A post-antibiotic era looms: can plant natural product research fill the void?

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ABSTRACT

Antimicrobial resistance is increasing among certain pathogenic bacteria to the extent that treatment efficacy is no longer always assured. According to the CDC, as few as six new antibiotics have been released for use over the past 30 years. Resistance has already been observed to each of these. Eleven plant natural products have been approved for therapeutic use during the same period – none of them being antimicrobial agents. We have learned through experience that some microorganisms will inevitably overcome antibiotic treatment in certain situations, and then spread. It is clear that the rate of new antimicrobial development is insufficient to meet our current and future needs, which should be addressed in order to guarantee the effective future of antimicrobial chemotherapy. However, in recent years there has been an increase in the number of peer-reviewed reports of antimicrobial efficacy among plant-derived secondary metabolites. A limitation with these reports is the wide range of modified *in vitro* methods used to determine antimicrobial efficacy of these products, showing an absence of the type of standardisation that is the norm when testing the efficacy of single- or combined-agent conventional antimicrobials in the laboratory, thereby making inter-study comparison difficult. Overall, despite the large diversity in preparation and testing strategies used currently for natural product plant-derived antimicrobials, our investigations suggest that the field shows promise in the provision of novel antimicrobial agents, even as exemplified by our selected example, *Inula helenium* (Elecampane).

KEY WORDS: Antimicrobial susceptibility testing.
Drug resistance, antimicrobial.
Inula helenium.
Natural product research.
Plant constituents.

Introduction

Antimicrobial activity may apply to both commercially-produced antibiotics and to certain bioactive natural products produced by living organisms found in nature, whether of plant, microbial or marine origin. Antimicrobial resistance to conventional antimicrobial agents is increasing among pathogens. For example, in 2013, approximately 480,000 new cases of multi-drug resistant tuberculosis were documented in over 100 countries.[1] Significant global increase in drug-resistant infections will potentially result in 10 million preventable deaths worldwide and an enormous financial cost reaching $100 trillion dollars by 2050.[2] The number of compounds that are currently being developed is still insufficient to control infectious disease global threats.[3]

This rise of antimicrobial resistance (AMR), particularly among significant pathogens, when considered in the light of few novel replacements over recent decades, suggests an urgent need to find effective antimicrobials. Only an estimated 15% of approximately 300,000 higher plant species have been investigated phytochemically – which suggests that plant-derived natural products provide potential for new agents.[4] The term 'chemical space' has been defined as the set of all possible molecular structures in one collection.[5] Combinatorial compound diversity, chosen by pharmaceutical companies in recent times as a means to generate active compounds, has shown considerably less diversity than have natural product compounds.[6] The structural diversity of natural products inevitably also surpasses that from synthetic or combinatorial compounds,[7] thus suggesting that plant-derived natural products provide potential for therapeutic agents.

Atanasov *et al*.[8] list all isolated plant natural products approved for therapeutic use in the last 30 years (1984–2014), none of them being antimicrobial in nature. Nevertheless, natural products are a continuing source of novel drug possibilities to tackle AMR, requiring interdisciplinary collaborations and vigorous exploration of all approaches to drug discovery for the effective development of novel therapeutics.[4]

Natural products may be utilised in numerous modified and unmodified forms. Cragg *et al*.[9] have analysed the major sources of new commercial drugs from 1981–2010, which may be summarised as follows:

- unmodified natural (pure) compound
- modified natural compound
- semi-synthetic compound
- synthetic compound with no natural product conception
- synthetic compound with natural product pharmacophore.

Secondary metabolites, while widely regarded as being synonymous with natural products, might be defined as organic compounds in the correct chiral configuration to exert bioactivity with no growth, development or reproductive function in an organism.[10] Occurrence of

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molecularly diverse secondary metabolites can be restricted to particular plant families, genera or single species.[11] Bioactive compounds or constituents elicit confirmed biological effects in human or animal systems. Drug discovery and development involves the identification of therapeutically important new chemical entities (NCEs). Koehn[12] describes advanced analytical and spectroscopic methods that allow the rapid identification and structural elucidation of complex natural products in crude or pre-fractionated plant extracts while profiling their bioactivity in a process coined bioassay-guided-fractionation. Advanced developments in the field of metabolite analysis have recently been reviewed by Wolfender *et al*.[13]

Methods of evaluating antimicrobial activity of natural products often involve the modification and adaption of standard *in vitro* methods designed for single constituent compounds, as shown in Figure 1. In this review the advantages, limitations and comparisons between each current diffusion, dilution and bioautographic method will be highlighted with illustration using the anti-staphylococcal plant *Inula helenium* L.

Conventional *in vitro* antimicrobial test method adaption in natural product research: what are the major problems?

Figure 1 depicts the array of antimicrobial assays available to researchers and since each method is not based on the exact same principle, the results obtained may be influenced profoundly by the method selected, inoculum size, choice of organisms, degree of sample solubility, compound stability, chemical complexity and stereochemistry.[14,7] Currently available antimicrobial screening methods in natural product research may be divided into three categories:

- Diffusion methods (qualitative)
- Dilution methods (quantitative)
- Chromatographic-coupled bioautographic methods (qualitative and quantitative).

Standardised procedures and approved guidelines (through agencies such as the British Society for Antimicrobial Chemotherapy, or the US Clinical Laboratory and Standards Institute) are the accepted practice for the antimicrobial susceptibility testing of conventional single constituent chemotherapeutics; however, these regulated methodologies may be unsuited to the direct investigation of multi-constituent agents (i.e. crude or fractionated plant extracts). Standard diffusion and dilution techniques initially employed for single constituent agents have been modified consequently.[15] The problem with such modification is that these methods are not universally standardised for natural products. Incomplete standardisation, redundant and out-dated methodology[16] and inconsideration of all of the components of the extracts and their physiochemical properties has led to a plethora of unsubstantiated reporting of results. This complicates efforts of inter-laboratory comparison between different plant extracts by authors and research groups worldwide.[16] No single assay or combination of assays is necessarily optimal given the diversity of complex multi-constituents present in different extracts,[17] even where methods might possibly be standardised between laboratories. Furthermore, evaluating

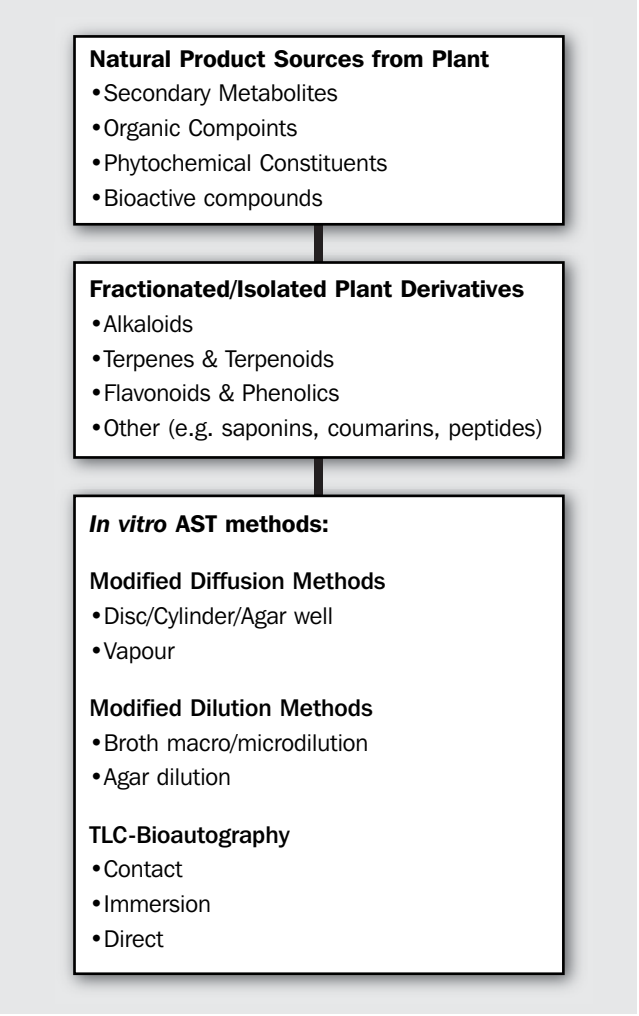

Fig. 1. Natural product sources from plants and categorisation of widely adopted antimicrobial susceptibility testing methods for their investigation.

the bioactivity of natural products – whether of plant, marine or animal origin – requires interdisciplinary collaboration rooted in both biology and chemistry specialties.

Rios *et al*.,[18] back in 1988, were among the first authors to review the application of standardised conventional antimicrobial susceptibility testing methods to natural product research. In addition to the antibacterial assay selected, many other factors can affect the overall outcome in natural product research, including environmental and climatic growing conditions, plant material, extract preparation, extraction method and choice of solvent,[19] thereby adding complication to efforts to standardise methods for natural product antimicrobial assessment.

Modified AST methods (multiple constituents)

Diffusion methods: disc diffusion

Diffusion methods are among the most widely adopted *in vitro* antibacterial screening techniques used today which provide what is often described as a qualitative (rather than quantitative) categorical interpretation of the degree of microbial susceptibility to an antimicrobial agent.[20,16] The

Table 1. Limitations of diffusion methods in plant natural product research.

Experimental variable	Associated limitation
Qualitative screening	Diffusion methods cannot generate accurate MICs or distinguish between bactericidal and bacteriostatic activity.[31]
Comparability issues	It is imperative that zones of inhibition are not assumingly correlated with the strength of antibacterial activity – a common misconception.[16]
Solubility issues	Diffusion and dilution-based methods are largely dependent on the availability of the active principle which is a function of the test compounds' solubility.[19]
Varied diffusion rates	Variability is introduced with the presence of crude extracts or multi-constituent fractions.[19] Particle size, shape and volume influence molecule diffusion rates.[14]
Compound adsorption	Cellulose filter paper is frequently used for disc preparation, (i.e. β-(1-4) glucose monomers). The presence of free hydroxyl groups on each glucose residue renders the disc surface hydrophilic. Cationic polar plant constituents would therefore be expected to adsorb to the disc surface thus influencing compound diffusion through the medium.[30]
Subjective interpretations	Greater dependence on the investigator to perform the procedures and interpret the results correctly is much higher in the absence of approved instrumentation and standardsation.[20]
Choice of solvent and controls	Solvents used for both extraction and/or solubilisation of extracts should be non-toxic; ensuring no interference to the bioassay.[19]
Factors influencing inhibitory zone diameter	Method sensitivity is influenced by microorganism species, growth medium pH and composition, agar thickness and disc adsorption.[32,33]

methods are easy to perform, economical and require little or no specialised instrumentation.[21,22] In the case of plant extract testing for antimicrobial activity, the disk diffusion procedure is performed similarly to the standard method using antibiotic impregnated disks. The method involves loading circular discs with a chosen plant extract which is then aseptically placed onto the surface of an inoculated agar medium. Minor variations have been reported in the literature including the following:

- refrigeration of the prepared plates at 4°C for 1–2 hours to allow prediffusion of the plant extract into the inoculated medium prior to incubation[23–25]
- extended soaking of the sterilised discs with plant extract for two hours before placing on the inoculated medium[26]
- drying of the impregnated discs under laminar air-flow overnight.[27]

Disc-diffusion methods generate qualitative data suitable for preliminary bioactivity screening of novel extracts and cannot be manipulated for the generation of quantitative data including minimum inhibitory concentrations (MICs) or minimum bactericidal concentrations (MBCs).[19] The compounds diffuse from the disc into the surrounding medium, any antibacterial activity is visually represented by the presence of a clear zone which depicts the interrupted growth of bacteria as a result of encounter with the agents.[28] Qualitative results are recorded upon measuring the difference in circumference from the disc to the surrounding clear zones.

Diffusion methods: agar-well (hole plate) and cylinder diffusion

The agar-well and cylinder diffusion assays are similar to the disc-diffusion method, with minor exceptions regarding the type of reservoir used.[19] In the agar-well method, defined wells of 6–8 mm are aseptically engraved into the agar forming holes using a sterile cork-borer, allowing at least 30 mm of space between adjacent wells. For the cylinder method, aluminium, glass or porcelain reservoirs are required. Pure plant extracts of defined concentration (i.e. 0.01–10 mg/mL) are introduced into the cylinders or newly-formed wells.[26,29] Valgas *et al*.[14] claim that the agar-well method is more sensitive when testing plant samples in comparison to the disc-variant methods – the premise of which is rooted in the potential constituent adsorption to the paper discs[30] as described in Table 1.

Diffusion methods: vapour diffusion (micro atmosphere-diffusion method or disc volatilisation method)

Plant volatile oils are naturally-occurring, highly complex plant compounds characterised by a potent aroma and which are produced by aromatic plants as secondary plant metabolites.[34] Some earlier studies have documented the evaluation of plant oils through direct contact (diffusion) methods or liquid [dilution] methods; however, compound diffusion and solubility factors largely affect the feasibility of these methods.[35]

Vapour diffusion is a modified agar diffusion procedure developed on the premise that plant oil vapours exert critical biological activity.[15] This method allows for a more uniform vapour distribution within the Petri dish headspace. Each individual constituent has its individual volatility, therefore when the constituents are introduced into a free, non-saturated state in a closed environment, the volatile constituents begin to disperse at differing rates according to their degree of volatility until they reach equilibrium.[36] At equilibrium the headspace composition is more homogeneous and may be reproducible, and this has led to the development of specifically designed air-tight acrylic chambers that allow for multiple exposures to plant oil vapours and the parallel assessment of bioactivity using multiple Petri dishes.[37] Limitations of the method include vapour loss owing to insufficient sealing, and some authors have documented the addition of 5 mL agar into the inner surface of the dish to help seal and prevent the adsorption of volatile vapours to the Petri dish material. These measures prevent interference with the internal environment.[38,36]

Table 2. Limitations of dilution methods in plant natural product research.

Experimental variable	Associated limitation
Non-polar compound precipitation	Reduces contact with the bacterial suspension.[43]
Membrane filtration Adsorption	Hydrophilic constituents and heat-labile water-based extracts can only be reliably sterilised via membrane filtration, which could potentially allow constituents to adsorb onto the membrane thus affecting bioactivity.[44]
Ambiguous MIC determination	Interference by turbid or coloured extracts can obstruct accurate visual or spectrophotometric measurements of the microtitre plates post-incubation, suggesting an advantage to using colorimetric methods.
Subjective visual results	Visual assessment of turbidity, when testing a variety of agents can be subjective.[45]
The inoculum effect (IE)	High inocula can induce false resistance and low inocula can result in false susceptibility,[45,46] in the absence of recommended guidelines by a competent authority.
Incomparability to other methods	Results obtained from the microdilution method are not always comparable to diffusion or bioautographic methods.[41]

Dilution methods: agar dilution

This method incorporates different concentrations of the sterilised sample into an agar medium, followed by inoculation of the surface of the agar with bacteria, and it has the same disadvantages as its alternative, the macro/micro-dilution method (Table 2). In addition it is more laborious than broth dilutions.[39,16]

Broth dilution methods: macrodilution and microdilution

Broth macrodilution is an older method than broth microdilution, and is less frequently used nowadays. It requires the manual preparation of serial dilutions using relatively high quantities of reagents and laboratory space. Disadvantages include the labour-intensive preparation of test solutions and the concurrent possibility of errors associated with such repeated preparations.[40]

The broth microdilution technique involves serial broth dilution of the sample, which is sequentially inoculated with bacteria in a 96-well microtitre plate.[41] Notable merits include rapid quantitative generation of MIC and MBC values, enhanced reproducibility, method feasibility and convenience. Miniaturisation of the technique greatly reduces expenditure on reagents and laboratory space required for implementing the technique.[40] Precise aliquots of diluted samples in broth are dispensed into each singular reservoir using an automated multi-pipette. MIC values are quantified post-incubation using either manual or automated viewing systems which inspect each microtitre panel for microbial growth. These automated panel or tray readers greatly assist researchers by generating computerised reports[40] Microdilution methods provide reliable and efficient assessment of antimicrobial agents, but they are not suitable for the assessment of essential oils, as solubility is an obvious requisite.[42]

Automated antimicrobial susceptibility testing methods

The merits of using automated instrumentation include non-subjective standardisation of end-points and higher through-put in comparison to manual readings. They also facilitate the analysis of large amounts of data, and monitoring of trends in resistance. Sensitive optical-detection systems permit detection of subtle bacterial growth patterns.[40] Early automation developments have included microtitre tray preparation, instrument-assisted readers and computer-assisted result databases. Rapid automated susceptibility testing systems frequently integrate robotics, micro-processors and micro-computers to rapidly identify susceptibility.[47] As an example, the FDA has approved the following automated instruments in the U.S. for conventional antimicrobial susceptibility testing: Vitek 2 System (bioMérieux, Durham, NC); MicroScan WalkAway

Table 3. Limitations of bioautographic methods in plant natural product research.

Experimental variable	Associated limitation
Active zones close to the origin	Clear inhibitory zones in close proximity to the origin must be separated further with a mobile phase of greater polarity to reveal active sub-fractions suitable for further evaluation and to reject non-active fractions.[51]
Active constituent(s) 'synergy' disruption	Some authors report the potential disruption of synergistic mechanisms between the active constituents in the plant extract thus potentially reducing the biological activity of the extract at large.[25,54]
Unsuitable for volatile Constituents	Volatile fractions may be lost through evaporation from the chromatogram.
Non-standardised method parameters	Criteria including mobile phase composition, stationary phase adsorbent, test species, TLC plate pre-conditioning and visualisation methods are all varying parameters that may potentially affect the results of these bioassays.[55]
Mobile phase pH	Strongly acidic or strongly alkaline solvents may remain on TLC plates thus potentially inhibiting bacterial growth.[57]

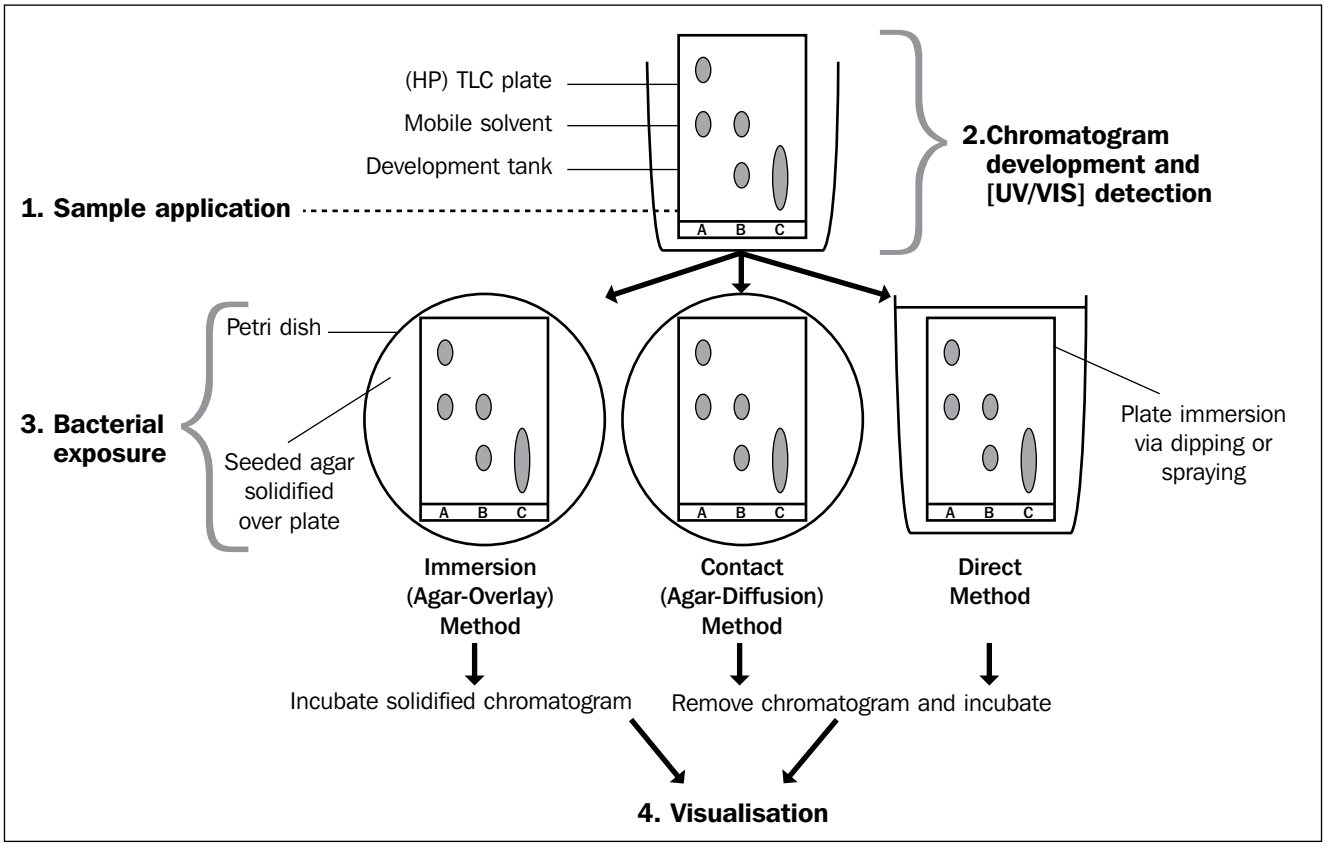

Fig. 2. Schematic diagram of available (HP)-TLC bioautographic methods.

System (Siemens Healthcare Diagnostics, Deerfield, IL); BD Phoenix Automated Microbiology System (BD Diagnostics, Sparks, MD); and the overnight Sensititre ARIS 2X (Trek Diagnostics Systems, OH).[40,47]

The predominant reported limitation of these rapid susceptibility testing methods is the reduced ability to detect emerging resistance mechanisms accurately, including inducible -lactamases, vancomycin-resistant staphylococci or carbapenem-resistant Gram-negative pathogens.40,47–49 However, guidelines for testing natural products for antimicrobial efficacy using these technologies have not been generated to date.

Chromatographic-coupled techniques: bioautography

Bioautography is an analytical phytochemical screening tool which detects bioactive portions of a complex extract based on the principle of bioassay-guided fractionation.[50,51] In natural product research, bioautographic detection is a functional microbial screening method typically combined with planar chromatographic techniques including thin-layer-chromatography (TLC), high-performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), over-pressured layer chromatography (OPLC) and planar electro-chromatography (PEC).[51]

Merits of these methods over modified diffusion and dilution assays include:

- *Easily applicable plant screening method*: hyphenated-bioautography remedies the issue of isolating antimicrobial compounds from complex crude plant extracts by offering a simple alternative isolation and identification process.
- *Reduced extract quantities*: minimised requirement for plant extract volume in comparison to other methods, which has both time- and money-saving benefits.
- *Target-directed isolation of active constituents*: the localisation of active compounds in a complex matrix combined with in situ bioactivity determination enables the guided isolation of targeted constituents.[52,53]
- *Parallel bioactivity comparison between samples*: samples of varied origin can be assayed on the one TLC plate allowing a direct comparison between extracts.[54]
- *Bio-guiding method*: bioautographic methods points towards active compounds that warrant further investigation via structural analysis.[51]
- *Spectroscopic input*: provides a broad range of information regarding the bioactivity and even the structures of the analytes.[51]
- *Preparative TLC Isolation*: separated components can be obtained directly from TLC plates by applying the sample in a wide band, which is scraped directly from the plate, eluted with appropriate solvents and structurally analysed via LC-MS, OPLC or other sophisticated methods for structural evaluation.[51]

Three versions of TLC-coupled bioautographic methods are currently used: contact, direct and immersion variants (Fig. 2). A listing of their disadvantages is shown in Table 3.

- *Contact (agar diffusion) bioautography*: involves placing the developed chromatogram onto the pre-inoculated agar medium surface for a specific period to allow diffusion. The next step requires the removal of the chromatogram and the subsequent incubation of the inoculated agar layer. Clear zones indicate growth inhibition.[55]

- *Direct bioautography*: involves the application of an antimicrobial agent directly onto developed TLC plates, followed by development in an appropriate solvent system and the direct application of the suspended bacterial test strain onto the TLC plate. Nutrients from the broth medium adhere to the TLC plate surface - which acts as a nutrient source for the bacterial strains thus enabling the direct growth of the bacteria on to the plate surface.[55] Similarly to the diffusion assays, clear inhibitory zones on the TLC plate indicate the presence of antimicrobial agents. Colorimetric indicators are used to visualise microbial growth, typically tetrazolium salts.[51] Microorganisms convert tetrazolium salts into formazan via the presence of dehydrogenases.[55]
- *Immersion (agar-overlay) bioautography*: This is a hybrid of both contact and direct procedures.[56] The technique requires total immersion of the pre-exposed TLC plate with seeded agar medium. In order to enhance diffusion rates,[55] it is suggested that plates should be kept at a low temperature for a few hours prior to incubation to allow greater diffusion of the antimicrobial agent into the medium, thus optimizing the procedure.

Plant-derived natural product drug discovery: current challenges

Aside from poor methodological set-up and analysis of data,[58] preclinical analysis of plant-derived natural products faces a variety of challenges, a selection of which are outlined in Table 4. Ultimately, the purpose of addressing these inherent challenges is to maximise accurate screening in plant natural product research.[59]

Novel antibacterial high-throughput screening modes for natural products

The principle of high-throughput screening (HTS) is the random and systematic evaluation of libraries of chemicals likely to modulate a specific biological target in cell-free-, phenotypic- or targeted cell-based assays.[63-65] Since the 1990s, HTS evaluation has accelerated in parallel with the HTS chemistry methods that initiated the development of synthetic compound libraries.[65]

Inula helenium L. (Elecampane): a case study

Inula helenium, which is a plant naturalised to Ireland, was selected for particular study in this review, both on the basis that it had been studied previously in our own laboratory and elsewhere, and that there have been several recent published studies of its antimicrobial efficacy.

From our earlier work,[66] when we reported 100% inhibition of 200 staphylococcial isolates encompassing methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) and coagulase-negative *Staphylococcus* (CNS), we recorded MBC values of 0.9–9.0 mg/mL against *I. helenium* extracts using both a crude drop-test, whereby a specified volume of extract was pipetted on a freshly inoculated bacterial lawn, and a microdilution method.

Deriu *et al*.[67] evaluated supercritical fluid (SCF) and hydro-distilled (HD) essential oil extracts of Italian *I. helenium* roots against a number of bacteria using disc-diffusion and microdilution methods. MICs ranged from 0.009 mg/mL to >14 mg/mL. An MIC of 0.6 mg/mL (HD extract) and 3.7 mg/mL (SCF extract) was reported against *S. aureus* (ATCC 29213); the streptomycin control MIC was 0.05 mg/mL – significantly lower than both *I. helenium* extracts. *Pseudomonas aeruginosa* was the most resistant of all strains tested (MIC 14.8 mg/mL). Both extracts significantly inhibited ampicillin-, erythromycin-, penicillin-, and tetracycline-resistant *Enterococcus faecium* strains (MIC >0.3 mg/mL). The authors remarked that HD-extracted oil is more active than SCF-extracts on the basis of this study.

A recent review by Seca *et al*.[68] stated that alantolactone is one of the most widely tested isolated constituents from *I. helenium* L. Stojanovi -Radi *et al*.[69] reported alantolactone, diplophyllin and isoalantolactone as the active anti-staphylococcal constituents of *I. helenium*. Further evidence suggests that 4 ,5 -epoxyalantolactone contributes to the antimicrobial function of *I. helenium*.[67]

Gökbulut *et al*.[70] states that (Turkish) methanol *I. helenium* root extracts were most active against Gram-positive bacteria in the (CLSI modified) agar-dilution method using a concentration range from 6.25 – 800 μg/mL. Lowest MICs were generally observed for the *I. helenium* root extracts over either flower or leaf extracts: *Enterococcus faecalis* (ATCC: 29212; MIC 100 μg/mL), *Escherichia coli* (ATCC: 25922; MIC 200 μg/mL), *S. aureus* (ATCC: 29213; M..IC 100 μg/mL) and *P. aeruginosa* (ATCC: 27853; MIC 400 μg/mL). In agreement with Deriu *et al*.,[67] *P. aeruginosa* exhibited strongest *in vitro* resistance to *I. helenium*.

Jiang *et al*.[71] evaluated the antibacterial activity of isolated Chinese *I. helenium*-derived compounds against *Bacillus cereus*, *E. coli*, *Erwinia carotovora*, *S. aureus* and *P. aeruginosa* using the broth microdilution method with reference to CLSI guidelines. A total of 16 extracted compounds were listed; 4α,15α-epoxyisoalantolactone demonstrated stronger activity (MIC 15.5 μg/mL) to *B. cereus* than the positive ampicillin control, while isoalantolactone exhibited moderate antibiosis to *B. cereus* (MIC 31.3 μg/mL). Compounds isoalantolactone, 4α,15α-epoxyisoalantolactone, macrophyllilactone, 4α, 15-epoxyisoalantolactone, telekin and 3α-hydroxyeudesm-4,11-dien-12,8β-olide demonstrated weak inhibition (MICs 62.5–125 μg/mL) to *Escherichia coli*. Again compound 3α-hydroxyeudesm-4,11-dien-12,8β-olide demonstrated weak inhibition to *B. cereus* and isoalantolactone to *S. aureus*, while compounds isoalantolactone, macrophyllilactone, telekin and 3α-hydroxyeudesm-4,11-dien-12,8β-olide inhibited *Erwnia carotovora* and compounds isoalantolactone and 3α-hydroxyeudesm-4,11-dien-12,8β-olide inhibited *B. subtilis*. All other (non-specified) compounds were inactive, showing MICs >250 μg/mL. This study is an example of critical, comprehensive and high-standard natural product research. The authors state that the bioactivity of the aforementioned compounds are characteristic of an eudesmane structure with an α,β-lactone and oxirane thus emphasising the link between molecular structures and configuration with constituent bioactivity – which further strengthens the importance of documenting plant chemical profiles in conjunction with bioassay evaluations in order to ascertain any claimed activity.

Stojanovi -Radi *et al*.[69] also demonstrated the structure-activity relationship of *I. helenium*, suggesting that the

Table 4. Plant-derived natural product drug discovery: current challenges.

Specific challenges	
Species-specific Compounds	Natural products occurring in many different plant species are favoured over species-specific compounds as this removes the supply constraints hindering successful research, development and commercialisation of natural product-derived drugs.[8,60]
Natural product compound resupply	Resupply from the original plant species is often unfeasible and economically non-viable to meet market demands upon commercialisation of naturally-occurring drugs. Emerging biotechnological and (total-, semi-) chemical synthesis strategies offer potential alternatives to overcome the precursor resupply obstacle.[8]
Illogical dosage/concentrations and erroneous IC_{50} value endpoints	The use of excessively high (unrealistic) concentrations of plant extracts for *in vitro* and *in vivo* testing to obtain a dose-response effect have been documented in the literature.[58,61] The need for stringent end-point criteria is imperative. Cos *et al*.[61] recommend *IC_{50} values ≤100 μg/mL for extracts and ≤25 μmol/L for pure compounds as a relative starting point for standardised endpoints.
Presence of nuisance compounds	Contamination of plant material with metals can cause defective bioassay outcomes.[8] Metal accumulating plant species can concentrate heavy metals (e.g., cadmium, zinc, copper, manganese, nickel, lead) up to 100 or 1000 times that of non-accumulating plants.[62]
Pro-drug evaluation	Some natural products are categorised as (inactive) pro-drugs that require metabolic activation to confer its pharmacological activity,[58] for example cyanogenetic glycosides.[63] This complicates identification methods when effector compounds are not present in the starting plant material, thus undetectable through typical identification methods.
General inherent challenges	
Cultivation, harvest and post-harvest neglect	The World Health Organization (WHO) released Industry Guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants to promote sustainable harvest and collection techniques and to reduce ecological issues pertaining to wild-crafting of medicinal plants.[8]
Botanical identification, authentication and nomenclature	Unequivocal plant material identification requires genetic (omic techniques) and chemical analysis, including hyphenation of GC or HPLC with various spectroscopic methods. Plant taxonomy and synonym issues can complicate this step further.[63]
Plant material availability	Limited compound quantity is problematic for post-pharmacological activity and subsequent characterisation.[8]
Ethnopharmacological and traditional use approaches	Numerous challenges face these approaches to plant selection on the basis of their traditional use. Legal-right claims can be provoked from specific county of origin or ethnical group from which the traditional knowledge was originally documented.[8]

*IC_{50} is the half maximal inhibitory concentration.

eudesmane core olefinic bonds together with the α,β-methylene-lactone ring and that these are vital structural features for anti-staphylococcal activity. Further conformational analysis demonstrated that diplophyllin's higher potency is due to optimal lactone moiety interaction with the binding region of targets.

Qiu *et al*.[72] concluded that isolated isoalantolactone did not exhibit anti-staphylococcal activity *in vitro* using the modified CLSI microdilution method. The authors did, however, note the compound's ability to inhibit α-toxin expression in *S. aureus* at very low concentrations and its protection of mice against pneumonia *in vivo*. α-toxin is a pore-forming toxin secreted by most *S. aureus* strains, which is essential for pneumonia pathogenesis. This is an example of where unremarkable in vitro antimicrobial results may be less significant than elucidation of (natural products') bioactive mechanisms of action. This may actually provide more vital information toward their use as anti-infective agents, than the initial results suggest.

Radulovi *et al*.[73] also reported the inactivity of Serbian *I. helenium* root-derived 3-methyl-2alkanones against *S. aureus* using the microdilution method in vitro (MIC >3.70 mg/mL) in comparison to the positive control nystatin (MIC 0.78 μg/mL). The authors imply that the bioactive sesquiterpene aldehydes of this fraction are still unidentified and that they occupy a mass spectrum resembling the compound bicyclogermacrenal.

Stojakowska *et al*.[74] reported moderate antibacterial activity of *I. helenium* root-derived isolate, 10-Isobutyryloxy-8,9-epoxythymol isobutyrate, against *S. aureus* (MIC 50–250 μg/mL), *Enterococcus faecalis* (MIC 1000 μg/mL), *Escherichia coli* (MIC 1000 μg/mL) and *P. aeruginosa* (MIC 1000 μg/mL) using the broth microdilution method *in vitro*. The authors comment on the role of thymol derivatives in the young roots of *I. helenium* potentially contributing to its antibacterial activity.

As an interesting sequel to the traditional use of *I. helenium* root as an antimycobacterial agent, Gautam *et al*.[75] reviewed *in vitro* antimycobacterial screening methods, including dilution, diffusion, radiorespirometry and reporter gene assays when testing *I. helenium* L. and *I. racemosa* Hook f. Prior to this, Cantrell *et al*.[76] had reported 100% inhibition of *Mycobacterium tuberculosis* (H382v ATCC 27294) by crude organic *I. helenium* root extracts (MIC: 0.1 mg/mL; South, Central and North American-derived) in vitro using a radiorespirometric assay. Percentage inhibition was unaffected by the choice of extraction solvent used (hexane, dichloromethane and methanol extracts).

Of the literature cited, it is clear that many authors in the field of natural products do not attempt to include vital information in their methodology, particularly descriptions of the plant material, extraction process, constituent profiling, MIC/MBC definitions, guidelines followed (if any) or verification of the assumed extract potency.

In vitro evaluation is important as a primary screen, but compounds exhibiting promising activity require further studies to validate or ascertain therapeutic potential. Confirmation of the pharmacological potential of, for example, *I. helenium* bioactive secondary metabolites must be subjected to *in vivo* studies; for example, using the zebrafish (Danio rerio) model. The zebrafish model has emerged as a biomedically relevant *in vivo* model for high-content drug screening and the simultaneous determination of multiple efficacy parameters including selectivity and toxicity in the content of the whole organism.[77]

An important parameter that appears to be often overlooked is not only the correlation between *in vitro* and *in vivo* results, but the dose- and route-of-administration-associated toxicity of plant extracts and isolations. The majority of the literature reviewed for this paper have cited compounds exhibiting high IC_{50} values (μmol/L) – thus representing perhaps unfeasibly high necessary dosages – when compared, for example, with commonly-used antibiotics such as amoxicillin, therapeutically quoted at nanomolar levels. Comprehensive toxicological studies are therefore also a vital consideration within the pre-clinical validation of potential therapeutic compounds.[67]

Recommendations towards method uniformity in natural product antimicrobial research

At the time of writing, no approved guidelines, standards or official recommendations exist, to our knowledge, for *in vitro* antimicrobial screening or susceptibility testing methodologies for natural products of plant origin. There are several points to be considered here. These might be expected to include botanical certainty of the identity of the plants to be used and their potential geographical and nutritionally-caused variations. Further considerations include unification of extraction protocols, assay choice, inoculum densities, results interpretation, and inter-laboratory reproducibility in determination of MIC and MBC.

The current challenges may be divided into two distinct areas. The first being the comprehensive identification of the constituents to be extracted for each species of plant, and a preliminary analysis of their respective concentrations, while seeking to maintain synergistic effects where they occur to the final production stage and testing thereafter. The second area concerns the *in vitro* susceptibility testing of these products, where methods and interpretive guidelines may be established to determine efficacy for treatment of infections, ultimately, similar to the methods used currently for conventional commercial antimicrobial agents. The investment costs to reach this stage with any natural plant-derived product will undoubtedly be very significant. Overall, despite the large diversity in preparation and testing strategies used currently for natural product plant-derived antimicrobials, our investigations suggest that the field shows promise in the provision of novel antimicrobial agents, even as exemplified by our selected example, *I. helenium*. However, our research of the topic suggests that much work is needed to confirm this.

Atanasov *et al*.[8] listed all isolated plant natural products approved for therapeutic use in the last 30 years (1984–2014), including artemisinin, capsaicin, colchicine, dronabinol, cannabidol, galanthamine, ingenol mebutate, masoprocol, omacetaxine mepesuccinate, paclitaxel and solagmargine – none of which are antimicrobial agents. Furthermore, according to the CDC,[78] just six commercial antibiotics have been developed in the last 30 years (1985–2010), including imipenem and ceftazidime (1985), levofloxacin (1996), linezolid (2000), daptomycin (2003), and ceftaroline (2010). Of these, linezolid and daptomycin are antibiotics with novel modes of action, but, even with these agents, bacterial resistance has been noted.[79]

We have learned that microorganisms will inevitably overcome antibiotic treatment in some situations, and then spread, and it is clear that the rate of new antimicrobial development is insufficient to meet our current and future needs. This underlines the importance of investigating and developing promising antimicrobial agents to guarantee the effective future of antimicrobial chemotherapy. □

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