The anti-platelet aggregation activity of *Rapanea melanophloeos* -A Zulu medicinal plant

By

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Supervisor : Prof. A.R Opoku

Co-supervisor:Prof. O.A Oyedeji

2011

Dedication

To

My family

DECLARATION

This is to certify that the work reported in the dissertation entitled “The anti-platelet aggregation activity of *Rapanea melanophloeos (L.) Mez (*Myrsinaceae*)* from South Africa” is an original work of Mr. Phiwamandla Emmanuel Gwala, carried out under our supervision and directions. The dissertation has been submitted to fulfill the requirements for the degree Master of Science (Msc) with the approval of the undersigned.

I, P.E Gwala, declare that the dissertation has not previously been submitted by me for a degree at this or any other University, that this is my own work in design and in execution, and that all the material contained therein has been duly acknowledged.

P.E. Gwala	Prof A.R. Opoku	Prof O.A. Oyedeji
(Student)	(Supervisor)	(Co-Supervisor)
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ABSTRACT

Rapanea melanophloeos (L.) Mez is a medicinal plant that is used by Zulu traditional healers to manage blood-clot related diseases.Various extracts (methanol, n-hexane, chloroform, ethyl acetate and water) prepared from the bark of *R. melanophloeos* were screened for phytochemicals, antioxidant, and anti-platelet aggregation activity.

Phytochemical screening of the plant showed the presence of tannins, terpenoids, alkaloids, saponins, cardiac glycosides, flavonoids and phlobatannins**.** Steroids and anthraquinone were however not detected. The extracts strongly (>70%) scavenged 1, 1′-diphenyl-2-picryhydrazyl and 2, 2-azinobis 3-ethyl-benzothiazoline-6-sulfonic acid free radicals. The extracts had 91% chelating effect on Fe^{2+} ions and exhibited concentration dependent reducing power.

The extracts showed varying degrees of inhibition (21% to 97%) of rat platelet aggregation induced separately by thrombin, adenosine diphosphate (ADP) and epinephrine. The extracts further exhibited antiplatelets aggregation activity on enzymes (trypsin, papain and bromelain) treated platelets.

The lethality of the extracts was tested on brine shrimps larvae. Hexane and chloroform extracts had LC_{50} values of 1068.731 mg/ml and 3648, 349mg/ml respectively.

A triterpene (3β-Hydroxylanosta-9, 24-dien-21-oic acid) wasisolated and characterized (through various chromatographic techniques, extensive 1D and 2D NMR spectroscopy) from the ethyl acetate extract.

The triterpene exhibited antiplatelet aggregation, and inhibition of acetylcholinesterase activity. The cytotoxicity of the triterpene on two cell lines (HEK293 and HEPG2) gave LC_{50} values of 851.5 µg/ ml and 796.0 µg/ ml respectively.

The results suggested that the extracts of *Rapanea melanophloeos* could be considered as herbal treatment for disease associated with blood clotting.

CONTRIBUTION TO KNOWLEDGE

(See Appendix E)

Lazarus GG, Mosa RA, **Gwala PE**, Oyedeji AO, Opoku AR. "*In vitro* anti-platelet aggregation activity of the extracts of some Zulu medicinal plants". Greengold Symposium. 14-16 September 2009. Pretoria, SA

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DPPH - 1, 1′-diphenyl-2-picryhydrazyl radical

ABTS - 2, 2-azinobis 3-ethyl-benzothiazoline-6-sulfonic acid radical

ADP - Adenosine diphosphate

IC_{50} - Inhibitory concentration at 50%

LC_{50} - lethal concentration at 50%

NMR - Nuclear magnetic resonance spectroscopy

HEK293 - Human embryonic kidney cells

HEPG2 - Human hepatocellular carcinoma cells

WHO - World Health Organisation

TXA_2 - Thromboxane A_2

HCl - Hydrochloric acid

MeOh - Methanol

TCA - Trichloroacetic acid

BHA - Butylhydroxyanisole

BHT - Butylhydroxytoluene

DMSO - Dimethylsulfoxide

MTT - 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide

NaCl - Sodium chloride

EDTA - Ethylenediaminetetraacetic acid

ADA - Adenosine deaminase

$CaCl_2$ - Calcium chloride

CHCl3 - Chloroform

H_2SO_4 - Sulfuric acid

DTNB - 5, S'Dithiobis (2nitrobemoic acid)

PBS - Phosphate buffered saline

PVPP - Polyvinypolypyrrolidone

GPIb/IX - Platelet glycoprotein GPIb/IX complex

Factor II - Prothrombin

Factor VII - Proconvertin factor

Fctor X - Stuart- Power factor

Factor XI - Plasma thromboplastin antecedent

Factor XIII - Fibrin stabilizing factor

Factor XIIIa - Fibrin stabilizing factor A

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Chapter three

Chapter One

Introduction

Blood clot is a mass of blood cells and blood components that form to stop the bleeding that occurs when a blood vessel is injured. When a blood vessel is broken, platelets in the blood become sticky and clump together at the site of the injury. Clotting is the body's normal response to prevent a person from bleeding to death. When an injury occurs, a clot usually forms within 5 minutes after a blood vessel wall has been damaged (Elliot and Elliot, 2005).

However, blood clot formation can be dangerous if they occur within healthy blood vessels, or if they are not degraded when their work is done. Diseases like heart attack, stroke, pulmonary embolism and many others have been associated with inappropriate blood clot formation (Rebecca and Heather, 2001). Although there are some blood thinners such as aspirin, heparin and many others that are used in Western medicine, there have been reports of side effects such as carcinogenicity caused by synthetic drugs (Sanchez-Lamar *et al*., 1999). Thus, the interest in natural products has increased considerably in finding natural drugs. Phytotherapy has been considered an alternative to alleviate side-effects associated with synthetic drugs (Sanchez-Lamar *et al*., 1999). The World Health Organization (WHO) estimates that about 80% of the population living in the developing countries relies on traditional medicine for their primary health care needs (Mukherjee and Wahile, 2006).These plant-based medicines are used for primary health care needs. Between 25-50% of modern drugs are derived from plants. Medicinal plants are increasingly in demand in both developing and developed countries (de Silva, 1997). In an effort to improve the efficiency of the practice and to possibly incorporate it into modern medicinal

practices, researchers are increasingly turning their attention to evaluating the therapeutic potential of medicinal plants (McGraw *et al*., 1997).

This project aims to investigate the antiplatelet aggregation activity of extracts prepared from *Rapanea melanophloeos;* an attempt will be made to identify and characterize the active constituent there in.

Chapter Two

Literature review

2.1 Blood clotting

Blood is one of the most important tissues in the body; it consists of cells floating in plasma. It carries oxygen and nutrients to every cell of the body and removes waste products from the tissues. It also plays a central role in the body's defence against infectious agents (Wilson, 1997). Within the cells of the blood there are platelets that prevent excessive bleeding by forming a clot (Campbell, 2008).

Blood- clotting is a sequence of complex reactions that convert fluid blood into a gel that provides a secure area to an injured blood vessel. About thirteen coagulation factors are involved in coagulation. Most of these factors are proteins such as factor ii, vii,xi and x. These proteins are released by the liver into blood; they require Vitamin K to be synthesised (Elliot and Elliot, 2005). The process of blood clotting and then the subsequent dissolution of the clot, following repair of the injured tissue, is termed homeostasis and it consists of four main events that occur in a set order following the loss of vascular integrity (Marieb and Hoehn , 2007):

(i) the initial phase is vascular constriction; this process limits the flow of blood to the area of injury.

(ii) platelets become activated by thrombin and aggregate at the site of injury (forming a loose, temporary platelet plug).

Platelet clumping is stimulated by fibrinogen. Platelets become clumped by binding to collagen that becomes exposed after the endothelial lining of vessels has been ruptured. After activation, platelets release ADP and thromboxane A_2 (which activate more platelets), serotonin, phospholipids, lipoproteins, and other proteins important for the coagulation cascade. In addition to induced secretion, activated platelets alter their shape to accommodate the formation of the plug.

(iii) to assure constancy of the initially loose platelet plug, a fibrin mesh (clot) forms and entraps the plug.

(iv) lastly, the clot should dissolve so that the blood can circulate normally and freely, and the dissolution of the clot occurs through the action of plasmin.

There are two pathways that lead to the formation of a fibrin clot: the intrinsic and extrinsic pathway (figure 2.1). Although they are initiated by different mechanisms, they join on a common pathway that leads to clot formation. A clot in response to an abnormal vessel wall in the absence of tissue injury is the result of the intrinsic pathway. A clot formation in response to tissue injury is the result of the extrinsic pathway. These pathways are complex and involve many different proteins called clotting factors (King, 2008).

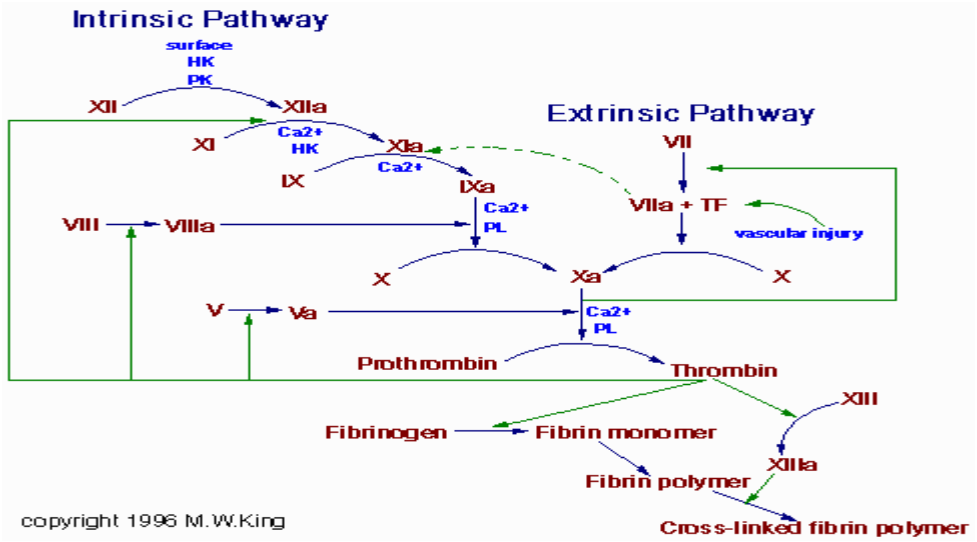

Figure 2.1 Schematic representation of both intrinsic and extrinsic pathways.

2.1.1 Platelet Activation

Platelets must be activated before they can aggregate and platelets get activated by adhering to the exposed collagen and releasing the contents of their granules, and then aggregate. The adhesion of platelets to the collagen exposed on endothelial cell surfaces is mediated by von Willebrand's factor (vWF), which is not present in von Willebrand disease (King, 2008).

The role of vWF is to work as a bridge between specific glycoprotein on the surface of the platelets (GPIb/IX) and collagen fibrils. It also a bridge between platelets and exposed collagen on endothelial surfaces, vWF binds to and stabilizes coagulation factor VIII. Binding of factor VIII by vWF is required for normal survival of factor VIII in the circulation.

Von Willebrand factor is a complex multimeric glycoprotein that is produced and stored in the a-granules of platelets. It is also synthesized by megakaryocytes and found associated with subendothelial connective tissue. The initial activation of platelets is induced by thrombin binding to specific receptors on the surface of platelets, thereby initiating a signal transduction cascade. The thrombin receptor is coupled to a G-protein that, in turn, activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol- 4,5-bisphosphate (PIP_2) leading to the formation of inositol trisphosphate$_3$(IP_3) and diacylglycerol (PLC-(DAG). IP_3 induces the release of intracellular Ca^{2+} stores, and DAG activates *protein kinase C* (PKC) (King, 2008).

2.1.2 Activation of Fibrinogen to Fibrin

Fibrinogen (factor I) consists of 3 pairs of polypeptides $([A\alpha][B\beta][\gamma])_2$. The six chains are covalently linked near their N-terminals through disulfide bonds. The A and B portions of the Aα and Bβ chains comprise the fibrinopeptides, A and B, respectively. The fibrinopeptide regions of fibrinogen contain several glutamate and aspartate residues imparting a high negative charge to this region and aid in the solubility of fibrinogen in plasma. Active thrombin is a serine protease that hydrolyses fibrinogen at four arg-gly (R-G) bonds between the fibrinopeptide and the **a** and **b** portions of the protein.

Thrombin-mediated release of the fibrinopeptides generates fibrin monomers with a subunit structure $(\alpha\beta\gamma)_2$. These subunits spontaneously aggregate in a regular array, forming a weak fibrin clot.

In addition to fibrin activation, thrombin converts factor XIII (fibrin stabilizing factor) to factor XIIIa (fibrin stabilizing factor a), a highly specific transglutaminase that introduces cross-links composed of covalent bonds between the amide nitrogen of glutamines and ε-amino group of lysines in the fibrin monomers (King, 2008).

Blood clots, however, can be potentially dangerous if they occur within healthy blood vessels, or if they do not dissolve when their work is done. Blood clots that develop in the arteries can cause heart attack, and stroke. Blood clots in the veins or venous system can cause deep venous thrombosis (DVT) in the pelvic, leg, and upper extremity veins. When these DVTs break off and travel through the bloodstream to the heart and then to the lung blood vessels, they cause acute pulmonary embolism (PE).

There are number of risk factors contributing to blood clots. Some of them include phlebitis (inflammation of a vein) , high cholesterol levels, diabetes, smoking, free radicals, family history of blood clotting and disorders in clotting or lack of mobility due to old age, being restricted to bed after surgery or illnesses, paralysis, obesity, pregnancy and body positions that slow blood circulation (Heather and Rebecca, 2001).

2.2 Free radicals

Free radicals are atoms or groups of atoms with unpaired number of electrons. Free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals (Shetti *et al,* 2009).

Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions which serve as sources of free radicals include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P450 system (Langseth, 1996). Some internally generated sources of free radicals are: mitochondria, phagocytes, xanthine oxidase, reactions involving iron and other transition metals, arachidonate pathways, peroxisomes, exercise, inflammation and ischaemia or reperfusion. Free radicals also arise in non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. Some externally generated sources of free radicals are: cigarette smoke, environmental pollutants, radiation, ultraviolet light, certain drugs, pesticides, anaesthetics, industrial solvents and ozone (Langseth, 1996). Fe^{2+} ions are known to stimulate the free radical reaction decomposing lipid peroxides to chain propagating alkoxyl radicals and also reacting with H_2O_2 to produce hydroxyl radicals and other highly reactive oxygen species (ROS) (Halliwell and Gutteridge, 1988).

Once formed these highly reactive radicals can start a chain reaction. Their destructive effects occur when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs.

Cancer and atherosclerosis, two major causes of death, are salient "free radical" diseases (Lea, 1966). Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances.

These compounds induce endothelial cell injury and produce changes in the arterial walls (Harman, 1992).

Platelets are activated upon exposure to Oxygen Free Radicals -generating systems, including those derived from polymorphonuclear leukocytes and red blood cells. Thus the presence of OFR in the surrounding medium stimulates platelet aggregation and may contribute to thrombus formation (Miller e*t al*., 1994).

2.2.1 Antioxidants

Antioxidants terminate the oxidation chain reaction initiated by free radicals by removing the free radical intermediate and inhibiting other oxidation reactions by being oxidized itself (Sies, 1997). Some antioxidants are produced in the body, while others must be obtained from the diet or supplemented. Antioxidants belong to different classes of compounds, such as caratenoids, polyphenolics, polyamides, garlic acid derivatives, tannins and catechins. Phenolic phytochemicals can be categorized into three namely: Non-flavonoid polyphenols, Flavonoids and Phenolic acids (Packer, 2002).

Recently, interest has increased in finding naturally occurring antioxidants to be used in food stuffs and medicinal materials, to replace synthetic drugs which are restricted due to their side effects. In addition natural antioxidants have the capacity to improve food quality and stability; they can also be used as nutriceuticals to terminate free radical chain reactions in biological systems and, may thus provide improved health benefits for the consumers (Brad-Williams *et al*.,1995).

A number of experimental models have been developed for the determination of antioxidant activities. They are classified into two major categories namely: measuring the potential of the sample to donate an electron or a hydrogen atom to a specific reactive species or to any electron acceptor and also measuring the ability to remove any source of oxidative initiation e.g. inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation (Vaya and Aviram, 2001).

Epidemiological (Stephens *et al* (1996) evidence strongly links high vitamin E intake to reduced risk of coronary heart disease possibly through its antioxidant activity, and its ability to inhibit prostaglandin synthesis. Stephens *et al* (1996) demonstrated that vitamin E treatment also significantly reduced the risk of cardiovascular deaths as well as non-fatal myocardial infarctions.

Western medicine provides certain anti-coagulants that are used as blood thinners (heparin, aspirin, warfarin, streptokinase and many others) (Sanchez-Lamar *et al*., 1999), but they are not locally available especially in rural areas. They are also not affordable to poor people and it is difficult to consult western doctors because it could be expensive (Heather and Rebecca, 2001). There is therefore a need for the research of new drugs that will be readily available and with fewer side effects.

2.3 Traditional healing

Traditional healing is the oldest form of structured medicine, that is, a medicine that has an underlying philosophy and set of principles by which it is practised.

Traditional healing makes use of locally available natural resources such as medicinal plants, soil, insects and animal parts to prevent and treat different ailments

and diseases (Deepak and Anshu, 2008). Traditional medicine is readily available and affordable (Mhame *et al*., 2000). Traditional healing is very diverse, it addresses a whole range of issues and it is based on the needs of individuals (Deepak and Anshu, 2008).

According to the World Health Organization, it is estimated that approximately 80% of the developing world's population meet their health care needs through traditional medicines (Mueller and Mechler, 2005).

Traditional healing has a holistic approach to health. Different people may receive different treatments even if, according to modern medicine, they suffer from the same disease. Traditional medicine is based on a belief that each individual has his or her own constitution and social circumstances which result in different reactions to causes of disease and treatment**.** A traditional healer makes a diagnosis based on a thorough examination of the patient and then prescribes a personalized medicine, usually consisting of a mixture of different ingredients. Studies have shown that an interest in alternatives to modern medicine is vastly increasing more than ever before, and a large part of that interest revolves around the use of medicinal plants (de Silva, 1997).

This is driven by an increasing demand worldwide of products that are 'all natural'. Assurance of the safety, quality, and efficacy of medicinal plant and herbal products has now become a key issue in industrialized and developing countries (WHO, 1997).

2.3.1 Medicinal plants

Medicinal plants are plants that are commonly used in treating and preventing specific ailments and diseases, and that are generally considered to play a beneficial role in health care (Srivastava *et al*.,1996). Traditional healers use different plants to treat and prevent different ailments and diseases. One plant may have one or more medicinal uses (Muthu *et al*., 2006). Most of the plants are collected from the wild and medicinal properties may be present in one or all of their parts: roots, stem, bark, leaf, fruit or seed (Jitendra *et al*.,1996).The therapeutic properties of many plants have been investigated (Gumede, 1989). Many plants have been scientifically proven to possess anti-platelet aggregation activity. Such plants include *Capsicum frutescens* (Wang *et al*., 1984), *Ginkgo biloba* (Gholam *et al., 2005) Vaccinium myrtillus* (Bottecchia, *et al*. (1987), *Curcuma longa* (Lee, 2005), and *Zingiber officinale* (Ying *et al*., 1998). Onion (*Allium sepa*) and garlic (*A. sativum*) help to reduce fibrin and platelet stickiness (Wood, 1988).

In trying to improve the efficacy and the use of medicinal plants, more researchers have turned their attention towards evaluation of the potential therapeutic activities of some Zulu medicinal plants. Zulu traditional healers were interviewed (see Appendix C) about medicinal plants used to treat platelet aggregation related diseases and *Rapanea melanophloeos* was highly recommended among others by different traditional healers. Traditional healers mentioned that most of these plants do not work alone; they either work in combination with other plants or other ingredients such as animal parts.

2.3.1.1 Rapanea melanophloeos (L) mez

Rapanea melanophloes (Figure 2.2) is an evergreen tree that grows from 4 up to 18 meter high. The mature leaves are leathery and dull, dark green, paler below. They are simple, oblong-lanceolate, about 100 mm long, with reddish leafstalks and are clustered mostly at the last part of the branches. When they are young, leaves are pale green and maroon. Small, whitish or creamy yellow clusters of flowers appear on the branchlets in June to December (Palmer and Pitman, 1972) .The fruit are thinly fleshed and round in shape, green when young and purple when matured. It is not uncommon to find flowers and fruit on the same tree. Fruit start appearing three months after the flowers. *R. melanophloes* is not threatened or endangered; this is attributed to its wide distribution (van Wyk *and van Wyk,* 1997).

Figure 2.2 *Rapanea melanophloeos* (Pooley, 1993)

R. melanophloes is widely distributed throughout southern Africa from the southern Cape to Zambia, and from the east coast to the tropics. It is found in damp areas of

mountain and coastal forests or swamps and bush clumps. It survives well in coastal areas where winds are strong; it is fairly drought tolerant (Pooley, 1993).

R. melanophloes and *R. gilliana* are the two known species of *Rapanea* in South Africa (Palmer and Pitman, 1972). In the coast of the Eastern Cape. *R. gilliana* is a small tree compared to *R. melanophloeos.* The flower attracts bees and flies, the fruit are eaten by birds, baboons and vervet monkeys (Van Wyk and van Wyk, 1997).

The grey bark or sometimes roots are used medicinally for respiratory problems, stomach, muscular and heart complaints. The bark contains tannin and is used as *iNtelezi* (a charm to protect against evil spirits) by Nguni people (van Wyk *et al*., 1997).

Triterpenoids saponins isolated from *Rapanea Melanophloeos* has been scientifically proven to have molluscicidal and antifungal activities (Ohtan *et al*, 1992). Anthelmintic activity against the nematode parasite, Haemonchus contortus, of sheep has been reported (Githiori *et al.*, 2002).

2.4 Acetyl choline

Acetylcholine (ACh) is a neurotransmitter in both the peripheral nervous system (PNS) and central nervous system (CNS) in many organisms including humans. (Whittaker, 1990).

In the peripheral nervous system, acetylcholine activates muscles.In the central nervous system, acetylcholine and the associated neurons form a neurotransmitter

system, the cholinergic system, which tends to cause anti-excitatory actions (Purves et *al*., 2008).

Acetylcholine reduces the supply of blood clotting substances into the blood stream (Sokratov and Skipetrov, 1977).

2.4.1 Acetyl choline esterase

Acetyl cholinesterase, (AChE), is an enzyme that hydrolyses acetylcholine to choline and acetate. It is mostly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to terminate synaptic transmission (Voet et al., 1995). Acetyl cholinesterase is also found on the red blood cell membranes, where it constitutes the Yt blood group antigen (Purves *et al*., 2008).

Acetyl cholinesterase is the target of many Alzheimer's Dementia drugs, nerve gases, particularly the organophosphates (e.g. Sarin) and insecticides (e.g.carbaryl). These agents are known as cholinesterase inhibitors because they block the function of acetyl cholinesterase and thus cause excessive acetylcholine to accumulate in the synaptic cleft. The excess acetylcholine causes neuromuscular paralysis (i.e. interminable muscle contractions) throughout the entire body, leading to death by asphyxiation (Purves *et al*., 2008).

Some plants (*Pistacia atlantica and P. lentiscus*) have been reported to have acetylcholinesterase (AChE) inhibitory activity (Benamar *et al*., 2010).

2.5 Isolation and Purification

2.10.1 Thin Layer Chromatography (TLC)

TLC is a technique used for separation, qualitative identification or for the semi quantitative visual analysis of samples.TLC uses a thin layer of a material such as silicon dioxide or alumunium oxide coated into a glass, plastic,or alumunium plate. The stationary phase is a solid and a mobile phase is a liquid. It has been widely used in various fields of study such as in the analysis of natural and synthetic steroids in various environmental materials focuses mainly on steroid analysis in environmental materials such as pharmaceuticals, plant products and other biological specimens (Bhawini *et al*., 2010).

2.10.2 Column chromatography (CC)

Column chromatography is a convenient technique for physically separating the components of a mixture. The stationary phase is packed into a vertical column and the mixture is applied to the top of the column, followed by solvent which runs through under gravity. The components of the mixture adsorb onto the surface of the solid to different extents. Different components emerge from the bottom of the column at different times. Column chromatography is widely employed in the various fields of research, for example, in the isolation and characterization of proteins, in the separation of vitamins, steroids, hormones and alkaloids and also to determine the amount of these substances in samples of body fluids (Dale, 1991).

2.10.3 Melting point

Melting point is used to identify a solid compound which has been isolated in the laboratory, by comparing its melting point with that of the true compound. It is widely used in different areas of research for example, it has been used in the synthesis, charatrization and biological activities of ureas and thioureas derivatives (Edrah, 2010).

2.10.4 IR (Infrared spectroscopy)

Infrared spectroscopy (IR) is a technique that is used in chemical analysis for the identification of substances and characterization of biomolecules. Infrared spectroscopy is based on the measurement of the molecular bond vibration compounds, excited by radiation of a suitable frequency, when given the conditions for energy absorption by the molecules. IR determines different functional group e. g OH, -C=O and other groups present in a molecule. IR have been used in many studies including validation of the presence of catechin in the plant extracts (Maoela *et al.,* 2009*)*.

2.10.5 Ultraviolet - visible spectrophotometer

Ultraviolet - visible spectrophotometer gives information on chromophores that are found in a molecule e. g isoquinoline alkaloids, flavonoids and others can be characterized from characteristic absorption peaks.UV-Vis have been used to validate the presence of catechin in the plant extracts (Maoela *et al.,2009*).UV was also applied in the determination of vitamin C(ascorbic acid) which is an important precursor of redox mechanisms which is used in medicine and in food product (Hussain et al.,2010).

2.10.6 NMR (Nuclear Magnetic Resonance)

NMR gives information about the number and types of protons and carbon found in a molecule and the relationships among them. The sample is placed in a very strong magnetic field and its absorption of electromagnetic radiation is measured. The nuclei of certain atoms, especially hydrogens, absorb strongly under this condition. The two nuclei that are often studied in NMR spectroscopy are hydrogen (^{1}H) and carbon (^{13}c). NMR has been successfully utilized in several areas such as the analysis of natural product extracts (flora and fauna), combinatorial peptides mixtures, food extracts and polymers (Albert, 1999).

2.6 Scope of the work

2.6.1 Aims:

This project aimed to investigate the antiplatelet aggregation activity of extracts prepared from *Rapanea melanophloeos;* an attempt was made to identify and characterize the active constituent therein.

2.6.2 Objectives:

The objectives of the research included the following:

- Interviewing of traditional healers;
- Collection and identification of the plant materials (preparation of voucher specimen);
- Preparation of the plant's extracts
- Screening of plant for phytochemicals;
- Screening of extracts for anti-oxidant and anti-platelet aggregation activity;
- Isolate and characterize the active components of the most active extract;
- Screening of isolated compound for anti-oxidant and anti-platelet aggregation activity
- Cytotoxicity of the crude extract and the isolated compounds will be investigated and the effect of the isolated compound on the activity of Acetylcholine esterase will also be investigated.

Chapter Three

Materials and Methodology

The materials and methods that were used to obtain and prepare plant extracts, screen for photochemicals, antioxidant activity, and anti-platelet aggregation activity are presented in this chapter. The chapter also describes briefly the isolation and characterization of a bioactive compound from the plant. The details of the reagent preparation and methods are presented in the Appendix A and B respectively.

3.1 Materials

3.1.1 Equipment

- Rotary evaporator—Laborota 4000, Heidolph **(**Polychem supplies**)**
- Spectrophotometer—Spekol 1300 **(**Polychem supplies**)**
- Centrifuge—5404R Eppendorf **(**Merck**)**
- Grinder—IKA)
- Freeze-driyer—VirTis Benchtop K (Polychem supplies)
- Platform shaker—Labcon (Polychem supplies)
- Biotek ELx 808 UI plate reader (Biotek Instrument supplies)
- U-bottom 96-well plate (Sigma)
- pH meter **(**Hanna Instruments**)**
- Barnstead or Electothermal digital melting point apparatus (Thermo Scientific).
- Columns of different sizes,(Merck)
- Nuclear magnetic resonance (Bruker),
- Infrared spectroscopy (Perkin Elmer),

- Ulta-Violet visible spectroscopy (Perkin Elmer)

3.1.2 Chemicals/reagents (See Appendix A for reagent details)

Unless otherwise stated, all the chemicals and reagents used were of the analytical grade. The solvents used for the extraction were double distilled.

These chemicals were obtained from Sigma-Aldrich Co., Ltd (Steinheim, Germany)

1,1′-diphenyl-2-pictyhydrazyl, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), Dimethyl sulfoxide, Papain, Trypsin, Bromelain, Thrombin , Epinephrine, Adenosine diphosphate, Methanol, n –hexane, Ethyl acetate, Glucose, Dextrose, Trizma HCl, 2-Thiobarbituric acid, Ferric chloride, Citric acid, Ferrozine (Benzenesulfonic acid, 4,4'-(3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl) bis -, disodium salt), Deuterated chloroform (Merck), Ferrous chloride, Acetic anhydride, Mercury (II) chloride, Potassium iodide, Subnitrate bismuth, Potassium persulfate. Potassium ferrocyanide.

Merck supplied the following chemicals

Glacial acetic acid, n-butanol, trichloroacetic acid, tulphuric acid, hydrochloric acid, tri-Sodium citrate, sodium hydroxide, sodium chloride, calcium chloride, chloroform, lead acetate, silica gel 60 0.040-0.063 mm (230-400 mesh ASTM), silica gel 60 0.063-0.200 mm (70-230 mesh ASTM), silica gel 60 0.2-0.5 mm (30-70 mesh ASTM), acid purified sand, TLC aluminium sheets 20x20 cm Silica gel 60 F_{254},

Chemicals and reagents obtained from other sources include:

- Benzene **(**SAAR Chem**)**
- Diethyl ether **(**NT laboratory supplies**)**
- Ammonium solution **(**NT supplies**)**
- S-2238 (Chromogenix) **(**Instrumentation Laboratorycompany**)**
- diPotassium hydrogen phosphate **(**Associated chemical enterprises**)**
- Ethylenediaminetetra-acetic acid **(**Associated chemical enterprises**)**
- Sodium dihydrogen phosphate **(**Lab. consumables and chemicals supplies**)**
- disodium hydrogen phosphate **(**Lab. consumables and chemicals supplies**)**

3.2 Methods (See Appendix B for details)

3.2.1 Collection and preparation of plant materials:

Dry bark of the *Rapanea melanophloeos (L.) Mez* were collected from the *muthi* market at Empangeni , KwaZulu Natal in South Africa and taken to the Botany Department, at the University of Zululand (UZ) for identification. Voucher specimen (PE001) was deposited at the herbarium.The plant materials were thoroughly washed, cut into smaller pieces, and dried at 50±2 ^{o}C for 24 hrs. The plant materials were ground into powder (2 mm mesh) and stored in sterile brown glass bottles at –4^{o}C until used.

3.2.2 Extraction:

The dry powdered plant material was extracted (1:5 w/v) sequentially with different solvents (n- hexane, chloroform, ethyl acetate, methanol and water) according to their increasing polarity (See figure 3.1). The plant material and solvent mixture was

incubated at room temperature on the platform shaker (150 rpm) for 24 hrs. The extracts were filtered through Whatman No. 1 filter paper.

The organic solvent filtrates were evaporated to dryness on a rotary evaporator at 45±2^{o}C and the aqueous extract was freeze-dried. Each extract was re-suspended in the respective solvent of extraction to yield a stock solution. The yields of the plant extracts were recorded and the extracts then stored in sterile glass bottles at 4^{o}C.

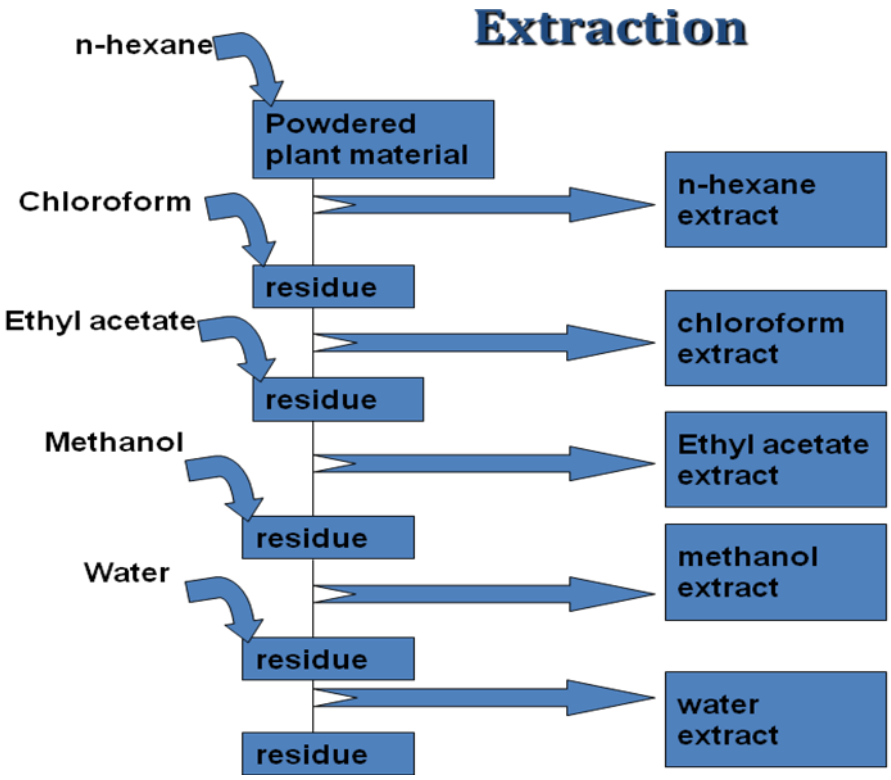

Figure 3.1 Sequential extraction method.

3.2.3 Phytochemical screening:

The extracts were screened for the following phytochemicals: saponins, tannins, steroids, terpenoids, alkaloids, anthraquinones, flavonoids, cardiac glycosides and

phlobatannins using methods described by Harbone, (1973),Odebiyi and Sofowora (1978).

3.2.3.1 Determination of total phenolics

The total phenolics in the extract were determined using Folin- Ciocalteu method as described by Kujala *et al*. (2000). To each sample solution (1.0 ml) and the standard (gallic acid) was added Folin-Ciocalteu (5 ml) and Sodium carbonate (7% w/v, 4 ml) and shaken. The solution was allowed to stand in the dark at room temperature, after which absorbance was measured at 765 nm using a spectrophotometer. The amount of total phenolics was expressed as gallic acid equivalent.

3.2.3.2 Determination of total flavonoid

Flavonoids were determined by preparing different concentration of quercetin (0.01-0.1 mg/ml) in diethyl ether the extracts (0.5 ml) was mixed with 1ml of diethyl ether. The residue was dissolved in 0.5 ml of 2% $ALCL_3$ (in 80% EtOH). The solution was allowed to stand for one 1 hour at room temperature (a yellow colour indicate the presence of flavonoids). The absorbance was measured at 420 nm. The amount of total flavonoids was expressed as quercetin equivalent in milligram per gram dry plant extract (Ordon *et al*., 2006).

3.2.3.3 Determination of Proanthocyanidin

Proanthocyanidin content in the extracts was determined by preparing cetechin in diethyl ether (0.01-0.1 mg/ml).The extract (0.5 ml) was mixed with diethyl ether. The residue was dissolve in 3ml of 4% vanillin-MeOH solution (80%) and 1.5 ml of 1%HCL. The solution was allowed to stand for 15 min at room temperature. The

absorbance was measured at 500 nm. The amount of total flavonoids was expressed as cetechin equivalent in milligram per gram dry plant extract (Sun *et al.*, 1998).

3.2 4 *In vitro* Antioxidant activity

3.2.4.1 Free radical scavenging activity

The potential of the plant extracts to scavenge free radicals was separately tested using DPPH, ABTS.

3.2.4.1. 1 1,1-diphenyl-2-picryl hydrazyl (DPPH) Scavenging activity

DPPH radical scavenging activity of plant extract was determined spectrophotometrically in the absence and presence of different concentrations (0-5mg/100ml) of plant extracts (Brad-Williams, 1995). A mixture of 2ml plant extract and 2ml DPPH (2mg% in methanol) was incubated for 30min and the absorbance read at 517nm.

3.2.4.1.2 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate (ABTS) radical scavenging activity

ABTS radical scavenging activity of the plant extracts was determined spectrophotometrically (Pellegrini, *et al,* 1999). ABTS* was generated by incubating in the dark 7 mM of ABTS and 2,45 mM potassium persulfate which resulted in the production of radical cation ABTS*. The ABTS* was diluted (1:60 methanol, v/v). Various concentrations (0-5 mg/100 ml) of the extracts (1 ml) were mixed with 1 ml of ABTS* and the absorbance at 734 nm read after 6 min incubation.

3. 2. 4. 2 Reducing power:

The reducing power of *the plant* extracts was evaluated according to the method described by Oyaizu (1986). To 2.5 mL of different concentrations (0-5 mg/100 ml) of extract in methanol was added 2.5 mL of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% potassium ferricyanide $K_3Fe(CN)_6$. The mixture was incubated at 50 ^oC for 20 min, 2.5 mL of 10% TCA was added to the mixture and centrifuged at 1000 rpm for 10 min.The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL,0.1%).The absorbance was measured spectrophotometrically at 700 nm. The higher the absorbance value the stronger the reducing power.

3. 2.4.3 Chelating activity on Fe^{2+}:

The Fe^{2+} chelating effect of the plant extracts was measured according to the method of Decker and Welch (1990). To 0.5 mL of various concentrations (0-5 mg/100ml) of extract in methanol, 1.6 mL of deionized water and 0.05 mL of $FeCl_2$ (2 mM) were added. After 30 s, the reaction was initiated by the addition of 5 mM ferrozine (0.1 mL). Then, the mixture was shaken and left at room temperature for 10 min. Absorbance of the mixture was measured spectrophotometrically at 562 nm. Citric acid (CA) and EDTA were used as standards.

3.2.4.4 Calculation of antioxidant activity

Unless otherwise stated, ascorbic acid, and BHA were used as standards. All assays were in triplicate and the mean± S.E reported.The inhibitory effect of the extract on each parameter was calculated as:

%inhibition = $\{(A_o-A_1)/A_o \times 100\}$

Where A_O is the absorbance of the control and A_1 is the absorbance of the extracts. The inhibitory concentration providing 50% inhibition (IC_{50}) was calculated from the graph of percentage inhibition against extract concentration.

3. 2.5 Reconstitution of extracts in DMSO:

The plant extracts were evaporated to dryness at 46 ± 2 ^{o}C and the extracts were reconstituted in minimal amounts of DMSO. The extracts were again stored in sterile glass bottles and kept in the fridge.

3. 2.6 Toxicity testing against the brine shrimp

The toxicity assay was determined using the brine shrimp lethality test according to Meyer *et al*. (1982). The shrimp**'**s larvae were placed in multiwelled culture plates. The cultured plate and test solution were added to each well. Saline solution with 3% Tween 20 and DMSO was used as a control solution without extract to which shrimp larvae were added. Lethality concentration (LC_{50} values) for each assay was calculated by taking average of three experiments using a Finney Probit analysis program on an IBM computer (McLaughlin *et al*., 1998).

3.2.7 MTT cell proliferation assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] cell proliferation assay (Mosman, 1983) was used to determine the cytotoxicity of the compound MS/12. The cytotoxicity of the compound MS12 was tested in human embryonic kidney (HEK293) and human hepatocellular carcinoma (HEPG2) cells. The cells were seeded in a 48-well plate at a density of 2.5 x 10^4 cells per well. Following an overnight incubation at 37^{o}C, the cells were incubated with the compound at different concentrations (50, 100, 150, 200, 250, 300, and 350 µg/200µl) in medium (MEM +

Gutamax + antibiotics + 10% fetal bovine serum) for 48 h. Thereafter, the medium was removed from the cells and 200µl MTT solution (5 mg/ml PBS) as well as 200 µl of cell culture medium was added to the corresponding wells. The cells were incubated at 37^{o}C for 4 h and the reaction was terminated by addition of DMSO (100/200/400 µl). The cells viability was determined spectrophotometrically (Biomate spectrophotometer) at 570 nm.

The experiment was done in triplicate and the results were expressed as mean ± SD. Percentage inhibition of cell growth was calculated as:

% cell death= $[(A_c – A_t)/A_c \times 100]$

where A_c is the absorbance of control and A_t is the absorbance of the extract.

Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics programme.

3. 2.8 Anti-Platelet aggregation activity

The plant extracts were tested at concentrations of 1, 3 and 10 mg (dry weight)/ml (DMSO).

3. 2.8.1 Anti-thrombin activity (Chromogenics: S2238)

The extracts, solubilized in DMSO were diluted to a final DMSO concentration of 1% (v/v) with Tris-HCl buffer (50 µl Tris -HCL, PH 7.4, containing 7.5 Mm EDTA and 175Mm NaCl). The plant extracts (50 µl) was added to 10 µl of thrombin. The mixture was left for 10 min at room temperature and 190 µl of 0.76 M s2238 was added. The reaction was recorded at 412 nm for 4 min at 10s intervals using a

Biotek ELx 808 UI plate reader plate reader (Rob *et al*, 1997). A negative control was done using 2% (v/v) DMSO in saline which represented 100% activity.

3. 2.8.2 Blood platelets:

Ethical clearance for the use of animals in this study was obtained from the research animal ethics committee of the University of Zululand (see Appendix C) The rats blood was collected using the method of Tomita *et al* (1983). The rats were killed by a blow to the head**.** The blood was immediately collected from the abdominal aorta and put into a centrifuge tube containing ADA (1 ml ADA: 5 ml blood). The blood was centrifuged at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively. Supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was discarded and sediment (platelets) obtained was resuspended in 5 ml of washing buffer (pH 6.5). This was centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the platelets suspended in a little volume of a resuspending buffer (pH 7.4). The platelets was kept in the fridge and used within 4 hrs.

3. 2.8.3 Enzyme treated platelets

The enzymes—trypsin, bromelain, papain (0.1mg) were incubated separately with 25ml of a 2% suspension of platelets for 60min at 25ºC. The cells were then washed (3 times) and a 2% suspension was prepared.

The platelets were divided into four (4) parts: untreated and enzyme (trypsin, bromelain, papain) treated platelets.

A 1: 10 dilution of the platelets in the resuspending buffer was made and each portion (the 4 parts of platelets) was treated as follows: to 0.4 ml of diluted platelets, was added 10 µl of $CaCl_2$ was added and this was mixed (Tomita *et al*., 1983).

3. 2.8.4 Antiplatelet activity:

The inhibitory activity of the plant extracts was tested separately on thrombin, ADP and epinephrine induced platelet aggregation. It was also tested on enzyme (trypsin, bromelain, papain) treated platelets. The plant extracts (40 µl) was added to rat platelets(100 µl) and left at room temperature for 5 min. Thrombin/ADP/epinephrine (20 µl) was added and the inhibition of blood clot was measured at 412 nm using a Biotek ELx 808 UI plate reader for 20 min at 30s intervals (Hauptmann *et al* 1980)**.** The activity of the extracts was expressed as % of mean inhibition ± standard deviation (n=2). IC_{50} values were obtained by probit analysis.

3. 2.9 Tannin removal

The tannins were removed following the method of Toth and Pavia (2001). Plant extracts were prepared at a concentration of 10 mg/ml in dH_20. PVPP was added to the extract at 10 mg/ml, shaken for 15 min at 4^oC, followed by centrifugation at 3645 g for 8 min at 4^oC. The pellet was discarded. Using the supernatant, the procedure was done several times to remove tannins to a negligible concentration.

The tannin-free extracts were then screened for anti-platelet aggregation activity.

3. 2.10 Isolation and identification of active compound

Ethyl acetate extract**,** as the most active extract**,** was subjected to isolation and purification techniques using Thin Layer and Column chromatography

3. 2.10.1 Thin Layer Chromatography

A small amount of the ethyl acetate extract was dissolved in 5 ml of the Ethyl acetate solvent. TLC of the extract was carried out, using different solvent systems of 100% n-hexane, 9:1, 8:2, 7:3, 5:5 and 1:9 n-hexanes: ethyl acetate. A maximum number of 11 compounds appeared on the TLC plate after spraying with 10% sulphuric acid and placing the TLC plate in the oven for about 10 min.

3. 2.10.2 Column Chromatography

Silica gel 60 0.063-0.200 (70-230 mesh ASTM) and silica gel 60 0.04-0.063 (230-400 mesh ASTM) were used for the column chromatography.The length of the column was 50 cm, the diameter was 50mm and the flow rate was 40 ml/5 min. About 80 fractions of 40 ml were collected using solvent systems of 100% n-hexane, 9:1, 8:2, 7:3, 5:5 (n-hexane:ethyl acetate)**.** The TLC of each fraction was done. Those with a corresponding R_f values were bulked and concentrated *in vacuo*. Fractions 45-53 (96:4 n-hexane: ethyl acetate) showed nine similar spots. These spots had a retention factor of 0.29.

3. 2.10.3 Purification of a compound

The solvent was allowed to dry completely and brownish yellow crystals with a mass of 165 mg were formed.The crystals were further purified by dissolving them in

methanol after which the yellow part dissolved in methanol and the white crystals were left.

3. 2.10.4 TLC of the white crystals

When dissolved in various solvents and spotted on the TLC plate, a single yellow spot was observed after spraying with 10% sulphuric acid and heating in the oven. The white crystals, weighing about 161 mg, were obtained. Melting point measurements were done and the compound was taken for further analyses with UV-Visible and NMR.

3. 2.11 General experimental

IR spectra were obtained from a Perkin Elmer Spectrum 100 FTIR with ATR Sampling Accessory instrument, using $CHCl_3$ (Merck) as solvent. ^{1}H (400 MHz) and ^{13}C NMR (600 MHz) spectra were recorded on a Bruker Avance lll 400 or 600 apparatus using $CDCl_3$ (Merck) as solvent. Chromatograms were examined under UV light (366 and 254 nm) using a UV-viewing cabinet and by spraying with 10% H_2SO_4.The melting point was done on a Barnstead or Electrothermal digital melting point apparatus using a powder sample.

3.2.12 Biological Activities of the isolate (3β-Hydroxylanosta-9, 24-dien-21-oic acid)

The purified compound was screened for Antioxidants **(3.2.4)**, antiplatelet aggregation activity **(3.2.7)** and Acetylcholine esterase activity **(3.2.10.1)**.

3.2.13 Effect of 3β-Hydroxylanosta-9,24-dien-21-oic acid on Acetylcholine esterase activity.

The effects of the isolate on acetylcholinesterase activity were performed using acetylthiocholine iodide as substrate following the method of Ellman *et al* (1961). The fish were pitched and brain was carefully removed. The brain tissues were weighed and then homogenized in phosphate buffer pH 8.The ground brain tissue was then diluted with phosphate buffer to obtain 20 mg of brain tissue per one millilitre of buffer. The tissue was centrifuged at 1000*g for 20 min and supernatant was analyzed immediately. Supernatant was pipetted into a test tube, phosphate pH, 8.0 buffer was added. Then DTNB reagent was added and mixed well. 20 µl of 5 mM s-acetyl- thiocholine iodide (ASChI) solution was added quickly and mixed well. The absorbance was recorded at 60 second intervals. The yellow colour produced by enzymatic reaction resulted in changes in absorbance. The reaction rate was calculated in units of changes in absorbance per minute.The absorbance was measured at 421 nm.

Chapter Four

Results

The results obtained from the phytochemical screening, anti-oxidative activity, cytotoxicity, and anti-platelet aggregation activity of the crude extracts; the isolation of active components, and the screening of the isolated compound for anti-oxidative activity, anti-platelet aggregation activity acetylcholinesterase inhibitory activity are presented here.

4.1 Extraction

The powdered bark of *R. Melanophloeos* was extracted sequentially at room temperature in hexane, chloroform, ethyl acetate, methanol and water.

The percentage yields of the plant extracts obtained from the sequential extraction are presented in table 4.1. The methanol extract gave the highest yield of 34%

Table 4.1: % Yields of extracts.

Plant extracts	% Yields (w/w, dry wt basis)
Hexane	4
Chloroform	1.5
Ethyl acetate	1.4
Methanol	34
Water	11.5

4.2 Phytochemical screening:

The table 4.2 presents the results of the phytochemical analysis**.** The plant contained all the phytochemicals that were screened for except steroids and anthraquinone. The quantitative values of total phenol, flavonoid and proanthocyanidin are presented in table 4.3. Flavonoids were the dominating phenolic class in water extracts, the phenolic contents was dominant in the methanol extract and proanthocyanidin content was dominant in the hexane extract.

Table 4.2 : Phytochemicals of *R melanophloeos*

Phytochemicals	*R.melanophloeos*
Saponins	+ +
Tannins	+ +
Steroids	_
Alkaloids	+ +
Antraquinones	_
Flavonoids	+ +
Cardiac glycosides	+ +
Phlobatannins	+ +
Terpenoids	+ +

(+ + strongly positive results) and (-negative results)

Table 4.3: The total phenol, flavonoid and proanthocyanidin contents of the extracts of *R. Melanophloeos.*

Extracts	Flavonoid (mg/g)	Total phenol (mg/g)	Proanthocyanidin (mg/g)
Hexane	0.8 ±0.006	0.4 ±0.060	0.8 ±0.046
Chloroform	0.8 ±0.016	1± 0.005	0.6 ±0.078
Ethyl acetate	0.7 ±0.004	1 ±0.020	0.5 ±0.095
Methanol	1.4 ±0.012	1.2 ±0.025	0.4 ±0.023
Water	0.9 ±0.078	1±0.098	0.2 ±0.045

4.3 Anti-oxidant activity

The antioxidant activities were assessed by measuring the ability of the plant extracts to scavenge the DPPH, ABTS radical as well as the ability to reduce ferric ions; the reductive potential of the extracts was also evaluated. The results are presented in table 4.4

Table 4 .4: IC_{50} (mg/100 ml) of the extracts and standards on DPPH, ABTS radical and chelating activity.

	Hexane	Chloroform	Ethyl acetate	Methanol	Water	BHT	Ascorbic acid	EDTA	Citric acid
DPPH	**3.05**	**4.32**	**4.07**	**3.31**	**4.83**	**4.57**	**4.06**		
ABTS	**3.31**	**3.22**	**2.79**	**1.44**	**5.69**	**3.43**	**3.37**		
Chelating activiy	**4.23**	**3.55**	**0.93**	**3.22**	**6.4**			**3.31**	**3.89**

Hexane extract exhibited the highest scavenging activity on DPPH radical compared to other extracts and standard antioxidants. Methanol extract strongly scavenged ABTS radical compared to other extracts and its scavenging activity was even higher than that of the standards. Ethyl acetate showed the highest chelating activity which was higher than that of other extracts and standards.

The potential of the plant extracts to donate electrons and neutralize free radicals was evaluated and the results are shown in fig 4.1

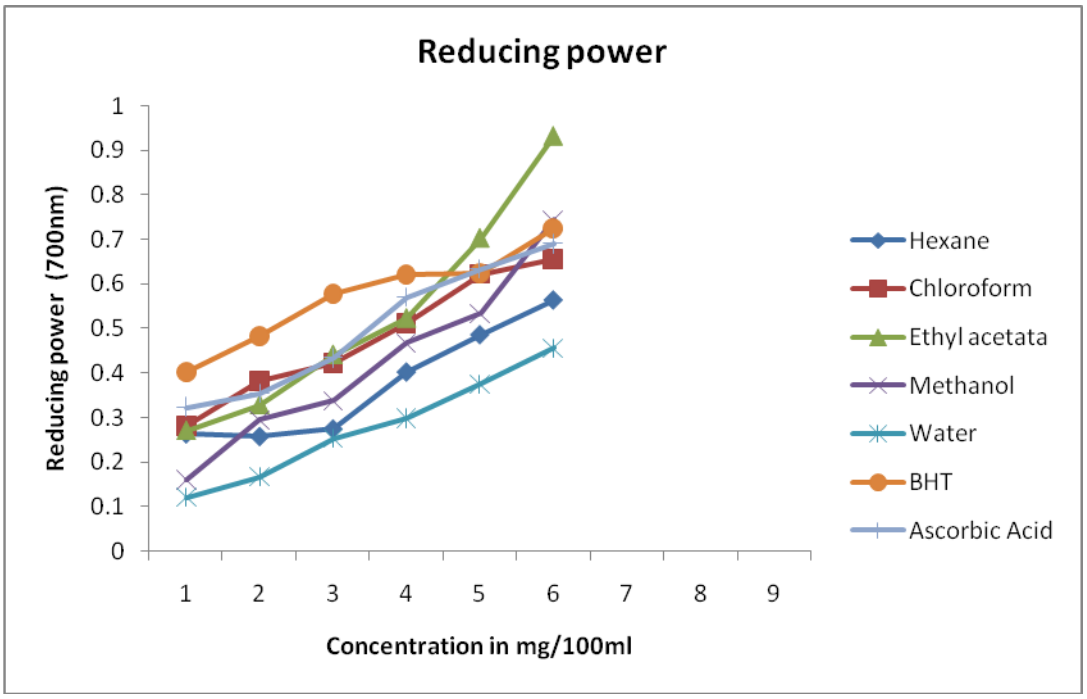

Figure 4.1: Reducing power of the extracts with BHT and ascorbic acid as standard antioxidants.

The graphs shows that the reducing power of the plant extracts increased as the concentration increased. Ethyl acetate had the highest reducing power compared to other extracts.

4.4 Cytotoxiity

The toxicity of the plant extracts were assessed using the brine shrimp lethality test. The lethal concentration at 50% was calculated and the results are presented in table 4.5

Table 4.5: Brine Shrimp lethality test. LC_{50} of extracts after 24hrs.

Extracts	LC_{50} (mg/ml)
Hexane	1068.731
Chloroform	3648.346
Ethyl acetate	41.58(9.28-2032)
Methanol	346.32
Water	30.930(15.42-66.48)

The LC_{50} of the extracts indicated that hexane and chloroform extracts were apparently not toxic to the shrimps**.** methanol extract was weakly toxic where as the water and ethyl acetate extracts were toxic to the shrimps.

4.5 Antiplatelet aggregation activity:

The extracts were first tested for anti-thrombin activity using an artificial thrombin substrate (S2238).The inhibitory activity of the extracts was also tested on thrombin, ADP, and epinephrine induced platelet aggregation. The extracts were further tested on enzyme (trypsin, bromelain, papain) treated platelets. The inhibitory activity of tannin-free extracts was also tested. The results are presented in table 4.6 – 4.18 as % inhibition± SD.

Table 4.6: The activity of the extracts on S2238.

S2238			
Extracts	Concentration (mg/10ml)	% Inhibition ± SD	IC_{50}
	control	0.0±0,346	0.0
Hexane	1	0.0±0,282	0.0
	3	0.0±0.324	
	10	0.0±0.057	
Chloroform	1	0.0±1.234	0.0
	3	0.0±0.0801	
	10	0.0±2.864	
Ethyl acetate	1	45±0.005	1.11
	3	84±0.082	
	10	98±0.043	
Methanol	1	0.0±0.001	2.4
	3	34±0.201	
	10	56±0.007	
Water	1	0.0±0,13	0.0
	3	0.0±0.11	
	10	0.0±0.105	

Ethyl acetate and methanol extracts inhibited thrombin activity. Hexane, chloroform and water did not show any activity against thrombin activity.

Table 4.7: Inhibitory activity of the extracts (tannins and without tannin) on thrombin induced platelets aggregation

Inhibitory activity of extracts with tannin and tannin free extracts on untreated platelets induced by thrombin					
Extracts	Concentration (mg/10ml)	% Inhibition ± SD (extracts with tannins)	% Inhibition ± SD (tannin free extracts)	IC_{50} (extracts with tannin)	IC_{50} (tannin free extracts)
	Control	0.0±0.00	0.0±0.001	0.0	0.0
Hexane	1	31±0.008	0.0±0.2		0.0
	3	35±0,002	0.0±0.003	7.1	
	10	51± 0,006	0.0±0.08		
Chloroform	1	43±0.03	0.0±0.005	1.45	0.0
	3	73±0.27	0.0±0.801		
	10	83±0.012	0.0±0.07		
Ethyl acetate	1	46±0.008	34±0.001	1.15	2.40
	3	94±0.043	56±0.121		
	10	97±0 011	78±0.097		
Methanol	1	0.0±0,001	0.0±0.03	0.0	0.0
	3	0.0±0.01	0.0±0.402		
	10	0.0±0.02	0.0±0.005		
Water	1	37±0.32	0.0±0.306	2.83	0.0
	3	51±0.16	0.0±0.45		
	10	63±0.43	0.0±0.45		

All the extracts were able to inhibit platelet aggregation induced by thrombin except methanol extract. Ethyl acetate had a highest activity and its IC_{50} was 1.15 mg/ml. Only ethyl acetate retained its inhibitory activity after the removal of tannins.

Table 4.8: Inhibitory activity of the extracts on papain treated platelets aggregation induced by thrombin.

Thrombin papain treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition ± SD	IC_{50}
	control	0.0±0,042	0.0
Hexane	1	0.0±0,054	0.0
	3	0.0±0.011	
	10	0.0±0.036	
Chloroform	1	0.0±0.044	2.67
	3	59±0.030	
	10	60±0.11	
Ethyl acetate	1	47±0.005	1.11
	3	86±0.082	
	10	98±0.043	
Methanol	1	0.0±0.001	0.0
	3	0.0±0.201	
	10	0.0±0.007	
Water	1	0.0±0,13	0.0
	3	0.0±0.11	
	10	0.0±0.105	

Only chloroform and ethyl acetate showed antiplatelet aggregation of papain treated platelets induced by thrombin. Methanol, hexane and water showed no inhibitory activity.

Table 4.9: Inhibitory activity of the extracts on bromelain treated platelets aggregation induced by thrombin.

Thrombin bromelain treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition ± SD	IC_{50}
	control	0.0±0.005	0.0
Hexane	1	0.0±0.031	2.63
	3	60±0.082	
	10	0.0±0.031	
Chloroform	1	0.0±0.129	2.21
	3	74±0,023	
	10	77±0.231	
Ethyl acetate	1	51±0.01	0.84
	3	58±0,03	
	10	60±0.04	
Methanol	1	0.0±0.129	7.22
	3	0.0±0.156	
	10	83±0.282	
Water	1	51±0.301	1.98
	3	58±0,011	
	10	60±0.082	

All extracts showed antiplatelet aggregation of bromelain treated platelets induced by thrombin.

Table 4.10: Inhibitory activity of the extracts on thrombin induced bromelain treated platelets.

Thrombin trypsin treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition ± SD	IC_{50}
	control	0.0±0.001	0.0
Hexane	1	0.0±0.207	0.0
	3	0.0±0.046	
	10	0.0±0.454	
Chloroform	1	0.0±0.342	0.0
	3	0.0±0.177	
	10	0.0±0.342	
Ethyl acetate	1	37±0,05	3.38
	3	48±0.22	
	0	67±0.9	
Methanol	1	1±0.461	41.6
	3	5±0.342	
	10	13±0.547	
Water	1	44±0.808	1.1
	3	86±0.237	
	10	0.0±0.425	

Ethyl acetate, methanol and water inhibited platelet aggregation of trypsin treated platelets induced by thrombin, but hexane and chloroform did not show any inhibitory activity.

Table 4.11: Inhibitory activity of the extracts with tannins and without tannins on ADP induced platelets.

Inhibitory activity of extract with tannins and tannin free extracts on untreated platelets induced by ADP					
Extracts	Concentration (mg/10ml)	% Inhibition ± SD (extracts with tannins)	% Inhibition ± SD (tannin free extracts)	IC_{50} (extracts with tannin)	IC_{50} (tannin free extracts)
	control	0.0±0.232	0.0±0.144	0.0	0.0
Hexane	1	33 ± 0.14	23 ± 0.403	5.25	9.87
	3	48±0.256	45 ± 0.112		
	10	54±0.934	50 ± 0.005		
Chloroform	1	24±0.323	34 ± 1.004	10.43	9.24
	3	28±0.156	24 ±0.991		
	10	56±0.218	54±1.225		
Ethyl acetate	1	46±0.148	42 ± 0.886	1.59	2.27
	3	58±0.401	54 ± 1.313		
	10	65±0.505	59 ± 0.143		
Methanol	1	0.0±0.17	0.0 ± 0.288	0.0	0.0
	3	0.0±0.618	0.0 ± 2.323		
	10	0.0±0.454	0.0 ± 0.8o8		
Water	1	0.0±0.176	16 ± 3.443	0.0	9.24
	3	37±0.778	0.0 ± 2.667		
	10	0.0±0.141	56 ± 0.114		

All the extracts showed antiplatelet activity except methanol which did not show any inhibitory activity and water extract which showed weak inhibitory activity at concentration of 3 mg/10 ml. All the extracts retained their inhibitory activity even after tannins had been removed.

Table 4.12: Inhibitory activity of the extracts on papain treated platelets induced by ADP.

ADP papain treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition± SD	IC_{50}
	control	0.0±0.342	0.0
Hexane	1	16±0.496	10.89
	3	27±0.514	
	10	38±0.05	
Chloroform	1	20±1.689	0.0
	3	38±1.977	
	10	0.0±2.914	
Ethyl acetate	1	41±0.323	1.12
	3	55±1.931	
	10	74±0.142	
Methanol	1	0.0±2.000	0.0
	3	0.0±2.914	
	10	0.0±2.217	
Water	1	21±2.208	0.0
	3	0.0±3.178	
	10	0.0±4.454	

All extracts showed weak inhibition of platelet aggregation induced by ADP on papain treated platelets except ethyl acetate which showed high inhibition.

Table 4.13: Inhibitory activity of the extracts on bromelain treated platelets induced by ADP.

ADP bromelain treated platelets			
Extracts	Concentration (mg/10ml)	% Inhibition± SD	IC_{50}
	control	0.0±0.218	0.0
Hexane	1	27±0.245	7.59
	3	34±0.148	
	10	58±0.082	
Chloroform	1	240.005	7.84
	3	36±0.282	
	10	56±0.156	
Ethyl acetate	1	33±0.301	3.41
	3	47±1.934	
	10	59±0.256	
Methanol	1	22±0.322	9.49
	3	35±0.409	
	10	51±0.618	
Water	1	26±0.188	6.22
	3	44±0,669	
	10	55±0.496	

All the extracts showed antiplatelet aggregation activity on bromelain treated platelets induced by ADP.

Table 4.14: Inhibitory activity of the extracts on trypsin treated platelets induced by ADP.

ADP trypsin treated platelets			
Extracts	Concentration (mg/10ml)	% Inhibition ± SD	IC_{50}
	control	0.0±0.003	0.0
Hexane	1	0.0±0.143	0.0
	3	0.0±0.781	
	10	0.0±0.226	
Chloroform	1	0.0±0.311	0.0
	3	0.0±0.568	
	10	0.0±0.617	
Ethyl acetate	1	28±0.018	4.93
	3	46±0.335	
	10	61±0.020	
Methanol	1	24±0.304	5.94
	3	44±0.226	
	10	58±0.210	
Water	1	37±0.412	4.17
	3	59±0.678	
	10	0.0±0.346	

Ethyl acetate, methanol and water extracts were able to inhibit platelet aggregation, but hexane and chloroform extracts did not show any inhibitory activity.

Table 4.15: Inhibitory activity of the extracts with tannins and without tannins and on epinephrine induced platelets.

Inhibitory activity of extracts with tannins and tannin free extracts on untreated platelets induced epinephrine					
Extracts	Concentration (mg /10ml)	% Inhibition ± SD (extracts with tannins)	% Inhibition ± SD (tannin free extracts)	IC_{50} (extracts with tannin)	IC_{50} (tannin free extracts)
	control	0.0±0.082	0.0±0.001	0	
Hexane	1	36±0.245	0.0±0.2	7.02	0.0
	3	47±0.148	0.0±0.03		
	10	52±0.129	0.0±0.008		
Chloroform	1	28±0.082	0.0±0.005	9.11	0.0
	3	43±0.005	0.0±0.801		
	10	51±0.282	0.0±0.007		
Ethyl acetate	1	46±0.156	34±0.002	1.64	2.4
	3	57±0.303	56±0.121		
	10	66±0.057	59±0.097		
Methanol	1	0.0±1.934	0.0±0.003	0	0.0
	3	0.0±0.256	0.0±0.402		
	10	0.0±0.008	0.0±0.005		
Water	1	21±0.781	0.0±0.306	>10	0.0
	3	37±0.226	0.0±0.645		
	10	47±0.143	0.0±0.364		

Hexane, chloroform and ethyl acetate extracts showed inhibition of platelet aggregation even though water showed low inhibitory activity. Methanol extract did not show any inhibitory activities. After the tannin removal it was only ethyl acetate extracts which retained its inhibitory activity.

Table 4.16: Inhibitory activity of the extracts on papain treated platelets induced by epinephrine.

Epinephrine papain treated platelets			
Extracts	Concentration (mg/10ml)	% Inhibition ± SD	IC_{50}
	control	0.0±0.074	0.0
Hexane	1	0.0±0.617	>10
	3	0.0±0.120	
	10	46±0.031	
Chloroform	1	27±1.380	8.98
	3	0.0±2.641	
	10	48±0.335	
Ethyl acetate	1	36±0.412	3.67
	3	49±0.245	
	10	58±0.074	
Methanol	1	0.0±1.614	0.0
	3	0.0±3.311	
	10	0.0±1.568	
Water	1	0.0±1.086	0.0
	3	0.0±2.362	
	10	0.0±3.226	

Ethyl acetate extract showed the highest inhibition of platelet aggregation. Chloroform and hexane extracts also showed inhibition but it was low. Methanol and water extracts did not show any activity.

Table 4.17: Inhibitory activity of the extracts on bromelain treated platelets induced by epinephrine.

Epinephrine bromelain treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition± SD	IC_{50}
	control	0.0±0.112	0.0
Hexane	1	0.0±1.143	0.0
	3	38±0.112	
	10	0.0±2.454	
Chloroform	1	0.0±3.404	>10
	3	27±1.323	
	10	39±2.114	
Ethyl acetate	1	31±0.033	3.79
	3	48±1.678	
	10	64±0.552	
Methanol	1	0.0±2.905	0.0
	3	0.0±1.447	
	10	0.0±3.323	
Water	1	26±0.911	>10
	2	34±1.332	
	10	41±2.608	

All the extracts showed weak inhibition except ethyl acetate extracts which showed high inhibition.

Table 4.18: Inhibitory activity of the extracts on trypsin treated platelets induced by epinephrine.

Epinephrine trypsin treated platelets			
Extracts	Concentration (mg/10ml)	%Inhibition± SD	IC_{50}
	control	0.0±0.003	0.0
Hexane	1	0.0±0.143	0.0
	3	0.0±0.781	
	10	0.0±0.226	
Chloroform	1	0.0±0.311	0.0
	3	0.0±0.568	
	10	0.0±0.617	
Ethyl acetate	1	39±0.018	3.67
	3	48±0.335	
	10	68±0.020	
Methanol	1	0.0±0.304	0.0
	3	0.0±0.226	
	10	0.0±0.210	
Water	1	27±0.412	7.59
	3	48±0.678	
	10	51±0.346	

Only ethyl acetate and water extracts showed inhibition. Hexane, chloroform and methanol did not show inhibition of platelet aggregation.

4.6: Isolation and characterization

The crude ethyl acetate extract which showed more consistent activity on the antiplatelet aggregation was subjected to isolation and purification. The compound (E/G 10) was obtained and analyzed through IR, UV, and NMR techniques. The 2D NMR (HSQC, HMBC, NOESY and COSY) were used to assign carbons and protons. Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR of the compound in comparison with literature values is presented in Table 4.19. The full NMR spectra for the compound are given in Appendix D.

Table 4.19: ^{1}H and ^{13}C NMR data of 3β Hydroxylanosta-9, 24-dien-21-oic acids.

Position	δ_H	δ_H (2)	δ_c	δ_c(2)
1			30.3	30.3
2			23.9	23.4
3	3.5 (1H,s, OH)	3.6 (1H,s, OH)	77.2	77.9
4			37.3	36.8
5			44.5	45.2
6			17.7	17.9
7			26.0	26.9
8			49.7	134.4
9			145.7	133.8
10			34.9	36.9
11	5.2 (1H,t)	_	118.2	20.8
12			29.3	28.9
13			43.3	44.2
14			51.0	49.5
15			31.2	30.8
16			27.3	27.0
17			47.2	47.1
18			16.5	16.0
19			18.5	18.8
20			48.2	47.7
21			181.5	183.3
22			32.4	32.4
23			25.3	25.9
24	5.33 (1H,m)	5.33 (1H,t)	123.6	123.2
25			132.2	17.6
26	1.62 (3H,s)	1.62 (3H,s)	17.6	25.7
27	1.65 (3H,s)	1.67 (3H,s)	25.7	21.7
28	1.21 (3H,s)	1.14 (3H,s)	21.8	21.8
29	0.92 (3H,s)	0.93 (3H,s)	27.7	27.5
30	1.04 (3H,s)	1.04 (3H,s)	22.7	24.3

The ^{1}HNMR of the isolated compound E/G 9 (white crystalline compound with the melting point of 114 -116^{0}c) (figure 4.2) followed the triterpenoid pattern with a large cluster of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8. The presence of four olefinic carbons between 145-118 ppm a carboxylic carbon, and five quaternary carbon assisted in suggesting a lanostaryl skeletal structure (Fig 4.2). Detailed assignment of the ^{13}C NMR and significant ^{1}HNMR is presented in table 4.19. These data were compared to those in literature of a 3β Hydroxylanosta-9,24-dien-21-oic acids (Killer AC *et al*,1996).

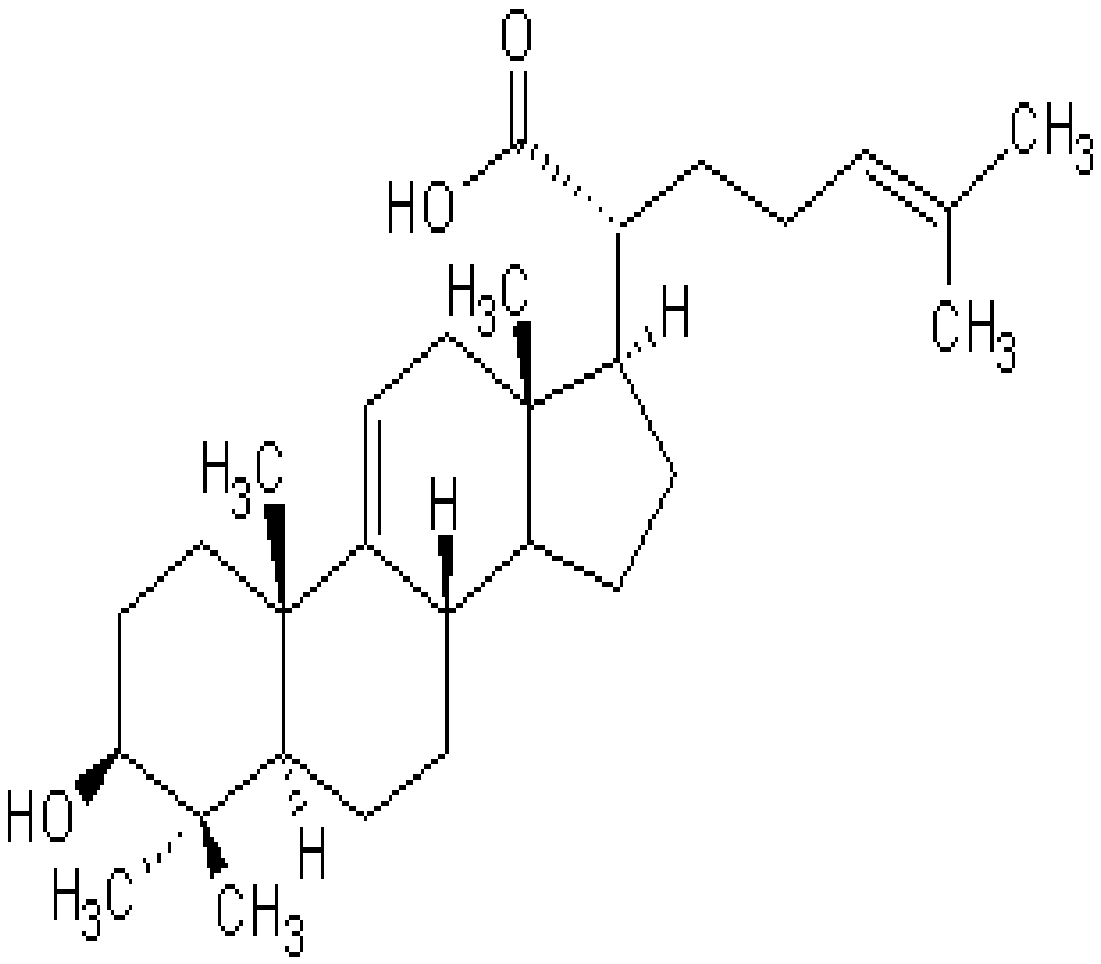

Figure 4.2: The structure of the isolated compound: 3β-Hydroxylanosta-9, 24-dien-21-oic acid

4.7 Anti-oxidant activity of 3β-Hydroxylanosta-9, 24-dien-21-oic acid

The ability of 3β-Hydroxylanosta-9, 24-dien-21-oic to scavenge free radicals is shown in figures 4.3. The 3β-Hydroxylanosta-9, 24-dien-21-oic acid did not show any observable scavenging activity on DPPH, any chelating activity (figure 4.4) or any reducing power (figure 4.5).

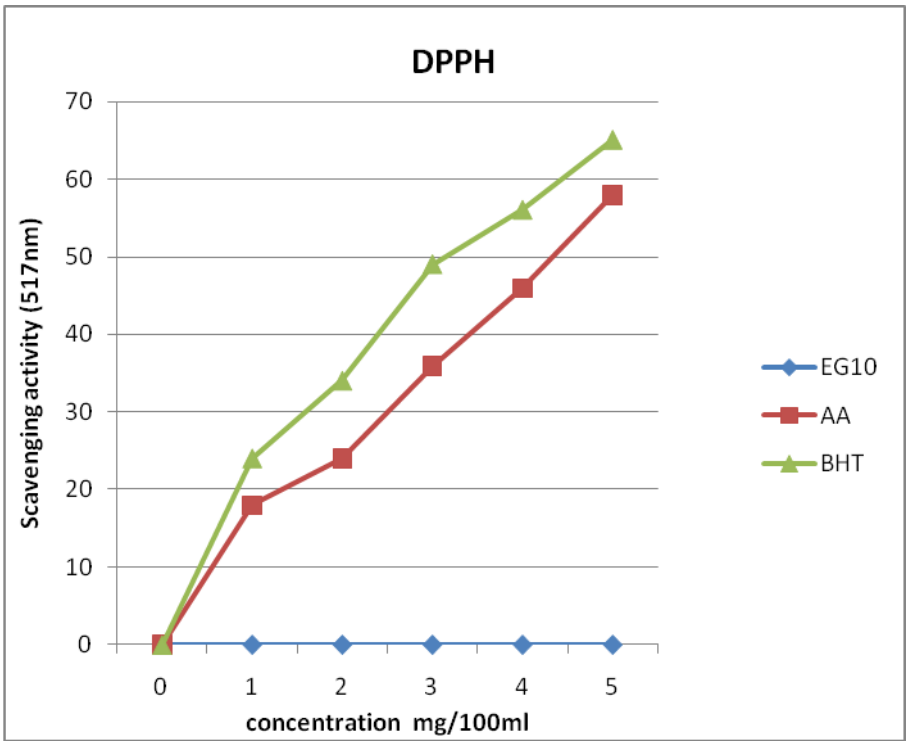

Figure 4.3: The DPPH scavenging activity of the 3β-Hydroxylanosta-9, 24-dien-21-oic.

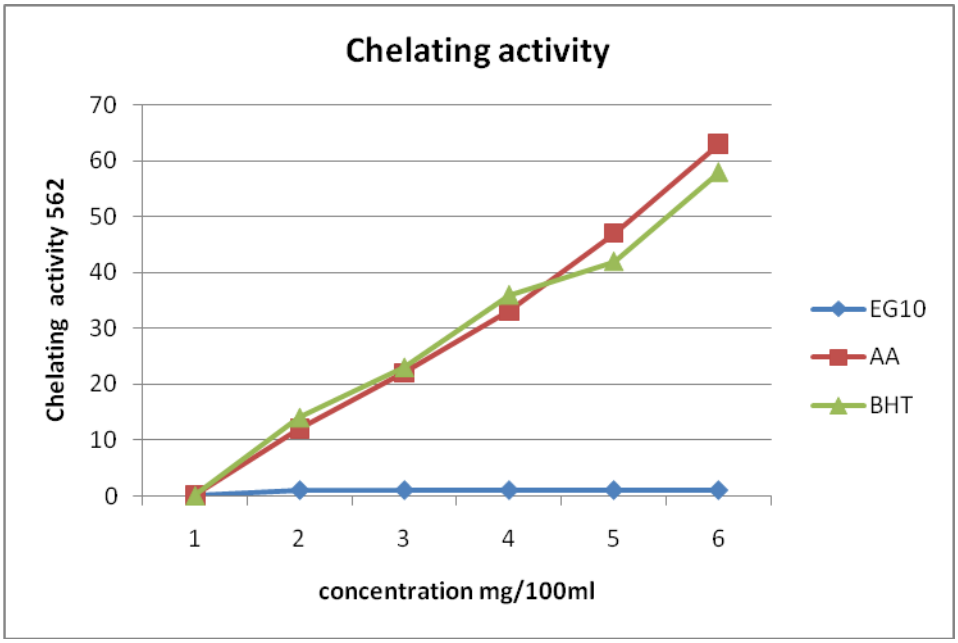

Figure 4.4: The chelating activity of the 3β-Hydroxylanosta-9, 24-dien-21-oic acid.

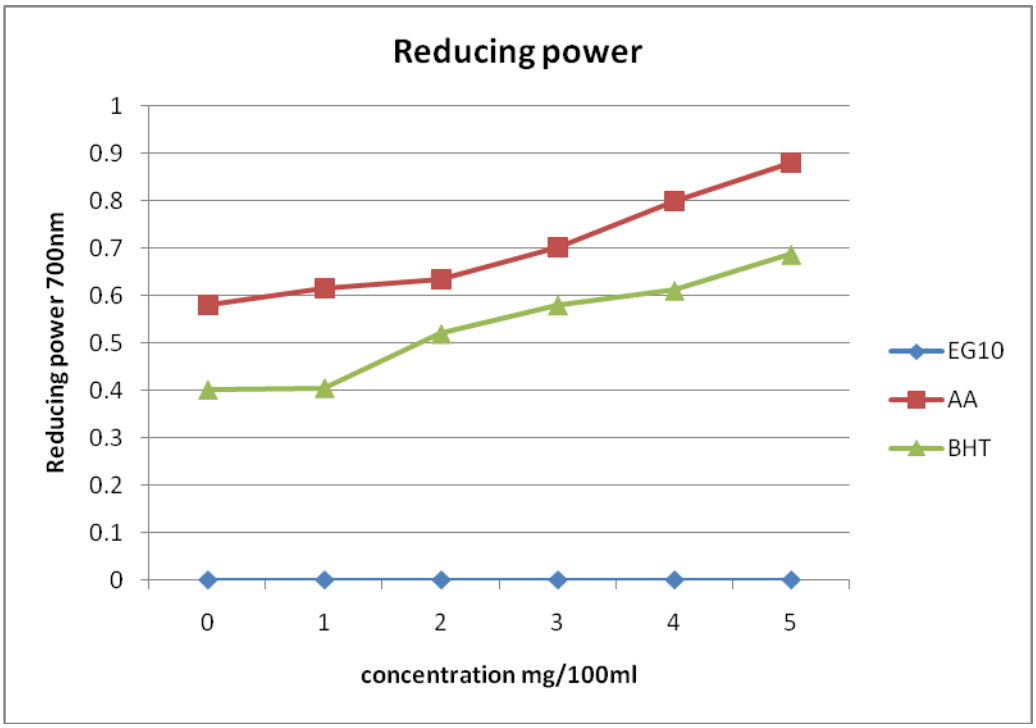

Figure 4.5: The reducing power of the 3β-Hydroxylanosta-9, 24-dien-21-oic acid.

4.8: Cytotoxicity on cell proliferation

The toxicity of the isolated compound on HEK293 (human embryonic kidney cells) and HEPG2 (human hepatocellular carcinoma cells) was tested. The results are presented in table 4.20.

Table 4.20: Toxicity **(**IC_{50}) of the isolated compound on HEK293 and HEPG2

Cells	IC_{50} (µg/ ml)
HEK293	851.5
HEPG2	796.0

The IC_{50} of the isolated compound suggests that the compound was not toxic to the two cell lines.

4.9 Antiplatelet aggregation of 3β-Hydroxylanosta-9, 24-dien-21-oic acid

The inhibitory activity of 3β-Hydroxylanosta-9, 24-dien-21-oic acid on thrombin, ADP, and epinephrine induced platelet aggregation was tested. The results are presented in table 4.21 – 4.23. The inhibition of platelet aggregation was concentration dependent in all the tests.

Table 4.21: Antiplatelet aggregation activity of 3β-Hydroxylanosta-9, 24-dien-21-oic acid induced by thrombin.

Isolate	concentrations	% Inhibition± SD	IC_{50}
3β-Hydroxylanosta-9,24-dien-21-oic acid	1	45±0.056	2.03
	3	50±2.546	
	10	55±3.862	

Table 4.22: Antiplatelet aggregation activity of 3β-Hydroxylanosta-9, 24-dien-21-oic acid induced by ADP.

Isolate	concentrations	% inhibition ± SD	IC_{50}
3β-Hydroxylanosta-9,24-dien-21-oic acid	1	34±5.764	4.74
	3	49±2.8.973	
	10	53 ±2.456	

Table 4.23: Antiplatelet aggregation activity of 3β-Hydroxylanosta-9, 24-dien-21-oic acid induced by epinephrine.

Isolate	concentrations	% Inhibition± SD	IC_{50}
3β-Hydroxylanosta-9,24-dien-21-oic acid	1	27±7.968	9.32
	3	34± 9,254	
	10	52± 4.675	

4.10 Effects of 3β -Hydroxylanosta-9, 24-dien-21-oic acid on acetylcholinesterase activity

The inhibition of acetylcholinesterase activity by 3β -Hydroxylanosta-9, 24-dien-21-oic acid is represented in figure 4.6

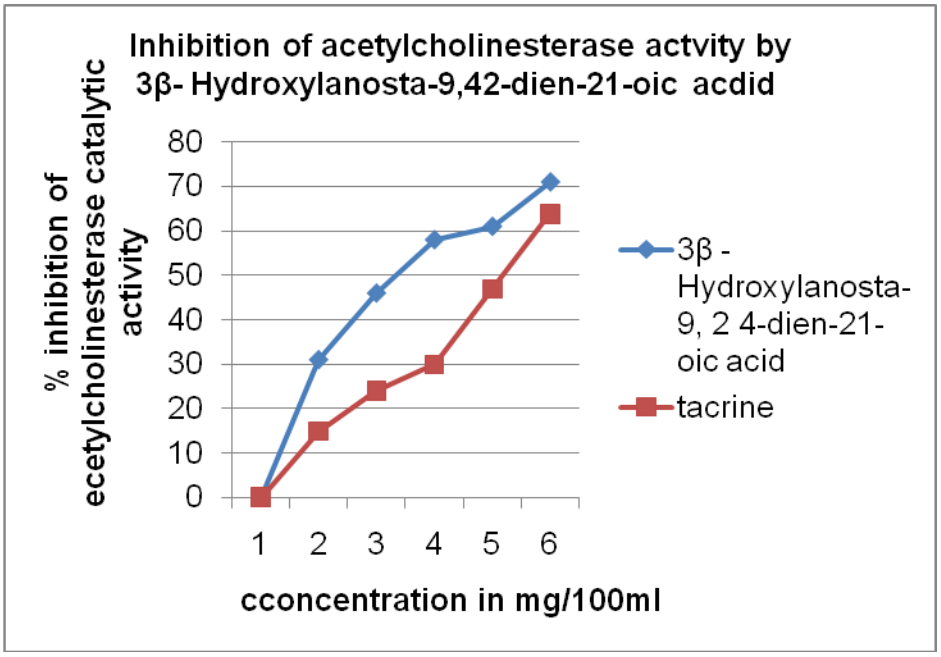

Figure 4.6: Inhibition of acetylcholinesterase catalytic activity by 3β -hydroxylanosta-9, 24-dien-21-oic acid.

The 3β -hydroxylanosta-9, 24-dien-21-oic acid inhibited acetylcholinesterase activity and the inhibition was concentration dependant. The inhibition was even higher than that of taccrine**,** a standard anti-cholinesterase drug.

Chapter Five

Discussion

Platelets dysfunctions significantly contribute to pathogenesis of cardiovascular diseases. Despite the progress that has been made in the management of such diseases as heart attacks, strokes, etc, atherothrombotic diseases continue to pose a threat to human health. The need for the discovery of new drugs cannot be overemphasized.

It is apparent from the results of this study that the medicinal plant, *R melanophloeos*, has constituents that prevent the aggregation of platelets (Tables 4.7 – 4.18). The proteolytic enzymes (trypsin, bromelain and papain) catalytically hydrolyze proteins (fibrinogen) into smaller fragments (fibrin monomers) with generation of new functional groups. The formation and exposure of the new functional groups stimulate aggregation (clot formation). The degree to which the extracts inhibited the aggregation of the enzyme-treated platelets does suggest that the extracts may not only be inhibiting thrombin and the other platelet agonist, but may also be preventing aggregation of degraded platelets. However, the platelets' loss of sensitivity to the agonists cannot be ruled out. The pre-incubation of platelets with the proteolytic enzymes has previously been reported to reduce platelet sensitivity to the agonists (Vellini *et al*., 1986; Metzig *et al*, 1999).

Oxidative stress occurs when the generation of reactive oxygen species (ROS, also called free radicals) in a system exceeds the system's ability to neutralize or eliminate them. Oxidative damage has been implicated in the irregular aggregation of platelets. The plant extracts exhibited antioxidant activities that were comparable

to the standards (ascorbic acid and BHT). The antioxidant activity could contribute to the observed antiplatelet aggregation activity of the plant's extracts.

The phytochemicals (alkaloids, flavonoids, tannins and saponins) that were identified in the plant (table 4.2) are known to have anti-platelet aggregation and antioxidant activities (Gilani, 2008). The ethyl acetate extracts however, retained their activity even after tannin removal (table 4.7, 4.11, and 4.15). It is apparent that the observed inhibitory activity was not due to the presence of tannins. The reduced activity of the tannin-free extracts was a possible indication of synergistic effect of the plant components.

The high and consistent antiplatelet aggregation activity exhibited by the ethyl acetate extract led to the isolation from this extract of the lanosteryl triterpenes- 3β-hydroxylanosta-9, 24-dien-24-oic acid (E/G 9).

There is a growing interest in natural triterpenoids. This interest has been attributed to the wide spectrum of biological activities exhibited by terpenes isolated from plants. These biological activities include anti-inflammatory (Potocka, 2003), antiulcerogenic (Ishikawa, 2008), analgesic (Costa, 2003), antimicrobial (Roberto, 2004), antiplasmodial (Moon, 2006), and hepatoprotective (Gao, 2004).

Antioxidant terpenes are rare (Maillard *et al*., 1996 and Markovic *et al*., 1996). However, a number of triterpenoids from various plants have been reported to have antiplatelet aggregation activity against thrombin, ADP and epinephrine (Xuemei *et al*., 2004, Lee *et al*., 1991 and Jin *et al*., 2004). The isolated compound (3β-Hydroxylanosta-9, 24-dien-21-oic acid) which is a triterpenoid, did not show any

antioxidant activity but it did show antiplatelet aggregation activity, even though its activity was lower than that of the crude extracts. The lower activity relative to the crude extracts could indicate the synergistic effect with other compounds, decomposition during fractionation, or removal of protective matrix.

Inhibition of AChE continues to be the best approach for the management of Alzheimer's disease, senile dementia, ataxia, myasthenia gravis and Parkinson's disease, and many research groups have put in considerable efforts in finding quality AChE inhibitors derived from natural sources (Mukherjee and Wahile 2007, Mills, 2004). Several plants derived triterpenoids have exhibited the ability to inhibit AChE enzyme (Calderon, 2009, Areche *et al*., 2009). From the results obtained from the present study, it is apparent that the isolated compound (3β-Hydroxylanosta-9,24-dien-21-oic acid) from *R. melanophloeos* could be used as medicines for the treatment of acetylcholinesterase hyperactivity.

Furthermore,the observed cytotoxic effect of the crude extracts on brine shrimps and the isolated compound on the human embryonic kidney (HEK 293) and hepatocellular carcinoma (HEPG2) cells indicate that they did not have any significant cytotoxicity. Despite the number of reports on the considerable cytotoxicity of triterpenes (Lee *et al*., 2007; Peteros and Uy, 2010), the isolated triterpene exhibited weak cytotoxic effects on HEK293 and HEPG2 (IC_{50} 8515 and 7960 µg/ml respectively). According to the American National Cancer Institute guidelines a compound is considered significantly active with IC_{50} value less than 30µg/ml (Suffness and Pezzuto, 1990). The results also encourage the development of this compound into a pharmacological antiplatelet aggregation drug.

Chapter Six

Conclusion

Medicinal plants are undoubtedly rich sources of biologically active compounds vital to human health. If used properly, medicinal plants are a solution to a wide range of diseases including atherothrombotic diseases.

- The plant extracts showed good antioxidant activity but isolated compound did not show any antioxidant activity.
- The plant extracts and the isolated compound inhibited antiplatelet aggregation activity.
- The isolated compound inhibited the activity of acetylcholine esterase.

These results suggest that the extracts of *Rapanea melanophloeos* could be considered as herbal treatment for disease associated with blood clotting and they support the use of this plant in folk medicine.

The isolated compound (3β -hydroxylanosta-9, 24-dien-21-oic acid) can be used for diseases related to acetylcholinesterase hyperactivity such as Alzheimer disease, neuromuscular paralysis and lewy body dementia.

6.1 Suggestion for the further studies

- It is suggested that further work be carried out on the mechanism of action of the isolated compound.
- The other biological (antimicrobial, anti-inflammatory, antiulcer etc) activities of the compound be tested

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Appendix A

Reagent Details

A1 Mayer's reagent:

1,358 g of silver chloride was dissolved in 60 ml of distilled water and poured into a solution of 5 g of KI in 10ml of distilled water and sufficient water was added to make 100 ml

A2 Dragendorff's reagent:

0,85g of Bismuth nitrate was dissolved in 40 ml of distilled water and 10ml of glacial acetic acid, followed by addition of 8g potassium iodide dissolved in 20ml of water.

Solutions A and B were stored separately in dark bottles. Just before use, 5 ml of solution A, 5 ml of solution B and glacial acetic acid were mixed and made up to 100 ml with distilled water in a volumetric flask.

A3 Resuspending buffer (pH 7.4):

8.18 g of 0.14 M NaCl; 2.36 g of 15 mM Tris-HCl and 0.9 g of 0.005 M glucose were dissolved and made up to 100 ml with distilled water.

A4 Washing buffer (pH 6.5):

32.77 g of 0.113 M NaCl; 3.053 g of 4.3 mM Na_2HPO_4; 3.741 g of 4.3 mM of K_2HPO_4; 14.64 g of 24,4 mM of NaH_2PO_4; 5.45 g of 5.5 mM glucose; and 1.86 g of 1 mM EDTA were dissolved in 5000 ml of distilled water.

A5 Tris buffer (pH 7.4):

7.88 g of 50 mM Tris-HC; 2.79 g of 7.5 mM EDTA; and 10.227 g of 175 mM NaCl were dissolved and made up to 1000 ml with distilled water and the pH adjusted to 7,4.

A6 Phosphate buffer (pH 6.6):

18 ml of 0.2M KOH and 50 ml of 0.2M KH_2PO_4 were mixed and made up to 100ml.

A7 ADA:

100 g of dextrose; 68.296 g of 0.065 M citric acid and 124.95 g of 0.085 M trisodium citrate were dissolved and made up to 5000 ml of distilled water.

A8 1 **% TBA**

A9 1% TBA was prepared in 50% glacial acetic acid

A10 ABTS*:

10mg ABTS was dissolved in 3 ml of distilled water and 0.002 g of potassium persulfate was added to the solution. The mixture was incubated at room temperature in the dark for 16 hrs. ABTS* was diluted with methanol (1 ml ABTS* : 60 ml methanol).

A11 DPPH

2 mg% DPPH was prepared in methanol.

Appendix B

Detail Methodology

B.1Extraction:

The dry plant material was extracted (1:5w/v) sequentially with different solvents (n-hexane, chloroform, ethyl acetate, methanol and water) according to their increasing polarity (See figure 3.1). The plant material was mixed with n-hexane and was incubated at room temperature on the platform shaker (150rpm) for 24 hrs. The plant material mixture was filtrated through Whatman no. 1 filter paper and evaporated to dryness on a rotary evaporator at 45±2^{o}C. The same procedure was also done to chloroform, ethyl acetate and methanol. Water extract was freeze-dried.The yields of each plant extracts were recorded and then reconstituted with their respective solvents. The extracts were stored in sterile glass bottles and kept at 4ºC.

B.2 Phytochemical screening

B.2.1Test for saponins:

2.5 ml of the plant material was extracted with boiling water. After cooling, the extract was shaken vigorously to froth and was then allowed to stand for 15 – 20 min and classified for saponin content as follows: no froth = negative (no saponins) and froth less than 1 cm = weakly positive (saponins present); froth 1.2 cm high = positive; and froth greater 2 cm high = strongly positive. (Harbone, 1973; Odebiyi and, Sofowora ,1978;Sofowora,1984)

B.2.2Test for tannins:

0.5 ml of methanolic extract was boiled with 10 ml of water for 15 min, filtered and made up to 10 ml. Few drops of 0.1 % $FeCl_3$ solution were added to 2 ml of the filtrate. Black-blue, green or blue-green precipitate was taken as preliminary evidence of the presence of tannins. (Harbone, 1973; Odebiyi and Sofowora, 1978; Sofowora, 1984)

B.2.3Test for steroids:

2 ml of acetic anhydride and 2 ml of 2 ml of concentrated sulphuric acid were added to 0.5 ml of extract. A colour change from violet to blue or green was taken as evidence of the presence of steroids. (Harbone, 1973; Odebiyi and Sofowora ,1978;Sofowora,1984)

B.2.4 Test for terpenoids (Salkwoski test):

0.5 ml of extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish-brown coloration of the interphase was taken as evidence of the presence of terpenoids. (Harbone, 1973;Odebiyi and, Sofowora ,1978;Sofowora,1984)

B2.5 Test for alkaloids:

0.5 ml of methanolic extract was acidified with 5 ml 1 % HCl (aq). The solution was stirred on steam bath and filtered. 1 ml of the filtrate was treated with Mayer's reagent. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent and a turbidity or precipitate was also take the evidence of the presence of alkaloids. (Harbone, 1973; Odebiyi and, Sofowora, 1978; Sofowora, 1984)

B.2.6 Test for anthraquinones:

0.5 ml of methanolic extract was dissolved and shaken with benzene. The mixture was filtered and 5 ml of 10 % ammonium solution was added to the filtrate. After shaking, the presence of a pink, red, or violet colour in ammonia solution (lower phase) was taken as evidence of the presence of anthraquinones. (Harbone, 1973;Odebiyi and, Sofowora ,1978;Sofowora,1984)

B.2.7 Test for flavonoids:

Three tests were used to determine the presence of cardiac glycosides in the extracts as follows:

B.2.8 Lead acetate test:

1 ml of extract was mixed with 1 ml of 10 % lead acetate. A reddish-brown colouration or precipitate was taken as an evidence of the presence of flavonoids.

B.2.9 Ferric chloride test:

1 ml of extract was mixed 1 ml of $FeCl_3$. A dark brown or dirty brown precipitate was taken as evidence of the presence of flavonoids.

B.2.10 Sodium hydroxide test:

1 ml of extract was mixed with 1 ml of dilute NaOH. A golden yellow precipitate was taken as evidence of the presence of flavonoids. .(Harbone, 1973;Odebiyi and, Sofowora ,1978;Sofowora,1984)

B.2.11 Test for cardiac glycosides:

Three tests were used to determine the presence of cardiac glycosides in the extracts as follows:

B.2.12 Lieberman's test:

0.5 ml of extract was mixed with 2 ml of acetic acid. This was cooled well in ice and 1 ml of concentrated sulphuric acid was carefully added down the sides. Colour change from violet to blue to green was taken as an indication of the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycoside.

B.2.13 Salkowski test:

0.5 ml of extract was mixed with chloroform and 2ml of concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at inter phase was taken as an indication of the presence of a steroidal ring i.e. a glycone portion of the cardiac glycoside.

B.2.14 Keller-Kiliani test:

0.5 ml of extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 10 % of $Fecl_3$ solution and this was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at interphase was taken as an indication of the presence of a deoxy sugar characteristic of cardenolides. Also a violet ring below a brown the brown ring or a greenish ring just above the brown ring (i.e. in the acetic acid layer) and gradually spread through the layer was taken as an indication of the presence of cardiac glycosides. (Harbone, 1973;Odebiyi and, Sofowora ,1978;Sofowora,1984)

B.2.15 Test for phlobatannins:

2.5 ml of the plant material was extracted with boiling water. The extract was boiled with1% HCl (aq).Deposition of a red precipitate was taken as evidence for the presence of phlobatannins. (Harbone, 1973;Odebiyiand Sofowora,1978; Sofowora,1984).

B.3 Determination of total phenols

The phenolic content of the extracts were determined using Folin- Ciocalteu method as described by Kujala et al. (2000). Various concentrations of Folin- Ciocalteu (0.01-0.1mg/ml) were prepared in diethyl ether. To each sample solution (1.0 ml) and the standard (gallic acid) was added 5 ml of Folin-Ciocalteu (and 4 ml Sodium carbonate (7% w/v) and shaken. The solution was allowed to stand for 30 min in the dark at room temperature, after which absorbance was measured at 765 nm using a spectrophotometer. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligram per gram dry plant extract.

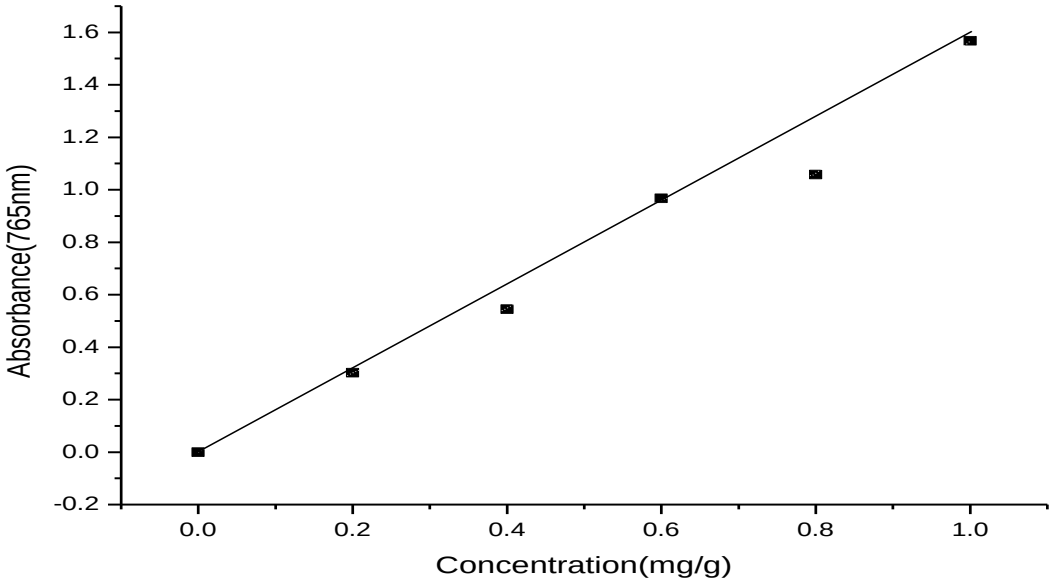

Figure B.1 Garlic acid Standard Curve

B.4 Determination of total flavonoid

Flavonoids were determined using the method described by Ordon et al. (2006). Various concentrations of quercetin were prepared (0.01- 0.1 mg/ml) in diethyl ether. 0.5ml of the extracts was mixed with 1 ml of diethyl ether. The residue was dissolve in 0.5 ml of 2% $ALCL_3$ ethanol solution (80% EtOH). The solution was allowed to stand for one 1 hour at room temperature (a yellow colour indicate the presence of flavonoids). The absorbance was measured at 420 nm. The amount of total flavonoids was expressed as quercetin equivalent in milligram per gram dry plant extract.

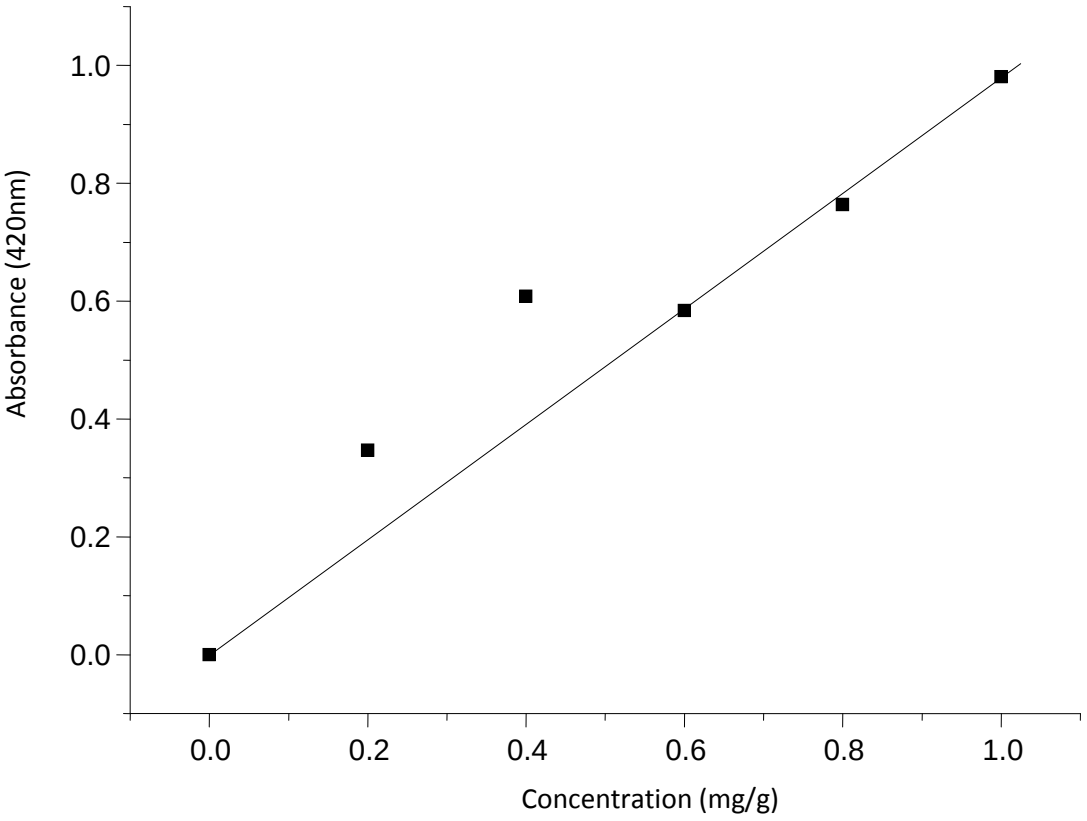

FIGURE B.2 Quercetin standard curve

B.5 Determination of Proanthocyanidin

Proanthocyanidin content in the extracts was determined following the method described by Sun et al. (1998). Various concentration of cetechin was prepared in diethyl ether (0.01-0.1 mg/ml) as phenols. 0.5 ml of the extracts was mixed with diethyl ether. The residue was dissolve in 3 ml of 4% vanillin-MeOH solution (80%) and 1.5 ml of 1%HCL. The solution was allowed to stand for one 15 min at room temperature. The absorbance was measured at 500 nm. The proanthocyanidin content was expressed as cetechin equivalent in milligram per gram dry plant extract.

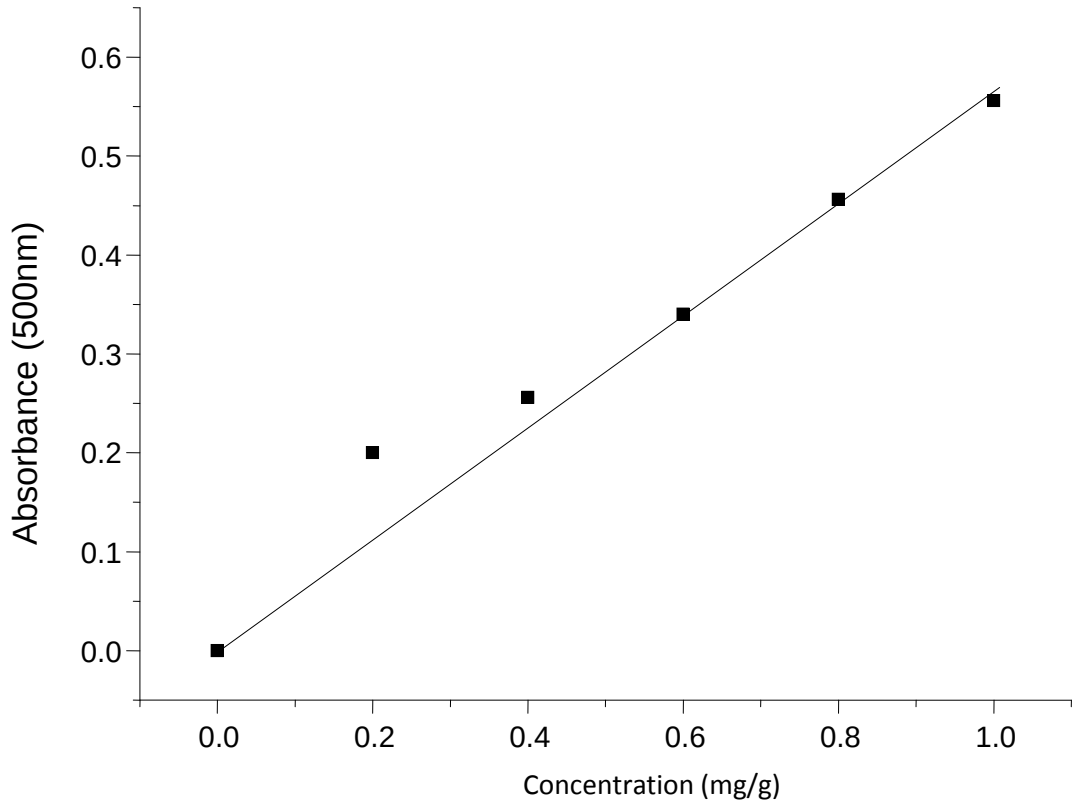

Figure B.3 Catichin standard curve

B.6 Antioxidative *In vitro*

B.6.1 Free radical scavenging:

DPPH: 2 mg/100 ml DPPH and different concentrations of extracts (0 – 5 mg/100 ml) were prepared in methanol. Six test tubes were set in duplicate for each extract (12 tubes). 2 ml of DPPH was put into each test tube and 2 ml of each extract was added into the corresponding test tubes. This was mixed and let to stand for 40 min with interval mixing. Absorbance was read at 517 nm. Methanol was used as blank and percentage scavenging activity was calculated;

$$\% \text{ scaveging activity} = [1-A_t/A_0] \times 100$$

where A_0 is the absorbance of the control and A_t is the absorbance of the extract. The graphs of percentage scavenging activity versus concentration of extract (mg/100ml) were constructed (Brad-Williams *et al*,1995).

B.6.2 ABTS*:

Different concentrations of methanolic extracts (0 – 5 mg/100 ml CH_3OH) were prepared. Test tubes were set as in the DPPH experiment. 1 ml of ABTS* was put into each test tube and 1 ml of each extract was added into the corresponding test tubes. This was mixed and allowed to stand for 6 min. The absorbance was read at 734 nm. Methanol was used as blank. Percentage scavenging activity was calculated from the formula above (DPPH) and graphs of percentage scavenging activity versus concentration of extract (mg/100 ml) were constructed (Pellegrin *et al*, 1986).

B.6.3 Reducing power:

Test tubes were set as in the DPPH experiment. 1 ml of extract was mixed with 2.5 ml phosphate buffer and 2.5 ml potassium ferrocyanide. The mixture was incubated for 20 min and 2.5 ml of 10% TCA was added. This was mixed and after 5 – 10 min, the mixture was centrifuged at 1000 rpm for 10 min. 2.5 ml of supernatant was collected and diluted with 2.5 ml of distilled water. This was mixed with 0.5 ml $FeCl_3$**.** Absorbance was read at 700 nm and distilled water was used as blank. The graphs of absorbance (nm) versus concentration of extract (mg/100 ml) were constructed (Oyaizu *et al* , 1986).

B.6.4 Chelating activity on Fe^{2+}:

Test tubes were set as in the DPPH experiment. 1 ml of extract was diluted with 3.75 ml deionized water and this was mixed with 0.1 of $FeCl_2$ and 0.2 ml of ferrozine. This was let to stand for 10 min with interval mixing. The absorbance was read at 562 nm and deionized water was used as blank. Percentage chelating activity was calculated from the formula above (DPPH) and graphs of percentage chelating activity versus concentration of extracts (mg/100ml) were constructed (Decker and Welch (1990).

B.7 Toxicity testing against the brine shrimp

B.7.1 Hatching shrimp

Brine shrimp eggs, *Artemia salina* leach were hatched in artificial seawater prepared by dissolving 38 g of sea salt in1l of distilled water. After 48 h incubation at room temperature (22–29 ◦C), the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with pipette. Nauplii were separated from eggs by aliquoting them three times in small beakers containing seawater.

B.7.2 Brine shrimp assay

Brine shrimp (*Artemia salina*) lethality test was carried out using the procedure described by Meyer *et al*. (1982) and McLaughlin (1992). Ten shrimps (counted on a slide) were introduced into the breakers containing different concentrations of the extracts (0 µl/ml, 5 µl/ml, 25 µl/ml, 50 µl/ml and 100 µl/ml) in DMSO solution and made up to 5 ml volume with the artificial sea water. Different concentration was used. Each beaker was shaken lightly to ensure a homogeneous test solution and was left at room temperature. The Saline solution with 3% Tween 20 and DMSO was used as a control without extract to which shrimp larvae were added. Each test was carried out on 4 different concentrations. The beakers were maintained under illumination and the survivors were recorded after 24 h, criteria for death was loss of locomotive action of nauplii. The percentages death at each concentration and control were determined. Lethality concentration (LC_{50} values) for each assay was calculated by taking average of three experiments using a Finney Probit analysis program on an IBM computer.

B.7.3 MTT CELL PROLIFERATION ASSAY

The cells that were used are HEK293 (human embryonic kidney cells) and HEPG2 (human hepatocellular carcinoma cells). The cells were grown to confluency in 25cm^2 flasks.This was then trypsinised and plated into 48 well plates at specific seeding densities.The cells were incubated overnight at 37 degrees celcius. Medium was then removed and fresh medium (MEN+ Glutmax+ antibiotics) was added. The extracts were then added in triplicate and incubated for 4 hrs. Then after the medium was removed and replaced by complete medium (MEM = Glutmax+ antibiotics + 10% Fetal bovine serum). After 48 hours cells were subjected to the MTT assay. The

concentration tested ranged from 50- 350 µl for all samples. All the samples were done in triplicate. The cell density (Samples 1- 8) = 2.5 x 10^4 cells per well in a well plate.The MTT solution of concentration 5mg/ml in PBS was prepared. The HEK293 (human embryonic kidney cells) and HEPG2 (human hepatocellular carcinoma cells) were incubated for 48 hrs. The medium was removed from cells in multiwall plate. 200 µl of MTT solution as well as 200 µl of medium was added to each well containing cells. The multiwell plate was incubated at 37^{o}c for 4 hours. Then after the medium and the MTT solution was removed from the wells. To each well 100/200/400 µl of DMSO was added. The reaction was stopped and insoluble formazan crystals were dissolved. The cells without the extracts were used as the control. The plate was read in a spectrophotometer at 570nm (Mosman T, 1983).

B.8 S-2238:

S-2238 (0.76 M) was prepared in distilled water and the extracts solubilised in DMSO were diluted to a final DMSO concentration of 1% (v/v) with Tris-HCl buffer (50 µl Tris -HCL, Ph 7.4, containing 7.5Mm EDTA and 175Mm NaCl). The plant extracts (50 µl) was added to 10 µl of thrombin. The mixture was left for 10 min at room temperature and 190 µl 0.76 M s2238 was added. The reaction was recorded at 412 nm for 4min at 10s intervals using a Biotek ELx 808 UI plate reader plate reader. (Rob *et al*, 1997). A negative control was done using 2% (v/v) DMSO in saline which represented 100% activity. Graphs of absorbance (nm) versus time (sec) were constructed and then the percentage inhibition and inhibitory concentration at 50% (IC50) was calculated.

B.9 Enzyme treated platelets:

0.1 mg of each enzyme (papin, bromelain and trypsin) was separately added to the platelets (100 µl). This was mixed and incubated at 25 ^{o}C for 60 min. A little volume (about 3 ml) of the washing buffer was added and this was centrifuged at 3200 rpm for 15 min and this was repeated three times. A 1: 1 dilution with a resuspending buffer was done (Tomita *et al*, 1983).

B.10 Antiplatelet activity:

The inhibitory activity of the plant extracts was tested separately on thrombin (5 U/ml), ADP (10 mM) and epinephrine (5 mM) induced platelet aggregation. It was also tested on enzyme (trypsin, bromelain, papain) treated platelets, where by the plant extracts (40 µl) was added to rat platelets (100 µl) and left at room temperature for 5 min. 20 µl of thrombin/ADP/epinephrine was added and the inhibition of blood clot was measured at the absorbance of 412 nm using a Biotek ELx 808 UI plate reader plate reader for 20 min at 30s intervals (Hauptmann *et al*., 1980).

B.11Tannin removal

The tannins were removed using the method described by Toth and Pavia (2001). Plant extracts was prepared at a concentration of 10 mg/ml in dH_2O. Insoluble PVPP was added to the extract at 10 mg/ml, shaken for 15 min at 4^{o}C, followed by centrifugation using an Eppendorf centrifuge model 5804 R, at 3645g for 8 min at 4^{o}C. The pellet was discarded. Using the supernatant, the procedure was done several times to remove tannins to a negligible concentration. The extracts were then screened for antiplatelet aggregation activity.

B.12 Isolation and identification of active compound

Ethyl acetate fraction was the most active extract and was thus subjected to isolation and purification techniques using Thin Layer Chromatography ,Column chromatography and taken to NMR for identification.

B.12.1 Thin Layer Chromatography (TLC)

A commercially prepared TLC aluminium sheets of 20 x 20cm Silica gel 60 F_{254} was used. The plate was cut to size of 5 x 5cm and the extract was spotted at the bottom the TLC plate (about 0.5cm from the base). The plate was then placed in a developing tank containing chosen solvent system. The initial solvent system used was 100% hexane. The polarity of solvent system was increased by adding ethyl acetate at various ratios of 95:5, 90:10, 85;15, 80:20 until 50:50 ratio of heaxane:ethyl acetate respectively was achieved. The spots were developed using UV light and 10% sulphuric acid . This method afforded 11 different spots.

B.12.2 Column chromatography (CC)

A glass tube with a diameter 50 mm and a height of 50 cm with a tap at the bottom was used for the column chromatography technique. A plug of cotton wool was placed at the bottom of the column very close to the tap so as to prevent the stationary phase from blocking the column.

The adsorbent which is the silica gel (70-230 mesh size) was used a ratio of 30 gm of the adsorbent to 1 gm of the crude extract. The column was filled with a little quantity of hexane. Slurry of weighted adsorbent was prepared using 100% hexane and then carefully poured into the column. Air bubbles were prevented from forming. A solution of the extract was then pipetted on top of the stationary phase. This layer

was topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the flow of the eluting solvents. The eluting solvent initially was 100% hexane and the polarity was gradually increase at a 95:5 hexane: ethyl acetate ratio until a 50;50 ratio was used. About 40 mls of eluting mixture was collect until there appeared to be no more solute in the column. The fractions collected were monitored by TLC and similar fractions were combined together. Solvent was then removed from the bulked fraction, allowed to dry and weighed. TLC analysis was carried out on the semi dry bulked fraction using various polar solvent to ensure the purity of the fraction.

B.12.3 Purification of a compound

The solvent was allowed to dry completely and brownish yellow crystals with a mass of 165 mg were formed. The crystals were further purified by dissolving them in methanol after which the yellow part dissolved in methanol and the white crystals were left.

B.12.4 TLC of the white crystals

When dissolved in various solvents and spotted on the TLC plate, a single yellow spot was observed after spraying and heating in the oven.

The white crystals, weighing about 161 mg, were obtained. Melting point measurements were done and the compound was taken for further analyses with IR, UV-Visible spectroscopy and NMR.

B.13 General experimental

IR spectra were obtained from a Perkin Elmer Spectrum 100 FTIR with ATR Sampling Accessory instrument, using CHC_{l3} (Merck) as solvent. ^{1} H (400 MHz) and ^{13}C NMR (600 MHz) spectra were recorded on a Bruker Avance lll 400 or 600 apparatus using CDCl3 (Merck) as solvent. Chromatograms were examined under UV light (365 and 254 nm) using a UV-viewing cabinet and by spraying with 10% H_2SO_4.The melting point was done on a Barnstead or Electothermal digital melting point apparatus using powder sample.

B.14 Effect of 3β-Hydroxylanosta-9,24-dien-21-oic acid on Acetylcholine esterase activity.

Acetylcholinesterase activity was estimated using acetylthiocholine iodide as substrate following the method of Ellman et al (1961). The fish were pitched and brain was carefully removed using scissor and forceps. The removal of the brain was achieve by making median incision in the dorsal side of the skull and excising brain free by cutting the optic nerves and spinal cord. Care was taken to avoid any blood clots. At the same time, it was made sure that all of the brain tissues were removed and weighed. The brain was homogenized in phosphate buffer pH 8. The ground brain tissue was then diluted with sufficient amount of phosphate buffer to obtain 20 mg of brain tissue per one millilitre of buffer. The tissue was further homogenized, and centrifuged at 1000*g for 20 min and supernatant was analyzed immediately. Supernatant was pipetted into a test tube, phosphate pH, 8.0 buffer was added. There after DTNB reagent was added and mixed well. 20~1 of 5 mM s-acetyl-thiocholine iodide (ASChI) solution was added quickly and mixed well. The absorbance was recorded at 60 second intervals. The yellow colour produced by

enzymatic reaction resulted in changes in absorbance. The reaction rate was calculated in units of changes in absorbance per minute.

The rate was calculated using. **R=574*A/C***.*** Where R = rate of µmoles, of substrate hydrolyzed /min/g brain tissue, A =change in absorbance per min and C= original concentration of brain tissue (mg/ml) in the homogenate (this value remains constant at 20 mg/ml throughout the study) and percentage inhibition was calculated. Tacrine was used as the standard.

Appendix C

Ethical clearance

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Ms Ronalda McEwan
Department of Biochemistry and Microbiology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6095
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28 November 2007

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **Prof AR Opoku** from the Department of Biochemistry and Microbiology at the University of Zululand submitted a research project proposal No. 2007-02 to the Ethics Committee of the University of Zululand. The research project will investigate **PLATELET ANTI-AGGREGANT PROPERTY OF SOME ZULU MEDICINAL PLANTS.**

Based on the research protocol stipulated the above-said Ethics Committee could find no reason to reject the proposed research provided that relevant internationally accepted procedures pertinent to the maintenance and experimental treatment of laboratory held rats are adhered to.

Ronalda McEwan
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand

Appendix D

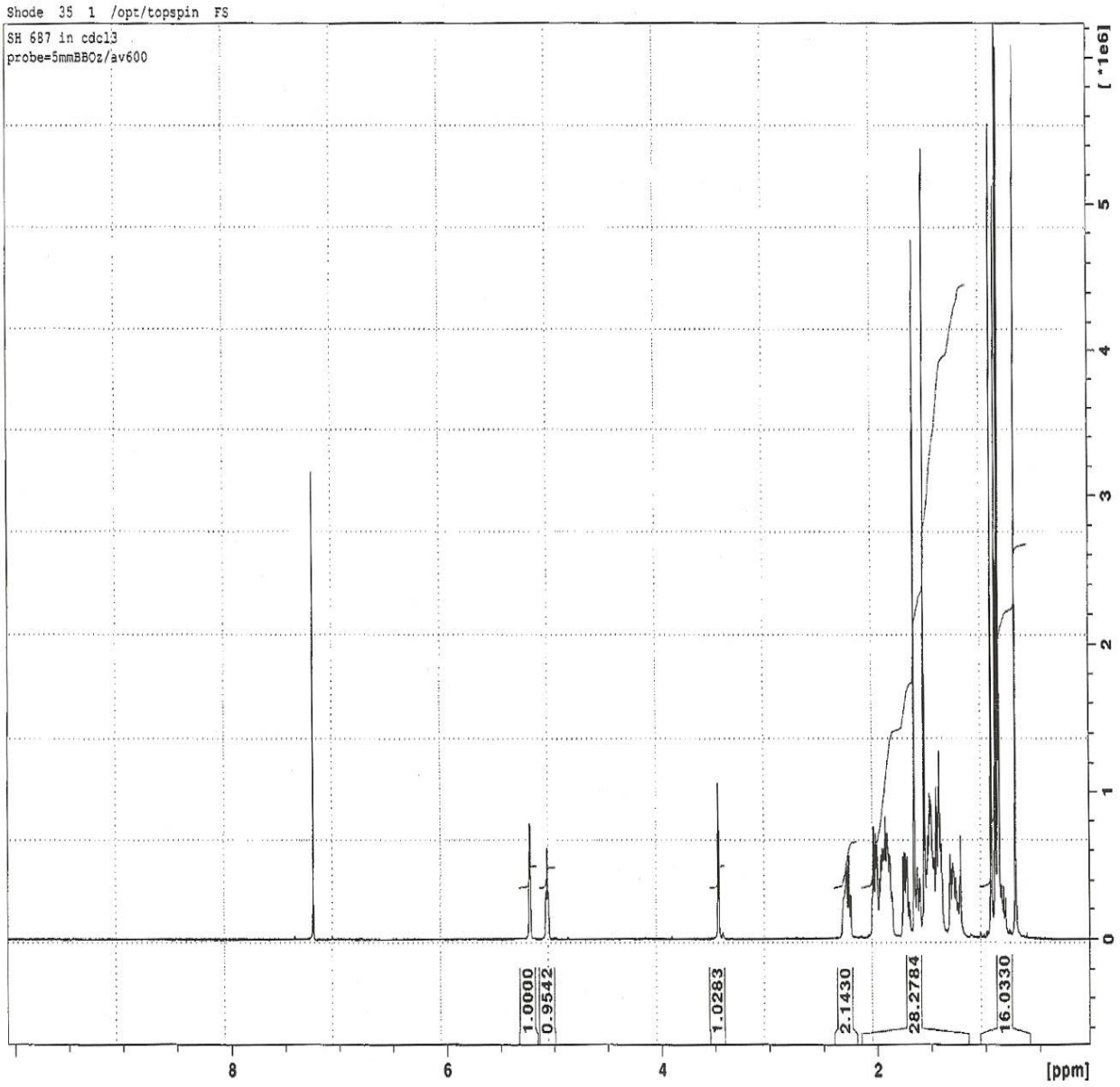

Figure D1: ^{1}H-NMR of Isolated Compound

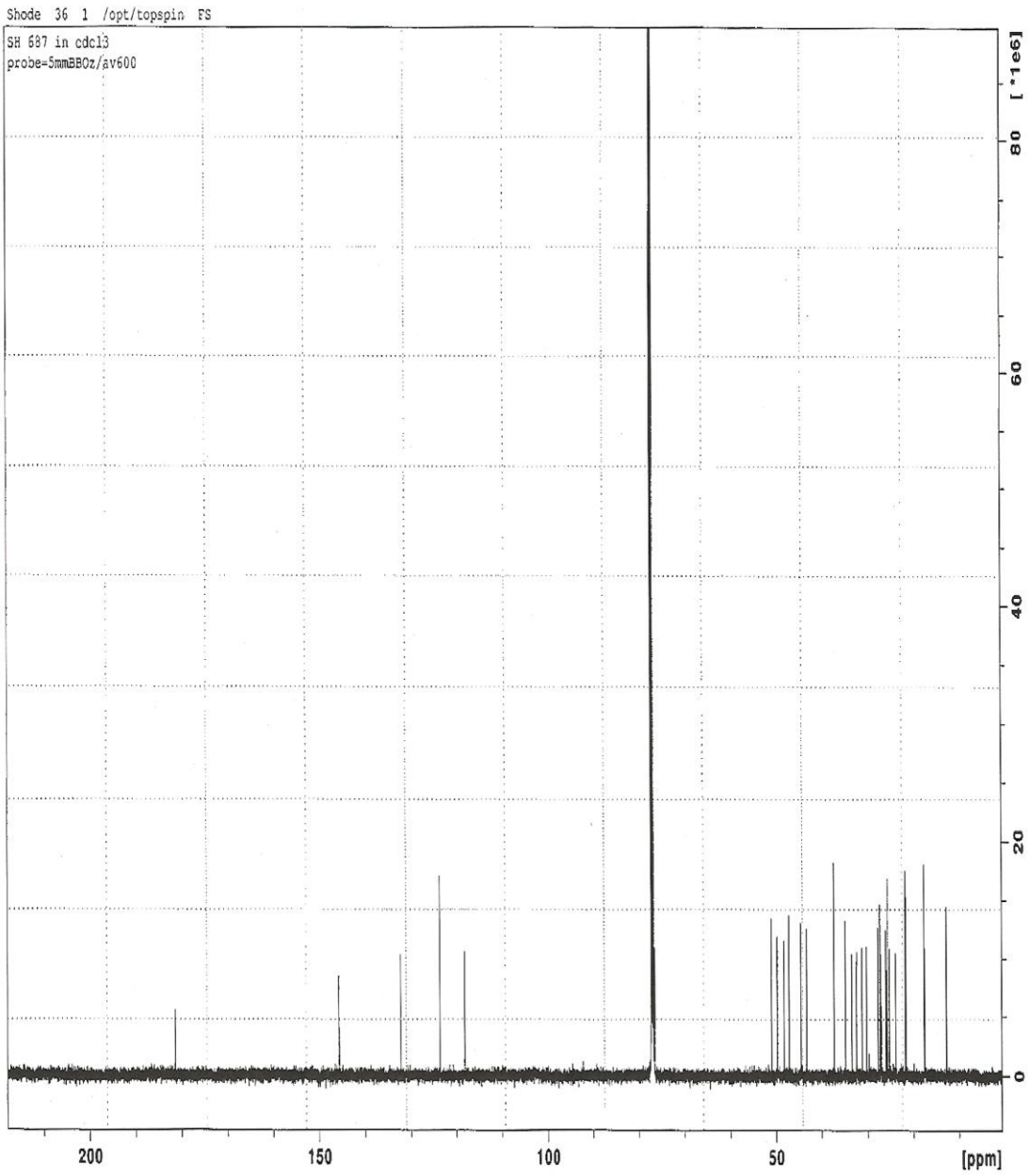

Figure D2: ^{13}C-NMR of Isolated Compound

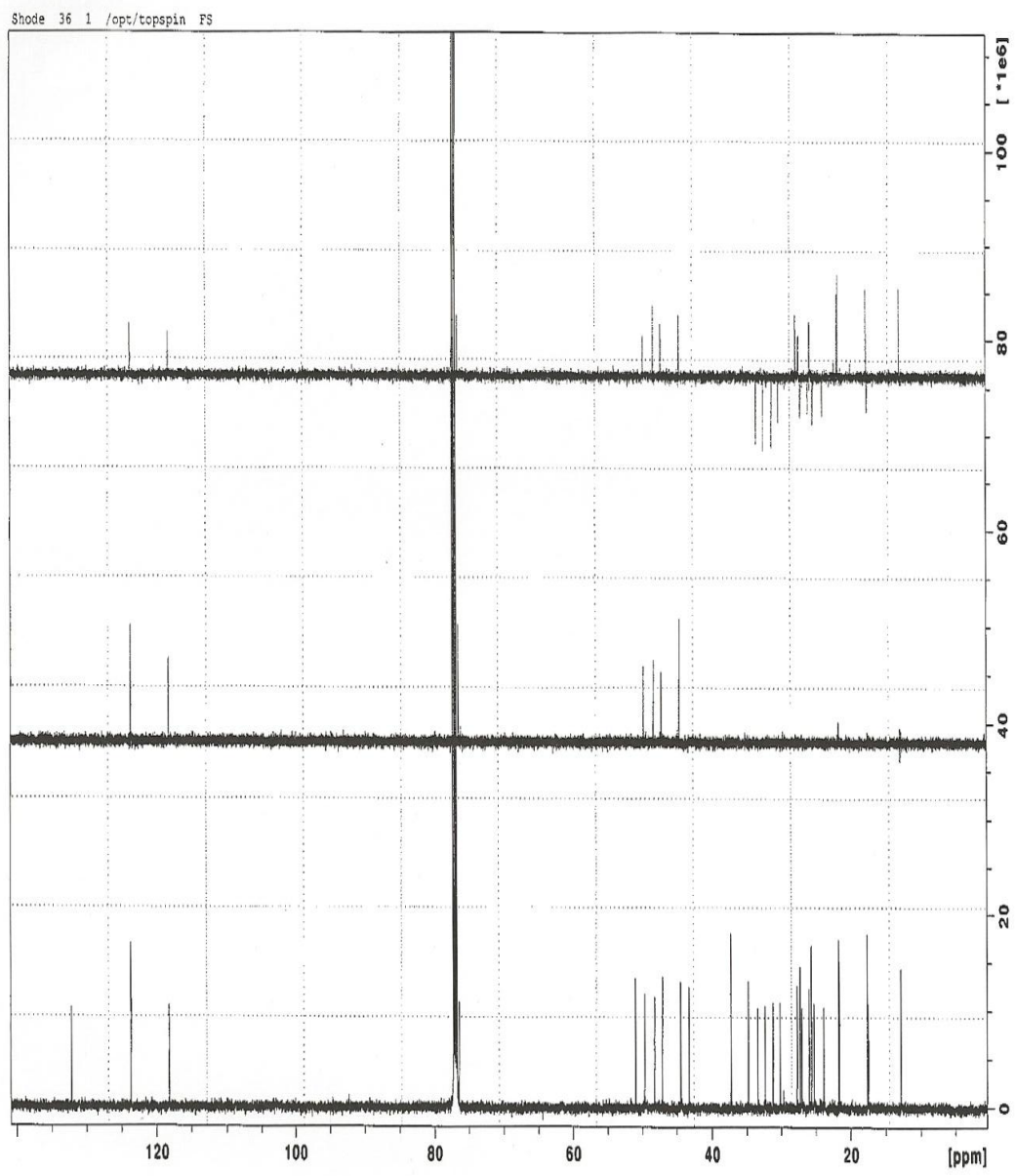

Figure D3: ^{13}C DEPT of Isolated Compound

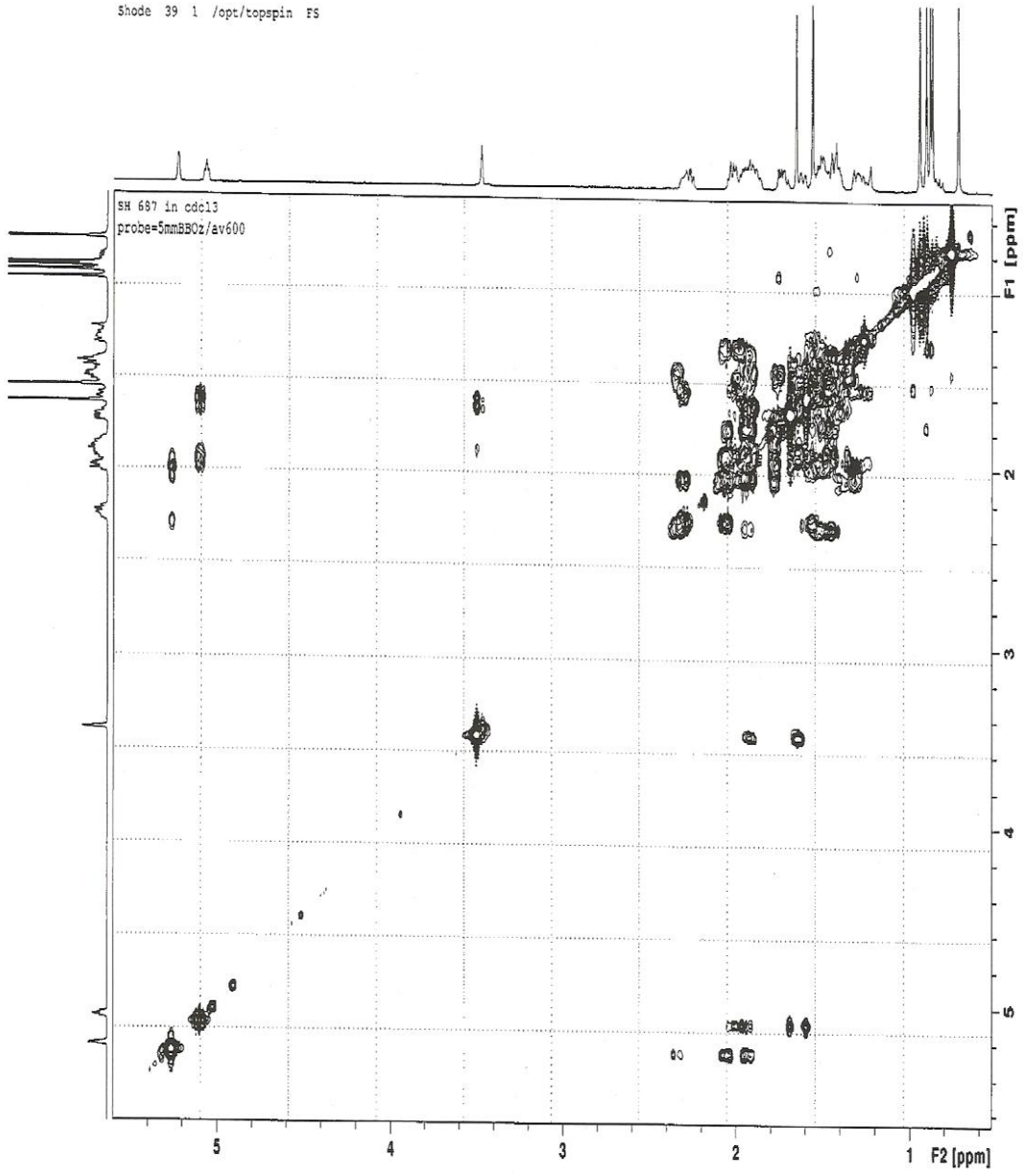

Figure D4: ^{1}H-^{1}H COSY NMR

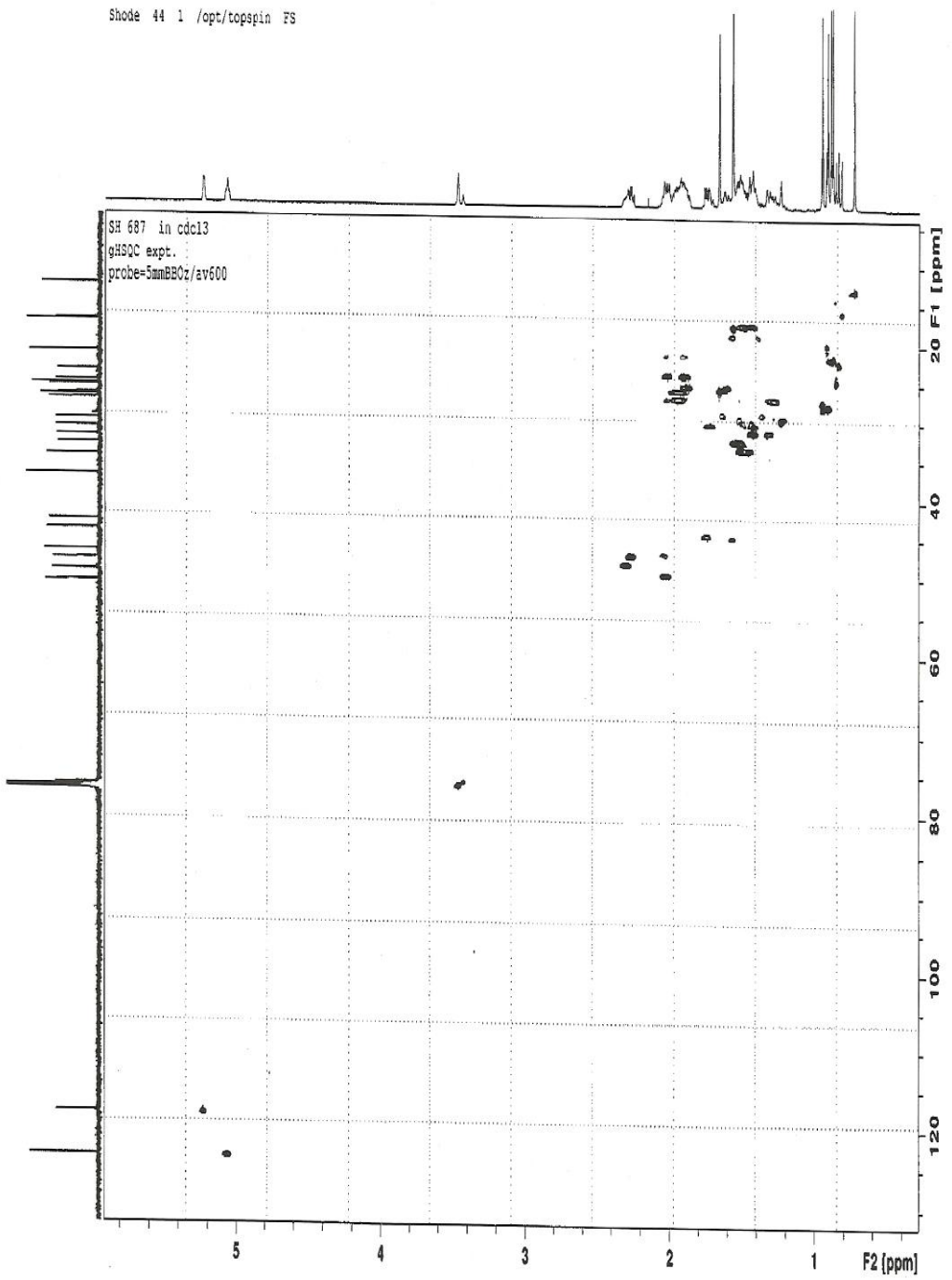

Figure D5: ^{1}H- ^{13}C COSY (HSQC) NMR of Isolated Compound

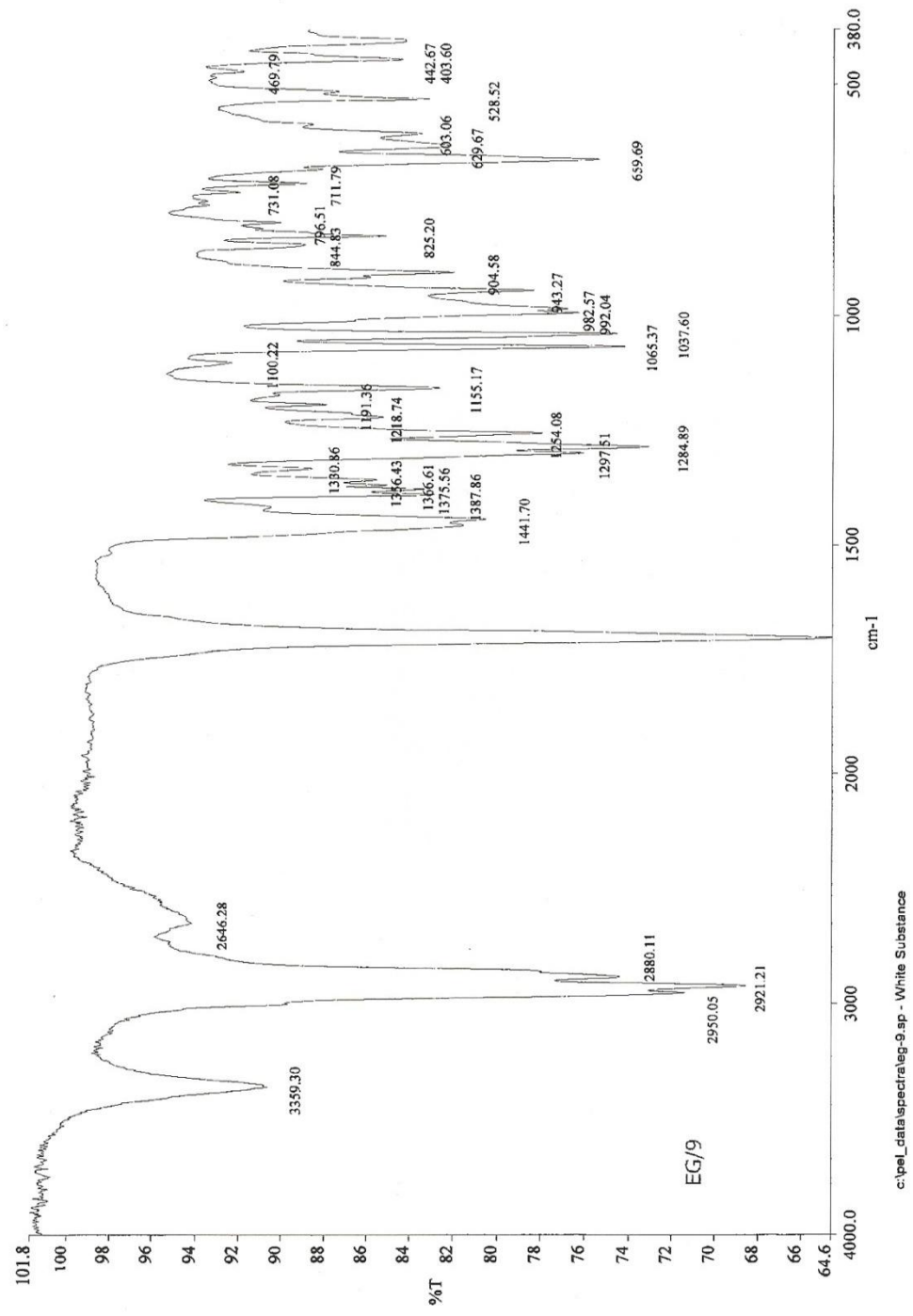

Figure D6: IR Spectrum of Isolated Compound

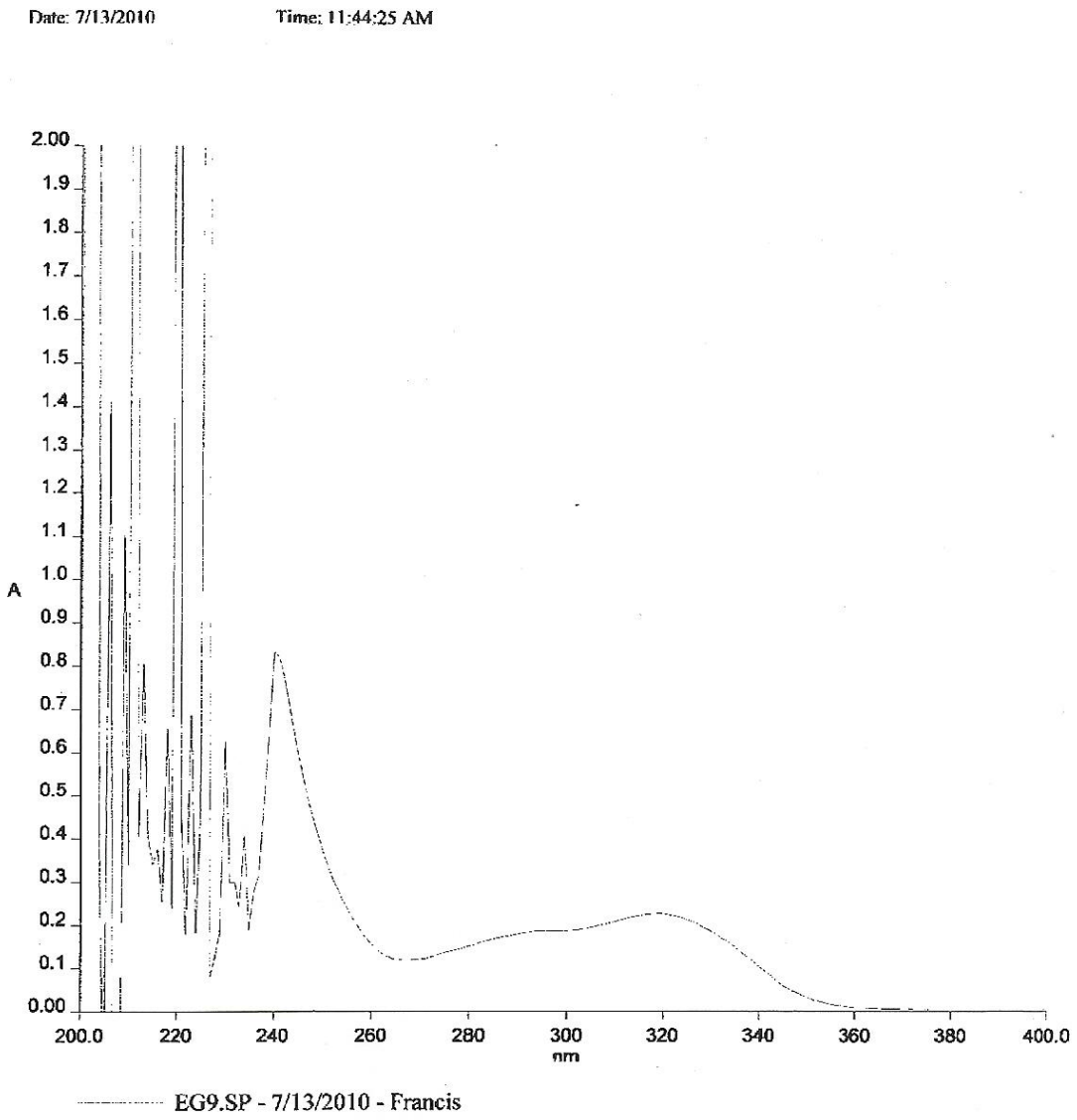

Figure D7: UV spectrum of isolated compound

Cgrthisformula. 1 - Sample absorbance

Appendix E: Contribution to knowledge

IN VITRO ANTI-PLATELET AGGREGATION ACTIVITY OF THE EXTRACTS OF SOME ZULU MEDICINAL PLANTS

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INTRODUCTION

➢Atherothrombosis is the cause of most cardiovascular diseases. The consequences of this could be the occurrence of an acute coronary accident, cerebrovascular stroke or peripheral arterial disease [1,13]

➢Three plants, *Bulbine natalensis, Protorhus longifolia, Rapanea melanophloes* that are commonly used by Zulu traditional healers to treat blood-clotting related diseases were screened for phytochemicals, cytotoxicity, and their anti-oxidant and anti-platelet aggregation activities.

➢*Bulbine natalensis* an aloe-like plant with clumping succulent rosettes, yellowish green leaves and yellow flowers. It is used by traditional healers to treat eczema, cracked lips, skin complaints, ringworm, rashes, burns, wounds, cuts and to stop bleeding. [7,15]

➢*Protorhus longifolia* is an evergreen, indigenous tree used to treat heart burn, bleeding from stomach, strengthen the heart and hemiphlegic paralysis.

➢*Rapanae melanophloes* is used to treat respiratory problems, stomach, muscular and heart complaints.

Bulbine natalensis

Protorhus Longifolia

Rapanae melanophloes

These medicinal plants were screened for anti-platelet aggregation activity.

MATERIALS AND METHODS

➢The plants were collected from Twinstream Nursery, Mtunzini. Plants were identified at the Department of Botany, University of Zululand and voucher specimens were prepared.

➢Air-dried and powdered plant parts were extracted sequentially with hexane, chloroform, ethyl acetate, methanol and water.

➢Phytochemical screening was carried out on the extracts using standard procedures to identify the bioactive constituents[5,11]

➢The anti-oxidant activity was determined by in vitro methods-DPPH, ABTS, REDUCING POWER and METAL CHELATING assay [3,4,12,14]

➢The methanolic extracts were tested for brine shrimp cytoxicity test activity . The shrimp larvae were subjected to the extracts in a multi welled culture plate. Number of survivors were counted and LC_{50} was determined. All experiments were carried out in triplicate[6,8]

➢ The anti platelet aggregation activity of the extracts was separately investigated on thrombin, ADP and epinephrine induced rat platelet aggregation; similar experiments were also carried out on enzyme (trypsin, bromelain, papain) treated platelets [8,9,10].

RESULTS AND DISCUSSION

TABLE 1

Phytochemical	*B natalensis*	*P longifolia*	*R melanophloes*
Anthraquinone	+	-	-
Saponin	+	+	+
Tannin	+	+	+
Flavonoid	+	+	+
Alkaloid	+	+	+
Cardiac Glycoside	+	+	+
Phlobatannin	-	-	+
Steroid	-	-	-
Terpenoid	-	+	+

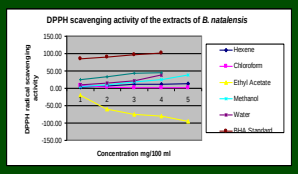
+ PRESENT ; - ABSENT

TABLE 2: LETHAL CONCENTRATION OF EXTRACTS LC_{50}

Solvent	*B natalensis* LC_{50}	95% CL µg/ml	*P longifolia* LC_{50}	95% CL µg/ml	*R melanophloes* LC_{50}	95% CL µg/ml
Ethyl Acetate	2.21	(1.87-2.52)	--	--	41,580	
Chloroform	2.55	(2.12-2.98)	54.7	(21.1 – 1668)	3698.349	
Water	4.30	(3.36-9.08)	--	--	30.930	
Hexane	5.23	(3.55-22.76)	39.6	(21.4 - 213.2)	1068,73	
Methanol	5.53	(4.692-10.08)	--	--	346.73	

ANTI OXIDATIVE ACTIVITY

Figure 1: DPPH activity for *B natalensis*



Extracts exhibited to varying degrees of efficiency, concentration and dependent anti oxidative properties as they scavenged DPPH, ABTS and chelated Fe^{2+} ions.

TABLE 3:

A: *Bulbine natalensis*

B *Protorhus Longifolia*

C:*Rapanae melanophloes*

IC_{50} (values I mg/100ml)

	DPPH			REDUCING POWER			METAL CHELATING			ABTS		
	A	B	C	A	B	C	A	B	C	A	B	C
HEXANE	>5	ND	>5	>3	ND	ND	>5	ND	4.21	ND	0.27	>5
ETHYL ACETATE	-	ND	4.83	>3	ND	ND	>5	ND	<1	ND	ND	4.99
CHLOROFORM	>5	ND	4.36	-	ND	ND	-	ND	3.57	ND	ND	2.4
METHANOL	>5	0.07	3.35	>3	ND	ND	>5	ND	>5	ND	ND	1.53
WATER	>5	ND	>5	>3	ND	ND	>5	ND	>5	ND	ND	>5
BHA	<1	ND	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND
BHT	ND	ND	4.54	ND	ND	ND	ND	ND	ND	ND	ND	3.72
ASCORBIC ACID	>5	ND	4.06	>5	ND	ND	ND	ND	ND	ND	ND	3.84
EDTA	ND	ND	ND	ND	ND	ND	<1	ND	3.42	ND	ND	ND
CITRIC ACID	ND	ND	ND	ND	ND	ND	3.81	ND	3.92	ND	ND	ND

ANTI PLATELET AGGREGATION STUDY: [3mg/ml]

Key: + (no aggregation)
- (aggregation)
ND: NOT DETERMINED

TABLE 4: Bromelain treated platelets

PLANT	Thrombin A	Thrombin B	Thrombin C	ADP A	ADP B	ADP C	Epinephrine A	Epinephrine B	Epinephrine C
HEXANE	+	+	ND	+	-	+	+	-	+
CHCl3	+	-	ND	+	+	+	+	+	+
ETHYL A.	+	+	ND	+	-	+	+	-	+
MeOH	+	+	ND	+	+	+	+	+	+
H_2O	+	+	ND	+	+	+	+	+	+

TABLE 5: Papain treated platelets

PLANT	Thrombin A	Thrombin B	Thrombin C	ADP A	ADP B	ADP C	Epinephrine A	Epinephrine B	Epinephrine C
HEXANE	ND	+	ND	+	+	+	+	-	-
CHCl3	ND	-	ND	+	+	+	+	+	-
ETHYL A.	ND	+	ND	-	+	+	+	+	+
MeOH	ND	-	ND	+	+	-	+	-	-
H_2O	ND	+	ND	+	-	-	+	-	-

➢Methanol and water extracts of *B natalensis,* Hexane, ethyl acetate and chloroform extracts of *P longifolia,* and Chloroform and ethyl acetate extracts of *R melanophloes provoked* a concentration dependent in vitro inhibition of rat platelet aggregation activity.

➢These results apparently support the use of these plants in managing blood clotting related diseases.

➢Phytochemical analysis are needed to characterize the active fractions responsible for the efect.

References will be provided on request

Appendix F

Research questionnaires

Interview of Traditional Healers

Date: Questionnaire No.

Name of the Interviewer:

Particulars of the area

GPS reading:

Name of the Area:

Name of the Village (Precise place):

Sociodemographic data

Gender: **Age:**

Male		15-24	
Female		25-34	
		35-44	
		45-54	
		55-64	

Plant Species particulars

Zulu name:

Plant 1:_____

Plant 2:_____

Plant 3: _____

Plant 4: _____

Scientific name:

Plant 1: _____

Plant 2: _____

Plant 3: _____

Plant 4: _____

English name:

Plant 1: _____

Plant 2: _____

Plant 3: _____

Plant 4: _____

Source of plant material:

Collected from the wild	
Cultivated (home-garden)	

What are the other uses of the plant?

_____

_____

Plant usage and collection

Question	Usage
Which part(s) are used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time of collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants	

Preparation Method:

a) How is the medicine taken (e.g. by mouth or as enema)?

b) How is the medicine prepared?

Storage Method:

Dosage:

a) What is the dosage (e.g. one cup three times a day)?_____

_____

b) For how many days is the medicine taken?_____

_____

c) Are there any known side effects? _____

_____

d) Where did the knowledge come from (e.g. grandmother, relative)?

_____

_____

Age Group:

Infants	
Children	
Adults	