Challenges and Considerations in the Preparation of Novel Antibiotic Phytochemicals

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors SE and MAB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MLV and LA managed the analyses of the study and review protocol. Authors MA and SA managed the literature searches. All authors read and approved the final manuscript.

ABSTRACT

Drug-resistant pathogenic and opportunistic bacteria pose a global health threat. Plants, particularly those employed in traditional (folk) medicine, may provide a source of antimicrobials compounds possessing novel mechanisms of action with which to combat current and emerging infectious diseases. However, experimental designs in ethnobotanical investigations of this type are complicated by several factors. Among these are chemotype variations among plants and plant parts, and potential antagonistic, additive or synergistic effects between plant compounds. These efforts are further hindered by a lack of standardized applicable chemical extraction methods in the

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1. INTRODUCTION

The search for novel antibiotic phytochemicals due to the evolution of antibiotic-resistant strains of microbes may be facilitated by the optimization of methods of extraction, isolation, and identification of natural products. The more preparation protocols account for key considerations in the preparation of antibiotic compounds, the more the barriers to optimization will be circumvented. Therefore, to further the refinement of efficacious and efficient antibiotic discovery methods, this review seeks to achieve five aims. First, it briefly describes the import of the dearth of antibiotic-resistant compounds and gives a brief overview of historical strategies to solve this dilemma. Next, it provides guidance for the design of preparation protocols by presenting an examination of key methodological considerations in the search for antibiotic phytochemicals. Third, it provides compound discovery guidance through a literature review of various antibiotic testing methods. Four, the importance of mutagenicity and toxicity testing is discussed to ensure the safety of compounds intended for human use. Fifth, it reviews methods of identification of the chemical constituents of extracts demonstrating antibacterial activity. Lastly, it provides insight into the literature of assays utilized to discern by what mechanism of action phytochemicals exert their antibacterial effects. Ultimately, through the fulfillment of these aims, this review seeks to provide insight into a framework through which the search for novel antibiotic phytochemicals can be increasingly standardized.

2. ANTIMICROBIAL RESISTANCE AND THE NEED FOR NOVEL ANTIBIOTIC COMPOUNDS

Infectious diseases, often caused by opportunistic or pathogenic antibiotic-resistant bacteria represent the world’s leading cause of human fatalities, accounting for approximately 700,000 deaths annually worldwide [1,2]. These endemic diseases can also indirectly render populations more susceptible to epidemic diseases, such as influenza, which could be caused by secondary bacterial infection [3]. In the search for novel antibiotics with which to combat drug resistance, “medicinal plants”, or plants extracts used in traditional medicine, may provide an expansive pool of unknown compounds from which to draw inspiration. Indeed, plants have already played a historical role in the promotion of human health, serving as the starting point for many of the modern pharmaceuticals in use today. In industrialized nations, more than a full quarter of prescribed medications derive their origins either directly or indirectly from plants. Over the last century, natural products (also known as phytochemicals or plant secondary metabolites) have served as the most successful source of drug leads for medications pertaining to a variety of diseases [4]. It is postulated that these compounds are selected for over time to fortify opportunistic vulnerabilities to various abiotic threats such as nutrient deficiency, drought, lack of oxygen, excessive temperature, ultraviolet radiation, or pollution, and as a co-evolutionary biodefense to biotic agents, which include vertebrates, insects, protists, fungi, and bacteria [5-8]. A causal relationship between microbial trespass and phytochemical synthesis is supported by the discovered synthesis of some of these compounds post-infection [9,10]. Further, it is known that plant tissues often possess secondary metabolites, which are produced and used by these plants for antibacterial purposes [4]. It is therefore reasonable to hypothesize that some of these plant compounds may also be beneficial in combating human bacterial infections.

Discovery of novel antibiotics in antiquity likely resulted primarily from inductive reasoning and were then passed down through oral transmission or codified in sacred texts or medicinal compilations. The Old Testament of the Bible refers in multiple passages to the antibiotic oils of the aromatic perennial herb *Origanum syriacum* [11,12]. Its Book of Leviticus states that, “If [the patients]…have been healed of their defiling skin disease the priest shall order...hyssop (*Origanum syriacum*) be brought...
for the person to be cleansed” [11]. The essential oils of *Origanum syriacum* showed antibacterial activity against 16 tetracycline-resistant *Brucella melitensis* isolates at a minimal inhibitory concentration of 3.125 µl/ml [13]. The Old Testament continues its antibiotic references when it mentions a botanical called wormwood, which possibly refers to either *Artemisia judaica* L. or *Artemisia herba-alba*, species present in Middle Eastern regions where the books of the Bible were written and compiled [5]. Isolated compounds from *Artemisia judaica* L. have exhibited antibacterial effects [5]. Likewise, the essential oils from four *Artemisia herba-alba* populations showed antibacterial activity in the concentration range of 1-2 mg/ml [14]. Over three-thousand years before Alexander Fleming’s discovery of penicillin in 1928, a Greek king of the sixteenth century B.C. is said to have mentioned that a peasant woman used mold scraped from cheeses to treat wounded soldiers [15]. Chinese sources also allude to the use of moldy soy beans to treat infected wounds over 3000 years ago [15]. These and other references to antibiotics and other therapeutics suggest the undertaking of drug discovery initiatives through the analysis of ancient medicinal and sacred texts.

3. THREE KEY CONSIDERATIONS REGARDING METHODS IN THE SEARCH FOR ANTIBIOTIC PHYTOCHEMICALS

Typically, studies of plants which investigate their therapeutic potential are broad screening analyses which evaluate their antiviral, antioxidant, anti-inflammatory, antiparasitic, antifungal or antibacterial properties. These properties are made possible due to the diverse phytochemicals with differing chemical compositions. Such investigations are usually based on ethnobotanical medicine leads [16], as those plants with a long-standing history of medicinal use constitute the most logical starting points [4]. Thus, the utilization of plants in various forms of traditional medicine by humans is a first consideration in the search for medicinal antibiotic natural compounds. For example, in an effort to identify medicinal plants used to treat general infectious diseases in the Kalahandi Indian district, Mishra et al., first ethnopharmacologically surveyed the Kandha tribe from the Eastern Indian mountains to identify and select nine medicinal plants for systematic screening with urinary tract infection causing bacteria [17]. In such studies, however, there are additional factors which may further complicate sample selection. First, potentially significant chemotype variations may exist, even among members of the same plant species due to geographical [18] or seasonal variations. Smida et al., for instance, in investigating the antibacterial activities of *Ludwigia peploides* and *Ludwigia grandiflora* extracts over a period of several months, reported time-dependent efficacy differences against multiple strains of bacteria, such as gram-positive *Staphylococcus aureus* and gram-negative *Salmonella enterica* [19]. Even the microenvironment in which a plant grows may differentially affect its chemical composition. According to optimal defense theory [20], the allocation of plant resources for growth versus defense, including the re-allocation of immunity, may differ depending upon the presence of stressors. For instance, when under both pathogenic and abiotic stress, younger leaves prioritize pathogenic defenses, while abiotic stress tolerance is re-allocated to older leaves [20]. Other studies like those performed by Vanitha et al., conversely showed no discernable differences between wild type and tissue cultured plant extracts of *Tylophora indica* suggesting that variations in chemical composition must be accessed on a species basis [9].

While it has been proposed that antibacterial compounds are more likely to be located within parts of annual growth, such as buds, young leaves or reproductive organs [20], it is quite evident from the literature that these compounds are not universally relegated to specific tissue types. Thus, plant part selection for bioactive compounds is a second key consideration in the search for novel antibiotics. Yasunaka et al., for example, noted differences among plant parts when examining the antibacterial properties of extracts created from the fruits, heartwoods, leaves, fruit peels, roots, stems and twigs of various Mexican medicinal plants [21]. Interestingly, several authors have observed that extracts prepared from different plant parts of the Annonaceae family using a variety of organic solvents showed apparent selective antimicrobial and antiparasitic activities [22-24]. As the inclusion and separate study of plants obtained from differing environments and times in search of efficacy would likely add substantially to workload, it may be wise to reference and mirror what plant parts were selected, and what methods were employed for procurement, in traditional medical practice.
The chemical extraction of plant material, in the pharmaceutical sense, refers to the separation of therapeutically active constituents, along with the elimination of insoluble material through treatment with selective solvents [25]. Another complicating factor is the inability to universally account for all possible chemotypes during the chemical extraction process. This is first evidenced in the literature with regards to the treatment of plant material prior to chemical extraction. Thus, plant part preparation and methodology constitute a third consideration. For example, starting plant material is often fragmented or pulverized, then dried, presumably to eliminate excess water and increase the concentrations of antibacterial compounds in the sample. However, plants may be dried in the light or shade, for varying periods of time, and at different temperatures, with no rationale provided by the authors for their method selection. Kuppusamy et al., for instance, cut Commelina nudiflora plant material into small pieces and dried it at room temperature in 12-hour cycles of light and darkness [26]. Voravuthikunchai similarly cut Thai medicinal plant material into small pieces, but this material was dried overnight at 60°C [27], while Kenny et al., sliced, then freeze-dried dandelion roots prior to extraction [28]. Further, the practice of drying plant material prior to chemical extraction is questionable, for as Ali-Alabri et al., reported, for example, extracts derived from fresh Datura metel leaves possess enhanced antimicrobial efficacy in comparison to those created from dry leaves [29]. Therefore, some additional considerations and experimentation may be necessary to determine whether plant material preparation affects antibacterial testing results.

Solvent choice and the methods employed in the chemical treatment of plant material also vary substantially, as there has been little effort to standardize the extraction procedure [25]. Further hindering standardization efforts in this regard is that extraction efficiency, the amount of extract produced per amount of starting material, may vary among plants and among solvents used, including when different methods are applied using the same solvent in treatment of the same plant material [25]. For the extraction of antibacterial compounds, ethanolic organic solvents, such as methanol and ethanol, are reported to be effective [17,30,31], and may constitute a good starting choice. However, the extent of their extractive abilities can differ by plant based on the biological and morphological characteristics of each plant. Andleeb et al., for instance, found ethanol extracts of Argemone Mexicana to be more potent against Bacillus subtilis, Staphylococcus aureus, Vibrio cholera, Enterobacter aerogenes, Klebsiella pneumonia, and Escherichia coli, followed by chloroform and lastly methanol [32]. Conversely, Chen et al., obtained greater antimicrobial activity from the water extract, as opposed to the methanol and ethanol extracts [33]. Additionally, special considerations must also be taken for the extraction of proteins in their native state as demonstrated by Akeel et al., who used sodium phosphate citrate buffer and sodium acetate buffer, the latter at six different pH values, in the extraction of peptides and proteins from medicinal plant seeds [34]. In a similar approach, Roy et al., used chloroform with added HCl in the chemical extraction of Andrographis paniculata plant material, claiming that this plant’s metabolites are extracted at higher yields in more acidic solvents [35]. However, given the large number of solvents available, it would again be reasonable to attempt to replicate the preparation methods used in traditional folk medicine or phytotherapy (also known as herbal medicine, botanical medicine, or phytomedicine) for the specific plant(s) under study [36], in order to best substantiate or refute historical claims of the plant’s medicinal value. For instance, in the antibacterial screening of twelve plants from northwestern Argentina, Soberon et al., prepared infusions, decoctions and tinctures in accordance with traditional uses, and indeed found that aqueous and alcoholic extracts of Tripodanthus acutifolius demonstrated antibacterial efficacies comparable to those of commercially available antibiotics against several strains of bacteria [36].

Though the extraction processes through which a solvent is applied to plant material also vary, the methods can be very straightforward. Voravuthikunchai, for instance, simply soaked crushed plant material in 95% ethanol for 7 days at room temperature [27]. Alternatively, Elddeen et al., sonicated powdered plant material for a period of only one hour [2]. Using a heat-based method, Stanoevic et al., created an aqueous extract by cooking dried plant material in a water bath at 80°C [37], while Ganie et al., produced a methanol extract from powdered plant material using a Soxhlet extractor at 60-80°C [38]. Though these heat-based methods may potentially increase extract yields, it is also possible that they can denature or destroy heat-labile proteins or compounds in the plant material. This is not necessarily the case, however, as Kousha reported enhanced
antibacterial efficacy of aqueous extracts of *Heracleum persicum* and *Heracleum mantegazzianum* prepared through boiling in comparison to extracts that were not boiled [39]. However, since no chemical analysis of the extracts was completed in this study, it is possible that boiling merely extracted the antibacterial compound(s) in the plant material at greater quantities. In general, these conventional extraction methods use a diversity of organic solvents and require a large volume of solvents and long extraction time. Thus, these processes are time-consuming, and intensely laborious where extraction, isolation and identification activities have been a limited step in drug development from natural products [40]. Some modern or more selective methods, including supercritical fluid extraction pressurized liquid extraction, and microwave assisted extraction have been applied in phytochemical extraction. Interestingly, they offer several benefits such as lower solvent consumption, shorter extraction time, higher selectivity and more importantly, greater conservation of natural product biological activities [40].

In one interesting comparative study of techniques, Kothari performed seed extractions using five different treatment methods: Soxhlet extraction, ultrasonication, continuous shaking at room temperature, and microwave extraction with or without intermittent cooling, and concluded the Soxhlet method to be superior in terms of extract efficiency [25]. However, similar to solvent selection, some trial and error using different methods of chemical extraction may be necessary to achieve optimal extract yields and to determine the effects on antibacterial testing results. Consultation of extract preparation methods for the plant(s) under study used in traditional medicine may help guide successful strategies here as well.

Once these plant extracts are obtained, the removal of insoluble material from a chemical extract is most routinely accomplished by means of filtration through cotton [41], cloth [10,41] filter paper, [42,43] or through centrifugation [25,44]. For sterility purposes, extracts can be passed through microporous syringe filters [44,45]. Following these steps, excess chemical solvent must be removed from the extract if the solvent is insoluble in media commonly used for antibacterial testing, or if the solvent itself is toxic to bacteria at low concentrations. Simple evaporation in an open environment or fume hood [29] is possible, but this may be very time consuming for water or other less volatile solvents. Freeze drying [16,46], water baths [47], and reduced pressure methods such as rotary evaporation [38,48] are also used, although again, heat based methods may damage sensitive compounds in the extract. Two unique methods of extract preparation appearing in the literature, which provide comparatively easy solvent removal, are those of essential oils and supercritical fluid extraction. Essential oils are mixtures of volatile compounds are obtained by steam distillation of the plant material, often using a Clevenger-type system [49]. Liquid, limpid, volatile and mostly colorless [50], they can be air dried over materials such as anhydrous sodium sulfate [46,47,51]. While this method may result in high extraction yields, potential drawbacks include potential thermal degradation of peptides and compounds, and the inability to obtain metabolites of larger molecular mass [49]. Alternatively, supercritical fluid extraction exposes solvents to extremely high temperatures and pressures, giving the solvent an increased density, while allowing it to retain its ability of diffusion. This process enhances extraction, as the solvent can more easily penetrate the plant material. A solvent-free sample is produced when pressure reduction converts the solvent to a gas, separating it completely from the liquid or semiliquid extract. The use of carbon dioxide, for example, which requires temperatures of only about 40°C, can prevent thermal degradation of peptides or other compounds [49]. Thus, as it potentially offers good yields and can circumvent the problem of thermal degradation, this latter method appears to have clear advantages over more commonly used chemical extraction methods and may see greater favor of usage in the future.

A final potentially complicating factor inherent to ethnobotanical investigations seeking antibiotic compounds is the possibility of additive, synergistic, or antagonistic effects between compounds in the plant material. For example, if multiple solvents are used, either in the individual treatment of separate samples of plant material, or the sequential treatment of a single sample of plant material, compounds possessing additive or synergistic effects may be allocated to separate solvents, possibly hindering the detection of these effects in antibacterial assays. Conversely, it is also possible that the use of one or a limited number of solvents could mask the antibacterial activities of a plant, if compounds exerting antagonistic effects, but normally physically sequestered in the plant, were
simultaneously extracted. For example, in investigating the antibacterial effects of compounds found in *Fructus Euodiae* on *Mycobacterium tuberculosis*, Hochfellner et al., reported antagonistic effects between indolquinazoline alkaloids and the quinolone alkaloid evocarpine, both of which were present in the original plant material [52]. Though additive or synergistic effects can be explored by the testing of separate extracts in combination, the detection of antagonistic effects is hypothetically much more difficult, with exhaustive investigation potentially requiring the isolation and testing of all compounds present.

4. ANTIBACTERIAL TESTING

The methods of antibacterial testing appearing in the reviewed literature are adopted from those used in synthetic antibiotic evaluation. The Kirby-Bauer method for example, or disc diffusion assay, represents the standard antibacterial assay in widest use [8]. However, this method has shortcomings. Obtaining zones of inhibition uniform in diameter requires even impregnation of the disc with extract [4], which is not necessarily done easily by hand. Second, the solubility of the extract and its subsequent rate of diffusion in the growth media being used affects the distance it travels from the disc, potentially diminishing the size of the observed zones of inhibition [6,48,53]. Thus, the disc diffusion assay is therefore not well suited to antimicrobial compounds or plant extracts which are insoluble or scarcely soluble in water [54]. Third, the initial bacterial inoculum level [4], and the growth rate of the microorganism being assayed [53] may also prejudice results, as an extract’s antibacterial efficacy, if limited, may be masked by higher inoculum levels or particularly virulent growth. Due to these limitations, the disc diffusion assay is considered an essentially qualitative method, primarily useful only for the preliminary screening of large numbers of samples [30]. Attempts to standardize the reading of inhibition zone diameters via automation have been undertaken, most notably by Idelevich et al., who evaluated the use of the ADAGIO automated system to read disk diffusion tests across a variety of bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *β-haemolytic streptococci* [32]. Idelevich et al., found that once the inhibition zone diameters were visually defined and adjusted, the ADAGIO system could be used to yield categorical data in agreement with the visual method without the subjectivity associated with previous methodologies.

The microbroth dilution method by comparison, measures the growth of bacteria in extract-containing liquid media, using either spectroscopic or visual means. This method allows for a more precise quantitation of inhibitory effects. For example, even when using a method as simple as visual inspection for a lack of turbidity in the culture, tetrazolium salts, which living bacteria convert to colored formazan derivatives, can be added directly to extract-treated microbroth dilution samples, and provide for a quantifiable means of assessment [33]. However, one common problem encountered when using visual or spectroscopic methods is that plant extracts tend to possess color [7], which complicates evaluation of test samples even when using spectroscopic methods. Measures can be taken to account for this effect, however, such as those utilized by Soberman et al., who included assay samples with the incubation mixture being tested, but excluded bacterial inoculum, and these optical density readings were then subtracted from those of test samples containing both the incubation mixture and inoculum [14]. Additionally, when using the microbroth dilution method, provided the extract, or the solvent in which it is suspended, is soluble in the media, even distribution of the sample should be obtained. Further, though initial inoculum level and virulence impacts on experimental results remain as concerns, starting bacterial levels are more easily titrated using this method.

Further, the microbroth dilution method is more economical in terms of time and resources required, enabling the simultaneous screening of combinations of different plant extracts and bacterial strains [55]. This exploration of extract combinations in search of additive or synergistic effects appears numerous times in the reviewed literature, and is justified when considering that traditional healers often use combinations of plants in disease treatment [56]. For example, Aql et al., reported synergistic antibacterial effects among plant extracts from India when tested against a number of clinical MRSA strains, as well as between these extracts and synthetic antibiotics [48]. Similarly, Adwan and Mhanna reported reductions in synthetic antibiotic minimum inhibitory concentration (MIC) values when applied in combination with extracts of Palestinian plants against *Staphylococcus aureus* clinical strains [43].
It is important to note that discerning bactericidal versus bacteriostatic effects, regardless of antibacterial assay method, requires observation for growth after transfer of extract-treated samples to fresh media. Yamaguchi et al., for example, plated microbroth dilution samples displaying no visible growth after incubation on agar plates for verification of bactericidal effects [57]. Still, the microbroth dilution method also provides the ability to perform, time-kill assays that allow for determination of the length of exposure necessary for bactericidal effects to be exerted. Leandro et al., for instance, removed aliquots from samples of Staphylococcus epidermidis treated with the plant compound dehydroabietic acid, at multiple time points up to a length of 24 hours, then serially diluted, plated, and observed these samples for visible growth [58].

It also appears to be a lack of agreement in the literature regarding what constitutes a meaningful or significant MIC value. For example, the crude state of the initial extract and the potentially low concentrations of the compounds responsible for the antibacterial activity [32], naturally results in higher experimental MIC values prior to sub-fractionation of the extract, or the isolation of individual compounds. Still, it has been suggested that for pharmaceutical use, only crude extracts and essential oils demonstrating antibacterial effects at or below concentrations of 100 µg/ml are promising candidates, with this number dropping to as low as 2 µg/ml for isolated compounds [19,58]. As a result, there exists a wide degree of variability in the working concentrations of extracts used in antibacterial assays in the literature, and this is problematic as it makes recognition of significant or meaningful results difficult. For example, in evaluating the antibacterial effects of sage extracts, Stanojevic et al., reported testing concentrations as high as 40 mg/ml [37]. Similarly, Hussain et al., assayed concentrations as high as 100 mg/ml in the evaluation of extracts of Pakistani plants used in traditional medicine [59], while Ugoh et al., reported testing concentrations as high as 500 mg/ml when evaluating the antibacterial efficacy of a Khaya senegalensis stem bark extract [60].

It is important to consider that besides inoculum level, MIC values may also be affected by factors such as temperature, volume, salt formation and precipitation, antioxidant properties or autofluorescence of the extract [6,54]. Consequently, the use of these extreme concentrations increases the likelihood that one or more of these variables will influence experimental results. It may be possible to account for some of these adverse effects on an individual basis however, as Chen et al., reported when he tested the extracts of Allium sativum over a range of pH values to determine the optimum pH of 3.0 that resulted in the greatest antibacterial activity [60].

5. MUTAGENIC AND TOXICITY TESTING

Mutagenicity and toxicity testing of plant extracts, though not always included in these investigations, do appear as well, and are certainly necessary components of a thorough investigation. As pointed out by Leandro et al., the extracts collected from these plant materials include phytochemicals that are synthesized by the plant as a defense mechanism against microorganisms and herbivores [6-8,61]. As such, these plant extracts may be toxic to other organisms, including humans. Emphasizing this concern are claims of plants used in traditional medicine displaying mutagenic effects in in vitro assays [61]. Direct toxicity of plant extracts towards eukaryotic cells can be tested with in vitro assays. These include the Ames test, which is a reverse mutation test used to evaluate the mutagenic potential of plant extracts [62]. Eldeen et al., used the Ames test to screen for mutagenic effects of extracts derived from trees used in South African traditional medicine [2]. Another possible test is the in vitro micronucleus assay in mammalian cells which is used to test for chromosome loss or breakage, typically used to assess genotoxicity [62]. In vitro cytotoxicity tests can also be used to quickly assess the toxicity of plant extracts. However, in the literature reviewed, this assay is more commonly used to determine the starting dose for future in vivo assays [61,62].

In the literature, a variety of cell types are used to assess toxicity using in vitro assays. Owais et al., for example, evaluated the safety of a Withania somnifera extract by monitoring for lysis of incubated human erythrocytes [42], whereas Miceli et al., tested for Borago officinalis and Brassica juncea extract toxicity by monitoring for lysis of sheep erythrocytes [63]. However, without animal model testing, which appears rarely in the reviewed literature, in vitro results alone are limited in their prediction of the usefulness of plant extracts as clinical antibiotics [10,61]. Therefore, a combination of both in vitro and in vivo assays is highly recommended [62]. Animal testing becomes particularly important
when defining optimal antibiotic activity conditions, especially in reference to oral antibiotics, as the gastrointestinal system can greatly affect bioavailability [16,49]. Some information in this regard can be obtained using simulated gastrointestinal fluids. For example, using the 1990 U.S. Pharmacopeia, Vermaak et al. simulated gastric fluid and intestinal fluid, and found that a wild camphor extract partially lost its antibacterial activity after exposure to simulated gastric fluid. However, they also reported that the *Agathosma betulina* extract became active only after exposure to simulated intestinal fluid. Therefore, in vitro screening results could either overestimate or underestimate *in vivo* antibacterial potential. Potential use in different applications also mandates that the extract be quite stable [64]. To evaluate extract stability, Miceli et al. again for example, assayed extracts prior to and after one year of being kept at -20°C, 4°C or room temperature, and reported the loss of antibacterial efficacy versus the bacterial strains showing greatest sensitivity prior to extract storage [63].

The intended application of a plant extract believed to possess antibacterial capabilities as a preservative may necessitate additional evaluations. Previous research has demonstrated that the intrinsic properties of food, including fat, protein or water content, water activity, pH, salt, other additives, antioxidants and preservatives, and extrinsic determinants such as temperature, vacuum packaging, air, gas and target microorganism characteristics, as well as interactions between these factors, can influence bacterial sensitivity, and thus require higher extract concentrations in comparison to those demonstrating efficacy in *in vitro* assays [50,54]. This may be the result of binding or inactivation of antibacterial compounds by food components or additives, or changes in extract solubility [55,63]. Another possibility is the enhancement of bacterial cell damage repair due to a greater nutrient availability in food in comparison to growth media [55]. Miceli et al. for example, tested the antibacterial efficacy of extracts using three food model systems (meat, fish, vegetable), and found that achieving similar results required ten times the concentrations used *in vitro* [63]. Similarly, Klancnik et al., evaluated rosemary extracts in food models of meat, vegetable, and dairy products, and reported that the type of food model used made a difference in MIC values, with higher numbers measured in high-protein and high-fat meat and dairy products as opposed to vegetable models [55]. Complicating this issue further is that the addition of plant extracts to food may change its organoleptic profile [63]. Extracts may alter food flavor [50,63] or smell, possibly making treated products less palatable to consumers or farm animals. Therefore, the intended application of plant extracts, be it as a medicine or as a preservative, warrants the use of these additional assays in such studies to better assess their practical utility.

### 6. METHODS OF IDENTIFICATION

A wide variety of chemical assays, chromatographic techniques, instrumental analyses, and combinations of these, are employed in efforts to identify the nature of the chemical constituents of extracts demonstrating antibacterial activity. For the identification of classes of compounds present in a plant extract, there exists a large number of readily available methods. While some of these are only qualitative, others allow for quantitative measurements as well.

In the literature, chemical methods used for the detection of alkaloids alone, for example, include reagents such as Dragendorff [26], Hager [10], Mayer and Wagner [42]. Similarly, flavonols can be detected using either the vanillin assay, or through the use of a p-dimethylaminocinnamaldehyde-based method [65]. More broadly, the colorimetric method can be used to determine the flavonoid content in methanol and ethanol extracted plant samples [33]. Though the presence or abundance of classes of compounds in an extract does not definitively illustrate responsibility for an extract's antibacterial efficacy, the use of these assays potentially narrows the pool of candidate compounds meriting identification using more advanced techniques or instrumental analyses.

Simple chromatographic techniques can be used to fractionate extract samples, allowing for enhanced identification using other means. Nemereshina et al., for example, combined one and two-dimensional paper chromatography to fractionate extracts prior to the use of chemical, light-based and fluorescence methods for compound identification [66]. Alternatively, using the more sophisticated and specialized chromatographic technique (i.e., high pressure liquid chromatographic-HPLC), Liu et al., fractionated extracts of Chinese plants used to treat snake bites, and created “high-resolution bio-chromatograms” correlating antibacterial
activity to retention time, in order to identify fractions meriting compound identification using another method [67]. However, chromatographic methods can serve as a means of compound identification themselves when extract samples are run in conjunction with reference compound standards. In thin layer chromatography (TLC) the distances migrated by plate spots, known as Rf values, can be compared to those of reference standards for tentative identification [10,68]. However, perhaps the greatest advantage TLC offers is “bioautography”, wherein completed gels can be overlaid with growth media containing bacterial cultures, and zones of growth inhibition can be observed on the plate directly atop individual spots, thus readily identifying those fractions with antibacterial activities [48,68]. In this regard, TLC lends itself particularly well to investigations of the antibacterial properties of plant extracts.

More advanced instrumental analyses usually preceded by, or coupled to a chromatographic method, can more accurately determine the identity of individual compounds present in a plant extract. Liquid chromatography-mass spectrometry (LC-MS) combines the physical separation capacity of liquid chromatography with mass analysis capabilities [4]. Mass spectrometry identification of compounds is achieved through comparison of spectral results to those of standards recorded in pre-existing databases. For example, Kuppusamy et al., compared Gas chromatography-mass spectrometry (GC-MS) spectra data from Commelina nudiflora extracts with those of the National Institute of Standards and Technology database [25], whereas Rodrigues et al. identified the components of essential oils from Mentha cervina by comparing retention indices to those of a standard hydrocarbon mixture, and GC-MS spectra to a home-made library constructed using laboratory-synthesized components and commercially available standards [69]. One advanced instrumental technique that may be particularly promising in the characterization of plant extracts was described by Regazzoni et al., in which flow injection analysis is coupled to high resolution mass spectrometry. This method, according to the authors, offers the advantages of requiring almost no chromatographic separation of extracts prior to analysis, needing no solvent consumption for liquid chromatography, and requiring generally short analysis times [70]. Compound identification using these instrumental analyses may then potentially allow for the isolation, synthesis or procurement of commercially available pure materials for further testing.

Recently, advances in metabolomics and gene sequencing have provided new tools for the screening of secondary metabolites, specifically the elucidation of biosynthetic gene clusters (BGC). In silico analysis of BGC’s has many advantages over previous in vitro methodologies, namely in the ability to identify many of the secondary metabolites that are capable of being produced not just the ones present in the plant samples at the time of cultivation that are constrained by environmental conditions. For example, Shan et al., was able to determine the synthesis of thalianol for the first time in the root epidermis of Arabidopsis thaliana by examining the role of several BGCs with formerly unknown functions [71]. The analysis of BGCs has been hampered in plants because of the larger genome size in comparison to bacteria and fungi where BGC analysis has proven to be a valuable resource in the drug discovery process. As genome sequencing becomes more accessible, the role of BGC analysis promises to unveil new opportunities as it did for bacteria [52].

7. MECHANISMS OF ACTION

Though rare, some assays used to discern mechanisms of action by which plant extracts or compounds exert their antibacterial effects are found in the literature. Ohene-Agyei et al., by focusing on a known bacterial drug-resistance mechanism, used in silico analysis to predict possible plant compound/drug efflux pump interactions, then confirmed some of these predictions using an efflux assay with Nile Red Dye [7]. Bodiba et al., used reverse transcription-polymerase chain reaction to compare changes in spaP and gtfB gene expression, through first using gel electrophoresis to assess RNA quality, then semi-quantitative polymerase chain reaction to determine mRNA levels in both treated and untreated cells [72].

However, general antimicrobial effects or the targeting of bacterial traits related to pathogenicity are more commonly explored. Lu et al., for instance, investigated extract effects on cell membrane permeability by means of transmission electron microscopy, through measuring treated cells for potassium leakage using atomic absorption spectrometry, and by using propidium iodide as an intercalation probe in flow cytometry [73]. Conversely, Chen et al.,
assessed the effects of garlic extracts on cell membrane integrity by testing for the leakage of proteins but conducted a colorimetric analysis using a spectrophotometer and calculated the protein concentration of the treated cells. Furthermore, the group examined changes in relative conductivity using a conductivity meter to determine the effects on membrane permeability [60]. In contrast, Wojnicz et al., investigated extract effects on cellular adhesion and biofilms, using hemagglutination of human erythrocytes as a method to assess P. fimbriae expression, the binding of Congo Red Dye to measure amyloid fiber curli expression, and a microtiter plate assay to quantify biofilm formation [54].

The comparison of an extract’s antibacterial testing results against bacterial species with different phenotypes may yield clues to its mechanism of action. For example, activity against both gram-positive and gram-negative species may indicate the presence of a broad spectrum of antibiotic compounds or general metabolic toxins [8]. On the other hand, reduced efficacy versus Gram-negative species and Gram-positive bacteria may suggest a mechanism of action obstructed by cell wall composition differences [74]. For example, in accordance with majority of the literature, da Silva Dannenberg et al., reported higher sensitivity in gram-positive bacteria against Schinus terebinthifolis plant extracts, suggesting that the dense lipopolysaccharide membrane of gram-negative bacteria is more resistant to the passage of small antimicrobial molecules [8]. Further, such comparisons may also yield clues as to the nature of a specific mechanism of action. For example, Ahmad et al., concluded that the increased antibacterial efficacy of Hedyotis root extracts on a DNA-repair deficient Bacillus subtilis strain in comparison to the wild-type B. subtilis suggest a possible DNA inhibitory mechanism of action [75]. Comparisons of results to standard antibiotics may be useful as well. Oskay et al., suggested that the greater efficacy demonstrated by several plant extracts from Turkey, opposed to synthetic antibiotics to which the bacteria tested were resistant, might indicate a unique mechanism of action [74]. Thus, the inclusion of different phenotypes and standard antibiotics in the antibacterial testing of a plant extract or compound may provide useful information. However, as the number of such assays in the literature appear infrequently, referencing techniques applied in the investigation of synthetic antibiotics is warranted for thorough mechanism of action investigations.

8. CONCLUSION

Of the 310,000 plants studied in the literature presented, less than 15% have been investigated phytochemically or pharmacologically. Encouragingly, there are a growing number of scientific reports accessing plant secondary metabolites in a myriad of applications including as antibacterials, antispasmodics, antivirals and anticancer agents. Significant forethought is commonly used in the selection of assays used to probe the underlying molecular mechanisms at play, but less consideration is usually given to the selection and preparation of plant tissues and extracts. As detailed above, considerable phytochemical variation can exist not only within plants of the same species collected from different areas, but within different parts of the same plant. These chemotype variations are the result of differences in stimuli that can be attributed to environmental conditions such as temperature, humidity, nutrient availability or external stimuli like predation, pollinators or even neighboring species releasing plant pheromones. Given the dynamic nature of plant chemotype variation it is imperative that future investigations carefully select plant tissues under conditions likely to give the best results. One method that can be employed to circumvent this predicament is the use of biosynthetic gene clusters, which should allow for the heterogenous expression of select pathways to produce secondary metabolites, often utilizing a bacterial expression system. While this technique avoids the undesirable investigation of plants not currently expressing potentially beneficial phytochemicals, it is limited in that only one metabolite is produced, and thus synergistic effects produced by the assay of plant extract fractions will not be observed. Conversely, antagonistic effects are likewise negated making observations of key interactions that would be inhibited or diminished possible without lengthy fractioning protocols to isolate metabolites.

The use of in silico techniques like BGCs analysis can also be used as a tool to elucidate structure and possibly the characteristics of compounds from the plant tissue itself. Beside the synergistic effects, practicality is currently the main advantage, as the larger genomes of plants make BGCs analysis difficult and time consuming. Additionally, the analysis of plant tissue through the utilization of social-ethnobotanical research can lead to the acquisition of beneficial information regarding the use of herbal medicines. The recording of the
use of plants by humankind to treat all matter of ailments in sacred texts thereby preserved ancient medicinal techniques and traditions which are so often erased by the passage of time.

The preparation of plant tissues after selection is also vital to the efficiency of the extraction. Pulverizing of plant tissue is beneficial because it increases the surface area available to interact with solvents. When particles become too small however, they may agglutinate and obstruct the flow of solvent. The effects of agglutination are dependent on the pressure available to push solvent through the sample and supercritical extraction techniques may be unthwarted by smaller particles. In general, the use of particles 0.5 mm in size seems amenable to many of the extraction techniques employed.

Likewise, the method of extraction warrants careful consideration. The use of solvent and the management of temperature, time and pressure all impact the quality and quantity of compounds that are retrieved. In this regard, the implementation of Mass Spectral analysis can be useful especially if authors publish chemical profiles so comparisons between plants of similar and differing species can be made. Metabolite profiles obtained by nuclear magnetic resonance are equally as informative and can also be used to analyze the differences between extraction techniques, treatments of plant samples and extraction efficiencies. Several comparisons of extraction techniques have demonstrated the effectiveness of Soxhlet extraction given ample time is allotted for repeated cycles of extraction. As such, Soxhlet extraction should be considered a starting point which more advanced methods are benchmarked against. In that context, other modern separation techniques such as molecular distillation, preparative-GC and multi-dimensional chromatographic separation have been developed with more automatic, sophisticated, rapid, and sensitive detection system to extract and separate natural products, which might reach the requirement of high-throughput screening.

There is a strong, clear and growing interest in the extraction, isolation and identification of natural products and their advantageous applications. The continued standardization of these methodologies will lead to superior quantitative controls, as well as reproducible results, in the search for antibiotic phytochemicals.

CONSENT
It is not applicable.

ETHICAL APPROVAL
It is not applicable.

ACKNOWLEDGEMENT
The authors are grateful to Dr. Barbieri laboratory's members for their insightful comments.

COMPETING INTERESTS
Authors have declared that no competing interests exist.

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