CHAPTER 4 RESULTS AND DISCUSSION

4.1 Isolation of Endophytic fungi

A total of fourteen Endophytic fungi were screened (CPR1- CPR14) for production of antimicrobial metabolites from root of *Calotropis procera*. Among these fourteen isolates, one isolate CPR5 was found to show maximum antimicrobial activity, compare to other isolates, against gram positive, gram negative bacteria and plant and human pathogenic fungi. Therefore only CPR5 was considered for further experiments. This is consistent with findings of earlier research work indicating that endophytic microorganisms can be found in virtually all higher plants (Strobel and Daisy, 2003), and that endophytic colonization differs with different parts of plant (Schulz and Boyle, 2005). The identification of the endophytic fungi was based on their characteristic morphology of colony, colour and pattern of spore production. Each isolate was then grown and examined to make certain that it is originated from single hyphae. All fungi present were isolated, sub cultured and preserved on PDA medium for further identification. The frequency of colonization (FC %) was calculated taking into account the number of morphologically distinct colony isolated (Ni) and the total number of sample fragments inoculated (Nf) (Araujo et al., 2002a, b).

FC% = (Ni / Nf) X100

Isolate CPR 1, CPR 2 CPR 7, CPR 8, CPR 9, CPR 11, and CPR 14 showed antibacterial activity against at least one test microorganism. CPR 3, CPR 4, CPR 5, CPR 10 CPR 12, and CPR 13 did not show any antibacterial activity and the results are listed in Table 3.

Table 3: Antibacterial activity (inhibition zone) of the crude aqueous extracts of 14 Isolates

Isolates	antibacterial activity of isolates (Diameter of inhibition zone in mm)					
	E.coli	*B.subtilis*	*P.aeroginosa*	*S.aureus*	*X. oryzae*	*R.solanacearum*
CPR1	0	3.5.0±0.0	0	0	0	0
CPR2	0	0	0	0	0	4.3±0.0
CPR3	0	0	0	0	0	0
CPR4	0	0	0	0	0	0
CPR5	13.0±0.1	9 ± 0.2	14. ± 0.3	12.0 ± 0.0	15. ± 0.3	14.0 ± 0.3
CPR5	0	0	0	0	0	0
CPR7	0	0	0	0	0	0
CPR7	5.0±0.1	0	0	0	0	0
CPR9	0	0	0	0	0	4.0±0.2
CPR10	0	0	0	0	0	0
CPR11	0	0	6.0±0.2	0	0	0
CPR12	0	0	0	0	0	0
CPR13	0	0	0	0	0	0
CPR14	0	0	0	0	3.0±0.3	0

Similarly, when these isolates tested for antifungal activity, CPR 1, CPR 2, CPR 7, CPR 9 and CPR 12 showed antifungal activity against at least one fungal species. CPR 3, CPR 4, CPR 6, CPR 8, CPR 10, CPR 13and CPR 14 did not show any antifungal activity and the results are enumerated in Table 2.

Table 4: Antifungal activity (inhibition zone) of the crude ethyl acetate extracts of 14 Isolates

Isolates	Antifungal activity of isolates (Diameter of inhibition zone in mm)				
	Penicillium sp.	*Fusarium species*	*Sclerotium rolfsii*	*S. scleratiourum*	*Candida albicans*
CPR1	0	0	0	3.0±0.2	0
CPR2	0	4.5.0±0.1	0	0	0
CPR3	0	0	0	0	0
CPR4	0	0	0	0	0
CPR5	4.0±0.4	13±09	15±.5	6.0±0.2	12.0±0.2
CPR6	0		0	0	0
CPR7	0	0	3.4.0±0.0	0	0
CPR8	0	0		0	0
CPR9	0	4.8.0±0.3	0	0	0
CPR10	0	0		0	0
CPR11	0		0	0	0
CPR12	0	0	0	4.6±0.4	0
CPR13	0	0	0	0	0
CPR14	0	0	0	0	0

Tested microorganisms were cultured on agar solidified medium. 15 µl crude extracts from all 14 isolates was applied on the disk (6 mm diameter). The bacterial cultures were placed in an incubator at 37 ^{0}C and fungal culture at 29 ^{0}C for overnight incubation. Inhibition zone was observed and diameter of inhibition zone was measured. Among all 14 isolates, crude metabolite of an endophytic fungus isolate the CPR5, displayed significant antimicrobial activity against all test pathogens; *Escherichia coli, Streptococcus pneumonia, Bacillus subtilis, Staphylococcus hyicus, B sphaericus, Staphylococcus aureus*, *pseudomonas aeruginosa,* plant pathogenic bacteria; *Xanthomonas oryzae* and human pathogenic fungus*, Candida albicans* and four plant pathogenic fungi *Sclerotium rolfsii, Sclerotinia scleratiourum, Fusarium species* and *Penicillin sp.* This result clearly indicates that the extract was significantly effective against both Gram positive and Gram-negative bacteria and plant pathogenic fungi. This showed the broad-spectrum nature of the metabolite for antimicrobial activity from root tissue of *Calotripis procera*.

Among another group of the 24 human pathogenic bacteria tested, maximum zone of inhibition was shown by *E. coli O157:H7* (28.mm) and *Klebsiella pneumonia* (25 mm*)* followed by remaining test bacteria *S epidermis (25.50* mm*), B cereus* (24.50 mm), *Pseudomonas aeruginosa* (24 mm), *B subtilis* (22.0 mm), *Staphylococcus aureus* (19. mm) *B megaterium* (19.50 mm), *Enterococcus faecalis* (19.50 mm*), Helicobacter pylori, M. D. R S aureus* (18. mm)*, Chlamydia pneumonia* (17 mm), *Streptococcus pyogenes* (14. mm), *Corynebacterium amycolatum (*14. mm*), Mycobacterium tuberculosis* (11. mm), *Listeria monocytogenes* (16. mm) *Salmonella typhimurium* (15. mm), *Salmonella typhi (*11. mm*), Streptococcus pneumonia* (16. mm) *Staphylococcus epidermidis* (18. mm), *Corynebacterium diphtheria* (17.85 mm), *Vibrio sp* (11. mm), *Clostridium difficile* (14.50 mm), *Enterococcus*

faecium (18. mm). Results of antimicrobial activity of aforesaid 24 human pathogenic bacteria are given in figure 8 and table 5. Since, Isolate CPR5 produced the maximum inhibition (28 mm) against *E. coli O157:H7*, a causal agent various human infection disease in humans, *Klebsiella pneumonia* (25mm) *w*hich is responsible for pneumonia, and it equally active against highly pathogenic bacteria; *M. D. R S. aureus, Mycobacterium tuberculosis, Helicobacter pylori Salmonella typhimurium, Salmonella typh* (Table 3). The extract showed antibacterial activity agaist *Xanthomonas, oryzae* which causes bacterial blight (BB) of rice which is one of the most important diseases of rice in most of the rice growing countries. Bacterial blight of rice has high epidemic potential and is destructive to high-yielding cultivars (cerials) in both temperate and tropical regions. Similarly, fungi, which were tested are highly plant pathogenic fungi and fungal extract showed strong antifungal properties against plant pathogenic fungi; *Sclerotium rolfsii, Sclerotinia scleratiourum, Fusarium species* and *Penicillin sp.* The antifungal activity shown against plant fungi was not stronger than bacterial counterpart (compare Table 5 and table 6). *Ralstonia solanacearum infects Potato (Solanum tuberosum); Tomato (Lycopersicum esculentum); Aubergine (egg plant) (Solanum melongena).* Therefore, metabolites from this fungal isolate could be an important chemical remedy in checking loss of crops caused by these bacteria. These experimental results showed the clear suggestion for the isolation of a strong antibacterial compound for this isolate. Which can provide a good alternative natural source for replacement of antibiotic like chloramphenicol, Fluconazole etc. Currently, there is demand for a search for new antimicrobial agents because of the development of pathogen resistance to available drugs. People are generating new synthetic drugs, but these may have an adverse effect on the environment.

Table 5 Antibacterial activity (inhibition zone) of the crude aqueous extracts of CPR5

Test bacteria	**Zone of inhibition(mm)**	**Minimum Inhibitory Concentration (MIC)**	
	Crud extract(1mg/ml)	**Crud extract** (µg/ml)	**Chloromphenicol (+ve controle)** (µg /ml)
E. coli O157:H7	28.0±0.2	130	*50*
B cereus	24.15±0.2	*230*	*40*
B subtilis	24.25±0.2	*230*	*45*
Staphylococcus aureus	19.0±0.2	*330*	*50*
B.megaterium	16.0±0.2	*200*	*35*
Helicobacter pylori	*15.12*±0.2	*270*	*70*
Streptococcus pyogenes	14±0.2	*310*	*100*
M. D. R S. aureus	*18.0*±0.2	*280*	*80*
Chlamydia pneumonia	*17.15* ±0.2	*220*	*70*
Corynebacterium amycolatum	*14.0*±0.2	*270*	*110*
Mycobacterium tuberculosis	*11.5*±0.2	*270*	*150*
S. epidermis	25.5±0.2	*322*	*45*
Pseudomonas aeruginosa	24.35±0.2	*330*	*30*
Listeria monocytogenes	*16.5*±0.2	*300*	*75*
Salmonella typhimurium	*15.20*±0.2	*300*	*130*
Salmonella typhi	*14.00*±0.2	*330*	*150*
Streptococcus pneumoniae	*16.85*±0.2	*280*	*75*
Staphylococcus epidermidis	*15.85*±0.2	*300*	*120*
Corynebacterium diphtheriae	17.0±0.2	*220*	*80*
Vibrio sp	12.00±0.2	*350*	*40*
Clostridium difficile	*14.55*±0.2	*300*	*120*
Klebsiella pneumonia	*25.38*±0.2	*220*	*45*
Enterococcus faecalis	*19.45±0.2*	*31*	*55*
Enterococcus faecium	18.05±0.2	*32*	*85*

ANOVA analysis was performed for analysis of correlation among inhibition of crud extract, MIC value and standered antibiotics for various bacterial species. An auto correation among crud extract among can yield high value of squireeven of poorly fitted models the results of T-test gave insignificant value (2.29717 E-8) at 95% of confidence level. An insignificant P-value is desirable because it empied crude extract are uncorrelated and and indicated goodness of model in predicting desirable response.

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	657809.30672	657809.30672	180.93126	0
Error	46	167241.57203	3635.68635		
Total	47	825050.87875			

Null Hypothesis: The means of all levels are equal
Alternative Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significantly different.

Fit Statistics

R-Square	Coeff Var	Root MSE	Data Mean
0.7973	0.4459	60.29665	135.22604

Means Comparisons

Tukey Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Level2	Level1	234.13125	17.40614	19.02268	9.29171E-8	0.05	1	199.09445	269.16805

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

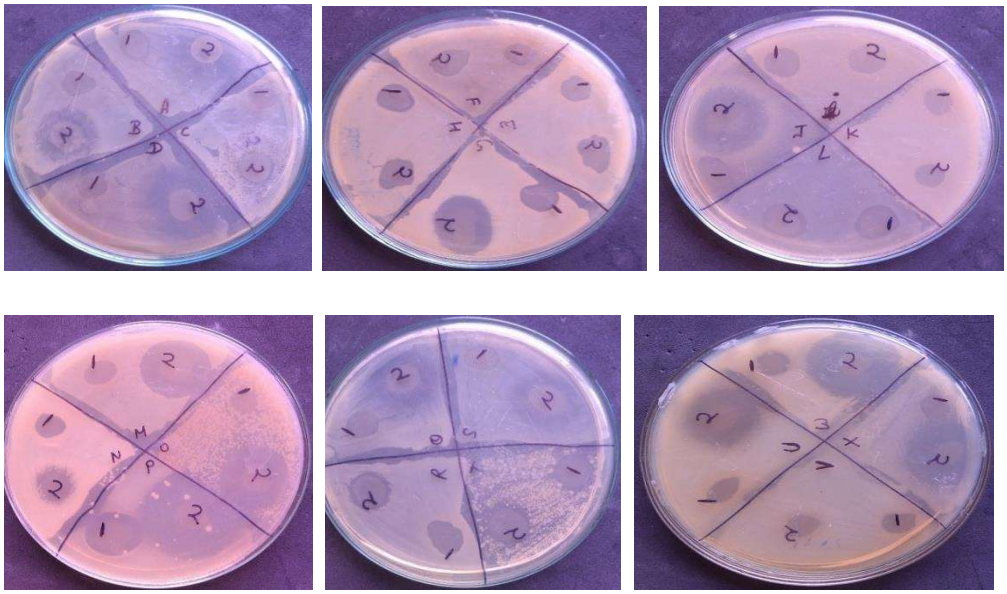


Fig. 8 Antibacterial activity test of 24 human pathogenic bacteria (In each part of four section made in each Petri plate only indication of '2' will be considered because two samples were simultaneously tested)

Table-6 Antifungal activity (inhibition zone) of the crude ethyl acetate extracts

Plant pathogenic fungi	**Zone of inhibition (mm)**		**Plant pathogenic bacteria**	**Zone of inhibition (mm)**	
	Crud extract	**Fluconazole(+ve controle)**		**Crud extract**	**Chloromphenicol (+ve controle)**
Penicillium chrysogenum,	3.35±0.2	10.5±0.2	*R.solanacearum*	7.15 ±0.2	18.4±0.2
Phoma exigua	2.21±0.2	12.4±0.2	*X. oryzae*	6.85±0.2	13.3±0.2
Sclerotium rolfsii	4.38±0.2	10±0.2	Xanthomonas campestris	8.38±0.2	16.3±0.2
Sclerotinia sclerationrum	5.15±0.2	11.3±0.2	Pseudomonas *spp*	6.0±0.2	16.3±0.2
Fusarium *oxysporum*	4.54±0.2	12.7±0.2			
Rhizoctonia *spp*	5.84±0.2	9.5±0.2			
Fusarium.verticilloides	3.45±0.2	14.5±0.2			
Penicillium expansum	2.21±0.2	8.9±0.2			
Human pathogenic fungus					
C.albicans	10. ±0.2	16±0.2			

ANOVA Test

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	389.16723	389.16723	53.14103	1.57067E-7
Error	24	175.759	7.32329		
Total	25	564.92623			

Null Hypothesis: The means of all levels are equal
Alternative Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significantly different.

Fit Statistics

R-Square	Coeff Var	Root MSE	Data Mean
0.68888	0.29364	2.70616	9.21577

Means Comparisons

Tukey Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Level2	Level1	7.73769	1.06144	10.30932	2.24906E-7	0.05	1	5.54697	9.92841

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

ANOVA analysis was performed for analysis of correlation among inhibition of crude extract, and standard antibiotics for various bacterial species. An auto correlation among crude extract can yield high value of R-square even for poorly fitted models. The results of t-test gave insignificant value (2.24906 E-7) at 95% of confidance level. An insignificant P-

value is desirable because it empied crude extract are uncorrelated and and indicated goodness of model in predicting desirable response.

4.2 Minimum Inhibitory Concentration (MIC)

MIC of crude extract from isolate CPR5 that showed inhibitory effect was determined by dilution methods The MIC values of crude extract from isolate CPR5 is shown in Table 3. The result indicated that fungal crude extract clearly showed antibacterial property against gram-positive and gram-negatives bacteria. The extract obtained from isolate CPR5 had MIC values of 240 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml and 140 µg/ml for *E.coli*, *B. subtilis, Penicillium chrysogenum, S. aureus* and *Xanthomonas oryzae* respectively. Similarly, MIC value for test fungi was determined; the extract in this case showed MIC value of 750 µg/ml, 600 µg/ml, 400 µg/ml, 450 µg/ml and 550 µg/ml fo*r C albicans, Penicillium chrysogenum, P exigua*, *Sclerotium rolfsii* and *Sclerotinia scleratiourum* respectively. Based on these results we can suggest that this CPR5 isolate could be excellent candidate for advance studies of their antibacterial bioactive compounds. The MIC values of the group of bacteria which were tested in department of microbiology IMS BHU where chloramphenicol was used as positive control in this group of bacteria and results are enlisted in Table 3.

The MIC value for bacteria and fungi showed prominent difference for bacterial and fungal group. It was higher for fungi as compared to bacteria. Composition and structural difference may be one of the possible reasons. The MIC value (Table 7) of crude extracts for both bacterial and fungal species explained that the molecules present in crude extract could be a good source of antimicrobial metabolites which can be effectively applied against human pathogenic and plant pathogenic microorganisms. Therefore further work should be done to explore structure and biological properties fungal bioactive metabolite

Table 7 Minimum inhibitory concentration (MIC, µg/ml) of the crude ethyl acetate metabolites produced by isolate CPR-5 which displayed the stronger antibacterial activity.

Minimum Inhibitory Concentration (µg/Ml) of Crude Extract			
bacteria		fungi	
E.coli	240	*C. albicans*	750
B.subtilis	150	*P.chrysogenum*	600
P.aeroginosa	200	*P. exigua*	400
S.aureus	250	*S. rolfsii*	450
X. oryzae	140	*S. sclerationrum*	550

ANOVA Test

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	388090	388090	26.50888	8.75745E-4
Error	8	117120	14640		
Total	9	505210			

Null Hypothesis: The means of all levels are equal
Alternative Hypothesis: The means of one or more levels are different
At the 0.05 level, the population means are significantly different.

Fit Statistics

R-Square	Coeff Var	Root MSE	Data Mean
0.76818	0.30788	120.99587	393

Means Comparisons

Tukey Test

		MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Level2	Level1	394	76.52451	7.28133	8.75787E-4	0.05	1	217.5345	570.4655

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals 0 indicates that the means difference is not significant at the 0.05 level.

ANOVA analysis was performed for analysis of correlation among inhibition zone of crude extract for various bacterial and plant pathogenic fungal species. An auto correlation among crude extract can yield high value of R-square even for poorly fitted models. The results of t-test gave insignificant value (8.757 E-7) at 95% of confidence level. An insignificant P-value is desirable because it empied crude extract are uncorrelated and and indicated goodness of model in predicting desirable response.

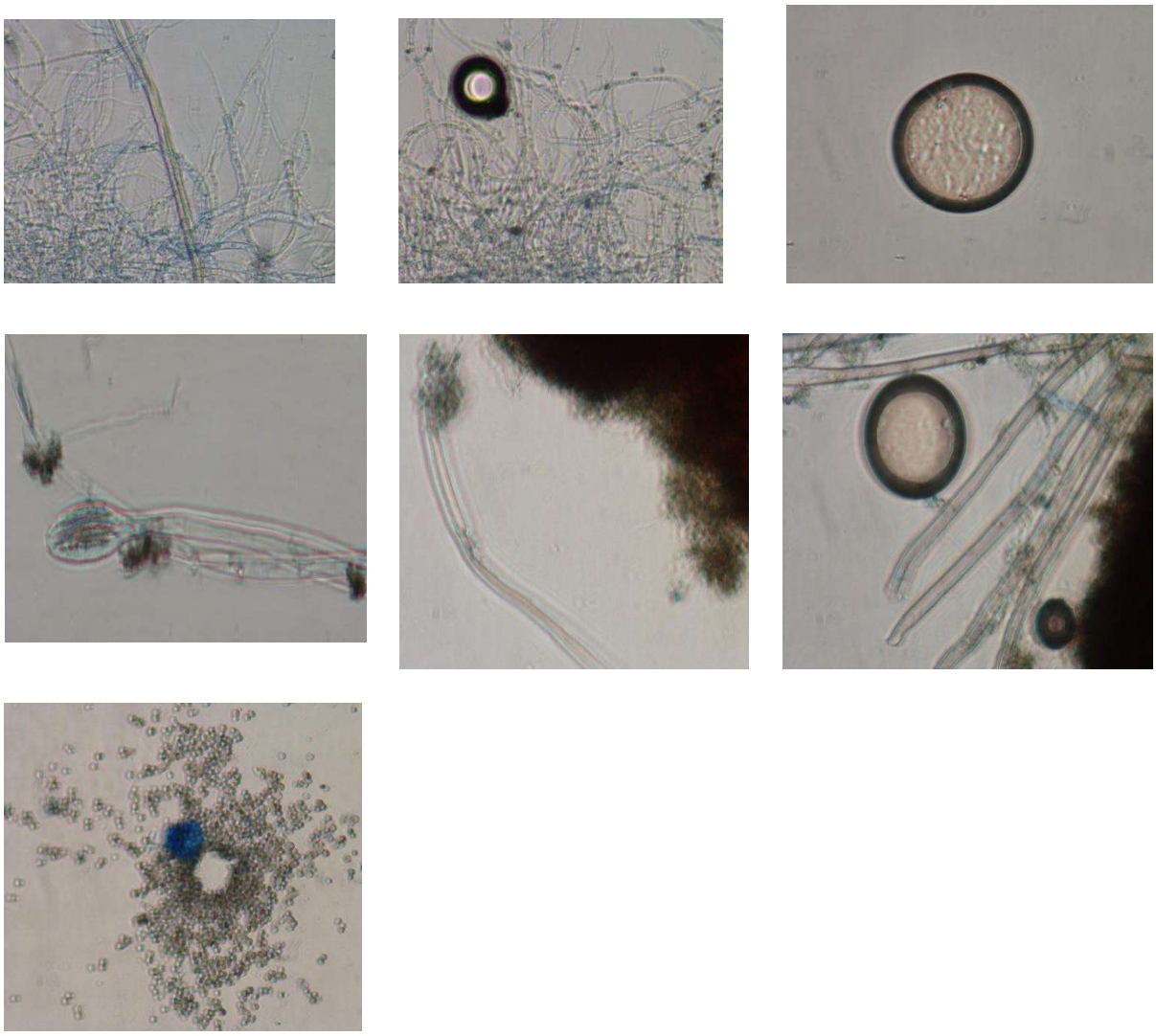


Fig. 9 (a) fungal hypha, (b) Metula in fungal mycelia (c) Single Spore, mycelia with conidia, (d) Pre matured cnidium , (e) Ruptured conidium, (f) Aspergillus isolate spores,

4.3 Morphological and Molecular Identification of Isolate CPR5

The endophytic fungal isolate was separated from healthy root of Calotropis procera and fungus was identified as dark brown to black colony, with pale brown margins on when grown on PDA medium. The colony on PDA is typically spherical blackish dark brown and mycelium is colourless and inconspicuous. Microscopic studies of the fungus isolate has shown the ova shaped conidiophores as smooth to finely rough walled, their size vary from

200 to 300 nm in length, up to 6-8 μm in diameter, enlarging gradually into vesicles of 18–20 mm diameter. Metulae has been clearly seen. The isolate produced septet, hyaline intermingled hyphae, simple or branched and numerous single-celled conidia. Conidia are mostly subglobose-globose to ellipsoidal, 2.0–2.5 mm in length, smooth surfaced, adhering in long compact columns. Identification of fungal species was done on the basis of cultural and morphological characteristics features like appearance of colony, colony colour, texture and margins, as well as microscopic observations, such as size of conidia and conidiophores and their arrangements were examined for species differentiation (Klich, 2002; Raper & Fennell, 1965). As a final we compared the morphological characteristics of tested Aspergillus isolates with those of the standard species and confirmed it as *aspergillus niger* species. Morphological characteristics have been shown in following below given figures (Fig.9). The Based on these typical features, the fungus has been identified as *Aspergillus niger*. The rDNA of ITS region was amplified and the PCR product was bidirectionally sequenced using forward (ITS4) and reverse (ITS5) primers. Based on BLAST search analysis the fungus was identified as *Aspergillus niger sp*. The 18S rRNA sequencing (Fig.10) followed by basic local alignment search tool (BLAST) and SeqMatch analysis was used for molecular conformation endophytic isolate.. The 18S rRNA sequence was submitted to the DNA Data Bank of Japan (DDBJ) and assigned an accession number (**LC062385**). The 18S rRNA gene sequence was used to build a Phylogenetic tree (Fig. 10), by performing automated BLASTN searches, to determine the closest type strain to the isolate under investigation. The percentage of identity was found to be 96%. Phylogenetic tree analysis indicated that 18S ribosome RNA sequence of CPR-5 strain was closely related to *aspergillus niger* species. The phylogenetic tree suggested that the local isolated strain is

definitely a member of the genus *aspergillus* and formed a common phylogenetic lineage that could be equated with a novel local species

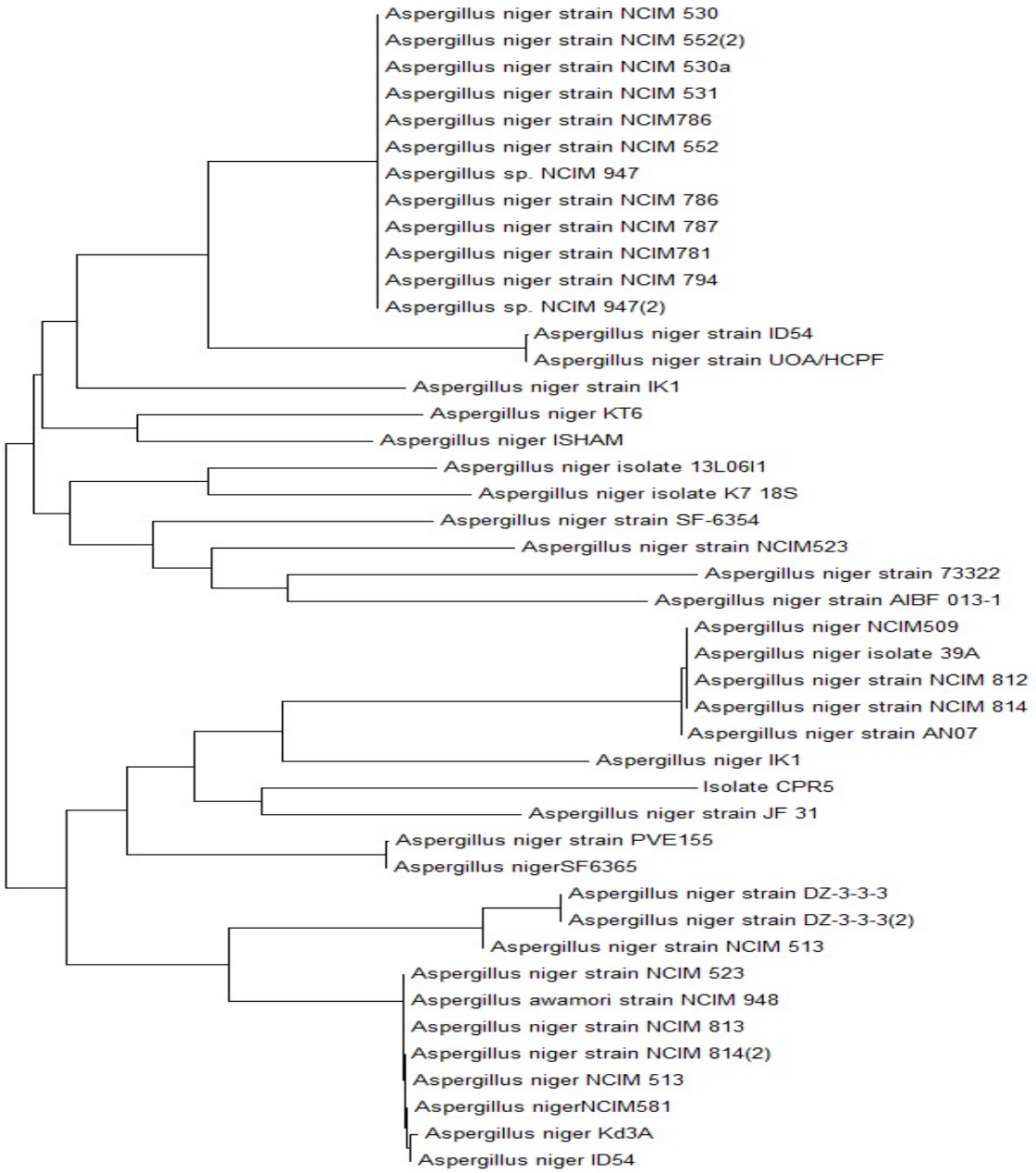


Fig. 10 Phylogenic tree based on 18S rRNA sequence showing the relationship between fungal endophytic isolate from *C. procera* (isolateCP R5) and reference strains, The evolutionary history was inferred using the UPGMA method.

Media optimization

4.4 Media optimization

The experiment was carried out to study optimization parameter of culture condition to maximize the production of antimicrobial agent. To design effective medium, it is necessary to evalue the effect of different carbon sources on the production of bioactive metabolite. Carbon is one of important souce for energy amd for synthesis of microbial cell membrane. Carcon sources which are rapidly metabolized, such as glucose, often lead to maximum groth of bio massbut a resuced production of metabolites. For optimization of different carbon sourse,1%(w/v) of all the carbon sources (glucose, Galactose, starch, fructose, maltose, lactose) were added in the production medium inoculated with CPR5 isolate and incubated for 8 days (Abbas, S., Subhan, M., Durrani, F., Mehmood, S., Khan, H. and Hameed, A. (2010). Starch was observed to support highest level of antimicrobial agent production (Fig. 11).Various yeast extract concentrations were tested (0.5 g/l to 4.5 g/l) to see the effect on growth and secondary metabolite production among the various concentration tested, yeast extract of 3 gm/l showed remarkable results followed by 3.5 g/l and 2.5 g/l concentrations (Fig. 12**).** Demain, A. L. and Fang, A. (1995)

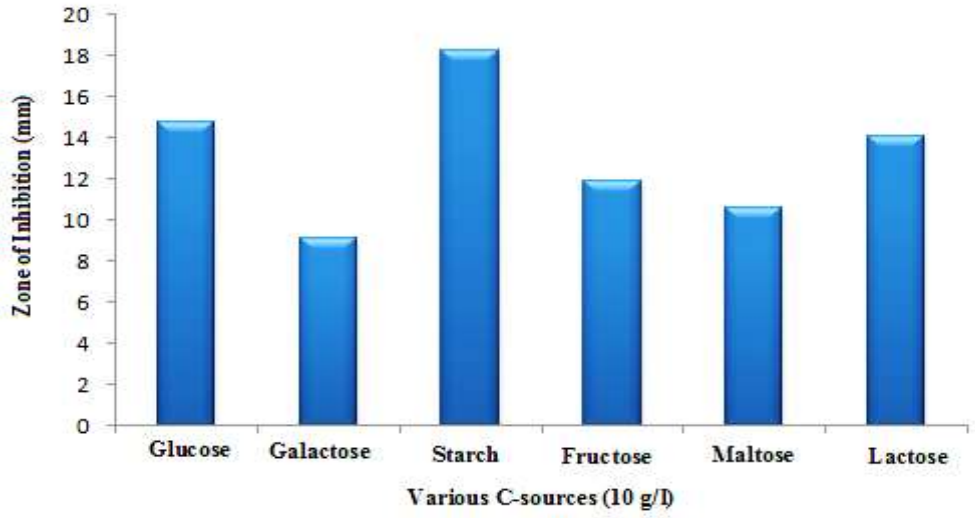


Fig. 11 Effect of various carbon sources on antimicrobial agent production

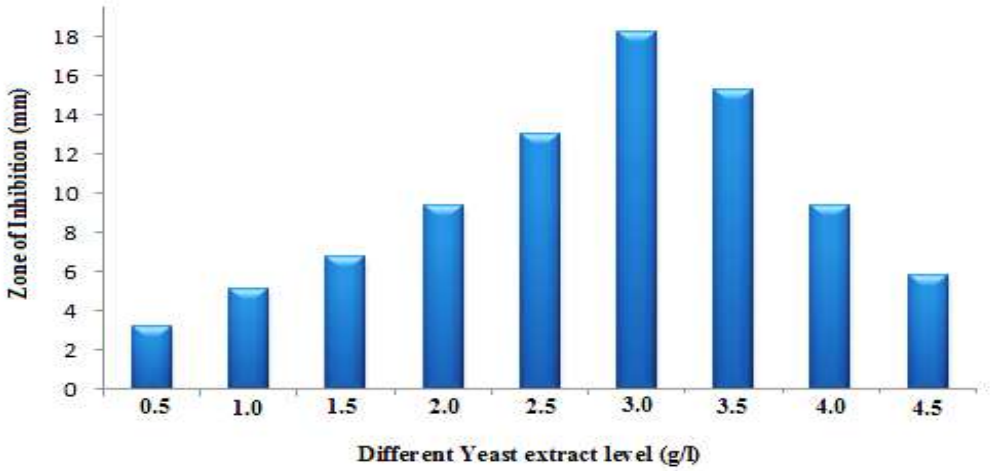


Fig.12 Effect of yeast extracts concentration on antimicrobial agent production

The incubation temperature directly affects the overall growth and development pattern of the organism and subsequently, the synthesis of various metabolites. Productions level of bioactive antibacterial compounds were observed at different temperatures like 15 ^{0}C, 20 ^{0}C, 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C. Maximum growth and bioactive metabolite production by

isolate CPR5 was recorded at incubation temperature 25 ^{0}C (±2) and it was followed by 30 ^{0}C, 35 ^{0}C, respectively (Fig. 12). Similar reports were reported by Griffith and Saker (2003); Lethimaki et al., 1997; Moita et al., 200, Noaman et al., 2004.

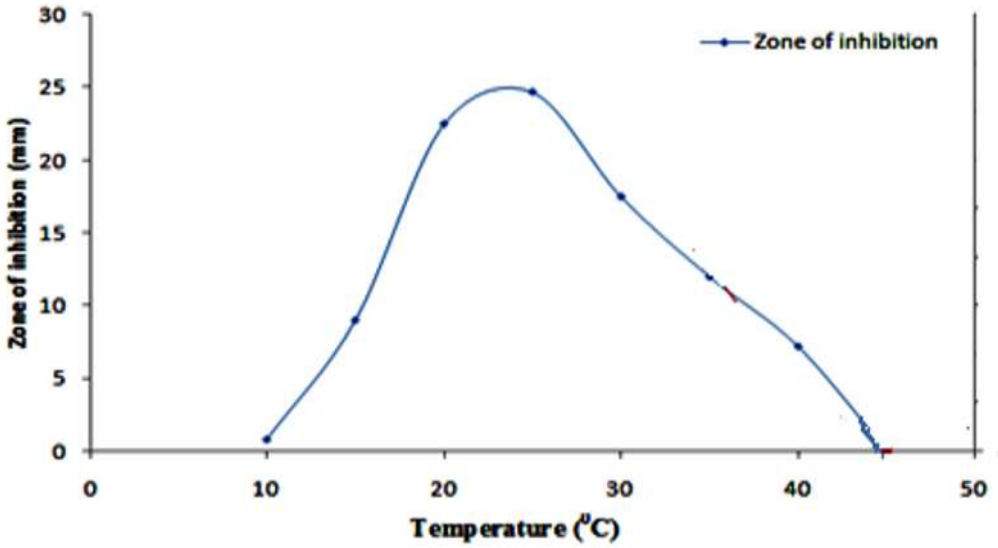


Fig. 13 Effect of temperature on production of antimicrobial metabolite

When effect of NaCl concentration was tested to see the effect of cell biomass production and bioactive metabolite production it was observed that NaCl concentration at 3%, 3.5% and 4% showed noticeable increase in both cell biomass and bioactive metabolite production and 3.5% of NaCl showed maximum observable values (Fig. 13). Similar reports were given by Sailer et al., 1993; Vahidi et al., 2004.

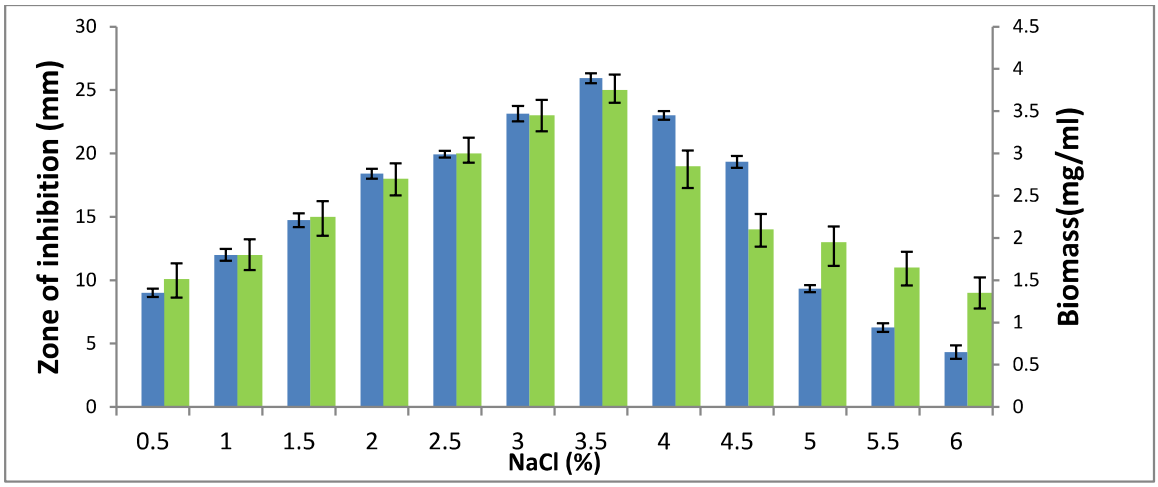


Fig. 14 Effect of various concentration of NaCl on production of antimicrobial metabolite

Initial pH 5.5 of the medium was observed to be the optimal for growth and bioactive metabolites production by CPR5 (Fig. 15). Although initial pH 7.0 also supported biomass production and bioactive metabolite production but lower yield was observed. No growth was observed at pH 3, 4 and pH 9, 11. Thongwai, and Kunopakarn, 2007; Digrak et al., (2001) reported that highest production of biomass by F. equiseti (1886) was at pH 8, whereas maximum toxic metabolite was produced at the pH 5. The pH of culture media is one of the very important determining parameter for the metabolism and hence for the biosynthesis of secondary metabolites. Permeability characteristics of the cell wall and membrane are also related to the cell environment pH value and thus have got effect on either ion uptake or loss to the nutrient medium (Hansen, 1968; Wang, 2000).

When incubation period was observed for antimicrobial metabolite production it was observed that maximum cell biomass and antimicrobial metabolit production was observed at 8 days and progressively decreases towords 9, 10 11 and 12 days. Although incubation period vary for different microbial soure beacuase there is diversity in metabolic pathway for antimicrobial metabolite production in different microbial source.

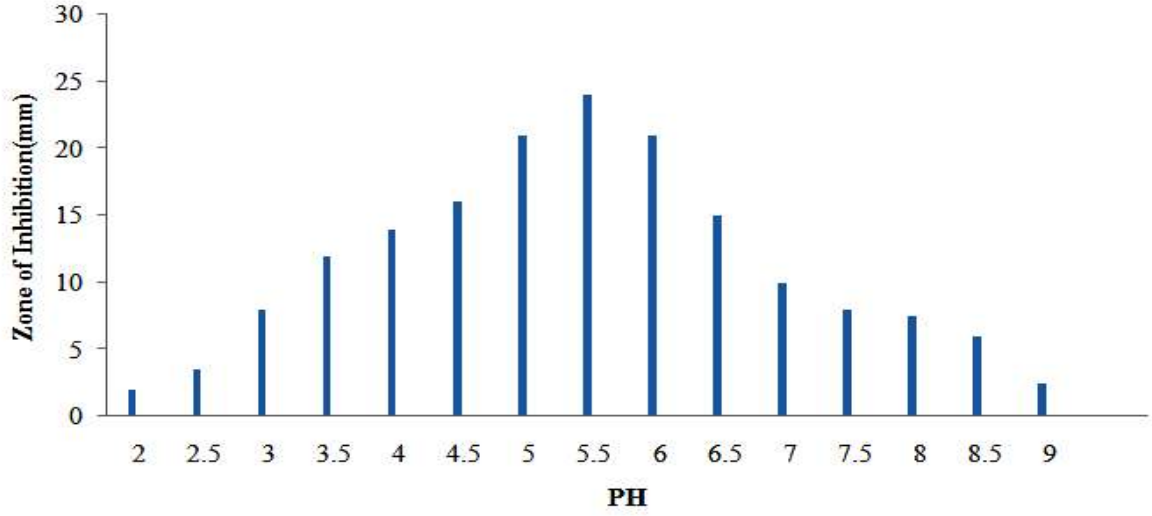


Fig. 15 Effect of initial pH on antimicrobial agent production

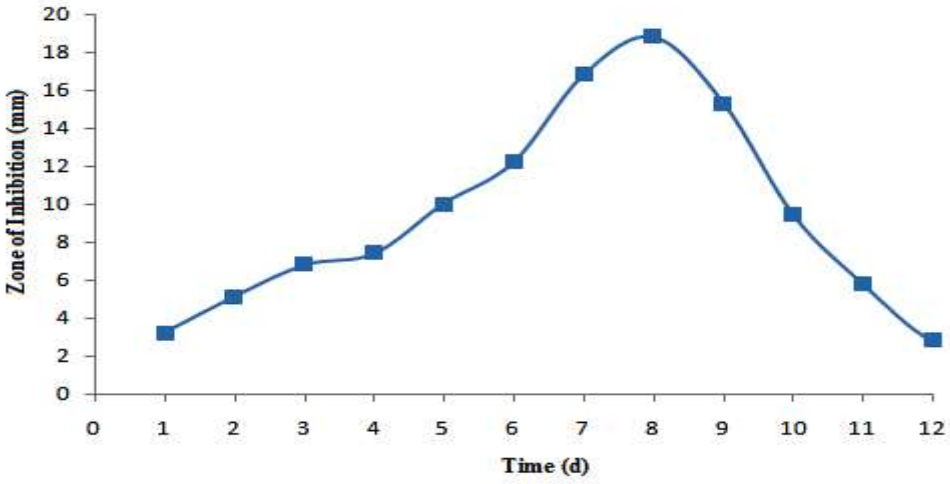


Fig. 16 Effect of incubation period on antimicrobial agent production

Effect of various Nitrogen sources was also studied. Equimolar concentration (2%, w/v) various nitrogen sources (sodium nitrate, potassium nitrate, yeast extract, peptone, and ammonium chloride and ammonium sulphate) were mixed in the production medium. The nature of nitrogen source used has a notable effect on the production of antimicrobial metabolite by fungal isolate. Depending on the biosynthetic pathway involved, nitrogen source may greatly influence production of antimicrobial metabolite production. Yeast extract was observed as the best nitrogen source for enhanced production of antimicrobial agent followed by potassium nitrate. The requirement of nitrogen source generally differs from one microorganism to another. In case of most microorganisms both inorganic and organic nitrogen source are metabolized to produce protein nucleic acid and various cell components. But it has also been observed that some carbon and nitrogen source have inhibitory effect of antimicrobial agent production. This negative result may be due to acid accumulation resulting in pH imbalance and oxygen depletion due to sugar catabolic repression.

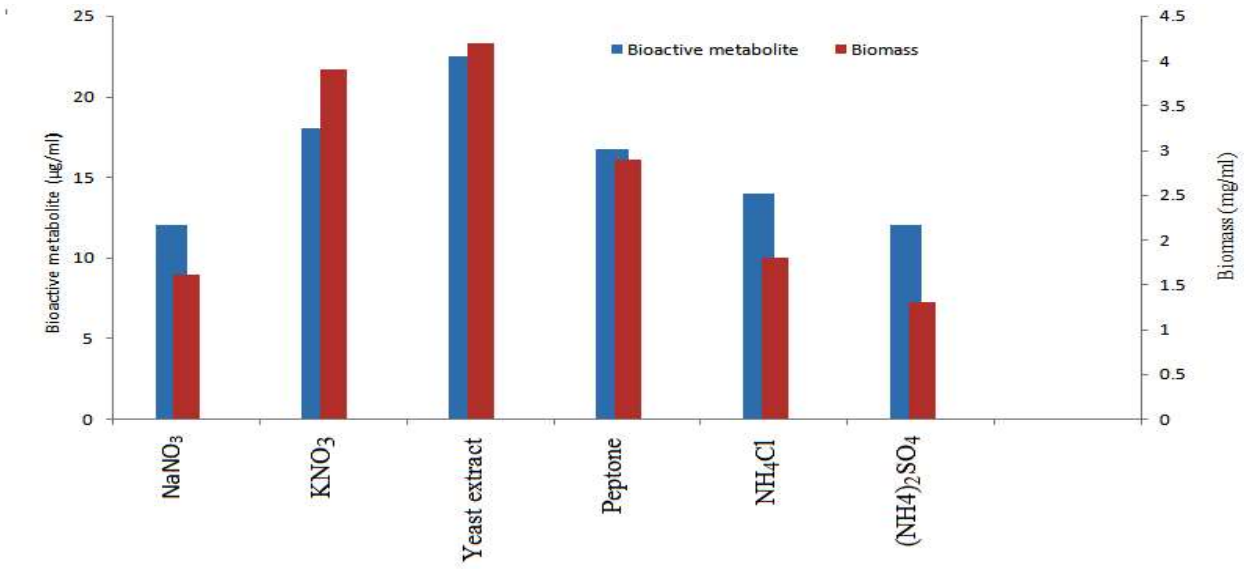


Fig. 17 Effect of nitrogen sources on antimicrobial agent production

Maximum antimicrobial agent was produced in starch medium followed glucose, by the isolate. In contrast low activity was observed with maltose, lactose with least activity was shown by galactose, these carbon sources may affect metabolic pathway of primary and secondary metabolite production. It has been reported that generally addition of glucose enhance the metabolite production but its significance in many fermentation process decrease because at higher concentration it has inhibitory effect.

4.5 SPECTROSCOPIC ANALYSIS OF COMPOUNDS

Compound 1

2-hydroxypropyl 5, 11-dihydroxy-8-(4-hydroxy-5-(hydroxyl methyl) tetrahydrofuran-2-yl) 17-methyleoctadecanate

Dark brown gum like, Composition (64.25%) H (10.39%) O (25.36%) molecular formula $C_{27}H_{52}O_8$, ESI-MS (m/z) 504.69698

^{1}H NMR (dmso-d_6,300 MHZ)1.25-145ppm(m,20H), 3.21(m,2H), 3.90 (m,1H), 0.90 (d,6H), 1.18 (d,3H), 1.79 (m,1H), 4.40-1.80 (m, H-pentose),3.58(m,3H), ^{13} C NMR(75

MHZ) DMSO-d_6; 172-174ppm(-C=O),163ppm(amid group),110ppm(C=C),27-38ppm (C-C),73ppm(C-OH), 85-93ppm(C-O-C), IR (KBr) υmax (cm^{-1}) 3250,1200

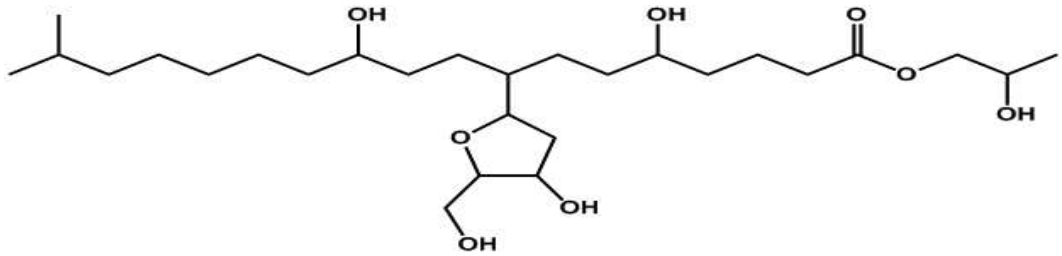


Fig. 18 2-hydroxypropyl 5, 11-dihydroxy-8-(4-hydroxy-5-(hydroxyl methyl) tetrahydrofuran-2-yl) 17-methyleoctadecanate

The results are in partial agreement with (Shaaban, M. (2004)

Compound 1 was observed active against- *E.coli, Streptococcus pneumonia, B. subtilis, Staphylococcus hyicus, B. sphaericus, Staphylococcus aureus,* and *pseudomonas aeruginosa, Candida albicans, Sclerotium rolfsii, Sclerotinia, scleratiourm, Fusarium species* and *Penicillin sp*

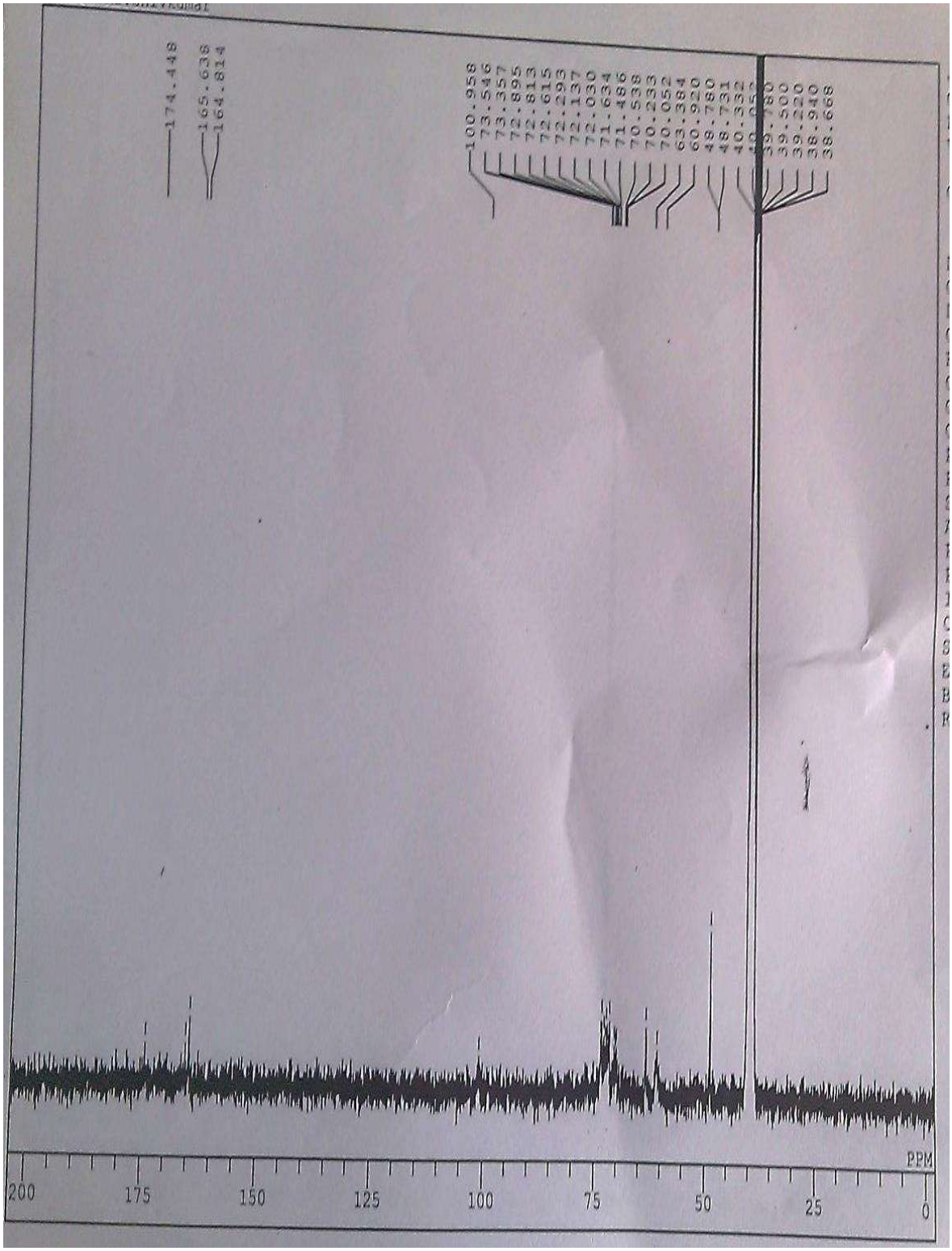


Fig. 19 ^{13}C MNR of Compound 1

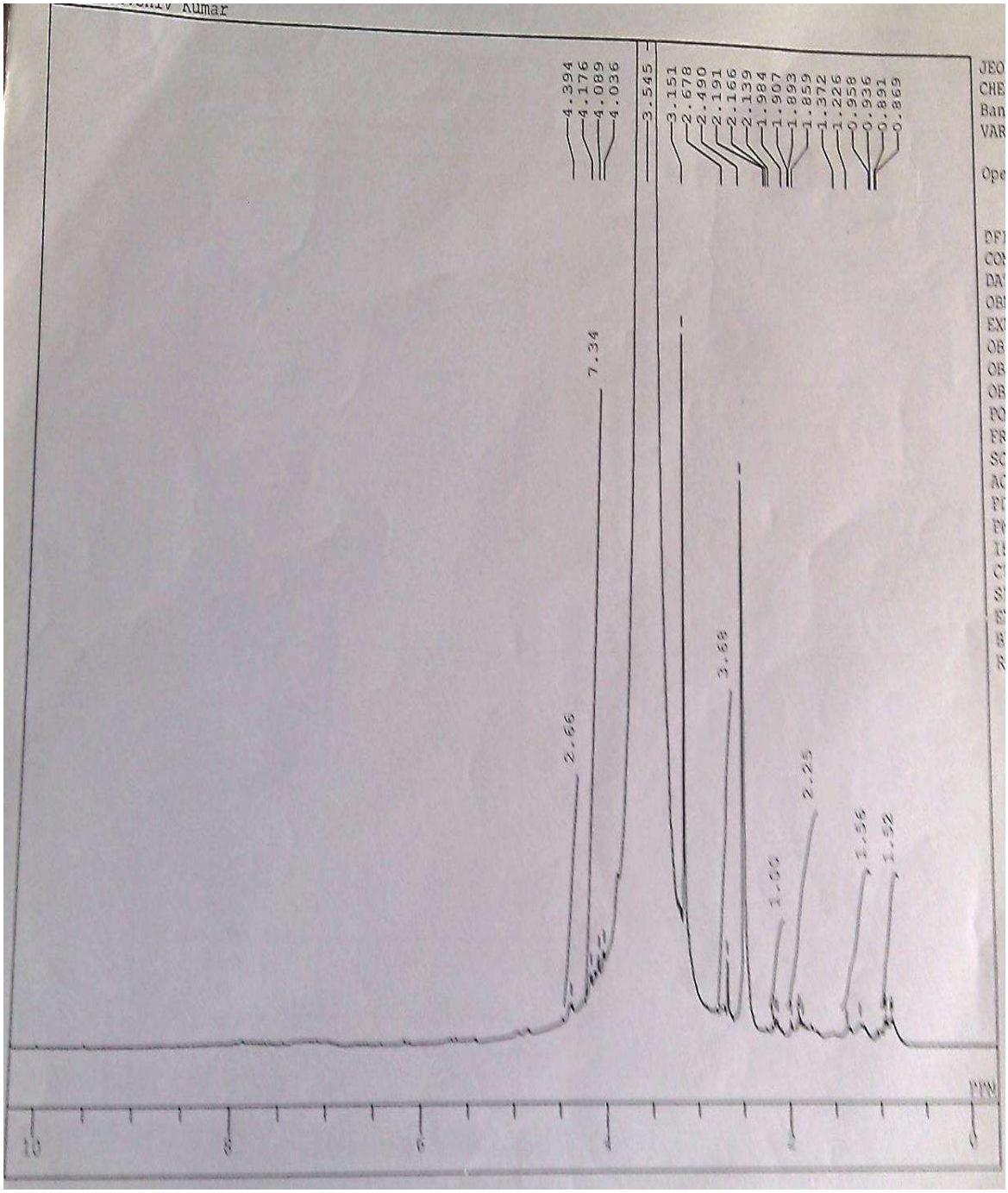
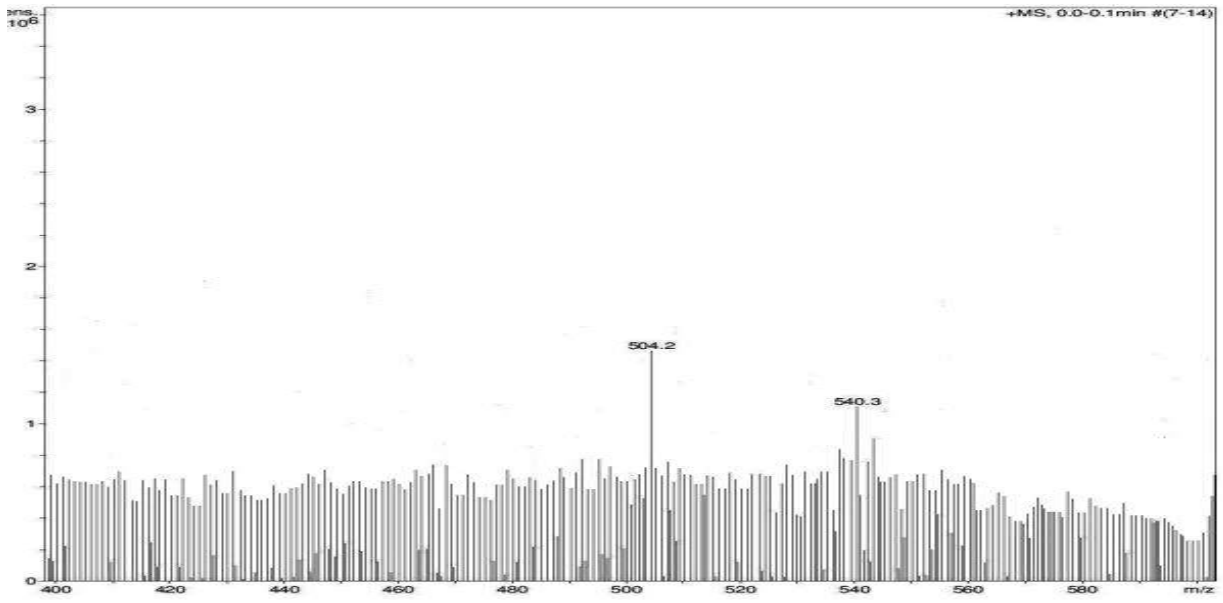


Fig. 20 ^{1}H NMR of Compound 1



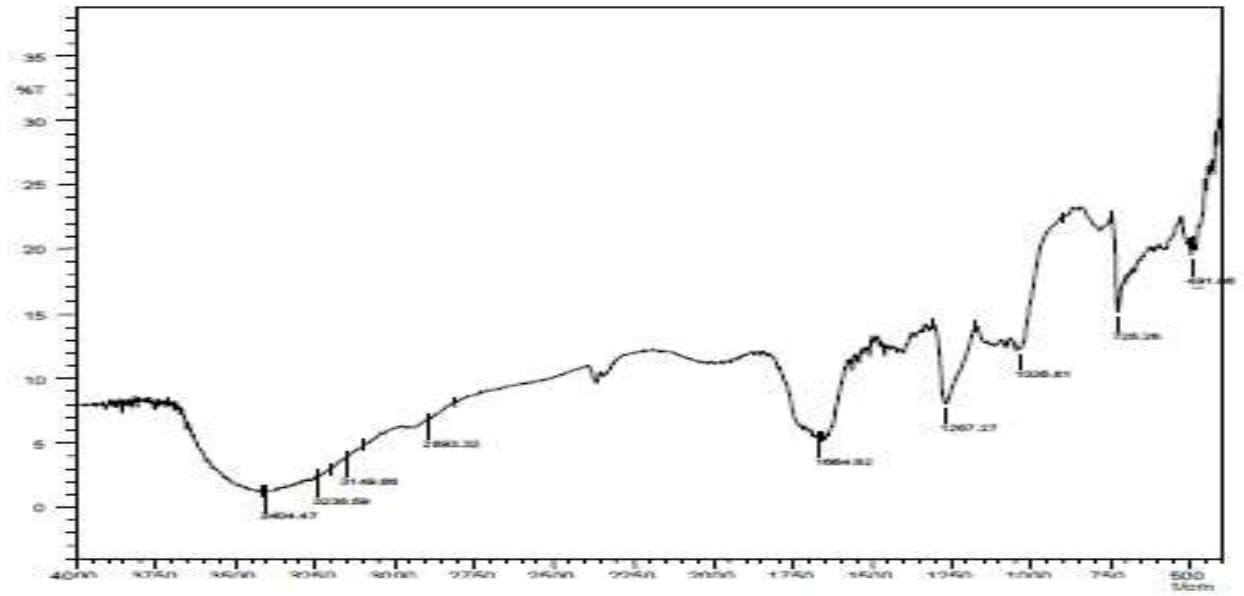


Fig. 21 ESI-MS and FT-IR of Compound 1

Compound 2

Black brown gum like, Composition: C(62.65%) H (9.62%) N(5.35%) O(22.39%) molecular formula $C_{41}H_{75}N_3O_{11}$, ESI-MS (m/z) 785.939612

^{1}H NMR (DMSO-d_6,300 MHZ), 3.3-3.8ppm (m, 2H-glucose), 4.43 (m,2H), 4.52(m,1H), 7.10-7.90 (m,4H- - NH_2), 1.96 (t,4H), 1.33(t,2H), 1.29 (m,10H), 1.25(m,12H), 5.62-5.7 (m,4H),4.85(s,1H), 0.96(d,3H), ^{13} C NMR (75 MHZ) DMSO; 172-174 ppm, (-C=O),163ppm(amide group),110ppm(C=C), 27-38ppm (C-C),73ppm(C-OH), 75ppm(C-O-C). IR (KBr) υ max (cm^{-1}) 3250, 1200, 1650, 1000, 1062

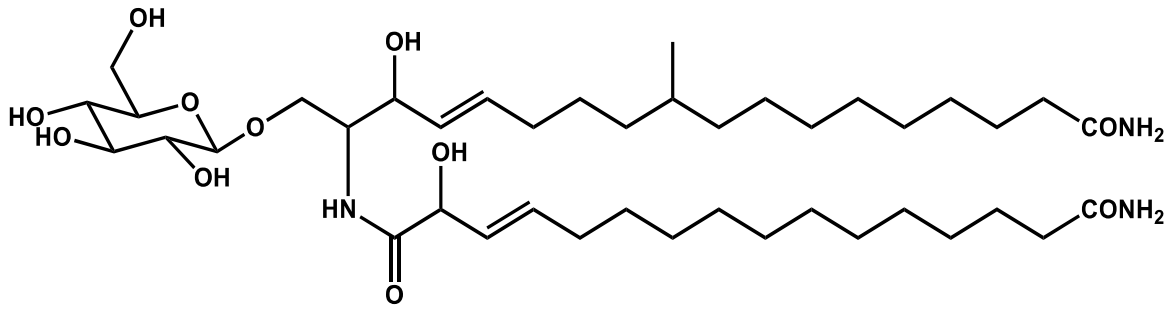


Fig. 22 (E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy)octadec-4-en-2-yl)-2-hydroxyhexadec-3-enediamide

Compound 2 was observed active against-*X.oryzae,* E.coli,*Streptococcus pneumonia, B.subtilis, Staphylococcus hyicus, B. sphaericus,Staphylococcus aureus*, and *pseudomonas aeruginosa, Candida albicans, Sclerotium rolfsii,* Phoma exigua,*Sclerotinia, scleratiourm*

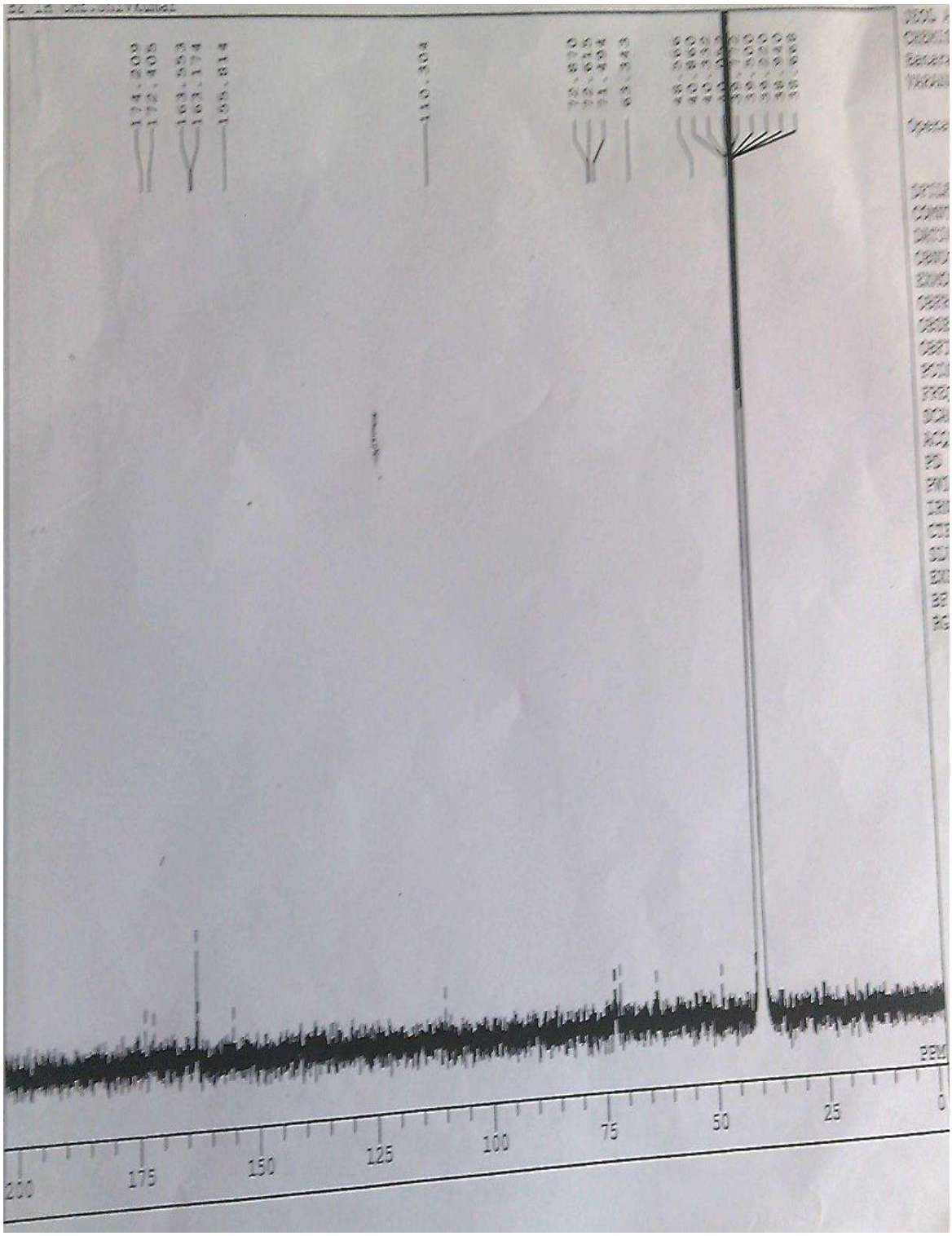


Fig. 23 ^{1}H NMR of Compound 2

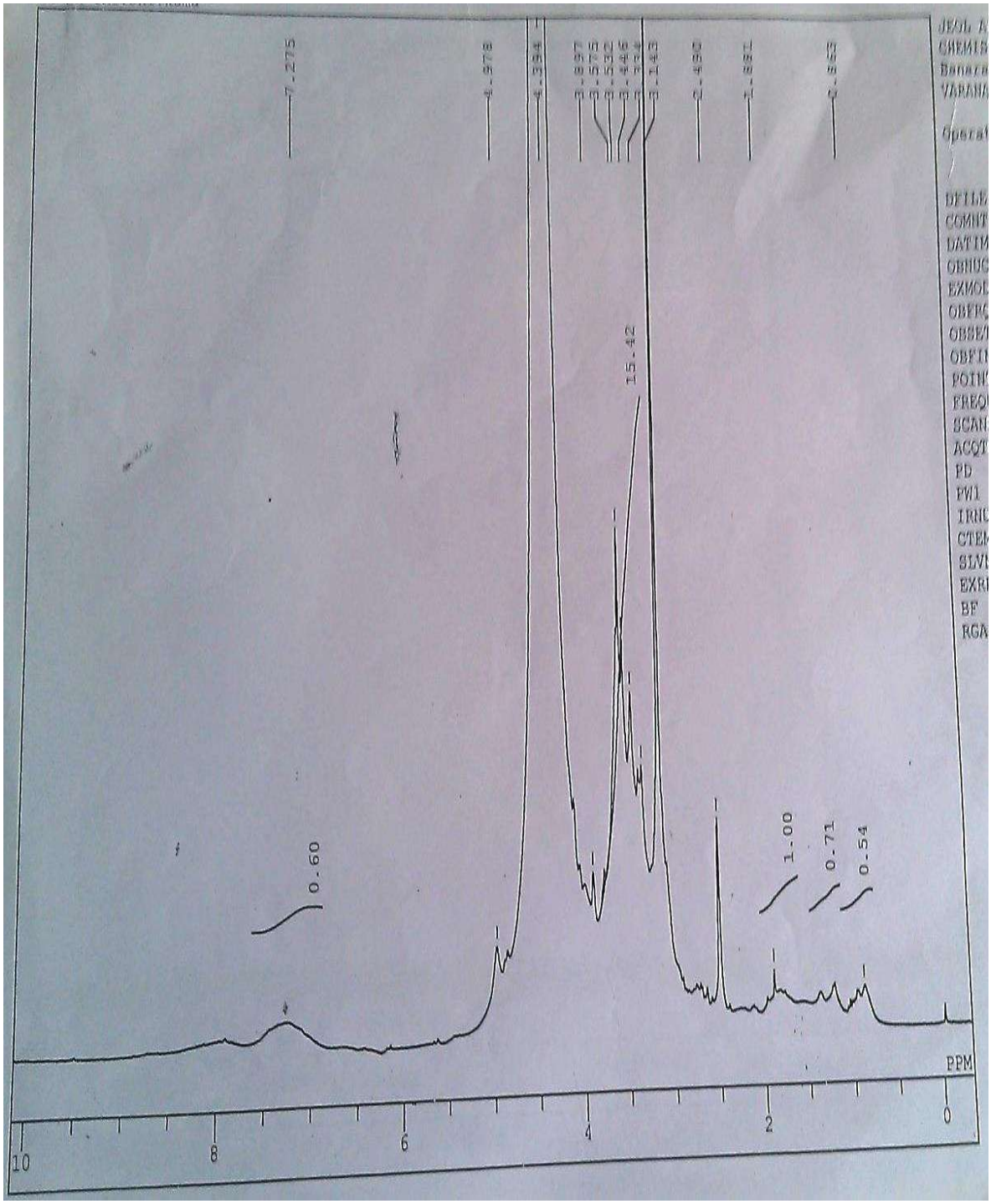
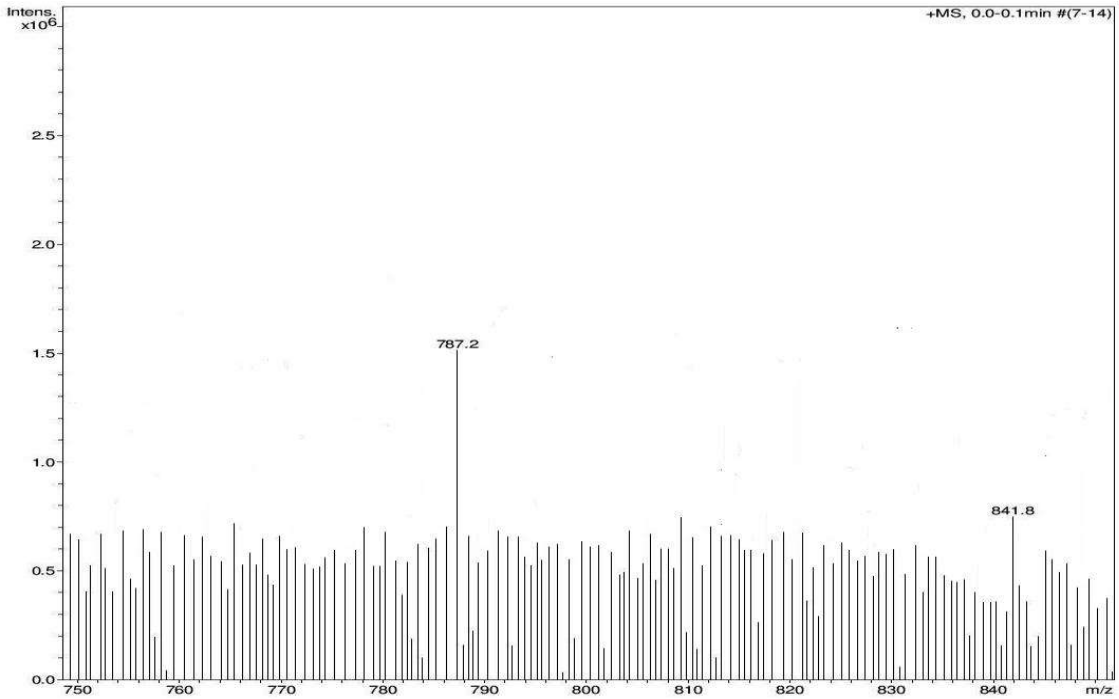


Fig. 24 ^{13}C NMR of Compound 2



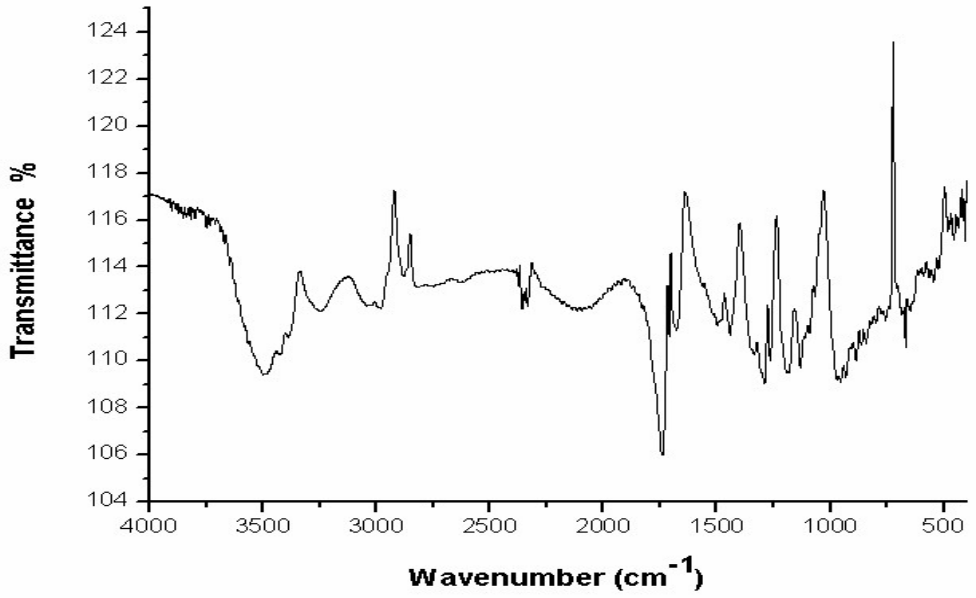


Fig. 25 ESI-MS and FT-IR of Compound 2

Compound 3

(E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)octadec-4-en-2-yl)-2-hydroxy-7-methylhexadec-3-enediamide.

Dark brown gum like, Composition: C(67.98%) H(10.73%) N(1.89%) O(19.40%) molecular formula $C_{42}H_{79}NO_9$, ESI-MS (m/z) 742.07796.

^{1}H NMR (dmso-d_6,,300 MHZ), 3.3-3.8ppm(m,2H-glucose), 4.43 ppm (m,2H), 4.52 ppm (m,1H), 1.96 (t,4H), 1.33(t,2H), 1.29 (m,10H), 1.25(m,12H), 5.62-5.7 (m,4H),4.85(s,1H), 0.96(d,3H),0.88 (m,6H), 1.31(m,4H), ^{13} C NMR(75 MHZ) DMSO-d_6;172-174ppm(-C=O),163ppm(amid group),110ppm(C=C),27-38ppm (C-C),73ppm(C-OH), 75ppm(C-O-C),134-135pppm(C=C). IR (KBr) υmax cm−13400, 1650, 3100, 1000, 1062.

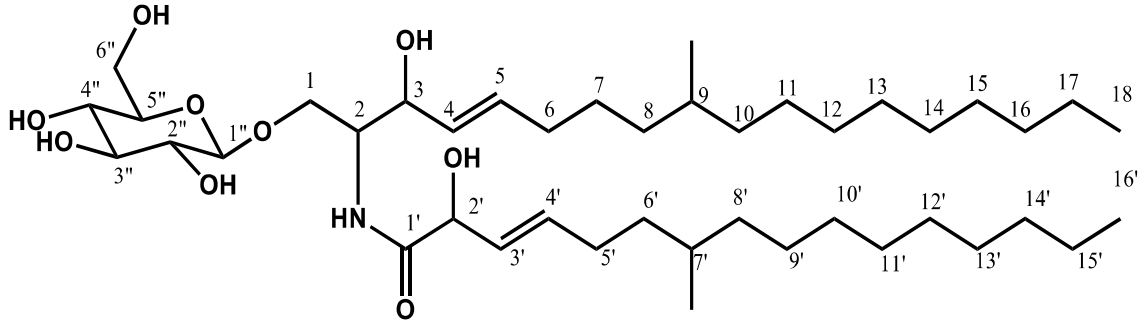


Fig. 26 (E)-N1-((E)-18-amino-3-hydroxy-9-methyl-18-oxo-1-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)octadec-4-en-2-yl)-2-hydroxy-7-methylhexadec-3-enediamide

Compound 3 was observed active against- *E.coli*, *Streptococcus pneumonia, B.subtilis, B. sphaericus, Staphylococcus aureus*, and *pseudomonas aeruginosa, Candida albicans, Sclerotium rolfsii, Phoma exigua*, *Sclerotinia, scleratiourm, scleratiourm*

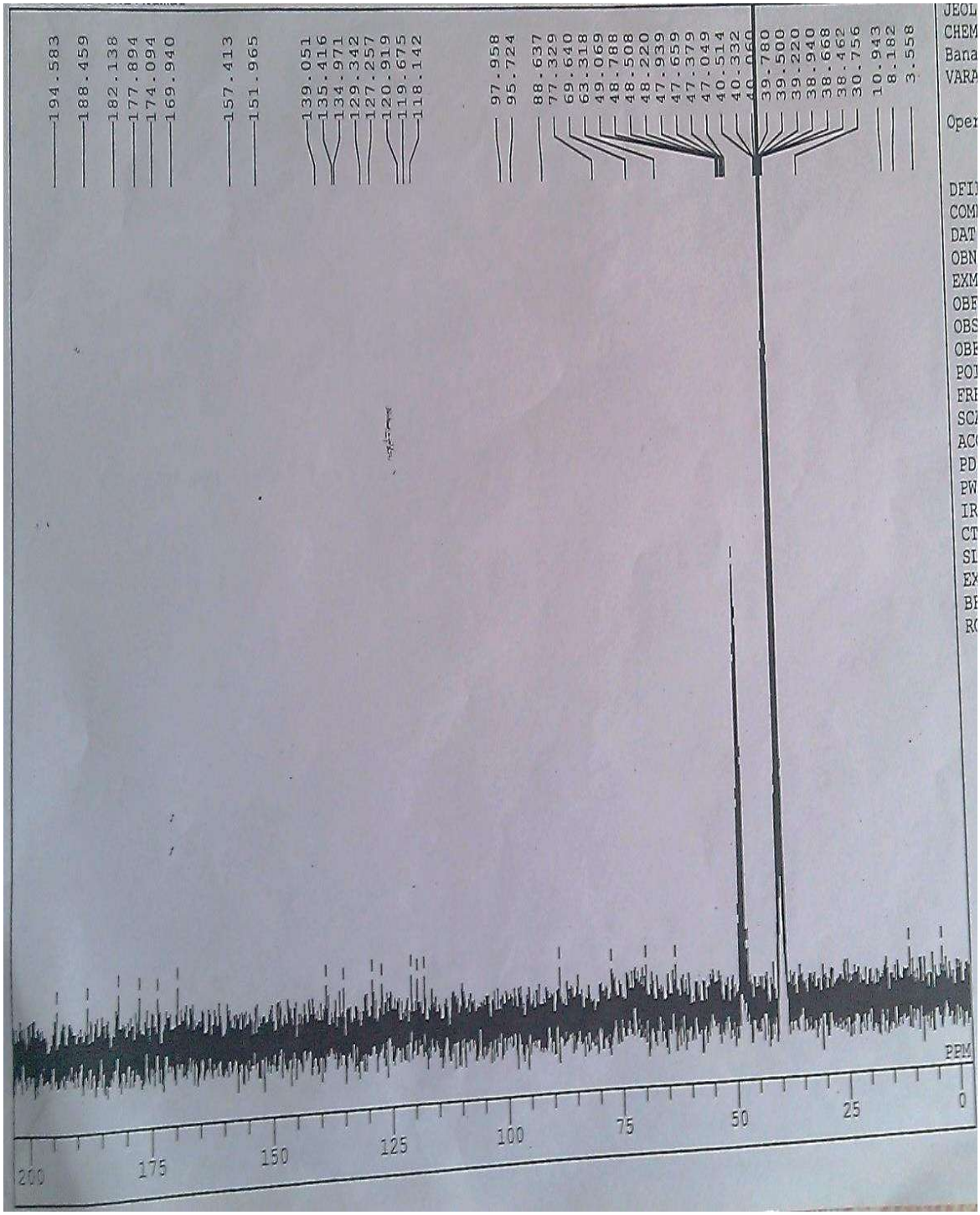


Fig. 27 ^{13}C NMR of Compound 3

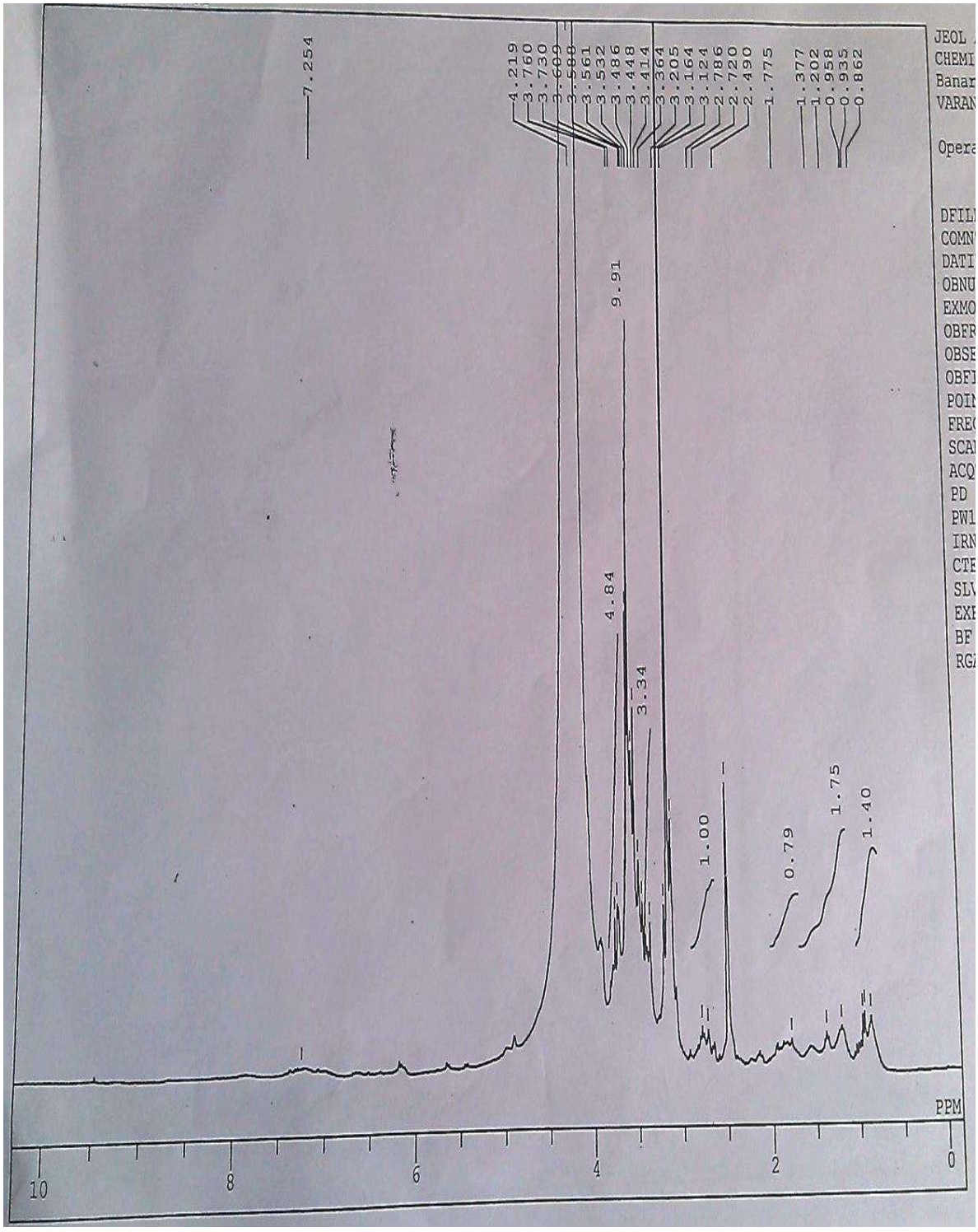


Fig. 28 ^{1}H NMR of Compound 3

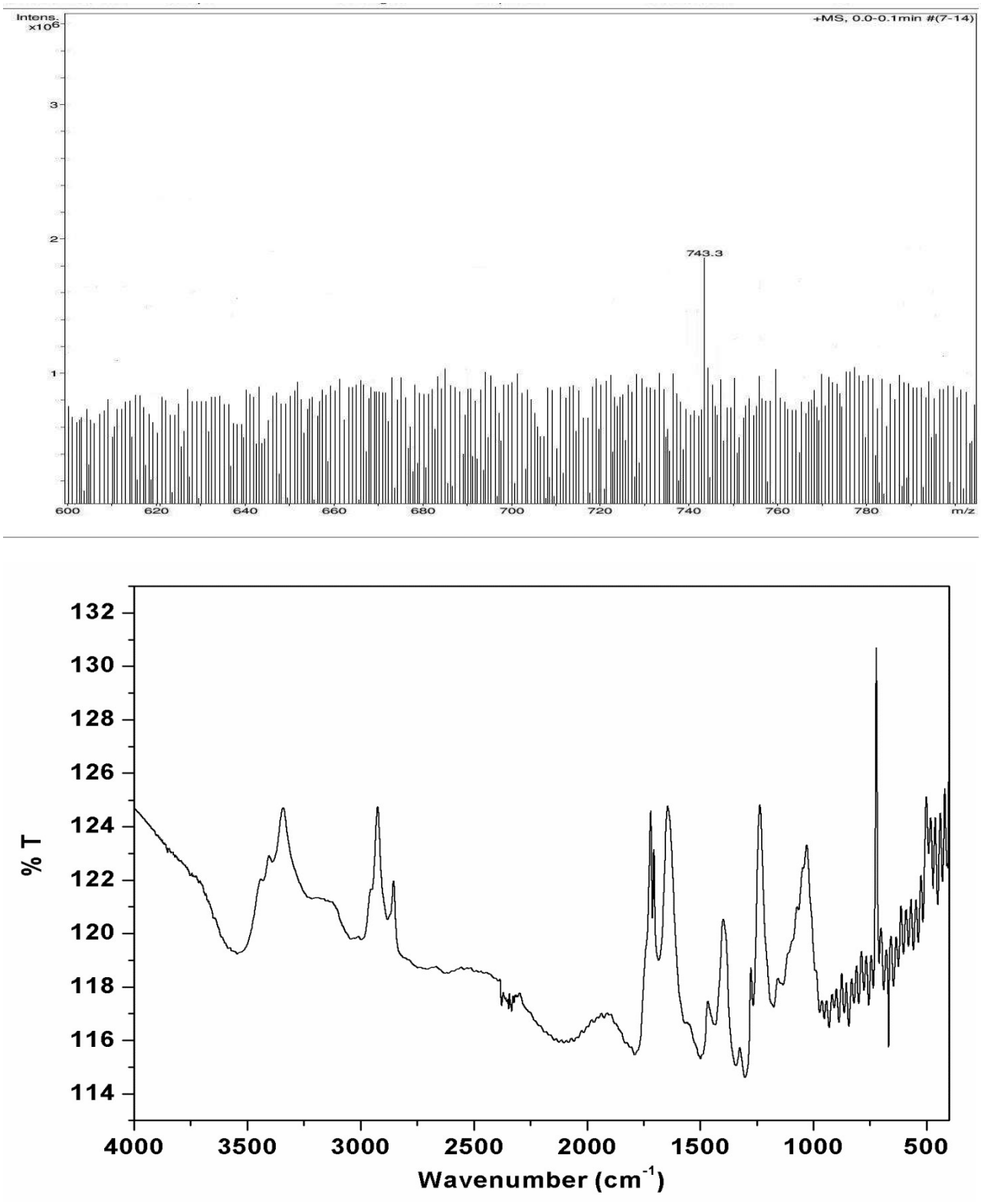


Fig. 29 ESI-MS and FT-IR of Compound 3

Compound 4

(E)-17-((E)-16-amino-2-hydroxy-7-methyl-16-oxohexadec-3-enamido)-16-hydroxy-10-methyl-18-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)octadec-14-enoic acid.

Dark brown gum like, Composition: C (62.97%) H (9.56%) N (3.50%) O (23.97%) molecular formula $C_{42}H_{76}N_2O_{12}$, ESI-MS (m/z) 801.05904

^{1}H NMR (dmso-d_6,,300 MHZ), 3.3-3.8ppm(m,2H-glucose), 4.43 (m,2H), 4.52(m,1H), 7.10-7.90 (m,4H- - NH_2), 1.96 (t,4H), 1.33(t,2H), 1.29 (m,10H), 1.25(m,12H), 5.62-5.7 (m,4H),4.85(s,1H), 0.96(d,3H), 2.30 (t,2H), ^{13} C NMR(75 MHZ) DMSO-d_6;172-174ppm(-C=O),163ppm(amid group),110ppm(C=C),27-38ppm (C-C),73ppm(C-OH), 75ppm(C-O-C),134-135pppm(C=C). IR (KBr) υmax (cm^{-1}) 1700, 1650, 1725, 3400, 1650, 3100,1000,1062

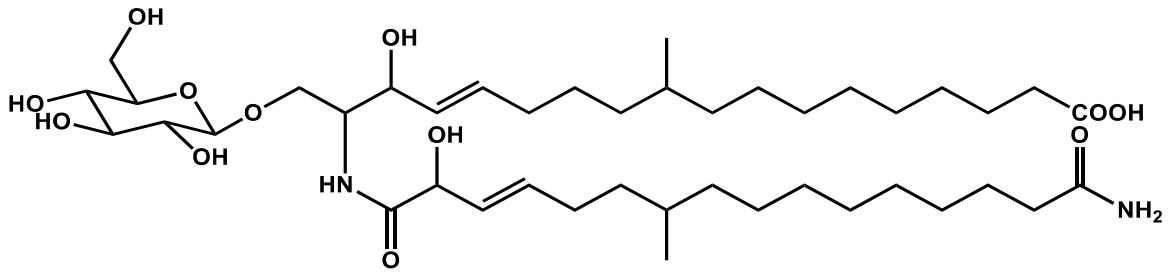


Fig 30 (E)-17-((E)-16-amino-2-hydroxy-7-methyl-16-oxohexadec-3-enamido)-16-hydroxy-10-methyl-18-(((2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)octadec-14-enoic acid.

Compound 4 was observed active against-

E.coli, Streptococcus pneumonia, B.subtilis, Staphylococcus hyicus, B. Sphaericus, Staphylococcus aureus, and pseudomonas aeruginosa Candida albicans, Phoma exigua, Sclerotium rolfsii, Sclerotinia, scleratiourm Fusarium sp. and Penicillin sp.

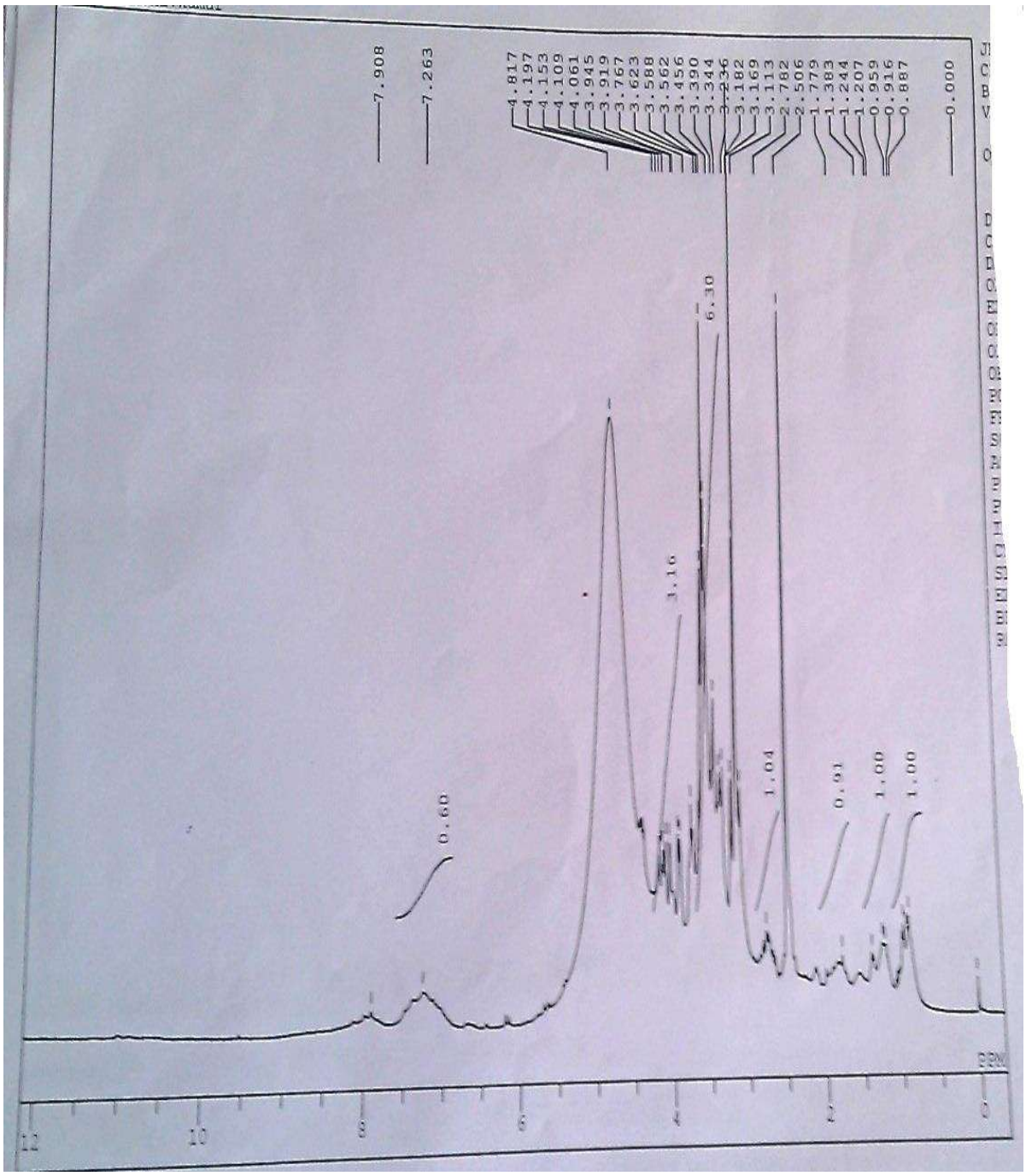


Fig. 31 ^{1}H NMR of Compound 4

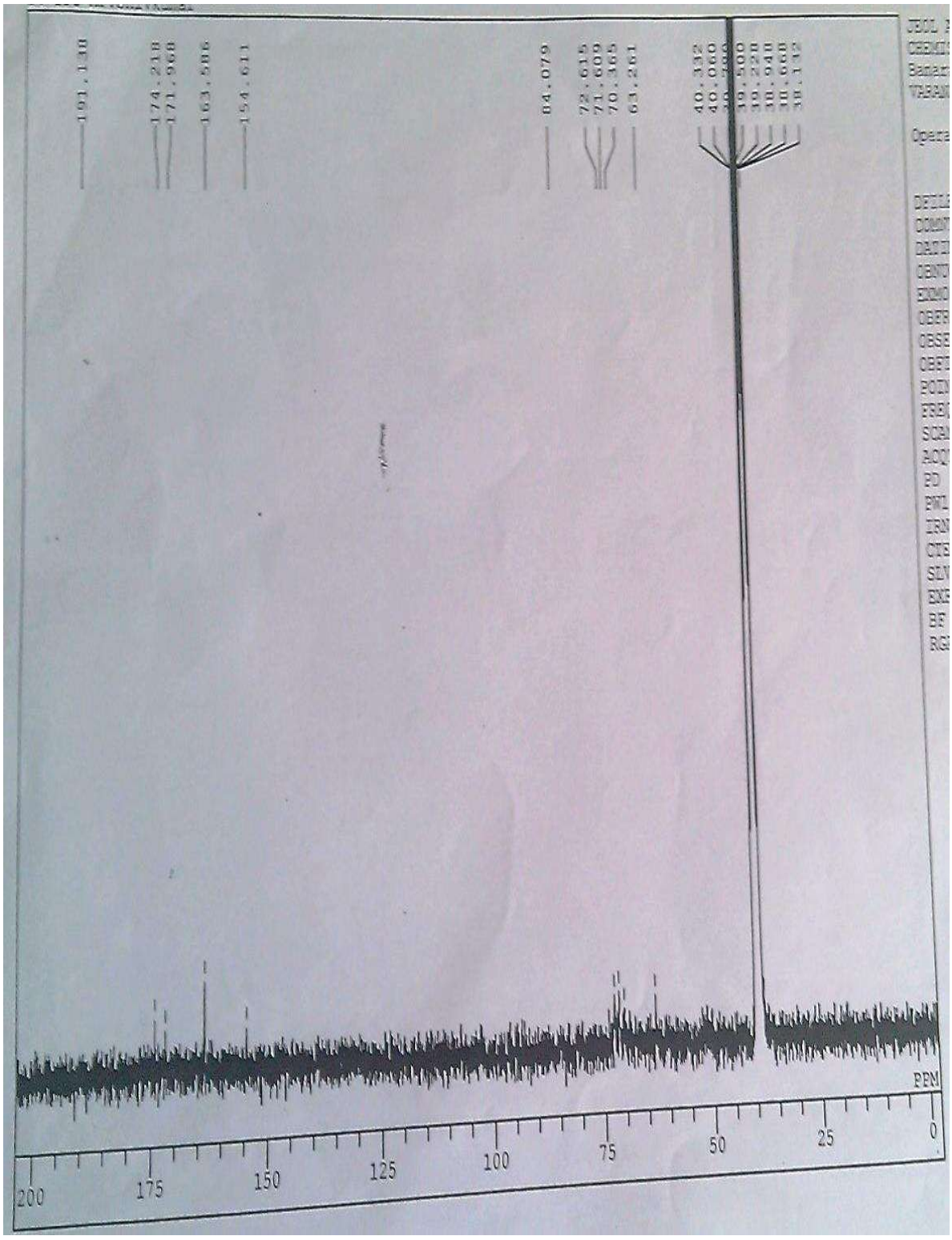


Fig. 32 ^{13}C NMR of Compound 4