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Challenges to antimicrobial susceptibility testing of plant-derived polyphenolic compounds

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As multidrug resistance gains momentum, the last two decades have seen an ever-growing interest in the antimicrobial properties of plant extracts and plant-derived compounds. Most of the focus is on polyphenols – a large and diverse group of phytochemicals with strong antibacterial activity. Testing methods provide reliable results as long as they follow standard procedures. However, methods and procedures used in antimicrobial susceptibility testing (AST) are often too diverse to allow comparison of results. The lack of uniformity and comparability is much owed to the absence of guidelines. The focus of this review is to give a critical overview of different methods used in the assessment of polyphenols antimicrobial efficacy and to highlight the importance of their standardisation.

KEY WORDS: antibiogram; AST; plant-derived compounds; polyphenols; standardisation

Over the past few decades, the search for antimicrobials derived from plants has accelerated, as bacterial resistance to antibiotics grows and new, clinically useful antibiotics fail to emerge. Research has focused on screening raw materials for new natural substances to replace synthetics. To protect themselves from microbial pathogens plants produce numerous secondary metabolites with antimicrobial properties, such as phenolic compounds, terpenoids, essential oils, alkaloids, lectins, and polypeptides (1). One of the largest and most widespread groups of plant chemicals with a diverse array of positive health effects are polyphenols. They exhibit significant antibacterial, antioxidant, anti-inflammatory, anticancer, and antihypertensive activity, largely attributed to interaction with cellular signalling pathways under normal and pathological conditions (2).

Polyphenols can be simple compounds with a single aromatic ring and low molecular weight or large and complex substances. Considering their chemical structure, they are generally divided in two groups: flavonoids and non-flavonoids. Flavonoids include flavones (luteolin), flavonols (quercetin), flavanones, flavanols (catechin, epicatechin), isoflavones, and anthocyanidins, while non-flavonoides include phenolic acids (benzoic and cinnamic acids), phenolic alcohols (tyrosol, hydroxytyrosol), stilbenes, and lignans (3). A number of phenolic compounds of plants used in traditional medicine (4) appear to have structures and modes of action that are different to those of antibiotics, which renders the risk of cross-resistance highly improbable makes them a potentially new class of antibiotic drug candidates or, more likely, resistance modifying agents in combination therapy with conventional antibiotics. Synergistic studies show promise against pathogens resistant to conventional antibiotics (5, 6). The last decade has seen growing evidence that plant-derived compounds can restore the clinical application of older antibiotics that are generally ineffective alone.

Even though their mechanisms of action are not fully clarified, polyphenols seem to damage bacterial cell membranes or interfere with the production of amino acids needed for bacterial growth (7). They also seem to modify bacterial resistance by inhibiting bacterial efflux pumps that expel antibiotics or by inhibiting enzymes that inactivate them (8–15).

Bacterial susceptibility to conventional antibiotics is determined by a number of phenotypic and genotypic antimicrobial susceptibility testing (AST) methods available in clinical laboratories. The selection of optimal method is based on factors such as flexibility, practicality, automation, cost, accuracy, reliability, and reproducibility. However, with plant-derived compounds additional problems arise, because natural products are complex mixes of many compounds that may not act as expected in the test system. For this reason, only a few AST methods have found application in determining antibacterial activity of natural products. However, AST methods provide reliable results only when they follow standardised procedures. Otherwise,

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differences in experimental conditions can lead to large variations (16, 17). As the use of AST methods faces numerous challenges with polyphenols, the aim of this mini-review is to take a critical look at them and highlight the importance of their standardisation.

FACTORS INFLUENCING AST METHODS

Solvents used for polyphenol extraction/solution and culture media

Plant extracts are natural products, and their chemical composition varies. Screening crude plant extracts for desired bioactivity is one of the most important operations in the study of medicinal plants, and extraction is the first crucial step. The quality of plant extracts depends on plant material (part of the plant used fresh or dried), which may vary with climate, time of harvest, extraction method, solvent used, and (in)stability of constituents (18). This variation is also true for polyphenolic extracts. Extraction methods usually vary in length, pH, temperature, particle size, and solvent-to-sample ratio, which also affects the quantity and quality of constituents obtained. This is why plant extraction methods should be standardised or harmonised to allow direct comparison of AST results.

Most phenolic compounds with antimicrobial activity are aromatic or saturated organic compounds and are usually obtained through initial ethanol extraction. Ethanol, however, can interfere with the bioassay. Its presence, even in very small amounts, can inhibit the growth of some microorganisms and affect test results. In concentrations >2.5 % it has toxic effects on some of food poisoning bacteria such as the *Listeria* and *Staphylococcus* genera (19, 20). Other solvents used are acetone, methanol, chloroform, water, and dimethyl sulphoxide (DMSO) (21–24). DMSO is frequently used because it dissolves both organic and inorganic compounds well. However, as an antioxidant it has also been reported to protect bacteria from death by antimicrobials in concentrations as low as 1 % (25) and that its use as an extraction solvent should be reconsidered. Recent research has focused on new, sustainable, and environmentally safe extraction solvents such as supercritical fluids. For example, pure supercritical carbon dioxide is safe and may significantly enhance the efficiency of ethanol extraction (26).

To account for the possible influence of solvents on AST results testing should include not only plant extracts but also the antibacterial effect of the growth medium containing solvent (negative control). In addition, testing should include a standard antibiotic as positive control against which to compare the activity of the extract or polyphenolic compound.

With respect to growth media used for AST, Müller-Hinton (MH) broth/agar is a standard for testing the susceptibility of non-fastidious microorganisms to antibiotics. The use of another culture medium may lead to discrepancy in results such as the one observed in two studies of phenolic resveratrol antimicrobial activity (27, 28). Although both used the same reference bacterial strain, the minimum inhibitory concentration (MIC) of resveratrol was 0.1 mg/mL when the MH broth was used (27) and over 1 mg/mL when the Luria-Bertani broth was used (28). Variations in results are possible even with the recommended MH culture media due to differences in their activity and composition. Even standardised components and procedures require quality control (QC) to ensure reliable results. QC testing involves reference bacterial strains to yield MIC and zone diameter values within recommended ranges. Ahman et al. (29) evaluated 21 internationally available brands of MH dehydrated media using a standardised disc diffusion method only to find that the inhibition zone diameters for QC reference strains were outside the European Committee on Antimicrobial Susceptibility Testing (EUCAST) QC criteria (30) for all but six tested brands. Authors also reported major differences in cation content and pH between the agars. MH agars of poor quality can affect susceptibility tests to the point that they produce false positive or negative findings. Therefore, users should evaluate the performance of the media they use and confirm that QC criteria published by national and international standard organisations are met.

AST guidelines and AST interpretative criteria (breakpoints)

Each AST method requires specific testing conditions, including media, incubation time, and temperature. These procedures have specifically been designed for assessing the activity of conventional antimicrobial agents (such as antibiotics). Various authorities, such as EUCAST and Clinical and Laboratory Standards Institute (CLSI), provide AST guidelines for antibiotics (31–34), which promote development and standardisation of *in vitro* AST methods and work towards international consensus and harmonisation of clinical breakpoints and testing performance. These guidelines are also used for determining antimicrobial activity of plant-derived compounds, as they have no standards of their own. However, problems arise because antibiotics are generally hydrophilic, and all standardised AST methods have been optimised to this condition, whereas most polyphenols are lipophilic and are not fully soluble in water. This requires adjusting conventional EUCAST and CLSI reference methods for testing phenolic extracts.

Another problem arising from the use of AST guidelines developed for antibiotics is the absence of interpretation criteria (breakpoints) for susceptibility testing of plant-derived substances. A breakpoint is the minimum drug concentration expected to be clinically effective against a microbe. It is used to describe bacterial isolates as susceptible, intermediate, or resistant. However,

interpretation criteria for polyphenol ASTs are yet to be established by standard-setting bodies. In the meantime, different criteria are used. Many studies rely on MIC. MICs of conventional antibiotics typically range between 0.01 and 10 μg/mL, whereas, plant compounds are routinely classified as antimicrobials if their MICs range between 100 and 1000 μg/mL. However, claiming positive activity for excessively high plant compound concentrations is a common mistake, and Rios et al. (35) propose that the MIC for antimicrobial activity of a plant extract and isolated compound to be considered significant should be below 100 μg/mL and below 10 μg/mL, respectively. Taguri et al. (36) propose the following cut-off MIC values of a pure compound/crude extract: <400 μg/mL for strong antimicrobial effect, 400–800 μg/mL for moderate, and >800 μg/mL for weak effect.

Bouarab-Chibane et al. (37), in turn, have taken a different approach, as they focus on polyphenol antimicrobial activity against food-spoiling bacteria. They propose polyphenol concentration of 1000 μg/mL for a breakpoint, as higher concentrations would have no practical use in the preservation of perishable foods. This parameter of antimicrobial activity is called bacterial load difference (BLD), and its significance (breakpoint) is set at 20 % decrease in bacterial growth, which is achieved with 1000 μg/mL of polyphenols.

Besides the EUCAST and CLSI standards widely used by clinical microbiology laboratories, European Standard EN 1276:2009 (38) is used to evaluate plant extracts as food sanitisers and European Standard EN 12054:1997 (39) to evaluate plant extracts as hand antiseptics.

Testing organisms and inocula

Regardless of the chosen AST method, it is necessary to use well-defined, standardised microbial strains obtained from a recognised source such as American Type Culture Collection (ATCC) or National Collection of Type Cultures (NCTC). The use of reference strains with known susceptibility against existing antimicrobial agents enables internal quality control and makes it possible to calibrate the test and compare results between laboratories. The choice of test microorganisms depends on the specific purpose of research. Drug-sensitive Gram-positive and Gram-negative reference strains of common pathogenic species are preferable for AST screening.

Bacterial inoculum size is the most important variable in antibacterial activity testing. Greater inoculum size and longer incubation time can result in higher antimicrobial MIC, which only underlines the importance of controlled and standardised conditions for susceptibility testing. The EUCAST standard recommends inoculum of $5x10^5$ colony forming units (CFU)/mL for broth microdilution method (31). This size can be assessed by counting viable colonies. To do that, 10 μL of inoculum should be removed from a growth-control well or tube immediately after inoculation, diluted in 10 mL of saline, and 100 μL spread onto a non-selective agar plate. After incubation, the number of colonies should be approximately 20–80. For results to be valid it is important that the bacteria used in AST testing come in the log phase of growth from a fresh culture.

In addition to growth media, bacterial strains, and inoculum size, there are many other factors specific for each type of assay (volume of agar/broth, size of wells/paper discs, incubation conditions, endpoints determination), which will be discussed with particular AST method.

COMMON *IN VITRO* AST METHODS

Phenotypic AST methods are either qualitative (disc diffusion technique) or quantitative (dilution methods, E-test). Phenotypic assays rely on measuring growth inhibition, metabolism, and viability in the presence of antibacterial compounds. Some methods provide quick results, while others allow better understanding of antibacterial impact on the viability and cell damage of tested microorganism. Conventional phenotypic AST methods include agar disc diffusion, well diffusion, and agar or broth dilution.

DIFFUSION METHODS

Agar disc diffusion assay

In the disc diffusion test (Kirby-Bauer method) the suspension of a microorganism in saline to the density of a McFarland 0.5 turbidity standard, approximately corresponding to 1×10^8 CFU/mL, is inoculated uniformly onto the surface of an agar plate. The density of the suspension is preferably measured with a photometric device that has been calibrated with a McFarland standard according to manufacturer's instructions. Alternatively, the density can be compared visually to a McFarland 0.5 and adjusted by addition of saline or more bacteria.

A paper disc containing a standard amount of an antimicrobial compound is applied to the surface of the plate, usually MH agar, and the substance is allowed to diffuse into the adjacent medium. Following incubation, bacterial growth appears on the plate. If the test isolate is susceptible to antimicrobial compound, a clear area of "no growth" will appear around the disc. This zone is referred to as the zone of inhibition. Its size will depend on the diffusion rate of the test solution and the degree of sensitivity of the microorganism. Strains resistant to an antimicrobial compound will reach the margin of disc. Inhibition zone diameter is therefore used to interpret inhibition, and standards for interpretation have been established for different antibiotics. The size of the inhibition zone in the disc diffusion test inversely correlates with MIC (40).

Pre-prepared, quality-controlled antibiotic discs provide reproducible and reliable findings. Unfortunately, this is not the case with extract-soaked filter paper discs, as they are not commercially available. Instead, filter paper discs or sterile commercial blank discs (about 6 mm in diameter) are impregnated with 0.01–0.02 mL of diluted test solution and allowed to dry before placing on top of agar plates (41). Drying time varies from 2 h to overnight under laminar flow.

The disc diffusion method for screening of natural products for antimicrobial activity owes its popularity to simple use and low cost. However, it involves a number of critical steps, such as the choice of the medium, pH, agar depth and moisture content, incubation conditions, and ensuring accurate inoculum density. The diameter of the inhibition zone may be affected by the solubility of the tested substance, diffusion range, and evaporation. Precipitation of water-insoluble substances in the disc will prevent any diffusion of antimicrobial substances into the agar. With mixtures of constituents with different diffusion rates, this may give unreliable results. In fact, no inhibition zone does not necessarily mean that the tested compound is ineffective, especially if we speak about less polar compounds, which diffuse more slowly in aqueous agar. It is therefore not surprising for phenolic compound AST to get inconsistent results. When a disc diffusion method is used, a correlation between a number of compound concentrations used and inhibition zone diameters needs to be established to consider it reliable (17, 42). The main disadvantage of the test, however, is that it is qualitative and does not distinguish between bactericidal and bacteriostatic effects. If used for antimicrobial screening, disc diffusion is better for low molecular weight compounds, whereas for high molecular weight compounds well diffusion is preferred (43).

Agar well diffusion assay

This method is frequently used for testing antimicrobial activity of plant-derived substances (44, 45). A bacterial isolate mixed with soft agar is poured onto a plate and cooled. Then wells are cut with a sterile borer (4–8 mm in diameter), and test substances placed into each well. After incubation, the plate is observed for clear zones of inhibition around the well. Another way is to spread inoculum suspension evenly on a solid agar plate and add antimicrobial substances into each well. Agar well diffusion seems to be more sensitive and convenient than the disc variant for testing cationic natural products, as they adsorb on the hydrophilic surface of the disc and not diffuse into the medium (43, 46).

Disc and well diffusion tests are qualitative, which means that they do not quantify the amount of the tested compound diffused in the agar medium. In view of known diffusion issues of non-polar compounds, both methods are a poor choice for AST of crude plant extracts, which often contain compounds with different polarities. Both have poor reproducibility and both often fail to yield a linear correlation between the inhibition zone diameter and antimicrobial concentration (17, 47). In such cases, antimicrobial activity should be determined with a quantitative dilution method.

Bioautography

Bioautography is a method of microbial detection relying on planar chromatography techniques. It can be used to test antimicrobial activity of plant extracts and pure compounds. Bioautography assays can be divided into three groups: (a) direct bioautography, (b) contact or agar diffusion bioautography, and (c) immersion or agar overlay bioautography. With direct bioautography microorganisms are grown directly on thin-layer chromatography (TLC) plates, which combine two uses: separation of analysed substances and antibacterial activity. Direct bioautography usually does not make use of agar gel. The plate is dipped in or sprayed with a seeded culture broth. Inhibition (bioautogram) is usually visualised thanks to tetrazolium salts converted to intensely coloured formazan by live aerobic and facultative anaerobic bacteria. For microaerophilic bacteria like *Campylobacter* spp. adenosine triphosphate (ATP) is a better choice, as it allows determination by bioluminescence (48).

Contact bioautography is a variant of agar diffusion often used to screen for antimicrobial activity of plant extracts (49–51). Unlike with other diffusion methods, the analyte diffuses to inoculated agar medium from the chromatographic layer. A sample to be analysed is first adsorbed on a TLC plate and then transferred from the TLC plate to inoculated agar surface (52).

Immersion (agar overlay) bioautography, combines direct and contact bioautography, as – unlike with contact bioautography – the seeded agar medium is applied onto the TLC plate (51).

Opinions about bioautography vary. Silva et al. (53) claim that it is practical, reproducible, and easy to perform. Rios et al. (54), in contrast, find bioautographic methods difficult to standardise, as there are many varying factors that affect results. Even so, bioautography has a number of advantages: it uses small amounts of a sample, it is suitable for evaluating complex lipophilic plant extracts, and it facilitates rapid, inexpensive, and easy evaluation (55).

Furthermore, bioautography provides information about antimicrobial activities of individual substances in a mixture. This is particularly true for TLC bioluminescence – a variant of direct bioautography used for rapid screening of complex mixtures (56). Complex mixtures are first separated by TLC. The TLC plate is then coated with bioluminescent bacteria to reveal antimicrobial activity as dark zones on a luminescent background. The advantage of this kind of separation combined with post-chromatography bioassays is that it provides rapid and

sensitive screening for individual antimicrobial compounds in various mixtures and can also be used for toxicity screening (57, 58).

DILUTION METHODS

In contrast to diffusion methods, dilution methods allow quantitation of MIC or minimal bactericidal concentration (MBC). Their main disadvantage is that they require relatively large amounts of reagents and space.

Agar dilution method

A tested substance is mixed in various concentrations with the MH agar before solidification. An inoculum of 0.5 McFarland standard density is then diluted in broth to give 10^7 CFU/mL (EUCAST/CLSI guidelines) (31, 32). 1-µL bacterial suspensions will give a final inoculum of 10^4 CFU/spot. Inoculated agar plates are then incubated and growth estimated by the naked eye. This technique, however, is tedious, labour-intensive, and takes large amounts of tested compounds. To address this issue Golus et al. (59) have proposed agar microdilution for testing oily and coloured plant extracts. This method combines convenience and time/cost effectiveness typical for microtitre methods with the advantages of the agar dilution of hydrophobic or coloured substances.

Broth dilution method

In the broth macrodilution tube assay serial dilutions of tested compounds prepared in a liquid medium (MH broth or water) are mixed with bacterial suspensions in a series of tubes. Broth microdilution assay (also known as microtitre/microwell plate method) uses microwell plates instead. Both assays show bacterial growth inhibition with various compounds by measuring turbidity after overnight incubation. Turbidity indicates how efficient specific concentrations of a tested compound are in inhibiting bacterial growth. The less turbid a sample is in each tube (well), the better the bacterial growth inhibition (efficiency). A growth medium without antimicrobial agents serves as control. At the same time as assays are performed, inoculum control has to be determined in a medium to ensure that an even and sufficient number of organisms is used in the test. Bacterial inoculum varies in this respect and can significantly affect the assessment of MIC. Inocula with cell counts lower than $5x10^5$ CFU/mL can show false susceptibility.

MBC is determined by subculturing the dilution that has shown no growth in the MIC assay. The selection of methods depends on the nature of compounds such as solubility and molecular weight. Although the broth microdilution method is the best way to establish the real potency of a pure compound, this will greatly depend on the solubility of the tested compound. Water-insoluble compounds disperse poorly in a liquid growth medium and separate to oil and water phases. Furthermore, if plant extract solution is turbid and coloured, it will be difficult to determine the endpoints of bacterial growth. If turbidity is measured visually, bacterial growth may be underestimated. In addition, precipitation of insoluble substances of the plant extract to the bottom of the well can sometimes make difficult visual or electronic turbidity measurement. This problem has been solved with redox indicators such as tetrazolium salts (60) or resazurin (61), which change colour with bacterial growth. The wells showing bacterial growth become pink, whereas the wells showing no growth remain colourless or blue, depending on the indicator. This procedure is referred to as colorimetric MIC method. In other words, broth microdilution methods have endpoints that can be determined either as a measure of turbidity or of cell viability indicators. Adding the indicator also helps to semi-automate broth microdilution through image processing. Using standardised bacterial inoculum Sarker et al. (62) have proposed a modified resazurin method, which corrects dilution inaccuracies, to achieve more accuracy in MIC determination for natural products. Recently, Veiga et al. (63) proposed another improved and validated colorimetric microdilution assay with triphenyltetrazolium chloride (TTC) as an indicator precise enough to correlate absorbance with the concentration of viable microorganisms and determine not only MIC but also 50 % and 90 % inhibitory concentrations (IC_{50} and IC_{90}, respectively. TTC is a salt that changes colour from clear to pink as it is metabolised by viable bacteria. Even with these indicators, however, strongly coloured/opaque pose media make reading colour changes difficult. In such cases, samples from each well are plated on a solid medium and colonies counted.

Another recent improvement to the standard microdilution method was an attempt to annul the risk of a medium inhibiting antimicrobial activity and improve extract solubility at the same time (64, 65). In the first step, bacteria in the mid-logarithmic growth phase are washed twice by centrifugation, re-suspended in Tris buffer, and incubated with a polyphenol without a growth medium. After five hours of incubation, follows the second step, in which Tryptic soy broth is added and microplates incubated for another six to twelve hours, that is, until controls form clearly visible pellets. At this point, viable cells are counted to identify growth. Microplates are then left to incubate for a total of 48 h to confirm by visual inspection that bacterial growth has completely been inhibited. This two-step microdilution assay offers more precise evaluation of antimicrobial efficacy of the tested compound. In addition, this assay has an option to use DMSO to enhance the solubility of more hydrophobic compounds, which facilitates identification of cationic and less polar bioactive compounds, whereas with other compounds it yields similar activity readings as the standard assay (65).

Although both agar (47, 66–68) and broth dilution (69–72) methods are commonly used to assess antimicrobial

activity of plant extracts and polyphenols, broth dilution produces the most consistent results (17).

The broth dilution method can also be used to assess the lethal effect of an antimicrobial agent and provide a dynamic picture of antimicrobial action and interaction over time (73).

ANTIMICROBIAL SYNERGY TESTING

Time-kill assay

Historically, the time-kill assay (TKA) has been used to evaluate bactericidal properties of a new drug or other product that is expected to have one by monitoring bacterial death caused by antimicrobials at a wide range of concentrations (0.5–4 × MIC) over time. Briefly, 10^6 CFU/mL of microorganisms are incubated with antimicrobials at 37 °C for predetermined sampling time (e.g. 0, 2, 4, 8, 12, and 24 h). Aliquots (1 mL, or appropriate volume) are then removed from each test suspension, serially diluted ten times (tenfold dilution) in sterile saline, and plated on agar plates for colony count. The rate of bactericidal activity is presented as the time-kill curve or survival curve. Generally, a 3-$\log_{10}$ drop in bacterial survival is considered the threshold for significant killing activity against a particular test microorganism.

TKA can also be used to evaluate synergistic effects of plant compound combinations (69, 74). Combination testing provides information about which antimicrobials can be combined to achieve optimal/maximal effect. In that respect, TKA and checkerboard assay are most commonly used to assess if there is synergy between phytochemicals and antibiotics (75, 76). Synergy is often defined as a ≥2 $\log_{10}$ decrease in the bacterial count (CFU/mL) compared to the most active compound tested alone (77).

Critical factors that affect the outcome of TKA include the preparation of an actively growing inoculum and the use of quantitative subcultures from drug-containing tubes. When high drug concentrations (≥4 x MIC) are tested, it is necessary to exclude the antibiotic carryover effect, which occurs when a tested compound transferred onto the agar plate with the subcultured aliquot is sufficient to inhibit the growth of viable microorganisms and results in a falsely low MBC. This phenomenon can be eliminated by widely streaking the transferred aliquot over at least one-half of an agar plate or by centrifugation and resuspension of the bacteria in antimicrobial-free media before plating.

Checkerboard assay

In checkerboard assay antibiotic is serially diluted along the abscissa and the plant compounds along the ordinate of microtitre plates. After inoculation and incubation, the plates are observed for bacterial growth and determination of MIC for a combination. Synergy is measured against the activity of individual components in a combination using the fractional inhibitory concentration (FIC) index, according to the following formula:

$$FICI = \frac{\text{MICa in combination}}{\text{MICa alone}} + \frac{\text{MICb in combination}}{\text{MICb alone}}$$

where MICa denotes the minimum inhibitory concentration of a plant extract and MICb of an antibiotic, if the synergy of the two is being evaluated.

The resulting index quantitatively denotes synergism if ≤0.5, additive effect if >0.5 to ≤1, or indifference if >1 (78). However, these indices are to be taken with reserve, as they involve one tube dilution variation and the possibility of reproducibility error. Due to inherent variability in MIC determination consequent inconsistent classification of the checkerboard assay results, Rand et al. (79) have proposed that a minimum of five replicates should be tested and ≥80 % agreement achieved between them for the effect to be categorised.

In any case, synergy testing results obtained with checkerboard assay may differ from those obtained with TKA, as the checkerboard method yields results at one time point, whereas killing curves measure changes over time.

MICROFLUIDIC AST METHODS

Screening for biological activity is often limited by the availability of active compounds. This can be overcome by techniques requiring them in low quantities. Developed in the last decade, microfluidic-based culture methods allow monitoring bacterial growth in channels/chambers that take no more than 20 μL of tested compounds (80). Li et al. (81) have recently demonstrated a promising adaptable microfluidic system that allows rapid phenotypic AST at the single-cell level. This miniaturisation made possible with new microfluidic technologies – described in detail in the review article of Schumacher et al. (82) – allows them to be integrated with an automatic device and therefore improve test speed and accuracy and achieve high throughput. Besides phenotypic, genotypic microfluidic ASTs have been developed for molecular detection of drug resistance to conventional antibiotics.

Recently, Parsley et al. (83) found significant differences in the bioactivity profiles of natural product peptides against *E. coli* between a microfluidic assay and a plate-based method. They suggested that the differences were owed to different bacterial growth conditions and different plastic materials involved in each assay and pointed out that microfluidic-based methods required significant optimisation to be reliably applied for AST.

Even so, microfluidics bring several advantages to AST over classical methods that use Petri dishes and multi-well plates, as they makes it possible to develop high throughput, real-time, low sample consumption assays. The downside is that such assays require expensive and specialised

equipment and samples need several preparation steps before analysis (84).

OTHER CONSIDERATIONS AND FUTURE NEEDS

Each of the presented AST methods has advantages and disadvantages (Table 1) and none can be regarded as the universal "best" method. Instead, each is best suited to different needs.

New bioassays and protocols should be selective and precise enough to detect bioactive effects of small amounts of natural compounds against target pathogens. They should also take less time to identify potential antimicrobial candidates.

Many new AST methods are automated and well suited for routine testing of conventional antibiotics with conveniently prepared and formatted microdilution drug panels, instrumentation, and automated reading. Novel instruments turbidimetry/nephelometry instruments measuring bacterial growth reduce technical errors and lengthy preparation times. Automation also allows inter-laboratory comparison and standardisation. However, these AST systems are not adapted to testing natural bioactive compounds and several attempts have recently been made to develop an automated system suitable for AST of plant extracts (85, 86) or adapt the existing ones. Recently, for example, Bouarab-Chibane et al. (37) successfully applied a self-contained incubation and high-throughput, microplate-based microbial growth tracking system relying on optical density (Bioscreen C™ Automated Microbiology Growth Curve Analysis System, Growth Curves USA, Piscataway, NJ, USA) to quickly screen 35 polyphenols for activity against six pathogens.

In contrast, genetic methods, which are increasingly used for AST of antibiotics, have not yet been adapted for assessing microbial resistance to plant-derived substances, because molecular assays can only detect what is already known. As genetic profiling for resistance to natural compounds has not yet been made for all the bacteria, these essays are not commercially available for the time being. For us to develop tests for detecting resistance genes we first need to learn more about the mechanisms of activity of polyphenols and about the molecular basis of microbial resistance to them.

Another issue that deserves consideration is the applicability of *in vitro* testing in developing plant derived

Table 1 Overview of commonly used i*n vitro* antimicrobial susceptibility testing methods

Method	Advantages	Disadvantages	Reference
Agar disk diffusion	simplicity of performance, low cost, flexibility, no special equipment required, suitable for lead identification	qualitative assay, poor level of reproducibility, diffusion of antimicrobial substances may be affected, applicable only to fast-growing bacteria	(17, 42, 43, 46, 47)
Agar well diffusion	simplicity of performance, low cost, more sensitive and more convenient than the disc variant for testing of cationic compounds	qualitative assay, poor level of reproducibility	(17, 43, 46,47)
Bioautography	simplicity of performance, little amount of sample required, rapid and inexpensive evaluation, suitable for screening of antimicrobials in mixtures	qualitative assay, difficult to standardise, not suitable for synergy studies, alteration of compounds during the fractional phase	(49–54)
Agar dilution	quantitative results, a number of bacterial species may be applied to a single dish	laborious and time consuming method, the large amount of reagents and space required	(47, 59, 66–68)
Broth microdilution	quantitative results, convenience and time/cost effectiveness, capacity to test opaque materials, possible automation, the most consistent results, the killing effect can be assessed	the possibility of errors in solution preparation, relatively high amount of space and reagents required	(17, 47, 60–64, 69–71)
Microfluidic methods	smaller volumes, short run time, higher sensitivity, potential for high throughput	specialised equipment needed, high-cost	(80–83)

substances as antimicrobial drugs. Namely, even though a plant compound may prove potent *in vitro*, it may have little biological activity *in vivo* if it does not reach the target tissues. Regardless of the testing method, *in vitro* measurement of antimicrobial activity does not address limited bioavailability for many of the larger polyphenols. Their activity may be affected by many host factors, such as biotransformation, pH, and local tissue properties (e.g. abscess cavity, cerebrospinal fluid, or intracellular location). Although most polyphenols are absorbed to some extent, each phenolic compound is absorbed differently. Polyphenols get cleaved by stomach acid and undergo extensive enzymatic and chemical modification during digestion and absorption. In fact, they reach the target in very low concentrations, and this is the main obstacle to be addressed by further research. Smaller phenolic molecules are metabolised so quickly that only their metabolites can be found in the blood. Moreover these metabolites are generally rapidly eliminated from the plasma (87, 88). Knowledge about phenolic bioavailability has increased over the last years, and we now know that oral bioavailability of polyphenols is generally between 2 and 10 % (89–94).

One promising solution to low oral bioavailability is the development of nanodelivery systems able to maintain the structural integrity of the bioactive molecules (95).

Furthermore, research should shift the focus to studying antimicrobial properties of phenolics *in vivo* (92–101). More animal and human experiments should be conducted in this field to establish general principles affecting their absorption *in vivo*.

Although naturally occurring polyphenols do not demonstrate toxic effects, any substance entered into the human body in excessive amounts can cause harm. The studies of polyphenols toxicity essential for the drug development process are also still lacking (102–105). Therefore, in any eventual uses of different polyphenols as antimicrobials, the safety aspects have to be taken into account as well.

To sum up, further research should focus on determining structure-function relationships, mechanisms of action, pharmacokinetic profiles, and interactions with antibiotics or other medicinal plants or compounds to better understand phenolic antimicrobial effects *in vivo* and optimise them through structural changes whether used alone or, more likely, as potentiators of other antibacterial agents.

Conflicts of interest

None to declare.

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Izazovi određivanja antimikrobnog učinka polifenola

Istraživanje antimikrobnih učinaka biljnih ekstrakata i spojeva u proteklih nekoliko desetljeća zaokuplja interes brojnih znanstvenika zbog sve veće otpornosti bakterija na antibiotike. Najčešći i najviše proučavani sekundarni biljni metaboliti su polifenoli – brojna i raznolika skupina fitokemikalija s dokazanim antibakterijskim djelovanjem. Međutim, istraživači koji proučavaju antimikrobni učinak prirodnih spojeva biljnoga podrijetla susreću se s brojnim preprekama, koje je potrebno prepoznati i izbjeći. Na rezultate ispitivanja antimikrobne osjetljivosti mogu utjecati brojni čimbenici, a izbor odgovarajuće metode ispitivanja od presudnog je značaja. Nepostojanje postupnika/smjernica za testiranje antimikrobnog učinka bioaktivnih prirodnih spojeva uvelike utječe na ujednačenost, analizu i usporedbu brojnih objavljenih rezultata. U načelu, samo primjena standardiziranih metoda osigurava dobivanje pouzdanih rezultata. Cilj je ovoga preglednog rada sustavno i kritički prikazati različite metode koje se koriste za ispitivanje antimikrobnog učinka polifenola te istaknuti potrebu za njihovom standardizacijom.

KLJUČNE RIJEČI: antibiogram; ispitivanje antimikrobne osjetljivosti; prirodni spojevi biljnoga podrijetla; standardizacija