SYNTHESIS, CHARACTERIZATION OF ANTIMICROBIAL ACTIVITY OF 22′DICHLOROHYDROBENZOXIN

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INTRODUCTION

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains which are multi-resistant. Consequently, new infections can occur in hospitals resulting in high mortality. The problem of microbial resistance is growing, and the outlook for the use of antimicrobial drugs in the future is still uncertain [1-6].

Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotics, to develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient.

Compounds having diol group are reported to exhibit a broad spectrum of biological activity such as antibacterial [7-9] and antifungal [10,11]. No diol compounds yet been synthesized with two electron withdrawing chloro groups as a substituents to our best knowledge. The structure of pure compound was characterized by mass, IR, and 1H nuclear magnetic resonance (NMR), and 13C NMR spectral analysis and single crystal X-ray diffraction (XRD) studies. The synthesized compound was evaluated for antimicrobial activity using agar well diffusion method.

METHODS

Synthesis and purification of 22′dichlorohydrobenzoin [22′CD]

The starting material, for the synthesis of 22′CD, was synthesized by benzoin condensation. About 4 g of KCN dissolved in 75 cc of water in a 1 L flask. 14 g of 2-chlorobenzaldehyde and 75 cc of 95% ethanol were added into the flask. The mixture formed into a solution at the boiling temperature and was refluxed for 1½ h. Steam was then passed through the solution until all the alcohol and nearly all the unreacted aldehydes were removed. The condensed water was decanted from the product and later set aside for crystallization. The product was then pressed as free as possible from oily material in an ice bath and constantly stirred. Concentrated HCl was added in dropwise and stirred until foaming ceases. To this, 10 g of 2, 2′-dichlorobenzil was added in acetone-d6 and MeOD as solvent, with TMS as an internal standard. The structure of the title compound was characterized by mass, IR, and NMR spectra. Mass spectrum was recorded using JEOL GC mate mass spectrometer. The infrared spectrum was recorded using Perkin Elmer spectrometer and alpha spectrometer in the frequency range of 4000–450/cm, using KBr pellet method. NMR spectrum was recorded using BRUKER 500 MHz AVANCE III instrument using CDCl3, DMSO-d6, acetone-d6 and MeOD as solvent, with TMS as an internal standard.

Antibacterial activity of 22′CD

The antibacterial activity of 22′CD was determined using the hole-in-plate bioassay procedure. The pure cultures of the microorganisms were inoculated onto Muller-Hilton nutrient broth incubated at temperature
of 37°C for 24 h [13]. Using a sterile cork borer of 5 mm diameter, three holes were made into the Petri dishes seeded with bacterial culture. The various concentrations of the synthesized compounds were inoculated in the wells prepared on the agar plates. The plates were incubated at temperature of 37°C for 18 h. Staphylococcus aureus (ATCC 25923), Escherichia coli 0157:H7 (PSSCMI 0032), Salmonella paratyphi (PSSCMI 0034), and Bacillus subtilis were used as the test microorganisms. All bacterial cultures were maintained on nutrient agar slants at temperature of 4°C and subcultured onto nutrient agar broth for 24 h before testing [14].

**Antifungal activity of 22’ CD**
Various concentrations of synthesized compounds were powered into the wells and examined against *C. albicans* and *Aspergillus niger*. Holes were made into the Petri dishes containing inoculated medium. The diameter of the clear zone around the wells (inhibition diameter) was measured at the end of the incubation period. The samples that presented high mean diameter were subjected to minimum inhibitory concentration analysis as described above. Three 22’CD doses in wells per plate against a single microorganism were used [15].

**Antimicrobial assay isolation and maintenance of cultures**
*E. coli*, *Pseudomonas aeruginosa*, *S. paratyphi*, *Klebsiella pneumoniae*, *S. aureus*, *Streptococcus pyogenes*, and *B. subtilis* were extracted from foodstuffs by serial dilution agar plate method. In this method, serial dilutions of samples obtained from foodstuffs were prepared, and aliquots from each dilution were added to the plates containing nutrient agar to allow the growth of microbes. All the bacterial isolates were identified by cultural, morphological biochemical characteristics (Gram and endospore staining). The plates were kept in an incubator at 37°C. The slants were prepared from the pure cultures obtained and kept in the refrigerator at 4°C for further use.

**Standardization of inoculum**
The microbial inoculum was standardized at 0.5 McFarland. In microbiology, McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. Original McFarland standards were made by mixing specified amounts of barium chloride and sulfuric acid together. Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate (BaCl₂•2H₂O), with 9.95 ml of 1% sulfuric acid (H₂SO₄). The standard could be compared visually to a suspension of bacteria in sterile saline or nutrient broth [16].

**RESULTS AND DISCUSSION**

**Mass spectral analysis of 22’CD**
Mass spectral data and elemental analysis were in good agreement with the assigned formula (Table 1 and Fig. 1).

**Fourier transform infrared (FTIR) spectral analysis of 22’CD**

![Scheme 1: Schematic representation of 22’dichlorohydrobenzoin](image)

![Table 1: Mass spectral fragmentation peaks for 22’CD](image)

![Fig. 1: Mass spectra of 22’dichlorohydrobenzoin](image)
In FTIR spectrum of the 22’CD, the –OH stretching modes were found at 3332/cm. The bands around 3000/cm in FTIR were assigned to the aromatic C–H stretching modes. The aliphatic -CH stretching vibration was assigned to the band at 2925/cm in FTIR with weak intensity (Table 2). The aromatic C=C symmetric stretching vibrations appear at 1439/cm as a strong intensity. The –OH out of plane deformation was observed as strong band around 1007/cm. The band at 741/cm in FTIR indicates the presence of disubstituted benzene ring (Fig. 2).

**FT NMR spectral analysis of 22’CD**

The 1H NMR and 13C NMR spectral signals of the 22’CD were observed (Fig. 3a and b). The corresponding datum was presented in Table 3. The spectra exhibit a multiplet at 6.8–7.5 ppm for the hydrogens of the aromatic rings hydrogen. The -CHOH hydrogen leads to a broad singlet of intensity equivalent to two hydrogens at 3 ppm. The spectra show doublet with an integration equivalent to two hydrogens at 5.8–5.9 ppm corresponding to the hydrogen of the –CH-CH group (the signal appears as a doublet of doublet in expanded spectra).

In the 13C NMR, aliphatic carbon appears in the range of 60–80 ppm and aromatic carbon atoms appear in the range of 127–137 ppm.

**Agar well diffusion method**

For determination of antimicrobial activity of 22’CD, different bacterial and fungal strains were used by agar ditch method. The pathogenic cultures were swabbed separately in each air-dried preincubated nutrient agar and Sabouraud dextrose agar plates with the help of sterile cotton swabs. Ditches were prepared in agar plates with the help of surface sterilized borer. After boring, the test drug of different concentrations was added separately to the ditches (50 µl) [17-20]. The commercial available ciprofloxacin Ranbaxy Laboratory Limited, New Delhi, was used for comparison study. The antibiotic ciprofloxacin and fungicidal agent miconazole were prepared and the concentrations (50 µg/ml) were impregnated into ditches in agar medium. The plates were incubated at 37°C. Controls were maintained. After 24 h diameter of clear zone produced around the ditches were measured to the nearest mm with the help of the micro scales.

Antibacterial activity of 22’CD was tested using agar well diffusion method. 200 μl of bacteria was aseptically introduced and spread using cotton swabs on the surface of gelled sterile Muller-Hilton agar plates. A well of about 6.0 mm diameter with sterile cock borer was aseptically punched on each agar plate. 22’CD in different three concentrations was introduced into the wells in the plates. A negative control well was too made with 50 µl of the sterile distilled water. A positive control was made by placing antibiotic disc (ciprofloxacin) on agar plate. Plates were kept in laminar flow for 30 min for prediffusion of 22’CD to occur and then incubated at 37°C for 24 h. Resulting zone of inhibition was measured using a Hi-Media zone scale.

The antibacterial activity of 22’CD was found to be effective against both *E. coli* with zone of inhibition of 10 mm (Table 4) and *B. subtilis* with zone of inhibition ranging between 8 and 10 mm (Table 5). The 22’CD lower concentration was ineffective against both the test organisms (Fig 4).

The inhibition zone formation of 22’CD was compared with the standard antibiotic miconazole (Table 6). The zone of inhibition at 100 µg/ml concentration was maximum. However, the high dose used presented significant activity against *C. albicans* and *A. niger*. From these results, it was observed that 22’CD bioactivity varied with the concentrations used (Fig 5).

**CONCLUSION**

Antimicrobial studies of 22’CD showed higher antimicrobial activity against Gram-positive bacteria compared to Gram-negative bacteria.

**Table 2: Vibrational assignments of the 22’CD**

| FTIR for 22’CD (wavenumber cm⁻¹) | Band assignments                  |
|---------------------------------|-----------------------------------|
| 3332/cm [vs]                    | –OH stretching                    |
| 3070/cm                         | Aromatic C-H stretching           |
| 2925/cm [w]                     | Aliphatic C-H stretching          |
| 1439/cm [s]                     | Aromatic sym C=C stretching       |
| 1007/cm                         | –OH out of plane deformation      |
| 741/cm                          | Disubstituted benzene ring        |

22’CD: 22’dichlorohydrobenzoin, FTIR: Fourier transform infrared, w: Weak; vs: Very weak, m: Medium, s: Strong, vs: Very strong

**Table 3: The chemical shift in 1H NMR and 13C NMR spectrum of 22’CD**

| Spectrum | Signal (ppm) | Group identification   |
|----------|--------------|------------------------|
| 1H NMR   | 2.0 ppm      | -CHOH                  |
|          | 5.8–5.9 ppm  | CH-CH                  |
|          | 6.8–7.5 ppm (multiplet) | Aromatic protons |
| 13C NMR  | 60 and 80 ppm | Aliphatic carbon atoms |
|          | 127–137 ppm  | Aromatic carbon atoms  |

22’CD: 22’dichlorohydrobenzoin, NMR: Nuclear magnetic resonance
AUTHORS’ CONTRIBUTION

Concept and writing of the article Thanuja B and review of the article Charles Kanagam.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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