ISSN 0974-3618 (Print) www.rjptonline.org
0974-360X (Online)

RJPT Research Journal of Pharmacy and Technology

REVIEW ARTICLE

Assessment of Antitubercular Activity- A Review

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ABSTRACT:
The chemotherapy of tuberculosis has been practiced for 2000 years but it has been a clinical reality for last few years. In tuberculosis there is an extensive tissue destruction and live virulent bacilli become isolated in the cavities and debris of necrotic tissue where they are relatively immune to chemotherapeutic attack. Thus a drug will be effective only if it can penetrate the tubercule and the debris and enter the phagocytes within which the parasites are growing and finally penetrate or attack the parasites. The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* underscores the need of continuous developments on new and efficient methods to determine the susceptibility of isolates of *M. tuberculosis* in the search for novel antimicrobial agents. Natural products and synthetic compounds constitute an important source of new drugs, but design and implementation of antimycobacterial susceptibility testing methods are necessary for evaluating the different extracts and syntheised compounds. A number of biological assay methodologies are in current use, ranging from the classical disk diffusion and broth dilution assay format, to radiorespirometric (BACTEC), dye-based, and fluorescent/luminescence reporter assays. This review presents an analysis on the *in vitro* susceptibility testing methods developed for determinate antitubercular activity in natural products and synthetic compounds and the criteria to select the adequate method for determination of biological activity. Different methods of analysing the antituberculosis is given below Anti-TB in vitro bioassays, Agar dilution method, Radiometricmethod, Micro broth dilution, Flow cytometry ,Reporter gene assays, High-performance liquid chromatography, mycolic acid analysis, Toxicology assays, Dormant tubercle bacilli assays ,Macrophages assays, Micro Almar Blue Assay (MABA):, Agar Micro Dilution Method, Cytotoxicity of Selected Compounds, Liquid culture method was used for detection of drug resistance, Filamenting temperature-sensitive technique.

KEYWORDS: *M. tuberculosis*, Susceptibility testing, Different methods of analysis of antitubercular, *invitro* analysis.

INTRODUCTION:
The chemotherapy of tuberculosis has been practiced for 2000 years It has been a clinical reality for last few years. By 1940, series of drugs effective against experimental and then clinical tuberculosis were developed. During next 30 years, beginning with dapsone and streptomycin and currently with rifampicin, capreomycin and ethambutol are being used Human pulmonary tuberculosis is predominantly caused by *Mycobacterium tuberculosis.*

Received on 17.12.2018 Modified on 29.01.2019
Accepted on 28.02.2019 © RJPT All right reserved
Research J. Pharm. and Tech 2019; 12(8):3973-3978.
DOI: 10.5958/0974-360X.2019.00684.X

The infectious character of the disease was established in year 1882 by Koch [1], who isolated tubercule bacillus, which causes tuberculosis. The organisms are rod-shaped,aerobic hard to strain.The organisms are difficult to decolorize even with acidic alcohol. The pulmonary form is the reason for about 90% of tuberculosis.

There are tuberculous-meningitis, enterlitis, laryngitis, ostemyelities and rapidly fatal form known as milliary tuberculosis or “galloping consumption”. In tuberculosis there is an extensive tissue destruction and live virulent bacilli become isolated in the cavities and debris of necrotic tissue where they are relatively immune to chemotherapeutic attack. The prevalence of tuberculosis is still very high in many parts of the world and in some

countries it is epidemic [2]. Though the antituberculosis drugs are applied since last 25 years, it has not declined as rapidly as anticipated [3]. It has been estimated that out of all infections caused by *mycobacterium tuberculosis*, 95% of those infected (tuberculin positive) live undisturbed by the presence of tubercule bacilli [4]. The screening of antitubercular drugs by *invitro* methods has helped the scientific committee and researchers to develop new drugs. Hence in this current review we are giving an insight in various methods of screening of anti tubercular drugs and its applications

EXPERIMENTAL:

It is important to develop new strategies for evaluation and discovery of antimycobacterial drugs, the review shows the different *in vitro* methods for discover antitubercular agents in natural products research, and offer a guide for researchers to select an adequate method for determinate biological activity.

The tubercular property of the compounds was tested by different methods.

susceptibility testing of M.tuberculosis are

1. The method of preparation:

The method of proportion is an agar-based method in which the number of colonies on the control medium is compared to the number of colonies on drug-containing media. Because growth of colonies is necessary for interpretation, this method requires three weeks of incubation.

2. The broth radiometric methods:

The radiometric broth method is much more rapid, the method is semi-automated, and generates radioactive waste. BacT/ALERT 3D Detection System:

The MB/BacT Detection System and the BacT/ALERT 3D employ a colorimetric sensor and reflected light to monitor the presence and production of carbon dioxide dissolved in the culture medium. Carbon dioxide is produced as the organisms metabolize the substrates in medium.When growth of the microorganisms produces CO2, the color of the sensor at the bottom of each culture bottle changes from dark green to lighter green or yellow. The lighter colour results in an increase in reflectance units as monitored by the system.

1. *in vitro* bioassays:

Agar diffusion:

The paper disk diffusion bioassay as well as agar diffusion assays were first developed for bacteria (5,6). Disk methods comprise the placing of filter paper disks containing test compounds on agar plate surfaces previously inoculated with the test organism. The test molecules or plant extracts then diffuse into the agar and inhibit growth of the test microorganisms. After incubation, mean diameters of growth inhibition zones are recorded. Recently, a *M. marinum* zone of inhibition assay has been developed as a method for screening of antimycobacterial compounds from marine organisms extracts, the use of *M. marinum*, which is a ubiquitous aquatic pathogen, makes of this specie a model for to evaluate antitubercular activity of this kind of natural products (8). Diffusion assays are recommended more for polar rather than non-polar molecules or mixture of compounds such as essential oils (7).

These assays employed in many antimicrobial assays for discovery of natural product leads are not quantitative when used to evaluate extracts or new compounds (9). A principle problem is that the mycobacteria cell wall is often more susceptible to less-polar compounds. Non-polar compounds will diffuse more slowly than polar compounds in the aqueous agar medium and, thus, giving the erroneous impression of weak activity. (10)

2. Agar dilution:

In the 1950s, Canetti *et al*. described the first Drug Susceptibility Testing (DST) method for *M. tuberculosis*, involving the preparation of a concentration series of drugs against *M. tuberculosis* complex in Lowenstein-Jensen medium, Inoculation of the bacterial cultures on the slants, and reading of the inhibition of growth by drugs at different concentrations (11,12). The agar dilution tests permit to determine the MIC, Volatilization of test material and activity in the vapor phase of a Petri dish may also occur and play a role in determining MIC by this method, affecting a real antimicrobial activity (13).

3. Radiometric:

BACTEC 460® instrument is a semi-automated radiometric drug susceptibility testing (RAD) method that measures the $^{14}CO_2$ produced by metabolic breakdown of (1-^{14}C) palmitic acid in a liquid Middlebrook 7H12 medium (14,15,16). With this method, multiple concentrations can be tested and an MIC calculated. Other radiometric assay system, which can be employed to screen for inhibitors of mycobacterial growth uses a strain of the rapidly growing saprophyte *Mycobacterium aurum* is used as the test organism. Inhibition of its growth is highly predictive of activity against *M. tuberculosis*, which cannot itself be used in screening because of its growth characteristics and highly infectious nature (17). The major disadvantages of these assays are the cost and the isotope disposal in some countries.

4. Micro broth dilution:

Dilution bioassays have one major advantage over diffusion bioassays, namely the test compound

concentration in the medium is defined. Consequently, dilution assays are regarded as the method of choice to compare MIC values (18,19). The advent of microtitre plates has led to significant reductions in test compound concentrations; furthermore, in combination with spectrophotometric or fluorometric plate readers it is possible to get enormously throughout. An important alternative can be the use of oxidation/reduction indicator dyes such as Alamar Blue (20,21,22) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (23,24), 2,3,5-triphenyltetrazolium chloride (TTC) and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide (XTT) (25), with which, the growth/inhibition can be read visually; and the reduced form of these dyes can also be quantitated colorimetrically by measuring absorbance at 570 nm, or fluorimetrically by exciting at 530 nm and detecting emission at 590 nm; the latter mode is more sensitive.

5. Flow cytometry:

The first experiments, in which flow cytometry was used to study the effects of antimicrobial agents in prokaryotes, were carried out at the beginning of the 1980s (26). In the 1990s, there were interesting and important advances in this field from microbiology laboratories, and consequently, the number of scientific articles addressing at the antimicrobial responses of bacteria (including mycobacteria), fungi, and parasites to antimicrobial agents, were considerably increased (27).

Fluorescein diacetate (FDA) (a nonfluorescent diacetyl fluorescein ester that becomes fluorescent upon hydrolysis by cytoplasmic esterases) staining and flow cytometry were used by Norden *et al*. (28) for the flow cytometry susceptibility testing of *M. tuberculosis*. Pina-Vaz *et al.* stained *M. tuberculosis* in the absence or presence of antimycobacterial drugs with SYTO 16 (a nucleic acid fluorescent stain that only penetrates into cells with severe lesion of the membrane) (29).

6.Reporter gene assays:

Genes encoding luciferase enzymes have been cloned from several species of firefly, beetle, crustacean, bacteria and the sea pansy (30) Other fluorescent proteins such as the red fluorescent protein (RFP) and green fluorescent protein (GFP) has been used, following their introduction in mycobacteria on plasmids, permits the rapid determination of bacterial viability by measuring the expression of an introduced fluorescent or luminescent protein (31,32).

7. High-performance liquid chromatography mycolic acid analysis:

Mycolic acid analysis using HPLC and *p*-bromophenacyl bromide derivatizing reagent for UV detection is a well-established method for identification of mycobacterial strains isolated from clinical specimens (33,34). It was found that a linear relationship between the total area under the mycolic acid (TAMA) chromatographic peaks of a culture of *M. tuberculosis* and log CFU per mL, suggesting the possibility of using TAMA as a good estimator of mycobacterial growth and also as a means of susceptibility testing of *M. tuberculosis* (35).

8.Toxicology assays:

Toxicity is a leading cause of attrition at all stages of the drug development process (36,37). *In vitro*toxicology assays can be divided on the basis of timing and purpose of the application into prospective assays and retrospective assays .Prospective *in vitro* toxicology assays are those assays that are conducted before the first *in vivo* toxicology studies, and attempt to predict toxicities that are development-limiting.

9. Dormant tubercle bacilli assays:

An important problem in tuberculosis control is the persistence of *M. tuberculosis* despite chemotherapy (38). A stage of latency in tubercle bacilli has been found as principle cause for most of the problems associated with the disease (41). There is still no specific drug available in the market, which could effectively kill this latent bacillus (39). The obstacle in the development of novel drugs is caused to the lack of a screening system, which can determine inhibitors of latent bacilli of tuberculosis. Wayne`s hypoxic model is used for *in vitro* evaluation of new compounds, but posses low throughput capability (40). Cho *et al.* (2007) implemented a high-throughput, luminescence-based low-oxygen-recovery assay for screening of compounds against nonreplicating *M. tuberculosis* (42).

10. Macrophages assays:

In vitro models of macrophage infection by *Mycobacterium* spp have been used to assay virulence and the intracellular activity of antimycobacterials. The species *M. tuberculosis* is cytotoxic for macrophages are known; the extent of the toxicity depends on all of the variables cited above. The results of macrophage cytotoxicity are most heavily infected die rapidly and become no adherent (43). The activity of selected compounds against intracellular *M. tuberculosis* can be determined using the murine macrophage cell line RAW 264.7 (ATCC TIB-71) infected with *M. tuberculosis* luciferase reporter strain pSMT1 (44). Measurement of luminescence has shown to provide a rapid alternative to the counting of colonies as a means of evaluate

mycobacterial viability. (45,46,47,48). Tosun *et al.* proposed to consider promising activity in a concentration of 200 µg/ml, but the literature reports that "antibacterial" compounds with MICs values greater than 100 µg/mL, which are poorly active and their clinical perspective has little relevance. (49,50,51).

11. Micro Almar Blue Assay (MABA):

The Synthesised compounds or natural products were evaluated against *M tuberculosis H37Rv* strains following micro almar blue assay (52). Briefly, two hundred microliters of sterile deionized water was added to all outer-perimeter wells of sterile 96-well plates to minimize evaporation of the medium in the test wells during incubation. The 96-well plates received 100 f..lL of the Middlebrook 7H9 broth and aserial dilution of the compounds was made directly on the plate. Plates were covered and sealed with parafilm and incubated at 37 *DC* for 5 days. After this time, 25 mL of a freshly prepared 1: 1 mixture of Alamar blue reagent and 10% Tween 80 was added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth, and a pink color was scored as growth. The MIC (Minimal Inhibition Concentration) was defined as the lowest drug concentration, which prevented a color change from blue to pink

12. Agar Micro Dilution Method:

Drug susceptibility and detennination of MIC of the test compounds/drugs against *M tuberculosis* H37Rv were perfonned by agar micro dilution method where serial two fold dilutions of each test compound were added into 7H 1 0 agar and *M. tuberculosis* H37Rv was used as test organism.(53) MIC was the concentration of the compound that completely inhibited the growth and colony forming ability of *M. tuberculosis.*

13. Cytotoxicity of Selected Compounds:

Cytotoxicity of the compounds can be checked by cell proliferation assay using VERO cells. In the assay numbers of viable cells were detennined colorimeterically with a reagent containing a tetrazolium compound (MTS, Owen's reagent) and an electron-coupling reagent (PES, phenazine ethosulphate). The MTS was bioreduced (by NADPH or NADH produced by dehydrogenase enzyme in live cells) into a coloured fonnazen that was soluble in tissue culture medium. VERO cells (104 cells/welVO.1 mL MEM containing antibiotics and 10% FBS) were seeded in 96-well tissue culture plate. After 24 h incubation the medium was replaced with fresh medium (5% FBS and no antibiotic) containing different concentrations of test compound lknown toxic compound/DMSO. After 24 h incubation MTS reagent (Promega Kit) was added and absorbance was read after 2 h at 490 nm. Absorbance shown by DMSO containing wells was taken as 100% survivors. A compound was considered toxic if it caused 50% inhibition at concentration 10-fold higher than its MIC.

14. Liquid culture method was used for detection of drug resistance:

The method involves 4 ml of media was taken in test tube (54). Then 256 mg product was dissolved, final concentration become 64 mg/ml. One tube without product was inoculated as growth control. M. tuberculosis colony was dissolved in both test tubes. Kept in incubation at 37°C for 10-14 days. Growth control tube was checked for growth by ZN stain. If growth positive in control tube, than test tube was also checked for growth by ZN stain25.

15. Filamenting temperature-sensitive technique:

Filamenting temperature-sensitive mutant Z (FtsZ), a tubulin homologue, is a highly conserved and ubiquitous bacterial cell division protein. Similar to the process of microtubule formation by tubulin, FtsZ polymerizes in a GTP-dependent manner, forming a highly dynamic cytokinetic structure, designated as the Z-ring, at the mid-point of the cell. The recruitment of the other cell division proteins leads to Z-ring contraction and results in septum formation (55). Because of the requirement of FtsZ in mycobacterial cytokinesis, inhibition of FtsZ is a promising target for antitubercular drug discovery. The validation of FtsZ as a novel antitubercular drug target has been confirmed by the work of various groups (56,57).Researchers have screened known tubulin inhibitors against *M. tb* and identified several benzimidazole, pyridopyrazine and pteridine based FtsZ inhibitors with potent antituberular activity (58,59). It is concluded that substituted benzimidazole derivatives interfered and delayed the *M. tb* cell division processes. Benzimidazole derivatives are also reported as potent antitubercular agents. Clubbed-[1,2,3] triazole by fluorine benzimidazoles are also very potent antitubercular agents against *M. tb* H37Rv.(60,61).

SUMMARY AND CONCLUSION:

Synthetic compounds and Natural products will continue to be a major resource for therapeutic products. Until now days, researchers consider them a source of diverse chemical structure that are virtually impossible to replicate in a synthetic chemistry laboratory. The above techniques have been refined into high throughput methods by the application of parallel processing procedures to the chromatographic and analytical steps. Following screening, active fractions are rapidly purified and the structure of the components determined using a rapid and sensitive combination of NMR and mass spectrometry with as little as 50 µg of sample. The continuous development of sensitive, rapid and inexpensive assay materials and detection equipment will ensure that researchers in all institutions are able to

contribute to the development of our understanding and utilization of natural product and synthetic compound resources.

ACKNOWLEDGEMENT:
The authors are thankful to the Vels Institute of Science Technology and advanced studies and management for providing us the necessary facilities and also utility of the library facilty online journal facilities and constant encouragement.

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