

Methodological Advancements in Antimicrobial Screening of Medicinal Plants: A Critical Appraisal of Extraction, Phytochemical, and Susceptibility Protocol

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ABSTRACT

Objective: This review provides a critical assessment of the experimental workflow adopted in medicinal plant antimicrobial screening from plant choice and extraction to phytochemical characterisation, in vitro susceptibility testing, anti-virulence and synergy testing, mode of action and toxicological assessment. **Method:** Traditional extraction methods including maceration, Soxhlet extraction and hydrodistillation are contrasted with more recent methodologies such as ultrasound-assisted extraction and green solvents comprising natural deep eutectic solvents in terms of yield, selectivity and stability of thermolabile components. Qualitative phytochemical tests and chromatographic identification techniques are reviewed along with their drawbacks in the verification of bioactivity. **Results:** weaknesses that were continually noted throughout the literature included: variable reporting of the concentration of extracts; non-standardised inoculum size and growth media; positive and negative controls not being used when applicable, and inadequate focus on reproducibility and specificity. **Novelty:** Methodological harmonisation to enhance the translational value of future investigations in this area is proposed. Recommendations are provided for the methodological harmonisation to enhance the translational value of futures studies in this area.

INTRODUCTION

Plants have supplied humanity with therapeutic agents for millennia, and a substantial proportion of the modern pharmacopoeia can be traced, directly or indirectly, to phytochemical origins. Interest in the antimicrobial properties of medicinal plants has intensified in recent years as clinically important pathogens have acquired resistance to multiple classes of antibiotics, narrowing the therapeutic options available to clinicians. The World Health Organization has documented a continuing rise in resistance across numerous pathogen-antibiotic combinations monitored through its Global Antimicrobial Resistance and Use Surveillance System, with resistance increasing in a substantial proportion of the combinations tracked between 2018 and 2023 [1]. This trend has renewed scientific and commercial interest in botanical sources as reservoirs of structurally novel antimicrobial scaffolds, either as stand-alone agents, as adjuvants capable of restoring the efficacy of existing antibiotics, or as anti-virulence agents that disarm rather than kill the pathogen [2].

In spite of the amount of published research on plant based antimicrobials, the discipline has been criticized for lacking methodological uniformity. They often vary in the type of solvents, extraction procedure, susceptibility testing method, inoculum size, growth media and the way of expression and interpretation of the activity. Klančnik et

al. [3] found that various diffusion and dilution methods can produce significantly different results of antibacterial activity of the same plant extract and warned that disc diffusion is not appropriate for determining a quantitative endpoint. Adopting a more critical perspective throughout the bioprospecting pipeline, Cushnie et al. [2] extended this critique, showing how misalignments of source material with screening strategy, inconsistent extract dissolution protocols, and failure to consider selectivity index calculations can prevent translation of the results in the most promising case. Thus, the current practice of methodology of experimental studies behind this literature needs to be critically reviewed.

In particular, we review relevant experimental strategies for *in vitro* investigation of antimicrobial activity of medicinal plants at each stage from the selection of starting materials, to extraction, to phytochemical characterization, to testing against bacteria and fungi, to assessment of anti-virulence and synergy, to mechanistic studies and to preliminary evaluation of safety. It does not aim to list individual plant species or their attributed activities in an exhaustive fashion, nor does it cover clinical or *in vivo* pharmacokinetic data, which are beyond the intended scope. The key gap in the literature to address here is the lack of an overarching, highly referenced guideline that integrates methodological advice for the entire experimental pipeline, rather than presenting extraction, bacterial testing, antifungal testing, and mechanistic work as discrete literatures. The goals of the review are fourfold: firstly, to analyze and contrast the principles and advantages/disadvantages of the main extraction and determination techniques presently employed, as well as newer green extraction chemistry techniques; secondly, to discuss the unique methodological aspects that are relevant when the target organism is a fungus rather than a bacterium, or when the outcome of interest is virulence attenuation and not growth inhibition; thirdly, to detect repeated vulnerabilities in methodology that may jeopardize comparability of studies, supported by examples for several host plant families; and fourthly, to suggest practical guidance which may enhance the rigor and translational impact of future experimental endeavours in this field.

RESEARCH METHOD

The literature was selected via structured searches of Web of Science, Scopus, PubMed and Google Scholar, supplemented by discipline-specific indexing platforms based on their natural product and microbiological research coverage: CAB Abstracts, AGRICOLA, the Directory of Open Access Journals, and the Cochrane Library for methodological support in the review process. The reference lists of included articles were also hand searched to identify further relevant studies not captured by the electronic search.

Search strings contained terms of plant material ("medicinal plant*" OR "plant extract*" OR "phytochemical*"), antimicrobial assessment ("antimicrobial screening" OR "antibacterial activity" OR "antifungal activity" OR "minimum inhibitory concentration" OR "disc diffusion" OR "broth microdilution" OR "biofilm"), anti-virulence strategy ("quorum sensing inhibitor*" OR "anti-virulence"), and method ("extraction method*" OR "green

extraction" OR "susceptibility testing" OR "bioautography" OR "synergy" OR "checkerboard" OR "selectivity index"). Boolean operators and truncation were applied consistently throughout all databases, and search fields were restricted to title, abstract and keywords. The literature was searched from 2005 to April 2026, a timeframe chosen to capture the era when Clinical and Laboratory Standards Institute standardized protocols were becoming the norm in natural product research, bearing in mind that classic methodological papers published before this period were kept if they still serve as the primary methodological reference for a particular technique.

Original research articles and review articles in indexed journals were included if they discussed any step of the experimental process for screening medicinal plants for antimicrobial activity. Where no corresponding peer-reviewed material was available, authoritative institutional reports were accepted, as long as a uniform resource locator was provided. Books, theses (unpublished), conference abstracts, trade publications, preprints, patents and literature which was not peer reviewed were excluded. Articles which were not available in English were excluded, along with those for which full-text was not accessible.

The narrative design rather than the systematic review design was adopted because the aim was to synthesise methodological concepts and technical considerations among a diverse group of laboratory studies, not to address a specific, narrowly defined clinical or effectiveness question for which quantitative pooling was feasible. Narrative reviews have been identified as a unique and valid form of knowledge synthesis, and are particularly well-suited to areas where the objective is to provide a broad interpretive and critical appraisal of methods rather than a pooling of like outcome measures [4]. This approach provided the needed flexibility to synthesise technical, chemical and microbiological information that would not easily fit within one systematic review protocol.

The titles and abstracts obtained from each database were screened for relevance to the topic, and duplicates across databases were removed before the full text was evaluated. Then, the full texts were reviewed according to the inclusion criteria described above. When a number of studies presented more or less the same methodological results, priority was given to the most cited among them in the field, to those appearing in journals with higher impact in the microbiology or pharmacognosy area, and to those with the clearest technical description of the method discussed, or with the clearest case-studies illustrating a specific plant family. Non-English records identified at screening were excluded based on title and abstract.

The accuracy of any follow-up antimicrobial tests is greatly influenced by the quality of the plant material utilized. Botanical authentication by a competent taxonomist with subsequent deposition of a voucher specimen in a recognized herbarium is regarded as mandatory practice as any error in identification renders the reproducibility of any reported activity doubtful. Plant part (leaf, bark, root, seed, flower or whole plant), collection season, geographical location of the plant and handling after harvest affect the content of secondary metabolites and hence the subsequently determined antimicrobial activity. The reliance on this was shown by Kebede et al. [5] in their study of seven Ethiopian medicinal plants, where percentage extraction yield among species varied starkly despite being Cooriginated from the same locality and processed using the same method. The associated legal and taxonomic due diligence in relation to source material, including adherence to access and benefit-sharing commitments, has become a mandatory aspect of ethical bioprospecting, and should be reported in conjunction with the botanical-related methods section of any report, Cushnie et al. [2] report.

Extraction represents the critical interface between raw plant material and the pool of bioactive constituents available for testing, and the choice of solvent and technique materially shapes the phytochemical profile obtained. Conventional methods, including maceration, Soxhlet extraction and hydrodistillation, remain widely used owing to their simplicity and low equipment cost, but are frequently criticised for prolonged extraction times, high solvent consumption and thermal degradation of heat-sensitive constituents such as certain flavonoids and terpenoids [6]. Modern techniques, including ultrasound-assisted extraction, microwave-assisted extraction, pressurised liquid extraction and supercritical fluid extraction, have been developed to address these shortcomings, generally offering shorter processing times, reduced solvent volumes and improved preservation of thermolabile compounds, although at greater capital cost and with a need for careful optimisation of parameters such as power, temperature and pressure [7], [6].

Another advancement of particular importance in anti-microbial screening is that of natural deep eutectic solvents (a continuum of deep eutectic solvents), obtained by mixing hydrogen-bond donors and acceptors such as choline chloride, organic acids and sugars at well-defined molar ratios. García-Roldán et al. [8] revealed that natural deep eutectic solvent extraction of polyphenols from spent coffee ground rendered extracts with an antimicrobial activity approximately ten times higher than that of analogous ethanolic or aqueous extracts, processing at milder temperature and without using flammable organic solvents. This is a demonstration of a broader concept in green chemistry of extraction: the enhanced ability of these solvents to sequester polyphenolic substances can be directly translated into enhanced antimicrobial activity, rather than simply representing an environmentally better way to produce extracts of the same potency. Solvent polarity more broadly governs class of secondary metabolites to be extracted, and general and comparative research show that hydroalcoholic solutions e.g. 70-80% methanol or ethanol in water are better at extracting diverse phenolic and flavonoid compounds than absolute aqueous or absolute organic solvents, which results in better antimicrobial activity for some plants. [5].

Table 1 summarises the principal extraction methods discussed above, comparing their underlying principle, typical yield or selectivity profile, and key limitations.

Table 1. Comparison of principal extraction methods used in antimicrobial screening of medicinal plants

Method	Principle	Typical yield/selectivity	Key limitations	Source references
Maceration	Prolonged soaking in solvent at ambient temperature	Moderate yield; broad-spectrum recovery	Slow (days); risk of microbial contamination of extract	[6], [5]
Soxhlet extraction	Continuous solvent reflux through plant matrix	High yield for non-polar/moderate polar constituents	Prolonged heating; thermal degradation of labile compounds	[6]

Method	Principle	Typical yield/selectivity	Key limitations	Source references
Hydrodistillation	Steam-mediated volatilisation of essential oil constituents	High selectivity for volatile terpenoids	Limited to volatile fraction; loses non-volatile actives	[9], [10]
Ultrasound-assisted extraction	Acoustic cavitation disrupts cell walls, enhancing solvent penetration	Reduced extraction time; improved yield of phenolics	Requires optimisation of frequency/power; scale-up challenges	[7], [6]
Natural deep eutectic solvent extraction	Hydrogen-bonded eutectic mixtures (e.g., choline chloride/organic acid) replace organic solvents	High polyphenol selectivity; markedly enhanced antimicrobial yield reported	Higher viscosity complicates downstream handling; optimisation of molar ratio required	[8]
Supercritical fluid extraction (CO ₂)	Solvation using fluid above its critical point	High selectivity; solvent-free extract	High capital cost; limited to non-polar/moderately polar targets without co-solvent	[6]

RESULT AND DISCUSSION

Results

After extraction, crude qualitative phytochemical screening is commonly performed to reveal the general types of secondary metabolites present, based on colorimetric and precipitation reactions of alkaloids, flavonoids, tannins, saponins, terpenoids, cardiac glycosides and phenolic compounds. Kebede et al. [5] utilized conventional reagent-based assays such as Dragendorff's reagent for alkaloids, ferric chloride for phenolics and the Salkowski test for terpenoids to show that methanolic extracts of Ethiopian traditional medicinal plants harboured more varieties of the secondary metabolites than the respective aqueous extracts, a trend which reflected in the enhanced antimicrobial activity of the methanolic fractions. Similar qualitative analyses were performed by Obistioiu et al. [11] in four Fabaceae species, where the

occurrence of flavonoids and tannins linked with activity against *Staphylococcus aureus* and *Streptococcus pyogenes*.

However, qualitative colour and precipitation reactions are non-specific and tell nothing about how much of the constituent is present and therefore authors are now being urged to confirm these tests chromatographically or spectrometrically. The gas chromatography (GC) with mass spectrometry (MS) analysis is the main strategy for characterization of volatile compounds from essential oils and similar materials, and it is known that validated methods are so far available which meet pharmacopoeial requirements of specificity, linearity and precision on single terpenoid marker quantification into a multicomponent antimicrobial formulation [9]. High-performance liquid chromatography also is used for the determination of individual phenolic acids and flavonoids and thus the phytochemical fingerprint of an extract can be more directly connected to its quantified antimicrobial activity than is possible with qualitative screening methodology. Table 2 sets out the reagent-based screening tests most commonly used at this stage, alongside their target constituent class and positive indicator.

Table 2. Common qualitative phytochemical screening tests and their target constituents

Test	Target constituent class	Positive indication	Source reference
Dragendorff/Mayer test	Alkaloids	Orange-brown or cream precipitate	[5]
Ferric chloride test	Phenolic compounds/tannins	Blue-black or greenish colouration	[5], [11]
Frothing test	Saponins	Persistent foam after agitation	[5]
Salkowski test	Terpenoids	Grey/reddish-brown interface colouration	[5]
Keller-Kiliani test	Cardiac glycosides	Reddish-brown interface ring	[5]
Gas chromatography-mass spectrometry	Volatile terpenoids, essential oil constituents	Quantified peak matched to reference spectral library	[9], [10]

In vitro assessment of antibacterial activity is based on a limited number of basic methodological designs, all having their power and limitations and none of them having become considered as the “gold standard” in the way the protocols for synthetic antibiotics have been established for a long time. The discor well diffusion method, which is based on the radial diffusion of a fixed volume or mass of the extract through an agar

containing the test organism, continues to be the most utilized primary screening method because of the ease of performance, low running cost and high throughput of sample analysis. However, the major drawback of this method is that the size of the inhibition zone depends on the diffusion rate of the tested substance in agar, consequently, hydrophobic, or high-molecular mass compounds with poor diffusion ability may be considered as inactive ones [3]. Thus, diffusion methods should be viewed as the qualitative or semi-quantitative step in screening for activity and not as a “gold standard,” and they should be complemented by a dilution-based assay in which the lowest concentration sufficient to inhibit growth can be determined.

Broth microdilution in a 96-well microtitre plate has been the favored quantitative method to determine the minimum inhibitory concentration and the minimum bactericidal concentration, subsequent to the serial two-fold dilution of the extract in a standard broth medium across a specific concentration range [12]. The use of a tetrazolium-based viability indicator, such as 2,3,5-triphenyltetrazolium chloride (TTC), also facilitates the visual determination of the end point in extracts that are coloured or turbid themselves, as bacterial dehydrogenase activity reduces the colourless TTC salt to a coloured formazan product, but only in viable cells [3]. Agar dilution, where the extract is mixed with molten agar before solidification and seeding, producing similar results for Gram positive bacteria as the broth microdilution method; is more resource demanding and slower [3]. Bioautography, which combines thin layer chromatographic separation of the extract components with either an overlaid agar or a direct contact bioassay, permits the chromatographic band related to an observed activity to be pinpointed and isolated before, thus holds promise for guiding activity focused fractionation of complex extracts [13].

Among all bacteria-based assays, a common methodological flaw among the reports is the lack of standardization or information of bacterial inoculum size, incubation temperature and time, identity, and concentration of positive control antibiotic which affect the comparability of the MIC values to be reported from different laboratories [12], [2]. The standard protocols established originally for antibiotic susceptibility testing by organizations such as Clinical and Laboratory Standards Institute have been similarly adapted for plant extracts, but the lack of interpretive breakpoints specific to natural products requires authors to apply judgment in classifying a plant extract as active, moderately active or inactive and therefore comparability between studies is lacking [5]. The bacterial and fungal assay formats that were mentioned throughout this review are summarised in Table 3 for ease of direct comparison at the end of Section 7.

Fungal targets, especially *Candida* spp, give rise to unique challenges that are not always adequately addressed when established protocols for bacteria are simply applied. The clinical broth dilution protocols for yeasts, which have been adopted from bacteria by the Clinical and Laboratory Standards Institute as document M27, incorporate the use of the RPMI 1640 medium buffered with morpholinepropanesulfonic acid, defined inoculum densities, and standardised readout time points and have changed

over time since they were published as a provisional document [14]. A similar reference method has been established by the European Committee on Antimicrobial Susceptibility Testing, further Berkow et al. [14] comment on the fact that not all of the interpretive breakpoints published by the two bodies for the same antifungal agents are matching. For instance, the European committee has issued breakpoints for amphotericin B against several *Candida* species which the Clinical and Laboratory Standards Institute has not, whereas for caspofungin it is the other way round, because an excessive variability in the results between laboratories obtained for the modal minimal inhibitory concentration range has so far hindered the European committee from publishing a breakpoint. This discrepancy between two internationally standardised reference methods for traditional antifungal agents highlights the caution that is generally required, when either method is to be applied - unadjusted - to chemically new plant-based antifungal leads since there are no comparable interpretive breakpoints available yet.

Formation of biofilm confers an additional level of resistance that planktonic susceptibility testing fails to predict, as *Candida* biofilms are surrounded by an extracellular matrix and are generally several orders of magnitude less susceptible to both traditional antifungal agents and those derived from plants than the planktonic cells of the same strain. The crystal violet staining assay, measuring the whole biofilm biomass after fixation and dye absorption, is still the most popular method to evaluate the antibiofilm activity and often used with metabolic viability based assays using reduction of tetrazolium salts to discriminate between reductions in biomass and reductions in cell viability [15]. A scoping review on the plant derivatives investigated against *Candida albicans* biofilms retrieved that crystal violet staining and tetrazolium-based metabolic assays were the most two common methodologies, applied in combination most of the time, while viable colony counts, dry weight measurement and microscopy are used less often as supplementary techniques [15]. Variation in the duration of biofilm formation and treatment (which most often is either twenty-four or forty-eight hours) was also reported in the same review, thus adding yet another barrier to comparison across studies as regards reported antibiofilm potency. Table 3 synthesizes the bacterial techniques (from Section 6) with the fungal-and biofilm-specific methodologies described previously in this section to illustrate where similar underlying concepts (diffusion, dilution, or biomass measurement) have been diverged in methodology between organism types.

Table 3. Comparison of principal in vitro susceptibility and biofilm testing methods for bacteria and fungi

Method	Organism/ endpoint	Advantages	Key limitations	Source references
Disc/well diffusion	Bacteria; zone of inhibition	Simple, inexpensive	Poor for hydrophobic/ large	[3]

Method	Organism/ endpoint	Advantages	Key limitations	Source references
		, high throughput	molecules; qualitative only	
Broth microdilution	Bacteria; minimum inhibitory /bactericidal concentration	Quantitative; low reagent volume; amenable to plate readers	Requires clear/soluble extract or viability dye	[12], [3]
Agar dilution	Bacteria; minimum inhibitory concentration	Reference- grade accuracy for Gram- positive organisms	High labour and material cost	[3]
Bioautography (agar overlay/direct)	Bacteria/fungi; localisation of active fraction	Links activity to specific constituent; guides isolation	Semi- quantitative; sensitive to plate/solvent conditions	[13]
CLSI M27/EUCAS ST broth microdilution	Candida spp.; minimum inhibitory concentration	Internationally recognised reference standards	Breakpoints differ between the two standards; no plant- specific breakpoints	[14]
Crystal violet/tetrazolium biofilm assay	Fungal (and bacterial) biofilm biomass/viability	Quantifies matrix- embedded, drug- tolerant populations	Variable exposure- time protocols; biomass ≠ viability	[15]

In addition to testing extracts alone, studies are increasingly addressing whether constituents from plants have the ability to enhance the activity of conventional antibiotics, providing a means to re-establish activity against the gram-negative,

multidrug-resistant pathogens. The checkerboard titration assay, in which two antimicrobials are mixed in different concentrations that follows a two-fold serial dilution, still holds as the common methodology to express such synergistic interactions, and the fractional inhibitory concentration index (FICI) is obtained by calculating the ratio of the MIC of each antimicrobials tested in combination to the MIC of each drug tested alone. An index of less than 0.5 is usually considered to be indicative of synergy, between 0.5 and 4 as additive or no interaction, and more than 4 as antagonistic effect. Time-kill kinetic studies that monitor the decline in a viable colony count over a fixed incubation interval offer a complementary dynamic perspective on the speed instead of the extent of bacterial killing, and should be considered as adjuncts rather than standalone tools when investigating combination effects [16]. An acetone extract of *Quercus infectoria* galls was also found to act synergistically with vancomycin against methicillin-resistant *Staphylococcus aureus* when tested using the checkerboard method evidence of synergistic fractional inhibitory concentration index values and time-kill assays showed that bactericidal activity was enhanced compared with the activity of vancomycin alone [16]. Table 4 presents the standard interpretation of the FIC index to define such interactions.

Table 4. Interpretation of the fractional inhibitory concentration index in checkerboard synergy assays

Fractional inhibitory concentration index	Interpretation	Typical confirmatory method	Source reference
< 0.5	Synergy	Time-kill kinetic assay	[16]
0.5–4.0	Additive or indifferent interaction	Time-kill kinetic assay	[16]
> 4.0	Antagonism	Time-kill kinetic assay	[16]

Another powerful and increasingly popular experimental strategy focuses on bacterial virulence instead of viability, based on the premise that causing a pathogen to lose its virulence is less likely to result in resistance than killing it. Many Gram-negative bacteria, including notably *Pseudomonas aeruginosa*, regulate the expression of virulence determinants including biofilm formation and production of elastase, pyocyanin and rhamnolipids through multiple cell-density-dependent quorum sensing systems, and targeting these systems rather than bactericidal activity is increasingly used as an anti-virulence screening end-point [17]. From a methodological perspective, this needs a different approach than traditional susceptibility testing: reporter strains (e.g., *Chromobacterium violaceum*, which synthesizes a detectable violet pigment in a quorum-sensing-dependent manner) can be used for screening of quorum-quenching compounds in a rapid colorimetric assay, while confirmation of downregulation of

specific quorum-sensing genes can be performed with reverse-transcription polymerase chain reaction or reporter-gene fusions [18].

Ahmed et al. [18] showed that the expression of the *las* and *rhl* quorum-sensing systems in *Pseudomonas aeruginosa* can be significantly downregulated by two naturally derived phytochemicals, trans-cinnamaldehyde and salicylic acid at sub-inhibitory levels, with concomitant decrease in the secretion of extracellular protease, elastase and pyocyanin, without any bactericidal action, a hall mark that differentiates a true anti-virulence agent from a mere anti-biotic. Maisuria et al. [19] adapted this methodology for a whole-organism infection model, indicating that proanthocyanidins isolated from cranberries decreased the generation of quorum-sensing signalling molecules, blocked the *LasI* and *RhlI* autoinducer synthase genes and acted as an antagonist to the *LasR* and *RhlR* transcriptional regulators, as well as conferred protection to *Drosophila melanogaster* against lethal *Pseudomonas aeruginosa* infection, a test design that bridges in vitro quorum-quenching information to a quantifiable survival outcome. D'Aquila et al. [20] utilized a similar screening strategy to assess bacterial growth, biofilm formation and quorums sensing simultaneously in *Chromobacterium violaceum*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*; analyzing ten oils, *Origanum vulgare* and *Foeniculum vulgare* significantly inhibited bacterial growth in *Chromobacterium violaceum* and *Enterococcus faecalis* in particular, demonstrating that a single screening panel can effectively differentiate between activity detrimental to growth and activity that modifies virulence using a single experimental model.

Showing that a plant extract is active against microbial growth constitutes only the initial step to define its therapeutic potential; elucidating the mechanism of action makes the proposal stronger for further development and helps in predicting the occurrence of resistance. The mechanisms of action of plant secondary metabolites on microbial cells are extremely varied, ranging from bacterial membrane integrity damage, efflux pump inhibition-mediated bacterial cell detoxification, interference with nucleic acids or protein synthesis, to quorum-sensing system inhibition (see Section 9) [17]. While alkaloids have been more frequently related to the inhibition of the bacterial efflux pumps, polyphenolic compounds like flavonoids and tannins appear to be more connected with modifications in the membrane permeability and with bacterial enzymes' inhibition via hydrogen bonding interactions [21]. These processes can be investigated using methods such as potassium ion or nucleic acid leakage assays for membrane damage, scanning and transmission electron microscopy to observe morphological changes in the bacterial cell wall, and assays for intracellular adenosine triphosphate concentration for metabolic interference. Tsamo et al. [22] demonstrated this mechanistic concept in their study of secondary metabolites from *Trifolium baccarinii* by using bacterial lysis and salt tolerance assays together with antioxidant enzyme activities to describe the mode of antibacterial effect of the isolated compounds. Despite this growing body of mechanistic literature, Jubair et al. [21] note that a clear mechanism of action has yet to be identified for a majority of documented plant antimicrobials, highlighting the

fact that mechanistic studies are more resource intensive than simple susceptibility testing.

A plant extract of true therapeutic relevance must also be safely administrable, however cytotoxicity assessment is either completely missing or is quite superficially presented in the scientific literature. The brine shrimp lethality assay, with nauplii of *Artemia salina* treated with different concentrations of the extract for 24 hours, is a simple, cost-effective and instrument-free reporter of general toxicity, and LC₅₀ values greater than 1 mg/mL are generally interpreted as indicative of non-toxicity. The tetrazolium-based colorimetric assay to mammalian cell lines allows a more physiologically relevant assessment of cytotoxicity and is being utilized increasingly concurrently with the brine shrimp assay to enhance confidence in a preliminary safety evaluation. Ghuman et al. [23] utilized both assays concurrently on 11 South African medicinal plants traditionally utilised for wound healing and they describe survival rates ranging from 90-100% in the brine shrimp assay and LC₅₀ values > 1 mg/mL in the tetrazolium-based assay for all the extracts studied which lends credence to the ethnomedicinal application of these species and also illustrates the benefits of segregate cytotoxicity endpoints.

Cushnie et al. [2] argue that the selectivity index, expressed as the ratio of the cytotoxic concentration to the minimum inhibitory concentration, should be calculated routinely and used, alongside the absolute minimum inhibitory concentration value, as the primary criterion for deciding whether an extract or isolated compound merits further development. A high antimicrobial potency accompanied by a low selectivity index offers limited therapeutic promise, since equivalent toxicity toward host cells is likely to accompany any dose sufficient to inhibit the pathogen, and the same review catalogues a series of avoidable pitfalls in extract dissolution and vehicle-control design that can otherwise produce spuriously favourable selectivity estimates.

A comparison of methodologically analogous studies among different plant families highlights both the commonality of the core experimental pipeline outlined above and the degree to which family-specific chemistry influences the selection of extraction and assay technique. Lamiaceae, a family abundant in volatile monoterpenes, is generally assessed via hydrodistillation to yield essential oils, which are then tested using broth microdilution against bacterial and fungal strains. Ghavam et al. [10] exploited this pipeline for four Iranian species of the Lamiaceae, reporting two entirely different categories of results based on endpoint used: by inhibition-zone diameter, the essential oil of *Thymus daenensis* produced the largest inhibition zones against the Gram-positive bacterium *Staphylococcus aureus* and the fungus *Aspergillus brasiliensis*, while minimum inhibitory concentration, the oils of *Hymenocrater incanus* and *Stachys inflata* recorded the highest activity specifically towards the Gram-negative bacterium *Pseudomonas aeruginosa*. This distinction is a salutary reminder that "most active" can mean different things depending on which endpoint a study reports, and that inhibition-zone and minimum-inhibitory-concentration rankings do not necessarily correlate.

Potente et al. [24] gave a more general overview of the anti-Candida potency of some Lamiaceae EOs stating that "besides simple growth inhibition, some oils also inhibited the expression of Candida virulence factors, including the yeast-to-hyphal transition and biofilm formation", reflecting the anti-virulence concept depicted for bacteria in Section 9.

By contrast, four species of Fabaceae were assessed by Obistioiu et al. [11] through traditional hydroalcoholic and not volatile-oil extraction based on the typical phenolic and flavonoid chemistry of the family and showed activity against a panel of bacterial and fungal test organisms comprised of *Staphylococcus aureus*, *Escherichia coli* and *Candida* species, the phenolic compounds responsible for the activity being identified by chromatographic analysis. Tsamo et al. also worked in the Fabaceae, applying organic solvent maceration of *Trifolium baccarinii* to prepare extracts assessed by broth microdilution and mechanistic lysis assays [22]. Overall, these family-level comparisons underline that the selection of extraction method are not random but should be guided by the dominant secondary metabolite chemistry anticipated within a plant family, an aspect rarely stated explicitly within the methods of individual primary studies. These five illustrative case studies are summarised in Table 5.

Table 5. Illustrative case studies of the experimental pipeline applied within specific plant families

Plant family	Extraction approach used	Principal testing methods	Key finding	Source references
Lamiaceae	Hydrodistillation (essential oil)	Broth microdilution; disc/zone diffusion	Zone-based ranking (<i>T. daenensis</i> strongest vs. <i>S. aureus</i> / <i>A. brasiliensis</i>) diverged from MIC-based ranking (<i>H. incanus</i> / <i>S. inflata</i> strongest vs. <i>P. aeruginosa</i>)	[10]
Lamiaceae	Hydrodistillation (essential oil)	Growth inhibition plus virulence-factor assays	Oils reduce <i>Candida</i> yeast-to-hyphal transition and biofilm	[24]

Plant family	Extraction approach used	Principal testing methods	Key finding	Source references
			formation, not only growth	
Fabaceae	Conventional hydroalcoholic extraction	Disc diffusion/microdilution; chromatographic constituent identification	Phenolic/flavonoid content linked to activity against Gram-positive and Gram-negative bacteria and <i>Candida</i>	[11]
Fabaceae	Organic solvent maceration	Broth microdilution; lysis and salt-tolerance mechanistic assays	Antibacterial action linked to membrane disruption and antioxidant enzyme modulation	[22]
Cross-family comparison (Apiaceae, Lamiaceae, Lauraceae, Myrtaceae, Rutaceae)	Hydrodistillation (essential oils)	Growth, biofilm and quorum-sensing inhibition assays in parallel	Oregano and fennel oils were most growth-inhibitory in two of the three test organisms; growth inhibition and virulence-modifying activity were assessed as separable endpoints within one design	[20]

Discussion

A few persistent problems lowball the comparability and reproducibility of the data used to screen for antimicrobial activity in the medicinal plant literature. First, the lack of internationally approved interpretive breakpoints for plant extracts as opposed to the breakpoints for routinely used antibiotics and antifungals, leads to the application

of different and sometimes arbitrary cut-off values for determining whether an extract is active or inactive [5], [14]. Second, differences in expression of extract concentration, as mass of crude extract per volume, or as the concentration of a particular marker compound, make difficult the comparison between studies and the meta-analytical synthesis. Third, inconsistent reporting of solvent controls, positive control antibiotics and the exact bacterial strain or American Type Culture Collection reference number utilized, raises questions regarding whether activity observed can be attributed to a true phytochemical effect, as opposed to residual solvent toxicity or susceptibility of a particular strain. Fourth, as described in Section 4, the extraction procedure itself has a significant impact on the phytochemical profile exposed to the test system, which can lead to a significant difference in reported activity for the same plant species when different solvents or methods are used.

The recurring issues are most clearly discussed by Cushnie et al. [2], who view poor practice in dissolution of extracts, in the nature and concentration of vehicle controls, and in not calculating or reporting a selectivity index as easily avoidable flaws that repeatedly appear in the bioprospecting literature regardless of the plant family or even organism under study. Addressing these challenges will require stricter adherence to established reporting guidelines, more frequent use of quantitative dilution-based endpoints rather than relying solely on measurements of diffusion zone, routine use of appropriate positive controls, vehicle controls, calculation of a selectivity index and incorporation of cytotoxicity data within a single publication, and, importantly, better separation, at least in biofilm and quorum-sensing research, of reductions in biomass, reductions in viability, and true attenuation of a defined virulence phenotype..

Future progress in this area could be assisted by the adoption of consensus reporting guidelines for plant-derived antimicrobial screening (similar to those that have been developed for reporting clinical antibiotic and antifungal susceptibility testing), to promote cross-study comparability. Stronger convergence of mechanistic and genomic methodologies, such as transcriptomic analysis of treated organisms, as well as molecular docking approaches that link defined phytochemical structures with putative bacterial or fungal targets, will further substantiate the scientific rationale used in prioritizing candidate extracts for continued development. More widespread adoption of combination and synergy testing with traditional antibiotics creates a pragmatic short-term translational path, especially when whole extracts or standardised fractions (rather than single isolated compounds) are investigated as adjuvants, and anti-virulence and quorum-quenching endpoints should be more widely adopted alongside traditional growth-inhibition assays due to their unique resistance-evolution landscape. The progress in green extraction processes, based on natural deep eutectic solvent systems, will bring about better extraction efficiency, reproducibility, environmental friendliness and sometimes even potency of the extract preparations, while intensified usage of chromatographic fingerprinting could assist in demonstrating batch-to-batch similarity, which is essential for any future regulatory dossier. Finally, increased collaboration

between phytochemists, microbiologists, and pharmacologists, as well as incorporation of cytotoxicity, selectivity index, and biofilm evaluation earlier in the experimental workflow available would provide further safeguards against promising in vitro results being chased to the ends of the earth with no realistic consideration of their translational feasibility.

CONCLUSION

Fundamental Finding: The in vitro evaluation of plant extracts for antimicrobial efficacy is a continuum of connected phases, starting from the origin of the plant material and extraction, to phytochemical profiling and susceptibility assays of bacteria and fungi, and, finally, anti-virulence, mechanistic and toxicity evaluation. A range of well-defined methodological options are available at each stage, none of which is without limitation, and, the decisions taken at any point will have a significant impact upon the results of subsequent stages. Despite its usefulness as an initial screening at the separation stage, disc diffusion method is insufficiently accurate as a stand-alone quantitative result and should be validated with broth microdilution or agar dilution for reliable minimum inhibitory concentrations and minimum bactericidal concentrations, considering fungal and biofilm targets have to be evaluated with reference methods specific and endpoints different to those suggested for planktonic bacteria. Example case studies from the Lamiaceae and Fabaceae families shows that extraction related methodological decisions are best guided by the family-specific chemistry of secondary metabolites rather than by considering unrelated plant families and that the order of "most active" extracts can change depending on which testing endpoint is reported. **Implication:** Synergy testing using checkerboard titration in conjunction with time-kill dynamics evaluation, provides a practical means for discovery of antibiotic-sparing combination therapies; Quorum-sensing inhibition assays represent a complementary anti-virulence approach with potentially reduced ability to induce resistance; and Mechanistic and cytotoxicity analyses, albeit less frequently conducted, are crucial for substantiating therapeutic promise claims. **Limitation:** This review is subject to several limitations. As a narrative rather than systematic review, the selection and weighting of included studies reflects the authors' judgement rather than a predefined, fully reproducible protocol, and the possibility of selection bias towards more prominently cited or more accessible studies cannot be excluded. The restriction to English-language, peer-reviewed sources may have excluded relevant methodological insights published in other languages or in grey literature. The rapidly evolving nature of extraction and analytical technology means that some emerging techniques may be underrepresented relative to their eventual significance. The case studies presented in Section 12 are illustrative rather than exhaustive and were selected to demonstrate methodological principles within two well-studied plant families; readers seeking a comprehensive account of antimicrobial findings across the full breadth of medicinal plant families should consult family- or species-focused reviews directly. Finally, because the review addresses methodology

rather than reported efficacy against specific pathogens, it does not provide a comprehensive assessment of the antimicrobial potential of any individual plant species.

Future Research: Alignment of methodology at these stages, facilitated by more uniform reporting style, and routine reporting of selectivity indices, would considerably strengthen the scientific and translational value of future research in this important field.

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