Review Article

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Bioprospecting for antituberculosis natural products – A review

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Abstract: There has been an increase in the reported cases of tuberculosis, a disease caused by *Mycobacterium tuberculosis*, which is still currently affecting most of the world's population, especially in resource-limited countries. The search for novel antitubercular chemotherapeutics from underexplored natural sources is therefore of paramount importance. The renewed interest in studies related to natural products, driven partly by the growing incidence of MDR-TB, has increased the prospects of discovering new antitubercular drug leads. This is because most of the currently available chemotherapeutics such as rifampicin and capreomycin used in the treatment of TB were derived from natural products, which are proven to be an abundant source of novel drugs used to treat many diseases. To meet the global need for novel antibiotics from natural sources, various strategies for high-throughput screening have been designed and implemented. This review highlights the current antitubercular drug discovery strategies from natural sources.

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Graphical abstract

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1 Introduction

Tuberculosis (TB), an infectious bacterial disease caused by *Mycobacterium tuberculosis*, has afflicted humans since ancient times. Although significant amounts of efforts have been made in the control of this disease at global and national levels, approximately one-third of the world's population is still infected with *M. tuberculosis* [1,2]. The global resurgence of TB and the development of drug resistance, multidrug-resistant (MDR), and extensively drug-resistant (XDR) strains present significant challenges to the management and control of TB. The alarming increase of MDR-TB cases requires the urgent development of new, more effective, and safer TB chemotherapeutics, which should be several months shorter than the currently adopted regimen [3].

Essentially, natural products constitute an important source of novel chemotherapeutics [4–7]. A huge amount of valuable antibiotics and metabolites of microbial sources have been derived from the soil. There has been a reduction in the considerable efforts made in this area since the late 1980s due to the perception that this resource has been extensively studied [8]. Subsequently, the research focus was switched to unexplored environments such as arid deserts, hot springs, and other unusual habitats for novel chemotherapeutic compounds against human pathogens [9]. Over the

past few decades, there have been reports of about 23,000 bioactive metabolic compounds produced by microbes with over 10,000 of these compounds synthesized by actinomycetes [10–12]. However, only a few plants from over 400,000 plant species with proven biological activity have been reported worldwide [13]. This makes it imperative to identify the unexplored plants' species for drug discovery research. Plant selection, which is the first and most crucial step for such studies, is based on several approaches such as random, ethnopharmacological, ethnobotanical, and traditional uses [14].

However, the need for novel chemotherapeutics still remains of prime concern due to the issue of resistance to multiple chemotherapeutics commonly exhibited by *M. tuberculosis*. It is therefore imperative to continue the search for novel natural products derived from plants as well as microbes from underexplored habitats including marine, estuarine, and even deep seas. Researchers have hypothesized that since these microorganisms could grow optimally in such unusual environments, some of these species produce new bioactive compounds, which apart from aiding their survival, could serve as new drugs [15].

2 Tuberculosis and current anti-TB chemotherapeutics

2.1 Tuberculosis

Tuberculosis is an ancient disease of humans caused by the *Mycobacterium tuberculosis* complex [16]. The disease

is known to be associated with immunosuppression, malnutrition, poverty, and overcrowding and results in the death of more than 1 million people annually around the world [17,18]. The genus *Mycobacterium* consists of several species, some of which are adapted to humans and various animals as shown in Figure 1. Those that cause human tuberculosis (*M. tuberculosis sensu stricto* and *M. africanum*) are obligate human pathogens. Although *M. bovis* and *M. caprae* have been found in several animals and humans, there is rarely human-to-human transmission [16].

M. tuberculosis is a very fastidious bacterium with a typical generation time of 12–24 h when growth conditions are optimal [19]. The bacterium is about 0.8–4 μm in size, acid-fast, non-motile, and aerobic [17]. A sizeable number of individuals will develop latent tuberculosis, which may be well contained in some persons and not progress to TB immediately. However, 5–15% of this group of individuals with latent tuberculosis will progress to active TB [20,21].

Tuberculosis is spread through the inhalation of infectious droplet nuclei that contain viable *M. tuberculosis* [22]. Churchyard et al. [23] gave a simple pathway by which tuberculosis is transmitted. This pathway entails a source case of tuberculosis, generation of infectious particles, survival of particles in the air, inhalation by susceptible individuals, and ultimately potential to develop tuberculosis.

Clinical manifestation of TB is presented in various forms. TB could manifest with mild and non-specific symptoms as observed in primary tuberculosis, which is

Figure 1: Species of *Mycobacterium* and their various adapted hosts. Some might infect multiple hosts but *M. africanum* and *M. tuberculosis sensu stricto* are known to infect only humans.

most often self-resolving. In pulmonary tuberculosis, patients present with night sweats, fever, and weight loss, and about 95% of individuals have non-remitting cough that may be accompanied by chest pain in patients with dyspnoea or subpleural involvement [22]. Besides pulmonary tuberculosis, extrapulmonary TB (EPTB), which is tuberculosis outside the lungs, also occurs. It is characterized by possible weight loss and fever and could manifest as lymphatic TB that involves any regional lymph node, meningeal TB that presents with headache and altered mental status or neurologic defects in advance, and TB of the joints and bones with swelling and persistent localized pain [24].

The paucibacillary nature of EPTB largely contributes to a setback in diagnosis due to lack of highly sensitive and specific testing kits, the emergence of multi- and extensively drug-resistant strains, limited vaccine efficacy, and HIV have exacerbated cases of tuberculosis globally and tend to stall eradication efforts [25].

2.2 Antituberculosis chemotherapies

Although the treatment strategy and guidelines of drug-sensitive TB (DS-TB) have not obviously evolved over the past 35 years, which requires a minimum of six months of treatment with a combination of four basic treatment regimens comprising isoniazid (INH) (1), rifampin (RIF) (2), pyrazinamide (PZA) (3), streptomycin (4) as well as ethambutol (EMB) (5), which are first-line antituberculosis drugs as shown in Table 1. While second-line drugs consist of fluoroquinolones (ofloxacin (6), moxifloxacin (7), levofloxacin

(8), and ciprofloxacin (9)), injectable (capreomycin (10), kanamycin (11), and amikacin (12)) and *p*-aminosalicylic acid (13), ethionamide (14) prothionamide (15) and cycloserine (16) terizidone (17) [26]. Bedaquiline (18) a diarylquinoline, linezolid (19) an oxazolidinone, delamanid (20) and pretomanid (21), which belong to the nitroimidazole class of antibiotics, and clofazimine (22) are newer and repurposed drugs used for tuberculosis treatment some of which are components of shorter-course regimen [27,28]. The treatment of multidrug resistance (MDR) and extensively drug-resistant (XDR) tuberculosis has, however, changed over time in repurposed and new drugs being introduced into the treatment regimens with treatment duration of up to 24 months [29,30]. The treatment of tuberculosis is in phases that consist of the initial phase that kills most of the actively growing bacteria with eradication of clinical symptoms and rapid sputum conversion. This is followed by the continuation phase of treatment that takes care of slow-growing strains or persistent tubercle bacilli [26]. All structures of antituberculosis chemotherapies are shown in Figure 2.

3 Antimicrobial resistance and resistance mechanism in *Mycobacterium tuberculosis*

Presently, TB is becoming the incurable disease it used to be in the past before the discovery of antibiotics due to limited treatment options arising from different levels of resistance including mono-drug resistance, MDR, XDR,

Table 1: Characteristics of first-line antituberculosis drugs

Drug	General information	Effect on <i>M. tuberculosis</i>	Administration
Isoniazid (1)	Hydrazide of isonicotinic acid	It is greatly bactericidal and used against replicating <i>M. tuberculosis</i>	It can be given orally, intramuscularly, or intravenously
Rifampicin (2)	Semisynthetic derivative of rifamycin	It is bactericidal both to actively growing and slow metabolizing <i>M. tuberculosis</i>	It can be administered orally or intravenously. It is always administered to patients along with other effective antimycobacterial agents because resistance to it can be easily developed
Pyrazinamide (3)	Synthetic analogue of nicotinamide	It is weakly bactericidal in the mode of action	It is administered orally. For adults, it is usually administered for the first 2–3 months of TB treatment
Streptomycin (4)	Aminoglycoside	It is bactericidal in the mode of action	It can be administered intramuscularly or intravenously
Ethambutol (5)	Synthetic congener of 1,2-ethenediamine	Ethambutol is bacteriostatic	It is administered orally together with other antimycobacterial drugs to forestall or delay resistance

Information in table adapted from ref. [31].

Figure 2: Structure of antituberculosis chemotherapies.

and total drug resistance exhibited by the pathogen. MDR-tuberculosis implies tuberculosis that is caused by *M. tuberculosis* strain that is resistant to at least both isoniazid (1) and rifampicin (2). While XDR-tuberculosis is caused by strains of *M. tuberculosis* resistant to rifampicin (2), isoniazid (2), one second-line injectable-like amikacin (12), capreomycin (10), or kanamycin (11), and a fluoroquinolone [32]. Drug-resistant *M. tuberculosis* is widespread globally with a continual rise in treatment failures. There are various targets of antituberculosis drugs but a seemingly uniform factor that mediates antimicrobial resistance in *M. tuberculosis* is mutation. Mutations noted in various genes that encode key enzymes, which in some cases transform pro-drugs to active forms as in the case of pyrazinamide (3) and isoniazid (1), contribute to the synthesis of components of the cell wall as with ethambutol (5)

or transcription, as shown in Table 2, have been associated with resistance. Besides mutation, possession of active efflux pumps has also been attributed as a factor that mediates antimicrobial resistance in tuberculosis. Drugs including isoniazid (1) and fluoroquinolones group, streptomycin (4), bedaquiline (18), ethambutol (5), clofazimine (22), and rifampicin (2) are extruded by Mycobacterial efflux pumps.

4 Antituberculosis natural product drug discovery

The need to explore nature (Figure 3) in the quest for newer antituberculosis drugs is imperative since available

Table 2: Mechanism of antimicrobial resistance and antituberculosis drugs

Drug	Mechanism of action	Mechanism of resistance	Reference
Isoniazid (1)	Inhibits mycolic acid synthesis, thereby making <i>Mycobacterium</i> lose its acid-fast nature	Mutation in <i>katG</i> , <i>inhA</i> , <i>kasA</i> , <i>ndh</i> , and <i>oxyR-ahpC</i> have been identified as mediators of isoniazid resistance. Principally, a mutation in the <i>katG</i> gene that encodes catalase and peroxidase results in the loss of ability to convert the pro-drug into the active form	[33]
Rifampicin (2)	Binds to the β -subunit of RNA polymerase, resulting in inhibition of transcription	Mutation in the <i>rpoB</i> gene (81 bp region) results in the amino acid alteration of codons within this region most, especially codon 526 or 531 causes high-level resistance	[34,35]
Pyrazinamide (3)	Pyrazinamide is a pro-drug that is enzymatically transformed to pyrazinoic acid which in turn disrupts membrane energetics and inhibits the membrane transport function	Mutation in the gene <i>pncA</i> that codes for Pzase (pyrazinamidase/nicotinamidase) results in the loss of Pzase activity with concomitant ineffectiveness of the drug	[26,36–38]
Ethambutol (5)	Ethambutol exerts its effect on the cell of <i>M. tuberculosis</i> by interfering with the biosynthesis of the cell wall arabinogalactan	Mutation in genes of the <i>embCAB</i> operon majorly <i>embB</i> and sometimes <i>embC</i> that encodes arabinosyltransferase results in loss of efficacy of the drug	[26,39]
Streptomycin (4)	Streptomycin acts by binding to the 16S rRNA gene where it inhibits protein synthesis	Mutation identified in genes that encodes ribosomal protein S12 (<i>rpsL</i>) and 16S rRNA (<i>rrs</i>) have been linked with streptomycin resistance	[40,41]
Fluoroquinolones group	Fluoroquinolones act by inhibiting bacterial topoisomerase IV and DNA gyrase	Resistance to fluoroquinolones is mediated by point mutations in <i>gyrA</i> and <i>gyrB</i> genes that encode the two subunits of DNA gyrase, consequently, blocking DNA replication	[39,42–44]
<i>p</i> -Aminosalicylic acid (13)	<i>p</i> -Aminosalicylic acid acts majorly against <i>M. tuberculosis</i> through poisoning of folate metabolism	Mutations in <i>thyA</i> , <i>folC</i> , <i>dfrA</i> , and <i>ribD</i> that in turn result in the disruption of the folate pathway have been linked to <i>p</i> -aminosalicylic acid Resistance in <i>M. tuberculosis</i> . Also, possession of an active efflux pump has been indicated in mediating <i>p</i> -aminosalicylic acid resistance	[45,46]
Ethionamide (14)	Ethionamide interferes with mycolic acid synthesis by inhibiting the enoyl-acyl carrier protein reductase enzyme	Mutation in <i>inhA</i> gene mediates resistance to ethionamide	[27]

Figure 3: Bioprospecting strategies for antituberculosis drug discovery.

drugs are plagued with resistance coupled with associated side effects suffered by patients that use them [47,48]. A few compounds such as bedaquiline (**18**) and delamanid (**20**) are new antituberculosis drugs that have been introduced after decades of dependence on older drugs [4].

4.1 Plant-derived antimycobacterial natural products

In many regions of the world, plant-derived anti-TB recipes are well known in folklore medicaments. Plants are an important source of natural products with diverse bioactivities and have the enormous therapeutic potential [14]. Numerous structurally unique bioactive compounds have been isolated from plant extracts [49]. This has driven a lot of drug-discovery research on plants that have previously been known to be used traditionally for the treatment of infections. Infusions of leaves from *Lippia javanica* (Verbenaceae), an aromatic plant, are used in Mozambique as a decoction to treat several ailments like flu symptoms, cough, stomach aches, and many others [48].

Triterpene euscaphic acid was obtained from this herb and found to exhibit antimycobacterial bioactivity against *M. tuberculosis* with a minimum inhibitory concentration recorded as 50 µg/mL [50]. Some compounds such as diospyrin (**23**), shinanolone (**24**), and 7-methyljuglone (**25**) were isolated from the roots of the plant *Euclea natalensis* (Ebenaceae) and displayed significant

activity against drug-sensitive and -resistant strains of *M. tuberculosis* [51]. This plant has been known historically to treat infections associated with chest conditions, toothache amongst other uses, and lends credence to its ethnomedicinal use [52]. *A. afrawas* was also reported to exhibit several biological activities such as antidepressant, antioxidant, cardiovascular, and antimycobacterial, *in vitro* [53]. This plant is commonly available in Eastern and Southern Africa and has been used traditionally to treat respiratory and oro-pharyngeal illnesses such as colds, coughs, asthmatic conditions, to mention a few [54]. Ursolic acid (**26**) and hydroquinone (**27**), which were isolated from *Artemisia capillaris* Thunb. (Asteraceae), a plant that was traditionally known for the treatment of malaria, was assessed for its antimycobacterial activity against MDR-TB strains and was found to have MIC values ranging from 0.0125 to 0.025 mg/mL [55]. Another plant, *Curcuma longa* L. (Zingiberaceae), which has been traditionally associated with the treatment of whooping cough, was assessed for its antimycobacterial potentials. Isoxazole (**27**) analogues of curcuminoids were isolated from this plant and were found to have bioactivity against MDR-TB at MIC values ranging from 0.0019 to 0.00312 mg/mL [56].

Knowltonia vesicatoria (Ranunculaceae), a plant commonly available in South Africa, has been traditionally known for the treatment of tuberculosis. Antimycobacterial drug discovery efforts led to the isolation of 2 compounds, 5-(hydroxymethyl)dihydrofuran-2(3H)-one (**28**) and 5-(hydroxymethyl)furan-2(5H)-one (**29**), with a minimum inhibition concentration of 50.0 mg/mL. Crude extracts of this plant were also used together with isoniazid (INH) (**1**) to determine

whether there would be improved antimycobacterial activity due to probable synergistic effect, thus validating its traditional use in the treatment of tuberculosis [52,57].

4.2 Microbes as sources of antimycobacterial natural products

Microorganisms have been recognized as robust sources of chemotherapeutics. However, their potentials as a repertoire of useful antibiotics have not been fully exploited as only a very limited number of microbial diversities is known globally [58]. The discovery of the compound streptomycin several decades ago (**4**) by Selman Waksman was a major breakthrough in drug discovery as the first effective antibiotic against tuberculosis [59]. Selman Waksman's commitment to the isolation and screening of soil bacteria in the search for bioactive small molecules, which could be potential antibiotics, was validated by the discovery of streptomycin [60].

Fungi have been implicated to exhibit antimycobacterial potentials. The fungus *Mortierella alpine* FKI-4905 isolated from Japanese soil was found to have antimycobacterial activity against *M. tuberculosis* and *M. smegmatis* with MIC values of 12.5 and 0.78 µg/mL [61]. Another natural anthraquinone compound, 4-deoxybostrycin (**30**), was obtained from the mangrove endophytic fungus *Nigrospora* sp., which was isolated from the South China Sea. This compound showed good anti-TB activity *in vitro*. Further studies on the mechanisms of action on *M. tuberculosis* H37Rv showed that 4-deoxybostrycin (**30**) could affect the expression of its genes that are involved in coenzymes, lipids, nucleotides, and energy to mention a few [62]. However, some other researchers [63] explored the antimycobacterial potentials of freshwater actinobacteria isolated from Lake Michigan through screening of a library of compounds derived from aquatic bacteria. A fraction from one of the bacteria (*Micromonospora* sp.) exhibited significant inhibitory activity *in vitro* in comparison to clinically used anti-TB agents. From this strain, two new secondary metabolites, diazaquinomycins H (DAQH) (**31**) and J (DAQJ) (**32**), were isolated and characterized with MIC values of 0.04 and 0.07 µg/mL, respectively, against *M. tuberculosis*. Manikkam *et al.* [64] also isolated actinomycetes from diverse Indian soil ecosystems with a significant number exhibiting antimycobacterial activity.

5 Dereplication strategies for novel anti-TB natural products

Identification of previously isolated or screened microorganisms and their bioactive metabolites, also known as dereplication, remains a major challenge for drug discovery studies. This method provides speedy identification of known bioactive metabolites in complex mixtures using very minimal amounts of crude metabolites, thereby limiting time-consuming isolation procedures that are related to the characterization and screening of an extensive array of known strains [65]. This is usually carried out following preliminary screening in the process of discovering new pharmacologically active substances from natural product mixtures. For this reason, systematic dereplication methods, such as LC-UV-(MS), and combined with high-throughput screening (HTS) for crude extract samples were developed [66]. However, the process of dereplication is not limited to the use of analytical chemistry tools alone. Currently, different strain dereplication strategies such as single-strand conformation polymorphism, rep-PCR, colour grouping including analytical chemistry tools (LC-MS, pyrolysis, MALDI-TOF) have been developed by natural products scientists to assess and thus evaluate the diversity of novel and already known strains. A combination of microbiological assessments, such as identifying producer microbes by morphology and gene analysis, are sometimes used exhaustively together with different bioinformatics techniques [65,67]. Pyrolysis mass spectrometry that involves the use of the whole-cell fingerprinting technique is also used as a dereplication strategy by enabling the rapid and reproducible categorization of microbes by utilizing limited samples in a completely automated system [68]. This method has been successfully adopted previously to discern nitrile-hydrolysing strains of actinomycetes, thus revealing a significant variation within pyrogroups containing strains with identical genotypic characteristics, thereby establishing its discriminatory capacity at the intraspecies level [69].

Other analytical methods such as MALDI-TOF mass spectrometry utilizing intact cells have been adapted for rapid screening for strain dereplication. Dieckmann *et al.* [70] used this rapid screening method for dereplication of bacterial isolates from marine sponges resulting in the efficient clustering of 456 strains hinged on their proteomes, allowing rapid dereplication and identification of unique species.

5.1 Uncultured microbes and novel anti-TB natural products

The number of microorganisms that have been isolated using traditional culturing techniques is less than 1% of microorganisms, making the extensive majority of microorganisms as well as their biochemical metabolic pathways unavailable [71–73]. The metagenomics approach remains promising in drug discovery from uncultured microorganisms. These microorganisms have proven an important source of novel antibiotics through analysis of their DNA isolated from environmental samples [65]. The advent of metagenomic procedures as a culture-independent technique has therefore made various tools available to evaluate the level of the uncultured microbial diversity in full, consequently, enabling access to the biochemical metabolic pathways in these uncultured microbes [74,75]. Culture-independent molecular approach has however confirmed the presence of indigenous actinobacteria through different studies [76–78]. These approaches focus on nucleic acids directly extracted from the samples [79] and typically involve the amplification of DNA from RNA extracted from environmental samples by PCR followed by further analysis of the diversity of the amplified molecules (community fingerprinting) [9]. These amplicons could otherwise be cloned, sequenced, and the rare/novel actinobacteria available in the sample identified and enumerated [80,81]. Monciardini et al. [76] developed selective primer sets for PCR amplification of the 16S rDNA obtained from Actinomycetales families Thermomonosporaceae, Streptosporangiaceae, Streptomycetaceae, and Micromonosporaceae as well as from *Dactylosporangium* sp. Each designed primer, evaluated on the basis of genomic DNA from reference strains, indicated high specificity and sensitivity. However, the use of the primers to process environmental samples indicated the discovery of these groups of actinomycetes in high frequency and also showed sequences that could be ascribed to novel actinobacterial groups [76]. Other molecular approaches such as terminal restriction fragment length polymorphism (T-RFLP) analysis, evaluates the terminal restriction fragments from a PCR-amplified marker based on the size polymorphism [82] and the method links at the minimum, three technologies such as comparative genomics/RFLP, PCR, and electrophoresis. Electrophoresis techniques such as the denaturing gradient gel electrophoresis (DGGE) as well as TGGE (thermal-GGE) are also procedures by which short segments of DNA of different sequences but the same length can be resolved electrophoretically [82]. In this case, separation of DNA fragments leverages on the decreased electrophoretic movement of a double-stranded DNA, which is

partially denatured either by a denaturing reagent or increased temperature and resolved on polyacrylamide gels. The PCR-DGGE/TGGE method was extensively utilized to determine the abundance and diversity of actinomycetes in environmental samples [73,77,82,83].

Metaproteomic and metagenomic technologies enable newer techniques towards gene, genome, protein as well as the discovery of metabolic pathways [72]. The number of yet-to-be-cultured marine microbiota that synthesizes secondary metabolites has greatly reduced accessibility to a vast genetic diversity linked to untapped chemical resources required in therapeutic and other industrial applications [9,84]. This includes complex marine (e.g. sponge, dinoflagellates, tunicates) also terrestrial (e.g. human gut, biofilm, plant-microbe) microbial consortia in which case the presence of huge populations of varying microbiota as well as related genomes that constitute natural product gene clusters yet are unexplored [72,73]. This novel chemical and metabolic diversity resource will help increase fundamental knowledge and make a contribution to current drug discovery efforts against several diseases.

6 Bioassays for evaluating anti-TB compounds

6.1 Agar diffusion method

The agar diffusion test of natural products is typically done on the agar medium surface by placing the filter paper disks previously immersed in extracts on the plate inoculated with the test organism and incubated at 37°C. This enables the test compounds to diffuse into the agar consequently inhibiting the growth of the inoculum. After incubation, the mean diameters of inhibition zones of the test compounds against the inoculum are recorded. The model strain, *M. marinum*, has been used for antimycobacterial natural products screening due to its ubiquitous nature. Diffusion assay is an ideal technique of choice for mixtures of compounds such as essential oils or polar molecules [85]. This method ensures rapid screening of crude extracts using *M. marinum* as a model strain [86,87].

6.2 Agar microdilution assay

This method developed by Golus et al. [88] involves the screening of natural products with different concentrations

against pathogenic test strains in a 96-well microplate containing the agar medium. The assay enables the easy, fast, and cost-efficient sample preparation including the provision of a stable and uniform dispersion of test samples without differentiation of the coloured plant extracts into oil–water phases, which is a frequent occurrence in methods involving liquid media. The agar microdilution method also enables the easy, reliable, and cost-efficient MIC determination of such agents.

6.3 LJ proportion method

This proportion method uses the Löwenstein–Jensen medium (LJ), which is an egg-based medium supplemented with malachite green dye. Incorporation of the antimycobacterial drugs in the LJ medium is done at varying concentrations before the medium is solidified by a process known as inspissation. The solid medium is later inoculated with 100 μ L of the inoculum of up to 1.0 MacFarland Standard and incubated at 37°C for 42 weeks. Natural products are generally not heat-stable, hence this method could serve as a disadvantage if the crude extracts are incorporated into the medium prior to inspissation, which requires heat of up to 80°C. Alternatively, the natural product could be added to the surface of the solid medium after inspissation and cooling. Although the advantage of using this method is its cost efficiency, particularly in resource-limited laboratories that are unable to afford more expensive culture media, the disadvantage of using this method is the long period of incubation to assess the resistance or susceptibility of the strain to the compounds through growth detection [86].

6.4 Micro-broth dilution assay

Bioactivity screening of natural products with the use of a 96-well microplate offers the advantage of minimal sample requirements, reduced cost, and high-throughput processes, including the potential for automation [89–92]. This method involves the growth of mycobacteria in growth media such as Middlebrook 7H9 broth supplemented with growth supplements including oleic acid, albumin, dextrose, and catalase. The growth of several mycobacterial strains could be determined quantitatively in a liquid medium by turbidity but a major drawback in this test is the possibility of mycobacteria clumping together. The metabolic compounds or extracts derived from natural products may also confer some turbidity on the culture medium thereby serving as a challenge in the determination of

results. The use of an indicator dye that works with the principle of oxidation–reduction such as Alamar blue, resazurin, tetrazolium, malachite green, and crystal violet, makes the micro-broth dilution a technique of choice for speed and sensitivity [93,94].

6.5 Radiometric BACTEC 460 assay

This assay determines the inhibition or growth of *M. tuberculosis* through the measurement of $^{14}\text{CO}_2$ generated by *M. tuberculosis* cultivated in broth containing ^{14}C -labeled palmitic acid. This liquid growth medium is Middlebrook 7H12 medium, and mycobacterial growth is measured using the BACTEC 460 instrument for HTS assay and the results can be observed in less than 2 weeks [95]. The percentage inhibition of $^{14}\text{CO}_2$ production relative to drug-free controls could be extrapolated, or large numbers of extract concentrations could be screened and their MIC values determined [92,96,97]. Results are recorded usually at 24 h intervals, thus, enabling accurate evaluation of growth kinetics or inhibition of the test mycobacterial. The main disadvantage of this assay is the cost such as in disposal of the isotope in certain countries as well as the substantial amount of growth medium required, which consequently utilizes a large quantity of samples for the assay [86].

6.6 BACTEC MGIT 960 assay

This assay requires the use of modified Middlebrook 7H9 broth containing a fluorescent sensor ruthenium chloride pentahydrate as a growth medium in a non-radiometric automated system. The method is an excellent, high-throughput procedure, which is advantageous in terms of provision of very fast and reliable results in less than 2 weeks. [55]. However, a major disadvantage to this method is the high costs as well as large volumes of the medium required to run the assay, which consequently requires a large amount of the test sample (metabolic extract) to be screened.

6.7 Reporter gene assays

These assays are generally centred on the use of reporter genes associated with the red fluorescent protein, luciferase, and green fluorescent protein. The proteins are typically derived from different species of insects (fireflies), crustaceans, beetles, and bacteria (*Vibrio harveyi*)

and allow the rapid determination of bacterial viability, which is extrapolated by expressing a luminescent or a fluorescent protein [92,98]. The assay can be applied in a multiwell format, which brings about improved high-throughput detection. A major advantage in the use of the reporter gene assays is its cost-effectiveness as it is inexpensive for routine use in the HTS of antitubercular compounds with minimal commercial applications [99,100]. The major disadvantage of using this method is that its use for commercial applications is usually restricted by patent challenges; hence, limiting the number of mycobacteriology laboratories adopting this method for susceptibility screening of natural products against *M. tuberculosis* [92,98].

6.8 Wayne's hypoxia model

For decades, latent tubercle bacilli of *M. tuberculosis* have continued to challenge TB therapy efforts, which has become a cause for concern thereby resulting in prolonged treatment of active TB. The currently available anti-TB drugs are ineffective against the dormant forms of *M. tuberculosis* and the fact that there was no screening bioassay for chemical molecules that have activity against dormant tubercle bacilli has been an impediment to the development of novel chemotherapeutics against latent TB [92]. A model was therefore developed by Wayne and Sramek [101] as the first *in vitro* model for *Mycobacterium tuberculosis* for a specific time frame (usually 24 days), thereby inducing hypoxic conditions thus leading to the sensitivity of hypoxia-induced latent bacilli for metronidazole with the introduction of a single stress factor.

6.9 The rapid anaerobic model

Some other modified versions of the Wayne model that are aimed to yield more consistent and accurate results have been developed such as the rapid anaerobic model [102,103]. The major difference between this model and the Wayne model is the size of stir bars, which are larger, as well as the much faster stirring rate in others to increase the homogeneous population of bacilli, and oxygen distribution within the culture. The available oxygen is depleted rapidly ensuring anaerobiosis within a limited period in comparison with the Wayne model [104].

6.10 Betts starvation model

A nutrient starvation model, which involves the simulation of *M. tuberculosis* in physiologically dormant states and the introduction of one stress factor, was developed by Betts et al. [105]. The procedures require the transfer of bacilli into a nutrient-deficient medium followed by incubation at 37°C for 6 weeks [105]. In this model, the rate of respiration is used to inhibit the growth of the test strain. Ultimately, this model provides evidence for deceleration of the energy metabolism, transcription apparatus, lipid biosynthesis, and cell division including induction of the stringent response and many other genes that may lead to long-term survival within the host.

6.11 Low-oxygen recovery assay (LORA)

The search for novel antimicrobials through screening for bioactivity is usually carried out using microbes that are actively replicating. It has however been established that antimicrobial tolerance in various bacterial infections is a result of a physiological state of non-replicating persistent (NRP) dormant forms of *M. tuberculosis*. For the treatment of tuberculosis, the possibility of shortening the typical 6-month regimen is highly dependent on the NRP subpopulation. LORA is an assay developed for the bioactivity of compounds against non-replicating *M. tuberculosis* utilizing an *M. tuberculosis* pFCAluxAB strain. This luminescence-based assay uses high-throughput measures to screen test bioactive compounds against NRP forms of *M. tuberculosis* [106,107]. In this assay, the MICs of antimicrobial agents are evaluated using the microplate method [106]. This is a multiple stress model that works by applying a combination of stress factors including high CO₂ (10%), low oxygen (5%), low acidic pH (5.0), and nutrients. The advantage of adopting this model is its efficiency in generating bacilli that meet all criteria of dormancy, and this multiple stress method is easily applicable to HTS of natural products [108,14].

6.12 A low pH and nutrient stress assay

The need for easy and more rapid assessment of novel anti-tubercular agents against non-replicating bacilli facilitated the development of a method for the detection of the bactericidal activity of new bioactive natural compounds

within 7 days by Early *et al.* [109]. This method utilized the incubation of *M. tuberculosis* strains at sub-optimal pH, bringing about induction of a non-replicating state. Antimicrobial susceptibility testing of bacteria against compounds is determined by the evaluation of luminescence. The advantages of this simple assay are that it does not require an outgrowth period, and results are easily extrapolated with minimal manipulations, which could be adapted for bactericidal activity screening of natural products against non-replicating *M. tuberculosis*.

6.13 Anti-TB *ex vivo* assays

This procedure involves the use of mouse/human macrophages (usually THP-1 cells, J774 cells), which evaluates the intracellular *M. tuberculosis* killing or sterilizing activity of test compounds [14]. In most cases, macrophages are infected with *M. tuberculosis* with a multiplicity of infection (MOI) of 10, and intracellular anti-TB activity is determined by exposure to test compounds for 3–7 days. The antimycobacterial effects of the test compounds are typically monitored by determining the bacterial load within macrophages by lysing them with hypotonic buffer (1.5 mM MgCl₂, 10 mM HEPES, and 10 mM KCl). The samples are spread on Dubos agar plates at varying time intervals and colonies are enumerated after incubation for 21 days. The bacterial viability is determined by the enumeration of colony-forming units [110–112].

7 Current approaches to the discovery of novel natural products

Application of natural products in the drug development pipeline for eventual use typically undergoes many challenges including (1) lack of elucidation of biological mechanisms, (2) difficulty in isolation of purified chemical compounds, (3) limited standardization procedures, and (4) documented clinical trials [113]. A combinatorial approach with the aid of modern technologies such as computational biology techniques, quantum computing, big data, profiling techniques, and artificial intelligence have been currently adopted to exploit the therapeutic properties of natural products [113,114]. Due to the complex nature of crude metabolic extracts of natural products, it is imperative to utilize some efficient strategies

towards the removal of impurities that interfere with screening processes [115]. These extraction and fractionation methods have aided the drug discovery process leading to optimum yield for hit drug leads [116]. The technologies adopted include microwave-assisted and molecular distillation methods [117], ultrasonic- and enzyme-assisted extraction, semi-bionic extraction [118], and supercritical fluid extraction [113].

8 Future prospects and conclusions

Natural products continue to remain a major resource for chemotherapeutic agents aimed to combat tuberculosis. Only recently have researchers considered them a source with diverse chemical structures whose structural compositions could be used as scaffolds for replication in synthetic chemistry. However, the minimal success rate of novel drug discovery from natural products requires a fundamental change of innovative drug development strategies. Several techniques have been refined and adopted as high-throughput strategies for efficient screening and dereplication to ensure isolation of novel drug leads and undoubtedly contribute to the supply of novel compound libraries through well-developed high-throughput screening programs. However, the continuous development of cost-efficient, sensitive and rapid assay materials has become imperative and this would consequently encourage scientists globally to contribute to the development of natural product resources. Therefore, to address global health challenges in the fight against drug-resistant tuberculosis, there is a need to channel more efforts in the search for novel antitubercular drugs from unique natural sources in order to achieve sustainable development goals on health.

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