# Bioprospecting for Antibacterial Drugs: A Multidisciplinary Perspective on Natural Product Source Material, Bioassay Selection and Avoidable Pitfalls

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**Suggested running headline**

Bioprospecting for antibacterial drugs

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**ABSTRACT**

Bioprospecting is the exploration, extraction and screening of biological material and sometimes indigenous knowledge to discover and develop new drugs and other products. Most antibiotics in current clinical use (eg. β-lactams, aminoglycosides, tetracyclines, macrolides) were discovered using this approach, and there are strong arguments to reprioritize bioprospecting over other strategies in the search for new antibacterial drugs. Academic institutions should be well positioned to lead the early stages of these efforts given their many thousands of locations globally and because they are not constrained by the same commercial considerations as industry. University groups can lack the full complement of knowledge and skills needed though (eg. how to tailor screening strategy to biological source material). In this article, we review three key aspects of the bioprospecting literature (source material and *in vitro* antibacterial and toxicity testing) and present an integrated multidisciplinary perspective on (a) source material selection, (b) legal, taxonomic and other issues related to source material, (c) cultivation methods, (d) bioassay selection, (e) technical standards available, (f) extract/compound dissolution, (g) use of minimum inhibitory concentration and selectivity index values to identify progressible extracts and compounds, and (h) avoidable pitfalls. The review closes with recommendations for future study design and information on subsequent steps in the bioprospecting process.

**KEYWORDS:** drug discovery; natural products; ecology; ethnomedicine; biolaw

**ABBREVIATIONS**

AAF-R110: bis-alanyl-alanyl-phenylalanyl-rhodamine

ATCC: American Type Culture Collection

BrdU: bromodeoxyuridine

CBD: Convention on Biological Diversity

$CC_{50}$: half-maximal cytotoxic concentration

CFU-GM: colony forming unit granulocyte/macrophage

CLSI: Clinical and Laboratory Standards Institute

CO-ADD: Community for Open Antimicrobial Drug Discovery

DMSO: dimethyl sulfoxide

EdU: ethynyl deoxyuridine

ELC: Eli Lilly and Company

ELISA: enzyme-linked immunosorbent assay

EMA: European Medicines Agency

EUCAST: European Committee on Antimicrobial Susceptibility Testing

EURL: European Union Reference Laboratory for Alternatives to Animal Testing

FDA: US Food and Drug Administration

GF-AFC: glycylphenylalanyl-aminofluorocoumarin

$HC_{50}$: half-maximal hemolytic concentration

HTD: highest tolerated dose

$IC_{50}$: half-maximal inhibitory concentration

ICTAM: International Cooperation on Alternative Test Methods

ISO: International Organization for Standardization

MAT: mutually agreed terms

MBC: minimum bactericidal concentration

MIC: minimum inhibitory concentration

NBD-PE: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine

NCTC: National Collection of Type Cultures

NIH: National Institutes of Health

NP: Nagoya Protocol

OECD: Organisation for Economic Co-operation and Development

OSMAC: one strain – many active compounds

PAIN: pan-assay interference

SAR: structure-activity relationship

SRB: sulforhodamine B

TLC: thin layer chromatography

**OUTLINE**

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## INTRODUCTION

Whilst antibiotic resistance continues to emerge and spread, the number of new antibacterial drugs being approved for use is declining. Linezolid (an oxazolidinone), daptomycin (a lipopeptide), bedaquiline (a diarylquinoline) and lefamulin (a pleuromutilin) represent the only new classes of systemic antibiotic introduced in the last 20 years [with the discovery of oxazolidinone (1) and pleuromutilin (2) antibiotics actually dating back much further]. Declining productivity has been attributed, in part, to an over-reliance on synthetic chemical libraries and sub-cellular target-based screening for drug discovery. Synthetic 'Lipinski-like' molecules, favored for their amenability to hit-to-lead optimization for other medical conditions, are now recognized to penetrate the bacterial cell envelope poorly (3, 4). Also, engineering cell permeability into inhibitors identified by *in vitro* or *in silico* target-based screening has proven more difficult than anticipated (5, 6). Commercial factors are at play too. Antibiotics, because they are typically used in short courses of treatment and can elicit resistance, provide a smaller, more risky return on investment than other drugs, and many multinational pharmaceutical companies have withdrawn from antibacterial research (7, 8).

During the 'golden age' of antibiotic discovery (1940 to 1970), the templates for most of the new drug classes (the aminoglycosides, tetracyclines, amphenicols, polymyxins, macrolides, pleuromutilins, glycopeptides, rifamycins, lincosamides, streptogramins and phosphonic acid antibiotics) were identified by screening natural products for activity against whole bacterial cells (9, 10). The discovery of the nitroimidazole and quinolone antibiotic classes was less straightforward, but can be traced back to natural products also (azomycin and quinine, respectively) (11, 12). Because natural products are natural metabolites, they are more likely than synthetic compounds to be substrates for the transporter systems that facilitate their entry into the bacterial cell (13, 14). Natural products are also more architecturally complex than synthetic chemicals, their many chiral centers, ring fusions and functional groups permitting greater specificity towards biological targets (15, 16). Whole cell screening, used exclusively until the 1980s (5), is very effective as an antibiotic discovery strategy too. In addition to identifying only

those compounds capable of penetrating bacterial cells, this approach is more likely to identify multitarget inhibitors (17), decreasing the ease with which bacteria can evolve antibiotic resistance (5, 18).

With Big Pharma reducing investment in antibacterial research, other sectors are attempting to fill the void (1, 19). The biopharmaceutical/biotechnology sector is an important example, and several companies (including Adenium Biotech, Motif Bio, NovaBiotics, Paratek Pharmaceuticals and Spero Therapeutics) now have drug candidates in clinical-stage development (20, 21). Drug research has also increased in academia, but is held in low regard by many in industry (22). Despite its considerable collective expertise and an unparalleled level of access to the natural resources so important for antibacterial drug discovery, individual research groups can lack the full complement of knowledge and skills needed to identify progressible compounds (23). One problem in particular is the frequent mismatch between natural product source material and screening strategy (24). To begin to redress these issues, we provide here an integrated multidisciplinary perspective on three key aspects of the current bioprospecting literature. Information on natural product source material and *in vitro* methods of antibacterial and toxicity testing are critically reviewed by specialists in ecology, ethnomedicine, biolaw, taxonomy, natural product chemistry, microbiology, pharmacology and drug development. Avoidable pitfalls, both legal and scientific, are described throughout the text and summarized at the end. *In vivo* testing is beyond the scope of this review, but useful methods such as the *Caenorhabditis elegans* rescue assay (25) should not be overlooked when designing bioprospecting programs.

## NATURAL PRODUCT SOURCES

### Source selection

Our planet is home to an estimated 1 to 6 billion species (26), of which many different sub-species and strains can exist. Given the limited pool of (often public or philanthropic) funding available to screen these organisms, it is incumbent on us to prioritize those sources most likely to yield therapeutically

useful compounds. Ecological, ethnomedical and historical information can all be used to predict which organisms might produce antibacterial compounds (Supplementary Table 1). Coprophilous fungi, for example, are screened on the basis that they compete with bacteria for a limited nutrient supply, and this creates a selection pressure for antibiotic production (27, 28). Medicinal plants are screened on the basis that they have traditionally been used to treat bacterial infection and may produce antibacterial compounds (29), and soil bacteria are screened on the basis that they have historically been a rich source of antibacterial drugs (17). Attempts can also be made to predict which sources are most likely to yield compounds selective in their toxicity (Supplementary Table 1). If, for example, a medicinal plant has been used for many years without reports of adverse effects, this might suggest its constituents will be safe and tolerable in humans (30). The same can be argued for products obtained from commensal and mutualistic species of the human microbiota (31, 32), human cells (33), and, to a lesser extent, the microbial symbionts of other eukaryotic organisms (34, 35). Such rationales do, of course, have their limitations. Ecological relationships are complex and theories must sometimes be revisited (36, 37), and the safety and efficacy attributed to traditional medicines are not always borne out by *in vivo* studies (38) or clinical trials (39).

Consideration can be given to other factors too (Supplementary Table 1). Prokaryotic diversity exceeds that of the eukaryotes (13) and, if the structural diversity of their natural products parallels this, then the likelihood of finding useful compounds from these organisms will be greater. The extent to which a habitat has been screened previously and whether or not it is aquatic is also relevant. Underexplored ecosystems (eg. deserts, caves, seas, oceans, permafrost soils, plant and animal microbiotas) will more readily yield novel organisms and novel natural products than those that have been heavily screened (35, 40-44), and the diluting effect of aquatic environments may, for secreted compounds, create a selection pressure for greater potency (45). Genome mining techniques such as the antiSMASH algorithm can be used to predict which organisms are likely to produce novel antibiotics too (46, 47). Lastly, source selection should take into account the facilities available at the research

institution. For example, the case for soil actinomycete screening is strong in settings with high throughput screening (17), but where this is absent it may make more sense to test organisms that can be rationally selected (species by species) based on ecological, ethnomedical and/or genomic considerations. The type of taxonomists (microbiologists, botanists, zoologists etc.) and repositories (culture collections, herbaria, zoological museums, cell banks etc.) available is pertinent too.

**Source collection (including legal issues)**

Under the United Nations 'Convention on Biological Diversity' (CBD; ratified in 1993), countries have sovereign rights over the genetic resources in their territories. Prior to collecting biological material from other countries, researchers must therefore obtain informed consent from the source country. This process involves agreement on how any benefits from the subsequent research will be shared with the source country in a fair and equitable way (48). The 'Nagoya Protocol' (NP) on Access and Benefit-sharing, a supplementary agreement (ratified in 2014), provides further clarification and extends the rights of the source country to any traditional knowledge associated with their national biota. In addition to providing source countries with legal recourse in the event of biopiracy, the above treaty seeks to facilitate access and research through the establishment of essential infrastructure, legal certainty for investment, etc. (49). An online platform, the 'Access and Benefit-Sharing Clearing-House', has been developed by the Secretariat of the Convention on Biological Diversity to connect providers (source countries) and users (researchers) (50). Publicly funded biobanks have also emerged as a means of facilitating access (51).

Collection of biological material from countries that have not signed the CBD or NP may be governed by national laws (52). Collection of biological material from international waters and territories is not covered by the CBD or NP, but by other treaties such as the United Nations 'Convention on the Law of the Sea' (Part XIII; ratified in 1994) (53) and the 'Antarctic Treaty' (Protocol on Environmental

Protection; ratified in 1998) (54). Collection of biological material within one's own country may also require formal permits if, as in the case of nature reserves or rare species, the land or species is protected (52, 55) or, in the case of human or animal samples, specific legislation is in place (56).

When biological material will be studied in a different country to where it was collected, researchers must ensure they have legally obtained the material under the appropriate legislation or regulatory requirements. With material obtained from countries that are signatories of the CBD and NP, for example, researchers must obtain 'prior informed consent' for the work they propose to do. Also, the provider country and researchers should arrange 'mutually agreed terms' (MAT) including any monetary and/or non-monetary benefits that they may share. For any subsequent work with the biological material not described in the original MAT, either by the original researcher or another research group, the terms and conditions of benefit sharing must be renegotiated with the provider country. Lastly, for researchers not arranging collection of source material directly but receiving this from collaborators, it is important to request a 'material transfer agreement' to ensure all material has been collected and will be used in compliance with the appropriate laws (57, 58).

Other advance planning is also needed. Contact with a repository curator should be made (with non-disclosure agreements in place if necessary) prior to collection to ensure sufficient biological material and supporting information is obtained for formal identification, voucher deposition (59-61), and/or any other biobanking requirements (56). Repositories, incidentally, should not be overlooked as a source of biological material themselves. Many microbial culture collections have not been systematically examined for bioactivity, and are becoming increasingly popular for large screening programs (52). An advantage of this approach is that microbial strains collected by the repository prior to ratification of the CBD (ie. strains pre-dating 29 December 1993) will be available without the regulatory restrictions described above. Ethical approval is required for research with higher animals (vertebrates and cephalopods) and, for all animals, consideration should be given to anesthesia, analgesia and/or euthanasia (62, 63). Collecting biological material from humans, in addition to requiring ethical approval,

requires informed consent (56, 64). As with any research, a health and safety assessment is essential (both for organisms that will likely be encountered during collection and processing and any chemicals used) (56, 63). Because intra-species variation and environmental factors can affect what chemical entities an organism produces, consideration should also be given to the collection of different sub-species and strains of the same organism (65, 66) and collection from different locations at different times (65, 67). Lastly, some organisms (eg. bacteria, fungi, algae) can lose viability within 1 to 24 hours of sampling (66, 68), may share habitats with faster growing species (28, 69) and/or will undergo genetic drift during passaging, so appropriate plans must be in place for transport, isolation, culture and storage [eg. specialized transport containers (70, 71), pre-prepared plates of selective media (68), facilities for dilution-to-extinction culturing (28), gravity separation (66) and other types of pretreatment (41, 72), and facilities for multi-temperature culture (41) and long-term preservation (73)].

**Confirmation of source identity and deposition of vouchers**

Confirmation of species identity by a qualified expert is essential if the subsequent research is to be accurate and reproducible. Chemical and pharmacological analyses will be a waste of time and money if the source material has been misidentified and, worse still, could mislead other researchers (65). The deposition of vouchers (Fig. 1) with a permanent, curated repository is also important (61, 65). A voucher is defined as "a specimen, a sample, or product thereof, and its associated data, that documents the existence of an organism at a given place and time in a manner consistent with disciplinary standards" (75). Deposits must be to publicly accessible repository collections if a new species or subspecies is being reported, whereas confidential deposits to an International Depositary Authority are recommended if there is a need to protect intellectual property through patent application (52, 76). With primary vouchers, there must be sufficient material to physically and visually document the existence of the organism (75). Guidelines differ between and within taxonomic groups but, by way of examples,

botanical specimens (incl. roots, stems, leaves, flowers and/or fruits) are pressed, dried and mounted on acid-free paper (65, 77), and entomological specimens are fixed (in cyanide, ethyl acetate or ethanol), then chemically-, critical point- or freeze-dried, and pin-, point- or card-mounted with their larvae fixed (in boiling water) and preserved in ethanol (60, 61). Associated data includes information such as scientific name, taxonomic authority, habitat, species size, population size, georeference, collection method, name(s) of the person(s) who collected and identified the species, date and time of collection, date of preservation (60, 61), and details of any collection permit or formal agreement with a landowner (52, 59). A primary voucher enables species identity to be re-appraised if there is difficulty reproducing a piece of work (eg. not being able to isolate the same active constituents) or reassigned in the event of a taxonomical revision (eg. the division of one species into two) (65). Secondary vouchers are products derived from the organism that provide supplementary information (75). In the case of organisms used in traditional medicine, a secondary voucher (properly cross-referenced against a primary voucher) might include a specimen(s) showing ethnobiologically important features (eg. juvenile leaves of edible herbs), together with a record of both the scientific and common name, cultural characteristics (in the original language and phraseology), etc. Vouchers in ethnobiological studies bridge the gap between folk knowledge and science (59). Repositories assign each voucher a unique accession number and this number, together with the names of the repository and taxonomist, should be included in any subsequent documents or publications describing work with the organism (59, 61). Although the practices described above are not always observed (compliance can be less than 10% for some taxonomic groups) (61), this is likely to change as the number of scientific journals rejecting submissions without these details increases (77).

**Preparation prior to extraction**

Whilst some organisms can be obtained in quite large quantities at the collection site (eg. plants or plant material), others may need to be cultivated to obtain sufficient quantities for extraction. This can be challenging for some species. For example, endophytes often have such a close relationship with their host plant species that it is necessary to culture them with freshly harvested plant tissue to achieve optimal growth (68). Even if a routine cultivation method has been established for an organism, it is worth considering alternative options. With fungi, potato dextrose and malt extract media are generally sufficient for maintaining laboratory cultures, but organisms synthesize few bioactive compounds in these media because of their low protein content (28). Ideally, multiple growth conditions should be tested as the profile of chemical compounds produced can vary depending on the mode of nutrition (autotrophic, heterotrophic or mixotrophic) (66), the nutrients supplied (66, 78, 79) or withheld (80, 81), whether the medium is solid or liquid (28), and factors such as pH (80, 82), temperature (79, 80, 82), salt concentration (83), the degree of aeration (78, 79), light intensity (78, 82) and growth phase (79). In microbiology, this is known as the "one strain – many active compounds" (OSMAC) phenomenon (28). Culture conditions (eg. culture medium, supplements, $O_2$ concentration) affect the compounds produced by mammalian cells too (33, 84). For the reasons above, consultation with someone experienced cultivating the organism or culturing the cells under investigation is generally recommended (28, 66). The development of innovative cultivation methods such as the iChip is also encouraged to increase the number of species capable of being cultured (40, 85).

When cultivating organisms or culturing cells prior to extraction, another consideration is whether pathogen attack, competition for resources, or other stresses can be simulated. Plants produce some of their antibacterial compounds constitutively (phytoanticipins), but others are only produced in the event of an infection (phytoalexins) (86). Likewise, insects produce some of their antimicrobial peptides constitutively (eg. stomoxyn), but others are only produced following an encounter with an invading pathogen (eg. attacins) (87). A similar pattern of results is observed when two microorganisms are

competing for nutrients (88). With *Aspergillus nidulans*, for example, polyketide production is only triggered in the presence of competing bacteria (88). Even when antibacterial compounds are produced constitutively, synthesis may be occurring at low levels if infection is absent (eg. cathelicidin LL-37 production by human mesenchymal stromal cells) (84). Such regulation is thought to have evolved as a means for organisms to minimize unnecessary or unbeneficial production of energetically costly antibacterial compounds (80, 89). Because it is desirable to detect and identify the full range of constitutive and inducible antibacterial compounds an organism produces, various methods have been developed to induce or simulate infection and competition. This can be achieved in plants by treatment with immune elicitors such as salicylic acid (89, 90), in insects by injection of entomopathogens or their components (eg. peptidoglycan, lipoteichoic acid, lipopolysaccharide) (91), in cells from higher animals (eg. cultured bovine, murine and human mesenchymal stromal cells) by co-incubation with bacterial products [eg. lipopolysaccharide (92), exotoxin (93)] or inflammatory cytokines [eg. interferon-γ, interleukin-12 (94)], and in bacteria by co-incubation with another organism (88, 95) or its products [eg. low concentrations of antibiotic (96, 97), siderophore or ionophore (34, 98)]. In addition to the biotic stressors above, abiotic stressors such as rare earth elements, heavy metals and gamma and ultraviolet radiation can be used to stimulate the expression of silent or less-active biosynthetic pathways (99, 100) or increase constitutive antibacterial production (84, 101).

Advance consideration should be given to what part(s) or product(s) of the organism will be used for extraction. With microorganisms, bioactive compounds are often exuded rather than stored intracellularly, so it is necessary to extract not just the microbial cells but the medium in which they have been cultured (102). With plants, it has been suggested that subterranean organs (eg. bulbs, roots) may be more likely to produce antibacterial compounds given their proximity to bacteria (incl. pathogenic bacteria) present in the soil (103). Also, some bioactive compounds can be limited to a single plant organ (104). Consideration should be given too to whether or how the raw material will be processed prior to extraction. Following harvest or euthanasia, degradation of constituent chemical compounds can occur

by the action of enzymes from the organism itself (eg. polyphenol oxidases in the case of plants) or by the action of enzymes from contaminant saprotrophs (eg. proteases in the case of fungi) (105), so raw material should be processed without delay. To minimize contamination, organisms such as plants and higher fungi are washed or gently brushed to remove soil and other debris (102). Also, to minimize enzymatic degradation of the constituent compounds, raw material is sometimes frozen (102), freeze-dried (66, 102), sun-dried (106) or oven-dried (102). Freeze-drying can be used to concentrate compounds too if, as in the case of secreted human or animal peptides or microbial products, they are present in culture media in low concentrations (33, 102). If the raw material is large in size or volume, it is advisable to first divide it into small pieces or aliquots to promote rapid and homogenous freezing and/or drying (102). Some compounds undergo structural alteration when exposed to heat or light and, if these are present, fresh, frozen or freeze-dried material will yield a different profile of chemical compounds to material that has been oven- or sun-dried (106, 107). Where available, ethnomedical information (108, 109) and knowledge of the classes of chemical compound likely to be present (102) should be used to guide the above decisions. For short-term storage, the frozen and/or dried material is sealed in a container, protected from light and put in a cool dry place (102). Long-term storage is not recommended (110).

## SCREENING FOR ANTIBACTERIAL ACTIVITY

When bioprospecting for a specific type of activity, bioassays are usually run in parallel to extraction and separation so that the isolated compounds will be not just new but active (111). Antibacterial screening may be classified according to a number of characteristics. Firstly, it may be classified according to the number of samples (compounds or extracts) tested per day, typically ‘high-throughput’ when 10,000 to 100,000 samples are examined per day, ‘medium-throughput’ when 1000 to 10,000 samples are examined per day, or ‘low-throughput’ when less than 1000 samples are examined per day (112). Screening may

also be classified as being either ‘whole-cell’ or ‘sub-cellular’ depending on whether sample activity is assessed against whole bacterial cells or a single molecular target such as an enzyme. Lastly, antibacterial screening can be classified according to the type of activity being detected. This may be direct antibacterial activity (ie. bacterial growth inhibition or bacterial killing), synergistic activity (ie. reduction of intrinsic or acquired resistance to an existing antibiotic), or antivirulence activity (ie. inhibition of a process that contributes to bacterial pathogenesis but is not required for bacterial growth or viability). In this review, we will focus on low-throughput antibacterial screening as medium- and high-throughput screening have been comprehensively discussed in excellent reviews by Fallarero *et al.* (112) and Niu and Li (113). We will focus on whole-cell rather than sub-cellular screening as this identifies only those compounds capable of either penetrating the cell envelope or exerting their antibacterial effect from outside the cell. Lastly, we will focus on assays of direct antibacterial activity rather than synergistic or antivirulence activities. Assays of synergistic activity often generate discrepant results (114) and, because pathogenesis is a multi-step process that varies between bacterial species, assays of antivirulence activity are so numerous and diverse they would justify a separate review in their own right.

**Antibacterial assays available and rationale for their selection**

Broadly speaking, two categories of assay are available for examining natural products for direct antibacterial activity – those that detect activity (ie. diffusion-based, bioautographic, and cell morphology-based) and those that quantify activity (ie. agar dilution, broth macrodilution, and broth microdilution). The use and description of diffusion-based techniques as semi-quantitative assays is, for bioprospecting purposes, almost always inappropriate (115). Disk diffusion can validly be considered semi-quantitative in a diagnostic laboratory setting, where the susceptibility of an unknown bacterial strain (a clinical isolate) to a known chemical compound (eg. an FDA- or EMA-approved antibiotic) is approximated based on foreknowledge of the compound’s diffusion characteristics in the assay (116,

117). In a bioprospecting laboratory, it is the chemical compound(s) (in the form of an extract or isolated constituent) that is being characterized rather than the bacterial strain, and no foreknowledge of the diffusion characteristics of this compound(s) exists. Because weakly antibacterial compounds that are small, polar and/or anionic diffuse through bacterial growth medium more quickly and can generate larger zones of inhibition than potently antibacterial compounds that are large, nonpolar and/or cationic, the activity of test compounds cannot reliably be approximated or compared based on the size of these zones of inhibition (115, 118). The only circumstance in which diffusion-based assays can validly be considered semi-quantitative in a bioprospecting laboratory is when the susceptibility of different (often paired) bacterial strains (eg. a drug-resistant mutant and its parent strain, or an overexpressed target strain and its parent strain) to a single extract or compound are being compared for the purpose of dereplication or target identification (119).

Both non-quantitative and quantitative assays can be used to guide the isolation of antibacterial compounds, and the category selected depends on the strategy of the research group and the resources available to them. If the priority is to isolate active compounds from source material as quickly as possible, and the research group has access to the human and technological resources to accomplish this, then an assay that detects rather than quantifies antibacterial activity can justifiably be used. If the priority is not to isolate compounds that are merely active but to isolate compounds that are sufficiently potent to justify further investigation, then a quantitative antibacterial assay should be used. Quantitative assays should also be used if a research group does not have the resources to isolate and identify active compounds because, without information on potency, any report describing antibacterial extracts is unlikely to generate sufficient interest for the species to be investigated further. Lastly, regardless of the category of assay(s) used to guide the isolation of antibacterial compounds from crude extract, the activity of those isolated compounds should be quantified.

Three key attributes both the above categories of assay should have are that they do not generate false negative results (eg. the assay is sufficiently sensitive to detect antibacterial compounds present at

low concentration, and does not mistake redox activity or autofluorescence for failure to inhibit bacterial growth) (24, 112, 119) or false positive results (eg. the assay does not mistake solvent activity for natural product activity) (120), and that the results obtained are reproducible (ie. similar results are obtained when the assay is performed on different days, by different users, in different locations, etc.) (24, 112). For quantitative assays, an additional requirement is that they generate accurate results [ie. the assay is able to produce the correct results when reference compounds of known antibacterial potency (usually FDA- or EMA-approved antibiotics) are tested against reference (culture collection) strains of bacteria] (112). Other desirable attributes for antibacterial assays are that they be simple, rapid (112, 120), inexpensive (108) and suitable for testing compounds of various polarity (115, 120). If possible, they should also be capable of dereplication (ie. able to differentiate novel natural products from known natural products) (24, 119), generating results from small quantities of extract or compound (119, 121), differentiating antibacterial activity from non-specific cytotoxicity (119), differentiating bacteriostatic activity from bactericidal activity (108, 115), detecting activity against mycobacterial species (103), and identifying antibacterial target (ie. able to identify what cellular structure the natural product interacts with to exert its antibacterial effect) (122). The relative importance of each of the above attributes will vary from one bioprospecting program to another. For example, when examining source material in which known antibiotics are more likely to be found than novel antibiotics [eg. soil actinomycetes (17)], an assay capable of dereplication will probably be the priority. However, when examining source material in which redox active compounds are likely to present [eg. plants (123)], an assay that does not mistake redox activity for failure to inhibit bacterial growth will probably be the priority, and when examining source material likely to harbor general cellular poisons [eg. cyanobacteria (124), animal venom (125)], an assay capable of differentiating antibacterial activity from non-specific cytotoxicity will probably be the priority.

**Non-quantitative antibacterial assays**

Diffusion-based, bioautographic, and cell morphology-based methods can all be used to detect antibacterial constituents present in source material. Diffusion-based assays involve inoculating the entire surface of an agar plate with a bacterial strain, adding small reservoirs of test extract to the surface-seeded agar plate (eg. on a disk or in a well), incubating for a predetermined time period, and then examining the agar plate for zones of bacterial growth inhibition around the test extracts (118, 119). Diffusion-based methods continue to be refined for the purpose of antibacterial bioprospecting, for example the use of susceptible-resistant pair screening as a means of dereplication (24, 119), and the use of over- and under-expressing strains as means of identifying the antibacterial target (119, 126). Diffusion-based methods were used extensively during the 'golden age' of antibiotic discovery, yielding many of the compounds in clinical use today (112, 119). Bioautographic methods use thin layer chromatography (TLC) to separate the compounds present in test extracts, with TLC plates then dipped in a bacterial suspension (direct bioautography), placed in contact with a surface-seeded agar plate (contact bioautography), or covered with molten, seeded agar (agar overlay bioautography), and antibacterial activity detected either by observation of zones of inhibition or the use of redox indicators to assess bacterial metabolic activity (127). Bioautographic methods also continue to be refined, for example adjustments that allow microaerophilic and obligately anaerobic bacteria to be tested (128), and coupling with mass spectrometry for the purpose of dereplication (129). Examples of antibiotics discovered by bioautography include lasalocid and salinomycin (127). Lastly, cell morphology-based assays work on the principle that antibacterial compounds inhibiting some bacterial processes induce an associated morphological change in the bacterial cell, the best known example being spheroplast formation (observable as large round refractile bodies) by peptidoglycan synthesis inhibitors (24). Such compounds can be detected by treating bacterial cells with a test extract and then examining them by light microscopy. Though information on this approach has only been disclosed relatively recently, spheroplasting assays were used by Merck and other pharmaceutical companies in the discovery of antibiotics such as fosfomycin, thienamycin,

moenomycin and mureidomycin (24, 119). The advantages and disadvantages of all three of the above assay types are presented in Table 1.

**Technical standards and guidelines for non-quantitative antibacterial testing**

With the exception of the disk diffusion method, standardized methods have not yet been developed for the above assays. The responsibility of ensuring these methods are capable of generating reproducible results therefore falls to the individual research groups using them. This can be achieved by, for example, regularly testing reference compounds of known stability and antibacterial potency against reference strains of bacteria of known susceptibility (108). Because the standardized disk diffusion methods were not developed for the purpose of bioprospecting [but for differentiating antibiotic-susceptible and -resistant clinical isolates in the diagnostic microbiology laboratory (116, 117)], researchers also need to verify that this and the other methods are not generating false negative results, false positive results etc. Detection limits can be determined by, for example, testing samples with known concentrations of antibacterial compound. Also, the possibility that false negative results are being caused by redox active natural products can be ruled out by testing extracts with the redox indicator in the absence of bacterial cells to ensure no color change is taking place (112).

**Quantitative antibacterial assays**

Agar dilution, broth macrodilution and broth microdilution methods can all be used to measure the inhibitory activity of test extracts and isolated compounds against bacterial growth. This activity is expressed as a minimum inhibitory concentration (MIC), the lowest concentration of test extract or compound required to inhibit bacterial growth (108, 118). The first method, agar dilution, involves preparing different concentrations of the test extract or compound in an agar medium, spot inoculating the

agar surface with bacterial strains, incubating for a predetermined time period, and then examining the agar surface for the growth of bacterial colonies (118). The other two methods, broth macrodilution and broth microdilution, involve preparing different concentrations of the test extract or compound in a broth medium (in tubes or microtiter plates, respectively), inoculating each of the tubes/wells with a bacterial strain, incubating, and then examining the tubes/wells for turbidity or bacterial pellets (118). The broth microdilution method has been used extensively for antibacterial bioprospecting (119). Unlike the agar dilution method, both broth dilution methods can also be used to measure bactericidal activity (118, 135). This is expressed as a minimum bactericidal concentration (MBC), the lowest concentration of test extract or compound required to kill 99.9% of bacterial cells (118, 135). MBCs are determined by transferring clear (growth-free) broth from the MIC and supra-MIC tubes/wells of the macrodilution/microdilution assay, inoculating onto an agar medium containing no test extract or compound, incubating, then performing colony counts and using these counts as a proxy measure of bacterial viability (118, 135). The advantages and disadvantages of all three of the above assays are presented in Table 2.

**Technical standards and guidelines for quantitative antibacterial testing**

Standardized methods have been developed for all three of the above assays by the Clinical and Laboratory Standards Institute (CLSI) (135, 139), with an additional International Organization for Standardization (ISO) standard available for the broth microdilution method (140, 141). Like the disk diffusion method, these guidelines were developed for use in the diagnostic microbiology laboratory rather than for bioprospecting, but there is a strong case to be made for their use. Firstly, because the CLSI standards have been in use for over thirty five years and undergone thirteen multicenter-coordinated iterations of review and revision in this time, they are fairly comprehensive in describing not just the many variables affecting antibacterial susceptibility test results (eg. inoculum size and age, growth medium, agar depth or broth volume, height to which agar plates or microtiter plates are stacked,

incubation time) but also what materials or values to use (139). Their employment for the purpose of bioprospecting is likely, therefore, to improve the reproducibility of test results. Another important feature of the CLSI standards is they include tables of test results that MIC and MBC assays should generate for reference (FDA-approved) antibiotics tested against reference (culture collection) strains of bacteria (135, 139). Such quality controls could help ensure MIC and MBC accuracy during bioprospecting. Test result accuracy is important because it helps funding bodies (and sometimes other researchers) prioritize the most potent antibacterial extracts and compounds for further investigation. Test result accuracy is also desirable in that it could allow the antibacterial activity of compounds isolated by different research groups to be directly compared, thereby facilitating the establishment of provisional structure-activity relationships (SARs) (142).

In most cases, the above standards can be used for bioprospecting without modification simply by including additional controls or testing additional bacterial strains as required. With broth macro and microdilution assays, for example, an uninoculated dilution series of test extract/compound and broth can and should be included to detect solubility problems and avoid false negative results due to compound precipitation (108). Also, dereplication can be achieved in all three assays by using paired strains (eg. a drug-resistant mutant and its parent strain) and comparing their susceptibility (24, 119). In other cases, small modifications of the technical standards may be justifiable. Because plant-derived antibacterial compounds are prone to bacterial efflux, for example, it has been suggested that plant extracts be screened for activity in combination with efflux pump inhibitors to increase assay sensitivity and reduce the likelihood of false negative results (89). Also, when testing panels of over-expressing or under-expressing strains [obtained by genetic manipulation (143) or antisense RNA technology (144)] for the purpose of target identification, it is necessary to include an inducer [eg. arabinose (143), xylose (144)] in the growth medium to regulate target protein levels. Any such modification to CLSI or ISO methods should be kept to a minimum and explained in publications. Lastly, CLSI and ISO standards are only suitable for measuring inhibition of exponentially growing cells. For measuring inhibition of bacterial

biofilms, other methods such as the microtiter plate-based resazurin assay (145), Lubbock chronic wound biofilm model assay (146), chronic wound biofilm infection assay (147) and microfluidic wound model assays (146) are required.

**Dissolution or solubilization of extracts and compounds for antibacterial testing**

When dissolving test extracts or isolated compounds prior to antibacterial testing, wasted effort can be minimized if consideration is given to the maximum concentration of test extract or compound that needs to be prepared. For antibacterial assays, this is generally 4000 µg/ml for crude or fractionated extracts and 400 µg/ml for isolated compounds [working on the basis that extracts and isolated compounds incapable of inhibiting bacterial growth at concentrations at or below 1000 µg/ml and 100 µg/ml respectively, are generally considered insufficiently active to merit further investigation (121, 148)]. Because most natural products have limited aqueous solubility, use of a solvent is almost always necessary. Many options are available [eg. acetone, dimethyl formamide, dimethyl sulfoxide (DMSO), ethanol, methanol, polyethylene glycol 400, sodium bicarbonate, sodium carbonate, sodium hydroxide (131, 149)], but it is DMSO that is most frequently used. Advantages of DMSO for bioassays include its ability to dissolve both polar and nonpolar compounds, and its miscibility with water and growth media (150). Also, the high boiling point of DMSO reduces room temperature evaporation, improving the accuracy of the test concentrations prepared (108). Lastly, pure DMSO is antimicrobial (151) so, if necessary, natural product extracts and compounds can (with adequate controls in place) be tested without filter sterilization, an advantage when working with compounds that might adsorb to the membrane filter (108). Bacterial tolerance to DMSO varies between species [MICs of 5 to 30% (v/v) (151)] but, in the interests of standardization, the final in-assay concentration of this solvent does not normally exceed 2.5% (v/v) (152) and is ideally just 1% (v/v) (108).

Regardless of which of the above solvents is used, some hydrophobic natural products will precipitate out (either immediately or eventually) upon dilution with water or growth media. Natural product solubility in these situations can sometimes be improved by using carriers such as cyclodextrins (137) or serum albumins (153). An alternative option is to prepare a colloid or, in the case of essential oils, an emulsion. This involves the use of a surfactant (eg. polysorbate 80, polyalkylene glycol) (115, 154) or emulsifier (eg. agar, lecithin) (155, 156) to disperse the natural product throughout the assay medium, promoting contact with bacterial cells and reducing the risk of false negative results. Mixed solvents are sometimes used too (eg. *N*-methyl-2-pyrrolidone and DMSO) (154). For low-throughput screening, the above solutions, colloids and emulsions are usually prepared fresh on the day of the experiment to reduce the risk of compound precipitation or degradation, or vehicle incompatibility or reactivity. Because solvents (151), carriers (157) and surfactants (158) can themselves be antibacterial or alter the activity of the compounds being tested, their use and concentration(s) should always be reported.

**Selection of target organisms**

According to the World Health Organization, future antibiotic research and development efforts should focus on certain key pathogens. Top of this list (critical priority) are *Mycobacterium tuberculosis* (multidrug- and extensively drug-resistant), *Acinetobacter baumannii* (carbapenem-resistant), *Pseudomonas aeruginosa* (carbapenem-resistant) and the Enterobacteriaceae (carbapenem- and third generation cephalosporin-resistant). Next (high priority) are *Enterococcus faecium* (vancomycin-resistant), *Staphylococcus aureus* (methicillin- and vancomycin-resistant), *Helicobacter pylori* (clarithromycin-resistant), *Campylobacter* spp. (fluoroquinolone-resistant), *Salmonella* spp. (fluoroquinolone-resistant) and *Neisseria gonorrhoeae* (third-generation cephalosporin- and fluoroquinolone-resistant), and lastly (medium priority) *Streptococcus pneumoniae* (penicillin non-susceptible), *Haemophilus influenzae* (ampicillin-resistant) and *Shigella* spp. (fluoroquinolone-resistant) (159). Testing wild-type (antibiotic-susceptible) reference strains (eg. American Type Culture Collection,

National Collection of Type Cultures) of the above species is usually advisable in the first instance as they are well characterized (making interpretation of results more straightforward), widely used (making inter-study comparisons of results possible) (108), and less likely to generate false negative results (due to efflux or other resolvable issues) (89, 119). As explained in Tables 1 and 2, there are also advantages with using paired strains, over-expressing or under-expressing strains, and reporter strains of bacteria (24, 119). Lastly, whilst the use of surrogate or model species can be very effective (eg. use of fast-growing *Mycobacterium smegmatis* instead of *M. tuberculosis* in the Janssen Pharmaceutica drug discovery program that yielded bedaquiline), it should be borne in mind that inter-species differences can cause false negative results (eg. up to 50% of *M. tuberculosis* inhibitors are not detected in screens using *M. smegmatis*) (160).

**Interpreting antibacterial test results**

Although the CLSI and ISO standards describe methods that can readily be repurposed for bioprospecting, they are on their own insufficient for interpreting results. Additional information is required to guide the isolation of antibacterial compounds from crude extracts, and to determine whether isolated compounds are sufficiently potent to merit further investigation. Universal consensus is lacking on what level of activity should be present before a crude extract is advanced for semi-purification, but it has been proposed that only extracts with MICs $\leq$1000 μg/ml should be considered active (108, 120, 121). Working on the principle that the antibacterial activity of a crude extract is due to the sum of the activities of its individual constituents, then semi-purified extracts obtained by fractionation should show an improvement in activity (lower MICs), allowing further purification and isolation of the active compound(s) (109). In practice, an improvement in activity is not always seen. This may be because compounds in the original crude extract were acting synergistically (108, 109), or because structural modification of the active compound(s) has occurred during fractionation (108, 109). Structural

degradation of active compounds or transformation to a less active form can occur if there is a reaction with the solvent being used (eg. esterification of acid groups on the molecule by an alcoholic solvent) or if oxidation occurs (eg. phenolic compounds reacting with oxygen dissolved in mobile phases) (109). If isolation of an antibacterial compound is achieved, it must generally have an MIC ≤100 μg/ml to be considered progressible (120, 121, 131, 148). This is because there are limitations to which structural modification can improve the antibacterial activity of isolated compounds, and because achievement of MIC (and preferably supra-MIC) levels of compound in the blood plasma must be feasible if it is to be effective as a systemic therapeutic agent (109, 118). Endpoint criteria are even more stringent within the pharmaceutical industry (119) and public-private partnerships (161), with isolated compounds unlikely to attract interest unless their MICs are <10 μg/ml (103) or <10 μM (161). For compounds that will be used topically rather than systemically (eg. for skin decolonization or the treatment of skin infections), slightly higher MICs may be acceptable as the compound can be delivered directly to the target site. If MBCs are also determined during testing, then compounds with an MBC no more than 4 times the MIC can provisionally be considered bactericidal (162, 163) unless they belong to a known cell-aggregating class of natural products such as the flavonoids (164, 165). Following the successful isolation of an antibacterial compound from a particular organism, consideration is sometimes given to the screening of other closely related species as minor evolutionary variations in the biosynthetic pathway of the compound can generate structural variants with improved activity (103).

## TOXICITY TESTING

When antibacterial compounds are isolated as part of a bioprospecting program, it is important they be tested for toxicity (166, 167). Contrary to views sometimes expressed in the literature, compounds from nature are no less likely to be toxic than those of synthetic origin. Many of the world's most lethal poisons, for example ricin, batrachotoxin, maitotoxin and botulinum, are actually naturally occurring

(168). Toxicity testing is particularly important when whole-cell screens (Tables 1 and 2) have been used for bioprospecting because, with the exception of some cell morphology-based assays and assays using under-expressing, over-expressing or reporter strains of bacteria, these screens do not distinguish between specific (bacterial) and nonspecific (general) cell toxicity (119). The number of *in vitro* assays available for toxicity testing has grown considerably in recent years and continues to grow, driven by public concern for animal welfare (169, 170) and legislative control of animal use (171, 172), and a desire on the part of pharmaceutical companies and regulatory agencies to reduce false-negative and false-positive test results caused by species-specific toxicity (173, 174), to reduce the cost and duration of toxicity testing (171, 175), to generate data for SAR analysis and pharmacokinetic and pharmacodynamic models (176), and to detect toxicity problems earlier in the drug development process (170, 177).

**Toxicity assays available and rationale for their selection**

At the time of writing, *in vitro* assays are available to detect cytotoxicity (including hepatotoxicity, nephrotoxicity, neurotoxicity, cardiotoxicity, immunotoxicity and hemotoxicity), mitochondrial toxicity, genotoxicity (including mutagenicity and carcinogenicity), phospholipidosis, steatosis and cholestasis (177). The type of toxicity initially tested for can be prioritized based on several factors. These include any known or predicted toxicity problems associated with the class of natural product being tested, the bacterial pathogen(s) being targeted, and whether a future drug derived from the natural product is likely to be used topically or systemically. Many of the pyrrolizidine alkaloids, for example, are hepatotoxic, many furoquinolones are mutagenic (104) and many cationic amphiphilic compounds induce phospholipidosis (178), so it makes sense to prioritize hepatotoxicity, mutagenicity and phospholipidosis testing with these classes of compound. Testing can also be prioritized based on the findings of predictive toxicity software such as Derek Nexus. Antimycobacterial compounds are usually tested for toxicity against macrophages (179, 180) because *Mycobacterium tuberculosis* resides in these cells during

infections. Lastly, drugs likely to be used topically are tested against epithelial, keratinocyte and/or dermal fibroblast cells (181, 182), while those intended for systemic use are tested against erythrocytes (183, 184) and cells from organs commonly affected by drug toxicity such as the kidney (185, 186), liver (187, 188) and heart (189, 190).

Key attributes toxicity assays should have are that they do not generate false negative or false positive results (177, 191), and that the results obtained are accurate [ie. the assay is able to produce the correct results when reference compounds of known toxicity are tested (192, 193)], reproducible (ie. similar results are obtained when the assay is performed on different days, by different users, in different locations, etc.) (191, 194), and comparable between studies (108). At this point, it is also important the toxicity test is fully quantitative (170) so that a selectivity index/indices (discussed later) can be calculated and a risk-benefit assessment made. The larger the value of the selectivity index, the more likely the compound will gain approval for development (161), and the more likely the final drug will benefit the patient without undue risk (18). Other desirable attributes for toxicity assays are that they be simple, rapid (174, 195), safe (196), inexpensive (174, 195), capable of generating results from small quantities of compound (197), not dependent upon ethical approval or difficult-to-source materials (173), and yielding information on mechanism of toxicity (167, 174). The relative importance of each of the above attributes will vary from one bioprospecting program to another. When competing for research funding, for example, it may be desirable to demonstrate superior selectivity over other antibacterial compounds, in which case an assay that generates reproducible results comparable to other studies will be the priority. However, when testing a class of natural products with known toxicity issues (eg. pyrrolizidine alkaloids), an assay that closely resembles *in vivo* conditions and generates fewer false negative results may be the priority.

Many different toxicity assays are available. These can be categorized according to the type of cells used, the measures and markers used to predict toxicity, and the technology used to monitor changes in the test cells. The two main cell types used are primary cells and immortalized cells (or cell lines),

primary cells being cells that have been isolated directly from human or animal tissue, and immortalized cells being cells that have been derived from tumors or treated with viruses to make them easier to culture (198). Potential target organs of toxicity can be identified by testing primary and immortalized cells of different origin (hepatocytes, cardiocytes etc.). Common measures of toxicity against such cells include reduced growth, reduced viability, reduced metabolic activity, reduced membrane integrity, and other forms of cellular disruption. Cell growth can be monitored using total protein (199, 200) or DNA synthesis (201, 202) as markers, cell viability can be monitored using intracellular protease activity (167, 195) or pH gradient (203, 204) as markers, metabolic activity can be monitored using redox activity or ATP as markers (167, 195), membrane integrity can be monitored using dye exclusion (205) or intracellular enzyme retention as markers (197, 206), and general cell health and integrity can be monitored using cell morphology as a marker (173, 207). Other more specialized tests, too numerous to describe here in detail, include the hERG potassium channel assays (for cardiotoxicity) (189, 190), the hemolysis (208) and colony forming unit granulocyte/macrophage (CFU-GM) (209) assays (for hematotoxicity), the measurement of mitochondrial membrane potential (for mitochondrial toxicity) (210), the Ames (211), chromosomal aberration, micronucleus (193) and mouse lymphoma (192, 193) assays (for genotoxicity), and the use of specialized stains such as *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE), Nile Red and LipidTox (for measuring phospholipidosis, steatosis and cholestasis) (177, 207). Lastly, in terms of technology, changes in the test cells can be monitored either spectrophotometrically (167) or by high content analysis (177, 207). The advantages and disadvantages of some of the most commonly used types of toxicity assay are presented in Table 3. In resource limited settings where these toxicity assays may not all be available, testing can be outsourced to initiatives such as the Community for Open Antimicrobial Drug Discovery (CO-ADD) or to contract research organizations.

**Technical standards and other useful protocols for toxicity testing**

Protocols have been published by the National Institutes of Health (NIH), Eli Lilly and Company (ELC) and others for many of the general toxicity assays including the protease assay (167, 195), neutral red uptake assay (204), tetrazolium and resazurin reduction assays (167), ATP assay (167), and trypan blue exclusion assay (214). These include useful guidance notes on reagent storage, cell density, positive and negative controls, temperature maintenance etc. Moving on to more specialized toxicity assessments, numerous technical standards have been developed, validated and published by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL), the International Cooperation on Alternative Test Methods (ICTAM) and the Organisation for Economic Co-operation and Development (OECD) including tests for ocular toxicity (eg. OECD Test Guidelines 491 and 492), skin irritation and corrosion (eg. OECD Test Guidelines 439 and 431), and genotoxicity (eg. OECD Test Guidelines 471, 473, 476, 487 and 490) (193, 218). As with the CLSI and ISO standards described for antibacterial testing, adoption of these NIH, ELC, EURL, ICTAM and OECD protocols and standards is likely to improve the reproducibility, accuracy, comparability and credibility of the toxicity data generated by bioprospecting programs.

**Dissolution or solubilization of compounds for toxicity testing**

As discussed earlier, DMSO has many properties that make it a useful bioassay solvent, and it is a popular choice for dissolving compounds for toxicity assays (172). However, it is important to verify experimentally that DMSO does not interfere with the human/mammalian cells being tested. With some cells and cell lines, low DMSO concentrations [$\leq$0.07 % (v/v)] can stimulate cell growth (219, 220), and higher DMSO concentrations [$\geq$0.5% (v/v)] can inhibit (221) or kill cells (220). If DMSO is found to affect the growth or viability of the human/mammalian cells being tested, then other solvents [eg. acetone, acetonitrile, ethanol, isooctane, isopropanol, sodium hydroxide, tetrahydrofuran (172, 219, 222, 223)],

surfactants (224), cosurfactants (225), cyclodextrins (226) or dendrimers (227) can be investigated as alternative means of dissolution or solubilization. Because concentration-dependent stimulatory and inhibitory effects have been detected with DMSO (219-221) and other solvents (222, 228), it is recommended that solvent concentrations always be kept constant in serial dilutions of test compounds (226).

**Interpreting toxicity test results**

While the decision to progress a compound beyond *in vitro* antibacterial testing is determined largely by potency cut-offs (eg. MICs <10 µg/ml or <10 µM for prospective systemic drugs), the subsequent decision to progress that compound beyond *in vitro* toxicity testing is based on a different measure. Referred to by some authors as the 'selectivity index' (229) [and others as the 'safety margin' (230), 'selectivity' (231) or 'selectivity window' (161)], this is the ratio of off-target (human/mammalian) to target (bacterial) toxicity (18, 230). In antibacterial research, the selectivity index is usually obtained by dividing a test compound's half-maximal cytotoxic concentration ($CC_{50}$; the concentration of compound required to reduce human/mammalian cell viability by 50%) by the compound's MIC against bacteria (229). Depending on the toxicity assay used, other values such as the half-maximal inhibitory concentration [$IC_{50}$; the concentration of compound required to inhibit a given biological process (eg. human/mammalian cell growth or enzyme activity) by 50%] (230) or half-maximal hemolytic concentration ($HC_{50}$; the concentration of compound required to lyse 50% of red blood cells) (231) may be used instead of the $CC_{50}$. Because multiple bacterial species and multiple human/mammalian cell types are tested as part of most bioprospecting programs, multiple selectivity indices are generated, and these can be presented in a 2-dimensional table called a 'heatmap grid' (230).

Evaluating selectivity indices is a critical juncture in the drug discovery process. If a cut-off is set too high, useful compounds may be discarded, but if a cut-off is set too low, then time and resources may

be wasted on a compound with no commercial or therapeutic value (161). Where the cut-off is set is a complex decision dependent on many factors. These include (a) whether the drug candidate addresses an unmet medical need, (b) whether the drug candidate is likely to have superior efficacy or safety over the current standard of care, (c) whether the drug candidate is intended for use as a short- or long-term therapy, (d) whether the drug candidate is intended for use in patients with co-infections or comorbidities that would put them at increased risk of drug-drug interactions or other adverse drug reactions, (e) the type of *in vitro* toxicity detected and (f) information on how predictive (sensitive and specific) the *in vitro* toxicity tests used are (230). It should be apparent from the above text that no single cut-off value is universally applicable to all antibacterial compounds (230). For prospective antimycobacterial drugs, for example, it has been proposed that the selectivity index should be greater than ten before they are considered for further development, but this value is usually higher for other indications (161). It should also be apparent from the above text that cut-off values for specific indications change over time because the competitive drug landscape changes over time (230). An important final point to make here is that the ratio of off-target to target toxicity (known as the 'therapeutic index' rather than the 'selectivity index' in subsequent *in vivo* and clinical studies) usually decreases as a compound progresses through the discovery and development pipeline (Fig. 2). This is, in part, because rarer toxicities are detected as animals and then human subjects are tested for longer durations and in increasingly large numbers (230). Efficacy decreases too, in many cases because antibacterial activity is growth rate-dependent and bacterial cells grow more slowly *in vivo* than they do *in vitro* (232). Such factors must also be borne in mind when deciding to progress a compound beyond *in vitro* toxicity testing.

## CONCLUDING REMARKS

Most antibacterial drugs in current clinical use are natural products (or semi-synthetic derivatives thereof), and these have the potential to re-emerge as an important starting point for drug discovery. Given the limited market incentives for development of antibacterial drugs however, and our increasing

reliance on public and philanthropic sources of funding for this type of research, it is imperative that future bioprospecting programs are optimized for efficiency. This can be achieved in a number of ways including the selection of study designs appropriate to institutional resources. In settings unequipped for high throughput screening, for example, random testing of soil actinomycetes is unlikely to be successful because less than 1 in 10 million bacteria produce novel antibiotics. In such settings, it may be more productive to select source material based on ecological, ethnomedical and/or genomic information, or to investigate the impact novel culture conditions (eg. different nutrients, simulated pathogen attack, abiotic stress) have on the chemical compounds organisms or cells produce. Antibacterial and toxicity assays should also be rationally selected based on any pertinent foreknowledge of the natural product source material. For example, when screening cyanobacterial extracts for antibacterial activity (or other source material likely to harbor general cellular poisons), the use of panels of over-expressing, under-expressing or reporter strains of bacteria should be considered to differentiate specific and non-specific cytotoxicity. Also, when an antibacterial compound belonging to a toxicity-prone class of natural products (eg. the pyrrolizidine alkaloids) has been isolated and is being assessed for toxicity, the inclusion of primary cells in the panel of test cells should be considered because these generate fewer false negative results than immortalized cells. Where technical standards or protocols are available for these bioassays (from the CLSI, ISO, EURL etc.), their use is recommended as this will improve data robustness and facilitate inter-study comparisons. Lastly, it is important to avoid the many pitfalls associated with each of the discussed aspects of antibacterial bioprospecting. These pitfalls are summarized in Supplementary Table 2.

In this review, we have provided a multidisciplinary perspective on some of the initial steps in natural product-based antibacterial drug discovery. All of the information included is correct at the time of writing, but new bioassays, bioassay guidelines and cell lines continue to be developed, and communication/collaboration with appropriate specialists is always good practice (167, 233, 234). Space restrictions prevented us from describing within this review all of the steps required for ‘hit validation’, the process of confirming isolated antibacterial compounds have the potential for modification to an entity

with properties suitable for clinical use. Hit validation involves additional laboratory and legal work including (a) preparation of concentration response curves and measurement of resistance frequency, (b) elucidation of antibacterial mechanism of action, (c) confirmation of compound identity and purity, (d) confirmation of the druggability of the chemical class (by assessing solubility, stability, reactivity, synthetic feasibility etc.) and (e) confirmation that no serious intellectual property conflicts exist and there is 'freedom to operate' (167). Readers are directed to other reviews (134, 235-237) and the NIH Assay Guidance Manual (167) for information on these steps, and a review by Katsuno *et al.* (161) for example hit validation criteria. Further testing, for example medicinal chemistry optimization and SAR assessment, screening for pan-assay interference (PAIN) behaviors, and screening for cytochrome P450 inhibition is not usually performed in depth until compounds have progressed into the subsequent 'hit to lead' stage of drug discovery (161, 238). However, it is useful to check the research literature for information on the bioactivity of the isolated compound and structurally related compounds, both to build up preliminary knowledge of SAR and assess the risk of PAIN behavior. If a class of natural products is reported to have many different biological activities, then this could suggest these compounds are promiscuous and lack target specificity (22).

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**Table 1** Advantages and disadvantages of different non-quantitative assays of direct antibacterial activity.

| Type of assay | Advantages | Disadvantages | References |
|---|---|---|---|
| **Diffusion-based** (eg. disk diffusion, well diffusion, cylinder diffusion, spot-on-lawn) | ▪ Simple, quick to perform (multiple extracts can be tested on the same plate), & requires no specialized equipment.<br>▪ False negative results can be minimized by using hypersensitive bacterial strains (ie. a panel of strains under-expressing conserved essential enzymes such as FabF) or using reporter strains (ie. a panel of strains which emit light when transcription of stress response genes or other genes of interest occurs).<br>▪ Dereplication can be achieved by using paired strains (eg. a drug-resistant mutant & its parent strain) & comparing their susceptibility.<br>▪ Target identification can be achieved by testing a panel of over- or under-expressing target strains & comparing their susceptibility to the parent strain, or by using reporter strains.<br>▪ False positive results caused by general cellular poisons (eg. membrane disruptors) can also be minimized by using a panel of over-expressing, under-expressing, or reporter strains. | ▪ Because test results are based on the observation of bacterial growth, this type of assay takes 18-24 h to generate results.<br>▪ False negative results are a possibility with all of these assays as nonpolar compounds can fail to diffuse through the agar & produce a zone of inhibition. With the disk diffusion method, false negative results can also occur when testing cationic compounds as these can adsorb to the disk.<br>▪ If dereplication is attempted using paired strains (eg. a drug-resistant mutant & its parent strain), then the assay can be confounded by the presence of multiple antibacterial compounds (eg. one known & one novel antibiotic in the same extract), resulting in further false negative results. | (10, 16, 24, 115, 119, 130, 131) |
| **Bioautographic** (ie. TLC-direct bioautography, TLC-contact bioautography, & TLC-agar overlay bioautography) | ▪ Quite simple to perform &, when a redox indicator (eg. resazurin or 2,3,5-tetrazolium chloride) is used to detect metabolically active bacteria, assays can generate results in ≤6 hours.<br>▪ Dereplication can be achieved because compounds separated by TLC & shown to have activity can be removed from the plate & analyzed by mass spectrometry or other methods to determine structure. | ▪ False negative results are a possibility with some redox indicators (eg. resazurin) because redox-active compounds can react with the indicator, giving the impression test bacteria remain metabolically active. False negative results are also a possibility with the contact & agar overlay methods because nonpolar compounds may not diffuse from the chromatogram to the plate or may migrate poorly through the agar & fail to produce a zone of inhibition.<br>▪ False positive results can occur because, even after extensive drying, some acidic & alkaline solvents remain on the TLC plate. | (112, 120, 127, 129, 132, 133) |
| **Cell morphology-based** (eg. spheroplasting assay) | ▪ Because test results are based on the observation of morphological changes (not bacterial growth), this type of assay can generate results within several hours.<br>▪ False negative results caused by low compound potency or concentration are minimized because morphological changes occur at concentrations less than those needed to inhibit bacterial growth.<br>▪ Target can provisionally be assigned based on a positive test result (eg. natural products inducing spheroplasts often target peptidoglycan synthesis). | ▪ False negative results are likely as assay only detects those antibacterial compounds inducing morphological changes. False negative results can also occur if multiple antibacterial compounds are present in the same extract, as the effects of one can mask the effects of the other (eg. a membrane disruptor lysing the spheroplasts generated by a peptidoglycan synthesis inhibitor). | (24, 119, 134) |

**Note:** Although the above assays cannot be used to determine the antibacterial potency of test extracts and compounds, they can be used to determine spectrum of activity. TLC, thin layer chromatography

**Table 2** Advantages and disadvantages of different quantitative assays of direct antibacterial activity.

| Type of assay | Advantages | Disadvantages | References |
|---|---|---|---|
| **Agar dilution** | ▪ Quite simple & quick to perform because multiple bacterial strains can be tested on the same agar plate.<br>▪ Can be used to test polar (hydrophilic) & nonpolar (hydrophobic) compounds.<br>▪ Can be used to test colored extracts & compounds, & colloids & emulsions as agar discoloration & turbidity are readily distinguishable from bacterial growth occurring on the agar surface.<br>▪ False negative results due to bacterial/ fungal contamination are unlikely as this is generally detectable by eye.<br>▪ Bacterial growth on a solid surface is considered more biologically relevant than planktonic growth in liquid. | ▪ Requires quite large quantities of extract or compound.<br>▪ Minimum bactericidal concentrations (MBCs) cannot be determined, so bacteriostatic & bactericidal activity cannot be differentiated. | (108, 112, 115, 118, 121, 131) |
| **Broth macrodilution** | ▪ Quite simple to perform.<br>▪ MBCs can be determined, so bacteriostatic & bactericidal activity can be differentiated. | ▪ Slow to perform because individual dilution series need to be prepared for each bacterial strain.<br>▪ Difficult to test nonpolar (hydrophobic) compounds using this method unless a carrier, surfactant or emulsifier is used.<br>▪ Difficult to test darkly colored extracts & compounds, colloids, & emulsions as broth discoloration & turbidity can, respectively, mask or resemble growth.<br>▪ Requires quite large quantities of extract or compound.<br>▪ False negative results can occur due to bacterial/fungal contamination because, unless occurring in uninoculated control tubes, it is not detectable by eye. | (108, 115, 118, 121, 131, 136, 137) |
| **Broth microdilution** (incl. tetrazolium- & resazurin-based assays for mycobacteria) | ▪ Can be performed with quite small quantities of extract or compound.<br>▪ MBCs can be determined, so bacteriostatic & bactericidal activity can be differentiated.<br>▪ Antimycobacterial activity can be measured by using a fast-growing model species (eg. *Mycobacterium phlei* or *M. smegmatis*), or by using redox indicators (ie. dyes such as tetrazolium or resazurin that assess the metabolic activity of test mycobacteria), or by using a reporter strain of *M. tuberculosis* (ie. a strain expressing green fluorescent protein that allows fluorescence to be used as a proxy measure of bacterial growth). | ▪ More difficult than other methods as multichannel pipette requires skill, & slow because a dilution series needs to be prepared for each bacterial strain.<br>▪ Difficult to test nonpolar (hydrophobic) compounds using this method.<br>▪ Difficult to test darkly colored extracts & compounds, colloids, & emulsions using this method.<br>▪ False negative results can occur due to contamination because it is not detectable by eye. False negative results can also occur with redox indicators (eg. resazurin) as redox-active compounds can react with them, & with reporter strains as some compounds autofluoresce. | (103, 108, 115, 118, 121, 131, 136-138) |

**Note**: As with diffusion-based assays, dereplication & target identification can be achieved & the risk of false-negative and false-positive results can be minimized in all the above assays by using under-expressing, over-expressing, drug-resistant &/or reporter strains of bacteria. Accurate results are also achievable with all the above assays if they are performed in compliance with CLSI or ISO & EUCAST guidelines (an advantage of this being that inter-study comparisons of results are then possible). MBCs, minimum bactericidal concentrations

**Table 3** Advantages and disadvantages of some of the most common types of toxicity assay.

| Type of assay | Advantages | Disadvantages * | References |
|---|---|---|---|
| **(a) Cell type †**<br>*Primary cells* | ▪ More of the key metabolic enzymes (eg. cytochrome P450 enzymes) are present in primary cells than in cell lines, so there is greater *in vivo*-like functionality & a lower risk of false negative & false positive results. | ▪ Requires ethical approval, & may be expensive & difficult to procure sufficient quantities of cells.<br>▪ Primary cells have a limited lifespan & begin to dedifferentiate after 24-48 h.<br>▪ There is donor-to-donor variability in primary cells, so test results may not be reproducible & inter-study comparisons of toxicity are not possible.<br>▪ Primary cells have a low proliferative rate & can undergo morphological changes during culture, so neither inhibition of cell growth nor morphological changes can readily be used as measures of toxicity. | (171, 173, 176, 191, 207) |
| *Immortalized cells (cell lines)‡* | ▪ Cell lines are readily available & ethical approval is not needed to use them.<br>▪ Cell lines remain viable for longer than primary cells.<br>▪ Test results are more reproducible with cell lines than primary cells & inter-study comparisons are possible.<br>▪ Cell lines have a high proliferative rate, so inhibition of cell growth can be used as a measure of toxicity. | ▪ Fewer of the key metabolic enzymes are present in cell lines than in primary cells, so there is less *in vivo*-like functionality & a higher risk of false negative & false positive results.<br>▪ Cell lines can become cross-contaminated during extended use, so cell line authentication is important. | (171, 173, 176, 191, 195) |
| **(b) Markers of toxicity**<br>*Total protein [sulforhodamine B (SRB) & Lowry photometric method]* | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.<br>▪ The SRB & Lowry photometric assays are not expensive.<br>▪ The SRB assay is simple & rapid.<br>▪ Cytostatic & cytocidal activity can be differentiated if cells are incubated with fresh medium after treatment with the test compound.<br>▪ Assay kits are commercially available. | ▪ The Lowry photometric method requires time-consuming dilution of samples if protein content is high. | (199, 200, 212) |
| *DNA synthesis [eg. bromodeoxy-uridine (BrdU) assay, ethynyl deoxyuridine (EdU) assay]* | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.<br>▪ The BrdU assay is not expensive.<br>▪ The EdU assay is quite simple because test cell DNA does not need to be denatured & incubated with antibody.<br>▪ DNA synthesis & cell morphology can be assessed in the same assay if an ELISA method is used.<br>▪ Assay kits are commercially available. | ▪ The EdU assay is quite expensive.<br>▪ The BrdU assay is quite time-consuming because test cell DNA must be denatured & incubated with anti-BrdU antibody.<br>▪ EdU can induce DNA damage & cell death in test cells during prolonged culture. | (201, 202) |
| *Intracellular protease activity (eg. GF-AFC assay)* | ▪ More rapid than redox reagent-based assays because cells can be incubated with GF-AFC for ≤ 1h.<br>▪ GF-AFC is non-toxic to cells, so assay duration can be optimized more easily than with redox reagents.<br>▪ In addition to quantifying viable cells, the assay can quantify non-viable cells if | ▪ More expensive than tetrazolium- & resazurin-based redox activity assays. | (167, 195) |

| | | | |
|---|---|---|---|
| | protease leakage is measured (eg. using AAF-R110).<br>▪ An assay kit is commercially available. | | |
| *pH gradient between cytoplasm & lysosomes (neutral red uptake assay)* | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.<br>▪ This assay is less expensive than total protein-, redox reagent- & lactate dehydrogenase-based assays. | ▪ Assay is slower than ATP-based assays as treated cells must be incubated with neutral red for 2h.<br>▪ Neutral red is itself toxic to cells, so reagent concentration & assay duration must be limited. | (196, 203, 204) |
| *Redox activity (tetrazolium- & resazurin-based assays)* | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.<br>▪ The tetrazolium- & resazurin-based assays are relatively inexpensive.<br>▪ Assay kits are commercially available. | ▪ Assays are slower than ATP-based assays as viable cells take 1-4h to induce color change or fluorescence of redox reagents (longer if cells have low metabolic activity).<br>▪ Tetrazolium & resazurin compounds are toxic if incubated with cells for longer than 3-4h, so (i) assay duration can be difficult to optimize (it must be long enough to detect redox activity, but short enough to avoid redox reagent-induced toxicity) & (ii) it may not be possible to test cells with low metabolic activity.<br>▪ False negative results are possible if the test compound reduces the redox reagents directly or, with resazurin, if the test compound is fluorescent.<br>▪ False positive results are possible if the test compound alters the pH of the culture medium. | (112, 167, 195, 213) |
| *ATP detection (luciferase-based assays)* | ▪ Small quantities of compound can be tested if microtiter plate-based assays are used.<br>▪ More rapid than redox reagent- & protease activity-based assays, as cells do not need to be incubated with reagent after treatment with test compound.<br>▪ Early cytotoxic events can be monitored & mechanism of toxicity can be studied because the assay is available in a real time continuous-read format in which RNA is left intact enough to detect stress response gene expression.<br>▪ Assay kits are commercially available. | ▪ The luciferase enzymatic reaction is affected by temperature, so temperature of assay must be carefully controlled.<br>▪ False positive results are possible if the test compound inhibits luciferase.<br>▪ False negative results are possible if the test compound inhibits ATPases.<br>▪ More expensive than tetrazolium- & resazurin-based redox activity assays. | (167, 195, 196) |
| *Dye exclusion (eg. propidium iodide staining, trypan blue staining)* | ▪ Dye exclusion assays are simple & inexpensive.<br>▪ When a fluorescent stain (eg. propidium iodide) is used, it is possible to apply a counterstain (eg. calcein AM) so that not just membrane-compromised cells but also membrane-intact cells are quantified.<br>▪ These assays can detect toxicity regardless of the rate of proliferation of test cells or whether or not the test cells are dividing at all. | ▪ Examination of treated cells is labor-intensive unless flow or image cytometry is available.<br>▪ False negative results are possible if (i) lethally damaged cells do not lose membrane integrity within the timeframe of the assay or (ii) cells do not just lose membrane integrity but disintegrate completely.<br>▪ Trypan blue is itself toxic to cells, so reagent concentration & assay duration must be limited.<br>▪ Propidium iodide is a suspected carcinogen & must be handled with care. | (196, 205, 214) |

| | | | |
|---|---|---|---|
| *Retention of intracellular enzymes (eg. lactate dehydrogenase assay, dead-cell protease assay)* | ▪ Small quantities of compound can be tested if microtiter plate-based assays (eg. dead-cell protease assay) are used.<br>▪ Data normalization is possible with the dead-cell protease assay, so the results generated are reproducible & comparable between studies.<br>▪ These assays can detect toxicity regardless of the rate of proliferation of test cells or whether or not the test cells are dividing at all.<br>▪ Assay kits are commercially available. | ▪ False negative results are possible if (i) the test compound inhibits the leaked enzyme being monitored or (ii) enzyme leakage is measured too early (before membrane damage has occurred). | (197, 206) |
| *Cell morphology [eg. morphological highest tolerated dose (HTD) assay, high-content analysis]* | ▪ Morphological changes (eg. cells becoming round, cells increasing or decreasing in size) occur before loss of cell viability & are therefore a more sensitive measure of toxicity. | ▪ Examination of treated cells is labor-intensive unless high-content analysis equipment & software is available. | (173, 207, 215-217) |
| **(c) Test system** *Spectrophoto-, fluoro- & luminometric analysis* | ▪ Can be performed with standard laboratory equipment such as a microplate spectrophotometer or flow cytometer. | ▪ Detects toxic compounds with low sensitivity (~46% according to one study), predominantly just those compounds disrupting cellular proliferation or viability. | (177) |
| *High-content analysis* | ▪ Detects toxic compounds with high sensitivity (~93% according to one study) due to ability to detect genotoxicity, phospholipidosis, steatosis & cholestasis.<br>▪ Information on mechanism of toxicity can be obtained by applying multiple stains simultaneously (eg. JC-10), monitoring the cells in real time, & identifying the event (eg. mitochondrial damage) that precedes cell death. | ▪ Requires specialized equipment & data analysis software. | (177, 207) |

**Notes**: *, One limitation common to all of these assays is that each batch of primary cells or cell line reflects the phenotype of just a single donor (171), this limiting the extent to which the toxicity data can be generalized. †, Studies are underway to determine if stem cell-derived cardiomyocytes, hepatocytes and other cells could be used for toxicity testing, an approach that could solve current challenges relating to cell procurement and *in vivo*-like functionality (174); ‡, Two of the most common cell lines in current use are Vero (normal monkey kidney) cells and Hep G2 (human hepatocellular carcinoma) cells, an advantage of including these being that toxicity results can then be compared between studies and antibacterial hits prioritized; AAF-R110, bis-alanyl-alanyl-phenylalanyl-rhodamine 110; GF-AFC, glycylphenylalanyl-aminofluorocoumarin

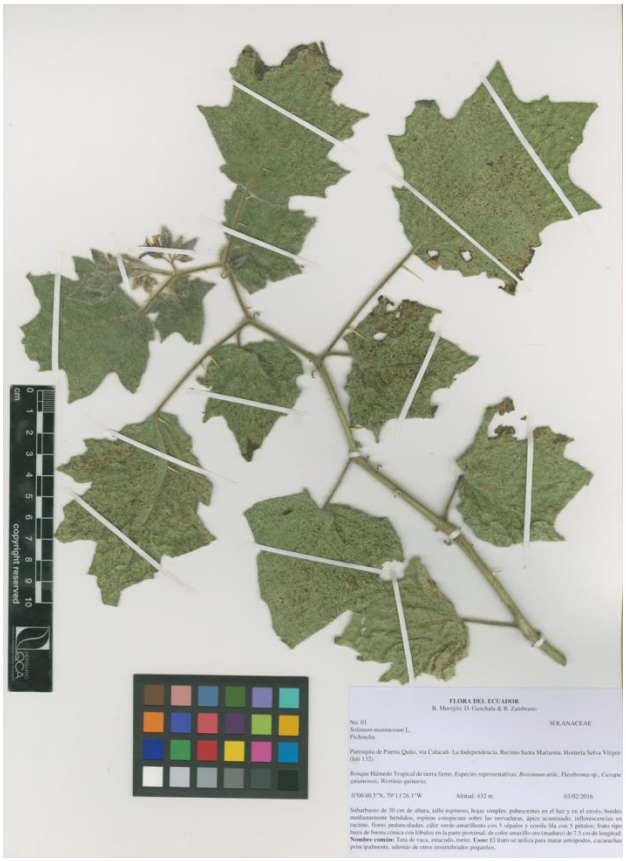


**Fig. 1** Photograph showing some of the key elements of a voucher, in this case a pressed, dried and paper-mounted botanical specimen with associated data such a scientific name, taxonomic authority, georeference, date of collection, common names and uses printed in the bottom right corner. Image from (74) by permission of PLOS ONE (Public Library of Science).

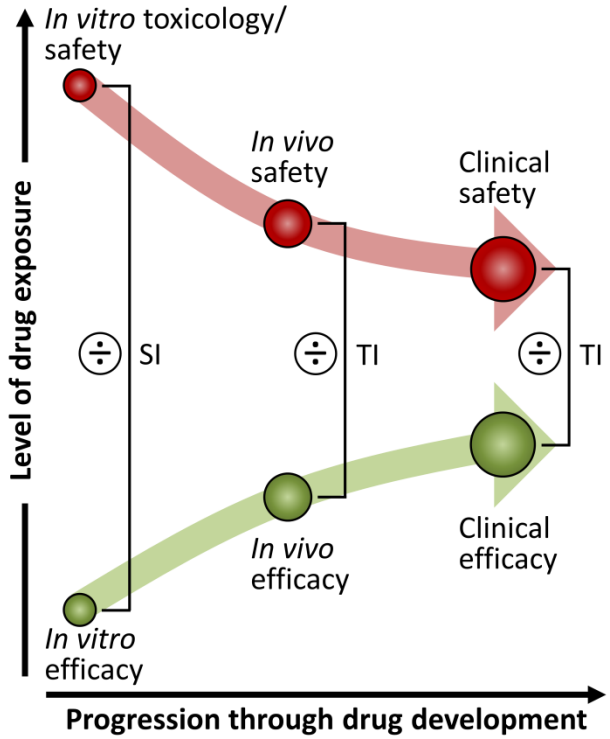


**Fig. 2** Selectivity index (SI) and therapeutic index (TI) values usually decrease as a prospective drug progresses through the development pipeline. This is because the quantity of safety and efficacy data (depicted by the differently-sized red and green circles) steadily increases during development, and problems relating to safety and efficacy are more likely to be detected. Image adapted from (230) by permission of Nature Reviews Drug Discovery (Springer Nature).

**REFERENCES**


1. Hutchings MI, Truman AW, Wilkinson B. Antibiotics: Past, present and future. Curr Opin Microbiol. 2019;51:72-80.
2. De Mattos-Shipley KMJ, Foster GD, Bailey AM. Insights into the classical genetics of *Clitopilus passeckerianus* - the pleuromutilin producing mushroom. Front Microbiol. 2017;8:Article 1056.
3. Brown DG, Lister T, May-Dracka TL. New natural products as new leads for antibacterial drug discovery. Bioorg Med Chem Lett. 2014;24(2):413-418.
4. Lewis K. New approaches to antimicrobial discovery. Biochem Pharmacol. 2017;134:87-98.
5. Chopra I. The 2012 Garrod Lecture: Discovery of antibacterial drugs in the 21st century. J Antimicrob Chemother. 2013;68(3):496-505.
6. Benedetto Tiz D, Kikelj D, Zidar N. Overcoming problems of poor drug penetration into bacteria: Challenges and strategies for medicinal chemists. Expert Opin Drug Discov. 2018;13(6):497-507.
7. Ribeiro da Cunha B, Fonseca LP, Calado CRC. Antibiotic discovery: Where have we come from, where do we go? Antibiotics. 2019;8(2):Article 45.
8. Jacobs A. Crisis looms in antibiotics as drug makers go bankrupt. The New York Times. 2019. Available from: https://www.nytimes.com/2019/12/25/health/antibiotics-new-resistance.html. Accessed on: 30 May 2020.
9. Silver LL. Challenges of antibacterial discovery. Clin Microbiol Rev. 2011;24(1):71-109.
10. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: History, methods and perspectives. Int J Antimicrob Agents. 2019;53(4):371-382.
11. Dingsdag SA, Hunter N. Metronidazole: An update on metabolism, structure-cytotoxicity and resistance mechanisms. J Antimicrob Chemother. 2017;73(2):265-279.
12. Andersson MI, MacGowan AP. Development of the quinolones. J Antimicrob Chemother. 2003;51(Supplement 1):1-11.
13. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov. 2015;14(2):111-129.
14. Wencewicz TA. New antibiotics from Nature's chemical inventory. Bioorg Med Chem. 2016;24(24):6227-6252.
15. Lee S, Siddiqui R, Khan NA. Animals living in polluted environments are potential source of antimicrobials against infectious agents. Pathog Glob Health. 2012;106(4):218-223.
16. Katz L, Baltz RH. Natural product discovery: Past, present, and future. J Ind Microbiol Biotechnol. 2016;43(2):155-176.
17. Baltz RH. Antimicrobials from actinomycetes: Back to the future. Microbe. 2007;2(3):125-131.
18. Singh SB, Young K, Silver LL. What is an "ideal" antibiotic? Discovery challenges and path forward. Biochem Pharmacol. 2017;133:63-73.
19. Genilloud O. The re-emerging role of microbial natural products in antibiotic discovery. Antonie van Leeuwenhoek. 2014;106(1):173-188.
20. British Society for Antimicrobial Chemotherapy (BSAC). AntibioticDB. BSAC. 2020. Available from: https://www.antibioticdb.com/. Accessed on: 30 May 2020.
21. National Library of Medicine. ClinicalTrials.gov. National Institutes of Health. 2020. Available from: https://clinicaltrials.gov/. Accessed on: 30 May 2020.
22. Baell JB. Screening-based translation of public research encounters painful problems. ACS Med Chem Lett. 2015;6(3):229-234.
23. Baell JB, Holloway GA. New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays. J Med Chem. 2010;53(7):2719-2740.
24. Silver LL. Rational approaches to antibacterial discovery: Pre-genomic directed and phenotypic screening. In: Dougherty TJ, Pucci MJ, editors. Antibiotic discovery and development. New York: Springer; 2012. p. 33-75.

25. Kong C, Eng S-A, Lim M-P, Nathan S. Beyond traditional antimicrobials: A *Caenorhabditis elegans* model for discovery of novel anti-infectives. Front Microbiol. 2016;7:Article 1956.
26. Larsen BB, Miller EC, Rhodes MK, Wiens JJ. Inordinate fondness multiplied and redistributed: The number of species on Earth and the new pie of life. Q Rev Biol. 2017;92(3):229-265.
27. Bills GF, Gloer JB, An Z. Coprophilous fungi: Antibiotic discovery and functions in an underexplored arena of microbial defensive mutualism. Curr Opin Microbiol. 2013;16(5):549-565.
28. Karwehl S, Stadler M. Exploitation of fungal biodiversity for discovery of novel antibiotics. In: Stadler M, Dersch P, editors. How to overcome the antibiotic crisis: Facts, challenges, technologies and future perspectives. New York: Springer; 2016. p. 303-338.
29. Adnan M, Ali S, Sheikh K, Amber R. Review on antibacterial activity of Himalayan medicinal plants traditionally used to treat pneumonia and tuberculosis. J Pharm Pharmacol. 2019;71(11):1599-1625.
30. Buenz EJ, Verpoorte R, Bauer BA. The ethnopharmacologic contribution to bioprospecting natural products. Ann Rev Pharmacol Toxicol. 2018;58(1):509-530.
31. Donia MS, Fischbach MA. Small molecules from the human microbiota. Science. 2015;349(6246):Article 1254766.
32. Chevrette MG, Currie CR. Emerging evolutionary paradigms in antibiotic discovery. J Ind Microbiol Biotechnol. 2019;46(3):257-271.
33. L. PK, Kandoi S, Misra R, S. V, K. R, Verma RS. The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine. Cytokine Growth Factor Rev. 2019;46:1-9.
34. Adnani N, Rajski SR, Bugni TS. Symbiosis-inspired approaches to antibiotic discovery. Nat Prod Rep. 2017;34(7):784-814.
35. Tanvir R, Javeed A, Bajwa AG. Endophyte bioprospecting in South Asian medicinal plants: An attractive resource for biopharmaceuticals. Appl Microbiol Biotechnol. 2017;101(5):1831-1844.
36. Davies J. Are antibiotics naturally antibiotics? J Ind Microbiol Biotechnol. 2006;33(7):496-499.
37. Pishchany G, Kolter R. On the possible ecological roles of antimicrobials. Mol Microbiol. 2020;113(3):580-587.
38. Dunnick JK, Nyska A. The toxicity and pathology of selected dietary herbal medicines. Toxicol Pathol. 2012;41(2):374-386.
39. Lam CL, Wong W, Fong DY. Chinese herbal medicine in the treatment of acute upper respiratory tract infection: A randomised, double blind, placebo-controlled clinical trial. Hong Kong Med J. 2009;15(Suppl 6):30-34.
40. Goodfellow M, Nouioui I, Sanderson R, Xie F, Bull AT. Rare taxa and dark microbial matter: Novel bioactive actinobacteria abound in Atacama Desert soils. Antonie van Leeuwenhoek. 2018;111(8):1315-1332.
41. Rangseekaew P, Pathom-Aree W. Cave actinobacteria as producers of bioactive metabolites. Front Microbiol. 2019;10:Article 387.
42. Stincone P, Brandelli A. Marine bacteria as source of antimicrobial compounds. Crit Rev Biotechnol. 2020;40(3):306-319.
43. Racine E, Gualtieri M. From worms to drug candidate: The story of odilorhabdins, a new class of antimicrobial agents. Front Microbiol. 2019;10:Article 2893.
44. Sayed AM, Hassan MHA, Alhadrami HA, Hassan HM, Goodfellow M, Rateb ME. Extreme environments: Microbiology leading to specialized metabolites. J Appl Microbiol. 2020;128(3):630-657.
45. Hughes CC, Fenical W. Antibacterials from the sea. Chemistry. 2010;16(42):12512-12525.
46. Foulston L. Genome mining and prospects for antibiotic discovery. Curr Opin Microbiol. 2019;51:1-8.
47. Ren H, Wang B, Zhao H. Breaking the silence: New strategies for discovering novel natural products. Curr Opin Biotechnol. 2017;48:21-27.
48. Secretariat of the Convention on Biological Diversity. The Convention on Biological Diversity. 1992. Available from: https://www.cbd.int/convention/. Accessed on: 30 May 2020.

49. Secretariat of the Convention on Biological Diversity. The Nagoya Protocol on Access and Benefit-Sharing. 2010. Available from: https://www.cbd.int/abs/. Accessed on: 30 May 2020.
50. Secretariat of the Convention on Biological Diversity. The Access and Benefit-Sharing Clearing-House (ABSCH). 2020. Available from: https://absch.cbd.int/. Accessed on: 30 May 2020.
51. Lee S, Jung PE, Lee Y. Publicly-funded biobanks and networks in East Asia. SpringerPlus. 2016;5(1):Article 1080.
52. Boundy-Mills KL, Glantschnig E, Roberts IN, Yurkov A, Casaregola S, Daniel H-M, et al. Yeast culture collections in the twenty-first century: New opportunities and challenges. Yeast. 2016;33(7):243-260.
53. United Nations Division for Ocean Affairs and the Law of the Sea (DOALOS). United Nations Convention on the Law of the Sea. DOALOS. 1982. Available from: http://www.un.org/depts/los/convention_agreements/convention_overview_convention.htm. Accessed on: 30 May 2020.
54. Secretariat of the Antarctic Treaty. The Protocol on Environmental Protection to the Antarctic Treaty. 1991. Available from: http://www.ats.aq/e/protocol.html. Accessed on: 30 May 2020.
55. Benson E. Endangered science: The regulation of research by the U.S. Marine Mammal Protection and Endangered Species Acts. Hist Stud Nat Sci. 2012;42(1):30-61.
56. Geraghty RJ, Capes-Davis A, Davis JM, Downward J, Freshney RI, Knezevic I, et al. Guidelines for the use of cell lines in biomedical research. Brit J Cancer. 2014;111(6):1021-1046.
57. Smith D, Da Silva M, Jackson J, Lyal C. Explanation of the Nagoya Protocol on Access and Benefit Sharing and its implication for microbiology. Microbiology. 2017;163(3):289-296.
58. Heinrich M, Scotti F, Andrade-Cetto A, Berger-González M, Echeverría J, Friso F, et al. Access and benefit sharing under the Nagoya Protocol – *quo vadis*? Six Latin American case studies assessing opportunities and risk. Front Pharmacol. in press.
59. Bye Jr RA. Voucher specimens in ethnobiological studies and publications. J Ethnobiol. 1986;6(1):1-8.
60. Huber JT. The importance of voucher specimens, with practical guidelines for preserving specimens of the major invertebrate phyla for identification. J Nat Hist. 1998;32(3):367-385.
61. Turney S, Cameron ER, Cloutier CA, Buddle CM. Non-repeatable science: Assessing the frequency of voucher specimen deposition reveals that most arthropod research cannot be verified. PeerJ. 2015;3:Article 1168.
62. Cooper JE. Anesthesia, analgesia, and euthanasia of invertebrates. ILAR J. 2011;52(2):196-204.
63. McDiarmid RW, Foster MS, Guyer C, Gibbons JW, Chernoff N, editors. Reptile biodiversity: Standard methods for inventory and monitoring. Los Angeles: University of California Press; 2012.
64. Caulfield T, Murdoch B. Genes, cells, and biobanks: Yes, there's still a consent problem. PLOS Biol. 2017;15(7):Article e2002654.
65. Eisenman SW, Tucker AO, Struwe L. Voucher specimens are essential for documenting source material used in medicinal plant investigations. J Med Act Plants. 2012;1(1):30-43.
66. Kiuru P, D'Auria MV, Muller CD, Tammela P, Vuorela H, Yli-Kauhaluoma J. Exploring marine resources for bioactive compounds. Planta Med. 2014;80(14):1234-1246.
67. Ncube B, Finnie JF, Van Staden J. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. S Afr J Bot. 2012;82:11-20.
68. Martinez-Klimova E, Rodríguez-Peña K, Sánchez S. Endophytes as sources of antibiotics. Biochem Pharmacol. 2017;134:1-17.
69. Tiwari K, Gupta RK. Rare actinomycetes: A potential storehouse for novel antibiotics. Crit Rev Biotechnol. 2012;32(2):108-132.
70. Mahajan GB, Balachandran L. Sources of antibiotics: Hot springs. Biochem Pharmacol. 2017;134:35-41.
71. Tortorella E, Tedesco P, Palma Esposito F, January GG, Fani R, Jaspars M, et al. Antibiotics from deep-sea microorganisms: Current discoveries and perspectives. Mar Drugs. 2018;16(10):Article 355.

72. Subramani R, Sipkema D. Marine rare actinomycetes: A promising source of structurally diverse and unique novel natural products. Mar Drugs. 2019;17(5):Article 249.
73. Tedeschi R, De Paoli P. Collection and preservation of frozen microorganisms. In: Dillner J, editor. Methods in biobanking. Totowa: Humana Press; 2011. p. 313-326.
74. Pilaquinga F, Morejón B, Ganchala D, Morey J, Piña N, Debut A, et al. Green synthesis of silver nanoparticles using *Solanum mammosum* L. (Solanaceae) fruit extract and their larvicidal activity against *Aedes aegypti* L. (Diptera: Culicidae). PLOS ONE. 2019;14(10):Article 0224109.
75. Kageyama M, Monk RR, Bradley RD, Edson GF, Baker RJ. The changing significance and definition of the biological voucher. In: Williams SL, Hawks CA, editors. Museum studies: Perspectives and innovations. Washington: Society for the Preservation of Natural History Collections; 2007. p. 257-264.
76. Smith D. Culture collections over the world. Int Microbiol. 2003;6(2):95-100.
77. Culley TM. Why vouchers matter in botanical research. Appl Plant Sci. 2013;1(11):Article 1300076.
78. Verma VC, Kharwar RN, Strobel GA. Chemical and functional diversity of natural products from plant associated endophytic fungi. Nat Prod Commun. 2009;4(11):1511-1532.
79. Besse A, Peduzzi J, Rebuffat S, Carré-Mlouka A. Antimicrobial peptides and proteins in the face of extremes: Lessons from archaeocins. Biochimie. 2015;118:344-355.
80. Cornforth DM, Foster KR. Competition sensing: The social side of bacterial stress responses. Nat Rev Microbiol. 2013;11(4):285-293.
81. Van der Meij A, Worsley SF, Hutchings MI, Van Wezel GP. Chemical ecology of antibiotic production by actinomycetes. FEMS Microbiol Rev. 2017;41(3):392-416.
82. Molnár K, Farkas E. Current results on biological activities of lichen secondary metabolites: A review. Z Naturforsch C. 2010;65(3-4):157-173.
83. Djinni I, Defant A, Kecha M, Mancini I. Actinobacteria derived from Algerian ecosystems as a prominent source of antimicrobial molecules. Antibiotics. 2019;8(4):Article E172.
84. Alcayaga-Miranda F, Cuenca J, Khoury M. Antimicrobial activity of mesenchymal stem cells: Current status and new perspectives of antimicrobial peptide-based therapies. Front Immunol. 2017;8:Article 339.
85. Manteca Á, Yagüe P. *Streptomyces* differentiation in liquid cultures as a trigger of secondary metabolism. Antibiotics. 2018;7(2):Article 41.
86. González-Lamothe R, Mitchell G, Gattuso M, Diarra MS, Malouin F, Bouarab K. Plant antimicrobial agents and their effects on plant and human pathogens. Int J Mol Sci. 2009;10(8):3400-3419.
87. Ratcliffe NA, Mello CB, Garcia ES, Butt TM, Azambuja P. Insect natural products and processes: New treatments for human disease. Insect Biochem Mol Biol. 2011;41(10):747-769.
88. Netzker T, Flak M, Krespach MKC, Stroe MC, Weber J, Schroeckh V, et al. Microbial interactions trigger the production of antibiotics. Curr Opin Microbiol. 2018;45:117-123.
89. Lewis K, Ausubel FM. Prospects for plant-derived antibacterials. Nat Biotechnol. 2006;24(12):1504-1507.
90. Kuć J. What's old and what's new in concepts of induced systemic resistance in plants, and its application. In: Tuzun S, Bent E, editors. Multigenic and induced systemic resistance in plants. Boston: Springer; 2006. p. 9-20.
91. Casanova-Torres ÁM, Goodrich-Blair H. Immune signaling and antimicrobial peptide expression in Lepidoptera. Insects. 2013;4(3):320-338.
92. De Moraes CN, Maia L, De Oliveira E, De Paula Freitas Dell'Aqua C, Chapwanya A, Da Cruz Landim-Alvarenga F, et al. Shotgun proteomic analysis of the secretome of bovine endometrial mesenchymal progenitor/stem cells challenged or not with bacterial lipopolysaccharide. Vet Immunol Immunopathol. 2017;187:42-47.
93. Hosseiniyan Khatibi SM, Kheyrolahzadeh K, Barzegari A, Rahbar Saadat Y, Vahed SZ. Medicinal signaling cells: A potential antimicrobial drug store. J Cell Physiol. in press.

94. Sutton MT, Fletcher D, Ghosh SK, Weinberg A, Van Heeckeren R, Kaur S, et al. Antimicrobial properties of mesenchymal stem cells: Therapeutic potential for cystic fibrosis infection, and treatment. Stem Cells Int. 2016;2016:Article 5303048.
95. Zhang C, Straight PD. Antibiotic discovery through microbial interactions. Curr Opin Microbiol. 2019;51:64-71.
96. Truong TT, Seyedsayamdost M, Greenberg EP, Chandler JR. A *Burkholderia thailandensis* acyl-homoserine lactone-independent LuxR homolog that activates production of the cytotoxin malleilactone. J Bacteriol. 2015;197(21):3456-3462.
97. Newman D. Screening and identification of novel biologically active natural compounds. F1000Research. 2017;6:Article 783.
98. Ueda K, Beppu T. Antibiotics in microbial coculture. J Antibiot. 2016;70:361-365.
99. Ochi K, Tanaka Y, Tojo S. Activating the expression of bacterial cryptic genes by *rpoB* mutations in RNA polymerase or by rare earth elements. J Ind Microbiol Biotechnol. 2014;41(2):403-414.
100. Wiese J, Imhoff JF. Marine bacteria and fungi as promising source for new antibiotics. Drug Dev Res. 2019;80(1):24-27.
101. Kasiri MM, Beer L, Nemec L, Gruber F, Pietkiewicz S, Haider T, et al. Dying blood mononuclear cell secretome exerts antimicrobial activity. Eur J Clin Invest. 2016;46(10):853-863.
102. Sarker SD, Latif Z, Gray AI, editors. Natural products isolation, 2nd edn. Totowa: Humana Press; 2006.
103. Gibbons S. Phytochemicals for bacterial resistance - strengths, weaknesses and opportunities. Planta Med. 2008;74(6):594-602.
104. Cushnie TPT, Cushnie B, Lamb AJ. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents. 2014;44(5):377-386.
105. Bucar F, Wube A, Schmid M. Natural product isolation - How to get from biological material to pure compounds. Nat Prod Rep. 2013;30(4):525-545.
106. Li S-L, Yan R, Tam Y-K, Lin G. Post-harvest alteration of the main chemical ingredients in *Ligusticum chuanxiong* Hort. (Rhizoma Chuanxiong). Chem Pharm Bull. 2007;55(1):140-144.
107. Lang K, Corrêa J, Wolff F, Da Silva GF, Malheiros A, Filho VC, et al. Biomonitored UHPLC-ESI-QTOF-$MS^2$ and HPLC-UV thermostability study of the aerial parts of *Sphagneticola trilobata* (L.) Pruski, Asteraceae. Talanta. 2017;167:302-309.
108. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. J Ethnopharmacol. 2006;106(3):290-302.
109. Houghton PJ, Howes M-J, Lee CC, Steventon G. Uses and abuses of *in vitro* tests in ethnopharmacology: Visualizing an elephant. J Ethnopharmacol. 2007;110(3):391-400.
110. Stafford GI, Jager AK, Van Staden J. Effect of storage on the chemical composition and biological activity of several popular South African medicinal plants. J Ethnopharmacol. 2005;97(1):107-115.
111. Cespedes CL, Alarcon J, Aqueveque PM, Lobo T, Becerra J, Balbontin C, et al. New environmentally-friendly antimicrobials and biocides from Andean and Mexican biodiversity. Environ Res. 2015;142:549-562.
112. Fallarero A, Hanski L, Vuorela P. How to translate a bioassay into a screening assay for natural products: General considerations and implementation of antimicrobial screens. Planta Med. 2014;80(14):1182-1199.
113. Niu G, Li W. Next-generation drug discovery to combat antimicrobial resistance. Trends Biochem Sci. 2019;44(11):961-972.
114. Doern CD. When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. J Clin Microbiol. 2014;52(12):4124-4128.
115. Tan JBL, Lim YY. Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. Food Chem. 2015;172:814-822.
116. Clinical Laboratory Standards Institute (CLSI). M02-A12 Performance standards for antimicrobial disk susceptibility tests; approved standard, 13th edn. Wayne: CLSI; 2018.

117. European Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST disk diffusion test methodology; version 8.0. European Society of Clinical Microbiology and Infectious Diseases. 2020. Available from: https://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/. Accessed on: 30 May 2020.
118. Amsterdam D, editor. Antibiotics in laboratory medicine, 6th edn. London: Wolters Kluwer; 2014.
119. Singh SB, Young K, Miesel L. Screening strategies for discovery of antibacterial natural products. Expert Rev Anti-infect Ther. 2011;9(8):589-613.
120. Moreno PRH, Da Costa-Issa FI, Rajca-Ferreira AK, Pereira MA, Kaneko TM. Native Brazilian plants against nosocomial infections: A critical review on their potential and the antimicrobial methodology. Curr Top Med Chem. 2013;13(24):3040-3078.
121. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100(1):80-84.
122. Li JW-H, Vederas JC. Drug discovery and natural products: End of an era or an endless frontier? Science. 2009;325(5937):161-165.
123. Rajniak J, Giehl R, Chang E, Murgia I, Von Wirén N, Sattely E. Biosynthesis of redox-active metabolites in response to iron deficiency in plants. Nat Chem Biol. 2018;14(5):442-450.
124. Niedermeyer THJ. Anti-infective natural products from cyanobacteria. Planta Med. 2015;81(15):1309-1325.
125. Fratini F, Cilia G, Turchi B, Felicioli A. Insects, arachnids and centipedes venom: A powerful weapon against bacteria. A literature review. Toxicon. 2017;130:91-103.
126. Genilloud O. Natural products discovery and potential for new antibiotics. Curr Opin Microbiol. 2019;51:81-87.
127. Dewanjee S, Gangopadhyay M, Bhattacharya N, Khanra R, Dua TK. Bioautography and its scope in the field of natural product chemistry. J Pharm Anal. 2015;5(2):75-84.
128. Kovács JK, Horváth G, Kerényi M, Kocsis B, Emödy L, Schneider G. A modified bioautographic method for antibacterial component screening against anaerobic and microaerophilic bacteria. J Microbiol Methods. 2016;123:13-17.
129. Choma MI, Jesionek W. TLC-direct bioautography as a high throughput method for detection of antimicrobials in plants. Chromatography. 2015;2(2):225-238.
130. Valgas C, Souza SM, Smânia EFA, Smânia Jr. A. Screening methods to determine antibacterial activity of natural products. Braz J Microbiol. 2007;38(2):369-380.
131. Ríos JL, Recio MC, Villar A. Screening methods for natural products with antimicrobial activity: A review of the literature. J Ethnopharmacol. 1988;23(2):127-149.
132. Fennell CW, Lindsey KL, McGaw LJ, Sparg SG, Stafford GI, Elgorashi EE, et al. Assessing African medicinal plants for efficacy and safety: Pharmacological screening and toxicology. J Ethnopharmacol. 2004;94(2):205-217.
133. Jaskiewicz M, Orlowska M, Olizarowicz G, Migon D, Grzywacz D, Kamysz W. Rapid screening of antimicrobial synthetic peptides. Int J Pept Res Ther. 2016;22:155-161.
134. Cushnie TPT, O'Driscoll NH, Lamb AJ. Morphological and ultrastructural changes in bacterial cells as an indicator of antibacterial mechanism of action. Cell Mol Life Sci. 2016;73(23):4471-4492.
135. Clinical Laboratory Standards Institute (CLSI). M26-A Methods for determining bactericidal activity of antimicrobial agents; approved guideline. Wayne: CLSI; 1999.
136. Delaquis PJ, Stanich K. Antilisterial properties of cilantro essential oil. J Essent Oil Res. 2004;16(5):409-414.
137. Oliveira FS, Freitas TS, Cruz RPD, Costa MDS, Pereira RLS, Quintans-Júnior LJ, et al. Evaluation of the antibacterial and modulatory potential of alpha-bisabolol, beta-cyclodextrin and alpha-bisabolol/beta-cyclodextrin complex. Biomed Pharmacother. 2017;92:1111-1118.
138. Liu X, Chen C, He W, Huang P, Liu M, Wang Q, et al. Exploring anti-TB leads from natural products library originated from marine microbes and medicinal plants. Antonie van Leeuwenhoek. 2012;102(3):447-461.

139. Clinical Laboratory Standards Institute (CLSI). M07 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 11th edn. Wayne: CLSI; 2018.
140. International Organization for Standardization (ISO). ISO 20776-1:2019 Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices - Part 1: Broth micro-dilution reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. Geneva: ISO; 2019.
141. European Committee on Antimicrobial Susceptibility Testing (EUCAST). MIC determination of non-fastidious and fastidious organisms. European Society of Clinical Microbiology and Infectious Diseases. 2020. Available from: http://www.eucast.org/ast_of_bacteria/mic_determination/. Accessed on: 30 May 2020.
142. Echeverría J, Opazo J, Mendoza L, Urzúa A, Wilkens M. Structure-activity and lipophilicity relationships of selected antibacterial natural flavones and flavanones of Chilean flora. Molecules. 2017;22(4):Article 608.
143. DeVito JA, Mills JA, Liu VG, Agarwal A, Sizemore CF, Yao Z, et al. An array of target-specific screening strains for antibacterial discovery. Nat Biotechnol. 2002;20(5):478-483.
144. Singh SB, Phillips JW, Wang J. Highly sensitive target-based whole-cell antibacterial discovery strategy by antisense RNA silencing. Curr Opin Drug Discov Dev. 2007;10(2):160-166.
145. Van den Driessche F, Rigole P, Brackman G, Coenye T. Optimization of resazurin-based viability staining for quantification of microbial biofilms. J Microbiol Methods. 2014;98(Supplement C):31-34.
146. Brackman G, Coenye T. *In vitro* and *in vivo* biofilm wound models and their application. In: Donelli G, editor. Advances in microbiology, infectious diseases and public health. New York: Springer; 2016. p. 15-32.
147. Brackman G, Garcia-Fernandez MJ, Lenoir J, De Meyer L, Remon J-P, De Beer T, et al. Dressings loaded with cyclodextrin-hamamelitannin complexes increase *Staphylococcus aureus* susceptibility toward antibiotics both in single as well as in mixed biofilm communities. Macromol Biosci. 2016;16(6):859-869.
148. Van Vuuren SF. Antimicrobial activity of South African medicinal plants. J Ethnopharmacol. 2008;119(3):462-472.
149. Lorian V, editor. Antibiotics in laboratory medicine, 5th edn. London: Lippincott, Williams, and Wilkins; 2005.
150. Balakin K, Savchuk N, Tetko I. *In silico* approaches to prediction of aqueous and DMSO solubility of drug-like compounds: Trends, problems and solutions. Curr Med Chem. 2006;13(2):223-241.
151. Basch H, Gadebusch HH. *In vitro* antimicrobial activity of dimethylsulfoxide. Appl Microbiol. 1968;16(12):1953-1954.
152. Mishra A, Dobritsa SV, Crouch M-L, Rabenstein J, Lee JXY, Dhakshinamoorthy S. Establishment and validation of a 384-well antibacterial assay amenable for high-throughput screening and combination testing. J Microbiol Methods. 2015;118:173-175.
153. Maes J, Verlooy L, Buenafe OE, De Witte PAM, Esguerra CV, Crawford AD. Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. PLOS ONE. 2012;7(10):Article e43850.
154. Hu J, Akula N, Wang N. Development of a microemulsion formulation for antimicrobial SecA inhibitors. PLOS ONE. 2016;11(3):Article e0150433.
155. Mayaud L, Carricajo A, Zhiri A, Aubert G. Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. Lett Appl Microbiol. 2008;47(3):167-173.
156. Ghaderi L, Moghimi R, Aliahmadi A, McClements DJ, Rafati H. Development of antimicrobial nanoemulsion-based delivery systems against selected pathogenic bacteria using a thymol-rich *Thymus daenensis* essential oil. J Appl Microbiol. 2017;123(4):832-840.
157. Dhondikubeer R, Bera S, Zhanel GG, Schweizer F. Antibacterial activity of amphiphilic tobramycin. J Antibiot. 2012;65(10):495-498.

158. Sutherland CA, Nicolau DP. To add or not to add polysorbate 80: Impact on colistin MICs for clinical strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* and quality controls. J Clin Microbiol. 2014;52(10):3810.
159. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318-327.
160. Grzelak EM, Choules MP, Gao W, Cai G, Wan B, Wang Y, et al. Strategies in anti-*Mycobacterium tuberculosis* drug discovery based on phenotypic screening. J Antibiot. 2019;72(10):719-728.
161. Katsuno K, Burrows JN, Duncan K, Van Huijsduijnen RH, Kaneko T, Kita K, et al. Hit and lead criteria in drug discovery for infectious diseases of the developing world. Nat Rev Drug Discov. 2015;14(11):751-758.
162. French GL. Bactericidal agents in the treatment of MRSA infections - the potential role of daptomycin. J Antimicrob Chemother. 2006;58(6):1107-1117.
163. Gonzalez N, Sevillano D, Alou L, Cafini F, Gimenez M-J, Gomez-Lus M-L, et al. Influence of the MBC/MIC ratio on the antibacterial activity of vancomycin versus linezolid against methicillin-resistant *Staphylococcus aureus* isolates in a pharmacodynamic model simulating serum and soft tissue interstitial fluid concentrations reported in diabetic patients. J Antimicrob Chemother. 2013;68(10):2291-2295.
164. Cushnie TPT, Lamb AJ. Recent advances in understanding the antibacterial properties of flavonoids. Int J Antimicrob Agents. 2011;38(2):99-107.
165. Green AE, Rowlands RS, Cooper RA, Maddocks SE. The effect of the flavonol morin on adhesion and aggregation of *Streptococcus pyogenes*. FEMS Microbiol Lett. 2012;333(1):54-58.
166. Moloney MG. Natural products as a source for novel antibiotics. Trends Pharmacol Sci. 2016;37(8):689-701.
167. Sittampalam GS, Coussens NP, Brimacombe K, Grossman A, Arkin M, Auld D, et al., editors. Assay guidance manual. Bethesda: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2019.
168. Cotton S. Handle with care – the world's five deadliest poisons. The Conversation. 2016. Available from: https://theconversation.com/handle-with-care-the-worlds-five-deadliest-poisons-56089. Accessed on: 30 May 2020.
169. Purchase IFH, Botham PA, Bruner LH, Flint OP, Frazier JM, Stokes WS. Workshop overview: Scientific and regulatory challenges for the reduction, refinement, and replacement of animals in toxicity testing. Toxicol Sci. 1998;43(2):86-101.
170. Bluemel J. Toxicity testing in the 21st century: Challenges and perspectives. J Drug Metab Toxicol. 2012;3(5):Article 1000e112.
171. Hammond T, Allen P, Birdsall H. Is there a space-based technology solution to problems with preclinical drug toxicity testing? Pharm Res. 2016;33(7):1545-1551.
172. Kreiling R, Gehrke H, Broschard TH, Dreeßen B, Eigler D, Hart D, et al. *In chemico*, *in vitro* and *in vivo* comparison of the skin sensitizing potential of eight unsaturated and one saturated lipid compounds. Regul Toxicol Pharmacol. 2017;90:262-276.
173. May JE, Xu J, Morse HR, Avent ND, Donaldson C. Toxicity testing: The search for an *in vitro* alternative to animal testing. Brit J Biomed Sci. 2009;66(3):160-165.
174. Csöbönyeiová M, Polák S, Danišovic L. Toxicity testing and drug screening using iPSC-derived hepatocytes, cardiomyocytes, and neural cells. Can J Physiol Pharmacol. 2016;94(7):687-694.
175. Pelkonen O. Predictive toxicity: Grand challenges. Front Pharmacol. 2010;1:Article 3.
176. Soldatow VY, LeCluyse EL, Griffith LG, Rusyn I. *In vitro* models for liver toxicity testing. Toxicol Res. 2013;2(1):23-39.
177. Schoonen WGEJ, Westerink WMA, Van de Water FM, Horbach GJ. High content screening for *in vitro* toxicity testing. Eur Pharm Rev. 2011;16(3):50-55.
178. Halliwell WH. Cationic amphiphilic drug-induced phospholipidosis. Toxicol Pathol. 1997;25(1):53-60.

179. Bernuci ZK, Iwanaga CC, Fernandez-Andrade MC, Lorenzetti BF, Torres-Santos CE, Faiões DV, et al. Evaluation of chemical composition and antileishmanial and antituberculosis activities of essential oils of *Piper* species. Molecules. 2016;21(12):Article 1698.
180. Guy CS, Tichauer E, Kay GL, Phillips DJ, Bailey TL, Harrison J, et al. Identification of the anti-mycobacterial functional properties of piperidinol derivatives. Brit J Pharmacol. 2017;174(14):2183-2193.
181. Chudzik B, Czernel G, Miaskowski A, Gagoś M. Amphotericin B-copper(II) complex shows improved therapeutic index *in vitro*. Eur J Pharm Sci. 2017;97:9-21.
182. Jaiswal S, Mishra P. Antimicrobial and antibiofilm activity of curcumin-silver nanoparticles with improved stability and selective toxicity to bacteria over mammalian cells. Med Microbiol Immunol. 2018;207(1):39-53.
183. Khara JS, Priestman M, Uhía I, Hamilton MS, Krishnan N, Wang Y, et al. Unnatural amino acid analogues of membrane-active helical peptides with anti-mycobacterial activity and improved stability. J Antimicrob Chemother. 2016;71(8):2181-2191.
184. Sowmya PV, Poojary B, Kumar V, Vishwanatha U, Shetty P. Fluorinated pyrrole incorporated 2-thiazolyl hydrazone motifs: A new class of antimicrobial and anti tuberculosis agents. Arch Pharm Res. in press.
185. Moreira W, Aziz DB, Dick T. Boromycin kills mycobacterial persisters without detectable resistance. Front Microbiol. 2016;7:Article 199.
186. Mitachi K, Yun HG, Kurosu SM, Eslamimehr S, Lemieux MR, Klaic L, et al. Novel FR-900493 analogues that inhibit the outgrowth of *Clostridium difficile* spores. ACS Omega. 2018;3(2):1726-1739.
187. Krátký M, Dzurková M, Janoušek J, Konečná K, Trejtnar F, Stolaríková J, et al. Sulfadiazine salicylaldehyde-based Schiff bases: Synthesis, antimicrobial activity and cytotoxicity. Molecules. 2017;22(9):Article 1573.
188. Kwon J, Mistry T, Ren J, Johnson ME, Mehboob S. A novel series of enoyl reductase inhibitors targeting the ESKAPE pathogens, *Staphylococcus aureus* and *Acinetobacter baumannii*. Bioorg Med Chem. 2018;26(1):65-76.
189. Li X, Zhang R, Zhao B, Lossin C, Cao Z. Cardiotoxicity screening: A review of rapid-throughput *in vitro* approaches. Arch Toxicol. 2016;90(8):1803-1816.
190. Lester RM, Olbertz J. Early drug development: Assessment of proarrhythmic risk and cardiovascular safety. Expert Rev Clin Pharmacol. 2016;9(12):1611-1618.
191. Roggen EL. *In vitro* toxicity testing in the twenty-first century. Front Pharmacol. 2011;2:Article 3.
192. Mei N, Guo X, Moore MM. Methods for using the mouse lymphoma assay to screen for chemical mutagenicity and photo-mutagenicity. In: Caldwell GW, Yan Z, editors. Optimization in drug discovery: *In vitro* methods. Totowa: Humana Press; 2014. p. 561-592.
193. Organisation for Economic Co-operation and Development (OECD). OECD iLibrary. OECD. 2020. Available from: https://www.oecd-ilibrary.org/oecd/about. Accessed on: 30 May 2020.
194. Anadón A, Martínez MA, Castellano V, Martínez-Larrañaga MR. The role of *in vitro* methods as alternatives to animals in toxicity testing. Expert Opin Drug Metab Toxicol. 2014;10(1):67-79.
195. Riss TL, Moravec RA, Niles AL. Cytotoxicity testing: Measuring viable cells, dead cells, and detecting mechanism of cell death. In: Stoddart MJ, editor. Mammalian cell viability: Methods and protocols. Totowa: Humana Press; 2011. p. 103-114.
196. Aslantürk ÖS. Chapter 1: *In vitro* cytotoxicity and cell viability assays: Principles, advantages, and disadvantages. In: Genotoxicity - a predictable risk to our actual world. London: IntechOpen; 2017. p. 1-17.
197. Cho M-H, Niles A, Huang R, Inglese J, Austin CP, Riss T, et al. A bioluminescent cytotoxicity assay for assessment of membrane integrity using a proteolytic biomarker. Toxicol in Vitro. 2008;22(4):1099-1106.
198. Vinken M, Vanhaecke T, Rogiers V. Primary hepatocyte cultures as *in vitro* tools for toxicity testing: *Quo vadis*? Toxicol in Vitro. 2012;26(3):541-544.

199. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Instit. 1990;82(13):1107-1112.
200. Orellana EA, Kasinski AL. Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. Bio-protocol. 2016;6(21):Article e1984.
201. Vega-Avila E, Pugsley MK. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc West Pharmacol Soc. 2011;54:10-14.
202. Kohlmeier F, Maya-Mendoza A, Jackson DA. EdU induces DNA damage response and cell death in mESC in culture. Chromosome Res. 2013;21(1):87-100.
203. Borenfreund E, Puerner JA. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol Lett. 1985;24(2):119-124.
204. Repetto G, Del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 2008;3(7):1125-1131.
205. Sadeh N, Oni-Biton E, Segal M. Acute live/dead assay for the analysis of toxic effects of drugs on cultured neurons. Bio-protocol. 2016;6(15):Article e1889.
206. Shukla SJ, Huang R, Austin CP, Xia M. The future of toxicity testing: A focus on *in vitro* methods using a quantitative high-throughput screening platform. Drug Discov Today. 2010;15(23):997-1007.
207. Sirenko O, Hesley J, Rusyn I, Cromwell EF. High-content assays for hepatotoxicity using induced pluripotent stem cell-derived cells. Assay Drug Dev Technol. 2014;12(1):43-54.
208. Tramer F, Da Ros T, Passamonti S. Chapter 15: Screening of fullerene toxicity by hemolysis assay. In: Reineke J, editor. Nanotoxicity: Methods and protocols. Totowa: Humana Press; 2012. p. 203-217.
209. Pessina A, Bonomi A. CFU-GM assay for evaluation of drug myelotoxic activity. Curr Protoc Toxicol. 2007;34(1):20.2.1-20.2.18.
210. Sakamuru S, Attene-Ramos MS, Xia M. Mitochondrial membrane potential assay. In: Zhu H, Xia M, editors. High-throughput screening assays in toxicology. New York: Springer; 2016. p. 17-22.
211. Mortelmans K, Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res. 2000;455(1-2):29-60.
212. Houghton P, Fang R, Techatanawat I, Steventon G, Hylands PJ, Lee CC. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. Methods. 2007;42(4):377-387.
213. Pace RT, Burg KJL. Toxic effects of resazurin on cell cultures. Cytotechnology. 2015;67(1):13-17.
214. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2015;111(1):A3B1-A3B2.
215. Borenfreund E, Borrero O. *In vitro* cytotoxicity assays. Potential alternatives to the Draize ocular allergy test. Cell Biol Toxicol. 1984;1(1):55-65.
216. Mennecozzi M, Landesmann B, Harris GA, Liska R, Whelan M. Hepatotoxicity screening taking a mode-of-action approach using HepaRG cells and HCA. ALTEX Proc. 2012;1(12):193-204.
217. Boubaker-Elandalousi R, Mekni-Toujani M, Kaabi B, Larbi I, Diouani M-F, Gharbi M, et al. Non-cytotoxic *Thymus capitata* extracts prevent Bovine herpesvirus-1 infection in cell cultures. BMC Vet Res. 2014;10:Article 231.
218. European Union Reference Laboratory for Alternatives to Animal Testing (EURL). Validated test methods. European Commission. 2020. Available from: https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/validated-test-methods. Accessed on: 30 May 2020.
219. Rodríguez-Burford C, Oelschlager DK, Talley LI, Barnes MN, Partridge EE, Grizzle WE. The use of dimethylsulfoxide as a vehicle in cell culture experiments using ovarian carcinoma cell lines. Biotech Histochem. 2003;78(1):17-21.
220. Singh M, McKenzie K, Ma X. Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. J Biotech Res. 2017;8:78-82.
221. Nygren P, Larsson R, Lindh E, Rastad J, Akerström G, Gylfe E. Dimethyl sulfoxide inhibits proliferation but not hypertrophy and functional dedifferentiation of bovine parathyroid cells in culture. Res Commun Chem Pathol Pharmacol. 1988;62(2):161-171.

222. Vandhana S, Deepa PR, Aparna G, Jayanthi U, Krishnakumar S. Evaluation of suitable solvents for testing the anti-proliferative activity of triclosan - a hydrophobic drug in cell culture. Indian J Biochem Biophys. 2010;47(3):166-171.
223. Yu KO, Fisher JW, Burton Jr GA, Tillitt DE. Carrier effects of dosing the H4IIE cells with 3,3',4,4´tetrachlorobiphenyl (PCB77) in dimethyl sulfoxide or isooctane. Chemosphere. 1997;35(4):895-904.
224. Mohamed EA, Abu Hashim II, Yusif RM, Suddek GM, Shaaban AAA, Badria FAE. Enhanced *in vitro* cytotoxicity and anti-tumor activity of vorinostat-loaded pluronic micelles with prolonged release and reduced hepatic and renal toxicities. Eur J Pharm Sci. 2017;96:232-242.
225. Piazzini V, Monteforte E, Luceri C, Bigagli E, Bilia AR, Bergonzi MC. Nanoemulsion for improving solubility and permeability of *Vitex agnus-castus* extract: Formulation and *in vitro* evaluation using PAMPA and Caco-2 approaches. Drug Deliv. 2017;24(1):380-390.
226. Timm M, Saaby L, Moesby L, Hansen EW. Considerations regarding use of solvents in *in vitro* cell based assays. Cytotechnology. 2013;65(5):887-894.
227. Xie Y, Yao Y. Octenylsuccinate hydroxypropyl phytoglycogen enhances the solubility and *in-vitro* antitumor efficacy of niclosamide. Int J Pharm. 2018;535(1):157-163.
228. Jamalzadeh L, Ghafoori H, Sariri R, Rabuti H, Nasirzade J, Hasani H, et al. Cytotoxic effects of some common organic solvents on MCF-7, RAW-264.7 and human umbilical vein endothelial cells. Avicenna J Med Biochem. 2016;4(1):Article e33453.
229. Kumar A, Boradia VM, Thakare R, Singh AK, Gani Z, Das S, et al. Repurposing ethyl bromopyruvate as a broad-spectrum antibacterial. J Antimicrob Chemother. 2019;74(4):912-920.
230. Muller PY, Milton MN. The determination and interpretation of the therapeutic index in drug development. Nat Rev Drug Discov. 2012;11(10):751-761.
231. King A, Chakrabarty S, Zhang W, Zeng X, Ohman DE, Wood LF, et al. High antimicrobial effectiveness with low hemolytic and cytotoxic activity for PEG/quaternary copolyoxetanes. Biomacromolecules. 2014;15(2):456-467.
232. Vaddady PK, Lee RE, Meibohm B. *In vitro* pharmacokinetic/pharmacodynamic models in anti-infective drug development: focus on TB. Future Med Chem. 2010;2(8):1355-1369.
233. Ekins S, Spektor AC, Clark AM, Dole K, Bunin BA. Collaborative drug discovery for More Medicines for Tuberculosis (MM4TB). Drug Discov Today. 2017;22(3):555-565.
234. Koehn FE. Drug discovery from natural products. Nat Rev Drug Discov. 2009;8(8):678.
235. Pope CF, O'Sullivan DM, McHugh TD, Gillespie SH. A practical guide to measuring mutation rates in antibiotic resistance. Antimicrob Agents Chemother. 2008;52(4):1209-1214.
236. Wagner BK, Schreiber SL. The power of sophisticated phenotypic screening and modern mechanism-of-action methods. Cell Chem Biol. 2016;23(1):3-9.
237. Pauli GF, Chen S-N, Simmler C, Lankin DC, Gödecke T, Jaki BU, et al. Importance of purity evaluation and the potential of quantitative $^{1}$H NMR as a purity assay. J Med Chem. 2014;57(22):9220-9231.
238. Baell JB. Feeling Nature's PAINS: Natural products, natural product drugs, and pan assay interference compounds (PAINS). J Nat Prod. 2016;79(3):616-628.

**Supplementary Table 1 for the Pharmaceutical Research journal article 'Bioprospecting for Antibacterial Drugs: A Multidisciplinary Perspective on Natural Product Source Material, Bioassay Selection and Avoidable Pitfalls'**

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**Supplementary Table 1** Different sources of natural products from the (a) bacterial, (b) archaeal, (c) fungal, (d) protozoan, (e) chromistan, (f) plant and (g) animal kingdoms, the rationale(s) for screening them for antibacterial activity, and associated advantages and disadvantages.

| Source | Rationale for screening | Advantages/Disadvantages | Reference(s) |
|---|---|---|---|
| **(a) Bacteria** | | | |
| *Soil bacteria* | *Ecological:* There is an argument that soil bacteria (especially nonmotile species) produce antibiotics to inhibit or kill microbial competitors growing in their proximity.<br>*Ethnomedical:* In Ancient Greece, 'medicinal soil' was used to treat gonorrhea & eye infections.<br>*Historical:* Soil bacteria have been the world's main source of antibacterial drugs, the actinomycetes alone providing 70-80% of antibiotics in current use (eg. streptomycin). | *Advantages:* Only a small fraction of soil actinomycetes (just $10^7$ out of more than $10^{26}$) have been screened for activity, so it is likely that many more antibiotics await discovery. Other even less explored soil bacteria (eg. obligate anaerobes, myxobacteria) have recently been shown capable of producing potent antibiotics too (eg. closthioamide, coralmycins A & B).<br>*Disadvantages:* With actinomycetes, high throughput screening & dereplication are necessary as less than 1 in 10 million bacteria produce novel antibiotics but 1 in 100 produce streptomycin. The multiplication time of some soil bacteria (eg. actinomycetes) is extremely slow. | (1-9) |
| *Symbionts of plants* | *Ecological:* Endophytic bacteria may have evolved chemical-based mechanisms of competing with other microorganisms (incl. bacteria) for the microhabitat within their host plants.<br>*Ethnomedical:* Endophytic bacteria associate with specific hosts, so the anti-infective activity ascribed to some medicinal plants may actually be due to bacterial natural products. Some epiphytic cyanolichens (eg. *Lobaria pulmonaria*) have been used in traditional medicine to treat bacterial infection. | *Advantages:* Endophytic bacteria do not usually harm their hosts, so natural products obtained from them may be less toxic to eukaryotic cells than those from non-symbionts.<br>*Disadvantages:* Endophytic bacteria can be difficult to culture *in vitro* if they have become dependent on their host plant for nutrients. | (10-13) |
| *Symbionts of insects* | *Ecological:* Insect 'farmers' (eg. pine beetles & attinine ants that, respectively, tend to the plants & fungi they eat) use antibiotic-producing bacterial symbionts to protect their crops from microbial pathogens. Carnivorous insects (eg. digger wasps) do likewise with their prey.<br>*Ethnomedical:* The antibacterial activity of honey, a traditional medicine used to treat wounds, has been partially attributed to compounds synthesized by bacterial symbionts of *Apis mellifera*. | *Advantages:* Thousands of insect species participate in agriculture, so this could potentially be a rich source of antibiotics.<br>*Disadvantages:* There are a growing number of ethical issues related to the use of insects in research (eg. declining insect populations & the need to conserve vulnerable & endangered species; emerging evidence that insects may experience emotive states comparable to those of vertebrates & cephalopods). | (13-19) |
| *Symbionts of higher animals* | *Ecological:* Microbiota bacteria produce bacteriocins & other types of antibacterial compound to inhibit the growth of competitors.<br>*Ethnomedical:* In fourth-century China, oral ingestion of fecal matter (the first documented use of fecal microbiota transplants) was sometimes prescribed to treat severe diarrhea.<br>*Historical:* The proven clinical efficacy of fecal microbiota transplants against *Clostridium difficile* infections (usually delivered by enema nowadays rather than oral ingestion) suggests that microbiota bacteria could be a useful source of antibacterial | *Advantages:* Some microbiota bacteria produce antibiotics that inhibit pathogenic but not commensal bacteria (eg. *Lactobacillus gasseri* produces lactocillin). Bacteriocins can have MICs in the picomolar to nanomolar range (eg. colicins), dual mechanisms of action (eg. microcins), & broad-spectrum (incl. Gram-negative) activity (eg. microcins, lantibiotics). Microbiome mining is now sufficiently advanced that antibacterial screening can be directed towards certain body sites & genera (eg. oral lactobacilli).<br>*Disadvantages:* Some bacteriocins (eg. colicins) have very narrow | (17, 18, 20-26) |

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| | compounds. | spectrum activity. Research with higher animals (vertebrates & cephalopods) requires ethical approval. | |
| *Marine bacteria* | *Ecological:* There is an argument that marine bacteria which dominate many environments (eg. *Pseudovibrio* spp.) may produce chemicals to outcompete other bacteria. The ecological argument for antibiotic production is less strong for autotrophs (eg. cyanobacteria) as they do not compete with heterotrophs for organic nutrients. | *Advantages:* Marine bacteria have been screened less extensively than terrestrial bacteria. Because they can be cultured in the laboratory, smaller samples are needed compared to other marine organisms. With autotrophs, natural product isolation is less complicated than other bacteria as they can be cultured using inorganic salt solutions. Several potent antibiotics have already been identified, including one (PM181104) with MICs in the nanomolar range. If an antibacterial lead is identified, subsequent scale-up of production is inexpensive because bacteria can be cultured in bioreactors.<br>*Disadvantages:* Marine actinobacteria are more difficult to culture than terrestrial actinobacteria. Many of the cyanobacterial natural products isolated so far have been toxic to mammalian cells. | (27-37) |
| **(b) Archaea** | *Ecological:* Archaea produce archaeocins to inhibit the growth of microbial competitors. | *Advantages:* Because they were discovered relatively recently, archaea have been underexplored as a source of antibacterial natural products.<br>*Disadvantages:* Most archaeocins isolated so far have had very narrow spectrum anti-archaeal activity. | (38, 39) |
| **(c) Fungi**<br>*Soil fungi* | *Ecological:* Some soil fungi (eg. coprophilous fungi) occupy highly competitive niches & may have evolved chemical-based mechanisms to inhibit their microbial competitors.<br>*Ethnomedical:* Some civilizations (eg. Ancient Egypt & Greece) applied molds to skin & wound infections.<br>*Historical:* Soil fungi such as *Penicillium chrysogenum* have been the source of several β-lactam antibiotics (incl. penicillin). Also, *Acremonium fusidioides* (formerly *Fusidium coccineum*), isolated from monkey dung, produces fusidic acid. | *Advantages:* The taxonomy of soil fungi is now sufficiently advanced that antibacterial screening can be directed towards the least-explored & most pathway-novel species.<br>*Disadvantages:* Because soil has been screened for antibiotic-producing fungi more than other sources, it can be challenging to isolate novel genera & species (special techniques such as dilution-to-extinction culturing & pre-pasteurization of soil have been developed to try & overcome this problem). | (40, 41) |
| *Symbionts of plants* | *Ecological:* Endophytic fungi may have evolved chemical-based mechanisms of competing with other microorganisms (incl. bacteria) for the microhabitat within their host plants.<br>*Ethnomedical:* Bioactive constituents have been isolated from the endophytic fungi of some medicinal plants, & may be responsible for the reported activities of these plants. Some epiphytic cyanolichens (eg. *Lobaria pulmonaria*) have been used in traditional medicine to treat bacterial infection. | *Advantages:* Endophytic fungal species differ considerably from non-symbiont species. The taxonomy of endophytic fungi is now sufficiently advanced that new species (which are more likely to produce novel compounds) can readily be distinguished from known species. Screening has already yielded several potent antibiotics (eg. altersetin, penicibrocazine C).<br>*Disadvantages:* Antibiotics isolated so far have not been sufficiently selective in their toxicity &, if progressible, will require considerable medicinal chemistry optimization. | (12, 41-44) |
| *Symbionts of insects* | *Ecological:* Insect 'farmers' (eg. the fungus-growing ant species | *Advantages:* Thousands of insect species participate in agriculture, | (16, 19) |

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| | *Cyphomyrmex cosatus*) use antibiotic-producing fungal symbionts (eg. *Lepiota* sp.) to protect their crops from microbial pathogens. | so this could potentially be a rich source of antibiotics.<br>*Disadvantages:* There are a growing number of ethical issues related to the use of insects in research (eg. need for conservation & humane treatment of insects). | |
| *Higher terrestrial fungi* | *Ecological:* Higher fungi produce antimicrobial peptides as part of their innate immune system. They produce antibacterial lactones (eg. calopins) too.<br>*Ethnomedical:* Some mushrooms (eg. *Ganoderma lucidum*) have been used in traditional medicine for infection treatment (incl. infections of possible bacterial etiology).<br>*Historical:* The mushroom *Citopilus passeckerianus* produces pleuromutilin, a natural product from which the pleuromutilin class of antibiotics was derived. | *Advantages:* Some antimicrobial peptides have been shown to target cell structures unique to bacteria (eg. plectasin inhibits peptidoglycan synthesis), reducing the risk of nonspecific toxicity sometimes associated with these compounds.<br>*Disadvantages:* Obstacles associated with antimicrobial peptides include susceptibility to proteolytic degradation, low bioavailability, & sensitivity to salt, pH & serum. | (45-51) |
| *Marine fungi* | *Ecological:* Seawater contains ~$10^6$ bacteria/ml, so marine fungi are more likely than their terrestrial counterparts to have evolved chemical means of inhibiting bacterial competitors & pathogens. | *Advantages:* Marine fungi have been screened less extensively than terrestrial fungi. Several potent antibiotics have already been identified (eg. pestalachloride D, spiromastixone J). If an antibacterial lead is identified, subsequent scale-up of production is inexpensive because fungi can be cultured in bioreactors.<br>*Disadvantages:* Fungi isolated from marine environments have thus far been very similar to their terrestrial counterparts, & their natural products merely derivatives of known molecules. | (27, 30, 32, 35, 36, 41, 52, 53) |
| **(d) Protozoa**<br>*Dinoflagellates* | *Ecological:* Marine protozoa (eg. *Amphidinium klebsii*) outcompete fungi & diatoms growing in their proximity by producing inhibitory compounds (eg. the amphidinols), & may have evolved a similar chemical-based mechanism to compete with the many bacteria ($10^6$/ml) also present. | *Advantages:* Protozoa have been screened less extensively than organisms in the bacterial, fungal & plant kingdoms. | (54) |
| **(e) Chromista**<br>*Brown algae, diatoms & golden algae* | *Ecological:* Seawater contains ~$10^6$ bacteria/ml, so marine organisms such as brown algae may have evolved chemical defenses to protect themselves against infection.<br>*Ethnomedical:* Some brown algae (eg. *Fucus vesiculosus, Saccharina latissima, Ulva lactuca*) have been used in traditional medicine for infection treatment (eg. acne, syphilis, tuberculosis) & prevention (eg. burn & wound infections). | *Advantages:* Chromista have been screened less extensively than organisms in the bacterial, fungal & plant kingdoms. At least one potent antimycobacterial compound has already been identified (the carotenoid fucoxanthin).<br>*Disadvantages:* Extracting bioactive compounds is challenging with some chromistan species as their cell walls contain polysaccharides (eg. cellulose, algin) & other compounds (eg. silica) that impede the passage of extraction solvents into cells. | (30, 52, 55-60) |
| **(f) Plants**<br>*Terrestrial plants* | *Ecological:* Plants produce both constitutive & inducible secondary metabolites (phytoanticipins & phytoalexins) to protect themselves from microbial infection (though it has been | *Advantages:* Individual species can be rationally selected for testing based on their ethnomedicinal use. Some potent compounds have been discovered (incl. an antistaphylococcal terthiophene & | (61-70) |

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| | hypothesized that these typically act in synergy & have little antibacterial activity on their own).<br>*Ethnomedical:* Many terrestrial plant species (eg. *Hydrastis canadensis*) have been used in traditional medicine to treat bacterial infections.<br>*Historical:* Early attempts at total quinine synthesis (an antimalarial alkaloid from cinchona bark) led to the discovery of nalidixic acid & development of the 4-quinolones. | antipseudomonal salicylic acid derivative). Plants also produce efflux pump inhibitors (eg. 5'-methoxy-hydnocarpin) that could be developed as antibiotic adjuncts.<br>*Disadvantages:* Several companies (incl. Merck) have conducted large scale screens for phytochemicals that could be developed as broad-spectrum antibacterial drugs without success. Some medicinal plant species have become rare or endangered due to overharvesting & other pressures (eg. *H. canadensis*). Activity is generally weak against Gram-negative bacteria. Scale-up of phytochemical production can be costly (requiring large quantities of plant material or plant tissue culture). | |
| *Marine plants (incl. green & red algae)* | *Ecological:* Seawater contains ~$10^6$ bacteria/ml (incl. pathogens), so marine plants are more likely than their terrestrial counterparts to have evolved chemical defenses against infection.<br>*Ethnomedical:* Some marine plants (eg. *Halophila* spp.) have been used in traditional medicine to treat bacterial infections. | *Advantages:* Several potent antibacterial compounds have already been identified (eg. bromophycoic acid A).<br>*Disadvantages:* Access & supply can be more difficult than for terrestrial organisms, & scale-up of production can be costly. Extracting bioactive compounds is challenging with some algal species as their cell walls contain sporopollenin-like biopolymers that impede the passage of extraction solvents into cells. | (27, 32, 60, 64, 71-73) |
| **(g) Animals**<br>*Insects (both solitary & social)* | *Ecological:* Insects produce antimicrobial peptides as part of their innate immune system (found in the hemolymph & on epithelial surfaces). For insects occupying niches with large numbers of microbes (eg. maggots, cockroaches), there is a strong selection pressure for chemical-based defenses. The same can be argued for social insects (eg. bees), which are more vulnerable to infection because they live in large numbers in confined spaces with elevated temperatures.<br>*Ethnomedical:* Whole insect extracts (eg. blister beetles) have been used in traditional medicine to treat bacterial infections. Bee products (eg. honey) have been used too.<br>*Historical:* Honey is sometimes used in modern medicine to treat burns & dress wounds. | *Advantages:* Individual species can be rationally selected for testing based on their ethnomedicinal use. Entomotherapies have been less extensively screened for antibacterial activity than medicinal plants. Antimicrobial peptides have been identified with activity against Gram-positive (eg. defensin) & also Gram-negative bacteria (eg. pyrrhocoricin). Some peptides (eg. pyrrhocoricin & its derivatives) have shown efficacy in *in vivo* studies.<br>*Disadvantages:* There are a growing number of ethical issues in insect research (eg. need for conservation & humane treatment). Some antimicrobial peptides (eg. drosocin) are unstable in mammalian blood. Other obstacles associated with antimicrobial peptides include susceptibility to proteolytic degradation, low bioavailability, & sensitivity to salt & pH. | (19, 48, 74-77) |
| *Venomous animals* | *Ecological:* Insects, arachnids & centipedes produce antimicrobial peptides as part of their innate immune system (some of which are found in their venom).<br>*Ethnomedical:* Venom from insects (eg. ants) & arachnids (eg. scorpions) has been used in traditional medicine to treat various infections (incl. tuberculosis). | *Advantages:* Individual species can be rationally selected for testing based on their ethnomedicinal use.<br>*Disadvantages:* Many of the antimicrobial peptides isolated from venom have been toxic not just to bacterial cells, but to mammalian cells also (eg. mastoparan, cupeinnin I). | (74, 78) |
| *Other terrestrial animals (incl. higher animals)* | *Ecological:* Humans & other animals produce antimicrobial peptides as part of their innate immune system (eg. human mesenchymal stromal cells secrete cathelicidin LL-37, β-defensin- | *Advantages:* Antimicrobial peptides (eg. cathelicidins, defensins) typically have broad-spectrum, bactericidal activity. In *in vivo* models of sepsis & cystic fibrosis-related infection, treatment with | (22, 48, 79-91) |

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| | 2 & hepcidin; human platelets secrete β-defensin-2 & thrombocidin-1; equine mesenchymal stromal cells secrete elafin; murine myeloid leukocytes secrete hepcidin). Some human growth factors (eg. heparin-binding EGF-like growth factor) & their proteolytic products (eg. GKR22) are antibacterial & may constitute part of the innate immune system too.<br>*Ethnomedical:* Some animals & animal products have been used in traditional medicine (eg. *Moschus chrysogas*ter gall bladder & umbilicus) for infection treatment (eg. upset stomach, diarrhea, tuberculosis). | antimicrobial peptide-producing mesenchymal stromal cells improves outcomes. Preliminary clinical studies (in the field of regenerative medicine rather than infectious disease) have shown the secretome of human mesenchymal stromal cells can be used safely. Because some antimicrobial peptides have enhanced activity at low pH (eg. hepcidin), their development could be useful for gastric, vaginal & skin infections. Antimicrobial peptide-producing cells (eg. mesenchymal stromal cells) can be isolated & cultured from sources such as breast milk & menstrual fluid, so research is possible without invasive collection procedures.<br>*Disadvantages:* Research with humans & other higher terrestrial animals (vertebrates) requires ethical approval. Some zootherapies use vulnerable or endangered species (eg. *M. chrysogaster*). Antimicrobial peptides are susceptible to proteolytic degradation, have low bioavailability, & are sensitive to salt, pH & serum. Some antimicrobial peptides (eg. cathelicidin LL-37) & antibacterial growth factors (eg. heparin-binding EGF-like growth factor) are thought to play a role in the pathogenesis or progression of diseases such as rosacea, psoriasis & cancer. | |
| *Marine animals* | *Ecological:* Because seawater contains ~$10^6$ bacteria/ml (incl. pathogens), marine animals are more likely than their terrestrial counterparts to have evolved chemical defenses. This is especially true of lower marine animals (eg. sponges) because they lack cell-based immune responses.<br>*Ethnomedical:* Some marine animals (eg. sponges) have been used as traditional medicines for infection treatment. | *Advantages:* Because the antibacterial activity of marine natural products (eg. fish peptides) is not inhibited by high salt concentrations, their development could be useful for cystic fibrosis lung infections. Several potent antibacterial compounds have already been identified (eg. cadiolide E).<br>*Disadvantages:* Access & supply can be more difficult than for other organisms. For some marine animal-derived natural products, it is not clear if they have been produced by the animal itself or by a symbiont. Research with higher marine animals (vertebrates & cephalopods) requires ethical approval. | (27, 30, 32, 71, 72, 92, 93) |

Note: In the above table, the description of an isolated antibacterial compound as 'potent' is restricted to those with a minimum inhibitory concentration (MIC) less than or equal to 10 µg/ml [based on recommendations in (68)].

**REFERENCES**


1. Sayed AM, Hassan MHA, Alhadrami HA, Hassan HM, Goodfellow M, Rateb ME. Extreme environments: Microbiology leading to specialized metabolites. J Appl Microbiol. 2020;128(3):630-657.
2. Baltz RH. Antimicrobials from actinomycetes: Back to the future. Microbe. 2007;2(3):125-131.
3. Li JS, Barber CC, Zhang W. Natural products from anaerobes. J Ind Microbiol Biotechnol. 2019;46(3):375-383.
4. Van Geelen L, Meier D, Rehberg N, Kalscheuer R. (Some) current concepts in antibacterial drug discovery. Appl Microbiol Biotechnol. 2018;102(7):2949-2963.
5. Mahajan GB, Balachandran L. Sources of antibiotics: Hot springs. Biochem Pharmacol. 2017;134:35-41.

6. Tiwari K, Gupta RK. Rare actinomycetes: A potential storehouse for novel antibiotics. Crit Rev Biotechnol. 2012;32(2):108-132.
7. Behnken S, Hertweck C. Anaerobic bacteria as producers of antibiotics. Appl Microbiol Biotechnol. 2012;96(1):61-67.
8. Retsas S. Geotherapeutics: The medicinal use of earths, minerals and metals from antiquity to the twenty-first century. Geological Society, London, Special Publications. 2017;452(1):133-139.
9. Cornforth DM, Foster KR. Competition sensing: The social side of bacterial stress responses. Nat Rev Microbiol. 2013;11(4):285-293.
10. Tanvir R, Javeed A, Bajwa AG. Endophyte bioprospecting in South Asian medicinal plants: An attractive resource for biopharmaceuticals. Appl Microbiol Biotechnol. 2017;101(5):1831-1844.
11. Martinez-Klimova E, Rodríguez-Peña K, Sánchez S. Endophytes as sources of antibiotics. Biochem Pharmacol. 2017;134:1-17.
12. Ahmadijian V. The lichens. London: Academic Press; 1973.
13. Van der Meij A, Worsley SF, Hutchings MI, Van Wezel GP. Chemical ecology of antibiotic production by actinomycetes. FEMS Microbiol Rev. 2017;41(3):392-416.
14. Ramadhar TR, Beemelmanns C, Currie CR, Clardy J. Bacterial symbionts in agricultural systems provide a strategic source for antibiotic discovery. J Antibiot. 2014;67(1):53-58.
15. Boomsma JJ, Aanen DK. Evolutionary ecology: Wasp mother's little helpers. Curr Biol. 2005;15(5):R163-R165.
16. Beemelmanns C, Guo H, Rischer M, Poulsen M. Natural products from microbes associated with insects. Beilstein J Org Chem. 2016;12:314-327.
17. Adnani N, Rajski SR, Bugni TS. Symbiosis-inspired approaches to antibiotic discovery. Nat Prod Rep. 2017;34(7):784-814.
18. Chevrette MG, Currie CR. Emerging evolutionary paradigms in antibiotic discovery. J Ind Microbiol Biotechnol. 2019;46(3):257-271.
19. Freelance CB. To regulate or not to regulate? The future of animal ethics in experimental research with insects. Sci Engin Ethics. 2019;25(5):1339-1355.
20. Mousa WK, Athar B, Merwin NJ, Magarvey NA. Antibiotics and specialized metabolites from the human microbiota. Nat Prod Rep. 2017;34(11):1302-1331.
21. Duan K, Sibley CD, Davidson CJ, Surette MG. Chemical interactions between organisms in microbial communities. In: Collin M, Schuch R, editors. Bacterial sensing and signaling. Basel: Karger; 2009. p. 1-17.
22. Liévin-Le Moal V, Servin AL. Anti-infective activities of *Lactobacillus* strains in the human intestinal microbiota: From probiotics to gastrointestinal anti-infectious biotherapeutic agents. Clin Microbiol Rev. 2014;27(2):167-199.
23. Donia MS, Fischbach MA. Small molecules from the human microbiota. Science. 2015;349(6246):Article 1254766.
24. Rebuffat S. Microcins in action: Amazing defence strategies of enterobacteria. Biochem Soc Trans. 2012;40(6):1456-1462.
25. Liubakka A, Vaughn BP. *Clostridium difficile* infection and fecal microbiota transplant. AACN Adv Crit Care. 2016;27(3):324-337.
26. Durand GA, Raoult D, Dubourg G. Antibiotic discovery: History, methods and perspectives. Int J Antimicrob Agents. 2019;53(4):371-382.
27. Mayer MSA, Guerrero JA, Rodríguez DA, Taglialatela-Scafati O, Nakamura F, Fusetani N. Marine pharmacology in 2014-2015: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, antiviral, and anthelmintic activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. Mar Drugs. 2019;18(1):Article 5.
28. Kiuru P, D'Auria MV, Muller CD, Tammela P, Vuorela H, Yli-Kauhaluoma J. Exploring marine resources for bioactive compounds. Planta Med. 2014;80(14):1234-1246.
29. Crowley SP, O'Gara F, O'Sullivan O, Cotter PD, Dobson ADW. Marine *Pseudovibrio* sp. as a novel source of antimicrobials. Mar Drugs. 2014;12(12):5916-5929.
30. Kang HK, Seo CH, Park Y. Marine peptides and their anti-infective activities. Mar Drugs. 2015;13(1):618-654.
31. Niedermeyer THJ. Anti-infective natural products from cyanobacteria. Planta Med. 2015;81(15):1309-1325.
32. Gribble GW. Biological activity of recently discovered halogenated marine natural products. Mar Drugs. 2015;13(7):4044-4136.
33. Dhakal D, Pokhrel AR, Shrestha B, Sohng JK. Marine rare actinobacteria: Isolation, characterization, and strategies for harnessing bioactive compounds. Front Microbiol. 2017;8:Article 1106.
34. Liu M, El-Hossary EM, Oelschlaeger TA, Donia MS, Quinn RJ, Abdelmohsen UR. Potential of marine natural products against drug-resistant bacterial infections. Lancet Infect Dis. 2019;19(7):e237-e245.
35. Nalini S, Sandy Richard D, Mohammed Riyaz SU, Kavitha G, Inbakandan D. Antibacterial macro molecules from marine organisms. Int J Biol Macromol. 2018;115:696-710.
36. Tortorella E, Tedesco P, Palma Esposito F, January GG, Fani R, Jaspars M, et al. Antibiotics from deep-sea microorganisms: Current discoveries and perspectives. Mar Drugs. 2018;16(10):Article 355.
37. Stincone P, Brandelli A. Marine bacteria as source of antimicrobial compounds. Crit Rev Biotechnol. 2020;40(3):306-319.
38. Besse A, Peduzzi J, Rebuffat S, Carré-Mlouka A. Antimicrobial peptides and proteins in the face of extremes: Lessons from archaeocins. Biochimie. 2015;118:344-355.
39. Mullis MM, Rambo IM, Baker BJ, Reese BK. Diversity, ecology, and prevalence of antimicrobials in nature. Front Microbiol. 2019;10:Article 2518.

40. Bills GF, Gloer JB, An Z. Coprophilous fungi: Antibiotic discovery and functions in an underexplored arena of microbial defensive mutualism. Curr Opin Microbiol. 2013;16(5):549-565.
41. Karwehl S, Stadler M. Exploitation of fungal biodiversity for discovery of novel antibiotics. In: Stadler M, Dersch P, editors. How to overcome the antibiotic crisis: Facts, challenges, technologies and future perspectives. New York: Springer; 2016. p. 303-338.
42. Verma VC, Kharwar RN, Strobel GA. Chemical and functional diversity of natural products from plant associated endophytic fungi. Nat Prod Commun. 2009;4(11):1511-1532.
43. Ancheeva E, Daletos G, Proksch P. Lead compounds from mangrove-associated microorganisms. Mar Drugs. 2018;16(9):Article 319.
44. Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, et al. Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol Res. 2010;165(6):437-449.
45. Wasser SP. Reishi or Ling Zhi (*Ganoderma lucidum*). In: Coates PM, Blackman MR, Cragg GM, Levine MA, White JD, Moss J, editors. Encyclopedia of dietary supplements. New York: Marcel Dekker; 2005. p. 603-622.
46. Schneider T, Kruse T, Wimmer R, Wiedemann I, Sass V, Pag U, et al. Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. Science. 2010;328(5982):1168-1172.
47. Beattie KD, Rouf R, Gander L, May TW, Ratkowsky D, Donner CD, et al. Antibacterial metabolites from Australian macrofungi from the genus *Cortinarius*. Phytochemistry. 2010;71(8):948-955.
48. Abedinzadeh M, Gaeini M, Sardari S. Natural antimicrobial peptides against *Mycobacterium tuberculosis*. J Antimicrob Chemother. 2015;70(5):1285-1289.
49. De Mattos-Shipley KMJ, Foster GD, Bailey AM. Insights into the classical genetics of *Clitopilus passeckerianus* - the pleuromutilin producing mushroom. Front Microbiol. 2017;8:Article 1056.
50. Sandargo B, Chepkirui C, Cheng T, Chaverra-Muñoz L, Thongbai B, Stadler M, et al. Biological and chemical diversity go hand in hand: Basidiomycota as source of new pharmaceuticals and agrochemicals. Biotechnol Adv. 2019;37(6):Article 107344.
51. Kombrink A, Tayyrov A, Essig A, Stöckli M, Micheller S, Hintze J, et al. Induction of antibacterial proteins and peptides in the coprophilous mushroom *Coprinopsis cinerea* in response to bacteria. ISME J. 2019;13(3):588-602.
52. Hughes CC, Fenical W. Antibacterials from the sea. Chemistry. 2010;16(42):12512-12525.
53. Hou X-M, Wang C-Y, Gerwick WH, Shao C-L. Marine natural products as potential anti-tubercular agents. Eur J Med Chem. 2019;165:273-292.
54. Houdai T, Matsuoka S, Matsumori N, Murata M. Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate. BBA-Biomembranes. 2004;1667(1):91-100.
55. Mayer AMS, Rodríguez AD, Taglialatela-Scafati O, Fusetani N. Marine pharmacology in 2009-2011: Marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. Mar Drugs. 2013;11(7):2510-2573.
56. European Medicines Agency (EMA). Assessment report on *Fucus vesiculosus* L., thallus. EMA. 2014. Available from: https://www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2015/07/WC500190398.pdf. Accessed on: 30 May 2020.
57. Šudomová M, Shariati MA, Echeverría J, Berindan-Neagoe I, Nabavi SM, Hassan STS. A microbiological, toxicological, and biochemical study of the effects of fucoxanthin, a marine carotenoid, on *Mycobacterium tuberculosis* and the enzymes implicated in its cell wall: A link between mycobacterial infection and autoimmune diseases. Mar Drugs. 2019;17(11):Article 641.
58. Pérez MJ, Falqué E, Domínguez H. Antimicrobial action of compounds from marine seaweed. Mar Drugs. 2016;14(3):Article 52.
59. Hoppe HA. Marine algae and their products and constituents in pharmacy. In: Hoppe HA, Levring T, Tanaka Y, editors. Marine algae in pharmaceutical science. Berlin: Walter de Gruyter; 1979. p. 29, 32.
60. Hernández-Ledesma B, Herrero M, editors. Bioactive compounds from marine foods: Plant and animal sources. Chichester: John Wiley & Sons; 2013.
61. Andersson MI, MacGowan AP. Development of the quinolones. J Antimicrob Chemother. 2003;51(Supplement 1):1-11.
62. Lewis K, Ausubel FM. Prospects for plant-derived antibacterials. Nat Biotechnol. 2006;24(12):1504-1507.
63. Ngo LT, Okogun JI, Folk WR. 21st century natural product research and drug development and traditional medicines. Nat Prod Rep. 2013;30(4):584-592.
64. Alvin A, Miller KI, Neilan BA. Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. Microbiol Res. 2014;169(7-8):483-495.
65. Cushnie TPT, Cushnie B, Lamb AJ. Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. Int J Antimicrob Agents. 2014;44(5):377-386.

66. Gibbons S. Phytochemicals for bacterial resistance - strengths, weaknesses and opportunities. Planta Med. 2008;74(6):594-602.
67. Salam AM, Quave CL. Opportunities for plant natural products in infection control. Curr Opin Microbiol. 2018;45:189-194.
68. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100(1):80-84.
69. Adnan M, Ali S, Sheikh K, Amber R. Review on antibacterial activity of Himalayan medicinal plants traditionally used to treat pneumonia and tuberculosis. J Pharm Pharmacol. 2019;71(11):1599-1625.
70. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Secretariat. Convention on International Trade in Endangered Species of Wild Fauna and Flora and Appendices I, II and III. 1983 and 2019. Available from: https://www.cites.org/eng/disc/text.php and https://www.cites.org/eng/app/appendices.php. Accessed on: 30 May 2020.
71. Engel S, Jensen PR, Fenical W. Chemical ecology of marine microbial defense. J Chem Ecol. 2002;28(10):1971-1985.
72. Li JW-H, Vederas JC. Drug discovery and natural products: End of an era or an endless frontier? Science. 2009;325(5937):161-165.
73. Kumar CS, Sarada DVL, Gideon TP, Rengasamy R. Antibacterial activity of three South Indian seagrasses, *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capensis*. World J Microbiol Biotechnol. 2008;24(9):1989-1992.
74. Ratcliffe NA, Mello CB, Garcia ES, Butt TM, Azambuja P. Insect natural products and processes: New treatments for human disease. Insect Biochem Mol Biol. 2011;41(10):747-769.
75. Lee S, Siddiqui R, Khan NA. Animals living in polluted environments are potential source of antimicrobials against infectious agents. Pathog Glob Health. 2012;106(4):218-223.
76. Nenadić M, Soković M, Glamočlija J, Ćirić A, Perić-Mataruga V, Ilijin L, et al. The pygidial gland secretion of the forest caterpillar hunter, *Calosoma (Calosoma) sycophanta*: The antimicrobial properties against human pathogens. Appl Microbiol Biotechnol. 2017;101(3):977-985.
77. Saikaly SK, Khachemoune A. Honey and wound healing: An update. Am J Clin Dermatol. 2017;18(2):237-251.
78. Fratini F, Cilia G, Turchi B, Felicioli A. Insects, arachnids and centipedes venom: A powerful weapon against bacteria. A literature review. Toxicon. 2017;130:91-103.
79. Chakravorty J, Meyer-Rochow VB, Ghosh S. Vertebrates used for medicinal purposes by members of the Nyishi and Galo tribes in Arunachal Pradesh (North-East India). J Ethnobiol Ethnomed. 2011;7(1):Article 13.
80. Bahroudi M, Bakhshi B, Soudi S, Najar-peerayeh S. Antibacterial and antibiofilm activity of bone marrow-derived human mesenchymal stem cells secretome against *Vibrio cholerae*. Microb Pathog. 2020;139:Article 103867.
81. Kasiri MM, Beer L, Nemec L, Gruber F, Pietkiewicz S, Haider T, et al. Dying blood mononuclear cell secretome exerts antimicrobial activity. Eur J Clin Invest. 2016;46(10):853-863.
82. Alcayaga-Miranda F, Cuenca J, Khoury M. Antimicrobial activity of mesenchymal stem cells: Current status and new perspectives of antimicrobial peptide-based therapies. Front Immunol. 2017;8:Article 339.
83. Tohidnezhad M, Varoga D, Wruck CJ, Podschun R, Sachweh BH, Bornemann J, et al. Platelets display potent antimicrobial activity and release human beta-defensin 2. Platelets. 2012;23(3):217-223.
84. Tanhaeian A, Mohammadi E, Mansury D, Zeinali T. Assessment of a novel antimicrobial peptide against clinically isolated animal pathogens and prediction of its thermal-stability. Microb Drug Resist. 2020;26(4):412-419.
85. Malmsten M, Davoudi M, Walse B, Rydengård V, Pasupuleti M, Mörgelin M, et al. Antimicrobial peptides derived from growth factors. Growth Factors. 2007;25(1):60-70.
86. Harman RM, Yang S, He MK, Van De Walle GR. Antimicrobial peptides secreted by equine mesenchymal stromal cells inhibit the growth of bacteria commonly found in skin wounds. Stem Cell Res Ther. 2017;8(1):Article 157.
87. L. PK, Kandoi S, Misra R, S. V, K. R, Verma RS. The mesenchymal stem cell secretome: A new paradigm towards cell-free therapeutic mode in regenerative medicine. Cytokine Growth Factor Rev. 2019;46:1-9.
88. Rendon A, Schäkel K. Psoriasis pathogenesis and treatment. Int J Mol Sci. 2019;20(6):Article 1475.
89. Rainer BM, Kang S, Chien AL. Rosacea: Epidemiology, pathogenesis, and treatment. Dermatoendocrinol. 2017;9(1):Article e1361574.
90. Coffelt SB, Marini FC, Watson K, Zwezdaryk KJ, Dembinski JL, LaMarca HL, et al. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. Proc Natl Acad Sci USA. 2009;106(10):3806-3811.
91. Yang C-C, Chang K-W. Eicosanoids and HB-EGF/EGFR in cancer. Cancer Metastasis Rev. 2018;37(2):385-395.
92. Sipkema D, Franssen MCR, Osinga R, Tramper J, Wijffels RH. Marine sponges as pharmacy. Mar Biotechnol. 2005;7(3):142-162.
93. Newman D. Screening and identification of novel biologically active natural compounds. F1000Research. 2017;6:Article 783.

**Supplementary Table 2 for the Pharmaceutical Research journal article 'Bioprospecting for Antibacterial Drugs: A Multidisciplinary Perspective on Natural Product Source Material, Bioassay Selection and Avoidable Pitfalls'**

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**Supplementary Table 2** Avoidable pitfalls associated with different steps in the antibacterial bioprospecting process, from selecting and securing source material to testing for antibacterial activity and toxicity.

| Bioprospecting step | Action or inaction on part of researcher | Consequence(s) |
|---|---|---|
| **(a) Selecting & securing source material** | ▪ No clear rationale (ecological, ethnomedical, historical or genomic) for why the source material was selected for antibacterial testing. | ▪ Active compounds are less likely to be discovered (1). |
| | ▪ Failure to secure correct permissions for collecting biological material or ethnomedical knowledge. | ▪ Researchers & their employers can receive intense negative publicity [even in cases where material & knowledge was collected before ratification of the Nagoya Protocol (2)].<br>▪ Prosecution in court is possible & patent applications based on the research may be rejected (3). |
| | ▪ Failure to have biological material formally identified or to deposit a voucher specimen with an appropriate repository for long-term preservation & storage. | ▪ Research with the material will be unverifiable (4, 5) & important discoveries may be unreproducible if the source material was misidentified (4), undergoes genetic drift, or loses viability (6). |
| **(b) Antibacterial testing** | ▪ Use of excessively high concentration of solvent, surfactant or emulsifier to dissolve or disperse test extract or compound. | ▪ Solvent may be antibacterial or may enhance activity of the test extract or compound, leading to false positive results (7-9). |
| | ▪ Failure to report what concentration of solvent, surfactant or emulsifier was used to dissolve test extract or compound. | ▪ Lack of transparency can raise doubts about the findings presented (10). |
| | ▪ Use of diffusion-based assays to compare the antibacterial potency of different extracts. | ▪ Extracts containing small, polar &/or anionic compounds will appear more active than those containing large, nonpolar &/or cationic compounds, so extracts containing less potent compounds may be incorrectly prioritized for further investigation (11, 12). |
| | ▪ Testing excessively high concentrations of extract or compound (>2 mg/ml or >200 µg/ml, respectively). | ▪ Solubility problems may be encountered at high concentrations, leading to false negative results (1, 10). |
| | ▪ Failure to (i) include reference antibiotics (sometimes referred to as 'positive controls') & reference strains of bacteria (eg. ATCC) during testing or to (ii) use standard methods (eg. CLSI). | ▪ It will be impossible to verify that the results obtained for the test extracts or compounds (MICs etc.) are accurate & difficult or impossible to compare results between studies. Also, some nonstandard methods are flawed (1, 10). |
| | ▪ Failure to include appropriate controls such as (i) inoculated culture medium with no solvent & no test compound, (ii) inoculated culture medium with solvent but no test compound & (iii) uninoculated culture medium containing just the solvent & test extract/compound [& assay reagent (eg. resazurin) if an assay reagent is being used]. | ▪ False positive results are possible due to (i) non-viable inoculum or (ii) solvent inhibiting inoculum (10, 13). False negative results are possible due to (iii) compound precipitation being misinterpreted as inoculum growth, compound reducing the redox indicator directly, or compound fluorescing (10, 13). |
| | ▪ Concluding that extracts with MICs >1000 µg/ml & isolated compounds with MICs >100 µg/ml are active. | ▪ Resources will be wasted investigating extracts & compounds with negligible development potential (1, 10, 14, 15). |

| | | |
|---|---|---|
| **(c) Toxicity testing** | ▪ Failure to determine the effect of the test solvent on the mammalian cells or cell line(s) being tested. | ▪ False positive results are possible if the test solvent inhibits cell growth or kills cells (16, 17). False negative results are possible if the test solvent stimulates the growth of cells (17, 18). |
| | ▪ Preparing a dilution series of test compound in which the solvent concentration also varies. | ▪ Results may be skewed because some solvents (eg. DMSO) stimulate mammalian cell growth at low concentrations & inhibit cell growth at higher concentrations (19). |
| | ▪ Failure to (i) document passage number of test cell lines, (ii) monitor cell line characteristics during repeated passaging & (iii) set limits (eg. 10-20 passages) before thawing & sub-culturing frozen stock of the cell lines. | ▪ Cell characteristics can change with repeated passaging, causing false positive & false negative results (20). |
| | ▪ Relying on just one assay or marker (eg. ATP detection) to evaluate test compounds for toxicity. | ▪ False positive results are possible if a test compound interferes with the assay reagent (eg. test compounds inhibiting the luciferase enzyme in ATP assays) (21). |
| | ▪ Failure to include appropriate controls such as (i) test cells with no solvent & no test compound, (ii) test cells with solvent but no test compound & (iii) cell-free culture medium containing just the solvent, test compound & assay reagent (eg. the redox indicator tetrazolium or resazurin). | ▪ False positive results are possible due to (i) test cells not growing. False positive or negative results are possible due to (ii) solvent inhibiting or stimulating the growth of test cells (17). False negative results are possible due to (iii) the test compound reducing redox indicators directly or fluorescing (22). |

**Note:** Most of the above pitfalls also decrease the likelihood of the research being publishable because they render the work ineligible for submission (5) &/or because they raise doubts about the accuracy or reproducibility of the data presented (4, 5).

**REFERENCES**


1. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100(1):80-84.
2. Bourdy G, Aubertin C, Jullian V, Deharo E. Quassia "biopiracy" case and the Nagoya Protocol: A researcher's perspective. J Ethnopharmacol. 2017;206:290-297.
3. Caulfield T, Murdoch B. Genes, cells, and biobanks: Yes, there's still a consent problem. PLOS Biol. 2017;15(7):Article e2002654.
4. Eisenman SW, Tucker AO, Struwe L. Voucher specimens are essential for documenting source material used in medicinal plant investigations. J Med Act Plants. 2012;1(1):30-43.
5. Turney S, Cameron ER, Cloutier CA, Buddle CM. Non-repeatable science: Assessing the frequency of voucher specimen deposition reveals that most arthropod research cannot be verified. PeerJ. 2015;3:Article 1168.
6. Tedeschi R, De Paoli P. Collection and preservation of frozen microorganisms. In: Dillner J, editor. Methods in biobanking. Totowa: Humana Press; 2011. p. 313-326.
7. Basch H, Gadebusch HH. *In vitro* antimicrobial activity of dimethylsulfoxide. Appl Microbiol. 1968;16(12):1953-1954.
8. Dhondikubeer R, Bera S, Zhanel GG, Schweizer F. Antibacterial activity of amphiphilic tobramycin. J Antibiot. 2012;65(10):495-498.
9. Sutherland CA, Nicolau DP. To add or not to add polysorbate 80: Impact on colistin MICs for clinical strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* and quality controls. J Clin Microbiol. 2014;52(10):3810.
10. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. J Ethnopharmacol. 2006;106(3):290-302.
11. Tan JBL, Lim YY. Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. Food Chem. 2015;172:814-822.
12. Amsterdam D, editor. Antibiotics in laboratory medicine, 6th edn. London: Wolters Kluwer; 2014.

13. Fallarero A, Hanski L, Vuorela P. How to translate a bioassay into a screening assay for natural products: General considerations and implementation of antimicrobial screens. Planta Med. 2014;80(14):1182-1199.
14. Moreno PRH, Da Costa-Issa FI, Rajca-Ferreira AK, Pereira MA, Kaneko TM. Native Brazilian plants against nosocomial infections: A critical review on their potential and the antimicrobial methodology. Curr Top Med Chem. 2013;13(24):3040-3078.
15. Van Vuuren SF. Antimicrobial activity of South African medicinal plants. J Ethnopharmacol. 2008;119(3):462-472.
16. Nygren P, Larsson R, Lindh E, Rastad J, Akerström G, Gylfe E. Dimethyl sulfoxide inhibits proliferation but not hypertrophy and functional dedifferentiation of bovine parathyroid cells in culture. Res Commun Chem Pathol Pharmacol. 1988;62(2):161-171.
17. Singh M, McKenzie K, Ma X. Effect of dimethyl sulfoxide on *in vitro* proliferation of skin fibroblast cells. J Biotech Res. 2017;8:78-82.
18. Rodríguez-Burford C, Oelschlager DK, Talley LI, Barnes MN, Partridge EE, Grizzle WE. The use of dimethylsulfoxide as a vehicle in cell culture experiments using ovarian carcinoma cell lines. Biotech Histochem. 2003;78(1):17-21.
19. Timm M, Saaby L, Moesby L, Hansen EW. Considerations regarding use of solvents in *in vitro* cell based assays. Cytotechnology. 2013;65(5):887-894.
20. European Collection of Authenticated Cell Cultures (ECACC). Passage numbers explained (report number: M219.20170127.v2). Public Health England. 2020. Available from: https://www.phe-culturecollections.org.uk/media/114565/m219_passage-numbers-explained.pdf. Accessed on: 30 May 2020.
21. Riss TL, Moravec RA, Niles AL. Cytotoxicity testing: Measuring viable cells, dead cells, and detecting mechanism of cell death. In: Stoddart MJ, editor. Mammalian cell viability: Methods and protocols. Totowa: Humana Press; 2011. p. 103-114.
22. Sittampalam GS, Coussens NP, Brimacombe K, Grossman A, Arkin M, Auld D, et al., editors. Assay guidance manual. Bethesda: Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2019.