PHARMACOGNOSTIC AND ANTIMICROBIAL STUDIES OF THE STEM BARK OF *DETARIUM MICROCARPUM* GUILL. & PERR. (FABACEAE)

BY

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AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

JANUARY, 2015.

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BY

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DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT,

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

JANUARY, 2015.

Declaration

I hereby declare that the work reported in this thesis titled "**Pharmacognostic and Antimicrobial Studies of the Stem Bark of *Detarium microcarpum* Guill. & Perr. (Fabaceae)**" was performed by me in the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria; under the supervision of Prof. A. Agunu and Dr. U. H. Danmalam. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project was previously presented for the award of another degree or diploma at any University.

ABUBAKAR Sani

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CERTIFICATION

This thesis entitled "Pharmacognostic and Antimicrobial Studies of "THE STEM BARK OF *DETARIUM MICROCARPUM* GUILL. & PERR. (FABACEAE)" by Sani ABUBAKAR, meets the regulations governing the award of the degree of Masters of Science in Pharmacognosy of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Abstract

Detarium microcarpum Guill & Perr. (Fabaceae) is used in parts or whole in Nigeria and several other West African countries for the treatment of various ailments; either as topical applications or oral administration. It is used alone or with other herbs and consumed to either alleviate pains or treat other form of diseases like malaria, pile, bacterial infection etc. The pharmacognostic studies were carried out on the stem bark of the plant. This comprises the marcroscopic studies, microscopic studies and the evaluation of the crude drug. The anatomical and powdered samples of this plant parts were investigated for their microscopic profiles. These revealed the presence of phloem tissues, parenchyma cells, cork cells, calcium oxalate crystals, starch grains and secretory ducts. The anatomical/transverse section revealed the presence of xylem tissues in addition to the tissues found on the bark. The following were the physical constants: moisture content (5.10), Ash value (4.3), acid insoluble extractive value (1.80), alcohol soluble extractive value (22.0) and water soluble extractive value (18.0). The phytochemical screening of the methanol extract of this plant indicated the presence of saponins (triterpenes), carbohydrates, flavonoids, cardiac glycosides, tannins and alkaloids. The total phenolic content of the methanol stem bark extract was determined in terms of tannic acid equivalent (TAE) as 16.00 mg/g; the tannin fraction as 13.86 mg/g and that of the residual fraction as 2.14 mg/g of bark weight of *Detarium microcarpum*. TLC studies of the pet-ether fraction, Crude extract, residual fraction and tannins fraction was done. Nine distinct spots were obtained on a TLC plate for the pet-ether fraction when the solvent system n-Hexane : Ethyl-acetate (9:1v/v) was used and three distinct spots resulted from the Methanol crude extract when the solvent system ethyl-acetate : MeOH : Water (10:1.6:1.4 v/v) was used. The co-chromatography of the MeOH extract and five different standard reference compounds (catechol, gallic acid, phloroglucinol, pyragallol and quercetin) revealed the presence of catechol and quercetin.

The Methanol crude extract showed an LD_{50} of 282.84 mg/kg; the tannin fraction had an LD_{50} >5000 mg/kg and the residual fraction revealed an LD_{50} of 269,300 mg/kg in mice. The crude Methanol extract was bactericidal. The isolates used were more susceptible to the crude methanol extract; this is likely due to some synergistic effect. The results obtained from this research could be used for the preparation of a monograph for the plant.

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Abbreviations and Symbols

%	Percent
+ve	Positive
±	Plus or Minus
µ	Micron
µl	Micro-litre
^0C	Degrees Celsius
ABU	Ahmadu Bello University
ABUTH	Ahmadu Bello University Teaching Hospital
AIDS	Acquired Immune Defficiency Syndrome
ANOVA	Analysis of Variance
ARI	Acute Respiratory Tract Infection
Bp	Boiling Point
BP	British Pharmacopoea
bw	Body Weight
cfu	Colony Forming Unit
cm	Centimeters
DNA	Deoxyribonucleic Acid
DPX	Disterene Pastilizer and Xylene
E. coli	*Escherichia coli*
e.g.	Example given
et.al.	and others
$FeCl_3$	Ferric chloride
FFA	Formalin Acetic Alcohol

GAE	Gallic Acid Equivalent
H_2SO_4	Hydrogen Tetraoxosulphate (IV) acid
HAART	Highly Active Anti-Retroviral Therapy
HCl	Hydrochloric Acid
HIV	Human Immuno Deficiency Virus
HPLC	High Performance Liquid Chromatography
Hrs	Hours
KOH	Potassium Hydroxide
L or Ltr	litres
LD_{50}	*lethal dose to 50% of the tested group of animals*
LTD	Limited
MA	*Mac Conkey Agar*
MBC	Minimum Bactericidal Concentration
MCE	Methanolic Crude Extract
MeOH	Methanol
RF	Residual fraction
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MR Test	Methyl Red Test
Min(s)	Minutes(s)
MSA	Manitol Salt Agar
NaCl	Sodium Chloride
NB	Nutrient Broth
№	Number

OPIs	Opportunistic Infections
PEPFAR	U.S President Emergency Programme For Aids Relief
PLWA	People Living With AIDS
PR	Protease
RT	Reverse Trascriptase
SDA	Sabouraud Dextrose Agar
Sig	Significance
Staph	*Staphylococcus*
Std	Standard
TF	Tannin Fraction
TLC	Thin Layer Chromatograph
UNAID	United Nationale des Architectes d'Intérieur, Designers (United Nations Joint Action Program on HIV/AIDS)
Uv	Ultra-violet
v/v	Volume by Volume
-ve	Negative
Viz	Namely
VP Test	Voges-Proskauer Test
w/w	Weight by Weight
WHO	World Health Organizations

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

Medicinal plants have been an important source of health care to millions of peoples across the globe especially the rural dwellers for which traditional medicine is the only source of remedy or healing to certain ailments (Evans, 2008). The practice of traditional medicine in the treatment of diseases and infections is increasingly gaining grounds in the scientific world. The use of plants in ethno-medicine is continually being employed worldwide as a guide to the possible therapeutic potentials of plants. This helps the researchers to ultimately come up with ways in which such plants could be used in treating diseases and infections or develop drugs for the orthodox medical practice (Evans, 2008).

The abundance and diversity of African flora, especially in the tropical rain forests, has prompted enormous efforts to investigate these plant resources for lead compounds in the development of new pharmaceutical agents and other compounds with economic potentials (Nkunya, 1994). Current estimates of the number of species of flowering plants range between 200,000 and 250,000 in some 300 families and 10,500 genera. Despite rapidly expanding literature on phytochemistry, only a small percentage of the total species has been examined chemically, leaving a large field for future research (Evans, 2008).

The available knowledge on the use of plant preparations in traditional medicines in most tropical countries allows a direct search for such compounds following scientifically established norms. In other cases, the medicinal usefulness of the

plant may not be known, but chemical investigations might reveal some interesting bioactive compounds (Nkunya, 1994).

Crude drugs may be arranged not only according to families and chemical constituents, but also into such morphological groups as barks, roots, leaves, seeds, etc. some drugs constitute more than one morphological part; for example, whole barks and commercial 'roots', which may consist of both rhizomes and roots (Evans, 2008).

1.2. The Importance of Natural Drugs Substances

In general, natural drug substances offer four vital and appreciable roles in modern system of medicine thereby adequately justifying their legitimate presence in the prevailing store of therapeutic substances. These vital roles are

i. Serve as extremely useful natural drugs
ii. Provide basic compounds affording less toxic and more affective drug molecules.
iii. Exploration of biologically active prototypes towards newer and better synthetic drugs.
iv. Modification of inactive natural products by suitable biological/chemical means into potent drugs (Kar, 2007).

1.3. Standardization of medicinal plants

This is a step taken towards a safer and a more rational use of plants by the traditional healer and medical scientists alike, as well as justification for pharmaceutical application of plant drugs. Standardization of medicinal plants is the process of prescribing a set of standards or characteristics adequately controllable which could form an official monograph or part thereof for a proposed

plant drug preparation. A monograph is therefore a set of standard characteristics of a crude drug or drug preparation with specific profile, pharmacognostically or pharmaceutically. Provision of a monograph for drug is an evidence of standardization for that drug. It carries with it an assurance of quality, safety, efficacy and reproducibility which are the most essential parameters in any national drug regulatory requirements for a dossier (African Pharmacopoeia, 1986).

It is generally believed that standardization of plant material is not required when used by the rural communities for their primary health care. But, regardless of whether the medicinal plant is to be used by local communities or by industries, a systematic approach is required for a plant identified from traditional medicine, as is done in modern medicine. It is necessary to focus on all aspects of medicinal plant research: from cultivation, ethno-pharmacology, utilization, isolation and identification of active constituents to efficacy evaluation, pharmacology, safety, standardization, formulation and clinical evaluation. Animal toxicity studies are required to establish the potential adverse effects. An outline of the entire process includes formulating an appropriate strategy and it has been estimated that the entire process would take more than 10–20 years. This approach is very demanding since there is an estimated 270,000 species of higher plants present on this earth (Encarta, 2009). However, this scenario would change with use of the high throughput advanced screening methods that are available today. Another approach that can prove to be a highly productive and cost effective in development of safe, effective and acceptable therapeutic agents is reverse pharmacology which is based on the documented therapeutic effects of plants in ancient texts.

The process of Standardization for medicinal plants can be classified into two main study groups viz:

1. Pharmacognostic standardization,
2. Biological standardization.

1.3.1. Pharmacognostic Standardization: This provides over 80% of the requirements of a crude plant drug monograph for African Pharmacopoeia and more than 50% in any Pharmacopoeia all over the world. The following procedures are required to standardize a plant species:

i. Sensory perception: consisting of careful judgment on odour/smell and taste sensations, which often provide the simplest and quickest indication for drug identity, purity and quality when compared with those perceptions described in the official monograph (African Pharmacopoeia, 1986)

ii. Marcroscopical description of vegetable drugs: This include the shape, size, morphological part, colour, surface characters, texture, fracture and characteristics of the broken surfaces, these are all useful in judging the identity, purity and to a certain extent, the quality of the crude drug (Evans, 2008)

iii. Microscopy: This is the most objective and most reliable among the pharmacognostic techniques. It is based on the fact that no two different plant species will possess exactly the same cellular patterns qualitatively and quantitatively, in all respects. The use of microscopic features, also known as diagnostic characters, is indispensible for the identification of crude drugs especially when in the powdered forms. Microscopic standards are divided into two viz: qualitative and quantitative microscopic standards. The qualitative

microscopic standards will include the types of epidermal cells, stomata, stone cells, fibers, trichomes vascular members and the cell contents; the quantitative microscopic standards involve cellular micrometry or measurements of all the above tissue materials which can be measured accurately (African Pharmacopoeia, 1986)

iv. Evaluation of Crude Drug (Physico-chemical analytical method): This method involves the determination of total ash values, acid insoluble ash values, moisture content and yields to solvents i.e. alcohol and water soluble extractive values. (African Pharmacopoeia, 1986)

It also involves determining the source of the plant material, selection, collection, processing and storage of the plant material.

v. Phytochemical Studies: Phytochemical studies of the plant preparations are necessary for standardization, which helps in understanding the significance of phytoconstituents in terms of their observed activities. Phytochemistry also helps in standardizing the herbal preparations so as to get the optimal concentrations of known active constituents, and in preserving their activities. Standardization can be carried out by obtaining a chemical fingerprint/profile or through bioactivity guided fractionation. Chemical fingerprints through chromatographic techniques are more commonly used for standardization and are obtained in terms of one or more marker compounds. It would be ideal to use the active constituent in the plant as the marker compound; however, in cases where active constituents are not known the marker compound can be independent of the therapeutic activity. Furthermore, the plant extracts can also be standardized to class of compounds e.g.

ginsenosides in ginseng, kava lactones in kava, or oxindole alkaloids in cat's claw (Roman, 2001). Such an approach would be suited to situations where though the active constituents are not known they are expected to belong to a particular class of compounds.

Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) are the most commonly used methods for obtaining chemical fingerprints and identification of the crude plant extracts. However, there are several possibilities that may arise while using these techniques for standardizing the crude extracts. It is possible that the plant material collected from the same plant in two different seasons can show different phytochemical fingerprint and therefore different biological activity or two plants with identical taxonomy collected under same environmental conditions can show different phytochemical fingerprint but similar biological activity. In such situations comparisons of the phytochemical profiles as an indicator of important constituents can act as a shortcut for identifying biologically active constituents.

DNA fingerprinting is another technique, which though still in its early years, seems to be of immense potential in identification of medicinal plants, particularly when profiling the genotypic differences (Vasudevan, 2004). Apart from identifying these genetic variations, it can also aid in identification of germplasms of important or endangered plants for future cultivation or conservation. Use of isolated compounds can result in better biological activity due to higher concentrations, but it can also lead to potential side effects e.g. the active constituent conessine isolated from *Holarrhena antidysenterica*, a plant commonly

used by Ayurvedic practitioners in the treatment of diarrhoea, was found to be toxic to the central nervous system (Chemexcil, 1992). More recent studies have also indicated at reduced biological activity with isolated active constituents compared to crude extracts (Kicklighter *et al*, 2003).

1.3.2. Biological Standardization: Biological screening is necessary to provide a scientific basis for validating the traditional utilization of medicinal plants. A great number of screening programs are on-going worldwide for new plant based bioactive molecules. Several researchers have worked on medicinal plants with activity against different ailments. Preclinical pharmacological studies and randomized clinical trials form an important part of the biological screening of medicinal plants. Preclinical studies usually serve to verify the data on mechanisms of action reported in animals or humans. However, a pharmacological effect observed in vitro or in animal models, for both safety and efficacy needs to be reconfirmed by clinical studies and the information obtained from the preclinical studies can form the basis for further clinical trials. (Lipsky and Sharp 2001, Bleicher *et al.* 2003, Dove 2003, Kenakin 2003, Knowles and Gromo 2003, Verkman 2004). Biological studies are thus divided into preclinical and clinical studies.

1.3.2.1. Clinical Studies: Clinical studies are necessary to confirm the pharmacological effects of medicinal plants before they can be integrated into conventional medical practice. Well established, randomized controlled clinical studies facilitate the acceptance of herbal medicines in different regions and in people with different cultural traditions. This would be especially true in case of

some unrelated effects of therapy contributing to efficacy that may be difficult to measure pre-clinically. These studies should be carried out on the basis of information obtained from official national compendia and relevant literature or traditional medical practitioners. The general principles for the clinical studies that apply to conventional drugs should be followed when testing a new herbal preparation, a new indication for an existing formulation, or a significantly different dosage form or route of administration (WHO, 2000). Well recorded case reports can contribute towards useful information at such times and put forward new hypothesis and stimulate further study (Morris 1989). However, double blind clinical trials may not be required when an extensive and detailed database of case studies is available. Such a database is especially important when a particular treatment is individualized.

The methods and guidelines used for clinical validation of modern medicines must be applied to herbal products even though the latter has a holistic approach to treatment. However, conventional concepts of clinical research design may be difficult to apply when using clinical research to evaluate various systems and practices of traditional medicine (WHO, 2000). This could be due to the fact that herbal remedies are individualized (each person has certain predispositions to disease and susceptible to factors like environment, genetics, dietary and lifestyle) therapies and hence depend on the proficiency (including the skills and experience) of medical practitioners. Pharmaological aspect of biological standardization involves evaluating the efficacy, toxicity and/or safety of medicinal plants.

i. Toxicity and safety: Here, safety is always emphasized as the most crucial requirement and must override all other criteria for the selection of traditional

medicine. As a rule, proper documentation of a long period use of traditional medicine should be taken into consideration for safety assessment; particularly if there is no detailed toxicological report and this should form the basis for risk assessment. The toxicity, of naturally occurring substances widely used in herbal medicine is a potential for systemic toxicity, carcinogenicity and teratogenicity (WHO, 1991).

ii. Efficacy: This is another useful criterion besides toxicity used in herbal selection for health care purposes. It implies the expression of a definite bioactivity profile in dose-response manner including the pharmacological effects on different body organs suggesting side effects, contraindications, effective and toxic dose-ranges. Pharmacological standardization most, often forms the bases for Biological studies/standardization.

1.3.2.2. Preclinical Studies: Preclinical testing helps in collection of important efficacy and safety data before clinical trials can be carried out. The preclinical evaluation and authentication of medicinal plants involves documentation and testing of their pharmacological efficacy *in vitro* (cells) and *in vivo* systems and studies of toxicology, specificity, biopharmaceutical properties and drug interactions. The preclinical studies help in determining the therapeutic effect of the plant in question and also elucidate the efficacy and/or the mechanism of action including cell interactions, cell-environment interactions, intracellular activity, and genetic studies. Plants with novel and/or multiple mechanism(s) of action can also be identified. The advantage of these studies is that one can easily study and compare the efficacy of different plants in a cost effective manner and design rational drug combinations. This requires proper designing of screening assays that

have significant impacts on the outcome of the overall drug discovery process. The selected assay should be able to mimic the *in vivo* dynamics as far as possible with high sensitivity and specificity. The basis for designing a screening assay is the identification of valid target. An estimated 30-40% of experimental drugs fail due to an inappropriate target (Butcher, 2003) and hence it is important to develop new screening assays with newer and more appropriate targets. It is crucial to establish the role of the target in question in the cause or symptoms of a disease (Williams, 2003). Pharmacological manipulation of the target should consistently lead to desired phenotypic changes. The desired changes must also be reproducible in at least one relevant animal model (Drews, 2003). Emphasis has to be placed on assessment of assay quality and validation of the parameters being used.

1.4. A General Overview of Traditional Medicine Practice

Before the introduction of modern medicines, disease treatment was entirely managed by herbal remedies. It is estimated that about 80% of the world population residing in the vast rural areas of the developing and under developed countries still rely mainly on medicinal plants. These medicinal plants are the only affordable and accessible source of primary health care for them, especially in the absence of access to modern medical facilities. Studies revealed that there are more traditional medicine providers than the allopathic providers especially in the rural areas (WHO, 2002).

The use of traditional medicine has increased in developed countries also, mainly due to the failure of modern medicine to provide effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites. The adverse effects of chemical drugs, questioning of the approaches and assumptions of allopathic medicine, their increasing costs and greater public access to information on traditional medicine has also led to an increase in interest in alternative treatments (WHO, 2002). Plant extracts have become

a source of hope as a wide group of medicinal plant preparations are available that have been used over the centuries almost exclusively on the basis of empirical evidence. Hence, it has become necessary to revisit the importance of these herbal medicines. Increasing interest by multinational pharmaceutical companies and domestic manufacturers of herbal-based medicines is contributing to a significant economic growth of the global medicinal plants sector. However, a large proportion of medicinal plant research is focused on nutraceuticals, chronic and metabolic disorders (diabetes, cardiovascular, etc.) and other diseases like HIV/AIDS, malaria, etc. whereas, the common diseases of resource poor communities such as diarrhoeal diseases and acute respiratory tract infections (ARI) are often not addressed. Moreover, unlike the rural communities who use fresh/dried plant material or their crude extracts, the industry lays importance on isolation of active principles or standardized fractions since crude extracts are not patentable. However, it is often seen that a crude extract is more active compared to the isolated active fractions e.g. *Cirriformia tentaculata* loses its activity upon fractionation with hexane (Kicklighter *et al* 2003).

1.5. Tannins

Tannins are phenolic phytochemical compounds having molecular weights between 500 and 3000 (Hagerman, 2002) or 1000 – 5000 (Evans, 2008) and giving the usual phenolic reactions as well as having special properties such as the ability to precipitate alkaloids, gelatine and other proteins (Hagerman, 2002).The term ‘tannin’ was first applied by Seguin in 1796. It denotes substances present in plant extracts. They are highly soluble in water and in most other polar solvents particularly Methanol and Ethanol. They are able to combine with protein of animal hides, prevent their putrefaction and convert them into leather (Evans, 2008). The word “tannin” is a more recently substituted term from the term

"polyphenol". The substitution is in an attempt to emphasize the multiplicity of phenolic groups which is characteristic of these compounds. This was based on the fact that molecular weights as high as 20,000 have been reported, and that tannins complex not only with proteins and alkaloids but also with certain polysaccharides (Hagerman, 2002). Preferably, the term tannin is used which emphasizes the character which sets tannins apart from all other phenolics: the ability to precipitate protein. Two main groups of tannins are usually recognised namely: hydrolysable and non-hydrolysable tannins (Evans, 2008).

Tannins are of wide occurrence in plants and are usually found in greatest quantity in dead or dying cells. They exert an inhibitory effect on many enzymes due to protein precipitation and, hence, they may contribute a protective function in barks and heartwoods. Commercial tannins are used in the leather industry, and are usually obtained from quebracho, wattle, chestnut and myrobalans trees. Pharmaceutical tannin is prepared from oak galls and yields glucose and gallic acid on hydrolysis; many commercial samples contain some free gallic acid. Some plants (e.g. Clove and cinnamon) contain tannin in addition to the principal therapeutic constituents. This may complicate extraction or produce incompatibilities with other drugs. For example, many alkaloids are precipitated by tannins (Evans, 2008).

1.5.1. Classification of Tannins

1.5.1.1 Hydrolysable Tannins: The hydrolysable tannins are formed from several molecules of phenolic acids such as gallic acids (fig 1.) and hexahydroxydiphenic acid (fig 3.) which are united by ester linkages to a central glucose molecule. These may be hydrolysed by acids or enzymes such as tannase. Like gallic acids, their

solutions turn blue with iron salts. They were formally known as pyrogallol tannins, because on dry distillation gallic acid and similar components are converted into pyrogallol. Two principal types of hydrolysable tannins are gallitannins (fig. 4) and ellagitannins which are, respectively, composed of gallic acid and hexahydroxy-diphenic acid (fig. 3) units. Gallitannins could be obtained from: rhubab, cloves, red rose petals, bearberry leaves, Chinese galls, Turkish galls, hamamelis, chestnut and maple. Ellagitanins could be obtained from pomegranate rind, pomegranate bark, myrobalans, eucalyptus leaves, kousso, some Ausstralian kinos, chestnut (*Castanea spp*) and oak bark (Evans, 2008

1.5.1.2. Condensed (Non-Hydrolysable) Tannins: The Condensed tannins (also called proanthocyanidins) are not readily hydrolyzed to simpler molecules and they do not contain a sugar moiety. They are related to the flavonoid pigments and have polymeric flavan-3-ol structures. Catechins, which also occur with the tannins and flavan-3, 4-diols (leucoanthocyanidins) are intermediates in the biosynthesis of polymeric molecules. On treatment with acids or enzymes, condensed tannins are converted into red insoluble compounds known as phlobaphenes. Phlobaphenes give the characteristic red colour to many drugs such as red cinchona bark, which contain these phlobatannins and their decomposition products. On dry distillation they yield catechol (fig. 5) and these tannins are therefore sometimes called catechol tannins. Like catechol itself, their solution turns green with ferric chloride (Evans, 2008). The following are rich in condensed tannins:

i. Barks: cinnamon, wild cherry, cinchona, willow, acacia (wattle, mimosa), oak and hamamelis
ii. Roots and rhizomes: krameria (rhatany) and male fern
iii. Flowers: lime and hawthorn

iv. Seeds: cocoa, guarana, kola and areca

v. Fruits: cranberries, grapes (red wines), hawthorn

vi. Leaves: hamamelis, hawthorn and tea especially green tea

vii. Extracts and dried juices: catechu, acacia and mangrove cutches, East Indian kino, butea gum and eucalyptus kino (Evans, 2008).

Other forms of tannins include:

1.5.1.3. Complex Tannins: These are group of tannins which are biosynthesized from both hydrolysable tannin (mostly a C-glucoside ellagitannin) and condensed tannin. The union occurs through a C-C bond between the C-1 of the glucose unit of the ellagitannin and the C-8 or C-6 of the flavan-3-ol derivative. The monomers are also involved in oligomer formation (Evans, 2008). To date, complex tannins have not great relevance to the mainstream in pharmacognosy; monomers have been isolated from the Combretaceae, Fagaceae (Queercus, Castanea), Myrtaceae, Polygonaceae (Rheum) and Theaceae (Camellia). It is anticipated that many more compounds of this group will be discovered (Evans, 2008).

1.5.1.4. Pseudotannins: These are compounds of lower molecular weight than true tannins and they do not respond to the goldbeater's skin test. (Evans, 2008). Below are some pseudotannins and plant materials where they could be obtained:

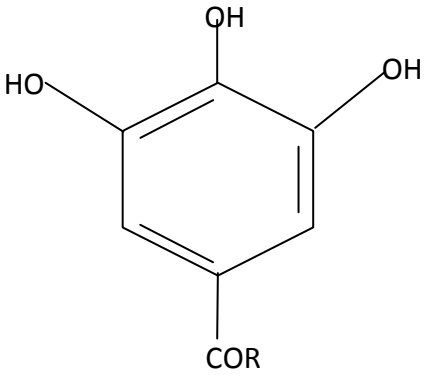
i. *Gallic acid (fig. 1)*: from rhubarb and most materials which contain gallitannins

ii.

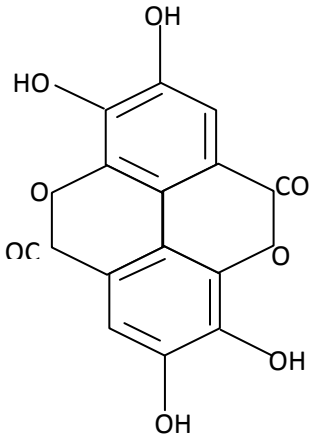
iii. *Catechins*: from catechu, acacia cutch, many Australian kinos, cocoa, guarana, and many other drugs containing condensed tannins.

iv. *Chlorogenic acid:* from mate, coffee, (particularly unroasted) and nux vomica (a small quantity only)

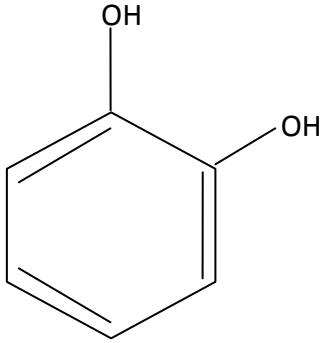
v. *Ipecacuanhic acid*: from ipecacuanha (Evans, 2008



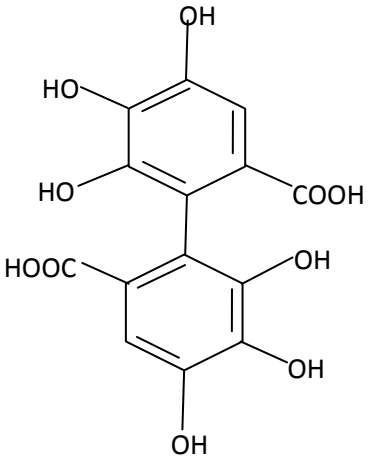
(a) Gallic Acid; R=OH



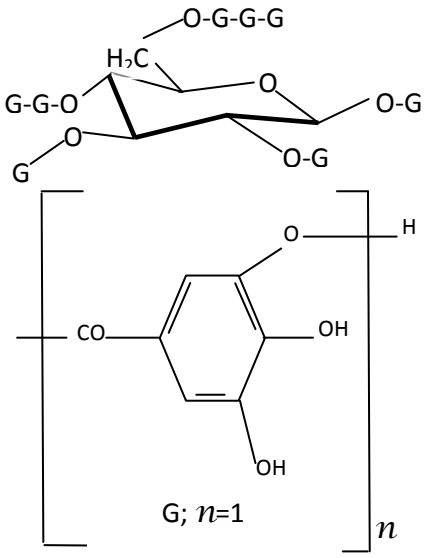
(b) Ellagic Acid (the depside of Gallic Acid)



(c) Catechol



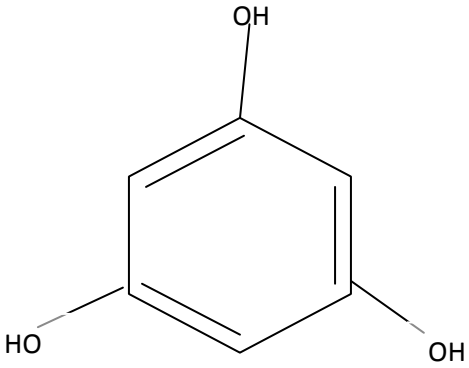
(d) Hexahydroxydiphenic acid



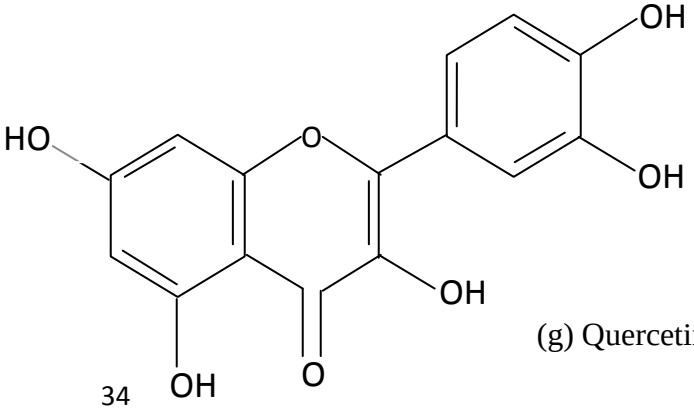
G-G; n=2

G-G-G: n=3

(e) Gallitannin of *Rhus sp.*



(f) Phloroglucinol



(g) Quercetin

Fig 1.1. (a-g): Chemical Structures of Some Tannins

1.5.2. General Properties and Tests of Tannins

Tannins are soluble in water, dilute alkalis, alcohol, glycerol and acetone, but generally only sparingly soluble in other organic solvents. Solutions of tannins precipitate heavy metals, alkaloids, glycosides and gelatin. With ferric salts, gallitannins (fig. 4) and ellagitannins give blue-black precipitates and condensed tannins give brownish-green precipitates. If a very dilute ferric chloride solution is gradually added to an aqueous extract of hamamelis leaves (which contains both types of tannin), a blue colour is produced which changes to olive-green as more ferric chloride is added (Evans, 2008).

The following are other useful tests for tannins.

1.5.2.1 Goldbeater's skin test: Soak a small piece of goldbeater's skin in 2% hydrochloric acid; rinse with distilled water and place in the solution to be tested for 15 minutes. Wash with distilled water and transfer to a 1% solution of ferrous sulphate. A brown or black colour on the skin denotes the presence of tannins. Goldbeater's skin is a membrane prepared from the intestines of the ox and behaves similarly to an untanned hide (a reference sample of hide powder, as used in the BP assay of the tannins of Rhatany Root, may be obtained from the European Pharmacopoeia Secretariat, Strasbourg (Evans, 2008).

1.5.2.2 Gelatin Test: Solutions of tannins (about 0.5- 1%) precipitate a 1%solution of gelatin containing 10% sodium chloride. Gallic acid and other pseudotannins also precipitate galatin if the solutions are sufficiently concentrated (Evans, 2008).

1.5.2.3 Phenazone test: To about 5ml of an aqueous extract of the drug add 0.5 g of sodium acid phosphate; warm, cool and filter. To the filtrate add 2% solution of phenazone. All tannins are precipitated, the precipitate being bulky and often coloured (Evans, 2008).

1.5.2.4 Test for catechin: Catechins on heating with acids form phloroglucinol and they can, therefore, be detected by a modification of the well-known test for lignin. Dip a match stick in the plant extract, dry, moisten with concentrated hydrochloric acid and warm near a flame. The phloroglucinol produced turns the wood pink or red (Evans, 2008).

1.5.2.5 Test for Chlorogenic Acid: An extract containing chlorogenic acid when treated with aqueous ammonia and exposed to air gradually developes a green colour (Evans, 2008).

1.5.3. Medicinal and Biological Properties

Tannin-containing drugs will precipitate protein and for this, it has been used traditionally as styptics (able to stop bleeding) and internally for the protection of inflamed surfaces of the mouth and throat. They act as antidiarrheal and have been employed as antidotes in poisoning by heavy metals, alkaloids and glycosides. In western medicine, their use declined after World War II when it was found that absorbed tannic acid can cause severe central necrosis of the liver. Recent studies have concentrated on the antitumor activity of tannins (Evans, 2008) and it has been shown that, to exhibit a strong activity, ellagitannin monomer units having galloyl groups at the *O-2* and *O-3* positions on the glucose core(s), as in the

tellimagrandins are required. Anti-HIV activity has also been demonstrated (Evans, 2008).

Proanthocyanidins (or condensed tannins) are associated with beneficial effects of various herbs and infusions produced from them. The anti-tumour activity of green and black tea has been extensively researched in recent years with positive findings. Of the components of tea, epigallocatechin-3-gallate, specifically, has been shown to prevent angiogenesis in mice. Cranberry juice has long been used for reducing bacterial infections of the bladder and these claims have now been supported by a randomized, double-blind, placebo-controlled trial carried out on 153 elderlywomen (Evans, 2008). Fructose has been implicated in this activity but recently, proanthocyanidins prepared from cranberries by reverse-phase and adsorption chromatography were shown to inhibit the adherence of P-fimbriated *E. coli* to uroepithelial-cell surfaces; other *Vaccinum spp.,* including blueberries had similar bioactivity, suggesting their contribution to the salutary effects in urinary tract infections (Evans, 2008).

CHAPTER TWO

2.0 Literature Review

2.1. The Taxonomic Classification of *Detarium microcarpum*

DOMAIN: *Eukaryota*

KINGDOM: *Plantae*

SUBKINGDOM: *Viridaeplantae*

PHYLUM/Division: *Magnoliophyta*

SUBPHYLUM/Subdivision: *Euphyllophytina*

INFRAPHYLUM: *Radiatopses*

CLASS: *Magnoliopsida*

SUBCLASS: *Rosidae*

SUPERORDER: *Fabanae*

ORDER: *Fabalos/Fabales*

FAMILY: *Fabaceae*

SUB FAMILY: *Caesalpinioideae*

TRIBE: *Detarieae*

GENUS: *Detarium*

SPECIFIC EPITHET: *microcarpum* – Guill & Perr

BOTANICAL NAME: *D. microcarpum* Guill. & Perr

(Bisby *et al*, 2007).

2.2 Botanical Description of the Plant

D. microcarpum Guill & perr. belongs to the family Fabaceae. It is commonly called Tallow tree in English. The Hausas from northern Nigeria call it *Taura*. It is a savannah tree, growing up to 9.114 m, with reddish-brown scaly bark; leaves glaucous (covered in grayish powdery substance) beneath; flowers cream in dense inflorescences; fruits edible, branchlets deciduous; a plant of the drier savannahs.

Leaflets rounded and usually emarginated at apex, oblong-elliptic, 6-12, alternate: calyx in bud densely pubescent outside; inflorescences congested; leaflets thickly coriaceous, usually about 7-11cm broad, but sometimes smaller, with numerous translucid gland-dots; fruits sub orbicular and flattened, about 4cm in diameter and 2.5cm thick, not very fleshy; often forming small abortive or galled fruits (Hutchison, 1966).

2.3 Family Fabaceae

The Fabaceae are herbs, vines shrubs, trees and lianas found in both temperate and tropical areas. They comprise one of the largest families of flowering plant numbering 630 genera and 18,000 species. The leaves are stipulate, nearly always alternate, and range from bipannately or palmately compound to simple. The petiole base is commonly enlarged into a pulvinus that commonly functions in orientation of the leaves (sometimes very responsively, as in the sensitive plant, *Mimosa pudica).* The flowers are usually bisexual, actinomorphic to zygomorphic, from slightly to strongly perigynous, and commonly in racemes, spikes, or heads. The androecium consists of commonly one or many stamens (most commonly 10), distinct or variously united, sometimes some of them reduced to staminodes. The pistil is simple, often stipulate, comprising a single style and a superior ovary with one locule containing two to many marginal ovules. The fruit is usually a legume, sometimes a samara, loment, follicle, indehiscent pod, achene, drupe, or berry. The seeds often have a hard seed coat with hourglass-shaped cells, and sometimes bear a u-shaped line called a pleurogram (Bisby *et al*, 2007).

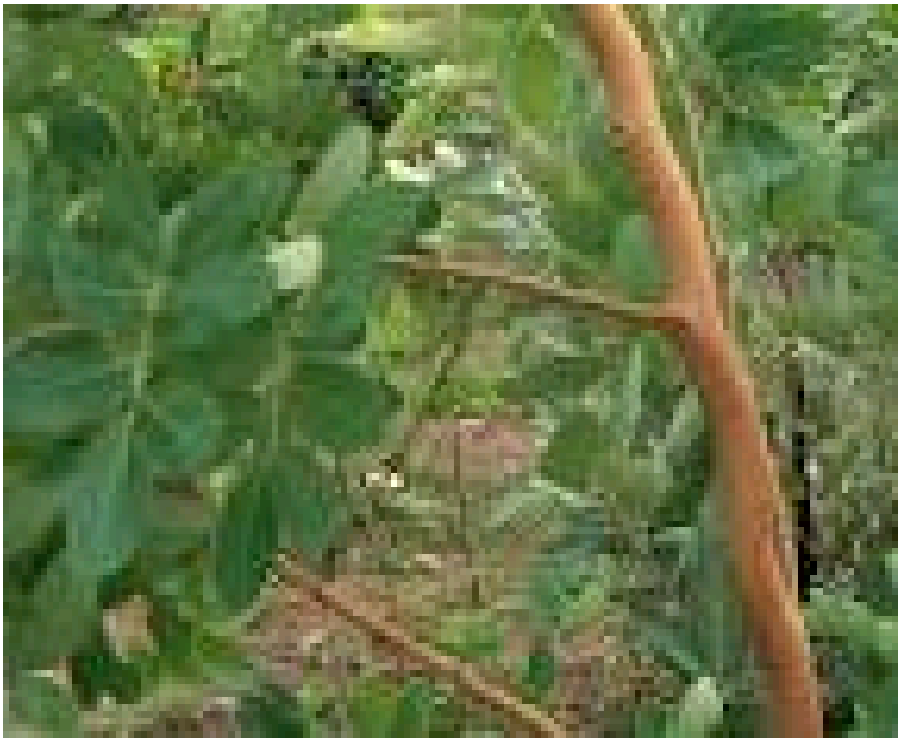


Plate I. *Detarium microcarpum* in its Natural Habit



Plate II. Fragments of the Barks of *D. microcarpum*

2.4. Other Species Belonging To the Same Genera

*D. senegalense (*J. F. Gmel). In Linn. Syst. Nat, *D. macrocarpum* Harms in Engl. Bot. Jahrb: and *D. Senegalensis: (*J.F. Gmelin) English: tallow tree (a corruption of The Gambian Mandinka name); Senegal dattock (from Wolof). (Burkill, 1995).

2.5. Ethnobotanical Survey of the Plant

Detarium microcarpum (Guill &Perr) is catalogued as a major African medicinal plant (Iwu, 1993).

The bark leaves and roots of *Detarium microcarpum* are widely used throughout its distribution area as diuretics and astrigents. They are prepared as infusions or a decoction to treat rheumantism, veneral diseases, urogenital infections, haemorrhoids, carries, biliousness, stomach-ache, intestinal worms and diarrhea including dysentery (Kouyaté, 2006).

2.5.1. Medicinal Uses

i. Fresh bark or leaves are applied to wounds, to prevent and cure infections; cutaneous, subcutaneous parasitic infection.
ii. In Mali the bark is also used to treat measles, nocturia, hypertension, itch and tiredness, while a decoction of the leaves or roots is taken against paralysis, meningitis, tiredness, cramps and difficult delivery.
iii. The powdered seeds are applied to skin infections and inflammations, whereas the fruit is eaten to cure meningitis and malaria.
iv. In Burkina Faso the fruit pulp is used for treating skin infections.
v. A preparation of the fruits is taken against dizziness in Niger and Togo.

vi. In Senegal a mixture of the leaves of *Detarium microcarpum*, *Sclerocarya birrea* (A. Rich.) Hochst. and *Acacia macrostachya* Rchb. ex DC. Pounded in milk is considered very efficient for snakebites.

vii. In Benin a decoction of the leaves is taken to treat fainting and convulsions.

viii. In West Africa the roots are part of a medico-magical treatment for mental conditions, and for protection against bad spirits.

ix. In veterinary medicine the leaves and roots are used to treat diarrhoea in cattle in southern Mali, and in Benin to treat constipation. In Niger cattle are made to inhale the smoke of the leaves to treat fever.

x. The plant as a whole is used in the treatment of arthritis, rheumatism, etc.; genital stimulants/depressants; leprosy; liver, etc. (Kouyaté, 2006).

2.5.2. Economic Uses

i. The fruit is sweet and commonly eaten fresh, while the pulp is used in the preparation of cakes and couscous. The pulp is used as a substitute for sugar. The seeds and leaves are eaten as a condiment and vegetable

ii. The seeds are used as frankincense and to make necklaces for women.

iii. The wood is hard and tough, with a regular grain, and is easy to work. It is used for carpentry, fence poles and joinery. It is durable and long-lasting even under water. The wood is well appreciated as firewood, as it lights quickly even if wet.

iv. *D. microcarpum* is well integrated in the traditional agroforestry systems of the Sahel, and it can be coppiced well. The leaves and flower are used as fodder, and the seeds as pig feed. In southern Mali the leaves are used as

roofing material, and as organic fertilizer. In Burkina Faso the leaves are used to make masks (Kouyaté *et al*, 2006).

2.6. Previous Research on *D. microcarpum*

2.6.1 Ninety percent aqueous methanol extract of the seeds coat (32.324%, w/w) of *Detarium microcarpum* was screened against clinical isolates of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomona aeruginosa, Klebsiella pneumonia, Salmonella paratyphi and Candida albicans*. A broad spectrum antimicrobial activity was observed. On bio-guided fractionation, the ethyl acetate soluble fraction retains the observed spectrum of antimicrobial activity. Preparative thin layer chromatographic separation on silica gel 60G using butan-2-one : chloroform : acetic acid : water (40:40:2:1 v/v/v/v) gave eight constituent bands: B1 (R_f = 0.000), B2 (R_f = 0.079), B3 (R_f = 0.250), B4 (R_f = 0.286), B5 (R_f = 0.500), B6 (R_f = 0.686), B7 (R_f = 0.814), and B8 (R_f = 1.000). B1 retained the observed antimicrobial activity spectrum, B2 and B3 showed residual inhibition against *B. subtilis* and B4 - B8 were inactive.

Phytochemical screening indicated the presence steroidal saponins and flavonoids in B1, and flavonoids. The UVvisible spectrum of B2 and B3 revealed the characteristic benzopyrone maxima (236 – 300 nm). (Ebi *et al*, 2011).

2.6.2. A 12-week study was conducted to investigate response of weaner rabbits to diets containing graded levels, 0, 5, 10 and 15%, of dietary *D. microcarpum* fruit pulp meal (DFPM). The results of the phytochemical assay shows that DFPM contained tannins (0.023%) whereas saponins, phytate, oxalates and hydrogen cyanides were not detected. Feed intake was significantly ($P < 0.05$) lower in 15% DFPM than in the control diet. Daily weight gain, nutrients intake, protein to gain

ratio and feed conversion ratio were not influenced by the dietary treatments. Similarly, nutrients digestibility was similar ($P > 0.05$) among the treatments. (Obun, 2010).

2.6.3. In this study, the chemical constituents of *D. microcarpum* plant were investigated for a comparison of the biological actions of the chemical composition of its leaves, stem and root barks. Extracts from the leaves, stem and root barks were analyzed for feeding deterrent and contact toxicity activities. All the solvent-based extracts from the various parts of *D. microcarpum* showed feeding deterrent and contact toxicity effects against *Tribolium casteneum*, Hbst with an LC50 value of 47µg/insect. IR and GCMS analyses have identified some saturated carboxylic acids and carbonyl compounds (Adeyemi, 2010).

2.6.4. A study was carried out to produce food products mainly concentrated juice and jam from the fruit pulp of *D. microcarpum* (DM), and compare them with a commercial sample as control. The proximate chemical analysis of the dried fruit pulp of DM showed high proportions of carbohydrate (4042.0%) and protein (29.1-30.9%); samples from Abu Gibaiha (AB) are the highest in both protein and carbohydrates in comparison with other samples from Ghibaish (GH) and Omdurman (OM). ICP-MS was used to determine the minerals (on mg/100 g dry weight basis) (Abdalbasit *et al*, 2009).

2.7. Research Problems

Despite the ethno-medicinal uses of this plant, no scientific evaluation has been provided for these uses.

2.8. Justification

Worryingly, bacteria related infections are increasingly becoming unresponsive to most antibiotics. This is due to so many resistant strains of these pathogenic microorganisms that resulted over the years. This therefore has unequivocally created more demands for antibiotics with better potency against those resistant strains.

2.9. Hypothesis:

Detarium microcarpum contains phytochemicals with antimicrobial activity

2.10. Overall Aim

To standardize and evaluate antimicrobial effect of Detarium *microcarpum*.

2.11. Objectives

i. To Evaluate Pharmacognostic charactersters of the stem bark of *D. microcarpum.*
ii. To detetrmine the phytochemical constituents in the stem bark of the plant.
iii. To investigate the antibacterial effect of the plant extract, and two other fractions.

iv.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Materials

Materials are those available in the Department of Pharmacognosy and Drug Development and that of the Department of Pharmaceutics and Pharmaceutical Microbiology. All reagents and chemicals were of laboratory standard/grade.

Materials and Reagents

Acetone
Ammonia
Ammonium
Animal's blood
Autoclave
Barfoed's reagent
Bojou bottles
Borer
Bunsen burner
Cabol fuchsine
Calcium Carbonate
Capillary tubes
Catechol
Chloroform
Choral Hydrate solution
Chromatographic glass tube
Ciprofloxacin
Compound light microscope
Cotton wool
Crucibles
Crystal Violet Stain
Disinfectants
Ethanol
Ethyl acetate
Fehling solutions
Ferric chloride solution
Filter papas
Forceps
Gelatin

Glycerin
Hager's reagent
Hydrochloric Acid
Incubator
Inoculating loop
Iodine solution
Iodine vapour
Kovacs' reagent
lead sub-acetate solution
Mac Conkey Agar
Magnesium chips
Manitol Salt Agar
Marker
Mayer's reagent
Methanol
Methylene blue
Mice
Micropipette
Microtone
Molisch's reagent
Mordant
Mueller-Hinton agar
Multidisc Antibioic
n-Hexane
Nutrient Broth
Oven
Paper discs
Paraffin wax
Peptone water
Petri dishes
Petroleum ether
pH indicator
Pipette teats
Plant materials
Ploroglucinol
Pyragallol
Pyridine
Quercetin
Chromatographic glass chambers
Resorcinol
Sabouraud Dextrose Agar
Sample botles
Sephadex LH-$_{20}$
Slides with cover slips
Sodium picrate paper
Sodium Chloride
Sodium Sulphate
Sudan IV
Sulphuric Acid

Syringes
Tannic acid
TLC chromatographic plates
Wagner's reagent

3.2. Collection, Identification and Preparation of the Plant Material

The plant material was collected from the wild in vegetation around the settlements in Giwa local Government Area located in Kaduna State, Nigeria. The collection was done in December 16th, 2008. The stem cuttings and twigs are the plant parts of interest. Thus; about 1.5 kg of these were collected. In addition, two fresh young twigs of the plant material were also collected. These were stored in a glass jar under refrigeration and used within 24hrs for the preparation of anatomical sections (Birdi *et. al.,* 2008).

Another fresh twig comprising all the essential parts used in the identification of plants was collected and taken to the Herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria for authentication/identification. A voucher number, 900676 was assigned to the plant sample on 17th December, 2008 by Mal. M. Musa and Mal. U.S. Gallah.

Preliminary processing of the plant material was done. This includes elimination of undesirable materials and contaminants, washing to remove soil, sorting and cutting (Evans, 2008; Birdi *et al.,* 2008). The bark was removed from the stem cuttings dried in an open air under shade. This was then powdered using mortar and pestle (Evans, 2008).

3.3. Microscopical Studies of the Stem Bark

The microscopical study was carried out using method described in the African Pharmacopoeia (1986). About 5g of the powdered stem bark was weighed in a test

tube and about 10ml of chloral hydrate solution was added. Slides were prepared using dilute glycerine as the mountant. The microscopic features were viewed using a compound light microscope. Microscopical measurements of the size dimentions of these features were also done using a stage micrometer in conjuction with an eyepiece micrometer (African Pharmacopoeia, 1986); (Kokate, 2001).

3.4. Anatomical Examination of *D. microcarpum*: It involves fixing sections of the stem of the plant in paraffin wax. The procedure was carried out as follows:

Firstly, the material was fixed in Formalin Acetic Alcohol (FAA) for at least 24 hours. Thereafter, the following were carried out:

i. *Rinsing*: The plant material was rinsed in water for 2 hours

ii. Dehydration: This tissue was fxed in 30%, 50%, 70%, 95%, alcohol each for 2hours. This is followed by further fixing in an absolute alcohol for two hours. This last step (i.e. in Absolute alcohol) is done twice.

iii. *Cleaning or de-alcoholization*: as soon as the tissue was removed from absolute alcohol, it was immediately fixed in Chloroform : Alcohol in the ratios 1:3, 1:1, 3:1, and finally in Absolute Chloroform. In each case, the tissue was allowed to stay for 2hours and the one in Absolute chloroform was done twice.

iv. *Impregnation of the Paraffin*: little paraffin wax chips molten at 60^0C were added; a little at a time by infiltration until chloroform no longer evaporates in which case impregnation process was expected to have taken place in molten paraffin wax at 60^0C for 24 hours (overnight).

v. Solidification: The impregnated tissue was kept in a watch glass to properly embed the tissue in the paraffin wax until the time for sectioning.

vi. Sectioning: The prepared tissue was then mounted on a microtone set at 5µ and then sectioned.

vii. *Preparation of Slides*: The sectioned tissue was placed on a clean slide and fixed on the slide using an adhesive (Glycerin and albumin)

viii. Staining Procedure: The sections were dawaxed in two stages using xylene for 5mins each. They were then treated as follows:

- In absolute alcohol twice for 2 mins in each case, then 95%, 70%, 50% and 30% alcohol for 2 mins in each case and finally in distilled water for 2 mins.
- It was then stained in sarafin for 30 mins and rinsed in tap water for 1min
- This was followed by de-staining using 70% Acid : Alcohol for 30 seconds.
- The de-stained tissue was then passed through 70%, 50%, and 30% Alcohol for 1min in each case. Thereafter, it was washed in distilled water for 1min.
- A counter staining was done using fast green for 1-2 mins, washed in tap water for 1min and in distilled water for another 1 min.
- It was later passed through 30%, 50%, 70%, 95%, and in absolute alcohol twice for 1min in each case.
- It was then passed through xylene in 2stages for 5mins in each case and finally mounted in D.P.X. which was a mixure of distrene (a polystyrene), a pastilizer (tricresyl phosphate) and xylene or Canada balsam. It was then covered with cover slips.

At this stage, the permanent slides prepared were then ready for viewing for the microscopical characters (Ruzin, 1999).

3.5. Chemomicroscopic Examination of the Powdered Stem Bark of *D. microcarpum*

The Chemomicroscopical studies was also done using methods in Evans, (2005) and African Pharmacopoeia, (1986)

3.5.1. Test for Cellulose

The powdered stem bark was moistened with N/50 iodine and allowed to stay until nearly dry. Then 80% w/w sulphuric acid was added. (Evans, 2008).

3.5.2. Test for Lignin

To a sample of the powdered stem bark, a few drops of phluroglucinol were added and allowed to stand for 2-3 mins until almost dry. A drop of concentrated hydrochloric acid was then added. A pink or cherry red colour indicates the presence of lignified cells; particularly sclerenchyma cells (A. P., 1986).

3.5.3. Test for Tannins

Here, the crude extract and the other two fractions were used. Firstly, 1g of the crude extract was dissolved in a test tube using 70% methanol. Thereafter, a very dilute solution of ferric chloride was gradually added to the solution of the extract which resulted in blue-black colouration. As more ferric chloride was added, the colour changes to olive-green. The same procedure was repeated for the methanol and tannins fractions (Evans, 2008).

3.5.4. Test for Mucilages

About 3 g of the powdered sample was moistened with a few drops of Ruthenium red. A reddish or dark pink coloration was shown (A. P., 1986; Evans, 2008).

3.5.5. Test for Starch

To about 5 g of the powdered sample, a few drops of N/50 iodine were added. (Kokate, 2001; Evans, 2008).

3.5.6. Test for Fats and Oils

About 1-2 drops of Sudan IV were added to the powdered sample and gently heated over a water bath. The fatty substances in the powdered sample are stained orange-red or brick-red (Evans, 2008).

3.5.7. Test for proteins

To about 3 g of the powdered plant sample was added a few drops of ninhydrin and the mixture was warmed gently over a water bath for about 5 mins. (Evans, 2008).

3.5.8. Test for Calcium Oxalate Crystals and Calcium Carbonates

For the calcium oxalate crystals, the powdered sample was first cleared using chloral hydrate solution. A little portion of the cleared sample was used to prepare a temporal slide with glycerine used as the moutant. When viewed under the light microscope, the crystal appears as bright structures of definite shapes and sizes. It was followed by adding 8% sulphuric acid and viewed again. This time, the calcium oxalate crystals disappear. This confirms their presence in the original powdered sample (Evans, 2008).

In order to confirm the presence of calcium carbonates, to about 5g of the powdered stem bark sample was added 5mls of acetic acid in a test tube. The

calcium carbonate dissolves with effervescence. When 50% sulphuric acid was used, needle-shaped crystals of calcium sulphate gradually separate (Evans, 2008).

3.6. Evaluation of Some Physical Constants of *D. microcarpum*

3.6.1. Determination of Moisture Content

The following procedure was carried out to determine the moisture content of the powdered stem bark of *D. microcarpum* using method described in (Brain and Turner, 1975).

An empty evaporating dish was heated to a constant weight, allowed to cool and placed in a desiccator. Thereafter, 3g of the powdered material was weighed into the dish; the dish with its content were placed in an oven at 105^0C and allowed to dry to a constant weight. Two identical weights read off consecutively confirm a constant weight. The total loss in weight was determined by subtracting the final weight of the dish and powder from the initial weight of the dish and the powdered stem-bark. Finally, the percentage of the moisture content was calculated with reference to the initial weight of the powdered drug (stem bark) as shown below (Brain and Turner, 1975)

$$\text{Percentage Moisture-Content} = \frac{\text{Final Weight of Powdered Stem Bark}}{\text{Initial Weight of Powdered Stem Bark}} \times 100\%$$

3.6.2. Determination of Total Ash Value

An empty nickel crucible was heated in an oven at 105^0C to a constant weight and the crucible was allowed to cool and kept in a desiccator. To the crucible was added 2 g of the powdered stem bark. The crucible and its content were heated gently until it was moisture free and completely charred. The heat was gradually increased until most of the carbon (black residue) was vapourized and then the heating was done strongly until the residue was free from carbon (i.e. almost white). The crucible and its content were then cooled, weighed and the weight accurately noted. The heating cooling and weighing was continued until the weight of the residue (ash) became constant. The weight of the ash was determined by subtracting the weight of the crucible from the final weight (i.e. weight of the crucible and the residue or ash). The ash value of the powdered drug (in percentage) was then calculated with reference to its initial weight as shown below:

$$\text{Percentage Total Ash Value} = \frac{\text{Weight of residual ash}}{\text{Weight of original sample}} \times 100\%$$

3.6.3. Determination of Acid-insoluble Ash Value

The crucible containing the ash from the Total-Ash-Value experiment was transferred to a beaker containing 25 ml of dilute hydrochloric acid. This mixture was boiled for 5 mins and filtered through an ash-less filter paper. The content of the beaker was rinsed with water through the filter paper. This washing/rinsing was repeated 3 times and each time, the washing was passed through the filter paper in a manner so that the residue is collected in the tip of the cone of the filter paper. The funnel was then dried along with the filter paper in an oven at 105^0C. A clean nickel crucible was heated and its weight was accurately measured and noted. The filter paper with the residue earlier dried in an oven was folded into a small cone

and transferred into the crucible. The crucible and its content was heated gently until the filter paper was completely charred then the heat was applied strongly for a few minutes. The crucible with its content was cooled and weighed. This final weight was noted accurately. The weight of the residue (the acid insoluble ash) was determined by subtracting the weight of the crucible from the final weight. The acid insoluble ash value (in percentage) was calculated with reference to the initial weight of the powdered drug from the Total-Ash-Value as shown below:

$$\text{Percentage Acid insoluble Ash Value} = \frac{\text{Weight of the acid insoluble ash}}{\text{Weight of the powdered drug}} \times 100\%$$

3.6.4. Determination of Extractive Values

3.6.4.1. Alcohol-soluble Extractive Value: This was carried out using the procedure the following procedure:

About 5g of the powdered stem bark was accurately weighed into a 250ml conical flask. 100ml of 90% ethanol was added and the stopper replaced firmly. The flask and its content was shaken on a mechanical shaker for 6 hours and then allowed to stand for 18 hours. The mixture was filtered quickly. The weight of a clean flat-bottomed evaporating dish was accurately determined. About 20ml of the filtrate was poured into the tarred evaporating dish and evaporated to dryness on a hot plate. The weight of the residue was determined from the 20ml extract by subtracting the weight of the evaporating dish from the final weight and the Alcohol-extractive value was calculated with reference to the initial weight of the powdered drug as follows

$$\text{Percentage Alcohol-soluble extractive value} = \frac{\text{Mass of Residue}}{\text{Mass of Powdered Drug}} \times 100\%$$

3.6.4.2. Water- soluble Extractive Value: The procedure of Alcohol-extractive-value above was repeated using chloroform-water (0.25% v/v chloroform in distilled pure water), in place of ethanol as the extracting solvent. The Water-soluble extractive value was determined using the method described in (Brain and Turner, 1975).

$$\text{Percentage Water-soluble extractive value} = \frac{\text{Mass of Residue}}{\text{Mass of Powdered Drug}} \times 100\%$$

3.7. Extraction Procedures

Firstly, 1000g of the powdered crude drug was defatted using 5 litre of petroleum ether (60-80^0 C). The marc obtained from this was subjected to extraction by cold percolation using 1 litre of 70% methanol. The filterate obtained was divided into two portions. Tannins were precipitated from one portion using 1% solution of gelatin containing 10% NaCl. This was filtered to separate the precipitated tannins and a filtrate is obtained. The tannins were recovered from the residue by adding another 1 ltr of 99% MeOH to the precipitate obtained and filtered. This dissolves the tannins leaving a residue of gelatin and NaCl. The filterate contains the tannins while the gelatin and the NaCl remains in the precipitates as the residue (or the marc). The filtrate was concentrated over water bath at a controlled temperature and MUST NOT be charred as some phenolics are unstable at certain high temperatures. The resulting extract constitutes the tannin fraction (TF). The filtrate obtained after precipitating the tannins was also concentrated in an evaporating dish over a water bath. This constitutes the Residual Fraction (RF). The second portion of the filterate obtained after defatting was concentrated to obtain the crude extract (MCE). The petroleum ether fraction obtained from the defatting procedure

was also concentrated in an evaporating dish over water bath and stored for chromatographic (TLC) analysis.

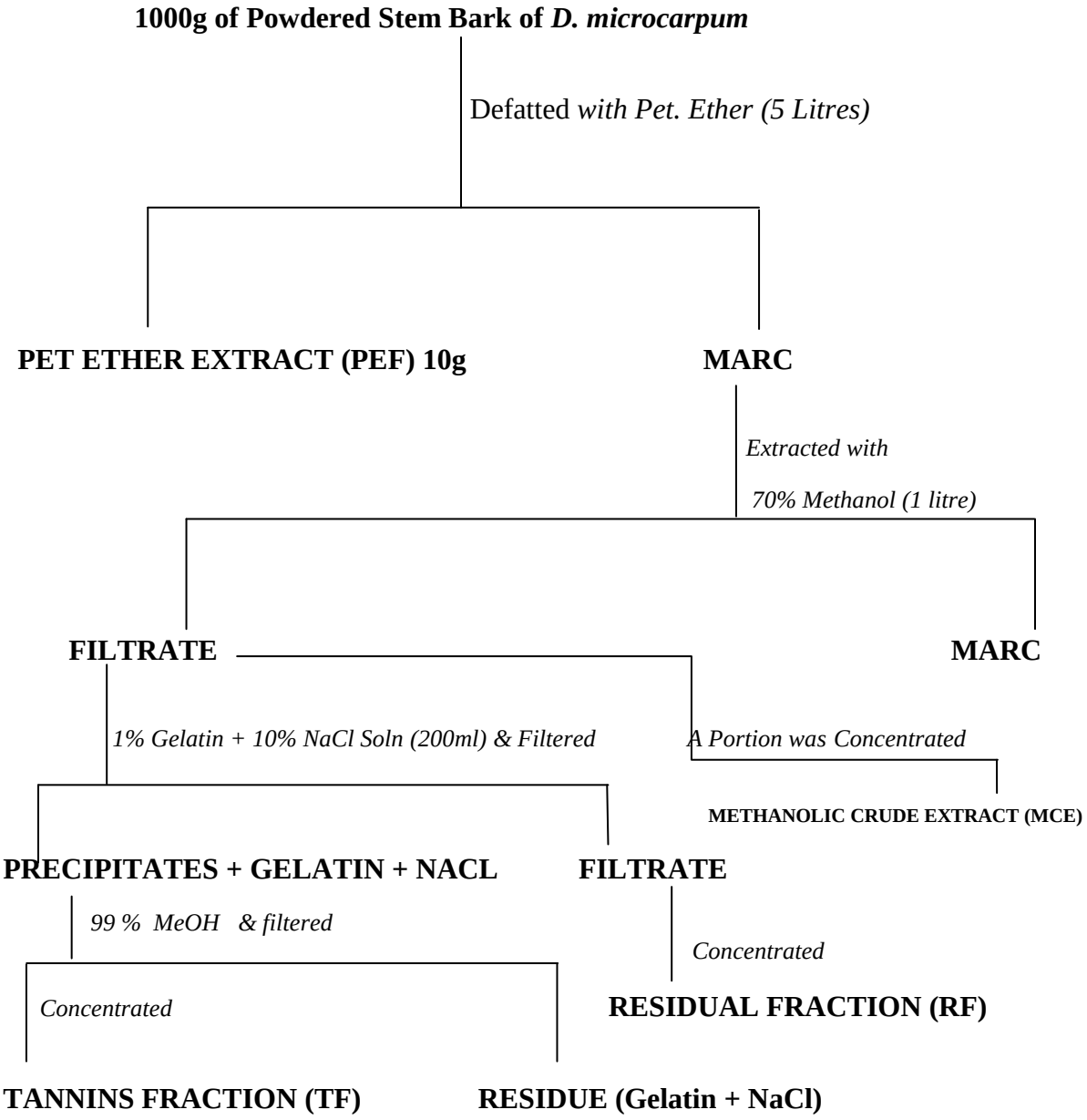


Fig.3.1. Flow Chart (General Procedure for Preparing Tannin Fraction, Residual Fraction and Crude Extract) (Cannell, 1998).

3.8. Phytochemical Screening of the Crude Extract, Tannin Fraction and Residual fraction

Standard screening tests of the crude extract and the other two fractions were carried out for the presence of various phytochemical constituents of the stem bark.

3.8.1. Test for Carbohydrates

3.8.1.1. Molisch's Test (General Test for Carbohydrates):

About 2g of the plant extract was dissolved in 5ml of 70% of Methanol in a test tube. About 3ml of this solution was dispensed in a separate test tube; a few drops of Molisch's reagent were added. Then a small amount of concentrated H_2SO_4 was carefully added to form a lower layer. A reddish colour at the interface indicates the presence of carbohydrate (Evans, 2008)

3.8.1.2. Fehling's Test (Test for Reducing Sugars/Combined Reducing Sugars):

A solution of the extract was prepared by dissolving 0.1g of the extract in 5ml of water. 2ml of an equal mixture of Fehling solutions A and B were added to about 5-6ml of the solution of the extract and the mixture was boiled in a water bath for 5mins. A brick-red precipitate indicates the presence of free reducing sugar. If the above test is negative, 0.2g of the drug solution was hydrolyzed in water by boiling with 5mlof dilute hydrochloric acid for 5mins. 5ml of 10% sodium hydroxide was

added to neutralize the solution of A and B and boiled. A brick red precipitate indicates the presence of combined reducing sugars (Evans, 2008)

3.8.1.3. Barfoed's Test:

About 5ml of a dilute solution of the drug sample was prepared by dissolving it in water in a test tube. 1ml of Barfoed's reagent was then added and heated in a water bath. A red precipitate indicates the presence of monosaccharide *(*Brain and Turner, 1975*).*

3.8.1.4. Test for Pentoses

An aqueous solution of the drug sample was prepared by dissolving 3g of the powdered drug in 5ml of distilled water. The solution was mixed with an equal volume of hydrochloric acid containing a little phloroglucinol. The formation of a red colour indicates the presence of pentoses (Evans, 2008).

3.8.1.5. Resorcinol Test (Selivanoff's Test):

To a dilute solution of the drug sample, a crystal of resorcinol and an equal volume of concentrated HCl was added and heated in a water bath. A rose colour produced indicates the presence of ketoses (Evans, 2008)

3.8.2. Test for saponins

3.8.2.1. Frothing Test:

About 25ml solution of the extract was prepared using 70% methanol and 10ml of distilled water was added in a test tube. The test tube was shaken vigorously for

about 30 seconds. It was then allowed to stand in a vertical position and observed for a period of 30minutes. A honey comb froth which persists for about 10- 15 minutes indicates the presence of saponins in the drug sample (Silva *et. al., 1998*)

3.8.2.2. Haemolysis Test

About 2ml of the solution of the extract in 70% methanol was dispensed in a test tube and 2ml of 1.8% aqueous solution of sodium chloride was added. Another test tube containing 2ml of distilled water which serve as the control was set up. Thereafter, 5 drops of animal's blood were added to each of the tests tubes and the content was mixed gently. The tubes were observed for 15 – 30 minutes for haemolysis. The presence of haemolysis in the tube containing extracts and its absence in the control test tube indicates the presence of saponins in the extract (Brain and Turner, 1975).

3.8.3. Test for Flavonoids:

3.8.3.1. Ferric Chloride Test:

About 2ml of the extract was measured and put in a test tube; a few drops of 10% ferric chloride solution were added. A green or blue colour indicates the presence of phenolic nucleus (Evans, 2008).

3.8.3.2. Shinoda's Test:

Four pieces of magnesium chips were added to 5ml of the extract followed by a few drops of concentrated HCl. A pink or red colour indicates the presence of flavonoids

3.8.3.3. Sodium Hydroxide Test:

About 5ml of the extract was measured and poured in a test tube; 5ml of 10% NaOH was added. A yellow solution indicates the presence of flavonoids. On addition of dilute hydrochloric acid, the solution becomes colourless (Evans, 2008).

3.8.3.4. Test for Cyanogenetic Glycosides

About 1g of the powdered drug was placed in a clean test tube and a few drops of water were added to moisten the drug. About 0.5ml of chloroform was also added to the test tube containing the drug sample to enhance the enzyme activity. A piece of freshly prepared sodium picrate paper was suspended above the drug by trapping the top edge between a cork and the test tube wall. The closed test tube was placed on a water bath for 15 minutes. A brick red or maroon colour on the paper due to the release of hydrocyanic acid vapour indicates the presence of cyanogenic glycosides (Silva *et al.*, 1998).

3.8.4. Test for Steroids / Triterpenoids:

About 0.2g of the extract was dissolved in 10ml of chloroform. This was stirred thoroughly for about 5 minutes and transferred into a test tube and 0.5mg of anhydrous Sodium Sulphate was added to it. The mixture was gently shaken and filtered into 3 clean and dry test tubes (I, II and III). These were used for the following tests.

3.8.4.1. Liebermann-Burchard Test:

To the filtrate in test tube I, equal volume acetic anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was then added down the side of the test tube to form a lower layer. Colour changes were observed immediately and over a period of one hour. The colour changes were compared with the control tube III.

Blue to blue-green colour in the upper layer and a reddish ring indicates the presence of steroids and red, pink or purple colour indicates the presence of triterpenoids (Silva *et al*., 1998).

3.8.4.2. Salkowski's Test (for steroidal ring):

The test tube II was at an angle 45^0 and 2-3 drops of concentrated sulphuric acid was added from the side of the test tube. A reddish brown ring at the interface indicates the presence of a steroidal ring (Sofowora, 1993).

3.8.5. Test for Anthraquinones

3.8.5.1. Free Anthraquinones: A small quantity of the extract was shaken with 10ml of benzene. The content was filtered and 5ml of 10% ammonia solution was added to the filtrate, the presence of a pink, red or violet colour in the ammonical (lower) layer indicates the presence of free anthraquinones (Evans, 2008).

3.8.5.2. Borntrager's Test: About 1g of the powdered drug was boiled with 5ml of 10% hydrochloric acid for 2-3 minutes. The hot solution was then filtered in a test tube and allowed to cool. 5ml of benzene was added to the filtrate in a separating funnel; the upper benzene layer was separated off and shaken gently in a test tube with half of its volume of 10% ammonium hydroxide. Rose pink to cherry red colour in the lower ammonia layer indicates the presence of anthraquinone (Evans, 2008).

3.8.6. Test for Tannins

3.8.6.1. Ferric Chloride Test:

To 2ml of the extract, in a test tube, 2-3 drops of 5% ferric chloride solution was added. A green or greenish-black precipitate indicates the presence of condensed tannins while a blue or brownish-blue precipitate shows the presence of hydrolysable tannins (Evans, 2008).

3.8.6.2. Lead Acetate Test:

To about 2ml of the extract, 2-3 drops of lead sub-acetate solution was added. A blue coloured precipitate indicates the presence of tannins *(*Brain and Turner, 1975*).*

3.8.6.3. Gelatin Test:

To about 0.5-1% of the solution, 1% solution of gelatin containing 10% sodium chloride was added. The presence of precipitates indicates the presence of tannins.

3.8.7. Test for Alkaloids

3.8.7.1. Extraction:

About 10ml of the methanol extract was dissolved in 5% sulphuric acid. This was filtered and placed in a 100ml separating funnel. The solution was made alkaline by the addition of dilute ammonia solution. An equal volume of chloroform was added shook gently and the layer was allowed to separate. The chloroform layer was allowed to run off into a test tube. This was extracted with two quantities each of 5ml of dil

ute sulphuric acid.

3.8.7.2. Test:

To 1ml of the extract, 3 drops of Mayer's reagent, Wagner's reagent, Dragendorff's reagent, Hager's reagent and Tannic acid were each added.

3.8.7.3. Observation/inference:

Turbidity or precipitation with either of the above reagents was taken as evidence for the presence of alkaloids in the extracts being evaluated (Evans, 2008).

3.8.8. Test for Cardiac Glycosides

3.8.8.1. Keller-Kiliani's Test:

To 5ml of the extract, 3 drops of strong lead sub-acetate solution were added and mixed thoroughly. The mixture was then filtered. The filtrate was extracted in a separating funnel with 10ml of chloroform. The chloroform layer was run off into a beaker. The residue was dissolved in 1ml of glacial acetic acid containing trace amount of ferric chloride solution, and transferred to a dry test tube. With the test tube held at 45^{O} angle, 1ml of concentrated sulphuric acid was allowed to run down the inside wall of the test tube and a layer was formed at the bottom. A reddish brown ring formed at the junction of the two layers and the upper layer turning bluish green indicates the presence of cardiac glycosides (Brain and Turner, 1975).

3.8.8.2. Kedde Test for Cardenolides:

About 3g of the extract was dissolved in pyridine and a few drops of sodium nitroprussade together with a few drops of 20% sodium hydroxide solution were added. A deep red colour, which fades to brownish yellow, indicates the presence of cardenolide (Kokate, 2001).

3.9. Chromatographic Analysis

3.9.1. Thin Layer Chromatographic Analysis of Methanolic Extract (ME) and the Pet-Ether Fraction (PEF) of the stem Bark of *D. microcarpum*

TLC chromatographic plates (20cm by 20cm pre-coated with silica gel) were used. They were cut into strips with dimensions of 10cm by 3cm. The TLC plates were then activated in an oven at 100^0C for 15mins

3.9.1.1. Sample spotting:

The crude plant extract and the tannin fraction were each separately dissolved in 70% Methanol while the pet-ether fraction was dissolved in petroleum ether; with each resulting in about 1-2% solution of the sample material. Each of the prepared solution of sample materials was spotted at the origin of the TLC plate (marked with a pencil) with the aid of a capillary tube. The Crude extract and the tannin fraction were spotted side by side on the same plate while the pet-ether fraction was spotted on a separate plate.

3.9.1.2. Solvent System:

Ethyl-acetate: Methanol: Water (10:1.6:1.4v/v) gave the best resolution for the crude extract and the tannins fraction while n-Hexane: Ethyl-acetate (9:1v/v) gave the best separation for the pet ether fraction. The spots were sprayed with the appropriate reagent (10% H_2SO_4) to determine the class of compounds contained in the plant. The R_f values were also calculated

3. 9. 2. Column Chromatographic Analysis of the Tannins Fraction

3.9.2.1. Packing of the column

The method of wet packing was employed. Firstly, 5g of the adsorbent (Sephadex –LH_{20}) was slurried in 200ml of absolute Ethanol to equilibrate it. A small plug of

cotton wool was fixed at the base of a clean chromatographic column which was gently and firmly clamped to a retort stand. The slurried sephadex was added in portions to the tube; allowing the packing to settle between additions in order to ensure a more homogenous column. The excess solvent (Ethanol) was allowed to drain out. A solution of the tannin fraction was prepared by dissolving 2 g of it 50 ml of Ethanol. This was filtered through a Whatman № 1 filter paper to obtain the ethanol-soluble portion and to remove any insoluble material. The filterate was then concentrated over water bath. The concentrate was gently applied to the top of the adsorbent column. The first solvent (pure and absolute ethanol) was allowed to percolate through the column. Subsequently, the eluate is collected in the collection bottles in small portons of about 3 ml. Altogether, 20 different portions of the eluates were collected while the ethanol was allowed to percolate through the column after which the second solvent (acetone) was then used. The acetone was likewise allowed to percolate the column and about 20 portions of the eluates were also collected (about 3ml each) in the collection bottles. Sephadex LH_{20} sorbs tannins in alcohol, and releases them in aqueous acetone; thus phenolics are applied to Sephadex in ethanol and ultimately eluted with acetone. The eluates were allowed to concentrate at room temperature (Hagerman, 2002); (Evans, 2008).

3.9.3. Co-chromatography Using TLC plates: The co-chromatography of the unidentified fractions obtained from the column chromatography was done alongside five (5) standard reference compounds. These reference compounds were as follows: catechol, gallic acid, phloroglucinol, pyragallol and quercetin.

3.9.3.1. Procedure

The solvent system was prepared and poured to a depth of 3cm in the chamber lined from inside with a filter paper. The plates were activated in an oven at 120^{o}C for 10minutes**.** Standard solutions of the reference compounds were prepared by dissolving about 2 mg of each in 2ml of distilled water. The spots of individual reference compound solutions were applied (0.005ml) as reference standards at a distance of 1-2cm from each other using of a capillary tube. Five different spots of acetone fractions were also applied at the other end of the plates. The spots were then allowed to dry at room temperature. The glass plates were then gently placed inside the chamber and developed by ascending technique till the solvent front moved about 10cm. The plates were taken out and the solvent front was marked and the plate dried at room temperature. The characteristic colorations were firstly observed in daylight. The first plate was exposed to iodine vapour in an iodine glass chamber for about 10minuites, the second plate was sprayed with 10% solution H_2SO_4 acid and heated at 100^{0}C for about 5-10mins while the third plate was sprayed with 5% solution of ferric chloride. The R_f values of each standard reference compound and those of the unknowns were calculated. These unknowns were then identified by comparing their colour and R_f values with that of the standard reference compounds (Kokate, 2001). The eluate cannot be monitored in the UV because of the strong UV absorbance of acetone (Hagerman, 2002).

3.10. Determination of Total Phenolics

The total phenolics of the stem bark of *D. microcapum* were determined by the UV-Visible Spectrophotometric Analysis method. Here, tannic acid was used as the standard or reference. Five different concentrations of the tannic acid (4, 8, 12, 16, and 20) g/dm^3 were prepared. Each was then reacted with a few drops of 1%

$FeCl_3$ solution in 50% Methanol at a pH of 7.12. The reaction mixture was allowed to stay at room temperature for about 8-10minutes after which the absorbences were measured using the Jenway 630 UV-visible spectrophotometer at 570nm against water: methanol blank. The various absorbances obtained were plotted against the corresponding concentrations to obtain a standard calibration curve. Thereafter, the plant sample was treated in the same way as the tannic acid (Singleton *et al.*, 1999).

A standard calibration curve was plotted using tannic acid (4-20 g/dm^3).

The total phenolic compounds were calculated by the following formula; $T=\frac{CV}{M}$

Where, T= total content of phenolic compounds in TAE/g

C= concentration of Extract established from the calibration curve in g/dm^3

V= volume of extract in ml

M= weight of plant extract in grams

The results were expressed as mg of tannic acid equivalents (TAE)/1g of extract weight.

3.11. Acute Toxicity Studies of Methanolic Crude Extract and the Tannin Fraction

The acute toxicity studies were carried out according to the method described by Lorke (1983). The experiment was conducted in three batches. A total of 13 mice were used for each batch. Each batch differs from the others based on the type of extract administered as well as the mode of administration. In batch A, the methanolic crude plant extract was used which was administered intraperitoneally (*i.p.*) in batch B, the tannin fraction was administered using the *i.p.* route of administration and in batch C, the residual fraction was used and this was administered intraperitoneally.

Table 3.1. Samples from Stem Bark of *D. microcarpum* and Route of Administration

Batch	Extract Administered	Route	Number of Mice Used
A	Crude Plant Extract	*i.p.*	13
B	Tannin Fraction	*i.p.*	13
C	Residual Fraction	*i.p.*	13

In each batch, the experiment was carried out in two phases. The first phase comprises 9 mice divided into three groups comprising 3 mice each. The volume administered to each mouse was calculated in proportion to their body weight. These groups received 10 mg/kg b.w., 100 mg/kg b.w., and 1,000 mg/kg b.w. The result obtained from the first phase, was used to determine the doses to be administered in the second phase. General signs and symptoms for toxicity and mortality were observed between 24 – 48 hours after each administration.

3.12. Antimicrobial Studies

3.12.1. Isolation of Microorganisms

3.12.1.1. Preparation of Nutrient Broth (NB): Exactly 3.25 g of NB was dissolved in 250 mls of distilled water (13 g ≡ 1 litre). The mixture was properly stirred and 5ml each were aseptically dispensed into forty different bijou bottles. The bottles and their contents were sterilized in an autoclave at 121^0C for 30 mins and kept in the refrigerator.

3.12.1.2. Samples Collection: Forty (40) urine samples (about 10mls each) from 20 different HIV positive patients and 20 different HIV negative patients were collected from the PEPFER clinic (Nasara clinic), ABUTH, Shika, Zaria with

special interest on those patients that have not been on any antibiotic or HAART for a minimum of two weeks before the collection. The samples were collected in 25ml sterile bottles and taken straight to the laboratory for inoculation

3.12.1.3. Inoculation: 5 ml of each urine sample was inoculated into a bottle containing 5 mls of sterile nutrient broth prepared prior to the samples collection; (a single sample per bottle). These were kept in the incubator at 37^0C for 18 hours.

3.12.1.4. Culturing: 30 clean glass plates (Petri dishes) were sterilized in an oven at 161^0C for 1hr; each was divided into 4 quadrants using permanent markers at the base. The following were then prepared and sterilsed in an autoclave according to the manufacturer's instructions:

10 bottles containing 10 ml each of MacConkey Agar (MA) – Oxoid LTD

10 bottles containing 10 ml each of Manitol Salt Agar (MSA) –Oxoid LTD

10 bottles containing 10 ml each of Sabouraud Dextrose Agar (SDA) –Oxoid LTD

The content of each bottle (10 ml of agar) was emptied into each plate in its molten state and allowed to solidify. Each sample earlier grown in Nutrient Broth was inoculated in the plates using an inoculating loop by the streaking method; each quadrant represents a sample. Each of the three groups of media has a quadrant representing a sample. The quadrants were labeled accordingly. These were kept in an incubator at 37^0C for 48 hours.

3.12.1.5. Sub Culturing: The colonies that resulted (or grew) from those plates were then sub cultured into prepared 5ml agar slants; Nutrient Agar slants were used for the organisms grown on MA and MSA while SDA slants were used for

the growths obtained from SDA plates. Each colony were grown on three different slants i.e. in triplicates and labeled appropriately.

3.12.1.6. Preparation for Microscopy: Nutrient broth was prepared and dispensed in bottles 5 ml each. The bacteria were then sub-cultured in the bottles and kept in the incubator for 18 hours at 37^0C.

3.12.1.7. Preparation of Slides: A glass slide was properly cleaned and a circular mark was made slightly off the center using a marker. A loopful of water was placed within the marked centre of the slide

3.12.1.8. Smearing: With the aid of an inoculating loop, a very small amount of the bacteria culture was aseptically picked up and mix into the drop of water. This was then spread out evenly within the marked circle to about ½ inch area. The name of the culture was marked with the wax pencil in the far left edge on the slide. This is also known as filming. (Prescott, 2002).

3.12.1.9. Air Drying: The smear on the slide was allowed to dry in air at room temperature.

3.12.1.10. Heat Fixation: The heat-fixing was done after the smear was dry. This was accomplished by gentle heating. Holding the smeared slide by one edge, it was passed slowly through a Bunsen burner flame several times through the hot portion of the flame of a Bunsen burner to fix the bacteria. This fixation step denatures bacterial proteins causing the cells to stick to the slide while also killing them thus, making them safe for the following steps (Prescott, 2002).

3.12.1.11. Primary Staining: In this procedure, the heat fixed slide was placed on a staining rack, covered with a small amount of crystal violet stain and incubated

for 30 seconds, the dye was then drained and rinsed off the slide with distilled water from squeeze wash bottle and, finally, blotted dry (Prescott, 2002).

3.12.1.12. Mordant (Gram's Iodine): The mordant is applied to cover the smear. About 5 drops of iodine was added to the same slide and allowed to stand for about 1 minute. Again, this was drained and rinsed off the slide with distilled water (Prescott, 2002).

3.12.1.13. Decolourization: The slide was held at 45^0angle. Over the staining rack and rinsed with 90% alcohol containing few drops of methylene blue drop wise. The ethanol was washed off the slide by rinsing with water for 5 seconds as it runs off. Blot dry with bibulous paper (Prescott, 2002).

3.12.1.14. Counterstain: Finally, a few drops of dilute cabol fuchsine were added to cover the bacterial smear on the slide. This was drained out and the slide rinsed after about 30 seconds. Finally, the slide was blotted with dry bibulous paper and examined under oil immersion (Prescott, 2002).

A slide was prepared for each sample of bacterial colony using the following procedure.

3.12.1.15. Microscopy: A few drops of immersion oil were placed on the smeared area on each slide shortly before viewing under the microscope.

3.12.2 Biochemical Tests

Although the morphological appearance of these bacteria as viewed under the microscope gives a clue to which is gram positive and which is gram negative, it is necessary to carry out some biochemical tests to confirm the claims of the gram staining results.

3.12.2.1 Indole Formation: This is a useful biochemical test used in distinguishing various species of the genera *Escherichia, Proteus* and *Vibrio* from other bacterial genera. The cells of the above named genera contain the enzyme, tryptophanase, which is capable of breaking down tryptophan. The amino acid tryptophan is found in nearly all proteins. Bacteria that contain the enzyme tryptophanase can hydrolyze tryptophan if present in the culture to indole to its metabolic products, namely, indole, pyruvic acid, and ammonia. The bacteria use the pyruvic acid and ammonia to satisfy nutritional needs; indole is not used and accumulates in the medium. The presence of indole can be detected by the addition of Kovacs' reagent. (Bhome's reagents A & B) Kovacs' reagent reacts with the indole, producing a bright red compound on the surface of the medium (Prescott, 2002).

3.12.2.1.1. Method for Indole Test: The test was carried out on a 5 day culture of the bacterial cells in peptone water. To each bottle containing the different sample of bacterial cells, few drops of Kovacs' reagent were added

3.12.2.2. Methyl Red (MR) Test

All enteric bacteria catabolize glucose for their energy needs; however, the end products vary depending on the enzyme pathways present in the bacteria. The pH indicator methyl red detects a pH change to the acid range as a result of acidic end products such as lactic, acetic, and formic acids. This test is of value in distinguishing between *E. coli* (a mixed acid fermenter) and *E. aerogenes* (a butanediol fermenter). Mixed acid fermenters such as *E. coli* produce a mixture of fermentation acids and thus acidify the medium. Butanediol fermenters such as *E. aerogenes* form butanediol, acetoin, and fewer organic acids. The pH of the medium does not fall as low as during mixed acid fermentation (Prescott, 2002).

3.12.2.3. Voges-Proskauer (VP) Test

The Voges-Proskauer test (named after Daniel Voges, German physician, and Bernhard Proskauer, German hygienist, in the early twentieth century) identifies bacteria that ferment glucose, leading to 2,3-butanediol accumulation in the medium. The addition of 40% KOH and a 5% solution of alpha-naphthol in absolute ethanol (Barritt's reagent) will detect the presence of acetoin-a precursor in the synthesis of 2,3-butanediol. In the presence of the reagents and acetoin, a cherry-red color develops. Development of a red color in the culture medium 15 minutes following the addition of Barritt's reagent represents a positive VP test; absence of a red color is a negative VP test (Prescott, 2002).

3.12.2.3.1. Procedure for MR and VP tests

i. The bacterial cells were cultured in MRVP medium three days prior to the tests
ii. Each culture was divided into equal portions
iii. To one portion, 5 drops of 0.04% methyl red solution was added. A magenta red colour is considered MR positive, a yellow colour MR negative or pink colour is equivocal.

iv. To the other portion a trace of creatinine and 5ml of 40% KOH solution were added and the tube was thoroughly shaken

v. A pink colour is VP-positive while no colour is VP-negative

3.12.2.4. Citrate Utilization Test

The citrate utilization test determines the ability of bacteria to use citrate as a sole carbon source for their energy needs. This ability depends on the presence of a citrate permease that facilitates transport of citrate into the bacterium. Once inside the bacterium, citrate is converted to pyruvic acid and CO_2. Simmons citrate agar slants contain sodium citrate as the carbon source, NH_4+ as a nitrogen source, and the pH indicator bromothymol blue. This test is done on slants since O_2 is necessary for citrate utilization. When bacteria oxidize citrate, they remove it from the medium and liberate CO_2. The CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate—an alkaline product. This raises the pH, turns the pH indicator to a blue colour, and represents a positive citrate test; absence of a colour change is a negative citrate test Citrate-negative cultures will also show no growth in the medium (Prescott, 2002).

3.12. 3. Antibiotic Sensitivity (Susceptibility) Tests

3.12.3.1. Paper Disc Technique:

A multidisc with eight different antibiotics (Abtek® Biologicals LTD) was used. This is a test of diffusion of the antibiotics.

Another method that is used to determine antibiotic susceptibility is the sensitivity paper disc method of Kirby-Bauer. In this method, antibiotics are impregnated onto paper disks and then placed on a seeded Mueller-Hinton agar plate using a mechanical dispenser or sterile forceps (Prescott, 2002).

3.12.3.1.1 Standardization of Organisms: Each bacterial sample was sub cultured in nutrient broth and incubated for 18hrs. The gram-negative bacteria (*E. coli*) were

standardized by diluting the overnight cultures based on 1 in 1000 dilution and the gram-positive bacteria (*S. aureus*) were standardized based on 1 in 500 dilution. The dilution was done using prepared sterile normal saline.

3.12.3.1.2. Preparation of Agar Plates: Twenty-one sterile plates were prepared and 20ml of sterile Mueller Hinton agar was aseptically dispensed into each plate and allowed to cool and solidify. Standard method (Prescott, 2002) was used.

3.12.3.1.3. Inoculation: The surface of each agar was inoculated with 5ml of an appropriate standardized (diluted) bacterial culture by flooding the surface of the solidified agar ensuring that the whole surface was evenly circulated over the entire plate and then the excess discarded. Each plate was labeled appropriately. This is also called seeding (Prescott, 2002). The seeded agar plates were allowed to stay on the plate for 10 minutes at room temperature with the top (lid) in place.

3.12.3.1.4. Dispensing the Antibiotic Discs: The antibiotics discs were dispensed individually onto the plates aseptically with forceps making sure that contact is made between the antibiotic disc and the culture by gently pressing the disk with alcohol-flamed forceps. The plates were allowed to stay for about 30mins then incubated for 16 to 18 hours at 35°C (Prescott, 2002)

3.12.3.1.5. Measurement of the Zones of Inhibition: After 18hrs of incubation, the zones of inhibition were measured from the antibiograms (antibiotic susceptibility patterns). The measurement was done for each of the antibiotics tested to the nearest mm. Two diameters were taken across each antibiogram and the average calculated and recorded (Prescott, 2002).

3.12.3.2. Well Diffusion Technique

Here, two extracts from the plant and a standard drug (ciprofloxacin) were used. A control was also set up in the operation to run alongside the tested extracts. Like the paper disc technique, the principle involved is also based on the diffusion of the antibiotic.

3.12.3.2.1. Standardization of Organisms: Each bacterial sample was sub cultured in nutrient broth and incubated for 18hrs. The gram-negative bacteria (*E. coli*) were standardized by diluting the overnight cultures based on 1 in 1000 dilution and the gram-positive bacteria (*S. aureus*) were standardized based on 1 in 500 dilution. The dilutions were done using prepared sterile normal saline.

3.12.3.2.2. Preparation of Agar Plates: Twenty-one sterile plates were prepared and 20ml of sterile Mueller Hinton agar was aseptically dispensed into each plate and allowed to cool and solidify.

3.12.3.2.3. Inoculation: The surface of each agar was inoculated with 5ml of an appropriate standardized (diluted) bacterial culture by flooding the surface of the solidified agar ensuring that the whole surface was evenly circulated over the entire plate and then the excess discarded. Each plate was labeled appropriately. This is also called seeding (Prescott, 2002)

The seeded agar plates were allowed to stay on the plate for 10 minutes at room temperature with the top (lid) in place.

3.12.3.2.4. Boring of Wells: Three holes per plate were punched-out in the agar using a borer. The borer was flamed before and after use on each plate.

3.12.3.2.5. Addition of Antibiotics: The appropriate concentrations of the three antibiotics were aseptically added using sterile syringes. The plates were allowed to stay for about 30mins then incubated for 16 to 18 hours at 35°C (Prescott, 2002).

3.12.3.2.6. Measurement of the Zones of Inhibition: After 18hrs of incubation, the zones of inhibition were measured from the antibiograms (antibiotic susceptibility patterns). The measurement was done for each of the antibiotics tested to the nearest mm. Four diameters were taken across each antibiogram and the average calculated and recorded (Prescott, 2002).

3.12.3.3 Well Diffusion Technique (With Only Plant Extracts)

Here, the crude plant extract and the tannin fractions were used. The procedure in the preceding test above was followed except that two wells were punched out instead of three.

3.12.4. Determination of Minimum Inhibitory Concentration (MIC)

3.12.4.1. Preparation of Nutrient Agar

500ml double strength Mueller Hinton Agar (Oxoid LTD) was prepared according to the manufacturer's instructions. This was dispensed in 10ml each into 50 clean sample bottles and sterilized by autoclaving at 121^0C for 15mins.

3.12.4.2. Preparation of Plant Extract Concentrations: A total of 10 different concentrations were prepared. Firstly, stock concentration of 100mg/ml was prepared by dissolving 5g of the Methanol extract into 45ml sterile distilled water. About 10ml of this stock concentration dissolved in 10ml of the prepared Mueller Hinton agar (MHA) gives a concentration of 50mg/ml.

This represents the first concentration and labeled concentration –A. A sub-stock conc. for each of the remaining nine concentrations was prepared from the stock concentration by serial dilution. 10ml of each sub stock concentration dissolved in 10ml molten MHA gives the respective concentrations from B-J.

3.12.4.3. Preparation of Sterile Agar Plates

Forty clean agar plates were arranged in canisters and sterilized in an oven at 160^0C for 1hour. The plates were allowed to cool at room temperature. The plates were then arranged

on a clean working lab table which was earlier swabbed with a disinfectant (JIK bleach). The arrangement was done in sets of four; with each set meant to run the test for a particular concentration. A permanent ink marker was then used to indicate clearly at the base of each plate the organisms to be inoculated and the concentration of the extract in each plate.

The sterile Mueller Hinton agar earlier prepared were melted in the autoclave; 10 ml of the agar was mixed with 10ml of each concentration (A-J) and aseptically poured into a plate labeled with the corresponding concentration; one plate at a time. The plates were allowed to solidify at room temperature with the lid of each plate in place (Prescott, 2002).

3.12.4.4. Inoculation

A good quantity paper discs punched-out from Watman No. 1 filter paper using an office punch. These were placed in a clean Petri dish and kept under UV light for 2-5hours to be sterilized. A pair of these discs were gently and aseptically placed on the agar plates where the organisms will be inoculated using a flamed forceps. An overnight broth culture of the test organisms was standardized and 10µl of these were dispensed onto the paper discs in the agar plates (seeding). The seeded agar plates were allowed to stay on the plate for 5 to 10 minutes at room temperature with the top (lid) in place. The plates were allowed to stay for about 30mins then incubated for 16 to 18 hours at 35°C. This procedure was carried out for the three different extracts (Prescott, 2002).

3.12.4.5. Determination of Minimum Inhibitory Concentrations (MIC)

After incubation, the series of plates were observed for microbial growth. The lowest concentration of the test agent that did not show any visible growth was considered the minimum inhibitory concentration (MIC) of the microbial agent. A positive control was also set up to ensure viability of test organisms and sterility of the media used (Prescott *et al*, 2005).

3.12.5. Determination of Minimum Bactericidal Concentration (MBC)

The paper discs that did not show any growth on or around them at the end of the MIC test procedure were carefully and aseptically transferred into bottles containing 5ml Nutrient Broth (NB) with 3% tween-80 using sterile (flamed) forceps. The addition of tween-80 to the NB was meant to stop the action of the microbial test agent and to allow the fast recovery of the injured bacterial cells. Each bottle containing a pair of the paper discs was labeled appropriately for test isolates (organisms) and the concentration of test antimicrobial agent. The bottles were then incubated at 37^0C for 72hours.

The lowest concentration of the test agent that did not show any visible growth in duplicates on the recovery broth medium was considered the minimum bactericidal concentration (MBC). A positive control was also set up to ensure viability of test organisms and sterility of the media used (Prescott *et al.*, 2005).

3.12.6. Determination of the Rate of Kill

Isolate number 5 was used to determine the rate of kill as this showed the highest value of MBC.

This also implies that it has the highest resistance as compared to the other isolates.

3.12.6.1. Preparation of Agar: About 450ml of Mueller Hinton agar with 3% tween-80 was prepared according to the manufacturers' instructions. This was dispensed in 10mls into clean macatney bottles and sterilized in an autoclave at 121^0C for 15mins (Ehinmidu, 2003).

3.12.6.2. Standardization of Organisms: The bacterial isolate was sub-cultured in 5ml nutrient broth and incubated for 18hrs. The overnight culture was standardized based on 1 in 1000 dilution using sterile normal saline to obtain 10^5 cfu/ml (Ehinmidu, 2003).

3.12.6.3. Ten-fold Dilutions and Inoculation: A fixed concentration of 100mg/ml of the plant extract (i.e. 5 x MIC for crude extract and 2 x MIC for the tannin and Residual Fractions) was prepared. 1ml of the standardized culture was added to the prepared concentration of the extract. About 1ml sample was removed from the reaction mixture using a micropipette at 0, 10, 30, 60, 120, 180 and 240 minutes, diluted serially in 9ml sterile distilled water containing 30% tween-80 and was mixed with 10ml of the prepared sterile MHA. This was poured aseptically in clean sterile plates, allowed to settle and incubated at 37^0C for 24hrs.

The control comprises of Mueller Hinton Agar without plant samples but seeded with the test inoculums

CHAPTER FOUR

4.0. Results

4.1. Microscopical Studies of the Stem Bark of *D. microcarpum*

Qualitative microscopical studies of the powdered stem bark revealed the following features:

4.1.1. Epidermal Cells

These are polygonal in shape. They occur at the epidermis in frequency of about 2-3 layers thick round the epidermis. They are about 11µm in length and about 3.4µm in width.

4.1.2. Round Parenchyma cells of the cortex

The diameter measured 21.6 - 23.8µm. The cells occurred numerously in the cortex.

4.1.3. Polygonal parenchyma cells of the phloem

The phloem parenchyma cells were polygonal in shape. The diameter measured 21.6 - 23.8µm in length. They occurred numerously.

4.1.4. Phloem Fibres

The phloem fibres were elongated in shape. The size ranges within 82 - 245µm in length and 2.8 - 10.3µm in width. The frequency of occurrence was moderate. They had tapering apices. The fibres were also slightly lignified.

4.1.5. Sclereids

These were of the horse shoe type, measuring about 11-21µm in length and 10-18µm in width. The frequency of occurrence was moderate.

4.1.6. Secretary Cells

Secretary cells contained mucilage, tannins or oils. Shapes were circular and quite few in occurrence

4.1.7. Starch Grains

The starch grains measured about 8 - 12µm in diameter. Some were almost circular in shape while most were irregular. The position of hilum was central. They occurred in aggregates as simple and the frequency of occurrence was occasional.

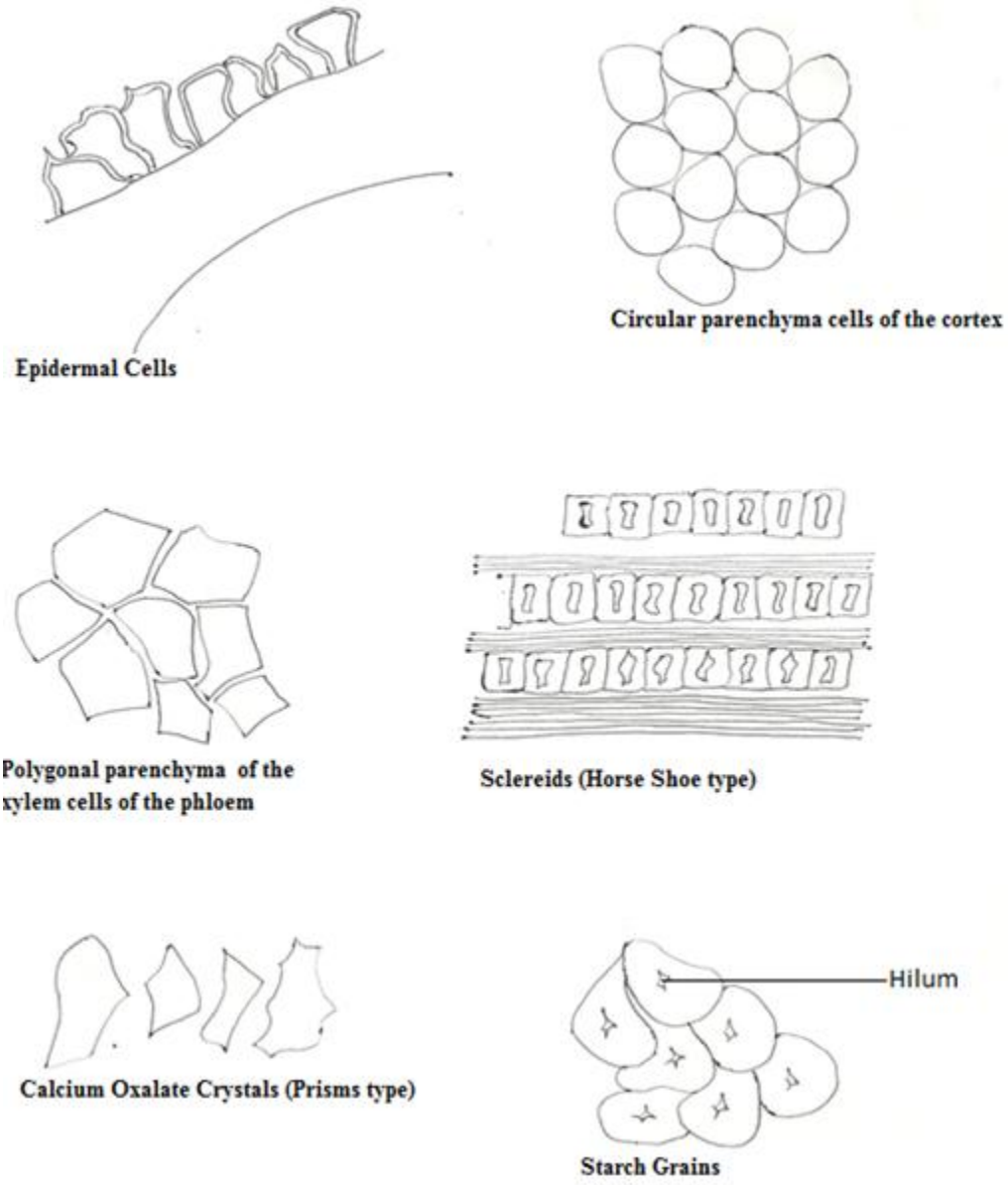


Fig 4.1. Microscopic Features of the Powdered Stem Bark of *D. microcarpum* (X400)

4.2. Transverse Section of the Stem of *D. microcarpum*

The transverse sections revealed the epidermal layer consisting of 1 to 2 rows of elongated parenchyma cells. Stomata were absent but lenticels were present at intervals. Trichomes were also observed. They were the clothing (non-glandular) type found on the epidermal layer. Next to these were 2 to 3 layers of collenchyma cells. The cortex comprises of parenchyma cells; (larger than those at the epidermis), secretory cells as well as sclerenchyma cells. This was followed by the phloem cells consisting of phloem fibres, phloem parenchyma and companion cells. The sieve tubes were not clearly differentiated. The cambium consists of thin walled elongated parenchyma cells in 2 – 3 rows. The xylem was observed to consist of xylem vessels, xylem fibres, xylem parenchyma and uniseriate medulary rays. The pith was the inner-most layer consisting of loosely packed almost circular parenchyma cells, with air spaces between them. Calcium oxalate crystals, starch grains, secretary cells and other cell inclusions were observed especially in the cortex (Plate III).

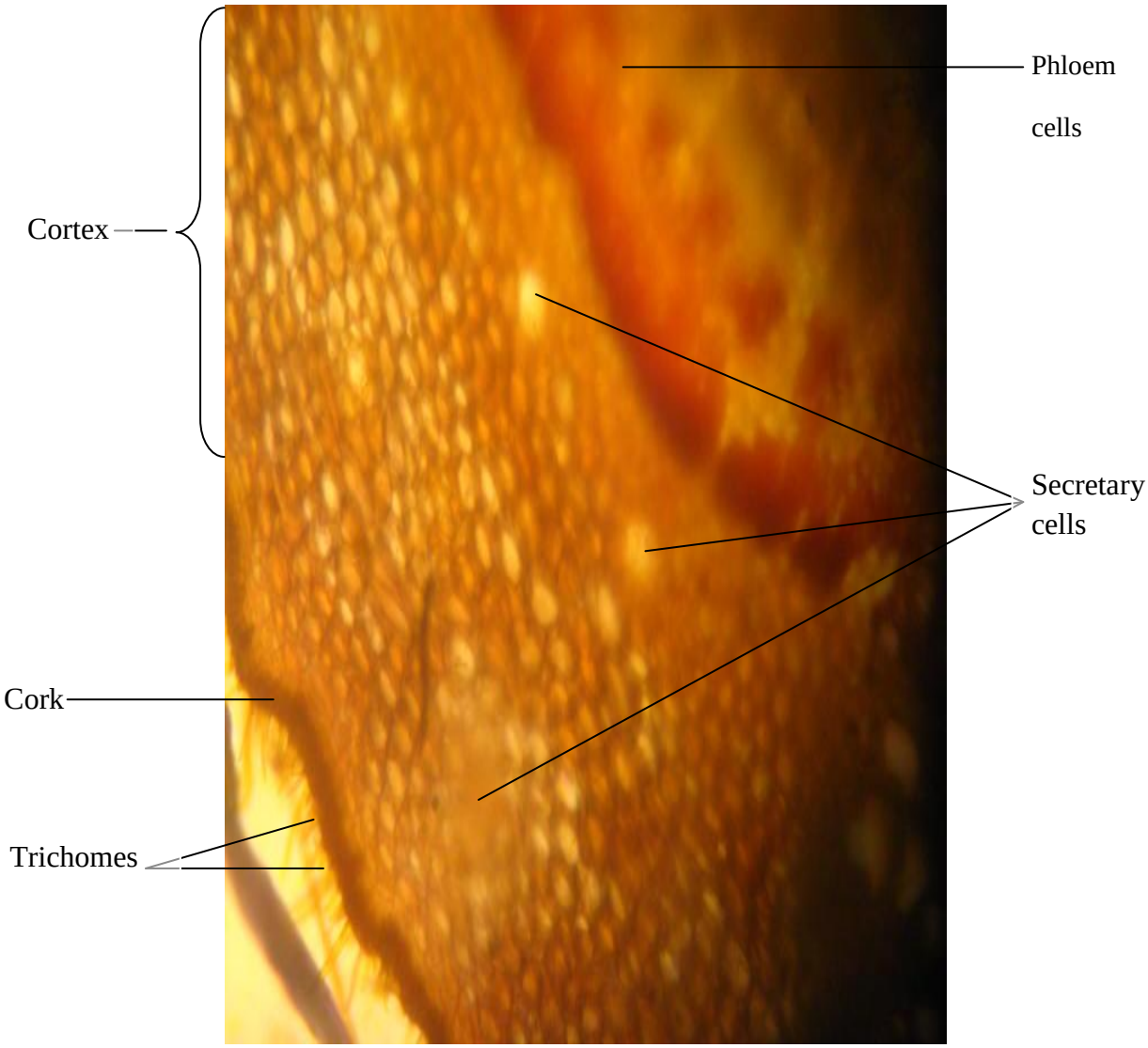


Plate III Transverse Section of the Stem of *D. microcarpum* (X400)

4.3. Chemomicroscopic Examination of the Powdered Stem Bark

The chemo-microscopical examination revealed the presence of cellulose, starch grains, oil, lignin, tannins, mucilage and suberin some in the cell wall and some others in the cell contents of the powdered bark sample (Table 4.1).

Table 4.1Chemomicroscopy of the Powdered Stem Bark of *D. microcarpum*

Constituents	Tissue where found	Results
Cellulose	Epidermal cells (cell wall)	+
Lignin	Around thickened cells (parenchyma of cortex and xylem)	+
Tannins or other phenolics	Secretory cells (storage cells)	+
Starch	Almost in all cells of the cortex and the epidermis	+
Oils or lipids	In cells of the cortex	+
Mucilage	Cell wall	+
Calcium oxalate crystals	Cells of the cell wall	+
Suberin	Walls of the cork cells	+

Key: + Present

- Absent

4.4. Physical Constants of the Powdered Stem Bark of *D. microcarpum*

The water soluble extractive value was 18.0% w/w while the acid insoluble extractive value had the lowest value of 1.8% w/w the result is summarized in table 4.2.

Table 4.2. Physical Constants of the powdered Bark

Parameters Evaluated	Mean /SEM (% w/w)
Moisture content	5.00 ± 0.3
Ash value	4.67 ± 0.3
Acid insoluble extractive value	1.75 ± 0.2
Alcohol soluble extractive value	22.00 ± 1.9
Water soluble extractive value	18.13 ± 0.1

n=3 (Mean Values of Three Counts)

SEM (Standard Error of Mean)

4.5. Phytochemical Screening of the Crude Extract, Tannin Fraction and Residual fraction

The phytochemical screening of the three fractions of the stem bark revealed carbohydrates and glycosides (saponins and flavonoids) in the methanolic crude extract (MCE), residual fraction (RF), and Tannin fraction (TF). However, cardiac glycosides occurred were detected in the MCE with mild occurrence in the RF . The alkaloids tested positive in the MCE and RF but was absent in the TF (Table 4.3).

Table 4.3. Phytochemical Constituents of the Powdered Stem Bark of *D. microcarpum*

CONSTITUENTS	OBSERVATION	Inferences		
		A	***B***	***C***
Carbohydrates	A ring of purple colour	+	+	+
	Brick red precipitate of cuprous oxide	+	+	+
Anthraquinones derivatives	No change	-	-	-
Cardiac glycosides	A reddish brown colour gradually turns blue	+	+	-
	A deep red colour, which fades to brownish yellow	+	+	-
Saponins	A honey comb froth persists for about 10- 15 minutes	+	+	+
	presence of haemolysis	+	+	+
	pink colour indicates the presence of triterpenoids	+	+	+
Flavonoids	A pink or red colour	+	+	+
	A yellow solution which becomes colourless in HCl	+	+	+
Tannins	blue coloured precipitate	+	+	+
Alkaloids	Turbidity or precipitation	+	+	-

KEY: *A* = Methanolic Crude Plant Extract (MCE)

B =Residual Fraction (RF)

C =Tannins fraction (TF)

\+ = Present

\- = Absent

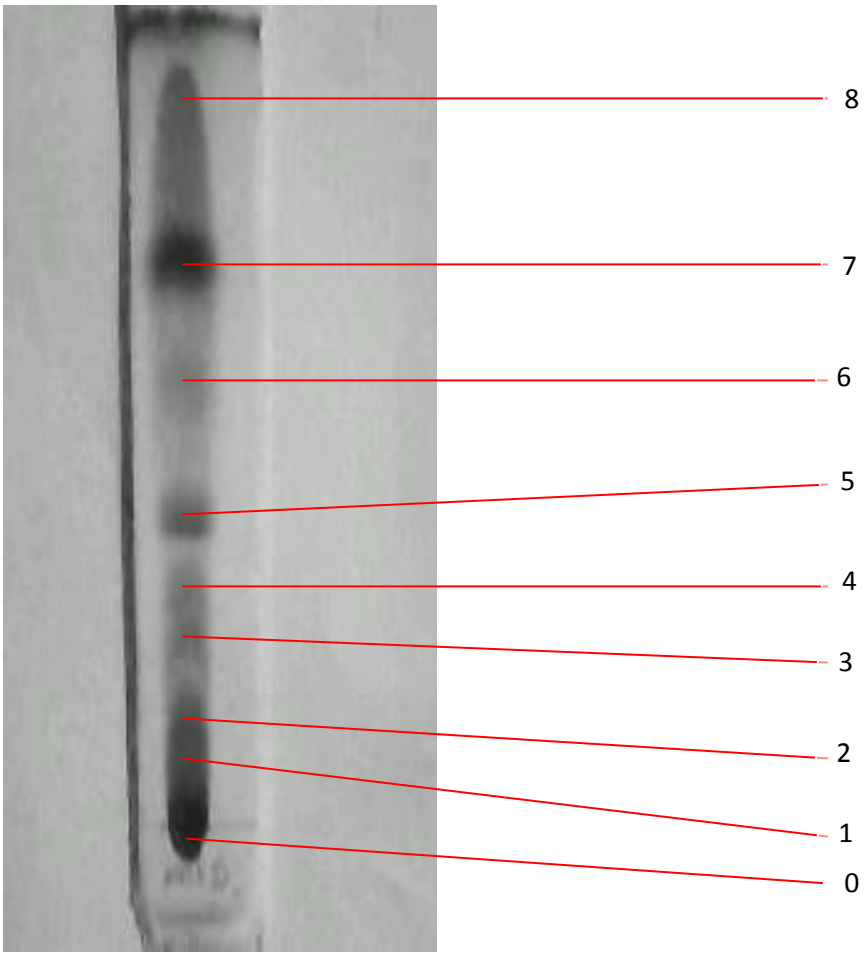
4.6. Chromatographic Analysis

4.6.1. TLC Analysis of the Pet-Ether Fraction of *D. microcarpum*

A tolal of nine spots were revealed when sprayed with H_2SO_4 the various R_f values calculated and the characteristic colouration obtained when sprayed with 10% H_2SO_4 are as shown in table 4.4.

Table 4.4. The TLC Analysis of Pet-Ether Fraction Sprayed with 10% Sulphuric Acid.

Spots	R_f value	Colour reaction
0	0.00	Brown-black
1	0.07	Orange-brown
2	0.14	Greenish-brown
3	0.23	Yellow
4	0.30	Purple
5	0.41	Light-purple (or pink)
6	0.50	Greenish
7	0.71	Blue-black
8	0.82	Purple



n-Hexane : Ethyl-acetate (9:1v/v)

Plate IV. TLC of Petroleum-Ether Fraction in 10% Sulphuric Acid.

4. 6.2. Column chromatography Using Tannin Fraction of Stem Bark of *D. microcarpum*

Eight of the collected portions of eluates (A2, A3, A4, A5, A6, A7, A8 and A9) were seen almost forming crystals. These were used to run a co-chromatography on TLC plates

4. 6.3. Co-chromatography

The co- chromatography revealed the presence of all the five reference standard compounds used and two distinct spots for the unknowns. The R_f values and the characteristic colours of the unknowns tallied with that of catechol

Table 4.5. Co-Chromatography of Reference Compounds and Tannin Fraction

REFERENCE COMPOUNDS AND FRACTIONS	COLOURS WTH DETECTION REAGENTS AND Rf VALUES							
	Daylight	**R_f**	**I_2 vapour**	**R_f**	**H_2SO_4**	**R_f**	**$FeCl_3$**	**R_f**
Catechol	Light-yellow	0.90	Greyish-brown	0.90	Grey	0.90	Black	0.90
Gallic acid	Yellowish-brown	0.78	Greenish-brown	0.78	Grey	0.78	Purple	0.78
Phloroglucinol	Colourless	0.83	Carton-brown	0.83	Orange	0.83	Brown	0.83
Pyragallol	Light-brown	0.85	Brown-black	0.85	Purple	0.85	Black	0.85
Quercetin	Greenish-brown	0.87	Brown	0.87	Brown	0.87	Green	0.87
A_2	Colourless		Light-green	0.83	Brown	0.83	Green	0.83
A_3	Colourless		Light-green	0.83	Brown	0.83	Colourless	
A_4	Colourless		Colourless		Brown	0.83	Colourless	
A_5	Colourless		Colourless		Brown	0.83	Colourless	
A_6	Colourless		Colourless		Brown	0.83	Colourless	
A_7	Colourless		Colourless		Brown	0.83	Colourless	
A_8	Colourless		Colourless		Brown	0.83	Colourless	
A_9	Colourless		Colourless		Brown	0.83	Colourless	

KEY: A_2-A_9 = Eluates from column using acetone as the eluting solvent after first using ethanol

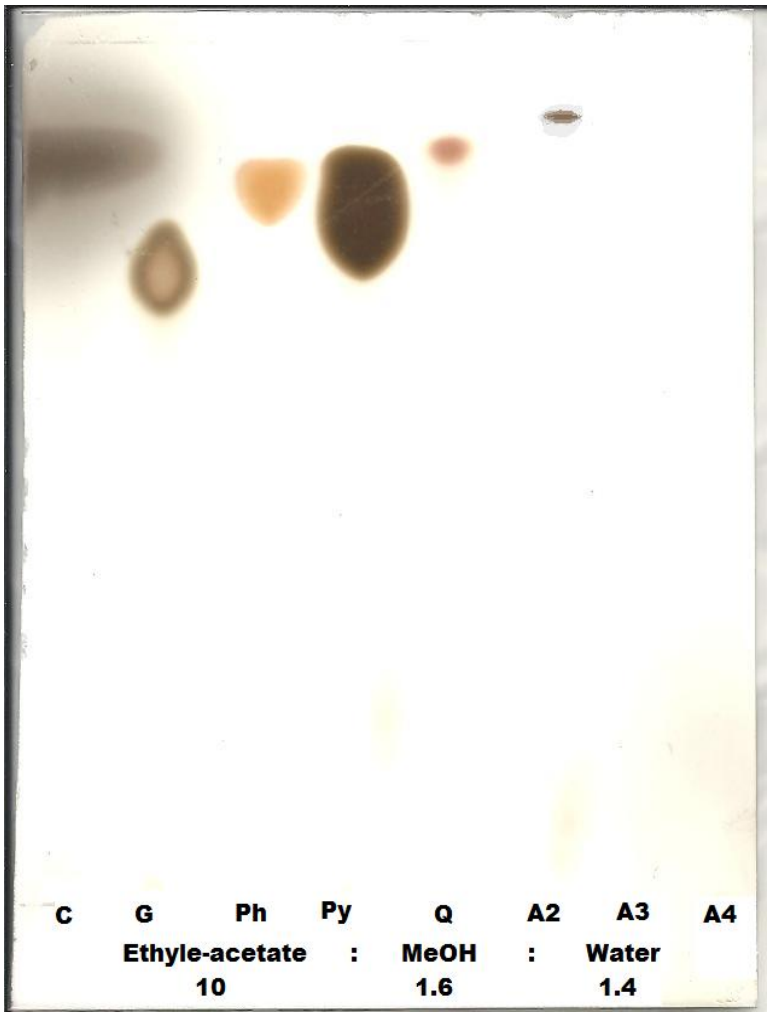


Plate V. Chromatogram of Reference Compounds and Unknown Fractions in Iodine Vapour

KEY: C = Catechol,

G = Gallic Acid,

Ph = Phloroglucinol,

Py = Pyragallol,

Q = Quercetine

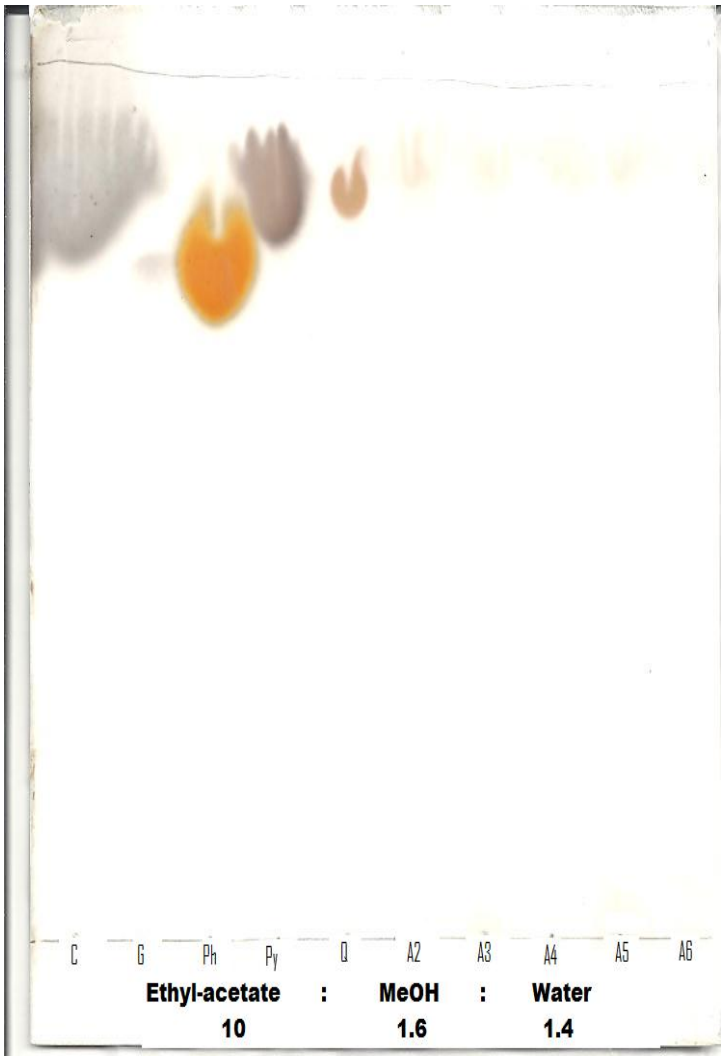


Plate VI. Chromatogram of Reference Compounds and Unknown Fractions when sprayed with 10% H_2SO_4

KEY: C = Catechol,

G = Gallic Acid,

Ph = Phloroglucinol,

Py = Pyragallol,

Q = Quercetine

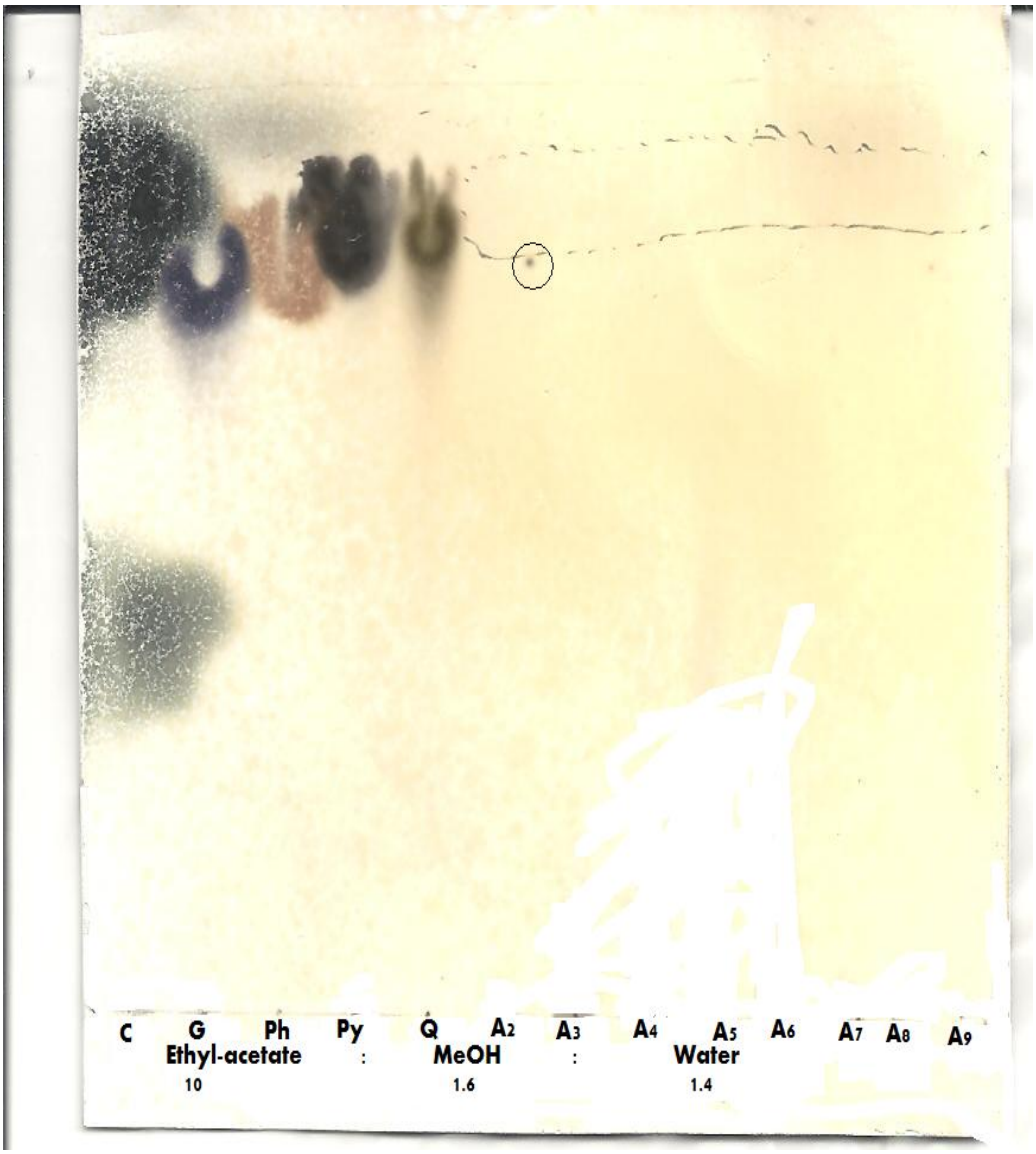


Plate VII. Chromatogram of Reference Compounds and Unknown Fractions of Extracts in Ferric Chloride

KEY: C = Catechol,

G = Gallic Acid,

Ph = Phloroglucinol,

Py = Pyragallol,

Q = Quercetine

4.7. Total Phenolic Content of the Powdered Stem Bark of *D. microcarpum*

The graph below was used to determine the values of x (concentrations) from the values of y (absorbances) measured from the UV-VIS Spectrophometer.

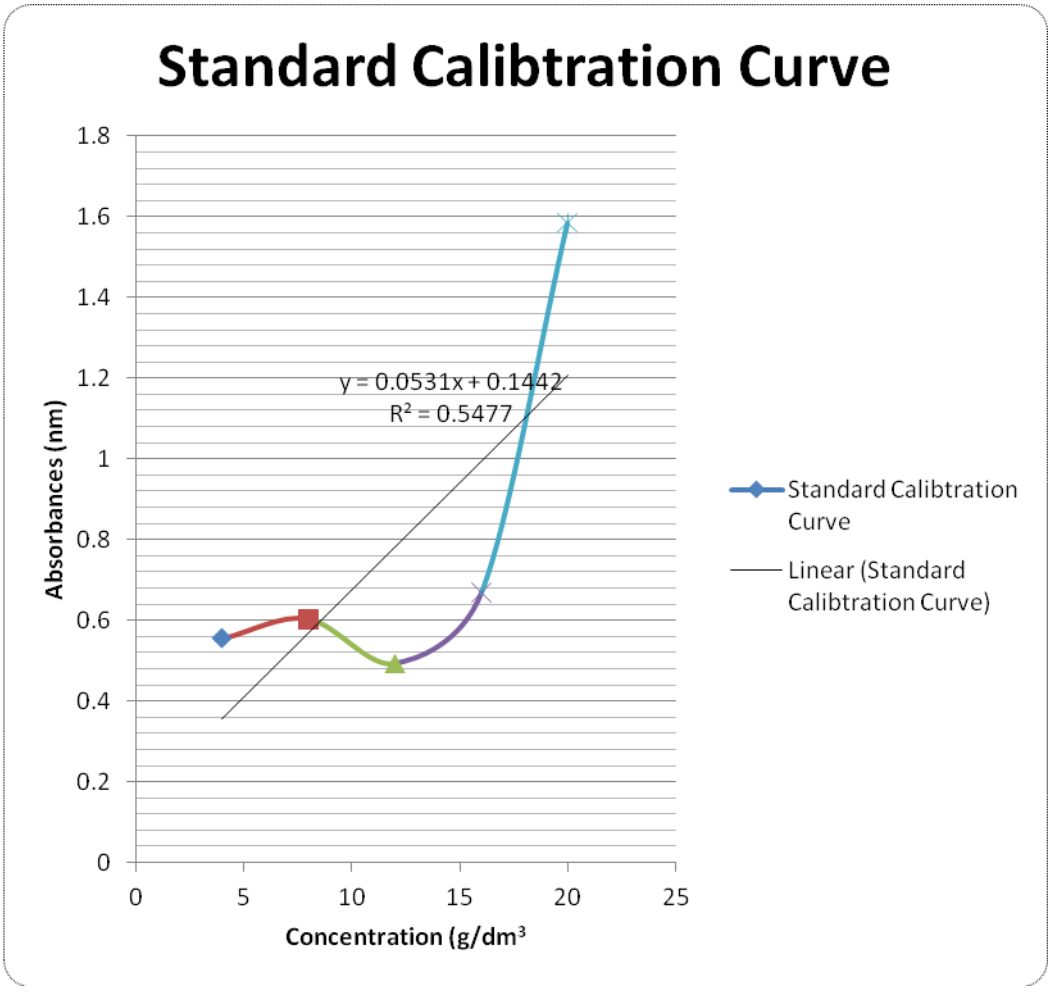


Fig 4.2. Graph of Absorbance against Concentration of the Samples

The R^2 value being up to 0.5 is an indication that there is a strong relationship between the absorbance and the concentrations. This means that the absorbance vary with or is dependent on the change in concentration. The crude methanolic plant extract (MCE) gave an absorbance of 1, tannin fraction (TF) had 0.87 and the residual fraction (RF) had 0.44

while the total phenolics were 16mgTAE/g, 9.86 mg TAE/g and 6.14 mg TAE/g respectively (Table 4.6)

Table 4.6. Total Phenolics of the three Samples from Powdered Stem Bark of *D. microcarpum*

Extract/Fractions	Absorbance	Total Phenolics (mgTAE/g)
Crude plant Extract	1.00	16.00
Tannin fraction	0.87	09.86
Residual fraction	0.44	06.14

4.8. Acute Toxicity Studies on the Three Samples

In phase 1 of batch 'A' the mice showed 100% survival at10mg/kg body weight, 66.67% survival for 100mg/kg b.w., and 0.0% survival for 1000mg/kg b.w. dose. However, in the 2^{nd} phase, the 1^{st} three doses (50, 100, and 200mg/kg b.w.) showed 100% survival; only the fourth dose 400mg/kg b.w. recorded no survival (00.00% survival). These are summarized in the table 4.7:

Table 4.7. Batch 'A' (Crude Plant Extract Administered in Mice Using *i.p.* Route)

Dose (mg/kg)	№ of Mice Per Group	Number of Deaths	Percentage Survival (%)
PHASE 1			
10	3	0	100
100	3	1	66.67
1,000	3	3	00.00
PHASE 2			
50	1	0	100
100	1	0	100
200	1	0	100
400	1	1	00.00

$LD_{50} = \sqrt{C \times D}$

Where C = survival dose and D = lethal dose

$LD_{50} = \sqrt{200 \times 400}$

$= 282.8427125$

$LD_{50} \approx \underline{282.84 \text{ mg/kg}}$

In phase 1 of batch 'B' the mice showed 100% survival for all the three doses used. Similarly, in the 2nd phase, the three doses administered also showed 100% survival; these are summarized in table 4.8.

Table 4.8. Batch 'B' (Tannins Fraction Administered in Mice Using *i.p*. Route)

Dose (mg/kg)	№ of Animals Per Group	Number of Deaths	Percentage Survival (%)
PHASE 1			
10	3	0	100
100	3	0	100
1,000	3	0	100
PHASE 2			
1,600	1	0	100
2,900	1	0	100
5000	1	0	100

LD_{50} > 5000 mg/kg

In phase 1 of batch 'C' the mice showed 100% survival for the three doses administered. However, in the 2nd phase, the 1st two doses showed 100% survival but the third dose of 500mg/kg b.w. recorded no survival (0% survival). These are summarized in table 4.9

Table 4.9. Batch 'C' (Residual Fraction Administered in Mice Using *i.p.* Route)

Dose (mg/kg)	№ of mice per group	Number of Deaths	Percentage Survival (%)
PHASE 1			
10	3	0	100
100	3	0	100
1,000	3	0	100
PHASE 2			
1,600	1	0	100
2,900	1	0	100
5000	1	1	00.00

$LD_{50} = \sqrt{2,900 \times 5,000}$

$= 3807.8865$

$LD_{50} \approx \underline{3807.89 \text{ mg/kg}}$

4.10. Antimicrobial Studies on *E. coli* and *S. aureus* Using the Three Samples

4.10.1. Microscopy of Organisms: *Staph. aureus* (L. *aureus,* golden) cells appeared spherical, 0.5 to 1.0 µm in diameter, occurring singly, in pairs, and in irregular clusters. *E. coli* (Gr. *colon,* large intestine) cells appeared as straight rods, 2.0 to 6.0 µm in length, occurring singly or in pairs.

4.10.2. Biochemical tests.

4.10.2.1. Indole Formation Test: The development of a red color within a few minutes after adding 10 drops of Kovacs' reagent—a positive test. Negative reactions are colorless

4.10.2.2. Methyl Red (MR) Test: the methyl red indicator turns red—a positive methyl red (MR) test. At a pH of 6, the indicator turns yellow—a negative methyl red (MR) test.

4.10.2.3. Voges-Proskauer (VP) Test: Development of a red color in the culture medium 15 minutes following the addition of Barritt'sreagent represents a positive VP test**;** absence of ared color is a negative VP test

4.10.2.4. Citrate Test: When bacteria oxidize citrate, they remove it from the medium and liberate CO_2. CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate—an alkaline product. This raises the pH, turns the pH indicator to a blue colour, and represents a positive citrate test.

4.11. Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of three Samples from *D. microcarpum*

The table below shows the zones of inhibitions for the MICs

Table 4.10. Readings on MIC of three Samples from *D. microcarpum*

S/No Orgs.	Concentration of extracts (mg/ml) and inferences																																			
	A (50)			B (40)			C (30)			D (20)			E (10)			F (5)			G(2.5)			H(1.25)			I(0.625)			J(0.3125)								
	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M						
1	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
2	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+						
3	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
4	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+						
5	-	-	-	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
6	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
8	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+						
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+						
10	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
11	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
12	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+						
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
14	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+						
16	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+						
17	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+						
18	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+						
19	--	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+						
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+						
	A			B			C			D			E			F			G			H			I			J								

KEY: X : Crude Extract, Y : Tannin Fraction, M : Residual Fraction, ++ : Presence of Growth and -- : Absence of Growth

The table below shows the zones of inhibitions for the MBCs

Table 4.11. Readings on MBC of three Samples from *D. microcarpum*

S/No. of Isolates	Concentration of extracts (mg/ml) and inferences																														
	A (50)			B (40)			C (30)			D (20)			E (10)			F (5)			G (2.5)			H(1.25)			I (0.625)			J(0.3125)		
	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M	X	T	M
1	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
3	-	+	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
5	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
9	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
13	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	-	-	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
16	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
18	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
19	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mg/ml ►	A (50)			B (40)			C (30)			D (20)			E (10)			F (5)			G(2.5)			H(1.25)			I(0.625)			J(0.3125)		

KEY:

X : Crude Extract

T : Tannin Fraction

M : Residual Fraction

- : No growth

Table 4.12. Summary of MIC and MBC results for Crude, Residual and Tannin Samples

S/No. of Isolates	Minimum Concentrations (mg/ml)					
	MIC			MBC		
	MCE	TF	RF	MCE	TF	RF
1	5.0	-	50	10.0	-	-
2	2.5	50	30	5.0	-	-
3	5.0	-	50	10.0	-	50
4	2.5	40	10	2.5	-	-
5	5.0	50	50	30.0	50	50
6	10.0	50	30	20.0	50	-
7	10.0	10	10	10.0	-	-
8	2.5	30	10	10.0	50	50
9	2.5	10	30	10.0	50	-
10	5.0	10	30	10.0	-	-
11	10.0	40	10	10.0	-	-
12	2.5	20	10	5.0	-	-
13	5.0	10	20	20.0	-	-
14	5.0	20	10	10.0	50	-
15	5.0	10	20	5.0	50	-
16	10.0	30	20	10.0	-	-
17	0.625	30	20	1.25	50	50
18	0.625	20	10	1.25	-	-
19	1.25	20	10	10.0	-	-
20	0.625	10	10	10.0	-	-

KEY

- No Minimum concentration (Growth occurred even at highest concentration of 50mg/ml)

Table 4.13. Rate of Kill of *E. coli* using the three Samples

TIME (MINS.)	LOG OF VIABLE COLONY COUNTS		
	Crude Extract	**Tannin Fraction**	**Residual Fraction**
0	2.06	2.16	2.06
10	1.98	2.08	2.04
30	1.61	2.05	2.05
60	1.34	2.04	2
120	0.6	2.1	2.06
180	0	1.72	1.66
240	0	1.11	1.63

n=3

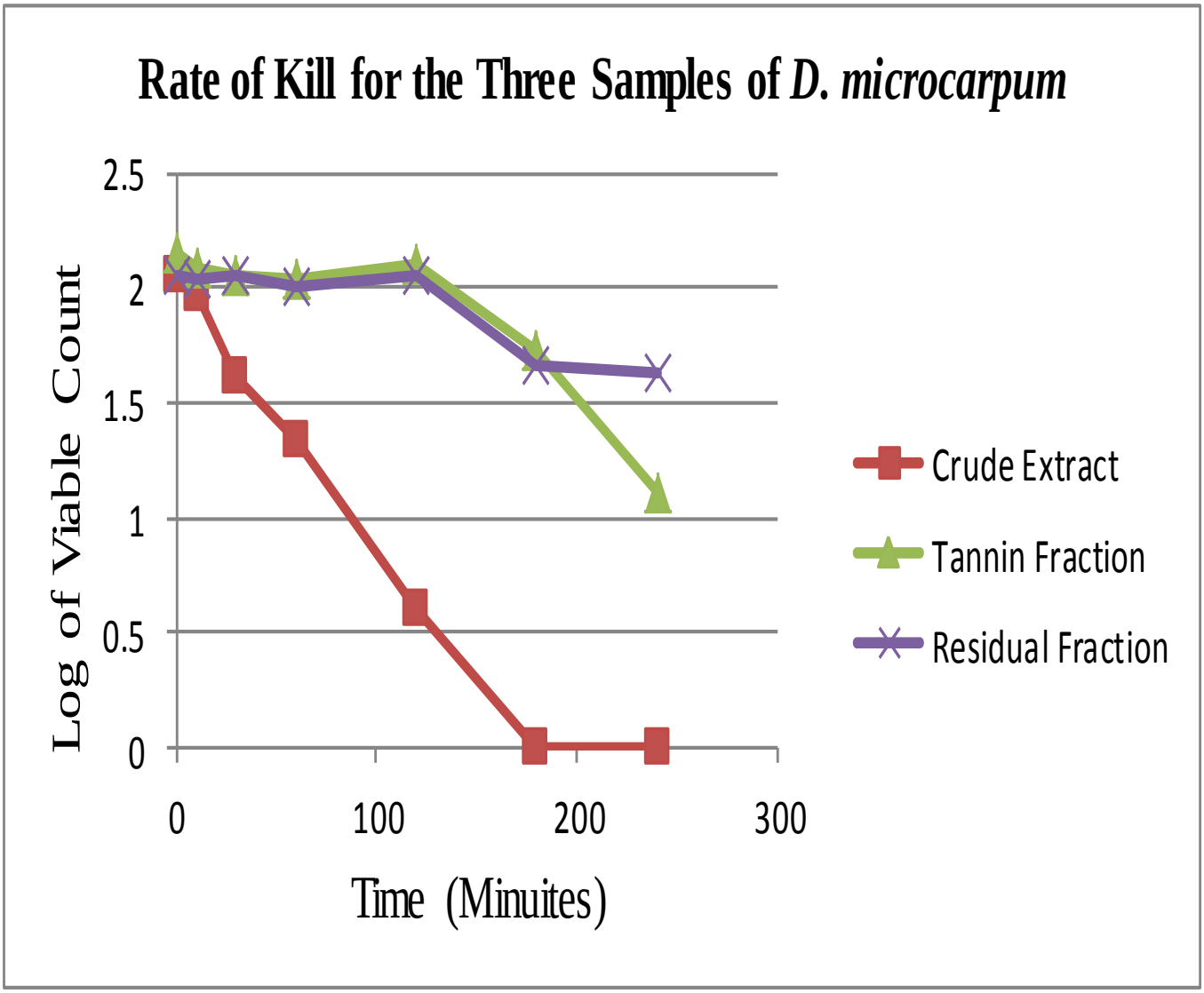


Fig. 4.3. Rate of Kill using Crude Extract, Tannin Fraction and Residual Fraction of *D. microcarpum*

CHAPTER FIVE

5.0. DISCUSSIONS

The stem-bark leaves and roots of *D. microcarpum* are widely used for their diuretic and astringent properties. Their use cut across medicine, pharmacy, veterinary medicine, traditional medicine, agroforestry etc. (Kouyaté and Damme, 2006). This wide application forms the basis for the pharmacognostic evaluation and the antimicrobial studies of the plant stem bark.

The marcroscopical features as observed revealed the thickness of the stem-bark measuring between 0.5-1.2 cm while its width was between 2-5 cm. the length of the bark was 14-18cm. It was generally curved and channeled in shape after drying. The outer surface had shades of milky-brown to grayish-brown colouration; with cracks (furrows) running transversely. The inner surface however, had shades of brown to ox-blood red colour and it is striated. (See plate II). The bark breaks with short granular fractures on the outside and short fibrous fractures with a few splinters on the inner side. When powdered, the bark was light-brown in colour with a mild aromatic smell and an astringent taste. These are also in conformity with previous findings on *D. microcarpum*.

The diagnostic features of the stem-bark observed microscopically, e.g. the phloem fibres which were elongated tapering at the ends and slightly lignified. The calcium oxalate crystals which are tetragonal, several groups of secretory cells and ducts, rounded sclerids and circular/ irregular shaped starch grains were observed. In addition to these, the anatomical sections (comprising the transverse, longitudinal and tangential sections) and the chemomicroscopical characters are important tools in the taxonomic classification of this

plant as well as its proper identification as shown in Fig. 10. Cellulose is the main constituents of plant cell wall. They are responsible for the rigid cellular frame work. Lignin is a strenghtening material which impregnates cell walls of tracheids, vessels, fibres, and sclerids of vascular plants (Evans, 2008). Present in the greatest concentration in middle lamellae and in primary walls. Tannins occur in mature dead cork cells.

Mucilages are polysaccharide complexes of sugar and uronic acid; usually formed from the cell wall; insoluble in alcohol but swell or dissolve in water. They are usually deposited in layers.

The moisture content of 5.0% w/w is an indication of the storage quality of the powdered sample which is within the acceptable range of not more than 12% (African Pharmacopoeia, 1986). This indicates its ability to retain its natural metabolites without any degradation from moisture. This is because when there is high moisture content in a particular drug sample in conjunction with suitable temperature, enzymes will be activated and it ultimately leads to the proliferation of decay causing living organisms (Evans, 2008), therefore, low moisture content reduces enzymatic activity and does not support the growth of micro-organisms (Brain and Turner, 1975; Evans, 2008).

This also implies that the crude drug can be stored for a long period of time without any significant level of degradation.

The total-ash value (4.67% w/w) is moderate. It indicates to some extent the amount of care taken in the preparation of the drug (Evans, 2008). This total-ash value and the acid insoluble ash value (1.75% w/w) are parameters showing the amount of organic and inorganic materials in the powdered plant sample. The

alcohol soluble and water soluble extractive values (22% w/w and 18.13% w/w respectively) indicate the extractive potency of the plant in alcohol and water. The higher value for the alcohol soluble extractive value, suggests alcohol as the better extraction solvent for the polar metabolites present in the plant material compared to water. The high values also indicates high amount of lipids (Evans, 2008).

The phytochemical screening/evaluation of the crude methanolic extract and the other two fractions (residual fraction and tannin fraction), revealed the presence of some phytochemicals (cardiac glycosides, saponins, flavonoids and tannins) present in the plant as shown in table 4.3. Carbohydrates occurred in all three samples (MCE, RF and TF) free anthraquinones were absent in the three samples, cardiac glycosides were found present in the MCE and RF but absent in the tannins fraction (TF), flavonoids as well as tannins occurred in all the samples however, the observation with the residual fraction was mild or faint; alkaloids were also absent in the TF but present in the MCE and RF. The screening also showed the occurrence of saponins in all the three samples. The presence of phenolic compounds; particularly the tannins gave credence to the antimicrobial activity of this plant as reported being used locally for antimicrobial activity (Kouyaté and Damme, 2006). The thin layer chromatography revealed the presence of different metabolites in the three samples used. The n-Hexane : Ethyl-acetate (9:1) solvent system gave the best separation for the pet-ether fraction (see plate III). When sprayed with sulphuric acid (general spraying reagent), a pink colour which indicates the presence of triterpenes (spot 5) and the red colour indicates steroids (spot 7) (Evans, 2008); A solvent system of Ethyl-acetate : Methanol : Water (10:1.6:1.4) was best used for the separation of the compounds in the MCE and the

TF (see plate 2) This also served as a guide for the choice of the solvent system for the co-chromatography. The green-blue/black colour when sprayed with ferric chloride indicates the presence of phenolic compounds (Evans, 2008).

The co-chromatographic study revealed the presence of all the five reference standard compounds used (catechol, gallic acid, phloroglucinol, pyragallol and quercetine) see plates V, VI and VII; catechol gave a light-yellow spot in daylight, grayish-brown in iodine vapour, grey colouration in H_2SO_4 and greenish-black colouration in $FeCl_3$ with an R_f value of 0.90; the spots of gallic acid gave yellowish-brown colouration in daylight, greenish-brown when exposed to iodine vapour, grey colour when sprayed with H_2SO_4 and a purple colouration when sprayed with $FeCl_3$ with an R_f value of 0.78. The phloroglucinol had an R_f value of 0.83 with a colourless spot in daylight, a brown colouration in iodine vapour, an orange spot when sprayed with H_2SO_4 and a brown spot when sprayed with $FeCl_3$. Pyragallol had an R_f value of 0.85 with a light-brown spot in daylight, a brown-black spot when exposed to iodine vapour, a purple coloured spot when sprayed with H_2SO_4 and a black spot when sprayed with $FeCl_3$. Quercetine had an R_f value of 0.87 with a greenish-brown spot in daylight, a brown spot when exposed to iodine vapour, a brown spot when sprayed with H_2SO_4 and a greenish spot when sprayed with $FeCl_3$ (Table 4.5). The Fractions co-chromatographed with these standards revealed two spots when sprayed with H_2SO_4 and $FeCl_3$. These spots had coloures and R_f values corresponding to those of catechol and quercetin, this therefore shows that catechol and quercetine are contained in stem bark of *D. microcarpum.* These are the simple phenolics (catechol) which also indicates the presence of condensd tannins because on dry distillation, condensed tannins yield

catechol and these tannins are therefore sometimes called catechol tannins. Like catechol itself, their solution turns green with ferric chloride (Evans, 2008). The quercetin (a flavonol) is the second type of phenolics shown or represented by the second spot. These two phenolics are themselves monomers or precursors of other phenols such as the tannins. The catechol and quercetin structures are compounds associated with condensed tannins (Evans, 2008). See fig. 1.1 (c and g respectively).

The total phenolics as determined in terms of Tannic Acid Equivalence (TAE) were 16 mg TAE/g of crude extract weight, 09.86mgTAE/g of tannin fraction weight and 6.14 mg TAE/g of residual fraction weight. This shows that the crude plant extract still has the highest phenolic content although only slightly different from the ones in the tannin fraction. The residual fraction has the least content of the phenolics. This obviously constitutes other phenolics apart from the tannins since the tannins have been separated from this fraction.

The LD_{50} using *ip* route for the crude plant exract was 282.84 mg/kg; the tannin fraction (*ip*), it was of no practical interest (>5000 mg/kg) and the residual fraction was 3807.89 mg/kg. According to Lorke (1983), LD_{50} <1mg/kg body weight is extremely toxic; LD_{50} of 1 – 50mg/kg body weight is highly toxic; LD_{50} of 50 – 500 mg/kg body weight is moderately toxic; LD_{50} 500 - 5000 mg/kg body weight is slightly toxic and LD_{50} > 5000 mg/kg body weight is practically nontoxic (Onaolapo and Udobi 2010). Thus the crude plant extract with LD_{50} = 282.84mg/kg is moderately toxic. Residual fraction with LD_{50} = 3807.89 mg/kg is slightly toxic whereas, tannin fraction with LD_{50} >5000 mg/kg is practically non-toxic and. It is therefore important to note that a cumulative toxic effect could occur if the crude

extract or the residual fraction is taken over time. This may however require a more advanced toxicity studies.

The susceptibility studies were done to establish the efficacy of the stem bark of this plant on the target organisms and also to compare the efficacy with that of standard antibiotics. In these studies, virtually all cultured bacterial cells grew indicating a high frequency of occurrence and viability of the cells particularly *in vivo* as they are clinical isolates. When grown against antibiotic discs, they were generally resistant to amoxicillin and augmentin but showed a high level of susceptibility to nitrofurantoin, gentamicin and ofloxacin. Out of the 8 samples of the *E. coli* isolates, most of them were resistant to Tetracycline as compared to the 13 samples of *S. aureus* isolates which had most of them susceptible to it. ANOVA test performed at 0.05 shows that there is generally a significant difference in their activity (Table C.2). However, for the multiple comparisons, where only two antibiotics are compared at a time, some showed a significant difference (table C.3). For the well diffusion technique with the plant extracts and the standard drugs, the statistical test showed that there was a significant difference between the standard drug and the samples (MCE, RF and TF) used. However the difference between any of the two samples wasn't significant. Also, for the well diffusion technique using only the plant extracts, only the multiple comparison between the crude plant extract and the methanolic extract showed a significant difference. The difference between the other two fractions (Tannins and methanol) wasn't significant.

The MIC and MBC showed activities against some of the bacterial isolates however isolate №5 (an *E.coli*), had MICs of 5mg/ml, 50mg/m and 50mg/ml for

the crude, tannin and Residual Fractions respectively and MBCs of 30mg/ml, 50mg/ml and 50mg/ml for the crude extract, tannin and Residual Fractions respectively. The *E. coli* №5 showed the highest MBCs of 30mg/ml, 50mg/ml and 50mg/ml for the crude extract, tannin and Residual Fractions respectively and was therefore used for the determination of the rate of kill (Ehinmidu, 2003).

The rate of kill at different time intervals of 0, 10, 30, 60 120, 180 and 240 minutes and at a fixed concentration of 100mg/ml of the plant extract (i.e. 5 x MIC for crude extract and 2 x MIC for the tannin and Residual Fractions) gave a graph pattern when the log of viable counts was plotted against the time intervals. There was a general gradual onset of kill for the three fractions. The rate of kill became rapid at about 10minutes for the crude extract, and at about 120 minutes for both the tannin and the Residual Fractions. The rapidity and intensity of this kill is a proof of the bactericidal action of this plant. However there was a little rise in the population in the tannin fraction before the crash while that of the MeOH extract had a slightly irregular pattern. This suggests a possible synergistic action with some other compounds for complete action. The drop in population of the bacterial cells continued until there were no viable counts at later times of the experiment. The shapes of the death curve produced are quite similar to the normal death curve pattern produced by most anti-bacterial drugs (Ehinmidu, 2003).

CHAPTER SIX

6.0. Summary, Conclusion and Recommendation

6.1. Summary: The detailed results of the macro and microscopical study, quantitative evaluative parameters, preliminary phytochemical screening and the chromatographic analysis were established for the powdered stem-bark of *D. microcarpum* and are useful parameter for the monograph of the plant. The TLC separates the different metabolites present in the two fractions and the crude extract. The spots obtained which matches well with that of the standards (catechol and quercetin) confirm the presence of these two compounds in *D. microcarpum* stem-bark. The LD_{50} of the methanolic extract of the stem bark (3807.89 mg/kg) and that of the crude plant extract (282.84 mg/kg) indicates that the RF and MCE are not safe because according to Lorke (1983), LD_{50} <1mg/kg body weight is extremely toxic; LD_{50} of 1 – 50mg/kg body weight is highly toxic; LD_{50} of 50 – 500 mg/kg body weight is moderately toxic; LD_{50} 500 - 5000 mg/kg body weight is slightly toxic and LD_{50} > 5000 mg/kg body weight is practically nontoxic (Onaolapo and Udobi, 2010). The crude extract has an MBC of 1.25mg/ml for isolates 17 and 18, 2.5mg/ml for isolate No. 4 and 5mg/ml for isolate No. 2, 12 and 15. The plant has shown anti-bacterial or bactericidal activity. Thus supporting and establishing that the plant, *D. microcarpum* has antimicrobial activity.

6.2. Conclusion

The pharmacognostic characters of the stem-bark of *D. microcarpum* have been successfully established which will enrich the monograph of the plant. The phytochemicals have also been determined specifically in the chromatography which shows that catechol and quercetine are contained in stem bark of *D.*

microcarpum. The safety margin of this plant has also been established. The methanolic extract showed activity against some bacterial clinical isolates used (*E.coli* and *S. aureus*).

6.3. Recommendations

It is therefore suggested that further work should be carried out on the crude methanol extract and the tannin fraction so as to ascertain the mode of action of the tannins in the antimicrobial activity. This will also help to know if the activity is synergetic with other compounds or otherwise. The identification of the chemical structures is also recommended. Chronic toxicity studies should also be carried out and the main toxic compounds identified.

Further biochemical work should also be done on the resistant *E. coli* to establish the reason for its resistance.

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APPENDICES

Appendix A. Concentrations and Absorbances in Determining Total Phenolic Content

Table A1. Concentrations and Absorbances of the Stem Bark of *D. microcarpum*

CONCENTRATIONS			ABSORBANCE
($g/50cm^3$)	(g/dm^3)	Molar (M)	(nm)
0.2	4	0.0024	0.556
0.4	8	0.0047	0.603
0.6	12	0.0071	0.494
0.8	16	0.0094	0.669
1.0	20	0.0118	1.585

Appendix B. Computations on Total Phenolics

Total Phenolics

From the graph above, $y = 0.0531x + 0.1442$

Thus, $x = \frac{y - 0.1442}{0.0531}$

i. For the plant extract, the absorbance was measured as 1nm = y

Thus x, (Concentration) $= \frac{1 - 0.1142}{0.0531}$

Thus, $x = 16.11576 g/dm^3$

≈ 16.12 g/L

The total phenolic compounds are then calculated by the following formular;

$$T = \frac{CV}{M}$$

Where, T= total content of phenolic compounds in TAE/g

C= concentration of Extract established from the calibration curve of Tannic acid in mg/ml

V= volume of extract in ml

M= weight of plant extract in grams

Thus, C = 16 g/dm (1.6mg/ml)

V = 5ml

M = 0.5g

$T = \frac{1.6 \times 5}{0.5}$ = 16 mg TAE/g of extract weight.

ii. For tannin fraction, the absorbance was measured as 0.87nm

C = 14.23g/dm^3 (1.4mg/ml)

V = 5ml

M = 0.5g

$T = \frac{1.4 \times 5}{0.5}$ = 14mgTAE/g of tannin fraction weight

iii. For the residual fraction, the absorbance was measured as 0.44nm

C = 6.14g/dm^3 (0.614mg/ml)

V = 5ml

M = 0.5g

$T = \frac{0.614 \times 5}{0.5}$ = 6.14mgTAE/g of residual fraction weight

Appendix C. Some Tables on Antimicrobial Studies

Table C.1. Paper Disc Technique Using Standard Antibiotics on Test Organisms:

S/No	Bacterial sample code	Diameter of zones of inhibition for each antibiotic disc (mm)							
		AMX (25µg)	**COT** (25µg)	**NIT** (300µg)	**GEN** (10µg)	**NAL** (30µg)	**OFL** (30µg)	**AUG** (30µg)	**TET** (30µg)
1	MA^{1+}	NZ	NZ	20.0 cz	18 Dotd	19 Dotd	18 Dotd	NZ	NZ
2	MA^{2+}	NZ	16 Dotd	11 cz	15 cz	19 cz	NZ	NZ	NZ
3	MA^{9+}	NZ	NZ	18 cz	NZ	NZ	25 cz	NZ	NZ
4	MA^{10+}	NZ	NZ	23 Dotd	NZ	NZ	NZ	NZ	NZ
5	MA^{13+}	NZ	NZ	25 cz	19 Dotd	NZ	11cz	NZ	NZ
6	MA^{17+}	NZ	NZ	NZ	14 cz	NZ	14 cz	NZ	NZ
7	MA^{20+}	NZ	NZ	NZ	20 cz	NZ	NZ	NZ	NZ
8	MSA^{2+}	NZ	22 Dotd	NZ	NZ	NZ	28 cz	NZ	16 Dotd
9	MSA^{4-}	NZ	6 cz	NZ	17 cz	NZ	24 Dotd	NZ	7 Dotd
10	MA^{5-}	NZ	NZ	28 cz	NZ	17 cz	28 cz	NZ	8 cz
11	MSA^{5-}	NZ	NZ	NZ	NZ	NZ	20 Dotd	NZ	10 Dotd
12	MSA^{6-}	NZ	NZ	8 Dotd	12 Dotd	NZ	NZ	NZ	NZ
13	MSA^{7-}	NZ	NZ	27 Dotd	NZ	NZ	30 cz	NZ	10 Dotd
14	MSA^{9+}	NZ	NZ	NZ	NZ	NZ	NZ	NZ	7 Dotd
15	MA^{10-}	NZ	24 Dotd	26 cz	18 cz	24 cz	32 cz	NZ	12 cz
16	MSA^{11+}	NZ	NZ	15 cz	19 cz	NZ	29 Dotd	NZ	12 Dotd
17	MA^{11+}	NZ	NZ	7 Dotd	10 cz	NZ	28 cz	NZ	NZ
18	MA^{14+}	NZ	NZ	25 Dotd	NZ	NZ	21 cz	NZ	NZ
19	MA^{15-}	NZ	NZ	15 cz	NZ	NZ	NZ	NZ	NZ
20	MA^{18+}	NZ	NZ	15 Dotd	NZ	NZ	NZ	NZ	NZ
21	MA^{18-}	NZ	NZ	NZ	12 cz	NZ	22 cz	NZ	14 Dotd

KEY:

Antibiotic Discs used

AMX = *Amoxycillin*
COT = *Cotrimoxazole*
NIT = *Nitrofurantoin*
GEN = *Gentamicin*
NAL = *Nalidixic acid*
OFL = *Ofloxacin*
TET = *Tetracycline*

Zones of Inhibition

CZ = *Clear Zone*
Dotd= *Dotted Zone (Mild drug resistance)*
NZ = *No Zone (Complete Drug resistance)*

Selective Media Used

MSA = *Manitol Salt Agar*
MA = *Mac Conkey Agar*

Table C.2 ANOVA For paper discs Using Standard Antibiotics on Test Organisms Zones of Inhibition

	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	2781.397	5	556.279	6.732	.000
Within Groups	9916.571	120	82.638		
Total	**12697.968**	**125**			

Table C.3. Multiple Comparisons For paper discs of Standard Antibiotics
Dependent Variable: Zones of Inhibition

(I) Antibiotics	(J) Antibiotics	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
COT(Cotrioxazole)	NIT(Nitrofurantoin)	-9.28571(*)	2.80540	.001	-14.8402	-3.7312
	GEN(Gentamicin)	-5.04762	2.80540	.074	-10.6021	.5069
	NAL(Nalidixic acid)	-.52381	2.80540	.852	-6.0783	5.0307
	OFL(Ofloxacin)	-12.47619(*)	2.80540	.000	-18.0307	-6.9217
	TET(Tetracycline)	-1.33333	2.80540	.635	-6.8878	4.2212
NIT(Nitrofurantoin)	COT(Cotrioxazole)	9.28571(*)	2.80540	.001	3.7312	14.8402
	GEN(Gentamicin)	4.23810	2.80540	.133	-1.3164	9.7926
	NAL(Nalidixic acid)	8.76190(*)	2.80540	.002	3.2074	14.3164
	OFL(Ofloxacin)	-3.19048	2.80540	.258	-8.7450	2.3640
	TET(Tetracycline)	7.95238(*)	2.80540	.005	2.3979	13.5069
GEN(Gentamicin)	COT(Cotrioxazole)	5.04762	2.80540	.074	-.5069	10.6021
	NIT(Nitrofurantoin)	-4.23810	2.80540	.133	-9.7926	1.3164
	NAL(Nalidixic acid)	4.52381	2.80540	.109	-1.0307	10.0783
	OFL(Ofloxacin)	-7.42857(*)	2.80540	.009	-12.9831	-1.8741
	TET(Tetracycline)	3.71429	2.80540	.188	-1.8402	9.2688
NAL(Nalidixic acid)	COT(Cotrioxazole)	.52381	2.80540	.852	-5.0307	6.0783
	NIT(Nitrofurantoin)	-8.76190(*)	2.80540	.002	-14.3164	-3.2074
	GEN(Gentamicin)	-4.52381	2.80540	.109	-10.0783	1.0307
	OFL(Ofloxacin)	-11.95238(*)	2.80540	.000	-17.5069	-6.3979
	TET(Tetracycline)	-.80952	2.80540	.773	-6.3640	4.7450
OFL(Ofloxacin)	COT(Cotrioxazole)	12.47619(*)	2.80540	.000	6.9217	18.0307
	NIT(Nitrofurantoin)	3.19048	2.80540	.258	-2.3640	8.7450
	GEN(Gentamicin)	7.42857(*)	2.80540	.009	1.8741	12.9831
	NAL(Nalidixic acid)	11.95238(*)	2.80540	.000	6.3979	17.5069
	TET(Tetracycline)	11.14286(*)	2.80540	.000	5.5884	16.6974
TET(Tetracycline)	COT(Cotrioxazole)	1.33333	2.80540	.635	-4.2212	6.8878
	NIT(Nitrofurantoin)	-7.95238(*)	2.80540	.005	-13.5069	-2.3979
	GEN(Gentamicin)	-3.71429	2.80540	.188	-9.2688	1.8402
	NAL(Nalidixic acid)	.80952	2.80540	.773	-4.7450	6.3640
	OFL(Ofloxacin)	-11.14286(*)	2.80540	.000	-16.6974	-5.5884

LSD

N.B the values of Amoxyciline and Augmentin were not used in this analysis because their values were zero throughout i.e. there were no zones of inhibition (NZ) shown by these antibiotics on the test organisms (Table C.1).

Table C. 4. Well Diffusion Technique (Using Plant Extracts with a Standard Drug)

S/No	Diameter of zones of inhibition for each antibiotic (mm)			
	A 100mg/ml	**B** 100mg/ml	**C** 50µg/ml	**Control**
1	NZ	NZ	23 Dotd	G
2	17 Dotd	11.5 Dotd	31 cz	G
3	NZ	NZ	24cz	G
4	17 Dotd	17 Dotd	25 cz	G
5	07 cz	06 cz	35 cz	G
6	16 Dotd	7.5 Dotd	32 Dotd	G
7	19 Dotd	06 Dotd	23 Dotd	G
8	18.5 cz	25.5 cz	26 cz	G
9	16.5 cz	12 cz	35 cz	G
10	22 cz	26 Dotd	35 cz	G
11	19 Dotd	12.5 Dotd	36 cz	G
12	17 Dotd	13 Dotd	24.5 Dotd	G
13	18 Dotd	12 Dotd	15.5 cz	G
14	17 Dotd	15.5 Dotd	25 Dotd	G
15	16 cz	12 cz	32 cz	G
16	19.5 cz	14 cz	35 cz	G
17	14 cz	13 cz	37.5 cz	G
18	20 cz	18 cz	45 cz	G
19	NZ	NZ	NZ	G
20	17.5 Dotd	12 Dotd	25 cz	G
21	19 cz	18 Dotd	38.5 cz	G

KEY: A = Crude Extract CZ = Clear Zone

B = Tannin Fraction Dotd = Dotted Zone (Mild drug resistance)

C = Ciprofloxacin NZ = No Zone (Complete Drug Resistance)

G = Growth Occurred

Table C.5. ANOVA for Well Diffusion Technique (Using Samples with a Standard Drug)

Zones of Inhibition

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3378.151	2	1689.075	26.837	.000
Within Groups	3776.333	60	62.939		
Total	7154.484	62			

Table C.6. Multiple Comparisons for Well Diffusion Technique

Dependent Variable: Zones of Inhibition
LSD

(I) Extracts and the standard drug	(J) Extracts and the standard drug	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
crude extract	Tannin fraction	2.78571	2.44830	.260	-2.1116	7.6830
	Ciprofloxacine	-13.95238(*)	2.44830	.000	-18.8497	-9.0550
Tannin fraction	crude extract	-2.78571	2.44830	.260	-7.6830	2.1116
	Ciprofloxacine	-16.73810(*)	2.44830	.000	-21.6354	-11.8408
Ciprofloxacine	crude extract	13.95238(*)	2.44830	.000	9.0550	18.8497
	Tannin fraction	16.73810(*)	2.44830	.000	11.8408	21.6354

* The mean difference is significant at the .05 level

Table C.7. Well Diffusion Technique with Plant Samples Only

S/No	Diameter of zones of inhibition for each antibiotic disc (mm)			
	A CONCENTRATION = 100mg/ml	**B** CONCENTRATION = 100mg/ml	**C** CONCENTRATION = 100mg/ml	
	Average Reading	**Average** Reading	**Average** Reading	**Control**
1	NZ	NZ	NZ	G
2	17 Dotd	11 Dotd	9	G
3	NZ	NZ	NZ	G
4	16.5 Dotd	17 Dotd	15	G
5	NZ	06 cz	NZ	G
6	15.5 Dotd	7.5 Dotd	NZ	G
7	18 Dotd	NZ	NZ	G
8	18.5 cz	25.5 cz	16.5	G
9	16 cz	11.5 cz	10	G
10	21 cz	26 Dotd	20	G
11	18.5 Dotd	12 Dotd	9.5	G
12	17 Dotd	11 Dotd	9	G
13	18 Dotd	11 Dotd	10	G
14	17 Dotd	15 Dotd	12.5	G
15	14	16	10	G
16	19 cz	13.5 cz	11.5	G
17	14.5 cz	13.5 cz	10.5	G
18	NZ	18.5 cz	NZ	G
19				
20	17.5 Dotd	11.5 Dotd	10	G
21	19 cz	18 Dotd	11.5	G

KEY: A = *Crude Plant Extract* CZ = *Clear Zone*

B = *Tannin Fraction* Dotd = *Dotted Zone (Mild Drug resistance*

C = Residual Fraction NZ = *No Zone (Complete Drug resistance)*

G = *Growth Occurred* *n=2*

Table C.8 ANOVA (Plant Samples Only)

Zones of inhibition

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	332.008	2	166.004	3.469	.038
Within Groups	2728.038	57	47.860		
Total	3060.046	59			

Table C.9 Multiple Comparisons (Plant Samples only)

Dependent Variable: Zones of inhibition
LSD

(I) Drug Extracts	(J) Drug Extracts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude extract (X)	Tannin Fraction (T)	1.62500	2.18770	.461	-2.7558	6.0058
	Residual Fraction (M)	5.60000(*)	2.18770	.013	1.2192	9.9808
Tannin Fraction (T)	Crude extract (X)	-1.62500	2.18770	.461	-6.0058	2.7558
	Residual Fraction (M)	3.97500	2.18770	.074	-.4058	8.3558
Residual Fraction (M)	Crude extract (X)	-5.60000(*)	2.18770	.013	-9.9808	-1.2192
	Tannin Fraction (T)	-3.97500	2.18770	.074	-8.3558	.4058

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

Table C.10 ANOVA (for the MICs)
MICs

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.530	2	.765	.073	.929
Within Groups	593.490	57	10.412		
Total	595.020	59			

Table C.11. Multiple Comparisons of MICs

Dependent Variable: MICs

LSD

(I) Drugs	(J) Drugs	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude Extract	Tannin fraction	.18601	1.00818	.854	-1.8328	2.2049
	Residual Fraction	-.20559	1.03374	.843	-2.2756	1.8644
Tannin fraction	Crude Extract	-.18601	1.00818	.854	-2.2049	1.8328
	Residual Fraction	-.39160	1.02167	.703	-2.4375	1.6543
Residual Fraction	Crude Extract	.20559	1.03374	.843	-1.8644	2.2756
	Tannin fraction	.39160	1.02167	.703	-1.6543	2.4375

Table C.12 ANOVA for MBCs

Minimum Inhibitory Concentrations

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	750.000	2	375.000	1.055	.355
Within Groups	20259.375	57	355.428		
Total	21009.375	59			

Table C.13 Multiple Comparisons of MBCs

Dependent Variable: Minimum Inhibitory Concentrations (MBCs)

LSD

(I) Type of Drug Extract/fractions	(J) Type of Drug Extract/fractions	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Crude drug exrtract	Tannin fraction	-7.50000	5.96178	.214	-19.4382	4.4382
	Residual Fraction	.00000	5.96178	1.000	-11.9382	11.9382
Tannin fraction	Crude drug exrtract	7.50000	5.96178	.214	-4.4382	19.4382
	Residual Fraction	7.50000	5.96178	.214	-4.4382	19.4382
Residua fraction	Crude drug exrtract	.00000	5.96178	1.000	-11.9382	11.9382
	Tannin fraction	-7.50000	5.96178	.214	-19.4382	4.4382