ISOLATION AND CHARACTERIZATION OF MEDICINAL PRINCIPLES OF *Pouteria alnifolia* BAKER ROBERTY (SAPOTACEAE)

By

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DEPARTMENT OF CHEMISTRY,

FACULTY OF SCIENCE,

AHMADU BELLO UNIVERSITY,

ZARIA.

2011

DECLARATION

I hereby declare that this thesis has been written by me and that it is a record of my own research work. It has not been presented in any previous application for a higher degree. All quotations are indicated and sources of information are adequately acknowledged by means of references.

---------- ----------

Anyam, John Vershima **Date**

CERTIFICATION

This thesis entitled "Scientific Evaluation of the Medicinal principles of *Pouteria alnifolia* (Baker) Pierre (Sapotaceae) by Anyam, John Vershima meets the regulations governing the award of the degree of Master of Science in Chemistry of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To Andrew and Phoebe Anyam: my parents, and every other parent.

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ABSTRACT

Phytochemical screening of the plant *Pouteria alnifolia* indicated the presence of tannins, sterols, cardiac glycosides, flavonoids but no alkaloids. Two triterpenes have been isolated from the stem bark and identified by GC-MS, and Carbon-13 NMR analyses as Betulin and Lupeol. The extracts from the stem back were found to be antifungal: effective against *Aspergillus nigre, Trichophyton rubrum,* and *Candida albicans* inhibiting their growths at minimum concentrations of 10mg/ml, 20mg/ml and 5mg/ml respectively; and completely killing *Aspergillus nigre* and *Candida albicans* at minimum fungicidal concentrations of30mg/ml, 40mg/ml and 10mg/ml, respectively. Antibacterial screening of the methanol and ethyl acetate extracts showed activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans,* at 0.625mg/ml, 0.625mg/ml, 1.25mg/ml, 1.25mg/ml, and 1.25mg/ml respectively for the ethyl acetate extracts but the methanol extract showed 1.25mg/ml for *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans*. Minimum bactericidal concentrations of the extracts against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans* were found to be 1.25mg/ml, 1.25mg/ml, 5mg/ml, 5mg/ml, and 5mg/ml respectively for ethyl acetate extract and a 5mg/ml for the methanol extract in turn, with *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans.*The methanol extract was found to be active against *Trypanosoma brucei brucei,* in an *in vitro* test, killing all the parasites at a minimum concentration of 5mg/ml in 10 minutes as opposed to the standard drug Diminaveto® 100mg/ml in 3 minutes. The above findings mark *Pouteria alnifolia* as an important medicinal plant.

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CHAPTER ONE

1.1 INTRODUCTION

With all the advancements made by mankind, man has continued to look at plants as important sources of medication for his ailments and new drugs are still being discovered. Hippocrates (460 – 377BC) was reported to have listed approximately 400 different plant species for medicinal purposes (Sarker and Lutfun, 2007). Today, in China alone, 7295 plant species are utilized as medicinal agents (Sarker and Lutfun, 2007).

To the organic chemist, plant constituents are merely a crude chemical brew that needs to be simplified to classifiable active principles (Mills, 1994). A reductionist approach is usually adopted for the following reasons: (i) the isolated principles provide a scientific window into the medicinal usage of the herb, (ii) understanding the active constituents can also provide answers to better isolation procedures and pharmacological behavior of a plant (Bruneton, 1995).

Sometimes, however, a straightforward natural product isolation route, irrespective of bioactivity, is also applied, which results in the isolation of a number of natural compounds (small compound library) suitable for undergoing any bioactivity screening(Sarker and Lutfun, 2007).The most extensively used plant components can be classified into alkaloids, tannins, steroids, phenolic compounds, terpenoids and carbohydrates(Hill, 1952).

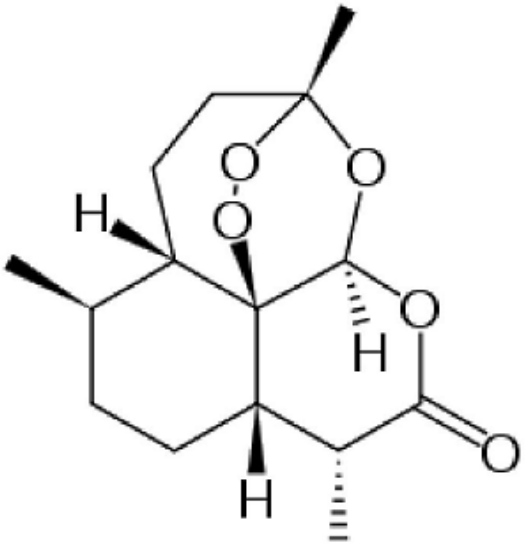
Herbal medicine plays a very important role in the healthcare system of the developing world especially those who do not have access to orthodox medicines, an inclination supported by the (WORLD HEALTH ORGANISATION, 2002). Although about 40% of modern pharmaceuticals are derived from plants, none is used against viruses. In contrast, traditional healers have long used phytomedicines to prevent or cure infectious conditions. Scientists are interested in antimicrobial plant extracts because (i) of dissatisfaction with antibiotics; (ii) of public inclination to take herbal medication; and (iii) viral diseases have no viable cure yet. The earth's resources are dwindling (Lewis, 1995) leading to critical losses of structurally diverse and potentially useful phytochemicals (Borris, 1996). It is against this backdrop that the need arises to investigate plant resources for medicinal uses.

1.2 PHARMACOLOGICAL PRODUCTS IN PLANTS

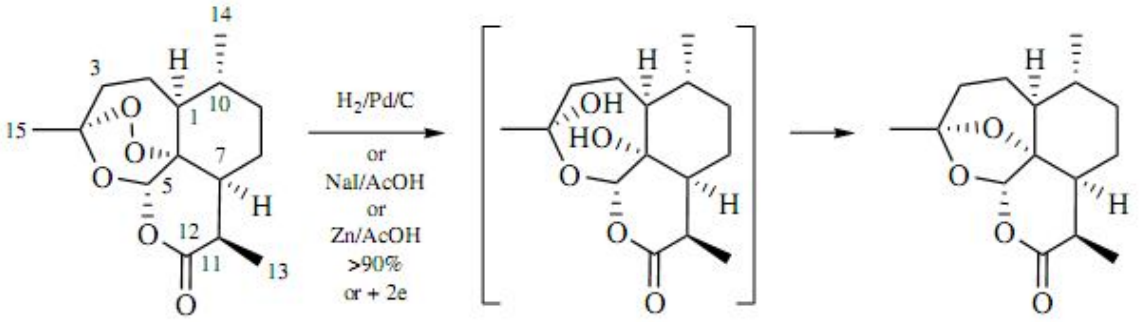
Alkaloids, tannins, steroids, phenolic compounds, terpenoids, proteins, waxes, glycosides, carbohydrates etc. are responsible for physiological activity of plant products. Some of the more important ones will be discussed piecemeal below.

1.2.1 Terpenoids

There are more than 23,000 known terpenoids. They are the largest class of natural products. The terpenoids find wide ranging applications in industry and beyond. For example, the antimalarial drug Artemisinin and the anticancer drug paclitaxel (Taxol®) are two of a few terpenes with established medicinal applications (FAO, 1995).Artemisinin and its derivatives are presently the most potent drugs against *Plasmodium falciparum* (White, 1997)



A



A B C

Figure 1.0 Conversion of Artemisinin to Dehydroartemisinin.

The group of functionalities namely peroxy, ketal, acetal, and lactone in the Artemisinin molecule (A) can be selectively reduced to deoxyartemisinin. Deoxyartemisinin(C) lacks the antimalarial properties of artemisinin indicating that the antimalarial character is conferred by the peroxy moiety(Zeng B.-B. , Wu, Yu, Wu, Li, and Chen, 2000)(Zeng B.-B. , *et al*., 2003).

1.2.2 Phenolic Compounds

This is a large group of structurally diverse naturally occurring compounds that possess at least a phenolic moiety in their structures. For example, quercetin is a flavonoid that has four phenolic hydroxyls at C-5, C-7, C-3^1 and C-4^1. The phenolic group of compounds encompasses various structural types, for example: phenylpropanoids, coumarins, flavonoids and isoflavonoids, lignans tannins etc. (Krishnamachari, Levine, and Pare, 2002)(Sarker and Lutfun, 2007).

Quercetin possesses various degrees of antioxidant or free radical scavenging properties (Sarker and Lutfun, 2007)

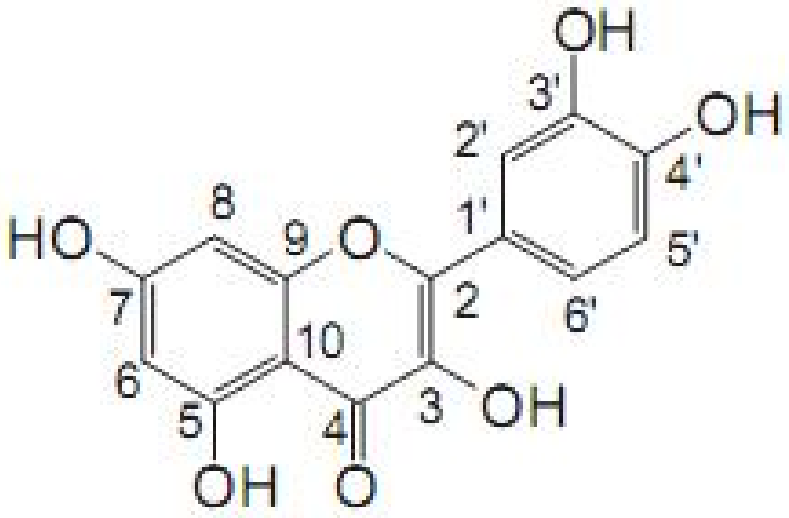
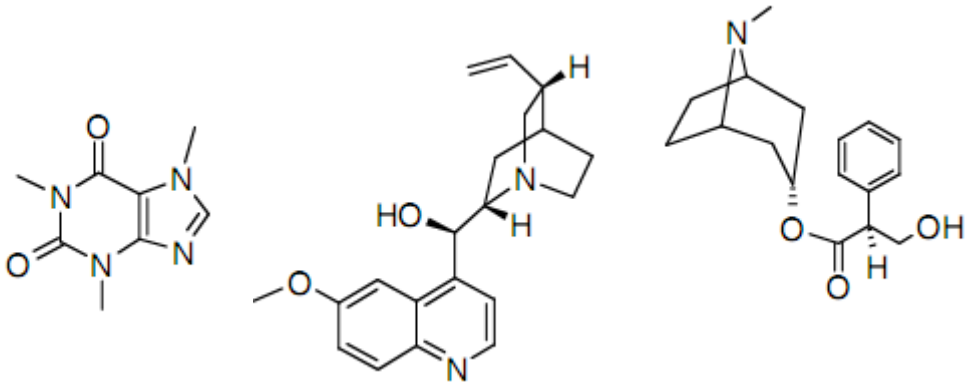

OH
3'
OH
2'
4'
8
1'
HO
9
O
5'
7
2
6'
10
6
3
OH
5
4
OH
O

Figure 2.0 Quercetin

1.2.3 Alkaloids

Most alkaloids are obtained from plants. They are pharmacologically active, basic compounds derived from amino acids. They may contain one or more heterocyclic nitrogen atoms in the molecule (Cseke, Kirakosyan, Kaufman, Warber, Duke, and Brielmann, 2006)(Cordell, 1981). In fact, most nitrogen-containing secondary metabolites are usually deemed alkaloids, but for amines or glucosamines may be grouped differently. The word "alkaloid"originated from the Arabic, *al-qali* (an early form of soda ash) (Cordell, 1981). Many alkaloids have a bitter taste, and a large number of them exhibit potent physiological effects on mammals. For example, morphine shows narcotic effects; reserpine is an antihypertensive agent; atropine is a smooth muscle relaxant; cocaine is a potent central nervous system stimulant; and strychnine is a nerve stimulant.



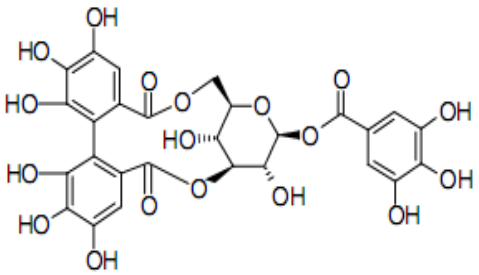
1. 2. 3.

Figure 3.0 Some important alkaloids 1. Caffeine (a pseudo-alkaloid) 2. Quinine 3. Atropine

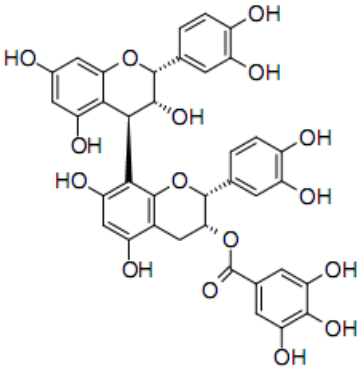
Alkaloids in plants serve as chemo-protective anti-herbivory agents or as growth regulators, such as the well-known plant hormone, indole-3-acetic acid, (IAA) which is an indole derivative synthesized from tryptophan (Buchanan, Gruissem, and Jones, 2000).

1.2.4 Tannins

Tannins are water-soluble oligomers, rich in phenolic groups, capable of binding or precipitating water soluble proteins (Hagerman and Butler, 1989). The tannins, common to vascular plants, exist primarily within woody tissues but can also be found in leaves, flowers, or seeds. Plant tissues that are high in tannin content have a highly bitter taste and are avoided by most feeders. Tannins fall into two groups: either condensed tannins or hydrolysable tannins.



1.



2.

Figure 4.0 Structures of representative hydrolysable tannins (1) and condensed proanthocyanidins (2)

Corilagin shows anti-human-immunodeficiency-virus (HIV) activity by inhibiting reverse transcriptase(Notka, Meier, and Wagner, 2004)(Cseke, Kirakosyan, Kaufman, Warber, Duke, and Brielmann, 2006).

1.2.5 Steroids

Steroids are chemical messengers. Some steroids are also hormones. All steroids are derived from the acetate biosynthetic pathway. Hundreds of distinct steroids have been identified in plants, animals and fungi, and most of them have interesting biological activity. They possess a perhydrocyclopentaphenanthrene nucleus (Figure 1.4).

The four rings are lettered A, B, C and D, and the carbon atoms are numbered beginning in the A ring as shown in figure 5.0. These fused rings can be trans fused or cis fused. In steroids, the B, C and D rings are always trans fused. In most naturally occurring steroids, rings A and B are also trans fused. Different steroids vary in the functional groups attached to these rings.

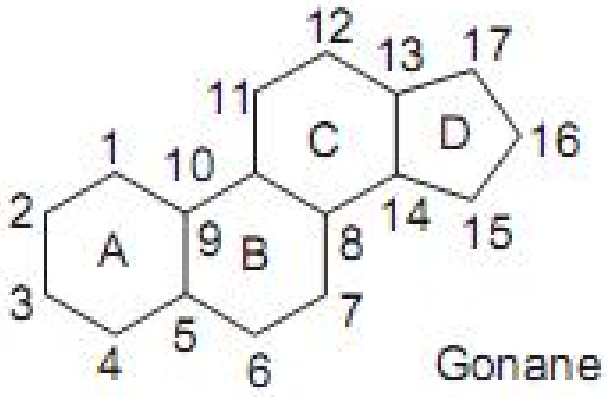


Figure 5.0 Gonane

Phytosterols found in plants have many applications ranging from food additives, medicine to cosmetics (Sarker and Lutfun, 2007).

1.3 AIMS AND OBJECTIVES OF THE RESEARCH

1.3.1 Aims

The aims of this project were to:

i. Authenticate or otherwise, the ethno medicinal claims on the vegetative parts of *Pouteria alnifolia.*

ii. Isolate and characterize some compound(s) that may be responsible for the claimed ethno medicinal values.

1.3.2 Objectives of the Research

1. Collection, proper botanical identification, drying and pulverizing of the vegetative part of the plant.

2. Extraction of the powdered plant material using different solvents based on the eluotropic series i.e. from non-polar pet-ether (60-80c) to methanol (polar).

3. Phytochemical screening for bio-active compounds using the crude extracts.

4. Antibacterial screening of the extracts.

5. Antifungal screening of the extracts.

6. Analytical separations involving several consecutive steps of chromatographic techniques and purification.

7. Verification of the purity of the isolated compound(s).

8. Structural elucidation and characterization of the possible isolated compounds using available spectroscopic techniques, ^{13}CNMR, ^{1}HNMR, IR and GC-MS etc.

1.4 RESEARCH JUSTIFICATION

Natural products present unparalleled chemical variety with structural complexity and biological properties (Verdine, 1996). Natural products can model the design of synthetic compounds (Breinbauer, Vetter, and Waldmann, 2002). Research on natural products has led to the discovery of novel mechanisms of action for example, those of immune-suppressants (Urizar, Liverman, Dodds, *et al*, 2002)(Zhang and Demain, 2005). Diseases like HIV/AIDS still have no viable cure. The World Health Organization (World Health Organization, 2011) estimates that 4 billion people, or 80 percent of the world's population, use herbal medicine for some aspect of primary health care.

It is against this background that *Pouteria alnifolia,* which is a plant used extensively as an herbal remedy in some parts of Nigeria, West Africa, and throughout sub-Saharan Africa is being investigated.

1.5 SCOPE AND LIMITATION OF THIS RESEARCH

The scope of this research work would be: the phytochemical screening, *in vitro* antibacterial/antifungal screening, isolation, characterization and structural elucidation of the active principles of *Pouteria alnifolia*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 THE FAMILY SAPOTACEAE

Sapotaceae is a family of flowering plants, belonging to order *Ebenales* .The family name is coined from *zapote*, a Mexican vernacular name for one of the plants (in turn derived from the *Nahuatl "tzapotl").* The name was Latinized by Linnaeus as sapota. The family includes about 800 species of evergreen trees and shrubs in approximately 65 genera (35-75, depending on generic definition). Members of the family are found throughout the tropics, many producing edible fruits, for example shea butter and the Australian native plum (USDA, ARS, National Genetic Resources Program, 2003). Many also have use in traditional medical practice throughout the world and have been supported by scientific evaluation (Ndukwe, Amupitan, Isah, and Adegoke, 2007)

2.1.1 The Genus *Pouteria*

The genus *Pouteria* is among the 57 genera of *Sapotaceae.* Commonly, this genus is known as ***Pouteria*** trees or, in some cases, **eggfruits**. There is a lot of uncertainty about the genus *Pouteria* and therefore its members are continually being reclassified. Many *Pouteria* species produce edible fruits and dense hard woods for timber and firewood but there the utility ends as the woods are too heavy for furnishings (UNITED STATES DEPARTMENT OF AGRICULTURE (USDA), 2009).

2.1.2 Chemical Composition of *Pouteria* Species

Triterpenes and flavonoids are the most abundant principles of this genus (Cíntia, Silva, and Simeoni, 2009). Below is a summary of the chemical composition of *Pouteria* species as reported by (Cíntia, Silva, and Simeoni, 2009) (Table 2.1).

Table 2.1Phytochemistry of Pouteria species showing compounds isolated

Species	Compound
Pouteria caimito (Ruiz and Pav.) Radlk.	**1, 3, 9, 15, 16, 16a, 16b, 17, 18, 26**
Pouteria cambodiana (Pierre exDubard) Baehni	**24**
Pouteria campechiana (Kunth) Baehni	**20, 21, 21a, 21b, 21c, 22, 23, 24**
Pouteria gardnerii (Mart. and Miq.)Baehni	**1, 2, 1a, 3a, 10, 11, 12**
Pouteria mammosa (L.) Conquest (sin Achras mammosa L. and Calocarpum mammosum Pierre)	**9, 42**
Pouteria pariry (Ducke) Baehni	**26**
Pouteria sapota (Jacq.) H. E. Moore et Stern (Calocarpum sapota (Jacq.) Merr. and Sideroxylon sapota Jacq.)	**20, 21, 21a, 21b, 21c, 23, 42, 43, 43a**
Pouteria splendens (A. DC.) Kuntze	**27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41**
Pouteria subrotata Cronquist	**42**
Pouteria venosa (Mart.) Baehni	**4, 11, 16, 18, 19, 19a**
Pouteria viridis (Pittier) Cronquist (sin. Calocarpum viride Pittier)	**20, 21, 21a, 21b, 21c, 22, 23**
Pouteria vitiensis (Gillespie) O.Deg.	**18, 24a, 25**
Pouteria tomentosa (Roxb.) Baehni	**1a, 2a, 10, 11,**
Pouteria torta (Mart.) Radlk.	**1, 1a, 2, 2a, 3, 3a, 4, 5, 6, 7, 8, 9a, 10, 11,13, 14, 22, 23, 24, 25**

Key to table 2.1

1	α-amyrin	**16b**	taraxenona	**32**	eremophilene
1a	α-amyrinacetate	**17**	β-sitosterol	**33**	δ-cadinene
2	β-amyrin	**18**	spinasterol	**34**	10-epi-α-cadinol
2a	β-amyrin acetate	**19**	miriantic acid	**35**	10-epi-α-muurolol
3	lupeol	**19a**	19α,23-dihydroxyursolic acid	**36**	epi-globulol
3a	lupeol acetate	**20**	gallic acid	**37**	globulol
4	taraxasterol	**21**	(+)-gallocatechin	**38**	ledene
5	pseudotaraxasterol	**21a**	(+)-catechin	**39**	palustrol
6	cycloartenol	**21b**	(-)-epicatechin	**40**	isophytol
7	lanosterol	**21c**	(+)-catechin-3-O-gallate	**41**	trans-phytol
8	lanosta-7,24-dien	**22**	dihydromyricetin	**42**	Lucumin
9	erythrodiol	**23**	myricitrin	**43**	lucuminic acid
9a	erythrodiolpalmitate	**24**	protocatechuic	**43a**	lucuminamide
10	betulinic acid	**24a**	vanilloyl sucrose		
11	ursolic acid	**25**	myo-inositol		
12	oleanolic acid	**26**	α-copaen		
13	α-friedelinol	**27**	cis,trans-α-farnesol		
14	β-friedelinol	**28**	trans-nerolidol		
15	dammarenediol	**29**	cis-β-elemene		
16	taraxerol	**30**	germacrene		
16a	Taraxerol acetate	**31**	β-selinene		

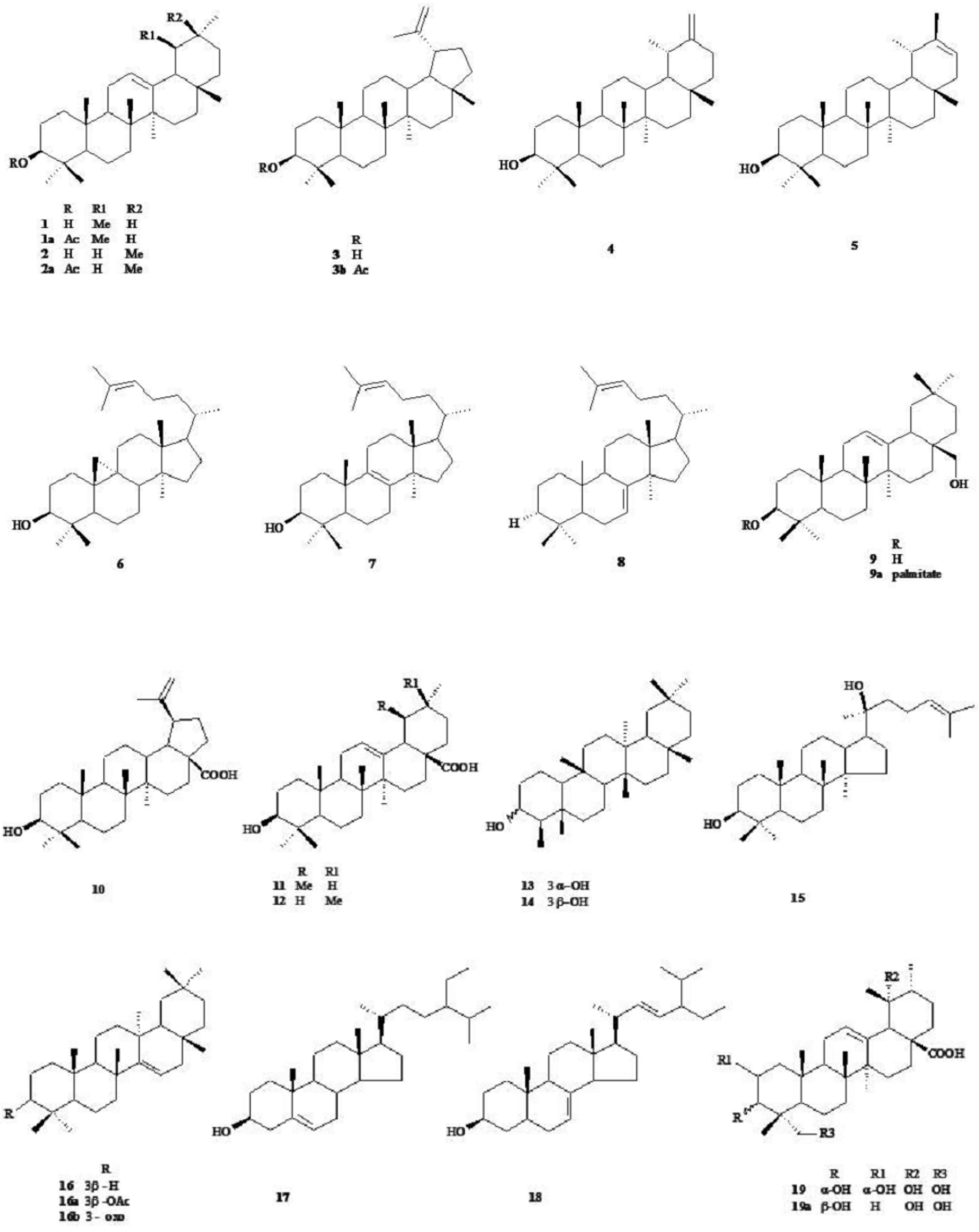


Figure 6.0 Isolated substances from species of genus *Pouteria* (Sapotaceae) (Cíntia, Silva, and Simeoni, 2009)

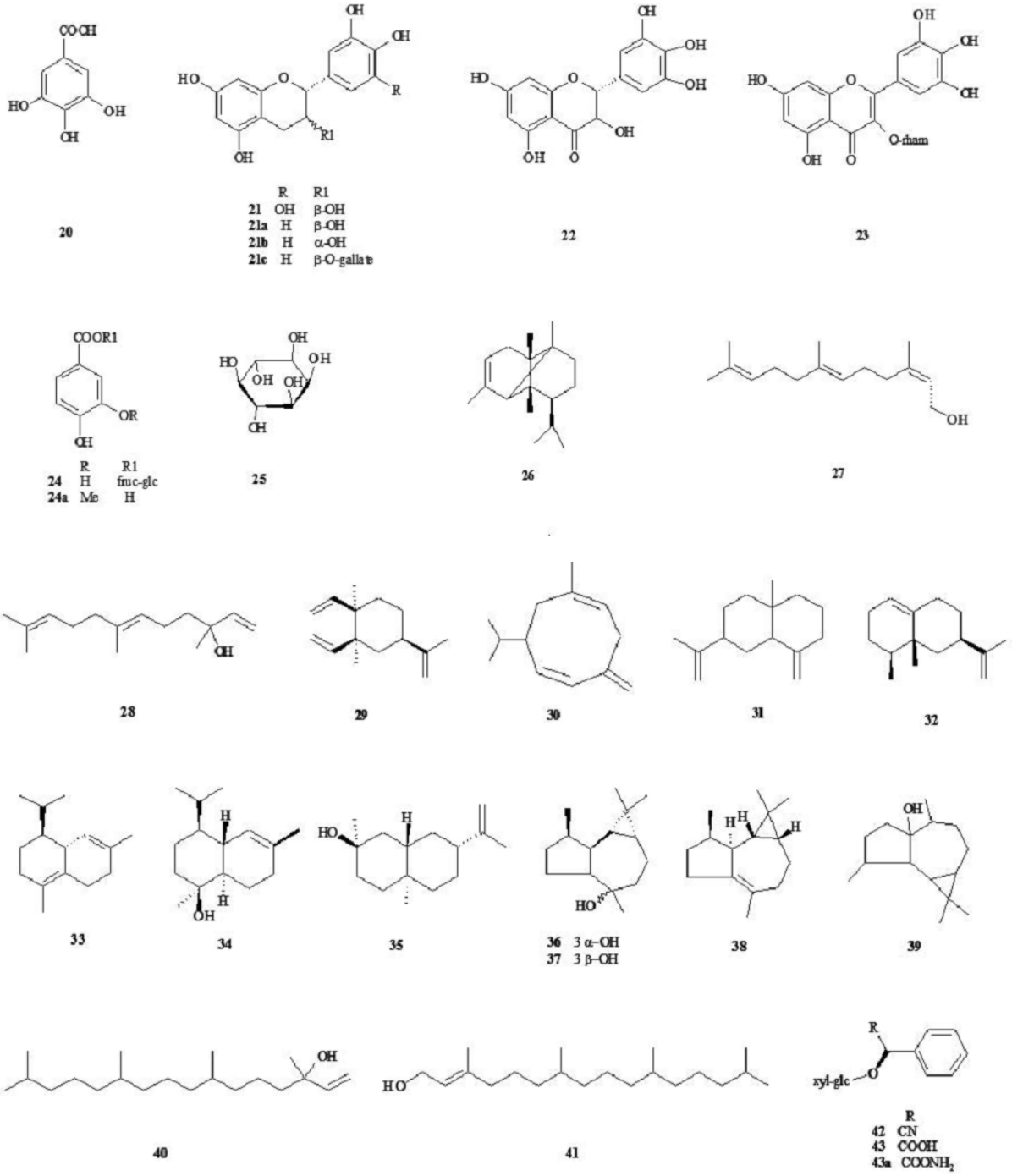


Figure 7.0 Isolated substances from species of genus *Pouteria* (Sapotaceae) (Cíntia, Silva, and Simeoni, 2009)

(Cíntia, Silva, and Simeoni, 2009)

2.1.3 Biological Activity of *Pouteria* Species

Many *Pouteria* species have been used in folk medicine for ailments ranging from fevers, diabetes, yaws, inflammation, ulcers, nausea, back pain, to promotion of lactation(Cíntia, Silva, and Simeoni, 2009)(Ma, Yang, Basile, and Kennelly, 2004)(Perfeito, Santos, Lopez, Paula, and Silveira, 2005)(Manosroi, Saraphanchotiwitthaya, and Manosroi, 2005).

Scientific data is gradually growing to support these claims: antioxidant and free radical scavenging activities have been reported to be present in the more polar extracts and fractions (Rice-Evans, Mille, and Paganga, 1996). The compounds present in the genus represent several biological activities ranging from anti-malarial, anti-cancer, anti-inflammatory among a host of many others(Ying, Rinehart, Simon, and Cheronis, 1991)(Pelzer, Guardia, Juarez, and Guerreiro, 1998)(Nijveldt, van Nood, van Hoorn, Boelens, van Norren, and van Leeuwen, 2001)(Hodges, Kweifio-Okai.G., and Macrides, 2003)(Cushnie and Lamb, 2005)(Fontanay, Grare, Mayer, Finance, and Duval, 2008) (Table 2.2).

Table 2.2 Biological activity of Pouteria species (Cíntia, Silva, and Simeoni, 2009)

Species	Parts	Biological activity
Pouteria caimito	leaves	Antioxidant
Pouteria cambodiana	stem bark	Imunomodulatory, antioxidant; cell antiproliferative
Pouteria campechiana	fruits juice	Antioxidant and antinitrosative, anti-mitotic
Pouteria grandiflora	leaves	antimicrobial and DNA-damaging subchronic embryotoxicity to *Crassostrea gigas*
Pouteria guianensis	wood	antimicrobial
Pouteria pallida	leaves	antimicrobial and DNA-damaging fungicidal
Pouteria psamophila	Leaves, wood	Antioxidant; anti-inflammatory and antinociceptive activity; toxicity to *Artemia salina* larvae
Pouteria ramiflora	leaves	antimycobacterial
Pouteria reticulata	Barks fruits juice	Antioxidant
Pouteria sapota	fruits juice stems	trypanocidal activity in vitro
Pouteria splendens	leaves	germination inhibition Antioxidant, toxicity to *Artemia salina* larvae, antibacterial and antifungal, germination and growth inhibition, estrogen antagonism
Pouteria torta	Leaves, seeds	antifungal and induction of human, rabbits and rats erythrocyte agglutination ability
Pouteria venosa	Leaves, leaves	Antioxidant, antimalarial activity in vivo anti-HIV
Pouteria viridis	fruits juice, Stem, stem bark	Antioxidant antitermite



1.



2.

Figure 8.0 *Pouteria campechiana* (1) and *Pouteria multiflora* (2) (phytoimages, 2010)



1.



2.



3.

Figure 9.0 *Pouteria pallida* (1) *Pouteria obovata* (2) *Pouteria splendens* (3) (phytoimages, 2010)



Figure 10.0 *Pouteria alnifolia* (Plant of Study)

2.1.4 REVIEW OF *POUTERIA ALNIFOLIA* (BAKER) ROBERTY

2.1.4.1 ***Taxonomic classification***

Kingdom: Plantae (Plants)

Subkingdom: Tracheobionta (Vascular plants)

Superdivision: Spermatophyta (Seed plants)

Division: Magnoliophyta (Flowering plants)

Class: Magnoliopsida (Dicotyledons)

Subclass: Dilleniidae

Order: Ebenales

Family: Sapotaceae (Sapodilla family)

Genus: *Pouteria*

Species: *Pouteria alnifolia* (Baker) Pierre [Not. bot. 61. 1891] =*Pouteria alnifolia* (Baker) Roberty) (BOTANICA SYSTEMATICA, 2007)

2.1.4.2 ***Common Names***

Afraba, Akala, Akara, Akonko, Akpafia, Banga, Banwa, Chepkabet, Dablaku, Fafaraha, Fuoda, Gigintshotsho, Kipwoibet, Lablaku, Luniondet, Muna, Mutunguru, Ngechebet, Owri, Pusum, Seite, Shoibuzozi, Tutoduodi(The Wood Explorer, 2009)

Pouteria alnifolia is a tropical tree found in an area in Africa spanning the coast of Senegal through Nigeria to south-western Ethiopia and eastern Kenya, and south to Mozambique(The Wood Explorer, 2009)(Lemmens, 2009)(ITHAKA, 2008).

2.1.4.3 ***Ethnobotany***

The wood is used locally for construction, implements, walking sticks and canoes, as firewood and for charcoal production. The sweet fruit pulp is eaten fresh.

The leaves are sometimes used as fodder for livestock. They are also used as food wrappers. The leaves are applied externally in traditional medicine in West Africa to treat yaws, wounds and conjunctivitis. The pulped bark is administered in embrocations to treat rheumatism and as an enema to treat diarrhoea in children (Lemmens, 2009). The leaf sap is used for eye treatments (ITHAKA, 2008)

2.1.4.4***Properties***

The heartwood is yellowish white and not clearly demarcated from the sapwood. The specific gravity of the wood is 0.45. The wood is fairly hard, durable and resistant to termites.

The leaves are well accepted as fodder by sheep, although they contain considerable amounts of tannin. The nutrient content per 100 g dry matter is: crude protein 13.3 g, crude fat 3.7 g, crude fibre 25.6 g and total ash 7.6 g Per 100 g dry matter. The total phenol content is 8.3 g of which tannin phenols is 6.8 g and extractable condensed tannins 6.6 g.

2.1.4.5***Ecology***

Pouteria alnifolia occurs in dry and gallery forests, often in the transition zone between savannah and forest, and also in disturbed forests. It is often found on rocky sites. In south-

western Burkina Faso *Pouteria alnifolia* is most common on moister soils. In East Africa it is often an under-storey tree in lowland rain and deciduous forests. It is very common, especially in West and Central Africa. *Pouteria alnifolia* is a pioneer species that tolerates fire. It regenerates abundantly in regularly burnt forests, where it is often more common than in unburnt forests. In Benin, vegetation communities in which *Pouteria alnifolia* plays an important role are considered an indicator of sites with great potential for growing teak (The Wood Explorer, 2009).

2.2 BACTERIAL AGENTS

2.2.1 Staphylococcus "Gram - Positive"

The genus *Staphylococcus* consists of cluster-forming, gram-positive cocci. *Staphylococcus* derived from Greek *staphyle* "bunch of grapes" and *kokkos* "grain or berry" the main pathogen within the genus. *Staphylococcus aureus* is the cause of a wide range of major and minor infections in man and animals. Currently, there are some 27 different species of *Staphylococci.* These fall into two main groups on the basis of their capability to clot blood plasma by action of the enzyme coagulase. *Staphylococcus aureus* is by far the most important coagulase positive species. The coagulase-negative *Staphylococci* are skin commensals, which are now recognized as important opportunistic pathogens that can cause infections associated with prosthesis, catheters and implants *(Staphylococcus epidermidis)* and urinary tract infections *(Staphylococcus saprophyticus.)*. It has not been common practice for medical microbiologists to identify coagulase-negative *Staphylococci* to the species level. However, methods are now available that facilitate identification and the

importance of other recently recognized coagulase-negative species is being assessed. (Pfaller and Herwaldt, 1988).

Staphylococcus aureus which is present in the nose and on the skin of a variable proportion of healthy people is an opportunistic pathogen, in that, it causes infection most commonly at sites of lowered host resistance such as damaged skin or mucous membranes or haematomas in the cancellous tissues of a long bone(Jones, Board, and Sussman, 1990)(Zakirai, 2009).

2.2.2 Streptococcus "Gram - Positive"

Streptococci are gram - positive bacteria arranged in chains of varying length each cell is approximately 1.0µm in diameter, non - motile, non- sporing and may be capsulate.

Streptococci form part of the normal flora of man and animals. They inhabit various sites notably the upper respiratory tract, and usually live harmlessly as commensals. Some species, of which *Staphylococcus pyogenes* is the most important, may adopt a more aggressive role. This organism can cause a wide spectrum of diseases, including tonsillitis, erysipelas, impetigo, scarlet lever, and septicaemia, which range in severity from the trivial to the life threatening. *Staphylococcus pyogenes* is also involved in the aetiology of rheumatic fever and acute glomerulonephritis. Other pathogenic species includes *Staphylococcus agalaectiate* an important cause of neonatal infection and *Streptococcus pneumoniae.* commonly called *Pneumococcus* which primarily causes disease of the middle ear, paranasal sinuses mastoids and the lung parenchyma but may spread to other sites, such as the joints, peritoneum, endocardium and biliary tract, in particular the meninges

2.2.3*Klebsiella pneumonia* "Gram-Negative"

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Ray, 2004). In recent years, klebsiellae have become important pathogens in nosocomial infections (Postgate , 1998). The Danish scientist Hans Christian Gram (1853–1938), developed the technique now known as Gram staining in 1884 to discriminate between *K. pneumoniae* and *Streptococcus pneumoniae*. *Klebsiella* was named after the German bacteriologist Edwin Klebs (1834–1913). Multiple-resistant *Klebsiella pneumoniae* have been killed *in vivo* via intraperitoneal, intravenous or intranasal administration of phages in laboratory tests (Podschun and Ullman, 1998). As with many bacteria, the recommended treatment has changed as the organism has developed resistances. Klebsiella organisms are often resistant to multiple antibiotics. Current evidence implicates a plasmid as the source of the resistant genes. Klebsiella with the ability to produce extended-spectrum beta-lactamases ESBL are resistant to many classes of antibiotics. The most frequent resistances include resistance to aminoglycosides, fluoroquinolones, tetracyclines, chloram-phenicol, and sulfamethoxazole-trimethoprim (Podschun and Ullman, 1998).

2.2.4 Salmonella "Gram – Negative"

Salmonellae are widely distributed in nature. All vertebrates appear capable of harbouring *Salmonellae* in their gut. It has also been isolated in a wide range of arthropods such as flies, cockroaches, fleas, and ticks. *Salmonella typhi* and *Salmonella paratvphi A. B.* and C are primary human pathogens which are rarely isolated from other animals. *Salmonellae paratyphi,* while essentially a human pathogen, is occasionally; isolated from cattle, pigs,

poultry, and other animals. The cycle of transmission in these hosts has not been fully demonstrated (Christie, 1987). The ability of *Salmonellae* to enter and survive within macrophages and other cells particularly in the liver and kidney occasionally leads to persistent infection and the chronic carrier state where sufferers from *Salmonella* infection continue to excrete the organism in their stools for days or weeks after complete clinical recovery.

In the treatment and clinical management of enteric lever due to Salmonellae the problem of bone marrow toxicity and widespread emergence of chloramphenicol resistance in *Salmonella typhi* in many parts of the world prompted the search for alternative drugs and among these are ampicillin and cotrimoxazole and they have been shown to be comparable in efficacy to chloramphenicol (Levine, Ferreccio, Black, Tacket, and Germanier, 1989).

2.3 FUNGI

Fungi constitute large diverse group of heterotrophic organisms which exist as saprophytes, parasites or commensals. Most are found as saprophytes in the soil and on decaying plant material.

About 180 of the 250,000 known fungi species are recognized as capable of causing diseases (mycosis) in man and animals (Rippo, 1988). Most of these are mould fungi but there are number of pathogenic yeast fungi and many are dimorphic. Dimorphic fungi usually assume the mould form when growing as saprophytes in nature and the yeast form when causing infection. In the laboratory, the tissue form can be induced by culture at 37 ^{o}C on rich media such as blood agar, whereas the mould form develops when incubated at a lower temperature (22-27^{0}C) on a less rich medium such as Sabouraud's agar (Rippo, 1988).

Yeast infections affecting skin and nails and mucous membranes of the mouth and vagina, are usually caused by *Candida albicans* which are found as commensals of man. Yeast infections are generally endogenous in origin but can be transmitted sexually.

There are relatively few therapeutically useful antifungal agents that are available. This is due mainly to the fact that fungi and man are both eukaryotes and most substances that kill or inhibit fungal pathogens are also toxic to the host. Most superficial Candida infections respond well to topical therapy with nystatin amphotencin B. or azoles.

Superficial *Candida* infections occasionally occur on the penis after intercourse with females with vaginal thrush. The yeast may also infect the outer ear (Roberts, Hay, and Mackenzie, 1984)

In oral candidosis, nystatin, ampholericin *B* and miconazole may be effective in lozenge or gel form. Most cases of vaginal candidosis can be treated successfully with a single application of an azoles derivative oral therapy with fluconazole or itraconazole (Odds, 1988)(Zakirai, 2009).

2.4 *TRYPANOSOMA BRUCEI BRUCEI*

2.3.1Scientific classification

Kingdom: *Excavata*

Phylum: *Euglenozoa*

Class: *Kinetoplastea*

Order: *Trypanosomatida*

Genus: *Trypanosoma*

Species: *T. brucei*

Binomial name: *Trypanosoma brucei*

(Barrett, *et al*., 2003)

Human African trypanosomiasis, sleeping sickness African lethargy or Congo trypanosomiasis is a parasitic disease of people and animals, caused by protozoa of the species *Trypanosoma brucei* and transmitted by the tsetse fly.

The disease is endemic in some regions of sub-Saharan Africa, covering about 36 countries and 60 million people. It is estimated that 50,000 to 70,000 people are currently infected, the number having declined somewhat in recent years.

It is believed that many cases go unreported. About 48, 000 people died of it in 2008. Four major epidemics have occurred in recent history: one from1896–1906 primarily in Uganda and the Congo Basin, two epidemics in 1920 and 1970 in several African countries, and a recent 2008 epidemic in Uganda. The term 'sleeping sickness' comes from the symptoms of the neurological phase. The symptoms include confusion, reduced coordination, and disruption of the sleep cycle, with bouts of fatigue punctuated with manic periods leading to daytime slumber and night-time insomnia.

Without treatment, the disease is invariably fatal, with progressive mental deterioration leading to coma and death. Damage caused in the neurological phase can be irreversible (Barrett, *et al.,* 2003).

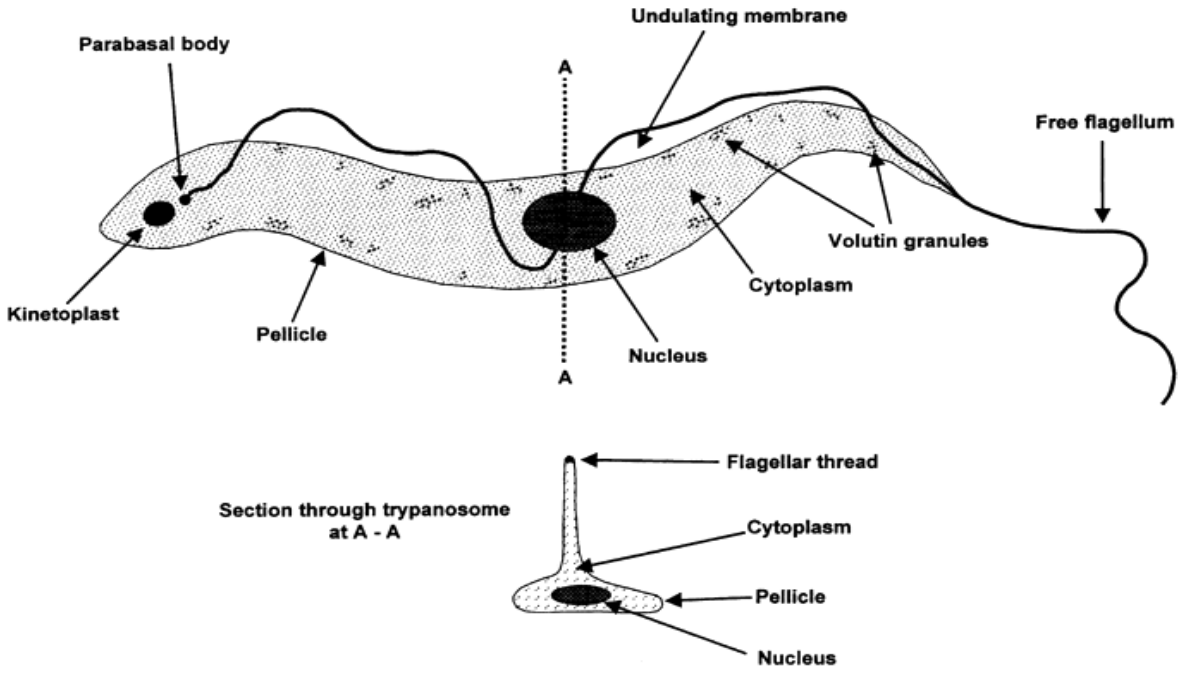


Figure 11 a Trypanosome

CHAPTER THREE

MATERIALS AND METHODS

3.1CHEMICALS/REAGENTS

The chemicals used were of high quality and the reagents used were of analytical grade. The solvents were distilled before being used. Silica gel 60G for thin-layer chromatography was used throughout.

3.1.2 Equipment

Agilent Gas Chromatography (6890N model) coupled to5973 Mass Selective detector (MSD) and NMR spectrophotometer:-Bruker- NMR-FT-400MHz was used for the characterization.

3.1.3 Plant Collection and extraction

The stem bark, root bark and leaves of the plant were collected from Birnin Gwari, Kaduna state, Nigeria, on May 21st 2010, identified and authenticated at the Herbarium in Department of Biological Sciences, Ahmadu Bello University, Zaria.

The plant material was dried indoors in a well-ventilated room for 3 weeks and then size reduced in a wooden mortar. The pulverised plant material was packaged and labelled appropriately.

About 800g of the plant material was extracted with redistilled methanol using soxhlet extractor. The extract was then concentrated using a rotary evaporator at about 40^{o}C.

Percentage recovery of the crude extracts was calculated as percentage crude extract of plant material used.

3.1.4. Preparation of stock solution for in vitro studies

Exactly 1g of the methanol extract was weighed into Eppendorf tubes and dissolved in 10ml Dextrose saline to produce extract solutions of 100 mg/ml stock solution. Three other extract concentrations (20 mg/ml, 40mg/ml and 80mg/ml) were prepared from the 'stock' extract solution by appropriate dilution with Dextrose saline. Extract solutions were prepared just before use (Atawodi *et al.,* 2005). These concentrations were used for the *in vitro* studies.

3.1.5 Phytochemical Screening

Chemical tests were carried out on the plant extract to identify the secondary metabolites such as alkaloids, flavonoids, saponins, tannins, anthraquinones, cardiac glycosides and steroids/terpenes using standard procedures as described by(Sofowora , 1982) (Sofowora ,1993)(Edeoga, Okwu, and Mbaebie, 2005).

3.1.5.1 ***Test for Carbohydrates***

Molisch's test (General test for Carbohydrates): The aqueous extract (2ml) in a test tube was added three drops of Molisch's reagent followed by three drops of concentrated sulphuric acid down the side of the test tube which was allowed to form a lower layer. A purple to violet coloured interface indicate the presence of carbohydrates. The mixture, when shaken gently and allowed to stand for two minutes, followed by dilution with water (5ml) would produce a dull violet precipitate-another indication of carbohydrates (Trease and Evans, Pharmacognosy, 1972) (Trease and Evans, 1989) (Trease and Evans, 2002).

Fehling's test for free reducing sugars:5ml of a mixture (1:1) of Fehling solution A and Fehlings solution B was added to the extract(2ml) dissolved in water and the mixture boiled on water bath for 5 minutes. A brick-red precipitate would indicate the presence of reducing sugars.

3.1.5.2 Test for Anthraquinone Glycosides.

To the extract (0.2g) was added benzene (10ml) and shaken for 5 minutes. 10% ammonia (5ml) solution was added and shaken. The presence of a pink, red or violet colour in the ammoniacal (lower layer) phase indicates the presence of free anthraquinones.

For the anthraquinone glycosides, the extract (0.2g) was boiled with dilute sulphuric acid (10ml) and filtered while hot. The filtrate was shaken with benzene (5ml), the benzene layer was separated and to it, 10% ammonia solution (3ml) was added and mixture shaken. The layers were allowed to separate. A pink, red or violet colouration in the lower layer shows the presence of anthraquinone glycosides (Sofowora, 1993).

3.1.5.3 Test for Saponin Glycosides

Frothing test: the extract (0.2g) was shaken with water (5ml) in a test tube for 30 seconds. A persistent froth for 15 minutes or when warmed on water bath indicates the presence of saponins (Sofowora, 1993).

3.1.5.4 Test for Steroids and Terpenoids

a) **Salkowski's test for steroids:** the extract (0.5g) was dissolved in chloroform and concentrated sulphuric acid (2ml) was carefully added down the side of the test tube to form

a lower layer. A reddish brown colour at the interface indicates the presence of steroidal ring (Sofowora, 1993).

b) **Liebermann - Burchard's test**: the extract (0.5g) was dissolved in chloroform (2ml) and filtered into a clean, dry test tube. Acetic anhydride (2ml) was added to the filtrate and shaken. Few drops of concentrated sulphuric acid were added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the zone of contact of the two liquids and the upper layer turning green denotes the presence of sterols and terpenes

3.1.5.5 Test for Tannins

a. **Ferric chloride test**: the extract (0.2g) was stirred with water (5ml) and filtered. To the filtrate (2ml) in a test tube, two drops of ferric chloride solution were added. A green or greenish black precipitate indicate the presence of condensed tannins, while a blue or bluish black precipitate shows the presence of hydrolysable tannins (Trease and Evans, 1989).

b. **Lead subacetate test**: To the aqueous extract (1ml), three drops of lead subacetate solution was added. A coloured precipitate indicates the presence of tannins (Trease and Evans, 2002).

3.1.5.6 Test for Alkaloids

General test: About (0.2g) of the extract was stirred with 1% hydrochloric acid (5ml) on a water bath. To the filtrate (1ml) was added, 2-3 drops of the following reagents were added:

Wagner reagent solution of iodine in potassium iodide,
Mayer’s reagent (potassium mercuric iodide solution)
Dragendroff's reagent (potassium bismuth iodide solution)

The presence of precipitate in most or all of the above reagents indicates the presence of alkaloids (Trease and Evans, 1989), that is

Light brown to brownish precipitate- Wagner's reagent

White or creamy white precipitate - Mayer's reagent

Orange or orange-yellow precipitate - Drangendroff's reagent

3.1.5.7 Test for Cardiac Glycosides

The extract (1.0g) was dissolved in water. Strong lead subacetate solution (1ml) was added and filtered. The filtrate was divided into two for the following tests:-

Keller-killiani Test (for Steroidal nucleus): To the first portion of the filtrate was added chloroform, the chloroform layer (lower) was separated and evaporated to dryness on water bath. The residue was dissolved in 3.5% ferric chloride (3ml) solution in glacial acetic acid. The mixture was allowed to stand for a minute before it was transferred to a test tube. Concentrated sulphuric acid (1.5ml) was added down the side of the test tube to form a lower layer on standing, the presence of a brown colour at the junction of the two liquids and a pale green colour in the upper layer indicates the presence of cardiac glycosides (Brain and Turner, 1975).

3.1.5.8 Test for Flavonoids

Shinoda's test: the extract (0.2g) was diluted with ethanol. Some pieces of magnesium filing were added followed by five drops of concentrated hydrochloric acid. A pink or red colour indicates the presence of flavonoids (Silva, Lee, and Kinghorn, 1998)

Sodium hydroxide test: the extract (0.2g) was dissolved in water (2ml) and filtered. 10% aqueous sodium hydroxide (2ml) solution was added. A Yellow solution indicates the

presence of flavonoids. On addition of dilute hydrochloric acid, the solution becomes colourless, (Brain and Turner, 1975).

Ferric Chloride Test: the extract (0.1g) was boiled with water and filtered. To the filtrate (2ml), 2 drops of 10% ferric chloride solution were added. A green or blue colour indicates the presence of phenolic nucleus.

3.1.6Antimicrobial Screening

Test Organisms

The bacteria for this research viz. *Staphylococcus aureus; Streptococcus pyrogenes; Escherichia coli; Salmonella typhi; Shigella dysenterea; Pseudomonas aeruginosa; Klebsiella pneumoniae* and the fungi *Trichophyton rubrum, Microsporum sp., Aspergillus fumigatus* and *Aspergillus nigre* were gotten from Ahmadu Bello University Zaria Teaching Hospital. All the micro-organisms were checked for purity and maintained in slants of agar.

3.1.7 Cultivation and Standardization of Test Organisms

A loop full of each of the test organisms was taken from the agar slant and sub cultured into test tubes containing sterile nutrient agar and sabouraud dextrose medium (20 ml) for the fungi. The test tubes were then incubated for 24 hours at 37^{o}C for two days. The broth culture was standardized using sterile normal saline to obtain a density of 106 cfu/ml for bacteria. A sporulated test fungal spore was harvested with 0.05%Tween80 in sterile Normal saline and standardized to 106 spores/ml.

3.1.8 Preparation of Culture Media

The prescribed quantities of the dehydrated bacteriological culture media was weighed and hydrated with distilled water according to the manufacturer's specification. Where necessary, gentle heat was applied to aid dissolution and the resultant suspensions were dispensed into clean bottles and sterilized at 121^{o}C for 15 minutes in an Adelphi bench autoclave.

3.1.9 Antimicrobial Profile (susceptibility test)

The antibacterial screening was carried out using the agar diffusion method as described by (Lino and Deogracios, 2006) with slight modifications. Overnight culture of the various bacteria in blood agar and the fungi in sabouraud dextrose agar slant media were sterilized to produce inoculums size of 10^{6}cfu/ml. The medium was seeded with standard inoculums (0.1 ml) of the micro-organism (mc-forland 0.5). The inoculums were sprayed evenly by the use of sterile swab over the surface of the medium; the seeded plants were allowed to dry at 37^{o}C for 30mins inside incubator. A standard cork borer (6mm) was used to cut a well at the centre of each seeded medium used and the solution of the extracts (10mg/ml of each) was then introduced into each hole on the surface of the medium. In one medium was introduced sterile water (0.1ml) to serve as negative control. The medium was incubated at 37^{o}C and 27^{o}C for 24 hours and 2-7 days for the bacteria and fungi respectively after which the plates were observed for zones of inhibition. The zones of inhibition were measured with a transparent rule and the result recorded.

3.2.0 Determination of Minimum Inhibitory Concentration

This was done using broth dilution method as described by (Vollekova, Kostalova, and Sochorova, 2001) modified by (Usman, Abdulrahman, and Ladan, 2007). In this method,

10ml nutrient broth (prepared using manufacturer's specifications) was dispensed into test tubes and sterilized at 121^{o}C for 10 minutes and allowed to cool. Mc-farland's turbidity standard scale number 0.5 was prepared to give a turbid solution. Normal saline was inoculated with each of the test micro-organism and incubated at 37^{o}C for 6 hours to make a turbid suspension of the micro-organisms. After incubations, dilution of the micro-organism in normal saline was done until the turbidity ($1.5x10^{6}$cfu/ml) matched that of the Mc-Forland scale by visual comparison. A solution of the extract (5 ml) (ethyl acetate extract and n-butanol portions of concentration 10, 5, 2.5, 1.25, 0.625, 0.312 mg/ml) was mixed with the nutrient broth (5ml). From the suspension of the micro-organism in normal saline, 0.1ml was inoculated into the different concentrations of the extract in the nutrient broth. The broths were incubated at 37^{o}C for 24hrs. The results after 24 hours were recorded.

3.2.1 Determination of Minimum Bactericidal Concentration (MBC)

Blood agar was prepared according to manufacturer's instruction, sterilized at 121^{o}C for 15 minutes. It was poured into sterile petri-dishes. The plates were allowed to cool and solidify. The contents of the MIC test tubes in the serial dilution were then sub-cultured on to the prepared plates and the plates were then incubated at 37^{o}C for 24 hours. The results were recorded after 24 hours (Vollekova, Kostalova, and Sochorova, 2001)(Usman, Abdulrahman, and Ladan, 2007).

3.2.2 *Trypanosoma brucei brucei*

The parasites were obtained from the Department of Veterinary Parasitology, Ahmadu Bello University, Zaria. Parasites were passaged into a white albino rat by intra-peritoneal inoculation.

3.2.3 Determination of Parasitaemia

The parasites were maintained in the laboratory by continuous passage in rats until it was required. The passage was with infected blood containing 1×10^3 parasites per field of view and was introduced intra-peritoneally into healthy rats in blood/physiological saline solution (0.1–0.2mL). The numbers of parasites was determined microscopically at magnification ×400 and Parasitaemia was monitored daily (Nathan, *et al*., 2010) in the inoculated rat by obtaining blood daily from pre sterilized tail.

3.2.4 *in vitro* studies

The Drug Incubation Infectivity Test (Delespaux, Geysen, Van den Bossche, and Geerts, 2008) was carried out with Wet blood films method (Chappuis, Loutan, Simarro, Lejon, and Uscher, 2005) to detect any motile trypanosomes and it was done in triplicates in a 96 well microtitre plate with infected blood (100µl) to be incubated with each extract concentration (25µl) (previously prepared). Parasitaemia was monitored between 1–20 minutes of incubation at 30◦C. Test mixtures (About 0.5ml–1ml) were observed every 1 minute under a microscope ×400. A set of Positive Control (PC) and Negative Control (NC) was set up to stand for 4hrs with the infected blood containing the parasites. The PC will contain DIMINAVETO$^{(R)}$ (25mg/ml)(1.05 g of 4.4'- diamidinodiazoaminobenzene diaceturate and

1.31g Antipyrine), the NC will contain only the infected blood suspended in heparin and Phosphate Buffer Saline Glucose (pH 7.2).

3.2.5 Identification and separation of the active principle

The methanol extract used in the *in vitro* studies, was defatted with n-hexane and partitioned into ethyl acetate. This was done by first dissolving the methanol extract in distilled/deionised water (200mL). The mixture was transferred into a 500ml separatory funnel and extracted with several 150ml portions of ethyl acetate. The ethyl acetate extract collected was concentrated *in vacuo* and allowed to dry.

The ethyl acetate extract was subjected to thin layer chromatographic analysis to find the solvent system that will give better separation of the components therein. This was achieved using pre-coated TLC plates. A micro quantity of the sample solution was spotted on TLC pre-coated (MERCK) F254 plates, and developed with various ratios of organic solvents (petroleum spirit, ethyl acetate, methanol, chloroform) taking into consideration their polarity. The solvent system that separated the components to high degree of resolution was considered. The plates were visualized under visible and UV-light (366 and 254 nm). The plates were sprayed with 10% sulphuric acid in methanol and heated at 100ºC for 2-5 minutes. Appropriate fractions which were collected from the column were monitored by TLC. The fractions with the same R_f values were pooled together and concentrated (Hostettmann, Hostettmann, and Marston, 1998).

3.2.5.1 Spraying Reagents:

Lieberman-Burchard reagent: mix 5ml acetic anhydride under cooling with 5ml concentrated sulphuric acid, this mixture was added cautiously to 50ml absolute ethanol with cooling. The chromatogram was heated for 10 minutes at 100^{o}C (Krebs, Hausser, and Wimmer, 1969).

Sulphuric acid (5%) was also used i.e. 5ml of concentrated sulphuric acid in 95ml of distilled water. This was used to spray the chromatogram and heated for 10 minutes at a temperature of 100 ^{o}C in an oven. The spots and colours observed were noted and recorded.

3.2.5 Vacuum Liquid Chromatography

The ethyl acetate extract was subjected to vacuum liquid chromatography (VLC) to fractionate the extract, (2.2g) which was dissolved in ethyl acetate and pre-adsorbed on celite(Taber, 1982). The pre-adsorbed sample was dried *in vacuo* into fine flowing powder using the rotary evaporator under reduced pressure. The sintered glass funnel (porosity 3) used for the (VLC) was loaded with silica gel under vacuum ensuring that it was compacted and uniformly spread (Leopold, 1982)(Hostettmann, Hostettmann, and Marston, 1998).

A non-polar solvent (petroleum ether) was run through the column under vacuum then the pre-adsorbed sample was evenly spread on the silica gel. Suction was applied to compress the sample to the silica gel and a piece of Whatman$^{(R)}$ filter paper was used to cover the surface to prevent disturbance during the course of the elution. Gradient elution was used: starting from 100% Petroleum Spirit then slowly inching up the concentration of the more polar ethyl acetate until 100% ethyl acetate and finally with 10% methanol in chloroform. 34

fractions were collected. The concentrated fractions were subjected to TLC and similar fractions were pooled together.

Fraction H_1 gave white feathery crystals. This was re-chromatographed three times to yield the final product.

3.2.6 Melting Point determination

Melting Point: it was determined using Electro thermal IA 9300 (Gallenkhamp capillary melting point apparatus with a thermometer).

3.2.7 Spectroscopic Measurement

The final product was analyzed using Agilent Gas Chromatography (6890N model) coupled to5973 Mass Selective detector (MSD), in chloroform as solvent at Faculty of Science, Usmanu Danfodiyo University Sokoto.

The results obtained were compared with an inbuilt main library C:/Database/NIST02.L) and this library enabled the confirmation of the compound(s) present in the plant.

NMR –spectrophotometer:-Bruker- NMR-FT-400MHz was used for the characterization.

CHAPTER FOUR

RESULTS

4.1 EXTRACTION

The powdered stem bark (650g) of *Pouteria alnifolia* after extraction and defatting with petroleum ether and methanol gave a dark brown residue weighing 30g. 22g of the methanol extract of the stem bark of *Pouteria alnifolia* on partitioning gave an amber coloured residue of ethyl acetate 2.3g Table 4.1

Table 4.1: Extractive values of methanol extract of the stem bark of *Pouteria alnifolia*

Extract	**Colour**	**Weight (g)**	**Percentage yield (%)**
Petroleum ether	Light brown	3.00	0.375
methanol	Brown	30.0	4.615
Ethyl acetate	Amber	2.40	0.369

4.2. PHYTOCHEMICAL SCREENING

Phytochemical screening of the petroleum ether extract, methanol extract of the Stem bark of *Pouteria alnifolia* was carried out along with the ethyl acetate fraction. The methanol extract was found to contain mainly saponins, flavonoids, and tannins as summarized in Table 4.2.

Table 4.2: Phytochemical constituents of the stem bark of *Pouteria alnifolia*

CONSTITUENTS	TEST	OBSERVATION	INFERENCE	
			CH_3OH	EtOAc
Carbohydrate	a. Molisch's test	Purple colour at interphase	+	+
		Brick red precipitate	+	+
	b. Fehling test	No precipitate	+	+
Anthraquinones	a. Borntrager's test	No change	-	-
	b. Combined Anthraquinone	No change	-	-
Saponin	a. Frothing test	Frothing which persist > 30 min	+	-
Steroid	a. Lieberman Burchard's test	Red colour ring	+	+
and triterpenes	b. Salkowski test	Red colour at interphase	+	+
Tannins	a. Lead subacetate	Yellow coloration	+	+
	b. $FeCl_3$ test	Greenish black coloration	+	+
Alkaloids	a. Meyer's test	No precipitate	-	-
	b. Dragendoff's test	No precipitate	-	-
		No precipitate	-	-
	c. Tannic acid test	No precipitate	-	-
Cardiac glycosides	a. Kella-killiani test	Purple ring at interphase	+	+
		Purple-blue ring	+	+
	b. Salkowski test	Cherry red ring	+	+
Flavonoid	a. Sodium hydroxide	Yellow coloration	+	+
	b. $FeCl_3$ test	Yellow coloration	+	+
	c. Shinoda's Test	A red coloration	+	+

Key: EtOAc = ethyl acetate extract, - = absent, + = present

4.2.1 Antimicrobial Screening

Table 4.3 shows the zones of inhibition for the ethyl acetate and methanol extract of the stem bark of *Pouteria alnifoli*a on *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Shigella dysenterea, Pseudomonas aeruginosa*, *Klebsiella pneumonia, Candida albicans, Aspergillus fumigatus, Microsporum sp., and Trichophyton rubrum.* Five of the organisms

showed sensitivity to the extracts. The table also shows that both the methanol and ethyl acetate extracts showed the same activity to microbes namely *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Candida albicans and Pseudomonas aeruginosa*.

Table 4.3: Zone of inhibitions ethyl acetate and the methanol extract of the stem bark of *Pouteria alnifolia* on ten micro-organisms.

Micro-organisms	**Zones of inhibition (mm)**		
	Sterile water	Ethyl acetate fraction	Methanol fraction
Staphylococcus aureus	0	28	24
Escherichia coli	0	26	22
Klebsiella pneumonia	0	0	0
Pseudomonas aeruginosa	0	20	21
Shigella dysenteria	0	0	0
Salmonella typhi	0	22	20
Candida albicans	0	24	20
Aspergillus fumigatus	0	0	0
Microsporum sp	0	0	0
Trichophyton rubrum	0	0	0

4.2.2 Minimum Inhibitory Concentration (MIC)

Table 4.4 shows the MIC of the ethyl acetate and methanol extracts of the stem bark of *Pouteria alnifolia* on the microbes.

Table 4.4: Minimum Inhibitory concentration (MIC) of the ethyl acetate and methanol extracts of the stem bark of *Pouteria alnifolia* on micro-organisms

Micro-organisms	**Concentration (mg/ml)**									
	Ethyl acetate fraction					Methanol fraction				
	5	2.5	1.25	0.62	0.312	5	2.5	1.25	0.62	0.312
Staphylococcus aureus	-	-	-	MIC	+	-	-	MIC	+	+
Escherichia coli	-	-	-	MIC	+	-	-	MIC	+	+
Klebsiella pneumonia	-	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	MIC	+	+	-	-	MIC	+	+
Shigella dysenteria	-	-	-	-	-	-	-	-	-	-
Salmonella typhi	-	-	MIC	+	+	-	-	MIC	+	+
Candida albicans	-	-	MIC	+	+	-	-	MIC	+	+
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	-
Microsporum sp										
Trichophyton rubrum										

Key: - = no turbidity, + = turbidity MIC= Minimum inhibitory concentration

From Table 4.4, it shows that the ethyl acetate fraction of the stem bark of *Pouteria alnifolia* has activity against most of the micro-organisms. While methanol fraction of same plant showed less activity.

Table 4.5 Minimum Fungicidal Concentration of the extract against the test microbes

Test Organism	Methanol extract						Ethyl acetate extract					
	40mg/ml	30mg/ml	20mg/ml	10mg/ml	5mg/ml	4mg/ml	40mg/ml	30mg/ml	20mg/ml	10mg/ml	5mg/ml	4mg/ml
Aspergillus nigre	-	MFC	+	++	+++	++++	-	-	-	-	-	-
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	-	-	-
Trichophyton rubrum	MFC	+	++	+++	++++	++++ +	-	-	-	-	-	-
Microsporum sp	-	-	-	-	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	MFC	+	++	-	-	-	-	MFC	+

Key - = no fungal growth, MFC = Minimum Fungicidal Concentration, + = Scanty fungal growth, ++ = moderate fungal growth, +++ = Moderate fungal growth

Table 4.6: Minimum Bactericidal Concentration (MBC) of the ethyl acetate and methanol extract of the stem bark of *Pouteria alnifolia* on micro-organisms

Micro-organisms	**Concentration (mg/ml)**									
	Ethyl acetate fraction					Methanol fraction				
	5	2.5	1.25	0.62	0.312	5	2.5	1.25	0.62	0.312
Staphylococcus aureus	-	-	MBC	+	+	MBC	+	+	+	+
Escherichia coli	-	MBC	+	+	+	MBC	+	+	+	+
Pseudomonas aeruginosa	MBC	+	+	+	+	MBC	+	+	+	+
Shigella dysenteria	-	-	-	-	-	-	-	-	-	-
Salmonella typhi	MBC		+	+	+	MBC	+	+	+	+
Candida albicans	MBC	+	+	+	+	MBC	+	+	+	+
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	-
Microsporum sp	-	-	-	-	-	-	-	-	-	-
Klebsiella pneumonia	-	-	-	-	-	-	-	-	-	-
Trichophyton rubrum	-	-	-	-	-	-	-	-	-	-

Key: - = no turbidity, + = turbidity

Table 4.7 *In vitro* effect of methanol extract of the stem bark of *Pouteria alnifolia* on *Trypanosoma brucei brucei*.

Concentration(mg/ml)	methanol extract (no of parasites)	Diminaveto®
100	**-**	**-**
50	**-**	**+9**
25	**-**	**+8**
10	**-**	**-**
5	**+5**	**-**

Key -= no parasite, +number = antilog

The methanol extract performed excellently against the standard drug Diminaveto®

in *in vitro* tests against *T. brucei brucei.*

4.2.4 Analysis of H_1

The feathery white crystalline precipitate weighing 84.2mg was subjected to repeated VLC using a lower gradient each successive time to eventually yield 55mg. Spectral data of H_1 was obtained using Carbon-13 and GC-MS. The melting point was found to be 230^{o}C.

4.2.5 GC-MS and ^{13}C-NMR Spectroscopy of H_1

Figure 11, Figure 12 and Figure 13 show the GC-MS data for H_1 while Figure 14 shows the ^{13}C-NMR of H_1.

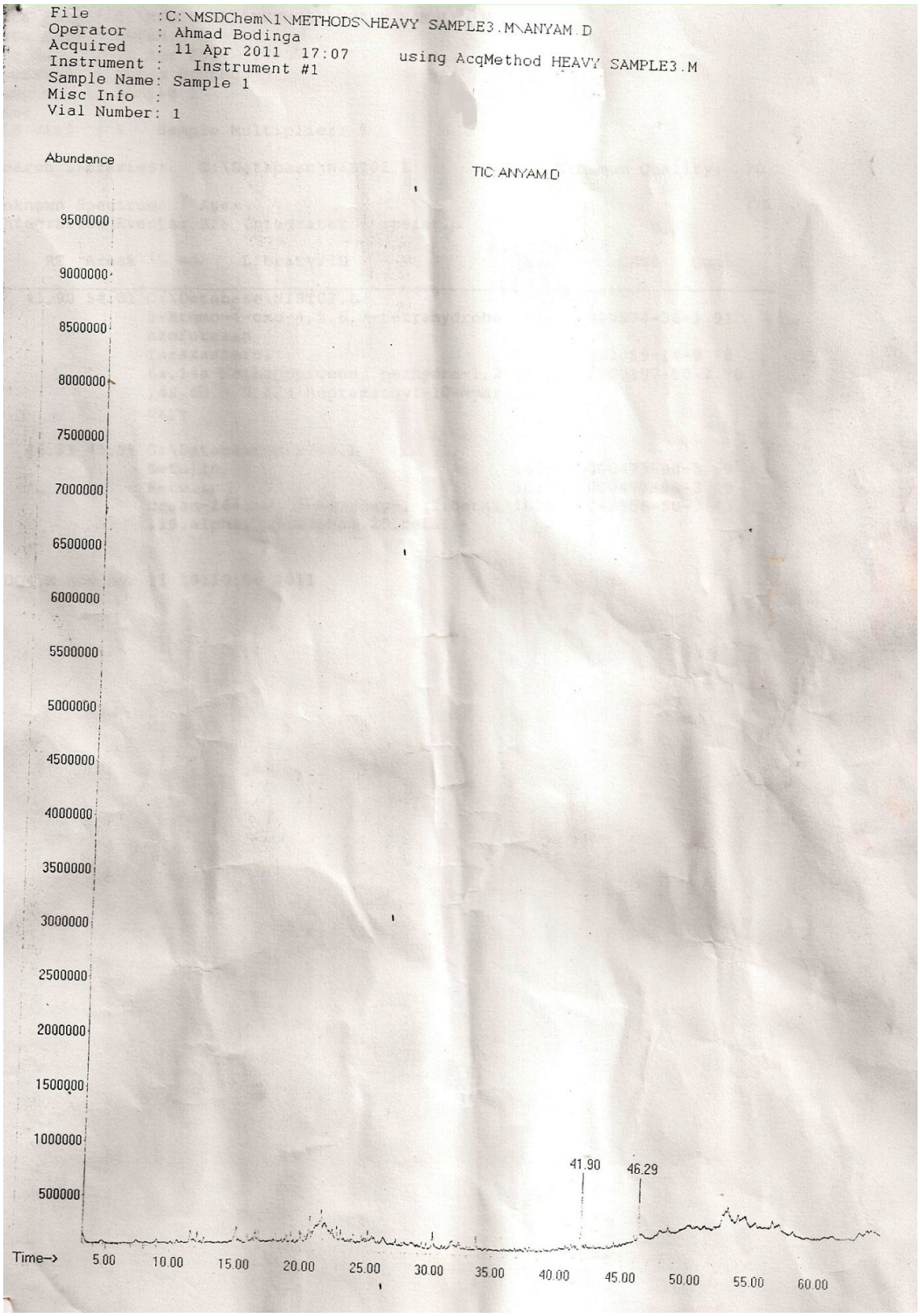


Figure 12: Gas chromatogram of H1

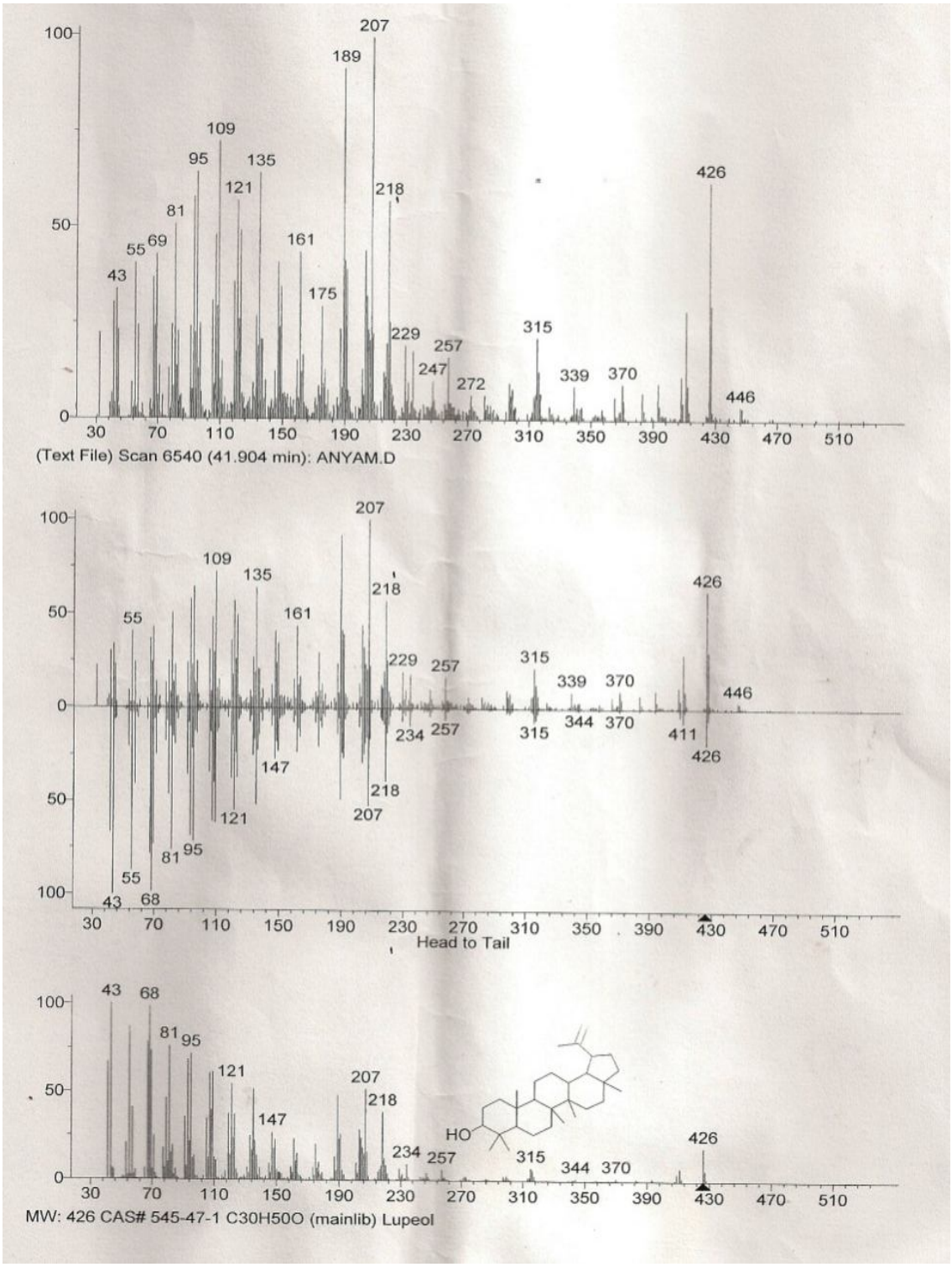


Figure 13: Mass spectrum of peak 41.904

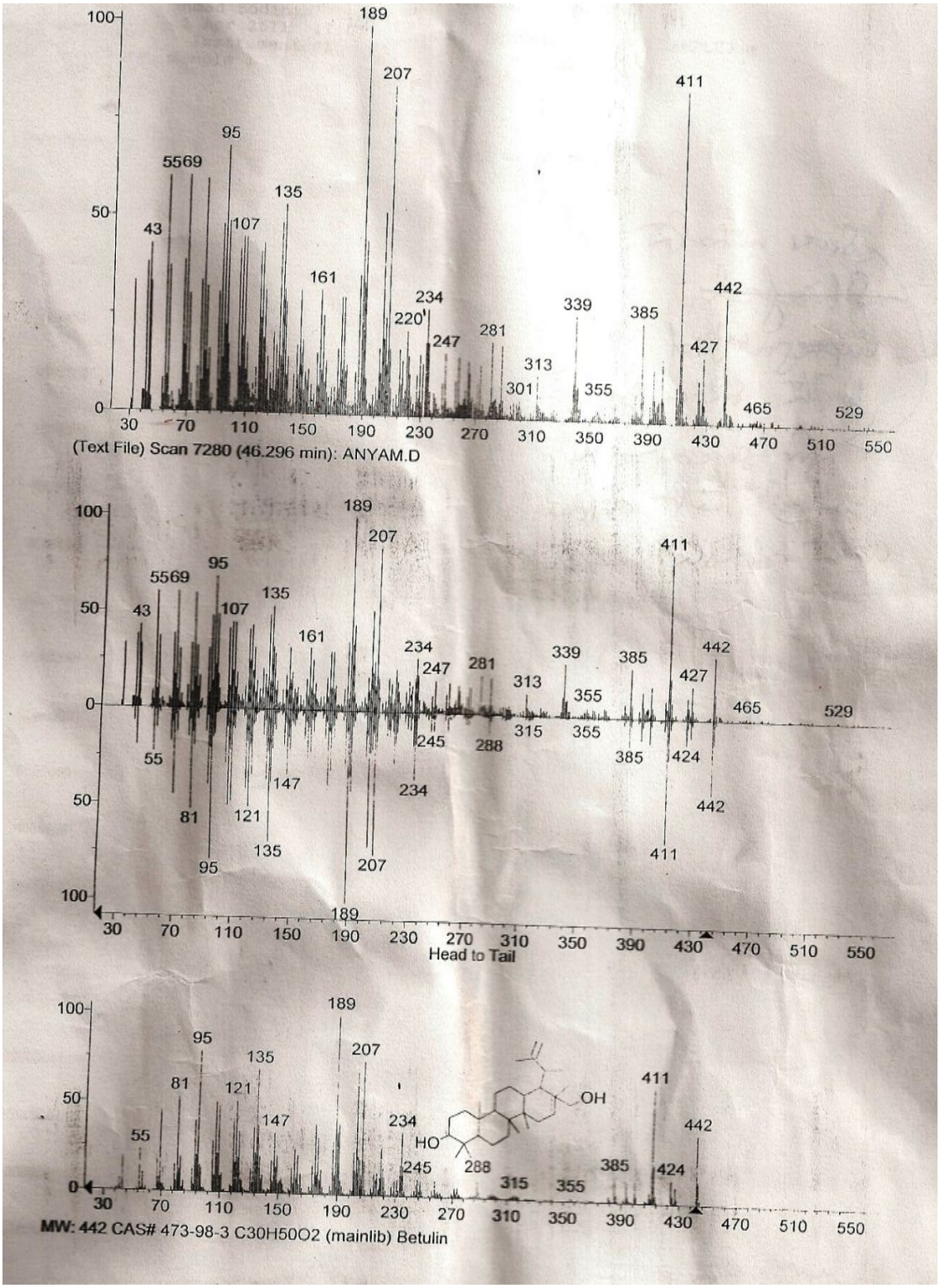


Figure 14 Mass spectrum of peak 46.296

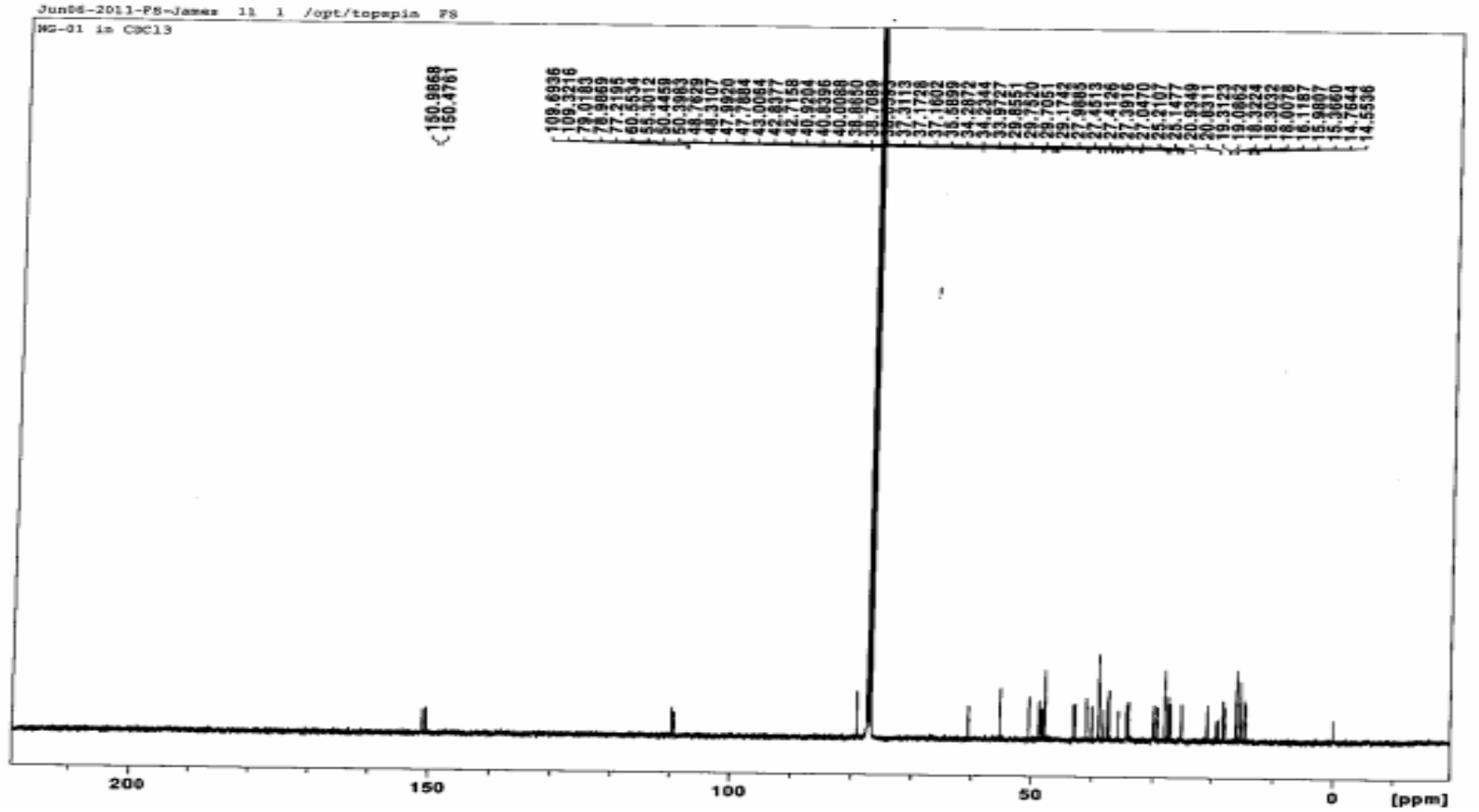


Figure 15: 13C NMR SPECTRUM FOR H_1

From the above observations (Figure 11, Figure 12, Figure 13 and Figure 14), the data in Figure 14 after a comprehensive literature survey can be tabulated as shown in Table 4.8, Table 4.9 and Table 5.0:

Table 4.8: ^{13}C NMR spectral data of the isolated compound lupeol in $CDCl_3$ (δ in ppm)

C	δc (Lupeol) (Lit.)	δc(Lupeol) (Lit.)	δc(Lupeol Isolated)	node
1	38.7	38.6	38.7	CH_2
2	27.4	27.3	27.4	CH_2
3	78.9	78.9	79.0	CH
4	38.8	38.9	38.9	C
5	55.3	52.2	55.3	CH
6	18.3	18.2	18.3	CH_2
7	34.2	34.2	34.2	CH_2
8	40.8	40.7	40.8	C
9	50.4	50.3	50.5	CH
10	37.1	37.1	37.2	C
11	20.9	20.8	20.8	CH_2
12	25.1	25.0	25.2	CH_2
13	38.0	33.7	38.7	CH
14	42.8	42.7	42.8	C
15	27.4	27.9	27.5	CH_2
16	35.5	35.4	35.6	CH_2
17	43.0	42.7	43.0	C
18	48.2	48.2	48.3	CH
19	47.9	47.9	48.0	CH
20	150.9	150.9	150.0	C
21	29.8	29.7	29.8	CH_2
22	40.0	39.9	40.0	CH_2
23	28.0	27.9	28.0	CH_3
24	15.4	15.3	15.4	CH_3
25	16.1	16.0	16.1	CH_3
26	15.9	15.9	16.0	CH_3
27	14.5	14.9	14.6	CH_3
28	18.0	17.9	18.0	CH_3
29	109.3	109.3	109.3	CH_2
30	19.3	19.3	19.3	CH_3

Table 4.9:^{13}C NMR spectral data of the isolated compound Betulin in $CDCl_3$ (δ in ppm)

H/C	δc (betulin) (Lit.)	δc(Betulin Isolated)	node
1	38.8	38.9	CH_2
2	27.2	27.4	CH_2
3	78.9	79.0	CH
4	38.9	39.0	C
5	55.3	55.3	CH
6	18.3	18.3	CH_2
7	34.3	34.3	CH_2
8	40.9	40.9	C
9	50.4	50.4	CH
10	37.2	37.2	C
11	20.9	20.9	CH_2
12	25.3	25.2	CH_2
13	37.3	37.3	CH
14	42.7	42.7	C
15	27.0	27.1	CH_2
16	29.2	29.2	CH_2
17	47.8	48.0	C
18	48.8	49.0	CH
19	47.8	48.0	CH
20	150.6	150.5	C
21	29.8	29.9	CH_2
22	34.0	34.0	CH_2
23	28.0	28.0	CH_3
24	15.4	15.4	CH_3
25	16.1	16.1	CH_3
26	16.0	16.0	CH_3
27	14.8	14.8	CH_3
28	60.2	60.6	CH_2
29	109.6	109.7	CH_2
30	19.1	19.1	CH_3

Therefore, the structures of the isolated compounds are probably Betulin and Lupeol as already pointed out by the gas chromatography – mass spectroscopy result (Figure 11, Figure 12 and Figure 13).

Table 5.0: δC values for Lupeol and Betulin from literature ((Reynolds, Mcleans, Poplawsky, Enriquez, Escobar, and Leon, 1986) (Tinto, Blair, Reynolds, and Macleans, 1992))

C	δc (Lupeol) (Lit.)	δ_c Lupeol) (Lit.)	δc(Lupeol Isolated)	δc (betulin) (Lit.)	δc(Betulin Isolated)	node
1	38.7	38.6	38.7	38.8	38.9	CH_2
2	27.4	27.3	27.4	27.2	27.4	CH_2
3	78.9	78.9	79.0	78.9	79.0	CH
4	38.8	38.9	38.9	38.9	38.9	C
5	55.3	52.2	55.3	55.3	55.3	CH
6	18.3	18.2	18.3	18.3	18.3	CH_2
7	34.2	34.2	34.2	34.3	34.3	CH_2
8	40.8	40.7	40.8	40.9	40.9	C
9	50.4	50.3	50.5	50.4	50.4	CH
10	37.1	37.1	37.2	37.2	37.2	C
11	20.9	20.8	20.8	20.9	20.9	CH_2
12	25.1	25.0	25.2	25.3	25.2	CH_2
13	38.0	33.7	38.7	37.3	37.3	CH
14	42.8	42.7	42.8	42.7	42.7	C
15	27.4	27.9	27.5	27.0	27.1	CH_2
16	35.5	35.4	35.6	29.2	29.2	CH_2
17	43.0	42.7	43.0	47.8	48.0	C
18	48.2	48.2	48.3	48.8	48.8	CH
19	47.9	47.9	48.0	47.8	47.8	CH
20	150.9	150.9	151.0	150.6	150.5	C
21	29.8	29.7	29.8	29.8	29.9	CH_2
22	40.0	39.9	40.0	34.0	34.0	CH_2
23	28.0	27.9	28.0	28.0	28.0	CH_3
24	15.4	15.3	15.4	15.4	15.4	CH_3
25	16.1	16.0	16.1	16.1	16.1	CH_3
26	15.9	15.9	16.0	16.0	16.0	CH_3
27	14.5	14.9	14.6	14.8	14.8	CH_3
28	18.0	17.9	18.0	60.2	60.6	CH_3 for L;CH_2 for B
29	109.3	109.3	109.3	109.6	109.7	CH_2
30	19.3	19.3	19.3	19.1	19.1	CH_3

Key: L=Lupeol; B=Betulin; Lit = Literature

4.2.6 ^{13}C NMR spectrum of Isolated Compound

The ^{13}C NMR spectrum points to two lupeol-type triterpene derivatives. The characteristic sp^2 carbon signals of lupeol are observed as shifts at δ 150.9868 and 109.3216ppm (Tinto, Blair, Reynolds, and Macleans, 1992) and shifts at δ 150.4761 and 109.6936ppm (Reynolds, Mcleans, Poplawsky, Enriquez, Escobar, and Leon, 1986) for betulin. Oxygenated carbon shifts for C-3 and C-28 for betulin and lupeol were observed at δ 78.9869 and 60.5534 ppm for betulin, δ 79.0183 and 18.0087 ppm for lupeol. Ultimately, the spectra revealed two compounds each with six methyl groups, thirty carbon atoms: a lupene-type triterpenoidal nucleus with two hydroxyl groups at C-3 and C-28 (C-28 is a methyl carbon for Lupeol) for betulin and a hydroxyl group at C-3 for lupeol. The NMR data of H_1 was compared to that reported in the literature and that simulated by MestReNova$^{(R)}$ (APPENDIX VII, VIII, IX) and ChemDrawUltra$^{(R)}$(APPENDIX II, X,); all ^{13}C shifts were within ±0.3 ppm(Reynolds, Mcleans, Poplawsky, Enriquez, Escobar, and Leon, 1986),(Tinto, Blair, Reynolds, and Macleans, 1992)(Jain and Bari, 2010).

The ^{13}C spectral data (Table 4.8, Table 4.9 and Table 5.0) suggests that the ^{13}C spectrum is a composite of lupeol and betulin spectra since every signal has been accounted for in terms of betulin and lupeol.

4.2.6.1 Mass Spectrum of H_1

The mass spectrum of peak at retention time 41.904 of the Gas Chromatogram showed the parent ion[M^+] peak at M/Z 426 which corresponds to the molecular formula, $C_{30}H_{50}O$ and the other peaks showing characteristics reminiscent of lupeol (Figure 12) (Jain and Bari, 2010). A library search on the database came back with a 99% match for lupeol. The mass spectrum of peak 46.29 from the Gas Chromatogram showed the parent ion [M^+] peak at M/Z 442 which corresponds to the molecular formula $C_{30}H_{50}O_2$ and the other peaks also showing

characteristics of mass spectra for betulin (Figure 13). A library search on the database also came back with a 99% match for betulin.

CHAPTER FIVE

DISCUSSION

5.1 PRELIMINARY PHYTOCHEMISTRY

From the phytochemical screening, it was found out that the plant contains the following secondary metabolites: tannins, flavonoids, saponins, resins but no alkaloids and anthraquinones.

5.2 INVESTIGATION OF THE ETHYL ACETATE EXTRACT OF *POUTERIA ALNIFOLIA*

Vacuum liquid chromatography on silica gel 60G of ethyl acetate extract using n-hexane-ethyl acetate gradiently and repeatedly gave H_1. H_1 was recrystallized from n-hexane and it gave a single spot with different solvent systems. The component had a sharp melting point at 230^{o}C, was not detected under UV light (254/366nm) and was detected only after staining with Liebermann-Burchard reagent during TLC. H_1, however, was found to be a mixture of two compounds.

Based on the results in the (Table 4.8, Table 4.9 and Table 5.0; Figure 12 and Figure 13) above, the following structures are therefore proposed for H_1.

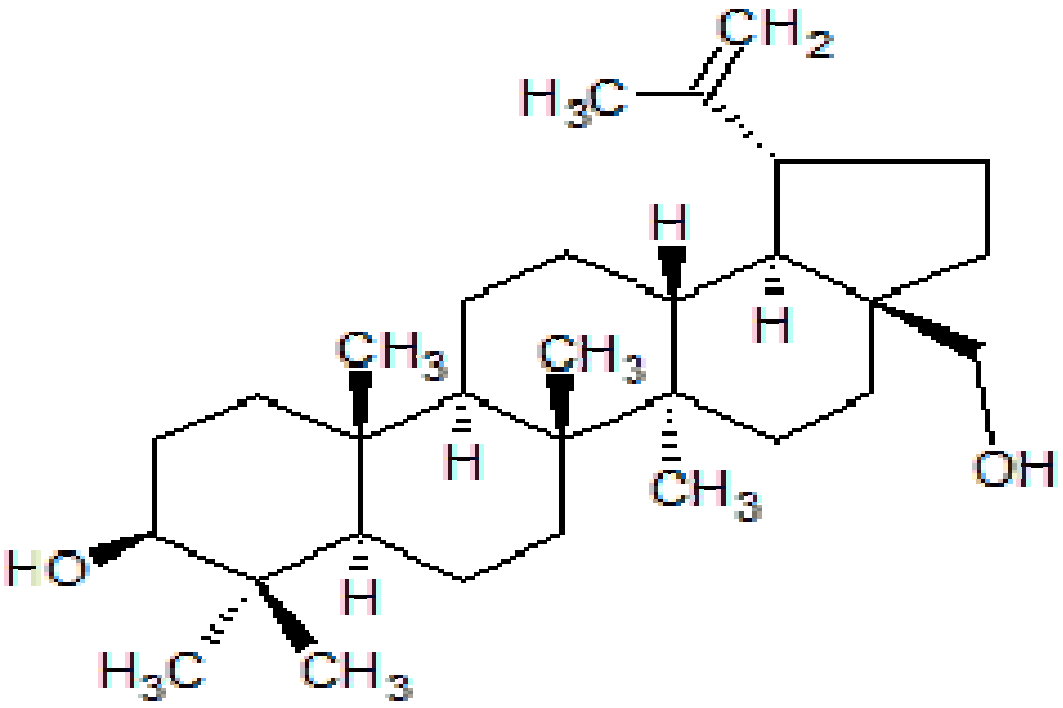


Figure 16. Betulin (Lup-20(29)-ene-3β, 28-diol),

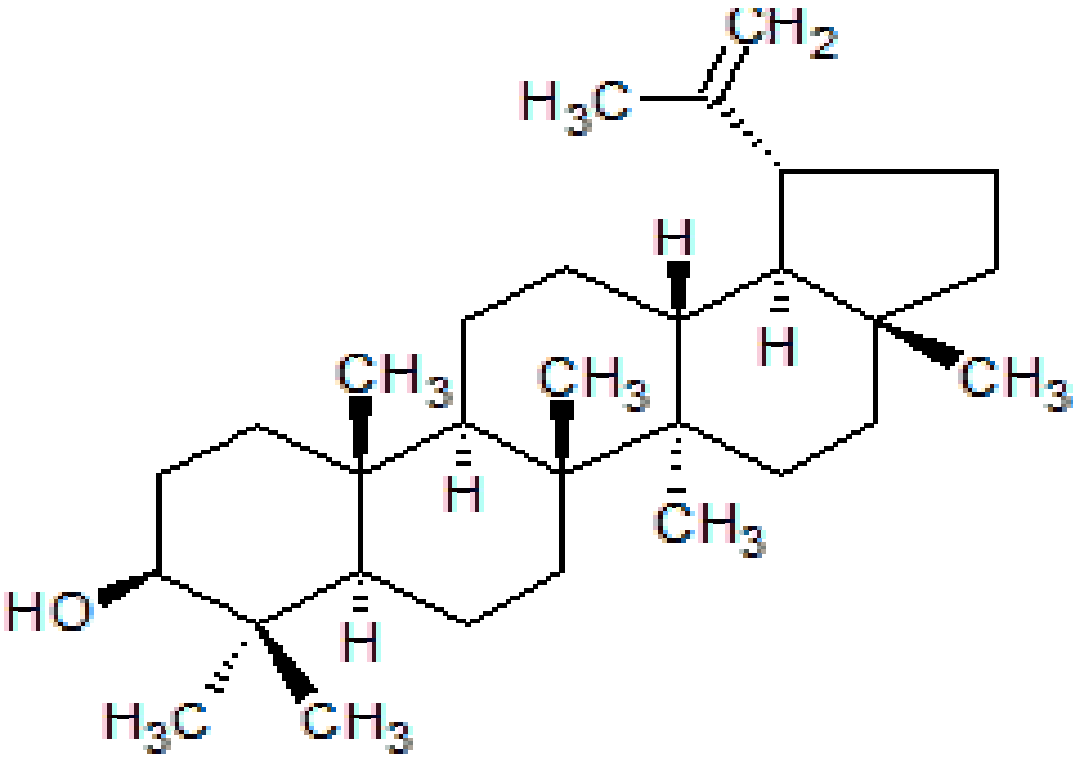


Figure17. Lupeol (3β, 13ξ)-Lup-20(29)-en-3-ol)

5.3 ANTIMICROBIAL PROFILE

The extracts from the stem back were found to be antifungal: effective against *Aspergillus nigre, Trichophyton rubrum,* and *Candida albicans* inhibiting their growths at minimum concentrations of 10mg/ml, 20mg/ml and 5mg/ml respectively; fungicidal against *Aspergillus nigre, Trichophyton rubrum,* and *Candida albicans* at minimum concentrations of 30mg/ml, 40mg/ml and 10mg/ml, respectively.

Antibacterial screening of the methanol and ethyl acetate extracts showed activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans,* at 0.625mg/ml, 0.625mg/ml, 1.25mg/ml, 1.25mg/ml, and 1.25mg/ml respectively for the ethyl acetate extracts and 1.25mg/ml for the methanol extract. Minimum bactericidal concentrations of the extracts against test organisms were found to be 1.25mg/ml, 1.25mg/ml, 5mg/ml, 5mg/ml, and 5mg/ml for ethyl acetate extract and 5mg/ml for the methanol extract with *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans* respectively.

The methanol extract was found to be active against *Trypanosoma brucei brucei,* in an *in vitro* test, killing all the parasites at a minimum concentration of 5mg/ml in 10 minutes as opposed to the standard drug Diminaveto® at 100mg/ml in 3 minutes. The plant *Pouteria alnifolia* is found abundantly in Nigeria (BOTANICA SYSTEMATICA, 2007)(ITHAKA, 2008), but no information on the MIC and MBC of the ethyl acetate and methanol extract of the plant has been reported.

Both lupeol and betulin, reportedly, have antiprotozoal, antimicrobial, anti-inflammatory, antitumor and chemo preventive properties (Gallo and Sarachine, 2009).

Motivated by the ethno medicinal uses of *Pouteria alnifolia,* this research was carried out to examine the claims. The results support the fact that the antimicrobial activity of plant extracts depends on the phytochemicals present in them.

RECOMMENDATIONS

The plant *Pouteria alnifolia* is promising. *In vivo* studies of the methanol extract on *Trypanosomiasis brucei brucei* need to be carried out. Investigations of its anti-malarial properties also need to be carried out since it has shown some anti-protozoan tendencies. Many more useful compounds can still be isolated from this plant.

H_1 should be subjected to further purification processes to isolate pure lupeol and pure betulin.

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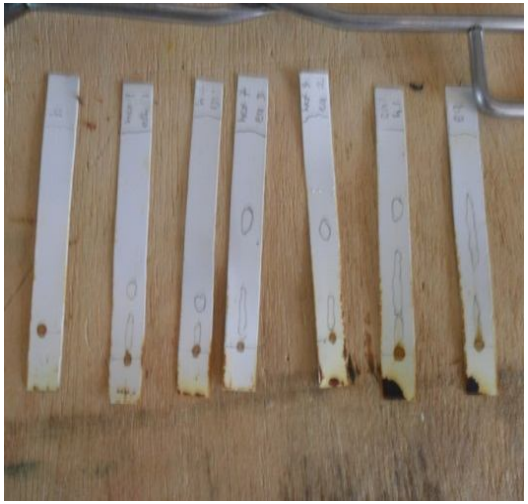
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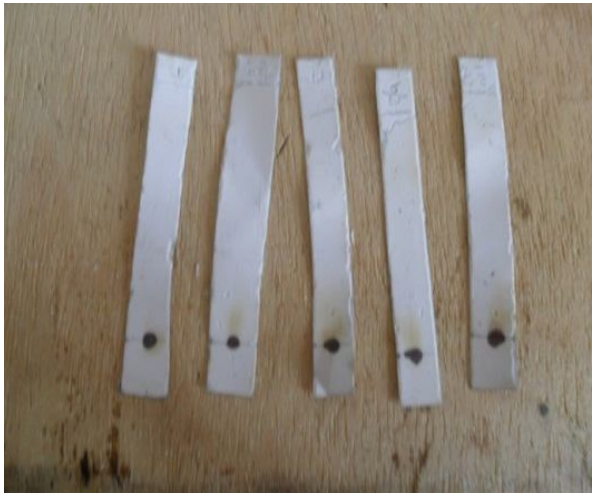
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Software used: ChemDrawUltra **CambridgeSoft Corporation;**http://www.cambridgesoft.com
MestReNova: Mestrelab Research | Chemistry Software for NMR and LC GC MS

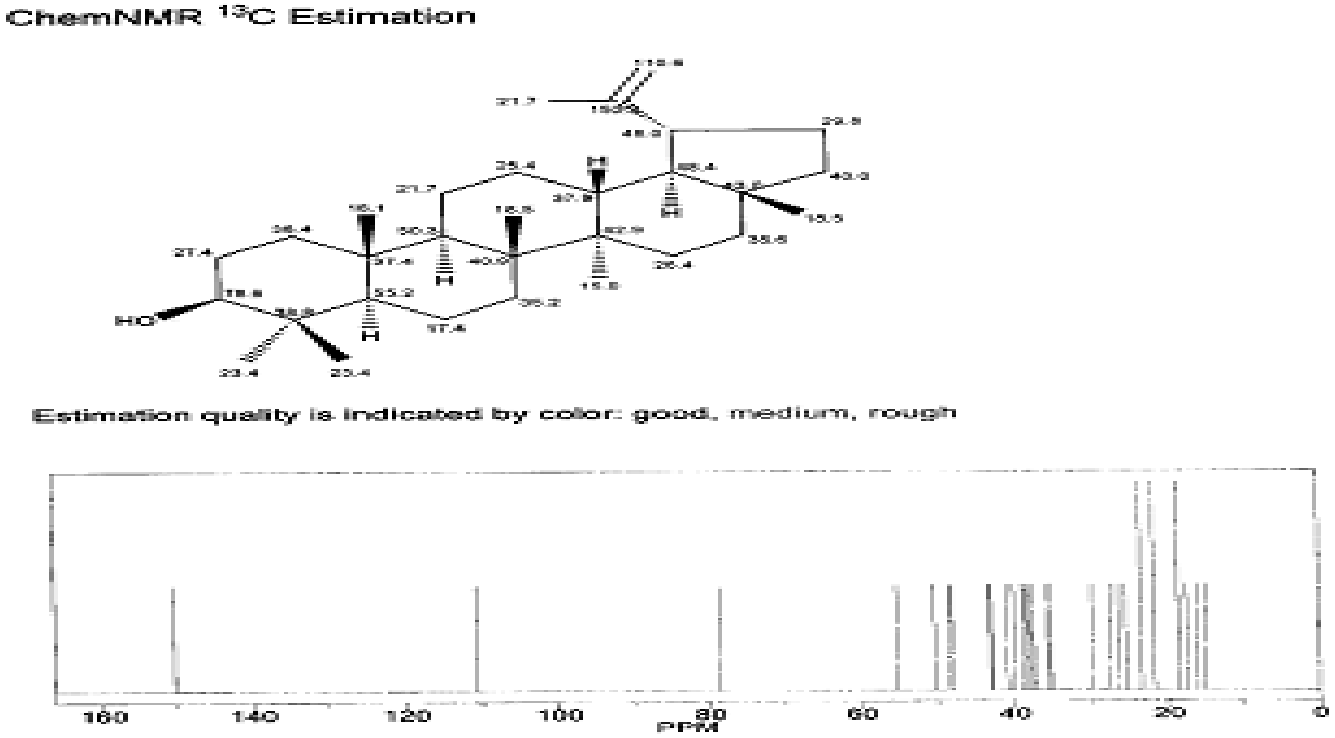
APPENDIX I: Stages of purification for H_1







APPENDIX II: ChemNMR ^{13}C estimation for Betulin based on spectra from H_1



APPENDIX III NMR spectral data of the isolated compound lupeol in $CDCl_3$ (δ in ppm)

C	δc (Lupeol) (Lit.)	δc (Lupeol) (Lit.)	δc(Lupeol Isolated)	δ_C(Lupeol) (ChemDrawUltra)	δ_C(Lupeol)MestReNova)	node
1	38.7	38.6	38.7	38.4	38.3	CH_2
2	27.4	27.3	27.4	27.4	27.2	CH_2
3	78.9	78.9	79.0	78.6	78.9	CH
4	38.8	38.9	38.9	38.8	39.3	C
5	55.3	52.2	55.3	55.2	54.4	CH
6	18.3	18.2	18.3	17.4	19.0	CH_2
7	34.2	34.2	34.2	35.2	34.7	CH_2
8	40.8	40.7	40.8	40.9	41.4	C
9	50.4	50.3	50.5	50.3	50.4	CH
10	37.1	37.1	37.2	37.4	37.5	C
11	20.9	20.8	20.8	21.7	21.6	CH_2
12	25.1	25.0	25.2	25.4	26.4	CH_2
13	38.0	33.7	38.7	37.9	37.9	CH
14	42.8	42.7	42.8	42.9	42.7	C
15	27.4	27.9	27.5	26.4	29.6	CH_2
16	35.5	35.4	35.6	35.6	36.3	CH_2
17	43.0	42.7	43.0	43.0	43.2	C
18	48.2	48.2	48.3	48.4	50.1	CH
19	47.9	47.9	48.0	48.0	48.4	CH
20	150.9	150.9	150.0	150.3	151.1	C
21	29.8	29.7	29.8	29.8	30.9	CH_2
22	40.0	39.9	40.0	40.0	40.1	CH_2
23	28.0	27.9	28.0	23.4	25.6	CH_3
24	15.4	15.3	15.4	23.4	16.8	CH_3
25	16.1	16.0	16.1	16.1	16.5	CH_3
26	15.9	15.9	16.0	18.5	17.0	CH_3
27	14.5	14.9	14.6	15.0	15.6	CH_3
28	18.0	17.9	18.0	18.5	19.6	CH_3
29	109.3	109.3	109.3	110.6	110.0	CH_2
30	19.3	19.3	19.3	21.7	21.3	CH_3

APPENDIX IV NMR spectral data of the isolated compound Betulin and $CDCl_3$ (δ in ppm)

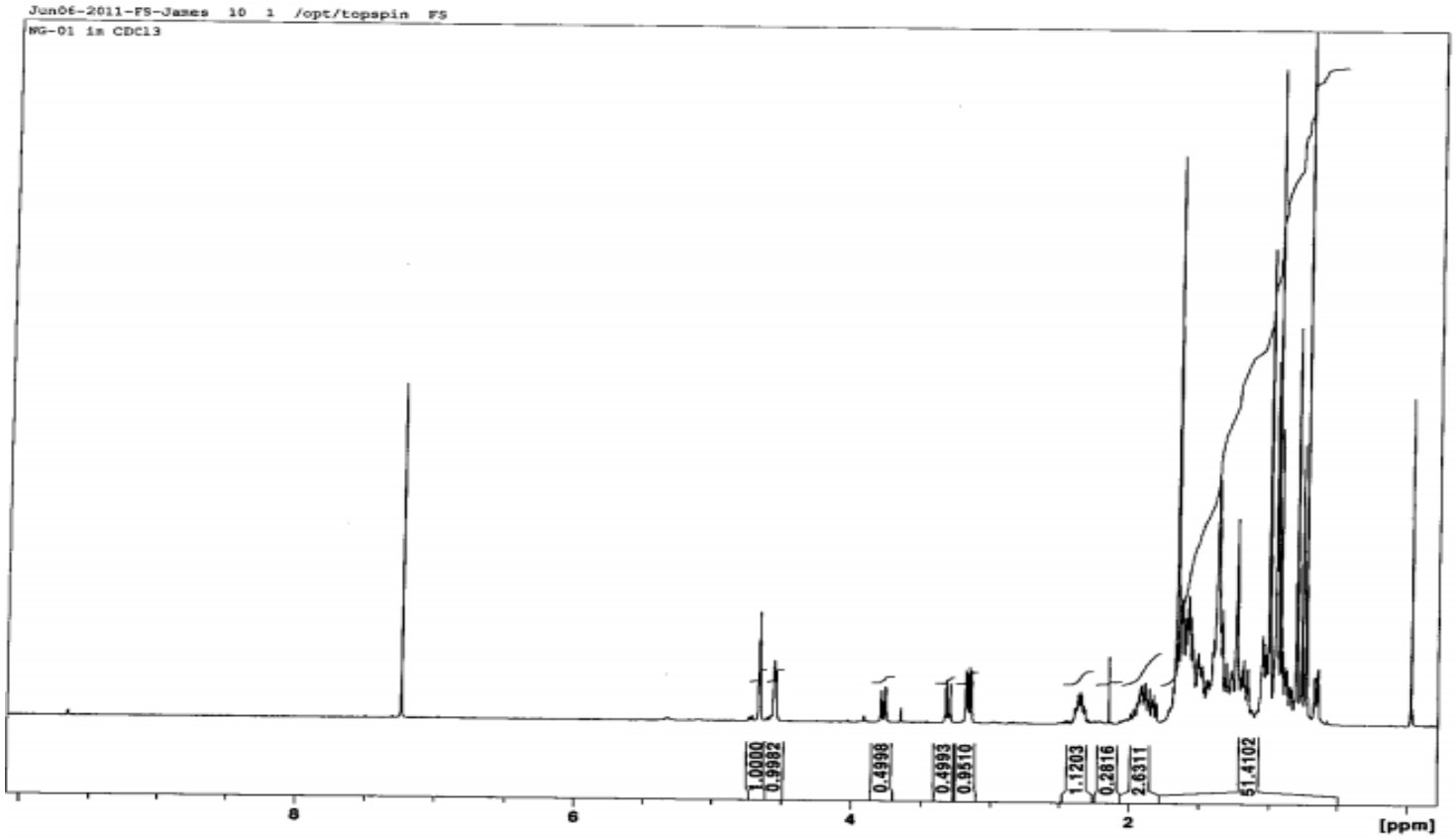
H/C	δc (betulin) (Lit.)	δc(Betulin Isolated)	δc(Betulin) ChemDrawUltra	δ_C (Betulin) (MestReNova)	node
1	38.8	38.9	38.4	38.2	CH_2
2	27.2	27.4	27.4	27.2	CH_2
3	78.9	79.0	78.6	78.9	CH
4	38.9	39.0	38.8	37.5	C
5	55.3	55.3	55.2	54.4	CH
6	18.3	18.3	17.4	19.0	CH_2
7	34.3	34.3	35.2	34.5	CH_2
8	40.9	40.9	40.9	41.4	C
9	50.4	50.4	50.3	50.4	CH
10	37.2	37.2	37.4	37.5	C
11	20.9	20.9	21.7	21.6	CH_2
12	25.3	25.2	25.4	26.4	CH_2
13	37.3	37.3	38.2	36.3	CH
14	42.7	42.7	42.9	42.7	C
15	27.0	27.1	26.7	29.3	CH_2
16	29.2	29.2	29.5	31.7	CH_2
17	47.8	48.0	47.8	48.0	C
18	48.8	49.0	48.7	47.6	CH
19	47.8	48.0	48.3	48.6	CH
20	150.6	150.5	150.3	151.1	C
21	29.8	29.9	30.1	30.4	CH_2
22	34.0	34.0	34.9	33.9	CH_2
23	28.0	28.0	23.4	25.6	CH_3
24	15.4	15.4	23.4	16.8	CH_3
25	16.1	16.1	16.1	16.5	CH_3
26	16.0	16.0	18.5	17.0	CH_3
27	14.8	14.8	15.0	15.6	CH_3
28	60.2	60.6	60.5	63.3	CH_2
29	109.6	109.7	110.6	110.0	CH_2
30	19.1	19.1	21.7	21.3	CH_3

APPENDIX V: δC NMR values for lupeol and Betulin from literature (Reynolds, Mcleans, Poplawsky, Enriquez, Escobar, and Leon, 1986) (Tinto, Blair, Reynolds, and Macleans, 1992)

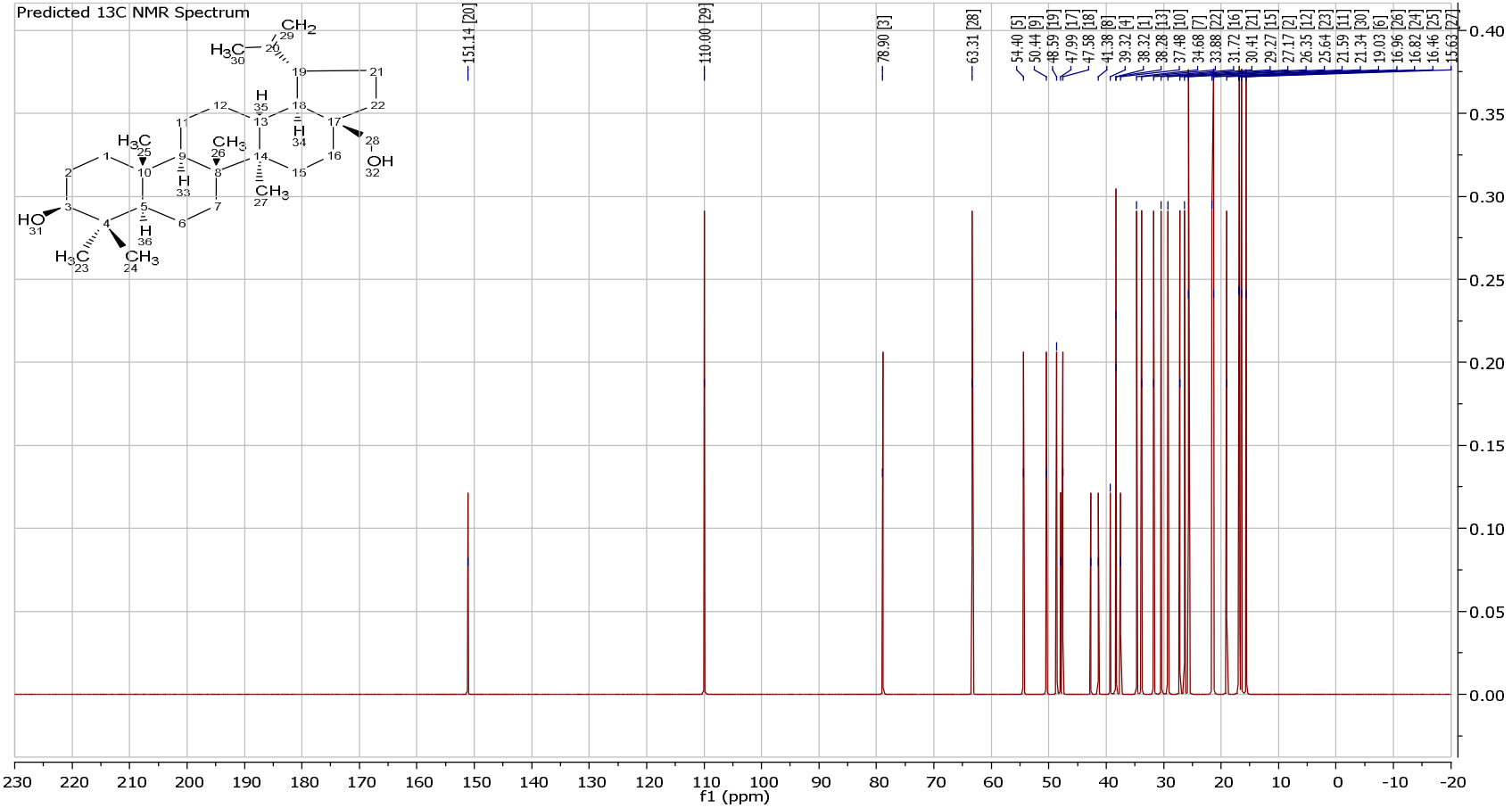
C	δc (Lupeol) (Lit.)	δ_cLupeol) (Lit.)	δc(Lupeol Isolated)	δc (betulin) (Lit.)	δc(Betulin Isolated)	node
1	38.7	38.6	38.7	38.8	38.9	CH_2
2	27.4	27.3	27.4	27.2	27.4	CH_2
3	78.9	78.9	79.0	78.9	79.0	CH
4	38.8	38.9	38.9	38.9	38.9	C
5	55.3	52.2	55.3	55.3	55.3	CH
6	18.3	18.2	18.3	18.3	18.3	CH_2
7	34.2	34.2	34.2	34.3	34.3	CH_2
8	40.8	40.7	40.8	40.9	40.9	C
9	50.4	50.3	50.5	50.4	50.4	CH
10	37.1	37.1	37.2	37.2	37.2	C
11	20.9	20.8	20.8	20.9	20.9	CH_2
12	25.1	25.0	25.2	25.3	25.2	CH_2
13	38.0	33.7	38.7	37.3	37.3	CH
14	42.8	42.7	42.8	42.7	42.7	C
15	27.4	27.9	27.5	27.0	27.1	CH_2
16	35.5	35.4	35.6	29.2	29.2	CH_2
17	43.0	42.7	43.0	47.8	48.0	C
18	48.2	48.2	48.3	48.8	48.8	CH
19	47.9	47.9	48.0	47.8	47.8	CH
20	150.9	150.9	151.0	150.6	150.5	C
21	29.8	29.7	29.8	29.8	29.9	CH_2
22	40.0	39.9	40.0	34.0	34.0	CH_2
23	28.0	27.9	28.0	28.0	28.0	CH_3
24	15.4	15.3	15.4	15.4	15.4	CH_3
25	16.1	16.0	16.1	16.1	16.1	CH_3
26	15.9	15.9	16.0	16.0	16.0	CH_3
27	14.5	14.9	14.6	14.8	14.8	CH_3
28	18.0	17.9	18.0	60.2	60.6	CH_3=L,CH_2=B
29	109.3	109.3	109.3	109.6	109.7	CH_2
30	19.3	19.3	19.3	19.1	19.1	CH_3

Key L=Lupeol; B=Betulin

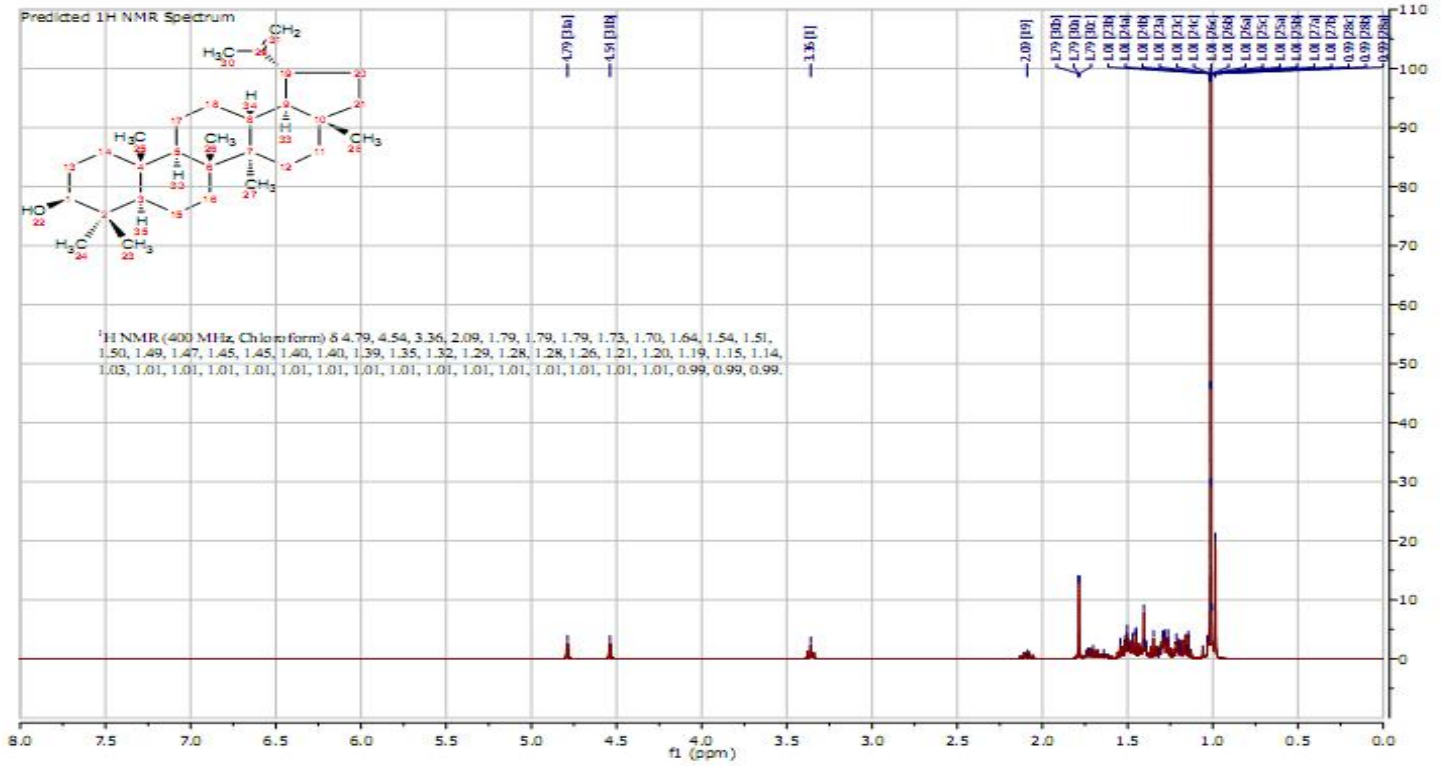
APPENDIX VI


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WG-01 in CDCl3
1.0000
0.9982
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4
2
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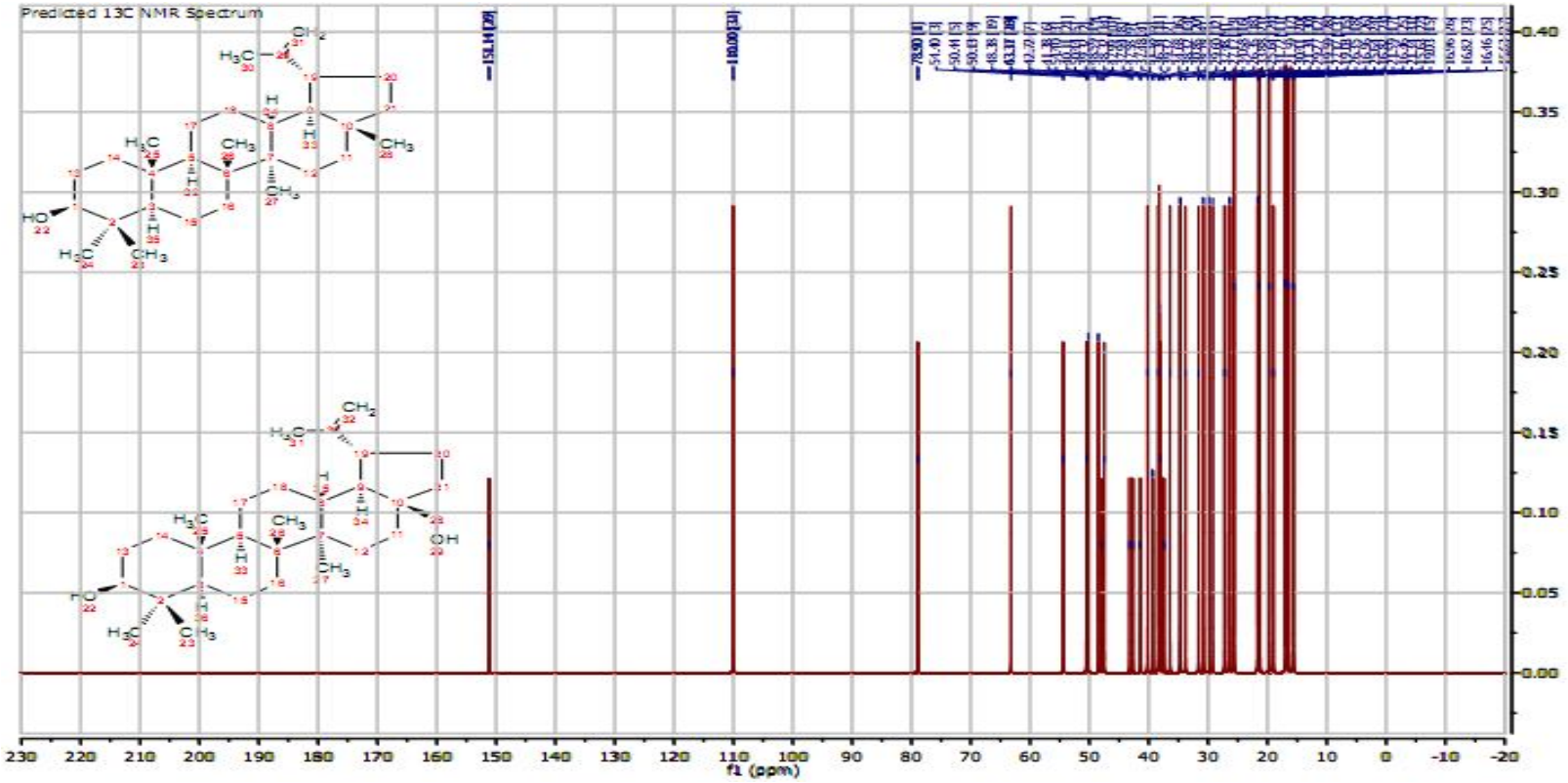
APPENDIX VII: ^{13}C NMR SPECTRUM SIMULATION FOR BETULIN BY MESTRENOVA®



APPENDIX VIII: ^{1}H NMR SPECTRUM SIMULATION FOR LUPEOL BY MESTRENOVA®



APPENDIX IX: ^{13}C NMR SPECTRUM SIMULATION FOR H_1 (Superimposed spectra of Lupeol and Betulin showing a striking similarity with that of H_1)



APPENDIX X: ^{13}C NMR SPECTRUM FOR BETULIN

