DESIGN, SYNTHESIS, AND BIOLOGICAL EVALUATION OF ANTIMYCOBACTERIAL AGENTS FROM PLANT DERIVED BETULINIC ACID, OLEANOLIC ACID AND THEIR DERIVATIVES

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2015

Dedicated

To

John-Mark Oreoluwa Oyedepo Adeniyi Fadipe

Declaration

This is to certify that the work reported in the dissertation entitled “Design, synthesis, and biological evaluation of antimycobacterial agents from betulinic acid, oleanolic acid and their derivatives” is an original work by Mr Victor Olugbenga Fadipe, carried out under our supervision and directions. The dissertation has been submitted in fulfillment of the requirement for the degree (PhD) with the approval of the undersigned.

I, V.O. Fadipe, declare that the dissertation has not been previously submitted by me for a degree at this or any other University, that this is my own work in design and in execution, and that all the material contained therein have been duly acknowledged.

V.O Fadipe	Prof A.R Opoku	Prof O.O.O Shode

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Abstract

Tuberculosis (TB) is a dangerous disease that has killed several millions of people globally in recent times. The available drugs for the treatment of the disease are not effective for complete cure and in most cases, usually come with side effects, as a result of which new set of potent drugs are needed.

In a quest to develop potent hit/drug leads for TB, betulinic acid (BA) and oleanolic acid (OA) were isolated respectively from *Curtisia dentata* and *Syzigum aromaticum*. The 3-O- acetyl analogue of BA and OA were synthesized. The cinnamic acid conjugates at C-28 position of the four (4) synthesized compounds were all characterized using IR, MS and ^{1}H and ^{13}C NMR. Co-crystal compound synthesized from the isolated BA and OA with foremost first line antitubercular drug isoniazid (INH) was carried out for the first time. The co-crystal compounds were synthesized using three different conditions: I. Solvent evaporation method, II. Solvent drop method and III. Dry co-grinding method. The synthesized co-crystal compounds were characterized by P-XRD, TGA, and SEM. The isolated triterpenes and their synthesized derivatives were then evaluated for anti-mycobacterial activity (MABA test, against $H_{37}RV$ [ATCC27294] strain), cytotoxicity (MTT test using human embryonic kidney [HEK293] and human hepato-cellular carcinoma [HepG2] cell lines), and DNA polymerase β (pol β) inhibitor activity (with the POLB human ELISA kit).

All the test compounds exhibited anti-TB activity, albeit to different levels of efficacy. The MIC values of the two pentacyclic triterpenes (BA and OA) against the mycobacterium ranged from >109.48 uM and 42.04 uM respectively. The acetylation of BA and OA at C-3 position did not observably improve their activity (MIC value of

39.70 uM and 100.26 uM) and neither did the cinnamic acid derivatives of BA and OA at C-28 position enhance the anti-TB activity (MIC value of >85.20 uM and 48.05 uM respectively). The di-substituted, 3-O-acetyl and 28- cinnamic acid ester of BA and OA however exhibited some enhanced anti-TB activity with MIC value of 17.88 uM. The co-crystallization of the triterpenes to INH drastically increased the efficacy of the triterpenes (MIC values in the range of 0.45 uM to 1.06 uM were obtained).

The DNA polymerase β inhibitor activity of oleanolic acid and betulinic acid, their acetate derivatives, along with their cinnamic acid hybrid indicated that their inhibition of pol β was concentration dependent. The cytotoxicity of the test compounds to the two human cell lines (HEK293 and HepG2) was in the range of $IC_{50} \geq 300$ µg, indicating low toxicity level.

Conclusively, BA and OA may be explored as template for the syntheses of potent anti-TB drug hit/lead when combined with other compounds with known moderate anti-TB activity index.

Key Words: *Curtisia dentata*, *Syzigum aromaticum*, BA, OA, molecular hybridization, co-crystal synthesis, solvent evaporation, solvent drop, co-grinding, anti-mycobacterial activity, DNA polymerase β inhibitors, cytotoxicity, HEK293 and HepG2.

Abbreviations

µM	micromoles
ATCC	American Type Culture Collection
BA	Betulinic Acid
DCM	Dichloromethane
DMAP	4-(-dimethylamino-)-pyridine
DNA	Deoxyribonucleic acid
EI-MS	Electron Ionization Mass Spectrometry
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
GDP	Gross Domestic Product
HIV	Human Immunodeficiency Virus
HR-MS	High Resolution Mass Spectroscopy
IC_{50}	50% inhibitory concentration
INH	Isoniazid
M/Z	mass-to-charge ratio
MDR-TB	Resistant to both isoniazid [INH] and rifampicin [RMP], two of the first-line drugs used in treating smear-positive pulmonary tuberculosis.
MIC	minimum inhibitory concentration

MTT	Colorimetric assay for assessing cell metabolic activity
NMR	Nuclear Magnetic Resonance
OA	Oleanolic Acid
POL β	Polymerase beta
SAR	Structure Activity Relationship
TB	Tuberculosis
TDR-TB	Totally Drug-Resistant tuberculosis
TLC	Thin Layer Chromatography
UCT	University of Cape Town
UKZN	University of KwaZulu- Natal
UV	Ultra Violet
UZ	University of Zululand
WHO	World Health Organization
XDR-TB	Extensively drug-resistant TB

Contributions to Knowledge

A. Publication

1. **Fadipe, V.O.,** Mongalo, N.I., Opoku, A.R. (2015). *In vitro* evaluation of the comprehensive antimicrobial and antioxidant properties of *Curtisia dentata* (Burm.f) C.A.Sm: toxicological effect on the human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines. *EXCLI Journal*, 14, 971-983.
2. **Fadipe, V.O**., Mongalo, N.I., Opoku, A.R. (2015). Antibacterial properties of *Curtisia dentata* leaves and some triterpenes/actives principles isolated from them. *South African Journal of Botany*, 18, 208 (Abstract). http://dx.doi.org/10.1016/ j.sajb.2015.03.148.
3. **Fadipe, V.O**., Mongalo, N.I., Opoku, A.R. (2015). Bioprospecting for betulinic acid among medicinal plants from South African origin. *South African Journal of Botany*. 2015; 98: 208 (Abstract). http://dx.doi.org/10.1016/ j.sajb.2015.03.148.
4. **Fadipe, V.O.,** Opoku, A.R., Shintre, S.A., Singh, P., Mongalo. N.I. (2016). Antimycobacterial, antiplasmodial and toxicological effect of oleanolic acid and its derivatives from Syzygium aromaticum Linn (Myrtaceae). South African Journal of Botany. (Abstract). http://dx.doi:10:1016/ j sajb.2016.02.043.
5. Opoku, A.R., **Fadipe, V.O.,** Mongalo, N.I. (2016). Selectivity index and Antioxidant properties of extracts and newly isolated active principle from *Curtisia dentata (Burm.f) C.A.Sm (Curtisiceae)* leaves (Abstract). South African Journal of Botany. http://dx.doi:1016/ j.sajb.2016.02.145.

6. Mongalo, N.I., **Fadipe, V.O.,** Mosa, R.A., Opoku, A.R. (2016). In vitro antimycobacterial activity of fractions, isolated compounds and derivatives from *Curtisia dentata (Burm.f) C.A. Sm leaves*. South African Journal of Botany. (Abstract). http://dx.doi: 1016/j.sajb.2016.02.118.

B. Oral communications

1. **Fadipe, V.O.,**Mongalo, N.I., and Opoku, A.R. Antimycobacterial, antiplasmodial and cytotoxicitity activities of oleanolic acid and its derivatives. October 28th 2014. 9th Annual Faculty of Science and Agriculture Research Symposium, Kwadlangezwa, South Africa.

C. Poster presentations

1. **Fadipe, V.O.,** Mongalo, N.I., and Opoku, A.R., *In Vitro* evaluation of the comprehensive antimicrobial and antioxidant properties of *Curtisia dentata* (Burm.f) C.A. Sm: Toxicological effect on the human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines. 4th International Conference and Exhibition on Immunology, Houston, USA, September 28th -30th 2015.
2. **Fadipe V.O.,** Mongalo N.I., and Opoku A.R. Antibacterial properties of *Curtisia dentata* leaves and some triterpenes/active principles isolated from them. 3rd International Conference on Medicinal Chemistry and Computer Aided Drug Designing, San Francisco, USA, December 08th - 10th 2014.

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Chapter 1

1.0 Introduction

Africa is plagued with many diseases viz, malaria, schistosomiasis, trypanosomiasis, leishmaniasis, onchocerciasis, filariasis, sickle cell anemia, diabetes, HIV/AIDS, hypertension, cancer, tuberculosis, to mention a few (Ibezim et al., 2009). Despite the availability of western medicine, most (~80%) Africans and peoples in developing countries depend on traditional (plant- based) medicine for the management of various diseases (Sasidharan et al., 2011). Medicinal plants have continued to gain prominence as cheaper source of drugs for the treatment and management of a variety of chronic ailments for several decades back (Mahomoodally et al., 2013) . Most of the medicinal plants involved were discovered by trial or error, while others were discovered as a result of the existing practice recorded in both folklore and books of early practitioners (Ayob and Tan., 2013).

In the early nineteenth century, medicines from plants were administered in crude forms usually as infusions (herbal teas), tinctures (alcoholic extracts), decoctions (boiled extract of roots or bark), syrups (extracts of herbs made with honey), or applied externally as ointments (poultices, balms and essential oils) and herbal washes to treat some diseases. The principle being that the disease is allowed to have enough contact with the medium conveying the active component(s) of the plant materials. A drawback of these methods of disease therapy is the lack of dosage instructions, among others (Soumya et al., 2009).

The end of the nineteenth and early twentieth centuries brought about changes, as there was interest by scientists to identify, isolate, and purify active ingredients from

medicinal plant extracts reportedly responsible for some observed biological activity the plants extracts displayed.

The curiosity to know and ascertain the chemical compounds responsible for some specific biological activity led to the discovery of some of the known important drugs in the market for the treatment of diseases today(Ngo et al., 2013). A typical example is the isolation of morphine from opium poppy (*Papaver somniferum*) which is regarded as a pain relieving drug. Similarly, the isolation of quinine from Cinchona plant species as an effective drug in the treatment of malaria. Taxol isolated from *Taxus brevifolia*, and vincristine isolated from *Catharanthus roseus* are highly potent drugs against certain types of cancer. Serpentine is another important compound isolated from the root of the Indian plant, *Rauwolfia serpentia,* which is used in the treatment of hypertension (Baker et al., 2007).

Aspirin, an ancient analgesic,was first isolated from *willow bark.* Similarly, penicillin was also isolated from *Penicillum notatum*. Penicillin is the first and most potent antibiotic drug that revolutionized the discovery of other important antibiotic drugs used for the treatment of infectious diseases(Hamed et al., 2012).

Medicinal plants have inspired the growth and development of modern drugs by providing lead compounds which are either used directly, or are further probed through chemical transformation synthesis to form new potent drugs which are in use today. It is reported that 75% of the drugs used in the treatment of infectious diseases can be traced to chemical compounds isolated from natural origins (Hamed et al., 2012).

The research on potential drugs from plants have increased tremendously in recent years, as plants still remain a major reservoir of novel chemicals, proven to be effective in the treatment of many diseases. This has led to the current drive of bio-prospecting of medicinal plants for potent phytochemicals that could serve as lead compounds to be developed directly or modified through chemical transformation into new drugs (Ayob & Tan, 2013).

Tuberculosis is a serious pandemic that is affecting humanity in recent times. In fact, it is second to HIV/AIDS as the greatest killer worldwide (Sharma and Mohanm, 2013) . This single infectious disease has no geographical boundary, and as such present in every corner of the whole world without any geographical restriction, as it is an airborne disease.

In 2013, nine (9) million people were infected with TB, out of which 1.5 million died. Over 95% of deaths associated with TB occur mostly in low- and middle-income countries; with sub-Saharan Africa being the worst hit. This is attributable to the emergence of HIV/AIDS and different types of resistant strains of the causative organism (Lawn et al., 2011).

Chemotherapy used in the treatment of TB dates as far back as 1944, but it has remained ineffective in the treatment of the disease lately (Mitra, 2012). There is thus an urgent need to develop new set of anti-mycobacterial agents with possibly better efficacy than the current sets of drugs in the market.

The concept of molecular hybridization of two or more molecules to form a single unit molecule with better drug-like properties than the individual units has featured

prominently in drug discovery in recently, and it has been suggested that it could as well aid the emergence of new set of drugs for the treatment of tuberculosis (Bosquesi et al., 2011; Dutra et al., 2011).

In the light of the above, this study was designed to hybridize two moderately active anti-mycobacterial agent molecules to form a single unit. In doing so, it is expected that the new compound will be more potent than the individual molecules.

To achieve this, two pentacyclic triterpenic acids from known medicinal plants were isolated and chemically transformed to enhance their efficacy against tuberculosis. The chemical transformed will be in two parts:-

a. The synthesis of the molecular hybrid of the isolated compounds and their acetate analogue with cinnamic acid and their subsequent evaluation against wild strain of mycobacterium $H_{37}RV$.

b. The co-crystal synthesis of the isolated triterpenic acid with a first-line TB drug isoniazid (INH) and followed by evaluation of the co-crystal compounds against wild strain of mycobacterium $H_{37}RV$.

The final part of the work was to subject all compounds to cytotoxicity study using human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines.

1.1 Structure of dissertation

The dissertation is set out in 6 chapters and appendices, as follows:

Chapter 1

- Provides a brief background to and motivation for the study.

Chapter 2

- Gives the general literature review on the entire study. This section provides in-depth information on tuberculosis (TB), with available cure and justification for new set of drugs for TB.
- It also summarizes the aims and objectives of the study.

Chapter 3

- Describes the materials and methods used in conducting the research. The stages are: collection of plants, extraction, isolation and characterization of triterpenes.
- The synthesis of the molecular hybridization and co-crystals products were described as well.
- Biological study of the compounds: anti-TB, anti-Poly β and cytotoxicity.

Chapter 4

- Presents the result of the experiments without discussing the implications. The data is organized in the form of tables, figures, and images.

Chapter 5

- This is the discussion part of the data obtained in the study and emphasizes the interpretation of the overall findings.

Chapter 6

- This is the overall conclusion drawn from the study and provides suggestions for further studies.

Chapter 2
Literature review

2.1 Tuberculosis

Tuberculosis is an infectious disease that has been with human being for thousands of years (Haradhan 2015). The early part of the 20th century witnessed a reduction in the mortality rate of TB related diseases because of the presence of the set of TB drugs in the market today. The gains of the early 20th century thus led to the declined of interest of the developed countries to further research into the development of new drugs to tackle TB and its control programmes (Haradhan 2015). Other factors like the emergence of HIV and resistant strains of the causative bacterial lead to the reappearance of the disease today and has played a great role in the new status of the disease worldwide.

TB is now a global disease (Fig 2.0) and is regarded as second to HIV/AIDS as the greatest killer worldwide. With 8.8 million people infected by *M. tuberculosis* in the year 2012, it is estimated to kill someone approximately every 25 seconds resulting in the death of 1.3 million (Zumla et al., 2013a; WHO, 2013). It is estimated that nearly 1 billion people will become infected, 200 million will become sick, and 70 million will die between now and 2020. In recent time, sub-Saharan Africa is the worst hit with a dramatically rising incidence of TB, which is attributable to HIV/AIDS co-infections and emergence of resistance type of TB (Coxon et al., 2012).

TB is caused by *Mycobacterium tuberculosis* (MTB), which in most cases enters the air from an infected person through coughing, sneezing, talking and spitting of sputum outside etc. Once the organism is in the air, it is carried on droplets and spreads round

to a new host or uninfected person through any available airway like ear, nose, skin, and mouth (Soni et al., 2009).

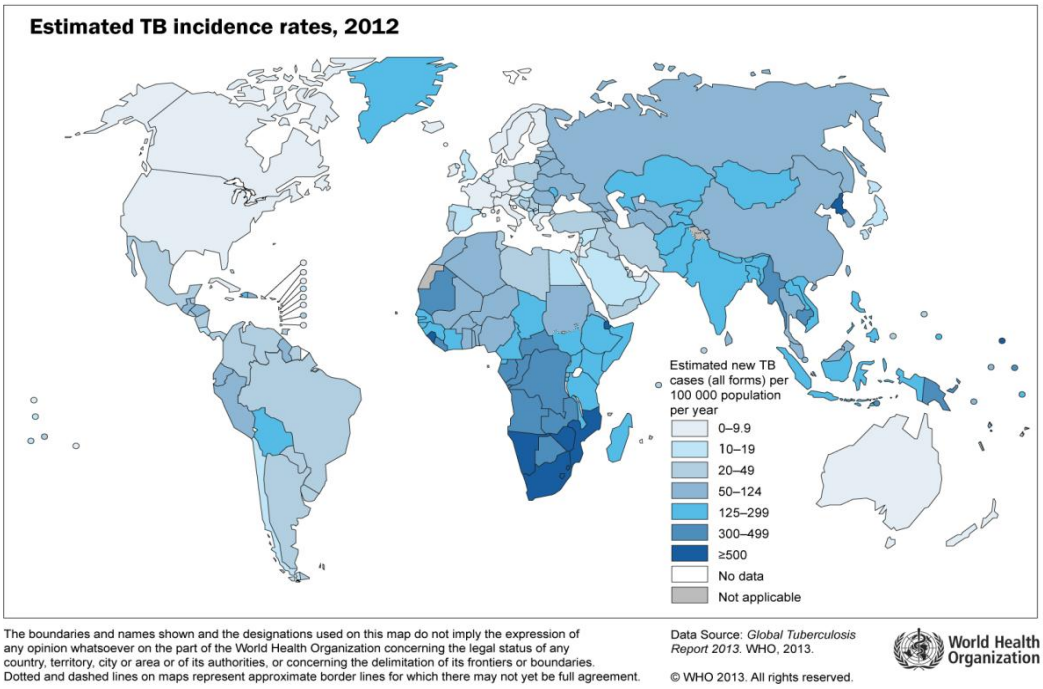

Fig. 2.0 Global Tuberculosis Report (WHO, 2013)

The initial stage of TB infection in most cases lasts for months, if not years, this depending on the immunity status of individual. The defense system of the body is therefore the determining factor as to when an individual will develop full blown tuberculosis disease that can easily be diagnosed after the first contact with the causative organism (Ma et al., 2010).

Mycobacterium tuberculosis (Fig. 2.1) is a large non motile rod –shaped bacterium. It is about 2-4 micrometers in length, and of about 0.2-0.5 micrometer in width (Welin, 2011). It thrives so well under moist aerobic condition, which is why TB develops faster in the lung than any other part of the human body because of the well-aerated upper

lobes of the lungs. The mycobacterium is therefore, a facultative intracellular parasite of macrophages, which has a slow generation time of about 15-20 hours, a physiological characteristic that aids its virulent nature(Welin, 2011).

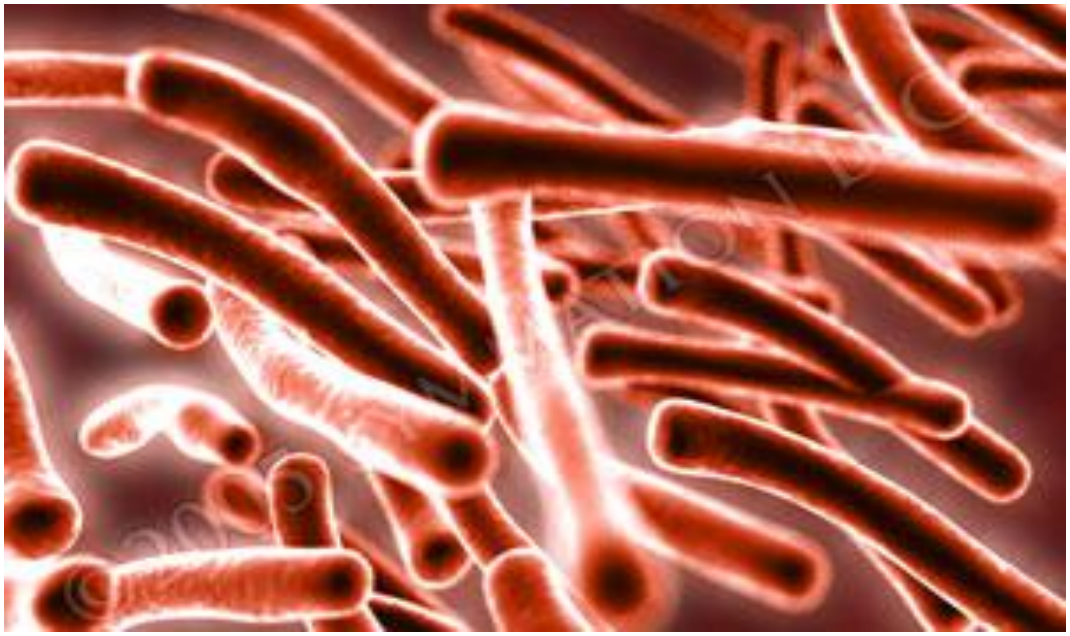

Fig. 2.1 Conceptual image of TB bacteria (Bryantfurlow, 2010)

M. tuberculosis (MTB) has a unique cell wall that is made of lipid (Fig.2.2). The lipid component of the bacterium is well above 60% of the mycobacterial cell wall (Khare et al., 2009). The lipid component of MTB's cell wall is made up of three major components, mycolic acid, cord factor and wax-D (Crick et al., 2001).

The mycolic acids component of the Mycobacterium tuberculosis is a very unique complex interwoven structure made of alpha –branched lipids in the cell walls of mycobacterium and corynebacterium. It constitutes up to 50% of the dry weight of the mycobacterial envelope (Crick et al., 2001). Mycolic acids are potentially strong hydrophobic molecules that constitute the main cell wall of the organism which enhance the permeability properties at the cell surface.

The high concentration of lipids in the cell wall of the *M. tuberculosis* has been discovered as the major factor for its virulent properties, and as such, rendering it impermeable to stains and dyes, resistant to many antibiotics, resistant to killing by acidic/alkaline compounds, and resistance to osmotic lysis via complement deposition (Ehebauer and Wilmanns, 2011).

Active TB disease present some signs and symptoms like slight fever, night sweats, weight loss, fatigue and various other symptoms, depending on the part of the body that is affected (Ehebauer and Wilmanns, 2011).

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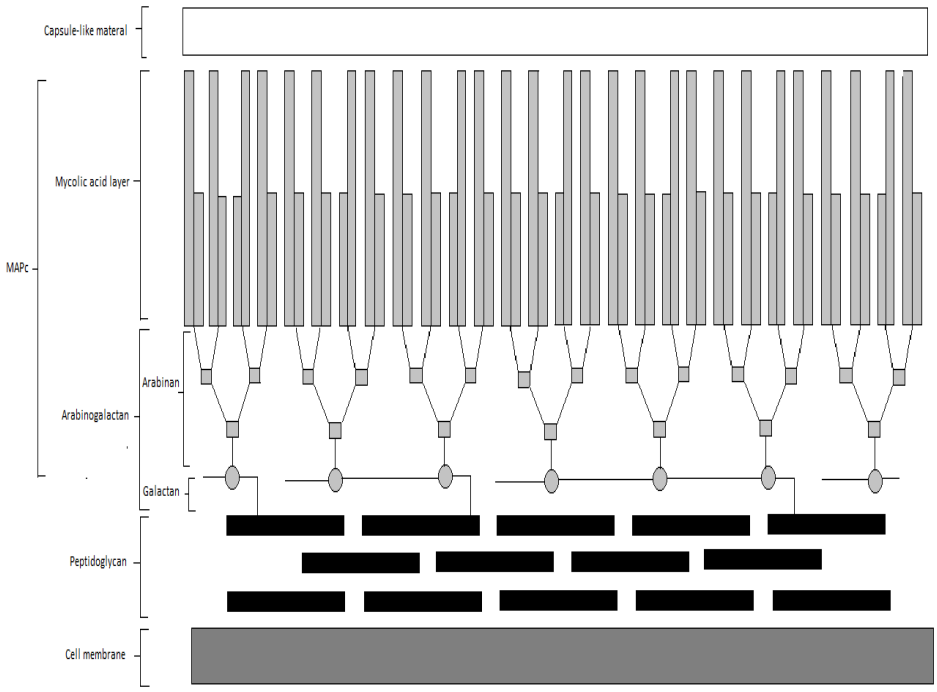


Fig.2.2 Schematic cross-section of the *M.tuberculosis* cell envelope (The model shows the mycolic acid- arabinogalactan- peptidoglycan complex) (Crick et al., 2001).

2.2 Economic implication of tuberculosis disease

The effect of TB pandemic is enormous. Apart from the high mortality rate, there is economic impact, which made the United Nations assembly, in its sixth millennium development goal meeting, to declare that there is also socio-economic impact of the devastating effect of the TB disease. The economic impact has been classified into world, country and family level in order to be able to examine the full implication of the scourge in human beings' life (Hossain et al., 2012).

TB has been discovered to have a firm hold, and will likely continue to rob the world's poorest countries of an estimated $1 to $3 trillion in the next 10 years unless immediate efforts are put in place to globally eradicate the disease (van der Werf et al., 2012). In all, the developing countries, most especially African and Asian countries are the worst hit, where about 94% of new TB cases and 98% of TB death rate occur. Out of this percentile, 75% of new TB cases are from the productive peer ages of 15-54 (Sharma et al., 2015).

Living in overcrowded environment which is common in most poor countries and this increase the chances of spreading and contacting TB, and this is the reason why poverty is a driving force in the management of tuberculosis. The most distressing recent report is that 2.7 billion people around the world sustain their living with $2 per day or less daily (Fonkwo, 2008; Hossain et al., 2012; Ataguba et al., 2010).

This amount is staggeringly inadequate to provide minimally acceptable living conditions. This corroborates the argument that the affected countries will lose an estimated 4-7 % of their GDP to TB (Bryde and Waheed, 2013).

Families are not left out, most especially in a typical endemic country, it has been reported that the burden will shift towards the women of the child rearing age within the next five years (Wampande et al., 2013). It is estimated that up to four million women will be lost to TB if there is no medical intervention in treatment of TB diseases (Tanimura et al., 2014). The annual estimate of the children that suffer from TB is estimated at 250,000 each year of which close to 100,000 will die (Bryde and Waheed, 2013).

2.3 Current tuberculosis chemotherapy

TB can be treated and possibly cured provided it is discovered early (Shehzad et al., 2013). The chemotherapy for the treatment of infectious diseases began shortly after the introduction of sulfonamide and penicillin for the treatment of bacterial infections and was later extended for the treatment of tuberculosis, though later discovered to be ineffective for its cure (Fallah and Abdolghafoorian, 2014).

From 1944 till date, more than twenty different types of anti-TB drugs have been introduced (Fig 2.3). Such drugs include streptomycin, *p*-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin [rifampicin] (1963). Aminoglycosides compounds such as capreomycin, viomycin, kanamycin and amikacin were later introduced and the very recent ones are the quinolones (e.g. ofloxacin and ciprofloxacin) which are strictly used in drug resistance situations (Kolyva and Karakousis, 2009; Manjunatha and Smith, 2014; Wong et al., 2013).

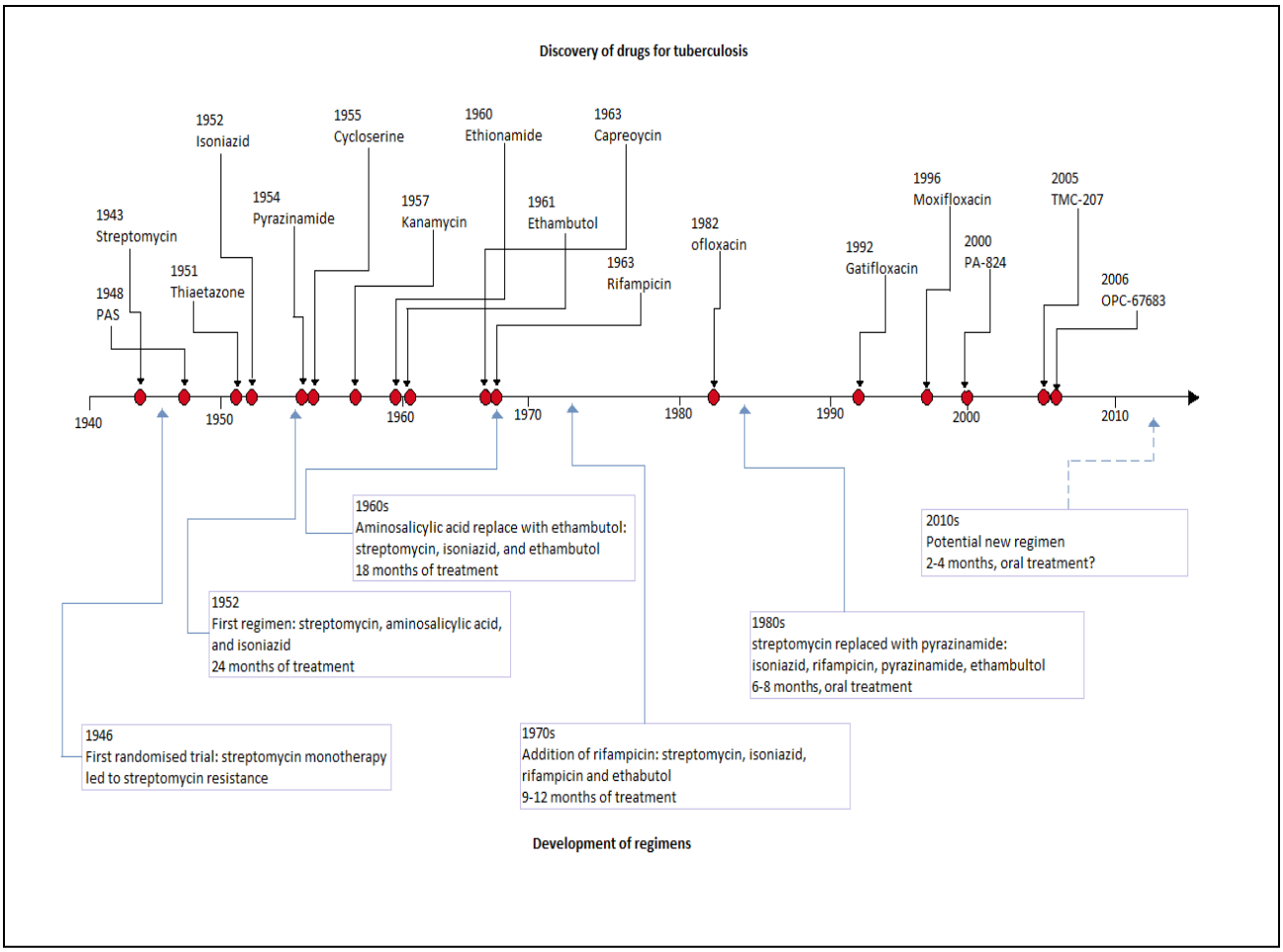


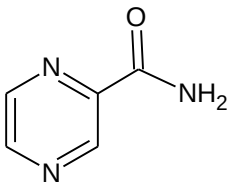
Fig. 2.3 Trends in discovery of drugs for tuberculosis (Ma et al., 2010)

The anti-TB drugs were divided as either a first-line or second-line. The first line serves dual purpose for prevention and treatment. While the second line drugs are used in the treatment. First lines are seldom used alone. When they are used alone, it is for prevention. When used in combination with other drugs, it is for curative purpose (Kolyva and Karakousis, 2009).

The five basic or "first line" TB drugs are (Table 2.4):

- Isoniazid
- Rifampicin
- Pyrazinamide
- Ethambutol

- and Streptomycin



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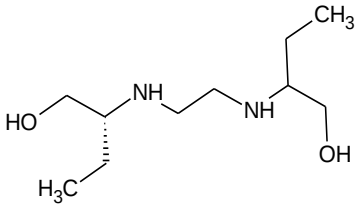

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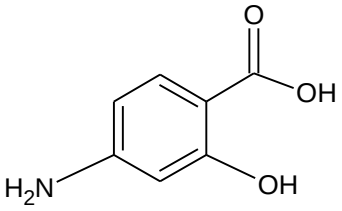

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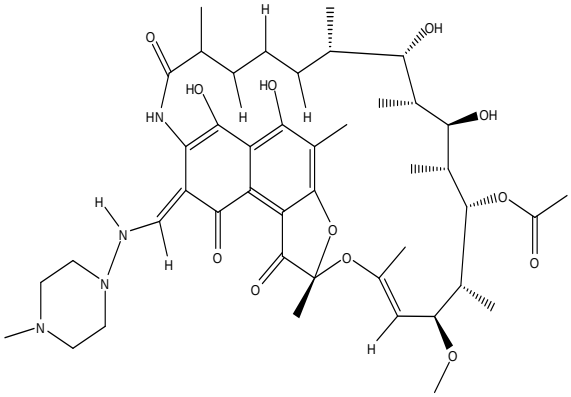

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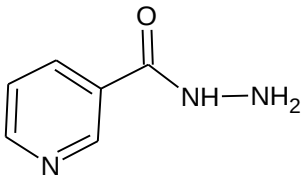

(3)

Fig. 2.4 Structure of the five basic tuberculosis drugs (Zumla et al., 2013)

(1, Pyrazinamide; 2, Streptomycin; 3, Isoniazid; 4, Ethambutol; 5, Rifampicin)

The anti-tuberculosis drugs have been further classified into synthetic and natural. The literature report indicated that close to 80% of the drugs were inspired by natural products and most especially of plant origin (Crag and Newman, 2013) (Table 2.0).

Table 2.0 Classification of the tuberculosis drugs into synthetic and natural (Ribon et al., 2012).

Drug (Year)	Source	Moeity/Pharmacophore	MIC (uM)	Class of TB drug
Streptomycin (1944)	Natural	Aminocyclitolglycosises – Amicocyclitol moiety glycosidially linked to a carbohydrate moiety.	1.72	First line
P- Aminosaliclic acid (1946)	Synthetic	Aminobenzoic acid derivative- Amine group attached to benzene moiety	1.9-6.5	Second line
Isoniazid (1952)	Synthetic	Pyridine carboxylic acids – Pyridine ring bearing carboxylic acid	0.182	First line
Pyrazinamide (1952)	Synthetic	Pyrazine carboxamides – Pyrazine ring which bears a carboxamide	490pH5.5	First line
Etheionamide (1956)	Synthetic	Pyridine analogue (ring) – six – member aromatic heterocyclic which consists of one nitrogen atom and five carbon atom	1.5	Second line
Kanamycin (1957)	Natural	Aminocyclitolglycosides – Amicocyclitol moiety glycosidially linked to a carbohydrate moiety	3.43	First line
Ethambutol (1961)	Synthetic	1,2- amino alcohol – Alkyl chain with amine group attached to C-l atom and alcohol group attached to C-2 atom	2.45	First line
Rifampicin (1966)	Semi-Synthetic	Naphthofuran-furan ring fused to naphthalene moiety	0.486	First line
Amikacin (1972)	Semi-Synthesis	Semi-synthetic amino glycoside derived from kanamycin	0.85-1.7	First line
Fluoroquinolones	Synthetic	Quinolone derivative – 4 – Quinolone moiety with 6-fluoro and 7 piperazine substituents	0.6-1.4	Second line

2.4 The need for new set of tuberculosis drugs

1. The current set of tuberculosis drugs are not potent enough to totally eradicate the disease due to the emergence of resistance strains (Bhowmi et al., 2009; Yew et al., 2011) . The failure to comply with the treatment regimen of these drugs, has led to the recent development and emergence of different resistant strains of TB such as MDR-TB and XDR-TB (Du Toit et al., 2006). In the early 2012, an outbreak of resistant TB was reported in India, which later spread to Iran and Italy. The "totally-drug-resistance strains (TDR-TB)" was only reported in South Africa in 2013 (Asif, 2012; Garg et al., 2014; Velayati et al., 2013).

2. Most of the current drugs have high toxicity profile level

The first line anti-tuberculosis drugs are known to be potent in the treatment of tuberculosis, but show some side effects like severe damage to the eighth cranial nerve, irreversible impairment of auditory function, hypersensitivity (streptomycin), hepatotoxicity and hepatitis (isoniazid, pyrazinamide and rifampicin, rifabutin, rifapentine) and thrombocytopenic purpura (rifampicin)(Yew et al., 2011).

The second line anti-tuberculosis drugs, are reportedly more toxic than first line drugs, and not as potent as the first line drugs in the treatment of tuberculosis (Shakya et al., 2012). Drugs such as amikacin and kanamycin are capable of damaging the kidney, and could cause loss of hearing after a prolong use. Viomycin and capreomycin cause nephrotoxicity and eighth cranial nerve toxicity. Fluoroquinolones, another versatile class of TB drugs (ciprofloxacin, moxifloxacin, ofloxacin, levofloxacin, gatifloxacin, trovafloxacin, enofloxacin, sparfloxacin etc) were introduced due to growing prevalence

of antibiotic resistance drugs (Yew et al., 2011). They cause gastrointestinal disturbances, headaches, skin rash and allergic reactions, seizures, hallucinations, tendon rupture, angioedema and photosensitivity (Shakya et al., 2012).

Ethionamide causes gastro intestinal tract disorders (anorexia, salivation, nausea, abdominal pain, and diarrhea), mental imbalance symptoms (depression, anxiety, psychosis, dizziness, drowsiness, and headache) and hypersensitivity (Shakya et al., 2012).

Cycloserine has serious side effects on the Central Nervous System (CNS) which have results into disorders like headache, irritability, depression, convulsions; while para - amino salicylic acid also causes gastro intestinal disorder problems which eventual develop to anorexia, nausea, epigastric pain, abdominal distress, diarrhea, ulcers and hypersensitivity (Shakya et al., 2012).

3. The regimens are too long for easy compliance (Sizemore et al., 2012).

4. Most of the current set of tuberculosis drugs are quite expensive and not commonly accessible (Tanimura et al., 2014).

2.5 Method for generation of new chemical entities as drugs

There are two common methods to generate new chemical entity for drugs viz: Target-based approach and phenotypic-based approach.

2.5.1 Target based

The target-based approach (Figure 2.5), which is sometimes called 'reverse pharmacology' or 'reverse chemical biology', starts with identification of an important enzyme relevant to a disease in question and then an inhibitor to the enzyme or

protein is then designed through the study of three-dimensional structural information of the target (Frank, 2005; Gilbert, 2013).

It is not fairly complex, as it involves the use of biochemical assays to assist in identifying relevant enzymes or proteins important to the survival of the disease in question(Hughes et al., 2011). These methods have been successful in drug discovery to some extent for various communicable (in particular viral) and non-communicable diseases(Gilbert, 2014).

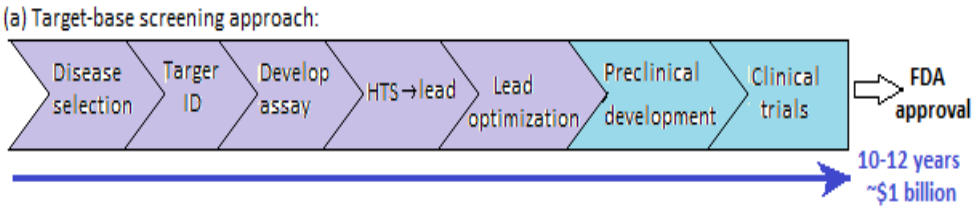

Fig. 2.5 Target-base screening approach

The failure rate sets in with most drugs discovered through this method via the emergence of resistance stains of disease and most especially TB drugs (Guzman et al., 2014).

2.5.2 Phenotypic-based approach

Phenotypic –based approach (Figure 2.6) is whole cell attack strategy on the diseases. It does not involve the identification of any enzyme or protein. The drug compound can be sourced synthetic or natural origin. What is important is that the compound must exhibit good activity profile against the whole organism. This approach ensures multiple targets action against the disease causing organism.

Phenotypic- based approach in drug discovery screening is a fast and straight forward method to discover relevant hits (Zheng et al., 2013).

The phenotypic-based screening takes place in a physiologically relevant environment of cells or whole organism, the resultant effect from such screens provide a more direct view of the desired responses as well as highlight potential side effects if any (Enna, 2014; Kotz et al., 2012). More importantly, phenotypic-based screening at times can lead to the multiple attack actions against multiple proteins/ enzymes or pathways that may not have been previously linked to a given biological output.

One of the sure advantages of phenotype-based approach is that it provides an unbiased way to find biologically active compounds in the context of complex biological systems (Enna, 2014). Recent statistical data reported that a substantial number of first-in-class drugs with novel mechanisms of action have been discovered from phenotypic-based screening leading to a resurgence of interest in this method that has earlier been abandoned for target –based (Enna, 2014).

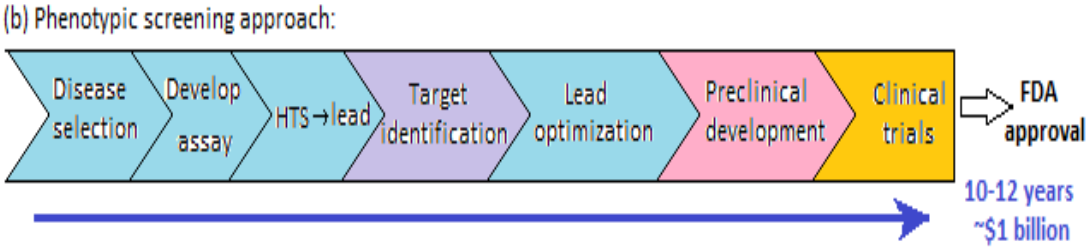

Fig. 2.6 Phenotypic screening approach

It has also been revealed in literature that most of all the antibiotics that are currently used as TB drugs and those on pipeline against TB have been developed based on their whole–cell activity (Guzman, et. al.,2014). Table 2.1 summaries the comparison of target based and *in vitro* phenotypic screens for lead discovery.

Table 2.1 Comparison of target based and *in vitro* phenotypic screens for lead discovery (Enna, 2014)

Features	Target-based screening		*in vitro* Phenotypic-based screening	
	Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
Molecular target of a disease	Known	Have to know	Do not need to know	Unknown
Screening throughput and assay	Higher; relatively easy to set up	Assay may be less biologically relevant	Medium or low; biologically relevant	Could be low; could have higher cost
Mechanism of action of lead compound	Known at onset, which can accelerate preclinical drug development	Limited possibility of identifying a new mechanism	Multiple targets and signaling pathways can be targeted; may involve native biological targets and complexes	Unknown at onset
Methods for confirmation of lead compound	Direct binding assay, modeling X-ray crystallography, or other biophysical methods	Need to be confirmed in cell-bases and phenotypic assays with native targets and complexes	Can move to *in vivo* study quickly	Target identification may be required; which can be complicated and time consuming
Methods for SAR optimization	Readily available and direct		Additional assays may need to support SAR	May need to develop a more targeted assay
Disease relevance of lead compound	Direct if it is relevant	Drug target may not be disease-relevant, as lack of human efficacy found in late-stage clinical trials	Usually disease relevant; may target more complex diseases	
Hypothesis limitation of lead compound		Limited by the hypothesis, simple	Less hypothesis-restricted	

2.6 Molecular hybridization method to generate new tuberculosis drug lead

Molecular hybridization is the combination of two or more pharmacophoric moieties from different bioactive substances to produce a new type of hybrid compound with different properties from the two parent compounds.

It is a common practice in drug discovery, done with the aim to increase efficacy. The new drug is expected to be better in physiochemical properties, adequate absorption, distribution, metabolism, and excretion, effective pharmacologic potency and lacking any toxicity than the old drug components (Dutra et al., 2012).

Molecular hybridization thus remains an important method in obtaining better drugs with better performance than individual drugs. This technique is gaining momentum in drug design and discovery, most especially for infectious diseases, particularly those that are easily prone to resistance strains which therefore require more potent drug for effective cure and treatment (Bosquesi et al., 2011).

2.6.1 The principle of molecular hybridization

The failure of one drug for a single target of the 1960s led to the rise of many diseases around which are difficult to treat today. The emergence of resistant forms of various diseases like tuberculosis, malaria, etc, is as a result of the fallout of such inadequate treatment. The challenge to design new drugs with better therapeutic and safety profile remain a challenge to be surmounted in view of the devastating effect of many human diseases in recent time (Medina-Franco et al., 2013).

The treatment of tuberculosis in recent time has defile simple methods of treatment, instead needing a more complex method of drug combinations. The strategy employed in tuberculosis treatment usually involves any of the followings:-

1. The use of more than one drug for the treatment. This method offers opportunity for multiple target attacks from the individual drugs. The only disadvantage of this method is that the compliance is extremely difficult to adhere to by the patient in most cases.

2. There is a fixed dose combination therapy with two or more drugs combined in a single tablet.

3. The hybridization of two biologically active molecules to form a single entity with multiple targets actions.

Designing of a single drug with multiple actions in the treatment of tuberculosis has been identified as a better method to treat the disease as well as in assisting to address the shortcomings of the current set of tuberculosis drugs viz

- Therapy compliance.
- More potent drugs than the existing with good safety profile.
- Reduced resistance tendencies of the new drugs.

The molecular hybridization concept can be illustrated by using drug A and drug B, as shown diagrammatically below. Figure 8, below represents different situations in the design a new drug (Mitra, 2012). The drug design of the hybrid compound must consider any of the three different parameters as necessary (Figure 2.7):

- The desired subunits (drug A and drug B) are linked by a spacer agent;
- Both subunits (drug A and drug B) are linked without spacer agent, and are fused;
- The desired activities from drug A and drug B are merged in a new structure.

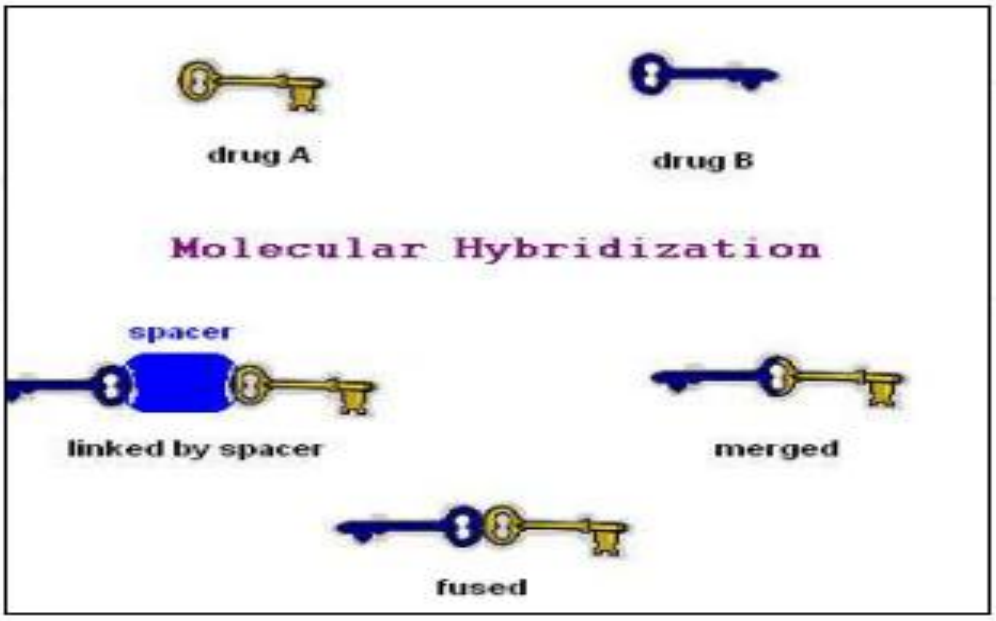

Fig. 2.7 Schematic diagram illustration of molecular hybridization between two compounds A and B(Leandro and Dutra et al., 2012)

2.6.2 Tuberculosis drugs obtained through molecular hybridization

A typical example using this strategy is found when isoniazid is hybridized with quinolone derivative as shown in Fig. 2.8

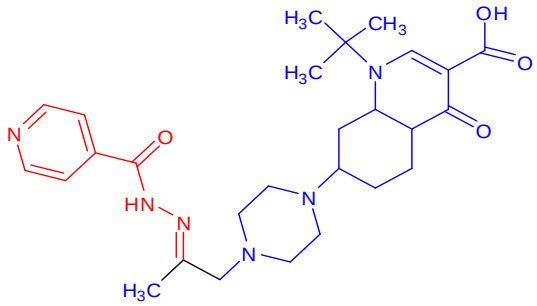

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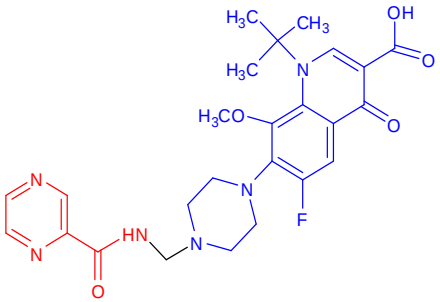
Fig. 2.8 Molecular hybridization between fluorquinole(blue skeleton) and isoniazid (red skeleton)(Shindikar and Viswanathan, 2005).

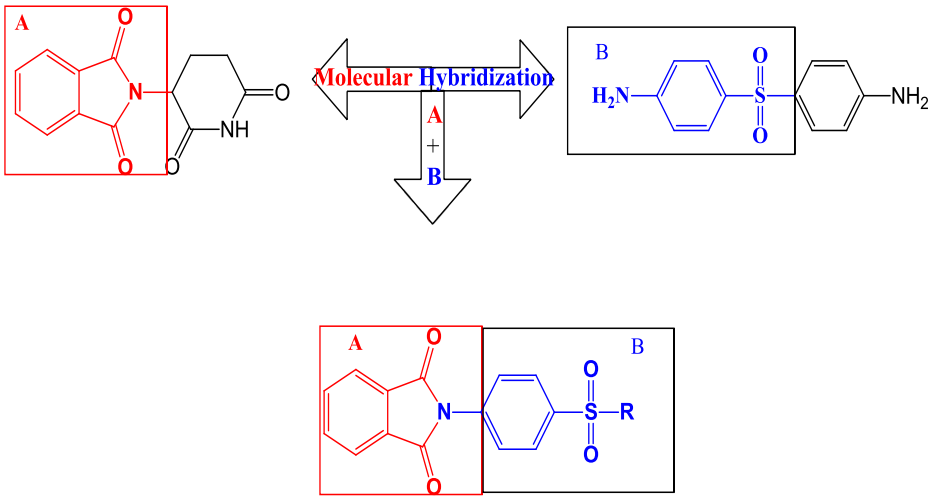


(7)

Fig.2.9 Molecular hybridization between fluorquinole (blue skeleton) and pyrazinamide (red skeleton) Sriram et al., 2006).

The two hybrid compounds (Fig 2.8 and 2.9) had better activity and were well tolerated by patients. The hybrid product from molecular hybridization, in most case does have better activity than the parent compounds because of the synergistic effect from the constituent compounds.

Lastly, the molecular hybridization between thalidomide and dapsone (Figure 2.10) showed good activity against *Mycobacterium tuberculosis* (Yamasaki et al., 2014).



(8)

Fig. 2.10 Molecular hybridization of dapsone with thalidomide.

2.7 Ancient tuberculosis drug- cinnamic acid

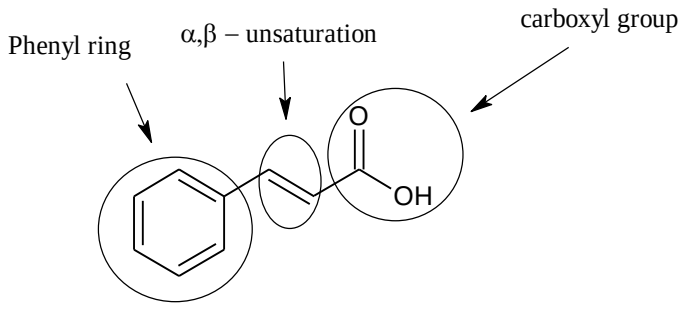
Cinnamic acid (CA), with the formula $C_6H_5CHCHCO_2H$, is a white crystalline compound that is lightly soluble in water, and freely soluble in many organic solvents. CA has melting point of 133°C, and boiling point 300°C.

They occur naturally in fruits, vegetables, flowers and several medicinal plants and can be synthesized in the laboratory as well (Gupta and Wakhloo, 2007; Sharma 2011). They have numerous biologically activities such as, anti-diabetic, antioxidant, antimicrobial, as fragrance materials, hepatoprotectvity, CNS depressant, anti-cholesterolemic, antifungal and fungitoxic, anti-hyperglycemic, anti-malarial, antiviral, anxiolytic, cytotoxic, anti-inflammatory, UV rays absorbent and anti-TB (Sharma 2011; Guzman , 2014).

The biological activity of cinnamic acid and its derivatives have been reported to depend mainly on three pharmacophores present in it (Figure 2.11), namely:-

- Carboxylic acid,
- α, β-unsaturation, and
- Phenyl ring.

The existence of the phenyl and α, β-unsaturation are necessary conditions for the anti-tubercular activity (De et al., 2011). The carboxylic acid functional group, offers the most convenient and excellent reactive site of this compound with other biologically active molecules through molecular hybridization(Lone and Koul, 2014). The substituent attached to the phenyl and α, β-unsaturation may increase or decrease the overall biological activity exhibited by cinnamic acid and its derivatives(Bosquesi et al., 2011; Dutra et al., 2012; Yempala et al., 2012).



(9)

Fig. 2.11 Structure of cinnamic acid pharmacophore

When cinnamic acid and its derivatives like ester, amide and aldehyde react with other compounds through the carboxyl acid group, it generates michael acceptor moiety easily(De et al., 2011).

Cinnamic acid is one of the few organic molecules that have paraded an excellent pharmacophore that is very useful in pharmacy and medicine. It can be easily hybridized with other organic acids to form hybrid compounds entirely different in properties from the parent compounds(Guzman, 2014).

Out of all the biological properties of cinnamic acid and despite the fact that it has been used as TB drug in the time past, the antituberculosis properties have not been properly harnessed, and therefore it has attracted the attention of scientific community in recent time (Balta et al, 2011; Guzman, 2014).

2.8 Co-crystallization synthesis in drug discovery and designing

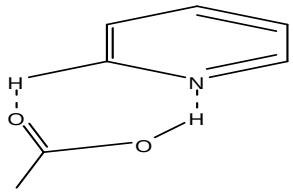
Co-crystallization is solid crystals that are held together by weak intermolecular forces. Most organic solid compounds contain molecules which are held together by intermolecular forces such as hydrogen bond that limit or restrict their mobility; the

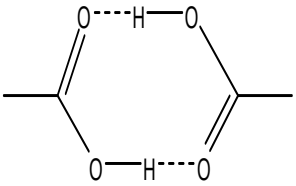
assembly of these molecules in solids creates single entities which are termed super molecule (Brown, 2012).

The structural arrangement in the crystal is usually determined by the sizes, shape, and functionalities of the constituent's organic molecules (Battle and Atkinson, 2011).

Co-crystal compound gained prominence in recent time in organic chemistry through the concept of "supramolecular synthons," The supramolecular synthons provides the ability to fine tune a compound's physical properties without altering its chemical identity. It has a lot of applications in pharmaceutical industry most especially in co-crystal systems formation. It has also been extensively used to improve several physical properties of a number of Active Pharmaceutical Ingredient (API), such as solubility, bioavailability, thermal stability etc (Brown, 2012).

In a co-crystal system, covalent bonding remains the major determining force between the molecules held in crystal. The covalent bond in the co-crystal system is the hydrogen bonds; and non-covalent bonds are van der Waals forces and π-π interactions. There are two types of synthons: homosynthon and heterosynthon (Figure 2.11). The homosynthon consist of the self complementary functional groups, while the heterosynthon is composed of complementary groups that differ in functionality (Bs, 2009; Sekhon, 2012; Steed, 2013).





Heterosynthon(a) **Homosynthon(b)**

Fig.2.12 Schematic representation of complementary hydrogen-bonded supramolecular homo- and heterosynthons

2.8.1 Co-crystal synthesis and application

A co-crystal can be prepared by dissolving the solid components in a solution and allowing co-crystallization via slow evaporation or sublimation. The solution-based crystallization has been reported to provide a well-defined and highly-ordered crystal formation (Muller et al., 2000).

The other method of preparation is mechanochemistry, which is the act of grinding or milling solids to induce the formation or breaking of a chemical bond (Konthur et al., 2012). Typically, the grinding is carried out using a mortar and pestle or an automated ball and mill. Substantial increases in heat and pressure are exerted on the solids. For a co-crystal formation, non-covalent interactions are, thus, formed and broken thus, there is no chemical transformation. Moreover, given the significance of co-crystals and obtaining these highly useful materials with little or no use of solvent makes the materials appealing from numerous perspectives(Konthur et al., 2012).

Discovery and development of new drugs has been identified as time consuming, expensive and highly risky venture (Zheng et al., 2013). Drug repositioning and improvement therefore provide alternative method to discover more new potent drugs

(Sekhon, 2013; Mahata et al., 2014). Co-crystal synthesis may therefore offer better opportunity to develop more potent new drugs, since the new compound is formed without altering its chemical identity. The ultimate goal of the application of co-crystal synthesis is to improve on one of the properties of the existing active pharmaceutical ingredients (API) for better drug like properties.

Table 2.2: Recent case studies of pharmaceutical co-crystals (Mahata et al., 2014)

API	Conformer	Preparation methods	mproved property
Piroxicam	Saccharin	Solvent evaporation	Physical stability
Carbamazepine	Nicotinamide, saccharin	Cooling crystallization	Physical stability, Dissolution rate and oral Bio-availability
Sulphamethazine	Aspirin, Benzoic acid, Trimethoprim, 4-Amino salicylic acid	Solvent evaporation	Physical stability
Indomethacin	Saccharin	Solvent evaporation or solvent assisted grinding	Physical stability and dissolution rate
Theophylline	Nicotinamide	Solvent evaporation	solubility
Caffeine	Maleic acid, Glutaric acid	Solution mediated phase transfer technique	Physical stability
Acetaminophen	Pyridine-2,4-dicarboxylic acid	Solution mediated phase transfer technique	Physical stability
Sulphamethazine	Theophylline	Solvent evaporation	Physical stability, Spectroscopic and X-ray diffraction properties

Evora et al., 2011 reported a 1:1 co-crystal synthesis involving pyrazinamide, an important TB drug with diflunisal, anti-inflammatory substances for the first time. When co-crystal compound of pyrazinamide with diflunisal were used in the treatment of tuberculosis, pains in large and small joints which are one of the major side effects of these drugs were reduced. Before the advent of these co-crystal compounds, the side

effects, which are the pains, were better managed by prescription of pyrazinamide along with aspirin. Aspirin is a strong analgesic which has its own side effect. The side effects from both drugs therefore constitute a serious problem. The co-crystal drug combination of pyrazinamide with diflunisal therefore offered a better treatment than ordinarily using pyrazinamide in the treatment of tuberculosis.

Vijayaraj et al. (2013) reported the co-crystal synthesis of para-amino salicylic acid with isoniazid, a second line and first line TB drugs. A clinical trial investigation of this co-crystal compound reported a better synergistic effect than taking the drugs individually in most cases (Schultheis and Newman, 2009).

Ge et al. (2010) reported that the combination of OA with isoniazid (INH), rifampicin (RMP) or ethambutol (EMB) showed favourable synergistic antimycobacterial effects.

Co-crystal synthesis has many practical applications in pharmaceutical industries in the development of target molecule with improved physical and chemical properties (Table 2.2).

2.9 Isoniazid

Isoniazid (INH, Pyridine-4-carboxyhydrazide) **(3)** is a foremost first line drug used in the treatment of tuberculosis (Vijayaraj et al., 2013). It is used either with other TB drugs or alone. In view of previous reported literature (Babu et al., 2013), it has ability to form supra molecular interactions with carboxylic acid in 1:1 stoichiometric ratio because of the presence of the pyridine ring in the INH **(3)** (Babu et al., 2013). Like most other TB drugs such as rifampicin and pyrazinamide, isoniazid induces serious hepatotoxicity and made compliance difficult when treating tuberculosis (Kumar et al., 2013).

2.10 Medicinal plants and drug discovery

Medicinal plants are made up of primary and secondary metabolites as the main constituents which are present in various amounts (Ramawat et al., 2009). The secondary metabolite components of most medicinal plants possess the observed biological activity (anti-malarial, anti-cancer, anti-tuberculosis etc.) in them (Medina-Filho, 2006). The large number of secondary metabolites makes the screening and separation of the medicinal plants into their various components a tedious task which involves a lot of methodology and technique which vary from plant to plant (Ramawat et al., 2009).

2.10.1 Isolation and characterization of active components

Extraction is the first and most important step in the analysis of medicinal plants constituents for drug discovery (Sasidharan et al., 2011). The most common methods and procedures for extraction are the conventional ones like soxhlet extraction, sonification and maceration (Table 2.3).

Table 2.3: Common methods of extraction from plant materials (Sasidharan et al., 2011)

	Soxhlet extraction	**Sonification**	**Maceration**
Common solvent used	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water
Temperature (^{O}C)	Depending on the solvent used	Can be heated	Room temperature
Pressure applied	Not applicable	Not applicable	Not applicable
Time required	3-18 hrs depending on the material	1 -5 hrs depending on the material	7 days
Volume of required (ml)	Depend on the starting material	Depend on the starting material	Depend on the starting material

The other methods of extractions; the non-conventional methods include microwave, ultrasound, supercritical fluid, pressure liquid, hydrotropic and enzyme assisted extractions are also used (Sasidharan et al., 2011). The choice of solvent further helps to fractionate the plant material into non-polar, weakly –polar, medium-polar and highly-polar fractions (Cosa et al., 2006).

The process is achieved in two ways, the first approach is to use either a single universal solvent like methanol or methanol / water (80:20) to extract these four classes of compounds together and later partition them sequentially, or the plant materials are extracted sequentially with all the four different solvents of increasing polarity e.g. n-Hexane is used for extraction of non-polar components, chloroform is used to extract weakly polar compounds, ethyl acetate is used to extract medium polar compound and methanol is used to extract highly polar compound as shown in Figure 2.14.

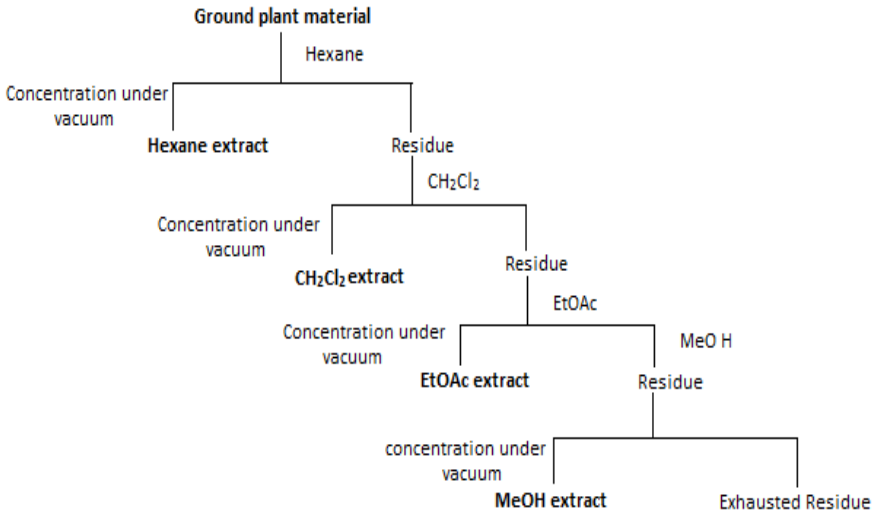


Fig. 2.13 Extraction method in plants material in order of increase polarity (Brusotti et al., 2013).

The isolation of the biologically active compounds comes after the extraction. The isolation depends on the profile of the crude extract under consideration. Plant crude extract is usually a combination of different types of biologically active compounds and different separation techniques such as TLC, column chromatography and HPLC are usually employed to obtain some of the compounds in pure form (Sasidharan et al., 2011). The compounds are then subjected to structural and biochemical characterization study (Figure 2.15).

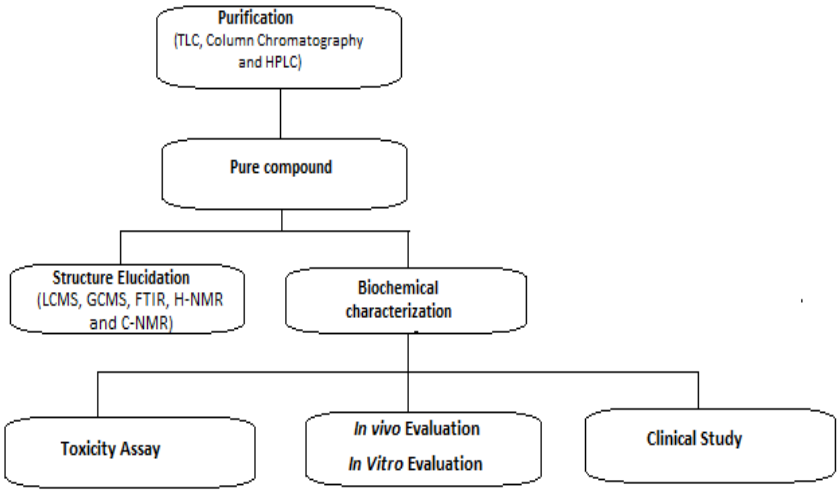


Fig. 2.14 Genera method of isolation and characterization of biologically active compounds from plant materials (Sasidharan et al., 2011).

The information obtained from the biological and chemical screening exercise is what actually gives each plant its constituent profile which is then used to validate the use of the plants in traditional medicine (Hostettmann, 1999). Records have it that, there are still many plants that have not been investigated for biologically active compounds. Considering the fact that a plant may contain thousands of constituents, this may

translate into high chance of discovering new molecules upon proper and exhaustive investigation of the plants (Cosa et al., 2006; Sasidharan et al., 2011).

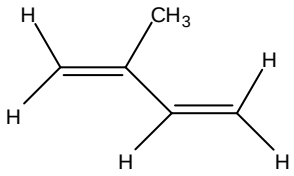
At least, not less than 13 classes of compounds have been isolated from plants among which are alkaloid, anthraquinone, cardiac glycoside, flavonoid, phenol, phlobatannin, Pyrrolizidine, alkaloid, reducing sugar, saponin, steroid, tannin, volatile oils and terpenoids. Almost all these classes of the compounds have been tested and some have been found active against tuberculosis in crude form (Ramachandra and Balasubramanian., 2014).

2.11 Terpenoids—As source of anti-tuberculosis drugs

Terpenoids are important class of natural products which are made up of several units of isoprene as the building block such as monoterpenoids which has two (2) units of isoprene. The numbers of the isoprene present are used to subsequently to classify this class of natural products (Ramawat et al., 2009). The isoprene's have a general formula $(H_5H_8)n$ as shown in Table 2.4.

Table 2.4: Classification of Terpeniods

Terpenes	Isoprene unit	Carbon atom
Monoterpenoids	2	10
Sesquiterpenoids	3	15
Diterpenoids	4	20
Sesterterpenoids	5	25
Triterpenoids	6	30
Carotenioid	8	40
Rubber	>100	>500



(10)

Fig. 2.15 Structure of a unit of isoprene

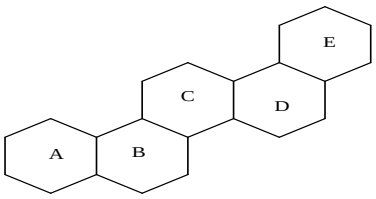
Terpeniods exist in nature in large amounts and they have been reported to show numerous ranges of biological activities against cancer, malaria, inflammation, tuberculosis (Wang et al., 2005). Several members of the terpeniod groups along with their derivatives and structural analogues have been studied extensively and have been reported to show moderate antimycobacterial activity that varies from 1 to >128 µM (Table 2.5) (Cantrell et al., 2001; Arya, 2011).

Table 2.5: Anti-tuberculosis activity of terpeniods (Wachter et al., 1999)

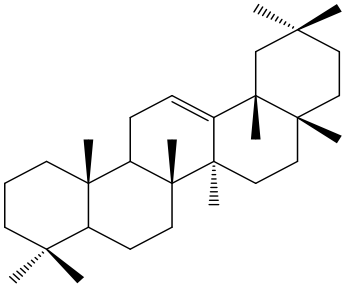
Compound	Chemical name	MIC value(μM)
1	Rifampin	0.3
2	Zeorin	8
3	Lupeol	64
4	7β-acetyl-22-hydroxphopane	>128
5	7β,22-dihydroxhopane	>128
6	Oleanolic acid (**17**)	64
7	Erythodiol	64
8	Ursolic acid	32
9	Uvaol	32
10	Betulinic acid (**15**)	32
11	Betulin	32
12	Epi-betulinic acid	64
13	Lupeol acetate	>128
14	Lupenone	>128
15	3-hydroxynorlupen-20-one	>128
16	3-acetoxynorlupen-20-one	>128
17	Friedelinol	>128
18	Pomolic acid(**25**)	64
19	Pomolic acid acetate	32
20	Tormentic acid**(26)**	>128
21	2-epi-tormentic acid	>128
22	Euscaphic acid	128
23	Niga-ichigoside F1 aglycone	>128

2.11.1 Pentacyclic triterpenes: Scaffold for the synthesis of anti- tuberculosis drug lead

Pentacyclic, acyclic and tetracyclic triterpenes are the three main members of triterpenoids, with pentacyclic triterpene (PT) as the only five member rings configuration (Fig 2.17) (Hostettman and Marston, 1995). The pentacyclic triterpenes (PT) are further divided into three main groups, the oleanane, ursane and lupane (Figure 2.17) (Alqahtani et al., 2013).

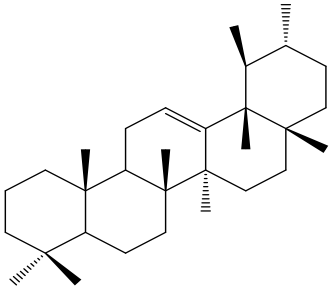


(11)



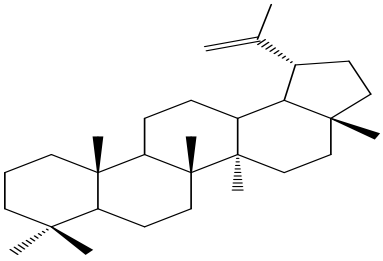
(12)

Oleanane



(13)

Ursane



(14)

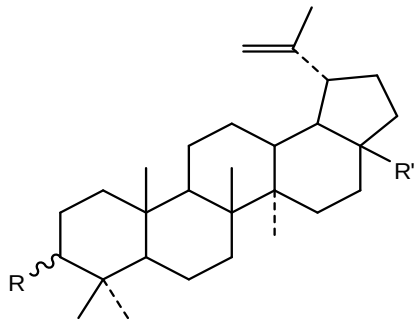
Lupane

Fig. 2.16 Basic structure of the main bioactive pentacyclic triterpenes

Pentacyclic triterpenes (PT) exist in the plant and animal kingdoms. They are reported to possess wide spectrum of biological and pharmacological actions because of the intricate nature of their structure (Sheng and Sun, 2011).

All the three classes of PT skeleton have C=C in C^{12}-C^{13} in oleanane, ursane and C^{20}-C^{30} in lupane. The acid form of PT has OH and COOH at C^{3} and C^{28} position in addition to the C=C to form the Pentacyclic triterpene acid (PTA) which is the stable form of PT in nature and as such do exist in various amounts in a large number of plant and medicinal plants (Alqahtani et al., 2013).

Most PTAs undergo interesting chemical reaction as a result of which they serve as templates in the synthesis of many of the potent drugs we have around in recent time (e Silva et al.,(2012)**.** Previous investigations on the antimycobacterial potentials of PT compounds indicated that they exhibit moderate to high *in vitro* antimycobacterial activity against *M. tuberculosis* (Wachter et al., 1999). It was reported that betulinic acid (**15**) was more active than its C-3 epimer, epi-betulinic acid (**16**). (Wachter et al., 1999).

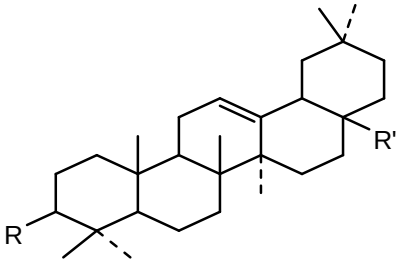


(14)

15: R =β-OH, R'=COOH,

16: R=α-OH, R'=COOH,

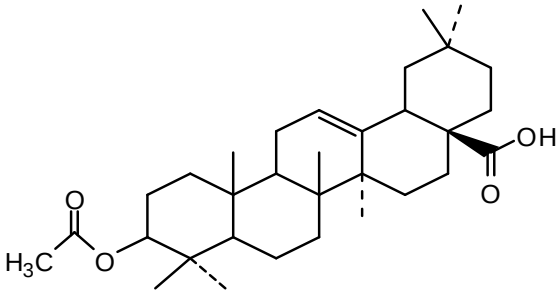
Oleanolic acid **(17)** was 4-fold less active than its C-3 epimer, 3-epi-oleanolic acid (**18**) whereas the acetate derivative (**19**) of compound (**17**) was as active as its parent compound (**17**) (Wachter et al., 1999). The 3-keto analogue (**20**), of oleanonic acid (**17**), exhibited an equivalent MIC to that of 3-epi-oleanolic acid (**18**). (Wachter et al., 1999).



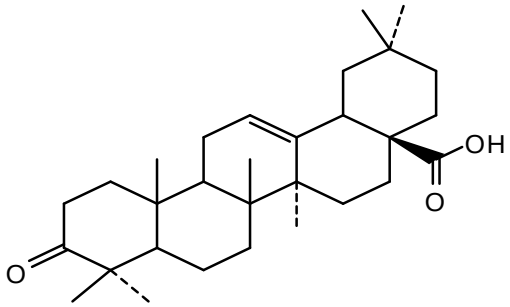
(12)

17: R = β-OH, R'=COOH

18: R= α-OH, R'=COOH



(**19)**



(20)

The presence of an ester function at C-22(**21)** of compound (**17**) resulted in a 2-fold decrease in activity (Wachter et al., 1999)

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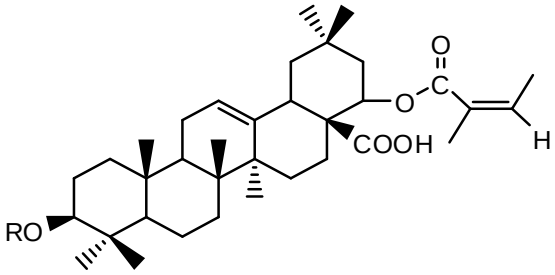
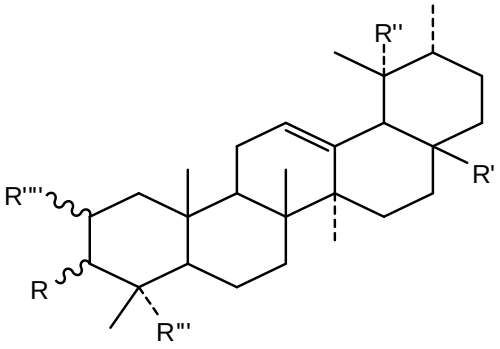


21: R=H,

The structural isomer of oleanolic acid (**17)**, ursolic acid **(22)** presented another interesting biological activity on the introduction of the hydroxylic functionality at the C^{-19}, and C^{-2} position respectively to give pomolic acid (**23**) and tormentic acid (**24)**, resulted in an equivalent or a 2-fold decrease in antimycobacterial activity (Valcic et al., 1997).

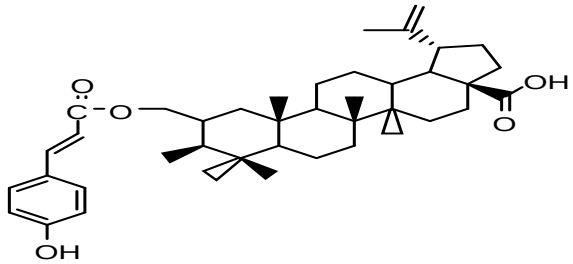


22: R= β-OH, R´= COOH, R´´= H, R´´´= Me, R´´´´= H

23: R= β-OH, R´= COOH, R´´= OH, R´´´ = Me, R´´´´= H

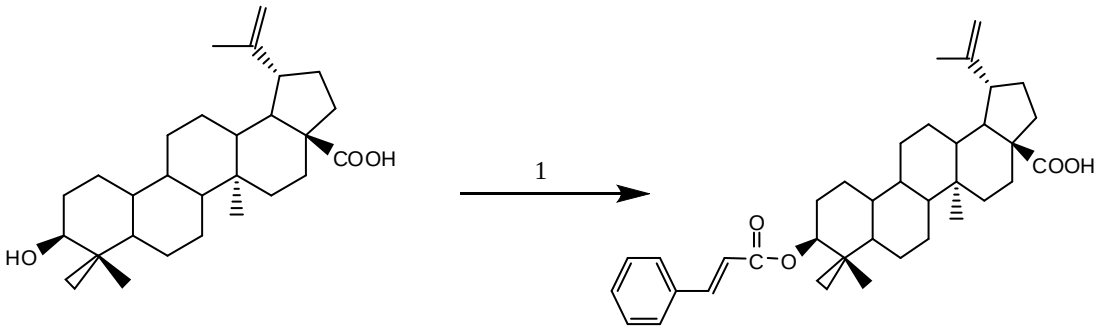
24: R= β-OH, R´ = COOH, R´´= OH, R´´´= Me, R´´´´= α-OH

Wachter et al., 1999, further concluded that modifications of any of the rings (A, B, C, D and E) could produce an appreciable effect in their antimycobacterial activity of this group of compounds. Most of the results observed for the various groups of triterpenes compounds are not straight forward as expected and therefore difficult to predict the structural requirements responsible for its antimycobacterial activity from this class of compounds. To buttress this and in the course of literature review, it was observed, that the presence of a p-coumarate moiety at the C-2 hydroxyl group of alphitolic acid (2a-hydroxybetulinic acid) (**25)** resulted in increased antimycobacterial activity (Tanachatchairatana et al., 2008).



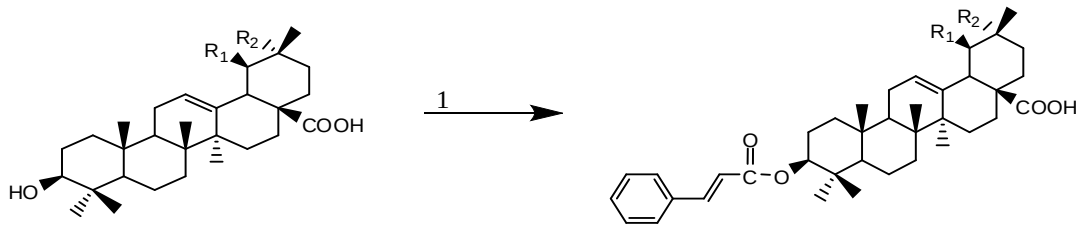
(25)

Similarly, when the triterpenic acids, betulinic acid **(15),** oleanolic acid **(17)** and ursolic acid **(22)** were modified by the introduction of the cinnamoyl moiety to replace hydroxyl group at the C-3 position, there was a loss in antimycobacterial activity when compared with the parent compound betulinic acid (**15)**, oleanolic acid (**17**) and ursolic acid (**22**) (Tanachatchairatana et al., 2008).



(15) **(26)**

Fig. 2.17 Synthesis of cinnamate-based ester of betulinic acid (**15**) Reagents and conditions: (i) RCOCl / DMAP/ Benzene, 60^{O} C, 0.5-1h (ii) 10%, K_2CO_3 / MeOH, 30 min (Tanachatchairatana et al., 2008).

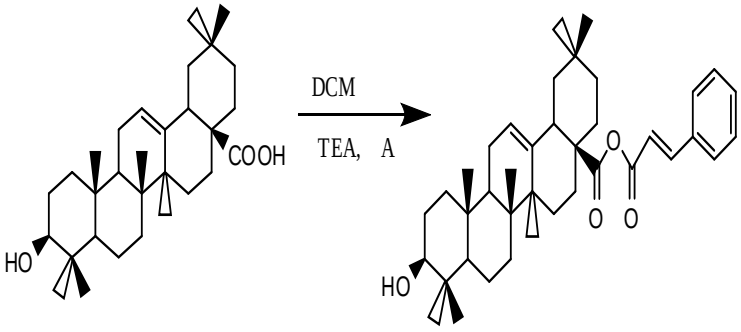


(17) R_1=H, R_2 =Me **(27) R_1=H, R_2 =Me**

(22) R_1=Me, R_2=H **(28) R1=Me, R_2=H**

Fig. 2.18 Synthesis of cinnamate-based esters of oleanolic acid (**8**) and ursolic acid (14), Reagents and conditions: (i) RCOCl / DMAP/Benzene, 60^{O} C, 0.5-1h (ii) 10%, K_2CO_3 / MeOH, 30 min (Tanachatchairatana et al., 2008).

In a similar manner, when 28-cinnamic acid hybrid of oleanolic acid **(29**) was synthesized from molecular hybridization from oleanolic acid (**17)** and cinnamic acid (**9),** it was observed that the hybrid product was more potent as an anti-malarial agent than chloroquine.



(17) **(29)**

Fig. 2.19 Synthesis of the cinnamic hybrid of oleanolic acid at C-28 position, Reagents and conditions: (i) RCOCl / TEA/DCM, rt, 24h (ii) 10% K_2CO_3 / MeOH, 30min (Habila et al., 2011).

2.12 Medicinal plants used in this study

Several medicinal plants have been reported to bioaccumulate triterpenes (Bishayee et al., 2011), however *Curtisia dentata* and *Syzigum aromaticum* will be the reference study plants for the isolation of the triterpenes under consideration in this study. An ethnobotanical survey conducted by this researcher recently, indicated that the two plants are used in the prevention, management and treatment of respiratory ailment particularly in tuberculosis infected patients in rural areas of KZN in South Africa, this is in addition to other various reported uses in literatures.

2.12.1 *Curtisia dentata*

Curtisia dentata (Burm.f.) C.A.Sm, locally named Assegaai (Afrikaans) and umLahleni (Xhosa, Zulu); umPhephelelangeni (Zulu) and mufthefhera (Venda) in South Africa; IiliNcayi, IsiNwati in Swaziland; modula-tshwene in northern Lesotho, belongs to the *Cornaceae* family. Geographically, the plant (Figure 2.21) in South Africa is found in the forest of Limpopo, Kwazulu-Natal, Eastern and Western Cape provinces. It has extended into the eastern Zimbabwe, Mozambique and Swaziland (Yembaturova et al., 2009).

Curtisia dentata, is an attractive woody and medium-sized ever green tree with dark like square patches at the stem bark. The leathery leaves are glossy, ovate and smooth; the plant's height above the soil level is about 10 cm (Yembaturova et al., 2009). In documented literature, the herbal utilization of this plant is well documented to include, sexually transmitted infections, stomach ailments, diarrhea, blood purifier, aphrodisiac, heart-water, diabetes and obesity (Cunningham, 1988; Huntchings et al., 1996). It has attained a status of endangered species in the area and is mostly found in reserve area of the forest because of bark harvesting for medicinal plant trade (Ndawonde, 2006).

Curtisia dentata had previously been reported to bioaccumulate four pentacyclic triterepene namely lupeol, betulinic acid, ursolic acid and 2α- hydroxursolic acid which may be responsible for the reported antibacterial and antifungal activity of the leaves of this plant (Shai et al., 2008; Fadipe et al., 2015).

Fig. 2.20 Leaves of *Curtisia dentata* (Picture taken by Mr John Burrows from Buffelskool Private Nature Reserve in Mpumalanga province, South Africa, in March 2014

2.12.2 *Syzygium aromaticum*

Syzygium aromaticum, commonly known as *"Cloves"* is an evergreen tree of the family Myrtaceae. Its flower bud (Figure 2.22) is used as a spice in India (Aparna et al., 2014). Besides being used as a condiment in foods, cloves may be used in the treatment of toothache, mouth, malaria, tuberculosis, cholera and throat inflammation (Bhowmik et al, 2012). The extracts and essential oils from this plant species are reported to possess antifungal activity against *Penicillium citrinum, Paecilomycetes variotii* and *Aspergillus niger* (Aiko and Mehtam 2013; Yazdanpanah and Mohamadi, 2014, Avasthi et al., 2010; Khewkhom and Shangchote, 2009) and antibacterial activity against some food and human pathogens which include; *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, Shigella *spp*, *Vibrio spp*, *Pseudomonas aeruginosa* and *Clostridium*

botulinum (Saeed et al., 2013; Dua et al., 2014; Srivastava et al., 2014). Its extracts are also known to contain antioxidant, anti-viral, larvicidal, anti-platelet, anti-stress, anti-pyretic, anesthetic, anti-cancer anti-inflammatory, anti-diabetic, anti-nociceptive and insecticidal activity amongst other pharmacological activities (Cortés-Rojas et al., 2014; Milind and Deepa, 2011).

Several compounds have been isolated from *S. aromaticum* namely 5, 7-dihydroxy-2-methylchromone-8-C--D-glucopyranoside, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid, orsellinic acid glucoside , oleanolic acid (Cai and Wu, 1996).

Fig. 2.21 Cloves bud (Picture supplied by Mr Ishamel Mongalo, Department of Botany, University of Zululand. South Africa)

2.13 Scope of the work

2.13.1 Aim

This study aims to design, synthesize and biologically evaluate the antimycobacterial activity of plant-derived betulinic acid (**15**), oleanolic acid (**17)** and their derivatives.

2.13.2 Objectives

- Collection and authentication of the plant materials.
- Extraction, isolation, purification and characterization of betulinic acid (**15)** and oleanolic acid (**17**).
- Synthesis of the acetate analogue of the isolated betulinic acid (**15)** and oleanolic acid (**17**).
- Synthesis of the cinnamic acid hybrid of the betulinic acid (**15)**, oleanolic acid (**17**) and their acetate.
- Evaluation of the antimycobaterial potentials of the betulinic acid (**15)**, oleanolic acid (**17**), and their acetate.
- Evaluation of the antimycobacterial potentials of the cinnamic acid (**9)** hybrid of betulinic acid (**15)**, oleanolic acid (**17**), and their acetate.
- Evaluation of the pol β inhibition of the cinnamic acid (**9)** and acetate hybrid of betulinic acid (**15)** and oleanolic acid (**17**).
- Cytotoxicity study on the betulinic acid (**15)**, oleanolic acid (**17**), and their acetate.
- Cytotoxicity study on the cinnamic acid (**32)** hybrid of betulinic acid (**15)**, oleanolic acid (**17**), and their acetate.

- Synthesis of the INH **(3)**-triterpenic acids betulinic acid **(15),** and oleanolic acid (**17**).
- Evaluation of the antimycobacterial potential of INH **(3)** –triterpenic acids betulinic acid (**15)**, oleanolic acid (**17**) co-crystals.
- Cytotoxicity study on the INH **(3) -** triterpenic acids betulinic acid (**15)**, and oleanolic acid **(17)** co-crystals.

Chapter 3

Materials and methods

In this chapter, the materials and methods used in this study are described.

3.1 Techniques and equipment for the isolation and synthesis

3.1.1 Column chromatography

Silica gel 60 (0.04 -0.063 mm; 230-400 mesh ASTM) supplied by Merck (Darmstadt, Germany) and columns of various sizes.

3.1.2 Thin layer chromatography

All isolated compounds were monitored and analyzed by TLC (F254-Merck, Whitehouse Station, NJ, USA) separation using different solvent systems to establish their purity and determine RF values. The developed plates were visualized under UV light, sprayed with H_2SO_4 and MeOH (80:20) solution and then scanned.

3.2 Physical characterization

3.2.1 Melting point determination

In addition to the state and colour of the materials isolated and synthesized, along with the melting points of the compounds were determined on a Stuart Scientific SMP3 apparatus.

3.3 Structural elucidation

Structure elucidation of pure isolates was carried out using spectroscopic techniques such as infra-red (IR) spectroscopy, ultra-violet (UV) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy (1D), and HR-MS.

3.3.1 Nuclear magnetic resonance (NMR)

^{1}H, ^{13}C NMR spectra were recorded on a Bruker Avance instrument operating at 400 MHz, Chemical shifts are reported as δ values (ppm) relative to an internal standard of tetramethylsilane (TMS) or to the solvent line of $CDCl_3$ ($^{\delta}H$ = 7.26 ppm, $^{\delta}C$ = 77.16 ppm).

3.3.2 Mass spectrometry (ES-MS)

The mass data of the isolated and synthesized compound were obtained using a Bruker micro TOF-Q II ESI instrument operating at ambient temperature. About 1.0 mg of the compounds was submitted for the analysis at Pietermaritzburg Campus, University of Kwa-Zulu Natal, South Africa.

3.3.3 Infrared spectroscopy (IR)

The infra-Red (IR) spectroscopy determination was carried out using Perkin Elmer Spectrum 100 FTIR spectrometer.

3.4 Techniques and equipment for the characterization of the co-crystals Compound

Unless otherwise stated the techniques and some equipment used in this section are similar to those stated above (Section 3.1).

3.4.1 Thermal analysis

Thermogravimetric analysis (TGA) measures the amount and rate of change in the weight of a material as a function of temperature or time in a controlled atmosphere. TGA are used to determine the composition of materials and to predict their stability at temperatures u to 1000 ^{O}C. TGA was carried out at 10 ^{O}C/min heating rate using a

Perkin-Elmer Pyris 6 TGA from 30 ^{O}C to 900 ^{O}C in a closed perforated aluminum pan and were ran under nitrogen gas at flow of 20 mL/min.

3.4.2 X-ray diffraction technique

X-ray diffraction (XRD) is a versatile, non-destructive method that reveals detailed information about the chemical composition, crystallographic and micro structure of materials. X-rays are electromagnetic radiation similar to light but with a much shorter wavelength (few angstrom). They are produced when electrically charged particles of sufficient energy are decelerated. In an X-ray tube the high voltage maintained across the electrodes draws electrons towards a metal target (the anode). X-rays are produced at the point of impact and radiate in all directions. Rays of wavelength comparable to the crystal lattice spacing are strongly scattered (diffracted). Analysis of the diffraction pattern allows for the obtaining of information such as lattice parameter, crystal structure, sample orientation, and particle size,

The lattice parameters are obtained from the Bragg equation (Sharma et al., 2012)

$$2\,d\sin\Theta = n\lambda;$$ where d is the lattice spacing.

In a typical set-up, a collimated beam of X-rays is incident on the sample. The intensity of the diffracted X-rays is measured as a function of the diffraction about the atomic basis. The sharpness and shape of the spots are related to the perfection of the crystal.

Powder diffraction patterns were recorded in the high angle 2Θ range of 20-70^{o} using a Bruker AXSD8 diffractometer equipped with nickel filtered CU Kα radiation (λ=1.5418 Å) at 40 kV, 40 mÁ and at room temperature.

3.4.3 Scanning electron microscope

Scanning Electron Microscope (SEM) uses a focused beam of high –energy electron to produce different types of signals based on the surface topography of solid specimens. The signals observed are as a result of the interaction between the electrons and the constituent in the sample provided information such as the external morphology (texture), chemical composition, and crystalline structure and orientation of the materials make up the sample. In most applications, data are collected over a selected area of the sample surface and a 2-dimensional image is generated that display spatial variations in these properties. The total area of about approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20 X to approximately 30,000 X, spatial resolution of 50 to 100 nm).

A ZEISS ULTRA plus FEG-SEM at 5 Kv with In Lens capabilities using nitrogen gas and ultra-high-resolution BSE imaging was used for the study.

3.5 Experimental

This study involved the isolation of betulinic acid **(15)** and oleoanic acid **(17)** from *Curtisia dentata* and *Syzyigum aromaticum* respectively**.** The two organic acids were then reacted with cinnamic acid **(9)** and INH **(3)** in a controlled reaction system.

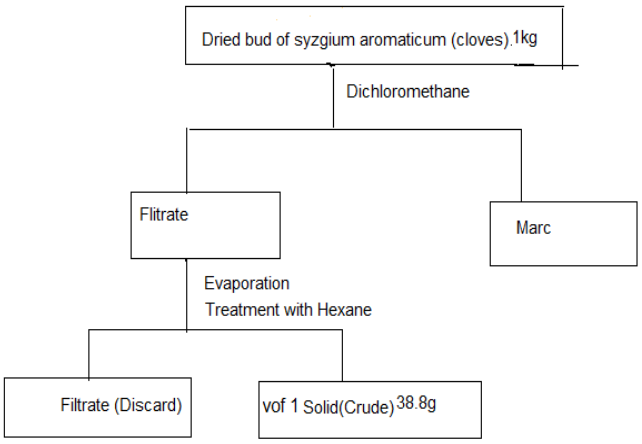
3.5.1 Plants collection

(a) The *Syzygium aromaticum* (Cloves) was purchased from an Indian spices shop in Richards Bay, South Africa. It was properly authenticated by a qualified Botanist, Mr Ishamel Mongalo, Department of Botany, University of Zululand. South Africa. Voucher specimen is deposited at the University Herbarium.

(b) Fresh leaves of Curtisia *dentata* (3 kg) were collected from Buffelskloof private nature reserve in Mpumalanga province (South Africa) in March 2014. The plant was authenticated and identified by Mr John Burrows, Botanist / Reserve Manager, Buffelskloof Private Nature Reserve and a voucher specimen (B.C.Turpin-2062) was deposited in the Herbarium of the Buffelskloof Private Nature Reserve, Mpumalanga, South Africa.

3.5.2 Isolation of oleanolic acid (17) from *S. aromaticum*:

The dried bud of *Syzigum aromaticum* (1 kg) was macerated in dichloromethane (2 x), filtered and concentrated *in vacuo.* The combined dichloromethane extract was then defatted using n-hexane (2 x) and the solid residue was collected (38.8 g). The defatted material (1 g) was dissolved in ethyl acetate (5 ml), packed into a silica gel column (65 cm / 4 cm) and eluted with a gradient of ethyl acetate: n-hexane (0:10), and ethyl acetate: n-hexane (3:7) ratio to afford white amorphous solid (879.23 mg) which was further re-crystallized in methanol to give vof1(799.25 mg) (Figure 3.1)



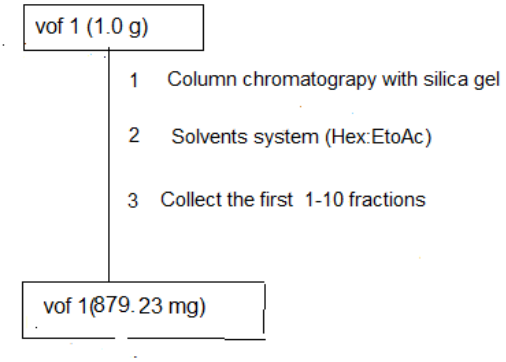


Fig.3.1 Isolation of oleanolic acid **(17)** from the Cloves

3.5.3 Extraction and isolation of betulinic acid (15) from *C. dentata*

The extractions were done in two parts:

1. The air-dried leaves of *Curtisia dentata* (50 g) was extracted repeatedly (3 x) over 7 days with ethanol (5 L) at room temperature. The combined ethanolic extracts were concentrated *in vacuo* to thick syrup, which was suspended in water and sequentially partitioned with hexane, methylene chloride and acetone (Table 3.1).

Table 3.1: Partitioning of the extracts into various solvents

S/N	Fractions	Dry weight(g)
1	n-Hexane	8.8
2	Methylene chloride	9.2
3	Acetone	6.4
4	Ethanol	17.4

2. The air- dried leaves of *Curtisia dentata* (1.0 kg) was extracted repeatedly (3 x) for 7 days with ethanol at room temperature. The combined extracts were concentrated to dryness under reduce pressure in a rotary evaporator (40±2°C). Dried extracts (104.5 g) were cleaned up to obtain clean ethanol extracts (CEE 81.2 g) as shown diagrammatically in Figure 3.2.

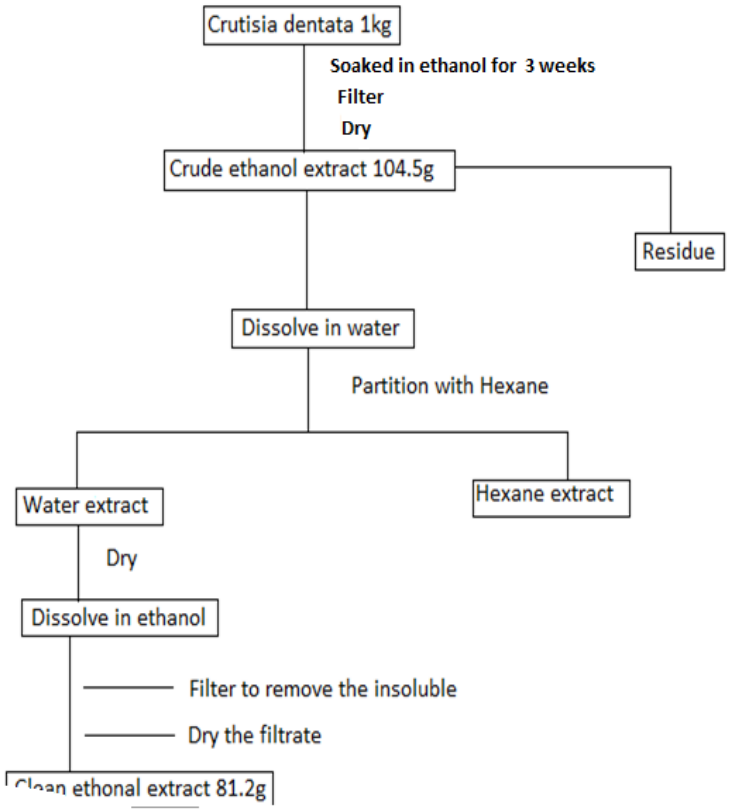


Fig.3.2 Isolation of betulinic acid **(15)** (from *Curtisa dentata* leaves)

Dried clean ethanol extracts (CEE; 2 g) were subjected to column chromatograph (40.5 X 530.5 mm) using silica gel 60 (180 g, 0.04 -0.063 mm; 230-400 mesh ASTM) supplied by Merck (Darmstadt, Germany). The clean ethanol extracts were chromatographed using a gradient elution of n-hexane-ethyl acetate in a 10% increase and collecting 80 mL fractions. Twenty-five (25) fractions were collected and monitored based on their TLC (F254-Merck, Whitehouse Station, NJ, USA) spots. Visualization was achieved by UV light (254 nm) and spraying with 20% H_2SO_4 acid in MeOH followed by heating in the oven (250 °C.). Crude compound vof2 (89.34 mg) was obtained from fractions 7-11 as single spot and was re-crystallized until a clean neat single spot was obtained as

pure vof2 (210.54 mg). In like manner at 30% increase of solvent system n-hexane: ethyl acetate vof 3 (154 mg) was obtained as one spot from fractions 23-28. This was further re-crystallized using methanol.

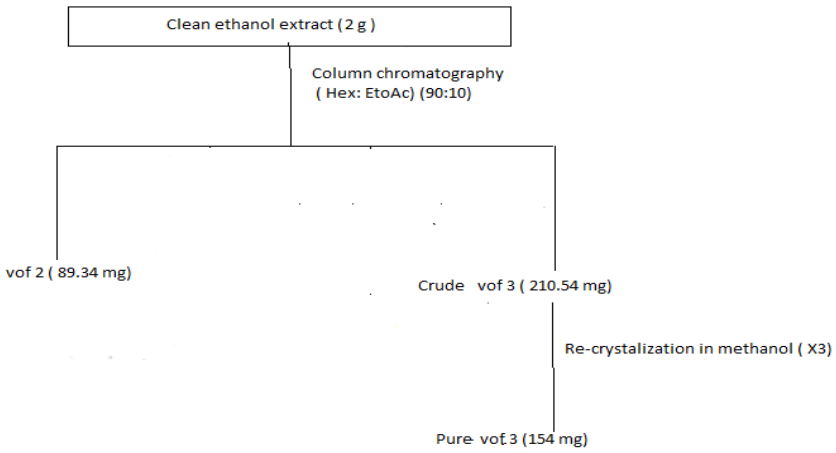


Fig. 3.3 Purification of betulinic acid (**15)** from clean ethanol extract

The % yield and % purity for isolated and synthesized compounds were calculated using the formula below.

$$\% \text{ Yield} = \frac{\text{Actual mass obtained}}{\text{Calculated mass}} \times 100$$

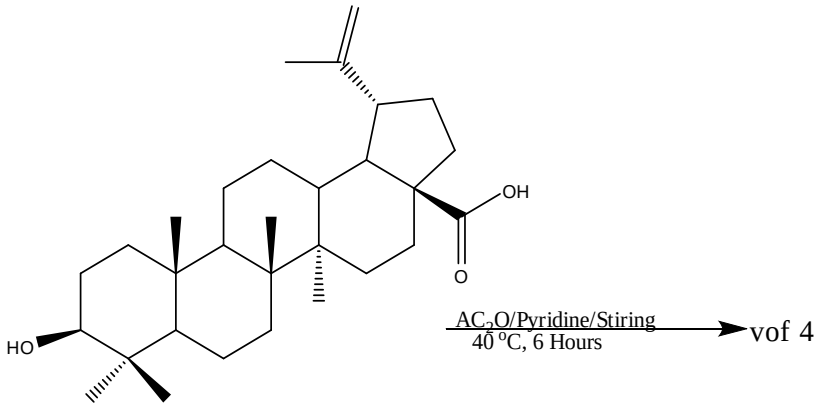
$$\% \text{ Purity} = \frac{\text{Mass of pure product}}{\text{Mass of impure product}} \times 100$$

3.6 Design and synthesis of 3-O-acetyl of betulinic and oleanolic acids

Betulinic acid **(15)** and oleanolic acid **(17)** are naturally occurring pentacyclic triterpenes with broad spectra of pharmacological activities viz, anti-HIV, antibacterials, antimalarial, anti-inflammatory, anthelmintic, antioxidant and anticancer (Yogeeswari and Sriram, 2005). Despite these biological activities, their medicinal uses that have been hampered by limited solubility (0.02 mg/mL) (Ahmad et al., 2010). This factor invariably makes it difficult to make injectable formulations from them, and subsequently decrease their bioavailability in human body (Ahmad et al., 2010). Transdermal formulation may as well be difficult to achieve. The introduction of a polar group like acetate ester is expected to aid the observed biological activity common with this class of compound most especially the antimycobacterial activity (Gauthier et al., 2008; Thibeault et al., 2007).

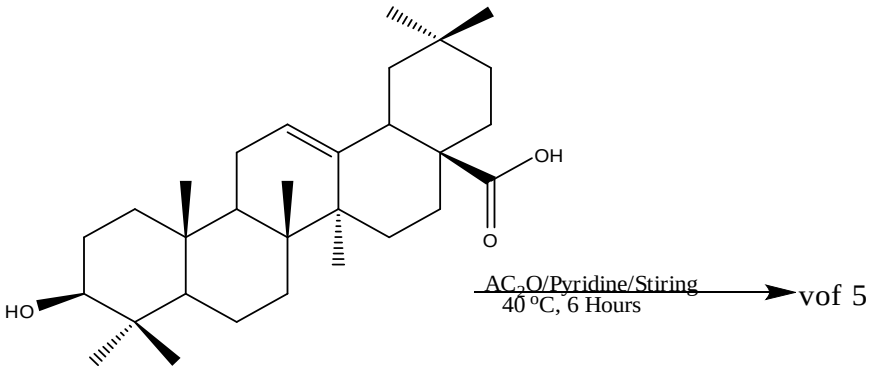
3.6.1 Preparation of 3-O-acetyl of betulinic (Vof 4) and oleanolic (Vof 5) acids

The preparations of the 3-O-acetyl derivatives of betulinic acid **(15)** and oleanolic acid **(17)** were similar: The two acids, betulinic / oleanolic (250 mg each) were separately mixed with acetic acid anhydride (10 ml) and pyridine (3 ml) and stirred at 40°C for 6 hours. The mixture was transferred into water and stirred for 1 hour at room temperature for hydrolysis. It was then filtered and diluted with hydrochloric acid (10%) to remove any traces of pyridine. The residue was dried, packed into a small column, and eluted with ethyl acetate: n-hexane (1:9) to afford a crude white amorphous powder-vof4 (BAA, 175.57 mg) / vof5 (OAA, 184.38 mg), and on re-crystallization in methanol to give a white pure form vof4 (112.24 mg) / vof5 (137.12 mg) (Ahmad et al., 2010).



(15)

Fig. 3.4 The synthesis of vof 4 (Ahmad et al., 2010).



(17)

Fig. 3.5 The synthesis of vof 5 (Ahmad et al., 2010).

3.7 Design and synthesis of 28-cinnamic acid hybrid of betulinic and oleanolic acids

The aim of this study was to validate the synthesis of the 28- cinnamic acid hybrid of oleanolic acid **(17)** earlier reported (Habila et al., 2011), and use the same synthetic methodology to synthesize the 28-cinnamic acid hybrid of betulinic acid for the first time

and then evaluate both products for antimycobaterial activity. This will hopefully provide an insight into the viability of the hybridization at C-28 position.

The reaction is a two-step synthesis:-

The first step is the conversion of the cinnamic acid to its chloride. The reaction is moisture and water sensitive and the rate of the reaction depends on it. Water and any traces of moisture were avoided completely.

The second step will involve the coupling of the cinnamic acid chloride with the betulinic acid (BA) **(15)** and oleanolic acid (OA) **(17).**

3.7.1 The synthesis of cinnamic acid chloride

Cinnamic acid (1.0 g) **(9)** was transferred to a 500 ml round bottom flask; thionyl chloride ($SOCl_2$, 3 ml) was added and refluxed for 2 h. It was then distilled to remove the excess $SOCl_2$, DCM (1.0 ml) was then added and the mixture concentrated *in vacuo* at 40°C to remove any trace of $SOCl_2$ (Habila et al., 2011; Jitareanu et al., 2013).

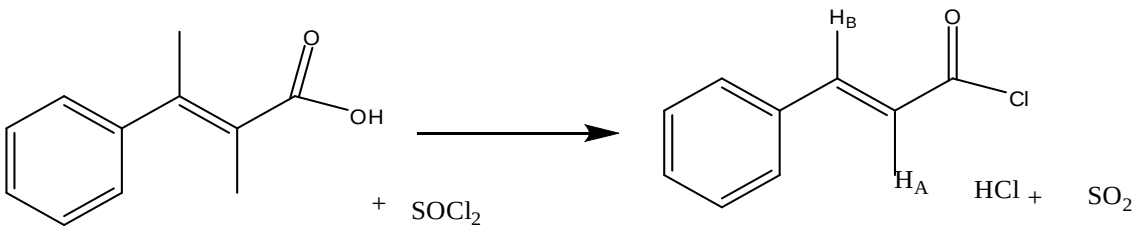
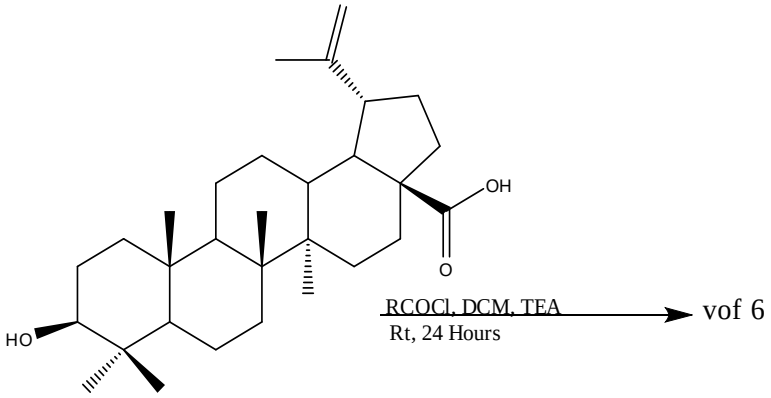


Fig. 3.6 Synthesis of cinnamonyl chloride (Habila et al., 2011; Jitareanu et al., 2013).

3.7.2 Synthesis of 28-cinnamic acid hybrid of betulinic and oleanolic acids

The two acids, betulinic and oleanolic (0.10 g each) were separately transferred to a beaker containing DCM (7 ml), and triethylamine (TEA, 1.0 ml). The mixture was stirred and then introduced to the content in the round bottom flask containing 0.10 g of the cinnamonyl chloride (already prepared in 3.7.1 above). This mixture was then stirred at room temperature for 24 h, after which it was diluted with 4 ml of DCM and was washed three times with 50 ml of distilled water (Habila et al., 2010 and 2011). The organic layer was separated and dried over anhydrous sodium sulphate (Na_2SO_4), and concentrated under pressure at 40°C. The final product vof 6 (95.83 mg) and vof 7 (97.23 mg) were re-crystallized using methanol to give a white amorphous powder vof 6 (CA-BA; 89.67 mg) vof 7(CA-OA; 85.57 mg).



(15)

Fig.3.7 Synthesis of 28-cinnamic acid hybrid of betulinic acid using -Habila et al., 2010 and 2011 synthetic protocol

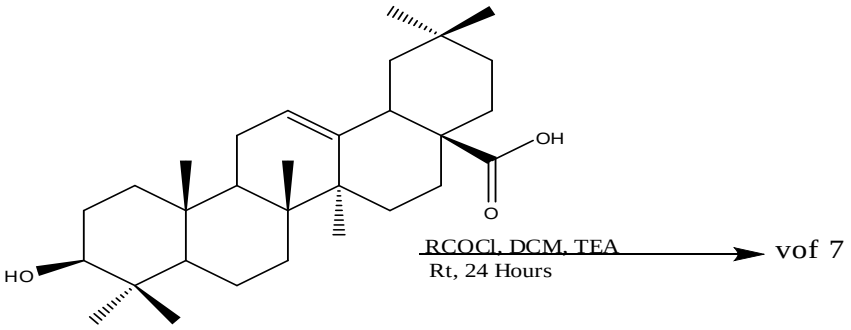


Fig. 3.8 Synthesis of 28-cinnamic acid hybrid of oleanolic acid **(31)** (Habila et al., 2010 and 2011).

3.8 Design and synthesis of 3- O- acetyl and 28-cinnamic acid hybrid of betulinic and oleanolic acids

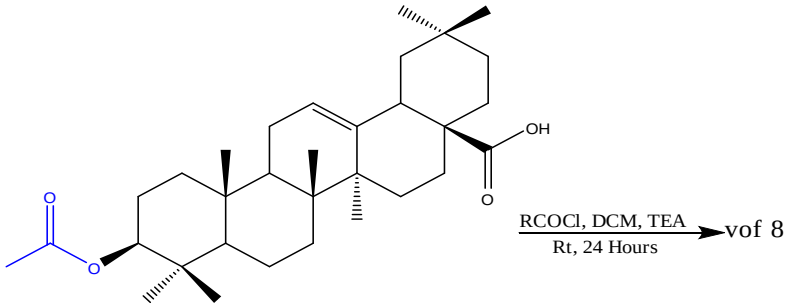
The idea to synthesis the cinnamic acid derivative of the ester of pentacyclic triterpene was conceived to test the effect of the disubstituted acetate at C-3 and cinnamonyl moiety at C-28 position on the betulinic and oleanolic acid on the antimycobacterial properties of the acids.

The hypothesis is to synthesize the cinnamic acid derivatives of the ester of oleanolic and betulinic acids and evaluate them for anti-tubercular activity. The design and chemistry is the coupling of the cinnamate moiety to position C-28 of the ester of the two pentacyclic triterpenic acid. This will unravel the structural variation along with the empirical anti-mycobacterial biological activity.

3.8.1 3- O-acetyl -28-cinnamic acid hybrid of betulinic and oleanolic acids

3-O-acetyl BA and OA (0.1 g each) were separately transferred to beakers containing DCM (7 ml), Triethylamine (TEA, 1.0 mL), and the stirred mixture was then subsequently introduced to the contents in the round bottom flask containing (0.1 g) of

cinnamonyl chloride already prepared in 3.7.1 above. This was then stirred at room temperature for 24 hours after which it was diluted with chloroform (4 mL) and washed three times with double distilled water (3 x 50 mL). The organic layer was separated and dried over anhydrous sodium sulphate (Na_2SO_4) and concentrated under pressure at 40 ˚C. The final product vof 8 (95.47 mg) and vof 9 (93.40 mg) was purified in column chromatography using n-hexane:ethyl acetate (9:1), until a cream white powder vof 8 (88.12 mg) and vof 9 (79.34 mg) was obtained.



(19)

Fig. 3.9 Synthesis of 28-cinnamic acid derivative of 3-O-acetyl-oleanolic acid

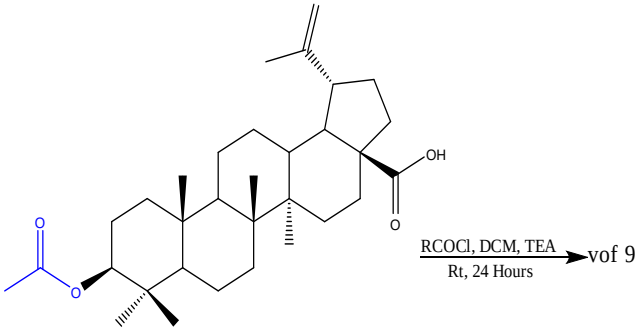


Fig.3.10 Synthesis of 28-cinnamic acid derivative of 3-O-acetyl-betulinic acid

3.9 Evaluation of physiochemical activity of co-crystal of isoniazid with betulinic and oleanolic acids

The hypothesis underlining the co-crystal synthesis of isoniazid (INH) **(3)** with betulinic acid **(15)** and oleanolic acid **(17)** is that, INH **(3)** a versatile supramolecular compound (former) is an excellent hydrogen bonding acceptor from carboxylic acid (conformer) to form supramolecular complex that can help improve on the undesirable properties in INH**(3).**

The co-crystals were prepared under three different conditions:

- Solvent Evaporation(SE)
- Solvent Drop(SD)
- Co-grinding(Cg)

3.9.1 Preparation of co-crystal form using solvent evaporation method

Isoniazid (0.137 g; 1m.mol) (INH) (3) and 0.457g (1m.mol) each of betulinic acid (15) or oleanolic acid (17) were dissolved separately in 5ml ethanol with warming and were mixed together. The solution was cooled to room temperature and kept for slow evaporation for 24 hours. The crystals were harvested by filtration and subsequently dried in the air under fume hood (Vijayaraj et al., 2013; Babu et al., 2013). The percentage yield was 79.66%.

3.9.2 Preparation of co-crystal form using solvent drop method

Isoniazid (0.137 g; 1m.mol) (INH)(3) and betulinic acid (15) or oleanolic acid **(17)** (0.457 g; 1m.mol) were transferred to pestle & mortar and milled for upward of 10 minutes. A few drops of the solvent (ethanol) were added dropwise. The resulting mixture again milled for 10 minutes and kept in fume hood to dry for 24 hours (Vijayaraj et al., 2013; Babu et al., 2013). The yield was 85.10%.

3.9.3 Preparation of co-crystal form using co-grinding method

Isoniazid (0.137g; 1m.mol)(INH) **(3)** and betulinic acid **(15)** or oleanolic acid **(17)** (0.457g; 1m.mol) were transferred to pestle & mortar and milled for 1 hour and kept in fume hood to dry for 24 hours (Vijayaraj et al., 2013; Babu et al., 2013). The yield was 83.54%.

3.10 Biological evaluation activities

All the compounds isolated were evaluated for:-

- Anti-tuberculosis using *in vitro* H_{37}RV strains (MABA)
- DNA Polymerase β inhibitor *in vitro* activity (ELISA)
- Cytototoxicity using *in vitro* HEK293 and *in vitro* HepG2

3.10.1 Antimycobacterial evaluation procedure:

The antimycobacterial activity of the isolated and synthesized compounds was done using the Microplate Alamar Blue Assay (MABA) protocol at the Institute for Tuberculosis Research, University of Illinois, Chicago, USA.

3.10.1.1 Bacterial Strains for anti-TB biological assays

For the preparation of the inoculum, a virulent strain of *M. tuberculosis*, H37Rv (ATCC 27294) was grown in 100 ml of Middlebrook 7H9 Broth (Difco, Detroit, MI), supplemented with 0.2% (v/v) glycerol (Sigma Chemical Co., St Louis, MO), 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco), an 0.05% (v/v) Tween 80 (Sigma), also referred to as 7H9GC-T80.

3.10.1.2 Microplate Alamar Blue Assay (MABA)

Anti-TB susceptibility testing of isolates and derivatives were determined in the fluorometric Microplate Alamar Blue Assay (MABA) as described by Collins and Franzblau (1997) and Franzblau *et al* (1998). The extracts MICs against MTB H_{37}RV (ATCC 27294) were assessed by the MABA using, rifampin and isoniazid as positive controls. Sample stocks were prepared in DMSO at concentration of 12.8 mM, and the final test concentrations range from 128 µM to 0.5 µM. Two fold dilutions of compounds were prepared in Middlebrook 7H12 medium (7H9 Broth containing 0.1% w/v casitone, 5.6 µg/mL palmitic acid, 5 mg/mL bovine serum albumin, 4mg/mL catalase, filter-sterilized) in a volume of 100 µL in 96-well Microplates (BD Optilux, 96- well Microplates, black/clear flat bottom). MTB cultures (100 µL inoculum of 2 x 105 cfu/ mL) were added, yielding a final testing volume of 200 µL. The plates were incubated at 37^0C. On the seventh day of incubation, 12.5 µL of 20% Tween 80, and 20 µL of Alamar Blue (Invitrogen BioSource^{TM}) were added to the wells. After incubation at 37 ^{0}C for 16-24 hrs, fluorescence of the wells was measured (ex 530, em 590 nm). The MIC was determined as the lowest concentration effecting a reduction in fluorescence of ≥ 90% relative to the mean of replicate bacteria-only controls.

3.10.2 MTT Cell proliferation assay procedure

The cytotoxicity studies were carried out using the MTT Cell Proliferation Assay as described by Mosman *et al*. (1983). Human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells were all grown to confluence in 25 cm^2 flasks. This was then trypsinized and plated into 48 well plates at specific seeding densities.

Cells were incubated overnight at 37 °C. Medium was then removed and fresh medium (MEM + Glutmax + antibiotics) was added. Isolates and synthesized compounds (50–350 µL) were then added in triplicate and incubated for 4 hrs. Thereafter, medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics + 10% Fetal bovine serum). Afterwards, the 48 hrs cells were subjected to the MTT assay. Data were evaluated through regression analysis using QED statistics program and from the linear equation the IC_{50} values representing the lethal concentration for 50% mortality was calculated.

3.10.3 DNA polymerase β Inhibitor Assay (ELISA)

Briefly, compounds were dissolved in DMSO (200 ul) and reconstituted to a working stock of 2 mg/ml in 0.1M PBS. HepG2 cells were seeded at a density of 15000 cells per well and allowed to attach overnight. Cells were then treated with compounds at 10, 100 and 500 ng/ml concentrations and incubated for 24 hours. Following incubation, the treatments were removed and the ELISA was conducted according to the manufacturer's instructions. The absorbance readings obtained were tabulated in a spread sheet where calculations and normalization were done according to the manufacturer's instructions.

Chapter 4
Results

The Plant-derived oleanolic acid (OA) and betulinic acid (BA), isolated from *S. aromaticum* and *C. dentata* respectively along with their synthesized 3-O-acetyl derivatives at C^{-3} position, 28-cinnamic acid hybrids at C^{-28} position, and 3-O-acetyl-3 position and cinnamic acid at C^{-28} position were screened for their anti-TB activity. The results so obtained for their synthesis and anti-TB are presented below:-

4.1 Compounds Identification

The isolated compounds and their derivatives were identified and their structures established through spectral (^{1}H and ^{13}C) analysis (Appendix A) and by comparison with literature values as indicated against each compound.

4.1.1 Characterization of compound vof 1 obtained from *S. aromaticum*

Physical state:

% Yield: 87.92

% purity: 90.90

State: White powder which was obtained after re-crystallized in MeOH,

Experimental melting point: 299-310^{0}c

Spectroscopic data:

Molecular Mass: m/z: 456.3570 (M); 455.3522$(M-H)^{+}$ (**Appendix A3**)

Molecular Formula: $C_{30}H_{48}O_{3,}$ MW = 456

FT-IR (KBr): 3425 cm^{-1}(due to O-H, alcohol), 2940 cm^{-1}(C-H stretching, alkane), 1686 cm^{-1} carbonyl of carboxylic acid, and 1462 cm^{-1}(CH_3, CH_2) (**Appendix A2**).

^{13}C-NMR: The proton decoupled ^{13}C-NMR ($CDCl_{3,}$ 100 MHz) spectrum, (**Appendix A1**; **Table 4.1**), showed thirty carbon signals; methine (CH) -5; methylene (CH_2 -ve) - 10; methyl (CH_3 +ve) -7; and quaternary(C) – 8, carbon atoms.

The chemical shifts at σ_c 182.6, 122.8 and 143.7 were the characteristic peaks of oleanolic type of skeleton, assigned to C^{-28}, C^{-12} and C^{-13} respectively. The oxygen de-shielding chemical shift at σ 79.2 was assigned to C^{-3}. A literature search revealed that compound vof 1 to be oleanolic acid (Onoja. et al., 2013).

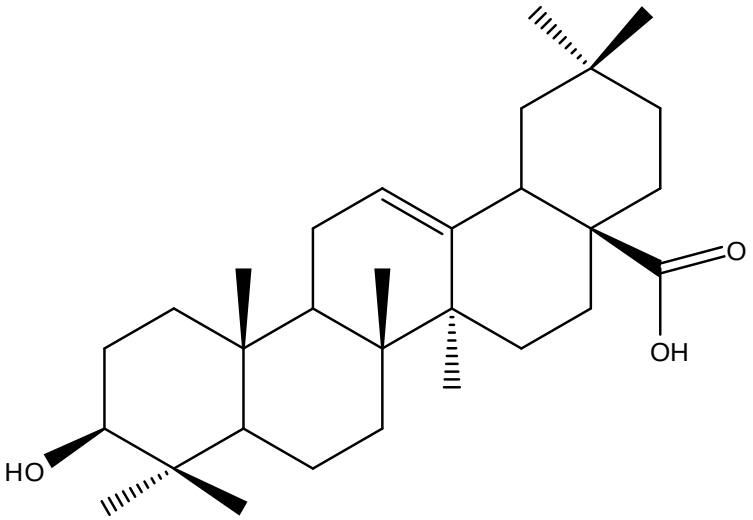


Fig. 4.1 Structure of oleanolic acid (17)

Table 4.1: ^{13}C-NMR (100 MH_Z) chemical shift assignments for oleanolic acid (Onoja et al., 2013) and compound vof 1 in $CDCl_3$

Carbon position	Type of Carbon	Oleanolic acid (Onoja et al, 2013)	Compound vof 1
1	CH_2	38.4	38.6
2	CH_2	27.2	27.3
3	CH	79.0	79.2
4	C	38.4	38.9
5	CH	55.2	55.4
6	CH_2	18.3	18.5
7	CH_2	32.7	33.2
8	CH_2	39.3	39.4
9	C	47.6	47.8
10	CH	37.1	37.2
11	C	23.0	23.1
12	CH_2	122.7	122.8
13	CH	143.6	143.7
14	C	41.6	41.8
15	C	27.7	27.8
16	CH_2	23.4	23.6
17	CH_2	46.5	46.7
18	C	41.1	41.2
19	CH	45.9	46.0
20	CH_2	30.7	30.8
21	C	33.8	34.0
22	CH_2	32.4	32.6
23	CH_2	28.1	28.3
24	CH_3	15.6	15.7
25	CH_3	15.3	15.5
26	CH_3	17.1	17.5
27	CH_3	25.9	26.1
28	COOH	182.4	182.8
29	CH_3	33.1	32.8
30	CH_3	23.6	23.7

4.1.2 Characterization of compound vof 2 obtained from *C.dentata*

Physical state

% Yield: 4.47

% Purity: 89.55

State: white and crystalline solid which was re-crystallized from MeOH

Experimental melting point: 135^0 C -136^0 C.

Spectroscopic data

Molecular Mass: m/z: 438.3787(M+H+Na); 437.37(M+Na) (**Appendix A6**)

Molecular Formula: C_{29} H_{50} O, MW = 414

FT-IR (KBr): 3449 cm^{-1}(due to O-H, alcohol), 2927 cm^{-1}(C-H due to alkane) and 1689 cm^{-1}(C=C), 1461 cm^{-1} (**Appendix A5**)

^{13}C-NMR: The proton decoupled ^{13}C-NMR ($CDCl_3$, 400 MHz) spectrum, (**Appendix A4** and **Table 4.2**), showed twenty nine carbon signals, methine (CH) -10; methylene (CH_2 -ve) - 11;methyl (CH_3 $_+$ve) -6;and quaternary (C) – 3 , carbon atoms. The C=C conjugated are at 140.9 and 121.9. A literature search revealed the compound vof 2 to be β-sitosterol (Isah et al., 2014)

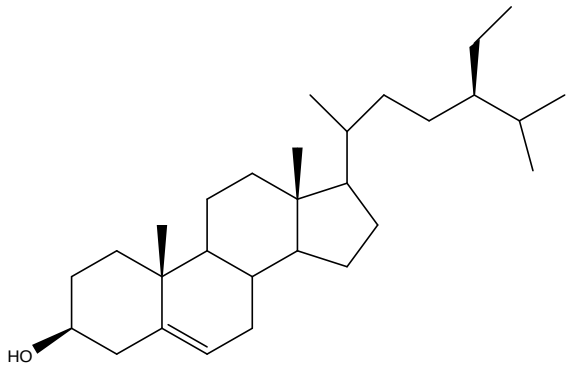


Fig. 4.2 Structure of β-sitosterol (32)

Table 4.2: ^{13}C-NMR (100 MH_Z) chemicals shift assignments for β-sitosterol (Isah et al., 2014) and compound vof 2 in $CDCl_3$

Carbon position	Type of Carbon	β-sitosterol (Yinusa et al., 2014)	Compound vof 2
1	CH_2	37.2	37.4
2	CH_2	31.7	31.8
3	CH	71.8	72.0
4	C	42.9	42.4
5	CH	140.8	140.9
6	CH_2	121.7	121.9
7	CH_2	31.9	32.1
8	C	31.9	31.8
9	CH	50.1	50.3
10	C	36.5	36.3
11	CH_2	21.1	21.2
12	CH	39.8	39.9
13	C	42.3	42.4
14	C	56.8	56.9
15	CH_2	24.3	24.5
16	CH_2	28.2	28.4
17	C	56.1	56.2
18	CH	12.0	12.0
19	CH_2	19.4	19.6
20	C	36.1	34.1
21	CH_2	19.0	19.2
22	CH_2	33.9	32.1
23	CH_3	29.1	26.2
24	CH_3	45.8	46.0
25	CH_3	26.0	29.3
26	CH_3	18.8	18.9
27	CH_3	19.8	19.9
28	C	23.1	23.2
29	CH_3	11.9	12.1

4. 1.3 Characterization of compound vof 3 obtained from *C.dentata*
Physical state

% Yield= 10.53

% Purity=73.15

State: White powder which was re-crystallized from MeOH.

Experimental melting point: 299^0 C -302^0 C

Spectroscopic data

Molecular Mass: m/z: 456.3556 (M); 455.3520$(M-H)^+$ (**Appendix A9**)

Molecular Formula: C_{30} H_{48} O_3; MW=456

FT-IR (KBr): 3424 cm^{-1}(due to O-H, alcohol), 2939 cm^{-1}(C-H due to CH_3 & CH_2) and 1687 (C=O) cm^{-1} (**Appendix A8**)

^{13}C-NMR

The proton decoupled ^{13}C-NMR ($CDCl_3$, 400 MHz) spectrum**,** (**Appendix A7; Table 4.3**), showed thirty carbon signals, methine (CH) -6; methylene (CH_2 -ve) - 12; methyl (CH_3 +ve) -6; and quaternary(C) – 6, carbon atoms. The chemical shifts at 177.2 (C^{-28}), 150.3 (C^{-20}), 109.6 (C^{-29}) were the characteristic peaks for betulinic type of skeleton. A literature search revealed the compound vof 3 to be betulinic acid (Uddin et al., 2011).

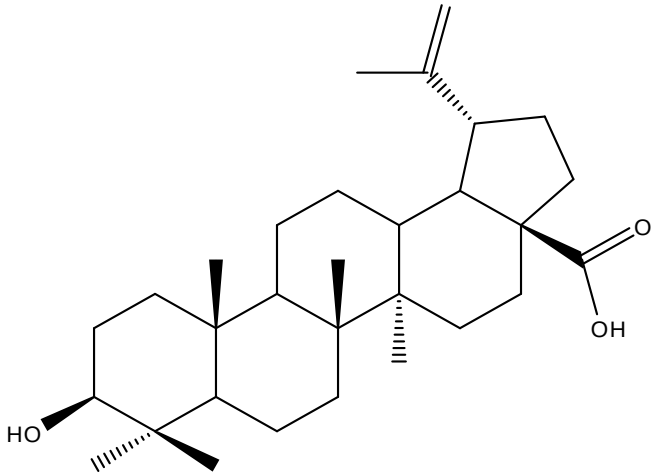


Fig. 4.3 Structure of betulinic acid (15)

Table 4.3: ^{13}C-NMR (100 MH_Z) chemical shift assignments for betulinic acid (Uddin et al., 2011) and compound vof 3 in $CDCl_3$.

Carbon postition	Type of carbon	Betulinic acid(Uddin et al., 2011)	Compound vof 3
1	CH_2	38.9	38.8
2	CH_2	27.9	27.1
3	CH	79.0	76.7
4	C	38.7	38.5
5	CH	55.5	54.9
6	CH_2	18.3	17.9
7	CH_2	34.3	36.7
8	C	40.9	40.2
9	CH	50.5	49.9
10	C	37.2	37.54
11	CH_2	20.8	20.4
12	CH	25.2	25.0
13	CH	38.4	38.2
14	C	42.4	42.0
15	CH_2	30.6	31.7
16	CH_2	32.1	33.9
17	C	56.3	55.4
18	CH	46.8	46.6
19	CH_2	49.2	48.5
20	C	150.4	150.3
21	CH_2	29.8	30.1
22	CH_2	34.1	36.3
23	CH3	28.0	29.2
24	CH_3	15.3	15.7
25	CH_3	16.0	15.9
26	CH_3	16.1	15.8
27	CH_3	14.8	14.3
28	COOH	180.3	177.2
29	CH_3	109.6	109.6
30	CH_3	19.4	18.9

4. 1.4 Characterization of compound vof 4 obtained from Figure 3.3

Physical state

% Yield=73.75

% Purity= 74.37

State: White powder which was obtained after re-crystallized in MeOH

Experimental melting point: 261-263^{0}c

Spectroscopic data

Molecular Mass: m/z: 498.3669(M); 497.3634$(M-H)^+$ (**Appendix 12**)

Molecular Formula: C_{32} H_{50} O_4, MW=498

FT-IR(KBr): Showed absorption at 3693 cm^{-1} which correspond to OH stretching bonded, the signal at 1779 cm^{-1} is due to carbonyl carbon (C=O, ester) while 1721 cm^{-1} is due to (C=O, acid). The signal at 2969 cm^{-1} to 2851 cm^{-1} is due to C-H, alkane which account for high degree of the saturation. The signal at 1455 cm^{-1} is due to (CH_3, CH_2), and 1024 cm^{-1} correspond to C-O. (**Appendix 11)**

^{1}H and ^{13}C NMR

The ^{1}H-NMR ($CDCl_3$, 400 MHz) spectrum**,** (**Appendix 10 and Table 4.4**), showed signal at σ_H 0.90, 0.86, 0.93, 0.75, 1.16, 0.85, 0.92 which indicate the presence of 7 methyl group of triterpenes skeleton. These were attributed to 23-CH_3, 24-CH_3, 25-CH_3, 26-CH_3 27-CH_3, 29-CH_3 and 30-CH_3 respectively. The signal at σ_H 2.14 (3H, s) was due to the methyl protons of acetyl group of the ester carbonyl carbon attached to C^{-3}. The signal at σ_H 5.55 ppm (brs) showed an olefinic proton attached to H-12, another

downfield signal appeared as triplet at σ_H 4.5 ppm (t) which is the proton at H-3. A proton double doublet was observed at σ_H 2.8 ppm (d, 13.8) and assigned to H-18.

The proton decoupled ^{13}C-NMR ($CDCl_3$, 100 MHz) spectrum, (**Appendix 13 and Table 4.4)**, shows 32 carbon signals, showed thirty two carbon signals, methine (CH) -5; methylene (CH_2 -ve) - 10;methyl (CH_3 +ve) -8;and quaternary (C) – 9, carbon atoms. The signals at σ_c 171.2 and 183.3 ppm indicated the presence of acetyl and carboxylic acid groups respectively. The signals at σc 81.2 ppm and 122.7 and 143.7 ppm suggested the presence of an oleanene triterpene type and were assigned to C^{-12} and C^{-13} respectively. A literature search revealed the compound to be oleanolic acid acetate. (Habila et al., 2012).

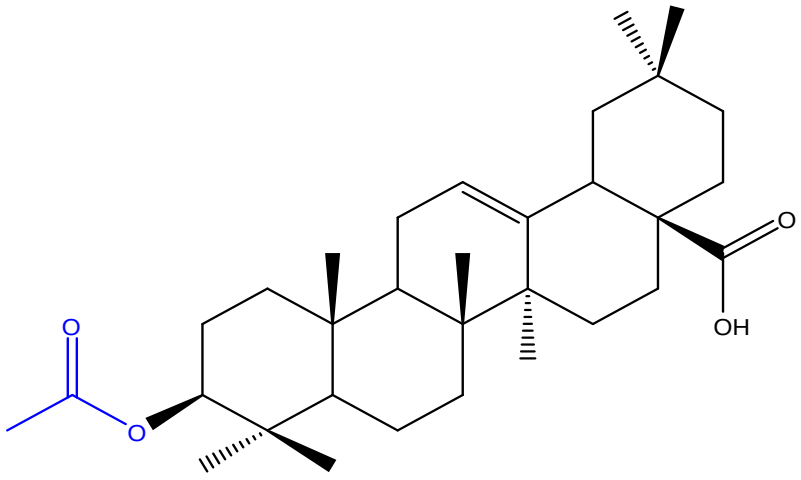


Fig.4.4 Structure of 3-O-acetyl oleanolic acid (19)

Table 4.4: ^{13}C-NMR (100 MH_Z) chemicals shift assignments for oleanolic acid acetate (Habila et al., 2012) and compound vof 4 in $CDCl_3$

Carbon postition	Type of carbon	Oleanolic acid acetate(Habila et al., 2012)	Vof 4
1	CH_2	39.3	39.4
2	CH_2	23.5	23.8
3	CH	80.9	81.1
4	C	37.7	38.0
5	CH	55.3	55.4
6	CH_2	17.2	17.2
7	CH_2	33.8	33.9
8	C	38.1	38.2
9	CH	47.5	47.7
10	C	37.0	37.1
11	CH_2	23.4	23.5
12	CH_2	122.6	122.7
13	CH	143.6	143.7
14	C	40.9	41.7
15	CH_2	25.9	26.0
16	CH_2	32.4	32.7
17	CH	45.8	46.0
18	CH	41.5	41.7
19	CH_2	46.5	46.7
20	CH_2	32.5	32.7
21	CH_2	33.1	33.2
22	CH_2	28.0	28.0
23	CH_3	22.7	23.1
24	CH_3	18.2	18.3
25	CH_3	15.3	17.3
26	CH_3	16.7	17.7
27	CH_3	23.6	23.8
28	C	184.1	183.3
29	CH_3	30.7	30.8
30	CH_3	27.7	27.8
1’	C	171.1	171.2
2’	CH_3	21.3	21.2

4. 1.5 Characterization of compound vof 5 obtained from Figure 3.4

Physical state

% Yield =70.23

% Purity= 63.93

State: White powder which was re-crystallized from MeOH

Experimental melting point: 283^0 C -285^0 C

Spectroscopic data

Molecular Mass: m/z: 498.3668 (M); 497.3628 $(M-H)^+$ (**Appendix A17)**

Molecular Formula: C_{32} H_{50} $O_{4,}$ MW=498

FT-IR (KBr): showed absorption at 3692 cm^{-1}(due to O-H, alcohol), 2962 cm^{-1} -2851 cm^{-1}(C-H stretch from CH_3 and CH_2), 1722 cm^{-1}(C=O, ester) and 1689 cm^{-1}(C=O) (**Appendix A16)**

The ^{1}H-NMR ($CDCl_{3,}$ 400 MHz) spectrum**,** (**Appendix A14 and Table 4.5**) showed singlets at σ_H 0.83, 0.84, 0.85, 0.93, 0.97, 1.69, which indicate the presence of 6 methyl groups of triterepene skeleton. These were attributed to 23-$CH_{3,}$ 24-CH_3, 25-$CH_{3,}$ 26-$CH_{3,}$ 27-$CH_{3,}$ 30-CH_3 respectively. The signal at σ_H 2.03 (3H, s) was due to the proton of acetyl group of the ester carbonyl carbon attached to C^{-3} respectively. The presence of two hydrogen at C^{-29} position was confirmed by the signals at σ_H 4.61(1H, brs) and σ_H 4.74(1H, brs). The double doublet at σ_H 4.50 was assigned as proton attached to the carbon bearing at C^{-3} position. The signal at σ_H 3.00(1H, m) was due to the hydrogen at C^{-19} position.

The proton decoupled ^{13}C-NMR ($CDCl_{3,}$ 100 MHz) spectrum, **(Appendix 15 and Table 4.5**), showed thirty two carbon signals, methine (CH) -6; methylene (CH_2 -ve) - 11;

methyl (CH_3 +ve) -7; and quaternary(C) – 8, carbon atoms. The presence of a signal at σ 78.0 ppm, which was assigned to C^{-3} indicate the presence of an ester group. The signal at σ 177.2 ppm indicates the presence of a carboxyl carbon while the signal at σ 170.1 ppm indicates the presence of ester carbonyl carbon. The signals at σ 150.3 ppm and 109.6 ppm were due to carbon-carbon double bond between C^{-20} and C^{-29} respectively. The signal at σ 20.4 ppm was due to the methyl group acetate. A literature search revealed the compound vof 5 to be betulinic acid acetate **(33).**

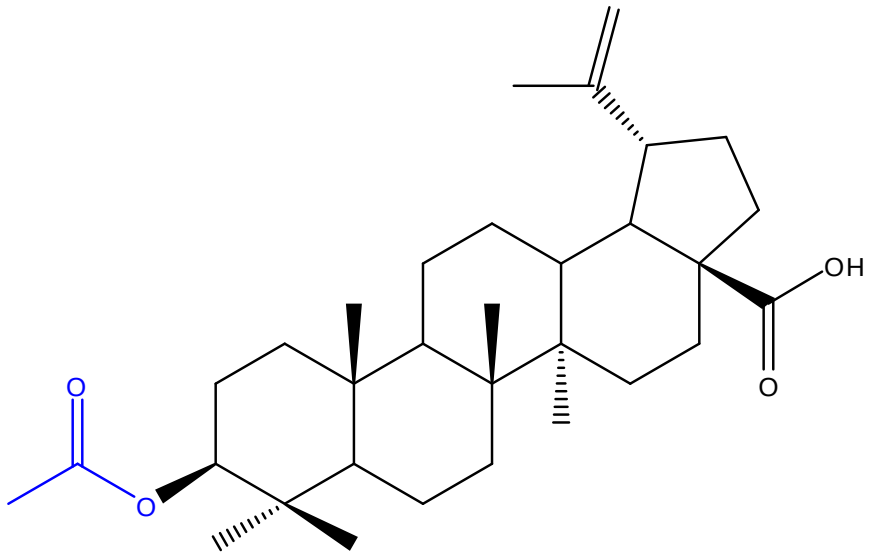


Fig. 4.5 Structure of 3-O-acetyl betulinic acid (30)

Table 4.5: ^{13}C-NMR (100 MHz) chemicals shift assignments for betulinic acid acetate (Raza et al., 2015) and compound vof 5 in $CDCl_3$.

Carbon postition	Type of carbon	Betulinic acid acetate(Raza et al.,2015)	Compound vof 5
1	CH_2	38.3	38.9
2	CH_2	23.7	23.3
3	CH	80.9	79.9
4	C_q	37.8	37.7
5	CH	55.4	55.4
6	CH_2	18.1	20.9
7	CH_2	34.2	33.7
8	C_q	40.6	40.2
9	CH	50.3	49.6
10	C_q	37.1	37.5
11	CH_2	20.8	23.3
12	CH_2	25.4	25.0
13	CH	49.2	38.9
14	C_q	42.4	42.0
15	CH_2	30.5	30.1
16	CH_2	32.1	31.7
17	C_q	56.3	55.4
18	CH	38.3	37.5
19	CH	46.9	46.6
20	C_q	150.4	150.3
21	CH_2	29.7	30.1
22	CH_2	37.0	37.3
23	CH_3	27.9	29.2
24	CH_3	16.0	15.7
25	CH_3	16.4	15.8
26	CH_3	15.2	16.4
27	CH_3	14.6	14.3
28	C_q	180.6	177.2
29	CH_3	109.8	109.6
30	CH_3	19.3	18.9
1’	C_q	171.1	170.1
2’	CH_3	21.3	20.9

4. 1.6. Characterization of compound vof 6 obtained from Figure 3.6

Physical state

%Yield: 97.23

%Purity: 88.00

State: White amorphous powder which was obtained after re-crystallized in MeOH

Experimental melting point: 181-183^{0}C (Literature: Habila et al., 2012).

Spectroscopic data

Molecular Mass (ES-MS): m/z: 603.3761$(M-NH_3)^+$ (**Appendix A21**)

Molecular Formula: C_{39} H_{54} $O_{4,}$ MW=586

FT-IR (KBr): 3397 cm^{-1}(due to O-H), 2941 cm^{-1}(C-H stretch), 1682 cm^{-1}(C=O, possibly from anhydride) (**Appendix A20).** A carbon-carbon double bond (C=C) signal is observed at 1630 cm^{-1}, the band at 1578 cm^{-1} corresponds to aromatic C=C stretch, the absorption at 1450 cm^{-1}-1421 cm^{-1} characterized the bending vibration of methylene group (CH_2) and the signal observed at 1068 cm^{-1}-1027 cm^{-1} is due to carbon-oxygen(C-O) bond.

The ^{1}H-NMR ($CDCl_{3,}$ 400MHz) (**Appendix A18 and Table 4.6**), showed trans coupled protons at σ_H : 6.44 1H d(J=16.0 H_2); 7.75 1H d(J=16.0 H_2); and 5 aromatic protons (7.52 2H m, 7.40 3H m).

The proton decoupled ^{13}C-NMR ($CDCl_{3,}$ 100MHz) spectrum, **(Appendix A19 and Table 4.6),** showed thirty-seven carbon signals on expansion; seven (7) methyl (CH_3), ten (10) methylene (CH_2), ten (10) methine (CH) and ten(10) quaternary (C) carbon atoms. The chemical shifts of the six- sp^2 at σ_c 146.7, 130.7, 130.7, 129.2, 128.0 and 117.6 are due

the benzene aromatic ring. When the data obtained for vof 6 is compared with data obtained by Habila et al, 2011, the compound is found to be cinnamic hybrid of oleanolic acid **(31).**

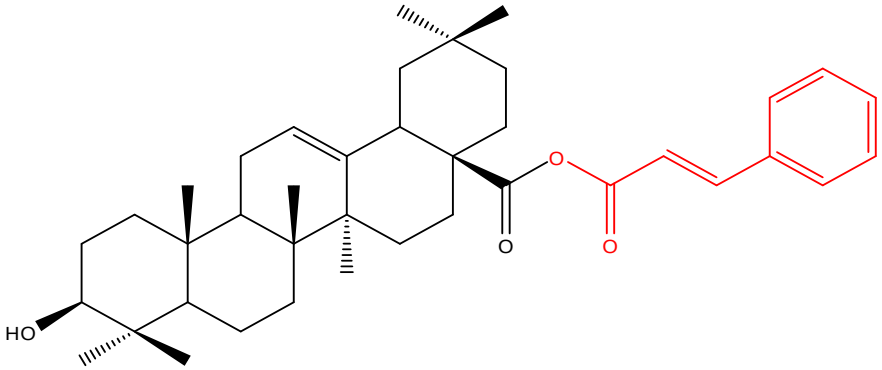


Fig. 4.6 Structure of 28-cinnamic acid hybrid of oleanolic acid (29)

Table 4.6: ^{1}H-NMR (400 MHz) and ^{13}C NMR (100 MHz) chemicals shift assignments for 28- cinnamic acid- oleanolic acid and compound vof 6 in $CDCl_3$.

Carbon position	^{13}C-NMR(Habila et al., 2011)	^{1}H-NMR-(vof6)	Type of carbon	^{13}C-NMR(vof6)
1	38.8		CH_2	37.2
2	27.7		CH_2	27.3
3	79.0	3.2(brs)	CH	79.2
4	38.5		C	38.6
5	55.3		CH	55.4
6	23.2		CH_2	18.5
7	33.7		CH_2	34.0
8	39.4		C	38.9
9	47.7		CH	47.8
10	37.0		C	38.9
11	25.1		CH_2	23.1
12	123.1	5.3(brs)	CH	117.6
13	143.2		C	143.7
14	48.2		C	41.2
15	30.7		CH_2	27.8
16	23.5		CH_2	23.1
17	46.0		C	41.8
18	41.3		CH	41.2
19	45.9		CH_2	46.0
20	32.8		C	30.8
21	34.3		CH_2	34.0
22	29.7		CH_2	32.6
23	23.5	0.98	CH_3	28.2
24	15.6	0.98	CH_3	15.6
25	17.1	0.77	CH_3	15.6
26	18.3	1.04	CH_3	17.0
27	27.2	1.13	CH_3	23.5
28	173.2		C	171.0
29	31.6	0.98	CH_3	34.0
30	28.1	0.98	CH_3	23.5
1’	162.8		CO	171.0
2’	117.3	6.45(d,16)	CH	117.6
3’	148.2	7.75(d,16)	CH	146.7
1”	133.8		C	130.7
2”/6”	128.6	7.55(m)	CH	128.0
3”/5”	129.1	7.40(m)	CH	129.2
4”	131.2		CH	130.7

4. 1.7 Characterization of compound vof 7 obtained from figure 3.7

Physical state

% Yield: 95.83

% Purity: 93.57

State: White amorphous powder which was obtained after re-crystallized in MeOH,

Experimental melting point: 181-183^{0}C

Spectroscopic data

Molecular Mass (Appendix A25): m/z: 603.3778 (M) $(M\text{-}NH_3)^+$

Molecular Formula: C_{39} H_{54} O_4, MW=586

FT-IR(KBr)(Appendix A24): Showed absorption at 3395 cm^{-1}(due to O-H), 3026 cm^{-1}, 2925 cm^{-1}, 2854 cm^{-1}(C-H-stretching), 1686 cm^{-1}, 1630 cm^{-1}(C=O,). A carbon-carbon double bond (C=C) signal is observed at 1579 cm^{-1}, the band at 1494 cm^{-1} corresponds to C=C aromatic stretching while the absorption at 1466 cm^{-1} characterized the bending vibration of methylene group (CH_2) and the signal observed at 1074 cm^{-1} is due to carbon-oxygen(C-O) bond.

The ^{1}H-NMR ($CDCl_3$, 400MHz) **(Appendix A22):** Showed trans coupled protons at σ_H : 6.45 1H d(J=16.0 H_2); 7.76 1H d(J=16.0 H_2); and 5 aromatic protons (7.55 2H m; 7.40 2H m).

The proton decoupled ^{13}C-NMR ($CDCl_3$, 100MHz) spectrum, (**Appendix A23)** showed thirty-seven carbon signals on expansion; six (6) methyl (CH_3), eleven (11) methylene (CH_2), eleven(11) methine (CH) and nine (9) quaternary (C) carbon atoms.

The chemical shifts of the six- sp^2 at σ_c 146.8, 134.3, 130.8, 129.1, 128.5 and 117.5 are due to the benzene aromatic ring. The proposed structure for 28-cinnamic acid hybrid of betulinic acid **(31)** is as shown in figure 4.7

The general observation from the signal of 1H-NMR and ^{13}C-NMR spectra of the compound is that, they are weak and many overlapping were observed at 400 MHz. In view of this, an NMR of higher field like 500MHz above may be needed to have good spectra.

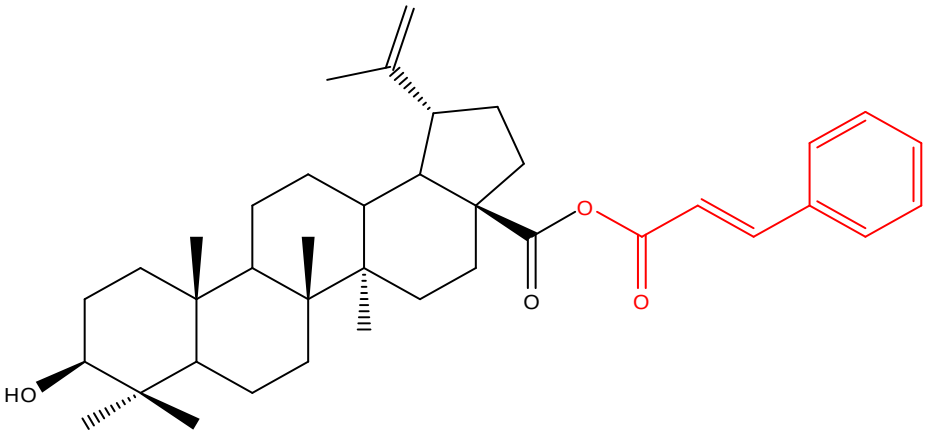


Fig. 4.7 Structure of 28- cinnamic acid hybrid of betulinic acid (31)

Table 4.7: ^{1}H-NMR (400 MHz) and ^{13}C NMR (100 MHz) chemicals shift assignments for compound vof 7

Carbon position	^{1}H-NMR-(Vof7)	Type of Carbon	^{13}C-NMR(Vof7)
1		CH_2	
2		CH_2	27.2
3	3.35	CH	81.1
4		C	
5		CH	55.5
6		CH_2	18.3
7		CH_2	34.0
8		C	
9		CH	
10		C	
11		CH_2	
12		CH	
13		C	
14		C	
15		CH_2	30.8
16		CH_2	32.7
17		C	55.4
18		CH	45.9
19	3.1(m)	CH_2	47.7
20		C	
21		CH_2	
22		CH_2	
23	1.32	CH_3	
24	1.25	CH_3	16.8
25	1.06	CH_3	
26	0.95	CH_3	
27	0.85	CH_3	
28		C	171.0
29	4.76(s)-29(a), 4.90(s)-29(b)	CH_3	117.5
30	1.4(s)	CH_3	18.3
1’		CO	170.9
2’	6.45(d,16)	CH	117.5
3’	7.75(d,16)	CH	146.8
1”		C	134.3
2”/6”	7.55(m)	CH	128.5
3”/5”	7.40(m)	CH	129.1
4”	7.26(m)	CH	130.8

4. 1.8 Characterization of compound vof 8 obtained from Figure 3.8

Physical state

% Yield= 93.40

% Purity=84.95

State: White amorphous powder which was obtained after re-crystallized in MeOH,

Experimental melting point: 161-163^{0}C

Spectroscopic data

Molecular Mass (ES-MS): m/z: 645.4005 $(M)^+$, $(M+NH_3)^+$ (**Appendix A29)**

Molecular Formula: C_{41} H_{56} $O_{5,}$ MW =628

FT-IR (KBr) (Appendix A28): 3374 cm^{-1}(due to O-H), 2944 -3017 cm^{-1}(C-H stretching), 1722 cm^{-1} -1684 cm^{-1}(C=O, possibly from anhydride). A carbon-carbon double bond (C=C) signal is observed at 1630 cm^{-1}, the band at 1579 cm^{-1} corresponds to C=C-H, the absorption at 1466 cm^{-1} characterized the bending vibration of methylene group (CH_2) and the signal observed at 1070 cm^{-1} is due to carbon-oxygen(C-O) bond.

The ^{1}H-NMR ($CDCl_{3,}$ 400MHz) (**Appendix A26 and table 4.8),** showed trans coupled protons at σ_H : 6.44 1H d(J=16.0); 7.75 1H d(J=16.0); and 5 aromatic protons (7.52 2H m; 7.38 2H m).

The proton decoupled ^{13}C-NMR ($CDCl_{3,}$ 100MHz) spectrum, **(Appendix A27 and table 4.8),** showed thirty-nine carbon signals on expansion; eight (8) methyl (CH_3), ten (10) methylene (CH_2), ten (10) methane (CH) and eleven (11) quaternary (C) carbon atoms. The chemical shifts of the six- sp^2 at σ_c 146.4, 134.3, 130.6, 129.2, 128.7 and 117.9 are

due the benzene aromatic ring and 183.4 due to ester moiety at C^{-3} position. In view of the above the structure below is proposed for 3-O-acetyl-28-cinnamic acid hybrid of oleanolic acid **(32)**

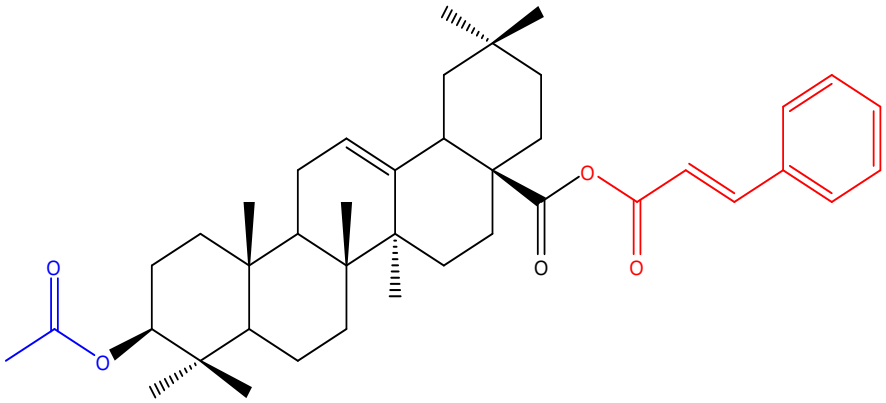


Fig. 4.8 Structure of 3-O-acetyl-28-cinnamic acid hybrid of oleanolic acid (32)

Table 4.8: ^{1}H-NMR (400 MHz) and ^{13}C NMR (100 MHz) chemicals shift assignments for compound vof 8 in $CDCl_3$

Carbon position	^{1}H-NMR-(vof8)	Type of Carbon	^{13}C-NMR(vof 8)
1		CH_2	39.1
2		CH_2	28.1
3	3.1(brs)	CH	81.1
4		C	39.6
5		CH	55.4
6		CH_2	17.1
7		CH_2	37.0
8		C	39.6
9		CH	48.1
10		C	37.8
11		CH_2	21.4
12	5.09(brs)	CH	125.8
13		C	138.1
14		C	42.1
15		CH_2	28.2
16		CH_2	21.4
17		C	47.7
18	2.04(s)	CH	42.1
19		CH_2	46.0
20		C	29.8
21		CH_2	37.0
22		CH_2	28.2
23	0.92	CH_3	28.2
24	0.89	CH_3	17.0
25	0.78	CH_3	15.7
26	1.02	CH_3	17.1
27	1.12	CH_3	28.1
28		C	183.4
29	0.95	CH_3	36.8
30	0.93	CH_3	21.4
1’		CO	171.2
2’	6.45(d,16)	CH	117.9
3’	7.75(d, 16)	CH	146.4
1”		C	134.3
2”/6”	7.55(m)	CH	128.7
3”/5”	7.40(m)	CH	129.2
4”		CH	130.6
1”’	2.04(s)	CH_3	
2”’		CO	183.4

:

4. 1.9 Characterization of compound v of 9 obtained from Figure 3.9

Physical state

% Yield=95.47

% Purity=92.30

State: White amorphous powder which was obtained after re-crystallized in MeOH,

Experimental melting point: 161-163^{0}c

Spectroscopic data

Molecular Mass: m/z: 645.4016$(M-NH_3)^+$ (**Appendix A33)**

Molecular Formula: C_{41} H_{56} O_5, MW=628

FT-IR(KBr)**(Appendix A32):** broad band at 2937 cm^{-1}(C-H stretching), 1682 cm^{-1} -(C=O,ester) and (C=C) signal is observed at 1630 cm^{-1}, the band at 1450 cm^{-1} corresponds to C=C-H, the absorption at 1422 cm^{-1} characterized the bending vibration of methylene group (CH_2) and the signal observed at 1027 $cm^{-1}$is due to carbon-oxygen(C-O) bond.

The ^{1}H-NMR (400 MHz, $CDCl_3$) **(Appendix A30)**, showed trans coupled protons at σ_H : 7.55 1H d(J=15.92 H_2); 7.40 1H d(J=16.0 H_2); and 5 aromatic protons (7.55 2H m, 7.40 2H m).

The proton decoupled ^{13}C-NMR (100 MHz, $CDCl_3$) spectrum, **(Appendix A31)** showed thirty-nine (39) carbon signals on expansion which are made of; seven (7) methyl (CH_3), eleven (11) methylene (CH_2), eleven (11) methane (CH) and ten (10) quaternary (C) carbon atoms against expected forty-one carbon signal (41). This suggests overlapping of signals of the same value. The chemical shifts of the six- sp^2 at σ_c 138.1, 130.7, 130.8, 128.4, 128.4 and 117.6 are due the benzene aromatic ring and 183.6 due to

ester moiety at C^{-3} position. Similarly, and in view of the above, the structure below is proposed for 3-O-acetyl-28-cinnamic acid hybrid of betulinic acid **(33)**

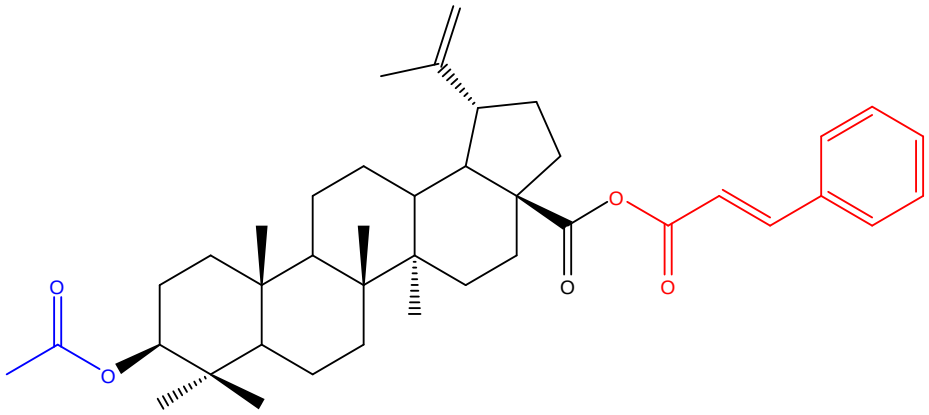


Fig. 4.9 Structure of 3-O-acetyl-28-cinnamic acid hybrid of betulinic acid (33)

Table 4.9: ^{1}H-NMR (400 MHz) and ^{13}C NMR (400 MHz) chemicals shift assignments for compound vof 9 in $CDCl_3$

Carbon position	^{1}H-NMR-(vof 9)	Type of carbon	^{13}C-NMR(vof 9)
1		CH_2	39.0
2		CH_2	18.3
3	3.45	CH	81.2
4		C	37.8
5		CH	55.4
6		CH_2	22.7
7		CH_2	33.0
8		C	39.7
9		CH	52.7
10		C	38.2
11		CH_2	23.7
12		CH	26.0
13		C	38.4
14		C	39.6
15		CH_2	42.1
16		CH_2	30.8
17		C	46.0
18		CH	52.7
19	3.2(m)	CH_2	47.7
20		C	146.8
21		CH_2	30.8
22		CH_2	30.7
23	1.07	CH_3	28.2
24	0.93	CH_3	17.1
25	0.85	CH_3	15.6
26	0.83	CH_3	17.0
27	0.79	CH_3	183.6
28		C	171.4
29	(a) 4.5, (b) 4.15	CH_3	117.6
30	1.25	CH_3	21.3
1’		CO	171.2
2’	6.45(d,15.92)	CH	117.6
3’	7.75(d, 16)	CH	138.1
1”		C	134.3
2”/6”	7.55(m)	CH	128.4
3”/5”	7.40(m)	CH	130.8
4”		CH	130.8
1”’	2.03(s)	CH_3	21.3
2”’		CO	183.6

4.2 Co-crystal synthesis of BA, OA with INH

Betulinic acid (**15)** and oleanolic acid (**17)** have limited application in drug discovery because of their poor aqueous solubility and good hepatoprotective profile, while isoniazid **(3)** has acute toxic profile but have high bioavailability in living cell. It is, therefore, apparent to improve on the undesirable properties of these molecules in other to aid their full utilization very well in view of their interesting biological activities.

Several methods have been employed in the past to improve on the drug-like nature profile of molecule such as the synthesis of the analogue of the molecule, molecular hybridization of two molecules and co-crystallization synthesis of two molecules together in recent.

Of all the methods, co-crystal synthesis remains straightforward and inexpensive to prepare and to improve on a number of properties that are deficient in any active pharmaceutical ingredients. It simply allows two or more molecules to be held together by weak hydrogen bonding to form a single compound that could enjoy synergistic cooperation to effect good biological activity in living cell.

The isoniazid is first line tuberculosis drug which sometimes used to manage HIV co-infection with tuberculosis. Similarly, betulinic acid and oleanolic acid have been report to show moderate anti-tuberculosis activity in addition to its anti-HIV activity.

The hypothesis underlining this study is that since isoniazid (INH) has been established as a versatile former molecule with excellent hydrogen bonding acceptor from carboxylic acid. The betulinic acid and oleanolic acid could, therefore, serve as conformer in the co-crystal synthesis involving both molecules.

From the foregoing and since both compound are solid at room temperature, the preparation of the co-crystal complexes of both molecule is therefore probable by studying the effect of water on the crystalline products (amorphous) that are formed using three different synthetic methods (solvent evaporation, solvent drop and co-direct grinding) and each products were then subjected to anti-tuberculosis activity to see any effect of the synthetic method on the biological activity.

All the compounds were characterized using P-XRD, TGA, and SEM and their images presented in Figure 4.10-4.19.

4. 2.1 Powered X-Ray diffraction (P-XRD), TGA and SEM images for Oleanolic acid (OA), Betulinic acid (BA) and Isoniazid (INH)

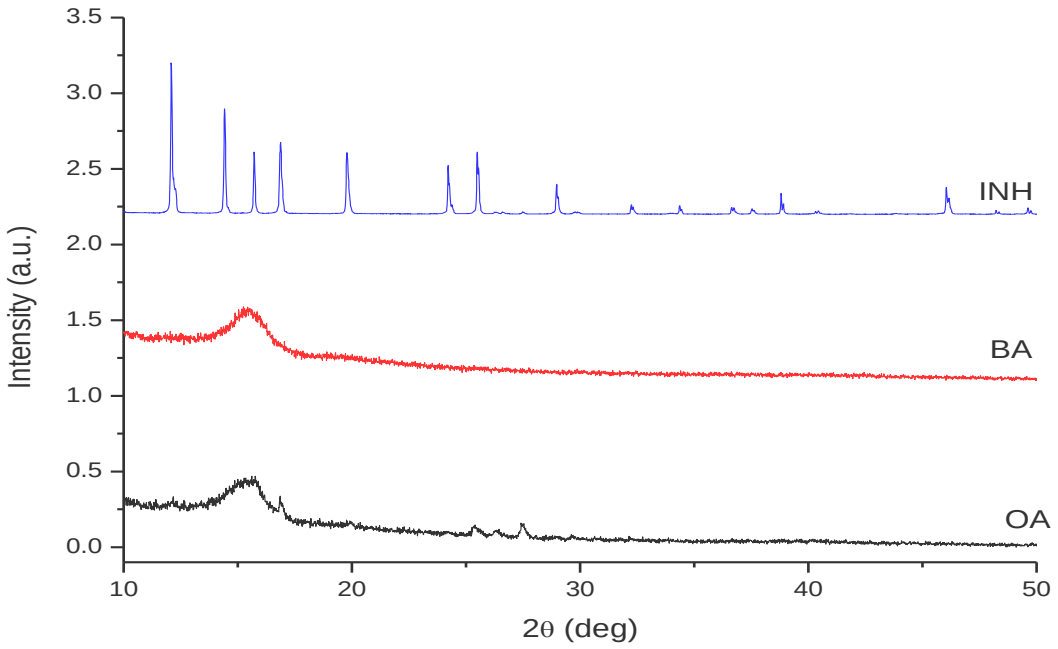


Fig. 4.10 The P-XRD pattern of OA, BA and INH

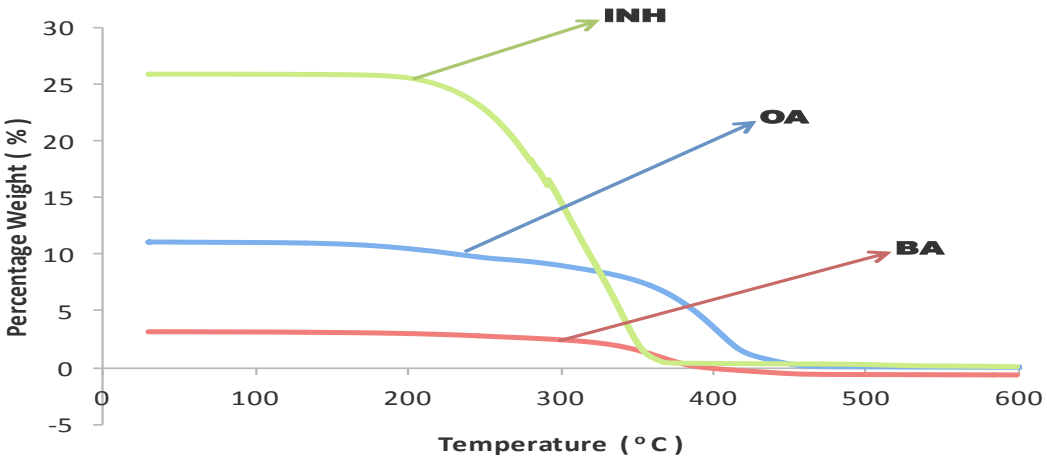


Fig. 4.11 TGA micrograph for OA, BA and INH

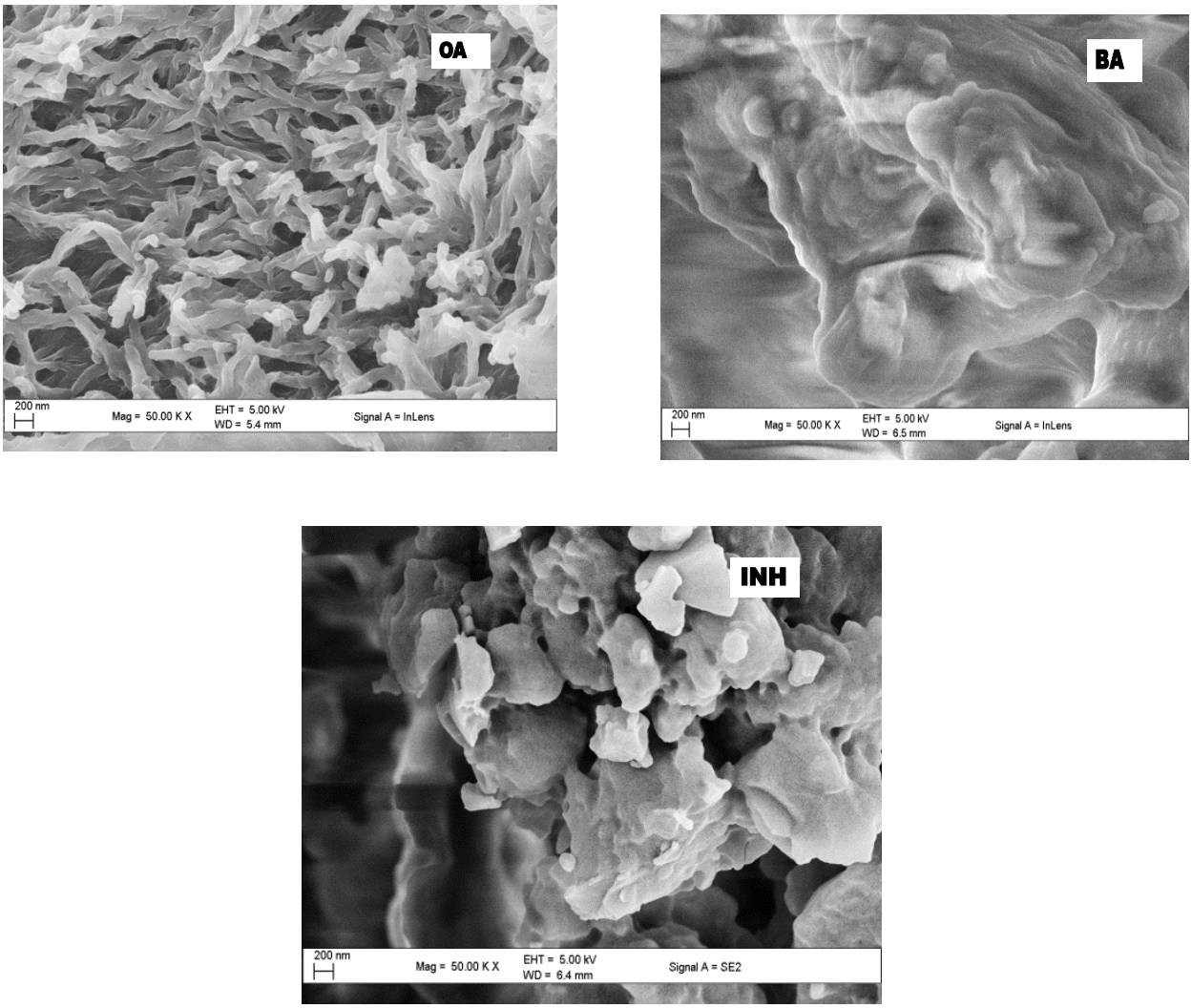


Fig. 4.12 SEM images of OA, BA and INH

4. 2.2 Oleanolic acid / betulinic acid co-crystallization with isoniazid (INH) prepared under three different conditions

4. 2.2.1 Effect of solvent evaporation method on OA/INH, BA/INH and INH

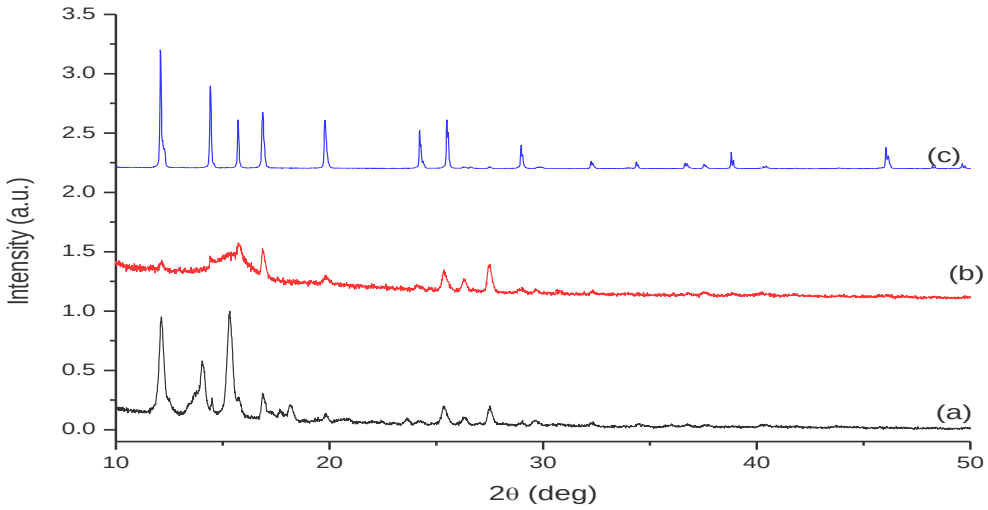


Fig. 4.13. The P-XRD pattern of solvent evaporation method on OA/INH (a), BA/INH (b) and INH(c)

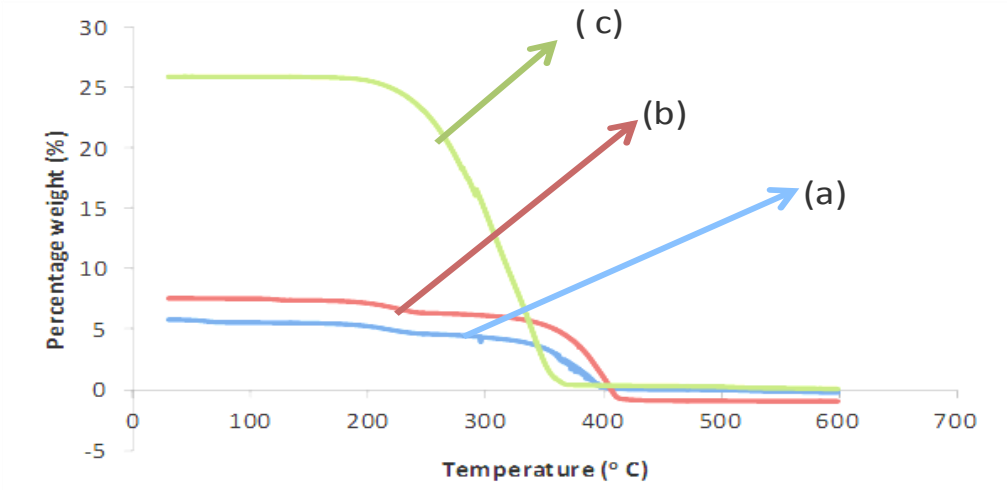


Fig. 4.14 TGA monograph of solvent evaporation on OA/INH (a), BA/INH (b) and INH(c)

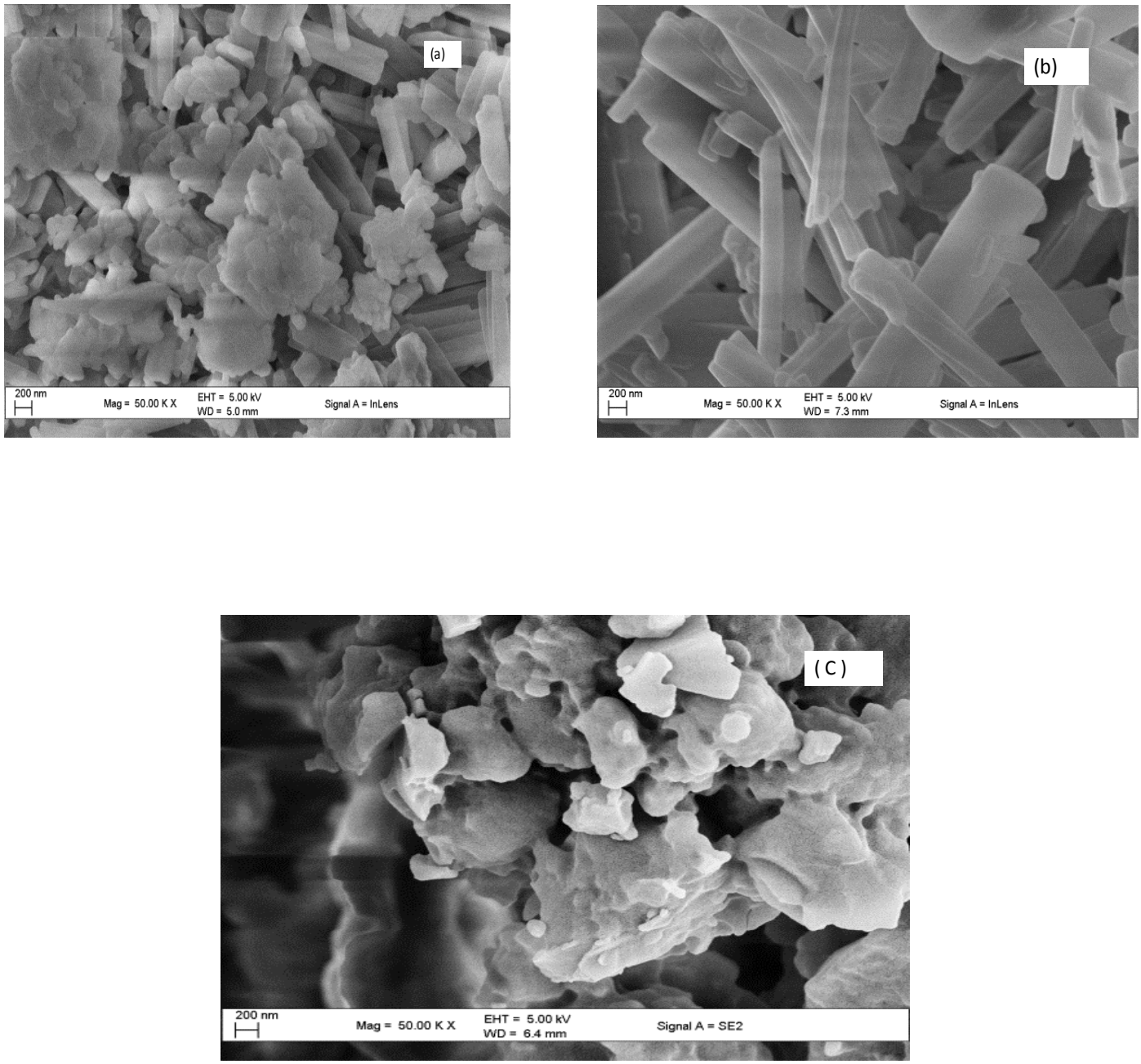


Fig. 4.15 SEM image of solvent evaporation on OA/INH (a), BA/INH (b) and INH(c)

4. 2.2.2 Effect of solvent drop method on OA/INH, BA/INH and INH

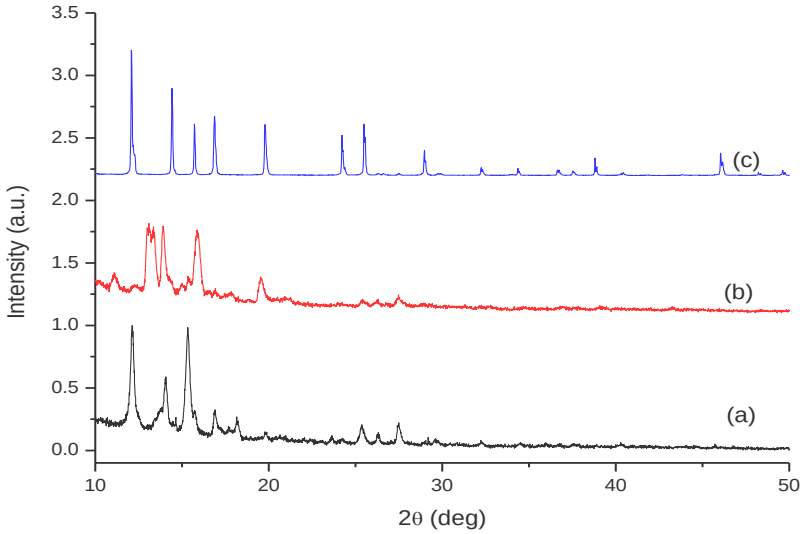


Fig. 4.16 The P-XRD pattern of solvent drop on OA/INH (a), BA/INH (b) and INH(c)

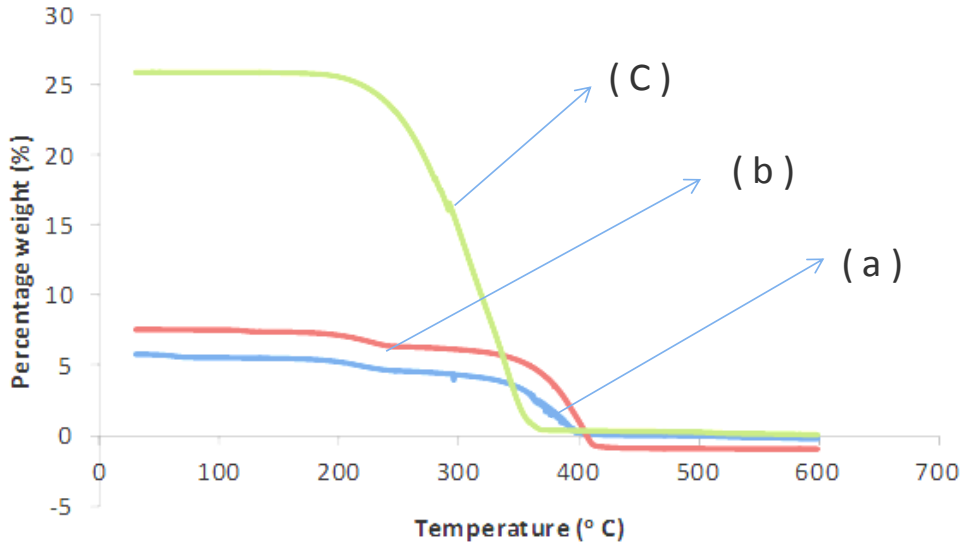


Fig. 4.17 The TGA monograph of solvent drop on OA/INH (a), BA/INH (b) and INH(c)

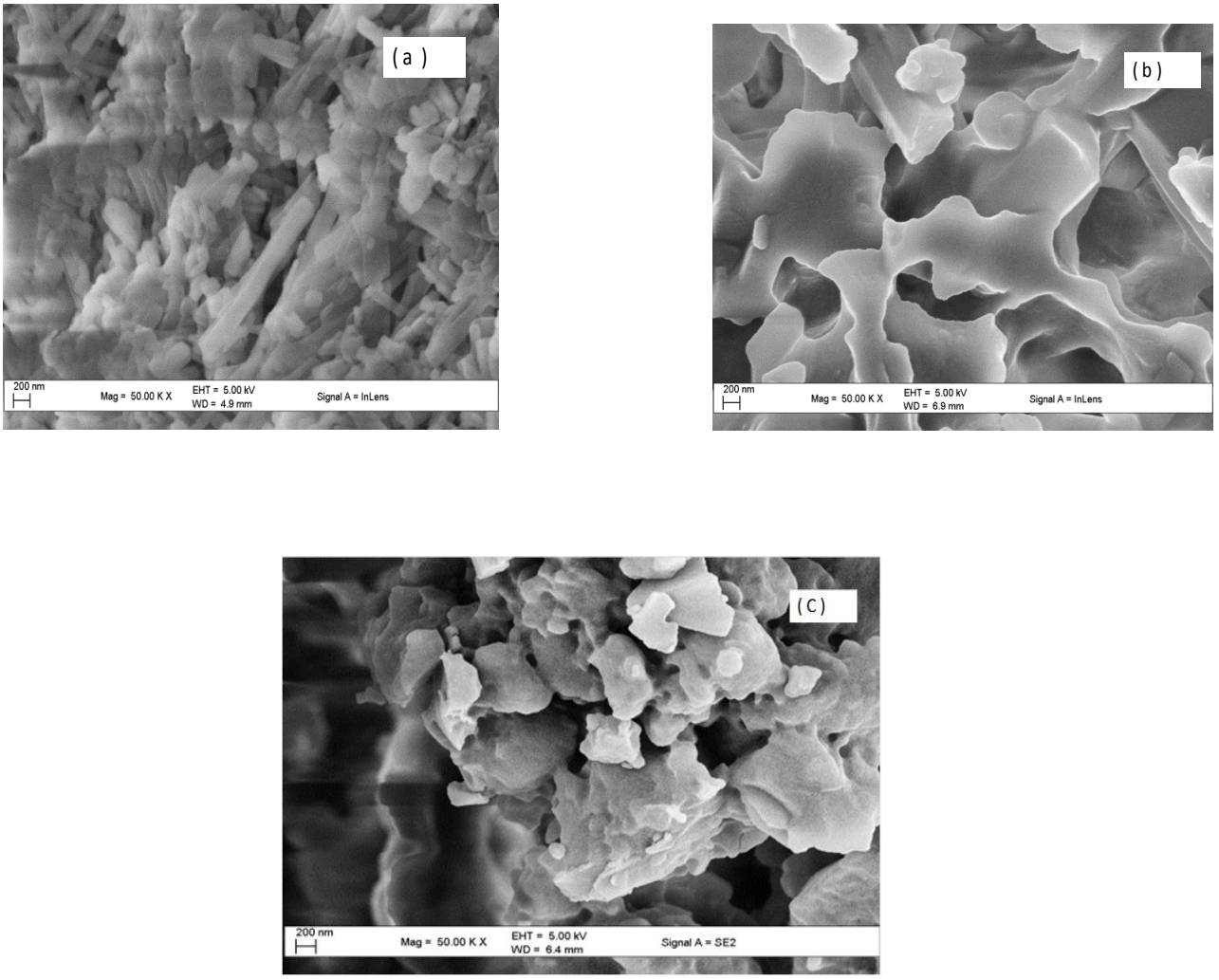


Fig. 4.18 The SEM image of solvent drop on OA/INH (a), BA/INH (b) and INH(c)

4. 2.3 Effect of co-grinding method on OA/INH, BA/INH and INH

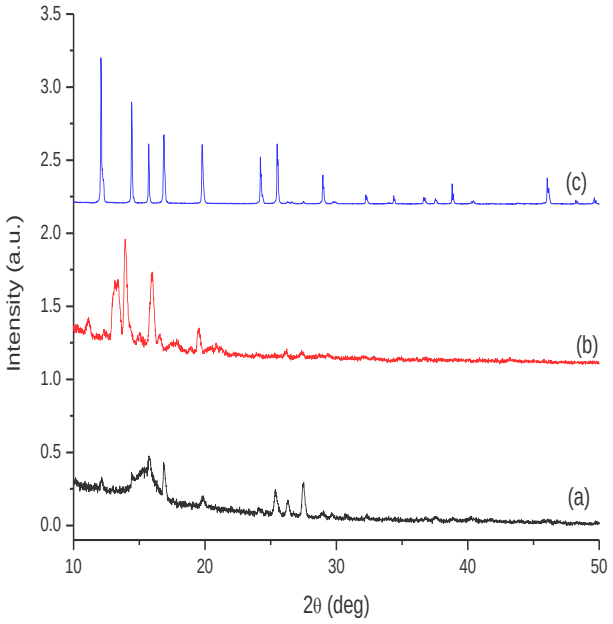


Fig. 4.19 The P-XRD pattern of co-grinding on OA/INH (a), BA/INH (b) and INH(c)

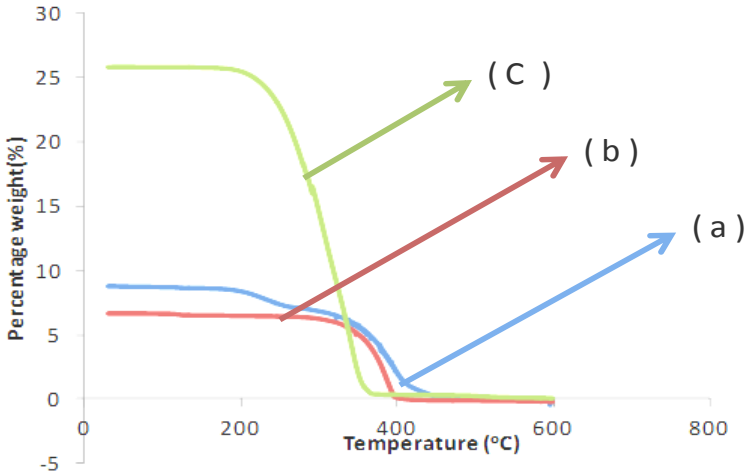


Fig. 4.20 The TGA monograph of the effect of co-grinding on OA/INH (a), BA/INH (b) and INH(c)

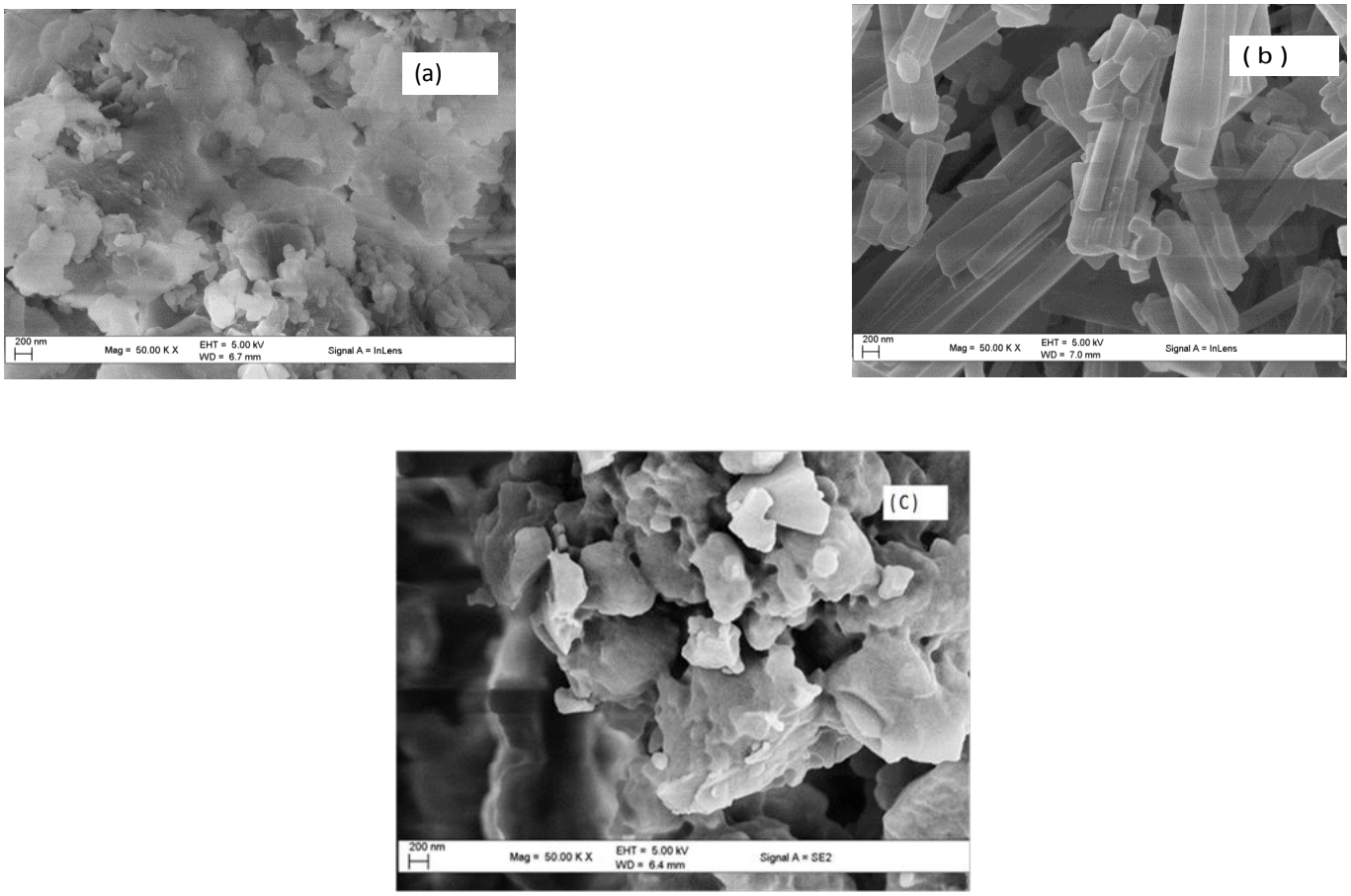


Fig. 4.21 The SEM image of the effect of co-grinding on OA/INH (a), BA/INH (b) and INH(c)

4. 2.3.1 Comparison of the three co-crystal products of OA/INH from the three synthetic methods along with OA and INH

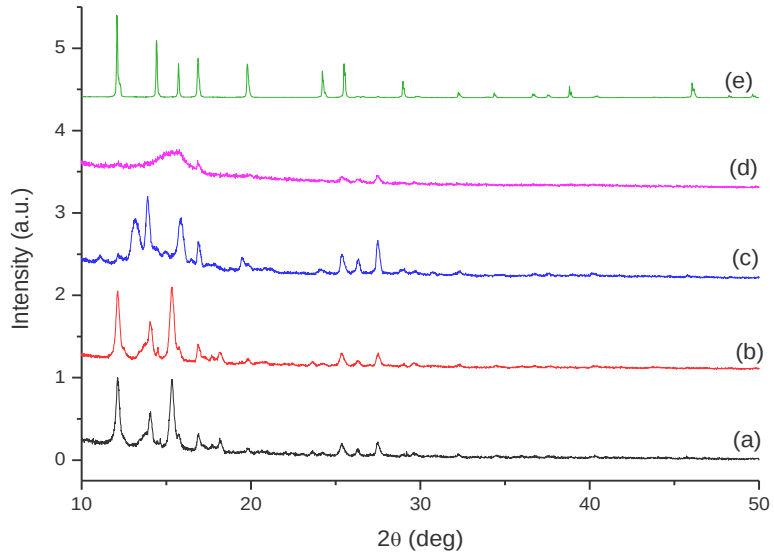
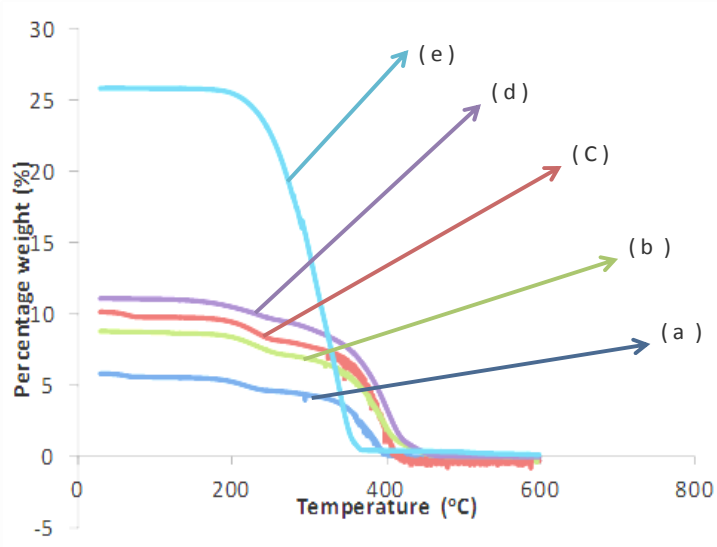
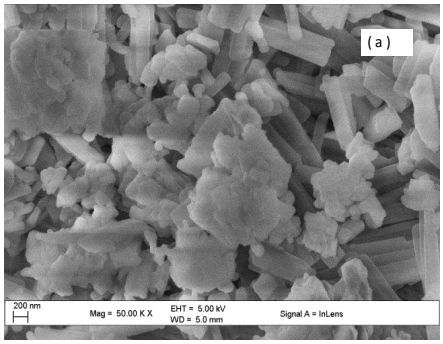
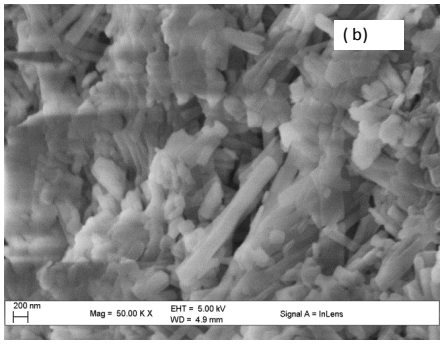


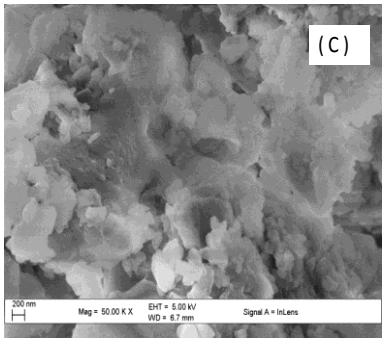
Fig.4.22 The P-XRD pattern of the three synthetic methods OA/INH [SE (d), SD(c), Dg (b)] with OA (a), INH (e)

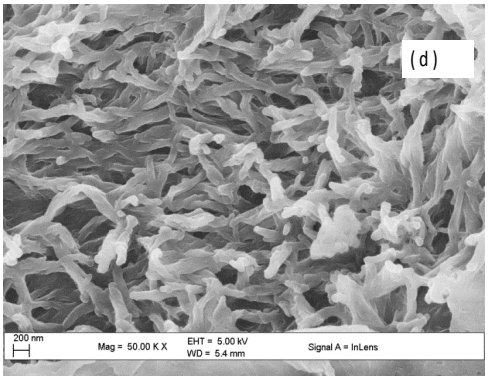


4.23The TGA monograph of the three synthetic methods OA/INH [SE (b), SD(c), Dg (d)] with OA (a), INH (e)









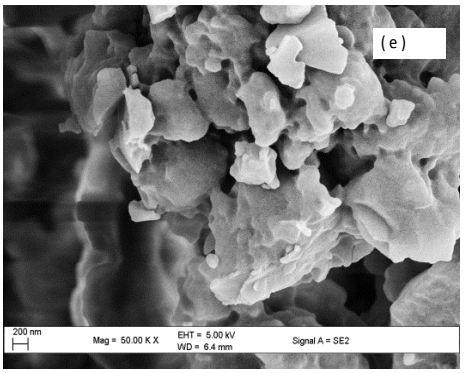


Fig. 4.24 The SEM image of the three synthetic methods OA/INH [SE (b), SD(c), Dg (d)], OA (a) with INH (e)

4. 2.3.2 Comparison of the three co-crystal product of BA/INH from the three synthetic methods along with BA and INH

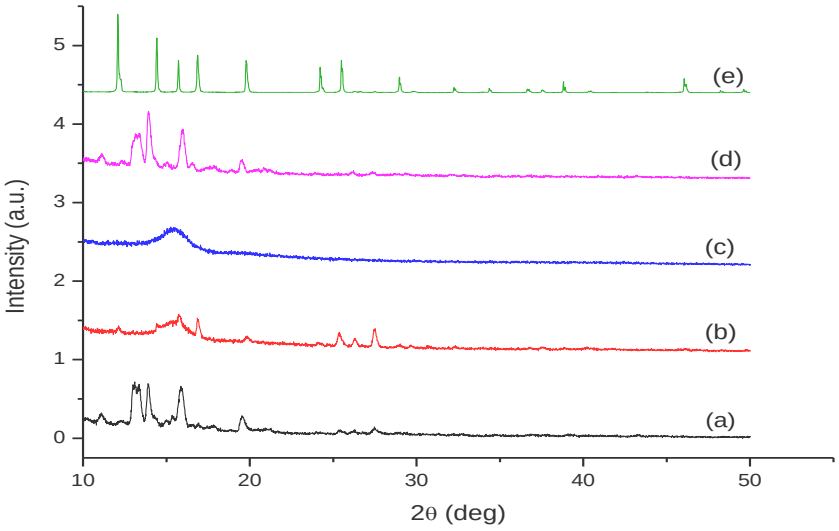


Fig. 4.25: The P-XRD pattern of the three synthetic methods of BA/INH [SE (d), SD (b), Dg (c)] with BA (a) and INH (e)

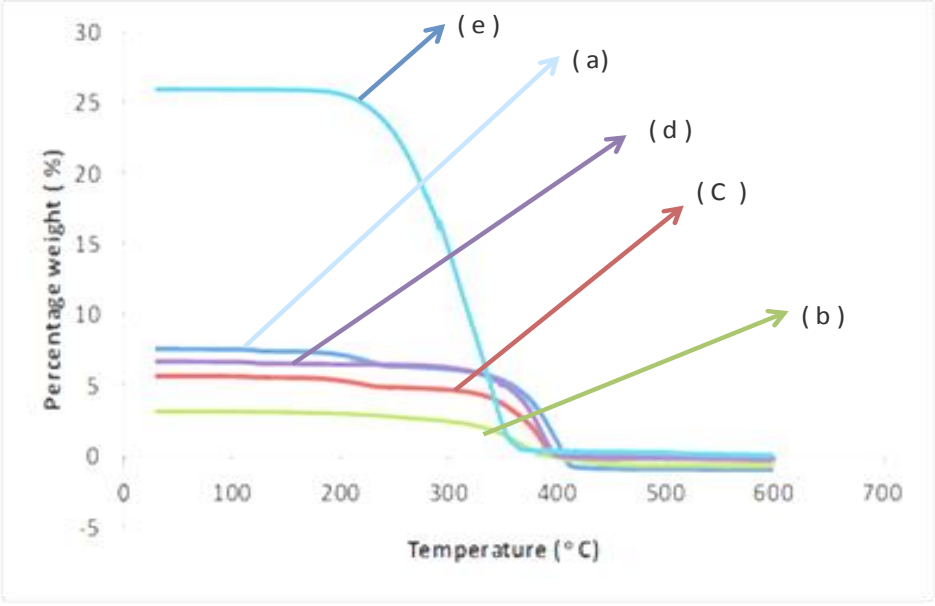


Figure 4.26: The TGA monograph of the three synthetic methods of BA/INH [SE (d), SD (b), Dg (c)] with BA (a) and INH (e)

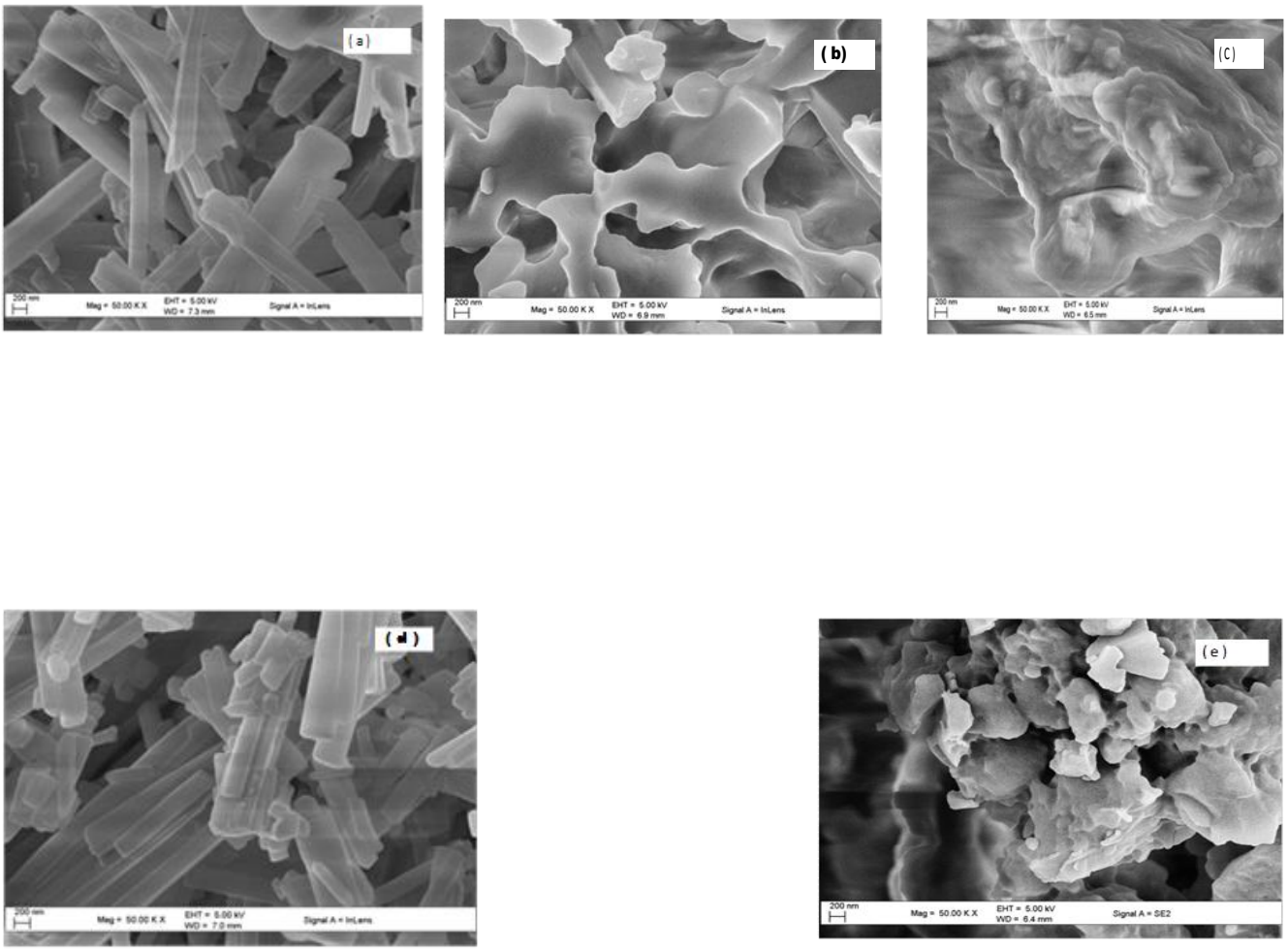


Fig. 4.27 The SEM images of the three synthetic methods of BA/INH [SE (d), SD (b), Dg (c)] with BA (a) and INH (e)

4.3 Biological activities

The results of the bio-activity (anti-TB, cytotoxicity, and DNA poly-β inhibition) of the triterpenes and their derivatives are presented in Tables 4.10 to 4.24

Table 4.10, summarizes the responses of the four (Hexane, dichloromethane, acetone and ethanol) extract to MABA Test. The results showed that the ethanol extract is the most active with MIC of 22.20 ug/ml, and all the four extracts were not as active as the reference compounds used.

Table 4.10: Anti-TB of crude extracts of C*urtisia dentata*

Compound ID	Amount(mg)	Stock(mg/ml)	$H_{37}RV$/(MABA) MIC(ug/ml)
Hexane extract	6	5	>50
Methylene chloride extract	5.5	5	19.8
Acetone extract	7	5	>50
Ethanol extract	12.2	5	22.20
Reference compound			**uM**
RMP			0.02
SM			0.25
TMC207			0.02

The results obtained, when OA and its derivatives (Table 4.11) and BA and its derivatives (Table 4.12) were tested for anti-TB activity indicate that OA's activity is lower than the crude ethanol extract, and the activity was even lowered with the chemical modification of OA. Even though BA did not show any appreciable anti-TB

activity, BAA-CA exhibited considerable activity, comparable to the crude ethanol extract. In general all the compounds had higher MIC values when compared with the reference compounds.

Table 4.11: Anti-TB of OA, its derivatives and cinnamic acid

Compound ID	MW/(g/mol)	Amount /(mg)	Stock/(mg/ml)	H_{37}RV/(MABA), MIC**(uM)**
Oleanolic acid	456	7.4	5	42.04
Oleanolic acid ester	498	2.4	5	>100.26
Oleanolic acid-cinnamic acid	586	8.35	5	48.05
Oleanolic acid ester-cinnamic acid	628	10.85	5	>79.51
Cinnamic acid	148	5.0	5	>337.50
Reference compounds				**uM**
RMP				0.02
SM				0.25
TMC207				0.02

Table 4.12: Anti-TB of BA, its derivatives and its cinnamic acid

Compound ID	MW/(g/mol)	Amount /(mg)	Stock/(mg/ml)	$H_{37}RV$/(MABA), MIC**(uM)**
Betulinic acid	456	3.6	5	>109.48
Betulinic acid ester	498	5.8	5	39.70
Betulinic acid-cinnamic acid	586	9.25	5	>85.20
Betulinic acid ester-cinnamic acid	628	7.9	5	17.88
Cinnamic acid	148.	5.0	5	>337.50
Reference compounds				**uM**
RMP				0.02
SM				0.25
TMC207				0.02

The co-crystallization of OA (Table 4.13) and BA (Table 4.14) with INH were carried out successfully and the anti-TB activities of both co-crystals were carried out as well. In all, it was observed that the anti-TB co-crystals of BA with INH had a more efficient activity than OA with INH co-crystals. Both complexes had close similar behavior when subjected to heat/ water / moisture as observed in P-XRD, TGA and SEM images obtained. The co-crystallization by the co-grinding method resulted in a more enhanced activity.

Table 4.13: Anti-TB of co-crystal synthesis of OA with isoniazid (INH)

Compound ID (Preparation methods)	MW/(g/mol)	Amount/ (mg)	Stock/(mg/ml)	$H_{37}RV$/(MABA) MIC**(uM)**
Solvent evaporation	593	2.3	5	1.06
Solvent drop	593	2.2	5	0.50
Co-grinding	593	4	5	0.61
INH	137	10.2	5	2.04

Table 4.14: Anti-TB of co-crystal synthesis of BA with isoniazid (INH)

Compound ID (Preparation methods)	MW/(g/mol)	Amount/(mg)	Stock/(mg/ml)	$H_{37}RV$/(MABA) MIC**(uM)**
Solvent evaporation	593	1.5	5	0.56
Solvent drop	593	1.1	5	0.51
Co-grinding	593	1.9	5	0.45
INH	137	10.2	5	2.04

The results of the effect of OA and its derivatives (Table 4.15) and BA and its derivatives (Table 4.16) indicate a concentration dependent inhibition of DNA polymerase β activity of both compounds. It is apparent that BA and its derivatives were stronger as DNA polymerase β inhibitor than the OA and its derivatives.

Table 4.15: DNA polymerase β activity of OA and its derivatives

Compound ID	10/ng/ml	100/ng/ml	500/ng/ml
Oleanolic acid	70.34	59.47	47.28
Oleanolic acid ester	50.05	64.11	71.69
Oleanolic acid –cinnamic acid	56.28	57.78	53.11
Oleanolic acid ester-cinnamic acid	53.70	59.55	61.54

Table 4.16: DNA polymerase β activity of BA and its derivatives

Compound ID	10/ng/ml	100/ng/ml	500/ng/ml
Betulinic acid	80.45	68.23	60.51
Betulinic acid ester	72.50	79.38	75.53
Betulinic acid-cinnamic acid	51.16	49.61	46.78
Betunlinic acid ester-cinnamic acid	54.44	60.07	58.23

The results of the cytotoxicity of the compounds on the two cell lines (HEK 293 and HepG2) are presented in Tables 4.17 to 4.24. With $IC_{50} \geq 300\mu g$ it is apparent that the compounds have low cytotoxicity.

Table 4.17: Toxicological effect on human embryonic kidney (HEK293) on cinnamic acid hybrid of oleanolic acid and its derivatives

	OA	OA-CA	OAA	OAA-CA
Control-only cells	0.693 +/- 0.036	0.92 +/-0.019	0.958+/-0.021	0.92+/-0.019
50 ug	13.28%	7.72%	11.95%	14.13%
100 ug	27.27%	17.72%	14.93%	15.43%
150 ug	38.81%	24.67%	18.06%	19.57%
200 ug	43.58%	32.39%	21.92%	23.59%
250 ug	39.39%	37.07%	29.65%	28.48%
300 ug	44.59%	45.43%	40.92%	34.89%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.18: Toxicological effect on human embryonic kidney (HEK293) on cinnamic acid hybrid of betulinic acid and its derivatives.

	BA	BA-CA	BAA	BAA-CA
Control –only cells	0.958+/-0.021	0.92+/-0.019	0.95+/-0.021	0.92+/-0.014
50 ug	10.33%	11.96%	5.01%	8.98%
100 ug	14.61%	15.43%	10.54%	13.37%
150 ug	14.82%	23.80%	19.83%	20.0%
200 ug	21.09%	30.87%	25.26%	24.13%
250 ug	30.38%	38.04%	25.68%	25.65%
300 ug	37.16%	45.65%	32.46%	30.43%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.19: Toxicological effect on human hepatocellular (HepG2) on cinamic acid hybrid of oleanolic acid and its derivatives

	OA	OA-CA	OAA	OAA-CA
Control-only cells	0.673 +/- 0.027	0.868 +/-0.014	0.826+/-0.019	0.868+/-0.014
50 ug	13.97%	5.76%	1.69%	91.94%
100 ug	20.80%	16.13%	4.84%	24.77%
150 ug	25.26%	21.08%	9.81%	27.42%
200 ug	30.76%	28.80%	15.50%	38.71%
250 ug	35.07%	37.67%	18.40%	47.58%
300 ug	40.56%	47.24%	28.33%	56.91%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.20: Toxicological effect on human hepatocellular (HepG2) on cinnamic acid hybrid of betulinic acid and its derivatives

	BA	BA-CA	BAA	BAA-CA
Control-only cells	0.826+/-0.019	0.868+/-0.014	0.826+/-0.019	0.868+/-0.014
50 ug	4.24%	17.97%	1.57%	7.95%
100 ug	9.81%	26.38%	9.20%	16.94%
150 ug	18.77%	32.4%	17.31%	20.97%
200 ug	26.91%	38.71%	25.79%	27.76%
250 ug	32.57%	60.60%	28.93%	37.90%
300 ug	38.62%	55.18%	38.01%	47.58%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.21: Toxicological effect on human embryonic kidney (HEK293) on oleanolic acid- isoniazid (INH) complexes.

	Solvent evaporation	Solvent drop	Co-grinding
control-(only cells)	0.958 +/-0.021	0.958 +/- 0.021	0.958 +/- 0.021
50 ug	4.91%	10.54%	4.70%
100 ug	11.17%	16.28%	9.08%
150 ug	10.44%	17.22%	16.60%
200 ug	18.37%	21.82%	23.70%
250 ug	-	-	27.14%
300 ug	-	-	35.28%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.22: Toxicological effect on hepatocellular (HepG2) on oleanolic acid- isoniazid (INH) complexes.

	Solvent evaporation	Solvent drop	Co-grinding
control-(only cells)	0.826 +/-0.019	0.826 +/- 0.019	0.826 +/- 0.019
50/ug	8.47%	11.74%	0.36%
100/ug	22.40%	15.74%	3.15%
150/ug	25.42%	23.12%	7.51%
200/ug	36.32%	28.45%	14.53%
250/ug	-	-	22.88%
300/ug	-	-	32.08%
IC_{50}	>300/ug/ml	>300/ug/ml	>300/ug/ml

Table 4.23: Toxicological effect on human embryonic kidney (HEK293) on betulinic acid- isoniazid (INH) complexes.

	Solvent evaporation	Solvent drop	Co-grinding
control-(only cells)	0.958 +/-0.021	0.958 +/- 0.021	0.958 +/- 0.021
50 ug	21.32%	10.33%	5.85%
100 ug	17.43%	14.61%	6.26%
150 ug	23.07%	14.82%	9.60%
200 ug	27.35%	21.08%	16.60%
250 ug	34.68	-	21.81%
300 ug	39.87	-	27.56%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml

Table 4.24: Toxicological effect on hepatocellular (HepG2) on betulinic acid- isoniazid (INH) complexes

	Solvent evaporation	Solvent drop	Co-grinding
control-(only cells)	0.826 +/-0.019	0.826 +/- 0.019	0.826 +/- 0.019
50 ug	6.62%	3.27%	11.99%
100 ug	21.67%	7.38%	19.98%
150 ug	28.45%	19.01%	33.17%
200 ug	39.95%	27.97%	40.44%
250 ug	44.55%	-	48.43%
300 ug	48.43%	-	50.24%
IC_{50}	>300 ug/ml	>300 ug/ml	>300 ug/ml

CHAPTER 5
DISCUSSION

5.1. Anti-TB activities for extracts, isolated BA/OA, their 3-O-acetyl and 28-cinnamic acid derivatives

The microbial resistance of various strains against common antibiotics, especially in developing countries has resulted in undue pressure on some antibiotic for the treatment of infectious diseases like tuberculosis. Plants have for long been identified as a source of reservoir of underutilized bioactive compounds with good profile that can be easily used as drugs (De et al., 2011).

Several varieties of extracts, essential oils and compounds of South Africa medicinal plants have been investigated for antimycobacterial activity (Lall etal., 1999; Mativandlela et al., 2008; McGaw et al., 2008; Green et al., 2011; Masoko et al., 2013).

The result obtained in this study (Table 4.10) indicates that the leaves of *Curtisia dentata* possess anti-TB activity, albeit, to low efficacy. This possibly justifies the use of the plant by the rural dweller to treat tuberculosis and other infectious diseases.

Betulinic acid **(15)** and oleanolic acid **(17)** are plant metabolites that have been studied for extensive use in medicine for a long time. Both compounds have been reported to possess broad spectrum of biological activities such as anti-inflammatory, antioxidant, anticancer, antibacterial and hepatoprotective effects (Jesus et al., 2015; Fontanay et al., 2008; Cerga et al., 2011; Yu et al., 2015; Bori et al., 2012).

The two triterpenes betulinic acid **(15)** and oleanolic acid **(17)** however showed poor activity in inhibiting the growth of the mycobacterium (Tables 4.11 and 4.12). It is

apparent that the active component of *C dentata* is not any of the isolated triterpene, but possibly other phytochemicals that could be present in low amounts that act synergistically. The literature indicate that betulinic acid **(15)** and oleanolic acid **(17)** exhibit moderate antimycobacterial activity (Watcher et al., 1999; Cantrell et al., 2001; Copp & Pearce et al., 2007; Okunade et al., 2004).

Chemical modification of drugs is known to increase the effectiveness of drugs. For example, the chemical modification of ursolic acid **(24)** to its acetate derivative is reported to have resulted in a drastic increase in the anti-malaria activity (Innocente et al., 2012; Simelane et al, 2013). *Trans*-cinnamic acid and its derivatives, INH **(3)** and rifamycin have been separately identified as the first set of TB drugs that have been utilized for a long time (De et al., 2011; Rastogi et al., 1998). The anti-TB activities of the hybrid product of the two TB drugs (INH **(3)** and rifampicin) with trans-cinnamic acid were better than the original two TB drugs themselves under consideration (Rastogi et al., 1998).

Tanachatchairatana et al., (2008), synthesized a cinnamic acid **(9)** hybrid of betulinic acid **(15),** oleanolic acid **(17)** and ursolic acid **(9)** at C^{-3} position and evaluated their antimycobacterial activity. The observed antimycobacterial activity obtained were poor (MIC>200 ug/ml) which indicated a loss in biological activity, further corroborating the earlier result of Watcher et al., (1999), that free OH group in C^{-3} position is a necessary condition for antitubercular activity of the triterpenes.

Targeting carbon positions C^{-3} and C^{-28} has been reported as suitable pharmacophores that promote increase in biological activity in triterpenes (Ban et al., 2010) and this depend on the biological activity being considered. For antimycobacterial, OH group at

the C^{-3} position is a must for good activity. The OH group close on the carbonyl functional group of the carboxylic acid may not have been investigated for its specific role in the antimycobacterial activity of the terpenoids.

The OH groups in position C^{-3} and C^{-28} positions are therefore the most favoured possible points of attack by the generated cinnamic acid chloride. The use of reagents plays a significant role in the direction of the reaction will go. Two different set of reagent have been used by two authors; RCOCl (TEA) DCM, Rt/24hrs by Habila et al., (2011) , while Tanachairatana et al., 2008 used RCOCl (DMAP) BENZENE/60^{O}C/1hr.

On critical appraisal of these two reaction medium, it is observed that the two major factors responsible for this disparity in the reaction system is the orientation position of the two different hydroxyl groups: The 3-OH group is hindered at β orientation position when both compounds betulinic acid **(15)** and oleanolic acid (**17**) are isolated from nature, while the 28-OH group is slightly acidic due to the presence of the carbonyl function group from the acid functional group. This therefore affects the rationale behind the use of the different reagents by the different schools of thought. In this study, and since the desire is to attach cinnmaic acid to C-28 position, the Habila et al. (2011) protocol was followed i.e. to utilize the C-28 position of both isolated betulinic acid **(15)** and oleanolic acid **(17)** along with their acetate for the molecular hybridization with cinammic acid **(9).**

As at the time of this report, this is the first time the anti-TB activity of the cinnamic acid hybrid of oleanolic acid, betulinic acid and their acetate is being reported (Tables 4.11 and 4.12).

5.2. Co-crystal complexes of BA and OA with INH

From the perspective of drug combination regimen commonly adopted in the treatment of other infectious diseases and tuberculosis, the synthesized co-crystal complexes may be an interesting pharmaceutical product which may likely arise from the synergistic activity of the component molecules. The P-XRD, TGA and SEM (Figures 4.10 - 4.19) images obtained confirm that the co-crystals were successful to some extent; the yield and purity levels were determined as well to an appreciable level in all.

Tables 5.1 and 5.2 provide information on the shape of the image of the crystalline product obtained. The cleavage temperatures for the crystals were reported, this help to suggest the possibility of improvement in the solubility of the co-crystals as against the starting materials.

The shape of the crystals will provides information on the lattice parameter of the co-crystals compounds that was formed.

What is easily observed in the two tables, shows that the complexes from the two compounds are independent of the synthetic methods used in its preparation simply because the cleavage occur nearly in the same range of the temperature. The shape of the crystal in all the synthetic methods are similar.

Table 5.1: Oleanolic acid-INH complex characterization

	Solvent evaporation (SE)	Solvent drop (SD)	Direct grinding (Dg)
PXRD	(Fig 4.5) Maintain similar crystalline nature like INH	(Fig 4.8) Maintain similar crystalline nature like INH	(Fig 4.11) Maintain similar crystalline nature like INH
TGA	(Fig 4.6) The cleavage occurs from 220^{o} -360^{o} C	(Fig 4.9) The cleavage occurs from 220^{o} -360^{o} C	(Fig 4.12) The cleavage occurs from 220^{o} -360^{o} C
SEM	(Fig 4.7) The images obtained appears rod-like in nature	(Fig 4.10) The images obtained appears rod-like in nature	(Fig 4.13) The images obtained appears rod-like in nature

Table 5.2: Betulinic acid-INH complex characterization

	Solvent evaporation (SE)	Solvent drop (SD)	Direct grinding (Dg)
PXRD	(Fig 4.5) Maintain similar Crystal-line nature like INH	(Fig 4.8) Maintain similar crystalline nature like INH	(Fig 4.11) Maintain similar crystalline nature like INH
TGA	(Fig 4.6) The cleavage occurs from 220^{o} -360^{o} C	(Fig 4.9) The cleavage occurs from 220^{o} -360^{o} C	(Fig 4.12) The cleavage occurs from 220^{o} -360^{o} C
SEM	(Fig 4.7) The images obtained appears rod-like in nature	(Fig 4.10) The images obtained appears rod-like in nature	(Fig 4.13) The images obtained appears rod-like in nature

The above result is expected since the compounds involved are organic compounds and water does not really have any serious effect once the compounds are dried before the analysis is carried out. Most organic compounds do have similar behavior when subjected to PXRD, TGA and SEM (Sharma et al., 2014; Song et al., 2014; Yu et al., 2015).

From table 4.11, which summarizes the anti-TB activity of oleanolic acid and its derivatives, the structure activity relationship (SAR) studies based on the antimycobacterial activity in a standard minimum inhibitory concentration (MIC) assay, indicated a loss from oleanolic acid (MIC=42.04 uM) to oleanolic acid acetate (MIC>100.26 uM).

This confirms the essentiality of OH group for antitubercular activity in triterpenes (Watcher et al., 1999). The cinnamic hybrid of the oleanolic acid and its acetate followed a similar pattern due to the presence of free OH at C^{-3} position which may have conferred antituberculosis activity advantage on the cinnamic-oleanolic acid (MIC= 48.05 uM) over the cinnamic- oleanolic acid acteate (MIC>79.51 uM). The melting point of the compounds decreased in the order of OA>OAA.>OA-CA>OAA-CA. This suggests that oleanolic acid's solubility is enhanced when a water loving moiety is attached to C^{-3} and C^{-28} position (Innocente et al., 2012). In all, the antimycobacterial activity of the compound is less than the antimycobaterial activity of all the reference compounds.

The structure activity relationship (SAR) studies of betulinic acid and its derivatives (Table 4.12) however, indicated an increase from betulinic acid (MIC>109.48 uM) to betulinic acid acetate (MIC=39.70 uM). This contradicts the earlier assumption that OH

moiety group is essential for antitubercular activity in triterpenes. There may be other explanations for this which may involve the presence of Michael acceptor moiety formation at C^{-28} carbon and cinnamic acid. This has been established to help trap an active intermediate in the biological cycle (Tiruveedhula et al., 2013). The rigidity of the Michael acceptor moiety may be observed more in betulinic acid than in the case of the oleanolic acid. Another factor worth considering is the difference in ring E of both oleanolic acid (6 member rings) and betulinic acid (5 member rings). The presence of the double bond between C^{-11} and C^{-12} in oleanolic acid while in betulinic acid is between C^{-28} and C^{-29}, and could also be a contributing factor worth mentioning. The cinnamic hybrid of the betulinic acid **(15)** and its acetate followed similar pattern because the presence of free OH at C^{-3} position does not confer antitubercular activity advantage on the cinnamic-betulinic acid (MIC>85.20 uM) over the cinnamic- betulinc acid acteate (MIC=17.88 uM) as expected. The melting point of the compounds decreased in the order of BA>BAA.>BA-CA>BAA-CA. These suggest that betulinic and oleanolic acid's solubility is enhanced when a water loving moiety is attached to C^{-3} and C^{-28} position. In all, the antimycobacterial activity of the compound is less than the antimycobaterial activity of all the reference compounds. Based on SWOT (Strength, Weakness, Opportunity, and Threat) analysis (Table 5.3), the 3-O-acetyl-28-cinnamic acid hybrid of betulinic acid (Figure 4.8) was the most potent as antimycobacterial agent of the entire synthesized compound

Table 5.3 SWOT Analysis

Strength	Weakness
• The *in vitro* study as anti-TB agent indicated to have moderate activity. • The study can be extend for malaria, cancer, HIV and HIV/TB co-infection • Non toxic in vitro	• No report on the in vivo study • No report on the mechanism of action • No report on the in vivo cytotoxicity
Opportunities	**Threats**
• The phenyl rings offer possibility for synthesis of other derivatives • Series of esters substituent at C^{-3} position may be investigated for potency against other diseases • The C_{20}=C_{29} offer potential site for further reactions	• Unknown at this stage

The cytotoxicity of the test compounds (Tables 4.17 to 4. 20) indicate IC_{50} values of >300 ug/ml which make them to be considered safe. Sahranavard et al. (2009) reported that a compound is assumed toxic if the IC_{50} is about 100 ug/ml or less. It is apparent that, when dilution in the bloodstream is taken into account, the triterpenes and their derivatives should be considered as non-toxic.

5.3. DNA polymerase β Inhibitory activity.

DNA polymerase β inhibition activity DNA repair is an essential multi-pathway process that protects the integrity of DNA. DNA polymerase β (pol β) is one of the enzymes

responsible in the base excision repair (BER) pathway (Kornberg and Baker, 1991). Pol β is therefore one of the targets for agents (drugs) that are aimed at destroying the DNA of invasive cells, e.g. cancer cells (Hasima and Aggarwal, 2012). This is consistent with the belief that the inhibition of pol β should enhance the cytotoxicity of DNA damaging agents used in chemotherapy. Rifampicin, a drug used in the treatment of TB targets RNA polymerase β (Shehzad et al., 2013). Mispyric acid, a triterpene isolated from *Mischocarpus pyriformis* (Mizushina et al, 2005), betulinic acid **(15)** and oleanolic acid **(17)** (Gao et al., 2008), and some other triterpenes (Murakami et al, 2002) have been established as strong inhibitors of DNA polymerase. Tables 4.15 and 4.16 indicate that the pure oleanolic acid **(17)** and betulinic acid **(15),** and their acetate and cinnamic acid **(9)** hybrid derivatives inhibited pol β in a concentration dependent manner.

Even though the studied triterpenes, betulinic acid **(15),** oleanolic acid **(17)** and their derivatives (acetate at C^{-3} position and cinnamic hybrids at C^{-28} position) and co-crystals with INH did not show activities higher than those of existing anti-TB drugs (e.g. INH), the results of cytotoxicity and the inhibition of pol β suggest that the compounds could be explored as lead for anti-TB drugs. Due to the flexible nature of the synthesized compound in term of the pharmcophore, more derivable products can be synthesized as well.

Chapter 6
Conclusion

Medicinal plants are rich sources of biologically active compounds (phytochemicals) often referred to as secondary metabolites. They are either individually or synergistically responsible for the various therapeutic properties of medicinal plants. Plants-derived triterpenes exhibit a wide spectrum of biological activities.

In this study, triterpenes, betulinic acid **(15)** and oleanolic acid **(17)** were isolated and characterized from medicinal plants. The isolated triterpenes along with their chemical derivatives were screened for bio-activity. Molecular hybridization was also employed as a tool for the development of potent antitubercular drugs from hybrid of cinnamic acid **(9)** and the betulinic acid **(15)** and oleanolic acid **(17)** (Dutra, et al 2012).

Even though the triterpenes exhibited little anti-TB activity, the conjugation at C^{-28} position of betulinic acid **(15),** oleanolic acid **(17)** and their 3-O-acetyl derivatives with cinnamic acid offered a direction of modification of both compounds as antimycobacterial agents. The preliminary result obtained from this work show that the synthetic reaction is probable with better anti-TB activity when compared with the anti-TB activity of starting materials betulinic acid **(15),** oleanolic acid **(17)** and cinnamic acid **(9).**

The advantage that is derivable from this study is the possibility of activating the broad spectrum of biological activities associated with triterpenes which could be harnessed to develop a drug lead for a multi-target approach as against the single target approach in the treatment of tuberculosis easily without any difficulty.

6.1 The limitations

Most plant derived biological active compounds are very difficult to isolate in pure forms in large quantity. Due to the smaller yields of the compounds, it was a bit difficult to carry out some more experiments as expected.

6.2 Suggestions for further studies

The synthesized bioactive compound should be subjected to the following studies to provide adequate information on the compounds:-

- Thorough *in vivo* studies to include its bioavailability studies.
- Mechanism of action of the active compound should be elucidated.
- The active compounds should be evaluated for the treatment of other diseases such as TB/HIV and Malaria/HIV.

REFERENCES

Ahmad, F. B. H., Ghaffari, M., Basri, M., and Abdul Rahman, M. B. (2010). Anticancer activity of 3-0-Acetyl-betulinic acid: An Antitumor Reagent. *Asian Journal* of *Chemistry,* **22**: 3186-3192.

Aiko, V., and Mehta, A. (2013). Inhibitory effect of clove *(Syzygium aromaticum)* on the growth of *Penicillium citrinum* and citrinin production. *Journal* of *Food Safety,* **33**:440- 444.

Ataguba, J.E., Akazili, J., and Di McIntyre,D.(2011). Socioeconomic-related health inequality in South Africa: evidence from General Household Surveys. *International Journal for Equity in Health,* **10**:10-48.

Aparna, Y., Kranthi, S.U., and Sarada, J. (2014). *Syzygium aromaticum*- A common food spice with potential quorom quenching activity on *Serratia sps* YAJS. *International Journal of Current Microbiology and Applied Sciences*, **3**: 93-102.

Arya, V. (2011). A Review on Anti-tubercular plants. *International Journal of Pharmaceutical and Technology Research*, **3**:872-880.

Alqahtani, A., Hamid, K., Kam, A., Wong, K.H., Abdelhak, Z., Razmovski-Naumovski, V., Chan, K., Li, KM., Groundwater, P.W., and Li, G.Q. (2013). The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. *Current Medicinal Chemistry,* **20**: 908-931.

Avasthi, S., Gautam, A.K., and Bhadauria, R. (2010). Antifungal activity of plant products against *Aspergillus niger.* A potential application in the control of a spoilage fungus. *Biological Forum- An International Journal,* **2**: 53-55.

Asif, M. (2012). A Review of Antimycobacterial Drugs in Development. *Mini-Reviews in Medicinal chemistry*, **12**: 1404-1418.

Ayob, M. K., and Tan, Y. N. (2013). Natural products-current and future promising source of novel drugs: A review on their antimicrobial mechanism of actions. *Microbiology*, **4**:1196 - 1208.

Babu, J.M.S., Sevukarajan, M., Thamizhvanan, K., Naveenkumar, B., Reddy, S. B., Vivekananda,U., and Shyamkumar, V.,(2013). Evaluation of physiochemical and anti- tubercular activity of co- crystal of isoniazid with Methyl Paraben. *International Journal of Innovative Drug Discovery*, **3**: 10-27.

Ban, H.S., Minegishi, H., Shimizuk, K., Maruyama, M., Yasui, Y., and Nakamura, H. (2010). Discovery of carbrances as inducers of 20S proteasome activity. *ChemMedChern*. **5**, 1236 - 1240.

Baker, D. D., Chu, M., Oza, U., and Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. *Natural Product Reports,* **24**: 1225-1244.

Baltas, M., De, P., and Bedos-Belval, F. (2011). Cinnamic acid derivatives as anticancer agents- A Review. *Current Medicinal Chemistry,* **1**: 1672-1703.

Battle, M., and Atkinson, J. (2011). "Fundamentals and applications of co-crystal methodologies: reactivity, structure determination, and mechanochemistry" PhD (Doctor of Philosophy) thesis, University of lowa.http://ir.uiowa.edu/etd/1197.

Bishayee, A, Ahmed, S., Brankov, N., and Perloff. M. (2011). Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer (triterpeniod with several biological activities). *Frontiers in Bioscience,* **16**:980-996

Bhowmik, D., Kumar, K.P.S., Yadav, A., Srivastava, S., Paswan, S., Dutta, A.S.(2012). Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. *Journal* of *Pharmacognosy and Phytochemistry,* **1**: 13-22

Bori, I. D., Hung, H.-Y., Qian, K., Chen, C.-H., Morris-Natschke, S. L., and Lee, K.-H. (2012). Anti-AIDS agents 88. Anti-HIV conjugates of betulin and betulinic acid with AZT prepared via click chemistry. *Tetrahedron Letters, **53**:1987-1989.*

Bosquesi, P. L., Melo, T. R. F., Vizioli, E. O., Santos, J. L. Dos, and Chung, M. C. (2011). Anti-Inflammatory drug design using a molecular hybridization approach. *Pharmaceuticals,* **4**: 1450-1474.

Brown, R. (2012)." Investigating pharmaceutical co-crystals as a means to improve the stability of a drug" M.Sc (Master of Science) thesis, University of Kansas.

Brusotti, G., Cesari, I., Dentamaro, A, Caccialanza, G., and Massolini, G. (2014). Isolation and characterization of bioactive compounds from plant resources: the role of analysis in the ethnopharmacological approach. Journal of Pharmaceutical and Biomedical Analysis, **87**: 218-28.

Bryantfurlow, B. A (2010). Tuberculosis. Radiologic Technology, **82**: 33-55

Bryde, L., and Waheed, U. (2013). Infectious diseases in developing countries. Scholars Journal of Applied Medical Sciences, **1**: 286-287

Bs, S. (2009). Pharmaceutical co-crystals - a review. ARS *Pharmaceutica, **50**: 99*- 117

Cai, L., and Wu, C.D. (1996). Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. *Journal of Natural Product,* **59** : 987-990.

Cantrell, C. L., Franzblau, S. G., and Fischer, N. H. (2001). Antimycobacterial plant terpenoids. *Planta Medica,* **67**: 685-694.

Cerga, O., Borcan, F., Ambrus, R., and Popovici, J. (2011). Synthesis of new cyclodextrin complexes with oleanolic and ursoic acids. *Journal of Agroalimentary Processes and Technologies,* **17**: 405-409.

Cragg, G. M., and Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta*, **1830**:3670-3695.

Collins, L. A, and Franzblau, S. G. (1997). Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrobial Agents and Chemotherapy,* **41**:1004-1009.

Copp B.R., and Pearce, A. (2007). Natural product growth inhibitors of *Mycobacterium tuberculosis*. *Natural product report*, **24**: 278-297.

Cortes-Rojas, D.F., de Souza, C.R.F., and Oliveia, W.P. (2014). Clove *(Syzygium aromaticum):* A precious spice. *Asian Pacific Journal of Tropical Biomedicine,* **4**: 90- 96.

Cosa, P., Vlietinck, A.J., Berghe, D.V., and Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *Journal of Ethnopharmacology,* **106**:290-302.

Cunningham, AB. (1988). An investigation of the herbal medicine trade in NatallKwaZulu. Invesigational report no 29. Institute of Natural Resources. University of Natal. Pietermaritzburg

Coxon, G. D., Cooper, C. B., Gillespie, S. H., and McHugh, T. D. (2012). Strategies and challenges involved in the discovery of new chemical entities during early- stage tuberculosis drug discovery. *The Journal of Infectious Diseases, **205**:* S258-64.

Crick, D. C., Mahapatra, S., and Brennan, P. J. (2001). Biosynthesis of the arabinogalactan-peptidoglycan complex of *Mycobacterium tuberculosis*. *Glycobiology*, **11**: 107-118

Dua, A, Garg, G., Nagar, S., and Mahajan. R. (2014). Methanol extract of clove *(Syzygium aromaticum* Linn) damages cells and inhibits growth of enteropathogens. *Journal of Innovative Biology*, **1**: 200-205.

De, P., Yoya, G. K., Constant, P., Bedos-belval, F., Duran, H., Saffon, N.,Daffe, M., and Baltas, M. (2011). Design, synthesis, and biological evaluation of new cinnamic derivatives as antituberculosis agents. *Journal of Medicinal Chemistry*, **54**:1449-1461.

Du Toit, L. C., Pillay, V., and Danckwerts, M. P. (2006). Tuberculosis chemotherapy: current drug delivery approaches. *Respiratory Research,* **7**: 1-18.

Dutra, L. A., Regina, T., du Melo, T.R.F., Chin, C. M., and do Santos, L.J. (2012). Antitubercular drug discovery: the molecular modification as promise tool. *International Research Journal of Pharmacy and Pharmacology*, **2**: 1-9.

Evora, A. (2011). Pyrazinamide-Diflunisal: A new dual-drug co-crystal. *Crystal Growth and Design*, **11**: 4780-4788.

Ehebauer, M. T., and Wilmanns, M. (2011). The progress made in determining the Mycobacterium tuberculosis structural proteome. *Proteomics,* **11**: 3128-3133.

Enna, S. J. (2014). Phenotypic drug screening. Journal of the Peripheral Nervous System, **19**(supp.,): S2-S9.

Fadipe, V.O., Mongalo, N.L, and Opoku, AR. (2015). *In vitro* evaluation of the comprehensive antimicrobial and antioxidant properties of *Curtisia dentata* (Burm.f) C.ASm: toxicological effect on the human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cell lines. *EXCLI Journal,* **14**:971-983.

Fallah, F., and Abdolghafoorian, H. (2014). The History of Tuberculosis and Bacillus Calmette - Guerin vaccine in Iran. *Archives of Pediatric Infectious Diseases,* **3**:1-5.

Franzblau,S.G., Witzig, R.S., McLaughlin, J.C., Torres, P., Madico, G., Hernandez, A., Degnan, M.T., Cook, M.B., Quenzer, V.K., Ferguson, R.M., and Gilman, R.H. (1998). Rapid, low- technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar blue assay. *Journal of Clinical Microbiology,* **36**: 362-366.

Fonkwo, P. N. (2008). The economic and health implications of infectious diseases. *EMBO Reports,* **9**(S13): 3-7.

Fontanay, S., Grare, M., Mayer, J., Finance, C., and Duval, R. E. (2008). Ursolic, oleanolic and betulinic acids: antibacterial spectra and selectivity indexes. *Journal of Ethnopharmacology,* **120**: 272-276.

Frank, S. (2005). Target-based drug discovery: is something wrong? *Drug Discovery Today,* **10**: 39 -147.

Gao, Z., Maloney, D.J., Dedkova, L.M., and Hecht, S.M. (2008). Inhibitors of DNA polymerase beta: activity and mechanism. *Bioorganic and Medicinal Chemistry,* **16**: 4331-4340.

Gauthier, C., Legault, J., Lavoie, S., Rondeau, S., Trembly, S., and Pichette, A. (2008). Synthesis of two natural betulinic acid saponins containing a-1-Rhamnopyranosyl, a-1- Arabinopyranose and their analogues. *Tetrahedron,* 64:7386- 7399.

Garg, M., Sidhu, N. K.,and Kakarala, K. K. (2014). In-silico analysis of Clp protease catalytic subunit-2 of Mycobacterium tuberculosis: Modeling and docking analysis*. International Journal of Advanced Research,* **2**: 11170-11203.

Ge, F., Zeng, F., Liu, S., Guo, N., Ye, H., Song, Y., Fan, J., Wu, X., Wang, X., Deng, X., Jin,Q., and Yu, L. (2010). In vitro synergistic interactions of oleanolic acid in combination with isoniazid, rifampicin or ethambutol against Mycobacterium tuberculosis. *Journal of Medical Microbiology,* **59**: 567-72.

Ghadi, R., Ghuge, A., Ghumre, S., Waghmare, N., and Kadam, D. V. J. (2014). Co-crystals: Emerging approach in pharmaceutical design. *Indo American Journal of Pharmaceutical Research*, **4**: 3881-3893.

Gilbert, I. H. (2013). Drug Discovery for Neglected Diseases: Molecular Target-Based and Phenotypic Approaches. *Journal of Medicinal Chemistry,* **56**: 7719-7726

Gilbert, I. H. (2014). Target-based drug discovery for human African trypanosomiasis: selection of molecular target and chemical matter. *Parasitology,* **141**: 28-36.

Green, E., Samie, A., Obi, C.L., Bessong, P.O., and Ndip, R.N. (2010). Inhibitory properties of selected South Africa medicinal plants against *Mycobaterium tuberculosis*. *Journal of Ethnopharmacology,* **130**: 151-157

Gupta, M., and Wakhloo, B.P. (2007). Tetrabutylammoniumbromide mediated Knoevenagel condensation in water: Synthesis of cinnamic acids. ARKIVOC, **207**: 94-98

Guzman, J. D. (2014). Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobialactivity*. Molecules,* **19**: 19292-19349

Guzman, J.D., Mortazavi, P.N., Munshi, T., Evangelopoulos, D., McHugh, T.D., Gibbons, S., Malkinson, J., and Bhakta, S. (2014). 2-Hydroxy-substituted cinnamic acids and acetanilides are selective growth inhibitors of *Mycobacterium tuberculosis*. *Medicinal Chemistry Communication,* **5**: 47-50.

Habila, J. D., Shode, F. O., Ndukwe, G. I., Amupitan, J. O., and Nok, A. J. (2011). Novel antimalarial agent (Cinnamic 3β-hydroxyolean-12-en-28-carboxylic anhydride): Synthesis, characterization and in vivo studies. *African Journal* of *Pharmacy and Pharmacology,* **5**: 2667-2675.

Habila,J.D., Shode,F.O., Ndukwe, G.I., Amupitan, J.O., and Nok, A.J. (2012). Effect of C-3 modification of oleanolic acid on *Candida spp.,* Trichophytontonsurans and Microsporum canis Inhibition. *Pharmacologia* **3**: 313-324

Hamed, H.K., Hoseini, E.S., Nikzad, H., and Aarabi, M. H. (2012). Pharmacological properties of medicinal herbs by focus on secondary metabolites. *Life Science Journal,* **9**: 509-520.

Haradhan, K. M. (2015). Tuberculosis is a fatal disease among some developing countries of the World. *American Journal* of *Infectious* Diseases *and Microbiology,* **3**: 18-31.

Hasima and Aggarwal (2012). Cancer-linked targets modulated by curcumin. *International Journal Biochemistry and Molecular Biology,* **3**:328-351.

Hostettmann, K. (1998). Strategy for the biological and chemical evaluation of plant extracts. *Pure and Appied Chemistry,* **70**: 1-9.

Hostettmann, K.A., and Marston A. (1995). Saponins. Chemistry and pharmacology of natural products. Cambridge University Press, Cambridge, United Kingdom. 239-284.

Hossain, S., AbdulQuaiyum, M., Zaman, K., Banu, S., Husain, M.A., Akramul Islam, M., Cooreman, E., Borgdorff, M., Lonnroth, K., Salim, A.H., and Leth, F. (2012). Socio- economic position in TB prevalence and access to services: results from a population prevalence survey and a facility-based survey in Bangladesh. *PloS One,* 7: e44980.

Hutchings, A., Scott, A.H., Lewis, G., and Cunningham, A.B. (1996). Zulu medicinal plants: An inventory. University of Natal Press, pp226-227.

Hughes, J. P., Rees, S., Kalindjian, S. B., and Philpott, K. L. (2011). Principles of early drug discovery. *British Journal* of *Pharmacology,* **162**:1239-49.

Ibezim, E.C., Abonyi, D.O., Adikwu, M.U., and Esimone, C. O. (2009). Plants as sources of antiviral agents. *African Journal of Biotechnology,* **8**: 3989-3994.

Isah, Y., Ndukwe, I.G., Rufai, Y., R.G. and Ayo, R.G. (2014). Characterization and microbial activities of 13-sitosterol and 13-sitosterione mixture isolated from stem bark of methanol fraction of Sarcocephalus Latifolius (Smith Bruce). *International Research Journal of Natural Sciences,* **2**: 1-13.

lshowmik, D., Chandira, R. M., and Pradesh, U. (2009). Recent trends of drug used treatment of tuberculosis. *Journal of Chemical and Pharmaceutical Research*, **1**: 113-133.

Innocente, A.M., Silva, G.N., Cruz, L.N., Moraes, M.S., Nakabashi, M., Sonnet, P., Gosmann, G., Garcia, C.R., and Gnoatto, S.C. (2012). Synthesis and anti-plasmodial activity of betulinic acid and ursolic acid analogues. *Molecules,* **17**:12003-12014.

Jitareanu, A., Tataringa, G., Zbancioc, A.M., Tuchilus, C., Balan, M., and Stanescu, U. (2013). Cinnamic acid derivatives and 4-Aminoantipyrine amides - Synthesis and evaluation of biological properties. *Research Journal of Chemical Sciences*, **3**: 9-13.

Jesus, J.A., Lago, J.H., Laurenti , M. D., Yamamoto, E. S., and Passero, L. F. (2015). Antimicrobial activity of oleanolic and ursolic acids: An update. *Evidence-Based, Complementary and Alternative Medicine*. **2015**:1-14.

Khare, S.K., Kumar, A., and Kuo, T.A. (2009). Lipase-catalysed production of bioactive fatty amide derivatives of 7, 1 0-dihydro-8(E)-Octandecenoic acid. *Bioresource Technology,* **7**:258- 261.

Khewkhom, N., and Shangchote, S. (2009). Postharvest antifungal activity of extracts and compounds from *Cinnamomum zeynalucum, Boesenbergia pandurata* and *Syzygium aromaticum* against *Colletotrichum gloeosporioides* and *Botryodiplodia theobromae. Asian Journal of Food and Agro-Industry, Special Issue,* 125-132.

JKolyva, A. S., and Karakousis, P. C. (2009). Old and New TB Drugs: Mechanisms of Action and Resistance, Understanding Tuberculosis-New approaches to fighting against drug resistance, Dr Pere-Joan Cardona (Ed.), ISBN: 978-953-307-948-6, . Science, Technology, and Medicine open access publishers.

Konthur, R. R., Swetha, A. S., and Bondili, N. P. (2012). An outline of crystal engineering of pharmaceutical co-crystals and applications: A Review. *International Journal of Pharmaceutical Research and Development,* **4**: 84-92.

JKotz, J. (2012). Phenotypic screening, take two, 1-3.Science –Business eXchange, **5**: 1-3.

Kornberg, A., and Baker, T. (1991). DNA replication, (2nd Edn), W. H Freeman & Company, New York.

Kumar, P., Rawat, P., and Singh, P. (2013). Medicinal chemistry and development of antitubercular drugs. *International Journal of Pharmaceutical Sciences and Research,* **4**: 2080-2093.

Lawn, S.D., Brools, S.V., Kranzer, K., Nicol, M.P., Whitelaw, A., Vogt, M., Bekker, L. and Wood, R. (2011). Screening for HIV-associated tuberculosis and rifampicin resistance before antiretroviral Therapy Using the Xpert MTB/RIF Assay: A prospective study. PLoS Med 8: e1001067.

Lall, N., and Meyer, J.J.M. (1999). In vitro inhibition of drug-resistant and drug-sensitive strains of Mycobacterium tuberculosis by ethnobotanically selected South African plants, Journal *of Ethnopharmacology*, **66**: 347-354.

Leandro do Santos, J., Dutra, L.A., Ferreira de Melo, T.R., and Chin, C.M. (2012). New antitubercular drugs designed by molecular modification. In: *Understanding Tuberculosis- New Approaches to fighting against drug resistance,* Dr. Pere-Joan Cardona(Ed), ISBN: 978-953-307-948-6, Science, Technology, and Medicine open access publishers.

Lone, R., Shuab, R., and Koul, K. K. (2014). Role of cinnamate and cinnamate derivatives in pharmacology. *Global Journal of pharmacology,* **8**:328-335.

Ma, Z., Lienhardt, C., McIlleron, H., Nunn, A.J., and Wang, X. (2010). Global tuberculosis drug development pipeline: the need and the reality. *Lancet,* **375**: 2100-2109.

McGaw, L. J., Lall, N., Meyer, J. J. M., and Eloff, J. N. (2008). The potential of South African plants against Mycobacterium infections. *Journal of Ethnopharmacology,* **119**: 482-500

Masoko, P., and Nxumalo, K.M. (2013). Validation of antimycobacterial plants used by traditional healers in three districts of the Limpopo Province (South Africa). *Evidence- Based complementary and Alternative Medicine*, **2013**:1-7.

Mativandlela, S. P. N., Meyer, J. J. M., Hussein, A. A., Houghton, P. J., Hamilton, C. J., and Lall, N.(2008). Activity against Mycobacterium smegmatis and M. tuberculosis by extract of South African medicinal plants. *Phytotherapy Research,* **22**: 841-845.

Mahata, G., Dey, S., and Chanda, J. (2014). Crystal Engineering: A Powerful Tool towards Designing Pharmaceutical Solids with Desirable Physicochemical Properties. *American Journal of Drug Discovery,* **1**: 1-9.

Mahomoodally, M. F. (2013). Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants. Evidence-Based Complementary and Alternative Medicine, **2013**:1-15.

Manjunatha, U. H., and Smith, P. W. (2014). Perspective: challenges and opportunities in TB drug discovery from phenotypic screening. *Bioorganic* and *Medicinal Chemistry*

Medina-Franco, J. L., Giulianotti, M. A., Welmaker, G. S., and Houghten, R. A. (2013). Shifting from the single - to the multitarget paradigm in drug discovery. *Drug Discovery Today,* **1B***:* 495-501

Mendonca-Filho,R.R.(2006).Bioactive phytocompounds: new approaches in the phytosciences. In: Ahmad, I., Aqil, F., Owais, M. (Eds.), Modern Phytomedicine: Turning Medicinal Plants into Drugs. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Mitra, P. P. (2012). Drug discovery in tuberculosis: a molecular approach. *Indian Journal of Tuberculosis,* **59**, 194-206.

Milind, P., and Deepa, K. (2011). Clove: A champion spice. *International Journal of Research in Ayurveda and Pharmacy,* **2**: 47-54.

Mizushina, Y., Takikawa, H., Imamura, Y., Sasaki, M., Mori, K., and Yoshida, H. (2005): Inhibitory effect of mispyric acid on mammalian DNA polymerases. *Bioscience Biotechnology Biochemistry;* **69**: 1534- 1538.

Mosmann, T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**: 55-63.

Muller, K.D., and Hobza, P. (2000). Noncovalent Interactions: A challenge for Experiment and theory. *Chemical Review,* **100**:143-167.

Murakami, S.K., Masuda, S., Campbell, A.E., Muzzin, O., and Darst, A.S. (2002). Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. *Science* **296**:1285-1290.

Ndawonde, B.G., (2006). Medicinal plant sales: A case study in Northern Zululand. MSc dissertation. University of Zululand, South Africa.

Ngo, L. T., Okogun, J. I., and Folk, W. R. (2013). 21st century natural product research and drug development and traditional medicines. *Natural Product Reports,* **30**: 584-592.

Onoja, E., and Ndukwe I.G. (2013). Isolation of oleanolic acid from chloroform extracts of Borreria stachydea (DC) Hutch and Dalziel. *Journal of Natural Product and Plant Resources,* **3**: 57-60

Okunade, A.L., Elvin-Lewis, M.P.F., and Lewis, W.H. (2004). Natural antimycobacterial metabolites: *Current status. Phytochemistry*, **65**: 1017-1032

Raza, R., Ilyas, Z., Ali, S., Nisar, M., Khokhar, M. Y., and Iqbal, J. (2015). Identification of Highly Potent and Selective a-Glucosidase Inhibitors with Antiglycation Potential, Isolated from Rhododendron arboreum. : *Records of Natural Products*, **9**: 262-266

Ramawat, K.G., Dass, S., and Mathur, M. (2009). The Chemical Diversity of Bioactive Molecules and Therapeutic Potential of Medicinal Plants, K.G.Ramawat(ed), Herbal Drugs: Ethnomedicine to Modern Medicine, © Springer-Verlag Berlin Heidelberg.

Ramachandran, S. S., and Balasubramanian, S. (2014). Plants: A source for New Antimycobacterial Drugs. *Planta medica,* **80**: 9-21.

Rastogi, N., Abaul, J., Goh, K. S., Devallois, A., Philogene, E., and Bourgeois, P. (1998). Antimycobacteriall activity of chemically defined natural substances from the Caribbean flora in Guadeloup. FEMS ImmunoIog and Medical Microbiology, **20**:267- 273.

Ribon, W. (2012). Biochemical isolation and identification of mycobacteria. In: Jimenez J. (ed) Biochemical testing. In Tech; Available from http://www.intechop-en.com/books/biochemical-testing/biochemical-isolation-and-identification-of-myco- bacteria, 21-52.

Saeed, M., Nadeem, M., Khan, R.M., Shabbir, M.A., Shehzad, A and Amir, R.M (2013). Antimicrobial activity of *Syzygium aromaticum* extracts against food spoilage bacteria. *African Journal of Microbiology Research*, **7**:4848- 4856.

Sahranavard, S., F. Naghibi, F., Mosaddegh, M., Esmaeili,S., Sarkhail, P., Taghvaei, M and Ghafari, S. (2009). Cytotoxic activities of selected medicinal plants from Iran and phytochemical evaluation of the most potent extract. *Research in Pharmaceutical Sciences,* **4**:133-137.

Sasidharan, S., Chen, Y., Saravanan,D., Sundram, K.M. and Yoga Latha, L.(2011). Extraction, isolation and characterization of bioactive compounds from Plants' Extracts. *African Journal of Traditional Complementary Alternative Medicine,* **8**: 1-10.

Silva, M. L., David, J. P., Silva, L. C. R. C., Santos, R. A F., David, J. M., Lima, L. S.,Reis,P.S., and Fontana, R. (2012). Bioactive oleanane, lupane and ursane triterpene acid derivatives. *Molecules,* **17**:12197-12205.

Sharma, R., Bisen, D.P., Shukla, U., and Sharma, B.G. (2012). A powerful method of characterizing nanomaterials. *Recent Research in Science and Technology*, **4**: 77-79.

Sharma, P.R., and Varma, A.J. (2014). Funtionalized celluloses and their nanoparticles: Morphology, thermal properties, and solubility studies. *Carbohydrate Polymers*, **104**:135-142.

Shehzad, A., Rehman, G., Ul-Islam, M., Khattak, W.A., and Lee, S.Y. (2013). Challenges in the development of drugs for the treatment of tuberculosis. *Brazilian Journal of Infectious Diseases*; **17**:74-81.

Sheng, H., and Sun, H. (2011). Synthesis, biology and clinical significance of pentacyclic triterpenes: a multi-target approach to prevention and treatment of metabolic and vascular diseases. *Natural Product Reports*, **28**:543-593.

Shindikar,A.V., and Viswanathan, C.L.(2005). Novel fluoroquinolones: Design, synthesis and in vivo activity in mice against *Mycobacterium tuberculosis* H_{37} RV. *Bioorganic Medicinal Chemistry Letters*, **15**:1803-1806.

Srivastava, P., Kasoju, N., Bora, U., and Chaturvedi, R. (2010). Accumulation of Betulinic, Oleanolic, and Ursolic acids in In vitro Cell Cultures of Lantana camara L. and their Significant Cytotoxic Effects on HeLa Cell Lines. *Biotechnology and Bioprocess Engineering,* **15**:1038-1046.

Sriram, D., Yogeeswari, P.,and Madhu, K. (2006). Synthesis and in vitro antitubercular activity of some 1-[(4-sub) phenyl]-3-(4-{1-[(pyridine-4-carbonyl) hydrazone] ethyl} phenyl) thiourea. *Bioorganic and Medicinal Chemistry Letter*s, **16**:876-878.

Song,N,R., Lee, K.W., and Lee, H.J. (2012). Molecular targets of dietary phytochemicals for human chronic diseases: cancer, obesity, and alzheimers diseases. *Journal of Food and Drugs Analysis*, **20**:342-345.

Song,J., Wang, Y., Song, Y., Chan, H., Bi, C., Yang, X., Yan, R., Wang, Y., and Zheng, Y (2014).Development and characterization of ursolic acid nanocrystals without stabilser having improved dissolution rate and in vitro anticancer activity. *American Association of Pharmaceutical Scientists*, **15**: 11-19.

Schultheiss, N., and Newman, A. (2009). Pharmaceutical Cocrystals and Their physicochemical properties. *Crystal Growth and Design*, ***9***: 2950–2967.

Sekhon, B. S. (2012). Pharmaceutical co-crystals- An update. *International Bulletin of Drug Research*, **1**: 24–39.

Shai, L.J., Mcgaw, L.J., Aderogba, M.A., Mdee, L.K., and Eloff, J.N. (2008). Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm leaves. *Journal of Ethnopharmacology*, **119**: 238-244.

Shakya, N., Garg, G., Agrawal, B., and Kumar, R. (2012). Chemotherapeutic interventions against tuberculosis. *Pharmaceuticals*, **5**: 690–718.

Sharma, P. (2011). Cinnamic acid derivatives: A new chapter of various pharmacological activities.*Journal of Chemical and Pharmaceutical Research*,**3**: 403–423.

Sharma, S. K., and Mohan, A. (2013). Tuberculosis : From an incurable scourge to a curable disease - journey over a millennium. *Indian Journal Medical Research*, **137**:455–493.

Simelane, M.B.C., Shonhai, A., Shode, F.O., Smith, P., Singh, M., and Opoku, A. (2013). Anti-plasmodial activity of some Zulu medicinal plants and of some triterpenes isolated from them. *Molecules*, **18**:12313–12323.

Sizemore, C. F., Schleif, A. C., Bernstein, J. B., and Heilman, C. A. (2012). The role of biomedical research in global tuberculosis control: gaps and challenges. *Emerging Microbes and Infections*, **1**: e9.

Soni, M. K. (2009). "Synthesis and Physico chemical studies of some organic compound." PhD thesis, Saurashtra University.

Soumya P. R., Choudary, K. A., Kar, D. M., Das, L., and Jain, A. (2009). Plants in tradiional medicinal system-future source of new drugs. *International Journal of Pharmacy and Pharmaceutical Sciences*, **1**: 1–23.

Steed, J. W. (2013). The role of co-crystals in pharmaceutical design. *Trends in Pharmacological Sciences*, **34**: 185–193.

Tanachatchairatana, T., Bremner, J.B., Chokchaisiri, R., and Suksamrarn, A. (2008). Antimycobacterial activity of cinnamate-based esters of triterpenes, betulinic, oleanolic and ursolic Acids, *Chemical and Pharmaceuticals Bulletin,* **56**:194-198.

Tanimura, T., Jaramillo, E., Weil, D., Raviglione, M., Lönnroth, K. (2014). Financial burden for tuberculosis patients in low- and middle-income countries: a systematic review. *The European Respiratory Journal*, **43**: 1763–1775.

Thibeault. D., Gauthier, C., Legault, J., Bouchard, J., Dufour, P., and Pichette, A. (2007). Synthesis and structure-activity relationship study of cytotoxic germanicane- and lupane-type 3beta-O-monodesmosidic saponins starting from betulin. *Bioorganic and Medicinal Chemistry,* **15**: 6144- 6157.

Tiruveedhula, V.V.N.P., Witzigmann,C.M., Verma, R., Kabir, M.S., Rott, M., Schwan, W.R., Medina-Bielski,S., Lane, M., Close, W., Polanowski, R.L., Sherman, D., Monte, A., Deschamps, J.R., and Cook, J.M. (2013). Design and synthesis of novel antimicrobials with activity against Gram-positive bacteria and mycobacterial species, including *Mycobaterium tuberculosis.* *Bioorganic and Medicinal Chemistry*, **21**:7830-7840.

Uddin, G., Aliullah, W., Siddiqui, B., Alam, M., Sadat, A., Ahmad, A., and Uddin, A (2011). Chemical constituents and phytotoxicity of solvent extracted fractions of stem bark of Grewia optiva Drummond ex Burret. *Middle-East Journal of Scientific Research*, **8**: 85-91.

Valcic, S., Wacher, G.A., Montenegro, G., and Timmermann, B.N (1997). Triterpenoids from Acaena pinnatifida R.et P. *Zeitschrift fur Naturforschung. C, Journal of biosciences,* **52c**:264-266.

Van der Werf, M. J., Giesecke, J., and Sprenger, M. (2012). Can the economic crisis have an impact on tuberculosis in the EU / EEA ? *Euro Surveill*, **17**: 17–18.

Velayati, A. A., Farnia, P., and Masjedi, M. R. (2013). The totally drug resistant tuberculosis (TDR-TB). *International Journal Clinical Experimental Medicine*, **6**: 307–309

Vijayaraj, S., Sangeetha, V., OM Shanthi, B., and Sampath Kumar, K.P. (2013). Development and validation of isoniazid and para-amino salicylic acid simultaneously in prepared cocrystal formulation by UV-spectrophotometry. *The Experiment,* **17:** 1205-1212.

Wang, G., Tang, W., and Bidigare, R. (2005). Terpenoids as therapeutic drugs and pharmaceutical agents. In Natural Products: Drug Discovery and Therapeutic Medicine; Zhang, L., Demain, A.L., Eds.; Humana Press: Totowa, NJ, USA.

Wachter, G.A., Valcic, S., Flagg, M.L., Franzblau, S.G., Montenegro, G., Suarez,E.and Timmermann,B.N. (1999). Antitubercular activity of pentacyclic triterpenoids from plants of Argentina and Chile. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, **6:** 341–345.

Wampande, E.M., Mupere,E., Debanne, S.M., Asiimwe, B. B., Nsereko, M., Mayanja, H., Eisenach, K., Kaplan, G., Boom, H. W., Gagneux, S. and Joloba, M. L. (2013). Long-term dominance of *Mycobacterium tuberculosis* Uganda family in peri-urban Kampala-Uganda is not associated with cavitary disease. *BMC Infectious Diseases*, **13**: 484.

Welin, A. (2011). *Survival strategies of Mycobacterium tuberculosis inside the human macrophage*.Doctoral thesis, Linkoping University.

Wong, E. B., Cohen, K. A, and Bishai, W. R. (2013). Rising to the challenge: new therapies for tuberculosis. *Trends in Microbiology*, **21**:493–501.

Yamasaki, P.R., do Nascimento, D.C., Chelucci, R.C., de Faria Fernandes Belone, A., Rosa ,P.S., Diório, S.M., de Melo, T.R., Barbieri, K.P., Placeres,M.C., Carlos, I.Z., Chung, M.C.,and dos Santos, J.L. (2014). Synthesis and evaluation of novel dapsone-thalidomide hybrids for the treatment of type 2 leprosy reactions. *Bioorganic and Medicinal Chemistry Letters*, **24**: 3084–3087.

Yazdanpanah, L.,and Mohamadi, N. (2014). Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Paecilomycetes variotii* agent of pistachio dieback. *Journal of Biodiversity and Environmental Sciences,* **4**: 42-45.

Yembaturova, E.Y., Van Wyk, B-E., and Tinley, P. M. (2009). A review of the genus *Curtisia* (Curtisiaceae). *Bothalia,* **39**: 87-96.

Yempala, T., Sriram, D., Yogeeswari, P., and Kantevari, S. (2012). Molecular hybridization of bioactives: synthesis and antitubercular evaluation of novel dibenzofuran embodied homoisoflavonoids via Baylis-Hillman reaction. *Bioorganic and Medicinal Chemistry Letters*, **22**: 7426–30.

Yew, W. W., Lange, C., and Leung, C. C. (2011). Treatment of tuberculosis: update 2010. *The European Respiratory Journal*, **37**:441–462.

Zheng, W., Thorne, N., and McKew, J. C. (2013). Phenotypic screens as a renewed approach for drug discovery. *Drug Discovery Today*, **18**: 1067–73.

Yogeeswari, P., and Sriram, D. (2005). Betulinic acid and its derivatives: a review on their biological properties. *Current Medicinal Chemistry*, **12**: 657-666.

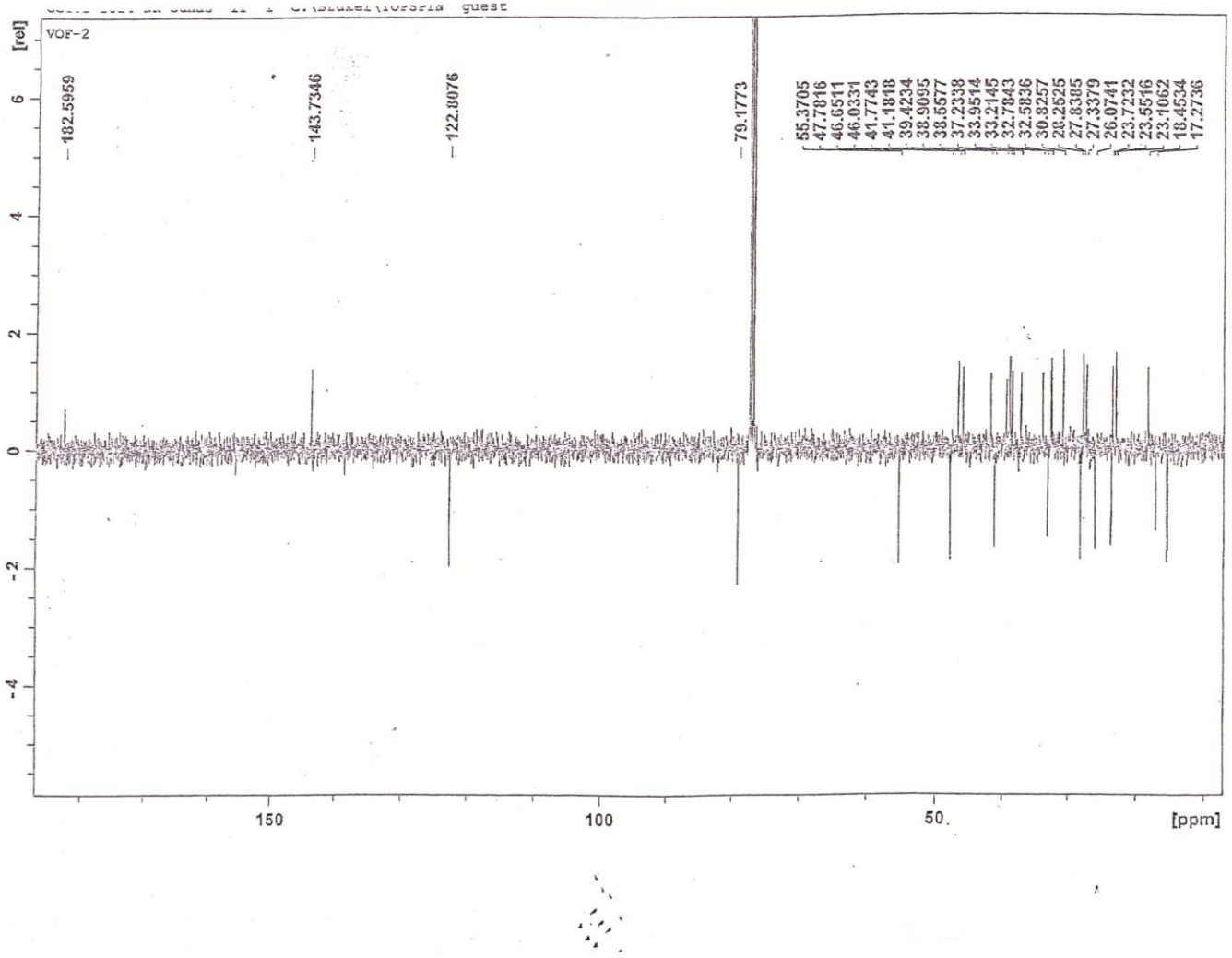
Yu, M., Ocando, E.J., Trombetta, L., and Chatterjee, P. (2015). Molecular interaction studies of amorphous solid dispersions of the antimelanoma agent betulinic acid. *American Association of Pharmaceutical Scientists.* **16**: 384-396

Zumla, A., Raviglione, M., Hafner, R., and Fordham von Reyn, C. F. (2013). Tuberculosis. *The New England Journal of Medicine*, **368**: 745–555.

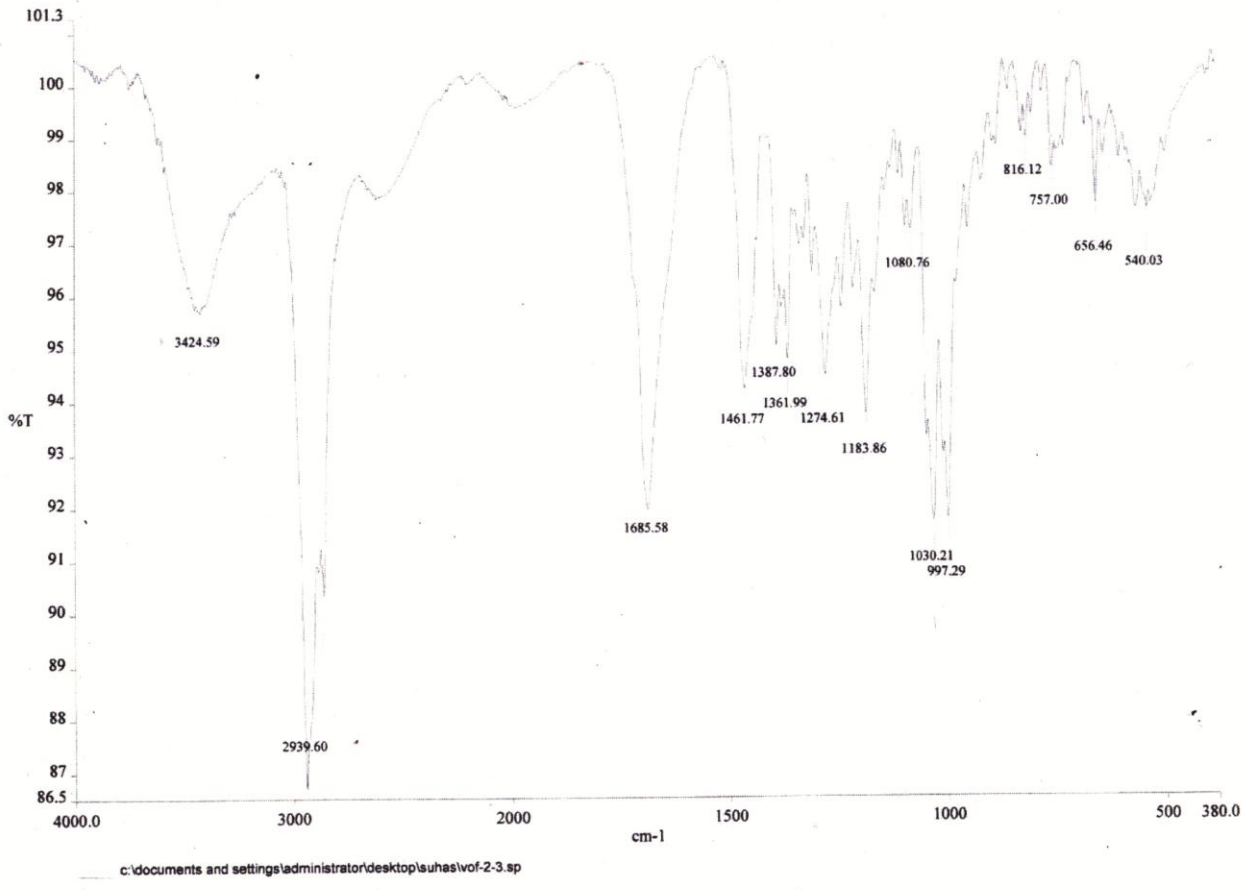
Zumla, A., George, A., Sharma, V., Herbert, N., B M of Ilton.(2013a). WHO's 2013 global report on tuberculosis: successes, threats, and opportunities. Lancet. **382** :1765-1767.

Appendix A

Spectra



Appendix A1: ^{13}C NMR (100 MHz) spectrum of oleanolic acid in $CDCl_3$

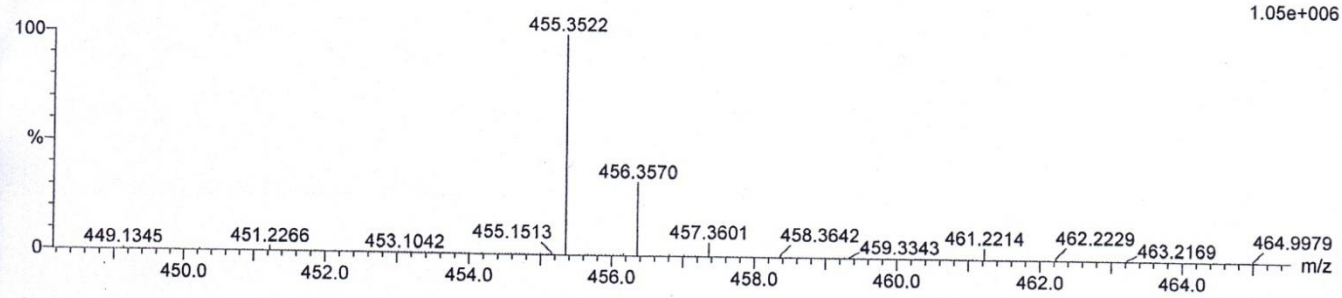


Appendix A2: IR spectrum of oleanolic acid

Elemental Composition Report **Page 1**

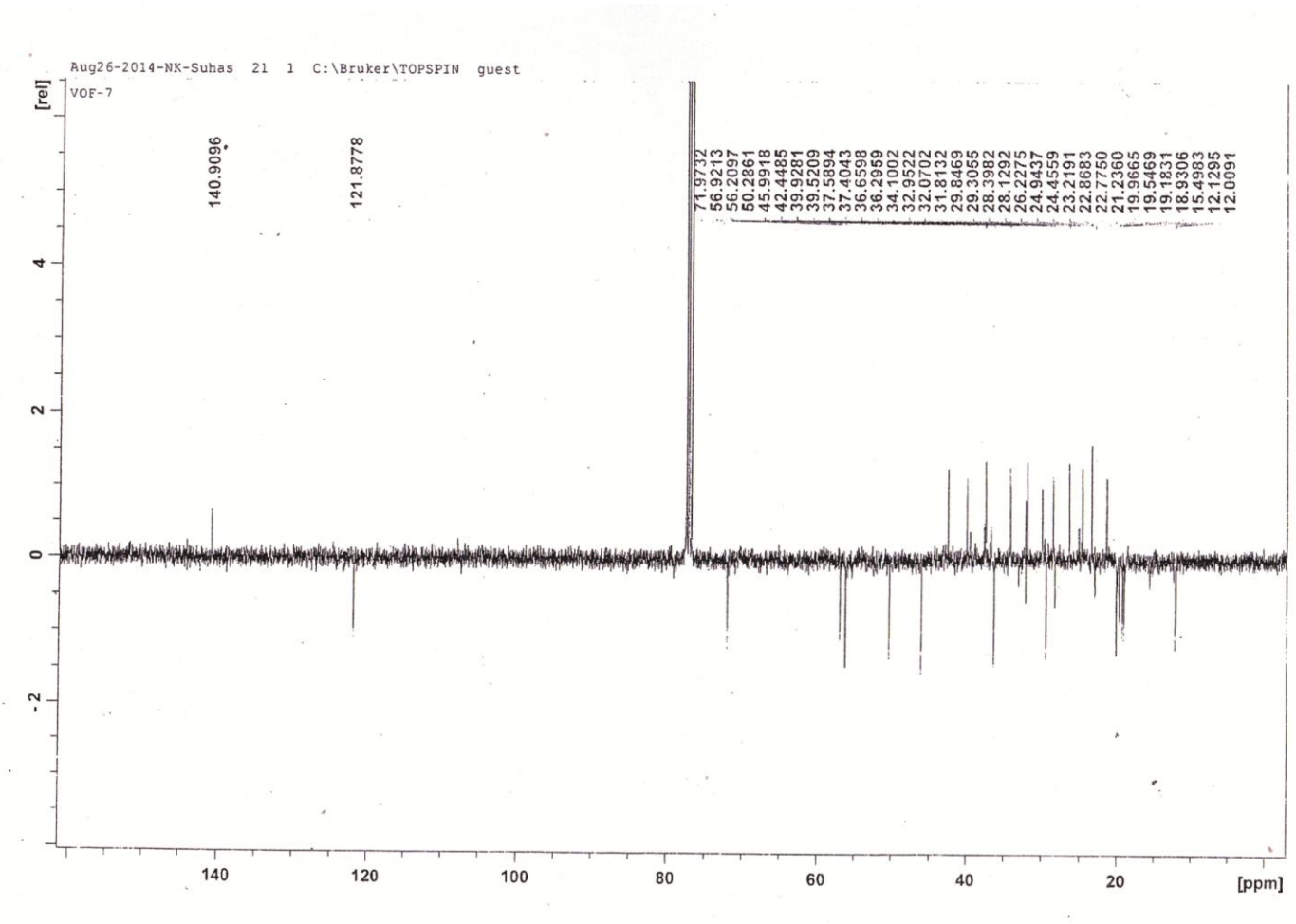
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
2 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 30-35 H: 45-50 O: 0-5
Compound 2 5 (0.135) Cm (1:61)
TOF MS ES-

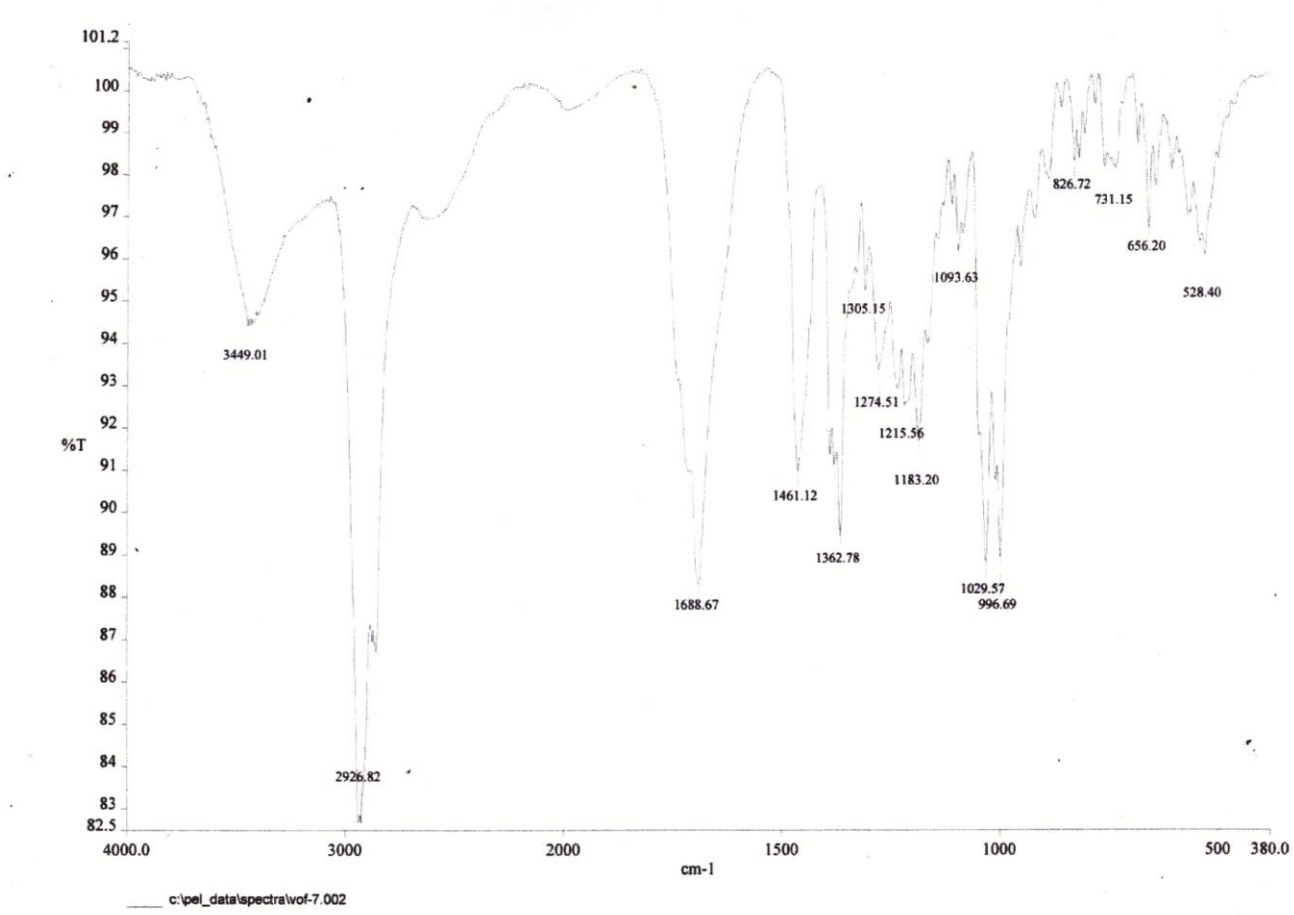


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
455.3522	455.3525	-0.3	-0.7	7.5	613.6	0.0	C30 H47 O3

Appendix A3: ES-MS spectrum of oleanolic acid



Appendix A4: 13C NMR (100 MHz) spectrum of β-sitosterol in $CDCl_3$

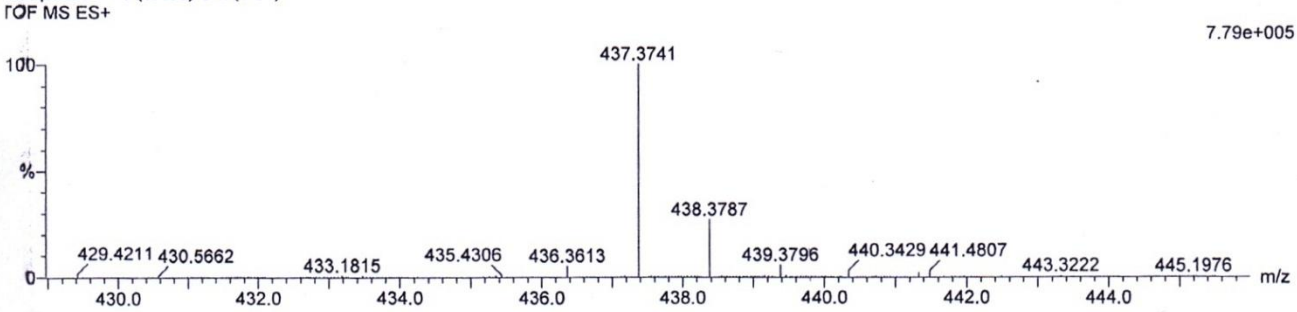


Appendix A5: IR spectrum of β-sitosterol

Elemental Composition Report **Page 1**

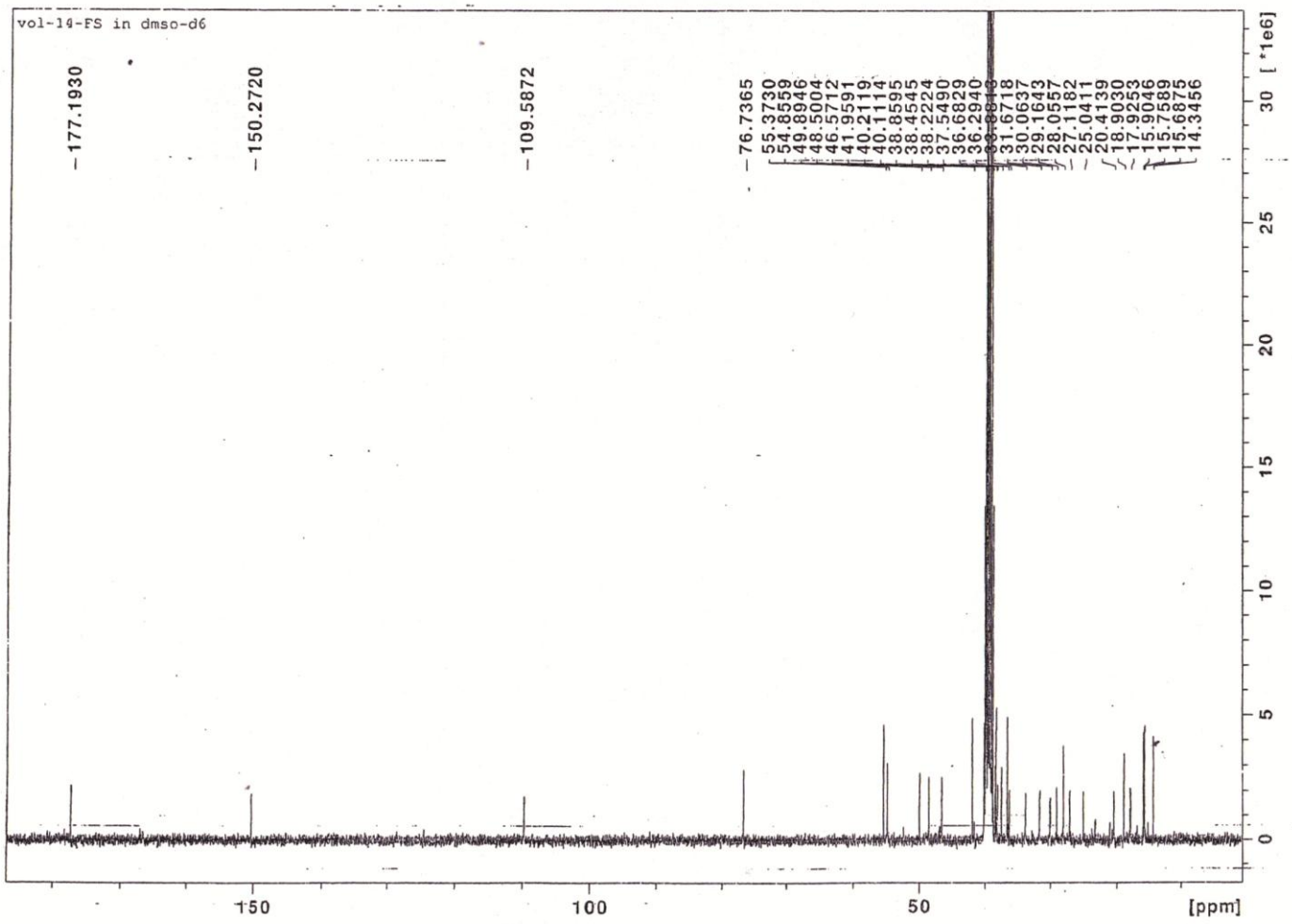
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
3 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 25-30 H: 45-50 O: 0-5 Na: 0-1
Compound 7 13 (0.405) Cm (1:61)
TOF MS ES+

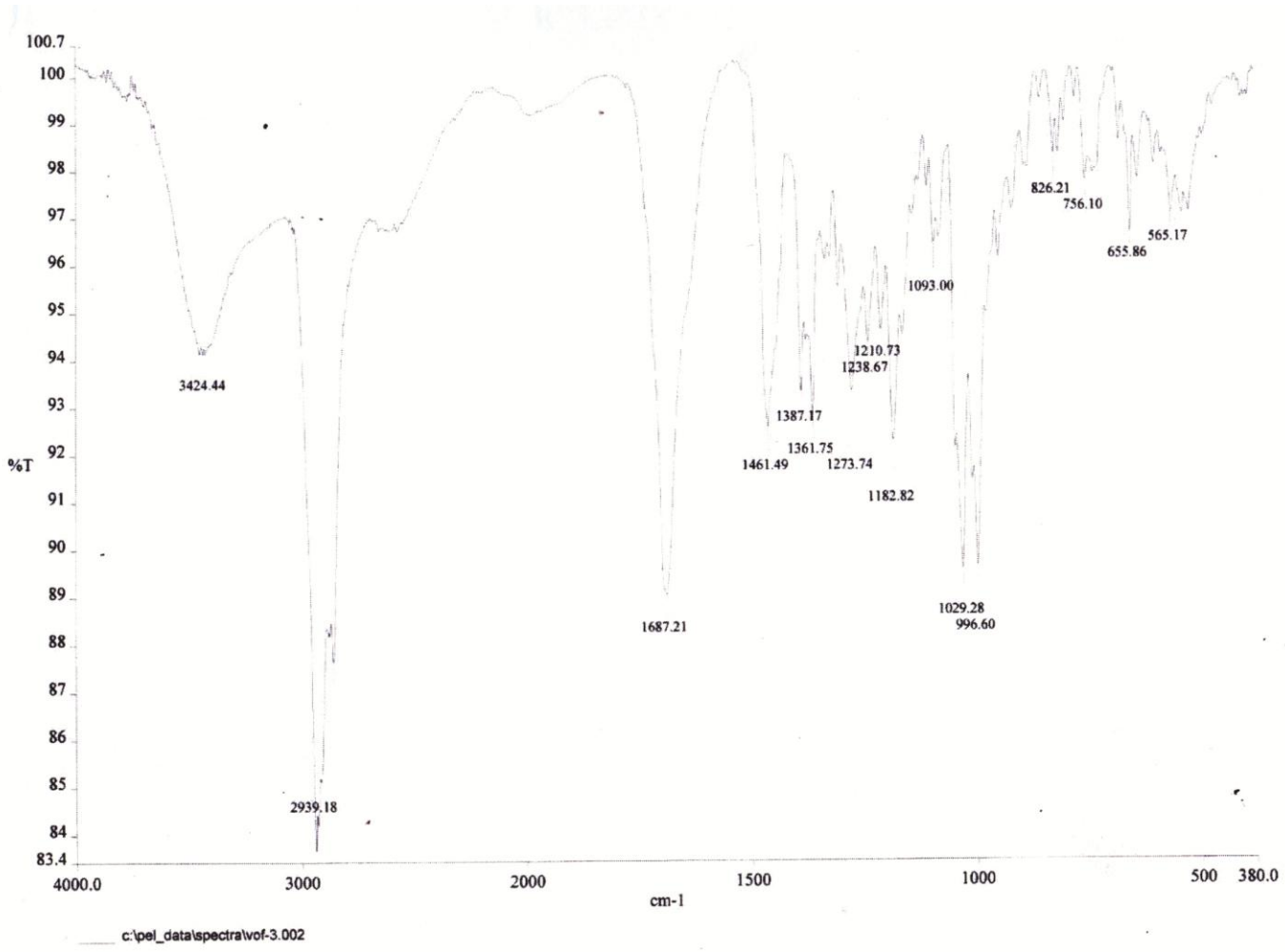


inimum:				-1.5			
aximum:		5.0	5.0	100.0			
ass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
37.3741	437.3759	-1.8	-4.1	4.5	677.6	0.0	C29 H50 O Na

Appendix A6: MS-ES spectrum of β-sitosterol



Appendix A7: 13C NMR (100 MHz) spectrum of betulinic acid in $CDCl_3$

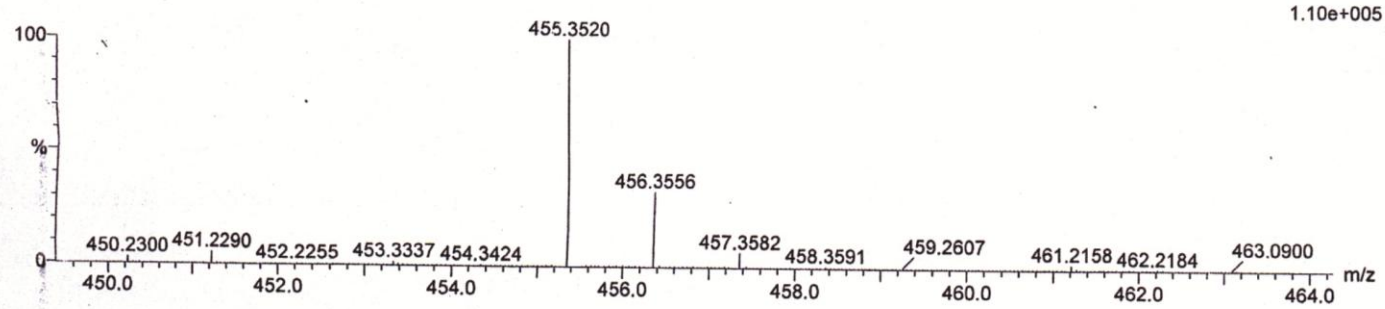


Appendix A8: IR spectrum of betulinic acid

Elemental Composition Report **Page 1**

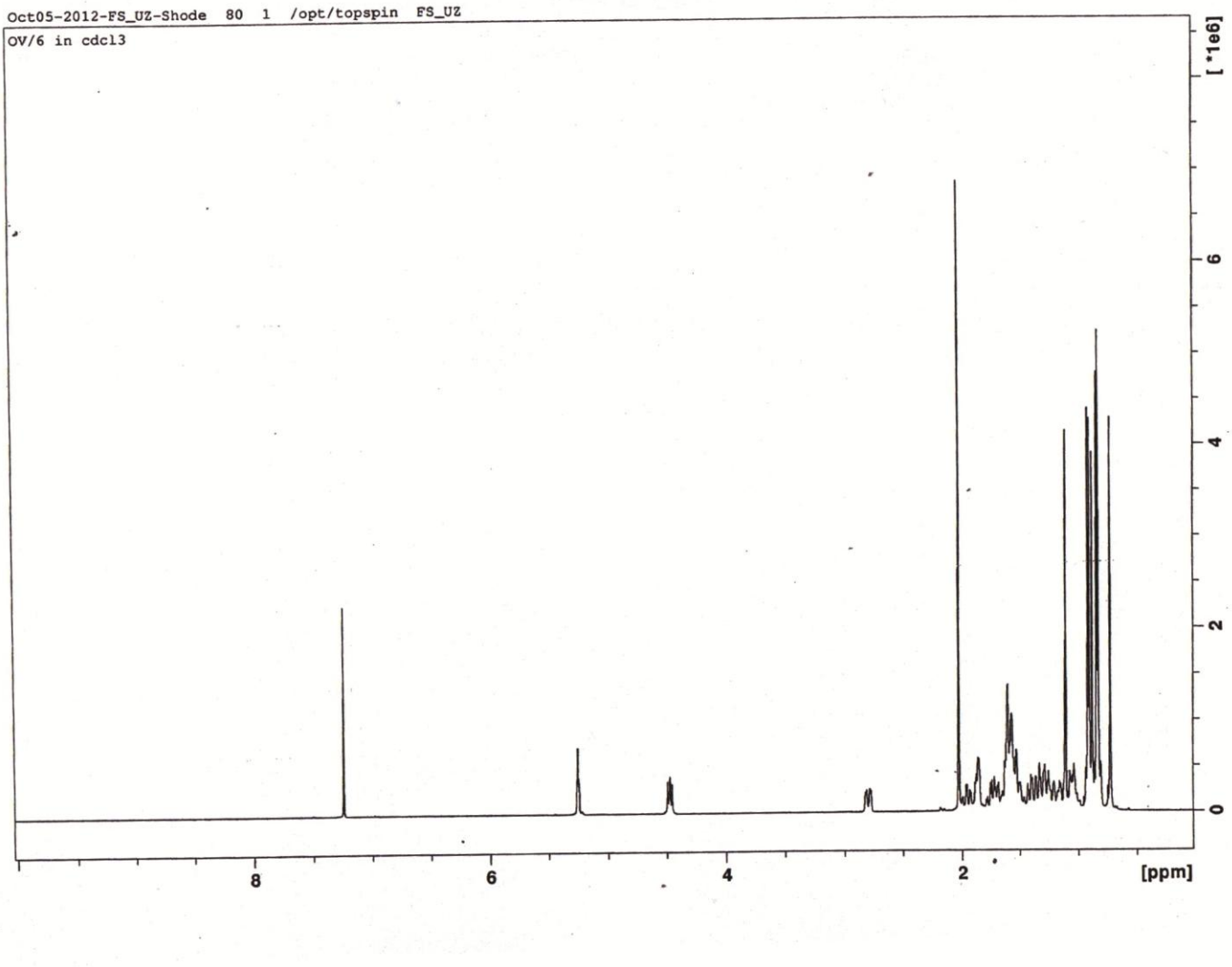
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
2 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 30-35 H: 45-50 O: 0-5
Compound 4 13 (0.406) Cm (1:61)
TOF MS ES-

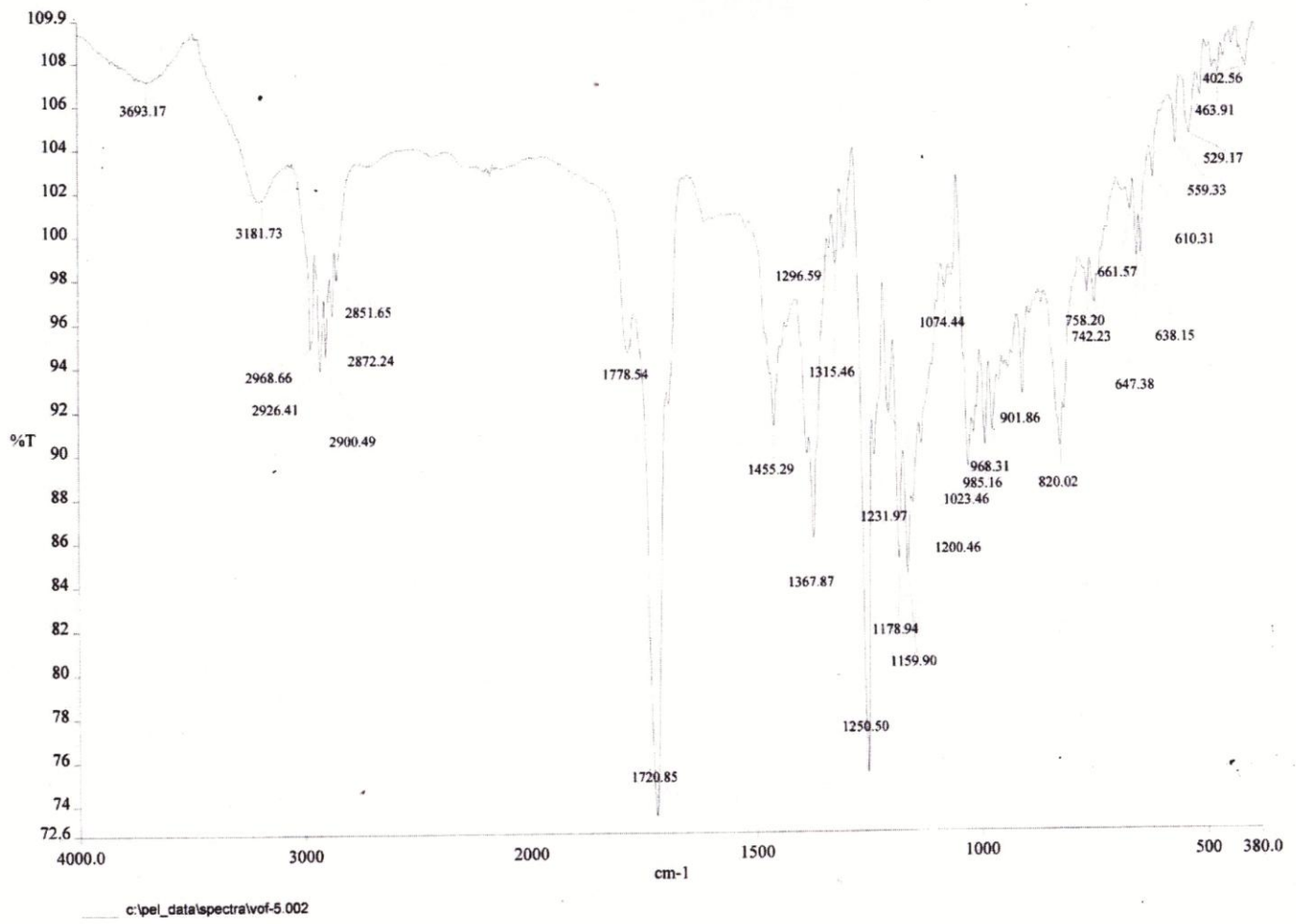


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
455.3520	455.3525	-0.5	-1.1	7.5	559.2	0.0	C30 H47 O3

Appendix A9: MS:ES spectrum of betulinic acid



Appendix A10: ^{1}H NMR (400 MHz) spectrum of oleanolic acid acetate in $CDCl_3$



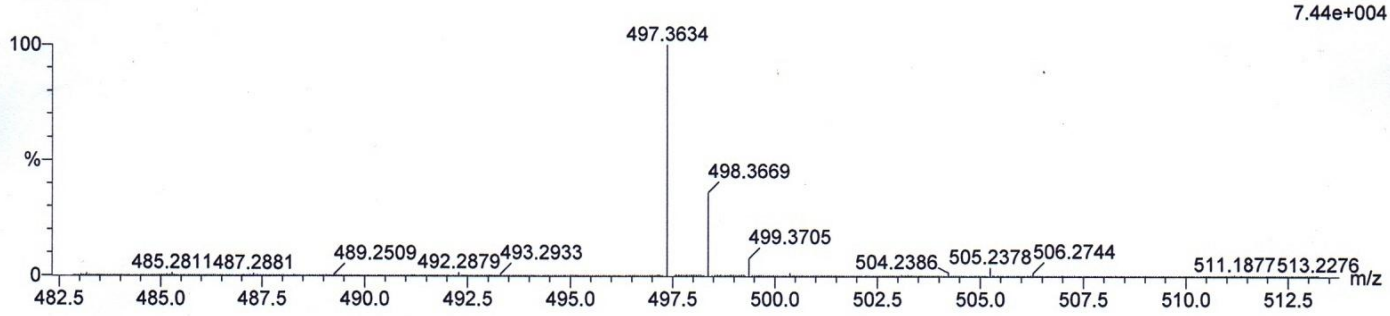
Appendix A11: IR spectrum of oleanolic acid acetate

OAA

Elemental Composition Report **Page 1**

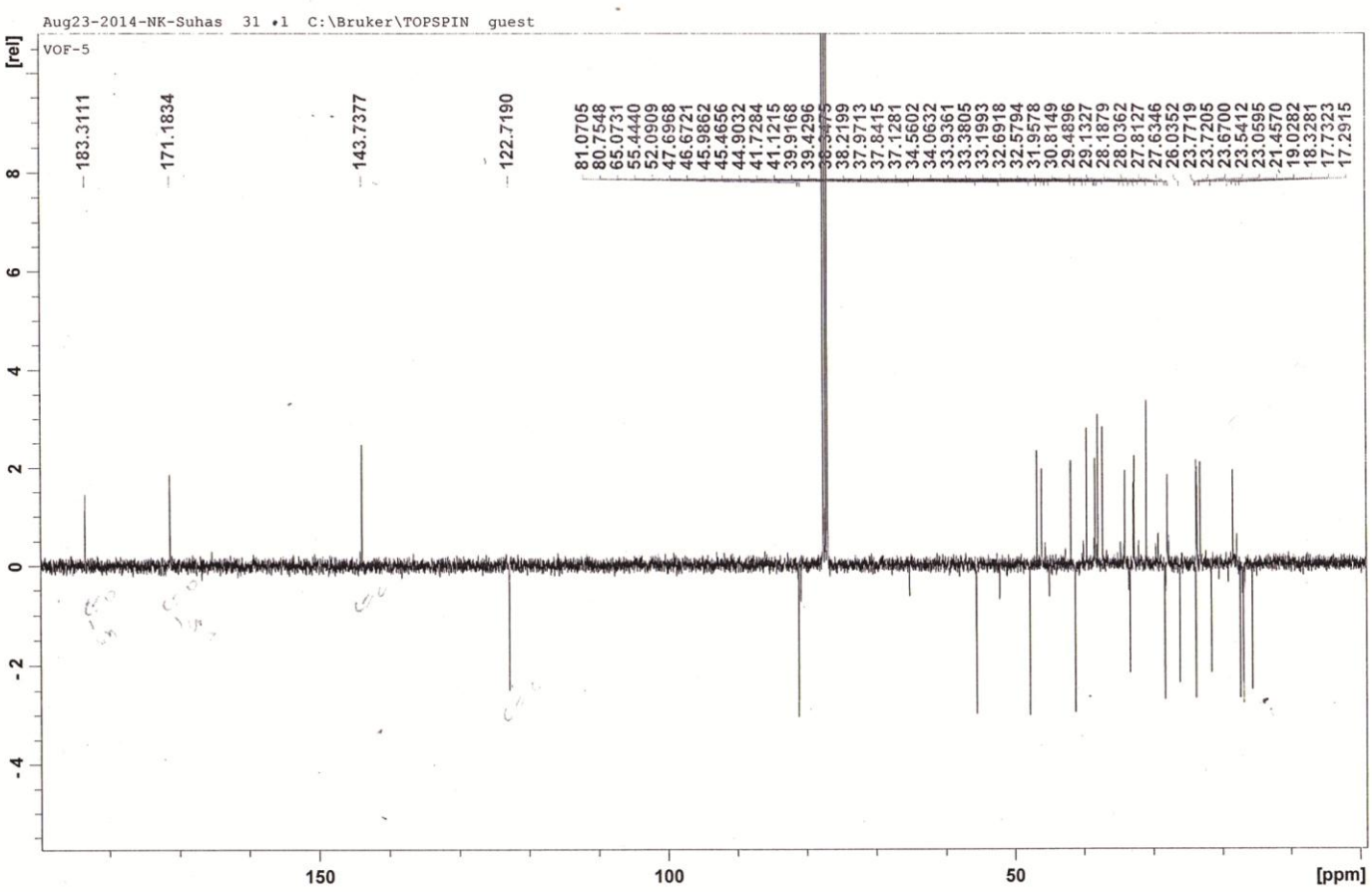
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
3 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 30-35 H: 45-50 O: 0-5
Compound 5 2 (0.034) Cm (1:61)
TOF MS ES-

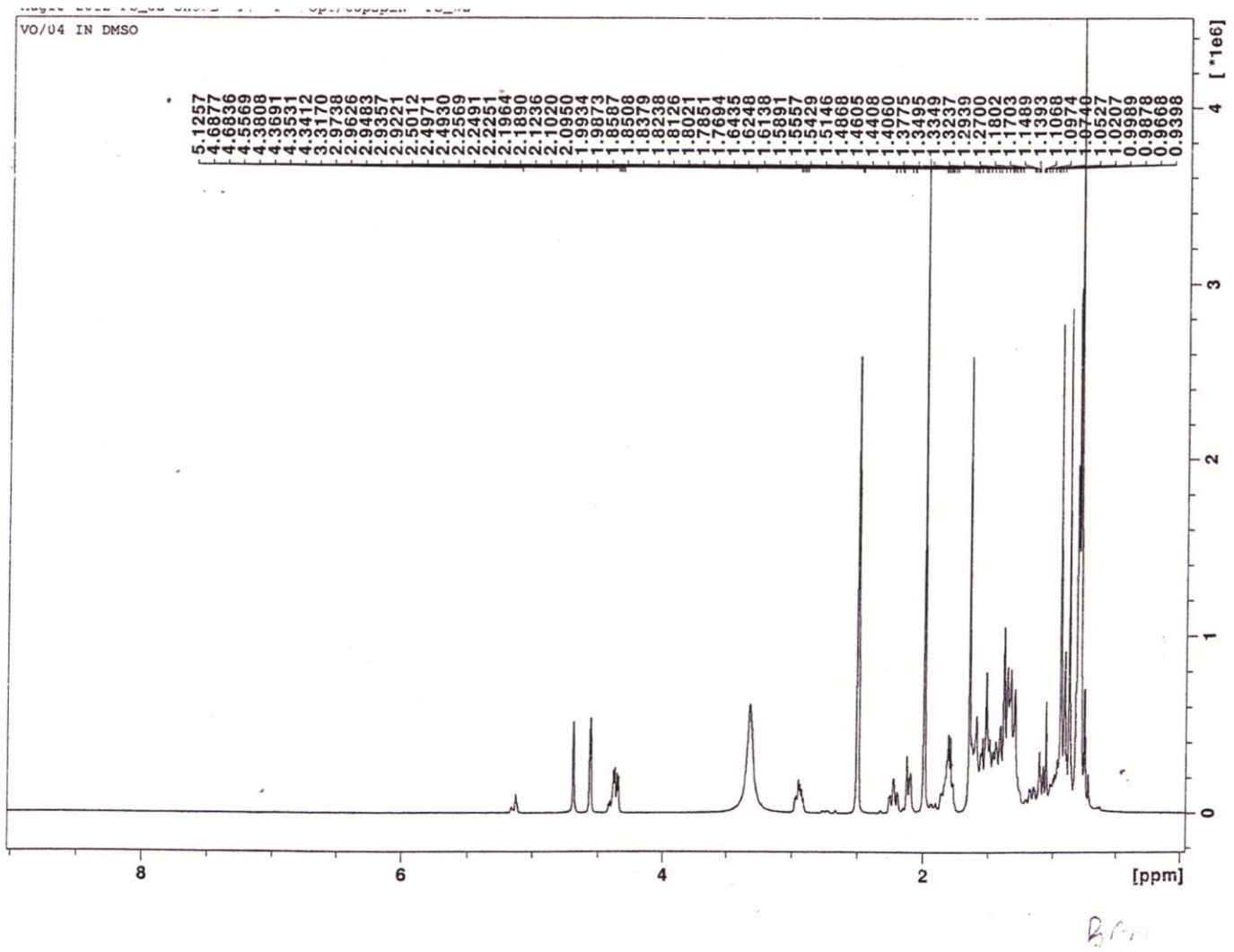


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
497.3634	497.3631	0.3	0.6	8.5	501.0	0.0	C32 H49 O4

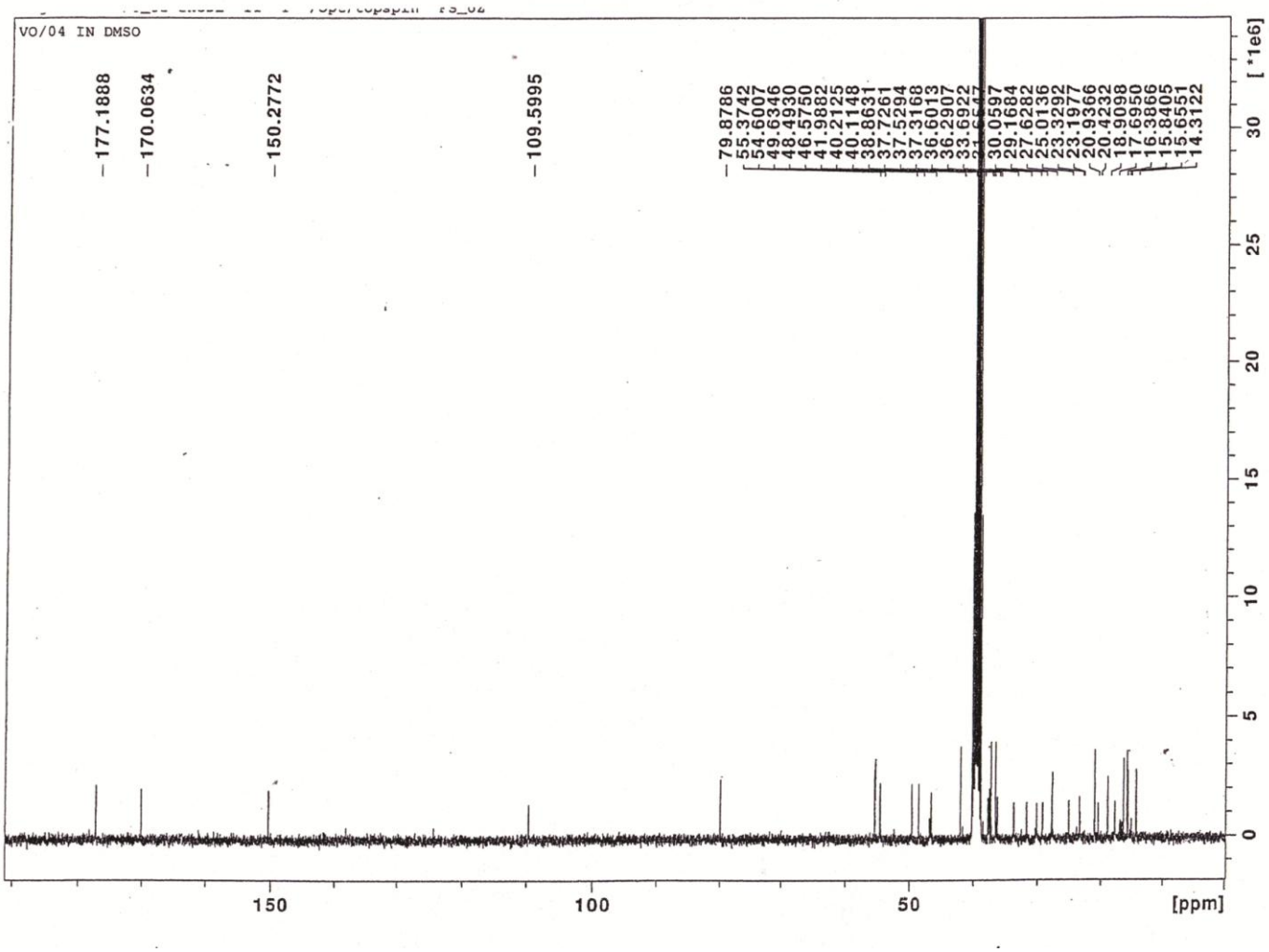
Appendix 12: MS:ES spectrum of oleanolic acid acetate



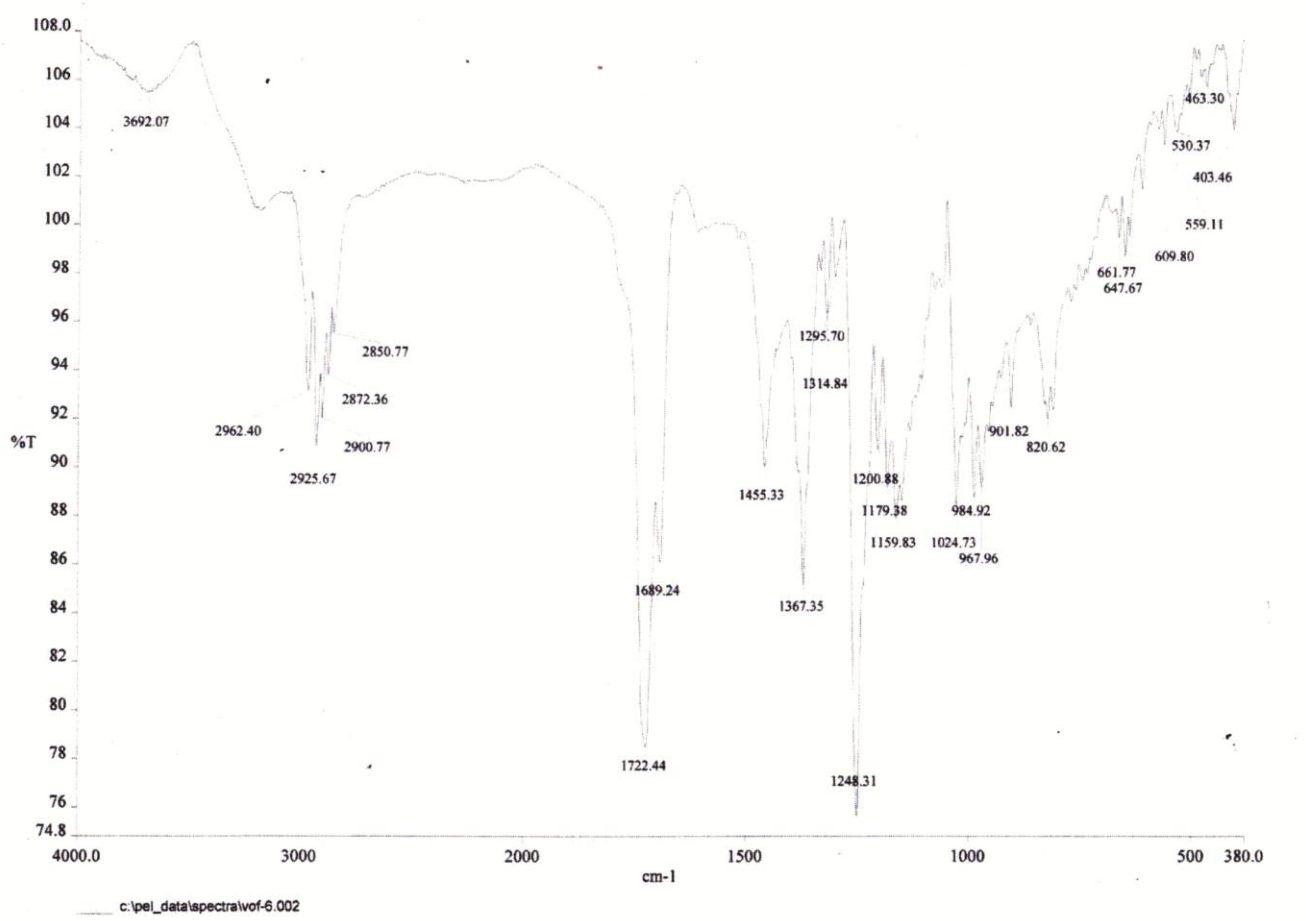
Appendix 13: ^{13}C NMR (100 MHz) spectrum of oleanolic acid acetate in $CDCl_3$



Appendix A14: ^{1}H NMR (400 MHz) spectrum of betulinic acid acetate in $CDCl_3$



Appendix A15: ^{13}C NMR (100 MHz) spectrum of betulinic acid acetate in $CDCl_3$

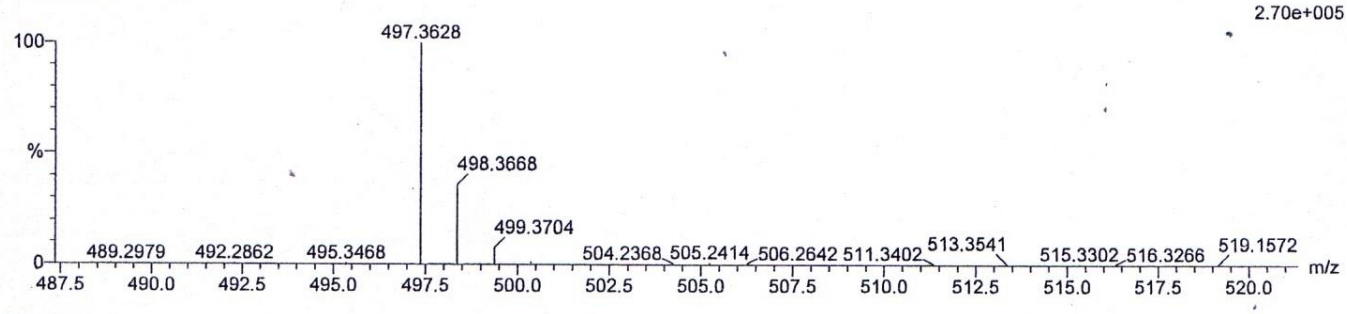


Appendix A16: IR spectrum of betulinic acid acetate

Elemental Composition Report **Page 1**

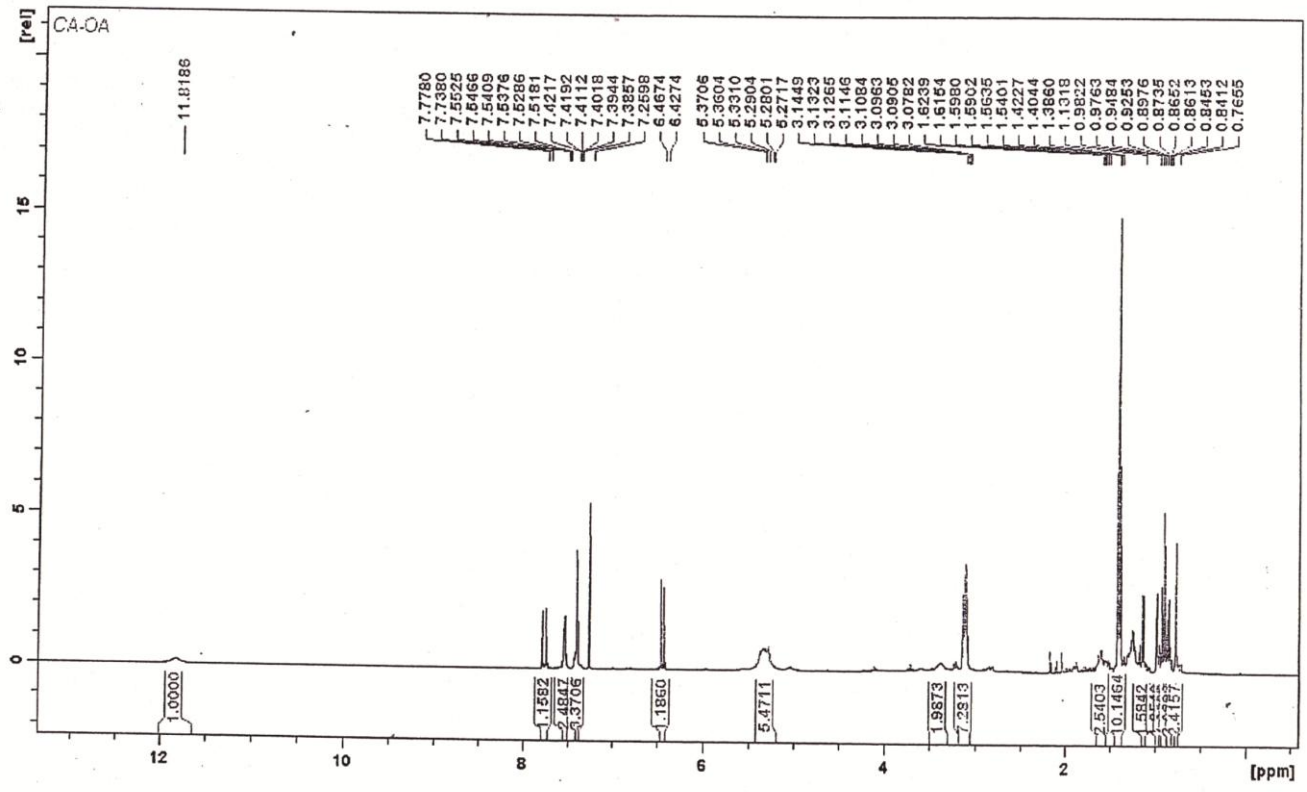
Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
3 formula(e) evaluated with 1 results within limits (up to 20 closest results for each mass)
Elements Used:
C: 30-35 H: 45-50 O: 0-5
Compound 6 50 (1.652) Cm (1:61)
TOF MS ES-

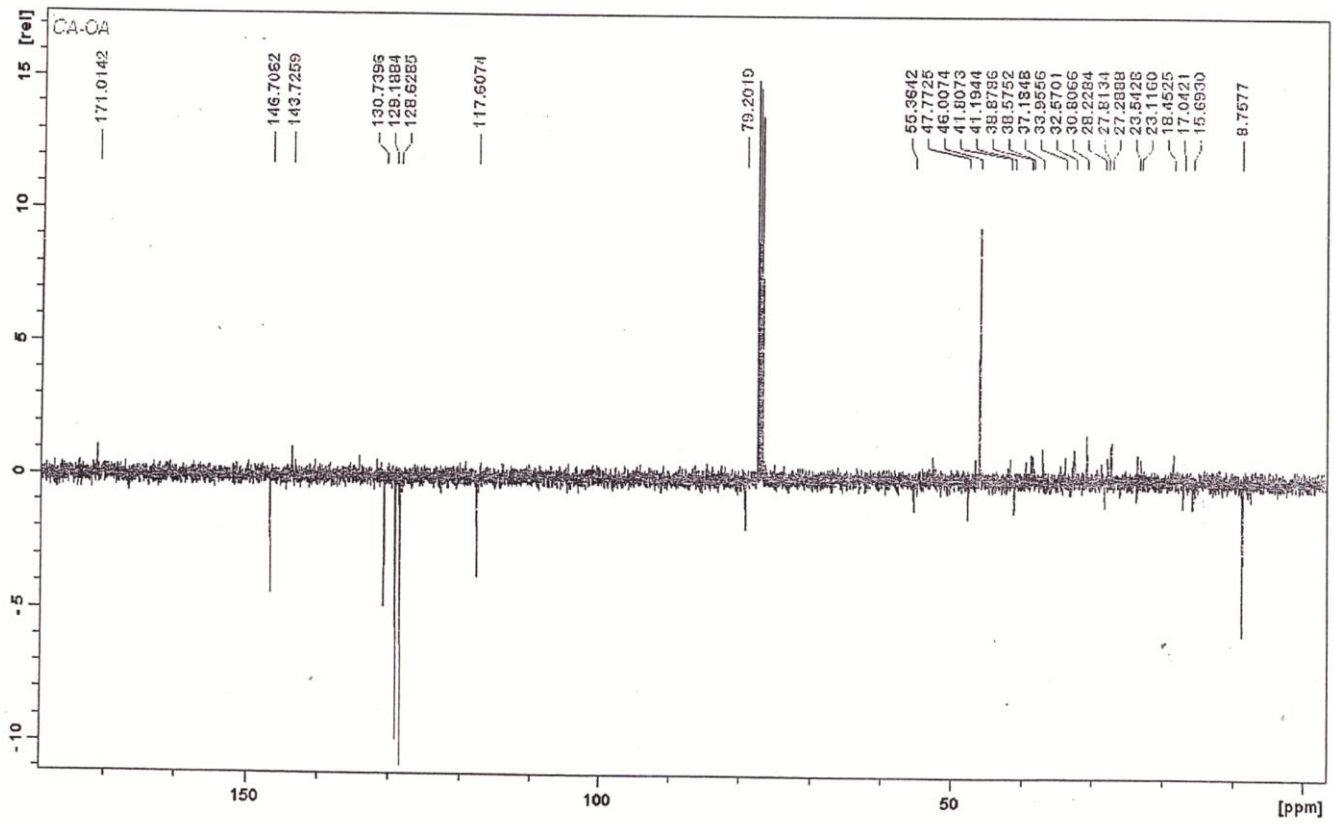


Minimum:				-1.5			
Maximum:		5.0	5.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
497.3628	497.3631	-0.3	-0.6	8.5	567.5	0.0	C32 H49 O4

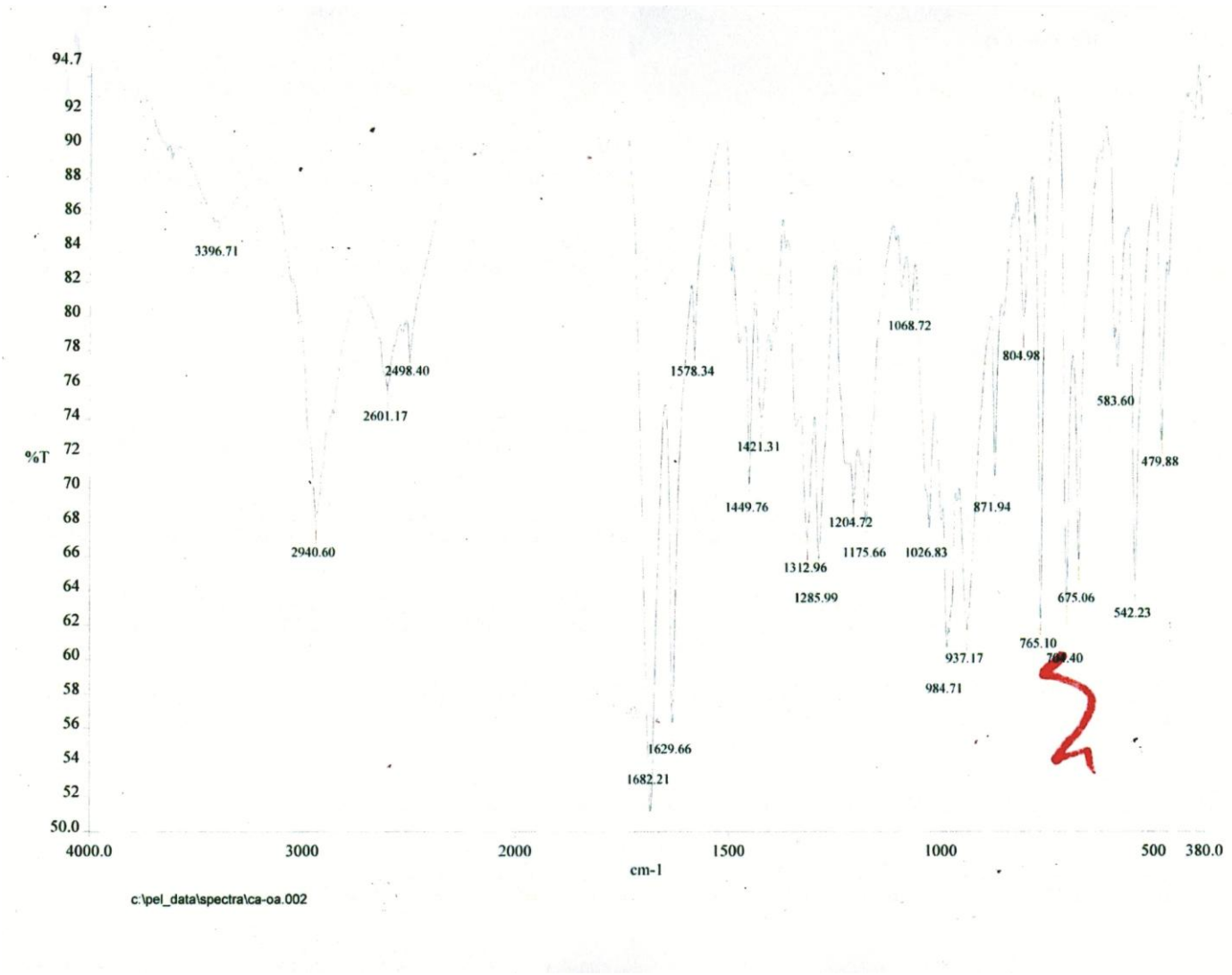
Appendix A17: MS:ES spectrum of betulinic acid acetate



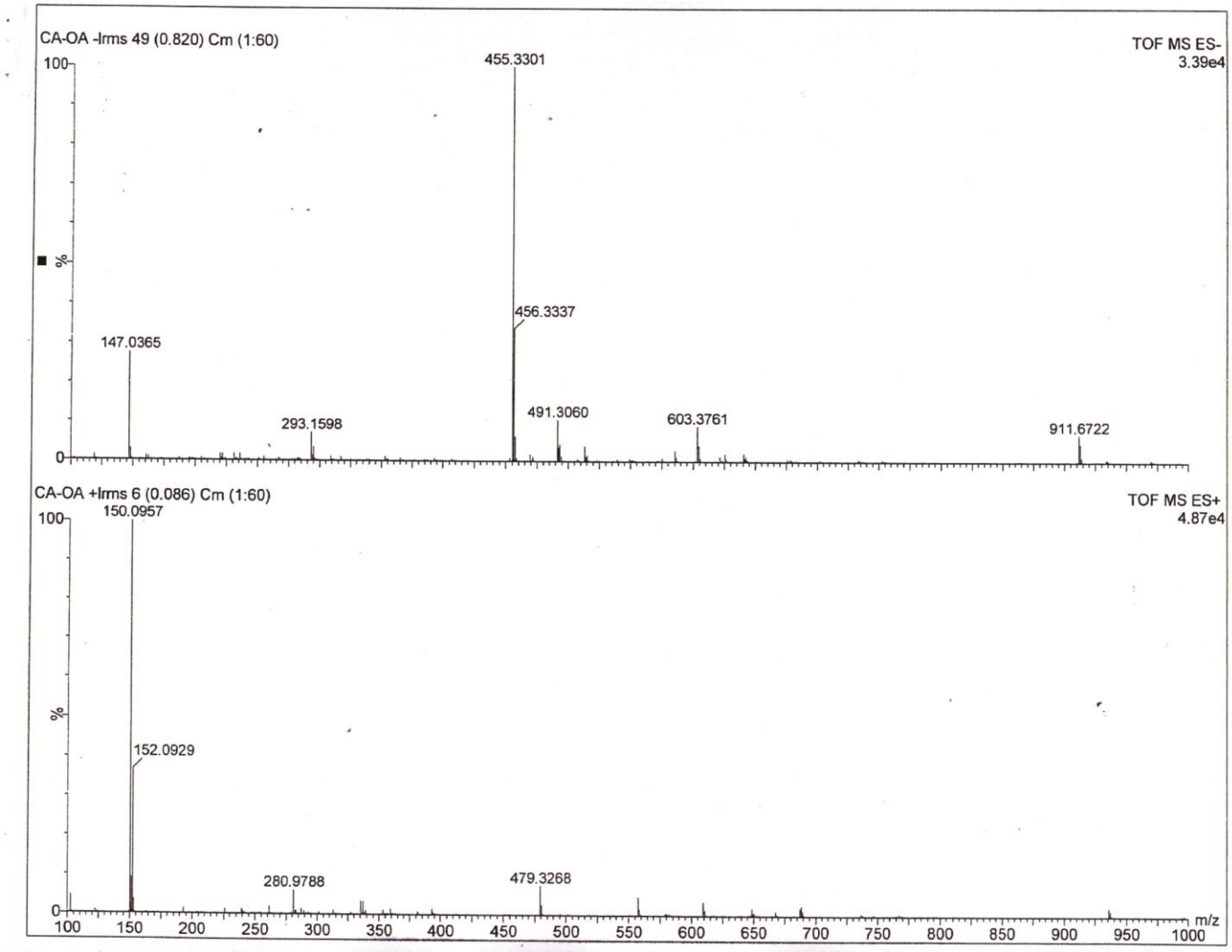
Appendix A18: ^{1}H NMR (400 MHz) spectrum of cinnamic acid hybrid of OA in $CDCl_3$



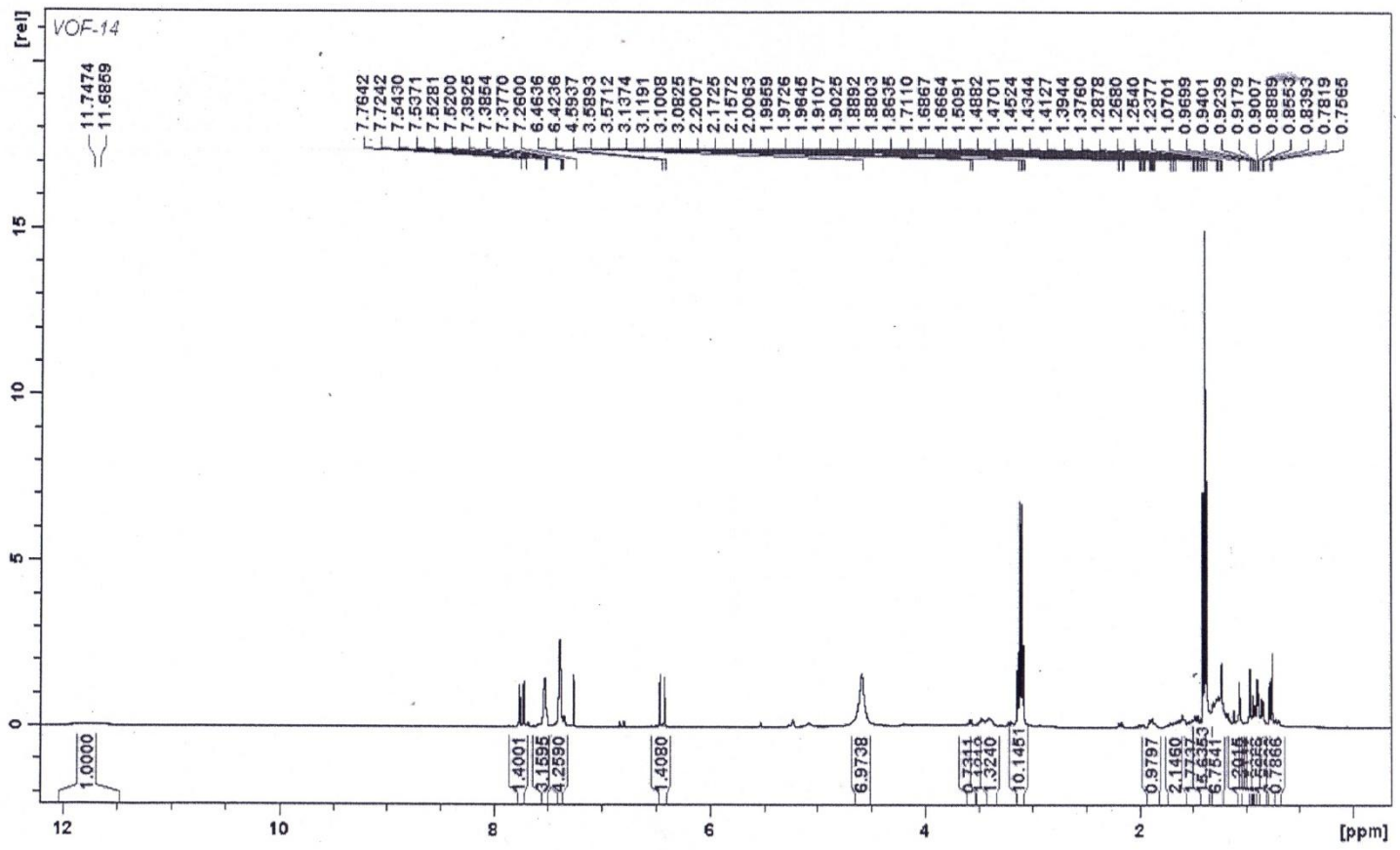
Appendix A19: ^{13}C NMR spectrum of cinnamic acid hybrid of OA



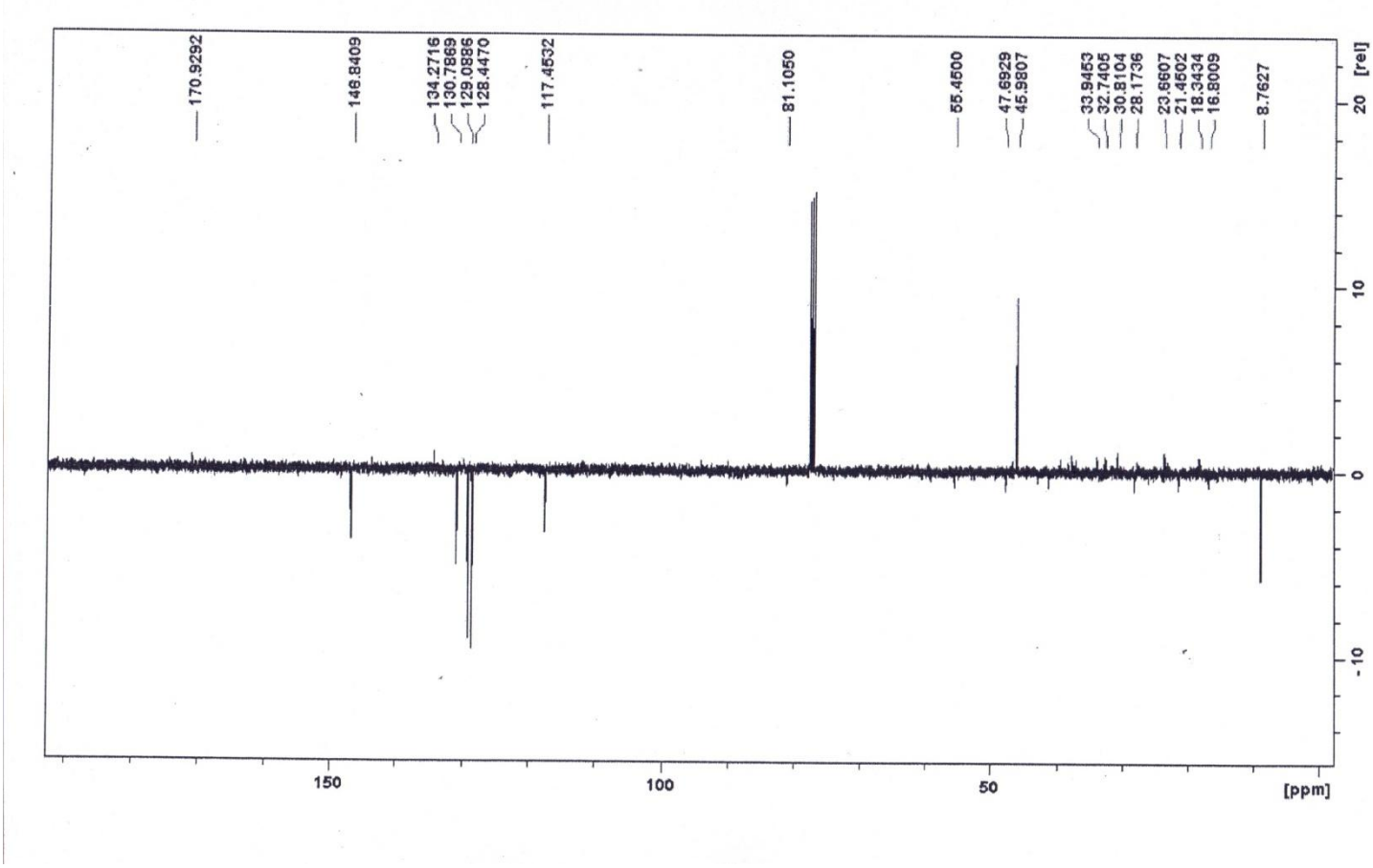
Appendix A20: IR spectrum of cinnamic acid hybrid of OA



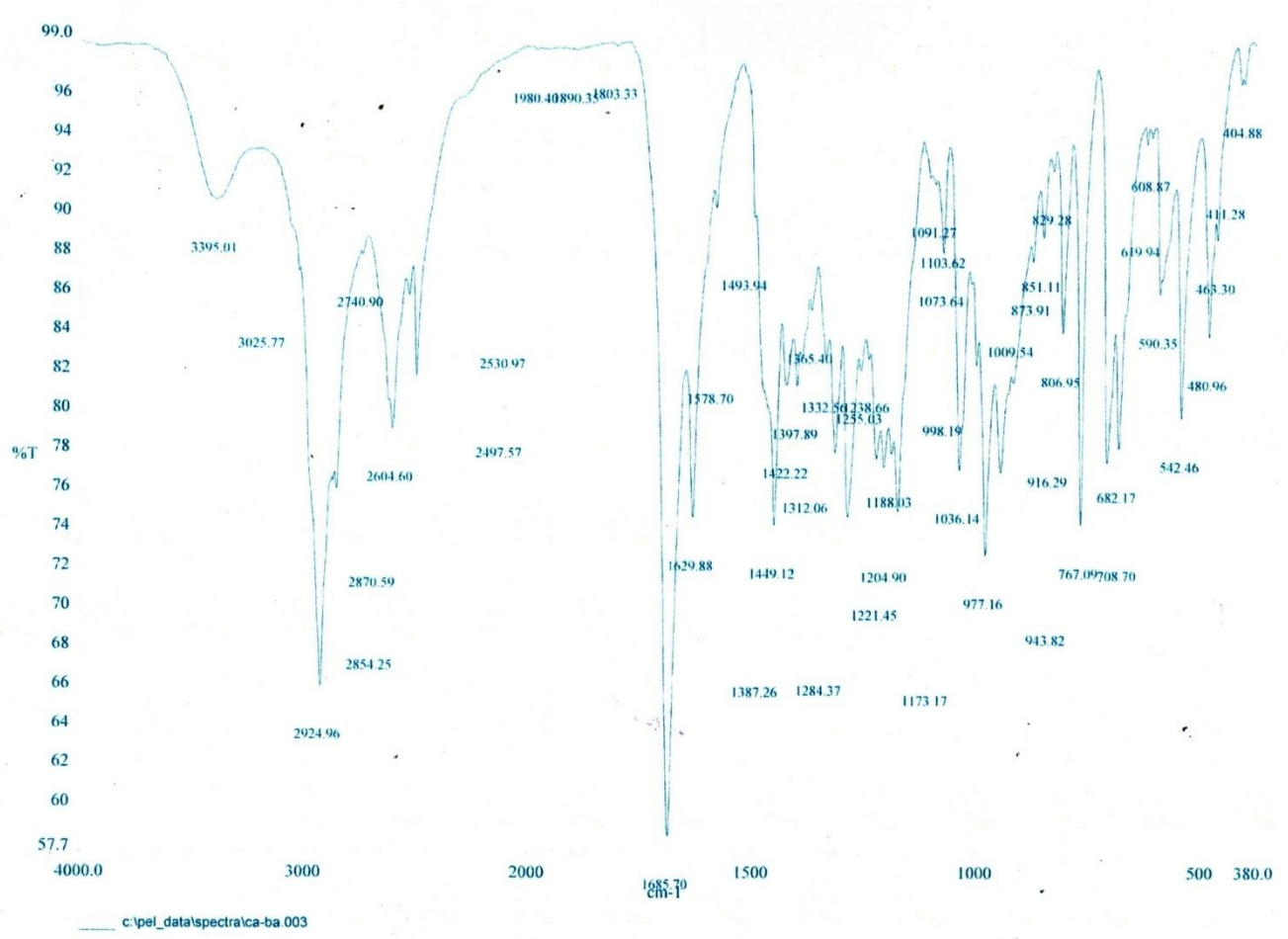
Appendix A21: ES-MS spectrum of cinnamic acid hybrid of OA



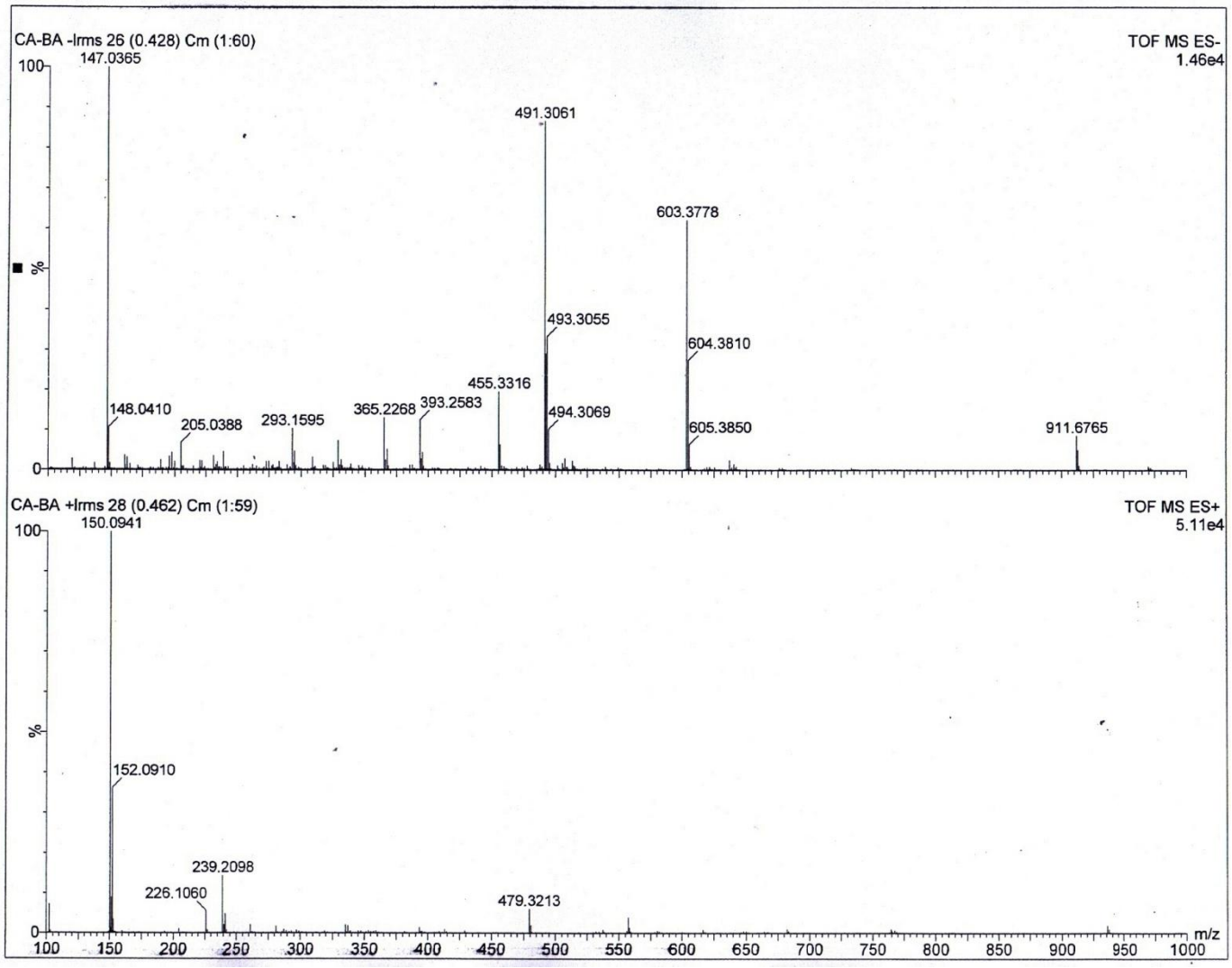
Appendix A22: ^{1}H NMR (400 MHz) spectrum of cinnamic acid hybrid of BA in $CDCl_3$



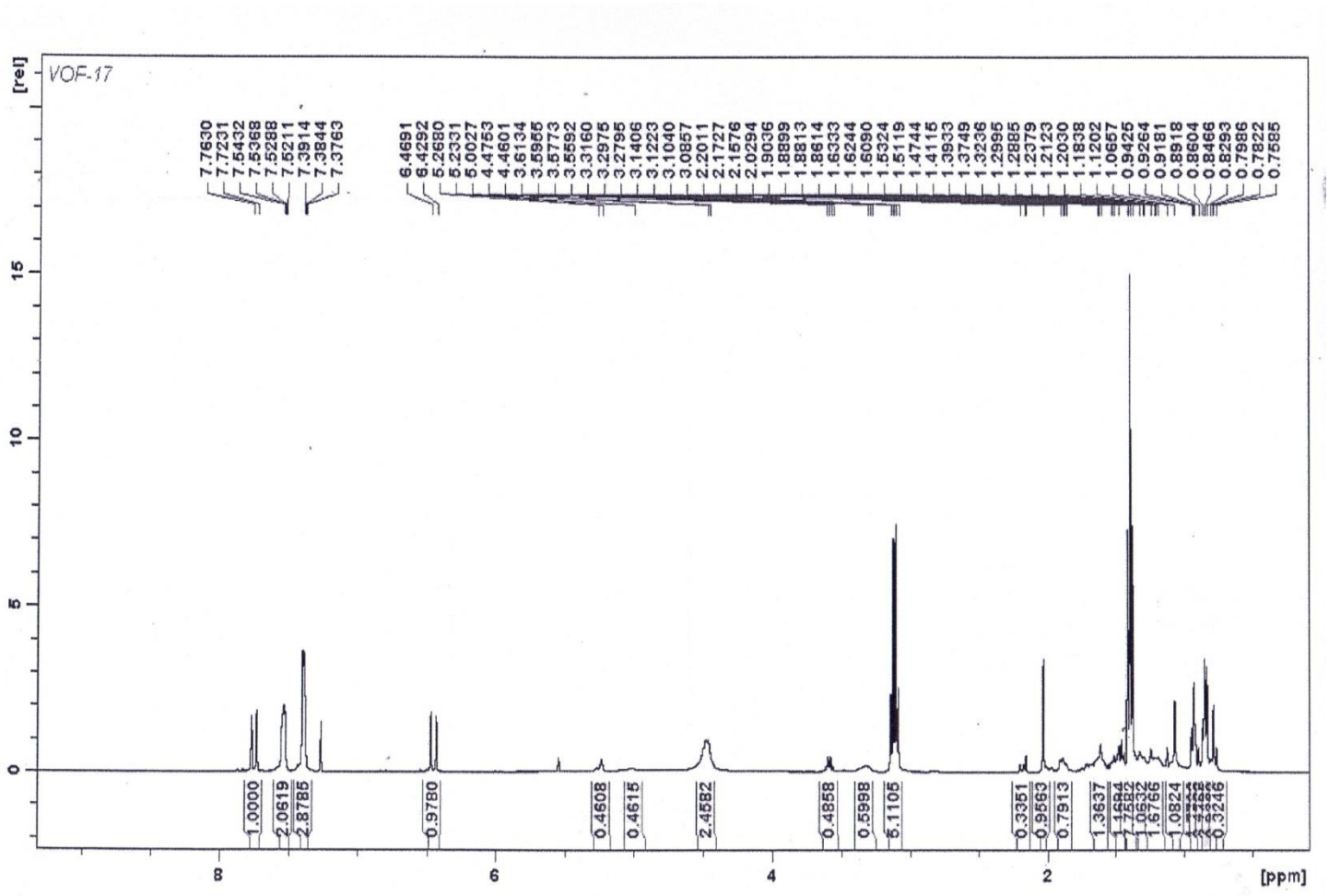
Appendix A23: ^{13}C NMR (400 MHz) spectrum of cinnamic acid hybrid of BA in $CDCl_3$



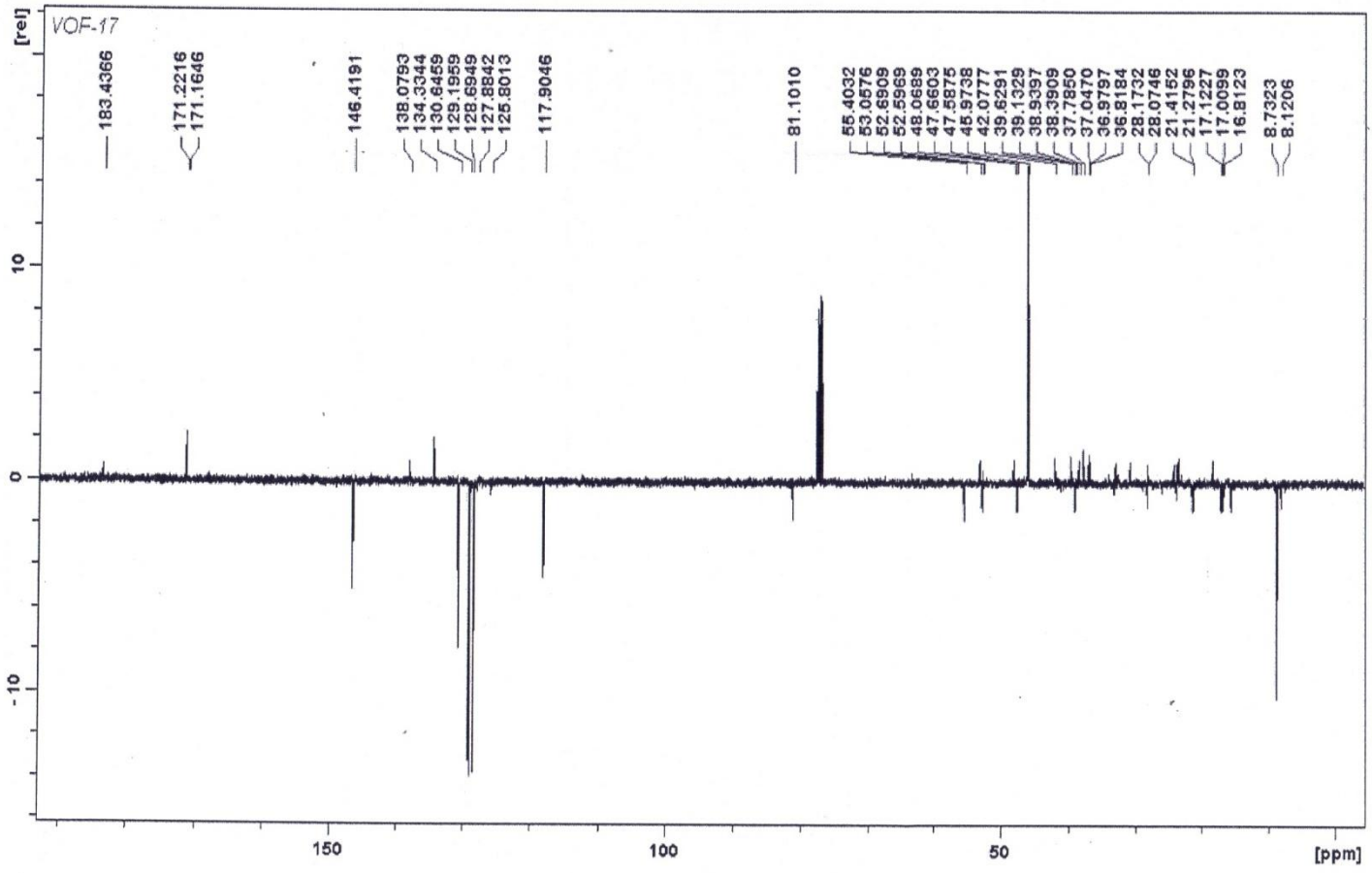
Appendix A24: IR spectrum of cinnamic acid hybrid of BA



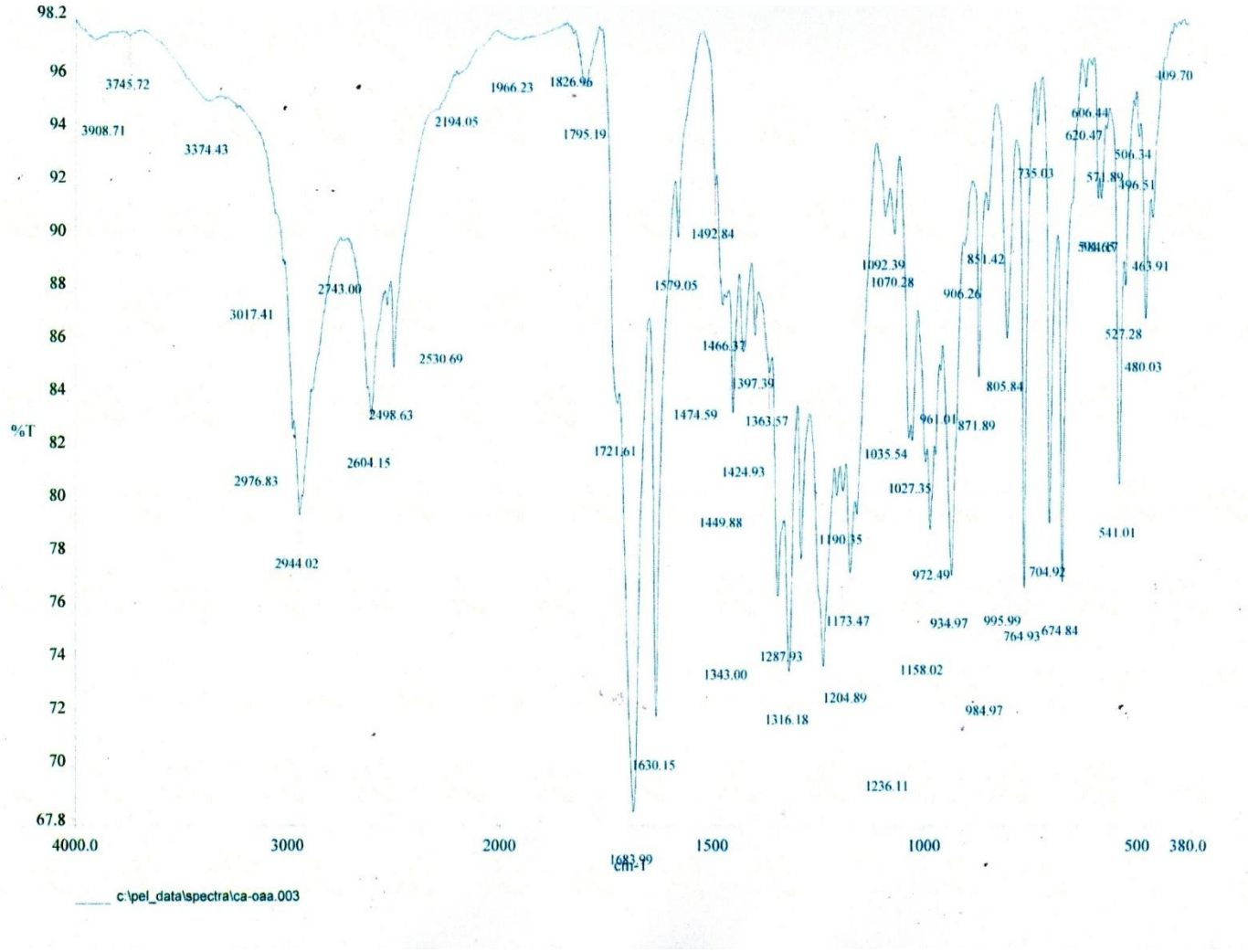
Appendix A25: ES-MS spectrum of cinnamic acid hybrid of BA



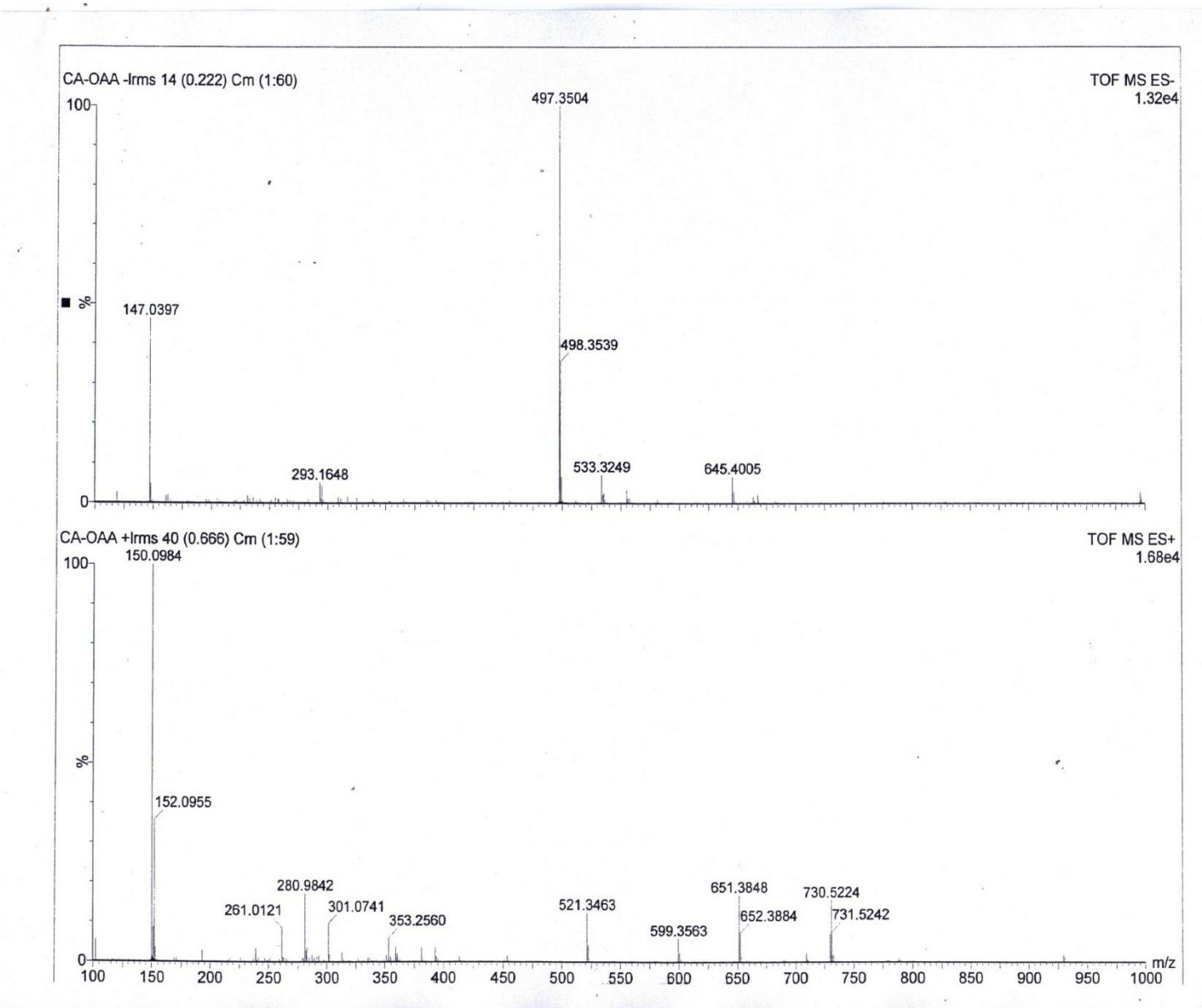
Appendix A26: ^{1}H NMR (400 MHz) spectrum of cinnamic acid hybrid of OAA in $CDCl_3$



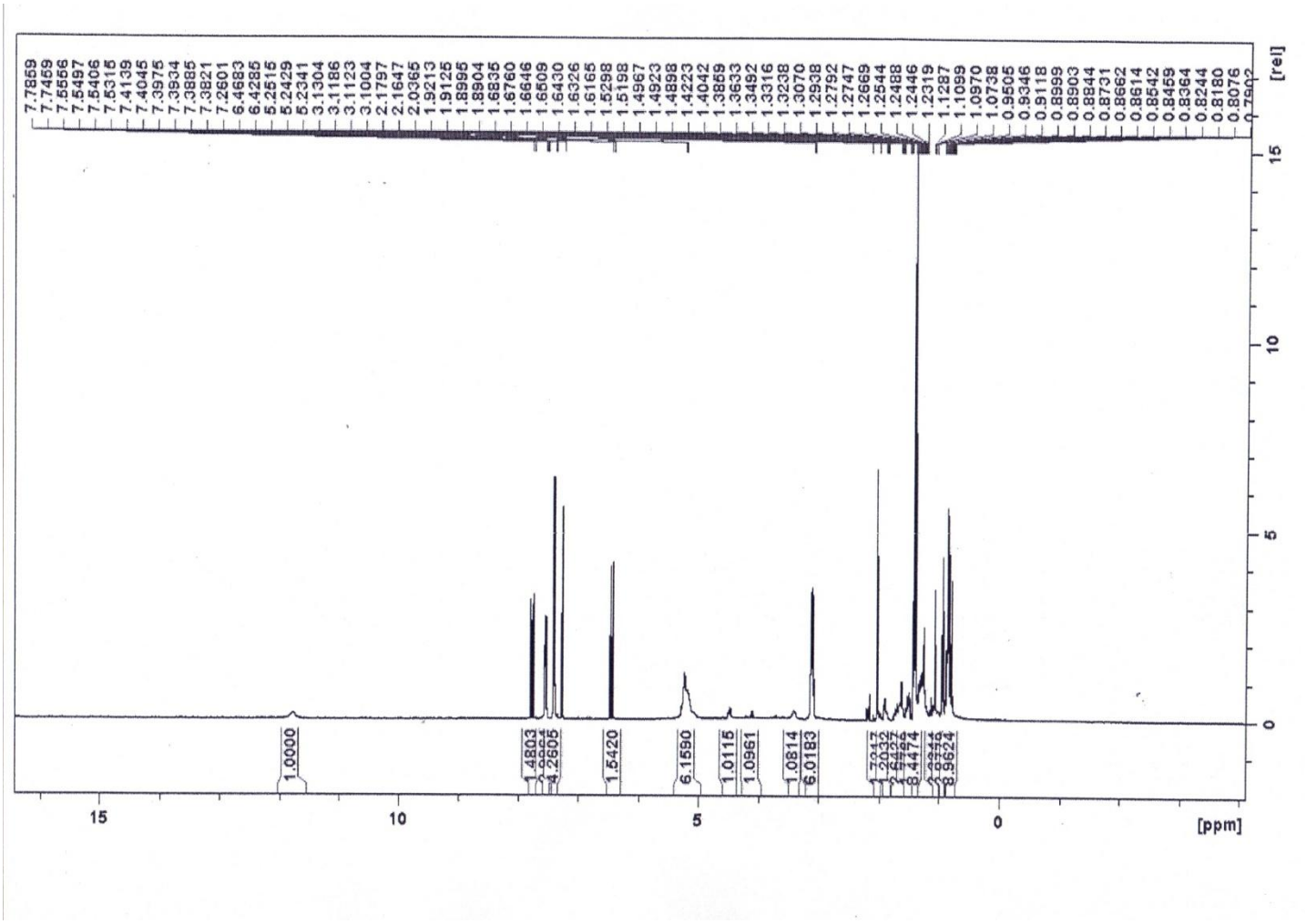
Appendix A27: ^{13}C NMR (400 MHz) spectrum of cinnamic acid hybrid of OAA in $CDCl_3$



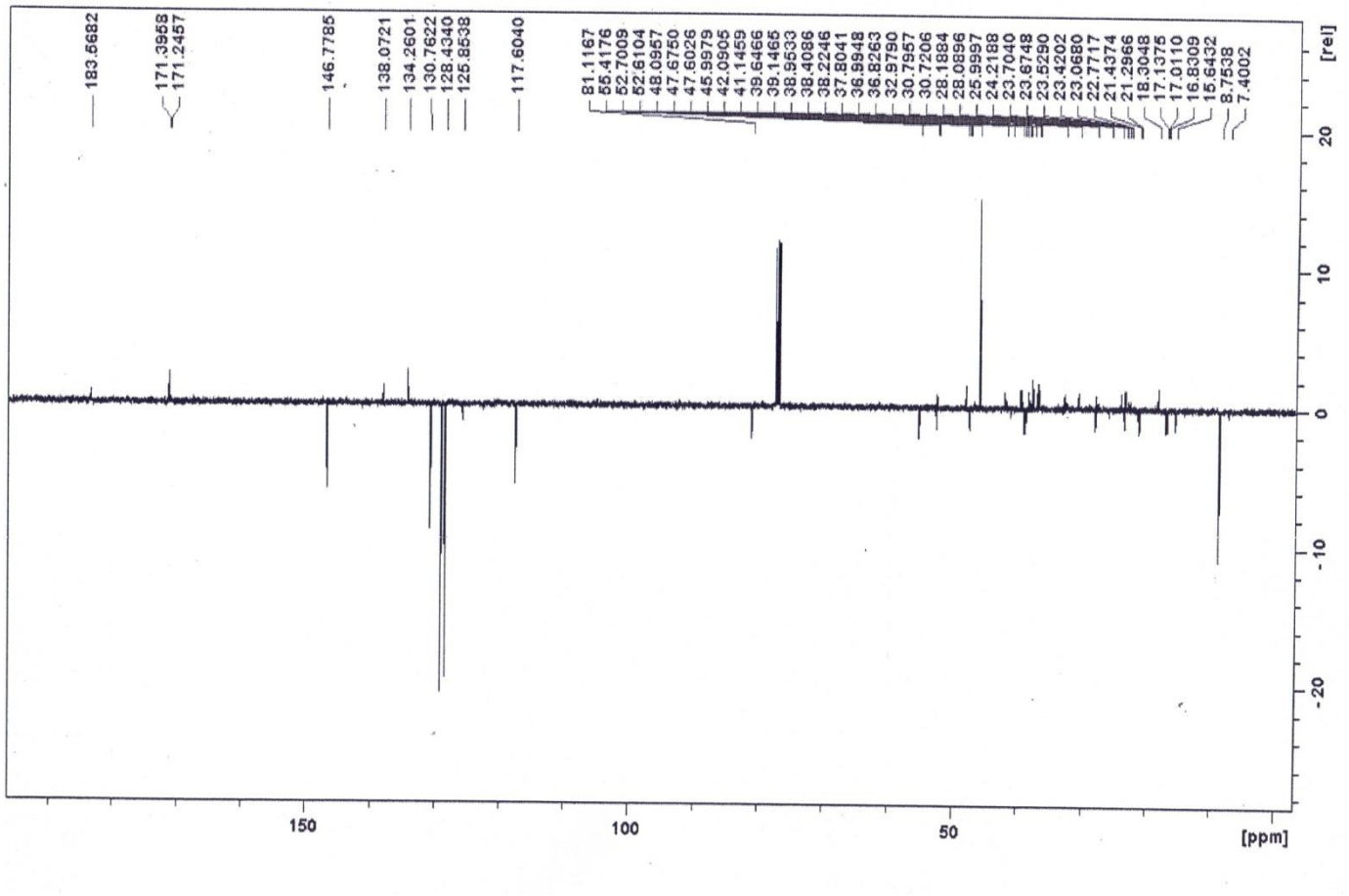
Appendix A28: IR spectrum of cinnamic acid hybrid of OAA



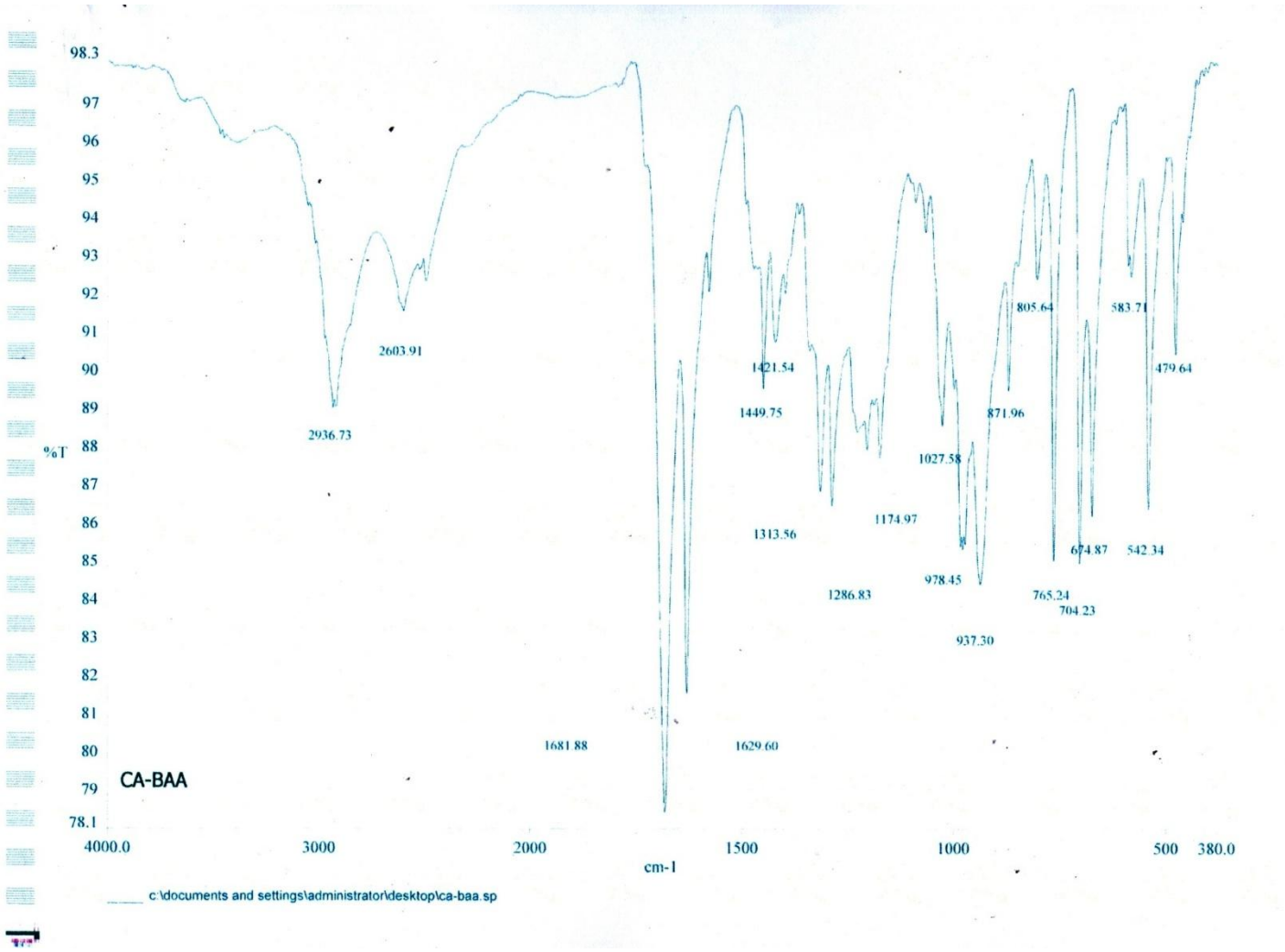
Appendix A29: ES-MS spectrum of cinnamic acid hybrid of OAA



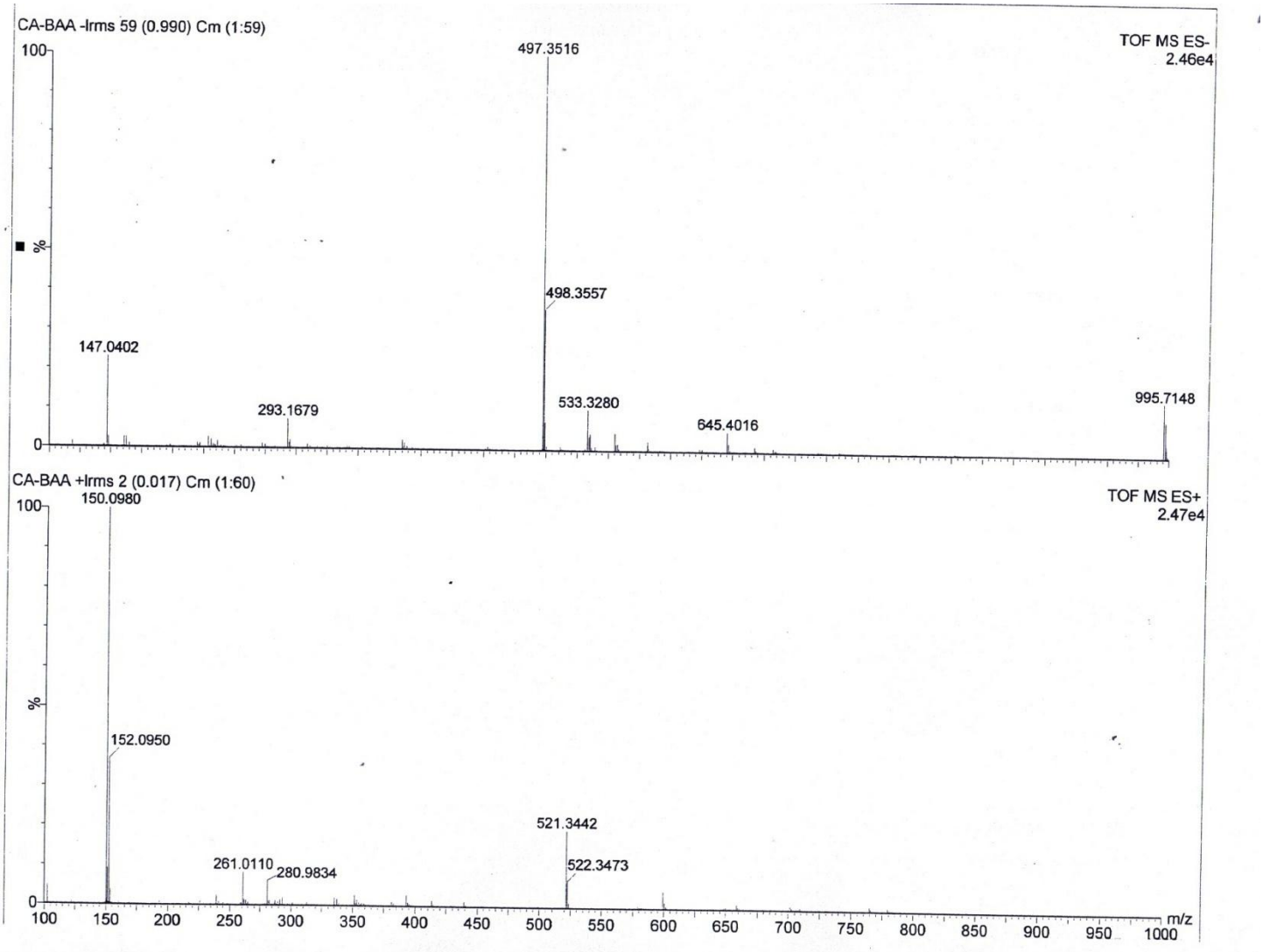
Appendix A30: ^{1}H NMR(400 MHz) spectrum of cinnamic acid hybrid of BAA in $CDCl_3$



Appendix A31: ^{13}C NMR (100 MHz) spectrum of cinnamic acid hybrid of BAA in $CDCl_3$



Appendix A32: IR Spectrum of cinnamic acid hybrid of BAA



Appendix A33: ES-MS Spectrum of cinnamic acid hybrid of BAA

Appendix B

Contributions to knowledge

EXCLI Journal 2015;14:971-983 – ISSN 1611-2156
Received: May 20, 2015, accepted: June 15, 2015, published: August 24, 2015

Original article:

IN VITRO EVALUATION OF THE COMPREHENSIVE ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF *CURTISIA DENTATA* (BURM.F) C.A. SM: TOXICOLOGICAL EFFECT ON THE HUMAN EMBRYONIC KIDNEY (HEK293) AND HUMAN HEPATOCELLULAR CARCINOMA (HEPG2) CELL LINES

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http://dx.doi.org/10.17179/excli2015-351

ABSTRACT

Curtisia dentata is used in African traditional medicine to treat variety of infections. *C. dentata* leaves were collected from Buffelskloof Nature Reserve, South Africa. The ethanol, chloroform, ethyl acetate and acetone extracts were evaluated for antimicrobial activity using micro dilution assay against *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis, Mycoplasma hominis, Candida albicans* and some clinical isolates of *Moraxella catarrhalis*, *Proteus mirabilis* and *Staphylococcus aureus* isolated from HIV patient. Acetone extract exhibited lowest MIC of 0.01 mg/ml against *Candida albicans* compared to other extracts. Besides lupeol, betulinic acid and ursolic acid, β-sitosterol was isolated for the first time from *C. dentata* leaves and exhibited antimicrobial activity with MIC values ranging from 0.20 to 6.25 mg/ml. Furthermore, the ethanol extract and the four isolated compounds revealed microbicidal effect, with MIC index of less than 4. Ethanol extract revealed the best total activity of 2400 ml/g against *Mycoplasma hominis*. Cytotoxicity of the isolated compounds was further investigated against the Human embryonic kidney (HEK293) and Human hepatocellular carcinoma (HepG2) cell lines using the MTT assay. Ursolic acid exhibited the lowest LD_{50} of 122.4 µg/ml against HEK293 cell line while lupeol exhibited LD_{50} of 278.8 and 289.4 µg/ml against HEK293 and HepG2 respectively. Lupeol exhibited low selectivity index. Ethyl acetate and acetone extracts were further investigated for antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH). The acetone extract exhibited potent inhibition of DPPH compared to ethyl acetate extract. The findings of the current work validate the use of the plant species in the treatment of various human infections.

Keywords: Curtisia dentata, β-sitosterol, ethnomedicine, cytotoxicity, antioxidant, antimicrobial

INTRODUCTION

Microbial resistance to common antibiotics is becoming a norm and an enormous threat to general health care facilities, especially in poorer countries with little or improper medical facilities and resources (Marasini et al., 2015; Chovanová et al., 2013;

ground can root and grow to form new plants. *Opuntia pubescens* can be confused with the jointed cactus (*Opuntia aurantiaca*) and the bur cactus (*Opuntia salmiana*). The impacts of *O. pubescens* are not yet known but it is suspected that they will be similar to the notorious jointed cactus (*O. aurantiaca*) which entails a decrease in the grazing area for livestock; injuries to humans and livestock; loss of vegetation; lowered value of pasture; displacement of indigenous plants and curtailed movement of animals and people in the infested area.

doi:10.1016/j.sajb.2015.03.146

Biosphere reserves as multi-use conservation landscapes: Perspectives from the Kruger to Canyons Biosphere Reserve, South Africa

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Biosphere reserves (BRs), as multi-use conservation landscapes, exemplify the landscape mosaic approach to environmental decision-making. With dual *biodiversity conservation* and *sustainable development* objectives, they provide a long-term perspective that aims to improve the relationship between traditional conservation and sustainable use; providing local communities with options to sustainably manage environmental resources into the future. South Africa has six BRs listed with UNESCO's Man and the Biosphere Programme (MaB), with the Kruger to Canyons Biosphere Reserve (K2C) in the north-eastern South Africa being one of the largest globally. K2C is a spatially structured socio-ecological system, with important economic sectors and world-renowned protected areas proximal to extensive, mostly rural, human populations engaged in informal livelihood practices. In this study, time-series remotely-sensed data (1993–2006–2012) was used to track landscape transformation across the subregion, analyzing spatial changes in cover relative to the theoretical MaB concept. The focus is on changes in the scale of land-cover change (spatial extent, rate, intensity of change) across the analysis period, simulating future changes to 2018 and 2024. Results indicate that the spatial distribution of these land-cover changes bear little regard for the prescriptive BR zonation and the relative limitations on 'use' that typifies successful BR implementation. The increased rate of change in the recent observation period (2.3% versus 5.7%) poses challenges for landscape management, with future predictions of escalating transformation likely to undermine BR sustainability long-term. Consequently, the thresholds for scheduling proactive management action have been identified, allowing for a timely detection of unfavorable transformations while practical options for intervention remain.

doi:10.1016/j.sajb.2015.03.147

Antibacterial properties of *Curtisia dentata* leaves and some triterpenes/active principles isolated from them

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Curtisia dentata leaves were collected from Buffelskloof Private Nature Reserve in Mpumalanga Province (South Africa) in April 2014. The leaves were dried and extracted separately with ethanol, chloroform, diethyl acetate and acetone. The extracts were evaluated for antibacterial activity using a micro dilution assay against ATCC strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis* and some clinical isolates (*Moraxella catarrhalis*, *Proteus mirabilis* and *Staphylococcus aureus*) obtained from HIV patients at the Nongoma District hospital in KwaZulu-Natal Province. Ethanol, chloroform and acetone extracts exhibited lowest minimum inhibitory concentration (MIC) of 0.78 mg/ml against *P. aeruginosa*, while diethyl acetate extract exhibited an MIC of 3.13 against *E. coli*, *M. catarrhalis*, *M. smegmatis* and *P. mirabilis*. Two pentacyclic triterpenes, ursolic acid (UA) and betulinic acid (BA) were isolated from the ethanol, ethyl acetate and chloroform extracts and characterized through spectral analysis (IR, NMR, GS-MS) also exhibited antibacterial activity. The BA and UA exhibited an MIC of 0.06 and 0.08 mg/ml against *S. aureus* and *P. mirabilis* respectively. The biological activity of the extracts and the isolated compounds reported, in a way, validates the use of the species in the treatment of various infectious diseases.

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Bioprospecting for betulinic acid among medicinal plants from South African origin

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[c]*Department of Biochemistry and Microbiology, University of Zululand, Private Bag X1001, KwaDlangezwa 3886, South Africa*

Betulinic acid is a pentacyclic triterpenoid that is widely distributed in plant kingdom. It has gained a lot of attention in recent years because of its broad spectrum of biological and medicinal properties. It is an important precursor in synthetic chemistry/pharmacy. Specifically, it is biologically known to possess anti-cancer, anti-HIV, anti-microbial, anti-malaria, anti-anthelmintic, anti-feedant, anti-obese, anti-nociceptive and inhibits DNA polymerase beta. From the available literature, it is observed that plants which bioaccumulate sufficient quantity of betulinic acid may add economic value to the economy if well harnessed. The main objective of this study was to identify plants of South African origin which bioaccumulate betulinic acid, isolate and quantify. The findings of these studies indicated that the leaves of *Melaleuca bracteata* (Johannesburg Gold), leaves of *Curtisia dentata* (umLahleni) and the stem bark of *Peltophorum africanum* (Mosetlha) have betulinic acid as part of their chemical components which has enhanced the medicinal utility potential of these plants in recent time. It is interesting to note that barring any change in climatic conditions, the leaves of *M. bracteata* bioaccumulate betulinic acid more than the leaves of *C. dentata* and the stem bark of *P. africanum*; while the leaves of *C. dentata* is higher in yield than the stem of *P. africanum*. The importance of this result is that it is possible to establish research collaboration involving botanist, biotechnologist, chemist etc. to develop a variety *M. bracteata* with a better yield of betulinic from the present state; in view of the significance of the compound in medicinal chemistry and drug discovery.

doi:10.1016/j.sajb.2015.03.149

Appendix C

Certificate from ethics committee

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

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

Private Bag X1001
KwaDlangezwa 3886

Tel: 035 902 6645
Fax: 035 902 6222
Email: dviljoen@pan.uzulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030 PGD 2012/8**								
Project Title	**Design, synthesis and biological evaluation of antimycobacterial agents from betulinic acid, oleanolic acid and their derivatives**								
Principal Researcher/ Investigator	VO Fadipe								
Supervisor and Co-supervisor	Prof. Shode				Prof. Opoku				
Department	Biochemistry and Microbiology								
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.