In Vitro Anti-platelet Aggregation Activity of the extracts of *Bulbine natalensis*

By

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DECLARATION

I hereby declare that the work on which this dissertation is based is my own work. The experimental work was carried out in the Department of Biochemistry and Microbiology at the University of Zululand. Further lab work was conducted at the School of Chemistry, University of KwaZulu Natal under the supervision of Prof. A.R.Opoku and Prof. A.O. Oyedeji. The duration of the study was from April 2009 to August 2010. Where I have quoted from the work of others, the source is always given and where I have consulted the published work of others this is always clearly attributed.

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DEDICATION

This project is dedicated to my late grandfather, **Mr. Isaac Matthew**, who shared my passion for medicine and science. I would also like to dedicate this to my family for their positive motivation and support through the trials and tribulations of my life. Thank you for having faith in me and my dreams.

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- To my parents whose tremendous support and understanding has been wonderful. I love you.
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Abstract

Bulbine natalensis Baker is a medicinal plant with succulent, aloe-like leaves from the Asphodelaceae family, commonly used by Zulu traditional healers to treat blood clotting-related diseases. The aim of this research was to test the anti-platelet aggregation effect of *B.natalensis'* extracts on rat platelet aggregation separately induced by thrombin, ADP, collagen, epinephrine, papain, bromelain and trypsin. Fresh plant material was extracted sequentially using hexane, chloroform, ethyl acetate, methanol, and water. The chloroform extract had the highest yield of 0.20%. Phytochemical screening revealed the presence of anthraquinones, cardiac glycosides, saponins, tannins, flavonoids and alkaloids. The hexane extract revealed the highest contents of total phenols (5.028 mg/g); flavonoid (3.293 mg/g) and proanthocyanidin (2.565 mg/g). Hexane extract also displayed the highest ABTS scavenging activity (IC_{50} 4.72 mg/ml) followed by the ethyl acetate extract (IC_{50} 5.39 mg/ml). The water extract exhibited the highest reducing power**;** the activity was even greater than the standard antioxidant BHA. Studies reveal that *B.natalensis* is a good antioxidant. Brine shrimp lethality test indicated that all the extracts were highly toxic to the larvae. The ethyl acetate extract was the most toxic (LC_{50} 2.21 mg/ml).

Anti-platelet aggregation activities on rat platelets were observed. The chloroform extract inhibited ADP-induced clotting by 100% at doses 1 and 3 mg/ml with IC_{50} values of 5.32 mg/ml before tannin removal and >10 mg/ml after tannin removal.

Two compounds (F6/1 and F6/5) were isolated and purified from the chloroform extract. It was apparent that the compounds were unstable and could not be

specifically identified and characterized by NMR analysis. However, based on the spectra obtained, and comparing to existing literature data, the isolated compounds were envisaged to be a knipholone (an anthraquinone).

The compounds inhibited (100%) ADP-induced platelet aggregation. In addition the isolated compounds also exhibited *in vitro* anticoagulant activity on the whole rat's blood. Acetylcholinesterase activities of the compounds showed that they are not inhibitors of acetylcholinesterase.

CONTRIBUTION TO KNOWLEDGE

See Appendix E for details

1. **Lazarus, G.G**., Mosa, R.A., Gwala, P.E., Oyedeji, A.O., Opoku, A.R. "*In vitro* anti-platelet aggregation activity of the extracts of some Zulu medicinal plants". Greengold symposium, 14-16 September 2009, Pretoria, South Africa

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AA	Ascorbic Acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADA	Acid-dextrose-anticoagulant
ADP	Adenosine diphosphate
BHA	Butylated Hydroxy-Anisole
CC	Column chromotography
$CHCl_3$	Chloroform
CL	Confidence limit
DEPT	Distortionless enhancement by polarization transfer
DPPH	1, 1'-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetra-acetic acid
EtoAc	Ethyl acetate
HPLC	High performance liquid chromotography
IR	Infrared
KZN	KwaZulu Natal
LC_{50}	Lethal concentration with 50% inhibition
MeOH	Methanol
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
UZ	University of Zululand
UV	Ultraviolet
VLC	Vacuum liquid chromatography
^{1}H-NMR	Proton spectra
^{13}C-NMR	Carbon spectra
H_2O	Water

Chapter 1

Introduction

The body's natural defense mechanism is to protect itself against cuts and bruises. The formation of blood clots prevents severe blood loss from damaged tissue, blood vessels, or organs. If blood did not clot, a simple cut would lead to death. Blood platelets play a pivotal role in the haemostatic process. They rapidly adhere to the exposed sub-endothelium macromolecules such as collagen and thrombin, and eventually form a platelet plug temporarily sealing off the damaged vessel wall. However, disorders that cause excessive clotting in the body can lead to clot formation in arteries that could completely block the supply of blood and thus oxygen and nutrients to a portion of the body. If left untreated, blood clots in arteries can cause myocardial infarction and cerebrovascular accidents (Lee, 2009). According to Smith (2004), cardiovascular diseases are the most common cause of mortality in developing countries. More than 17 million people die from blood clotting related diseases in western countries. The risk factors include smoking, ageing, hypertension insulin resistance, hypercholesterolemia and free radical formation (Amrani, 2009).

The approach to treating these disorders is to inhibit the aggregation of platelets (Gerike and Van wyk, 2000). Common anti-platelet aggregation drugs such as aspirin and ticlopidine are used to protect against strokes, myocardial infarctions, cardiovascular death and other atherosclerosis diseases (Hankey *et al*, 2003). These drugs, however, have some restrictions in their mode of efficacy and action (Fabre and Gurney, 2010;

Patrono *et al,* 2004; Wiviott *et al*, 2007; Mega *et al,* 2009; Bhatt *et al*, 2007; Wiviott *et al*, 2007).

There is therefore a critical need for new anti platelet therapies that do not increase risk of bleeding. A cheaper alternative to treating blood clots and preventing their formation is medicinal herbs. Since the beginning of civilisation people have used plants to treat hypertension, wounds, stomach ailments and venereal diseases. In many developing countries, a large percentage of the population still rely heavily on traditional practitioners and medicinal plants to meet primary health needs. Although modern medicine may be available in these countries, herbal medicines have often maintained popularity for historical and cultural reasons.

Many plants are used by traditional healers in treating blood related disorders. *Crinum macowanni* is used by Zulus to increase blood supply in the body (Haouari *et al*, 2000). *Commelinaceae benghalensis* is used to reduce high blood pressure (Harborne *et al,* 1973). *Allium sativum* (Garlic) has been used to reduce the hardening of arteries and blood clotting by inhibiting platelet aggregation (Edoega *et al*, 2005).

A medicinal plant, *B. natalensis,* used traditionally by the Zulus to provide healing has not received scientific validation for its medical application as an anti-agglutinant.

This study investigates the anti-platelet aggregation activity of *B. natalensis* against rat platelet aggregation separately induced by thrombin, epinephrine and ADP.

Chapter 2

Literature Review

Blood is a specialized fluid in the body that is of vital importance for physiological processes to take place. It transports food, nutrients, O_2, CO_2, water and waste around the body. It is the medium through which all life processes flow. In order for this to happen normally and to prevent excessive bleeding, there is a defense mechanism in the body called hemostasis that stops bleeding (Verhamme and Hoylaerts, 2009). Other synonyms for solidification of blood include aggregation, coagulation, clotting or thrombosis.

2.1 The blood clotting cascade

Blood platelet aggregation plays a critical physiological role in the human body and platelets, also called thrombocytes, are directly involved in the haemostatic processes (Gerike *et al*, 2000; Van Wyk *et al*, 1997)**.** When damage is caused to a blood vessel, solidification takes place by the platelets aggregating to the site of injury, sealing off further blood loss and reducing the possibility of bleeding to death.

The formation of a blood clot occurs at the site of injury where vascular endothelium is disrupted. In the case of the sub-endothelium being damaged by atherosclerosis the thrombogenicity of the vascular wall increases and the fibrous cap that covers the atherosclerosis plaque is ruptured and breaks the endothelial barrier. The plaque fissure exposes the collagen matrix to the flowing blood and allows the adherence of platelets, thereby triggering the formation of a thrombus (Fabre and Gurney, 2010). There are two

haemostatic processes that are initiated in the coagulation cascade ultimately leading to the formation of an insoluble blood clot:

i) The tissue factor pathway also known as the extrinsic pathway that activates platelets and initiates enzymatic production of thrombin (Furie *et al*, 2008).

ii) The contact activation pathway also known as the intrinsic pathway which initiates blood coagulation (Cazenave *et al*, 2004).

The pathways are a series of reaction in which zymogens of a serine protease and its glycoprotein co-factor are activated to become active components that then catalyze the next reaction in the cascade. Fibrinogin is a protein that stops bleeding by helping blood clots to form. Fibrinogin is converted to cross-linked fibrin clots by thrombin. Subsequently, these pathway factor interactions take place and then converge at the same point, appropriately named the common pathway which involves the activation of factor X to Xa (Figure 2.1). The factor Xa is responsible for conversion of prothrombin to thrombin (proteolytic enzyme). From the common pathway, clotting continues initiating a series of events that ultimately lead to the formation of an insoluble, stabilized fibrin clot. Ca^{2+} ions play an important role in the activation of many proteins involved in the coagulation cascade. It participates in the activation of prothrombin to thrombin and it also activates phospholipase A_2, the enzyme responsible for arachidonic acid synthesis. Cyclooxygenase1 (COX-1)-catalysed arachidonic acid metabolism produces thromboxane A_2 (Fabre and Gurney, 2010), a potent platelet aggregator. The formation of a thrombus takes approximately 12-16 sec in a normal individual (Obdoni and Ophuku 2001). The blood clot is only a temporary solution to stop bleeding; vessel repair is therefore needed. The aggregated platelets help this process by secreting

chemicals that promote the invasion of fibroblasts from surrounding connective tissue into the wounded area to form a scar. When the blood clot is no longer required for haemostasis, the fibrinolytic system dissolves it and clears it by phagocytosis (Arnout *et al*. 2006)

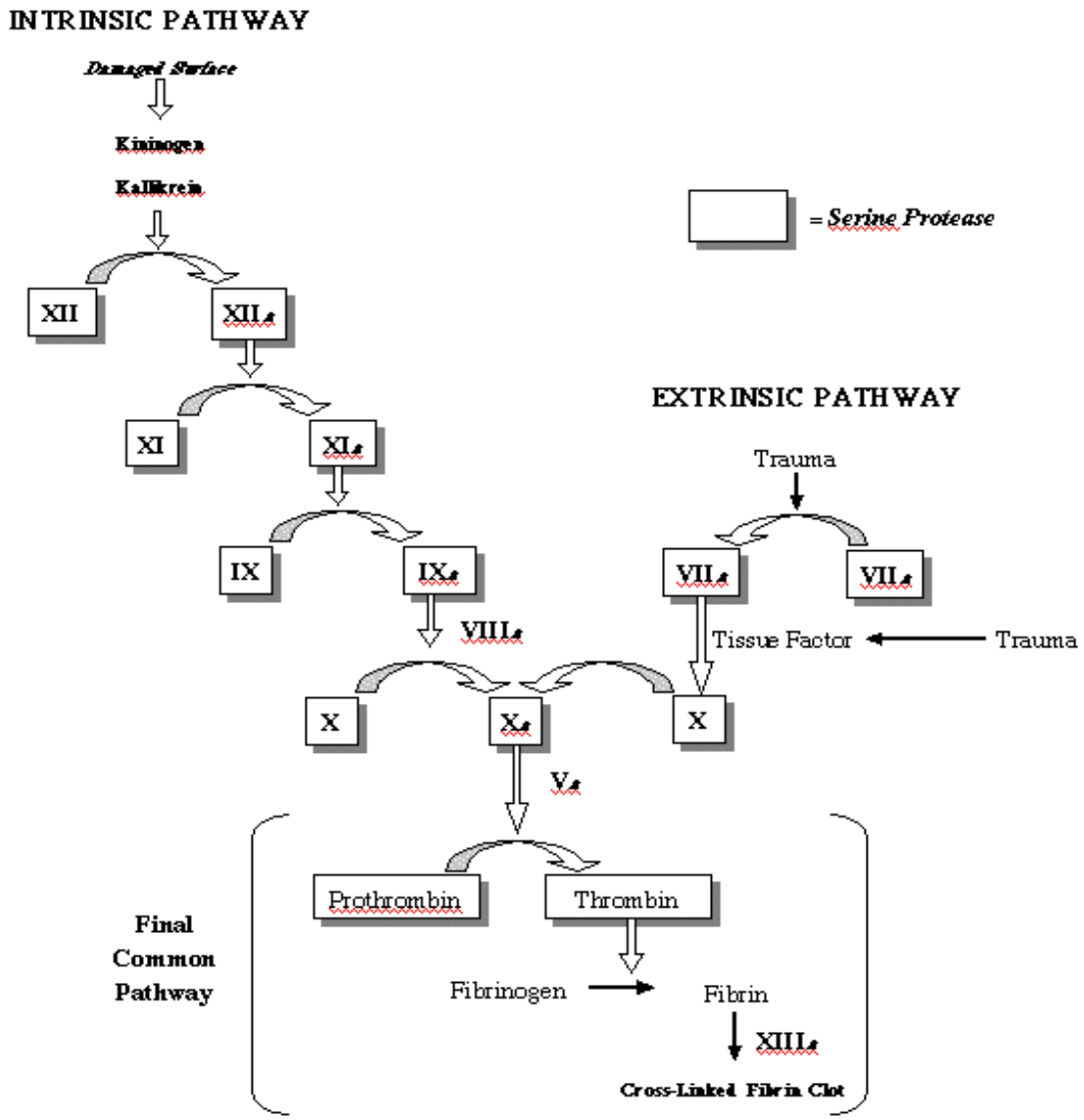


Figure 2.1 Blood clotting cascade (King, 1996).

2.1.1 Platelet activation and aggregation

Platelet activation is complicated and normally involves multiple signaling pathways and molecules which are responsible for the different biochemical interactions (Xiang *et al*, 2008). Platelet activation and aggregation is induced by the binding of agonists on the various receptors found on the platelet surfaces. The agonists include thrombin, epinephrine, thromboxane $A_{2,}$ collagen, platelet-activating factor and adenosine-5'-diphosphate (ADP) all of which have specific receptors to bind to. The various agonists bind to their specific receptors on the platelet surface and mediate the activation and aggregation of platelets through these receptors. The available anti-platelet agents are designed in such a way that they act by targeting specific pathways.

2.1.1.1 Thrombin

Thrombin is very important in both haemostasis and thrombosis. Its effects are mediated through membrane-bound G-protein coupled receptors. There are two pathways through which thrombin activate platelets (Soslau *et al*, 2001). The first pathway involves hydrolysis of protease-activated receptor-1 (PAR-1) followed by glycoprotein IIb/IIIa (GP IIb/IIIa) dependent platelet aggregation, while the second one does not hydrolyse PAR-1, it is GP Ib dependent and uses fibrin polymer instead of fibrinogen. Recent reports have shown that thrombin acts through PAR-1 and PAR-4 receptors and these receptors work cooperatively in the activation of platelets (Fabre and Gurney, 2010). Platelet activation by thrombin can be characterized by the activation of membrane receptors, shape change, granular secretion, cytoskeletal remodeling and aggregation (Jardin *et al*, 2007).

2.1.1.2 ADP

The ADP-induced platelet activation is autocatalytic in that upon activation by ADP platelets it releases other ADP molecules that act on nearby platelets thereby amplifying the reaction. ADP acts through G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$. The two receptors work closely together to ensure a complete activation and aggregation of platelets. The platelet activation and aggregation is initiated through $P2Y_1$, amplified and sustained through $P2Y_{12}$ (Storey, 2006). The activity of ADP-induced platelet activation requires the availability of Ca^{2+} and is inhibited by cAMP.

Therefore, increased intracellular Ca^{2+} and a decrease in cAMP level are crucial for ADP-induced platelet activation and aggregation. Gurney and Fabre (2010) have shown that ADP acts through the $P2Y_{12}$ and gq pathway to inhibit adenhyl cyclase formation of cAMP. Platelet morphological change, granular release, increased intracellular Ca^{2+} and decreased cAMP are the characteristics of ADP-induced platelet aggregation (Puri and Colman, 1997).

2.1.1.3 Epinephrine

Epinephrine is a weak platelet agonist reported to exert its effects on human platelets through α2-adrenergic receptors and also potentiates aggregation induced by other platelet agonists (Lanza *et al*, 1988; Choi, 2002). Lanza *et al.* (1988) further suggested the ability of epinephrine to potentiate all types of aggregating agents on aggregation such as intracellular Ca^{2+} mobilization, fibrinogen binding, or protein phosphorylation and granular release.

2.1.2 Blood clotting related disorders

Disorders that cause excessive clotting in the body can lead to clot formation in the arteries that could completely block the supply of blood and subsequently oxygen to a portion of the body, for example, blockages may occur in the heart or brain which may lead to swelling, inflammation or death (Fansworth, 1996 and Himmelheber *et al*, 2000; Amrani and Harnafi, 2009). If left untreated, a blood clot that forms in a coronary artery supplying blood to the heart muscle can cause myocardial infarctions. Blood clots in the vein can cause deep vein thrombosis (DVT) in the pelvic leg, deep veins of the legs or upper limb (Bates and Ginsberg, 2004). A stroke is the rapidly developing loss of brain function due to a disturbance in the blood supply to the brain. Pulmonary embolism is a blockage of the pulmonary artery or one of its branches, usually occurring when a deep vein thrombosis (blood clot from a vein) becomes dislodged from its site of formation and travels to the arterial blood supply of one of the lungs. Predisposition to endothelial impairment is increased by risk factors such as atherosclerosis, hypercholesterolemia, hypertension insulin resistance, aging and smoking (Amrani and Harnafi, 2009). Other studies in hypertension patients have shown that platelets were more sensitive to thrombin and showed an elevation in their intracellular free calcium which may later initiate platelet activity and increase the risk of thromboembolic diseases (Dogne *et al*, 2002).

Research has shown that inflammation and platelet aggregation activate each other (Fabre and Gurney, 2010). One characteristic of a damaged vessel is inflammation. Atherosclerosis is the most common inflammatory disease of the vascular wall

(Hansson *et al*, 2006). It results when low-density lipoproteins (LDL) become oxidized by free radicals causing the arteries to be inflamed. As a result LDL transport cholesterol in the bloodstream and expose them to oxidation. Oxidation lipids promote endothelial cells to express adhesion molecules for inflammatory cells (Hansson *et al*, 2006). Atherothrombosis which involves interaction between atherosclerotic plaque and arterial thrombosis may result when vulnerable, lipid-rich atherosclerotic plaque erode or rupture. This triggers the formation of a platelet-rich thrombus that may partially or completely block the artery (Lee *et al*, 2009).

2.1.3 Free Radicals

Free radicals are atoms or molecules with an unpaired electron which makes them highly unstable. In an effort to achieve stability, free radicals attack nearby molecules to obtain another electron and in doing so damage those molecules which lead to cellular and genetic mutations. Types of free radicals include the hydroxyl radical (O.H), the superoxide radical ($O._2$), the nitric oxide radical (NO.) and the lipid peroxyl radical (LOO). Free radicals and other reactive oxygen species are derived from external sources, for example, x-rays, ozone, cigarette smoking, and environmental pollutants or from normal metabolic processes in the body such as reactions involving iron and other transition metals, inflammation, ischaemia (Langseth, 1996). Enzymatic and non-enzymatic reactions stimulate the formation of free radicals in cells. Enzymatic reactions include those involved in the respiratory chain, phagocytosis, prostaglandin synthesis and the cytochrome P_{450} system. Non-enzymatic reactions of oxygen with organic compounds give rise to free radicals as well as those initiated by ionizing radiations

(Langseth, 1996). Two major causes of death: cancer and atherosclerosis are silent free-radical diseases. Emerging studies on atherosclerosis reveal that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxidases and other substances. These compounds induce endothelial cell injury and produce changes in arterial wall (Harman, 1992). Free radicals can thus cause inflammation in the arteries which leads to swelling and internal platelet aggregation.

Free radical scavengers or antioxidants protect against free radical damage. These compounds inhibit the oxidation of other molecules by binding with the free radicals before they can do their damage (Mole and Waterman, 1987). They absorb all the excess reactivity that free radicals have and render them harmless for removal from the body (Adedayo *et al*, 2010). Antioxidants found in nature are glutathione, vitamin C, as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Recent studies show that one of the practical ways through which the activity of free radicals could be managed in the body is through dietary means (Oboh and Rocha, 2007). Dietary antioxidants act as radical scavengers, reducing agents, forming complexes with pro-oxidant metals and quenchers of singlet oxygen formation (Obah, 2005).

2.2 Acetylcholine

Acetylcholine (ACh) is a chemical compound that activates muscles, and is a major neurotransmitter in the autonomic nervous system. In the central nervous system,

acetylcholine and the associated neurons form a neurotransmitter system, the cholinergic system, which tends to cause anti-excitatory actions. Recent studies have correlated nicotinic acetylcholine receptor (nAChR) dysfunction with the neurodegeneration and cognitive deficits of Alzheimer's disease, epilepsy, schizophrenia and Parkinson's disease (Nashmi *et al*, 2003).

Alzheimer's disease is a progressive neurodegenerative disorder that primarily affects the elderly and accounts for 70% of all dementia. Alzheimer patients have reduced synthesis of acetylcholine which causes damage to the cholinergic system in the brain resulting in memory deficits. Those with the disease display a progressive deterioration of memory and cognitive function that eventually leads to severe dementia and death (Georgi, 2005).

Research in the late 1980's indicated that a major feature of Alzheimer's disease is decreased cholinergic (acetylcholine-based) activity in the brain. In addition, it was found that patients had up to 90% decrease in acetylcholinesterase activity. These two enzymes, acetylcholinesterase and cholineacetyltransferase activity are respectively involved in the degradation and synthesis of acetylcholine (Small and Fodero, 2002). Acetylcholinesterase inhibitors are chemical compounds that bind to and deactivate acetylcholinesterase, thus compensating for post-receptor loss with a higher level of synaptic acetylcholine. These inhibitors have been successful and there are currently several such drugs on the market including Tacrine, Riva stigmine and Donepezil. While these drugs can be beneficial, they cannot prevent the progression of the disease. Because acetylcholine transmission is not affected in muscles, these drugs may lead to

an over stimulation of cholinergic systems outside of the brain and cause a variety of side effects including continuous stimulation of the muscles leading to muscle cramping, nausea, vomiting, anorexia, diarrhea, bradycardia, weakness, nightmares, insomnia and agitation (Grutzendler and Morris, 2001).

Pharmacological manipulation of cholinergic function has been found useful in the treatment of CNS disorders including Parkinson's disease (Srikumar *et al*, 2004). Blocking or hindering the action of acetylcholine has many uses in medicine. Thus, assessing cholinergic function is considered as an important tool in neuroscience research. There are several approaches to evaluate cholinergic function indirectly. Estimating the expression of choline acetyl transferase (ChAT) and acetylcholinesterase (AchE) by immunochemical and histochemical techniques provide information on the cholinergic function, but are time-consuming. A relatively easy and valuable assessment of choliergic function is estimating the AchE activity.

2.3 Current anti-platelet therapy and its limitation

The approach to treating disorders such as deep vein thrombosis, strokes and heart attacks is to inhibit the aggregation of platelets (Gerike; Van wyk, 2000). Common anti-platelet drugs such as aspirin and ticlopidine are used to protect against strokes, myocardial infarctions, cardiovascular death and other atherosclerosis diseases (Hankey *et al*, 2003). Oral doses of aspirin are absorbed in the stomach and the upper small intestine where esterases in the gastrointestinal membrane and liver rapidly hydrolyze it to salicylic acid. Aspirin is rapidly cleared from the circulation with a half life

of 15-25 minutes. Systemic exposure to low-dose aspirin thereby is limited (Fabre and Gurney, 2010). Clinical doses of Clopidogrel and Prasugrel have greater impact on bleeding than does low-dose aspirin (Fabre and Gurney, 2010; Patrono *et al,* 2004; Wiviott *et al*, 2007; Mega *et al,* 2009; Bhatt *et al*, 2007). Recent studies have shown that for each cardiovascular death prevented by Prasugrel as compared to Clopidogrel, there was approximately one additional fatal haemorrhage (Fabre and Gurney, 2010). Therefore, the risk of bleeding time limits the benefit of all these drugs (Wiviott *et al*, 2007).

There is therefore a critical need for new anti platelet therapies that do not increase risk of bleeding. This will have important benefits for stroke patients, for heart patients undergoing coronary artery bypass surgery (CABG) (Gadi and Bnouham, 2009; Hsieh *et al*, 2007). New medicines of plant origin are continually being discovered (Kee *et al*, 2007; Van Wyk *et al*, 1997; Rahman *et al*, 2007; De Medeirois *et al*, 2000).

2.4 Traditional medicine

Since the beginning of civilization, plants have been used to treat infections and diseases. A medicinal plant is any plant that has medical effects or medical properties. The chemical components present in plants have medicinal values which produce definite physiological actions in the body. These components are called phytochemicals, the most important of these bioactive groups being: alkaloids, steroids, terpenoids, flavonoids, tannins and phenolic compounds (Edeoga *et al,* 2005).

Traditional healers are able to prescribe medicine in the form of plants that grow in their surrounding areas. In some Asian and African countries, 80% of the population depends

on traditional medicine for primary health care (Hutchings, 1996). Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained popularity for historical and cultural reasons. Plants have been used to treat diseases including heart attack, stroke, and stomach ailments (Hutchings, 1996; Breyer and Watt, 1932).

Herbal remedies prepared from garlic *(Allium sativam)* are believed to inhibit platelet activation (Edoega *et al*, 2005; Rahman and Billington *et al*, 2000). Tomatoes (*Lycopersicum esculentum*) may protect against cardiovascular diseases by inhibiting platelet aggregation (Dutta-Roy *et al*, 2001). *Melicope semecarpifolia* has shown anti-platelet aggregation properties (Hutchings, 1996). *Melilotus albus* contains the chemical coumarin that exhibits anti-clotting activities (Sofoware *et al*, 1993). Few other plant species that provide medicinal value have been scientifically evaluated for their possible medical application. *Ocimum basilicum* and *Petrolselinum crispim,* are among other plants being used for their haemostatic and cardiovascular effects (Amrani and Harnafi, 2009; Gadi and Bnouham, 2009). *Panax notoginseng* and *Panax quinquefoilum* are good sources of lead compounds for novel anti-platelet and anti-coagulant therapeutics (Lau *et al*, 2009). Plants species that have been used to treat blood diseases including arterial hypertension are *Arbutus unedo* (Ericaceae) and *Urtica dioica* (Urticaceae), (Goldhaber and Morrison, 2002). Other anti-clotting medicinal plants include angelic root, anise, borage, devils claw, papain, ginseng, gingko, horse chestnut, alfalfa, red clover, fever few, passionflower herb and garlic (Van Wyk *et al*, 1997). Interestingly, some natural compounds in the diet may inhibit platelet activation (Gadi and Bnouham,

2009). Traditional healers in Zululand were interviewed (see Appendix C) and one of the plants that are commonly used to treat blood related diseases is *Bulbine natalensis*.

2.5 *Bulbine natalensis* Baker

B.natalensis Baker also known as ibhucu (Zulu); rooiwortel (Afrikaans) is a frost tender evergreen plant with broad, sharp-pointed, fleshy, yellow-green leaves (Figure 2.2). This succulent is widely distributed in eastern and northern South Africa. Its soft, clumping, fleshy, yellowish green leaves grow up to 35cm long and is drought-tolerant and need regular to moderate water (Boham and Kocipal, 1974). The leaf is filled with a clear gel and is used traditionally by the Zulus in a similar way that aloe vera is used. It is used for the treatment of wounds, burns, rashes, itches, ringworm, cracked lips (Pujol, 1990; Rood, 1994; Watt and Breyer, 1962) and herpes. The roots are taken two or three times a day orally in the form of infusions (or sometimes a brandy tincture) to ease vomiting and diarrhea (Pujol, 1990) and also to treat convulsions, venereal disease, diabetes, rheumatism, urinary complaints and blood disorders (Pujol, 1990; Rood, 1994; Watt and Breyer, 1962).

Figure 2.2 *Bulbine natalensis* Baker (Van wyk *et al*, 1997).

Pharmacological studies done on rats have shown that aqueous extracts of *B. natalensis* have various activities, for example, in the management of male sexual dysfunction (Yakubu and Afolayan, 2010), disorders of libido and sexual behaviour (Boham and Kocipal, 1974; Hsieh, 2005; Yakubu and Afolayan, 2009c) and for use by females during the organogenic period of pregnancy (Yakubu and Afolayan, 2009d).

Recent studies have also investigated the effect of *B. natalensis,* on the haematological and serum lipid profile (Yakubu and Afolayan, 2009a), hepatic and renal functions (Yakubu and Afolayan, 2009b), of wistar rats and the inappropriate use of the plant's extracts in the treatment of HIV/AIDS due to the inaccessibility of antiretroviral drugs (du Plessis-Stoman, 2009).

Stems and roots of Bulbine species contain anthraquinones such as chrysophanol and knipholone (Van staden and Drewes, 1994; Van wyk, 1995). The antibacterial activity of the plant is most likely due to glycoproteins in the leaf gel, such as aloctin A and aloctin B (Bruce, W.G.G. 1975).

2.6 Review of Methods

A phytochemical is a naturally occurring bioactive compound present in plants. There are more than a thousand known phytochemicals (secondary metabolites). These compounds have no nutritive value in plants. Their major role is in defense against herbivores, microorganisms and insects (Policegoudra *et al*, 2010). Humans have found use of these chemicals in food flavouring, medicine or recreational drugs (Schultz, 2002).

Since the plant material contains numerous chemicals, there is a need for more advanced standard methods or techniques that can, at the same time, perform both qualitative and quantitative analysis. Several different standard phytochemical screening methods have been employed in order to know about the phytochemical composition of plant material.

2.6.1 Isolation and Purification Methods

Isolation and characterization of bioactive compounds from crude extracts are a tedious and time consuming process. A relatively low yield of the bioactive compounds is a major concern that also needs to be addressed. The recent advancements in isolation, separation and analytical techniques of the active constituents of crude extracts have

led to an increase in the number of isolated compounds from various medicinal plants (Harvey, 2007; George *et al*, 2001).

Chromatography plays an important role in the separation of compounds from complex mixtures such as natural product extracts. Various techniques are employed such as thin layer chromatography (TLC), vacuum layer chromatography (VLC), column chromatography (CC) and high performance layer chromatography (HPLC). Separation is achieved when the solutes (or sample compounds) in the mobile phase demonstrate different affinities for the stationary solid phase, the mobile phase, or both, resulting in different retention times for the various sample compounds.

2.6.1.1 Thin Layer Chromatography (TLC)

TLC is fast, simple and cost effective. It is a qualitative identification of a sample and gives us a basic idea as to how many components are in the extract. The stationary phase is a thin layer on a glass plate or a plastic film. The thin layer may be an adsorbent such as silica gel or alumina which is made into a slurry placed in a layer on a sheet and then dried. Usually the silica is fluorescent. The sample is placed 0.5cm from the edge of the plate as a small spot solution. The TLC plate is placed in a chromatography tank and the solvent is allowed to flow up the plate by capillary action up to about 1cm from the end of the plate. As the solvent moves up, it causes separation of the materials. Once the solvent has evaporated off the plate, the spots may be visualized under UV light. A spray agent is applied to the plate. This is followed by heating for optimal colour development.

2.6.1.2 Column Chromatography (CC)

This type of chromatography is used to obtain pure chemical compounds from a mixture of compounds. It is also a solid-liquid technique in which the two phases are a solid (stationary phase) and a liquid (moving phase). Dry packing is used for bonded silica gel. The stationary phase is “wetted” using an appropriate solvent by allowing the solvent to flow through the column. The column is then equilibrated with the mobile phase required for the sample. Slurry packing is the easiest and most common. Glass columns with a tap at the bottom are used of different sizes (5cm x 50cm; 20cm x 150 cm) depending on the size of the sample. The slurry is prepared by mixing the adsorbent with the solvent to be used for elution. The mixture of materials to be separated is placed at the top of the column and is slowly washed down with a suitable mobile phase. Each type of material moves down the column at different rates, depending on its solubility and its tendency to be adsorbed. The solid phase usually consists of silica gel or alumina. The choice of silica gel is important because it affects the flow of the solvent through the column. The mobile phase is the solvent. A mixture of solvent can be used and the eluting power results mostly from adsorption of the solvent. Combinations of solvent systems are used to help separate compounds of varying polarities. In many cases compounds with biological activity are relatively non-polar (Brendelar *et al*, 2010). It is advisable to first test which solvent system best separates the compounds by using the simple TLC method. Usually solvent systems of increasing polarity are employed, thus allowing for optimum elution of the column. As the eluants are collected as fractions, the polarity of the eluting solvent is increased.

The fractions are then analyzed by TLC and the similar fractions are combined and their chemical composition investigated.

2.6.1.3 Vacuum layer chromatography (VLC)

The vacuum is used to achieve a compact packing of the column. After the sample has been added, the mobile phase is carefully applied. The flow is activated by vacuum. The solvent is removed under vacuum in a rotary evaporator leaving the sample adsorbed onto the silica gel. This dry silica gel containing the sample can be transferred to the top of the column bed and wetted with a little of the initial mobile phase to remove air bubbles.

2.6.1.4 High performance layer chromatography (HPLC)

HPLC is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres which makes it much faster than previous methods. Small particle sizes are considered for the column which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture. It has detection methods which are highly automated and extremely sensitive making it one of the most powerful tools in analytical chemistry.

2.6.2 Structural elucidation and identification

Structural elucidation of the isolated compound can be determined through advanced techniques such as nuclear magnetic resonance (NMR), ultraviolet spectroscopy (UV), infrared spectroscopy (IR) and mass spectrometry (MS). The different techniques provide different information on the structure of the compound. NMR is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon and others do not, depending on whether they possess a property called a spin. NMR consists of two types 1D (^{1}H-NMR, ^{13}C-NMR, ^{13}C-DEPT) which involves studying the chemical structure. They have one frequency axes and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HMQC) which determines the structure of more complicated molecules. It has two frequency axes which can correspond to similar nuclei or different nuclei (Hornak, 1997-1999).

2.6.3 Cytotoxicity

Despite an increased interest in natural products and their derivatives, safety of their use is still a big concern. A plant extract or active compound may show very good biological activity but at the same time exhibit strong potential cytotoxic properties. Brine shrimp lethality test is a common bioassay employed for preliminary screening of the cytotoxicity of plant extracts. Although the brine shrimp is considered a very simple and fast method to determine the cytotoxicity of the extracts, it is also very time consuming and subjective (Ripa *et al*, 2009).

2.7 Aims

The aim of this study was to investigate the *in vitro* anti platelet aggregation activity of the extracts of *B. natalensis* and to isolate and partially characterize the active compound therein.

2.8 Objectives

- Collection and identification of the plant material
- Extraction and preparation of the extracts
- Phytochemical screening of the plant
- Screening the plant extracts for anti-oxidant, anti-platelet aggregation activities and cytotoxicity
- Determination of the rate of clot formation induced by $CaCl_2$
- Tannin removal from the extracts and screening of the tannin-free extract for their anti-platelet aggregation activity
- Isolation and characterization of active constituent(s) from the extract
- Anti-oxidant and antiplatelet aggregation activity of isolated compound
- Effect of the isolated compound on acetylcholinesterase activity

Chapter 3

Materials and Methods

This chapter gives a brief description of materials and methods used to prepare plant materials, screen for phytochemicals, antioxidant, anti-platelet aggregation and acetylcholinesterase inhibition activity. It also includes a brief description of the materials and methods that were used to isolate and characterize active compounds. The details of the preparation of reagents and the methods are presented in the Appendix A and B respectively.

3.1 Materials

3.1.1 Reagents (See appendix A for details)

All chemicals, reagents and solvents were of the analytical grade.

The following chemicals were obtained from SIGMA Co., Ltd (Steinheim, Germany):

ABTS, Acetythiocholine (ATC), ADP, Ascorbic Acid, Atropine, Benzene, Bile salts, Bromelain, Butylated Hydroxy-Anisole, Chloroform-d, Dimethyl sulfoxide 5,5'-dithiobis-(2-nitrobenzoic acid) DTNB, Epinephrine, (5,6-Diphenyl-3-(2-pyridyl)-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) FerroZine, Ethylenediaminetetracetic acid, Papain, Polyvinylpolypyrrolidone (PVPP), Subnitrate Bismuth, Tacrine (9-Amino-1,2,3,4-Tetrahydroacridine hydrochloride hydratge), Thiobarbaturic acid, Thrombin, Tris-HCI, Trypsin, Tween 20

These chemicals were obtained from MERCK:

Acetone, Acetic anhydride, Ammonium hydroxide, Calcium chloride, Chloroform, Dichloromethane, Diethyl ether, Dragendorff, Ethanol, Ethyl acetate, Ferric chloride, Folin Ciocalteu reagent, Glacial acetic acid, Hexane, Hydrogen sulphate, Lead acetate, Mayers Reagent, Methanol, *n*-butanol, Olive oil, Potassium ferrocyanide, Potassium iodide, Potassium persulfate, Pyridine, Silica Gel 60 0.063-0.200 mm (70-230 mesh ASTM), Silica gel 60 0.040-0.063 mm (230-400 mesh ASTM), Silica gel 60 0.2-0.5 mm (30-70 mesh ASTM), Sodium carbonate, TLC aluminium sheets 20x20cm Silica gel 60 F_{254}, Trisodium citrate

The chemicals obtained from ASSOCIATED CHEMICAL ENTERPRISES include:

Sodium chloride, Sodium dihydrogen phosphate monohydrate, *Di*-potassium hydrogen orthophosphate, *Di*-sodium hydrogen orthophosphate

SAARCHEM supplied the following chemicals:

Glucose, Sodium Hydroxid, Sodium sulphite anhydrous, Citric acid monohydrate

Other supplies included:

Trisamminomethane (BIORAD LABORATORIES), Chromogenic substrate (S2238) (INSTRUMENTATION LABORATORY COMPANY), *Sprague dawley* rats (UNIZUL: SCIENCE DEPARTMENT, ANIMAL HOUSE), Brine shrimp eggs (A*rtemia salina*) (Fish Designs, Mtunzini, SA

3.1.2 Equipment

Drying oven (GALLENKAMP), Centrifuge 5404R (Eppendorf) (MERCK), Hammer mill (IKA) (POLYCHEM SUPPLIES), Platform shaker (Labcon) (POLYCHEM SUPPLIES), NMR 400 (ULTRASHIELD BRÜKER), Rotavapor (Laborota 4000-Heidolph) (POLYCHEM SUPPLIES), UV Spectrometer (Analytik jena Spekol 1300) (PERKIN ELMER), Microtitre plate reader (ELx808 GEN5 Software) (BIOTEK INSTRUMENT SUPPLIES), Platform shaker (LABCON POLYCHEM

SUPPLIES), Freeze dryer—VirTis Benchtop K (POLYCHEM SUPPLIES), Nuclear Magnetic Resonance 400 (ULTRASHIELD BRUKER), Purified sand, Columns of different sizes.

3.1.3 Plant material

Bulbine natalensis Baker plants were obtained from the Twin stream Nursery, Mtunzini (KZN, SA) and identified by Mrs Ntuli, of the Botany Department at the University of Zululand. A voucher specimen (GGL 01) was prepared. The leaves were washed and air-dried.

3.1.4 Animals

The study was conducted after obtaining ethical clearance from the Research Animal's Ethics Committee of the University of Zululand on animal use and care (see Appendix C).

3.2 Methodology (See Appendix B for details)

3.2.1. Preparation of plant extracts

The fresh leaves were sequentially extracted (1:5 w/v) with different solvents according to their increasing polarity that is, hexane, chloroform, ethyl acetate, methanol and distilled water (figure 3.1). The plant material and respective solvents were left to incubate on a platform shaker (150 rpm) for 24 hours at room temperature. The extracts were filtered through Whatman's No 1 filter paper. The residue was re-extracted successively with the different solvents mentioned. The organic solvent filtrates were concentrated in *vacuo* using the rotary evaporator (45ºC) while the aqueous extract was freeze-dried. Extracts were reconstituted in their respective solvents and stored at 4ºC for further use.

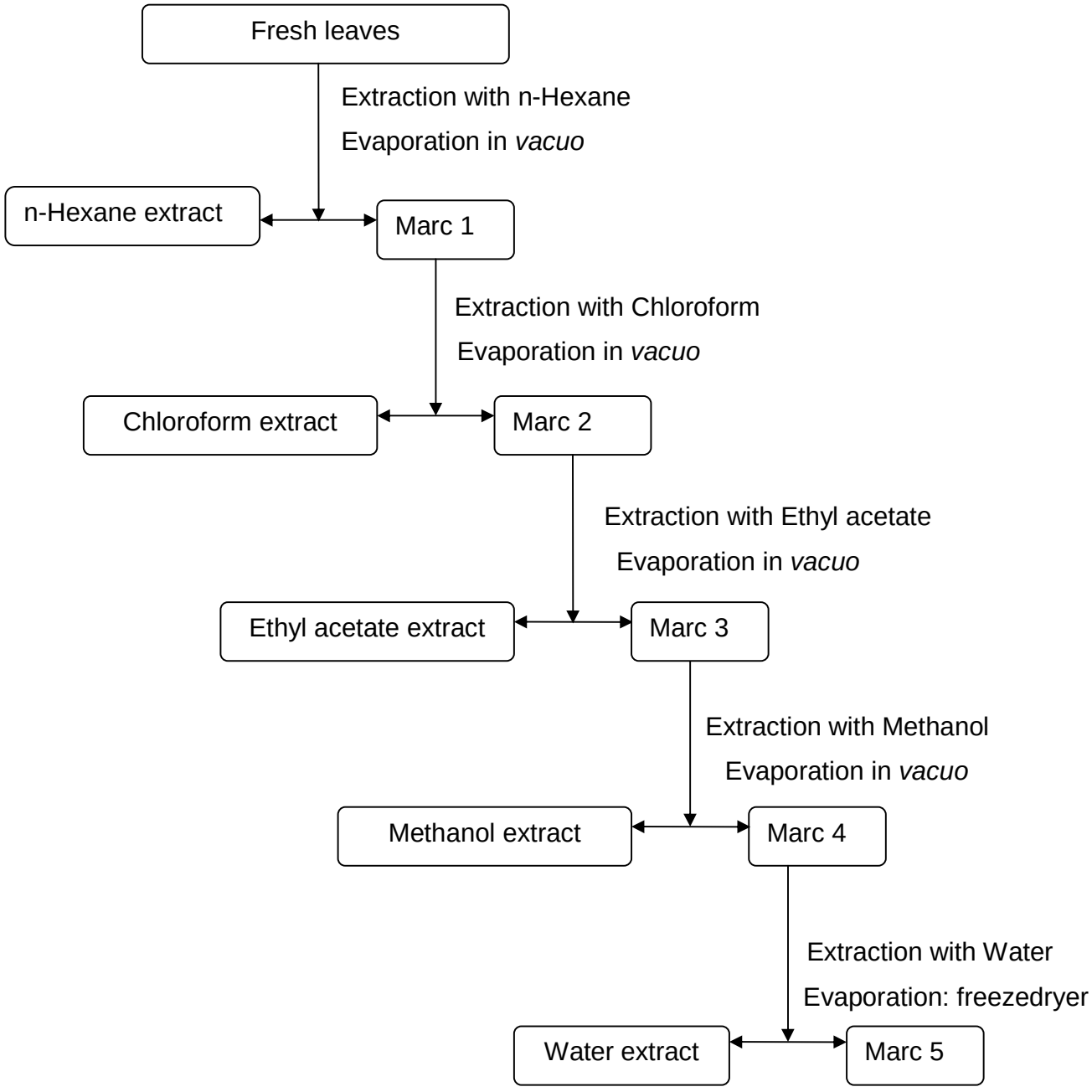


Figure 3.1: Schematic presentation of the sequential extraction of the fresh leaves of *B. natalensis.*

3.2.2. Phytochemical Screening

Phytochemical screening of the plant material was carried out using standard procedures.

The presence of tannins, phlobatannins, saponins, flavonoids and steroids were determined by adapting the methods of Sofoware *et al* (1993) and Harbone *et al* (1973). The Salkowski test and Keller-Killani test were used to determine terpenoids and cardiac glycosides (Sofoware *et al*, 1993 and Harbone *et al*, 1973). Formation of precipitates and colour changes upon the corresponding tests were taken as preliminary evidence of the presence of the various phytochemicals in the plant material.

3.2.3 Determination of total phenols

The method of Kähkönen *et al* (1999) was adapted for the determination of the total phenol content and expressed as gallic acid equivalent.

Different concentrations (0.01-0.1 mg/ml) of gallic acid were prepared in diethyl ether. The different plant extracts were dissolved in diethyl ether. The diethyl ether was evaporated off and the residue treated with Folin Ciocalteu (1 ml: 10 ml dH_20) reagent and sodium carbonate (7% w/v). This was kept in the dark for 30 minutes and the absorbance read at 765 nm against a blank containing the Folin-Ciocalteu reagent and sodium carbonate solution. The phenol concentration was extrapolated from the gallic acid standard curve (see Appendix B).

3.2.4 Determination of total flavonoid contents

Flavonoid content was measured by using the method of Ordon-Ez *et al*, (2006). Different concentrations of quercetin (0.01-0.1 mg/ml) were prepared in diethyl ether. The extracts (0.5 ml) were mixed with 1ml of diethyl ether. The residue was dissolved in 0.5 ml of 2 % $AlCL_3$ (prepared in 80 % ethanol) solution. It was allowed to stand for 60 minutes at room temperature. A yellow colour indicated the presence of flavonoids. The absorbance was read at 420 nm. The amount of total flavonoids was expressed as quercetin equivalent in milligram per gram dry plant extract.

3.2.5 Determination of Proanthcyanidin

Standard cetechin was prepared in diethyl ether (0.01-0.1 mg/ml) (Sun *et al*, 2002). 0.5ml of the extracts was mixed with diethyl ether. The residue was dissolved in 3 ml of 4% vanillin-MeOH solution (80%) and 1.5 ml of 1% HCL. The solution was allowed to stand for 15 minutes at room temperature. The absorbance was measured at 500 nm. The amount of total flavonoids was expressed as catechin equivalent in milligram per gram dry plant extract.

3.2.6 *In vitro* antioxidant Activity

3.2.6.1 DPPH free radical scavenging assay

The free radical scavenging activity of plant extracts against DPPH free radical was evaluated spectrophotometrically as described by Brad-Williams *et al* (1995). Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared. Aliquots (2 ml) of 2 mg % DPPH were added to 2 ml of each concentration and allowed to stand in the

dark for 30-60 minutes. Absorbance was read at 517 nm and the percentage scavenging activity calculated.

3.2.6.2 ABTS

Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared for the measurement of ABTS radical scavenging activity of plant extracts (Pellegrini *et al,* 1999). ABTS (7 mM) was prepared and the equivalent of 2.45 mM potassium persulfate was added. The mixture was incubated in the dark, at room temperature for 16 hours. ABTS radical was diluted with MeOH [1 ml ABTS: 60 ml MeOH]. To 1 ml of ABTS, 1 ml of different concentrations of the extract was added. It was mixed and allowed to stand for 6 minutes. The absorbance was read at 734 nm and the percentage scavenging activity was calculated.

3.2.6.3 Reducing Power

The reducing power ability of the plant extracts was evaluated by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu *et al*. (1986). Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared. 1ml of plant sample was mixed with 2.5 ml of (0.2 M, pH 6.6) phosphate buffer and 2.5 ml (1%) ($K_3(Fe(CN)_6$). The mixture was incubated at 50℃ for 20 minutes . Thereafter 2.5 ml (1%) TCA was added. It was mixed and allowed to stand for 10 minutes. The whole mixture was centrifuged for 10 minutes, 2.5 ml of the supernatant was removed and mixed with 2.5 ml dH_2O and 0.5 ml (0.1%) $FeCl_3$. This was allowed to stand for 30 minutes and

the absorbance was measured at 700 nm. The higher the absorbance observed, the higher the reducing power of the extract.

3.2.6.4 Chelating activity on Fe^{2+}

The method of Decker and Welch (1990) was followed to measure the ability of the extract to chelate Fe^{2+} ions. Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared. To 1 ml of the extracts, 3.75 ml deionized H_2O, 0.1 ml of $FeCl_2$ (2 mM) and 0.2 ml Ferrozine (5 mM) was added. It was mixed vigorously and allowed to stand for 10 minutes. The absorbance of Fe^{2+} ions was measured at 562 nm. The chelating activity of the extracts for Fe^{2+} was then calculated. Citric acid and EDTA were used as standards.

Calculation of the percentage inhibitory effect of extract

Unless otherwise stated, ascorbic acid, BHA and BHT were used as standards. All assays were repeated three times and the mean ± S.E reported. The inhibitory effect of *B. natalensis* extracts on each parameter was calculated as:

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where, A_0 is the absorbance value of the fully oxidized control and A_1 is the absorbance of the extract. The inhibitory concentration providing 50% inhibition (IC_{50}) was calculated from the graph of the percentage inhibition against *B. natalensis* extract concentrations.

3.2.7 Brine shrimp Lethality Test

The method of Meyer *et al*. (1982) was used with slight modification. The brine shrimp assay involves incubating test substances with freshly hatched brine shrimp larvae and observing the larvae for mortality after incubation (Meyer *et al*, 1982; Solis *et al*, 1993; McLaughlin *et al*, 1998; McGaw and Eloff, 2005). Brine shrimp larvae were placed in multi-welled culture plates. Different concentrations (0-15 mg/ml) of the plant extract were prepared and the experiment was carried out in triplicate for each concentration at room temperature. The respective extracts were added to each well of the brine suspension and the plates were maintained under illumination. For the control, MeOH was used instead of the plant extract. The numbers of survivors were counted after 24 hours and 48 hours and the percentage mortality rate of the shrimp larvae was calculated. The lethality concentration (LC_{50} values) for each assay was calculated by using a Finney Probit analysis program on an IBM computer (McLaughlin *et al*. 1991).

3.2.8 *In vitro* anti-platelet aggregation study

The extracts were separately dissolved in 1% DMSO for use in the anti-platelet aggregation study.

3.2.8.1 Preparation of platelets

Washed platelets were prepared as described by Tomita *et al* (1983). *Sprague Dawley* rats were slightly anaesthetized with ether and blood was drawn from the abdominal aorta and placed into a centrifuge tube containing (5:1 v/v) acid dextrose anticoagulant (ADA). It was centrifuged at 1200 rpm for 15 minutes and consecutively at 2200 rpm

for 3 minutes. The supernatant was taken and again centrifuged at 3200 rpm for 15 minutes. The sediment was re-suspended in washing buffer (WB), centrifuged at 3000 rpm for 15 minutes and the sediment obtained suspended in re-suspending buffer (RB). It was kept in the fridge and used within 4 hours.

Enzyme treated platelets were obtained by separately incubating 0.1mg of the respective enzyme (papain, bromelain and trypsin) with 25 ml of a 2% suspension of cells for 60 minutes at 25 ℃. It was washed 6 times with washing buffer and a 2% suspension in re-suspending buffer was prepared.

3.2.8.2 Test with Chromogenix

The anti-thrombic activity of the plant extracts was determined using the modified method of Rob *et al.* (1997). The chromogenic substrate, S2238 (H-D phenylalanyl-L-pipecolyl-*p* nitroanilide dihydrochloride, Chromogenix), was used. Plant extracts were screened at concentrations of 1 and 3 mg (dry weight/ml buffer). The extracts were solubilised in DMSO and the volume was made up with resuspending buffer to yield a final DMSO concentration of 1% (v/v). The extract (50 µl) was added to (10 µl) thrombin at 30 U/ml in dH_20. This mixture was left to incubate for 10 minutes at room temperature and 190 µl 0.76 M S2238 was added. The reaction was monitored at 412 nm for 4 minutes at 10 second intervals using a BioTek microtitre plate reader (GEN 5 software).

3.2.8.3 Anti-platelet aggregation activity

The method of Hwang *et al*. (1974) as modified by Mekhi *et al*. (2004) was adopted for the measurement of platelet aggregation. A 1: 20 dilution of platelets was prepared in RB. A sample of washed platelets (0.4 ml) was mixed with $CaCl_2$ to a final concentration of 1.3 mM.

Platelet aggregation was separately induced by adding thrombin (5 U/ml), ADP (5 mM) and epinephrine (10 mM) to platelets. Similar experiments were also carried out on enzyme (trypsin, bromelain, papain) treated platelets (0.1 mg/25 ml). The platelets (100 µl) were incubated for 5 minutes with different concentrations of the crude extracts (1, 3 and 10 mg/ml) and an aggregation inducer (20 µl) was introduced to the mixtures. Anti-platelet aggregation activity was observed at 415 nm. DMSO (2%) was used as a negative control.

3.2.8.4 Thrombin-induced clotting time assay

The thrombin-induced clotting time assay was measured by adopting the Kee *et al*, (2008) method. Plant extracts were tested at 1, 3 and 10 mg/ml in saline containing a DMSO concentration of 2% (v/v). The plant extract (50 µl) was added to rat platelets (100 µl) and incubated at room temperature for 5 minutes. Thrombin (5 U/ml) was added and the rate of clot formation was determined with the Biotek plate reader using GEN 5 software by following the increase of the absorbance at 412 nm for 20 minutes at 30 second intervals. A negative control was performed using 2% (v/v) DMSO in saline, which represented 100% activity.

3.2.8.5 $CaCl_2$-induced clotting time assay

This assay was carried out to determine a 50% clotting time and the effect on fibrin formation. The extracts were screened at (1, 3 and 10 mg/ml) using 2% DMSO (v/v) to solubilize the plant extracts. To 100 µl rat platelet, 50 µl plant extract was added. The reaction was mixed and left to incubate for 5 minutes at room temperature. Clotting was induced by adding 20 µl 0.16 M $CaCl_2$. The reaction was monitored and followed at 412 nm with a microtitre plate reader for 2 hours at 3 minute intervals (Kee *et al*, 2008).

3.2.9 Tannin removal

Tannin removal was carried out using polyvinylpolypyrrolidone (PVPP). The method was modified from that described by Toth and Pavia (2001). Plant extracts were prepared at concentrations of 1, 3, 10 mg/ml in dH_2O. PVPP was added to the extracts and shaken for 15 minutes at 4 ℃. It was then cent rifuged (3500 xg) for 8 minutes at 4℃. The pellet was discarded. The supernatant was removed and the process repeated twice to remove tannins.

Calculation of the percentage inhibitory effect of plant extracts on platelet aggregation

All assays were repeated three times and the mean slope (A) ± SD reported. Unless otherwise indicated, the inhibitory effect of the extract on each parameter was calculated as:

$$\% \text{ Inhibition} = \{(A_0 - A_1)/A_0 \times 100\}$$

where, A_0 is the mean slope of control and A_1 is the mean slope of the extract. The inhibitory concentration providing 50 % inhibition (IC_{50}) was determined using statistical package Origin 6.1.

3.2.10 Isolation, purification and characterization of active compounds

The crude chloroform and ethyl acetate fractions showed consistent anti-platelet aggregation activity and were further purified through column chromatography (CC), thin layer chromatography (TLC) and characterized by Nuclear Magnetic Resonance Spectroscopy (NMR). The isolated compounds were bioassayed for activity.

3.2.10.1 Isolation of active compounds

In order to identify the active compounds that showed consistent activity in the anti-platelet aggregation study, the crude ethyl acetate extract (1.12 g) was subjected to silica gel column chromatography (20 mm x 500 mm; Silica gel 60; 0.063–0.2 mm; 70-230 mesh ASTM), eluted with hexane:ethyl acetate solvent system (gradient) to yield a total of 12 combined fractions (Fig 3.2). Thin layer chromatography (TLC) (silica gel 60, TLC aluminium sheets 20 cm x 20 cm, F_{254}, hexane:ethyl acetate solvent system 9:1-3:7) was used to analyze the fractions. The TLC plates were first viewed under ultraviolet (UV) light and developed using a 10% H_2SO_4 spray agent and then heated. The fractions with similar profile were combined, concentrated *in vacuo* and their weights were determined. The third fraction was separately recrystalised in ethyl acetate to obtain compound D3. The chloroform extract (2 g) was subjected to the same procedure to yield 11 combined fractions (Fig 3.3).

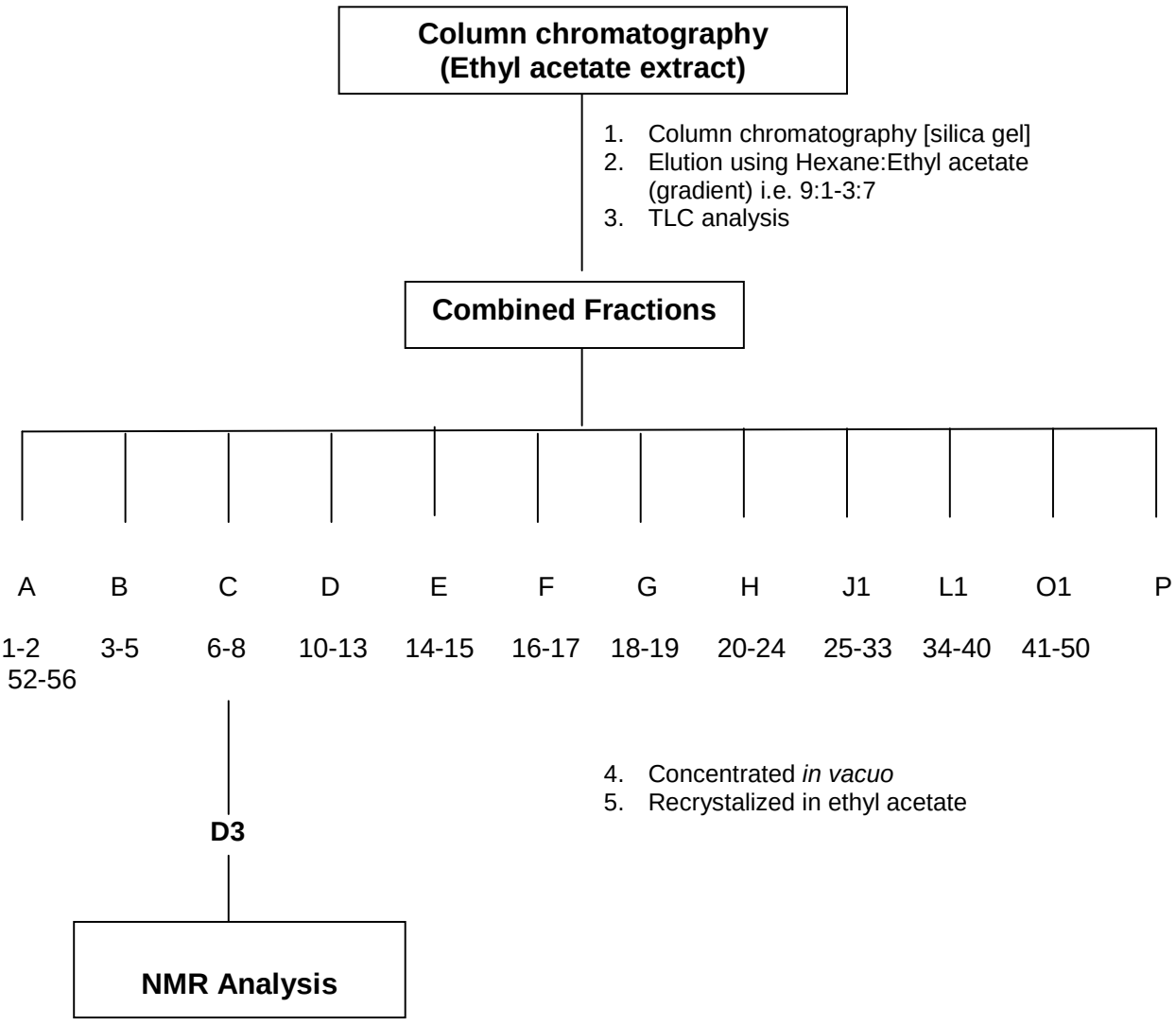


Figure 3.2: Schematic presentation of the isolation and purification of compound D3 through column chromatography. Compound D3 showed 2 prominent brown spots and was prepared to be sent for NMR analysis.

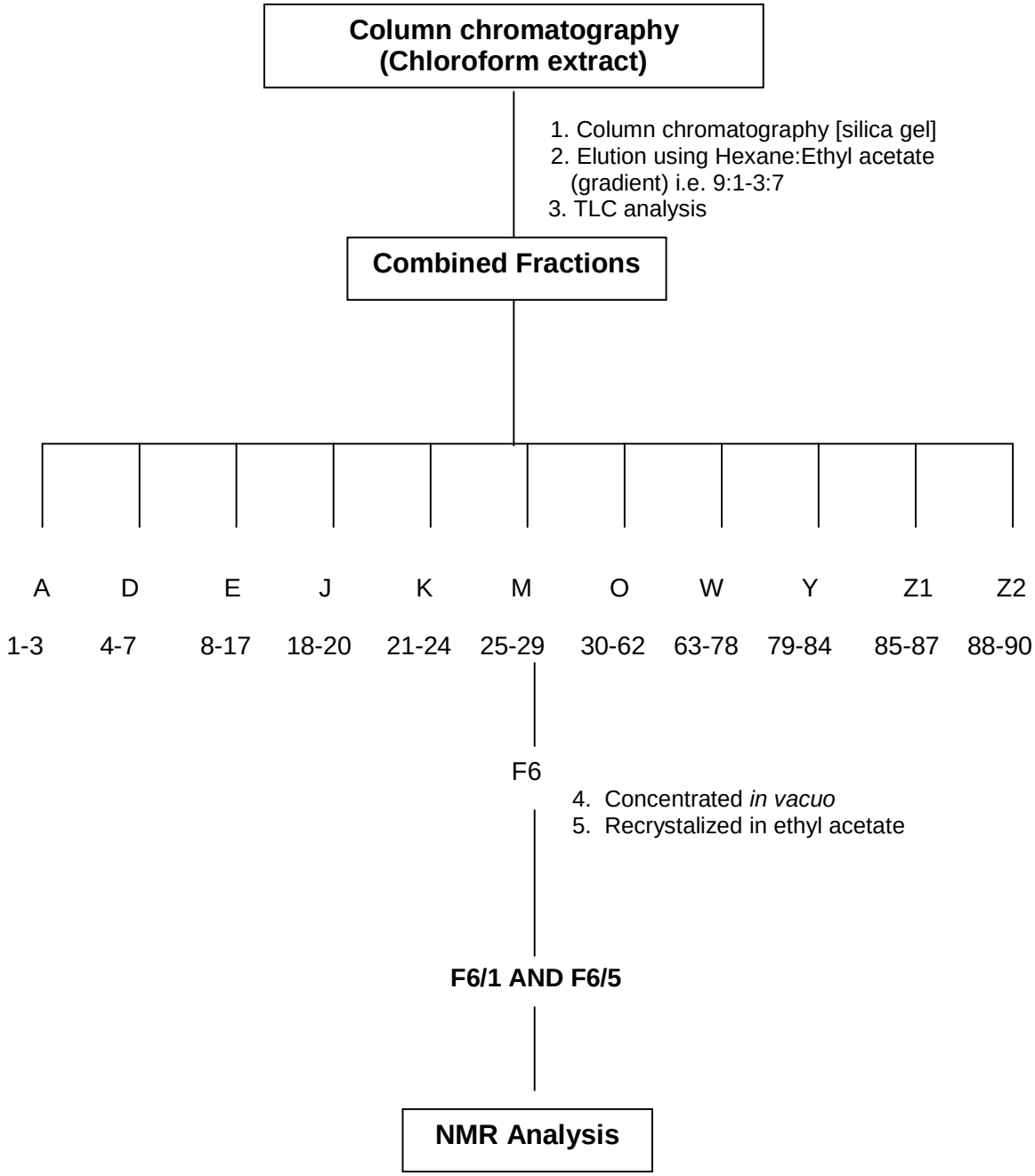


Figure 3.3: Schematic presentation of the isolation and purification of compound F6/1 and F6/5 through column chromatography. Each compound showed a distinct green spot and was prepared to be sent for NMR analysis

3.2.10.2 Characterization of active compound

There was not enough of compound D3 to carry out NMR analysis. Compound F6/1 and F6/5 in were prepared in $CDCl_3$ and analyzed using 1D and 2D NMR techniques (^{1}H- ^{1}H, ^{13} C^{13} C, DEPT spectrum). The resulting spectra (see Appendix D) were analyzed and the compounds identified by comparing them with standards and library materials.

3.2.11 Acetylation

An acetylation reaction was carried out to confirm the compounds identity. The reaction involved the replacement of the hydrogen atom of a hydroxyl group with an acetyl radical (CH_3CO) to yield a specific ester, the acetate. Acetic anhydride was used as an acetylating agent reacting with free hydroxyl groups. The three similar fractions, previously isolated, were combined and 5 ml acetic anhydride and 5 ml pyridine added to it. It was left overnight with a magnetic stirrer for 24 hours. Thereafter hydrolysis was initiated to remove acetic anhydride. The acetylated product was removed and TLC was carried out to ensure its purity before NMR spectroscopic analysis.

3.2.12 Anticoagulant activity of the Isolates (F6/1 and F6/5)

The anti-coagulant activity of F6/1 and F6/5 were investigated on rat whole blood. The isolate was dissolved in olive oil. The anti-coagulant activity of the isolate (1, 3 and 7 mg/ml) was separately tested against thrombin (5 U/ml), ADP (5 mM), epinephrine (10 mM) and arachidonic acid (10 mM). A blank was set up; it contained 100 µl whole blood and 20 µl oil. The control contained 100 µl whole blood and 20 µl clotting agonist.

Blood was drawn from the abdominal aorta of the rat and 100 µl was immediately mixed with clotting agonist (20 µl) and the isolate (50 µl) in the corresponding wells. The reaction was monitored by visualization 4-5 minutes to record the time it took the blood to clot. The experiment was done in duplicate.

3.2.13 Acetylcholinesterase inhibition activity

The method of Srikumar *et al* (2004) was used to measure the acetylcholinesterase activity. Different concentrations of the isolate (0.01-0.05 mg/100 ml olive oil) were prepared. *Sprague Dawley* rats were decapitated and the brains were quickly removed and placed in ice-cold saline. The tissues were weighed and homogenized (20 mg/ml) in 0.1 M phosphate buffer (pH 8). To a cuvette containing 1.3 ml phosphate buffer and 50 µl of DTNB, 0.2 ml aliquot of the brain homogenate was added. The contents were mixed and the absorbance measured at 412 nm using a UV Spectrometer (Analytik jena Spekol 1300). Thereafter 10 µl of the substrate that is, acetylthiocholine was added and 10 µl of the isolated compound. The change in absorbance was recorded for a period of 10 minutes at intervals of 2 minutes. Atropine and Tacrine were used as the negative and positive controls respectively. The blank contained the brain homogenate and olive oil. The change in the absorbance per minute was determined and enzyme activity was calculated using the following formula: $R= 5.74 \times 10^{-4} \times A/CO$ where:

R= Rate in moles of substrate hydrolyzed/ minute/ gm tissue

A= Change in absorbance / min

CO= Original concentration of the tissue (mg/ml)

Chapter 4

Results

The hexane, chloroform, ethyl acetate, methanol, and aqueous extracts of the leaves of *B. natalensis* were screened for antioxidant and anti-platelet aggregation activity. An attempt was made to isolate and characterize the active constituent in the chloroform extract. The results are presented in this chapter.

4.1 Yield of extract

Table 4.1 shows the percentage yield obtained from the sequential extraction of the fresh leaves of *B. natalensis*. The chloroform extract gave the highest yield of 0.20%.

Table 4.1 Percentage yield from sequential extraction of the plant.

Plant extract	% Yield
$CHCl_3$	0.20 %
n-Hexane	0.11%
MeOH	0.09 %
EtoAc	0.03 %
H_2O	0.002 %

4.2 Phytochemical Screening Results

The phytochemical analysis of the leaves of *B natalensis* revealed the presence of anthraquinones, cardiac glycosides, saponins, tannins, flavonoids and alkaloids (table 4.2).

Table 4.2: Qualitative analysis of *B. natalensis*.

Phytochemical	Presence
Anthraquinone	+
Cardiac Glycoside	+
Saponin	+
Tannin	+
Flavonoid	+
Alkaloid	+
Terpenoid	-
Phlobatannin	-
Steroid	-

+ =Presence of constituents; - = absence of constituent

4.2.1 Total Phenol, Flavonoid and Proanthocyanidin contents

The quantitative values of the total phenol, flavonoid and proanthocyanidin contents of the extracts are presented in table 4.3. Hexane extracted more of the compounds than any of the other solvents used. The values decreased with increasing polarity of the solvents.

Table 4.3: Quantitative values of the total phenol, flavonoid and proanthocyanidin contents of the extracts of *B. natalensis.*

	Concentration (mg/g)		
Plant extract	**Total Phenol**	**Flavonoid**	**Proanthocyanidin**
Hexane	5.028 ± -0.044	3.293 ± 0.046	2.565 ± 0.020
EtoAc	4.625 ± 0.153	2.840 ± 0.095	1.975 ± 0.025
$CHCl_3$	4.545 ± 0.670	2.421 ± 0.074	1.652 ± 0.098
MeOH	3.396 ± 0.146	1.783 ± 0.056	1.384 ± 0.081
H_2O	1.151 ± 0.048	1.894 ± 0.021	1.463 ± 0.045

Values are expressed as mean ± SD (n=2)

4.3 Anti-oxidant Activity of extracts

B.natalensis was evaluated for its antioxidant capacity against DPPH and ABTS radical-scavenging, ferrous ion reducing, and ferrous ion chelating activity.

Table 4.4 shows the 50% inhibitory concentration (IC_{50}) of the extracts' antioxidant capability. The extracts showed a varying degree of efficiency in scavenging free radicals. The plant extracts were less efficient than the standard antioxidants tested.

Table 4.4: IC_{50} (mg/ml) values of antioxidant activity of extracts.

Extract	DPPH	Metal Chelating	ABTS
Hexane	>5	>5	4.72
Ethyl Acetate	<5	>5	5.39
$CHCl_3$	>5	>5	<5
MeOH	>5	>5	<5
H_2O	>5	>4	>5
BHA	1.29	-	1.52
Ascorbic Acid	3.10	-	>2
EDTA	-	0.73	-
Citric acid	-	3.81	-

4.3.1 Reducing Power

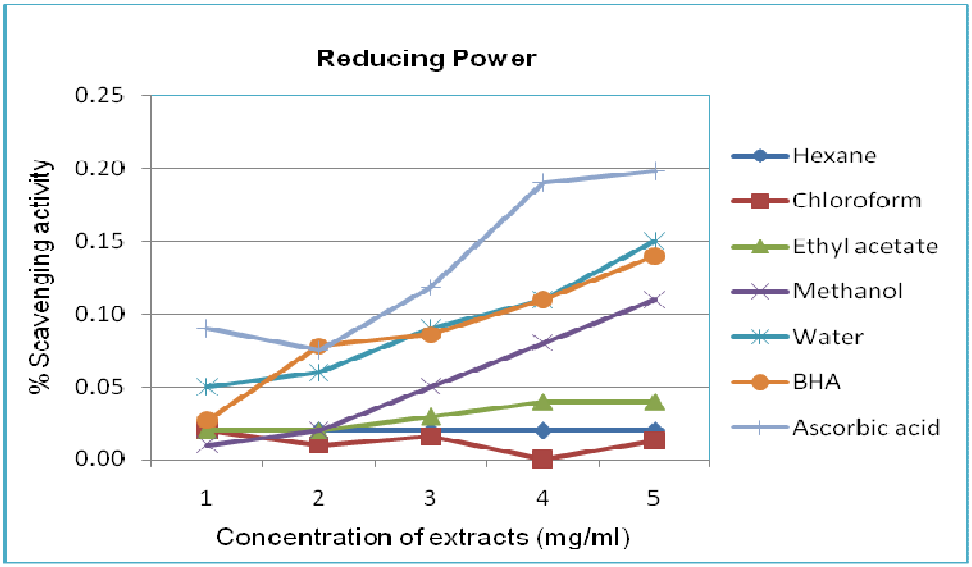


Figure 4.1: Reducing power of the extracts and standard anti-oxidants.

The graph (Figure 4.1) represents the reduction potential of the extracts. The activity was concentration dependent. The water extract exhibited the highest reducing power and the activity was even greater than the standard antioxidant BHA.

4.4 Cytotoxicity test

Table 4.5 shows the toxicity of the plant extracts to brine shrimp larvae after 24 hours of exposure. It is apparent that all the extracts were highly toxic to the larvae.

Table 4.5: LC_{50} values (mg/ml) extracts.

Extract	LC_{50} (mg/ml)	95% CL
EtoAc	2.21	1.87-2.52
$CHCl_3$	2.55	2.12-2.98
H_2O	4.30	3.36-9.08
Hexane	5.23	3.55-22.76
MeOH	5.53	4.692-10.08

CL: Confidence Limit

4.5 Measurement of platelet aggregation

The extracts were first tested on an artificial thrombin substrate, Chromogenix (S2238). Percentage inhibition of $CaCl_2$ induced clotting time was conducted. 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 extracts was also tested on tannin free extracts.

4.5.1 Anti-thrombin Activity

Fig 4.2 shows the anti-thrombin activity of the extracts on Chromogenix S2238. Only the water extract inhibited (9%) thrombin activity at a dose of 10 mg/ml.

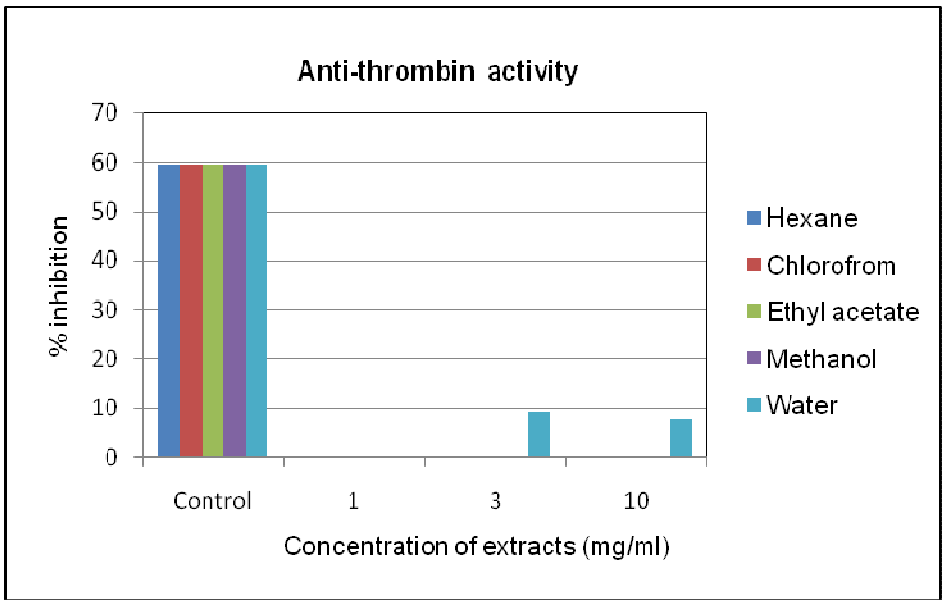


Figure 4.2 Anti-thrombin activity of the extracts on Chromogenix S2238.

4.5.2 $CaCl_2$ induced clotting time assay

$CaCl_2$ induced clotting time assay was carried out to determine the rate at which the extracts inhibited clot formation and their effect on fibrin formation. The result is shown in table 4.6. The extracts showed poor abilities to inhibit the $CaCl_2$ induced clotting time.

Table 4.6: Percentage inhibition of $CaCl_2$ induced clotting time.

Extract	Conc. (mg/ml)	IC_{50} (mg/ml)	Percentage Inhibition
Control		N/A	0± 0.680
Hexane	1	2.41	2 ± 0.987
	3		3 ± 0.232
	10		0 ± 2.442
ChCl3	1	6.53	0± 1.635
	3		0 ± 1.017
	10		14± 0.236
EtoAc	1	7.89	0 ± 0.837
	3		0 ± 0.931
	10		0± 0.901
MeOH	1	2.13	0 ± 0.203
	3		22 ± 1.610
	10		35 ± 0.081
H_2O	1	6.16	0 ± 0.365
	3		0 ± 0.442
	10		0 ± 0.014

4.5.3 Anti-platelet aggregation activity induced by Thrombin, ADP and Epinephrine

4.5.3.1 Activity of the extracts before tannin removal

The effect of the plant extracts on thrombin, ADP and epinephrine induced rat platelet aggregation is presented in table 4.7.

In the thrombin-induced platelet aggregation only hexane showed 100% inhibition at 1 mg/ml. In the ADP-induced platelet aggregation chloroform displayed 100% activity at 1 and 3 mg/ml with IC_{50} value of 5.32 mg/ml. Chloroform showed 100% inhibition at doses

of 1 and 10 mg/ml with IC_{50} value <5 mg/ml for the epinephrine-induced platelet aggregation.

Table 4.7: IC_{50} values (mg/ml) of the plant extracts (before tannin removal) on platelet aggregation.

Extract	Conc. (mg/ml)	IC_{50} (mg/ml)			Percentage Inhibition		
		Thro	ADP	Epin	Thrombin	ADP	Epinephrine
	Control	N/A	N/A	N/A	26± 0.178	2.3±1.009	4.6±2.839
Hexane	1	2.69	>10	5.46	100 ± 0.196	0 ± 0.494	100± 1.898
	3				45 ± 0.073	7 ± 0.053	67 ± 2.349
	10				0 ± 0.336	27 ± 0.301	33 ± 1.623
$CHCl_3$	1	N/A	5.32	<5	0 ± 0.223	100 ± 1.623	100± 0.031
	3				0 ± 1.475	100 ± 0.807	59 ± 1.212
	10				0 ± 2.546	20 ± 2.150	100± 0.317
EtoAc	1	>10	1.90	2.52	0 ± 0.071	100 ± 0.432	100 ± 2.596
	3				6 ± 0.319	0 ± 0.807	100 ± 0.383
	10				42 ± 0.100	0 ± 0.997	100 ± 0.149
MeOH	1	N/A	1.81	<2	0 ± 0.097	25 ± 0.966	100 ± 3.554
	3				0 ± 0.136	100 ± 1.956	0 ± 0.132
	10				0 ± 0.001	65 ± 0.281	100 ± 2.401
H_2O	1	N/A	2.07	>10	0 ± 0.028	69 ± 0.089	100± 0.956
	3				0 ± 0.458	35 ± 0.372	0 ± 0.571
	10				0 ± 0.573	45 ± 0.004	91± 0.064

The results obtained after tannin removal is presented in table 4.8. The chloroform and ethyl acetate extracts retained their activities even after the tannin were removed.

Table 4.8: IC_{50} (mg/ml) values of the plant extracts on platelet aggregation after tannin removal.

Extract	Conc. (mg/ml)	IC_{50} (mg/ml)			Percentage Inhibition		
		Thro	ADP	Epin	Thrombin	ADP	Epinephrine
	Control	N/A	N/A	N/A	0 ± 21.625	0 ± 0.985	0 ± 0.235
Hexane	1	>10	N/A	1.77	100± 0.511	0 ± 0.573	0 ± 2.843
	3				75 ± 1.983	0 ± 0.548	100 ± 0.243
	10				76 ± 1.202	0 ± 0.706	100 ± 0.403
$CHCl_3$	1	>10	>10	>10	100± 0.937	100 ± 0.941	100± 0.087
	3				89 ± 0.003	100 ± 1.599	100± 2.916
	10				83 ± 1.220	100 ± 0.053	100± 0.013
EtoAc	1	>10	<2	>10	100± 0.142	100 ± 3.164	100± 2.007
	3				76± 0.656	100 ± 0.046	95± 0.747
	10				87± 0.404	100 ± 0.591	100± 0.344
MeOH	1	>10	2.73	>10	98 ± 1.296	0 ± 0.200	70 ± 0.018
	3				61 ± 2.972	0 ± 1.165	72 ± 0.808
	10				70 ± 0.453	70 ± 0.116	63 ± 0.795
H_2O	1	<2	N/A	10.6	64 ± 0.675	0 ± 0.700	0 ± 1.061
	3				88 ± 1.940	0 ± 0.263	7 ± 0.901
	10				84 ± 3.093	0 ± 0.448	47± 0.236

4.5.4.1 Activity of the extracts before tannin removal on trypsin treated platelets

Fig 4.3 and table 4.9 illustrate the activity of the extracts before tannin removal, on trypsin treated platelets separately induced by thrombin, ADP and epinephrine. The extracts that displayed 100% inhibition on trypsin treated platelets induced by ADP were methanol (3 and 10 mg/ml) and water (1 and 10 mg/ml). The water extract showed 50% inhibition at 1.94 mg//ml. It was the best potential inhibitor followed by methanol (<3 mg/ml). Epinephrine induced platelet aggregation was inhibited (100%) by water at the

test concentrations and methanol (1 and 3 mg/ml). The polar solvents displayed relatively good inhibitory activity.

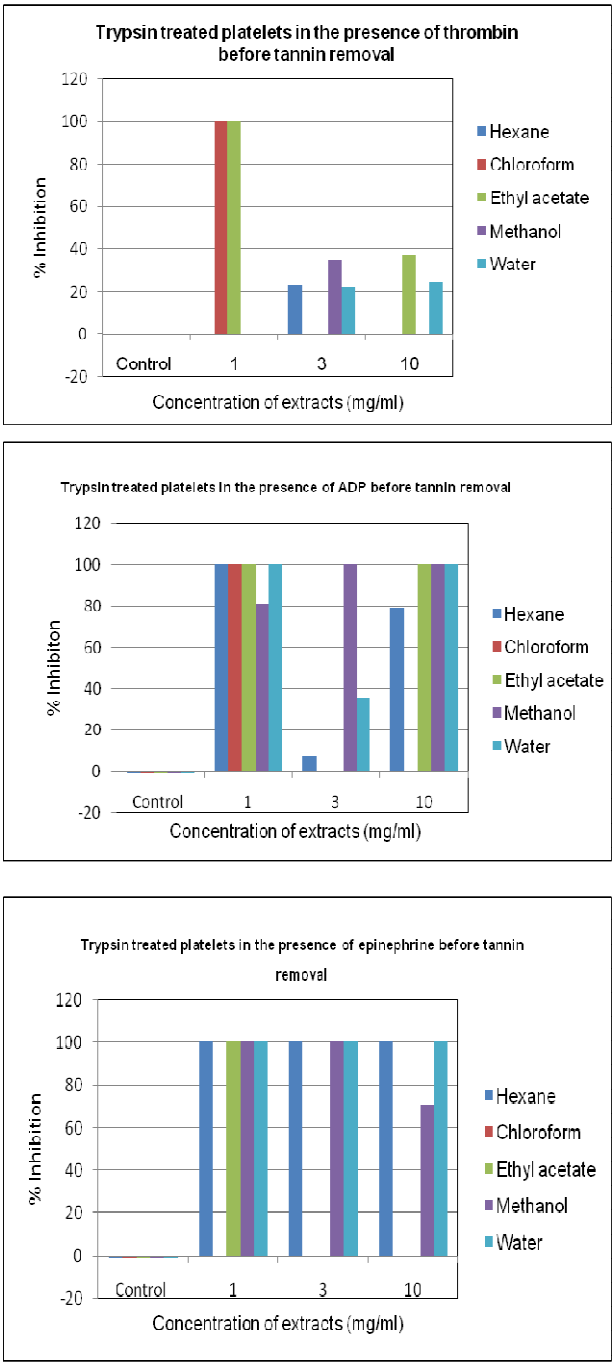


Figure 4.3: Inhibitory activity of the extract (with tannins) on trypsin treated platelet aggregation induced by: a) Thrombin b) ADP c) Epinephrine.

Table 4.9: IC_{50} (mg/ml) values of the plant extracts (before tannin removal) on trypsin treated platelets. Aggregation induced by thrombin, ADP and epinephrine.

IC_{50} (mg/ml)			
Extract	**Throm**	**ADP**	**Epinep**
Hexane	6.77	>10	6.47
$CHCl_3$	9.14	2.60	5.46
EtoAc	2.30	2.73	2.38
MeOH	1.85	<3	2.52
H_2O	1.07	1.94	2.47

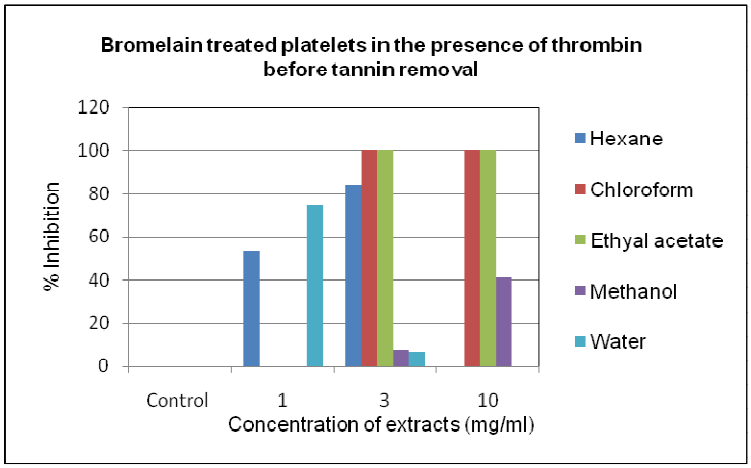
4.5.4.2 Activity of the extracts after tannin removal on trypsin treated platelets

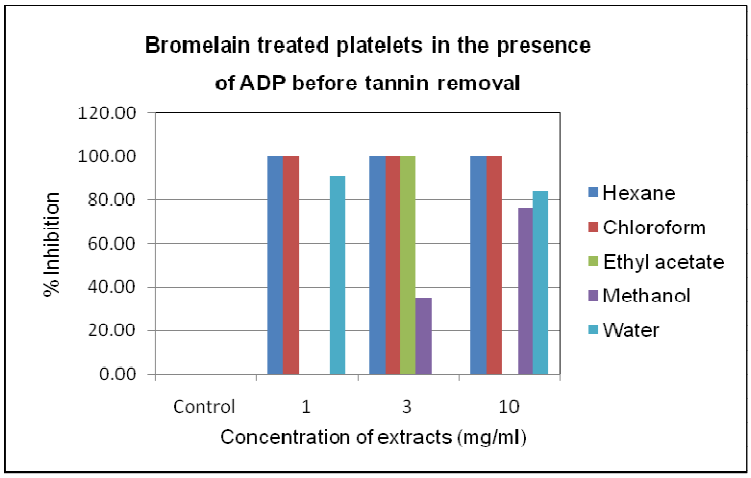
AFTER tannin was removed, the platelets were treated with trypsin and clotting was separately induced by ADP and epinephrine. None of the extracts showed inhibition at the tested concentrations.

4.5.5 Activity of the extracts (with tannins) on bromelain treated platelets

The platelets were treated with bromelain and clotting was separately induced by thrombin, ADP and epinephrine. The activities of the extracts (with tannins) are shown in table 4.10 and fig 4.4.

Both the chloroform and ethyl acetate extracts showed 100% inhibition against thrombin induced clotting at doses of 3 and 10 mg/ml with very low IC_{50} values 1.9 mg/ml and 0.98 mg/ml respectively. ADP-induced platelet aggregation was inhibited by chloroform and hexane by 100% at the tested concentrations with IC_{50} values of 3.92 mg/ml and <1 mg/ml respectively.





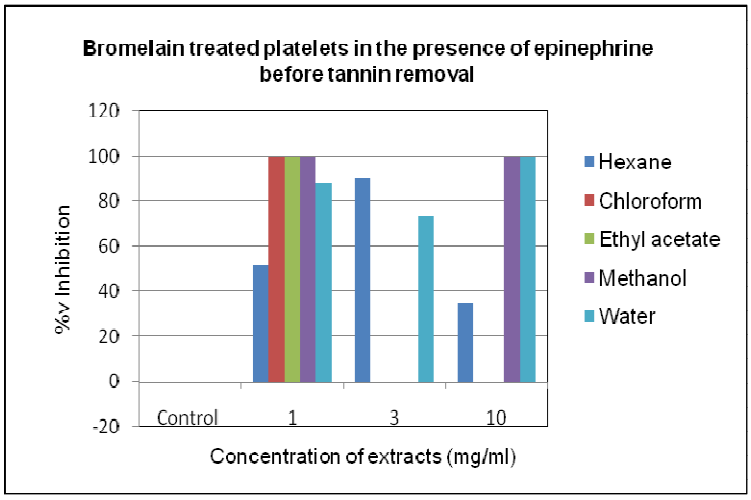


Figure 4.4: Inhibitory activity of the extract (with tannins) on bromelain treated platelets. Aggregation induced by: a) Thrombin b) ADP c) Epinephrine.

Table 4.10: IC_{50} (mg/ml) values of the plant extracts (with tannins) on bromelain treated platelets. Aggregation induced by thrombin, ADP and epinephrine.

IC_{50} (mg/ml)			
Extract	**Throm**	**ADP**	**Epinep**
Hexane	<1	<1	8.09
$CHCl_3$	1.9	3.92	1.77
EtoAc	0.98	7.78	2.12
MeOH	9.51	5.59	>10
H_2O	1.68	>10	>10

4.5.6 Thrombin-induced aggregation on papain treated platelets

The result of the activity of the extracts (with tannins) on papain treated platelets separately induced by thrombin is seen in table 4.11. Hexane and ethyl acetate displayed 100% activity and had low IC_{50} values of 2.53 mg/ml and 2.69 mg/ml respectively.

Table 4.11: Thrombin-induced aggregation assay on papain treated platelets.

Extract	Conc. (mg/ml)	IC_{50} (mg/ml)	Percentage Inhibition
Hexane	1	2.53	100 ± 6.493
	3		100 ± 2.207
	10		100 ± 4.033
$CHCl_3$	1	2.21	100 ± 1.886
	3		21 ± 0.819
	10		0 ± 4.687
EtoAc	1	2.69	100 ± 3.327
	3		100 ± 0.232
	10		100 ± 0.015
MeOH	1	<2	100 ± 0.476
	3		0 ± 0.473
	10		0 ± 0.430
H_2O	1	9.89	0 ± 0.977
	3		0 ± 0.176
	10		0 ± 3.698

4.6 Isolation of the active compound

The ethyl acetate and chloroform extracts displayed consistent anti platelet aggregation activity. They were therefore subjected to isolation and purification. Only 1 fraction (GL/2/C/C) of the ethyl acetate extract exhibited bioactivity. It showed 1 spot on the TLC however, the ^{1}H NMR analysis showed an impure compound and the sample was too small to carry out any further purification.

The chloroform extract gave 2 fractions (F6/1 and F6/5). These fractions gave single spots on TLC but, ^{1}H NMR and ^{13} C-NMR spectroscopic data of the samples (see Appendix D) indicated chemical shifts that were similar to those obtained for a similar

compound. An acetylation reaction was carried out on the compounds to confirm its identity. A distinct dark green colour was noted throughout purification and the compound had a sticky texture. TLC revealed 1 spot, yet 2 spots were observed the following day. It was apparent that the compound was unstable and possibly underwent oxidation. Therefore, the compound could not be specifically identified. However, based on the spectral data obtained, and comparing with existing literature data, (F6/1 and F6/5) was envisaged to be a knipholone (an anthraquinone) (Figure 4.5).

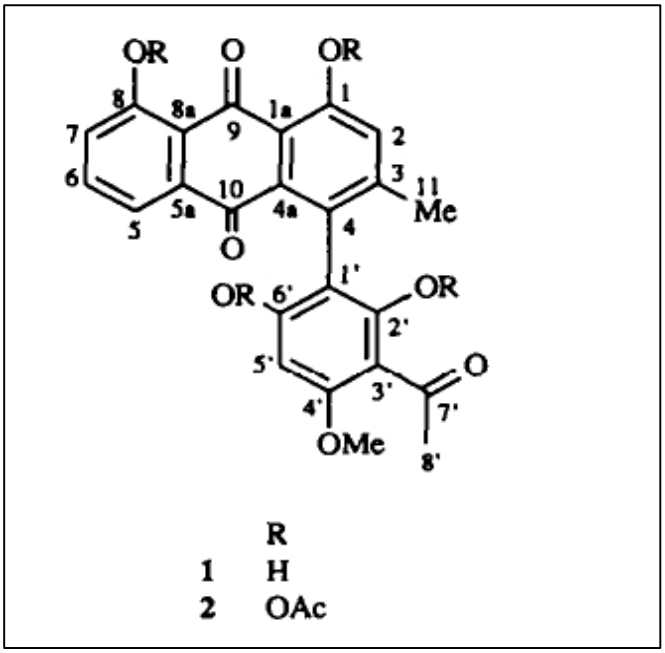


Figure 4.5 Knipholone (Frances van Staden and Drewes, 1994).

4.7 Anti-oxidant activity of the Isolated compounds (F6/1 and F6/5)

The isolated compounds (F6/1 and F6/5) were investigated for their antioxidant activities. Figure 4.6 shows the result of the reducing power. Results of their metal chelating capabilities and DPPH scavenging activity are given in Figure 4.7 and 4.8 respectively.

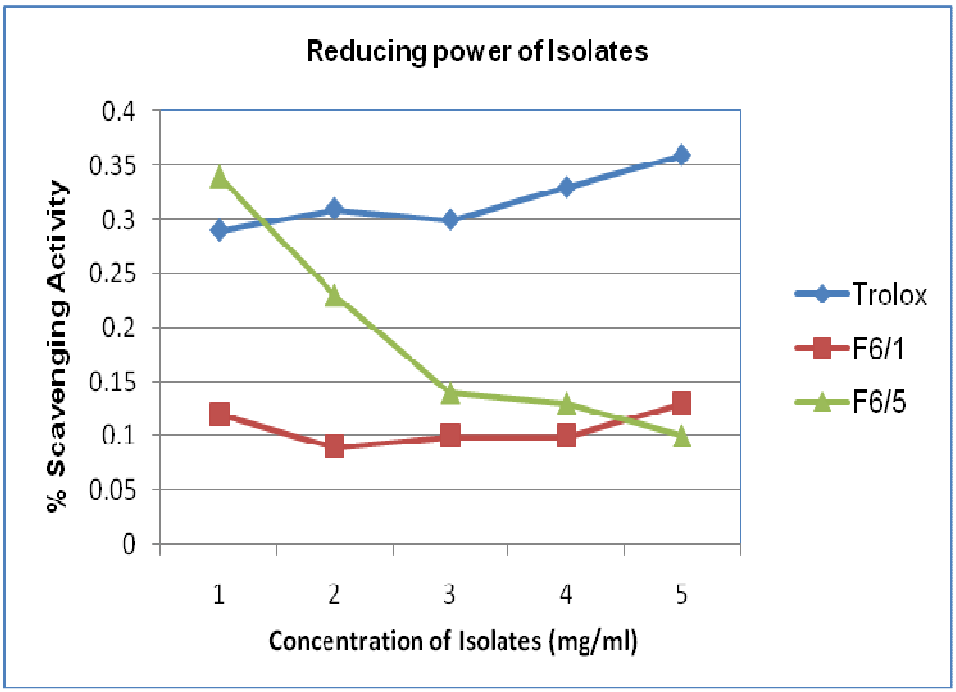


Figure 4.6: Reduction potential of the compounds F6/1 and F6/5 isolated from the crude chlorofoextract of *B.natalensis.* Trolox was used as a standard antioxidant.

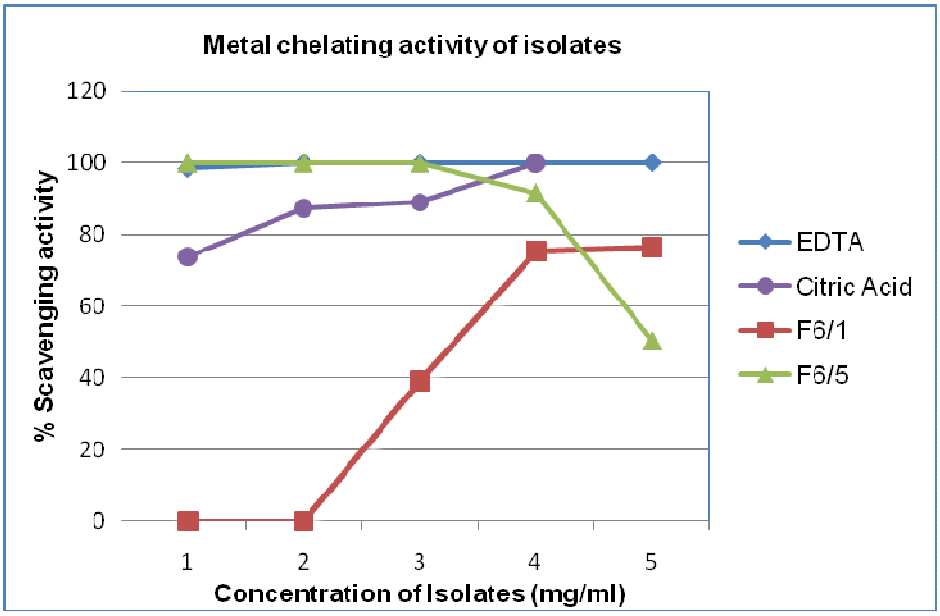


Figure 4.7: Metal chelating potential of the compounds F6/1 and F6/5. EDTA and citric acid was used as standard antioxidants.

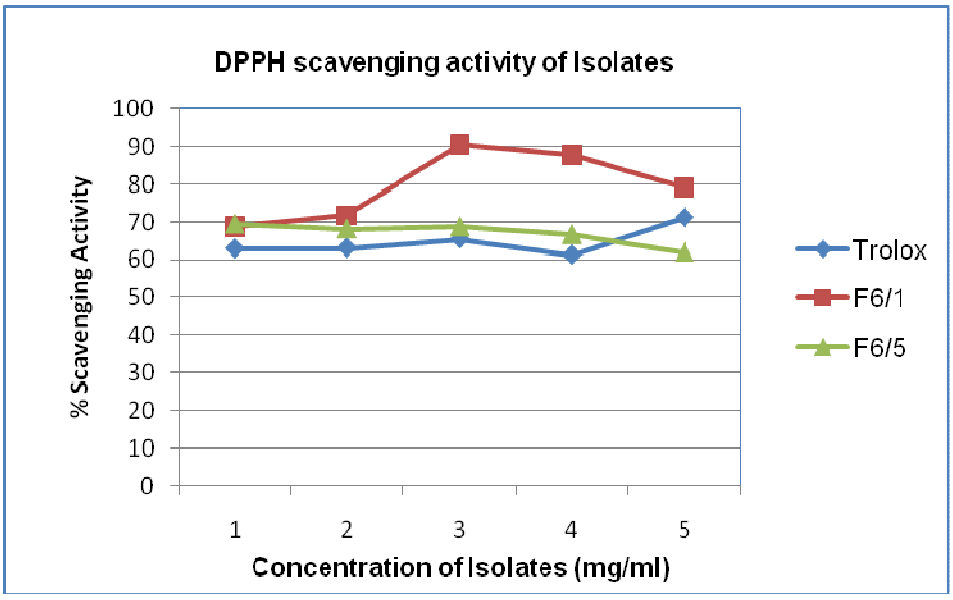


Figure 4.8: DPPH radical scavenging activity of the compounds F6/1 and F6/5. Trolox was used as standard antioxidant.

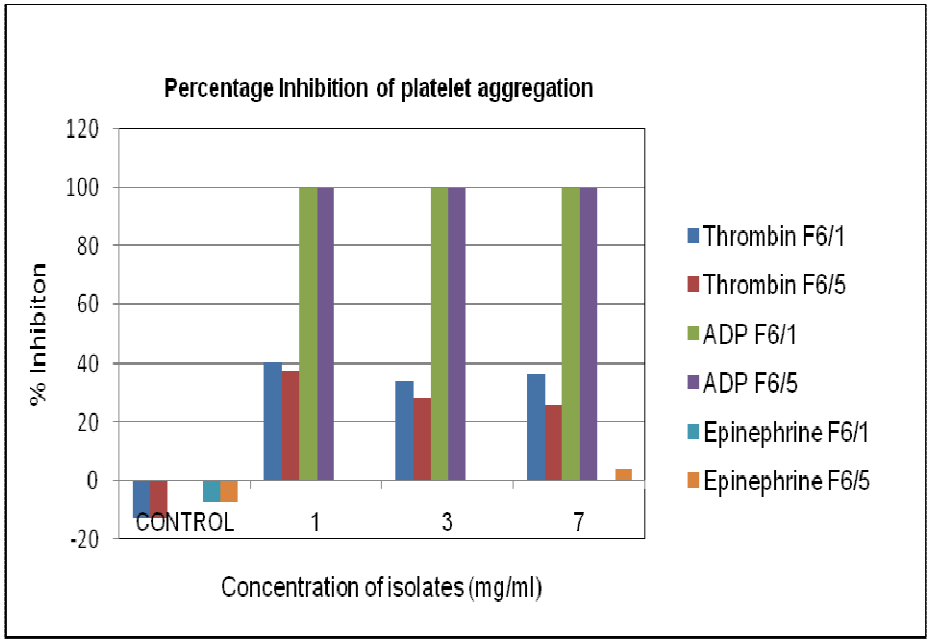


Figure 4.9: Percentage Inhibition of Isolated compounds (F6/1 and F6/5).

The isolated compounds (F6/1 and F6/5) exhibited good antioxidant activity. F6/1 exhibited high DPPH scavenging activity compared to the standard antioxidant.

4.8 Anti-platelet aggregation activity of the Isolates: F6/1 +F6/5

In the ADP induced aggregation, both F6/1 and F6/5 displayed 100% inhibition. There was no activity in the presence of epinephrine. Both the compounds had low IC_{50} values F6/1 (<1 mg/ml) and F6/5 (0.84 mg/ml). It is apparent that they are both good inhibitors.

4.9 Effect of the Isolates (F6/1 and F6/5) on Acetylcholinesterase activity

The results obtained from the assay of the effect of the isolates (F6/1 and F6/5) on acetylcholinesterase activity are presented in figure 4.10.

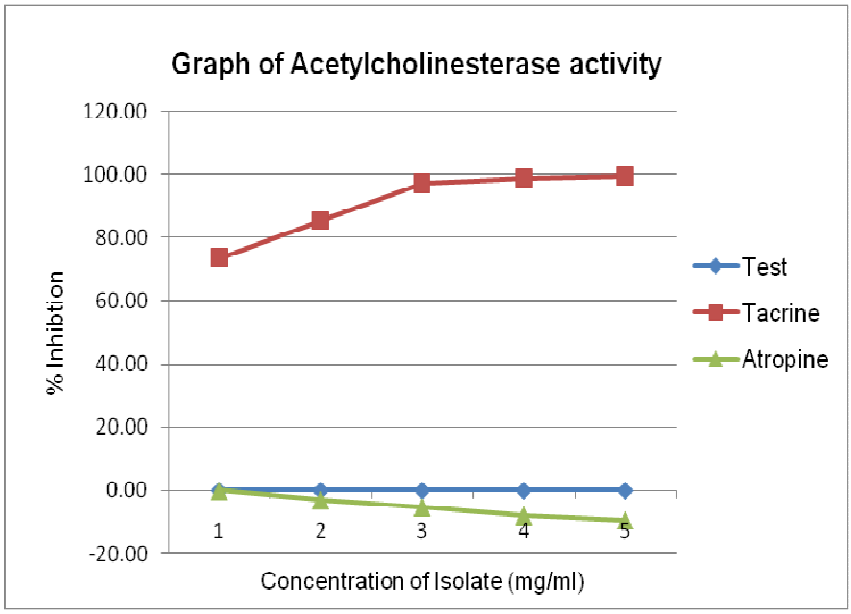


Figure 4.10: Acetylcholinesterase inhibition activities of the combined Isolates (F6/1 and F6/5).

Table 4.12: the IC_{50} values of the isolate acetylcholinesterase activity.

IC_{50} (mg/ml)		
Tacrine (+ve)	**Atropine (-ve)**	**Isolate**
<5	5.45	5.39

The IC_{50} value (mg/ml) of the isolates on the acetylcholinesterase activity is presented in table 4.12: Tacrine is an agonist and inhibits the action of AchE activity and was used as the positive control. Atropine is an antagonist and stimulates the degradation of acetylcholine (Himmelheber, 2000; Gulledge and Stuart 2005). It was used as the negative control. The isolates did not inhibit any appreciable acetylcholinesterase activity.

Table 4.13 *In vitro* anticoagulant activity.

Concentration	Thrombin	Epinephrine	ADP	Arachidonic acid
Control	1 min	1 min	1 min	1 min
1 mg/ml	2 min	2 sec	1 min	1 min
3 mg/ml	3 min	>3 min	2 min	2 min
10 mg/ml	> 5 min	4 min	4 min	3min

The anticoagulant activities of the isolate were investigated on rat' whole blood to determine the time it took for blood to clot. The compound showed anti-coagulant activity as it delayed blood clotting time in a concentration dependent manner compared to the control in which blood clotting occurred under a minute.

Chapter 5

Discussion

The ability of the body to form blood clots is a critical physiological mechanism that must take place for normal healthy living. However, excessive clotting is fatal to ones health and platelets, with the help of coagulation factors, are directly involved in this process. Besides their physiological function in stopping bleeding, platelets play a central part in the pathogenesis of some cardiovascular diseases (Wilson and Ferguson, 1999). Blood flow disturbances at the sites of atherosclerotic plaque promote platelet activation and arterial thrombus formation (Lee *et al*, 2009). Other diseases include myocardial infarction and peripheral artery disease (Dogne *et al*, 2002). Medicines are available to prevent abnormal platelet hyperactivity but these are expensive and not accessible to all rural people. Therefore, the use of alternate therapeutic methods is being used including the consumption of natural products. The screening of *B.natalensis* indicated that the extracts exhibited anti-platelet aggregation abilities.

The extracts of the plant inhibited the activity of thrombin on the artificial substrate (chromogenix), and then also inhibited rat platelet aggregation induced with thrombin, ADP and epinephrine (fig 4.2 and table 4.7). The ability of the plant extracts to increase the $CaCl_2$-induced clotting time (table 4.6) suggests that they can substantially inhibit Ca^{2+} ions from activating clotting factors resulting in a weak clot. $CaCl_2$-induced clotting time assay allows for the determination of a 50% clotting time and effect on fibrin formation (Kee *et al*, 2008).

The proteolytic enzymes (trypsin, bromelain and papain) catalytically hydrolyze proteins (fibrinogen) into smaller fragments (fibrin monomers) with generation of new functional groups. Clot formation (platelet aggregation) results from the formation and exposure of the new functional groups. The degree to which the extracts inhibited the aggregation of the enzyme treated platelets (fig 4.4 and table 4.10) does suggest that the extracts may not only be inhibiting other platelet agonists 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 and Metzig *et al*, 1999). The chloroform extract showed 100% inhibition against bromelain treated platelet aggregation induced by the agonists.

The plant *(B natalensis)* contained most of the phytochemicals that were screened for (table 4.2). These phytochemicals are known to have anti-inflammatory, anti-oxidative, anti-spasmodic, anti-allergic and anti-viral activities (Bruneton, 1995; van-Vyk *et al*., 1997; Cowan, 1999). Anthraquinones have been reported to be present in *Bulbine* species and are known to have anti-bacterial and healing effects (Bruce, 1975; Van staden and Drewes, 1994; van Wyk, *et al*. 1995).

The anti-coagulant or anti-platelet aggregation activity of tannins has been demonstrated by various researchers (Dong *et al*, 1986; Mekhi *et al,* 2006, Kee *et al*, 2008). The anti-platelet aggregation activity of the chloroform and ethyl acetate extracts could be attributed to the tannins present in these extracts (Bruneton, 1995

and Cowan 1999). These extracts however, retained the bioactivity after tannin removal. The observed activity of the extracts could thus be due to components other than tannin. The reduced activity of the tannin-free extracts (table 4.8) was an indication of synergistic effect of the plant components.

Factors that contribute to platelet activation include the formation of free radicals. Free radicals cause damages to arteries and stimulate platelet aggregation leading to inflammation. Antioxidants bind to free radicals and neutralize them. They act as scavengers by helping to prevent cell and tissue damage that could lead to cellular damage and disease (Mole and Waterman, 1987). The beneficial effects of antioxidants on the inhibition of platelet activation and aggregation have been reported by Krotz *et al*. (2004) and Sobotkva' *et al.* (2009).

From the anti-platelet aggregation activity studies it was observed that the ethyl acetate and chloroform extracts displayed the highest inhibitory activity (table 4.7, table 4.8 and fig. 4.4), high phenolic and flavonoid contents (table 4.3), high ABTS scavenging activity but poor ferric reducing antioxidant properties and abilities to chelate ferrous ions (fig 4.1 table 4.4). The polar solvents, for example the water extract which displayed low anti-platelet aggregation activities showed strong anti-oxidant properties. Polar compounds donate an electron from their hydroxyl group, making them good antioxidants. It is thus apparent that the anti-platelet aggregation activity of the plant components was not primarily due to the antioxidant activity.

Depending on the intended biological or medicinal activity, a good drug has to be active but not toxic. There is little or no documentation on the safe use of traditional medicinal plants. According to Svensson *et al*, (2005) toxicity of plant extracts is dependent on the concentration of specific compounds. Brine shrimp (*Artemia salina*) are fast growing organisms commonly used in the preliminary screening of cytotoxicty of plant extracts. The cytotoxicity of the plant was determined by observing the LC_{50} values of the extracts. The results (table 4.5) illustrated that the chloroform and ethyl acetate extracts, with the lowest LC_{50} values, were highly potent. LC_{50} values lower than 1000 µg/ml are considered bioactive (Meyer *et al*, 1982).

The high and consistent anti-platelet aggregation activity exhibited by the chloroform extract led to the isolation from this extract of two compounds, F6/1 and F6/5. The isolated compounds showed instability. It was therefore difficult to identify and characterize them. Previous isolation studies carried out on *B.natalensis* also revealed an unstable compound (Yakubu, 2009c).

The anthraquinone, knipolone (fig 4.5), has been isolated from fresh bulbs of *B.latifolia* and *B. frutescens* (Van staden and Drewes, 1994; Bringmann *et al*, 1990). The presence of chrysophanol in *Bulbine* (van Oudtshoorn, 1964) and the isolation of knipholone from *Bulbine* species indicate a close chemical relation between the genera *Bulbine* and *Kniphofia* (Frances van Staden and Drewes, 1994). It appears that chrysophanol is a common denominator for the Asphodeloideae and the knipholone-type compounds have been established to occur in the genera of the Asphodeloideae and may be present in other members of this sub-family (Yenesew and Dagne, 1994).

The activities of the isolated compounds, F6/1 and F6/5, were relatively as high as the crude extract. It showed strong anti-platelet aggregation activity especially ADP-induced platelet aggregation. The ability of the compounds (F6/1 and F6/5) to inhibit the arachidonic acid-induced platelet aggregation indicates its potential to be developed into a good pharmacological drug. Arachidonic acid-induced platelet aggregation is medicated by thromboxane A_2 and prostaglandins H_2 (Parise *et al*, 1984). Consequently, the prostaglandins H_2 is also a precursor for the prothrombic thromboxane A2. The efficiency of the isolates to prevent blood clotting is further evidenced by its ability to considerably delay the *in vitro* clotting time of the rat whole blood (table 4.13).

Acetylcholinesterase breaks down acetylcholine into choline and acetic acid and prevents continued muscle contraction in the absence of additional nervous stimulation (Whittaker, V. 1990). AChE inhibitors play an important role in nervous system disorders owing to their potential as pharmacological and toxicological agents. AChE inhibitors are useful in the treatment of myasthenia gravis (Srikumar *et al*, 2004), Alzheimers disease and Parkinson's disease (Ceravolo *et al*, 2006). Most indirect acting acetylcholine (ACh) receptor agonists work by inhibiting the enzyme acetylcholinesterase. The resulting accumulation of acetylcholine causes continuous stimulation of the muscles, glands and central nervous system (Purves *et al*, 2008). The isolate of *B.natalensis* showed no inhibition on acetylcholinesterase activity (fig 4.10).

Overall, the results revealed that the organic crude chloroform extract and the two isolated compounds, F6/1 and F6/5, have high anti-platelet aggregation activity, low antioxidant activity and high toxicity levels against brine shrimp. The extract also possessed high polyphenolic contents that could be responsible for the inhibitory effect and preventing platelet functions. The anti-platelet aggregation activity of the plant had no correlation to the low anti-oxidant activities. (Ebrahimzaheh *et al*, 2008). Extracts of natural products provide a useful source of bioactive compounds which can be developed into drugs for use against cardiovascular and atherothrombotic diseases. With the increase in thrombembolic disorders the result of this study scientifically validates the use of *B.natalensis* in treating blood clots.

Chapter 6

Conclusion

The results from this study suggest that the chloroform extracts and the isolated compounds (F6/1 and F6/5) from *B.natalensis* have anti-platelet aggregation activity. The isolated compounds displayed good anti-oxidant activity. It was not an inhibitor of acetylcholinesterase (AChE) and cannot be used to treat nervous system disorders. Ethyl acetate which was toxic to the brine shrimp larvae showed high anti-platelet aggregation activity. The water extract exhibited high scavenging activity, reducing power and metal chelating abilities.

The results give a rationale for the use of *B.natalensis* in folk medicine to manage blood clotting related diseases. Synergism of the plant components could be the basis for its traditional medicinal use.

6.1 Suggestions for further work

Further studies could be to isolate and stabilize these constituents in higher quantities and to screen them for:

1) Anti-ulcer activity
2) Anti-bacterial activity
3) Anti-inflammatory effects
4) Effect of *B.natalensis* on carcinogenic chemicals
5) The mechanism through which the compounds exert their therapeutic effects and to elucidate these effects

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Appendix A

Preparation of Reagents

A1. ABTS

One ABTS tablet (10 mg) was dissolved in 3ml distilled water and the equivalent of 2.4 mM potassium persulfate (2 mg) was added to the solution. The mixture was incubated at room temperature in the dark for 16 hours. ABTS* was diluted with methanol (1 ml ABTS*: 60 ml methanol)

A2. ADA

24.9 g of 0.085M trisodium citrate, 13.65 g of citric acid monohydrate, and 20 g of dextrose was dissolved in 1000 ml of distilled water. It was refrigerated until use (1ml of anticoagulant was added for every 5 ml of blood)

A3. Chromogenix

47.54 g was dissolved in 100ml of 1% DMSO in Tris-Buffer

A4. 1% DMSO in Tris buffer

1 ml DMSO was made up to 100 ml Tris-buffer

A5. DPPH

2 mg DPPH was dissolved in 100 ml MeOH. It was stored in a brown bottle and kept in the dark to prevent oxidation

A6. DTNB Reagent

39.6 mg of DTNB with 15 mg $NaHCO_3$ was dissolved in 10ml of 0.1 M phosphate buffer (pH 7.0)

A7. 0.1 M Phosphate Buffer pH 8

Solution A: 5.22 g of K_2HPO_4 and 4.68 g of NaH_2PO_4 was dissolved in 150 ml of distilled water. Solution B: 6.2 g of NaOH was dissolved in 150 ml of distilled water. Solution B was added to solution A to get a pH of 8.0 and the final volume was made up to 300 ml dH_20

A8. 0.2 M Phosphate Buffer pH 6.6

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

A9. Re-suspending Buffer

41 ml of 0.03 M HCl was added to 50 ml of 0.03 M tris amminomethane. Thereafter, 8.12 g of 0.14 M NaCl and 0.99 g of 0.005 M glucose was dissolved in it. The solution was made up to 1000 ml distilled water at pH 7.4

A10. Tris-HCl buffer

2.36 g of 50 mM Tris-HCl was prepared in 100 ml dH_2O, thereafter 0.837 g of 7.5 mM EDTA and 3.068 g of 175 mM NaCl was dissolved in it and the solution was made up to 300 ml dH_2O at pH 7.4

A11. Washing Buffer

13.2 g of 0.113 M NaCl, 1.22 g of 4.3 mM Na_2HPO4, 1.49 g K_2HPO4, 5.85 g NaH_2PO4, 2.18 g glucose, and 0.744 g EDTA was dissolved in 2000 ml of distilled water at a pH 6.5. It was refrigerated until use

Appendix B

Details of Methods

B1. Sequential Extraction

The fresh leaves were extracted (1:5 w/v) sequentially with different solvents (n-hexane, chloroform, ethyl acetate, methanol and water) according to their increasing polarity. The leaves were diced into smaller pieces and blended with n-hexane solvent. The plant leaves and solvent mixture were incubated at room temperature on the platform shaker (150 rpm) for 48 hours. Thereafter, the mixture was filtered through Whatman No. 1 filter paper. The filtrate was labeled and refrigerated. The residue was re-extracted with $CHCl_3$. This was done sequentially with ethyl acetate, methanol and distilled water. The organic solvent filtrates were concentrated 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 and the percentage yields were recorded. The extracts were stored in sterile glass bottles and refrigerated.

B2. Phytochemical Screening

B2.1. Test for Tannins

The method of Sofoware *et al*. (1993) and Harbone *et al*. (1973) were used. 5 g dried powdered sample was added to 10 ml of water. It was stirred and filtered. A few drops of 0.1 % ferric chloride was added to 2 ml of the filtrate and observed for a blue-green or blue-black colouration.

B2.2. Test for Phlobatannin

The method of Sofoware *et al*. (1993) and Harbone *et al*. (1973) were used. Deposition of a red precipitate was taken as evidence for the presence of phlobatannin when 0.2 g of the powdered sample was boiled in 1% aqueous HCl.

B2.3 Test for Saponin

The method of Sofoware *et al*. (1993) and Harbone *et al*. (1973) were used. 0.5 g of the powdered sample was added to 2.5 ml distilled water. It was shaken vigorously warmed and observed for a stable persistent froth.

B2.4 Test for Flavonoids

3 tests were carried out for the presence of flavonoid: The method of Sofoware *et al*. (1993) and Harbone *et al*. (1973) were used.

i) Lead Acetate Test:

1 ml of the MeOH extract was added to 1 ml of 10% lead acetate. A reddish-brown colouration of the precipitate indicated a positive test.

ii)) Ferric Chloride Test:

1 ml of the MeOH extract was added to 1 ml of 10% ferric chloride. A dark brown precipitate was observed as evidence for Flavonoid.

iii) Sodium Hydroxide Test:

1 ml extract was added to 1ml diluted sodium hydroxide. A golden yellow precipitate was observed indicating the presence of Flavonoid.

B2.5 Test for Steroids

The method of Sofoware *et al.* (1993) and Harbone *et al*. (1973) were used. 2ml of acetic anhydride was added to 0.5 g ethanolic extract of the sample with 2 ml H_2SO_4. The colour changed from violet to blue or green indicating the presence of steroids.

B2.6 Test for Terpenoids

The method of Sofoware *et al*. (1993) and Harbone *et al*. (1973) were used. To 5ml of methanolic extract, 2 ml of chloroform was mixed and 3 ml H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of Terpenoid.

B2.7 Test for Cardiac Glycosides

Three tests were carried out for the presence of cardiac glycosides: The method of Sofoware *et al.* (1993) and Harbone *et al*. (1973) were used.

i) Liebermans Test

To 0.5 g extract, 2 ml acetic anhydride was mixed. It was cooled well in ice and H_2SO_4 was carefully added to form a layer. A violet colour which faded to blue and green indicated the presence of a steroidal nucleus ie. the aglycone portion of the cardiac glycoside.

ii) Salkowski Test

0.5 g methanolic extract was dissolved in 2 ml of chloroform and H_2SO_4 was carefully added to form a layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring.

ii) Keller-killiani Test

0.5 g powdered sample was treated with 2 ml glacial acetic acid containing one drop of 10% ferric chloride solution. This was underlayed with 1ml concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just gradually throughout this layer.

B2.8 Total Phenolic Determination

Total phenolic compound contents were determined by using the Folin Ciocalteu method (Kahkonen *et al*, 1999) Different concentrations of gallic acid (0.01; 0.02; 0.04; 0.08; 0.1 mg/ml) were prepared in diethyl ether. The different concentration of plant extracts were treated with diethyl ether (0.5 ml/ml) and mixed. The diethyl ether was evaporated off and the residue kept. The residue was dissolved in 1.5 ml (5 ml/50 ml dH_2O) Folin Ciocalteu Reagent and 1.2 ml (7.5 g/100 ml dH_20) Sodium Carbonate (Na_2CO_3) was then added. It was stored in the dark for 30 minutes and the absorbance was measured at 765 nm against a blank containing the Folin Ciocalteu reagent and sodium carbonate solution. A graph of absorbance versus concentration was plotted (figure B.1), and total phenol values were estimated as gallic acid equivalent (mg/g of dry mass).

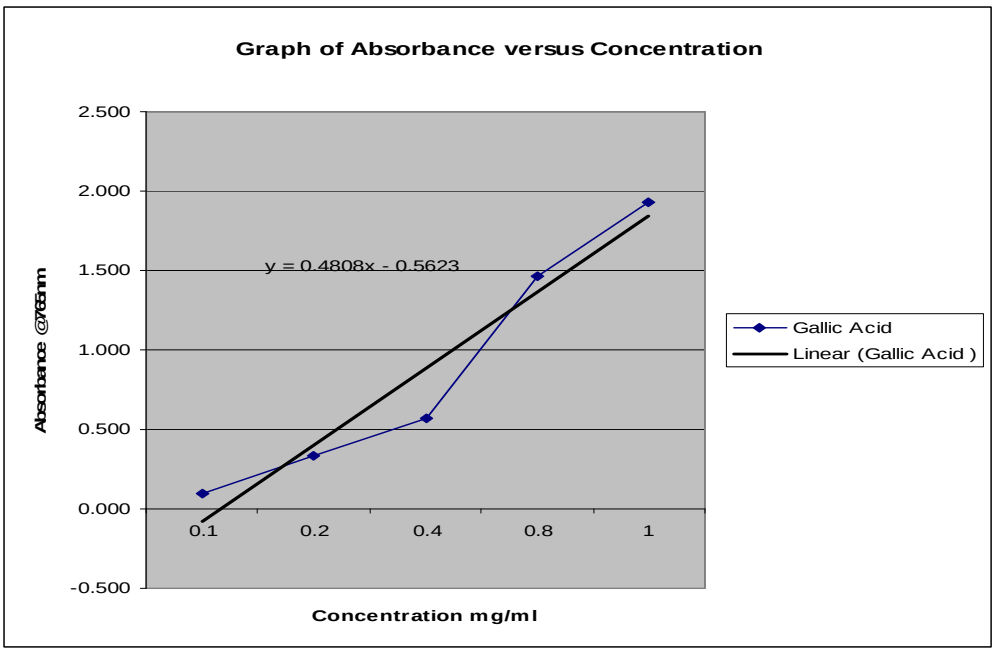

Figure B.1—Standard gallic acid graph

B2.9 Determination of total flavonoid contents

Flavonoid content was measured by the method of Ordon-Ez *et al*, (2006). Different concentrations of quercetin (0.01; 0.02; 0.04; 0.08 0.1 mg/ml) were prepared in diethyl ether. 0.5ml of the extracts was mixed with 1ml of diethyl ether. The residue was dissolved in 0.5 ml of 2% $AlCL_3$ in ethanol (80 % EtOH). It was allowed to stand for 60 minutes at room temperature. A yellow colour indicated the presence of flavonoids. The absorbance was read at 420 nm and the standard graph was plotted (figure B.2). The amount of total flavonoids was expressed as quercetin equivalent in milligram per gram dry plant extract (Ordon *et al*, 2006).

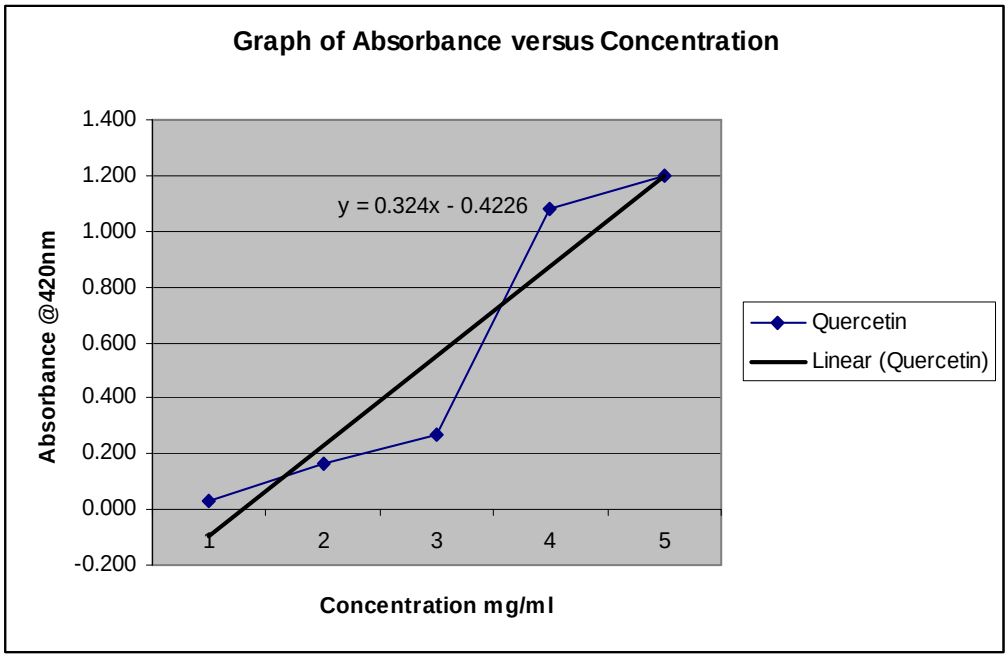

Figure B.2—Standard quercetin graph

B2.10 Determination of Proanthcyanidin

The standard cetechin was prepared in diethyl ether (0.01; 0.02; 0.04; 0.08 0.1 mg/ml) Sun *et al*, (2002). 0.5 ml of the extracts was mixed with diethyl ether. The residue was dissolved in 3 ml of 4% vanillin-MeOH solution (80%) and 1.5 ml of 1%HCL. The solution was allowed to stand for 15 minutes at room temperature. The absorbance was measured at 500 nm. The amount of total flavonoids was expressed as quercetin equivalent in milligram per gram dry plant extract (figure B3).

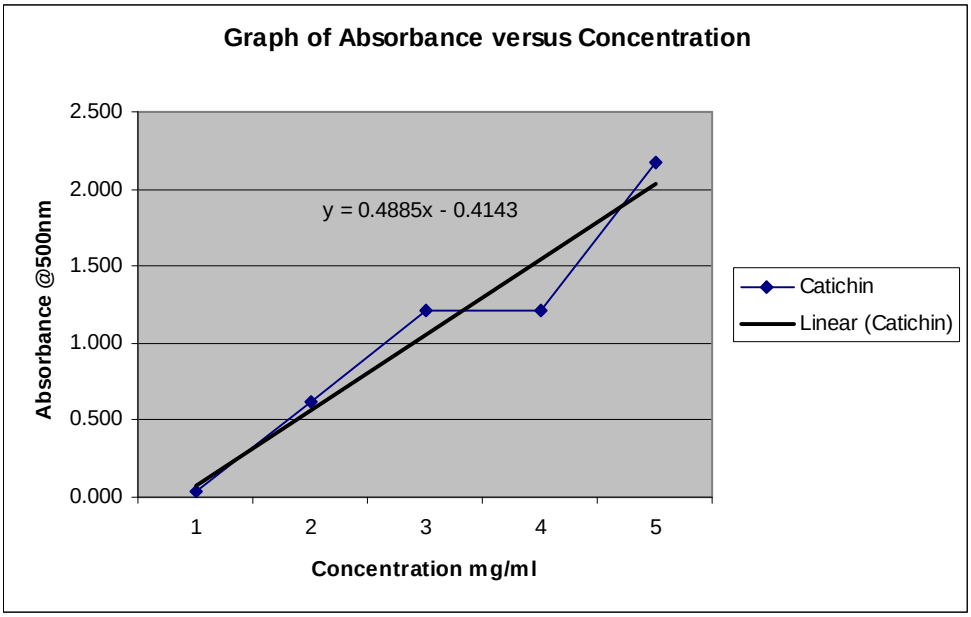

Figure B.3—Standard cetechin graph

B3. Antioxidant Activity *in vitro*

B3.1 DPPH Free Radial Scavenging Activity

DPPH free radical scavenging activity of plant extracts was determined spectrophotometrically by using the method of Brad-Williams *et al* (1995). Aliquots (2 ml) of 2 mg% DPPH were added to 2 ml of each concentration of the extracts. BHA (2 mg/100 ml MeOH) and Ascorbic Acid (2 mg/100 ml MeoH) were used as the standards. A control was set up containing 2 ml DPPH and 2 ml MeOH. It was mixed with a vortex mixer and allowed to stand in the dark for 30-60 minutes. Absorbance was read at 517 nm.

B3.2 ABTS

ABTS radical scavenging activity of plant extracts was determined spectrophotometrically by the using method of Pellegrini *et al* (1999). To 1 ml of ABTS, 1ml of different concentrations (0-5 mg/100 ml) of the extract was added. It was mixed and allowed to stand for 6 minutes. BHA (2 mg/100 ml MeOH) and Ascorbic Acid (2 mg/100 ml MeoH) were used as the standards. A control was set up containing 1ml ABTS and 1ml MeOH. The absorbance was read at 734 nm and the percentage scavenging activity was calculated.

B3.3 Reducing Power

The reducing power ability of the plant extracts was evaluated according to the method of Oyaizu *et al*. (1986). Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared. 1 ml of plant sample was mixed with 2.5 ml of (0.2 M), ph 6.6 phosphate buffer and 2.5 ml (1%) (K_3 ($Fe(CN)_6$). It was incubated at 50℃ for 20 minutes. Thereafter 2.5 ml (1%) TCA was added. It was mixed with a vortex mixer and allowed to stand for 10 minutes. The whole mixture was centrifuged for 10 minutes @ 1000 rpm; 2.5 ml of the supernatant was removed and mixed with 2.5 ml dH_2O and 0.5 ml (0.1) % $FeCl_3$. This was allowed to stand for 30 minutes and the absorbance was measured at 700 nm. BHA (2 mg/100 ml MeOH) and Ascorbic Acid (2 mg/100 ml MeOH) were used as the standards. A control was set up containing all of the reagents and MeOH was added in place of the extract. The high absorbances were recorded has having a strong reducing power.

B3.4 Chelating activity on Fe^{2+} [7]

The ability of the extract to chelate Fe^{2+} ions was evaluated according to the method of Decker and Welch (1990). Different concentrations of the extract (0-5 mg/100 ml MeOH) were prepared. To 1 ml of the extracts, 3.75 ml deionized H_2O, 0.1 ml of $FeCl_2$ (2 mM) and 0.2 ml Ferrozine (5 mM) was added. It was mixed vigorously and allowed to stand for 10 minutes. The absorbance of Fe^{2+} ions was measured at 562 nm. EDTA (5 mg/100 ml MeOH) and Citric acid (5 mg/100 ml MeoH) were used as the standards. A control was set up containing all of the reagents and MeOH was added in place of the extract. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as:

$$\% \text{ chelating activity} = \{(A_0 - A_1)/A_0 \times 100\}$$

B4. Measurement of platelet aggregation

B4.1 Preparation of untreated platelets

Washed platelets were prepared as described by Tomita *et al* (1983). *Sprague dawley* rats were anaesthetized with diethyl ether and sacrificed. Blood was drawn from the abdominal aorta and placed into a centrifuge tube containing (5:1 v/v) acid dextrose anticoagulant (ADA). It was centrifuged at 1200 rpm (230 g) for 15 minutes and consecutively at 2200 rpm (800 g) for 3 minutes. The supernatant was taken and again centrifuged at 3200 rpm (1700 g) for 15 minutes. The sediment was re-suspended in 5 volumes washing buffer (WB), centrifuged at 3000 rpm (1500 g) for 15 minutes and the sediment obtained suspended in re-suspending buffer (RB). It was kept in the fridge and used within 4 hours.

B4.2 Preparation of enzyme treated platelets

0.1 mg of the respective enzyme (papain, bromelain and trypsin) was separately incubated with 25 ml of a 2% suspension of cells for 60 minutes at 25 ℃. It was washed 6 times with washing buffer and a 2% suspension in resuspending buffer was prepared. It was used within 4 hours.

B4.3 Test with Chromogenix

The anti-thrombic activity of the plant extracts was determined using the modified method of Rob *et al*. (1997). The chromogenic substrate, S 2238 (H-D phenylalanyl-L-pipecolyl-*p* nitroanilide dihydrochloride, Chromogenix), was used. Plants extracts were screened at concentrations of 1; 3; 10 mg (dry weight)/ml buffer. The extracts were solubilised in dimethyl sulfoxide (DMSO) and the volume was made up with resuspending buffer to yield a final DMSO concentration of 1% (v/v). 50 µl of the extract was added to 10 µl of thrombin in a 96 multiwell plates. This mixture was left to incubate for 10 minutes at room temperature and 190 µl 0.76 M S2238 was added to each well. The blank contained chromogenix (190 µl) and DMSO (50 µl) in place of the extract whilst the control contained chromogenix (190 µl) and thrombin (10 µl). The multi well plate was then placed in the BioTek microtitre plate reader. It was shaken for 30 seconds and the reaction was monitored at 412 nm (temperature 37° C), for 4 minutes at 10 second intervals using the GEN 5 software.

B4.4 Thrombin-induced clotting time assay

The method of Kee *et al*. (2008) was adopted for the measurement of thrombin-induced clotting time assay. The maximum concentration that was used without significantly affecting clot formation was 2% (v/v). Plant extracts were tested at 1; 3; 10 mg/ml in saline containing a DMSO concentration of 2% (v/v). The plant extract (50 µl) was added to human plasma (100µl) in a 96 multiwell plate and incubated at room temperature for 5 minutes. Thrombin (20 µl) was added to each well and the 96 multiwell plates were placed into the BioTek microtitre plate reader. The plate was shaken for 30 seconds and the rate of clot formation was determined by following the increase of the absorbance at 412 nm (temperature 37℃), for 20 minutes at 30 second intervals using the GEN 5 software. The blank contained human plasma (100 µl) and DMSO (50 µl) in place of the extract whilst the control contained human plasma (100 µl) and thrombin (10 µl).

Plant extracts that inhibited the rate of clot formation was further tested at a range of concentrations to determine a concentration that inhibited that rate by 50% (IC_{50}).

A negative control was performed using 2% (v/v) DMSO in saline, which represented 100 % activity.

B4.5 $CaCl_2$-induced clotting time assay

The method of Kee *et al* (2008) was adopted for the measurement of $CaCl_2$-induced clotting time assay. The assay was carried out to determine a 50% clotting time and the effect on fibrin formation. The extracts were screened at (1, 3, 7 mg/ml) using 2% DMSO (v/v) to solubilise the plant extracts. 100 µl human plasma were added to a 96

multiwell plate, thereafter 50 µl plant extract was added. The reaction was mixed and left to incubate for 5 minutes at room temperature. Clotting was induced by adding 20 µl 0.16M $CaCl_2$. The blank contained human plasma (100 µl) and DMSO (50 µl) in place of the extract whilst the control contained human plasma (100 µl) and thrombin (10 µl).The plate was put into the BioTek microtitre plate reader and shaken for 30 seconds. The reaction was monitored and followed at 412 nm (temperature 37℃), for 2 hour at 3 minute intervals using the GEN 5 software.

B4.6 Whole blood clotting time assay

The $ChCl_3$ extract was insoluble in MeOH and DMSO. Therefore, it was dissolved in olive oil. A blank was set up and it contained 100 µl whole blood and 20 µl oil. The control contained 100 µl blood, 20 µl coagulant and 40 µl extract. It was carried out in duplicate. The blood was separately treated with thrombin, ADP and epinephrine. The extract was added to the blood and clotting time was observed for 4 minutes.

B5. Tannin removal

Tannin removal was carried out using PVPP. PVPP is insoluble and binds to tannins, thereby removing it from the extracts before assaying. The method of Toth and Pavia (2001) was modified. Plant extracts were prepared at concentrations of (1, 3, 10 mg/ml) in dH_2O. PVPP was added to the extracts at 10 mg/ml and shaken for 15 minutes at 4℃. It was then centrifuged using the Eppendorf ce ntrifuge model 5404 R at 4℃ for 8 min. The pellet was discarded. The supernatant was removed and the process repeated twice to remove tannins. The tannin-removed extracts were then solubilised in DMSO

and the volume was made up with resuspending buffer to yield a final DMSO concentration of 1% (v/v). The tannin-removed extract (50 µl) was added to human plasma (100 µl) in a 96 multiwell plate and incubated at room temperature for 5 minutes. Thrombin (20 µl) was added to each well and the 96 multiwell plates were placed into the BioTek microtitre plate reader. The blank contained human plasma (100 µl) and DMSO (50 µl) in place of the extract whilst the control contained human plasma (100 µl) and thrombin (10 µl).The plate was shaken for 30 seconds and the rate of clot formation was determined by following the increase of the absorbance at 412 nm (temperature 37℃), for 20 minute at 30 second intervals using t he GEN 5 software.

B6. Brine shrimp Lethality Test

The method of Meyer *et al*. (1982) was used with slight modification. Brine shrimp eggs were hatched in a container filled with artificial sea water (40 g of coarse non-iodized salt per 1L of distilled water). Eggs were added to the container and aeration was supplied. It was left in a dark room and the temperature of the hatching water between 27℃ to 30℃ using a 100 W incandescent light bulb over the hatching container. After 48 hours, the phototropic nauplii were collected using a Pasteur pipette and transferred to a fresh container to separate the egg shells from the unhatched eggs. Different concentrations (0-5 mg/100 ml MeOH) of the extracts were prepared and the experiments were done in triplicate for each concentration at room temperature. The number of brine shrimps was determined by placing, with a pipette, two drops of water with brine shrimps under the microscope (two drops contained 10 larvae), 25 ml degassed water and 100 µl of plant extract in MeOH into a Petri dish. For the control,

MeOH was used instead of the plant extract. When control deaths occured, the percent death values were corrected using Abbott's formula as expressed by Rasoanaivo and Ratsimamanga-Urverg (1993). Mortality numbers were recorded and the number of survivors was counted after 24 hours and 48 hours. Lethality concentration (LC_{50} values) for each assay was calculated by taking the average of three experiments using a Finney Probit analysis program on an IBM computer (McLaughlin *et al*., 1991)

B7. Isolation and Characterization of active component

Crude solvents (hexane, ethyl acetate and chloroform) were distilled by using simple distillation for the use in column chromatography and TLC. Open column chromatography of the crude chloroform extract was carried out. Wet packing was done for the column chromatography and

slurry was prepared by mixing silica gel 60 0.063-0.200 mm (70-230 mesh ASTM) (75 g) with an initial solvent system (hexane:ethyl acetate; 9:1) to be used for elution.

The column was packed by adding the slurry to the column and it was allowed to reach a constant level before it could be sealed with a little amount of sand (about 0.1-0.3 mm; 50-150 mesh). Crude chloroform extract (2 g) was loaded on to the column and it was again sealed with a little amount of the sand. The initial solvent system was run through the column several times to equilibrate the column and also to ensure a tight packing as well as to remove any air bubbles. After about 150 ml of the eluent was collected, series of 40 ml fractions were collected into beakers. Elution was done using hexane:ethyl acetate solvent system starting with 9:1 to 3:7. A total of 90 of 20 ml fractions were collected. TLC analysis of the collected fractions was performed to identify those with

the common profile. The TLC plates were first viewed under UV light and developed using a 10% H_2SO_4 spray agent and then heated. The fractions with similar profile were combined as A (1-3), B (4-7), C (8-17,…,D (18-20) to give 11 combined fractions. The combined fractions were separately concentrated *in vacuo* and their weights were determined. The compounds F6/1 and F6/5 were separately dissolved in ethyl acetate to recrystalize. The mixtures were then filtered to obtain filtrates. The samples were analyzed by using thin layer chromatography techniques and the filtrates showed purity.

B8. Characterization of active compound

The compounds were subjected to NMR techniques for characterization. There was not enough of compound D3 to carry out NMR analysis. Compound F6/1 and F6/5 were prepared in $CDCl_3$ and analyzed using 1D and 2D NMR techniques (^{1}H- ^{1}H, ^{13} C^{13} C, DEPT spectrum). The resulting spectra (see Appendix E) were analyzed and the compounds identified by comparing them with standards and library materials.

B9. Acetylation

The reaction involved the replacement of the hydrogen atom of a hydroxyl group with an acetyl radical (CH_3CO) to yield a specific ester, the acetate. Acetic anhydride was used as an acetylating agent reacting with free hydroxyl groups. The 3 similar fractions, previously isolated, were combined and 5 ml acetic anhydride and 5 ml pyridine added to it. It was left overnight with a magnetic stirrer. The following day a small amount of H_2O was added to the reaction to initiate hydrolysis and remove acetic anhydride. The

acetylated product was removed and TLC was carried out to ensure its purity before NMR spectroscopic analysis.

B10. Acetylcholinesterase Inhibition Activity

The method of Srikumar *et al* (2004) was used to measure the acetylcholinesterase activity in the brain. Different concentrations of the isolate (0.01-0.05 mg/100 ml olive oil) were prepared. Two *Sprague dawley* rats were decapitated and the brains were quickly removed and placed in ice-cold saline. The tissues were weighed and homogenized (20 mg/ml) in 0.1M phosphate buffer (pH 8). To a cuvette containing 1.3 ml phosphate buffer and 50 µl of DTNB, 0.2 ml aliquot of the brain homogenate was added. The contents were mixed and the absorbed measured at 412 nm). When the absorbance reached a stable value, it was recorded as the basal reading. Thereafter, 10 µl of the substrate that is, acetylthiocholine was added and 10 µl of the isolated compound. The change in absorbance was recorded for a period of 10 minutes at intervals of 2 minutes. Atropine was set up as the negative control in serial dilutions and Tacrine was set up as the positive control in the same way. The blank contained the brain homogenate and olive oil. The change in the absorbance per minute was determined and the enzyme activity was calculated using the following formula: $R= 5.74 \times 10^{-4} \times A/CO$ where: R= Rate in moles of substrate hydrolyzed/ minute/ gm tissue, A= Change in absorbance / min, CO= Original concentration of the tissue (mg/ml)

Appendix C

C1 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
Email: rvande@pan.uzulu.ac.za

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.

RM van.

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

C2. Research Questionnaire

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 (for example,. by mouth or as enema)?

b) How is the medicine prepared?

Storage Method:

Dosage:

a) What is the dosage (for example, 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 (for example, grandmother, relative)?

Age Group:

Infants	
Children	
Adults	

Appendix D

D1. Spectral data for compounds F6/1 and F6/5

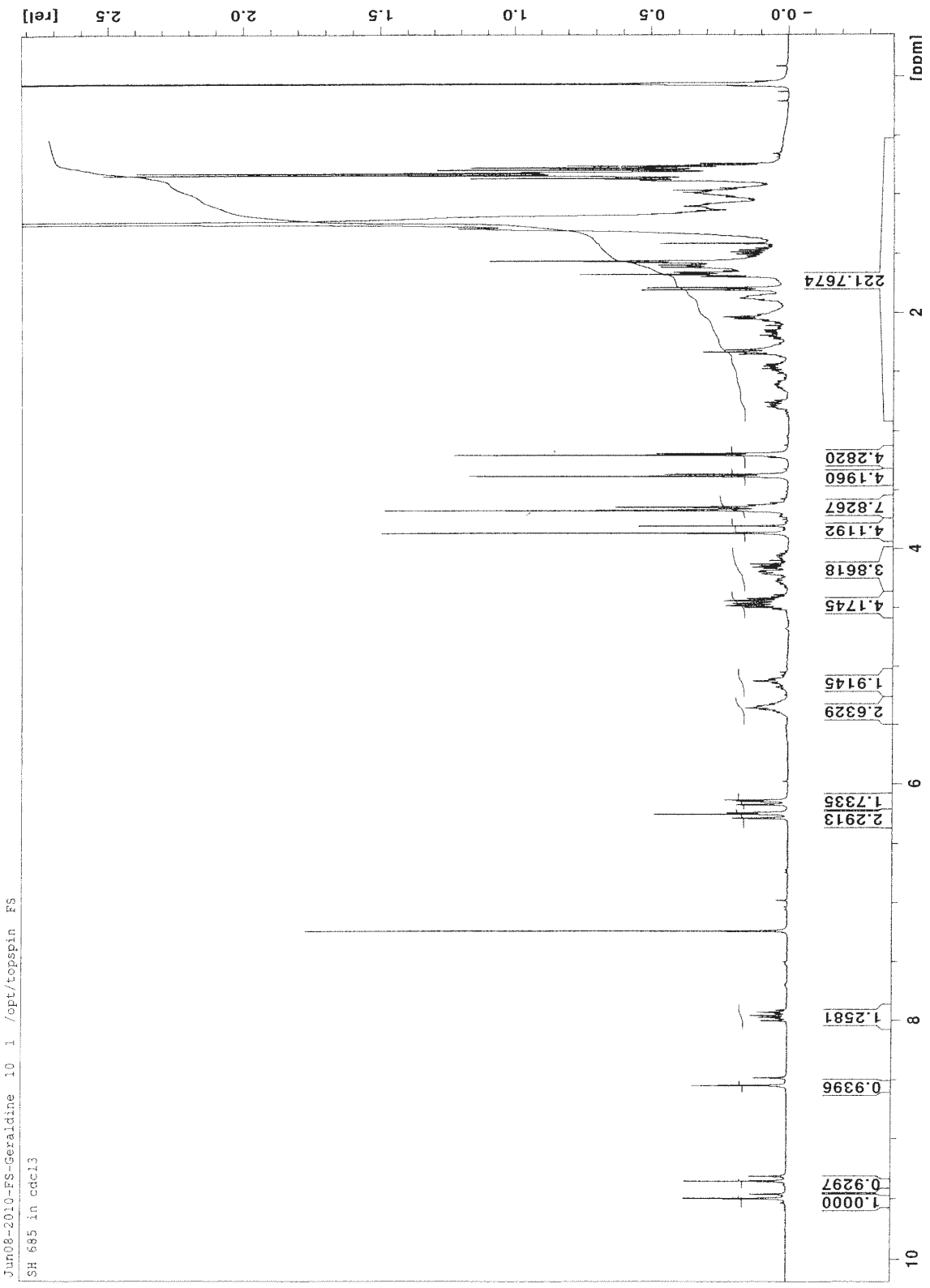

Figure D1.1 ^{1}H-NMR spectrum of compound F6/1

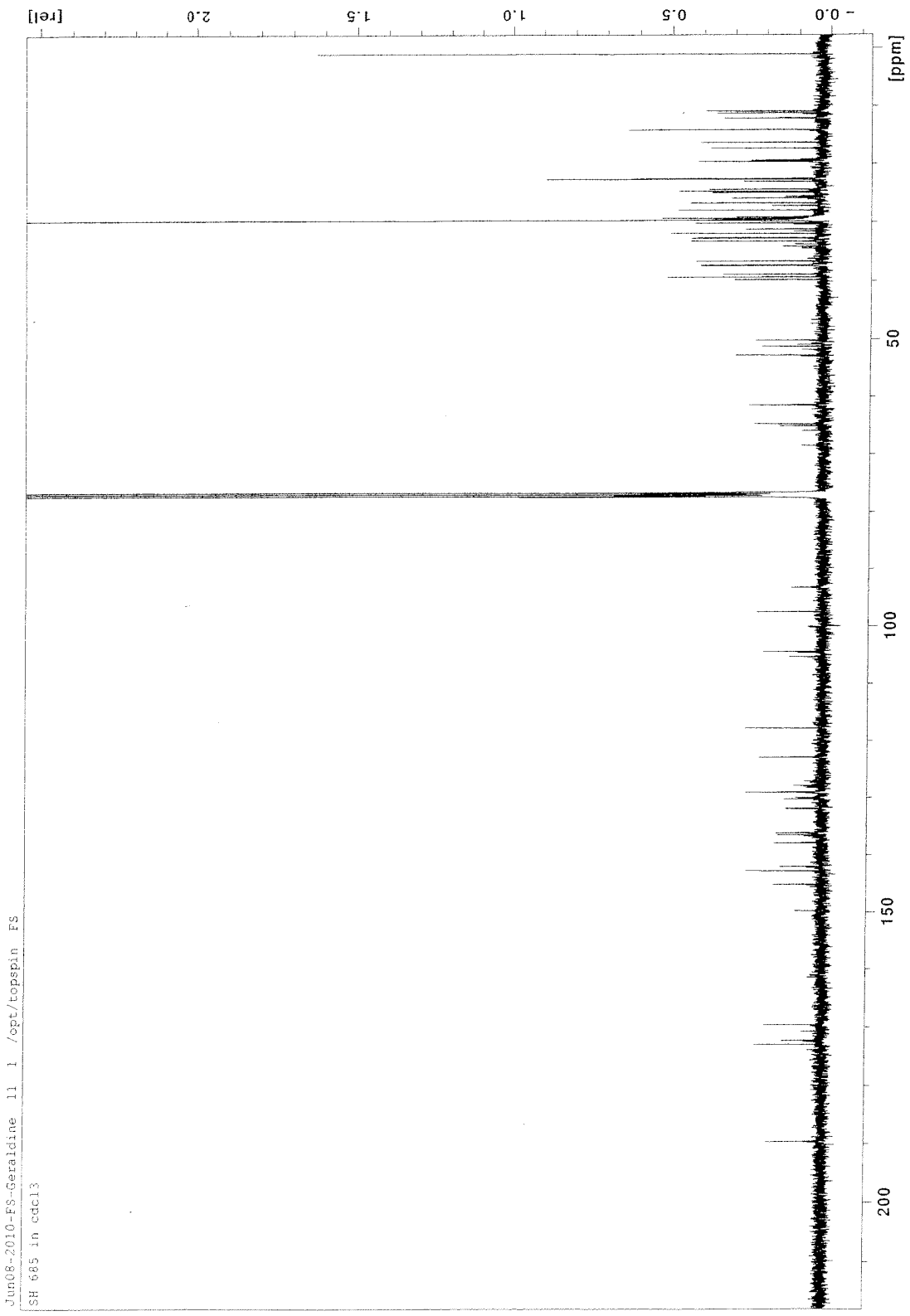

Figure D1.2 ^{13}C-NMR spectrum of compound F6/1

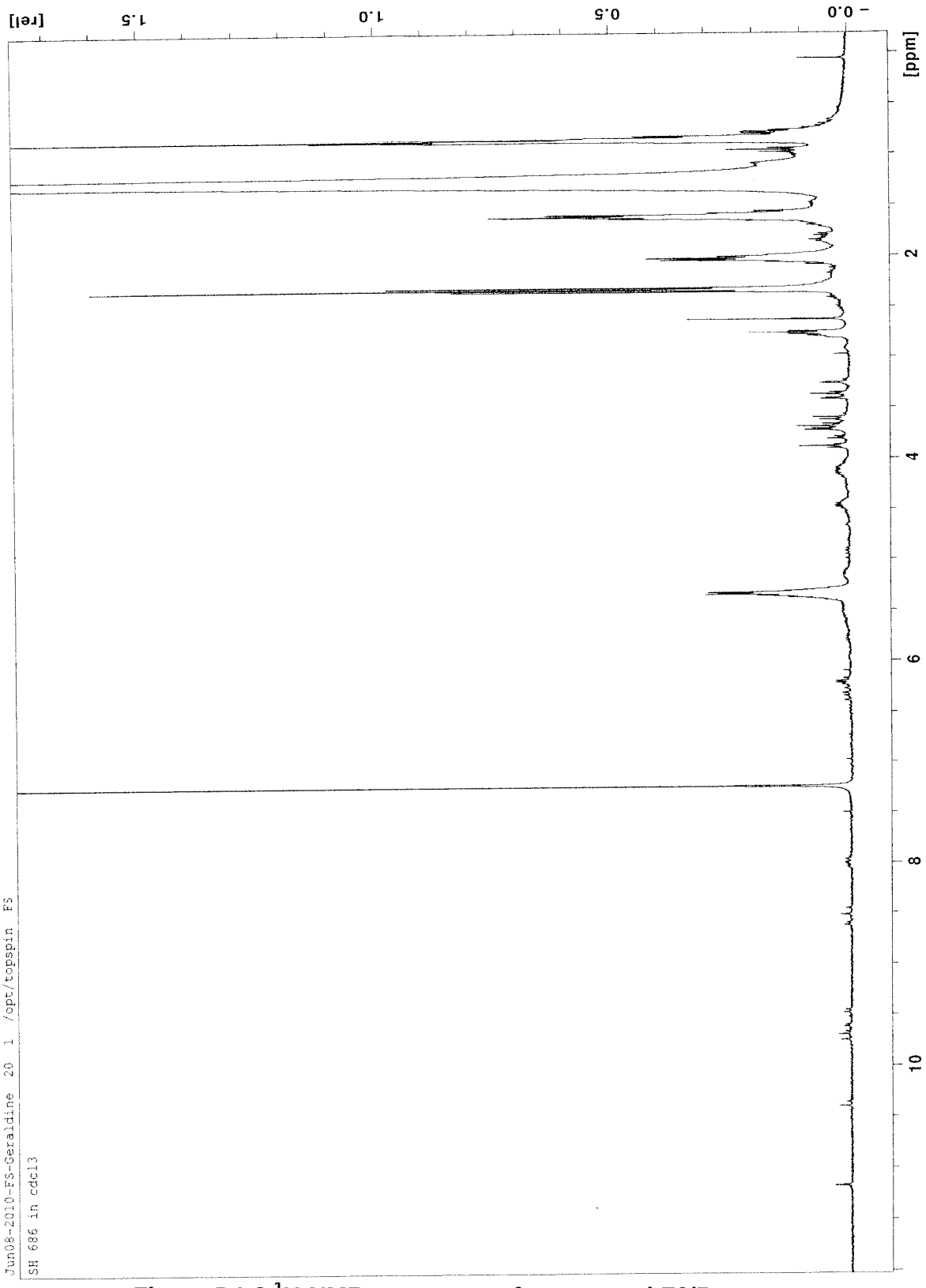

Figure D1.3 ^{1}H-NMR- spectrum of compound F6/5

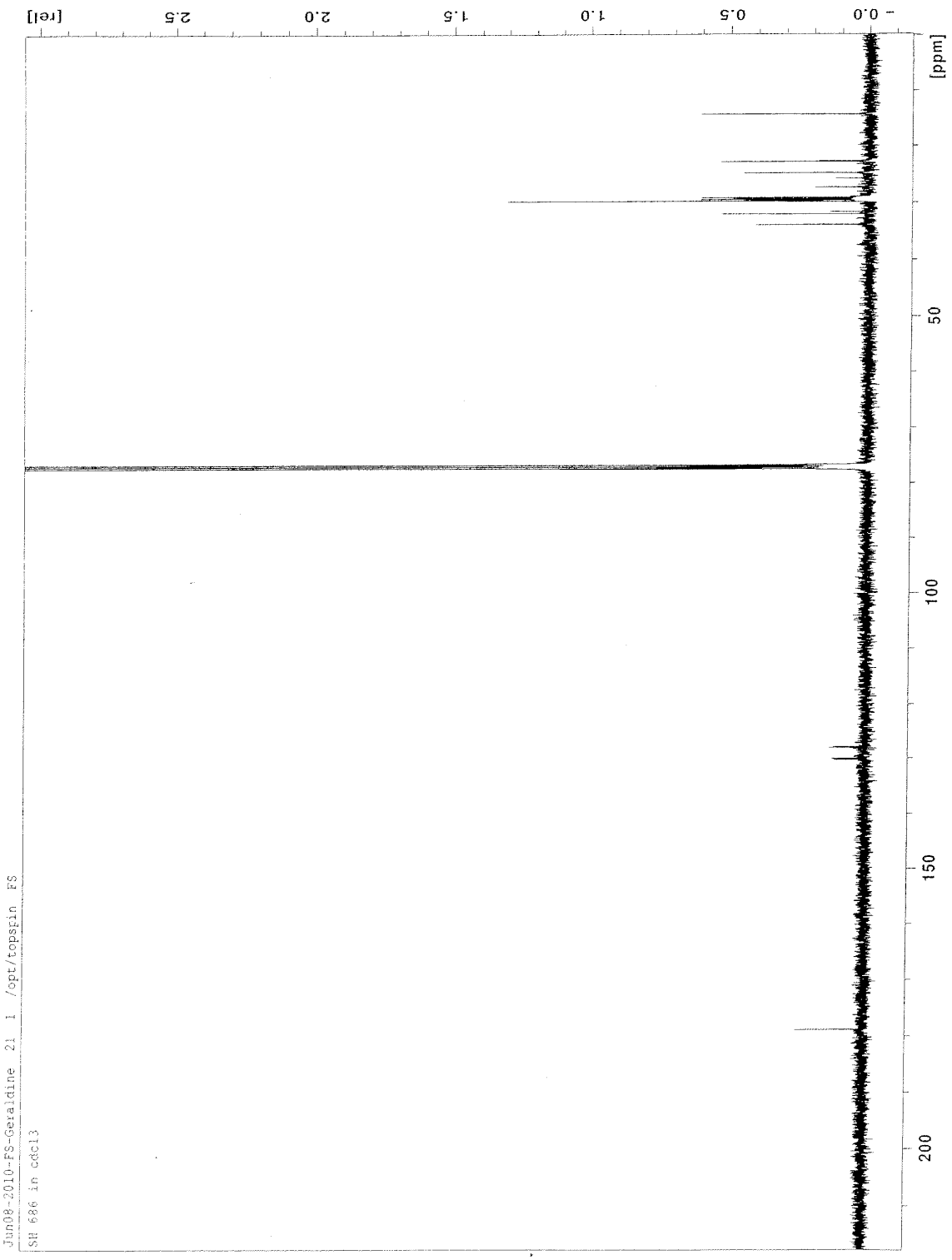

Figure D1.4 ^{13}C-NMR spectrum of compound F6/5

APPENDIX E

E.1 CONTRIBUTION TO KNOWLEDGE

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

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INTRODUCTION

➢Disorders that cause excessive clotting in the body can lead to clot formation in the arteries that could completely block the supply of blood and thus oxygen to a portion of the body. [1,13]

➢Three plants, *Bulbine natalensis, Protorhus longifolia, Rapanae 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. Distributed in Eastern and northern South Africa it is used 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. It is found in the open woodland in Eastern Cape and KZN. It used to treat heart burn, bleeding from stomach, strengthen the heart and hemiphlegic paralysis.

➢*Rapanae melanophloes* is widely distributed in Southern Africa and in the east coast to tropics. It 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.

EXTRACTION

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

PHYTOCHEMICAL SCREENING [2,5,11]

➢Phytochemical screening was carried out on the extracts using standard procedures to identify the bioactive constituents.

ANTIOXIDANT ASSAY [3,4,12,14]

➢The anti-oxidant activity was determined by in vitro methods-DPPH, ABTS, REDUCING POWER and METAL CHELATING assay.

CYTOTOXICITY TEST [6,8]

➢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.

MEASUREMENT OF PLATELET AGGREGATION [8,9,10]

➢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.

RESULTS AND DISCUSSION

TABLE 1

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

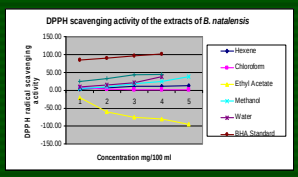
+ ve : PRESENT - ve : ABSENT

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
HEXENE	>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	+	-	-	+	-	-

TABLE 6: Untreated platelets after tannin removal

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

➢Methanol and water extracts of *B natalensis,*

➢Hexane, ethyl acetate and chloroform extracts of *P longifolia*

➢Chloroform and ethyl acetate of *Rapanae melanophloes* exhibited a concentration dependent anti platelet aggregation activity. These extracts also showed lipid per oxidation inhibitory activity. These results apparently support the use of these plants in managing blood clotting related diseases.

References will be provided on request