CHAPTER -6
ANTIMICROBIAL STUDIES
6.1. Introduction

Azo compounds are well known to be involved in number of biological reactions such as DNA, RNA, and protein synthesis, nitrogen fixation and carcinogenesis. These are having staining properties, medicinal properties, antibacterial and antifungal activities against pathogenic bacteria. Azo compounds exhibit pharmacological activities. The use of colorants in bio-chemistry for visual identification of histological structures is of great importance as the selective coloration of tissue with synthetic dyes is necessary for their microscopic characterization. Selective staining is most important, since biological tissue generally consists of morphologically different units [1-3]. Azo compounds are significant due to their use as models for metal-enzyme interactions and transport of metal ions in biological fluids.

Photodynamic Therpy (PDT) of cancer is a promising application of photodynamic action. PDT is a new type of treatment for tumours and certain other diseases. Indocyanine green (a tricarbocyanine dye) and other cyanine dyes, Procion blue (an amino anthraquinone derivative), Sudan black (a diazo dye) and some of other mono, disazo and polyazo dyes have also been suggested as the possible photothermal sensitizers and show selective thermal damage of some forms of cancer and other diseases on illumination with a pulsed laser. Recently, newer approaches have been introduced in which polymers are azo linked to various drug molecules. Many bacteria possess the ability to reduce azo compounds. Sulfasalazine, which is used for the treatment of rheumatoid arthritis, was found to have potential effect in the treatment of inflammatory bowel diseases IBD [4-7].

Co-ordination compounds have also been studied as antitumour, antiviral and antimalarial agents. The ability of metal ions to form complexes with ligands containing nitrogen, oxygen and sulphur donor atoms is related to such bioactivity. Complexes of amino acids with sulphonated azo dyes have been used as model systems for understanding of biomolecular recognition of glycosaminoglycans for proteins. Further,
sulphonato azo dyes, Evans blue and Congo red are being used as HIV inhibitors of viral replications. The latter effect is believed to be caused by binding of azo dyes to both protease and reverse transcriptase of this virus [8-9].

Transition metal complexes with azo ligand have been studied widely in the past few years since they are found to be important as biochemical, analytical and antimicrobial reagents. Metal complexes have been proved to be more fungitoxic compared to the basic organic compound [10]. Transition metal complexes with dyes e.g. copper (II) complex of methyl orange have shown pronounced antifungal and antibacterial activity. Azo metal chelates also exhibit bacteristatic and other biochemical activities.

Aromatic amines are essential intermediates for the synthesis of azo dyes. It was recognized that some of them are carcinogenic, when certain workers in dye manufacturing plants developed bladder cancer as a consequence of their occupational exposure to aromatic amines like benzidine, 2-naphthylamine, 4-aminobiphenyl, and 2-aminofluorene. Aromatic amines become carcinogenic upon metabolic conversion to electrophilic species that interact with DNA to form a covalent bond. A two-stage metabolic activation is often associated with this process. The arylnitrenium ion is regarded as the ultimate carcinogenic form of aromatic amines. Hence, the observed mutagenicity can be a function of the ease of elimination of the acyloxy group and the strength of the N–O bond. A compound will have high carcinogenic potential when the reactive electrophile is stabilized by resonance. This stabilization gives the reactive species a better chance of remaining active during transfer from the activation site to the bonding site on DNA [11-20].

The mutagenic behaviour of aniline compounds is impacted by the electronic character of ring substituent groups. Ring activating groups ortho or para to the amino group of mono-substituted aniline enhance the stability of the corresponding arylnitrenium ions, while ring deactivating groups destabilize arylnitrenium ions and as a result lower mutagenicity. Mutagenicity of mono-substituted anilines can be influenced by increasing their hydrophilicity.

Phenylenediamines are commonly used in the formation of hair dyes [21] or as coupling components in the synthesis of azo dyes. Some of the most widely used
Antimicrobial studies

examples are meta-phenylenediamine, 2,4-diaminotoluene, 2,4-diaminoanisole and para-phenylenediamine all of which are indirect-acting mutagens [18-22] and animal carcinogens [23]. Shahin and co-workers [24-26] and Freeman and co-workers [27] reported that the mutagenicity of meta- and para-phenylenediamines can be lowered or removed by the incorporation of bulky alkyl or alkoxy groups ortho to one of the amino groups [28-31].

M. Tuncel and co-workers [32] reported the histological activity of novel polydentate azo ligands and their cobalt (II), copper (II) and nickel (II) complexes. The azo ligands effectively stained the selected tissue structures.

Gavali and Hankarep [33] studied the antimicrobial activity of transition metal complexes of 4-[2^1^-hydroxy salicylidene 5\(^1\^-[2^{11}^-thiazlazo\)] chlorobenzene and reported that the metal complexes were more active than their ligands because metal complexes may serve as a vehicle for activation of ligand as the principle cytotoxic species.

G.G. Mohammed et al. [34] investigated the antimicrobial activity of transition metal complexes of Schiff bases. The metal complexes were found to be more potent antibacterial than the parent ligand and claimed that the metal complexes have a possible antitumor effect since gram-negative bacteria were considered a quantitative microbiological method for testing beneficial and important drugs in both clinical and experimental tumour chemotherapy.

Gajanan Pandey and K.K. Narang [35] studied the antifungal activity Mn(II), Ni (II), Cu (II) and Zn (II) complexes with (2,4-sulphophenyl azo)-1,8-dihydroxy-3,6-naphthelene disulphonic acid tri sodium salt and the metal complexes have been proved to be more fungitoxic compared to the basic organic ligand.

Nurcan Kurtoglu et al. [36] reported the antimicrobial properties of transition metal complexes of 4-[(E)-phenyldiazenyl]-2-[(E)-phenylimine methyl] phenol dye against eight bacteria and three fungi and the [Ni (dmp) Cl(H\(_2\)O)] chelate exhibited high activity against all the bacteria and fungi.

M.A. Zayed and co-workers [37] investigated the biological activities of novel azo dyes o-phenylazo-(C\(_{14}\)H\(_{13}\)N\(_3\)O\(_2\)), p-bromo, p-methoxy, p-nitro-o-phenylazo-p-acetamidophenol.
Antimicrobial studies

H.M. Shukla et al. [38] studied the microbial activity of co-ordination polymers based on the bis ligand $2,2^1-(3,3^1$-dimethoxy biphenyl-4,4$^1$-diyl) bis (diazene-2,1-diyl) bis (4,1-phenylene) bis (oxo methylene) dibenzoic acid with transition metal ions viz. Cu$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ and Co$^{2+}$. All the polymers have good microbial activity.

Nasr M. Regeh et al. [39] investigated the antimicrobial activity of some 7-aryl-2, 5-diamino-3(4-hydroxyphenylazo) pyrazolo 1, 5-pyrimidine-6-carbonitriles and their copper (II) complexes.

Shen Liu et al. [40] reported the antimicrobial effect of cationic monoazo dyes featuring different chain length alkyl quaternary ammonium salts as diazo components and N,N-dimethyl benzene amine as coupling components and that alkyl chains of different length placed in the dye structure provided antimicrobial efficacy.

6.2. Experimental

6.2.1. Antibacterial activity

The synthesized ligand and metal complexes were screened for their antimicrobial activity by cup-plate method in nutrient agar (antibacterial activity) and sabouraud dextrose agar (antifungal activity). The invitro antibacterial activity was carried against 24h old cultures of pathogenic bacteria like gram (+) S-aureus and gram (-) Salmonella typhi, Klebisella pneumoniae and Bacillus subtilis at 37° C. Antifungal activity was carried out against 72h old cultures of fungal strains like Candida albicans, C.lipolytica, Cryptococcus neoformenas and Saccharomyces cerevisiae. In order to ensure that solvent had no effect on bacteria or yeast growth, a control test was performed with DMSO and found inactive in culture medium.

The media as per Indian Pharmocopoeia was prepared by dissolving bacteriological peptone (6g), pancreatic digest of casein (4g), yeast extract (3g), beef extract (1.5g), dextrose (1g) and agar (15g) in distilled water to produce 1 litre of medium. The $\text{pH}$ of the solution was adjusted to 6.5-6.6 by using 1M sodium hydroxide and 1M hydrochloric acid. Then it was sterilized for 30 minutes at 15 lbs pressure.

The nutrient broth was prepared by dissolving bacteriological peptone (6g), pancreatic digest of casein (4g), yeast extract (3g), beef extract (1.5g), dextrose (1g) in distilled
Antimicrobial studies

To produce 1 litre of broth, water was adjusted to pH 6.2 and sterilized by autoclaving for two hours, at 15 lbs pressure. The organisms used in the present study for evaluating antibacterial activity of test compounds were obtained from the microbiology department of Sahyadri Science College, Shivamogga. On the day of testing, the organisms were sub cultured into sterile nutrient broth. After incubating the same for three hours, the growth thus obtained was used as inoculums for the test. All the test compounds were dissolved in DMSO to get the final concentration of 1mM.

6.2.2. Method of testing

Cup-plate method depends on the diffusion of an antibiotic from a cavity through the solidified agar layer in a Petri dish to an extent such that the growth of the added microorganism was prevented entirely in a circular area or zone around the cavity containing a solution of antibiotic.

A previously liquefied medium was inoculated appropriate to the assay with the requisite quantity of the suspension of microorganisms at 40-50° C and the inoculated medium was poured into Petri dishes to give a depth 3 to 4 mm. ensured that the layers of medium were uniform in thickness by placing the dishes on a levelled surface. With the help of a sterile cork borer, five cups of each 6 mm diameter were punched and scooped out the set agar in each Petri dish. The sample solutions of known concentrations were fed into the bored cups.

The dishes were left standing for one to four hours at room temperatures as a period of pre-incubation diffusion to minimize the effects of variation in time the applications of different solutions. These were then incubated for 24 hrs at 37° C. The zone of inhibition developed, if any, was then accurately measured. The results were tabulated in Table 6.1. The photographs showing antibacterial activity were given in figure-6(a) and 6(b).

6.2.3: Results and discussions

Microorganisms used for test were pathogenic bacteria like gram (+) S-aureus and gram (-) Salmonella typhi, Klebisella pneumoniae, Bacillus subtilis. Pseudomonas auregenosa and Escheritia coli. All the bisazo ligands showed excellent antibacterial
activity against *S. typhi* and *K. pneumoniae*. The dye PdBaAB exhibited a good inhibiting effect on *S. aureus* and *Bacillus subtilis* and PdBaAn has shown higher efficacy for *Salmonella typhi*. The azo ligand PdBaNA and all the metal complexes showed good activity against gram (+) *S-aureus* and gram (-) *Salmonella typhi, Klebsiella pneumonia*. While Mn(II), Ni(II) and Zn(II) complexes have a moderate effect on *Bacillus subtilis*. Bisazo ligands were found to be more potent than their metal complexes. All the dyes have shown excellent antibacterial activity for *K. pneumoniae* and *S. typhi*.

### Table 6.1: Antibacterial activity of azo dye PdBaNA and their complexes

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Test Bacteria</th>
<th>Zone of inhibition in mm</th>
<th>Control DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Co</td>
<td>Cu</td>
</tr>
<tr>
<td>1</td>
<td><em>Samonella typhi</em></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td><em>Staphylococcus aureus</em></td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus subtilis</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>Klebsella pneumonia</em></td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>

### Table 6.2: Antibacterial activity of azo dyes

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Antibacterial activity (zone of inhibition in mm )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.aureus</td>
</tr>
<tr>
<td>PdBaAn</td>
<td>17</td>
</tr>
<tr>
<td>PdBaClA</td>
<td>-</td>
</tr>
<tr>
<td>PdBaTd</td>
<td>13</td>
</tr>
<tr>
<td>PdBaDA</td>
<td>-</td>
</tr>
<tr>
<td>PdBaAPh</td>
<td>-</td>
</tr>
<tr>
<td>Std ( Cefatoxime)1mg/ml</td>
<td>40</td>
</tr>
</tbody>
</table>
## Table 6.3: Antibacterial Activity (Zone of Inhibition in mm Diameter)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>PdBaAPh 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Co 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Cu 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Mn 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Ni 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Zn 0.5 mg/ml</th>
<th>1 mg/ml</th>
<th>Standard (Chlormphenicol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escheritia coli</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Staphylococcus Aureus</td>
<td>7</td>
<td>8</td>
<td>7</td>
<td>9</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>8</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>Pseudomonas Auregenosa</td>
<td>10</td>
<td>13</td>
<td>7</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>PdBaAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escheritia coli</td>
<td>8</td>
<td>7.6</td>
<td>5.4</td>
<td>7.2</td>
<td>6.6</td>
<td>6.8</td>
<td>0</td>
<td>4.9</td>
<td>9.6</td>
<td>10.4</td>
<td>5.6</td>
<td>5.8</td>
<td>25</td>
</tr>
<tr>
<td>Staphylococcus Aureus</td>
<td>11.4</td>
<td>11.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>8</td>
<td>8.0</td>
<td>7.6</td>
<td>0</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>10.5</td>
<td>10.8</td>
<td>9.5</td>
<td>10.4</td>
<td>8.6</td>
<td>8.8</td>
<td>5.6</td>
<td>7.9</td>
<td>8.2</td>
<td>10.6</td>
<td>7.8</td>
<td>7.6</td>
<td>27</td>
</tr>
<tr>
<td>Pseudomonas Auregenosa</td>
<td>9.4</td>
<td>9.2</td>
<td>7.5</td>
<td>7.8</td>
<td>7.7</td>
<td>7.9</td>
<td>10.2</td>
<td>11.4</td>
<td>6.6</td>
<td>7.2</td>
<td>9.3</td>
<td>10.3</td>
<td>25</td>
</tr>
</tbody>
</table>

Well Dia: 6mm
Antimicrobial studies

Fig. 6(a): Antibacterial activity of dye PdBaAPh and its complexes against K. pneumonia

6(b): Antibacterial activity of dye PdBaAPh and its complexes against E. coli
Antimicrobial studies

6(c): Antibacterial activity of complexes of PdBaAB against Pseudomonas auregenosa
6.3. Antifungal activity- Effect on fungal growth

The target chemical bis azo dyes and their metal chelates were tested to study the biological effect. The ligands and all the complexes synthesized in the present investigation and the respective metal salts were evaluated for antifungal activity. *Candida albicans*, *C.lipolytica*, *Cryptococcus neoformenas* and *Saccharomyces cerevisiae* *Aspergillus flavus* which are known to deteriorate vegetables, backery products, dairy products, stored food grains and common contaminants in the laboratory experiments [41-56].

Preparation of potato dextrose agar (PDT): The PDA media added with test complexes were prepared as follows:

- **Potato**: 200mg
- **Dextrose**: 25g
- **Agar**: 25g
- **PH**: 5.8
- **Test complex 0.1- 200mg**

200g of fresh potato tubers were washed and outer skin was peeled off, cut into small pieces and boiled in a required quantity of water for 20 minutes. The thick paste like decoction was filtered through cheese cloth and diluted to about 450ml. To the above liquid 25g of dextrose and 25g agar-agar was added with constant stirring to homogenize the mixture, and made up to 1000ml.

The *Candida albicans*, *C.lipolytica*, *Cryptococcus neoformenas* and *Saccharomyces cerevisiae were* studied for their growth and sporulation colour characteristics in the presence of the selected metal azo complexes and were procured from The Department of Microbiology, Sahyadri science college, Shimoga. Solutions of different metal azo complexes were prepared by dissolving required amount of complexes in 2ml of DMSO and 98ml of PDA medium and sealed with aluminum foil and sterilized in an autoclave at a temperature 120 °C and 15 psi pressure for 15 min. The hot sterilized medium was poured into sterile petriplates in an aseptic chamber and cooled. The *Candida albicans*, *C.lipolytica*, *Cryptococcus neoformenas* and *Saccharomyces cerevisiae were inoculated
Antimicrobial studies

on solidified medium as a point at the centre of the plate. Inoculated plates were incubated at 23 ±1°C for one week, and the observations were made.

6.3.1. Results and discussion

Effect of azo dyes and complexes of Cu(II), Co(II), Ni(II), Zn(II) and on Candida albicans, C. lipolytica, Cryptococcus neoformenas and Saccharomyces cerevisiae in different ppm concentrations was studied.

The bisazo ligands PdBaAn and PdBaDA were found to be active against C. lipolytica. The dye PdBaAn has been more potent on the fungi Cryptococcus neoformenas, whereas the ligand PdBaClA has proved to be a very good fungicidal material.

The fungi Saccharomyces cerevisiae was affected vigorously by azo ligand PdBaNA, Co (II), Cu (II) and Zn (II) complexes whereas C. Lipolytica was moderately affected by the ligand, Cu (II) and Ni (II) complexes. The synthesized compounds have no effect on C. albicans and C. neoformens. The results in Table 6.5 reveal that the synthesized compounds were potent as bacteriostatic agents and have a good inhibitor effect on the growth of fungi Saccharomyces cerevisiae.

Table 6.4: Antifungal activity of dyes

<table>
<thead>
<tr>
<th>SI No</th>
<th>Antifungal activity (zone of inhibition in mm )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. neoformens</td>
</tr>
<tr>
<td>PdBaAn</td>
<td>21</td>
</tr>
<tr>
<td>PdBaClA</td>
<td>-</td>
</tr>
<tr>
<td>PdBaTd</td>
<td>12</td>
</tr>
<tr>
<td>PdBaDA</td>
<td>-</td>
</tr>
<tr>
<td>PdBaAPh</td>
<td>-</td>
</tr>
<tr>
<td>Std (Flucanozole) 1mg/ml</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 6.5: Antifungal activity of the dye PdBaNA and its complexes

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Test Yeasts</th>
<th>Zone of inhibition in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Co</td>
</tr>
<tr>
<td>1</td>
<td><em>Candida albicans</em></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Candida lipolytica</em></td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td><em>Cryptococcus neoformens</em></td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 7: Antifungal activity of bisazo ligands \( A_3 \)-PdBaClA, \( A_4 \)-PdBaCTd, \( A_5 \)-PdBaDA, \( A_6 \)-PdBaAPh. Against a) C. lipolytica and b) C. neoformens
Fig. 7(b): Antifungal activity of complexes of PdBaNA using a) Saccharomyces and b) Candida albicans
6.4. Activity of dyes and complexes against anthracnose disease

6.4.1. Introduction

The sustainability of chilli-based agriculture is threatened by a number of factors. Main biotic stresses such as bacterial wilt, anthracnose, viruses and several insect pests have been reported to impair the crop productivity. Anthracnose disease is a major problem in India and one of the more significant economic constraints to chilli production worldwide, especially in tropical and subtropical regions.

The disease causes both pre- and post-harvest fruit decay. Chilli anthracnose usually develops under high humid conditions when rain occurs after the fruits have started to ripen with reported losses of up to 84%. Economic losses caused by the disease are mainly attributed to lower fruit quality and marketability. Although infected fruits are not toxic to humans or animals, severely affected fruits showing blemishes are generally considered unfit for human consumption. This is because the anthracnose causes an unpleasant colour and taste in chilli products. Management of the disease under the prevailing farming systems in India has become a recurrent problem to chilli growers. Chilli anthracnose was first reported in India on chillies from the Coimbatore of Madras Presidency.

The disease has been identified in all the chilli producing regions of the world and has become a serious constrain in chilli production whenever the crop is grown. Different species of *Colletotrichum*, namely *C. capsici*, *C. gloeosporioides* and *C. acutatum* were known to cause anthracnose in chilli in India. Recent surveys conducted revealed *C. capsici* as the most predominant species in the major chilli growing states namely Karnataka and Andhra Pradesh in India. Seed-borne infections appear to be common in seed samples collected from affected fields. The most serious infection of *C. capsici* was reported from seed samples of the Mulabagilu village of the Kolar district of Karnataka.

A traditional identification and characterization of *colletotrichum* species have been based on morphological characters such as size and shape of conidia and aspersoria existence of setae, the teleomorph state and cultural characters such as colony color, growth rate and texture. These criteria are not always adequate for species identification.
due to overlap in morphological characters and phenotypic variations among species under different environmental conditions [57-64].

Conidial shapes has been applied as reliable means of discriminating certain species, identification can be complicated because of overlapping ranges of conidial morphology and variation in colony characteristics. Correct taxonomic identification is very impotent in disease management such as choosing appropriate fungicides.

*C. capsici* is both internally and externally seed born, such infected seeds and infected seedlings acts as primary source of inoculums. *Colletotrichum* species belongs to fungal kingdom; they grow usually on Sabaroutdes Dextrose Agar media, potato dextrose agar media. But any media rich with carbon source that’s sugars (glucose maltose, dextrose, sucrose), and nitrogen source enhances luxuriant growth of *Colletotrichum* species. Richards’s medium was also best preferred medium for inoculation of *Colletotrichum* species.

On proper inoculation and incubation at 26 ± 1 °C for about 7 days luxuriant growth of *Colletotrichum* species will be observed. The colonies were almost circular or elliptical pale brown, sometimes blackish sporulations arranged radially around the seed and even white mycelium with pinkish pigmentation was observed. Mycelia were watery or slimy on malt extract agar media. A healthy and completely developed mycelium measures about 3.5 cm in diameter.

Anthracnose is found to be one of the major economically threatening diseases to plant breeder material and introduced varieties can be screened for resistance to diseases before adopted by growers. There are several methods to control anthracnose disease; among them some experimentally proved best methods were fruit injection and Fruit Spray.

The disease can be chemically controlled by seed and foliar spray treatement with Azoxystrobin, chlorothalonil, copper difenoconazole, famoxadone, iprodione, procymidione. The fungicide traditionally recommended for Anthracnose management in chilli was Mancozeb (2.5g/l), although it doesn’t consistently control the severe form of Anthracnose on chilli fruit.

In biological methods, the antagonistic pseudomonas species as seed treatment as well as spray treatment were found to be effective against *Colletotrichum* species. Tricho
dermal species were able to effectively control *Colletotrichum* infection in chilli [65-68]. Other biological control agents that have been tested for efficiency against *Colletotrichum* include *Bacillus subtilis* and *Candida oleophila*.

**6.4.2. Anthracnose disease**

Anthracnose, derived from a Greek word meaning ‘coal’, was the common name for plant diseases characterized by very dark, sunken lesions, containing spores (Isaac, 1992). Generally, anthracnose disease was caused by *Colletotrichum* species which belongs to the kingdom Fungi; Phylum Ascomycota, Class Sordariomycetes; Order Phyllachorales; and Family Phyllachoraceae. The anamorphs are Glomerella species. Anthracnose of chilli was first reported from New Jersey, USA, by Halsted (1890) who described the causal agents as *Gloeopsorium piperatum* and *Colletotrichum nigrum*. These taxa were then considered as synonyms of *C. gloeosporioides*. (Von Arx 1957).

Anthracnose causes extensive pre- and post-harvest damage to chilli fruits causing anthracnose lesions. Even a small anthracnose lesion on chilli fruits reduces their marketable value. Many post-harvest diseases of fruit exhibit the phenomenon of quiescence in which symptoms do not develop until the fruit ripens. *Colletotrichum* species were the most important pathogens that cause latent infection. Appressoria are known to form adhesive disks that adhere to plant surfaces and remain latent until physiological changes occur in fruits. Appressoria that formed on immature fruits may remain quiescent until ontogenic changes occur in the fruits. Anthracnose disease can also occur on leaves, stems, and both pre- and post-harvest fruits. Typical fruit symptoms were circular or angular sunken lesions, with concentric rings of acervuli that were often wet and produce pink to orange conidial masses. Under severe disease pressure, lesions may coalesce. Conidial masses may also occur scattered or in concentric rings on the lesions. Many studies have concluded that disease management practices were often inadequate to eliminate the diseases. Breeding to develop the long-lasting resistant varieties has also not been successful due to involvement of multiple *Colletotrichum* species in anthracnose infection [69-71].
6.4.2: Causal agents of chilli anthracnose

In the *Colletotrichum* patho-system, different *Colletotrichum* species can be associated with anthracnose of the same host. The different species cause diseases of different organs of the chilli plant; for example, *C. acutatum* and *C. gloeosporioides* infect chilli fruits at all developmental stages, but usually not the leaves or stems, which were mostly damaged by *C. coccodes* and *C. dematium*. Different *Colletotrichum* species may also play an important role in different diseases of mature stages of chilli fruit as well.

*Colletotrichum* species can survive in and on seeds as acervuli and micro-sclerotia. Survival of mycelia and stomata in colonized chilli seeds had been reported. It has been shown that the pathogen readily colonizes the seed coat and peripheral layers of the endosperm even in moderately colonized seeds. Heavily colonized seeds had abundant inter- and intracellular mycelia and acervuli in the seed coat endosperm and embryo, showing disintegration of parenchymatous layers of the seed coat and depletion of food material in endosperm and embryo.

Fungi can over winter on alternative hosts such as other solanaceous or legume crops, plant debris and rotten fruits in the field. *Colletotrichum* species naturally produce micro-sclerotia to allow dormancy in the soil during the winter or when subjected to stressful conditions and these micro-sclerotia can survive for many years. During warm and wet periods, conidia from acervuli and micro-sclerotia were splashed by rain or irrigation water from diseased to healthy fruit and foliage. Diseased fruit acts as a source of inoculum, allowing the disease to spread from plant to plant within the field.

Initial infection by *Colletotrichum* species involves a series of processes including the attachment of conidia to plant surfaces, germination of conidia, production of adhesive appressoria, penetration of plant epidermis, growth and colonization of plant tissue and production of acervuli and sporulation. Anthracnose was mainly a problem on mature fruits, causing both pre- and post-harvest fruit decay resulting severe economic losses. Appressoria that formed on immature fruits may remain quiescent until the fruits mature or ripen.
Environmental factors play a major role in the development of disease epidemics. The relationships among rainfall intensity, duration and crop geometry and the dispersal of inoculum possibly lead to different levels of disease severity. The effects of temperature often interact with other factors, such as leaf surface wetness, humidity, light or competitive microbiota. The duration of the surface wetness, however, appears to have the most direct influence on the germination, infection and growth of the pathogen on the host. Generally infection occurs during warm, wet weather. Temperatures around 27 °C and high humidity (a mean of 80%) were optimum for anthracnose disease development.

*Colletotrichum* species utilize diverse strategies for invading host tissues, which vary from intracellular hemibiotrophy to subcuticular intramural necrotrophy. *Colletotrichum* species produce a series of specialized infection structures such as germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae. These pathogens infect plants by either colonizing subcuticular tissues intramurally or being established intracellularly. The pre-infection stages of the both were very similar, in which conidia adhere to and germinate on the plant surface, producing germ tubes that form appressoria which in turn penetrate the cuticle directly. Following penetration, the pathogens that colonize the intramural region beneath the cuticle invade in a necrotrophic manner and spread rapidly throughout the tissues. There was no detectable biotrophic stage in this form of parasitism [72-74]. In contrast, most anthracnose pathogens exhibit a biotropic infection strategy initially by colonizing the plasmalemma and cell wall intracellularly. After the biotrophic state, intracellular hyphae colonize one or two cells and subsequently produce secondary necrotrophic hyphae. These pathogens were therefore regarded as hemibiotrophs or facultative biotrophs. For example, *C. gloeosporioides* on avocado, chilli and citrus can produce both types of colonizations: intracellular biotrophy at an early stage and intramural necrotrophy later. Subcuticular intramural necrotrophy with hyphal development within periclinal and anticlinal walls of epidermal host cells which were swollen and wider apart. Hemibiotrophic interaction with infection vesicles and broaden primary hyphae within host cells [75]. Inter- and intracellular hyphal growth could be seen as the subsequent necrotrophic phase (e.g., combination of biotrophy and necrotrophy but mostly a biotrophic disease on chilli).
Antimicrobial studies

There was no biotrophic infection vesicle found during the infection process of *C. gloeosporioides* in susceptible chilli. Epidermal cytoplasm became condensed and small vacuoles increased and cell destruction extended to the sub epidermal cells of the plant, which were likely to be damaged by the pathogen enzymes. At later stages of infection, tissues were colonized inter- and intracellularly by the pathogen. This structural feature indicated that the infection was governed by necrotrophic fungal growth.

6.4.4. Materials and methods

i) Soil Sampling

Soil is a natural reservoir for almost all kinds of micro-organisms and they lived in a mixed population. Therefore it was often very difficult to separate from each other. Many suitable methods have been developed to isolate these microorganisms, among these serial dilution technique was widely used. It is a method for analyzing quantitative presence of microorganisms and it can also be used for obtaining pure cultures. In this method original inoculum was serially subjected to successive serial dilution so that concentration of microorganisms gradually becomes less and less. When dilutions were plated discrete colonies appear on the plates when incubated.

The soil samples for selective isolation of suitable actinomycetes and fungi were selected from the Gejjenahalli region of Shimoga district. The quadrant method was used to selectively collect samples, where specific points in hectare area (one curve in this context) were selected and the soil was collected. The litter on the surface must be previously removed. Nearly 15cm of the upper surface was dig horizontally from four sides (appearing as “square” shaped) and it was then cleared from the roots and other forest wastes.

The samples were collected in sterilized “Zip-Lock Covers”, made air tight and brought into the lab. The drying process was done by spreading the collected sample on a blotting sheet. Later the samples were further used for the serial dilution technique for the isolation of strains.
Antimicrobial studies

ii) Isolation from infected parts of chilli fruits

The small black circular spots on the skin of the fruit that spread in the direction of long axis, the spots get diffused and black, greenish or dirty grey in color. In some cases, the lesions are brown, and turn black from the formation setae and sclerotia. When a diseased fruit was cut open, the lower surface of the skin covered with minute, spherical, black stromatic masses of the fungus, a mat of fungal hyphae covers the seed. Such seeds turn rusty in color. Affected fruits were deformed, white in color and lose their pungency. Ultimately, the disease fruit shrivels and dry up. The infected chilli fruits were taken for the isolation of *Colletotrichum* species.

Serial dilution method: This method was used for the isolation of *Colletotrichum* organism. Collected soil sample was subjected to serial dilution, sterile water and seven sterile test tubes and Petri plates were prepared and 9ml of sterile water was poured into each test tube. 1g of collected soil sample was weighed and dissolved in first test tube. This sample was serially diluted in about ten test tubes using sterile pipettes. 1ml of each sample was poured into respective Petri plates. Sabouraud’s dextrose agar media with acidic pH which was previously prepared was sterilized and poured into each petri plate. The media in petriplate was well mixed with serially diluted sample. These plates were kept for incubation at 26° ± 1 °C for 7 days. These plates were observed after 7 days for isolated colonies of *Colletotrichum*.

6.4.5. Effect of synthetic compounds

The effect of synthesized bisazo compounds and their metal chelates on Anthracnose disease in turn against *Colletotrichum* Sp were studied. Primarily required number of petri plates, sterile media and sterile water were prepared. SDA media was selected for this purpose. For each series of synthetic compounds six petri plates were used, five plates for seeds treated with synthetic compounds and one as control. Media was poured into plates and kept for solidification, on solidified media infected seeds dipped in synthetic compounds were serially placed using sterile forceps under sterile conditions and kept for incubation at 26 ±1 °C for 7 days. Observation was made on seventh day and the results were collected.
Characterization of selected isolates

The colonies were picked from the SDA plate using sterile forceps and were placed on the slide having water. Then cotton blue stain was added to the slides containing inoculum, cover slip was placed, the slides were observed under high power objective of the microscope. *Collatotrichum gleosporoides* and other species of *Colletotrichum* were isolated from sampling. Small white and pale green sporulated fungal mycelia were grown around the seeds and were placed on blotting paper for 7 days of incubation. Through the spores colour may be similar and even conidia shape are similar to that of *Fusarium*, *Colletotrichum* Spp differ from them through in length and size of conidia. *Colletotrichum* Spp produce very large conidia compared to *Fusarium* and even the clonal character/symptoms produced by *Fusarium* on chilli was completely different from *Colletotrichum* Spp.

Synthetic dyes and metal azo chelates used for screening technique showed excellent result in controlling the growth of *Colletotrichum gleosporoides*. Among the synthetic compounds, complexes of PdBaDA and PdBaTd were found to be most effective. The result showed by dyes and metal complexes against the growth of *Colletotrichum* were appended in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>PdBaAn</th>
<th>PdBaNA</th>
<th>PdBaClA</th>
<th>PdBaTd</th>
<th>PdBaDA</th>
<th>Co- PdBaAn</th>
<th>Cu- PdBaAn</th>
<th>Mn- PdBaAn</th>
<th>Ni- PdBaAn</th>
<th>Zn- PdBaAn</th>
<th>Co- PdBaDA</th>
<th>Cu- PdBaDA</th>
<th>Mn- PdBaDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.5</td>
<td>Co-PdBaTd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PdBaAn</td>
<td>2.1</td>
<td>Cu-PdBaTd</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PdBaNA</td>
<td>2.5</td>
<td>Mn-PdBaTd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PdBaClA</td>
<td>1.7</td>
<td>Ni-PdBaTd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PdBaTd</td>
<td>2.9</td>
<td>Zn-PdBaTd</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PdBaDA</td>
<td>3.2</td>
<td>Co-PdBaDA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Co- PdBaAn</td>
<td>1.5</td>
<td>Cu-PdBaDA</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cu- PdBaAn</td>
<td>-</td>
<td>Mn-PdBaDA</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mn- PdBaAn</td>
<td>0.2</td>
<td>Ni-PdBaDA</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
### Antimicrobial studies

<table>
<thead>
<tr>
<th></th>
<th>Ni-PdBaAn</th>
<th>Zn-PdBaDA</th>
<th>Co-PdBaClIA</th>
<th>Cu-PdBaClIA</th>
<th>Mn-PdBaClIA</th>
<th>Ni-PdBaClIA</th>
<th>Zn-PdBaClIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni-PdBaAn</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zn-PdBaAn</td>
<td>2.0</td>
<td>Co-PdBaAPh</td>
<td>1.2</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co-PdBaNA</td>
<td>1.5</td>
<td>Cu-PdBaAPh</td>
<td>0.9</td>
<td>0.8</td>
<td>1.3</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Cu-PdBaNA</td>
<td>-</td>
<td>Mn-PdBaAPh</td>
<td>0.8</td>
<td>1.2</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mn-PdBaNA</td>
<td>1.7</td>
<td>Ni-PdBaAPh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni-PdBaNA</td>
<td>-</td>
<td>Zn-PdBaAPh</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zn-PdBaNA</td>
<td>0.8</td>
<td>Co-PdBaAPB</td>
<td>1.2</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Co-PdBaClIA</td>
<td>-</td>
<td>Cu-PdBaAPB</td>
<td>1.2</td>
<td>1.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cu-PdBaClIA</td>
<td>0.8</td>
<td>Mn-PdBaAPB</td>
<td>1.9</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mn-PdBaClIA</td>
<td>1.3</td>
<td>Ni-PdBaAPB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ni-PdBaClIA</td>
<td>1.9</td>
<td>Zn-PdBaAPB</td>
<td>1.1</td>
<td>1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zn-PdBaClIA</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Sabouraud's Dextrose Agar media

**Ingredients**  | **Concentration**
--- | ---
Dextrose | 40g
Peptone | 15g
Agar | 20g
Distilled water | 1000ml
PH | 5.6

### 6.4.6. Results and discussion

Sustainability and crop yield in chilli is majorly affected by fungal diseases called by *Colletotrichum* species like *C. capsici, C.gelosporoides* etc. Anthracnose in chilli pods has devastating effect on chilli yield by causing rotting of these fruit. Remedial measures to control this fungal pathogens was carried out by pathologist in different parts of India resulted in variable result. Antagonistic fungi like *T.herzianum and Aspergillus* were screened as good bio control agents like *Trichoderma* and *Aspergillus*.
isolated from forest soils of Shimoga district. The fungi were inhibited by both the bio
control agents, with a greater inhibition by *Aspergillus* than *Trichoderma*.

The present work utilized synthetic compounds like azo metal complexes which are
known for their antimicrobial activity against human bacterial and fungal pathogens.
These complexes have significantly reduced the mycelial growth when compared with
control plates. Promising results of dyes inhibiting *Colletotrichum* species promoted us
to check the combination of these dyes with already known effective fungicides for
*Colletotrichum* species. Combination was known to enhance the inhibitory effect when
compared to individual treatment of synthetic colonies or fungicides. Thus the
synthesized compounds have bio enhancing effect on the fungicides. Further studies are
needed to test the lowest concentration and range of concentrations of these dyes,
inhibiting the *Colletotrichum* species as present work has used only 1 % concentration of
fungicides as well as azo dyes. Further studies can be carried out to find out Minimum
Inhibitory Concentration (MIC) of synthetic dyes and metal complexes in inhibiting the
fungal pathogen.
Antimicrobial studies

Figure 8(a): Isolation of *Colletotrichum* species.
Figure 8(b): Antifungal activity of complexes of PdBaAn, PdBaTd, PdBaAPh and PdBaDA on Colletotrichum gleosporoides
Antimicrobial studies

Figure 8(c): Antifungal activity of complexes of PdBaAB and PdBaClA on Colletotrichum gleosporoides
Antimicrobial studies

References

Antimicrobial studies


Antimicrobial studies


28. J.L. Epler and A. W. Hsie, “Epidermal Carcinogenicity of Bis(3,3-epoxyclopentyl) ether, 2,2-Bis(p-glycidyloxphenyl) propane, and m-Phenylenediamine in C3H and C47B/6 Inbred Male and Female Mice”, ORNL Biology Division Report 5375, 48, 1978.


Antimicrobial studies


