Antiplatelet aggregation and cytotoxic activity of betulinic acid and its acetyl derivative\ from *Melaleuca bracteata* var.revelovtion gold

F. O Osunsanmi, F.O Shode, and A.R Opoku

Department of Biochemistry and microbiology, Faculty of Science. University of Zululand, Private Bag x10001, KwaDlangezwa,3886, South Africa

2015

INTRODUCTION

- More than 17.6 million people die of cardiovascular diseases and this number is expected to grow to more than 23.6 million by 2030 (Mozaffarian et al., 2014)

- In 2007, Medical Research Council of South Africa reported that for every women that die of cardiovascular diseases , two men die.

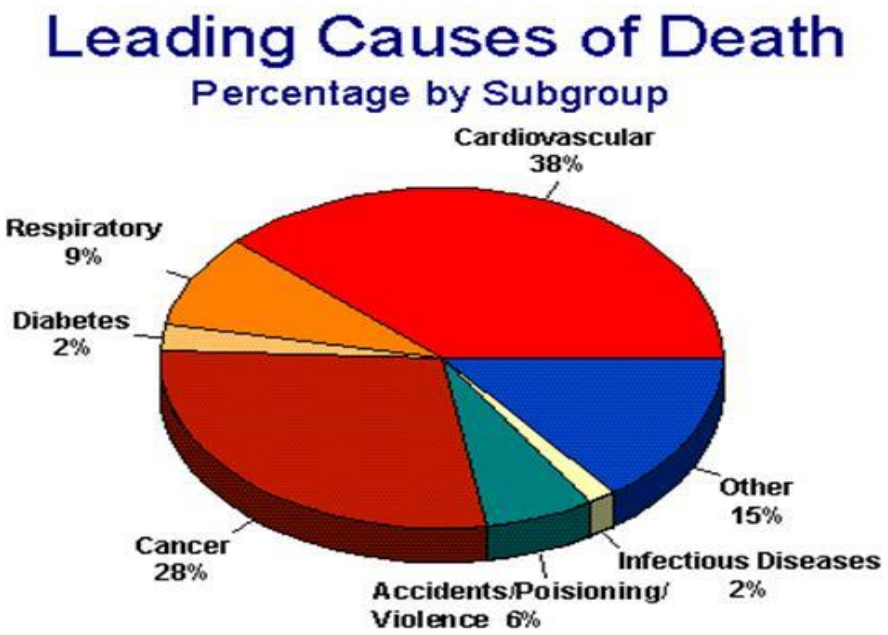
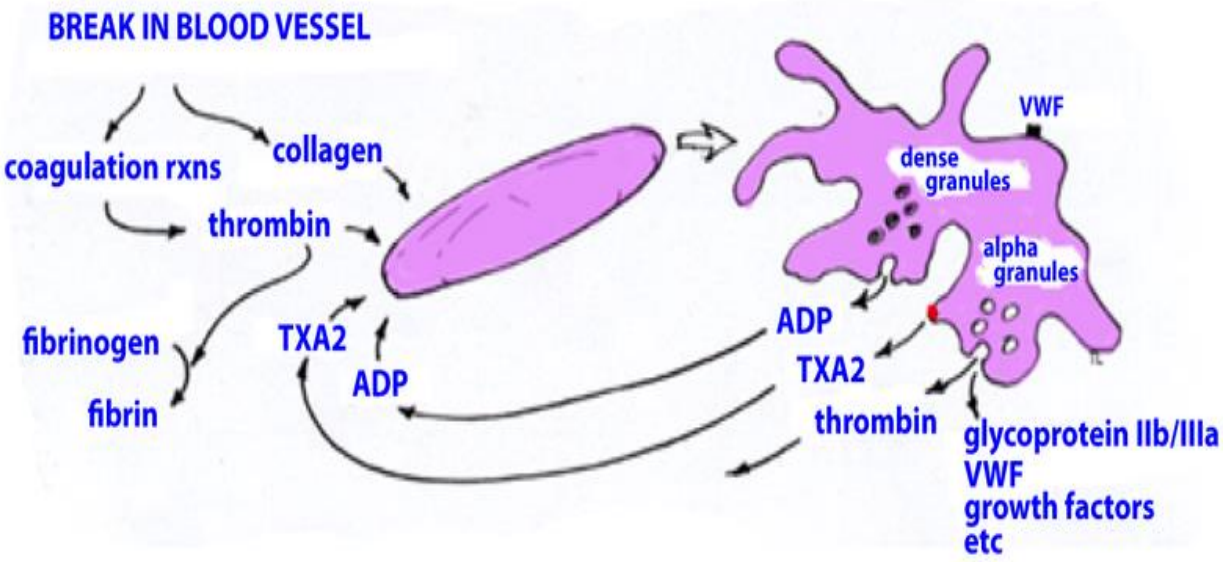


Figure 1: Leading causes of death

- A substantial number of the deaths can be attributed to pathological platelet aggregation



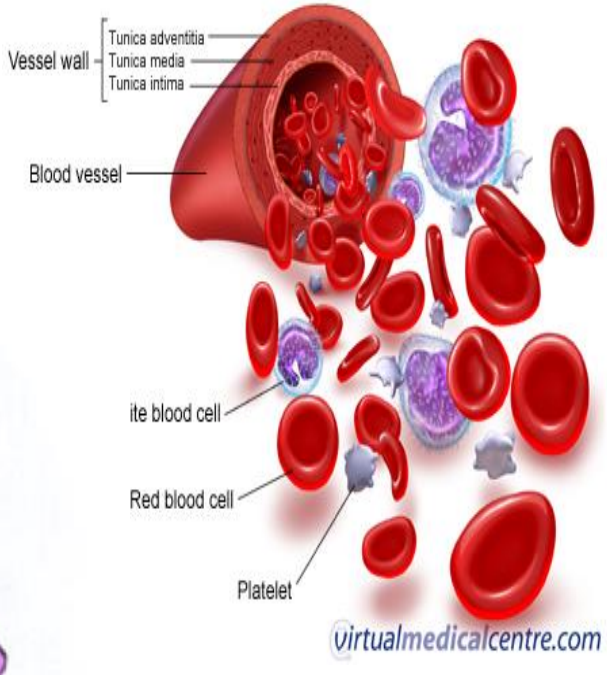


Figure 2: Mechanism of Platelet activation (adapted from Jagroop *et al.*, 2000)

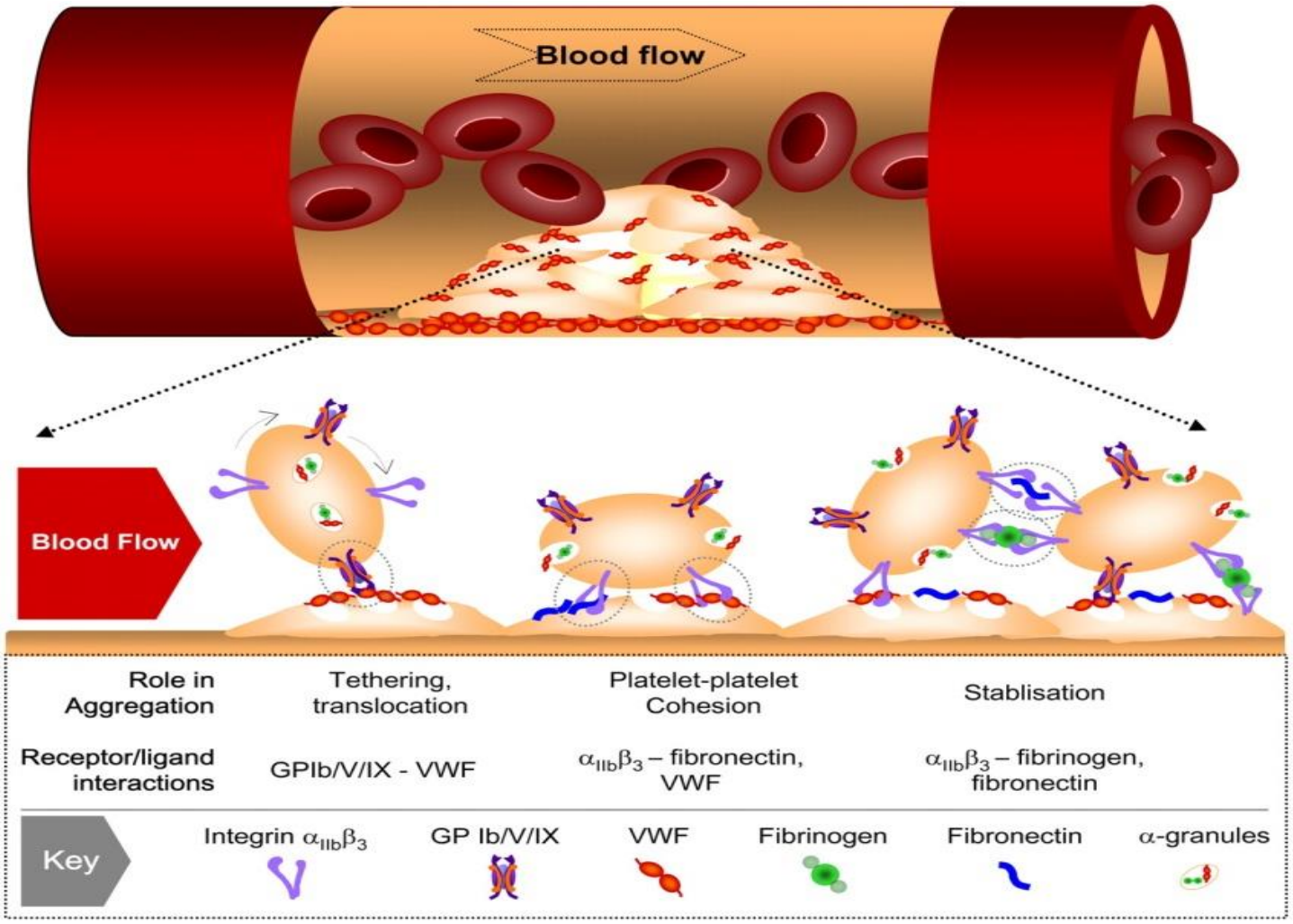
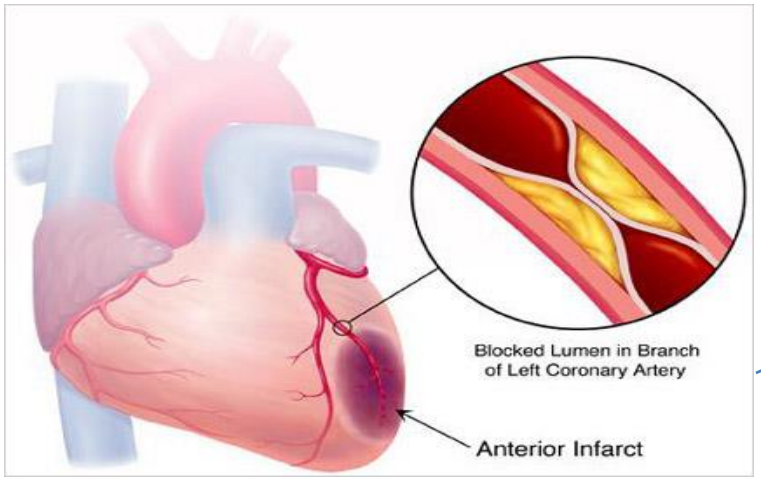
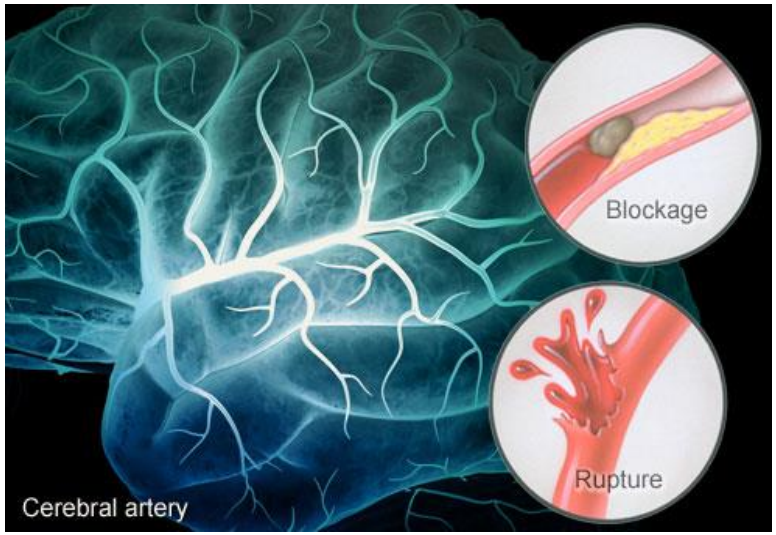
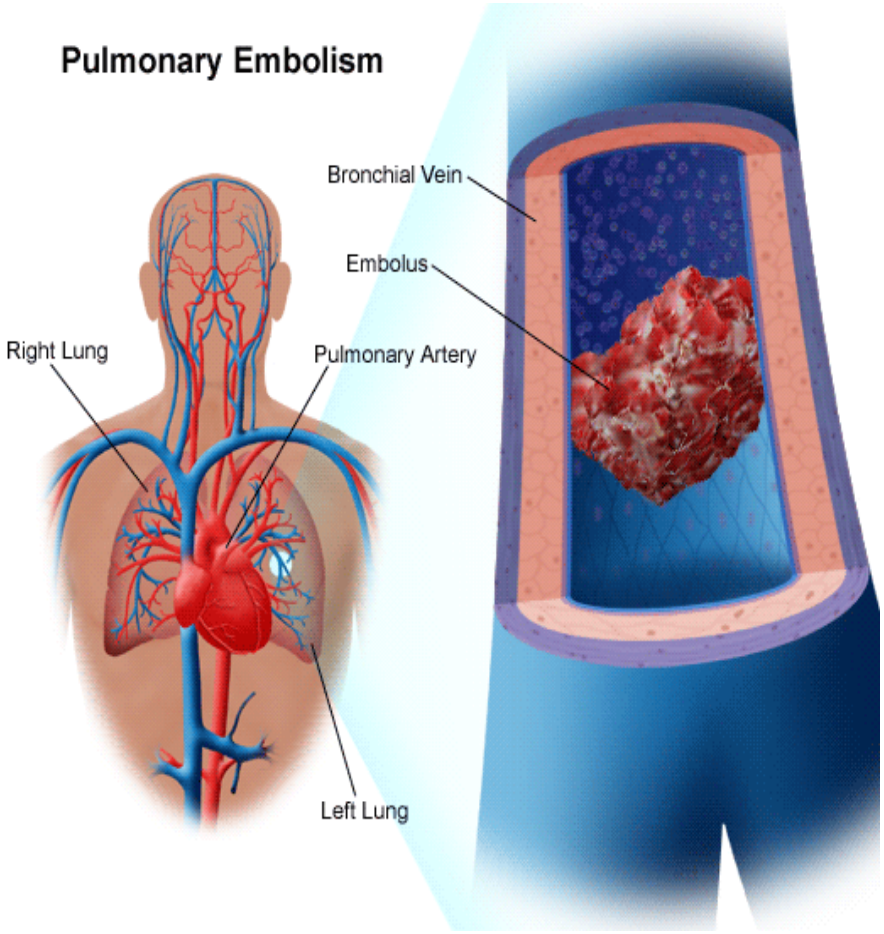


Figure 3: Processes of platelets aggregation (adapted from Jackson, 2007)

.

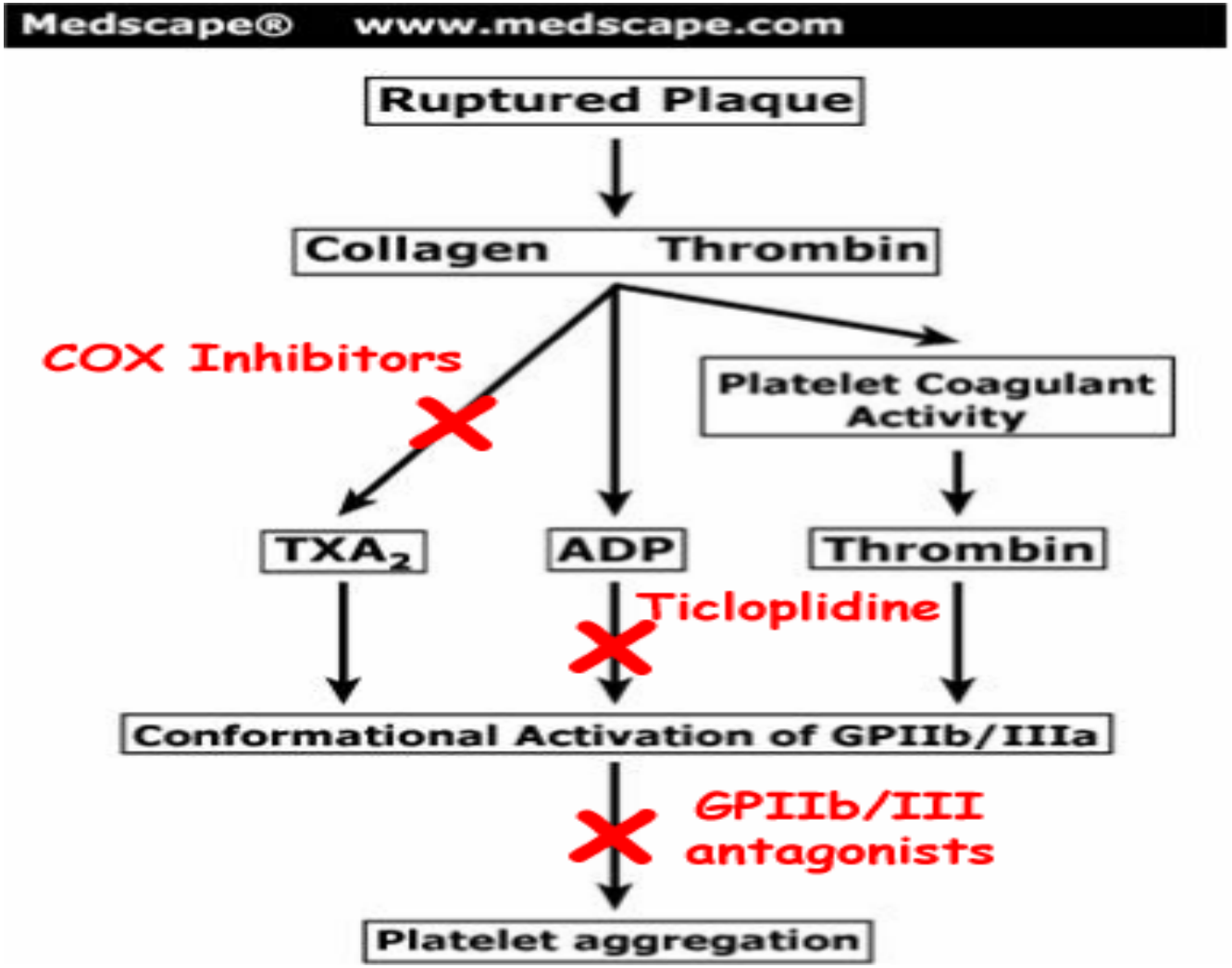

Blocked Lumen in Branch
of Left Coronary Artery
Anterior Infarct


Blockage
Rupture
Cerebral artery


Pulmonary Embolism
Bronchial Vein
Embolus
Right Lung
Pulmonary Artery
Left Lung

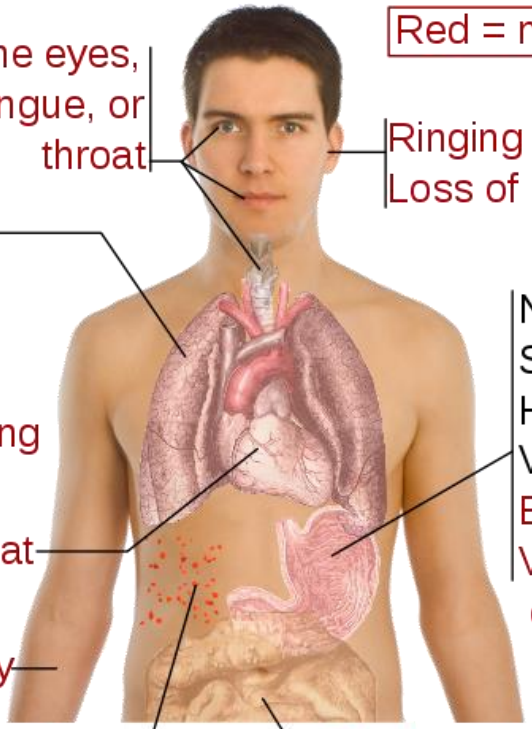
Antiplatelet Drugs

The crown and it's clones !


Medscape® www.medscape.com
Ruptured Plaque
Collagen Thrombin
COX Inhibitors
Platelet Coagulant Activity
TXA2
ADP
Thrombin
Ticloplidine
Conformational Activation of GPIIb/IIIa
GPIIb/III antagonists
Platelet aggregation

Side effects of

Aspirin



Red = more serious

Swelling of the eyes, face, lips, tongue, or throat

Ringing in the ears
Loss of hearing

Wheezing
Difficulty breathing
Hoarseness
Fast breathing

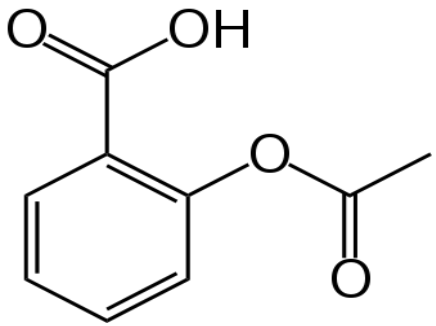
Nausea
Stomach pain
Heartburn
Vomiting
Bloody vomit
Vomit resembling coffee grounds

Fast heartbeat

Cold, clammy skin

Hives
Rash

Bright red blood in stools
Black or tarry stools



Medicinal Plant

➢ The use of Medicinal plants for the treatments of various chronic diseases are attributed to the present of **flavonoids, phenols, alkaloids, glycosides, terpenoids** and other secondary metabolites.

➢ Many medicinal plants have been scientifically proven to possess anti-platelet aggregation activity such include ***Ginkgo biloba*** (Gholomet *et al.,* 2005), ***Protorhus longifolia*** *(Mossa,2011),* ***Rapanea melanophloeos*** **(**Gwala**,**2011**)**

➢ Pentacyclic triterpenes were reported to possessed wide range of activities such as **antioxidant, antiplatelet, antihelmintes, antiviral, antifungal and antibacterial** (Ibrahim *et al.,* 2013)

Betulinic acid

- Betulinic acid is among the naturally occurring classes of pentacyclic triterpenoid

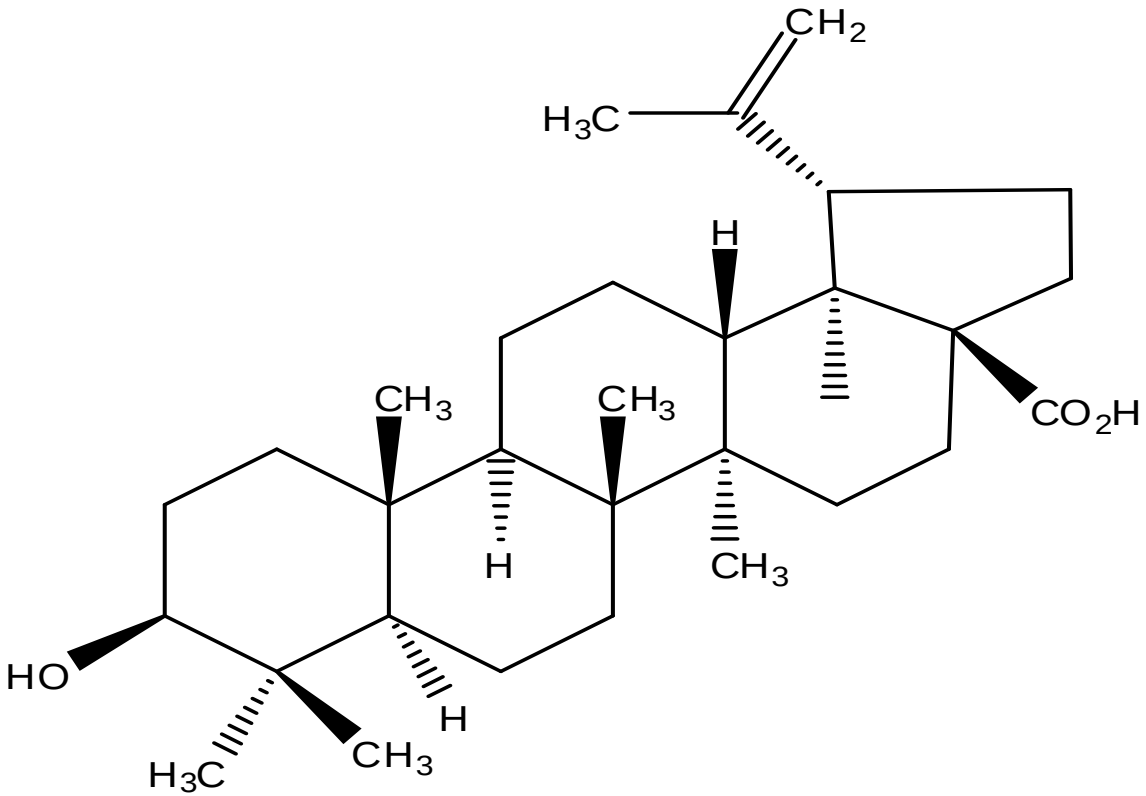


Figure 6: Structure of Betulinic acid (adapted from Osunsanmi et al., 2015)

Melaleuca bracteata var.revolution gold

- *Melaleuca bracteata var.revolution gold* is a genus plants in the myrtle family.

- Most of it are endemic to Australia (Craven, 2008).

- They are commonly called Black tea tree or River tea tree, Golden bottle brush, snow in the summer and white cloud tree.

- *Melaleuca bracteata* var.revolution gold is widely cultivated mostly in regions of South Africa and are commonly called Johannesburg gold

- It is commonly use as antiseptic, anti-fungal, pain killer (Habilla, *et al.,* 2011)

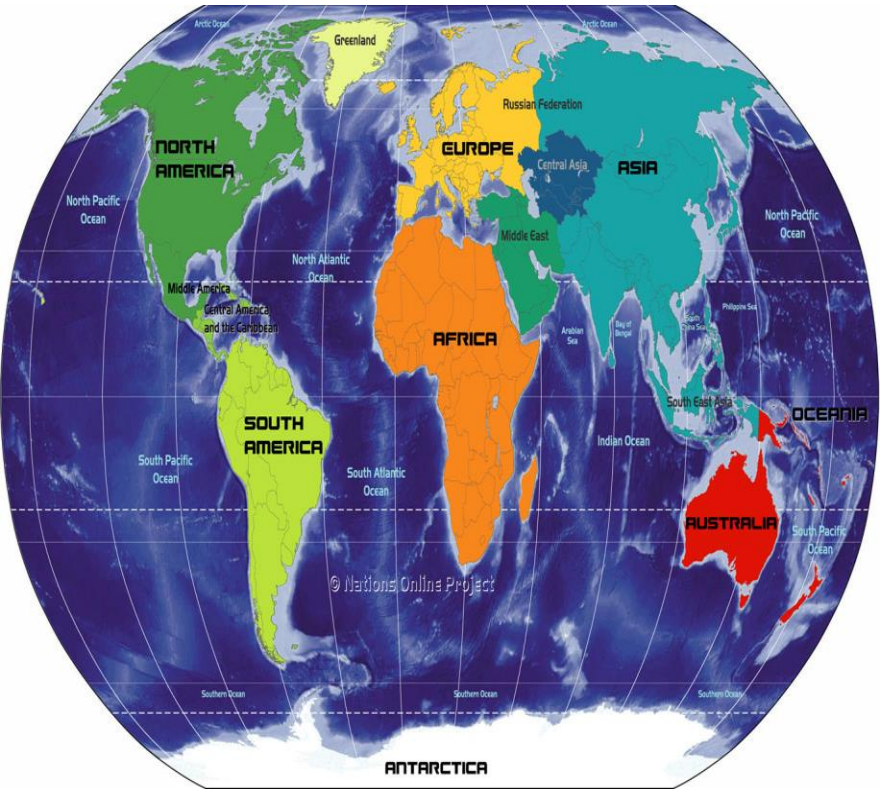


Figure 7: Picture of *Melaleuca braceteata*

Research hypothesis

Pentacyclic tritepenes have antiplatelet aggregation activities, therefore Betulinic acid and its derivatives are potent platelet aggregation inhibitors.

Aims

This Project aims to extract and isolate betulinic acid from *Melaleuca bracteata* var. revolution gold, and synthesize its acetyl derivatives, which will be evaluated for their antiplatelet aggregation and cytotoxicity.

Objective

- Collect and identify of *Melaleuca bracteta* var. revolution gold.
- Extract and isolate betulinic acid from medicinal plant
- Synthesize betulinic acid derivates.
- Characterized the compound.
- Determine the antiplatelet aggregation activities of betulinic acid and its' derivates.
- To determine the cyctoxicity of betulinic acid and its ' derivates

Materials and methods

Collection and identification of medicinal plant

- Leaves of *Melaleuca bracteata* var. revolution gold were collected from trees growing on the KwaDlangezwa campus of University of Zululand.

- The plant was taken to Department of Botany for identification and voucher specimen deposited at the University herbarium.

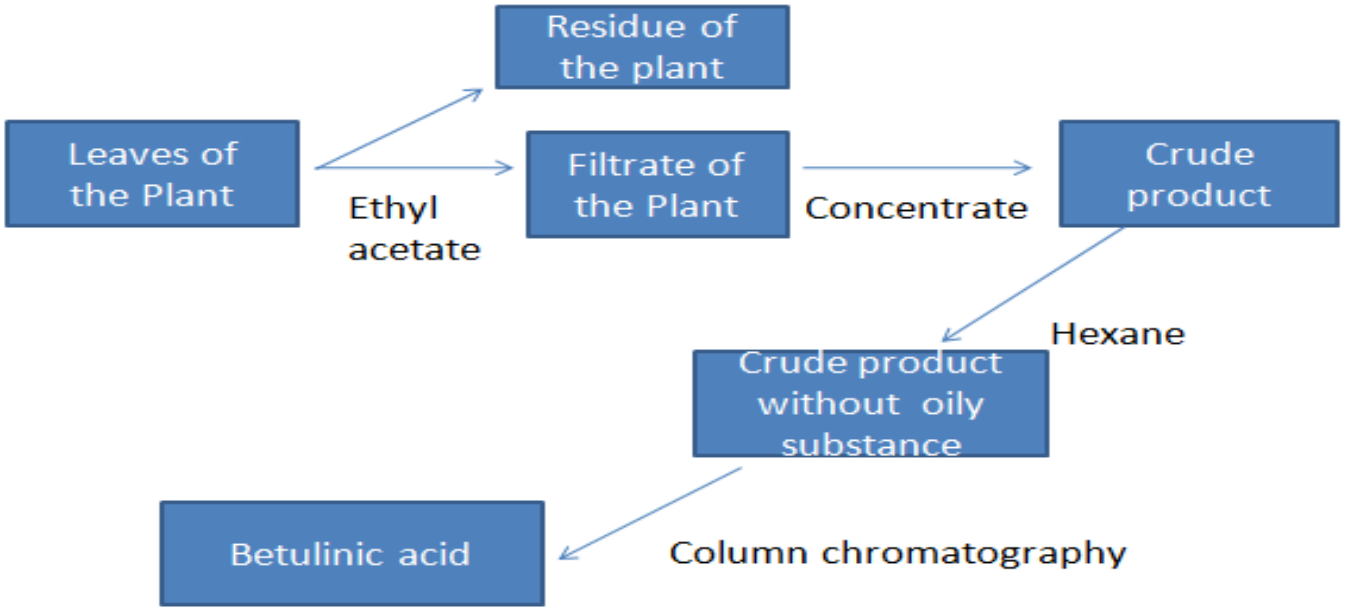


Figure 8: Schematic representation of the isolation of betulinic acid from *Melaleuca bracetata var revolution gold*

- **The method of Mosa *et al.*, (2011) was adopted**
- **Percentage yield (10%)**
- **mp 315-316 ^{o}C**
- **Spectral analysis (NMR. MS & IR)**

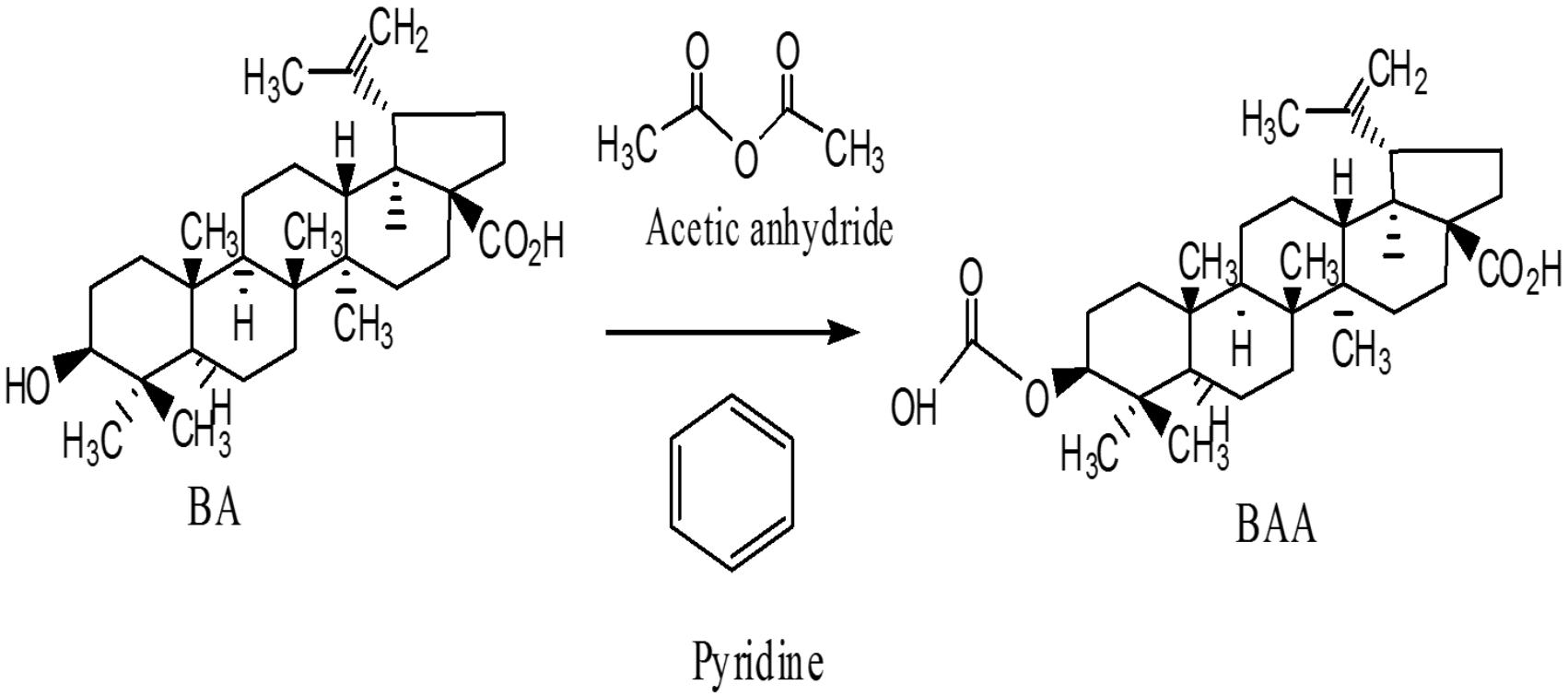


Figure 9: Schematic diagram for synthesis of BAA

- **The method of Habila *et al*., (2011) was adopted with slight modification.**
- **Percentage yield (12%)**
- **mp 258-260 ^{o}C**
- **Spectral (NMR, MS & IR) analysis**

Experimental animals

- The ethic clearance (UZREC 171110-030 PGD 2014/53) was obtained from the Research Animal Ethical Clearance committee (RAEC) of University of Zululand.

- Dawley rats of either sex (8weeks, 220 -250kg) were collected from the Animal house at the Department of Biochemistry and Microbiology, University of Zululand.

- The Animals were acclimatized to the standard laboratory facility and maintained using Standard Ethic protocol with asses to clean water and pellet feeds.

In *vitro* antiplatelet aggregation study

➢Preparation of blood platelets

The blood platelet was collected according to the method described by Mosa et al., (2011).

➢Anti-platelet aggregation evaluation

The method of Mekhfi *et al*., (2004) was used with slight modification

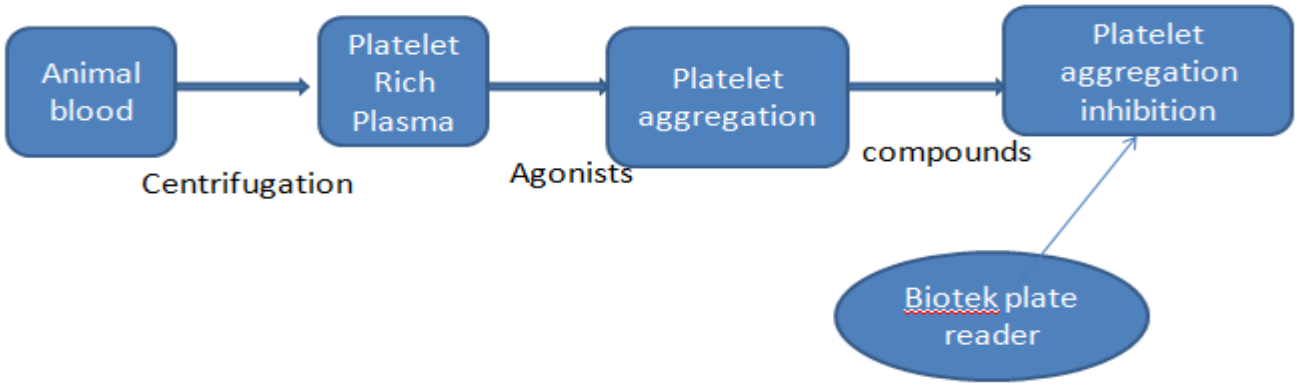


Figure 10: Schematic representation of platelet aggregation studies

Cytotoxity test

- The MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetraz oliumbromide]cytotoxicity proliferation assay will be used to measure the toxicity (Mosman, 1983).

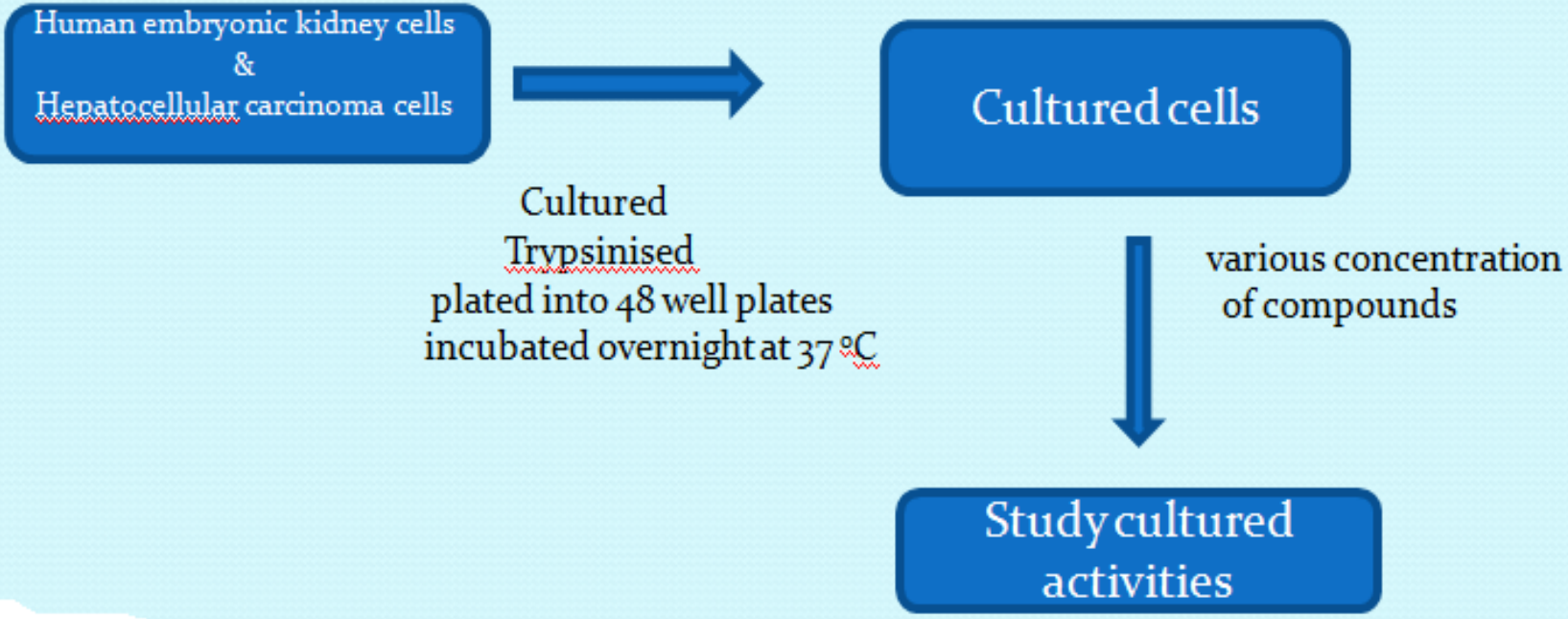


Figure 11: Schematic respresentation of cytototoxity test

Statistical analysis

- All assays were performed in triplicates and data expressed as **mean ± SEM**

- **Anova (One way)** and **post hoc Dunnett's** test were used to analyse the data using Graphpad prism version 5.03.

- The statistically significant was given as **P< 0.05.**

Compounds Identification

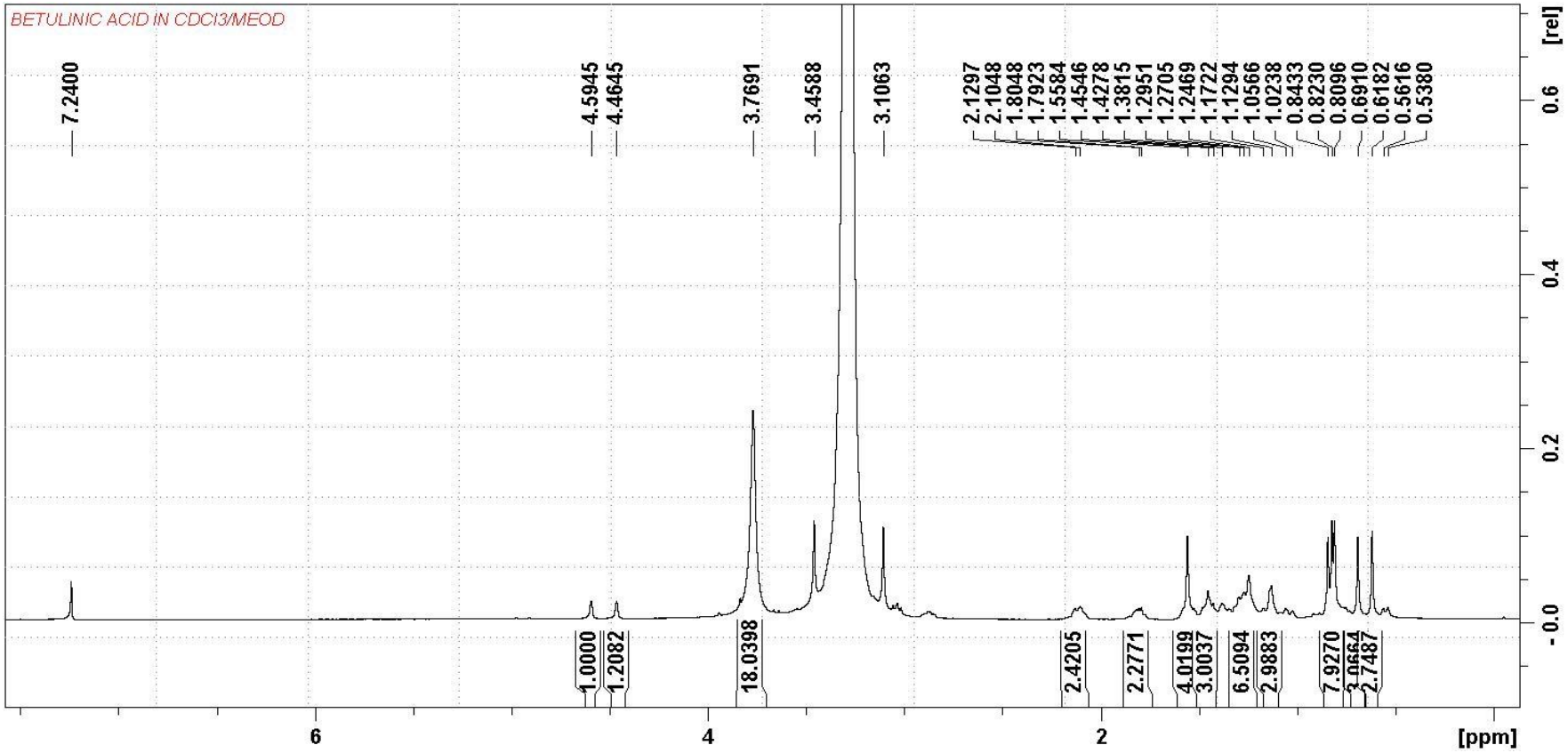


Figure 12: The IHNMR spectrum of BA revealed various peaks corresponding to the **methyl groups at around 0.80 to 1.17 ppm** and **terminal methylene protons at 4.46 to 4.59 ppm,** which is indicative of the presence of **48 hydrogenatoms in BA**

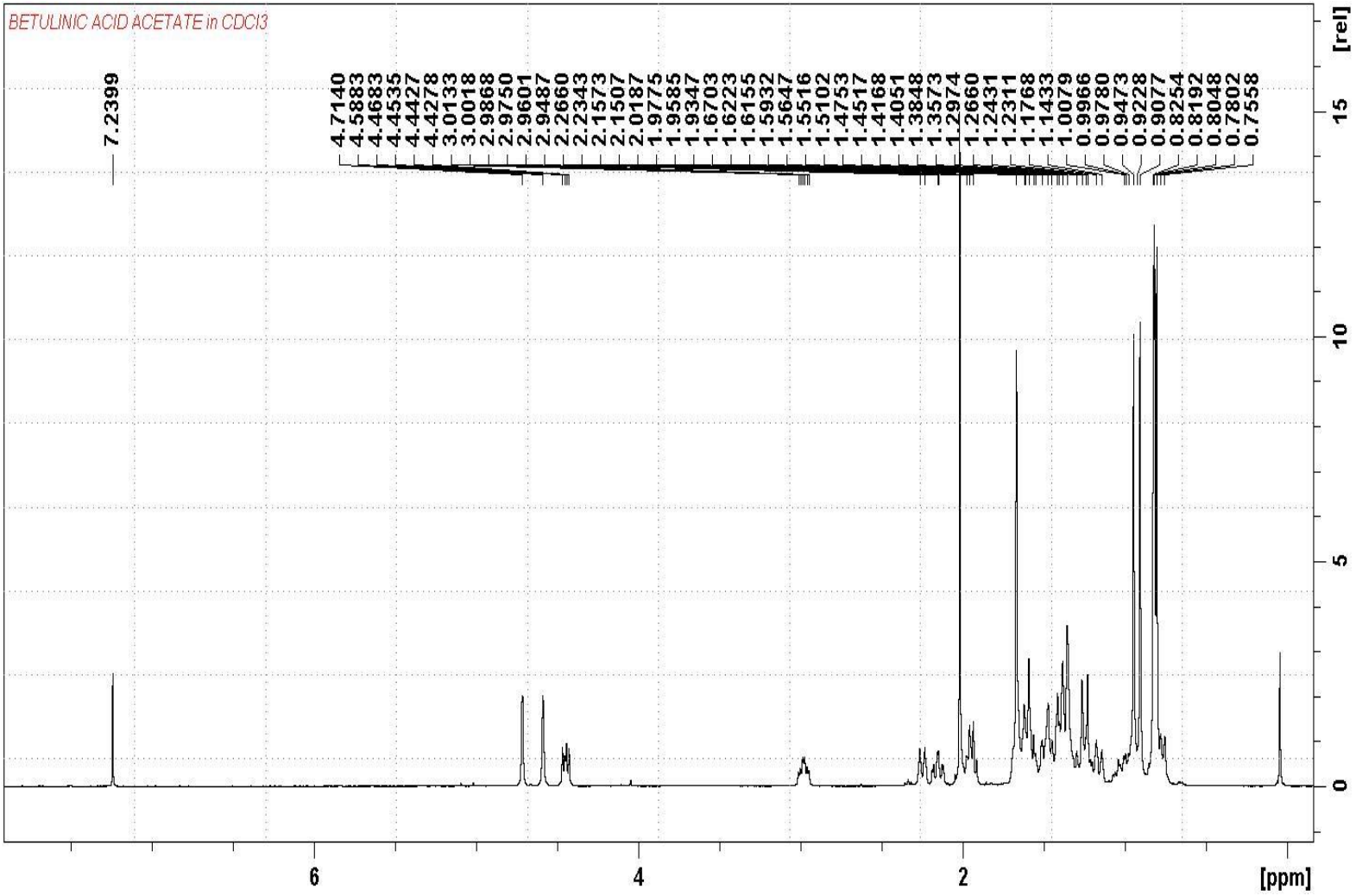


Figure 13: As expected, the IH NMR spectrum of BAA showed the presence of 50 hydrogen atoms, which agrees with literature for previously reported values (Habila et al., 2011).

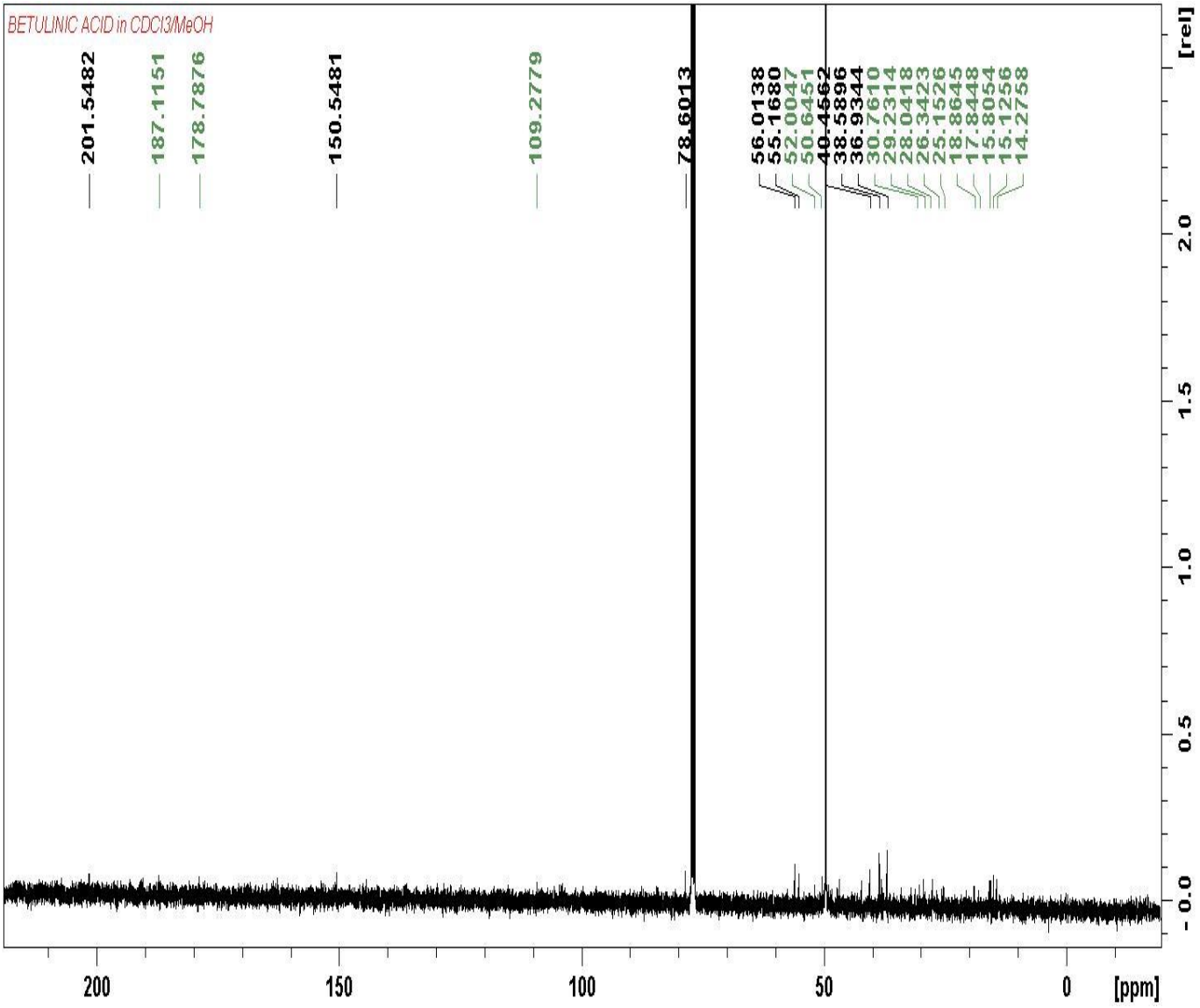


Figure 14: ^{13}C-NMR spectrum of BA

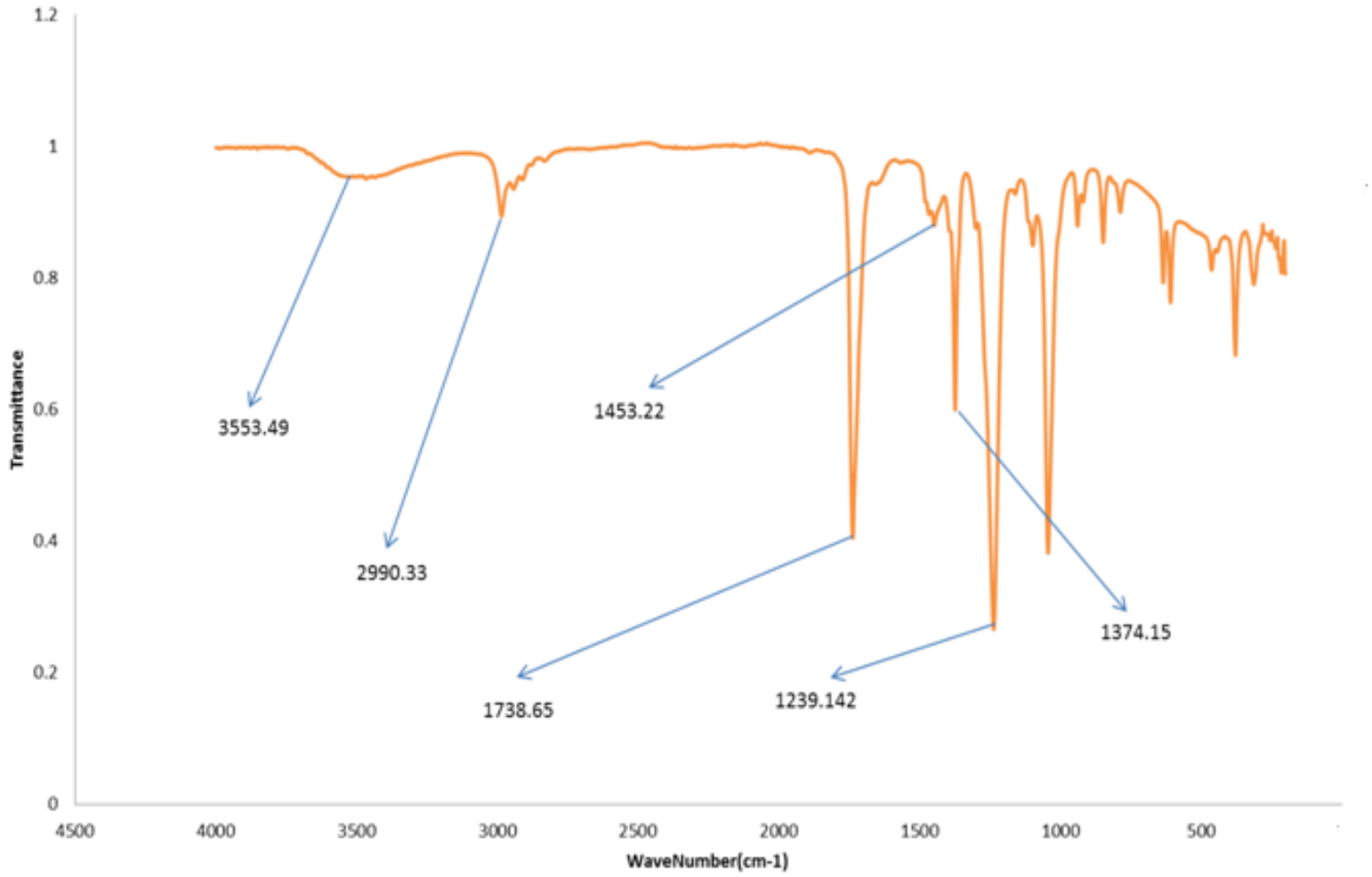


Figure 15: The presence of hydroxyl groups in the compounds was indicated by the appearance of an absorption band between 3424 to 3456 cm-1 in the IR Spectra

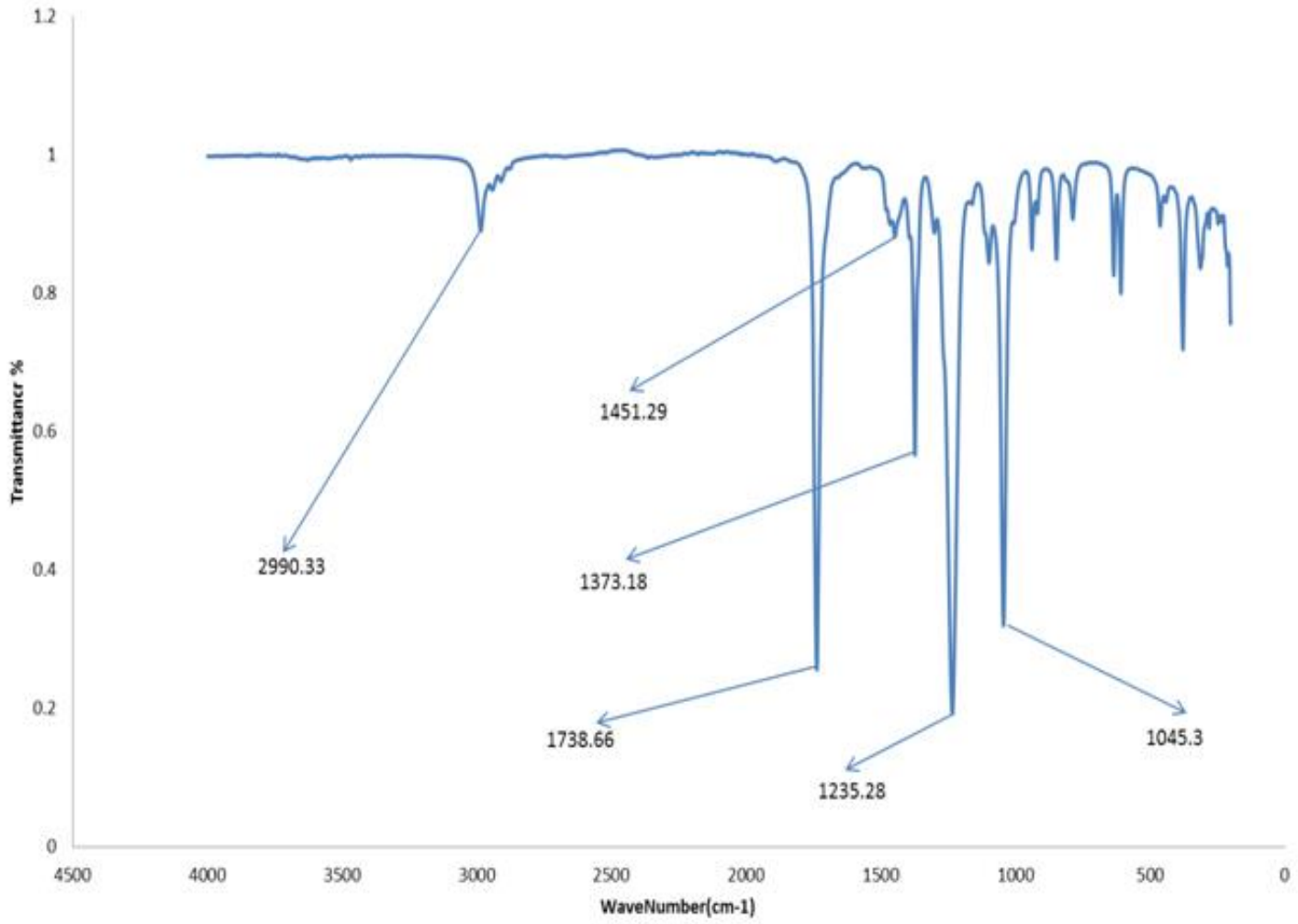


Figure 16: IR Spectra for BAA

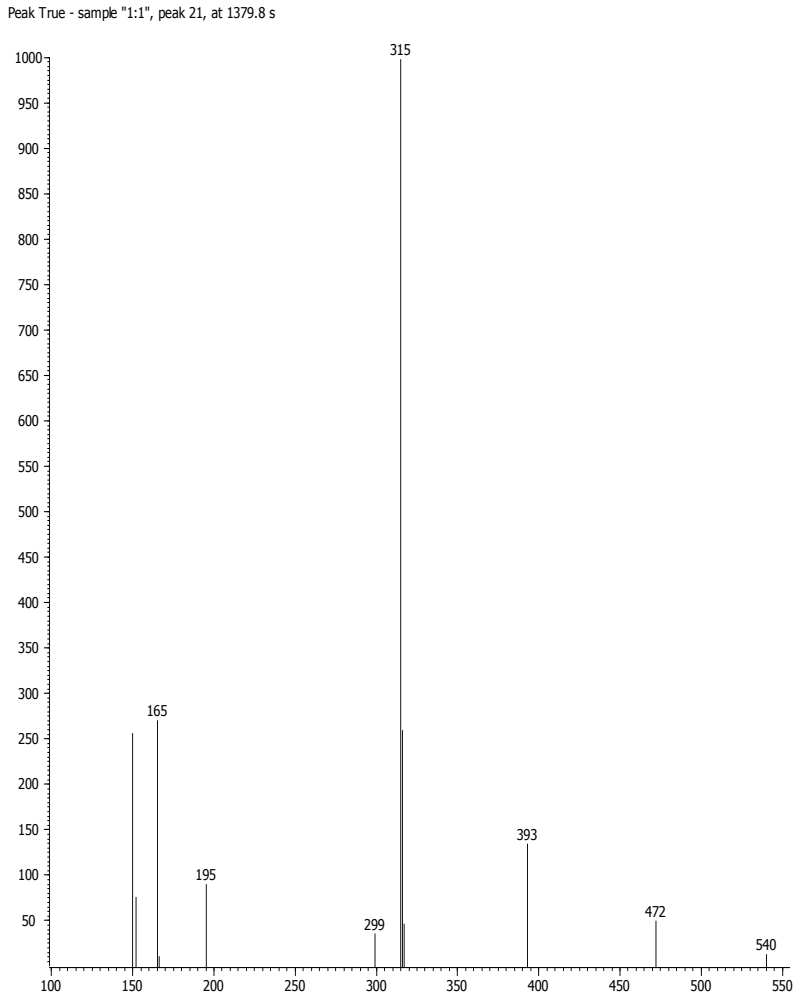


Figure 17:Mass spectroscopy for BA

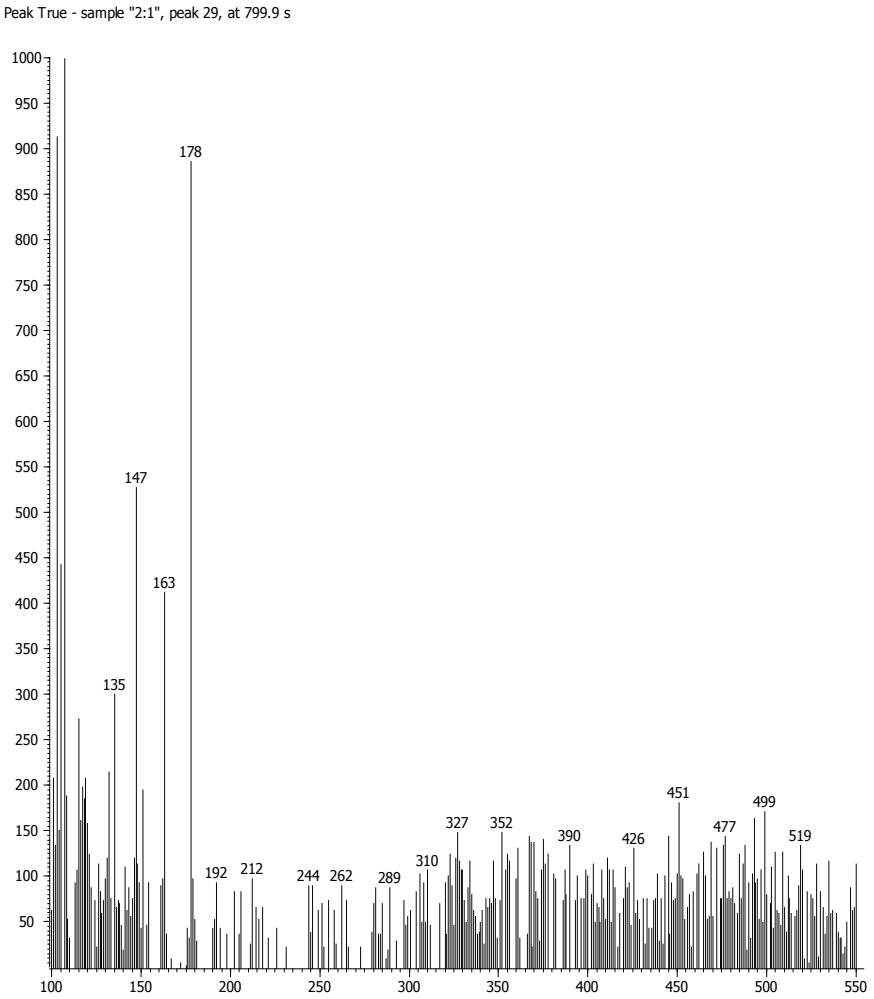


Figure 18:Mass spectroscopy for BAA

- 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.

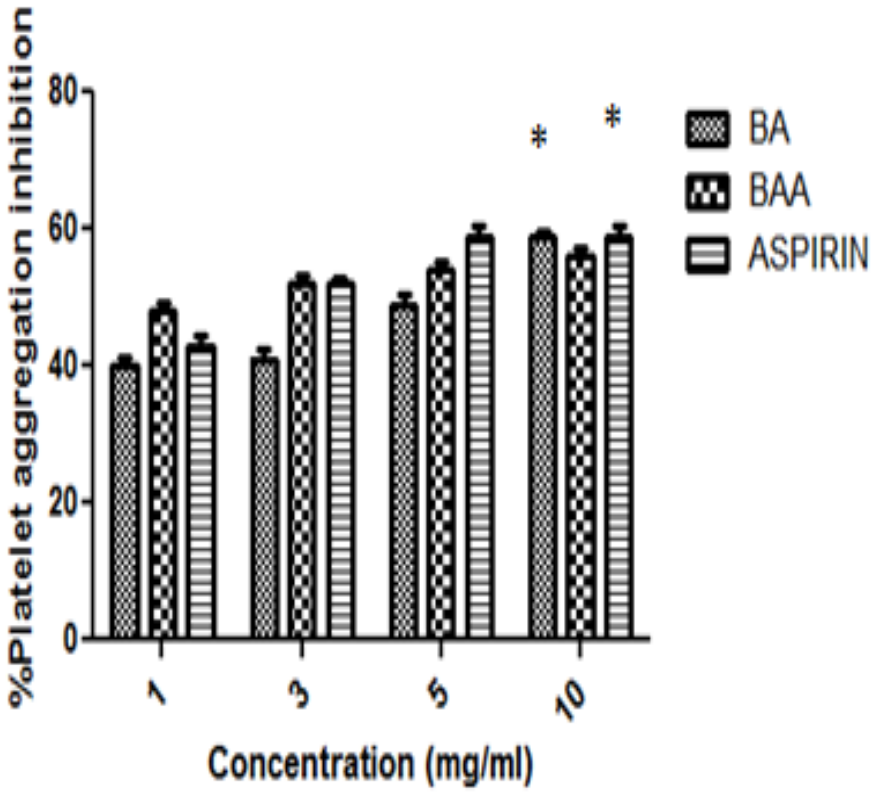


Figure 19: Platelet aggregation inhibition induced by collagen Data were expressed as mean ± SD. *P<0.05, **P<0.01.

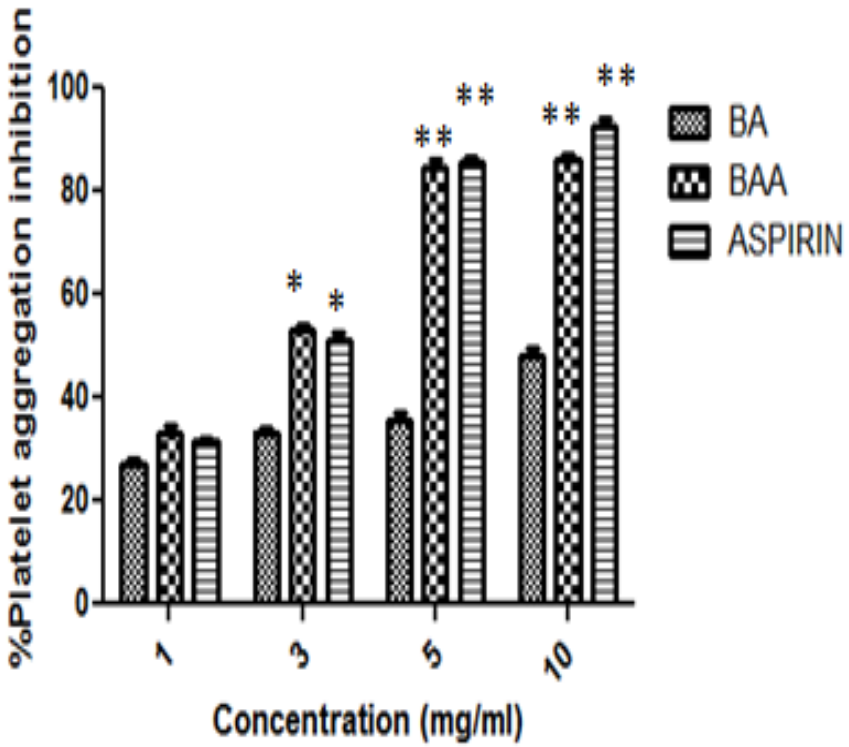


Figure 20: Percentage platelet aggregation inhibition induced by ADP Data were expressed as mean ± SD. *P<0.05, **P<0.01.

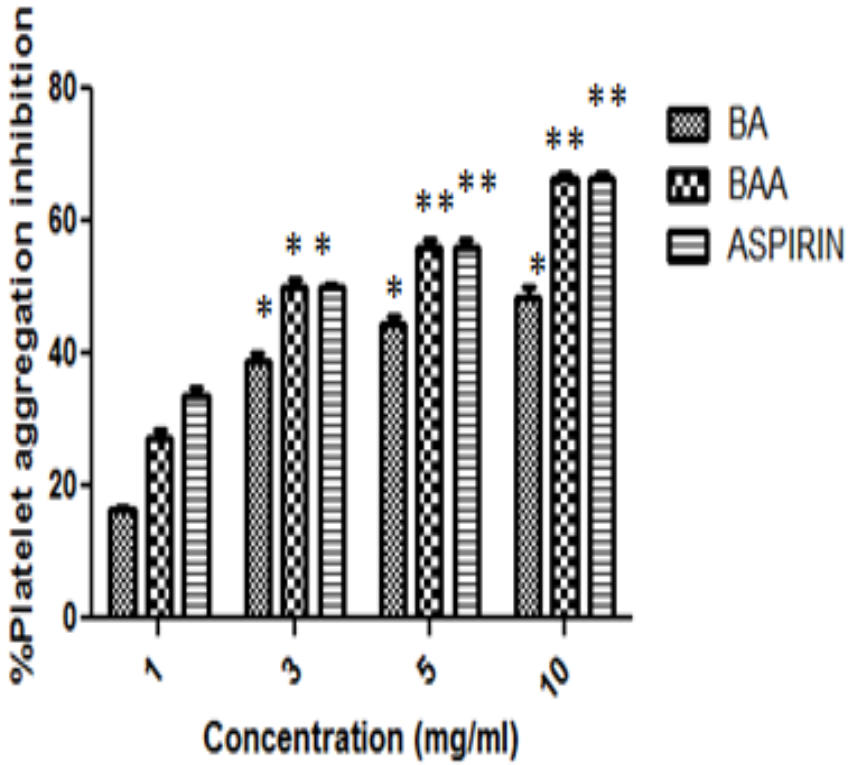


Figure 21: Percentage platelet aggregation inhibition induced by thrombin Data were expressed asmean ± SD. *P<0.05, **P<0.01.

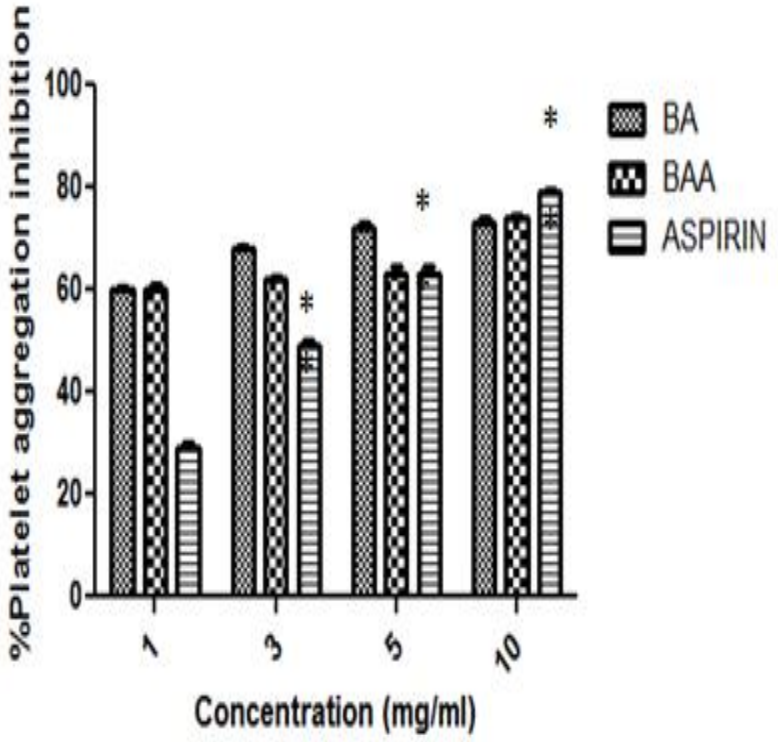


Figure 22: percentage platelet aggregation inhibition induce by epinephrine Data were expressed asmean ± SD. *P<0.05, **P<0.01.

Table 1: The platelet aggregation inhibition IC_{50} for betulinic acid and acetyl derivative induced by agonists

Compounds	IC_{50} (mg/ml)			
	Collagen	Thrombin	ADP	Epinephrine
Betulinic acid	5.45	11.6	11.1	0.78
3-β acetylbetulinic acid	1.72	2.92	2.72	0.85
Aspirin	2.58	2.72	2.72	2.98

Table 2: The IC_{50} MTT Cytototoxicity assay for betulinic acid and 3-β acetylbetulinic acid using HEK293 and HEPG2 cells

Compound	IC_{50} (µg/ml)	
	HEK 293	HEPG2
Betulinic acid	1027	448
3-β Acetylbetulinic acid	1051	672

Discussion

- The results showed that BA and BAA **inhibited platelet aggregation regardless of the agonists (thrombin, collagen, ADP and epinephrine).**

- Antiplatelet aggregation activities of some other pentacyclic triterpenes against platelet agonists (ADP, thrombin and epinephrine)have previously been reported (Jin et al., 2004; Kim et al., 2010; Xuemei et al., 2010).

- **Targeting carbon positions 3 and 28 are new pharmacophores** for increasing biological activity (Ban et al., 2010).

- The relatively higher antiplatelet aggregation activity of BAA could be attributed to the **acetyl modification at carbon-3 (C-3) position**.

- The **American National Cancer Institute guidelines** consider a pure compound as cytototoxic with **IC50 < 30 µg/ml** (Suffness and Pezzuto, 1990).

- Despite the weak cytotoxic effect exhibited by the two triterpenes, a relatively higher activity on HEPG2 than HEK293 implies **the compound could selectively inhibit the proliferation of cancer cells at higher concentration**

- Betulinic acid has previously been reported to selectively inhibit tumour cells (Pisha et al., 1995).

- The **weaker cytotoxic effect of BAA** on the two cells used in this study could also be attributed **to the acetyl modification of C-3 position**.

Conclusion

- The in-vitro antiplatelet aggregation activity reveled that betulinic acid and derivative are potent antiplatelet aggregation inhibitors **regardless of different agoinsts**

- In addition to efficacy, the weak cytotoxic effect showed by the compounds indicated their potential use as templates for **synthesis of safe pharmacologically active antiplatelet agents**

- For further study, elucidation of the **mechanism of action** of the compounds is recommended

Acknowledgement


THANK
YOU