ANTI-PLATELET AGGREGATION ACTIVITY OF *MELALEUCA BRACTEATA* VAR.REVOLUTION GOLD DERIVED BETULINIC ACID AND ITS DERIVATIVES

BY

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DECLARATION

The experimental work described in this dissertation was conducted in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, University of Zululand, from January 2013 to November 2015, under the supervision of Prof. A.R. Opoku and co-supervision of Prof. F.O. Shode

This study represents the original work by the author which has not been submitted in any form to another University. Any use of the work of others has been properly cited and acknowledged in the text.

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DEDICATION

This research work is dedicated to the Almighty God, my beloved late father Deacon David Olanrewaju Osunsanmi, my mother Mrs Benedicta Omolara Osunsanmi, my dearest wife Mrs Adesola Sekinat Osunsanmi and my children Obanioluwa and Olaloluwa.

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LIST OF ABBREVIATIONS

12-KETE	12-ketoeicosatetraenoic acid
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BA	Betulinic acid
BAA	3-β acetylbetulinic acid
$CaCl_2$	Calcium chloride
COX-1	Cyclooxgenase 1
COX-2	Cyclooxgenase 2
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetra-acetic acid
EETs	Epoxyeicosatrienoic acids
Factor II	Prothrombin
Factor VII	Proconvertin factor
Factor XI	Plasma thromboplastin antecedent
Factor XIII	Fibrin stabilizing factor
Factor XIIIa	Fibrin stabilizing factor A
Fctor X	Stuart- Power factor
FLAP	5-lipoxygenase-activating protein
GPIb/IX	Platelet glycoprotein GPIb/IX complex
H_2SO_4	Sulfuric acid

HEK293	Human embryonic kidney cells
HEPG2	Human hepatocellular carcinoma cells
HETEs	Hydroxyeicosatetraenoic acids
HPETE	Hydroxyperoxyeicosatetraenoic acid
HSC	Human stem cell
IC50	Inhibitory concentration with 50%
IR	Infra-red Spectroscopy
LD	Lethal dose
LOX	Lipoxygenase
LT	Leukotriene
MK	Megarkaryocytes
MS	Mass Spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
NMR	Nuclear magnetic resonance spectroscopy
NMR	Nuclear Magnetic Resonance
OA	Oleanolic acid
OAA	3-β acetyloleanolic acid
PDE	Phosphodiesterase
PG	Prostaglandin
RANTES	Regulated on Activation Normal T Expressed and Secreted
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TPO	Thrombopoetin

TXA_2	Thromboxane A_2
VWf	Von Willebrand factor
WHO	World Health Organisation

ABSTRACT

Abnormal Platelet aggregations are implicated in the onset of cardiovascular diseases which are the leading cause of death and disability globally. Management of pathological platelet aggregation with medicinal plants is a promising approach in treatment of cardiovascular diseases.

In this study, betulic acid (BA) and a mixture of betulinic acid and oleanolic acid (BA/OA) isolated from *Melaleuca bracteata* leaf extract and their acetyl derivatives (3-β acetylbetulinic acid) (BAA), (3-β acetylbetulinic acid and 3-β acetyloleanolic acid mixture) (BAA/OAA) were investigated for their antiplatelet aggregation, anti-inflammatory, anticoagulant, anti-oxidant and cytotoxicity activity. The compound structures were confirmed through spectral nuclear magnetic resonance (NMR), mass (MS) and infrared (IR) spectroscopy data analysis. The antiplatelet aggregation activities of the compounds were evaluated against four agonists (thrombin, collagen, adenosine diphosphate and epinephrine) used separately to induce platelet aggregation. The ability of the compounds to separately inhibit the hydrolysis of chromogenic substrate was used for antithrombin activity of the triterpenoids. The release of ATP and calcium mobilization from the cytosol, as platelets aggregate, was investigated using a commercial kit and Fura 2/AM respectively. The anti-acetylcholinesterase activity of the triterpenes was also investigated using a commercial kit. The compounds were fed to rats and the tail bleeding time was used to determine the *ex vivo* anticoagulation activity of the triterpenoids. The anti-inflammatory activity of the triterpenes was investigated using the cotton pellet-induced granuloma model in rats. The homogenates from the granuloma tissues were used to determine the effect of the test compounds on catalase (CAT) and superoxide dismutase (SOD) activities. The *in vitro* effect of the triterpenes on cyclooxygenase COX-1 and COX-2 activity was investigated. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was used to investigate the cytotoxic effect of the triterpenoids against carcinoma (HEPG2) and human embryonic (HEK293) cell lines.

All the test compounds exhibited significant anti-platelet aggregation activity, albeit to

different degrees of efficacy. BAA showed the highest antiplatelet aggregation activity regardless of the agonists. Coupled with its anti-platelet aggregation activity, BAA also exhibited significant anti-inflammatory, antithrombin, acetylcholinesterase inhibition, phosphodiesterase inhibition, calcium mobilization inhibition, inhibition of the release of ATP from dense granules, anticoagulant, cyclooxgensase (COX-2) activity inhibition, and iron chelating activities. BAA also significiantly stimulates SOD and CAT activity. In addition to the efficacy, the weak cytotoxicity of triterpenoids indicated their safety as an antiplatelet agent.

It was concluded that BAA could be served as a template for the synthesis of safer anti-platelet agent.

CONTRIBUTION TO KNOWLEDGE

Publications

- **Osunsanmi, F.O., Soyinbe,** O.S. Ogunyinka, I.B.,Mosa, R.A.,Ikhile, M.I., Ngila, J.C., Shode, F.O., Opoku, A.R. (2015). Antiplatelet aggregation and cytotoxic activity of betulinic acid and its acetyl derivative from *Melaleuca bracteata.* Journal of Medicinal Plant Research. 9(22): 647-854.
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Chapter one

1. Introduction

More than 17 million people die of cardiovascular diseases (such as pulmonary hypertension, stroke, heart attacks, and angina pectoris) annually and this number is expected to grow to more than 23.6 million by 2030 (Mozaffarian et al., 2014). A substantial number of the deaths can be attributed to pathological platelet aggregation which forms clots (thrombosis) within the blood vessel and disrupts the ease of blood circulation. A clot within the vessel can break and begin to travel around the body leading to an embolus formation (Furies and Furies, 2008). Unfortunately, most of the currently used antiplatelet agents have been reported with undesirable side effects and drugs resistance (Armani et al., 2009). Therefore provision of optimal protection from thrombosis or embolism with no risk of side effects on the body system is the new frontier of research in antiplatelet therapy. This requires the identification of agents that can block undesired pathological thrombosis without altering the physiological protection of homeostasis.

Natural products, which are chemical compounds or substances produced by living organisms offer new opportunities for the treatment of antiplatelet aggregation. The medicinal properties of various plants traditionally used to cure different ailments have been well documented (George et al., 2001). In South Africa, which is a developing nation, indigenous African medicinal plants are used alongside western allopathic medicine to treat ailments (Van Wyk et al., 2004).

This present study investigated the antiplatelet aggregation activity of betulinic acid and its acetyl derivatives from *Melaleuca bracteata* Revolution Gold.

1.1Structure of the thesis

This thesis consist six chapters and appendices:

Chapter One gives a brief background and motivation of the study

Chapter Two gives the literatures review and. also described the aim and objectives of the study.

Chapter Three gives the materials and methods used to conduct all the experiments in the study.

Chapter Four gives the results obtained from the study.

Chapter Five gives the overall discussion of the results

Chapter Six gives the conclusion obtained from the results and suggestion for further studies.

Chapter two

2. Literature review

Blood platelet hyperactivity is implicated in atherosclerosis plaques which are the major cause of cardiovascular diseases such as stroke, heart attack and pulmonary hypertension. Cardiovascular diseases are one of the leading causes of mortality (Patrono, 2001).

Platelets, initially called “dust of the blood”, were discovered by James Homer Wright to be produced by megakaryocytes from bone marrow. He used Wrights’ stain to distinguish the similarities in the morphology between megakaryocytes and platelets (Kuter, 1996). In 1837, Osler described the structure of platelets, whereas Bizzozero described their anatomy and was the first to identify megarkaryocytes in bone marrow, but never identified them as the precursor of platelets (Kuter, 1996).. Bizzozero recognized that platelets were responsible for hemostatic and thrombosis formation and demonstrated that platelets adhere to ruptured endothelial blood vessels to form aggregates (Kuter, 1996)..

Platelets are anucleated cells that have a discoid shape, with a diameter of 1- 3 µm. They are formed from the cytoplasm of megakaryocytes (Kellie *et al*., 2013). The megakaryocytes are the largest cell (50- 100 µm) in which 0.01 % of nucleated cells are accounted for in the bone marrow (Pease, 1956). During platelet formation, megakaryocytes undergo two major stages. In the first stage, megakaryocytes’ DNA replicates without cellular in a process known as endomitosis. This stage requires megakaryocytes growth factors but takes longer days to complete. The MKs cytoplasma proliferate and enlarge as it is filled with platelet specific granules, protein cytoskeletals and membranes for the platelet assembly process. The second stage takes an hour for completion, the MKs firstly remodel their cytoplasm to form proplatelets, which later transform into preplatelets. The matured preplatelets then elongate and divide repeatedly to form discoid platelets from their tips (Richardson *et al.,* 2005). During the development of Platelets, their granular contents are received from the MK cell body. Platelets are then released into the blood circulation along with the red blood cells

(RBC) and white blood cells (WBC) (May *et al.,* 1998). Human platelets have a 7-9 day life span within the blood stream after formation, whereas rodents' platelets only have 4-5 days to survive (Aster, 1967; Jackson and Edward, 1977).

2.1 Platelet structure

Platelets are anucleated cells that comprise of organelles. These organelles are divided into three zones, each with specific functions (Figure 2.1).

The first is a peripheral zone containing glycocalyx, which is a thick coat around the platelet membranes (Moake *et al.,* 1988). The platelet membranes consist of a lipid bilayer that comprises of lipoproteins, glycolipids and glycoproteins. These glycoproteins are reported to be responsible for platelet antigenicity, cellular tissue compatibility and blood group (Matzdorff, 2005). The platelet glycoproteins function as receptors that aid in the transmission of external impulses through the cell membrane. The glycoprotein Ib (GPIb) enhances platelet adherence to the sub-endothelium through von Willebrand factor (vWF) binding. The platelet membranes house glycoprotein GP IIb/IIIa which serves as a receptor for fibrinogen, fibronectin and vWF during high shear to initiate platelet aggregation. They also house the receptors serotonin, ADP, thrombin, collagen and epinephrine that further strengthen platelet aggregation.

The second zone, called the Sol-Gel zone or cytoskeleton, contains microtubules and microfilaments. The microtubules encase each platelet, thereby giving them a discoid shape. The microfilaments are found in the cytoplasm, and they contain contractile protein (actin and myosin). The actin accounts for 20 - 30 % of platelet protein, whereas myosin composes 2 - 5%.

The third zone, called organelle, is the site of most platelet metabolic activity. Platelets have three types of storage granule; these include Alpha granules, dense granules and lysosomes. The Alpha granules are most abundant (20 - 200 per platelet) whereas the dense granules are only at about 2 - 10 per platelet. The storage granules have substance with mitogenic, angiogenesis, platelet proaggregatory and vasoconstriction stimulating effects (Hartwig, 1991). Alpha granules are composed of different membrane proteins. Proteomic studies indicated that alpha granules contain numerous

proteins (Coppinger et al., 2004). These proteins include angiogenic, and antiangiogenic, pro-inflammatory and anti-inflammatory, coagulant and anticoagulant, proteases and proteases inhibitor. The contradictory action of the alpha granules component has raised a lot of questions on how they effectively manage their biological functions (Italiano et al., 2008; Blair and Flaumenhaft, 2009). It was reported that different alpha granules have distinct components. The Immunofluorescence microscopy was used to demonstrate that fibrinogen and Von Willebrand factor (vWf) are located in different granules (Sehgal and Storrie, 2007). The dense granules contain polyphosphates, adenine nucleotide, cations, and amines such as histamine and serotonin. These are released during platelet activation to recruits more platelets to the site of damaged subendothelium (Sigel and Corfu, 1996). Platelets cargo two types of lysosome (primary and secondary lysosomes). They contain cathepsin, acid hydrolases, CD63 and LAMP-2. They play major roles in endosomal digestion by breaking down of substances ingested by pinocytosis and phagocytosis (Flaumenhaft, 2013).

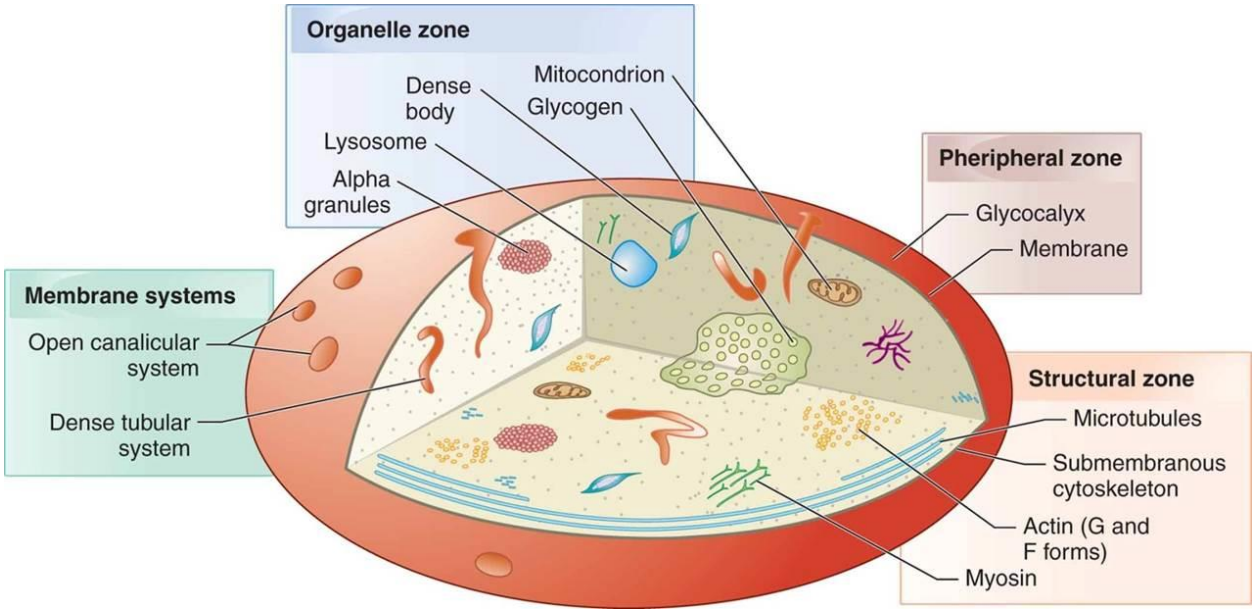


Figure 2.1 Platelet structure (adapted from www.blogspot.co.za)

2.2 Platelet activation

In a healthy endothelium vessel, microtubules help to keep platelets inactive by maintaining their discoid shape. The healthy endothelium releases prostacyclin (prostaglandin I2) and inhibits the release of activating factors from platelet granules. But, once the endothelials are insulted through rupture, platelets lose shape, adhere and spread over the injury site to stop bleeding or initiate the healing process. Platelet activation entails (a) platelet aggregation, (b) secretion, (c) stimulation of biochemical pathways to produce thromboxane and other agonists that amplify more platelet aggregations, and (d) activation of major integrin (αIIbβ3) and Von Willebrand factor (vWF) receptors (Harrison *et al.,* 1997).

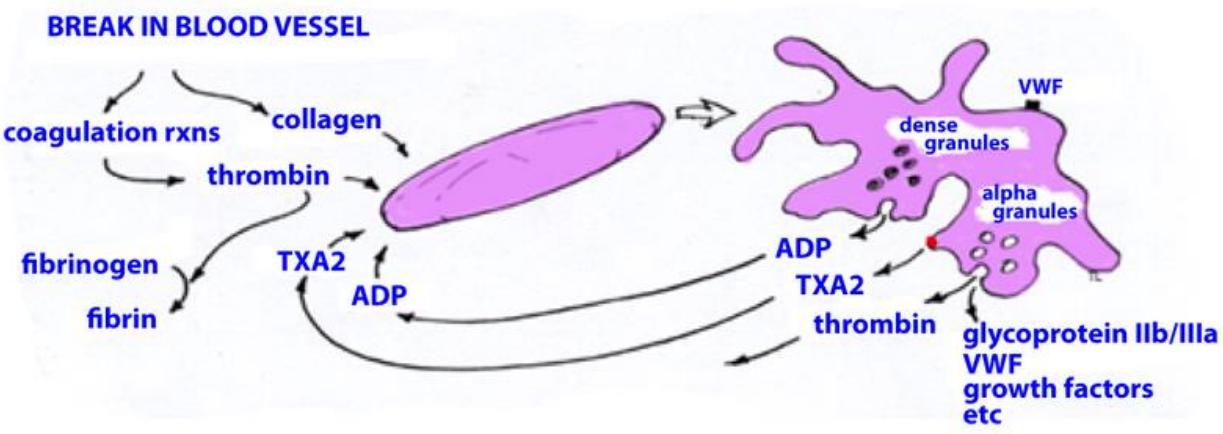


Figure 2.2: Mechanism of platelet activation (adapted from Jagroop et al., 2000)

During platelet activation (Figure 2.2) biochemical events are stimulated, due to an increase in Ca^{2+} mobilization into the cytoplasm. The platelets lose their disc shape to form finger–like projections (pseudopodia) from the cell peripheral and extended broad lamellae (Jagroop *et al.,* 2000). The flattened platelets cause the organelle and granules to concentrate to the centre giving a "fried egg" appearance (Figure 2.3). The compacted platelets, along with their extended dendrites, enhance adhesion to the damaged endothelium (Grundmann *et al.,* 2003). The influx of Ca^{2+} into the cytoplasm

are instigated by the activation of the phospholipase C pathway. The phospholipase hydrolyzes polyphosphoinostide (P_1P_2) into inositotriphosphate (IP_3) and diacylglycerol (Knofler et al., 1998). The soluble IP3 diffuses into the cytoplasm to bind to dense granules and release stored calcium (Escolar et al., 1999). The G-proteins (βγ), coupled with serpentine receptors, also activate the phospholipase C pathway. The serpentine receptors include ADP receptors (P_2Y_1 and P_2Y_{12}), 5HT receptors and protease activated receptors (PAR_1 and PAR_3).

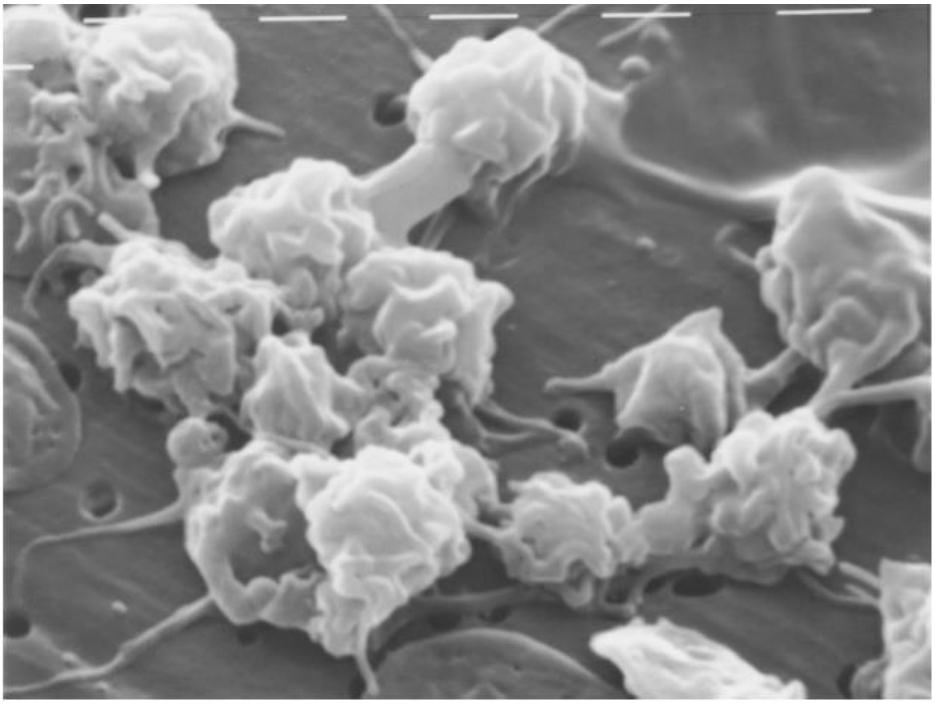


Figure 2.3: Representative scanning electron microscopic (ScEM) showing activated platelets (5HT and ET1) (adapted from Jagroop *et al.,* 2000).

2.3 Platelet receptors

Platelet receptors (Figure 2.4), molecular functions, and signaling pathways have been widely studied by researchers. A variety of transmembrane receptors are found on platelet membranes, such as integrins ($\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_2\beta_1$), immunoglobulin superfamily (GP VI, FcγRIIA), tyrosine kinase receptors (Gas-6, thrombopoietin receptor, ephrins and Eph kinases), G-protein coupled transmembrane receptors

(GPCR) ($P2Y_1$ and $P2Y_{12}$ ADP receptors, PAR-1 and PAR-4 thrombin receptors TPα and TPβ TxA_2 receptors), leucine-rich repeated (LRR) receptors (Toll-like receptors, Glycoprotein [GP] Ib/IX/V), C-type lectin receptors (P-selectin), and other types (like CD63, CD36, P-selectin ligand 1, TNF receptor type) (Rivera et al., 2009).

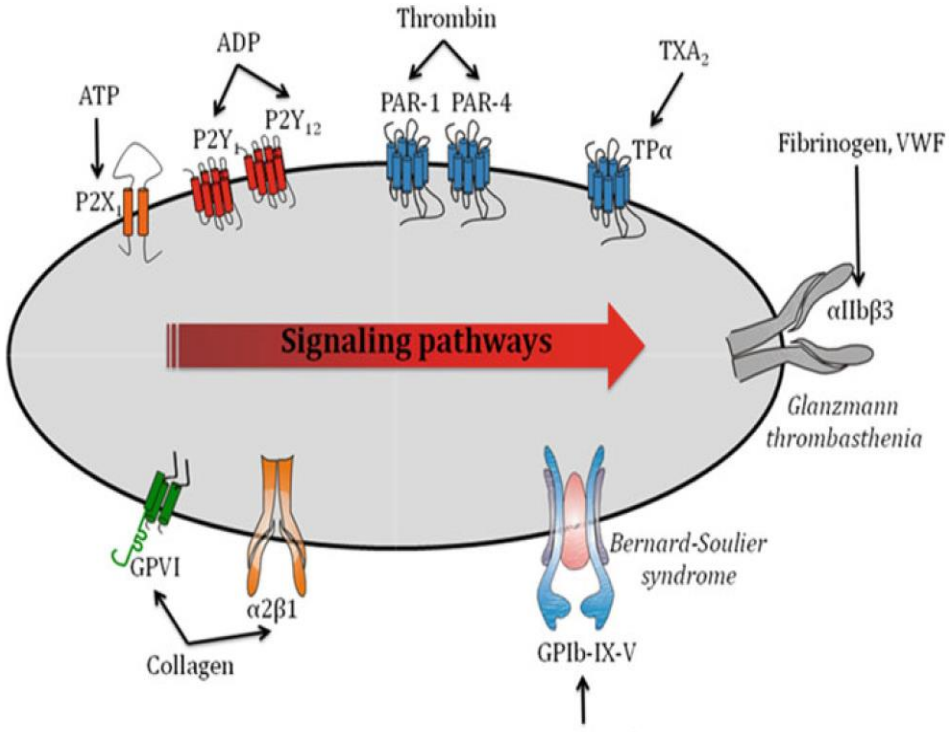


Figure 2.4: Platelet receptors-ligand interactions. Overview of well-known receptors on platelets and mode of activation (adapted from Kauskot and Hoylaerts, 2012).

2.3.1 **Collagen receptors**

In sub-endothelial matrix, collagen makes up between 20 - 40% of total protein constituents in the aorta. Apart from its contribution to vascular strength, collagen supports platelet plug formation and platelet adhesion. There are nine types of collagen, of which only the fibrillar type (I, III, V and VI) and non-fibrillar type (IV and VIII) show thrombogenic properties. Likewise, platelets have six collagen receptors (α2β1, p47,

p67, GPIV, THICBP and GPVI), but only GPVI and α2β1 integrin are crucial for binding to collagen and subsequent platelet activation (Nuyttens et al., 2011).

2.3.1.1 Glycoprotein VI receptors

Glycoprotein VI (GPVI) receptor (63 kDa) is a member of the transmembrane receptor family. It consists of two immunoglobulin subunits (Ig) connected to a glycosylated linker, cytoplasmic tail and a transmembrane domain. They are expressed in megakaryocytes and platelets (3,700 per platelet). The GPVI is found to associate with transmembrane adaptor FcRγ. The stabilization of FcRγ enhances the surface expression of GPVI receptors on platelet membranes. This is made possible via the salt bridge between FcRγ (Asp residue) and the GPVI domain (Arg residue). FcRγ is a homodimer containing one copy of an Immunoreceptor Tyrosine-based Activation Motif (ITAM), which is defined by two YxxL sequences separated by seven amino acids (Clemetson and Clemeston, 2001). ITAM motifs, phosphorylated by Src kinase (Lyn and Fyn) are in contact with GPVI; they trigger platelet signaling and lead to platelet activation. Monomeric GPVI displays low affinity for collagen, whereas dimeric GPVI has high affinity for collagen thus initiating platelet signaling (Miura et al., 2002). The defect of GPVI receptors are characterized with moderate bleeding. Studies of various mouse models lacking GPVI receptors are shown to lack collagen induced platelet aggregation (Mangin et al., 2006; Massberg et al., 2003; Konstantinides et al., 2006).

2.3.1.2 $\alpha_2\beta_1$ integrins

The $\alpha_2\beta_1$ integrin is a collagen receptor with a 130-kDa β1 chain and a 150-kDa α_2 chain. It is expressed in platelets (1,730 per platelet). The α_2 chain houses only one domain with a 200 residue sequence (Clemetson and Clemetson, 2001). Platelet activation by classical agonists causes the rearrangement of $\alpha_2\beta_1$ domains, which increase appropriate ligand sequence affinity on collagen fibrils (Siljander et al., 2004). The defects of $\alpha_2\beta_1$ integrin allelic polymorphism (G873A and C8O7T) are implicated in thrombotic episodes such as stroke and myocardial infarction (Moshfegh et al., 1999).

2.3.2 **Platelet CD148 receptors**

Platelet CD148 receptor is a tyrosine phosphatase on platelet transmemembrane. It is expressed in epithelial, fibroblast, endothelial cell and hematopoietic cells. CD148 is reported to regulate the expression of GPVI/FcRγ. Mouse models lacking CD148 showed reduced GPVI expression. Likewise, Scr family kinase (SFK) platelet activity was attenuated. This result showed poor response to agonists that stimulate SFK, such as collagen and fibrinogen (Senis et al., 2009; Ellison et al., 2010). The defect of CD148 receptors in some carcinoma diseases has been reported (Austschbach et al., 1999).

2.3.3 **C-type lectin-like receptor 2 (CLEC-2)**

C-type lectin-like receptor 2 (CLEC-2) is a transmembrane receptor that is mapped to chromosome 12. It consists of thirty amino acid cytoplasmic tails that house YxxL sequences known as (hem) immunoreceptor tyrosine based activation motifs (hem ITAM). It is expressed in platelets, megakaryocyte and neutrophil. CLEC-2 was first isolated from snake venom rhodocytin in the Malaya (Suzuki-Inoue et al., 2006). Rhodocytin was demonstrated not to bind to integrin GPVI, $\alpha_2\beta_1$ and GPIbα but through CLEC-2 receptors to activate platelet aggregation (Suzuki-Inoue et al., 2006). CLEC-2 plays a crucial role in the stabilization of thrombosis via hemophilic intersection (Hughes et al., 2010).

2.3.4 **Platelet integrin $\alpha II_b\beta_3$ receptor**

The platelet integrin $\alpha II_b\beta_3$ receptor is found in abundance on the platelet membrane (40,000= 80,000 per platelet). The subunits αIIb and β3 have 128 kDa and 95 kDa proteins respectively. The $\alpha II_b\beta_3$ receptor binds to RGD containing ligands such as vitronectin, fibronectin, fibrin, thrombospondin, Von Willebrand factor and fibrinogen. Fibrinogen shows the highest binding affinity to αIIbβ3 by recruiting more intracellular proteins and triggers the clustering of $\alpha II_b\beta_3$ receptors (Salsmann et al., 2006). The changes of $\alpha II_b\beta_3$ receptors from low to high affinity are reported to be a fairly common pathway for platelet activation. Defects in $\alpha II_b\beta_3$ receptors result in a diseased condition known as Glanzmann Thrombasthenia (GT). GT is characterized by gastrointestinal

bleeding, hematuria, epistaxis, gingival hemorrhage (Coller and Shattil, 2008; Fiore et al., 2011).

2.3.5 **Integrin $\alpha_v\beta_3$**

The integrin $\alpha_v\beta_3$ is a widespread member of the transmembrane family. They are expressed in leukocytes, smooth muscle, osteoblasts and endothelial cells. $\alpha_v\beta_3$ binds to RGD ligands such as adeno-virus penton, vitronectin fibronectin, fibrinogen, thrombospondin and osteopontin. Vitronectin was demonstrated to have the highest affinity for $\alpha_v\beta_3$, and is thus referred to as the preferred ligand for $\alpha_v\beta_3$. Activated $\alpha_v\beta_3$ integrins are found on atherosclerolsis plaque and on injured endothelial lining, but not on normal vascular endothelials (Kasirer-Friede et al., 2007; Nurden, 2006).

2.3.6 **P2Y1 receptors**

The P2Y1 receptor (42 kDa) is widely distributed and contains 377 amino acid residues. It is found in smooth muscle, blood vessels, the testes, neural tissue, ovaries, the heart, the prostrate, blood vessels and platelets. P2Y1 receptors are released from the platelet α granules. P2Y1 receptors are induced by ADP to initiate platelet aggregation and shape changes. They act via G_q coupled receptors for ADP. P2Y1 receptors account for 20-30 % of the binding sites for ADP on platelet membranes (Gachet, 2008).

2.3.7 **P2Y12 receptors**

$P2Y_{12}$ receptor gene is mapped at chromosome 3q21-q25 and contains 342 amino acid residues. These receptors are found in smooth muscle, glial cells, endothelial cells and platelets. ADP stimulates $P2Y_{12}$ receptors, whereas ATP and some of its trisphosphate analog inhibits $P2Y_{12}$ receptor activity. They act through G_i-coupled receptors for ADP which reduce adenylase cyclase activity. Defects in $P2Y_{12}$ receptors are characterized by mucocutaneous bleeding, post-traumatic and post-surgical hemorrhage (Gachet, 2008).

2.3.8 **P2X1 receptors**

The P2X1 genes are mapped at chromosome 17p13.2. It has 399 amino acid residues with two transmembrane domains (TM1 and TM2). An extracellular domain consisting of ten cysteine moieties separates the two transmembrane domains of P2X1. It is expressed in platelets and megakaryocytes. ATP released from dense granules binds to the extracellular domain of P2X1 receptors. This triggers a shape change of platelets and increases the permeability of ions such as Na^+, K^+ and Ca^{2+} in the extracellular matrix. P2X1 was demonstrated to amplify platelet activation even at lower concentrations of other agonists (Oury et al., 2004). P2X1 has been considered as a safe target for the treatment of thrombotic episodes, due to its mild effects on platelet functioning (Hu and Hoylaert, 2010).

2.3.9 **Thromboxane (TXA_2) receptors**

Thromboxane (TXA_2) is synthesized from arachidonic acid via the cyclooxygenase pathway that bind with TXA_2 receptors (Patron et al., 2005). TXA_2 receptors (or TP) (57kDa) have two isoforms (TPα and TPβ), which differ in the C-terminal domains. The two isoforms reside in the platelet membrane. TPα is expressed in platelets whereas TPβ is expressed in endothelial cells (Habib et al., 1999). They activate platelets through G- coupled proteins such as G_q and $G_{12/13}$ (Hirata et al., 1996).

2.3.10 **Prostaglandin E_2 (PGE_2) receptors**

Prostaglandin E_2 (PGE_2) is highly expressed during inflammation of endothelilal cells, smooth muscle and macrophages. PGE_2 exhibits biphasic effects on platelet function, depending on its concentration. At low concentration platelet activation is enhanced, whereas at high concentrations platelet activtation is inhibited. Four G-coupled receptors (EP_1, EP_2, EP_3 and EP_4) activate PGE_2. Each of these receptors possesses a distinct intracellular signal and pharmacological signature. EP3 receptors enhance platelet activation by increasing the influx of intracellular calcium and P-selectin, whereas EP2 and EP4 inhibit platelet activation by increasing cAMP intracellular levels via Gαs protein (Ma et al., 2001). In mouse models, the EP3 receptor has been

implicated in atherosclerotic plaque (Gross et al., 2007). EP4 receptors enhance the platelet inhibitory action of Aspirin and can be used more effectively as an antagonist (Philipose et al., 2010).

2.3.11 **Prostaglandin I_2 (PGI_2)**

Prostaglandin I_2 (PGI_2) is synthesized from arachidonic acid. It is widely expressed on vascular endothelial cells and platelets. PGI_2 serves as a vasodilator, an inhibitor of platelet aggregation and maintains vascular smooth muscle integrity. It binds to the prostaglandin receptor (IP receptor) to inhibit platelet aggregation. The IP receptor (37-41 kDa) is a member of the prostanoid G-receptor family and, upon activation, it enhances the expression of adenyl cyclase which increases intracellular CAMP (Stitham et al., 2007). Synthestic salt of PGI_2 (Epoprostenol) was demonstrated to inhibit the interaction of platelet-leucocyte and platelet microparticle formation. Therefore, PGI_2 might be a potent inflammatory therapy for thrombosis (Tamburrelli et al., 2011).

2.3.12 **Thrombin receptors**

The ability of thrombin receptors to stimulate platelet aggregation is partially dependant on GPIb-IX-V, but it prefers the two protease-activated receptors (PAR-1 and PAR-4). GPIb has been demonstrated as the enhancer of thrombin to PAR-1 and PAR- 4. Patients lacking GPIb show poor thrombin response (Adam et al., 2003). PAR-1 and PAR- 4 are activated by irreversible proteolytic cleavage of the extracellular loop; this exposed the N-terminal that serves as the tethered ligand. PAR-1 is the major thrombin receptor which is stimulated at low concentrations, whereas PAR-4 serves as a back up receptor, stimulated at high concentrations. PAR-4-triggered Ca^{2+} influx into the intracellular matrix is slow but prolonged, whereas PAR1 Ca2+ mobilization is fast and easy to switch off (Oestreich, 2009).

2.3.13 **Eph kinases**

Eph kinases are a member of tyrosine kinase receptors with ephrin as the preferable ligand. It is expressed on the cell surface and has an extracellular tyrosine kinase

domain and an intracellular domain. The interaction between Eph kinases and ephrin are implicated in vasculogenesis and neuronal patterning (Prevost et al., 2003). The human cell expresses three isoforms of ephrin (Eph A4, ephrin B1 and EphB1). Eph A4 or Eph B1 enhances platelet adhesion to fibrinogen during platelet aggregation, whereas ephrin B1 interacts with αIIbβ3 in both a resting and an activated state. The activities of the isoforms are enhanced through the activation of Raph1, a member of the Ras family that stimulates the activation of platelet integrins (Prevost et al., 2003).

2.3.14 **Gas 6 (growth arrest-specific gene 6)**

The Gas 6 (growth arrest-specific gene 6) is a vitamin K dependent protein integrin. Gas 6 plays an important role in cell adhesion, growth and migration through its association with TAM family receptors (Tyro 3, Mer and Axl tyrosine receptor). In humans, Gas 6 is mostly expressed in plasma, whereas in mice it is predominantly found in plasma and platelets. Gas 6 has been demonstrated to play a crucial role in vascular homeostasis and thromobogenesis. Gas 6 interacts with αIIbβ3 outside-in signaling through the activation of AKt and PI-3K which trigger β3 phosphrylation (Angelillo-Scherrer et al., 2005). These processes describe the mechanism of ADP thrombus stabilization during platelet aggregation. (Cosemans et al., 2006, 2010).

2.3.15 **P-selectin receptors**

P-selectin (140 kDa) is a member of selectin family with a high adhesion affinity to the extracellular matrix. P-selectin preferable ligand is P-selectin glycoprotein ligand-1 (PSGL-1) which is predominantly found in leukocytes (Abdullah et al., 2009). The interaction between P-selectin and PSGL-1 enhances the tethering of activated platelets and leukocytes on the surface of endothelial lining. Tissue factors are shown to play important roles in the fibrin network during the activated coagulation cascade. This assists in the stabilization of thrombin formation. Tissue factors have been shown to be dependent on monocytes carried by microvesicles. The microvesicules are attached to the ongoing thrombus formation through the interaction of P-selectin and PSGL-1, thus delivering tissue factor to the thrombus (Morel et al., 2008). Mice models with a

deficiency in either P-selectin or PSGL-1 have been demonstrated to show a reduced thrombus size (Ramacciotti et al., 2009).

The receptor–ligand interactions are important for the recruitment of platelets in circulation in the damaged endothelials, thus enhancing platelet activation and platelet aggregation. During high shear stress in arterioles, the interaction between platelet receptors (GPIb) and Von Willebrand factor is important for platelet function. Von Willebrand factor is produced and stored in the endothelial matrix. Likewise, Von Willebrand factor is recruited to bind to exposed collagen fibres during damage to endothelial lining (Savage et al., 1998). The Von Willebrand factor –GPIb complex forms a weak adhesion of platelet and collagen receptors (GPVI) complementing the action of the complex by strengthening the adhesion. This process triggers conformational changes in β integrin on platelet membranes, thus increasing its affinity to their ligands. The platelet adhesion binds to collage via α2β1 intergin to initiate platelet spreading, whereas platelet adhesion binds via αIIβ3 to initiate platelet aggregation by increasing fibrinogen binding affinity (Varga-Szabo et al., 2008). Platelet receptors are also implicated in platelet interactions in inflamed endothelial cells and leukocyte interactions during pro-inflammatory activities (Pitchford et al., 2003).

During damage to the vascular endothelial, the subendothelail collagen fibrils are exposed to endothelial factors in circulation such as the fibronectin, vitronectin, laminin, proteoglycans and Von Willebrand factor (Hoylaerts et al., 1997). Sub-endothelial VWF binds to collagen VI and to exposed collagen I and III to enhance the recruitment of more Von Willebrand factor and afford multimetric VWF strands. This tethers the platelet receptor GPIbα to initiate platelet rolling on ruptured endothelial lining (Wu et al., 2000). The GPIbα (135 kDA) along with GPIbβ (26 kDa), GPV (82kDa) and GPIX (20 kDa) constitute the members of GPIb family. The four subunits of the GPIb family are encoded to gene mapping of chromosome 17p12 (GPIBA), 22q11.2, (GPIBB), 3q21 (GP9) and 3q29 (GP5) respectively. Most of the encoded genes are expressed in platelets, and those that remain are expressed in endothelial cells (Wu *et al.,* 1997). The GPIb domain sites accommodate the binding for mac-1, P-selectin and Von Willebrand factor. The GPIb domain serves as a receptor for coagulation factors (XI, XII), kininogen

and thrombin (Bradford et al., 2000; Baglia et al., 2002; Lanza, 2006). GPIbα favours the binding of thrombin to its appropriate receptor (a protease activated receptor). The thrombin cleavage of GPV exposes the GPIbα-IX, and thus enhances the binding affinity of GPIbα to thrombin (Ramakrishnan *et al.,* 2001). GPIb receptors are implicated in platelet inflammatory pathways by binding to endothelial cell P-selectin (Romo et al., 1999). Defects in GPIb receptor genes can result in a diseased condition known as Bernard-Soulier Syndrome (BSS). This is characterized by larger platelets, prolonged bleeding times and thrombocytopenia (Poujol et al., 2002).

2.4 Platelet aggregation

Platelet aggregation (Figure 2.5) is a natural process in which thrombocytes cluster together to help prevent bleeding at the site of vascular injury. The human body is in equilibrium with the factors that prevent bleeding and the factors that prevent excessive blood clotting in the hemostatic system. The hemostatic system helps the body to maintain free vascular pathways by aiding the formation and breakdown of blood clots. Platelets play an important role in the maintenance of homeostasis.

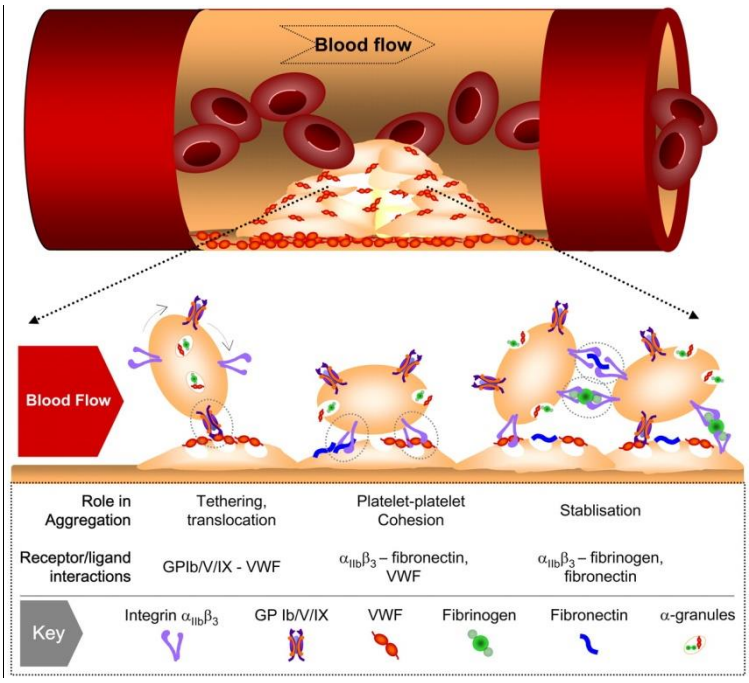


Figure 2.5: Processes of platelets aggregation (adapted from Jackson, 2007)

2.5 Hemostatic system

Hemostatic system (Figure 2.6) is an instinctive response of the body to prevent bleeding, thereby preventing the loss of blood. Four steps occur in a rapid sequence during this process. These steps are listed below:

Vascular spasm: This is the first response to damaged blood vessels, causing vessels to constrict and prevent blood loss. It is triggered by factors such as the direct injury to vascular smooth muscle. Cell signaling molecules such as cytokines, P-selectin, prostaglandins are released by endothelial cells, and platelets and refluxes initiated by local pain receptors all contribute to vascular spasm. The spasm response increases according to the level of damage to the vessels (Marieb, 2012).

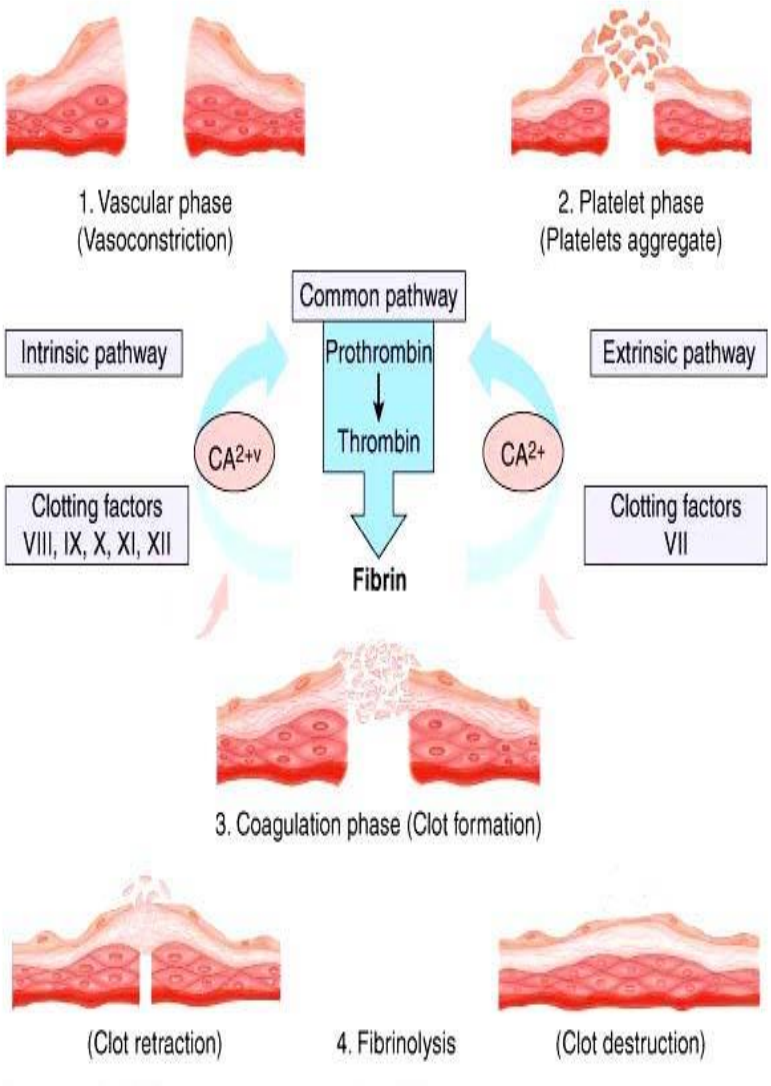
Formation of platelet plugs: This is the second step, in which platelets stick together to form a temporary seal over the ruptured vessels and degranulate. The degranulated platelets release cell signaling substances such as adenosine di-phosphate (ADP), serotonin and thromboxane A2. These cause more platelets to stick to the region of damage and release their contents. As more chemicals are released, more platelets stick together to form larger platelet plugs. Platelets are responsible for preventing vascular damage under the skin on a daily basis (Clemerison, 2012). Platelet plug formation is activated by a glycoprotein called Von Willebrand factor (VWF), which is found in the blood plasma (Lassila et al., 2012).

Coagulation or blood clothing: This is the third step, in which coagulation strengthens the platelet plugs with the fibrin threads that act as a “molecular glue” (Marieb, 2010). There are two pathways for blood coagulation: intrinsic and extrinsic. This converts pro-thrombin into thrombin, which in turn converts fibrinogen into the fibrin that forms the insoluble mesh during blood clotting (Porth, 2005).

Within the coagulation pathways, the anti-thrombin regulator inhibits the effect of serine proteases. This is achieved by preventing the conversion of zymogens into active factors. The pro-coagulant and anticoagulant factors maintain the balance in the vascular system (Stassen et al., 2004).

Fibrinolysis. This is the fourth step in the hemostatic system, in which the break down of

blood clots is regulated and occlusion of the vascular system prevented. This occurs when plasminogen activators are present in the endothelium and plasminogen is converted into plasmin which is responsible for the degradation of insoluble fibrin (Stassen et al., 2004).



Biology of hemostasis. The four physiologic processes that interrelate to limit blood loss from an injured vessel are illustrated and include vascular constriction, platelet plug formation, fibrin clot formation, and fibrinolysis.

Figure 2.6: Biology of homeostasis system www.press.com/2010/06/biology-hemostasis

The activation of platelets causes changes in the shape of platelet morphology and conformational changes in the glycoprotein IIb/IIIa receptors, thereby transforming the receptors from a ligand-unreceptive state to a ligand-receptive state. Ligand-receptive glycoprotein Iib/IIIa receptors bind fibrinogen molecules, which form bridges between adjacent platelets and facilitate platelet aggregation. Inhibitors of glycoprotein IIb/IIIa receptors also bind to glycoprotein Iib/IIIa receptors, blocking the binding of fibrinogen and thus preventing platelet aggregation (Yerem, 2000).

The formation of blood clots (thrombus) within blood vessels can be physiological or pathological. Physiological thrombus formation is controlled by a variety of receptors on the platelet surface. The receptors participate in the process of thrombosis, i.e. the platelet–mediated formation of a hemostatic plug. There are four steps in the formation of a thrombosis. These are: (1) the activation of platelet glycoproteins IIb/IIIa (GpIIb/IIIa) by different agonists, such as blood proteins and enzymes; (2) platelet adhesion to vascular lesions by means of cell receptors; (3) platelet aggregation into a large mass called thrombus; and (4) coagulation due to the platelet aggregation that facilitates the formation of an impervious three dimensional meshwork (Cook *et al.,* 1994).

Pathological thrombus formation is caused by platelet hyperactivity and, when unchecked, could result in atherothrombotic diseases such as stroke, heart attacks, and pulmonary embolism (Huo and Ley, 2004).

Platelet dysfunctions are contributed to by adenosine diphosphate (ADP), thrombin, arachidonic acid, collagen, epinephrine and other factors such as free radicals, inflammation, stress and hypercholesterolemia. The thrombin activates platelets through two protease–activating receptors (PAR), PAR 1 and PAR4, belonging to G protein–coupled receptors. ADP can only bind to the G protein–coupled P2Y1 receptor and activates phospholipase C, thus resulting in an increase in the intracellular concentration of Ca^{2+} (Donna et al., 2001).

2.6 Acetylcholinesterase

Acetylcholinesterase (Figure 2.7) is a member of carboxylesterase family that hydrolyzes the neurotransmitter acetylcholine (ACh) into acetate and choline. They are

predominantly found in conducting fibres, such as muscle, sensory and motor fibres, nerve, noncholinergic and cholinergic fibres, and central and peripheral tissues (Massoulié et al., 2008). They have a very high turnover: a molecule of acetylcholinesterase hydrolyzes 2500 molecues of ACh per second (Taylor, 1994). The active binding site of the enzyme is divided into two sub-sites: the esteratic site and the anionic site. The esteratic site is where the acetylcholine is hydrolyzed to acetate and choline, whereas the anionic site is where the inhibitor, the quaternary amine of ACh and the cationic substrate bind (Tripathi, 2008). During nerve signal transmission, ACh is released from the pre-synaptic vesicle of nerve cells into the synaptic cleft to bind ACh receptors at the post-synaptic membrane and relay the nerve signal. ACh at the post-synpatic nerve terminates the signal by hydrolyzing the Ach into acetate and choline. The liberated choline is used to synthesise more ACh at the pre-synaptic nerve by combining with acetyl-CoA in the presence of choline acetyltransferase (Purves et al., 2008).

In maintaining the signal transmission, the ACh receptors must always be vacant to accommodate another ACh. This occurs when there is a reduction in ACh concentration at the synaptic cleft. The inhibition of ACh at the post synaptic nerve leads to the accumulation of ACh at the synaptic cleft, thus impeding nerve transmission (Pohanka, 2012).

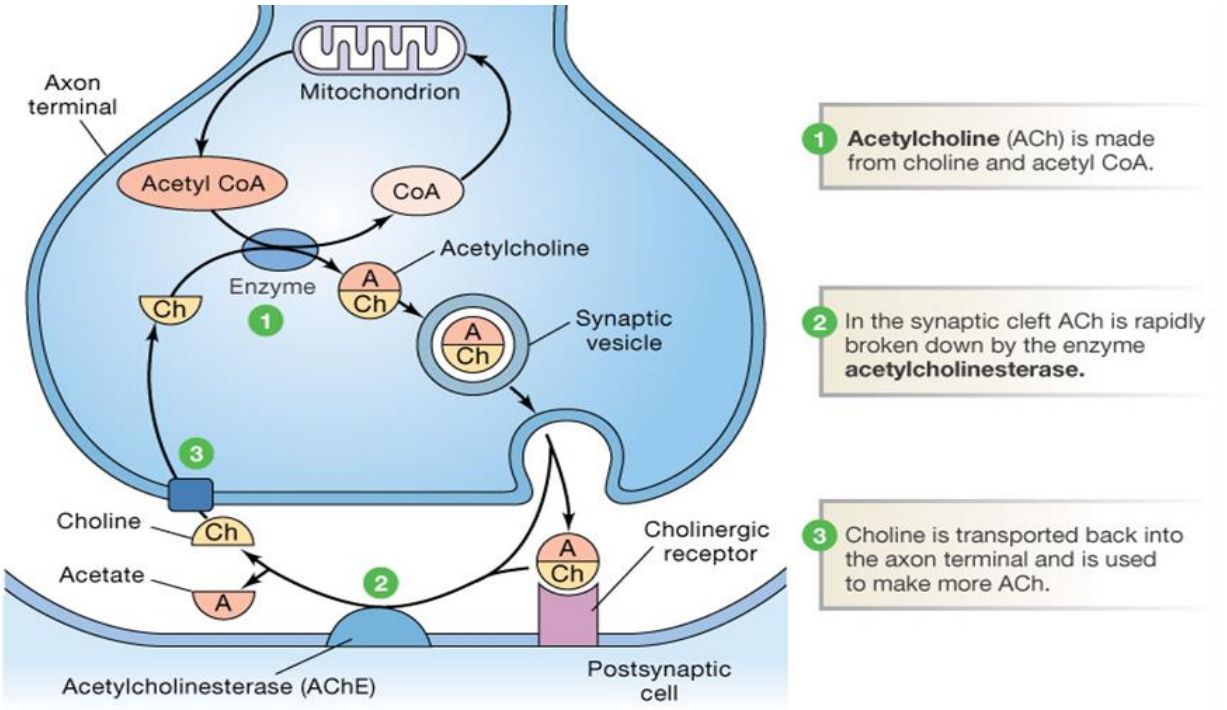


Figure 2.7: Mechanism of acetylchilinesterase adapted from www.peaknootropics.com

The loss of nerve signal transmission can result in neurodegenerative diseases characterized by the progressive loss of function or structure of neurons, particularly those of the central nervous system (CNS). Protein aggregation in neurons could also contribute to the progression of neurodegeneration (Park, 2010). Alzheimer's disease (AD) is one of well-known neurodegeneration diseases. AD is characterized by memory loss, poor judgment, language deterioration, and human motor and perceptual spatial skill deterioration. Cholinergic neurotransmission dysfunction in the brain contributes to prominent cognitive decline in AD. The damage to cholinergic cells results in a decrease in concentration of ACh. This also leads to the accumulation of β-amyloid (Aβ) which disrupts synaptic functioning and neural networking. The amyloid fibres trigger pathological cascades, which ultimately culminate in neuronal death (Cramer et al., 2012).

Acetylcholine stimulates the NO released from the endothelial vasculature, which leads

to the relaxation of smooth muscle and vasodilation by increasing the soluble cGMP level. This mechanism inhibits platelet aggregation and adhesion (Andrew et al., 2001). Likewise, prostacyclin a potent antiplatelet agent is also released from endothelial vasculature in response to ACh. This stimulates the G-protein stimulators (Gs) that stimulate the adenylate cyclase activity which increase the production of cyclic AMP (Moncada, 1982). Acetylcholinesterase is also found in red blood cells. It constitutes the Yt blood group antigen (Purves et al., 2008). Acetylcholinesterase functions by terminating nerve signals and also reduces the supply of clotting factors into the blood stream (Sokratov and Skipetrov, 1977).

Reversible AChE inhibitor drugs such as tetrahydroaminoacridine, rivastiggmine and donepezil are used in the treatment of AD. These drugs bind to the esteratic site of the AChE for a short time, activating their therapeutic effect (Shaked et al., 2009). Some medicinal plants such as *Pistacia atlantica* and *P. lentiscus* have been demonstrated to inhibit AChE activity (Benamar et al., 2010).

2.7 Phosphodiesterase

Phosphodiesterase (PDE) (Figure 2.8) is an enzyme that degrades the phosphodiester bond of secondary messengers such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). They regulate the activity of the cyclic nucleotide signal within the cellular matrix. They are therefore important in the transduction of signals mediated by secondary messengers (Conti, 2000). PDE are predominantly found in the brain and tissues. It is classified into 11 families (PDE1-PDE11) based on their regulation properties, substrate specificities, amino acid sequences, tissue distribution and pharmacological properties. The PDE family shows substrate selectivity; PDE (1, 2, 3, 10 and 11) hydrolyzed cAMP and cGMP, PDE (4, 7 and 8) hydrolyzed cAMP whereas PDE (5, 6 and 9) hydrolyzed cGMP. The binding of either cyclic nucleotides to the GAF-B regulatory domain enhances the activity of alternate nucleotides (Iffland, et al., 2005).

The two intracellular secondary messengers, cAMP and cGMP, provide potent inhibitory activity on platelet aggregation. cAMP inhibits platelet aggregation by stimulating cAMP

dependent intracellular Ca^{2+} influx whereas cGMP enhanced the production of the vasorelaxin factor that inhibits adhesion and aggregation of platelets (Gresele et al., 2011). The pharmacological inhibition of PDE prolongs the physiological effects mediated by the cyclic nucleotides (cAMP and cGMP). Sildenafil (Viagar) selectively inhibits PDE5, which regulates the vasodilation of the artery to the corpus cavernosum through cGMP. This process enhances prolonged erection and could thus be used to treat erectile dysfunction (Jeon, 2005). Similarly, Sildenafil could also be used for the treatment of benign prostatic hyperplasia and Duchenne Muscular Dystrophy by enhancing myo and cardioprotective effects (Khairallah et al., 2008; Wang, 2010).

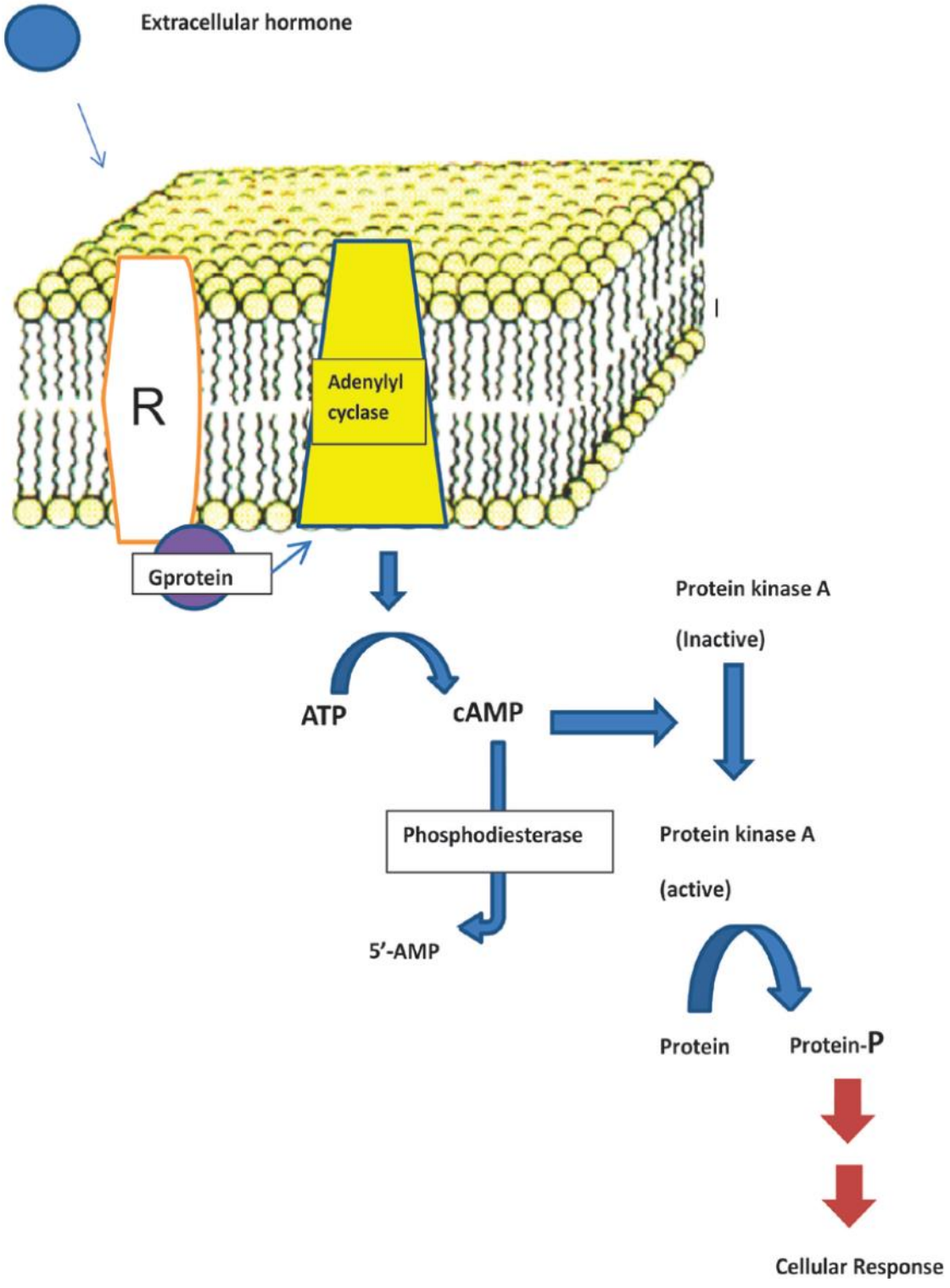


Figure 2.8: The Cyclic AMP Pathway (adapted from Moustafa and Feldman, 2014)

2.8 Inflammation

Inflammation is the complex biological response of vascular tissue to harmful stimuli such as damaged cells, microbial activities and irritants (Ferrero, 2007). Inflammation is characterized by the interaction between platelets, endothelial cells and leukocytes. During infection, the endothelial cell is activated to release the adhesion molecules, such as endothelial adhesion molecules 1 and intercellular adhesion molecules, which enhance the rolling of activated leukocytes to the site of infection. This process causes the generation of reactive oxygen specie by leukocytes which trigger the secretion of stored pro-inflammatory cytokines and chemokines such as P-selectin, E-selectin, CD40-L, and RANTES from activated platelet granules. The pro-inflammatory cytokines further amplify the recruitment of more leukocytes to the site of infection (Denisa and Peter, 2003).

Inflammation also contributes to the platelet aggregation process through arachidonic acid pathways (Figure 2.9). Once the arachidonic acid is released from the membrane, phospholipid is converted into prostaglandins and leukotriene by phospholipase. The prostaglandin produced is then converted into thromboxane A2, prostaglandin I2, prostaglandin E_2 and prostaglandin D_2 by cyclooxygenases that interfere with the platelet aggregation process (Murphy, 2004).

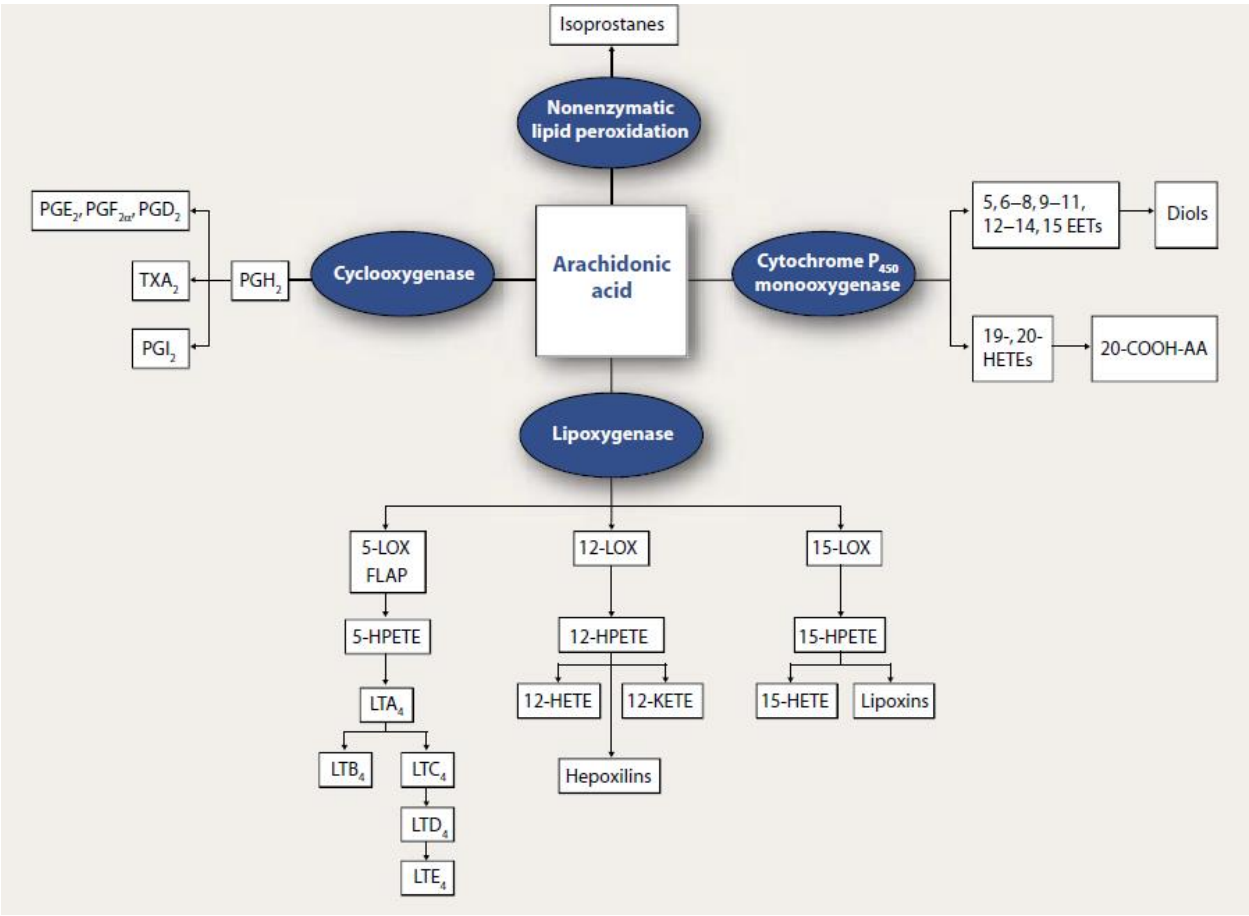


Figure 2.9: The four major pathways for Arachidonic acid metabolism (adapted from Smith, 2006).

2.8.1 **Cyclooxygenase (COX-1 and COX-2)**

Cyclooxygenase (COX) is an enzyme that plays an important role in the formation of prostanoids, including thromboxane, prostaglandins and prostacyclin. The inhibition of COX by non-steroidal anti-inflammatory drugs (NSAID) such as aspirin and ibuprofen reduce the symptoms of inflammation, pain and fever. COX is classified into two types (COX-1 and COX-2) (see Figure 2.10). COX-1 acts as a constitutive enzyme. It is required for normal physiological function, whereas COX-2 is an inducible enzyme that is produced during inflammation (Funk and Fitz-Gerald, 2007).

The COX-1 and COX-2 have similar molecular weights (70 and 72 respectively),

identical binding sites and 65% homologous amino acid sequences. The major difference between the COX isoenzymes is the type of amino acid at position 523; COX-1 has isoleucine, whereas COX-2 has valine. The smaller Val_{523} residue in COX-2 allows access to a hydrophobic side-pocket in the enzyme (which Ile_{523} sterically hinders) (Hawkey et al., 2001; Schachte, 2003).

NSAIDs are considered nonselective because they inhibit both COX-1 and COX-2. The inhibitions of thromboxane and prostaglandin synthesis by NSAID have analgesic, antithrombotic, antipyretic and anti-inflammatory effects. Gastrointestinal tract disorder is the common side effect of NSAID, resulting from reduced protection from prostaglandin on the gastrointestinal mucosa (Wallace, 2008). The inhibition of COX-2 by NSAIDs accounts for the anti-inflammatory effect of the drugs. COX-2 inhibition decreases prostacyclin formation, thus increasing the thromboxane imbalance which leads to cardiovascular diseases such as stroke, heart attack and thrombosis (Kearney et al., 2008).

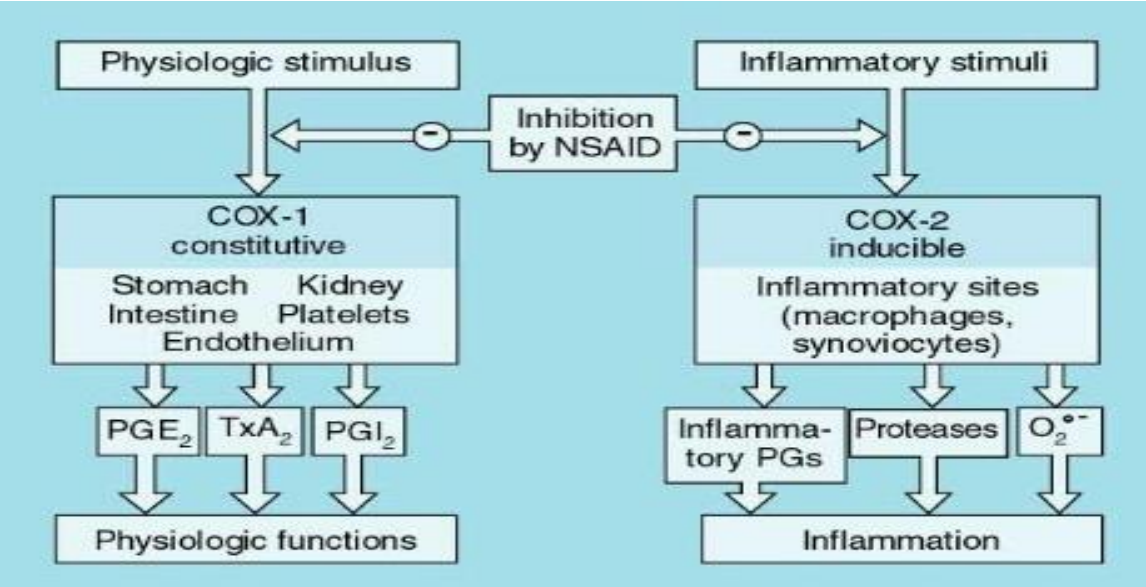


Figure 2.10: The difference between COX-1 and COX-2 (adapted from Dubois *et al.*, 1998).

2.9 Reactive oxygen species

Reactive Oxygen Species (ROS) are reactive chemical molecules possessing oxygen. Examples of ROS include oxygen ions and peroxides like superoxide anion (O_2^-), hydroxyl radical (OH), hypochlorous acid (HClO) and hydrogen peroxide (H_2O_2) (Valko et al., 2007). These are formed as a by-product of normal cellular metabolism. ROS plays a dual role: it is either beneficial or harmful to living systems (Valko et al., 2006). The beneficial role of ROS occurs at low concentrations; this includes cellular signaling, immue responses and mitogenic responses. The harmful role of ROS occurs at high concentrations, which lead to cellular damage due to oxidative stress (Kovacic et al., 2005; Ridnour et al., 2005). The oxidative stress (Figure 2.11) is caused by an imbalance of peroxidant and antioxidant enzymes in the living organism (Betteridge, 2000). This results in cellular damage to proteins, lipids and DNA, resulting in a reduction in their normal physiological functioning. Redox homeostasis protects the living organism against oxidative stress damage by maintaining the ROS and antioxidant equilibrium (Droge, 2002).

The inter-relationship among ROS, cancer and inflammation has been demonstrated over the years by epidemiologic and experimental research (Gupta *et al.,* 2012). Inflammation is induced by the expression of COX-2 by ROS, pro-inflammatory transcription factors (NF-κB), inflammatory cytokines (interleukin 6 (IL-6), chemokines (IL-8, CXCR4), and interleukin 1 (IL-1), tumor necrosis factor alpha (TNFα) (Gupta et al., 2012).

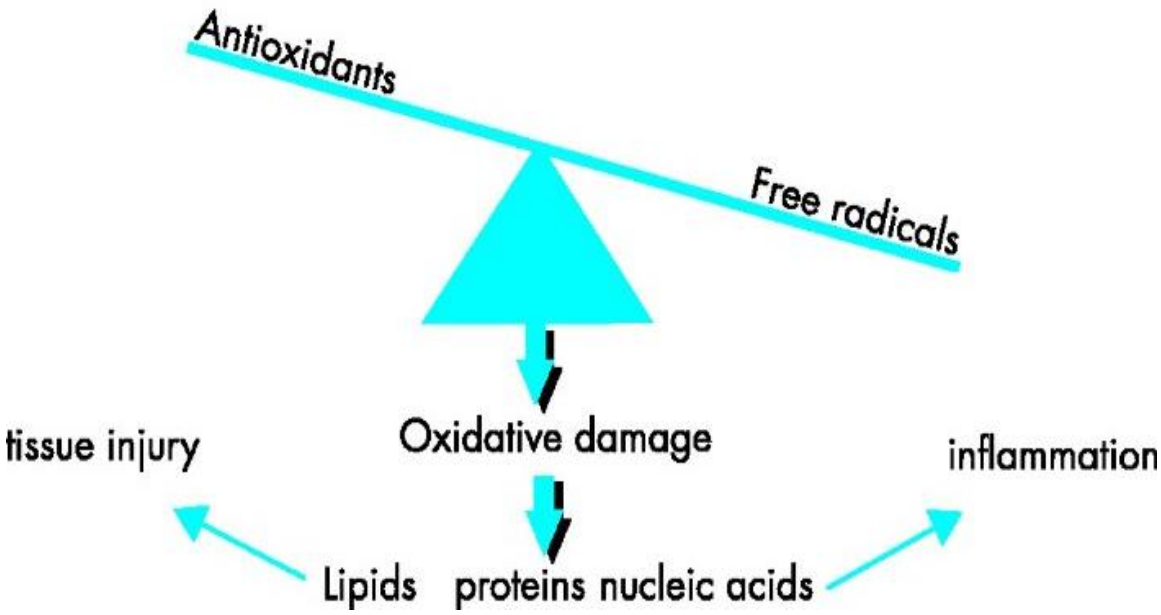


Figure 2.11: Oxidative stress (adapted from Conner and Grisham, 1996)

2.9.1 **Antioxidants**

Antioxidants are molecules that prevent the oxidation of other molecules. Oxidation involves the generation of ROS which develop other chains of reaction that could eventually result into the damage of cellular components such as proteins, lipids and DNA (Vertuani et al., 2004). Antioxidants terminate the chain of reaction by removing ROS intermediates and preventing other oxidation. ROS includes the free radical O_2^- and OH, HClO and H_2O_2 (Valko et al., 2007). ROS demonstrates involvement in redox signaling, and antioxidants are therefore expected not to totally remove ROS but to reduce ROS to optimum levels (Rhee, 2006).

Antioxidants are classified into two categories based on lipid or water solubility. The lipid soluble antioxidants protect the cell membranes from lipid peroxidation, whereas water soluble antioxidants prevent oxidation in blood plasma or cell cytosols (Sies, 1997). Most of the antioxidant may be synthesized by the body system or derived from diet (Vertuani et al., 2004). Antioxidants could be vitamins (A, C and E), enzymes (CAT, SOD and perioxidases) and other low molecular weight substances (e.g. Uric acid) (Figure 2.12).

2.9.1.1 **Superoxide dismutase**

SOD (Figure 2.12) is a metalloprotein enzyme that catalyses the dismutation of the superoxide radical (O_2^-) to oxygen or hydrogen perioxide (H_2O_2). Superoxide radicals are chemical toxins that are produced as by-product of the metabolism of oxygen, which causes cell damage (Alscher et al., 2002). SOD is divided into three groups based on metal cofactors and protein folds. These include the Ni type (binds to nickel), Cu/Zn type (binds to copper and zinc) and Fe and Mn type (binds to Fe or Manganese). The Cu/Zn type is found predominantly in cytosol, whereas the Mn type is common in mitochondria. The Ni type is usually found in the prokaryotic cells (Barondeau et al., 2004; Borgstahi et al., 1992).

Superoxide dismutase is necessary to reverse the negative effects of superoxides on the citric cycle. Superoxide inactive aconitase is an enzyme that converts citric acid to iso-citric acid in the citric acid cycle. This could result in metabolic poisoning and the

release of toxic iron into the extracellular tissue (Gardner, 1995). SOD also serves as an anti-inflammatory agent by inhibiting endothelial activation, thus preventing endothelial-leukocyte interactions and the expression of adhesion molecules (Segui et al., 2004).

SOD occurs naturally in all living organisms. It is also available in dietary supplements and in injectable form. It is used in the treatment and prevention of diseases related to ROS damage (Lien et al., 2008), including inflammatory diseases such as colitis, inflammatory bowel disorders and cancer (Beaugerie and, Itzkowitz, 2015).

2.9.1.2 **Catalase**

Catalase (Figure 2.12) is an important antioxidant enzyme predominantly found in living organisms. It protects cells from oxidative stress damage by reactive ROS. Catalase enhances the decomposition of hydrogen peroxide to form water and oxygen (Chelikani et al., 2004). Catalase also acts as a reducing agent of various toxins and metabolites such as formaldehyde, alcohols, phenol, acetaldehyde and formic acid (Ogura and Yamazaki, 1983). Cyanide acts as a competitive inhibitor of catalase at high concentrations of hydrogen peroxide, whereas heavy metals such as copper and cations in copper (II) sulphate exhibit non-competitive inhibitors (Ogura and Yamazaki, 1983). Arsenate serves as an activator of catalase at a high concentration of hydrogen peroxide (Kertulis-Tartar et al, 2009).

The biological importance of catalase in living organisms is dispensable. Mice deficient in catalase are found to be phenotypically normal. However, catalase deficiency has been demonstrated to cause type 2 diabetes mellitus (Ho et al., 2004). Hydrogen peroxide is used by antimicrobial agents against pathogen invasions in the host. However, some pathogens, such as *Campylobacter jejuni, Legionella pneumophila* and *Mycobacterium tuberculosis* are able to survive in the host by producing catalase which decomposes the hydrogen peroxide to form water and oxygen (Srinivasa-Rao et al., 2003).

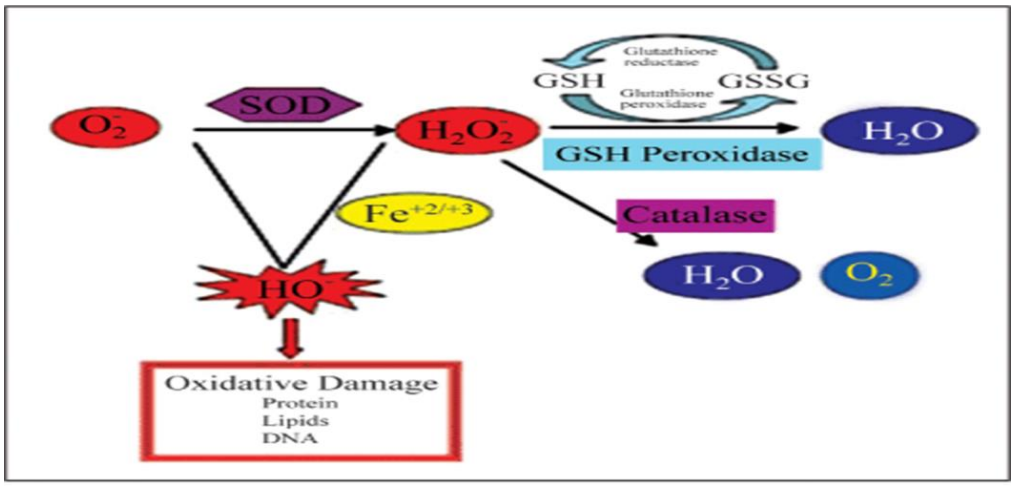


Figure 2.12: Diagram that shows how SOD and Catalase carry out their functions(adapted from www.final-yearproject.com)

2.10 Treatment of platelet aggregation

Various antiplatelet drugs decrease platelet aggregation, and thus inhibit thrombus formation. They are effective in arterial circulation where anticoagulants have effect. They are widely used in the primary and secondary prevention of thrombotic cerebrovascular or cardiovascular diseases. Most antiplatelet drugs are able to prevent the formation of blood clots, significantly contributing to the management of pathogenesis in cardiovascular diseases (Halt and Chandra, 2002). However, drug toxicity occurs when multiple antiplatelet drugs are used in treatments. The most common symptom is bleeding of the gastrointestinal tract (Shehab et al., 2012). Therefore there is an urgent need to search for more potent drugs from natural origin (Amani et al., 2009).

2.10.1 **Aspirin**

Aspirin (Figure 2.13) is one of the non-steroidal anti-inflammatory drugs (NSAIDs) widely used as an antiplatelet agent. The Aspirin mechanism of action differs from other NSAIDs, in that it irreversibly inhibits cyclooxygenase 1 (COX-1) instead of cyclooxygenase 2 (COX-2), as is the case for other NSAIDs (Burke et al., 2006). Cyclooxygenase is crucial in the production of thromboxane A_2, a potent platelet agonist

and vasoconstrictor. Thromboxane A_2 triggers platelet aggregation which impedes the normal flow of blood in the arteries. This process might lead to cardiovascular diseases such as stroke, heart attack and pulmonary thrombosis. Low doses of Aspirin are administered to patients immediately after a heart attack to prevent a further heart attack. Aspirin could also serve as an anticancer agent, especially in colorectal cancer (Algra and Rothwell, 2012).

Aspirin (ASA) is known to have three additional mechanisms of action. First, it uncouples oxidative phosphorylation in hepatic mitochondria by diffusion from the inner membrane into the mitochondria matrix where it ionizes to release protons (Somasundaram et al. 2000). Second, it enhances the production of NO radicals in the body which reduce the adhesion of leukocytes, thus preventing inflammation (Paul-Clark et al., 2004). Third, it modulates the activity of NF-kB kappa, a transcription factor which plays a major role in inflammation (McCarty and Block, 2006).

Aspirin is associated with some side effects. The prolonged use of Aspirin increases the incidence of gastrointestinal bleeding (Aster et al., 2004). ASA disrupts normal platelet function, reduced cytoprotection of gastrointestinal mucosa by Prostaglandin E_2 and enhances ulcerogenic effects through the stimulation of gastric mucosa, thus leading to gastrointestinal tract bleeding (Liberopoulos et al., 2006). The action of Aspirin on thromboxane A2 are dose-dependent (>30mg/day), as are its side effects (Patron et al., 2005). It was demonstrated that lower doses of Aspirin (30mg/day) resulted in less gastrointestinal tract complications than higher doses (1200mg/day) (Farrelln et al., 1991).

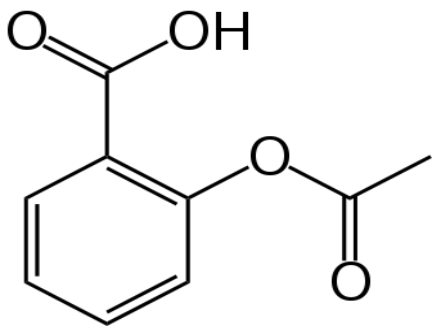


Figure 2.13: Structures of Aspirin (adapted from Neault et al., 2000)

2.10.2 **Clopidogrel**

Clopidogrel (Figure 2.14) belongs to the thienopyridine class of antiplatelet agents. It is widely used to prevent heart attacks, cerebrovascular diseases and coronary artery disease (Anderson et al., 2010). Clopidogrel irreversibly inhibits $P2Y_{12}$ receptors, thus reducing the activity of adenosine diphosphate (ADP) as an agonist in platelet aggregation (Duerschmied et al., 2010). Clopidogrel can serve as an alternative antiplatelet drug for patients with aspirin-induced gastric ulcers (Chan et al., 2005). Clopidogrel is a prodrug; it is metabolized to its active form in the liver by cytochrome P450 2 C19 (CYP2C19) to serve as an antiplatelet agent. The CYP2C19 enzymes are among the superfamily of cytochrome p450. They are monooxgenases that play a crucial role in xenobiotic and lipid metabolism (Mistry et al., 2011).

Clopidogrel has been associated with adverse side effects such as thrombocytopenia purpura and hemorrhaging (Zakarija et al., 2004)**.** This drug interacts with proton pump inhibitors such as esomeprazole or omeprazole, thereby reducing the antiplatelet potential. Clopidogrel exhibits less antiplatelet aggregation effects on patients who are on proton pump inhibitors (John and Koshy, 2012). Studies indicate that patients with pulmonary arteriosclerosis show aspirin resistance and clopidogrel might be the best alternative antiplatelet agent (Diehm et al., 2004).

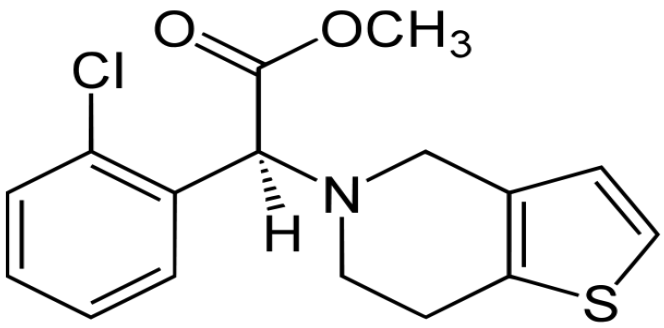


Figure 2.14: Structure of Clopidogrel (adapted from Shaw et al., 2015)

2.10.3 **Dipyridamole**

Dipyridamole (Figure 2.15) is a drug with vasodilator and antiplatelet properties, derived from pyrimidopyridine. Dipyridamole inhibits the activities phosphodiesterase, enzymes that break down cAMP. This increases the intracellular level of cAMP and reduces the binding affinity of ADP to its receptor to initiate platelet aggregation. The drugs also plays a crucial role in triggering the release of prostaglandin (PGI_2) from the endothelial cells and inhibit the reuptake of adenosine by platelets, endothelial cells and red blood cells leading to an increase of adenosine in the extracellular matrix (Sudlow,2005). Dipyridamole is used to lower pulmonary hypertension without a change in systemic blood pressure (De Schryver, 2003). The drug has been implicated in preventing the release of pro-inflammatory cytokines (MMP-9, MCP-1) and in the inhibition of smooth muscle proliferation (Dixon et al., 2009).

Dipyridamole absorption into the gastrointestinal tract is pH-dependent, therefore the use of proton pump inhibitor in the treatment of a gastric ulcer might prevent absorption (Derendorf, 2005). Dipyridamole is usually used along with aspirin as a dual therapy to effectively combat transient ischaemic attacks and stroke (Halkes et al., 2006). In a recent study, stroke patients treated with dipyridamole showed a significant reduction in thrombotic episodes when compared with a control. In addition, the combination of dipyridamole and aspirin showed a more significant reduction in thrombotic episodes in comparison to dipyridamole alone (Leonardi-Bee et al., 2005).

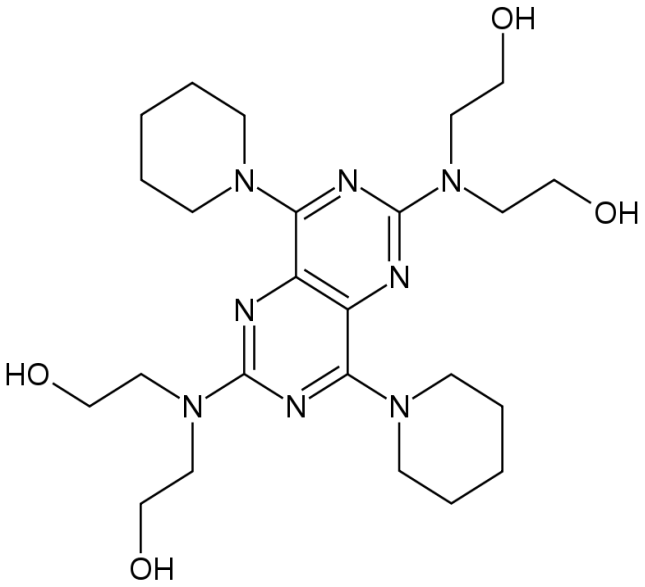


Figure 2.15: Structure of Dipyridamole (adapted from Lin and Buolamwini, 2007)

2.10.4 **Ticlopidine**

Ticlopidine (Figure 2.16) is an antiplatelet agent in the family of thienopyridine. It inhibits ADP receptors on platelet membranes. This prevents the activation of glycoprotein llb/llla integrins which increases the binding affinity of fibrinogen to platelets. It is commonly used in patients who do not tolerate aspirin and clopidogrel. However, the drug is implicated in the onset of neutropenia and aplastic anaemia (Bortolotti et al., 2002).

Ticlopidine, in combination with aspirin as a dual therapy, is found to be more effective in the treatment of recurrent vascular episodes in patients with vascular diseases (Tran et al., 2004).

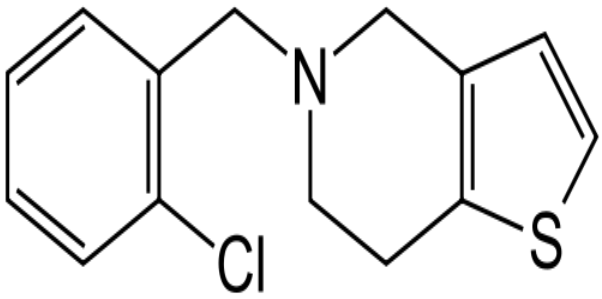


Figure 2.16: Structure of Ticlopidine (adapted from Quinn and Fitzgerald, 1999)

2.10.5 **Cilostazol**

Cilostazol (Figure 2.17) is a derivative of quinolinone which is commonly used as an antiplatelet, vasodilator and anti-thrombotic (Chapman et al., 2003). Cilostazol is reported to relieve intermittent claudication in patients with peripheral vascular diseases (Robless et al., 2008). Cilostazol selectively inhibits phosphodiesterase-III (PDE3) which increases the level of cAMP in the extracellular matrix. This leads to an increase in activated protein kinase A (PKA) which prevents platelet aggregation by reducing calcium mobilization from the saroplasmic reticulum. PKA also inhibits the activation of myosin light-chain kinase, the enzyme responsible for vasoconstriction, thereby exerting vasodilatatory effects. The main function of cilostazol is the vasodilation of the arteries of peripheral and anti-platelet aggregation. The drug was reported to improve walking distance and cardiovascular episodes in patients with a stable inspiratory capacity (Storey, 2002).

Cilostazol is well tolerated, but has unexpected side effects such as headaches, palpitations, increased heart rate, rhinitis, diarrhea, peripheral oedema and abnormal stool (Barnett et al., 2004).

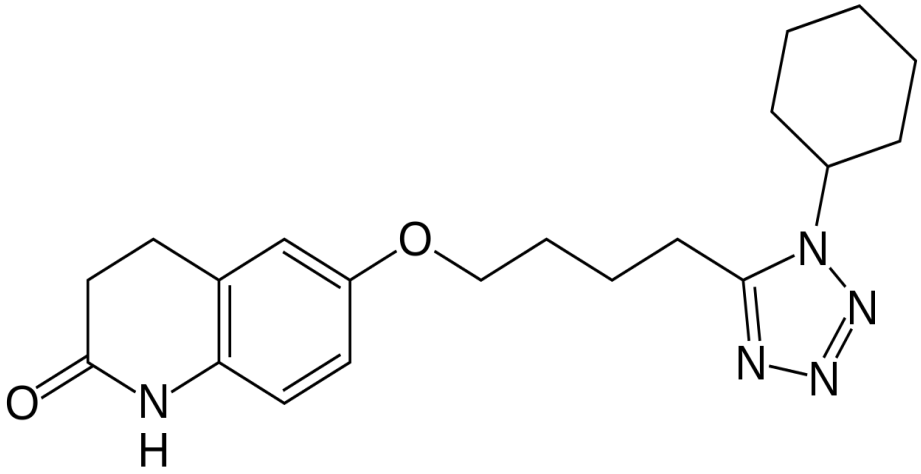


Figure 2.17: Structure of Cilostazol (adapted from Basniwal et al., 2010)

2.10.6 **Sarpogrelate**

Sarpogrelate (Figure 2.18) is a drug that acts as an antagonist at serotonin receptors (5HT2A and 5HT2B) thereby inhibiting platelet aggregation induced by serotonin (Muntasir et al., 2007). In mammals, the drug is metabolized to (R-S)-1-[2-[2-(3-methoxyphenyl)ethyl[phenoxy]-3-(dimethylamino)-2-propanol-M-1through the hydrolysis of its succinate ester moiety in the liver (Saini et al., 2004). Serotonin plays an important role in the onset of atherothrombosis. The stored serotonins in the dense granules of platelets are released during platelet activation. This stimulates smooth muscle proliferation, endovascular contraction and subsequent thrombus formation, which may lead to vessel occlusion (Nishihira et al., 2006). Sarpogrelate was demonstrated to improve endothelial function in patients with peripheral arterial diseases after oral administration for 12 weeks (Miyaza et al., 2007).

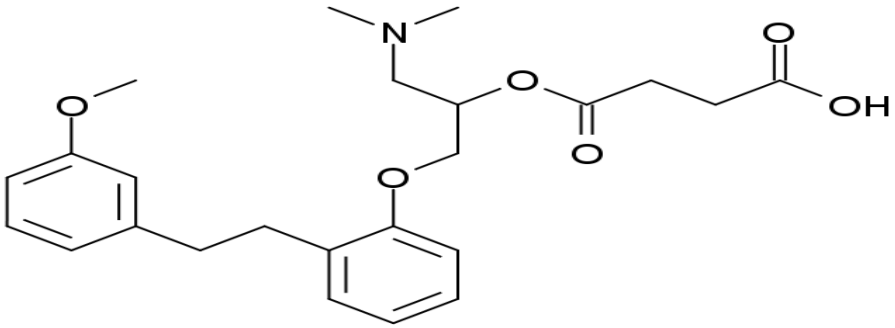


Figure 2.18: Structure of sarpogrelate (adapted from Park et al., 2010)

2.10.7 **GPIIb/IIIa Receptors Antagoinst**

GPIIb/IIIa Receptors are found on the platelet membrane. They are triggered during platelet activation to bind fibrinogen or VWF and form crosslinks with platelets which are the common final pathway to platelet aggregation (Auer et al., 2003). GPIIb/IIIa receptor antagonists are divided into three classifications: monoclonal anti-body fragments (abciximab), peptide inhibitors (eptifibatide) and non-peptide inhibitors (lamifiban and triofiban) (Auer et al., 2003). These drugs are appropriate for diabetes and renal related diseases, but unsuitable for patients scheduled for surgery due to prolonged bleeding (Tcheng et al., 2003).

2.10.8 **Picotamide**

Picotamide (Figure 2.19) is an antagonist of the thromboxane A_2 receptor (TXA_2) and prostaglandin H (PGH_2). It is a derivative of methoxy-isophtalic acid and has been reported to inhibit thromboxane A2 synthase (Neri- Sereneri et al., 2004). The efficacy of picotamide as an antiplatelet is attributed to its dual action, which includes the inhibition of platelet aggregation and the increase in production of antiplatelet aggregation and prostaglandins such as prostaglandin I2 (PGI2) (Gresele et al., 1991). Picotamide was reported to significantly reduce the mortality rate (to 23%) in patients with peripheral arterial diseases when compared to a placebo (Balsano et al., 1993).

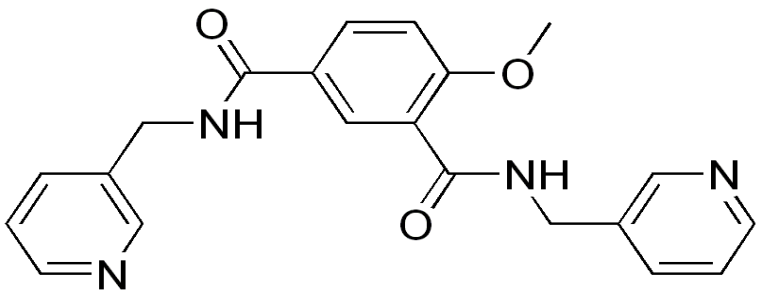


Figure 2.19: Structure of picotamide (adapted from Modesti et al., 1993)

2.10.9 **Beraprost**

Beraprost (Figure 2.20) is an orally administered drug analogue, with similar pharmacodynamic properties to prostaglandin I_2 (PGI_2) (Melian et al., 2002). The beraprost mechanism of action includes: vasodilation, which leads to lower blood pressure; dispersion of abnormal platelet aggregates; and the disruption of platelet aggregation (Nishio et al., 2001). Beraprost binds to PGI2 receptors on the platelet membrane. This stimulates production of the adenylate cyclase and guanylate cyclase which increase the levels of cAMP and cyclic guanosine monophosphate (cGMP), respectively. The cAMP and cGMP inhibit calcium influx into the transmembrane from the intracellular matrix. This process leads to the relaxation of the smooth muscle cell, thereby initiating vasodilaton (Melian et al., 2002). Beraprost is an appropriate drug for pulmonary hypertension and for the reperfusion of injured patients (Barst et al., 2003).

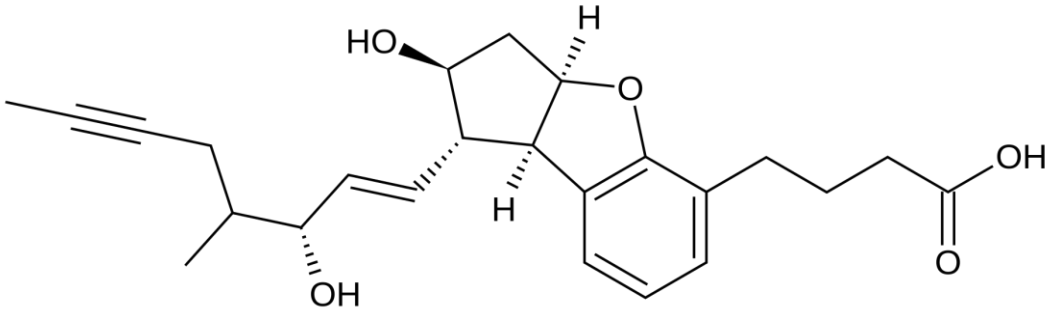


Figure 2.20: Structure of Beraprost (adapted from Morrison et al., 2010)

2.10.10 **Trapidil**

Trapidil (triazolopyrimidine) (Figure 2.21) is an antiplatelet drug widely used to reduce restenosis after percutaneous coronary angioplasty (Maresta, 1994). Trapidil is a platelet derived growth factor antagonist (PDGF) and thus serves as a vasodilator. PDGF is triggered during vascular injury and plays a crucial role in smooth muscle cell proliferation, extracellular matrix formation and inflammatory cell chemotaxis. Trapidil was demonstrated to reduce the incidence of cardiovascular episodes and to improve the prognosis in cardiovascular diseases (Hirayama et al., 2003).

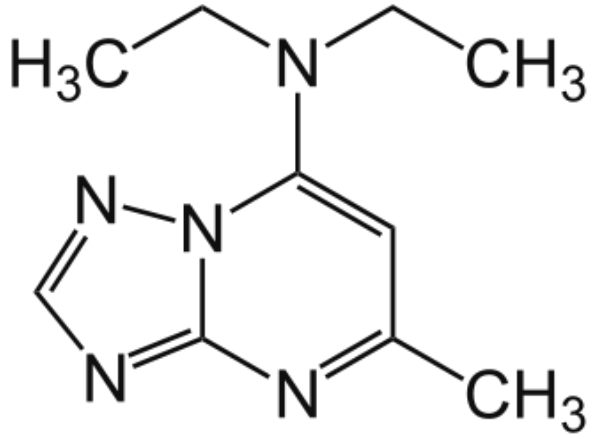


Figure 2.21: Structure of Trapidil (adapted from Vijaya et al., 2013)

2.11 Recent developments in platelet aggregation inhibitors

Explortation of medicinal plants extract as antiplatelet agents have gain new frontline in pharmaceutical research. Some of the recent investigated antiplatelet aggregation potential of medicinal plants include; **ginisenosides** isolated from processed ginseng (Lee *et al*., 2010); **flavonoids** from *Leuzea carthamoides* (Koleckar et al., 2008); **sulfur** containing compounds extracted from *Scorodocarpus borneensis* (Lim *et al*., 1999); **non–glycosidic iridoids** from the leaves of *Canpsis grandifora* (Jin *et al*., 2005); **aporphine alkaloids** isolated from leaves of *Magnolia obovata* (Pyo *et al*., 2003); crude extracts of *Euchresta formosana* (Lo *et al.*, 2003); **phenolic** and **furan** type compounds isolated from *Gastrodia elata* (Pyo *et al*., 2004); extracts of *Cinnamomum*

cassia (Kim *et al*., 2010); **amides** isolated from *Pipe taiwanese* (Chen *et al*., 2007); and **dihydrochalcones** isolated from the leaves of *Muntingia calabura* (Chen *et al*., 2007); Pentacyclic triterpenoids (Oleanolic, Hederagerin, Ursolic acid, Tormentic acid Myrianthic acid) isolated from the leaves of *Campsis grandiflora* (Jin et al., 2010).

2.12 Medicinal plants in traditional medicine

World Health Organization (WHO) defines traditional medicine as the sum total of skills, practices and knowledge based on experiences, beliefs, and theories indigenous to various cultures that are used to diagnose, prevent and treat any forms of ailment (WHO, 2009). Several known medicinal plant species currently used today have been part of traditional medicine going as far back as 2000 BC (Holt and Chandra, 2002). In most developing countries, the use of traditional medicine among the people is based on availability and affordability (Payyappallimana, 2010). The WHO estimates that 80% of the world populace depends on medicinal plants for their basic health care (George et al., 2001).

Africa is reported to be endowed with enormous medicinal plant resources. It is estimated that over 500,000 species can be found in forest regions alone (Farnsworth et al., 1985). A large number of scientific publications are made available on the uses of some medicinal plants (Hutching et al. 1996; Opoku et al., 2002; Iwalewa et al., 2007; Bibhabasu et al.,2008, Baccelli, 2010; Fasola, 2011; Osunsanmi et al., 2015).

A large proportion of modern drugs are derived from medicinal plants (Govaerts, 2001; Thorne, 2002). Medicinal plants are therefore the best source for new drug discovery (Anthony, 2005). For example, *Cinchona succiruba* yields quinine, an important source of antimalalrial (Fabiano-Tixier et al., 2011); *Rauwolfia vomitoria* produces reserpine, an antihypertensive and tranquilizer (Yu et al., 2013); the Calabar bean yields serine or physostigmine, which is used in the treatment of ophtalamia diseases (Triggle et al., 1998); *Chrysanthemum cinerariifolium* produces pyrethrins, which are used as insecticide (Bradberry et al., 2005); *Zingiber officinale* produces gingerol, which is used as a carminative (Ghosh et al., 2011); *Agave sisalana* produces hecogenin, which is used in contraceptives (Elujoba, 2005).

Medicinal plants produce chemical compounds called secondary metabolites, such as phenol, saponin, alkaloid, flavonoid and others (Tapsell *et al*., 2006). These secondary metabolites protect the plants against the attacks of predators and also play an important role in their biological activities. The secondary metabolites are also used to fight a wide range of human diseases. Over 12 000 compounds have been isolated from medicinal plants (Tapsell *et al*., 2006). Herbal medicines do not differ from modern drugs in their mechanisms of action. This is based on the fact that the chemical compounds in medicinal plants mediate their effects on the human body in a manner identical to modern drugs. This makes herbal medicine as effective as modern drugs (Lai et al., 2004). Over 122 of the chemical compounds identified by scientific researchers are derived from medicinal plants and used in modern drugs (Fabricant et al., 2001).

2.13 Triterpenoids

Triterpenes are a class of natural compounds consisting of sterols and steroids. They are abundantly found in plants and animals. Most triterpenes consist of a C-30 carbon skeleton and are biosynthesized from squalene (Figure 2.22). Most triterpenes are produced from squalene through cyclization, ring expansions and molecular losses. An example of such triterpenes is cholesterol (Figure 2.23). There are over twenty groups of triterpenes.

The cyclization of squalene in a chair boat conformation produces the protostane cation. Lanostrane (Figure 2.24), derived from this cation, forms most of the precursor of steroids found in animals, whereas cycloartane (Figure 2.25), derived from a cation by cyclization between C9-C19, forms most of the terpenoids in plants (Buckingham, 1996). These triterpenoids are widely called phytosterol (Buckingham, 1994).

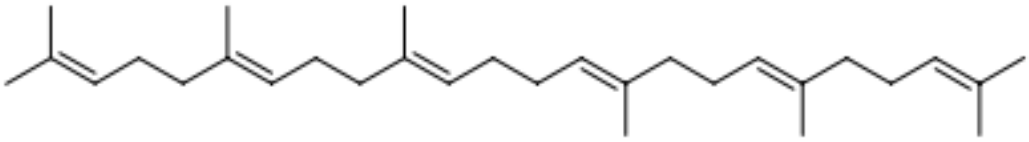


Figure 2.22: Structure of squalene (Buckingham, 1994)

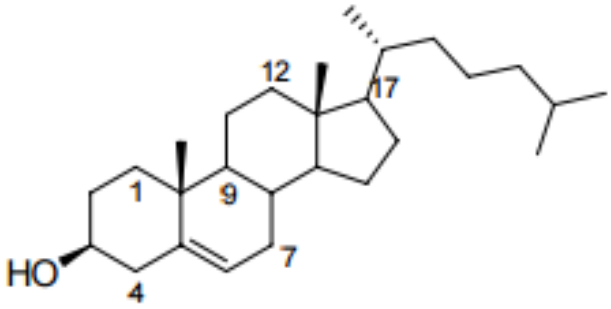


Figure 2.23: Structure of Cholesterol (Buckingham, 1994)

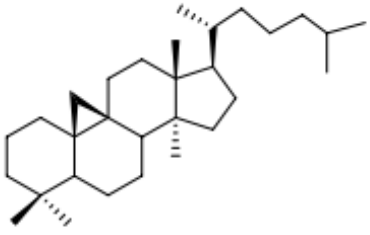


Figure 2.24: Structure of Lanostrane(Buckingham, 1994)

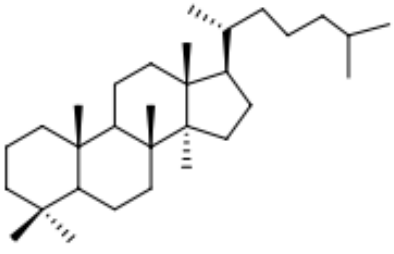


Figure 2.25: Structure of Cycloartane (Buckingham, 1994)

Over 2500 triterpenes have been investigated for their biological and pharmacological activities. These include antimicrobial, antimutagenic, anti-inflammatory, anti-hiv, antitumor and anticancer activities (Connoly et al., 2008, Zaidi et al., 2005, Lin et al., 2003, Qian et al., 2007). The activities of some triterpenes were demonstrated to inhibit the action of multi-drug resistance (Molnar et al., 2006). Tanachatchairatana et al., (2008) demonstrated that a triterpene esterified by cinnamic acid inhibited the activities of *Mycobacterium tuberculosis*.

2.14 Betulinic acid

Betulinic acid (Figure 2.26) is found in the outer bark of several species of plants, but is common in the white birch (*Betula pubescens)* from where its name is derived. It yields up to 22 % dry weight (Tan *et al*., 2003). Betulinic acid is among the naturally occurring classes of pentacyclic triterpenoids which reportedly show anti-neoplastic (Fulda et al., 1999), anti-angiogenesis (Mukherjee et al., 2004), antiplasmodial (Ziegler et al., 2004) antiretroviral (Huang et al., 2006; Qian et al., 2007), anti-viral (Parlova et al., 2003; Baltina et al., 2003), antioxidant, anti-tumour, anthelmintic, anti-inflammatory and antiplatelet (Mukherjee et al., 1997; Liu et al., 2004; Habila *et al.,* 2011; Habila et al., 2013) activities**.**

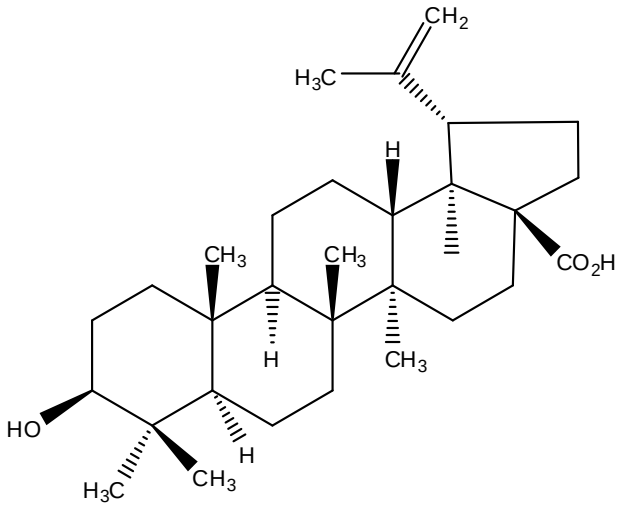


Figure 2.26: Structure of Betulinic acid (adapted from Osunsanmi et al., 2015)

2.15 Melaleuca bracteata

Melaleuca bracteata is a genus in the myrtle family Myrtaceae with fine scented foliage and profuse white flowers appearing in all seasons. It is commonly called black tea tree, paper bark, river tea tree, punk tree, honey myrtle, golden bottle brush, snow in the summer tree, and white cloud tree. There are well over 2000 recognized species, most of which are endemic to Australia (Craven, 2008). *Melaleuca bracteata* var. revolution gold (Figure 2.27) is widely found in South Africa where it is commonly referred to as Johannesburg gold. These species are shrubs and trees growing from 2 - 30 m tall, often with exfoliating bark. The leaves are evergreen, alternately arranged, ovate to lanceolate, 1- 25 cm long and 0.5 - 7 cm broad with an entire margin, dark green to grey-green in colour. The flowers are produced in dense clusters along the stems, each flower has a small petal and a tight bundle of stamens about 7- 8 mm long fused into five bundles (each containing about 20 stamens) opposite the petals; flower colour varies from white to pink, red, pale yellow or greenish. The fruit is a small capsule about 2 - 3 mm in diameter containing numerous minute seeds about 0.5 – 0.8 mm long. The fruits aggregate into cylindrical masses along sections of the twigs. They are found in woodlands and open forests along watercourses and on the edges of swamps (Craven, 2008).

Figure 2.27: *Melaleuca bracteata* var. revolution gold (adapted from Osunsanmi et al., 2015)

2.15.1 **Scientific classification of *Melaleuca bracteata***

Kingdom:	Plantea
Plant subkingdom:	Tracheobionta
Super division:	Spermatophyta
Division:	Magnoliopsida
Class:	Magmoliopsida
Subclass:	Rosidae
Order:	Myrtales
Family:	Myrtaceae
Genus:	*Melaleuca L*
Species:	*Melaleuca bracetata*

(Barlow, 1998)

2.15.2 **Some other Melaleuca genuses**

There are various melaleuca genuses from the family of Myrtaceae. These include;

- *Melaleuca acuminate*
- *Melaleuca lternifolia*
- *Melaleuca agathosmoides*
- *Melaleuca adnata*
- *Melaleuca acacioides*
- *Melaleuca acerosa*
- *Melaleuca amydra*
- *Melaleuca alsophila*
- *Melaleuca adenostyla*
- *Melaleuca bracteata*

*(*Aboutabl et al., 1998)

2.15.3 **Economic importance of *Melaleuca bracteata* var. revolution gold**

Melaleuca bracteata var. revolution gold (Figure 2.26) is cultivated widely because of its compact shape and ability to grow in different environmental conditions. The plant is pest and disease free, and is cultivated mostly in tropical regions of South Africa and other tropical areas worldwide. In Australia, it is used as a food plant and an ornamental plant (Craven and Lepschi, 1999).

The leaves of *Melaleuca bracetata* are commonly used by traditional healers for the treatment and prevention of diseases; the leaves are chewed to alleviate headache and other ailments. The flexibility and softness of the stem bark of this plant made it an important tree for aboriginal people. The stem bark is used as a sleeping mat, food wrapper, a raincoat, for bandages, and for sealing holes in canoes (Byrnes, 1986).

The wood is hard, heavy and durable, and it could thus be used for poles and posts. The trees also serve as good shelter and could potentially be used in the control of erosion (Craven and Lepschi, 1999).

The essential oil from *Melaleuca bracteata* has been demonstrated to have good antifungal and antibacterial properties, and eliminates warts and the human papilloma virus (Cribb and Cribb, 1981; Oliva *et al.,* 2003). The *Melaleuca bracteata* oil is a major component in “Burn aid”, a commonly used first aid treatment for minor burns. This *Melaleuca bracteata* oil is also used in pet fish medications, such as Bettafix and Melafix (Takarada, 2004). These medications are use for the treatment of fungal and bacterial infections (Hammer, 2003; Mondello, 2003). The *Melaleuca bracetata* oil is also used as a germicidal, insecticidal and as an antiseptic (Yatagai, 1997).

2.16 Problem statement

The diseases associated with platelet aggregation are among the leading causes of death in the world today; these include heart attacks, stroke, pulmonary hypertension and angina pectoris. Antiplatelet aggregation therapy treatment and prevention has

undergone dramatic changes and improvement over the years. Various synthesized drugs have been formulated for the treatment of diseases associated with platelet aggregation which have been shown to be effective, but are unfortunately not without side effects. These problems have created the need to formulate new drugs with improved clinical safety and efficacy at a reduced cost. This study aims to investigate the antiplatelet aggregation activities of betulinic acid and derivatives from *Melaleuca bracetata* var. revolution gold.

2.17 Aims and objectives

2.17.1 Aims

This project aims to extract and isolate betulinic acid from *Melaleuca bracteata* var. revolution gold, and synthesize some of its derivatives, which will be evaluated for their antiplatelet aggregation, anti-inflammatory activities and possibly determine the apparent mechanism of action.

2.17.2 Objectives

The objectives of this study are to:

Collect and identify *Melaleuca bracteta* var. revolution gold

Extract, isolate and characterize betulinic acid

Synthesize betulinic derivatives

Investigate the antithrombin properties of the compounds

Investigate the antiplatelet aggregation activities of the compounds using blood platelets from Wistar rats

Investigate the anti-inflammatory activities of the compounds

Investigate the mechanisms of action of the compounds

Evaluate the cytotoxicity potential of the compounds using human hepatocellular carcinoma (HepG2) and human embryonic kidney (HEK293)

2.18 Research hypothesis

Pentacyclic triterpenes have platelet aggregation inhibitory potential, therefore Betulinic acid and its derivatives exhibit anti platelet aggregation activities.

Chapter three

3. Materials and methods

The materials used for this research work are listed below and a brief methodology is also given. The full details of reagent preparations and the methodology used are presented in Appendix A and B respectively.

3.1 Materials

3.1.1 List of equipment

- Incubator (Labcom)
- Rotor evaporator (Heidolph—Laborota 4000)
- pH meter (Hanna Instruments)
- Centrifuge- 5404R Eppendorf **(**Merck**)**
- Platform shaker - Labcon (Polychem supplies)
- BiotekElx 808 UI plate reader (Biotek Instrument Suppliers)
- U-bottom 96-well plate (Sigma)
- Spectrophotometer—Spekol 1300 (Polychem supplies)
- Barnstead or Electothermal digital melting point apparatus (Thermo Scientific)
- Dissecting set (Laboratory and Scientific Equipment Company (PTY) (Lasec)
- Columns of different sizes (Merck)
- Nuclear magnetic resonance (Bruker)
- Infrared spectroscopy (Perkin Elmer)
- Ulta-violet visible spectroscopy (Perkin Elmer)
- Grinder—IKA (Werek)
- Microwave oven – Defy model DMO 353
- Light microscope

3.1.2 **Chemicals and reagents (see Appendix A for reagent details)**

The chemical solvents and regents for this project were all of analytical grade.

3.1.2.1 **Chemicals supplied by sigma-Aldrich, St Louis, MO, USA**

Deuterated dimethyl sulfoxide d_6, deuterated Chloroform, Thrombin, Adenosine diphosphate, Citric acid, Methanol, Ethyl acetate, Glucose, Epinephrine, Dextrose, Trizma HCl, 2-Thiobarbituric acid, Ferric chloride, Ferrozine (Benzenesulfonic acid, 4,4'-(3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl) bis- disodium salt), n–hexane, Ferrous chloride, Acetic anhydride, Mercury (II) chloride, Potassium iodide, Potassium persulfate, and Potassium ferrocyanide.

3.1.2.2 **Chemicals supplied by Merck, Darmstadt, Germany**

Glacial acetic acid, calcium chloride, hydrochloric acid, tri-Sodium citrate, sodium hydroxide, sodium chloride, silica gel 60 0.040-0.063 mm (230-400 mesh ASTM), silica gel 60 0.063-0.200 mm (70-230 mesh ASTM), sulphuric acid silica gel 60 0.2-0.5 mm (30-70 mesh ASTM), acid purified sand, chloroform, hexane, ethylacetate, TLC aluminium sheets 20x20 cm, and Silica gel 60 F254.

3.1.2.3 **Chemicals supplied by other sources**

- disodium hydrogen phosphate (Lab. Consumables and Chemical Supplies)
- dipotassium hydrogen phosphate (Associated Chemical Enterprises)
- ammonium solution (NT Supplies)
- ethylenediaminetetra-acetic acid (Associated Chemical Enterprises)
- S-2238 (Chromogenix) (Instrumentation Laboratory Company)
- sodium dihydrogen phosphate (Lab. Consumables and Chemical Supplies)

3.2 Methods (see Appendix B for details)

3.2.1 **Collection and identification of plants**

The leaves of *Melaleuca bracteata* var. revolution gold were harvested from trees growing around the University of Zululand, KwaDlangezwa campus, South Africa.

These leaves were taken to the Department of Botany, University of Zululand for identification and voucher specimen (VN 0256) was deposited at the University herbarium.

3.2.2 **Extraction and isolation of betulinic acid**

The method described by Habila and colleagues (2011) was used to extract betulinic acid from *Melaleuca bracteata* var. revolution gold. The leaves were allowed to air dry at room temperature and then extracted by cold maceration in dichloromethane (1.5 w/v) at room temperature (5L X 3) for 24 hours. The combined filtrate was concentrated under a reduced pressure by using the rotator evaporator at 40 ^{o}C and allowed to air dry at room temperature, yielding 0.6 % of the crude extract. The mass obtained from the crude extract was washed with n-hexane (80 %) twice to remove oily materials. A portion (5 g) of the residue was subjected to chromatographic separation on a silica gel (60-120 mesh) column (20 x 5.5 cm) and eluted with a gradient of hexane/ethyl acetate (8:2 to 7:3) for the isolation of betulinic acid. Eighty fractions of eluates (20 ml) were collected and monitored with thin-layer chromatography. Similar fractions containing the desired compound were combined. These were further concentrated by a rotator evaporator at 40 ^{o}C and recrystallized in methanol. The isolated compound was characterized by spectral analysis (NMR, IR and MS).

3.2.3 **Preparation of betulinic derivatives**

The method described by Adrine et al (2012) was adopted, with slight modification, to synthesize acetyl derivatives of BA (Figure 3.1). A portion of betulinic acid (2 g) was mixed with pyridine (10 ml) and acetic anhydride (12 ml) in a round bottom flask. This mixture was then refluxed in a fume cupboard for 8 hours at 40 ^{0}C. Distilled water (25 ml) was used to terminate the reaction. The mixture was stirred for 45 minutes and filtered. The filtrate was rinsed with HCl (12 %) to remove pyridine and then air-dried at room temperature. The compound was purified by subjecting it to chromatographic separation on silica gel (60 x 120 mesh) columns (20 x 5.5 mm) and by using the n-hexane and acetyl acetate solvent system (8:2 to 7:3) to elute. A total of 50 fractions of

eluates (20 ml) were collected and similar fractions (based on thin-layer chromatography) were combined and concentrated *in vacuo* at 40 ^{o}C. The compound was recrystallized in methanol to form a yellowish powder. The spectral analysis (NMR, IR, and MS) was used to confirm the compound.

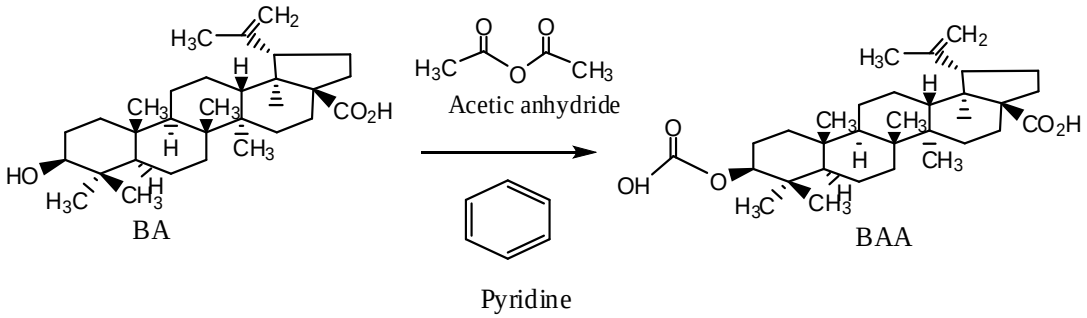


Figure 3.1: Schematic diagram for synthesis of BAA

3.2.4 **Isolation of betulinic and oleanolic acid**

The method described by Habila *et al.,* 2011 was followed, with slight modification, to extract a mixture of betulinic and oleanolic acid from *Melaleuca bracetata* var. revolution gold. The leaves (600 g) were allowed to air dry and were extracted by cold maceration in ethyl acetate at room temperature (5L X 3) for 72 hours. The filtrate was concentrated on a rotator evaporator at 40^{o}C and dried at room temperature (25 ^{o}C) to yield 8 % of the crude extract. The crude extract was defatted using n-hexane. The crude extract (6g) was then subjected to chromatographic separation on silica gel (60x120 mesh) columns (20 x 5.5 cm) and solvent system hexane/ethylacetate (8:2 to 7:3). A total of 46 fractions of eluates (2 ml) were collected. Similar fractions were combined by monitoring with thin-layer chromatography. This fraction was concentrated and recrystallized with methanol. Spectral analysis (NMR, IR, and MS) was carried out on the compounds for confirmation.

3.2.5 **Preparation of betulinic and oleanolic acids derivatives**

The method described by Andrine *et al.,* 2012, as modified and described in section 3.2.3 above, was employed to prepare the acetyl derivatives of the two acids mixtures (Figure 3.2). The spectral analysis (NMR, IR, MS) was carried out on the compound for

confirmation.

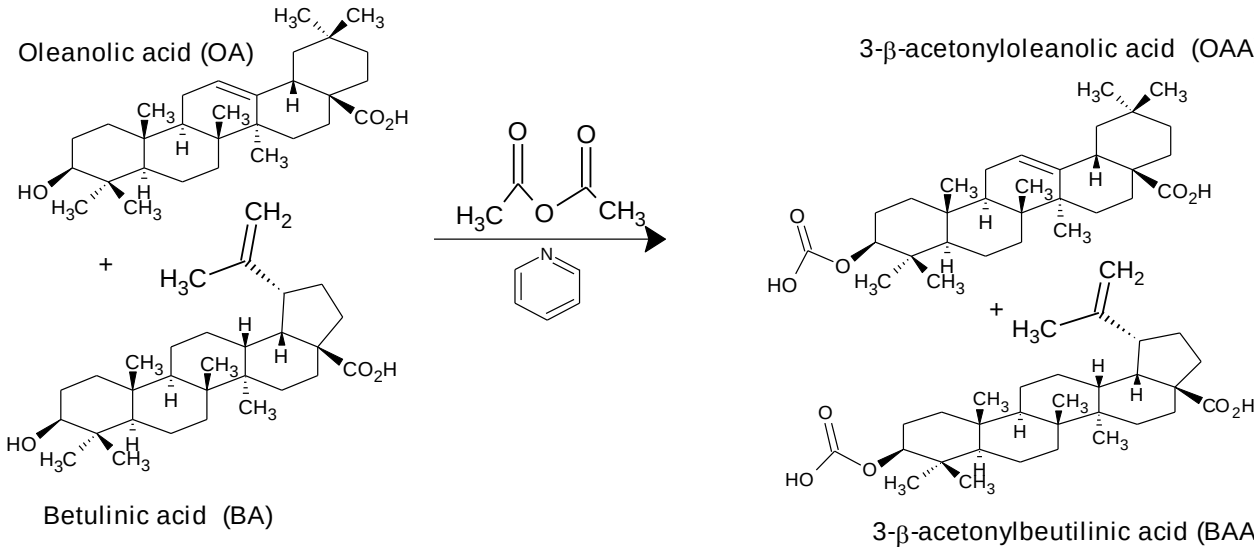


Figure 3.2: Schematic diagram for synthesis of BAA/OAA from BA/OA

3.2.6 **Structural elucidation**

All NMR experiments were conducted on a 400 MHz Bruker Ultrashield spectrometer. BA and the BA/OA mixture were dissolved separately in a mixture (1:2) of deuterated chloroform and methanol-d4, whereas BAA and BAA/OAA were separately dissolved separately in deuterated chloroform. Infrared spectra were recorded with a PerkinElmer Spectrum FTIR spectrophotometer. Mass data was run on the Agilent 1100 series LC/MSD trap system Electrospray ionization. All solvents and reagents were purchased from Sigma-Aldrich and were used as received. Melting points were recorded on an Electrothermal (Thermoscientific) digital melting point apparatus and were uncorrected

3.2.7 **Experimental animals**

Ethical clearance (UZREC 171110-030 PGD 2014/53; see Appendix D) for experimental animals was obtained from the Research Animal Ethic Committee (RAEC) of the University of Zululand. The guidelines for the proper care of animals and conducting of animal experiments were followed. Sprague–Dawley rats (9 weeks, 230-260 kg) of both sexes were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand. The animals were housed in

standard cages and were maintained under standard environmental conditions (room temperature, with 12: 12 light: dark cycle), having access to normal pellet feeds and drinking water.

3.2.8 **In vitro antiplatelet aggregation study**

3.2.8.1 **Preparation of compounds and derivatives for anti-platelet aggregation study**

Various concentrations of the compounds (1 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml) were prepared by dissolving the compounds and their derivatives in a 1 % DMSO that contained a few drops of tween 20.

3.2.8.2 **Preparation of blood platelets**

The blood platelets were obtained according to the method described by Tomita et al. (1983), with slight modification. Eight Sprague-Dawley rats were sacrificed by cervical dislocation, and blood (5 ml) was collected surgically from the abdominal aorta and mixed (5:1 v/v) with an anticoagulant (acid-dextrose anticoagulant, 0.085 mM citric acid, 2 % dextrose). The blood was centrifuged at 1200 rpm for 15 minutes and at 2200 rpm for 3 minutes consecutively. The supernatant was collected and centrifuged again at 3200 rpm for 15 minutes. The supernatant was discarded and the sediment (platelets) re-suspended in 5 ml washing buffer (PH 6.5). The washed platelets were centrifuged again at 3000 rpm for 15 minutes and the supernatant discarded. The platelets were suspended in a buffer (at pH 7.4, containing 0.14 M NaCl, 15 mM Tris- HCl, 5 mM glucose). The working solution was prepared by further diluting (1:10) the platelets with resuspending buffer and supplementary calcium chloride (0.4 ml, 10 µl $CaCl_2$).

3.2.8.3 **Anti-platelet aggregation evaluation**

The method of Mekhfi and co-workers (2004) was used, with slight modification. The antiplatelet aggregation activity of the compounds and their derivatives was separately tested on collagen (10 µg/ml), thrombin (5 µg/ml), ADP (10 µg/ml), and epinephrine (10 µg/ml) induced platelet aggregation. The platelets (200 µl) and 20 µl of the different concentrations of the compounds and derivatives (1, 3, 5 and 10 mg/ml) were

separately pipetted into 96-well micro plates and pre-incubated at 37 ^{o}C for 5 minutes. The agonist (20 µl) was then added to the mixture to induce platelet aggregation. Platelet aggregation inhibitory activity of the compounds was determined by the Biotek plate reader by following the changes in absorption at 415 nm for 20 minutes at 30 second intervals. DMSO (1 %) served as a negative control, whereas aspirin served as the positive control.

3.2.9 **Antithrombin activity (chromogenic: S2238)**

The method described by Rob et al., (1997) was adopted, with slight modification. The various test compounds (1, 3, 5, 10 mg/ml) were prepared for antithrombin activity by separately solubilizing them in 5 % DMSO. These were then diluted to a final concentration of 1 % DMSO with a Tris-HCl buffer (175 mM NaCl, 50 µl Tris-HCl, 7.5 mM EDTA, pH 7.4). A portion (50 µl) from the prepared solution was then added to thrombin (10 µl) in 96-well plates. The mixture was left for 10 minutes at room temperature before adding chromogenic substrate (190 µl; 0.76 M). The reaction was read at 412 nm for 8 minutes at 1 minute intervals using the Biotek ELx 808 UI plate reader. DMSO (2 % v/v) in saline solution was used as a negative control.

3.2.10 **Determination of calcium levels in cytosol**

The level of calcium in cytosol was determined with Fura-2/AM following the method of Kim *et al.,* (2006). The platelets were incubated with 5 µM of Fura-2/AM for 30 minutes at 37 ^{o}C and then washed. The Fura-2-loaded, washed platelets were pre-incubated with the compounds (1, 3, 5 and 10 mg/ml) for 3 minutes at 37 ^{o}C in the presence of 1 mM $CaCl_{2.}$ The Platelets were stimulated to aggregate with thrombin for 5 minutes. The Fluorescence Spectrofluorometer was used to measure the fluorescence signals from the platelet suspension. The fluorescence emission was determined at 510 nM, with continuous excitation at 340nM and 380nM and changing every 0.5s.

The following formula was used to determine the Ca^{2+}:

Ca^{2+} in cytosol= 224nM x (F-Fmin) / (Fmax-F)

224nM was the dissociation constant of the Fura-2-Ca^{2+} complex, while F minimum and F maximum represent the fluorescence level of intensity at very low and high intensity concentrations and Ca^{2+} concentrations respectively. Fmax was given a fluorescence intensity at 510 nm of the Fura-2- Ca^{2+} complex after the platelet suspension containing 1 mM of $CaCl_2$ had been solubilized by Triton X-100 (0.1 %). Fmin was the fluorescence intensity at 510nm of the Fura-2-Ca^{2+} complex after the platelet suspension containing 20 mM Tris/3 mM of EDTA had been solubilized by Triton X-100 (0.1%). F was the fluorescence intensity of the Fura-2-complex at 510 nm after the stimulation of platelet suspension containing 1 mM $CaCl_2$ with thrombin, with and without the compounds.

3.2.11 **ATP release assay**

Platelets were pre-incubated for 3 minutes with various concentrations of the compounds (1, 3, 5 and 10 mg/ml) and were then stimulated to aggregate by collagen following the method of PaI et al*.,* (2011). The reaction was terminated and the samples were centrifuged. The supernatants were then used for the assay. The ATP release was measured in an Luminometer (Biotek plate reader ELx808) using the ATP assay kit.

3.2.12 **Determination of phosphodiesterase activity**

The phosphodiesterase inhibitory activity of the compounds was determined by following the method of Razzell (1963), with slight modification. The reaction mixtures consist of 50 µl of 0.5 mM p-Nph-5′-TMP, 100 µl of 0.1 M TrisHCl (pH 8.9), 25 µl of various concentrations (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml) of the compound and 25 µl of the enzyme. The mixture was then incubated at 37°C for 15 minutes. The reaction was stopped with 50 µl of 0.2 N NaOH. Absorbance was read at 400 nm and percentage inhibition of the enzyme calculated. Caffeine served as a positive control.

3.2.13 **Tail bleeding time assay**

Rat tail bleeding time was measured by following the method of Wang et al., (2004), with slight modification. Twenty Sprague-Dawley rats were divided into four groups of five each. Groups one to four were administered with the compounds (10 mg/kg, 50 mg/kg, and 250 mg/kg) and aspirin (40 mg/kg) respectively, and after 2 hours the animals were anaesthetized with sodium pentobarbital (50 mg/kg). Anesthetized rats were placed on a hotplate and the tails thermostated at 37^{o}C. The bleeding time was assessed by amputating 5mm of the tail tip with a scalpel and was blotted onto filter paper every 30 seconds until the paper no longer stained with blood. The period between amputation and when bleeding stopped was taken as bleeding time.

3.2.14 **Cytotoxity test**

The MTT [3-(4, 5-dimethylthiazol-2yl)-2-5-diphenyltetrazoliumbromide] cytotoxicity proliferation assay was used to measure the toxicity of betulinic acid and its derivatives by determining the absorbance of the cells in a culture (Mosman, 1983). The cells which were used for this assay are the human embryonic kidney cells (HEK293) and human hepatocellular carcinoma cells (HepG2). The cells were cultured in a 25 cm^2 flask to confluence, which was then trypsinised and plated into 48-well plates, a 2.5 x 10^4 seeding density per well and incubated overnight at 37 ^{o}C. Two exposure periods of 24 hours and 48 hours were chosen to determine the toxicity of betulinic acid and its derivatives along with the positive control containing the cultured cells and the medium. The negative control contained the medium and samples. The percentage cell growths were calculated against the medium as a triplicate reading mean ± SD. The results were stated as concentrations to reduce the absorbance of treated cells by 50 % with reference to the control (untreated cells) or cancer cell growth inhibitor (D_{50}) and the lethal dose IC_{50} values (µg/ml) of the compound derived from the growth curve.

3.2.15 **Anti- acetylcholine esterase activity of betulinic acid and its derivate**

The effects of the betulinic acid and its derivatives on acetylcholinesterase activity were determined using acetylthiocholine kits based on manufacturer (sigma-Aldrich, St Louis,

MO, USA) procedure.

3.2.16 **Iron (Fe^{2+}) chelation**

The iron chelation effect of the compounds was determined by following the method described by Decker and Welch (1990), with slight modification. In a beaker, 0.5 ml of compounds (0 - 5 mg/ml in methanol), were separately mixed with 0.05 ml of $FeCl_2$ (2 mM) and 1.6 ml of dilute water. After 45 seconds, the reaction was intiated by the addition of 0.1 ml of ferrozin (5 mM). The mixture was further shaken and kept at room temperature (25 ^{o}C) for 10 minutes. The absorbance of the mixture was read at 562 nm. Citric acid and EDTA were used as positive controls.

3.2.17 **Anti-inflammatory evaluation**

3.2.17.1 **Cotton pellet-induced granuloma test**

The proliferation phase of inflammation was investigated using the cotton pellet-induced granuloma model (Penn and Ashfor*,* 1963). Twenty Sprague-Dawley rats were divided into four groups of five rats each and allowed to acclimatize for 5 days (Figure 3.3). The animals were pre-administered with the compounds thirty minutes before interscapular implantation of pre-weighed (20 mg) sterile cotton pellets.

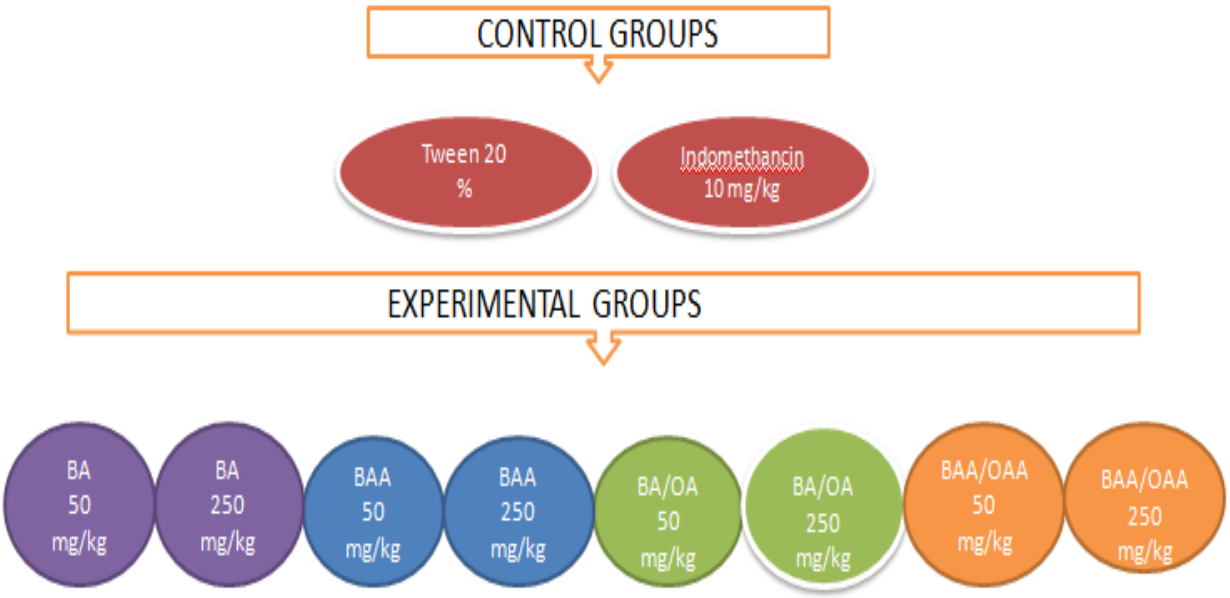


Figure 3.3: Animal model chart for inflammation evalution

The rats were anesthetized (diethyl ether) prior to cotton pellet implantation. Inflammation was induced by making an intrascapular incision and then implanting the sterilized cotton pellet (20 mg) subcutaneously between the scapulae on each rat.

The different concentrations of the compounds dissolved with DMSO (1 ml) were orally administered to the animals in the respective groups for seven consecutive days, starting from the day of sterilized cotton pellet implantation. On the eighth day, the rats were anesthetized and the implanted cotton pellets were carefully dissected out. The cotton pellets were made free of extraneous tissue. The wet pellets were separately weighed and dried in an oven at 60 ^{o}C for 24 hours. The differences between the dry and wet pellets were taken as measurements of the formed granuloma weight. The anti-proliferation effects of the compounds were compared with the control.

Percentage inhibition was calculated using the following formula:

% inhibition = (Wc –Wt/ Wc) x 100

Wc = pellet weight of the control group rats

Wt = pellet weight of drug treated rats.

The dried cotton wool pellets were digested and the hydrolysate used to estimate the catalase and superoxide dismutase (SOD) stimulating potential of the compounds.

3.2.18 **In vitro cyclooxygenase (COX-1 and COX-2) inhibition assay**

The *in vitro* cyclooxygenase (COX-1 and COX-2) inhibitory activity of various concentrations of the compounds and their derivatives were determined using the COX assay kit based on the manufacturer (sigma-Aldrich, St Louis, MO, USA) procedure. This kit measures the peroxide activity of cyclooxygenase (COX). COX is also known as prostaglandin H synthase (PGHS), this is a bifunctional enzyme showing both cyclooxygenase and peroxidase activities. COX converts arachidonic acid to a hydroperoxy endoperoxide (prostaglandin G2; PGG2) and the peroxide component reduces the endoperoxide to the corresponding alcohol (Furse et al., 2006). There are two isoforms of COX: COX-1 is expressed in a variety of cell types and is involved in normal cellular homeostasis, whereas COX-2 is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions (Bakhle, 2001). This inducible COX-2 is believed to be the target enzyme for the anti-inflammatory activity of NSAIDS.

Unless otherwise stated the percentage inhibition of the compound on each parameter was calculated as:

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

Where At is the absorbance of the compounds and Ac is the absorbance of the control.

3.2.19 **Superoxide dismutase (SOD) activity**

The effects of various concentrations of compounds and the derivatives on superoxide dismutase activity were determined using the SOD kit, based on the manufacturer's (sigma-Aldrich, St Louis, MO, USA) procedure. SOD catalyses the breakdown of superoxide anions into hydrogen peroxide and molecular oxygen. It is one of the most important antioxidant enzymes. The SOD Assay kit-WST allows very convenient SOD assaying by utilizing Dojindo's highly water soluble tetrazolium salt.

3.2.20 **Catalase activity**

The effects of various concentrations of compounds and their derivatives on catalase activity were determined using the catalase assay kit, based on the manufacturer's procedure. Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian cells. It catalyses the breakdown of hydrogen peroxide into water and oxygen.

3.2.21 **Statistical analysis**

All assays were performed in triplicates and data expressed as a mean ± SD. Anova (one way) and the post hoc Dunnett's tests were used to analyse the data using Graphpad prism, version 5.03. The statistical significance was given as $P< 0.05$.

Chapter four

4. Results

Betulinic acid and a mixture of betulinic and oleanolic acids (isolated from *M bracetacta*), along with their prepared acetyl derivatives, were screened for antiplatelet aggregation activities. The results obtained are presented below.

4.1 Compound Identification

The isolated compounds and their derivatives were identified and their structures established through spectral NMR (1H and 13C), IR, MS analysis (Appendix C) and by comparison with literature values (Habila et al., 2013).

BA (Figure 4.1) Colourless crystal; mp 315-316 ^{o}C; IR (KBr) v_{max} 3456, 2920, 2851, 1724 cm^{-1}; m/z (ESI) 455.2 (M^{+}-1); δH (400 MHz, $CDCl_3$ and CH_3OD): 4.59 (1H, s), 4.46 (1H, s), 3.10 (2H, d), 2.13 (2H, dd), 1.80 (2H, s), 1.45 (8H, m), 1.38 (11H, m), 0.80-1.17 (21H, m); δC (100 MHz, $CDCl_3$ and CH_3OD): see Table 4.1.

BAA (Figure 4.2) white powder; mp 258-260 ^{o}C; IR (KBr) v_{max} 3424, 2919, 2851, 1724, 1692, 1642, 1240 cm^{-1}; m/z (ESI) 496.8 (M^{+}-1); δH (400 MHz, $CDCl_3$): 4.71 (1H, s), 4.59 (1H, s), 4.45 (1H, m), 2.98 (1H, m), 2.25 (1H, d), 2.15 (1H, d), 1.94 (5H, d), 1.59 (9H, m), 1.43 (3H, s), 1.40 (4H, m), 1.24 (3H, d), 1.17 (2H, s), 1.00 (8H, m), 0.80 (10H, m); δC (100 MHz, $CDCl_3$): see Table 4.1.

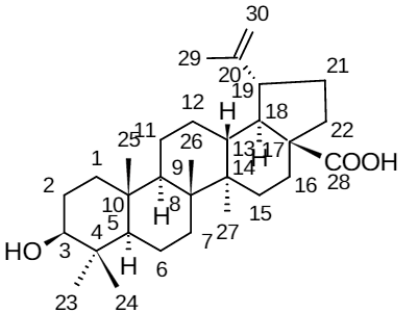


Figure 4.1: Chemical structure of betulinic acid

The presence of hydroxyl groups in the compounds was indicated by the appearance of an absorption band between 3424 - 3456 cm^{-1} in the IR Spectra. The C-H stretching frequencies were observed around 2851-2920 cm^{-1}, and the bands that are characteristic of the presence of carboxylic acid in a molecule were observed around 1642 and 1729 cm^{-1} (Mahato and Kundu,1994). The proton NMR spectroscopy further confirmed the structure of BA and BAA. The ^{l}H NMR spectrum of BA revealed various peaks corresponding to the methyl groups at around 0.80-1.17 ppm and terminal methylene protons at 4.46–4.59 ppm, which is indicative of the presence of 48 hydrogen atoms in BA. As expected, the ^{l}H NMR spectrum of BAA showed the presence of 50 hydrogen atoms, which agrees with the literature regarding previously reported values (Habila et al., 2011). In ^{13}C NMR spectra, the appearance of two additional carbons for compound BAA assigned as C-31 and C-32 (Table 4.1) further confirmed the formation of BAA. The carboxylic acid carbon assigned as C-28 (Table 4.1) appeared as the most deshielded around 178.8 and 182.2 ppm for both BA and BAA respectively, which is also in agreement with literature (Habila et al., 2011). Further evidence for the isolation of BA and BAA was provided by the ESI-MS spectra which showed intense molecular ions corresponding to M^{+}-1 at 455.2 and 496.8 respectively. The calculated molecular weight for BA and BAA is 456 (molecular formula – $C_{30}H_{48}O_3$) and 498 (molecular formula – $C_{32}H_{50}O_4$) respectively.

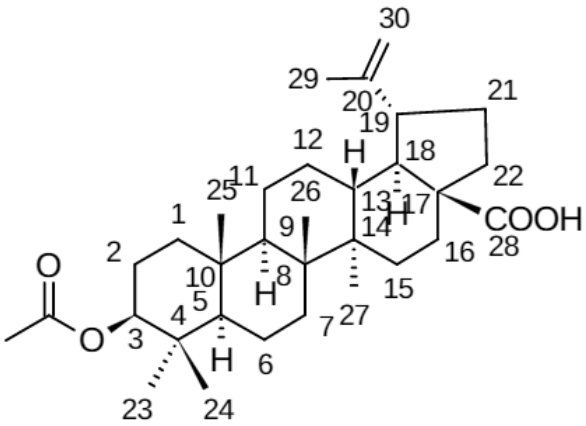


Figure 4.2: Chemical structure of 3-β acetylbetulinic acid

BA/OA **(**Figure 4.1, 4.3**)** white powder; mp 290–292 ^{o}C; IR (KBr) v_{max} 3553, 2990, 1739, 1453, 1374, 1239, 1087 cm^{-1}. δH (400 MHz, $CDCl_3$ and CH_3OD): 4.96 (s, 1H), 4.44 (1H, s), 4.43 (1H, s), 3.11, 2.73, 1.98, 1.43, 1.29, 0.79-1.24 (21H, m); δC (100 MHz, $CDCl_3$ and CH_3OD): see Table 4.2.

BAA/OAA (Figure 4.2, 4.4) white powder; mp 297-299^{o}C; IR (KBr) v_{max} 2990, 1739, 1451, 1373, 1235, 1045 cm^{-1}; δH (400 MHz, $CDCl_3$): 5.18 (s, 1H), 4.60 (1H, s), 4.47 (1H, s), 4.34 (1H, s), 2.88, 2.11, 1.95, 1.93, 1.81, 1.57, 0.72 – 1.49 (m); δC (100 MHz, $CDCl_3$): see Table 4.2.

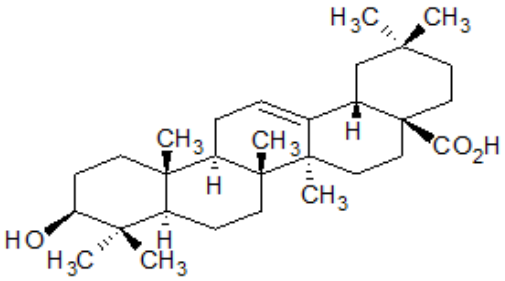


Figure 4.3: Chemical structure of Oleanolic acid

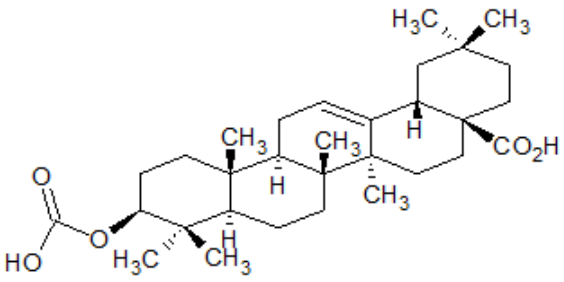


Figure 4.4: Chemical structure of 3-β acetyloleanolic acid

The isolation of the BA/OA mixture from *Melaleuca bracteata* and the synthesis of BAA/OAA mixture were confirmed by the ^{1}H, ^{13}C NMR and IR spectroscopy. BA and OA are isomers and have Rf values of 0.62 and 0.68 respectively. Attempts to obtain each as a pure compound were unsuccessful (Ibrahim et al, 2013). The melting point of the compounds mixture (BA/OA and BAA/OAA) was also determined and was consistent with previously reported values (Ibrahim et al., 2013). The presence of hydroxyl groups

in the compounds was indicated by the appearance of an absorption band around 3553 cm^{-1} in the IR Spectra. The C-H stretching frequencies were observed around 2990 cm^{-1}, and the bands that are characteristic of the presence of carboxylic acid in a molecule were observed around 1739 cm^{-1} (Mahato and Kundu, 1994). The ^{l}H NMR spectrum of samples 3 and 4 both revealed various peaks corresponding to the methyl groups, at around 0.72-1.49 ppm, and terminal methylene protons, at 4.34–5.18 ppm. Further confirmation for the isolation of sample 1 and for the formation of sample 2 was provided by ^{13}C NMR spectroscopy. The ^{13}C NMR spectra of sample 1 and 2 actually showed that they are mixtures. This is as a result of the appearance of 60 carbons in ^{13}C NMR spectra of sample 1 (30 each for BA and OA as assigned in Table 4.2) and 64 carbons in ^{13}C NMR spectra of sample 2 (32 each for BAA and OAA as assigned in Table 4.2), which agrees with reported data for related compounds (Seebacher et al., 2003; Habila et al., 2013). For sample 2 the appearances of four additional carbons in the ^{13}C spectrum (two each for BAA and OAA), assigned as C-31 and C-32 respectively (Table 4.2), further confirmed the formation of BAA/OAA.

Table 4.1: ^{13}C-NMR (100 MHz) spectral data for 1 and 2

Position	BA (1)	BAA (2)
1	38.4 (CH_2)	38.1
2	26.5 (CH_2)	27.6
3	78.4 (CH)	80.7
4	38.5 (C)	37.5
5	55.0 (CH)	55.1
6	17.9 (CH_2)	17.8
7	33.9 (CH_2)	33.9
8	40.2 (C)	40.4
9	50.1 (CH)	50.1

10	36.7 (C)	37.5
11	20.5 (CH_2)	20.5
12	25.1 (CH_2)	25.1
13	37.9 (CH)	38.1
14	42.0 (C)	42.1
15	30.2 (CH_2)	30.3
16	31.9 (CH_2)	31.8
17	55.8 (C)	56.1
18	46.6 (CH)	46.6
19	48.8 (CH)	48.9
20	150.3 (C)	150.1
21	29.2 (CH_2)	29.4
22	36.7 (CH_2)	36.8
23	27.4 (CH_3)	27.6
24	14.9 (CH_3)	15.7
25	15.4 (CH_3)	15.9
26	15.6 (CH_3)	16.1
27	14.2 (CH_3)	14.3
28	178.8 (C)	182.2
29	18.8 (CH_3)	19.0
30	109.3 (CH_2)	109.8
31	-	170.8 (C)
32	-	23.4 (CH_3)

Data reported in ppm.

Table 4.2: ^{13}C-NMR (100 MHz) spectral data for samples 3 and 4

Position	Sample 3 (BA/OA)	Sample 4 (BAA/OAA)
1	38.5 (BA) 38.7 (OA) (CH_2)	38.1 (BAA) 38.2 (OAA)
2	27.3 (BA) 29.2 (OA) (CH_2)	27.7 (BAA) 29.5 (OAA)
3	78.2 (BA) 78.2 (OA) (CH)	81.2 (BAA) 81.2 (OAA)
4	38.7 (BA) 40.2 (OA) (C)	38.2 (BAA) 40.5 (OAA)
5	55.0 (BA) 55.9 (OA) (CH)	55.1 (BAA) 55.2 (OAA)
6	17.8 (BA) 18.6 (OA) (CH_2)	18.0 (BAA) 19.0 (OAA)
7	33.9 (BA) 32.6 (OA) (CH_2)	34.0 (BAA) 32.1 (OAA)
8	40.2 (BA) 39.0 (OA) (C)	40.5 (BAA) 38.2 (OAA)
9	50.1 (BA) 48.9 (OA) (CH)	50.3 (BAA) 49.9 (OAA)
10	36.7 (BA) 37.8 (OA) (C)	37.0 (BAA) 38.1 (OAA)
11	20.5 (BA) 23.7 (OA) (CH_2)	20.7 (BAA) 23.5 (OAA)
12	25.1 (BA) 125.10 (OA) (CH_2)	25.3 (BAA) 125.0 (OAA)
13	37.9 (BA) 137.8 (OA) (CH)	37.6 (BAA) 143.0 (OAA)
14	42.0 (BA) 42.1 (OA) (C)	42.2 (BAA) 42.3 (OAA)
15	30.2(BA) 27.6(OA) (CH_2)	30.4(BAA) 27.7 (OAA)
16	31.8 (BA) 23.7 (OA) (CH_2)	32.1 (BAA) 23.5 (OAA)
17	55.8 (BA) 46.7 (OA) (C)	56.0 (BAA) 46.8 (OAA)
18	46.6 (BA) 42.0 (OA) (CH)	46.8 (BAA) 42.2 (OAA)
19	48.8 (BA) 46.7 (OA) (CH)	49.0 (BAA) 46.8 (OAA)
20	150.2 (BA) 31.8 (OA) (C)	150.5 (BAA) 32.1 (OAA)
21	29.2 (BA) 33.9 (OA) (CH_2)	29.5 (BAA) 34.0 (OAA)
22	36.5 (BA) 32.6 (OA) (CH_2)	36.9 (BAA) 32.1 (OAA)

23	27.5 (BA)	29.2 (OA)	(CH_3)	27.7 (BAA)	29.5 (OAA)
24	14.8 (BA)	16.4 (OA)	(CH_3)	14.4 (BAA)	16.2 (OAA)
25	15.1 (BA)	15.6 (OA)	(CH_3)	15.7 (BAA)	15.9 (OAA)
26	15.3 (BA)	17.8 (OA)	(CH_3)	15.6 (BAA)	18.0(OAA)
27	14.1 (BA)	26.2 (OA)	(CH_3)	14.4 (BAA)	25.3 (OAA)
28	178.6 (BA)	180.2 (OA)	(C)	178.9 (BAA)	178.9 (OAA)
29	18.6 (BA)	33.9 (OA)	(CH_3)	19.0(BAA)	34.0 (OAA)
30	108.9 (BA)	23.7 (OA)	(CH_2)	109.3 (BAA)	23.5 (OAA)
31	-			171.6 (BAA)	171.6 (OAA) (C)
32	-			23.5 (BAA)	23.5 (OAA) (CH_3)

Data is reported in ppm. BA (Betulinic acid), OA (Oleanolic acid), BAA (Betulinic acid acetate), OAA (Oleanolic acid acetate).

4.2 Cytotoxicity assay

The cytotoxicity of the compounds was investigated and the results are presented in Table 4.3.

Table 4.3: The IC_{50} (µg/ml) of betulinic acid and 3-β acetylbetulinic acid on HEK293 and HEPG2 cells

Compound	IC_{50} (µg/ml)	
	HEK 293	HEPG2
BA	1027	448
BAA	1051	672
BA/OA	724	585
BAA/OAA	499	269

The cytotoxicity of the compounds was investigated and the results are presented in Table 4.3. The compounds (BA, BAA, BA/OA and BAA/OAA) displayed poor cytotoxicity activity against HEK293 (IC_{50} values of 1027 µg/ml, 1051 µg/ml, 724 µg/ml, 499 µg/ml respectively) and HepG2 cell lines (IC_{50} values of 448 µg/ml, 672 µg/ml, 585 µg/ml, 269 µg/ml respectively). The compounds showed more cytotoxic effects on cancerous cells HEPG2 compared with normal cells HEK 293. BAA (IC_{50} value of 1051 µg/ml) showed the weakest cytotoxic effects on normal cells HEK 293, whereas BAA/OAA (IC_{50} value of 269 µg/ml) shown the highest cytotoxic effect on cancerous cells.

4.3 Anti-thrombin activity

Chromogenic, S-2238 (H-D-phenylalanyl-L-pipecolyl-p-nitroanilide dihydrochloride) is an artificial substrate of thrombin. The effect of the test compounds in inhibiting the hydrolysis of chromogenic by thrombin was investigated and the percentage antithrombin activity of the compounds is presented in Figure 4.5.

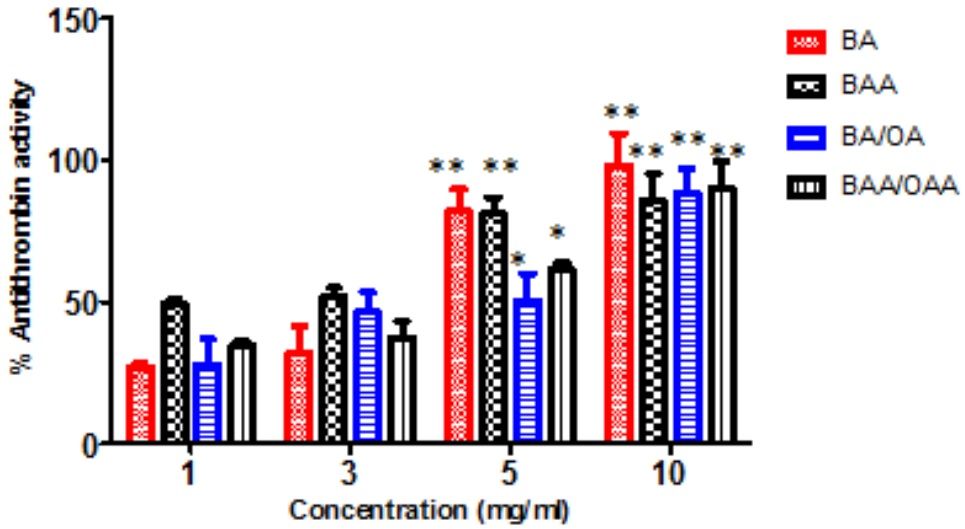


Figure 4.5: Antithrombin activity of the isolated compounds. Data was expressed as mean SD. *P < 0.05, **P < 0.01.

Table 4.4: The IC_{50} values of antithrombin activity in the isolated compounds

Compound	BA	BAA	BA/OA	BAA/OAA
IC_{50} mg/ml	3.85	1.25	4.79	3.98

The compounds showed significant dose-dependent antithrombin activity. BA exhibited the highest inhibition at 10 mg/ml. BAA showed better antithrombin activity than other compounds, with an IC_{50} value of 1.25 mg/ml (Table 4.4). BA with IC_{50} values of 3.85 mg/ml showed higher antithrombin activity when compared with BA/OA (IC_{50} value of 4.79 mg/ml). The BAA/OAA mixture, with an IC_{50} value of 3.98 mg/ml also showing better antithrombin activity than the BA/OA mixture (IC_{50} value of 4.79 mg/ml).

4.4 The Anti-Platelet aggregation studies

The anti-platelet aggregation activity of the compounds was tested separately on thrombin, ADP, collagen, or epinephrine induced rat platelet aggregation. The results are summarized in Figures 4.6a-d and Table 4.5.

Table 4.5: The IC_{50} values of betulinic acid and 3-β acetylbetulinic on platelet aggregation inhibition

Compound	IC_{50} (mg/ml)			
	Collagen	ADP	Thrombin	Epinephrine
BA	5.45	11.1	11.6	0.78
BAA	1.72	2.72	2.92	0.85
BA/OA	6.92	2.38	2.58	0.68
BAA/OAA	3.38	2.72	2.85	1.18
Aspirin	2.98	2.83	2.98	2.9

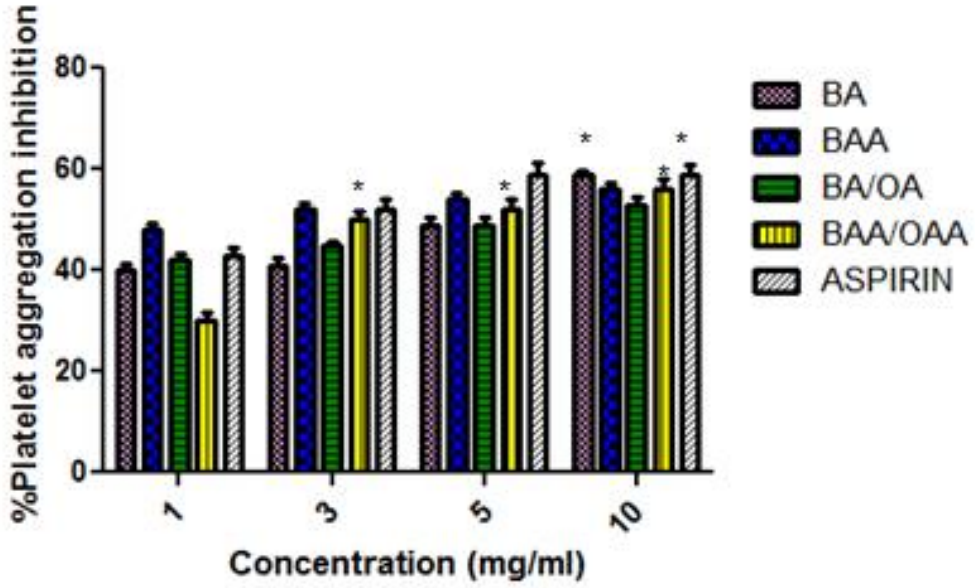


Figure 4.6a: Platelet aggregation induced by collagen Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

The compounds showed significant (p< 0.05) dose-dependent activity in collagen induced platelet aggregation (Figure 4.6a). The compounds also showed higher platelet aggregation inhibition at 10 mg/ml. BAA showed the IC_{50} value (1.72 mg/ml) in comparison to BA, BA/OA and BAA/OAA (with IC_{50} values of 5.45 mg/ml, 6.92 mg/ml and 3.38 mg/ml respectively) (Table 4.5). The compound (BAA) also showed a better IC_{50} value (1.72 mg/ml) compared to Aspirin (IC_{50} value of 2.98). BAA/OAA exhibited twice the IC_{50} values of BA/OA.

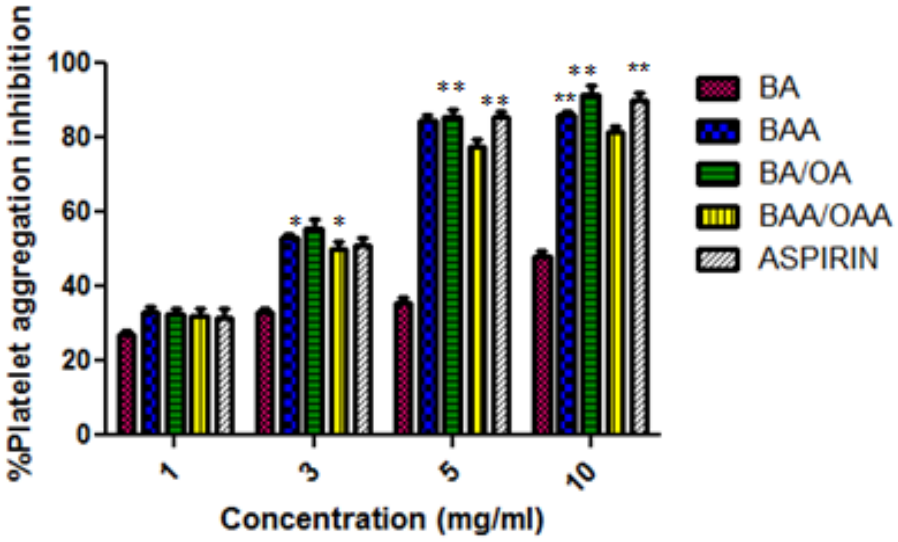


Figure 4.6b: Platelet aggregation induced by ADP Data is expressed as mean ± SD.*P < 0.05, **P < 0.01.

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The results revealed that the compounds showed significant ($p < 0.05$) dose dependence in ADP induced platelet aggregation (Figure 4.6b). The compounds also showed the highest platelet aggregation inhibitory activity at 10 mg/ml. BAA showed significant ($p< 0.01$) platelet aggregation inhibitory activity when compared with BA at 10 mg/ml (Figure 4.7). The compounds (BAA, BA/OA and BAA/OAA), with IC_{50} values of 2.72 mg/ml, 2.38 mg/ml and 2.72 mg/ml respectively, showed better antiplatelet aggregation activity compared to BA (with an IC_{50} value of 11.1 mg/ml). The compounds (BAA, BA/OA and BAA/OAA) also showed similar IC_{50} values to Aspirin (Table 4.5).

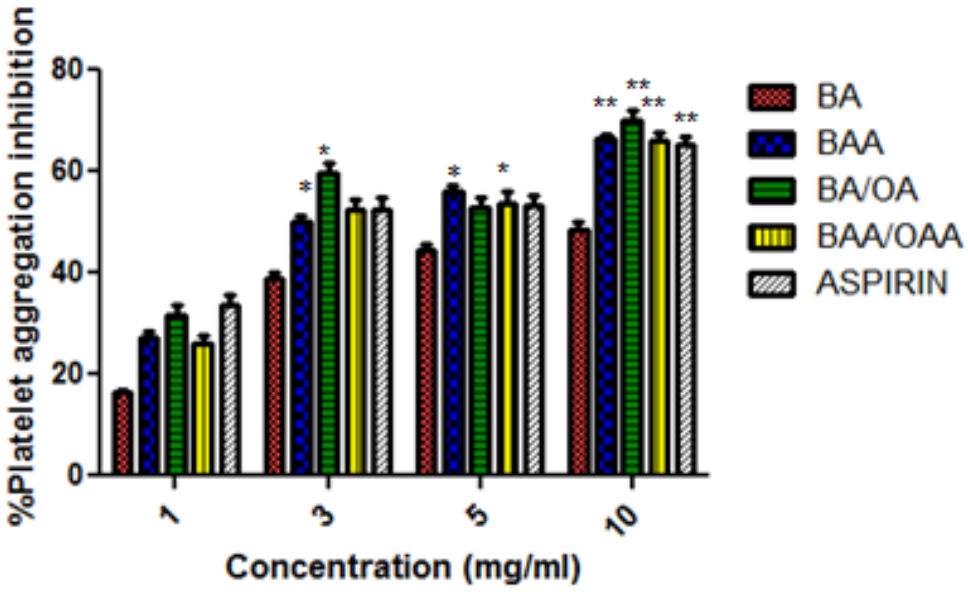

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Figure 4.6c: Platelet aggregation induced by thrombin Data is expressed as mean SD. *P < 0.05, **P < 0.01.

The results showed that the compound exhibited significant ($p < 0.05$) dose-dependent platelet aggregation inhibitory activity against thrombin induced platelet aggregation (Figure 4.6c). The BA/OA mixture showed the highest activity at 10 mg/ml. The compound (BAA) showed better antiplatelet aggregation activity than B (Figure 4.8). The compounds (BAA, BA/OA and BAA/OAA) with IC_{50} values of 2.92 mg/ml, 2.58 mg/ml and 2.85 mg/ml respectively exhibited better platelet aggregation inhibitory activity than BA (with an IC_{50} value of 11.6 mg/ml). The compounds (BAA, BA/OA and BAA/OAA) have similar IC_{50} values to Aspirin (Table 4.5).

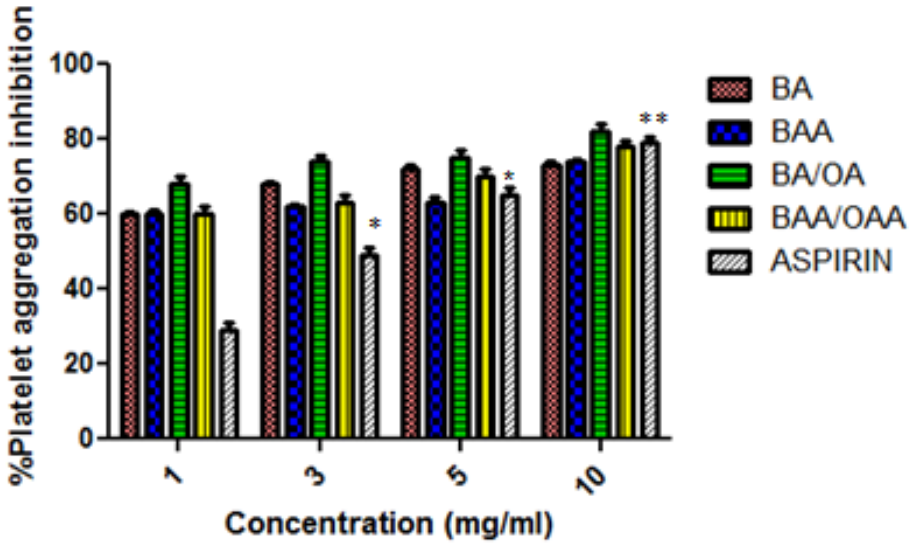

Figure 4.6d: Platelet aggregation induced by epinephrine Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

The results showed that the compounds exhibited dose-dependent platelet aggregation inhibitory activity against epinephrine induced platelet aggregation (Figure 4.6d). The BA/OA mixture showed the highest inhibitory activity at 10 mg/ml whereas Aspirin showed the lowest platelet aggregation activity at 1 mg/ml. The compounds (BA, BAA, BA/OA and BAA/OAA), with IC_{50} values of 0.78 mg/ml, 0.85 mg/ml, 0.68 mg/ml and 1.18 mg/ml respectively, were better when compared with Aspirin (IC_{50} values of 2.98 mg/ml) (Table 4.5).

4.5 ATP Assay

The release of ATP from the platelet granules as the platelet aggregate was investigated and the results presented in Figure 4.7

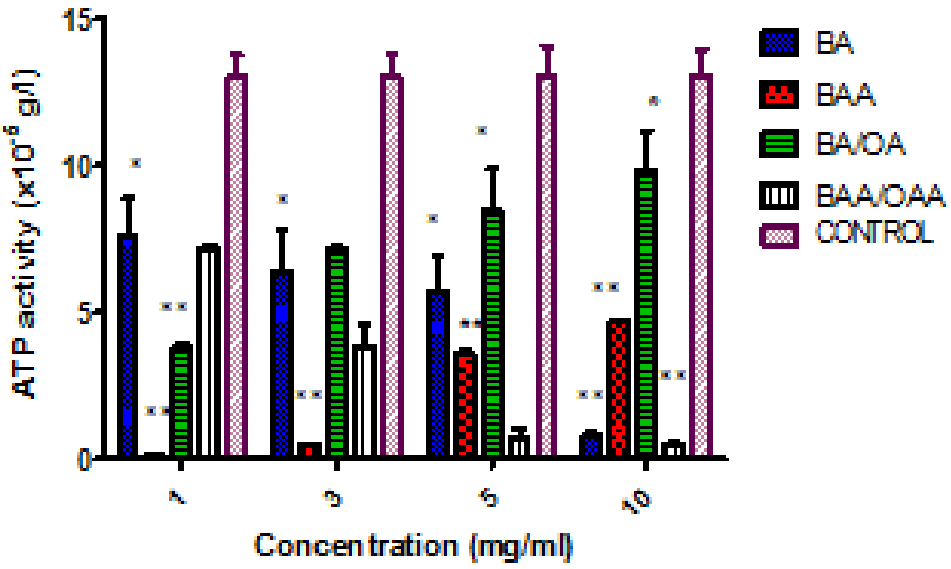

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Figure 4.7: ATP activity of the isolated compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

It is apparent that the compounds are significantly ($p < 0.05$) lower in ATP release compared to the control. BAA showed the highest inhibition of ATP release at 1 mg/ml. BA/OA showed higher significanceant inhibitory activity than BA at 1 mg/ml. BA/OA also showed reverse patterns of ATP release in comparison to BAA/OAA.

4.6 The Anti-acetylcholinesterase activity

The results of the acetylcholinesterase inhibition activity of the compound are presented in Figure 4.8

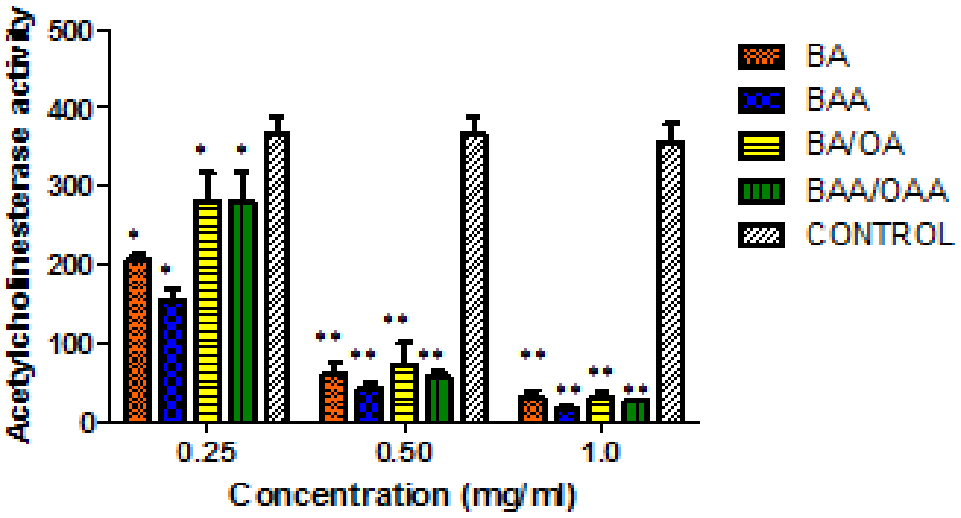

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Figure 4.8: Acetylcholinesterase inhibition activity of the compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

The compounds showed significant dose-dependent acetylcholinesterase inhibition activity. BAA showed the highest acetylcholinesterase inhibition at 1.0 mg/ml. BA showed better activity than the BA/OA mixtures. The BA/OA and BAA/OAA mixtures showed similar acetylcholinesterase inhibition activity at 0.25 mg/ml.

4.7 Phosphodiesterase inhibitory activity

The percentage phosphodiesterase inhibition activity of the compounds presented in Figure 4.9 indicates a significant ($p < 0.05$) dose-dependent activity. BAA showed the highest inhibitory activity, with an IC_{50} value of 1.58 mg/ml, when compared to other compounds (Table 4.6). BA and BAA, with IC_{50} values of 2.72 mg/ml and 1.58 mg/ml respectively, showed better inhibitory activity than the positive control, caffeine (IC_{50} values of 2.98 mg/ml). BA, with an IC_{50} value of 2.72 mg/ml, also showed better inhibition activity than the BA/OA mixture (3.82 mg/ml).

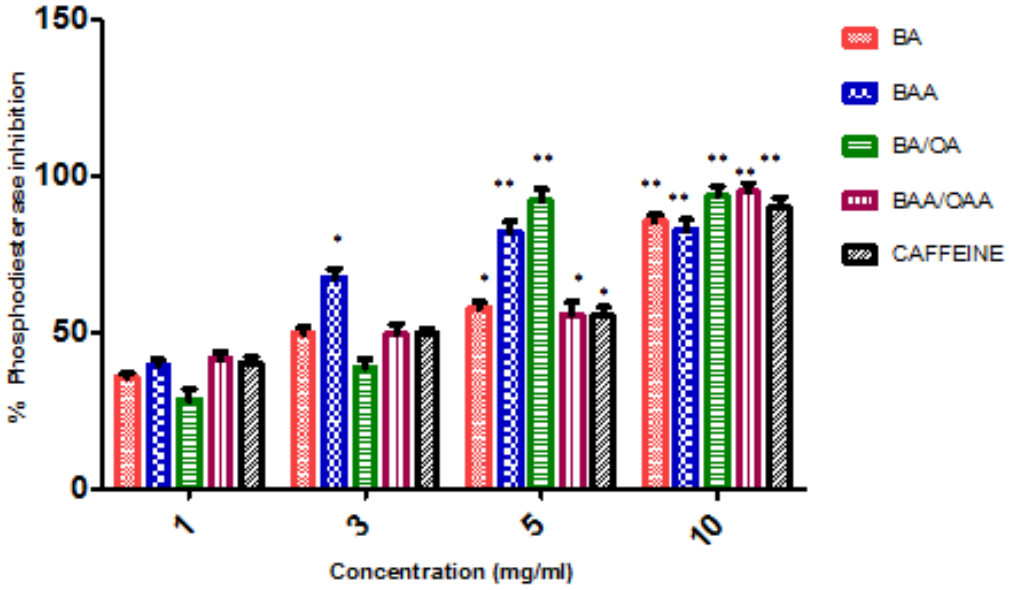

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Figure 4.9: Percentage phosphodiesterase inhibition activity of the compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

Table 4.6: The IC_{50} values of percentage phosphodiesterase inhibition of the compounds

Compound	BA	BAA	BA/OA	BAA/OAA	Caffeine
IC_{50} mg/ml	2.72	1.58	3.82	3.20	2.98

4.8 Calcium levels in cytosol

The inhibitory effects of compounds on the release of calcium into cytosol are presented in Figure 4.10

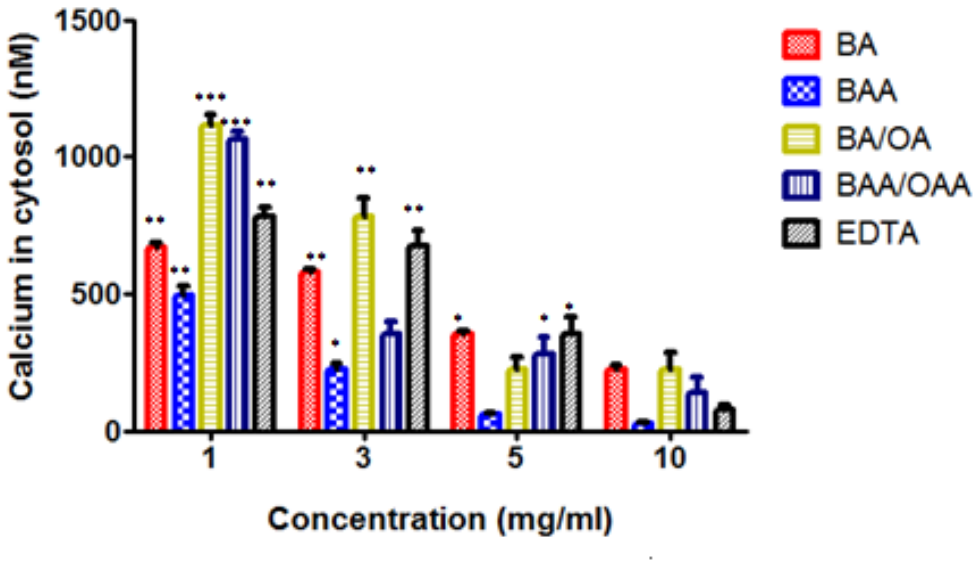

Figure 4.10: The compound inhibition of Calcium levels in cytosol Data is expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

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The compounds showed a significant dose dependent calcium level decrease in cytosol. BAA showed the highest inhibition at 10 mg/ml, when compared with other isolated compounds. BAA also showed better inhibition than EDTA, the positive control. BA showed better inhibition than the BA/OA mixtures, at 1 and 3 mg/ml. The BAA/OAA mixture showed better inhibition of calcium levels in cytosol than the BA/OA mixture.

4.9 Tail Bleeding time (*ex vivo*)

The *ex vivo* anticoagulant activity of the test compounds was evaluated using the rat's tail bleeding time assay. It is apparent that the compounds prolonged the bleeding time (Figure 4.11).

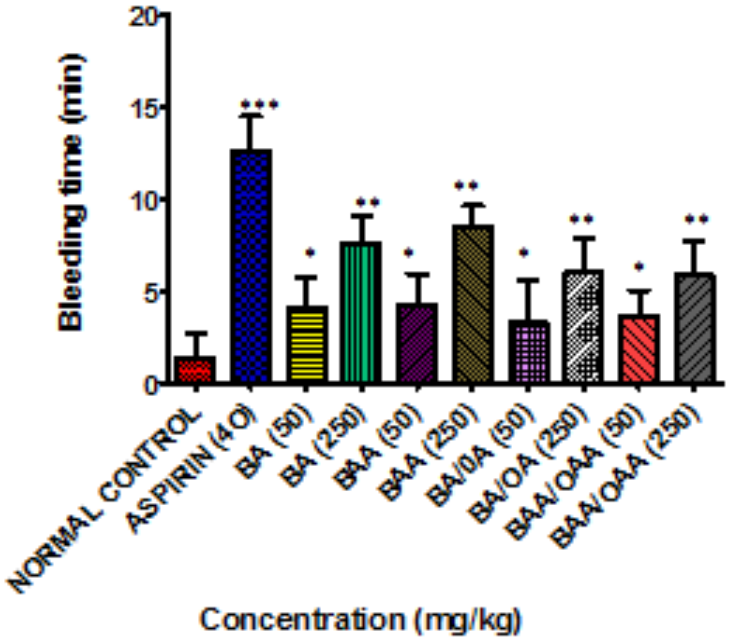

Figure 4.11: Bleeding time for the isolated compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

All the compounds exhibited significant dose-dependent bleeding times. BAA at 250 mg/kg showed the highest bleeding time in comparison to other compounds. Aspirin exhibited the longest bleeding time in comparison to all the compounds.

4.10 Anti-inflammation activity

The anti-inflammatory activity of isolated compounds was presented in Figure 4.12

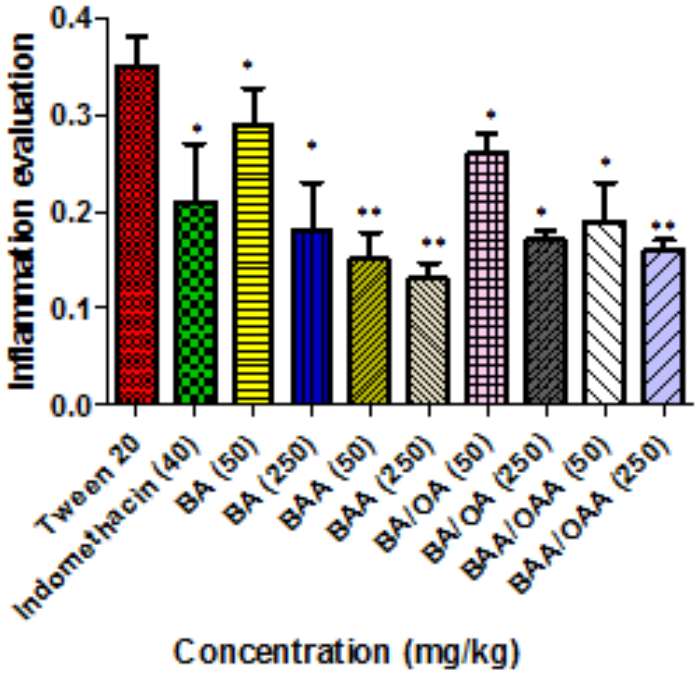

Figure 4.12: Inflammation evaluation of the isolated compounds. Data is expressed as mean SD. *P < 0.05, **P < 0.01.

The isolated compounds showed significant dose-dependent anti-inflammatory activity. BAA showed better anti-inflammatory activity in comparison to the positive control indomethacin. BAA also exhibited the highest inhibition, at 250 mg/kg, when compared to other isolated compounds. BAA/OAA showed higher anti-inflammatory activity when compared to BA/OA at 50 mg/kg.

4.11 Cyclooxygenase evaluation

The percentage inhibition of *in-vitro* COX activity was investigated and presented in Figure 4.13

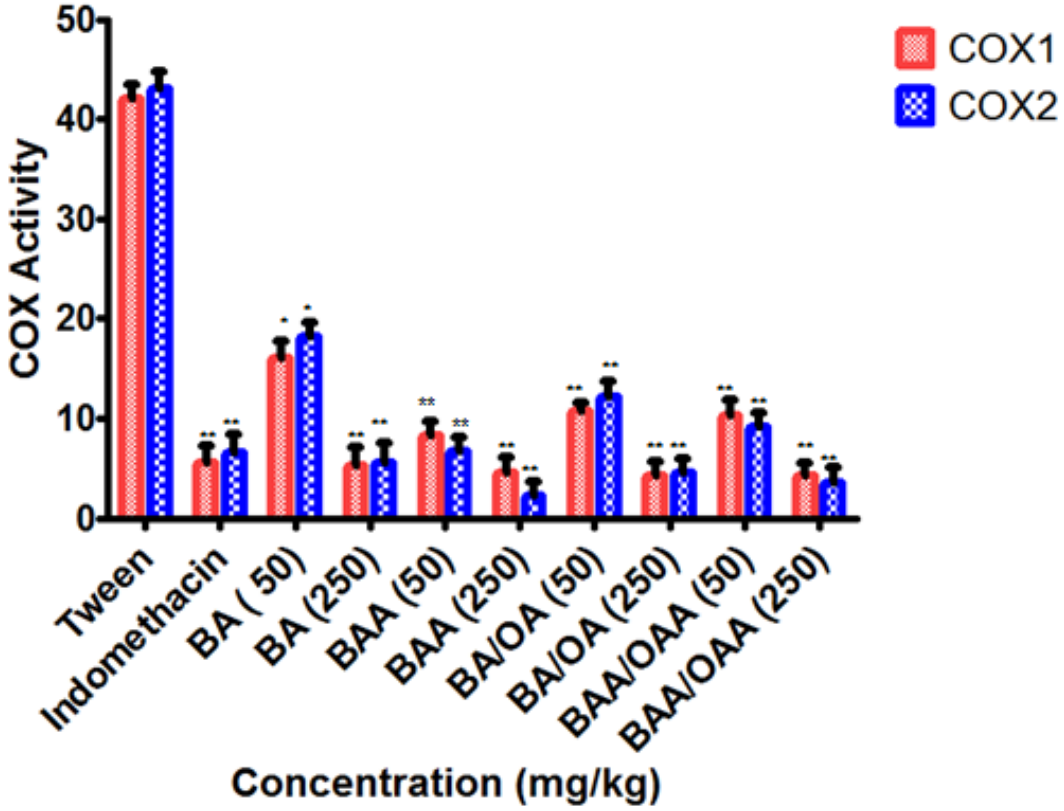

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Figure 4.13: Percentage COX inhibitory activity of the isolated compounds Data is expressed as mean SD. *P < 0.05, **P < 0.01.

The compounds showed significant dose-dependent inhibition on both COX-1 and COX-2 when compared to the negative control. The BA and BA/OA mixtures along with Indomethacin, the positive control, showed better inhibitory activity on COX-1 than COX-2, whereas the BAA and BAA/OAA mixtures showed better inhibitory activity on COX-2 than COX-1. BAA showed the highest COX 2 inhibition at 250 mg/kg.

4.12 Superoxide Dismutase activity

The SOD stimulatory activity of the compounds was investigated and is presented in Figure 4.14

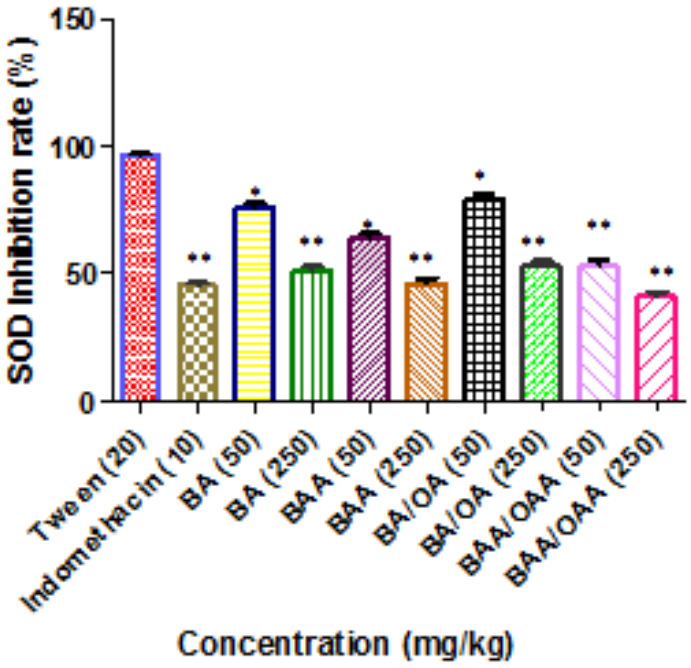

Figure 4.14: Percentage SOD stimulatory activity of the isolated compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

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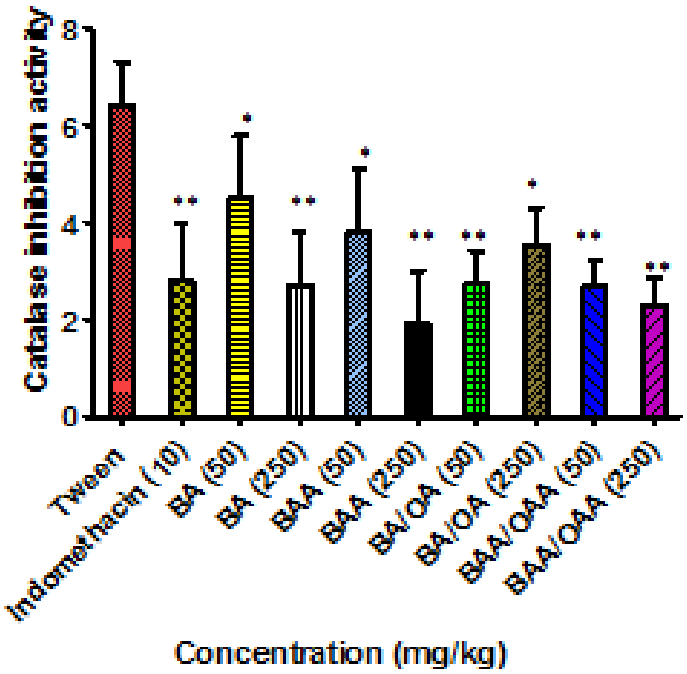
The compounds showed significant dose-dependent SOD stimulatory activity. BAA showed better SOD activity when compared to BA at 50 mg/kg. BAA/OAA also showed better SOD activity than BA/OA at 50 mg/kg, BAA and BAA/OAA showed similar SOD activity to the positive control, indomethacin, at 250 mg/kg,

4.13 Catalase activity

The catalase stimulatory activity of the compound was investigated and is presented in Figure 4.15.



Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

Figure 4.15: Catalase stimulatory activity of the isolated compounds.

The compounds showed significant dose-dependent catalase stimulatory activity. BAA showed better catalase activity than BA. BAA/OAA mixtures showed better catalase activity than BA/OA at 250 mg/kg. BAA also showed better catalse activity than the indomethacin positive control at 250 mg/ml.

4.14 Iron chelation

The results of iron chelation activity of the compounds are presented in Figure 4.16.

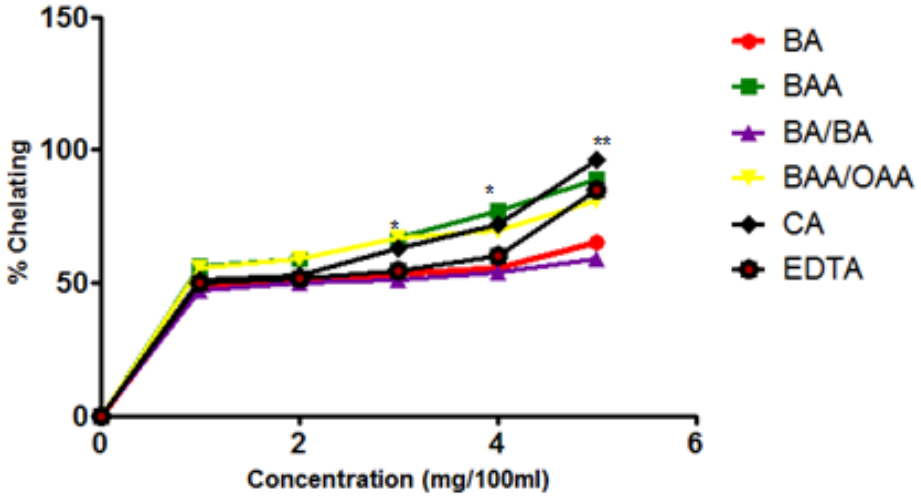


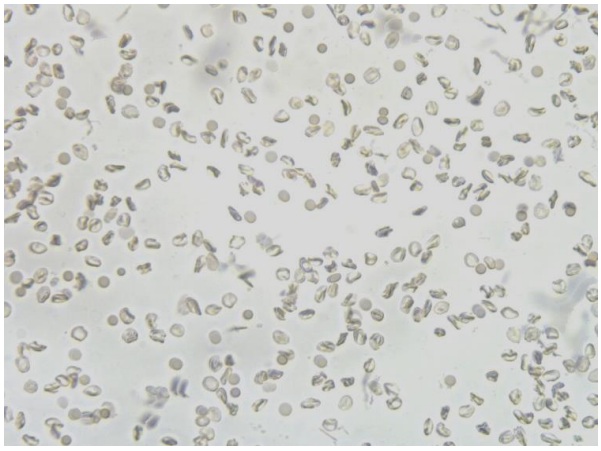
Figure 4.16: Percentage iron chelation of the isolated compounds.

Compound	IC_{50} (µg/ml)
BA	1.62
BAA	0.88
BA/OA	2.16
BAA/OAA	0.92
CA	0.96
EDTA	1.04

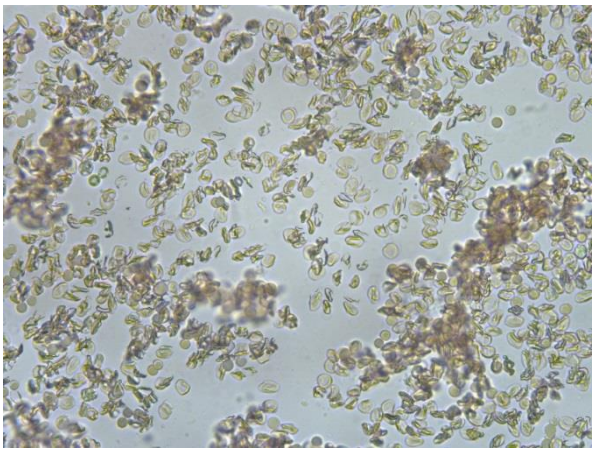
Table 4.7: The IC_{50} values of percentage iron chelation of the isolated compounds Data is expressed as mean ± SD. *P < 0.05, **P < 0.01.

The compounds showed significant dose-dependent iron chelation activity. BAA showed the highest activity with IC_{50} values of 0.88 mg/ml when compared to other compounds (Table 4.7). BAA and BAA/OAA, with IC_{50} values of 0.88 mg/ml and 0.92 respectively, also exhibited better IC50 values than the positive control CA (IC_{50} of 0.96 mg/ml) and EDTA (with an IC_{50} value of 1.04 mg/ml).

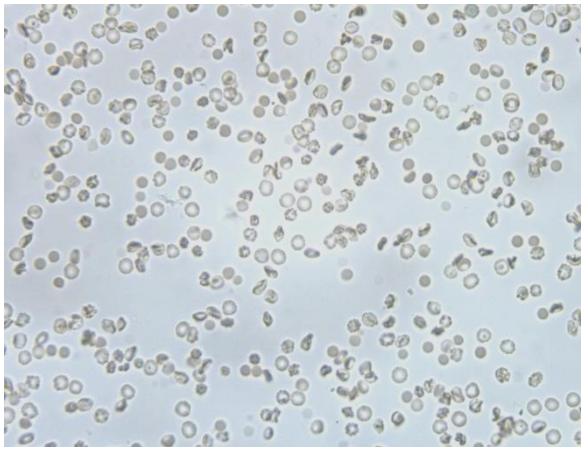
4.15 The microscopic pictures of platelet aggregation treated with isolated compounds



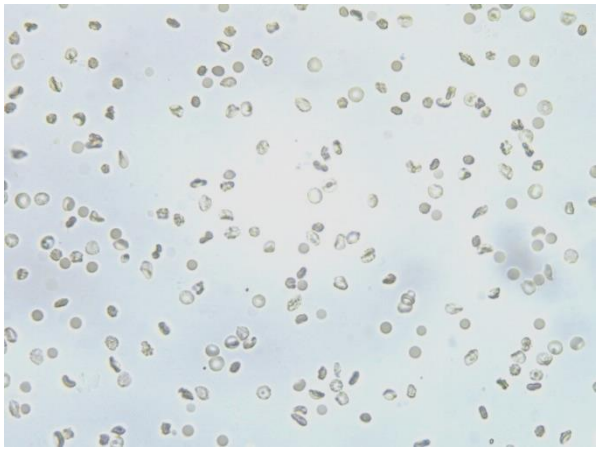
A



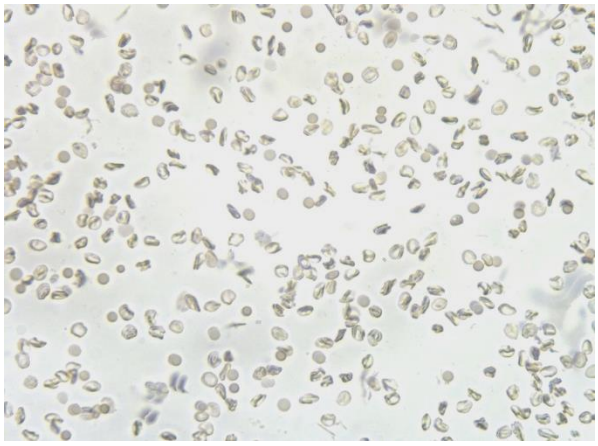
B



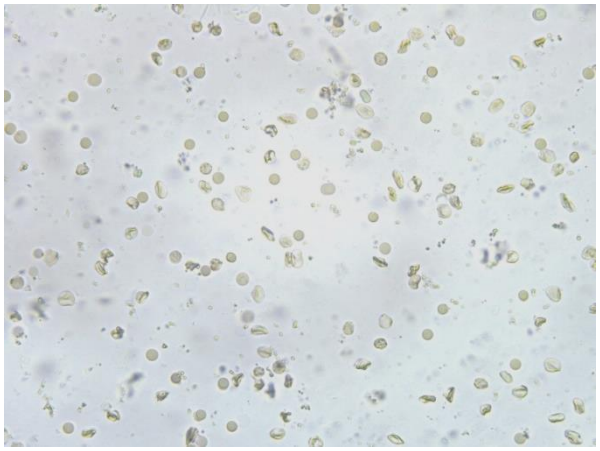
C



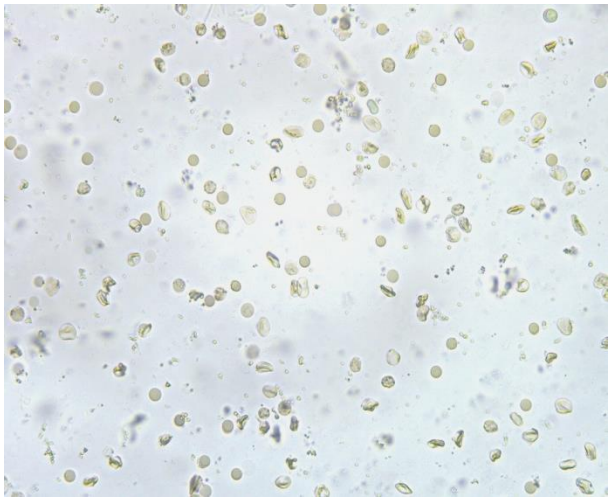
D



E



F



G

Figure 4.17: The microscopic pictures of platelet aggregation treated with isolated compounds (10mg/ml) at a magnification of x1500.

(A) resting platelets, (B) platelet aggregation induced by thrombin, (C) platelet aggregation induced by thrombin and treated with BA, (D) platelet aggregation induced by thrombin and treated with BAA), (E) platelet aggregation induced by thrombin and treated with BA/OA, (F) platelet aggregation induced by thrombin and treated with BAA/OAA, and (G) platelet aggregation induced by thrombin and treated with Aspirin.

The microscopic pictures of antiplatelet aggregation activity of the compounds are presented in Figure 4.17. The compounds inhibit the platelet aggregation induced by thrombin (Figure 4.17 C, D, E, F).

Chapter Five

5. Discussion

Blood platelets are important in the maintenance of hemostatic systems and in the repair of damaged endothelium tissues during rupture. However, pathological platelet aggregations have been implicated in cardiovascular diseases (Dickneite et al., 1995). Therefore, targeting platelet aggregation could be the right approach to minimizing cardiovascular disease-related mortality. Medicinal plants are currently the source of diverse bioactive compounds vital to human health. The multi-therapeutic properties of plant-derived triterpenes have made them potential candidates for the development of new drugs. The results obtained in this study revealed that the triterpenes isolated from *M bracetacta* and their derivatives exhibit anti-platelet aggregation activities (Table 4.5, Figuure 4.6 and Figure 4.17) regardless of the agonists used (thrombin, collagen, ADP and epinephrine). Some pentacyclic triterpenes have previously been reported to display antiplatelet aggregation activities (Jin et al., 2010; Kim et al., 2010; Xuemei et al., 2010; Tzakos et al., 2012; Habila et al., 2013; Ibrahim et al., 2013).

The apparent mechanism by which the triterpenes in this study exhibited the observed anti-platelet aggregation activity could be attributed to various factors, which include, among others: anti-thrombin activity, the potential to inhibit the release of ATP from platelet granules, the inhibition of acetylcholine esterase and phosphodiesterase activity, chelation of Ca^{2+} ions, as well as anti-inflammatory and anti-oxidant properties.

Thrombin plays an important role in abnormal coagulation formation (Dickneite et al., 1995). Thrombin converts fibrinogen into insoluble fibrin during the coagulation process. It also activates coagulation factors V, XIII, and XI, thus increasing the number of activated platelets in the circulation of blood. This results in the activation of arachidonic pathways that release phospholipids, which in turn trigger platelet aggregation (Tortora and Grabowski, 2000). Apart from this, thrombin exhibits pro-inflammatory properties by enhancing the proliferation and migration of smooth muscle cells (Borissoff et al., 2011). Antithrombin agents induce the release of prostaglandin I2 from vasculoendothelial cells, which inhibits platelet aggregation (Mizutani et al., 2003). Findings from the

present study indicate that the compounds possessed antithrombin activity (Table 4.4, Figure 4.5). The antithrombin activity of diterpenes isolated from *Dictyota menstrualis* have been reported previously (Moura et al., 2014)

Platelets comprise of cellular granules which store chemical components possessing extraordinary potential. During platelet activation, the content of the granules is released (Gehani, 1998). ATP is released via exocytosis from platelet dense granules along with other components, such as ADP and thromboxane A_2. This amplifies the progression of platelet aggregation and the mobilization of more active platelets (Jacqueline, 2005). There is thus a connection between antiplatelet aggregation activity and ATP release (Ban et al., 2007). The findings obtained from this study show that the compounds inhibited ATP release (Figure 4.7), possibly by preventing the degranulation of platelet-dense granules. Aspirin has been reported to inhibit ATP release (Raul, 1999). The increase in ATP released as the concentrations of BAA and BA/OA increase could indicate that the compound affects the loss of membrane integrity in platelet-dense granules as concentration increased (Haubelt, 2008).

Acetylcholine is a neurotransmitter which co-ordinates the transmission of nerve impulses between nerve cells or neuromuscular junctions. Acetylcholine enhances the production of NO and prostacyclin, which are potent platelet aggregation inhibitors by preventing platelet adhesion and aggregation (Moncada, 1982; Andrew et al., 2001). Acetylcholine regulates the mobilization of clotting factors for homeostasis during tissue injury, as well as maintaining vasculoendothelial tissue tone (Sakrov and Skipetrov, 1977). At the cholinergic synaptic cleft, acetylcholinesterase (AChE) hydrolyses acetylcholine to choline and acetate (López, 2010), thereby regulating the transmission of impulses across cholinergic synapses. The inhibition of AChE has been considered to be a promising approach for the treatment of neurodegenerative diseases such as senile dementia, Alzheimer's diseases, myasthenia gravis, ataxia and Parkinson's disease (Mukherjee et al., 2007). Platelet dysfunction has also been implicated in Alzheimer's disease (Larlori et al., 2005). AChE inhibitors derived from medicinal plants have previously been reported on (Mills, 2004; Mukherjee and Wahile 2006; Dohi et al., 2009). The results obtained from this study revealed that the compounds possessed

anti-acetylcholinesterase activity (Figure 4.8). Triterpenes isolated from medicinal plants have been reported to inhibit AChE activity (Areche *et al*., 2009; Calderon, 2009). BAA exhibited the highest acetylcholinesterase inhibition activity in this study.

Platelet aggregation could be inhibited either by antagonists of membrane receptors coupled with intracellular signaling pathways, or by inhibition of enzymes, such as phosphodiesterase which is directly involved in intracellular signaling pathways (Kalantzi et al., 2012). Phosphodiesterase hydrolyzes cAMP and cGMP intracellular secondary metabolites which are important to platelet functioning. Increased intracellular levels of these secondary metabolites inhibit platelet activation (Page and Spina, 2011). In the present study, the isolated compounds exhibited potent phosphodiesterase inhibitory activity (Table 4.6; Figure 4.9). Triterpenes isolated from *Vochysia pacifica* were reported to inhibit phosphodiesterase activity (Weniger et al., 2005). Better inhibitory activity of phosphodiesterase by BAA than BA could be attributed to the modification of hydroxyl groups at the C-3 position with acetyl moiety (Ban, 2010). BA and BAA also showed better inhibitory activity than caffeine, the positive control. Caffeine is a member of the methylated xanthines, have previously been reported as a non-selective PDE inhibitor (Moustafa and Feldman, 2014). The non- selective PDE-inhibitor also acts as an antagonist of adenosine receptors and prevents adenosine uptake for platelet aggregation. Adensoine is widely used during supraventricular tachycardia to prevent heart attacks or stroke (Konrad et al., 2013).

The concentration of intracellular calcium in the platelet cytosol determines the status of platelet activation. This drives the conformational changes in platelet structures and subsequent degranulation that enhances platelet activation (Nesbitt et al., 2002). Calcium ions activate the integrin αIIbβ3 that binds to fibrinogen, thus stimulating platelet aggregation (Warwick et al., 2003). Calcium ions also play an important role in the activation of coagulation cascade by enhancing the binding of coagulation factors (Johan et al., 2002). The level of intracellular calcium is maintained by cAMP dependent calcium pumps (Yip et al., 2005). The protein tyrosine kinases antagonist elevates the cAMP level by enhancing the adenylate cyclase activity and leads to the chelation of intracellular calcium (Johan et al, 2002). The cAMP enhances the calcium effluxes,

which reduces the calcium concentration that prevents platelet activation (Johan et al., 2002). The fura-2 dye is used to measure platelet calcium flux due to platelet fluorescence capacity (Yap et al., 2000). This study revealed that the compounds are dose-dependent and inhibit calcium concentration in the platelet cytosol (Figure 4.10), possibly through their potential to elevate cAMP through the inhibition of phosphodiesterase activity. Oleanolic acid, a pentacyclic triterpene, has previously been reported to inhibit the calcium mobilization in extracellular matrix (Lee et al., 2007). BAA shows better calcium mobilization inhibition than the EDTA positive control.

Tail bleeding time is used to assess the anticoagulation potential of medication. Abnormal coagulation contributes to thromboembolic disorders such as stroke, deep venous thrombosis, heart attack and pulmonary emboli (Nalise et al., 2008). Hypercoagulation has also been implicated in the progression of cancer (Rickles and Falanga, 2001). The compounds exhibited a prolonged bleeding time (Figure 4.11), indicating anticoagulant properties. Lanosteryl triterpenes, isolated from *Protorhus longifolia* stem bark, have previously been reported to prolong tail bleeding time (Mosa et al., 2015). The test compounds displayed lower activity when compared with Aspirin, the positive control. This could be due to the poor bioavailability of the compounds. However, it has been documented in previous studies that there is no correlation between antiplatelet aggregation activity and bleeding time (Mekhfi et al., 2008; Gadi et al., 2009).

Chronic inflammation is implicated in thromboembolic disorder such stroke, pulmonary embolism and myocardiac infarction. These disorders have been reported to cause over 20% of world mortality (Cohen et al., 2011). The relationship between inflammation and hemostatic systems in both physiological and pathological conditions has been established (Hirsh et al., 2007; Verhamme and Hoylaerts, 2009). In this study, cotton pellet-induced granuloma was used for a chronic inflammation investigation. The compounds showed significanceant anti-inflammatory properties compared to the control (Figure 4.12). The anti-inflammatory potential of some triterpenes has previously been reported on (Yadav et al., 2010; Mosa et al., 2011, 2015). BAA again exhibited better anti-inflammatory properties than BA, which again is attributed to the modification

of C-3 hydroxyl group with acetyl. BAA also has higher anti-inflammatory properties compared to indomethacin, a widely used non-steroidal anti-inflammatory drug (NSAID). These findings validated the report of Mosa et al., (2015) that triterpenes isolated from *protorhus longifolia* stem bark possessed higher anti-inflammatory potential than indomethacin. BA/OA has higher anti-inflammatory properties compared to BA at 50 mg/kg. This could be attributed to the synergistic action of the compound mixture. BAA/OAA also showed better activity compared to BA/OA. This could be attributed to the modification of the C- 3 hydroxyl group position within the acetyl group.

The cyclooxygenase (COX) enzymes play major roles in the formation of thromboxanes and prostaglandins. They have been implicated in virtually all physiological and pathological activities (Fitzpatrick, 2004). The inhibition of COX enzymes has effectively been used in the treatment and management of inflammation related ailments such as cardiovascular diseases, cancer, diabetes, neurodegenerative diseases, pulmonary diseases and autoimmune diseases (Shacter and Weitzman, 2002). There are two types of COX enzymes (COX-1 and COX-2). COX-1 is a constitutive enzyme which is important for cellular housekeeping, whereas COX-2 is an inductive enzyme, induced during inflammation. Non–selective NSAIDs, such as Aspirin, inhibit both iso-enzymes. This could also attribute to their frequent side effects. The use of Aspirin in treatments has also been implicated in gastrointestinal bleeding. This prompts the search for selective COX-2 inhibitors in others, to spare the COX-1 housekeeping effects (Rainsford, 2007). The present study has shown that, with the inhibition of COX enzymes, all the compounds exhibited anti-inflammatory properties (Figure 4.13) Some pentacyclic triterpenes, isolated from *protium kieinii*, have previously been reported to inhibit COX enzymes (Otuki et al., 2005). Betulinic acid, isolated from *Scoparia dulcis*, has been demonstrated to possess anti-inflammatory properties in attenuating COX enzymes (Tsai, 2011). Indomethacin is a commonly used NSAID that has been reported to selectively inhibit COX-1 enzymes (Stichtenoth et al., 1997). BAA possessed higher inhibitory activity compared with other compounds and indomethacin. The inhibitory activity of BAA and BAA/OAA mixtures on COX-2 was better than COX-1.

Superoxide dismutase is an endogenous antioxidant that hydrolyzes superoxide

radicals into hydrogen peroxide and oxygen, which are lesser toxins. The present study showed that the compounds exhibited potent SOD stimulatory activity (Figure 4.14) Triterpenes isolated from *Ganoderma lucidum* have been reported to possess potent SOD stimulatory activity (Smina et al., 2011). The potent SOD stimulatory activity of Lanosteryl triterpene, isolated from *Protorhus longifolia* stem bark, has previously been reported on (Mosa et al., 2015). BAA at 250 mg/kg also possessed a similar SOD stimulatory activity with indomethacin, a commonly used NSAID drug. Indomethacin has been reported to possess a strong SOD stimulatory activity (Dillon et al., 2003).

Inflammation enhances the formation of excess reactive oxygen species (ROS), which could lead to oxidative stress. This triggers the release of inflammation mediates from the macrophages, which further intensifies the inflammation process (Porfire et al., 2009). The relationship between oxidative stress and abnormal platelet aggregation has previously been reported on (Violi and Pignatelli, 2012). High concentrations of hydrogen peroxide have been demonstrated to stimulate platelet aggregation (Lopez et al., 2007). Catalase is an antioxidant found in all living cells that catalyzes the hydrolysis of hydrogen peroxide into water and oxygen (Chelikani et al., 2004). Antioxidant drugs have been found to be effective in the treatment of inflammation and abnormal platelet aggregation diseases such as stroke, heart attack and pulmonary embolism (Freedman, 2008). The present study showed that all the compounds possessed potent catalase activity (Figure 4.15). Triterpene isolated from *protorhus longifolia* stem bark have previous been reported to posses high catalase stimulatory activity (Mosa, 2015). The compound (BAA) showed higher catalase stimulatory activity at 250 mg/kg compared to other compounds. This could be attributed to the modification of the C-3 position of hydroxyl within the acetyl group.

Abnormal iron homeostasis has been implicated in the onset of cardiovascular diseases, asthma and cancer (Lee et al., 2004). Iron overload leads to fenton reactions which encourage the production of free radicals such as hydrogen peroxide (H_2O_2). The H_2O_2 reacts with more Fe^{2+} to produce hydroxyl radicals that result in lipid peroxidation in the membrane (Thirupathi *et al.,* 2011). This process could also trigger abnormal platelet activation and inflammation. The present results indicated that the isolated

compounds could prevent ROS formation through iron chelation (Table 4.7; Figure 4.16). The highest iron chelation activity of BAA and BAA/OAA, when compared with the other isolated compounds and the positive control, could be as a result of the modification of the C-3 position of the hydroxyl group within the acetyl moiety.

In the search for alternative potent antiplatelet drugs, clinically safe drugs are desirable. Potent antiplatelet drugs are expected to display weak cytotoxic effects on normal cells. The American National Cancer Institute guidelines consider a compound with IC_{50} values of < 30 µg/ml as cytotoxic (Suffness and Pezzuto, 1990). The findings from this present study revealed (Table 4.3) weak cytotoxic effects of the compounds on both normal cells (HEK293) and cancerous cells (HEPG2). However, some triterpenes have been reported to have strong cytotoxic effects (Lee et al., 2007; Peteros and Uy, 2010). Despite the weak cytotoxic effects displayed by the compounds, the higher cytotoxic effects on HEPG2, compared to HEK293, implies that the compounds could inhibit cancer cell proliferation. Betulinic acid has been demonstrated to selectively inhibit tumour cells (Pisha et al., 1995). BA and its derivatives, isolated from *Melaleuca cajuput*, have also been reported to selectively inhibit myeloid leukemia (HL-60) cell line (Faujan, 2010).

BAA and the other acetyl derivatives exhibited the highest anti-platelet aggregation activity compared to other compounds. The acetyl derivatives were also the most active in all the parameters measured. This could be attributed to the modification of C-3 hydroxyl with acetyl moiety (Ban et al., 2010). The chemical modification of drugs is known to increase the effectiveness of drugs (Simelane et al., 2013). Targeting carbon positions 3 and 28 has been reported as the new pharmacophores for increasing biological activity (Ban et al., 2010). It is worth noting that Aspirin, a potent antithrombin agent, has acetyl moiety as its major functional group (Undas et al., 2007). The BA/OA and the BAA/OAA mixtures exhibited a lower antithrombin activity when compared with BA, which is possibly indicative of the synergistic effects of the compounds.

Chapter Six

6. Conclusion

The present study revealed that BAA possessed the highest anti-platelet aggregation activity, regardless of the agonists, in comparison with Aspirin, which is a known non-steroidal, anti-inflammatory drug (NSAID). BAA also possessed anti-inflamatory activity which complemented platelet functions. The possible mechanisms of action of BAA as a platelet aggregation inhibitor include: antithrombins, acetylcholinesterase activity inhibition, phosphodiesterase activity inhibition, calcium mobilization inhibition, the inhibition of release from dense granule content, anticoagulants, cyclooxgensase (COX-2) activity inhibition, iron chelators, and enhanced SOD and CAT activity. These processes attenuate thrombosis formation and the progression of cardiovascular diseases (Robbin et al., 2006). In addition to their efficacy, the poor cytotoxicity of BAA indicated their safety as antiplatelet agents. To the best of the researcher's knowledge, this is the first time that the antiplatelet aggregation activity and possible mechanisms of action of BA and the acetyl derivatives isolated from *Melaleuca bracetata* were investigated.

6.1 Recommendation for further studies

The following suggestions are made for further studies:

- Synthesise betulinic acid derivatives by modifying compounds at C-28 moiety.
- Investigate the activity of compounds on G protein coupling receptors.
- Investigate the activity of compounds on the binding affinity of fibrinogen.
- Investigate the activity of compounds on the biosynthesis of thromboxane $A_{2.}$
- Investigate the activity of compounds on Glycoprotein IIb/IIIa for platelet aggregation.
- Investigate the activity of compounds on cytokines such as P-selectin, CD40L and interleukin.

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APPENDIX A

A. Details of preparation of some reagents

A1. Resuspending buffer (pH 7.4)

8.18 g of 0.14 M NaCl; 2.36 g of 15mM Tris-HCI and 0.9 g of 0.005 M glucose were dissolved and made up to 1000 ml with distilled water.

A2. Washing buffer (pH 6.5)

32.77 g of 0.113 M Nacl; 3.053 g of 4.3 mM Na2HPO4; 3.741 g of 4.3 mM of K2HPO4; 14.64 g of 24.4 mM of NaH2PO4; 5.45 g of 5.5 mM glucose; and 1.86 g of 1 mM EDTA were dissolved in 5000 ml of distilled water.

A3. Tris buffer (Ph 7.4) containing EDTA and NaCl

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

A4. ADA (acid dextrose – anticoagulant)

100 g of dextrose; 68.296 g Citric acid and 124.95 g trisodium citrate were dissolved in 5000 ml of distilled water.

A5. Phosphate buffer (pH 6.6, 0.2M)

18 ml of 0.2 M KOH and 50 ml of 0.2 M KH2PO4 were mixed and made up to 100ml with distilled water.

A6. Homogenisation buffer pH 7.0

87.65g of of 250 mM of sucrose, 1 crushed protease inhibitor tablet and 7.88g of Tris-HCl (50 mM) were mixed together and made up to 100 ml with distilled water.

APPENDIX B

B. Details of some methodologies

B1. Extraction and isolation of betulinic acid

The leaves of *Melaleuca bracteata* var. revolution gold were plucked from the stuck and allowed to air dry at room temperature. The leaves (500 g) were weighed in a beaker and were then extracted by cold maceration in dichloromethane (1.5 w/v) at room temperature (5L X 3) for 24 hours. The combined filtrate was concentrated under reduced pressure by using a rotator evaporator at 40 ^{o}C and allowed to air dry at room temperature, yielding 0.6 % of the crude extract. The mass obtained from the crude extract was defatted with n-hexane. A portion (5 g) of the residue was subjected to chromatographic separation on silica gel (60-120 mesh) columns (20 x 5.5 cm) and was eluted with a gradient of hexane/ethyl acetate (8:2 to 7:3) for the isolation of betulinic acid. Eighty fractions of elutes (20 ml) were collected and monitored with thin-layer chromatography. The TLC plates (aluminium sheets 20 cm × 20 cm) were first viewed under ultraviolent light, before being developed by spraying them with 10% H_2SO_4 and then dried with a hot dryer. Similar fractions containing the desired compound were combined. These were further concentrated by using a rotator evaporator at 40 ^{o}C and recrystallized in methanol. The isolated compound was characterized with spectral analysis (NMR, IR and MS).

B2. Synthesis of acetyl derivative

The betulinic acid isolated from *Melaleuca bracteata* was dissolved in a conical flask containing pyridine (10 ml) and acetic anhydride (12 ml). The mixture was refluxed under a fume cupboard for 8 hours at 40° C. The reaction was stopped by transferring the mixture into beakers containg 250 ml of water. This was then filtered using Whatman No. 1 filter paper. The filtrate was air dried and weighed to calculate the percentage yield. The acetylate compound was confirmed using spectral analysis (NMR, IR and MS).

B3. Chelating activity of Fe^{2+}

The various concentrations (0 -.5 mg/100 ml CH3OH) of the compounds 5mM ferrozine and 2mM $FeCl_2$ were prepared. Test tubes were set up in triplicate for each compound. One milliliter of compound was diluted with 3.75 ml of distilled water and then mixed with 0.2 ml of ferrozine and 0.1ml of $FeCl_2$. The mixture then stood for 10 minutes and mixed at intervals. The mixture was read at 562 nm to determine their chelating activity. EDTA and citric acid served as a positive control and distilled water served as the negative control. The percentage chelating activity of the compounds was calculated as;

% chelating activity = [1-At/Ac] x100

At is the absorbance of treated sample whereas Ac is absorbance of control.

The IC_{50} of the compounds was calculated using statistical package origin 6.1

B4. MTT cell proliferation assay

MTT (5 mg/ml) was prepared with a phosphate buffer.The cells were cultured in a 25 cm^2 flask to confluency and trysinsed. They were then pipetted into a 48-wells plate at specific seeding densities (2.5 x 10_4 cells per well). The cells were incubated for 24 hours at 37°C and fresh medium (MEN + Glutmax + antibiotic) was added. The compounds were then added in triplicate and further incubated for 4 hours. The medium was removed and replaced with complete medium (MEM + Glutmax + antibiotics + 10 % Fetal bovine serum). After 48 hours the complete medium was replaced with MTT (200 µl) and the cell culture medium (200 µl) was incubated for another 4 hours. The MTT and cell culture medium was removed from the 48-wells plate after the 4 hours. The reaction in each plate was stopped by DMSO and dissolved in formazan crystal. The absorbance of mixture on the plates was read at 570 nm using the Biotek plate reader ELx808 IU. The graph of the cell survivial was plotted against compound concentrations. The LC50 (Lethal concentration at 50 % cytotoxicity mortality) was calculated by regression analysis using the QED statistics program.

B5. chromogenic substrate

The chromogenic substrate (0.008 M) was prepared with distilled water and various concentrations of compounds (1-5 mg/ml) were prepared with tris buffer. A 96-wells plate was used and the experiment was triplicated. Compounds (50 µl) and 10 µl of thrombin (30 U/ml) were incubated in corresponding wells for 10 minutes at 25 °C. Chromogenic substrate (190 µl) was then added and changes in absorbance at 415 nm for 4 minutes at 12 second intervals were read by using the Biotek plate reader ELx808 IU. DMSO (1 %) served as a negative control. The percentage antithrombin was calculated using the following formula;

% Antithrombin activity = [1-At/Ac] x100

At is the absorbance of treated sample and Ac is the absorbance of control.

The IC_{50} of the compounds was calculated using the statistical package Origin 6.1.

B6. Acetylcholinesterase assay

Different concentrations of the compounds (0.25, 0.5, 1.0 mg/ml) were prepared for anti-acetylcholinesterase studies. The acetylcholinesterase assay kit was used following the instructions described by the manufacturer. The acetylcholinesterase inhibitory activity of the compound was calculated using the following formula;

$$\text{AChE Activity (units/L)} = \frac{(A412)\ \text{final} - (A\ 412)\ \text{initial} \times n \times 200}{(A\ 412)\ \text{calibrator} - (A412)\ \text{blank}}$$

(A412) finial = absorbance measurement after 10 minutes of incubation of the samples at room temperature

(A412) intial = absorbance measurement after 2 minutes of incubation of the samples at room temperature

n = the dilution factor (10, 20 and 100)

200 = the equivalent activity (units/L) of the Calibrator. When assayed, it is read at 2 minutes and 10 minutes.

(A412) calibrator = the absorbance of the calibrator at 10 minutes

(A412) blank = absorbance of the blank at 10 minutes

B7. Cotton pellet-induced granuloma

The anti-inflammatory activity of the compound was investigated by the cotton pellet-induced granuloma model. Twenty Sprague-Dawley rats (220 ± 20 g) were divided into four groups of five rats each and allowed to acclimatize for 5 days. The animals were pre-administered with the compounds thirty minutes before interscapular implantation of (20 mg) pre-weighed sterile cotton pellets. The rats were anesthetized prior to implantation of the cotton pellets. Inflammation was induced by making an intrascapular incision and then implanting the (20 mg) sterilized cotton pellet subcutaneously between the scapulae of each rat. The incisions were covered with medical plaster in order to secure the implanted cotton pellets.

The compounds were orally administered to the animals in the respective groups for seven consecutive days, starting from the day of sterilized cotton pellet implantation. On the eighth day, the rats were anaesthetized and the implanted cotton pellets were carefully dissected out. These were made free from extraneous tissues. The wet pellets were separately weighed and dried in an oven at 60 ^{o}C for 24 hours. The differences between the dry and wet pellets were taken as measurements of the formed granuloma weight. The anti-proliferation effects of the compounds were compared with the control. The percentage inflammation inhibition was calculated using the following formula:

% inhibition = (Wc –Wt/ Wc) x 100

Wc = the pellet weight of the control group rats

Wt = the pellet weight of drug treated rats

The dried cotton pellets were digested and the hydrolysate was used to investigate the catalase and superoxide dismutase (SOD) activity of the compounds

B8. COX activity

The COX activity of the homogenate was determined using the COX activity assay kit (item NO. 760151, Cayman chemical) following the manufacturer's instructions. The reaction mixture, consisting of assay buffer (120 µl), homogenate or COX standard (40 µl), heme (10 µl), and either inhibitor of COX-1 or COX-2 (40 µl), were pipetted into a 96-wells tray. This mixture was incubated for 5 mintes at room temperature. Colorimeter substrate (20 µl) was then added to the mixture along with arachidonic acid (20 µl) to intiate the reaction. The mixture was further incubated for 5 minutes at room temperature. The oxidation of calorimeter substrate was read at 590 nm using Biotek plate reader ELx808 IU. The experiment was triplicated and the percentage of COX inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Total COX activity - COX activity}}{\text{Total COX activity}} \times 100$$

B9. Determination of SOD content

The various concentrations (0.01, 0.1, 0.5, 1.0, 2.0, 5.0, 10 mg/ml) of diethyl ether were prepared and absorbances were read at 420 nm using the Biotek plate reader ELx808 IU. These values were used to plot the standard curve.

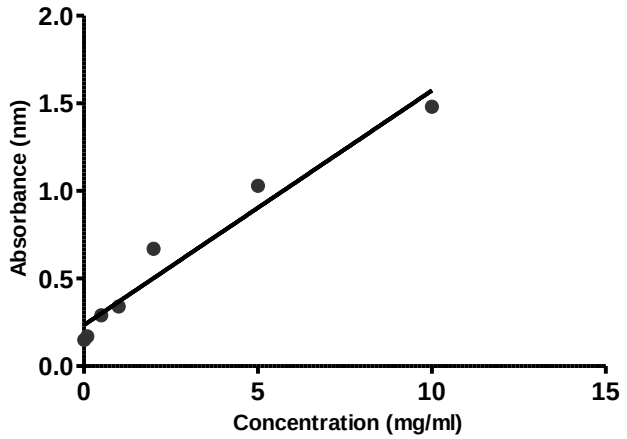

Figure B1: Calibration curve of SOD concentration (mg/ml) against absorbance (nm).

APPENDIX C

C.Spectra

C1. Spectra for BA

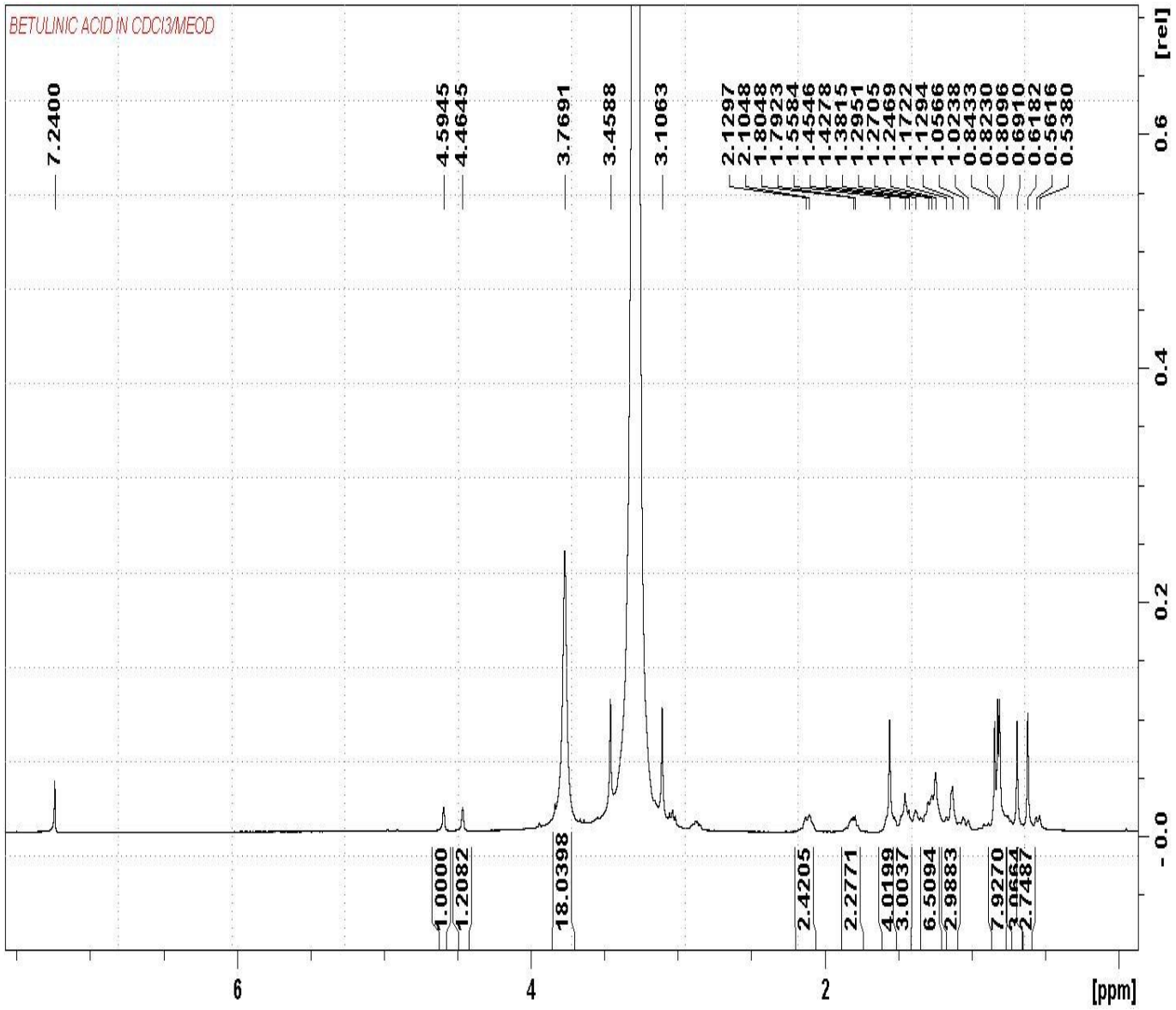

Figure C1.1a: ^{1}H-NMR spectrum of BA

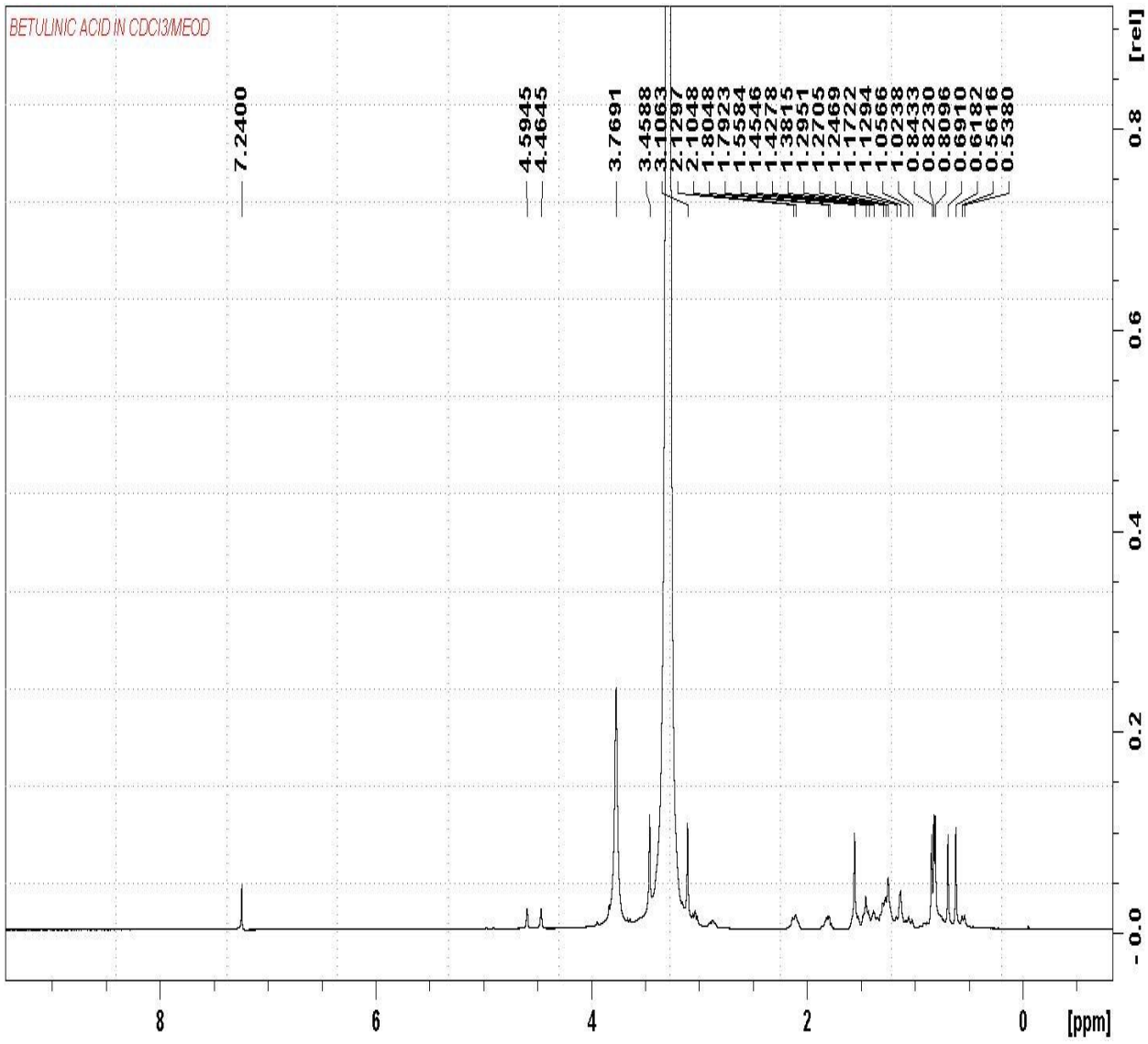

Figure C1.1b: ^{1}H-NMR spectrum of BA

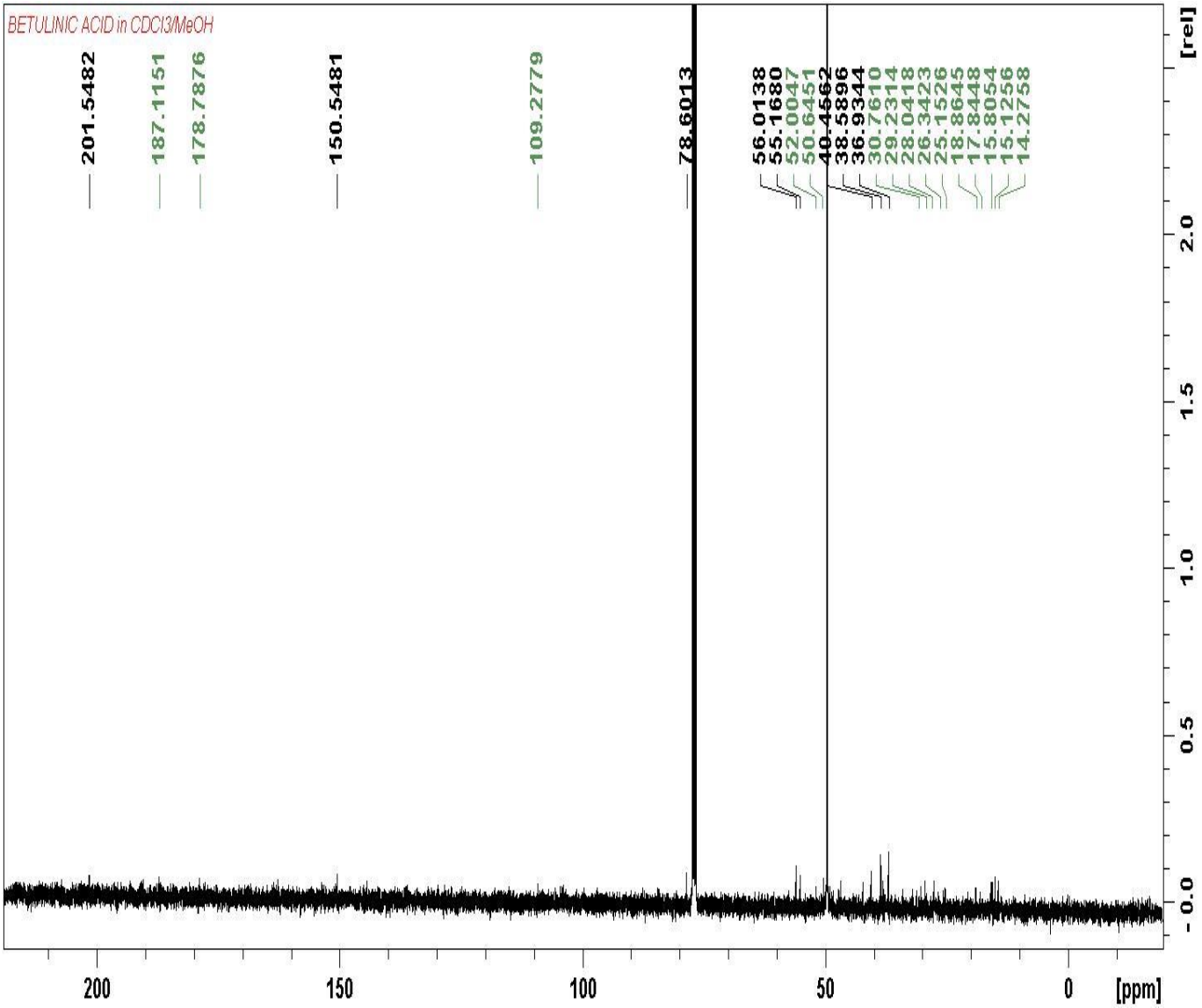

Figure C1.2a: ^{13}C-NMR spectrum of BA

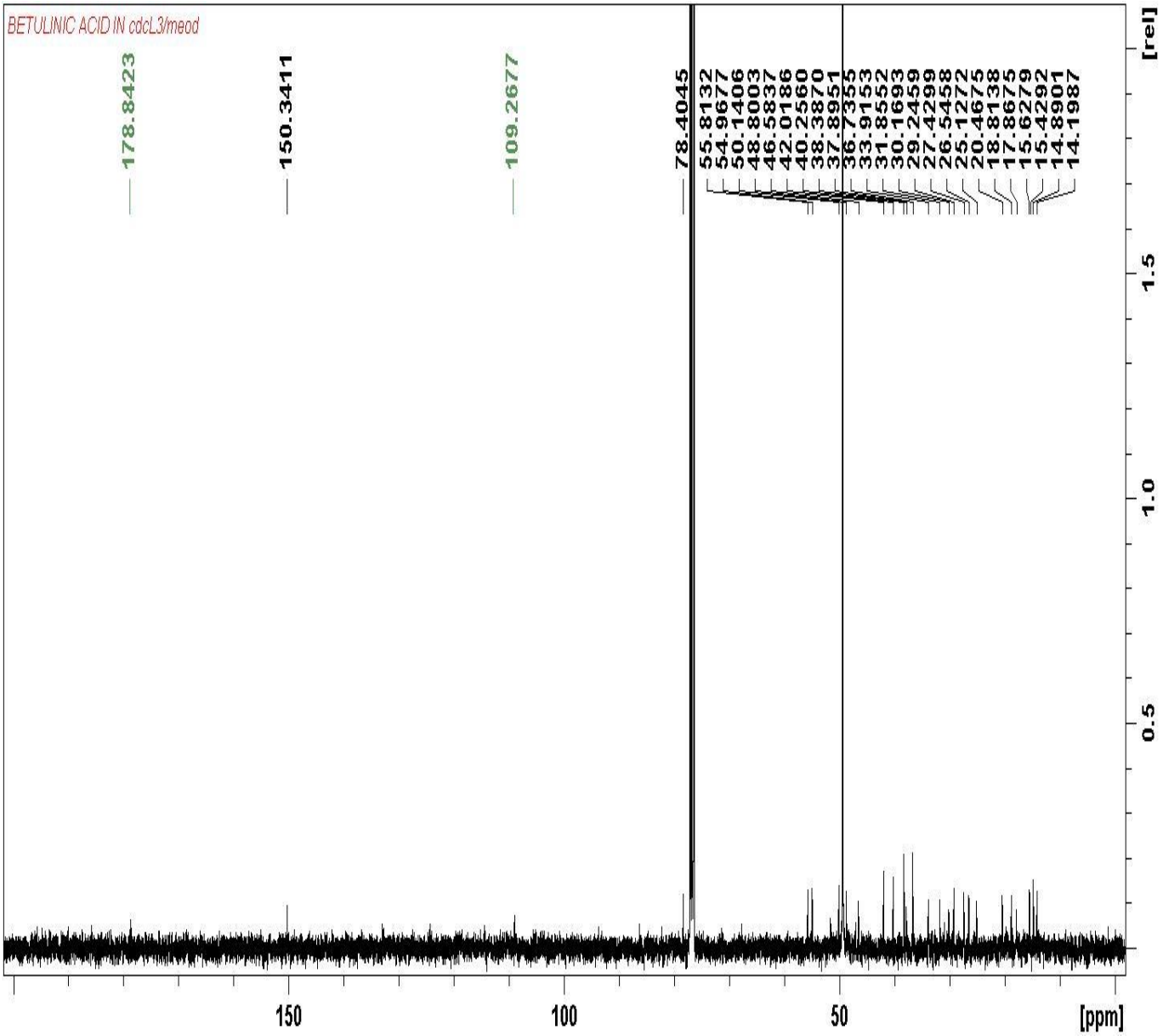

Figure C1.2b:^{13}C-NMR spectrum of BA

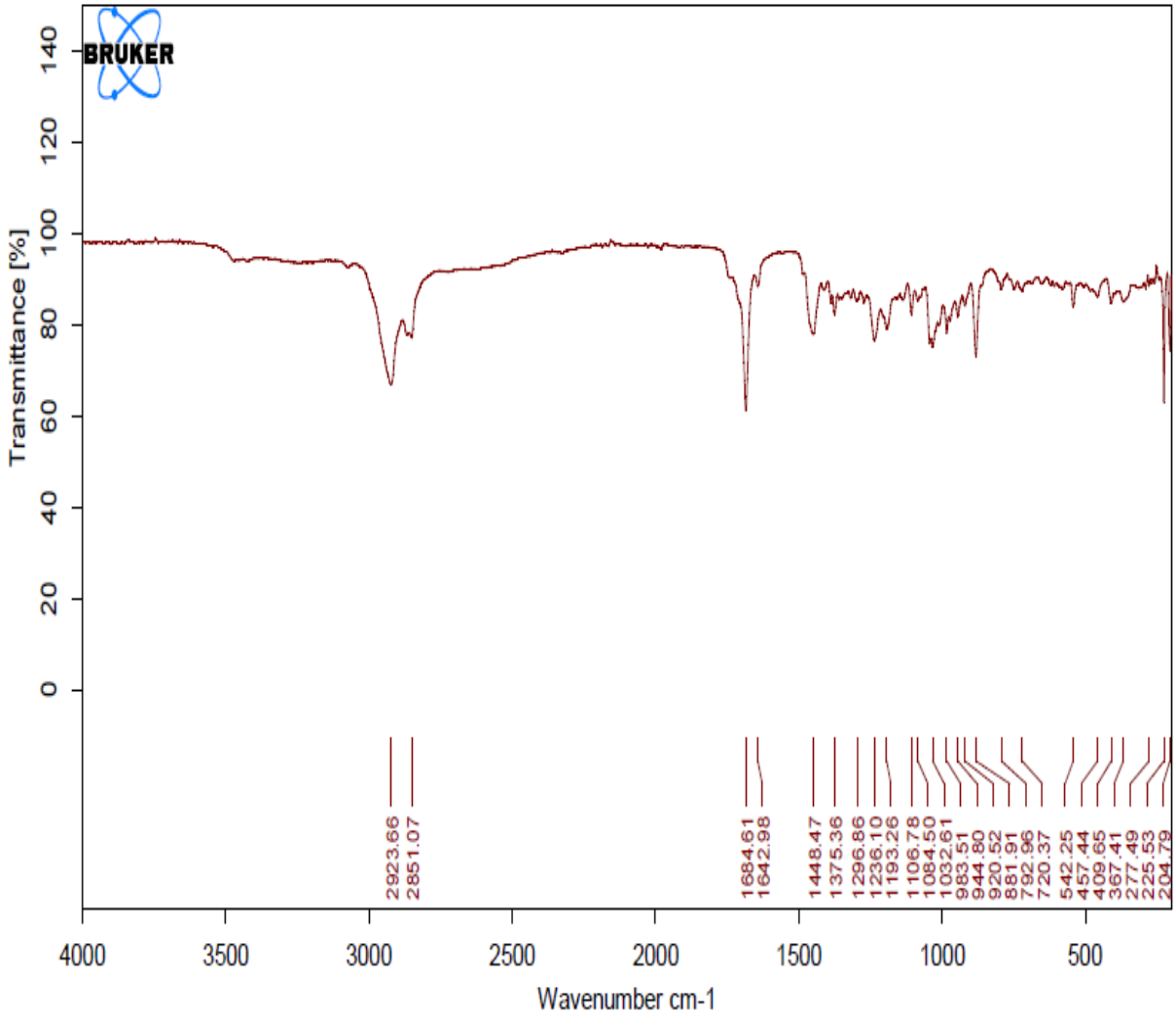

Figure C1.3a: IR spectrum of BA

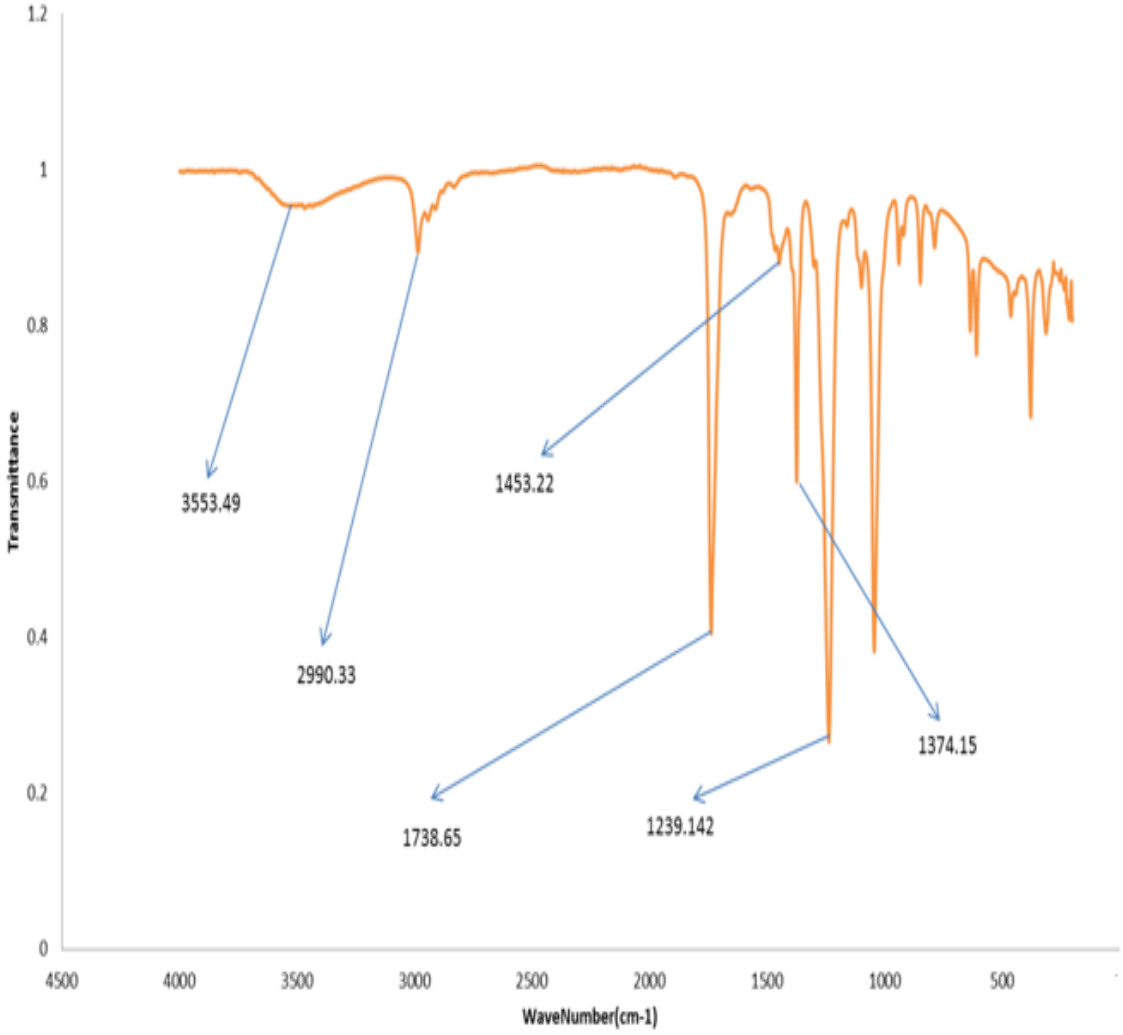

Figure C1.3b: IR spectrum of BA

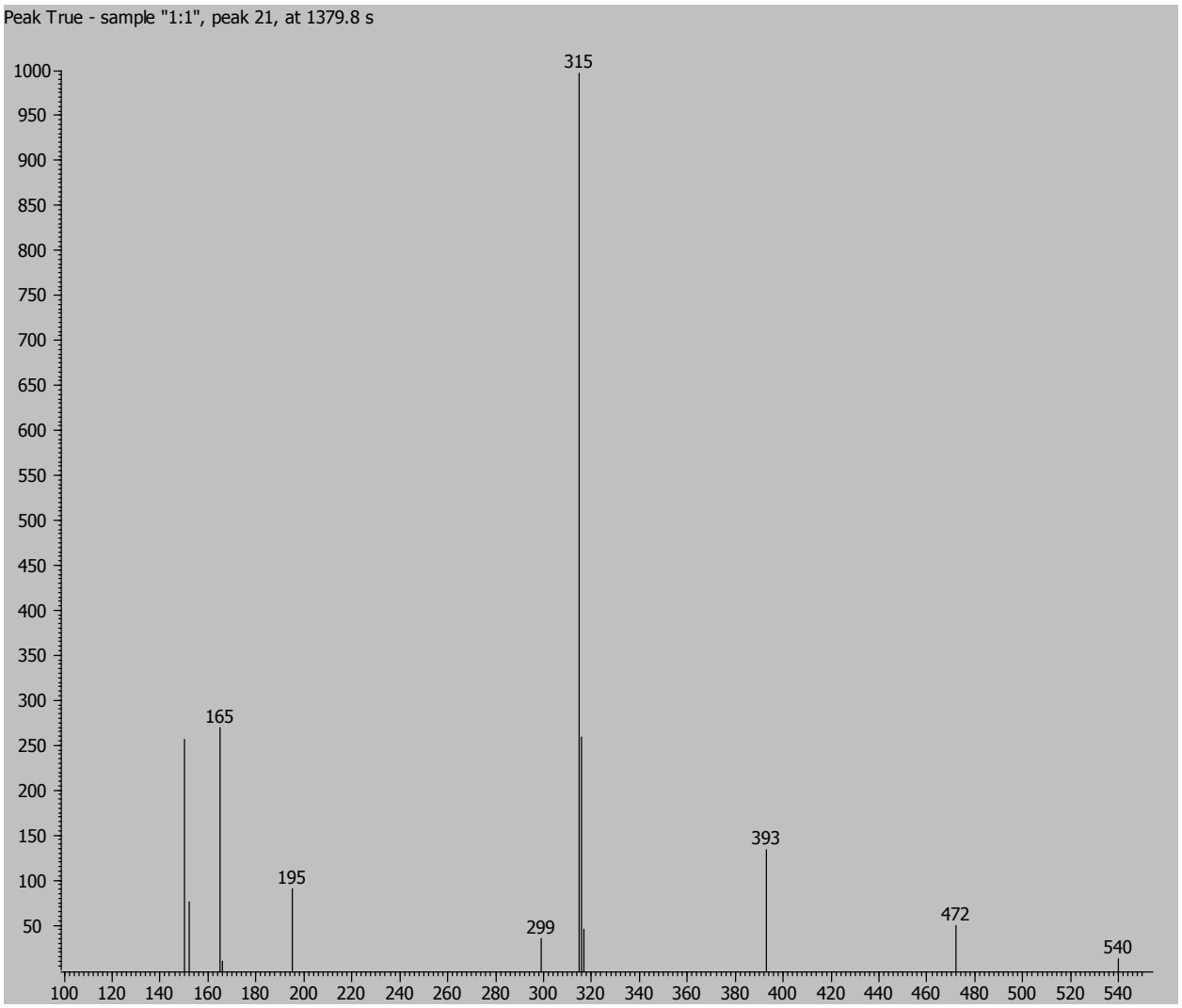

Figure C1.4a: Mass spectroscopy of BA

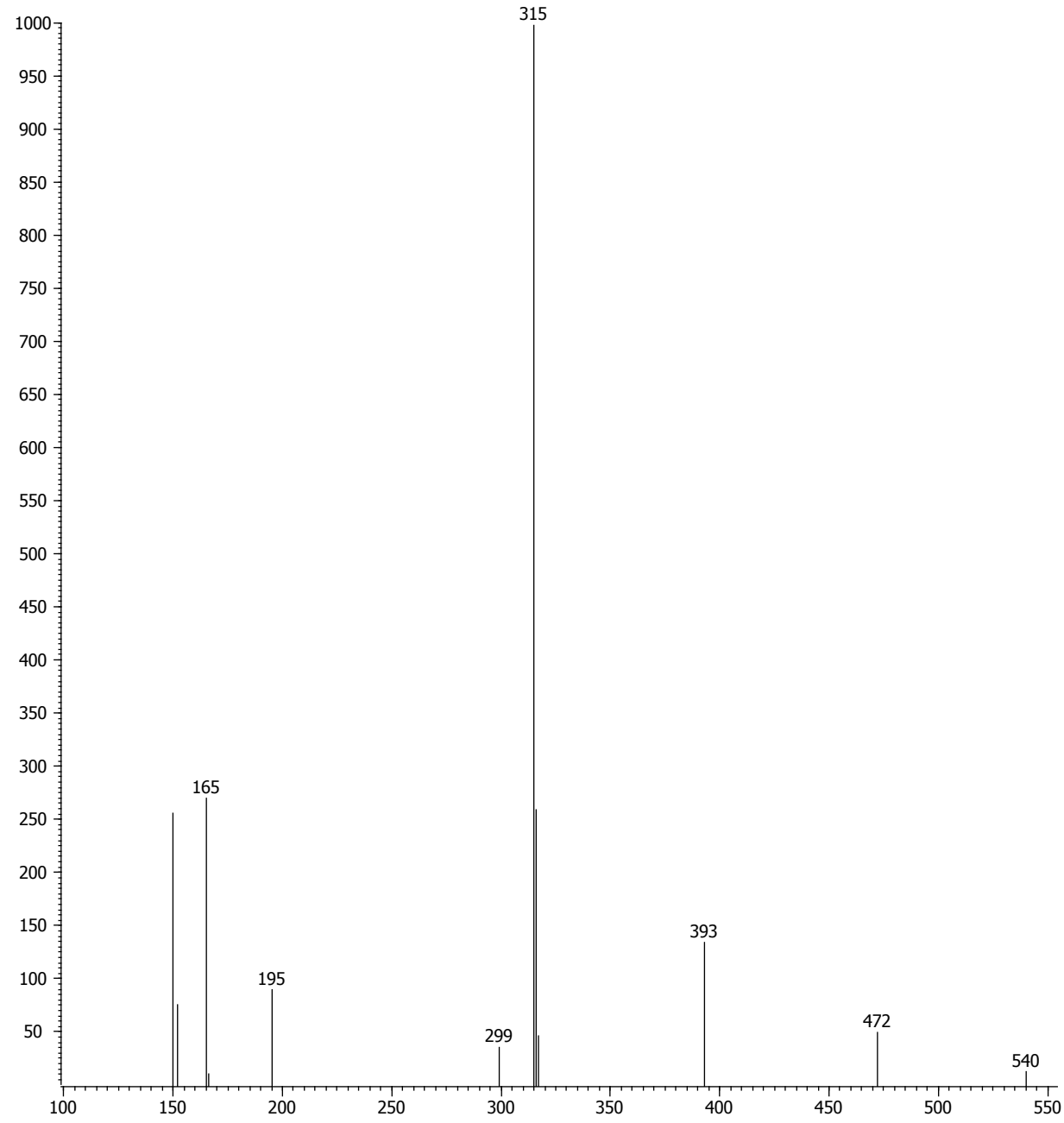

Figure C1.4b: Mass spectroscopy of BA

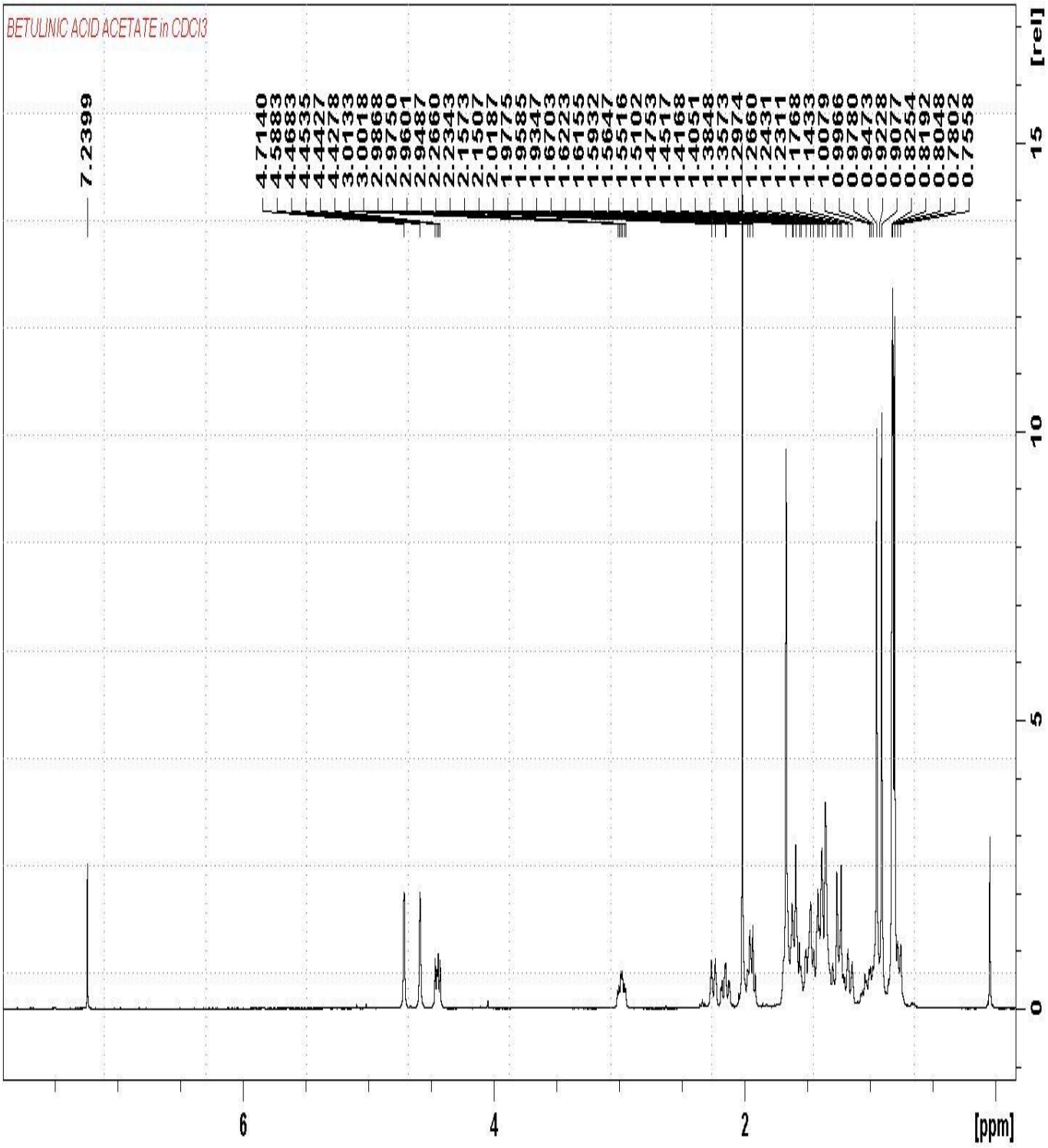

Figure C2.1a: ^{1}H-NMR spectrum of BAA

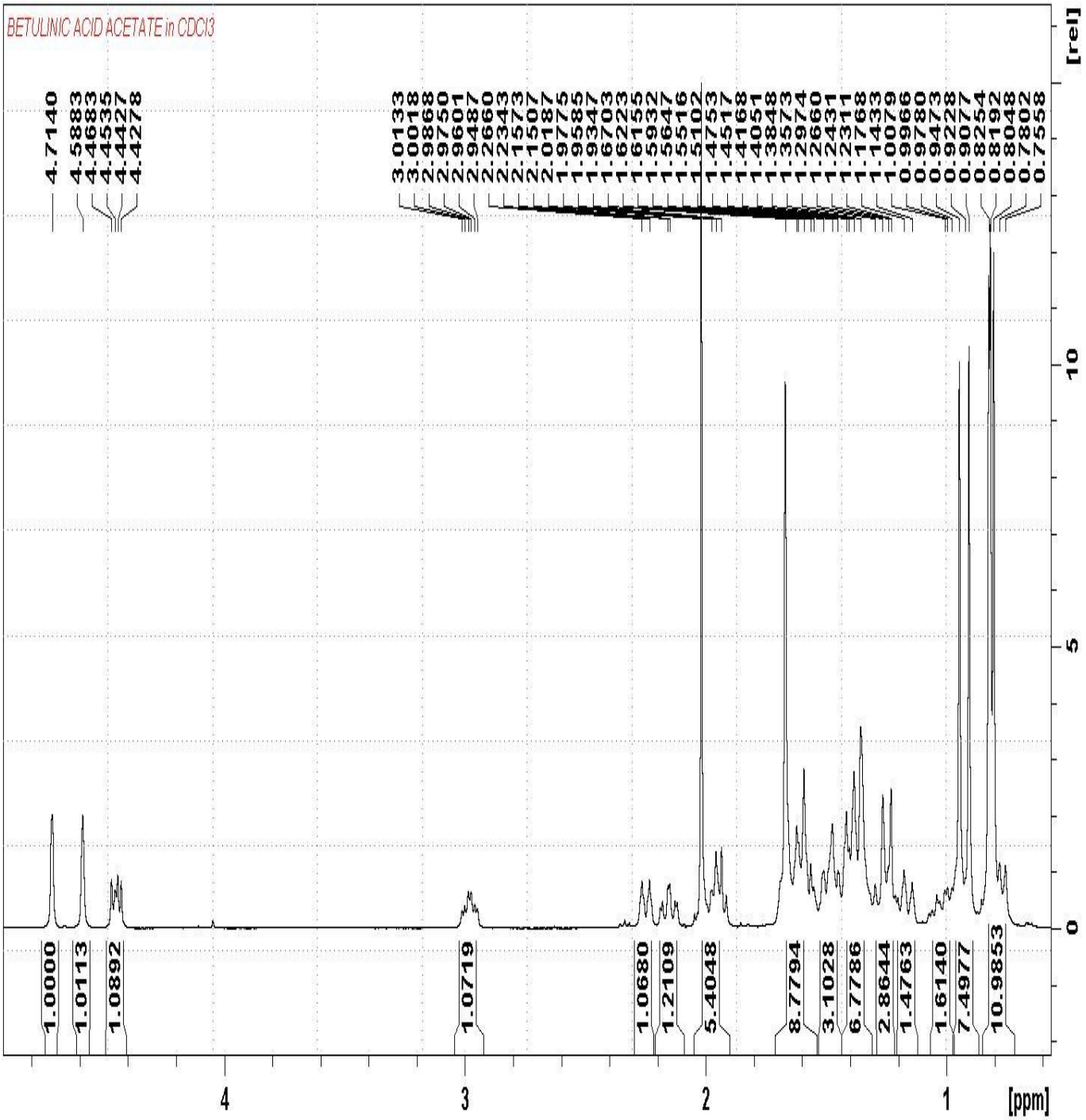

Figure C2.1b: ^{1}H-NMR spectrum of BAA

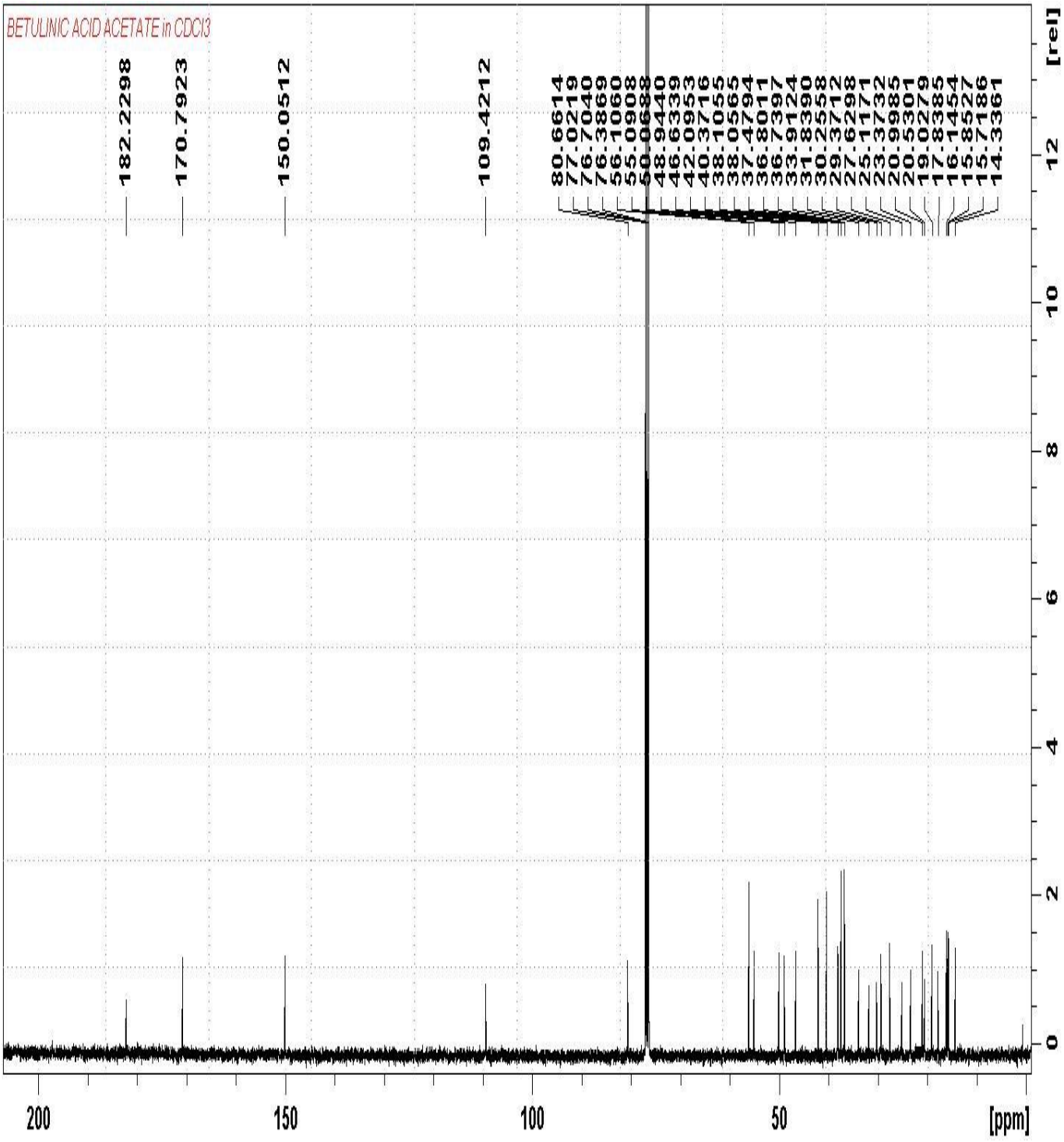

Figure C2.2: ^{13}C-NMR spectrum of BAA

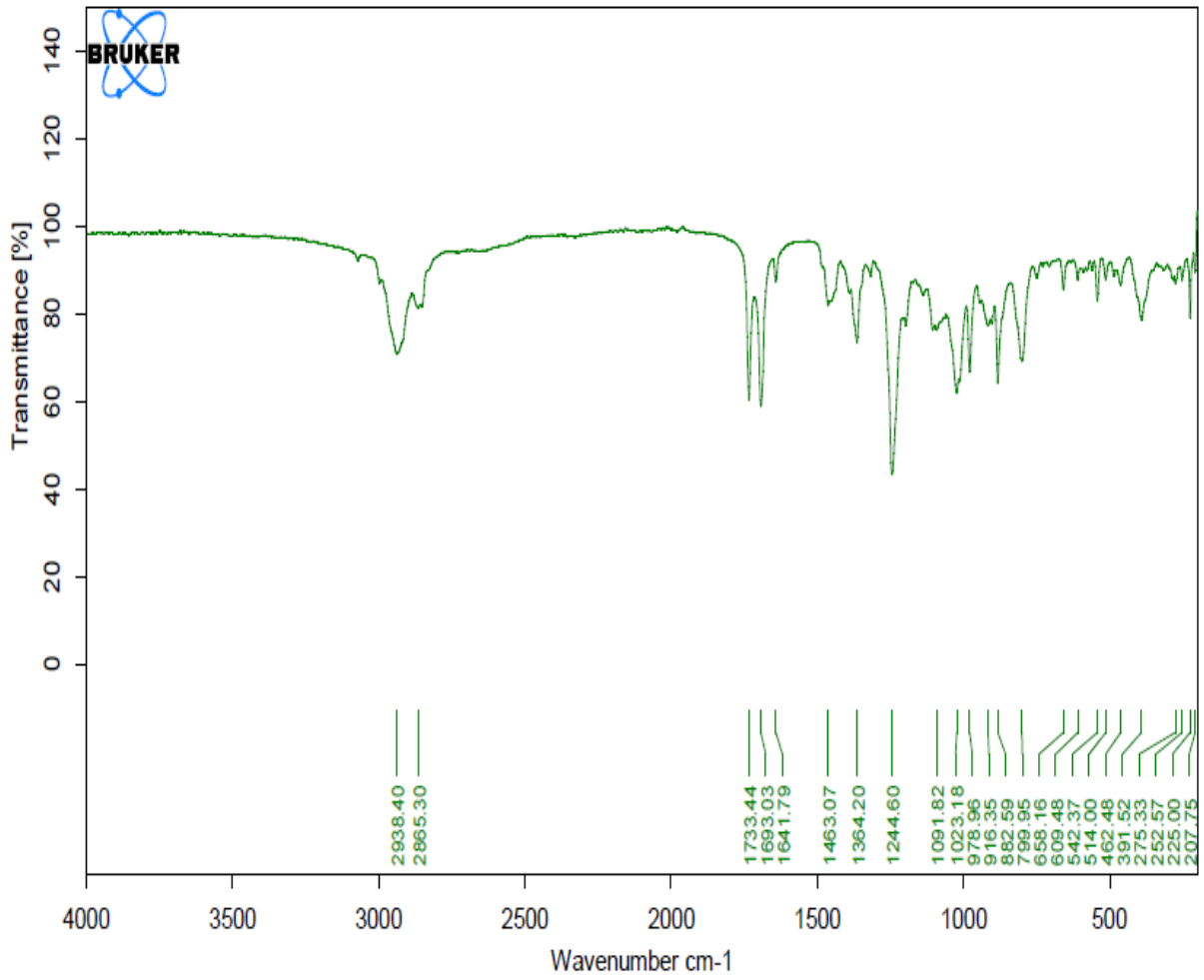

Figure C2.3a: IR spectrum of BAA

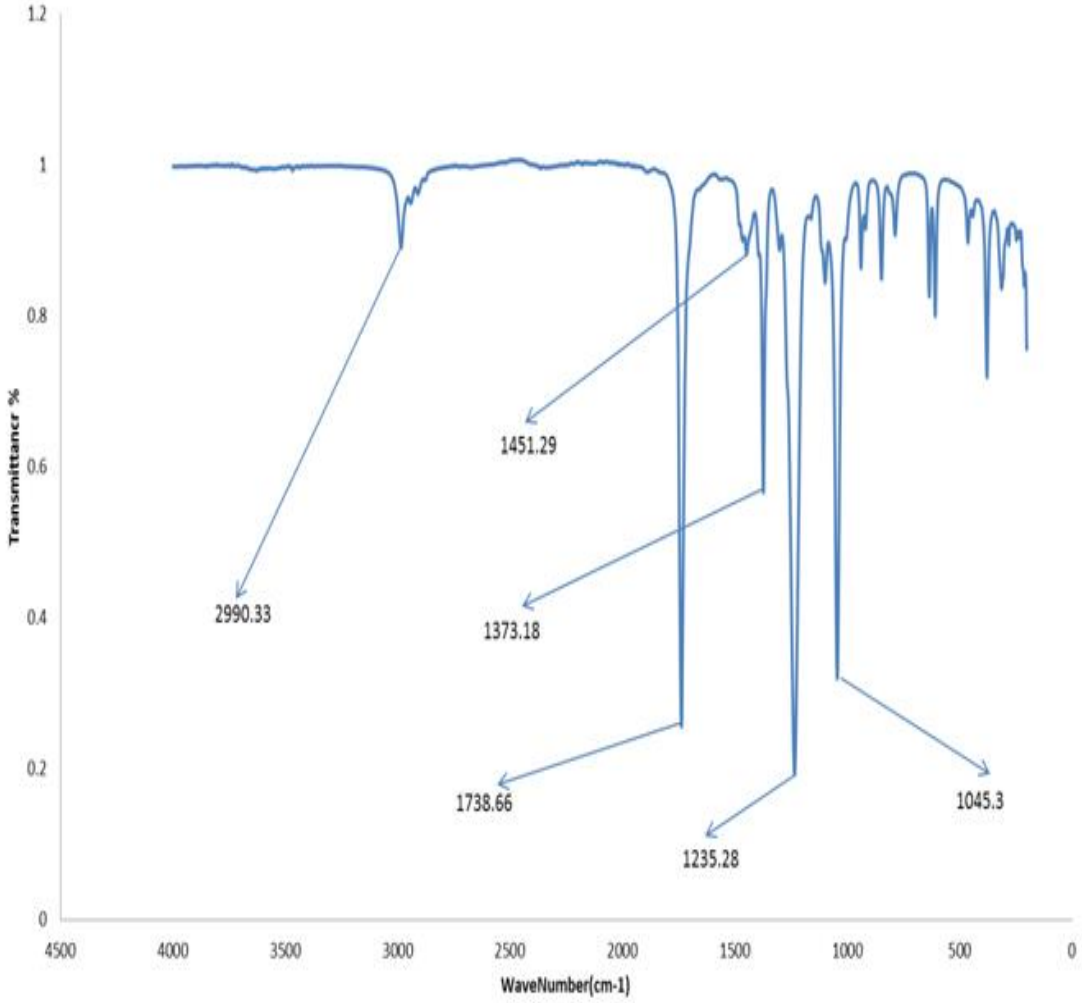

Figure C2.3b: IR spectrum of BAA

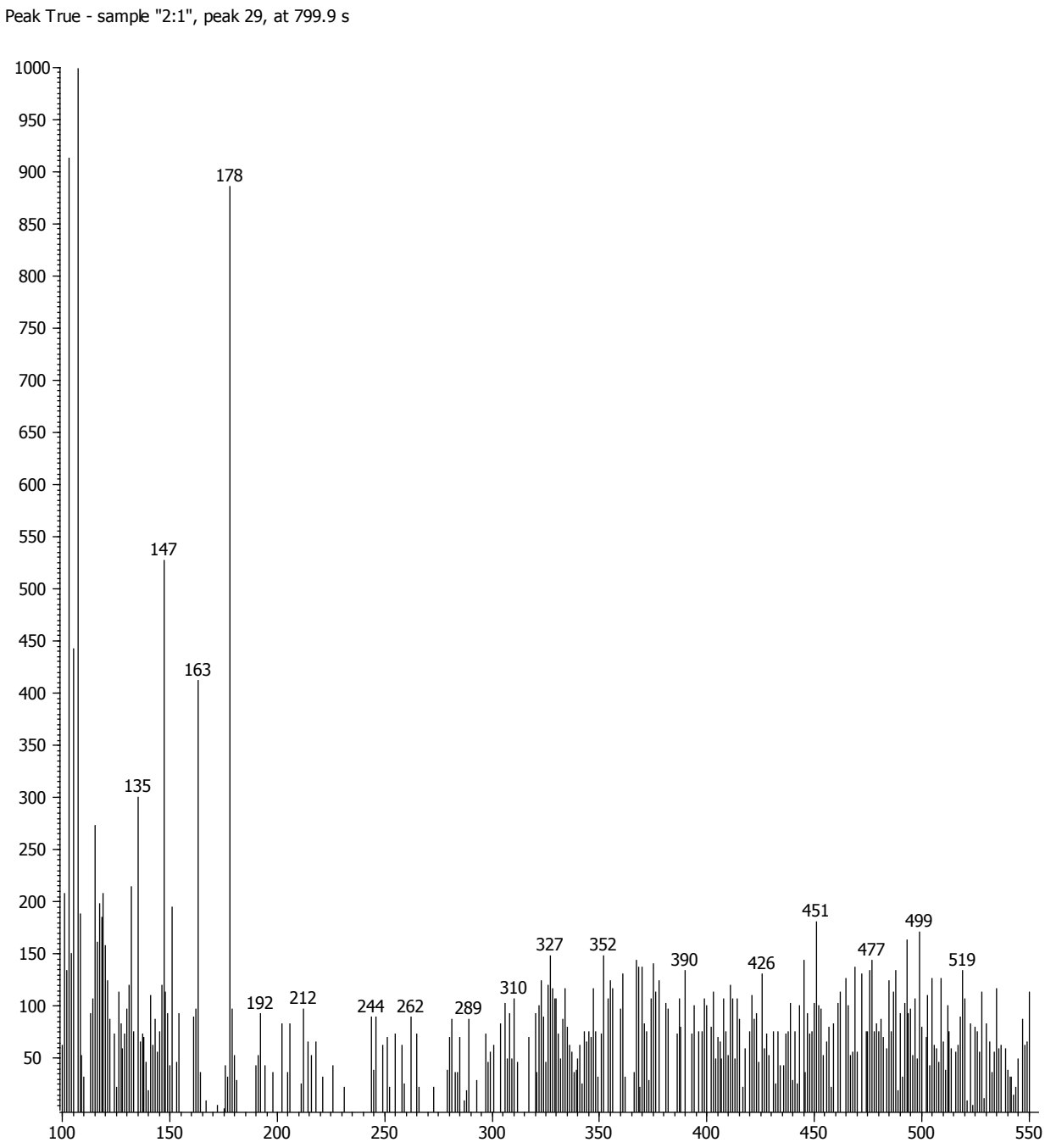

Figure C2.4a: Mass spectroscopy of BAA

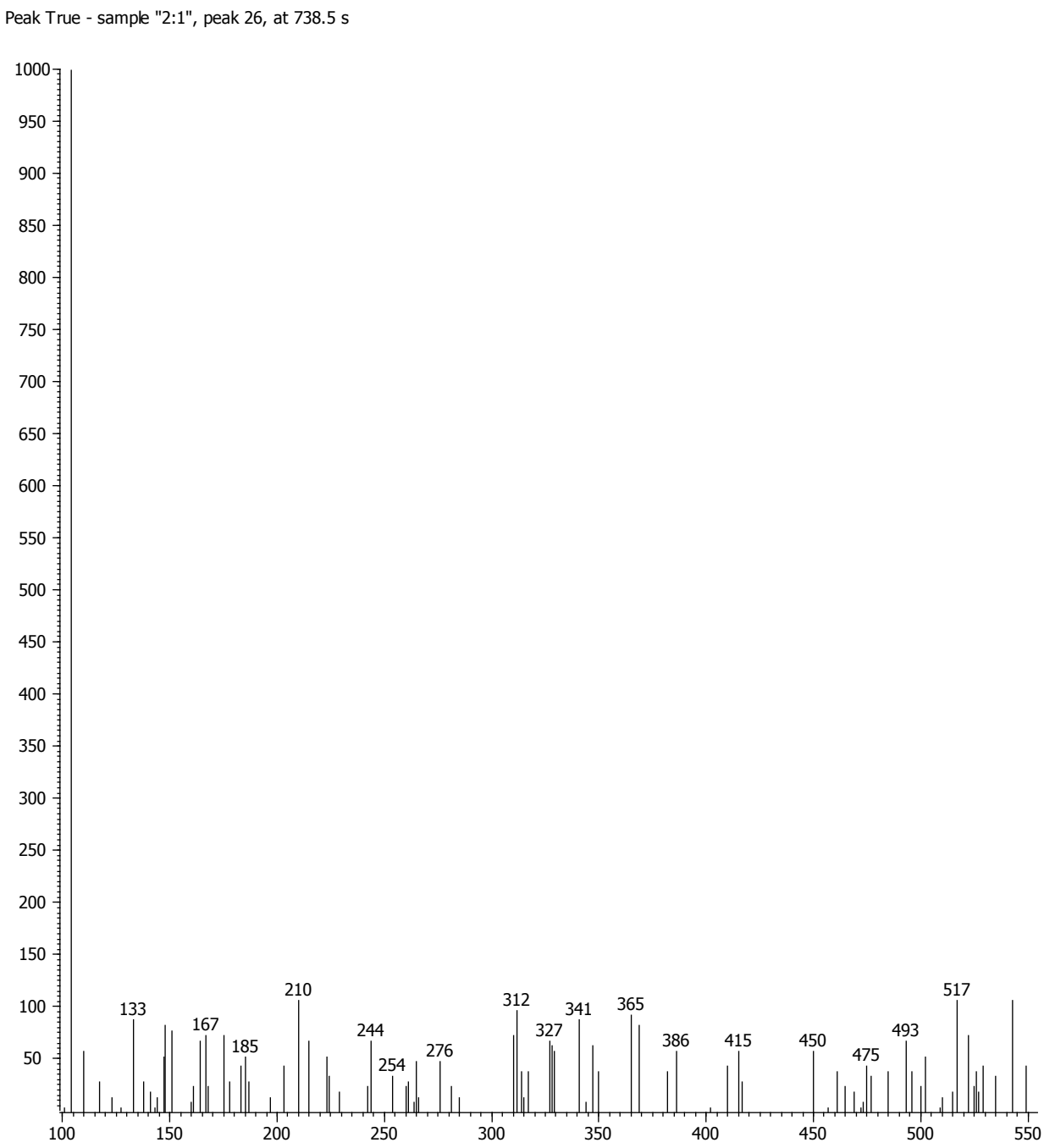

Figure C2.4b: Mass spectroscopy of BAA

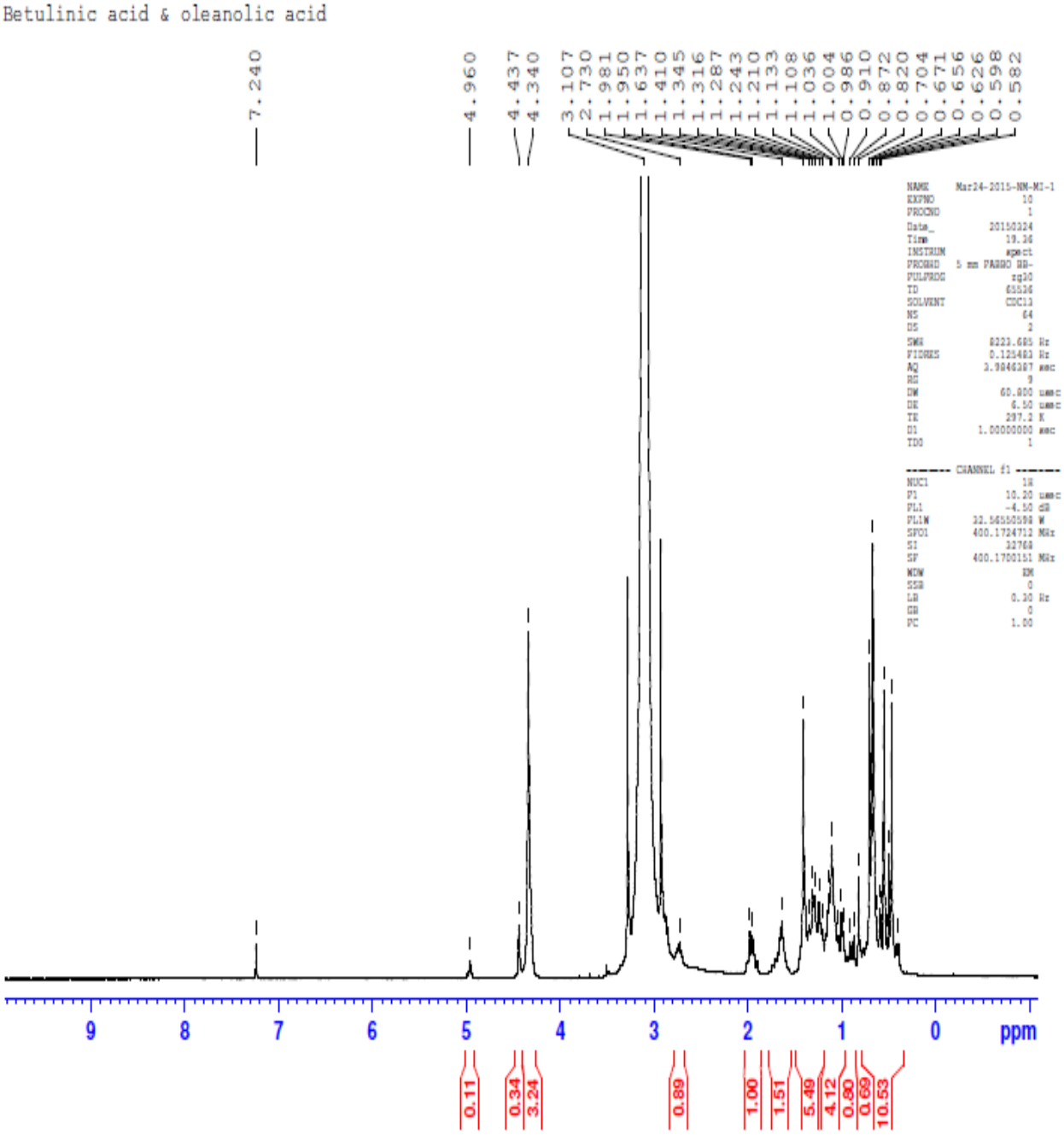

Figure C3.1a: ^{1}H-NMR spectrum of BA/OA

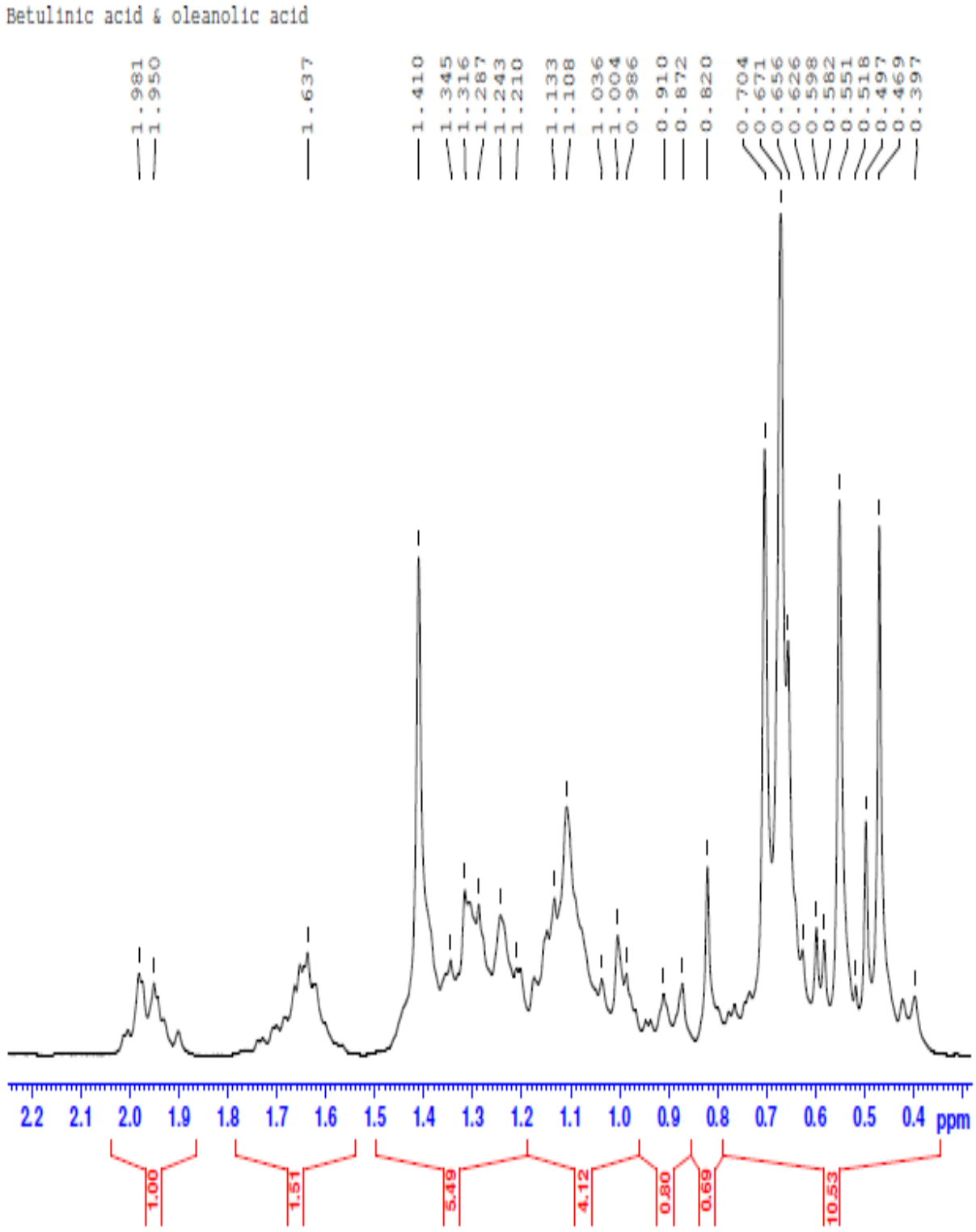

Figure C3.1b: ^{1}H-NMR spectrum of BA/OA

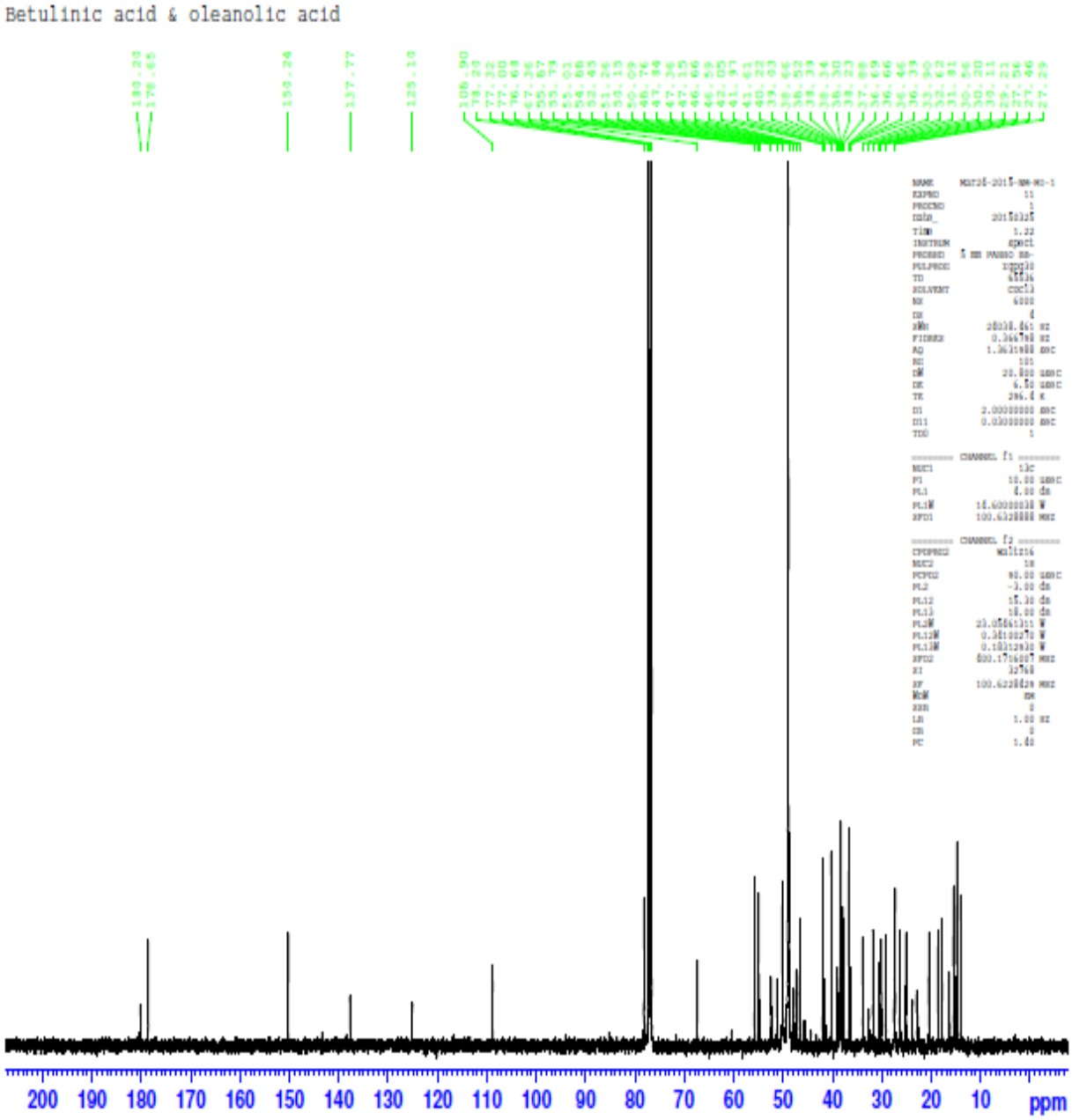

Figure C3.2a: ^{13}C-NMR spectrum of BA/OA

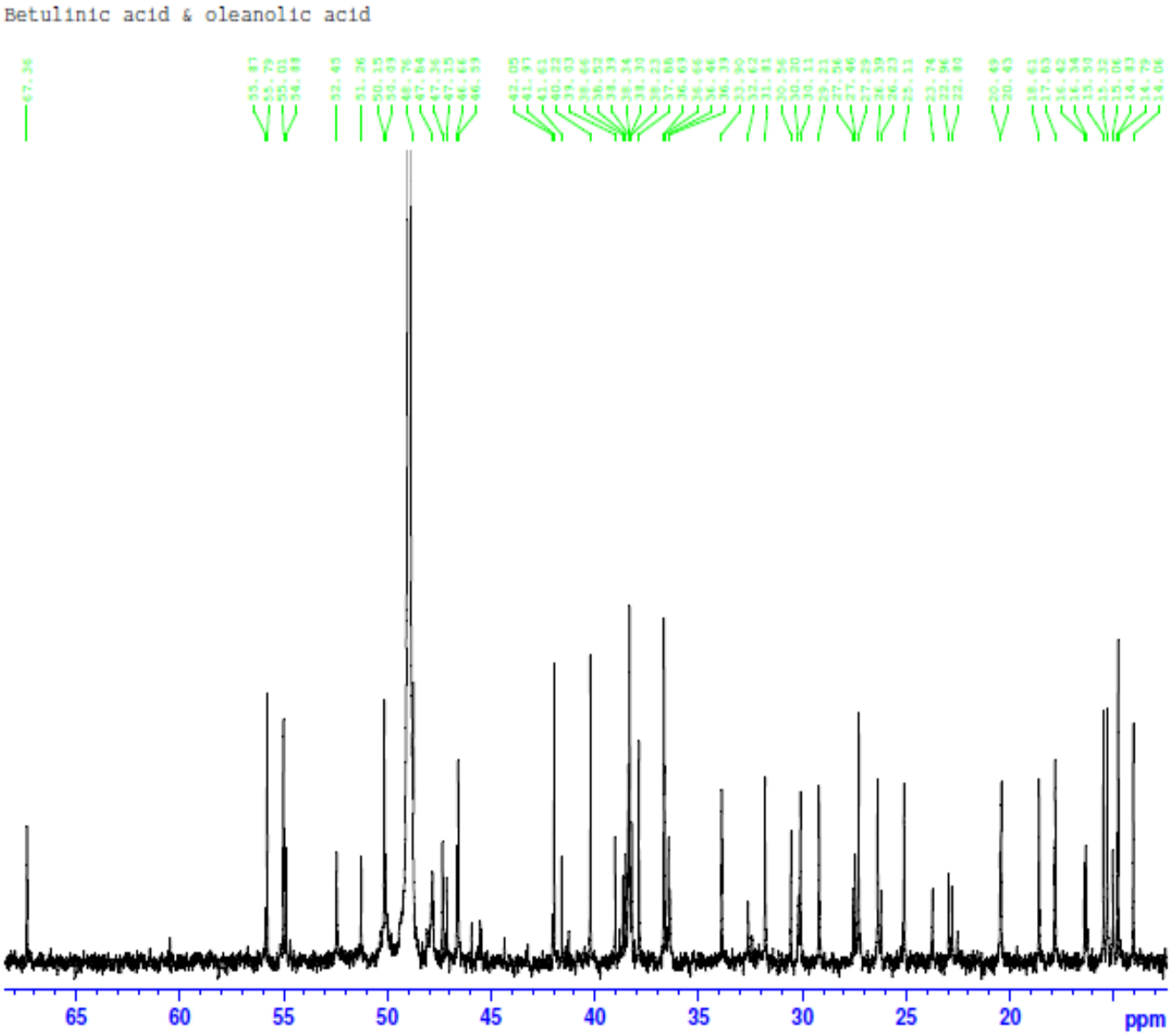

Figure C3.2b: ^{13}C-NMR spectrum of BA/OA

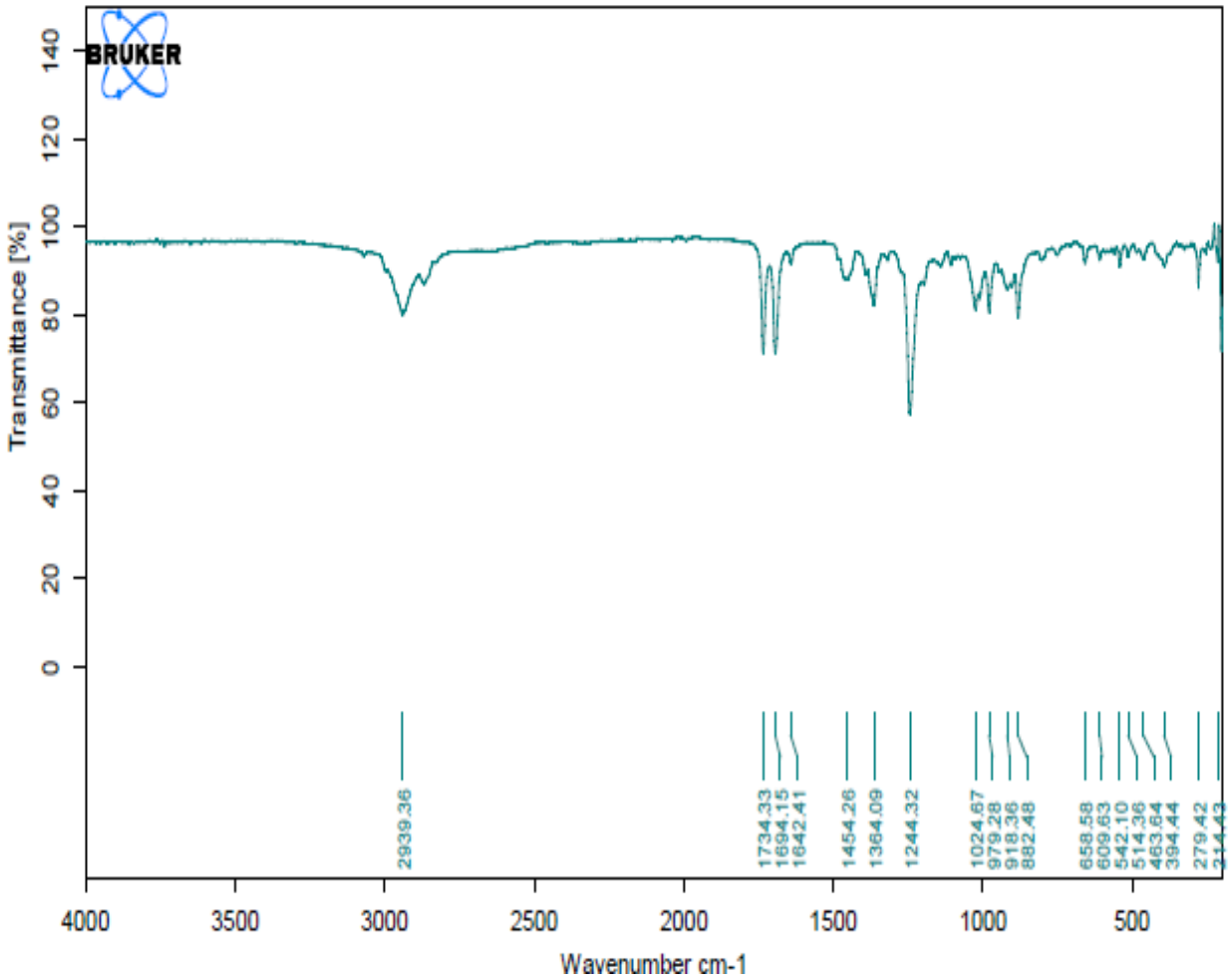

Figure C3.3a: IR spectrum of BA/OA

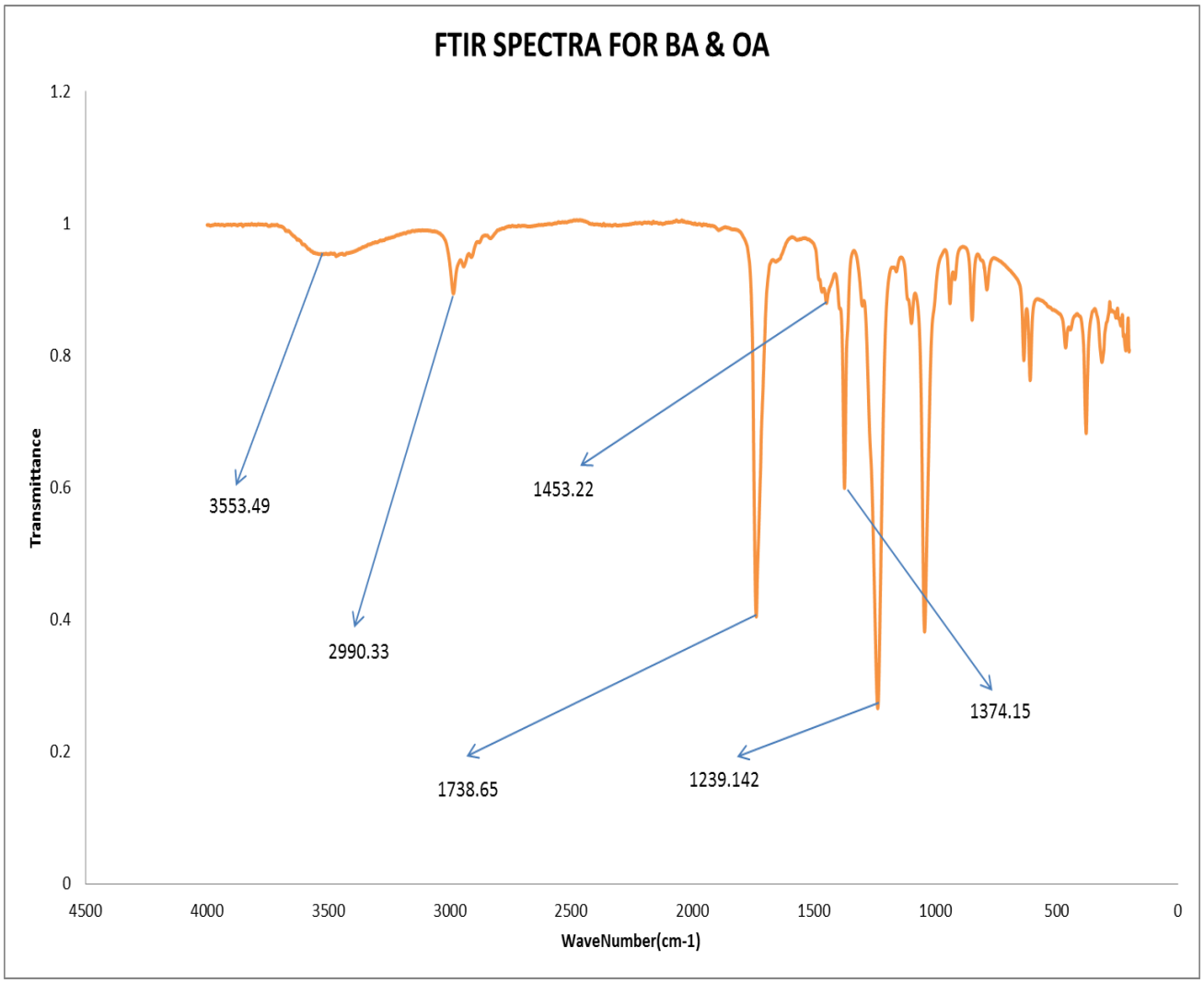

Figure C3.3b: IR spectrum of BA/OA

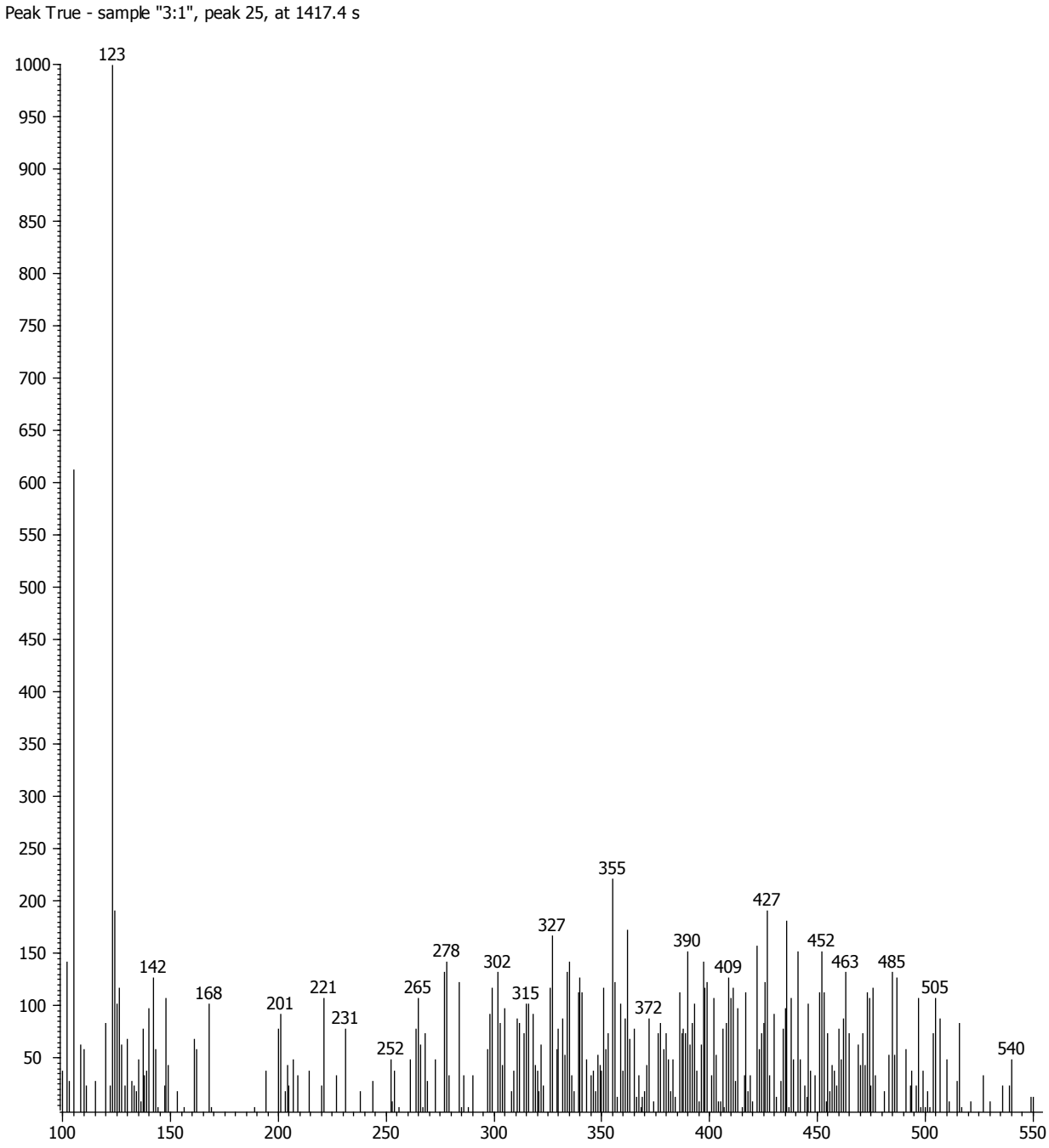

Figure C3.4a: Mass spectroscopy of BA/OA

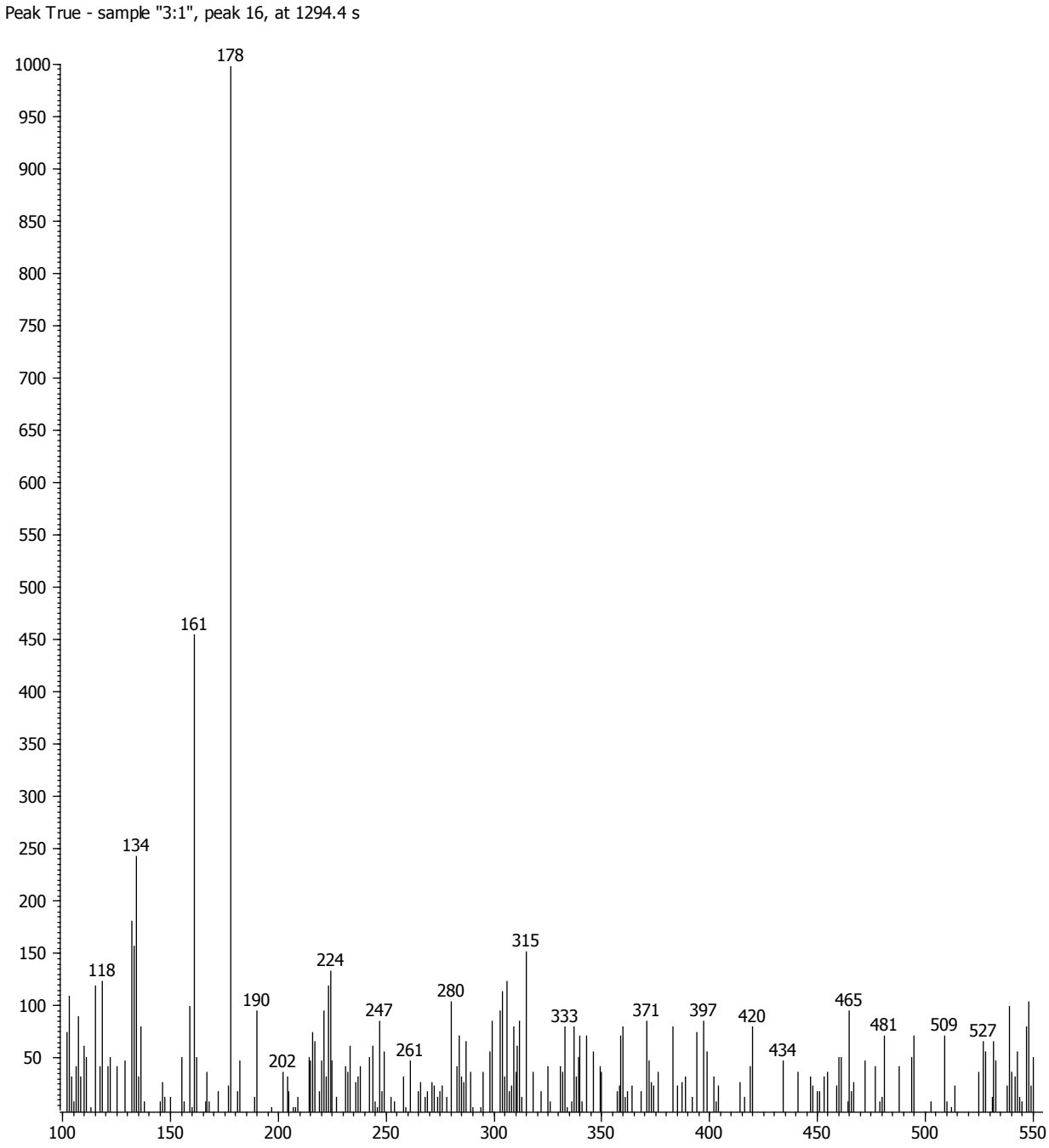

Figure C3.4b: Mass spectroscopy of BA/OA

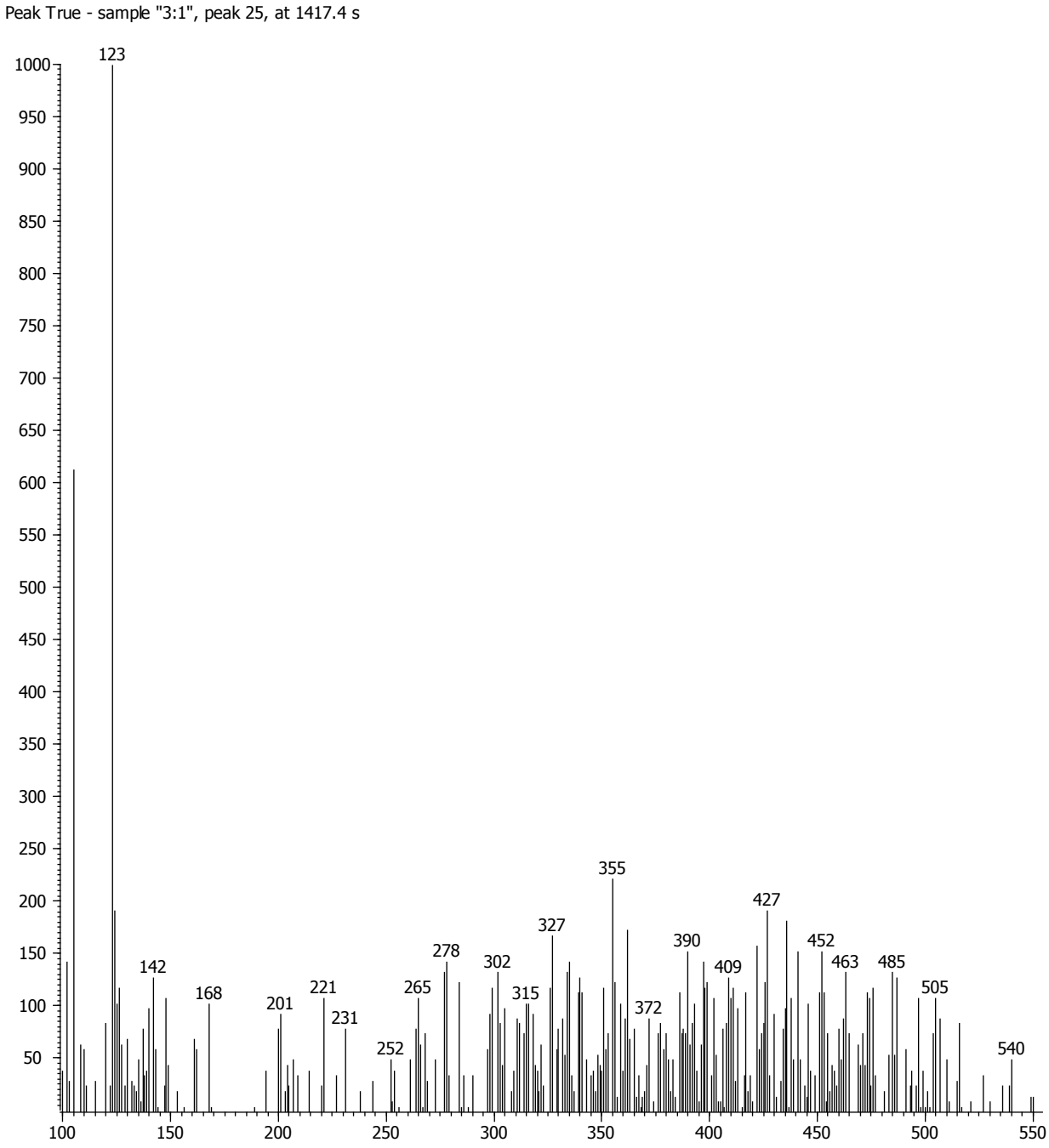

Figure C3.4c: Mass spectroscopy of BA/OA

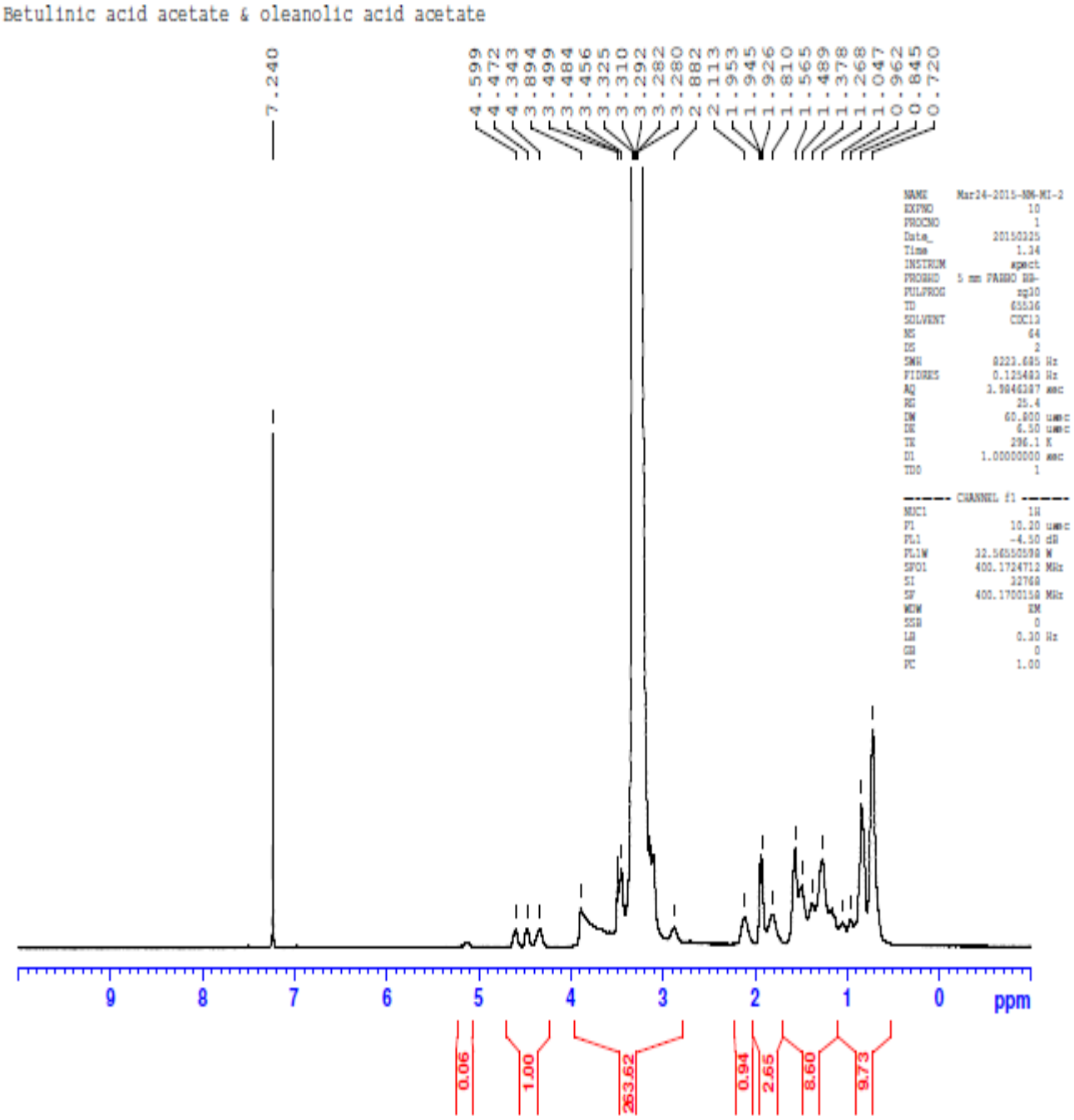

Figure C4.1: ^{1}H-NMR spectrum of BAA/OAA

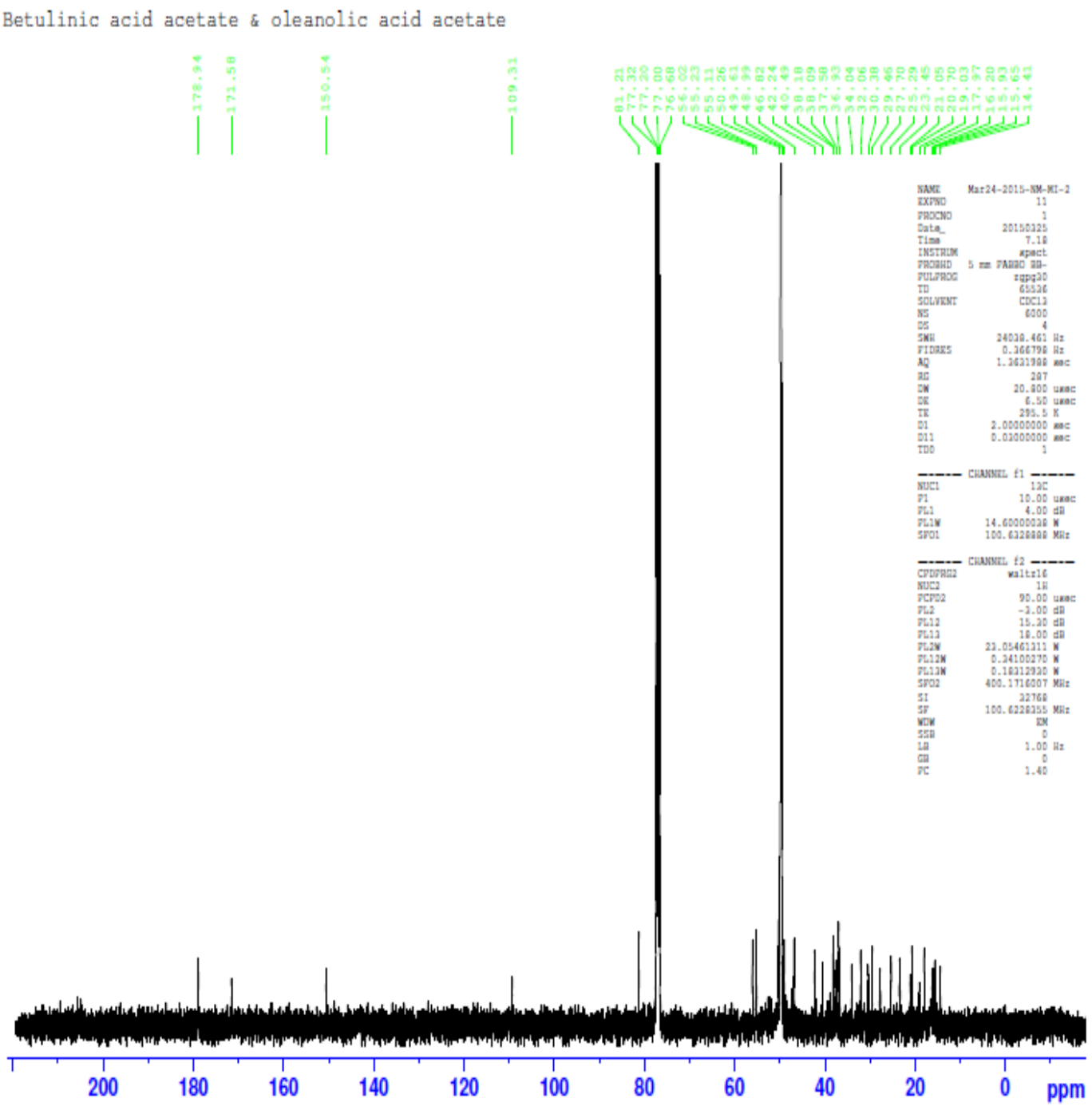

Figure C4.2a: ^{13}C-NMR spectrum of BAA/OAA

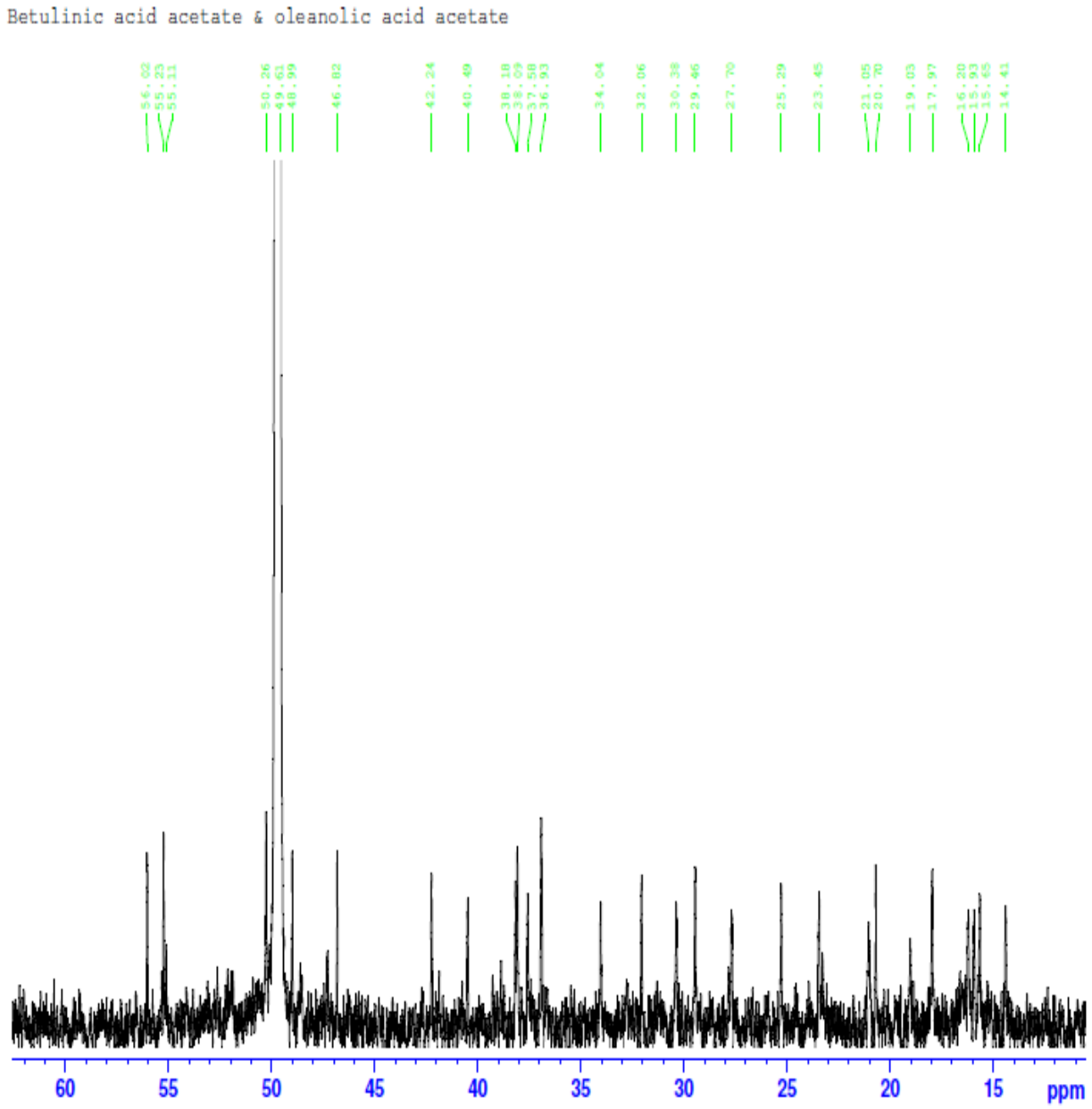

Figure C4.2b: ^{13}C-NMR spectrum of BAA/OAA

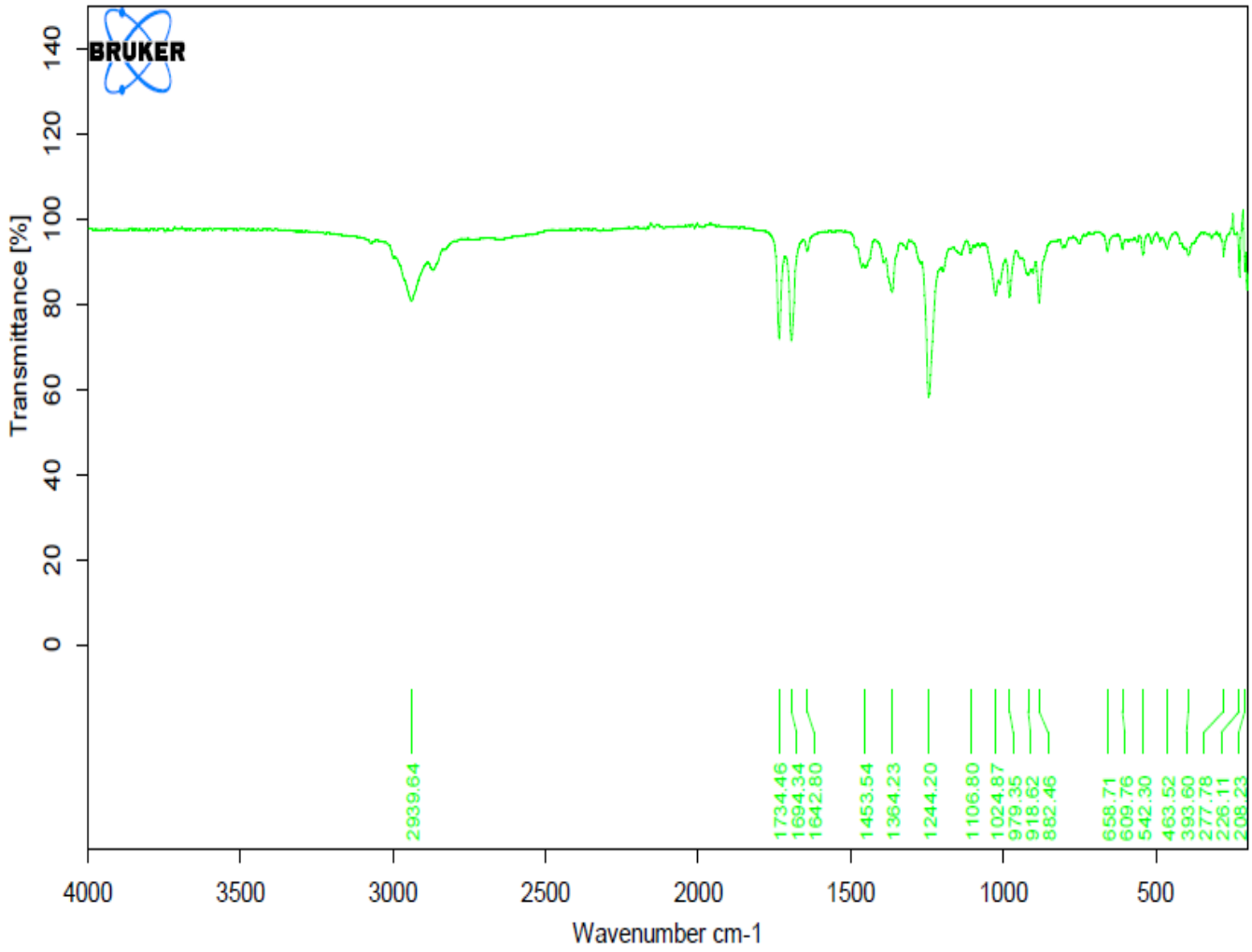

Figure C4.3a: IR spectrum of BAA/OAA

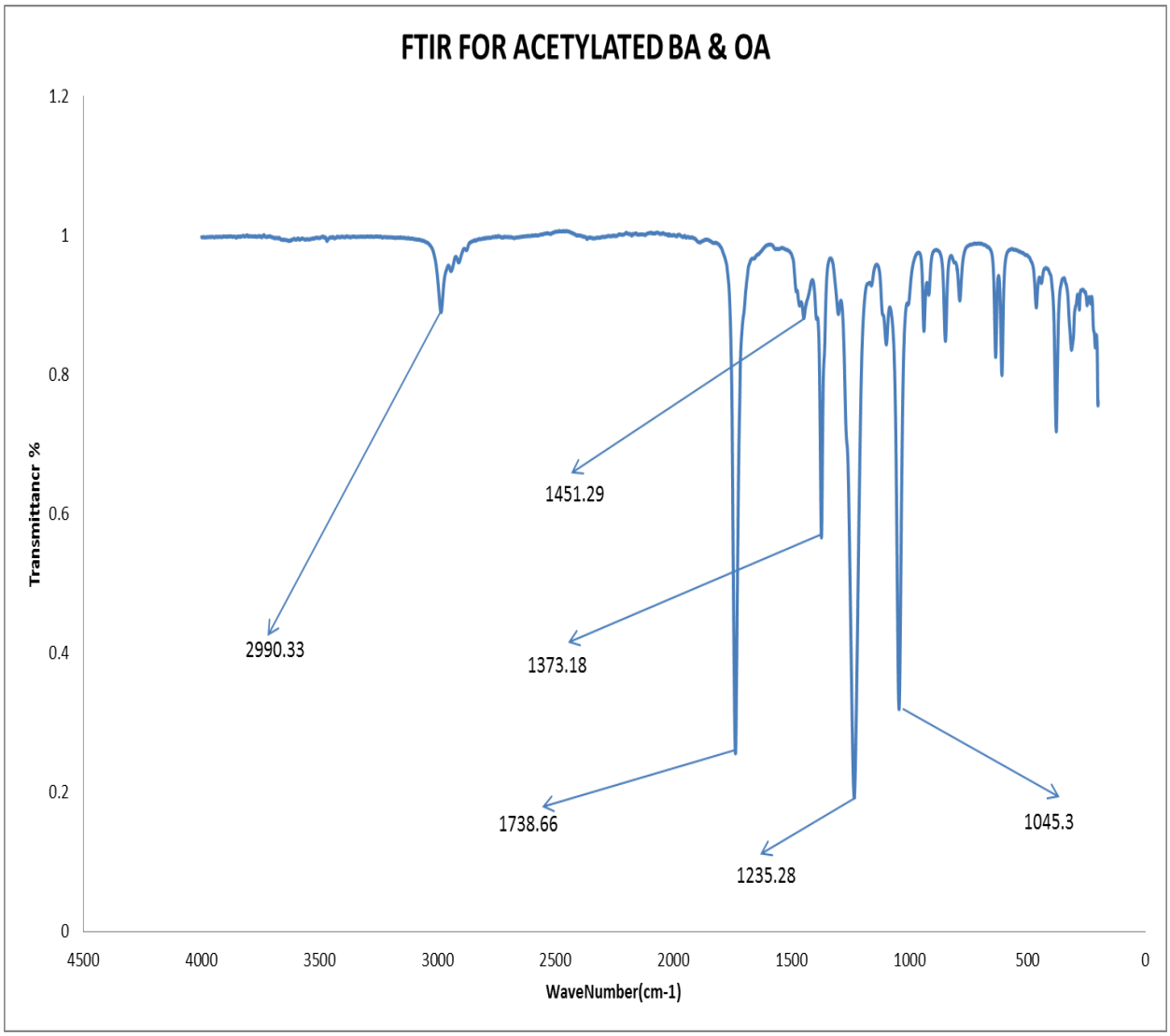

Figure C4.3b: IR spectrum of BAA/OAA

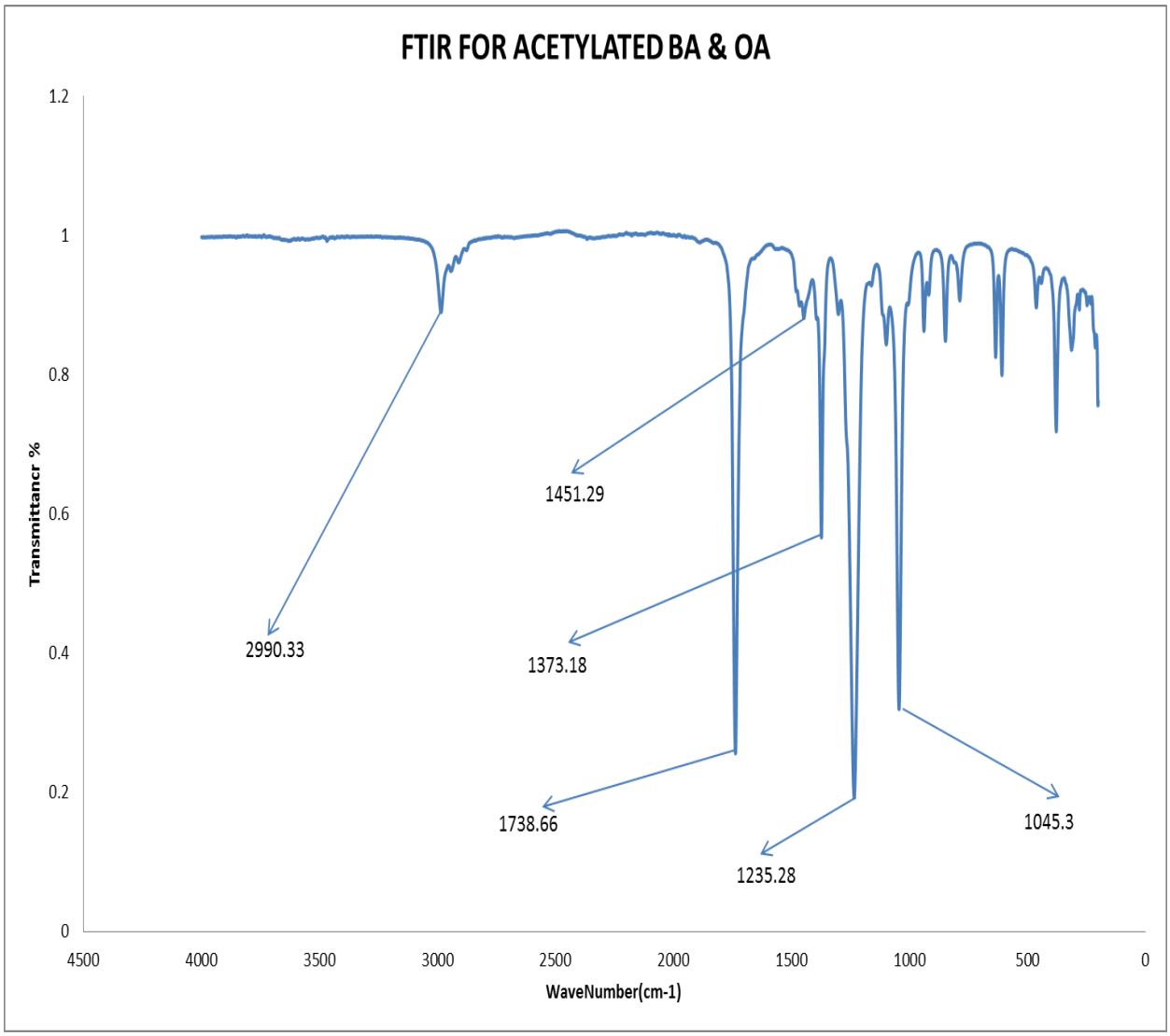

Figure C4.3b: IR spectrum of BAA/OAA

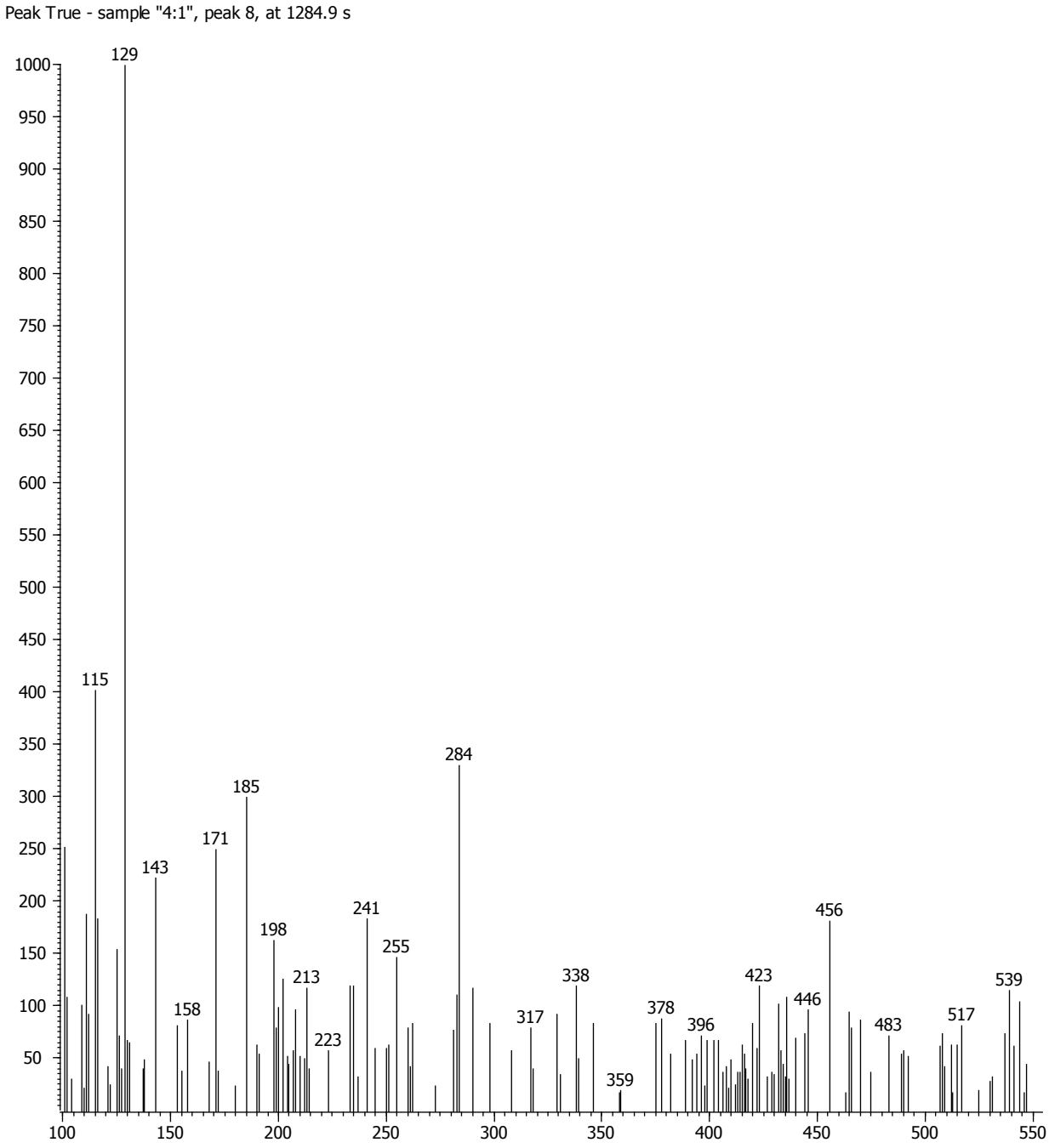

Figure C4.4a: Mass spectroscopy of BAA/OAA

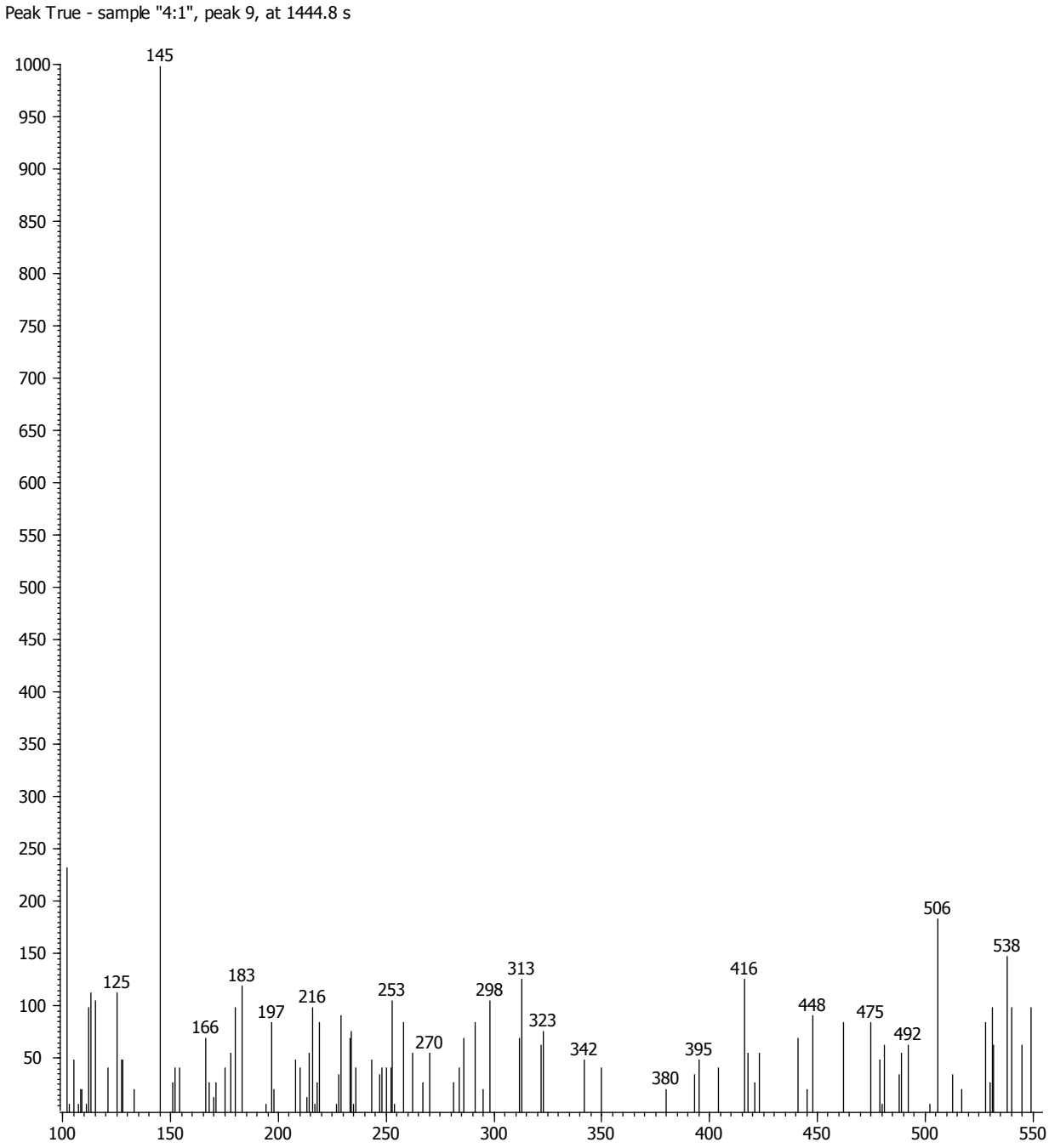

Figure C4.4b: Mass spectroscopy of BAA/OAA

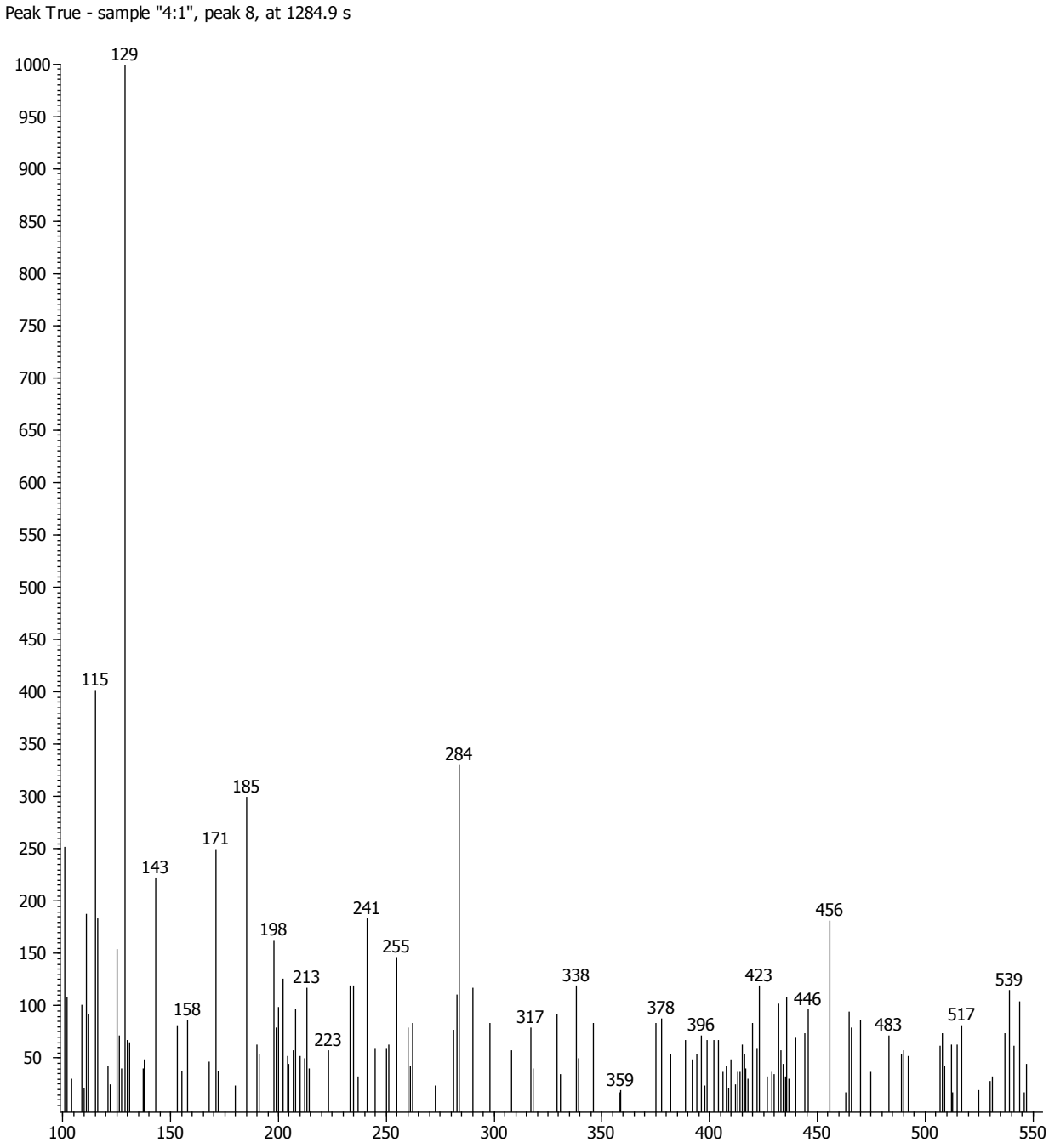

Figure C4.4a: Mass spectroscopy of BAA/OAA

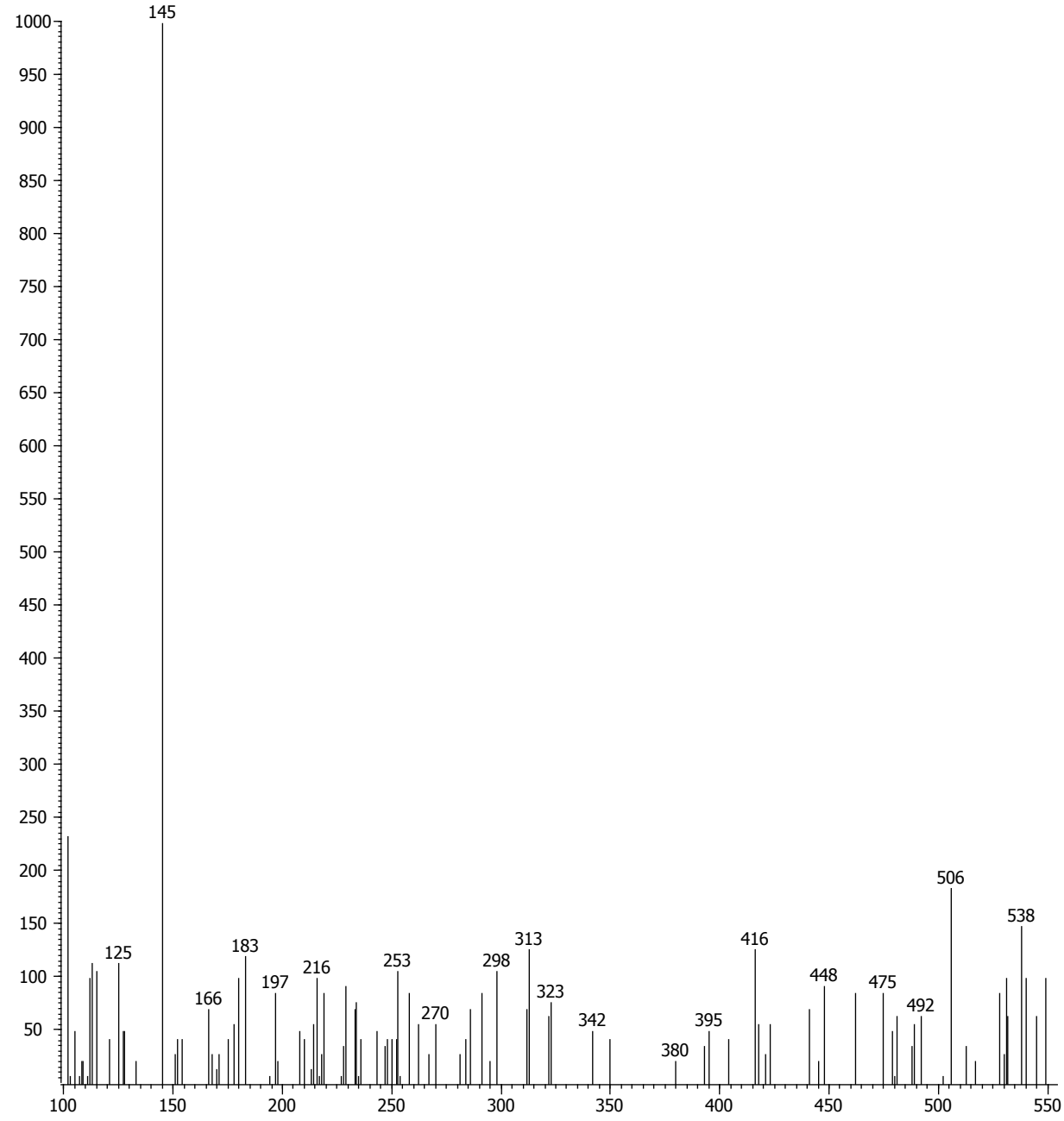

Figure C4.4b: Mass spectroscopy of BAA/OAA

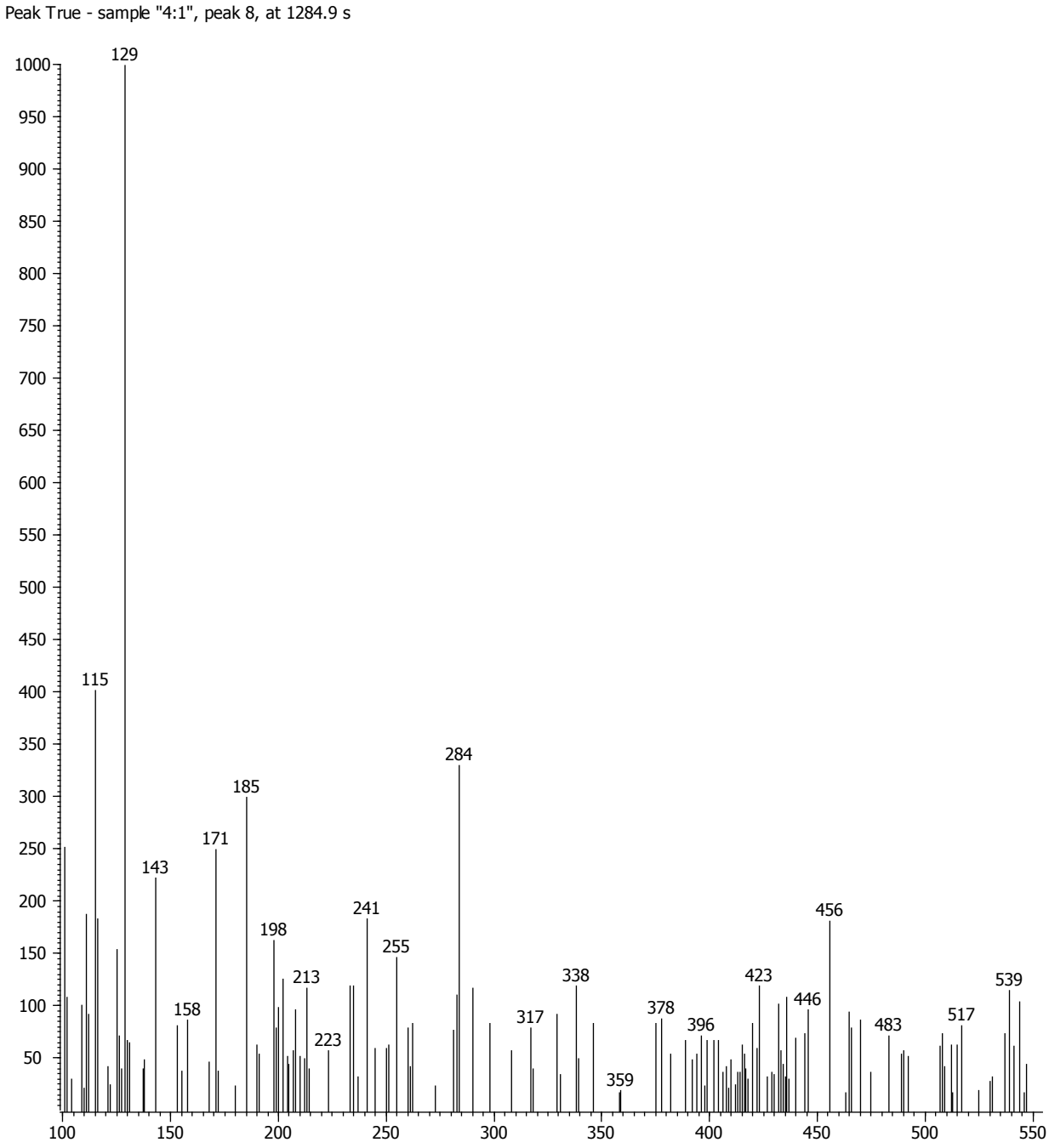

Figure C4.4c: Mass spectroscopy of BAA/OAA

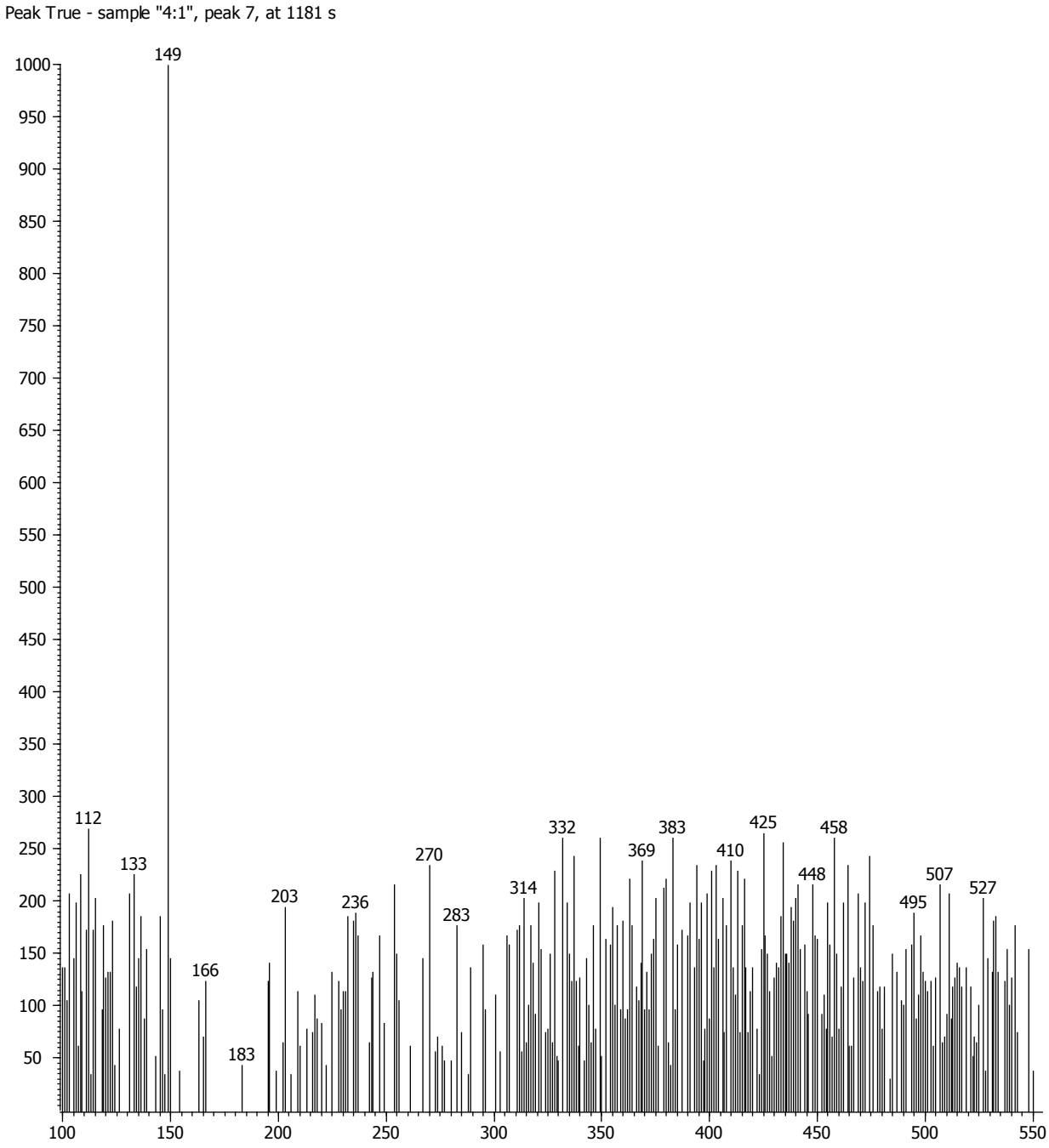

Figure C4.4d: Mass spectroscopy of BAA/OAA

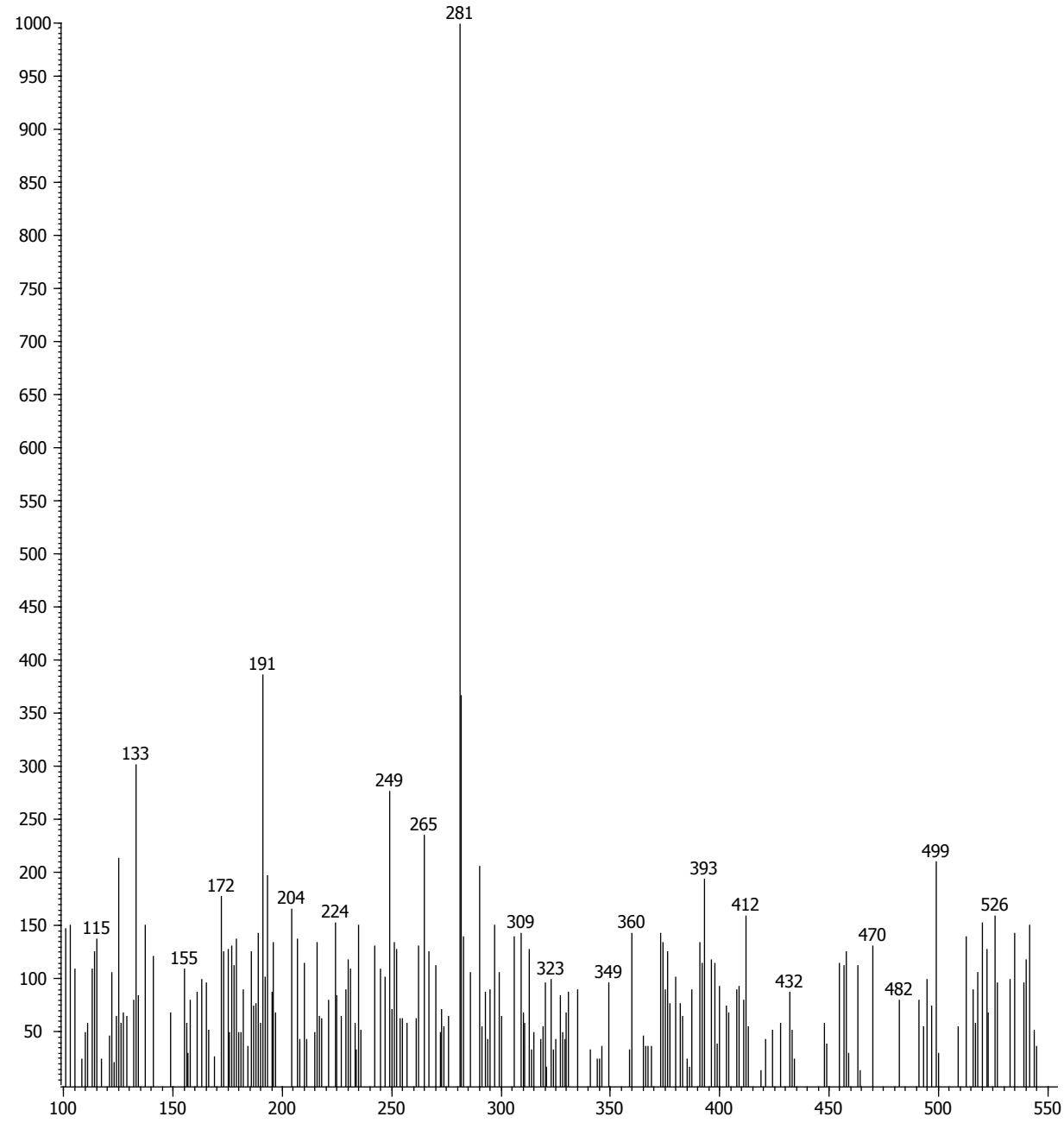

Figure C4.4d: Mass spectroscopy of BAA/OAA

APPENDIX D

D. Ethics Clearance

UNIVERSITY RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-30)

UNIVERSITY OF ZULULAND
Website: http://www.unizulu.ac.za

Private Bag X1001
KwaDlangezwa 3886

Tel: 035 902 6887
Fax: 035 902 6222
Email: MangeleS@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	UZREC 171110-030 PGD 2014/53								
Project Title	Anti-platelet aggregation activity of Mecaleuca Bracteata Var. Revolution gold derived betulinic acid and its derivatives								
Principal Researcher/ Investigator	FO Osunsanmi								
Supervisor and Co-supervisor	Prof. AR Opoku								
Department	Biochemistry								
Nature of Project	Honours/4th Year		Master's		Doctoral	x	Departmental		

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate. Special conditions, if any, are also listed on page 2.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

The Principal Researcher must report to the UZREC in the prescribe format, where applicable, annually and at the end of the project, in respect of ethical compliance.