CHAPTER VI

ANTIMICROBIAL STUDIES

6. Antimicrobial studies

6.1. General

One important area of research in fighting diseases is the synthesis of new drugs and best ways to use the available drugs. Although some general guide-lines for the therapy of diseases caused by microbes are accepted everywhere, it is still necessary to search for new antimicrobial agents. The bioactivity of molecules is reported to be a combination of steric, electronic and pharmokinetic factors and can be understood by chelate theory. It has been demonstrated that inert, highly stable metal chelates have considerable activity in the presence of tissue, body fluid and infective exudates against a wide range of microorganisms, most of which show high resistance to various antibiotics. The greatest biological activity is generally associated with complexes whose considerable lipid solubility in vitro would favour penetration of biological membranes which at the same time would have a high capacity to bind by coulombic and van der Waals forces to diverse
biological surfaces having suitable complementing steric factors. In enzyme reactions the metal ion plays an important role by providing proper stereochemical orientation and bringing the reacting molecules, the enzyme and the substrate closer so that the reaction may occur. Coordination compounds can act upon biological systems in several ways. Applications of inorganic complexes in medicine are wide and varied and include antiviral, anti-tumour, anticancer and antibacterial agents etc. Complexes are active against a range of bacteria, fungi and viruses. The proposed mechanism of action are (1) agents that inhibit the synthesis of bacterial cell walls, (2) agents acting directly on the cell membrane leading to leakage of intercellular compounds, (3) agents causing irreversible inhibition of protein synthesis, (4) agents altering protein synthesis leading to cell death and (5) agents that affect nucleic acid metabolism. Various antimicrobial agents can build tunnels through cell membrane or act as ion carriers thus disrupting normal concentration gradients leading to cell death. An antimicrobial agent should show selectivity against bacterial cell over animal cell, otherwise it would be useless as a medicine. Antimicrobial studies of some metal chelates indicated that the following trends are observed. 

(1) Metal complexes show increased activity than the corresponding ligands. It is known that chelation reduces polarity of metal ion because of partial sharing of its positive charge with the donor groups of ligands. Such chelation increases the lipophilic character of metal complex, which is necessary to
cross the permeability barrier of cells resulting in interference with normal process of microorganisms. (2) Compounds with \( \pi \) electron delocalisation exhibit more toxicity than the corresponding compounds which lack \( \pi \) electron delocalisation. (3) The general trend of growth inhibition against all the bacteria is found to be in the order \( Ni > Cu > Co > Zn \)

It is well known that most of the antibiotic drugs have N, O and S containing groups situated at the site ideal for formation of strain free five or six membered ring. They may give 1:1 or 1:2 (metal to drug) chelates with biologically important metal ions such as Cu(II), Co(II), Zn(II), Mn(II), Mg(II) and Fe(II) mostly in octahedral (or occasionally square planar) stereochemistry. On chelation the polarity of the metal ion will be reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of positive charge of metal ion with donor groups. Further it increases the delocalisation of \( \pi \) electrons over the whole chelate and enhances the lipophilicity of the complexes. Chelation with metal ions gives some important physical properties to these drugs, which are helpful in their biological activity, such as low dissociation constant, special redox potential, electron distribution and solubility. Chelation leads to increase in the hydrophobic character of the metal chelate favoring its permeation through the lipid layer of microorganism. Attempts have been made to correlate the stability of the metal drug chelates with their antibacterial activity.\(^{236-240}\)
The studies on drug-metal ion equilibrium show that the ligands containing N and S as donor atoms form metal-drug bonds with sufficient degree of covalent character resulting in high lipid solubility and hence high bio-availability and antibiotic activity of the drug. It may be anticipated that a stable metal chelate will produce its effect by structural or functional activation or inactivation of a susceptible biological site.

An antibacterial substance should possess selective toxicity and should be capable of absorption and distribution throughout the tissues of the host. Drug resistance in bacteria is often measured in relation to the smallest amount of drug, which completely prevents growth or inhibits visible growth in culture tubes. These end points are designated as minimum lethal (or bactericidal) and minimum inhibitory (or bacteriostatic) concentrations abbreviated as MLC or MIC respectively.

6.2. Brief Description of Microorganisms

6.2.1. Bacteria

6.2.1.1. Staphylococcus aureus

Staphylococcus aureus is a Gram positive pathogenic bacteria present in the skin and mucous membrane of man and is commonest cause of suppuration. They show exceptional ability to appear in multiply drug resistant form. Thermal death point for the cocci is 60°C for 30 mts. They survive in dried pus for 2-3 months and grow well in presence of 10% sodium chloride. These potential pathogens are transmitted from the nasal membrane
of a symptomatic carrier to a susceptible host. Usually they cause skin infection, cardiac and intestinal infections and deep infections in bones, joints and respiratory track. S. aureus are usually sensitive to antibiotics but rapidly develops resistance to these antimicrobial agents.

6.2.1.2. Escherichia Coli

Escherichia Coli, a Gram-negative bacteria is a consistent resident of small intestine in man and animals. They are killed by exposure to chlorine or to temperature of 55°C for 1 h. Some strains of E.coli are pathogens that cause intestinal infections, urinary tract infection and neonatal meningitis. They are usually sensitive to many antibiotics such as ampicillin and tetracycline.

6.2.2. Fungus

Fungus is a eukaryotic organism that is a member of the kingdom fungi. Fungi are heterotrophic organisms possessing a chitinous cell wall. The majority of species grow as multicellular filaments called hyphae forming mycelium. Some fungal species also grow as single cells. Fungi can cause a wide variety of infections in people, animals and plants. Spores of some fungi present in air, when inhaled by man can cause infections in the lungs. Certain types of fungi present on body surfaces or in the intestines cause local infections of the skin, nails, mouth etc. With the exception of few fungal infections like histoplasmosis, blastomycosis and coccidioidomycosis other fungal infections seldom cause serious harm in people.
6.2.2.1. Saccharomyces Cerevisiae (Yeast)

Yeast like many fungi are particularly useful in the fermentation of carbohydrates. They are unicellular and particularly abundant in sugary substances such as nectar of flowers and the surface of fruits like grapes. Some species are pathogenic to plants while others are even pathogenic to man and animals.

6.3. Methods Employed for Evaluating Antimicrobial Activity

Among the variety of tests that are available, turbidimetric and diffusion methods are most commonly used.

6.3.1. Turbidimetric method

The presence or absence of growth of the test organism is examined by measuring the turbidity. There are several types of turbidimetric methods, which differ in the manner by which the responses of the test organisms are measured. The two most popular methods are the serial dilution assay and turbidimetric tube assay. Serial dilution assay is examined visually for the presence or absence of growth of the test organism. In the turbidimetric tube assay, the concentration of organisms is usually measured in a photometer, the scale readings of which may be converted by a calibration curve. These tests in liquid media are more direct than those employing solid media because factors such as diffusion and reaction with agar are avoided, but they are generally more time consuming to carry out. In the serial dilution type of test, organisms are contacted with graded concentrations of the test substance in a
nutrient medium and the minimum concentration preventing detectable growth is taken as a measure of bacteriostatic activity.

### 6.3.2. Diffusion method

Different diffusion techniques are reviewed by Gavin. In the diffusion method, a zone of growth or inhibition of the test organism is formed around an application point or area. The zone sizes are affected by the diffusion coefficient (a function of molecular weight). The theory of the diffusion method was developed by Cooper and co-workers for linear diffusion. In this method the samples are held in holes in the agar, in cups, in fish-spine beads or in paper discs. Based on this there are different diffusion methods such as agar cup method, ditch plate method, fish-spine method and disc diffusion method.

### 6.4. Antimicrobial Studies of Zinc(II) and Cadmium(II) Complexes

Zinc(II) and cadmium(II) complexes of diethylenetriamine, pyridine and 2-amino pyridine were prepared and subjected to 800 kGy gamma radiation. Antimicrobial activity of the complexes before and after gamma irradiation has been investigated against Escherichia coli, Staphylococcus aureus, Mycobacterium smegmatis and Saccharomyces cerevisiae by disc diffusion susceptibility test.

### 6.4.1. Experimental

#### 6.4.1.1. Preparation of solutions of the compounds.
Stock solutions were prepared by dissolving the compounds in DMSO to give a concentration of 10 mg/mL. Working solutions at 50, 100 and 200µg/mL were prepared by diluting the stock solutions with the same solvent and sterilized by passing them through 0.22 nm membrane filters.

6.4.1.2. Preparation of Discs

Discs (6 mm diameter) were prepared by punching Whatman No.1 filter paper with an office punch and the discs were sterilized in an autoclave. Discs impregnated with the compounds at appropriate concentrations were prepared at the time of the experiment in a laminar flow hood by dipping the discs in the sterile working solutions and draining off excess solution.

6.4.1.3. Assay of Antimicrobial Activity

Antimicrobial activity was tested on the following prokaryotic and eukaryotic organisms:

Escherichia coli (Gram negative bacterium)

Staphylococcus aureus (Gram positive bacterium)

Mycobacterium Smegmatis (Fast growing non pathogenic mycobacterium)

Saccharomyces cerevisiae (yeast, Fungus, Eukaryotic Microbe).

One hundred micro litres of the late lag phase cultures of these organisms were spread on appropriate agar media in Petri plates. Once the liquid is absorbed, the discs were placed on the agar. The plates were incubated at 37°C for 24 hours. A clear circular zone around the disc in the lawn of the bacterium / yeast indicates inhibition. The diameter of the clear
zone is directly proportional to the potency of the compound as an antimicrobial agent against that particular organism.

6.5. Results

Results of antimicrobial studies are shown in table 34 and figures 43, 44 and 45. All the samples under investigation were inactive against bacterial strains. However antifungal activity was observed in a few samples.

6.5.1. Zinc(II) and cadmium(II) complexes of diethylenetriamine

6.5.1.1. Bis (diethylenetriamine) zinc (II) nitrate

Both unirradiated and irradiated samples were inactive against all microbial strains.

6.5.1.2. Bis (diethylenetriamine) cadmium(II) nitrate

The unirradiated sample was inactive while the irradiated sample exhibited antifungal activity. Further the zone of inhibition is found to be increased with increasing concentration of the test solution (Table 34) Fig(43).

6.5.2. Zinc(II) and cadmium(II) complexes of pyridine

6.5.2.1. Dinitratobis(pyridine) zinc (II)

Unirradiated sample remained inactive whereas the irradiated sample showed antifungal activity. Here also the zone of inhibition increased with increasing concentration of the test solution. (Table 31) Fig(44).

6.5.2.2 Dinitratobis(pyridine) cadmium (II)

Both unirradiated and irradiated samples exhibit antifungal activity. The unirradiated sample showed higher activity compared to the irradiated
sample. However zone of inhibition increased with increasing concentration of
the test solution. (Table 34) Fig(45).

6.5.3 Bis(2-aminopyridine)dinitrato zinc(II)

Due to insoluble nature of the complex it could not be screened for
antimicrobial activity.

6.6. Discussion

The interaction of metal chelates with biomolecules and the function of
metal ions in physiological systems is quite complex and the precise
mechanism is almost unknown. In general drug binding interactions involve
intermolecular bonding interactions such as ionic bond, hydrogen bond,
van der Walls interaction and dipole-dipole interaction. Some drugs may form
covalent bonds to their targets. Three physical features of particular
importance are hydrophobicity, electronic factors and steric factors. There are
several reasons why the physical properties of a compound should be
important to biological activity. The overall hydrophobic character of a
compound influences how efficiently it can cross cell membranes; the
hydrophobic character and size of the individual substituents may influence
how well the compound interacts and fits into the binding site. Electronic
character of substituents can influence the basicity of the compound affecting
both absorption and receptor binding. The electronic properties of different
substitutes can also play an important role in affecting biological activity.
Coordinately saturated complexes operate by physical interaction. The biological activity therefore depends on size, charge distribution, shape and redox potential of metal chelate. There are well known methods in literature for enhancing existing binding interactions. These methods include tuning the relative position of binding group or altering the electronic properties, increasing $pK_a$, changing the size of alkyl or aryl group, effecting ring contraction or expansion, chain contraction or extension etc. These operations can lead to changes in physical and chemical features of a drug. Modifying the polarity or $pK_a$, can lead to changes in relative solubility which in turn may influence the pharmokinetic properties. If a drug contains an amine functional group its relative solubility in polar and non polar solvents can be altered by varying the $pK_a$. Higher the $pK_a$ the more the amine is ionized and the more water soluble it will be. Lower the $pK_a$, more amine will be present as the free base and more soluble it will be in non polar environments.

In general, amines are potentially bioactive and the same can be improved by complexation with transition metals. In the present study it is seen that irradiation induced antifungal activity in bis(diethylenetriamine) cadmium(II) nitrate and dinitratobis(pyridine)zinc(II). Also antifungal activity of dinitratobis(pyridine) cadmium(II) is found to be slightly diminished upon irradiation. In all cases it is found that antifungal activity follows the order $200 \mu g/mL > 100 \mu g/mL > 50 \mu g/mL$. Changes in antimicrobial activity upon irradiation have been observed in some systems.\textsuperscript{173,185,187} Cases in which
irradiation induced antimicrobial activity have also been reported. In certain other systems effect of irradiation on antimicrobial property is insignificant.

Upon irradiation lattice defects are produced. These defects include interstitials, vacancies, electrons, holes, etc. Atoms or ions liberated thus may occupy some non-equilibrium interstitial positions. The localised dissipation of energy can result in lattice oscillations terminating in some reorientation of the local regions in the crystal lattice. This may lead to the generation of transient regions where there is a slight excess of or deficit of electrons, i.e., a random fluctuation of electron density. This situation allows mild interaction between molecules such that an area of transient electron deficiency in one molecule can interact with a transient electron rich area leading to changes in vander Walls interaction. At the same time dipole-dipole interaction, which represents relative orientation of electron density may also operate in the irradiated sample. Gamma irradiation can create atomic disorder. The term atomic disorder includes high concentrations of point defects in a crystal lattice, vacancies, line defects such as dislocations, interstitial atoms, amorphous regions, grain and sub grain boundaries and the like relative to its normal ordered crystalline state. Atomic disorder leads to irregularities in surface topography and inhomogenities in the structure on a nanometer scale. In addition to lattice defects, radiation induces chemical damage also. Thus the overall effects of irradiation lead to changes in physical and chemical
features of the material. If a structural change results in significantly enhanced affinity it suggests that an additional interaction with the target is established or operating. Difference in activity may be due to difference in receptor binding interaction or they may be due to altered pharmokinetics. Further as each system is unique, the changes due to irradiation in physical and chemical properties will be different in different systems.

**Summary**

Zinc(II) and cadmium(II) complexes of diethylenetriamine and pyridine and zinc(II) complex of 2-aminopyridine were prepared and characterized. The well dried samples were exposed to 800 kGy gamma radiation from $^{60}$Co $\gamma$-ray source. In vitro antimicrobial studies were carried out for the irradiated and unirradiated samples by disc diffusion method. Due to the insoluble nature of 2-aminopyridine complexes in DMSO, they could not be screened for antimicrobial activity. The complexes exhibited antifungal property and no activity was observed against bacterial strains. Most of the complexes gave inhibition zone ranging from 6mm to 12mm. Gamma irradiation induced antifungal activity in cadmium(II) complex of diethylenetriamine and zinc(II) complex of pyridine. Antifungal property increased with increase in concentration of the test solution. It was observed that gamma irradiation decreased antifungal property of cadmium(II) complex of pyridine. Both irradiated and unirradiated samples of zinc(II) complex of diethylenetriamine were inactive against all microorganisms screened.
Fig. 43. Antifungal activity of [Cd(dien)₂(NO₃)₂]
Fig. 44. Antifungal activity of $[\text{Zn(py)}_2(\text{NO}_3)_2]$
Fig. 45. Antifungal activity of [Cd(py)$_2$(NO$_3$)$_2$]

Table 1d

Results of antimicrobial studies

Saccharomyces Cerevisiae, Zone of inhibition (mm)

<table>
<thead>
<tr>
<th>Concentration μg/mL</th>
<th>Unirradiated</th>
<th>Irradiated</th>
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<tr>
<td>50</td>
<td>10</td>
<td>9.5</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>11.4</td>
</tr>
<tr>
<td>200</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Concentration range: 0-200 μg/mL
Table 34
Results of antimicrobial studies
Sacchromyces Cerevisiae, Zone of inhibition (mm)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of test solution</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>50µg/mL</td>
</tr>
<tr>
<td>Unirradiated [Cd(dien)₂(NO₃)₂]</td>
<td>-</td>
</tr>
<tr>
<td>800kGy [Cd(dien)₂(NO₃)₂]</td>
<td>9</td>
</tr>
<tr>
<td>Unirradiated [Zn(py)₂(NO₃)₂]</td>
<td>-</td>
</tr>
<tr>
<td>800kGy [Zn(py)₂(NO₃)₂]</td>
<td>9</td>
</tr>
<tr>
<td>Unirradiated [Cd(py)₂(NO₃)₂]</td>
<td>9.3</td>
</tr>
<tr>
<td>800kGy [Cd(py)₂(NO₃)₂]</td>
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</tr>
</tbody>
</table>
Conclusion

The investigations reported in this thesis are concerned with the effect of gamma radiation on cobalt(II), nickel(II) zinc(II) and cadmium(II) complexes of diethylenetriamine, pyridine and 2-amino pyridine.

From thermal studies, it is seen that irradiation enhanced thermal decomposition lowering thermal as well as kinetic parameters. Thermograms of each pair i.e., unirradiated and irradiated sample of each complex are essentially of the same pattern except in the case of dinitratobis(pyridine) cadmium (II), where irradiation modifies the decomposition pattern.

XRD studies showed change in lattice constants and lattice parameters upon irradiation. It is observed that intensities of diffracted lines were changed. In [Ni(dien)$_2$] (NO$_3$)$_2$ powder lines corresponding to higher 2θ values are not observed in the irradiated sample. This may be due to contraction of the unit cell caused by irradiation. X-ray diffractograms of irradiated [Zn(py)$_2$(NO$_3$)$_2$] and [Ni(py)$_2$(NO$_3$)$_2$] showed broadening of lines.

Spectral studies revealed that irradiation affect the spectral parameters. Bands in the UV-visible spectra of irradiated complexes appeared broad and shift in $\lambda_{max}$ were observed. Irradiation decreased LFSE and increased Racah parameter $B_1$ and covelency factor $\beta$. Increase in $\beta$ value suggests lesser covalent character in the metal-ligand bond of irradiated samples. Lowering in LFSE is more pronounced in pyridine and
2-aminopyridine complexes, since the damaged nitrate ion is inside the coordination sphere. In \([\text{Ni(dien)}_2(\text{NO}_3)_2]\), lowering in LFSE is insignificant. The nitrate ion is not coordinated to the metal ion and therefore the effect of damaged nitrate is negligible. Compared to cobalt (II) complexes, the effect of irradiation is more in nickel (II) complexes.

From antimicrobial studies it is found that antifungal property of the investigated complexes is sensitive to radiation treatment. Irradiation induced antifungal activity in cadmium(II) complex of diethylenetriamine and zinc(II) complex of pyridine. Antifungal activity of cadmium(II) complex of pyridine decreased upon irradiation. Zinc(II) complex of diethylenetriamine was inactive against all microorganisms before and after irradiation.