CHAPTER-5

PURIFICATION AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUND ISOLATED FROM Enterococcus faecium (MCC2729)
5.1 INTRODUCTION:

Bioactive secondary metabolites of microorganisms and their synthetic derivatives are among the most frequently used therapeutics in human and veterinary medicine. Natural products have several advantages over compounds derived from combinatorial synthetic approaches. While synthetic molecules can be rapidly generated, their structural diversity is limited due to the lack of chiral centers, aromatic rings, oxygen-containing substituents and structural rigidity (Feher & Schmidt, 2003). The diversity of natural products on the other hand can provide physicochemical properties that are hardly found in molecules derived from combinatorial synthesis (Koehn & Carter, 2005; Nussbaum et al., 2006). As metabolic products of living organisms, natural product-derived antibiotics have the unpretentious advantage of being active in target cells *in vivo* (Baltz, 2008). However, natural product-based drug discovery is a complex, time-consuming and expensive endeavor. High investments, short therapy times and the limited lifespan of antibiotics due to emerging resistances have led to decreased or abandoned antibacterial drug discovery efforts in most of the major pharmaceutical companies (Norrby et al., 2005; Projan, 2003; Shales et al., 2004), leaving the field mainly to academia and smaller biotechnology firms (Nussbaum et al., 2006). Various biological compounds obtained from diverse natural habitats have been found to be the most consisted source of novel bioactive molecules acting against a wide range of assay targets. Active compounds from only less than 10% of the world biodiversity have been evaluated for the bioactivity and structural diversity. Many useful compounds may be awaiting in nature for proper evaluation and characterization. Several microbial strains isolated from the soil samples belonging to diverse prospective areas were screened for antimicrobial activity. Fermentation broth was tested against bacterial test organisms attempts were made to characterize the antibiotics producing strains. Crude extracts obtained from the fermentation broth were subjected to purification to isolate the active compound for chemical characterization.

Microorganisms can be found in diverse environment and are remarkably adaptive as they can be found in all sorts of seemingly inhospitable environments. In the course of the adaptation in the harsh environment they have evolutionarily come across distinct metabolic pathways and secondary metabolites could be produced due to ecological stresses, eventually.
One of these secondary metabolites is the antibiotics. Antibiotics are the most important microbially produce metabolites and used as the special chemotherapeutic agents which have lethal or inhibitory effects on microbes. But in therapeutic concentrations they have little or no toxic effect on the tissues. Antibiotics have played a major role in the increase in life expectancy in the last 50 years and reduced morbidity and mortality due to infectious diseases. The search for new, safer and broad-spectrum antibiotics with novel mechanism of action has been progressing slowly.

Nearly all antibiotics have been discovered in empirical approaches using simple antagonistic assays against different bacteria by assessing the inhibition of growth of different target organisms by whole broths or extracts of microbial fermentations (Singh & Barrett, 2006). One of the reasons why natural products lost its popularity in industry stems from the perception that extracts are prone to produce artifacts and interferences in the sensitive assays or are simply undetected because of low titers (Koehn & Carter, 2005; Schmid et al., 1999). One way to overcome these issues is by including a fractionation step after the extraction (Koehn & Carter, 2005; Schmid et al., 1999) and the physiobiochemical properties which distinguish the desired antibiotic from other fermentation products must be exploited by using an appropriate separation technique. The purification and identification of the compounds responsible for the biological activity detected in the extract is a crucial step. At this point it is critical to have an efficient system to identify already known antibiotics and to be able to focus the resources on the important ones which are not identified upto now. These “dereplication” issues are of paramount importance, since many known antibiotics are produced by different species and they will be found repeatedly in antimicrobial screens (Liu et al., 2010). This process of dereplication has made enormous progress by the use of databases containing LC–MS (Koehn & Carter, 2005; Pannell & Shigematsu, 1998) or LC–NMR data (Bobzin et al., 2000). The extraction of the extracellular antibiotic compounds from microbial culture involves the solvent extraction of the cell broth based on the solubility of the antibiotics. Most of the extracellular antibiotics have high degree of solubility in non-polar organic solvents, whereas the intracellular antibiotics could be extracted with both polar and non-polar solvents. Further, antibiotic extract is subjected to different analytical techniques to obtain the pure fraction. The purified compound is used for elucidation of structure by various techniques IR and NMR analysis. The present study was
undertaken for the purification and structural elucidation of extra cellular antibiotic metabolite produced by *Enterococcus faecium* CST-1 strain.

5.2 MATERIALS & METHODS:

5.2.1 Inoculum Preparation:

The indigenous isolate *Enterococcus faecium* inoculum was prepared as described in chapter-iv (4.2.2 Inoculum preparation).

5.2.2 Fermentation and isolation of crude bioactive compound:

*Enterococcus faecium* inoculum was prepared as described earlier and subjected for batch fermentation. The batch fermentation (12 lit) was conducted by shake flask method in 1000ml Erlenmeyer flasks. Each flask contained 400ml of the production medium.

A 10% (v/v) of 48h aged inoculum was transferred to 400ml of production medium having composition 10.0g/L Proteose peptone; 10.0 g/L Beef extract; 5.0 g/L Yeastextract; 40.0 g/L Dextrose; 25.0g/L Tryptone; 1.0g/L Polysorbate80; 2.0g/L Ammoniumcitrate; 5.0g/L Sodium acetate; 0.1g/L Magnesium sulphate; 0.05g/L Manganese sulphate; 2.0g/L Dipotassium phosphate ; pH (6.5±0.2). All the flasks were incubated on a rotary shaker (120 rpm) at 37°C for 72 h and then harvested. The pooled culture broth (12 lit) was centrifuged at 8000 rpm for 20 min, at 4°C and then separated the culture filtrate.

5.2.3 Solvent Extraction of crude bioactive compound:

The extracellular bioactive compounds which are present in the culture filtrate were extracted by Solvent extraction method (Shahed et al., 2008). In order to select suitable solvent, an effort has made to extract the bioactive compound from the minimum quantity of culture filtrate using various immiscible organic solvents. The culture filtrate was shaken vigorously with immiscible organic solvents like benzene, n-hexane, ethyl acetate, chloroform, ethanol, n-butanol, methanol. The culture filtrate (500ml) was extracted twice with positive organic solvent (2x500ml). The positive solvent extracts were pooled and evaporated to dryness under reduced pressure at 45°C in a rotary evaporator. The biological activities of crude extract at 1mg/ml were tested against test organisms. Further, the crude antibiotic residue was subjected to thin layer
chromatography and bioautography to get an idea regarding various compounds present in the crude antibiotic mixture and their antibiotic activities.

5.2.4 Purification of crude extract by column chromatography:

Silica gel of 100 - 200μm particle size was selected for column chromatography. The silica gel was suspended in petroleum ether for packing the column. The column consisted of a 40cm long corning glass tube having an internal diameter of 2.5cm with a glass stopper at the bottom. The lower end of the tube contained stinted disc and the washed silica gel suspension was introduced gradually to obtain an air-bubble free continuous column. The final size of the column was 25x2.5cm. The column was equilibrated with methanol. The sample not exceeding 5ml was passed through the column keeping the flow rate at 0.2ml/min, with gradient solvent system consisting of chloroform: methanol (9:1, 7:3, 1:1). Finally the column was washed with methanol. Fractions of 10ml with each solvent system were collected and all the individual fractions were analyzed by TLC for homogeneity. The chromatograms were run and the colored spots were detected by visual detection while colorless substances were detected by iodine vapors. Fractions with similar Rf values were pooled together, and evaporated to dryness under reduced pressure at 45°C. All the pooled fractions were subjected to antimicrobial studies using the test pathogenic organisms.

The active fractions which showed high antimicrobial activity against pathogenic organisms were selected for further detailed studies. While selecting this fraction, the total weight of the residue as well as the pattern of TLC spots were taken into consideration. The active fractions were dissolved in methanol and were applied on a Sephadex LH 20 column (25x2.5cm), which was pre-equilibrated with methanol at a flow rate of 20ml/h. The active fraction was eluted with methanol and 10ml fractions were collected. All these fractions were chromatographed on TLC and fractions with similar Rf values were pooled together. The biological activities of these fractions were evaluated as described earlier.

5.2.5 Determination of homogeneity of the antibiotic metabolite:
The homogeneity of the isolated antibiotic was determined by paper and thin-layer chromatography. For this purpose, one dimensional ascending chromatography was performed on paper and thin layer chromatographic plates. The spots on papers and TLC plates are visualized by keeping them in a closed chamber containing iodine vapor. The antibiotic activities of the spots were confirmed by bioautography using test pathogenic organisms. The Rf values of the antibiotically active spots were determined. The homogeneous antibiotic metabolite was designated as CPY-1 and used for characterization and for further studies.

5.2.5.1 Thin layer chromatography (TLC):

Bertina and Barath (1964) and Bickel et al., (1962) used the combination of thin layer chromatography and bioautography for the detection of antibiotics. Initially pure solvents i.e. hexane, chloroform, ethyl acetate and methanol were used to get a preliminary idea about different components present in the crude mixture and their polarities. Pre silica gel coated TLC sheets (20x20cm, E-Merk) were used in the present study. Twenty μl of crude ethanol extract (2.5mg/ml) was applied with a micropipette. Single glass chamber was used for development of chromatograms. After development in each solvent system, the chromatograms were scanned under UV light for the detection of colorless spots while the colored spots were detected by visual observation. All the chromatograms were later bio autographed.

5.2.5.2 Bio-autography:

The bio autographic glass plates (32cm X 23cm) were sterilized by dry heat. 250ml of sterile nutrient agar was seeded with 2ml of pathogenic test organism, thoroughly mixed, poured into bio autographic plate and allowed to solidify. The detection of active spots on developed chromatogram (bio autography) was done by keeping the pre-coated TLC sheet directly onto the seeded agar medium. The sheet was removed after 6 hours from the agar surface and the plates were incubated at 37°C for 24 hours.

5.2.6 Determination of antimicrobial activity:

The fractions obtained from column chromatography were dissolved, each in a specific volume of ethanol to give 1 mg/ml concentration and tested for their antimicrobial activities using test organisms by agar well plate method. Aqueous solutions of the purified antibiotic
metabolite (250-1000μg/ml) were used. Different test organisms *Pseudomonas aeruginosa* (MTCC4676), *Escherichia coli* (MTCC1652), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 10403), were used as test organisms. Assay plates were prepared, upon solidification, 6mm diameter wells were scooped out with sterile cork borer and to each well 50μl of the antibiotic metabolite solution of different concentrations were added. For each concentration of antibiotic metabolite, three replicates were made. The plates were incubated at 37°C for 24 h and the inhibition zones were measured to the nearest mm using antibiotic zone reader.

5.2.7 Determination of purity by High pressure Liquid Chromatography (HPLC):

The active fraction was further checked for purity by using HPLC analysis using Waters Alliance 2695 HPLC model. The active fraction was loaded onto the Zorbax SB C18 column (150 x 4.6mm) and eluted by applying 6000 psi pressure at 25°C using 0.1% orthophosphoric acid in ethanol and KH₂PO₄. The flow rate was maintained at 1.0 mL/min. The wavelength of the detector was maintained at 254nm using photo diode array (PDA) detector (Waters 2998).

5.2.8. Structural elucidation of the antibiotic metabolite:

5.2.8.1 Physio-chemical properties of purified antibiotic compound:

5.2.8.1.1 Solubility:

The solubility pattern of the purified compound was determined by using various organic polar and non-polar solvents.

5.2.8.1.2 Melting point:

The melting point was determined by Fisher-Johns melting point apparatus as described by (Ganghi *et al.*, 1976).

5.2.8.2 Spectroscopic analysis of antibiotic compound:

5.2.8.2.1 Mass spectrum:
The mass spectrometric measurements of purified compound were performed on an AGILENT 6410 Triple Quad LC-MS. The software used for viewing and editing the data was executed by MASSHUNTER WORKSTATION VERSION B.03.01; Build 3.1.346.0.

5.2.8.2.2 Elemental analysis:

The ratio of the C, H, O, N and S of the purified antibiotic was analyzed according to the method of (Atta et al., 2011) by using elemental analyzer (Elementar, Vario EL).

5.2.8.2.3 FTIR spectrum:

The Infrared (FTIR) absorption spectrum of the purified antibiotic was recorded using a Bruker Alpha-T FT-IR Spectroscopy. The purified antibiotic was taken in KBr discs and spectrum was scanned between wave number 500 to 4000 cm\(^{-1}\), at a resolution of 4 cm\(^{-1}\). The OPUS 6.5 software was used for viewing the resulting data.

5.2.8.2.4 \(^1\)H and \(^{13}\)C NMR Spectrum:

The NMR data of purified compound was acquired using an AMX-400 spectrometer (Bruker, Rheinstetten, Germany). \(^1\)H NMR spectra were obtained at 400.13 MHz and \(^{13}\)C NMR spectra were obtained at 100.6 MHz. All NMR spectra were recorded in CDCl\(_3\). The chemical shifts were expressed in δ (ppm) using CDCl\(_3\) as solvent and TMS (Tetra Methyl Saline) as internal reference. The multiplicities of the \(^{13}\)C-NMR values were assigned by attached proton test (APT) coupling constants J (Hz); s (singlet); d (doublet), t (triplet), q (quartet).

5.2.9. Minimum inhibitory concentration (MIC):

The minimum inhibitory concentration (MIC) of the antibiotic metabolite against bacteria was determined by broth dilution method (NCCLS, 2000). An aqueous solution of the purified antibiotic was added to nutrient broth to attain the desired concentrations. The antibiotic metabolite containing broths were inoculated with the respective test organisms such as Staphylococcus aureus (MTCC 3160), Bacillus cereus (MTCC 430) Bacillus Subtilis (MTCC 441) Eischeria coli (MTCC 443) Pseudomonas aeruginosa (MTCC 424) and incubated at 37°C for 24h. The results were expressed as the minimum concentration required for total inhibition of bacterial growth.
5.2.10. Statistical analysis:

The results analyzed in this chapter were the mean or SD (Standard Deviation) of three independent experiments. The data was statistically analyzed by one way ANOVA and the means were assessed by DMRT (Dunken Multiple Range Test) at 0.5% level of significance.

5.3 RESULTS AND DISCUSSION:

5.3.1. Solvent Extraction of crude bioactive compound:

The active substance present in the culture filtrate was extracted with various immiscible solvents. Among them, the compound was extracted with the solvent ethanol. The ethanol extract was then evaporated to dryness under reduced pressure at 45°C in a rotary evaporator. The compound appeared as white to pale yellow colored solid material. About 2.5 gm of the active substance was obtained from the culture filtrate (12 lit).

5.3.2 Purification of ethanol fraction by column chromatography:

The ethanol extract was purified by using silica gel column chromatography. About 2.5g of crude ethanol extract was dissolved in 25ml of methanol and was chromatographed on silica gel column and eluted with gradient solvent system consisting of 100% CHCl3, chloroform: methanol in the ratio of (9:1,7:3, 1:1) and followed by 100% methanol. A total of 115 fractions of 10ml each were collected (Table 5.1). The purity of all the fractions was checked by TLC studies. Fractions with similar Rf values were pooled together, which finally resulted in five major fractions. Each of these five fractions was evaporated to dryness under vacuum at 45°C.

The silica gel column chromatography profile was shown in Table 5.1. Among all fractions tested for antimicrobial activity, only 7:3 fraction showed antimicrobial activity against pathogenic test organisms. Hence it was selected for further purification. Fraction (7:3) was further purified by sephadex column chromatography, in order to remove the minor impurities and other unsolicited metabolites. A total of 20 minor fractions of 2 ml each were collected. All
the fractions were subjected to TLC analysis and the fractions with similar Rf values were pooled together. Finally three major fractions were obtained i.e, Fraction I (232mg), fraction II (24mg) and fraction III (11mg) as shown in the Table 5.2. Among the three fractions, only the Fraction I exhibited antibacterial activity against the test organisms and designated as CPY-1. Similarly, Zhang 2011, reported that pyroglutamic acid is isolated from the ethanol extracts by the silica gel column and Sephadex LH-20 column chromatography.

**5.3.3 Homogeneity of the antibiotic metabolite:**

Thin layer chromatographic methods have been used to check the identity and homogeneity of the CPY-1. The Rf values of the antibiotically active spots were determined. The ethanol fraction was found to form a single band on TLC with Rf values 0.63 and the variation in the purity of the antibiotic and the experimental conditions used often lead to variation in the Rf values of the antibiotic. It was observed that the purified compound formed single active spot on all chromatograms. Results of paper and thin layer chromatography using number of solvent systems have confirmed the homogeneous nature of CPY-1 and also confirmed with bioautography (Figure 5.1). Hence it was analyzed for its purity using HPLC.

**5.3.4 Determination of Purity by HPLC:**

The purity of the antibiotic CPY-1 was analyzed by HPLC and the chromatogram was shown in figure 5.2. The sample was run for a run time of 20min and a single major peak along with few minor peaks were obtained. The major peak was obtained at a retention time of 8.208 with purity of about 96%. Since the compound was more than 95% pure, it was further investigated for structural elucidation.

**5.3.5 Properties and Structural Elucidation of antibacterial compound CPY-1.**

The compound was obtained in the form of a white to pale yellow coloured solid material. It was soluble in water, dimethyl sulfoxide and ethanol. The melting point of the purified compound was found to be 180°C.
5.3.5.1 FT-IR absorption spectra:

The IR absorption spectrum of the antibiotic was shown in **Figure 5.3**. In IR spectrum it exhibited absorptions at 3301, 3013, 2993, 2391, 1715 cm\(^{-1}\). The absorption spectrum showed presence of a hydroxy group (3301 cm\(^{-1}\)); amide group (3013 cm\(^{-1}\)); carboxylic acid (1715 cm\(^{-1}\)); amide group (1438 cm\(^{-1}\)); aromatic alkenes (C=C) 975 cm\(^{-1}\) and aromatic hydrogen’s (878 cm\(^{-1}\), 831 cm\(^{-1}\), 758 cm\(^{-1}\), 706 cm\(^{-1}\), 642 cm\(^{-1}\), 573 cm\(^{-1}\), 545 cm\(^{-1}\)). From the above observations, the compound may contain amide group, hydroxy group and aliphatic hydrogens and carboxylic acid group.

5.3.5.2 Mass spectrum and elemental analysis:

The molecular mass (m/z) of the compound was found to be 129.99kDa. The Mass spectral of the antibiotic CPY-1. From the elemental analysis calculated the results are as followed: C 46.51%; H 5.46%; N 10.85%; O 37.18%.

5.3.5.3 NMR analysis of spectral data:

\(^1\)H NMR was obtained at 400.13 MHz and \(^1\)C NMR spectra were obtained at 100.5 MHz and were shown in **Figure 5.5** and **Figure 5.6** respectively.

5.3.5.3.1 \(^1\)H NMR Spectrum:

From the \(^1\)H NMR spectrum of the compound peaks were observed at \(\delta\) 1.83 (1H, m), 2.02 (2H,m), 2.18 (1H,m), 4.00(1H,m), 2.00 (1H,m), 1.28 (1H,m) .The \(^1\)H NMR spectrum showed single peak in the carboxylic acid region[\(\delta\) 1.073(1H); and \(\delta\) 1.079 (1H, m), \(\delta\) 2.02 (2H, m), \(\delta\) 1.063 (1H, m), \(\delta\) 1.083 (1H, m),and \(\delta\) 1.040 (1H, m), ] indicating the presence of 7 protons in the 2-pyrrolidone region.

5.3.5.3.2 \(^1\)C NMR Spectrum:

From the \(^1\)C NMR spectrum of the compound peaks were observed at \(\delta\) 29.01, \(\delta\) 24.55, \(\delta\) 54.71 ,\(\delta\) 174.36 and \(\delta\) 176.97. The \(^1\)C NMR spectrum showed 3 carbon atoms in the aliphatic region[\(\delta\) 29.01(1C), \(\delta\) 24.55(1C) and \(\delta\) 54.719(1C)] and and 2 quaternary carbon atoms \(\delta\) 174.36 (1C) carboxylic group region and \(\delta\) 176.97 (1C) in the amide group region indicating the presence of 5 carbon atoms.
These chemical assignments obtained from the IR, LC-MS, NMR suggested to be CPY-1 as **2-Pyrrolidone-5-Carboxylic acid**. The molecular formula was determined as \( \text{C}_5\text{H}_7\text{NO}_3 \). and the proposed structure was shown in Figure 5.7.

**Table 5.1: Silica gel Chromatographic profile of crude ethanol extract.**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Eluent</th>
<th>No. of fractions</th>
<th>fractions Pooled fraction No. after TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloroform</td>
<td>1-20</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>CHCl₃:CH₃OH (9:1)</td>
<td>21-50</td>
<td>II</td>
</tr>
<tr>
<td>3</td>
<td>CHCl₃:CH₃OH (7:3)</td>
<td>51-72</td>
<td>III</td>
</tr>
<tr>
<td>4</td>
<td>CHCl₃:CH₃OH (1:1)</td>
<td>73-93</td>
<td>IV</td>
</tr>
<tr>
<td>5</td>
<td>Methanol</td>
<td>94-115</td>
<td>V</td>
</tr>
</tbody>
</table>

**Table 5.2: Rechromatographic profiles of Fraction III on silica gel column.**

<table>
<thead>
<tr>
<th>S.NO</th>
<th>No. of fractions</th>
<th>fractions Pooled fraction No. after TLC</th>
<th>Weight of residue (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-20</td>
<td>I</td>
<td>232</td>
</tr>
<tr>
<td>2</td>
<td>21-30</td>
<td>II</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>31-40</td>
<td>III</td>
<td>11</td>
</tr>
</tbody>
</table>

Eluent: Methanol
Figure 5.1: (a) Thin Layer Chromatography and (b) Bio-autography showing single peak & bioactivity.
Figure 5.2: Chromatogram of the antibiotic CPY-1 by HPLC
Figure 5.3: Infrared absorption spectrum of the purified antibiotic CPY-1
Figure 5.4: $^1$H NMR spectrum of the antibiotic CPY-1
Figure 5.5: $^{13}$C NMR spectrum of the antibiotic CPY-1
2-Pyrrolidone-5-carboxylic acid

Chemical Formula: C₅H₇NO₃

Exact Mass: 129.99

Elemental analysis: C 46.51%; H 5.46%; N 10.85%; O 37.18%,

Figure 5.6: Structure of CPY-1.

5.3.6 Determining the antimicrobial potential with purified antibiotic (CPY-1) Minimum inhibitory concentration (MIC):

The antibiotic metabolite CPY-1 showed broad spectrum antibacterial activity inhibiting both gram positive and gram negative bacteria. The MIC values for the antibiotic were shown in the Table 5.3. The MIC required to inhibit the growth of bacteria was identified to be 10µg/mL to 24µg/mL. The gram positive bacteria B.subtilis and S.aureus were inhibited at 10µg/ml and 12 µg/ml concentration. While gram negative bacteria P.aeruginosa inhibited at 20µg/ml and E.coli was inhibited at 24µg/ml concentration respectively. Overall results indicated that the purified antibiotic CPY-1 showing a broad antimicrobial activity against gram positive bacteria.
Table 5.3: Minimum Inhibitory concentrations of the antibiotic metabolite CPY-1

<table>
<thead>
<tr>
<th>Test organism</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Positive Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> (MTCC 3160)</td>
<td>12 ± 0.266</td>
</tr>
<tr>
<td><em>B. Subtilis</em> (MTCC 441)</td>
<td>10 ± 0.614</td>
</tr>
<tr>
<td><em>Bacillus cereus</em> MTCC 430</td>
<td>14 ± 0.232</td>
</tr>
<tr>
<td><strong>Gram negative bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> (MTCC 443)</td>
<td>24 ± 0.333</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (MTCC 424)</td>
<td>20 ± 0.577</td>
</tr>
</tbody>
</table>

Values expressed are a mean of the three replicates ± SD [ P ≤ 0.05 (DMRT)].

5.3.7. Testing the putative compound 2-pyrillodine-5-carboxylic acid for anti-proliferative activity:

The putative compound CPY-1 isolated from *Enterococcus faecium* (MCC 2729) was tested for the anti-proliferative activity.

5.3.7.1 Antiproliferative activity of the purified antibiotic compound:

Natural products have afforded a rich source of compounds that has found many applications in cancer chemotherapy. Global cancer rates could increase by 50% to 15 million by 2020 (WHO, 2010). Chemotherapy is one of the potent treatments for prolonging the patient’s life (Sirinet, 2010). Over 70% of the anticancer compounds are either natural products or natural product derived substances (Karikas, 2010) and the therapeutic applications of microbial metabolites provides the opportunity for the discovery of anticancer agent for example: Disorazole A1, doxorubicin, bleomycin, mitomycin, lipopeptide and dactinomincines (Grever, 2001; Elnakady et al., 2004).
5.3.7.2 Materials & Methods:

5.3.7.2.1 Cell culture:

   Human cancer cell lines (HeLa and k-562) used in this study were procured from National Centre for Cell Science, Pune. All cells were grown in Minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37°C in 5% CO₂ for 48h in CO₂ incubator.

5.3.7.2.2 MTT assay:

   The standard MTT assay developed (Mosmann, 1983) was modified and used to determine the inhibitory effects of test compounds on cell growth in vitro. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5x10³ cells/well in growth medium and cultured at 37°C in 5% CO₂ to adhere. After 48hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (20, 40, 60, 80 and 100 μg/ml) in triplicates to achieve a final volume of 100μl and then cultured for 48hr. The compound was prepared as 1.0mg/ml concentration stock solutions in PBS. Culture medium and solvent are used as controls. Each well then received 20μl of fresh MTT (0.5mg/ml in PBS) followed by incubation for 4hr at 37°C. The supernatant growth medium was removed from the wells and replaced with 100μl of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 492nm on an ELISA reader, Anthos 2020 spectrophotometer.

5.3.7.2.3 Statistical analysis:

   The results analyzed in this chapter were the mean or SD (Standard Deviation) of three independent experiments. The data was statistically analyzed by one way ANOVA and the means were assessed by DMRT (Dunken Multiple Range Test) at 0.5% level of significance.
5.3.7.3 Results & Discussion:

In the present study antibacterial compound CPY-1 which was isolated from *Enterococcus faecium* MCC2729 was found to be inhibiting the proliferation of HeLa and K-562 cell lines in a dose dependent manner (20 to 100) µg/ml. A linear correlation was observed between CPY-1 concentration and percentage of inhibition of cell proliferation. The CPY-1 compound causes the cell aggregation and cell sinkage of HeLa and K-562 cell lines and most of the cells contain uncharacterized bodies, this may due to the induction of apoptosis (Figure 5.10 & 5.11).

The compound CPY-1 exhibits anti-proliferative activity on the cervical cancer cell lines (HeLa) and Leukemia cell lines (K562) (Table 5.4) (Table 5.5). The IC$_{50}$ value for HeLa and K-562 cell lines were found to be 60.32µg/mL and 82.41µg/mL respectively (Figure 5.8 & 5.9). The survival rate of K-562 cells was higher than that of HeLa cells at the same concentration of compound CPY-1. These results suggest that the compound CPY-1 can be used as an anti-proliferative agent. However, the antitumor effects of low molecular weight substances like sodium pyroglutamate, isolated from the basidiomycete fungus as antitumor and anti-angiogenic substances were reported by Kimura, *et al.*, 2004.
Table 5.4: Effect of *Enterococcus faecium* metabolite-CPY-1 on HeLa cell lines (Cervical Cancer Cell lines)

<table>
<thead>
<tr>
<th>Conc. In µg/ml</th>
<th>% Cell Survival</th>
<th>% of Inhibition activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>77.1</td>
<td>22.9</td>
</tr>
<tr>
<td>40</td>
<td>73.3</td>
<td>26.7</td>
</tr>
<tr>
<td>60</td>
<td>62.7</td>
<td>37.3</td>
</tr>
<tr>
<td>80</td>
<td>54.4</td>
<td>45.6</td>
</tr>
<tr>
<td>100</td>
<td>20.7</td>
<td>79.3</td>
</tr>
</tbody>
</table>

IC$_{50}$ = 82.41 ug/mL

Correlation coefficient = 0.8805253

Intercept = 26.5516666

Slope = 0.20932042

Values expressed are mean of the three replicates ± SD

Values indicate significant difference at P ≤ 0.05.
Table 5.5: Effect of *Enterococcus faecium* metabolite CPY-1 on K-562 (Leukemia)cell lines

<table>
<thead>
<tr>
<th>Conc. in µg/ml</th>
<th>% Cell Survival</th>
<th>% of Inhibition activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>80.2</td>
<td>19.8</td>
</tr>
<tr>
<td>40</td>
<td>77.4</td>
<td>22.6</td>
</tr>
<tr>
<td>60</td>
<td>49.6</td>
<td>50.4</td>
</tr>
<tr>
<td>80</td>
<td>39.7</td>
<td>60.3</td>
</tr>
<tr>
<td>100</td>
<td>27.6</td>
<td>72.4</td>
</tr>
</tbody>
</table>

**CPY-1 action on K-562 cell lines (Leukemia)**

- IC\(_{50}\) = 60.32 µg/mL
- Correlation coefficient = 0.94446820
- Intercept = 8.8433332
- Slope = 0.2305377

Values expressed are mean of the three replicates ± SD

Values indicate significant difference at \(P \leq 0.05\).
Figure 5.7: Inhibiton activity of 2-pyrollidine-5-carboxylic acid on Hela cell lines.

Data represented are a mean of the three replicates ± SD [P≤0.05 (DMRT)]

Figure 5.8: Inhibiton activity of 2-pyrollidine-5-carboxylic acid on K-562 cell lines.

Data represented are a mean of the three replicates ± SD [P≤0.05 (DMRT)]
Figure 5.9: Activity of Enterococcus faecium metabolite CPY-1 on Hela (Cervical cancer) cell lines before and after addition of metabolite.

Figure 5.10: Activity of Enterococcus faecium metabolite CPY-1 on K-562 cell lines (Leukemia) cell lines before and after addition of metabolite.
5.4 CONCLUSION:

The structure was elucidated after the analysis of the available data from the graphs, HPLC, LC-Mass, FT-IR, $^1$H & $^{13}$C NMR. The proposed structure of the antibiotic named as **2-Pyrrolidone-5-Carboxylic acid (CPY-1)**. Antimicrobial compounds which are produced by lactic acid bacteria *sps* have also been described and currently being used due to their wider spectrum activity. For example *Lactobacillus reuteri* produces reutericyclin and reuterin (Talarico and Dobrogosz 1989; Ganzle *et al.* 2000), and *Lactobacillus casei ssp.casei* produces 2-pyrillodine-5-carboxylic acid (Huttunen *et al.* 1995). Nardi *et al.*, 2005 also isolated pyroglutamic acid from *Lactobacillus murinus* strain L1. Pyroglutamic acid (PCA), is also produced by *Streptococcus bovis*. Pyroglutamic acid has a stronger antimicrobial activity and its mechanism of action is similar to that of organic acids and inhibits *Bacillus subtilis*, *Enterobacter cloacae*, *Pseudomonas putida* and *Pseudomonas fluorescens* (Nardi *et al.*, 2005).

The MIC required by **2-Pyrrolidone-5-Carboxylic acid (CPY-1)** to inhibit the growth of both gram positive and gram negative bacteria was identified to be 10µg/mL to 24µg/mL. The compound CPY-1 exhibits antiproliferative activity on the Cervical cancer cell lines HeLa cells (Table 5.4, Figure 5.8 & 5.10) and Leukemia cell lines K562 (Table 5.5, Figure 5.9 & Figure 5.11). The percentage of inhibitory activity for the cervical cancer cell line was 82.41% at 100µg/ml concentration and for the Leukemia it was 60.32% at 100 µg/ml concentration. It can be concluded as the inhibitory effect was more on the cell line HeLa (cervical cancer lines) when compared to the K562 (Leukemia cancer cell lines).