INHIBITORY ACTIVITY OF TRITERPENOIDS, EUGENOL AND EUGENOL ACETATE IN THROMBIN, ADENOSINE DIPHOSPHATE AND EPINEPHRINE-INDUCED PLATELET AGGREGATION

BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, IN PARTIAL FULFILMENT FOR THE AWARD OF MASTER OF SCIENCE (M.Sc) IN BIOCHEMISTRY,

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DECLARATION

I declare that the thesis entitled "INHIBITORY ACTIVITY OF TRITERPENOIDS, EUGENOL AND EUGENOL ACETATE IN THROMBIN, ADENOSINE DIPHOSPHATE AND EPINEPHRINE-INDUCED PLATELET AGGREGATION", has been performed by me in the Department of Biochemistry under the Supervision of DR. I. A. Umar and Prof. S. E. Atawodi. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

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CERTIFICATION

This project thesis entitled "INHIBITORY ACTIVITY OF TRITERPENOIDS, EUGENOL AND EUGENOL ACETATE IN THROMBIN, ADENOSINE DIPHOSPHATE AND EPINEPHRINE-INDUCED PLATELET AGGREGATION" by Habila, Amaya Jobin meets the regulations governing the award of Master of Science (MSc) degree of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Platelet hyper-aggregation is the underlying cause of cardiovascular diseases. The clinical limitation and adverse side effect associated with currently used anti-platelet aggregation agents have fuelled the search for more effective agents of natural origin. Oleanolic acid, maslinic acid and eugenol from *Syzigium aromaticum* and betulinic acid from *Melaleuca bracteata* were isolated. The isolated compounds were modified to their acetates to mimic the functionality of aspirin. The isolated and modified compounds were characterized using nuclear magnetic resonance (NMR) spectroscopic techniques to ascertain the structure of the compounds. The isolated and modified compounds were evaluated for platelet aggregation inhibition induced by Thrombin, Adenosine diphosphate (ADP) or epinephrine. The result of the platelet aggregation inhibition using thrombin, ADP and epinephrine as agonist on the test compounds showed that the highest inhibition exhibited by betulinic acetate on the thrombin-induced platelet aggregation (54.5±0.01 at 1mg/ml, 63.5±0.17 at 3mg/ml and 73.5±0.15 at 10mg/ml, IC_{50} 0.81mg/ml) which was similar ($P<0.05$) to the values of Aspirin (65.4±0.07 at 1mg/ml, 72.1±0.03 at 3mg/ml and 76.5±1.22 at 10mg/ml, IC_{50} 0.33mg/ml) and Heparin (65.9±0.59 at 1mg/ml, 66.9±0.03 at 3mg/ml and 70.6±0.16 at 10mg/ml, IC_{50} 0.26) used as standards. Antioxidant activity was investigated using 1,1′-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{+}$)) assay to suggest a mechanism of action of the test compounds. The anti-oxidant activities of the compounds showed that eugenol possessed significant ($P<0.05$) free radical scavenging activity with highest activity in the $ABTS^{+}$ assay (57.7±0.002, at 0.0625mg/100ml, 75.3±0.003, at 0.125mg/100ml, 89±0.002, at 0.25mg/100ml, 89.7±0.001, at 0.5mg/100ml, 90.7±0.001, at 1mg/100ml and an IC_{50} 0.05mg/100ml) which is more than those of the standards Ascobic acid (9.7±0.001, at 0.0625mg/100ml, 19.5±0.003, at 0.125mg/100ml, 26.0±0.001, at 0.25mg/100ml, 47.7±0.001, at 0.5mg/100ml, 60.9±0.002, at 1mg/100ml and an IC_{50} 0.66mg/100ml) and Butylated hydroxyanisole (9.7±0.005, at 0.0625mg/100ml, 15.4±0.001, at 0.125mg/100ml, 21.9±0.002, at 0.25mg/100ml, 52.0±0.001, at 0.5mg/100ml, 65.8±0.001, at 1mg/100ml and an IC_{50} 0.48mg/100ml)) used. The study suggests that the isolated and modified compounds be used as anti-platelet aggregation agents in the management of blood-clotting related diseases.

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LIST OF ABBREVIATIONS

AA: Ascorbic acid

ABTS: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

ADA: Acid-dextrose-anticoagulant

ADP: Adenosine diphosphate

A2AR: Alpha-2-adrenergic recptor

BA: Betulinic acid

BAA: Betulinic acetate

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

cAMP: Adenosine-3′,5′-cyclic monophosphate

CMC: Carboxymethyl cellulose

COSY: Correlation spectroscopy

COX: Cyclooxygenase

DMSO: Dimethyl sulfoxide

DEPT: Distortionless enhancement by polarisation transfer

DNA: Deoxyribonucleic acid

DMAPP: Dimethylallyl pyrophosphate

DPPH: 1,1′-diphenyl-2-picrylhydrazyl

EDTA: Ethylenediaminetetra-acetic acid

EU: Eugenol

EUA: Eugenol acetate

GPP: Geranyl pyrophosphate

HMBC: Heteronuclear multiple bond correlation

HSQC: Heteronuclear multiple quantum coherence

IR: Infra-red

IC50: Inhibitory concentration with 50%

IPP: Isopentenyl pyrophosphate

MS: Mass spectroscopy

MAA: Maslinic acetate

NMR: Nuclear magnetic resonance

NOESY: Nuclear overhauser enhancement spectroscopy

OA: Oleanolic acid

OAA: Oleanolic acetate

PAR: Protease- activated receptor

PAF: Platelet activating factor

PBS: Phosphate buffer saline

PCTs: Pentacyclic triterpenes

PGs: Prostaglandins

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

TBHQ: Terbutylhydroquinone

TCA: Trichloroacetic acid

TLC: Thin-layer chromatography

UV: Ultraviolet light

CHAPTER ONE

1.0 INTRODUCTION

Platelets are disk-shaped, non-nucleated blood cells that help to make blood clot. They are also called thrombocytes with a very fragile membrane. They play a major role in blood clotting process and tend to adhere to uneven or damaged surfaces. They average about 250,000 per cubic millimeter of blood and are formed in the red bone marrow by fragmentation of megakaryocytes, the largest of the bone marrow cells. Platelet production is controlled by a hormone thrombopoietin, and regulatory lymphocytes acting at the stem cell level. At any given time about one-third of the total blood platelets can be found in the spleen; the remaining two-thirds are in the circulating blood (Holt and Chandra, 2002). It is well known that platelets play an important role in the physiology of primary hemostasis and pathophysiological processes such as thrombosis (Shattil *et al*., 1998; Stouffer and Smyth, 2003). Blood clots can become a problem especially if they are in the heart, brain or other arteries in the body (Ruggeri, 2002). Blood clotting forms an important part of haemostasis; 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 (Murray *et al*., 2003)

Platelet hyperactivity and consequent hyper aggregation is 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 (Valko *et al*., 2005), heart attack, and

pulmonary embolism (Valko *et al*., 2007). Aberrant intravascular thrombosis is the main cause of a wide variety of cardiovascular diseases (Grenache *et al*., 2003; Huo and Ley, 2004). Thrombin, Adenosine diphosphate, epinephrine, arachidonic acid, collagen and other risk factors, such as free radicals, inflammation, stress and hypercholesterolemia, significantly contribute to platelet dysfunctions (Ambrosio *et al*., 1997; Bakdash and Williams, 2008; Verhamme and Hoylaert, 2009; Davi and Patrono, 2007; Grenache *et al*., 2003; Huo and Ley, 2004). Anti-platelet drugs stop blood from clotting and this helps to prevent heart attack and stroke (Willoughby *et al*., 2002). The clinical limitations and adverse side effects associated with the currently used anti-platelet aggregation agents such as Aspirin, dypridamol clopidogrel, ticlopidine, prasugrel have fuelled the search for more effective agents of natural origin (Coller *et al*., 1995)

Natural products are generally either of prebiotic origin or originate from microbes, plants, or animal sources (Jarvis, 2000; Holt and Chandra, 2002). As chemicals, natural products include such classes of compounds as terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA), deoxyribonucleic acid (DNA) among others. Natural products are not just accidents or products of convenience of nature. More than likely they are a natural expression of the increase in complexity of organisms (Cragg and Newman, 2001).

The interest in natural sources to provide treatments for pain, palliatives, or curatives for a variety of maladies or recreational use dates back to the earliest points of history. Nature has provided many resources for humankind over the years; including the tools

for the first attempts at therapeutic intervention. Thus, the search for naturally occurring anti-platelet aggregation inhibitors from plants creates opportunities to discover new ethno pharmaceuticals for developing potent anti-platelet aggregation drugs.

World Health Organization has estimated that 80% of people in developing countries meet their primary health care need through use of plants (Gumede, 1989). *Syzigium aromaticum* also called cloves posses antimicrobial, antihelmintic activity (Balch and Balch, 2000). It is used in aromatherapy when stimulation and warming are needed especially for digestive problems (Alqareer *et al*., 2006). *Melaleuca bracteata* is reported to have remedy against skin infection (Goldstein *et al*., 1990; Belousova and Denisova, 1992; Howes, 1974; Oliver, 1960; Irvine, 1961).

Triterpenoids are metabolites of isopentenyl pyrophosphate (IPP) and are found in plants. They protect the plants from both constitutive and induced defensive responses against insects and environmental stress (Keeling and Bohlmann, 2006; Franceschi *et al.,* 2005). Triterpenoids are found to have the potential ability to limit the development of certain cancers and inflammatory diseases (Sporn and Suh, 2000).

1.1 STATEMENT OF RESEARCH PROBLEM

i. Aberrant intravascular thrombosis is the main cause of a wide variety of cardiovascular diseases.

ii. Some of the currently used drugs take several days for their maximum effect to be realised.

iii. According to WHO, the most immediate improvements in cardiovascular diseases is achieved with the commonly used drug, Aspirin which has some adverse side effects such as gastrointestinal ulcers and stomach bleeding. Also, some patients still suffer thrombosis despite aspirin therapy.

1.2 JUSTIFICATION

Platelet hyper-aggregation is the underlying cause of atherothrombotic diseases, heart attack or stroke and high blood pressure (Valko *et al*., 2007; Valko *et al*., 2005). The clinical limitations and adverse side effects associated with the currently used anti-platelet aggregation drugs have fuelled the search for more effective agents of natural origin. Hence, the proposed study.

1.3 AIMS

The aim of this project is to evaluate the inhibitory activity of naturally occurring compounds as potential targets for the development of new anti-platelet aggregation drugs.

1.4 RESEARCH OBJECTIVES

i. To isolate Oleanolic acid and eugenol from the leaves and fruits of *Syzigium aromaticum*.

ii. To isolate Betulinic acid from the leaves of *Melaleuca bracteata*.

iii. To modify Oleanolic acid, eugenol and Betulinic acid to their 3β-acetoxy-oleanolic acid, eugenol acetate and 3β-acetoxy-betulinic acid respectively.

iv. To characterize the isolated and modified compounds.

v. To determine the anti-platelet aggregation activity of the isolated and modified compounds.

vi. To determine the antioxidant activity of the isolated and modified compounds.

1.5 HYPOTHESES

The isolated (oleanolic acid, betulinic acid, maslinic acid and eugenol) and modified (oleanolic acetate, betulinic acetate, maslinic acetate and eugenol acetate) compounds do not posses anti-platelet aggregation activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BLOOD CLOTTING

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. 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 usually 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 (Ruggeri, 2002). 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.

2.1.1 Platelet Activation and Aggregation

Resting platelets circulate as discoid shape in the blood which is changed upon activation. When there is damage or injury to blood vessel, platelets rush to the site of **i**njury as they adhere to exposed collagen surface which is mediated by von Willebrand factor (vWF) **(**a complex multimeric glycoprotein that is produced by and stored in the α-granules of platelets**)**. The stoppage of bleeding is facilitated by platelets 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 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 2.1). 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 crosslinking

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.

Activation of platelets is required for their consequent aggregation to a platelet plug. However, equally significant is the role of activated platelet surface phospholipids in the activation of the coagulation cascade.

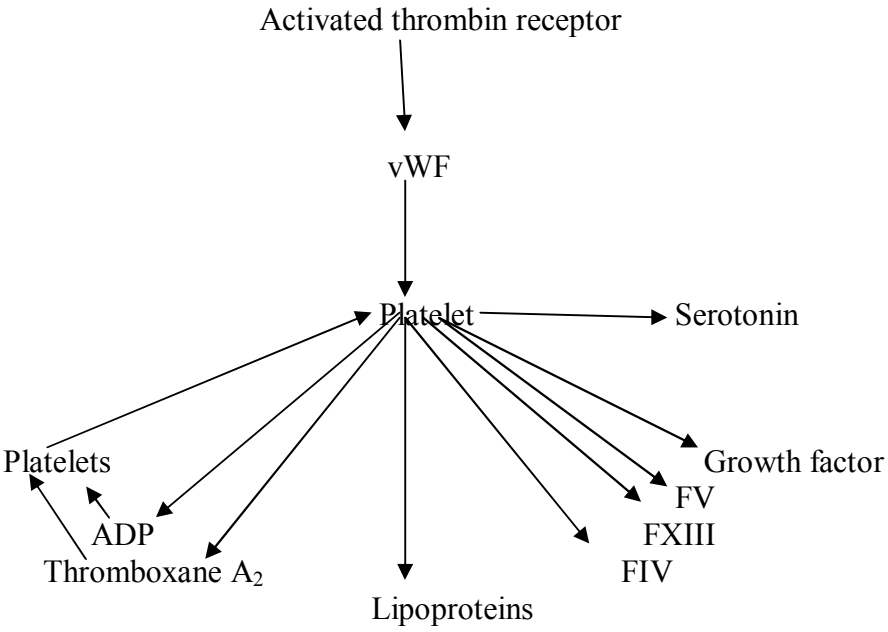


Figure 2.1: Events that occur upon platelet activation followed by platelet aggregation. (Source: Devlin, 2006)

2.1.2 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 2.2). 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. Calcium ion (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.

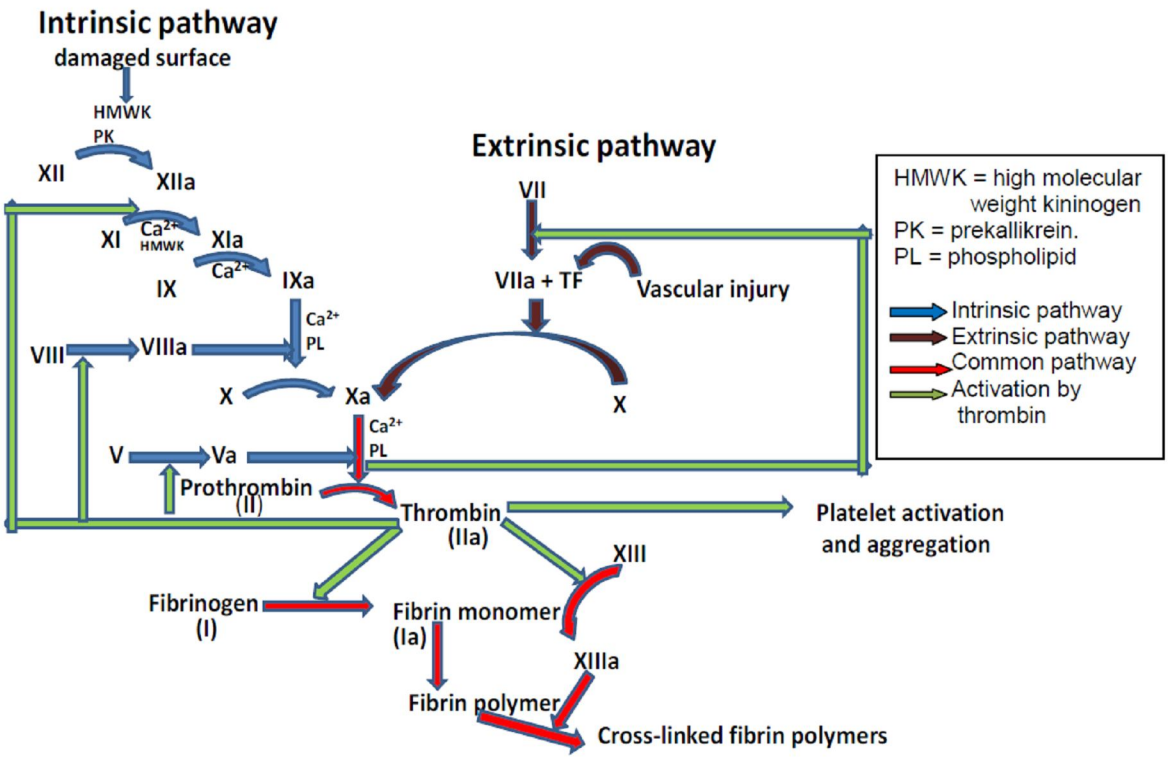


Figure 2.2: Coagulation cascade showing activation of the intrinsic and extrinsic pathways which converge at the final common pathway involving activation of factor X to Xa and generation of thrombin. Activated factors are designated with small letter a. (Source: Murray *et al*., 2003).

Table 2.1: Numerical nomenclature of blood clotting factors.

Factors	Name
I	Fibrinogen
II	Prothrombin
III	Tissue factor
IV	Ca^{2+}
V	Labile factor
VII	Proconvertin
VIII	Antihemophilic factor A
IX	Antihemophilic factor B (Christmas factor)
X	Stuart-Prower factor
XI	Plasma thromboplastin antecedent
XII	Hageman factor
XIII	Fibrin stabilizing factor

(Source: Murray *et al*., 2003)

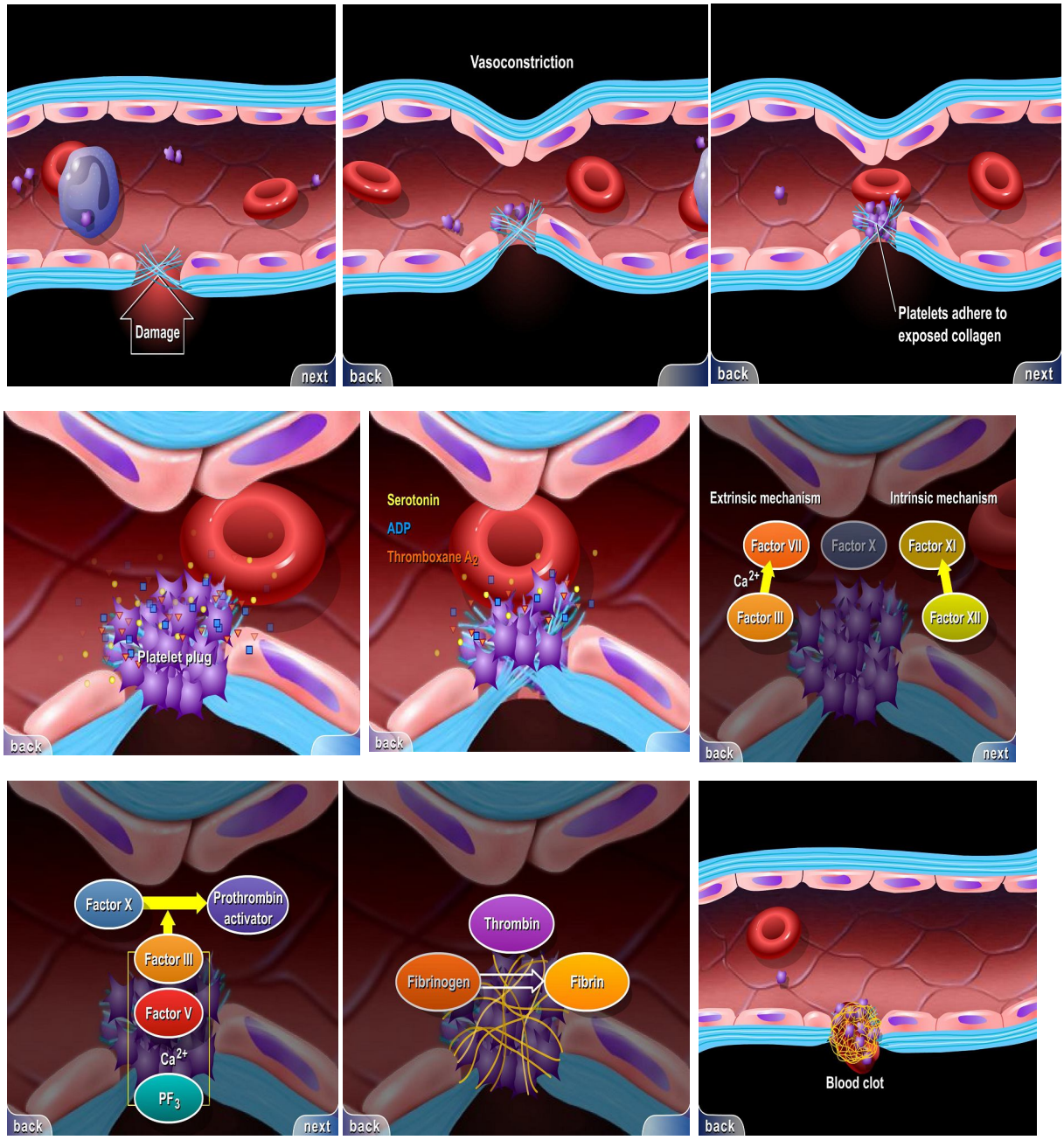


Figure 2.3: Pictoral representation of processes involved in blood clotting (Source: Anonymous, 2002).

Damage to blood vessel leads to immediate reflex that promotes vasoconstriction, exposed collagen from the damaged site will promote the platelets to adhere to the damaged vessel and undergo degranulation releasing its cytoplasmic granules, which contain serotonin, a vasoconstrictor, ADP and Thromboxane A_2. The ADP attracts more platelets to the area, and the thromboxane A_2 promotes platelet aggregation, degranulation, and vasoconstriction. Thus ADP and thromboxane A_2 promote more platelet adhesion and more ADP and thromboxane. The positive feedback promotes the formation of a platelet plug.

The final haemostatic mechanism is coagulation where damaged tissue releases factor III, which with the aid of Ca++ will activate factor VII, thus initiating the extrinsic mechanism. Factor XII from active platelets will activate factor XI, thus initiating the intrinsic mechanism. Both active factor VII and active factor XI will promote cascade reactions, eventually activating factor X. Active factor X, along with factor III, factor V, Ca++, and platelet thromboplastic factor (PF_3), will activate prothrombin activator. Prothrombin activator converts prothrombin to thrombin. Thrombin converts fibrinogen to fibrin. Fibrin initially forms a loose mesh, but then factor XIII causes the formation of covalent Cross links, which convert fibrin to a dense aggregation of fibres. Platelets and red blood Cells become caught in this mesh of fibre, thus the formation of a blood clot.

2.2 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. Examples of platelet agonists include thrombin, adenosine-5′-diphosphate (ADP) and epinephrine. The available anti-platelet agents are designed in such a way that they act by targeting specific pathways.

2.2.1 Thrombin

Thrombin is important in both haemostasis and thrombosis. Its effects are mediated through membrane-bound G-protein coupled receptors. 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. The second one does not hydrolyse PAR-1, but 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).

2.2.2 Adenosine Diphosphate (ADP)

The ADP-induced platelet activation is autocatalytic in that upon activation by ADP, platelets release other ADP molecules that act on nearby platelets, amplifying the

reaction. ADP acts through G-protein coupled receptors P2Y1 and P2Y12 (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 P2Y1, amplified and sustained through P2Y12 (Storey, 2006). In addition to platelet activation and consequent thrombus formation, P2Y12 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. Fabre and Gurney (2010) have shown that ADP acts through the P2Y1 to mobilise intracellular Ca^{2+} and through P2Y12 and the Gq-linked activation of phospholipase C to inhibit adenylyl cyclase formation of cAMP and is also supported by Jianguo *et al.,* (1998). Platelet morphological change, granular release, increased intracellular Ca^{2+} and decreased cAMP are the characteristics of ADP-induced platelet aggregation (Puri and Colman, 1997; Jianguo *et al*., 1998)

2.2.3 Epinephrine

Epinephrine is a weak platelet agonist reported to exert its effects on human platelets through α_2-adrenergic receptors (A2AR) and also potentiates aggregation induced by other platelet agonists (Lanza *et al*., 1988; Choi, 2002). They further suggested that epinephrine has the ability 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 high blood pressure, hypercholesterolemia, diabetes, smoking, stress, sedentary lifestyle and age (Verhamme and Hoylaert, 2009; Davi and Patrono, 2007; Grenache *et al*., 2003; Huo and Ley, 2004). Inflammation and exposure to high amounts of free radicals also have a significant contribution (Chang *et al.,* 2010 ;).

2.2.4 Free Radicals

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 are implicated in various pathophysiological conditions such as neurodegenerative diseases (Beal, 1995), autoimmune diseases (Pham-Huy *et al*., 2008), arthritis (Valko *et al*., 2007), inflammation (Halliwell and Gutteridge, 2007; Bahorun *et al*., 2006; Valko *et al*., 2004; Valko *et al*., 2007; Droge, 2002; Willcox *et al*., 2004) and cardiovascular diseases (Bahorun *et al*., 2006; Droge, 2002; Halliwell, 1996; 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 *et al*., (1994) demonstrated that a reaction between xanthine and hydrogen peroxide enhanced platelet aggregation. Free radicals can be classified as reactive oxygen species (ROS) (e.g. O_2^-, $OH^\bullet$) and reactive nitrogen species (RNS) (e.g. $NO^\bullet$, $\bullet OONO^-$). Superoxide (O_2^-) can give rise to a non-radical but potent oxidant, hydrogen peroxide (H_2O_2), and the most powerful hydroxyl radical ($OH^\bullet$) (Ambrosio *et al*., 1997; Hsu *et al*., 2007). Free radicals are by-products of normal 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 (Pacher *et al*., 2007; Genestra, 2007; Young and Woodside, 2001; Valko *et al*., 2004) through oxidation. Free radicals attack stimulate cellular autoxidation that 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).

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 ANTI-PLATELET THERAPY, LIMITATIONS AND FUTURE PROSPECTS

Platelets aggregation, beyond the purpose of haemostasis is the mechanism underlying atherothrombotic diseases (Gadi *et al*., 2009). Circulatory events such as myocardiac infarctiions and srokes account for high rate of mortality and morbidity worldwide, particularly in developed countries (Hsieh *et al*., 2007; Morrell *et al*., 2008; Fabre and Gurney, 2010). Their prevalence is becoming important in developing countries as a

result of changing lifestyle, industralization and urbanization (Yusuf and Ounpuu, 2001). Morrell *et al.,* (2008) reported thrombosis as the underlying cause of cardiovascular diseases and stroke. The need to maintain balance between haemostasis and thrombosis has lead 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, heparin 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 seems to be the major limitation to their efficacy and safety. For example aspirin and Y2Y12 antagonists (clopidogrel, ticlopidine, prasugrel) only inhibit platelet activation induced by thromboxane A_2 and ADP respectively. Due to their irreversible action they are associated with the risk 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 associated with problems such as drug-drug interaction, anti-platelet resistance and affordability (Xiang *et al*., 2008). New anti-platelet agents with mild, reversible inhibition and multiple targets may be ideal. It can be of multiple beneficial effects such as anti-inflammatory and anti-platelet activity. These ideal drugs may come from plants bioactive compounds.

2.3.1 Mechanism of Action of Aspirin

Acetyl salicylic acid (ASA), the major active ingredient of Aspirin interferes with the formation of Thromboxane A_2. It irreversibly acetylates and thus inhibits the Platelet cyclooxygenase system involved in the formation of thromboxane A_2 (Figure 2.4) a potent aggregator of Platelets and also a vasoconstrictor. prostaglandin, intensifies platelet aggregation and constriction of blood vessels. Once platelets is in contact with ASA they become unable to produce Thromboxane A_2 for the rest of their lifetime of 7 to 10 days, while the endothelial cells regain their ability to produce prostacyclin after a few hours. Therefore, ASA helps to ensure the vessel's integrity and to prevent the formation of platelet-rich clots.

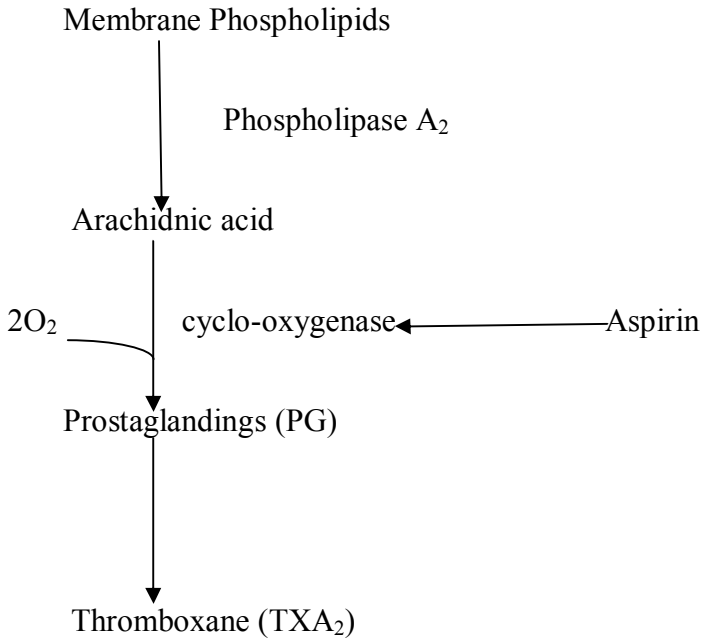


Figure 2.4: Conversion of Arachidonic acid from Membrane Phospholipids to Prostaglandins and Thromboxane showing the Target point of Aspirin.

2.4 *SYZIGIUM AROMATICUM*



Plate I: *Syzigium aromaticum* plant and its dried flowers

Syzigium aromaticum (Common name; Cloves) belongs to the Myrtaceae family. The aromatic dried flowers buds of this tree called cloves is native to Maluku islands in Indonesia and used as a spice in cuisines all over the world. Cloves are called Kanafaru in Yoruba, Osasagbogbo in Igbo and Kanumfari in Hausa. They are harvested mainly in Indonesia, India, Madagascar, Zanzibar, Paskitan and Sri Lanka. Cloves find wide application in traditional medicinal uses especially in Indian Ayurvedic medicine, Chinese medicine and Western herbalism. Aqueous cloves infusion was found to inhibit the growth of *Bacillus subtillis, Salmonella enterides* and *Eschericia coli.* The essential oil is used in aromatherapy when stimulation and warming are needed especially for digestive problems (Alqareer *et al*., 2006) Cloves are also a natural antihelmintic (Balch and Balch, 2000).

Some of the active chemical compounds of cloves include eugenol which comprises 72-90 % of the essential oil from cloves and is the compound that is mostly responsible for the cloves aroma. Other essential oil components include acetyl eugenol, beta-caryophyllene and vanillin. Crategolic acid; tannins, gallotannic acid, methyl salicylate (painkiller); the flavonoids eugenin, kaemferol, rhamnetin and eugenitin; triterpenoids like oleanolic acid, stigmasterol and campesterol and several sesquiterpenes (Bensky *et al*., 2004) The methanolic extract of *S. aromaticum* has been shown to have potent antioxidant potential and radical scavenging activity (Atawodi *et al*., 2011). Eugenol, a principle component from clove has been shown to prevent lipid peroxidation and also known as scavengers of active Oxygen radicals (Das, 2004). Compounds from *S. aromaticum* which have inhibitory activity against oral pathogen have been identified as 5,7-dihydroxy-2-methylchromone-8-C-β-D-glucopyranoside, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid and oleanolic acid (Nassar, 2006). The polyphenolic constituents of *S. aromaticum* were detected as ellgic acid, ellagic acid derivative, gallic acid and quercetin glucoside (Atawodi *et al.,* 2011).

2.5 *MELALEUCA BRACTEATA*



Plate II: A picture of *Melalueca bracteata* plant

Melaleuca species are another member of the Myrtaceae family. The genus *Melaleuca* is entirely Australian, and has more than 100 species of trees and shrubs with leathery leaves and spicate inflorescences of red, white and yellow flowers and itt is referred to as Johannesburg gold in South Africa. The trees and shrubs are mostly cultivated for the beauty of the flowers due to their conspicuous stamens, which are united in bundles. The leaves and their volatile oils are used in enthnomedicine of West Africa as a remedy for numerous diseases such as skin infection (Goldstein *et al*., 1990; Belousova and Denisova, 1992; Howes, 1974; Oliver, 1960; Irvine, 1961). *Melalueca bracteata* are evergreen shrubs that grow up to 6m in height with tall tree having white flowers in spring. The essential oil obtained from the fresh leaves and twigs are antiseptic in nature and do have germicide and insecticidal potentials (Cribb and Cribb, 1981; Yatagai, 1997).

2.6 TRITERPENES

Natural compounds have been used extensively in the treatment of many diseases and are of interest to researchers both in their natural forms and as templates for synthetic modification. Natural compounds currently used in medicine exhibit a very wide chemical diversity, and together with their analogues and several other natural products, they demonstrate the importance of compounds from natural sources in modern drug discovery efforts. Evidence from tissue culture, animal, and clinical studies suggests that more than 20,000 triterpenoid-rich fruits are found in nature and have the potential ability to limit the development and severity of certain cancers and inflammatory diseases (Sporn and Suh, 2000). These triterpenoids, along with their close chemical relatives the steroids, are members of a larger family of related structures called cyclosqualenoids. Triterpenoids, synthesized in many plants by the cyclization of squalene (Phillips *et al*.**,** 2006), are widely used in Asian medicine. More than 100 prescribed drugs in the United States are obtained from natural sources and represent one fourth of the total drugs used. Apart from these drugs that originate from natural sources, other phytochemicals also serve as potential drugs after structural modification (Kinghorn and Balandrin, 1993).

Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers that are chemically related to squalene, which is a large group of compounds having 30 carbon atoms arranged in five rings with several oxygen atoms attached. Triterpenoids are part of the largest group of plant products, Saponins can be chemically biosynthesized when one or more sugar moieties attach to aglycone. There are two types of saponins, steroidal

aglycone and triterpenoid aglycone. Both steroid and triterpenoid systems are found to be biosynthesized from a common precursor such as squalene (Liu, 1995). Triterpenoids are synthesized from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). For this cyclization, three prenyltransferases synthesize the linear prenyl pyrophosphates geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Squalene is in turn derived biosynthetically by the cyclization of a number of units of isoprene, $(C_5H_8)n$, which undergo folding through 20 different patterns in the presence of prenyl pyrophosphates to form monocyclic, dicyclic, tricyclic, tetracyclic, or pentacyclic derivatives (Bouvier *et al*., 2005).

A family of oxidosqualene cyclases may produce only a single product, such as lupeol cyclases, but there are also multifunctional oxidosqualene cyclases that use dammarenyl cation intermediates to produce many products. Once squalene undergoes cyclization, it goes through the cytosolic mevalonate pathway to make a proximate tetracyclic C30 compound, lanosterol, which further undergoes oxidation and catabolic metabolism to form cholesterol.

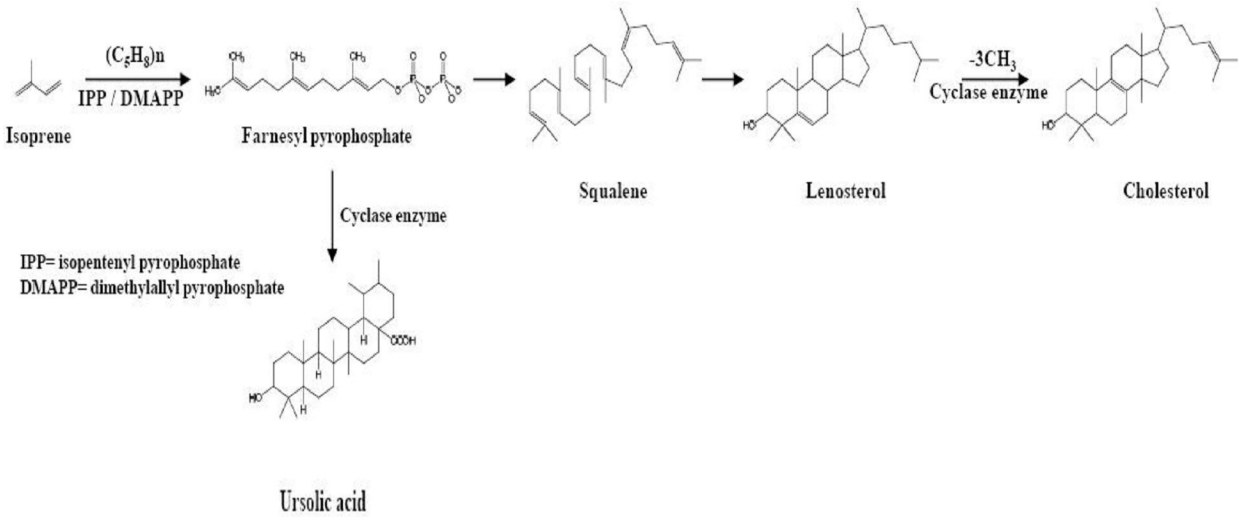
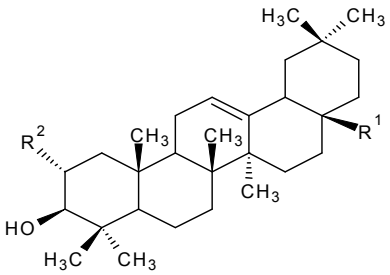


Figure 2.5: Different patterns of cyclization of squalene to form triterpenoids. (Source: Vivek *et al*., 2010)

Terpenes and their metabolites are widely distributed in various plant systems that depend on various biotic and abiotic environmental factors. Terpenes and their metabolites are used in several developmental and physiological functions on the basis of the differential expression profiles of terpene synthase genes. Terpenes and their metabolites play a very important role in a plant's defense mechanism. They protect the plants from both constitutive and induced defensive responses against insects and environmental stress (Keeling and Bohlmann, 2006; Franceschi *et al.,* 2005). Hence, triterpenoids provide a very good protection shield for plants, indicating their potential for use in the prevention of various inflammatory diseases in humans. Triterpenoids affect multiple signalling pathways, and the clinical properties of triterpenoids, particularly those of pentacyclic triterpenoids, have been shown in various studies. The structure activity relationships indicate that the presence of -unsaturated carbonyl moieties significantly enhance the potency of these pentacyclic triterpenoids.

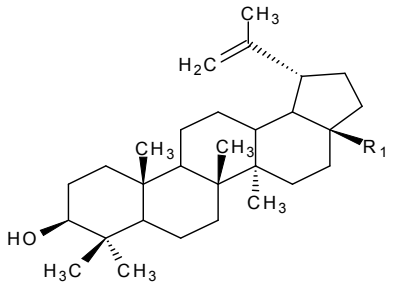
2.6.1 Pentacyclic Triterpenes from Nature

Several pentacyclic triterpenes (PCTs), for example, compounds (**1 – 10**) have been isolated and reported as biologically active compounds (Vivek *et al*., 2010).



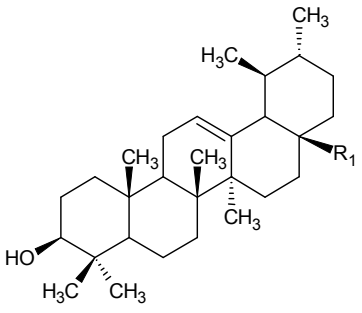
Oleanane

R^1	R^2	
1: COOH	H,	Oleanolic acid
4: CH_2OH	H,	erythrodiol
7: CH_3	H,	β-Amyrin
10: COOH	OH,	maslinic acid



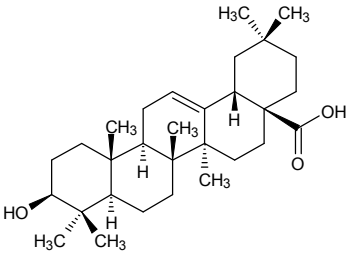
Lupane

R^1	
2: COOH	betulinic acid
5: CH_2OH	betulin
8: CH_3	lupeol



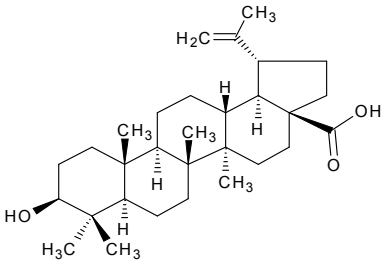
Ursane

R^1	
3: CH_3	α-Amyrin
6: CH_2OH	uvaol
9: COOH	ursolic acid



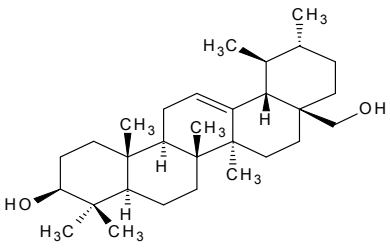
Oleanolic acid

1



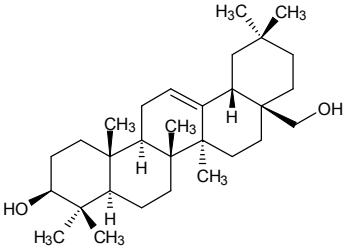
betulinic acid

2



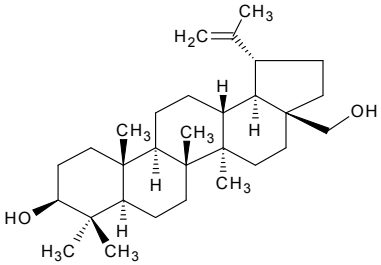
uvaol

3



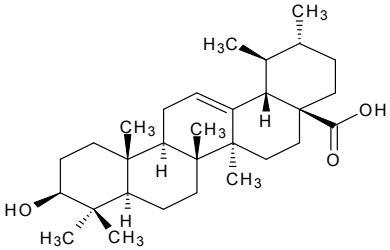
erythrodiol

4



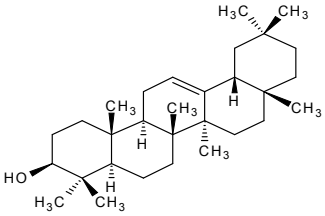
betulin

5



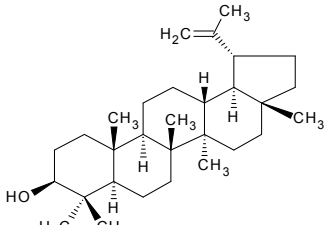
ursolic acid

6

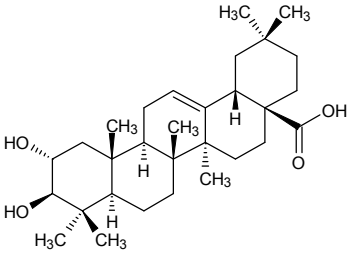


beta-Amyrin

7

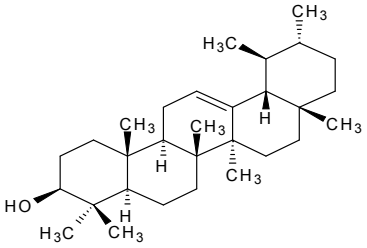


8



maslinic acid

9



alfa-Amyrin

10

Figure 2.6: Structures of different classes of triterpenes

2.6.2 Betulinic Acid (BA)

The reported biological activities of BA include the following; anticancer (Amico *et al.*, 2006; Huang *et al.*, 2007), HIV-1 maturation inhibitor (Fukoja *et al.*, 1994), antibacterial activity with inhibitory effects against *Bacillus subtilis* (Chandramu *et al.*, 2003).

2.6.3 Oleanolic Acid (OA)

Oleanolic acid occurs in numerous varieties of plants and possesses many biological properties including anti-inflammatory, trypanocidal, anti-HIV and cytotoxic activities. (Saleem *et al*., 2005; Sultana and Ata, 2008).

2.6.4 Maslinic Acid (MA)

Maslinic Acid is reported to have biological activities which include the following; antitumor (Li *et al*., 2010), antioxidant (Jager *et al*., 2009), anti-inflammatory (Vivek *et al*., 2010), gastroprotective (Tati *et al*., 2008) effects among others.

2.6.5 Eugenol (EU)

Eugenol is one of the mojor component of cloves, it is an essential oil reported to have biological activities such as; Antioxidant (Abdulrahim *et al.,* 2011; Lee and Shibamato, 2001), cytotoxic, apoptotic, antiangiogenic (Zheng *et al.,* 1992; Chaieb *et al*., 2007), antimicrobial (Himejima and Kubo, 1992) activities.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

i. Eppendorf centrifuge 5804 R (Merck)

ii. Biotek ELx 808 UI plate reader (Biotek Instrument supplies).

iii. 96-well microtitre plates (Sigma-Aldrich Chemicals)

iv. Magnetic stirrer/Heater (Thermolyne Nouva)

v. Ernst Leitz Wetzlar melting point apparatus

vi. Bruker-Advance 400MHz FT-NMR Spectrometer

vii. Top loading Balance (Precisa 3100C)

viii. Aluminuim Pre-coated TLC paper (Silica gel 60 F_{254}, Merck)

ix. Perkin Elmer Spectrum 100 FTIR Spectrometer

x. Agilent Technologies 1200 Series Binary SL (LC-MS)

xi. Digital Plethysmometer LE 7500 (Panlab, Spain).

xii. Spectrophotometer (Spekol 1300)

3.1.2 Chemicals

i. Ehylacetate (Searchem)

ii. Hexane (BDH)

iii. Ethanol (BDH)

iv. Methylsulfoxide-d_6 (DMSO- d_6) (Sigma-Aldrich Chemicals)

v. Deuterated Chloroform ($CDCl_3$) (Sigma-Aldrich Chemicals)

vi. 1,1'-diphenyl-2-pictyhadrazyl (DPPH) (Sigma-Aldrich Chemicals)

vii. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Sigma-Aldrich Chemicals)

viii. Thrombin (Sigma-Aldrich Chemicals)

ix. Adenosine 5'-phosphate (ADP) (Sigma-Aldrich Chemicals)

x. Epinephrine (Sigma-Aldrich Chemicals)

xi. heparin (Sigma-Aldrich Chemicals)

xii. butylated hydroxyanisole (BHA) (Sigma-Aldrich Chemicals)

xiii. Ascorbic acid (AA) (Sigma-Aldrich Chemicals)

xiv. Aspirin (Sigma-Aldrich Chemicals)

xv. Tween 20 (Sigma-Aldrich Chemicals)

3.1.3 Plant Material

3.1.3.1 *Syzygium aromaticum* and *Melaleuca bracteata*

Syzygium aromaticum and *Melaleuca bracteata* were supplied by Prof. F.O Shode of the School of Chemistry, University of KwaZulu-Natal Durban, South Africa. The plant was identified and voucher specimens with herbarium number (Glow 001 and SA 023) respectively, were deposited in the herbarium.

3.1.4 Animals

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

3.2 METHODS

3.2.1 Extraction and Isolation of Oleanolic Acid and Maslinic Acid from *S. aromaticum* (Method described by Shode *et al*., 2010)

The flower bud of *S.aromaticum* was macerated in dichloromethane (DCM) (1kg:2L) for 48 hrs and the procedure was repeated five times and filtered. The filtrate was concentrated under reduced pressure and defatted using hexane (Vol, 1:2), to give a solid residue and hexane soluble extract, the hexane soluble extract was steam distilled. The distillate was re-extracted with ethyl acetate (1:1), dried with a little quantity of anhydrous sodium sulphate and the residual solvent was allowed to evaporate at room temperature to give an oil (AJ/3). The hexane solid crude extract (AJ/2, 10 g) was subjected to column chromatography using 2500ml hexane:ethylacetate (8:2) as the eluting solvent system to give two white amorphous powder oleanolic acid (10 g) and a mixture (4 g) of oleanolic acid (OA) and masilinic acid (MA).

3.2.2 Formation of Oleanolic and Maslinic Acetates (Method described by Shode *et al*., 2009)

The mixture of Oleanolic acid and Maslinic acid (4 g) from above was acetylated by the addition of pyridine (5 ml) and acetic anhydride (5 ml) in a round bottom flask and stir for 24 hrs, after which water (10 ml) was added and stirring continued for another 30 minutes. The mixture was then filtered and washed thoroughly with enough quantity of 10 % HCl. The solid residue was then subjected to column chromatography to yield oleanolic acetate (OAA, 1.5 g) and maslinic acetate (MAA, 750 mg).

3.2.3 Isolation of Eugenol and Formation of Eugenol Acetate (Method described by Shode *et al*., 2010)

The oil (AJ/3, 50 ml) was introduced into a separating flask, 5% solution of potassium hydroxide (500ml) was added mixed thoroughly and extracted with enough quantity of ethyl acetate which gave an oil on drying identified as eugenol (AJ/3/1). To the aqueous layer, 10 % hydrochloric acid was added and extracted with ethyl acetate to give a mixture of eugenol and eugenol acetate (AJ/3/2). To the mixture, AJ/3/2 (5 ml) in a round bottom flask, was added pyridine (5 ml) and acetic anhydride (5 ml) and stirred at room temperature for 24 hr. Then water (10 ml) was introduced and stirred for a further 30 min. the mixture was then introduced into a separating flask containing about 50ml ethylacetate and the organic layer was separated and washed with 25ml of 10% HCl, re-extracted in ethylacetate, dried using sodium sulphate and allowed to concentrate at room temperature to give an oil which was identified to be eugenol acetate (2 ml).

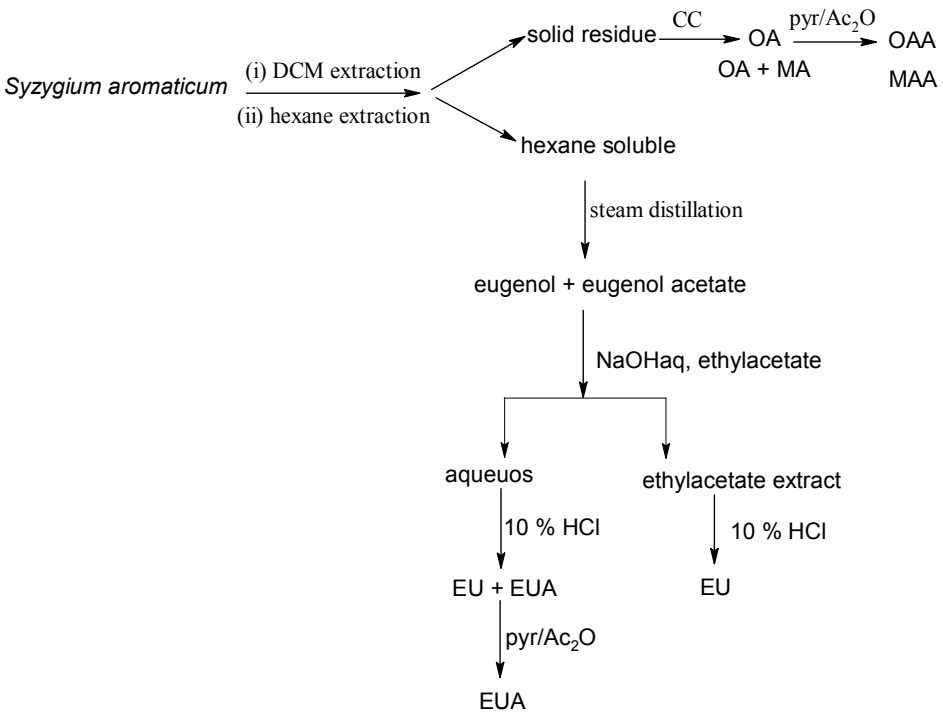
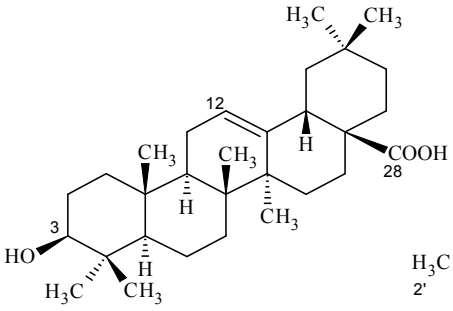


Figure 3.1: Schematic extraction, Isolation and synthesis of Oleanolic acid (OA), Oleanolic acetate (OAA), Maslinic acetate (MAA), Eugenol (EU) and Eugenol acetate (EUA)

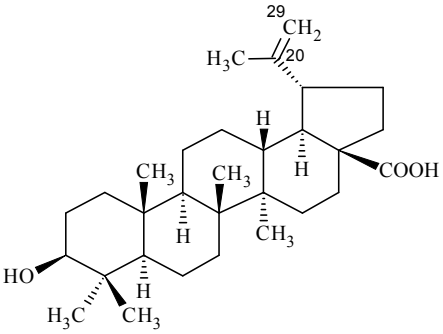
3.2.4 Extraction and Isolation of Betulinic Acid (BA) and Preparation of Betulinic Acetate (BAA) (Shode *et al*., 2010 and Shode *et al*., 2009)

Leaves of *M. bracteata* (1kg) was macerated repeatedly for five times in dichloromethane (2L) for 48 hrs, filtered and concentrated under reduced pressure. The residual crude extract was then defatted with hexane (Vol, 1:2), concentrated and allowed to stand at room temperature to dry. The solid residue (10 g) was then subjected to column chromatography on a silica gel bed using 3000ml hexane:ethylacetate (8:2) for elution to give a white amorphous powder which was identified as BA (1.0 g) using spectroscopic techniques (NMR, IR GC-MS). Betulinic acid (2.0 g) was acetylated as described above to give BAA.

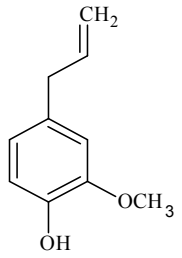
Isolated Compounds



1. Oleanolic acid (OA)

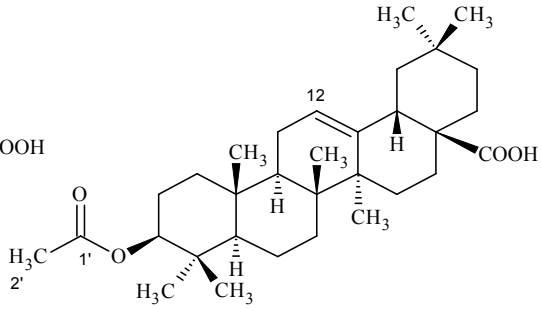


3. Betulinic acid (BA)

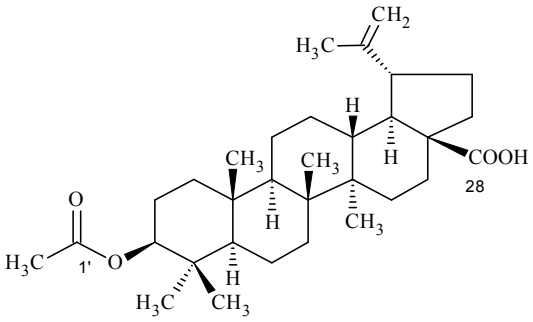


5. Eugenol (EU)

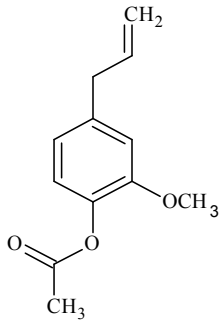
Modified Compounds



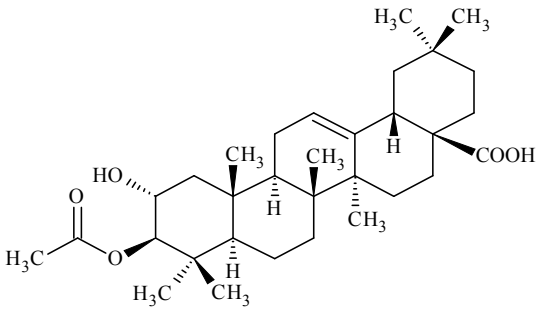
2. Oleanolic acetate (OAA)



4. Betulinic acetate (BAA)



6. Eugenol acetate (EUA)



7. Maslinic acetate (MAA)

Figure 3.2: Structures of isolated and modified compounds (1-7)

3.2.5 Spectroscopy

The compounds isolated and modified were characterized using the following spectroscopic techniques:

Nuclear Magnetic Resonance (NMR) for the determination of total number of carbon and protons in each molecule. This was analyzed using a 400MHz bruker advanced NMR Spectrometer. Samples (5mg) were run in a liquid form using 1ml deuterated Dimethysulfoxide (DMSO) and Chloroform to give the NMR spectra results which was used in the determination of total number of carbon, proton and hence the molecular structure of compounds.

Liquid Chromatography Mass Spectroscopy (LC-MS) was used to determine the molecular weight of the molecule using the Agilent Technologies 1200 Series Binary SL machine. Sample (0.5mg) in 0.2µl HPLC graded methanol was used to carry out this analysis.

Infra Red (IR) Spectroscopy was used to determine the functional group present in the molecule using the Perkin Elmer Spectrum 100 FTIR Spectrometer. The sample (1mg) was used in its original form.

3.2.6 Acute Toxicity

The oral acute toxicity of eugenol (EU) was reported by Sober *et al.,* (1950) to be 1930mg/kg. Astudillo *et al.,* (2002) reported the intraperitoneal acute toxicity of OA to be >600mg/kg, zero toxicity for BA and 1670mg/kg for EUA. For MAA, BAA and OAA concentrations where kept below the reported toxicity of OA in the entire test carried out.

3.2.7 *In Vitro* Antiplatelet Aggregation Study

The compounds were separately dissolved in 1% dimethyl sulfoxide (DMSO) and a few drops of tween 20 to obtain various concentrations for the anti-platelet aggregation study.

3.2.7.1 *Preparation of Blood Platelets*

The blood platelets were collected according to method described by Tomita *et al*., (1983). The rat was placed in a container containing diethylether, the vapour rendered the rat unconscious and blood was surgically collected from the heart immediately. The blood was mixed (5:1 v/v) with an anticoagulant (acid-dextrose-anticoagulant, trisodium citrate (0.085M), citric acid (0.065M), 2% dextrose). The platelets were obtained by a series of centrifugation at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively, the supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was collected and discarded and the sediment (platelets) was resuspended in 5 ml washing buffer (pH 6.5). This was centrifuged again at 3000 rpm for 15 min after which the supernatant was discarded and 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

diluted in the resuspending buffer (1:10) the resulting suspension was mixed with calcium chloride (0.4 mL: 10µL $CaCl_2$).

3.2.7.2 *Anti-platelet Aggregation Activity*

The method of Mekhfi *et al*., (2004) was used with some modifications**.** The antiplatelet aggregation activity of the compounds were separately tested on thrombin (5 U/mL), ADP (5 mM) and epinephrine (10 mM) induced platelet aggregation; The platelets (150 µL) were incubated in a 96-well microtitre plate at 37^{o}C for 5 min, with 50 µL of different concentrations of the compounds (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 for 20 min at 30 seconds intervals. Dimethyl Sulfoxide (1%) and aggregation inducer were used as negative control while heparin and aspirin were used as positive controls.

3.2.7.3 *Calculation of Percentage Inhibitory Effect of the Compounds on Platelet Aggregation*

All assays were performed in triplicates. The inhibitory effects of the compounds on each parameter were calculated as:

% inhibition = {(1-(At/Ac) x 100}

Where, **At** is the absorbance of the mixture containing the compounds and **Ac** is absorbance of the control without the test compound.

3.2.8 *In Vitro* Antioxidant Activity

3.2.8.1 *Thin Layer Chromatography Bioautography using DPPH and Beta-carotene methods*

DPPH Method: Preliminary test for antioxidant activity of the compounds was carried out using the DPPH method as described by Bors *et al.,* (1992) and Choi *et al.,* (1994). The compounds were spotted on a TLC plate and developed with hexane:ethylacetate (7:3) solvent system, after which the TLC plate was dried then spray with 0.2 % DPPH solution in methanol. The plate was examined after 30 min. of spraying; antioxidant active compounds appeared as pale yellow against the purple DPPH background (Plate X).

β-carotene Method: This was carried out using the method described by Pratt *et al.,* (1984) and Cavin *et al.,* (1998). The compounds were spotted on a TLC plate and developed with hexane:ethylacetate (7:3) solvent system, after which the TLC plate was dried then spray with 0.05 % solution of β-carotene in chloroform. The plate was exposed to ultraviolet light, at 254 nm the appearance of bleached spot against a yellow background indicates antioxidant activity (Plate XI).

3.2.8.2 *1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Assay*

Free radical scavenging activities of the compounds were determined using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Brad-Williams, (1995). Decolourisation of DPPH (purple) upon addition of the compounds indicated radical scavenging activity and this was measured after 30-60 min at 514 nm.

3.2.8.3 *2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate (ABTS) Radical Assay*

The method described by Re *et al.,* (1999) was adopted to determine ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) 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.

3.2.8.4 *Calculation of Percentage Radical Scavenging Activity of the Compounds*

Ascorbic acid and BHA were used as standards. All assays were performed in triplicates. The radical scavenging activities of the compounds are calculated as:

% scavenging activity (SA) = {(1-(At/Ac) x 100}

Where, **Ac** is the absorbance value of the control without the compounds and **At** is the absorbance of the mixture containing the compounds.

Statistical Analysis

The data are presented as mean ± SD of triplicates. The data were statistically analyzed by using a statistical software program SPSS (SPSS for windows, version 18, SPSS Science, Chicago, IL, USA). The one-way analysis of variance (ANOVA) followed by the Tukey's multiple range post-hoc test was employed to find the differences. The data were considered significantly different at $p < 0.05$. Student t-test was used to analyse statistical difference

between groups of different concentrations.

The inhibitory concentration providing 50% inhibition (IC_{50}) was determined using statistical package Origin 6.1.

CHAPTER FOUR

4.0 RESULTS

4.1 ISOLATION OF BIOACTIVE COMPOUNDS

Oleanolic acid: This was isolated as a white amorphous powder from column chromatography of the solid residue obtained from *Syzigium aromaticum.*

Eugenol: The oil was isolated from the hexane soluble extract of *Syzigium aromaticum.*

Maslinic acid: Maslinic acid was isolated as a mixture of Oleanolic acid and Maslinic acid as a white amorphous powder from column chromatography of the solid residue obtained from *Syzigium aromaticum.*

Betulinic acid: A white amorphous powder isolated from column chromatography of the solid residue extracted from *Melaleuca bracteata*.

4.2 MODIFICATION OF ISOLATED COMPOUNDS

All the isolated compounds (Oleanolic acid, Maslinic acid, Betulinic acid and Eugenol) were modified by acetylation to give Oleanolic acetate, Maslinic acetate, Betulinic acetate as a white amorphous powder and Eugenol acetate as a yellow oily liquid.

4.3 CHARACTERIZATION OF ISOLATED AND MODIFIED COMPOUNDS

4.3.1 Characterization of Oleanolic Acid (olean-12-en-28-oic acid)

Oleanolic acid (OA): The compound was identified as oleanolic acid by spectra comparism with literature values (Mahato and Kundu, 1994). The Proton decoupled ^{13}C – NMR spectrum showed 30 Carbon atom (Table 4.1, Appendix 1-7), DEPT-90 (Distortionless enhancement polarization transfer) sub-spectrum indicate five methine (CH) Carbon while DEPT-135 indicates 10 methylene (CH_2, negative), Seven methyl (CH_3, positive) groups. Quaternary carbons do not contain attached protons hence do not appear in the DEPT sub-spectrum, they may be identified as the signals which appear additionally in the proton broadband decoupled ^{13}C –NMR spectra. Therefore eight quaternary carbons were identified. The Electron impact mass spectroscopy (EI-MS, Appendix 8) showed a molecular ion $[M+2H]^+$ at m/z 454. The Infra-Red (IR $\square_{max,}$ cm^{-1}, Appendix 9) Spectra shows absorption at 3392 this corresponds to (O-H) stretch for hydroxyl hydrogen bonded, the signal at 1686 is due to (C=O, acid) stretch of a carbonyl carbon. The signal at 2922 is due to (C- H) stretch for alkane. The signal at 1451 is due to (CH_3-, CH_2-).

Table 4.1: ^{13}C-NMR ($CDCl_3$) Assignment of the Test Compounds.

Carbon position	Oleanolic acid	Oleanolic acetate	Betulinic acid	Betulinic acetate	Maslinic acetate	Eugenol	Eugenol acetate
1	38.37	39.27	38.66	38.42	43.61	131.74	139.01
2	27.09	23.52	27.26	25.44	73.26	111.21	112.74
3	78.61	80.94	78.42	80.97	80.84	146.63	150.92
4	38.69	37.69	38.76	37.80	38.34	144.08	138.05
5	55.16	55.28	55.26	56.39	47.55	115.45	120.67
6	18.28	17.17	18.20	16.47	18.20	121.16	122.54
7	33.08	33.78	34.25	34.24	33.78	39.87	40.10
8	39.25	38.05	40.62	40.70	39.30	137.90	137.08
9	47.55	47.54	50.42	50.40	46.50	114.53	116.16
10	36.96	36.99	37.06	37.12	39.75	55.75	55.78
11	22.96	23.39	20.78	19.35	23.46		169.17
12	122.06	122.55	25.43	27.95	122.28		20.65
13	144.00	143.61	38.10	38.38	143.67		
14	41.65	40.90	42.32	42.42	55.10		
15	28.10	25.90	30.56	29.69	30.67		
16	23.29	32.44	32.22	30.57	22.81		
17	45.99	45.83	55.99	55.42	41.58		
18	40.31	41.54	46.80	49.27	40.86		
19	46.15	46.54	49.09	46.95	45.82		
20	30.63	32.51	150.76	150.38	32.43		
21	33.85	33.06	29.61	32.16	33.06		
22	32.41	28.04	37.06	37.05	28.51		
23	28.10	22.86	27.67	23.70	23.56		
24	15.64	18.17	15.42	21.32	25.93		
25	15.27	15.34	15.94	16.18	17.14		
26	17.00	16.65	16.04	16.04	16.61		
27	25.85	23.57	14.58	14.66	16.33		
28	180.44	184.10	178.43	181.86	184.07		
29	33.08	30.66	109.27	109.74	27.61		
30	23.56	27.66	19.27	18.16	32.40		
1’	-	171.07	-	171.08	171.69		
2’	-	21.31	-	20.85	21.38		

4.3.2 Characterization of Oleanolic Acetate (3β-acetyloleanolic acid)

Oleanolic acetate (OAA): The Proton decoupled ^{13}C –NMR spectrum showed 32 Carbon atoms (Table 4.1, Appendix 10-16), DEPT-90 sub-spectrum showed five methine (CH) Carbon while DEPT-135 showed 10 methylene (CH_2, negative) and eight methyl (CH_3, positive) groups. Nine quaternary carbons were identified in the signals which appeared additionally in the proton broadband decoupled ^{13}C –NMR spectra. The electron impact mass spectroscopy (EI-MS) gave a molecular ion $[M+Na]^+$ for $C_{32}H_{50}O_4Na$ at m/z 521.2 (Appendix 17). Hence the molecular mass was determined as 498.2. The IR-spectra ($IR_{\square max}$ Cm^{-1}; Appendix 18) showed absorption at 3400 and this corresponds to (O-H) stretch for hydroxyl hydrogen bonded, the signals at 1721 is due to a Carbonyl carbon (C=O, ester) while 1686 is due to (C=O, acid). The signal at 2921 is due to (C-H) stretch for an alkane. The signal at 1455 is due to (CH_3-, CH_2-), 1631 is due to (C=C) and 1024 corresponds to (C-O).

4.3.3 Characterization of Betulinic Acid (3β-hydroxylup-20(29)-en-28-oic acid)

Betulinic acid (BA): BA was assigned the molecular formula $C_{30}H_{48}O_3$ by EI-MS (electron impact mass spectroscopy) which showed a molecular ion at m/z 457.1 $[M^+ +H]$ (Appendix 26). ^{13}C –NMR spectrum (Table 4.1, Appendix 19-25) reveals 30 Carbon signals, which were assigned by DEPT experiments as five methine (CH) eleven methylene (CH_2) six methyl (CH_3) and seven quaternary carbons. The two sp^2 carbons observed at δ_C 150.76 and δ_C 109.27 in the ^{13}C –NMR spectrum shows the $\Delta^{20,29}$ functionality of a lupene skeleton. Spectra data (IR and NMR) with the application of

COSY, NOESY, HSQC and HMBC were used to assign the structure 3β-hydroxyllup-20(29)-en-28-oic acid (Betulinic acid).

4.3.4 Characterization of Betulinic Acetate (3β-acetyl-betulinic acid)

Betulinic acetate (BAA): The EI-MS results (Appendix 34) showed a molecular ion peak at m/z 475. The IR spectra(IR $\square_{max,}$ cm^{-1} Appendix 35) results showed signals at 3400 (OH), 2922 (CH) alkane, 1733 (C=O, ester), 1688 (C=O, acid); the NMR spectra (Table 4.1;Appendix 27-33) showed 32 carbon atoms, the DEPT experiment showed 6 methine (CH) carbon, 11 methylene (CH_2) carbons, 7 methyl carbons and 8 quartenary carbons.

4.3.5 Characterization of Maslinic Acetate (3β-acetylmaslinic acid)

Maslinic acetate (MAA): The Proton decoupled ^{13}C –NMR spectrum showed 32 Carbon atoms (Table 4.1, Appendix 36-42), DEPT-90 sub-spectrum showed six methine (CH) Carbon while DEPT-135 showed eight methylene ($CH_{2,}$ negative) and eight methyl (CH_3, positive) groups. Ten quaternary carbons were identified in the signals which appeared additionally in the proton broadband decoupled ^{13}C –NMR spectra. The electron impact mass spectroscopy (EI-MS) gave a molecular ion $[M]^+$ at m/z 528 (Appendix 43). The IR-spectra ($IR_{\square max}$ Cm^{-1}; Appendix 44) showed absorption at 3393 this corresponds to (O-H) stretch for hydroxyl hydrogen bonded, the signals at 1686 is due to a Carbonyl carbon (C=O, ester) while 1631 is due to (C=O, acid). The signal at 2921 is due to (C-H) stretch for an alkane. The signal at 1451 is due to (CH_3-, CH_2-), 1631 is due to (C=C) and 1045 corresponds to (C-O).

4.3.6 Characterization of Eugenol

Eugenol (EU): **T**he 13-Carbon NMR spectra (Table 4.1,Appendix 45-51) showed a total of 10 carbon atoms, the DEPT experiments showed 4 methine (CH) carbons, 2 methylene carbons, one methyl and 3 quartenary carbons.The EIMS result showed a molecular ion m/z at 164 (Appendix 52). The IR spectra (IR $\square_{max,}$ cm^{-1}; Appendix 53) showed signal at 3434 which corresponds to O-H stretching vibration, 2922 for alkane (CH) stretching, 1685 for C-O absorption.

4.3.7 Characterization of Eugenol Acetate

Eugenol acetate (EUA): Its molecular formula was determined as $C_{12}H_{14}O_3$, by analysing its NMR spectra (Table 4.1,Appendix 54-60) which showed a total of 12 carbon atoms, the DEPT experiment showed 4 methine (CH), one methylene (CH_2), 2 methyl (CH_3) and 4 quartenary carbons. The IR spectra (IR $\square_{max,}$ cm^{-1}; Appendix 62) result showed signals at 3442 assigned to O-H stretching vibration, 2923 for CH stretching vibration of alkane, 1686 for (C=O, ester), 1637 for C-O.

4.4 ANTI-AGGREGATION ACTIVITY OF ISOLATED AND MODIFIED COMPOUNDS

4.4.1 Anti-aggregation Activity of Isolated and Modified Compounds in Thrombin-induced Platelet Aggregation

In the Thrombin-induced platelet aggregation (Table 4.2), The percentage inhibitory activity of the compounds (OA,BA,OAA,BAA,MAA,EU,EUA+EU) showed an increase in the inhibition of platelet aggregation with increase in concentration of the compound except for the compound, EUA which showed a decrease in the inhibition of platelet

aggregation as the concentration of the compound increases (79.6±0.53% at 1mg/ml, 61.1±0.45% at 3mg/ml and 46.3±0.71% at 10mg/ml, IC_{50} 3.72mg/ml) showing that there is an optimal concentration for inhibiting platelet aggregation of EUA after which the percentage inhibition starts to decrease.

Betulinic acetate in the Thrombin-induced platelet aggregation is the most active compound to inhibit platelet aggregation with percentage inhibitory activity of 54.5±0.01% at 1mg/ml, 63.5±0.17% at 3mg/ml and 73.5±0.15% at 10mg/ml. with IC_{50} of 0.81mg/ml, followed by OA with percentage inhibitory activity of 50.0±0.11% at 1mg/ml, 68.4±0.04% at 3mg/ml and 83.0±0.23% at 10mg/ml, having an IC_{50} of 1.00mg/ml. MAA was next with percentage inhibitory activity of 49.6±0.35% at 1mg/ml, 55.4±0.66% at 3mg/ml, and 55.4±1.39% at 10mg/ml, IC_{50} 1.17 mg/ml, followed by BA with percentage inhibitory activity of 38.2±1.01% at 1mg/ml, 62.9±0.65% at 3mg/ml, and 75.8±0.12% at 10mg/ml, IC_{50} 1.99 mg/ml, then EU with IC_{50} 2.23mg/ml, EUA+EU with IC_{50} 2.76mg/ml and EUA with IC_{50} 3.72mg/ml. The least activity was exhibited by OAA which showed a percentage inhibitory activity of 40.5± 0.24% at 1mg/ml, 44.2±1.04% at 3mg/ml and 64.1±0.50% at10mg/ml with an IC_{50} of 5.00mg/ml.

Table 4.2: Percentage Inhibitory Activity of the Isolated Compounds on Thrombin-induced Platelet Aggregation

	Percentage inhibitory activity (%)			
Compounds	**1mg/ml**	**3mg/ml**	**10mg/ml**	**IC_{50}mg/ml**
Oleanolic acid	50.0±0.11^{a,1}	68.4±0.04^{a,2}	83.0±0.23^{a,3}	1.00
Betulinic acid	38.2±1.01^{b,1}	62.9±0.65^{b,2}	75.8±0.12^{b,3}	1.99
Oleanolic acetate	40.5±0.24^{b,1}	44.2±1.04^{c,2}	64.1±0.50^{c,3}	5.00
Betulinic acetate	54.5±0.01^{a,1}	63.5±0.17^{b,2}	73.5±0.15^{b,3}	0.81
Maslinic acetate	49.6±0.35^{c,1}	55.4±0.66^{d,2}	55.4±1.39^{d,3}	1.17
Eugenol	39.8±0.31^{b,1}	56.3±1.66^{d,2}	81.6±0.35^{a,3}	2.23
EU+EUA	47.5±0.32^{c,1}	50.3±0.30^{d,2}	75.0±0.49^{b,3}	2.76
Eugenol acetate	79.6±0.53^{d,1}	61.1±0.45^{b,2}	46.3±0.71^{e,3}	3.72
Heparin	65.9±0.59^{e,1}	66.9±0.03^{a,2}	70.6±0.16^{b,3}	0.26
Aspirin	65.4±0.07^{e,1}	72.1±0.03^{a,2}	76.5±1.22^{b,3}	0.33

Data are presented as means ±SD of triplicate. $^{a-e}$Values with different superscript letters down the columns and $^{1-3}$Values of different superscript numbers along the rows are significantly different ($p<0.05$)

EU+EUA=Mixture of eugenol and eugenol acetate.

4.4.2 Anti-aggregation Activity of Isolated and Modified Compounds in Adenosine-diphosphate-induced Platelet Aggregation

The result of ADP-induced platelet aggregation (Table 4.3) showed that the compounds (OA,BA,OAA,MAA,EUA) had anti-aggregation activity. The compounds showed an increase in percentage inhibition of platelet aggregation as their concentration increases except for BAA which showed a decrease in the inhibition of platelet aggregation as the concentration of the compound increases. EU and EU+EUA did not show any inhibitory activity at the concentrations that were used. This could be due to synergistic effect of the compounds.

The most active compound to inhibit platelet aggregation was exhibited by BAA with percentage inhibitory activity of 59.9±1.19% at 1mg/ml, 15.4±0.05% at 3mg/ml and 0.1±0.09% at 10mg/ml. with IC_{50} value of 1.39mg/ml, followed by EUA with percentage inhibitory activity of 0.0±0.12% at 1mg/ml, 66.9±084% at 3mg/ml and 73.3±0.42% at 10mg/ml. with IC_{50} of 2.50mg/ml. Oleanolic acetate (OAA) was next with IC_{50} of 2.89mg/ml, followed by BA with IC_{50} 4.20mg/ml, then MAA with IC_{50} 7.4mg/ml. The least activity was exhibited by OA which showed a percentage inhibitory activity of 0.6± 0.43% at 1mg/ml, 13.1±0.39% at 3mg/ml and 69.7±1.06% at10mg/ml with an IC_{50} of 7.62mg/ml.

Table 4.3: Percentage Inhibitory Activity of the Isolated Compounds on ADP-induced Platelet Aggregation

	Percentage inhibitory activity (%)			
Compounds	**1mg/ml**	**3mg/ml**	**10mg/ml**	**IC_{50} mg/ml**
Oleanolic acid	$0.6\pm0.43^{a,1}$	$13.1\pm0.39^{a,2}$	$69.7\pm1.06^{a,3}$	7.62
Betulinic acid	$0.0\pm0.11^{a,1}$	$44.9\pm0.47^{b,2}$	$74.3\pm0.58^{a,3}$	4.20
Oleanolic acetate	$8.7\pm0.19^{a,1}$	$53.0\pm0.01^{c,2}$	$62.9\pm0.47^{a,3}$	2.89
Betulinic acetate	$59.9\pm1.19^{b,1}$	$15.4\pm0.06^{a,2}$	$0.1\pm0.09^{b,3}$	1.39
Maslinic acetate	$0.0\pm0.98^{a,1}$	$29.5\pm0.09^{d,2}$	$61.9\pm1.04^{c,3}$	7.42
Eugenol	$0.0\pm0.24^{a,1}$	$0.0\pm0.45^{e,1}$	$0.0\pm0.43^{b,1}$	ND
EU+EUA	$4.7\pm0.76^{a,1}$	$4.8\pm0.38^{e,2}$	$4.3\pm0.56^{b,3}$	ND
Eugenol acetate	$0.0\pm0.12^{a,1}$	$66.9\pm0.84^{f,2}$	$73.3\pm0.42^{a,3}$	2.50

Data are presented as means ±SD of triplicate. $^{a-e}$Values with different superscript letters down the columns and $^{1-3}$Values of different superscript numbers along the rows are significantly different ($p<0.05$)

EU+EUA=Mixture of eugenol and eugenol acetate.

ND= not determined

4.4.3 Anti-aggregation Activity of Isolated and Modified Compounds in Epinephrine-induced Platelet Aggregation

In the epinephrine-induced platelet aggregation the compounds (OA, BA, OAA, MAA, EU and EUA) showed varying degrees of inhibition of platelet aggregation with the exception of EU+EUA which had no inhibitory effect on platelet aggregation at the concentrations of the compounds used. OA,BA,BAA,EU andEUA exhibited an increase in percentage inhibition of platelet aggregation as their concentration increases while for OAA and MAA showed a decrease in the inhibition of platelet aggregation as the concentration of the compound increases.

Betulinic acetate (BAA) is shown to be the most active compound with percentage inhibitory activity of 44.9±1.27% at 1mg/ml, 58.6±0.55% at 3mg/ml and 66.7±0.16% at 10mg/ml having the lowest IC_{50} value of 1.81mg/ml. The next active compound OAA with percentage inhibitory activity of 72.6±0.10% at 1mg/ml, 28.1±0.19% at 3mg/ml and 6.1±0.08% at 10mg/ml with IC_{50} value of 2.17mg/ml, followed by BA with IC_{50} of 4.63mg/ml.Oleanolic acid (OA) with IC_{50} of 7.14mg/ml, MAA with IC_{50} 7.38mg/ml, then EUA with IC_{50} 11.20mg/ml. The least active compound was EU with percentage inhibitory activity of 0.0± 1.10% at 1mg/ml, 0.0±0.32% at 3mg/ml and 33.8±0.34% at10mg/ml with the highest IC_{50} of 12.21mg/ml.

Table 4.4: Percentage Inhibitory Activity of the Isolated Compounds on Epinephrine-induced Platelet Aggregation

	Percentage inhibitory activity (%)			
Compounds	**1mg/ml**	**3mg/ml**	**10mg/ml**	**IC_{50} mg/ml**
Oleanolic acid	$21.4\pm0.68^{a,1}$	$23.0\pm0.75^{a,2}$	$67.9\pm0.75^{a,3}$	7.14
Betulinic acid	$31.7\pm1.32^{a,1}$	$39.3\pm0.31^{b,2}$	$86.3\pm0.56^{b,3}$	4.63
Oleanolic acetate	$72.6\pm0.10^{b,1}$	$28.1\pm0.19^{a,2}$	$6.1\pm0.08^{c,3}$	2.17
Betulinic acetate	$44.9\pm1.27^{c,1}$	$58.6\pm0.55^{c,2}$	$66.7\pm0.16^{a,3}$	1.81
Maslinic acetate	$59.6\pm0.10^{d,1}$	$51.4\pm0.12^{d,1}$	$49.1\pm0.17^{d,2}$	7.38
Eugenol	$0.0\pm1.10^{e,1}$	$0.0\pm0.32^{e,2}$	$33.8\pm 0.34^{e,3}$	12.21
EU+EUA	$0.0\pm1.12^{e,1}$	$0.0\pm0.02^{e,1}$	$0.0\pm0.36^{c,1}$	ND
Eugenol acetate	$8.2\pm0.21^{e,1}$	$15.9\pm0.14^{a,2}$	$42.0\pm0.66^{d,3}$	11.20

Data are presented as means ±SD of triplicate. $^{a-e}$Values with different superscript letters down the columns and $^{1-3}$Values of different superscript numbers along the rows are significantly different ($p<0.05$)

EU=Eugenol, EU+EUA=Mixture of eugenol and eugenol acetate.

ND= not determined

4.5 ANTIOXIDANT ACTIVITY OF ISOLATED AND MODIFIED COMPOUNDS.

4.5.1 Thin Layer Chromatography Bioautography of Isolated and Modified Compounds

Table 4.5 shows the ability of the isolated and modified compounds to scavenge free radicals using DPPH and β-carotene methods. Base on the test concentration, the compounds with negative sign do not possess the ability to scavenge free radicals while those with positive sign possess free radical scavenging ability.

Table 4.5: Result of Antioxidant Bioautography of the Isolated and modified Compounds by Thin Layer Chromatography

COMPOUNDS	DPPH	β-CAROTENE
Oleanolic acid	-	-
Oleanolic acetate	-	-
Maslinic acetate	-	-
Betulinic acid	+	-
Betulinic acetate	-	-
Eugenol	++	++
Eugenol acetate	++	++
Mixture of eugenol and eugenol acetate	+	++

Key: + = positive, ie compound with antioxidant activity.

- = negative, ie compound without antioxidant activity.

4.5.2 Antioxidant Activity of Isolated Compounds using DPPH Assay

The result of the DPPH assay (Table 4.6) showed the percentage scavenging activity of the compounds (EU,EU+EUA and EUA) increased as the concentration of the compounds increased. The most active compound is EU with a percentage scavenging activity ranging from 28.1±0.005% at 0.0625mg/100ml to 79.9±0.002% at 1mg/100ml with lowest IC_{50} value of 0.54mg/100ml, followed by EU+EUA with IC_{50} 0.69mg/100ml and the least active compound EUA with IC_{50} of 5.86mg/100ml.

Table 4.6: Percentage Scavenging Activity of Isolated Compounds using DPPH Assay

	Percentage scavenging activity (%)					
Compound	0.0625 mg/100ml	0.125 mg/100ml	0.25 mg/100ml	0.5 mg/100ml	1 mg/100ml	IC_{50} mg/ 100ml
Eugenol	$28.1\pm0.005^{a,1}$	$30.2\pm0.002^{a,2}$	$30.9\pm0.007^{a,3}$	$46.8\pm0.004^{a,4}$	$79.9\pm0.002^{a,5}$	0.54
EU+EUA	$10.8\pm0.007^{b,1}$	$15.1\pm0.019^{b,2}$	$17.9\pm0.002^{b,3}$	$35.9\pm0.006^{b,4}$	$71.2\pm0.003^{b,5}$	0.69
*Eugenol acetate	$39.8\pm0.006^{c,1}$	$43.1\pm0.006^{c,2}$	$44.3\pm0.003^{c,3}$	$45.5\pm0.018^{c,4}$	$47.6\pm0.018^{c,5}$	5.86
Ascorbic acid	$0.6\pm0.003^{d,1}$	$35.6\pm0.005^{a,2}$	$45.0\pm0.008^{d,3}$	$45.6\pm0.010^{a,4}$	$66.3\pm0.005^{b,5}$	0.60
butylated hydroxyan-isole	$50.5\pm0.053^{e,1}$	$61.4\pm0.004^{d,2}$	$69.3\pm0.004^{e,3}$	$80.2\pm0.003^{d,4}$	$85.1\pm0.002^{a,5}$	0.05

Key:* The concentrations used where 1 mg/100ml, 2 mg/100ml, 3 mg/100ml, 4 mg/100ml, 5 mg/100ml respectively.

Data are presented as means ±SD of triplicate. $^{a-e}$Values with different superscript letters down the columns and $^{1-3}$Values of different superscript numbers along the rows are significantly different ($p<0.05$)

EU+EUA=Mixture of eugenol and eugenol acetate.

4.5.3 Antioxidant Activity of Isolated Compounds using ABTS Assay

The result of the ABTS assay (Table 4.7) showed the percentage scavenging activity of the compounds (EU,EU+EUA and BA) were increasing as the concentration of the compounds increased. The most active compound is shown to be EU which exhibited a percentage scavenging activity ranging from 57.7±0.002% at 0.0625mg/100ml to 90.7±0.001% at 1mg/100ml with lowest IC_{50} value of 0.05mg/100ml, followed by EU+EUA with IC_{50} 0.07mg/100ml and the least active compound BA with IC_{50} of 2.30mg/100ml.

Table 4.7: Percentage Scavenging Activity of Isolated Compounds using ABTS Assay

	Percentage scavenging activity (%)					
Compound	**0.0625 mg/100ml**	**0.125 mg/100ml**	**0.25 mg/100ml**	**0.5 mmg/100ml**	**1 mg/100ml**	**IC_{50} mg/100ml**
Eugenol	$57.7\pm0.002^{a,1}$	$75.3\pm0.003^{a,2}$	$89\pm0.002^{a,3}$	$89.7\pm0.001^{a,4}$	$90.7\pm0.001^{a,5}$	0.05
EU+EUA	$48.5\pm0.001^{b,1}$	$64.5\pm0.001^{b,2}$	$71.1\pm0.002^{b,3}$	$73.2\pm0.001^{b,4}$	$77.3\pm0.003^{b,5}$	0.07
*Betulinic acid	$39.0\pm0.001^{c,1}$	$47.2\pm0.001^{c,2}$	$55.3\pm0.003^{c,3}$	$57.7\pm0.002^{c,4}$	$57.7\pm0.003^{c,5}$	2.30
Ascorbic acid	$9.7\pm0.001^{d,1}$	$19.5\pm0.003^{d,2}$	$26.0\pm0.001^{d,3}$	$44.7\pm0.001^{d,4}$	$60.9\pm0.002^{d,5}$	0.66
butylated hydroxyanisole	$9.7\pm0.005^{d,1}$	$15.4\pm0.001^{d,2}$	$21.9\pm0.002^{d,3}$	$52.0\pm0.001^{d,4}$	$65.8\pm0.001^{d,5}$	0.48

Key:*The concentrations used where 1 mg/100ml, 2 mg/100ml, 3 mg/100ml, 4 mg/100ml, 5 mg/100ml respectively.

Data are presented as means ±SD of triplicate. $^{a-e}$Values with different superscript letters down the columns and $^{1-5}$Values of different superscript numbers along the rows are significantly different ($p<0.05$)

EU+EUA=Mixture of eugenol and eugenol acetate.

CHAPTER FIVE

5.0 DISCUSSION

The result of the spectral data (IR and NMR) with the application of correlation spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) were used to assign the structures of the isolated compounds (oleanolic acid, oleanolic acetate, betulinic acid, betulinic acetate, maslinic acetate, eugenol and eugenol acetate) as compared with literature values reported by Mahato and Kundu, (1994). All the electron impact mass spectroscopy (EI-MS) values of the various compounds was determined and the molecular ion shown for the various to be m/z 454 for oleanolic acid, 498.2 for oleanolic acetate, 457.1 for betulinic acid, 475 for betulinic acetate, 528 for maslinic acetate and 164 for eugenol which also corresponds to literature values reported by Mahato and Kundu, (1994).

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 the anti-platelet aggregation study suggest that the isolated compounds possess the ability to inhibit the aggregation of platelets. The compounds inhibited rat platelets aggregation induced with thrombin, ADP and epinephrine.

A number of triterpenoids from various plants with anti-platelet aggregation activity against thrombin, ADP and epinephrine have been reported (Jin *et al*., 2004; Yang *et al*., 2009; Sankaranarayanan *et al*., 2010). The result of the Thrombin-induced platelet aggregation showed betulinic acetate to be the most active compound with percentage inhibition from (54.5±0.01 at 1mg/ml, 63.5±0.17 at 3mg/ml and 73.5±0.15 at 10mg/ml, IC_{50} 0.81mg/ml) while oleanolic acetate gave the least activity with percentage inhibition of (40.5±0.24 at 1mg/ml, 44.2±1.04 at 3mg/ml, and 64.1±0.50 at 10mg/ml, IC_{50} 5.00 mg/ml). The effectiveness of betulinic acetate in inhibiting platelet aggregation is similar ($p<0.05$) when compared to that of Heparin and Aspirin that were used as standards, there is a significant difference ($p<0.05$) in the inhibition of platelet aggregation between the different concentration of betulinic acetate that were used.

When ADP was used to induce platelet aggregation, the compounds showed varying activities with the exception of eugenol and a mixture of eugenol and eugenol acetate. This is an indication that eugenol does not inhibit platelet aggregation via the mechanism that ADP induces platelet aggregation. Betulinic acetate showed the highest inhibition of platelet aggregation (59.9±1.19 at 1mg/ml, 15.4±0.06 at 3mg/ml, 0.1±0.09 at 10mg/ml, IC_{50} 1.39) even though there was a decrease in percentage inhibition with increase in concentration, it suggest that there is an optimal concentration of activity. Oleanolic acid exhibited the lowest activity with IC_{50} 7.62mg/ml. In the epinephrine-induced platelet

aggregation, the highest inhibition was also exhibited by betulinic acetate with IC_{50} 1.81mg/ml and the lowest activity was exhibited by eugenol with IC_{50} 12.21mg/ml.

Overall result of the anti-platelet aggregation studies reveals that the compounds inhibit platelet aggregation through different routes. While some inhibit platelet aggregation using a more specific mechanism example, eugenol inhibited platelet aggregation only when thrombin was used as agonist, others inhibit platelet aggregation through different mechanisms example, betulinc acetate inhibited platelet aggregation in all the three agonist used. All the compounds showed a significant difference ($p<0.05$) between the different concentrations used in thrombin, ADP and epinephrine-induced platelet aggregation. It implies that the isolated and modified compounds are as good anti-platelet aggregation agents as the standards (Aspirin and Heparin) used in this experiment.

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 have been implicated in various diseases including cardiovascular diseases (Atawodi, 2005) due to interference 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); Sobotkvá *et al.,* (2009)] have reported the beneficial effects of antioxidants on the inhibition of platelet activation and aggregation. Furthermore, some plants such as *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 isolated compounds (oleanolic acid, oleanolic acetate, betulinic acid, betulinic acetate and maslinic acid) exhibited poor antioxidant activity, it was apparent that the anti-platelet aggregation activity of the compounds was not primarily due to antioxidant activity. Zhou *et al*., (2008) also reported weak free radical scavenging activity exhibited by the some triterpenes isolated from *Poriacocos* which had anti-platelet aggregation activity. Some triterpenes are, however, known to exhibit antioxidant activities (Sekiya *et al*., 2003; Russel and Paterson, 2006). The overall results revealed that the anti-platelet aggregation activity of the isolated compounds could be exerted through variable mechanisms other than antioxidant activity.

Inflammation suppresses the natural anticoagulant system which favors the expression and synthesis of prothrombogenic molecules (Verhamme and Hoylaerts, 2009). Some anti-inflammatory agents act 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). There is a large body of evidence on the anti-inflammatory activities of triterpenes (Ríos *et al*., 2000; Biswas *et al*., 2009; Shiao *et al.,* 1994*;* Siqueira *et al*., 2007; Ko *et al*., 2007). The anti-platelet aggregation activity of some of the compounds could be as a result of the anti-inflammatory potentials of the compounds. Like aspirin, anti-platelet aggregation agents can also inhibit inflammation. 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.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Medicinal plants are undoubtedly rich sources of biologically active compounds vital to human health which could be solutions to a wide range of diseases including atherothrombotic diseases. The result obtained from this study reveals;

i. The compounds Oleanolic acid, Eugenol,Maslinic acid were isolated from *S. aromaticum*, while Betulinic acid was isolated from *M. bracteata.* The 3β-acetoxy-oleanolic acid, 3β-acetoxy-maslinic acid, Eugenol acetate and 3β-acetoxy-betulinic acid were formed from the isolated compounds above and these compounds were fully characterized.

ii. The isolated and modified compounds (Oleanolic acid, Eugenol, Oleanolic acetate, Eugenol acetate and Maslinic acetate) from *S. aromatic* and from *M. brateata* (Betulinic acid and Betulinic acetate) possess anti-platelet aggregation activity.

iii. The isolated and modified compounds (oleanolic acid, oleanolic acetate, betulinic acid, betulinic acetate and maslinic acetate) are poor antioxidants which suggest that mechanisms other than the antioxidant activity could be mediating their anti-platelet aggregation activity while the anti-platelet aggregation activity of eugenol and eugenol acetate could be due to their antioxidant properties. The anti-platelet aggregation activity of some of the compounds may be linked to their ant-inflammatory activity.

Considering the increasing demand on the discovery and development of new pharmacologically active drugs to substitute the currently used anti-platelet aggregation drugs, the compounds therefore could be potential pharmacologically active anti-platelet aggregation agents in the management of blood-clotting related diseases.

6.2 RECOMMENDATIONS

i. We wish to recommend that the test compounds OA, OAA, BA, BAA, MAA, EU and EUA be incorporated into nano-carriers and their anti-platelet aggregation effects studied.

ii. The mechanism(s) through which the compounds exert therapeutic effects should be clearly elucidated.

iii. Other biological activities such as analgesic, antimicrobial effects of the compounds should be evaluated.

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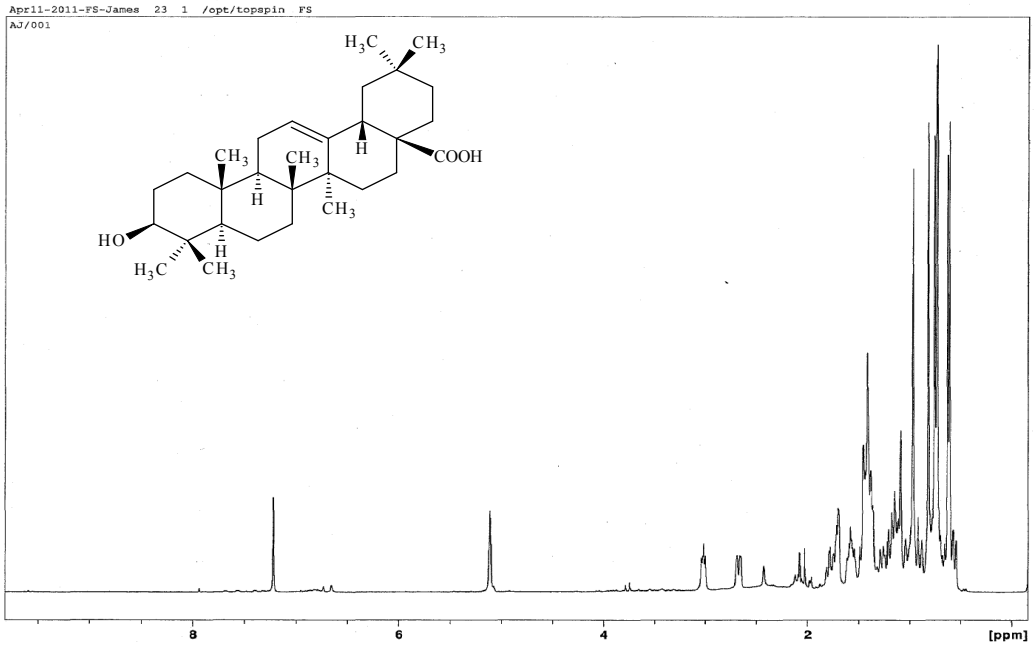
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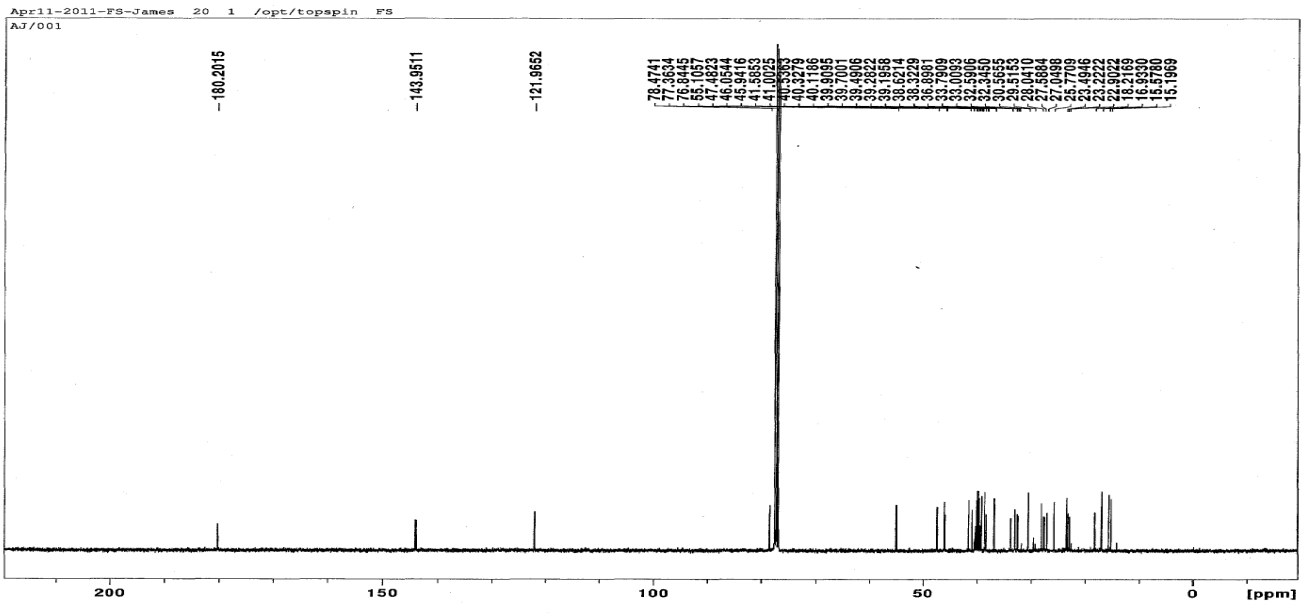
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APPENDICES

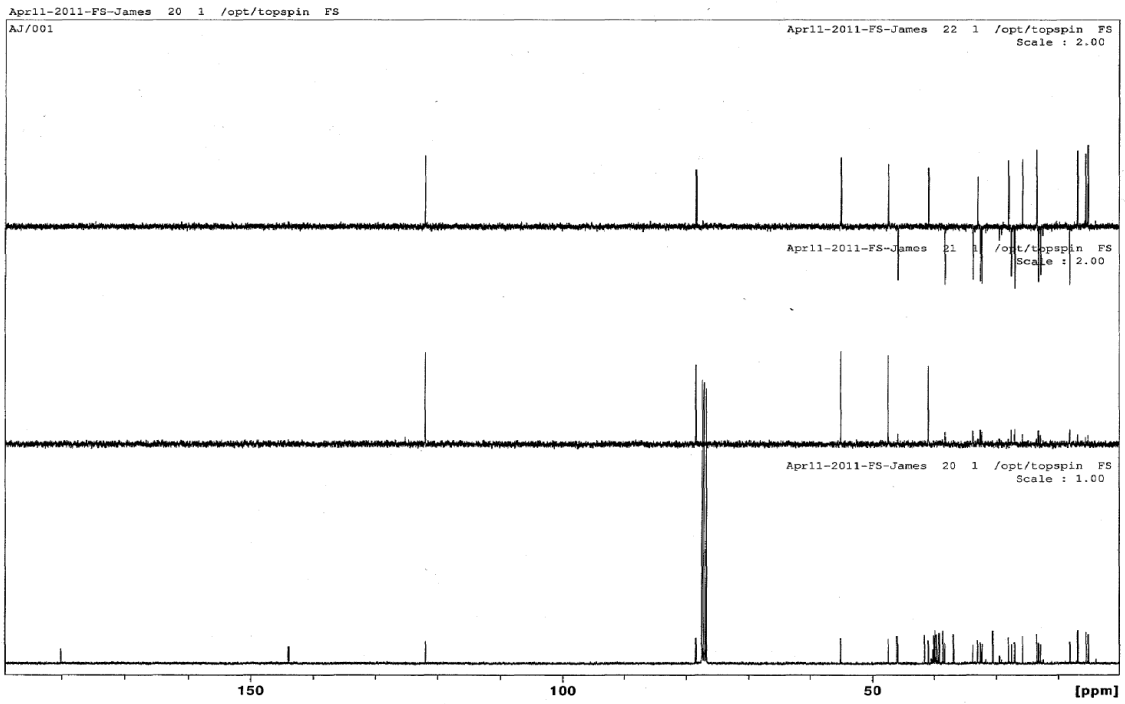
Appendix 1: H-NMR Spectrum of OA



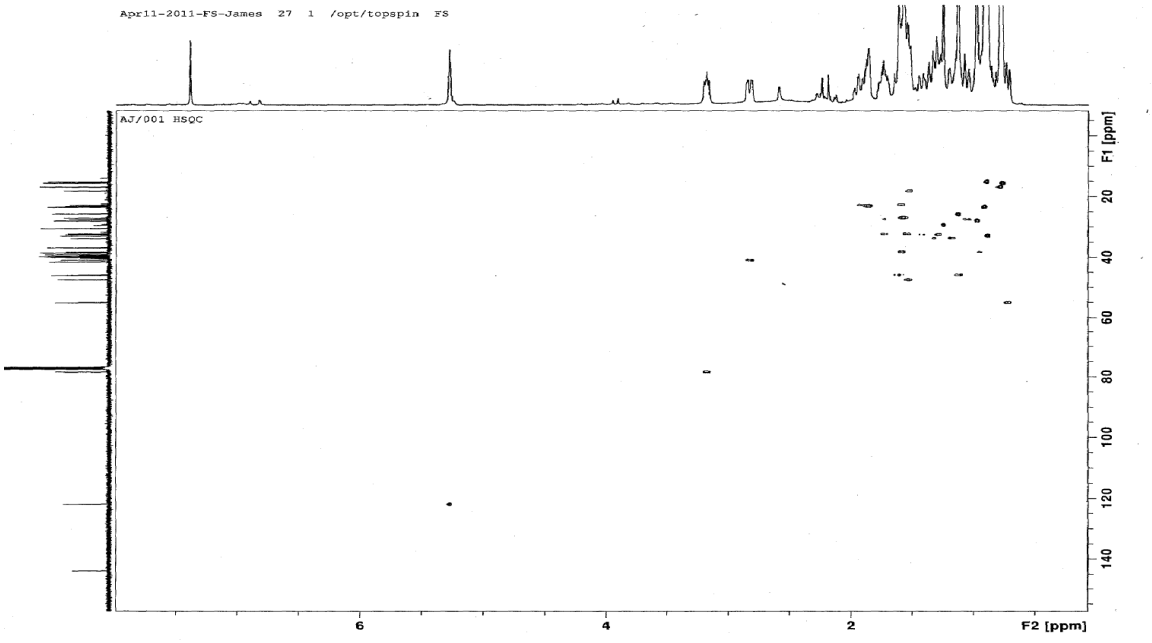
Appendix 2: C- NMR Spectrum of OA



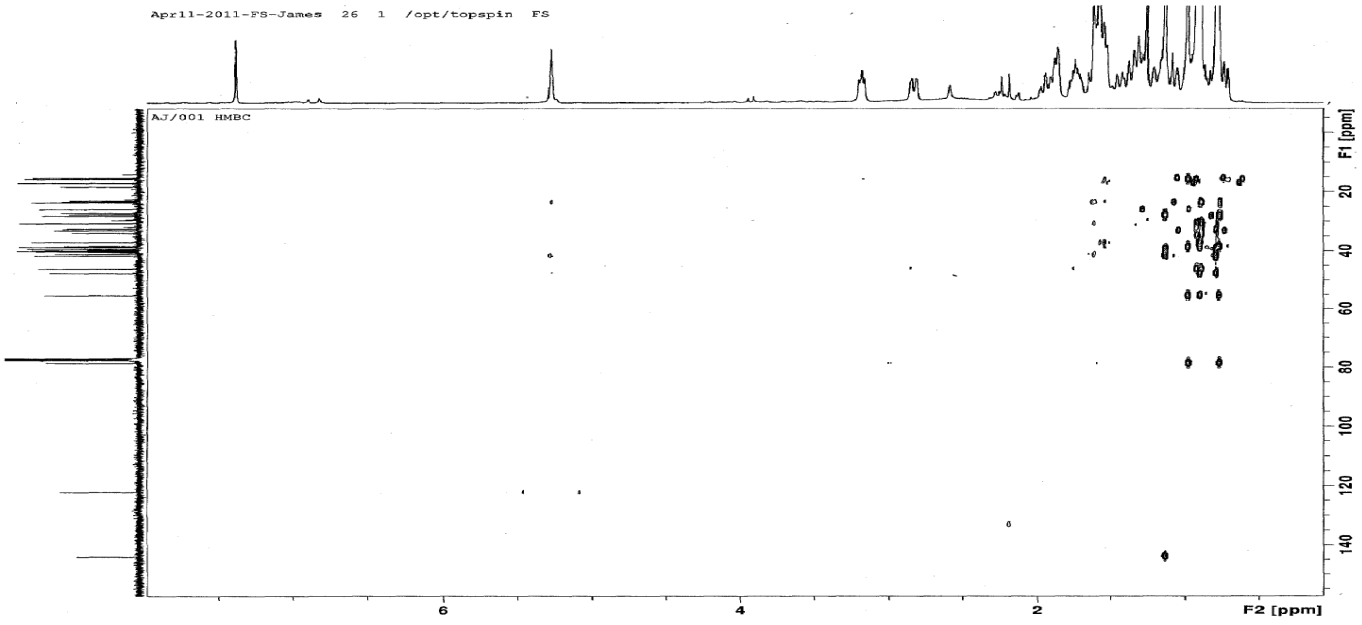
Appendix 3: C-NMR, DEPT 90 and DEPT 135 Spectrum of OA



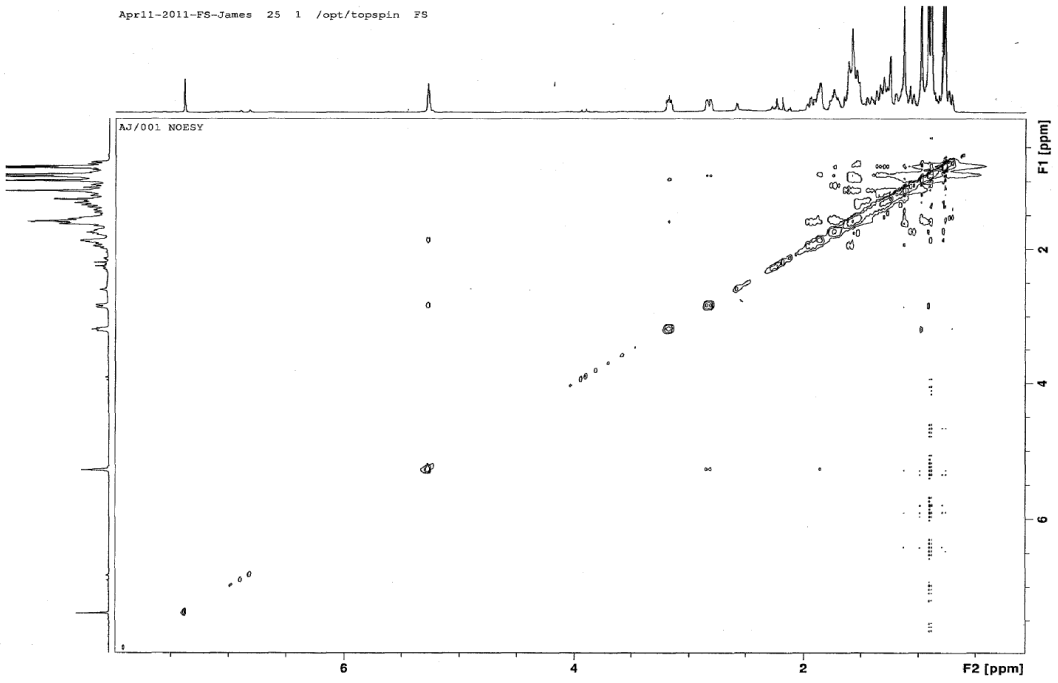
Appendix 4: HSQC-NMR Spectrum of OA



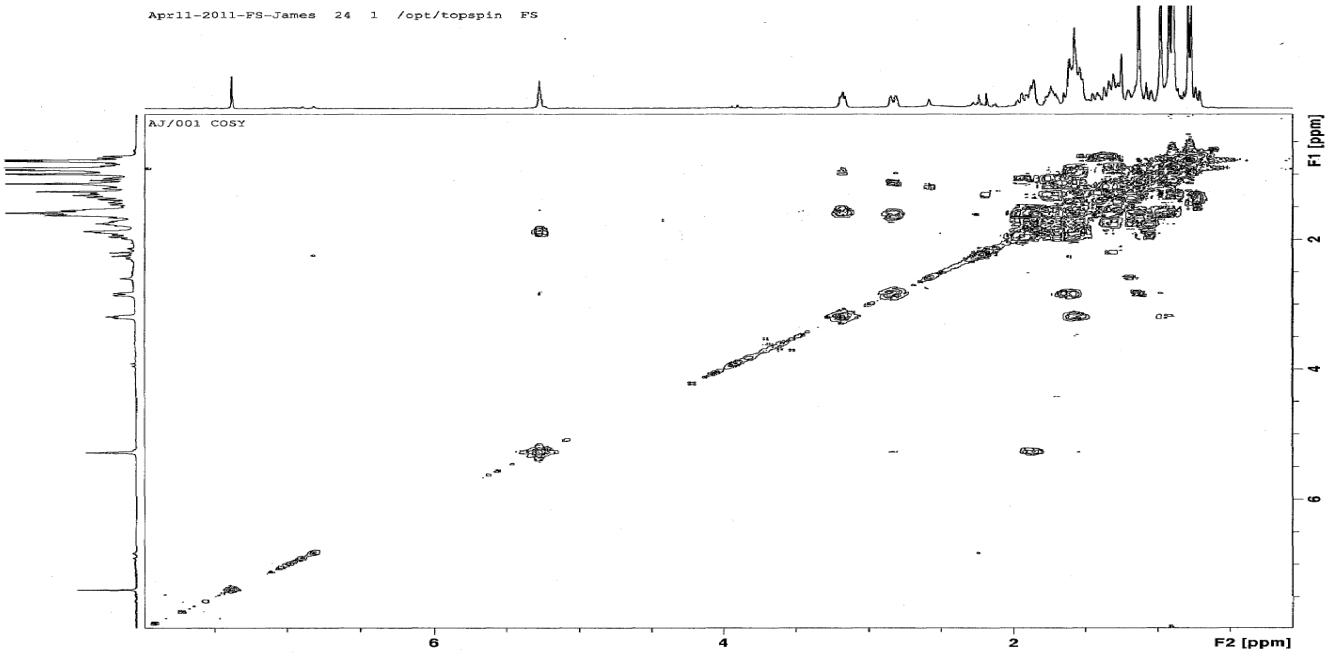
Appendix 5:HMBC-NMR Spectrum of OA



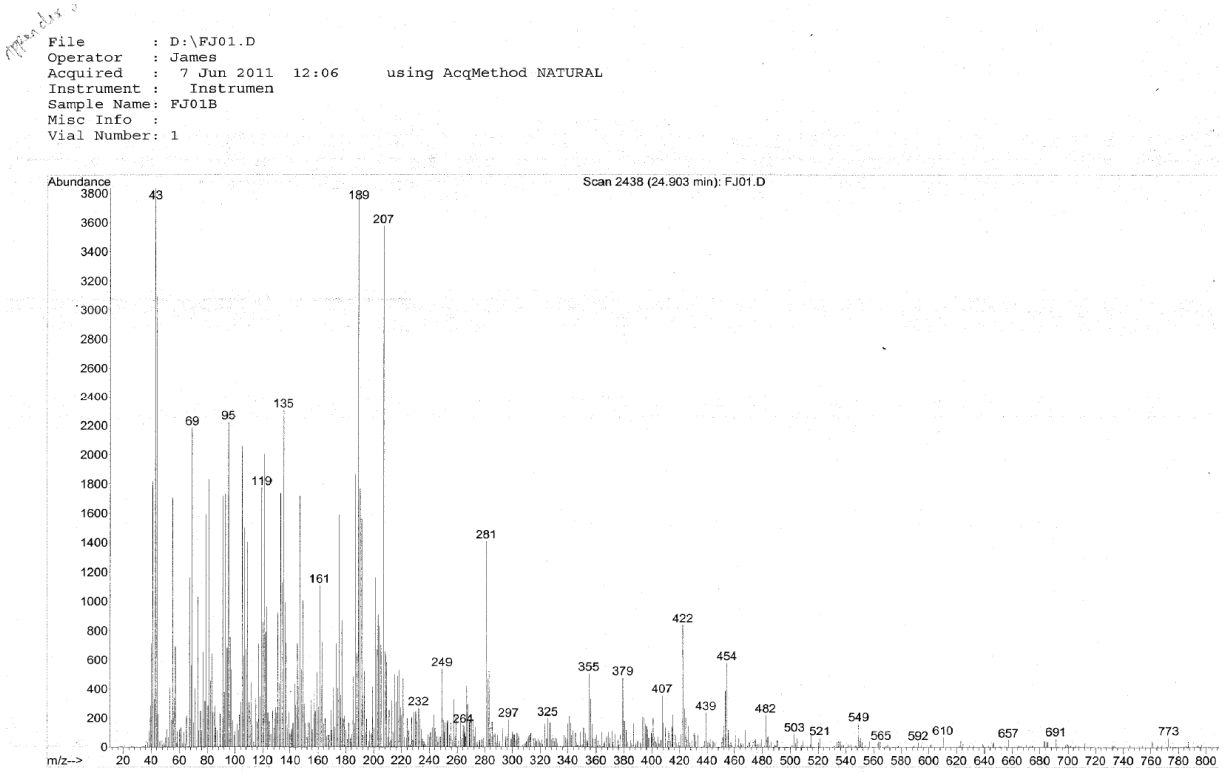
Appendix 6: NOESY-NMR Spectrum of OA



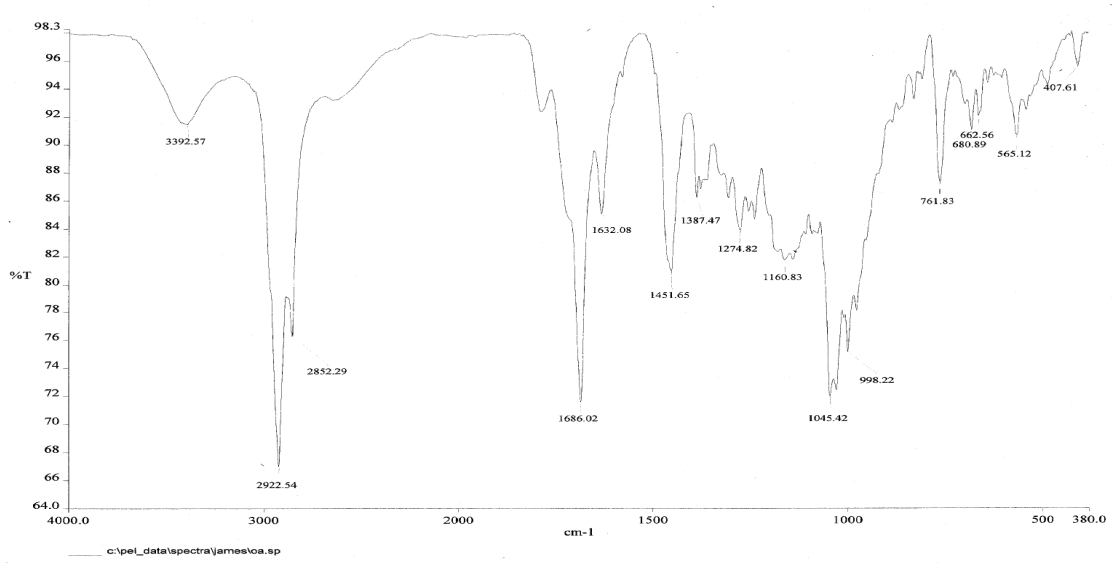
APPENDIX 7: COSY-NMR Spectrum of OA



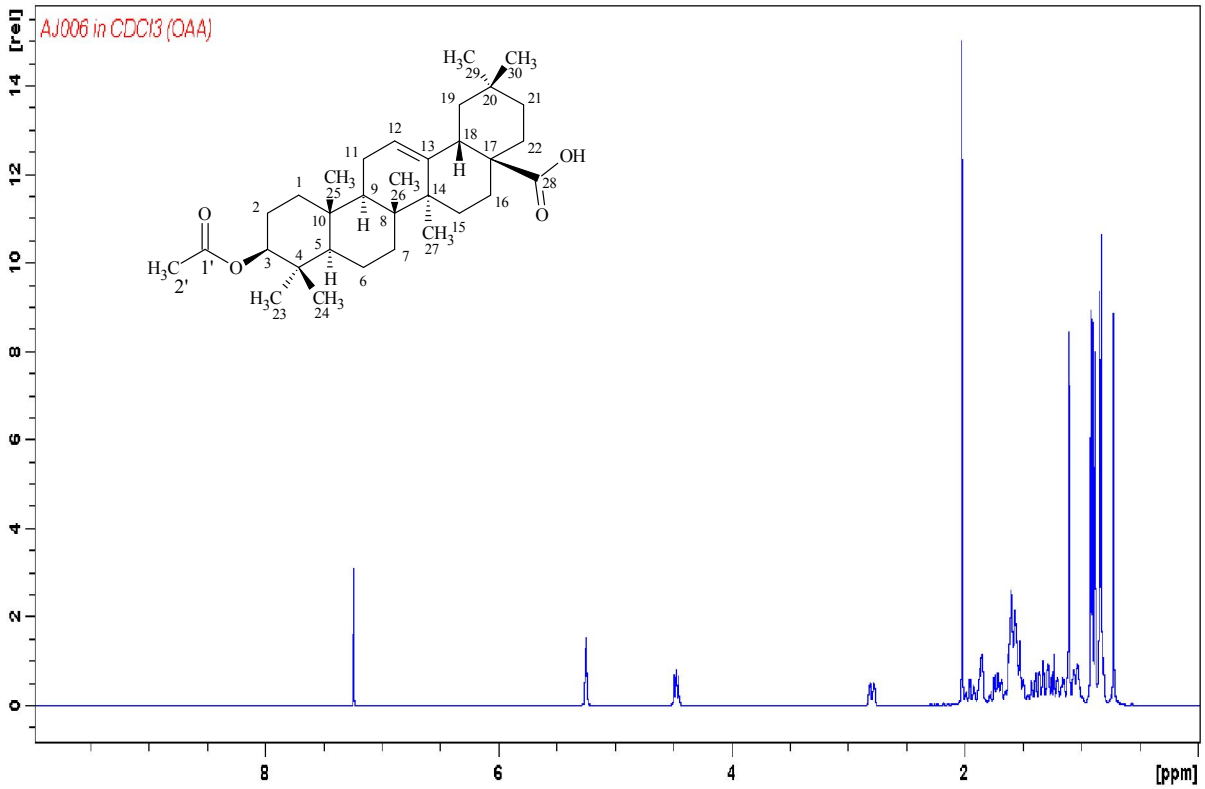
Appendix 8: EI-MS Spectrum of OA



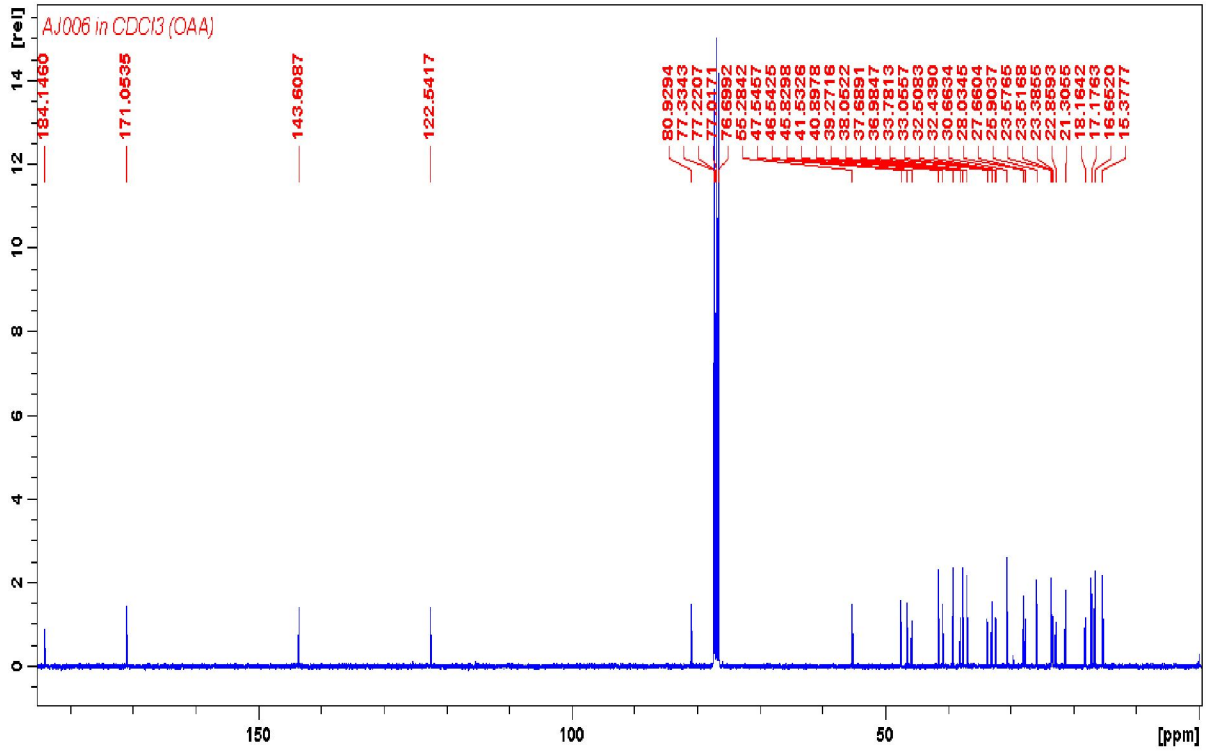
Appendix 9: IR Spectrum of OA



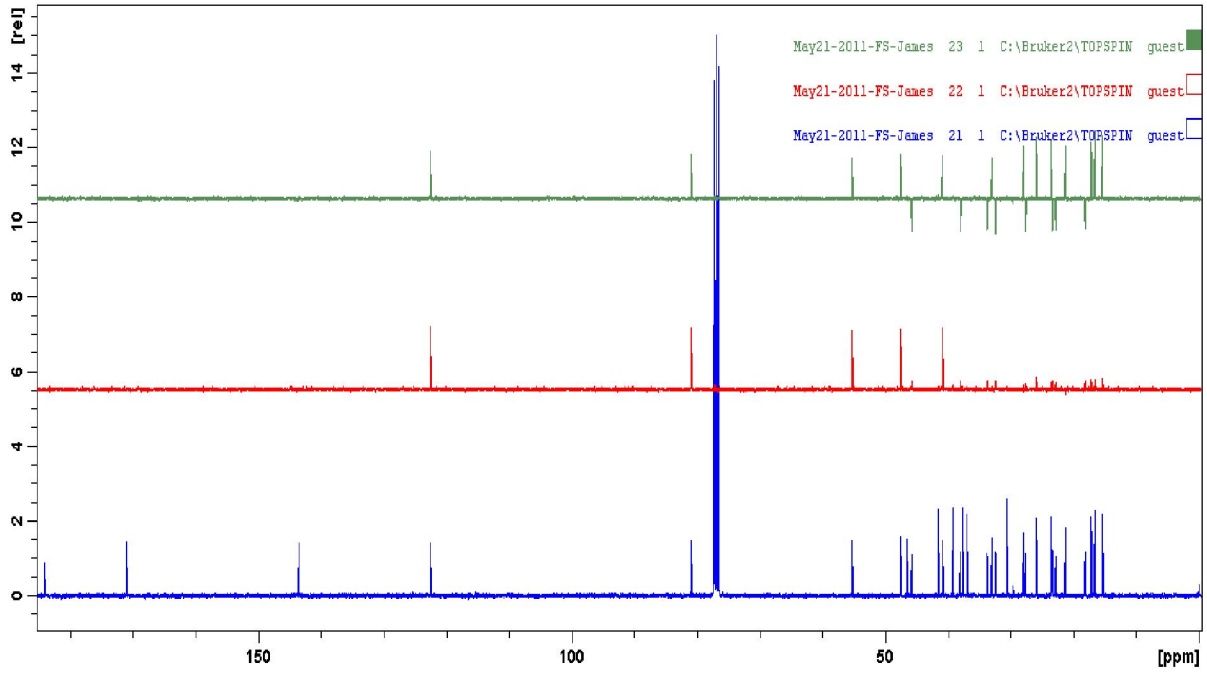
Appendix 10: H-NMR Spectrum of OAA



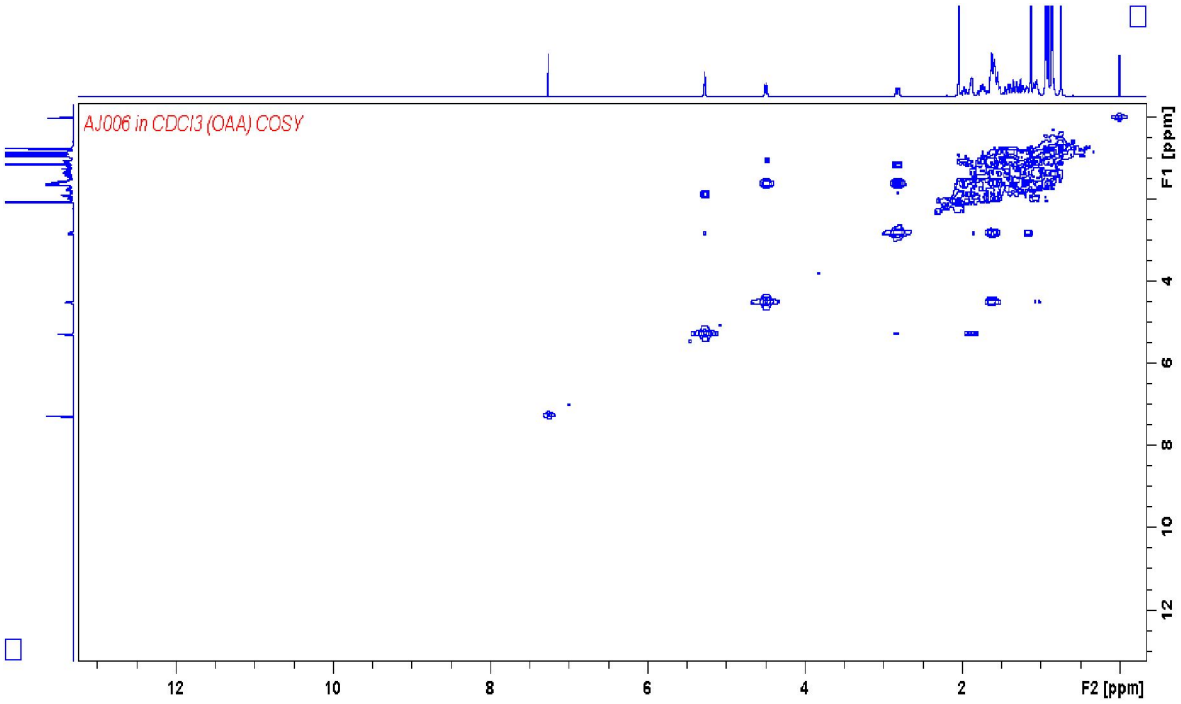
Appendix 11: C- NMR Spectrum of OAA



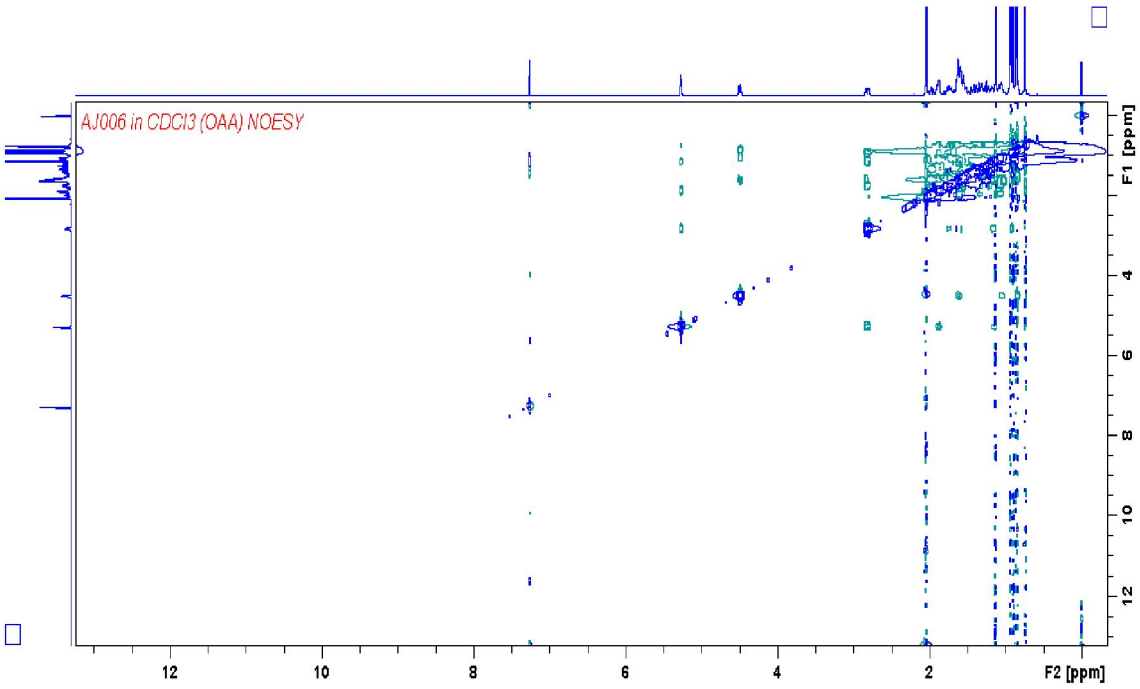
Appendix 12: C-NMR, DEPT 90 and DEPT 135 Spectrum of OAA



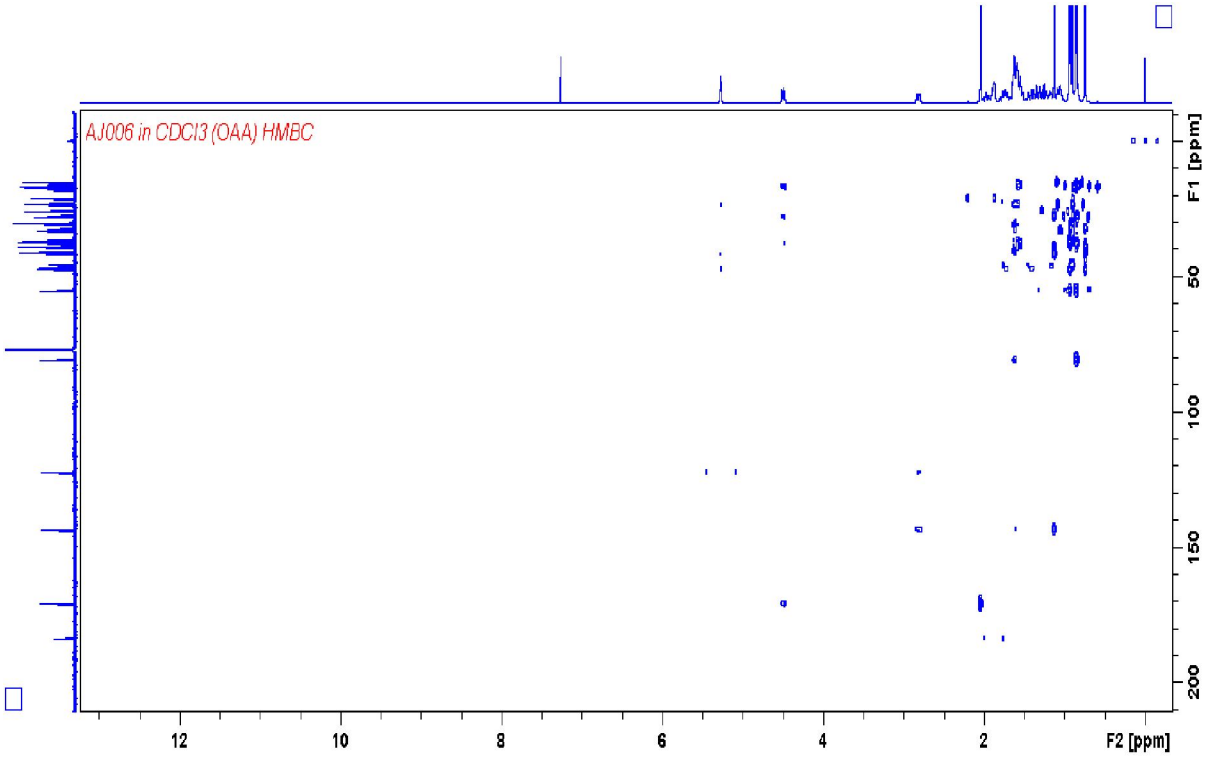
Appendix 13: COSY-NMR Spectrum of OAA



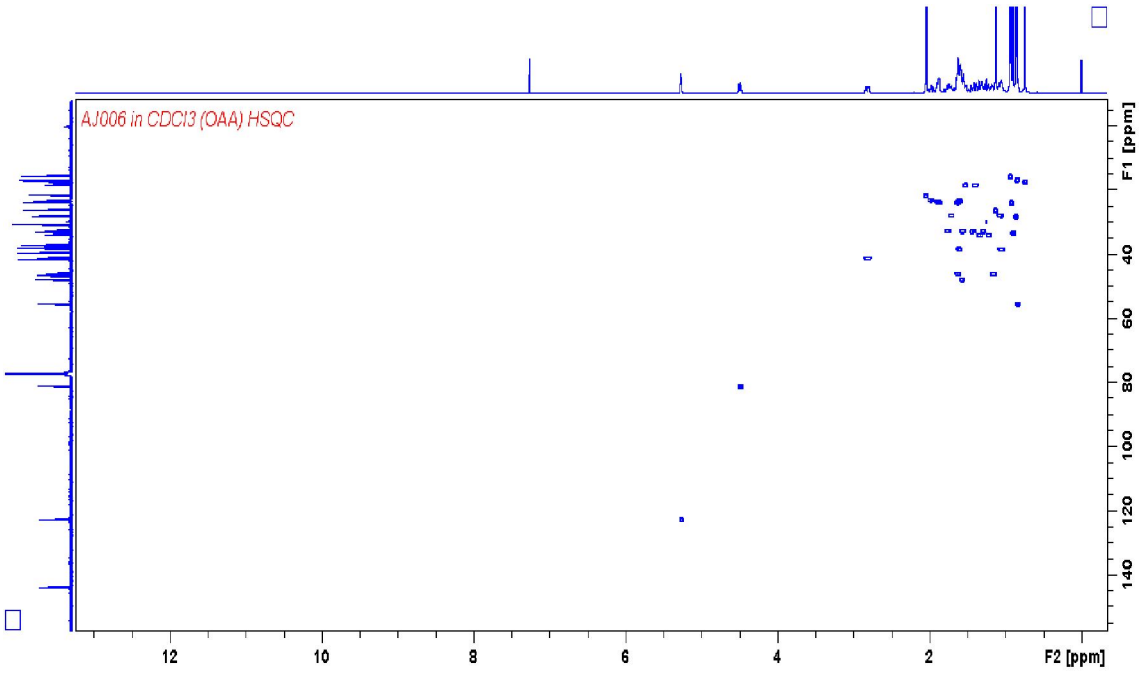
Appendix 14: NOESY-NMR Spectrum of OAA



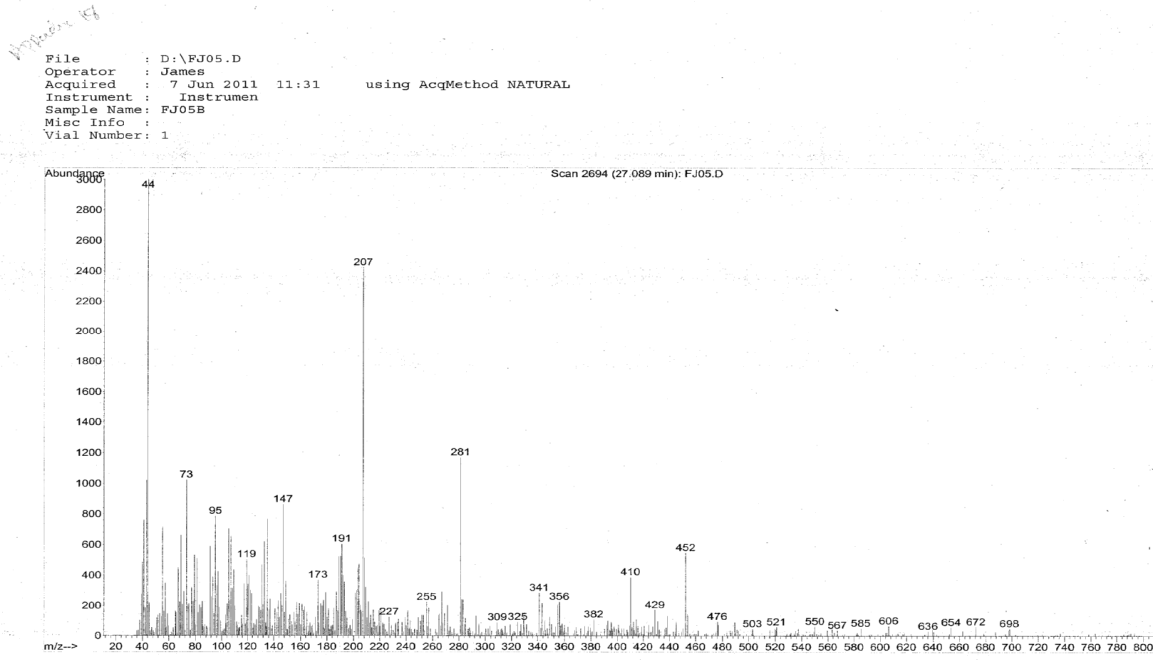
Appendix 15:HMBC-NMR Spectrum of OAA



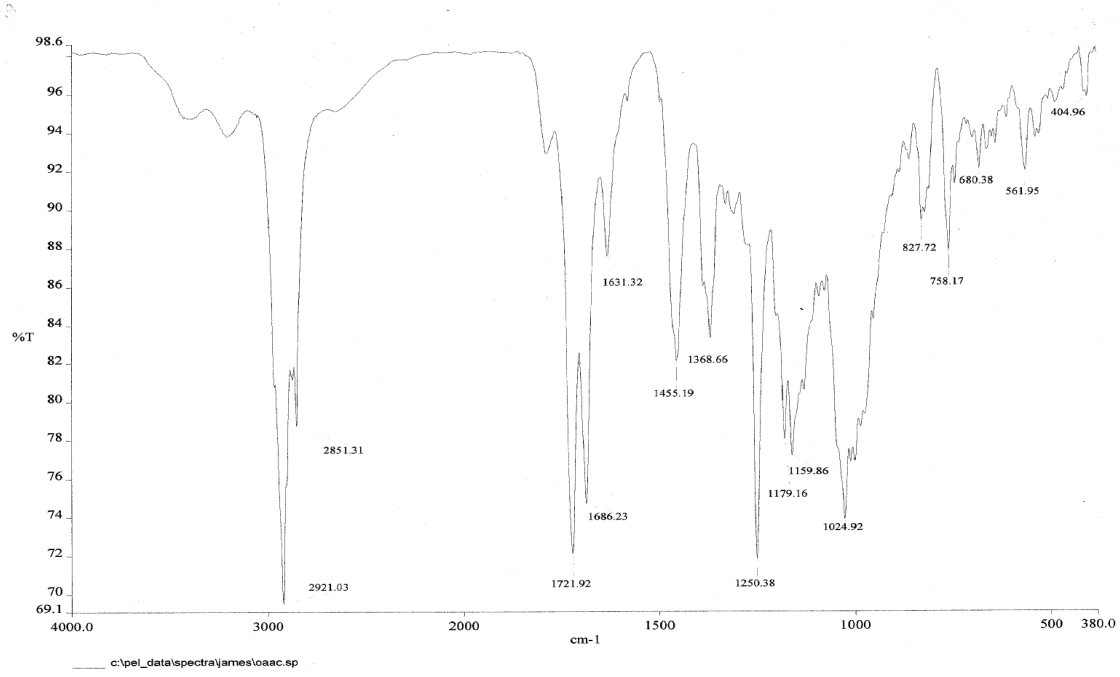
Appendix 16: HSQC-NMR Spectrum of OAA



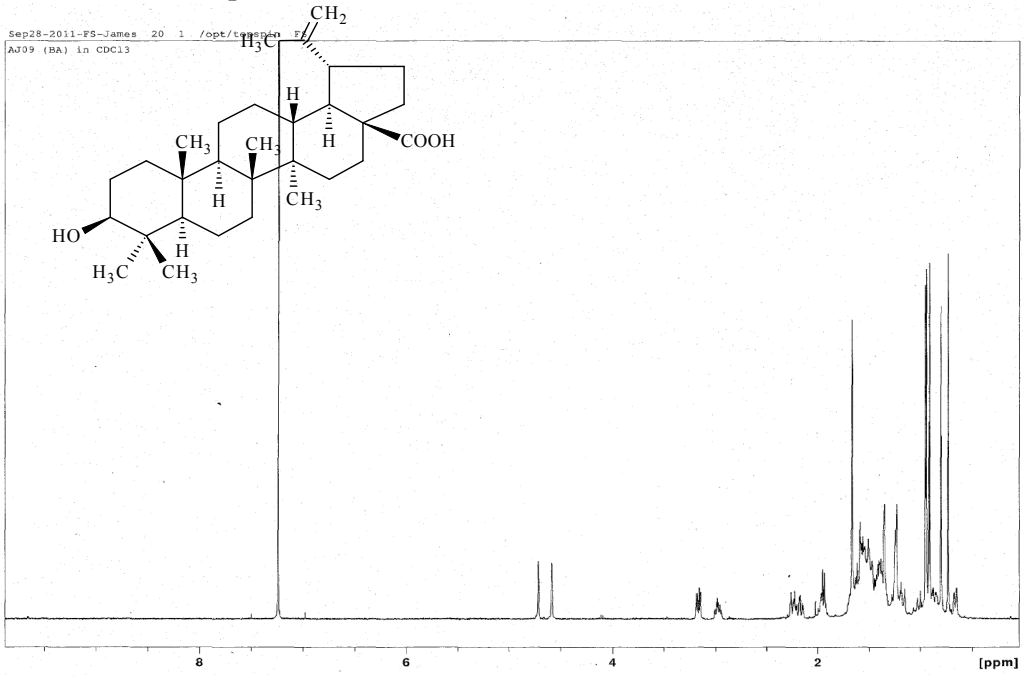
Appendix 17: EI-MS Spectrum of OAA



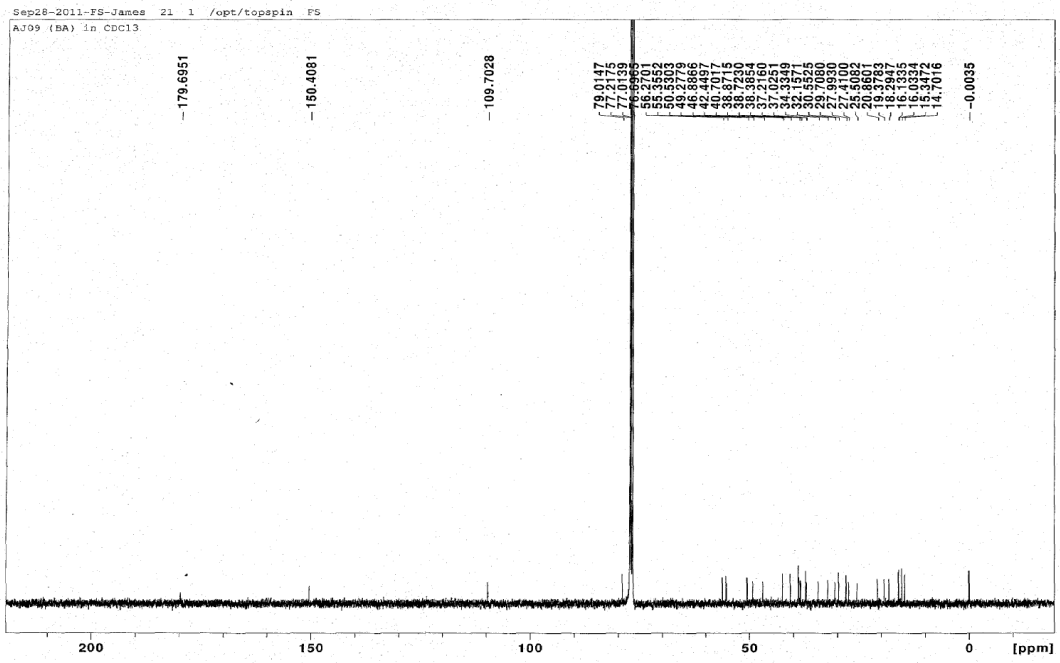
Appendix 18: IR Spectrum of OAA



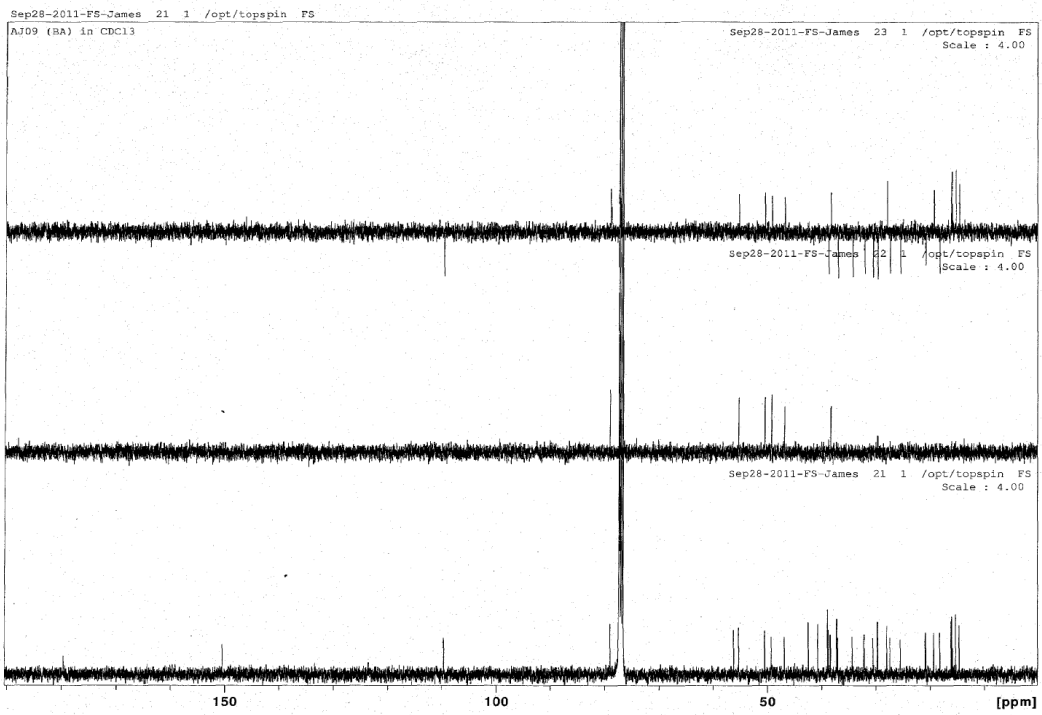
Appendix 19: H-NMR Spectrum of BA



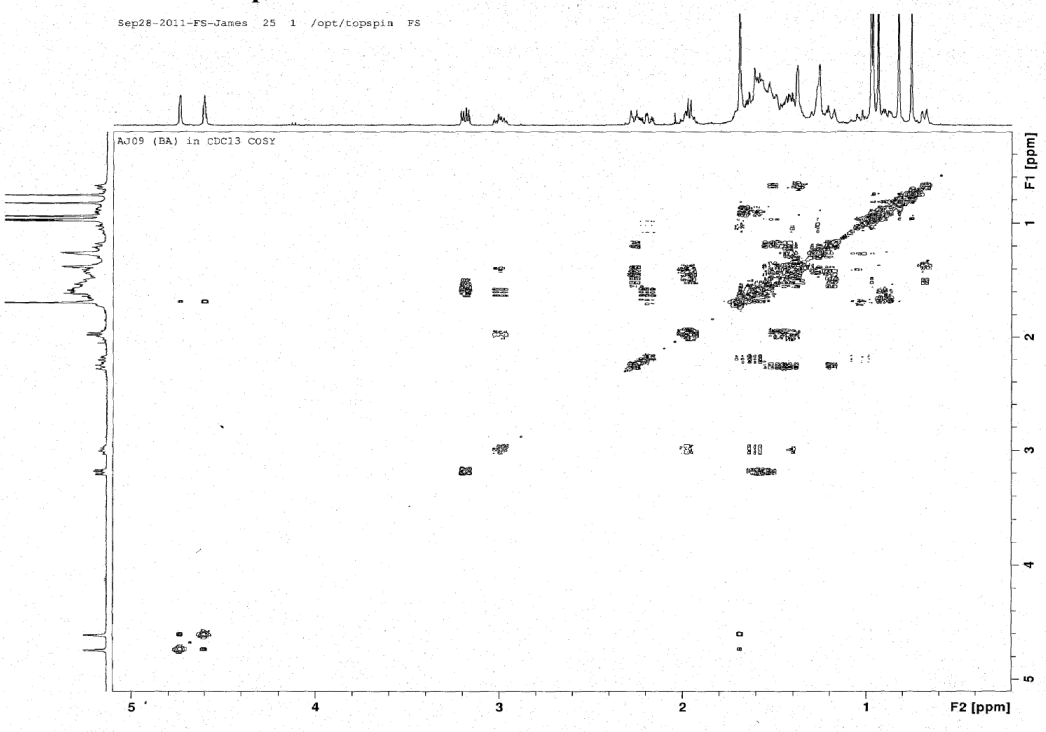
Appendix 20: C- NMR Spectrum of BA



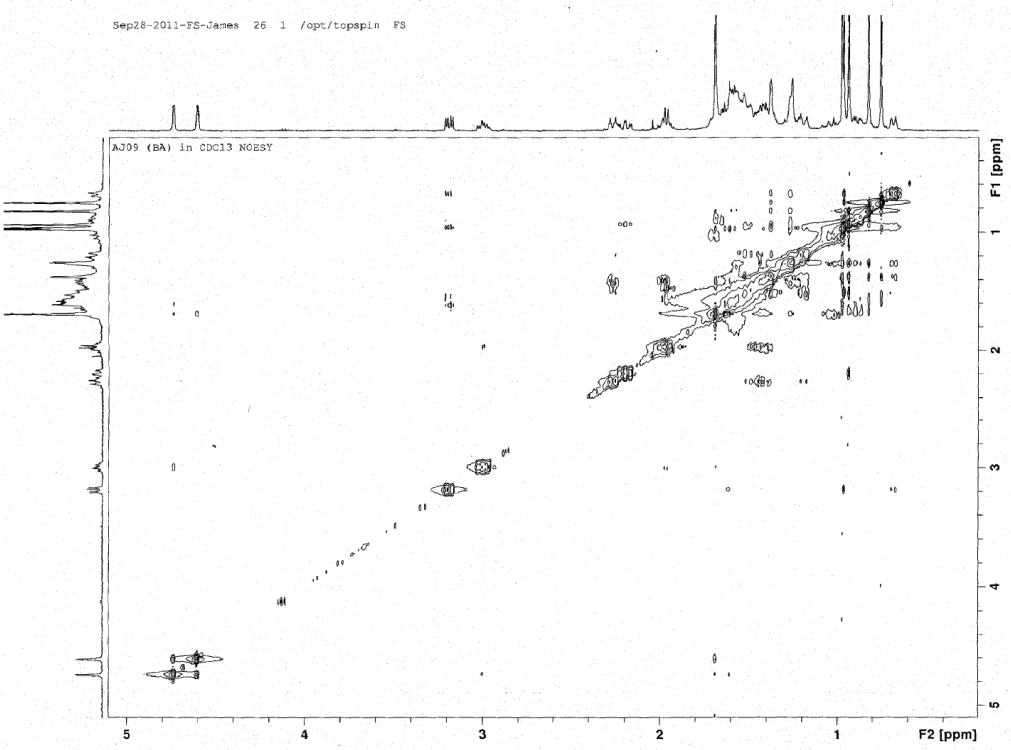
Appendix 21: C-NMR, DEPT 90 and DEPT 135 Spectrum of BA



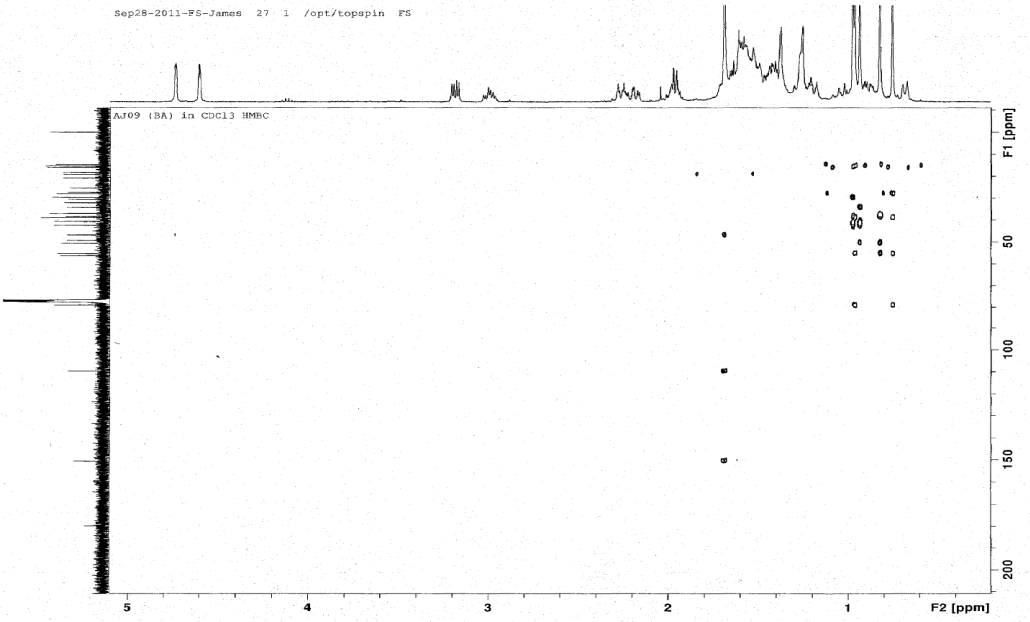
Appendix 22: COSY-NMR Spectrum of BA



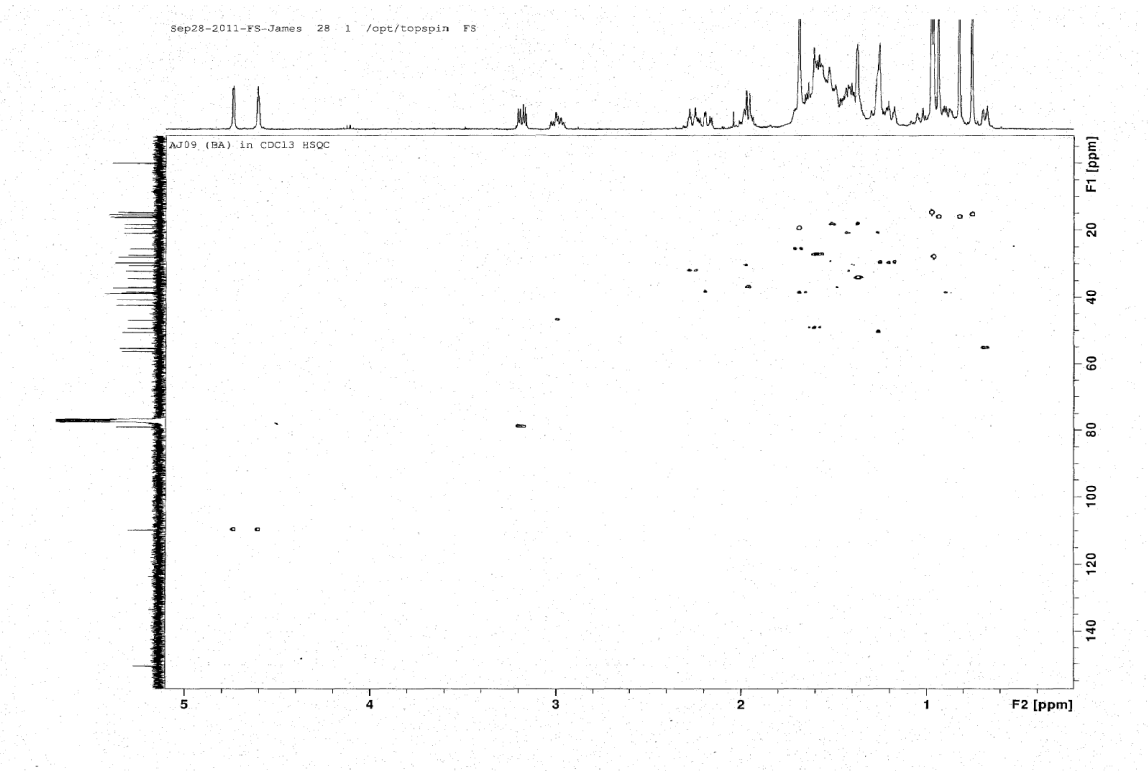
Appendix 23: NOESY-NMR Spectrum of BA



APPENDIX 24:HMBC-NMR Spectrum of BA



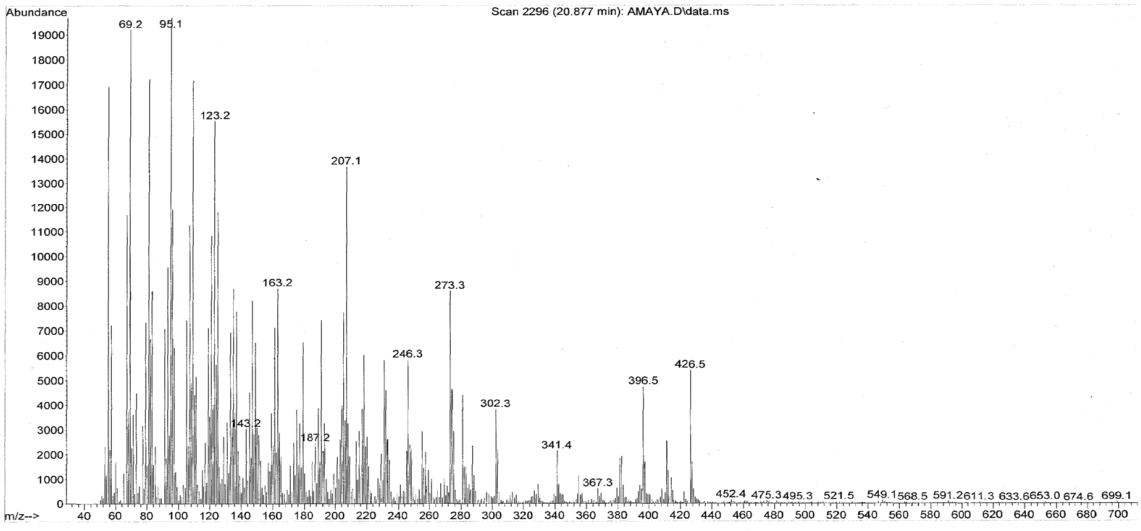
Appendix 25: HSQC-NMR Spectrum of BA



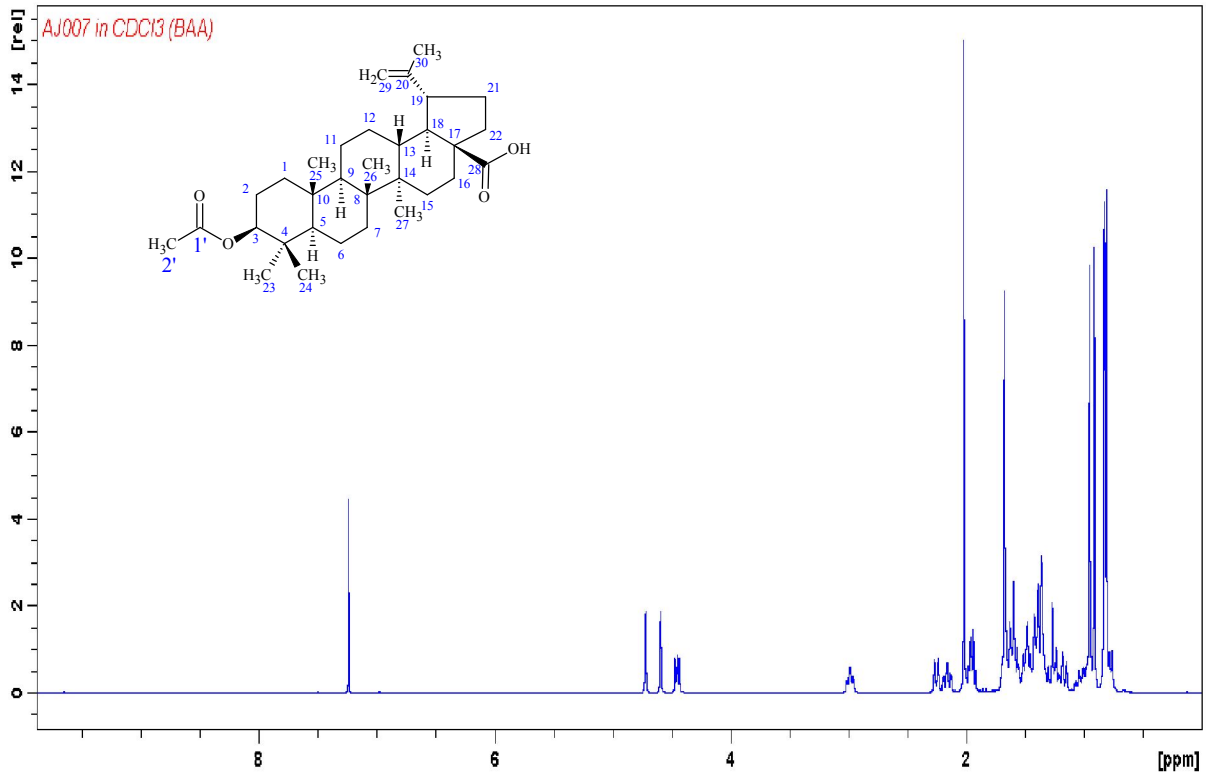
Appendix 26: EI-MS Spectrum of BA

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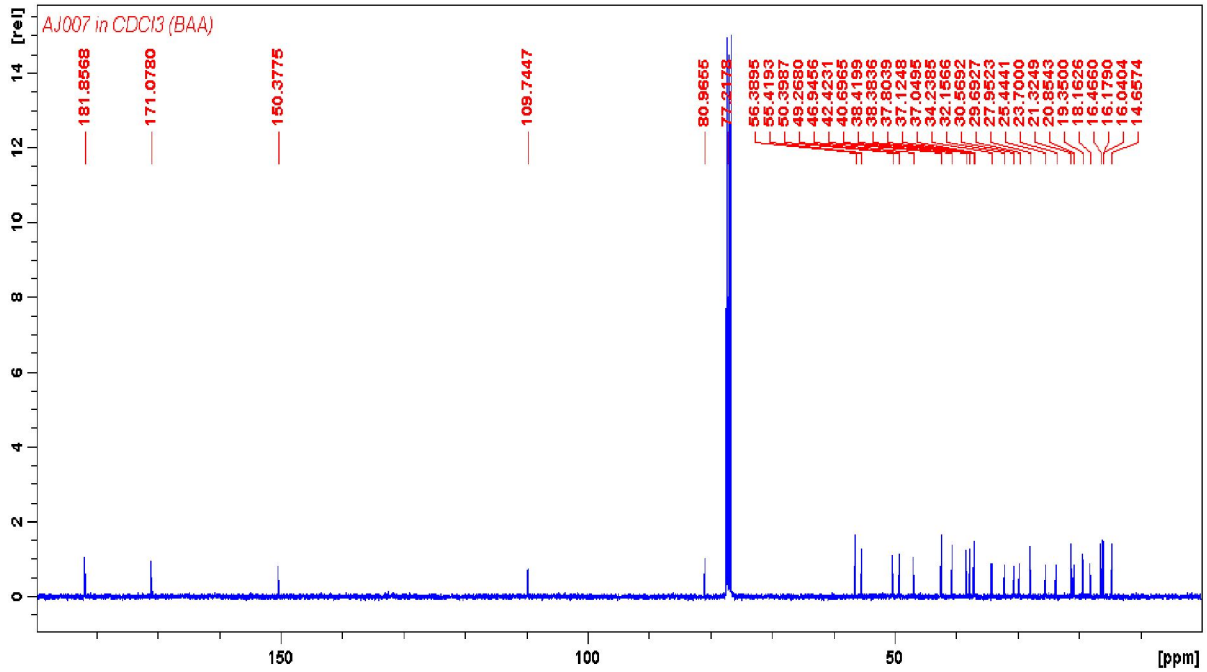
File :C:\msdchem\1\data\James\AMAYA.D
Operator : NB
Acquired : 9 Dec 2011 15:26 using AcqMethod NATPRODUCTS MANUAL INJ.M
Instrument : 5973N
Sample Name: BA
Misc Info :
Vial Number: 1



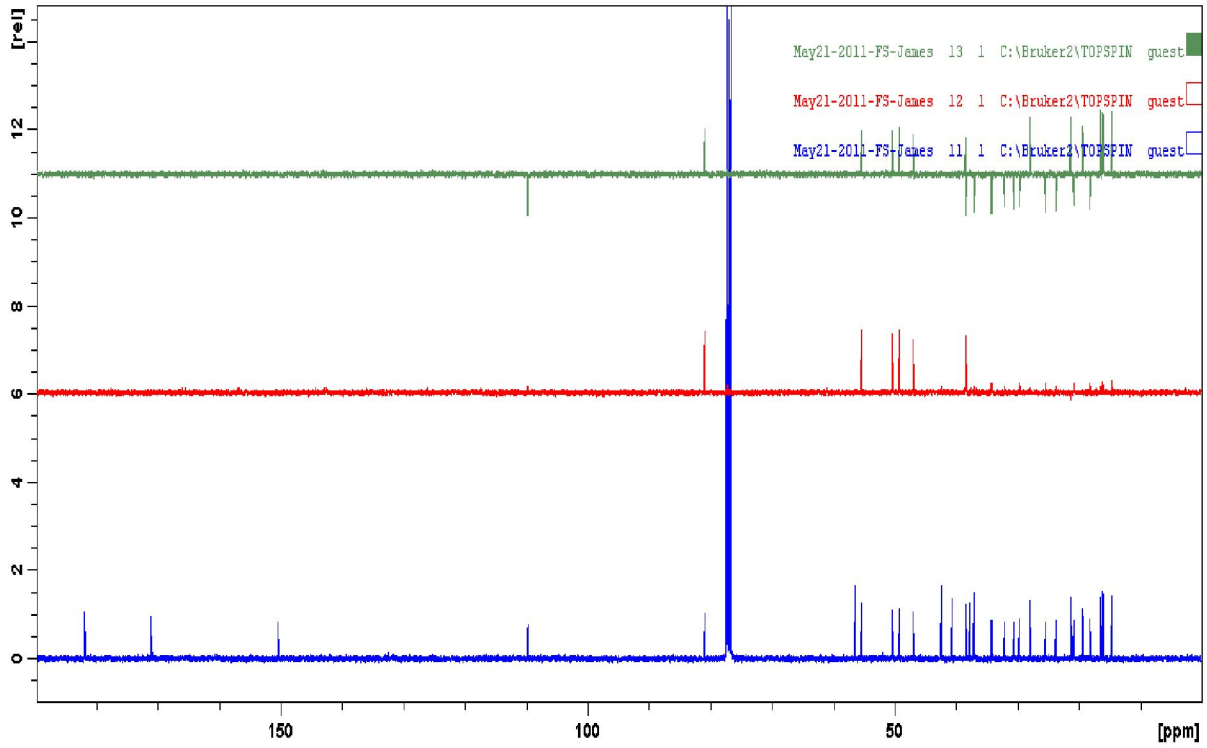
Appendix 27 : H-NMR Spectrum of BAA



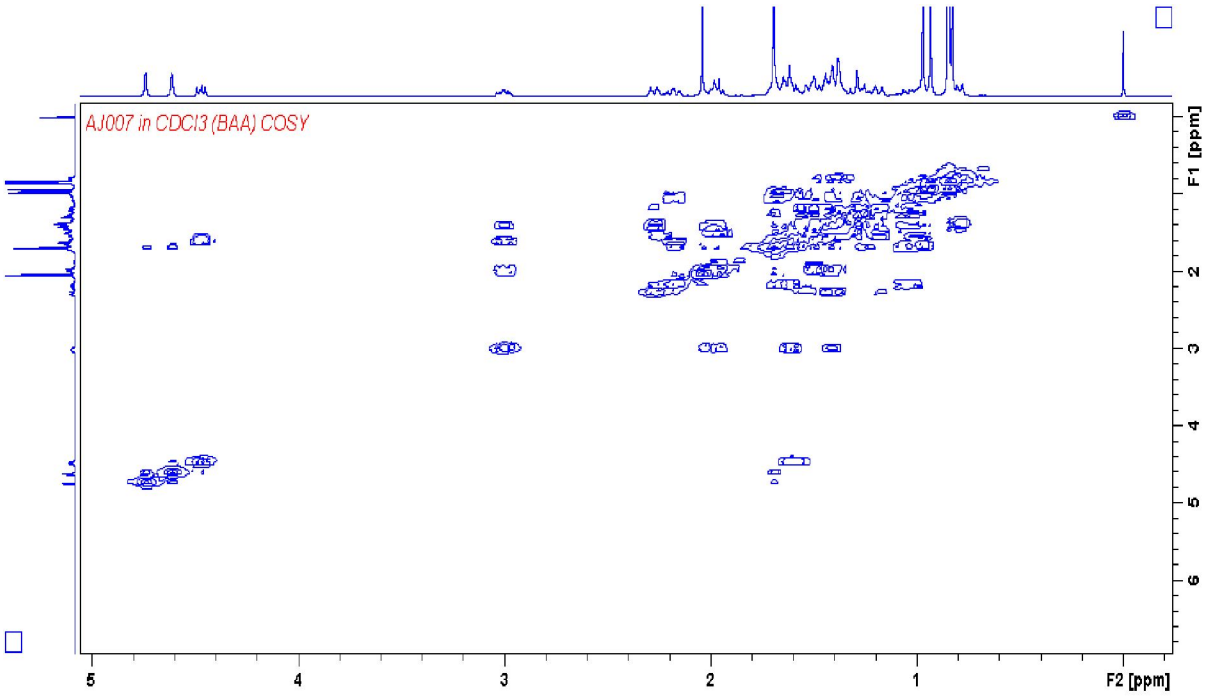
Appendix 28: C- NMR Spectrum of BAA



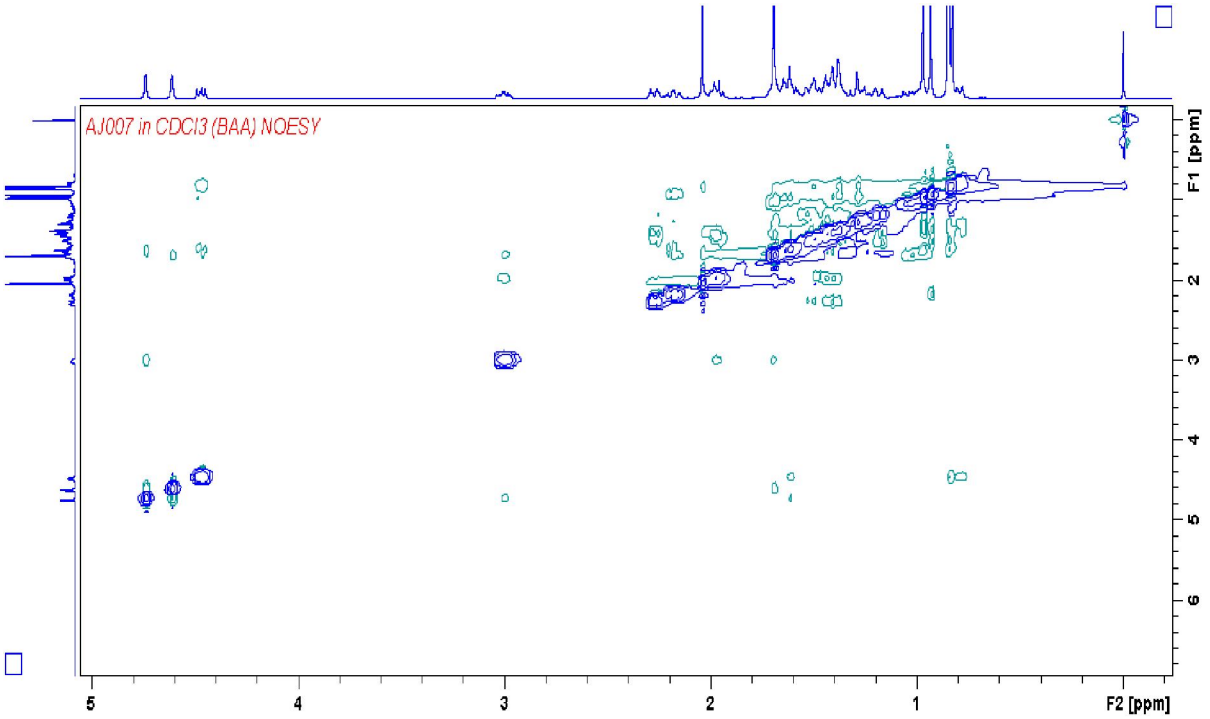
Appendix 29: C-NMR, DEPT 90 and DEPT 135 Spectrum of BAA



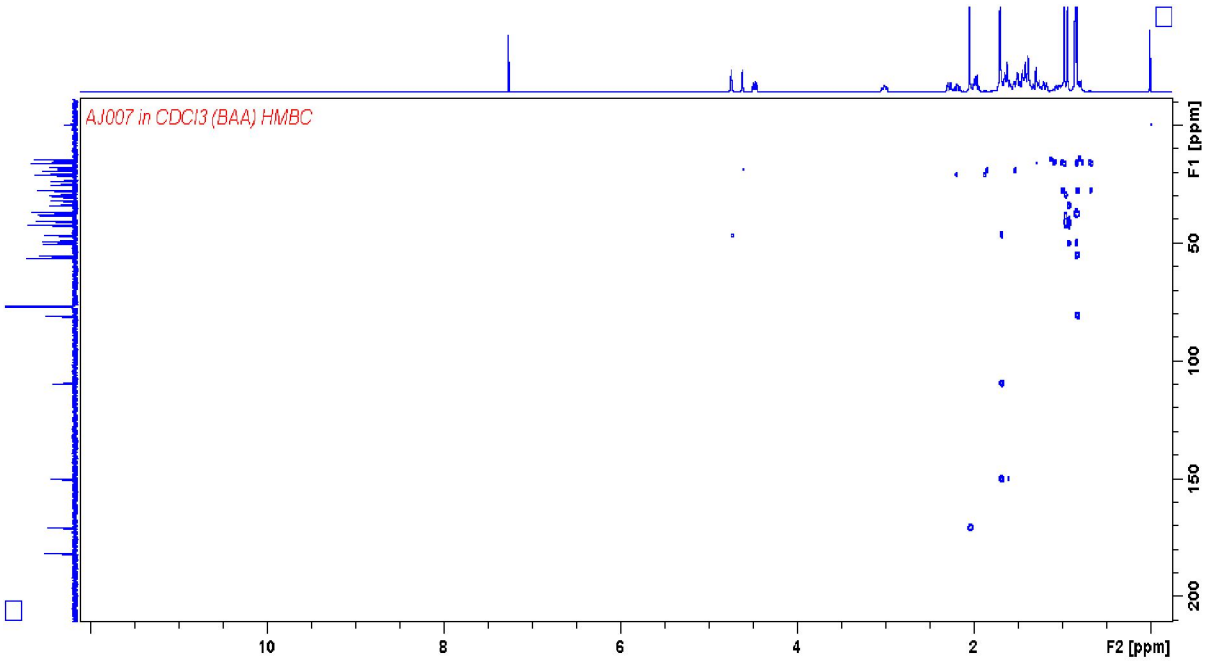
Appendix 30: COSY-NMR Spectrum of BAA



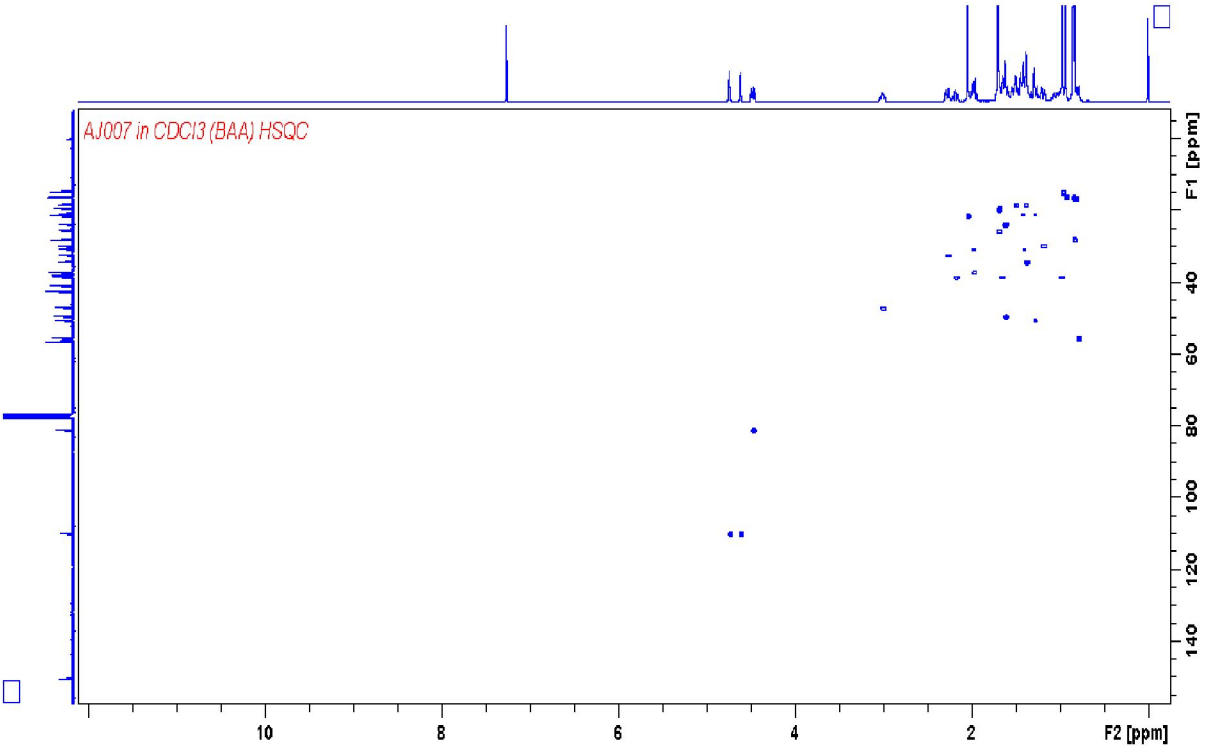
Appendix 31: NOESY-NMR Spectrum of BAA



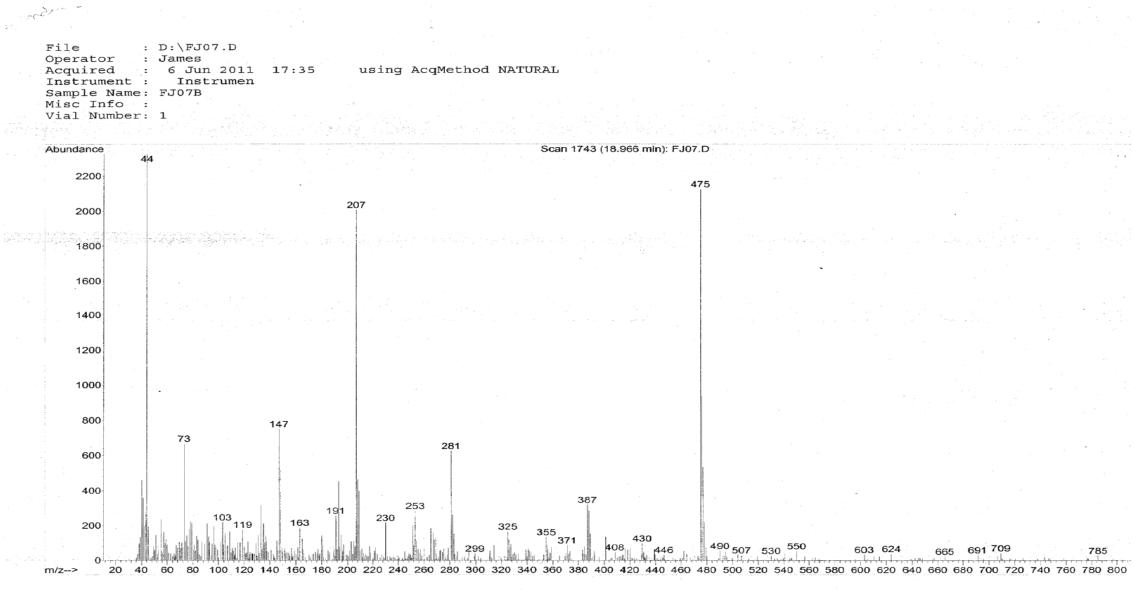
Appendix 32:HMBC-NMR Spectrum of BAA



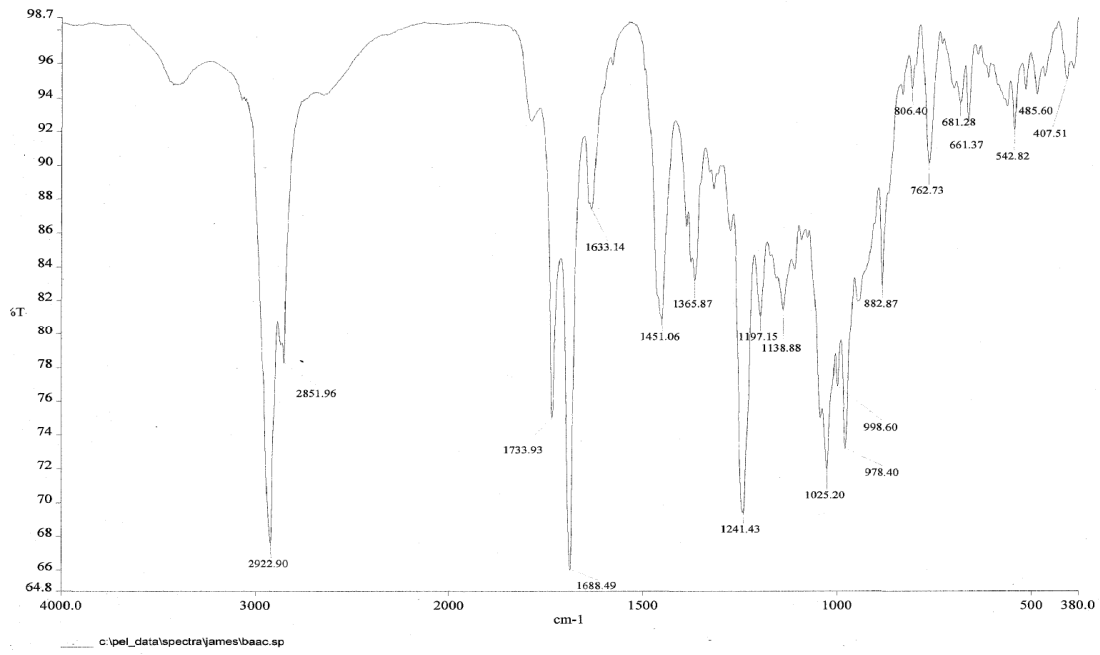
Appendix 33: HSQC-NMR Spectrum of BAA



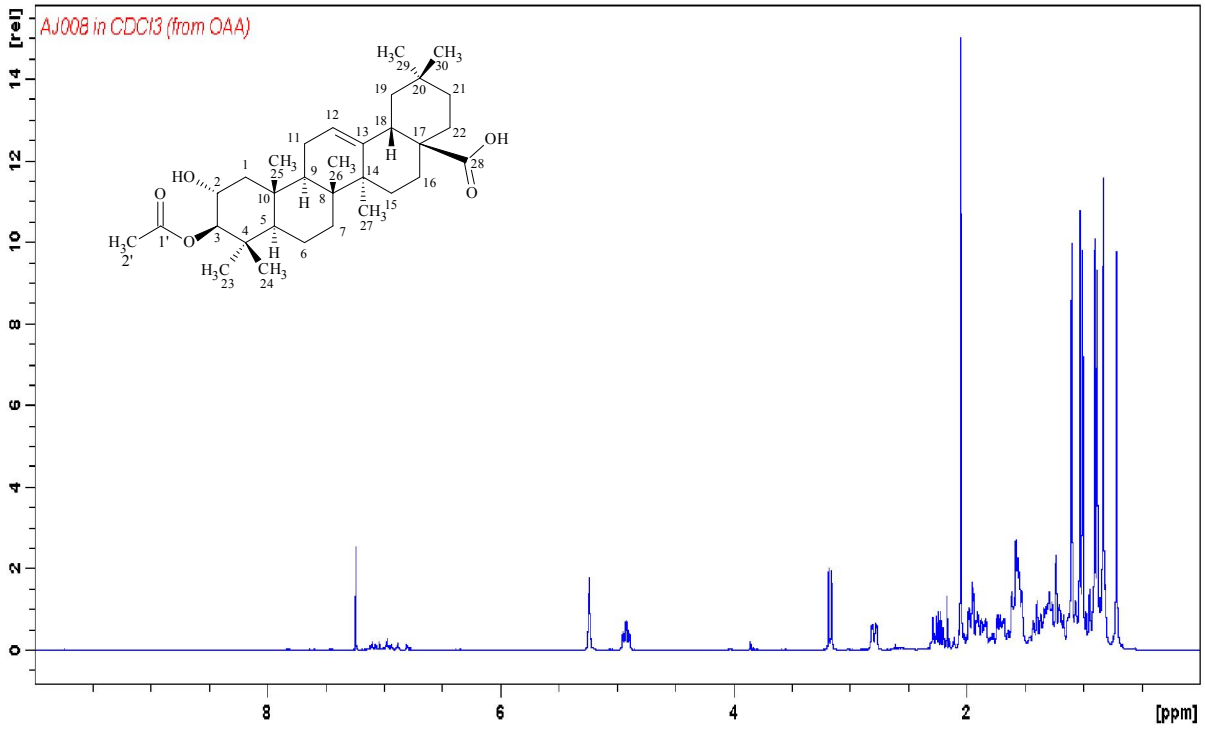
Appendix 34: EI-MS Spectrum of BAA



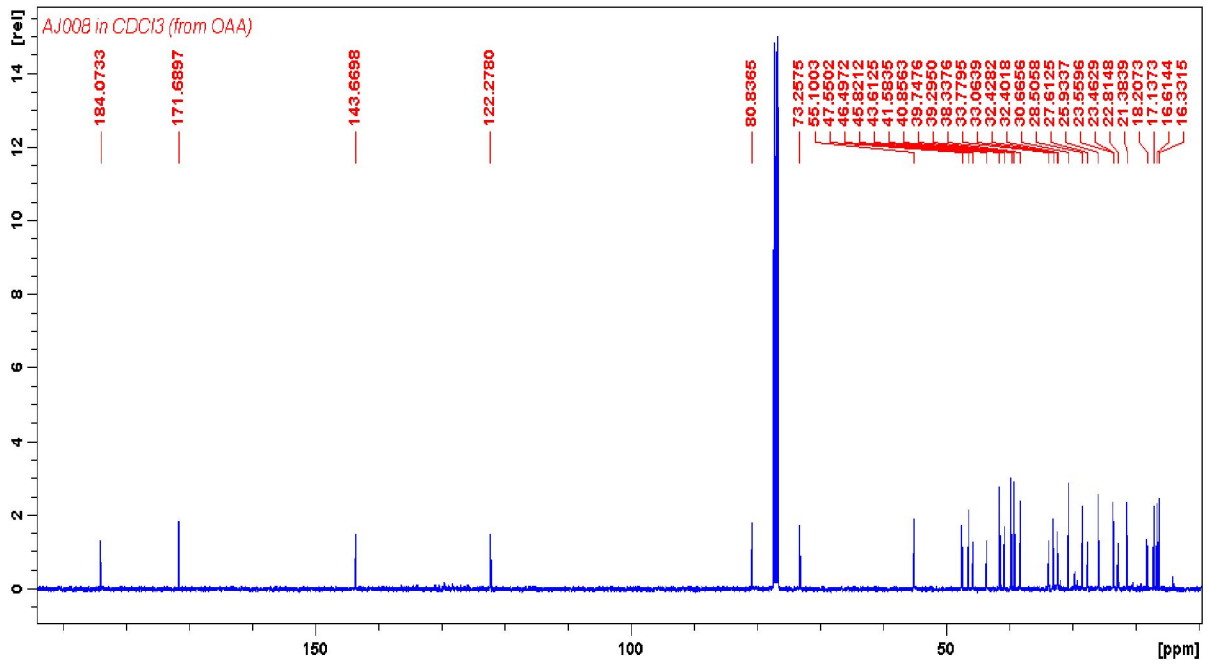
Appendix 35: IR Spectrum of BAA



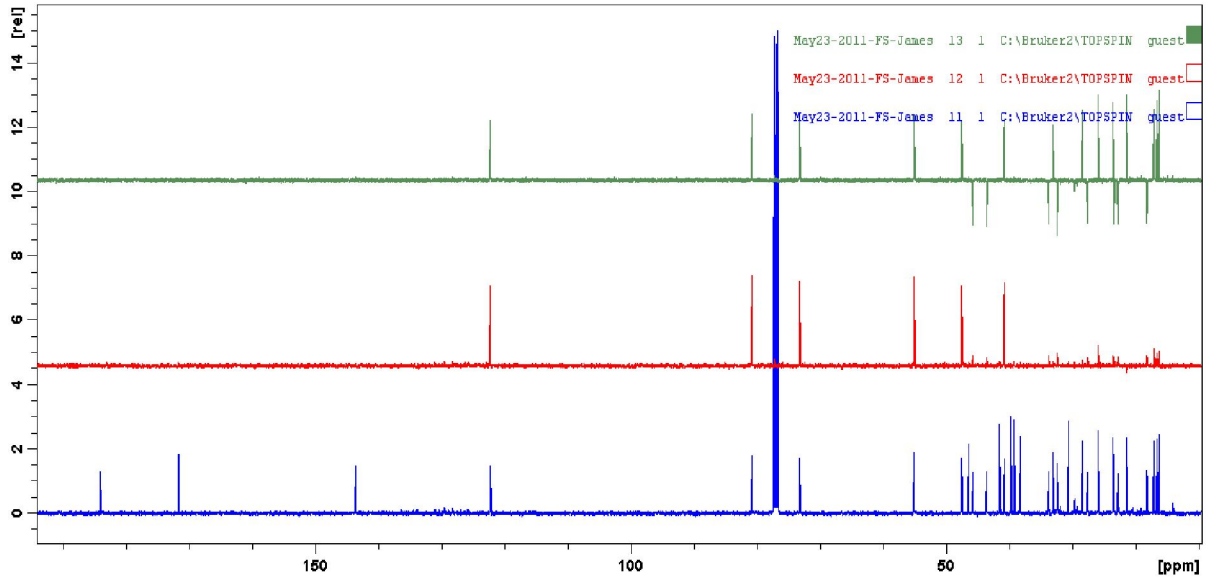
Appendix 36: H-NMR Spectrum of MAA



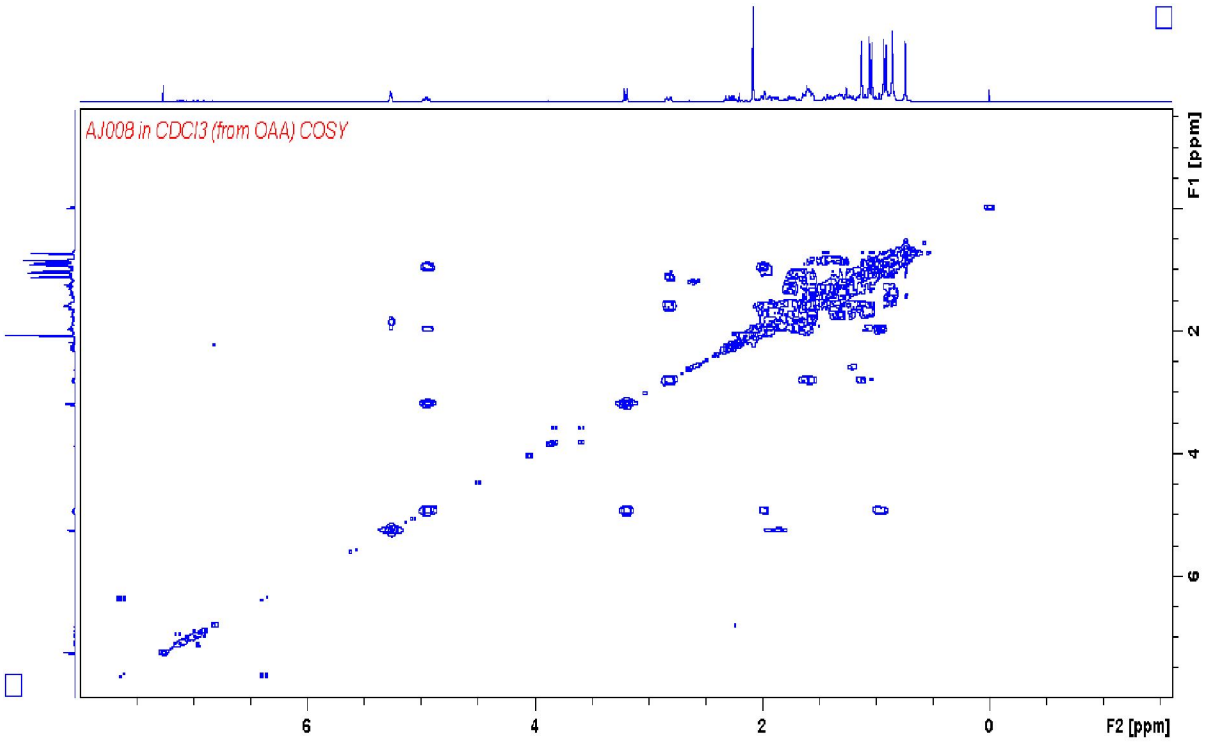
Appendix 37: C- NMR Spectrum of MAA



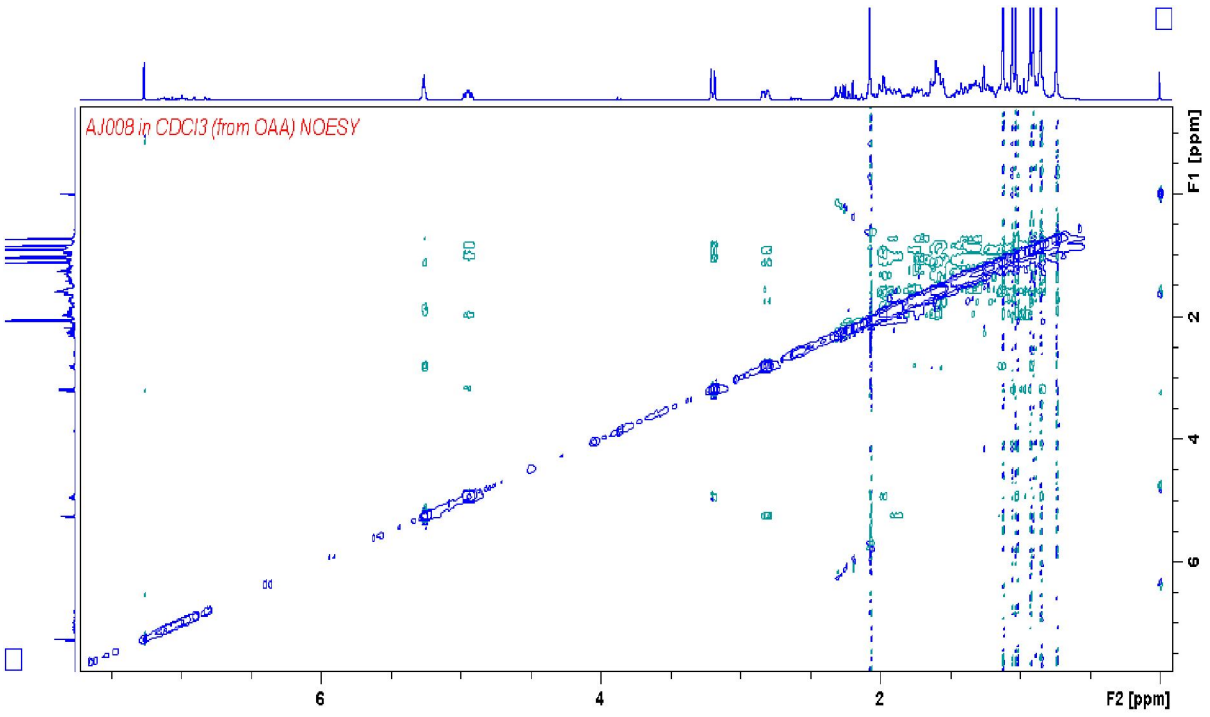
Appendix 38: C-NMR, DEPT 90 and DEPT 135 Spectrum of MAA



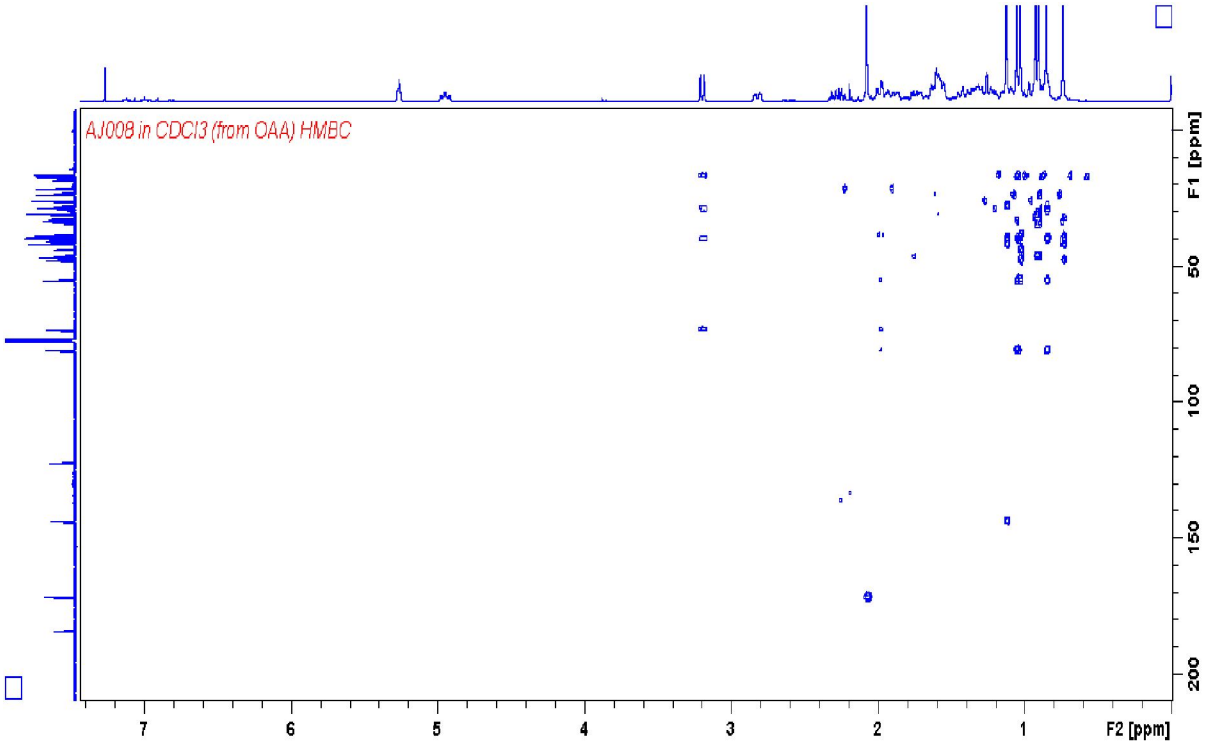
Appendix 39: COSY-NMR Spectrum of MAA



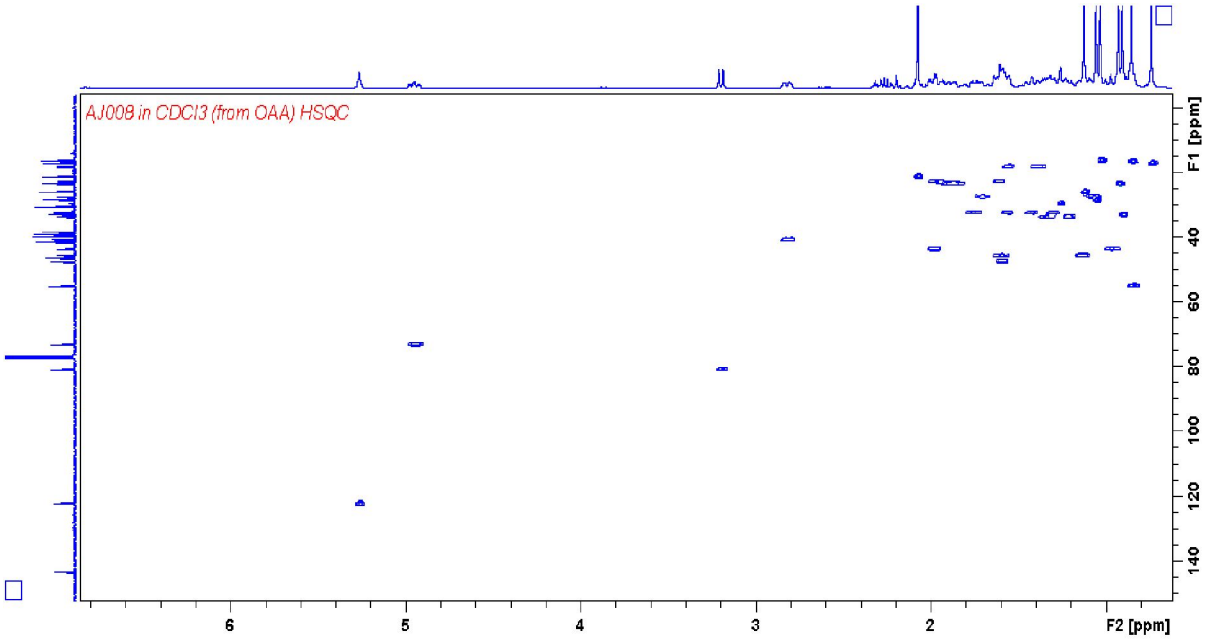
Appendix 40: NOESY-NMR Spectrum of MAA



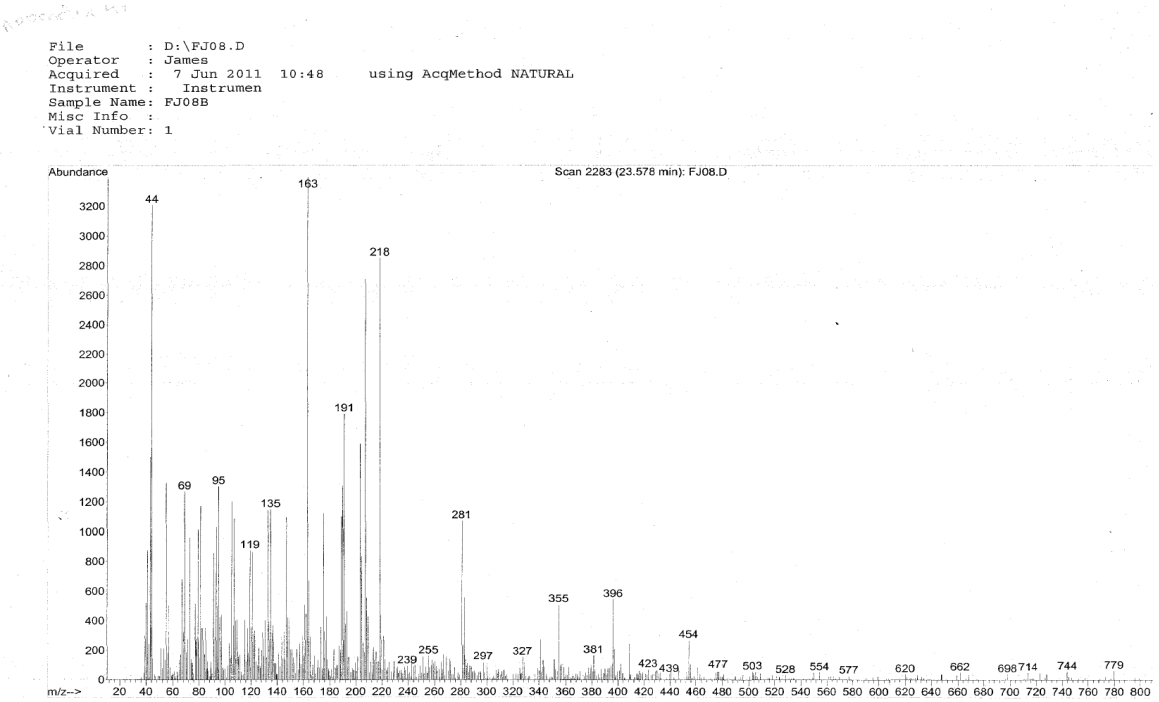
Appendix 41:HMBC-NMR Spectrum of MAA



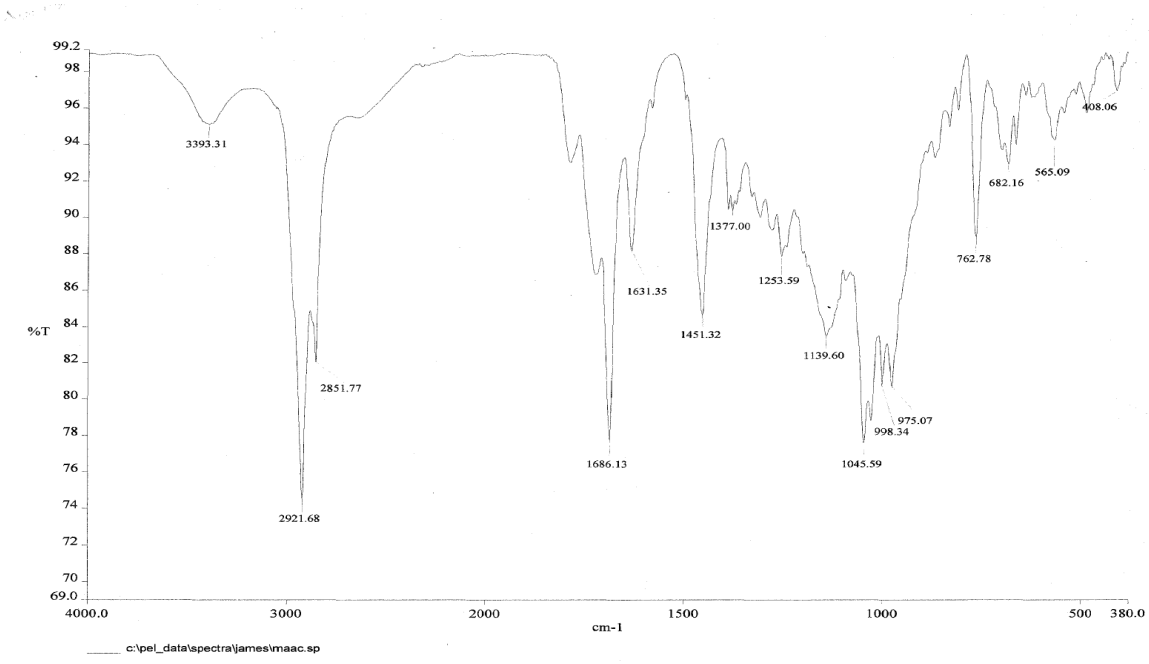
Appendix 42: HSQC-NMR Spectrum of MAA



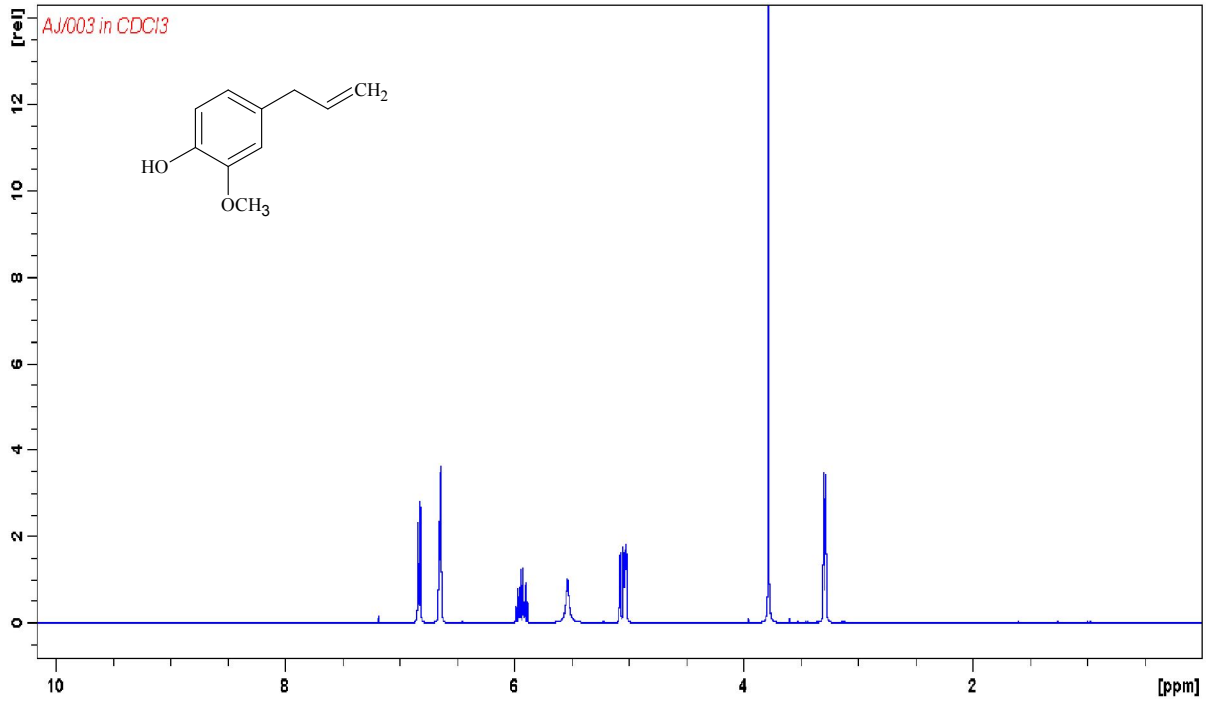
Appendix 43: EI-MS Spectrum of MAA



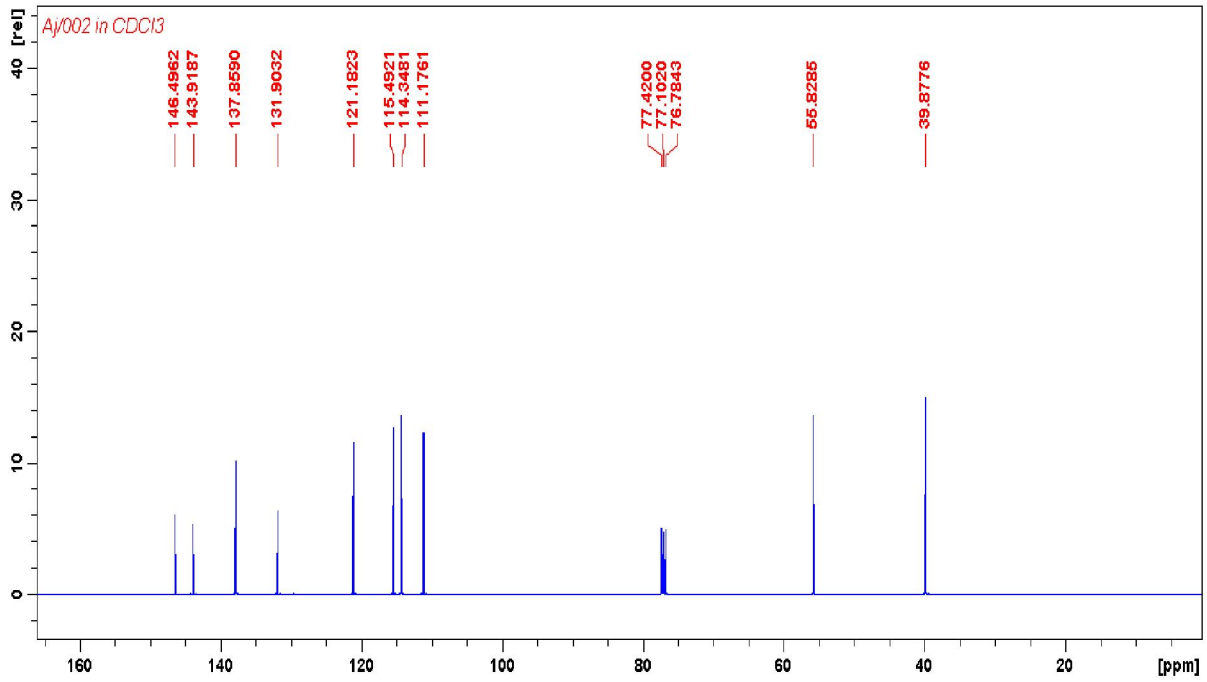
Appendix 44: IR Spectrum of MAA



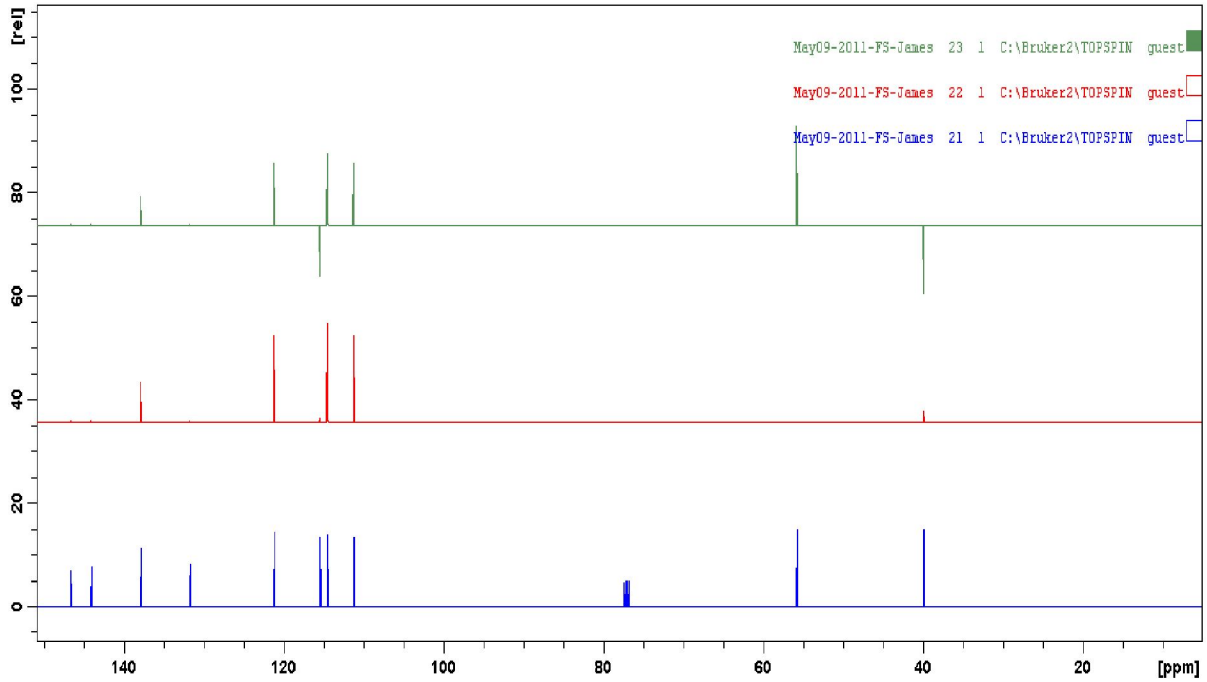
Appendix 45: H-NMR Spectrum of EU



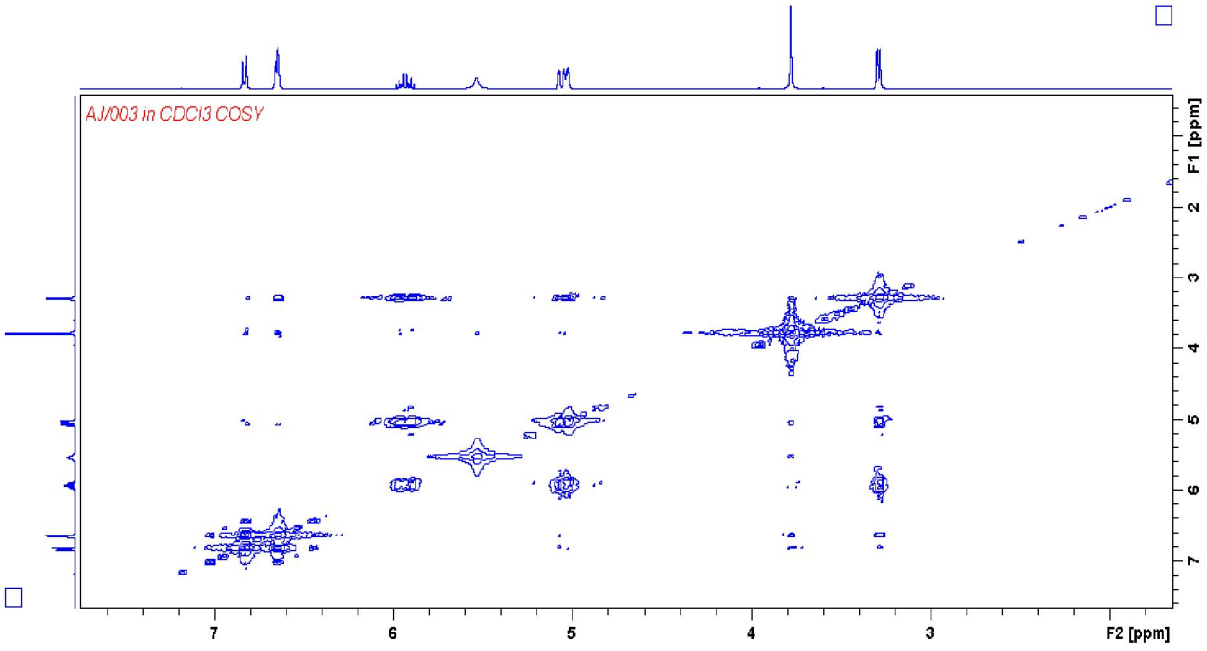
Appendix 46: C- NMR Spectrum of EU



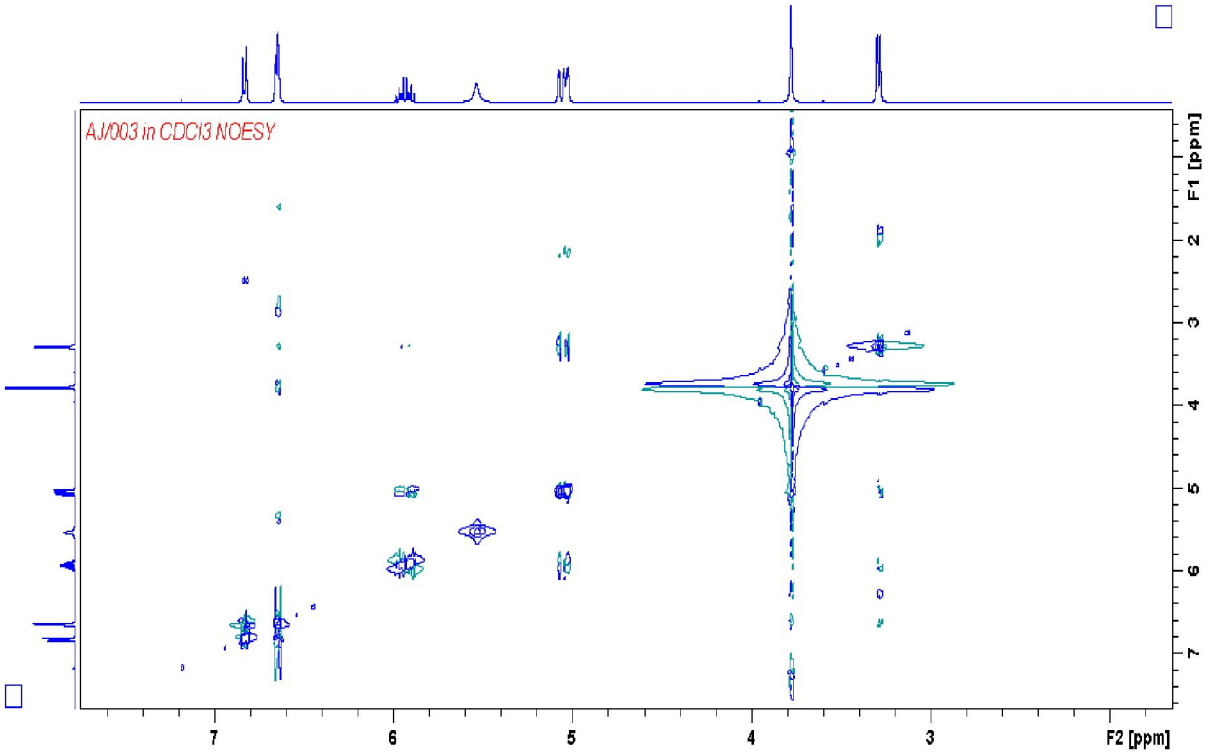
Appendix 47: C-NMR, DEPT 90 and DEPT 135 Spectrum of EU



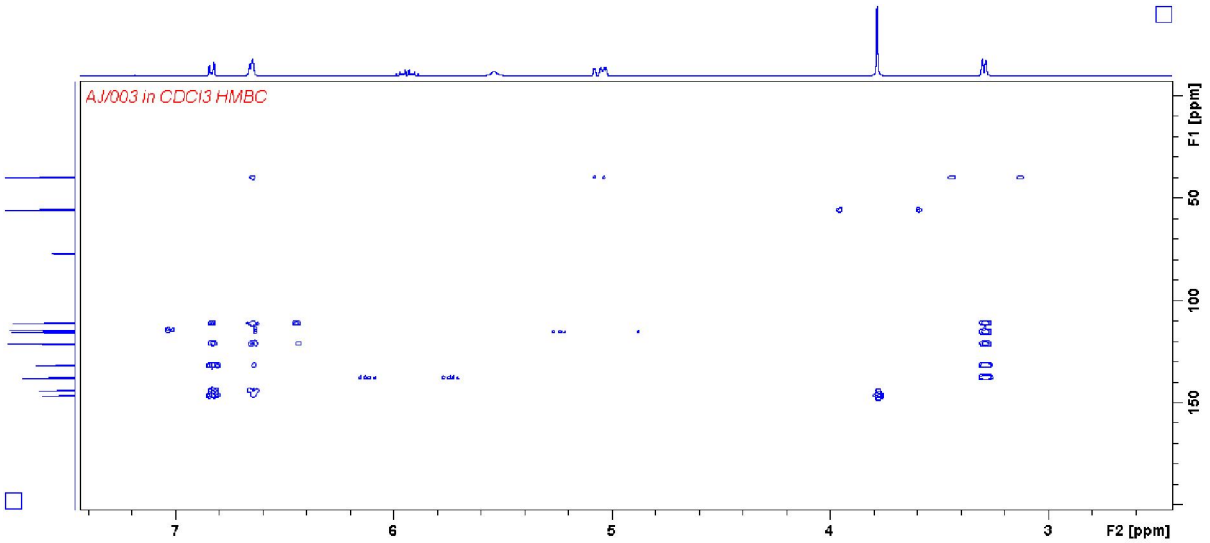
Appendix 48: COSY-NMR Spectrum of EU



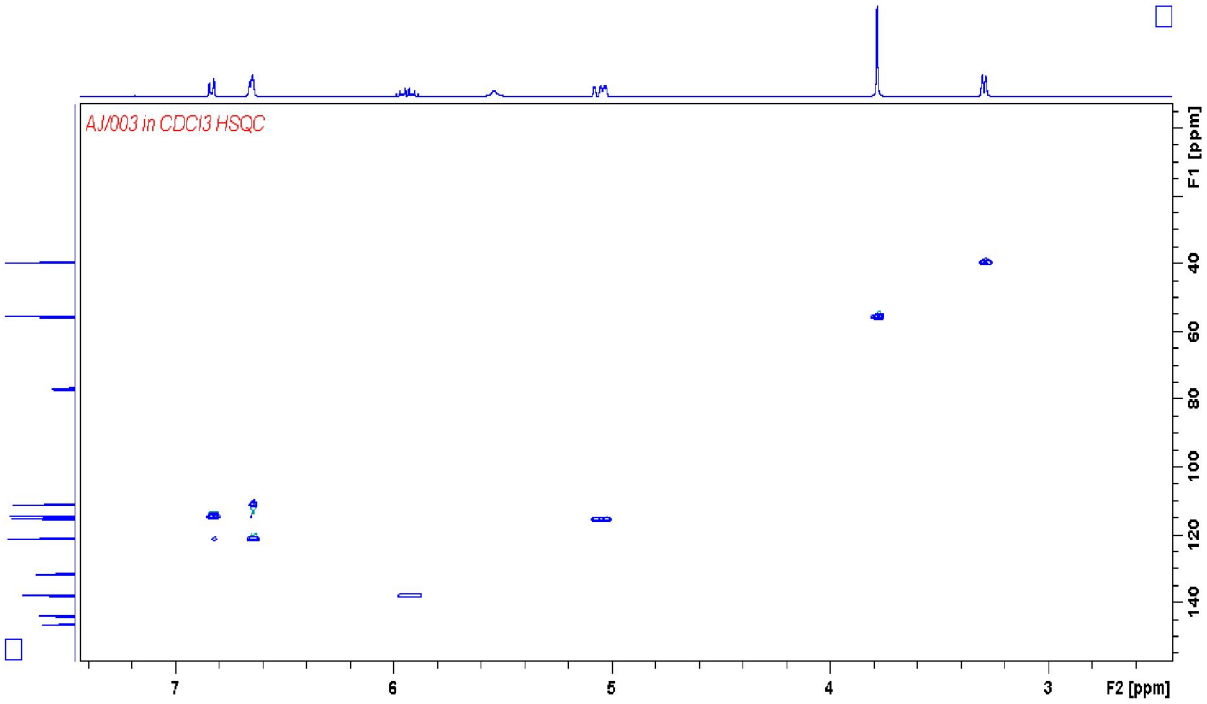
Appendix 49: NOESY-NMR Spectrum of EU



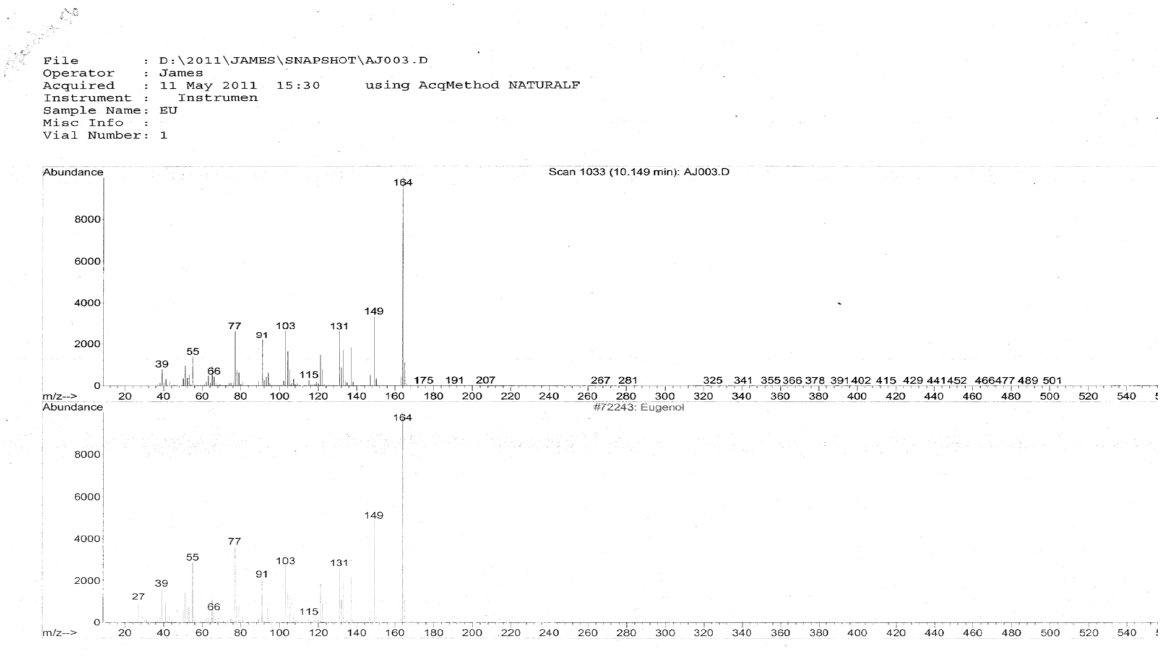
Appendix 50:HMBC-NMR Spectrum of EU



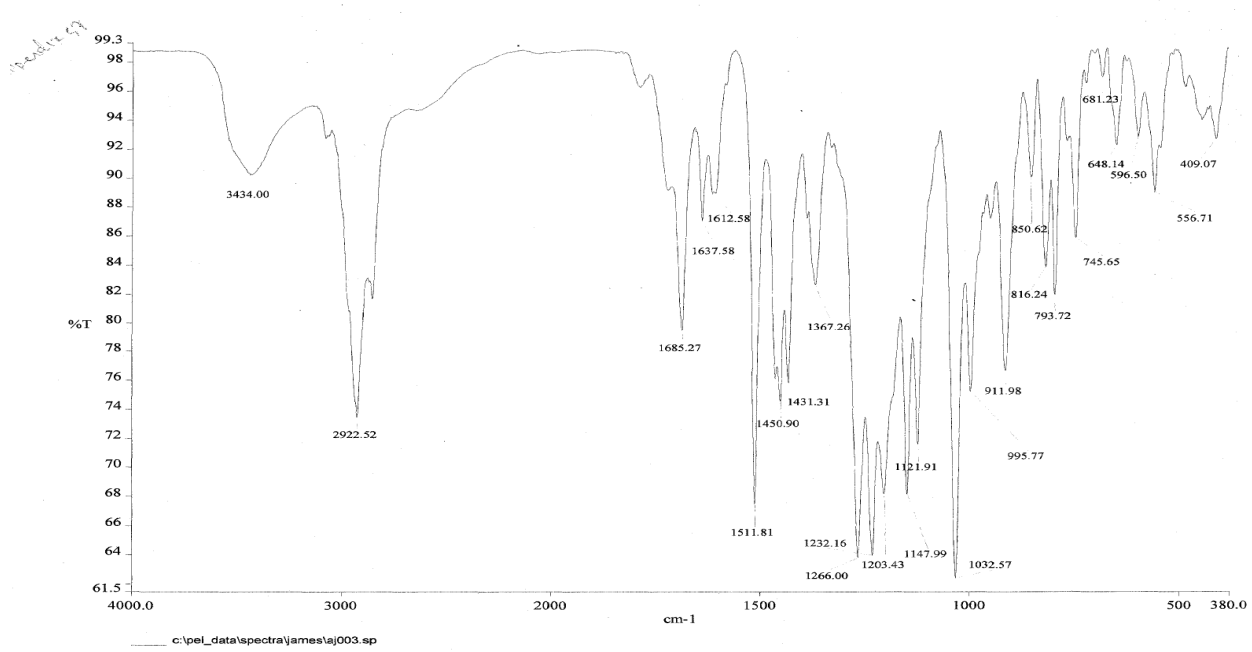
Appendix 51: HSQC-NMR Spectrum of EU



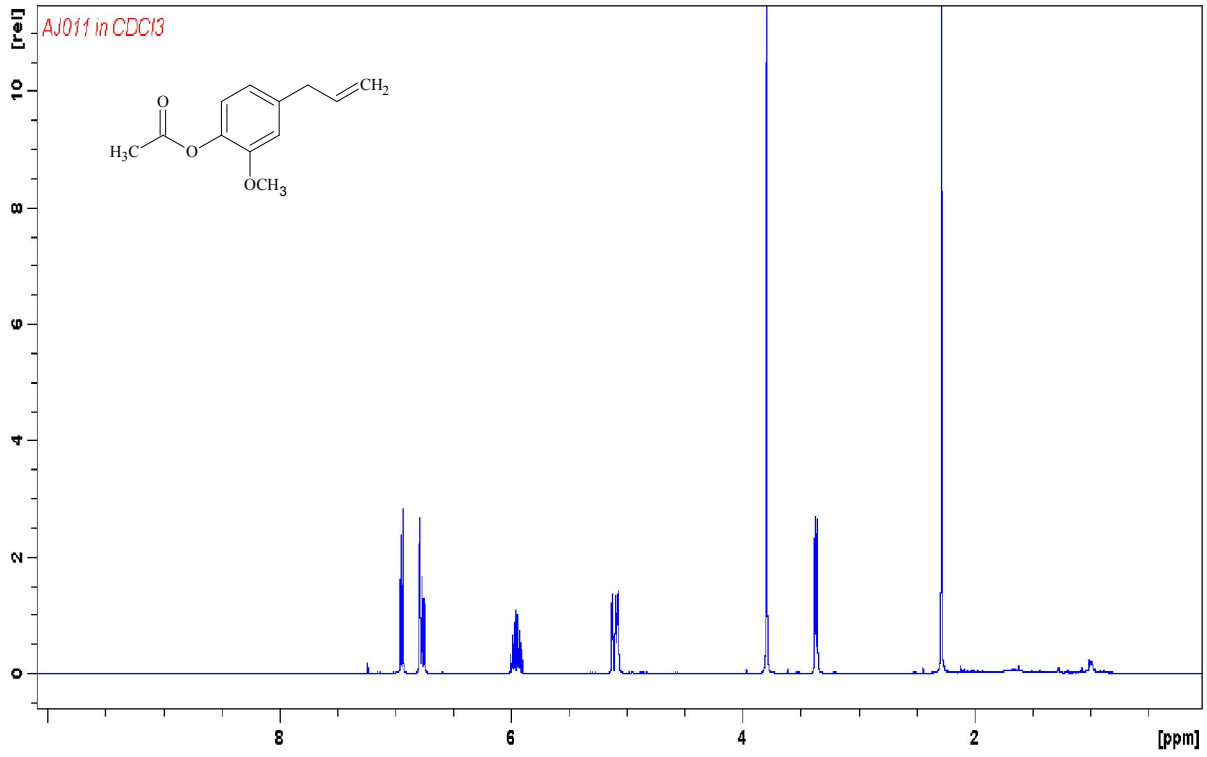
Appendix 52: EI-MS Spectrum of EU



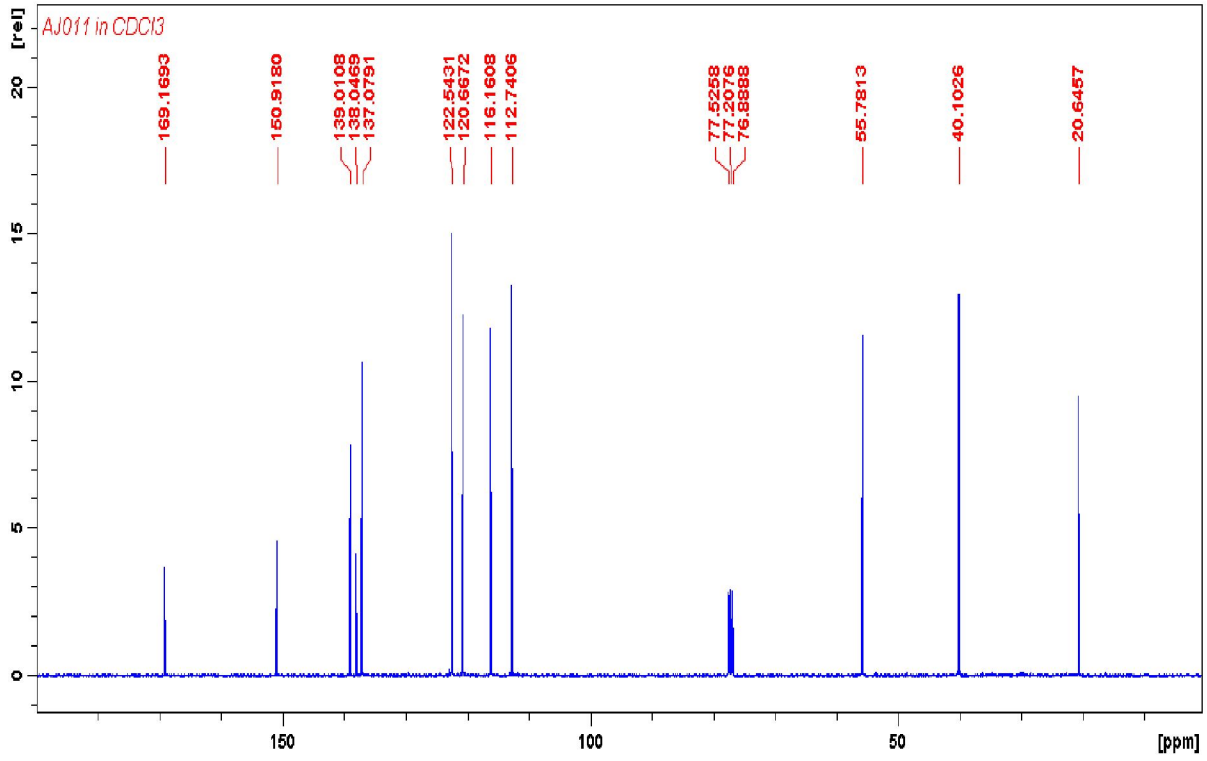
Appendix 53: IR Spectrum of EU



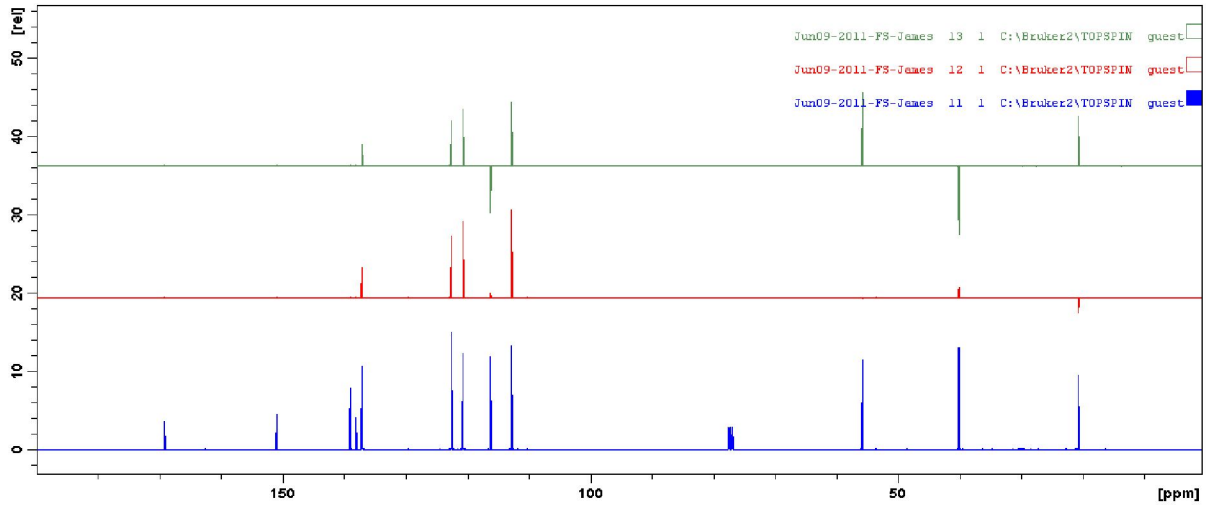
Appendix 54: H-NMR Spectrum of EUA



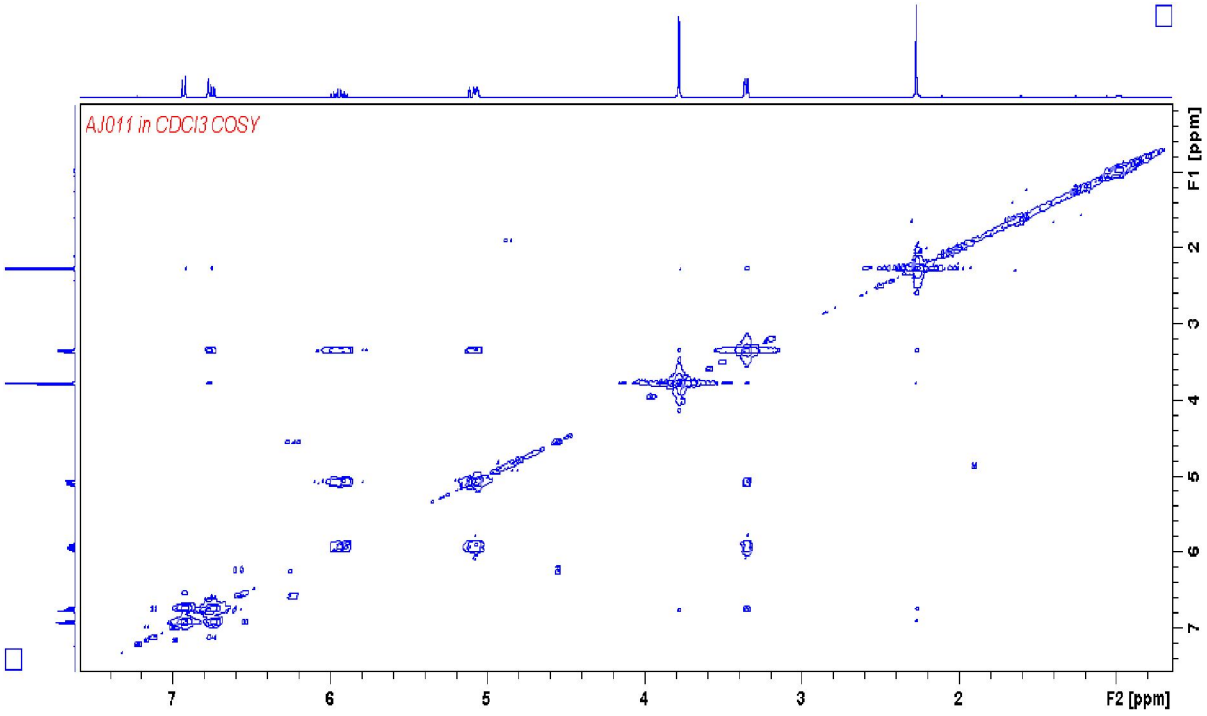
Appendix 55: C- NMR Spectrum of EUA



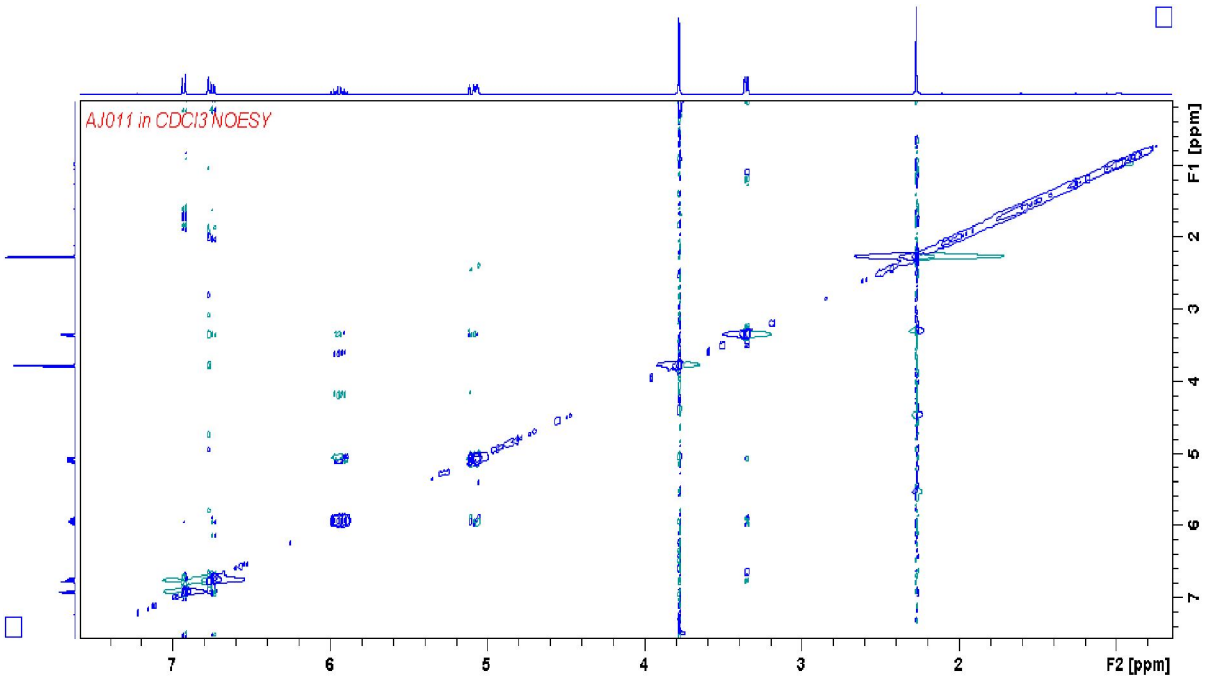
Appendix56: C-NMR, DEPT 90 and DEPT 135 Spectrum of EUA



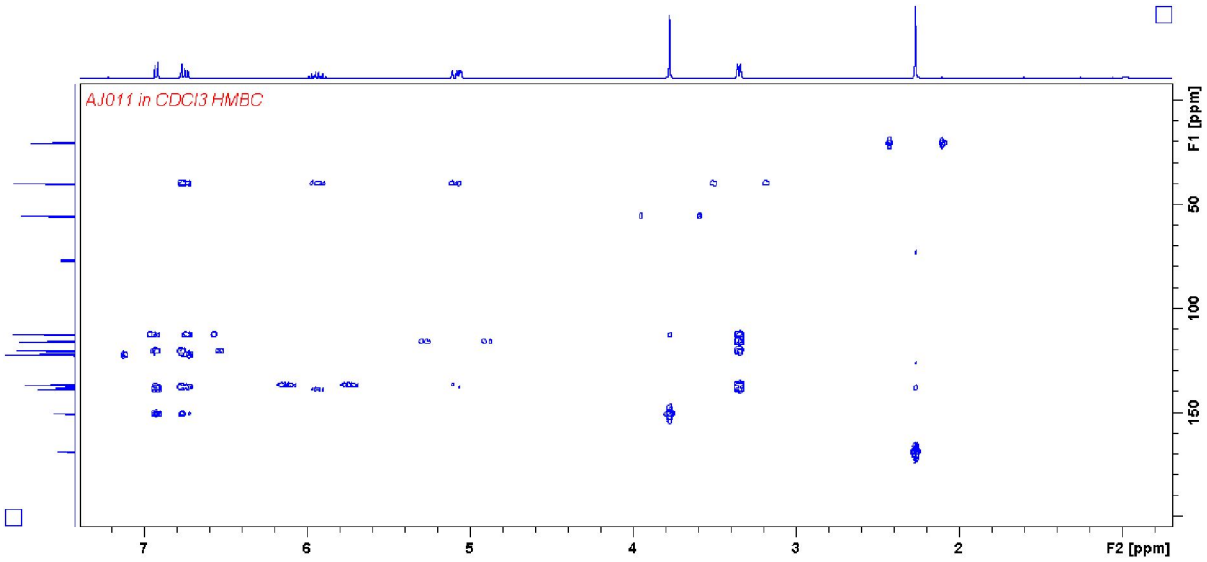
Appendix 57: COSY-NMR Spectrum of EUA



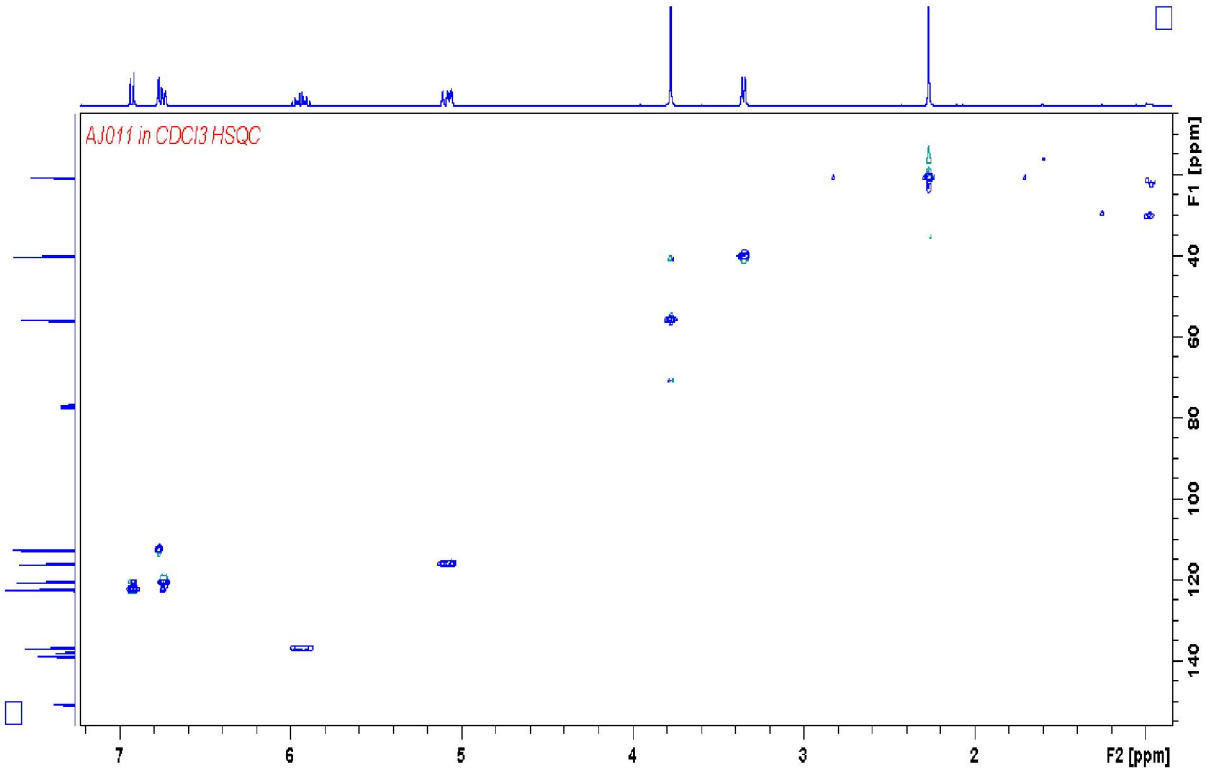
Appendix 58: NOESY-NMR Spectrum of EUA



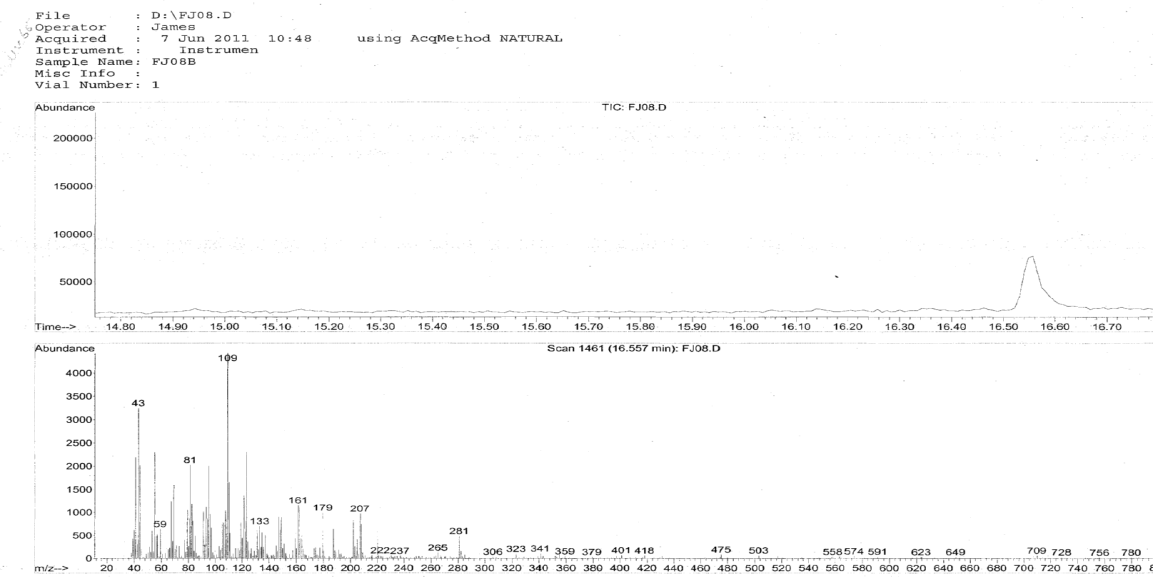
Appendix 59:HMBC-NMR Spectrum of EUA



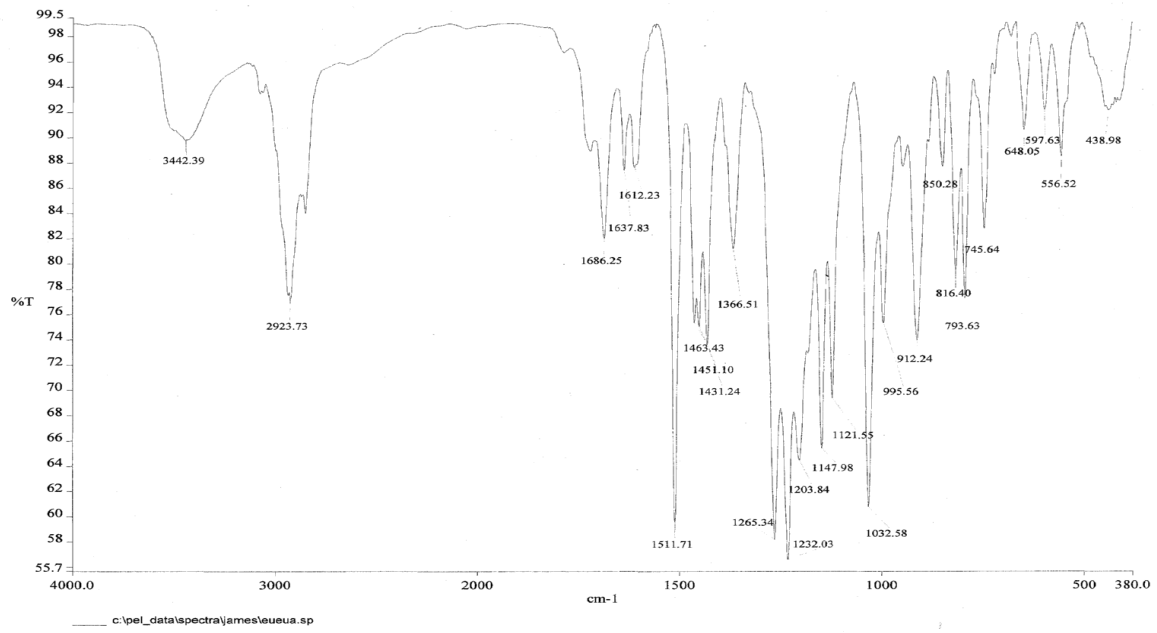
Appendix 60: HSQC-NMR Spectrum of EUA



Appendix 61: EI-MS Spectrum of EUA



Appendix 62: IR Spectrum of EUA



Appendix 63: Thin Layer Chromatography of Isolated and Modified Compounds.

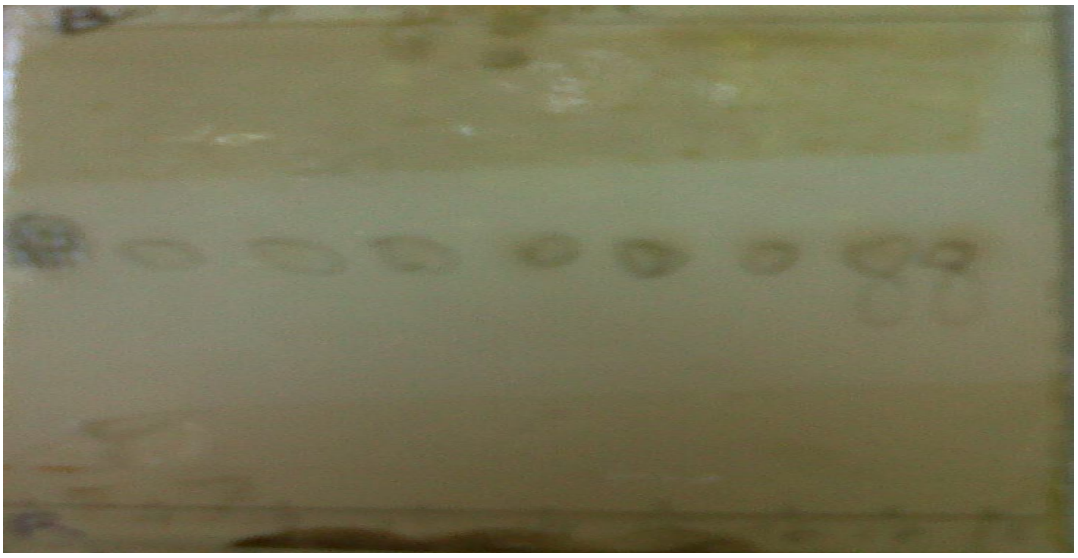


Plate III: TLC Confirming pure fraction of Betulinic Acid (BA). (R=Reference, 11-16= BA)

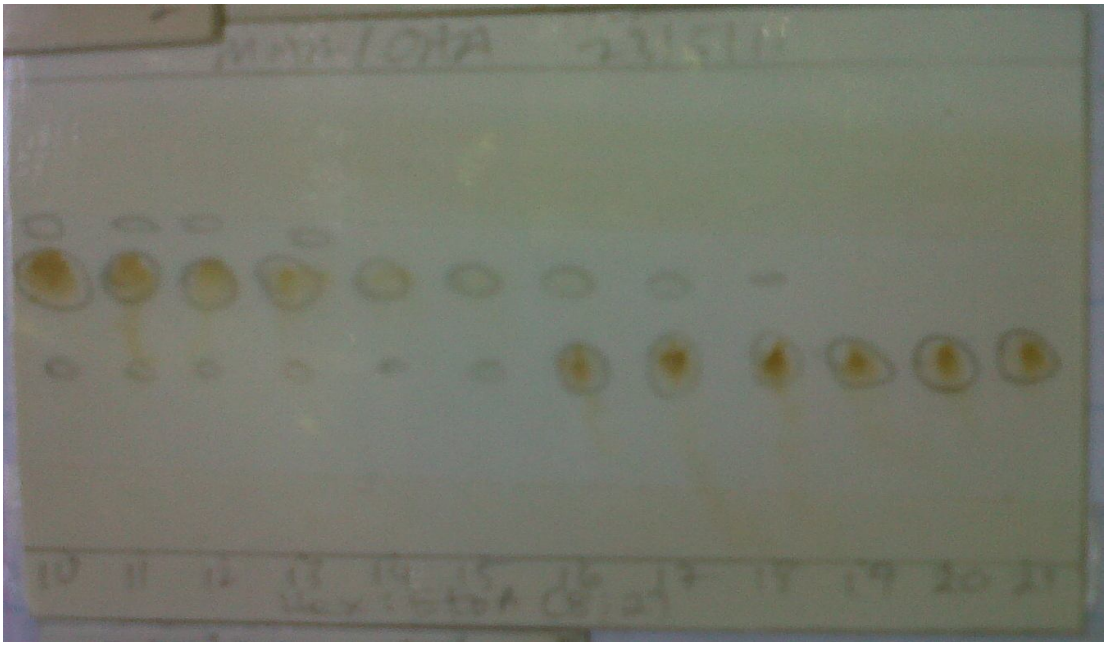


Plate IV: TLC Confirming pure fraction of Maslinic Acatate (MAA). (21= Reference, 18-20= MAA)

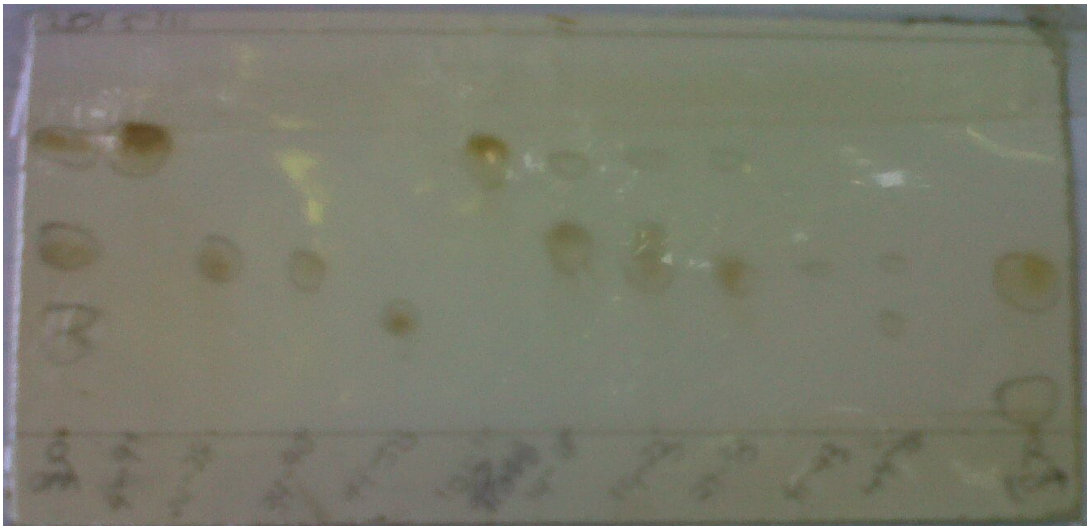


Plate V: TLC Confirming pure fraction of Oleanolic acid (OA) and Oleanolic Acetate (OAA). (10 and 11= OAA Reference, R (OA)= OA Reference, 24-28 and 34-40= OA, 4-9= OAA)

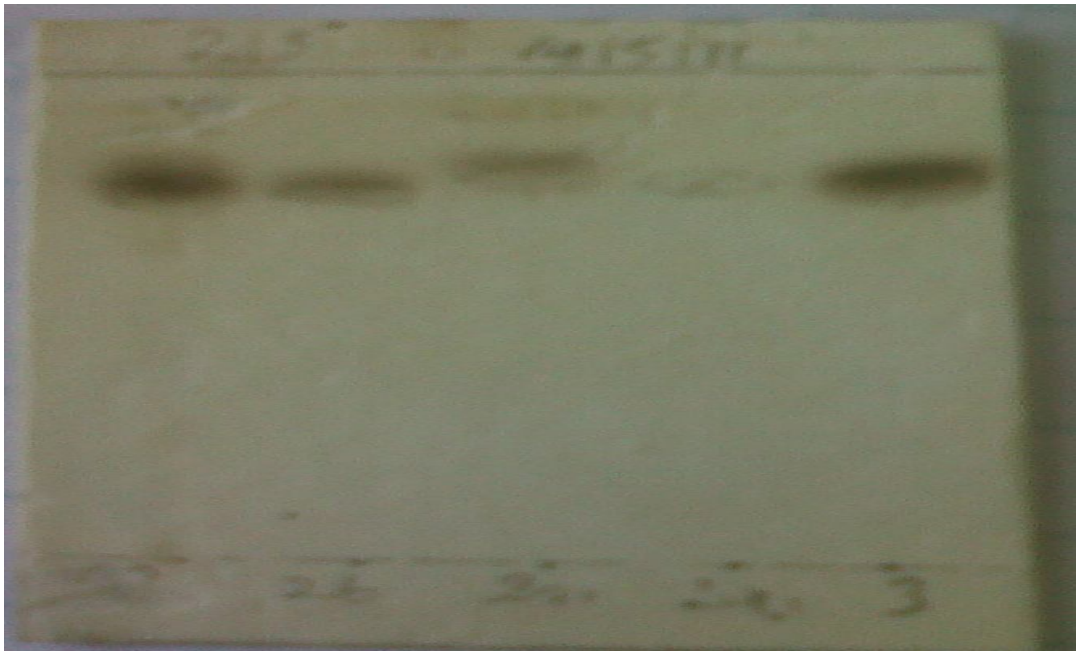


Plate VI: TLC Confirming pure fraction of Eugenol and a mixture of Eugenol and Eugenol Acetate. (R= Reference, 2b,3 and 2a= Eugenol, 2a'= Miture of Eugenol and Eugenol Acetate)



Plate VII: TLC Confirming pure fraction of Betulinic Acid. (R= Reference for Betulinic acid, 1= Betulinic acid)

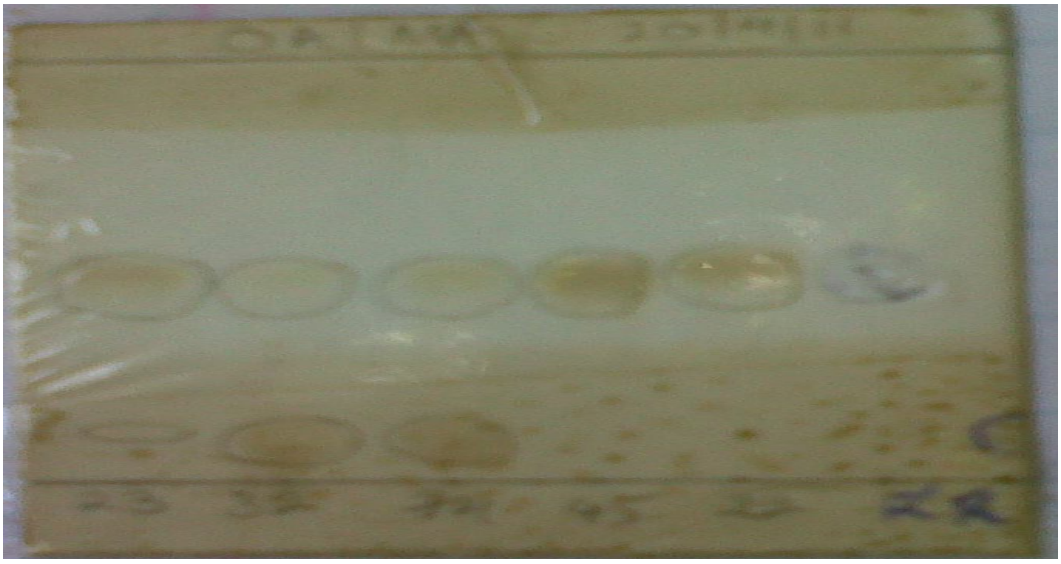


Plate VIII: TLC Confirming pure fraction of Oleanolic Acid (OA) and a mixture of OA and Maslinic Acid (MA). (R= MA Reference, R'= OAReference, 22 and 45=OA, 23,32 and 74= mixture of OA and MA)

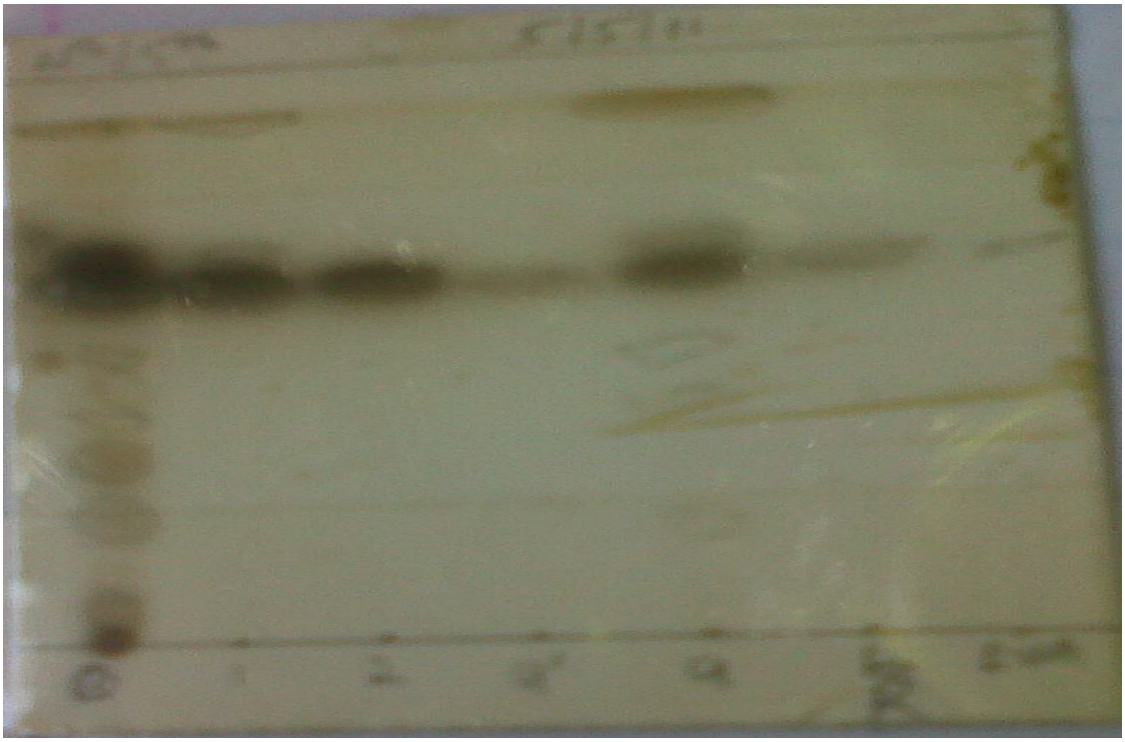


Plate: IX: TLC Confirming pure fraction of Eugenol Acetate. (R= Reference, EUA, 2,3= Eugenol Acetate)

Appendix 64: Thin Layer Chromatograhy Bioautography Showing Qualitative Antioxidant Activity of Isolated and Modified Compounds.

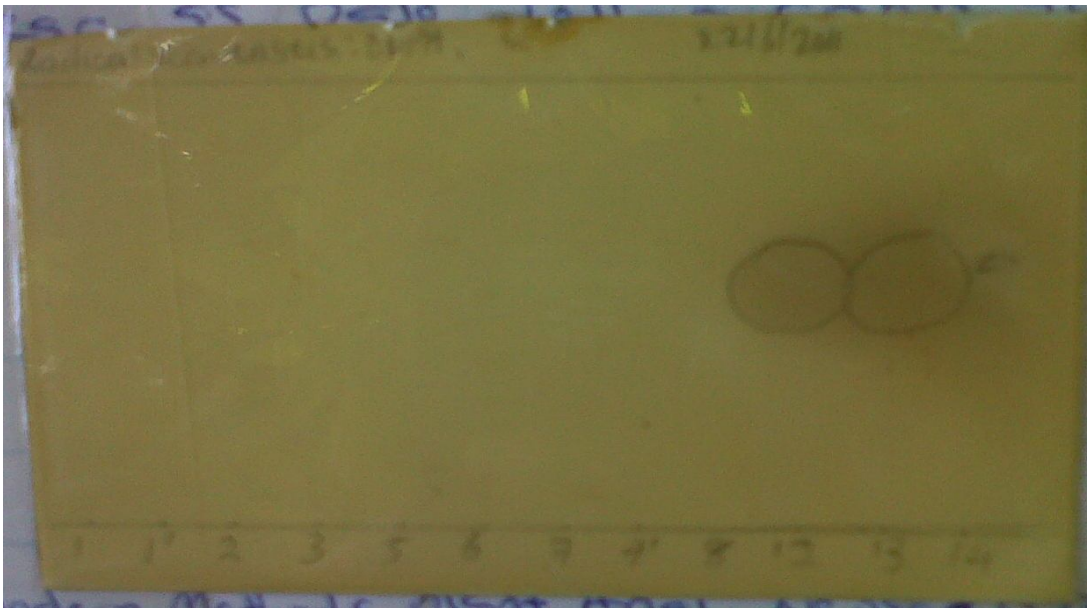


Plate X: Antioxidant Activity of Compounds Using DPPH Method. (12=Eugenol+Eugenol Acetate, 13=Eugenol, 14=Eugenol Acetate)

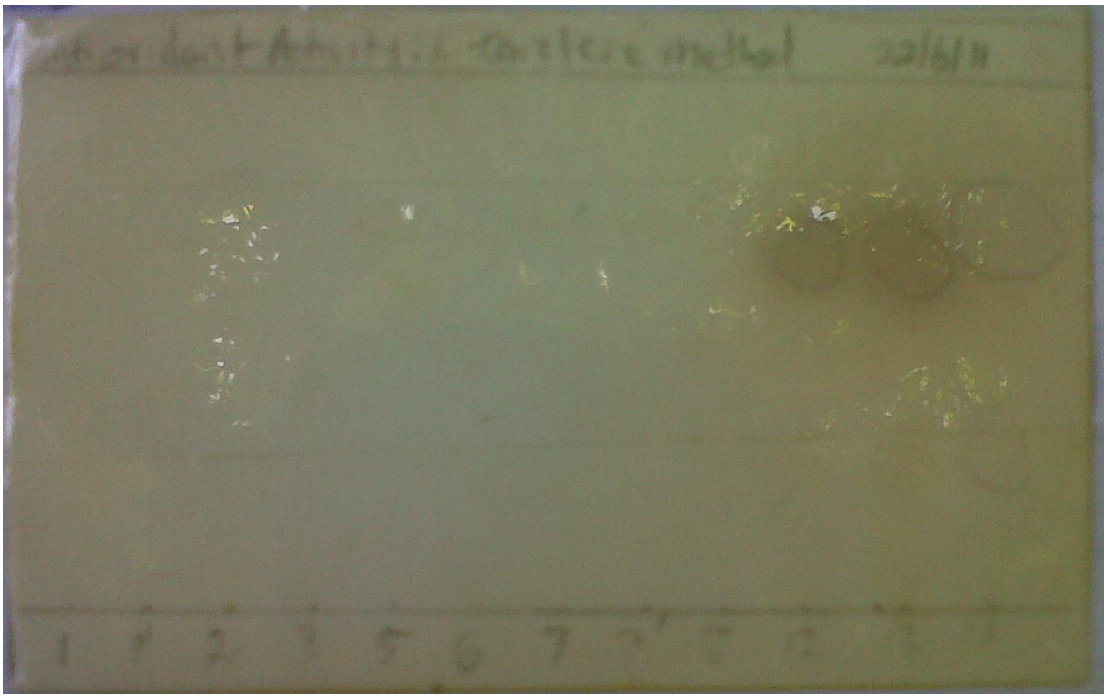


Plate XI: Antioxidant Activity of Compounds Using β-Carotene Method. (12=Eugenol+Eugenol Acetate, 13=Eugenol, 14=Eugenol Acetate)