GCMS, FTIR and NMR analysis of chloroform extract of *Azadirachtaindica*

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Abstract

Azadirachtaindica, tree of Meliaceae family contains a flexible source of bioactive compounds for controlling plant diseases. But nowadays, people have become more aware of the dangerous side effects of these chemicals, so using biocontrol agents is becoming more and more important. Researchers have started to focus on using plants and microbes as a biocontrol agent as the introduction of some ecofriendly and secure alternative control tactics for agriculture is currently a key problem in the field of plant pathology.It is well known for its therapeutic and ethnomedicinal relevance since it has a variety of pharmacological effects. The goal of the ongoing study is to use GC-MS, FTIR, and NMR investigation to identify the chemical components of *Azadirachtaindica*chloroform extract. The Soxhletmethod was used forextraction process. To isolate and identify the antibacterial fraction from *Azadirachtaindica* chloroform extract, TLC-bioautography was used. Phenol 3,5-bis (1,1-dimethyl ethyl), Phthalic acid bis (7-methyloclyl), Dido decyl phthalates, Oxalic acid, allylhexadecyl ester, and 2-Piperidinone, N-(4-bromo-n-butyl) were the five primary antibacterial chemicals identified by the GC-MS study. The findings of the FTIR study revealed the presence of functional groups C-H str, C=O str, and C=C str as well as alcohol and the carboxylate ion. While the ^{13}C NMR data demonstrated the existence of carbonyl, aromatic carbon, quaternary carbon, olefinic carbon, and methyl group, and the ^{1}H NMR results revealed the presence of aliphatic OH, methyl, aromatic OH, and olefinic proton. The phytoconstituents found using GC-MS analysis showed a wide range of pharmacological properties, including antioxidant, antibacterial, antifungal, anti-inflammatory, and antimalarial effects. Thus, Azadirachta*indica* plant has a high concentration of medicinal chemicals constituents that can be used to make antimicrobial medications to fight against plant pathogenic bacteria.

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Keywords: *Azadirachtaindica*, TLC-bioautography, GC-MS, FTIR, NMR.

Introduction

Secondary metabolites are the broad category of organic chemicals that are created by the plants, many of which don't seem to directly contribute to growth and development (Hartmann, 1991). The unique source of supply that these secondary metabolites provide for medications, food additives, flavour, and other industrial materials is crucial (Zhao et al. 2005). These substances are a very diverse set of organic materials produced by a wide range of organisms, including plants, fungi, bacteria, algae, and mammals. The lack of secondary metabolites has no detrimental effects on a plant's ability to survive, but it does have a significant impact on its defence mechanisms (Stamp and Nancy, 2003). It has been estimated that, between 14 and 28 percent of higher plant species are used for medicinal purposes, and from that 74% of plant species are utilised pharmacologically, based on research into the ethnomedical uses of the plants (Ncube et al. 2008). Many therapeutic benefits are demonstrated by the diverse array of phytochemicals that medicinal plants produce, including phenolic acids, flavonoids, tannins, and other substances (El-Najjar et al. 2007). Terpenes, phenolics, and chemicals with nitrogen are the three primary categories of secondary metabolites. In this, phenolics include phenolic acids, coumarins, flavonoids, tannins, and lignin, while nitrogen-containing chemicals include alkaloids and glycosylates. Terpenes include plant volatiles, cardiac glycosides, carotenoids, and sterols.

Many efforts have been made to find novel antimicrobial chemicals from a variety of sources, including microorganisms, animals, and plants. Plants now defend themselves against microbial infection and degeneration because of the discovery to novel, powerful antibacterial chemicals from plants (Cowan, 1999). To prevent plant pathogenic infections, there is increased interest in employing natural antibacterial chemicals, particularly those obtained from plants. As a result of the increased public knowledge of the dangerous side effects of chemicals, the employment of biocontrol agents is becoming increasingly important. Researchers have started to focus on using plants and microbes as a biocontrol agent due to the introduction of some ecofriendly and secure alternative control tactics in agriculture, which is currently a key problem in the field of plant pathology.

The plant *Azadirachtaindica*, also known as neem, belongs to the Meliaceae family and has already risen to the top of the list of potential biocontrol agents. Many pharmacological activities, including antibacterial, antifungal, antiulcer, antifeedant, repellant, pesticide, inhibitor, and sterilant, have been linked to the neem leaf, bark, fruit, stem, and flower. The presence of bioactive chemicals makes it commercially viable and

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2. What constitutes the problem to plant pathology in this statement?

enables it to be employed against a variety of plant diseases historically. Due to the plant's medicinal and bioactive properties, researchers from around the world are interested in studying its bioactive components (Luo et al. 1999). It has been claimed that bioactive substances derived from natural sources can be used to diagnosis a variety of diseases (Hamoburger and Hostettmann, 1991).The general screening of plants to find those with bioactivity against pathogenic organisms serves as the first step in the isolation and characterization of bioactive chemicals (Oyewale et al. 2004).

Alkaloids, tannin, flavonoids, phenolic compounds, dicarboxylic acid, and plasticizer-type chemicals are among the bioactive substances mostly found in *Azadirachtaindica* (Hill, 1985).These groups of substances exhibit a variety of pharmacological properties, including antimicrobial and antimalarial activity (Elaiyaraja and Chandramohan, 2016), antioxidant activity (Ajayi et al., 2011), anti-inflammatory activity (Chandrashekar et al., 2015), antifouling activity (Kumari et al., 2012), anti-hypersensitive activity (Mallika Devi et al., 2012), cancer (Lee et al. 2000).

Thus, the aim of this paper is to report on the isolation and partial characterization of a bioactive antimicrobial compound from chloroform extract of *Azadirachtaindica* using GCMS, FT-IR and NMR (^{1}H and ^{13}C).

Materials and methods

Preparation of plant extract

The fresh leaves of *Azadirachtaindica* were collected and thoroughly washed under running tap water to get rid of dirt and other contaminants. The leaves were ground into a powder using a grinder, then stored in an airtight container for later use. The leaves were dried individually under shade with occasional shifting for around 3 to 4 weeks. The Soxhlet's method proceduregiven by Gupta et al. 2013, was carried out for extraction of leaves. Following extraction, the supernatant was collected in the flask individually by filtering it usingWhatman No. 1 filter paper and allowed to evaporate at room temperature. Air dried extracts were weighed separately and kept in little tubesat 5 °C in the refrigerator.

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Thin Layer Chromatography

To determine the chemical composition of *Azadirachtaindica* leaf chloroform extract, thin layer chromatography was used. The TLC plates were made by combining 25 g of silica gel-G (Hi media) with 50 ml of distilled water, and then using a spreader to evenly spread the resulting slurry across the plates with a thickness of 0.25 mm. The plates were

heated in an oven at 110 ˚ C for one hour after being allowed to dry at room temperature. With the aid of capillary tubes, a 10-l sample of the *Azadirachtaindica* chloroform extract was put on TLC plates at identical distances after being diluted in DMSO. The TLC plate was retained and allowed to run until it reached the 3/4 position in the hexane: ethyl acetate (1:1) solvent system. The produced chromatogram on the TLC plates was examined with visible and ultraviolet light after being allowed to air dry. The distance travelled by the solvent front and the solute front was used to calculate the bands'Rf values (Relative front).

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Bioautography

According to Ahmed and Beg 2001, the bioautography approach was used to isolate the bioactive chemicals. On TLC plates, the zone of inhibition was visible as a transparent spot on a red background. The position of the compound exhibiting antibacterial activity was confirmed using bioautographytechnique on the TLC plate. Later the antibacterial fraction was scraped out from the silica geland was thoroughly dissolved in chloroform. It was then centrifuged for 10 minutes at 10,000 rpm. For complete solvent evaporation, the supernatant was evaporated at 60°C for 50 min using a vacuum concentrator.

FT-IR, GC-MS, ^{1}H and ^{13}C NMR analysis

Nuclear Magnetic Resonance (NMR), Gas Chromatography-Mass Spectrometry (GC-MS), and Fourier Transform Infrared (FT-IR) spectroscopy (FT-IR) techniques were used to conduct additional study on the purified chemical compound. For this analysis, separate 70 mg active compound was stored in tiny, sterile glass vials. The samples were sent to the Sophisticated Analytical Facility, IIT, Powai, Bombay, for chemical analysis using FT-IR, GC-MS, and ^{1}H and ^{13}C NMR. Tables and Figures showed the interpreted data.

Results

Extraction yield of *Azadirachtaindica*

Extraction yield of *Azadirachtaindica*leaves in different solvents are presented in Table 1 and

Extraction yield of *Azadirachtaindica*

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Distilled water exhibited (9.08%) maximum extraction from *Azadirachtaindica* leaves whereas minimum extraction yield was observed in petroleum ether (2.9%).**Thin Layer Chromatography (TLC)**

Thin layer chromatography was used for separation of different chemical constituents present in chloroform extract of *Azadirachtaindica.*

TLC-Bioautography

To assess the antibacterial activity of isolated compounds against the tested bacterium, bioautography technique was utilized on TLC plates run in hexane: ethyl acetate (1:1). The TLC plate displayed a transparent zone of inhibition against a red background around the band that contained the active ingredient responsible for the antibacterial activity after being sprayed with 2, 3,5-tri phenyl tetrazolium chloride. One compound from *Azadirachtaindica* extract demonstrated well-resolved suppression of *Xanthomonasaxonopodis*pv. *citri* at Rf- 0.74 while emitting a pink color when illuminated by UV light.

GCMS analysis of *Azadirachtaindica*

Chloroform leaf extract from *Azadirachtaindica* revealed the existence of five major components together with their retention times, peak areas, and molecular weights. Phenol 3, 5-bis (1,1-dimethyl ethyl) at retention time 9.88, mol. wt. 206, and peak area 659085.64 was the first compound. Phthalic acid bis (7-methyl octyl) ester was second compound at retention time 15.58, mol. wt. 418, and peak area 1188911.27. The third compound wasDodecyl phthalates with retention time 20.17, mol. wt. 502 and peak area 3732124.99. The fourth and fifth compoundare Oxalic acid, allylhexadecyl ester, and 2-Piperidinone, N-(4-bromo-n-butyl) at retention times of 27.24 and 31.34, mol. wt. 354 and 233,their peak areas were 1528807.42 and 2565012.55, respectively, (Table 2 and Figure 1,2,3,4 and 5).

FTIR analysis of *Azadirachtaindica*

The following peaks and functional groups were identified using FT-IR analysis. Peak 3427 indicates the existence of a hydroxyl methyl group, while peak 2952 and peak 1734 indicate C-H stretching and carboxylic acid-like C=O stretching, respectively. C-H bending was found at 1089.46 and 801.61 peak, and C=C stretching was seen at 1465.19. (Table 3 and Figure 6).

^{1}HNMR analysis of *Azadirachtaindica*

Different signals for various proton types were discovered by ^{1}H NMR analysis. The initial signal indicated the existence of an aromatic proton between 6.95 and 7.78.The existence of olefinic proton was indicated by the second signal, 6.67 to 6.81. At 4.51 to 4.72 signal, phenolic hydroxyl group presence was evident. While the 4.21 to 3.31 signal indicated the presence of a hydroxyl group, or a methylene group connected to an electronegative atom. At 3.62 to 3.70 signal, the ester group was present, and at 2.62 signal, the ketone group. And the final signal (0.9-1.48) indicated the existence of a methyl group (Table 4 and Figure 7).

^{13}C NMR analysis of *Azadirachtaindica*

Carbonyl group was present at signals 169.50, 67.1, and 67.3 according to ^{13}C NMR study. Quaternary carbons were observed at signals 129.7 to 132.4 while aromatic carbons were present at signals 116.7 to 127.57. Likely at 27.85 to 23.79 and 14.52 signals, methyl groups were present respectively, (Table 4a and Figure 8).

Discussion

Extraction

Table 1 provides the extraction yields of the various solvents employed in this study. The polarity and capacity of a solvent to extract additional chemical compounds from the *Azadirachtaindica* plant determines how extractable it is.*Azadirachtaindica* was recognised to yield more bioactive compounds when extracted with distilled water compared with other solvents. The findings were like those of Raja Pandiyan et al. (2011), who found that water extract had the maximum extraction yield (4.5g) due to the presence of highly polar alkaloids, flavones, and sugars. Babu et al. (2016) also previously recorded the highest extraction yield (0.6882g) from *Azadirachtaindica* leaves in water extract.

Thin Layer Chromatography

Several antibacterial fractions or secondary metabolites responsible for antibacterial activity were separated using thin layer chromatography. The findings of the above investigation, revealed the retention factors (Rf) of ethanol extracts of *Azadirachtaindica* in various solvent systems, are consistent with those of Mondali et al. (2014). In hexane: ethyl acetate (1:1) solvent system, the ethanol extracts generated nine fractions with Rf 0.09, 0.10, 0.19, 0.22, 0.38, 0.48, 0.58, 0.66, and 0.91. In the current study, the same solvent system hexane: ethyl acetate (1:1) produced the maximum band separation using chloroform extract. As a result, the TLC results show that chloroform extracts contain a variety of chemical components.

Bioautography

The distinct antibacterial fraction of *Azadirachtaindicia* chloroform extract eluted on TLC plates in a hexane:ethyl acetate (1:1) solvent system was identified using the bioautography method. The 2,3,5-triphenyl tetrazolium chloride was sprayed onto TLC plates, which displayed a whitish or translucent zone of inhibition against a pink or red background at Rf-0.74. Kruzselyi et al. (2016) used High Performance Liquid Chromatography (HPLC) and electrosprayionization mass spectrometry to identify the active chemicals present in *Azadirachtaindica* oil and designated them as linoleic and oleic acid. Using TLC-bioautography and spectroscopic analysis, Shubham et al. (2016) also reported the existence of an active chemical tetra nor-triterpenoidlimonoid with an Rf- 0.56 and a retention period of 3.8 minutes in *Azadirachtaindica* leaves.

GCMS analysis

The several bioactive components found in *Azadirachtaindica* chloroform extract are identified using Gas Chromatography and Mass Spectrometry (GC-MS) technique. Five main chemicals that are responsible for *Azadirachtaindica*therapeutic potential were discovered using GC-MS research. Table 4 lists the recognised compounds along with their retention time, molecular weight, molecular formula, and peak area. These components fall under the categories of alkaloid, phenolic, dicarboxylic, and plasticizer chemicals.

Phenol, 3,5, bis-(1,1-dimethyl ethyl), Dodecyl phthalate, 2-piperidinone, N-(4-bromo-n-butyl), Oxalic acid, allylhexadecyl ester, and Phthalic acid, bis (7-methyloctyl) ester were the most prevalent of the above-mentioned described compounds.The first compound Phenol, 3,5, bis-(1,1-dimethyl ethyl), was discovered in the leaves of *Indoneesiellaechiodes* (Elaiyaraja and Chandramohan, 2016), *Azadirachtaindica* (Sandanasamy et al. 2014), *Hibiscus micranthus* (Kumar et al. 2011), *Nerium oleander* (Dey and Chaudhary, 2016), and *Ninbapatradichooram*(Chandrasekhar et al. 2015).This phytochemicaldemonstrated a variety of activities, including antimicrobial (Elaiyaraja and Chandramohan 2016, Sandanasamy et al. 2014; Lawal et al. 2016; Wagay and Rothe 2016; Arora et al. 2017; Rai et al. 2016 and Chandrasekhar et al. 2015), antioxidant (Ajayi et al. 2011; Victoria and Samrot 2016; Rai et al (Rai et al. 2016).Lawal et al. 2016 and Rukhsana et al. 2015 and Govindappa et al. 2014), antimalarial (Elaiyaraja and Chandramohan 2016), anti-inflammatory (Chandrasekhar et al. 2015), analgesic, anesthetic, antiseptic, antiviral, cancer preventive and fungicidal (Rai et al. 2016).

Dodecyl phthalate, the second compound, was recognised for its plasticizing properties (Priya and Vijayalakshmi, 2011). These phthalates are present in cosmetics, detergents, lubricating oils, alternatives for polychlorinated biphenyls, carriers in pesticide formulations, solvents, and building materials like flooring, sheeting, and films (George and Prest, 2002). This phytocompound was identified in a variety of plant extracts, including *Mukiamaderaspatana*(Mallikadevi*et al*. 2012), *Sarcostemmasecamone*(Kumari*et al*. 2012), *Blighiasapida*(Ojo*et al*. 2018),*Viola odorata* (Jasim*et al*. 2018) flower and *Trigonellafoenum*(Priya and Vijayalakshmi, 2011). Additionally, a variety of activities, including antimicrobial and antifouling (Kumari et al. 2012; Priya and Vijayalakshmi 2011; Chandel and Kumar 2015 and Kalaiarasan et al. 2012), anti-hypersensitive, vasodialator,

diuretic, and angiotensin ATZ receptor antagonist, were demonstrated by this compound (Mallikadevi et al. 2012).

The third compound, 2-piperidinone, N-(4-bromo-n-butyl), was reported to be found in a variety of plants, including the leaves extract of *Microcosmusexaspeatus* (Meenakshi et al. 2012), *Asparagus racemosus* (Selvam et al. 2014), sesame seed (Olaleye et al. 2018), the leaves, fruit, and latex of *Croton bonplandianum*((Vennila and Udayakumar, 2015), and in *Aspergillustamarii* and*Penicilliumislandium*(Hady et al. 2016). This compoundshowed antibacterial, anti-inflammatory (Vennila and Udayakumar, 2015; Meenakshi et al., 2012), and antioxidant properties (Meenakshi et al. 2012).

The presence of fourth antibacterial compound Oxalic acid, allylhexadecyl ester was found by various researchers in other plant species like in *Laurenciabrandenii* (Manila*et al*. 2011), *Aloe vera* plants (Arunkumar*et al*. 2009), Nigerian rice (Adekoyeni*et al*. 2018) and in *Pongamiapinnata* (Anuradha and Krishnamoorthy 2012).This compound produced a variety of activities such as antimicrobial (Sathya*et al*. 2012), acaricide, irritant, pesticidal, renotoxic and varroacidial (Zayed and Samling 2016).

The final compound,Phthalic acid,bis (7-methyloctyl) ester was similarlyknown for its plasticizing properties (Santhi et al. 2016; Ramakrishnan and Venkataraman 2011). Later, different plant taxa including *Tabebuiaargentea* (Melappa et al. 2017), *Aporosalindleyana* (Ramakrishnan and Venkataraman 2011), *Calotropisgigantea* (Singh and Javed 2015), *Purpurapersica* (Santhi et al. 2016), and *Centratherumpunctatum*(Sivasubramanian and Brindha, 2013). were shown to contain this compound. According to Ramakrishnan and Venkataraman (2011), the antibacterial compound exhibited antibacterial and antifouling properties as well as tumour-fighting properties against mice sarcoma 180 cell lines (Lee et al. 2000).

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FTIR analysis

Azadirachtaindica chloroform extract was further analysed using Fourier Transform Infrared Spectroscopy to detect the various functional groups that were present. Table 5 presents the findings of the discovered functional groups by FTIR analysis together with their peaks and average range. The results of the FTIR analysis discussed above were consistent with those of Shaikh et al. (2017), who found that O-H str was present at peak 3456.55, C=O str was present at peak 1653.05, and alkene and alkyl halide group were present at peak 675.11. Moreover, the existence of C=O str and C=C str was noted at maxima in 1730 and 1452, respectively (Reshmi et al. 2014). At peaks 873.75 and 721.38, 2922.26

and 2852.72, 1463.97 and 1741.72, C-H bend, C-H str, C=C, and C=O were all detected. (Banerjee*et al*. 2016).

NMR analysis

To determine the types of protons and carbon contained in various compounds from the *Azadirachtaindica* plant, the Nuclear Magnetic Resonance technique was used. The first type of NMR is ^{1}H, which identifies the proton type, and the second type is ^{13}C, which identifies the carbon type contained in the corresponding plant extracts. Tables 5 and 6 show the chemical shifts, types of protons, and carbon atoms. Similar findings were made in the study of ^{1}H NMR analysis by Kumar et al. (2012), who indicated the existence of an aromatic proton at peak 7.2–6.8, a CH_2 group at peak 3.1–3.8, a CH_3 group at peak 2.2–2.9, and a methyl group at peak 1.28. Furthermore, the methyl groups at 0.77–0.78 and 1.14–1.33 (Patil*et al*. 2017) and aromatic protons at 6.19-7.2, 7.54, 6.40 and 6.88 peaks were given by Sambadam*et al.* 2016.

The above mentioned FTIR and NMR (^{1}H and ^{13}C) data revealed various functional groups, proton types, and carbons, which as a result are responsible for producing various pharmacological actions as demonstrated by the bioactive compounds from the *Azadirachtaindica* plant that were found. These chemical constituents and the groups they were placed in the study play a significant part in having various activities against plant pathogens, which will be more crucial in the future for controlling the most serious horticultural and agricultural diseases.

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Plant	Solvent	Yield in %

Tables

Azadirachtaindica	Petroleum ether	2.9
	Chloroform	6.7
	Dichloromethane	4.64
	Distilled water	9.08

Table1. Effect of different solvents on per cent extraction yield from dry weight of leaves

Table 2. Identification of compounds from chloroform extract of *Azadirachta*indica leaves by GCMS

Sr. no.	Name of compound	Formula	MW	Retention time	Peak area
1	Phenol 3,5-bis (1,1-dimethyl ethyl)	$C_{14}H_{22}O$	206	9.88	659085.64
2	Phthalic acid bis (7-methyloclyl) ester	$C_{25}H_{42}O_4$	418	15.58	1188911.27
3	Didodecyl phthalates	$C_{32}H_{54}O_4$	502	20.17	3732124.99
4	Oxalic acid, allylhexadecyl ester	$C_{21}H_{38}O_4$	354	27.24	1528807.42
5	2-Piperidinone, N-(4-bromo-n-butyl)	$C_9H_{16}BrN_0$	233	31.34	2565012.55

Table3. Identification of functional group from chloroform extract*Azadirachta*indica leaves by FTIR analysis

Sr.no.	Peak	Functional group	Average range
1	3427	R-CH2OH, R_2-CHOH R_3-C-OH	3400-3600
2	2952	C-H str. Hydrocarbons aliphatic aromatic	2850-3000
3	1734	C=O str. Carbonyl group	1650-1800
4	1465.19	C=C str. Aromatic compounds	1450-1600
5	1089.46 and 801.61	C-H bending hydrocarbons,aliphatic aromatic	650-1000

Table 4. Identification of types of protons from *Azadirachtaindica* leaves by Proton NMR analysis (deuterated methanol)

Sr. no.	(δ) Chemical shift	Type of proton
1	6.95-7.78	Aromatic proton
2	6.67-6.81	Olefinic proton
3	4.51-4.72	Phenolic OH
4	4.21-4.31	CH_2 or CH attached to electronegative atom
5	3.62-3.70	CH_2 or CH or Ar-O=CH_3 or R-O-C-CH_3 (ester)
6	2.62	Ar-CH_3 or O=C-CH_3 (ketone)
7	0.9-1.48	R-CH_3

Table 4a. Identification of types of carbon from *Azadirachtaindica* leaves by ^{13}C NMR analysis (deuterated methanol)

Sr. no,	(δ) Chemical shift	Type of carbon

1	169.50	C=O
2	129.7-132.4	Quaternary carbon aromatic
3	116.7-127.57	Aromatic carbon Olefinic carbon
4	31.9-49.67	CH or CH_2 attached to electronegative atom, O-CH_3
5	27.85 and 23.79	R-CH or R-CH_2
6	14.52	R-CH_3

Fig. 1 Mass spectra of compound Phenol 3, 5, bis (1,1-dimethyl ethyl) from chloroform extract of *Azadirachtaindica*leaves

Fig. 2 Mass spectra of compound Phthalic acid bis (7-methyloctyl) ester from chloroform extract of *Azadirachtaindica*leaves

Fig. 3 Mass spectra of compound Didodecyl phthalate from chloroform extract of *Azadirachtaindica*leaves

Fig. 4 Mass spectra of compound Oxalic acid, allylhexadecyl ester from chloroform extract of *Azadirachtaindica*leaves

Fig. 5 Mass spectra of compound 2-piperidinone, N- (4-bromo-n-butyl) from chloroform extract of *Azadirachtaindica*leaves

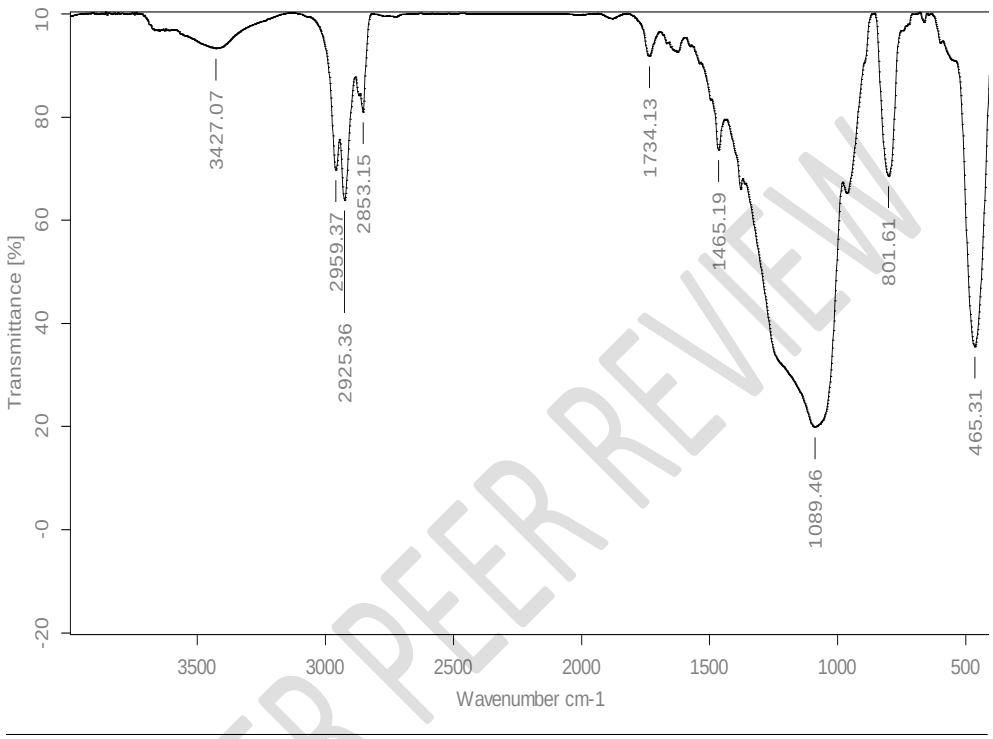

***Fig. 6 FTIR spectra of compound isolated from chloroform extract of Azadirachtaindica*leaves**

***Fig. 7 ^{1}H NMR spectra of compound isolated from Azadirachtaindica*leaves (deuterated methanol)**

***Fig. 8^{13}C NMRspectra of compound isolated fromAzadirachtaindica*leaves (deuterated methanol).**