Antimicrobial study

An antimicrobial agent which kills microorganisms or inhibits their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against, bacteria and fungi. They can also be classified according to their function; the agents that kill microbes are called microbicidal, and when they inhibit their growth are called microbiostatic.

The main classes of antimicrobial agents are disinfectants, which kill a wide range of microbes on non-living surfaces to prevent the spread of illness, antiseptics (which are applied to living tissue and help reduce infection during surgery), and antibiotics (which destroy microorganisms within the body). The term “antibiotic” originally described only those formulations derived from living organisms but is now also applied to synthetic antimicrobials, such as the sulphonamides, or fluoroquinolones. Use of substances with antimicrobial properties is known to have been common practice for at least 2000 years. Ancient Egyptians and ancient Greeks used specific molds and plant extracts to treat infection. More recently microbiologists such as Louis Pasteur and Jules Francois Joubert observed antagonism between some bacteria and discussed the merits of controlling these interactions in medicine. In 1928, Alexander Fleming became the first to discover a natural antimicrobial fungus known as penicillium rubens. He named the substance extracted from the fungus penicillin and in 1942 it was successfully used to treat a streptococcus infection. Many antimicrobial agents exist for use against a wide range of infectious diseases.

The remarkable success of antimicrobial drugs generated misconception in the late 1960s and early 1970s that infectious diseases had been conquered. However, 40 years later, infectious diseases remain the third-leading cause of death in the United States and the second-leading cause of death worldwide. Furthermore, the emergence of multidrug-resistant bacteria has created a situation in which there are few or no treatment options for infections with certain microorganisms. The spectre of bioterrorism, which gained widespread public attention after 11 September 2001, has magnified the problem, because genetic engineering of pathogens could render them resistant to currently available antimicrobials.
Although the need for new antimicrobials is increasing, development of such agents faces significant obstacles. Pharmaceutical research and development costs, which are estimated to be $400–$800 million per approved agent, pose a considerable barrier to new drug development in general. A number of factors make antimicrobial agents less economically attractive targets for development than other drug classes. For example, the aging of the US population has shifted drug discovery efforts towards agents that treat chronic medical conditions that are more prevalent among elderly persons, such as hypercholesterolemia, hypertension, mood disorders, dementia, and arthritis. Conversely, antimicrobials are usually used for short-course therapies that cure disease and thus eliminate their own need in a given patient. In addition, the large number of antimicrobials already approved results in a high level of competition for newly developed agents. Finally, the appropriate public health need to limit use of broad-spectrum antimicrobials, thereby minimizing the pressures driving resistance, causes the medical community to discourage the first-line use of newly developed antimicrobials, negatively impacting sales. For these reasons, some large pharmaceutical companies have indicated that they are curtailing—or abandoning completely—anti-infective research. The purpose of this study is to evaluate the impact of these research cutbacks on the availability of new antimicrobial agents.

Antimicrobials are used worldwide in human medicine, food, agriculture, livestock and household products. Many medicinal plants are considered to be potential antimicrobial crude drugs as well as a source for novel compounds with antimicrobial activity, with possibly new modes of action. Research and development of antimicrobial materials for food applications such as packaging and other food contact surfaces is expected to grow in the next decade with the advent of new polymer materials and antimicrobials. Antimicrobial packaging can take several forms such as addition of sachets containing volatile antimicrobial agents into packages; incorporation of volatile and non-volatile antimicrobial agents directly into polymers by ion or covalent linkages; and use of polymers that are inherently antimicrobial. At least 17 classes of antimicrobial agents, including tetracycline, penicillin, macrolides, lincomycin, and virginiamycin are approved for growth promotion of livestock. Antimicrobial coating of household products has obtained a wide acceptance in the past years. To control the growth of microorganisms, antimicrobials are used in cotton.
fibers and a wide range of plastic applications, such as telephones, PVC leather for furniture, wall covering, flooring, escalator rails, roof and pool liners, film and sheathing. They are also used in plastic products where infection is a concern, such as hospital furniture.

**Antibacterials study**

Antibacterials are used to treat bacterial infections. Bacteria were first identified in the 1670s by van Leeuwenhoek, following his invention of the microscope. The relationship between bacteria and diseases gradually set up in the nineteenth century. Since then, researchers started to try and find effective antibacterial agents.

The development of antibacterial agents is one of the greatest successes of 20th century medicine. Since the serendipitous discovery of penicillin by Alexander Fleming in 1928, an arsenal of antibacterial agents have been developed and found widespread clinical application. However, bacteria have quickly become resistant to commonly prescribed antibiotics. Combined with the lack of fundamental antibiotic research carried out by pharmaceutical companies over recent decades we are left with a legacy of relatively few efficacious drugs. Indeed, there is little doubt amongst many involved in antibacterial research that the existing drugs we have in hand for the treatment of infectious diseases are insufficient to protect us in the long term. Thus, bacterial infection, particularly from multi-drug resistant strains, remains a serious threat to human lives and there is a clear and critical medical need for the discovery of novel antibacterial agents, as small molecular mass compounds are certain to play a prominent role.

Small organic molecules have always been of interest in chemistry and biochemistry due to their ability to exert powerful effects on the functions of macromolecules that comprise living systems. Indeed, the underlying approach of using small molecules to treat disease represents the basis for medicinal chemistry as we know it. There are several benefits associated with the use of small molecules as therapeutic agents including improved stability over peptides in oral administration (where peptide bonds are easily cleaved by proteases), synthetic accessibility and, perhaps most notably, optimisation of compound bioactivity is considerably easier for a small
molecule than for complex macromolecules.

Methods which utilise small molecules as ‘chemical probes’ to modulate biological systems can be described by the umbrella term\textsuperscript{20} chemical genetics\textsuperscript{21}. Whereas traditional genetics uses gene knockouts (or knock-ins) on the level of the DNA, chemical genetics uses biologically active small molecules to directly attenuate the corresponding biological macromolecular product and thus affect a biological response, for example, the inhibition of bacterial growth. The first step of a chemical genetics experiment involves the identification of a small molecule which induces a desired phenotype (forward chemical genetics) or modulates the function of a specific protein of interest (reverse chemical genetics). Thus, in the former case, investigations proceed from phenotype to protein, whereas in the latter case investigations progress from protein to phenotype. Small molecules that exhibit biological effects can be discovered by both forward and reverse chemical genetics approaches through the screening of collections (or ‘libraries’) of small molecules to identify those with the desired characteristics (so-called ‘hits’).

**Antifungals study**

Antifungals are used to kill or prevent further growth of fungi. Over the past 30 years, the importance of antifungal drugs to the practice of modern medicine has increased dramatically. Because the vast majority of life-threatening fungal infections affect people with altered immune function, the increased incidence of invasive fungal infections can be correlated with an expansion in the number of people living with conditions or treatments that affect immune function, examples of which include HIV/AIDS, primary immune deficiency, cancer chemotherapy, hematologic and solid organ transplantation, prematurity, and immune-modulatory medications\textsuperscript{22}. It is, therefore, sobering to consider that two of the three classes of antifungal drugs (azoles and polyenes) in current use had already been introduced into the clinics by 1980 and the third class (echinocandins) had been discovered\textsuperscript{23}. Furthermore, even with these newest therapies, the clinical outcomes for most invasive fungal infections are far from ideal. Indeed, infections caused by species of molds for which there is no reliable medical therapy are emerging as are strains of the more common organisms such as Candida albicans and Candida glabrata that are resistant to currently used
drugs. It therefore seems fairly clear that the tempo of antifungal drug development has not kept pace with the clinical needs.

Fungi are nonphotosynthetic eukaryotic rising either as colonies of single cells (yeasts) or as filamentous multicellular aggregate (molds). The fungi are heterotrophic organisms—they require organic compounds for nutrition. When they feed on dead organic matter, they are known as saprophytes. Saprophytes decompose complex plant and animal remains, breaking them down into simpler chemical substances that are returned to the soil, thereby increasing its fertility. Thus they can be quite beneficial to humans. But they can also be undesirable when they decompose timber, textiles, food and other materials. Saprophytic fungi are also important in industrial fermentations: for example the brewing of beer, the making of wine, and the production of antibiotics such as penicillin. The leavening of dough and the ripening of some cheeses also depend on fungal activity. As parasites (i.e., when living in or on another organism), fungi cause diseases in plants, humans, and other animals. Although fungal diseases are less commonly encountered than bacterial or virus diseases in humans and other animals, they are of great importance in causing diseases of plants.\textsuperscript{24}

All the synthesized compounds were evaluated for antimicrobial activities by using E-test Method\textsuperscript{25}. Antibacterial activity of all the synthesized compounds were carried out by using two Gram positive bacteria (Staphylococcus aureus and Bacillus subtilis) and two Gram negative bacteria (Escherichia coli and Pseudomonas aeruginosa). Antifungal activity of all the synthesized compounds were carried out by using one fungi (Candida albicans). Antimicrobial activity was estimated by minimum inhibitory concentration (MIC).
Antimicrobial Sensitivity Test Protocol

Dilution and Diffusion Test

E test also known as the epsilometer test is an ‘exponential gradient’ testing methodology which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium.

Reagents for the Diffusion Test

1. Müeller-Hinton Agar Medium

Of the many media available, Müeller-Hinton agar is considered to be the best for routine susceptibility testing of nonfastidious bacteria for the following reasons:

- It shows acceptable batch-to- batch reproducibility testing.
- It is low in sulphonamide, trimethoprim, and tetracycline inhibitors.
- It gives satisfactory growth of most nonfastidious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Although Müeller-Hinton agar is reliable generally for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of a test organism, zones obtained in a disk diffusion test will usually be larger than expected and may exceed the acceptable quality control limits. Only Müeller-Hinton medium formulations that have been tested according to, and that meet the acceptance limits described in, NCCLS document M62-A7- Protocols for Evaluating Dehydrated Müeller-Hinton Agar should be used.

Preparation of Müeller-Hinton Agar

Müller-Hinton agar preparation includes the following steps.

1. Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions of Hi-Media.

2. Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath.

3. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of
approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with
diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.

4. The agar medium should be allowed to cool to room temperature and, unless the
plate is used the same day, stored in a refrigerator (2 to 8°C).

5. Plates should be used within seven days after preparation.

6. Each batch of plates should be examined for sterility by incubating at 30 to 35°C
for 24 hours or longer.

2. Preparation of Synthesized compounds stock solutions

Synthesized compounds may be received as powders (solids) form. Powders must be
accurately weighed and dissolved in the appropriate diluents (DMSO, DMF, Ethanol, etc
suggested by Antimicrobial agent provider) to yield the required concentration, using
sterile glassware. Standard strains of stock cultures should be used to evaluate the
antimicrobial agent stock solution.

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 $V$
**Turbidity standard for inoculum preparation**

To standardize the inoculum density for a susceptibility test, a BaSO$_4$ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent (e.g., latex particle suspension), should be used. A BaSO$_4$ 0.5 McFarland standard may be prepared as follows:

1. A 0.5-ml aliquot of 0.048 mol/L BaCl$_2$ (1.175% w/v BaCl$_2$ . 2H$_2$O) is added to 99.5 ml of 0.18 mol/L H$_2$SO$_4$ (1% v/v) with constant stirring to maintain a suspension.

2. The correct density of the turbidity standard should be verified by using a spectrophotometer with a 1-cm light path and matched cuvette to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard.

3. The Barium Sulfate suspension should be transferred in 4 to 6 ml aliquots into screw-cap tubes of the same size as those used in growing or diluting the bacterial inoculum.

4. These tubes should be tightly sealed and stored in the dark at room temperature.

5. The barium sulfate turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance. If large particles appear, the standard should be replaced. Latex particle suspensions should be mixed by inverting gently, not on a vortex mixer.

6. The barium sulfate standards should be replaced or their densities verified monthly.

**Procedure for Performing the Dilution and Diffusion Test**

**Inoculum Preparation**

**Growth Method**

The growth method is performed as follows

1. At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture of known culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a brain heart infusion broth.
2. The broth culture is incubated at 35°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours)

3. The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain a turbidity optically comparable to that of the 0.5 McFarland standard.

**Inoculation of Test Plates**

1. Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

2. The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

3. The lid may be left ajar for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed.

4. 7 well having 10 mm size to be made on agar plates by bore having standard size of 10 mm.

**Application of Antimicrobial agent to Inoculated Agar Plates**

1. The predetermined battery of antimicrobial agents is dispensed in each well with manner of increasing concentration of antimicrobial agent.

2. The plates are incubated in an incubator at 37°C within 15 minutes in upright position after the antimicrobial agent applied.
Reading Plates and Interpreting Results

1. After 16 to 18 hours of incubation, each plate is examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the well.

2. The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye.

3. MIC point is determined where zone of inhibition started

Minimum Inhibitory Concentration of Standard Drugs as MIC(µg/ml).

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Staphylococcus aureus</th>
<th>Bacillus subtilis</th>
<th>Escherichia coli</th>
<th>Pseudomonas aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENTAMYCIN</td>
<td>0.25</td>
<td>1</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>AMPICILLIN</td>
<td>250</td>
<td>125</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>CHLORAMPHENICOL</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>CIPROFLOXACIN</td>
<td>10</td>
<td>05</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>NORFLOXACIN</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLUCANAZOLE</td>
<td>10</td>
</tr>
<tr>
<td>NYSTATIN</td>
<td>100</td>
</tr>
<tr>
<td>GRESEOFULVIN</td>
<td>500</td>
</tr>
</tbody>
</table>
Antituberculosis Activity

Tuberculosis (TB) is an airborne disease caused by Mycobacterium tuberculosis (M. tb), an organism that was discovered by a German physician Robert Koch in 1882. TB is a disease that affects a number of different organs of the human body, but primarily the lung. The mode of spread is from an infected person to an uninfected person by inhalation of infected droplet nuclei with Mycobacterium tuberculosis. The period from which a person is infected by Mycobacterium tuberculosis up to the development of active disease varies from individual to individual. The development of infection to active disease can take many years, or the infection may remain dormant for life. Only 10% to 20% of all individuals who are infected with Mycobacterium tuberculosis end up with active disease in their life time.

Every year, 1.7 million people die of tuberculosis, a curable disease. The poor are disproportionately affected, and tuberculosis further impoverishes individual people and societies. Goal 6 of the Millennium Development Goals of the United Nations includes the halting and reversal of the rising incidence of tuberculosis, and stop TB Partnership aims to halve the prevalence of tuberculosis and resulting deaths by 2015.

Infection with Human Immuno deficiency Virus [HIV] is the most potent risk factor for development of tuberculosis. This is also a leading cause of death among people who are HIV-positive 13% of AIDS death worldwide.

Sputum-smear-based diagnosis under the Direct Observation of Therapy (Short Course) (DOTS) strategy of the World Health Organization for global tuberculosis control misses half of incident cases at first presentation. Transmission continues until cases are detected with more advanced (smear-positive) disease and are correctly treated. Multidrug-resistant tuberculosis increases morbidity and mortality and, through treatment failure, facilitates continuing transmission from patients who (like their health care providers) wrongly believe they are being cured. The use of treatment failure to prompt drug susceptibility testing relies on the same illness threshold effect as waiting for smears to become positive in patients with negative smears. Both scenarios could be addressed by appropriate new diagnostics.
The current chemotherapy is based on age-old drugs like pyrazinamide, Isoniazid and Rifampicin for tuberculosis. TB treatment requires long-term treatment with combination of drugs as recommended by the world health organisation (WHO). The available treatment establishes a multidrug regime lasting a minimum of six months, although there is no guarantee that the complete sterilization of the infection will be obtained. Furthermore, the increase in TB cases caused by MDR and XDR strains and coinfection with HIV have pointed out the urgent need to develop new antitubercular drugs which will not only effectively kill MDR strains but also should be less toxic, shortened duration of therapy and induced rapid mycobacterial mechanism of action and the ability to penetrate host cells and exert antimycobacterial effects in the intracellular environment.

Optimal methods for identifying drug resistant Mycobacterium tuberculosis in a timely and affordable way in resource-limited settings are not yet available. The currently available drug susceptibility testing (DST) methods with solid media are inexpensive but slow and laborious and can not meet such a demand. Liquid automated commercial systems\(^{36-37}\), such as the BACTEC MGIT 960 (Becton Dickinson Sparks, MD) are rapid but require heavy, expensive equipment have high running costs, and are technically complex. Molecular genetic methods\(^{38-40}\) are fast but too expensive and require well-trained manpower in order to be used in resource-poor settings. In addition, not all mutations conferring resistance to anti-TB drugs are known. Several low technology methods\(^{41-42}\) are also available. Most are indirect DST methods requiring more time and considerable laboratory expertise. The microscopic observation drug susceptibility assay (MODS) is a low-technology, direct DST method which detects resistance to INH and RIF directly from sputa, with positive results available within 2 weeks in most cases.

Some of the selected compounds were evaluated for antituberculosis activity by using MODS method\(^{43}\).
Microscopic Observation of Drug Susceptibility (MODS):

Procedure:

- Middlebrook 7H9 broth was prepared by using a Middlebrook 7H9 broth base, 0.31% of glycerol and 10% of OADC containing PANTA antibiotic.
- A stock of 100 mg/mL was prepared by dissolving the plant extract in minimum amount of DMSO (20%) and Middlebrook broth 7H9 containing 10% OADC was used to make up the volume.
- This stock was serially diluted to obtain a dilution of 0.5 mg/mL.
- Antibiotic stock solutions were added to give final critical concentrations of 2 mg/ml for Isoniazid (Isoniazid was used as standard).
- In a sterile 24-well plate, 1 ml of drug-free broth was distributed in the each row. Then the plant extract in desired concentrations were serially added in to the plate.
- M. tuberculosis H37Rv strain was cultured at 37°C in Lowestein-Jensenn medium until log phase growth was obtained.
- Suspension of M. tuberculosis H37Rv strain was made in sterile normal saline. The cell density was adjusted to >3×10^8 cfu/ml by comparing it with McFarland Std 1.
- A One hundred microliters of these suspension was then added to the wells containing serially diluted test compounds.
- Isoniazid (1mg/mL) was used as a standard. A well containing only suspension of H37Rv and containing no drug/plant extract was used as negative control. DMSO (20%) was used as vehicle control.
- The plate was then covered with its lid and securely sealed throughout its edge using polyethylene tape. The date of inoculation and plate number were recorded on the plate. The dates, plate numbers, and corresponding plant extract were noted on a plate layout laboratory worksheet prepared for the recording of results.
- Each plate was placed in a polyethylene bag to prevent evaporation and incubated at 37°C.
- Observation of drug-free control wells was commenced on day 3 of incubation and performed each day, using an inverted-light microscope at 20x.
magnification, except on holidays. After 15 days of incubation, observation was limited to once or twice a week.

- Growth was defined as the emergence of visually detectable serpentine clusters of bacteria. Drug susceptibility results were interpreted on the same day that distinct growth was visualized in control wells. A sample was considered as susceptible (i.e. having anti TB activity) if growth was visible in the drug-free well but not in the drug-containing well.

- A sample was considered resistant if both the drug-free and the drug-containing wells showed visible growth.
References
Chapter - 5  Biological Evaluation


