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Piperine and isoflavan-4-one from the stems of *Piper chaba* Hunter and their *In vitro* antimicrobial activities

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Abstract
A known piperine (1), along with two new isoflavan-*4*-one (2, 3) were isolated from the ethanol extract of ethyl acetate fraction of *Piper chaba* H. stems. The chemical structure of these compounds were established by detailed spectral data and in comparison with their spectral data reported earlier. The study was also performed to determine the *in vitro* antimicrobial activity by disc diffusion method. Among them, the pure compound 1 showed good antimicrobial activity against all of the test micro-organisms, fungi and standard Kanamycin.

Keywords: *Piper chaba* H., isoflavan-*4*-one, piperine, antimicrobial activity, NMR, IR, UV

1. Introduction

Piper species are widely distributed in the tropical and subtropical regions of the world[1]. The plant *Piper chaba* Hunter (*Piperaceae*) is a climbing, glabrous shrub available in various parts of India and Malay Islands[1]. In Bangladesh, it is grown in plenty in the southern part particularly in Jessore, Khulna, Satkhira and Bagerhat areas[2]. Popularly known as Java long pepper or choi[3], it is used as spices in meat curry and other dishes and believed to have medicinal value in a wide variety of disease conditions including arthritis, asthma, bronchitis, piles, colic pain, dyspepsia and gastralgia[4, 5]. The crude extracts of *Piper chaba* have also been found to exhibit anti-diarrhoeal and diuretic activities[6] and possess antibacterial, carminative, stimulant, expectorant, analgesic, anti-hypertensive and smooth muscle relaxant properties[2, 7, 8, 9]. Recently, 85% aqueous acetone extract from the fruit and leaf of *Piper chaba* as well as some isolated alkamides were found to be protective against ethanol and indomethacin induced gastric lesions in rats[10]. Stem bark of *Piper chaba* produced a significant anti-inflammatory effect in rat model[11]. Previous phytochemical investigation of stem bark of *Piper chaba* had revealed the presence of Lignan[12] and alkaloids such as piperamine-*2*, *4*-decadienoic acid piperidide, kusunokinin and pellitorine[13]. A unique piperine dimer Chabamide has also been isolated from stem bark [14]. Besides *β*-caryophyllene, caryophyllene oxide and few monoterpene hydrocarbons, a moderate content of sesquiterpenes and high amount of aliphatic hydrocarbons have been found in the fruit oil of the plant[15]. It has the major alkaloid in piper species, has been shown to have antimycobacterial activity[16] and several pharmacological activities such as antihyperlipidemic[17], antiandrogenic[18], immunoregulatory [19], antidepressant[20]. Piperine has also been shown to have certain serious toxicities such as antifertility[21], respiratory paralysis, hemorrhagic necrosis and edema in gastrointestinal tract, urinary bladder and adrenal glands[22] and immunotoxicological effects[23]. Biological investigations on *Piper chaba* have shown that chloroform and methanol extracts of the plantshow anti-amoebic and anti-giardial activities against *Entamoeba histolytica* (IC_{50} value of 72.4 µg/ml) and *Giardia intestinalis* (IC_{50} value of <100 µg/ml)[24, 25]. The fruit of *Piper chaba* is used as an anti-flatulent, gastro-protective, appetizing property, as an expectorant, anti-tussive, anti-fungal agent and also possesses cholesterol lowering properties [26]. Ethanolic extract of fruit of *Piper chaba* has also been shown to possess erythropoietic effects [27], CNS depressant and anxiolytic effects[28]. Stem is used to reduce post-delivery pain in mothers and also useful in rheumatic pains and diarrhea [29]. In this study, is described the isolation and structure elucidation of two new compounds 2-3 together with isolation of a known compound 1 from the stem of *Piper chaba*. Along with antimicrobial activity are performed for pure compounds 1-3.

2. Materials and Methods

2.1 General experimental section

Melting points were determined with a Köfler type melting point apparatus and are uncorrected. UV and IR spectra were obtained on a Shimadzu UV-1601 UV spectrophotometer and a Shimadzu IR prestige-21(FT-IR) spectrometer (Organic Research Laboratory, Department of Chemistry, Bangladesh University of Engineering and Technology, Dhaka, Bangladesh). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_3OD on a BRUKER NMR DPX-400 MHz instrument (Bangladesh Council of Scientific and Industrial Research Lab, Dhaka, Bangladesh) at 400 MHz for protons and 100 MHz for carbons using TMS as the internal standard. All NMR spectra were obtained using the standard Bruker software. The ultraviolet lamp used in visualizing TLC plates was a Mineralight® device, multiband UV-254/365 nm obtained from UVP, Inc., USA. Column chromatography (CC) was carried out over silica gel (230-400 mesh, ASTM, Merck) and TLC was carried out with silica gel 60 pre-coated plates F-254 (Merck Germany).

2.2 Plant material

The fresh plant was collected from Kurigram (Bangladesh) on July, 2013. The taxonomy of the plant was confirmed consulting with Botanist of Bangladesh National Herbarium.

2.3 Extraction and isolation

The air dried fresh stems (286.32 g) of *Piper chaba* were exhaustively extracted with EtOH at room temperature. A solid residue (12.7 g) was obtained after removal of the solvent using rotary vacuum evaporator and fridge dryer. The dried ethanol extract was then suspended in H_2O and partitioned by separating funnel successively with *n*-hexane, EtOAc and MeOH, to give *n*-hexane (2.28 g), ethyl acetate (4.32 g), methanol (2.7 g) and aqueous (3.4 g) part.

2.4 Isolation of compounds 1 to 3 from ethyl acetate part

The ethyl acetate (4.32 g) part, was subjected to silica gel column eluted with *n*-hexane and increasing the polarity with dichloromethane till 100% dichloromethane then with methanol, 32 fractions were collected (100 ml each), the significant fractions were then repeated CC on silica gel eluted with *n*-hexane as eluent, then increasing the polarity by using dichloromethane and methanol to give three different components from different fractions. These components were purified by small glass column afforded compound 1 (4.2 mg) in white crystalline solid from 2% CH_2Cl_2 in *n*-hexane eluate, compound 2 (5.3 mg) in white crystalline solid from 60% CH_2Cl_2 in ethyl acetate eluate and compound 3 (4.4 mg) in white crystalline solid from 5% MeOH in ethyl acetate eluate.

Compound 1: white crystalline solid (4.2 mg); R_f= 0.6 [(dichloromethane: ethyl acetate ≡ 4:1)]; UV Vis (MeOH): 248 and 338 nm; IR (KBr): 2993, 2855, 1636, 1540, 1193 and 922 cm^{-1}; ^{1}H and ^{13}C NMR data were similar to reported data[30].

Compound 2: white crystalline solid (5.3 mg); R_f= 0.42 [(dichloromethane: ethyl acetate ≡ 3: 2)]; UV Vis (MeOH): 230 and 320 nm; IR (KBr): 3163, 2929, 1715, 1546, 1360, and 1155 cm^{-1}; ^{1}H and ^{13}C NMR (TMS): Table 1.

Compound 3: white crystalline solid (4.4 mg); R_f= 0.36 [(dichloromethane: ethyl acetate ≡ 3: 2)]; UV Vis (MeOH): 230 and 260 nm; IR (KBr): 3178, 3032, 1711, 1490, 1247, and 1064 cm^{-1}; ^{1}H and ^{13}C NMR (TMS): Table 1.

Table 1: $^{13}C$and ^{1}H NMR spectral data of 2 and 3 (100, 400MHz, DMSO-d_6, TMS)

Position	δ_c	Compound 2 δ_H(*J* in Hz)	δ_c	Compound 3 δ_H(*J* in Hz)
2	60.6	4.04 (2H, m)	60.8	4.05 (2H, m)
3	51.6	5.91 (1H, s)	53.7	5.37 (1H, s)
4	178.4	-	178.5	-
5	165.3	-	146.4	-
6	104.3	-	140.7	-
7	133.0	-	134.0	-
8	140.5	-	106.6	-
9	147.9	-	165.5	-
10	104.3	-	106.6	-
1′	138.0	-	140.3	-
2′, 6′	130.2	7.36 (2H, m)	129.0	7.16 (2H, m)
3′, 5′	128.8	7.51 (2H, m)	128.9	7.45 (2H, m)
4′	127.6	7.25 (1H, m)	127.8	6.85(1H, m)
CH_3 at C-6	13.9	1.06(3H, t, *J*= 7.2)	-	-
CH_3 at C-7	18.6	2.22 (3H, s)	18.7	2.12 (3H, m)
CH_3 at C-8	-	-	14.1	1.22 3H, m)

2.5 Antimicrobial activities

Agar diffusion method[31]: The total ethyl acetate extract and pure compound 1-3 of *P. chaba* H. were dissolved in sufficient amount of respective solvents and evaluated by agar diffusion method at a dose of 300 µg/disc and the results were reported as shown in Table-2. The tested fungi were *Candida albicans*, *Aspergillus niger*, and *Sacharomycescerevacae*; gram positive bacteria were *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Sarcina Lutea*, and *Staphylococcus aureus* while gram negative bacteria were *Escherichia coli*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigellaboydii*, *Shigelladysenteriae*, and *Vibrio mimicus*. Kanamycin (30µg/disc) disc was used as standard antifungal and antimicrobial agents; the diameter of zone of inhibition was measured in mm. The microorganisms were obtained from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh.

Table 2: Antimicrobial activitiesof ethyl acetate extract and pure compound 1-3 of *P. chaba* H.

Test bacteria and fungi	ethyl acetate extract	Comp 1	Comp 2	Comp 3	Kanamycin
	300 (µg/disc)				30 (µg / disc)
Gram-positive bacteria					
Bacillus cereus	6.5	7	---	---	32
Bacillus megaterium	6.5	13	---	---	32
Bacillus subtilis	13	---	---	---	32
Staphylococcus aureus	11	14	---	---	33
Sarcina lutea	12	---	---	---	33
Gram-negative bacteria					
Escherichia coli	11	---	---	---	33
Salmonella paratyphi	10	---	---	---	33
Salmonella typhi	11	---	---	---	33
Shigellaboydii	9	---	---	---	33
Shigelladysenteriae	11	---	---	---	33
Vibrio mimicus	9	---	---	---	32
Pseudomonas aeruginosa	6.5	---	---	---	33
Fungi					
Candida albicans	10	---	---	---	32
Aspergillus niger	18	14	---	---	32
Sacharomycescerevacae	9	---	---	---	32

"---" Indicates 'No activity'

3. Result and Discussion

The EtOH extract from the stems of *Piper chaba* was suspended in water and partitioned between n-hexane, ethyl acetate and methanol successively. Ethyl acetate fraction on being subjected to silica gel column chromatography (CC) afforded compounds 1 to 3.

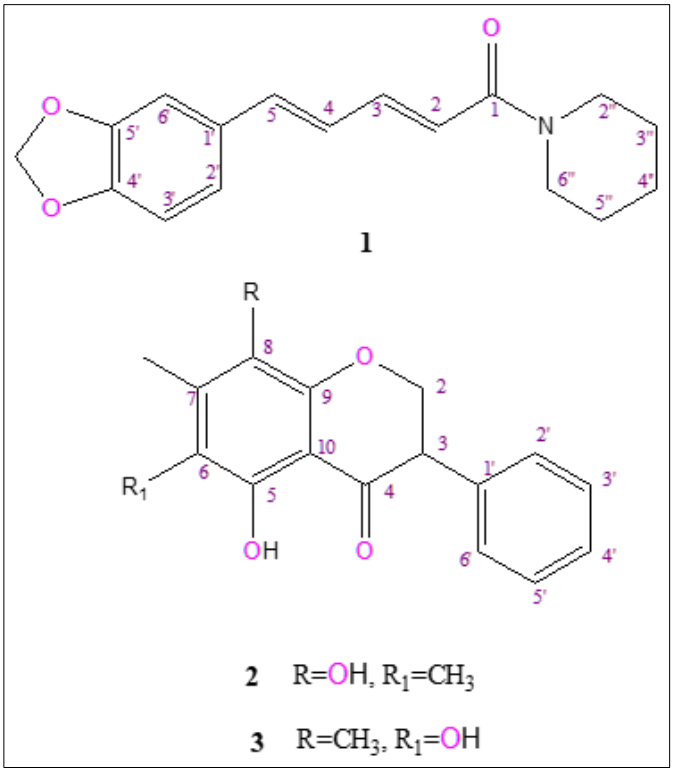

Fig 1: Structure of 1-3

Compound 1 (Figure-1), $C_{17}H_{19}O_3N$ (M^+ 285) was obtained as a white crystalline solid, m.p. 126^0 C. It was identified as piperine 1 by comparison of its spectral data with literature [32]. The detailed IR and UV data are provided in the Experimental Section.

Compound 2 (Figure-1), $C_{17}H_{16}O_4$ (M^+ 284) was isolated as a white crystalline solid. Its IR spectrum showed the presence of hydroxyl (3163 cm^{-1}), carbonyl group (1715 cm^{-1}) and aromatic C-H stretching (3036 cm^{-1}). Its UV spectrum in MeOH showed absorption at 230 and 320 nm, characteristic of isoflavonoid[32]. The ^1H NMR spectrum exhibited two methyl groups on the aromatic ring [δ_H1.06 (3H, t), 2.22 (3H, s)], a methylene and a methine protons at C-2 and C-3 of isoflavanone moiety [δ_H4.04 (2H, m), 5.91 (1H, s)], a phenyl ring of *o*-, *m*-, *p*- protons [δ_H7.36 (2H, m), 7.51 (2H, m), 7.25 (1H, m)] and hydroxyl protons were not showed significant spectrum[33]. The ^{13}C NMR spectrum showed 17 carbon signals, which on DEPT experiments, represented 2 methyl (δ_C 13.9, 18.6), 1 methylene (δ_C 60.6), 6 methine (δ_C 51.6, 130.2, 128.8, 127.6) and 8 quaternary (δ_C 104.3, 165.3, 133.0, 140.5, 147.9 and 178.4) carbons[33]. The upfield quaternary carbon signals at δ_C 178.4, 165.3 and 147.6 represented carbonyl group at C-4, hydroxyl carbon at C-5 and a carbon (C-9) near oxygen atom. The ^{13}C NMR spectral data of compound 2 was similar to that of dihydrodaidzein except hydroxyl position and introduced two methyl groups[34]. These result indicated the compound 2 was isolated as 5, 8-dihydroxy-6, 7-dimethyl isoflavan-4-one.

Compound 3 (Figure-1), $C_{17}H_{16}O_4$ (M^+ 284) was isolated as a white crystalline solid. Its IR spectrum showed the presence of hydroxyl (3178 cm^{-1}), carbonyl group (1711 cm^{-1}) and aromatic C-H stretching (3032 cm^{-1}). Its UV spectrum in MeOH showed absorption at 230 and 360 nm, characteristic of isoflavonoid[32]. The ^1H NMR spectrum exhibited two methyl groups on the aromatic ring [δ_H1.22 (3H, t), 2.12 (3H, s)], a methylene and a methine protons at C-2 and C-3 of isoflavanone moiety [δ_H4.07 (2H, m), 5.37 (1H, s)], a phenyl ring of *o*-, *m*-, *p*- protons [δ_H7.16 (2H, m), 7.45 (2H, m), 6.87 (1H, m)] and hydroxyl protons were not showed significant spectrum[33]. The ^{13}C NMR spectrum showed 17 carbon signals, which on DEPT experiments, represented 2 methyl (δ_C 14.1, 18.7), 1 methylene (δ_C 60.8), 6 methine (δ_C 53.7, 129.0, 128.9, 127.8) and 8 quaternary (δ_C 106.6, 165.5, 134.0, 140.7, 146.4 and 178.5) carbons[33]. The upfield quaternary carbon signals at δ_C 178.5, 165.5 and 146.4 represented carbonyl group at C-4, a carbon (C-9) near oxygen atom and hydroxyl carbon at C-5. The ^{13}C NMR spectral data of compound 3 was similar to that of dihydrodaidzein except hydroxyl position and introduced two methyl groups at C-7 and C-8[34]. These result indicated the compound 3 was isolated as 5, 6-dihydroxy-7, 8-dimethyl isoflavan-4-one.

4. Conclusion

A detailed phytochemical analysis has been carried out on the herb *Piper chaba* Hunter. During this investigation five compounds were isolated from this plant extract. Among them structural elucidation of the three compounds were performed. The structural elucidation of the remaining compound was not carried out as its ^{1}H-NMR gives evidence for being a fatty material. All the compounds were identified preliminarily by chemical methods and then the structural elucidations of the compounds were performed by various spectral methods (UV, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-135 etc.) and were confirmed by the comparison with the results that are available in the published literatures. The three compounds, piperine alkaloid type and two flavanoid type compounds 5, 8-dihydroxy-6, 7-dimethyl isoflavan-4-one and 5, 6-dihydroxy-7, 8-dimethyl isoflavan-4-one were isolated from the ethyl acetate part. The pure compound piperine and ethyl acetate extract have also anti-microbial activity. Since the plant *Piper chaba* contains various bioactive compounds, so it may be concluded that this plant might play a vital role as a medicinal plant.

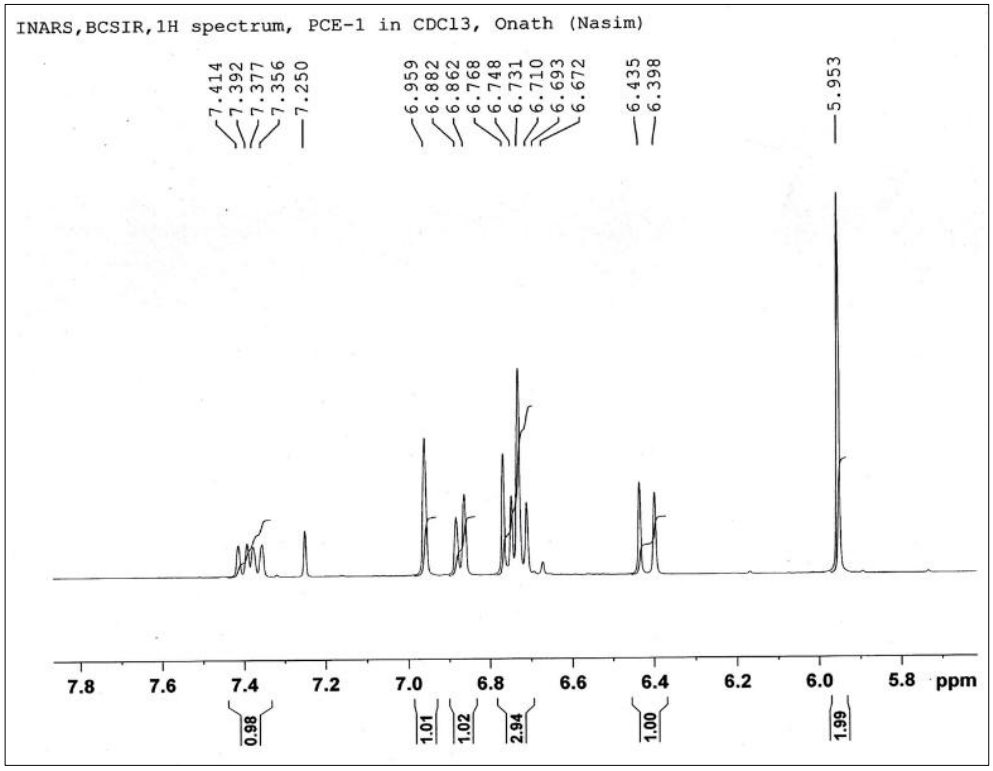

Fig 2: ^{1}H NMR Spectrum of compound-1

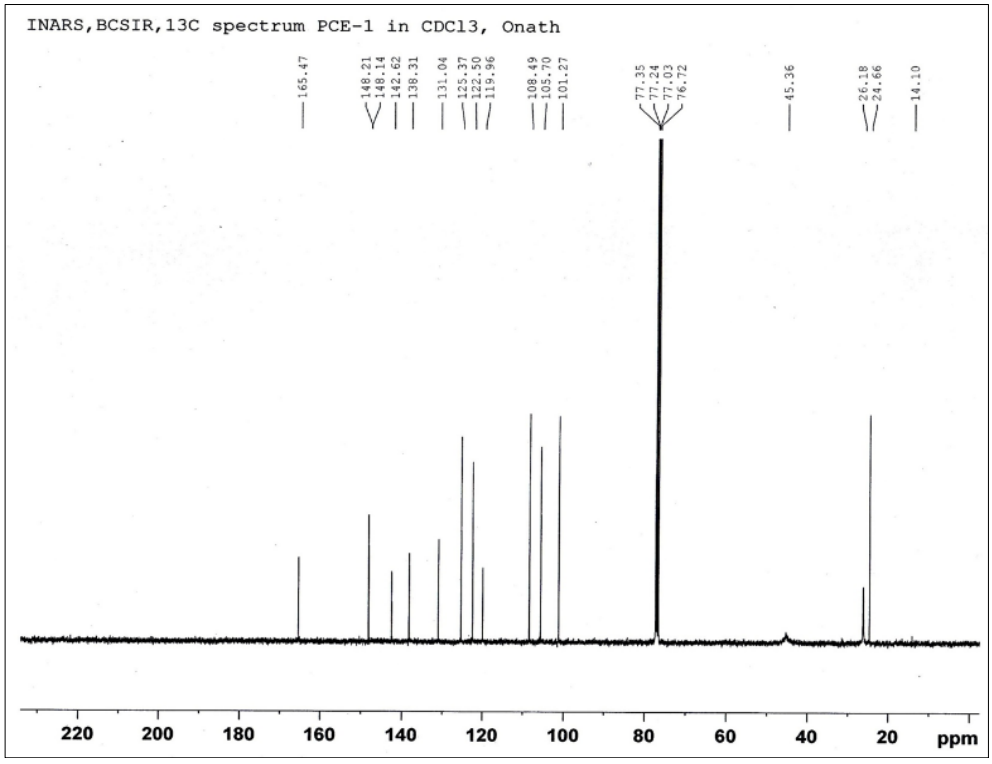

Fig 3: ^{13}C NMR Spectrum of compound-1

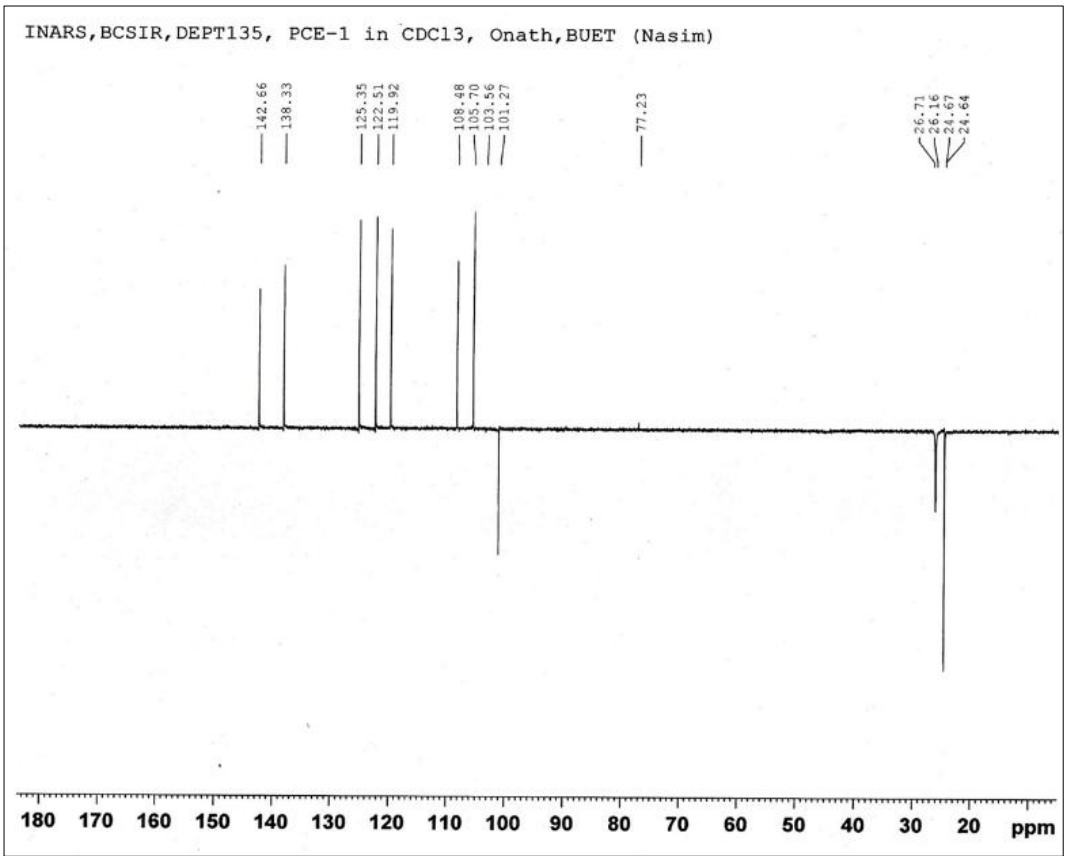

Fig 4: DEPT-135 NMR Spectrum of compound-1

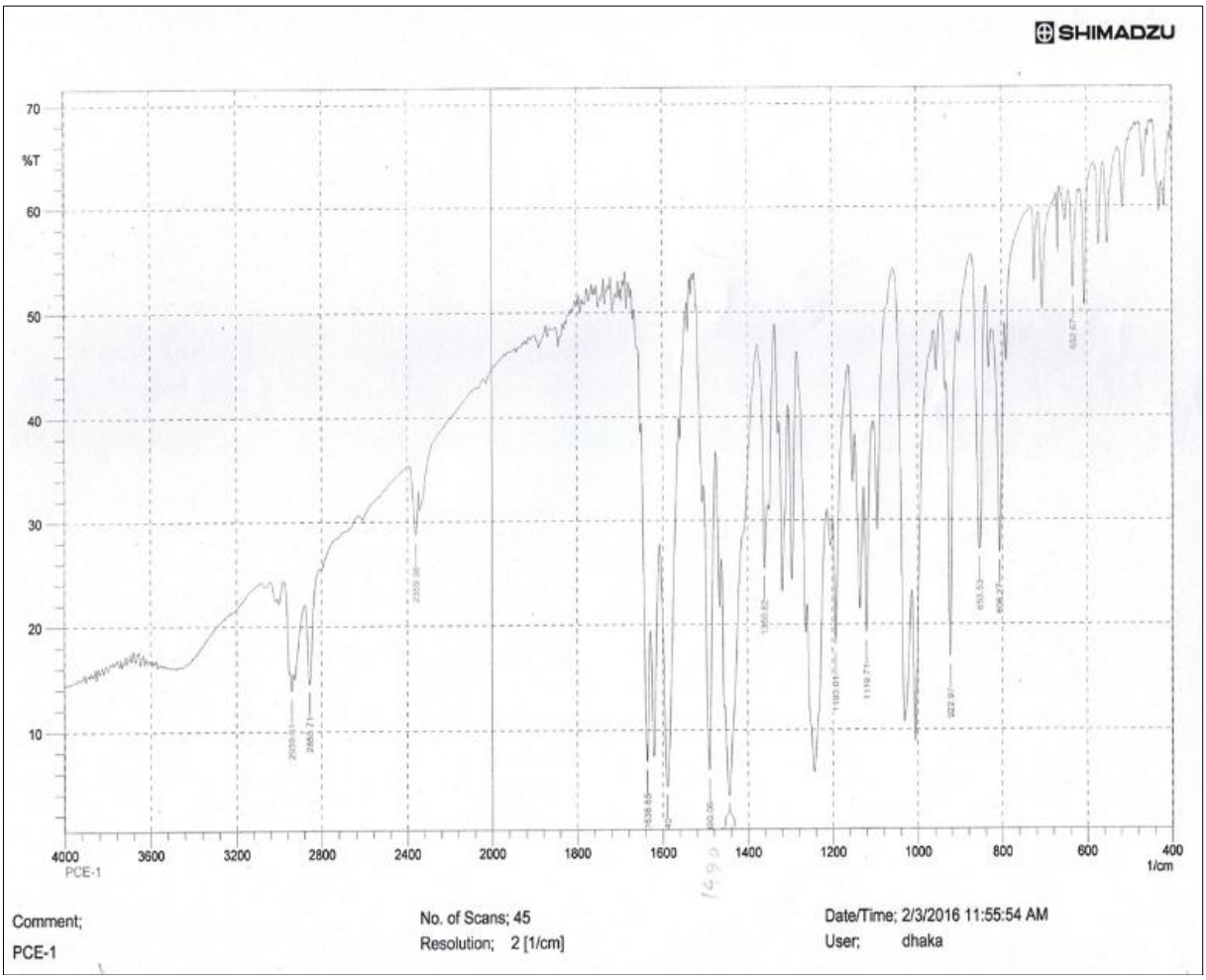

Fig 5: IR Spectrum of compound-1

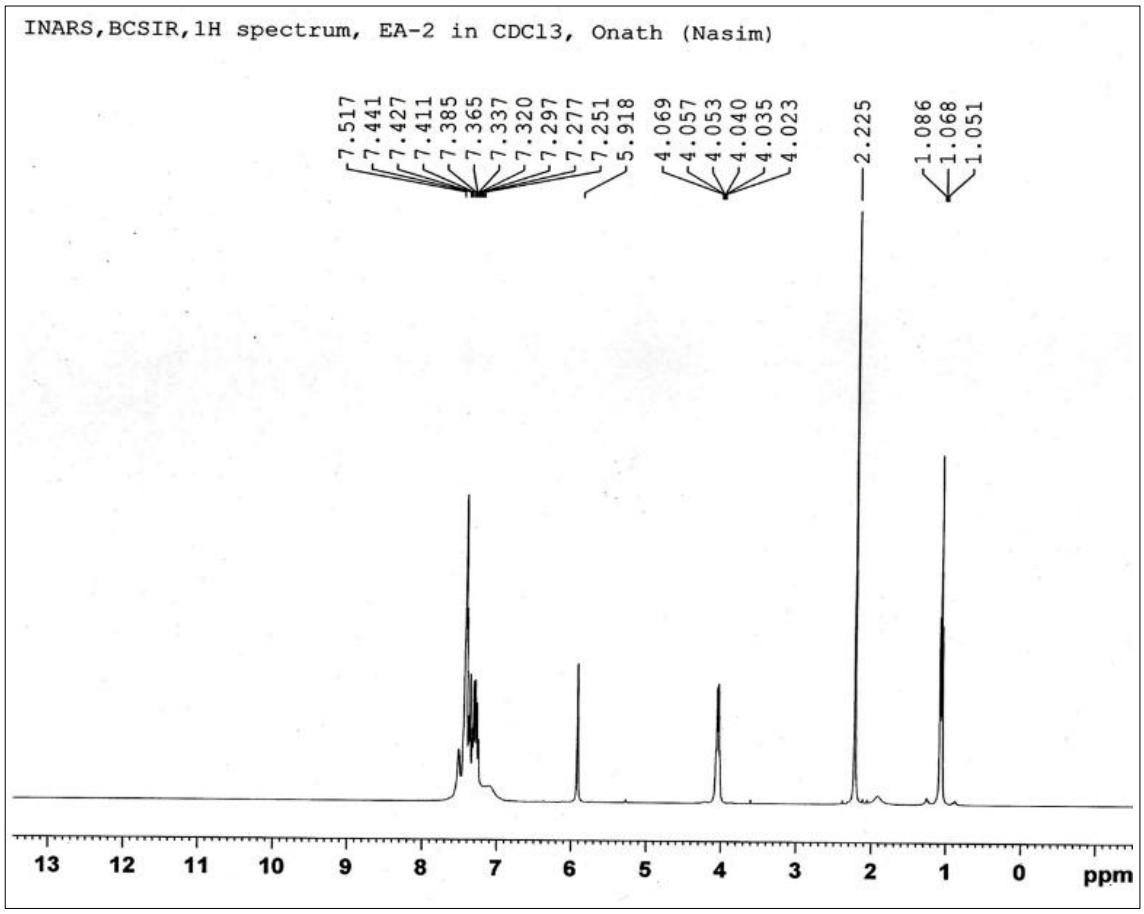

Fig 6:^{1}H NMR Spectrum of compound-2

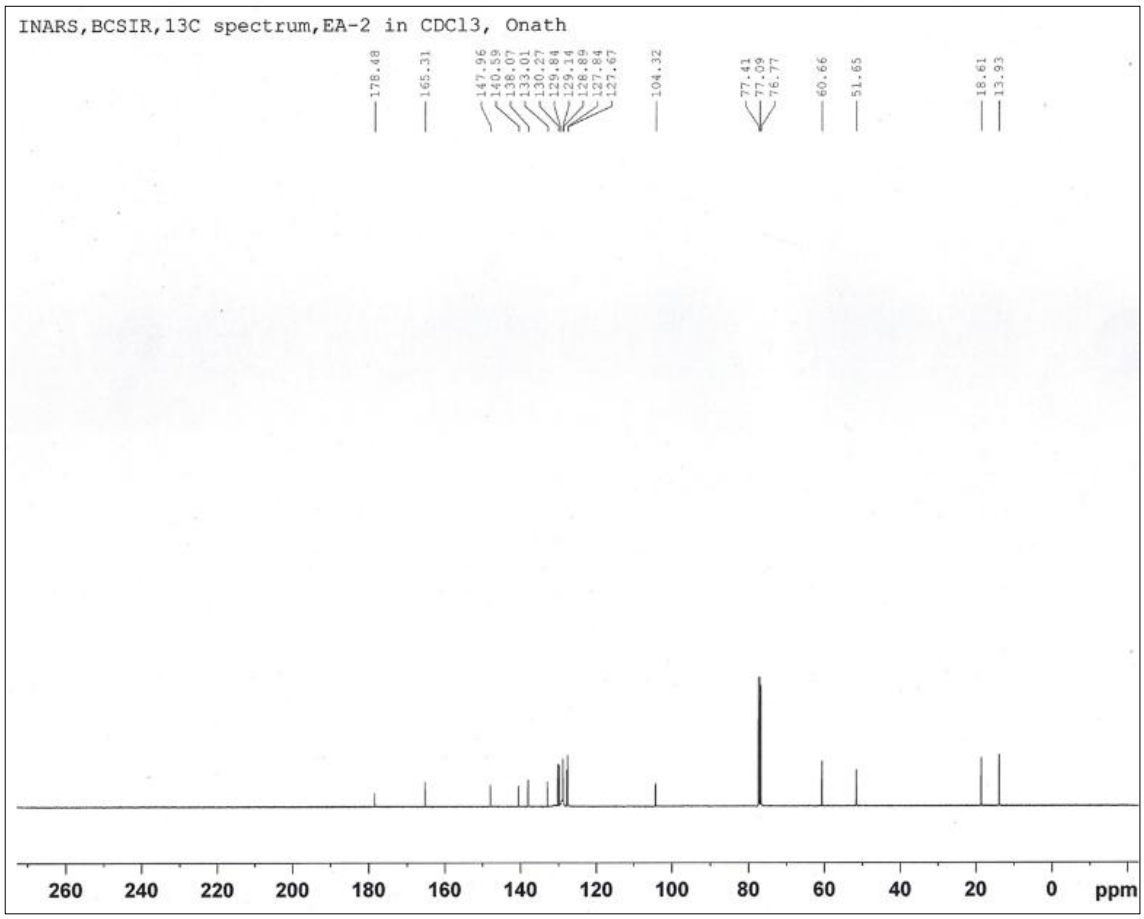

Fig 7:^{13}C NMR Spectrum of compound-2

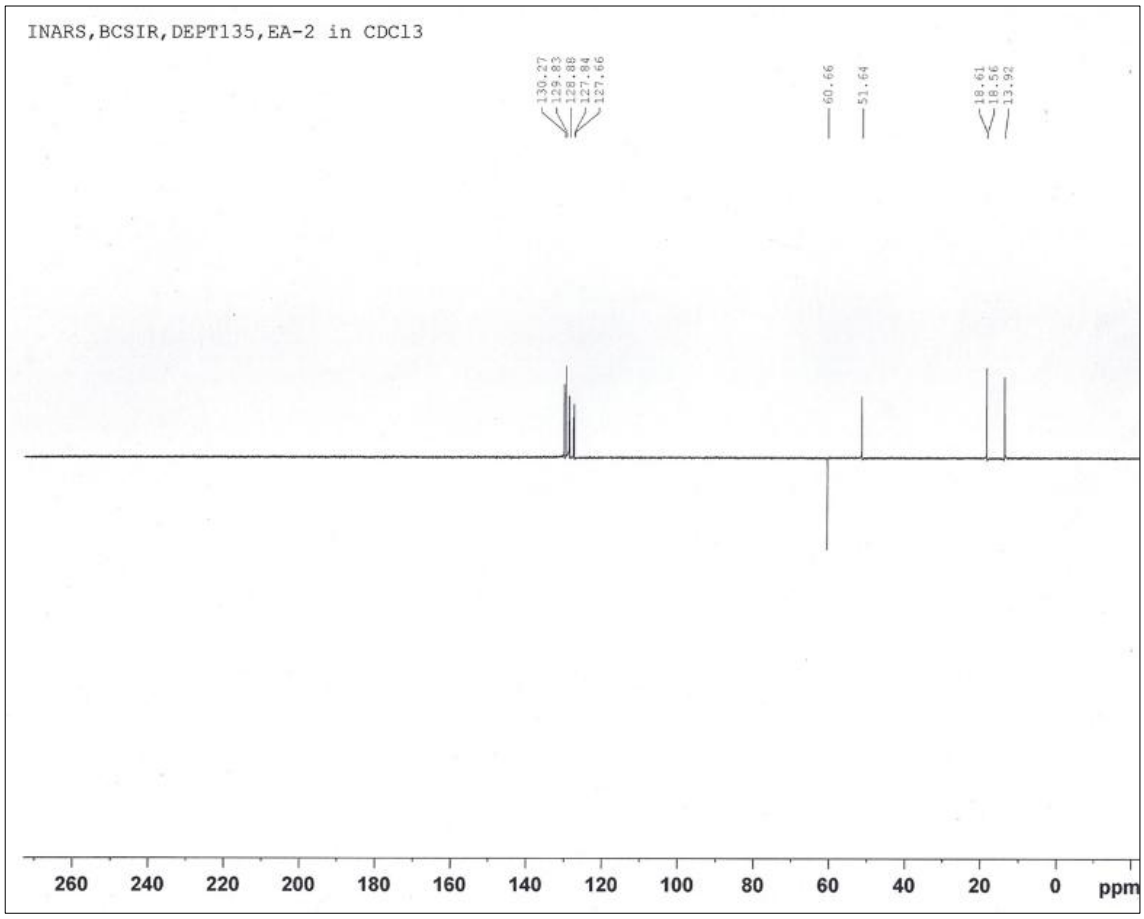

Fig 8: DEPT-135 NMR Spectrum of compound-2

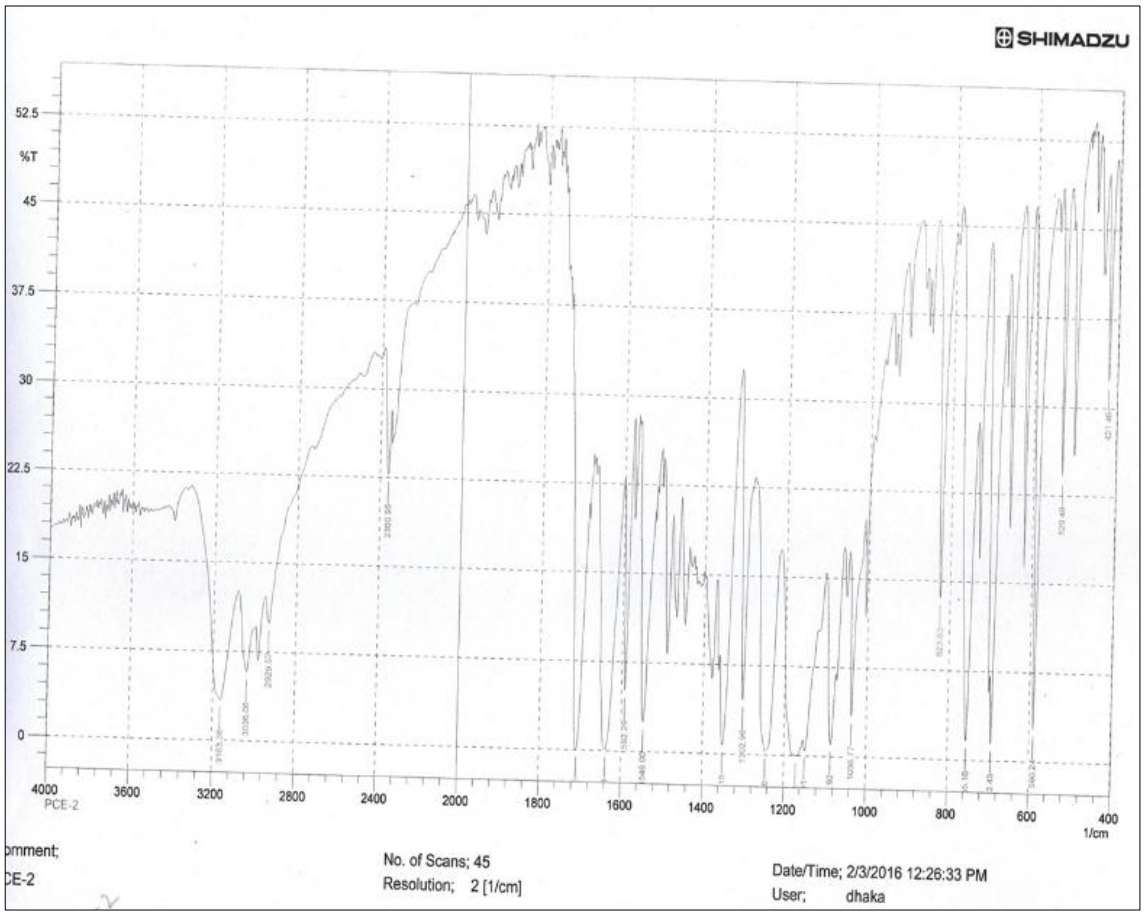

Fig 9: IR Spectrum of compound-2

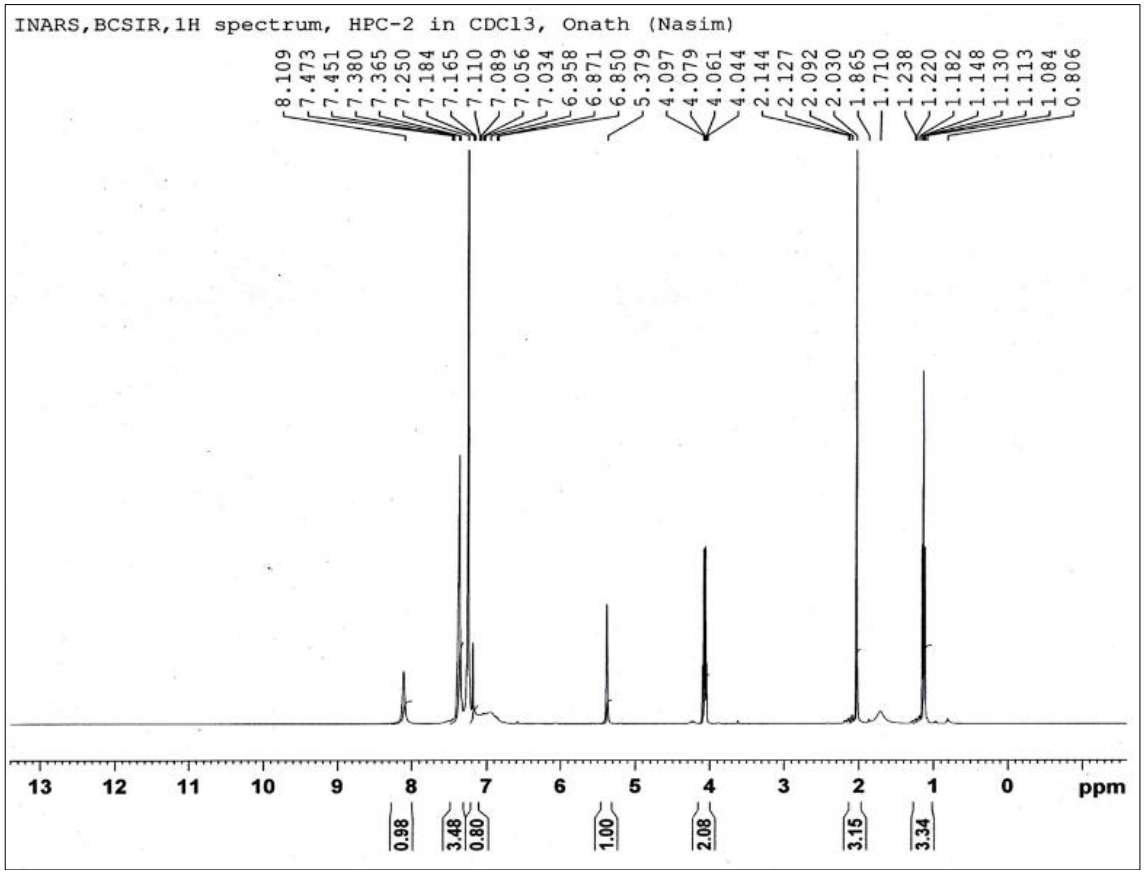

Fig 10: ^{1}H NMR Spectrum of compound-3

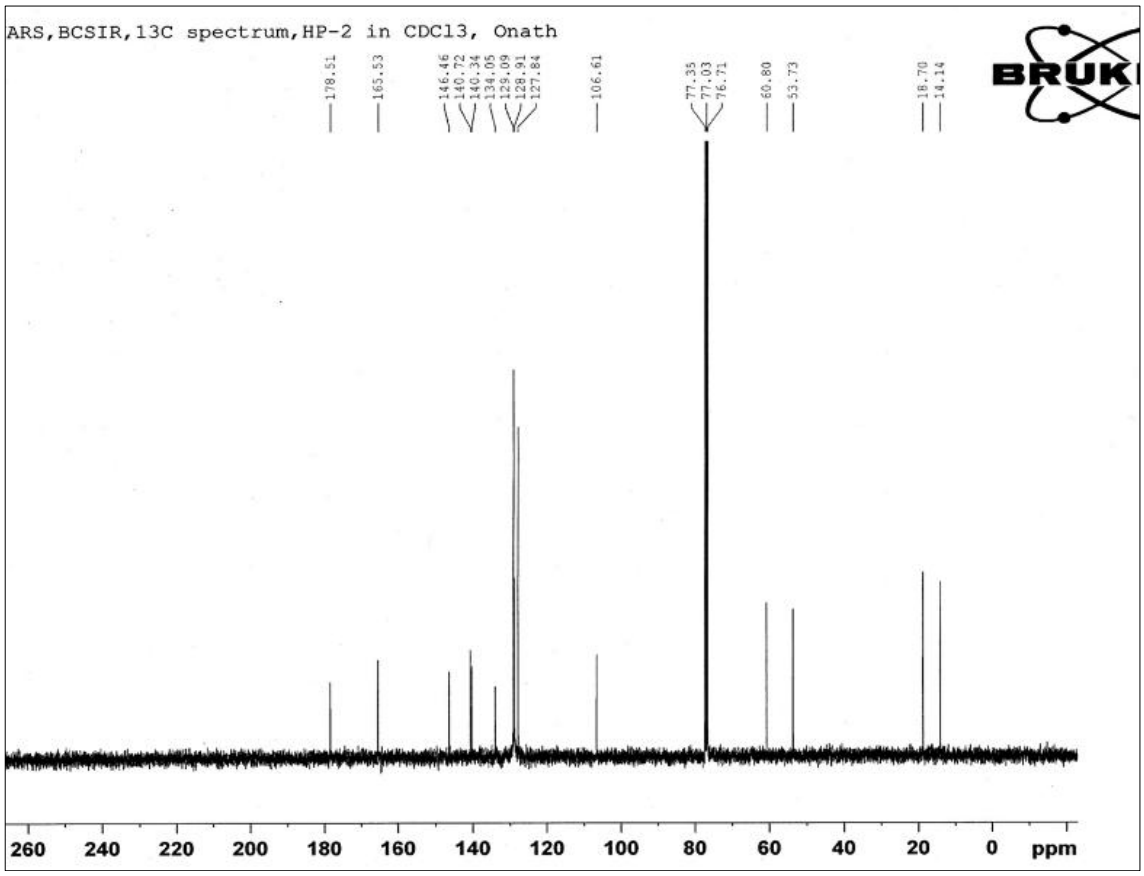

Fig 11: ^{13}C NMR Spectrum of compound-3

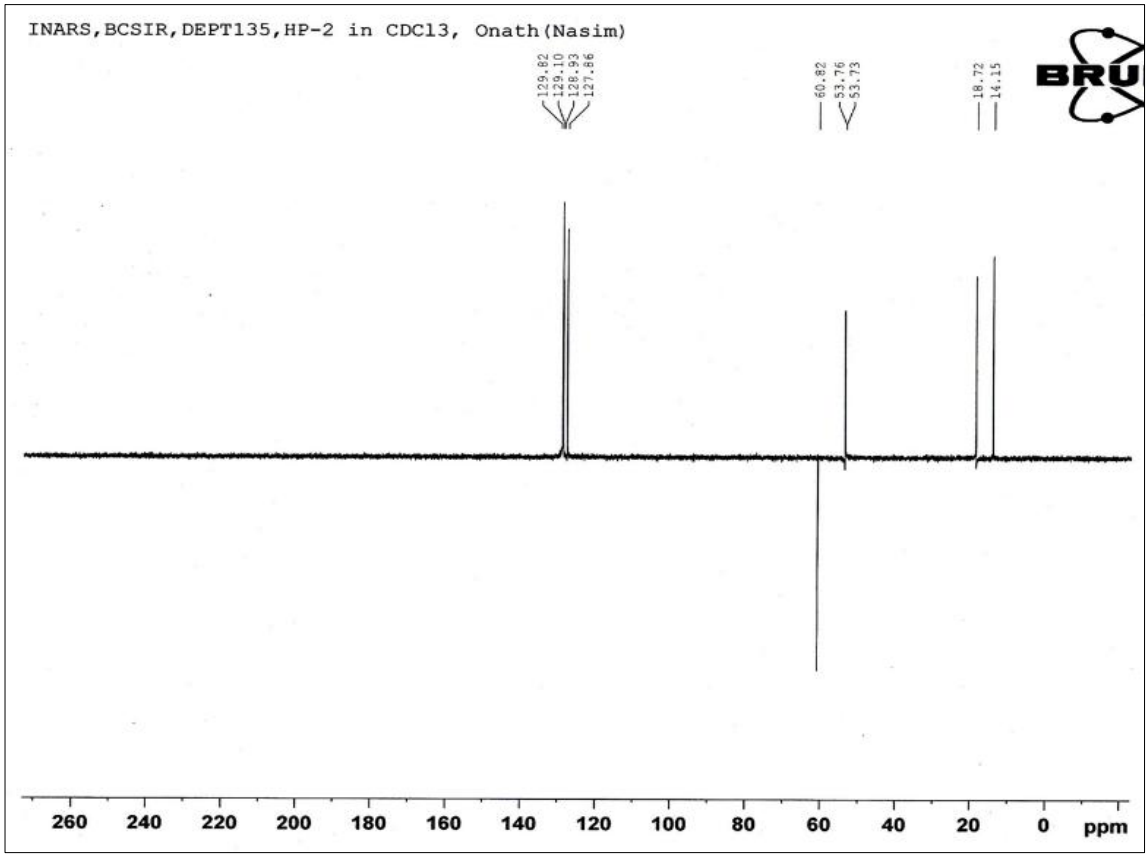

Fig 12: DEPT-135 NMR Spectrum of compound-3

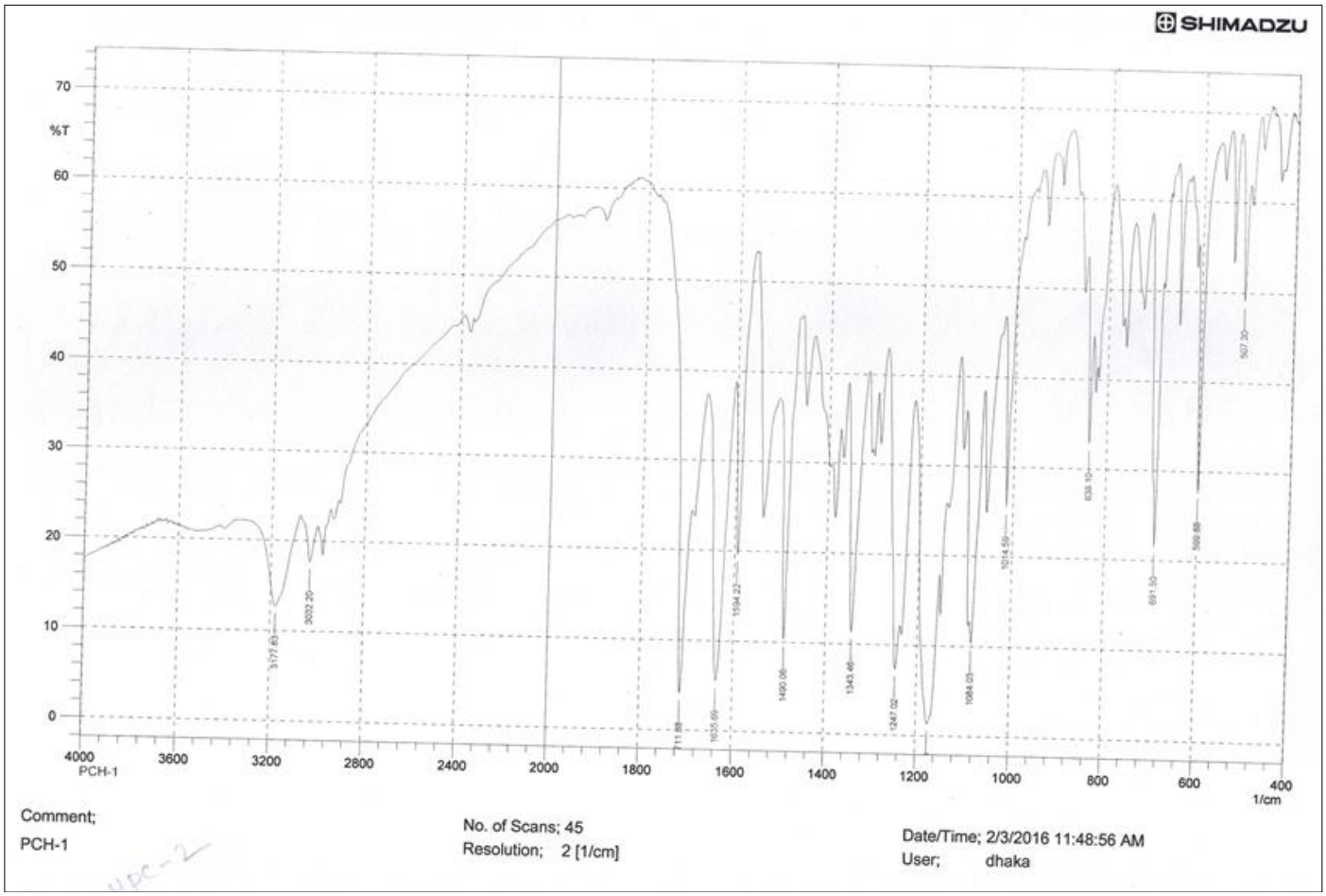

Fig 13: IR Spectrum of compound-3

5. Acknowledgement
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