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SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

DEPARTMENT OF CHEMISTRY

A study of the chemical components of extracts from *Kirkia wilmsii* and an investigation into their properties.

MSc Research Project Dissertation

By

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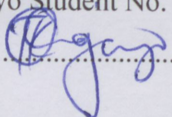

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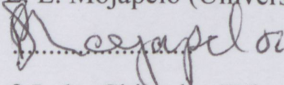

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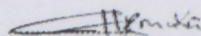

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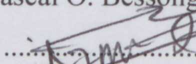

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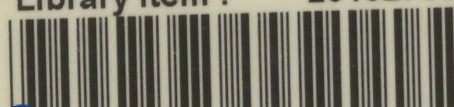

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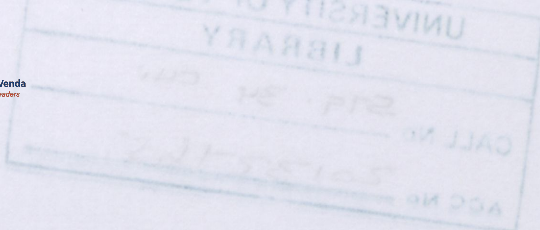
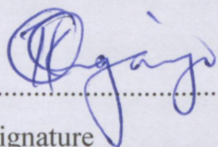
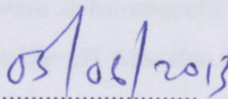


Declaration

I Kayini Chigayo hereby declare that the whole of this study is my own work and has not been copied from any other source, where use has been made of the work of others, it has been duly acknowledged. This work was done at the University of Venda under the supervision of Mr P. E.L. Mojapelo and Professors L. Chimuka and P. O. Bessong.





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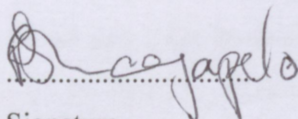

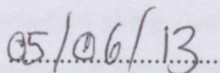

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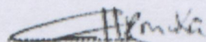

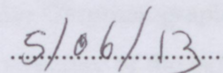

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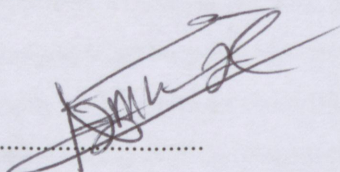

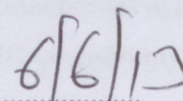

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Abstract

Aqueous extraction was performed on the plant *Kirkia wilmsii* roots at room temperature. The extracts were filtered and stored at 4 °C. An analytical high performance liquid chromatographic (HPLC) method was developed for determination of the components of *Kirkia wilmsii* roots. A gradient elution programme was developed that employed two mobile phases. Mobile phase A water while the pH was adjusted with trifluoroacetic acid (TFA). Mobile phase B was organic, made up of acetonitrile, where the pH was also adjusted with TFA. A C18 column and a photo diode array (PDA) detector were used. Flow rate was changed continuously during the run, to ensure the best resolution of the peaks. The resolved peaks were then collected into different containers using the fraction collector on the HPLC. The peaks that were chosen for this research were those at 16.7, 20.3, 28.2, 31.5, 40.4, 50.1, 56.5 and 64.2 mins. Collected components were then freeze dried as opposed to using a rotary evaporator, to avoid heating samples whose chemical properties were not yet known. The net dry weight of each component was determined.

The purity of the components was checked by gas chromatography (GC), where each component was dissolved in acetone and then run on the GC.

Electrochemical properties of the components were investigated using cyclic voltammetry and anti-oxidant properties were exhibited by four components. All four components showed that they could undergo reversible reactions. The I_{pc}, E_{pc}, I_{pa}, E_{pa}, E, ΔE_p, I_{pc}/I_{pa} and the number of electrons involved in the process for each component were calculated.

Possible structures of bioactive compounds were identified using Gas Chromatography – Mass Spectrometry (GC-MS). These results indicated the possible structure of the isolated compounds. Compound 1 contained triphenylphosphine oxide, (carbethoxymethyl)-triphenylphosphonium bromide or formadehyde, triphenylphosphoranylidene) hydrazone. Compound 5 was made up of diisooctyl phthalate, phthalic acid, di(2-propylpentyl) ester orphthalic acid octyl 2-propylpentyl ester. Compound 7 contained 1,2,3-benzenetriol, 1,2,4-benzenetriol or 3-methyl-pyrazole-5-carboxylic acid, while compound 8 contained catechol, resorcinol or hydroquinone.

All the components analysed by GC-MS showed possible structures that have functional groups that can undergo reversible reactions. This was confirmed by the cyclic voltammetric results.

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Raw fresh extract was shown to have antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Candida albicans and Enterobacter aerogenes*.

Separated and dried components were each dissolved in 2 ml of water and antimicrobial activity tests were repeated. Component 1 exhibited antimicrobial activity against *Shigella dysenteriae, Aeromonas hydrophilia, Salmonella thyphii, Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus.* The minimum inhibitory concentration (MIC) was calculated to be 3.445 ng/µL for this extract against all these microorganisms. Component 5 was only active against *Proteus mirabilis* and the MIC was calculated to be 0.08 ng/µl. Component 7 was active against *Shigella, Escherichia coli* 10, 11 and *Staphylococcus aureus*. The MIC for component 7 against *Shigella* was 0.365 ng/µl, 0.046 ng/µl against *Escherichia coli* 10, and the 0.091 ng/µl against *Escherichia coli* 11. Component 8 was active against *Shigella, Aeromonas hydrophilia, Salmonella, Proteus mirabilis, Escherichia coli* 10 and 11. The MIC against all these microorganisms was found to be 0.155 ng/µl.

It was concluded that some the components of *Kirkia wilmsii* possess antimicrobial and anti-oxidant properties. Chemical properties and possible structures of the biologically active components were investigated. Further tests still need to be done on the plan, especially the stuctures, which have to be identified.

Keywords: *Kirkia wilmsii*, method development, gradient elution, broad spectrum drug, biological activity, anti-oxidant, cyclic voltammetry.

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Dedication

This work is dedicated to God who strengthens me every day. He has blessed me with a wonderful family, relatives, wife Zahara, son Tauya Stephen and daughter Zuhura. Surrounded by such company, I am motivated to work even beyond my imagined capabilities.

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I would like to thank the Holy Spirit which guides us every day in everything we do.

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Abbreviations

AAS	Atomic Absorption Spectrophotometry
AIDS	Acquired Immune Deficiency Syndrome
APCI	Atmospheric Pressure Chemical Ionization
CI	Chemical ionisation
ECD	Electron Capture Detector
EI	Electron Ionisation
GC	Gas Chromatograph
GC-MS	Gas Chromatography – Mass Spectrometry
HMDE	Hanging Mercury Drop Electrode
HPLC	High Performance Liquid Chromatography
IC	Ion Chromatography
INT	Iodonitrotetrazolium chloride
λ	Wavelength
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
NCE	New Chemical Entities
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
PDA	Photo Diode Array
SNR	Signal to Noise Ratio
TLC	Thin Layer Chromatography
TFA	Trifluoroacetic acid
TM	Traditional Medicines
UV/Vis	Ultraviolet/Visible
WHO	World Health Organisation

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Chapter one: Introduction

1 Background and justification

1.0 Introduction

In this study, an HPLC analytical method for the analysis of components of *Kirkia wilmsii* was developed. Methods of analysis should be reproducible and able to be used for further studies on the plant. Extracts were tested for minimum inhibitory concentrations against microbes.

This research will assist in the development of new drugs. The use of Analytical Chemistry is a tool that can be used as part of pharmaceutical industry research, in the quest to regulate the use of traditional medicines through the production of regulated and validated compedial methods of analysis.

During the traditional use of these medicinal plants, the active ingredients and the quantities administered to patients, are erratic. Further, the natural resources from which traditional medicines are derived, are continuously being depleted, without replacement, although, of late, efforts are being made to resuscitate these natural resources.

Therefore, is has become imperative to find a solution to this problem. There is a need to engage in this type of research so as to gain an in-depth understanding of the structure and to synthesise the components of the plants.

In this study the following activities were undertaken:

- The first step was to identify a plant that is currently used for being prevention/curative purposes in local communities, after which the plant was then taken to the laboratory for the initial extraction and antimicrobial activity tests.
- This was followed by extracting the active compound(s) from the plant using several different solvents.
- Then the study focussed on the development of HPLC methods for the separation and isolation of the various extracts that were obtained.
- Biological activity studies were done, followed by the identification of compounds that are found to be bioactive.
- Finally, the chemical properties of the bioactive, identified compounds were investigated.

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1.1 General Objective

The major objective of this study was to isolate and identify bioactive molecule(s) from the plant *Kirkia wilmsii*.

1.2 Specific objectives

The specific objectives were:

- To develop and optimise analytical HPLC methods for the separation and isolation of compound(s) from *Kirkia wilmsii.*
- To conduct bioactivity studies on the separated compounds.
- To determine the structure of the isolated molecule with the aid of the an analytical GC-MS method, the structures being confirmed by NMR.
- To determine the chemical properties of the compound(s) by using Voltammetry/Polarography, Ion Chromatography (IC) and Gas Chromatography (GC).

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Chapter two: Literature review

2.0 Literature Review

2.1 Introduction

Plant preparations have been used for thousands of years as remedies for many ailments, especially in developing countries where primary health care facilities are limited (Balunas and Kinghorn, 2005). Some of these are used with little or no processing, in the form of tinctures, teas, powders, poultices or other forms.

The isolation and purification of opium from morphine in the early 19th century probably initiated more research into drug discovery from medicinal plants, which then led to the isolation of drugs such as cocaine, codeine, quinine and others. Although drugs like cocaine and morphine have been abused, they still find use as analgesics and anaesthetics. In the 1950s, medicinal remedies from plants such as cinchona, digitalis and opium were replaced by the purer isolated active forms (Phillipson, 2007).

There are hundreds of thousands of plant species that are found in many places around the world, however, only a small fraction of these plants have been phytochemically and pharmacologically investigated (Hostettmann, 1999). A direct consequence of this is that if there are many plants that can be investigated. For this to occur, an efficient system for the rapid chemical and biological screening of the plants must be put in place.

Scientifically, even processed herbal remedies can be considered to be crude, as not much is known about them. Further, the knowledge of these medicinal plants that are slowly disappearing, is not documented and the information is only passed down from one generation to another, and hence may not be reliable.

The pharmaceutical industry spends money every year on research and development, and yet a little of this money is used to discover new drugs from plants.

According to the World Health Organisation (WHO, 2008), about 80% of the population in the developing world depends on traditional medicines, mainly plant extracts for the treatment of diseases (Harvey, 2000, WHO, 1999). It is estimated that about 4 billion people in the world rely on the use of plants (Farnsworth, 1988). Farnsworth also states that Hong Kong has the largest herbal market in the world, with Japan and China also claiming large volumes passing through herbal markets. A survey carried out in India illustrated that documented scientific research has been on-going since the 1930s (Rao, 2007).

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The WHO defines traditional medicines (TM) as knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness (WHO, 2008).

The WHO encourages research into TM so that they can be used safely and effectively. By so doing, the use of TM can be regulated. When all the information about the TM becomes available, it is backed by research and scientific facts.

The use of TM for the development of new drugs has been necessitated by the advent of new diseases, for example, Acquired Immune Deficiency Syndrome (AIDS) and related illnesses, chronic diseases such as cancer and the need for cheaper medicines. Closely related to this is the emergence of drug resistant TB strains (Pauli et al., 2005). Thus, in the quest to find new drugs, natural products offer a good alternative.

A good example is the occurrence of opportunistic infections, which are more pronounced in immune compromised individuals, for example, candidiasis, cryptococcosis and others, for which there are a limited number of effective antifungals (Hostettmann, 1999). Natural products can then be used as a solution for such problems.

Some of the synthesised drugs that are currently used for treatment of ailments, are not always effective and may have side effects, which can be fatal. In Central and South America, Chagas disease is an example. The drugs used for the treatment of this disease are only effective in the acute stage (Düsman Tonin et al., 2009). To exacerbate the situation, those drugs also have serious side effects like cardiac toxicity.

The search for new plant drugs has followed a wide range of routes. The most effective has been found to be the testing of plants that are currently in use in some communities. In this way, the chances of success are higher subject to the availability of sufficient funding for the research (Farnsworth, 1988).

One issue that has to be seriously considered is whether it is better to continue using TM or to invest in research in the hope of obtaining purer compounds. One very distinct fact that has discourage most scientists from pursuing this route is that the isolation of pure, biologically active components from plants is a long and tedious process. To add to the woes of most of the developing countries, the costs of conducting this scientific research are astronomical (Harvey, 1999). Further, the cost of large scale importation of pure drugs is high. Therefore, several factors have to be scrutinized, depending on the individual medication. For example, a chemically standardized tincture of *Atropa belladonna* used in treating stomach ulcers has

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therapeutic efficacy equivalent to that of a standard dose of atropine sulfate (Farnsworth et al., 1985). A further advantage is that the plant can easily be cultivated, which would then reduce the intake of atropine and save money for most countries, especially through importation.

An analysis of new drugs introduced between 1982 and 2002, showed that of the new chemical entities (NCE), 52% originated from natural products (Chin et al., 2006). This clearly indicates that natural products are a very important factor in the production of new drugs.

Despite all the positives that have been realised from the development of drugs from natural products, there are factors that hinder research into TM.

The greatest challenge in discovering new drugs from plants, is that the process requires a multidisciplinary approach, requiring chemical, biological, botanical, ethnobotanical and phytochemical skills. This is a very distinct disadvantage as it may take as long as ten years to produce one drug (Jachak and Saklani, 2007).

The other challenge is that most of these plants are found in developing countries, which are commonly not politically stable. This presents a challenge in that the continued supply of the raw material is not guaranteed. It is very rare for a plant to be restricted to one particular geographical location, hence this can be circumvented. However, some scientists claim that there are biological variations in plants from different areas (Farnsworth, 1988).

Sometimes the cost is very high and wasteful if some of the routes are abandoned after utilising a large proportion of the research money and time. Therefore, options should be first weighed, to assess whether the costs of developing the drug will be recovered. Further, a solid patent must be secured so that the costs can be recovered.

Another setback that has also led to the reduction of research into plant materials, is the length of time that the drug discovery process takes to complete. It has been reported that this can take any length of time from 10 years or even more (Balunas and Kinghorn, 2005).

In conclusion, there are other factors that reduce the amount of research into natural products, apart from the above, which include; very low yields are obtained, complex mixtures are found with multiple stereoisomers, follow up studies are rarely done and potent activity is rarely exhibited (Pauli et al., 2005).

2.2 *Kirkia wilmsii*

The *Kirkia wilmsii* is a tree which can grow to between 8 and 10 m in height.

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From the pictures in Figures 2.1 and 2.2, it can be seen that the tree has brilliant autumn colours which make it easy to be identified particularly during the months of April and May when the leaves develop a bright red colour from their normal pale green.

Figure 2.1: *Kirkia wilmsii* tree found in Lebowakgomo, Limpopo Province

The genus name *Kirkia* was coined from kirkii, which was used to refer to Dr John Kirk (1832-1922), an English doctor and plant collector, who travelled with David Livingstone on the Zambezi expeditions. The species name *wilmsii* came from F. Wilms (1848 -1919), a German Pharmacist, plant collector and botanist.

This tree has the common names of wild pepper or Mountain Seringa (English) and Modumela (Northern Sotho). The *Kirkia wilmsii* belongs to the *Sapindales* family and yet it was wrongly placed in the *Simaroubaceae* family (Bachelier and Endress, 2008). This family (Kirkiaceae) includes a wide range of small to medium sized trees, mainly found in eastern tropical Africa, Madagascar and South Africa.

Kirkia wilmsii is a medium to large tree, which can grow on granite or dolomitic soils in dry areas and prefers rocky places (Figure 2.2). In South Africa, the tree is mainly found in the Mpumalanga and Limpopo provinces. In fact, the *Kirkia wilmsii* is the main vegetation that is found in the Leolo Mountain range, situated in Sekhukuneland, Limpopo Province (Venter et al., 2007).

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Figure 2.2: *Kirkia wilmsii* tree in autumn season environment

This tree is one of the plants that have underground storage organs (such as rhizomes, tubers, corms, bulbs and caudex) (Laden and Wrangham, 2005). It can thus survive short periods of droughts as it can store water in its roots, which are in fact, tubers. Storage of plant resources in tubers has been caused naturally by the adaptation and need to survive unfavourable conditions during some growth phase, or season. A direct consequence of that is the nutritional value of the underground storage organs varies widely within species and between species, depending on the area, season and energy (Pillay et al., 2008). Other factors that influence the content are water, toxins and the presence of other nutrients in the environment. Indigenous people eat the bulb root for both medicinal and thirst quenching purposes (Smith, 1991).

This tree has generally been found to be free from pests (Grant and Thomas, 2001). Thus, there is need to investigate the insect repellent properties of the tree.

It has also been found that the plant can also be used for the treatment of malaria and feverish conditions (Suleiman et al., 2010).

Very little research has been done on the *Kirkia wilmsii* tree. The leaves have been investigated and dichloromethane/methanol and water extracts have been found to have antiplasmodial properties (Clarkson et al., 2004, Pillay et al., 2008). Another study that was done on the leaves showed biological activity against *Staphylococcus aureus* (acetone, methanol, hexane and dichloromethane extracts), *Enterococcus faecalis* (acetone, methanol

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and hexane extracts), *Escherichia coli* (acetone, hexane and dichloromethane extracts), *Pseudomonas aeruginosa* (acetone, hexane and dicholoromethane extracts), *Sporothrix schenckii* (methanol and hexane extracts), *Microsporum canis* (acetone extract), *Cryptococcus neoformans* (acetone, hexane and dichloromethane extracts) and *Candida albicans* (acetone and hexane extracts) (Suleiman et al., 2010).

The leaves further showed biological activity against the animal fungal pathogen *Aspergillus fumigatus* (Suleimana et al., 2010).

Hexane, dichloromethane and methanol extracts of the dried and powdered bark of the *Kirkia wilmsii* were investigated and found to contain secondary metabollites (Mulholland et al., 2003). These were identified as lignans, isocoumarins, flavanoids and nor-carotonoids.

Other than these few cases of documented research, little literature has been published on *Kirkia wilmsii,* yet it has been widely used amongst the Sotho people for the treatment of a wide range of diseases.

The study will help to fill the gaps in knowledge about *Kirkia wilmsii*. Further, the Sotho people use the bulb as a general cure for many diseases, which motivated research on this part of the plant.

Other species of the Kirkia genus, such as *Kirkia acuminate,* have been shown to contain tannins, which are naturally occurring polyphenolic compounds that form complexes with proteins (Aganga and Mosase, 2001).

2.3 Drug Discovery from Plants

For centuries, herbs have been used as medicines (Abu-Dahab and Afifi, 2007). Originally, plants were the only medicinal agents that were available to humans (Ganesan, 2008). However, at that time, the available information about the chemistry of these natural products was limited, whereas today, sophisticated instruments and techniques are available to investigate and characterise the active components in medicinal plants. An encouraging discovery is that natural products are more readily absorbed by the body more than synthetic drugs as most of them are small molecules (Harvey, 2008).

Most of the time, plants synthesise only one enantiomeric form of a secondary metabolite (Demetriou et al., 1993). Such enantiomers do not have the same chemical properties and consequently, they have different physiological responses. However, in some instances, both enantiomers can be found but only one will dominate, a case in point is the production of

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both enantiomers of α-pinene from the pine tree. Chiral chromatography, which was being used for the analysis of enantiomers, is being replaced with reverse phase C18 analysis, that is much cheaper. A good example is the pain relieving morphine, produced from the poppy plant, *Papaver somniferum*. These secondary metabolites are produced by plants in response to external stimuli such as nutritional changes, infection and competition (Strohl, 2000). They are produced as a characteristic mix of the chemicals that plants produce. These can be used to classify the plants. Human beings use some of these compounds as medicines, flavourings, or recreational drugs.

A majority of the drugs being produced today come from natural compounds, their derivatives and analogues, or are inspired by natural compounds. Today it is estimated that 50% of all the drugs being used come from natural products with the higher plants contributing about 25% of this total (Balandrin et al., 1993). Drugs obtained from the natural plants include reserpine, codeine, morphine, cocaine, ephedrine, atropine and many others. Sometimes, new chemical entities may not be found in the plants but the compounds found may provide good leads for developing new drugs. Despite all these encouraging facts, majority of plants have as yet not been investigated (Farnsworth, 1988, McChesney et al., 2007). This is alarming to scientists, as forests, including tropical floras and ecosystems, are being destroyed at an alarming rate, before the plants can be investigated for possible drug potential. If the current trend continues, opportunities for research will be lost.

The process generally starts with the collection and identification of the herbal plants of interest by a botanist, ethnobotanist, ethnopharmacologist or plant ecologist. The plants researched may be constituents of herbal preparations being used by local communities for the relief of common ailments (traditionally), or may involve a large class of plants randomly collected for a large screening programme.

Herbs are then taken to the laboratory, where phytochemists or chemists, perform solvent extractions to isolate the possible active compounds. Solvents, like chloroform, methanol, water, acetone and others are used in the laboratory extractions.

After extraction, biological screening, using the relevant biological activity assays, is done. Active compounds are then isolated, purified and characterised in the laboratory, using modern analytical techniques, such as chromatography and nuclear magnetic resonance.

Computational chemistry can then be employed to see if the identified compounds show any biological activity towards several organisms using theoretical simulations.

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Despite drawbacks, natural products have been used as lead material for drug development by multinational pharmaceutical companies (Borris, 1996, Turner, 1996). These two authors looked at perspectives of pharmaceutical companies like Glaxo and Merck. They pointed out that although significant, research from natural products only plays a small part in drug development.

In summary, three approaches have been employed in drug discovery, that is, traditional, empirical and molecular (Harvey, 1999). The traditional method is haphazard and drugs are discovered by chance. The empirical method follows naturally lead molecules. The molecular approach, is based on the molecular target for the medicinal agent.

Several steps might be followed during the development of a new drug.

2.3.1 Screening of natural compounds for biological activity

The discovery of penicillin was the launch pad of this field of Pharmacology, as it generated interest into research in this direction (Graham, 1994, Strohl, 2000). Many antibacterial agents were investigated. In their published research (Fabricant and Farnsworth, 2001), some scientists suggested several approaches that could be used for drug development with the highest chances of success. Some of the methods include random selection followed by one or more biological assays, follow up of biological activity reports where bioactivity was confirmed but the active principles were not studied and many other approaches.

2.3.2 Isolation and purification of the active compounds

Care has to be taken in this stage of drug development as the isolation and purification of the active compounds can be affected by structure, stability and quantity of the compound. For instance, penicillin took a long time to develop because it was unstable after isolation and only progressed with the advent of freeze drying (Farnsworth, 1988).

Developments in technology have also helped to make this step easier to achieve, such as the HPLC, which makes the process faster and more efficient, as compared to isolation from columns.

However, natural products are typically isolated and purified in very small quantities, which may not be enough to use in other tests such as lead optimisation, lead development and clinical trials. Therefore, further collaboration may be needed with synthetic organic

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chemists, who may determine the feasibility of synthesis or semi-synthesis. This can further be supported by the creation of natural product libraries (Bindseil et al., 2001). However, pharmaceutical companies have slowed down the screening of natural products, because the natural product libraries are not compatible with high-throughput screening methods (Lam, 2007).

Also, most medicinal plants can be found in the many regions, other than those where they are first investigated naturally (Farnsworth et al., 1985). Therefore, some scientists in one part of the world might have already studied the same plant that other scientists are studying elsewhere. This duplication of research could be avoided if collaboration is done and all the information on all the current research on medicinal plants made available to all.

2.3.3 Structure Determination

Structure determination of new compounds was a tedious process, that often required the breakdown of molecules into known fragments. Alternatively, total synthesis was used to make molecules and compare them with the newly found molecule. This process was time consuming. Thanks to new technology, the determination of structure now takes much less time and is more accurate due to instrumentation and methods such as NMR, IR, X Ray Crystallography and GC-MS that are now being used, either individually, or in combinations' to obtain accurate structures. This was not available to scientists historically. For instance, cholesterol was analysed in 1888 and only identified in 1932 (Krogsgaard-Larsen et al., 1996).

The use of hyphenated techniques, such as HPLC-NMR, have also been employed to increase the efficiency of the drug discovery process. However, there are some drawbacks, for instance, the sensitivity of the NMR analysis is compromised, if the residence time is determined by the HPLC flow rate instead of signal to noise ratio (Bross-Walch et al., 2005). However the technique works well if the sensitivity is high enough.

It is important to note that no single spectroscopic technique can be used for the purposes of structure determination, but all the information gathered from the techniques gives conclusive information about the structure of a molecule.

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2.3.4 Structure-activity relationships

Structure-activity relationship is a way of relating the effect of a drug or toxic substance on its receptor, based on the molecular structure of the drug. Thus, an investigation into which parts of the molecule cause biological activity and which parts do not, is undertaken.

This can be done by investigating a series of molecules, which differ slightly in structure, and observing the resultant biological activity. When these experiments are performed, the effects of each structural variation on biological activity is noted. These relationships are more frequently investigated in drug development as well as toxicity studies, particularly in environmental chemistry.

2.3.5 Synthesis of analogues

This is a very important stage of drug development, where similar chemical entities to the one that was discovered, are synthesised. They are synthesised to check if they also have biological activity, besides that of the discovered lead molecule. The motive is to increase biological activity while reducing side effects. If the analogues are biologically active, then molecules that have side effects are eliminated. At this stage occassionally only the analogues will be used. Several variants of the lead compound are normally synthesised to increase its efficacy, in order to improve the toxicity profile, modify susceptibility to degradative pathways, or modify pharmacokinetics of the lead molecule. Small changes are made to the structure, and experiments are performed to determine if those changes had a beneficial or detrimental effect on the efficacy of the drug. This process is called synthesis of analogues. It is an effective process, although it is expensive and time consuming. Analogue synthesis is the basis for medicinal chemistry, and remains an important part of drug discovery today.

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2.3.6 Receptor theories

Drugs exhibit effects on animals through interactions with specific receptors in their bodies and these effects depend on the ability of the drug to find access to a particular organ tissue in the body. A direct implication of this is that drugs must be specific and selective. This stage involves computer aided modelling of 3D models of the binding sites. Computer models can reveal more information about the molecules, their similarities, properties, active sites, the determination of the pharmacophore and many others. No further details will be discussed here as this is out of the scope of this research.

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2.3.7 Design and synthesis of novel drugs.

Although this stage is beyond of the scope of this research, it is the final stage in drug design. After careful consideration of the advantages and disadvantages of the drugs and modifications, synthesis can now commence.

2.4 High Performance liquid Chromatography (HPLC)

Chromatography is a group of methods that allow separation, identification and determination of even closely related components of complex mixtures.

In all chromatographic separations, a sample is carried through the column by a mobile phase or eluent. The stationary phase is fixed in a column. The sample components distribute themselves between the mobile and stationary phase to varying degrees. The components that are strongly retained by the stationary phase move slowly with the flow of the mobile phase and components that are weakly held by the stationary phase travel rapidly.

Consequently, because of these different migrations rates, sample components separate into discrete bands that can be analysed qualitatively (identification) and quantitatively (concentration).

Liquid chromatography was developed through the early work of the Russian, Tswett, who coined the name chromatography. His early work involved analysis that took a very long time to complete.

To increase column efficiency, particle size has to be reduced, thus the need to introduce pressure. Glass could not withstand the pressure and metal columns were developed. This new method was then named HPLC.

The mobile phase is a liquid and it can be dispensed through multi reservoirs. When one eluent is used, the method is called isocratic and when the eluent is changed, with time, during analysis, then the term gradient elution is used.

Dissolved gasses must be removed as they can lead to irreproducible flow rates, cause band spreading or interfere with the performance of the detector.

Dust and small particles must be removed from the mobile phase by filtration. Filtration through a millipore filter under vacuum can be done before introduction of the mobile phase into the system.

A pressure of up to 6000 psi can be reached.

Stainless steel columns are usually used, with supports prepared from silica, or silica based compositions such as hydrolysed silica with chemically reactive silanol groups. Heavy walled

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glass tubing and polymer tubing can also be used to withstand the high pressures generated by the pump. Most columns range from 5 to 25 cm long with an internal diameter of 3 to 5 mm.

In HPLC, control of column temperature may be needed and more reproducible chromatograms are usually obtained by maintaining the temperature.

Methods tend to be complex in liquid chromatography because the sample components interact with both the stationary phase and the mobile phase.

Separation of the components of natural products has been done by column chromatography, which is tedious and takes a long time to complete. Further, complete separation has to be confirmed by other methods, such as thin layer chromatography (TLC). Most often, separations are not completed in one run. The column chromatography/TLC cycle has to be repeated several times until satisfactory results are obtained. Recent advances in HPLC have seen the development of fraction collectors, which can be attached to an HPLC system that can collect the separated components. The advantages associated with the use of HPLC in the analysis of natural products include; accuracy, precision and the method is not limited by the nature of the sample, that is, volatility or stability of the sample (Jandera et al., 2005, Lee et al., 2008). However, TLC can be used as a preliminary technique before HPLC is employed, for instance the polarity of each component can be checked by TLC when designing a gradient elution programme (Mahler and Thomason, 2007). TLC is more widely used in qualitative analysis, whereas HPLC is a better tool it quantitative analysis is needed. Further, TLC is more likely to miss co-products than HPLC (Lapkin et al., 2009)

The HPLC method is much faster and the resultant chromatogram can be seen, clearly showing whether the components are separated or not. When the components are separated, then fraction collection can commence.

Recently, many HPLC methods have been developed for the analysis of natural products. The methods can be simple, that is, if samples that do not contain many compounds as is the case for mangostin (Pothitirat and Gritsanapan, 2009), which required gradient elution in order to achieve complete resolution. In this case, the method has been both developed and validated.

Isocratic HPLC methods have also been reported for carotenes (Sathish et al., 2009), which improved on the gradient elution methods that were already available. The method offers a faster way of determining carotenes. However, this may not always be possible particularly with multi-component natural products, which require more sophisticated gradient elution methods.

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Some HPLC techniques that are not common, have also been used for the analysis of natural compounds. Lipids have been separated on HPLC, using the silver ion HPLC in normal phase (Christie, 1988). Separation in this technique, is based on strong interactions between the silver ions and π-electrons from the double bonds. The advantage of this method is that it can separate lipids with different positions and numbers of double bonds (Holčapek et al., 2005). In addition, many other HPLC techniques can be used, for example, capillary electro-chromatography, supercritical fluid chromatography, subcritical fluid chromatography and many others.

If HPLC is coupled to a MS, this is one of the most useful techniques for the analysis of natural products. This technique, known as HPLC-MS, has the advantage of combining the "good" qualities of HPLC with those of MS. The HPLC will be used to separate components of the sample, then the library of the MS is used to identify the separated components. Atmospheric pressure chemical ionization (APCI), has been used for the analysis of triacylglycerols, instead of normal chemical ionisation (CI) or electron ionisation (EI) (Holčapek et al., 2005), which has the advantage of being very simple. However, the number and position of double bonds, the differences in chain lengths and other structural differences may lead to varying responses, especially at low wavelengths for the APCI and UV detectors. Thus, calculations that do not take into account response factors, will be misleading. Studies on the same plant materials using the charged aerosol detector have yielded more stabilised results (Lísa et al., 2007), which proved that it could be used as a universal detector for non-volatile compounds, especially used in combination with gradient elution methods.

In other studies, isoflavones were rapidly separated on HPLC, where retention times have been reduced to as low as 5 mins (Klejdus et al., 2007). The method was also adapted for the analysis of pesticides and similar results were achieved. The reduction in retention times were achieved through the use of extremely high pressure.

The HPLC technique has found application in many fields and has also been applied to many natural products. This is of very little surprise, as the technique is very versatile. In the food industry, studies on proanthocyanidins found in natural plants are also done by HPLC (Hellström and Mattila, 2008). However, the direct separations are not easy because the proanthocyanidins naturally occur as complex mixtures. Therefore it is necessary to depolymerise the samples prior to analysis.

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2.5 Gas chromatography-Mass Spectrometry (GC-MS)

A combination of a Gas Chromatograph (GC) coupled to a Mass spectrometer (MS) is called GC-MS. The GC separates components in a sample and the MS acts as the detector and at the same time helps to identify the separated components. The MS detects the separated components individually using their mass to charge ratio

In a GC, the mobile phase is a gas, called the carrier gas, which must be chemically inert. Helium, argon, nitrogen and hydrogen are used as carrier gases. They come in pressurized containers; which have pressure regulators, gauges and flow meters to control the flow rate. The carrier gas system contains molecular sieves to remove impurities and water.

A sample is introduced into a heated sample port located at the head of the column. The sample port is ordinarily set at about 50 °C above the boiling point of the least volatile component of the sample. Thus the sample is immediately volatized. Very low volumes (1 μℓ) of the samples are injected into the GC.

Two types of columns are used in GC; packed columns and open tubular columns (also called capillary) columns. In the development stage, GC analysis used packed columns only. Recently, packed columns have been replaced by more efficient, open tubular columns.

Packed columns vary in length from 1 m to 5 m in length and capillary columns can range from a few meters to 100 m long. Most columns are constructed from fused silica or stainless steel although glass and teflon are also used. To fit into the oven for thermostating, they are formed as coils with diameter of 10 to 30 cm.

Column temperature is very important and must be closely controlled for precise work. Thus, the column is housed in a temperature controlled oven. The temperature depends on the boiling points of the sample and the degree of separation required. For samples with components that greatly vary in boiling point, temperature programming is employed. Here, column temperature is increased, either continuously, or in steps, as the separation proceeds.

An MS analysis involves:

1. Vaporisation is the conversion of liquid sample to gas
2. Conversion of the molecules to ions (usually singly charge positive ions)
3. Separation of the formed ions on the basis of mass-to-charge ratio
4. Counting the number of ions of each type or measuring the ion current produced when the ions strike the transducer. Since most of the ions are singly charged, m/z is usually the mass of the ion (m/z is the mass to charge ratio).

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Initially, the coupling of the GC and MS was delayed because the packed columns needed special interfacing devices, which in turn prevented the rapid growth of the technique (Segura et al., 1998). In GC-MS, the MS scans the masses repetitively during a chromatographic experiment. A scan can be taken each second, thus if a chromatographic run is 10 min, 600 mass spectra are recorded. This data can then be analyzed by the data system.

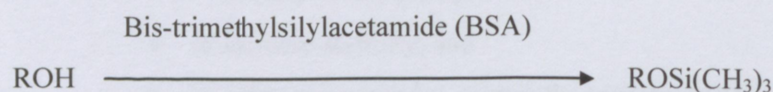
Thus, incomplete separation can be analysed by MS. Various unresolved components can be identified. Normally the GC-MS is equipped with a library. This enables the instrument to analyse the obtained data and identify the components.

In GC-MS, there are two popular ionisation techniques; electron ionisation and chemical ionisation. In conventional systems, only electron ionisation is employed, which measures only the more abundant positively charged ions (Halket et al., 2005). The energy supply can be kept constant, which gives reproducible mass spectra.

2.5.1 Derivatization

In the most common forms of GC and GC-MS, the sample under investigation must be in the gaseous form. In GC-MS analysis, the sample must remain in the gaseous phase in the GC as well as the MS. To be amenable to analysis by GC-MS, samples must therefore, be easily vaporised and must be thermally stable. Unfortunately, many chemical substances do not meet these requirements as some of them have polar functional groups and are not easily vaporised (Johnstone and Rose, 1996). These functional groups are difficult to analyse by GC or GC-MS because they are not sufficiently volatile, are too strongly attracted to the stationary phase, tail badly, are thermally unstable or decompose.

Derivatization is a technique that is used to chemically convert a compound so that it can be easily analysed by GC. Large relative molecular masses or strong intermolecular forces or both, cause molecules to be non-volatile. If the molecular mass is large derivatization may incerease the mass but then this is often offset by the reduction in the intermolecular forces. The strong interactions are hydrogen bonding and the electrostatic association of zwitterions (Johnstone and Rose, 1996). Hydrogen bonding can be reduced by the active hydrogen atoms by alkylation while the zwitterionic groups can be acetylated and methylated i.e.



$$ROH \xrightarrow{\text{Bis-trimethylsilylacetamide (BSA)}} ROSi(CH_3)_3$$

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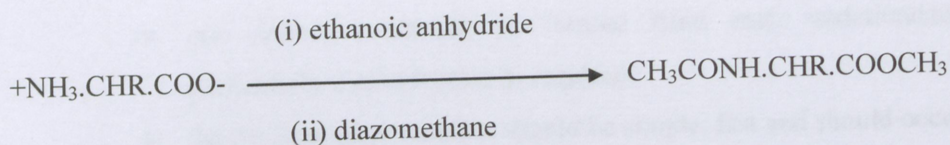

There are many useful reagents that are used for the purpose of derivatizing polar functional groups. The groups that are likely to give problems are hydroxyl, ketones, carboxylic acids and amines. The main methods used to derivatize these groups are silylation, acylation, alkylation formation of cyclic derivatives and chiral derivatization. These method have to be chosen taking into consideration ionisation mode, mass spectrometer resolution and increasing spectrometric selectivity (Scott, 1998).

The main purpose for performing this technique is to increase volatility and thermal stability to otherwise non-volatile and thermally unstable compounds. Non-volatility is normally found in large molecules. There are times when small compounds are also not volatile because of the strong intermolecular attractions of polar groups. On the other extreme, derivatization can be done to reduce the evaporation of some very volatile compounds so that the sample loss can be minimised and also to facilitate separation from the solvent peak. Derivatization can also help to increase resolution as compared to the unseparated closely related compounds in the underivatized form. More importantly, derivatization can improve the mass spectra's fragmentation pattern, especially at high mass numbers (which have more diagnostic value) can also be more enhanced. This is preferred because the higher masses are less susceptible to background interference and that are used for the determination of molecular mass.

One disadvantage of derivatization is that side reactions can occur, yielding multiple derivatives with polyfunctional compounds, which happens if reaction conditions are not correctly controlled. Multiple derivatives can be formed and they are useful as they help in identification but can reduce sensitivity of the technique.

Derivatization can be performed for the following reasons (Touchstone et al., 1986):

- to increase volatility to permit analysis of compounds with inadequate volatility or stability;
- to increase detectability;
- to increase stability; and
- to enhance sensitivity, especially with the ECD.

Successful derivatization has to meet the following requirements;

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- one derivative should be formed from each underivatized compound particularly if quantitation is required;
- the derivatization reaction should be simple, fast and should occur under mild conditions; and
- a high and reproducible yield that is stable in the reaction medium should be obtained. The same result must be obtained if the test is repeated.

Most derivatization procedures require the use of high temperatures for the completion of the reaction. This would not be ideal when dealing with unknown samples, whose decomposition temperatures may not be high. A procedure that did not require the use of heat was used in the analysis of deposits from Roman age amphorae (Petit-Dominguez and Martinez-Maganto, 2000). The method was also accomplished very rapidly with only a small amount of sample needed. Archaeological samples were analysed to investigate birch bark tar and were performed by GC-MS (Ribechini et al., 2011). The investigation hinged on the fact that tar and pitch were once produced from resins, barks and wood. Thermal derivatization was then used to convert the samples to enable analysis by GC.

Studies conducted on carbamate pesticides showed that derivatization was necessary as the carbamates in use were not stable under gas chromatographic conditions, as they decompose in the hot injector to form phenols and isocyanates (Stan and Klaffenbach, 1991). Acylation was used to achieve the derivatization.

In-vials derivatization of phenolic acids and flavonoids from plant extracts were also performed for rapid analysis using phase transfer catalysis (Fiamegos et al., 2004). In their paper, they discuss how m

ethylated derivatives were synthesised before analysis, which yielded low detection limits. Methods have also been reported for the analysis of environmental samples where in situ derivatization was performed on water samples (Henriksen et al., 2001). Acidic pesticides and their phenol degradation products were analysed. This shows the wide applicability of the derivatization methods.

The GC-MS technique with derivatization has wide applicability. In environmental chemistry, studies have been done where the determination of endocrine disrupting chemicals were investigated (Zhang et al., 2006). These chemicals are found in the environmental pollutants that interfere with the endocrine systems of wildlife and people. In this experiment, it was found that silylation was the best method for derivatization. Similarly, silylation

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followed by cyclisation yielded good result when the analysis of dihydrostreptomycin was carried out (Preu and Petz, 1999). More reproducible results were obtained although more runs had to be performed.

Microwave analysis has been known to catalyse reactions. In one research the microwave assisted derivatization method was done, where the samples were simply irradiated with microwaves (Athanasios et al., 2007). The microwaves were used instead of the more documented heating. Results were highly comparable to those performed on heat and yet the microwave reactions took much less time to perform.

Besides GC, derivatization has also been performed on HPLC methods. It was reported that fatty acids were analysed after conversion to their ester by HPLC using a fluorescence detector (You et al., 2001). Thus the derivatization is not unique to GC methods only.

2.6 Ion Chromatography (IC)

IC refers to the methods for separating and determining ions on columns with relatively low ion-exchange capacity. Ion exchange processes are based on exchange equilibria between ions in solution and ions of like sign on the surface of an essentially insoluble, high molecular weight solid. The most common active sites for cation exchange resins are the sulphonic acid groups ; $-SO_3^- H^+$, a strong acids, and the carboxylic acid group : $-COO^-H^+$. Anionic exchangers contain strongly basic tertiary amine groups:$-N(CH_3)^+OH^-$ or weakly basic primary amine groups: $-NH_3^+OH^-$.

In ion chromatography, the eluent also contributes to the conductivity detected which causes interference. To eliminate this, the conductivity of the eluting electrolyte has to be prevented from interfering with the measurement of the analyte conductivities

There are two types of systems that can be used to achieve this:

(i) Suppressor based IC

An eluent suppressor column was introduced to solve the problem created by the high conductance of eluent. These are introduced immediately following the ion exchange column. The suppressor column is packed with a second ion-exchange resin, that effectively converts the ions of the eluting solvent to a molecular species of limited ionization, without affecting the conductance due to analyte ions for example in cation analysis and HCl is chosen as the eluting reagent, the suppressor column is an ion-exchange resin in the hydroxide form The product of the reaction is water that is:

(ii) Single – column IC

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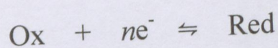
Here no suppressor column is used. This approach depends on the small different in conductivity between the sample ions and the prevailing eluent ions. To amplify these differences, low-capacity exchangers are used that permit elution with solutions with low electrolyte concentrations. Further, eluents of low conductivity are chosen. This method has the advantage of not requiring special equipment for suppression. However it is less sensitive especially for anions, than the suppressor column methods.

The conductivity detector is the obvious choice. It is highly sensitive, universal for changed species and they respond in a predictable way to concentration changes

2.7 Voltammetry

This is an electrochemical method for that can be used for the analysis of electroactive compounds. In this method, the current that develops in the electrochemical cell is measured, when voltage is applied to cause oxidation or reduction. The applied voltage is known as the excitation signal as it produces a characteristic current in the reaction cell. `
The applied potential determines the amounts of redox species at the surface of the electrode and the Nernst equation is used to find the concentrations (C_o and C_r) while the Butler-Volmer equation is used to determine the rate constants (k), where C_o and C_r are oxidation and reduction concentrations whereas k is the reaction rate (Kounaves, 1999).
For a reversible reaction, the equation;



$$Ox + ne^- \leftrightharpoons Red$$

where Ox and Red represent the oxidised and reduced species respectively, the potential forces concentrations to comply with the Nernst equation:

$$E = E^o + [RT/nF] \ln (C_o/C_r)$$

R = molar gas constant (8.3144 $Jmol^{-1}K^{-1}$), n = number of electrons transferred, T= absolute temperature (K), F= faraday constant (96485 C/equiv) and E^o = standard reduction potential for the redox couple.
Figure 2.3 shows the four types of excitation signals can be imparted, that is, linear scan, square wave, differential pulse and triangular:

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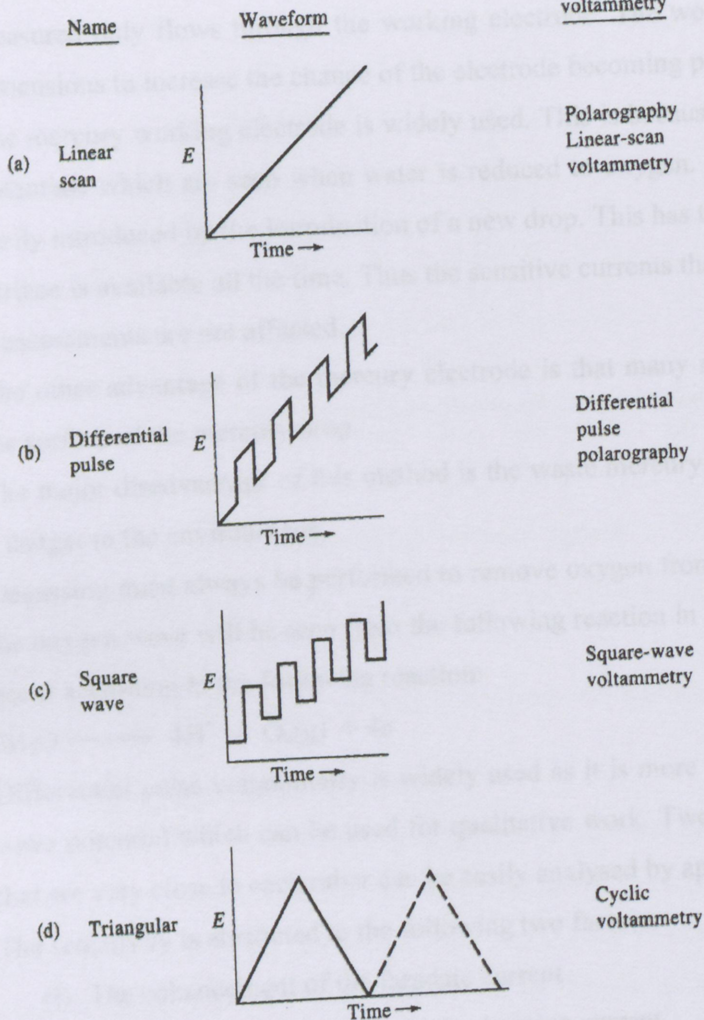

Figure 2.3: Types of voltammetry according to the excitation signals

The technique is not limited to metals. Voltammetry is widely used to monitor any species that can undergo oxidation and reduction.

The major advantage of this technique is that there is minimal consumption of the analyte. In this technique a variable potential is applied to the reaction cell, which produces a current. The current is then measured and used for the purposes of identification, quantification and investigation of other electrochemical properties, such as, reversibility processes.

In the cell there are three electrodes immersed in solution containing the analyte and the support electrolyte. The electrodes are the working electrode, the reference electrode and the counter electrode. The counter electrode is used to take away the current from the reference

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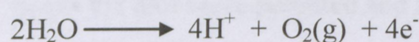
so that the current does not flow through the reference electrode. Therefore the current that is measured only flows through the working electrode. The working electrode has very small dimensions to increase the chance of the electrode becoming polarised.

The mercury working electrode is widely used. This is because it can stand the high negative potentials which are seen when water is reduced to oxygen. Further, a fresh surface can be easily introduced by the introduction of a new drop. This has the added advantage that a clean surface is available all the time. Thus the sensitive currents that are involved in these kinds of measurements are not affected.

The other advantage of the mercury electrode is that many metals are reduced reversibly at the surface of the mercury drop.

The major disadvantage of this method is the waste mercury that it produces that might pose a danger to the environment.

Degassing must always be performed to remove oxygen from the reaction vessel. If not done the oxygen wave will be seen from the following reaction in which the oxidation of water can occur according to the following reaction:



$$2H_2O \longrightarrow 4H^+ + O_2(g) + 4e^-$$

Differential pulse voltammetry is widely used as it is more sensitive and it can give the half wave potential which can be used for qualitative work. Two peaks with half wave potentials that are very close to each other can be easily analysed by applying the differential pulse.

The sensitivity is attributed to the following two factors:

(i) The enhancement of the faradaic current

(ii) The decrease in the nonfaradaic charging current.

A typical voltammogram showing different parameters is shown in Figure 2.4 below,

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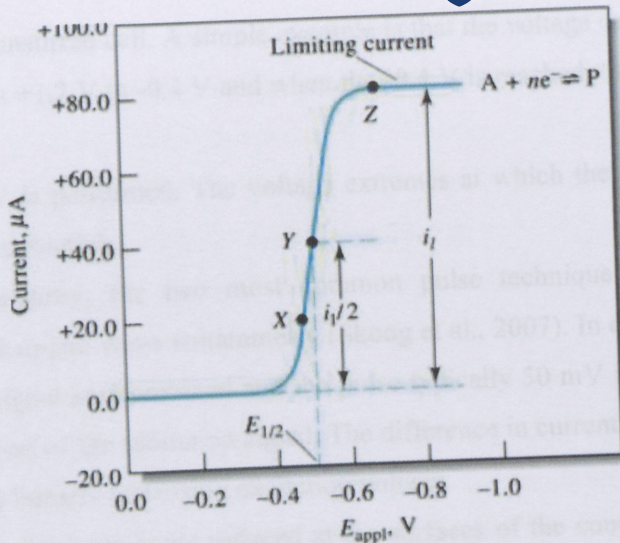

Figure 2.4: Current against voltage voltammogram

The limiting current, i_l, is the current found at the plateau of the graph and is proportional to the concentration

$E_{1/2}$ is the half wave potential and $i_{1/2}$ is the current at half wave potential.

2.7.1 Antioxidant Activity

The presence of an excess of oxygen in the human body has some negative effects as it can trigger radical chain reactions in the presence of reactive species. This can cause health problems, such as aging and cell destruction (Korotkova et al., 2002). Antioxidants have been found to be the solution to this problem as they interrupt these chain reactions to form radicals that can easily be removed from the human body thereby generally improving health, assist cell rejuvenation and cancer prevention (Boldyrev et al., 1993). Cells use different types of defence mechanisms against the reactive oxygen species, one of which is the low-molecular weight antioxidants (most often found in edible plants) (Chevion et al., 2000). Therefore antioxidants are now being widely added to food stuffs and medicines because of their benefits to life (Barros et al., 2011).

2.7.2 Cyclic Voltammetry

Cyclic voltammetry is one of the most effective electro-analytical techniques used to study the mechanisms of redox reactions, understanding reaction intermediates and assessing stability of reaction products (Kounaves, 1999). In this technique, a triangular voltage form

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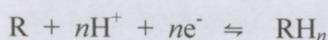
is applied to the unstirred cell. A simple example is that the voltage can be increased linearly, for instance, from +1.2 V to -0.4 V and when the -0.4 V is reached, the scan is reversed and a scan from

-0.4 V to +1.2 V is performed. The voltage extremes at which the reversals take place are called switching potentials.

In cyclic voltammetry, the two most common pulse techniques are differential pulse voltammetry and square wave voltammetry (Skoog et al., 2007). In differential pulse, a pulse and a staircase signal are combined and the pulse typically 50 mV is applied during the last 50 ms of the period of the excitation signal. The difference in current per pulse is measured as a function of the linearly increasing excitation voltage.

A lot of organic compounds are reduced at the surfaces of the common working electrodes like gold, platinum carbon and mercury. The reactions can be typically represented by the equation;



$$R + nH^+ + ne^- \leftrightharpoons RH_n$$

R is the oxidised and RH_n is the reduced organic molecule.

Peak potentials (E_{pc} and E_{pa}), peak currents (i_{pc} and i_{pa}) are the most important parameters to measure in cyclic voltammetry. The reaction is said to be electrochemically reversible if the electron transfer is fast and the peak separation is

$$\Delta E_p = |E_{pa} - E_{pc}| = 2.303RT/nF$$

At 25°C, and after incoperating the other constants R and F, the equation reduces to

$$\Delta E_p = |E_{pa} - E_{pc}| = 0.0592/n$$

and this value is 0.0592 V if only one electron is involved., which is practically difficult to attain because of factors such as slow electron transfer.

The formal reduction potential for a reversible couple is calculated using the equation

$$E^o = (E_{pc} + E_{pa})/2$$

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For a reversible reaction, the voltammogram recorded has certain well defined characteristic(Andrienko, 2008), which are listed below:

1. The voltage separation is approximately 0.0592/n;
2. The voltage scan rates should not change the positions of peak voltages;
3. The ratio of peak currents should be equal to one;
4. The peak currents are currents are proportional to the square root of the scan rate.

Redox properties play an important role in drug discovery, especially in the study of bioactivity profiles. Electrochemical properties can also help to understand how living organisms metabolise the compounds (Masek et al., 2011). Several natural products have been analysed for redox properties for these reasons. A few examples of the studies done will be given in the next few paragraphs.

Flavonoids, natural vegetable dyes are responsible for the colour of blooming plant portions and have biological activity as radical receptors. Electro-oxidation kinetic properties of flavonoids were assessed on cyclic and differential pulse voltammetry (Masek et al., 2011). The flavonoids showed irreversible oxidation at the platinum electrode and some oxidation of the hydroxyl groups was clearly demonstrated.

Studies on marine natural products belonging to the pseudopterosin group showed that the molecules are electro-active (Zhong and Little, 2009). The studies elucidated on the reaction mechanisms.

A group of drugs called calcium antagonists have a wide range of functions besides the normal blockage of calcium from entering into cells. These drugs were studied using cyclic voltammetry and they found the reactivity of the reduction products with biological targets and that certain conditions, such as pH, can affect the reactions in voltammetric techniques (Núñez-Vergara et al., 1996).

Carotenoids, pigments synthesised in nature by plants, were investigated for their electro-analytical properties by cyclic voltammetry and square wave voltammetry (Liu et al., 2000). This research gave an insight into the reactions that carotenoids undergo, focussing on redox reactions. The information that was found from this study showed the number of oxidation states and their stability together with the rate constants of the reactions.

Some studies were also conducted on the synthesised molecules, for example, ferrocene derivatives were synthesised and studied by electrochemical studies (Damljanović et al., 2009). In this study, where all the three aldehydes exhibited one electron redox couple at

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almost the same potential, it was shown that the ferrocenes met the requirements of reversible reactions.

The stability of the generated radicals, their redox properties and ability to oxidise substrates is important in the assessment of laccase-mediator systems (González Arzola et al., 2009). The experiments conducted allowed the researchers to clearly distinguish between mediators and enhancers and also the catalytic efficiency in the oxidation of natural compounds.

The cyclic voltammetry technique has been also used in the veterinary field, where erythrocyte superoxide dismutase from pigs was analysed and found to undergo irreversible reactions on the modified mercury electrode surfaces (Qian et al., 2004). Electrodes were modified to increase the rates of electron transfer and the reversibility of the reactions.

The most important concept is that it has been proved that the redox potentials of the active sites in a compound, affect the antibacterial activity of the compound (Düsman Tonin et al., 2009).

2.8 Freeze drying

There are many different ways of removing solvents from samples but not all of them can be used at any one point. The fastest method of removing solvents is by using heat, such as open air heating on a heating mantle, evaporation under reduced pressure on a rotary evaporator. However these methods are not always desirable. Heating unknown samples can be detrimental to research efforts, as sometimes the sample analysis can be completely destroyed by the high temperatures involved. There are two problems associated with normal evaporation procedures:

(i) It is difficult to remove the solvent completely, and hence a higher temperature such as 105 °C if water is to be removed (unless the pressure has been reduced),

(ii) The heat supplied may change the structure, shape and composition (Tsinontides et al., 2004).

Freeze drying can provide a very useful alternative in this regard. It completely removes the solvent, such as, water, from samples while leaving the basic structure and composition of the sample intact. The process of freeze drying is normally used to remove solvents especially water from samples. Volatile organic solvents can be easily removed by other methods such as blowing nitrogen over the samples. The process works through sublimation, which converts water directly from the solid state to vapour, without going the through the liquid phase. Two factors normally affect sublimation and any other phase transformation process;

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temperature and pressure. In the phase diagram, there are two very interesting points, the critical point and the triple point. Of the two, the triple point is found where the three lines meet and at that temperature and pressure, all the three phases, that is, solid, liquid and vapour, are all found in the system.

In the freeze-drying process, a vacuum is created by the compressor and this lowers the pressure and at the same time a small amount of heat is supplied. This causes the water to move directly from the solid phase to the liquid phase. This is depicted in the graph in Figure 2.5 where it is shown that at sufficiently low pressure and the right temperature, the solvent will move directly from the solid phase to gas (sublimation). Freeze drying takes advantage of this concept (Franks, 1998).

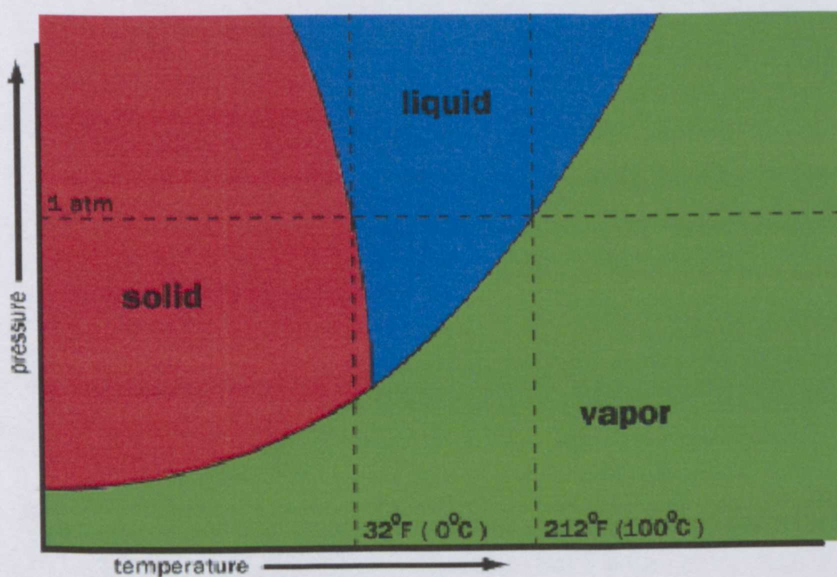

Figure 2.5: Pressure against temperature graph showing different phases

From this graph, it can be seen that, by manipulating the temperature and pressure, the required phase can be obtained. As long as the temperature and pressure are kept below the triple point, the solid/vapour equilibrium is established.

2.9 Microbiological Activity

Biological activity (pharmacological activity), describes the beneficial or adverse effects of a substance on living things (Jackson et al., 2007). However, the activity is only restricted to the beneficial effects of the compounds and is dosage dependent. However in the initial stages of the research, the quantities are not very important. The isolated compounds are then

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taken to microbiology laboratory where the biological activity tests are done to determine which organisms the isolated compounds can kill.

The ones that generate interest after the theoretical study of the molecules, can then be synthesised in the laboratory. The synthesis routes can also be aided by computational studies. Synthesis of the molecules of interest can then commence and biological activity studies can be used to confirm the initial findings. If positive, the results can then be used for laboratory synthesis of compounds that were initially only identified in plants.

Other studies must also be carried out, that is, bioavailability, adverse effects, metabolic half-life to mention just a few.

Natural products have been shown to exhibit microbiological activity against several microorganisms (Mann and Markham, 1998). In Limpopo, medicinal plants were used to show activity against diarrhoea and as many as twenty plants proved to be effective (Mathabe et al., 2006). Antibacterial, antifungal, antiviral and antiprotozoal activity in medicinal plants was also extensively studied (Sher, 2009).

Many other studies including drugs that are anti-diabetic (Chadwick et al., 2007), antibacterial (Ajay Kumar et al., 2002, Erasto et al., 2006, Mahesh and Satish, 2008, Masoodi et al., 2008, Smyth et al., 2009), effective against erythromycin resistant bacterial strains (Kreander et al., 2005), anti-cyanobacterial (Lürling and Beekman, 2010, Yang et al., 2009), or antioxidant (Fernandes et al., 2004), show the importance of plant extracts on the activity against several microorganisms.

2.9.1 Minimum Inhibitory Concentration (MIC)

In biological activity studies, the effectiveness of a molecule is quoted in terms of the MIC, which is the lowest concentration of that particular molecule capable of stopping the growth of a particular organism (Mann and Markham, 1998). This procedure is performed through serial dilutions until the lowest effective concentration is found. This measurement is very important as it is loosely considered to be a standard for the determination of susceptibility of organisms to antimicrobials (Andrews, 2001). The MIC is further used to confirm border line cases of microbial activity, as the lowest concentration that can stop the growth of microorganisms is determined. The microtitre plate method is now preferred to the other methods. However the traditional method is still being used.

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2.9.2 *Escherichia coli* (*E. coli*)

E. coli belong to a group of bacteria that are normally found in the intestines of mammals where they help in digestion. They are classified under the Gram negative, gamma proteobacteria (Doyle et al., 2001). They are straight, round ended bacilli and about 2 µm in length (Singleton, 1999).

They are not always harmful. However, some strains of the bacteria can be harmful. These are spread through contamination of food or water, especially if it contaminated with faeces of animals or human beings. Further, any food that comes into contact with the contaminated food, also become contaminated. This is made worse by poor sanitation practices.

The most common diseases that is caused by *E. coli* are gastroenteritis, dysentery, haemolytic uremic syndrome, urinary tract infection, septicaemia, pneumonia and meningitis (Bhunia, 2008). Most humans affected by *E. coli* normally do not take any medication as they recover unaided by drugs because the diarrhoea can be stopped by rehydration therapy. Drugs like bismuth subsalicylate, atropine and diphenoxylate are sometimes used in acute cases, however this may increase symptoms and trigger complications such as digestive system slow down which makes it more difficult for the body to get rid of the toxins (Singleton, 1999).

2.9.3 *Staphylococcus aureus*

The *Staphylococcus* genus are small, spherical, Gram positive bacteria, about 1µm in diameter, nonmotile and often found in grape like clusters. *Staphylococcus aureus* is the most important species in the *Staphylococcus* genus and they are normally found naturally on the skin of humans and animals (Bhunia, 2008).

Staphylococci normally affect humans through food poisoning as they produce toxins and usually cause vomiting and diarrhoea (Singleton, 1999) and it is the most common cause of gastroenteritis throughout the whole world (Doyle et al., 2001). Their effects are far more pronounced in immunodeficient people. Staphylococci cause skin infections such as boils, carbuncle and furuncle, headaches, cramps, severe vomiting and diarrhoea (Bhunia, 2008). Staphylococcal food poisoning is often self-limiting and can be reversed by rehydration. The following drugs can also be used; penicillins, clindamycin, quinolone and cotrimoxazole (Tortora et al., 1995).

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2.9.4 *Shigella*

Shigella are Gram-negative, nonsporulating rod, anaerobic and nonmotile.. The genus contains four species, of which the *Shigella dysenteriae* is the one known to cause deadly diseases, especially dysentery, whose symptoms are anorexia, fever, colitis, mucopurulet bloody stools, abdominal cramps and tenesmus (Doyle et al., 2001). Shigellae are commonly found in water and food contaminated with human faeces and mainly affect children under 5 year old (Bhunia, 2008). They can survive for days under harsh physical and chemical conditions, such as refrigeration, freezing, 5 % NaCl solution and even at pH 4.5 (Singleton, 1999), however they can be killed by heating and pasteurization.

Use of very high sanitary standards can help to prevent food and water contamination. Rehydration therapy with electrolyte containing fluids are administered to help reduce the effects of *Shigella* infection. Antibiotic treatment is not preferred as *Shigella* develops resistance against antibiotics. However , for severe cases, rehydration alongside antibiotics such as ampicillin, trimethoprim/sulphamethoxazole, nalidixic acid or ciprofloxacin can be administered. Vaccination also can be undertaken to alleviate the symptoms.

2.9.5 *Aeromonas hydrophilia*

The genus *Aeromonas* is made up of Gram negative, facultative anaerobic, glucose fermenting, mortile rod shaped bacteria (Doyle et al., 2001). This is a big genus, however generally the name *Aeromonas hydrophilia* has been used generally for the whole group.

The most common source of *Aeromonas* infection is contaminated water, especially drinking untreated water, which causes gastroenteritis (Dart, 1996). It is also common to catch the bacteria through open wounds when they come into contact with contaminated water, hence it has been dubbed the "flesh eating bacteria" as it causes severe problems especially in limbs. In some rare instances, infections from food contamination have been recorded. They produce toxins, some which are heat resistant and can induce fluid accumulation in animal intestines (Doyle et al., 2001).

Symptoms of infection range from mild where there is fever and chills to extreme where abdominal pains, nausea, vomiting, and diarrhoea are experienced (Singleton, 1999).

For diarrhoea infection, usually treatment is not required. However for the other conditions, antibiotic treatment has been successfully used, with antibiotics such as gentamycin, penicllin, ciprofloxacin amoxicillin/clavulanate being used.

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2.9.6 *Salmonella*

These are Gram-negative, rod shaped facultatively anaerobic bacteria that belong to the *Enterobacteriaceae. Salmonella* has been singled out as the leading cause of foodborne bacterial illness and the problem of salmonellosis is increasing worldwide (Doyle et al., 2001).

Salmonella is present in the intestinal tracts of poultry, animals and humans and it can easily be spread through poultry.

Salmonella infections can cause typhoid fever and uncomplicated enterocolitis whose sympotoms may include diarrhoea, prolonged and spiking fever, abdominal pains, headaches and prostration.

The infections can be treated by chloramphenicol, ampicillin and sulphamethoxazole/trimethoprim. However drug resistance has been reported (Doyle et al., 2001).

2.9.7 *Proteus mirabilis*

Proteus mirabilis are Gram-negative, facultatively anaerobic and rod shaped bacteria. They produce high levels of urease, which is responsible for the production of kidney stones through the hydrolysis of urea to ammonia, catalysed by urease. In that process the alkaline conditions (ammonia) lead to the stone formation. This may lead to renal failure (Singleton, 1999).

Drugs such as ceftriaxone and cefotaxime have been used for the treatment of *Proteus mirabilis* infections.

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Chapter three: Methodology

3.1 Reagents, Standards and Glassware

Before use all the glassware and plastic containers were thoroughly cleaned as follows:

All the glassware and plastic containers were soaked in chromic acid cleaning solution overnight, followed by washing and rinsing with de- ionised water.

To eliminate the possibilities of contamination, the clean glassware and plastic containers were then rinsed with dilute nitric acid before thoroughly rinsing with de ionised water again. The glassware and plastic containers at this stage were now ready for use.

Only ultra-pure de-ionised water was used for all dilutions. Ultrapure water was obtained from the Milli-Q Gradient Millipore deioniser that produced ultra-pure water with an electrical conductivity of 0.054 µS.

For the chromatographic analysis, the solvents were Omni Solv HPLC grade and were purchased from Sigma-Aldrich.

3.2 Samples

Roots of *Kirkia wilmsii* were collected in Lebowakgomo in Polokwane. The pictures of the collected roots are shown in Figure 3.1 below:

Figure 3.1: Picture of fresh *Kirkia wilmsii* bulbs

Some samples were extracted immediately upon arrival at the laboratory when the samples were still fresh. The other samples which were not needed immediately were frozen. The hard bark of the samples were removed before extraction.

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3.3 Sample Extraction

Only the bulbs of *Kirkia wilmsii* were used in the study. The bark was removed from the roots as traditionally the indigenous people remove the bark from the tubers before eating them. The peeled roots were first cut into small pieces using an ordinary kitchen knife and a small amount of water was added to the cut pieces. This mixture was transferred into a heavy duty blender which produced a fine paste from the mixture. More water was added so that the level of the water was above that of the meshed roots. This was left to stand in the refrigerator (4 °C) for at least 48 hours after which it was assumed that extraction was complete. Only cold deionised water was used and extractions were performed at room temperature.

The extracts were filtered through a 0.45 µm filter paper to remove the residues. Only the extracted supernatant liquid was used in the tests. The unused extracts were returned to the refrigerator (4 °C).

To concentrate the sample before separation on the HPLC, the solvent was removed from the supernatant liquid by the use of a Vacutex Flexi-Dry µp freeze dryer. This process reduced the volume of the liquid and significantly reduced the concentration of the extracts.

3.4 HPLC Analysis

A Waters HPLC 2535 with a quaternary Gradient Elution Module, Water 2707 Autosampler, Waters Fraction Collector III, Photo Diode Array (PDA) Detector and a Waters 2707 Autosampler was used for the separation of the components of the extracts.

The picture of the HPLC is shown in Figure 3.2:

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Figure 3.2: Picture of the Waters HPLC

A procedure was developed for the analytical mode of the HPLC. In this mode normal analysis takes place. After optimisation, the procedure was then adapted to the HPLC preparative method. The preparative method was then used to collect the different fractions of the plant extract.

The best method was found from the following the optimisation of the various methods that were developed. A gradient elution system was used. The method consists of two mobile phases: Mobile phase A; 0.5 %v/v trifluoroacetic acid (TFA) in water whereas mobile phase B was 0.25 %v/v TFA in acetonitrile.

The PDA was set to run from 200 to 800 nm and the total run time was 60 mins.

The first trial injection was done with an isocratic system with the eluent made up of 95 % mobile phase A and 5 % mobile phase B and run for 60 mins at 0.5 ml/min. The results were not good and gradient elution programmes were tried and the best of which was the one shown on in Table 3. Subsequently the gradient system was performed according Table 3.1;

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Table 3.1: The initial gradient programme used on the HPLC

Time (min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	2.0	95	5	0	0
20.00	2.0	5	95	0	0
28.00	2.0	5	95	0	0
28.10	2.0	95	5	0	0
40.00	2.0	95	5	0	0
55.00	2.0	95	5	0	0
60.00	2.0	95	5	0	0

The analytical column used for this stage was Waters BEH300, C18, 5 µm , 4.6 x 2500 mm.
The resolution was not so good, thus the flow rate was reduced to 0.5 ml/min.
The gradient elution programme was adapted from the analytical mode to the preparative mode using the prep calculator. The syringes, columns and sample vials are different in the analytical and preparative mode of the HPLC. In the preparative mode, all these accessories are much larger and hence take more volumes of samples and eluents. A prep calculator gives the optimum conditions that must be used when moving from the analytical to the preparative mode and vice versa.
The conditions obtained from the prep calculator where then used for the analysis. The following prep method was used and the gradient elution is shown in Table 3.2.
The mobile phases were not changed, that is, mobile phase A: 0.5 %v/v TFA in water whereas mobile phase B was 0.25 %v/v TFA in acetonitrile.
The chromatographic system consisted of Waters BEH300 19 x 150 mm 5 µm Prep C18 column, PDA detector set to scan from 200 to 400 nm and the run time was 36 mins;

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Table 3.2: Improved gradient elution programme

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	8.53	95	5	0	0
12.00	8.53	5	95	0	0
16.80	8.53	5	95	0	0
16.86	8.53	95	5	0	0
24.00	8.53	95	5	0	0
26.40	8.53	95	5	0	0
36.00	8,53	95	5	0	0

The chromatograms that were obtained from this adapted method were not satisfactory and the method optimisation was repeated.

The method was then further optimised to give the following conditions, that were then used for the final separation of the components, followed by collection .

This method consisted of the same two mobile phases, mobile phase A: 0.5 %v/v TFA in deionised water whereas mobile phase B was 0.25 %v/v TFA in acetonitrile.

The gradient system was adjusted according to Table 3.3;

Table 3.3: Further improved gradient elution programme

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	4	95	5	0	0
24.0	4	5	95	0	0
28.0	4	5	95	0	0
32.0	4	95	5	0	0
32.1	4	95	5	0	0
48.0	4	95	5	0	0
60.0	4	95	5	0	0

Subsequently satisfactory chromatograms were obtained and an example is shown below. From the above mentioned method and chromatograms, the fraction collection parameters were set.

The gradient elution system was then adjusted to the conditions shown in Table 3.4;

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Table 3.4: Continuously improved gradient elution method

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	2	95	5	0	0
30.00	2	95	5	0	0
31.00	2	40	60	0	0
48.00	2	40	60	0	0
50.00	2	40	60	0	0
51.00	8	95	5	0	0
52.00	4	95	5	0	0
64.00	4	95	5	0	0
65.00	0.2	95	5	0	0

Mobile phases A and B remained the same, the PDA scanned from 200 to 400 nm and the total run time was increased to 65 minutes.

The obtained chromatograms showed better resolution, as can be seen in Figure 4.4 in the results section.

The spectra gave the average value of the wavelength to be considered, as the different components absorb at different wavelengths.

There were some unresolved peaks at the end and the chromatograms showed that the run time had to be increased and hence the method was also further developed to the most satisfactory chromatograms that could be obtained. First, it was clear that the run time had to be increased as there were some peaks that would appear to be coming from the previous injection which were overlapping with the subsequent injection. Secondly, although the chromatogram was generally much improved, some of the peaks were not completely resolved. Using the conditions shown in Table 3.5 below, the residual overlap was eliminated and the peak resolution was greatly improved as well. The method was now finalised and the fraction was now to be conducted. Subsequently, fraction collection parameters were set as is shown in Table 3.6.

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Table 3.5: Final optimised gradient elution method

Time(min)	Flow(ml/min)	PumpA (%)	Pump B (%)	Pump C (%)	Pump D (%)
Initial	2.0	95	5	0	0
48.00	2.0	70	30	0	0
50.00	2.0	5	95	0	0
54.00	2.0	5	95	0	0
55.00	2.0	95	5	0	0
70.00	2.0	95	5	0	0
71.00	2.0	5	95	0	0
75.00	4.0	5	95	0	0
88.00	4.0	5	95	0	0
89.00	2.0	5	95	0	0
90.00	4.0	0	0	50	50
110.00	4.0	0	0	50	50
110.01	2.0	95	5	0	0
129.00	2.0	95	5	0	0
130.00	0.2	95	5	0	0

Table 3.6: Fraction collection parameters

Start Time(min)	End time(min)	% Collection
15.00	17.0	80
19.00	22.00	80
27.00	29.00	80
29.20	33.00	80
39.00	42.00	80
49.00	52.00	80
54.00	58.00	80
63.00	65.00	80

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The peaks at 50 and 58 mins were not collected, as they were not well resolved.

After the final optimisation of the HPLC method, the plant extract was concentrated before injection into the HPLC. The concentration was done by evaporation of the solvent on the freeze dryer. This step was done to increase the yield of the collected fractions from the collector. To further enhance the yields, the fractions were collected several times as the amount of injected sample was only 350 µl.

The separated components were all collected into different containers and the solvent was removed using the Vacutex Flexi-Dry µp freeze dryer.

The tare mass of the freeze drying tubes were taken before the samples were transferred into them. The mass of the freeze drying tubes and the dried samples were also recorded so that the difference between these two masses gave the yield for each component obtained.

These solutions were taken for further investigations in the microbiology laboratory where the antimicrobial activity and MIC tests were conducted.

3.5 Voltammetry

Tests for antioxidant properties and cyclic voltammetric properties were both conducted using the Metrohm 757 VA Computrace coupled with a 863 Compact autosampler, 843 pump station and 800 Metrohm Dosino system .

For both the antioxidant activity and the cyclic voltametric experiments, the samples were dissolved in 4ml of deionised water and the resultant concentrations are indicated below. 1ml of each component was added to the reaction vessel in both instances when each run was performed.

3.5.1 Antioxidant properties

Antioxidant and cyclic voltammetric properties were both tested using the Metrohm 757 VA Computrace, coupled with a 863 Compact autosampler, 843 pump station and 800 Metrohm Dosino system*.

The samples were dissolved in water to give various concentrations. The solutions were filtered and used without any further preparation.

Component One	:	17.23 mg/ml
Component Two	:	16.03 mg/ml
Component Three	:	1.55 mg/ml

*Supplied by Metrohm South Africa, 1 Bridle Close, Woodmead Office Park, Van Reenen Avenue Woodmead, Sandton, South Africa

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Component Four	:	1.20 mg/ml
Component Five	:	0.40 mg/ml
Component Six	:	2.23 mg/ml
Component Seven	:	1.83 mg/ml
Component Eight	:	0.78 mg/ml

The conditions that were used in this part of the research were:

Electrode	:	Hanging Mercury Drop Electrode (HMDE)
Drop size	:	4
Stirrer speed (rpm)	:	2000
Initial purge time (sec)	:	0
Deposition time (sec)	:	90
Equilibration time (sec)	:	5
Start potential (mV)	:	0.3
End potential (mV)	:	-1.7
Pulse amplitude (mV)	:	0.050
Voltage step (mV)	:	0.005
Voltage step time	:	1.0
Sweep rate (mV/sec)	:	0.005

To get the oxygen curve, 20 ml of 0.1M potassium nitrate was added to the reaction vessel and was not degassed. The run was done to record the oxygen curve. The solution was then purged for 300 sec to remove all the oxygen in the reaction vessel. The resultant solution was then subjected to the same potential. The reaction vessel was then emptied and both the vessel and the electrodes were thoroughly cleaned with deionised water.

To determine the antioxidant activity of the extracts,

1. 20.0 ml of potassium nitrate was added to the reaction vessel
2. 1.0 ml of extract 1 was added.
3. The solution was then stirred without purging for 300 seconds.
4. After that, the experiment was run and the voltammogram was obtained.
5. The reaction vessel was emptied and both vessel and electrodes were thoroughly cleaned again.

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6. The procedure was repeated for all the other extracts in exactly the same way and the voltammograms were also recorded.

3.5.2 Cyclic voltammetry

Ultrapure water and 3M KCl were used in all the investigations. The conductivity of the water was 0.054 µS. The 3 M KCl was prepared by dissolving 223.64 g of KCl in 1000 ml of ultrapure water.

The samples were dissolved in water to give various concentrations. The solutions were filtered and used without any further preparation.

Component One : 17.23 mg/ml
Component Two : 16.03 mg/ml
Component Three : 1.55 mg/ml
Component Four : 1.20 mg/ml
Component Five : 0.40 mg/ml
Component Six : 2.23 mg/ml
Component Seven : 1.83 mg/ml
Component Eight : 0.78 mg/ml

The conditions that were used in this part of the research were:

Electrode : Hanging Mercury Drop Electrode (HMDE)
Drop size : 6
Stirrer speed (rpm) : 2000
Initial purge time (sec) : 300
Deposition time (sec) : 90
Equilibration time (sec) : 5
Start potential (mV) : -200
First vertex potential (mV) : 800
Second vertex potential(mV) : -200
Voltage step(mV) : 0.006
Sweep rate (mV/sec) : 0.100

10 ml of ultrapure deionised water was added to the reaction cell followed by 2 ml of 3 M KCl and 1 ml of the sample (each component individually).

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The contents of the reaction cell were purged with nitrogen gas for five minutes. Equilibration was allowed to take place for five seconds and then the reaction was allowed to continue with the above-mentioned conditions.
The voltammograms were recorded and the current and potentials were calculated.

3.6 Gas chromatography

The GC analysis was conducted to check the purity of the separated components from the HPLC. Each component was run individually, to confirm whether complete separation had been performed. Further, GC methods used were then used to develop the method that was finally used on the GC-MS. The GC model was the Perkin Elmer Clarus 500 GC.
For all the components, the samples were dissolved in methanol and the following method was used to run the GC program.

Column : RTX-5MS
Column length : 30 m
Column diameter : 3 µm
Splitter : 20:1
Flow rate : 1 ml/min
Hydrogen flow rate : 45 ml/min
Air flow rate : 450 ml/min
Detection : Both ECD and FID
Injector temperature : 150 °C
Run time (min) : 32 mins

The temperature gradient programme was run as shown in Table 3.7 below:

Table 3.7: GC running conditions:

Oven ramp	Rate(°/min)	Temperature(°C)	Hold(min)
Initial	10	110	2
1	10	150	2
2	10	200	2
3	10	250	3

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3.7 Gas chromatography-Mass Spectrometry

The GC method used above was adapted for use in the GC-MS method. The GC column used is RTX-5MS which has the dimensions, 30 m length x 0.32 mm. The GC-MS model was the Perkin Elmer Clarus 580 GC coupled to a Clarus SQ8S MS.

After minor adjustments, all the components were analysed using the following conditions (see Table 3.8).

Table 3.8: GC-MS running conditions:

Oven ramp	Rate(°/min)	Temperature(°C)	Hold(min)
Initial	10	110	4
1	10	150	4
2	10	200	4
3	10	250	10

Solvent delay: 0.0 minutes

Column	:	Elite 1 column
Column length	:	30.0 m
Column diameter	:	320 µm
Split	:	5:1
Flow rate	:	1 ml/min
Helium flow rate	:	2 ml/min
Injector temperature	:	300 °C
Transfer temperature	:	200 °C
Source temperature	:	200 °C
Scan range	:	30 to 450 Da
Run time (min)	:	36 mins

3.8 Antimicrobial Activity

The extracts were tested for biological activity before the separation of their components. The extracts that gave positive results in the biological activity tests were further used in the study.

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The extracts that tested positive in the biological activity test were then taken to the HPLC where an analytical method for the separation of the components of each extract was developed. This involved the development of a new analytical HPLC method that was able to separate the several components of the extracts.

After the separation of the peaks, fraction collection was then conducted. Each fraction was again tested for microbiological activity. The peaks that were not active were discarded whereas the peaks that gave positive results were then characterised using the GC-MS and NMR.

The following part of the project was carried out in the Microbiological Laboratory, at the University of Venda.

3.8.1 Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations were determined using the method detailed below, which was adapted from (O'Donnell et al., 2010):

1. 13 g of nutrient broth was dissolved in water and diluted to 1000 ml with ultrapure deionised water.
2. The broth and all the other equipment were autoclaved for one hour at 121 °C, the pressure was allowed to drop and the autoclave was opened and the autoclaved broth and the equipment were stored in a sterile chamber.
3. 180 µl of nutrient broth was added to all the wells of the three microplates.
4. 20 µl of each separated component was added to the first 8 wells of row A and E of the first microplate (that is, row A for Component One and row E for Component Two).
5. Similarly, the second and third microplates were filled with components three, four, five and six as follows: the second microplate was filled with 20 µl of component three in the first 8 wells of row A and 20 µl of component four on row E while the third microplate was filled with 20 µl of component five in the first 8 wells of row A and 20 µl of component six in the first 8 wells of row E.
6. The contents of rows A and E of each microplate were thoroughly mixed.

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7. Four serial dilutions were done for each component as follows: 100 µl was taken from each well of row A and row E and added to row B and row F respectively. This was done successively up to row D and row H respectively.
8. 100µl of organisms were added to the microplates with different organisms occupying different columns from 1 to 8.
9. 20µl of the positive control was added to column 9 of all the plates and 100 µl of the organisms were added to column 9 from row A down to row H.
10. Column 10 was used as the negative control as it contained only the broth and the organisms.
11. 100 µl of media was added to all wells to give the total volume in each well of 400 µl.
12. Columns 11 and 12 contained only the media.
13. The microplates were covered with parafilm and then incubated at 37 °C for 24 hours in an incubator.
14. After 24 hours, iodonitrotetrazolium chloride (INT) was added to all the wells in all the microplates.
15. The results were observed visually. The wells which exhibited a purple pink colour showed that there was microbiological activity going on. The wells were the microorganisms were dead, became colourless or showed lack of colour.

The extracts were screened for antibacterial activity against the following organisms; *Vibrio cholera, Shigella, Enterobacter, Salmonella, Proteus mirabilis, Escherichia coli, Candida albicans* and *Staphylococcus aureus*.

The choice of these organisms was based on availability in the microbiology laboratory and incubation was done in a Labcon incubator at 37 °C for 24 hours.

The positive control was kanamycin with a concentration of 250 mg/1000 ml.

MICs were determined from the above mentioned method and the concentrations of the samples that were dissolved in 2 ml of water.

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Chapter four: Results and discussion

4.1 HPLC Analysis

The first analytical method was developed using the initial chromatographic conditions, in Table 1 on page 34 and using a Waters BEH300 4.6 x 250 mm 5 µm C18 column, the following chromatogram was obtained (Figure 9). The flow was run at a constant value of 0.5 ml/min and the PDA was set to run from 200 to 800 nm and the total run time was 60 minutes. The resultant chromatogram, Figure 4.1 below, did not show good resolution and an improvement was needed. Therefore it was decided that gradient elution was to be employed.

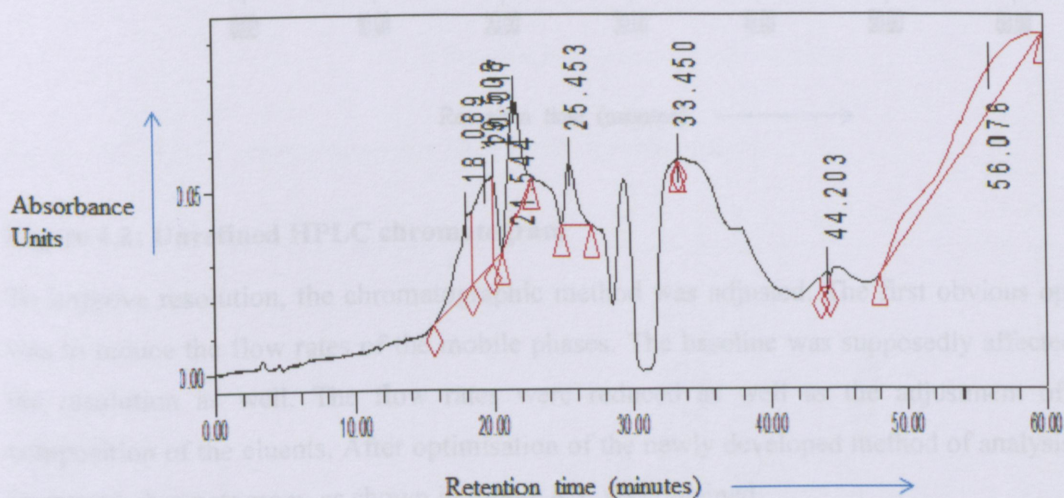

Figure 4.1: Chromatogram from isocratic run showing retention times

The gradient programme was initially chosen, so as to vary the mobile phase from initially high in aqueous (Mobile phase A) and low in organic content (Mobile phase B). The two mobile phases were constituted as: Mobile phase A; 0.5 %v/v trifluoroacetic acid (TFA) in water whereas mobile phase B was 0.25 %v/v TFA in acetonitrile. The content of the mobile phase was slowly changed to high organic and then back to high aqueous.

An analysis of the chromatogram shows that there are several peaks which are very poorly resolved. All the peaks are heaped together within the time spanning from around 26 minutes to about 46 minutes (Figure 4.2). Replicate injections gave the same result, to show that the method was reproducible.

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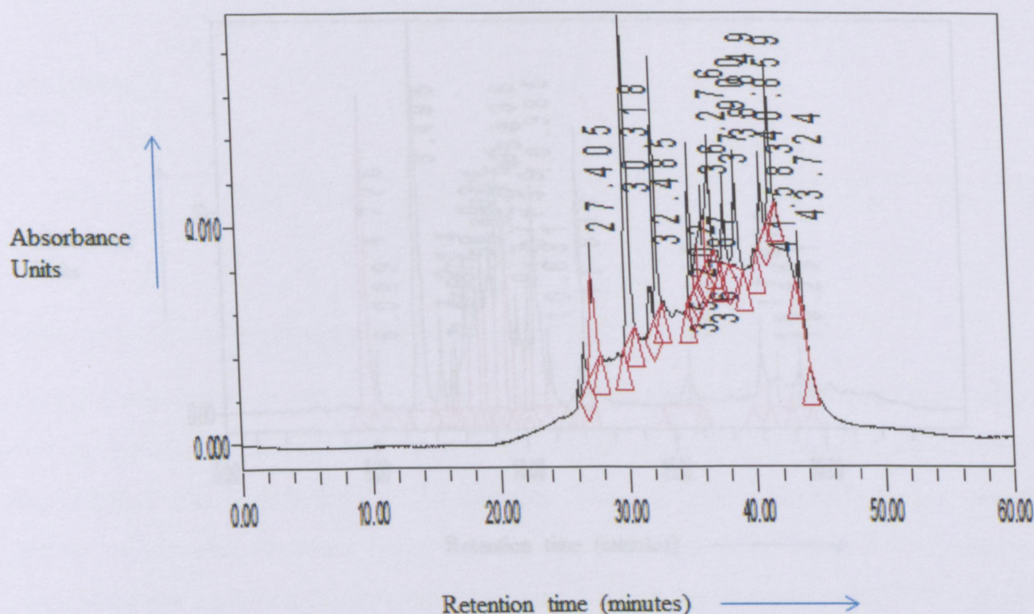

Figure 4.2: Unrefined HPLC chromatogram

To improve resolution, the chromatographic method was adjusted. The first obvious option was to reduce the flow rates of the mobile phases. The baseline was supposedly affected by the resolution as well. The flow rates were reduced as well as the adjustment of the composition of the eluents. After optimisation of the newly developed method of analysis, an improved chromatogram, as shown in Figure 4.3, was obtained.

The corresponding UV spectrum for the first peak at 27.405 minutes is shown below (Figure 4.5). For all the peaks, the UV/Vis spectra are attached in the appendix. From the maxima of each component, the average wavelength (λ) was calculated.

However as can be seen from the chromatogram in Figure 4.4, below, the resolution was not very satisfactory, especially in the time range between 30 and 40 minutes. In this range, most of the peaks were crammed in to a short span of time and there was further need to separate the said peaks so that the resolution was improved. This was done by the development of a further method that was developed from the previous method.

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Figure 4.3: HPLC chromatogram after method development

Figure 4.4: Optimised HPLC chromatogram

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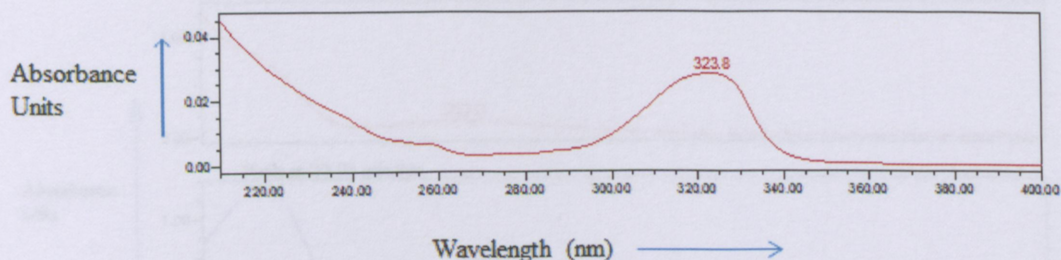

Figure 4.5: UV-VIS absorption spectrum of peak at 27.402 mins

After the improvement of the last used gradient programme, the flow rates were adjusted, as well as the manipulation of the organic and aqueous phases, to achieve the desired results. The method was lengthened to 130 minutes. The run time was much longer, however the desired result was obtained using the conditions outlined in Table 3.5 in chapter 3.The chromatogram obtained is depicted in Figure 4.6 and the corresponding UV-Vis spectra of each component are shown in Figure 4.7, showing the maximum absorption wavelength. The rest of the chromatograms and the corresponding performance tables are attached in Appendix B.

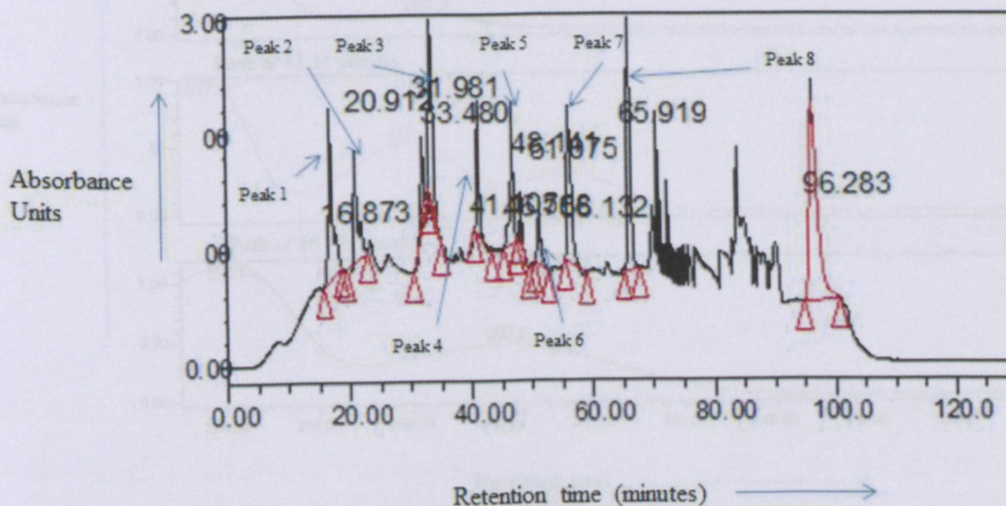

Figure 4.6: Final optimised chromatogram

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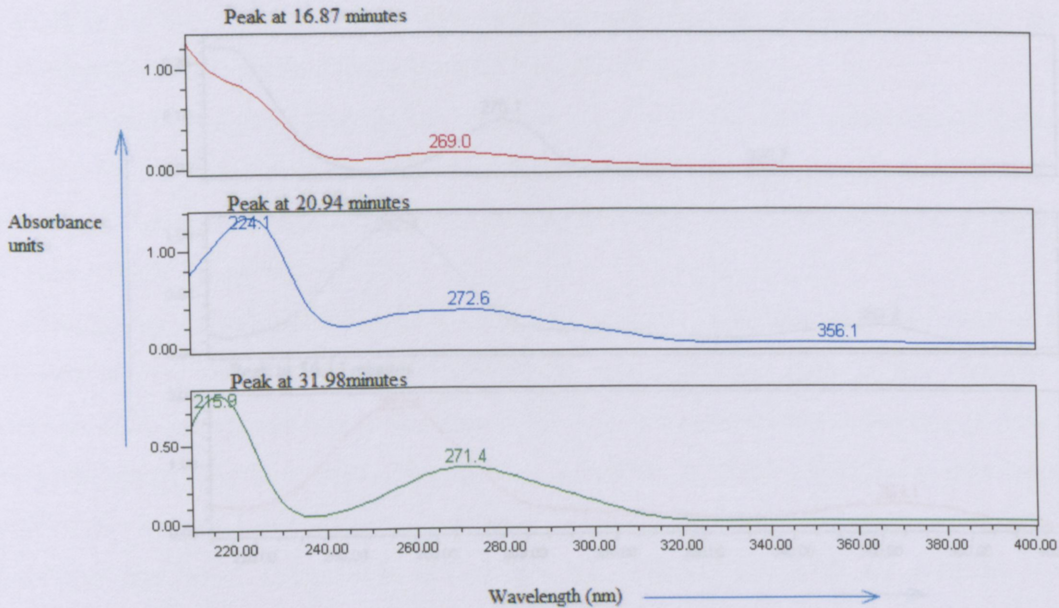

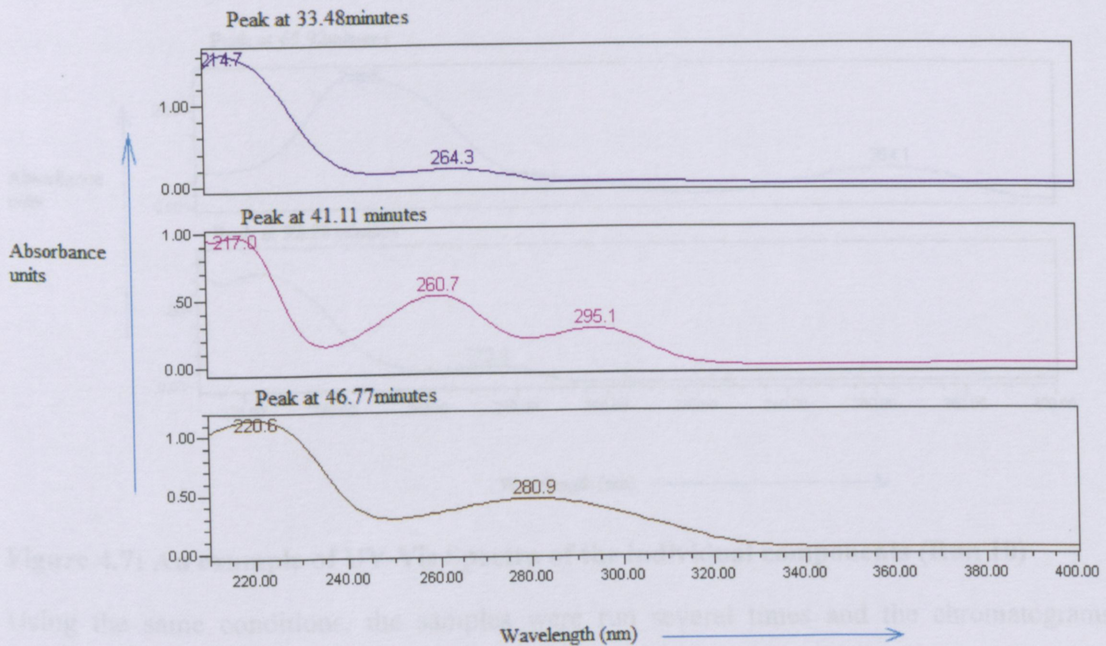

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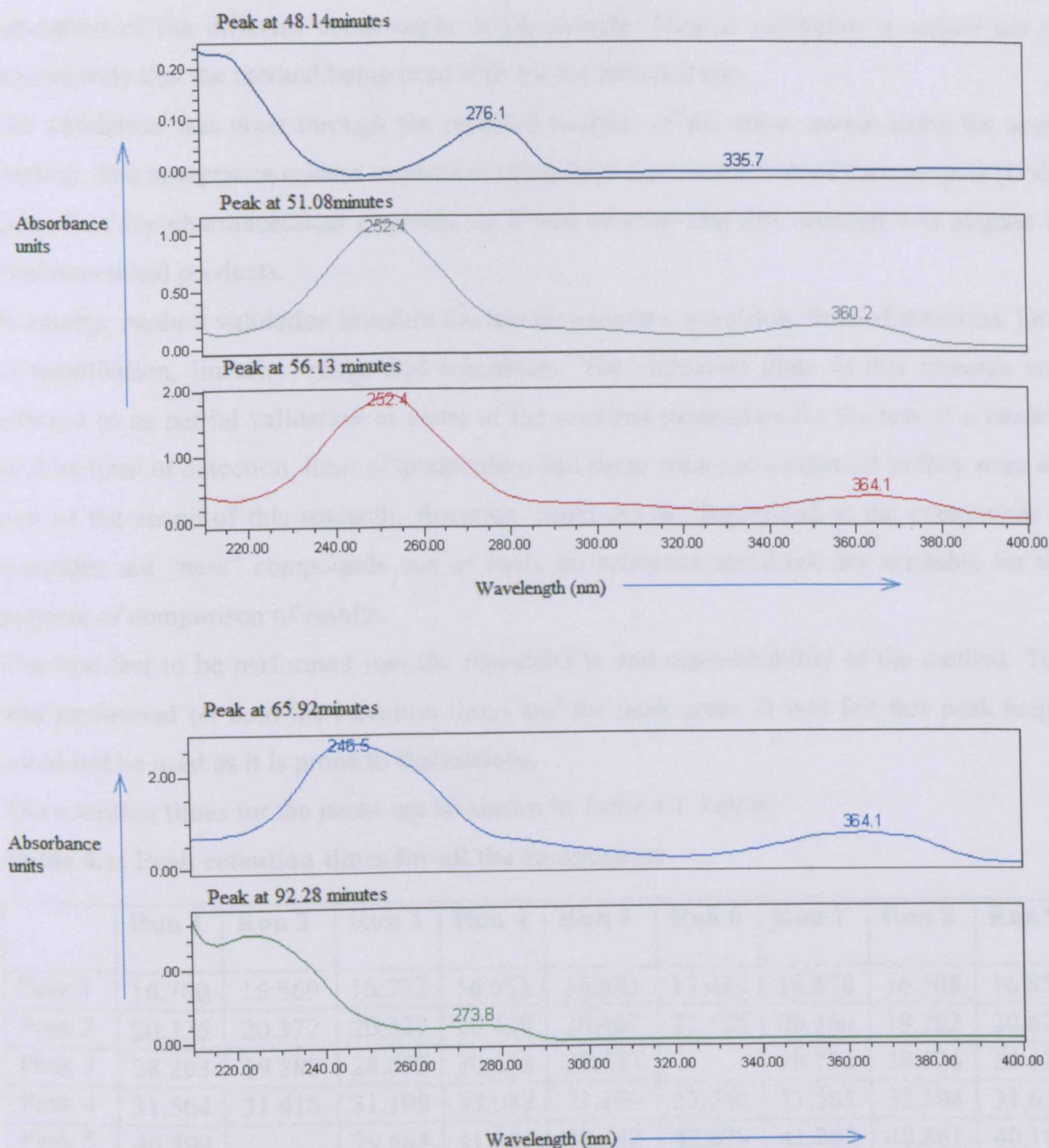

Figure 4.7: An example of UV-Vis Spectra of the individual components (Run 10)

Using the same conditions, the samples were run several times and the chromatograms obtained were reproducible and consistent. A decision was made after analysing the chromatograms that the following peaks would be used in this project; 16.700, 20.325, 28.203, 31.504, 40.399, 50.113, 56.451 and 64.237 mins. These peaks are labelled peak 1, peak 2 up to peak 8 in Chromatogram 1, Appendix B. Therefore those components were collected from the HPLC using the fraction collector and used for further tests. Further, a partial method validation was also performed on the developed method that was used for the

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separation of the different components of the sample. Method validation is carried out to demonstrate that the method being used if fit for the intended use.

The validation was done through the repeated analysis of the same sample using the same method. The acceptance criteria used were taken from the United States Pharmacopeia (USP) guidelines for pharmaceutical products, as it was deemed that this research was aligned to pharmaceutical products.

Normally, method validation involves the test for accuracy, precision, limit of detection, limit of quantitation, linearity, range and robustness. The validation done in this research was referred to as partial validation as some of the required parameters for the test of a method such as limit of detection, limit of quantitation and range were not conducted as they were not part of the scope of this research. Accuracy could not be determined as the compounds in questions are "new" compounds and as such, no reference standards are available for the purpose of comparison of results.

The first test to be performed was the repeatability and reproducibility of the method. This was performed on both the retention times and the peak areas. It was felt that peak height could not be used as it is prone to fluctuations.

The retention times for the peaks are all shown in Table 4.1 below;

Table 4.1: Peak retention times for all the components

	Run 1	**Run 2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**	**Run 7**	**Run 8**	**Run 9**	**Run 10**
Peak 1	16.700	16.569	16.722	16.651	16.593	17.024	16.658	16.508	16.852	16.873
Peak 2	20.325	20.372	20.327	20.429	20.407	21.525	20.360	19.362	20.676	20.912
Peak 3	28.203	29.380	28.852	30.358	28.743		29.732	30.426	29.625	31.981
Peak 4	31.504	31.415	31.199	32.048	31.159	33.744	31.583	32.594	31.630	33.480
Peak 5	40.399		39.863	41.300	40.442	42.679	41.287	40.861	40.186	41.105
Peak 6	50.113	52.269		49.842	49.480	50.290	49.652	50.160	49.027	48.141
Peak 7	56.451	57.162	56.653	55.489	53.764	54.858	53.791	53.204	53.725	51.075
Peak 8	64.237	62.930	63.916	66.911	64.681	65.077	62.470	64.777	64.720	65.919

For each peak, the averages and standard deviations were calculated as follows;

the average = $\Sigma x_i/n$

$$\text{Standard deviation} = \sqrt{\frac{(x_i-\bar{x})^2}{(n-1)}}$$

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x_i is any statistival value or measurement,

n, is the number of measurements,

and $\bar{x}$ is the mean or average

All the averages, standard deviation and relative standard deviations were calculated using the formulae above and the results are shown in Table 4.2 below. For all the peaks, both the retention times and the peak areas were used in the calculations.

Table 4.2: Calculation of average retention times and standard deviation

	Average mins)	Std dev
Peak 1	16.715	0.16
Peak 2	20.470	0.54
Peak 3	29.700	1.13
Peak 4	32.036	0.93
Peak 5	40.902	0.83
Peak 6	49.886	1.12
Peak 7	54.617	1.87
Peak 8	64.564	1.30

According tothe USP guide for method validation, the acceptance criteria for the standard deviation is not more than 2.0. Therefore the repeatability of retention times of the peaks obtained was satisfactory and the method can be used for the analysis of the *Kirkia wilmisii* with a high degree of confidence.

The UV-Vis spectra of all the peaks were also derived from the 3D data of the chromatogram. It can be demonstrated from the maximum wavelength values that all the collected eight components absorb at different wavelengths, although some of the wavelengths are very close to each other (Table 4.3). Thus, after the identification of the structures of the different components, the UV-Vis method can be used as a quick assessment tool in the analysis of *Kirkia wilmisii.*

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Table 4.3: Maximum wavelengths against retention times

	Average mins)	λ_{Max1}(nm)	λ_{Max2}(nm)	λ_{Max13}(nm)
Peak 1	16.715	269.0		
Peak 2	20.470	224.1	272.6	356.1
Peak 3	29.700	215.9	271.4	
Peak 4	32.036	214.7	264.3	
Peak 5	40.902	217.0	260.7	295.1
Peak 6	49.886	252.4	360.2	
Peak 7	54.617	252.4	364.1	
Peak 8	64.564	246.5	364.1	

The retention times were used to draw up a fraction collection programme, as they were shown to be reproducible as demonstrated by the standard deviations.

The maximum injection volume on the HPLC is 350 μL, which is a very small amount of sample. The fraction collector was used for the separation of the components of the sample. Therefore it was necessary to do repeated injection so that the total amount of the samples could be increased. The samples were then collected in different containers and after several collections, the samples shown in Figure 4.8, were collected before the removal of the solvents.

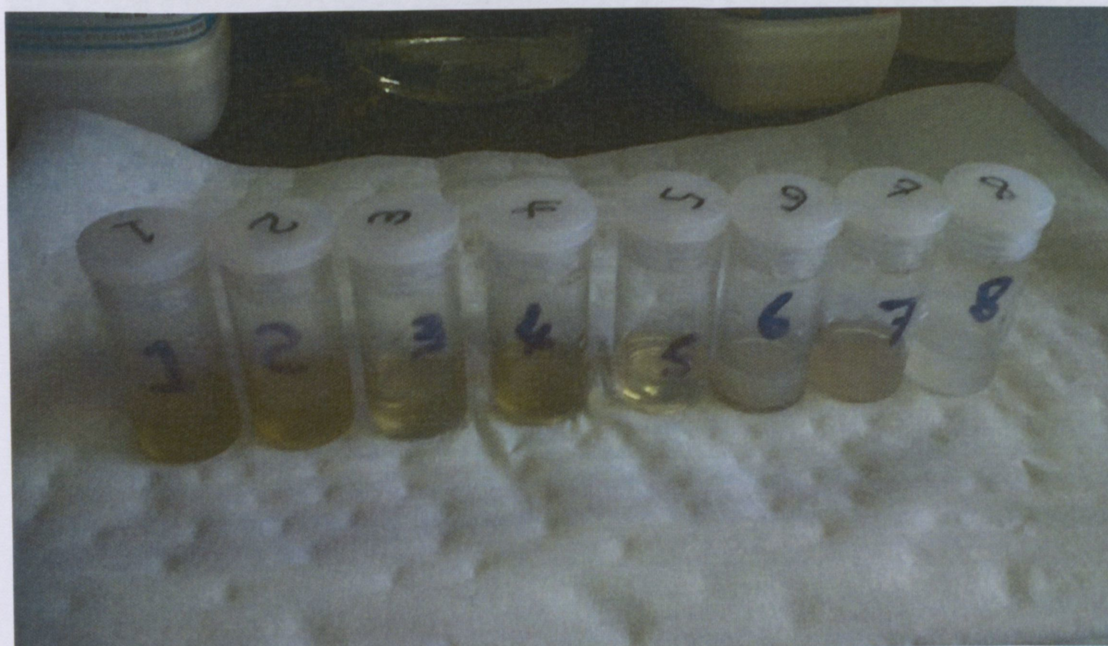

Figure 4.8: Separated components of the *Kirkia wilmsii* (1-8) where each tube represents one peak (See chromatogram 1 in Appendix B).

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Repeated fraction collections were performed, where all the separated components were collected into different containers. Components with the same retention time were then mixed and poured into the freeze drier tube, where the solvent was to be removed. The same was done for all the other components. Extracts were freeze dried. After solvent removal, the samples were clearly different as can be seen in Figure 4.10 below. This was partial proof of the separation of the components.

The solvents were removed from the sample using a freeze drier, Figure 4.9 below, other than using the conventional drying methods such as rotary evaporation. The major reason for this choice is that the chemical composition of the different components was not yet known, therefore it was felt that to maintain the integrity of the samples heating the samples (to remove the solvents) was not the best choice. Instead freeze drying was used as it does not affect the samples, for instance, if the boiling point of some of the components was low.

Figure 4.9: Samples being dried by the freeze drier

After drying, the dried extracts are shown in the freeze drier tubes (Figure 4.10). The tubes containing the samples were weighed. Since the tare mass of the freeze drier tubes had been

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obtained prior to drying, the masses of purified dried extracts were obtained. The masses obtained are shown in Table 4.4.

Figure 4.10: Samples of the dried components from *Kirkia wilmsii* and their component numbers in freeze drier tubes.

Table 4.4: Sample weight for dried components

Component	Empty weight (g)	Sample+container (g)	Sample weight (g)	Sample weight (mg)
1	9.9975	10.0684	0.0709	70.9
2	9.7406	9.8047	0.0541	64.1
3	9.6846	9.6908	0.0062	6.2
4	9.8055	9.8103	0.0048	4.8
5	9.8177	9.8193	0.0016	1.6
6	9.8431	9.8520	0.0089	8.9
7	9.9378	9.9451	0.0073	7.3
8	9.8468	9.8499	0.0031	3.1

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Solubility tests were done on these separated components of *Kirkia wilmsii* and the components dissolve in methanol, ethanol, water and acetone.

The dried extracts were weighed and dissolved in the solvent of choice, to give different concentrations which were used for further tests, such as, dissolving in water for microbial activity tests and the organic solvent for GC and GC-MS analysis.

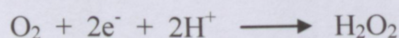
4.2 Voltammetry

In this study, the electrochemical properties of the separated components were done using voltammetry. Two major points of interest were cyclic voltammetry and the antioxidant properties of the components of *Kirkia wilmsii*. For these tests, only those separated components that exhibited antimicrobial activity were used. According to the results, only components 1, 5, 7 and 8 were investigated in this section.

First we look at the results of the antioxidant properties of the separated components. Antioxidant activity is a very important characteristic of food supplements as they have very important health benefits.

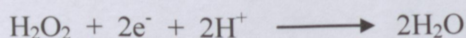
4.2.1 Antioxidant Activity

A 20.0 ml aliquot of 0.1 M KNO_3 was added to the reaction vessel and the system was run without purging the solution.The oxygen waves were obtained without purging and the results are shown in Figure 4.11. There are two clear peaks depicting how the two stages of oxygen reduction on the mercury surface, that is,



$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

and,



$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O$$

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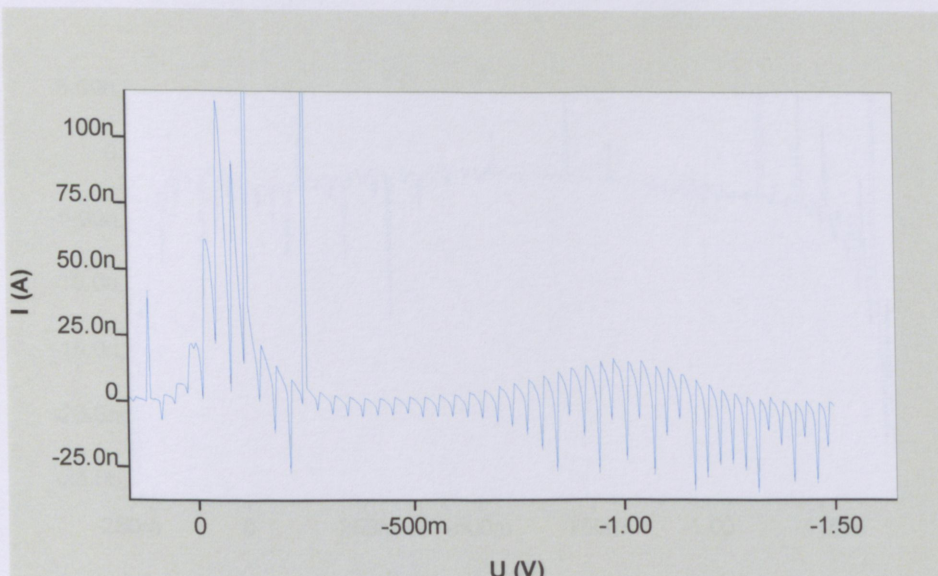

Figure 4.11a: Differential pulses for oxygen, one around 0 V and the other one around -1.0 V.

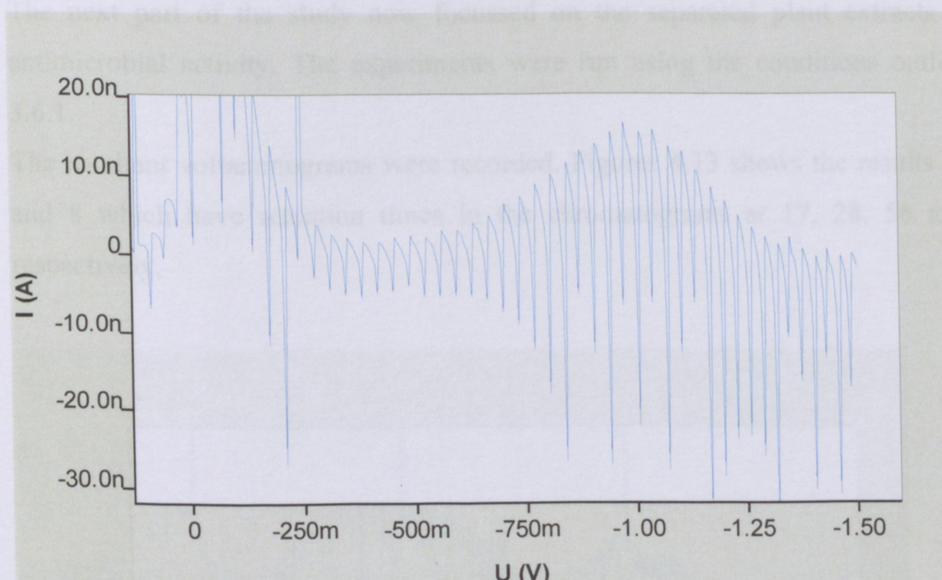

Figure 4.11b: Amplified picture of Figure 4.10a to show the peak at around -1.00 V more clearly.

To demonstrate the effect of oxygen removal, the next stage was run with 20.0 ml of 0.1 M KNO_3 as well. However this time, the oxygen was removed by purging with nitrogen gas for 300 secs. The resultant voltammogram is shown in Figure 4.12, where the oxygen is removed and the curve is more linear.

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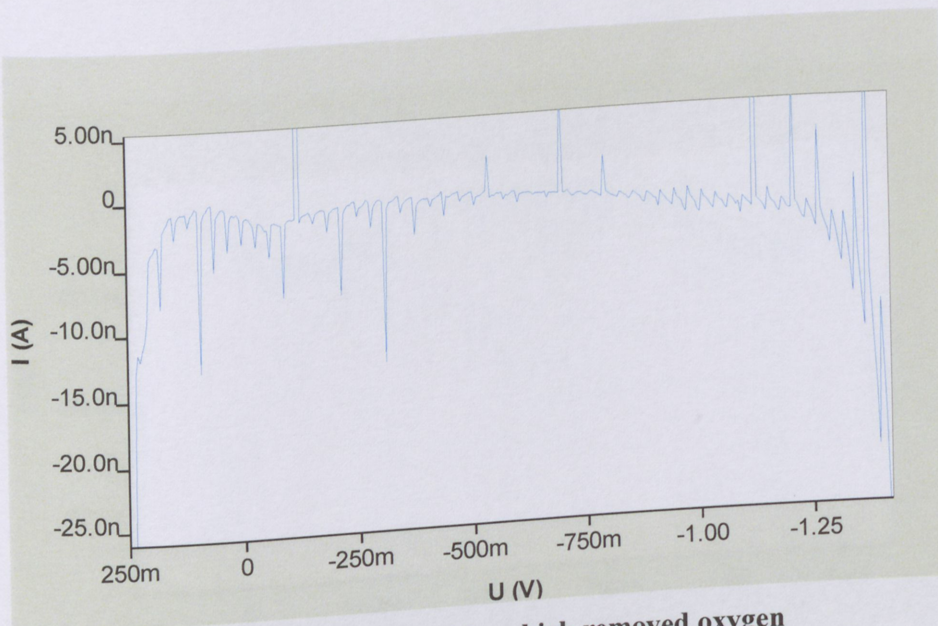

Figure 4.12: Solution purged for 300 s which removed oxygen

The next part of the study now focussed on the separated plant extracts that exhibited antimicrobial activity. The experiments were run using the conditions outlined in section 3.6.1.

The resultant voltammograms were recorded. Figures 4.13 shows the results of peak 1, 5, 7 and 8 which have retention times in the chromatograms at 17, 28, 56 and 64 minutes respectively.

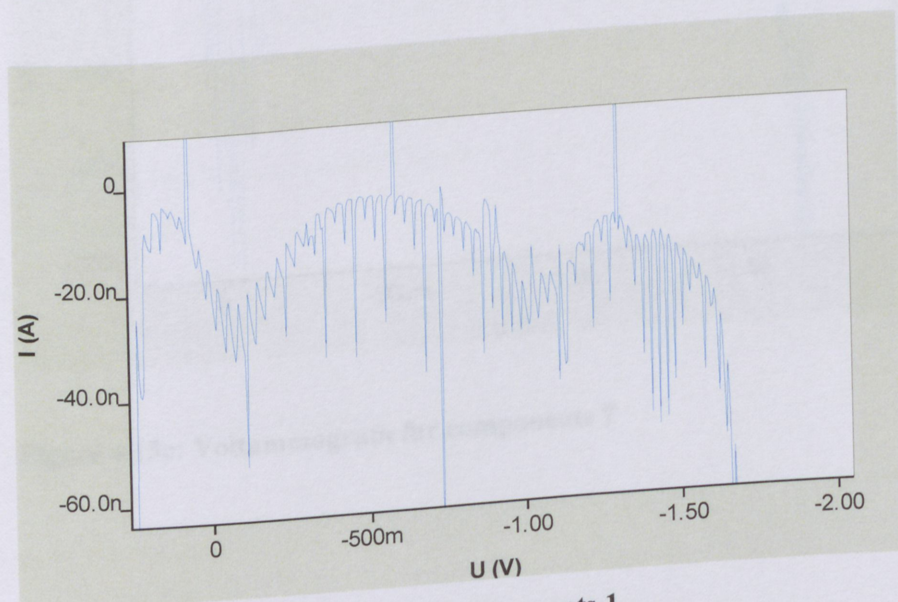

Figure 4.13a: Voltammogram for components 1

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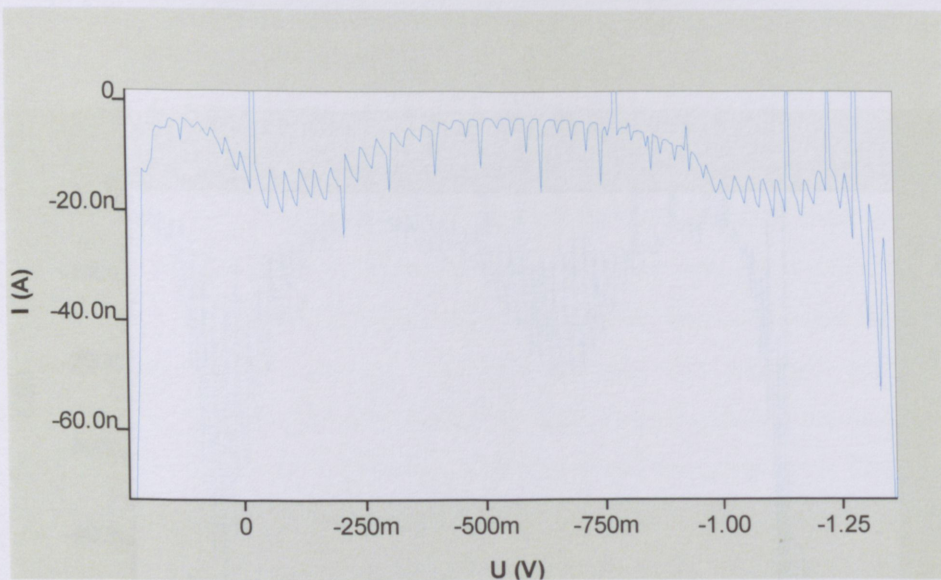

Figure 4.13b: Voltammogram for components 5

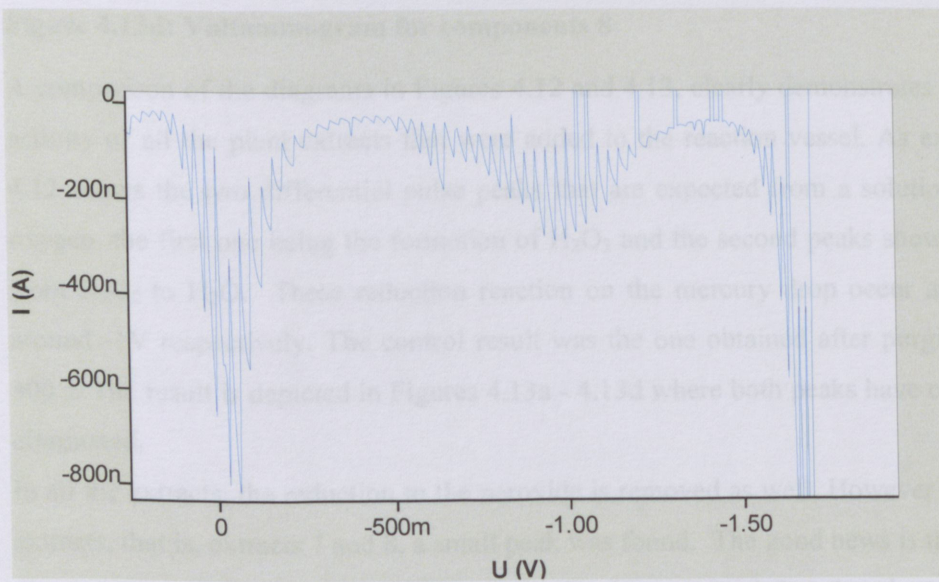

Figure 4.13c: Voltammogram for components 7

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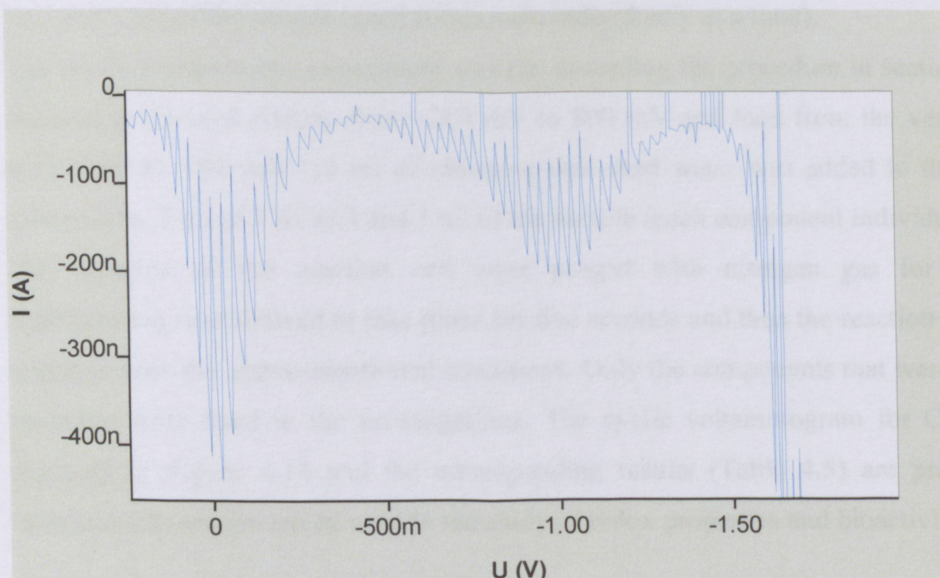

Figure 4.13d: Voltammogram for components 8

A comparison of the diagrams in Figures 4.12 and 4.13, clearly demonstrates the antioxidant activity of all the plant extracts that were added to the reaction vessel. As expected, Figure 4.12 shows the two differential pulse peaks that are expected from a solution that contains oxygen, the first one being the formation of H_2O_2 and the second peaks shows the reduction from H_2O_2 to H_2O. These reduction reaction on the mercury drop occur around 0 V and around -1V respectively. The control result was the one obtained after purging with N_2 for 300 s. The result is depicted in Figures 4.13a - 4.13d where both peaks have completely been eliminated.

In all the extracts, the reduction to the peroxide is removed as well. However for some of the extracts, that is, extracts 7 and 8, a small peak was found. The good news is that the peak had also been greatly reduced. Therefore, it can be demonstrated that the plant extracts can be used for the removal of oxygen.

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4.2.2 Cyclic Voltammetry

10 ml of ultrapure deionised water was added to the reaction cell followed by 2 ml of 3 M KCl and 1 ml of the sample (each component individually at a time).

The cyclic voltammetric experiment was run according the procedure in section 3.5.2. Each run was performed starting from -200 mV to 800 mV and then from the vertex point (800 mV) back to -200 mV. 10 ml of ultrapure deionised water was added to the reaction cell followed by 2 ml of 3 M KCl and 1 ml of the sample (each component individually).

The contents of the reaction cell were purged with nitrogen gas for five minutes. Equilibration was allowed to take place for five seconds and then the reaction was allowed to continue with the above-mentioned conditions. Only the components that were active against microbes were used in the investigations. The cyclic voltammogram for Component 1 is depicted in Figure 4.14 and the corresponding results (Table 4.5) are presented. Cyclic voltammetric results can be used in the study of redox properties and bioactivity profiles.

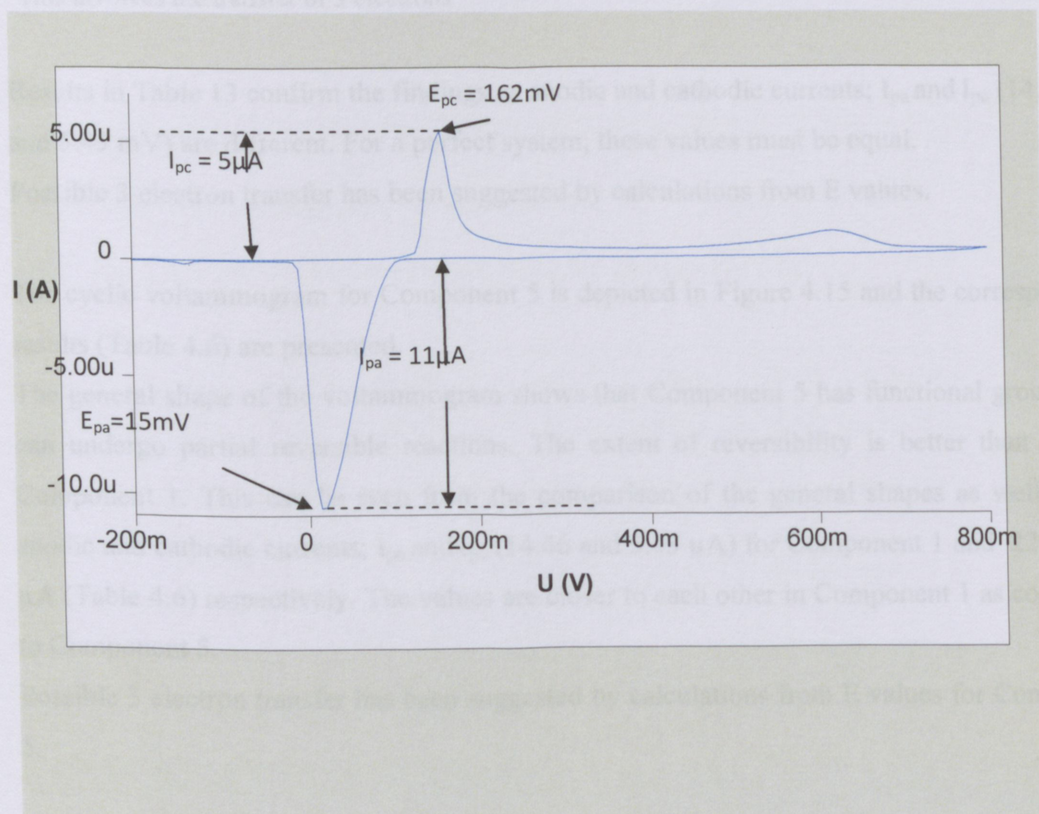

Figure 4.14: Voltammogram of component 1

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Information about the functional groups from such studies is important in building the structure of the molecules. The general shape of the voltammogram shows that Component 1 has functional groups that can undergo partial reversible reactions. If it was completely reversible, the shape would be perfectly symmetrical.

Table 4.5: Current and voltage calculations for component one

I_{pc}(cm)	E_{pc}(cm)	I_{pa}(cm)	E_{pa}(cm)	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)
3.80	3.35	7.50	0.30	5.43	161.45	10.71	14.46

For current, 3.5cm = 5μA and For voltage, 8.3cm = 400mV

$E^{0'} = (E_{pa} + E_{pc})/2 =$ 87.95 mV

$\Delta E_p = |E_{pa} - E_{pc}| =$ 146.99 mV $= 0.0592V/n$

$n =$ 2.4829046 $\approx$ 3.00

This involves the transfer of 3 electrons

Results in Table 13 confirm the findings as anodic and cathodic currents; I_{pa} and I_{pc} (14.46 and 5.43 mV) are different. For a perfect system, these values must be equal.
Possible 3 electron transfer has been suggested by calculations from E values.

The cyclic voltammogram for Component 5 is depicted in Figure 4.15 and the corresponding results (Table 4.6) are presented.
The general shape of the voltammogram shows that Component 5 has functional groups that can undergo partial reversible reactions. The extent of reversibility is better than that of Component 1. This can be seen from the comparison of the general shapes as well as the anodic and cathodic currents; I_{pa} and I_{pc} (14.46 and 5.43 μA) for Component 1 and 22 and 16 μA (Table 4.6) respectively. The values are closer to each other in Component 1 as compared to Component 5.
Possible 3 electron transfer has been suggested by calculations from E values for Component 5.

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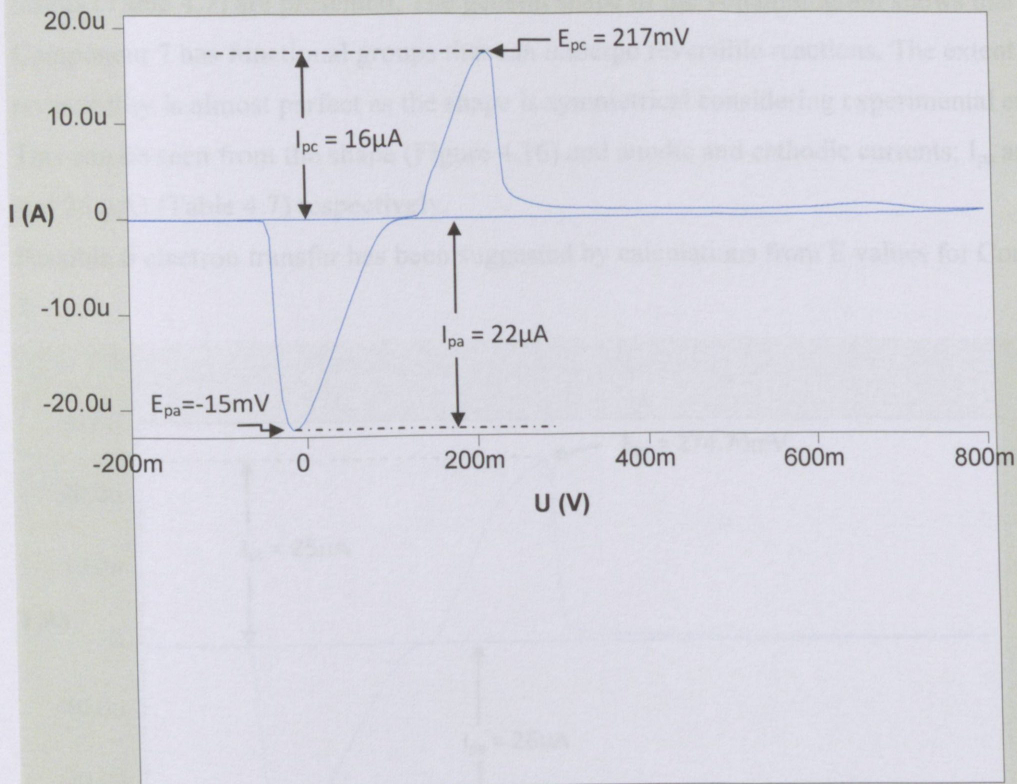

Figure 4.15: Voltammogram of component five

Table 4.6: Current and voltage calculations for component five

I_{pc}(cm)	E_{pc}(cm)	I_{pa}(cm)	E_{pa}(cm)	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)
4.95	4.50	6.60	-0.30	16.23	216.87	21.64	-14.46

For current, 6.1 cm = 20 μA and For voltage, 8.3cm = 400 mV

$E^{0'} = (E_{pa} + E_{pc})/2 =$ 101.20 mV

$\Delta Ep = |E_{pa} - E_{pc}| =$ 231.33 mV = 0.0592V/n

n = 3.907522 ≈ 4.00

This involves the transfer of 4 electrons

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The cyclic voltammogram for Component 7 is depicted in Figure 4.16 and the corresponding results (Table 4.7) are presented. The general shape of the voltammogram shows that Component 7 has functional groups that can undergo reversible reactions. The extent of reversibility is almost perfect as the shape is symmetrical considering experimental errors. This can be seen from the shape (Figure 4.16) and anodic and cathodic currents; I_{pa} and I_{pc} (26 and 25 μA) (Table 4.7) respectively.

Possible 6 electron transfer has been suggested by calculations from E values for Component 7.

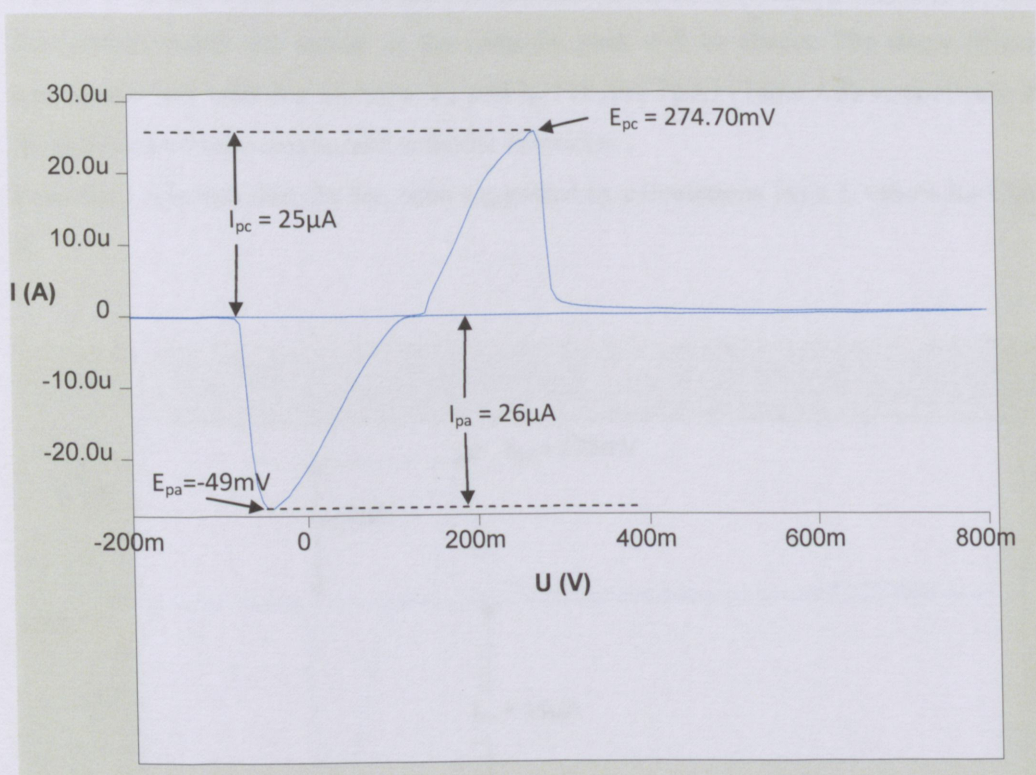

Figure 4.16: Voltammogram of component seven

Table 4.7: Current and voltage calculations for component seven

I_{pc}(cm)	E_{pc}(cm)	I_{pa}(cm)	E_{pa}(cm)	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)
5.55	5.70	5.80	-0.90	25.04	274.70	26.17	-48.65

For current, 6.65 cm = 30 μA and For voltage, 8.3 cm = 400 mV

$$E^{0'} = (E_{pa} + E_{pc})/2 = 113.03 \text{ mV}$$

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$\Delta Ep = \left| E_{pa} - E_{pc} \right| = 323.35 \text{ mV} = 0.0592V/n$

$n = 5.4619501 \approx 6.00$

This involves the transfer of 6 electrons

The cyclic voltammogram for Component 8 is depicted in Figure 4.17 and the corresponding results (Table 4.8) are presented. The general shape of the voltammogram shows that Component 8 has functional groups that can undergo partial reversible reactions, as the shapes of forward and reverse reactions are not the same. However if a system is completely irreversible, either the anodic or the cathodic peak will be absent. The shape (Figure 4.17) and anodic and cathodic currents; I_{pa} and I_{pc} (15 and 7µA) (Table 4.8) respectively illustrate the difference in the anodic and cathodic reactions. .

Possible 3 electron transfer has been suggested by calculations from E values for Component 8.

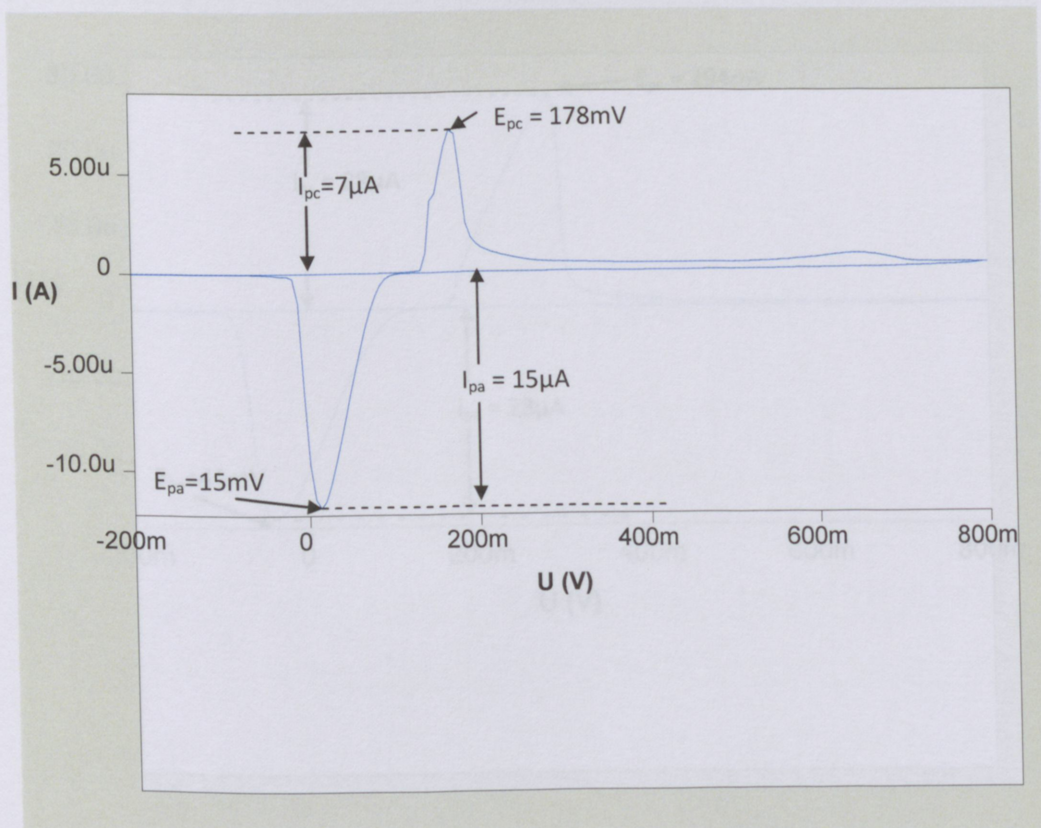

Figure 4.17: Voltammogram of component eight

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Table 4.8: Current and voltage calculations for component eight

I_{pc}(cm)	E_{pc}(cm)	I_{pa}(cm)	E_{pa}(cm)	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)
4.25	3.70	7.05	0.30	7.08	178.31	11.75	14.46

For current, 3.0cm = 5μA and For voltage, 8.3cm = 400mV

$$E^{0'} = (E_{pa} + E_{pc})/2 = 96.39 \text{ mV}$$

$$\Delta Ep = |E_{pa} - E_{pc}| = 163.86 \text{ mV} = 0.0592/n$$

$$n = 2.7678281 \approx 3.00$$

This involves the transfer of 3 electrons

The cyclic voltammogram for the unresolved extract is depicted in Figure 4.18 and the corresponding results (Table 4.9) are presented.

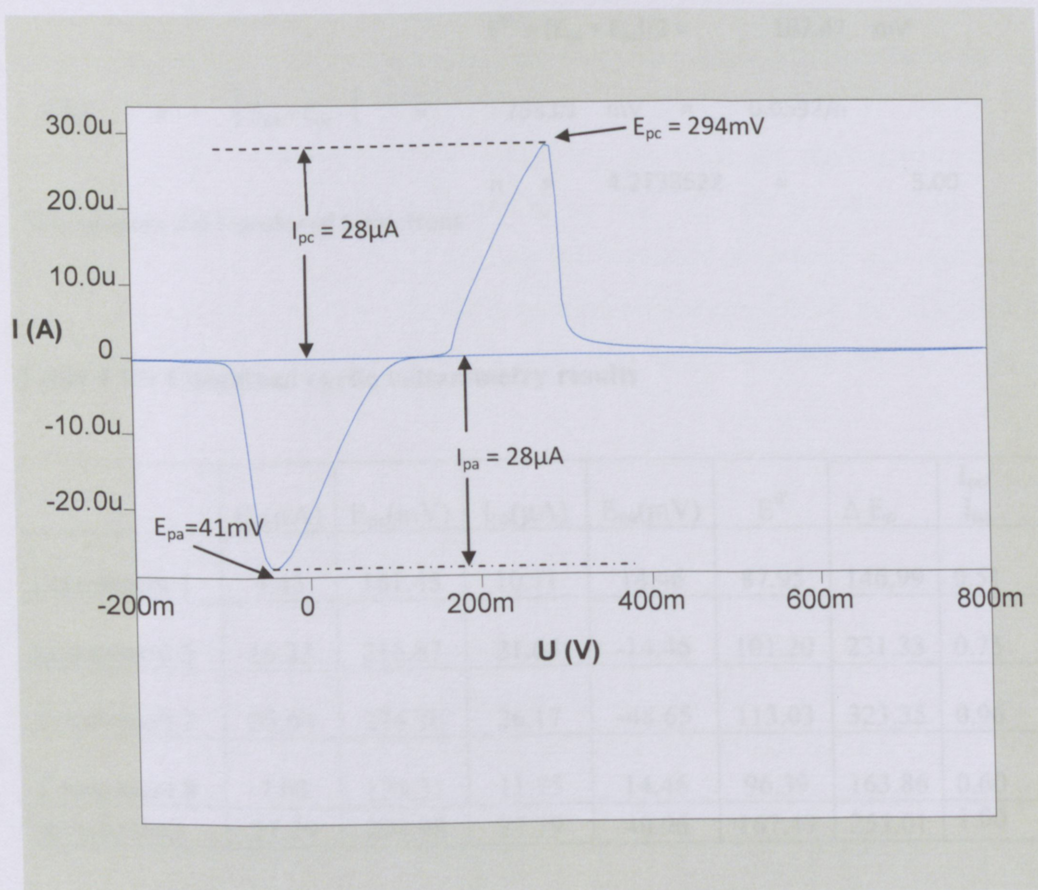

Figure 4.18: Voltammogram of unresolved extract

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The general shape of the voltammogram shows that the functional groups that can undergo reversible reactions. The shape is perfectly symmetrical (Figure 4.18), anodic and cathodic currents; I_{pa} and I_{pc} (28 and 28μA) (Table 4.9) respectively.

The raw extract shows a possible 5 electron transfer reaction which was concluded from the calculations from E values for the raw extract.

Table 4.9: Current and voltage calculations for raw extract

Raw Exctract

I_{pc}(cm)	E_{pc}(cm)	I_{pa}(cm)	E_{pa}(cm)	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)
5.65	6.10	5.65	0.85	27.79	293.98	27.79	40.96

For current, 6.1cm = 30μA and For voltage, 8.3cm = 400mV

$$E^{0'} = (E_{pa} + E_{pc})/2 = 167.47 \text{ mV}$$

$$\Delta Ep = \left| E_{pa} - E_{pc} \right| = 253.01 \text{ mV} = 0.0592/n$$

$$n = 4.2738522 \approx 5.00$$

This involves the transfer of 5 electrons

Table 4.10: Combined cyclic voltammetry results

	I_{pc}(μA)	E_{pc}(mV)	I_{pa}(μA)	E_{pa}(mV)	$E^{0'}$	ΔE_p	I_{pc}/I_{pa}	No. of e^-
Component 1	5.43	161.45	10.71	14.46	87.95	146.99	0.51	3
Component 5	16.23	216.87	21.64	-14.46	101.20	231.33	0.75	4
Component 7	25.04	274.70	26.17	-48.65	113.03	323.35	0.96	6
Component 8	7.08	178.31	11.75	14.46	96.39	163.86	0.60	3
Raw Extract	27.79	293.98	27.79	40.96	167.47	253.01	1.00	5

For a reversible reaction,

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$\Delta E_p = |E_{pa} - E_{pc}| = 0.0592/n$. If there is only one electron this value is reduced to 0.0592V.

From the results shown above, it can be demonstrated that all the extracts can undergo reversible reactions as the forward reaction is very similar to the backward reaction in all the instances. The shapes might not be similar, but they are very close.

However combined extract has a perfect match for a reversible system as the anodic current and the cathodic current are both exactly the same (Figure 4.17). Component 7 (Figure 4.15) comes very close as well as the difference between the anodic and cathodic peaks is very small.

A comparison of the potentials (Table 4.10) gives an insight into the compounds under investigation. Compounds with a lower potential are said to be better reducing agents and at the same time have higher antioxidant activity (Piljac-Žegarac et al., 2010). Therefore from the cyclic voltammetry results, it can be estimated that in order of antioxidant activity are components 1>8>5>7.

To assess the degree of reversibility, a combination of the peak potential separation (ΔE_p) and the ration of the anodic and the cathodic currents (I_{pc}/ I_{pa}) are used (Piljac-Žegarac et al., 2010). From the results in Table 4.10 above, it appears that only the unseparated extract can undergo completely reversible reactions as its (I_{pc}/ I_{pa}) ratio is 1.00, which was closely followed by component 7 which we can also estimate to undergo completely reversible reactions as its ratio was 0.96. For the other three components, it can be concluded that they can undergo quasi reversible reactions as their ratios range from 0.51 to 0.75.

4.3 Gas chromatography

GC analysis was performed for two reasons;

1. To check the purity of the separated compounds
2. To develop method to be used in the GC-MS analysis.

The first objective (purity of the compounds) was met as can be seen in the diagrams in Figures 4.19. The chromatograms show that only one main peak was found in all of the runs while the chromatograms for components 1and 5 show that further purification of those components was needed. This was done using preparative TLC.

The same conditions were to be used for the GC-MS analysis.

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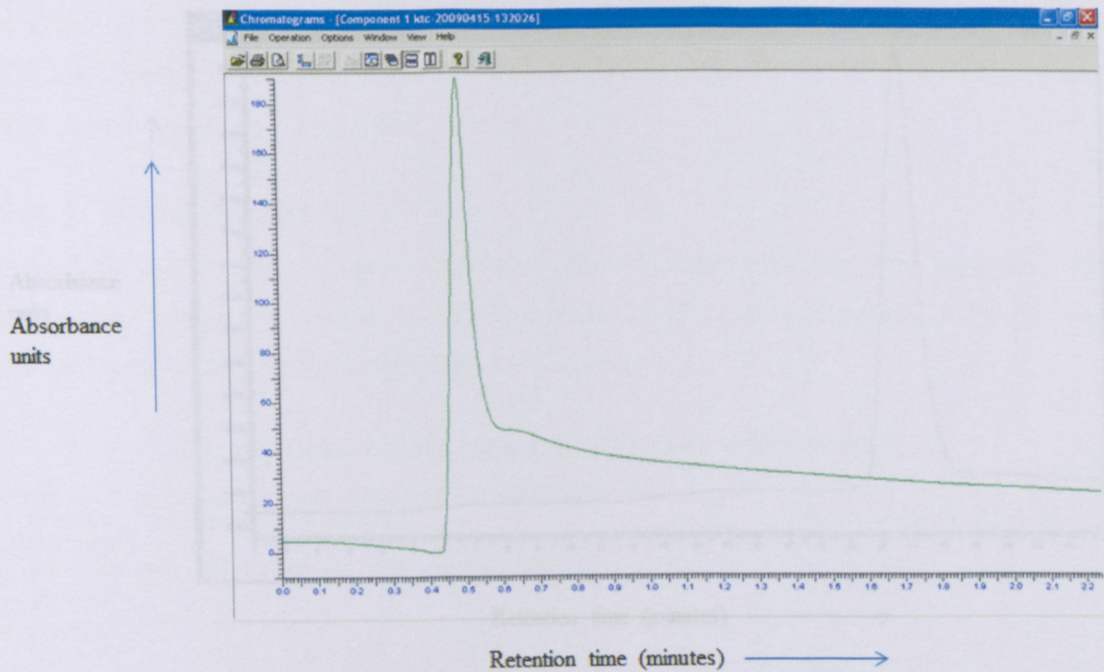

Figure 4.19a: GC chromatogram for components 1.

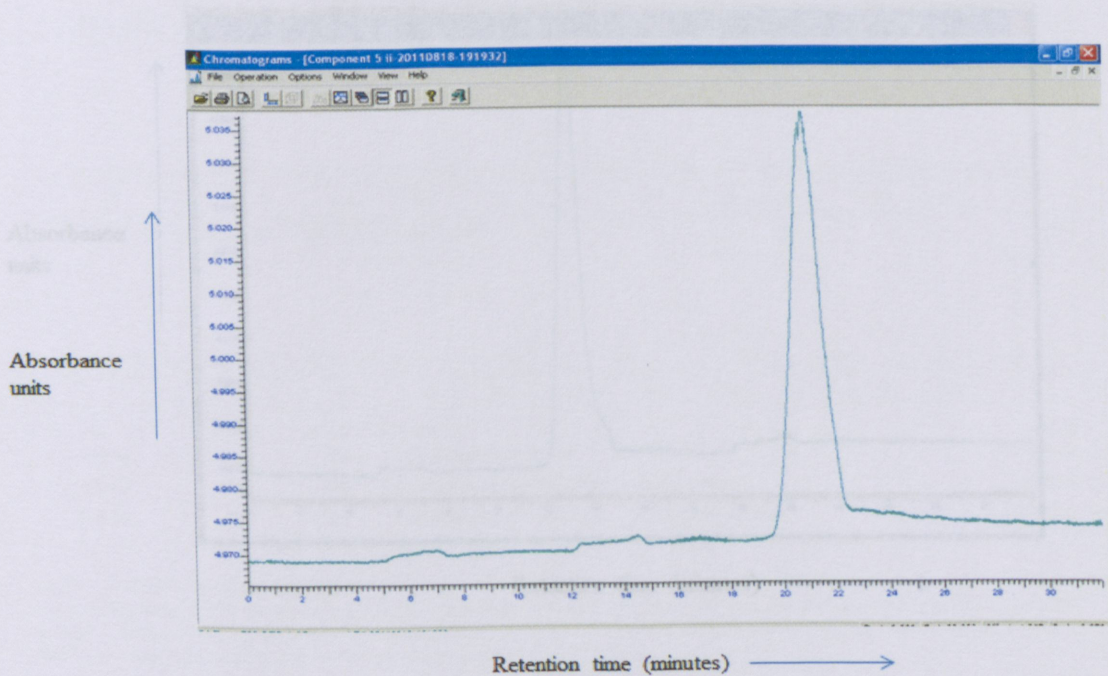

Figure 4.19b: GC chromatograms for components 5.

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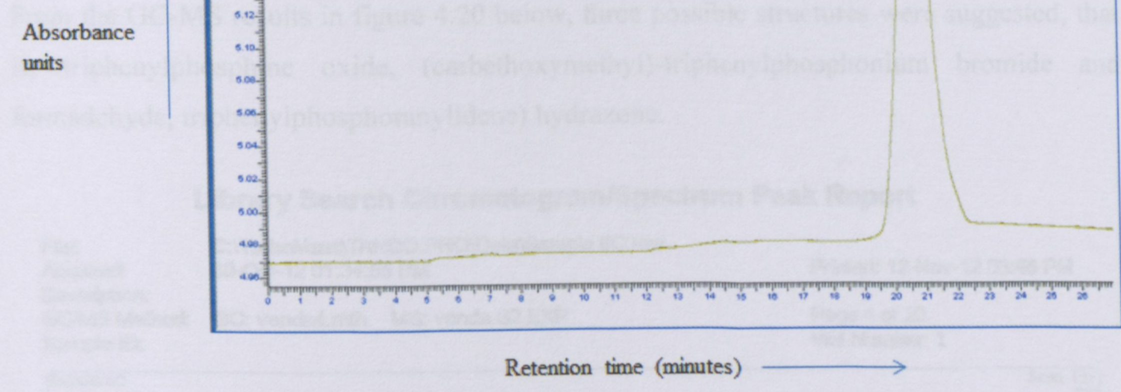

Figure 4.19c: GC chromatograms for components 7.

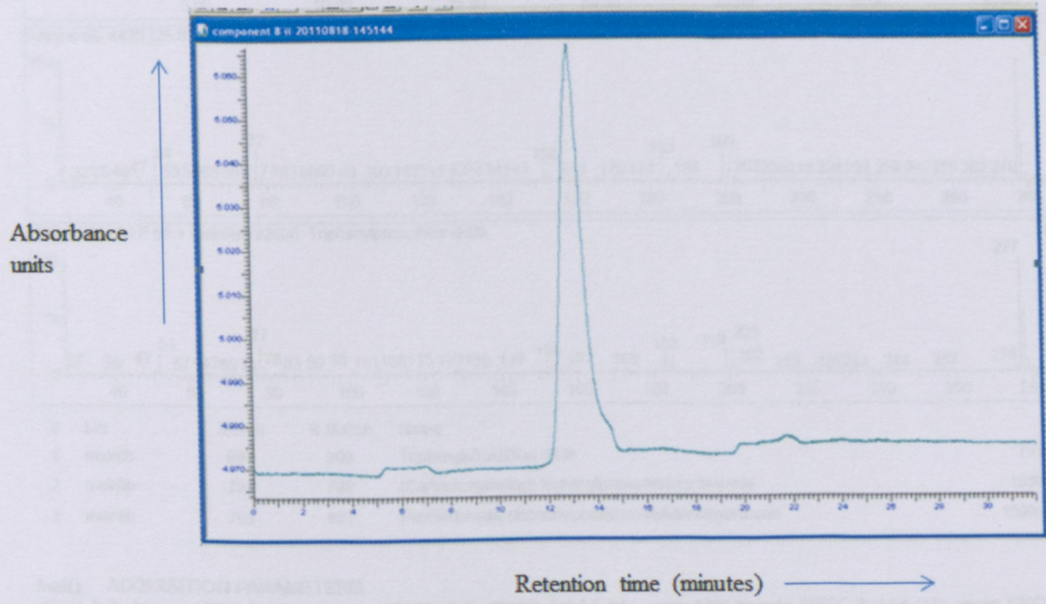

Figure 4.19d: GC chromatograms for components 8

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4.4 Gas chromatography-Mass Spectrometry

Eight samples were collected from the fraction collector on the HPLC. The components that exhibited antimicrobial activity (1, 5, 7 and 8) were analysed on the GC-MS to identify possible chemical structures. Structures were identified using the GC-MS National Institute of Standards and Technology (NIST) library.

4.4.1 Component 1

From the GC-MS results in figure 4.20 below, three possible structures were suggested, that is, triphenylphosphine oxide, (carbethoxymethyl)-triphenylphosphonium bromide and formadehyde, triphenylphosphoranylidene) hydrazone.

Library Search Chromatogram/Spectrum Peak Report

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Printed: 12-Nov-12 03:46 PM
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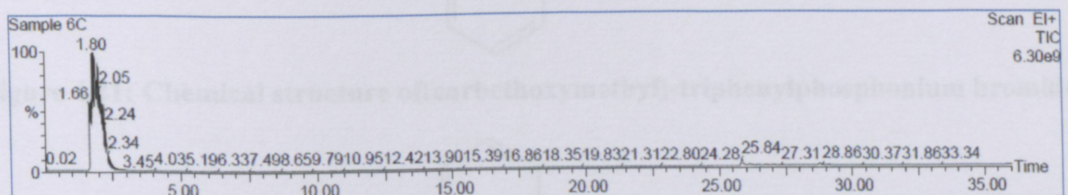
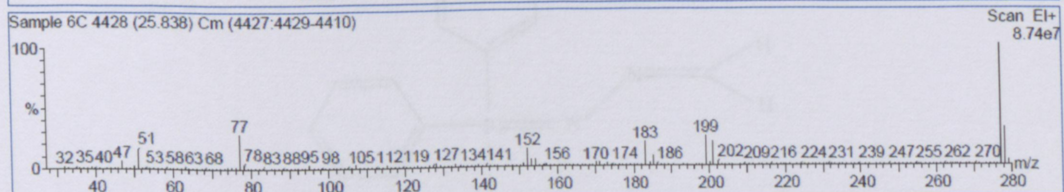
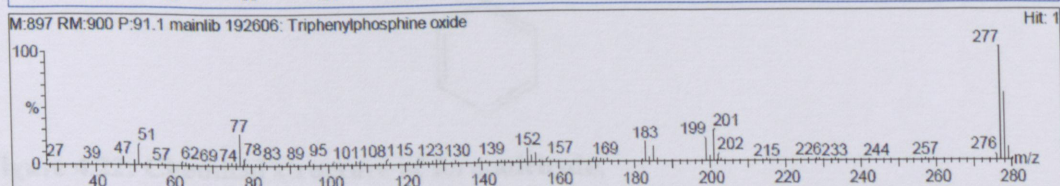
Page 4 of 20
Sample ID:
Vial Number: 1







#	Lib	Match	R.Match	Name	CAS#
1	mainlib	897	900	Triphenylphosphine oxide	791-28-6
2	mainlib	797	798	(Carbethoxymethyl)-triphenylphosphonium bromide	1530-45-6
3	mainlib	768	831	Formaldehyde, (triphenylphosphoranylidene)hydrazone	15990-54-2

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 110°C for 4 min, ramp 10°C/min to 150°C, hold 4 min, ramp 10°C/min to 200°C, hold 4 min, ramp 10°C/min to 250°C, hold 10 min, InjAauto=300°C, Volume=0 µL, Split=5:1, Carrier Gas=He, Solvent Delay=0.00 min, Transfer Temp= 200°C, Source Temp=220°C, Scan: 30 to 450Da, Column 30.0m x 320µm

Figure 4.20: GC-MS spectrum and splitting pattern of Component 1

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For all the compounds, the forward and backwards scans on the GC-MS have an excellent match.

Triphenylphosphine oxide is mostly used as a solvent, activation of crystallisation of chemical compounds and in the production of nanomaterials and therefore it was deemed not be an interesting compound. The other two chemical compounds, (carbethoxymethyl)-triphenylphosphonium bromide (Figure 4.21) and formadehyde, triphenylphosphoranylidene) hydrazone (Figure 4.22), are more likely to be the active ingredients in Component 1 extract. Their chemistry is not well documented.

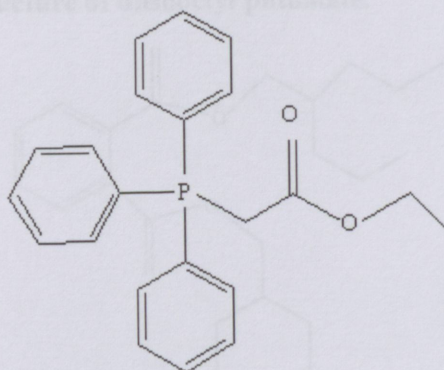

Figure 4.21: Chemical structure of(carbethoxymethyl)-triphenylphosphonium bromide

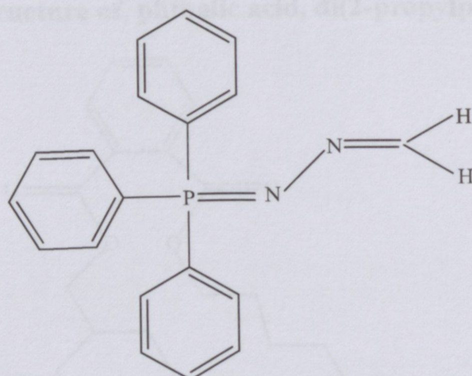

Figure 4.22: Chemical structure of formadehyde, riphenylphosphoranylidene)hydrazone

4.4.2 Component 5

The GC-MS spectrum and the subsequent splitting patterns (Figure 4.26) show the three possible structures, diisooctyl phthalate (Figure 4.23), phthalic acid, di(2-propylpentyl) ester (Figure 4.24) and phthalic acid octyl 2-propylpentyl ester (Figure 4.25). The forward and backward GC-MS scans and the splitting patterns also match very well.

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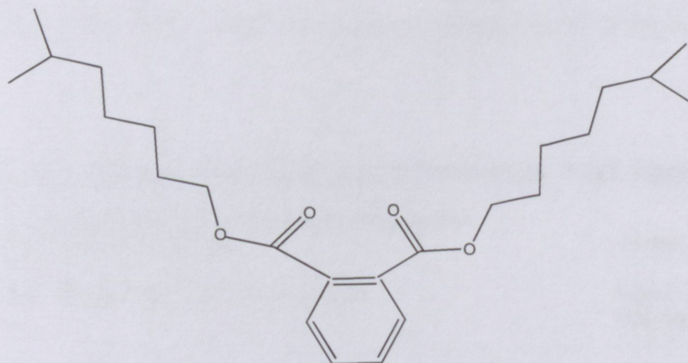

Figure 4.23: Chemical structure of diisooctyl phthalate.

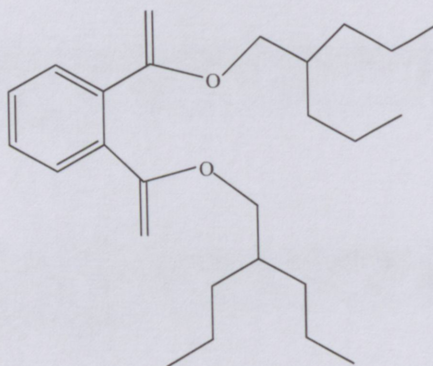

Figure 4.24: Chemical structure of, phthalic acid, di(2-propylpentyl) ester.

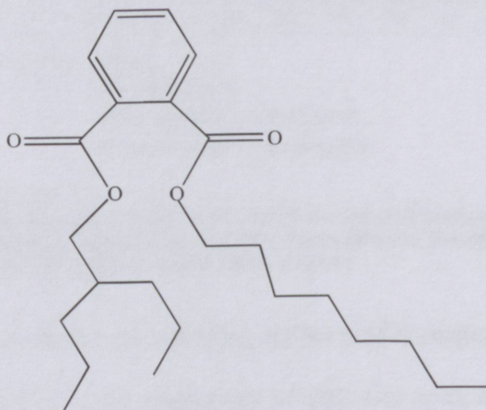

Figure 4.25: Chemical structure of phthalic acid octyl 2-propylpentyl ester.

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Diisooctyl phthalate is used as a crosslinking agent, plasticizer or dying carrier for polyesters

Library Search Chromatogram/Spectrum Peak Report

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Description:
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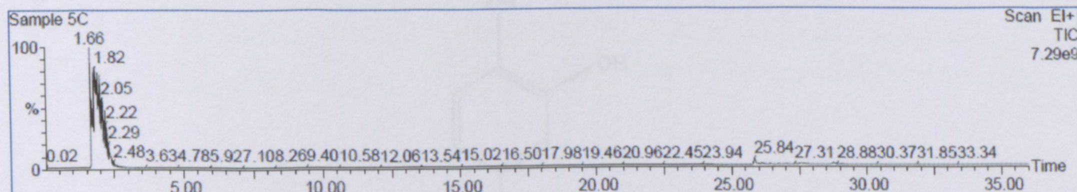
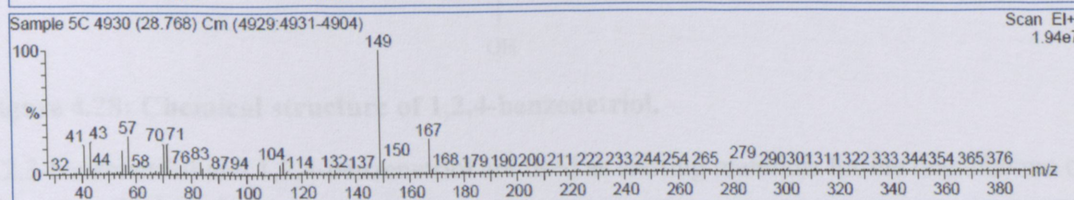
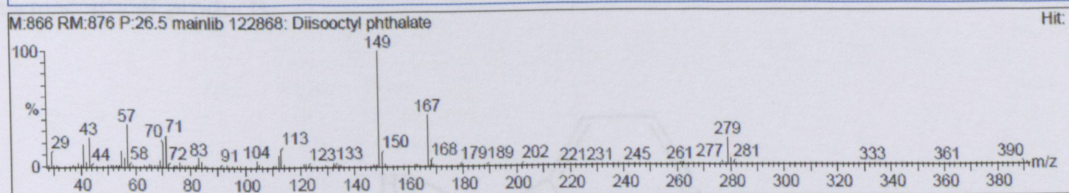
Sample ID:

Printed: 12-Nov-12 03:45 PM
Page 17 of 20
Vial Number: 5







#	Lib	Match	R.Match	Name	CAS#
1	mainlib	866	876	Diisooctyl phthalate	131-20-4
2	mainlib	862	884	Phthalic acid, di(2-propylpentyl) ester	
3	mainlib	831	857	Phthalic acid, octyl 2-propylpentyl ester	

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 110°C for 4 min, ramp 10°C/min to 150°C, hold 4 min, ramp 10°C/min to 200°C, hold 4 min, ramp 10°C/min to 250°C, hold 10 min, InjAauto=300°C, Volume=0 µL, Split=5:1, Carrier Gas=He, Solvent Delay=0.00 min, Transfer Temp= 200°C, Source Temp=220°C, Scan: 30 to 450Da, Column 30.0m x 320µm

Figure 4.26: GC-MS spectrum and splitting pattern of Component 5.

As is the case in Component 1, the chemistry of phthalic acid, di(2-propylpentyl) ester and phthalic acid octyl 2-propylpentyl ester is not well documented and further research on them is needed and we think that one of the two is more likely to be the compound of interest.

4.4.3 Component 7

The GC-MS results (Figure 4.30) show three possible structures of our compound of interest, that is, 1,2,3-benzenetriol (Figure 4.27), 1,2,4-benzenetriol (Figure 4.28) and 3-methyl-pyrazole-5-carboxylic acid (Figure 4.29).

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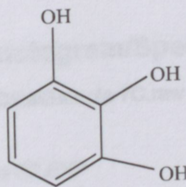

Figure 4.27: Chemical structure of 1,2,3-benzenetriol.

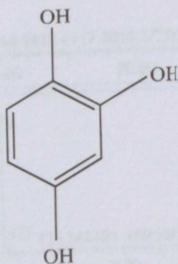

Figure 4.28: Chemical structure of 1,2,4-benzenetriol.

1,2,3-benzenetriol and 1,2,4-benzenetriol are used as starting materials for the manufacture of pharmaceutical products.

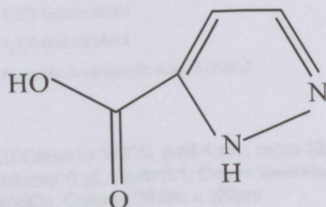

Figure 4.29: Chemical structure of 3-methyl-pyrazole-5-carboxylic acid.

It has been shown that 3-methyl-pyrazole-5-carboxylic acid can be used for the treatment of Schizophrenia, a serious neuropsychiatric disease (Haruta et al., 2011).

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Library Search Chromatogram/Spectrum Peak Report

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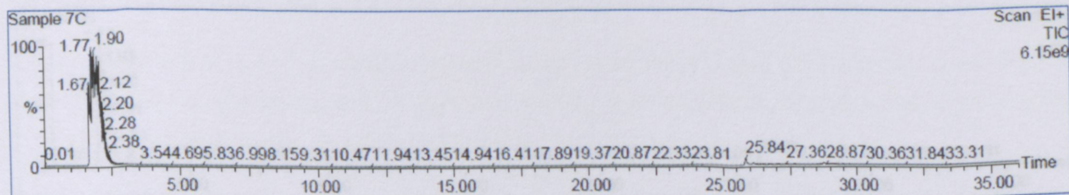

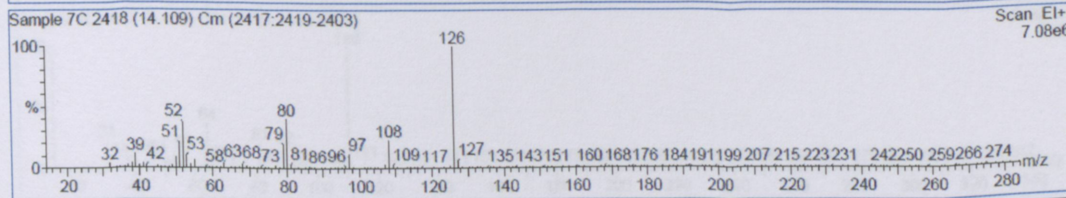

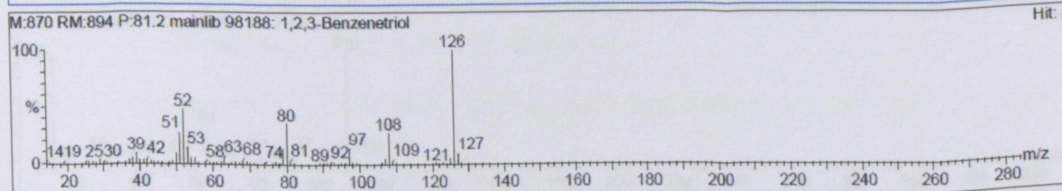

#	Lib	Match	R.Match	Name	CAS#
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2	mainlib	819	840	1,2,4-Benzenetriol	533-73-3
3	mainlib	708	734	Pyrazole-5-carboxylic acid, 3-methyl-	696-22-0

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 110°C for 4 min, ramp 10°C/min to 150°C, hold 4 min, ramp 10°C/min to 200°C, hold 4 min, ramp 10°C/min to 250°C, hold 10 min, InjAauto=300°C, Volume=0 μL, Split=5:1, Carrier Gas=He, Solvent Delay=0.00 min, Transfer Temp= 200°C, Source Temp=220°C, Scan: 30 to 450Da, Column 30.0m x 320μm

Figure 4.30: GC-MS spectrum and splitting pattern of Component 5.

4.4.4 Component 8

From the GC-MS splitting pattern (Figure 4.31), the best three possible structures suggested are catechol (Figure 4.32), resorcinol (Figure 4.33) and hydroquinone (Figure 4.34). These also comply with the cyclic voltammetry results.

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Library Search Chromatogram/Spectrum Peak Report

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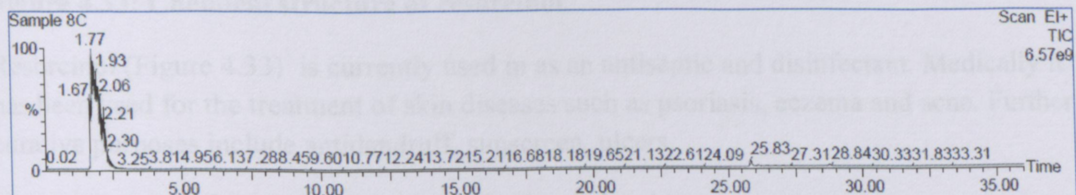
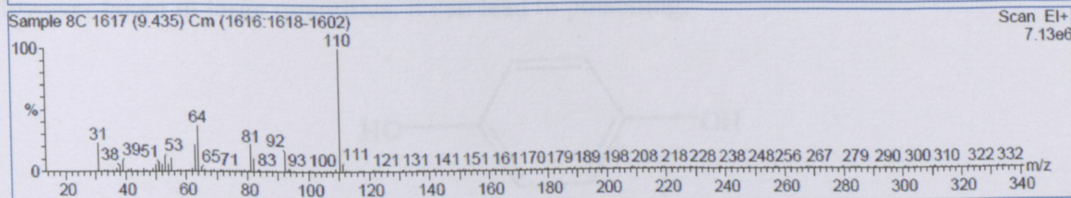
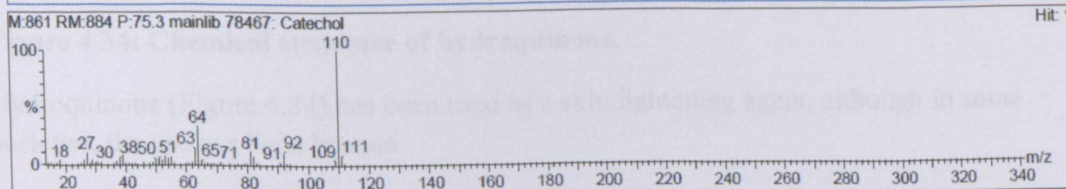
Sample ID:		Vial Number: 8







#	Lib	Match	R.Match	Name	CAS#
1	mainlib	861	884	Catechol	120-80-9
2	mainlib	802	821	Resorcinol	108-46-3
3	mainlib	787	808	Hydroquinone	123-31-9

Inst() ACQUISITION PARAMETERS

Oven: Initial temp 110°C for 4 min, ramp 10°C/min to 150°C, hold 4 min, ramp 10°C/min to 200°C, hold 4 min, ramp 10°C/min to 250°C, hold 10 min, InjAauto=300°C, Volume=0 µL, Split=5:1, Carrier Gas=He, Solvent Delay=0.00 min, Transfer Temp= 200°C, Source Temp=220°C, Scan: 30 to 450Da, Column 30.0m x 320µm

Figure 4.31: GC-MS spectrum and splitting pattern of Component 5

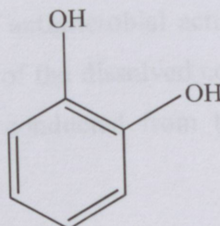


Figure 4.32: Chemical structure of catechol.

Industrially, catechol (Figure 4.32) is used in the manufacture of pesticides, perfumes and pharmaceuticals

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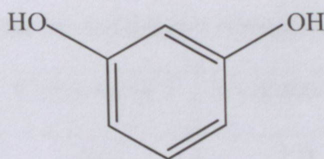

Figure 4.33: Chemical structure of resorcinol.

Resorcinol (Figure 4.33) is currently used in as an antiseptic and disinfectant. Medically it has been used for the treatment of skin diseases such as psoriasis, eczema and acne. Further curative purposes include antidandruff, sunscreen, ulcers.

However taken in large quantities, it can lead to poisoning.

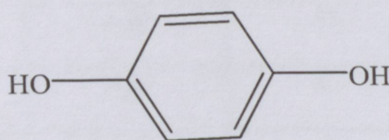

Figure 4.34: Chemical structure of hydroquinone.

Hydroquinone (Figure 4.34) has been used as a skin lightening agent, although in some societies, the use has been banned

All the components that have been analysed by GC-MS show possible structures that have functional groups that can undergo reversible reactions, which was confirmed by the cyclic voltammetry results.

4.5 Antimicrobial Activity

The raw plant was extracted with water and it had antimicrobial activity against *E. coli, Staphylococcus aureus, Candida albicans and Enterobacter aerogenes*.

The pure extracts were then dissolved in 2ml of ultrapure deionised water to give concentrations that were prepared from masses shown in Table 4.4. Of the pure extracts obtained, the extracts that exhibited antimicrobial activity are shown below in Table 4.11. The subsequent bulk concentrations of the dissolved components ranged from 1.55mg/ml to 34.45mg/ml. Serial dilutions were conducted from those concentrations to obtain lower concentrations.

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Table 4.11: Antimicrobial results for separated components

Organism	Component 1	Component 5	Component 7	Component 8
V.cholera	>A	>A	>A	>A
Shigella	A	>A	A	A
A.hydrophilia	A	>A	>A	A
Salmonella	>A	>A	>A	A
P.murabilis	A	A	>A	A
E.coli 10	A	>A	D	A
E.coli 11	>A	>A	C	A
S. Aureus	A	>A	A	>A

The microtitre plates were visualised by adding INT solution. The wells where microorganisms were viable were coloured whereas the wells where microorganisms were not viable did not change colour when compared to the negative controls. This can be observed in the microtitre plates that are shown in Figure 4.35;

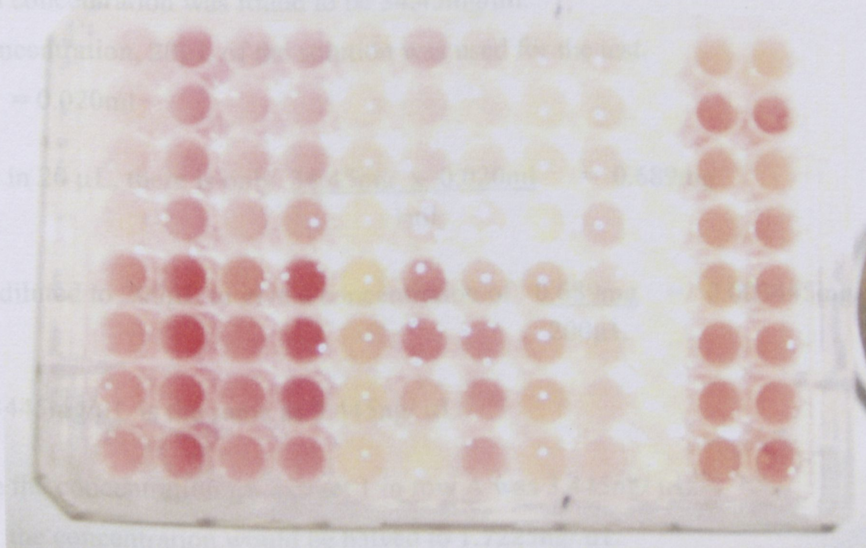

Figure 4.35: Microtitre plate of extracts soon after incubation and visualisation

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These results combined with the concentration of the dissolved extracts that are calculated in table 12 show that;

1. Component 1 exhibits antimicrobial activity for *Shigella, Aeromonas hydrophilia, Salmonella, Proteus mirabilis, Escherichia* coli and *Staphylococcus aureus*. The activity exhibited for this extract was at 3.445ng/µL, which turns out to be the MIC for these microorganisms.
2. Component 5 was only active against *Proteus mirabilis* at a concentration of 0.08ng/µl and the MIC was also calculated to be 0.08ng/µl.

3. Component 7 was active against *Shigella, Escherichia* coli 10, 11 and *Staphylococcus Aureus*. The MIC for Shigella was0. 365ng/µl, whereas the MIC for *Escherichia* coli 10,was found to be 0.046ng/µl and the MIC for *Escherichia* coli 11, 0.091ng/µl.

4. Component 8 was active for *Shigella, Aeromonas hydrophilia, Salmonella, Proteus mirabilis, Escherichia coli*10 and 11.The MIC for all these was found to be 0.155ng/µl

The calculations for all the MIC concentration was performed as follows, using component 1 as an example;

The initial concentration was found to be 34.45mg/ml.

Of this concentration, 20µL of the solution was used for the test.

But 20µL = 0.020ml

Therefore in 20 µL, there is only $\frac{34.45\text{mg} \times 0.020\text{ml}}{1\text{ml}} = 0.689\text{mg}$

This was diluted to 200µL to give a concentration of $\frac{0.689\text{mg}}{200\mu\text{L}} = 0.003445\text{mg}/\mu\text{L}$

But 0.003445mg/µL is the same as 3.445ng/ µL

Therefore the concentration for extract 1 in row A was 3.445ng/ µL

In row B, the concentration would be halved to 1.7225ng/ µL

Row C concentration was then 0.86125ng/ µL

And finally row D concentration would be calculated to be 0.430625ng/ µL

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The serial dilution from row A going down to rows B, C and D was 50% all the time.

All the other calculation were performed in the same manner.

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Chapter five: Conclusions

(i) The HPLC method of analysis for the separation of the components of the *Kirkia wilmsii* was developed. The developed method of analysis was also shown to have reproducible peak times and the resolution was good. The gradient elution analytical procedure was developed by employing two mobile phases, where mobile phase A was an aqueous and mobile phase B was an organic eluent made up of acetonitrile and the pH was also adjusted with TFA. However the retention time needs to be drastically reduced as it is too long.

(ii) The UV-Vis spectra for all the peaks were also extracted, which can be used as an alternative rapid method for the analysis of the different components.

(iii) Since bioactive compounds were found, the plant can be pursued and expected to make a contribution towards herbal medicines According to earlier studies done on other parts of the plants, bioactivity was recorded against malaria, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Sporothrix schenckii*, *Microsporum canis*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida albicans*. In this study activity was also recorded against three of the above microbes, that is, *Staphylococcus aureus, Escherichia coli* and *Candida albicans,* which tallies with the other studies done. The other microbes could not be compared as they are not available. However the root proved to be active against *Enterobacter aerogenes, Shigella, Aeromonas, hydrophilia,Salmonella, Proteus mirabilis* and *Shigella* which were not documented in other studies.

(iv) GC-MS results indicate that the possible isolated compounds: are triphenylphosphine oxide, (carbethoxymethyl)-triphenylphosphonium bromide, formadehyde, triphenylphosphoranylidene) hydrazine, diisooctyl phthalate, phthalic acid, di(2-propylpentyl) ester orphthalic acid octyl 2-propylpentyl ester 1,2,3-benzenetriol, 1,2,4-benzenetriol or 3-methyl-pyrazole-5-carboxylic acid, catechol, resorcinol and hydroquinone .

(v) The components also showed antioxidant activity and can be used as food additives after further tests and the cyclic voltammetric results can be linked to the structure of the components.

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5.1 Recommendations

(i) All the other peaks, for example, at 68 and 97 minutes must also be investigated.

(ii) Further method development is needed to try and separated the congested peaks between about 70 to around 90 minutes.

(iii) Toxicity studies must also be undertaken before the separated components can be considered for addition as food supplements.

(iv) Studies must also be undertaken to check the presence of heavy metals in the plant.

(v) Other methods, such as, nuclear magnetic resonance and fourier-transform infrared spectrometry, proton and carbon must be used to characterise the compounds.

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Appendices

Appendix A

Glossary

Derivatization - the process of chemically modifying a compound to produce a new compound which has properties that are suitable for analysis using a GC.

Pharmacognosy - the branch of pharmacology that deals with drugs in their crude or natural state and with medicinal herbs or other plants.

Pharmacology - the science of drugs, including their composition, uses, and effects.

Phytochemistry - the branch of organic chemistry dealing with the chemistry of plants.

Secondary metabolites - chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions.

Proanthocyanidins - present in the fruits, bark, leaves and seeds of many plants, where they provide protection against predation. At the same time they give flavour and astringency to beverages such as wine, fruit juices and teas

Zwitterion - compound with no overall electrical charge, but which contains separate parts which are positively and negatively charged e.g. amino acids

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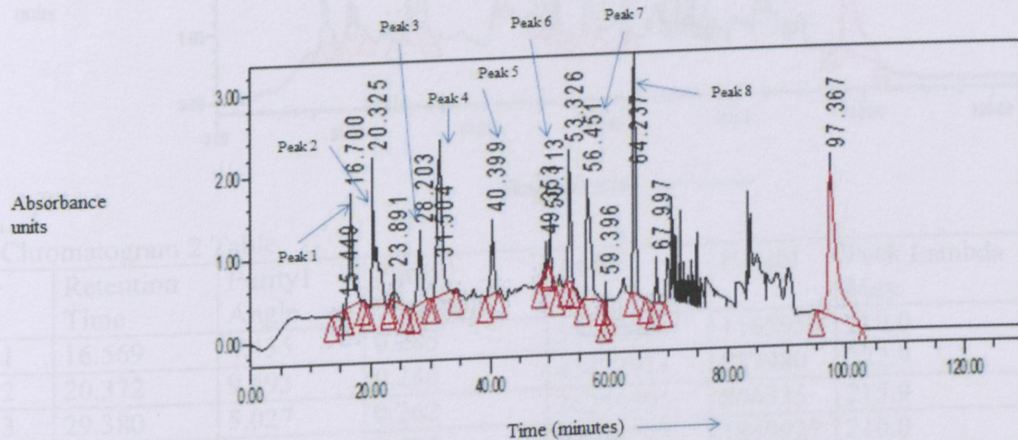
Appendix B

Chromatogram 1



Chromatogram 1 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	15.449	7.334	17.298	6376083	-123096	387.0
2	16.700	5.251	2.710	52069239	1155754	210.0
3	20.325	11.199	2.148	74623171	1576213	222.9
4	23.891	14.771	16.222	7819567	116208	210.0
5	28.203	5.184	2.643	38065516	672472	215.9
6	31.504	4.876	2.084	106015588	1672711	210.0
7	40.399	5.480	2.574	30117786	701407	217.0
8	49.353	48.649	9.634	5558030	133691	210.0
9	50.113	8.276	3.171	11039055	415944	224.1
10	53.326	1.243	1.274	42421553	1401414	252.4
11	56.451	3.026	1.359	70802880	1247939	253.6
12	59.396	26.496	90.000	6631098	201320	353.7
13	64.237	3.069	8.419	89866267	2769378	250.1
14	67.997	6.198	2.604	10198463	348041	247.7
15	97.367	3.683	1.797	189002111	1757950	210.0

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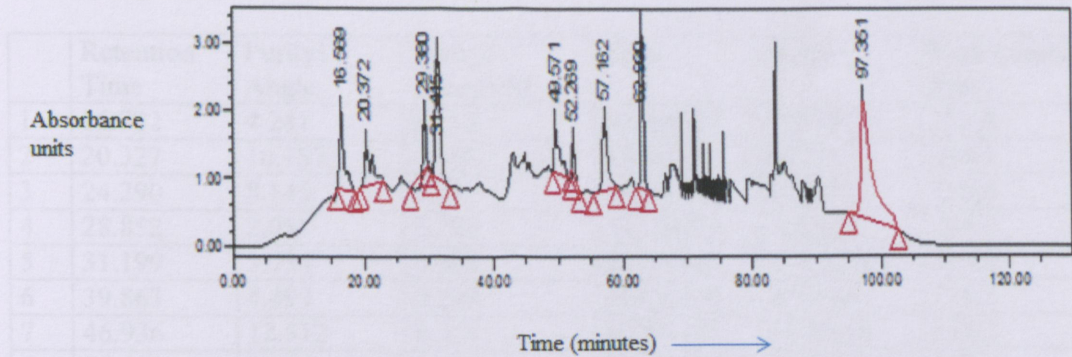
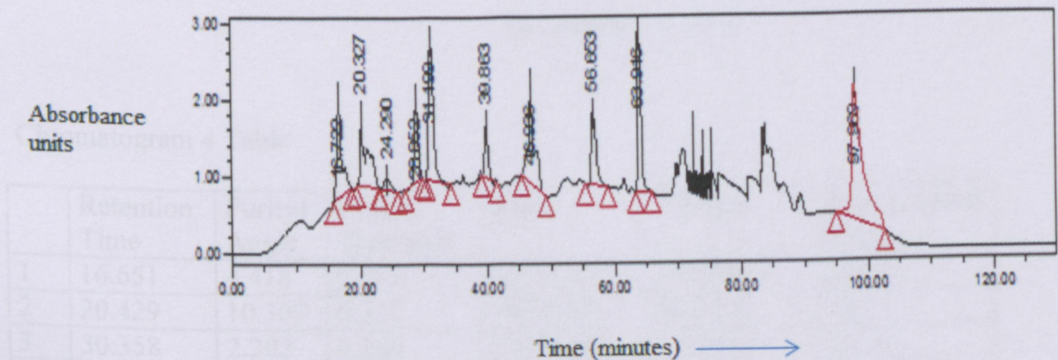
Chromatogram 2



Chromatogram 2 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.569	4.435	0.285	59380081	1165959	210.0
2	20.372	9.493	0.280	45432911	637980	222.9
3	29.380	5.027	0.262	36259595	806335	215.9
4	31.415	3.606	0.694	96358272	1849927	210.0
5	49.571	51.628	0.254	39059663	712828	219.4
6	52.269	1.847	0.236	13540916	450382	251.3
7	57.162	0.799	0.543	59678931	1012754	252.4
8	62.930	2.212	6.824	81625714	2500531	245.3
9	97.351	1.839	0.563	217741963	1739104	210.0

Chromatogram 3



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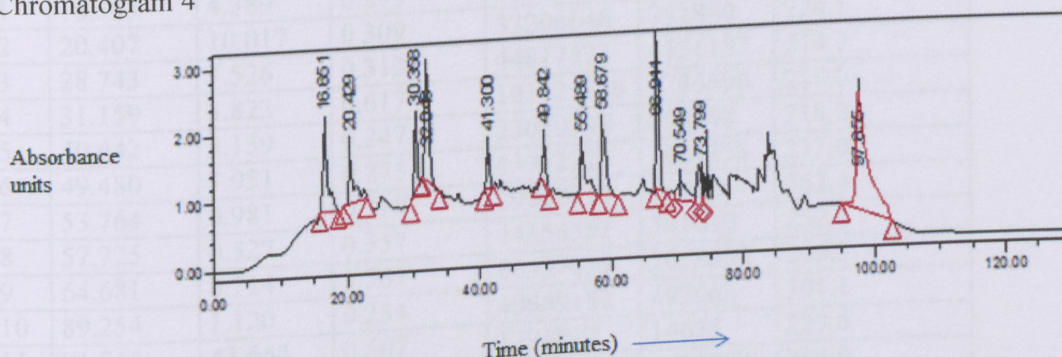
Chromatogram 3 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.722	4.241	0.312	68104397	1297807	210.0
2	20.327	10.757	0.366	68465412	932753	224.1
3	24.290	8.145	0.227	10791343	140291	210.0
4	28.852	2.058	0.296	44219675	1084414	214.7
5	31.199	3.934	0.628	98180400	1795878	210.0
6	39.863	4.499	0.249	26420520	676186	218.2
7	46.936	12.512	0.324	80294161	1290158	218.2
8	56.653	0.634	0.518	50292734	937710	252.4
9	63.916	0.938	4.061	84246329	2142438	246.5
10	97.969	1.950	0.586	217093354	1760128	210.0

Chromatogram 4



Chromatogram 4 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.651	4.418	0.293	62191830	1209861	210.0
2	20.429	10.307	0.322	39845181	813074	224.1
3	30.358	2.202	0.286	33045207	1008805	215.9
4	32.048	5.693	0.465	78629523	1611057	212.3
5	41.300	2.185	0.241	19551666	561868	218.2
6	49.842	6.021	0.247	17804148	595770	220.6
7	55.489	1.649	0.337	42130410	652747	252.4
8	58.679	0.548	0.513	52029561	978768	252.4
9	66.911	1.360	2.912	76864978	2204782	246.5
10	70.549	16.395	0.220	33085340	261237	210.0
11	73.799	2.057	0.242	4961548	419987	313.0

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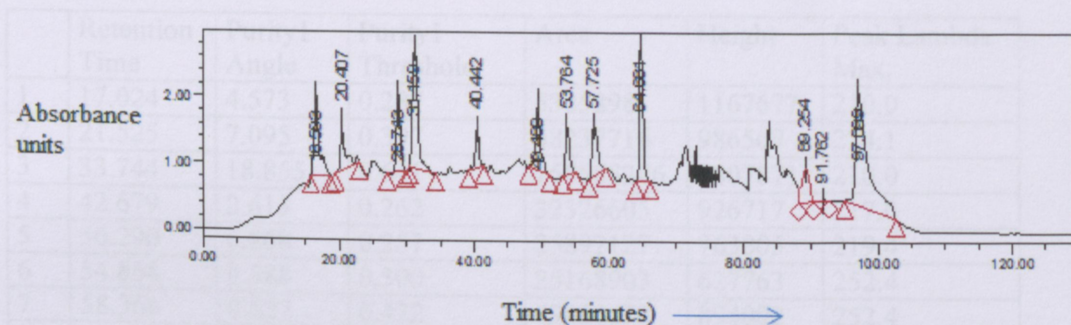
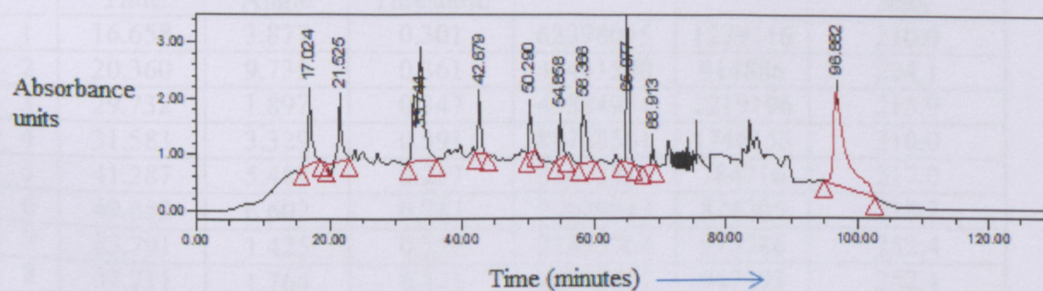
Chromatogram 5



Chromatogram 5 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.593	4.389	0.293	60986896	1202380	210.0
2	20.407	10.017	0.309	33206646	751840	224.1
3	28.743	1.526	0.312	44817373	1151157	214.7
4	31.159	3.823	0.617	101121496	1785698	210.0
5	40.442	3.159	0.247	23030799	598348	218.2
6	49.480	7.951	0.275	43912725	1021353	217.0
7	53.764	0.981	0.318	29068014	707826	252.4
8	57.725	0.527	0.357	44354552	741012	252.4
9	64.681	0.714	3.567	80337433	2058038	246.5
10	89.254	1.120	0.234	40659182	492962	301.1
11	91.762	43.664	0.507	1294817	14633	227.6
12	97.099	2.177	0.518	218672589	1689889	210.0

Chromatogram 6



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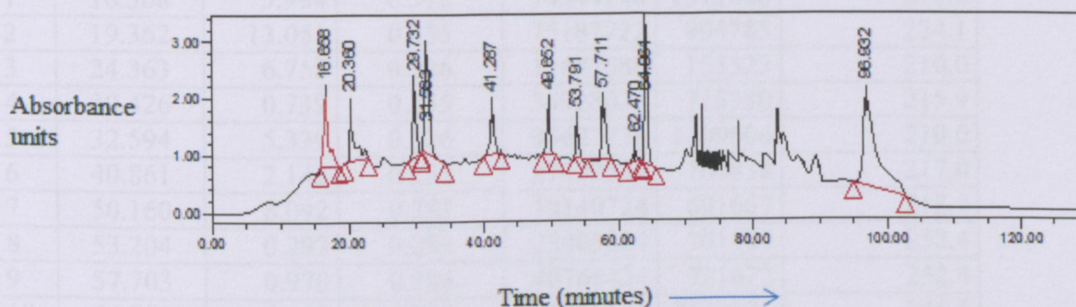
Chromatogram 6 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	17.024	4.573	0.287	53258981	1167677	210.0
2	21.525	7.095	0.397	48237716	986569	224.1
3	33.744	18.855	0.641	158588246	1807871	210.0
4	42.679	2.613	0.262	32326605	926717	217.0
5	50.290	6.868	0.257	25892127	763805	219.4
6	54.858	0.588	0.300	25168903	627763	252.4
7	58.366	0.527	0.422	49454678	894002	252.4
8	65.077	2.256	6.846	80966353	2483726	246.5
9	68.913	2.138	0.238	9483926	346778	247.7
10	96.882	2.055	0.521	226182873	1694843	210.0

Chromatogram 7



Chromatogram 7 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.658	3.877	0.301	62398045	1239316	210.0
2	20.360	9.735	0.361	44441590	914886	224.1
3	29.732	1.897	0.347	43884954	1219196	215.9
4	31.583	3.329	0.591	88768501	1746155	210.0
5	41.287	5.487	0.257	32672580	784716	217.0
6	49.652	6.602	0.263	27029843	824205	218.2
7	53.791	1.425	0.256	21455764	572786	252.4
8	57.711	1.764	0.321	48772001	947393	252.4
9	62.470	3.337	0.253	8560485	210757	246.5
10	64.061	2.359	6.748	81522802	2475655	246.5
11	96.932	1.962	0.414	221053813	1548635	210.0

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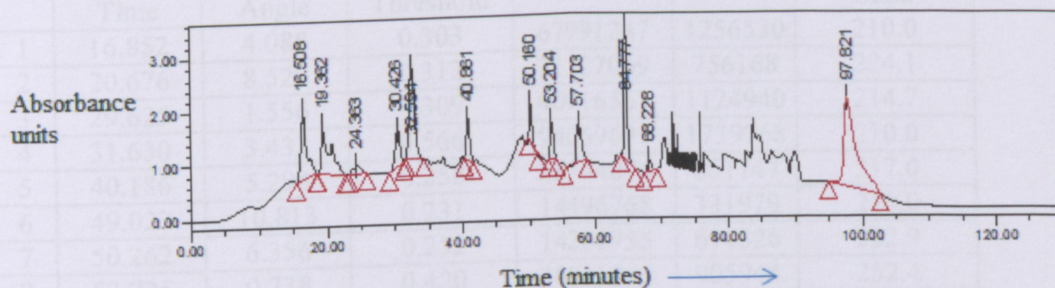
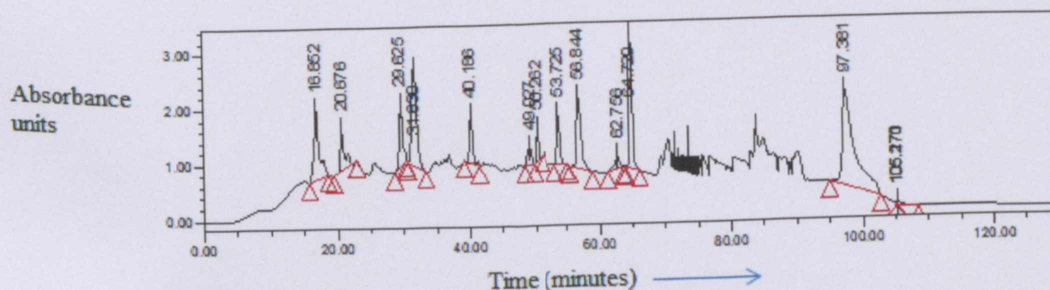
Chromatogram 8



Chromatogram 8 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.508	3.994	0.315	74344148	1313008	210.0
2	19.362	13.051	0.355	75187722	904785	224.1
3	24.363	6.758	0.226	11687088	153523	210.0
4	30.426	0.739	0.255	34928026	715330	215.9
5	32.594	5.339	0.536	95627736	1709606	210.0
6	40.861	2.142	0.251	27439524	766838	217.0
7	50.160	8.092	0.253	18140724	691667	218.2
8	53.204	0.293	0.295	23405224	701704	252.4
9	57.703	0.970	0.286	40768424	721472	252.4
10	64.777	3.689	8.837	80566821	2542171	246.5
11	68.228	1.159	0.226	7817892	215611	247.7
12	97.821	1.892	0.488	217620388	1656153	210.0

Chromatogram 9



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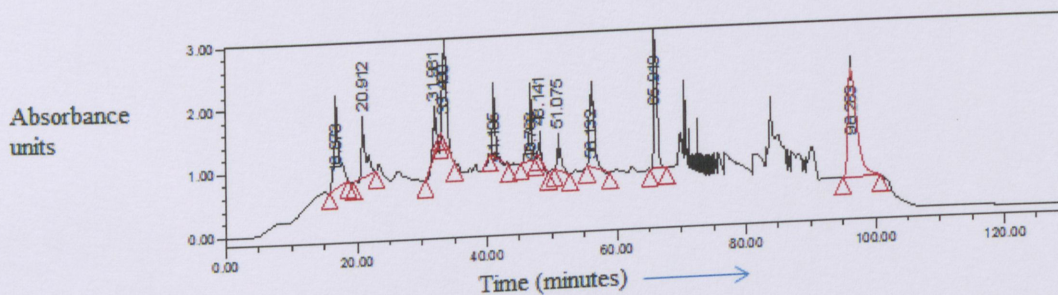
Chromatogram 9 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.852	4.088	0.303	67991247	1256530	210.0
2	20.676	8.523	0.312	31327069	756168	224.1
3	29.625	1.556	0.309	40916561	1124940	214.7
4	31.630	3.433	0.566	94069697	1739768	210.0
5	40.186	5.294	0.258	28544074	851747	217.0
6	49.027	10.813	0.231	14496768	331979	210.0
7	50.262	6.356	0.252	14200735	614326	222.9
8	53.725	0.738	0.420	30831842	905964	252.4
9	56.844	0.931	0.758	60696276	1274557	252.4
10	62.756	3.546	0.248	10932878	233026	246.5
11	64.720	2.168	7.053	82417742	2459361	246.5
12	97.381	1.930	0.533	217288813	1706765	210.0
13	105.171	30.694	11.405	404545	68436	210.0
14	105.270	20.712	10.432	4771036	66357	210.0

Component 10



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Component 10 Table

	Retention Time	Purity1 Angle	Purity1 Threshold	Area	Height	Peak Lambda Max.
1	16.873	4.198	0.307	68334827	1281821	210.0
2	20.912	9.556	0.323	39220792	792180	224.1
3	31.981	6.104	0.255	28425281	622018	215.9
4	33.480	8.557	0.477	67718979	1462638	214.7
5	41.105	4.466	0.265	28847793	962613	217.0
6	46.768	10.328	0.280	35961763	1032321	220.6
7	48.141	13.001	0.230	8980013	237070	210.0
8	51.075	0.484	0.273	18034485	389436	252.4
9	56.132	0.817	0.790	63551578	1150801	252.4
10	65.919	0.851	3.736	78730136	2038708	246.5
11	96.283	0.279	0.535	161970009	1709986	210.0

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