CHAPTER-4

BIОLOGICAL EVALUATION OF THE COMPOUNDS
4.1 General

In pharmacology, biological activity or pharmacological activity describes the beneficial or adverse effects of a drug on living matter. When drug is a complex chemical mixture, this activity is exerted by the substance’s active ingredient or pharmacophore but can be modified by the other constituents. Activity is generally dosage-dependent and it is not uncommon to have effects ranging from beneficial to adverse for one substance when going from low to high doses. Activity depends critically on fulfillment of the ADME criteria. ADME is an acronym in pharmacokinetics and pharmacology for absorption, distribution, metabolism, excretion, and describes the disposition of a pharmaceutical compound within an organism. Whereas a material is considered bioactive if it has interaction with or effect on any cell tissue in the human body, pharmacological activity is usually taken to describe beneficial effects, i.e. the effects of drug candidates. The main kind of biological activity is a substance’s toxicity.

4.1.1 Antimicrobial activity

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, or protozoans. Antimicrobial drugs either kill microbes (microbicidal) or prevent the growth of microbes (microbiostatic). Disinfectants are antimicrobial substances used on non-living objects or outside the body.

The history of antimicrobials begins with the observations of Pasteur and Joubert, who discovered that one type of bacteria could
prevent the growth of another. They did not know at that time that the reason one bacterium failed to grow was that the other bacterium was producing an antibiotic. Technically, antibiotics are only those substances that are produced by one microorganism that kill, or prevent the growth, of another microorganism. Of course, in today’s common usage, the term antibiotic is used to refer to almost any drug that attempts to rid your body of a bacterial infection. Antimicrobials include not just antibiotics, but synthetically formed compounds as well.

The discovery of antimicrobials like penicillin and tetracycline paved the way for better health for millions around the world. Before penicillin became a viable medical treatment in the early 1940s, no true cure for gonorrhea, strep throat, or pneumonia existed. Patients with infected wounds often had to have a wounded limb removed, or face death from infection. Now, most of these infections can be cured easily with a short course of antimicrobials. However, with the development of antimicrobials, microorganisms have adapted and become resistant to previous antimicrobial agents. The old antimicrobial technology was based either on poisons or heavy metals, which may not have killed the microbe completely, allowing the microbe to survive, change, and become resistant to the poisons and/or heavy metals.

Antimicrobial nanotechnology is a recent addition to the fight against disease causing organisms, replacing heavy metals and toxins and may someday be a viable alternative.
4.1.2 Antioxidant activity

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols [1].

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation [1]. These compounds may be synthesized in the body or obtained from the diet [2]. The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors [3].
The relative importance and interactions between these different antioxidants is a very complex question, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another [4,5]. The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system. The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts [2].

Some compounds contribute to antioxidant defense by chelating transition metals and preventing them from catalyzing the production of free radicals in the cell.

4.1.3 Antitubercular activity

Tuberculosis is a highly infectious disease with about one third of the world’s population including 40 per cent from India estimated to be infected it [6]. However, this problem has become serious as *Mycobacterium tuberculosis* developed resistance against both the first line as also the second line drugs. Due to this, there is emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* all over the world including India [7].

Medicinal plants offer a great hope to fulfill these needs and have been used for curing diseases for many centuries. These have been used extensively as pure compounds or as a crude material. Only a few plant species have been thoroughly investigated for their
medicinal properties [8]. India is one of the few countries in the world which has unique wealth of medicinal plants and vast traditional knowledge to use herbal medicine for cure of various diseases [9]. So far, few plants have been tested against mycobacteria and a few plants which showed anti-TB activity such as *Salvia hypargeia*, *Euclea natalensis*, etc. [10,11]. The increasing incidence of MDR and XDR-TB worldwide highlight the urgent need to search for newer antituberculosis compounds/drugs.

4.2 **Bacteria**

Bacteria are often maligned as the causes of human and animal. However, certain bacteria, the actinomycetes produce antibiotics such as streptomycin and nocardicin; others live symbiotically in the guts of animals (including humans) or elsewhere in their bodies, or on the roots of certain plants, converting nitrogen into a usable form. Bacteria put the tang in yogurt and the sour in sourdough bread; bacteria help to break down dead organic matter; bacteria make up the base of the food web in many environments. Bacteria are of such immense importance because of their extreme flexibility, capacity for rapid growth and reproduction, and great age - the oldest fossils known, nearly 3.5 billion years old, are fossils of bacteria-like organisms.

Bacteria are organisms made up of just one cell. They are capable of multiplying by themselves, as they have the power to divide. Their shapes vary, and doctors use these characteristics to separate them into groups. The bacteria (singular: bacterium) are a
group of unicellular microorganisms. They are very small, most being approximately 0.5 to 2.0 \( \mu \text{m} \) in diameter. Most of these organisms have a relatively simple morphology and cellular arrangement. Bacteria are ubiquitous in every habitat on Earth, growing in soil, acidic hot springs, radioactive waste, seawater, and deep in the Earth’s crust. There are typically 40 million bacterial cells in a gram of soil and a million bacterial cells in a milliliter of fresh water; in all, there are approximately five nonillion \( (5 \times 10^{30}) \) bacteria on Earth, forming much of the world’s biomass. Bacteria are vital in recycling nutrients, and many important steps in nutrient cycles depend on bacteria, such as the fixation of nitrogen from the atmosphere. However, most of these bacteria have not been characterized, and only about half of the phyla of bacteria have species that can be cultured in the laboratory.

Bacteria may be found on top of mountains, in the guts of animal, in the frozen rocks, and ice of Antarctica. One feature that has enabled them to spread so far and so long is their ability to go dormant for extended period. The cell walls of bacteria are made out of peptidoglycan while that of fungi and plants are made of chitin and cellulose respectively. The cell wall is used in characterizing bacteria into groups. Bacteria have been grouped in two groups. Gram-positive of cell wall having thick peptidoglycan or murein layer and teichoic acid; while Gram-negative of cell wall having thin peptidoglycan and lipopolysaccharide-containing membrane.
In the present study, bacterial and fungi strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*, *Aspergillus clavatus* for antimicrobial activity and *M. tuberculosis* were used as test bio-organism for the antituberculosis activity testing. Accordingly their general information about their history and living style are briefly discussed.

**Staphylococcus aureus**

*Staphylococcus aureus* is a bacterial species named from Greek meaning the "golden grape-cluster berry". Also known as "golden staph" and Oro staphira, it is a facultative anaerobic Gram-positive coccal bacterium. It is frequently found as part of the normal skin flora on the skin and nasal passages [12]. It is estimated that 20% of the human population are long-term carriers of *S. aureus* [12]. *S. aureus* is the most common species of staphylococcus to cause Staph infections. *S. aureus* is a successful pathogen due to a combination of bacterial immuno-evasive strategies. One of these strategies is the production of carotenoid pigment staphyloxanthin, which is responsible for the characteristic golden colour of *S. aureus* colonies. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant which helps the microbe evade the reactive oxygen species which the host immune system uses to kill pathogens [13,14]. *S. aureus* may occur as a commensal on skin; it also occurs in the nose frequently.
Bacillus subtilis.

*Bacillus subtilis*, known also as the hay bacillus or grass bacillus, is a Gram-positive catalase-positive bacterium [15,16]. A member of the genus *Bacillus*, *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct [17]. Although this species is commonly found in soil, more evidence suggests that *B. subtilis* is a normal gut commensal in humans. Recent study compared the number of spores carried by the soil (~10^6 spores/g) versus the levels found in human feces (~10^4 spores/g). The number of spores found in the human gut is too high to be attributed solely to consumption through food contamination.
Soil simply serves as a reservoir, suggesting that *B. subtilis* inhabits the gut and should be considered as a normal gut commensal [18]. *B. subtilis* is commonly used as a model organism in laboratory studies directed at discovering the fundamental properties and characteristics of Gram-positive spore-forming bacteria [19].

*Escherichia coli*

*Escherichia coli* commonly abbreviated *E. coli* is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans, and are occasionally responsible for product recalls due to food contamination. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing
vitamin K2 [20], and by preventing the establishment of pathogenic bacteria within the intestine [21]. *E. coli* and related bacteria constitute about 0.1% of gut flora [22], and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination. There is, however, a growing body of research that has examined environmentally persistent *E. coli* which can survive for extended periods of time outside of the host. The bacterium can also be grown easily and inexpensively in a laboratory setting, and has been intensively investigated for over 60 years.
**Pseudomonas aeruginosa**

*Pseudomonas aeruginosa* is a common bacterium that can cause disease in animals, including humans. It is found in soil, water, skin flora, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also in hypoxic atmospheres, thus colonized many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or those with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonizations occur in critical body organs, such as the lungs, the urinary tract, and kidneys, the results can be fatal [23]. Because it thrives on most surfaces, this bacterium is also found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics. It is implicated in hot-tub rash. It is also able to decompose hydrocarbons and has been used to break down tar balls and oil from oil spills [24].

*P. aeruginosa* is often preliminarily identified by its pearlescent appearance and grape-like or tortilla-like odor in vitro. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of both pyocyanin and fluorescein, as well as its ability to grow at 42°C.
**Candida albicans**

*Candida albicans* is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans [25]. Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immuno compromised patients. *C. albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns. *C. albicans* is commensal and a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract. *C. albicans* lives in 80% of the human population without causing harmful effects, although overgrowth of the fungus results
Biological evaluation of the compounds

in candidiasis (candidosis). Candidiasis is often observed in immunocompromised individuals such as HIV-infected patients.

Aspergillus niger

Aspergillus niger is a fungus and one of the most common species of the genus Aspergillus. It causes a disease called black mold on certain fruits and vegetables such as grapes, onions, and peanuts, and is a common contaminant of food. It is ubiquitous in soil and is commonly reported from indoor environments, where its black colonies can be confused with those of Stachybotrys [26]. Some strains of A. niger have been reported to produce potent mycotoxins called ochratoxins [27], but other sources disagree,
claiming this report is based upon misidentification of the fungal species. Recent evidence suggests some true *A. niger* strains do produce ochratoxin A [26,28]. It also produces the isoflavone orobol.

**Aspergillus clavatus**

*Aspergillus clavatus* is a species of *Aspergillus* with conidia dimensions 3-4.5 x 2.5-4.5 micrometres. It is found in soil and animal manure. This fungus was first described scientifically in 1834 by the French mycologist John Baptiste Henri Joseph Desmazières [29]. It can produce the toxin patulin which may be associated with disease in humans and animals. This species is only occasionally pathogenic. Other sources have identified many species of *Aspergillus* as producing dry, hydrophobic spores that are easily
inhaled by humans and animals. Due to the small size of the spores, about 70% of spores of *Aspergillus fumigatus* are able to penetrate into the trachea and primary bronchi and close to 1% into alveoli. Inhalation of spores of *Aspergillus* is a health risk. *Aspergillus clavatus* is allergenic, causing the occupational hypersensitivity pneumonitis known as Malt Worker’s Lung.

**Mycobacterium tuberculosis**

*Mycobacterium tuberculosis* (MTB) is a pathogenic bacterial species in the genus Mycobacterium and the causative agent of most cases of tuberculosis (TB) [25]. First discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy coating on its cell surface (primarily mycolic acid), which makes the cells impervious to Gram staining, so acid-fast detection techniques are used, instead. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system,
MTB infects the lungs. The most frequently used diagnostic methods for TB are the tuberculin skin test, acid-fast stain, and chest radiographs [25].

4.3 Biological agents

4.3.1 Antimicrobial agents

A variety of techniques and agents are available; which act in many different ways and each has its own limits of application. As we have used chemical agents for inhibition of growth of bacteria and fungi, we need a brief review of antimicrobial agents so far used for this purpose. Emphasis is given to antibacterial and antifungal activity particularly the manner in which they inhibit or kill microbial cells. The treatment of diseases with a chemical substance is known
as “Chemotherapy” and the chemical substance is called “Chemotherapeutic Agent.” Chemotherapy has been practiced for centuries. For example, syphilis was the first known disease for which mercury was used to cure it. In 1910 Paul Eldrick synthesized an arsenical compound known as Salvarsan that had potent microbial properties, low toxicity for humans and good chemical stability. It was the first systematic and deliberate search for a compound capable of curing diseases without great danger to the patient. He used the word “Chemotherapy” and wrote-

“In order to use chemotherapy successfully, we must search for substances which have an affinity for the cells of the parasites and a power of killing them greater than the damage such substances caused to the organism itself... This means... we must learn to aim, learn to aim with chemical substances.”

Paul Eldrick

(Father of Chemotherapy)

Then only in the last century, chemotherapy was revolutionized in the field of medicine by the discoveries of two new potent classes of antibacterially active chemotherapeutic agents: Sulfa drugs and Antibiotics- for the treatment of certain bacterial diseases have been reported for their biological activity. Since the past decay number of sulfonamides have been examined, sulfaisoxazole is currently the most popular as it has a comparatively broad antimicrobial spectrum in vitro. Then following the intensive efforts of half a century ago, that produced thousands of analogs, the sulfonamide class has
progressively fallen out of fashions and the field is quiescent today. Subsequently, however trimethoprim was developed by George Hitchings and Gertrude Elion (who shared a Nobel Prize for this and other contributions to chemotherapy in the 1980).

4.3.1.1 Classification of Antimicrobial Agents

They can be classified in several ways as follows:

Type of organism against which antimicrobial agents are active:

- **Antibacterial agents**: active against bacterial organisms
- **Antiviral agents**: active against viral organisms
- **Antifungal agents**: active against fungal organisms
- **Antiprotozoal agents**: active against protozoa

According to mode of action of antimicrobial agents:

- **Bacteriostatic**: act primarily by arresting bacterial multiplication
- **Bactericidal**: act primarily by killing bacteria

According to activity of antimicrobial agents against the range of bacteria or other organisms:

- **Broad spectrum**: Effective against prokaryotes which kill or inhibit a wide range of Gram positive and Gram negative bacteria.
- **Narrow spectrum**: Effective against Gram positive or Gram negative bacteria.
- **Limited spectrum**: Effective against single organism or disease.
4.3.1.2 Characteristics of useful Antimicrobial agents

There is not a single chemical agent which is best for the control of microorganisms for any and all the purposes. A general purpose of chemical antimicrobial agent would have an extremely elaborate array of characteristics as mentioned below:

- Broad spectrum of antimicrobial activity at lowest concentration
- Solubility, Stability, Homogeneity, Availability
- Non combination with extraneous organic materials, Capacity to penetrate
- Toxic to microorganisms at room or body temperature
- Deodorizing ability, Detergent capacities

4.3.2 Antioxidant agents

- Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves.
- The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines.
- Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which
is the cause of rancidity. Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms.

- The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with antioxidative activity is likely to be one that is itself readily oxidized. Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they can damage cells.

### 4.3.2.1 Application of antioxidant agents

- Three different antioxidant agents (sodium ascorbate, malvidin chloride, and pelargonidin chloride) were applied to the specimens of BSA, BMC and BPC groups immediately after the bleaching procedure.

- The ascorbic acid sodium salt (C\textsubscript{6}H\textsubscript{7}NaO\textsubscript{6}, Sigma-Aldrich Brazil Ltda., São Paulo, SP, Brazil) in crystalline form (m.w. = 198.11) to produce the 10% sodium ascorbate solution. The malvidin chloride solution was prepared with 200 mg malvidin chloride (C\textsubscript{17}H\textsubscript{15}ClO\textsubscript{7}, molecular weight of 366.75, Sigma-Aldrich Brazil Ltda., São Paulo, SP, Brazil) per ml distilled water [30]. The
same proportion was used with pelargonidin chloride (C₁₅H₁₁ClO₅, molecular weight of 306.70, Sigma-Aldrich Brazil Ltda., São Paulo, SP, Brazil) to create the antioxidant solution.

- The specimens were immersed and irrigated with antioxidant solutions for 10 minutes at a flow rate of 1 mL/min. After the antioxidant treatment was performed, the specimens were washed with distilled water for 30 seconds.

4.3.2.2 Antioxidant photoprotective agents

- The harmful effects of ultraviolet (UV) radiation from the sun or other sources are well established. Sunburn is often the first sign of excessive exposure to these damaging rays, whilst long term consequences may include photoaging and skin cancer. Some people need to be particularly careful because they are photosensitive.

- Currently the main method of protection against UV radiation is the use of topical sunscreens. However, there are several limiting factors with regards to the protection they provide. They need to be applied regularly (every 2 hours or immediately after swimming or strenuous activity) and getting uniform coverage over the entire body is often difficult to achieve.

- In recent years, there has been much interest in the use of oral and topical antioxidants as photoprotective agents. These antioxidants work against the harmful effects of UV radiation via a number of ways that may include:
- Scavenging free radicals and reactive oxygen species (ROS) that are harmful to the body
- Decreasing the number of UV-induced sunburn cells forming

Preserving Langerhans cells

➢ Antioxidant photoprotective agents that are available in oral and/or topical preparations include:
  - Vitamin C
  - Vitamin E
  - Carotenoids, such as beta-carotene
  - Green tea
  - Extract from fern plant Polypodium leucotomos

4.3.2.3 Classification characteristics of antioxidants

➢ The major antioxidants currently used in foods are monohydroxy or polyhydroxy phenol compounds with various ring substitutions. These compounds have low activation energy to donate hydrogen. The resulting antioxidant free radical does not initiate another free radical due to the stabilization of delocalization of radical electron.

➢ The resulting antioxidant free radical is not subject to rapid oxidation due to its stability.

The antioxidant free radicals can also react with lipid free radicals to form stable complex compounds.
4.3.3 Specific drugs of antitubercular activity

Antituberculosis drugs kill *M tuberculosis* complex organisms or inhibit multiplication of the organism, thereby arresting progression of LTBI and preventing most complications of early tuberculosis disease. Chemotherapy does not cause rapid disappearance of already caseous or granulomatous lesions. For treatment of tuberculosis disease, these drugs always must be used in recommended combination to minimize emergence of drug-resistant strains. Use of nonstandard regimens for any reason should be undertaken only in consultation with an expert in treating tuberculosis.

**Isoniazid**

Isoniazid is bactericidal, rapidly absorbed, and well tolerated and penetrates into body fluids, including cerebrospinal fluid (CSF). Isoniazid is metabolized in the liver and excreted primarily through the kidneys.

**Rifampin**

Rifampin is a bactericidal agent in the rifamycin class of drugs that is absorbed rapidly and penetrates into body fluids, including CSF. Other drugs in this class approved for treating tuberculosis are rifabutin and rifapentine. Rifampin is metabolized by the liver and can alter the pharmacokinetics and serum concentrations of many other drugs. *M tuberculosis* complex isolates that are resistant to rifampin are uncommon in the United States. Rifabutin is a suitable alternative to rifampin in children with HIV infection receiving highly active antiretroviral therapy that proscribes the use of rifampin. Rifapentine
is a long-acting rifamycin that permits weekly dosing in select adults, but it has not been evaluated in pediatric patients.

**Ethambutol**

Ethambutol is well absorbed after oral administration, diffuses well into tissues, and is excreted in urine. However, concentrations in the CSF are low. At 20 mg/kg per day, ethambutol is bacteriostatic, and its primary therapeutic role is to prevent emergence of drug resistance. Ethambutol can cause reversible or irreversible optic neuritis, but reports in children with normal renal function are rare.

**Streptomycin**

Streptomycin is regarded as a "second-line" drug and is available only on a limited basis. It is administered intramuscularly. When streptomycin is not available, kanamycin, amikacin, or capreomycin are alternatives that can be prescribed for the initial 4 to 8 weeks of therapy. Patients who receive any of these drugs should be monitored for otic, vestibular, and renal toxicity.

### 4.4 Evaluation or screening of antimicrobial activity.

#### 4.4.1 Factors influencing antimicrobial susceptibility testing:

**pH**

The exact method used will depend largely on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature after gelling. If the pH is too low, certain drugs will appear to lose potency, while other agents may appear to have excessive activity. If the pH is too high, the opposite
effects can be expected. The pH can be checked by one of the following means:

- Macerate a sufficient amount of agar to submerge the tip of a pH electrode.
- Allow a small amount of agar to solidify around the tip of a pH electrode in a beaker or cup.
- Use a properly calibrated surface electrode.

**Moisture**

The plates should be placed in an incubator (35°C) or a laminar flow hood at room temperature with lids a jar until excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the petri dish covers when the plates are inoculated.

**Temperature**

The effect of temperature on the antimicrobial activity of the peptides was tested by determining the MIC of the peptides after they were heated to a variety of temperatures. The peptides were heated to temperatures between 60 and 90°C for 30 min and then cooled, or were autoclaved (121°C, 15 psi, 20 min).

**Effects of Thymidine or Thymine**

Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which may result in false-resistance reports. Satisfactory media will provide
Biological evaluation of the compounds

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essentially clear, distinct zones of inhibition 20 mm or greater in diameter. Unsatisfactory media will produce no zone of inhibition, growth within the zone, or a zone of less than 20 mm.

Effects of Variation in Divalent Cations

Variation in divalent cations, principally magnesium and calcium, will affect results of aminoglycoside and tetracycline tests with \( P. aeruginosa \) strains. Excessive cation content will reduce zone sizes, whereas low cation content may result in unacceptably large zones of inhibition. Excess zinc ions may reduce zone sizes of carbapenems. Performance tests with each lot of Müeller-Hinton agar must conform to the control limits.

4.4.2 Factors affecting activity of Antimicrobial agents

Microorganisms are not simple physical targets. Many biological characteristics influence the mode of action by various agents and factors must be considered in the application of chemical agent used to inhibit or destroy microbial populations. The main factors which influence the efficiency of antimicrobial agents are:

- Nature of the chemotherapeutic agent
- Types of microorganisms
- Environmental conditions—such as chemical and physical properties of medium or substance carrying the organisms, presence of extraneous matter, temperature control.

4.4.3 Action of Antimicrobial agents on microorganisms

The manner in which antimicrobial agents inhibit or kill the microbial growth can be attributed to the following kinds of action:
➤ Inhibition of cell wall or damage to the cell wall.

➤ Damage to the cytoplasmic membrane. - Alteration in the permeability of the cytoplasmic membrane.

➤ Inhibition of nucleic acid and protein synthesis.

➤ Change in the physical and chemical state of proteins and nucleic acids.

➤ Inhibition of specific enzyme action.

4.4.4 Conditions necessary for inhibition or control of microorganism

The following conditions must be met for the screening of antimicrobial activity:

➤ There should be an intimate contact between test organisms and substance to be evaluated

➤ Required conditions should be provided for the growth of microorganisms

➤ Aseptic/sterile environment should be maintained

➤ Conditions should be same throughout the study

4.4.5 Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

➤ Disk diffusion
  - Stokes method
  - Kirby-Bauer method
4.5 Roll of Antioxidants

The key role played by antioxidants in the body is their ability to react with radicals. When this happens the destructive properties of the radical is eliminated. A free radical is a chemical compound that contains one or more unpaired electrons. Radicals can be produced by exposure to energy such as radiation or may be the product of incomplete reactions in the cells that produce electrons that haven escaped. The step that produces a radical is called the initiation step. Radicals are usually represented in chemical formula by a single dot. Below are three examples of initiation steps that illustrate the production of radicals.

\[
\text{RH + Energy} \rightarrow \text{R}^\cdot + \text{H}
\]

\[
\text{ROOH + Energy} \rightarrow \text{RO}^\cdot + \text{H}
\]

In nature electrons are usually paired. In radicals they are not, and so radicals generally are more reactive than non-radicals. Because they are reactive, radicals search out ways of pairing up their odd electron. In their haste to pair up their electron, radicals often attack nearby chemical compounds. These chemical compounds may
be involved in important enzyme reactions, may be components of cell walls or may be part of a DNA molecule. If their chemical structure is changed, their function in the body may be lost and the result can be disease or infection.

A radical can donate its odd electron to another molecule, it can rob an electron from another nearby molecule or it can combine with another radical. When these two radicals combine is called a termination step.

\[ R\cdot + R\cdot \rightarrow R - R \]

If the initial radical donates or steals an electron, a second radical is produced which can then in turn react. This can continue in a series of propagation steps, until termination occurs.

\[ R_1\cdot + R_2 \rightarrow R_1 + \cdot R_2 \]
\[ R_2\cdot + R_3 \rightarrow R_2 + \cdot R_3 \]
\[ R_3\cdot + R_4 \rightarrow R_3 + \cdot R_4 \]

### 4.6 Evaluation or screening of antituberculosis activity

#### 4.6.1 Purpose of antituberculosis drug

Antituberculosis drugs are medicines used to treat tuberculosis, an infectious disease that can affect the lungs and other organs.

Tuberculosis is a disease caused by Mycobacterium tuberculosis, bacteria that are passed between people through the air. The disease can be cured with proper drug therapy, but because the bacteria may become resistant to any single drug, combinations of antituberculosis drugs are used to treat tuberculosis (TB) are normally required for effective treatment. At the start of the 20th Century, tuberculosis was
the most common cause of death in the United States, but was largely eliminated with better living conditions. It is most common in areas of crowding and poor ventilation, such as crowded urban areas and prisons. In some areas, the AIDS epidemic has been accompanied by an increase in the prevalence of tuberculosis.

Some antituberculosis drugs also are used to treat or prevent other infections such as Mycobacterium avium complex (MAC), which causes disease throughout the bodies of people with AIDS or other diseases of the immune system.

4.6.2 Description

Antituberculosis drugs are available only with a physician’s prescription and come in tablet, capsule, liquid and injectable forms. Some commonly used antituberculosis drugs are cycloserine (Seromycin), ethambutol (Myambutol), ethionamide (TrecatorSC), isoniazid (Nydrazid, Laniazid), pyrazinamide, rifabutin (Mycobutin), and rifampin (Rifadin, Rimactane).

**People who have certain medical conditions may have problems if they take antituberculosis drugs. For example:**

- Cycloserine or isoniazid may increase the risk of seizures (convulsions) in people with a history of seizures.
- The dosage of cycloserine may need to be adjusted for people with kidney disease.
- Ethambutol or pyrazinamide may cause or worsen attacks of gout in people who are prone to having them.
- Ethambutol may cause or worsen eye damage.
Diabetes may be harder to control in patients who take ethionamide.

Isoniazid may cause false results on some urine sugar tests, and pyrazinamide may cause false results on urine ketone tests. Diabetic patients who either of these medicines should discuss the possibility of false test results with their physicians.

People with liver disease or a history of alcohol abuse may be more likely to develop hepatitis when taking isoniazid and are more likely to have side effects that affect the liver when taking rifampin.

In people with kidney disease, ethambutol, ethionamide, or isoniazid may be more likely to cause side effects.

Side effects are also more likely in people with liver disease who take pyrazinamide.

4.7 Literature survey

In-vitro antibacterial, antifungal and cytotoxic activities of some coumarins and their metal complexes have been performed by Rehman et al [31]. Synthesis and identification of b-aryloxyquinolines and their pyrano[3,2-c] chromene derivatives as a new class of antimicrobial and antituberculosis agents reported by Divyesh C. Mungra et al [32]. Gokhan Ceyhan et al reported Antioxidant, electrochemical, thermal, antimicrobial and alkane oxidation properties of tridentate Schiff base ligands and their metal complexes [33]. Jamadar et al reported Synthesis, characterisation and
antitubercular activities of a series of pyruvate-containing aroylhydrazones and their Cu-complexes [34].

4.8 Present study

In-vitro antimicrobial activity of transition metal(II) complexes have been tested against the microorganisms such as two Gram(+)ve Staphylococcus aureus (SA), Bacillus subtilis (BS) and two Gram(−)ve Escherichia coli (EC), Pseudomonas aeruginosa (PA), where antifungal against Candida albicans (CA), Aspergillus niger (AN) and Aspergillus clavatus (AC); and most of the metal chelates exhibit higher antimicrobial activity than the free ligands. The Antioxidant power was specifically the ability of transfer a single electron for compound and antioxidant capacity of complexes was determined by a FRAP method. And anti- tubercular activities of all the synthesized compounds were assessed against M. tuberculosis H37Rv.

4.9 Experimental

2.9.1 Antimicrobial activity

So far as microorganisms concerned with the study of biological activity of complexes, parental ligands and metal salts, the Bacterial organisms used in present study were:

Micro organisms:

Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger and Aspergillus clavatus.
4.9.1.1 Materials

- Sterile graduated pipettes of 10ml, 5ml, 2ml and 1ml sterile capped 7.5 x 1.3 cm tubes / small screw-capped bottles
- Pasteur pipettes
- Overnight broth culture of test and control organisms (same as for disc diffusion tests)
- Required micro organism.
- Required solvent for the micro organism.
- Sterile distilled water - 500ml and suitable nutrient broth medium
- A suitable rack to hold 22 tubes in two rows

4.9.1.2 Stock solution preparation

Stock solution can be prepared using the formula

\[
\frac{1000}{P} \times V \times C = W
\]

Where,

P=Potency given by the manufacturer in relation to the base
V= Volume in ml required
C=Final concentration of solution (multiples of 1000)
W= Weight of the antimicrobial to be dissolved in the volume V

Method

All the ATCC culture was collected from institute of microbial technology, Bangalore. 2% Luria broth solution was prepared in distilled water while, pH of the solution was adjusted to 7.4±0.2 at
room temperature and sterilized by autoclaving at 15 lb pressure for 25 min. The tested bacterial and fungal strains were prepared in the luria broth and incubated at 37 °C and 200 rpm in an orbital incubator for overnight. Sample solutions were prepared in DMSO for concentration 200, 150, 100, 50, 25, 12 and 6µg/mL. The standard drug solution of Streptomycin (antibacterial drug) and Nystatin (antifungal drug) were prepared in DMSO. Serial broth micro dilution was adopted as a reference method. 10 µl solution of test compound was inoculated in 5 mL luria broth for each concentration respectively and additionally one test tubes was kept as control. Each of the test tubes was inoculated with a suspension of standard microorganism to be tested and incubated at 35 °C for 24 h. At the end of the incubation period, the tubes were examined for the turbidity. Turbidity in the test tubes indicated that microorganism growth has not inhibited by the antibiotic contained in the medium at the test concentration. The antimicrobial activity tests were run in triplicate.

4.9.2 Antioxidant study

Requirement

- Switch water baths & spectrophotometer on
- Defrost samples & standards
- Prepare FRAP reagent:
  - 200ml acetate buffer
  - 20ml TPTZ solution
  - 20ml FeCl₃ solution
  - 24ml distilled water
Keep in a dedicated plastic bottle. The solution should be straw coloured, if it is tinged with blue discard and prepare fresh solution after rinsing bottle thoroughly with demonised water. Mix; keep in water bath at 37 °C.

4.9.2.1 Materials And Methods

Reagent preparation: Reagents included 300 mmol/ liter acetate buffer, pH 3.6 (3.1 g C₂H₃NaO₂•3H₂O (Rie del-de Haen, Germany) and 16 ml C₂H₄O₂ (BDH Laboratory Supplies, England) per liter of buffer solution); 10 mmol/liter TPTZ (2,4,6-tripyridyl-s-triazine, Fluka Chemicals, Switzerland) in 40 mmol/liter HCl (BDH); 20 mmol/liter FeCl₃•6H₂O (BDH). Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml FeCl₃•6H₂O solution.

Method

Ferric reducing antioxidant power (FRAP) was determine using an adapted method [35]. The antioxidant potentials of the compounds were examine by their reducing power of the TPTZ-Fe(III) complex to TPTZ-Fe(II) complex for the total antioxidant capacity of tested samples, This method was employed because of its simple, fast and also results can be obtain was reproducible. Initially following solutions were prepared, A) acetate buffer, 300 mM pH 3.6 (3.1g sodium acetate trihydrate and 16 ml conc. acetic acid per L of buffer solution), B) 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl, C) 20 mM FeCl₃•6H₂O in distilled water, D) 1mM of ascorbic acid dissolved in 100 mL distilled water. FRAP working solution was prepared by
mixing the above (A), (B) and (C) solutions in the ratio of 10:1:1 respectively. A mixture of 40.0 µL, 0.5 mM sample solution and 1.2 mL FRAP reagent was incubated at 37 °C for 15 min. The working solution was necessary to use as freshly prepared. The ascorbic acid was used as a standard antioxidant compound and results were expressed with compared to ascorbic acid.

4.9.3 Anti-tubercular activity

Method

Test compounds were evaluated for in vitro anti-tubercular activity. The MICs were determined and interpreted for *M. tuberculosis* H37Rv according to the procedure of the approved micro dilution reference method of antimicrobial susceptibility testing [36]. Compounds were taken at concentrations of 100, 50, 25 and 12 µg/mL in DMSO, 1.0 ml of each concentration was used for the study. To this, 9.0 ml of Lowenstein-Jensen medium was added. A sweep from *M. tuberculosis* H37Rv strain culture was discharged with the help of nichrome wire loop with a 3 mm external diameter into a vial containing 4 ml of sterile distilled water. The vial was shaken for 5 min. Then using nichrome wire loop suspension was inoculated on the surface of each of Lowenstein-Jensen medium containing the test compounds. Further test media was incubated for four weeks at 37 °C. Readings were taken at the end of fourth week. The appearance of turbidity was considered as bacterial growth and indicates resistance to the compound. Test compounds were compared to reference drugs Isoniazid (MIC = 0.025µg/mL), Streptomycin (MIC = 6.25µg/mL) and
Ethambutol (MIC = 20µg/mL). Lowenstein-Jensen medium containing standard drugs as well as DMSO was inoculated with *M. tuberculosis* H37Rv strain. The anti-tubercular activity tests were run in triplicate.
**Table 4.1** Antimicrobial, anti-tubercular and antioxidant results of ligand (L1-L10)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimal Inhibition Concentration a of microorganisms (µg/mL)</th>
<th>Anti-tubercular activity a</th>
<th>Antioxidant activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>BS</td>
<td>EC</td>
</tr>
<tr>
<td>L1</td>
<td>&gt;200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L2</td>
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<td>150</td>
</tr>
<tr>
<td>L6</td>
<td>200</td>
<td>&gt;150</td>
<td>200</td>
</tr>
<tr>
<td>L7</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>L8</td>
<td>100</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>L9</td>
<td>100</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>L10</td>
<td>150</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>CQ</td>
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</tr>
<tr>
<td>Isoniazide</td>
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<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
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<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

**Antimicrobial Activity:**

*Staphylococcus aureus*: CQ > L4, L7, L9 > L2, L3, L5, L10 > L6 > L1

*Bacillus subtilis*: CQ > L7, L8, L10 > L3, L5 > L4, L2, > L6 >, L9, L1

*Escherichia coli*: CQ > L4, L7, L10 > L5, L8, L9 > L1, L2, L3, L6

*Pseudomonas aeruginosa*: CQ > L4 > L7 > L9 > L2 > L3, L5, L8 > L1, L6, L10

*Candida albicans*: CQ > L8, L10 > L4 > L3, L7 > L3 > L1, L2, L9, > L6

*Aspergillus niger*: CQ > L10 > L3 > L2, L4, L8, L9 > L5 > L1, L6, L7

*Aspergillus clavatus*: CQ > L4, L9 > L5, L8, L10 > L2 > L1, L3, L6, L7

**Antioxidant activity**: NT

**Anti-tubercular activity:**

Isoniazide > Streptomycin > Ethambutol > L4 > L8 > L2 > L3, L5 > L7, L10 > L1, L6 > L9
Table 4.2 Antimicrobial, anti-tubercular and antioxidant results of compound (C1-C10)

<table>
<thead>
<tr>
<th>Compounds C1-C10</th>
<th>Minimal Inhibition Concentration a of microorganisms (µg/mL)</th>
<th>Anti-tubercular activity a</th>
<th>Antioxidant activity b</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
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</tr>
<tr>
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<td>SA</td>
<td>BS</td>
<td>EC</td>
</tr>
<tr>
<td>C1</td>
<td>200</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>C2</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>C3</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>C4</td>
<td>50</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>C5</td>
<td>50</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C6</td>
<td>&gt;100</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>C7</td>
<td>25</td>
<td>&gt;25</td>
<td>25</td>
</tr>
<tr>
<td>C8</td>
<td>25</td>
<td>25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>C9</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>C10</td>
<td>100</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>CQ</td>
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<td>12</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Animicrobial Activity:

**Staphylococcus aureus**: CQ > C7, C8, C9 > C4, C5, > C2, C3, C10 > C6 > C1

**Bacillus subtilis**: CQ > C8, C10 > C7 > C3, C5, C9 > C2, C4 > C1, C6

**Escherichia coli**: CQ > C7 > C8 > C4, C9, C10 > C5 > C3, C6 > C1, C2

**Pseudomonas aeruginosa**: CQ > C4, C7, C8 > C3 > C5 > C2, C6, C9, C10

**Candida albicans**: CQ > C7, C8 > C4, C5, C9 > C10 > C2, C6 > C1, C3

**Aspergillus niger**: CQ > C8 > C3, C7, C10 > C5 > C2, C4, C9 > C2

**Aspergillus clavatus**: CQ > C7, C8 > C4 > C5, C9 > C3, C10 > C2 > C1, C6

Antioxidant Activity: C7 > C5 > C4 > C8 > C2 > C10 > C9 > C6 > C3 > C1

Anti-tubercular activity:

Isoniazide > Streptomycin > Ethambutol > C4, C7, C8 > C3, C5, C10 > C2, C9 > C1, C6
Table 4.3 Antimicrobial, anti-tubercular and antioxidant results of compound (C11-C20)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimal Inhibition Concentration (^a) of microorganisms (µg/mL)</th>
<th>Anti-</th>
<th>Antioxidant activity (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>BS</td>
<td>EC</td>
</tr>
<tr>
<td>C11</td>
<td>150</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C12</td>
<td>100</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>C13</td>
<td>50</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>C14</td>
<td>25</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C15</td>
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<td>25</td>
<td>50</td>
</tr>
<tr>
<td>C16</td>
<td>150</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>C17</td>
<td>&gt;12</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>C18</td>
<td>25</td>
<td>&gt;25</td>
<td>12</td>
</tr>
<tr>
<td>C19</td>
<td>50</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C20</td>
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<td>100</td>
<td>25</td>
</tr>
<tr>
<td>CQ</td>
<td>12</td>
<td>12</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Antimicrobial Activity:

*Staphylococcus aureus*: CQ > C17 > C18, C14 > C13, C15, C19, C20, > C12, C11, C16

*Bacillus subtilis*: CQ > C13, C15, C17 > C18 > C14, C19 > C11, C16, C20 > C12

*Escherichia coli*: CQ > C18 > C17, C20 > C13, C15 > C14, C19 > C11, C12 > C16

*Pseudomonas aeruginosa*: CQ > C17 > C14, C18 > C15 > C11, C12, C19, C20 > C13 > C16

*Candida albicans*: CQ > C17 > C13, C18, > C14, C15, C19, C20, C11, C12, C16

*Aspergillus niger*: CQ > C18 > C14, C15, C17 > C11, C13 > C12, C19, C20 > C16

*Aspergillus clavatus*: CQ > C17 > C13, C18, C20 > C15 > C14, C19 > C11, C12, C16

Antioxidant Activity: C17 > C18 > C14 > C15 > C11 > C16 > C12 > C20 > C13 > C19

Anti-tubercular activity:

Isoniazide > Streptomycin > Ethambutol > C17 > C13, C14, C18 > C20 > C11, C15, C19, C16 > C12
Table 4.4 Antimicrobial, anti-tubercular and antioxidant results of compound (C21-C30)

<table>
<thead>
<tr>
<th>Compounds C21-C30</th>
<th>Minimal Inhibition Concentration a of microorganisms (µg/mL)</th>
<th>Anti-tubercular activity a</th>
<th>Antioxidant activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria (SA, BA, EC, PA, CA, AN, AC)</td>
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<td></td>
</tr>
<tr>
<td>C21</td>
<td>50 25 &gt;25 50 &gt;25 50 25 50 342.28</td>
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<tr>
<td>C22</td>
<td>25 100 50 25 &gt;25 25 50 50 332.40</td>
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</tr>
<tr>
<td>C24</td>
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</tr>
<tr>
<td>C25</td>
<td>25 50 50 25 50 25 50 25 380.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C26</td>
<td>50 25 &gt;25 50 &gt;25 25 &gt;25 25 321.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C27</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C28</td>
<td>&gt;12 25 &gt;12 25 &gt;12 25 25 &gt;12 398.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C29</td>
<td>50 &gt;50 25 &gt;25 &gt;12 25 &gt;25 &gt;12 309.61</td>
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<td></td>
</tr>
<tr>
<td>C30</td>
<td>25 25 &gt;25 &gt;12 25 &gt;25 &gt;12 25 331.82</td>
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</tr>
<tr>
<td>CQ</td>
<td>12 12 &gt;6 12 &gt;12 NT NT NT NT NT NT NT 0.025 NT</td>
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<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT NT NT NT NT NT NT NT NT NT NT NT NT NT NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12 6 12 12 NT NT NT NT NT 6 25 NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT NT NT 6 12 NT NT NT NT NT NT NT NT NT NT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Antimicrobial Activity:

**Staphylococcus aureus**: CQ > C24, C28 > C22, C23, C25, C27, C30, > C21, C26, C29

**Bacillus subtilis**: CQ > C21, C24, C26, C28, C30, > C27 > C25, C29, C22

**Escherichia coli**: CQ > C27, C28 > C24, C29, > C21, C26, C30 > C22, C23 > C25

**Pseudomonas aeruginosa**: CQ > C27, C30, C22, C23, C25, C28 > C24, C29 > C21, C26

**Candida albicans**: CQ > C24, C27, C30 > C21, C22, C26, C28 > C25, C29

**Aspergillus niger**: CQ > C23, C24, C27, C30 > C21, C22, C26, C28 > C25, C29

**Aspergillus clavatus**: CQ > C27 > C21, C24, C26, C28 > C30 > C23, C29, C22, C25

Antioxidant Activity: C27 > C28 > C24 > C25 > C21 > C22 > C30 > C26 > C23 > C29

Anti-tubercular activity:

Isoniazide > Streptomycin > Ethambutol > C27 > C24 > C25 > C21 > C22 > C30 > C26 > C23 > C29
Table 4.5 Antimicrobial, anti-tubercular and antioxidant results of ligand (L11-L20)

<table>
<thead>
<tr>
<th>Compounds L11-L20</th>
<th>Minimal Inhibition Concentration a of microorganisms (µg/mL)</th>
<th>Anti-tubercular activity a</th>
<th>Antioxidant activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>BS</td>
<td>EC</td>
</tr>
<tr>
<td>L11</td>
<td>200</td>
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<td>&gt;200</td>
</tr>
<tr>
<td>L12</td>
<td>100</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>L13</td>
<td>200</td>
<td>150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>L14</td>
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<td>100</td>
<td>&gt;150</td>
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</tr>
<tr>
<td>L18</td>
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<td>&gt;50</td>
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</tr>
<tr>
<td>L19</td>
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<td>&gt;150</td>
</tr>
<tr>
<td>L20</td>
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<td>&gt;100</td>
</tr>
<tr>
<td>CQ</td>
<td>12</td>
<td>12</td>
<td>&gt;6</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

**Antimicrobial Activity:**

*Staphylococcus aureus*: CQ > L12, L18, L20 > L14, L17, L19 > L11, L13, L15 > L16

*Bacillus subtilis*: CQ > L18 > L14, L15 > L17 > L11, L13, L16, L20 > L12, L19

*Escherichia coli*: CQ > L16, L20 > L12, L15, L18 > L13, L14, L19 > L17 > L11

*Pseudomonas aeruginosa*: CQ > L13, L15, L19 > L14 > L11, L17, L18, > L12 > L16, L20

*Candida albicans*: CQ > L12 > L11, L13, L16, L18, L20 > L15, L19 > L14, L17

*Aspergillus niger*: CQ > L14, L15, L17, L18, L19 > L13 > L12, L16 > L11, L20

*Aspergillus clavatus*: CQ > L14, L18 > L11, L15, L17 > L12, L19 > L13, L16, L20

**Antioxidant Activity**: NT

**Anti-tubercular activity:**

Isoniazide > Streptomycin > Ethambutol > L12 > L14, L18 > L11, L15, L17, L20 > L13, L19 > L26
### Table 4.6 Antimicrobial, anti-tubercular and antioxidant results of compound (C31-C40)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimal Inhibition Concentration a of microorganisms (µg/mL)</th>
<th>Antitubercular activity a</th>
<th>Antioxidant activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>SA</td>
<td>BS</td>
<td>EC</td>
</tr>
<tr>
<td>C31</td>
<td>50</td>
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<td>25</td>
</tr>
<tr>
<td>C32</td>
<td>&gt;12</td>
<td>25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>C33</td>
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<td>50</td>
</tr>
<tr>
<td>C35</td>
<td>&gt;25</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>C36</td>
<td>100</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C37</td>
<td>50</td>
<td>&gt;25</td>
<td>100</td>
</tr>
<tr>
<td>C38</td>
<td>50</td>
<td>&gt;25</td>
<td>100</td>
</tr>
<tr>
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<td>&gt;6</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

**Antimicrobial Activity:**

*Staphylococcus aureus*: CQ > C32 > C38, C40 > C35 > C31, C34, C37 > C33, C36, C39

*Bacillus subtilis*: CQ > C38 > C32, C34 > C37 > C33, C35, C36, C39, C40 > C31

*Escherichia coli*: CQ > C38 > C31, C35 > C32, C40 > C34 > C33, C36, C39 > C37

*Pseudomonas aeruginosa*: CQ > C32, C38, C40 > C33 > C31, C35, C37 > C34, C36, C39

*Candida albicans*: CQ > C40 > C36, C38, C39 > C32, C33, C37 > C31, C35 > C34

*Aspergillus niger*: CQ > C32 > C35, C37, C38 > C31, C33, C34, C36, C39, C40

*Aspergillus clavatus*: CQ > C32, C34, C38, C40 > C35 > C31, C37 > C33, C36

**Antioxidant Activity:** C32 > C38 > C40 > C34 > C35 > C37 > C31 > C39 > C33 > C36

**Anti-tubercular activity:**

Isoniazide > Streptomycin > Ethambutol > C32 > C34, C38 > C31, C35, C37, C40 > C33, C36, C39
### Table 4.7 Antimicrobial, anti-tubercular and antioxidant results of compound (C41-C50)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Minimal Inhibition Concentration ( \mu \text{g/mL} ) of microorganisms</th>
<th>Anti-tubercular activity ( \text{a} )</th>
<th>Antioxidant activity ( \text{b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>50</td>
<td>100</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C42</td>
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<td>12</td>
</tr>
<tr>
<td>C43</td>
<td>50</td>
<td>&gt;50</td>
<td>100</td>
</tr>
<tr>
<td>C44</td>
<td>25</td>
<td>50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>C45</td>
<td>50</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>C46</td>
<td>50</td>
<td>&gt;50</td>
<td>25</td>
</tr>
<tr>
<td>C47</td>
<td>25</td>
<td>&gt;25</td>
<td>50</td>
</tr>
<tr>
<td>C48</td>
<td>&gt;12</td>
<td>25</td>
<td>&gt;12</td>
</tr>
<tr>
<td>C49</td>
<td>50</td>
<td>&gt;25</td>
<td>100</td>
</tr>
<tr>
<td>C50</td>
<td>25</td>
<td>50</td>
<td>&gt;25</td>
</tr>
<tr>
<td>CQ</td>
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<td>&gt;6</td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

**Antimicrobial Activity:**

*Staphylococcus aureus*: CQ > C42, C48 > C44, C47, C50 > C41, C43, C45 > C46, C49  
*Bacillus subtilis*: CQ > C42, C48 > C47, C49 > C44, C50 > C43, C46 > C41, C45  
*Escherichia coli*: CQ > C42 > C48 > C45, C46 > C50 > C47 > C41, C44 > C43, C49  
*Pseudomonas aeruginosa*: CQ > C42, C48 > C43, C44 > C49 > C45 > C41, C46, C47, C50  
*Candida albicans*: CQ > C41, C42, C45, C48 > C44, C46, C50 > C47, C49 > C43  
*Aspergillus niger*: CQ > C42, C48 > C46, C50 > C41, C45, C47 > C43, C44 > C49  
*Aspergillus clavatus*: CQ > C42 > C45, C48 > C47, C49 > C43, C44, C46, C50 > C41  

**Antioxidant Activity:** C48 > C42 > C50 > C45 > C44 > C43 > C11 > C47 > C49 > C46  

**Anti-tubercular activity:**

Isoniazide > Streptomycin > Ethambutol > C42, C49 > C44 > C43, C45, C47, C50 > C41, C46, C59
### Table 4.8 Antimicrobial, anti-tubercular and antioxidant results of compound (C51-C60)

<table>
<thead>
<tr>
<th>Compounds C51-C60</th>
<th>Minimal Inhibition Concentration $^a$ of microorganisms (µg/mL)</th>
<th>Anti-tubercular activity $^a$</th>
<th>Antioxidant activity $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SA    BS  EC  PA  CA  AN  AC</td>
<td>SA    BS  EC  PA  CA  AN  AC</td>
<td></td>
</tr>
<tr>
<td>C51</td>
<td>100   50 &gt;25 &gt;50 50 100 50 &gt;25 345.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C52</td>
<td>12    25 25 12 25 25 12 &gt;12 425.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C53</td>
<td>100   50 &gt;50 100 25 50 &gt;50 330.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C54</td>
<td>25    &gt;50 &gt;25 50 100 25 25 345.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C55</td>
<td>50    100 50 &gt;25 100 &gt;25 50 &gt;25 370.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C56</td>
<td>100   50 100 &gt;50 50 100 50 &gt;50 300.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C58</td>
<td>&gt;12   25 12 25 25 &gt;12 12 420.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C59</td>
<td>&gt;50   100 50 &gt;25 100 50 25 &gt;25 309.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C60</td>
<td>100   &gt;50 &gt;25 100 &gt;25 50 &gt;50 50 390.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CQ</td>
<td>12    12 &gt;6 12 12 12 NT NT 0.025 NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazide</td>
<td>NT    NT NT NT NT NT NT NT NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NT    NT NT NT NT NT NT NT NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>12    6 6 12 NT NT NT NT 6.25 NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td>NT    NT NT NT 6 12 12 NT NT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antimicrobial Activity:**

*Staphylococcus aureus*: CQ > C52 > C58 > C54 > C55 > C59 > C51, C53 > C56, C60

*Bacillus subtilis*: CQ > C42, C48 > C51, C53, C56 > C54, C57, C60 > C55, C59

*Escherichia coli*: CQ > C58 > C52 > C51, C54, C57, C60 > C55, C59 > C53 > C56

*Pseudomonas aeruginosa*: CQ > C52 > C58 > C54 > C55, C59 > C54 > C51, C56 >, C53, C57, C60

*Candida albicans*: CQ > C52, C58 > C53, C60 > C51, C57 > C54, C55 > C59

*Aspergillus niger*: CQ > C52, C58 > C55, C57 > C53, C54, C59, C60 > C51

*Aspergillus clavatus*: CQ > C52 > C58 > C54, C59 > C51, C55 > C53, C57, C60 > C56

**Antioxidant Activity:** C52 > C58 > C60 > C55 > C51 > C54 > C53 > C57 > C59 > C56

**Anti-tubercular activity:**

Isoniazide > Streptomycin > Ethambutol > C58 > C52 > C54 > C51, C55, C57, C59 > C53, C56, C60
Where,

\[ a = \text{Average value of triplicate results} \]
\[ b = \text{FRAP results expressed in mM of ascorbic acid per 100 g of sample i.e. mmol/100g} \]
\[ NT = \text{Not Tested} \]

All the synthesized ligands and complexes were evaluated for their antibacterial and antifungal studies. The antibacterial and antifungal tests were carried out using the serial broth dilution method. The \textit{in vitro} antimicrobial activities of the investigated compounds were screened against the bacterial species \textit{SA, BS, EC, PA} and fungal species \textit{CA, AN} and \textit{AC}. The minimum inhibitory concentration (MIC) values of the compounds are summarized in above tables. A relative study for MIC values of the ligands and their complexes signify that complexes display higher antimicrobial activity than the free ligands. In present investigation, the antimicrobial activity of the ligands may be due to the heteroaromatic residues. Compounds containing \textit{C=N} group have improved antimicrobial activity than \textit{C=C} group. The growth of certain microorganisms takes place even in the absence of oxygen. Hence, compounds containing \textit{C=C} group still capable of absorbing oxygen which are not related with the growth of microorganisms. The greater activity of the complexes can be clarified on the basis of Overtone’s concept [37] and Tweedy’s chelation theory [38]. According to Overtone’s concept of cell permeability, the lipid membrane surroundings in the cell was permit only the lipid-soluble resources, which makes liposolubility a key part that control the antimicrobial activity. During complexation, the polarity of the metal ion will be decrease up to a certain level due to
the overlapping of the ligand orbital and partial sharing of the positive charge of the Cu(II) ion with hetero atoms. Furthermore, it enhances the delocalization of π-electrons around the entire complex ring and enhances the lipophilicity of the complexes. This increased lipophilicity which enhances the permeation of the complexes into lipid membranes and blocking of the metal binding sites in the enzymes of microorganisms. These complexes also bother the respiration process of the cell and hence block the synthesis of proteins, which restricts additional growth of the organism and as a result microorganisms die. The experimental deviation in the activity of the Cu(II) complexes across the different classes of organisms studied may be characteristic to differences in cell wall and/or membrane construction (Gram-positive bacteria, Gram-negative bacteria and fungi). It is expected that the more extensive heteroaromatic ring system of clioquinol and the presence of the lipophilic group C=N would give better lipophilicity on the Cu(II) complexes and permit it to penetrate the cell wall and inhibit intracellular interactions. At the same time, hydroxylated derivatives had excellent antibacterial activity it was unexpected that the other Cu(II) complexes were really moderate against all of the microbial species tested. Although the range of functionalities on the aromatic ring of the coumarin core is significant only in the presence of a hydroxyl group on the aromatic ring shown antimicrobial activity for the subsequent Cu(II) complexes. The role of the hydroxyl group in this activity is complicated to find out but the metal complexes of other hydroxylated
derivatives of coumarin have been earlier shown to have excellent antimicrobial activity. Examples contain Cu(II) and Ni(II) complexes of 4-hydroxycoumarins [39]. In a previous study on the antimicrobial activity of catechols, the position and number of hydroxyl groups on the aromatic ring were responsible for their relative toxicity towards microorganisms, with facts that increasing hydroxylation results in an enhance in antimicrobial activity [40]. The mechanism recommended being liable for catechol toxicity to microorganisms contain enzyme inhibition by the oxidized compounds. The results would be indicate that substitution of the hydroxyl groups on the aromatic ring of the coumarin ligand was also vital for giving antimicrobial activity onto the Cu(II) complexes.

In fact, coordination, locking the polar electronegative atoms in the inner core around the metal and confining the apolar residues in an external lipophilic envelope, favor diffusion through biomembranes [41]. It is also suspected that factors such as solubility, conductivity, dipole moment may be the possible reasons for the increase in activity. Since all complexes have not shown enhanced activity as compared to parent ligand which rationalizes the fact that, steric and pharmacokinetic factors also play a decisive role in deciding the potency of an antimicrobial agent [42]. In review, the antimicrobial testing results reveal that complexes possess higher activity at lower concentration compared to parent ligand.

Antioxidant power was specifically the ability of transfer a single electron for compound. The antioxidant capacity of complexes C_{1}-C_{60}
was determined by a FRAP method. The FRAP results was expressed as an equivalent of standard antioxidant ascorbic acid (mmol/100 g of dried compound). FRAP values indicate that all the compounds have a ferric reducing antioxidant power. Among the tested compounds hydroxyl group substituted derivatives possess more ferric reducing power compared to that of chloro and methoxy group substituted derivatives. Thus from the data obtained, the potency order on the basis of various substitutions on $p$- & $m$- position of the phenyl ring are given the next after all activity tables. However, none of the compounds have been found to show excellent activity with compared to standard ascorbic acid.

The anti-tubercular activities of all the synthesized compounds were charge against $M.\, tuberculosi$ $s\, H37Rv$ at 12, 25, 50 and 100 $\mu$g/mL. The Minimum Inhibitory Concentrations of compounds compared with Isoniazid, Streptomycin and Ethambutol, the standard drugs and are summarized in above tables. Ligands show inhibition at concentration 100$\mu$g/mL. omplexes exhibits higher activity than ligands. None of the tested compounds have the inhibition more than standards.

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