In Vitro Anti-platelet Aggregation Activity of the Extracts of *Protorhus longifolia*

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By

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DECLARATION

The experimental work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand and at the School of Chemistry, University of KwaZulu-Natal, Westville Campus between April 2009- June 2010, under the supervision of Prof. A.R. Opoku and Prof. A.O. Oyedeji.

This study represents the original work by the author. Where use was made of the work of others, it has been duly acknowledged in the text.

I declare the above statement to be true.

Mosa Rebamang Anthony

Prof. A.R. Opoku

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DEDICATION

This work is dedicated to my mother ‘Mankeletseng Lydia Mosa, who has always been my pillar of support throughout all the trying times.

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ABSTRACT

Platelet aggregation beyond purpose of haemostasis is the underlying cause of blood-clotting related diseases. Concoctions of *P. longifolia* are used by Zulu traditional healers to manage such diseases. This work aimed at investigating the anti-platelet aggregation activity of the extracts of this plant and to identify and characterise the active components present and responsible for the anti-platelet aggregation activity. Phytochemical screening of the plant material revealed the presence of various secondary metabolites (tannins, flavonoids, alkaloids and terpenoids). Crude extracts (obtained by sequential extraction of plant material with hexane, chloroform, ethyl acetate, methanol and water) and two triterpenes (3-oxo-5α-lanosta-8,24-dien-21-oic acid, and 3β-hydroxylanosta-9,24-dien-24-oic acid) isolated and characterized (using various chromatographic and spectrometric techniques-IR, MS, ^{1}H-NMR, and ^{13}C-NMR) from the crude chloroform extract were screened for antioxidant, anti-platelet aggregation, anti-inflammatory activities, and cytotoxicity. The antioxidant activity of the plant components was determined on DPPH and $ABTS^{+}$ radicals. Their reduction potential and chelating activity on Fe^{2+} were also determined. Except the methanol extract (IC_{50} of 0.07 and 0.16 mg/ml), the crude extracts and the isolated compounds showed poor (< 50%) antioxidant activities as they weakly scavenged DPPH and $ABTS^{+}$ radicals, exhibited low reduction potentials and poor Fe^{2+} chelating activities.

The anti-platelet aggregation activity of both the crude extracts and isolated compounds was separately investigated on thrombin, ADP and epinephrine induced rat platelet aggregation. The extracts and the isolated triterpenes exhibited a concentration dependent anti-platelet aggregation activity induced by the three agonists. The highest activity by the hexane extract (IC_{50} of 0.59 mg/ml) was observed on the thrombin-induced platelet aggregation. In addition, the isolated compound also exhibited *in vitro* anticoagulant activity on the whole rats' blood.

The acute anti-inflammatory activity of the isolated triterpene was determined using the carrageenan- induced rat paw oedema model. The compound (500 mg/kg body weight) significantly ($p<0.05$) inhibited the acute inflammation of rat paw. The hexane and chloroform extracts showed weak cytotoxic effects on brine shrimps with LC_{50} 39.6 and 54.7mg/ml respectively. Also the pure compound- 3β-hydroxylanosta-9,24-

dien-24-oic acid exhibited weak cytotoxic effects on HEK293 and HEPG2 cell lines (IC_{50} 8520 and 7960 µg/ml respectively).

These results support the use of *P. longifolia* in folk medicine in the management of blood-clotting related diseases.

LIST OF ABBREVIATIONS USED

AA	Ascorbic acid
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADA	Acid-dextrose-anticoagulant
ADP	Adenosine diphosphate
BHT	Butylated hydroxytoluene
cAMP	Adenosine-3′,5′-cyclic monophosphate
CC	Column chromatography
CCM	Cell culture medium
CE	Chloroform extract
CL	Confidence limit
CMC	Carboxymethyl cellulose
COSY	Correlation spectroscopy
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DEPT	Distortionless enhancement by polarisation transfer
DPPH	1,1′-diphenyl-2-picrylhydrazyl
EDTA	Ethylenediaminetetra-acetic acid
EAE	Ethyl acetate extract
HE	Hexane extract
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear multiple quantum coherence
IR	Infra-red
IC_{50}	Inhibitory concentration with 50%
LC-MS	Liquid chromatography-mass spectroscopy
LC_{50}	Lethal concentration with 50% inhibition
MDA	Malondialdehyde
ME	Methanol extract
MEM	Modified Eagles Medium
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
NSAIDs	Non steroidal anti-inflammatory drugs
PAR	Protease- activated receptor
PAF	Platelet activating factor
PBS	Phosphate buffer saline
PVPP	Polyvinylpolypyrrolidone
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin-layer chromatography
TME	Total methanol extract
UV	Ultraviolet light
VLC	Vacuum liquid chromatography
WE	Water extract
WHO	World Health Organisation

CONTRIBUTION TO KNOWLEDGE

(See Appendix F for details)

a. 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, S.A.

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TABLE OF CONTENTS

CHAPTER 3

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1.0 INTRODUCTION

Blood is a vital tissue in the body. Among its various physiological functions blood is important in the delivery of oxygen and nutrients to all parts of the body. A smooth flow of blood in the vessels is of utmost importance, but equally important is the process of blood-clotting. It is a normal and necessary process that helps prevent loss of life even from minor injuries by forming clumps or clots in an attempt to stop bleeding from a damaged or injured vessel. It is a complex process that needs to be carefully controlled. It involves platelets, enzymes and clotting factors in a cascade of reactions that lead to the catalytic conversion of a soluble fibrinogen by thrombin to insoluble fibrin polymers. The fibrin polymers form a meshwork around platelet to form a fibrin clot. Blood-clotting forms an important part of haemostasis.

Platelet hyperactivity and consequent hyperaggregation is, however, the cause of internal clots. Internal formation of clots, if not checked, can be fatal and are indeed the main cause of atherothrombotic diseases such as strokes, heart attack, and pulmonary embolism. Thrombin, ADP, epinephrine, arachidonic acid, collagen and other risk factors such as free radicals, inflammation, stress and hypercholesterolemia significantly contribute to platelet dysfunctions.

Western medical practitioners prescribe blood thinning agents to manage the atherothrombotic diseases. However, despite great progress made so far in finding effective treatment for the atherothrombotic diseases, strokes and other cardiovascular diseases are still causes for concern. The clinical limitations and adverse side effects associated with the currently used anti-platelet agents have fuelled the search for new**,** safer and effective anti-platelet aggregation agents of natural origin. Zulu Traditional healers use different concoctions prepared from medicinal plants to manage blood-clotting related diseases. Screening of such plants, as used by the traditional healers, will give scientific validation to this traditional practice. This could also give a lead to the discovery of new pharmacologically active drugs.

Ethnobotanical survey revealed that among the various medicinal plants used by the Zulu traditional healers to manage blood-clotting related diseases, *Protorhus longifolia* stood out as the most commonly used. The medicinal activity of this plant has not been scientifically validated and thus the aim of this work was to investigate the anti-platelet aggregation activity of the extracts of this plant and to partially identify and characterise the active components present and responsible for the anti-platelet aggregation activity.

CHAPTER 2

2.0 LITERATURE REVIEW

Blood is a mixture of cells (red blood cells, white blood cells, and platelets) and plasma. A constant smooth flow of blood in the vessels is vital because it supports the life of all other tissues and organs. Blood delivers oxygen and nutrients to different parts of the body and it also removes metabolic waste products from the tissues.

2.1 Blood-clotting

Though the smooth flow of blood in the blood vessels is important, equally important is the process of blood-clotting. Under physiological conditions blood-clotting becomes an important part of haemostasis—a condition in which the processes of clot formation, anticoagulation and clot dissolution are all in balance (Devlin, 2006). It is a complex process where liquid blood thickens and forms a clot in an attempt to stop excessive bleeding after a damage or injury to blood vessel. It is a normal and necessary process that needs to be carefully controlled. Inappropriate blood clotting is responsible for a large number of deaths (Elliot and Elliot, 2005). Under physiological conditions a clot forms in response to damage to the vessel and dissolves away after the damage has been controlled. However, under pathophysiological conditions the clot becomes permanently intact (thrombus), enlarges and hinders the blood flow. Sometimes it breaks off (embolus), travels in the blood stream and lodges in a different part of the body, resulting in further complications.

The process of blood-clotting (coagulation) involves platelets, enzymes and clotting factors. Platelets are regarded as key regulators of both haemostasis and pathogenesis of cardiovascular diseases (Bakdash and William, 2008; Xiang *et al*. 2008; Morrell *et al*., 2008; Lee *et al*., 2009; Fabre and Gurney, 2010). Therefore, as important as platelet activation and aggregation is in maintaining haemostasis, it is equally important to prevent irregular activation of platelet that could lead to cardiovascular events.

2.2 Mechanism of blood-clotting (coagulation cascade)

Blood-clotting is a very complex process that involves intertwined activation of both platelet and coagulation cascades. The coagulation cascade is a stepwise process that involves enzymes and coagulation factors. The coagulation cascade consists of intrinsic pathway which is initiated when blood contacts a foreign surface, and extrinsic pathway which is initiated upon vascular injury (Figure 2.1). The two pathways converge at the final common pathway which involves the activation of factor X to Xa. The factor Xa is responsible for conversion of prothrombin to thrombin (proteolytic enzyme) that converts a soluble fibrinogen into insoluble fibrin polymers. The fibrin polymers create a meshwork that cements platelets and other blood components together to form a clot (Porth, 2005). The thrombin can also activate factors V, VIII, XI and XIII (Strukova, 2001). Factor XIIIa cross-links the fibrin polymers thus stabilising the clot. Ca^{2+} plays an important role in the activation of many proteins involved in the coagulation cascade. It participates in the activaion 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.

Platelet activation cascade is very important in this process because activation of both the factor X and prothrombin to generate thrombin that eventually converts the fibrinogen into fibrin polymers occurs on the surface of activated platelets.

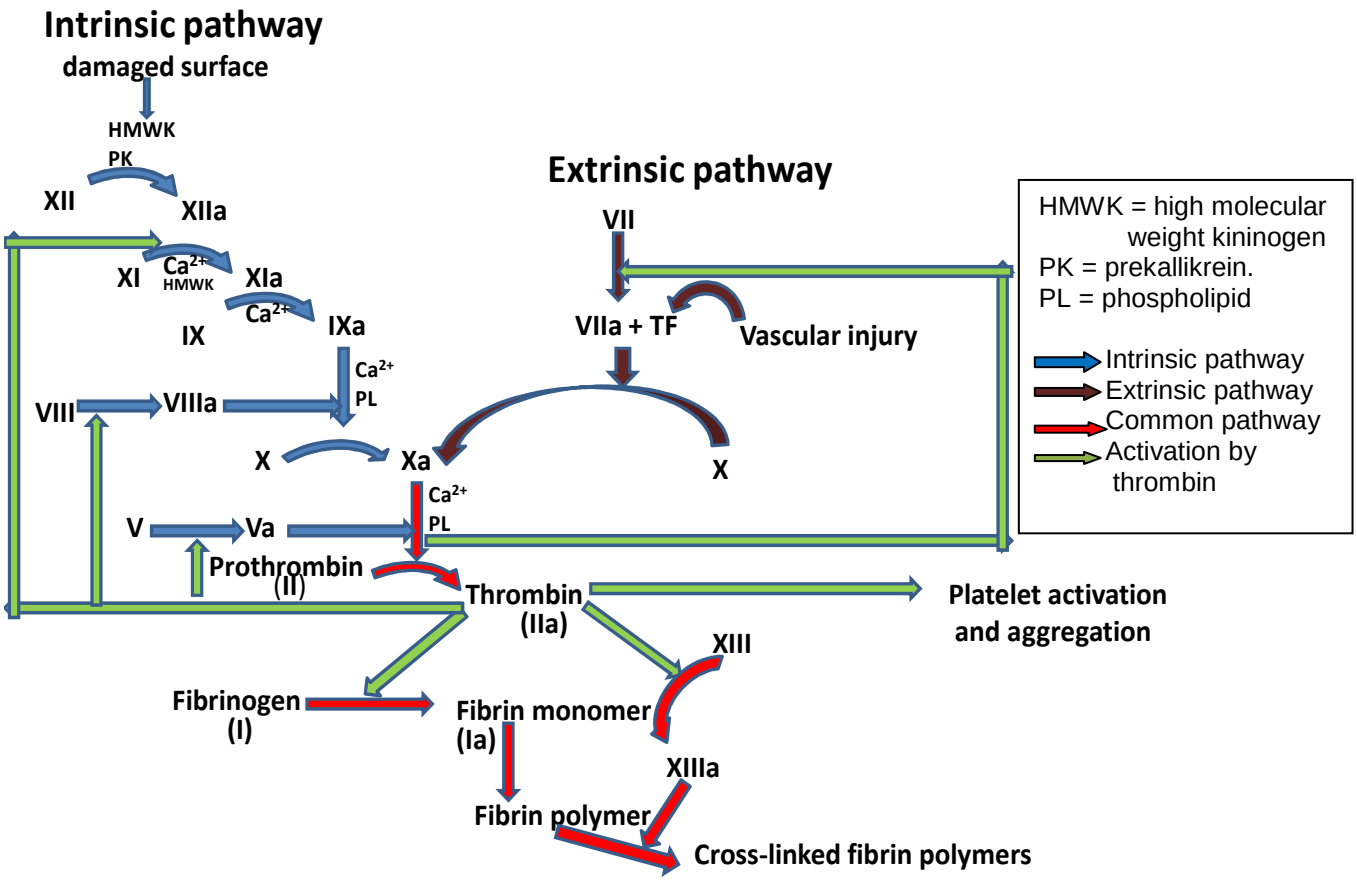


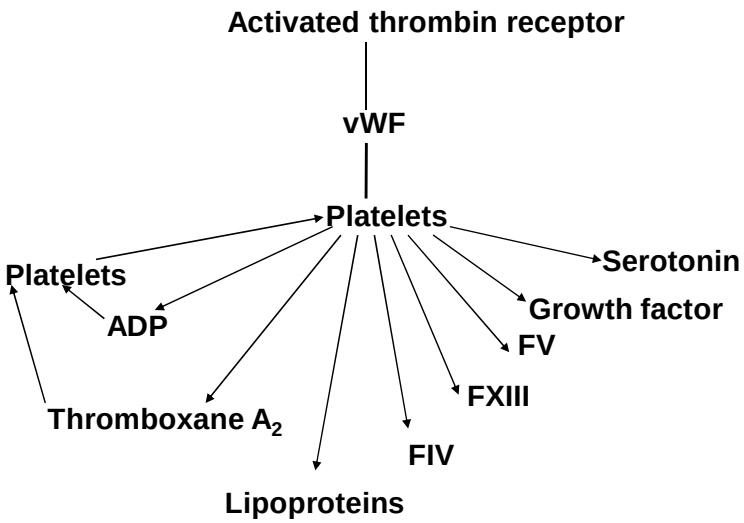
Figure 2.1: Coagulation cascade showing activation of the intrinsic and extrinsic pathways which converge at the final commomn pathway involving activation of factor X to Xa and generation of thrombin. Activated factors are designated with a. (Source: King, 1996)

2.3 Platelet activation and aggregation

Platelets (thrombocytes) are small irregularly-shaped anuclear cells. Resting platelets circulate in blood in discoid shape which is changed upon activation. Upon blood vessel damage or injury platelets rush to and form aggregate at the site of injury to stop bleeding and this is facilitated by their binding to the exposed activated thrombin receptor.

Platelet activation is complex, normally involves multiple signalling pathways and molecules which are responsible for the different biochemical interactions (Xiang *et al*., 2008). Platelet activation and aggregation is induced by binding of agonists on the various receptors found on the platelet surface. The agonists include thrombin, epinephrine, thromboxane A_2, collagen, platelet-activating factor and ADP all of which have specific receptors to bind to. Platelet activation results in morphological

change, release of granules including ADP, thromboxane A_2, coagulation factors and inflammatory mediators (Figure 2.2). There is also activation and exposure of glycoprotein IIb/IIIa (fibrinogen receptor) (Storey, 2006; Devlin, 2006). The release of ADP and thromboxane A_2 stimulates recruitment of even more platelets thus amplifying the aggregation of platelets. The binding of fibrinogen facilitates cross-linking of platelets resulting in platelet-rich thrombus formation. Mature platelet-rich thrombus is stabilised by fibrin polymers from the proteolytic activity of thrombin on the fibrinogen.



Source: Devlin, T.M. (2006). Text book of Biochemistry and Clinical Correlations

Figure 2.2: Events that occur upon platelet activation followed by platelet aggregation. Platelets undergo a morphological change and release granules (ADP, thromboxane A_2), some phospholipids and proteins that aid in coagulation (FIV, FV, FXIII).

2.3.1 Platelet agonists

The various platelet 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. Platelet agonists include thrombin, adenosine-5′-diphosphate (ADP) and epinephrine.

2.3.1.1 Thrombin

Thrombin is important in both haemostasis and thrombosis. Its effects are mediated through membrane-bound G-protein coupled receptors. Literature (Soslau *et al*., 2001) has proposed two distinct pathways through which thrombin activate platelets. 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 hydrolyze PAR-1, it is GP Ib dependent and utilises fibrin polymers instead of fibrinogen. Recent reports have shown that thrombin acts through PAR-1 and PAR-4 and these receptors work cooperatively in the activation of platelets (Fabre and Gurney, 2010). Platelet activation by thrombin can be characterised by activation of membrane receptors, shape change, granular secretion, cytoskeletal remodelling, and aggregation (Jardin *et al.* (2007).

2.3.1.2 ADP

The ADP-induced platelet activation is autocatalytic in that upon activation by ADP platelets release other ADP molecules that act on near platelets, amplifying the reaction. ADP acts through G-protein coupled receptors $P2Y_1$ and $P2Y_{12}$ (Davi and Patrono, 2007). 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). In addition to platelet activation and consequent thrombus formation, $P2Y_{12}$ receptor has also been reported to be very important in inflammatory responses (Evans *et al*., 2009).

The activity of ADP-induced platelet activation requires the availability of Ca^{2+} and it 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 $P2Y_1$ to mobilise intracellular Ca^{2+} and through $P2Y_{12}$ and the G_q pathway to inhibit adenyl 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).

2.3.1.3 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+} mobilisation, fibrinogen binding, or protein phosphorylation, and granular release.

In addition to the known physiological platelet agonists, there are various risk factors associated with platelet hyperactivity and the consequent inappropriate blood-clotting. These include hyperhomocysteinemia, high blood pressure, hypercholesterolemia, diabetes, smoking, stress, sedentary lifestyle and age. Inflammation and exposure to high amounts of free radicals also have a significant contribution (Chang *et al.,* 2010).

2.3.1.4 Free radicals

Free radicals are implicated in various pathophysiological conditions such as neurodegenerative diseases, autoimmune diseases, arthritis, inflammation, cardiovascular diseases and even aging (Atawodi, 2005). Apart from the exogenous reactive oxygen species (ROS), production of ROS by activated platelets (Krotz *et al*., 2004; Guzik *et al*., 2006) has brought conflicting ideas about their role in platelet function. While reactive nitrogen species (RNS) inhibit platelet activation and aggregation, it has been reported that exposure to ROS stimulates platelet aggregation by affecting several key steps of platelet functions (Ambrosio *et al*., 1997; Bakdash and Williams, 2008). Ikeda (1994) demonstrated that a reaction between xanthine and hydrogen peroxide enhanced platelet aggregation.

A free radical is any atom or a molecule with unpaired electron in its outermost shell as a result of addition or subtraction of electrons. This makes free radicals to be highly unstable and reactive. Free radical stabilises itself by abstracting an electron or proton from neighbouring molecule, thus creating another free radical. This process sets off a chain reaction whereby a molecule abstract electron or proton from the nearby molecule(s). Free radicals can be classified as reactive oxygen

species (ROS) (e.g. $^{\cdot}O_2^-$, $OH^{\cdot}$) and reactive nitrogen species (RNS) (e.g. $NO^{\cdot}$, $^{\cdot}OONO^-$). Superoxide (O_2^-) can give rise to a non-radical but potent oxidant hydrogen peroxide (H_2O_2), and the most powerful hydroxyl radical ($OH^{\cdot}$) (Ambrosio *et al*., 1997; Hsu *et al*., 2007).

Free radicals are by-products of the normal ongoing biochemical reactions in the body such as mitochondrial respiration and liver mixed function oxidases, through xanthine oxidase activity. Atmospheric pollutants, drugs and xenobiotics contribute to the production of free radicals (Saha *et al*., 2008). Free radicals have damaging effects to cell membranes, proteins and DNA through oxidation. Free radicals' attack; stimulate cellular autoxidation. This is well demonstrated in lipid peroxidation, a process in which an initial attack of polyunsaturated fatty acid by a free radical leads to self-propagatory events resulting in cellular damage (Moore and Roberts, 1998). Malondialdehyde (MDA), a major product of lipid peroxidation is usually used to measure or determine the lipid peroxidation *in vitro* (Nielsen *et al*., 1997, Del Rio *et al*., 2005, Grucka-Mamczar *et al.*, 2009).

Human body is equipped with various defence systems that deal with the destructive effects of the free radicals; these consist of enzymes and antioxidants. These defence systems prevent, slow or minimise the damage caused by the free radicals. A good antioxidant does not only inhibit production of free radicals, but also scavenges and reduce the existing free radicals. Antioxidants usually work by donating the much needed electron or proton to the free radical. Once stabilised or neutralised, the free radical loses its oxidant potential and become harmless to other chemical and/or biological structures.

Antioxidants comprise some vitamins (A, C, E), minerals, coenzymes and enzymes (superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidases). The common synthetic antioxidants include butylated hydroxytoluene (BHT), tertbutylhydroquinone (TBHQ) and butylated hyroxyanisole (BHA). A recent health concern about the use of these synthetic compounds has led to an increased interest in natural antioxidants (Akular and Odhav, 2008). Plant secondary metabolites are the major sources of the natural antioxidants. Among the various plant secondary metabolites, polyphenolic compounds are the most powerful natural

antioxidants. They are singlet oxygen quenchers, free radical scavengers, metal ion chelators and reducing agents (Hsu *et al*., 2007; Akular and Odhav, 2008).

The antioxidant activity of various plants traditionally used to cure different ailments has been well documented (Opoku *et al*., 2002, Adedapo *et al*., 2008). The involvement of antioxidants in the inhibition of platelet activation and aggregation (Krotz *et al*., 2004; Sobotkvá *et al*., 2009) can be important in managing thrombosis.

2.3.1.5 Inflammation

Inflammation can be described as the body's response to protect itself against harmful or injurious stimuli such as pathogens, toxic chemicals, irritants, and allergens. This response results in accumulation of plasmatic fluid and blood cells at site of injury (Lu *et al*., 2009). The main purpose of the whole process of inflammation is to remove the injurious stimuli and initiate the healing process (Verhamme and Hoylaerts, 2009).

Inflammation can be acute or chronic. The difference generally lies on the cells involved and time taken to heal. Acute inflammation is a rapid short-term process that takes only a few hours and it is mediated by granulocytes. Acute inflammation is basically characterised by redness and heat (due to increased blood flow to the inflamed site), swelling (caused by accumulation of fluid), pain (due to release of chemicals that stimulate nerve endings) and loss of function of the inflamed part (Underwood, 2000; Rote, 1994). Unlike acute inflammation, which self-limiting, chronic inflammation is self-perpetuating, prolonged (May last for weeks, months or years) and usually is caused by persistant irritants, most of which are insoluble and resistant to phagocytosis and other inflammatory mechanisms. Also chronic inflammation is mediated by monocytes and lymphocytes rather than granulocytes (Porth, 2005).

Inflammation is mediated by release of chemicals from tissues and migrating cells. These chemicals include histamine, bradykinin, interleukin-1, platelet-activating factor (PAF), arachidonic acid metabolites- prostaglandins (PGs), leukotrienes (LTs) and hydroxyperoxy-eicosatetraenoic acids (HPTETE's) (Lu *et al.*, 2009; Akular and

Odhav, 2008; Devlin, 2006). ROS have recently been implicated in inflammation as they stimulate production of inflammatory mediators from macrophages (Chang *et al.*, 2010).

There is an established linkage between inflammation and blood coagulation. Platelets play an important role in linking the two processes. Arachidonic acid and PAF mediate both inflammation and platelet aggregation and also activated platelet release cytokines and pro-inflammatory mediators (Blair and Flaumenhaft, 2009). This link is observed under both physiological and pathophysiological conditions. During endothelial injury, the two processes become autocatalytic in that inflammation triggers the activation of the coagulation system and also coagulation triggers inflammatory reactions (Strukova, 2001). Also during tissue injury and inflammation there is an increased production of thromboxane A_2, a potent platelet activator. To emphasise the relationship between these processes, Verhamme and Hoylaerts (2009) indicated that inflammation suppresses the natural anticoagulant system, which favours the expression and synthesis of prothrombogenic molecules.

Currently available anti-inflammatory drugs include steroids such as dexamethasone, prednisone and betamethasone, and non-steroidal anti-inflammatory drugs (NSAIDs) – aspirin, ibuprofen, acetominaphen and indomethacin. Aspirin is also a well known anti-platelet aggregation agent. Both classes of drugs have different mechanisms through which they exert their therapeutic activities. Generally the steroidal anti-inflammatory drugs prevent arachidonic acid synthesis by inhibiting phosholipase A_2. The NSAIDs act by inhibiting cyclooxygenase (COX) thereby preventing arachidonic metabolism. These drugs inhibit the production of prostaglandins which are mediators of inflammation (Devlin, 2006).

The prolonged use of anti-inflammatory drugs is also associated with some adverse side effects. The non-selective inhibition of COX by aspirin and other NSAIDs is associated with gastric ulcers and bleeding risks (Fendrick *et al*., 2008). There is a need for search of effective and safe anti-inflammatory drugs.

2.3.2 Antiplatelet therapy, limitations and future prospects

The whole process of blood-clotting and clot dissolution is carefully controlled by physiological anticoagulant and fibrinolytic systems. While clotting could be beneficial in stopping bleeding, unchecked internal formation of blood clots could be fatal. Platelet aggregation (blood clot formation), beyond the purpose of haemostasis is the mechanism underlying atherothrombotic diseases (Gadi *et al*., 2009).

Circulatory events such as myocardial infarctions and strokes account for high rate of mortality and morbidity worldwide, particularly in developed countries (Hsieh *et al*. 2007; Morrell *et al*., 2008; Amrani *et al*., 2009; Fabre and Gurney, 2010). Their prevalence is becoming important in developing countries as a result of changing life styles, urbanisation and industrialisation (Yusuf and Ôunpuu, 2001). Morrell *et al*. (2008) reported thrombosis as the underlying cause of the cardiovascular diseases and stroke.

The need to maintain balance between haemostastis and thrombosis has led to the development of various anti-platelet aggregation therapies. Various anti-platelet aggregation drugs are currently available to manage and help protect against the devastating cardiovascular diseases and other known risk factors. In addition to already clinically approved anti-platelet aggregation agents such as aspirin, ticlopidine, clopidogrel, prasugrel and also the anticoagulants, heparins and bivalirudin, there is a large number of new agents undergoing clinical trials (Xiang *et al*., 2008).

Among others, the single-target and/or irreversible inhibition character of the currently available anti-platelet aggregation agents seem to be a major limitation to their efficacy and safety. For example, aspirin and $Y2Y_{12}$ antagonists (clopidogrel, ticlopidine, prasugrel) only inhibit platelet activation induced by thromboxane A_2 and ADP respectively. Due to their irreversible actions they are associated with the risks of excessive bleeding (Hsieh *et al.*, 2007; Fabre and Gurney, 2010). In addition to their efficacy and safety, the currently available anti-platelet agents are still associated with problems such as drug-drug interaction, anti-platelet resistance and affordability (Xiang *et al*., 2008).

Despite the great progress made so far to find better and effective anti-platelet agents, blood-clotting related diseases are still a cause for concern. It is apparent that anti-platelet agents with mild, reversible inhibition and multiple targets may be ideal. The ideal therapeutic agent will be one with multiple beneficial effects such as anti-inflammatory and antithrombotic or anti-platelet aggregation activity. These ideal drugs may come from plants' bioactive compounds. The anti-inflammatory activity of some secondary plant metabolites such as terpenoids and polyphenols have been reported (Cuéllar *et al.*, 1998; Yang *et al.*, 2010). Plants bioactive compounds may become handy in providing compounds with multiple targets. A single bioactive compound**,** for example flavonoid**,** may have various biological activities such as anti-inflammatory, antioxidant and anticoagulant activities (Engler and Engler, 2004). Also the synergistic actions of medicinal plants constituents may help improve their efficacy.

2.4 Traditional healing/medicine

Traditional medicine (TM) is one of the indigenous knowledge systems. Trivedi (2007) defines traditional healing as the sum total of the knowledge and practices based on theories, beliefs and experiences indigenous to different cultures and used in the maintenance of health, as well as in the prevention, diagnosis and treatment of physical and mental illness. According to WHO (2008) TM is referred to as the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being. It is estimated that about 80% of the population of developing countries meet their primary health care needs mainly through the plant-based traditional healing (Gumede, 1989; Schemincke, 1997; WHO, 2008).

Traditional healing is very diverse and it approaches health in a holistic manner (Chan, 2005; Rajadurai *et al*., 2009). Different people may receive different treatments even if, according to modern medicine, they suffer from the same disease. Traditional medicine is based on a belief that each individual has his or her own constitution and social circumstances, which result in different reactions to causes of disease and treatment. According to Verpoorte *et al*. (2006) the recent

introduction of pharmaceutical disciplines such as pharmacogenomics and pharmacogenetics has shifted modern health approach from general into a more personalised medication. In response to the health disparities particularly in developing countries integration of the traditional medicine into the modern health system has become a burning issue. Bandaranayake (2006) has emphasised the need for a holistic approach to health care and utilisation of the untapped potential of traditional medicines.

Traditional medicine based on plants has been used for so many years (Abu-Rabia, 2005; Amrani *et al*., 2009). Most often the traditional healer's medications, often a mixture of different ingredients, are administered as hot or cold infusions, powders which are rubbed into the body where incisions have been made, lotions, ointments, emetics and enemas. If used properly there are no known side effects associated with traditional medicines. Different parts of the medicinal plant, rarely whole plant, are mostly used in preparation of traditional medicines (Van Wyk *et al*., 1997).

There are still mixed ideas and perceptions about the safety and efficacy of traditional medicines compared to the western medicine. Even though it is perceived as efficient, safe, cost effective, and easily accessible to the poor and those living in remote areas (Trivedi, 2007; Yineger and Yewhalaw, 2007), according to Ahmad *et al*. (2006) plant-based traditional medicines are neither completely safe nor poisonous. It all depends on the correct quantities and mode of administration. Different agencies, international, national and governmental are trying their best to ensure that safe, effective and affordable treatments are available to the vulnerable population (Trivedi, 2007).

Despite criticisms for so many years traditional healing has recently gained a tremendous revival. Not only do traditional healers provide immediate health care to rural population, they also play a very important role in providing leads to the discovery of pharmacologically active plant-derived compounds. Without traditional healers' contribution it would be very difficult and tedious to select plants with the desired biological activity (Rajadurai *et al*. 2009; Van Wyk *et al*., 2009).

2. 5 Medicinal Plants

Medicinal plants are those that have medicinal properties to cure or treat different ailments. A recent high demand in both developed and developing countries on natural products and their derivatives has resulted in an upsurge interest in medicinal plants as an alternative medicine (Hanson, 2005). Medicinal plants are rich sources of biologically active compounds (Palombo, 2006) often referred to as secondary metabolites. These compounds are commonly found at varying concentrations in one or more different parts of a plant. They are either individually or synergistically responsible for the various therapeutic properties of medicinal plants (Purohit and Vyas, 2004).

Despite the popular use of medicinal plants, their benefits are still controversial since there is no proper documentation of their toxicicology and pharmacological activities (Burta *et al*., 2008). The historical use of medicinal plants for various diseases and the emergence of the so-called incurable diseases upon the use of the modern medicine have led to the recognition of what nature can offer. These have led to a global interest in the study of medicinal plants and their traditional uses (Rajadurai *et al*., 2009). Researchers (Lin *et al*., 1999, 2002; Opoku *et al*., 2000, 2002, 2007; Musabayane *et al*., 2005; Simelane *et al*., 2010) are increasingly engaging in scientific validation of the therapeutic potential of medicinal plants (Kumar *et al*., 2008).

The continuous threat posed by blood-clotting related diseases has stimulated researchers to turn their attention into evaluating the anti-platelet aggregation properties of various medicinal plants (Amrani *et al*., 2009). Kee *et al*. (2008) screened the anticoagulant or antithrombotic activity of some South African medicinal plants. The anti-platelet aggregant effects of various plants, *Ocimum basilicum* (Amrani *et al*., 2009), lignoids from *Calamus quiquesetinervius* (Chang *et al*., 2010), *Arbutus unedo* (El Haouari *et al*., 2007), *Phellinus gilvus* (Chang *et al*., 2008), *Nepeta juncea* (Hussain *et al.*, 2009) and some Moroccan medicinal plants (Mekhfi *et al.,* 2004) have been reported. The platelet aggregation inhibitory effects of compounds from *Zingiberaceae* species (Jantan *et al*., 2008) and acidamides from the fruits of *Piper longum L.* (Park *et al*., 2007) have also been determined.

Interestingly, the anti-platelet aggregation activity of some of the plants or herbs that are usually used as food has also been reported. These include *Petroselinum crispum* (parsely) (Gadi *et al*., 2009), *Allium cepa* (onion) (Moon *et al*., 2000, *Allium sativum* (garlic) (Rahman and Billington, 2000) and *Lycopersicum esculentum* (tomato) (Dutta-Roy *et al*., 2001).

An ethnobotanical survey (see Appendix C) indicated that Zulu traditional healers use various plants to manage blood-clotting related diseases. The survey revealed that *Protorhus longifolia (unhlangothi)* (Figure 2.3) is one of the commonly used plants and was highly recommended by Zulu traditional healers.

2. 5.1 *Protorhus longifolia*

Figure 2.3: A picture of *Protorhus longifolia*

Protorhus longifolia (Benrh.) Engl. (Anacardiaceae) also known as red beech (English), *unhlangothi* (Zulu) and *Uzintlwa* (Xhosa) is an ever green, indigenous tall tree (up to 15 m). It usually has some yellow or red coloured leaves. It is very resistant to drought and is also not suitable for cultivation. *P. longifolia* is the only species in the genus *Protorhus* that is found in Southern Africa while about 20 of the other species are found in Madagascar (Archer, 2000). In South Africa it is predominantly found in KwaZulu Natal, Eastern Cape, Northern Province and Drakensburg escarpment in Mpumalanga. It grows in the forest, on river banks, and woodland. Pericarp structure, pachychalazal seed of *P. longifolia* and its taxonomic significance have been documented (Vonteichman, 1991a, 1991b). The tree has also been documented for its commercial value as its bark is sold in most *muthi* markets in South Africa (Keirugni and Fabricius, 2005; Dold and Cocks, 2002). *P. longifolia* is ranked number 4 in the 17 most important medicinal plants in Ngabara

area and number 7 in the 60 most frequently traded plants in the Eastern Cape Province (Keirugni and Fabricius, 2005; Dold and Cocks, 2002).

The bark of *P. longifolia* has been traditionally used to cure various diseases such as heartwater and diarrhoea in cows (Dold and Cocks, 2001); hemiplegic paralysis, heart burn, bleeding from the stomach, and unspecified parts have been used to strengthen the heart (Gerstner, 1941, 1939; Pujol, 1990). A decoction from the dried powdered stem-bark is mixed with some other ingredients to cure strokes and clean blood. Other medicinal uses of the plant include treatment of victims of lightning shock, cramps and swollen legs. The leaves extracts of *P. longifolia* have been reported to possess antimicrobial activity (Suleiman *et al*., 2009; 2010). A 10.2 – 18% tanning material and 7% tannin from the bark of *P. longifolia* has been reported (Hutchings *et al*., 1996).

Though *P. longifolia* is valued for its traditional medicinal purposes, very little scientific work has been done to evaluate its biological activities. Little has been done to evaluate therapeutic properties of *P. longifolia* and its anticoagulant properties have not been reported. Therefore, this project aimed at investigating the anti-platelet aggregation activity of the bark-extracts of *P. longifolia* and to partially identify the active components present and responsible for the anti-platelet aggregation activity.

Scientific evaluation of biological activities of a plant involves a series of steps starting with phytochemical screening of the plant material. This is often the first step in preliminary bioassay screenings followed by extraction of the plant with appropriate solvent depending on the intended bioassays. Also to obtain a biologically active compound, various separation and purification techniques are employed, followed by characterisation and identification of the active compound.

2.6 Review of Methods

2.6.1 Phytochemical screening

A phytochemical is a naturally occurring bioactive compound present in plant. There are more than a thousand known phytochemicals (secondary metabolites). These compounds have no nutritive value in plants. Their major role is in defence mechanism against herbivores, microorganisms, insects (Policegoudra *et al*., 2010). Human has found use of these chemicals in food flavouring, medicine or recreational drugs (Schultz, 2002). The common phytochemicals include alkaloids, tannins, terpenoids, flavonoids, and cardiac glycosides. The quality and quantity of these chemicals can largely be affected by time of collection and harvesting (George *et al*., 2001).

Research has revealed a diverse biological activity of polyphenolics most importantly as antioxidants (Panovska *et al*., 2005; Adedapo *et al*., 2008). Medicinal activities of flavonoids, a polyphenolic compound has gained more attention (Zabri *et al*., 2008). Therefore, analysis of the phytochemical constituents of plants more especially the plant understudy is very important. Mojab *et al*. (2003) pointed out that the phytochemical analysis is not only important for drug discovery, but also in discovering the actual value of traditional medicines. 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. Most of these methods are not specific and they only act as a guide**,** and false positive results are likely to occur. The presence or absence of a phytochemical in the plant material is generally tested by addition of appropriate chemical reagent to the plant material. Ferric chloride test is generally used for phenol tests. For example, it is employed to test for tannins as well as flavonoids. Alkaloids give a precipitate with heavy metal iodides. They give coloured precipitates in Mayer’s reagent and in Dragendroff’s reagent. The presence of saponins is tested by frothing (Trease and Evans, 1983).

2.6.2 Extraction

Different methods can be employed when drying the plant material, but most preferably is the use of air. The method and temperature employed to dry the plant material can have a great effect on the quality of the material (Mendonça-Filho, 2006). Also to avoid loss of some sensitive components (heat-labile or oxidisable), George *et al*. (2001) recommend a direct extraction of whole plant material not the powdered material.

Successful extraction begins with a good choice of a suitable solvent which depends on the intended uses of the extract. Though traditional healers extract with water, researchers use various solvents, mostly organic solvents with different polarities. Fresh or preferably dried plant materials can be used in the extraction. Extraction of fresh plant materials is associated with problems of water and chlorophyll interference in cases of liquid-liquid extraction (George *et al.*, 2001). Depending on the desired extract sequential or separate solvent extraction can be employed. The sequential extraction enables extraction of plant components with the same polarity in individual extracts. The separate extraction enables extraction of non-polar and/or polar plant components. While the sequential extraction may reduce the synergistic effects of the plant components, it makes it easy to isolate and purify active component(s) and the opposite is true for the separate extraction.

2.6.3 Isolation, purification and structural elucidation

Isolation and characterisation of bioactive compounds from crude plant extracts is a tedious and time consuming process. Also a relatively low yield of the bioactive compounds is a major concern 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).

2.6.3.1 Isolation and purification

The use of various separating techniques such as column chromatography (CC), vacuum liquid chromatography (VLC), high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) can be used to obtain pure

compound(s). Chromatography is probably the most widely used technique for isolation and purification of the chemical composition of samples. The separation of components in chromatography is based on the differential distribution of the components between a stationary phase and a mobile phase.

a) Column chromatography (CC)

Column chromatography is a method used to separate and purify individual components from mixtures of compounds. With the increasing interests in natural products, this technique is usually used for preparative applications for sample purifications. Columns (usually glass with a tap at the bottom) of different sizes (i.e. 2 cm x 50 cm, 30 cm x 100 cm) are employed depending on the amount of sample. Column chromatography can be categorised into gravity and flash chromatography. The difference is generally on the flow of the mobile phase. In the gravity column chromatography, the solvent is allowed to move down the column by percolation while in the flash chromatography air pressure is applied to push the solvent down the column.

Open column chromatography can be prepared using dry packing or wet packing. For the dry packing, the column is first filled with dry adsorbent powder (stationary phase), followed by addition of the solvent (mobile phase). For the wet packing, the slurry is first prepared by thoroughly mixing the adsorbent powder with the solvent to be used for elution. Silica gel or alumina is usually is usually used as solid. Choice of appropriate particle size of the adsorbent is important since the size greatly influences the flow of the solvent through the column. Mobile phase is a solvent of choice depending on the nature of the sample to be purified. Organic solvent of varying polarities are commonly used. A proper choice of the solvent or solvent system is also very important for a good separation. A failure to do so can result into no or very poor separation. A series of increasingly polar solvent system is usually employed to elute the column. Eluents are collected as fractions which are then analysed by TLC for their chemical composition.

b) Vacuum liquid chromatography (VLC)

VLC is another form of column chromatography in which the flow is activated by vacuum. This should not be confused with flash chromatography. VLC involves a step by step gradient elution with a suction (Coll and Bowden, 1986) being used to pull down the mobile phase. Also the column is allowed to dry up after each fraction is collected.

c) High performance liquid chromatography (HPLC)

HPLC is the most advanced of all the chromatographic techniques. Its introduction seems to have solved many problems which could not be addressed by the other separating techniques. It is now regarded as one of the most powerful tool in analytical chemistry with it diverse abilities (Marston, 2007). Besides, its ability as a separating technique, it can also identify and quantify the chemical composition of any liquid soluble sample.

d) Thin-layer column chromatography (TLC)

TLC is a common chromatographic technique used in the qualitative chemical analysis of a sample. Only a small amount (micrograms) of the sample is needed for this technique. Despite its lack of automation and reproducibility, TLC is fast, simple and cost effective. Multiple samples can be run at the same time on the TLC and it is so far considered the only technique in which all components of the sample are included in the chromatogram. It has also found use in biological activities (Marston, 2007).

Besides its use in analysing the eluents of column chromatography, TLC is also used to determine the proper solvent system for separation of compounds in the column chromatography. The appropriate solvent system is one that moves all components of the mixture off the baseline, but not put anything on the solvent front. Usually fluorescent silica gel pre-coated aluminium sheets are used as adsorbent (stationary phase). The spotted plate is placed in a chromatogram chamber with the spotted end down and the solvent is allowed to move up the adsorbent by capillary action up to about 1 cm from the to end of the plate. Spots can first be visualised under UV light

and then be developed by a spray reagent followed by heating to give clear coloured spots fro for visualisation.

2.6.3.2 Structural elucidation and Identification

The structural elucidation of the isolated pure compound can be achieved through the use of the advanced analytical techniques such as nuclear magnetic resonance (NMR), ultraviolet (UV), infra red (IR) and mass spectrometry (MS). Melting point can also be used to add valuable information about the unknown compounds when compared with literature.

The different analytical techniques provide different information about the structure of the compound. The different pieces of information are put together to propose the possible structure. NMR which consists of two types: 1D (^{1}H-NMR, ^{13}C-NMR, ^{13}C-DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HMBC, ^{1}H-^{13}C HMQC) provides information on the number and types of hydrogen and carbon atoms present in the compound and the relationship among these atoms (Van de Ven, 1995). MS tells about molecular mass, molecular formula and fragmentation pattern. The different functional groups present in the unknown molecule are identified by IR spectroscopy. UV gives information about the chromophores present in the compound. Also X-ray crystallography can provide information about the crystal structure of the unknown molecule.

Marston (2007) believes that a combination of these techniques (i.e. LC/UV LC/MS and LC/NMR) in one setup can permit a complete spectroscopic characterisation in a single analysis. This could encourage more work on the isolation and identification of compounds and possible discovery of novel compounds.

2.7 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 a very good biological activity but at 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. On the one hand, the brine shrimp is considered a very

simple and fast method to determine the cytotoxicity of the extracts, but on the other, it is very tedious and subjective. A correlation has been observed between cytotoxicity of plant extracts against brine shrimp and cell line assay (Ripa *et al*., 2009). Therefore, if an extract shows cytotoxic properties against brine shrimp larvae it is further recommended for cell line assay.

Cell lines are viable cultured cells from specific tissues. Cell line based bioassays are easy to perform and they are believed to provide reliable and valid results (Mire-Sluis *et al*., 1995)**.** They are also considered a good replacement of animal based methods (Betrabet *et al*., 2004). The use of cell line bioassays is more and more becoming the most popular and important technique in determining plant extracts bioactivities. The cytotoxicity of the extract is determined by exposing these cell cultures to the extracts after which the viability (growth) of the cells is determined. Cell lines have also been mostly used in research and drug development as models of normal and cancer tissues *(*Nature Reviews Cancer, 2010*).* Despite the vast popular use of the cell lines in research, problem of cross-contamination and authentication is still a challenge (Dunham and Guthmiller, 2008).

2.8 Objective and scope of the work

2.8.1 Objective

The aim of this study was to investigate the anti-platelet aggregation activity of the extracts prepared from the stem bark of *Protorhus longifolia* and to identify the active components present and responsible for the anti-platelet aggregation activity.

2.8.2 Scope of the work

- Collection and identification of the plant.
- Phytochemical screening of the plant material.
- Sequential extraction of the plant material using hexane, chloroform, ethyl acetate, methanol and water.
- Determination of antioxidant activity of the plant extracts.
- Determination of cytotoxicity of the plant extracts.
- Determination of antithrombin activity of the plant extracts using a chromogenix – an artificial substrate of thrombin.
- Investigation of anti-platelet aggregation activity of the plant extracts on thrombin, ADP and epinephrine-induced rat platelets and also on the enzyme (trypsin, bromelain and papain) treated platelets.
- Determination of MDA formation.
- Determination of rate of clot formation induced by thrombin and $CaCl_2$.
- Tannin removal from the extracts and screening of the tannin-free extract for their anti-platelet aggregation activity.
- Isolation and characterisation of active compounds from the extract that showed consistent anti-platelet aggregation activity.
- Investigation of anti-inflammatory activity of the isolated and purified compounds
- Testing the cytotoxicty of the isolated constituent on cell lines.

CHAPTER 3

3.0 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 anti-inflammatory activity. It also includes the brief description of materials and methods that were used to isolate and characterise active compounds. The details of the preparations of reagents and the details of the methodology are given in the Appendix A and B respectively.

3.1 Materials (See Appendix A for details)

3.1.1 Equipment

Rotary evaporator (Heidolph—Laborota 4000), Spectrophotometer (Spekol 1300), Grinding mill (IKA), Platform shaker (Labcon), were supplied by Polychem supplies.
Eppendorf centrifuge 5804 R, Micropipettes (Eppendorf AG)—Merck.
Biotek ELx 808 UI plate reader (Biotek Instrument supplies).
96-well microtitre plates—Sigma.
TLC plates (silica gel 60 TLC aluminium sheets 20cm x 20 cm, F_{254}) (Merck).
Digital Plethysmometer LE 7500 (Panlab, Spain).

3.1.2 Chemicals and reagents

1,1'-diphenyl-2-pictyhadrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), thrombin, adenosine 5'-phosphate (ADP), epinephrine, heparin, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), ascorbic acid (AA), quercetin, gallic acid, polyvinylpolypyrrolidone (PVPP), $FeCl_2$, $FeCl_3$, $4,4^1$-[3-(2-pyridinyl)-1,2,4-triazine-5,6-dryl] bisbenzene sulphonic acid (ferrozine), potassium ferricyanide, potassium persulfate, triz-HCl, dimethyl sulfoxide (DMSO), carboxymethyl cellulose (CMC), indomethacin, carrageenan, trichloroacetic acid (TCA) and arachidonic acid were all purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

Folin-ciocalteu's phenol reagent, silica gel 60 0.063 - 0.2 mm (70-230 mesh ASTM), sand, mercury (II) chloride, tri-Sodium citrate, lead acetate, ethylenediaminetetra-acetic acid (EDTA) were supplied by Merck Chemical Supplies, Damstadt, Germany. H-D-phenylalanyl-L-pipecolyl-p-nitroanilide dihydrochloride (S2238); *Chromogenix* (Instrumentation Laboratory Company). Cell culture medium (CCM), Modified Eagles Medium (MEM).

Brine shrimp (*Artemisia salina*) eggs (Fish Designs Mtunzini, SA).

HEK293 and HEPG2 (UKZN)

All the chemicals used including the solvents, were of analytical grade.

3.2 Methods (See Appendix B for details)

3.2.1 Animals

Ethical clearance for the use of animals in this study was obtained from the research animal ethics committee of the University of Zululand (see Appendix C) and *Sprague-Dawley* rats were collected from the animal house in the Department of Biochemistry, University of Zululand.

3.2.2 Plant material

The stem bark of the plant (*Protorhus longifolia* (Berhn.) Engl.) was obtained from the traditional *muthi* market at Empangeni, KwaZulu-Natal. Identification and preparation of voucher specimen (RA01UZ) was done in the Department of Botany, University of Zululand. The plant material was thoroughly washed, air-dried and ground to powder (2 mm mesh). The powder was stored in a brown bottle until use.

3.2.3 Phytochemical screening

The plant material was qualitatively screened for saponins, tannins, steroids, terpenoids (Salkowski test), alkaloids, anthraquinones, flavonoids (lead acetate, ferric chloride and sodium hydroxide test), cardiac glycosides (Lieberman, Salkowski, Keller-Kiliani test), and phlobatannins, using the standard methods of Sofowara (1984), Harborne (1973), Odebiyi and Sofowara (1978). Formation of precipitates and colour changes upon the corresponding tests were taken as preliminary evidence of presence of the various phytochemicals in the plant material.

3.2.4 Extraction

The powdered plant material was extracted (1:5 w/v) sequentially with hexane, chloroform, ethyl acetate, methanol and water (24 h on platform shaker per solvent, at room temperature, 160 rpm). See Figure 3.1. Another lot of the plant material was extracted separately with methanol to obtain a separate crude methanol extract. The organic solvent extracts were separately concentrated in *vacuo* using rotary evaporator at 35 ± 2^{o}C while the water extract was freeze-dried yielding crude hexane extract (HE), chloroform extract (CE), ethyl acetate extract (EAE), methanol

extract (ME), water extract (WE) and the total methanol extract (TME). The concentrated/dried crude extracts were stored in sterile bottles at 4^{o}C.

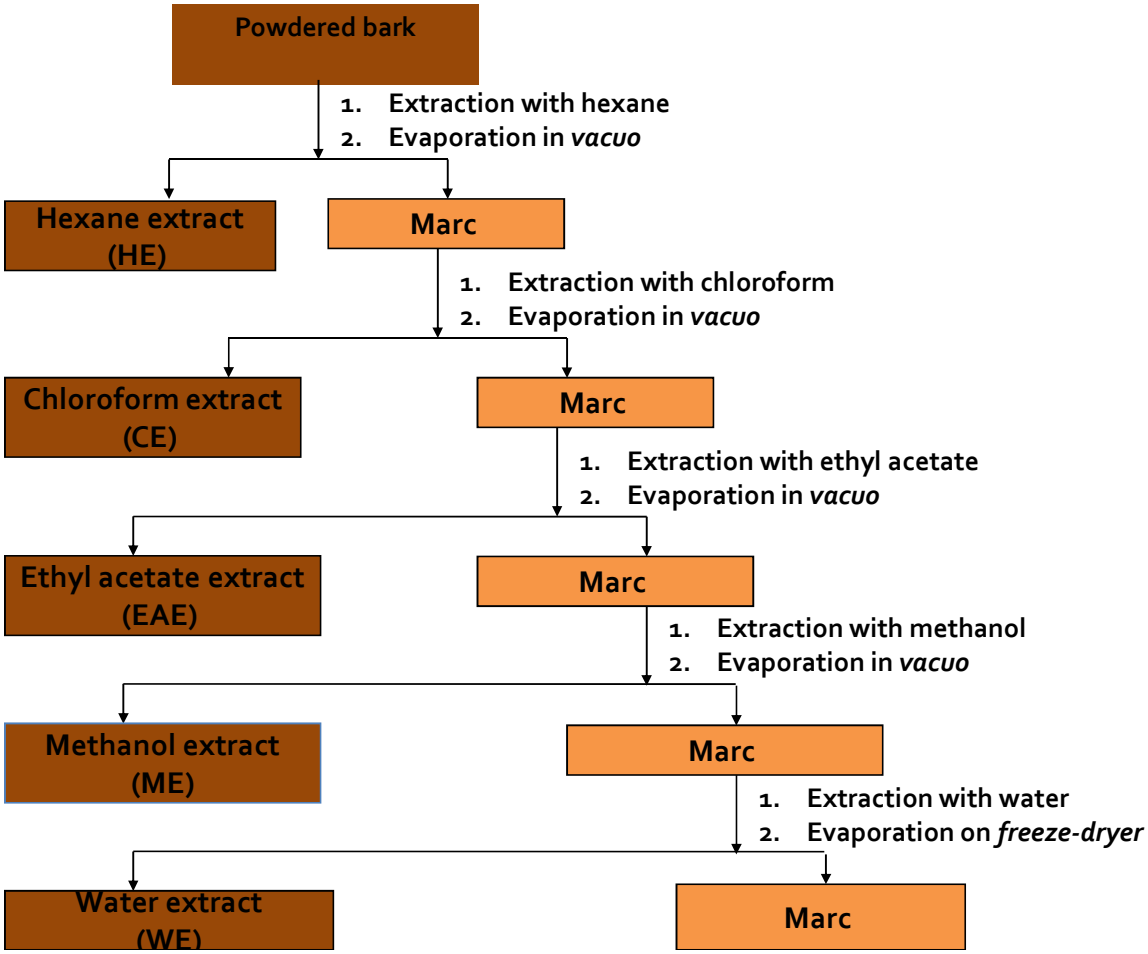


Figure 3.1: Schematic presentation of the sequential extraction of the powdered plant material.

3.2.5 Total phenolic content

Phenolic content of extracts of *P longifolia* was determined by the Folin-Ciocalteu reagent method (Kähkönen *et al*., 1999). Crude extracts (0.5 ml) were separately mixed and incubated with 1.5ml Folin-Ciocalteu reagent and 1.2 ml of 7.5% sodium carbonate solution. Absorbance of blue coloured mixtures was recorded at 765 nm against a blank containing the Folin-Ciocalteu reagent and sodium carbonate solution. The total phenolic content of the extracts was calculated as gallic acid equivalent (GAE) from the calibration curve of gallic acid (see figure B1.1) and expressed as mg/g dry plant material.

3.2.6 Flavonoid content

The method reported by Ordon-Ez *et al.* (2006) was used to determine flavonoid content of the plant extracts. The extracts (0.5 ml) were separately mixed and incubated with alcoholic aluminium chloride (2%, 0.5 ml). Absorbance of the yellow coloured mixtures was read at 420 nm against a blank containing alcoholic aluminium chloride solution. The flavonoid content of the extracts was determined as quercetin equivalent (QE) from the calibration curve of quercetin (see figure B1.2) and expressed as mg/g dry plant material.

3.2.7 *In vitro* antioxidant activity

The extracts were separately dissolved in methanol for use in the antioxidant activity study.

3.2.7.1 DPPH radical assay

Free radical scavenging activity of the plant extracts was determined using DPPH by the method of Brad-Williams (1995). Decolourisation of DPPH (purple) upon addition of the extract indicated radical scavenging activity and this was measured after 30-60 min at 514 nm.

3.2.7.2 ABTS radical assay

The method described by Re *et al.* (1999) was adopted to determine ABTS radical scavenging activity of the plant extracts. Decolourisation of $ABTS^{+}$ (green) upon addition of the extract indicated radical scavenging activity and this was measured after 6 min at 734 nm.

3.2.7.3 Reducing power

The method described by Oyaizu (1986) was followed to measure the reducing power of the plant extracts. The extract (1 ml) was mixed with 2.5 ml of phosphate buffer (0.2 M pH 6.6), and 1% potassium ferricyanide. The reaction was terminated by addition of 10% TCA (2.5ml), followed by centrifuging (1000 rpm, 10 min) to obtain supernatant (2.5 ml). The supernatant was diluted (1:1) with distilled water and mixed with 0.5 ml of 0.1% $FeCl_3$. The reducing power was determined

spectrophotometrically at 700 nm. The higher the absorbance value, the higher the reducing power of the extract.

3.2.7.4 Chelating activity on Fe^{2+}

The metal ion chelating activity of the plant extracts was tested on Fe^{2+} using the method reported by Decker and Welch (1990). The plant extract (1 ml) was diluted with deionised water (3.75 ml). This was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The chelating activity was determined by reading absorbance at 562 nm. EDTA and citric acid were used as standards.

Calculation of percentage inhibitory effect of plant extracts

Unless otherwise stated, ascorbic acid and BHT were used as standards. All assays were repeated three times and the mean ± SEM reported. 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 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 determined using statistical package Origin 6.1.

3.2.8 Cytotoxicity assay (Brine shrimp lethality test)

The cytotoxicity of the crude extracts of *P longifolia* was determined using the brine shrimp lethality test as described by Meyer *et al*. (1982) with some modification. The shrimp larvae were treated with different concentrations (0-25 mg/ml) of the plant extracts. After 24 h, and 48 h, the numbers of alive and dead brine shrimps were recorded and percentage mortality rate of the shrimp larvae was calculated. In the case of mortality in the control, the corrected percentage mortality was calculated using Abbott's formula (Abbot, 1925). Lethal concentration of the plant extracts resulting in 50% mortality of the brine shrimp (LC_{50}) was determined using probit analysis software (Probit Program Version 1.5, USA). The experiment was replicated thrice. DMSO (1%) was used as negative control.

3.2.9 Anti-inflammatory activity

The anti-acute inflammatory activity of the isolated compound (RA/04/I_1) obtained from the crude chloroform extract of *P. longifolia* was determined using the model of Carrageenan-induced rat paw edema as described by Carvalho *et al*. (1999) with some modification. Two groups of *Sprague-Dawley* rats (200 ± 20 g) were orally administered daily for four days before the experiment (50 and 500 mg/kg body weight respectively) with the compound suspended in 0.5% CMC. Negative and positive control animals were administered with 0.5 % carboxymethyl cellulose (CMC) and indomethacin (10 mg/kg) respectively (Figure 3.2).

One hour after the last administration, acute inflammation was produced in all the rats by subcutaneous injection of 0.1 ml (1% w/v) carrageenan solution into the plantar surface of the right hind paw; the left paws were injected with normal saline for comparison. The paw volume until knee joint was measured by a water displacement method using a digital plethysmometer (EL 7500) immediately and at 1 h interval for 4 h following the carrageenan injection. The anti-inflammatory activity was calculated by measuring the volume difference between the right and left paws in comparison with the control group. Statistical difference between the groups was analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The results were presented as mean ± SEM. Statistical difference was accepted at $p<0.05$. Student's t-test was used to analyse statistical difference between control and treated groups.

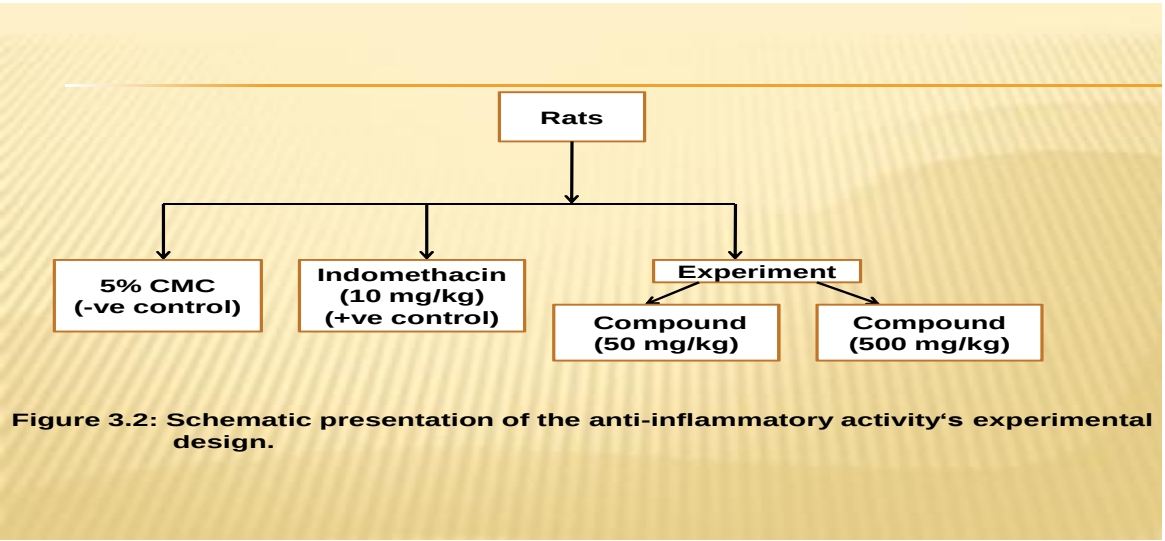


Figure 3.2: Schematic presentation of the anti-inflammatory activity's experimental design.

3.2.10 *In vitro* Anti-platelet aggregation study

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

3.2.10.1 Thrombin assay

Antithrombin activity of the crude extracts was first tested on a chromogenix, S-2238 (H-D-phenylalanyl-L-pipecolyl-p-nitroanilide dihydrochloride) - an artificial substrate of thrombin following the method of Rob *et al*. (1997). Crude plant extracts were solubilised in DMSO before making up the volume with 50 mM Tris-HCl buffer (pH 7.4; containing 7.5 mM EDTA and 175 mM NaCl) to a final 1% DMSO concentration. Then 50 µl of different concentrations (1, 3, and 10 mg/ml) were separately mixed with 10µl of thrombin (30 U/ml), and 190 µl of S-2238 (0.8 M) was added to the mixture. The inhibitory activity of the extracts on thrombin was determined with a Biotek plate reader using Gen5 software by reading absorbance at 412 nm for 4 min at 12 sec interval.

3.2.10.2 Blood platelets

The method of Tomita *et al.* (1983) was followed to obtain platelets. A rat was killed by a blow to the head and blood was immediately collected from abdominal aorta. The blood was mixed (5:1 v/v) with an anticoagulant (acid-dextrose-anticoagulant—0.085M trisodium citrate, 0.065 citric acid, 2% dextrose). The platelets were obtained by a series of centrifugation and washing of the blood. The platelets were finally suspended in a buffer (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose). The platelets were then divided into untreated and enzyme (trypsin, bromelain, and papain) treated platelets.

Enzyme treated platelets

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

3.2.10.3 Anti-platelet aggregation activity

The method of Mekhfi *et al*. (2004) was followed with some modifications**.** The anti-platelet aggregation activity of the extracts was separately tested on thrombin (5 U/ml), ADP (5 mM) and epinephrine (10 mM) induced platelet aggregation; similar experiments were also carried out on enzyme (trypsin, bromelain, papain) treated platelets. The platelets (100 µl) were incubated for 5 min with different concentrations of the crude extracts (1, 3, and 10 mg/ml) and an aggregation inducer (20 µl) was introduced to the mixtures. Aggregation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm. DMSO (1%) was used as negative control and heparin was used as positive control.

3.2.10.4 Malondialdehyde determination

Formation of malondialdehyde (MDA) was determined following the method of Okhawa *et al*. (1978) with some modification. Portion (100 µl) of the anti-aggregation reaction mixture was mixed with 1.5ml of 10% trichloroacetic acid (TCA), allowed to stand for 10 min and then centrifuged (3500xg; 20 min), after which 2 ml of 1% 2-thiobarbituric acid (TBA) was added to supernatant. The MDA formation was also determined with the Biotek plate reader using Gen5 software by reading absorbance at 490 nm.

3.2.10.5 Tannin Removal

The method described by Toth and Pavia (2001) was adapted to remove tannins from the crude extracts. The extracts were separately mixed (10 mg/ml) with polyvinylpolypyrrolidone (PVPP) and the mixtures were incubated for 15 min and centrifuged (3000xg; 10 min) several times to reduce tannin to a negligible concentration. The tannin-free extracts were screened for the anti-platelet aggregation activity by the method of Mekhfi *et al*. (2004), described above.

3.2.10.6 Thrombin and calcium chloride induced clotting time assays

The method described by Kee *et al.* (2008) was followed to determine the rate of clot formation induced by thrombin and calcium chloride ($CaCl_2$). The platelets were separately incubated with the different concentrations of the crude extracts, and

thrombin (5 U/ml) and/or $CaCl_2$ (0.16 M) was added. The rate of clot formation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm.

Calculation of percentage inhibitory effect of plant extracts on platelet aggregation

All assays were repeated three times and the mean slope (A) ± S.E 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.

3.2.11 Isolation, purification and characterisation of compounds

3.2.11.1 Isolation

In order to identify the active components present in the extract that showed consistent activity in the anti-platelet aggregation study, the crude chloroform extract (8 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 16 combined fractions (Figure 3.3). Thin layer chromatography (TLC) (silica gel 60 TLC aluminium sheets 20 cm x 20 cm, F_{254}, hexane:ethyl acetate solvent system 9:1 – 7:3) was used to analyse the fractions. The TLC plates were first viewed under ultraviolet (UV), developed using a 10% H_2SO_4 spray reagent and then heated. The fractions with similar profile were combined, concentrated *in vacuo* and their weights were determined. The seventh (RA/04/G) and ninth (RA/04/I) fractions were separately recrystalised in methanol and hexane respectively to obtain compounds ($RA/04/G_1$; 0.23 g) and ($RA/04/I_1$; 0.37 g).

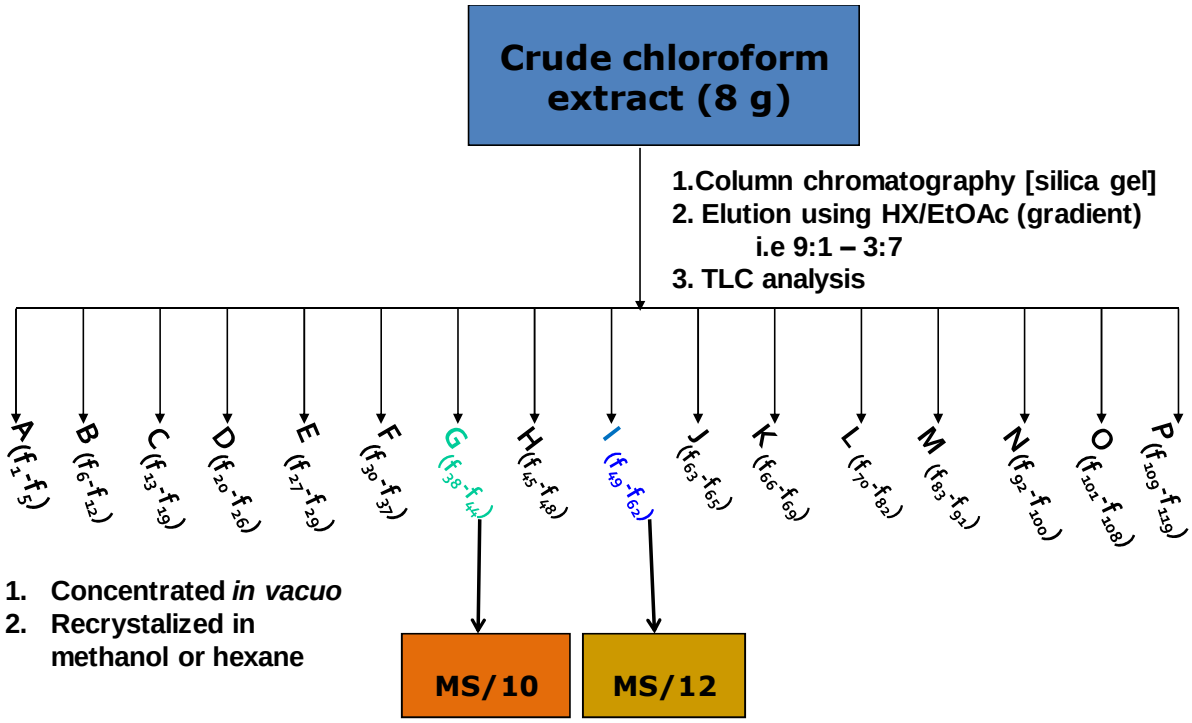


Figure 3.3: Schematic presentation of the isolation and purification of RA/04/G_1 and RA/04/I_1 through column chromatography.

3.2.11.2 Structure elucidation

RA/04/G_1 (MS/10) and RA/04/I_1 (MS/12) were analyzed using 1D and 2D NMR techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in $CDCl_3$, Bruker 600 MHz), infrared (IR) (Perkin-Elmer 100 FTIR), UV (in $CHCl_3$, Varian - Cary 50 UV-visible spectrophotometer) and liquid chromatography mass spectrometry (LC-MS) and the molecular formula was identified by ESI-MS (positive mode), $[M + H]^+$ (in DCM, Waters API Q-TOF Ultima). Melting point (Reichert Thermovar) of the compounds was also determined. The resulting spectra (see Appendix E) were analysed and the compounds identified by comparing them to standards and library materials.

The anti-platelet aggregation, antioxidant and anti-inflammatory activity of the pure compounds were also investigated following the same methods used for the crude extracts.

3.2.12 Anticoagulant activity of MS/12

The anticoagulant activity of the MS/12 was investigated on rat whole blood. The compound was solubilised in DMSO before making up the volume with 50 mM Tris-HCl buffer (pH 7.4; containing 7.5 mM EDTA and 175 mM NaCl) to a final 1% DMSO concentration. The anticoagulant activity of the compound (1, 3 and 10 mg/ml) was tested against thrombin (5 U/ml), ADP (5 mM), epinephrine (10 mM), and arachidonic acid (10 mM) as controls. 1% DMSO was used as blank. The compound (50 µl) and the clotting agonist (20 µl) were separately put in the corresponding wells. Blood was drawn from abdominal aorta of a rat and was immediately mixed with either the clotting agonist or the compound in the corresponding wells. The reaction was monitored by visualisation for 4-5 min to record the time it took the blood to clot. The experiment was done in duplicate.

3.2.13 Cytotoxicity of MS/12

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] cell proliferation assay (Mosman, 1983) was used to determine the cytotoxicity of the compound MS/12. The cytotoxicity of the compound MS12 was tested in human embryonic kidney (HEK293) and human hepatocellular carcinoma (HEPG2) cells. The cells were seeded in a 48-well plate at a density of 2.5×10^4 cells per well. Following an overnight incubation at 37^{o}C, the cells were incubated with the compound at different concentrations (50, 100, 150, 200, 250, 300, and 350 µg/200µl) in medium (MEM + Gutamax + antibiotics + 10% fetal bovine serum) for 48 h. Thereafter, the medium was removed from the cells and 200µl MTT solution (5 mg/ml PBS) as well as 200 µl of cell culture medium was added to the corresponding wells. The cells were incubated at 37^{o}C for 4 h and the reaction was terminated by addition of DMSO (100/200/400 µl). The cells viability was determined spectrophotometrically (Biomate spectrophotometer) at 570 nm.

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

$$\% \text{ cell death} = [(A_c - A_t)/A_c \times 100]$$

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

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

3.2.14 Statistical analysis

Statistical difference between groups treated with different extracts was analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. The results were presented as mean ± SEM. Statistical difference was accepted at $p<0.05$. Student's t-test was used to analyse statistical difference between control and treated groups.

CHAPTER 4

4.0 RESULTS

Extracts (hexane-HE, chloroform-CE, ethyl acetate-EAE, methanol-ME, and water-WE) obtained from the bark of *P logifolia* were screened for antioxidant, cytotoxicity and anti-platelet aggregation activity. The chloroform extract was subjected to isolation, purification and characterisation of the active constituents. The isolated compounds were also screened for bioactivity.

4.1 Extraction and percentage yields

After the extraction, the weights of the crude extracts were separately determined and their percentage yields were calculated (Table 4.1).

Table 4.1: Weights (g) and percentage yields (w/w dry weight bases) of the crude extracts

Extract	Weight (g)	Yield (%)
HE	0.75	1.88
CE	0.71	1.78
EAE	1.29	3.23
ME	7.00	17.5
WE	5.30	2.38
TME	0.95	26.5

Methanol was able to extract more of the plant components than the other solvents.

4.2 Phytochemical screening

The plant material was qualitatively screened for its chemical composition and the results are given in Table 4.2.

Table 4.2: Phytochemicals of the plant material of *P longifolia*

Phytochemical	*P longifolia*
Saponins	**+**
Tannins	**+**
Steroids	**-**
Terpenoids	**+**
Alkaloids	**+**
Anthraquinones	**-**
Flavonoids	**+**
Cardiac glycosides	**+**
Phlobatannins	**-**

Key: **+** *= present*

-- *= absent*

The phytochemical screening revealed the presence of most of the phytochemicals that were screened for. Anthraquinones, steroids and phlobatannins were, however, not detected in the plant material.

4.3 Total phenolic and flavonoid content

Total amount of phenols and flavonoids in the extracts of *P longifolia* were quantified as gallic acid and quercetin equivalent, respectively (Figure 4.1).

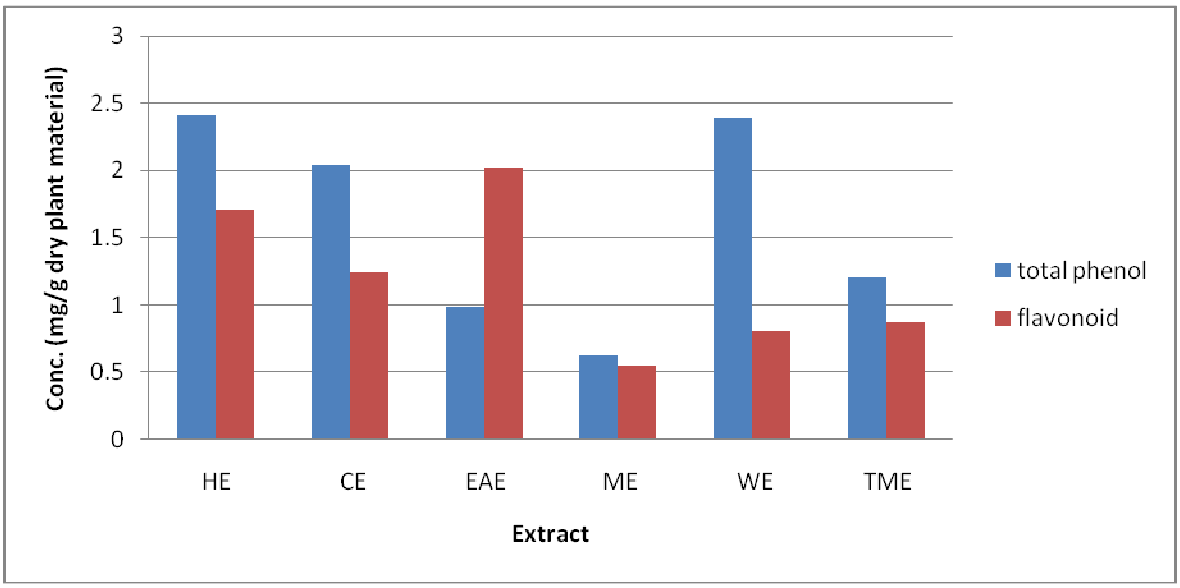


Figure 4.1: The total phenolic (gallic acid equivalent) and flavonoid (quercetin equivalent) content of the extracts of *P longifolia*.

Although water was able to extract as much as 2.4 mg/g of phenol, the total phenolic and flavonoid content seemed to decrease with increase in polarity. The two groups of compounds were extracted more by the non-polar solvents.

4.4 *In vitro* antioxidant activity

The radical scavenging activity was determined using DPPH and $ABTS^{+}$ radicals (Table 4.3). Their reducing power (Figure 4.2) and Fe^{2+} chelating activity were also tested.

Table 4.3: Percentage free radical (DPPH and $ABTS^+$) scavenging activity and IC_{50} (mg/ml) values of the extracts of *P longifolia* (BHT and AA were used as the standards).

Extract	DPPH		$ABTS^+$	
	% scavenging	IC_{50}	% scavenging	IC_{50}
HE	2.0 ± 0.01	>50	79.0 ± 0.08	0.27
CE	15.8 ± 0.00	>50	42.7 ± 0.01	>50
EAE	45.3 ± 0.00	>50	45.6 ± 0.01	>50
ME	84.0 ± 0.01	0.07	67.3 ± 0.01	0.16
WE	28.6 ± 0.10	>50	66.8 ± 0.03	2.0
TME	52.6 ± 0.04	0.12	80.1± 0.10	0.18
BHT	57.8 ± 0.00	1.43	85.7 ± 0.05	0.42
AA	52.7 ± 0.11	0.49	87.2 ± 0.07	0.15

Values are expressed as mean ± SEM, (n=3).

The extracts exhibited to varying degrees of efficiency, a concentration dependent DPPH and $ABTS^+$ scavenging activities. The methanol extracts exhibited the highest radical scavenging activity and their IC_{50} values were even less than or comparable to those of the standards (AA and BHT). Hexane extract (IC_{50} 0.27 mg/ml) also showed a good scavenging activity of $ABTS^+$ radical.

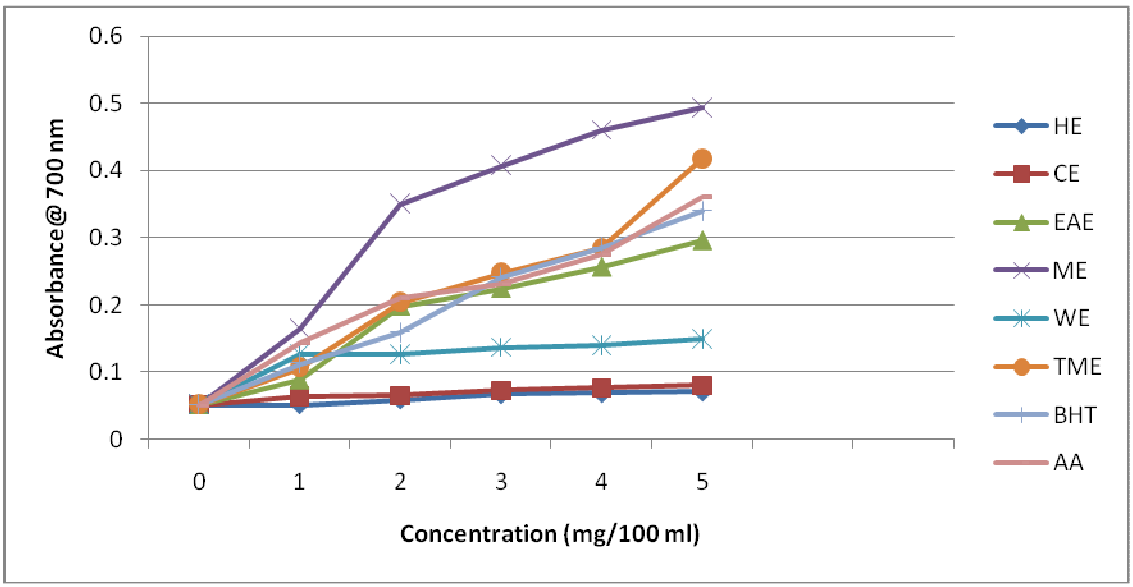


Figure 4.2: Reduction potential of the extracts of *P longifolia* along with commercial antioxidants BHT and AA. Data is expressed as mean ± SEM, (n=3).

The extracts exhibited a concentration dependent reducing power with the methanol extracts again showing the highest potential; the reducing power was also even higher than those of the BHT and AA. Except the hexane extract that exhibited a weak (27.7%) chelating activity, the other extracts showed no chelating activity on Fe^{2+}. EDTA and citric acid exhibited 63.1% and 54.8% chelating effect respectively.

4.5 Cytotoxicity test (Brine shrimp lethality test)

The cytotoxic effects of the extracts of *P longifolia* on brine shrimps were tested. The results of the 24h exposure are given in Table 4.5.

Table 4.4: Summary of results of brine shrimp lethality test (% mortality rate and LC_{50} (mg/ml))

	Concentration (mg/ml)				
Extract	1	5	25	LC_{50}	CL
HE	3.33 ± 5.77	10.0 ± 0.00	36.7 ± 15.3	39.6	21.1-1668
CE	6.67 ± 5.77	10.0 ± 0.00	40.0 ± 10.0	54.6	21.4-213.2
EAE	3.33 ± 5.77	10.0 ± 0.00	16.7 ± 5.77	nd	
ME	3.33 ± 5.77	6.67 ± 11.5	20.0 ± 17.3	nd	
WE	0.00 ± 1.59	3.33 ± 5.77	6.67 ± 11.5	nd	
TME	0.00 ± 5.77	3.33 ± 5.77	10.0 ± 17.3	nd	

nd- not detected; Values are expressed as mean ± SEM, (n=3).

The extracts exhibited a concentration dependent cytotoxic effect. Only HE and CE showed weak cytotoxic effects on the brine shrimps with the IC_{50} values of 39.6 mg/ml and 54.6 mg/ml respectively.

4.6 Thrombin assay

Thrombin is the main platelet activator and this makes it the target in anti-platelet therapy. Therefore, the antithrombin activity of the plant extracts was first tested on chromogenix (S2238) which is an artificial substrate of thrombin. The results are presented in Figure 4.3. Only the ethyl acetate, water and total methanol extracts exhibited a concentration dependent inhibitory activity on thrombin. They showed up to about 50% inhibitory activities.

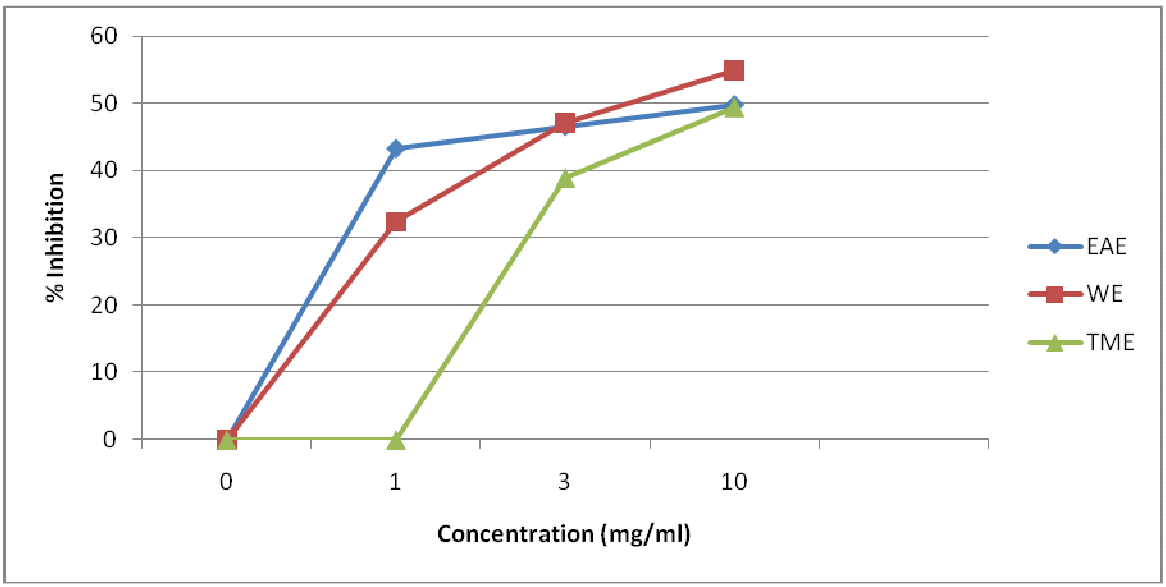


Figure 4.3: The extracts of *P longifolia* (EAE, ME, TME) inhibit the activity of thrombin on S2238. Data are expressed as mean ± SEM, (n=3).

4.7 Anti-platelet aggregation activity

The potential ability of the plants extracts to prevent platelet aggregation was then investigated on rat platelets. The anti-platelet aggregation activity of the extracts (with tannins and without tannins) was separately tested on thrombin, ADP or epinephrine induced rat platelet aggregation (Table 4.5). A similar experiment was carried out on enzymes (trypsin, bromelain, and papain) treated rat platelets. The results are shown in Figure 4.5 and Table 4.6.

Table 4.5: Inhibitory activity (%) of the extracts (with and without tannin) of *P longifolia* on platelet aggregation.

(a) *Thrombin-induced platelet aggregation*

Extract	Concentration (mg/ml)							
	1		3		10		IC_{50} (mg/ml)	
HE	*78.2 ± 1.60	0.00 ± 0.04	*81.3 ± 8.20	5.72 ± 0.02	*82.0 ± 4.09	56.7 ± 0.04	0.59	9.10
CE	0.00 ± 0.07	0.00 ± 0.01	0.00 ± 0.31	0.00 ± 0.02	23.1 ± 0.16	4.69 ± 0.41	>10	>10
EAE	23.3 ± 0.12	0.00 ± 0.02	*70.5 ± 0.30	0.00 ± 0.06	0.00 ± 0.22	0.00 ± 0.02	2.13	nd
ME	6.47 ± 0.22	20.3 ± 0.14	0.00 ± 0.14	40.5 ± 0.04	0.00 ± 4.29	20.0 ± 0.01	nd	>10
WE	0.00 ± 0.11	2.65 ±0.01	0.00 ± 0.14	15.9 ± 0.04	0.00 ± 0.24	20.0 ± 0.01	nd	>10
TME	0.00 ± 0.03	41.4 ±0.34	0.00 ± 0.21	35.0 ± 0.28	0.00 0.31	0.00 ± 0.19	nd	nd
Heparin	11.9 ± 0.24		22.1 ± 0.09		66.3 ± 1.01		7.40	

(b) *ADP-induced platelet aggregation*

Extract	Concentration (mg/ml)							
	1		3		10		IC_{50} (mg/ml)	
HE	42.3 ± 0.02	18.5 ± 0.13	*79.4 ± 0.32	44.0 ± 0.11	66.9 ± 1.15	0.00 ± 0.25	1.56	10>
CE	0.00 ± 0.50	27.8 ± 0.00	41.1 ± 0.04	37.2 ± 0.04	*81.3 ± 0.01	69.4 ± 0.09	4.48	5.86
EAE	20.3 ± 0.01	0.00 ± 0.49	*56.4 ± 0.19	0.00 ± 0.16	0.00 ± 0.19	0.00 ± 0.14	2.62	nd
ME	7.70 ± 0.10	35.6 ± 0.07	0.00 ± 0.86	30.7 ± 0.18	0.00 ± 5.66	7.09 ± 0.04	nd	nd
WE	6.79 ± 0.07	11.5 ± 0.20	0.32 ± 0.06	9.04 ± 1.12	0.00 ± 3.44	4.81 ± 0.20	nd	nd
TME	0.00 ± 0.60	40.4 ± 0.03	0.00 ± 0.35	29.6 ± 0.25	0.00 ± 0.17	20.6 ± 0.14	nd	>10

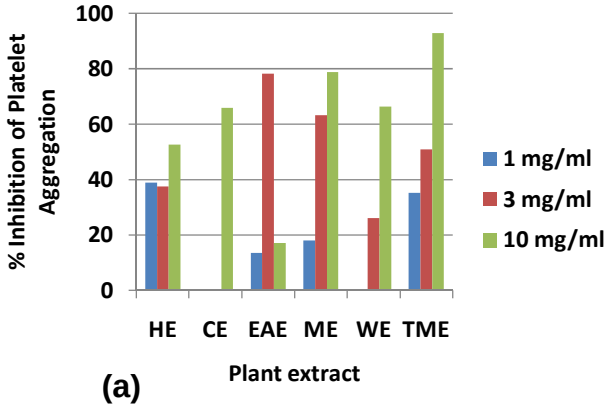
(c) *Epinephrine-induced platelet aggregation*

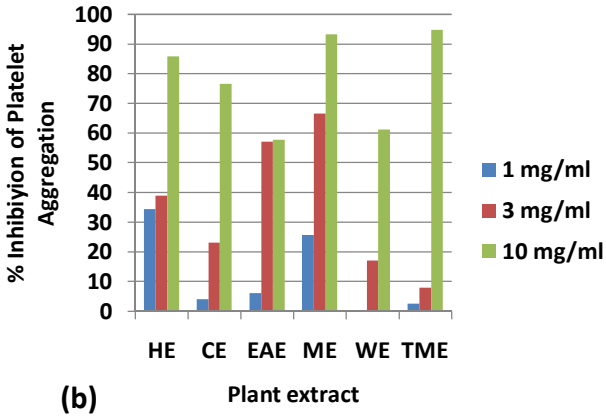
	Concentration (mg/ml)							
Extract	1		3		10		IC_{50} (mg/ml)	
HE	**45.8 ± 0.12**	**34.5 ± 0.28**	**46.4 ± 0.79**	**47.0 ± 0.27**	**51.1 ± 0.18**	**0.00 ± 0.06**	**>10**	**>10**
CE	**6.06 ± 0.54**	**17.1 ± 0.10**	***69.7 ± 0.14**	**42.2 ± 0.41**	***89.7 ± 0.41**	**77.8 ± 0.25**	**2.38**	**4.57**
EAE	**36.0 ± 0.12**	**0.00 ± 0.24**	***62.1 ± 0.29**	**0.00 ± 0.15**	**0.00 ± 1.11**	**0.00 ± 0.03**	**2.09**	**nd**
ME	**31.4 ± 0.05**	**20.5 ± 0.76**	***60.4 ± 0.50**	**26.0 ± 0.19**	**0.00 ± 0.19**	**24.2 ± 0.11**	**2.21**	**nd**
WE	**16.1 ± 0.11**	**34.5 ± 0.03**	**20.5 ± 0.08**	**23.4 ± 0.05**	**18.7 ± 0.01**	**19.3 ±0.05**	**>10**	**nd**
TME	**37.9 ± 0.30**	**51.8 ± 0.02**	**55.2 ± 0.03**	**57.8 ± 0.10**	**0.00 ± 0.01**	**0.00 ± 0.05**	**2.46**	**0.96**

*Values are expressed as mean ± SEM, (n=3), * p<0.05 compared to control.*
Before tannin-removal**; **after tannin-removal
nd- (not detected)

A concentration dependent inhibitory activity on the aggregation induced by thrombin, ADP and epinephrine was observed for all the extracts. The activity of the extracts was higher than that of heparin (1 mg/ml), a commercial anticoagulant. Most extracts showed anti-platelet aggregation activity in the presence of tannins and for those that retained their activity after the tannin removal, the activities were reduced. The hexane extract exhibited relatively the lowest IC_{50} values (0.59 and 9.10 mg/ml before and after tannin removal respectively) on the thrombin-induced platelet aggregation.

4.7.1 Antiplatelet aggregation activity of the extracts on the enzyme treated platelets





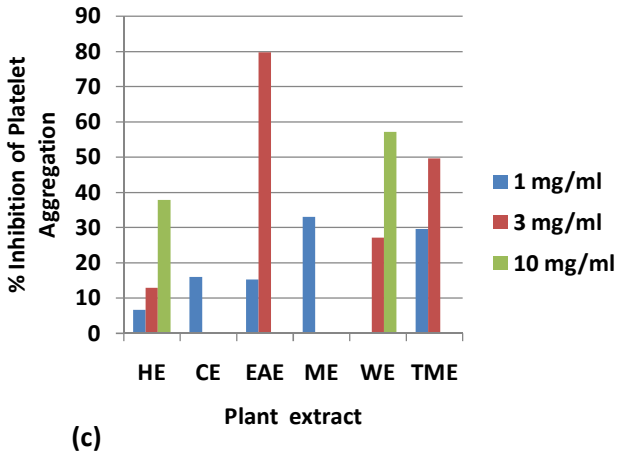


Figure 4.4a: Inhibitory activity of the extracts on (a) trypsin, (b) bromelain and (c) papain treated platelets. Aggregation was induced with thrombin. ***Data are expressed as mean ± SEM, (n=3).***

Table 4.6a: IC_{50} values (mg/ml) of the plant extracts on the thrombin-induced-enzyme-treated platelet aggregation.

Extract	Trypsin	Bromelain	Papain
HE	9.19	5.19	10<
CE	8.21	6.61	10<
EAE	2.17	2.63	2.66
ME	2.42	2.01	10<
WE	7.52	8.11	8.46
TME	2.94	8.28	10<

Platelet aggregation was induced by thrombin. Ethyl acetate extract with relatively low IC_{50} values showed the highest activity at 3 mg/ml. Only the ethyl acetate and water extracts showed a considerable inhibitory activity on the papain-treated platelets.

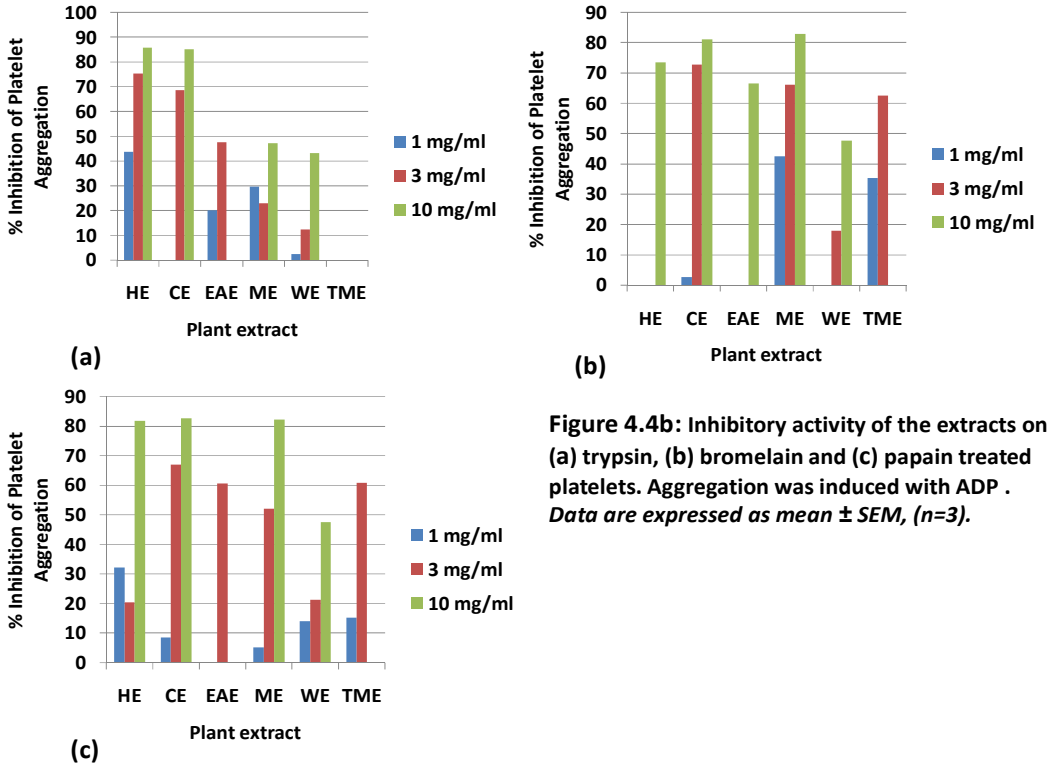
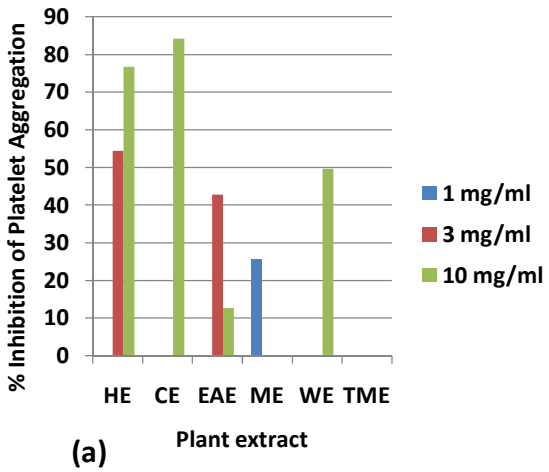


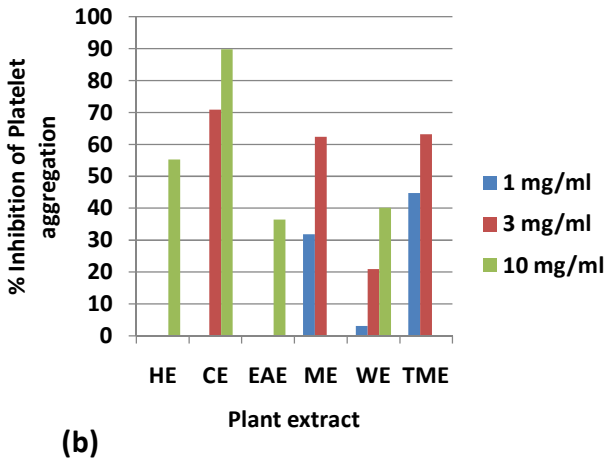
Figure 4.4b: Inhibitory activity of the extracts on (a) trypsin, (b) bromelain and (c) papain treated platelets. Aggregation was induced with ADP . *Data are expressed as mean ± SEM, (n=3).*

Table 4.6b: IC_{50} values (mg/ml) of the plant extracts on the ADP-induced-enzyme-treated platelet aggregation.

Extract	Trypsin	Bromelain	Papain
HE	1.36	7.77	5.54
CE	2.82	2.34	2.46
EAE	10<	8.29	2.66
ME	10<	1.61	2.90
WE	10<	10<	10<
TME	nd	2.13	2.50

When the aggregation is induced with ADP, the extracts also showed varying degrees of anti-platelet aggregation activity on the enzymes treated platelets. The chloroform extract exhibited the highest activity with relatively the lowest IC_{50} values. The hexane, ethyl acetate and methanol extracts also showed a considerable inhibitory activity.





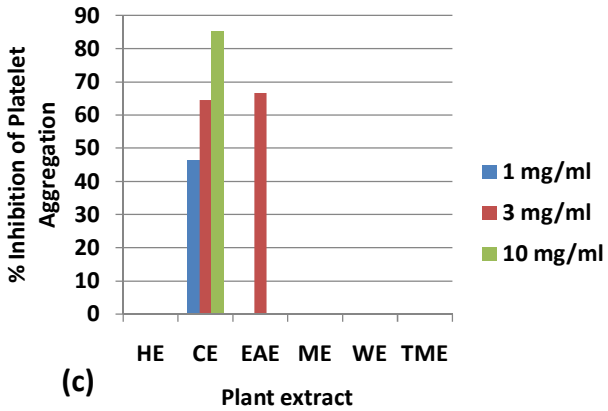


Figure 4.4c: Inhibitory activity of the extracts on (a) trypsin, (b) bromelain and (c) papain treated platelets. Aggregation was induced with epinephrine . ***Data are expressed as mean ± SEM, (n=3).***

Table 4.6c: IC_{50} values (mg/ml) of the plant extracts on the epinephrine-induced-enzyme-treated platelet aggregation.

Extract	Trypsin	Bromelain	Papain
HE	2.42	9.43	10<
CE	7.12	2.42	2.46
EAE	10<	10<	2.66
ME	10<	2.17	nd
WE	10<	10<	nd
TME	nd	1.61	nd

In the epinephrine-induced platelet aggregation, most of the extracts showed anti-platelet aggregation activity on the trypsin and bromelain treated platelets, only the chloroform and ethyl acetate (at 3mg/ml) extracts inhibited platelet aggregation on the papain treated platelets.

Overall, the extracts showed a varying degree of efficiency in preventing the enzyme-treated-platelet aggregation induced by the three agonists. While the chloroform, methanol and hexane extracts showed the more concentration dependent activity, the highest activity of ethyl acetate extract was observed only at concentration 3 mg/ml. The water extract was the least active. The total methanol extract was more active on the thrombin-induced platelet aggregation in the platelets treated with the three enzymes.

4.7.2 MDA determination

Since MDA (the major product of lipid peroxidation) production is known to accompany platelet aggregation (Tomita *et al*., 1983), it was important to determine the ability of the extracts to inhibit lipid peroxidation. The results are shown in Figure 4.5. All the extracts showed concentration dependent inhibition of MDA formation.

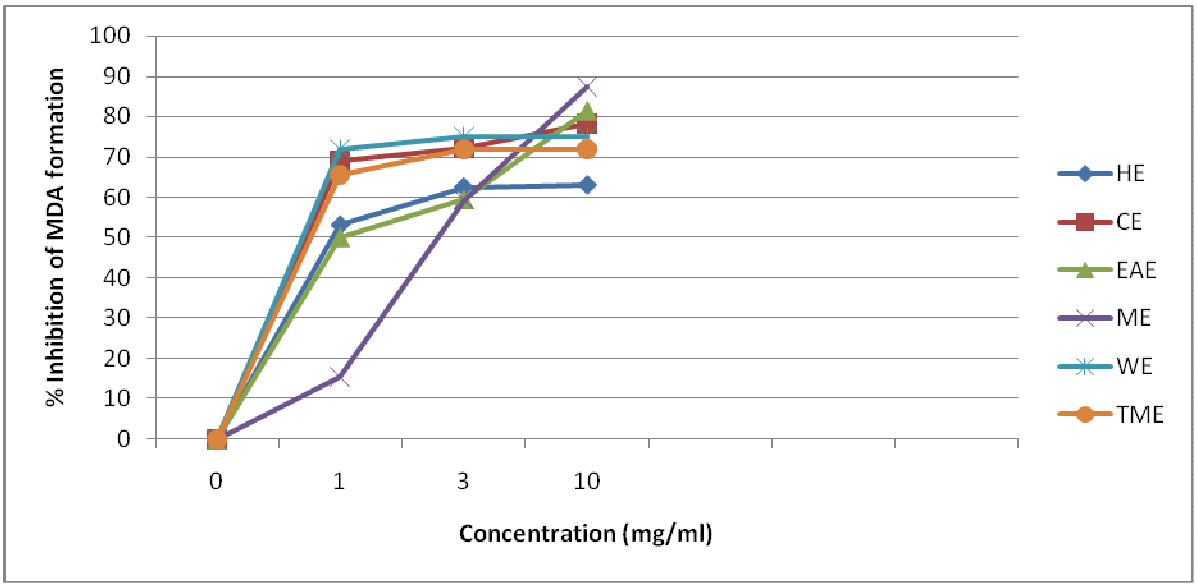


Figure 4.5: Inhibitory activity (%) of the extracts of *P longifolia* on the formation of MDA. Data are expressed as mean ± SEM, (n=3).

4.7.3 Thrombin and $CaCl_2$ induced clotting time

The thrombin and $CaCl_2$ induced clotting time assays were conducted in order to determine the rate at which the extracts inhibited clot formation and their effect on fibrin formation. The results are presented in Table 4.7.

Table 4.7a: Percentage inhibition of Thrombin-induced clotting time

	Concentration (mg/ml)			
Extract	1	3	10	IC_{50} (mg/ml)
HE	67.4 ± 0.51	24.1 ± 0.22	64.5 ± 0.31	7.56
CE	0.00 ± 0.15	0.00 ± 1.09	78.5 ± 0.59	7.61
EAE	31.3 ± 0.24	65.5 ± 0.07	0.00 ± 0.60	2.09
ME	0.00 ± 0.20	0.00 ± 0.34	0.00 ± 0.76	nd
WE	0.00 ± 0.91	0.00 ± 0.85	78.6 ± 1.15	7.44
TME	0.00 ± 0.24	0.00 ± 0.07	62.4 ± 2.80	8.62

Values are expressed as mean ± SEM, (n=3).

Table 4.7b: Percentage inhibition of $CaCl_2$-induced clotting time

	Concentration (mg/ml)			
Extract	1	3	10	IC_{50} (mg/ml)
HE	12.9 ± 0.51	10.6 ± 0.22	25.2 ± 0.31	nd
CE	0.00 ± 0.12	27.9 ± 0.07	35.3 ± 0.59	nd
EAE	8.18 ± 0.02	17.2 ± 0.05	0.00 ± 0.06	nd
ME	14.4 ± 0.11	47.4 ± 0.15	42.9 ± 1.02	nd
WE	0.00 ± 4.16	8.18 ± 0.02	37.7 ± 0.03	nd
TME	24.6 ± 0.02	0.00 ± 0.07	0.00 ± 1.15	nd

Values are expressed as mean ± SEM, (n=3).

The extracts exhibited the ability to increase the thrombin-induced clotting time in a concentration dependent fashion. The extracts hardly inhibited the $CaCl_2$-induced clotting time.

4.8 Isolation and characterization

The crude chloroform extract which showed more consistent activity on the antiplatelet aggregation was subjected to isolation and purification. The two compounds RA/04/G_1 (**MS/10**) and RA/04/I_1 (**MS/12**) were obtained and analysed through IR, UV, NMR and/or MS spectral techniques. The 2D NMR (HSQC, HMBC, NOESY and COSY) were used to assign carbons and protons. Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR of MS/10 and MS/12 in comparison with

literature values is presented in Table 4.8 and 4.9 respectively. The full NMR spectra for both compounds are given in Appendix E.

Table 4.8: ^{1}H-NMR and ^{13}C-NMR spectral data of compound **MS/10**.

Position	δ_H (ppm)	δ_H (ppm) [RC]	δ_C (ppm)	δ_C (ppm) [RC]
1			35.6	36.1
2			34.5	34.6
3			217.7	216.0
4			47.3	47.3
5			51.5	51.2
6			20.1	19.5
7			25.9	26.5
8			134.4	134.6
9			132.2	NA
10			37.2	37.0
11			21.1	21.3
12			29.3	29.2
13			44.0	44.8
14			49.7	49.8
15			29.7	30.9
16			27.4	27.4
17			47.0	47.7
18	0.98 (3H,s)	0.99 (3H,s)	15.8	16.8
19	1.04 (3H,s)	1.06 (3H,s)	19.6	18.6
20			47.4	49.0
21			181.3	178.6
22			32.4	33.2
23			26.9	26.7
24	5.33 (1H,t)	5.34 (1H,t)	123.5	124.9
25			132.7	131.6
26	1.62 (3H,s)	1.63 (3H,s)	17.6	17.7
27	1.67 (3H,s)	1.68 (3H,s)	25.7	25.8
28	1.14 (3H,s)	1.14 (3H,s)	21.3	21.3
29	0.99 (3H,s)	1.00 (3H,s)	26.6	26.3
30	1.05 (3H,s)	1.06 (3H,s)	24.5	24.5

NA= not available in literature, RC= reference compound

Compound MS/10 was obtained as a white amorphous powder with melting point 192-194^{o}C. The IR spectrum showed absorption band for the hydroxyl group at 3431 cm^{-1}(Appendix E). The compound produced a protonated ion at *m/z* 455.3524 (calculated 455.3525) in the LCMS, corresponding to the molecular formula of $C_{30}H_{46}O_3$ (Appendix E). The molecular formula of this compound was identified by ESI-MS (positive mode) *m/z* % 455 $[M+H]^+$; 154; 81; 45; 141; 95; 69; 154; 119; and fragmentation pattern is shown in Figure 4.6.

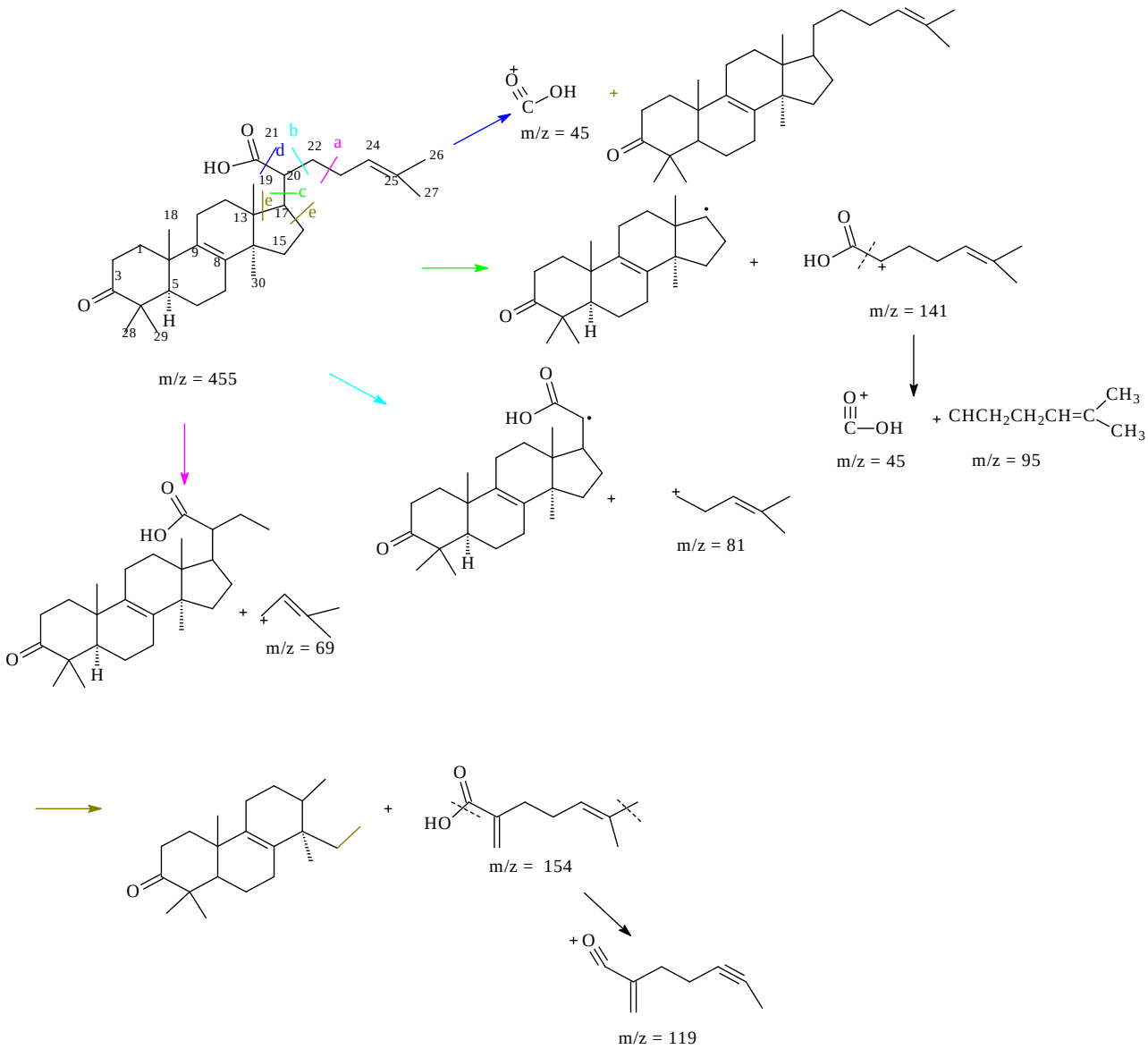


Figure 4.6: Mass fragmentation pattern of MS/10.

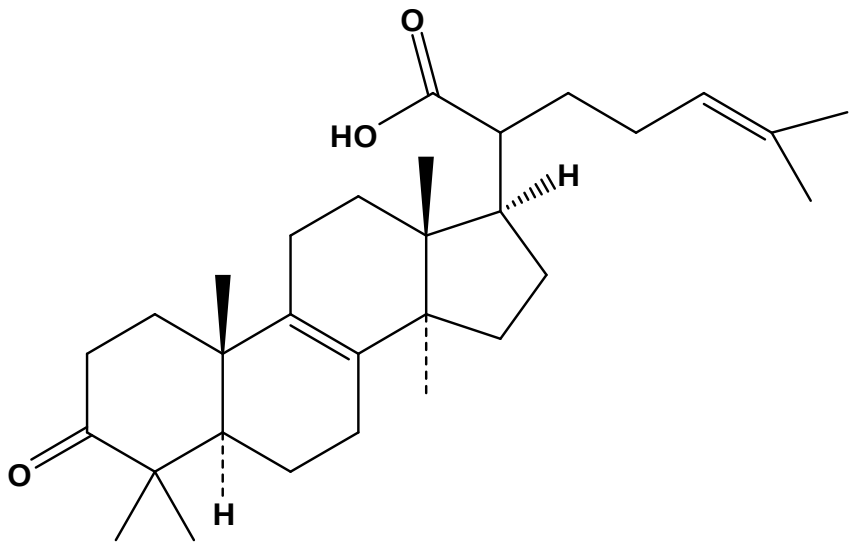
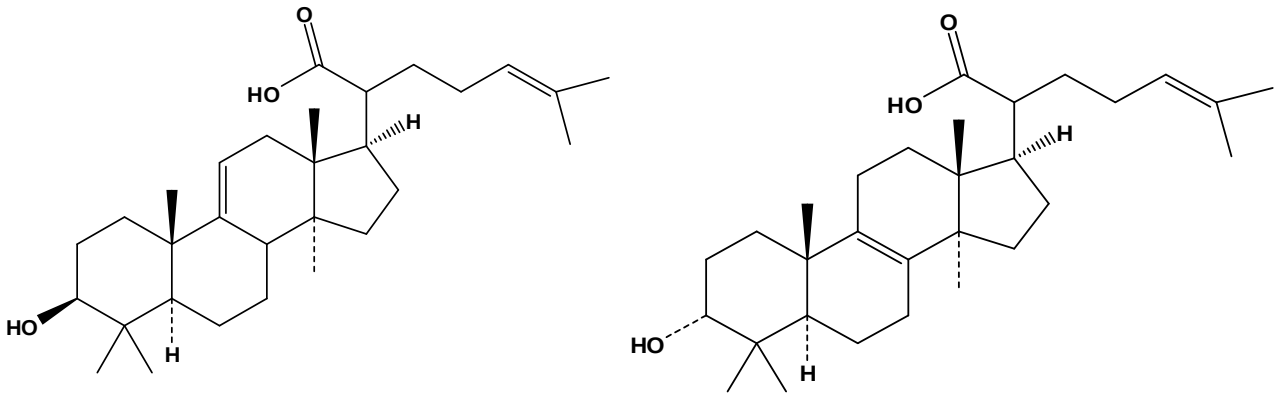


Figure 4.7: Chemical structure of 3-oxo-5α-lanosta-8,24-dien-21-oic acid (MS/10).

The ^{1}HNMR spectra of compound MS/10 (figure 4.7) had a triterpenoid proton pattern with large clusters of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8. However, one olefinic proton was identified at δ 5.33. The ^{13}C-NMR did help to further analyse the compound. The presence of carbonyl ketone at 217.7 ppm, four olefinic carbons between 134.4-123.5 ppm, a carboxylic carbon at 181.3 ppm and four quaternary carbons assisted in suggesting a lanosteryl skeletal structure (Vincken *et al*., 2007). The ^{13}C-NMR DEPT further indicated the presence of 11 CH_2 which also helped in elucidating the structure along with the 2D NMR and mass spectra (Appendix E). Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR is presented in Table 4.8. The melting point of the compound was 192-194^{o}C while the reference compound had 196-200^{o}C (Keller *et al.,* 1996).These data are in correlation with those of 3-oxo-5α-lanosta-8,24-dien-21-oic acids reported in the literature (Keller *et al.,* 1996; Ko *et al*., 2007).

Table 4.9: ^{1}H and ^{13}C NMR spectral data of compound **MS/12**

Position	δ_H (ppm)	δ_H (ppm) [RC]	δ_C (ppm)	δ_C (ppm) [RC]
1			30.3	30.3
2			23.9	23.4
3	3.5 (1H,s,OH)	3.6 (1H,s, OH)	77.2	77.9
4			37.3	36.8
5			44.5	45.2
6			17.7	17.9
7			26.0	26.9
8			49.7	134.4
9			145.7	133.8
10			34.9	36.9
11	5.2 (1H, t)		118.2	20.8
12			29.3	28.9
13			43.3	44.2
14			51.0	49.5
15			31.2	30.8
16			27.3	27.0
17			47.2	47.1
18			16.5	16.0
19			18.5	18.8
20			48.2	47.7
21			181.5	183.3
22			32.4	32.4
23			25.3	25.9
24	5.33 (1H,m)	5.33 (1H,t)	123.6	123.6
25			132.2	132.2
26	1.62 (3H,s)	1.62 (3H,s)	17.6	17.6
27	1.65 (3H,s)	1.67 (3H,s)	25.7	25.7
28	1.21 (3H,s)	1.14 (3H,s)	21.8	21.8
29	0.92 (3H,s)	0.93 (3H,s)	27.7	27.5
30	1.02 (3H,s)	1.04 (3H,s)	22.7	24.3



3β-Hydroxylanosta-9.24-dien-21-oic acid

3α-Hydroxylanosta-8.24-dien-21-oic acid

Figure 4.8: Chemical structural difference between 3β-hydroxylanosta-9,24-dien-21-oic acid (MS/12) and 3α-hydroxylanosta-8,24-dien-21-oic acid.

Compound MS/12 was obtained as white flakes (paper-like solids) with melting point 134-136^{o}C. The IR spectrum showed absorption band for the hydroxyl group at 3360 cm^{-1} (Appendix E). The data suggested the molecular formula $C_{30}H_{47}O_3$**.**

The ^{1}H-NMR of the isolated compound MS/12 (figure 4.8) followed by the triterpenoid pattern with a large clusters of signals of CH_3, CH_2 and CH between the δ 2.5 and 0.8. The ^{13}C-NMR did help to further analyse the compound. The presence of four olefinic carbons between 145-118 ppm, a carboxylic carbon, and five quaternary carbons assisted in suggesting a lanosteryl skeletal structure (Vinken *et al*., 2007). Detailed assignment of the ^{13}C-NMR and significant ^{1}H-NMR is presented in Table 4.9. These data were compared to those in literature of a 3α-Hydroxylanosta-8,24-dien-21-oic acids (Keller *et al*., 1996).

4.8.1 Antioxidant activity of the isolated compounds (MS/10 and MS/12)

The isolated compounds (MS/10 and MS/12) were investigated for their antioxidant activities. The results of their DPPH scavenging activity and reducing power are given in Figure 4.9 and 4.10 respectively.

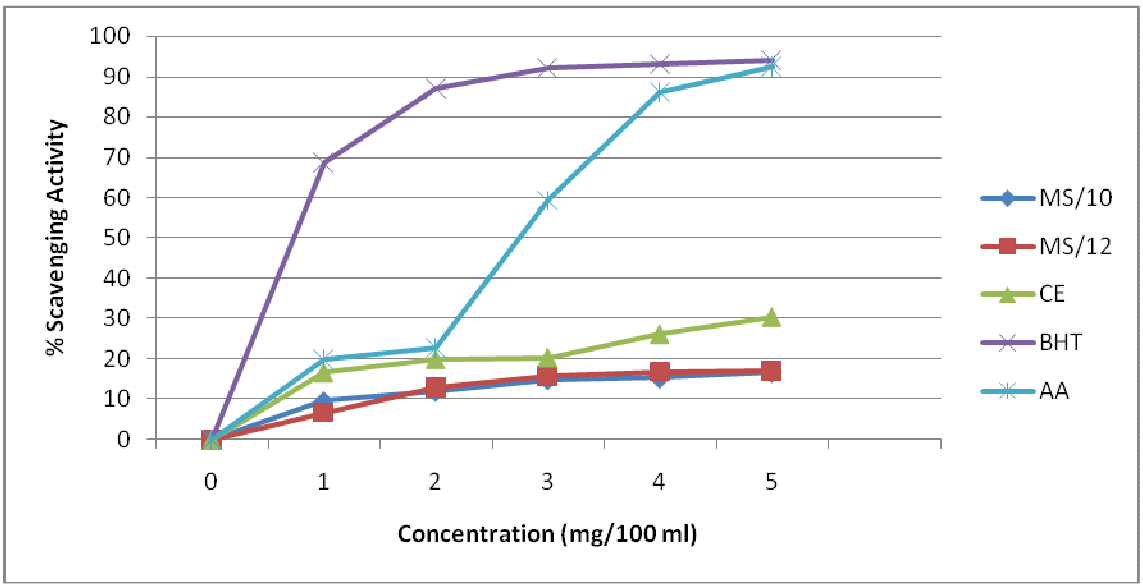


Figure 4.9: DPPH radical scavenging activity of MS/10 and MS/12 from the crude chloroform extract (CE) of *P longifolia*. BHT and AA were used as standard antioxidants. *Data is expressed as mean ± SEM, (n=3). * p<0.05 compared to control.*

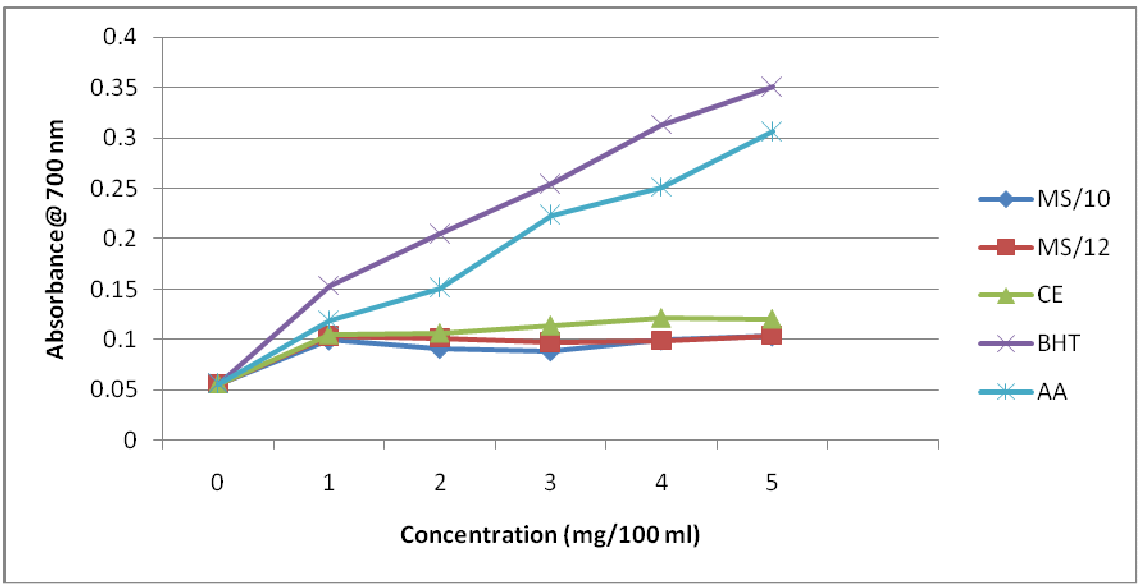


Figure 4.10: Reduction potential of the compounds MS/10 and MS/12 isolated from the crude chloroform extract of *P longifolia*. CE was used for comparison along with BHT and AA as standard antioxidants. *Data are expressed as mean ± SEM, (n=3). * p<0.05 compared to control.*

4.8.2 Cytotoxicity of MS/12

The cytotoxicity of the compound MS/12 was tested on HEK293 and HEPG2 cells. The results are given in Table 4.10.

Table 4.10: Inhibitory activity (%) of cell growth of MS/12 on HEK293 and HEPG2 cells.

Compound (µg/200µl)	HEK293	HEPG2
0	0.00 ± 2.59	0.00 ± 2.23
50	2.20 ± 2.55	-6.00 ± 4.66
100	-1.50 ± 0.62	1.40 ± 2.50
150	2.00 ± 1.80	11.2 ± 1.31
200	10.6 ± 2.30	12.2 ± 2.83
250	10.5 ± 3.08	19.3 ± 2.47
300	26.0 ± 3.48	22.0 ± 4.94
350	37.2 ± 3.46	26.2 ± 1.97
IC_{50} (µg/ml)	**8520**	**7960**

Control (cells without the compound) is represented by 0 µg/200µl. *Values are expressed as mean ± SEM, (n=3).*

The compound exhibited weak concentration dependent cytotoxic effects on HEK293 and HEPG2 cells as it inhibited cells growth only up to 37.2% at 350 µg/200µl.

4.8.3 Anti-inflammatory activity of MS/12

The acute anti-inflammatory activity of the compound MS/12 isolated from the crude chloroform extract was investigated using the carrageenan model. The antiinflammatory activity of MS/10 was not determined (had run short of the sample). The results are presented in Figure 4.11. The compound exhibited anti-inflammatory activity in a concentration and time dependent fashion as it reduced the rat paw oedema volume. The most significant activity ($P<0.05$) relative to the control group was observed after 2 to 3 h with the 500 mg/kg sample. A similar response was observed in the reference drug treated group.

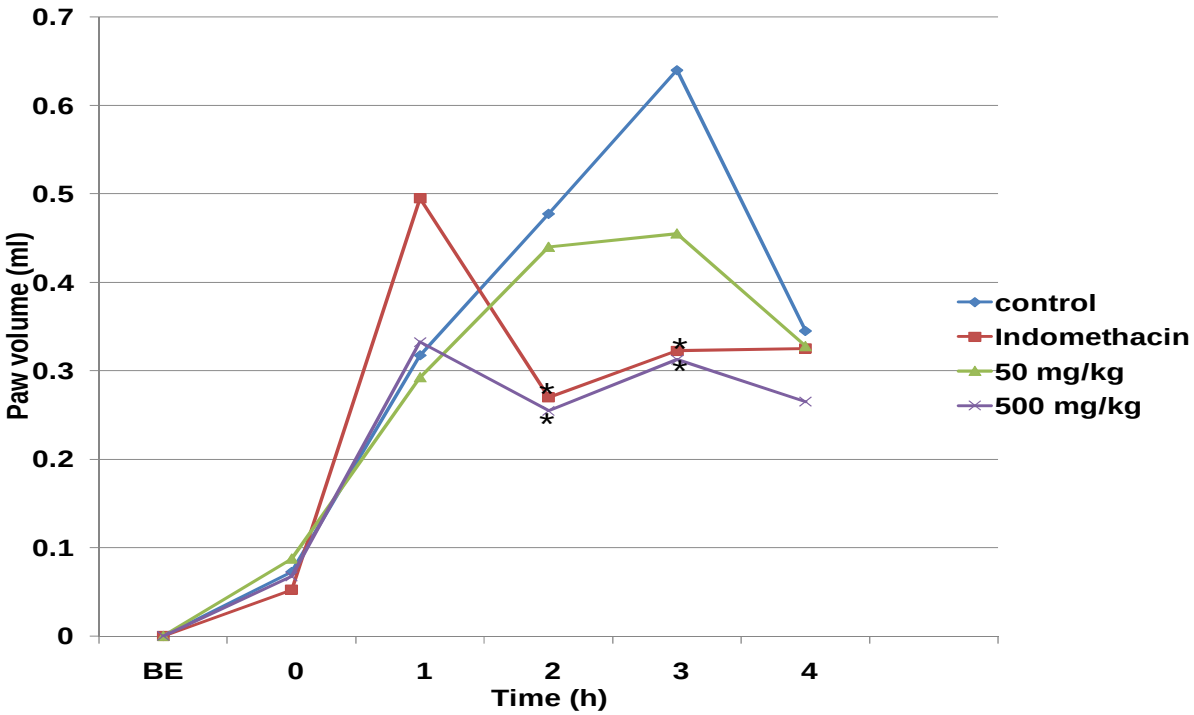


Figure 4.11: Effects of MS/12 from the chloroform extract of *P longifolia* on carrageenan-induced paw oedema in rats. Paw oedema volume was measured immediately before and at 1h interval for 4h following carrageenan injection. Data is expressed as mean ± SEM, (n= 4). ***** $p<0.05$ compared to the control group. (BE-before experiment).

4.8.4 Anti-platelet aggregation activity of MS/10 and MS/12

The anti-platelet aggregation activity of the tritepenes MS/10 and MS/12 was investigated on thrombin, ADP and epinephrine induced rat platelet aggregation. The inhibitory activity of MS/12 was also investigated on arachidonic acid-induced platelet aggregation. The results are presented in Figure 4.12 as percentage inhibition of aggregation.

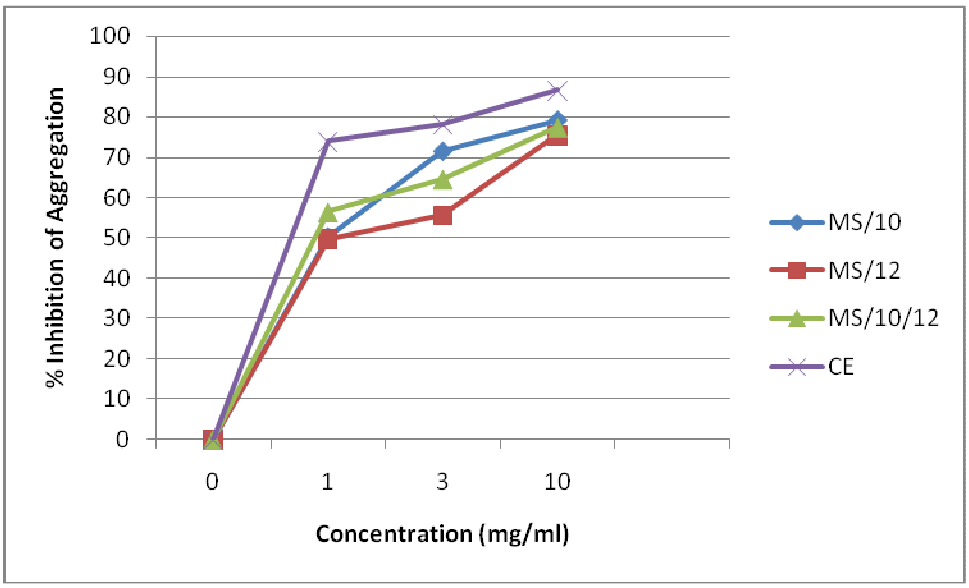


Figure 4.12 a: Effects of MS/10 and MS/12 on the thrombin-induced rat platelet aggregation. The crude chloroform extract (CE) and the combined mixture of the two compounds were used for comparison. The extracts exhibited varying IC_{50} values (mg/ml) of MS/10 (0.99), MS/12 (1.04), MS/10/12 (0.88) and CE (0.67). Data is expressed as mean ± SEM, (n=3).

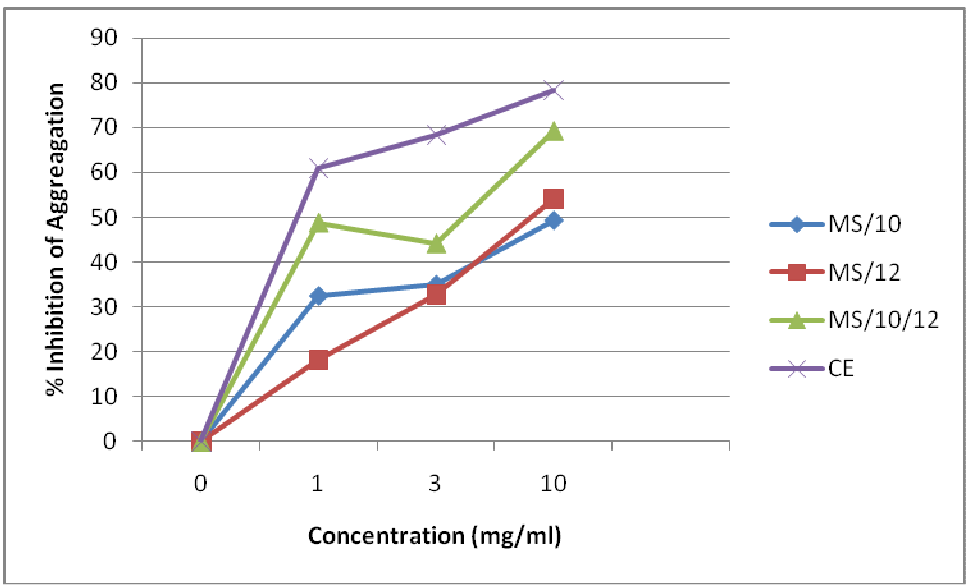


Figure 4.12 b: Effects of MS/10 and MS/12 on the ADP-induced rat platelet aggregation. The chloroform extract (CE) and the combined mixture of the two compounds were used for comparison. The IC_{50} (mg/ml) of CE (0.84) was lower than those of MS/12 (8.54), and MS/10/12 (4.53). Data is expressed as mean ± SEM, (n=3).

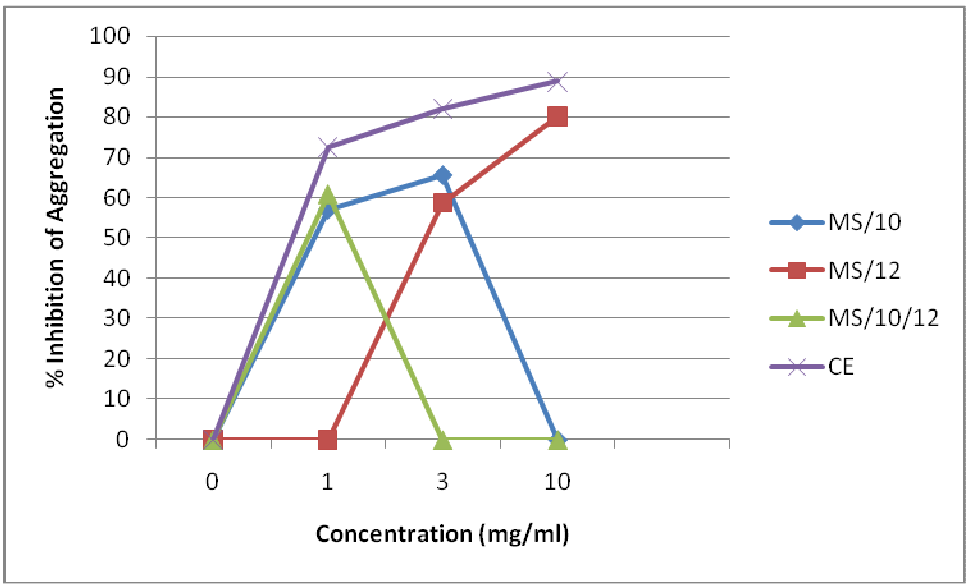


Figure 4.12 c: Effects of MS/10 and MS/12 on the epinephrine-induced rat platelet aggregation. The crude chloroform extract (CE) and the combined mixture of the two compounds were used for comparison. The IC_{50} (mg/ml) of CE (0.72) and MS/10 (0.88) were higher than that of MS/12 (2.70). Data is expressed as mean ± SEM, (n=3).

The compounds showed a concentration dependent anti-platelet aggregation activity on the aggregation induced by the three platelet agonists. Though the crude extract (CE) showed the highest inhibitory activity, there was no significant difference observed between the activity of the individual compounds and when combined (MS/10/12). MS/12 showed a weak inhibition (33.6%) only at the highest concentration (10 mg/ml) on the arachidonic acid induced platelet aggregation.

4.8.5 *In vitro* anticoagulant activity of MS/12

The anticoagulant activity of MS/12 was also determined on the rat whole blood (Figure 4.13); due to the shortage of the sample, the anticoagulant activity of MS/10 was not investigated.

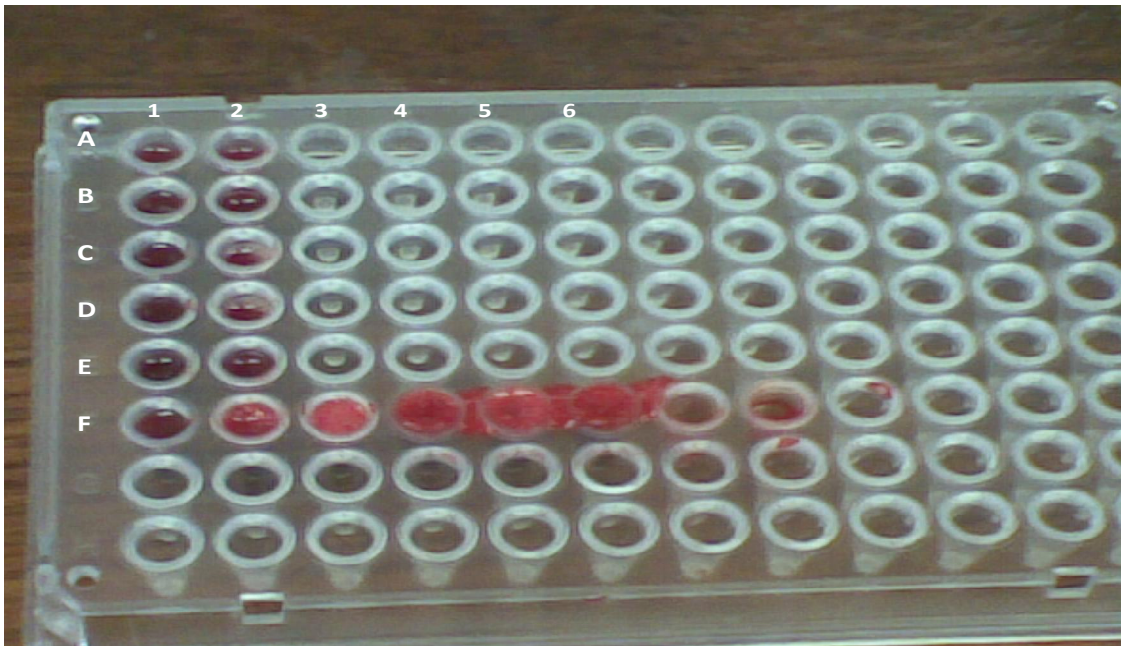


Figure 4.13: The anticoagulant activity of MS/12 on the rat whole blood. The blood was added to A (1% DMSO-blank); B (thrombin); C (ADP); D (epinephrine); E (arachidonic acid); and F_{1-6} (compound at 1, 3 and 10 mg/ml). The experiment was done in duplicates.

The compound showed the anticoagulant activity as it delayed blood clotting time in a concentration dependent manner (more than 5 min, wells F_{3-6}) compared to the control groups (wells A-E) in which clotting occurred within a minute.

CHAPTER 5

5.0 DISCUSSION

Blood clotting is a normal and necessary process that needs to be carefully controlled. Platelet aggregation is the key event in the process of blood-clotting under both physiological and pathophysiological conditions. While clotting could be beneficial in stopping bleeding, unchecked internal formation of blood clots could be fatal. Platelets dysfunctions significantly contribute to pathogenesis of cardiovascular diseases (Mekhfi *et al*., 2004; El Haouari *et al*., 2007). Different therapies are made available to prevent irregular activation and aggregation of platelets. But despite their availability atherothrombotic diseases continue to pose a threat to human health.

The results obtained from this study suggest that the medicinal plant, *P. longfolia*, possesses the ability to inhibit the aggregation of platelets: The extracts of the plant inhibited the activity of thrombin on the artificial substrate (chromogenix), and then also inhibited rat platelets aggregation induced with thrombin, ADP and epinephrine (Table 4.5 and Figure 4.3). It is noteworthy that the inhibition of platelet aggregation was accompanied by the inhibition of lipid peroxidation (Figure 4.5), a process that is associated with blood clotting (Tomita *et al*., 1983). Reduction in MDA formation suggests the potential of the extracts to inhibit phospholipid degradation, cyclooxygenase and thromboxane synthase**;** this is also characteristic of non-steroidal anti-inflammatory drugs (Kosela *et al*., 1986). The ability of the plant extracts to increase the thrombin-induced clotting time (Table 4.7a) suggests that they can substantially decrease the formation of fibrin resulting in a weak clot. Thrombin-clotting time measures time taken by thrombin to catalytically convert fibrinogen to fibrin clot (Gou *et al.*, 2003).

The proteolytic enzymes (trypsin, bromelain and papain) catalytically hydrolyze proteins (fibrinogen) into smaller fragments (fibrin monomers) with generation of new functional groups. The formation and exposure of the new functional groups stimulates aggregation (clot formation). The degree to which the extracts inhibited the aggregation of the enzyme-treated platelets (Table 4.6 and Figure 4.4) does suggest that the extracts may not only be inhibiting thrombin (Figure 4.3) and the

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; Metzig *et al*. 1999).

The anti-platelet aggregation activity was observed mainly in the extracts of the non-polar solvents with the hexane extract showing the highest activity (Table 4.5a). The results indicate that the most active anti-platelet aggregation compounds in the plant extracts could be non-polar in nature. Knowledge of phytochemical composition of plant understudy is important in understanding its biological or medicinal activities. The higher anti-platelet aggregation activity of the hexane and chloroform extracts could partly be attributed to their high phenolic and flavonoid content (Figure 4.1). Various researchers (Rein *et al*., 2000; Mekhfi *et al*., 2006; Tognolini *et al*., 2006; Kim and Choi, 2008) have reported the beneficial effects of these compounds on platelet functions. The flavonoid isolated from *Urtica dioica* inhibited thrombin, ADP, epinephrine and collagen induced platelet aggregation (El Haouari *et al*., 2006). However, the synergistic effects of the other components cannot be ruled out, since alkaloids also have anti-platelet aggregation activity (Cheng *et al*., 1996; Teng *et al*., 1997; Huang *et al*., 2008)

The anticoagulant or anti-platelet aggregation activity of tannins has been demonstrated by various researchers (Dong *et al*., 1998; Mekhfi *et al*., 2006; Kee *et al.,* 2008). Except the ethyl acetate extract that completely lost its activity, the reduced activity of the other tannin-free extracts (Table 4.5) was an indication of synergistic effect of the plant components, and also that the observed activity of the extracts was due to components other than tannin.

The high and consistent anti-platelet aggregation activity exhibited by the chloroform extract led to the isolation from this extract of the two lanosteryl triterpenes- 3-oxo-5α-lanosta-8,24-dien-21-oic acid (MS/10), 3β-hydroxylanosta-9,24-dien-24-oic acid (MS/12). To the best of this researcher's knowledge, this is the first time these compounds are being isolated from this plant species. Despite the fact that the activity of the isolated compounds was relatively not as high as those of the crude

extracts, the isolated triterpenes also showed good anti-platelet aggregation activity induced by the three platelet agonists (Figure 4.12). A number of triterpenoids from various plants have been reported to have anti-platelet aggregation activity against thrombin, ADP and epinephrine (Jin *et al*., 2004; Yang *et al*., 2009; Sankaranarayanan *et al*., 2010). The lower activity relative to the crude extract could indicate the synergistic effect with other compounds, decomposition during fractionation, or removal of protective matrix.

The ability of the compound MS/12 to inhibit the arachidonic acid-induced platelet aggregation indicates its potential to be developed into a good pharmacological anti-platelet and anti-inflammatory drug. Arachidonic acid-induced platelet aggregation is mediated by thromboxane A_2 and prostaglandin H_2 (Parise *et al.*, 1984). Consequently the prostaglandin H_2 is also a precursor for the prothrombotic thromboxane A_2. The efficiency of the MS/12 to prevent blood-clotting is further evidenced by its ability to considerably delay the *in vitro* clotting time of the rat whole blood (Figure 4.13).

In addition to the known physiological platelet agonists, free radicals and inflammation are some of the factors that significantly contribute to irregular platelet aggregation. Free radicals are implicated in various diseases including cardiovascular diseases (Atawodi, 2005). They stimulate platelet aggregation by interfering with several key steps of platelet functions (Ambrosio *et al*., 1997; Bakdash and Williams, 2008). Antioxidants, as the name implies, have the ability to combat the destructive effects of free radicals. Krotz *et al*. (2004) and Sobotkvá *et al.* (2009) have reported the beneficial effects of antioxidants on the inhibition of platelet activation and aggregation. Furthermore, some plants (*Hemidesmus indicus* (Mary *et al*., 2003), *Salvia miltiorrhiza* (Lin and Hsieh, 2010); *Hippophea rhamnoides* (Anjana *et al.,* 2010)) with antioxidant activity have shown anti-platelet aggregation activity. Since the active crude extracts and the pure compounds exhibited poor antioxidant activity (Table 4.2; Figures 4.2, 4.9 and 4.10), and the methanol extract which exhibited a good antioxidant activity poorly inhibited platelet aggregation (Table 4.5), it was apparent that the anti-platelet aggregation activity of the plant components was not primarily due to antioxidant activity. It is thus apparent that the inhibition of MDA production associated with the observed platelet aggregation was not the

results of the inhibition of oxidation, but rather the direct inhibition of clot formation. Zhou *et al*. (2008) also reported weak free radical scavenging activity exhibited by the lanostane-type triterpenes isolated from *Poria cocos.* Some triterpenes are, however, known to exhibit antioxidant activities (Sekiya *et al*., 2003; Russel and Paterson, 2006).

Inflammation suppresses the natural anticoagulant system which favours the expression and synthesis of prothrombogenic molecules (Verhamme and Hoylaerts (2009). Carrageenan-induced paw oedema is believed to involve two phases in which the first phase (1 h) involves the release of inflammatory mediators, histamine and serotonin while the second phase (over 1 h) is mediated by prostaglandins (Vinegar *et al*., 1969). The carrageenan-induced paw oedema model is suitable for the evaluation of acute anti-inflammatory agents acting by inhibition of cyclooxygenase (COX) which is involved in the synthesis of prostaglandins (Seibert *et al*., 1994; Mossa *et al*., 1995; Sawadogo *et al*., 2006). The significant anti-inflammatory activity exhibited by MS/12 at 2- 3h (Figure 4.11) is typical of inhibitors of arachidonic acid metabolites synthesis. Since the compound inhibited the inflammatory process in a way similar to that of the NSAID (indomethacin) known to be COX-1 and 2 inhibitor, the compound could possibly be exerting its therapeutic activity through the inhibition of COX-1 and/or COX-2 (Esteves *et al*., 2005).

There is a large body of evidence on the anti-inflammatory activities of triterpenes (Ríos *et al*., 2000*,* Biswas *et al*., 2009). Most importantly the anti-inflammatory activity of lanostane-type-triterpenoids has been reported (Shiao *et al.,* 1994*;* Siqueira *et al*., 2007; Ko *et al*., 2007)*.* It is apparent that the anti-platelet aggregation activity of the isolated compound (MS/12) could partly contribute to its anti-inflammatory activity. Also based on the overlap between mediators of inflammation, pain and fever the isolated triterpene may possibly have other biological activities such as analgesic, antipyretic and also acetylcholinestrase inhibitory activity.

Depending on the intended biological or medicinal activity, a good drug has to be active, but not toxic. There is little to 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 shrimps (*Artemia salina*) are fast growing organisms commonly used in the preliminary screening of cytotoxicity of plant extracts. Owing to the correlation that has been observed between cytotoxicity of plant extracts against brine shrimp and cell line assay, the extracts that show activity on brine shrimp are recommended for a further analysis on cell lines (Ripa *et al*., 2009). The cell lines are considered a good replacement of animal based methods (Betrabet *et al*., 2004). The hexane and chloroform extracts showed weak cytotoxic effects (Table 4.4). According to Rieser *et al.* (1996) only extracts with LC_{50} values less than 250 µg/ml are considered significantly active. The little to no toxicity of the extracts of *P. longifolia* encourages their use in the concoctions that manage blood clotting related diseases.

Furthermore, the cytotoxic effect of the compound (MS/12) isolated from the chloroform extract was investigated on the human embryonic kidney (HEK 293) and hepatocellular carcinoma (HEPG2) cells. Despite the number of reports on the considerable cytotoxicity of triterpenes (Lee *et al*., 2007; Peteros and Uy, 2010), the isolated triterpene exhibited weak cytotoxic effects on HEK293 and HEPG2 (IC_{50} 8520 and 7960 µg/ml respectively). According to the American National Cancer Institute guidelines, a compound is considered significantly active with IC_{50} value less than 30µg/ml (Suffness and Pezzuto, 1990). The results also encourage the development of this compound into a pharmacological anti-platelet aggregation drug.

While triterpenes are considered potential anticancer agents (Lin *et al*., 2003; Gonzalez *et al*., 2002; Su *et al.,* 2000; Zhou *et al*., 2008; Cheng *et al*., 2010) the isolated compound's weak inhibition of hepatocellular carcinoma growth may indicate lack of anticancer properties. An insignificant cytotoxicity of some lanostane triterpenoids against tumour cell lines has also previously been reported (Kima *et al.*, 2004; Shao *et al*., 2005).

Overall the results revealed that the organic crude extracts and the two isolated triterpenes have anti-platelet aggregation activity. Their anti-platelet aggregation activity could be exerted through variable mechanisms other than antioxidant activity. The significant anti-inflammatory activity exhibited by 3β-hydroxylanosta-9,24-dien-

24-oic acid suggests a link between anti-platelet aggregation and anti-inflammation of the compound. Like aspirin, anti-platelet aggregation agents can also inhibit inflammation. Most importantly the weak cytotoxicity exhibited by the crude extracts and the isolated triterpene suggest their safe use in the management of blood clotting related diseases.

Medicinal plants are undoubtedly rich sources of biologically active compounds vital to human health. If used properly medicinal plants are a solution to a wide range of diseases including atherothrombotic diseases. The results of this work scientifically validate the traditional use of *P. longifolia* in the management of blood clotting related diseases. Considering the increasing demand on the discovery and development of new pharmacologically active drugs to substitute the currently used anti-platelet aggregation drugs, the results reported in this dissertation suggest the potential use of these compounds in the management of blood-clotting related diseases.

CHAPTER 6

6.0 CONCLUSION

The results from this study suggest that the organic extracts and the two lanosteryl triterpenes (3-Oxo-5α-lanosta-8,24-dien-21-oic acid (MS/10) and 3β-Hydroxylanosta-9,24-dien-24-oic acid (MS/12)) from *P. longifolia* have anti-platelet aggregation activity. The active components are poor antioxidants which suggest that mechanisms other than antioxidant activity could be mediating their anti-platelet aggregation activity. The anti-platelet aggregation activity of MS/12 could be linked to its anti-inflammatory activity which is indicative of cyclooxygenase inhibitor. The two triterpenes could be potential pharmacologically active anti-platelet aggregation agents.

The results suggest rationale for the use of *P. longifolia* 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 studies

i. *In vivo* anticoagulant activity study is necessary to further confirm the anticoagulant activity efficiency of the isolated compounds.

ii. It is recommended that the mechanism(s) through which the compounds exert their therapeutic effects be elucidated.

iii. It is also important to evaluate other biological activities such as analgesic, antimicrobial and acetylcholinesterase inhibitory activity.

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APPENDIX A

DETAILS OF REAGENTS

A1 Mayer's reagent

Solution A: 1.36 g mercury (II) chloride was dissolved in 60 ml of distilled water.
Solution B: 5.0 g potassium iodide was dissolved in 20 ml of distilled water.
Both solutions were mixed and the mixture was made up to 100 ml with distilled water.

A2 Dragendorff's reagent

Solution A: 0.85 g of subnitrate bismuth and 20 ml of glacial acetic acid were dissolved in 40 ml of distilled water.
Solution B: 0.8 g potassium iodide was dissolved in 20 ml of distilled water.
Solutions A and B were stored separately in dark bottles. Just before use, 5 ml of solution A, 5 ml of solution B and glacial acetic acid were mixed and made up to 100 ml with distilled water in a volumetric flask.

A3 Resuspending buffer (pH 7.4)

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

A4 Washing buffer (pH 6.5)

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

A5 Tris buffer (pH 7.4) containing EDTA and NaCl

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

A6 Phosphate buffer (pH 6.6)

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

A7 ADA (acid-dextrose-anticoagulant)

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

A8 1% TBA

50 ml of glacial acetic acid was made up to 100ml with distilled water and 1 g of TBA was dissolved and the solution was made up to 100 ml.

A9 $ABTS^+$

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

A10 2 % $AlCl_3$

80 ml ethanol was made up to 100 ml with distilled water.
2 g $AlCl_3$ was dissolved in 50 ml of 80 % ethanol and was made up to 100 ml with 80 % ethanol.

A11 Artificial sea water

120 g of sea salt was dissolved in 4 L of distilled water (3:1).

A12 1% Carrageenan solution

2 g Na_2CO_3 was dissolved in distilled water and made up to 100 ml with distilled water.
1 g carrageenan was dissolved in 2% Na_2CO_3 and was made up to 100 ml with 2% Na_2CO_3.

APPENDIX B

DETAILS OF METHODOLOGY

B1 Extraction

Stem bark of *Protorhus longifolia* was washed, air-dried and ground to powder (2mm mesh). The powdered plant material (40 g) was extracted sequentially with hexane, chloroform, ethyl acetate, methanol and water in a platform shaker machine for 24 h per each solvent, at room temperature (157 rpm). The ratio of the plant material to the solvent was 1:5. A separate extraction with methanol only was also carried out with 20 g of the plant material to obtain a separate crude methanol extract. The extracts were separately filtered through Whatman no.1 filter paper. Except for the aqueous extract, all the other extracts were concentrated *in vacuo* at 37 ^{o}C ± 2 ^{o}C. The water extract was freeze dried. The extracts were stored in sterile glass bottles and kept in the fridge (4^{o}C) until used.

B2 Phytochemical screening

B2.1 Test for saponins

The plant material (2.5 g) was extracted with boiling water and was allowed to cool. The extract was shaken vigorously to froth and then allowed to stand for 15 – 20 min. The extract was then classified for saponin content as follows: no froth = negative (no saponins) and froth less than 1 cm = weakly positive (saponins present); froth 1.2 cm high = positive; and froth greater than 2 cm high = strongly positive.

B2.2 Test for tannins

The plant material (0.5 g) was boiled with 10 ml of water for 15 min, filtered and made up to 10 ml with distilled water. Two millilitres of the filtrate was put into another test tube and a few drops of 0.1 % $FeCl_3$ solution were added to the 2 ml of the filtrate. Black-blue, green or blue-green precipitate was taken as preliminary evidence of the presence of tannins.

B2.3 Test for steroids

Acetic anhydride (2 ml) and concentrated sulphuric acid (2 ml) were added to 0.5 g of the plant material and mixed. A colour change from violet to blue or green was taken as evidence of the presence of steroids.

B2.4 Test for terpenoids (Salkwoski test)

The plant material (0.5 g) was mixed with 2 ml of chloroform, and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish-brown colouration of the interface was taken as evidence of the presence of terpenoids.

B2.5 Test for alkaloids

The plant material (0.5 g) was dissolved in 5 ml 1 % HCl (aq). The solution was stirred on steam bath and filtered. One millilitre of the filtrate was treated with Mayer's reagent. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent and turbidity or precipitate was also taken as evidence of the presence of alkaloids.

B2.6 Test for anthraquinones

The plant material (0.5 g) was dissolved and shaken with benzene. The mixture was filtered and 5 ml of 10 % ammonium solution was added to the filtrate. After shaking, the presence of a pink, red, or violet colour in ammonia solution (lower phase) was taken as evidence of the presence of anthraquinones.

B2.7 Test for flavonoids

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

B2.7.1 Lead acetate test

The plant material (1 g) was mixed with 1 ml of 10 % lead acetate. A reddish-brown colouration or precipitate was taken as an evidence of the presence of flavonoids.

B2.7.2 Ferric chloride test

The plant material (1 g) was mixed with 1 ml of $FeCl_3$. A dark brown or dirty brown precipitate was taken as evidence of the presence of flavonoids.

B2.7.3 Sodium hydroxide test

The plant material (1 g) was mixed with 1 ml of dilute NaOH. A golden yellow precipitate was taken as evidence of the presence of flavonoids.

B2.8 Test for cardiac glycosides

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

B2.8.1 Lieberman's test

The plant material (0.5 g) was mixed with 2 ml of acetic acid and this was cooled well in ice. Concentrated sulphuric acid (1 ml) was carefully added down the sides. Colour change from violet to blue to green was taken as an indication of the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycoside.

B2.8.2 Salkowski test

The plant material (0.5 g) was mixed with 2 ml of chloroform, and 2 ml of concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at interface was taken as an indication of the presence of a steroidal ring i.e. aglycone portion of the cardiac glycoside.

B2.8.3 Keller-Kiliani test

The plant material (0.5 g) was dissolved in 2 ml of glacial acetic acid containing 1 drop of 10 % of $Fecl_3$ solution. One milliliter of concentrated sulphuric acid was carefully added on the side of the tube to form a lower layer. A brown ring at interface was taken as an indication of the presence of a deoxy sugar characteristic of cardenolides. Also a violet ring below the brown ring or a greenish ring just above the brown ring (i.e. in the acetic acid layer) and gradually spread through the layer was taken as an indication of the presence of cardiac glycosides.

B2.9 Test for phlobatannins

The plant material (2.5 g) was extracted with boiling water. The extract was boiled with 1 % HCl (aq). Deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

B3 Total phenolic content (gallic acid equivalent - GAE)

Different concentrations of gallic acid (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethyl ether) were prepared. Each plant extract (0.5 ml) was dissolve in 1 ml of diethyl ether and mixed. The diethyl ether was evaporated off to leave behind the residues. Sodium carbonate (7.5 g/100ml) was prepared and Folin-ciocalteus' phenol reagent (FC) was diluted with distilled water (1:10).

Test tubes were set in duplicates. To each test tube containing the residue, 1.5 ml FC and 1.2 ml Na_2CO_3 were added and they were well mixed to obtain solutions. The solutions were kept in the dark for 30 min. Absorbance of the blue coloured mixtures was read at 765 nm with the mixture of FC and Na_2CO_3 used as blank. The results were recorded and they were translated into a standard curve of absorbance (nm) versus concentration of gallic acid (mg/ml). See Figure B1.1. The total phenolic content of the extracts was calculated as gallic acid equivalent from the calibration curve of gallic acid and expressed as mg/g dry plant material. The experiment was replicated twice.

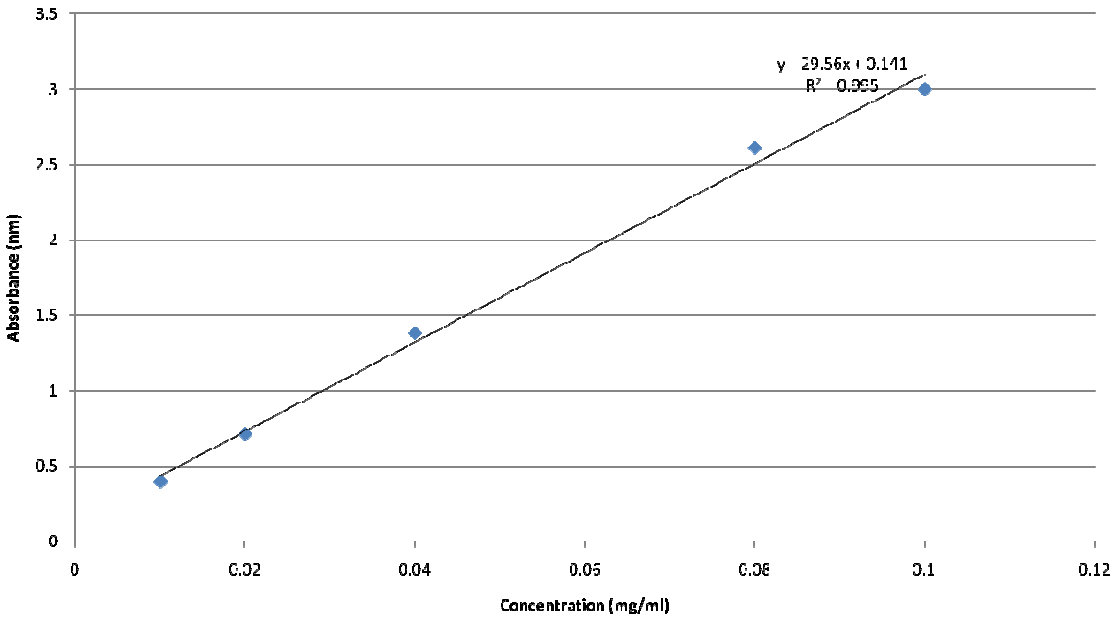

Figure B1.1: Calibration curve of gallic acid concentration (mg/ml) against absorbance (nm). The curve was used to determine the total phenolic content of the extract of *P. longifolia* as gallic acid equivalent.

B4 Flavonoid content (quercetin equivalent – QE)

Different concentrations of quercetin (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethyl ether) were prepared. Each plant extract (0.5 ml) was dissolve in 1 ml of diethyl ether and mixed. The diethyl ether was evaporated off to leave behind the residues.

$AlCl_3$ ethanol solution (2%) was prepared. Test tubes were set in duplicates. The residues were dissolved in 0.5 ml 2% $AlCl_3$ ethanol solution. The solutions were allowed to stand for 1h at room temperature (a yellow colour indicated presence of flavonoids). Absorbance was read at 420 nm against a reagent blank (2% $AlCl_3$ ethanol solution). The flavonoid content of the extracts was determined as quercetin equivalent from the calibration curve of quercetin (figure B1.2) and expressed as mg/g dry plant material.

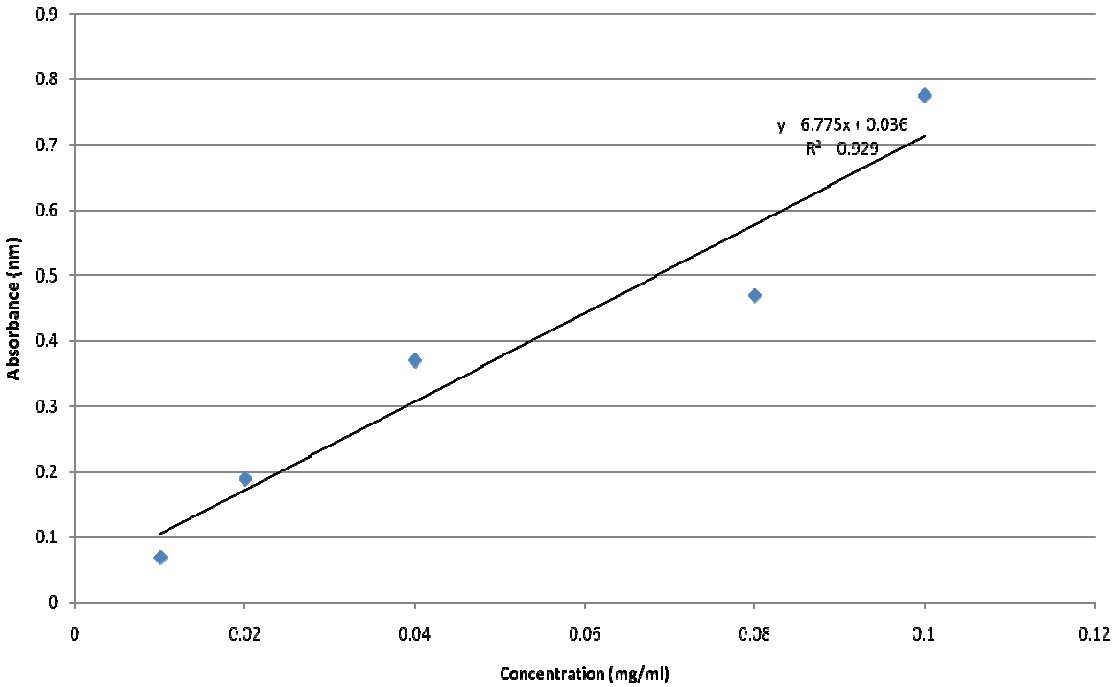

Figure B1.2: Calibration curve of quercetin concentration (mg/ml) against absorbance (nm). The curve was used to determine the flavonoid content of the extracts of *P. longifolia* as quercetin equivalent.

B5 Antioxidative Activity *In vitro*

B5.1 Free radical scavenging

B5.1.1 DPPH assay

DPPH (2 mg/100ml) and different concentrations of the extracts (0 – 5 mg/100 ml) were prepared in methanol. Six test tubes were set in duplicate (12 test tubes) for each extract.

Two millilitres of DPPH (purple) was put into each test tube and 2 ml of each extract was added into the corresponding test tubes and mixed. The mixture was allowed to stand for 30 – 60 min with interval mixing. Decolourisation of the DPPH indicated scavenging activity of the extracts which was determined by reading absorbance at 517 nm. Methanol was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards.

Percentage scavenging activity was calculated using the following formula;

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

Where, A_t =absorbance of test, A_0 = absorbance of control.

Graphs of percentage scavenging activity versus concentration of extract (mg/100ml) were constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B5.1.2 ABTS assay

ABTS (7 mM) was prepared in 3 ml of distilled water and mixed with 2.45 mM potassium persulfate. The mixture was incubated at room temperature in the dark for 16 h. The working solution was diluted with methanol (1 ml $ABTS^+$: 60 ml CH_3OH). The test tubes were set in duplicates (12 test tubes) for each extract.

One ml of the crude extract (0-5 mg/100 ml CH_3OH) was added to 1 ml of 7 mM $ABTS^+$ (green) in the corresponding test tubes and the samples were thoroughly mixed. The mixture was allowed to stand for 6 min. Decolourisation of the $ABTS^+$ indicated scavenging activity of the extracts which was determined by reading absorbance at 734 nm. Methanol was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards. Percentage scavenging activity of the extracts was calculated using the formula:

$$\% \text{ scavenging activity} = (1 - A_t/A_0)100$$

Graphs of percentage scavenging activity versus concentration of extract (mg/100ml) were constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B5.2 Reducing power

The different concentrations (0 – 5 mg/100 ml CH_3OH) of the extracts, PB, 1 % PF, 10 % TCA and 0.1 % $FeCl_3$ were prepared. Test tubes were set in duplicates for each extract.

One millilitre of the extract was mixed with 2.5 ml PB and 2.5 ml PF. The mixture was incubated for 20 min at 50^oC. TCA (2.5 ml) was added to the mixture and this was well mixed. After 5 – 10 min, the mixture was centrifuged at 1000 rpm for 10 min. Supernatant (2.5 ml) was collected and diluted with 2.5 ml of distilled water.

$FeCl_3$ (0.5 ml) was added and well mixed with the supernatant. The reducing power was determined spectrophotometrically at 700 nm and distilled water was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards. The graphs of absorbance (nm) versus concentration of extract (mg/100 ml) were constructed. The reducing power was determined from the graph, the higher the absorbance the higher the reducing power.

B5.3 Chelating activity on Fe^{2+}

The different concentrations (0 – 5 mg/100 ml CH_3OH) of the extracts, 2 mM $FeCl_2$ and 5 mM ferrozine were prepared. Test tubes were set in duplicates for each extract.

One millilitre of extract was diluted with 3.75 ml deionised water and this was mixed with 0.1 of $FeCl_2$ and 0.2 ml of ferrozine. The mixture was allowed to stand for 10 min with interval mixing. The chelating activity was determined by reading the absorbance at 562 nm. Deionised water was used as blank. EDTA and citric acid were used as standards. Percentage chelating activity was calculated using the formula;

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

The graph of percentage chelating activity versus concentration of extract (mg/100ml) was constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B6 Brine shrimp cytotoxicity assay

Brine shrimp eggs were hatched in artificial sea water and incubated at 24-28^oC. 120 g of sea salt was dissolve in 4 L of warm water. This was put in a dark covered container (bucket) and light was provided from one source. 4 g of shrimp eggs was added to the artificial sea water. The other bucket containing fresh water was provided and the million air device was used to aerate the water. After 48 h, the eggs were hatched giving a large number of larvae (brine shrimps) and the brine shrimps were used to for the cytotoxicity assay.

Different concentrations (0, 1, 5, and 25 mg/ml) of the plant extracts were prepared in 1% DMSO. Four petri-dishes were set in triplicates (12 petri-dishes) for each extract. Fifty microlitres (50 µl) of a different concentration of each extract was mixed with 25 ml of artificial sea water in each petri-dish and 10 shrimps were separately put in the corresponding petri-dishes. DMSO (1%) was used as negative control. After 24 h and 48 h, numbers of alive and dead shrimp larvae were recorded and mortality rates were calculated (table B1 and B2). In a case where there was mortality in the control, corrected percentages were calculated using Abbot's formula:

Corrected %= (1- n in T after treatment ***/*** n in Co after treatment) x 100
Where; n = organisms population, T= treated, Co= control

Lethal concentration determination: the lethal concentration of plant extract resulting in 50% mortality of the brine shrimp (LC_{50}) and 95% confidence intervals was determined from the 24 h count using probit analysis.

Table B1.1: Results of brine shrimp lethality test (% mortality rate) after 24 h exposure

(a) Hexane extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	27	19
Dead population after treatment	0	3	11
Percentage mortality (%)	3.33 ± 5.77	10.0 ± 0.00	36.7 ± 15.3

(b) Chloroform extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	28	27	18
Dead population after treatment	2	3	12
Percentage mortality (%)	6.67 ± 5.77	10.0 ± 0.00	40.0 ± 10.0

(c) Ethyl acetate extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	29	27	25
Dead population after treatment	1	3	5
Percentage mortality (%)	3.33 ± 5.77	10.0 ± 0.00	16.7 ± 5.77

(d) Methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	29	28	24
Dead population after treatment	1	2	6
Percentage mortality (%)	3.33 ± 5.77	6.67 ± 11.5	20.0 ± 17.3

(e) Water extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	29	28
Dead population after treatment	0	1	2
Percentage mortality (%)	0.00 ± 1.59	3.33 ± 5.77	6.67 ± 11.5

(f) Total methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	29	27
Dead population after treatment	0	1	3
Percentage mortality (%)	0.00 ± 5.77	3.33 ± 5.77	10.0 ± 17.3

Table B2.2: Results of brine shrimp lethality test (% mortality rate) after 48 h exposure

(a) Hexane extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	17	15	4
Dead population after treatment	13	15	26
Percentage mortality (%)	9.72 ± 8.67	36.2 ± 19.2	81.5 ± 22.0

(b) Chloroform extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	23	19	13
Dead population after treatment	7	11	17
Percentage mortality (%)	2.64 ± 18.4	20.0 ± 11.5	45.8 ± 3.75

(c) Ethyl acetate extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	22	20	19
Dead population after treatment	8	10	11
Percentage mortality (%)	7.87 ± 6.85	16.3 ± 5.17	20.0 ± 11.5

(d) Methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	18	13
Dead population after treatment	9	12	17
Percentage mortality (%)	12.6 ± 1.59	24.8 ± 10.9	28.4 ± 12.4

(e) Water extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	19	18
Dead population after treatment	9	11	12
Percentage mortality (%)	12.6 ± 1.59	28.8 ± 18.8	34.7 ± 20.7

(f) Total methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	20	17
Dead population after treatment	9	10	13
Percentage mortality (%)	12.6 ± 1.59	17.4 ± 9.70	37.0 ± 22.1

B7 Anti-inflammatory activity

Sprague-Dawley rats (weighing 200 ± 20 g) were let to acclimatise for four days before experiment. They were kept and maintained under standard environmental conditions where they had free access to standard diet and water as outlined in the guide for the care and use of laboratory animals.

The rats were orally administered with the compound (50 and 500 mg/kg) daily for four days before the experiment. An hour before the experiment, negative and

positive control animals were administered with 0.5% carboxymethyl cellulose (CMC) and indomethacin (10 mg/kg) respectively. One hour after the last day of administration, all the rats were subcutaneously injected into the plantar surface of the right hind paw with 0.1 ml (1% w/v) carrageenan solution, whereas the left paws were injected with normal saline for comparison.

The animals were divided into four groups of four rats each as follows:
Group I (negative control): received saline solution
Group II (positive control): received indomethacin
Group III (treatment): received 10 mg/200kg compound
Group IV (treatment): received 100mg/200kg compound

The paw volume until knee joint was measured by a water displacement method using a plethysmometer immediately and at 1 h interval for 4 h following the carrageenan injection. The anti-inflammatory activity was calculated by measuring the volume difference between the right and left paws in comparison with the control group. Data was expressed as mean ±SEM and student's t-test was applied to determine the significance of the difference between the control groups and rat treated with the compounds. Values of $p \leq 0.05$ were taken as statistically significant.

B8 Anti-platelet aggregation study

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

B8.1 S-2238

S-2238 (0.008M) was prepared in distilled water and different concentrations of plant extracts (0, 1, 3 mg/ml) dissolved in DMSO were prepared in Tris buffer. A 96-well microtitre plate was used and the experiment was replicated twice. Plant extract (50 µl) was incubated with 10 µl thrombin (30U/ml) in the corresponding wells for 10 minutes at room temperature. S-2238 (190 µl) was added to the wells and the reaction was monitored with Biotek plate reader ELx808 IU using Gen5 software by following change in absorbance at 415 nm for 4 min at 12 sec interval. 1% DMSO was used as negative control.

B8.2 Preparation of Blood platelets

A rat was killed by a nock on the head. Blood was immediately collected from abdominal aorta of the rat and was put in a centrifuge tube containing ADA (1 ml ADA: 5 ml blood). The blood was centrifuged (Eppendorf centrifuge 5804 R) at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively. Supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was discarded and sediment (platelets) obtained was resuspended in 5 ml of washing buffer (pH 6.5). This was centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the platelets were suspended in a little volume of a resuspending buffer (pH 7.4). The platelets were divided into four (4) parts: untreated and enzyme (trypsin, bromelain, and papain) treated platelets. A 1:10 dilution of the platelets in the resuspending buffer was taken.

B8.3 Enzyme treated platelets

An enzyme (0.1 mg) was added to the platelets (25 ml) and this was well mixed and incubated at 25^{o}C for 60 min. A little volume (about 3 ml) of the washing buffer was added. The mixture was centrifuged at 3200 rpm for 15 min and the supernatant was discarded. This (addition of the buffer and centrifuging) was repeated three times. A 1:1 dilution of the treated platelets with a resuspending buffer was done.

B8.4 Anti-platelet aggregation activity

Different concentrations of the plant extracts (1, 3 and 10 mg/ml) dissolved in DMSO were prepared in Tris buffer (pH 7.4). Diluted platelets were mixed with $CaCl_2$ (0.4 ml:10 µl $CaCl_2$). The 96-well micro plate was used in the experiment and all the experiments were replicated twice.

Platelets (200 µl) were pre-incubated with the different concentration of the plant extract in the corresponding well for 5 min at 37^{o}C. Then 20 µl of thrombin (5U/ml) was added to the mixture. The reaction was monitored at 415 nm for 20 min at 30 sec interval with the Biotek plate reader using Gen5 software. Heparin was used as a standard and 1% DMSO was used as negative control. Thrombin plus 1% DMSO was used as positive control.

B8.5 Measurement of MDA

After the anti-platelet aggregation test, 100 µl of the solution from each well was collected and 1.5 ml of trichloroacetic acid (TCA) was added. The mixture was allowed to stand for 10 min and then centrifuged at 3500 rpm for 20 min. Supernatant was collected and 1.5 ml of 2-thiobarbuturic acid (TBA) was added. The mixture was heated in boiling water bath for 30 min and was allowed to cool. Two millilitres of n-butanol was added and this was mixed and allowed to separate. Top layer was collected and the MDA formation was determined with the Biotek plate reader using Gen5 software by reading absorbance at 490 nm. n-butanol was used as blank. Percentage inhibition of MDA formation was calculated;

$$\% \text{ inhibition} = \frac{\Delta A \text{ control} - \Delta A \text{ plant extract}}{\Delta A \text{ control}} \times 100$$

Where, ΔA = change of absorbance

And graphs of % inhibition versus concentration of extract (mg/ml) were constructed. The same method was repeated with ADP and epinephrine on both untreated and enzyme treated platelets.

B8.6 Tannin removal

The plant extracts were prepared at a concentration of 10 mg/ml in distilled water. PVPP was added to the extracts at 10 mg/ml, shaken for 15 min at 4^{o}C, and the mixture was centrifuged at 5000 rpm for 8 min at 4^{o}C. The pellet was discarded and supernatant was collected. Using the supernatant, the procedure was repeated three times so as to remove tannins to a negligible concentration. The tannin-free extracts were re-dissolved in DMSO for use in subsequent tests for the anti-platelet aggregation activity.

B8.7 Thrombin-induced clotting time assay

The different concentrations (1, 3 and 10 mg/ml) of the extract were used in the assay. Platelets (200 µl) were mixed and incubated with 50 µl of the extract at room temperature. Twenty microlitres (20 µl) of thrombin (5U/ml) was added to the mixture and the rate of clot formation was determined with the Biotek plate reader using

Gen5 software by following change in absorbance at 415 nm for 20 min at 30s intervals. The experiment was replicated twice and 1% DMSO was used as a negative control.

B8.8 $CaCl_2$-induced clotting time assay

The different concentrations (1, 3 and 10 mg/ml) of the extract were used in the assay. Platelets (200 µl) were mixed and incubated with 50 µl of the extract at room temperature. Twenty microlitres (20 µl) of 0.16 M $CaCl_2$ was added to the mixture and the rate of clot formation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm for 2 h at 3 min intervals. The experiment was replicated twice and 1% DMSO was used as a negative control.

Calculation of percentage inhibitory effect of plant extracts

All assays were repeated three times and the mean V ± 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.

B9 Isolation, purification and characterisation

B9.1 Isolation and purification

Crude solvents (hexane, dichloromethane, ethyl acetate and methanol) 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) (210 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 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 extract (8.0 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 collection of 20 ml fractions was collected into beakers. Elution was done using hexane:ethyl acetate solvent system starting with 9:1 to 3:7. A total of 119 of 20 ml fractions were collected. TLC analysis of the collected fractions was performed to identify those with common profile. The TLC plates were first viewed under UV, developed using a 10% H_2SO_4 spray reagent and then heated. The fractions with similar profile were combined as RA/04/A (f_1-f_5), RA/04/B (f_6-f_{12}), RA/04/C (f_{13}-f_{19}),…, RA/04/P (f_{108}-f_{119}) to give 16 combined fractions. The combined fractions were separately concentrated *in vacuo* and their weights were determined.

Based on the nature of RA/04/G and RA/04/I, the two compounds were separately dissolved in methanol (RA/04/G) and hexane (RA/04/I) to recrystalise. The mixtures were then separately filtered to obtain residues (RA/04/G_1; RA/04/I_1) and filtrates (RA/04/G_2; RA/04I_2) respectively. The samples were analysed by TLC and the filtrates still contained impurities while both the residues (RA/04/G_1 and RA/04/I_1) showed purity. The pure compounds were subjected to NMR techniques for characterisation and structural elucidation.

B9.2 Structural elucidation

In order to characterise and identify the compounds, RA/04/G_1 (MS/10) and RA/04/I_1 (MS/12) were further analysed using 1D and 2D NMR techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in $CDCl_3$, Bruker 600 MHz with solid state probe), IR (Perkin-Elmer 100 FTIR with ATR sampling accessory), UV ($CHCl_3$, Varian - Cary 50 UV-visible spectrophotometer) and LC-MS (DCM, Waters API Q-TOF Ultima). The molecular formula was identified by ESI-MS (positive mode), $[M + H]^+$. In order to get more information about the structure and composition of compound, fragmentation of the compound was also done. Melting point (Reichert Thermovar) of the compounds was also determined. The resulting spectra were analysed and the compounds were identified by comparing them to similar compounds in literature.

The anti-platelet aggregation, antioxidant and anti-inflammatory activity of the pure compounds were also investigated following the same methods used for the crude extracts. Cytotoxicity of MS/12 was also determined using MTT cell proliferation assay.

B10 MTT cell proliferation assay

Cells were all grown to confluenecy in 25 cm^2 flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities (2.5 x 10^4 cells per well). Cells were incubated over overnight at 37^oC. Medium was then removed and fresh medium (MEM + Gutamax + antibiotics) was added. Compound at different concentrations (50, 100, 150, 200, 250, 300, and 350µg/200µl) was then added in triplicate and the mixture was incubated for 4 hrs. Thereafter medium was removed and replaced by complete medium (MEM + Gutamax + antibiotics + 10% fetal bovine serum). After 48 hrs cells were subjected to the MTT assay.

MTT solution was prepared at a concentration of 5 mg/ ml PBS. At the end of incubation period (48 hrs), the medium was removed from the cells in multiwell plate. MTT solution (200µl) as well as 200 µl of cell culture medium was added to each well containing the cells. The multiwell plate was incubated at 37^oC for 4 hrs. Thereafter the medium and MTT solution were removed from the wells and 100/200/400µl of DMSO was added to each well (stops reaction and dissolves insoluble formazan crystals). The plate was read in a plate reader or spectrophotometer at 570 nm.
The experiment was replicated thrice and the results were expressed as mean ± SD. Percentage inhibition of cell growth was calculated as:

$$\% \text{ cell death} = [(A_c - A_t)/A_c \times 100]$$

Where A_c is the absorbance of control and A_t is the absorbance of the extract.
Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics programme.

APPENDIX C

C1. Ethic 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.

RJ Ewan.

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

C2 Interview of Traditional Healers

Research Questionnaires

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	
Female	

15-24	
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) is used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time of collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants	

Preparation Method:

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

b) How is the medicine prepared?

Storage Method:

Dosage:

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

_____

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

_____

c) Are there any known side effects? _____

_____

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

_____

_____

Age Group:

Infants	
Children	
Adults	

APPENDIX D

GRAPHS

D1 Antioxidant Activity- free radical scavenging activity

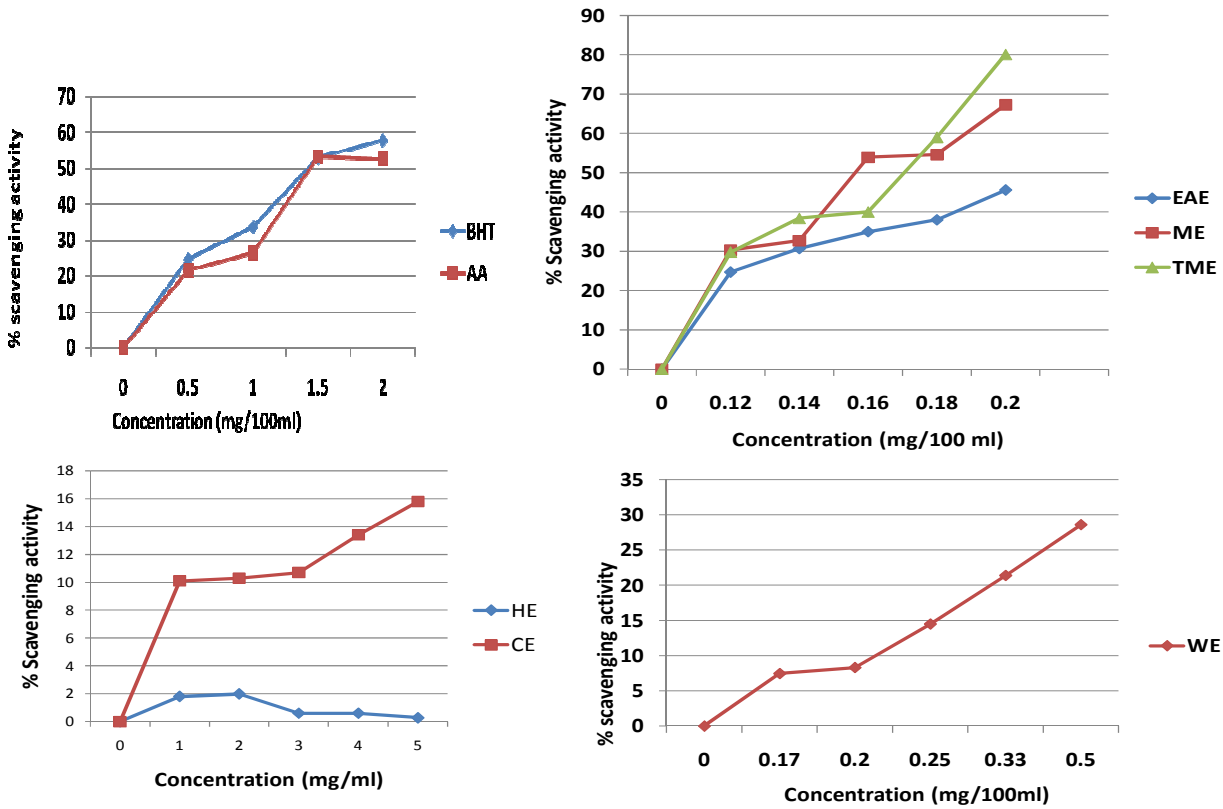

Figure D1.1: Percentage scavenging activity of the extracts of *P longifolia* on DPPH radical

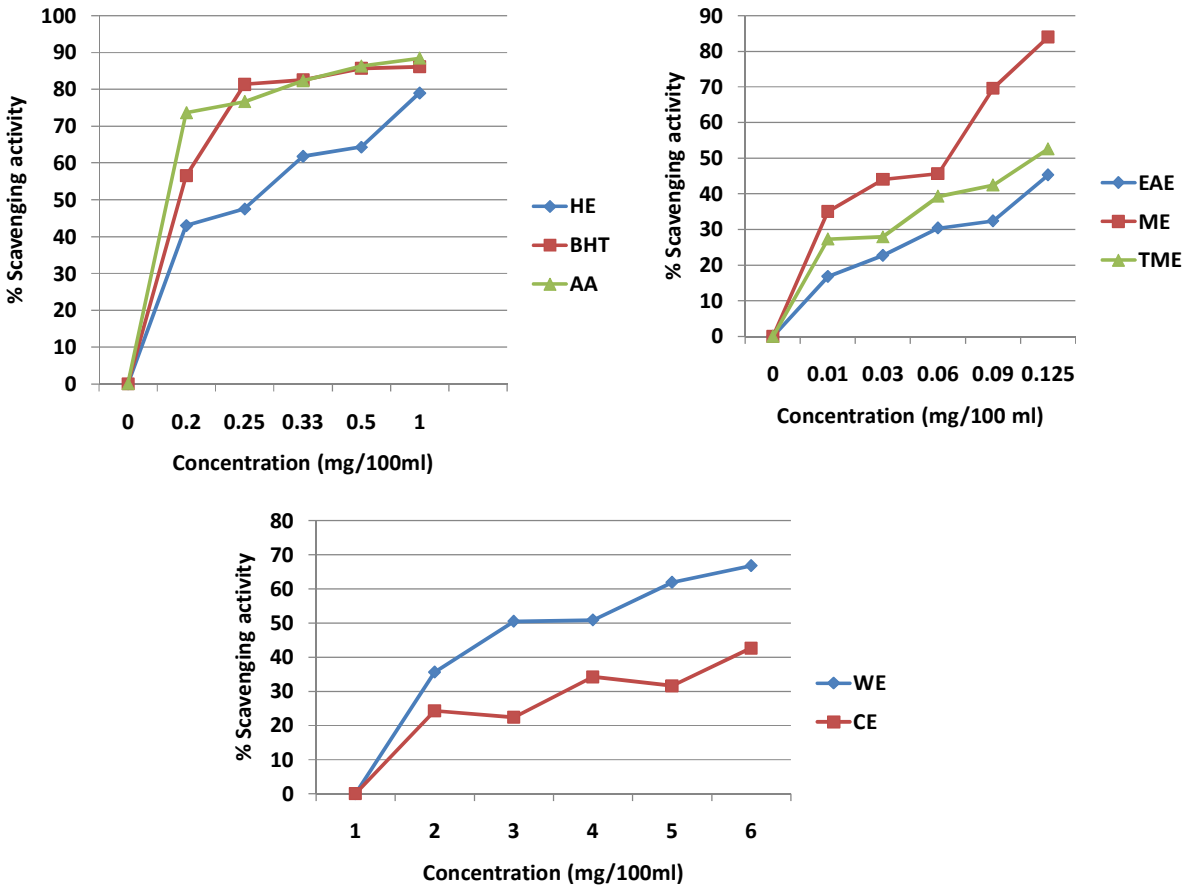

Figure D1.2: Percentage scavenging activity of the extracts of *P longifolia* on ABTS radical

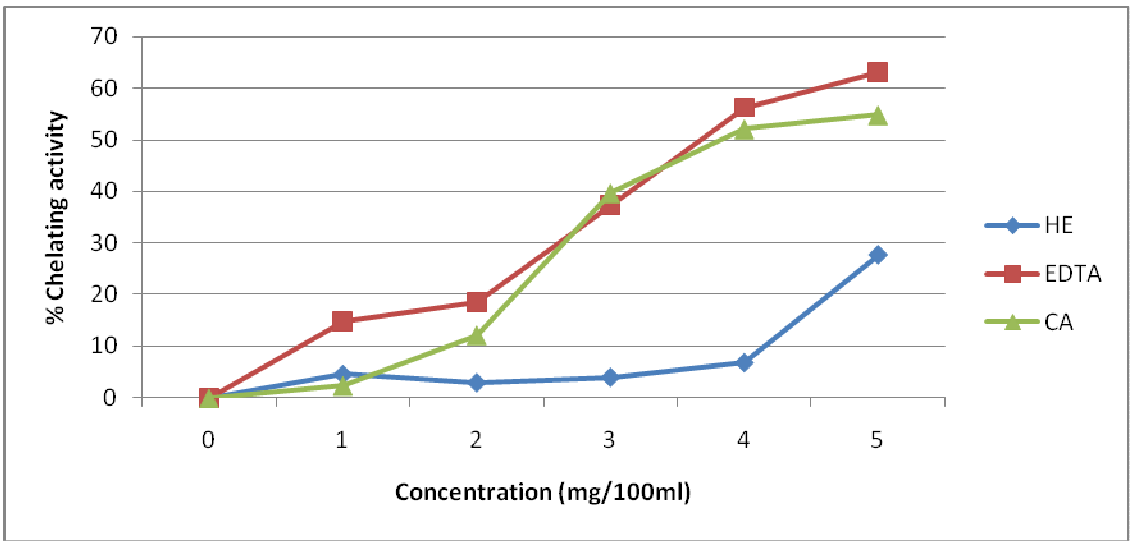

Figure D1.3: Percentage chelating activity of the extracts of *P longifolia* on Fe^{2+} Ethylenediaminetetra acetic acid (EDTA) and citric acid (CA) exhibited IC_{50} values of 3.68 and 3.85mg/ml respectively.

D2 Anti-platelet Aggregation Activity- untreated platelets

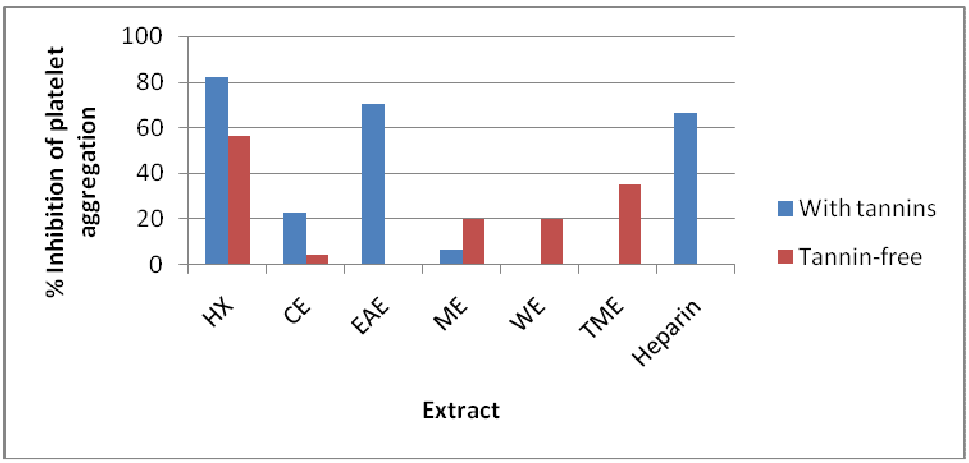

Figure D2.1a: Anti-platelet aggregation activity of the extracts of *P longifolia* on the thrombin-induced platelet aggregation.

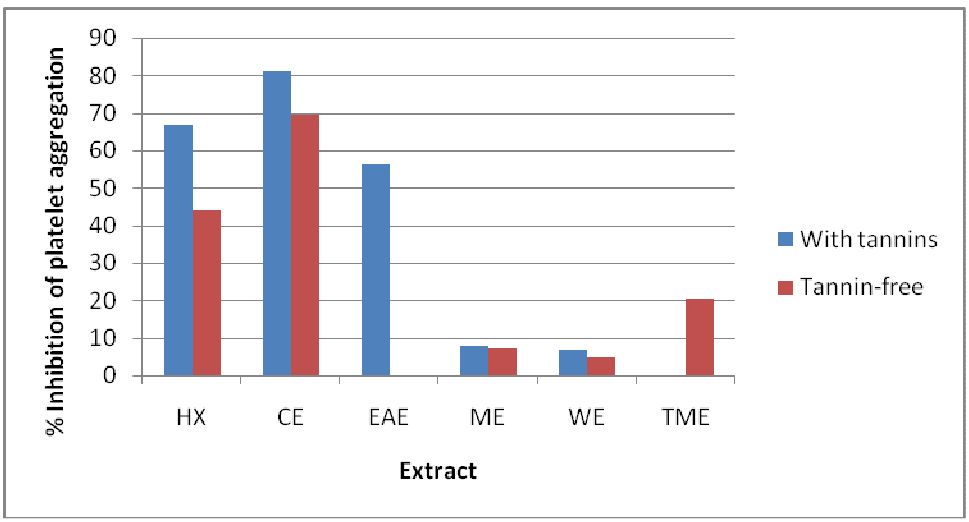

Figure D2.1b: Anti-platelet aggregation activity of the extracts of *P longifolia* on the ADP-induced platelet aggregation.

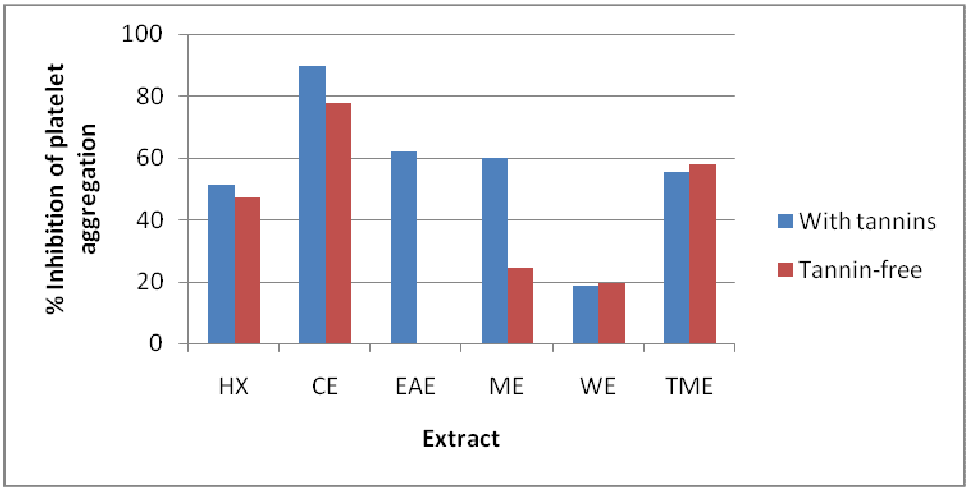

Figure D2.1c: Anti-platelet aggregation activity of the extracts of *P longifolia* on the epinephrine-induced platelet aggregation.

APPENDIX E

SPECTRA

E1 Spectral data for compound MS/10

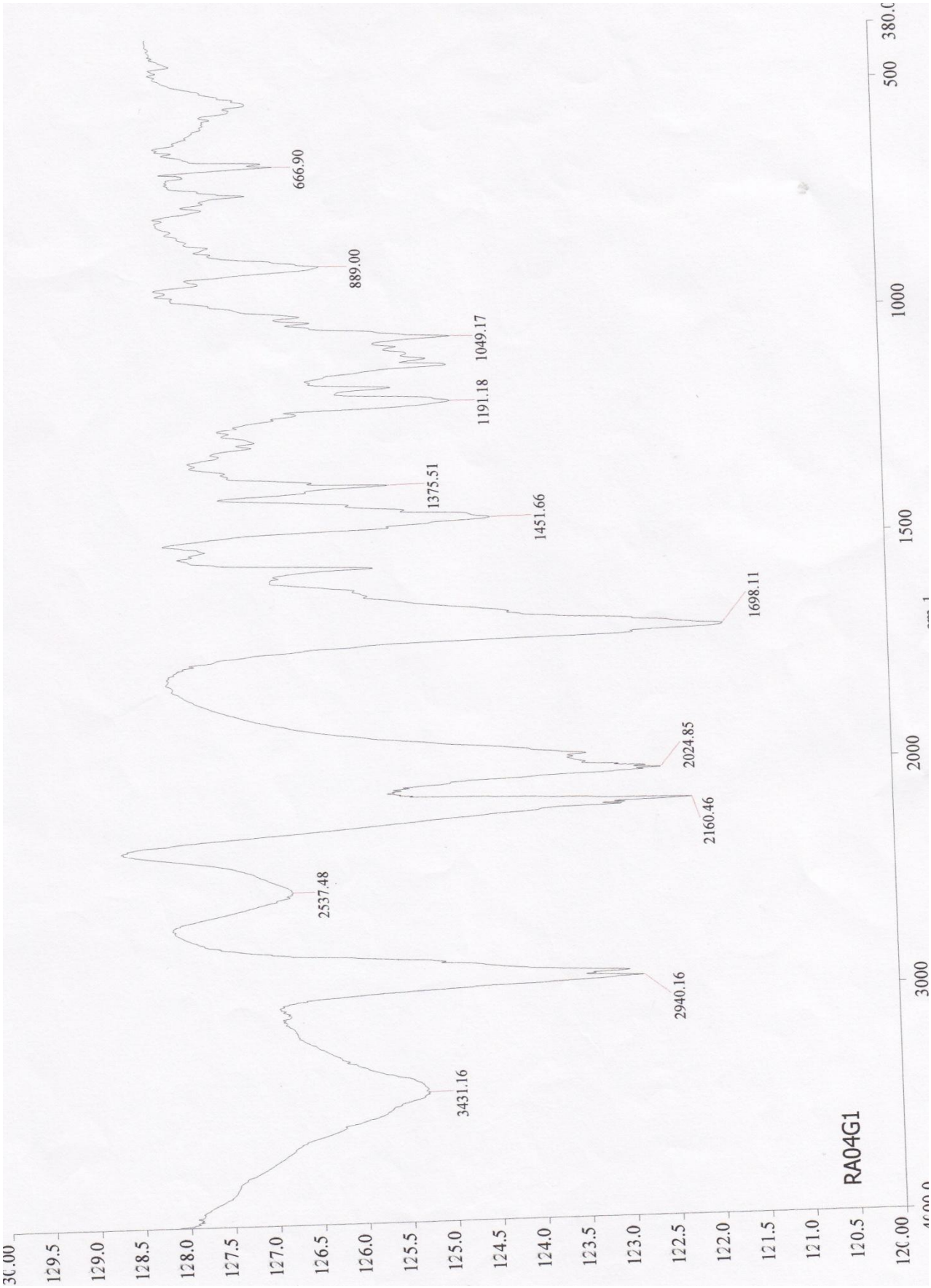

Figure E1.1: IR spectrum of compound MS/10

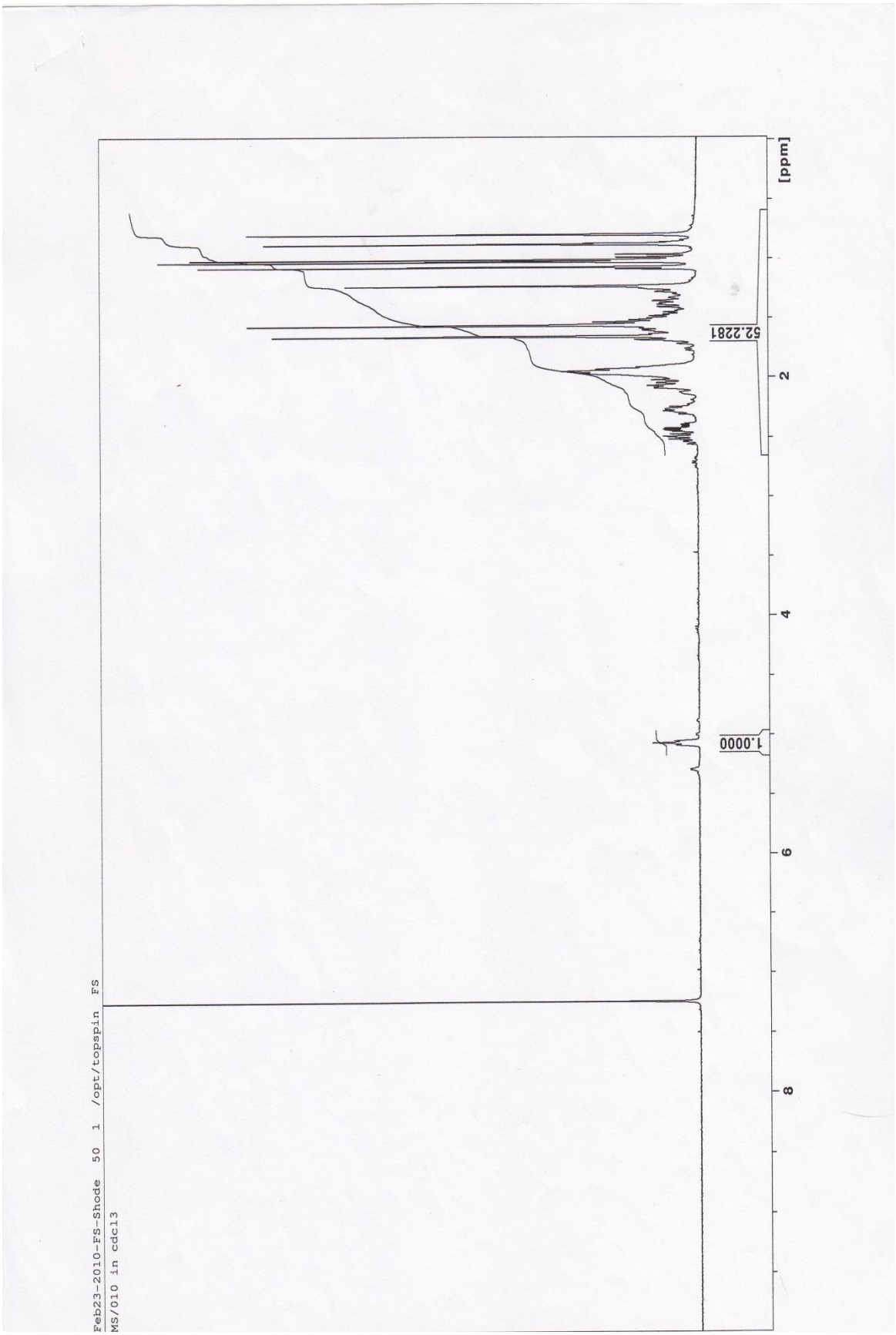

Figure E1.2: ^{1}H-NMR spectrum of compound MS/10

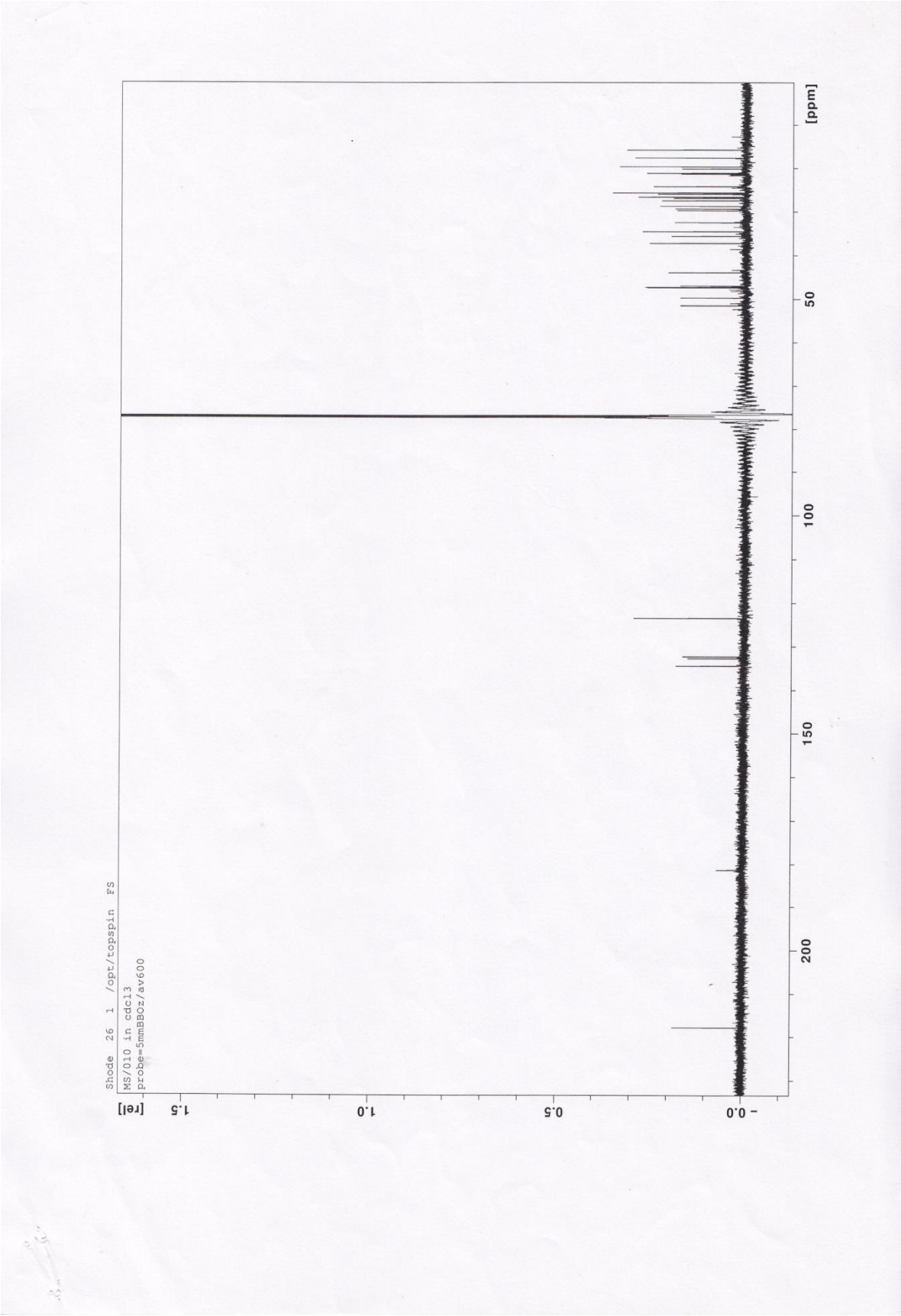

Figure E1.3: ^{13}C-NMR spectrum of compound MS/10

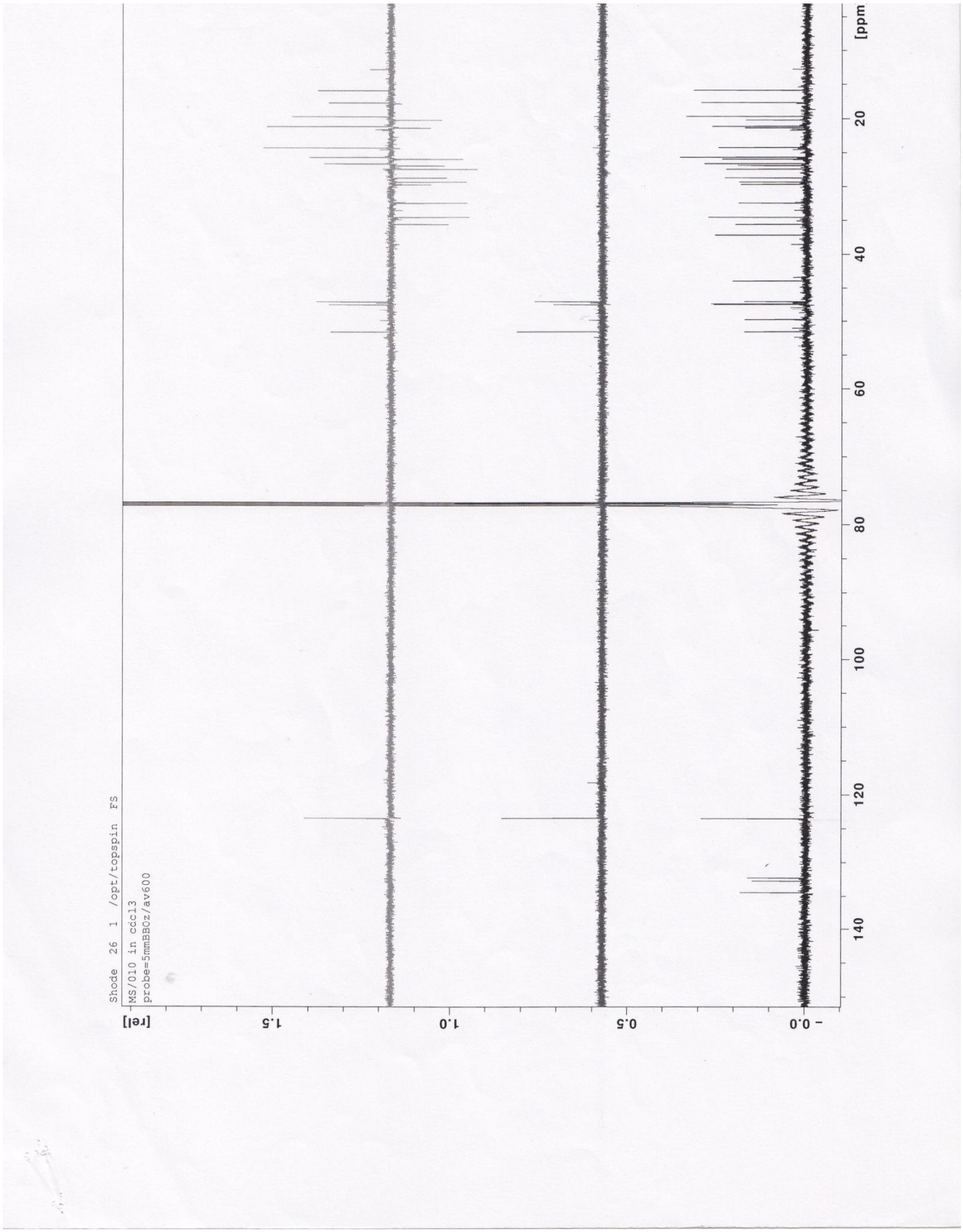

Figure E1.4: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound MS/10

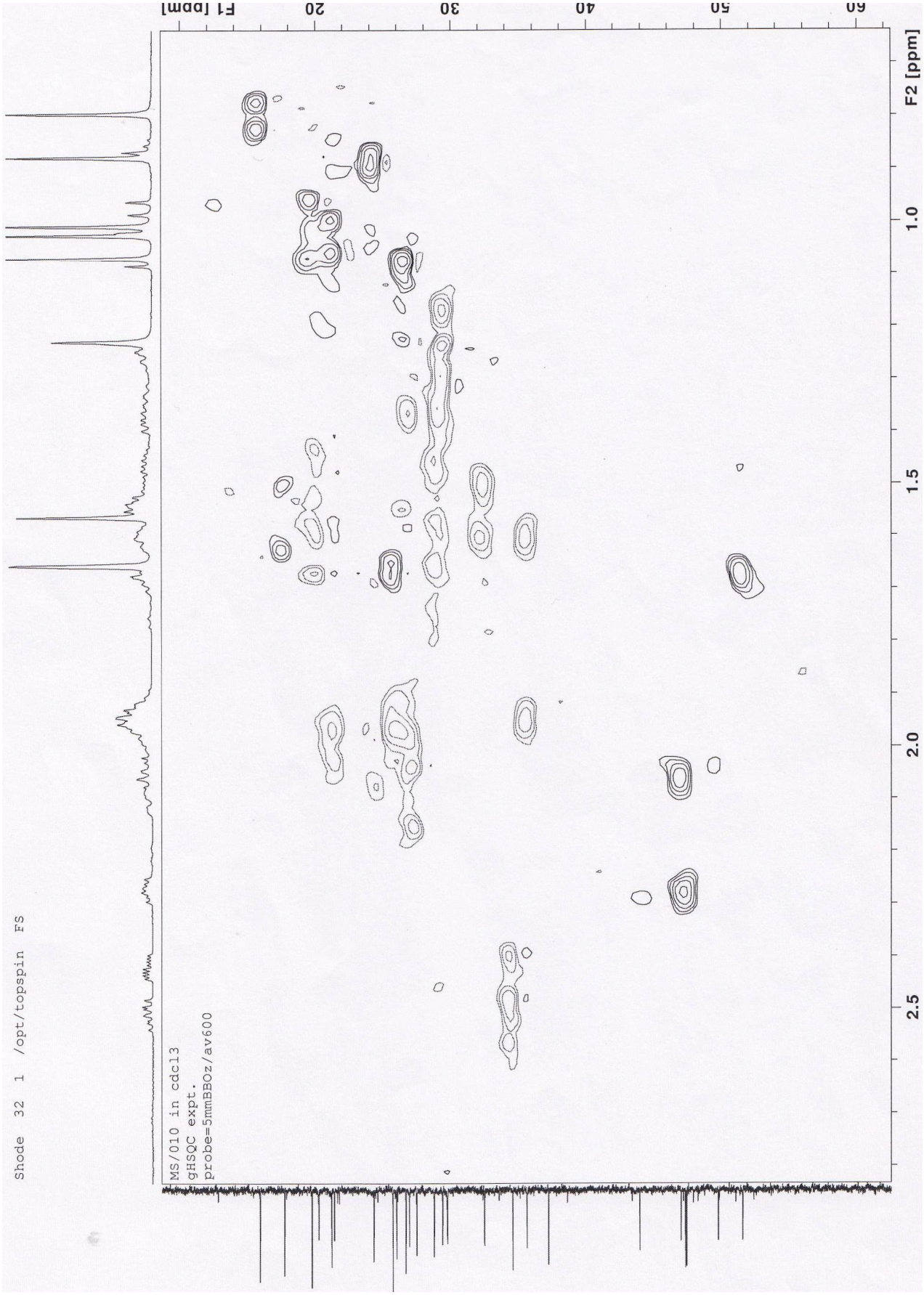
Shode 32 1 /opt/topspin FS
MS/010 in cdc13
gHSQC expt.
probe=5mmBBOz/av600
F1 [ppm]
F2 [ppm]
60
50
40
30
20
2.5
2.0
1.5
1.0

Figure E1.5: HSQC NMR spectrum of compound MS/10

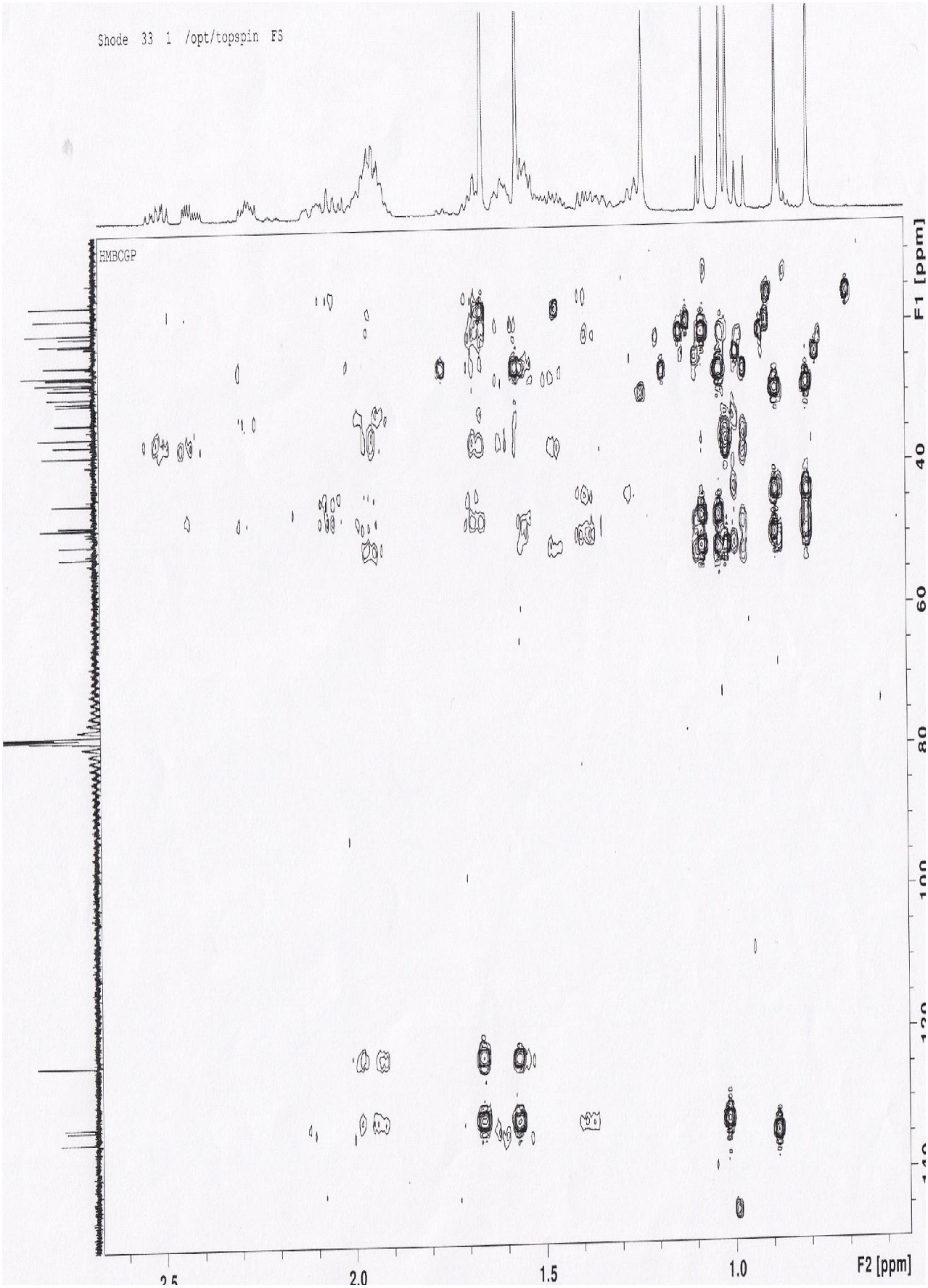

Figure E1.6: HMBC NMR spectrum of compound MS/10

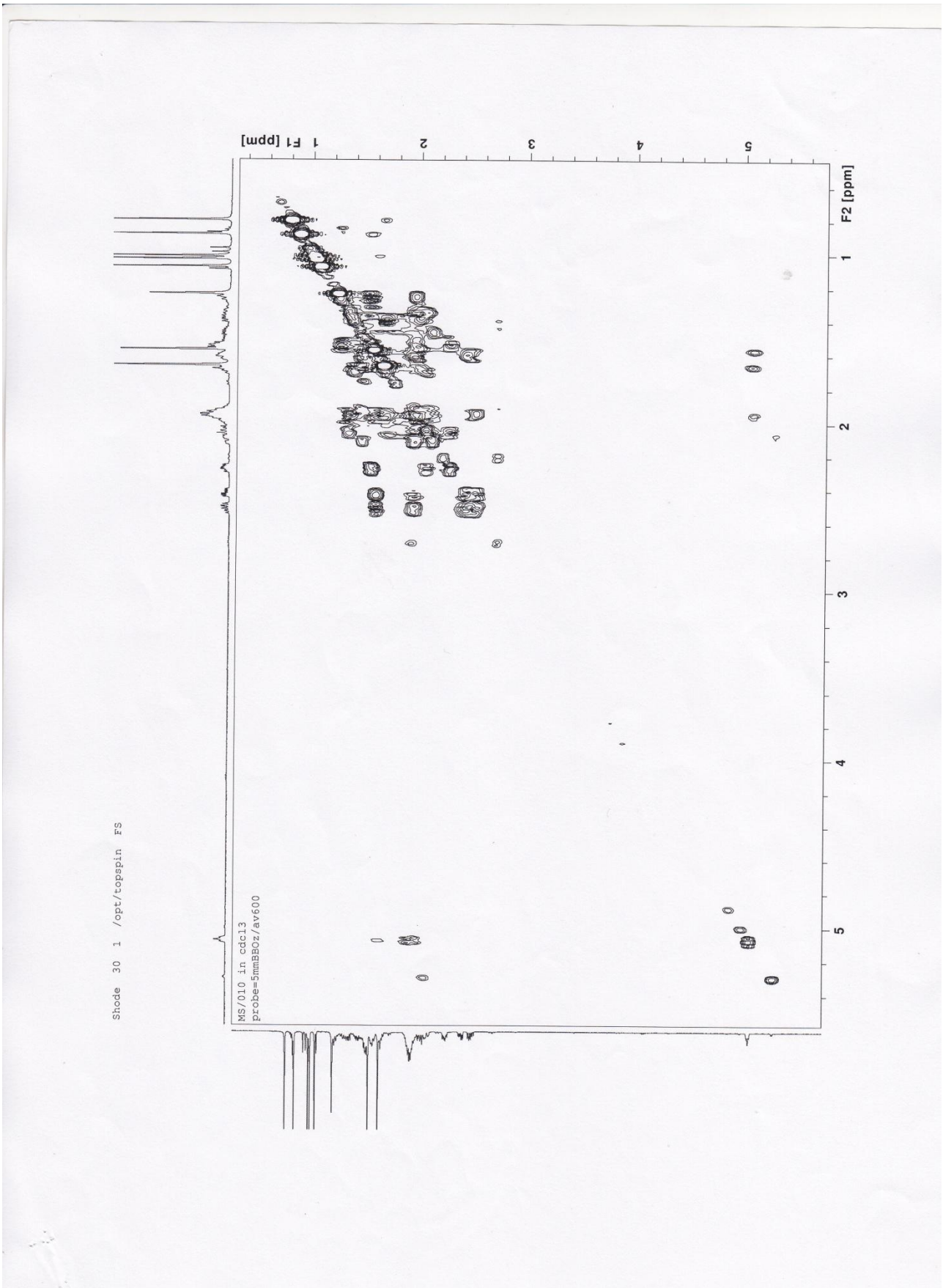

Figure E1.7: COSY NMR spectrum of compound MS/10

Figure E1.8: NOESY NMR spectrum of compound MS/10

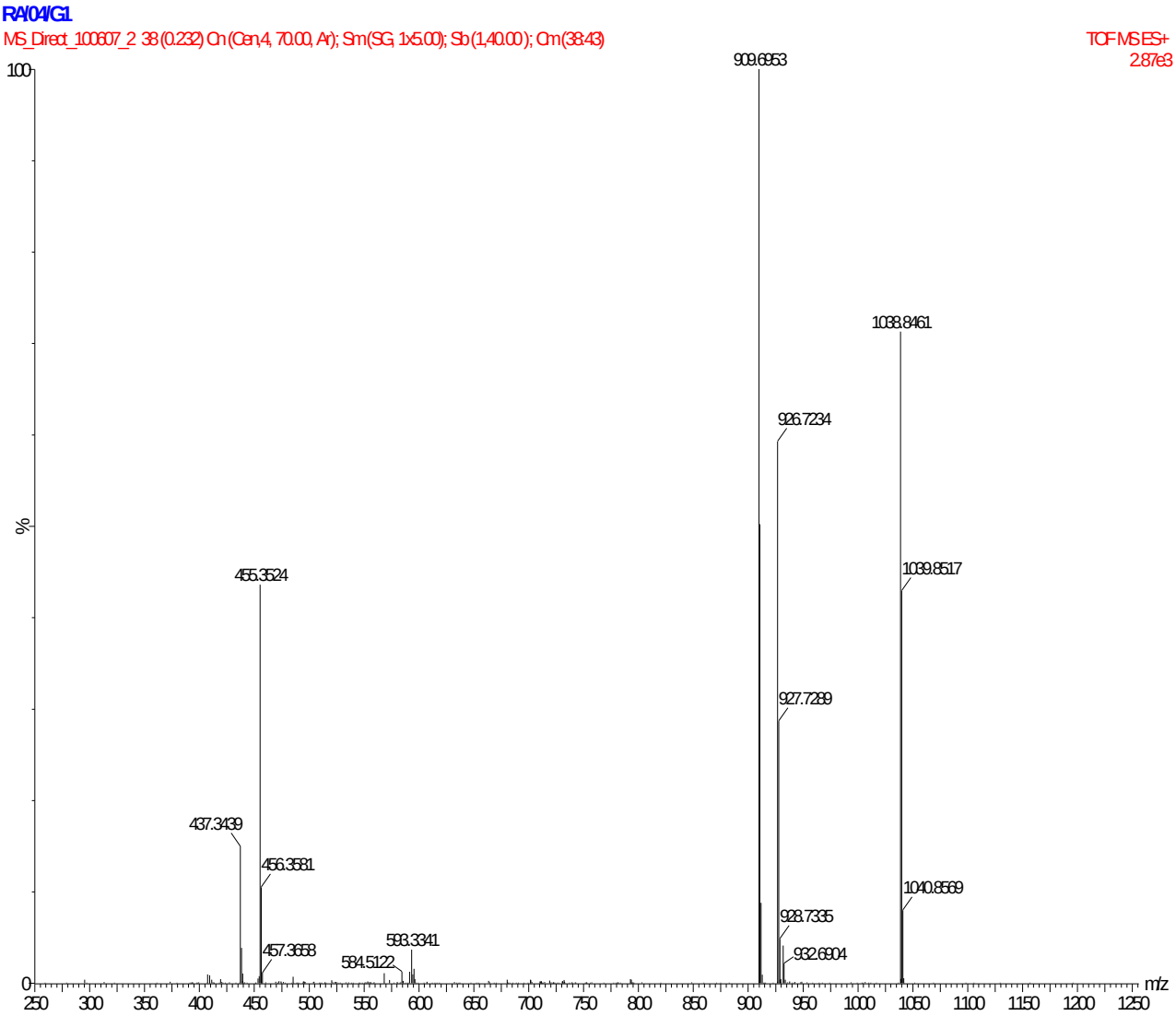

Figure E1.9a: MS spectrum of compound MS/10

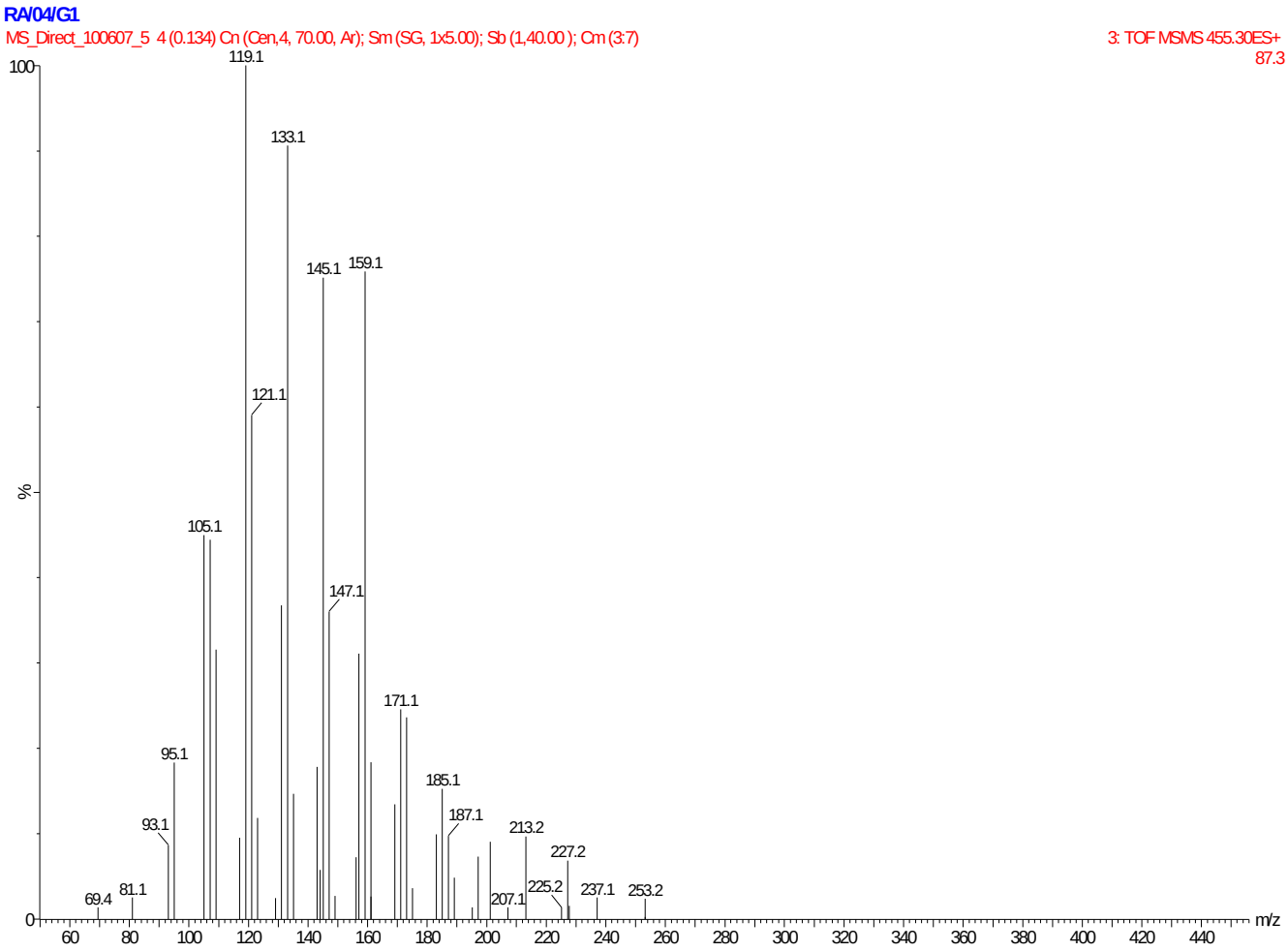

Figure E1.9b: MS spectrum of compound MS/10

E2 Spectral data for compound MS/12

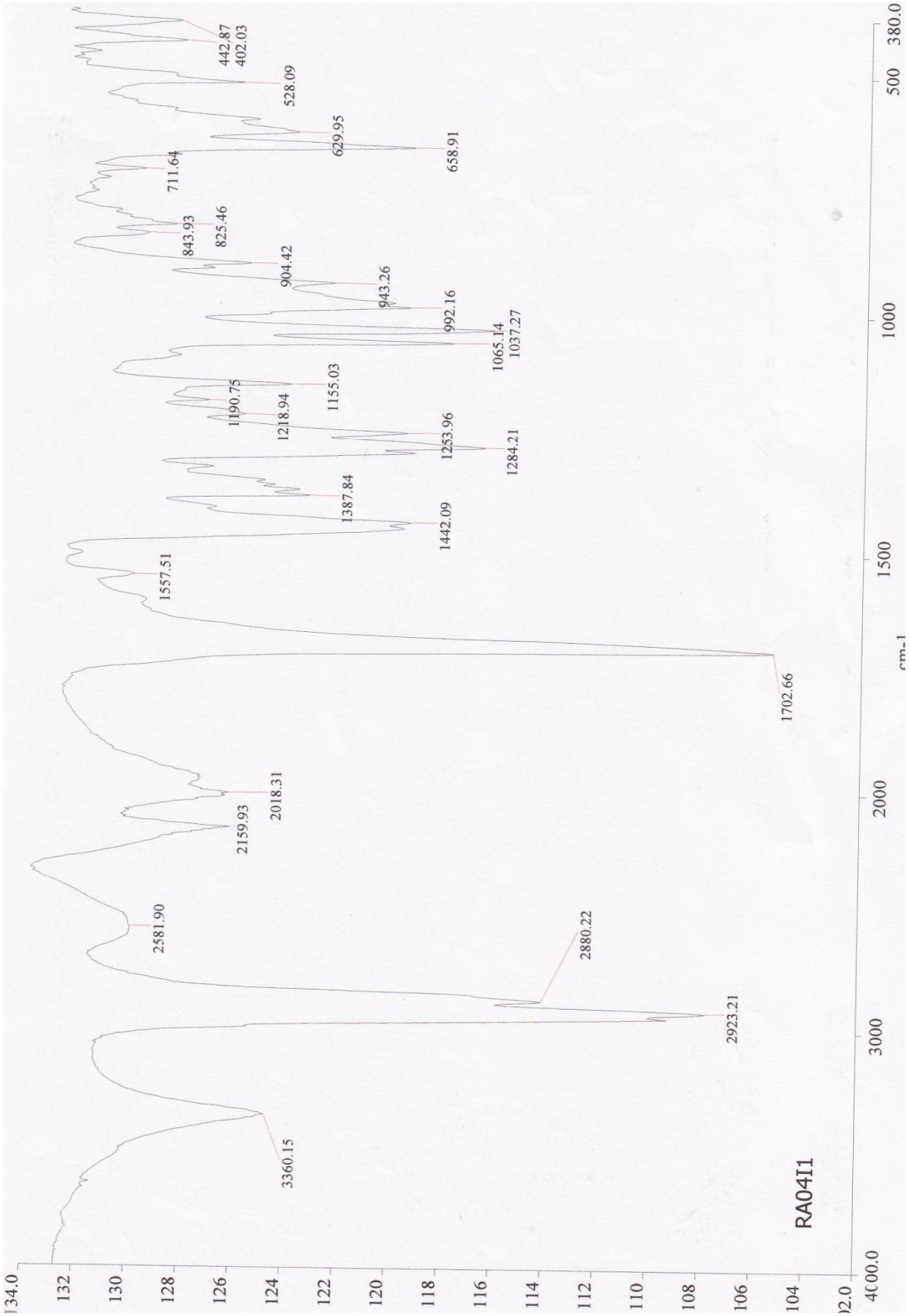

Figure E2.1: IR spectrum of compound MS/12

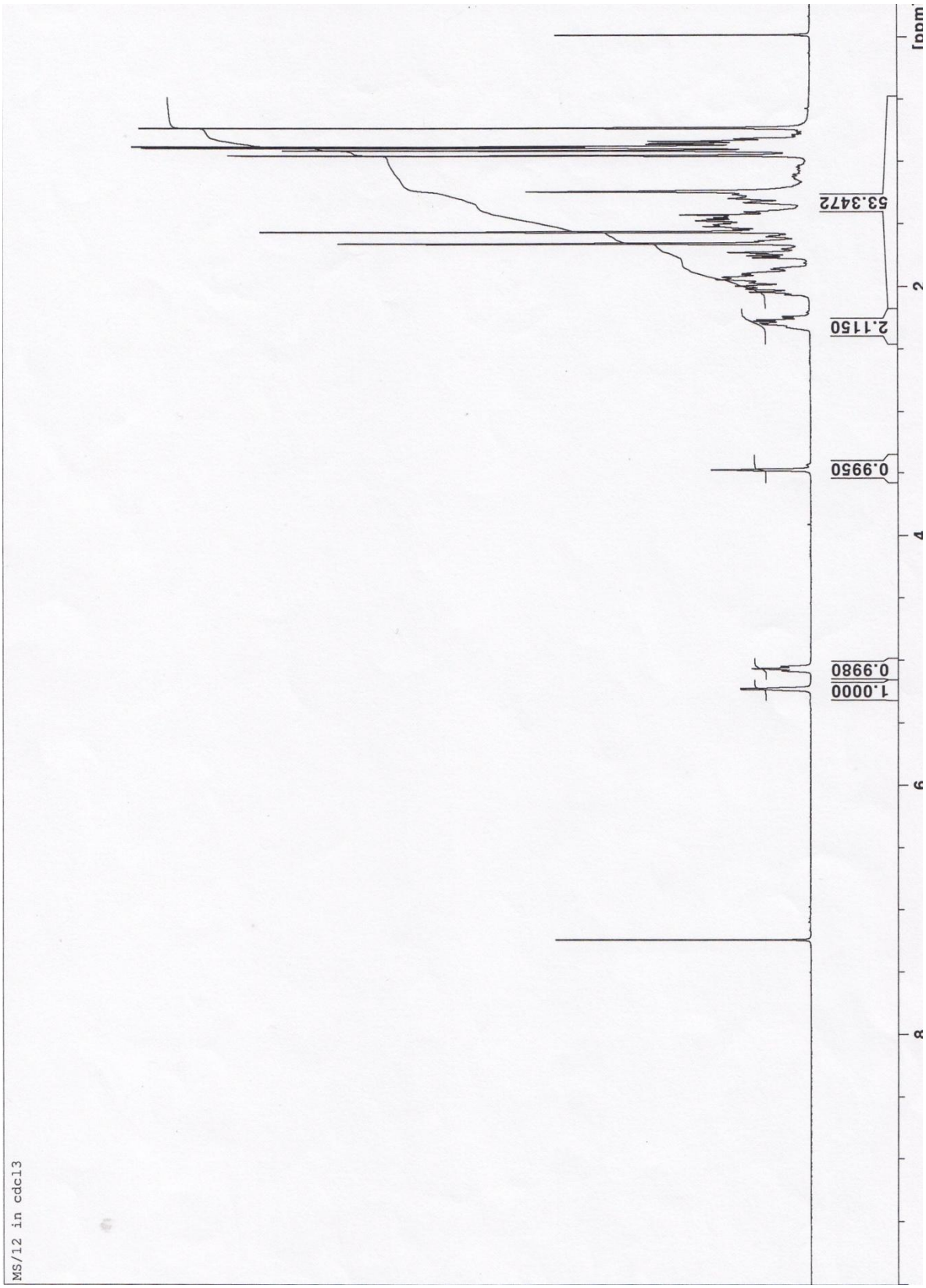

Figure E2.2: ^{1}H-NMR spectrum of compound MS/12

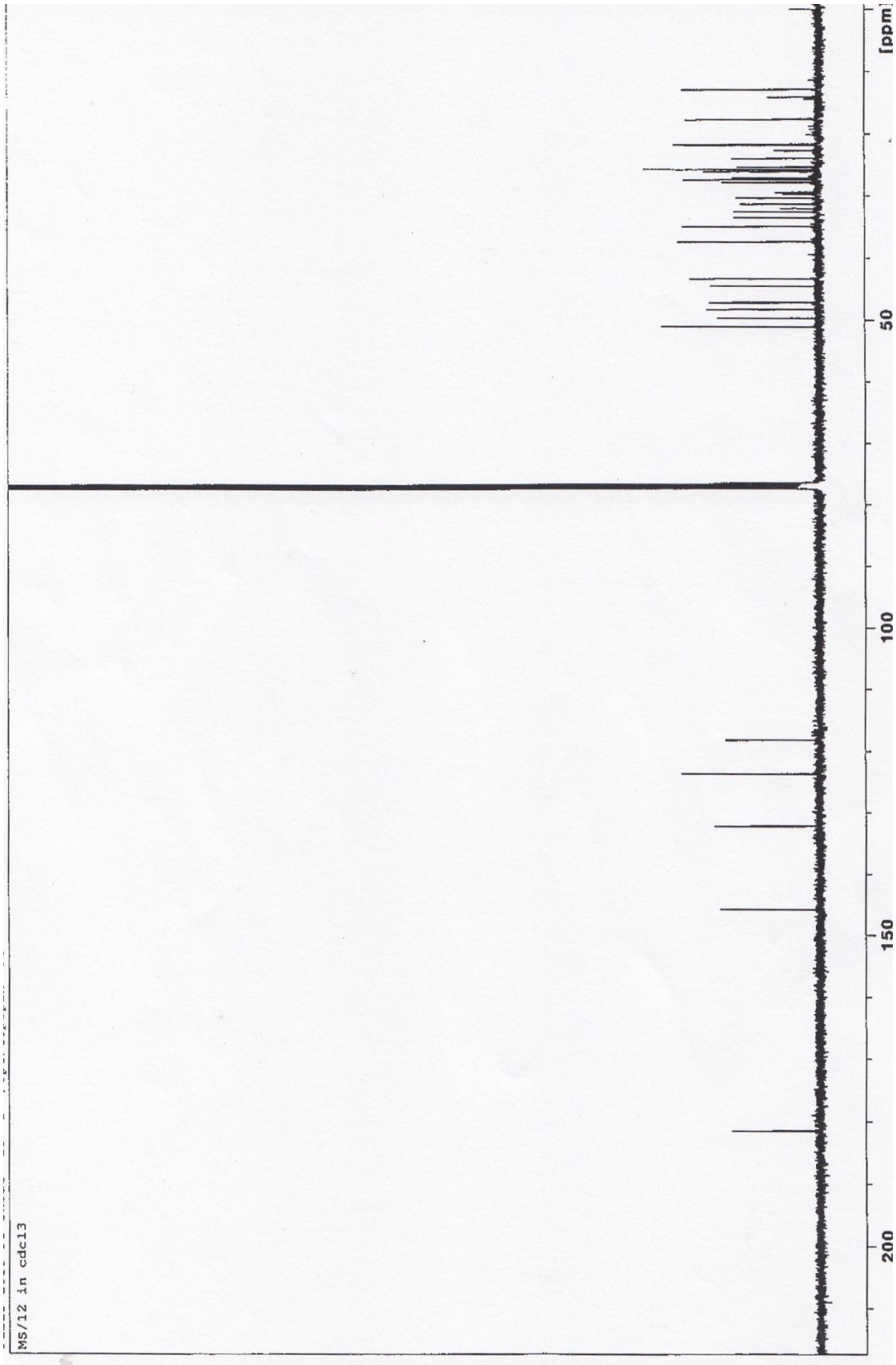

Figure E2.3: ^{13}C-NMR spectrum of compound MS/12

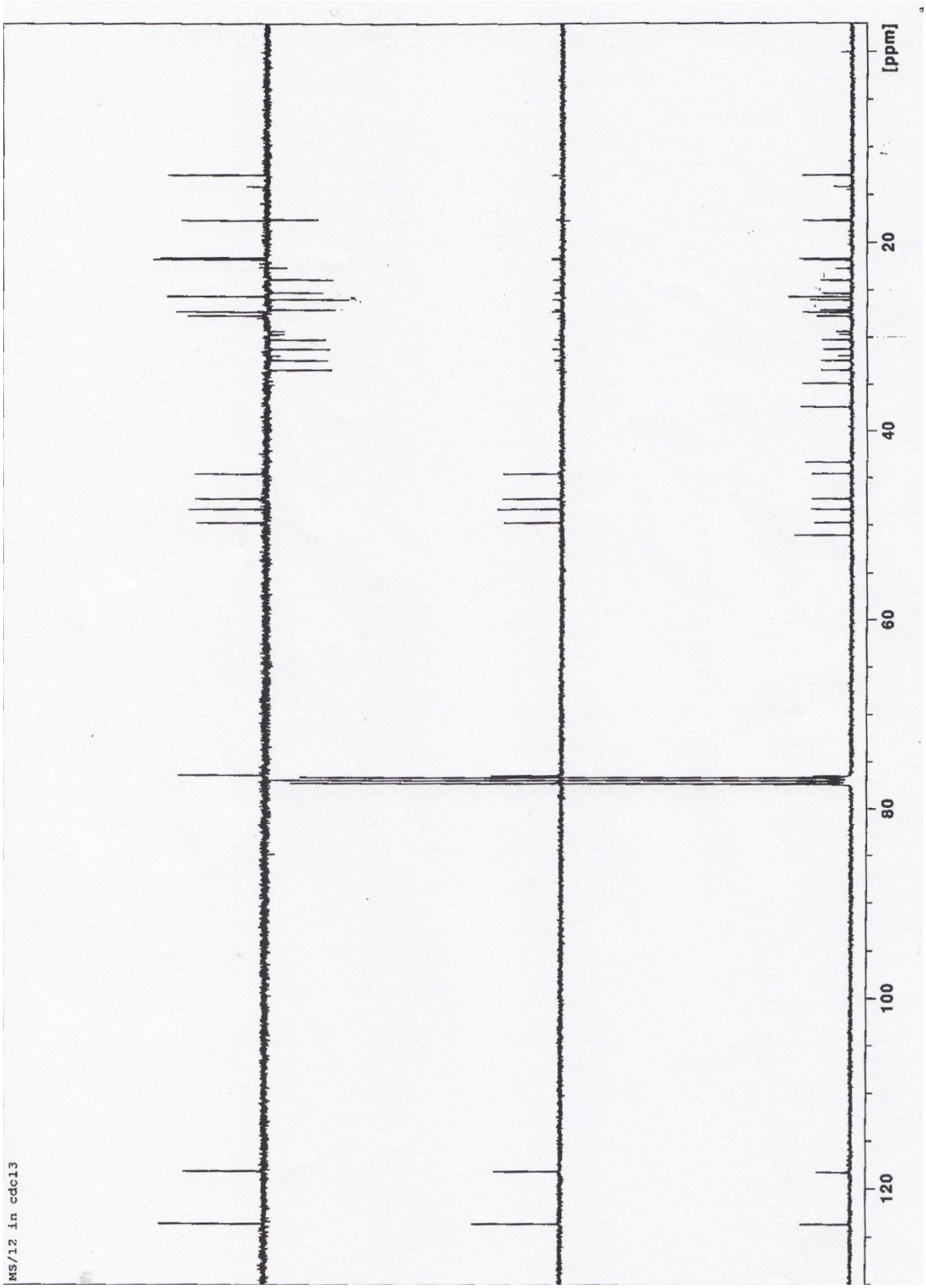

Figure E2.4: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound MS/12

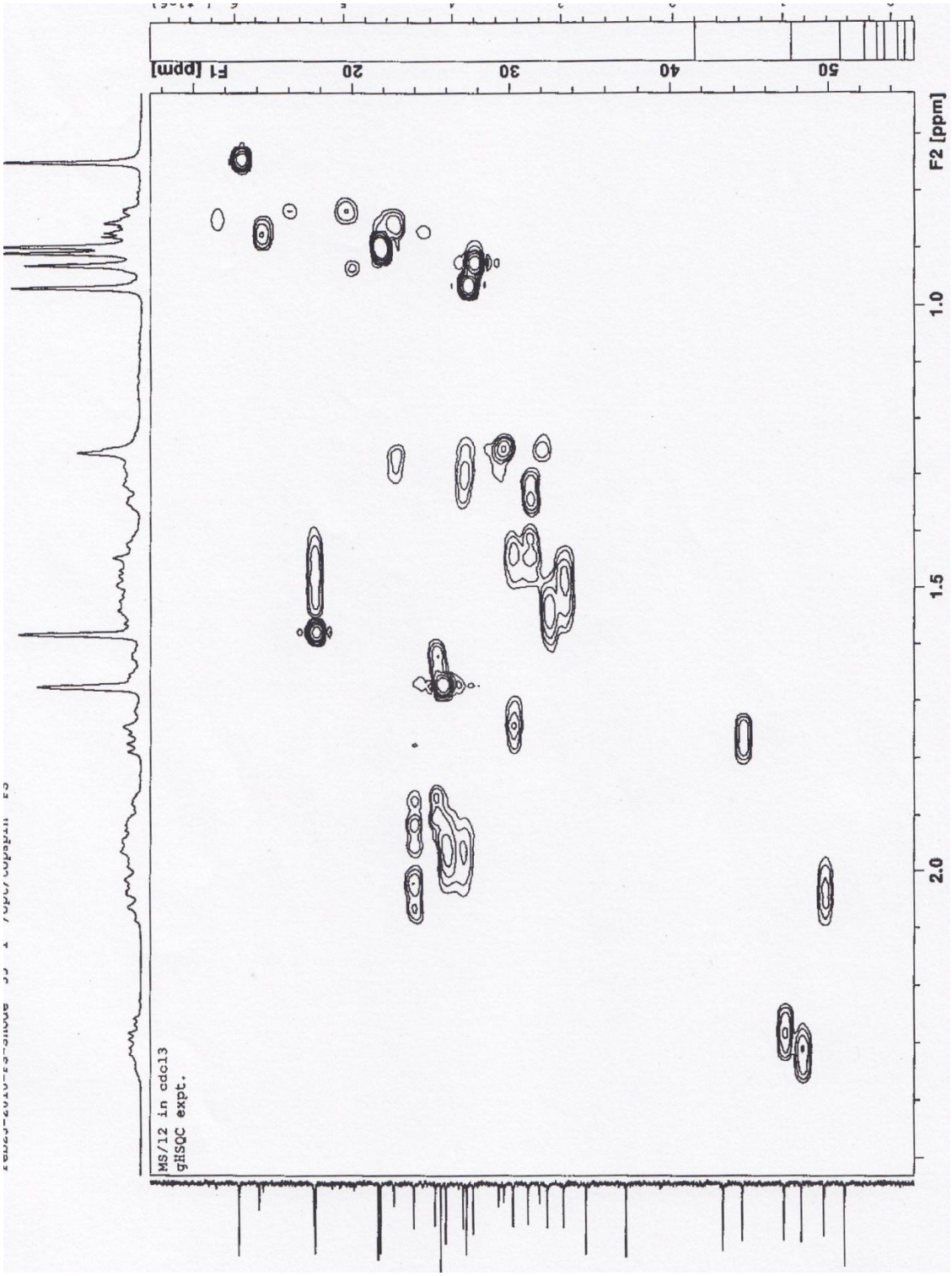

Figure E2.5: HSQC NMR spectrum of compound MS/12

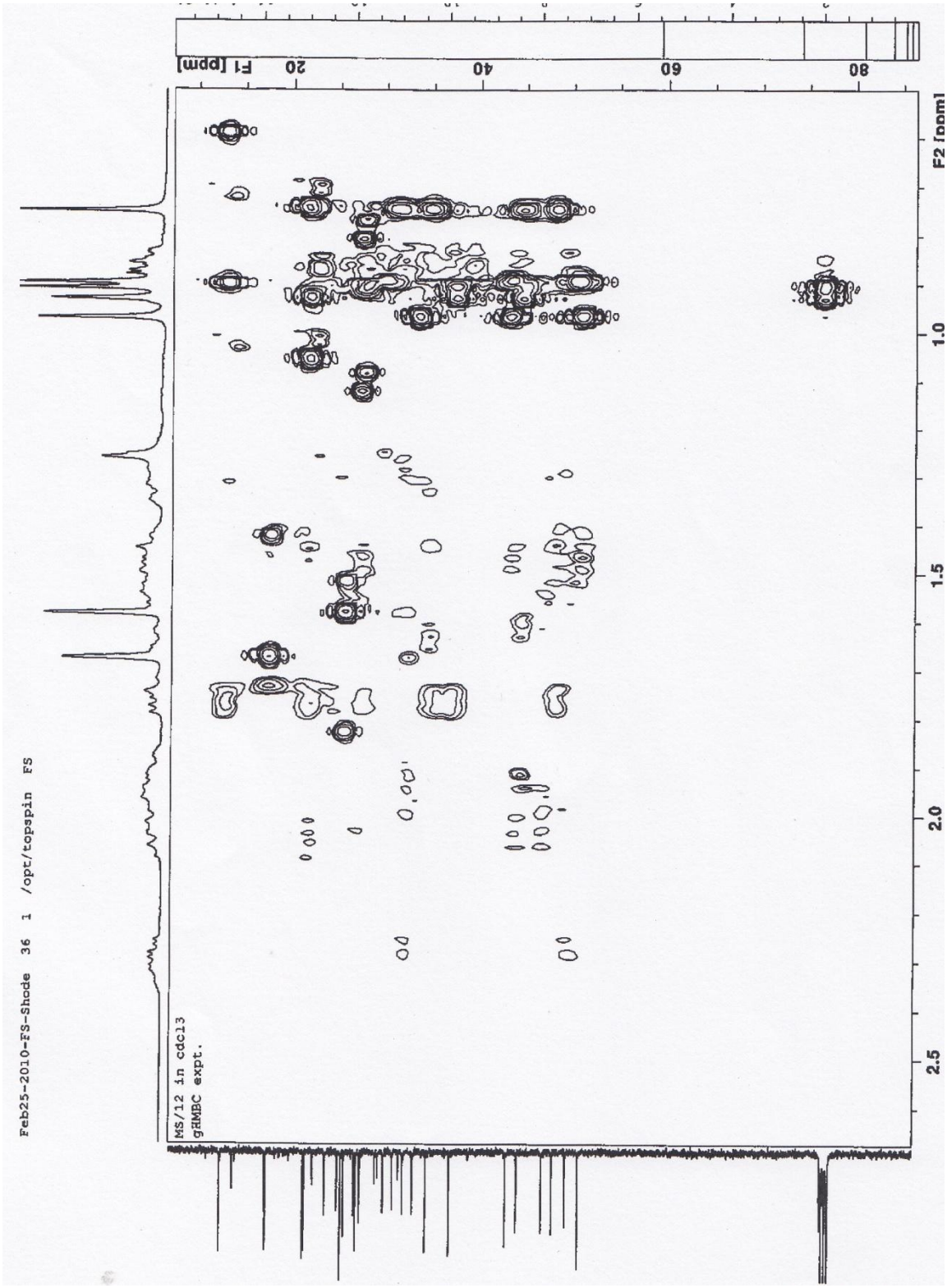

Figure E2.6: HMBC NMR spectrum of compound MS/12

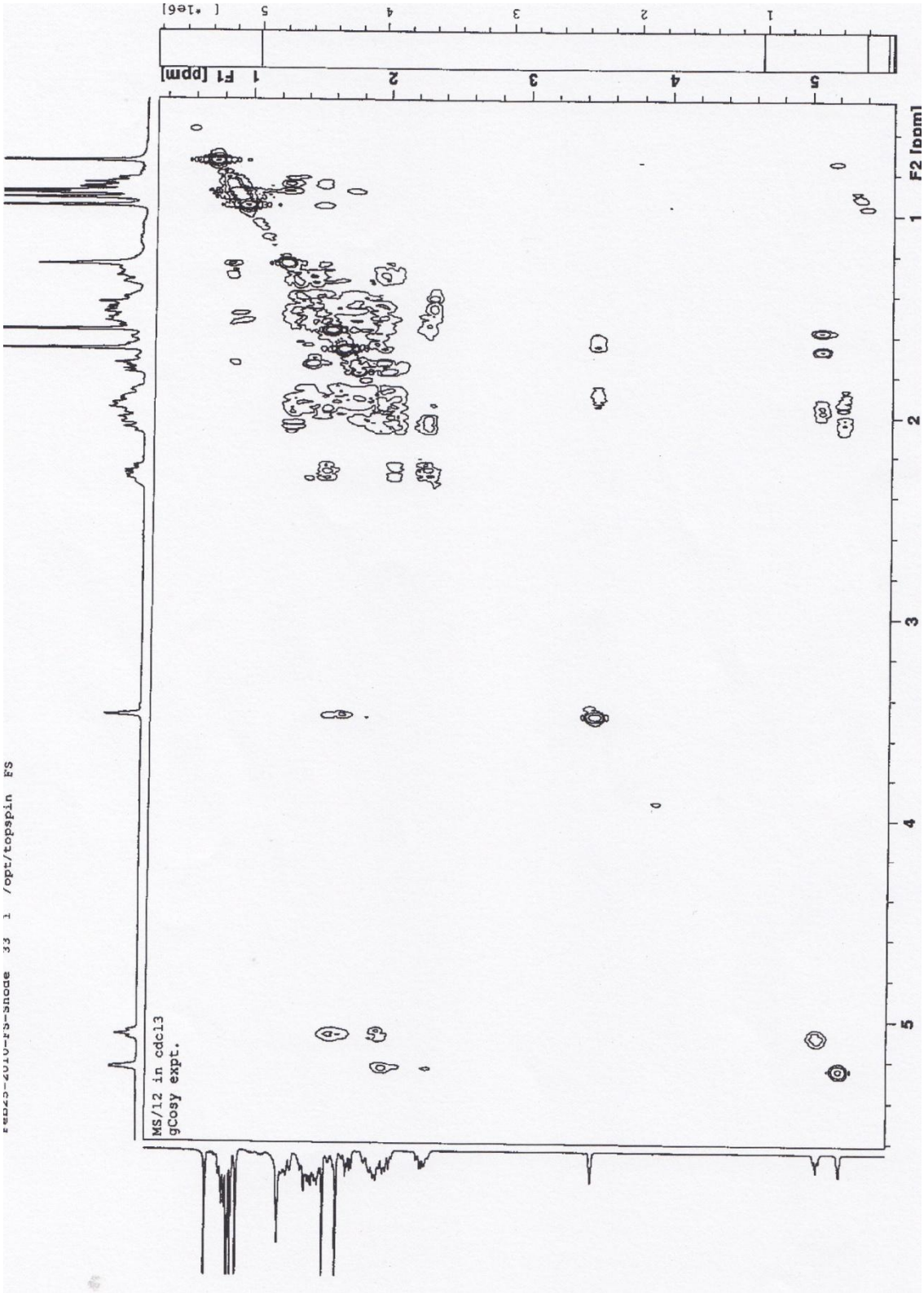

Figure E2.7: COSY NMR spectrum of compound MS/12

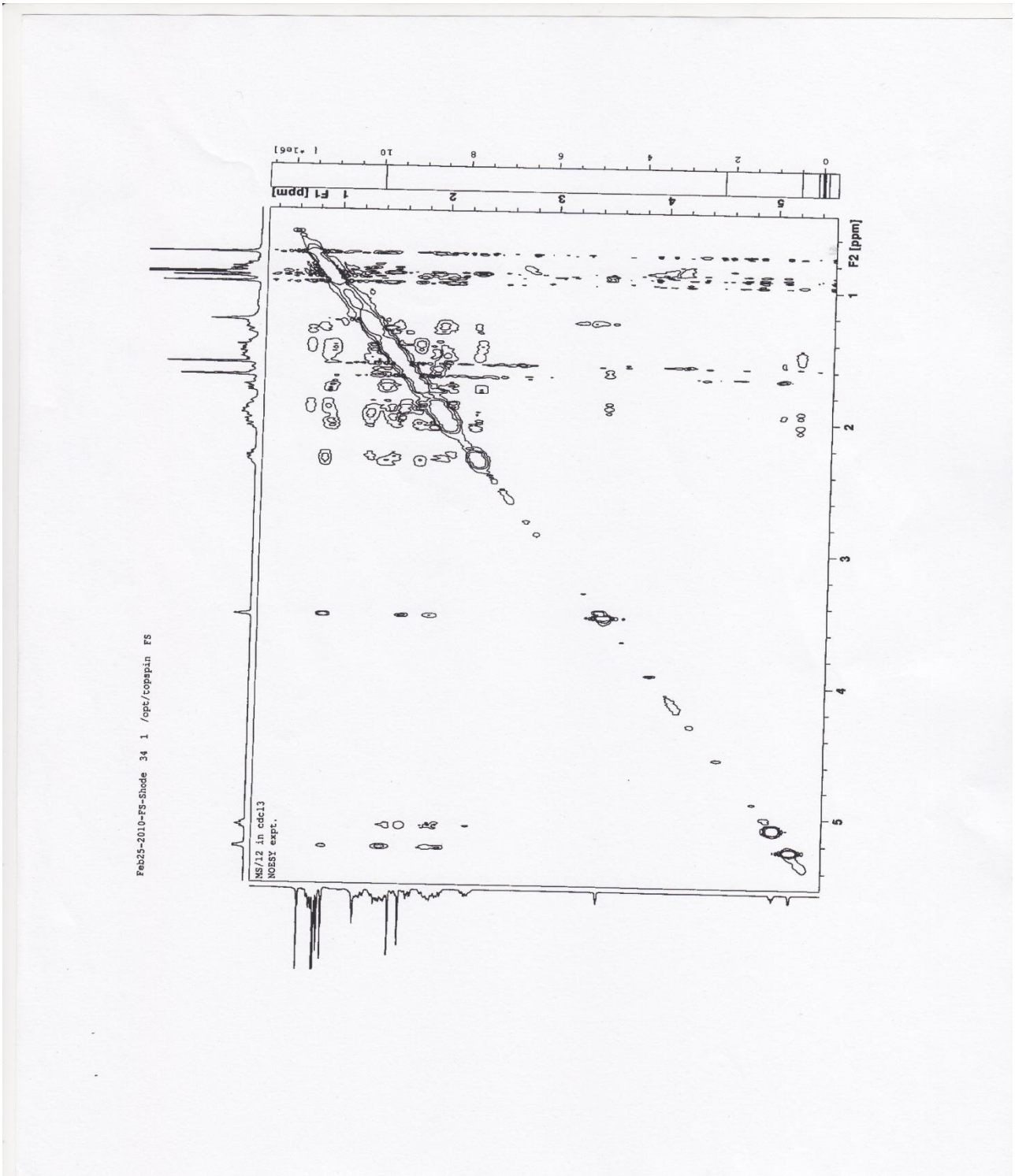

Figure E2.8: NOESY NMR spectrum of compound MS/12

APPENDIX F

CONTRIBUTION TO KNOWLEDGE

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

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INTRODUCTION

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

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

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

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

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

Bulbine natalensis

Protorhus Longifolia

Rapanae melanophloes

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

MATERIALS AND METHODS

➢The plants were collected from Twinstream Nursery, Mtunzini. Plants were identified at the Department of Botany, University of Zululand and voucher specimens were prepared.
➢Air-dried and powdered plant parts were extracted sequentially with hexane, chloroform, ethyl acetate, methanol and water.
➢Phytochemical screening was carried out on the extracts using standard procedures to identify the bioactive constituents[2,3,11]
➢The anti-oxidant activity was determined by in vitro methods-DPPH, ABTS, REDUCING POWER and METAL CHELATING assay [3,4,12,14]
➢The methanolic extracts were tested for brine shrimp cytoxicity test activity . The shrimp larvae were subjected to the extracts in a multi welled culture plate. Number of survivors were counted and LC_{50} was determined. All experiments were carried out in triplicate[6,8]
➢ The anti platelet aggregation activity of the extracts was separately investigated on thrombin, ADP and epinephrine induced rat platelet aggregation; similar experiments were also carried out on enzyme (trypsin, bromelain, papain) treated platelets [8,9,10].

RESULTS AND DISCUSSION

TABLE 1

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

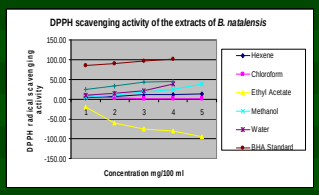
+ PRESENT ; - ABSENT

TABLE 2: LETHAL CONCENTRATION OF EXTRACTS LC_{50}

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

ANTI OXIDATIVE ACTIVITY

Figure 1: DPPH activity for *B natalensis*



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

TABLE 3:

A: *Bulbine natalensis*

B Protorhus Longifolia

C:Rapanae melanophloes

IC_{50} (values I mg/100ml)	DPPH			REDUCING POWER			METAL CHELATING			ABTS		
	A	B	C	A	B	C	A	B	C	A	B	C
HEXANE	>5	ND	>5	>3	ND	ND	>5	ND	4.21	ND	0.27	>5
ETHYL ACETATE	-	ND	4.83	>3	ND	ND	>5	ND	<1	ND	ND	4.99
CHLOROFORM	>5	ND	4.36	-	ND	ND	-	ND	3.57	ND	ND	2.4
METHANOL	>5	0.07	3.35	>3	ND	ND	>5	ND	>5	ND	ND	1.53
WATER	>5	ND	>5	>3	ND	ND	>5	ND	>5	ND	ND	>5
BHA	<1	ND	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND
BHT	ND	ND	4.54	ND	ND	ND	ND	ND	ND	ND	ND	3.72
ASCORBIC ACID	>5	ND	4.06	>5	ND	ND	ND	ND	ND	ND	ND	3.84
EDTA	ND	ND	ND	ND	ND	ND	<1	ND	3.42	ND	ND	ND
CITRIC ACID	ND	ND	ND	ND	ND	ND	3.81	ND	3.92	ND	ND	ND

ANTI PLATELET AGGREGATION STUDY: [3mg/ml]

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

TABLE 4: Bromelain treated platelets

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

TABLE 5: Papain treated platelets

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

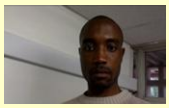
➢Methanol and water extracts of *B natalensis,* Hexane, ethyl acetate and chloroform extracts of *P longifolia,* and Chloroform and ethyl acetate extracts of *R melanophloes provoked* a concentration dependent in vitro inhibition of rat platelet aggregation activity.

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

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

References will be provided on request

TRITERPENES FROM *PROTORHUS LONGIFOLIA* EXHIBIT ANTI-PLATELET AGGREGATION ACTIVITY



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INTRODUCTION

Platelet hyperaggregation is the underlying cause of atherothrombotic diseases[1]. The clinical limitations and adverse side effects associated with the currently used antiplatelet aggregation agents has fuelled the search for more effective agents of natural origin[2,3]. Medicinal plant-based traditional healing does not only provide immediate health care to rural population but also a lead to discovery of new pharmacologically active drugs.

Stem-bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae) is commonly used by Zulu traditional healers to treat blood clot related diseases. The aim of this work was to investigate the antiplatelet aggregation activity of the stem-bark extracts of *Protorhus longifolia* and to partially identify active components present and responsible for the antiplatelet aggregation activity.

MATERIALS AND METHODS

❖The plant material was collected from Empangeni, KwaZulu-Natal. Dried, powdered plant material was sequentially extracted with hexane (HE), chloroform (CE), ethyl acetate (EAE), methanol (ME) and water (WE).

❖ The crude chloroform extract was subjected to isolation and purification using chromatographic techniques (Fig. 1). Structures of the isolated compounds were analyzed and confirmed through IR, NMR, MS spectral data and literature[4,5,6].

❖*In vitro* antiplatelet aggregation activity was investigated on thrombin, ADP and epinephrine induced rat platelet aggregation[7].

❖*In vitro* anticoagulant activity was determined on rat whole blood.

❖Acute anti-inflammatory activity was determined using carrageenan-induced rat paw oedema model[8].

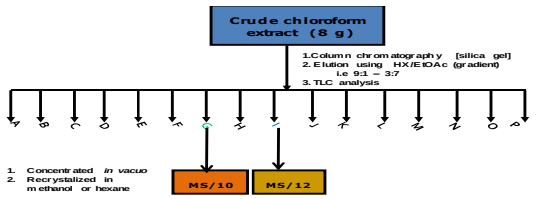


Fig. 1: Isolation and purification of MS/10 and MS/12 from the crude chloroform extract

Table 1: IC_{50} values (mg/ml) of the crude extracts of *P longifolia* on rat platelet aggregation

Agon\Extrct	HE	CE	EAE	ME	WE	Heparin
Thrombin	0.59	>10	2.13	Nd	Nd	7.40
ADP	1.56	4.48	2.62	Nd	Nd	
Epinephrine	>10	2.39	2.09	2.21	>10	

Nd- not detected

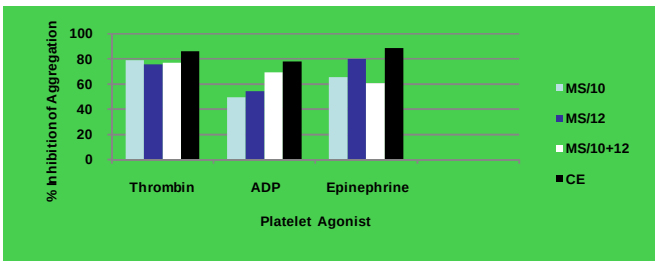


Fig. 3: Antiplatelet aggregation activity of MS/10 and MS/12 on the platelet aggregation induced by thrombin, ADP and epinephrine. Crude chloroform extract (CE) and the mixture (MS/10+12) were used for comparison. IC_{50} values (mg/ml) on the thrombin-induced platelet aggregation: MS/10 (0.99) , MS/12 (1.04), CE (0.67) and MS/10+12(0.88)

RESULTS

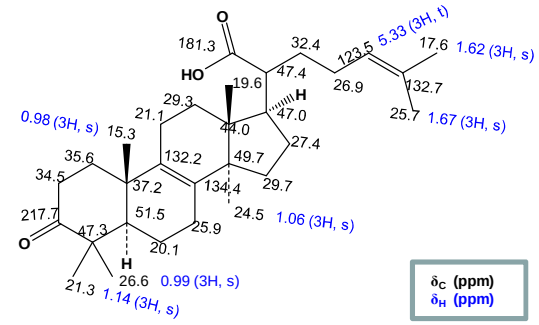


Fig. 2a: 3-Oxo-5α-lanosta-8,24-dien-21-oic acid (**MS/10**)

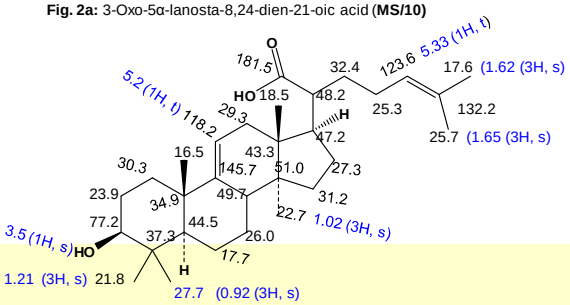


Fig. 2b: 3β-Hydroxylanosta-9,24-dien-24-oic acid (**MS/12**)

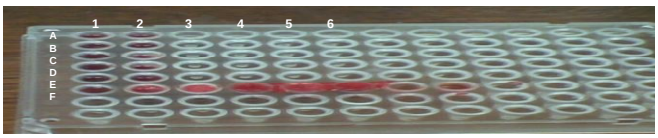


Fig. 4: The anticoagulant activity of MS/12 on the rat whole blood. The blood was added to A (1% DMSO-blank); B (thrombin); C (ADP); D (epinephrine); E (arachidonic acid); and F_{1-6} (compound at 1, 3 and 10 mg/ml). In the controls the blood clotted within a minute while in the presence of the compound it took 5±1 minutes to clot.

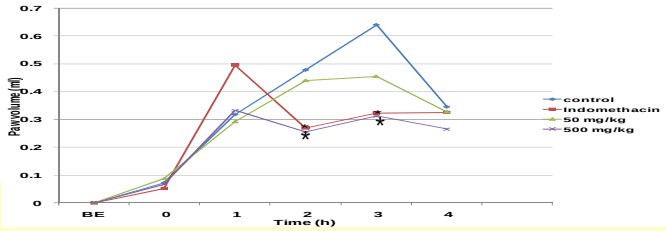


Fig. 5: Anti-inflammatory activity of MS/12 on carrageenan-induced paw oedema in rats. Paw oedema volume was measured immediately and at 1h interval for 4h following carrageenan injection. * P<0.05 compared to the control group.

DISCUSSION AND CONCLUSION

The organic extracts and the two triterpenes (Fig. 2) isolated from the chloroform extract prevent platelet aggregation in a concentration dependent manner. This is the first time the compounds are isolated from *P longifolia*. The activity of MS/12 was supported by its ability to delay clotting of whole blood. MS/12 further inhibited inflammation in a way similar to that of NSAIDs indicative of cyclooxygenase inhibitor. The triterpenes could be potential pharmacologically active antiplatelet aggregation agents. The results support the use of *P longifolia* in folk medicine to manage blood clotting related diseases. Further work is required to elucidate the possible mechanism in which these compounds exert their therapeutic activities. Also other biological activities need to be investigated.

References: will be provided on request.

Acknowledgements: Medical Research Council (MRC) for funding this project
University of Zululand Research Office for support

APPENDIX A

DETAILS OF REAGENTS

A1 Mayer's reagent

Solution A: 1.36 g mercury (II) chloride was dissolved in 60 ml of distilled water.
Solution B: 5.0 g potassium iodide was dissolved in 20 ml of distilled water.
Both solutions were mixed and the mixture was made up to 100 ml with distilled water.

A2 Dragendorff's reagent

Solution A: 0.85 g of subnitrate bismuth and 20 ml of glacial acetic acid were dissolved in 40 ml of distilled water.
Solution B: 0.8 g potassium iodide was dissolved in 20 ml of distilled water.
Solutions A and B were stored separately in dark bottles. Just before use, 5 ml of solution A, 5 ml of solution B and glacial acetic acid were mixed and made up to 100 ml with distilled water in a volumetric flask.

A3 Resuspending buffer (pH 7.4)

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

A4 Washing buffer (pH 6.5)

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

A5 Tris buffer (pH 7.4) containing EDTA and NaCl

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

A6 Phosphate buffer (pH 6.6)

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

A7 ADA (acid-dextrose-anticoagulant)

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

A8 1% TBA

50 ml of glacial acetic acid was made up to 100ml with distilled water and 1 g of TBA was dissolved and the solution was made up to 100 ml.

A9 $ABTS^+$

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

A10 2 % $AlCl_3$

80 ml ethanol was made up to 100 ml with distilled water.
2 g $AlCl_3$ was dissolved in 50 ml of 80 % ethanol and was made up to 100 ml with 80 % ethanol.

A11 Artificial sea water

120 g of sea salt was dissolved in 4 L of distilled water (3:1).

A12 1% Carrageenan solution

2 g Na_2CO_3 was dissolved in distilled water and made up to 100 ml with distilled water.
1 g carrageenan was dissolved in 2% Na_2CO_3 and was made up to 100 ml with 2% Na_2CO_3.

APPENDIX B

DETAILS OF METHODOLOGY

B1 Extraction

Stem bark of *Protorhus longifolia* was washed, air-dried and ground to powder (2mm mesh). The powdered plant material (40 g) was extracted sequentially with hexane, chloroform, ethyl acetate, methanol and water in a platform shaker machine for 24 h per each solvent, at room temperature (157 rpm). The ratio of the plant material to the solvent was 1:5. A separate extraction with methanol only was also carried out with 20 g of the plant material to obtain a separate crude methanol extract. The extracts were separately filtered through Whatman no.1 filter paper. Except for the aqueous extract, all the other extracts were concentrated *in vacuo* at 37 ^{o}C ± 2 ^{o}C. The water extract was freeze dried. The extracts were stored in sterile glass bottles and kept in the fridge (4^{o}C) until used.

B2 Phytochemical screening

B2.1 Test for saponins

The plant material (2.5 g) was extracted with boiling water and was allowed to cool. The extract was shaken vigorously to froth and then allowed to stand for 15 – 20 min. The extract was then classified for saponin content as follows: no froth = negative (no saponins) and froth less than 1 cm = weakly positive (saponins present); froth 1.2 cm high = positive; and froth greater than 2 cm high = strongly positive.

B2.2 Test for tannins

The plant material (0.5 g) was boiled with 10 ml of water for 15 min, filtered and made up to 10 ml with distilled water. Two millilitres of the filtrate was put into another test tube and a few drops of 0.1 % $FeCl_3$ solution were added to the 2 ml of the filtrate. Black-blue, green or blue-green precipitate was taken as preliminary evidence of the presence of tannins.

B2.3 Test for steroids

Acetic anhydride (2 ml) and concentrated sulphuric acid (2 ml) were added to 0.5 g of the plant material and mixed. A colour change from violet to blue or green was taken as evidence of the presence of steroids.

B2.4 Test for terpenoids (Salkwoski test)

The plant material (0.5 g) was mixed with 2 ml of chloroform, and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish-brown colouration of the interface was taken as evidence of the presence of terpenoids.

B2.5 Test for alkaloids

The plant material (0.5 g) was dissolved in 5 ml 1 % HCl (aq). The solution was stirred on steam bath and filtered. One millilitre of the filtrate was treated with Mayer's reagent. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent and turbidity or precipitate was also taken as evidence of the presence of alkaloids.

B2.6 Test for anthraquinones

The plant material (0.5 g) was dissolved and shaken with benzene. The mixture was filtered and 5 ml of 10 % ammonium solution was added to the filtrate. After shaking, the presence of a pink, red, or violet colour in ammonia solution (lower phase) was taken as evidence of the presence of anthraquinones.

B2.7 Test for flavonoids

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

B2.7.1 Lead acetate test

The plant material (1 g) was mixed with 1 ml of 10 % lead acetate. A reddish-brown colouration or precipitate was taken as an evidence of the presence of flavonoids.

B2.7.2 Ferric chloride test

The plant material (1 g) was mixed with 1 ml of $FeCl_3$. A dark brown or dirty brown precipitate was taken as evidence of the presence of flavonoids.

B2.7.3 Sodium hydroxide test

The plant material (1 g) was mixed with 1 ml of dilute NaOH. A golden yellow precipitate was taken as evidence of the presence of flavonoids.

B2.8 Test for cardiac glycosides

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

B2.8.1 Lieberman's test

The plant material (0.5 g) was mixed with 2 ml of acetic acid and this was cooled well in ice. Concentrated sulphuric acid (1 ml) was carefully added down the sides. Colour change from violet to blue to green was taken as an indication of the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycoside.

B2.8.2 Salkowski test

The plant material (0.5 g) was mixed with 2 ml of chloroform, and 2 ml of concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at interface was taken as an indication of the presence of a steroidal ring i.e. aglycone portion of the cardiac glycoside.

B2.8.3 Keller-Kiliani test

The plant material (0.5 g) was dissolved in 2 ml of glacial acetic acid containing 1 drop of 10 % of $Fecl_3$ solution. One milliliter of concentrated sulphuric acid was carefully added on the side of the tube to form a lower layer. A brown ring at interface was taken as an indication of the presence of a deoxy sugar characteristic of cardenolides. Also a violet ring below the brown ring or a greenish ring just above the brown ring (i.e. in the acetic acid layer) and gradually spread through the layer was taken as an indication of the presence of cardiac glycosides.

B2.9 Test for phlobatannins

The plant material (2.5 g) was extracted with boiling water. The extract was boiled with 1 % HCl (aq). Deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

B3 Total phenolic content (gallic acid equivalent - GAE)

Different concentrations of gallic acid (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethyl ether) were prepared. Each plant extract (0.5 ml) was dissolve in 1 ml of diethyl ether and mixed. The diethyl ether was evaporated off to leave behind the residues. Sodium carbonate (7.5 g/100ml) was prepared and Folin-ciocalteus' phenol reagent (FC) was diluted with distilled water (1:10).

Test tubes were set in duplicates. To each test tube containing the residue, 1.5 ml FC and 1.2 ml Na_2CO_3 were added and they were well mixed to obtain solutions. The solutions were kept in the dark for 30 min. Absorbance of the blue coloured mixtures was read at 765 nm with the mixture of FC and Na_2CO_3 used as blank. The results were recorded and they were translated into a standard curve of absorbance (nm) versus concentration of gallic acid (mg/ml). See Figure B1.1. The total phenolic content of the extracts was calculated as gallic acid equivalent from the calibration curve of gallic acid and expressed as mg/g dry plant material. The experiment was replicated twice.

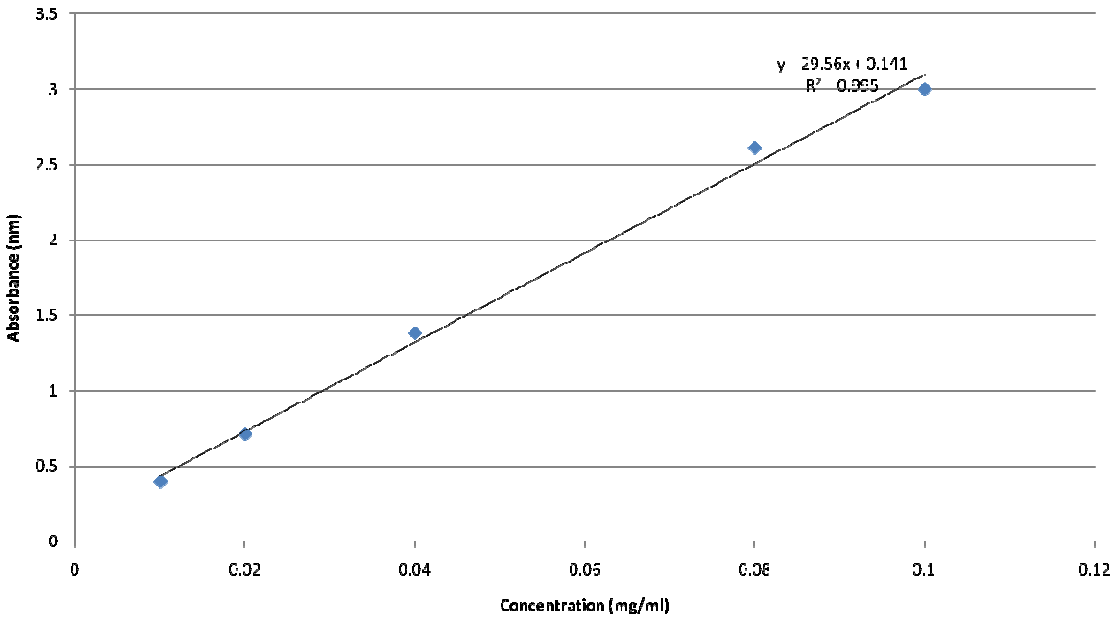


Figure B1.1: Calibration curve of gallic acid concentration (mg/ml) against absorbance (nm). The curve was used to determine the total phenolic content of the extract of *P. longifolia* as gallic acid equivalent.

B4 Flavonoid content (quercetin equivalent – QE)

Different concentrations of quercetin (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethyl ether) were prepared. Each plant extract (0.5 ml) was dissolve in 1 ml of diethyl ether and mixed. The diethyl ether was evaporated off to leave behind the residues.

$AlCl_3$ ethanol solution (2%) was prepared. Test tubes were set in duplicates. The residues were dissolved in 0.5 ml 2% $AlCl_3$ ethanol solution. The solutions were allowed to stand for 1h at room temperature (a yellow colour indicated presence of flavonoids). Absorbance was read at 420 nm against a reagent blank (2% $AlCl_3$ ethanol solution). The flavonoid content of the extracts was determined as quercetin equivalent from the calibration curve of quercetin (figure B1.2) and expressed as mg/g dry plant material.

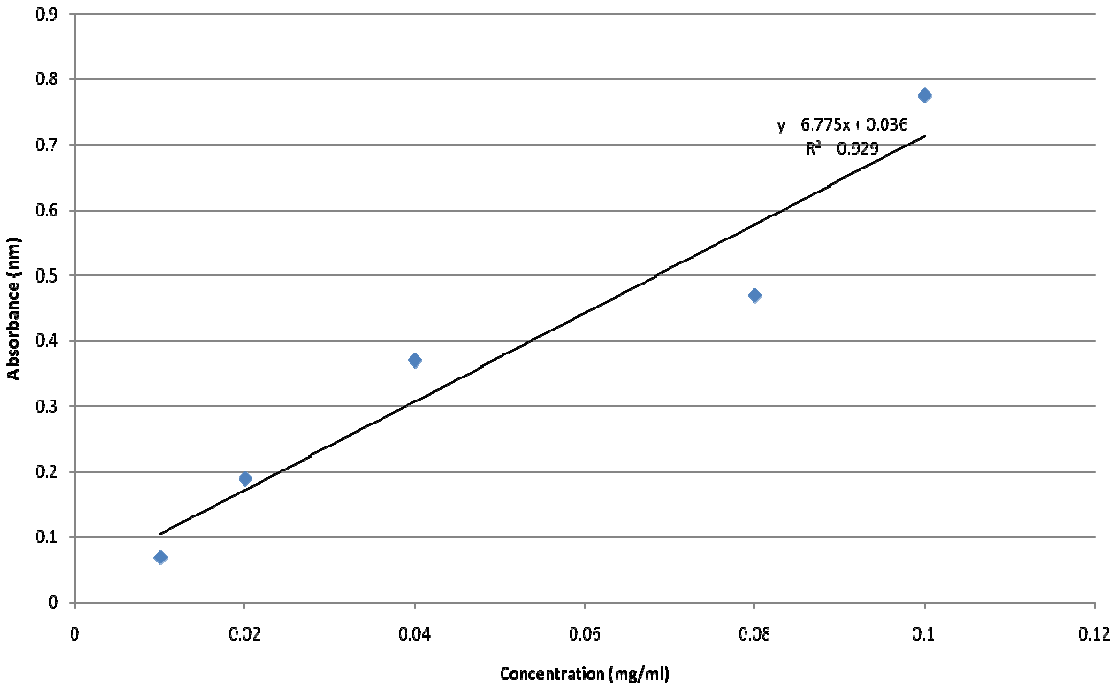


Figure B1.2: Calibration curve of quercetin concentration (mg/ml) against absorbance (nm). The curve was used to determine the flavonoid content of the extracts of *P. longifolia* as quercetin equivalent.

B5 Antioxidative Activity *In vitro*

B5.1 Free radical scavenging

B5.1.1 DPPH assay

DPPH (2 mg/100ml) and different concentrations of the extracts (0 – 5 mg/100 ml) were prepared in methanol. Six test tubes were set in duplicate (12 test tubes) for each extract.

Two millilitres of DPPH (purple) was put into each test tube and 2 ml of each extract was added into the corresponding test tubes and mixed. The mixture was allowed to stand for 30 – 60 min with interval mixing. Decolourisation of the DPPH indicated scavenging activity of the extracts which was determined by reading absorbance at 517 nm. Methanol was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards.

Percentage scavenging activity was calculated using the following formula;

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

Where, A_t =absorbance of test, A_0 = absorbance of control.

Graphs of percentage scavenging activity versus concentration of extract (mg/100ml) were constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B5.1.2 ABTS assay

ABTS (7 mM) was prepared in 3 ml of distilled water and mixed with 2.45 mM potassium persulfate. The mixture was incubated at room temperature in the dark for 16 h. The working solution was diluted with methanol (1 ml $ABTS^+$: 60 ml CH_3OH). The test tubes were set in duplicates (12 test tubes) for each extract.

One ml of the crude extract (0-5 mg/100 ml CH_3OH) was added to 1 ml of 7 mM $ABTS^+$ (green) in the corresponding test tubes and the samples were thoroughly mixed. The mixture was allowed to stand for 6 min. Decolourisation of the $ABTS^+$ indicated scavenging activity of the extracts which was determined by reading absorbance at 734 nm. Methanol was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards. Percentage scavenging activity of the extracts was calculated using the formula:

$$\% \text{ scavenging activity} = (1 - A_t/A_0)100$$

Graphs of percentage scavenging activity versus concentration of extract (mg/100ml) were constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B5.2 Reducing power

The different concentrations (0 – 5 mg/100 ml CH_3OH) of the extracts, PB, 1 % PF, 10 % TCA and 0.1 % $FeCl_3$ were prepared. Test tubes were set in duplicates for each extract.

One millilitre of the extract was mixed with 2.5 ml PB and 2.5 ml PF. The mixture was incubated for 20 min at 50^oC. TCA (2.5 ml) was added to the mixture and this was well mixed. After 5 – 10 min, the mixture was centrifuged at 1000 rpm for 10 min. Supernatant (2.5 ml) was collected and diluted with 2.5 ml of distilled water.

$FeCl_3$ (0.5 ml) was added and well mixed with the supernatant. The reducing power was determined spectrophotometrically at 700 nm and distilled water was used as blank. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as standards. The graphs of absorbance (nm) versus concentration of extract (mg/100 ml) were constructed. The reducing power was determined from the graph, the higher the absorbance the higher the reducing power.

B5.3 Chelating activity on Fe^{2+}

The different concentrations (0 – 5 mg/100 ml CH_3OH) of the extracts, 2 mM $FeCl_2$ and 5 mM ferrozine were prepared. Test tubes were set in duplicates for each extract.

One millilitre of extract was diluted with 3.75 ml deionised water and this was mixed with 0.1 of $FeCl_2$ and 0.2 ml of ferrozine. The mixture was allowed to stand for 10 min with interval mixing. The chelating activity was determined by reading the absorbance at 562 nm. Deionised water was used as blank. EDTA and citric acid were used as standards. Percentage chelating activity was calculated using the formula;

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

The graph of percentage chelating activity versus concentration of extract (mg/100ml) was constructed. The concentration of the extract at which 50% of the reaction was inhibited (IC_{50}) was also determined for each extract using statistical package Origin 6.1.

B6 Brine shrimp cytotoxicity assay

Brine shrimp eggs were hatched in artificial sea water and incubated at 24-28^oC. 120 g of sea salt was dissolve in 4 L of warm water. This was put in a dark covered container (bucket) and light was provided from one source. 4 g of shrimp eggs was added to the artificial sea water. The other bucket containing fresh water was provided and the million air device was used to aerate the water. After 48 h, the eggs were hatched giving a large number of larvae (brine shrimps) and the brine shrimps were used to for the cytotoxicity assay.

Different concentrations (0, 1, 5, and 25 mg/ml) of the plant extracts were prepared in 1% DMSO. Four petri-dishes were set in triplicates (12 petri-dishes) for each extract. Fifty microlitres (50 µl) of a different concentration of each extract was mixed with 25 ml of artificial sea water in each petri-dish and 10 shrimps were separately put in the corresponding petri-dishes. DMSO (1%) was used as negative control. After 24 h and 48 h, numbers of alive and dead shrimp larvae were recorded and mortality rates were calculated (table B1 and B2). In a case where there was mortality in the control, corrected percentages were calculated using Abbot's formula:

Corrected %= (1- n in T after treatment ***/*** n in Co after treatment) x 100
Where; n = organisms population, T= treated, Co= control

Lethal concentration determination: the lethal concentration of plant extract resulting in 50% mortality of the brine shrimp (LC_{50}) and 95% confidence intervals was determined from the 24 h count using probit analysis.

Table B1.1: Results of brine shrimp lethality test (% mortality rate) after 24 h exposure

(a) Hexane extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	27	19
Dead population after treatment	0	3	11
Percentage mortality (%)	3.33 ± 5.77	10.0 ± 0.00	36.7 ± 15.3

(b) Chloroform extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	28	27	18
Dead population after treatment	2	3	12
Percentage mortality (%)	6.67 ± 5.77	10.0 ± 0.00	40.0 ± 10.0

(c) Ethyl acetate extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	29	27	25
Dead population after treatment	1	3	5
Percentage mortality (%)	3.33 ± 5.77	10.0 ± 0.00	16.7 ± 5.77

(d) Methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	29	28	24
Dead population after treatment	1	2	6
Percentage mortality (%)	3.33 ± 5.77	6.67 ± 11.5	20.0 ± 17.3

(e) Water extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	29	28
Dead population after treatment	0	1	2
Percentage mortality (%)	0.00 ± 1.59	3.33 ± 5.77	6.67 ± 11.5

(f) Total methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	30	29	27
Dead population after treatment	0	1	3
Percentage mortality (%)	0.00 ± 5.77	3.33 ± 5.77	10.0 ± 17.3

Table B2.2: Results of brine shrimp lethality test (% mortality rate) after 48 h exposure

(a) Hexane extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	17	15	4
Dead population after treatment	13	15	26
Percentage mortality (%)	9.72 ± 8.67	36.2 ± 19.2	81.5 ± 22.0

(b) Chloroform extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	23	19	13
Dead population after treatment	7	11	17
Percentage mortality (%)	2.64 ± 18.4	20.0 ± 11.5	45.8 ± 3.75

(c) Ethyl acetate extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	22	20	19
Dead population after treatment	8	10	11
Percentage mortality (%)	7.87 ± 6.85	16.3 ± 5.17	20.0 ± 11.5

(d) Methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	18	13
Dead population after treatment	9	12	17
Percentage mortality (%)	12.6 ± 1.59	24.8 ± 10.9	28.4 ± 12.4

(e) Water extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	19	18
Dead population after treatment	9	11	12
Percentage mortality (%)	12.6 ± 1.59	28.8 ± 18.8	34.7 ± 20.7

(f) Total methanol extract

	Concentration (mg/ml)		
	1	**5**	**25**
Total population treated	30	30	30
Live population after treatment	21	20	17
Dead population after treatment	9	10	13
Percentage mortality (%)	12.6 ± 1.59	17.4 ± 9.70	37.0 ± 22.1

B7 Anti-inflammatory activity

Sprague-Dawley rats (weighing 200 ± 20 g) were let to acclimatise for four days before experiment. They were kept and maintained under standard environmental conditions where they had free access to standard diet and water as outlined in the guide for the care and use of laboratory animals.

The rats were orally administered with the compound (50 and 500 mg/kg) daily for four days before the experiment. An hour before the experiment, negative and

positive control animals were administered with 0.5% carboxymethyl cellulose (CMC) and indomethacin (10 mg/kg) respectively. One hour after the last day of administration, all the rats were subcutaneously injected into the plantar surface of the right hind paw with 0.1 ml (1% w/v) carrageenan solution, whereas the left paws were injected with normal saline for comparison.

The animals were divided into four groups of four rats each as follows:
Group I (negative control): received saline solution
Group II (positive control): received indomethacin
Group III (treatment): received 10 mg/200kg compound
Group IV (treatment): received 100mg/200kg compound

The paw volume until knee joint was measured by a water displacement method using a plethysmometer immediately and at 1 h interval for 4 h following the carrageenan injection. The anti-inflammatory activity was calculated by measuring the volume difference between the right and left paws in comparison with the control group. Data was expressed as mean ±SEM and student's t-test was applied to determine the significance of the difference between the control groups and rat treated with the compounds. Values of $p \leq 0.05$ were taken as statistically significant.

B8 Anti-platelet aggregation study

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

B8.1 S-2238

S-2238 (0.008M) was prepared in distilled water and different concentrations of plant extracts (0, 1, 3 mg/ml) dissolved in DMSO were prepared in Tris buffer. A 96-well microtitre plate was used and the experiment was replicated twice. Plant extract (50 µl) was incubated with 10 µl thrombin (30U/ml) in the corresponding wells for 10 minutes at room temperature. S-2238 (190 µl) was added to the wells and the reaction was monitored with Biotek plate reader ELx808 IU using Gen5 software by following change in absorbance at 415 nm for 4 min at 12 sec interval. 1% DMSO was used as negative control.

B8.2 Preparation of Blood platelets

A rat was killed by a nock on the head. Blood was immediately collected from abdominal aorta of the rat and was put in a centrifuge tube containing ADA (1 ml ADA: 5 ml blood). The blood was centrifuged (Eppendorf centrifuge 5804 R) at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively. Supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was discarded and sediment (platelets) obtained was resuspended in 5 ml of washing buffer (pH 6.5). This was centrifuged at 3000 rpm for 15 min. The supernatant was discarded and the platelets were suspended in a little volume of a resuspending buffer (pH 7.4). The platelets were divided into four (4) parts: untreated and enzyme (trypsin, bromelain, and papain) treated platelets. A 1:10 dilution of the platelets in the resuspending buffer was taken.

B8.3 Enzyme treated platelets

An enzyme (0.1 mg) was added to the platelets (25 ml) and this was well mixed and incubated at 25^{o}C for 60 min. A little volume (about 3 ml) of the washing buffer was added. The mixture was centrifuged at 3200 rpm for 15 min and the supernatant was discarded. This (addition of the buffer and centrifuging) was repeated three times. A 1:1 dilution of the treated platelets with a resuspending buffer was done.

B8.4 Anti-platelet aggregation activity

Different concentrations of the plant extracts (1, 3 and 10 mg/ml) dissolved in DMSO were prepared in Tris buffer (pH 7.4). Diluted platelets were mixed with $CaCl_2$ (0.4 ml:10 µl $CaCl_2$). The 96-well micro plate was used in the experiment and all the experiments were replicated twice.

Platelets (200 µl) were pre-incubated with the different concentration of the plant extract in the corresponding well for 5 min at 37^{o}C. Then 20 µl of thrombin (5U/ml) was added to the mixture. The reaction was monitored at 415 nm for 20 min at 30 sec interval with the Biotek plate reader using Gen5 software. Heparin was used as a standard and 1% DMSO was used as negative control. Thrombin plus 1% DMSO was used as positive control.

B8.5 Measurement of MDA

After the anti-platelet aggregation test, 100 µl of the solution from each well was collected and 1.5 ml of trichloroacetic acid (TCA) was added. The mixture was allowed to stand for 10 min and then centrifuged at 3500 rpm for 20 min. Supernatant was collected and 1.5 ml of 2-thiobarbuturic acid (TBA) was added. The mixture was heated in boiling water bath for 30 min and was allowed to cool. Two millilitres of n-butanol was added and this was mixed and allowed to separate. Top layer was collected and the MDA formation was determined with the Biotek plate reader using Gen5 software by reading absorbance at 490 nm. n-butanol was used as blank. Percentage inhibition of MDA formation was calculated;

$$\% \text{ inhibition} = \frac{\Delta A \text{ control} - \Delta A \text{ plant extract}}{\Delta A \text{ control}} \times 100$$

Where, ΔA = change of absorbance

And graphs of % inhibition versus concentration of extract (mg/ml) were constructed. The same method was repeated with ADP and epinephrine on both untreated and enzyme treated platelets.

B8.6 Tannin removal

The plant extracts were prepared at a concentration of 10 mg/ml in distilled water. PVPP was added to the extracts at 10 mg/ml, shaken for 15 min at 4^{o}C, and the mixture was centrifuged at 5000 rpm for 8 min at 4^{o}C. The pellet was discarded and supernatant was collected. Using the supernatant, the procedure was repeated three times so as to remove tannins to a negligible concentration. The tannin-free extracts were re-dissolved in DMSO for use in subsequent tests for the anti-platelet aggregation activity.

B8.7 Thrombin-induced clotting time assay

The different concentrations (1, 3 and 10 mg/ml) of the extract were used in the assay. Platelets (200 µl) were mixed and incubated with 50 µl of the extract at room temperature. Twenty microlitres (20 µl) of thrombin (5U/ml) was added to the mixture and the rate of clot formation was determined with the Biotek plate reader using

Gen5 software by following change in absorbance at 415 nm for 20 min at 30s intervals. The experiment was replicated twice and 1% DMSO was used as a negative control.

B8.8 $CaCl_2$-induced clotting time assay

The different concentrations (1, 3 and 10 mg/ml) of the extract were used in the assay. Platelets (200 µl) were mixed and incubated with 50 µl of the extract at room temperature. Twenty microlitres (20 µl) of 0.16 M $CaCl_2$ was added to the mixture and the rate of clot formation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm for 2 h at 3 min intervals. The experiment was replicated twice and 1% DMSO was used as a negative control.

Calculation of percentage inhibitory effect of plant extracts

All assays were repeated three times and the mean V ± 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.

B9 Isolation, purification and characterisation

B9.1 Isolation and purification

Crude solvents (hexane, dichloromethane, ethyl acetate and methanol) 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) (210 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 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 extract (8.0 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 collection of 20 ml fractions was collected into beakers. Elution was done using hexane:ethyl acetate solvent system starting with 9:1 to 3:7. A total of 119 of 20 ml fractions were collected. TLC analysis of the collected fractions was performed to identify those with common profile. The TLC plates were first viewed under UV, developed using a 10% H_2SO_4 spray reagent and then heated. The fractions with similar profile were combined as RA/04/A (f_1-f_5), RA/04/B (f_6-f_{12}), RA/04/C (f_{13}-f_{19}),…, RA/04/P (f_{108}-f_{119}) to give 16 combined fractions. The combined fractions were separately concentrated *in vacuo* and their weights were determined.

Based on the nature of RA/04/G and RA/04/I, the two compounds were separately dissolved in methanol (RA/04/G) and hexane (RA/04/I) to recrystalise. The mixtures were then separately filtered to obtain residues (RA/04/G_1; RA/04/I_1) and filtrates (RA/04/G_2; RA/04I_2) respectively. The samples were analysed by TLC and the filtrates still contained impurities while both the residues (RA/04/G_1 and RA/04/I_1) showed purity. The pure compounds were subjected to NMR techniques for characterisation and structural elucidation.

B9.2 Structural elucidation

In order to characterise and identify the compounds, RA/04/G_1 (MS/10) and RA/04/I_1 (MS/12) were further analysed using 1D and 2D NMR techniques (^{1}H-^{1}H, ^{13}C-^{13}C, DEPT, COSY, HMQC, HMBC and NOESY) (in $CDCl_3$, Bruker 600 MHz with solid state probe), IR (Perkin-Elmer 100 FTIR with ATR sampling accessory), UV ($CHCl_3$, Varian - Cary 50 UV-visible spectrophotometer) and LC-MS (DCM, Waters API Q-TOF Ultima). The molecular formula was identified by ESI-MS (positive mode), $[M + H]^+$. In order to get more information about the structure and composition of compound, fragmentation of the compound was also done. Melting point (Reichert Thermovar) of the compounds was also determined. The resulting spectra were analysed and the compounds were identified by comparing them to similar compounds in literature.

The anti-platelet aggregation, antioxidant and anti-inflammatory activity of the pure compounds were also investigated following the same methods used for the crude extracts. Cytotoxicity of MS/12 was also determined using MTT cell proliferation assay.

B10 MTT cell proliferation assay

Cells were all grown to confluenecy in 25 cm^2 flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities (2.5 x 10^4 cells per well). Cells were incubated over overnight at 37^oC. Medium was then removed and fresh medium (MEM + Gutamax + antibiotics) was added. Compound at different concentrations (50, 100, 150, 200, 250, 300, and 350µg/200µl) was then added in triplicate and the mixture was incubated for 4 hrs. Thereafter medium was removed and replaced by complete medium (MEM + Gutamax + antibiotics + 10% fetal bovine serum). After 48 hrs cells were subjected to the MTT assay.

MTT solution was prepared at a concentration of 5 mg/ ml PBS. At the end of incubation period (48 hrs), the medium was removed from the cells in multiwell plate. MTT solution (200µl) as well as 200 µl of cell culture medium was added to each well containing the cells. The multiwell plate was incubated at 37^oC for 4 hrs. Thereafter the medium and MTT solution were removed from the wells and 100/200/400µl of DMSO was added to each well (stops reaction and dissolves insoluble formazan crystals). The plate was read in a plate reader or spectrophotometer at 570 nm.
The experiment was replicated thrice and the results were expressed as mean ± SD. Percentage inhibition of cell growth was calculated as:

$$\% \text{ cell death} = [(A_c - A_t)/A_c \times 100]$$

Where A_c is the absorbance of control and A_t is the absorbance of the extract.
Lethal concentration of the compound that results in 50% cell death (LC_{50}) was determined by regression analysis using QED statistics programme.

APPENDIX C

C1. Ethic 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.

RJ Ewan.

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

C2 Interview of Traditional Healers

Research Questionnaires

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	
Female	

15-24	
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) is used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time of collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants	

Preparation Method:

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

b) How is the medicine prepared?

Storage Method:

Dosage:

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

_____

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

_____

c) Are there any known side effects? _____

_____

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

_____

_____

Age Group:

Infants	
Children	
Adults	

APPENDIX D

GRAPHS

D1 Antioxidant Activity- free radical scavenging activity

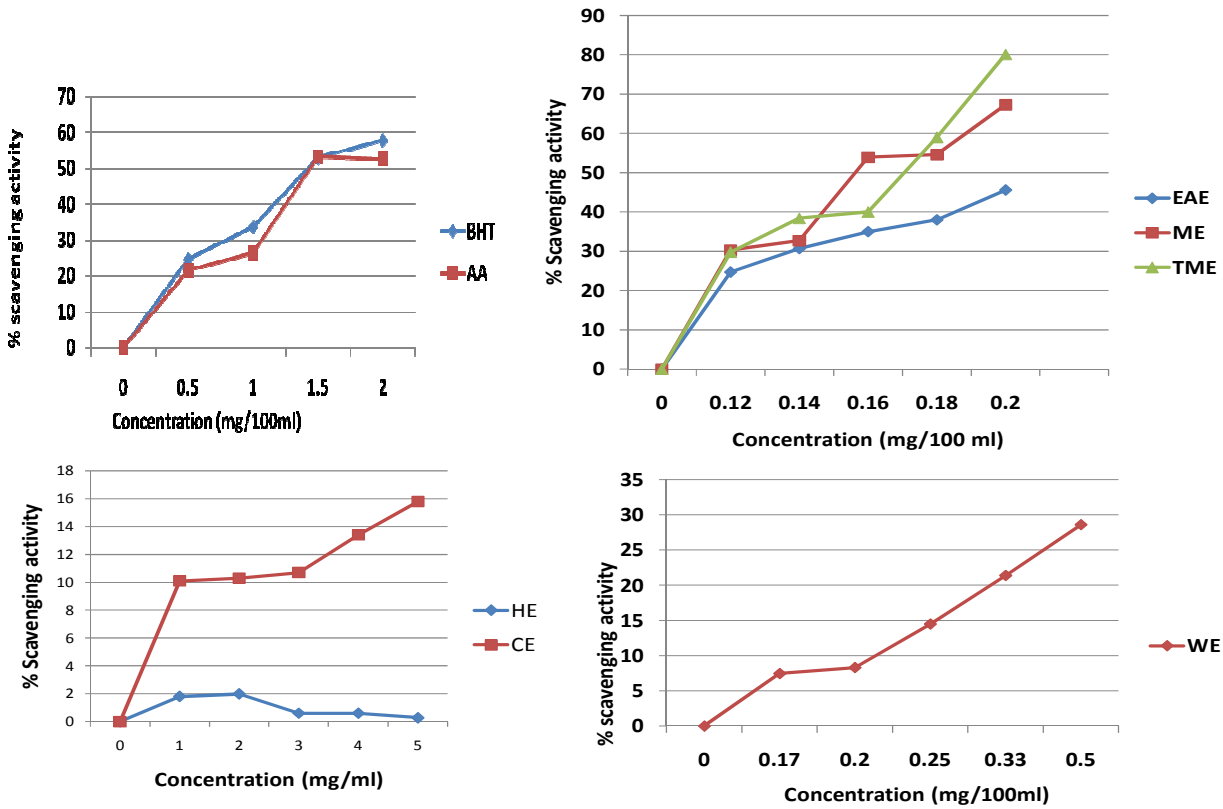


Figure D1.1: Percentage scavenging activity of the extracts of *P longifolia* on DPPH radical

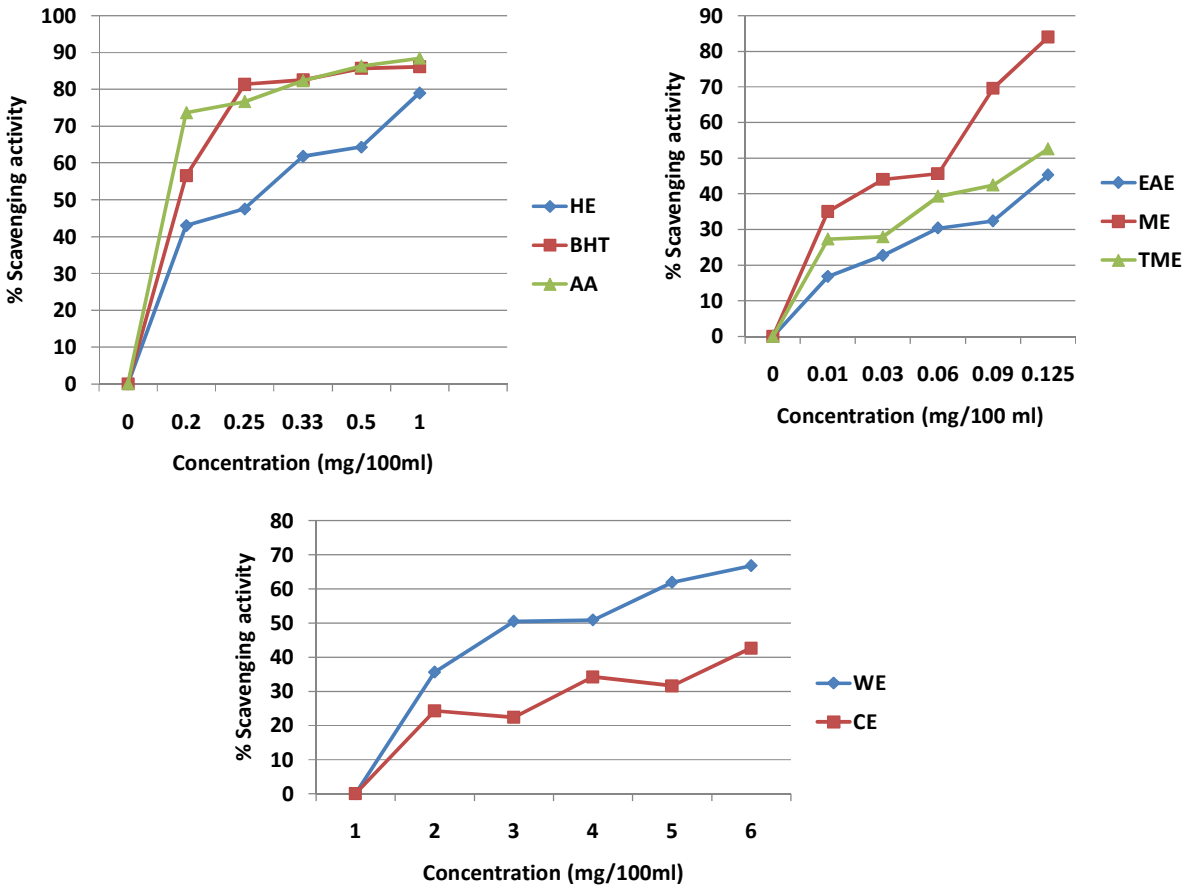


Figure D1.2: Percentage scavenging activity of the extracts of *P longifolia* on ABTS radical

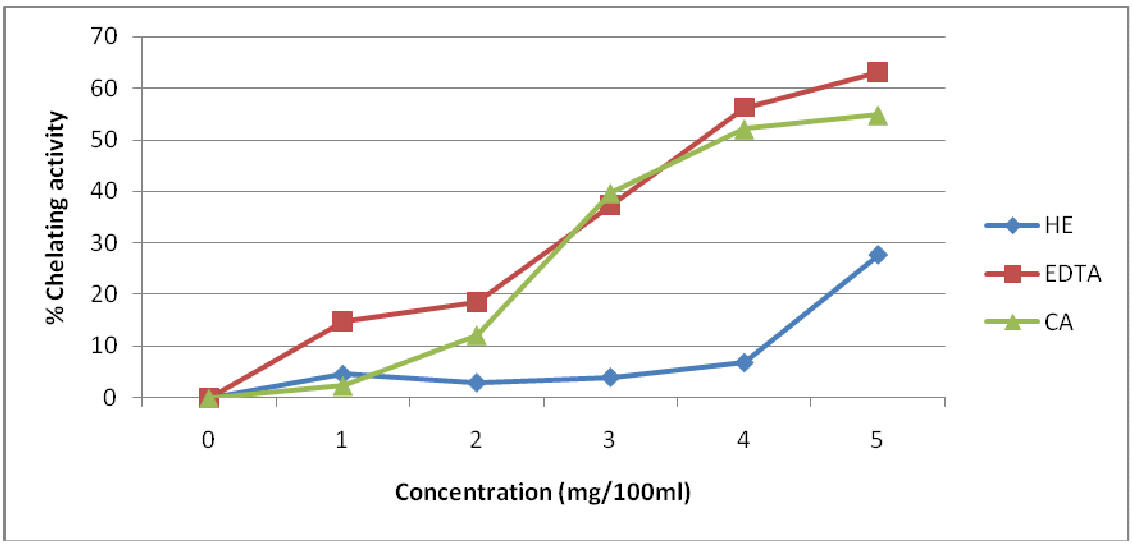


Figure D1.3: Percentage chelating activity of the extracts of *P longifolia* on Fe^{2+} Ethylenediaminetetra acetic acid (EDTA) and citric acid (CA) exhibited IC_{50} values of 3.68 and 3.85mg/ml respectively.

D2 Anti-platelet Aggregation Activity- untreated platelets

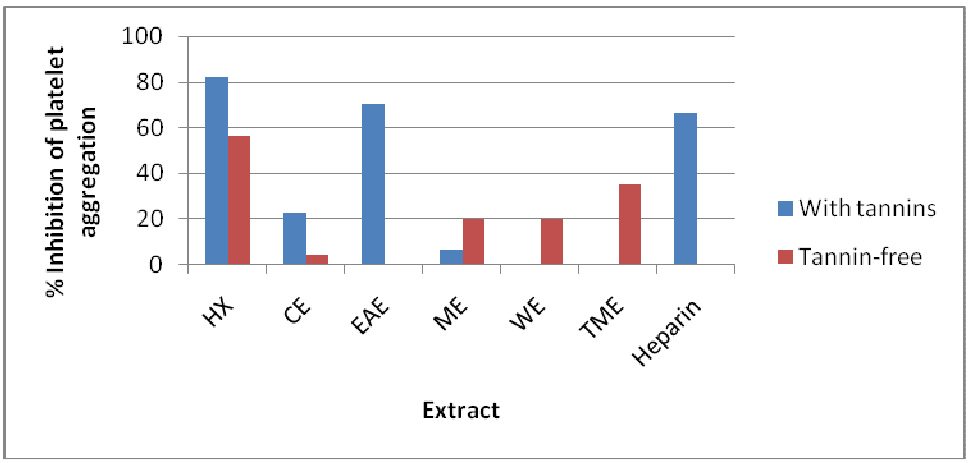


Figure D2.1a: Anti-platelet aggregation activity of the extracts of *P longifolia* on the thrombin-induced platelet aggregation.

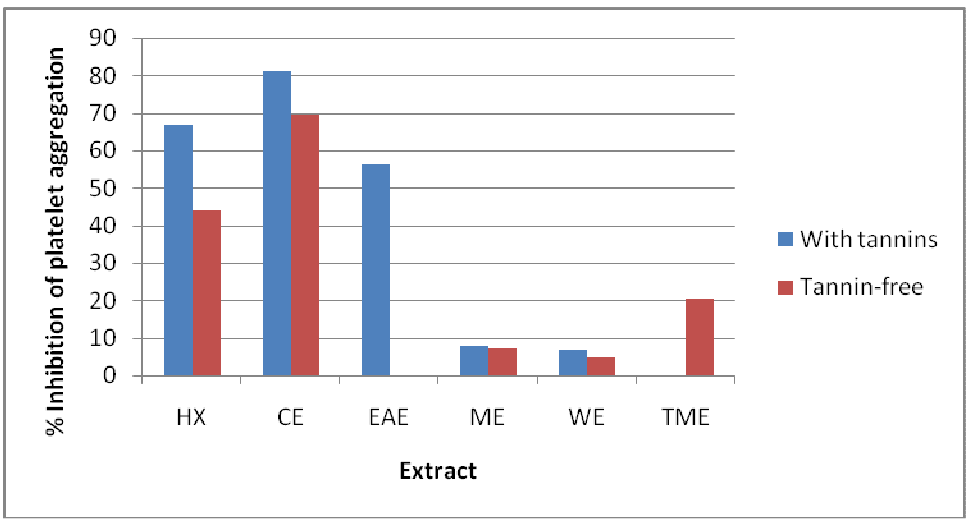


Figure D2.1b: Anti-platelet aggregation activity of the extracts of *P longifolia* on the ADP-induced platelet aggregation.

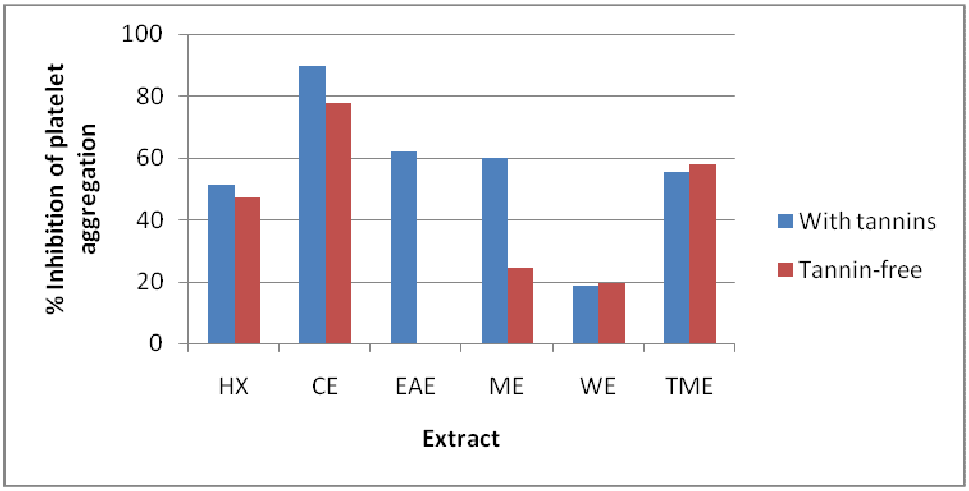


Figure D2.1c: Anti-platelet aggregation activity of the extracts of *P longifolia* on the epinephrine-induced platelet aggregation.

APPENDIX E

SPECTRA

E1 Spectral data for compound MS/10

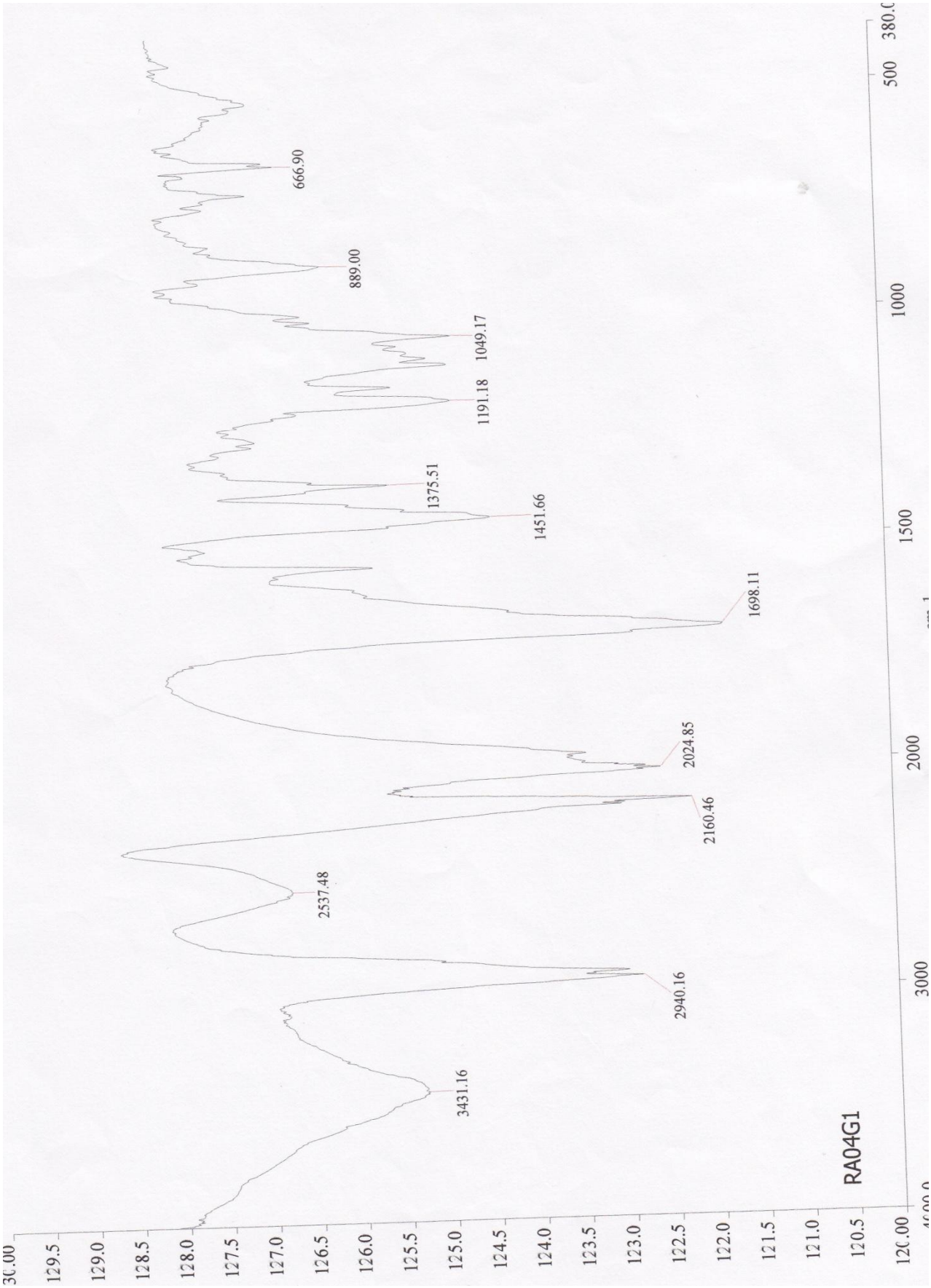


Figure E1.1: IR spectrum of compound MS/10

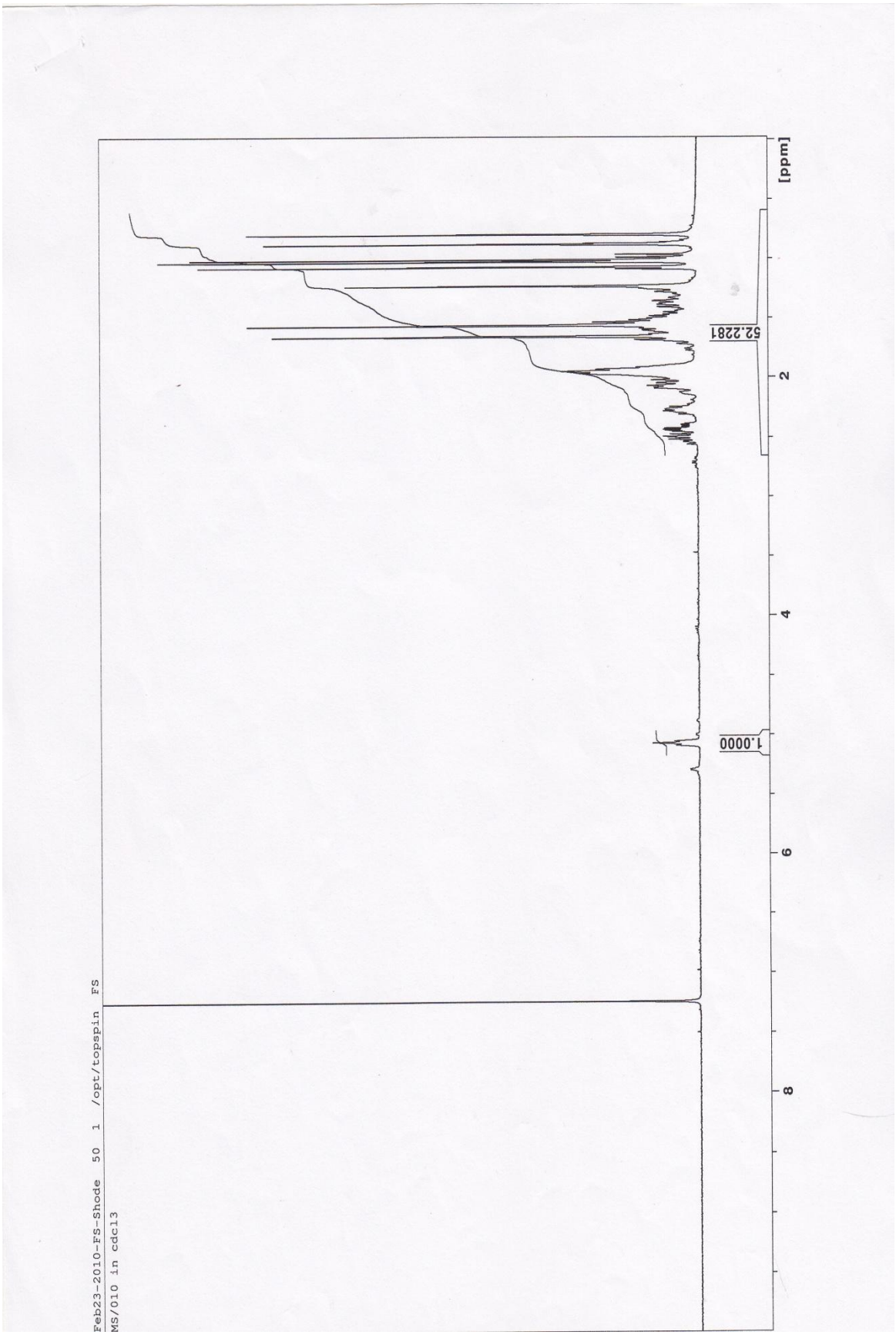


Figure E1.2: ^{1}H-NMR spectrum of compound MS/10

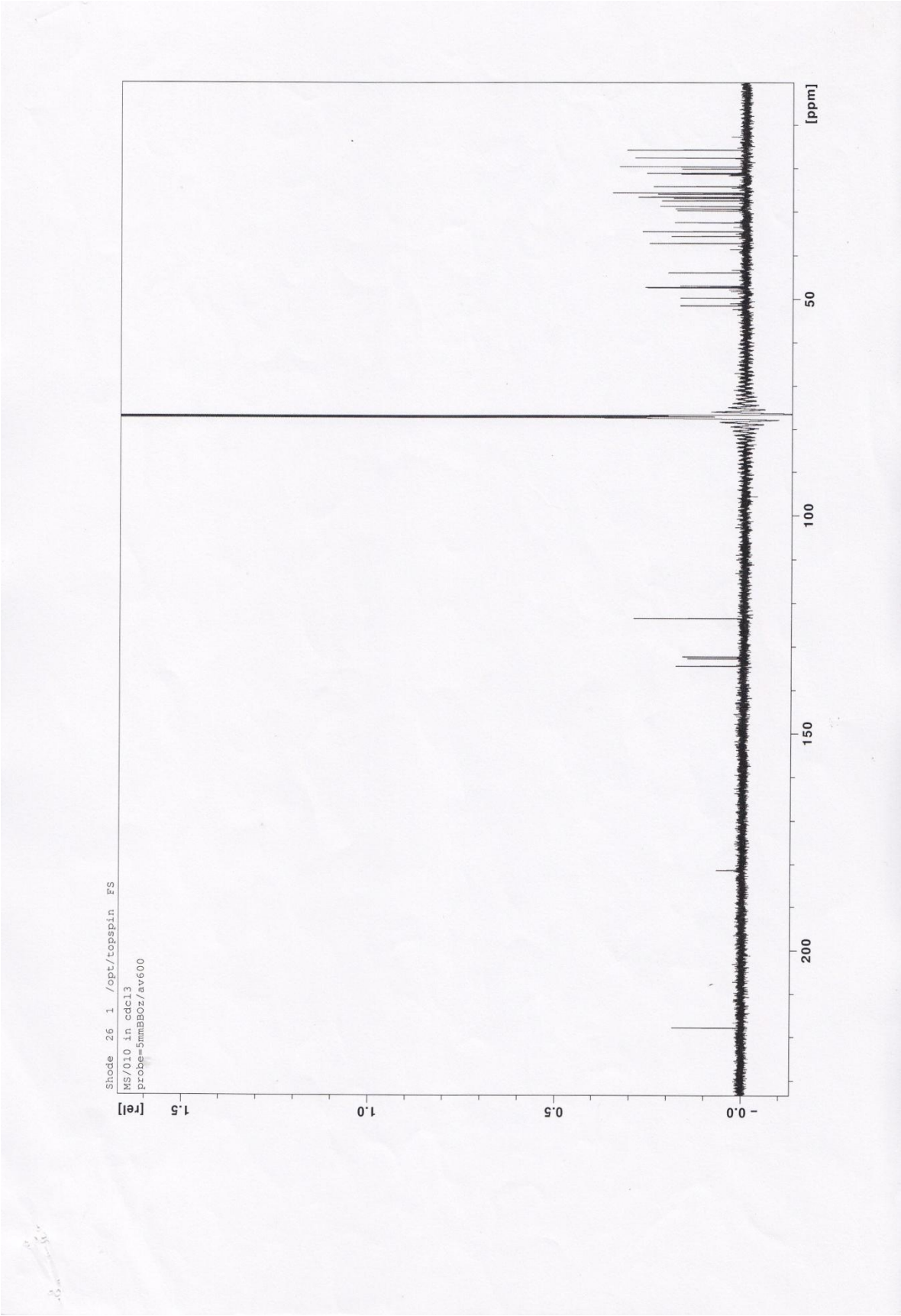


Figure E1.3: ^{13}C-NMR spectrum of compound MS/10

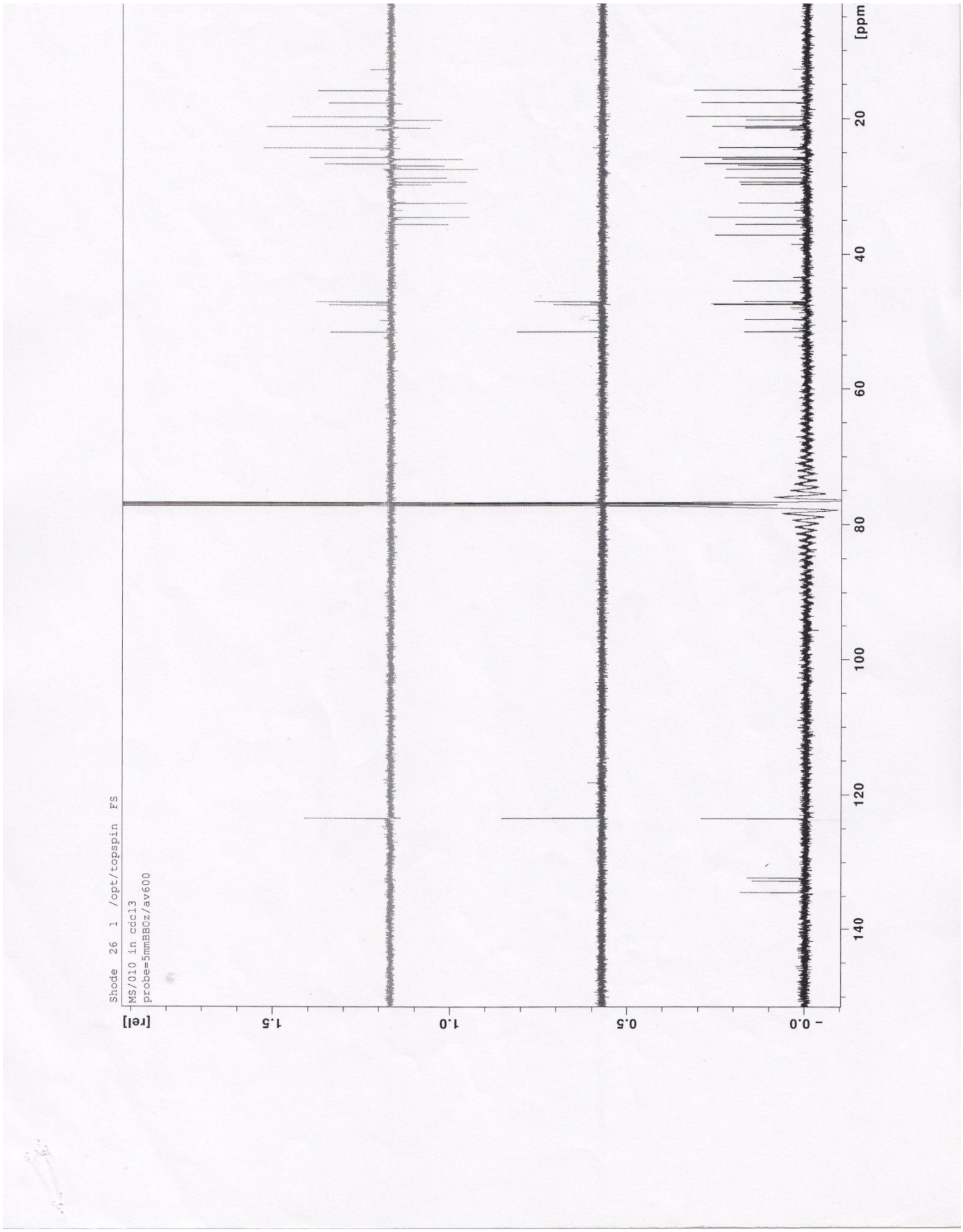
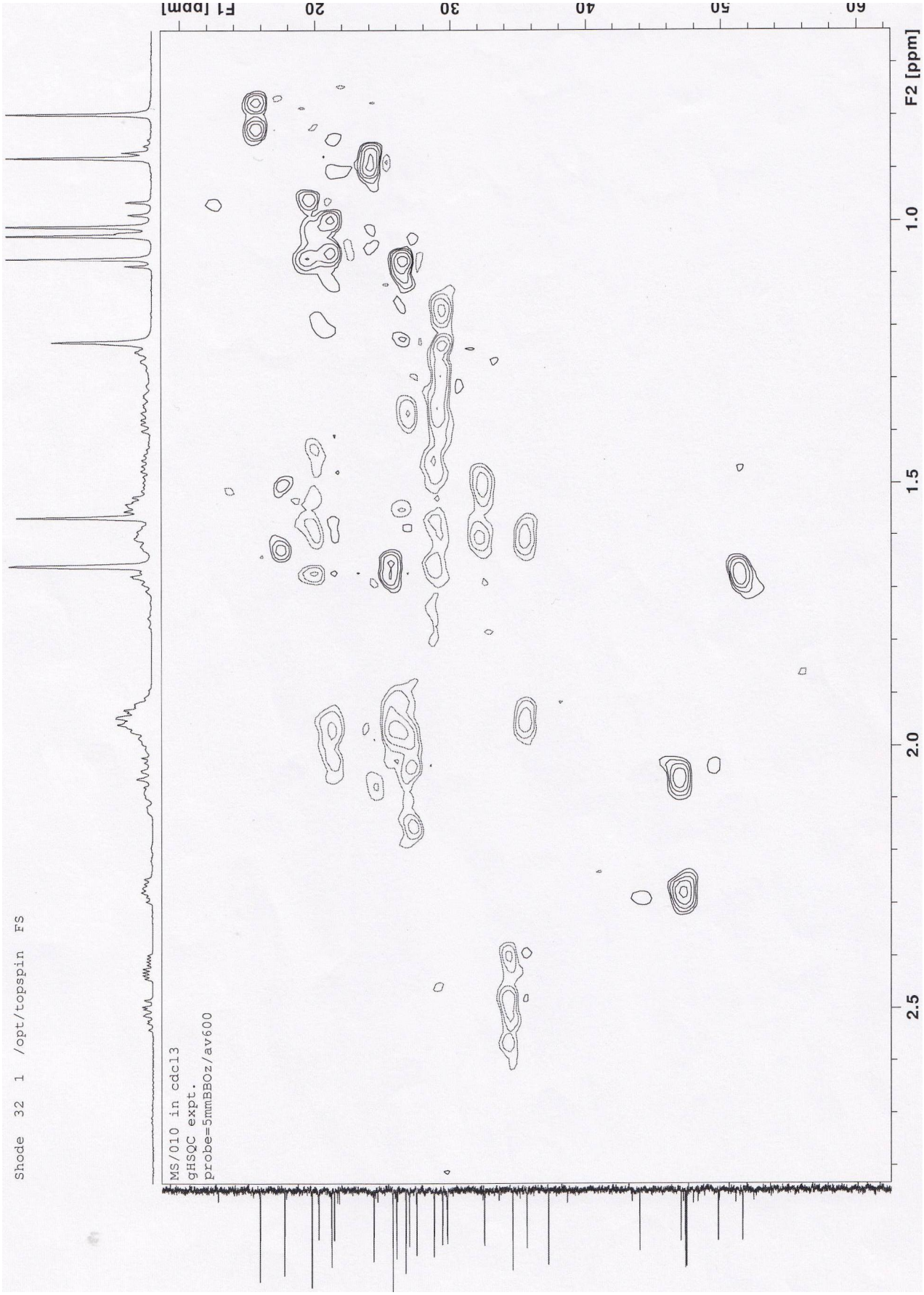


Figure E1.4: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound MS/10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MS/010 in cdc13
gHSQC expt.
probe=5mmBBOz/av600
F1 [ppm]
F2 [ppm]
60
50
40
30
20
2.5
2.0
1.5
1.0

Figure E1.5: HSQC NMR spectrum of compound MS/10

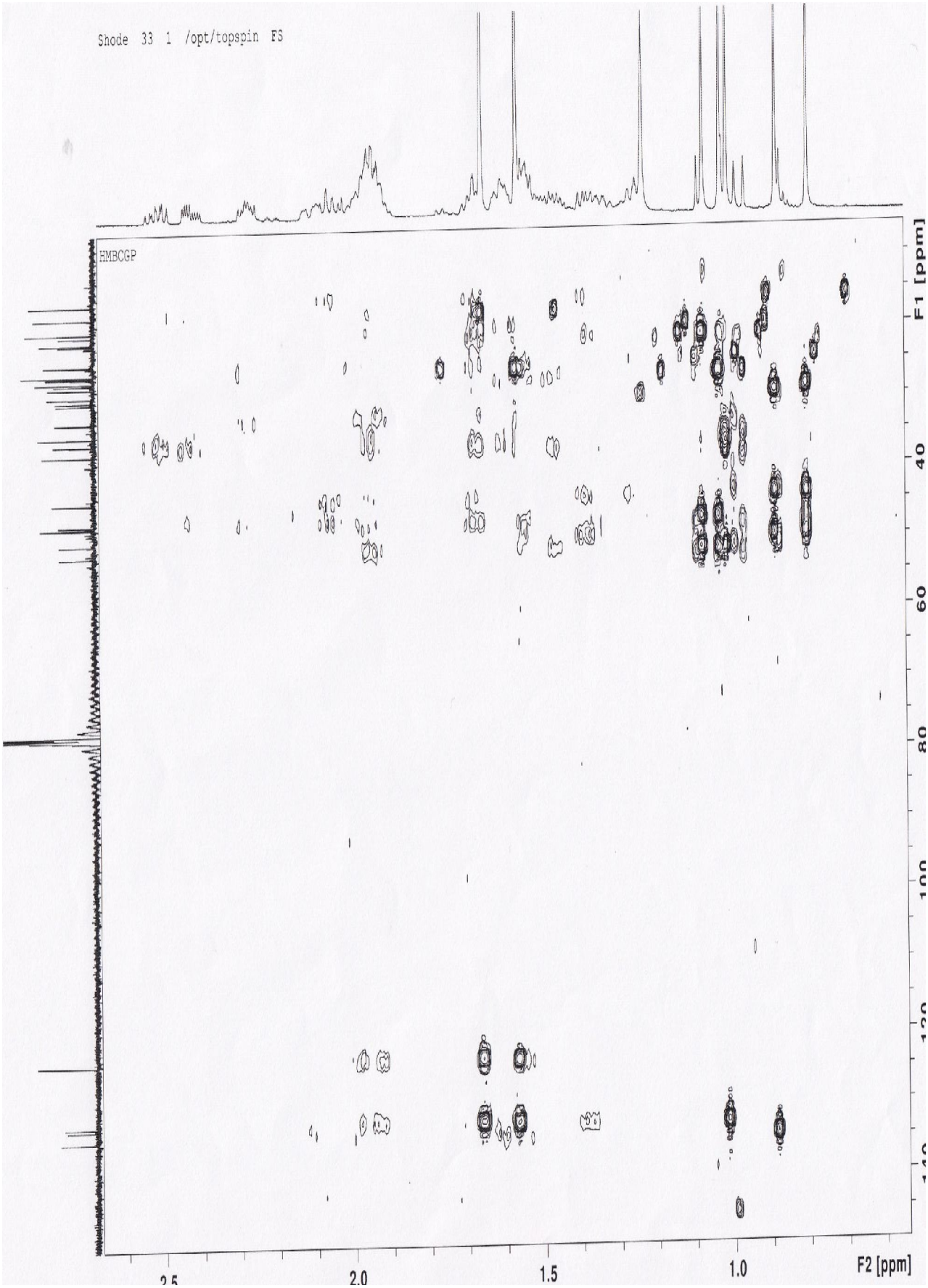

Figure E1.6: HMBC NMR spectrum of compound MS/10

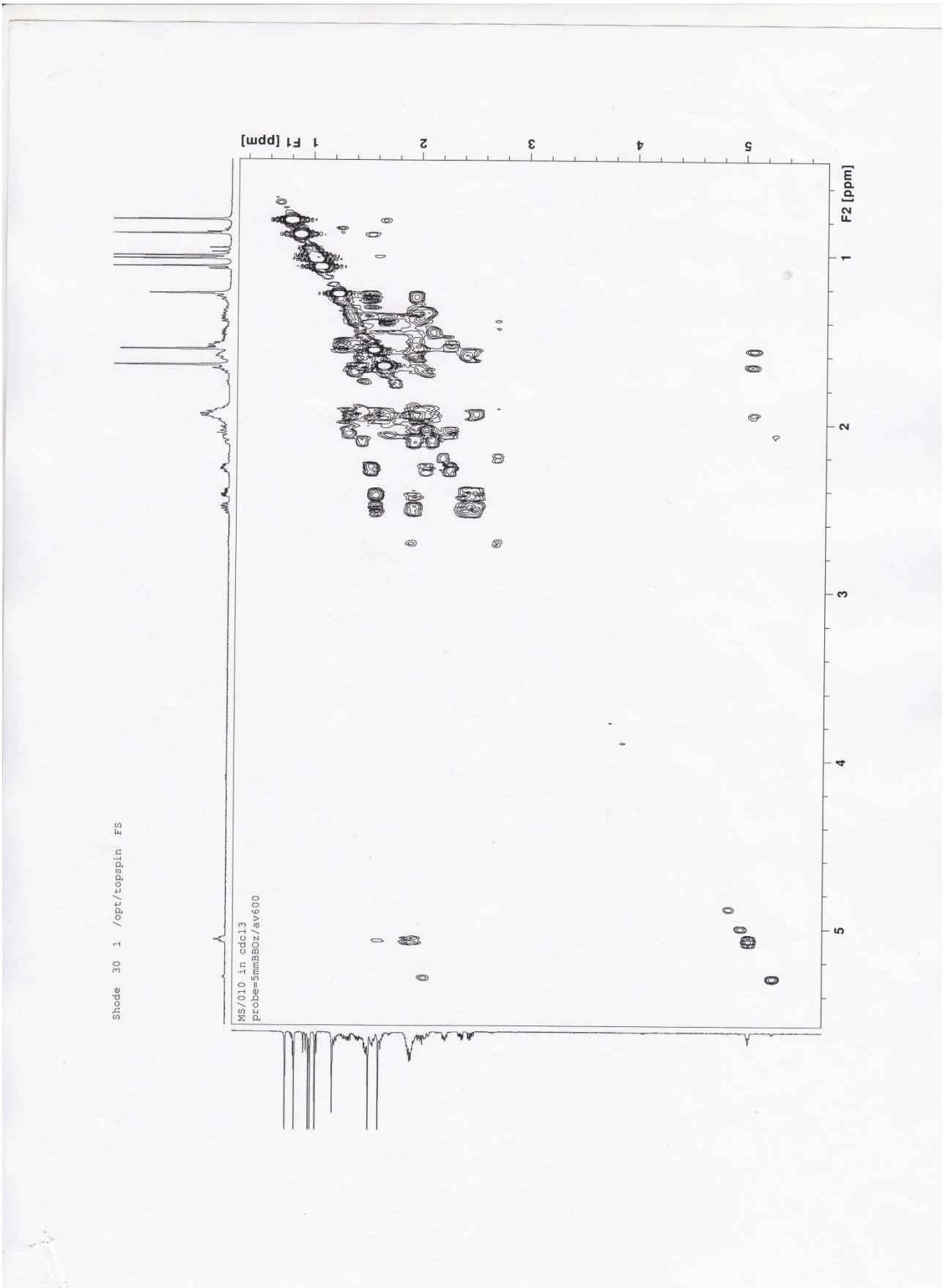

Figure E1.7: COSY NMR spectrum of compound MS/10

Figure E1.8: NOESY NMR spectrum of compound MS/10

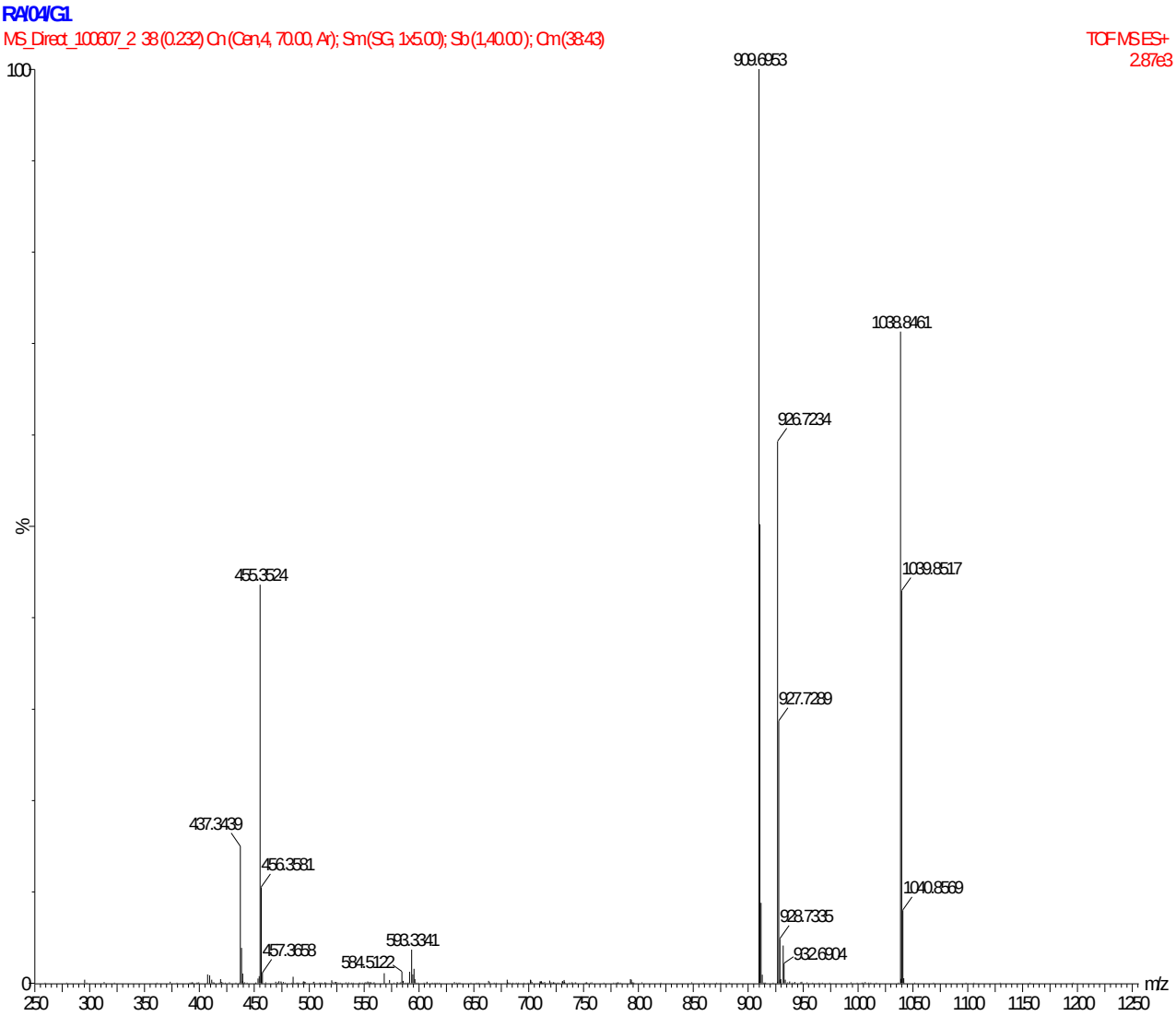

Figure E1.9a: MS spectrum of compound MS/10

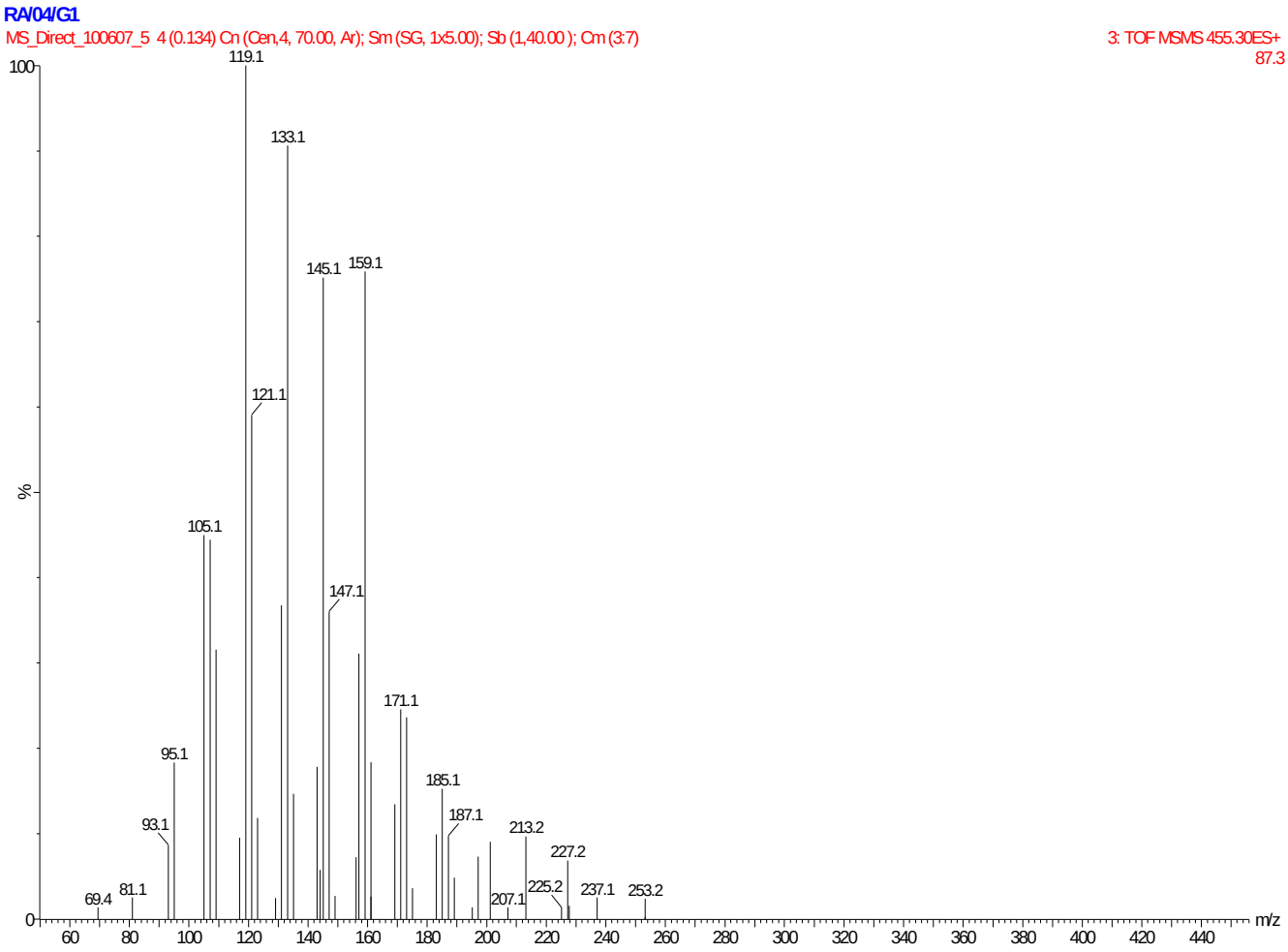

Figure E1.9b: MS spectrum of compound MS/10

E2 Spectral data for compound MS/12

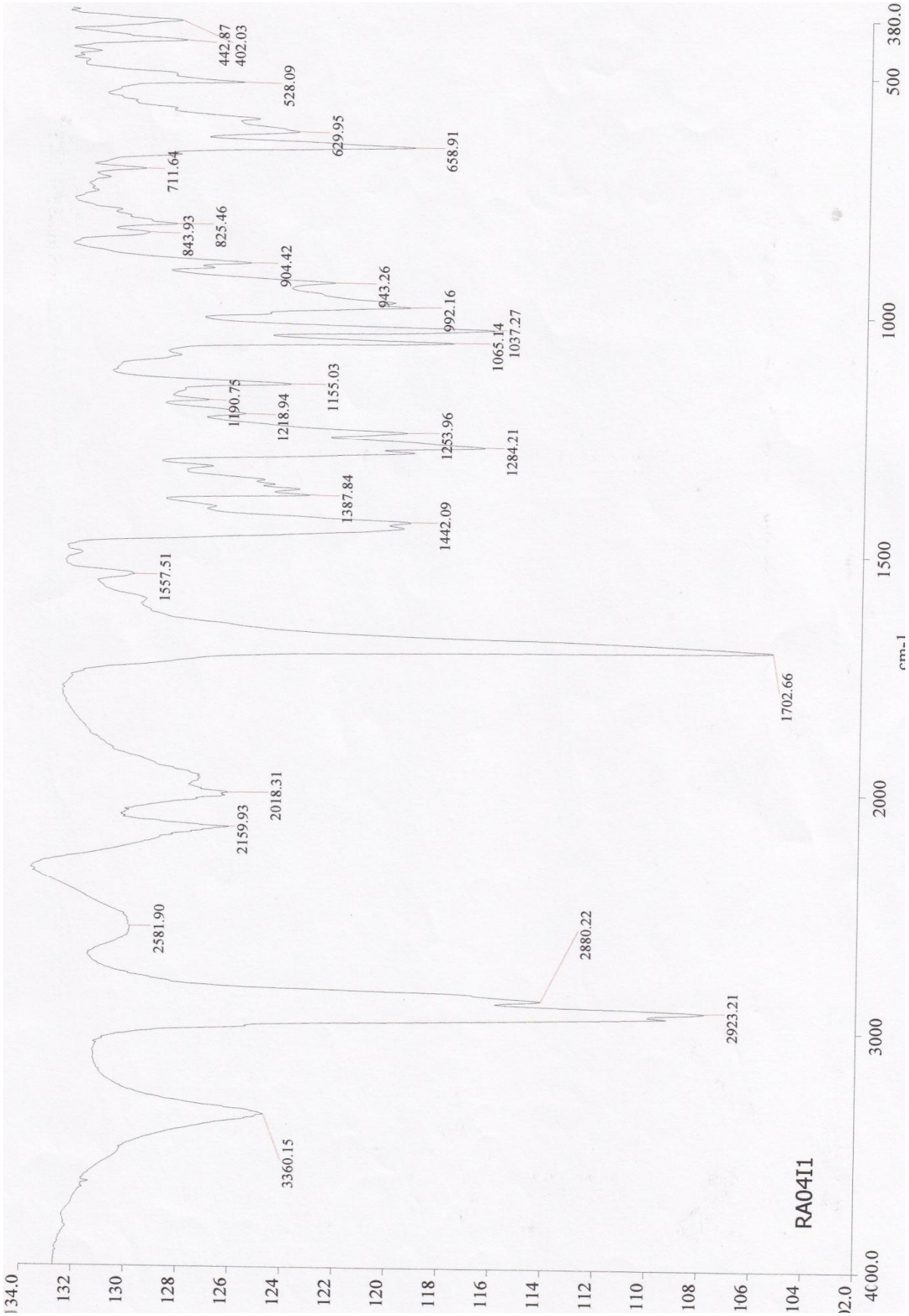

Figure E2.1: IR spectrum of compound MS/12

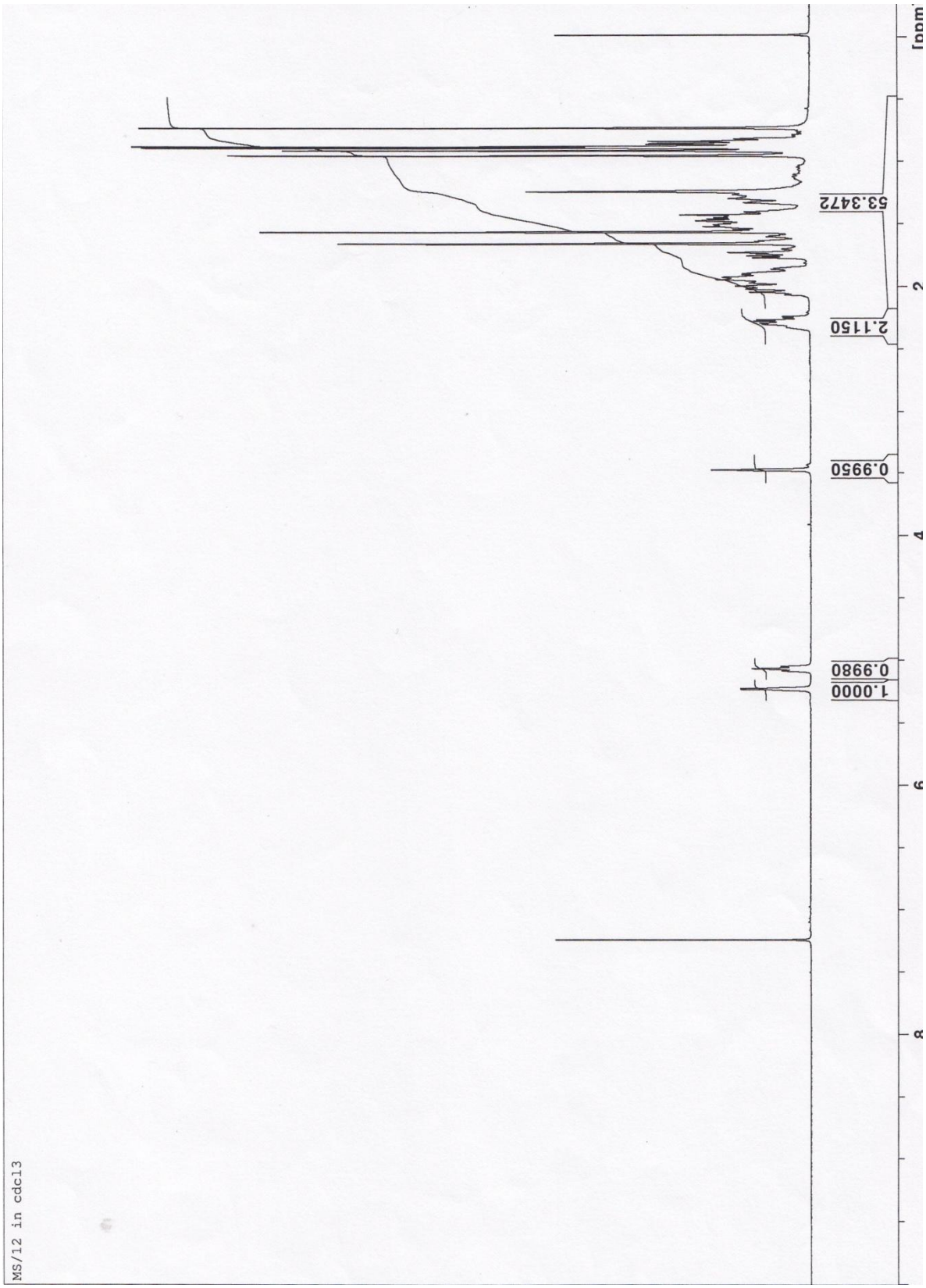

Figure E2.2: ^{1}H-NMR spectrum of compound MS/12

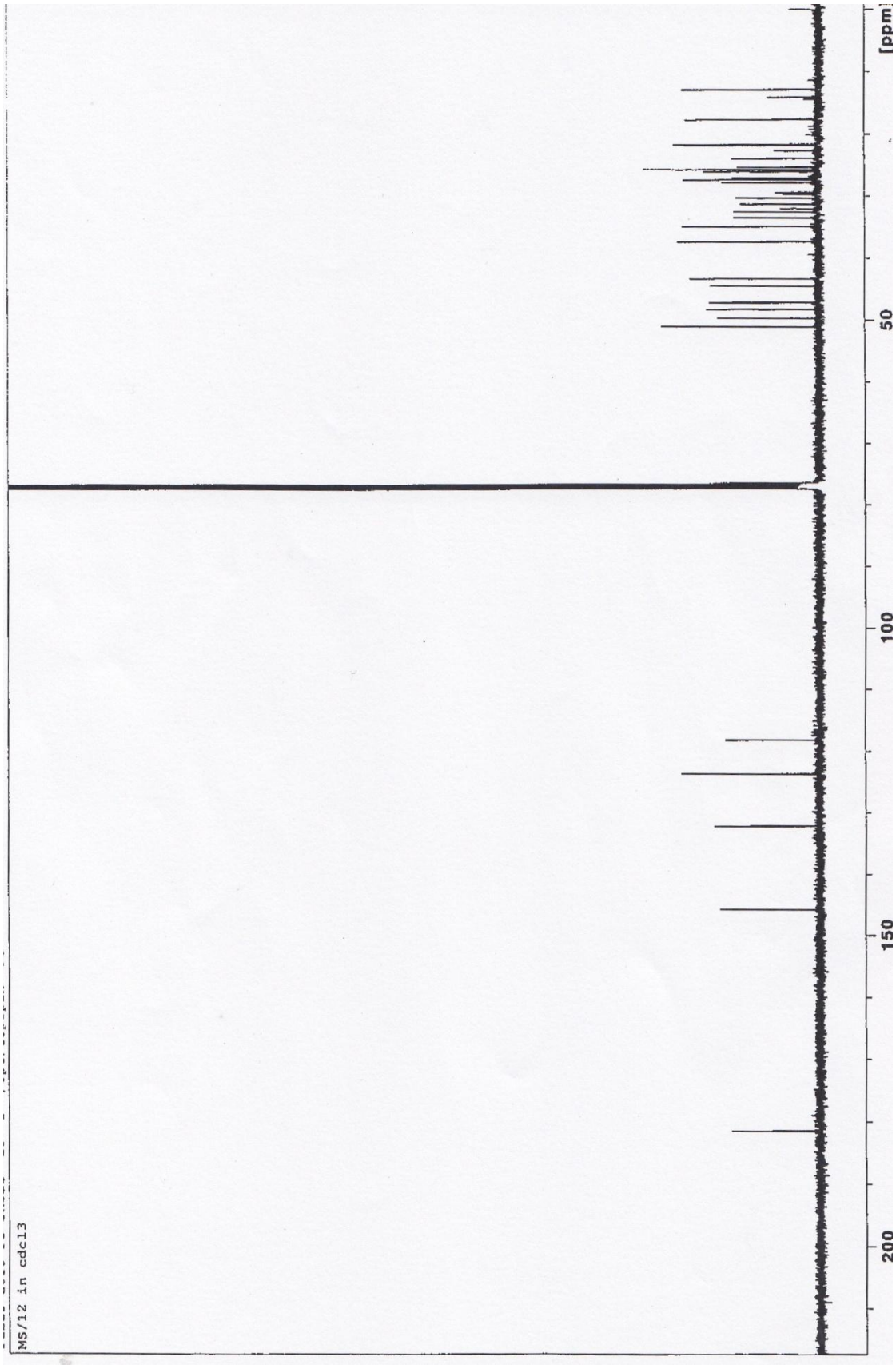

Figure E2.3: ^{13}C-NMR spectrum of compound MS/12

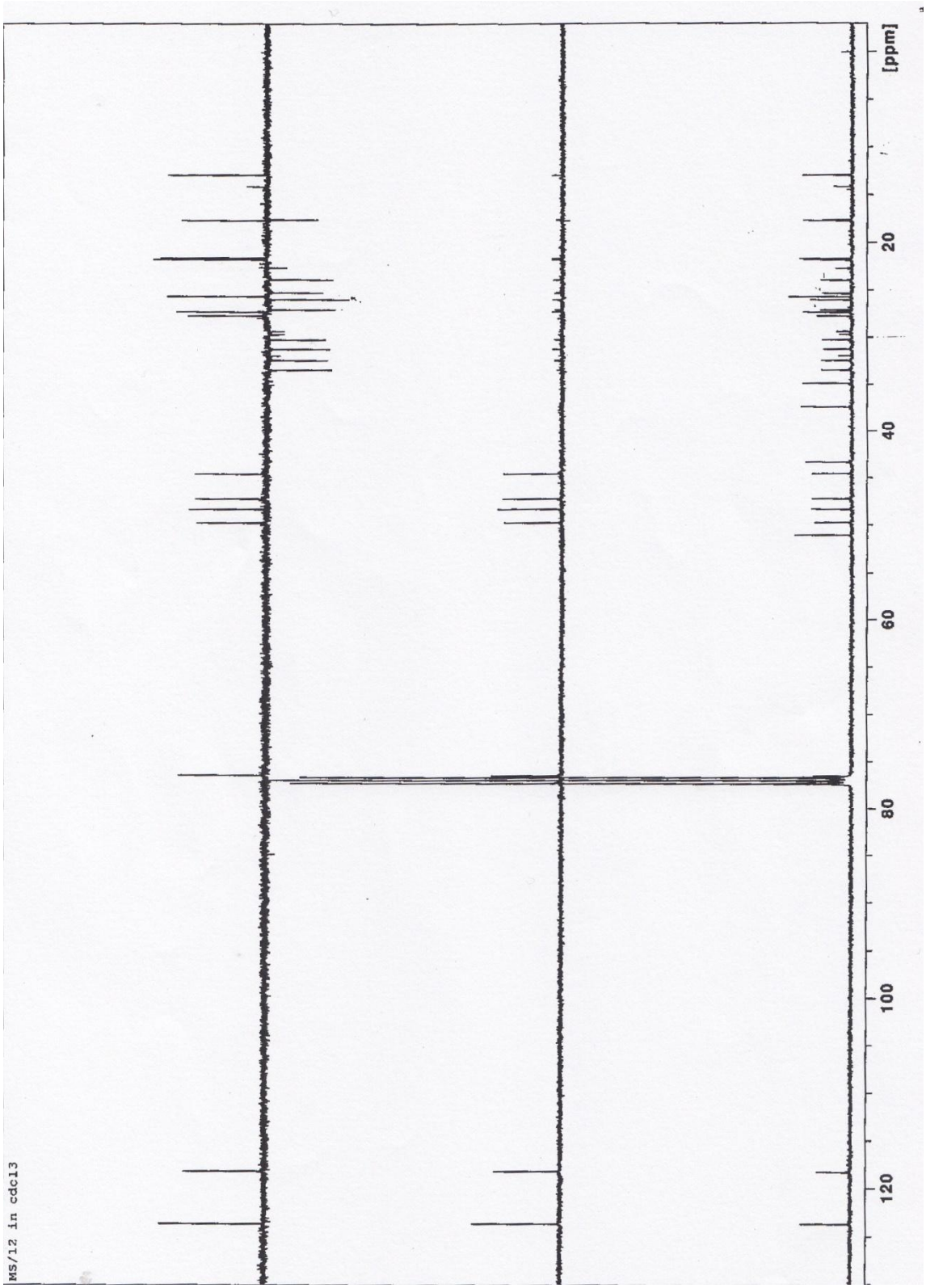

Figure E2.4: ^{13}C-NMR, DEPT 90 and DEPT 135 spectra of compound MS/12

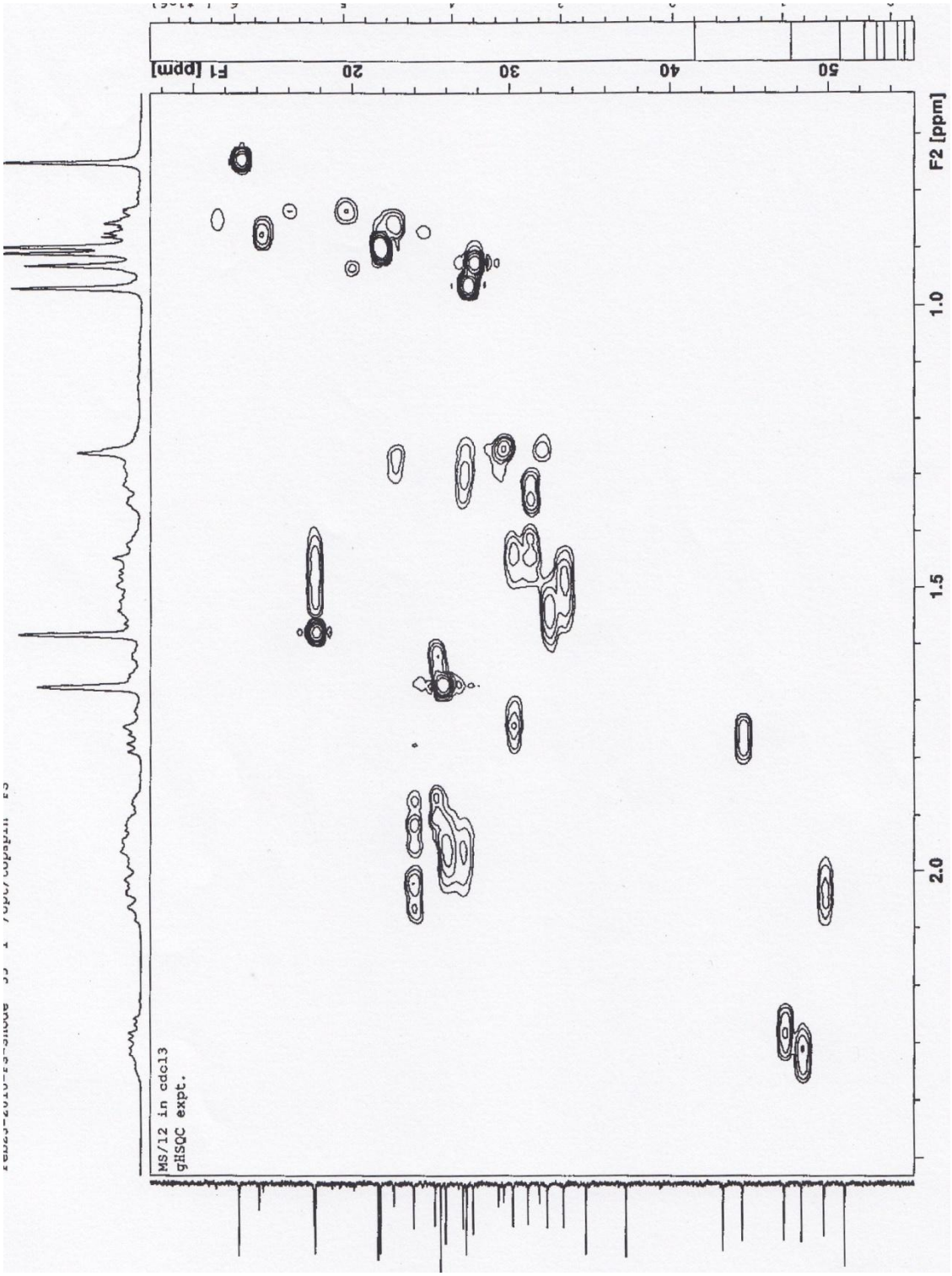

Figure E2.5: HSQC NMR spectrum of compound MS/12

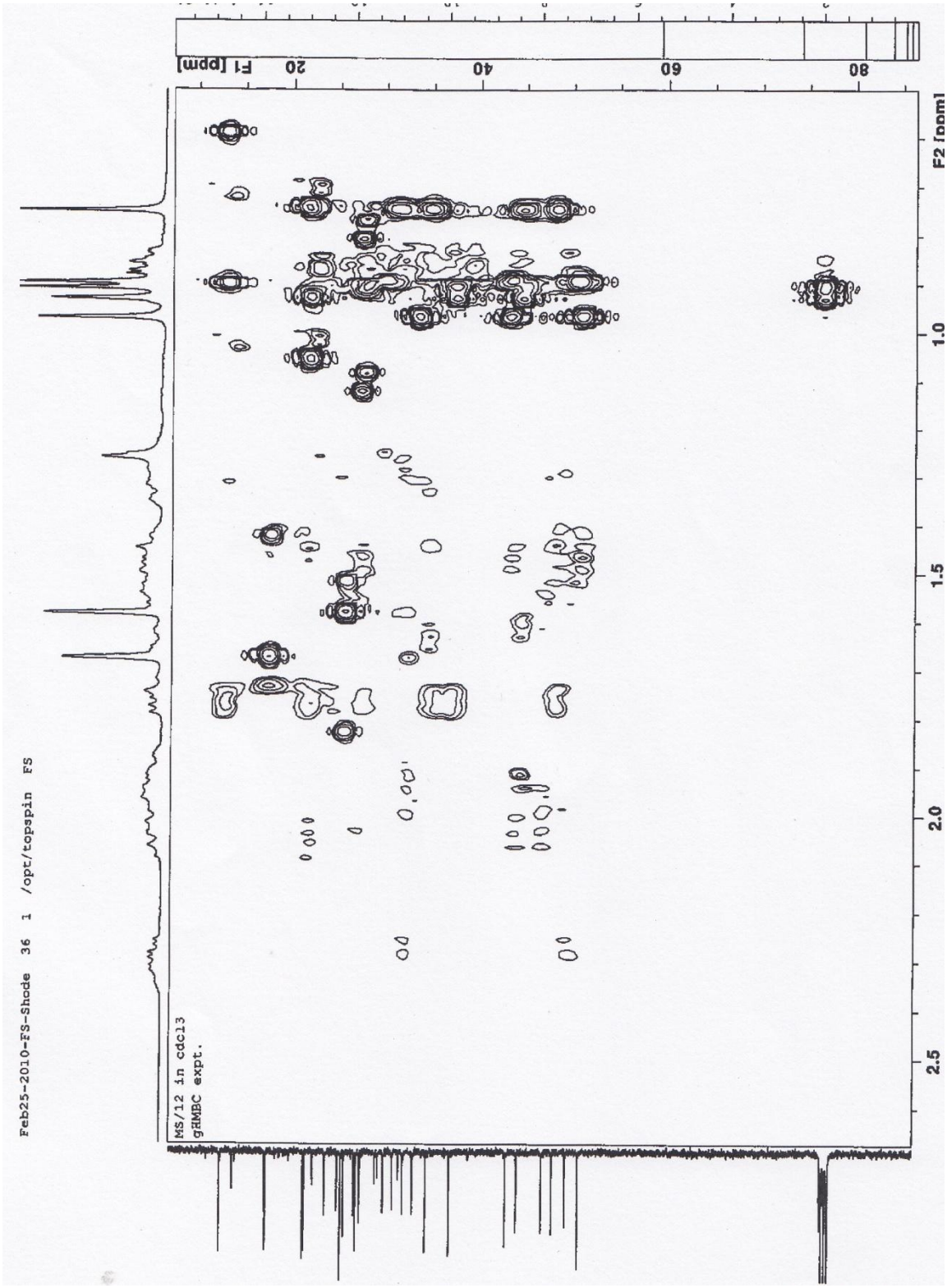

Figure E2.6: HMBC NMR spectrum of compound MS/12

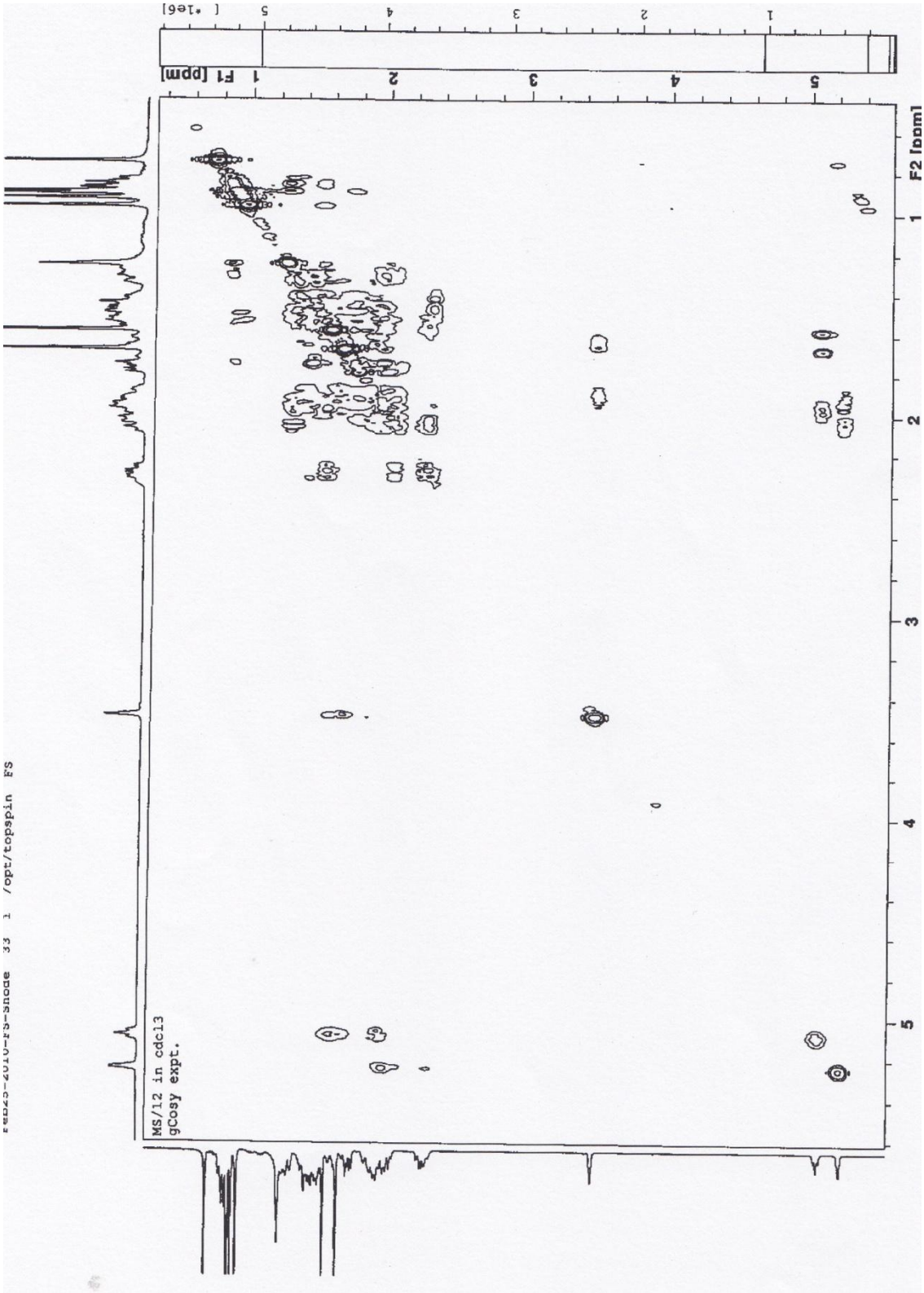

Figure E2.7: COSY NMR spectrum of compound MS/12

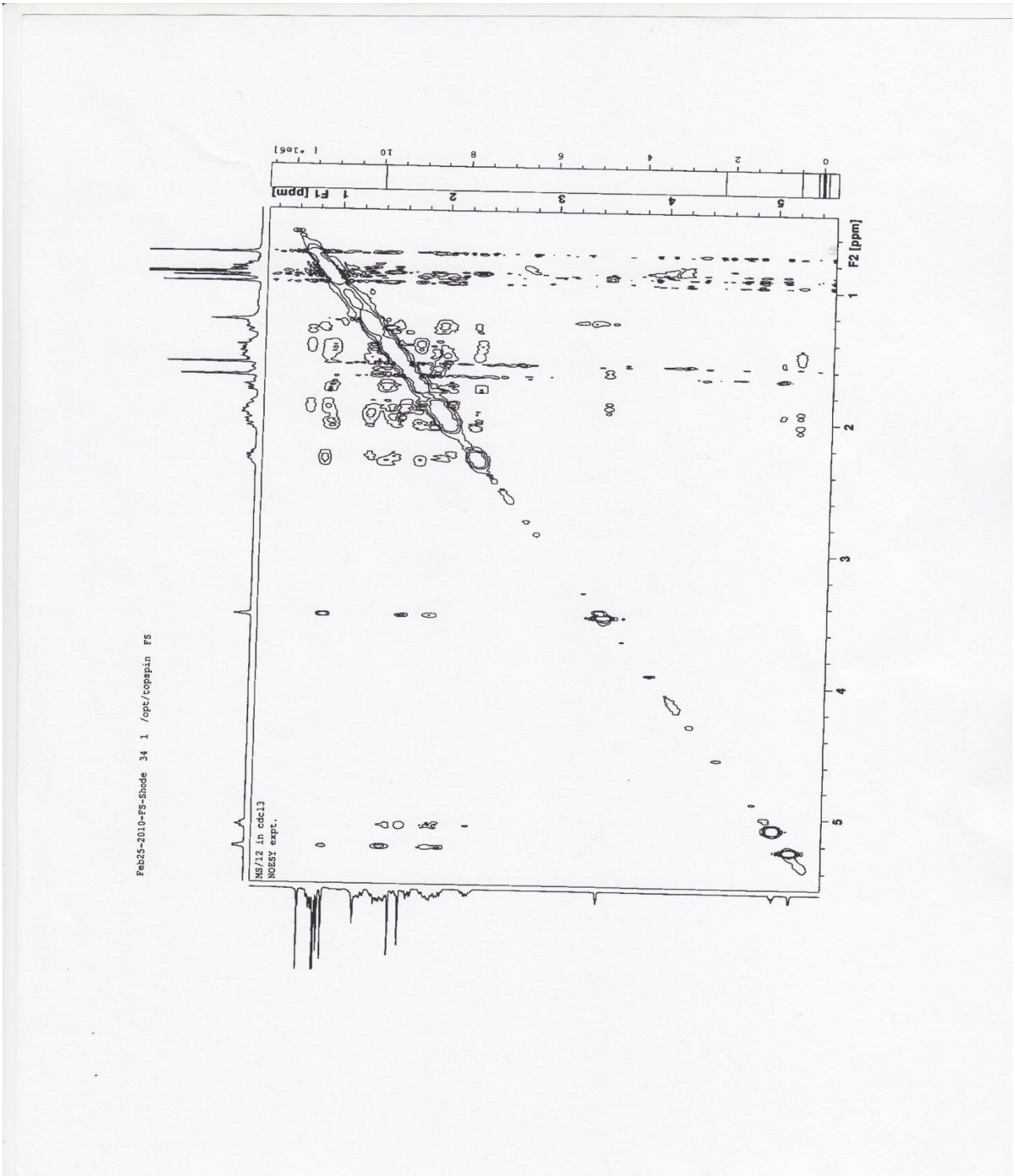

Figure E2.8: NOESY NMR spectrum of compound MS/12

APPENDIX F

CONTRIBUTION TO KNOWLEDGE

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

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[1]Department of Biochemistry and Microbiology and [2]Department of Chemistry, University of Zululand, Private Bag X1001, ZwaDlangezwa, 3886, Republic of South Africa
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INTRODUCTION

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

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

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

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

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

Bulbine natalensis

Protorhus Longifolia

Rapanae melanophloes

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

MATERIALS AND METHODS

➢The plants were collected from Twinstream Nursery, Mtunzini. Plants were identified at the Department of Botany, University of Zululand and voucher specimens were prepared.
➢Air-dried and powdered plant parts were extracted sequentially with hexane, chloroform, ethyl acetate, methanol and water.
➢Phytochemical screening was carried out on the extracts using standard procedures to identify the bioactive constituents[2,3,11]
➢The anti-oxidant activity was determined by in vitro methods-DPPH, ABTS, REDUCING POWER and METAL CHELATING assay [3,4,12,14]
➢The methanolic extracts were tested for brine shrimp cytoxicity test activity . The shrimp larvae were subjected to the extracts in a multi welled culture plate. Number of survivors were counted and LC_{50} was determined. All experiments were carried out in triplicate[6,8]
➢ The anti platelet aggregation activity of the extracts was separately investigated on thrombin, ADP and epinephrine induced rat platelet aggregation; similar experiments were also carried out on enzyme (trypsin, bromelain, papain) treated platelets [8,9,10].

RESULTS AND DISCUSSION

TABLE 1

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

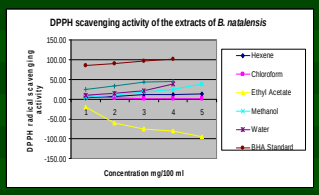
+ PRESENT ; - ABSENT

TABLE 2: LETHAL CONCENTRATION OF EXTRACTS LC_{50}

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

ANTI OXIDATIVE ACTIVITY

Figure 1: DPPH activity for *B natalensis*



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

TABLE 3:

A: *Bulbine natalensis*

B Protorhus Longifolia

C:Rapanae melanophloes

IC_{50} (values I mg/100ml)	DPPH			REDUCING POWER			METAL CHELATING			ABTS		
	A	B	C	A	B	C	A	B	C	A	B	C
HEXANE	>5	ND	>5	>3	ND	ND	>5	ND	4.21	ND	0.27	>5
ETHYL ACETATE	-	ND	4.83	>3	ND	ND	>5	ND	<1	ND	ND	4.99
CHLOROFORM	>5	ND	4.36	-	ND	ND	-	ND	3.57	ND	ND	2.4
METHANOL	>5	0.07	3.35	>3	ND	ND	>5	ND	>5	ND	ND	1.53
WATER	>5	ND	>5	>3	ND	ND	>5	ND	>5	ND	ND	>5
BHA	<1	ND	ND	<1	ND	ND	ND	ND	ND	ND	ND	ND
BHT	ND	ND	4.54	ND	ND	ND	ND	ND	ND	ND	ND	3.72
ASCORBIC ACID	>5	ND	4.06	>5	ND	ND	ND	ND	ND	ND	ND	3.84
EDTA	ND	ND	ND	ND	ND	ND	<1	ND	3.42	ND	ND	ND
CITRIC ACID	ND	ND	ND	ND	ND	ND	3.81	ND	3.92	ND	ND	ND

ANTI PLATELET AGGREGATION STUDY: [3mg/ml]

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

TABLE 4: Bromelain treated platelets

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

TABLE 5: Papain treated platelets

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

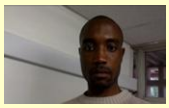
➢Methanol and water extracts of *B natalensis,* Hexane, ethyl acetate and chloroform extracts of *P longifolia,* and Chloroform and ethyl acetate extracts of *R melanophloes provoked* a concentration dependent in vitro inhibition of rat platelet aggregation activity.

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

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

References will be provided on request

TRITERPENES FROM *PROTORHUS LONGIFOLIA* EXHIBIT ANTI-PLATELET AGGREGATION ACTIVITY



[1]Mosa RA, [2]**Oyedeji OA,** [3]**Shode FO**, [1]**Opoku AR**

[1]Department of Biochemistry & Microbiology, University of Zululand, P. Bag X1001, KwaDlangezwa, 3886, South Africa
[2]Department of Chemistry, Walter Sisulu University, P/B X1, Mthata 5117, South Africa
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E-mail: rebamang@gmail.co.za

INTRODUCTION

Platelet hyperaggregation is the underlying cause of atherothrombotic diseases[1]. The clinical limitations and adverse side effects associated with the currently used antiplatelet aggregation agents has fuelled the search for more effective agents of natural origin[2,3]. Medicinal plant-based traditional healing does not only provide immediate health care to rural population but also a lead to discovery of new pharmacologically active drugs.

Stem-bark of *Protorhus longifolia* (Benrh.) Engl. (Anacardiaceae) is commonly used by Zulu traditional healers to treat blood clot related diseases. The aim of this work was to investigate the antiplatelet aggregation activity of the stem-bark extracts of *Protorhus longifolia* and to partially identify active components present and responsible for the antiplatelet aggregation activity.

MATERIALS AND METHODS

❖The plant material was collected from Empangeni, KwaZulu-Natal. Dried, powdered plant material was sequentially extracted with hexane (HE), chloroform (CE), ethyl acetate (EAE), methanol (ME) and water (WE).

❖ The crude chloroform extract was subjected to isolation and purification using chromatographic techniques (Fig. 1). Structures of the isolated compounds were analyzed and confirmed through IR, NMR, MS spectral data and literature[4,5,6].

❖*In vitro* antiplatelet aggregation activity was investigated on thrombin, ADP and epinephrine induced rat platelet aggregation[7].

❖*In vitro* anticoagulant activity was determined on rat whole blood.

❖Acute anti-inflammatory activity was determined using carrageenan-induced rat paw oedema model[8].

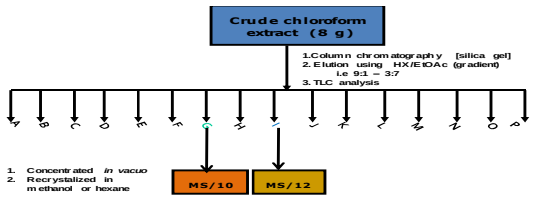

Fig. 1: Isolation and purification of MS/10 and MS/12 from the crude chloroform extract

Table 1: IC_{50} values (mg/ml) of the crude extracts of *P longifolia* on rat platelet aggregation

Agon\Extrct	HE	CE	EAE	ME	WE	Heparin
Thrombin	0.59	>10	2.13	Nd	Nd	7.40
ADP	1.56	4.48	2.62	Nd	Nd	
Epinephrine	>10	2.39	2.09	2.21	>10	

Nd- not detected

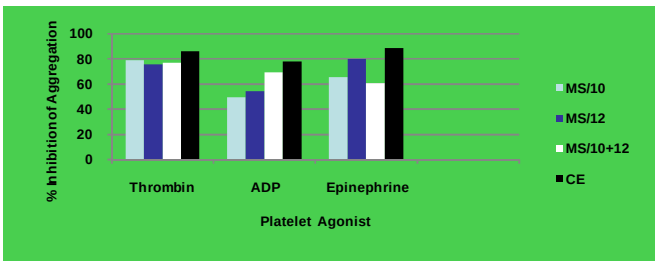

Fig. 3: Antiplatelet aggregation activity of MS/10 and MS/12 on the platelet aggregation induced by thrombin, ADP and epinephrine. Crude chloroform extract (CE) and the mixture (MS/10+12) were used for comparison. IC_{50} values (mg/ml) on the thrombin-induced platelet aggregation: MS/10 (0.99) , MS/12 (1.04), CE (0.67) and MS/10+12(0.88)

RESULTS

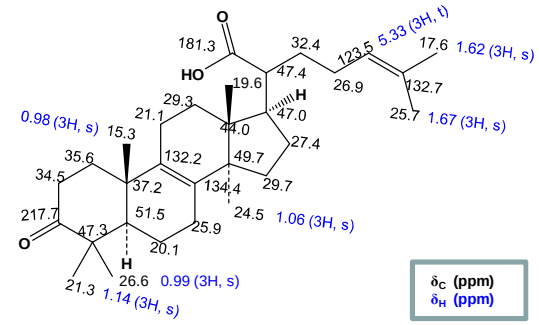

Fig. 2a: 3-Oxo-5α-lanosta-8,24-dien-21-oic acid (**MS/10**)

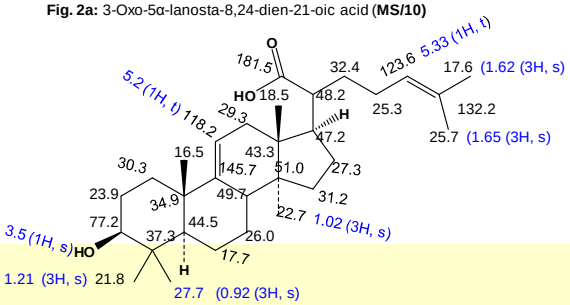

Fig. 2b: 3β-Hydroxylanosta-9,24-dien-24-oic acid (**MS/12**)

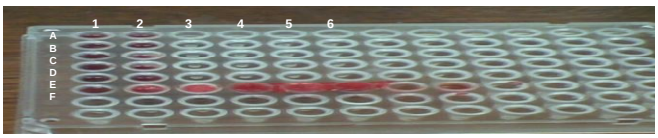

Fig. 4: The anticoagulant activity of MS/12 on the rat whole blood. The blood was added to A (1% DMSO-blank); B (thrombin); C (ADP); D (epinephrine); E (arachidonic acid); and F_{1-6} (compound at 1, 3 and 10 mg/ml). In the controls the blood clotted within a minute while in the presence of the compound it took 5±1 minutes to clot.

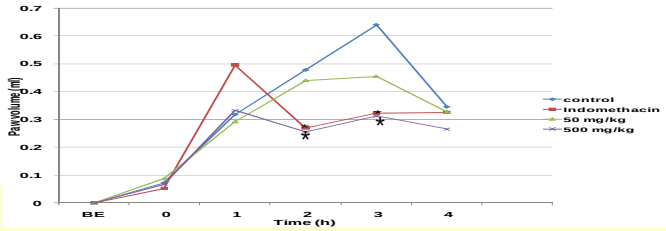

Fig. 5: Anti-inflammatory activity of MS/12 on carrageenan-induced paw oedema in rats. Paw oedema volume was measured immediately and at 1h interval for 4h following carrageenan injection. * P<0.05 compared to the control group.

DISCUSSION AND CONCLUSION

The organic extracts and the two triterpenes (Fig. 2) isolated from the chloroform extract prevent platelet aggregation in a concentration dependent manner. This is the first time the compounds are isolated from *P longifolia*. The activity of MS/12 was supported by its ability to delay clotting of whole blood. MS/12 further inhibited inflammation in a way similar to that of NSAIDs indicative of cyclooxygenase inhibitor. The triterpenes could be potential pharmacologically active antiplatelet aggregation agents. The results support the use of *P longifolia* in folk medicine to manage blood clotting related diseases. Further work is required to elucidate the possible mechanism in which these compounds exert their therapeutic activities. Also other biological activities need to be investigated.

References: will be provided on request.

Acknowledgements: Medical Research Council (MRC) for funding this project
University of Zululand Research Office for support