CHAPTER 4

4. EXTRACTION, PURIFICATION AND STRUCTURAL ELUCIDATION OF BIOACTIVE COMPOUNDS FROM MARINE ACTINOMYCETES

4.1 INTRODUCTION

Antibiotics are antimicrobial compounds produced by living microorganisms. These compounds were used therapeutically and some times prophylactically in the control of infectious diseases. Over 4,000 antibiotics have been isolated before, but only 50 have achieved wide usage. The other antibiotic compounds failed to achieve commercial importance for some reasons such as toxicity to human and animal ineffectiveness or high production costs. Filamentous soil bacteria belonging to the genus *Streptomyces* is identified as a major source of bioactive natural products representing some 70-80% of the all isolated compounds (Bull *et al*., 1992; El-Naggar *et al.*, 2003; Berdy, 2005; El-Naggar *et al.,* 2006).

In the late 1990s there had been about 40 new bioactive microbial products from marine organisms reported (Jensen and Fenical 1994; Bernan *et al.,* 1997) near half of them were found in actinomycetes, demonstrating that the hit rate of new antibiotic from marine actinomyces is higher than from other microbial species. The majority of these marine actinomycetes were isolated from sea sediments. Marine actinomycete isolate M097 from two anthraquinone compounds that structures were elucidated as Aloesaponarin II and 1,6-dihydroxy-8-hydroxymethylanthraquinone. It is the first time, reported as a secondary metabolite from a wild-type strain. That strain could be a promising material for studying the biosynthetic pathway of polyketides and the production of novel recombinant polyketides. Researches have shown that actinomycetes isolated from the marine environment gave a broad spectrum of interesting metabolites (Ismet *et al.,* 2004; Jensen *et al.,* 1991).

Actinomycetes are an amazingly productive source of structurally diverse secondary metabolites including many that possess pharmaceutically relevant biological

activities (Berdy, 2005). Marine organisms associated with actinomycetes could be a promising source for antitumor and antimicrobial bioactive agents. (Zhonghui *et al.*, 2000) reported approximately 43.6%, 20% and 3% of the tested marine actinomycetes cultures displayed antimicrobial activity, Cytotoxic activity and inducing activity respectively. The macrolactams isolated from two strains of *Actinomadura* sp. (SCC 1776 and SCC 1777) are members of a new class of antifungal antibiotics characterized by the presence of a 14 membered macrocyelic lactams. These metabolites showed good activity against *Candida albicans* bat only weak activity against dermatophytes (Hedge *et al.*, 1991; 1992).

The first marine actinomycete taxon, *Salinispora* was eventually described in 2005 (Maldonado *et al.*, 2005). *Streptomyces* sp. strain NI80 was obtained from an unidentified Japanese sponge (Imamura *et al.*, 1993). Analysis of the supernatant resulted in the identification of two new antimycins called urauchimycin A (**1**) and B (**2**), the first antimycins to possess a branched side-chain moiety. Urauchimycin A and B are active against the fungus *C.albicans* at the concentration of 10μg/ml. The *Streptomycetes* form a distinct clade within the radiation encompassed by the high-GC Gram-positive bacteria in the 16S rDNA tree. There is evidence that specific metabolites, such as clavulanic acid, may be synthesized by strains in a specific clade and that the ability to synthesize, for example, streptomycin and related metabolites appear to be randomly distributed across the whole genus (Chater, 1998).

Actinobacteria include many organisms that exhibit, or have a tendency towards, mycelial growth. 16S rRNA gene sequencing has led to the recognition of 39 families and 130 genera, which also include high G-C gram-positive bacteria with simpler morphology, such as bifidobacteria and micrococci. The deepest branch separates bifidobacteria from all other known families. The divergence of actinobacteria from other bacteria is so ancient that it is not possible to identify the phylogenetically closest bacterial group to Actinobacteria with confidence (Embley and Stackebrandt, 1994).

The first actinobacterial genome to be sequenced was that of the paradigm strain of the human tuberculosis agent, *M.tuberculosis* H37Rv (Cole *et al.,* 1998). Hopwood *et al.,* (1985) reported for the first time the production of "hybrid" antibiotics by genetic engineering of *Streptomyces* strains, i.e. through the transfer of biosynthetic genes between strains producing different members of the same class of antibiotics in order to combine structural features of both compounds. The complete genome of *S.avermitilis*, which produces the anti-parasitic agent avermectin, has also been sequenced recently (Ikeda *et al.,* 2003). Its genome (linear, 9Mb, G+C content of 70.7%) shows a similar division between core and arm regions and provides further support for the idea that the arms have a separate origin from the core and an adaptive function (Hopwood, 2003; Ikeda *et al.,* 2003).

The most interesting finding in the two *Streptomyces* genomes is the abundance of genes that would encode enzymes for secondary metabolism. Before the genome was sequenced, three antibiotics and a spore pigment were known tobe encoded in the *S. coelicolor* chromosome, but the sequence revealed 24 clusters for secondary metabolites. For *S.avermitilis*, 30 such clusters, nearly all encoding different compounds in the two species, were described (Hopwood, 2003). The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of bacterial DNA gyrase produced by different *Streptomyces* strains (Lewis *et al.*, 1996; Maxwell, 1993; Maxwell, 1997). Novobiocin was first reported in the middle of 1950s (called then streptonivicin) (Hoeksema *et al.*, 1955; Smith *et al.,* 1956), whereas coumermycin and clorobiocin were found between the 1960s and the 1970s (Kawaguchi *et al.*, 1965; Ninet *et al.*, 1972). In the literature, two producers of novobiocin are described, i.e. *S.spheroids* NCIMB 11891 (Steffensky *et al.*, 2000). However, Lanoot *et al.* (2002) have proposed that these two strains are synonyms of *S.caeruleus* LMG 19399T. Clorobiocin is produced by *S.hygroscopicus*, *S.albocinerescens* and *S.roseochromogenes* var. *oscitans* (Mancy *et al.*, 1974). Coumermycin A_1 is obtained from several *Streptomyces* strains, i.e. *S.rishiriensis*, *S. hazeliensis* var. *hazeliensis*, *S.spinichromogenes* and *S.spinicoumarensis* (Berger and Batcho, 1978). So far, two further aminocoumarin antibiotics, simocyclinones of the D class (Schimana *et al.*, 2000) and rubradirin (Sohng *et al.*, 1997) have been discovered

which are also produced by different *Streptomyces* strains. The citreamicins first polycyclic xanthones isolated from a *Micromonospora (Micromonospora citrea*, strain LL-E 19085*)*, are characterized by a quinine ring fused to a pyrone. They were discovered in a specific screen against anaerobic bacteria and showed good activity against *Clostridia* (Maiese *et al.*, 1989).

In this chapter, actinomycetes strains was isolated from Muthupet mangrove habitats were selected and tested for their capacity to produce compounds active against gram negative and gram positive pathogenic microbes and identified bioactive compounds functional groups.

4.2 MATERIALS AND METHODS

4.2.1.1 Extraction of bioactive compounds

Representative actinomycetes isolates (+18, +50 and +118) were inoculated in to starch casein broth and incubated in strandard optimum conditions. As maximum antibiotic production was observed on the 8th day of incubation (Augustine *et al.,* 2004), fermentation was terminated on the 8th day and the broth was centrifuged at 10,000 rpm for 20 min to separate the mycelial biomass. Different solvents *viz*., n-butanol, n-hexane, ethyl acetate, petroleum ether, chloroform, benzene, methanol and xylene were used for the extraction of the bioactive compounds from the culture supernatant to determine the ideal solvent for extraction of the bioactive compounds from the culture supernatant. The solvent was added to the supernatant in 1: 1 ratio. Solvent-supernatant mixture was agitated for 45 min. with homogenizer. The solvent was separated from broth by separating funnel. Solvent was centrifuged at 5000 rpm for 15 min. to remove traces of fermentation broth.

The solvent, methanol was evaporated by subjecting to the sample to rotating flash evaporator at 40°C (50 rpm) under vacuum. The dark brown gummy substance obtained was dissolved in ethyl acetate and concentrated, following which the crude bioactive compound was obtained. The crude antibiotic was collected and dried in vacuum oven at 40°C overnight. The residue obtained (crude antibiotic) was subjected to purification.

4.2.1.2 Separation and purification of bioactive compounds

The crude bioactive compound was screened for number of components present using precoated Thin-Layer Chromatography (TLC) plates using n-butanol: ethyl acetate:water: (9:9:1),Chloroform: methanol (9:1), Chloroform: methanol (9.2:0.8), Ethanol: water: chloroform (4:4:2), Ethanol: water: chloroform (4:2:4) solvent system. Purification of the antibiotic was carried out by column chromatography using silica gel

(60–120 mesh) of column chromatography grade. Column (35×10 mm) was cleaned using water and rinsed with acetone. After drying, a small piece of cotton was placed at the bottom of the column. Silica gel was then packed in the column by using ethanol: water (50:50) as solvent system. The crude antibiotic was loaded at the top of the column and eluted using ethanol: water (50:50) as solvent system. Fractions were collected at 20 min interval. TLC of each fraction was performed using precoated. TLC plates and simple glass plates to detect the antibiotic. The TLC plates were exposed to iodine vapors to develop the antibiotic, if any. The fractions having same *R*f value were mixed together and the solvent evaporated at 40°C in a vacuum oven. These fractions were tested for their antimicrobial activity by using the agar well diffusion method. The powder obtained was stored in an ampoule at 4°C.

4.2.2 CHARATERIZATION OF BIOACTIVE COMPOUNDS FROM MARINE ACTINOMYCETES NOCARDIOPSIS +18 AND STREPTOMYCES +50 AND +118

4.2.2.1 Ultraviolet and Fourier transform infrared spectra

Ultraviolet (UV) spectrums were recorded on Shimadzu UV-170 spectrophotometer. One milligram of sample was dissolved in 10 ml of water and the spectra were recorded at 200–400 nm range. The infrared spectra were recorded on Shimadzu IR-470 model. The spectra were scanned in the 400 to 4000 cm^{-1} range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide was dried under vacuum at 100°C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wave number.

4.2.2.2 Nuclear Magnetic Resonance

The bioactive compound was subjected to ^{1}HNMR (500 MHz, Brucker Biospin, Switzerland) studies. The bioactive compound was dissolved (3 mg for ^{1}HNMR) in 3 ml of chloroform and analysed by Nuclear Magnetic Resonance (NMR).

4.2.3 ANTIMICROBIAL SENSITIVITY OF PURE COMPOUNDS FROM NOCARDIOPSIS +18 AND STREPTOMYCES +50

4.2.3.1 Disc diffusion methods

Antibiotic sensitivity test will be performed by the commonly used agar diffusion method. which is designed to determine the smallest amount of the bioactive secondary metabolites needed to inhibit the growth of the microorganism. The medium of choice is Muller – Hinton agar with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5mm and allowed to solidify. Prior to use, the plates are transferred to an incubator at 37˚C for 10 to 20 minutes to dry off the moisture that develops on the agar surface. Overnight growth of pathogens broth culture was swabbed on the surface of the agar media and further well was prepared by using well cutter. To each well, 50µg of bioactive secondary metabolites from actinomycetes was added and incubated in a thermostat incubator for 24 hrs. After incubation, the zone of inhibition around the well was calculated and expressed as zone of inhibition in millimeter in diameter.

4.3 RESULTS

Separation and purification of antimicrobial compounds

The fermentation broth of actinomycetes was extracted with different solvents. All the extractsfrom +18, +50 and +118 were found to dissolved in ethyl acetate and methanol. Methanol was found to be better than the ethyl acetate.

Antimicrobial sensitivity

Antimicrobial activities of the extracts from actinomycetes were tested against eleven different pathogenic bacteria and four different fungi. Methanol crude extract from +18 strains showed 46mm zone of inhibition against *E.coli,* 12 mm against *S.epidermidis,* and moderate activity was observed aganist *S.aureus* (10 mm) *S.typhi* (9 mm) and *Vibrio paraheamolyticus* (8 mm). Methanol crude extract from +50 strain showed sensitivity (42 mm) against *E.coli,* 13 mm against *C.albicans* and *C. glabrata,* 12 mm against *S.epidermidis,* and moderate activity was observed *S.aureus* (10 mm) and *Salmonella typhi* (10 mm).The methanol crude extract from +118 strains showed maximum zone of inhibition by 21mm against *E.coli,* 16 mm against *C.albicans* and *C.glabrata,* 12 mm against *S.epidermidis,* and moderate activity was observed *S.aureus* (10 mm) *S.typhi* (10 mm) and *C.neoforman.* Finally +18 and +50 were selected for the characterization of antimicrobial compounds based on the antibacterial and antifungal activity. (Table 4.1 & Plate 11 & 12)

Characterization of antimicrobial compounds

The bioactive compounds from +18 strain revealed that the absorption maximum was 263 and 312 nm in methanol. The UV spectrum of +18 compounds was showed in (Fig 2.1). IR Spectrum of selected +18 compounds showed Asymmetric (NH_2) amino group stretching shows the strong absorption at 3427 cm^{-1}, and the symmetric medium stretching at 1640 cm^{-1} shows a peak for NH_2 group along with 1566 cm^{-1} and this

characteristic aromatic stretching at 771cm^{-1} for aromatic ring absorption (Fig 2.3). NMR spectrum has strong broad peak at 1.58 ppm which shows that the amino group with two protons and a peak with little lower intensity at 2.6 ppm for acetyl group in the aromatic ring or aliphatic linear chain (Fig 2.5).

The bioactive compounds from +50 strains revealed that, the maximum absorption at 263 and 329nm in methanol. The UV spectrum of compounds was showed in Fig 2.2. IR Spectrum of +50 compounds showed the maximum absorption at 3436 cm^{-1} and absorption peak shows the NH_2 Asymmetric stretching containing with the aromatic ring and 1639 cm^{-1} for NH_2 medium symmetric stretching, 768 cm^{-1} aromatic stretching which contains NH_2 group in the ring carbon (Fig 2.4). NMR spectrum showed only one strong peak at 1.5 ppm might be due to the presence of amino group in the aromatic ring (Fig. 2.6).

The purified antibiotics were analysed for antimicrobial activity (for selected *E.coli*) The +18 and +118 strains recored the inhibitions of 13mm, 11mm and 26mm against *E.coli* (Table 4.2).

Table 4.1 Antimicrobial efficacy of crude extract of actinomycetes isolates

S.No	Strains	Human pathogens														
		S.f	*V.p*	*B.s*	*S.t*	*C.c*	*E.f*	*P.a*	*V.c*	*S.a*	*E.c*	*S.e*	*A.sp*	*C.a*	*C.g*	*C.n*
1	+18	-	8mm	-	9mm	-	-	-	-	10mm	46mm	12mm	-	-	-	-
2	+50	-	-	-	10mm	-	-	-	-	10mm	42mm	12mm	-	13mm	13mm	-
3	+118	-	-	-	10mm	-	-	-	-	10mm	21mm	12mm	-	16mm	16mm	7mm

S.f- ***Shigella flexneri***, V.p- ***Vibrio paraheamolyticus***, B.s- ***Bacillus subtilis***, S.t- ***Salmonella typhi***, C.c- ***Camphylobacter coil***,, ***Pseudomonas aeruginosa***, V.c- ***Vibrio cholerae***, S.a- ***Staphylococcus aureus***, E.c- ***Escherichia coli***, S.e- ***Staphylococcus epidermidis*** , A.n- ***Aspergillus flavus***, C.a- ***C.albicans***, C.g- ***Candida glabrata***, C.n- ***C.neoforman***

Table 4.2 Antimicrobial efficacy of pure compounds from actinomycetes strain +18 and +50

S. No.	Strain No.	Inhibition Zone (mm diameter)	
		Peak No1	Peak No 2
1	+18	11	13
2	+50	11	NP

NP - No peak, NIZ - No inhibition zone

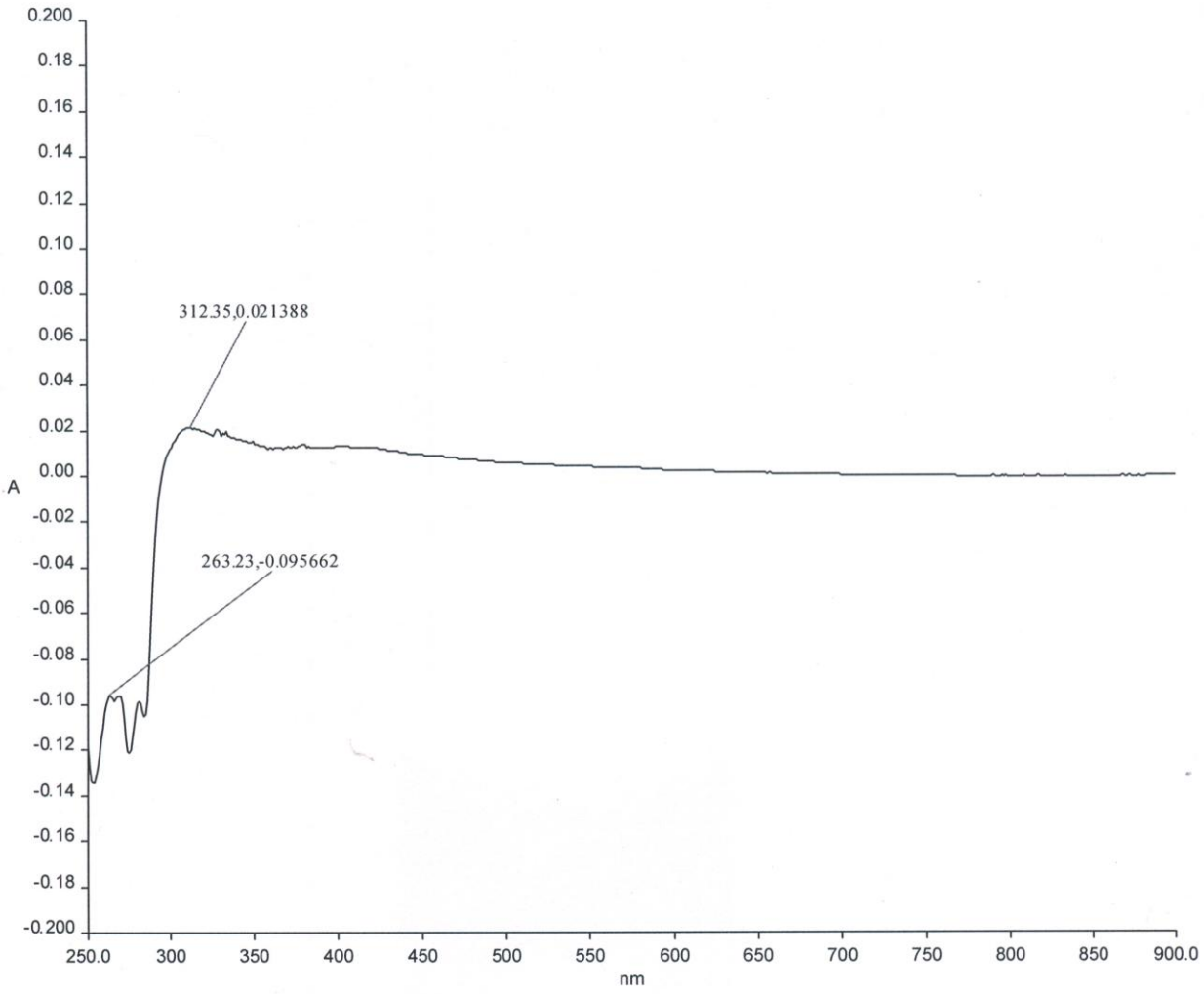


Fig. 2.1 UV-Vis spectrum of bioactive compounds from actinomycete +18 strain

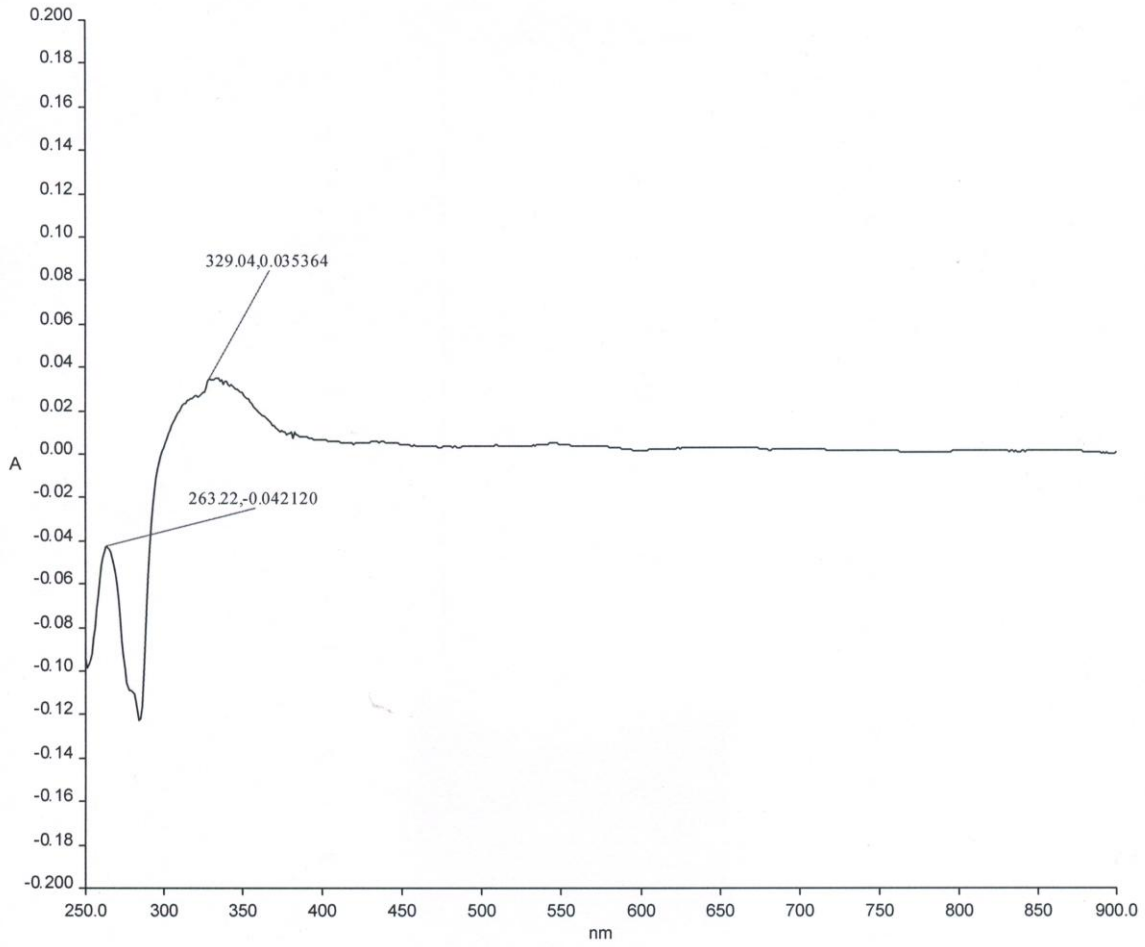


Fig. 2.2 UV-Vis spectrum of bioactive compounds from actinomycete +50 strain

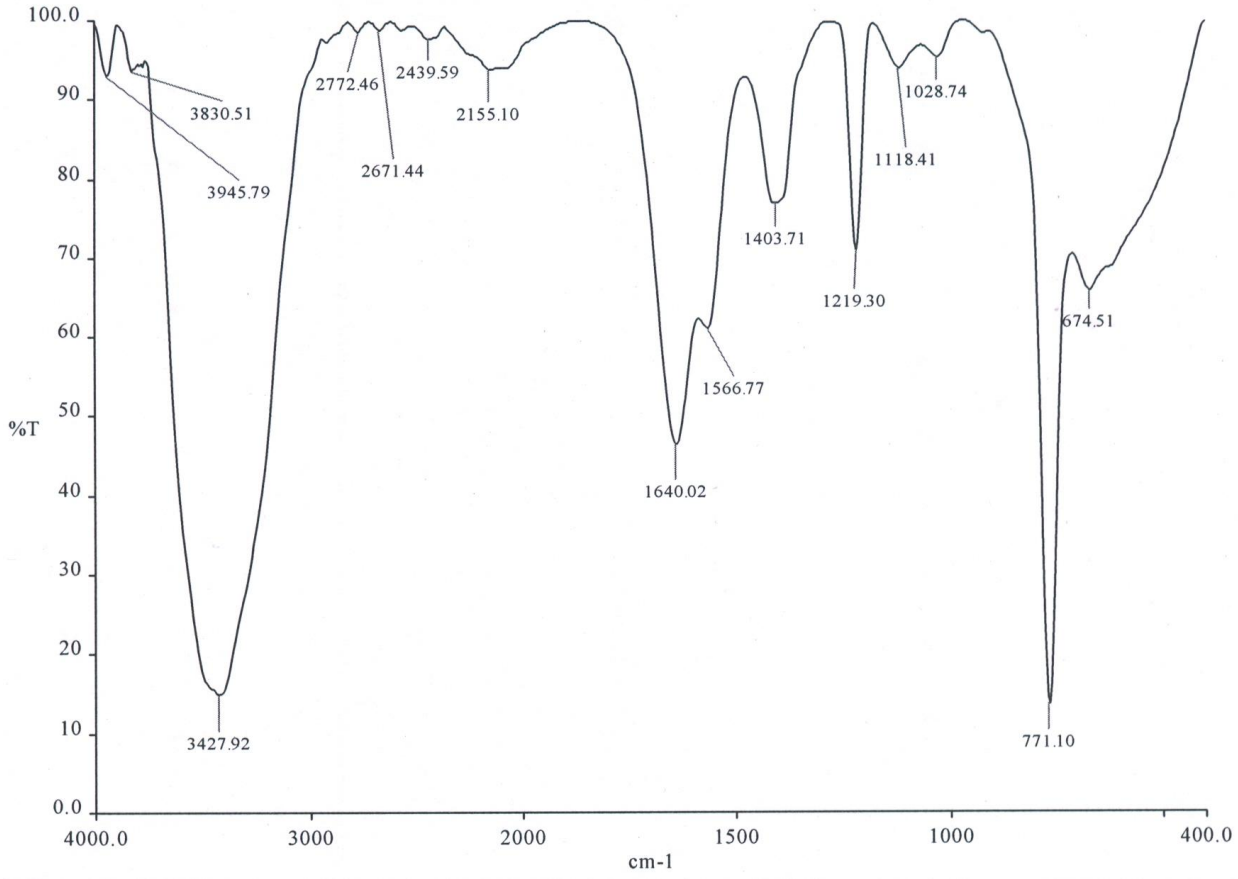


Fig. 2.3 FTIR spectrum of bioactive compounds from actinomycete +18 strain

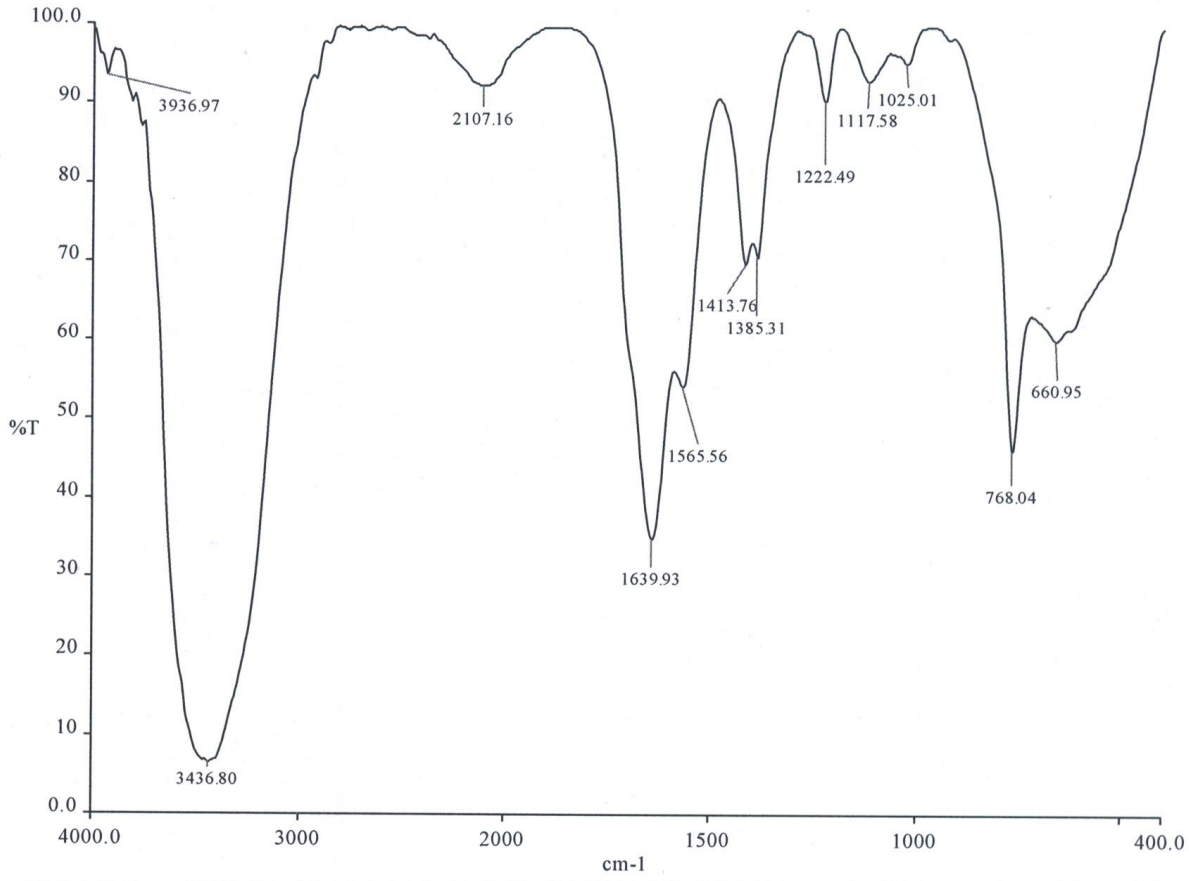


Fig. 2.4 FTIR spectrum of bioactive compounds from actinomycete +50 strain

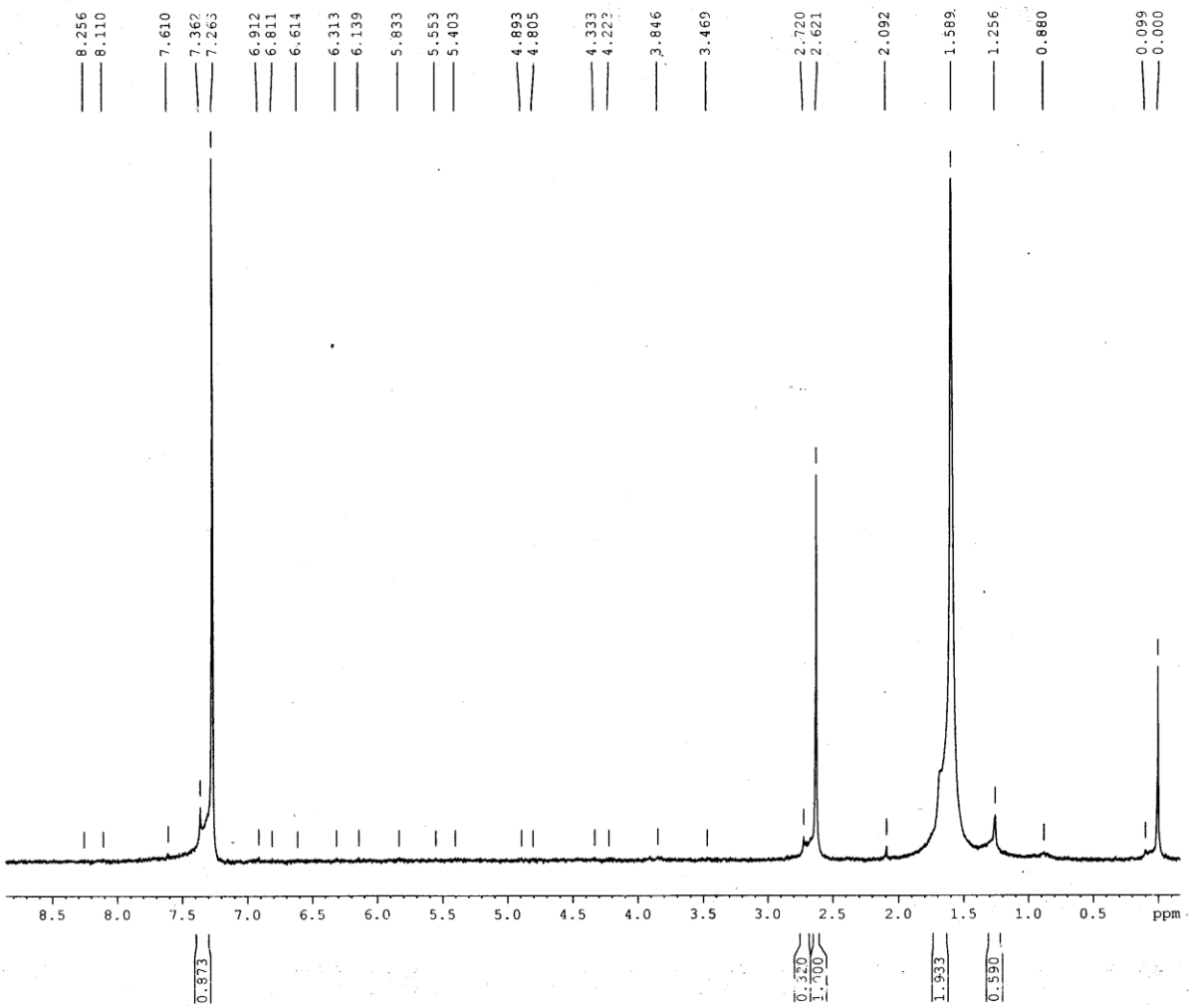


Fig. 2.5 NMR spectrum of bioactive compounds from actinomycete +18 strain

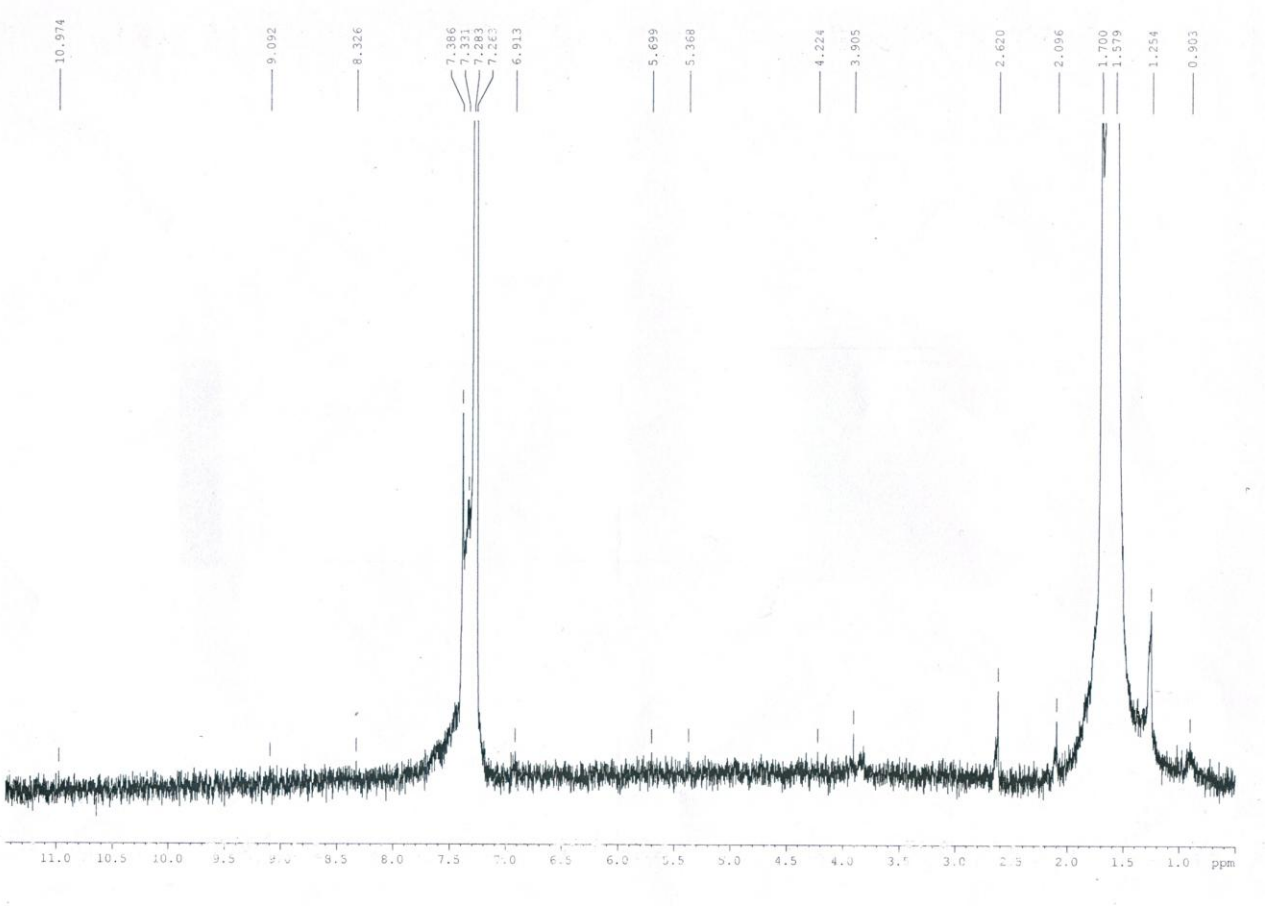
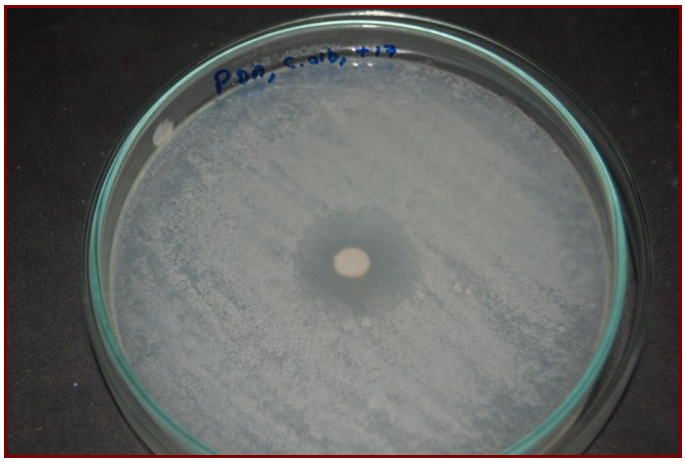
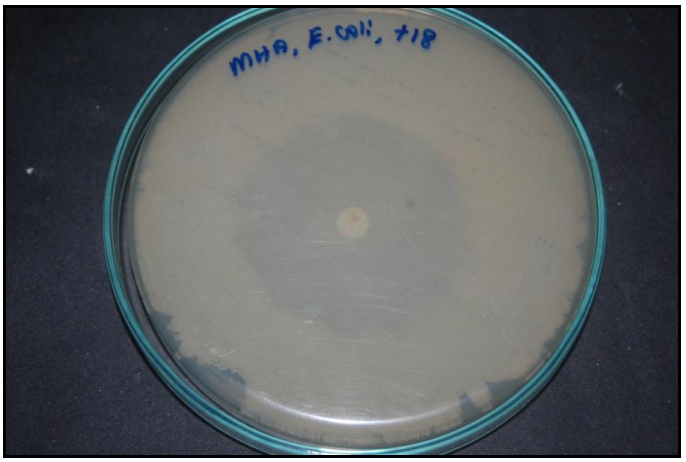


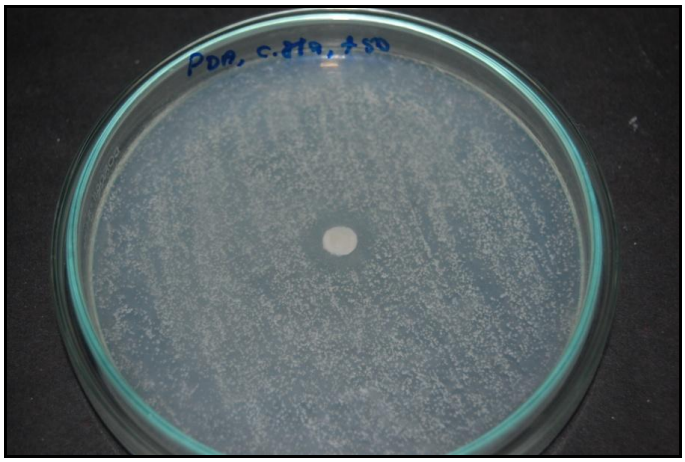
Fig. 2.6 NMR spectrum of bioactive compounds from actinomycete +50 strain



+18 inhibited against ***Candida albicans***



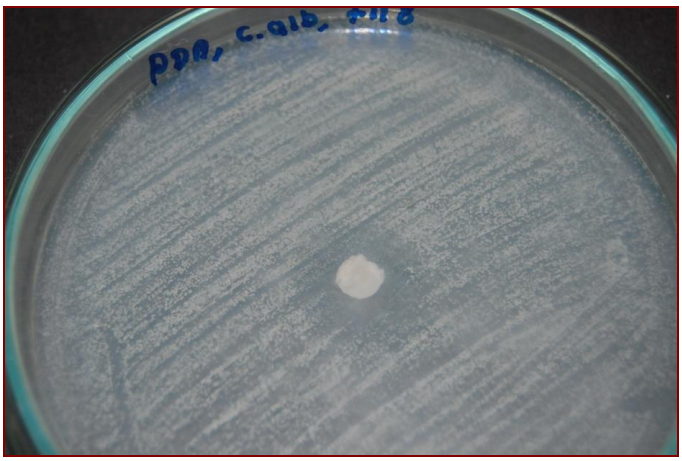
+18 inhibited against ***E.coli***



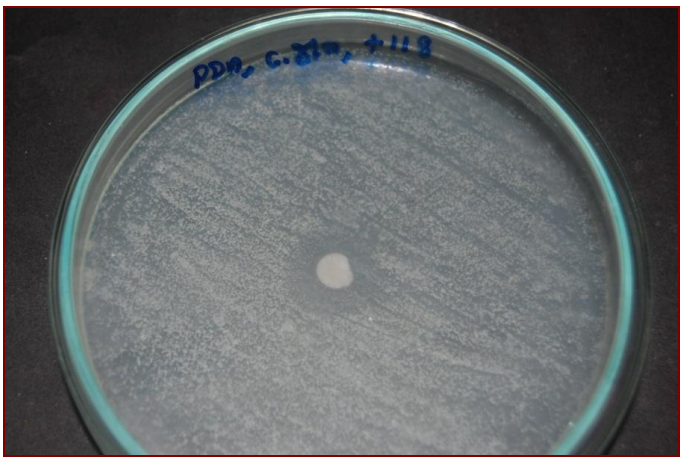
+50 inhibited against ***Candida glabrata***

+50 inhibited against ***E.coli***

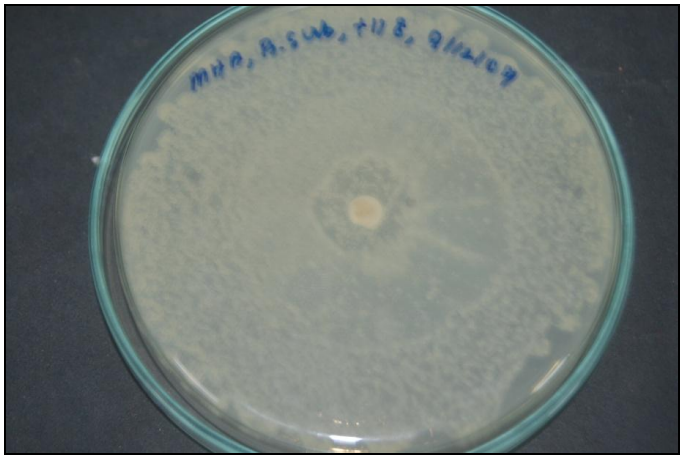
Plate 11 Antimicrobial activity of crude extracts from chosen actinomycetes strains (+18 &+50) against chosen bacterial and fungal pathogens



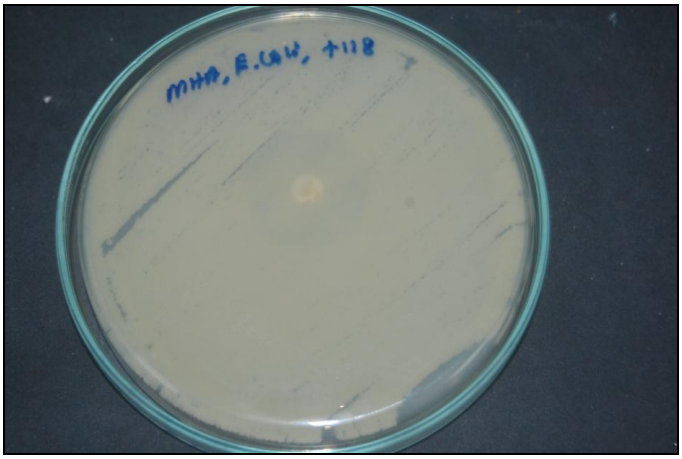
+118 inhibited against ***Candida albicans***



+118 inhibited against ***Candida glabrata***



+118 inhibited against ***Bacillus subtilis***



+118 inhibited against ***E.coli***

Plate 12 Antimicrobial activity of crude extracts from chosen actinomycetes strain (+118) against bacterial and fungal pathogens

4.4 DISCUSSION

Marine actinomycetes have been traditionally a rich source for biologically active metabolites. Although heavily studied over the past three decades, actinomycetes continue to prove themselves as reliable sources of novel bioactive compounds. Among the well-characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically relevant natural products (Jensen and Fenical, 2000).

In the present investigation, the +18, +50 and +118 supernatant was extracted with methanol solvent and subjected for the separation of compounds. Thin layer chromatographic technique was carried out for the separation of antimicrobial compounds of actinomycetes. Similar types of works have been reported by many workers (Sen and Nandi, 1958; Siewart and Kieslich, 1971; Riva *et al.*, 1989; Augustine *et al.*, 2005; Dhanasekaran, 2005; Vijayakumar, 2006).

The present study revealed that, the antimicrobial crude extract from strain +17 was reported maximum zone of inhibition with 30mm against *C.glabrata* and minimum inhibition of 7mm against *S.typhi*. The methanol crude extract from strain +18 has resulted maximum zone of inhibition of 46 mm against *E.coli* and minimum (8 mm) aganist *V.paraheamolyticus*. The crude extracts from strain +50 expressed maximum inhibition (42mm) against *E.coli* and minimum inhibition zone aganist *S.typhi* (10 mm). The crude extracts from +112 strain resulted maximum inhibition (40 mm) against *E.coli* whereas showed minimum zone of inhibition aganist *S. typhi* (10 mm). In case of +118 strain the maximum zone of inhibition (21mm) was noticed against *E.coil* whereas minimum activity was observed aganist *S.aureus*, *S.typhi* and *C.neoforman* (10 mm).

Based on the activity, the strains +18 and +50 was selected for the characterization of antimicrobial compounds. The identification of antimicrobial compounds was carried out by using UV, IR and ^{1}H NMR spectral analyis.

The bioactive compounds from actinomycetes strain +18 revealed that, the maximum absorption was obtained as 263 and 312 nm in methanol. The IR Spectrum showed asymmetric (NH_2) amino group stretching the strong absorption at 3427 cm^{-1}, and the symmetric medium stretching at 1640 cm^{-1} shows a peak for NH_2 group along with 1566 cm^{-1} and this characteristic aromatic stretching at 771cm^{-1} for aromatic ring absorption. The NMR spectrum reveals strong broad peaks at 1.58 ppm showed that amino group with two protons and a peak with little lower intensity at 2.6 ppm for acetyl group in the aromatic ring or aliphatic linear chain. The bioactive compounds from actinomycetes strain +50 revealed the maximum absorption (263) and (329 nm) in methanol. The IR Spectrum of the compounds showed the maximum absorption at 3436 cm^{-1}. The absorption peak showed that, the NH_2 asymmetric stretching containing with the aromatic ring and 1639 cm^{-1} for NH_2 medium symmetric stretching, 768 cm^{-1} aromatic stretching which contains NH_2 group in the ring carbon. In actinomycetes +50 strain, NMR spectrum showed only one strong peak at 1.5 ppm may be due to the presence of amino group in the aromatic ring. The physiochemical properties of antimicrobial compounds of actinomycetes have also been investigated and reported (Munro *et al.,* 1982; Nishio *et al.,* 1989; Riva *et al.,* 1989; Skoog *et al.,* 1997; Harada *et al.,* 2004; Augustine *et al.,* 2005).