



# Medicinal Plants as a Reservoir of New Structures for Anti-infective Compounds

Akram M. Salam and Cassandra L. Quave

## Abstract

The continued emergence of antimicrobial resistance across a spectrum of infectious pathogens presents a clear and urgent threat to human health across the globe. This trend has been further complicated by a decline in the discovery of novel chemical classes for anti-infective development. Natural products – primarily microbial in origin – have historically served as a key resource for anti-infective drug discovery efforts. On the other hand, natural products from the plant kingdom have served as a source of traditional medicine for millennia, and yet they remain relatively unexplored. The aim of this chapter is to provide an overview of plant natural products and discuss their potential as a resource for ongoing and future drug discovery efforts to fill the anti-infective pipeline and combat antimicrobial-resistant infections.

## Keywords

Antibiotic resistance · Pharmacognosy · Medicinal plants · Ethnobotany · Phytochemistry · Secondary metabolites

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A. M. Salam

Molecular and Systems Pharmacology program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA, USA

C. L. Quave (✉)

Center for the Study of Human Health, Emory University College of Arts and Sciences, Atlanta, GA, USA

Department of Dermatology, Emory University School of Medicine, Atlanta, GA, USA

Antibiotic Resistance Center, Emory University, Atlanta, GA, USA

Emory University Herbarium, Atlanta, GA, USA

e-mail: [cquave@emory.edu](mailto:cquave@emory.edu)

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277

## 1 Introduction

Antimicrobial resistance (AMR) has become a major point of concern in healthcare across the globe. In a recent report on AMR, it was estimated that it presently causes 700,000 fatalities annually and is projected to cause 10 million annually by the year 2050 (O'Neill 2016). The rate of discovery for new anti-infectives has slowed tremendously since the golden era of antibiotic discovery, and we have especially faced a drought in the discovery of new antibiotic classes since the 1980s (Silver 2011). Over the past two decades, all of the “new” drugs approved and brought to market were either initially discovered in the 1980s or prior or are “re-do” compounds – representing structural modifications of existing antibiotic core scaffolds. As a result, these antibiotics have also succumbed to common mechanisms of resistance – both intrinsic and acquired. Innovation in anti-infective drug discovery is recognized as one of the most important pieces in the strategy to addressing the burgeoning difficulties of infection control moving forward (van der Meer et al. 2014). The natural products contained in medicinal plants are very well-positioned to meet this need for innovation. This is due not only to the intrinsic chemical properties of plant natural products but also to their largely untapped diversity, the targeted approach of ethnobotany through which this diversity can be explored, and new technologies that further enable ethnobotanical drug discovery.

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## 2 Traditional Medicine and the Ethnobotanical Approach to Drug Discovery

Of the estimated 390,900 species of plants on Earth, at least 28,187 species – or 7% – have been documented in the literature as having a medicinal use (Willis 2017). While there is no precise tally of how many of these species have been investigated in a comprehensive manner for their full pharmacological potential, based on knowledge of the current literature one can estimate that this number is in the low hundreds, if that. The prioritization of medicinal species for drug discovery efforts is a key component of the ethnobotanical approach to drug discovery (Cox and Balick 1994). The field of ethnobotany – or the study of how people relate to and use plants – has also been referred to as the science of survival (Prance 2007). This is because it is the study of how people use plants as a source of food, tools, shelter, musical instruments, toys, medicine, and more.

Wherever people have inhabited the planet, a form of indigenous medicine has thrived. In some cases, traditional systems of medicine – referred to as such in contrast to Western medicine – continue to serve as the primary modality of healthcare for people, and plants are a key ingredient to their pharmacopoeias. Indeed, a 2002 World Health Organization report estimated that in some parts of the world, up to 80% of the population uses traditional medicine to meet their medical needs (WHO 2002). These forms of medicine may be based in shamanism, with healers

trained via individual apprenticeships, while others ascribe to a more structured form of knowledge transmission through schools, such as in Ayurveda, Traditional Chinese Medicine, Tibetan medicine, and more. In all cases, knowledge of the natural world – particularly knowledge of plants – is key to the process of becoming a healer or traditional medicine practitioner.

## 2.1 Plant Secondary Metabolites

Why do plants play such a key role in traditional medicine? Plants manufacture a vast array of bioactive secondary metabolites – or natural products – for the purposes of enhancing their own chances of survival, and sometimes these metabolites are active against targets of interest to human medicine. Secondary metabolites are differentiated from primary metabolites in that they are not required for primary metabolic processes (e.g., photosynthesis and growth) but rather serve specialized roles as tools of communication with other organisms in the environment. For example, some secondary metabolites are responsible for plant colors, scents and flavors. Plants use other secondary metabolites to attract pollinators and seed dispersers; to protect themselves from microbial pathogens, predatory insects, and overzealous herbivores; and to compete with other species for resources essential to their survival such as access to light, water, and nutrients. The production of secondary metabolites is regulated by plants in response to environmental cues.

This chemical perspective can be applied to any practice of traditional medicine. A plant preparation that demonstrates therapeutic activity against a given indication contains at least one chemical at a high enough dose to pharmacologically perturb the disease state. Often, it is a combination of chemicals that work additively or synergistically to exert a pharmacological effect, whether that be inhibition of angiogenesis in a tumor or inhibition of an efflux pump in multidrug-resistant bacteria. This is the foundation of the ethnobotanical approach to drug discovery – the end goal of which is the identification and study of these chemicals that contribute to the therapeutic effects of a medicinal plant preparation.

## 2.2 The Ethnobotanical Approach to Drug Discovery

Ethnobotanical drug discovery begins with a targeted approach to selecting plants for study: traditional knowledge is consulted to identify plants with a history of use against an indication of interest (Cox and Balick 1994). This initial step confines the study to plants that have already been shown to carry the therapeutic potential of interest with relevance to the disease state being targeted within traditional systems of medicine. Thus, only plants with a very high likelihood of containing chemicals of interest are included in the study. There are a number of databases that can be consulted to identify medicinal plants of interest, including NAPRALERT, Dr. Duke's

Phytochemical and Ethnobotanical Database, and the Native American Ethnobotanical Database (Duke 1992–2016; Farnsworth 2018; Moerman 2018). In other cases, ethnobotanists working with a drug discovery team may also undertake primary research in collaboration with communities that have a recent history of medicinal plant use or currently use them. This approach of looking to nature for medically relevant chemistry is also known as bioprospecting. It often requires expertise across a number of disciplines, such as anthropology, botany, chemistry, ecology, linguistics, medicine, molecular biology, microbiology, and pharmacology. A multidisciplinary team-based strategy is thus often the most effective strategy.

### 2.2.1 Ethical Bioprospecting

Bioprospecting and the ethnobotanical approach to drug discovery requires implementation of rigorous standards of ethics in the project design and implementation. The International Society of Ethnobiology has designed guidelines for ethical research, and this has been adopted by a number of academic societies in this field, including the Society of Ethnobiology and Society for Economic Botany, with the expectation that their members adhere to the Code (ISE 2006). The Code focuses on 17 principles that embody the concept and implementation of traditional resource rights; these include principles of prior rights and responsibilities, self-determination, traditional guardianship, active participation, full disclosure, educated prior informed consent, confidentiality, respect, active protection, precaution, reciprocity, mutual benefit and equitable sharing, supporting indigenous research, the dynamic interactive cycle, remedial action, acknowledgment and due credit, and diligence. Similar to the Code of Ethics for these organizations, guidelines for bioprospecting research in genetically rich source countries have also been a topic of attention among the membership of the American Society of Pharmacognosy (Cragg et al. 1997).

Both the guidelines share common principles outlined in further detail in the United Nations Convention on Biological Diversity (CBD) and the Nagoya Protocol (UN 2011). The CBD is a multilateral treaty widely considered the key document regarding sustainable development. It was signed by many countries in the international community in 1992 and has three aims: (1) the conservation of biological diversity, (2) the sustainable use of its components, and (3) the fair and equitable sharing of benefits arising from genetic resources. The CBD asserted provider countries, their people, and representatives as stakeholders to be included in negotiations for plant-based drug discovery programs, providing a framework for the regulation and defining bioprospecting. The CBD treaty left many open questions as well, especially with respect to access and benefit sharing. To bring clarity to this issue, the Nagoya Protocol, a supplementary agreement to the CBD, was adopted in 2010. Importantly, it outlines mechanisms for equitable access and benefit sharing with genetic resource source countries (UN 2011). Legally binding, it serves to further clarify the issues of access and benefit sharing by setting out obligations for its contracting parties to take measures in relation to them. There are a number of case studies and useful resources for reference on this topic available on the CBD website (UN 2018).

### 2.2.2 Collection of Medicinal Plants

Following their identification either by literature or database searches, or through primary field research, the next step in the drug discovery process is to access the plant material. In the majority of cases these materials are not commercially available and must be wild-crafted, or found and harvested from wild populations. Such endeavors require expertise in botanical taxonomy and an understanding of which ecosystems the plants may be found in. Furthermore, the optimal collection time for plant identification – when it is in a reproductive state with fruits or flowers – must also be considered. The World Health Organization's Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants covers a number of crucial protocols for collecting from wild populations, such as avoiding areas contaminated by environmental pollutants like road or agricultural field runoff and avoiding collection of CITES-protected species or species that are otherwise listed as threatened or endangered (WHO 2003).

Once the plants are identified in the wild, there are four types of samples that require collection: herbarium voucher specimens, DNA specimens, bulk specimens, and retention vouchers. Herbarium voucher specimens include vegetative and reproductive parts of the plant (e.g., a segment of a flowering tree branch or a whole small herb in fruit). Specimens are field pressed, or placed into a folded sheet of newspaper that has been given a collection number, and then squeezed flat in a plant press – a rectangular unit composed of two sets of wooden slats held together with thick straps. Upon return to the field research base station, the plants are more carefully arranged and subjected to drying under low heat. Multiple copies of vouchers for a single species are often collected in order to make deposits of the final pressed and labeled specimen in the scientist's home institution as well as at local institutions in the country or region where the collections took place.

DNA specimens are collected by taking leaf samples (roughly 1 square inch of leaf material) and storing them in labeled coin envelopes. All samples are numbered according to the same collection identification scheme for linking each of the samples (herbarium voucher, retention voucher, DNA, and bulk). The DNA samples can then be stored in sealed plastic bags with desiccant until they are taken to the lab for DNA extraction and characterization. This adds an additional level of evidence toward species identification and may be of use to other scientists engaged in various conservation efforts, such as in tracking the trade of medicinal plant products.

Bulk specimens are made of different plant tissues, often guided by traditional medical uses reported. For example, if the leaves of a plant are reported as being the key ingredients in a traditional medicine for a disease of interest, then the leaves should be the main focus of the bulk collection. Each plant tissue serves a different purpose for the plant as a whole organism, and as a result, each tissue also exhibits a different chemical profile than the others. In most cases, 40 g of dry material is plenty for an initial study on the chemical makeup and potential biological activity of each tissue. If the plant later becomes the subject of more in-depth studies aimed at the isolation of multiple individual compounds, then many kilograms of material may eventually be required, depending on the abundance, or percent yield, of the active compound(s) in the plant tissue.

In the field, bulk materials are typically collected and processed on the same day to avoid sample loss due to decay or mold. This may involve rapid field collection and later separation of plant tissues at the research base camp, where they are then chopped up into smaller segments and dried either in a desiccating cabinet at low heat or spread out in the shade in more arid countries, where they can dry without the aid of a heat source or dehumidifying machine. Once dried, samples may be packed up in vacuum-sealed bags with desiccant sachets for shipment back to the lab. Retention vouchers of this chopped up and then ground material are also collected upon return to the lab and stored in small plastic bags as a record of how the semiprocessed and ground material appears.

### 2.2.3 Extraction of Medicinal Plants

Bulk plant materials are processed according to a wide variety of techniques that have been developed for this purpose. Some examples include extraction in organic solvents either by maceration at room temperature or under pressure, with sonication, under heat, or under both heat and pressure. Other methods include extraction with water or by steam distillation for the extraction of essential oils. In some cases, the materials may be extracted in plant or animal-based fats. All methods have their own advantages and disadvantages with regard to extraction efficiency and the variety of compound classes that emerge from each technique. In any of these cases, the first level of extraction produces a “crude extract,” which is composed of many different compounds – sometimes including hundreds to thousands of unique molecular entities. As such, any crude extract is in itself a chemical library, representing multiple core scaffolds and many derivatives of each scaffold.

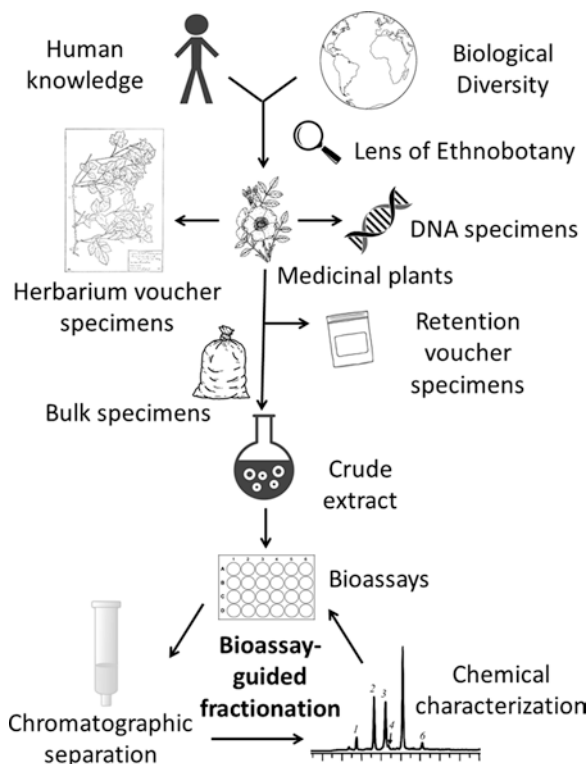
In order to identify bioactive compounds from the crude extract, the framework of bioassay-guided fractionation is followed. Based on this strategy, chromatographic techniques are employed, such as partitioning, column chromatography, flash chromatography, and high-performance liquid chromatography (HPLC), in order to produce fractions of the crude extract for testing in the biological model of interest. The most bioactive fractions are selected for further fractionation, the fraction characterized, and the cycle of chromatographic separation and biological testing repeats until the most active fraction or single compound is identified (Fig. 1).

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## 3 Characteristics of Plant Natural Products

With regard to anti-infective drug development, which until the 2010s focused heavily on classical growth-inhibitory mechanisms of action, microbial natural products as a resource were found to be extremely rewarding. Indeed, while 69% of all US FDA-approved antibacterials were natural products or derivatives thereof, as of 2016, 97% of these were contributions from microbes while a mere 3% came from plants (Patridge et al. 2016). During this time, however, plant natural products instead enjoyed exploration for various other indications, leading to several indispensable contributions to drug development. Some notable examples for cancer include vincristine and vinblastine from the Madagascar periwinkle (*Catharanthus roseus*), paclitaxel and derivatives thereof from yew (*Taxus*) species, and camptothecin and

**Fig. 1** Schematic representation of the ethnobotanical approach to drug discovery and bioassay-guided fractionation to identify bioactive molecules found in plants



derivatives thereof from *Camptotheca acuminata* (Cragg 1998; Moudi et al. 2013; Thomas et al. 2004). Also of note are the antimalarial artemisinin, isolated from sweet wormwood (*Artemisia annua*), and the Alzheimer's drug galanthamine, isolated from *Galanthus nivalis* (Heinrich 2010b; White et al. 2014).

It must be noted that the compounds contained in medicinal plants represent one of several chemical frontiers in which anti-infective drug discovery is being undertaken. Other very promising frontiers include marine natural products, microbial natural products, and recently developed complex synthetic small molecule libraries bearing the complexity of natural products (Gogineni et al. 2015; Rossiter et al. 2017). With that said, plant natural products remain largely underexplored (Kenny et al. 2015). Only about 15% of higher plant species have been phytochemically investigated, with only a tiny fraction having been studied for anti-infective potential (Cragg and Newman 2013).

### 3.1 Innovation in Plant Natural Product Research

There are many attributes of plant natural products that make them highly desirable for drug discovery, particularly for anti-infectives. Among the chief chemical attributes is that they have a high tendency to occupy regions of the biologically relevant chemical space, which refers to all chemicals that are biologically active (Kellenberger



et al. 2011; Quinn et al. 2008; Wetzal et al. 2011). Indeed, plant natural products have already interacted with a number of proteins during their biosynthesis, and following this, they often function to interact with yet other proteins found in organisms in the ecosystem. Accordingly, their core scaffolds are privileged structures, occupying a region of the chemical space predestined for protein interaction (Maier 2015). Considering that plants have adapted the production of secondary metabolites to interact with their biological surroundings, this in fact makes sense.

Because plant natural products (and natural products in general) are metabolite-like and hence are substrates for biological transporters, they represent an exemption to Lipinski's rule of five, as stated by Lipinski (Gedek et al. 2010; Harvey et al. 2015; Koehn and Carter 2005; Lipinski et al. 1997). The observation that led to this exemption was that only four categories of orally active drugs fell outside the fold of Lipinski's rules (antibiotics, antifungals, vitamins, and cardiac glycosides), and thus, these exemptions were summed up as "compound classes that are substrates for biological transporters" (Lipinski et al. 1997). Plant natural products, along with drug-likeness, also possess vast structural and chemical diversity in excess of many synthetic small molecule libraries (Harvey et al. 2015; Shen 2015). A large contributor to this diversity is the complexity of a large portion of plant natural products. This complexity in itself is yet another advantage, as it has been observed that infectious diseases remain one of the areas that often require chemically and structurally complex molecules (Morrison and Hergenrother 2014). Finally, screening of plant natural products, as well as natural products in general, has been found to be particularly relevant to therapeutic development against "non-druggable" targets (Keseru and Makara 2009).

In addition to providing innovation by way of unique chemistries, plant natural products also show promise with respect to novel mechanisms of action. This includes their potential for the development of both new classes of growth-inhibitory antibiotics and antivirulence drugs, and it is especially in these areas where plant natural products demonstrate great potential. The latter classes of anti-infective, antivirulence drugs have received the attention of research groups more recently, as the approach has been recurrently cited in the literature as a promising anti-infective method that could slow the development of AMR (Pieren and Tigges 2012; Wright 2016). In theory, and as supported preliminarily by a number of *in vitro* and *in vivo* studies of multiple microbial pathogens, antivirulence drugs would attenuate pathogenicity of the target microbe, relieving symptoms of infection and allowing for host immunity to clear the pathogen (Johnson and Abramovitch 2017; Salam and Quave 2018). In doing so, antivirulence drugs would exert less selective pressure for the development of resistance than growth-inhibitory drugs, as the latter clears non-resistant organisms, making way for resistant organisms to enrich the population. A few recent review articles demonstrate the strong promise of plant natural products in antivirulence drug discovery by covering the numerous such compounds



discovered to date (Dickey et al. 2017; Silva et al. 2016). Many antivirulence drug candidates are under investigation as both stand-alone treatments and adjuvants to classical antimicrobials. In the latter case, antivirulence drugs are predicted and have preliminarily been shown to potentiate antimicrobial efficacy.

### 3.2 Current Development of Anti-infectives

Epigallocatechin gallate, the most abundant catechin in tea, was found to inhibit virulence in *Streptococcus pneumoniae* in a nonbactericidal manner by preventing the oligomerization of pneumolysin and reducing the activity of sortase A, which helps anchor to the cell wall surface proteins that contribute to virulence (Song et al. 2017). Derivatives of hamamelitannin, isolated from American witch hazel (*Hamamelis virginiana*), are under investigation for enhancing vancomycin activity in biofilm-associated MRSA infections (Brackman et al. 2016; Vermote et al. 2017a, b). A derivative of 8-hydroxyquinoline, synthesized in roots of the diffuse knapweed (*Centaurea diffusa*), called INP1855 is being investigated for antivirulence activity against *Pseudomonas aeruginosa* (Anantharajah et al. 2016). INP1855 inhibits the injectisome and flagellar type III secretion systems and, very interestingly, was found in a synthetic small molecule library screen (Enquist et al. 2012).

We previously mentioned that one frontier besides plant natural products for anti-infective exploration is the frontier of complex synthetic small molecules that mimic the complexity of other natural products. To this extent, there are examples of such antivirulence small molecules that resemble plant natural product pharmacophores. For instance, Compound 22 is an isoquinolone mannoside, and virstatin is an isoquinoline, and they have been found to target the pili of *Escherichia coli* and *Acinetobacter baumannii*, respectively (Cushnie et al. 2014; Jarvis et al. 2016; Nait Chabane et al. 2014). As for growth-inhibitory plant natural products, up until now most of those that have been isolated from medicinal plants tend to exhibit weak potency and selectivity. An exception to this are a set of acylphloroglucinols from St. Johns Wort species (*Hypericum* spp.), which have demonstrated submicromolar MICs in clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) (Rahman et al. 2018).

On the way to isolating bioactive single compounds from plant extracts, bioassay-guided fractionation produces bioactive fractions. Our lab is currently studying fractions of two plant extracts for the isolation of compounds that inhibit the Agr system in *S. aureus*. The Agr system codes for the organism's quorum sensing system, which is its main mediator of virulence (Salam and Quave 2018). One enriched fraction of a European Chestnut (*Castanea sativa*) extract, as well as an enriched fraction of a Brazilian Peppertree (*Schinus terebinthifolia*) extract, demonstrated high bioactivity against *S. aureus* in vitro and no detectable resistance after drug passaging (Muhs et al. 2017; Quave et al. 2015). In a mouse skin infection model,

co-administration of either fraction with MRSA impaired pathogenesis without manifesting local or systemic toxicity. Of note, both plants were initially selected for study in primary screens of Agr inhibition due to their reported traditional medicinal uses for the treatment of topical infections.

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## 4 Challenges in Studying Plant Natural Products

Medicinal plants are gaining renewed interest as sources of new therapeutics, although this has come about only after several decades of scientists' attention favoring combinatorial chemistry, synthetic small molecules libraries, and high-throughput screening (HTS) (Atanasov et al. 2015; Harvey et al. 2015; Li and Vederas 2009). The diminished attention to plant natural products had been a result of an embrace of the aforementioned resources as novel and promising, legal challenges, difficulty of chemical manipulation, challenges in the compatibility of plant natural products with HTS, and other inherent obstacles in ethnobotanical drug development.

### 4.1 Legal Challenges

Prior to the 1990s, there was little legal guidance as to plant access, sharing of benefits, and patenting with local governments, and many developing countries have been previously exploited. These issues created barriers to the access of genetic resources – including plant materials – in biodiversity-rich countries and was one major factor that discouraged pharmaceutical companies from plant natural product drug discovery (David et al. 2015; Kingston 2011). Furthermore, the synthetic compound libraries became more attractive because they did not include the many access and intellectual property issues that accompanied work on plant natural products (Butler 2004). Fortunately, many of these issues have been addressed by the CBD and Nagoya Protocol, which we have previously discussed.

### 4.2 Chemical Manipulation

Chemical synthesis approaches and derivatization of plant natural products were difficult challenges in lead optimization and resupply (Butler 2004). Total synthesis of a natural product or semisynthesis from another natural product of similar structure are extremely useful because they represent production options that may be more economical and efficient than isolation from plant material. As for derivatization, it is almost always performed on lead natural products in order to perform structure-activity relationship (SAR) studies and to develop analogs with enhanced pharmacological properties. And since 2014, it is a required step in patenting a natural product after new guidelines were issued by the United States Patent and Trademark Office that state that a patent claim must demonstrate a

“marked difference” from a known natural law, material, or phenomenon (Atanasov et al. 2015). In either case, the chemistry has often been difficult to implement due to the innate complexity of plant natural products, particularly in those with numerous oxygen-containing substituents and chiral centers. Whereas classical medicinal chemistry is dominated by C–N bond formation, natural product syntheses rely heavily on C–C bond forming reactions, often accompanied by the formation of hydroxyl groups which contribute to the increased number of asymmetric centers (Maier 2015). Other general characteristics contribute to the chemical complexity of natural products, such as partial or complete saturation of ring structures. Compared to plant natural products, synthetic small molecule libraries tend to include compounds of lesser complexity which are easier to synthesize or modify (Atanasov et al. 2015).

### 4.3 Compatibility with High-Throughput Screening

As mentioned above, a major obstacle for the inclusion of plant natural products in drug discovery programs had been the compatibility of plant natural products – and natural products in general – with HTS (Henrich and Beutler 2013; Koehn and Carter 2005). Often, HTS is performed using plant extracts or fractions thereof rather than single compounds as a component of a wide bioassay-guided fractionation approach. Investigating such a large number of botanical compositions via HTS presents many challenges such as sample preparation and assay design must complement each other, extracts and fractions must be carefully handled so as to minimize compound degradation and precipitation, and attention must be given to the possibility of assay interference and nonspecific effects. In all of these respects, plant natural products are more prone to failure than synthetic compounds. For example, plant extracts can contribute to the optical density of wells, they have a tendency to form precipitants, and they are likely to contain nuisance compounds such as tannins that nonspecifically bind to proteins and saponins which can interfere with cell-based assays via cell lysis (Barbehenn and Peter Constabel 2011; Hostettmann and Marston 1995).

Plant extracts also have a tendency to contain fluorescent or fluorescence-quenching compounds, interfering with the output of assays such as fluorescence gene reporter assays, often employed in HTS (Gul and Gribbon 2010; Henrich and Beutler 2013; Zou et al. 2002). Certain plant natural product classes such as chlorophylls, polyphenols, flavonoids, and fatty acids have also demonstrated a propensity to interfere with several HTS assays (Henrich and Beutler 2013). False-positive results can also be obtained if inorganic molecules such as heavy metals end up in the samples tested (Fernando et al. 2013; Hermann et al. 2013). This can occur, for example, in extracts if plants were collected from busy roadsides exposed to automobile exhaust, or it can occur in synthesized natural products if the synthesis utilized metals which then remained in the sample (Guan and Peart 2006; Zhai et al. 2016).

## 4.4 Deeper Challenges

As we will discuss in the next section, the vast majority of the challenges above have been overcome. There are several other challenges native to ethnobotanical drug discovery that present an intrinsic barrier to entry, a characteristic shared with all other drug discovery approaches. All tasks related to plant collection cannot be automated and must include the expertise of botanists for unambiguous identification, documentation, and herbarium preservation. Collecting sufficient amounts of plant material for isolation, especially if the plant was obtained abroad, can prove difficult when the need for more plant material increases substantially following lead identification, advanced preclinical testing, and especially with demonstration of clinical efficacy. Care must be taken to source plant material in a sustainable way in order to avoid situations similar to the classical “taxol supply crisis” (Cragg et al. 1993). Loss of biodiversity and loss of traditional ethnobotanical knowledge (TEK) also lead to accessibility issues where (1) isolation of a natural product of interest is no longer possible due to the endangered status of the plant of origin, and (2) this drug discovery approach loses its inherent advantage of being a targeted approach when the knowledge guiding this advantage ceases to exist. Unlike many other challenges in ethnobotanical drug discovery, these two appear to be growing worse over time, as climate change prevention efforts and ethnobotanical surveys have yet to gain sufficient momentum. It is because of these collection and resupply challenges, as well as the aforementioned accessibility issues, that microbial natural product drug discovery has often been preferred by pharmaceutical companies (Butler 2004).

The complexity of medicinal plant extracts also presents challenges. The chemical composition of plant material can vary, especially between different collection times, presenting challenges for chemical assessment. A bioactive agent may be present at such low concentrations that it is not detected. An assay may also fail to detect it if it is unstable in mixture or separated by fractionation from a synergist (Wagner and Ulrich-Merzenich 2009). If detected, often only small quantities of the bioactive agent are present, and with that, structurally related molecules are usually present as well and must be distinguished. Considerable time is often required to structurally characterize natural products to determine whether the molecule is already known. Due to these challenges and those described above, a prevailing sentiment in the field is that plant natural product drug discovery requires tremendous effort, hits are theoretically easy to miss, and the probability of duplication is high (Li and Vederas 2009). In the 2000s and into the 2010s, along with plant natural products, microbial and marine natural product research have seen a decline (Beutler 2009; David et al. 2015; Ortholand and Ganesan 2004). With many large- and medium-sized pharmaceutical companies having terminated their natural products programs, academic universities and start-up companies have been left to move forward the bulk of the research and development in this space.

## 5 Re-emergence of Plant Natural Products for Drug Discovery

Pharmaceutical companies had for the past three decades largely avoided the difficulties of natural product-based drug discovery, turning instead toward combinatorial and synthetic small molecule libraries for the discovery of new anti-infective drug leads via HTS (Beutler 2009; David et al. 2015). The results of these decades of drug discovery, however, did not meet expectations (Scannell et al. 2012). The hype for combinatorial chemistry and synthetic libraries was that drug leads would be delivered quickly and in vast amounts for any therapeutic area of interest, as compared to previous drug discovery methods (Butler 2004). In fact, as evidenced by a declining number of new drugs reaching the market, the hype was not realized (David et al. 2015; Kingston 2011; Scannell et al. 2012). Indeed, while 45 new drugs were approved by the US Food and Drug Administration (FDA) in 1990, less than half that amount, 21, were approved in 2010 (David et al. 2015; Kingston 2011). An important contributor to this low turnout is the narrow region of chemical space occupied by these libraries. At present, the decrease in drug approvals is in part revitalizing interest in natural product drug discovery, notwithstanding its complexity (Heinrich 2010a). A recent analysis of PubMed publication trends in this research area reflects a rapid increase in plant natural product research (Atanasov et al. 2015). This renewed interest coincides with specific major scientific and technological advances, which have addressed many of the challenges of ethnobotanical drug discovery: improved understanding of disease pathogenesis, improved natural product medicinal chemistry, increased compatibility of natural products with HTS, and advances in phytochemical analysis.

### 5.1 Advances in Medicinal Chemistry for Natural Products

Over the past 20 years, the field of synthetic chemistry has seen tremendous progress in the ability to synthesize and modify natural products (Szychowski et al. 2014). Modern synthetic methods have opened doors to transformations that had traditionally been difficult due to their selectivity and high yield. These synthetic methods can be divided into two categories based on what they aim to create: (1) large numbers of complex and diverse small molecules for building libraries or (2) small quantities of derivatives of a given natural product (Morrison and Hergenrother 2014). Successful strategies for the former include diversity-oriented synthesis (Cui et al. 2011; Schreiber 2000), diverted total synthesis (Szpilman and Carreira 2010), function-oriented synthesis (Wender et al. 2008), biology-oriented synthesis (Wetzel et al. 2011), complexity to diversity (Ciardiello et al. 2017), and biosynthesis-inspired synthesis (Baskar et al. 2011).

As for producing derivatives in a more controlled fashion, such methods can be divided into two further categories: the use of a single reactive group to alter structure or the use of multiple reactive groups (Maier 2015). Traditional methods that target a single reactive group generate multiple products in one reaction, making separation difficult and yields low (Appendino et al. 2001). In the mid-2000s, reagent control allowed for the selective synthesis of product (de la Torre et al. 2003, 2005), and in the 2010s, the process was systemized to yield more predictable product (Balthaser et al. 2011; Hutt et al. 2013; Ignatenko et al. 2013; Ignatenko and Tochtrop 2013). As for methods that use multiple reactive groups, recent work has formalized the process of multiple structural transformations to produce several highly complex derivatives (Huigens et al. 2013). Thanks to such synthetic methods, there are chemistries we can access now that may very well aid in the development of new therapeutics (Szychowski et al. 2014).

## 5.2 Improvements in HTS Compatibility with Natural Products

For HTS, particularly in campaigns that involve natural products, one must pay attention to many sample details, including maintenance of integrity, potential to interfere with assay readouts, and nonspecific and off-target effects. As discussed in the prior section, these were among the most challenging aspects of incorporating natural products into HTS campaigns. Now, most of these potential problems can be addressed by careful experimental design and management. Extracts have been and should continue to be screened in HTS due to the sheer biodiversity they contain, which is much less accessible in the form of isolated single compounds due to the difficulty of isolations at that scale (Henrich and Beutler 2013). The problems of low concentrations of bioactive compounds and high concentrations of nuisance compounds in extracts have been addressed by the trend of prefractionation (Harvey et al. 2015; Henrich and Beutler 2013). Prefractionation aims to split an extract into a small number of fractions of reduced complexity, concentrating nuisance compounds (such as highly hydrophilic or hydrophobic compounds) into some fractions, while other metabolites, more likely to be pharmacologically active, become enriched in others (Harvey et al. 2015). Additionally, these fractions are more amenable to chromatographic analysis than their parent extracts, facilitating bioassay-guided fractionation.

HTS assays can be categorized as either biochemical or cell-based. In both types, false activities can be identified by utilizing parallel, nonspecific, or off-target assays so as to examine effects such as the actual detection enzymes, reagents, and cell survival (Henrich and Beutler 2013). In biochemical assays, assay interference has been shown to be reduced by protocol modifications such as more stringent washes for certain assays, testing multiple doses, carefully selecting detection reagents, and addition of agents to reduce aggregation or nonspecific binding. In the last 15 years, such adjustments have led to the identification of modulators of a number of enzymatic targets via natural products HTS. Cell-based assays can be categorized as targeted (assays examining reporter strains, enzymatic activities, etc)

or phenotypic (assays examining growth, differentiation, etc). In these assays, there is a strong need for eliminating nonspecifically cytotoxic compounds. The presence of such compounds can be effectively assessed by running a cytotoxicity assay in parallel with the reporter assay (Ruocco et al. 2007; Woldemichael et al. 2006). Following cytotoxicity assays, sample libraries as well as cell lines can be cataloged with acquired data to record the cytotoxicities exerted and experienced, respectively, aiding in identifying potentially problematic samples (Schulze et al. 2013). Additionally, secondary orthogonal screens, perhaps examining multiple doses, can help identify true active samples.

Indeed, the above advances make HTS of natural products more accessible than ever before. To boost access even further, the National Cancer Institute (NCI) has launched the NCI Program for Natural Product Discovery (NPNPD) in 2018 (Thornburg et al. 2018). Under this program, the NCI will create a prefractionated library using over 125,000 natural product extracts from the NCI's Natural Product Repository. This library will consist of over 1,000,000 fractions and will be made available free of charge in 384-well plates for screening by researchers against any disease. Importantly, NCI's Natural Product Repository is made up of extracts acquired through NCI Letters of Collection agreements with participating countries or their representatives, ensuring mechanisms for equitable access and benefit sharing in line with the spirit of the CBD and the Nagoya Protocol (UN 2011).

### 5.3 Emergence of Metabolomics and Ethnophytotechnology

Metabolomics and ethnophytotechnology represent two fields that stand to become strong enablers of ethnobotanical drug discovery thanks to recent technological advances. Metabolomics in its most general sense is the analysis of all metabolites in a biological sample. Advancements in chromatography and spectroscopy over the past decade have allowed for the acquisition of high-quality data, particularly when coupled in the form of with ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS) (Breton and Reynolds 2013; Rathahao-Paris et al. 2015; Seger et al. 2013). As for ethnophytotechnology, it refers to “the use of plant biotechnology to improve or enhance the inherent economic or culturally valuable traits of plants as described and influenced by ethnobotany” (de la Parra and Quave 2017). The field allows for the improved production, manipulation, and scientific understanding of natural products through advancements in technologies such as bioreactors and metabolic engineering.

In a metabolomics analysis of a plant extract, MS data provides a set of molecular features, which include molecular ions, adducts, and in-source fragments, among other data points (Allard et al. 2017). Molecular formulae can be deduced from an analysis of these data. MS fragmentation (MS/MS) data can then be used to link metabolites based on spectra or structure via an MS organization tool, and other information can be mapped to this organized data, including bioactivities, taxonomy, and gene sequences. This data set, now a molecular network, can be annotated against existing MS/MS databases, whether built experimentally or *in silico*, in order to



identify metabolites contained in the extract (Allard et al. 2016; Klein-Junior et al. 2017). Such a metabolomics analysis performed on fractions of a bioassay-guided fractionation campaign can inform researchers on a number of issues of interest, such as whether there are common pharmacophores among potentially bioactive compounds or whether certain compounds may act synergistically (Ngo et al. 2013). Such analyses can also be used in dereplication, the process by which already-known compounds in a natural product composition are identified to avoid duplication (Wolfender et al. 2015). Effectively, metabolomics transforms traditional bioassay-guided fractionation into a rapid identification process of valuable plant natural products.

While metabolomics facilitates the drug discovery process, ethnophytotechnology facilitates the resupply of plant natural products of interest. A major focus is their standardized and controllable production *in vitro* by way of bioreactors, where plant cell and tissue cultures reproducibility generate high yields (O'Connor 2015). To help increase yields further, TEK can be consulted to understand (1) timing and conditions when a medicinal plant is traditionally collected and (2) characteristics of populations of the medicinal plant that are traditionally favored for use (de la Parra and Quave 2017). Metabolomics can also be used to identify favorable timing and conditions by monitoring gene function and biochemical status of a plant (Harvey et al. 2015). By attempting different types of exogenous administration of hormonal elicitation or by genetically modifying cells to better meet optimal plant RNA or protein levels, appreciable improvements in yield can be achieved (Atanasov et al. 2015). Through metabolic engineering, enzymes can be altered or introduced to produce metabolite derivatives for SAR and lead optimization studies (Ochoa-Villarreal et al. 2016). *Agrobacterium*-mediated gene transfer and CRISPR technologies are being studied to this end (Chumakov et al. 2012; Loyola-Vargas and Avilez-Montalvo 2018). However, production of plant natural products need not only occur in plant cells and tissues; cultivable microbes including yeast have been successfully utilized (Galanie et al. 2015; Nakagawa et al. 2016). These technologies open up drug development opportunities for rare, endemic, or endangered medicinal plants, whose populations are too small for sustainable bulk collections (de la Parra and Quave 2017).

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## 6 Conclusions

We have presented an overview of the current state of the ethnobotanical approach to drug discovery and the emerging opportunities for the discovery of new anti-infectives from medicinal plants. Although this pathway to discovery was once burdened by numerous hurdles in the access and examination of plant natural products, today scientists can benefit from the establishment of clear international platforms for ethical access to materials as well as from advancements in fields such as medicinal chemistry, high-throughput screening, metabolomics, and ethnophytotechnology. This progress is of great importance as it has further enabled the pharmacological exploration of the chemical space encompassed by medicinal plants. Indeed, the biological resource and potential for anti-infective drug development are there, and now, it is more accessible than ever before. We predict that with the spread of these technologies, plant natural products will prove an important source for anti-infective drug development.

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