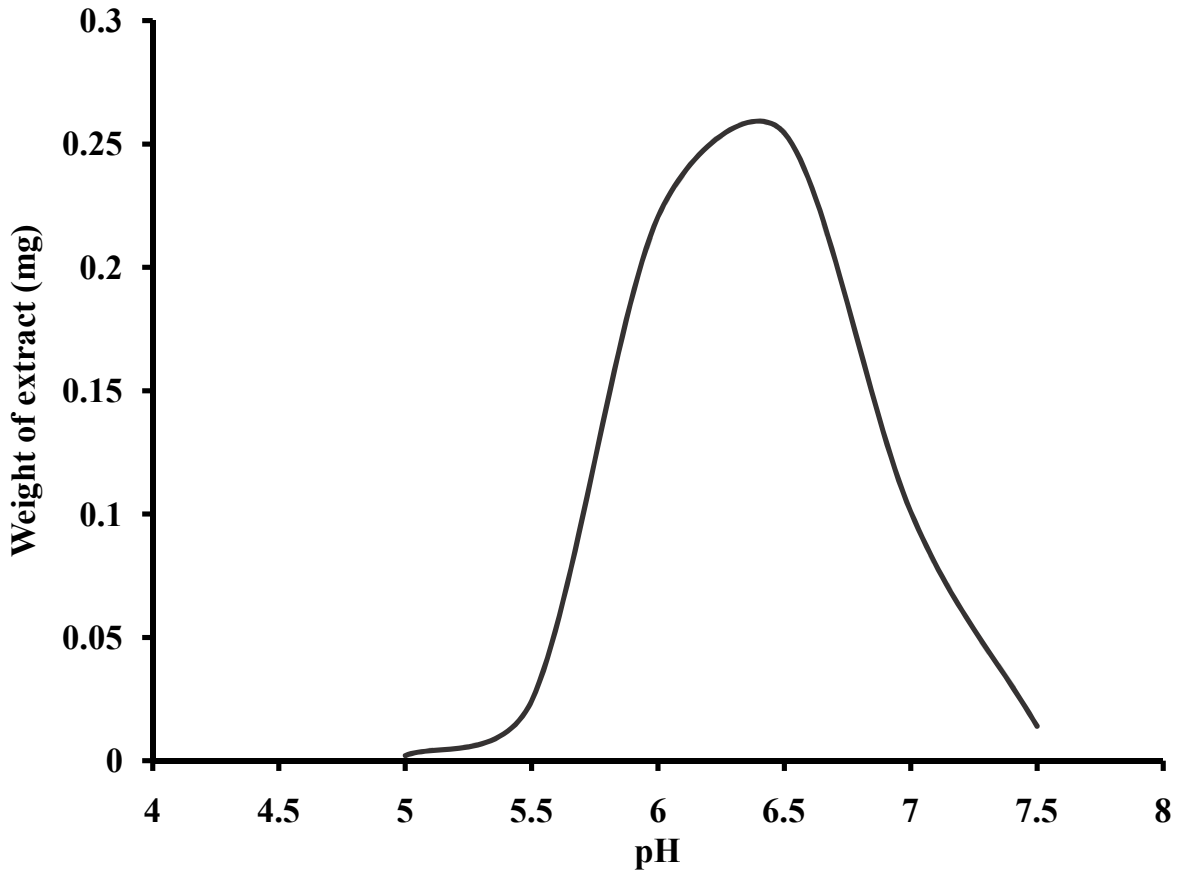


Figure 4.9: Optimum pH of production of extract with antibacterial activity by *P. chrysogenum* grown on cassava peel media

4.5 PHYSICAL PROPERTIES OF THE CRUDE AND PARTIALLY PURIFIED EXTRACT

4.5.1 Thin layer chromatogram (TLC) of the crude extract, partially purified extract and reference drug

Plate 4.2 shows the TLC plate with the crude extract, partially purified extract and reference drug using solvent system toluene:ethylacetate:acetic acid (2: 2: 1) v/v/v as mobile phase and detected at UV 256 and 345 nm. The plates were placed in Iodine chamber for one minute for spot development before viewing under the UV scan. Two spots were detected on the crude fraction and one spot each on the purified extract and control. R_f values when calculated shows 0.81 for the single spot in the reference drug, 0.35 for spot A and 0.81 for spot B in the crude extract and 0.80 for the single spot in the partially purified extract.

4.5.2 HPLC analysis of the crude extract, partially purified extract and reference drug

HPLC analysis of the crude extract (Plate 4.3), partially purified extract (Plate 4.4) and Reference drug (Plate 4.5) revealed the presence of two pronounced peaks in the crude extracts. The multiple smaller peaks might be due to the presence of hydrolytic enzymes. A single peak was observed in the partially purified extract as well as the reference drug. The retention time of the suspected secondary metabolite in the partially purified extract is similar with that of the reference drug. Thus HPLC results confirm the corresponding spots on the chromatogram in Plate 4.2. Penicillin eluted at approximately 2.35 minutes.

Plate 4.2: **Thin layer chromatogram of partially purified, crude extract of *P. chrysogenum* and reference drug (Penicillin)**

Stationary phase: Silica gel

Mobile phase: Solvent system toluene:ethylacetate:acetic acid (2:2:1) v/v/v

R_f value : 0.81

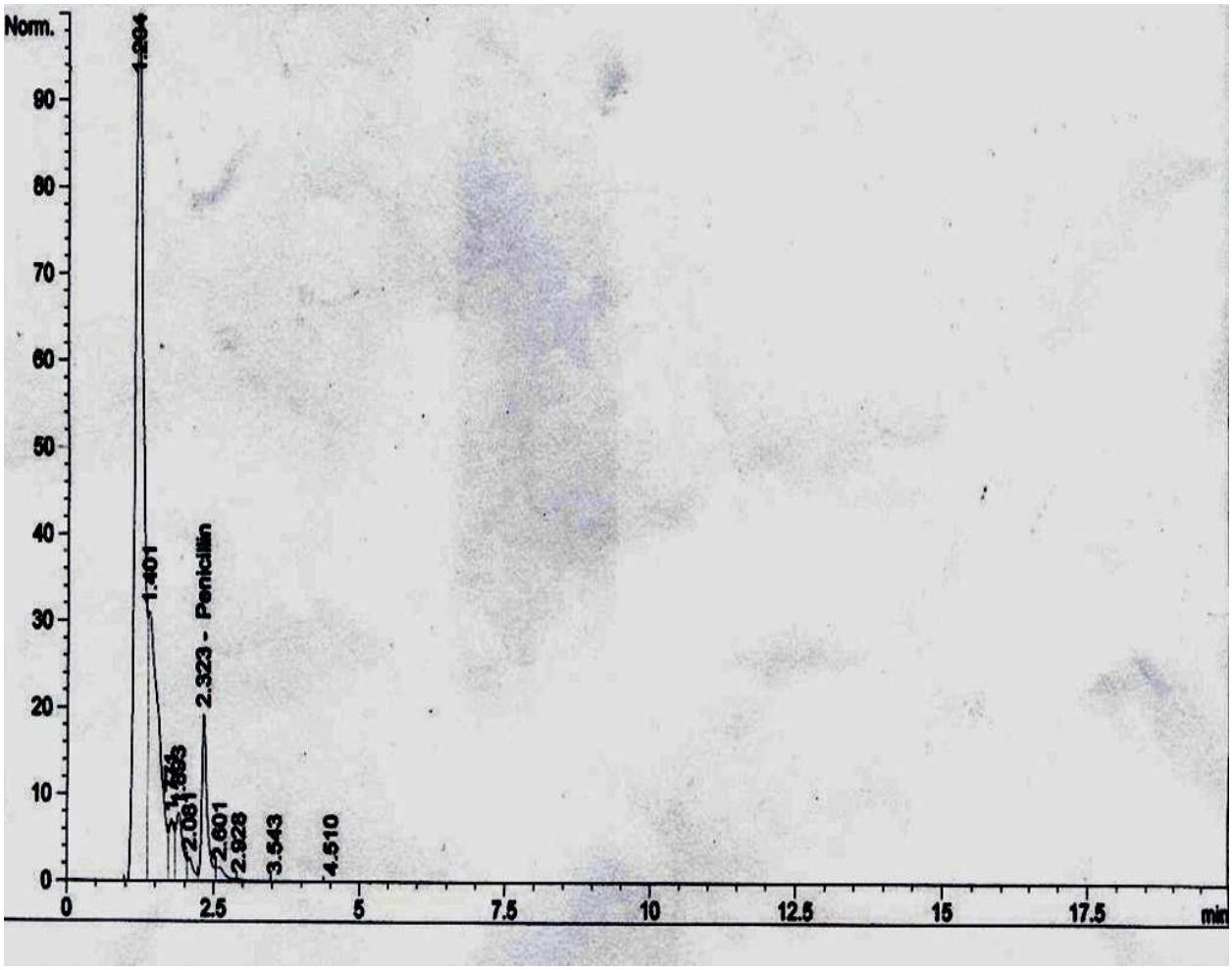


Plate 4.3: HPLC of crude extract of *P. chrysogenum* cultivated on cassava peel media

Gravity column: C_{18}

Buffer A: 50mM sodium acetate, pH 4.5

Buffer B: Acetonitrile.

Flow rate: 1.0ml/min,

Penicillin eluted at approximately 2.35minutes

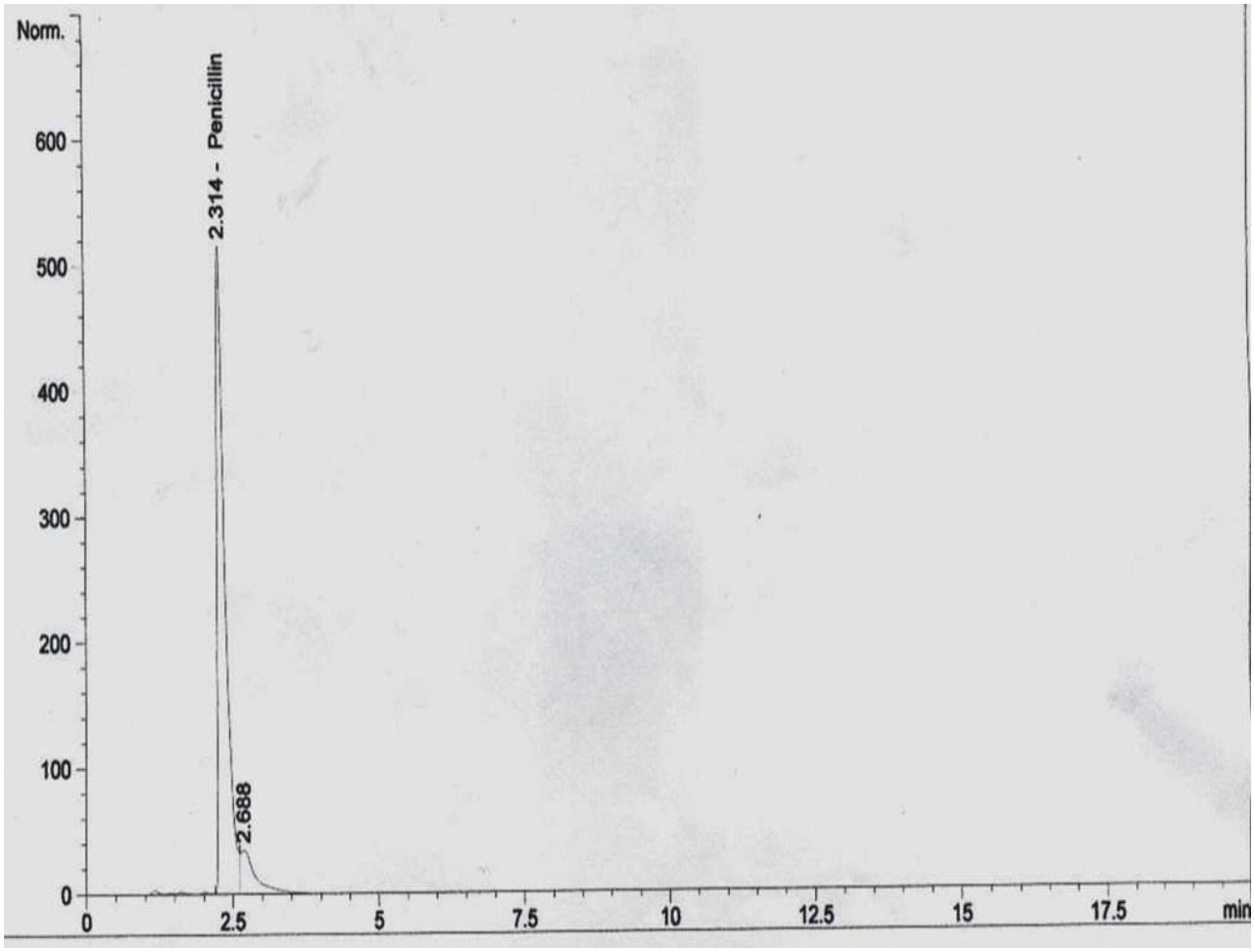


Plate 4.4: **HPLC of partially purified extract of *P. chrysogenum* cultivated on cassava peel media**.

Gravity column: C_{18}

Buffer A: 50mM sodium acetate, pH 4.5

Buffer B: Acetonitrile.

Flow rate: 1.0ml/min,

Penicillin eluted at approximately 2.35minutes

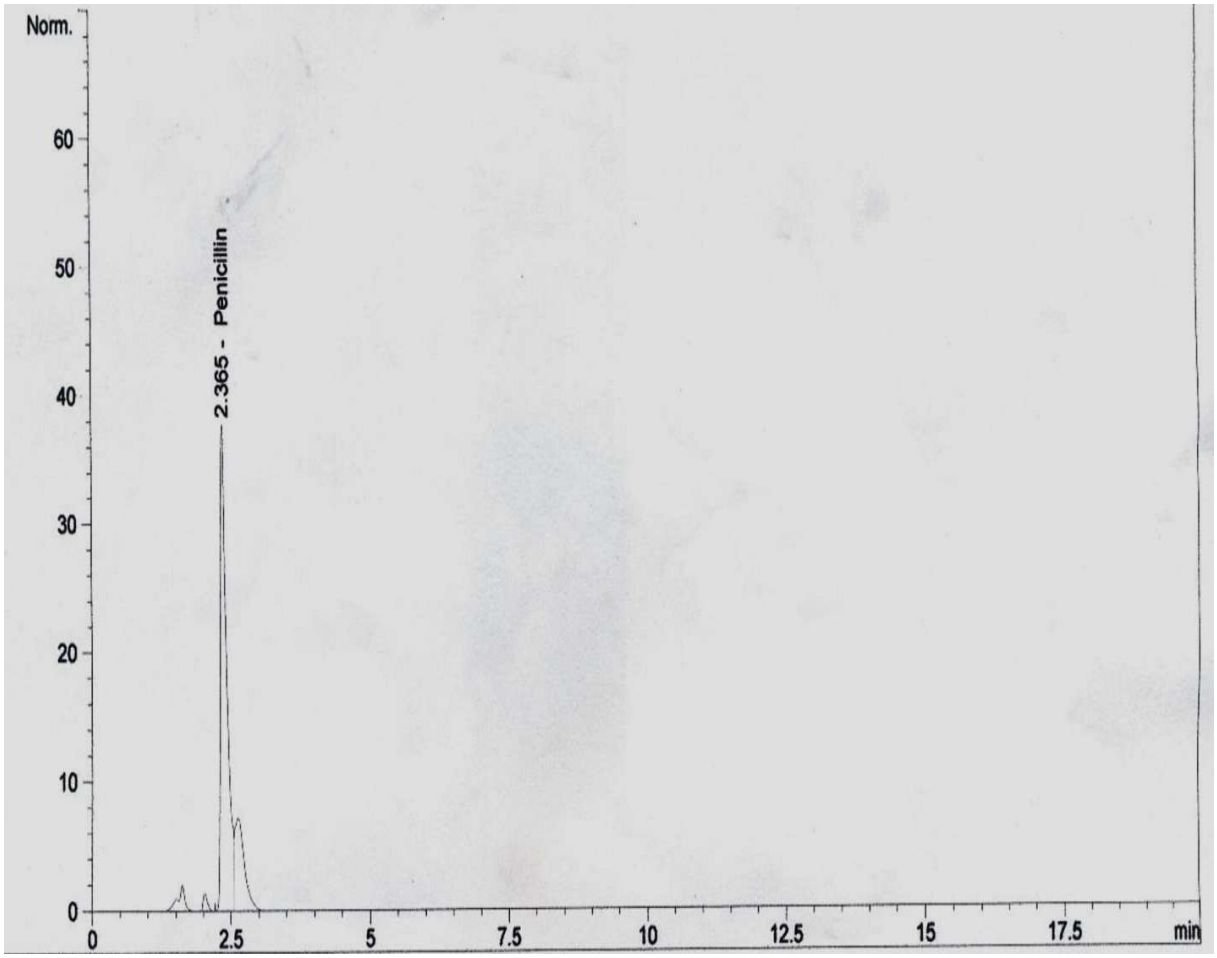


Plate 4.5: **HPLC of Benzyl Penicillin (Reference Drug)**

Gravity column: C_{18}

Buffer A: 50mM sodium acetate, pH 4.5

Buffer B: Acetonitrile.

Flow rate: 1.0ml/min,

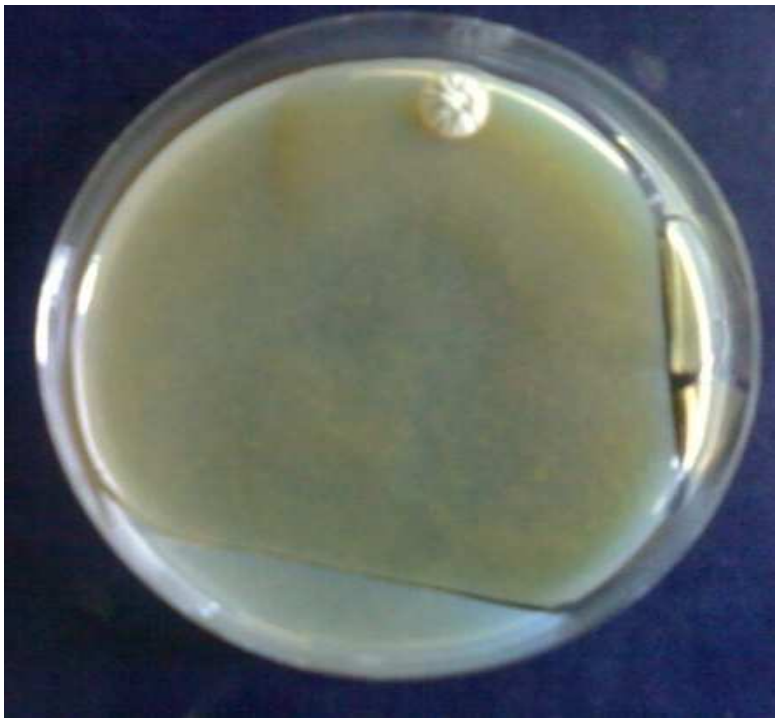
Penicillin eluted at approximately 2.35minutes

4.5.3 Modification of *P. chrysogenum* (wild strain) to obtain transformed strains with higher antibiotic yeilds

When *P. chrysogenum* wild strain grown on agro-waste was modified using Ultra Violet radiation and $NaNO_3$, the results show that UV modified strains of *P. chrysogenum* UV2 and UV3 were obtained after exposing the culture plate with cell suspension to UV light for 20 and 25minutes respectively with 1% survival rate of the organism and was therefore considered transformed strains (Plates 4.6 A and B). Plate C (HNP4) were strains obtained after treatment of cell suspension with $NaNO_3$ for 20minutes while Plates D (HNP2) and E (HNP1) were strains obtained after treatment of cell suspension with $NaNO_3$ for 40 and 50minutes respectively. There was more growth in plates treated with $NaNO_3$ for 10minutes compared to the wild strain. This could be attributed to the fact that the organism was using the nitrate present in the solution as a source of nitrogen for growth. Antibacterial production rate of transformed strains was monitored using HPLC.

A

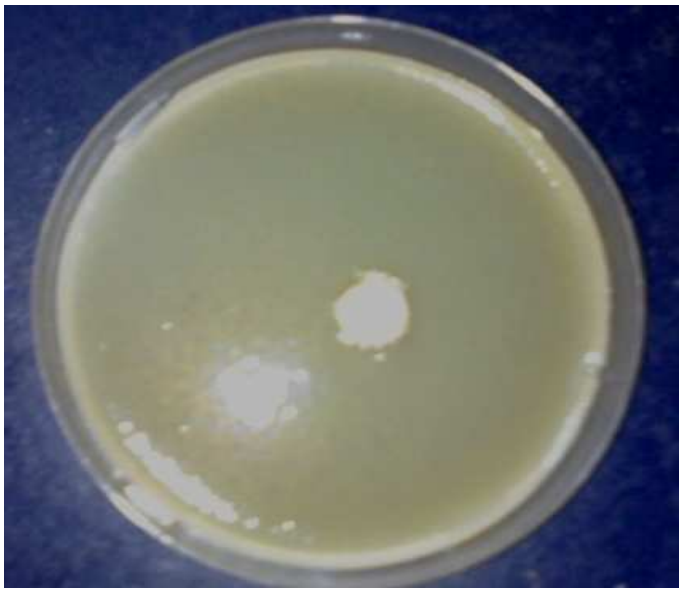
B



Plates 4.6: A & B – Transformed strains of *P. chrysogenum* obtained after exposure to UV light for 20 & 25minutes

C

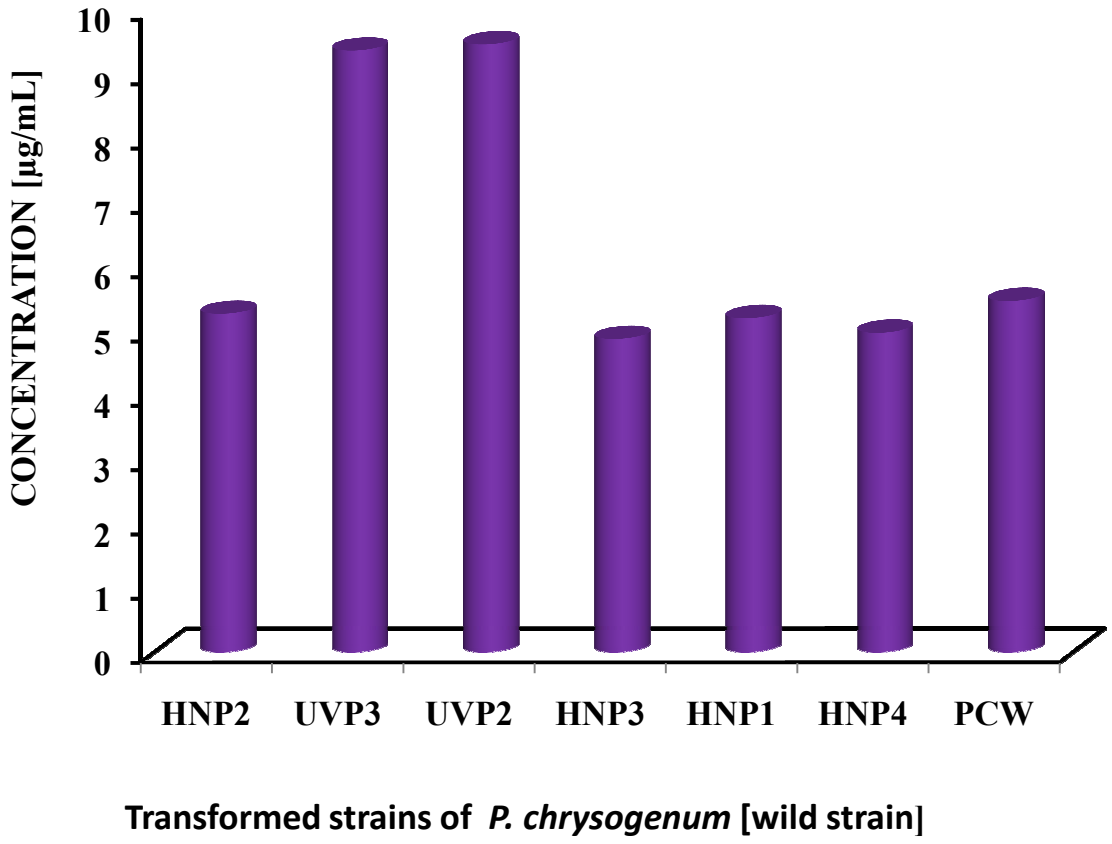
D



Plates 4.6: C and D - Strains of *P. chrysogenum* obtained after exposure to $NaNO_3$ for 40 and 50 minutes respectively

4.5.4 HPLC analysis of the potential of the transformed strains of *P. chrysogenum* to produce antibiotics

Figure 4.10 shows the rate of production of antibiotics monitored for five days by the UV transformed strains and the different strains obtained after treating the wild strain with $NaNO_3$ - a chemical mutagen. Results show that the quantity of antibiotics produced by $NaNO_3$ treated organism is 20% lower when compared to the quantity produced by the wild strain (PCW). Determination of the quantitative value of the potential of UV2 and UV3 transformed strain to produce antibiotics gave a 70% increase over the wild strain (PCW).



Legend

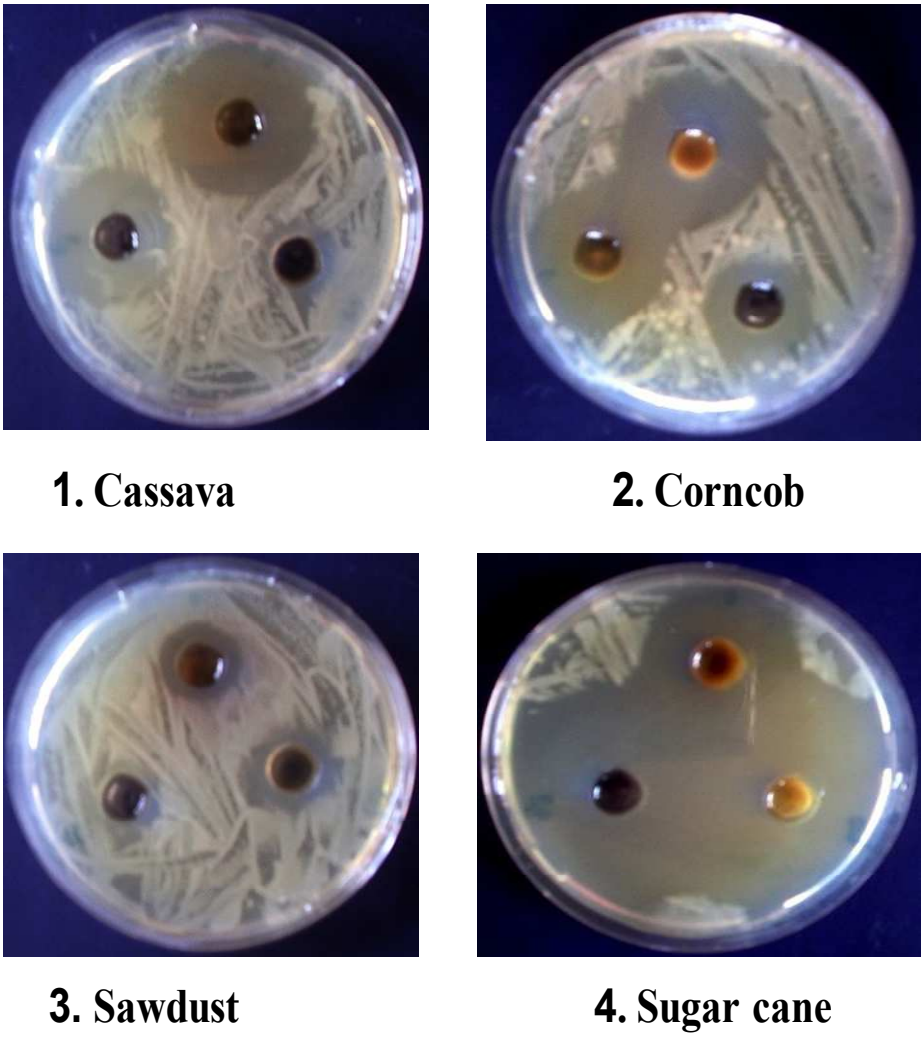
PCW -*P.chrysogenum* wild strain	HNP1 - $NaNO_2$ transformed strain 1
UVP3 – UV transformed strain 3	HNP2 – $NaNO_2$ transformed strain 2
UPV2 - UV transformed strain 2	HNP3 - NaNO2 transformed strain 3
	HNP4 - $NaNO_2$ transformed strain 4

Figure 4.10: Penicillin production by transformed strains of *P. chrysogenum* [wild strain] monitored for 5days using HPLC

4.6 BIOCHEMICAL PROPERTIES OF FUNGAL ANTIBACTERIAL EXTRACT (*IN VITRO*)

4.6.1 Zone of inhibition of clinical isolates of bacteria by extract of *P. chrysogenum* grown on agro waste

Plates 4.7:1 – 8 show the i*n vitro* effect of culture extracts of *P. chrysogenum* and commercial benzyl penicillin (Retarpen, Sandox Austria) as a Reference drug on the clinical isolates of *E. coli, B. subtilis, P. aeruginosa* and *P. mirabilis*. The reference drug and all the culture extracts of *P. chrysogenum* inhibited the growth of *E. coli* and *B. subtilis* but had no effect on *P. aeruginosa* and *P. mirabilis*. The degree of inhibition of pathogenic bacteria (observed when culture extracts of *P. chrysogenum* grown on different agro-waste was used as substrate) is in descending order of: Sugarcane > Cassava > Corncob > Sawdust.



Plates 4.7 (1-4): ***In vitro*** **effect of culture extract of** ***P. chrysogenum*** **grown on selected agro-waste against** ***E.coli*****. (1&2)** ***B. subtilis*** **(3&4). Areas around the cup shows zone of inhibition. SC (sugar cane), CAS (cassava peels), RSP (Reference standard drug - Penicillin).**

5. Reference drug on *P. aeruginosa*

6. CAS/SC extract on *P. aeruginosa*

7. CAS, SC & RSP extracts on *P. mirabilis*

8. Reference drug on *E. coli*

Plates 4.7 (5-8): ***In vitro*** **effect of culture extract of** ***P. chrysogenum*** **grown on selected agro-waste against** ***P. aeruginosa*** **(5&6)** ***P. mirabilis*** **(7) and Reference drug on** ***E. coli*** **(8). Areas around the cup shows zone of inhibition. SC (sugar cane), CAS (cassava peels), RSP (Reference standard drug - Penicillin).**

4.6.2 Qualitative determination of potential inhibition of clinical isolates of bacteria by extract of *P. chrysogenum* grown on agro waste

Table 4.2 shows the summary of the i*n vitro* activity of culture extracts of *P. chrysogenum* from various agro-wastes, glucose and lactose controls tested against clinical isolates of *E. coli, P. aeruginosa*, *P. mirabilis* and *B. subtilis* with commercial benzyl penicillin (Retarpen, Sandox Austria) as a reference drug. All the culture extracts, synthetic sources of carbon and the reference drug exhibited positive (+) effects on *E. coli* and *B. subtilis* and negative (-) effect on *P. aeruginosa* and *P. mirabilis*.

4.6.3 Quantitative determination of the potency of the extract of *P. chrysogenum* grown on agro waste on clinical isolates of bacteria

Table 4.3 shows the quantitative value of the extent to which the extract inhibits the clinical isolates of *E. coli* and *B. subtilis*. The degree of inhibition on the growth of the susceptible isolates varied with the diameter of zone of inhibition and potency of the extracts from sugarcane pulp and cassava peels giving values very close to that of the reference drug.

4.6.4 Determination of the value of the lowest concentration of the extract which can inhibit susceptible organisms

The lowest concentration of the extract inhibiting the visible growth of susceptible organism is defined as the minimum inhibitory concentrations (MIC). Table 4.4 shows the MICs of the culture extracts and the reference drug against clinical isolates of *E. coli* and *B. subtilis*. MIC against the susceptible organisms was 0.20mg/ml for the standard drug; it ranged from 0.40 to 2.0mg/ml for the culture extracts. It was 0.4 - 0.6mg/ml for cassava peels and sugarcane pulp, 0.6mg/ml for glucose and lactose, 0.8mg/ml for corncob and 2.0mg/ml for sawdust.

Table 4.1: Effect of extracts of *P. chrysogenum*, glucose, lactose and Reference drug on the growth of the clinical isolates of *E. coli, B. subtilis, P. aeruginosa* and *P. mirabilis*

Antibiotic	Carbon Source of culture media	Antibacterial activity on clinical isolates of bacteria			
		E. coli	***B. subtilis***	***P. aeruginosa***	***P. mirabilis***
Culture extracts of *P. chrysogenum*	Cassava peels	+	+	-	-
	Corncob	+	+	-	-
	Sawdust	+	+	-	-
	Sugarcane pulp	+	+	-	-
	Glucose	+	+	-	-
	Lactose	+	+	-	-
Reference Drug	Not applicable	+	+	-	-

(+ = inhibition; - = No visible zone of inhibition)

Table 4.2: Average diameter of inhibition zone and potency of culture extracts of *P. chrysogenum* and reference drug against clinical isolates of *E. coli* and *B. subtilis*

Source of antibiotics	Carbon Source of culture media	*E. coli*		*B. subtilis*	
		Diameter of inhibition zone (mm)	Quantitative value of Potent Extract (mg/ml)	Diameter of inhibition zone (mm)	Quantitative value of Potent Extract (mg/ml)
Extract of *P. chrysogenum*	Cassava peel	15.12 ± 0.03*	11.76*	26.6 ± 0.14*	10.43*
	Corncob	13.63 ± 1.59	11.50	14.75 ± 0.35	5.47
	Sawdust	6.25 ± 0.35	3.04	6.75 ± 0.35	3.57
	Sugarcane pulp	17.88 ± 0.35*	17.95*	28.3 ± 1.77*	11.41*
	Glucose	15.00 ± 0.35	8.26	15.75 ± 1.06	5.78
	Lactose	14.75 ± 1.41	11.04	17.25 ± 1.06	6.27
Reference drug	Not applicable	16.93 ± 0.50	16.00	34.46 ± 0.50	16.00

*=Significant values ($p<0.05$) Values are expressed as mean ± SEM

Potency of extract was calculated using the standard dose response

$$\underline{b} = \frac{n\sum XY(\sum X)(\sum Y)}{n\sum X^2 - (\sum X)^2}$$

$\underline{b}$ = Regression coefficent (Gordon, 1977).

Table 4.3: Minimum inhibitory concentrations (mg/ml) of culture extracts of *P. chrysogenum* and reference drug against clinical isolates of *E. coli* and *B. subtilis*

Antibiotic	**Carbon Source of culture media**	***E. coli***	***B. subtilis***
Culture extracts of *P. chrysogenum*	Cassava peels	0.4-0.6	0.4-0.6
	Corncob	0.8-1.0	0.8-1.0
	Sawdust	1.0-2.0	1.0-2.0
	Sugarcane pulp	0.4-0.6	0.4-0.6
	Glucose	0.6-0.8	0.6-0.8
	Lactose	0.6-0.8	0.6-0.8
Reference drug	Not applicable	0.2-0.4	0.2-0.4

4.6.5 Determination of the presence of β-lactam ring in the antibacterial extract

A 24 hour cell free culture of *P. aeruginosa* was qualitatively tested with β-lactamase identification sticks (Oxoid, Wesel, Germany) with nitrocefin as the substrate. There was a colour change from yellow to red indicating the presence of β-lactamase. Figure 4.11 shows that β –lactamase of *P. aeruginosa* effectively hydrolysed the β –lactam ring present in the culture extract and the reference drug (Benzyl Penicillin – a synthetic β –lactam compound).

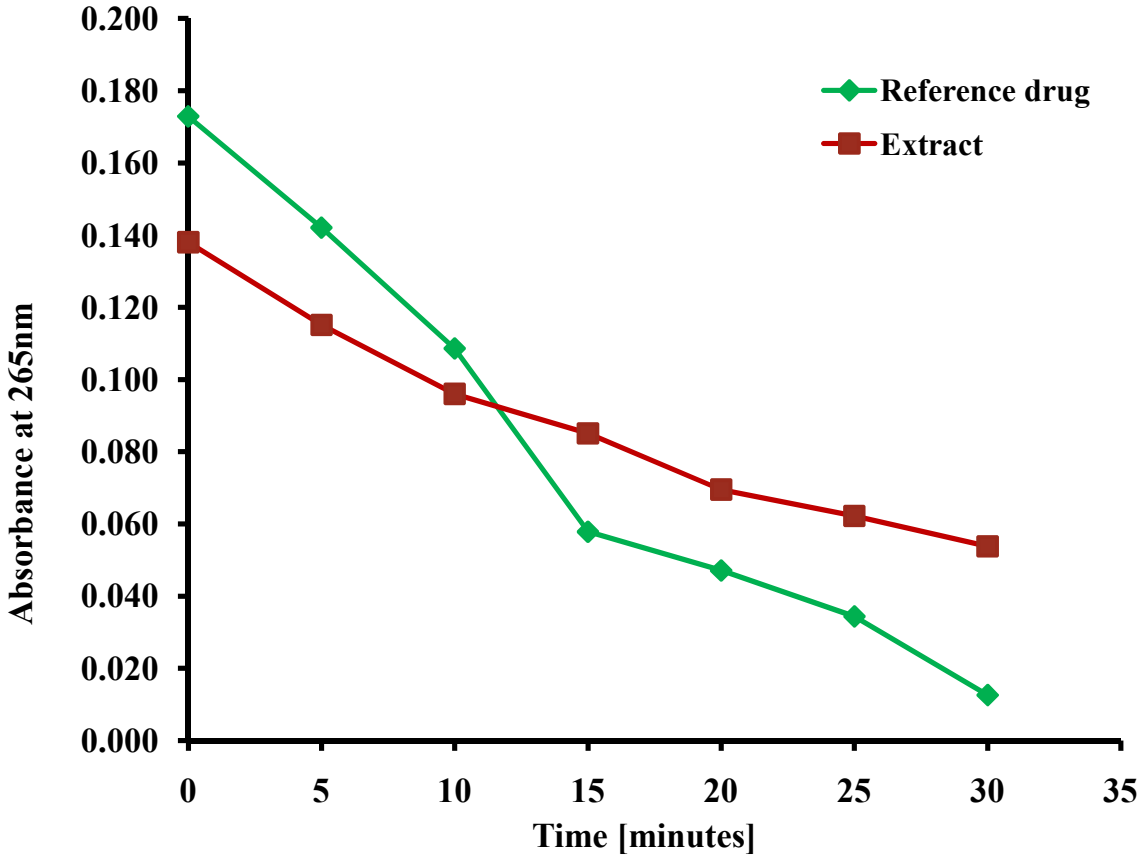


Figure 4.11: Hydrolysis of β-lactam compound present in culture extract of *P. chrysogenum* by β -lactamase of *P. aeruginosa.*

4.7 TOXICITY STUDY

4.7.1 Acute toxicity

The data shown in Table 4.5 represents the toxicity study carried out on mice with the culture extract of *P. chrysogenum*. No significant alterations were observed for the acute toxicity groups. The culture extract was found to be relatively safe at a maximum dose of 1500mg/kg body weight of the mouse since there were no death of the animals recorded in 24hrs.

4.7.2 Sub-acute toxicity study to evaluate *in vivo*, the effect of the extract on some biochemical analytes

4.7.2.1 Biochemical changes in chemical analytes involved in liver function

The result shows graphical representations of data obtained after administration of the extract and reference drug to some experimental animals. It was observed that the total protein value for the extract is within normal range, it is, however, lower than the reference drug and control Figure 4.12A). There were no significant difference ($p>0.05$) observed in the value of the albumin fraction of the extract, reference drug and control. Also there were no significant difference ($p>0.05$) in liver enzymes activity (Figure 4.12B) as well as bilirubin level (Figure 4.12C) seven days after the administration of the extract. This indicates that the extract as well as the reference drug did not cause any hepatocellular damage.

Table 4. 4: Acute toxicity results using varying dose range

GROUP	1	2	3	4	5	CONTROL
Dose (mg/kg)	500	750	1000	1250	1500	0
No of Mice	5	5	5	5	5	5
No. of death	Nil	Nil	Nil	Nil	Nil	Nil

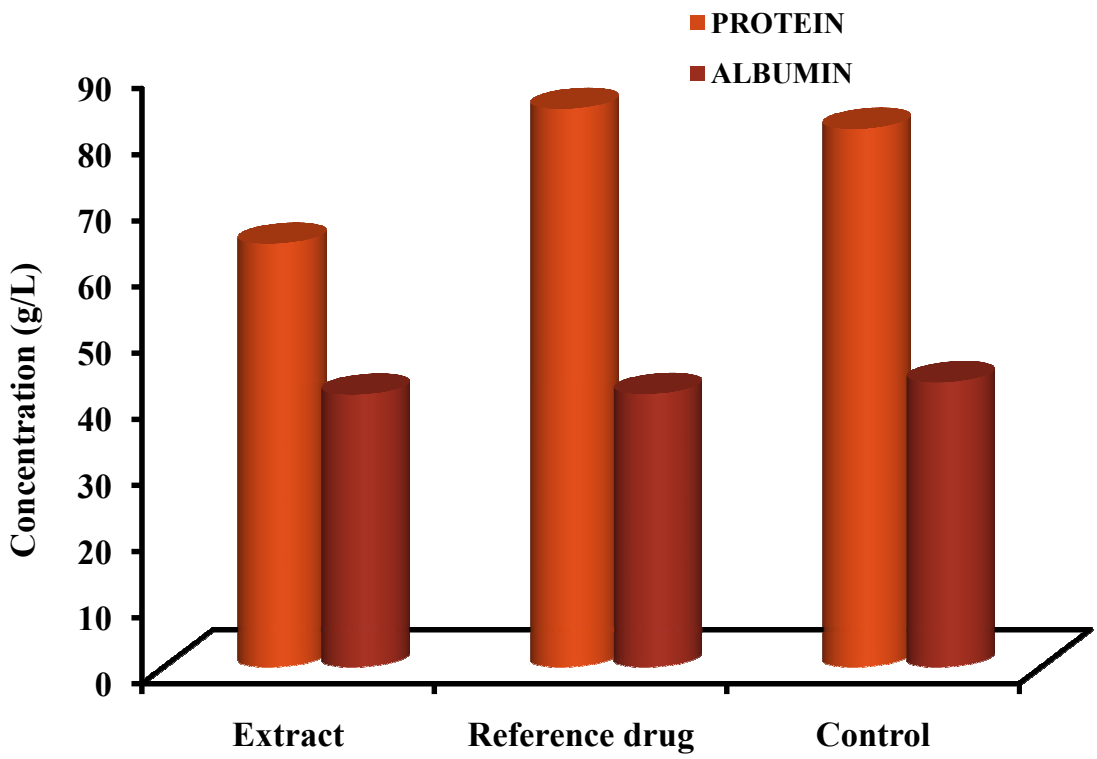
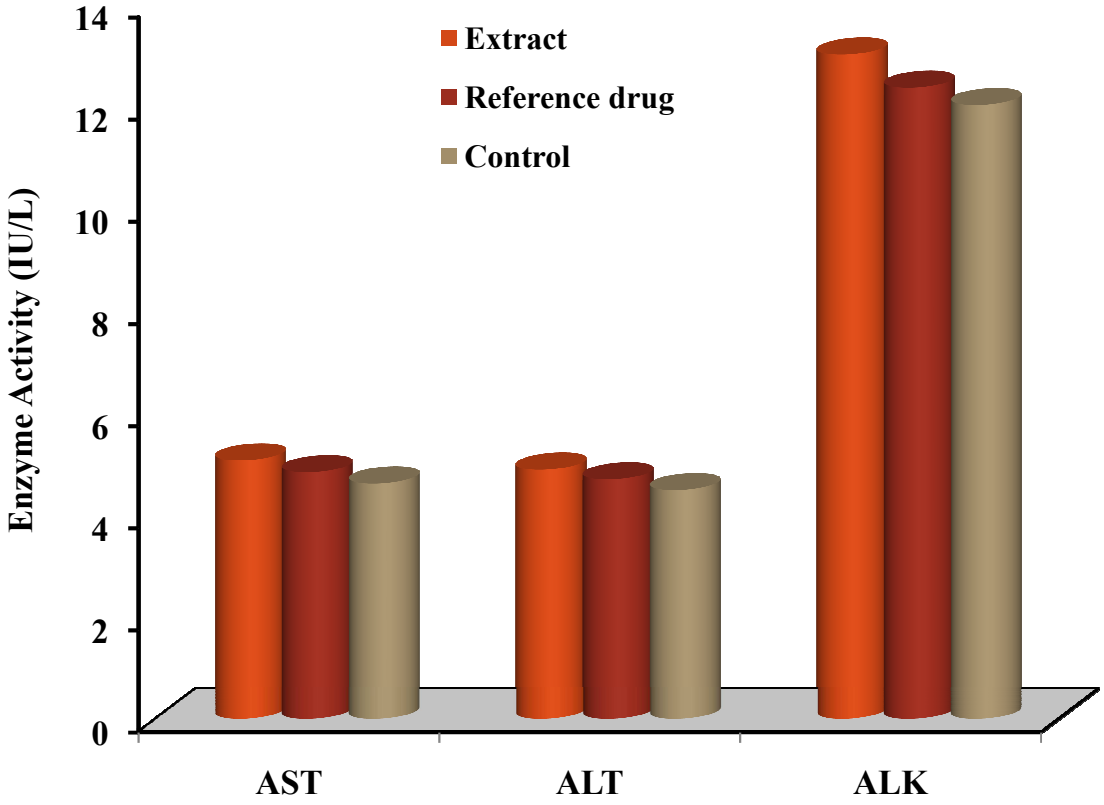


Figure 4.12A: ***In vivo*** **effects of extract and reference drug on Protein and Albumin (Sub-acute toxicity study) Values are expressed as mean ± SEM**



AST – Aspartate Transaminase
ALT - Alanine Transaminase
ALK - Alkaline Phosphatase

Figure 4.12B: *In vivo* effects of extract and reference drug on liver enzymes (Sub-acute toxicity study). Values are expressed as mean ± SEM

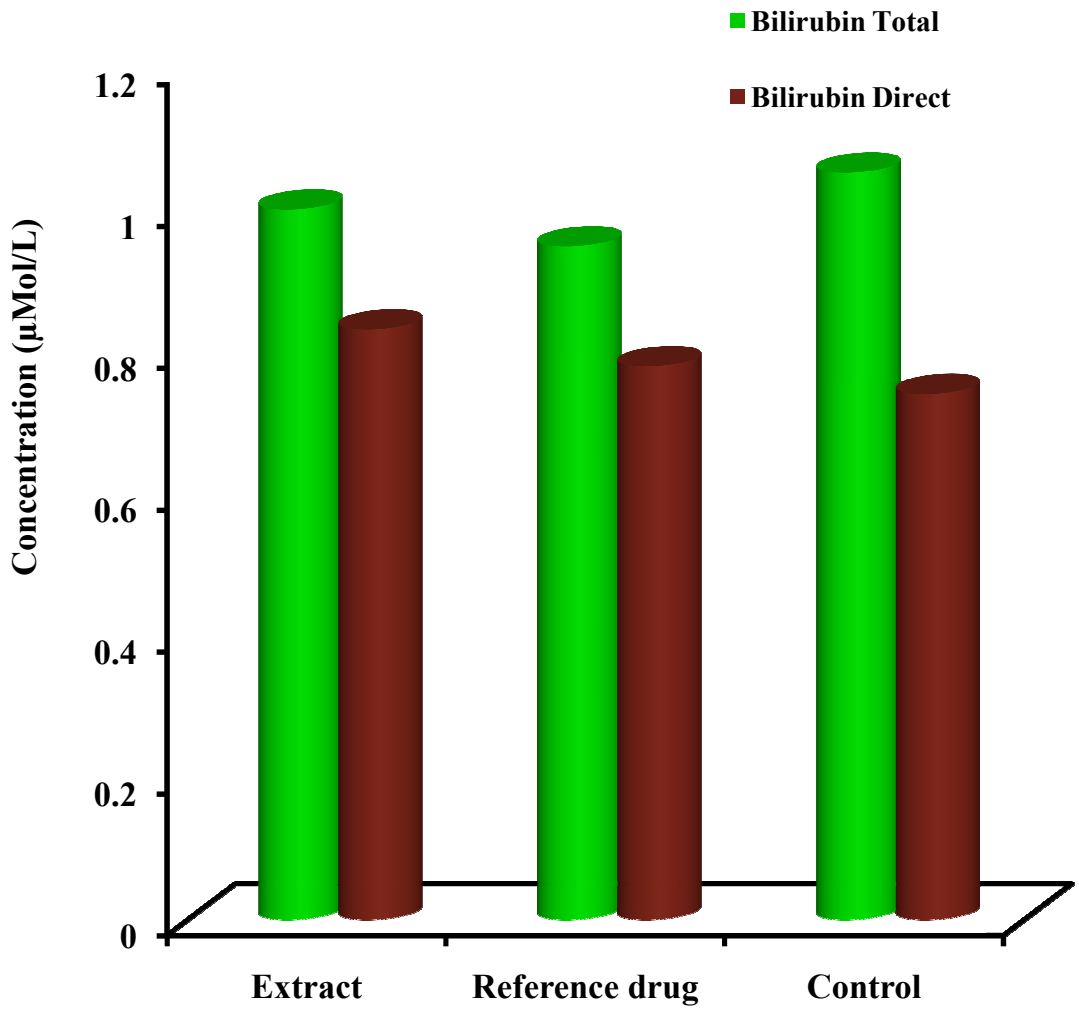


Figure 4.12C: *In vivo* effects of extract and reference drug on Total and Direct Bilirubin (Sub-acute toxicity study). Values are expressed as mean ± SEM

4.7.2.2 Biochemical changes in chemical analytes involved in kidney function.

In vivo sub-acute toxicity study of chemical analytes involved in kidney function shows a significant increase ($p<0.05$) in sodium, urea (Figure 4.13A) and creatinine (Figure 4.13B) values in the group administered with reference drug compared to the control group. There were no significant difference ($p>0.05$) in groups where the extract was administered. This indicates that the body handles natural products better than synthetic drug. There were no changes in chloride concentration for all the groups monitored (Figure 4.13C).

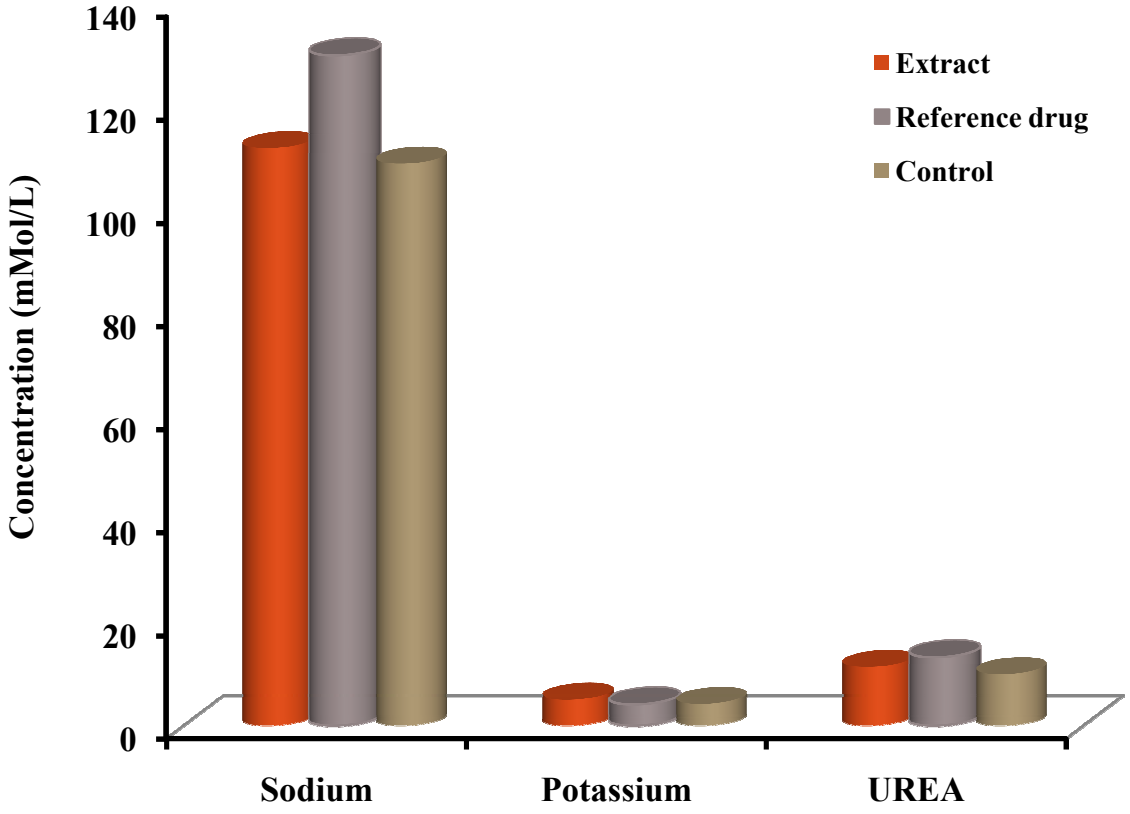


Figure 4.13A: *In vivo* effects of extract and reference drug on renal function (Sub-acute toxicity study). Values are expressed as mean ± SEM

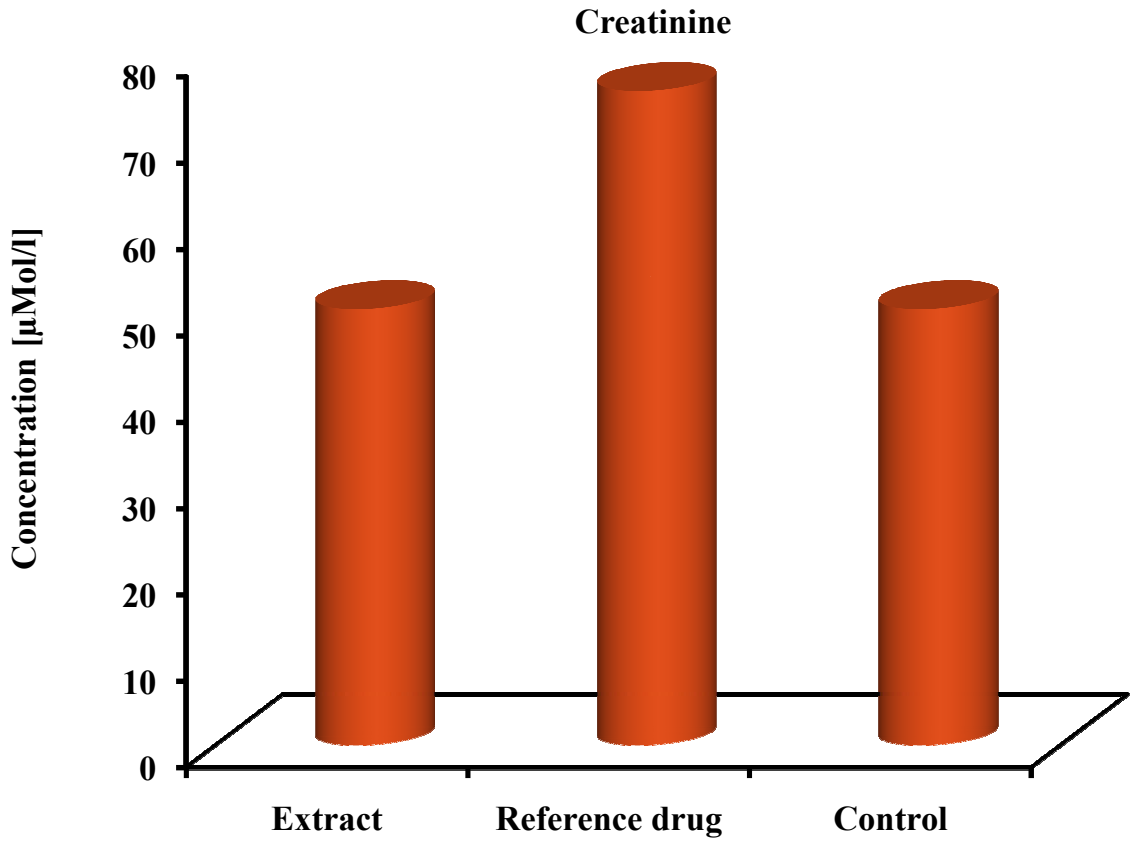


Figure 4.13B: ***In vivo*** **effects of extract and reference drug on creatinine (Sub-acute toxicity study). Values are expressed as mean ± SEM**

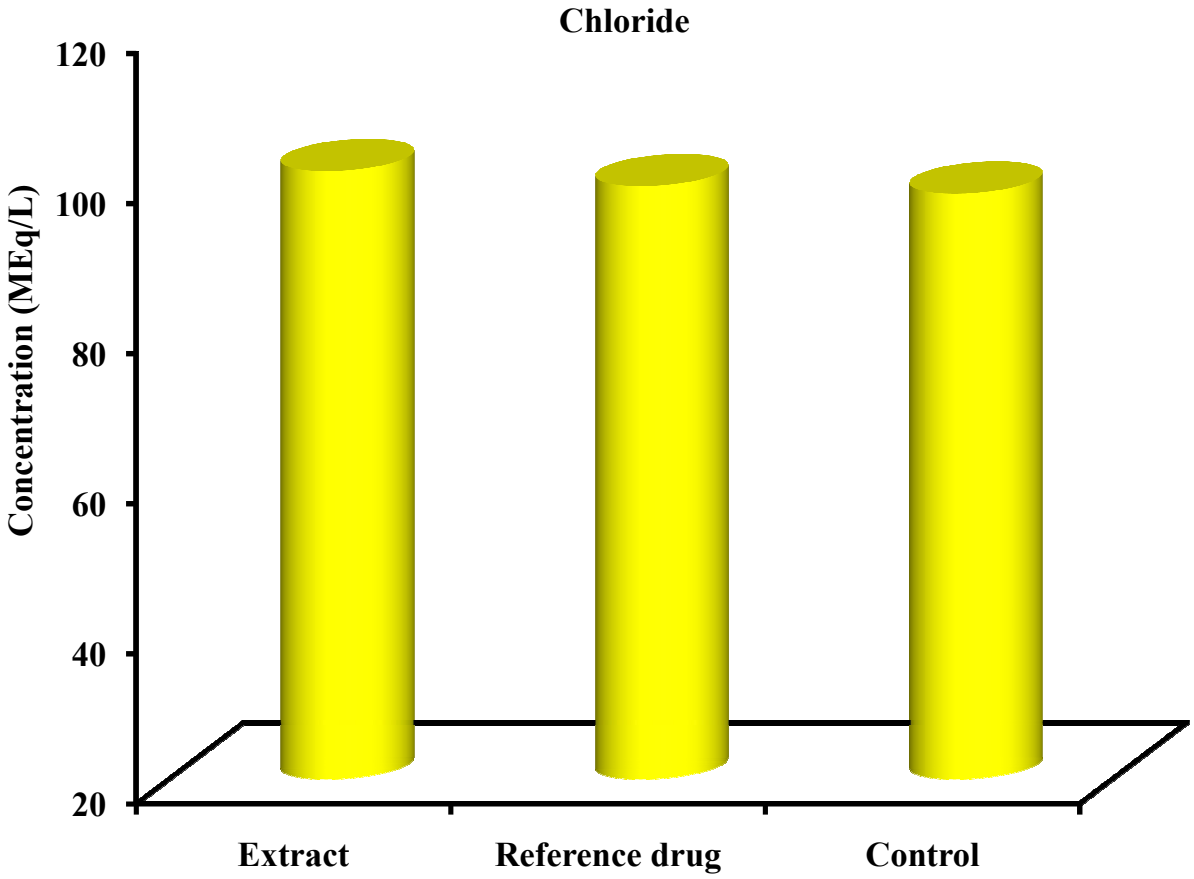
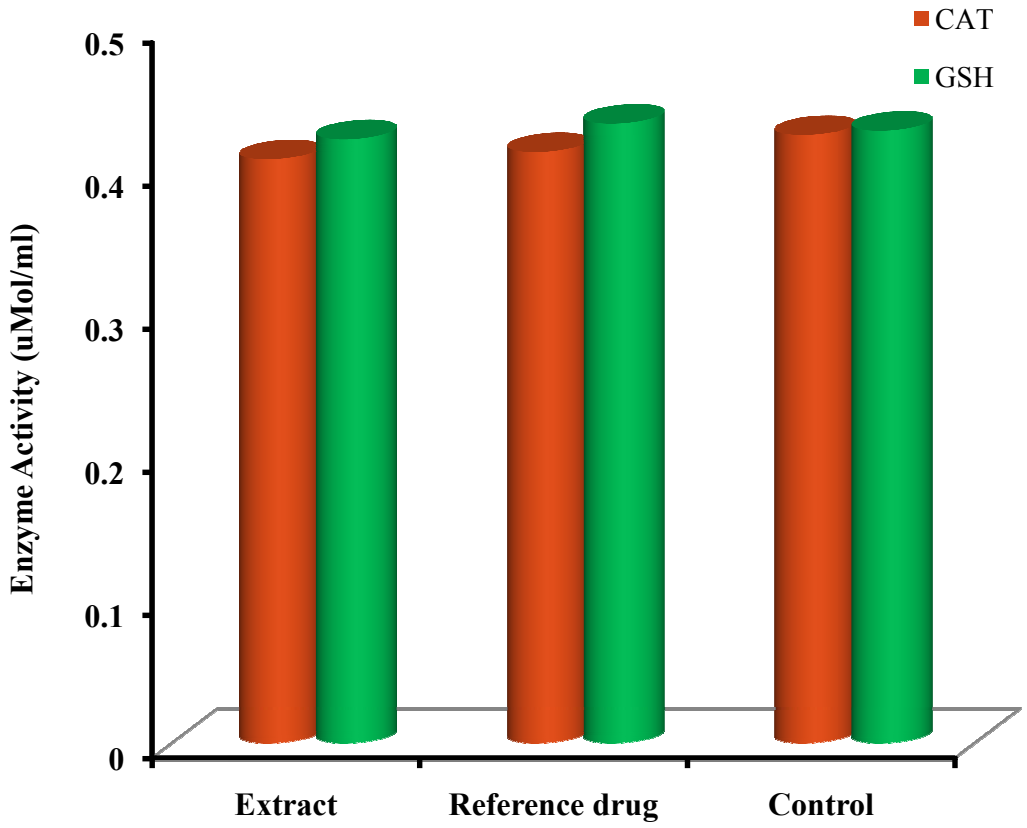


Figure 4.13C: *In vivo* effects of extract and reference drug on chloride (Sub-acute toxicity study). Values are expressed as mean ± SEM

4.7.2.3 Biochemical changes in chemical analytes involved in oxidative stress enzymes and oxidation products.

Biochemical parameters used in monitoring oxidative stress due to increased generation of reactive oxygen species include the enzymes - catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and oxidation product due to lipid peroxidation - malonyldialdehyde (MDA). From the results in Figures 4.14A, 4.14B and 4.14C, there is no significant difference ($p>0.05$) in all the parameters assayed when the extract and reference drug groups were compared with the control group.



GSH – Reduced Glutathione
CAT - Catalase

Figure 4.14A: *In vivo* biochemical effects of extract and reference drug on CAT, and GSH (sub-acute toxicity study). Values are expressed as mean ± SEM

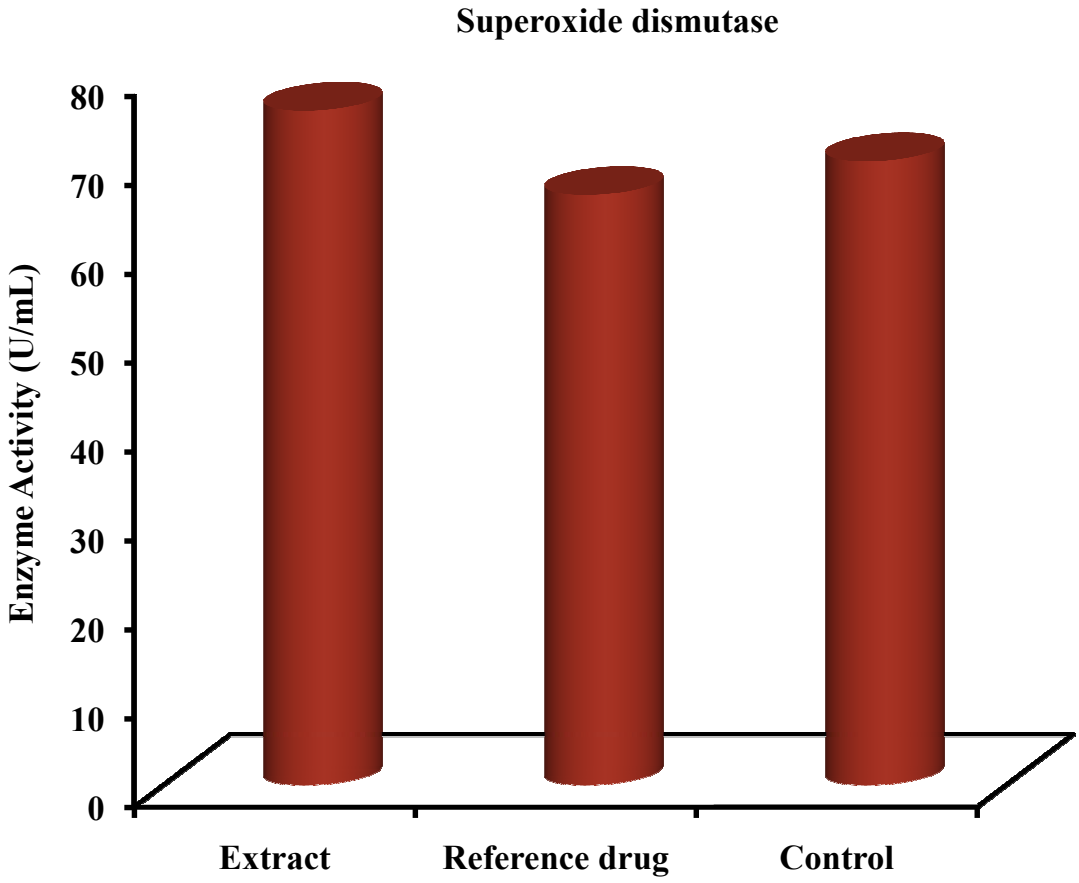


Figure 4.14B: Effects of extract and reference drug on superoxide dismutase (sub-acute toxicity study). Values are expressed as mean ± SEM

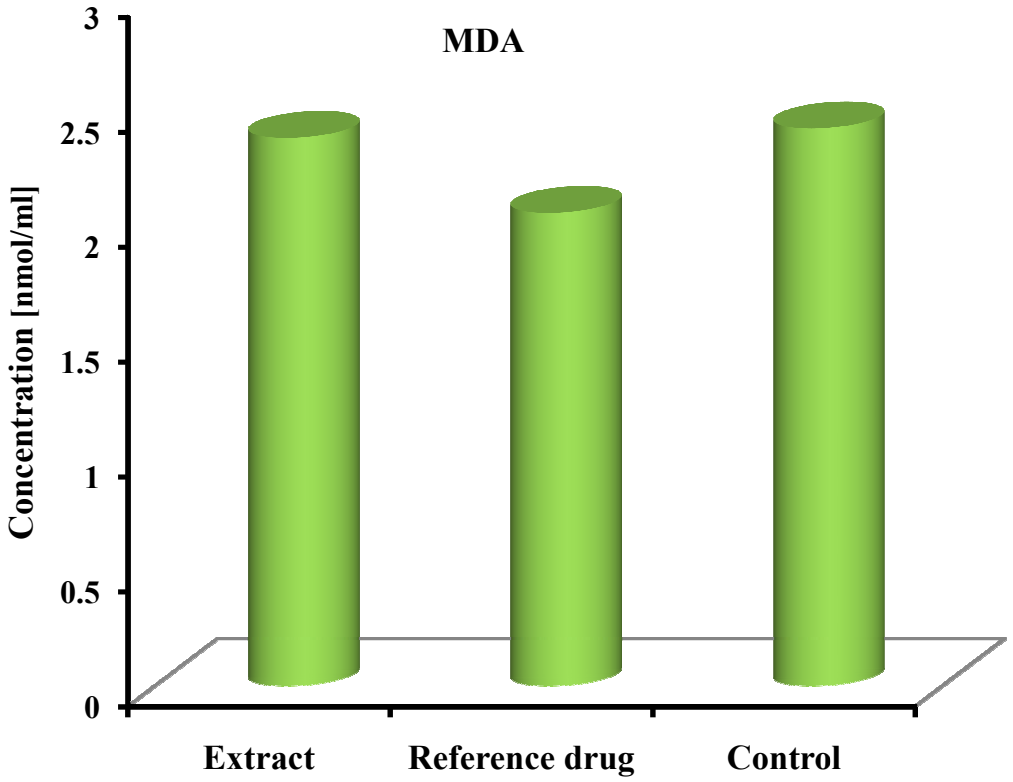
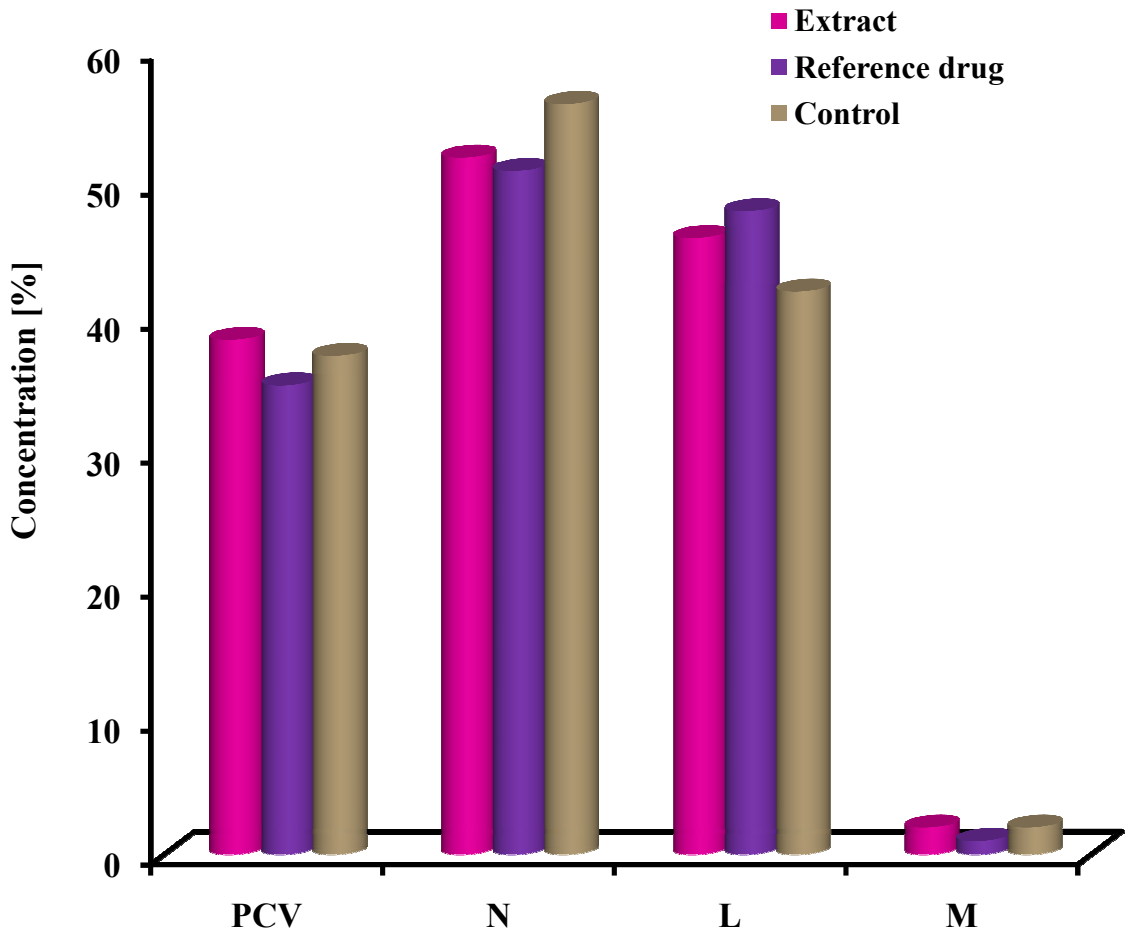


Figure 4.14C: Effects of extract and reference drug on malondialdehyde (sub-acute toxicity study). Values are expressed as mean ± SEM

4.7.2.4 Changes in haematology indices

Changes in haematological indices involving haemoglobin, Packed cell volume, platelet count, total white blood cell count and white cell differentials are represented in Figure 4.23 There were no significant changes ($p>0.05$) in the PCV level, WBC differentials (Figure 4.15A) and total WBC count (Figure 4.15B). However, in rats administered with the extract and reference drug compared to the control, there was a significant decrease ($p<0.05$) in Platelets count (Figure 4.15C) indicating thrombocytopenia.



Values are expressed as mean ± SEM

PCV – Packed Cell Volume N– Neutrophils L – Lymphocytes M - Monocytes

Figure 4.15A: *In vivo* effect of extract and reference drug on haematological indices (Sub-acute toxicity study)

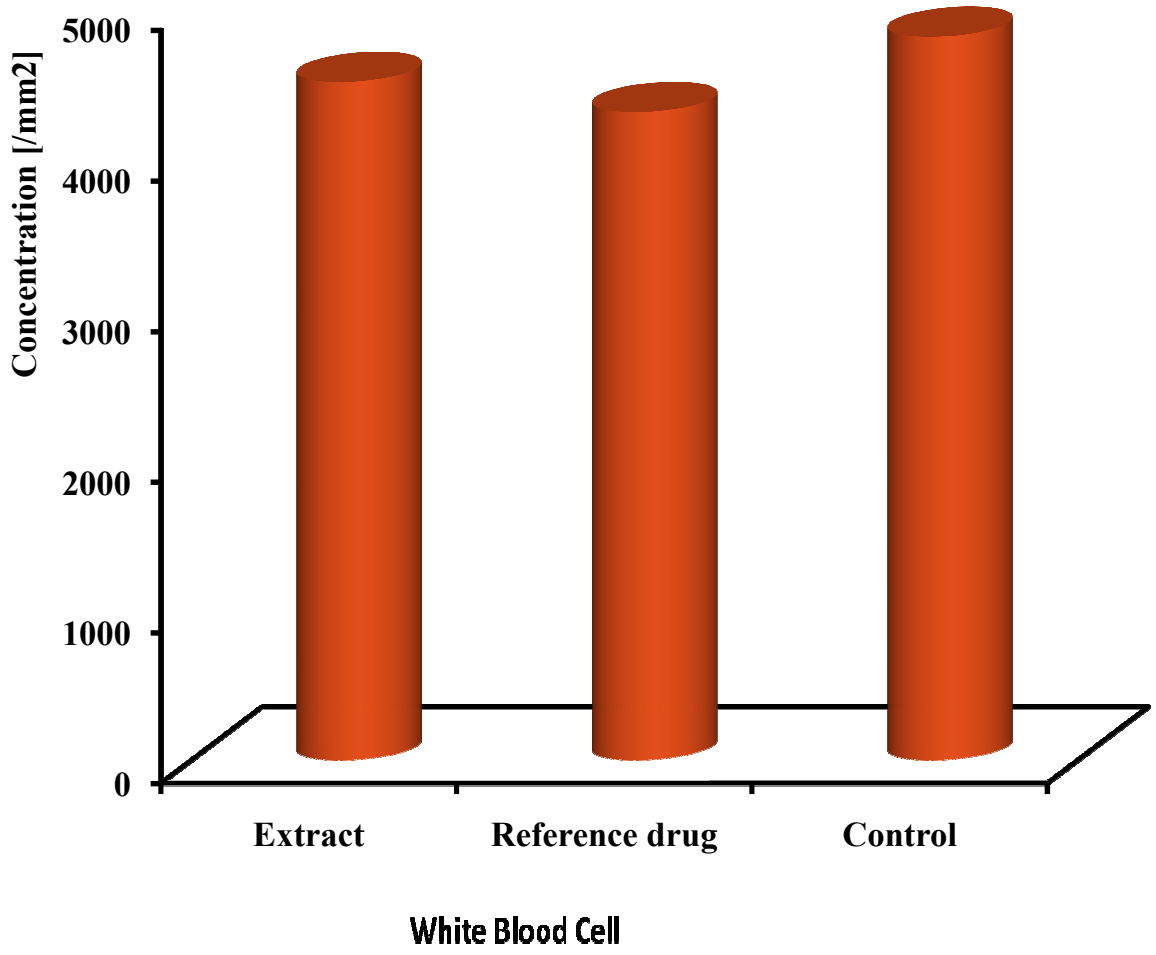
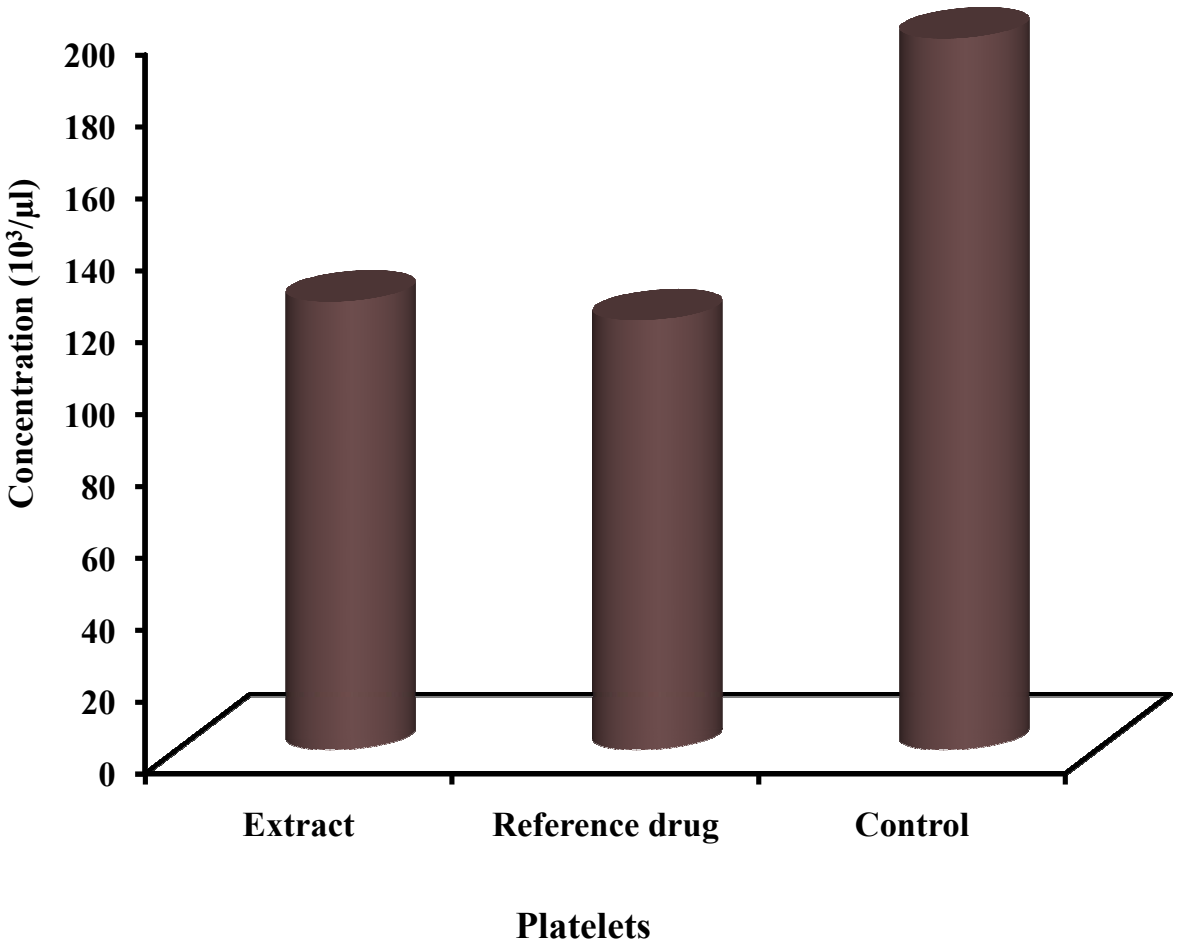


Figure 4.15B: *In vivo* effect of extract and reference drug on WBC (Sub-acute toxicity study). Values are expressed as mean ± SEM



Values are expressed as mean ± SEM

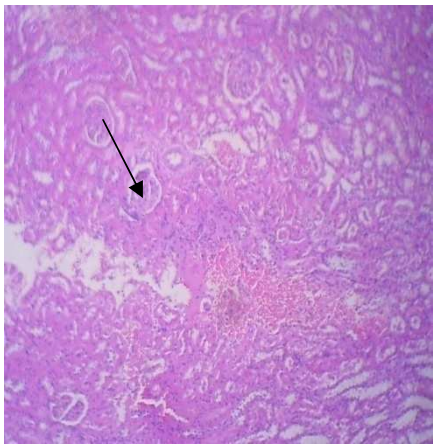
Figure 4.15C: *In vivo* effect of extract and reference drug on Platelets count (Sub-acute toxicity study)

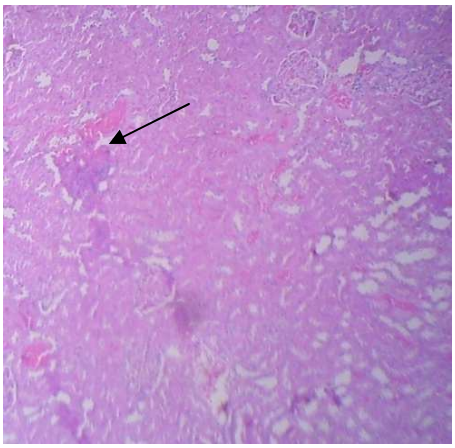
4.7.2.5 Results for histology

Table 4.6 is the histology result and Plates 4.24A and 4.24B are the photomicrographs of the effect of intramuscular administration of culture extract from *P. chrysogenum* and reference drug group for one week on rat kidney and liver as compared to the control group**.** A summary of the histology report of the kidney suggests papillary necrosis in groups administered with extract and mild oedema and congestion in groups administered with the reference drug. There was diffuse microvesicular change in the liver common to G1 (control) and G2 (extract) groups; this infers no damage or toxicity to the liver. However, there was portal aggregation of inflammatory cells as well as diffuse moderate microvesicular change in G3 (reference drug) group; the reference drug is a synthetic drug. However, results obtained 21 days post treatment from the groups treated with extract and reference drug shows mild oedema. This suggests that the extract did not cause an irreversible damage to the liver and kidneys and therefore is relatively safe for use.

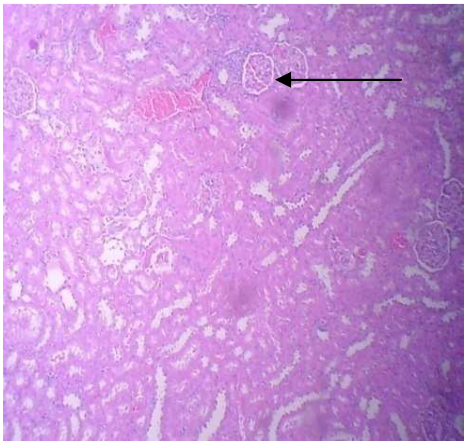
Table 4.5: Histology report of the effect of culture extract from *P. chrysogenum* and reference drug administration on rat kidney and liver (Sub-acute toxicity study)

ORGANS	GROUPS	OBSERVATION
Kidney	G1 (Control)	Vascular congestion
	G2 (Extract)	Papillary necrosis
	G3 (Reference drug)	Mild Oedema and congestion
Liver	G1 (Control)	Diffuse moderate microvesicular change
	G2 (Extract)	Diffuse moderate microvesicular change
	G3 (Reference drug)	Portal aggregation of inflammatory cells and diffuse moderate microvesicular change



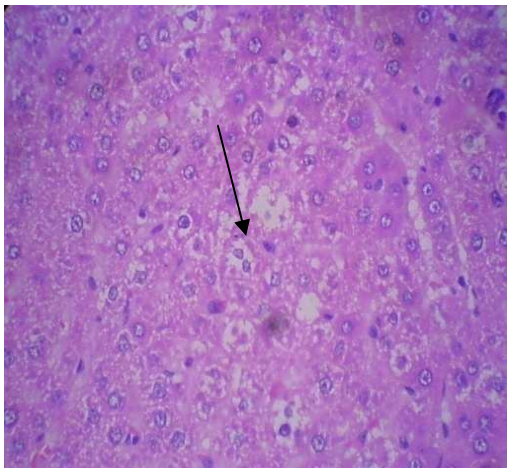


G1 **G2**

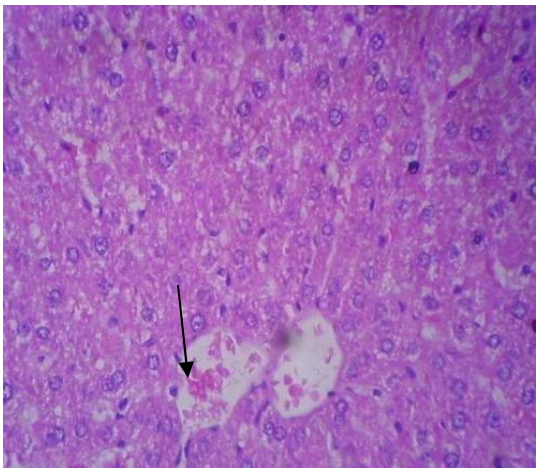


G3 **x100**

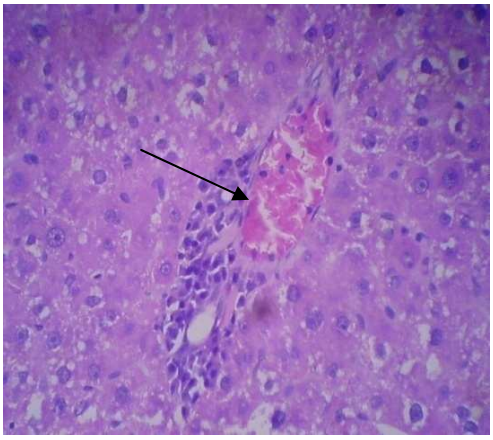
Plates 4.8A: Photomicrograph of the Kidney G1 (control) showing vascular congestion, G2 (extract) showing mild papillary necrosis, G3 (reference drug) showing mild oedema and vascular congestion.



G1



G2



G3 **x400**

Plates 4.8B: Photomicrograph of Liver G1 (Control) showing diffuse mild Microvesicular Change, G2 **(Extract) showing diffuse moderate micro vesicular change, G3 (Reference drug) showing portal aggregation of inflammatory cells and diffuse moderate micro vesicular change**

4.8 *IN VIVO* BIOCHEMICAL STUDIES TO DETERMINE THE EFFECT OF THE EXTRACT FOLLOWING *E.COLI* INFECTION AND EGG ALBUMIN- INDUCED INFLAMMATION

Results from *in vivo* biochemical study of the effects of the culture extract and reference drug on normal Wister albino rats following infection with *E.coli* and egg albumin induced inflammation shows that there were recorded deaths in all the animals not treated with the extract and reference drug (negative control). Cage side observation of the *E.coli* infected rats shows that there was diarrhoea, sleepiness and discharge from their skin and subsequently death. On the other hand, there was an increase in size up to 50% of the rats injected with egg albumin- an allergen which caused inflammation. Cage side observation shows difficulty in movement, sleepiness, drooping and subsequently death. However, there were no recorded deaths in the groups treated with the extract and reference drug.

4.8.1 *In vivo* biochemical effects of the extract on chemical analytes involved in liver function

Biochemical test of serum enzymes used as indicators of liver function – Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), bilirubin (total and direct), alkaline phosphatase (ALP), protein and albumin, were assayed. Results obtained after 24hours post-infection with *E.coli* and egg albumin induced inflammation(Table 4.7A and B) shows an increase ($p<0.05$) in the liver enzymes AST, ALT, ALP, as well as bilirubin (Total and Direct). These parameters gradually decreased in the course of treatment and returned to normal range by the eight day post treatment. There were no significant ($p>0.05$) differences in all the liver function markers when the tests were repeated 14days post treatment. Similarly, the protein and albumin levels were not altered.

Table 4.6A: Biochemical changes in liver function indices following *E.coli* infection, egg albumin induced inflammation and treatment with extract and reference drug.

	AST IU/L	ALT IU/L	BILIRUBIN µmol/L		ALK.PHOS IU/L
			Total	Direct	
Control	3.86 ± 1.02	3.86 ± 0.16	54.89 ± 0.06	41.38 ± 0.05	11.58±0.34
DAY 1					
E.coli	51.16 ± 0.84*	19.33 ± 0.50*	93.02 ± 0.13*	61.22 ± 0.06*	39.41±0.96*
Egg alb	45.92 ± 0.85*	21.74 ± 0.59*	85.16± 0.14*	41.72 ± 0.14*	43.41±0.62*
DAY 4					
EcRd	36.24 ± 0.18*	10.62 ± 0.08*	57.97 ± 0.08*	53.35 ± 0.15*	26.42±0.34*
EcEx	38.64 ± 0.91*	12.12 ± 0.37*	60.53 ± 0.10*	48.05 ± 0.04*	29.62±0.96*
EaRd	33.82 ± 0.77*	21.72 ± 0.41*	56.94 ± 0.14*	43.43 ± 0.16*	32.70±0.52*
EaEx	28.98 ± 0.79*	24.18 ± 0.93*	60.19± 0.09*	40.70± 0.13*	34.62±0.55*
DAY 8					
EcRd	12.08 ± 0.27*	4.13 ± 0.22*	53.35 ± 0.05	24.28 ± 0.04	12.39±0.17
EcEx	14.49 ± 0.87*	7.72 ± 0.32*	48.91± 0.10	22.74± 0.02	13.16±0.26
EaRd	15.80 ± 0.45*	3.86 ± 0.25*	52.84 ± 0.05	35.06± 0.06	13.33±0.30
EaEx	12.08 ± 0.27*	8.72 ± 0.34*	56.94± 0.14	40.01 ± 0.09	13.59±0.32
DAY 14					
EcRd	4.08 ± 0.79	4.01± 0.10	54.55 ± 0.13	34.78 ± 0.06	11.89±0.31
EcEx	3.99 ± 0.37	5.12 ± 0.37	53.77± 0.18	40.702± 0.10	11.33±0.33
EaRd	3.80 ± 0.56	3.97 ± 0.50	50.94 ± 0.15	39.88± 0.13	11.02±0.28
EaEx	4.58 ± 0.77	4.02 ± 0.22	55.02± 0.12	41.21 ± 0.04	12.09±0.34

*=Significant values ($p<0.05$) Values are expressed as mean ± SEM

Legend

E.coli – group infected with *E.coli*

Egg albumin – group induced with egg albumin

EcRd – group treated with Reference drug after infection with *E. coli*

EcEx – group treated with extract after infection with *E. coli*

EaRd – group treated with Reference drug after induction of inflammation with egg albumin

EaEx – group treated with extract after induction of inflammation with egg albumin

Table 4.6B: Biochemical changes in liver function indices following *E.coli* infection, egg albumin-induced inflammation and treatment with extract and reference drug.

	PROTEIN g/L	ALBUMIN g/L	BILIRUBIN µmol/L	
			Total	Direct
Control	60.73 ± 0.45	41.28 ± 0.31	54.89 ± 0.06	41.38 ± 0.05
DAY 1				
E.coli	59.76 ± 0.51	39.44 ± 0.37	93.02 ± 0.13*	61.22 ± 0.06*
Egg alb	51.15 ± 0.82	42.71 ± 0.92	85.16± 0.14*	41.72 ± 0.14
DAY 4				
EcRd	61.82 ± 0.56	39.49 ± 1.04	57.97 ± 0.08*	53.35 ± 0.15*
EcEx	56.18 ± 1.39	37.94 ± 0.39	60.53 ± 0.10*	48.05 ± 0.04*
EaRd	59.76 ± 0.57	40.40 ± 0.66	56.94 ± 0.14*	43.43 ± 0.16*
EaEx	54.31 ± 1.23	39.34 ± 0.42	60.19± 0.09*	40.70± 0.13
DAY 8				
EcRd	60.12 ± 0.22	41.81 ± 0.55	53.35 ± 0.05	24.28 ± 0.04
EcEx	62.07 ± 0.55	35.73 ± 0.88	48.91± 0.10	22.74± 0.02
EaRd	58.67 ± 1.27	41.53 ± 0.55	52.84 ± 0.05	35.06± 0.06
EaEx	59.77 ± 0.53	33.42 ± 0.84	56.94± 0.14	40.01 ± 0.09
DAY 14				
EcRd	64.12 ± 0.41	46.81 ± 0.55	51.35 ± 0.04	34.28 ± 0.01
EcEx	65.07 ± 0.51	42.73 ± 0.88	47.91± 0.11	32.74± 0.08
EaRd	68.67 ± 1.37	45.53 ± 0.55	48.84 ± 0.06	35.06± 0.03
EaEx	69.77 ± 0.43	43.42 ± 0.84	52.94± 0.12	39.01 ± 0.04

*=Significant values ($p<0.05$) **Values are expressed as mean ± SEM**

Legend

E.coli – group infected with *E.coli*

Egg albumin – group induced with egg albumin

EcRd – group treated with Reference drug after infection with *E. coli*

EcEx – group treated with extract after infection with *E. coli*

EaRd – group treated with Reference drug after induction of inflammation with egg albumin

EaEx – group treated with extract after induction of inflammation with egg albumin

4.8.2 *In vivo* Biochemical effects of extract on chemical analytes involved kidney function

In vivo biochemical changes in renal function markers - urea, creatinine and the electrolytes (Na^+, K^+, and Cl^-) following infection of experimental animals were monitored for 14 days. Results obtained 24 hours post-infection with *E.coli* and egg albumin induced inflammation (Table 4.8) shows a significant increase ($p<0.05$) in all the parameters assayed. These parameters gradually decreased in the course of treatment and returned to normal range when compared to the control groups by the end of the eight day post treatment. Tests results repeated 14 days post treatment shows no significant difference ($p>0.05$) between the control and the experimental groups.

Table 4.7: Biochemical changes in renal function indices following *E.coli* infection, egg albumin induced inflammation and treatment with extract and reference drug

	Na^+ mMol/L	K^+ mMol/L	Cl^- MEq/L	Ur mMol/L	Cr µMol/L
Control	109.2 ± 1.30	4.2 ± 0.08	98 ± 0.84	9.72 ± 0.09	75.46 ± 0.65
DAY 1					
E.coli	120.2 ± 1.48*	4.2 ± 0.07	107 ± 0.55	12.77 ± 0.08*	120.74 ± 0.53*
Egg alb	128.4 ± 1.14*	3.1 ± 0.13	102 ± 0.55	12.66 ± 0.09*	112.12 ± 0.06*
DAY 4					
EcRd	140.2 ± 1.31*	4.6 ± 0.11	96 ± 1.14	12.52 ± 0.08*	112.12 ± 0.44*
EcEx	123.2 ± 0.84*	5.1 ± 0.08	112 ± 1.14	11.61 ± 0.10*	103.49 ± 0.84*
EaRd	117.2 ± 0.84*	4.6 ± 0.11	98 ± 0.84	12.22 ± 0.09*	125.05 ± 0.64*
EaEx	128.2 ± 0.84*	4.3 ± 0.14	94 ± 0.84	11.76 ± 0.25*	120.74 ± 0.62*
DAY 8					
EcRd	128.4 ± 1.14*	4.3 ± 0.22	95 ± 0.84	10.05 ± 0.27*	94.87 ± 0.29*
EcEx	115.2 ± 1.30*	4.3 ± 0.07	92 ± 0.70	9.98 ± 0.26	81.93 ± 0.66*
EaRd	122.4 ± 1.14*	4.5 ± 0.08	95 ± 0.84	11.21 ± 0.12*	97.02 ± 0.77*
EaEx	127.2 ± 0.84*	4.5 ± 0.08	108 ± 1.14	9.98 ± 0.21	88.43 ± 0.66*
DAY 14					
EcRd	118.4 ± 1.10	4.2 ± 0.22	95 ± 1.14	9.05 ± 0.07	78.87 ± 0.10
EcEx	110.1 ± 1.30	4.1 ± 0.07	92 ± 0.70	9.18 ± 0.10	74.93 ± 0.09
EaRd	112.4 ± 0.84	4.3 ± 0.08	95 ± 0.70	9.21 ± 0.09	76.02 ± 0.08
EaEx	111.8 ± 0.84	4.5 ± 0.08	98 ± 0.84	9.58 ± 0.11	75.43 ± 0.15

*=Significant values ($p<0.05$) **Values are expressed as mean ± SEM**

Legend

E.coli – group infected with *E.coli*

Egg albumin – group induced with egg albumin

EcRd – group treated with Reference drug after infection with *E. coli*

EcEx – group treated with extract after infection with *E. coli*

EaRd – group treated with Reference drug after induction of inflammation with egg albumin

EaEx – group treated with extract after induction of inflammation with egg albumin

4.8.3 *In vivo* Biochemical effects of extract on antioxidant enzymes and oxidation product

Test results after 24 hours post-infection with *E.coli* and egg albumin induced inflammation (Table 4.9) shows a significant increase ($p<0.05$) in the activities of enzymes involved in oxidative stress following infection when compared to the control. There was also an increase in MDA- a product of lipid peroxidation and is indicative of oxidative stress. These enzymes and MDA gradually decreased in the course of treatment and returned to normal when compared with the control group by the end of the eight day post treatment. However tests repeated 14 days after shows no significant ($p>0.05$) difference in all the enzymes and MDA monitored.

Table 4.8: Biochemical changes in antioxidant enzymes and oxidation product following *E.coli* infection, egg albumin induced inflammation and treatment with extract and reference drug

	CAT µMol/ml	SOD mg/ml	GSH µMol/mL	MDA nMol/ml
Control	2.75 ± 0.36	117.63 ± 0.99	1.54 ± 0.03	5.29 ± 0.14
DAY 1				
E.coli	7.45 ± 0.22*	311.08 ± 0.64*	2.32 ± 0.11*	10.64 ± 0.16*
Egg albumin	6.24 ± 0.09*	269.34 ± 0.94*	2.13 ± 0.06*	7.73 ± 0.15*
DAY 4				
EcRd	3.93 ± 0.21*	193.47 ± 0.58*	1.05 ± 0.06*	10.56 ± 0.06*
EcEx	5.55 ± 0.09*	208.64 ± 0.77*	1.31 ± 0.13*	14.15 ± 0.46*
EaRd	3.75 ± 0.21*	166.92 ± 1.00*	1.13 ± 0.01*	15.69 ± 0.35*
EaEx	5.12 ± 0.16*	185.88 ± 0.39*	2.07 ± 0.03*	25.5 ± 0.32*
DAY 8				
EcRd	2.85 ± 0.14	128.98 ± 0.39*	0.68 ± 0.02	3.33 ± 0.07
EcEx	3.14 ± 0.21	144.15 ± 0.57*	0.94 ± 0.01	4.46 ± 0.08
EaRd	3.15 ± 0.05	121.39 ± 0.40*	0.61 ± 0.01	2.69 ± 0.08
EaEx	2.68 ± 0.08	132.77 ± 0.42*	1.08 ± 0.03	2.69 ± 0.10
DAY 14				
EcRd	2.65 ± 0.11	118.98 ± 0.17	1.38 ± 0.02	4.23 ± 0.25
EcEx	3.01 ± 0.09	114.15 ± 0.27	1.64 ± 0.03	5.46 ± 0.22
EaRd	3.11 ± 0.04	119.39 ± 0.30	1.54 ± 0.02	3.99 ± 0.34
EaEx	2.48 ± 0.12	122.77 ± 0.62	1.48 ± 0.01	4.19 ± 0.16

*=Significant values ($p<0.05$) Values are expressed as mean ± SEM

Legend

E.coli – group infected with *E.coli*

Egg albumin – group induced with egg albumin

EcRd – group treated with Reference drug after infection with *E. coli*

EcEx – group treated with extract after infection with *E. coli*

EaRd – group treated with Reference drug after induction of inflammation with egg albumin

EaEx – group treated with extract after induction of inflammation with egg albumin

4.8.4: *In vivo* biochemical effect of extract and reference drug on haematology indices

Twenty four-hour post-infection analysis of haematology indices shows a significant decrease ($p<0.05$) in PCV, Hb and platelet values and a significant increase ($p<0.05$) in WBC and WBC differentials count in groups infected with *E.coli* and egg albumin-induced inflammation (Table 4.10). Results obtained four days after the commencement of treatment in the group when compared to the 24hour results show a significant decrease ($p<0.05$) in PCV and Hb concentration indicating anaemia, also a significant decrease in platelets count was observed indicating thrombocytopenia. A significant increase ($p<0.05$) in PCV, Hb and platelets count was observed eight days from the administration of the extract. Results obtained 14 days after shows normal haematology indices.

Table 4.9: Changes in haematology indices following *E. coli* infection, egg albumin induced inflammation and treatment with extract and reference drug

	PCV	HB	Platelets	WBC	N	L	M
	%	g/L	103/L	/mm2 (x103)	%	%	%
Control	40.2±1.30	13.4±0.43	196 ±1.92	4.1 ± 70.71	52 ±1.41	48 ±1.41	0±0.00
DAY 1							
E.coli	22.4±0.55*	7.5±0.18*	173 ±2.88*	6.4 ±70.71*	33 ±1.22*	65 ±1.22*	2 ±0.0
Egg alb	34.2±0.84*	11.4±0.28*	178 ±2.24*	5.1 ±70.71*	38 ±1.41*	61 ±1.22*	1 ±0.0
DAY 4							
EcRd	26.4±2.07*	8.8±0.69*	159 ±2.12*	6.8 ±70.71*	48 ±1.00*	52 ±1.00*	0 ±0.0
EcEx	25.4±1.14*	8.5±0.38*	161 ±1.30*	8.5 ±70.71*	40 ±1.41*	59 ±0.71*	1 ±0.0
EaRd	24.4±0.89*	8.1±0.30*	161 ±1.14*	9.1 ±70.73*	51 ±1.58*	47 ±0.71*	2 ±0.0
EaEx	21.4±0.89*	7.1±0.30*	162 ±1.30*	8.2 ±70.74*	31 ±1.73*	69 ±1.22*	0 ±0.0
DAY 8							
EcRd	27.2±0.84*	9.1±0.28*	150 ±2.13*	4.4 ±70.71	50 ±1.58	49 ±1.22	1 ±0.0
EcEx	29.4±1.52*	9.8±0.51*	158 ±1.14*	5.3 ±70.71	39 ±0.71	61 ±1.22	0 ±0.0
EaRd	31.2±0.84*	10.4±0.28*	154 ±1.52*	8.7 ±70.71	49 ±1.22	50 ±1.41	1 ±0.0
EaEx	26.4±1.14*	8.8±0.38*	159 ±1.30*	5.5 ±70.71	33 ±1.58	67 ±0.71	0 ±0.0
DAY 14							
EcRd	36.6±1.30	12.1±0.43	191 ±1.92	4.5 ±70.71	48 ±1.41	51 ±1.41	1 ±0.0
EcEx	38.3±1.31	12.8±0.44	197 ±1.93	4.6 ±70.72	51 ±1.42	49 ±1.42	0 ±0.0
EaRd	37.2±1.32	12.4±0.45	193 ±1.94	4.2 ±70.73	56 ±1.43	44 ±1.43	0 ±0.0
EaEx	39.3±1.33	13.1±0.46	199 ±1.95	4.4 ±70.74	57 ±1.44	42 ±1.44	1 ±0.0

*=Significant values ($p<0.05$) Values are expressed as mean ± SEM

Legend

E.coli – group infected with *E.coli*

Egg albumin – group induced with egg albumin

EcRd – group treated with Reference drug after infection with *E. coli*

EcEx – group treated with extract after infection with *E. coli*

EaRd – group treated with Reference drug after induction of inflammation with egg albumin

EaEx – group treated with extract after induction of inflammation with egg albumin

4.9 COAGULATION TEST

4.9.1 Whole blood

Thrombin is a normal activation product of prothrombin; it is a platelet activator in the coagulation cascade and also converts fibrinogen to fibrin. Figure 4.16 shows the effect of varying concentrations of the culture extract of *P. chrysogenum* and reference drug on whole blood clotting process. Normal blood clotting time was observed at 6 minutes. However addition of different concentrations of the extract and reference drug caused an increase in clotting time. At 5 mg/mL of the reference drug, clot formation was not observed. Similarly, initiation of clot formation was prevented at 6 mg/mL of the extract added.

4.9.2 Thrombin

Figure 4.17 shows the effects of varying concentrations of "HemoStat" (Bovine) thrombin on citrated plasma on the initial increase in absorbance per 3 seconds during coagulation determined at room temperature. The absorbance of 1: 10 diluted solutions of plasma coagulation control was shown to increase rapidly after the addition of thrombin in the reaction medium the rate of increase in absorbance at 400 nm was found to be proportional to the amount of thrombin added. This allows a simple and accurate procedure of thrombin activities in test procedures.

Figure 4.18 shows the effect of varying concentrations of the culture extract of *P. chrysogenum* and reference drug when incubated with thrombin – a serine protease involved in thrombosis, an inflammatory disorder. Normal thrombin time (visual observation for clot formation) was obtained at 14 – 16 seconds with repeated samples at 37^{O}C. However when these samples were incubated with different concentrations of the extract, there was an increase in thrombin coagulation time to 248.5 second and 293.8 second at concentrations of 10 and 20 mg/mL for the reference drug and extract respectively. There was no visible clot

formation observed at concentrations above 10 and 20 mg/mL of the reference drug and extract respectively.

Similarly, absorbance of clot formation monitored at room temperature of each thrombin time test shows a significant decrease ($p<0.05$) in absorbance with corresponding increase in the concentration of extract used (figure 4.19). Therefore, results obtained using a mechanical aid for thrombin time (spectrophotometer) corresponded with the visual aid.

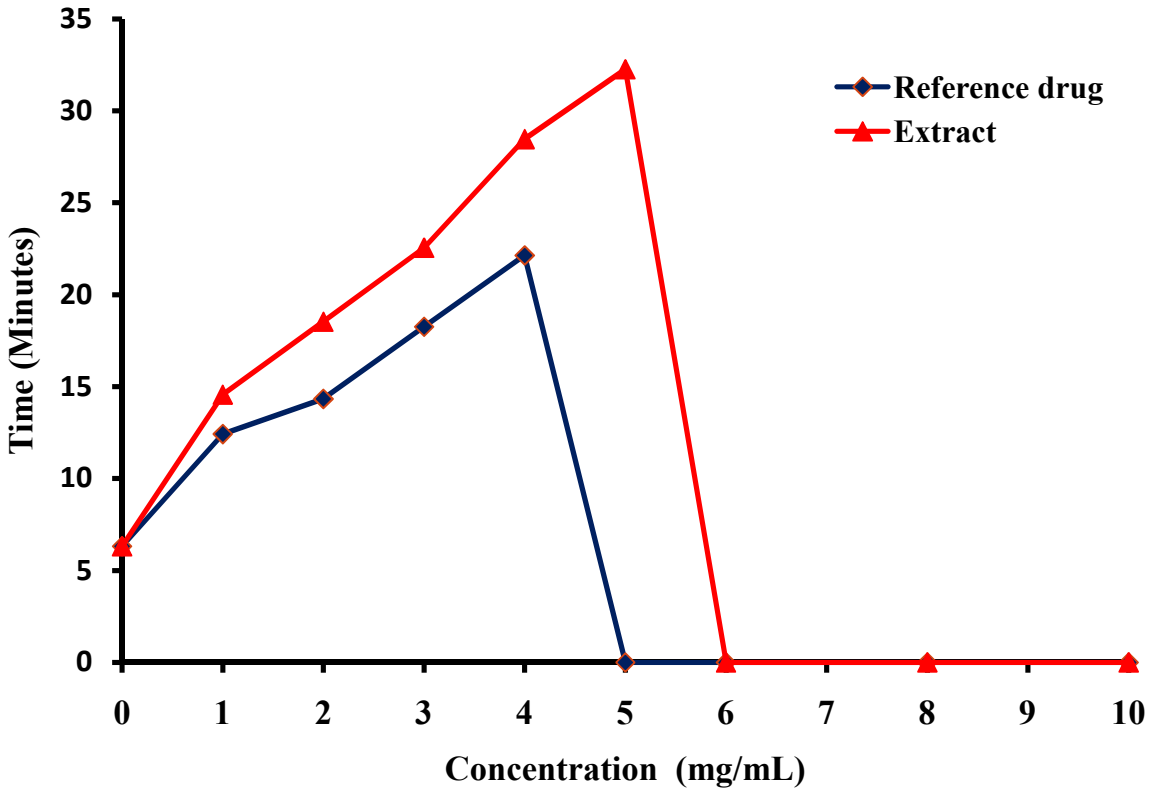


Figure 4.16: Inhibitory effect of culture extract of *P. chrysogenum* grown on selected agro-waste and Reference drug on Whole Blood clotting process.

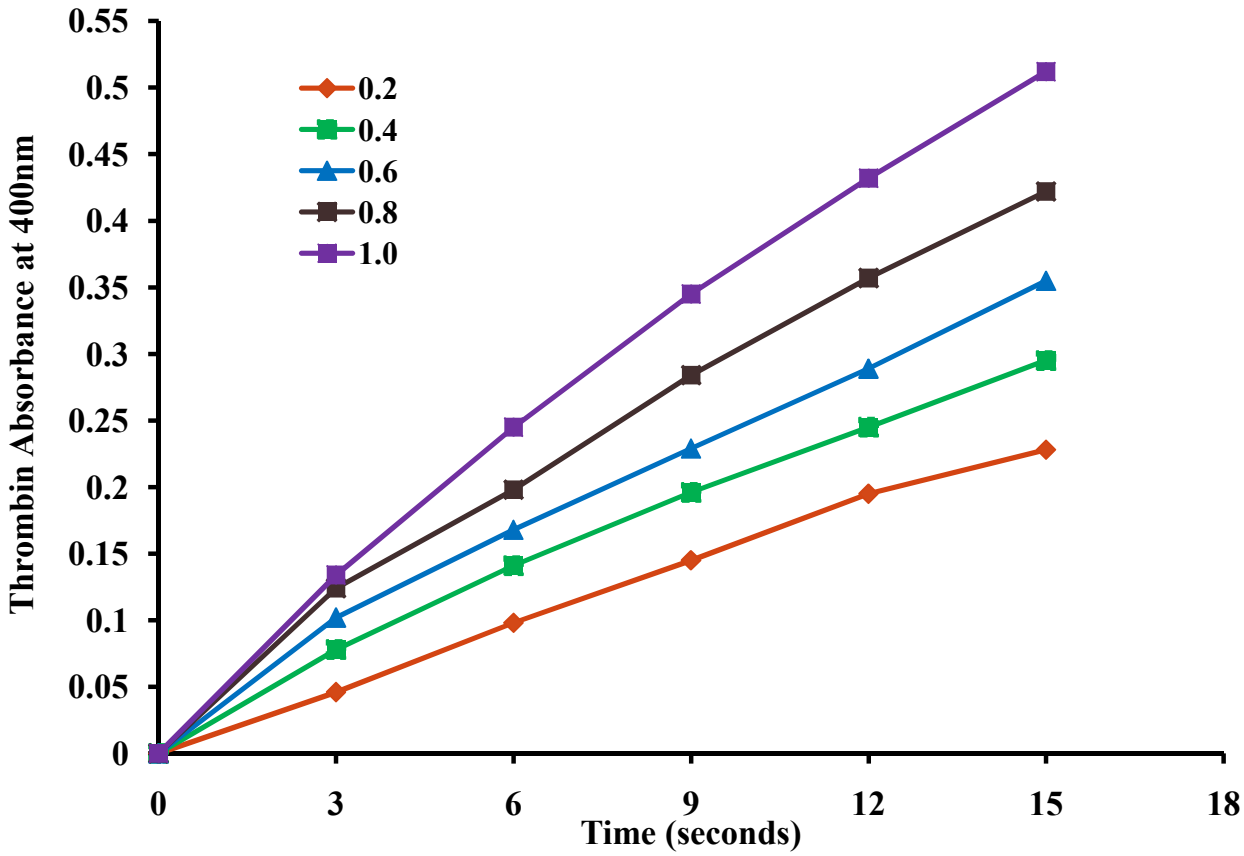


Figure 4.17: Effect of increasing "HemoStat thrombin" on the initial rate clot formation on citrated plasma at room temperature monitored at 400 nm at intervals of 3 seconds for 15 seconds.

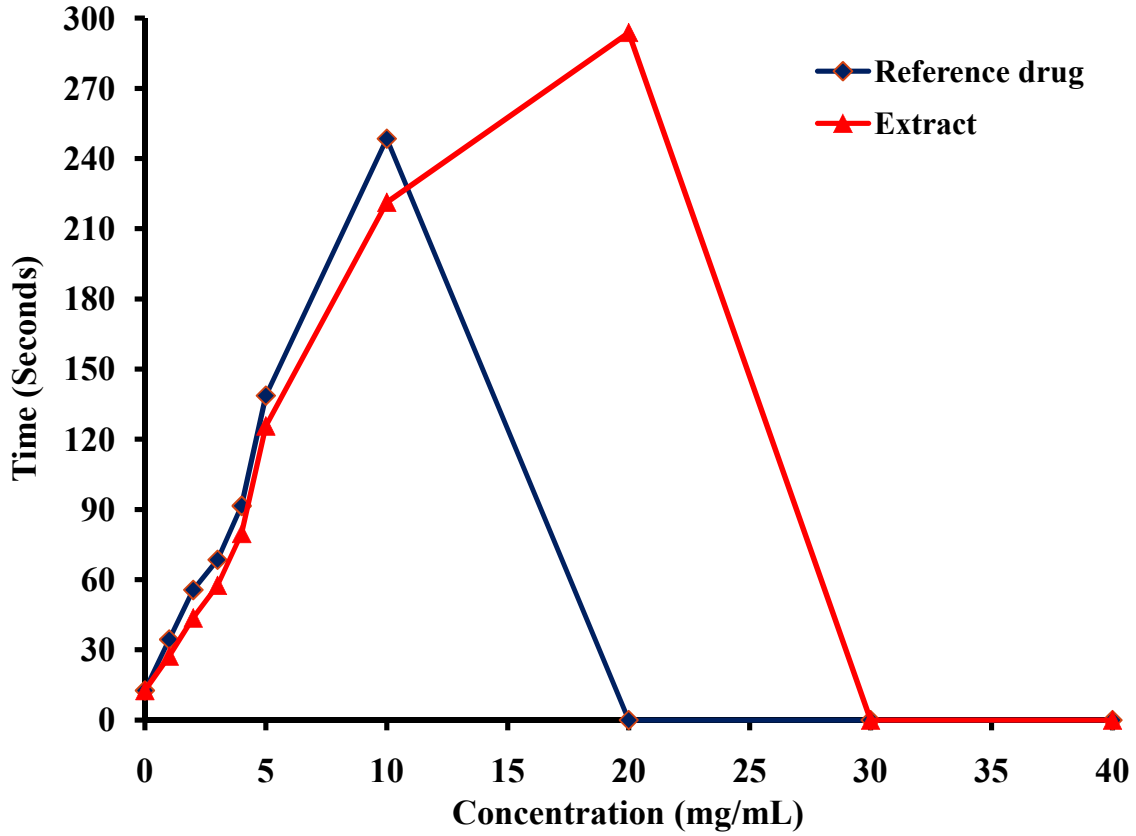


Figure 4.18: Inhibitory effect of extract of *P. chrysogenum* grown on selected agro-waste and Reference drug on Thrombin monitored at 37^{O}C.

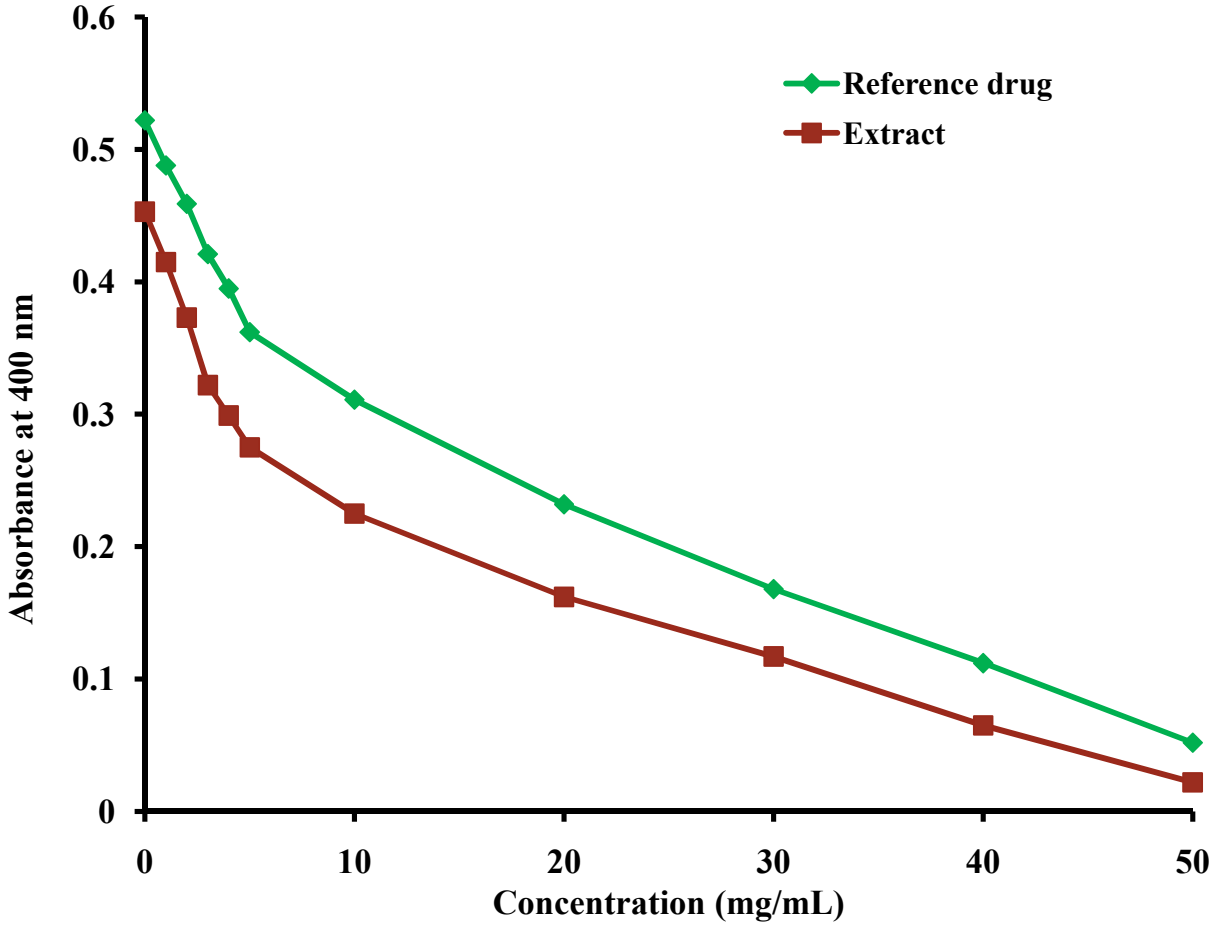


Figure 4.19: Inhibitory effect of varying concentrations extract of *P. chrysogenum* grown on selected agro-waste and Reference drug on Thrombin monitored at room temperature.

CHAPTER FIVE

DISCUSSION

The main focus of this research project is to add value to some agro-wastes, by biotransformation through biotechnology. This technique involves fermentation using microorganisms which results in production of useful metabolites that can be of benefit to humanity. Such microorganisms include fungi or bacteria. However micro fungi is of primary interest in this project because the products of fermentation including enzymes and other metabolites are easily obtained in the culture media as against bacteria in which some of the metabolites are membrane bound and more difficult to obtain.

In the process of growth, some micro-fungi produce primary metabolites (e.g. enzymes) which they use to digest the substrate as energy source for growth and subsequently produce secondary metabolites (e.g. antibiotics) which serve other purposes like defence. Some of the secondary metabolites used by the organisms are also beneficial to man. One of such secondary metabolite is penicillin, specifically, produced by *P.chrysogenum*. *P. chrysogenum* was the first micro fungus discovered to have antibacterial activity, it is used in the industrial production of antibiotics e.g. penicillin (Prescott *et al.,* 2002). In the course of other studies with biomass (wood wastes) in the department of Biochemistry, University of Lagos, it was observed that the extract from the culture medium of *P. chrysogenum* grown on agro-wastes has antibiotic activity. This triggered off the interest in this research in which carbon and energy sources for the growth of *P. chrysogenum* grown are agro-wastes as against industrial production of penicillins in which synthetic chemicals like glucose and lactose are used as carbon and energy sources. The substrates used in this study are more cost effective, sustainable and at the same time environmentally friendly.

The approach was to isolate *P.chrysogenum* from wood waste which the micro fungus uses as its source of nutrients for growth. Then the isolated *P.chrysogenum* was grown on agro-wastes (sawdust, cassava peel, corncob and sugarcane pulp) for the production of the antibacterial extract. The antibiotic so produced was characterized by physical and chemical methods. The physical methods used include TLC and HPLC while the chemical methods involved *in vivo* analysis of changes in chemical analytes in the blood and plasma of experimental rats after administration of the extract under well defined experimental conditions.

ISOLATION OF *P. CHRYSOGENUM*

Colonies of micro fungi were observed in Potato dextrose agar (PDA) and Czapek-Dox agar CDA media inoculated with stock cultures of decomposing saw dust. Sub-culturing of the respective colonies in the same commercial culture media resulted in production of pure isolates. It was observed in the course of experimentation that PDA supported the growth of the microorganisms better than CDA. This informed the decision to use PDA for sub-culturing and maintaining the organisms on slant at $4^{O}C$. Since incubation of the culture was done aerobically, all the emergent organisms on the agar plates would be aerobic or probably facultative in nature. Physical observation of plates containing colonies of *P.chrysogenum* morphologically, showed that they were initially white, but became flaky and ash (blue-green) in colour as they mature.

THE USE OF AGRO-WASTES AS SUBSTRATES FOR THE GROWTH OF *P. CHRYSOGENUM*

In order to make the project cost effective, modified culture media were formulated for the growth of *P.chrysogenum* for subsequent work using cassava peels, corncob, saw dust and sugarcane pulp as carbon and energy sources. Different parameters measured to monitor the growth of the microorganism include changes in the turbidity of the culture media, protein

yield, mycelia weight measurement and analysis of consumption of carbohydrate by the organism. The information derived from these measurements is discussed subsequently.

Turbidity of media monitored against a cell-free blank for 7 days showed remarkable increase in the turbidity of the various media containing different agro-wastes. This is as a result of increase in the population of the organism. This was confirmed by appreciable changes in daily optical density measurements which were comparable to the synthetic media enriched with glucose and lactose. The growth rate of the microorganism in the culture medium containing saw dust and corn cob showed lag phase in the first two days of incubation. This is probably due to the fact that saw dust and corn cob have more complex structures than others, such that simple sugars were not available for immediate use for energy and growth as against the others which may not be as complex. This is clearly affirmed by the observation of lag phase in the first two days of experimentation as mentioned earlier, which indicates that the enzymes needed for the breakdown of both substrates are inducible and the delay in hydrolytic activity corresponds to the period when chemical processes of chemical induction were operative. Interestingly, as the hydrolysis of both substrates (corncob and sawdust) started, turbidity measurement show that both of them supported the growth of the organism optimally on the 3rd day well above others, with the corn cob giving the highest value.

In the case of protein measurement, cassava peel gave the highest value followed by corncob and saw dust. Changes in protein values of cultures containing cassava peels, corn cob and sawdust were 1.9, 1.5 and 1.23 times higher than that obtained for glucose respectively. Also, mycelia measurement showed that highest values were obtained with media containing corncob, cassava and saw dust. These are positive indicators that these agro-wastes are suitable substrates for the growth of *P. chrysogenum*, particularly cassava peels and corncob which consistently gave optimum values within 3 days of the respective measurements. It is noted in this study that glucose and lactose used commercially as substrates for growth of *P.*

chrysogenum in industrial production of penicillin are not as efficient as agro-wastes based on the quantitative values of the parameters used in evaluating the growth rate. This lies in the fact that many complex enzyme processes come into effect in the process of breakdown of the agro-wastes to simple sugars. Therefore, the sugar needed for energy and growth are generated in more gradual and sustainable manner as against glucose or lactose which will use specific enzymes whose activities started much faster than in the case of the agro-wastes and which could be subject to product inhibition.

The differences in the complexity of the carbon sources could account for the disparity in the growth of the organism in the different media. In a comparative study on the growth and β-galactosidase activity of the penicillin producer industrial *P. chrysogenum* NCAIM 00237 strain using different carbon sources, good growth was observed using glucose, sucrose, glycerol and galactose, while growth on lactose was substantially slower (Nagy et al., 2001). However in this work, mycelia production by *P. chrysogenum* was found to be higher in media containing corn cob and cassava peels (30 and 80%) when compared to glucose and lactose respectively. It is interesting to note that the days where mycelia growth was highest did not record a corresponding increase in protein yield. This shows that cellular activity (e.g. enzyme activity) is not directly proportional to protein concentration.

Analyses of cell-free media enriched with agro waste shows that they all contain sugars. This is more of a metabolic process whereby the organism utilizes the carbohydrate present in the media as energy and carbon sources for growth. Cassava peels contain the highest amount of total sugar compared to all the other substrates used in this study. Total sugar content of all the culture media steadily decreased as fungal incubation and mycelia weight increased and was still available in culture media containing cassava peels and sugarcane pulp 21 days after fermentation by the organism. This indicates that the organism hydrolyzed the carbohydrates and utilized the available simple sugars for growth. Since glucose is more readily assimilated

and metabolized by cells, there is greater tendency for organisms to grow very rapidly in media containing the simple sugar than that which contain cellulose or sawdust. A similar observation was reported by Park *et al*., (2002) in the study of exo-biopolymer production and mycelia morphology in *Cordyceps militaris*. The least growth was obtained with sawdust. Very rapid decrease in sugar content of the culture media containing sawdust was noted during the growth of the fungus; it was such that there was no detectable sugar in the media after the 15^{th} day of incubation. This is consistent with the downward trend observed in mycelia weight and protein yield after 15 days. Also it shows that the organism has stopped growing and is beginning to deteriorate hence a slowdown in metabolic activities.

From the growth studies above, cassava peels is the most viable substrate for the growth of the fungus used in this work. This is very significant considering the fact that cassava is a staple food in Nigeria and its shavings are produced in very large quantities during processing. Therefore, rather than using synthetic media containing glucose, low-cost microbial media can be formulated with cassava peels or corncobs as the sole carbon and energy sources. The advantage over the industrial synthetic substrates is that cassava peel is readily available, sustainable, cost-effective and environmentally friendly.

OPTIMUM CONDITIONS FOR GROWTH OF *P. CHRYSOGENUM* AND PRODUCTION OF ANTIBIOTICS

The average mycelia weight of the organisms and change in turbidity were measured as indicators of growth and were used to determine the optimum pH and temperature of growth for the organism. Measurement of these parameters at various pH and temperature shows that maximum values were obtained at pH 6.5 and 25^0C. This is therefore considered as the Optimum pH and temperature of growth for the organism. Temperature and pH are the principal abiotic parameters used for determining the viability of life as a whole including microorganism (Plaza *et al*., 2003). This result agrees with the findings of Nagamune *et al*.,

(1988) that the maximum production of extract of *P. chrysogenum* with antibacterial activity was achieved when cultivation temperature were kept at 25±1°C.

The growth of *P. chrysogenum* is related to its production of penicillin. Optimum production of antibiotics was achieved in culture media containing sugar cane pulp and cassava peels at pH 6.5 and temperature of 25^0C.

PHYSICAL PROPERTIES OF THE CRUDE AND PARTIALLY PURIFIED EXTRACT

TLC analysis of the crude fraction of the extract indicated the presence of two peaks. But after partial purification and repeat of the TLC on silica gel, one spot each on the partially purified extract and control was observed. This was confirmed using HPLC. HPLC analysis of the crude extract, partially purified extract and reference drug revealed the presence of 2 pronounced peaks in the crude extracts, a single peak in the partially purified extract as well as the reference drug. The multiple smaller peaks observed in the crude extract might be due to the presence of hydrolytic enzymes.

MODIFICATION BY GENETIC MUTATION FOR HYPER-PRODUCTION OF ANTIBIOTIC

A high yielding strain is a prerequisite of any good antibiotic producing process. Continuous modification of process conditions will result in steady increases in yield. Random mutagenesis and subsequent screening are key strategies in strain yield improvement.

Efforts at improving penicillin yields have centered on growth optimization, development of available strains of *P. chrysogenum* by classical mutagenesis procedures, and the search for better strains of the organism (Chuan-Bao *et al.,* 2002; Veehuis, 2002). Although, these have led to the availability of cheaper and effective penicillins, the strategy in this study is to transform the *P. chrysogenum* which is a wild strain from our environment and use the low-

cost fermentation substrates to investigate its potential for higher yield of the antibiotics. UV radiation is a classical technique used for genetic modification (Karanam and Medicherla, 2008) and has been used in this study. The products of modification UVP2 and UVP3 had a 1% survival rate after UV radiation for 20 and 25 minutes respectively and therefore were considered transformed strains. HNP1 and HNP2 were strains obtained after treatment of cell suspension with $NaNO_3$ for 40 and 50 minutes respectively. There were more growth in plates treated with $NaNO_3$ for 10 and 20 minutes compared to the wild strain. This could be attributed to the fact that the organism was using the nitrate present in the solution as a source of nitrogen for growth, instead of as a mutagen.

Antibacterial production by UVP2 and UVP3 transformed strains gave a higher yield of 70% more than the parent strain which indicates that the modification was effective. A similar method of UV radiation was used by Karanam and Medicherla (2008) to enhance lipase production by mutation induced *Aspergillus japonicas.*

BIOCHEMICAL PROPERTIES OF FUNGAL ANTIBACTERIAL EXTRACT *IN VITRO*

In order to determine the biochemical properties of the extract, qualitative and quantitative analysis of its potential to inhibit clinical isolates of bacteria were carried out. Culture extracts of both the wild and modified (mutant) strains were tested against some clinical bacteria isolates (*E. coli* and *B. subtilis*) and observed for antibacterial activity. The extracts exhibited significant antibacterial effects against clinical isolates of *E. coli* and *B. subtilis*. It is interesting to note that the organism produced effective antibiotics in media containing refined sugars (glucose and lactose) as well as those containing the agro-wastes (cassava peels, sugarcane pulp, corncob and sawdust) as sole carbon and energy sources. Extracts from media containing sugarcane pulp and cassava peels, had higher antibacterial effects than culture extracts from media containing equivalent amount of glucose and lactose. This

confirms the previous conclusion stating that the agro-wastes are superior fermentation substrates than the synthetic ones.

The results also show that both the reference drug and culture extracts are not effective against the clinical isolates of *Pseudomonas aeruginosa* and *Proteus mirabilis*. This result is explained by the fact that several strains of *P. aeruginosa* and *P. mirabilis* isolated in different hospitals have been reported to produce β-lactamases which cleaves the β-lactam ring in penicillins and hence render them ineffective as antibiotics (Mugnier *et al*., 1996; Naas *et al*., 1999; Iyobe *et al*., 2002, Hermandez *et al.,* 2003; de Cueto *et al.,* 2006).

In order to establish the presence of β-lactam ring in this extract, qualitative test with β-lactamase identification sticks coated with nitrocefin as substrate were used to confirm the presence of β-lactamase in a 24 hour cell free culture of *P. aeruginosa* and *P. mirabilis.* The two bacteria were found to produce β- lactamase enzymes which cleave the β-lactam ring and thereby nullify the effect of the β-lactam antibiotics. The beta-lactam ring is part of the structure of β-lactam antibiotics, principally the penicillins, cephalosporins, carbapenems and monolactams. This implies that *P. chrysogenum* produces β-lactam antibiotics. Several strains of *Pseudomonas aeruginosa* have been reported to produce β-lactamases. This also confirms the presence of β-lactamase producing bacteria amongst clinical isolates from Nigeria. This is consistent with the fact that most strains of *P. chrysogenum* produce β-lactam antibiotics, mainly penicillins. These results show that *P. chrysogenum* used for this study produces β-lactam antibiotics and the failure of the extract to have effect on the clinical isolates of *P. aeruginosa* and *P. mirabilis* is explained by the presence of β –lactamase produced by these bacteria. These findings are consistent with the fact that most strains of *P. chrysogenum* produce β-lactam antibiotics, mainly penicillin (de Hoog *et al.,* 2000).

Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of the extract inhibiting the visible growth of each organism. The MIC against the susceptible organisms was higher for the standard drug, followed by antibiotics produced in media containing sugarcane pulp and cassava peels, the least was that obtained in media containing sawdust. The fact that the antibacterial effect of some of the extracts is comparable with that obtained with commercial benzyl penicillin indicate the prospect of *P.chrysogenum* isolated in Lagos as potential source of commercial antibiotics.

ANALYSIS OF BIOCHEMICAL CHANGES IN PLASMA ANALYTES FOLLOWING ACUTE AND SUB-ACUTE TOXICITY STUDIES, *E. COLI* INFECTION AND EGG ALBUMIN INDUCED INFLAMMATION

While the earlier determinations of the biochemical characteristics of the extract were carried out *in vitro*, using microorganisms, the studies in this section deal with experiments that evaluated the effect of the extract in living organisms (*in vivo*). The first experiment, known as acute toxicity study, was carried out by administering the extract on the animals to obtain information useful in choosing doses for repeat-dose studies. It is also aimed at providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity.

Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. It is usually necessary for any pharmaceutical intended for human use. The acute toxicity test carried out on mice with the extract provides evidence that the extract is relatively non-toxic at a given dose of 1500 mg/kg body weight; this produced neither side-effect nor death of the animals. In the acute toxicity study of the extract, no changes in the behavior and the sensory nervous system responses were observed in the animals. Also no adverse gastrointestinal effects were observed in male and female mice used in the experiment. Since there were neither visible side effects nor

death in the acute toxicity study, a sub-acute toxicity study was carried out to find out the long term effect of administration of the extract under normal conditions. Similarly, the antibiotic and anti-inflammatory properties of the extract were monitored after infecting a group of the animals with *E.coli* and inducing inflammation with egg albumin in another group. Parameters used to monitor the effect of the extract include biochemical assay of plasma analytes involved in liver and kidney functions, haematological indices as well as histological studies of the hepatic and renal organs of the animals. The observed findings are diccussed subsequently:

Effect of the extract on chemical analytes involved in liver function

Because the liver is the seat of protein synthesis and drug metabolism, the measurement of the activities of marker and diagnostic enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in liver tissues plays a significant role in diagnosis and disease investigation and in the assessment of drug or plant extract safety or toxicity. It is well known that an increase in the activity of liver enzymes in the serum is indicative of cellular leakage and loss of functional integrity of cellular membrane of the liver (Iweala and Okeke 2005). Among the different isoenzymes of ALP, the one originating from the liver has been shown to be the main component of the serum (Tietz, 2000). Serum ALT levels increase rapidly when the liver is damaged by any cause including: hepatitis, hepatic cirrhosis, liver tumor, obstructive jaundice or hepatotoxicity of certain drugs. Although serum levels of ALT and AST are elevated whenever disease processes affect the liver cells, ALT is the more liver-specific enzyme (Smith *et al.,* 2002; Yakubu *et al.,* 2005).

In this research, it was observed in the sub-acute toxicity study that there were no alterations in liver enzymes (AST, ALT and ALK) in the group administered with the extract and

reference drug when compared to the control group (i.e. group that did not receive the extract or the reference drug). This shows that the extract and reference drug did not have any toxic effect on the liver, it is also an indication of hepatic cellular activity without necrosis.

However, these enzymes were raised in animals infected with *E.coli* and egg albumin induced inflammation. The reason for some of the changes observed had been explained by Singh *et al.,* (2005) who stated that high levels of the aminotransferase (i.e. both AST and ALT) indicate the level of inflammation of liver cells due to hepatocyte injury. These parameters gradually decreased in the course of treatment with the extract and reference drug groups and returned to normal by the 8^{th} to 14^{th} day post treatment. But the protein and albumin levels were not altered. The extract significantly lowered the level of the serum enzymes in the animals infected with *E. coli* and helped to bring down the swelling due to inflammation induced with egg albumin. This reaffirms the earlier conclusion that the extract is an antibiotic as well as an anti-inflammatory agent. These findings agree with those of Vinothkumar *et al*., (2010), who reported that serum levels of transaminases returned to normal level with healing of hepatic parenchyma and regeneration of hepatocytes. Similarly, a study by Scott, (1998) has shown that certain plant extracts significantly lowered serum liver enzymes in humans with liver cirrhosis.

Changes in bilirubin values were also examined under these conditions because the liver is the seat of conjugation of bilirubin; therefore, increase in the amount of circulating unconjugated bilirubin is suggestive of hepatobiliary dysfunction (Tietz, 2000). The normal range for unconjugated bilirubin is 3.42 – 17.1 µmol/l while the normal range for conjugated bilirubin is 1.7 - 3.42 µmol/l (Tietz, 2000). The results obtained from the assay of bilirubin shows that the bilirubin total was less for the extract and reference drug administered groups in the sub-acute toxicity study when compared to the control group. This indicates that the extract had

no effect on biochemical processes that should give rise to increase in bilirubin levels in the blood.

However, bilirubin values obtained from the animals infected with *E.coli* and egg albumin induced inflammation were higher than that of the control. This could be due to red cell destruction caused by the infecting organism as well as egg albumin which the host cell recognizes as a foreign substance (antigen). These are stress conditions that may manifest as jaundice. There is a growing body of evidence that prolonged use of some β-lactam antibiotics (amoxicillin-clavulanic acid), which is a widely used antibiotic, has been associated with an increased incidence of cholestatic jaundice and acute hepatitis during therapy or shortly after, particularly in men and those aged over 65 years. The associated jaundice is usually self-limiting and very rarely fatal. Until 1993 the UK Committee on Safety of Medicines (CSM) has received 138 reports of hepatobiliary disorders (three fatal) with amoxicillin-clavulanic acid. 12% of all suspected adverse reactions reported with amoxicillin-clavulanic acid were in the hepatobiliary system. They, therefore, recommended that treatments such as amoxicillin/clavulanic acid preparations should be reserved for bacterial infections likely to be caused by amoxicillin-resistant β-lactamase-producing strains, and that treatment should not normally exceed 14 days (Joint Formulary Committee, 2004). Bilirubin result in this study does not suggest the extract to cause any biochemical abnormality or liver insufficiency.

Effect of extract on chemical analytes involved in kidney function and inflammation

Physiological functions of the body are regulated by electrolytes while the excretion of drugs and their metabolite and regulation of electrolyte balance are some of the important functions of the kidney (Tune *et al.,* 1997). The kidney also controls the levels of chloride in the blood and flush out potassium, magnesium as well as sodium. Therefore a shift in physiological levels of these electrolytes may be related to kidney function. When the level of electrolytes

in the body changed, the water content in the cells also changes. These changes can be associated with dehydration (shrinking) or excess fluid (oedema), due to inflammation of the tissues. Similarly, in the presence of normal renal blood flow, any increases in urea (17.8 to 35.0 mmol/L) and creatinine values (110 to 150µmol/L) are suggestive of moderate to severe kidney damage (Tietz, 2000). Therefore this study, has taken steps to examine the effect of the extract on kidney functions.

In the sub-acute toxicity study, increase in sodium (Na^+), urea, and creatinine levels were observed in the group administered with the reference drug when compared to the control group while normal Na^+ ion, urea and creatinine values were observed in the group administered with the culture extract. But no changes were observed in potassium (K^+) ions and chloride (Cl^-) ion. This shows that renal clearance of the natural extract was much faster compared to the reference drug which is a synthetic compound. It also indicates that the body handles natural products (like this extract) better than synthetic drug. On the other hand increase in Na^+ level in the reference drug group may be as a result of the sodium salts used in the preparation of the drug.

Similarly, results obtained 24 hours post-infection with *E.coli* and egg albumin-induced inflammation showed increase in Na^+ ion, urea and creatinine levels and no elevation in K^+ ions and Cl^- ion values were observed. These elevated parameters gradually decreased in the course of treatment with the extract and returned to normal range when compared to the control groups by the end of the 14th day post treatment. This result suggest that the increases in the levels of renal function parameters observed was due to the presence of infection, this is a transient event which did not cause any renal insufficiency. It also indicates that the extract had influence on the kidney in regulating and maintaining electrolyte, urea and creatinine balance in the body.

Influence of the extract on some enzymes of oxidative stress and inflammation

Cells have a comprehensive array of antioxidant defence mechanisms to reduce free radical formation or limit their damaging effects. These include enzymes such as superoxide dismutase (SOD) and catalase CAT) to degrade superoxide and peroxides respectively, and essential radical scavengers such as glutathione reductase (GSH) (Sabari, 2002).

In this study, the analysis of free radical enzymes and oxidation product (malonate dialdehyde) (MDA), shows that there were no changes in all the oxidative stress enzymes and oxidation product measured in sub-acute toxicity study. The data obtained provide evidence that the extract does not have detectable effect that could induce oxidative stress which leads to generation of free radicals resulting in cell death. This result corroborates the findings of Yogita *et al*., (2001) where the effect of penicillin treatment on SOD, CAT activity, GSH levels and MDA in islets was studied and found to have no significant change in oxidative stress enzyme levels of the islets.

However, in the presence of infection with *E.coli* and egg albumin-induced inflammation, activities of these enzymes and oxidation product were increased. These parameters gradually decreased in the course of treatment with the extract and reference drug and returned to normal range by the end of the eight day post treatment. The observed elevation indicates an increase in generation of free radicals in the system of the infected rats beyond the capacity of the body system to handle as it does in normal cell metabolism hence the manifestation of inflammation. The significantly elevated activities of SOD, CAT, GSH and MDA values in all the rats infected with *E.coli* and egg albumin-induced inflammation agree with previous studies that these enzymes are inducible in mammals and microorganism (Elchuri *et al*., 2005), and their levels will increase with increasing need to protect against toxic oxidation (Muller *et al*., 2006; Sentman *et al*., 2006). Similarly, Soliman, (2008) observed that antioxidant enzyme-dependent defenses play an important role in scavenging free radicals

produced under oxidative stress. Approximately 99% of Glutathione in brain cells exist in the reduced form (GSH). By serving as an electron donor, GSH plays an important role by removing hydrogen peroxides, thus protecting cells from oxidative stress. A decreased level of glutathione indicates decreased scavenging capacity of glutathione-dependent anti-oxidant defensive system against elevated lipid peroxidation processes. Antioxidant enzyme-dependent defenses play an important role in scavenging free radicals produced under oxidative stress.

Effect of extract on haematological indices

Haematology includes the study of aetiology, diagnosis, treatment, prognosis, and prevention of blood diseases. Any disease affecting the blood also affects the production of its components, such as blood cells, haemoglobin, the mechanism of coagulation, etc. The most commonly performed test in haematology laboratory is the complete blood count (CBC) also called full blood count (FBC), which includes; packed cell volume (PCV), haemoglobin level (Hb), white blood cell count (WBC), white cell differentials and platelet count.

Analysis of these parameters in the sub-acute toxicity study showed that there were no changes in the values of rats treated with the extract and reference drug compared with the control group. However, there were decreases in platelets counts both in the extract group and in the group treated with reference drug compared with the control. Similarly, these parameters were decreased in the presence of infection with *E. coli* and egg albumin induced inflammation. These increases in values gradually returned to normal after treatment with the extract and reference drug, but low platelet values were observed even after the treatment was stopped. Low platelet values depict thrombocytopenia caused by the extract and reference drug in the host cells as observed in both the sub-acute study group and the infected groups. Blood normally contains 150,000 to 350,000/µl. If this value should drop much below 50,000/µl, there is a danger of uncontrolled bleeding. This is because platelets aggregation is

an important step during blood clotting. Cazenave *et al*., (2008) observed that penicillin G and related antibiotics may be inhibitory to platelets function because they coat the platelet surface. Their effects on platelet functions are probably responsible for excessive bleeding and increased bleeding times observed in patients and volunteers receiving high doses of these antibiotics.

Whereas biochemical characterization of the extract shows that it is a β-lactam compound, toxicity profile and changes in plasma chemical analytes and haematology shows a remarkable decrease in platelet counts both in the presence and absence of disease and inflammation. The low platelet values recorded in this study necessitated further search into the effects of the extract on the coagulation cascade.

Effect of extract on coagulation cascade

Essentially, blood coagulation test measure the time taken for a sample of a patient's blood plasma to form a fibrin clot in a test tube after the addition of a reagent that initiates the coagulation cascade. Thrombin time test specifically looks at the final stage of the coagulation pathway – conversion of fibrinogen to fibrin. Effects of varying concentrations of "HemoStat" (Bovine) thrombin on citrated plasma shows that the rate of increase in absorbance was found to be proportional to the amount of thrombin added. This allows a simple and accurate procedure of thrombin activities in test procedures. Whole blood and thrombin coagulation tests in this study shows that normal blood clotting time was observed at 5 - 6 minutes. However addition of different concentrations of the extract and reference drug caused an increase in whole blood clotting time. At 6 mg/ml of the extract, clot formation was not observed. Also initiation of clot formation was prevented at 5 mg/mL of the reference drug. Similarly, normal thrombin time was observed at 14 – 16 seconds; however, there was an increase in thrombin time to 248.5 second and 293.8 second at concentrations of 10 and 20 mg/ml for the reference drug and extract respectively. There was complete inhibition of

thrombin (visual observation) at concentrations above 10 and 20 mg/ml of the reference drug and extract respectively. Absorbance of clot formation using various concentrations of extract on reaction mixtures showed a decrease in values with increase in the concentration of extract added. This result shows that the mechanism of action of the extract is similar to that of the reference drug which contains a β-lactam compound. Therefore, the suggestive mechanism of action of this extract in lowering platelet value is by, possibly, inhibiting thrombin formation which is known to be a platelet activator. According to Cazenave *et al*., (2008), platelet aggregation is required whenever fibrin formation is required to achieve haemostasis (blood coagulation) and vice versa and that thrombin – a serine protease - is a potent platelet activator and therefore constitutes an interesting target for drugs that would prevent the formation of fibrin and platelet rich thrombi induced by thrombin. An abnormally long thrombin time indicates a deficiency of factor 1 (Fibrinogen). These findings agree with Konaklieva (2000) that β - Lactam derivatives have been shown to inhibit a range of other enzymes with nucleophilic serine residues, including mammalian serine proteases such as elastase and prostate specific antigen by acylation of serine residues at the active site of enzymes (Penicillin-binding proteins). The centrality of thrombin to this system makes it an extremely powerful enzyme in coagulation and makes it the most suitable enzyme to be a target for anticoagulant drug therapy (Hongbao *et al*., 2008), as to control the action of thrombin would be to control the entire coagulation system.

The biological roles of thrombin are diverse. It is evident that thrombin is a molecule with an extremely wide range of biological roles, and an extremely high amount of regulation associated with it due to its potency as a pro-coagulant and pro-inflammatory mediator. New functions of thrombin are being discovered at a rapid rate and research is continuing into how it can be used to control those functions to human advantage, for example with the development of thrombin-specific anticoagulants such as the extract used in this research.

HISTOLOGY REPORT OF THE EFFECT OF THE EXTRACT ON LIVER AND KIDNEY

Histological examination of the kidney shows that the rats in the control group and the group treated with the extract had evidence of vascular congestion. This does not signify any pathological condition. However, mild papillary necrosis, a disease condition, was observed in the kidney of the group treated with the extract. The reference drug caused mild accumulation of fluid in the kidney (i.e. oedema) with congestion of blood cells. This reflects the mild elevation of Na^+ values observed in the group administered with reference drug which may be due to the fact that the reference drug was prepared using Sodium salts. However, a shift in electrolyte balance has been associated with oedema - an inflammatory disorder. Twenty-one day post examination of the kidney shows no end organ damage in all the groups.

The toxicity profile of the culture extract indicates mild toxicity to the organs or tissues at a dose of 2500 mg/kg body weight, but no possible end organ damage [especially in the kidney and liver]. A study conducted by James (2000) showed that among the penicillins, the semi synthetic agents have proved to be more hepatotoxic than native Penicillin. Also cholestatic injury has been described with oxacillin and its derivatives with a few instances of chronic cholestasis associated with the vanishing bile duct syndrome been recorded (Joint Formulary Committee, 2004).

This study has demonstrated that the extract of *P.chrysogenum* is relatively safe and because it is a natural product, the body was well able to handle its metabolism and excretion resulting in no end organ damage. It is evident that the *P.chrysogenum* (wild strain) isolated from our environment was made to undergo a transformation to yield two hyper natural antibiotic producing strains. The property of this antibiotic was suggestive to have anti-inflammatory,

anticoagulant and antithrombic activities and is relatively safe for use in health care management. Residual plant materials in urban refuse can serve as cheap carbon and energy sources for fermentation instead of refined sugars such as glucose and lactose (Solomon *et al.,* 1999; Howard *et al.,* 2003). This could turn the recalcitrant waste plant biomass into a valuable resource and reduce the pollution problem caused by its accumulation in the environment (Rahman *et al.,* 2000). The use of cassava peels and sugar cane pulp for both isolation and production of the antibacterial extract is an innovation which is not only cost effective and sustainable but also an environmentally friendly approach to natural penicillin production.

CONCLUSION

This study reveals that Indigenous strain of *P. chrysogenum* was isolated from wood waste. Organism grew well on cassava peels and corn cob. Optimum pH and temperature of growth of the organism and antibiotic production were obtained at pH 6.5 and 25^0C respectively, using sugar cane pulp and cassava peels to formulate the culture media. TLC and HPLC of analysis of the crude and partially purified extract confirm the presence of a peak which corresponds to Penicillin – the reference drug.

Genetic modification of wild strain *P. chrysogenum* using UV radiation yielded a 70% increase in natural antibiotic production compared to the wild strain. *In vitro* antibacterial activity of the agro waste culture extracts when tested against some clinical bacterial isolates, namely*, B. subtilis, E. coli, P. mirabilis* and *P. aeruginosa*, confirmed that the extract is an antibiotics of the β-lactam family. The minimum inhibitory concentration of culture extracts from cassava peels and sugarcane pulp compared favourably with that of the standard reference drug.

Toxicity study showed that the extract is safe for use as there were no visible changes or recorded deaths 24 - 72 hours after administration of the extracts. *In vivo* studies showed that the extract also has anti inflammatory property.A summary of the histology report shows that there was no possible end organ damage; therefore, the extract is relatively safe for use in the health care management.

Haematological evaluation of the albino rats showed that the extract and reference drug has influence in platelet function. It was shown to have a potent antithrombic and anticoagulant effects against thrombin and whole blood respectively**.** This would be valuable both in the treatment of inflammatory diseases and management of thrombotic disorders.

CONTRIBUTIONS TO KNOWLEDGE

1. This study has isolated an indigenous wild strain of *P. chrysoge*num from industrial agro waste in Lagos, Nigeria; under optimum conditions, the strain was modified genetically by UV treatment to give two hyper producing mutants with a 70% increase in natural antibiotic production compared to the wild strain. It was also established that the extract has inhibitory activity against *B. subtilis,* and *E. coli* and belongs to the β-lactam family, like penicillin because it was found to be hydrolyzed by β-lactamase of *P. aeruginosa* and *P. mirabilis*.

2. The antibacterial extract has anti-inflammatory property because it effectively corrected egg albumin-induced inflammation in treated Wistar albino rats while the group that were not treated after induction of inflammation died. Furthermore, *in vivo* biochemical studies and histology profile of the liver and kidney show that the extract is relatively safe as there were no damage to liver and kidney.

3. It is established that the extract has antithrombic activity and anticoagulant effects. Thrombin is a serine protease; therefore the inhibition of thrombin activity shows that the extract acts as an antiprotease agent. This would be valuable both in the treatment of inflammatory diseases and management of thrombotic disorders.

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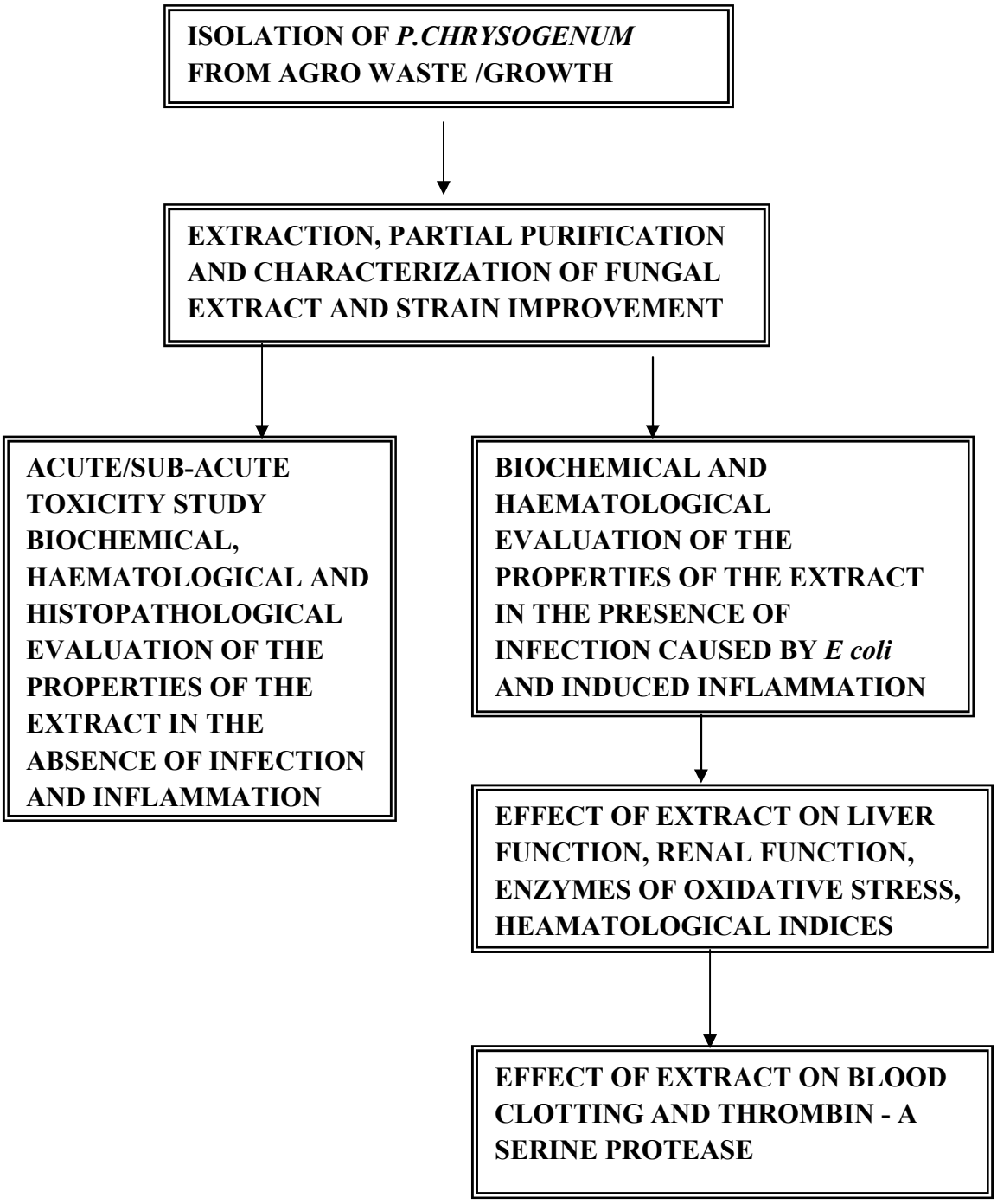
APPENDIX A

RESEARCH SCHEME



APPENDIX B

FORMULAE FOR CALCULATION OF BIOCHEMICAL PARAMETERS

1. Calculation of sample potency mg/mL

$$\underline{b} = \frac{n\sum XY(\sum X)(\sum Y)}{n\sum X^2 - (\sum X)^2}$$

X = concentrations of reference drug

Y = mean zone of diameter for each concentration

n = number of samples (5)

$\underline{b}$ = Regression coefficient

Determination of Potency of the extract was calculated using the formula below

$$\text{Concentration of unknown} = \frac{Yu-Ys}{b}$$

Where Yu = mean response of unknown sample

Ys = mean response of reference standard drug

2. Dosage Control formula

$$\text{mL of extract/drug given} = \frac{\text{mg of drug/extract per kg body weight}}{1000} \times \frac{\text{weight of animal (g)}}{\text{Concentration (mg/mL)}}$$

3. WBC

Calculation:

$$\frac{N \times 20 \times 10^6}{4 \times 0.1} \text{ per litre}$$

Where N = number of cells counted

Dilution factor = $^1/_{20}$

Area counted – 4 x 1 mm^2

4. Platelet count

N = Number of cells counted

Total platelet count = N x $^{1}/_{0.02}$ x 20 (dilution)

= N x 1000 µL

= N x 10^3/L

5. Total and Direct (Conjugate) Bilirubin

Calculation:

ABSA x 13 = mg/dL x 17.1 = µmol/L

Where ABSA = Absorbance of Test Sample

6. Alkaline Phosphatase

Enzyme activity (IU/L) = $\frac{\text{Absorbance of test}}{\text{Absorbance of Control}}$ x Concentration of Standard

Standard value = 50IU/L

7. Determination of Total Protein

Calculation:

$\frac{\text{ABSA}}{\text{ABSTD}}$ x 80

Where ABSA = Absorbance of Test Sample

ABSTD = Absorbance of standard Albumin

80 = Concentration of Standard Protein (g/L)

8. Determination of Albumin

Calculation:

$$\frac{ABSA}{ABSTD} \times 5$$

Where ABSA = Absorbance of Test Sample

ABSTD = Absorbance of standard Albumin

5 = Concentration of Standard Albumin (g/L).

9. Urea concentration

$$\text{Urea (mmol/l)} = \frac{\text{Absorbance of test}}{\text{Absorbance of urea standard}} \times \text{Concentration of urea standard}$$

10. Creatinine concentration

$$\text{Creatinine (µmol/l)} = \frac{\text{Absorbance of test}}{\text{Absorbance of creatinine standard}} \times \text{Concentration of creatinine standard}$$

11. Chloride (Cl^-)

Calculation:

$$\frac{ABSA}{ABSTD} \times 100 = MEqCl^-/L$$

Where ABSA = Absorbance of Test Sample

ABSTD = Absorbance of standard Chloride

100 = Concentration of Standard Chloride ($MEqCl^-/L$)

12. Determination of Catalase Enzyme Activity

Calculation:

$$\text{Catalase Enzyme Activity (µmol/ml)} = \frac{\Delta OD/min}{\Sigma} \times \frac{V}{v}$$

Where: ΔOD/min = change in absorbance/minute

Σ = Molar extinction coefficient ($40.0 M^{-1} Cm^{-1}$)

V = Total volume of reacting sample

v = volume of sample

13. Determination of Superoxide Dismutase (SOD) Activity

Calculation:

$$\text{SOD Enzyme Activity (m/ml)} = \frac{\Delta OD_{480}/min}{\Sigma} \times \frac{V}{v}$$

Where: ΔOD_{480}/min = change in absorbance at 480 nm per minute

Σ = Molar extinction coefficient ($4020 M^{-1} Cm^{-1}$)

V = Total volume of reacting sample

v = volume of sample.

14. Determination of Thiobabituric acid reactions (TBARS)

Calculation:

$$\text{MDA (nmol/1ml)} = \frac{\Delta OD/min}{\Sigma} \times \frac{V}{v}$$

Where: OD = Absorbance (Optical density) of sample

Σ = Molar extinction coefficient ($1056 \times 10^5 M^{-1} Cm^{-1}$)

V = Total volume of reacting sample

v = volume of sample.

15. Determination of Reduced Glutathione (GSH)

Calculation:

Actual absorbance reading = OD TEST – OD BLANK reagent.

$$\text{GSH } (\mu\text{mol/ml}) = \frac{OD}{\sum} \times \frac{V}{v}$$

Where OD = Actual absorbance reading of sample

$\sum$ = Molar extinction coefficient ($13{,}600 M^{-1}Cm^{-1}$)

V = Total volume of reacting mixture

V = Volume of sample in the reaction mixture.