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ON

PHYTOCHEMICAL INVESTIGATION OF SEEDS OF *Azadirachta indica* AND ITS EVALUATION FOR ANTIMICROBIALACTIVITIES.

BY

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PHYTOCHEMICAL INVESTIGATION OF SEEDS OF *Azadirachta indica* AND ITS EVALUATION FOR ANTIMICROBIAL ACTIVITIES

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List of Abbreviations

CFU	Colony forming unit
^{13}C-NM	^{13}Carbon Nuclear Magnetic Resonance
DMAPP	dimethylallyl diphosphate
GBDS	Global Burden of Disease Study
GPP	geranyl pyrophosphate
^{1}H-NMR	Proton Nuclear Magnetic Resonance
IPP	Isopentenyl diphosphate
MIDS	Microbial infectious diseases
NAD	New and Approved Drugs
NCDS	non-communicable diseases
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
TM	Traditional Medicine
UV-Vis	Ultraviolet-Visible spectra
WHO	World Health Organization

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Abstract

Natural products isolated from plants have been providing noble and clinically active drugs. The traditional medicinal system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care. Thus, the main objective of this study was to isolate and characterizes bioactive natural products from the seeds of *Azadirachta indica.* The plant material was collected from Gambella region of Pugnewdo district and was allowed to dry in the shade area, and then powdered using pestle and mortar. The powdered material was extracted by maceration method first using petroleum ether followed by chloroform/methanol (1:1).The extract was then filtered and evaporated using a rotary evaporator. The isolation of pure compound was made by Column chromatography controlled by TLC using petroleum ether in increasing amount of ethyl acetate for polarity. This led to the isolation of two compounds, characterized as, Azadiradione and Epoxyazadiradione: which their structures were established based on their NMR data comparison with values in the literature. 100mg/ml the crude extract and isolated compounds were evaluated for antibacterial and antifungal activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, penicillium* and *Aspergillus flavus* to show zone of inhibition ranging from (15-29 mm). Highest activity was recorded against *Escherichia coli* (29 mm) and least for *Aspergillus flavus* (15 mm). Compound A has better activity than compound B against *Staphylococcus aureus* but the two compounds have similar activity for fungal species. Generally two triterpenoids Meliaceae family were isolated and characterized from seeds of *Azadirachata indica*. The PE/EA (96:4) affords 166 mg of compound A a known triterpenoids azadiradione and (80:20) gives 186 mg of the second triterpenoids, compound **B** as epoxy derivative of compound **A** and assigned the name, 14, 15-epoxyazadiradione. However, further study on chemical compotation recommended including the usable seed of Azadirachata indica for future use is important for the development and production of new drugs and the biological activity should be also done on another bacterial and fungal strain.

Key words: - Azadirachata, Azadiradione, Epoxyazadiradione, Maceration, Mortar, Pestle.

1. Introduction

1.1 Background of the study

Plant synthesizes a wide variety of chemical compounds, which can be stored by their chemical class, biosynthetic origin and functional groups into primary and secondary metabolites. Secondary metabolites do not seem to be vital to the immediate survival of the organism that produces them and are not an essential part of the process of building and maintaining living cells [1]. They consist of a number of biologically active ingredients such as alkaloids, flavonoids, steroids, glycosides, terpenes, tannins and phenolic compound. These phytochemicals are synthesized in all parts of the plant body and are mainly attributed to the pharmacological actions. Medicinal plants are usually screened for phytochemicals that may lead to its further isolation, purification and characterization of active principle. The active compound can be then used as the basis for a new pharmaceutical product [2, 3].

According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Use of herbal medicines in Africa represents a long history of human interactions with the environment. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance [4].

Developing countries like Ethiopia depend on plant resources mainly for herbal medicines, food, forage, construction of dwellings, making household implements, sleeping mats, and for fire and shade. The use of medicinal plants as traditional medicines is well known in rural areas of many developing countries [5]. Traditional healers claim that their medicine is cheaper, more effective and impart least side effects as compared to synthetic medicines. In developing countries, low-income people such as farmers, people of small isolate villages and native communities use folk medicine for the treatment of common infections [6] and different phytochemicals components can be found from

plants. In relation to drug development, an emerging global market for pharmaceuticals raises concerns about a greater focus on conditions and markets deemed most profitable, regardless of the global burden of disease. How drugs will be developed for infectious diseases afflicting the poorest population groups with in such a context remains unclear.

For example, only 13 of the 1223 new chemical entities commercialized between 1975 and 1997 were for tropical diseases [7] and no new drugs for tuberculosis have been developed for over 30 years because, despite its enormous toll, only 5% of the 16 million infected can afford medication [8]. These inequities contribute to the 10/90 gap in which 90 percent of research funds address the health needs of 10 percent of the world's population [9]. For population mobility and mortality, the most direct impact has been the spread of infectious disease. Human migration has been a source of epidemics throughout history, and several of these have influenced the outcome of war or changed whole societies [10, 11].

Moreover, there is a gap between the burden of infections due to multidrug-resistant bacteria and the development of new antibiotics to tackle the problem. There is a particular lack of new agents with new targets or mechanisms of action against multidrug-resistant Gram-negative bacteria. To such agents with new or possibly new targets and documented activity were identified with isolation, structural elucidation constituents on the seed of neem *(Azadirachta indica)* both in early phases of development

Therefore, the main objective of this study was to carry out phytochemical investigation and evaluation of the antimicrobial activities of *Azadirachta indica seeds.*

1.2. Statement of the problems

In the past years, the pharmacological industries have produced a number of antibiotics and antifungal agents. Despite this effort however, the incidences of drug resistance by microorganisms has increasing. Many infectious diseases are increasingly becoming difficult to treat because of antimicrobial-resistant organisms, including *Staphylococcal* infection, tuberculosis, influenza, gonorrhea, Candida infection, and many others. The problem of microbial resistance is growing and the outlook for the use of the current antimicrobial drugs in the future is still uncertain. Therefore, the urgent need to find an alternative antimicrobial agent to address the current problem of drug-resistance is required. Studies on other species of the genus *Azadirachata* have revealed that the plants have constituents that are important in medicine and the chemical studies have done worldwide. Many of these plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms. A number of these agents appear to have structures and mode of action that are distinct from the antibiotics current use. So it is worthwhile to study plant and plant products for activity against resistant bacteria. Traditionally people in Ethiopia have used medicinal plants to treat different diseases and also it has great contribution in primary health care systems. However, most of the existing medicinal plants are not well investigated to identify the chemical principles for their claims. In most azadirachtin and their derivatives such as salannin, melianlriol and nimbin were common chemical compounds in this species.

1.3. Objective of the study

1.3.1. General objective

- The general objective of this study was to investigate bioactive secondary metabolites from the seed of *Azadirachta indica* effective against microbial agents.

1.3.2. Specific objective:

- To isolate secondary metabolites from the seed of *Azadirachta indica* using chromatographic techniques.
- To elucidate the structure of the isolated compounds using NMR data.
- To evaluate effectiveness of the crude extract and isolated compounds against *Staphylococcus aureus (ATCC 6538), Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 15442)* and *Bacillus cereus (ATCC 14579)* for antibacterial activity and *penicillium* and *Aspergillus flavus* for antifungal activity.

1.4. Significance of the Study

The bioactive phytochemical constituents in the plant play significance role in the development of medicines and drug discovery. Therefore phytochemical investigation is a very important for isolation and characterization of bioactive molecules from seed of *Azadirachta indica*. The outcome of this study is:-

- To identify and isolate some compounds that could be used as in the discovery of antimicrobial agents.
- To give information about the constituents of the plant seed.
- To document the obtained result from the plant material for further study.

2. Review of Related Literature

2.1 Microbial Infectious Problem

Historically, microbial infectious diseases (MIDs) have been the most important contributor to human morbidity and mortality until relatively recent times, when non-communicable diseases (NCDs) began to rival, and sometimes exceed, infections. Today, IDs still account for a large proportion of death and disability worldwide and in certain regions remain the most important cause of ill health. The Global Burden of Disease Study (GBDS) estimates that, in the past year, infectious diseases were responsible for 22% of all deaths and 27% of disability-adjusted life years worldwide [12]. Although infectious diseases can affect people of all ages, they impose a particular burden on the young, notably on children under five. This is not only because younger age groups have a lower prevalence of NCDs, because they are more susceptible than adults to new infections, lacking the protective mechanisms to reduce the impact of these illnesses. Consequently, in regions where a high proportion of the population is made up of young people Africa, Latin America and many other are developing regions. Infectious diseases usually extract a relatively high toll on the population. For example, GBDS estimates for 2002 were that infectious diseases were responsible for 52 and 50% respectively of all deaths and DALYs in sub-Saharan Africa but only 11 and 5% in the established market economies [13].

The term ‘infectious disease’ does not refer to a homogeneous set of illnesses but rather to a broad group of widely varying conditions. The relative and absolute importance of particular infections or groups of infections varies dramatically across regions. In high-income countries, deaths from IDs are overwhelmingly due to respiratory infections and HIV/AIDS. In sub-Saharan Africa, respiratory infections, diarrheal diseases, HIV/AIDS, TB and malaria account for roughly similar proportions of total ID deaths [14]. In addition, rates of specific infectious diseases are generally much higher in poor countries, regardless of the relative importance of these diseases. Therefore, in both relative and absolute terms, IDs are a considerably higher burden in low-income than high-income countries. An analysis of GBDS data concludes that the poorest 20% of the world’s

population experiences a far higher burden of infectious disease compared to the remaining 80% of the world's population [15].

It should be noted that estimates of the burden of infectious disease at regional or global level can obscure the importance of specific infections in particular populations. For instance, tropical diseases impose a heavy burden on some of the poorest populations in the world but, since they occur almost exclusively in certain climates, can be recorded as making a proportionately small contribution to the overall infectious disease burden [16]. Also, certain diseases such as dengue fever vary greatly with environmental and other determinants, and can rapidly assume epidemic proportions. This is often poorly represented in estimates of average annual disease incidence and prevalence.

Finally, in most assessments, certain illnesses with a strong infectious component, such as liver cancer and several important neurological diseases, are not considered part of the infectious diseases burden [17].

Estimates of the future burden of infections fall into two categories: predictions for individual diseases, and calculations of the overall impact of infectious diseases in the future. Examples of the former include those for HIV/AIDS and multidrug resistant tuberculosis, which often predict significant rises in prevalence and mortality [18]. The most influential summation of the likely over-all future burden of infectious diseases predicts a gradual decline in both the rates of infection and their proportionate contributions to overall disease burden in all regions of the world [19]. However, these estimates assume that disease patterns will evolve in much the same way as they have in high-income countries over the past 100 or so years, and that current efforts to contain their Impacts will be sustained. One finding from this review is that the future impact of many infections is uncertain because globalization is impacting on human societies and the natural environment in ways hitherto not experienced. Also, cases of certain infectious diseases (particularly vector-borne infections) have either been rare or entirely absent in most high-income countries during the last 100 years [20]. Finally, shifts in political and economic values may lead to changes in current resources for infectious disease control, particularly in low and middle-income countries [21].

Having the above idea, natural products have been the most successful source of potential drug leads [22]. However, their recent implementation in drug discovery and development efforts have somewhat demonstrated a decline in interest [22]. Nevertheless, natural products continue to provide unique structural diversity in comparison to standard combinatorial chemistry, which presents opportunities for discovering mainly novel low molecular weight lead compounds. Since less than 10% of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds await discovery with the challenge being how to access this natural chemical diversity [23].

Generally, medicinal plants have many characteristics when used as a treatment, as follow [24]: Synergic medicine- The ingredients of plants all interact simultaneously, so their uses can complement or damage others or neutralize their possible negative effects. Support of official medicine- In the treatment of complex cases like cancer diseases the components of the plants proved to be very effective. Preventive medicine- It has been proven that the component of the plants also characterize by their ability to prevent the appearance of some diseases. This will help to reduce the use of the chemical remedies which will be used when the disease is already present i.e., reduce the side effect of synthetic treatment.

2.2. Botanical Information *Azadirachata indeca*

Azadirachata indeca is a member of the family *Meliaceae*. It is broad-leaved evergreen tree which can reach heights of 30 meters with a trunk girth of 2.5 meters and can live for over two centuries. Its deep root system is well adapted to retrieving water and nutrients from the soil profile, but this deep root system is very sensitive to water logging. The neem tree thrives in hot, dry climates where the temperatures often reach 40°C and annual rainfall ranges from 400 to 1,200 mm. The tree can withstand many environmental adversities including drought and infertile, stony, shallow or acidic soils. These fruits contain kernels that have high concentrations of secondary metabolites [24].

Figure 1. The leaves and seed of neem at Gambella taken by Yirga kassa.

2.3. Uses of Azadirachata Indica

Neem fruits, seeds, leaves, roots, and bark has long been used in the traditional medicine [25, 26]. Thus, over thousands of years, millions of people have used neem medicinally. In addition, in places where the tree has been introduced in recent times, such as in Gambella region of Gog woreda pugnewdo district, it has also established a reputation as a useful cure for various ailments. Today, the best-established and most widely

recognized uses were based on its merits as antimalaria and antimicrobial activity. Neem had proved to be effective against certain fungi that infect the human body such as *Aspergillus flavus*, which causes increasing problems that difficult to be controlled by synthetic fungicide [27]. Users for many more ailments have claimed but have not been independently confirmed by trials under controlled conditions. Nonetheless, there are intriguing indications that neem might in future be used much more widely. These promising applications include antimicrobial, anti-inflammatory, hypertensive, and anti-malaria treatments

It has been also used traditionally for centuries in both agriculture and medicine [28]. Although it was one of the most ancient and most widely used herbs on earth, intense scientific investigations of the properties of neem are now being undertaken. Studies were quickly verifying the efficiency of its traditional uses and even more uses for neem could be applicable. From the very beginning of recorded human history, people have taken advantage of the remarkable neem tree. Even before ancient herbalists discovered the analgesic qualities of the willow tree from which aspirin is derived, people used branches, its fruit and leaves cure many diseases. Its medicinal qualities are outlined dated back to very remote times. Up to date, rural throughout the world refer to the neem tree as a village pharmacy because it cures diseases including malaria [29]. Its part is commonly used for medicine, shade, building materials, fuel, lubrication, and most of all as pesticides. Its use as an insecticide that now draws great interest from industrialized countries as it is considered as an environmentally safe alternative to synthetic pesticides [30]. To date over 195 species of insects are affected by its extracts at concentrations ranging from 0.1 to 1,000 ppm, and many insects that have become resistant to synthetic pesticides could be controllable with these extracts [31, 32]. Many modern researchers have investigated its remarkable tree. The seeds bark and leaves contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, and antifungal use [32].

2.4. Phytochemical Information of *Azadirachata indica*

Many biologically active compounds have been identified from neem, including terpenoids, phenolic compounds, carotenoids, steroids, flavonoids etc. Terpenoids were the majority among the secondary metabolites present in the neem (Table 1) and they were categorized into various classes which are detailed in the following sections [33].

Table1. Major groups of neem secondary metabolites based on their numbers and diversity, terpenoids appeared to be capturing major portion and much potential in having assortment of industrial and medicinal application.

N^{o}	secondary metabolites	% abundance
1	Nortriterpenoids	50.6
2	Triterpenoids	9.5
3	Fatty acids and their derivatives	8.3
4	Flavonoids	6.8
5	Hydrocarbons	6.2
6	Protolimonoids	3.6
7	Steroids	3.6
8	Carbohydrates and proteins	3.3
9	Coumarins and chalcones	2.7
10	Acids and their derivatives	2.4
11	Sesquiterpenoids	1.8
12	Phenolic constituents	1.4
13	Monoterpenoids	0.3

2.5. Compounds from *Azadirachata indica* and their biological activity

Compounds isolated from the aerial part of *Azadirachata indica* such as Melianol (**1**) is mononortriterpenoids isolated from neem leaves [34]. Meliacinin (**2**), azadiradionol (**3**) and azadirolic acid (**4**) are dinortriterpenoids isolated from fruit coats [35]. Meliacinolactol (**5**), Zeeshanol (**6**) and Limbonin (**7**) are the ones considered to be part of trinortriterpenoids[36]. Nimbandiol (**8**), 6-O-acetylnimbandiol (**9**), 4α–

Benzoylnimbandiol (**10**), Nimbinene (**11**), and 6-deacetylnimbinene (**12**) are pentanortriterpenoid compounds were reported so far [37].

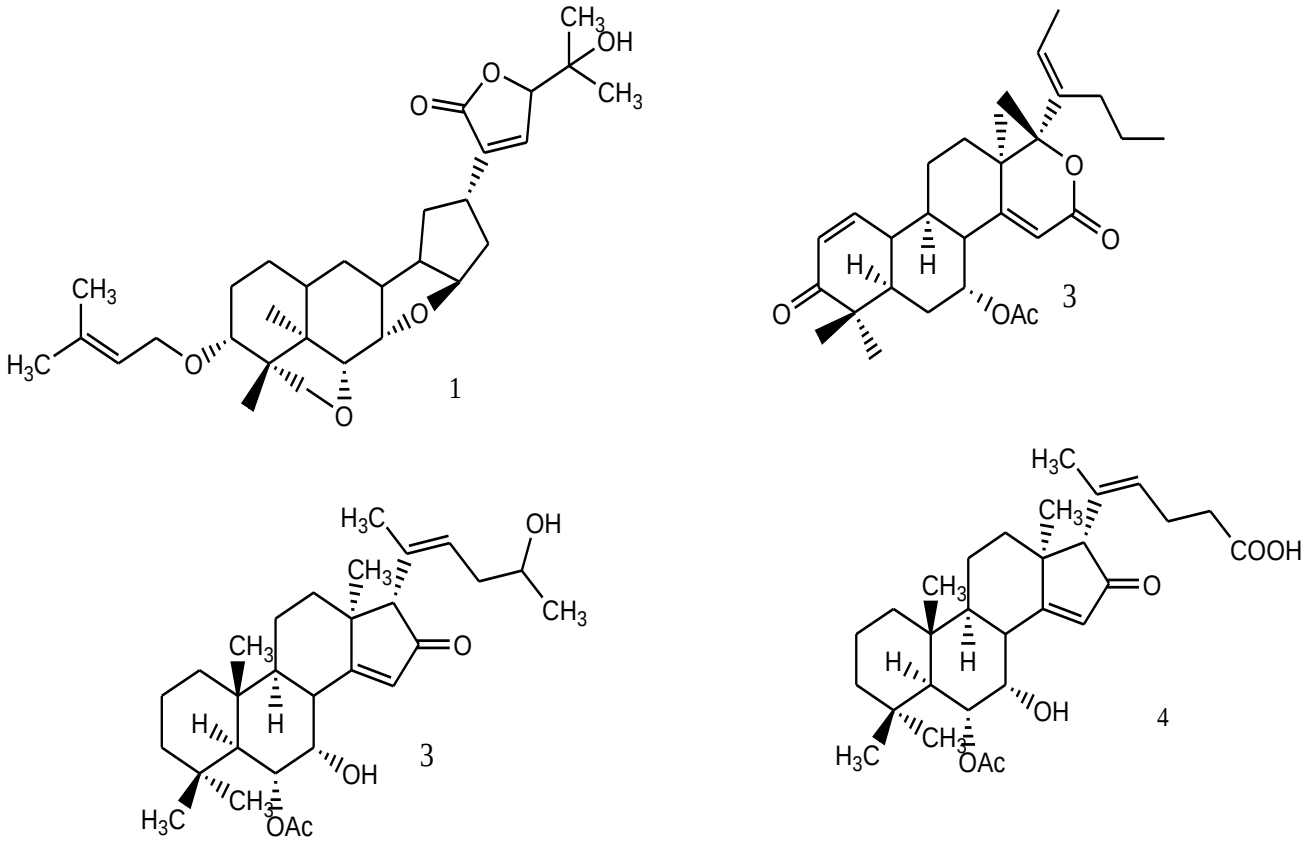


Fig. 2. Structures of mononortriterpenoids and dinortriterpenoids.

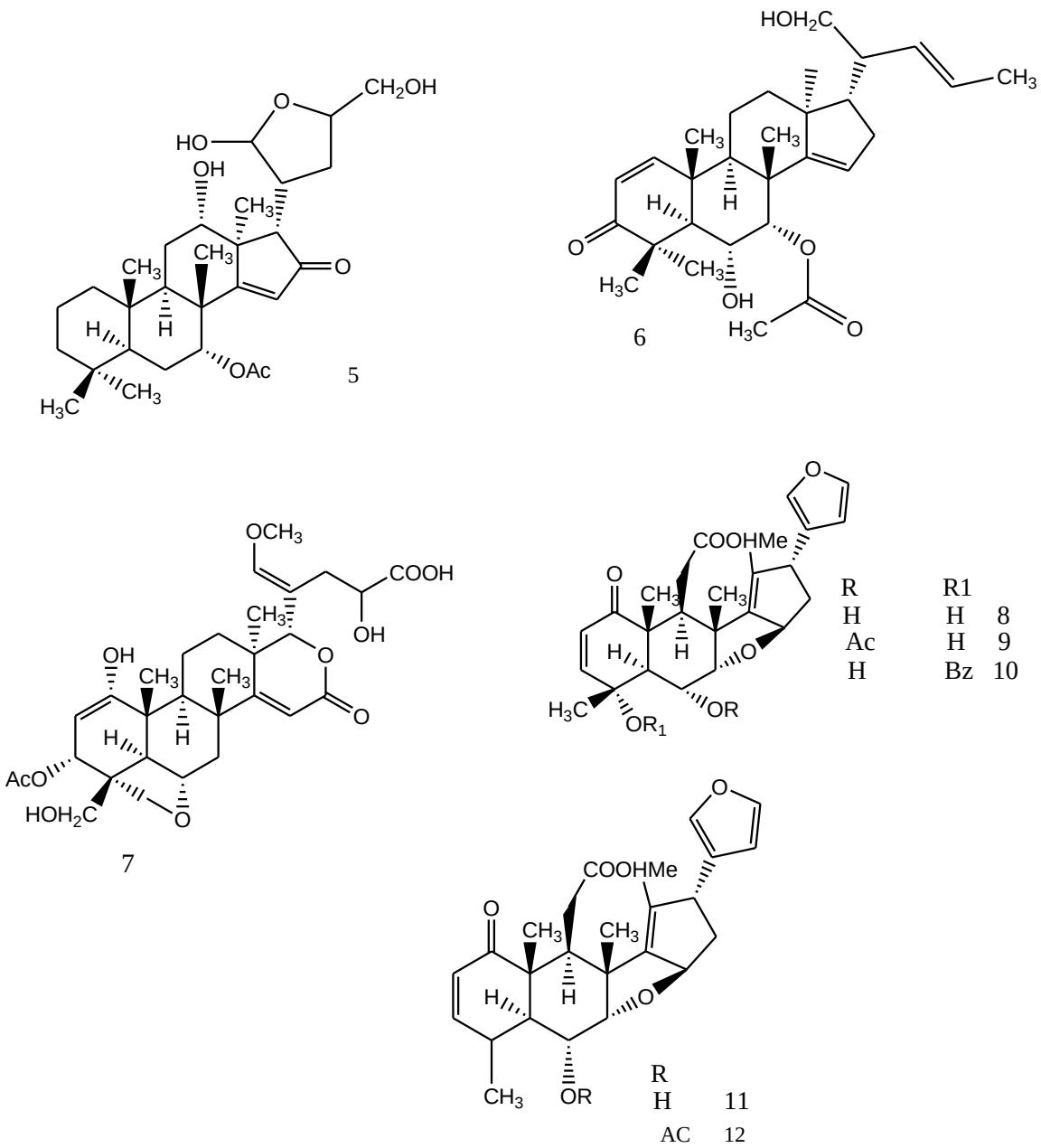


Fig. 3. Trinortriterpenoids and pentanortriterpenoids.

Whereas, Azadirones has unique feature due to the presence of oxygen function at C-3 and C-7 with no change in all the four rings of the basic skeleton. Meliacins this family are further sub grouped into azadirone (**13**), 7-Deacetyl azadirone (**14**), Nimocin (**15**), Nimocinol (**16**), Meldenindiol (**17**). Meldenin (**18**), Isomeldenin (**19**), Meldenindiol (**20**), and Azadiradione (**21**) 17-Epi azadiradione (**22**), 17-β-Hydroxy azadiradione (**23**), 17-B-

Hydroxyl azadiradione (**24**), Nimbocinol (**25**), 17-β-Hydroxy nimbocinol (**26**) in which oxidation reactions makes them related to each other [38].

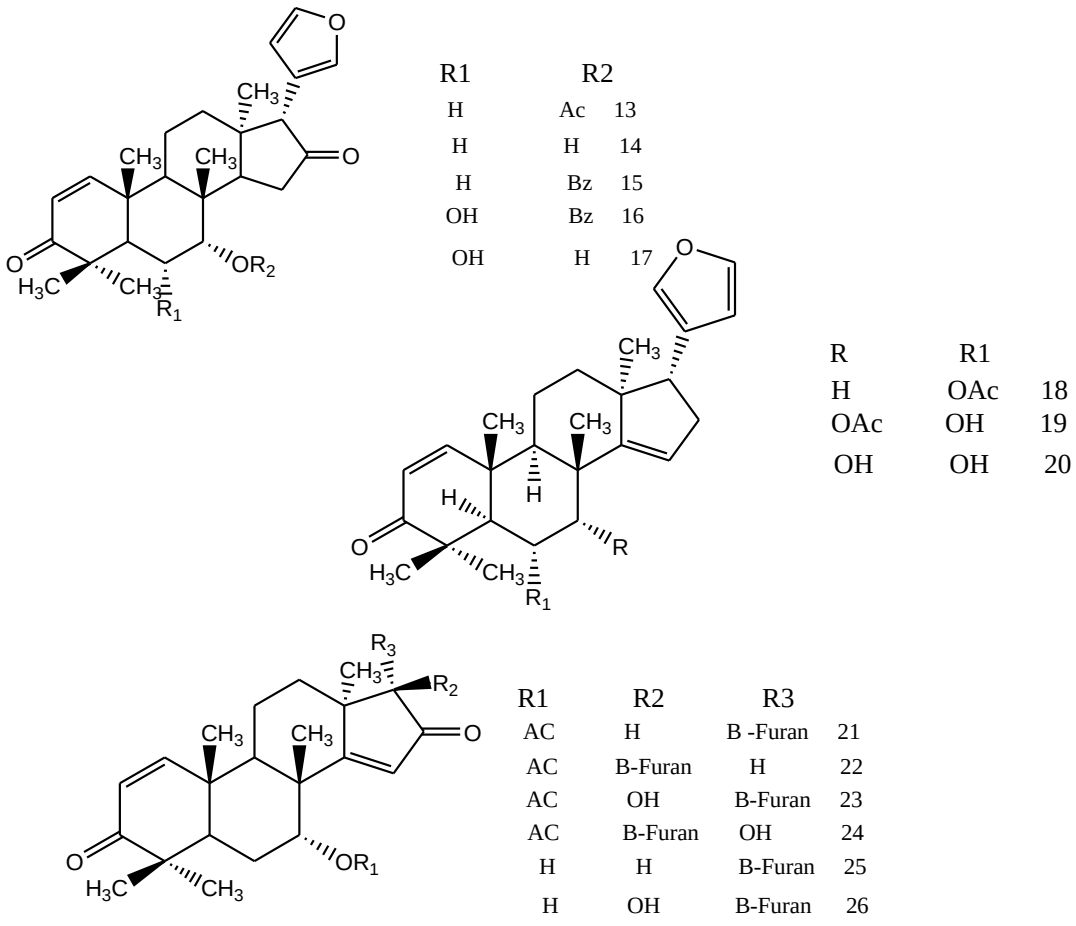
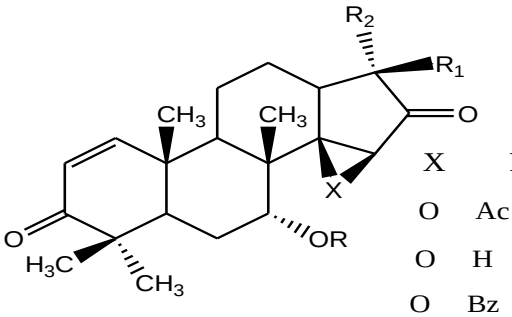


Fig. 4. Structures of azadirone family.

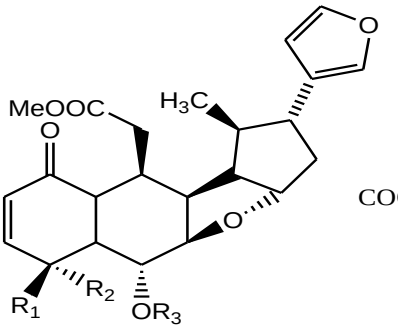
Hence, Gedunin is one of the potent bioactive tetranorterpenoids of neem reported to have anticancer, antifungal and antimalarial properties. The D-ring in parent structure of compounds epoxy Azadiradione (**27**), 7-Deacetyl (Gn) (**28**), 7-Deacetyl-7-benzoyl (Gn) (**29**) are considered to be oxidized probably by Baeyer Villiger type of reaction to give rise to compound **27** [39]. Also Nimbin (**30**), 6-Diacetyl nimbin (**31**), 4-Epinimbin(**32**),6-Deaceiyl nimbinal (**33**), Nimbinal (**34**) , Nimbinol (**35**) and Nimbic acid (**36**) were tetranortriterpenoid class of compounds. Amoorstatin (**37**) and vepinins (**38**)**:-** The biogenic precursor for these compounds could be nimocinol or other azadirones formed

through addition of 17-OH to the C-14/15 double bond and formation of 7a and 17a-oxygen bridge. Amoorstatins possess a C-19/29 oxygen bridge and reported for its hypoglycemic activity [40]

Nimolicinolic acid (**39)** and nimbocin (**40**) were hexanortriterpenoid**s** isolated from fruits and root bark, respectively. Nimolicinolic acid is considered to be the unique compound isolated from neem has apo-euphane or apo-tirucallane skeleton while other plants possess cucurbitacin, dammarane and lanostane skeletons [41]. While azadironol (**41**), desfurano-6a-hydroxyazadiradione (**42**) and desfurano- Desacetylnimbin (**43)** were reported to belong to octanortriterpenoids class [42] and β-Nimalactone **(44**) and a-nimalactone (**45**) are the only two compounds reported to belong to nonanortriterpenoids class [43]



X	R	R1	R2	
O	Ac	H	B-Furan	27
O	H	H	B-Furan	28
O	Bz	H	B-Furan	29



R1	R2	R3	
CH3	COOMe	Ac	30
CH3	COOMe	H	31
COOMe	CH3	Ac	32
CH3	CHO	H	33
CH3	CHO	Ac	34
CH3	CH2OH	H	35
CH3	COOH	H	36

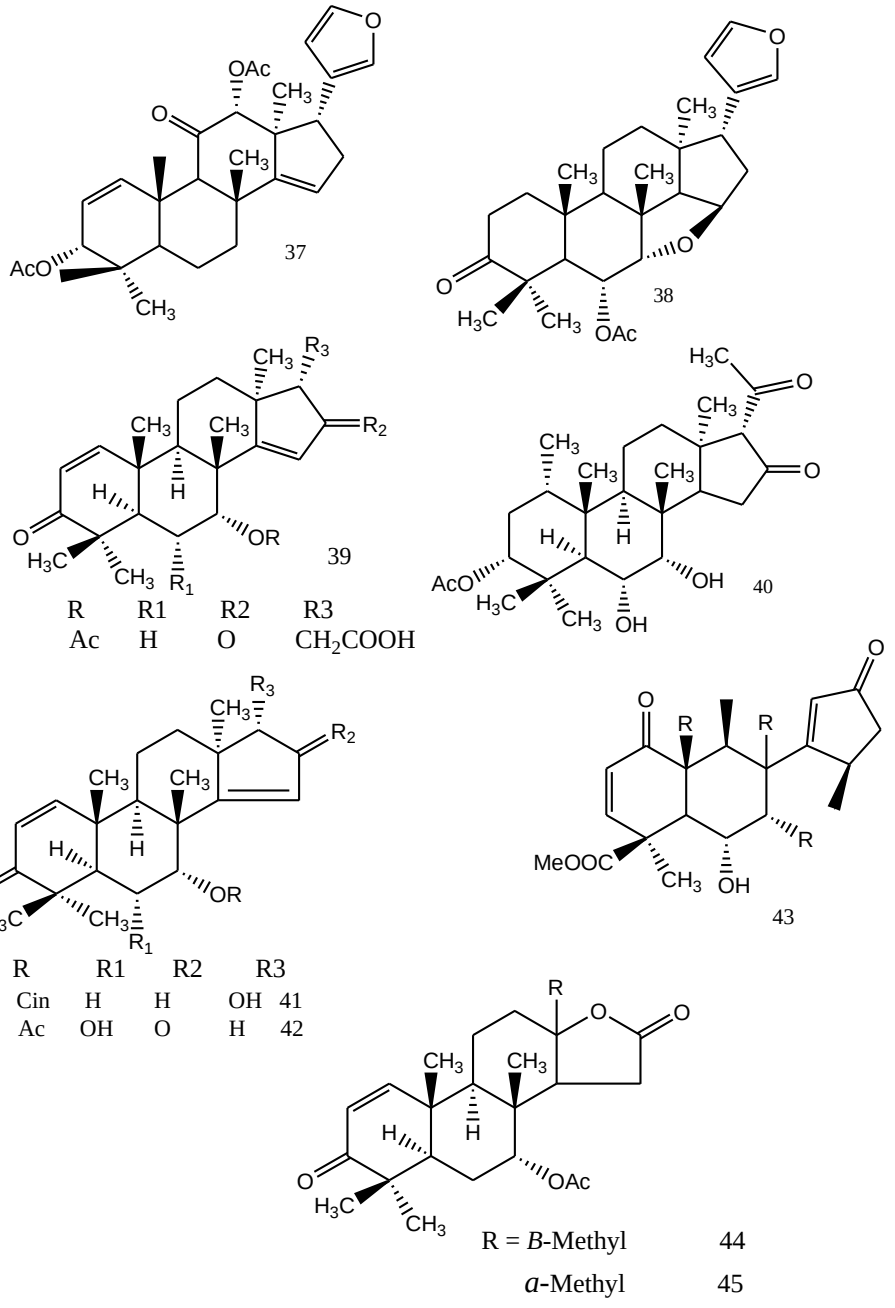
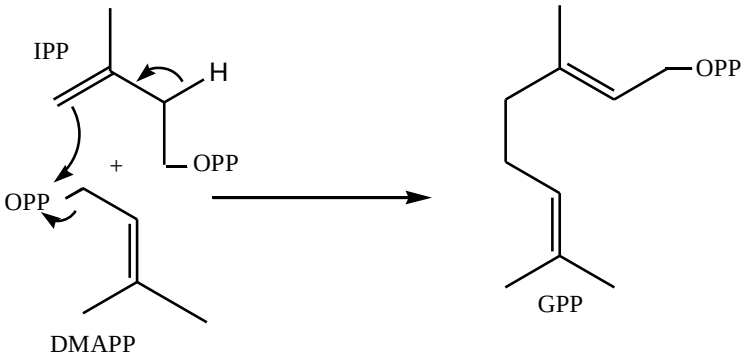


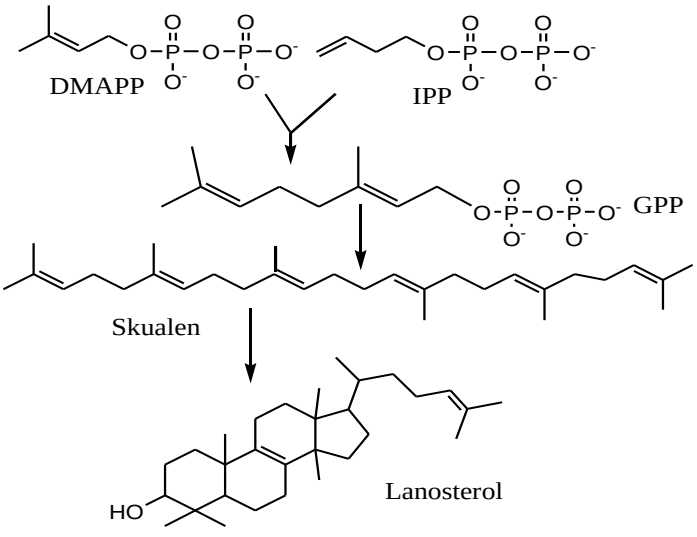
Fig. 5. Structures of some of tetranortriterpenoid class of compounds

2.5. Biosynthesis of the Terpenoides

Terpenoids are extraordinarily diverse but they all originate through the condensation of the universal phosphorylated derivative of hemiterpene, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) giving geranyl pyrophosphate (GPP) [44].



In higher plants, IPP is derived from the classic mevalonic acid pathway in the cytosol but from the methylerythritol phosphate pathway in plastids. It is generally accepted that the cytosolic pool of IPP serves as a precursor of sesquiterpenes, triterpenes, sterols and polyterpenes whereas the plastid pool of IPP provides the precursors of mono -, di - and tetraterpenes. IPP and DMAPP are the end-products in the pathway, and are the precursors of isoprene, monoterpenoids (10-carbon), diterpenoids (20-caotenoids (40-carbon), chlorophylls, and plastoquinone-9 (45-carbon). Synthesis of all higher terpenoids proceeds via formation of geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) [45].



Terpenes have also been defined as compounds whose carbon skeleton can be dissected into isoprenoid units joined in a regular linkage (e.g. head-to tail), or in an irregular linkage (e.g. head-to-head).These arrangements can readily be seen in the carbon skeletons of the monoterpene, terpinolene; the sesquiterpene, farnesol, the triterpene e.t.c.

3. Material and Methods

3.1. Chemical

Chemicals that were used for this study include methanol, chloroform, petroleum ether , ethyl acetate, Silica gel 60-120 mesh size, Iodine, distilled water, diethyl sulfoxide (DMSO), Mueller Hinton agar and nutrient broth as culture media were used for antibacterial activity test were also used in this study.

3.2. Apparatus and equipment

Rotary evaporator, TLC plate, round bottom flask (50, 100 and 250 mL), measuring cylinders, flasks, pestle and mortar, filter papers, weighing balances, oven, glass columns for column chromatography, UV-254 and 365 nm chamber (UV-techic) were used for the study. Spectral recording was done using Bruker 400 MHz advance NMR spectrometer with solvent residual as internal standard at Addis Ababa University.

3.3 Collection and Preparation of the plant Sample

The matured seeds of *Azadirachata indica* were collected from pugnewdo district Gog woreda, in Gambella region, 757 km away from Addis Ababa. Then it was authenticated (voucher number 3) at the Herbnarium, department of Biology Jimma University, Jimma, Ethiopia. The plant materials were then air dried in the shade and grounded into powder, and followed by extraction with petroleum ether to remove its fat-soluble components. Meanwhile, it was extracted with mixtures of CH_2Cl_2/MeOH (1:1). And the crude extracts were concentrated using rotary evaporator.

3.4. Extraction and Isolation of compounds

3.4.1. Extraction of seed of *Azadirachata indica*

The powdered seed of *Azadirachata indica* (1 kg) was first macerated with petroleum ether for 24 hours to remove out the oil part of the sample. Then filtered with volumetric flask to get the solvent free mark. The solvent free mark was then extracted with mixture of $CH_3OH/CHCl_3$ for 24 hours for three times and the resulting extract was evaporated under the reduced pressure using Rota vapor at 50 ^{0}C and found 42 g crude sample.

3.4.2 Isolation of seed of *Azadirachata indica*

Appropriate solvent was selected for elution after carrying on TLC analysis of the extracts. Accordingly petroleum ether combined with ethyl acetate different polarity was found to be suitable for elution of column. The column that eluted with petroleum ether and ethyl acetate combination increasing polarity summarized as in the following Table

Table 2. The solvent system for column elution during isolation of compounds.

Solvent system	Ratio by %	Fraction
PE	100	1-5
PE/EA	98:2	6-10
PE/EA	96:4	11-16
PE/EA	94:6	17-20
PE/EA	92:8	21-25
PE/EA	90:10	26-30
PE/EA	88:12	31-33
PE/EA	84:16	34-38
PE/EA	80:20	39-41
PE/EA	76:24	42-44
PE/EA	70:30	45-47
PE/EA	66:34	48-52
PE/EA	60:40	53-56
PE/EA	54:46	57-60
PE/EA	50:50	61-64
PE/EA	45:55	65-70
PE/EA	35:65	71-73
PE/EA	25:75	74-75
EA	100	76

Note: EA: Ethyl acetate and PE: petroleum ether

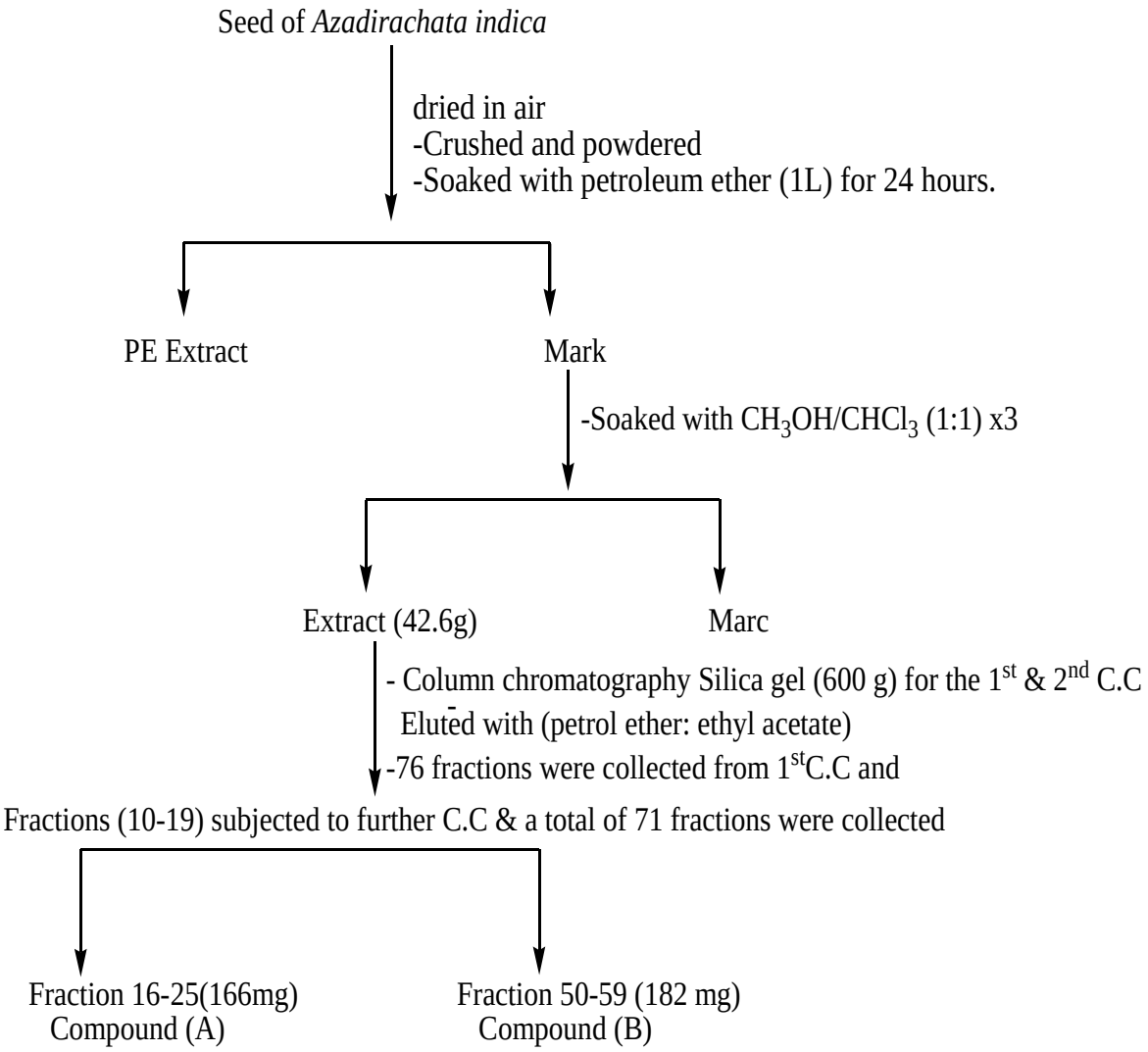
A total of 76 fractions were collected by increasing solvent polarity using PE/EA.

From first C.C the fractions 10-19 showed the same TLC profile and were combined and dried (18g). The combined fractions were then subjected to further purification on column chromatography over silica gel using petroleum ether in ethyl acetate as follows which found a total of 71 fractions and led to the isolation of two compounds based on their TLC profile. The isolated pure compounds obtained were identified using spectral (1H, 13C-NMR and DEPT-135) analyses.

Table 3. The solvent system for further column elution during isolation of compounds.

Solvent system	Ratio by %	Fraction	Compounds
PE	100%	1-10	
PE/EA	98:2	11-15	
PE/EA	96:4	16-20	Compound A (166 mg)
PE/EA	94:6	21-25	
PE/EA	92:8	26-30	
PE/EA	90:10	31-34	
PE/EA	88:12	35-39	
PE/EA	86:14	40-44	
PE/EA	84:16	45-49	
PE/EA	80:20	50-55	Compound B (186 mg)
PE/EA	78:22	56-59	
PE/EA	65:45	60-64	
PE/EA	50:50	65-67	
PE/EA	60:40	68-70	
EA	100	71	

The general scheme of the extraction and isolation of compounds.



Scheme 1: Method used to extract plant material

3.5. Procedures for Phytochemical analysis of MeOH /CHCl3 crude extract of *Azadirachata indica.*

Phytochemicals are compounds that occur naturally in plants. They contribute to the color, flavor and smell of plants. In addition, they form part of a plant's natural defense mechanism against diseases. Their therapeutic values to human health and disease prevention have been reported so far**.**

Test for alkaloids (Meyer's Test):- Two milliliter of crude extract solution of *Azadirachta indica* was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Meyer's reagent. The solution was observed for the presence of yellow precipitation [46]. This confirmed the presence of alkaloids.

Test for flavonoid:-Five milliliter of crude extract solution was treated with methanol solution. Then, the solution was warmed and metal magnesium was added. To this solution, 6 drops of concentrated hydrochloric acid was added and red colour was observed for flavonoids as indicated by the literature [47].

Test for saponins :- Two milliliter of crude extract was taken and treated with hot water and vigorously shaken for 30 sec. Thick forth was formed which confirmed the presence of saponins [48].

Test tannins: - Two milliliter of crude Extract was diluted with water and 3-4 drop of 10% ferric chloride solution was added. Appearance of the blue-green or black colour indicated the presence of phenol and tannins.

Test for polyphenol: - One milliliter of crude extract of plant was mixed with 1 ml water and 3 drops of 1 %(w/v) ferric chloride solution was added. Presence of polyphenol was confirmed from literature by appearance of greenish blue colour [46].

Test for terpenoids: - terpenoids detected by five milliliter of each extract was mixed with 2 ml chloroform. 3 ml of conc. sulfuric acid (H_2SO_4) was carefully added to form a layer. A reddish-brown coloration of the interface was formed to prove the presence of terpenoid.

Test for Quinononones: -One milliliter of crude extract of plant was dissolved in 2ml of CHCl3 and evaporated to dryness. Then 2ml of Conc H_2SO_4 was added for 2 minutes but no new colour was observed. This showed the absence of quinononones in the plant *Azadirachata indica.*

3.6. Evaluation of Antimicrobial activity.

The antimicrobial activity testes were carried out using *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis* for bacterial activity and *penicillium* and *Aspergillus fluves* for antifungal activity using standard procedure [49]. All bacterial and fungus cultures were first grown on agar plates at 37^0C for 24 hr prior to inoculation on to the nutrient agar. Few colonies (4-5) of similar morphology of the respective bacteria and fungus were transferred with a sterile inoculating loop, a nutrient broth liquid medium and this liquid culture was incubated until adequate growth of turbidity standard was obtained. The turbidity of the activity growing broth culture was adjusted with sterile saline solution to obtain turbidity optically comparable to that of 0.5 McFarland standards that was resulted in a suspension containing approximately $1.2x10^8$CFU/ml for the test strain. The respective bacterial and fungus culture was streaked on the Muller-Hinton agar Petri plates using a sterile swab to insure through coverage of the plates and a uniform thick lawn of growth following incubation. The 6 mm diameter sterile discs of what man N^o-3 paper was placed on the surface of the inoculated agar approximately at equal distance of corners in Petri plates in a 100mg /ml concentration that were prepared by dissolving 100 mg of crude and isolated compounds in 1ml of DMSO using micro pipette on the discs. After addition of test solution on the discs, it was allowed to diffuse for 5 minutes and the Petri plates then kept in an incubator at 32^0C for 24 and 72 hrs for bacteria and fungus respectively. The antimicrobial activity was evaluated after 24 and 72 hrs by measuring the diameter of growth inhibition surrounding the discs (in mm) using transparent ruler results were expressed as mean of test [50]. Gentamicine and Mancozeb were used as a standard for anti bacterial and antifungal agents respectively and DMSO as negative control. All the test strains were obtained from Biology department, Jimma University.

4. Result and Discussion

4.1. Extraction and purification data

The powdered material was first extracted with petroleum ether with intermittent agitation for 24 hrs. Then, the marc was successively extracted with mixture of methanol and chloroform in equal ratio. (1:1) for 3 rounds and found to be 42g crude extract. This crude organic extract was subjected to column chromatography, resulted in the identification of two known compounds azadiradione as compound A and epoxyazadiradione as compound B. These Compounds were identified on the basis of NMR spectroscopic data and comparing the result with reported value from literature as shown their TLC bellow.

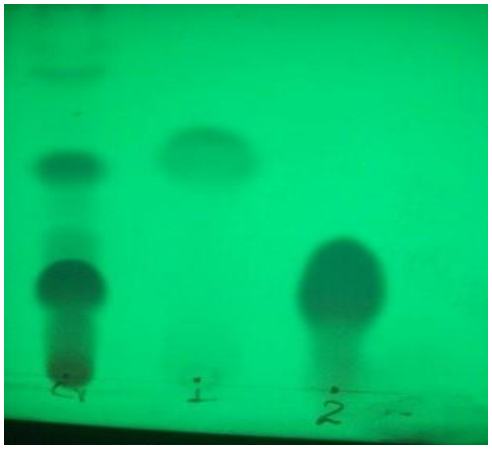


Figure 6.TLC for combined fractions (10-19), compound (1) as A and compound (2) as B

4.2. Phytochemical screening data

Phytochemical screening of *Azadirachata indica* showed the presence of most important phytochemicals. The medicinal value of *Azadirachata indica* can be correlated due to the presence of various bioactive chemical constituents (Table 4).The CH_3OH/CHCl3 (1:1) extract confirms the presence of terpenoides, saponins, tannins, alkaloids, flavonoids, polyphenol and show negative result to quinines as shown in the table below.

Table4: Qualitative analysis of phytochemicals in methanolic and chloroform extract of *Azadirachata indica* is shown bellow.

Components	Scoring
Alkaloids	+
Flavonoid	+
Saponin	+
Tannins	+
Polyphenol	+
Quinonones	—

Key:_ = not detected , + =detected

4.3. Spectral data and Characterization process

4.3.1. Characterization of Compound A

Compound **A** was obtained as a colorless solid mp 168-170^{o}C and *RF* valve 0.76 using petroleum ether and ethyl acetate (7:3) as a solvent. It showed a color change to violet when the TLC was sprayed with in iodine spray. The ^{1}H NMR spectrums (Appendix-1) of compound A , showed that a six methyl signals ^{1}H NMR(300Hz,$CDCl_3$) δ 0.99 (*s*, 3H), δ 1.27 (*s*, 3H), δ 1.05 (s, 3H), δ 1.14 (s, 3H), δ 1.30 (s, 3H) and δ 1.95 (s, 3H)) ppm indicating the presence of protons of aliphatic methyl groups , two one-proton doublets at δ 7.12 (*d*, 1H) ppm, and δ 5.85(*d*, 1H) ppm, due to H-1 and H-2 of 1-ene-3-one system of the ring, three one-protons multiplies at δ 6.24 (*m*, 1H), δ 7.54 (*m*, 1H), δ 7.43 (*m*, 1H) and two proton singlets at δ 5.89 (s, 1H) and δ 3.48 (s, 1H). The signal at (δ 3.48) was observed to be broadened showed that its allylic coupling with furanic protons. The result obtained here is comparable with ^{1}H NMR spectral data of azadiradione from literature.

Table5: Comparison of the observed ^{1}H NMR (400 MHz, $CDCl_3$) spectroscopic data of Compound **A** with the reported value of azadiradione [43, 44]

Position of H	Observed data (δ ^{1}H in ppm)	Reported data (δ in ppm)	Remark
1-H	7.12(*d*)	7.14(*d*), *J*=10.04	
2-H	5.85(*d*)	5.90(*d*) , *J*=10.04	
3	_	_	*Quat.*C
4	_	_	*Quat.*C
5-H	2.16(*m*)	2.21(*m*)	
6-2H	1.62-2.37(m)	1.79-2.06(m)	
7-H	5.29(*m*)	5.33(*m*)	
8	_	_	*Quat.*C
9-H	2.45(*m*)	2.50(*m*)	*Quat.*C
10	_	_	
11-2H	1.74-2.05(*m*)	1.79-2.06(*m*)	
12-2H	β 2.47(*m*), α1.55(m)	β2.56 (*m*), α1.57(*m*)	
13	_	_	*Quat.*C
14	_	_	*Quat.*C
15-H	5.85(*m*)	5.89(*s*)	
16	_	_	*Quat.*C
17-H	3.48(s)	3.43(*s*)	
18-Me	0.99(*s*)	1.04(*s*)	
19-Me	1.22(s)	1.26(s)	
20	_	_	*Quat.*C
21-H	7.54(m)	7.48(m)	
22-H	6.38(m)	6.28(m)	
23-H	7.39(*m*)	7.44(*m*)	
24-Me	1.05(*s*)	1.10(*s*)	
25Me	1.14(*s*)	1.11(*s*)	
26-Me	1.30(*s*)	1.35(*s*)	
27-Me	1.91(*s*)	1.96(*s*)	
28	_	_	*Quat.*C

Key: Quat.C= Quaternary Carbon

The ^{13}C NMR spectrum (Appendix-2) showed signals of six methyl C within the chemical shift values ranging δ_C 16-27. That is at (δ 26.42, C-18), (δ 18.99, C-19),(δ 26.96, C-24),(δ 21.26, C-25), (δ 26.24, C-26) and (δ 20.94, C-27), three carbonyl carbon oxygen double bond carbons at (δ 203.93, C-3), (δ 205.00, C-16) and (δ169.56, C-28), the acetate functional group observed δ 73.86, six methyl substituted carbon at (δ 44.02, C-4), (δ 44.52, C-8), (δ 39.96, C-10), (δ 47.95, C-13) and (δ 169.56, -$\underline{C}OCH_3$), and eight alkenes group at (δ 111.15, C-22), (δ 118.44, C-20), (δ 123.19 , C-15), (δ 156.83, C-1), (δ 125.83 , C-2) , (δ 192.41, C-14) ,(δ 142.71,C-23) and(δ 141.59, C-21).

In DEPT spectrum (Appendix-3) of compound **A**, the presence of six methyl proton at (δ 26.24, C-26), (δ 18.99, C-19), (δ 21.26, C-25), (δ 20.94, C-27), (δ 26.42, C-18), (δ 26.96, C-24), ten CH signals at (δ 156.83, C-1), (δ 125.83, C-2), (δ 46.06, C-5), (δ 73.86, C-9), (δ 123.19, C-15), (δ 60.66, C-17), (δ 141.59, C-21), (δ 111.15, C-22), (δ 142.71, C-23),(δ 38.13,C-9) and, three methylene (-CH_2) group at (δ 15.75, C-11), (δ 23.41, C-6) and (δ 30.23 C-12) and nine quaternary carbon atoms were observed.

Table6: Comparisons of observed ^{13}C NMR and DEPT data of Compound **A** with the reported value of Azadiradione [51, 52]

C-No	Observed δ-in ppm	Reported δ-in ppm	DEPT-135 δ in ppm	Remark
C-1	156.83	156.67	156.83	CH
C-2	125.83	125.96	125.83	CH
C-3	203.93	203.96	-	C
C-4	44.02	40.09	-	C
C-5	46.06	46.15	46.06	CH
C-6	23.23	23.46	23.23	CH_2
C-7	73.86	73.92	73.86	CH
C-8	44.52	44.56	-	C
C-9	38.13	38.23	38.13	CH
C-10	39.96	40.01	-	C
C-11	15.75	15.83	15.75	CH_2
C-12	30.23	30.36	30.23	CH_2
C13	47.95	47.97	-	C
C-14	192..41	192.24	-	C
C-15	123.19	123.33	123.19	CH
C-16	205.00	204.96	-	C
C-17	60.66	60.76	60.66	CH
C-18	26.42	26.45	26.42	CH_3
C-19	18.99	19.02	19.00	CH_3
C-20	118.44	118.44	-	C
C-21	141.59	141.67	141.59	CH
C-22	111.15	111.13	111.15	CH
C-23	142.71	142.80	142.71	CH
C-24	26.96	27.00	26.96	CH_3
C-25	21.26	21.28	21.26	CH_3
C-26	19.37	19.39	19.37	CH_3
C-27	20.94	20.95	20.94	CH_3
C-28	169.56	169.58	-	C

Based on the above ^{1}H NMR, ^{13}C NMR and DEPT-135 spectral data, the proposed structure of compound **A** was identified as azadiradione.

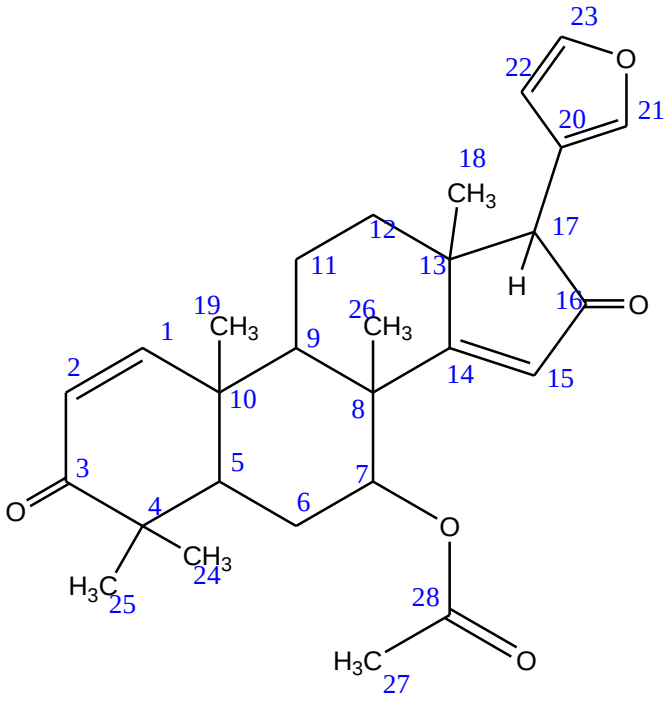


Figure 7: Proposed Structure of Compound 21

4.3.2. Characterization of Compound B

Compound B was also obtained as a colorless solid with mp 187-189 ^{o}C and it has RF value of 0.42 using 7:3 petroleum ether and ethyl acetate. It also showed a color change to violet up on spraying the TLC with iodine. The NMR spectra of compound B are closely related to that of compound A with the only notable difference being the epoxidation of the C-14/C-15 double bond in the case of compound B. In line with this, the ^{1}H NMR spectrum (Appendix 4) of compound B showed that a six methyl signals^{1}H NMR (300Hz,$CDCl_3$) at δ 1.03 (*s*, 3H), δ 1.25 (*s*, 3H) ,δ 1.04 (*s*, 3H), δ 1.08 (*s*, 3H) ,δ 1.21 (*s*, 3H) and δ 2.08 (*s*, 3H) ppm , two one-proton doublets at δ 7.16 (d, 1H) and δ 5.85(d, 1H) ppm attributed to H-1 and H-2 of 1-ene-3-one system, three one-protons multiplies at δ 6.24(*m*, 1H) , δ 7.52(*m*, 1H), δ 7.39(*m*, 1H). The signal at δ 3.38 was broadened showed its allylic coupling with furanic protons. The result obtained here is comparable with ^{1}H NMR spectral data of 14, 15-epoxy-azadiradione in the literature.

Table7: Comparison of the observed ^{1}H NMR (400 MHz, $CDCl_3$) spectroscopic data of Compound **B** with the reported value of epoxyazadiradione [51, 52].

Position	Observed (δ in ppm)	Reported (δ in ppm)	Remark
1	7.16 (d)	7.11, *d J*=10.38,	
2	5.85 (d)	5.87 (d) ,*J*=10.07	
3	_	_	*Quat.*C
4	_	_	*Quat.*C
5	2.17 (m)	2.18 (*m*)	
6	1.91 (m) , 2.03 (*m*)	1.90 (*m*) , 2.03 (*m*)	
7	4.69 (m)	5.31 (m)	
8	_	_	*Quat.*C
9	2.610 (m)	2.49 (m)	
10	_	_	*Quat.*C
11	2.57 (m) , 1.91 (m)	H α 2.53 (m), H β 1.90 (m)	
12	2.47(*dd*), 1.55(*dd*)	2.48(*dd*), 1.65(*dd*)	
13	_	_	*Quat.*C
14	_	_	*Quat.*C
15	3.37 (m)	3.41 (m)	
16	_	_	*Quat.*C
17	3.85 (s)	4.31 (s)	
18	1.03 (s)	0.99 (s)	
19	1.25 (s)	1.29 (s)	
20	_	_	*Quat.*C
21	7.52 (m)	7.46 (m)	
22	6.24 (m)	6.27 (m)	
23	7.39 (m)	7.40 (m)	
24	1.04 (s)	1.07 (s)	
25	1.08 (s)	1.08 (s)	
26	1.21 (s)	1.48 (s)	
27	2.08 (s)	1.94 (s)	
28	_	_	*Quat.*C

Key: Quat.C= Quaternary Carbon

The ^{13}C NMR spectrum (Appendix-5) also showed signals for six methyl carbon at (δ 24.82, C-18), (δ 19.81, C-19), (δ 27.02, C-24), (δ 20.99, C-25), (δ 19.37, C-26) and (δ 21.25, C-27), three carbonyl carbon-oxygen double bond carbon atoms at (δ 204.11, C-3), (δ 208.38, C-16) and (169.70, -COCH$_3$, and six alkenes group at (δ 110.91, C-22), (δ 116.56, C-20), (δ 125.70, C-2), (δ 141.5, C-21), (δ 142.39, C-23) and (157.51, C-1). The spectra of compound B exhibited the presence of ester group at δ 169.70 and signals of two carbon-oxygen single bond both at δ 72.55, for C-14 and 15 that is due to epoxy group. Similarly, the DEPT spectrum (Appendix-5) of compound B indicated the presence of six methyl at (*δ* 19.37, C-26), (*δ* 19.81, C-19), (*δ* 20.99, C-25), (*δ* 21.25, -COCH3), (*δ* 24.82, C-18), (*δ* 27.02, C-24), ten CH signals at (*δ* 157.51, C-1), (*δ* 125.70, C-2), (*δ* 46.63, C-5), (*δ* 73.60, C-7), (*δ* 39.63, C-9), (*δ* 116.56, C-15), (*δ* 57.16, C-17), (*δ* 141.50, C-21), (*δ* 110.91, C-22), and (*δ* 142.39, C-23) and, three methylene (-CH$_2$) groups at (*δ* 16.06, C-11), (*δ* 24.19, C-6) and (*δ* 29.07, C-12) and nine quaternary carbon.

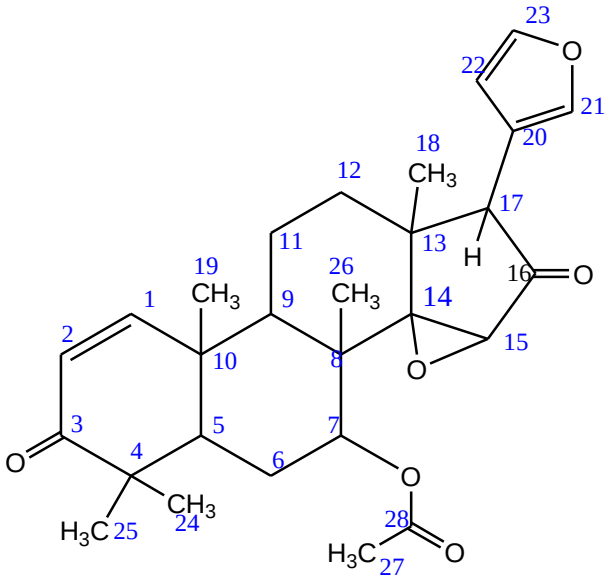


Figure 8: Proposed structure of Compound (B)

Table8: Comparison of observed ^{13}C NMR and DEPT spectroscopic data of Compound B with the reported value of epoxyazadiradione [51, 52]

Position of C	Observed NMR	Reported NMR	DEPT 135	Remark
C-1	157.51	157.47	157.50	CH
C-2	125.70	125.76	125.71	CH
3	204.11	204.22	-	*Quat.C*
4	44.20	44.22	-	*Quat.C*
5	46.63	46.67	46.64	-CH
6	24.19	24.20	24.19	-CH2
7	73.60	73.63	73.60	-CH
8	43.11	43.12	-	*Quat.C*
9	39.63	39.69	39.67	-CH
10	39.67	39.65	-	*Quat.C*
11	16.04	16.06	16.04	$-CH_2$
12	29.07	29.09	29.07	$-CH_2$
13	42.52	42.53	-	*Quat.C*
14	72.55	72.54	-	*Quat.C*
15	57.16	57.18	57.17	-CH
16	208.38	208.4	-	*Quat.C*
17	50.92	50.94	50.92	-CH
18	24.82	24.82	24.80	$-CH_3$
19	19.81	19.82	19.80	$-CH_3$
20	116.56	116.51	-	*Quat.C*
21	141.50	141.53	141.55	-CH
22	110.91	110.87	110.91	-CH
23	142.39	142.43	142.38	-CH
24	27.02	27.02	27.02	$-CH_3$
25	20.99	20.99	20.98	$-CH_3$
26	19.37	19.39	19.37	$-CH_3$
27	21.25	21.23	21.25	$-CH_3$
28	169.70	169.74	-	*Quat.C*

Key: Quat.C= Quaternary Carbon

Based on the above ^{1}H and ^{13}C NMR spectral data, compound **B** was identified as epoxy derivative of compound **A** and assigned the name, 14, 15-epoxyazadiradione with its structure shown above

4.4. Evaluation of Antimicrobial activities for the isolated compounds and its crude seed and leaves of *Azadirachata indica*

Two isolated compounds Azadiradione, Epoxyazadiradione, seed and leafe were evaluated for their effect on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus cereus* for antibacterial activity and penicillium and Aspergillus flavus for antifungal activity by disc diffusion method. The growth inhibition zones (in mm) of compounds and crude extracts were given in (table 7 and 8). The result indicated that the crude extract of leaves and seeds of *Azadirachata indica* is found to be more active on *Escherichia coli* and no inhibition zone is observed for crude extract of leave on *Bacillus cereus* of bacterial species. However, show the same result for antifungal activity on Aspergillus flavus. Additionally compound A found to be more active on *Staphylococcus aureus* (15 mm), *Pseudomonas aeruginosa* (10 mm) and moderately active on *Escherichia coli* and *Bacillus subtilis* (7 mm) for each. However, compound A and B show similar activity for antifungal on Aspergillus flavus (10mm) for each and moderately active on penicillin (7 mm) for each (Appendix 1.7 and 1.8).

Table 9: Results of antimicrobial activities of crude extracts (100 mg/ml) and isolated compounds (100 g/ml).

	Diameter of zone of inhibition in mm					
Bacterial strain	A	B	crude seed	crude leave	Gentamicine	DMSO
S.aureus	15	7	10	7	25	NI
E. coli	7	10	15	20	29	NI
P.aeruginosa	10	5	10	10	17	NI
B. Cereus	7	7	10	NI	19	NI

NI= No inhibition

Compound **A** showed comparatively higher antibacterial activity than compound **B** on the tasted bacterial strain (Appendix 1.7) The most susceptible organisms are S.aureus

and E.coli where as B.subtilis was found to be most resistant bacteria against compound A and B.

Table10. Results of antifungal activities of crude extracts and isolated compounds in zone of inhibition in mm.

	Diameter of zone of inhibition in mm					
Fungal strain	A	B	Crude seed	Crude leave	Mancozeb	DMSO
penicillium spp	7	7	10	NI	20	NI
Aspergillus Flaves	10	10	13	NI	15	NI

From the above table both compound A and B showed the same antifungal activity on the tasted fungal strain (appendix 1.8). Aspergillus flavus was easily susceptible for crude seed fungal activity. Whereas both as both penicillum spp and Aspergillus flavus were found to be more resistant fungal against the crude leave or no inhibition zone is observed.

The overall results of this study provide evidence that *Azadirachata indica* seed and leave extract as well as isolated compounds exhibit anti fungal and antibacterial activity for both gram negative and gram positive pathogens.

4.5. Spectral data of the isolated compounds

Compound (A): ^{1}H NMR (400 MHz, $CDCl_3$); δ(ppm): 7.53 (1H, *m*, H-21), 7.43 (1H, *m*, H-23), 7.12 (1H, *d*, *J* = 10.18Hz, H-1), 6.24 (1H, *m*, H-22), 5.85 (1H, *d*, *J*=10.18 Hz, H-2), 5.83 (1H, *s*, H-15), 5.29 (1H, *m*, H-7), 3.48 (1H, *s*, H-17), 2.47 (1H, *m*, H-9), 2.45 (1H, *m*, H-5), 1.95 (3H, *s*, -CO$\underline{C}H_3$), 1.30 (3H, *s*, H-26), 1.27 (3H, *s*, H-19), 1.14 (3H, *s*, H-25), 1.05 (3H, *s*, H-24), 0.99 (3H, *s*, H-18). ^{13}C NMR (100 MHz, $CDCl_3$); δ (ppm): 205.00 (C-16), 203.93 (C-3), 192.41 (C-14), 169.56 (C-$\underline{C}OCH_3$), 156.83 (C-1), 142.71 (C-23), 141.59 (C-21), 125.83 (C-2), 123.19 (C-15), 118.44 (C-20), 111.15 (C-22), 73.86

(C-7), 60.66 (C-17), 47.95 (C-13), 46.06 (C-5), 44.52 (C-8), 44.02 (C-4), 39.96 (C-10), 38.13 (C-9), 30.23 (C-12), 26.96 (C-24), 26.42 (C-18), 26.24 (C-26), 23.41 (C-6), 21.26 (C-25), 20.94 (C-CO<u>C</u>H_3), 18.99 (C-19), 15.83 (C-11).

Compound (B): ^{1}H NMR (400 MHz, CDCl3); δ (ppm): 7.52 (1H, *m*, H-21), 7.39 (1H, *m*, H-23), 7.16 (1H, *d*, *J*=10.04 Hz, H-1), 6.24 (1H, *m*, H-22), 5.85 (1H, *d*, *J*=10.04 Hz, H-2), 4.69 (1H, *m*, H-7), 3.86(1H, *s*, H-17), 3.37 (1H, *s*, H-15), 2.61 (1H, *m*, H-9), 2.17 (2H, *s* H-5), 3.85 (*d*)-7.36 (d) H-12), 2.03 (3H, *s*, -CO<u>C</u>H3), 1.25 (3H, *s*, H-19), 1.21 (3H, *s*, H-26), 1.08 (3H, *s*, H-25), 1.04 (3H, *s*, H-24), 1.03 (3H, *s*, H-18). ^{13}C NMR (100 MHz, CDCl3); δ(ppm**):** 208.38 (C-16), 204.11 (C-3), 169.70 (-<u>C</u>OCH_3), 157.51 (C-1), 142.39 (C-23), 141.50 (C-21), 125.70 (C-2), 116.56 (C-20), 110.91 (C-22), 73.60 (C-7), 72.55 (C-14), 57.16 (C-15), 50.92 (C-17), 46.63 (C-5), 44.20 (C-4), 43.11 (C-8), 42.52 (C-13), 39.67 (C-9), 39.63 (C-10), 29.07 (C-12), 27.02 (C-24), 24.82 (C-18), 24.19 (C-6), 21.25 (C-CO<u>C</u>H_3), 20.99 (C-25), 19.81 (C-19), 19.37 (C-26), 16.04 (C-11).

5. Conclusion and Recommendation.

5.1 Conclusion

Two triterpenoids were isolated and characterized from the seed of *Azadirachata indica***.** The petroleum ether-ethyl acetate (96:4) affords 166 mg of compound A a known triterpenoids azadiradione. The petroleum ether-ethyl acetate (80:20) gives 186 mg of the second triterpenoids, compound **B** as epoxy derivative of compound **A** and assigned the name, 14, 15-epoxyazadiradione. These two Compounds are identified by comparing their 1H NMR, 13C NMR and DEPT-135 spectroscopic data for similar compound from literature. The two compounds have different Rf value indicating that compound **B** is more polar than compound **A** .The antibacterial activity test result showed that the crude leave and seed extracts are potentially active specially against E. coli but the crude leave extract is less active for antifungal activity. Whereas compound **A** is potentially active spatially against S. aureus than compound **B** .However, both the two compounds have similar effect for antifungal activity. Thus, the observed antimicrobial activities of the crude extract and the isolated compounds could justify the traditional use of the plant for treatment of different bacterial and fungal infections. The observed antimicrobial activity of the isolated compounds as hit compound in the development of antimicrobial drugs. However, further tests are recommended on large number of bacterial and fungal strains to decide their potential as candidates in development of antimicrobial drugs.

5.2 Recommendation

- Further study on chemical compotation including the usable seed of *Azadirachata indica* for future use is important for the development and production of new drugs.
- Further purification should be carried out on the seed part of *Azadirachata indica.*
- Absolute configuration of the isolated compounds should be established.
- The biological activity should be also done on another bacterial and fungal strain.

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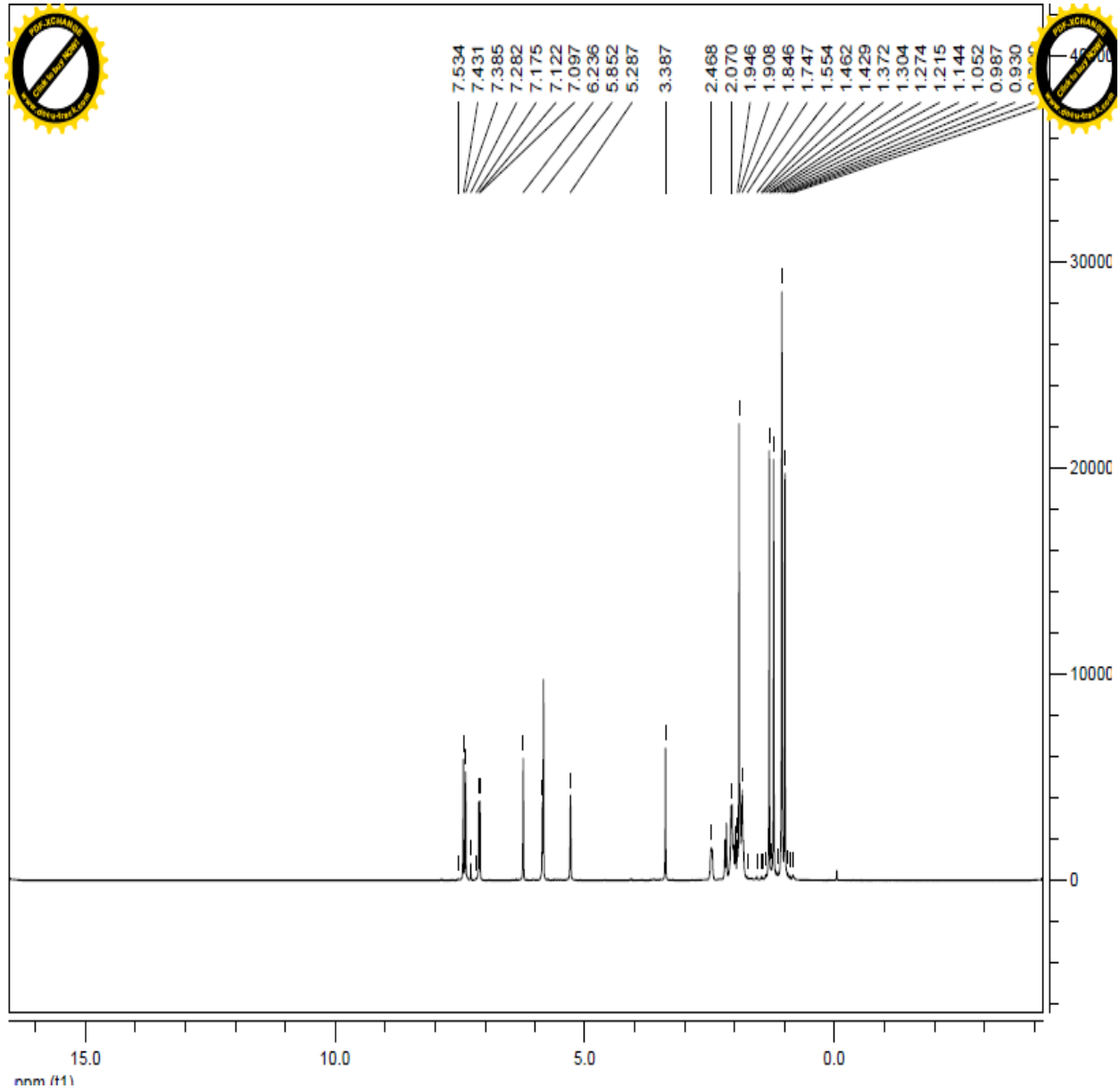
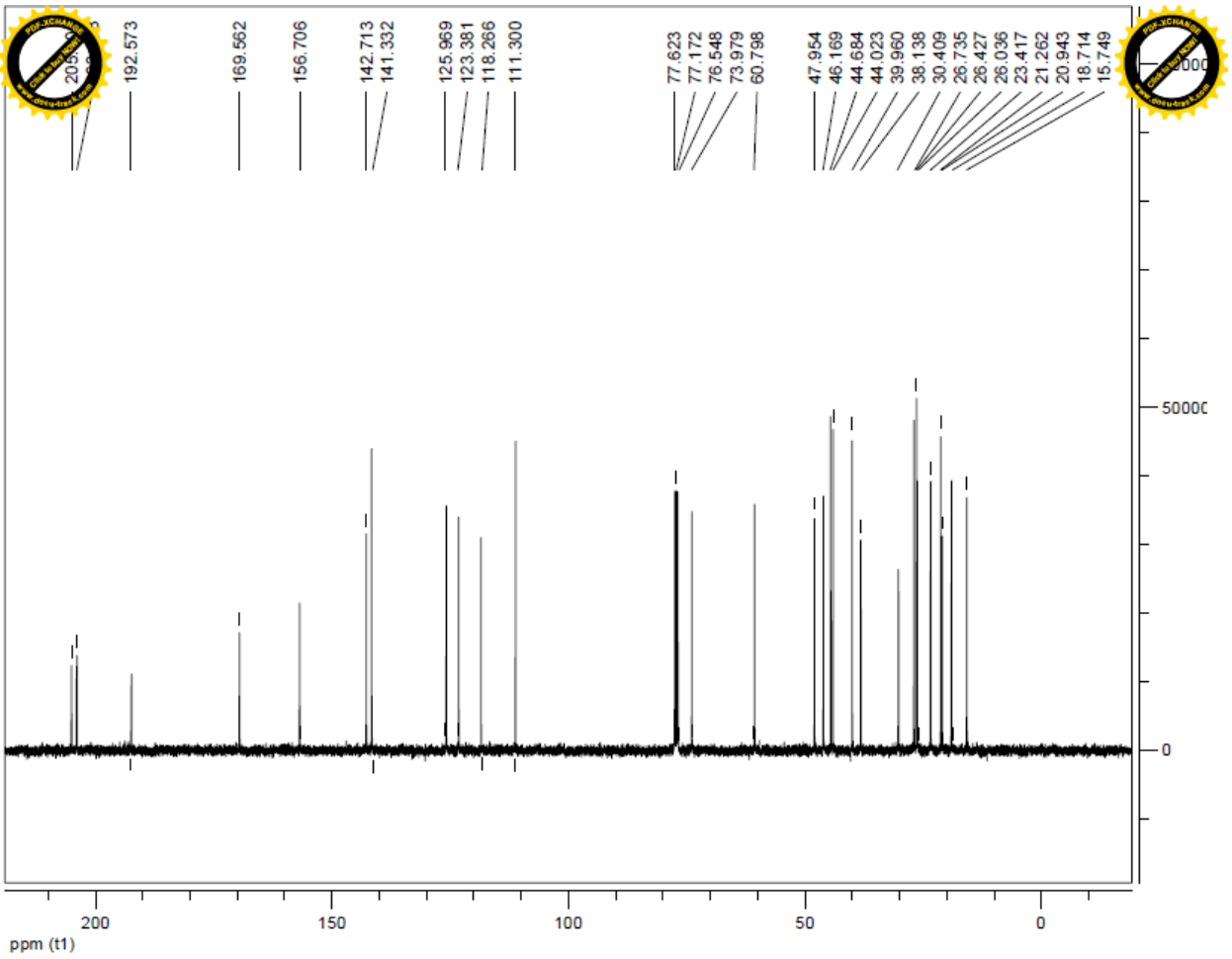
Appendix

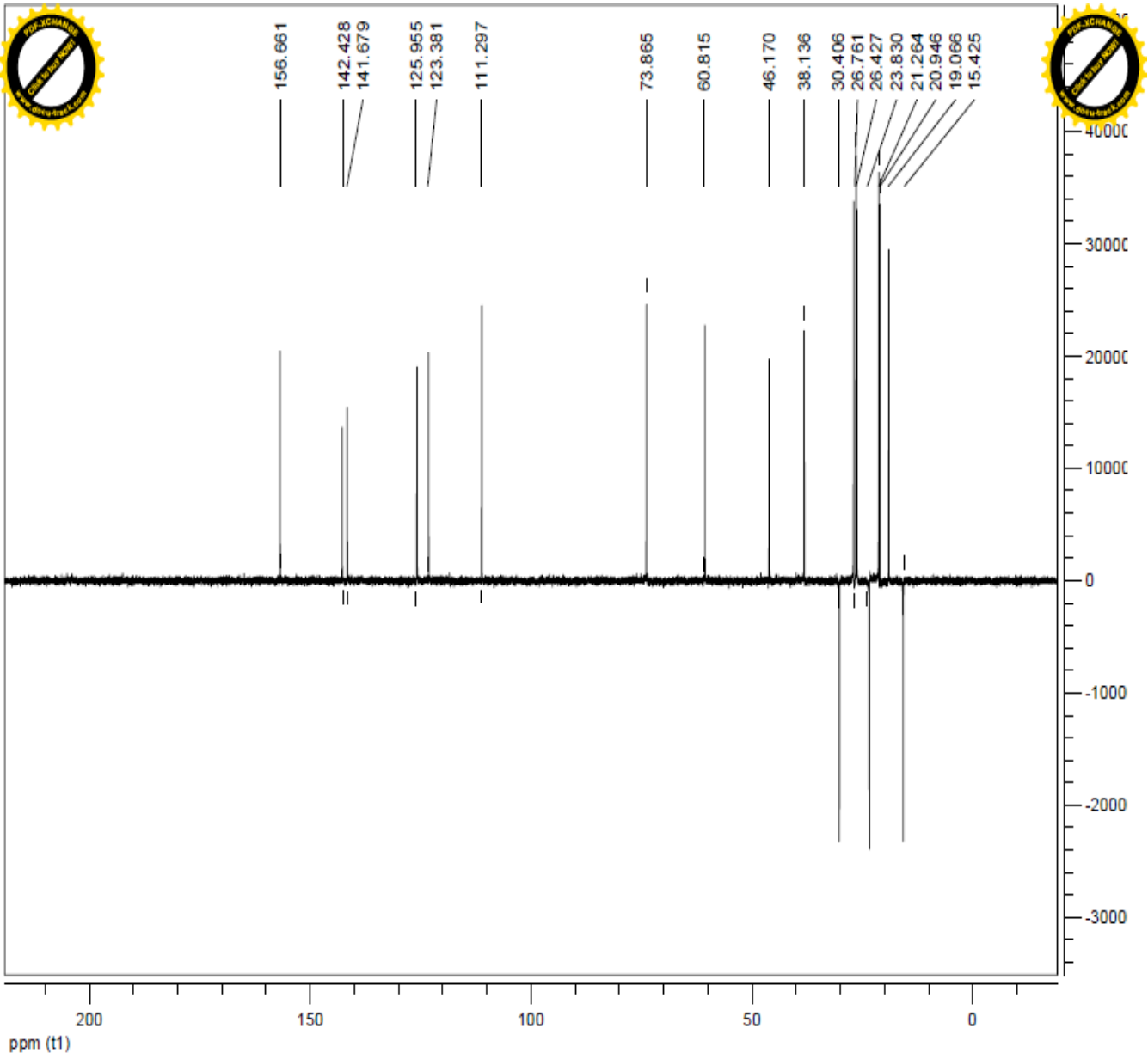
Appendix 1: ^{1} HNMR for compound A



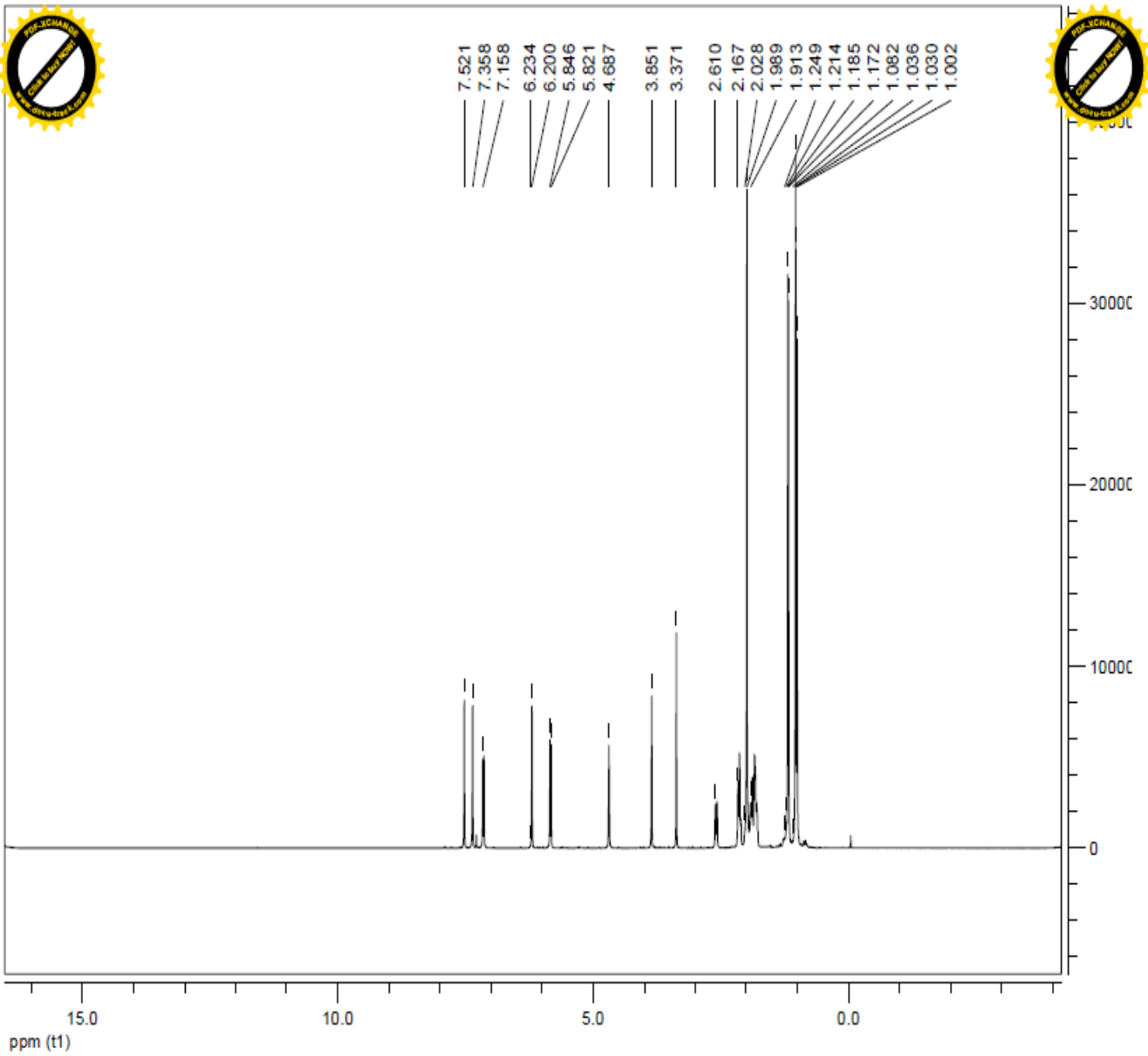
Appendix 2: ^{13}C NMR for compound A



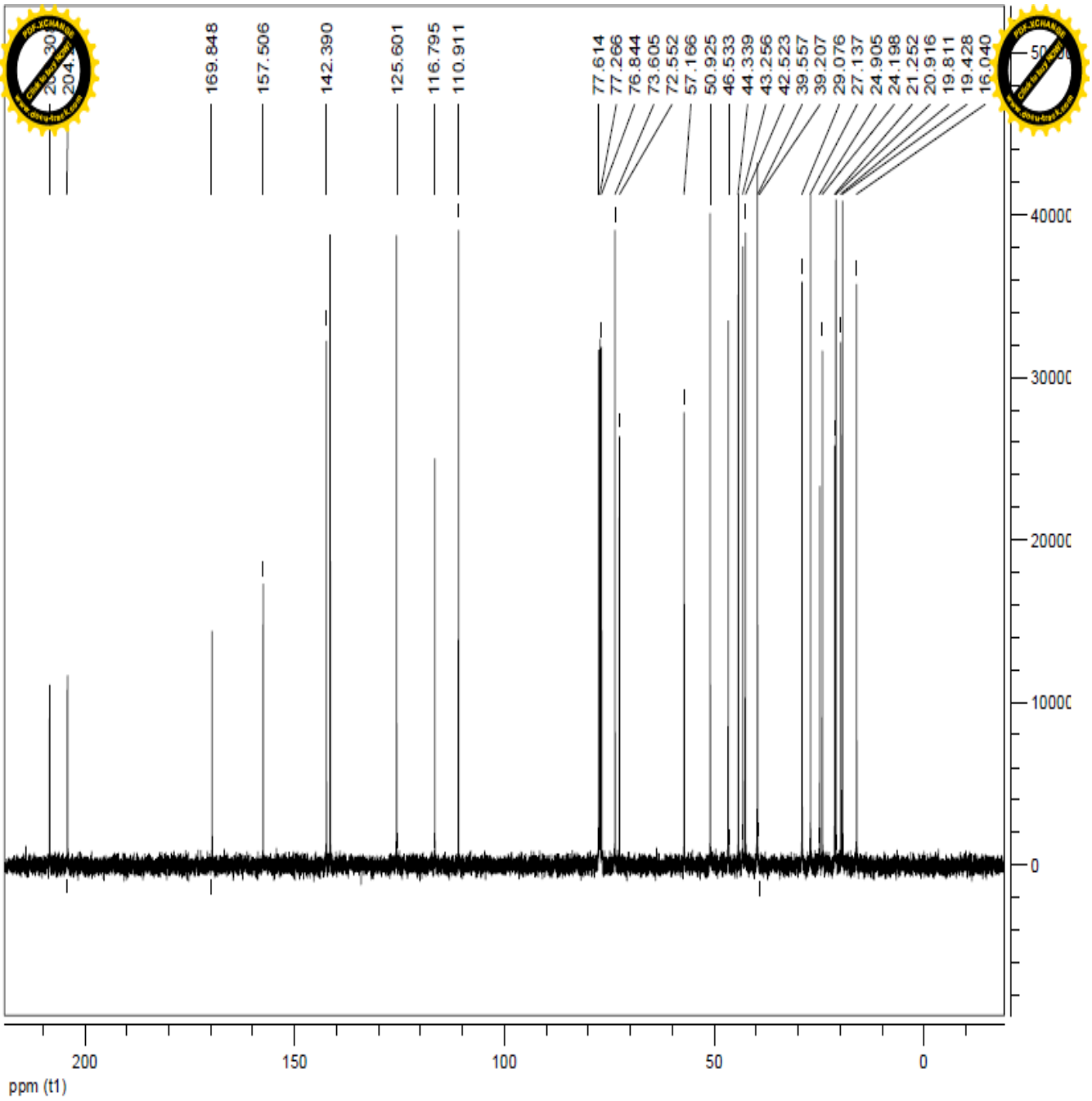
Appendix 3: DEPT-135 for compound A



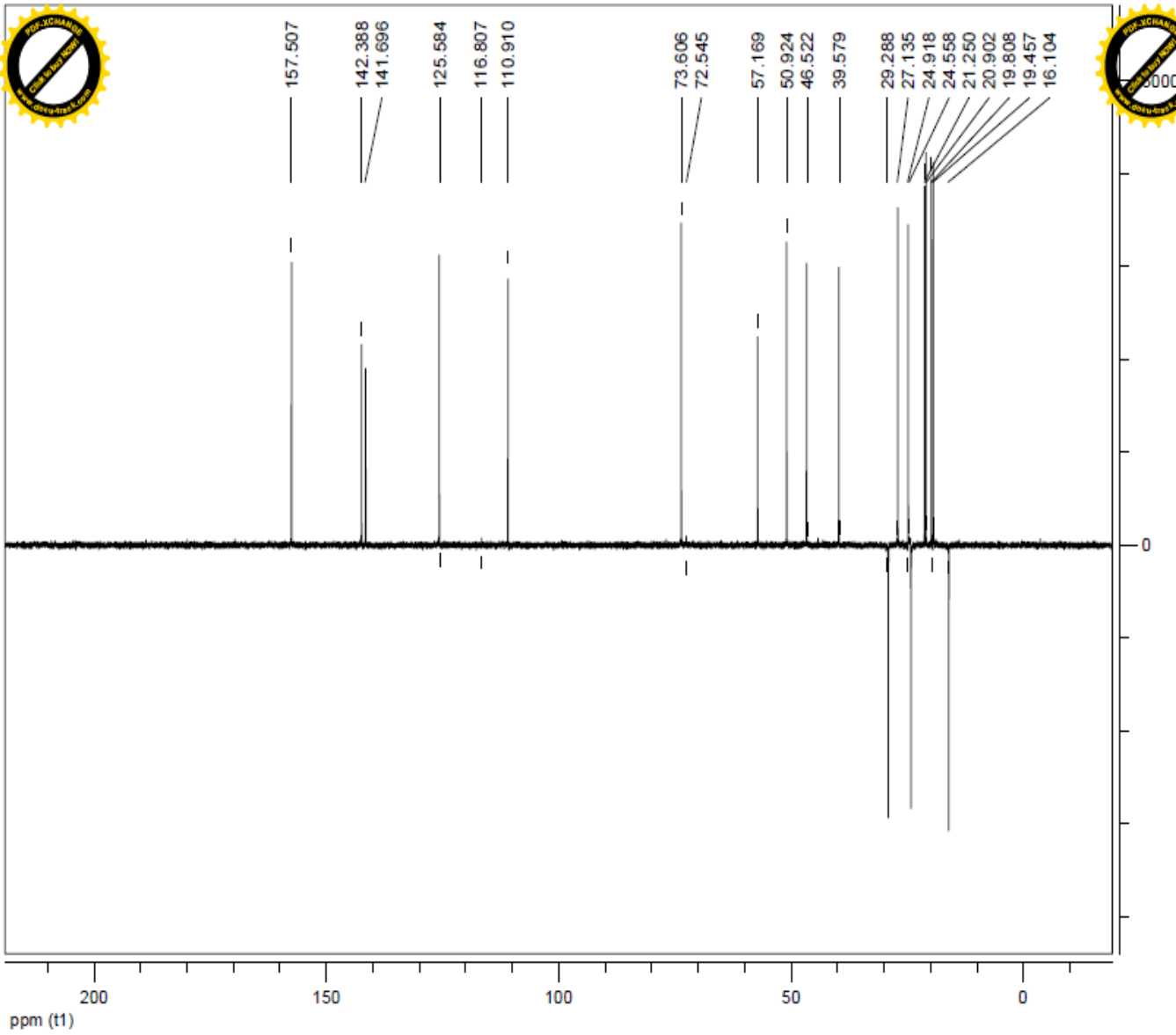
Appendix4: ^{1} H NMR for compound B



Appendix 5: ^{13}C- NMR for compound B

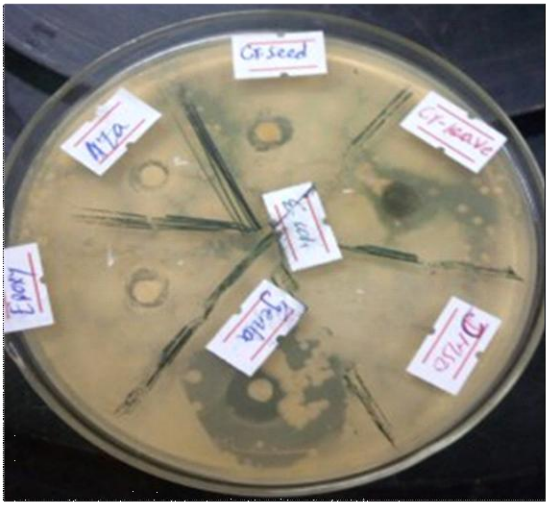


Appendix 6: DEPT-135 for compound B



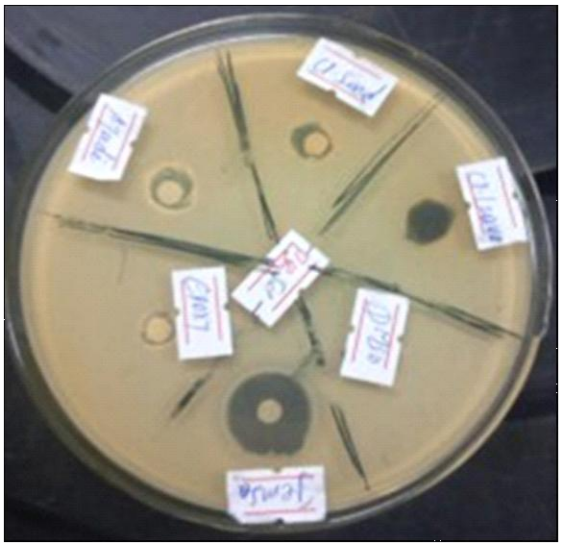
Appendix 7: Zone inhibition of compound A, B, crude seed and leave extracts on 4 bacterial strains

S.aureus



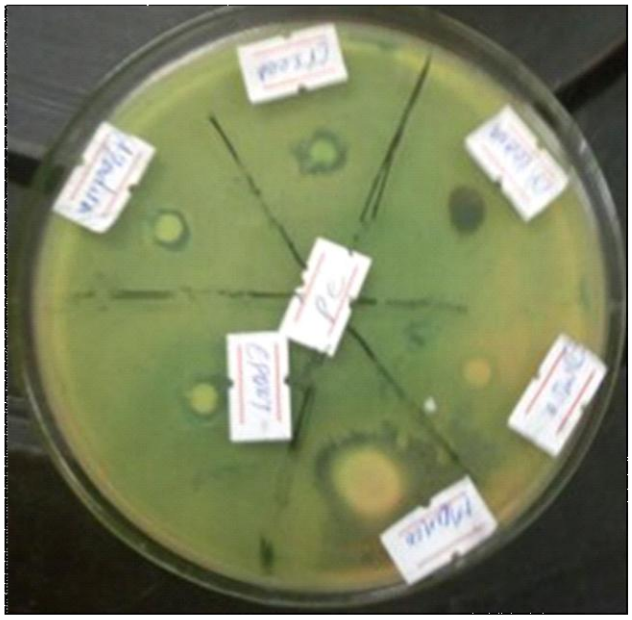
E.coli

P.aeruginosa

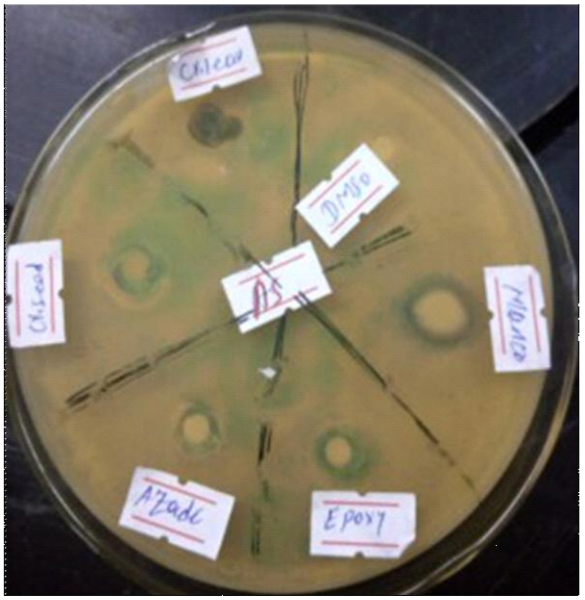


B.subtilis

Appendix8: Zone inhibition of compound A, B, crude seed and leave extracts on 2 fungal strains



Penicillium



Aspergillus flaves