CHAPTER-IV

BIOLOGICAL

EVALUATION
The development of chemotherapeutic agents has had a greater impact on clinical medicine than any other discovery. Although a variety of natural chemical agents had been used earlier, the real advances in work with chemotherapeutic agents began with the German scientist Paul Ehrlich. In the early 1900s, Ehrlich developed the concept of selective toxicity. The microorganisms comprising the bacteria, fungi, virus, cause a lot of known and unknown infections to humankind. Antimicrobial agent is a chemotherapeutic substance that destroys or inhibits the growth of microorganisms in living tissues while damaging the host as little as possible. Agents that kill organisms are often called cidal agents, with a prefix indicating the kind of organism killed. Thus, we have bacterial, fungicidal and viricidal agents. A bactericidal agent kills bacteria and it may or may not kill other kinds of microorganisms. Agents that do not kill but only inhibit growth are called static agents, and we can speak of bacteriostatic, fungistatic, and viristatic agents.

The synthetic antibacterial agents are comprised of two major classes of compounds. Those effective systemically and those effective topically. The systemically active antimicrobials have been divided into three groups; the Sulphonamides, antimycobacterial agents, and agents for the treatment of urinary tract infections. Those antibacterial agents that are used non-systemically are commonly termed as antiseptics, disinfectants or preservatives based on how they are used. There is a considerable degree of overlap on usage among these three groups.

The various microbial infections occurring now-a-days can be classified based on the type of the organism which is responsible for the infection. Antibacterial chemotherapy has played a vital role in the treatment of human infectious diseases. However, the repeated use of antibiotics often leads the organisms to become ineffective due to development of drug resistance. However, in the past several years, the rapid emergence of bacterial resistance to antibiotics has been observed and has become essentially an emerging threat in the chemotherapy of infectious diseases.

The purine derivatives are more important for chemists as well as to biologists as they are found in large variety of naturally occurring compounds and also in clinically useful molecules having diverse biological activities. They are known to possess antitubercular, antiulcer, antimicrobial, antitumor, antiviral and cardiotonic properties.
A very large number of antibiotics have been discovered, but probably less than 1% of them have been of practical value in medicine. Those that have been useful have had dramatic impact on the treatment of infectious diseases. The sensitivity of microorganisms to antibiotics and other chemotherapeutic agents varies. Gram-positive bacteria are usually more sensitive to antibiotics than are gram negative bacteria, although some antibiotics act only on gram-negative bacteria. An antibiotic that acts on both gram positive and gram negative bacteria is called a broad-spectrum antibiotic. In general, a broad-spectrum antibiotic finds wider medical usage than a narrow-spectrum antibiotic, which act on only a single group of organisms. A narrow-spectrum antibiotic may, however, be quite valuable for the control of microorganisms that fail to respond to other antibiotics. Some antibiotics have an extremely limited spectrum of action, being effective for only bacterial species.

Some of the infections caused by Gram positive (G +ve) and Gram negative (G -ve) bacteria are

<table>
<thead>
<tr>
<th>Infection</th>
<th>Organism</th>
<th>Symptom</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharyngitis</td>
<td>Streptococci</td>
<td>Sore throat, Fever, nausea, headache etc.</td>
<td>Benzathin, penicillin, Erythromycin, Azithromycin.</td>
</tr>
<tr>
<td>Skin infections</td>
<td>Streptococci</td>
<td>Impetigo Erysipelas</td>
<td>Penicillin, cefazolin, Vencomycin</td>
</tr>
<tr>
<td>Skin &amp; soft tissue infections</td>
<td>Staphylococcus epidermidis</td>
<td>Myositis</td>
<td>Nafcillin, oxacillin, cefazolin</td>
</tr>
<tr>
<td>Staphylococcal Bacteremia</td>
<td>S.aureus</td>
<td>Invades the bloodstream</td>
<td>Methicillin, vancomycin</td>
</tr>
<tr>
<td>Bordetallapertussis (Whoopingcough)</td>
<td>Bordetta pertussis</td>
<td>Lacrimation sneezing anorexia</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Meningococcal meningitis</td>
<td>Neissaria meningitidis</td>
<td>Higher fever abdominal pain</td>
<td>Ceftriaxone, Dexamethasone</td>
</tr>
</tbody>
</table>
Evaluation of antibacterial activity

The antibacterial activity of substituted purine derivatives were tested against skin disease causing bacteria like Salmonella abony, Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis, for their inhibitory activity, using a Muller-Hinton broth micro dilution method using abacavir as the standard drug.

Materials:

1. Muller-Hinton agar
2. McFarland turbidity standards
3. Scrupulously clean, acid-washed borosilicate glass tubes
4. Micropipette
5. Nutrient agar

Sterilization of media and glass ware:

The media in the present study, Muller-Hilton agar and nutrient agar were sterilized in conical flasks of suitable capacity by autoclaving at 15 lb pressure for about 20 minutes. The test tubes and pipettes were sterilized in hot air oven at 160° C for 1 hour.

Preparation of solution of test compounds:

Serial dilutions of the test compounds and reference drugs were prepared in Muller-Hinton agar. Samples (100 mg) were dissolved in dimethylsulfoxide (DMSO 10 ml). Further progressive dilutions with melted Muller-Hinton agar were performed to obtain the required concentrations of 50 and 500µg/ml.

Preparation of the Inoculum:

Test organisms were subcultured onto nutrient agar and incubated for 18 hrs at 35-37 °C, the tubes that contain 2 ml of Muller-Hinton agar were inoculated with five or more colonies from the agar plate and turbidity was adjusted to match a McFarland standard (10⁵ CFU/ml) and incubated at 37 °C for 18 hours, the MIC was the lowest concentration of the tested compound that yields no visible growth on the plate. To ensure that the solvent had no effect on the bacterial growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments and DMSO had no effect on the microorganisms in the concentrations studied.
Method:

In a first step 50μl of Muller-Hinton broth were distributed from the second to various test tubes according own requirements, extracts were initially dissolved in 100μl of dimethyl sulfoxide (DMSO) and then in Mueller-Hinton broth, to reach final concentration of 500 mg/ml. 100 μl of these suspensions were added to the first test well and each microtiter line and then 50 μl of serial dilution were transferred form the second to the ninth well. The tenth well was considered a growth control, since no extract solutions were added. Then 50 μl of microbial suspension (10⁵ CFU/ml) obtained from an overnight growth at 37 °C, were added to each well. The final concentration of the extract adopted is 0.025 to evaluate the antibacterial activity was included from 300 mg/ml.

The results obtained are tabulated in Table IV-1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Escherichia coli</th>
<th>Salmonella abortus</th>
<th>Staphylococcus aureus</th>
<th>Staphylococcus epidermidis</th>
<th>Staphylococcus epidermidis</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>6a</td>
<td>14.1</td>
<td>9.9</td>
<td>14</td>
<td>9.9</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>6b</td>
<td>13.8</td>
<td>10.1</td>
<td>14.1</td>
<td>9.6</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>6c</td>
<td>14.1</td>
<td>10.2</td>
<td>13.9</td>
<td>9.9</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>6d</td>
<td>14.2</td>
<td>10.4</td>
<td>14.1</td>
<td>10.1</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>6e</td>
<td>14.1</td>
<td>10.1</td>
<td>14</td>
<td>9.9</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>Std</td>
<td>16.1</td>
<td>12.4</td>
<td>13.8</td>
<td>9.8</td>
<td>NG</td>
<td>NG</td>
</tr>
</tbody>
</table>

Std: Abacavir  NG: No Growth  High:500mcg/ml  Low:50mcg/ml
Results:

The MIC values presented in Table IV-1 revealed that the minimal concentration varies from bacterial species to species. Out of 5 compounds tested for antibacterial activity, all shown inhibitory activity on only two species (Escherichia coli, Salmonella abony). Two compounds shown inhibitory activity on the species Staphylococcus aureus. Whereas all the compounds shown no activity on the species Staphylococcus epidermidis.

Antifungal activity

In the not so distant past in fact, only about 15 years ago fungi were generally considered of little consequence as relates to human disease. It was paradoxical situation considering that a fungus was among the first proven ethologic agents of an infectious disease, albeit, in silk worm. The general population certainly knew well the ever present, cosmopolitan, nuisance causing fungal skin problems such as "athlete's foot", and there were few women who had not experienced or had a friend, relative, or acquaintance who had not suffered from "yeast" vaginal infection. General medical practitioners and especially dermatologists saw these superficial fungal skin and mucous membrane infections quite often in their daily practices. Every so often, a cancer patient would develop a more deep seated fungal infection; likewise, those early kidney transplant recipients were occasionally diagnosed with invasive mycoses, which could at times be very severe and even fatal.

Medical Problems Caused By Fungi:

1. Allergic Diseases: Fungi certainly rank among the major inciters of allergy around the earth. While allergies are clearly major medical problems for many and are omnipresent, note that such fungal involvement represents an immunological aberration and not an infection.

2. "Mushroom" Poisoning: The consequences of ingesting toxin producing mushrooms may range from mild gastrointestinal upset to coral liver shutdown and death. Once again, the effects if these fungi are ones related to toxicology rather than infection.
3. Mycotoxins: some fungi, notably species aspergillus and fusarium, produce secondary metabolites that are inherently toxic to humans and animals such toxins have been collectively called mycotoxins and have been involved in the distinct scenarios following their ingestion or inhalation, after which the manifestations and illness, including serious neurologic sequelae and death. Again, as with the two categories above, mycotoxicoses are problems with chemical toxins and not infectious disorders.

4. Mycoses (mycosis, singular): Mycoses caused by pathogenic saprophytic yeasts, which are contagious and usually superficial infections involving the skin and mucous membrane. Some species of saprophytic yeasts (Asperigillus, Blastomyces, Candida, Cocudioles, and Histoplasma) under favorable conditions are capable of invading deeper body cavities and causing systematic mycosis. Such infections may become serious and occasionally life threatening and they are frequently difficult to treat.

Perhaps the single most significant and consequential event influencing the advent of the golden age of clinical mycology and the importance of fungi as infectious agents was the human immunodeficiency virus and the pandemic of acquired immunodeficiency syndrome. Many individuals with advanced HIV associated disease and frank AIDS epitomize the "living Petri dish" concept. As the AIDS era progressed, it became clear that HIV + individuals experienced significant problems with fungal infections.

Treatment of fungal infections generally has been less successful than that of bacterial infections largely because eukaryotic cells are much more similar to human cells than are bacteria. Many drugs that inhibit or kill fungi are therefore quite toxic for humans. In addition, most fungi have detoxification system that modifies many antibiotics, probably by hydroxylation. As a result the added antibiotics are fungistatic only as long as repeated application maintains high levels of unmodified antibiotic. Despite their relatively low therapeutic index, a few drugs are useful in treating many major fungal diseases.

A number of fungal infections have arisen newly even though a lot of anti fungal agents are existing, some of the fungal infections are:
Infection Organisms Drug of choice

<table>
<thead>
<tr>
<th>Infection</th>
<th>Organisms</th>
<th>Drug of choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candidiasis</td>
<td>Candida albicans</td>
<td>Fluconazole, Itraconazole</td>
</tr>
<tr>
<td>Miconozole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillosis</td>
<td>Aspergillus, fumigatus</td>
<td>Itraconazole</td>
</tr>
<tr>
<td>Ampotericin B.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucormycosis</td>
<td>Members of the genera</td>
<td>Amphotericin</td>
</tr>
<tr>
<td></td>
<td>Rhizopus, mucor, absidis etc.</td>
<td></td>
</tr>
</tbody>
</table>

So many remedies have been employed against fungi and the research over them still continues, which would lead to conclude that the ideal antifungal agent have not yet been found. The majority of the fungal infections (mycoses) involve superficial invasion of the skin or mucous membrane of body orifices. The diseases which can usually be controlled by local application of an antifungal agent are divided in to two etiological groups.

(I) The dermatophytes (tinea infections) which are contagious superficial epidermal infections caused by various Epidermophyton, Microsporum and Trichophytonsp.

(ii) Mycoses caused by pathogenic saprophytic yeasts, which are contagious and usually superficial infections involving the skin and mucous membrane. Some species of saprophytic yeasts (Aspergillus, Blastomyces, Candida, coccidioides, Histoplasma) under favorable conditions are capable of invading deeper body cavities and causing systematic mycosis. Such infections may become serious and occasionally life threatening and they are frequently difficult to treat.

**Evaluation of antifungal activity:**

The antifungal activity of the synthesized compounds was carried out on the organisms, Saccharomyces cerevisiae, Candida albicans, Aspergillus niger for their inhibitory activity, using a Muller-Hinton broth by a broth micro dilution method using Abacavir as the standard drug.
Materials:

1. sabouraud dextrose agar
2. McFarland turbidity standards
3. Scrupulously clean, acid washed borosilicate glass tubes
4. Micro pipette

Preparation of solution of test compounds:

Serial dilutions of the test compounds and reference drugs were prepared in Sabouraud dextrose agar. Drugs (100 mg) were dissolved in dimethylsulfoxide (DMSO, 10 ml). Further progressive dilutions with sabouraud dextrose agar were performed to obtain the required concentrations of 50 and 500 μg/ml.

Preparation of the Inoculum:

Test organisms were subscribed on to sabouraud dextrose agar and incubated for 24-48 hrs at 22-25°C. The MIC was considered to be the lowest concentration of the compound that inhibited the visible growth of fungi.

Method:

In a first step 50μl of Muller-Hinton broth were distributed from the second to various test tubes according own requirements, extracts were initially dissolved in 100μl of dimethyl sulfoxide (DMSO) and then in Mueller–Hinton broth, to reach final concentration of 500 mg/ml. 100μl of these suspension were added to the first test well and each microtiter line and then 50μl of serial dilution were transferred from the second to the ninth well. The tenth well was considered a growth control, since no extract solutions were added. Then 50μl of microbial suspension (10⁵ CFU/ml) obtained from an overnight growth at 37 °C, were added to each well. The final concentration of the extract adopted is 0.025 to evaluate the antifungal activity was included from 300 mg/ml. The results obtained are tabulated in Table IV-2.
### Table IV-2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Saccharomyces cerevisiae</th>
<th>Saccharomyces cerevisiae</th>
<th>Candida arbicans</th>
<th>Candida arbicans</th>
<th>Aspergillus niger</th>
<th>Aspergillus niger</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>High</td>
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<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
<tr>
<td>Std</td>
<td>12.1</td>
<td>9.2</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
<td>NG</td>
</tr>
</tbody>
</table>

STD: Abacavir  
NI: No Inhibition  
High: 500mcg/ml  
Low: 50mcg/ml  

**Results:**  
MIC values obtained for anti fungal activity of same 5 compounds were presented in Table IV-2. All 5 compounds have shown very good growth inhibitor activity with all the fungal strain Saccharomyces cerevisiae used in the study. But All 5 compounds have shown no growth inhibitor activity with all the fungal strain Candida arbicans, Aspergillus niger used in the study at both high and low Concentrations.
REFERENCES

1. M.T.Madigan, J.M.Martinko and J.Parker, "Biology of Microorganisms"

2. L.M.Prescott, "Microbiology"

3. E.M.Wolff, "Burger's Medicinal Chemistry"

4. Cremer A., "Microbiological methods"

5. D.A.Sutton, A.W.Fothergill and M.G.Rinaldi, "Guide to Clinically Significant Fungi"
   Williams & Wilkins, London, 1998, p-3-4


7. R.J.Hamill, "Medicinal Diagnosis and Treatment"

8. Koneman, EW, Testo atlante di microbiologia

9. Young-Tae Chang, Nathanael S Gray, Gustavo R Rosania, Daniel P Sutherlin, Soojin Kwon, Thea C Norman, Radhika Sarohia, Maryse Leost, Laurent Meijer and Peter G Schultz

10. Leire Aguado, Hendrik Jan Thibaut, EvaMaria Priego, Maria-Luisa Jimeno, Maria-José Camarasa, Johan Neyts, and María Jesús Perez-Perez

11. Steven R. Schow Richard L. Mackman et al.

12. Yu Lin Hu, Xiang Liu, and Ming Lu

13. Y. Murti, N. Badal, D. Pathak

14. Shaker Youssif, Fatmah Agil and Sahera F. Mohamed

15. Mourinta Koley, Xaver König, Karlheinz Hilber, Michael Schnürch, Peter Stanetty, and Marko D. Mihovilovic

17. Manish M. Jeni, Bhavesh R. Nathani and K. S. Pandya

18. Yu Lin Hu, Qiang Ge, Ming Lu and Hong Fei Lu