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Identification of Antimicrobial Compounds from *Streptomyces* sp. Isolated from Western Ghats Soil in Tamil Nadu

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Abstract

Three antibacterial compounds namely Dibutyl phthalate, 8-Hydroxyquinoline and 2-amino-3-chlorobenzoic acid were isolated from *Streptomyces* sp. (ERI-15 strain) ethyl acetate extract by bioassay-guided fractionation using a silica gel column chromatography. Totally 12 fractions were eluted from the crude extract using different combinations of hexane, ethyl acetate, acetone and methanol solvents. Bioassay screening of fractions was carried out against *Staphylococcus aureus* (ATCC 25923), Methicillin Resistant *S. aureus* (MRSA), *Bacillus subtilis* (ATCC 441) and *Escherichia coli* (ATCC 25922) by disc diffusion and bioautography methods. Fractions 7, 8 and 9 showed significantly higher antibacterial activity against the tested bacteria. Three compounds namely Dibutyl phthalate, 8-Hydroxy quinolone and 2-amino-3-chlorobenzoic acid were identified and purified from the active fractions. These compounds can be probed further for their use as antibiotics.

Keywords: *Streptomyces sp ERI-15; Bioassay Guided Separation, Bioautography, Dibutyl Phthalate, 8-Hydroxy Quinoline, 2-Amino-3-Chlorobenzoic Acid*

Introduction

Isolation of antimicrobial agents from natural resources is attaining more importance among researchers worldwide. Secondary metabolites of many microorganisms, especially the actinomycetes, possess biologically active molecules that can be used for the treatment of many microbial infections. The percentage of actinomycetes and fungal strains which are showing antimicrobial activities in standard agar diffusion assays ranges between 30 - 80% depending on the ecological or taxonomic groups [1,2].

Actinomycetes are the most useful group in antibiotic production and a good number of commercial antibiotics are from these microbes. Literature shows that many antimicrobial compounds have been isolated from *Streptomyces* spp. [3-5]. Based on a mathematical model, it is estimated that the genus *Streptomyces* is capable of producing 100,000 antimicrobial compounds; but a tiny fraction has been discovered so far [6]. In our previous study, we isolated a potential antimicrobial *Streptomyces* sp. (strain: ERI 15) with antimicrobial property from Western Ghats soil in Tamil Nadu. This strain showed good antimicrobial activity against pathogenic bacteria and fungi [7].

The present study was undertaken to isolate the antimicrobial compound from ethyl acetate extract of *Streptomyces* sp. (ERI 15) by bioassay-guided isolation and to characterize the compounds by spectroscopic methods.

Materials and Methods

Streptomyces sp. collection

The active isolate Streptomyces sp. was obtained from stock culture maintained at Entomology Research Institute, India. This active culture was previously collected from Western Ghats soil sample in Kanyakumari District, India.

Test Microorganisms

The test microorganisms used in the present study were procured from American type culture collection (ATCC) and Microbial type culture collection (MTCC) Chandigarh, India.

Fermentation

The fermentation was carried out in Modified Nutrient glucose (MNG) medium using *Streptomyces* sp. (ERI-15) in a 3.0 L fermentor of working volume 2.0 L. Suitable fermentation medium and conditions were previously optimized by Devadass et al. [7] and the same method was followed. The composition (gm/L) of the fermentation medium was: Peptone 5; glucose 20; sodium chloride 3; calcium carbonate 1.5; Yeast extract 3 and antifoam 204 (Sigma) 0.1. The pH of the medium was adjusted to 7.0 before sterilization. After sterilization and subsequent cooling, 1% (v/v) inoculum containing 2×10^6 cfu/ml was used to inoculate the fermentation medium using a peristaltic pump. Fermentation was carried out at 30°C, 350 rpm and at an aeration rate of 1.0 vvm for 7days. After 7 days of incubation, the culture broth was collected and cell free supernatant was extracted with ethyl acetate and concentrated by vacuum evaporator. The crude extract obtained was subjected to column chromatography to isolate the bioactive compound(s).

Column Chromatography and Compound Isolation

The concentrated crude ethyl acetate extract of *Streptomyces* sp ERI-15 was packed in silica gel chromatography. The column was eluted with stepwise gradient of Hexane (100%), hexane-ethyl acetate (95:5 to 5:95 ratio, respectively), acetone-ethyl acetate (10:90 to 100:0, respectively) and methanol (100%). The collected fractions were pooled together based on the TLC profile. Each fraction was tested for its antimicrobial activity against test pathogens by disc diffusion method according to NCCLS standards and the major bioactive fractions were tested against MRSA using bioautography method. The fractions showing antimicrobial activity were further purified by preparative HPLC and bioactive compounds were isolated. The isolated bioactive compounds were structurally analysed by GC-MS, IR, ^{1}H NMR and ^{13}C NMR.

Spectral studies of the isolated bioactive compounds

UV-Visible Spectrum: The UV-Visible spectral analysis of the purified antimicrobial compounds was examined using Shimadzu UV-2450 UV-Visible spectrophotometer. The compound was dissolved in methanol and the spectrum was recorded in the range of 200 - 800 nm.

Gas Chromatography-Mass Spectrometry analysis (GC-MS): GC-MS chromatograms of the purified compounds were recorded using Shimadzu GC-MS-QP 2010 equipped with DB-5 ms column (30 m x 0.25 mm i.d., 0.25 µm film thickness) with the following operating conditions: The temperature of the column (39.6 mts total) was held initially at 50°C for 1 min, then raised to 300°C with a hold time of 10 min and at the rate of 10°C min^{-1}.The carrier gas was helium (99.9995% purity) with a flow rate of 1.50 ml min^{-1} with split mode of injection of sample and the injector temperature was maintained at 280°C. The mass spectrometer was operated with ion source temperature at 200°C and interface temperature at 240°C and scanned from 40 to 1000 Da.

FT-IR spectrum: The FT-IR spectra of the purified antimicrobial compounds were recorded using Spectrum one model spectrophotometer (Perkin-Elmer Co., USA) using KBr pellet as standard.

Nuclear magnetic resonance spectroscopy: ^{1}H NMR and ^{13}C NMR of the purified compounds were recorded in $CDCl_3$ or deuterated DMSO with tetramethylsilane (TMS) as internal standard. The sample concentration was adjusted to 0.5% by weight for compound 1, compound 2 and compound 3.

High-resolution 1H spectra were recorded in Jeol ECA500 MHz spectrophotometer with δ version Iris platform. The operating parameters were as follows: 15 ppm to -5 ppm X, 16384 points, 6.575 (us) pulse, 5 (s) relaxation delay, 1.308 (s) acquisition time and 11.747 (T) field strength. High-resolution ^{13}C NMR spectra were recorded in Jeol ECA500 MHz spectrophotometer with δ version Iris platform. The operating parameters were as follows: 225 ppm to -25 ppm X, 32768 points, 3.4 (us) pulse, 2 (s) relaxation delay, 0.83361 (s) acquisition time and 11.747 (T) field strength.

Results

Extraction, purification and bioassay-guided isolation of active compounds

The ethyl acetate extract of *Streptomyces* sp. ERI-15 cell free supernatant was sequentially extracted with hexane, ethyl acetate and methanol solvents. A total of 650 fractions (50 ml/fractions) were collected, and were combined into 12 fractions based on the TLC band profiles. All the 12 fractions were tested for their antimicrobial activity against selected test pathogens. Fractions 7, 8 and 9 showed prominent antimicrobial activity against all the tested pathogens. Fraction 7 showed the highest zone of inhibition of 16 mm against *B. subtilis* and 14 mm against *S. aureus*, MRSA and *E. coli* (Table 1; Figure 1). Fraction 8 showed 14 mm zone of inhibition against *B. subtilis*. The active fractions 7 and 8 were eluted by the hexane-ethyl acetate solvent combinations and fraction 9 was eluted by acetone-ethyl acetate solvent combination. The active fractions were further purified by preparative TLC and three compounds were obtained.

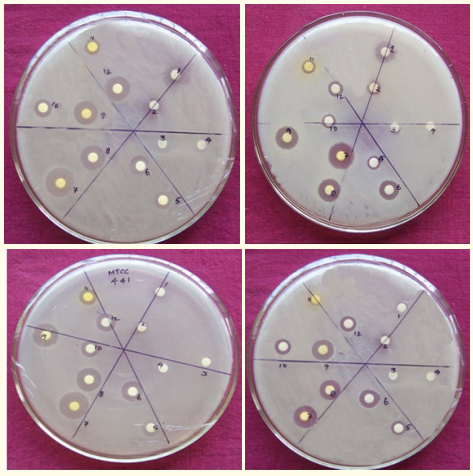

Figure 1: *Antibacterial activity of 12 fractions of ethyl acetate extract of Streptomyces sp. (ERI 15 strain) against Staphylococcus aureus (A), Methicillin-resistant Staphylococcus aureus (MRSA) (B), Bacillus subtilis (c) and Escherichia coli (D).*

Fractions	Zone of inhibition of growth (mm)			
	***S. aureus* ATCC 25923**	**MRSA**	***B. subtilis* MTCC 441**	***E. coli* ATCC 25922**
1	8	Nil	Nil	Nil
2	Nil	Nil	Nil	Nil
3	Nil	Nil	Nil	Nil
4	Nil	Nil	Nil	Nil
5	7	7	7	7
6	10	9	10	11
7	14	14	16	14
8	12	13	14	13
9	12	12	13	13
10	9	7	9	8
11	8	7	9	8
12	9	9	8	Nil

Table 1: *Antibacterial activity of 12 fractions collected from ERI 15 against selected bacterial strains.*

Compound 1

In bioautography experiment, the silica gel thin-layer chromatography of reddish-brown coloured crude ethyl acetate extract showed the presence of one component with antimicrobial activity (Figure 2). The hexane: methanol (9:1) solvent system that showed good separation of compounds of ethyl acetate extract was used as the developing solvent and the Rf value of the compound was found to be 0.83. In the UV spectrum, the purified compound-1 showed absorbance maxima at 263.5 nm, 225 nm and 213.5 nm and there was no appreciable absorbance above 300 nm.

Figure 2: *Bioautography of active fractions 7, 8 and 9 against Methicillin-resistant Staphylococcus aureus (MRSA).*

The FT-IR spectrum of the purified compound-1 (Figure 3a) in methanol clearly showed peaks at (cm^{-1}) 2924.46, 2855.13 characteristic of sp^3 —CH stretch and —CH aliphatic stretch. The peaks at 1728.48 and 1285.26 indicate ester moiety; 1599.68, 1580.54, 1465.67 corresponding to —C=C and 743.70 indicate —CH stretch of aromatic region.

In the GC-MS chromatogram of Compound-1 the retention time was 18.758 min (Figure 3b). The mass spectra of the compound were recorded and the spectrum revealed the mass of Compound-1 to be 278. The compound was identified by comparison of mass spectra with library data and interpretation of mass spectrometric fragmentation patterns. Fragmentation pattern of compound-1 indicated a peak of very less intensity at 278, molecular ion and one major fragment with a relative intensity of 100 at m/z 149, the major fragmentation ion of phthalates confirming its presence. The other fragments with relative intensity in parentheses that match the fragmentation pattern of dibutyl phthalate with reference to literature are as follows: 205 (5), 223 (4), 135 (1), 121 (4), 105 (5), 93 (4). The peak at m/z 149 denotes the fragment ion formed by the elimination of OC_8H_{18} from the molecular ion dibutyl phthalate. The fragment ions at m/z 205, m/z 223 are produced by the loss of C_4H_9 and OC_4H_9 from the molecular ion. The fragment ion at m/z 149 further dissociates to produce fragment ion at m/z 121, m/z 93 by the elimination of CO. The GC-MS pattern of compound-1 confirmed the structure of dibutyl phthalate produced by *Streptomyces* sp. ERI-15.

The possible structure of the isolated compounds from *Streptomyces* sp. ERI-15 was further decoded with ^1H and ^{13}C NMR spectroscopy. Figures 3c and 3d depict the NMR spectrum of Dibutylpthalate. The 1H spectrum of Dibutylpthalate showed signals at 7.68 (dd), 7.47 (dd), 4.28 (t), 1.69 (tt), 1.42 (q) and 0.93 (t). The signals at 7.68 and 7.47 revealed the presence of hydrogen atoms linked to sp^2 aromatic carbon at C7 and C10; C8 and C9 position, respectively. The peaks at 0.93 denote the methyl hydrogen groups at C1 position and that at 4.28, 1.69 and 1.42 indicates methine hydrogen groups at C4, C3 and C2, respectively. The presence of electronegative atom adjacent to C4 methine group was indicated by chemical shift of the signal -CH_2 further upfield by~3 ppm. The doublet of triplets' signal at 1.69 ppm for hydrogen groups in the C3 position confirms the presence of two adjacent methine and therefore the presence of -CH2 group at C2 and C4 were also confirmed. The –CH_3 groups' presence at C1 was confirmed by the splitting pattern (triplet) by adjacent methine group.

It was clear that the carbon signals of the spectrum corresponded to methyl, methine, sp^2 aromatic ring and quaternary, and carbonyl groups. The quartet signal at 13.9 ppm showed the presence of methyl carbon and the methylene carbon was indicated by the triplet signal

at 18.9 ppm and 30.4 ppm. The methylene carbon atom attached to carbonyl group showed a shift to the low field region (triplet at 65.68 ppm). The singlet signal at 132.5 ppm indicates the presence of quaternary carbon of the aromatic ring at C6 position and doublets at 128.8 ppm and 130.6 ppm refers to sp^2 carbon atoms attached to hydrogen group at C7 and C8 position, respectively. The singlet signal at 167.9 ppm showed the presence of carbonyl carbon atom at C5 position. The inferences on the spectra were all drawn by comparison on available spectrum of dibutyl phthalate from library data and Organic Spectroscopy. The resolved spectrum of the compound was shown to be dibutyl phthalate.

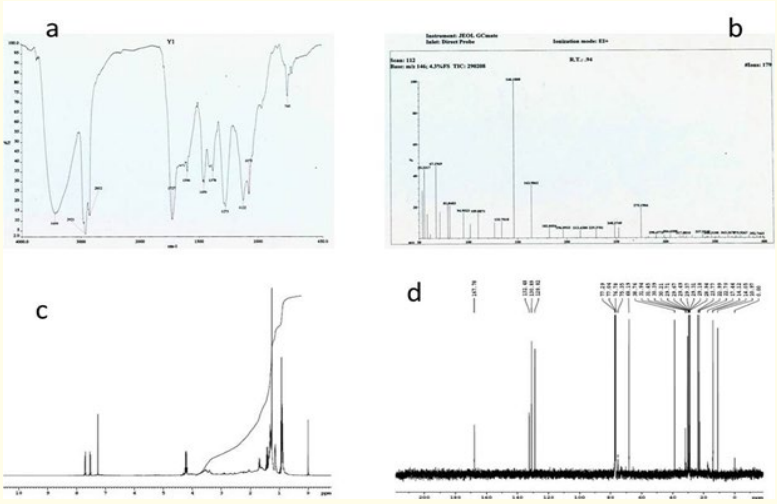

Figure 3: *FTIR (a), EI-Mass (b), ^{1}H NMR (c) and ^{13}C NMR (d) spectra of Dibutylpthalate (Compound 1).*

Compound 2

The hexane: ethyl acetate (7:3) solvent system that showed good separation of compounds of ethyl acetate extract was used as the developing solvent and the Rf value of the compound was calculated as 0.83.

The UV absorption spectrum of the purified compound 2 in methanol (Figure 4b) exhibited maximum absorption peak at 220 nm and also showed absorption in the visible range at 529 nm.

The IR spectral data of compound 2 (Figure 4a) exhibited peaks at V_{max} 3469 cm^{-1} which could be assigned to hydroxyl or NH stretch. The absorption band V_{max} 2924 cm^{-1} and V_{max} 2851 cm^{-1} corresponding to C-H stretching of the CH_2 groups and these peaks may also be characteristic for the presence of aliphatic CH groups in this compound. A peak at Vmax 1643 corresponding to the C=C stretching of the phenyl ring. A peak at V_{max} 1295 cm^{-1} corresponding to the vinylidene in C-H in plane bend.

The ^{1}H NMR spectrum possessed a quartet at 8 0.8 (J = 6), which corresponded to the aliphatic methyl protons and peak at 8 1.24 due to the methylene protons. A triplet at 8 2.32 resonates for the methylene in the ester or acid. A sharp singlet and one doublet at 83.6 and 83.7, respectively, resonate the hydroxylated methines. A triplet resonates at 8 4.1 corresponding to the H-C-OH protons. A sharp peak at 87.2 regions corresponds to aromatic protons (Figure 4c). The ^{13}C NMR spectrum showed methyl carbon resonating at 22.7 ppm. Two sharp peaks at 27.5 and 28.0 resonance is due to CH_2. A multiplet peak at 29.7 ppm resonance may due to methylene in chain. A sharp peak at 39.1 ppm resonance is due to quinolozidine ring (Figure 4d). The ESI-MS spectrum showed the m/z at 394.33 (100, M+) indicating the molecular mass of the purified compound (Figure 4e).

The mass fragmentation search in the Mass Bank and molecular weight search between 392 and 396 in the Novel Antibiotic database, PubChem, NISI and SDBS databases revealed that there was no compound with similar physicochemical properties to that of the puri-

fied compound. However, based on the UV absorption at 529 nm, IR and NMR spectral data, the purified compound was determined as hydroxyquinoline derivative.

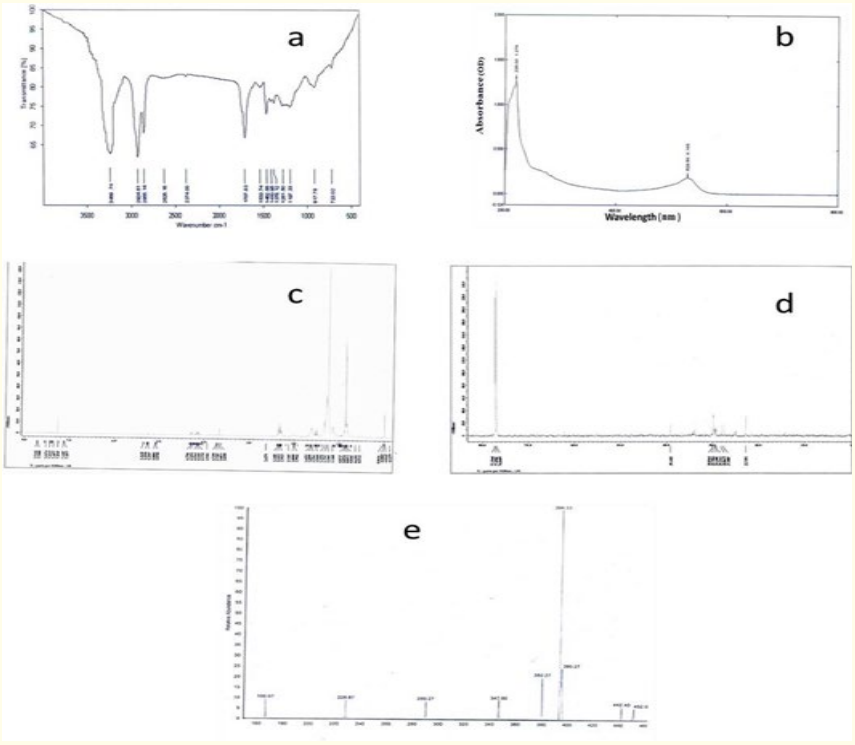

Figure 4: *FTIR (a), UV (b), 1H NMR (c), 13C NMR (d) and Mass (e) spectra of 8-Hydroxyquinoline (Compound 2).*

Compound 3

The hexane: ethyl acetate (4:6) solvent system that showed good separation of compounds of ethyl acetate extract was used as the developing solvent and the Rf value of the compound was calculated as 0.22.

The UV absorption spectrum of the purified compound in methanol (Figure 5b) exhibited maximum absorption peak at 324 nm. No absorption was found in the visible region. The IR spectrum of purified compound (Figure 5a) exhibited a peak at V_{max} 3482 cm^{-1} which could be assigned to hydroxyl or NH stretch. The absorption peaks V_{max} 1594 cm^{-1} and V^{max} 1554 cm^{-1} corresponding to N-H and N-H bend. A peak at V^{max} 1492 cm^{-1} may also be characteristic for the presence of aliphatic CH_2 stretch. A peak V_{max} 1256 cm^{-1} corresponded to the C-O stretching in the compound. A peak at V^{max} 752 cm^{-1} could be assigned to C-CI stretch found in the molecule.

The IH NMR spectrum of the purified compound showed a sharp peak at 86.78 and 87.41 which corresponded to C-H in the benzene ring. A singlet resonance at 811.61 corresponded to carboxylic acid proton (Figure 5c). The ^{13}CNMR spectrum showed peaks at 119.6, 129.5 and 133.9 ppm assigned for methine in the benzene ring. Quaternary carbon resonates at 147.2 ppm and carboxyl carbon resonates at 169.7 ppm (Figure 5d).

The GC-MS spectrum of the purified compound showed peak at m/z 171.56 g/mol indicating the molecular weight (Figure 5e).

The molecular weight search between 170 and 172 in the Novel Antibiotic database, PubChem, NIST and SDBS databases with antibacterial activity revealed a most similar compound with molecular mass of 171.58. Based on the physico-chemical properties of the purified compound, it was determined as 2-amino-3-chlorobenzoic acid.

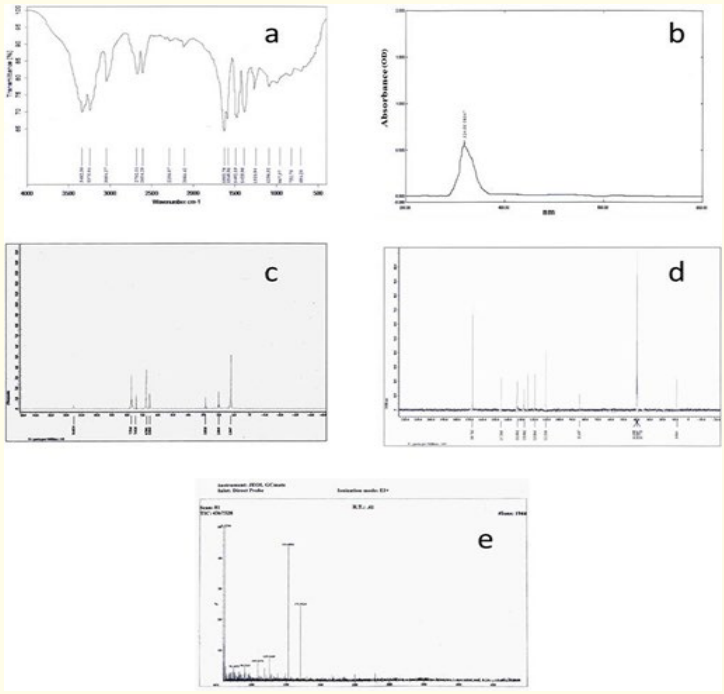

Figure 5: *FTIR (a), UV (b), 1H NMR (c), 13C NMR (d) and Mass spectra (e) of 2 amino 3 chlorobenzoic acid (Compound 3).*

Molecular formulae and physical properties

The molecular structures of the three isolated compounds are given in Figure 6. The molecular formula of dibutyl phthalate is $C_{16}H_{22}O_4$. The molecular formulae of 8-Hydroxyquinoline and 2 amino 3 chlorobenzoic acid are C_9H_7NO and $C_7H_2C_1N0_2$, respectively.

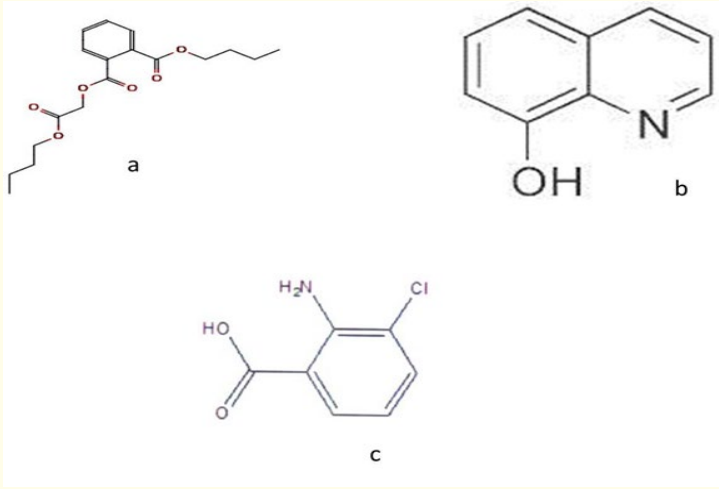

Figure 6: *Chemical structures of Dibutyl Phthalate (a), 8-Hydroxyquinoline (b) and 2-amino-3-chlorobenzoic acid (c).*

Dibutyl phthalate was a colorless to faint yellow oily liquid with aromatic odor; soluble in various organic solvents like alcohol, ether and benzene; melting point was -35°C. 8-Hydroxyquinoline was a dark pink thick amorphous compound, UV reactive with a Rf value of

0.83 (7:3, hexane: ethyl acetate). It was soluble in methanol, chloroform and ethyl acetate but poorly soluble in water. The melting point of the purified compound was 228 - 230°C (decomposition). 2 amino 3 chlorobenzoic acid was pale yellow fine crystal compound, UV reactive with the Rf value of 0.22 (4:6, hexane:ethyl acetate). It was soluble in methanol, DMSO and ethyl acetate, but poorly soluble in water. The melting point of the purified compound was 193 - 194°C.

Discussion

Three antimicrobial compounds namely Dibutyl phthalate, 8-Hydroxyquinoline and 2 amino 3 chlorobenzoic acid were isolated and purified from the ethyl acetate extract of *Streptomyces* sp (ERI-15). Dibutyl phthalate is classified by Environmental Protection Agency (EPA), America as a Group D, not classifiable as to human carcinogenicity. It is commonly used as plasticizer and as an additive to adhesives or printing inks. It is also used as an ectoparasiticide.

The genus *Streptomyces* is considered as an important actinomycetes group due to its ability to produce various antimicrobial compounds. Mehdi., *et al*. [5] have reported four bioactive molecules produced from *Streptomyces* sp.TN97 strain that belong to three different families of compounds namely diketopiperazines, isocoumarin and n-acetyltyramine. Similarly, different species of *Streptomyces* produced different types of bioactive molecules. For example, *S. rochei* produced the antibiotic lankacidin [3] and borrelidin [8]; a peptide antibiotic streptothricin [4] and cis-2-amino-1-hydroxycyclobutane-1- acetic acid, a free aminoacid herbicide were produced by *S. rochei. S. fradiae* produced aminosugar antibiotic phosphoamide [9], macrolide antibiotic tylosin and aminoglycoside antibiotic neomycin [10].

In the present study, the compound 1 was identified as dibutyl phthalate. Dibutyl phthalate has previously been reported from *Streptomyces albidoflavus* 321.2 [11] and *Desulfo vibriodesulfuricans* [12]. Dibutyl phthalate and some other phthalate derivatives namely dioctyl phthalate and phthalate ester of alkylated 9-hydroxynonanoic acid have been reported from the bacterium *Burkholderia cepacia* K87 by Sultan., *et al* [13]. Dibutyl phthalate has also been isolated from the methanol extracts of marine algae viz., *Undaria pinnatifida, Laminaria japonica* and Ulva sp. [14] and from a marine *Pseudomonas* strain and used as cathepsin B inhibitor [15]. It was also isolated from *S. melanosporofaciens* and used as a glucosidase inhibitor [16]. The efficacy of the compound dibutyl phthalate as a topical application for demodicidosis was proved effective without adverse reactions by Yuan., *et al.* [17].

The second and third compounds isolated from *Streptomyces* sp. in this study were hydroxyquinoline derivative and 2-amino-3-chlorobenzoic acid, respectively. Hydroxyquinoline is a dark yellowish amorphous compound and 2-amino-3-chlorobenzoic acid is a pale yellow fine crystal compound. Previously some investigators have reported that 8-Hydroxyquinoline had antimicrobial activity against Gram positive and Gram negative bacteria [18]. The third compound 2-amino-3-chlorobenzoic acid was a potent antibacterial agent against MRSA. An analog of this compound was produced by certain halophytic bacteria, containing a single phenyl ring with small substituents like 3-chloroanthranilic acid with some bioactivity [19].

Conclusion

Three antibacterial compounds namely Dibutyl phthalate, 8-Hydroxyquinoline and 2 amino 3 chlorobenzoic acid were isolated from *Streptomyces* sp. (ERI 15) by bioassay-guided isolation using silica gel column chromatography. These three compounds are promising antimicrobial agents, because they show good antibacterial activity against important pathogenic bacteria including methicillin resistant *S. aureus*. These compounds can be included in the list of antibiotics as natural agents.

Acknowledgments

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