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ANTIMICROBIAL SUSCEPTIBILITY BIOASSAYS: A COMPREHENSIVE REVIEW ON ADVANCED METHODOLOGIES AND TECHNIQUES

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Microbial, specifically fungal and bacterial infections are the prime cause of diseases in humans, animals, and plants. Technological advancements with molecular protocols have undoubtedly broadened the research areas against global microbial infections. High antifungal and antibacterial drugs usage with resistance development, host-specific toxicity, mutational alterations, and several other factors bring forth the urge for new antimicrobial agents. New protocols related to antimicrobial activities and developing novel drugs against severe infections are highly recommended in future research areas. Dilution, diffusion, bioautography protocols, and several others are frequently used to determine the antimicrobial activities. The aim of this review is to highlight and assemble the information regarding the various anti-fungal activity techniques. Additionally, their principle, methodology, advantages, and disadvantages, with information regarding solvents and microbial organisms tested, has also been reviewed.

Keywords: Antimicrobial, Diffusion, Dilution, Diseases, Infections.

Introduction

Antimicrobial infections and related diseases to living beings has emerged as a global threat and concern of the present century[1,2,3]. The infections caused by microorganisms including bacteria, fungi, nematodes, parasites, and viruses, has undergone evolutionary stages which has led to serious adaptations, mutations, and resistance against present antimicrobial drugs. Since ages, development of epidemic and pandemic diseases has led to overuse of antibiotics and antifungal medications. For agricultural practices as well, plants have been overexposed to harmful pesticides, fungicides, and several other hazardous chemicals[3]. For animals, clinical treatments, food manipulations, and chemical drugs are some of the factors responsible for antimicrobial drug resistance. Therefore, with worldwide challenges and threats, development of novel antimicrobial compounds focusing on natural products related to plants, animals and beneficial microorganisms could be a great alternative to infections and resistance[4,5]. For successful evaluations of natural as well as chemical antimicrobial drugs, appropriate optimized methodologies and techniques are highly essential. To understand the methodologies, qualitative as well as quantitative examinations are recommended and need to be updated with time. The mechanism of action and molecular background plays another important role in disease control and resistance mechanisms. Series of actions from susceptibility tests to molecular investigations will help in developing novel antimicrobial products against the growing threats on the living entities[5].

This review article focuses on the comprehensive study of antimicrobial activities including bacteria and fungi. The aim is to combine the protocols for the researchers and young scientists towards new approaches and techniques. The evaluation and assessment of novel antimicrobial agents as alternatives to

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chemical antimicrobial drugs is of prime importance in the new era. These new strategies related to therapeutics and nutraceuticals could not only play a beneficial role against microbial infections but would additionally provide high resistance, less toxicity, and genetic mutations compared to the synthetic antimicrobial drugs[6,7]. In the past few years, research areas have focused on developing antimicrobial agents from multiple sources such as plants and their metabolites for resistance and neutral to negative toxic effects. This led the scientific attention to larger scale screening of plants, specifically medicinal plants for antimicrobial activities. Bioassays related to both antifungal and antibacterial have been focused including diffusion and dilution assays which require minimum requirements, equipment, and laboratory technologies in comparison to other protocols such as flow cytofluorometric and bioluminescent methods[8].

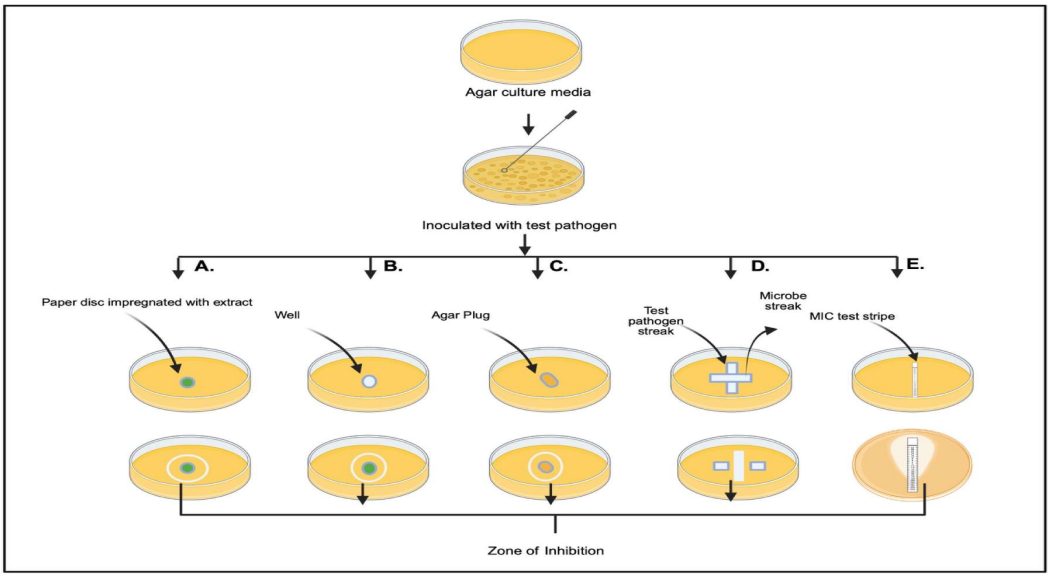

Figure 1. Diffusion assays for antimicrobial susceptibility test.
A.) *Disc diffusion.* ***B.)*** *Well diffusion.* ***C.)*** *Plug diffusion.* ***D.)*** *Cross streak assay.* ***E.)*** *Gradient diffusion or Etest assay.*

Antimicrobial Bioassay

1. Diffusion Methods

Diffusion methods for antimicrobial tests follow the procedure of diffusion of paper strips or discs of specific measurements diffused with antimicrobial drugs inoculated into culture medium. The second stage follows the inoculation of targeted pathogens of interest on the culture media petri plate. The inoculation of the pathogen can be performed by several techniques including spread plate or pour plate method. An overall "lawn-like" spread of the tested pathogen is performed for maximum growth and interpretation[9]. Furthermore, the diffusion methods can be broadly studied under several categories including gradient diffusion, well diffusion, disk diffusion, and plug diffusion, contact method, and cross streak diffusion.

i. Disk Diffusion Assay

Kirby-Bauer antibiotic method or commonly known as agar disc diffusion assay can be described as an ideal antimicrobial susceptibility test which predominantly requires minimum equipment with low-cost laboratory requirements[10,9]. The bioassay involves the diffusion of antimicrobial drugs impregnated on sterilized filter paper discs. The pathogen inoculation procedure is the

same as described in the diffusion assay. These paper discs when placed on agar culture medium forms a concentration gradient, the highest closer to the core site, consequently decreasing to the farthest. These impregnated discs determine the potential of the antimicrobial substance in accordance with the microorganisms or pathogen of interest[11]. A single disc can be placed at the center of the agar petri plate for evaluation of the zone of inhibition (Fig. 1). While several discs in a single petri plate may require a mechanical disc-dispenser device for simultaneous and uniform arrangement. The results are evaluated based on the zone of inhibition around the impregnated paper discs. The more is the zone of inhibition in measurement, the higher is the potential of the antimicrobial drug against the tested microorganism. Therefore, the zone of inhibition is defined as the circular inhibitory growth zone formed around the impregnant paper disc with respect to the organism[9].

For evaluating the disc diffusion results, MIC (Minimum Inhibitory Concentration) is analyzed. The lowest concentration of any antimicrobial agent expressed in µg/mL (mg/L) is defined as MIC in antimicrobial bioassay[12,13]. This testing is conducted under in vitro conditions to determine the susceptibility of the tested organism. The susceptibility results are based on three main categories mainly susceptibility, resistant, and intermediate susceptibility referred to as 'S', 'R', an 'I' respectively. The intermediate susceptible show response comparatively lower than the susceptible strains[9]. The determination of MIC and susceptibility test based on various antimicrobial bioassays are predicted through standard regulatory committees such as CLSI (Clinical and. Laboratory Standards Institute) which release cutoff MIC standards known as breakpoints which are regularly revised and published by the authentic agencies[9,14]. The organism-drug susceptibility tests and the MIC pattern determine the breakpoints.

The disc diffusion assay proved to show positive results for antimicrobial susceptibility tests. The assay showed a positive response to understanding the phenotypic traits of the microorganisms, synergistic effects of antimicrobial drugs, and detecting the susceptibility as well as the resistance mechanism of organisms. However, not all antifungal agents were available in the form of discs. These discs showed limitations of expenses, availability, and techniques as well. In contrast, the advantages include short-time assay with clear in vitro prediction of interpretations of microorganism's susceptibility tests.

ii. Well Diffusion Assay

The limitations of the disc diffusion assay aroused a need for a new methodology which does not depend upon the paper discs for bioassays. Magaldi in 1997, presented a modified diffusion assay and nomenclature it as agar well diffusion assay[15]. The initial procedure of culture media plate with inoculation of the target pathogen will be similar as described initially in the diffusion assay. A fixed diameter hole is developed in the culture media plate with a sterilized cork borer. This hole is known as the well for testing (Fig. 1). The well is filled with a measured quantity of antifungal compound to be tested against the pathogen. The zone of inhibition will be measured with a similar protocol described in the disc diffusion assay[16]. The well diffusion assay has advantages with simple protocol, minimum laboratory requirements, and quick results. However, it is known as a qualitative traditional technique which does not allow interpreting the MIC results of susceptibility tests[17].

iii. Plug Diffusion Assay

The diffusion assay highlights the diffusion technique by antagonism between two organisms or molecular compounds of organisms and antimicrobial products[18,19,20]. The agar culture media petri plate is streaked with the antimicrobial compound or drug of interest and is thus further known to diffuse in the media petri

plate. After the incubation period, a plug of the antimicrobial substance along with the culture media is extracted aseptically with the help of a sterilized cork borer (desired diameter) and placed on another petri plate with agar culture media (Fig. 1). This fresh media plate contains already inoculated test microorganisms or pathogens of interest. The plug diffusion assay works on the principle of taking a diffused plug of antimicrobial agent and analyzing its potential against the pathogen susceptibility. The zone of inhibition described in the previous protocols is recorded and thus evaluated[20].

iv. Cross Streak Assay

The cross-streak assay is a potential antimicrobial susceptibility and antagonism test[21,20]. The method is another improved variation of diffusion bioassay where the antimicrobial agent is tested against the tested microbe or pathogen of interest. This method involves streaking the test antimicrobial agent and the pathogen against each other and further evaluating the results based on the zone of inhibition. It is defined as a technique where two different microorganisms are streaked perpendicular to each other on a culture media plate test[22,23,5]. The assay involves the first streak of test antimicrobial agent on the agar culture medium petri plate. This streak will be preferably placed in the center of the petri plate for clear evaluations. After the incubation period, a second single or double streak of test pathogen will be formulated perpendicular on the first streak of the antimicrobial compound streak (Fig. 1). This pattern of cross streaking will allow diffusion of the microbial compounds in the agar medium, thus inhibiting the growth of the test pathogen. The zone of inhibition will develop from the intersection point of the streaks[5].

The cross-streak assay is a cost-effective, simple, minimal laboratory requirements protocol for antimicrobial activity. The resources and laboratory equipment are comparatively easier with lesser technical expertise. The major advantage of the assay lies with clear visualization of the zone of inhibition at the intersection site, thus allowing clear interpretation of the antagonistic activity[21,5]. The cross-streak assay is limited to the microorganism's diffusion potential tested for antimicrobial activity. Additionally, the concentration used also plays a major role in the assay. Like other diffusion assays discussed earlier, this assay is also interpreted on the qualitative data rather than quantitative, and thus limits the potential of the technique[24,5].

v. Gradient Diffusion Assay (Etest)

The Etest or the Gradient diffusion assay include combined properties of dilution and diffusion method[20,25,5]. The commercially available version of the gradient diffusion assay is known as the Etest® or BioMérieux. The protocol is highly specialized to investigate the antimicrobial properties of bacteria and fungi using the MIC technique. A petri plate of agar culture media is inoculated with organisms of interest. A strip of antimicrobial drugs with increasing concentration (to determine the MIC) is inoculated on the agar petri plate from one end to another[20,26,25] (Fig. 1). The intersection of the strip is considered as the evaluation site for the zone of inhibition and MIC analysis.

The gradient diffusion assay plays an important role in determining the antagonistic effects of microorganisms. However, previous studies have shown its role in determining the synergistic effects as well. The MIC values can be easily determined and used for comparative study as well[27,25]. To study the synergistic effects and MIC determination of two drug compounds, the first Etest strip and the second Etest strip (drug A and B) impregnated with the antimicrobial substance will be placed on the agar petri plate. The agar petri plate will be pre-inoculated with the pathogen to be tested. The first strip when placed will be removed after an hour, followed by the second strip.

The zone of inhibition is recorded followed by determination of MIC in combination and single antimicrobial substance[28,25].

The formula to calculate Fractional Inhibitory Concentration Index (FICI) is:

$$\sum \textbf{FICI=FIC(A) + FIC(B)}$$

$$\boldsymbol{FIC\ (A)} = \frac{MIC\ (A)\ in\ combination}{MIC\ (A)\ alone}$$

$$\boldsymbol{FIC\ (B)} = \frac{MIC\ (B)\ in\ combination}{MIC\ (B)\ alone}$$

For Synergistic interpretation: FICI ≤ 0.5. For antagonistic interpretations: FICI > 44.
The FICI between 0.5 and 1 was considered as addition and between 1 and 4 as indifference substance[15,20,25].

2. Dilution Methods

Dilution methods are widely used to isolate fungi from various samples such as soil, water, and air. The main goal is to reduce the concentration of fungal spores or cells in the sample, allowing individual colonies to grow separately on culture media, which can then be isolated and identified[29,30]. The dilution method is used to determine antifungal agents' MIC (Minimum Inhibitory Concentration) and is standardized for antimicrobial susceptibility testing. The MIC is the lowest concentration of an antimicrobial that inhibits visible growth of a microorganism under defined conditions[31]. However, they are essential in evaluating the effectiveness of antifungal compounds against isolated fungal strains. Agar and broth dilution methods are similar techniques used to determine the MIC of antimicrobial agents against bacteria or fungi.[32,33].

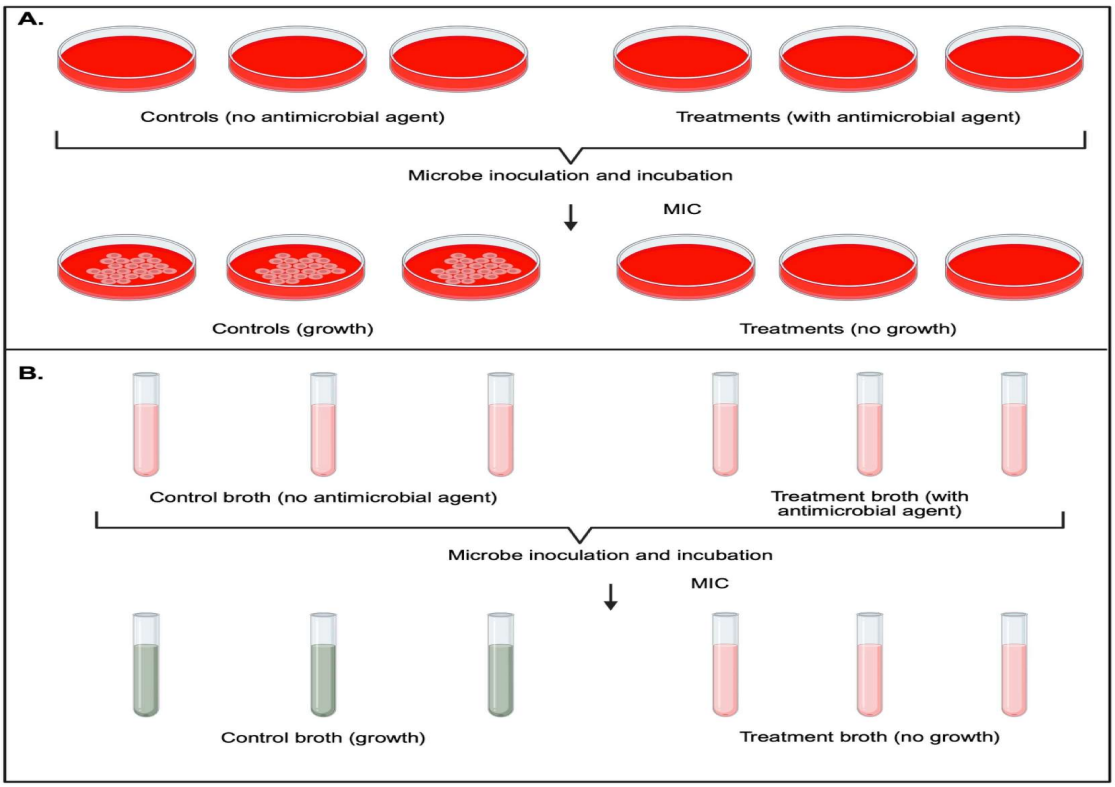

Figure 2. Dilution assays for antimicrobial susceptibility test.
A.) *Agar dilution assay.* ***B.)*** *Broth macrodilution assay. The test microbe is inoculated on agar plates and broth tubes with different concentrations of antimicrobial agent. MIC is determined in both the assays at the lowest concentration of growth visibility.*

i. Broth Dilution Assays

Broth dilution methods are essential for determining the antifungal susceptibility of fungi, including both yeasts and filamentous fungi. The primary goal is to identify the lowest concentration of an antimicrobial agent that inhibits visible fungal growth. These methods are standardized by the CLSI (Clinical and Laboratory Standards Institute) and are crucial for guiding clinical therapy, monitoring resistance, and aiding in drug development. The CLSI provide guidelines (e.g., M27-A3 for yeasts, M38-A for filamentous fungi) for performing broth dilution antifungal susceptibility testing[34,35]. These methods, including macrodilution and microdilution, are standardized procedures that provide reliable results for susceptibility testing.

Broth Macrodilution: Involves preparing a series of test tubes with liquid growth medium (broth) containing serially diluted concentrations of an antifungal agent. A standardized inoculum of the fungal isolate is added to each tube. After incubation, the lowest concentration that inhibits visible growth (Minimum Inhibitory Concentration, MIC) is recorded[36,37] (Fig. 2).

Broth Microdilution: Similar to microdilution but performed in small microplates (usually 96-well plates), allowing high-throughput screening and parallel testing of multiple compounds or fungal isolates testing[35]. MIC is determined visually or spectrophotometrically after incubation[38].

ii. Agar Dilution Assay

Agar dilution methods are also widely used in microbiology for both the isolation of microorganisms (fungi and bacteria) and for determining the MIC of antimicrobial agents[31]. Agar dilution can be adapted for the isolation of fungi and bacteria from mixed samples by preparing agar plates with serial dilutions of the sample and creating a series of concentrations. For fungi, specific media such as SDA (Sabouraud Dextrose Agar) with antibiotics may be used to selectively isolate fungal species. For bacteria, nutrient-rich agar or selective media can be used, depending on the target organism[39]. A standardized fungal/bacterial inoculum is applied to the agar surface. Plates are incubated under suitable conditions (typically 16–24 hours at 37°C for bacteria, or as appropriate for fungi). After incubation, plates are examined for growth. The MIC is recorded as the lowest concentration of antimicrobial agent that completely inhibits visible growth of the microorganism (Fig. 2). Agar dilution method allows simultaneous testing of multiple organisms, suitable for colored extracts or compounds that interfere with broth methods and is recommended for fastidious organisms[20].

3. Time-kill kinetics method

Time-kill kinetics are in vitro methods used to evaluate the rate and extent of microbial killing by an antimicrobial agent over time. They help determine whether an agent is bactericidal (kills bacteria) or bacteriostatic (inhibits growth) and provide insight into the concentration- and time-dependent effects of antimicrobials on specific microorganisms[40,41].

To evaluate time-kill kinetics, prepare a series of test tubes or wells containing a growth medium, and add the antimicrobial agent at different concentrations in each tube or well. After that, each tube or well is inoculated with the test microorganism. For a specified time duration, the tubes or wells are incubated. Samples are removed from each tube or well at predetermined time points (e.g., 0, 1, 2, 4, 6, 8, and 24 hours). Then, plated onto appropriate growth media to determine viable colony-forming units (CFU/mL) [41,5] (Fig. 3).

The effect of the antimicrobial agent on the growth and viability of the microorganism over the predetermined period (time interval) was examined. The killing kinetics were visualized by plotting

log CFL/mL against time[42,5]. The change (reduction or increase) of the microbial population with the initial inoculum was assessed by calculating the log decrease and percentage reduction from an initial microbial population over time. The change was calculated as follows[43]:

$$\% \textbf{\textit{Reduction}} = \frac{Initial\ Count - Count\ at\ X\ interval}{Initial\ Count} \times 100$$

Time-kill assays can be adapted for different microorganisms (bacteria, fungi) and a wide range of antimicrobial agents, including antibiotics, antiseptics, and novel compounds[44]. The assay can be used to assess the combined effects of multiple antimicrobial agents, helping to identify synergistic or antagonistic interactions that may influence treatment strategies[45].

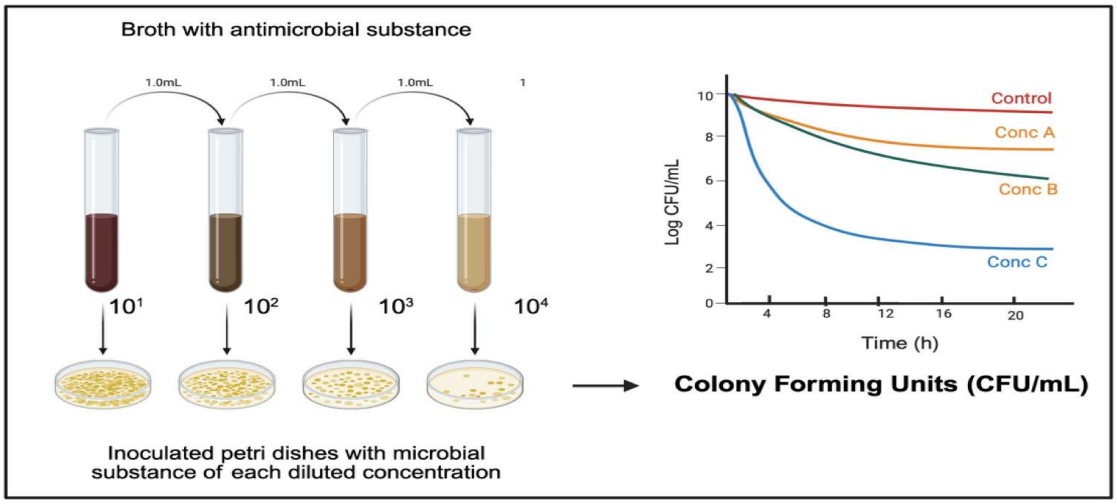

Figure 3. Time-kill kinetics assay.

Antimicrobial substances of different concentrations are cultured in agar media petri dishes with pre-inoculated test pathogen. The log CFU/mL is determined of all groups at variable time from 0-24 hrs.

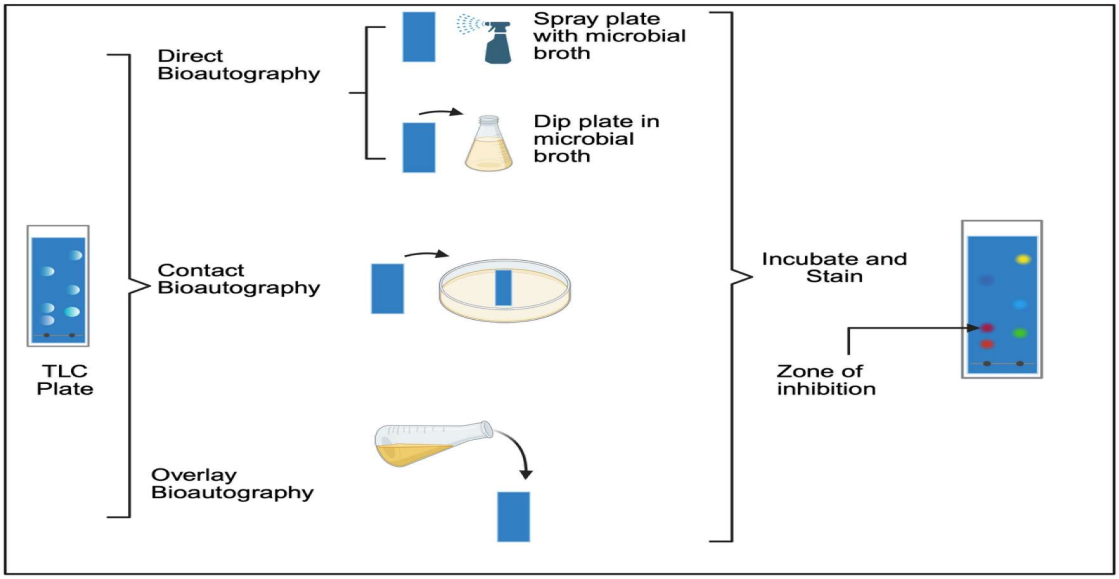

Figure 4. Bioautography Assay.

Comparative analysis of three bioautography techniques: Direct, Contact, and Overlay Bioautography.

4. Poisoning Food Techniques

The poisoning food technique is a laboratory assay primarily used to evaluate the antimicrobial activity of chemicals or plant extracts against pathogenic microorganisms. It is particularly valued for its simplicity, cost-effectiveness, and suitability for screening large samples against filamentous fungi[20,5]. In this method, the test substance is incorporated into a growth medium (such as Potato Dextrose Agar, PDA), and the growth of the target fungus is measured and compared to controls[46].

The antifungal agent or extract is added to the heated agar at a specified concentration and thoroughly mixed. The medium is then poured into Petri dishes. Agar discs (3–6 mm) embedded with the test fungus are excised from the margins of actively proliferating colonies and meticulously positioned at the middle of the petri dishes[20]. The plates are subsequently incubated at a suitable temperature for a designated period. After the incubation period, the microbial growth on both plates is assessed. If the treated plate shows a notably reduced microbial growth compared to the control, it suggests that the tested substance inhibits the growth of microorganisms[47,5]. The antifungal effect (%) is represented as a percentage, reflecting the level of fungal growth inhibition resulting from the test substance, calculated using the following equation[48]:

$$\textbf{\textit{Antifungal activity}}\ (\%) = \frac{Dc - Ds}{Dc} \times \mathbf{100}$$

Dc = diameter of growth in the control plate
Ds = diameter of growth in the plate containing the tested antifungal agent.

Detection bioassay of antimicrobial activity

1. Bioautography Assay

Bioautography can be defined as a living or biological sample detection using chromatographic evaluation, specifically planar chromatographic technique[49,50]. The phytochemical analysis of plant extracts with their chemical screening as antimicrobial agents can be easily evaluated through the bioautographic technique. Paper chromatography-bioautography (PCB) was introduced by Goodall and Lev, 1947 for the study of penicillin[51]. However, later in 1961, TPCB (Thin Paper Chromatography-Bioautography) was introduced by Fisher and Lautner, and to estimate the purity of penicillin.[52,53]. Therefore, planar chromatography-bioautography can be broadly categorized into PCB (Planar Chromatography-Bioautography) and TPCB. Moreover, with advanced research tools and scientific requirements, HPTLC (High Performance Thin Layer Chromatography) was introduced[50]. Later other techniques such as OPLC (Overpressure Layer Chromatography), and PLC (Planar Electro-Chromatography) were extensively used for antimicrobial screening.

The bioautography assay is based on the principle of screening the bioactive compounds of botanical extracts for antimicrobial properties[52,53,5]. The TPCB can be categorized into three main variants including direct bioautography, contact or agar diffusion bioautography, and overlay bioautography[55,5] (Fig. 4). The initial stages of all the three categories follow a common protocol. The samples are purified and filtered followed by application to the TPCB plate where the components in the sample are separated based on chemical properties. The TPCB plate is prepared and directed to the three sub-techniques for further analysis accordingly.

i. **Direct Bioautography:** TPCB plate inoculated directly into the microbial broth or suspension, incubated, and analyzed for zone of inhibition[5] (Fig. 4).
ii. **Contact or Agar Diffusion Bioautography:** The diffusion of microbial pre-inoculated culture petri plate with the TPCB plate. The plate is removed after 30 minutes, and the agar

petri plate is incubated for antimicrobial susceptibility analysis. The bioactive compounds diffuse from the TPCB plate into the agar plate; thus, growth inhibition mechanism is activated, and evaluations are conducted[5] (Fig. 4).

iii. **Overlay Bioautography:** Also known as immersion bioautography involves the overlaying or immersion mechanism of culture medium petri plate with the TPCB plate (Fig. 4). The incubation allows growth of inhibitory bioactive compounds of the antimicrobial substances with the zone of inhibition. The advantage to the technique lies with its simple, effective, and cost-effective analysis of botanical extracts with minimum laboratory equipment and resources[55,50]. The technique provides direct analysis of bioactive compounds for identification of known and unknown compounds of plant extracts for antimicrobial activity[56,5].

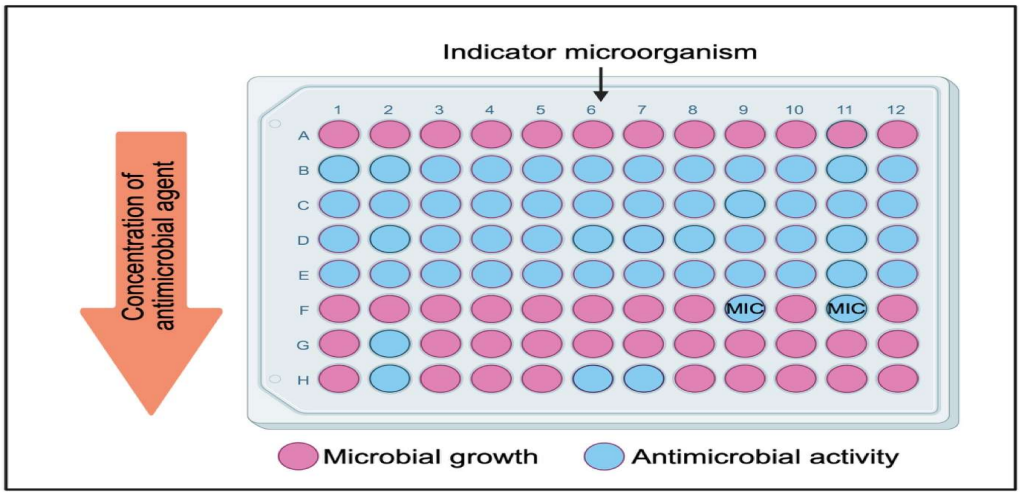

Figure 5. Alamar Blue assay.

The antimicrobial agent added to the wells followed by the target microbe and incubated. Resazurin is added to the wells and the color change is observed from blue to pink. The MIC is determined for interpretation.

2. Automated and Rapid Bioassay Techniques

Automated and rapid bioassay techniques are essential in modern laboratories for high-throughput, precise, reducing manual labor, and enhancing reproducibility. These methods are validated for various microbial states, including planktonic cells, biofilms, and agar cultures. Automation reduces manual errors and variability, and the use of robotic liquid handlers enables rapid processing of large sample numbers[57].

i. Colorimetric-Fluorescent Assays

Colorimetric-fluorescent assays are widely used to assess antimicrobial activity due to their sensitivity, speed, and suitability for high-throughput screening. Colorimetric assays rely on visible color changes, often using enzyme reactions (e.g., peroxidase, alkaline phosphatase) or nanoparticle aggregation to indicate microbial growth or inhibition[58]. These assays are simple, scalable to 96- or 384-well plate formats, and suitable for automation. They are widely used for routine analyses, especially when high throughput is needed. Commonly used in ELISA (Enzyme-Linked Immunosorbent Assay) and direct detection platforms, enabling rapid, cost-effective, and often instrument-free readouts[59].

Fluorescent Assays use fluorophores that emit light upon excitation or genetically encoded reporters (such as fluorescent proteins in bacteria) to monitor

cell viability or growth inhibition. Offer high sensitivity and allow real-time or endpoint measurements, often correlating fluorescence intensity with antimicrobial effect. They offer higher sensitivity and multiplexing capability compared to colorimetric assays[60]. Combining both colorimetric and fluorescent signals can be used to detect bacterial contamination and antimicrobial resistance in clinical and research settings. These assays are essential tools for rapid, reliable, and scalable evaluation of antimicrobial agents[5].

ii. Alamar Blue Assay

Alamar Blue assay (also known as the resazurin assay) is a simple, rapid, sensitive, and cost-effective method for measuring cell viability and metabolic activity in mammalian cells, bacteria, fungi, and other organisms[61]. This assay uses a redox indicator (resazurin) that changes color and fluoresces in response to cellular metabolic activity, providing a quantitative measure of cell viability. Viable microorganisms reduce Alamar Blue, resulting in a color/fluorescence change, while antimicrobial agents that inhibit growth prevent this reduction[62]. The assay is frequently employed in microbiology to assess bacterial proliferation and antibiotic sensitivity, often in conjunction with the microdilution method. Alamar Blue's versatility, sensitivity, and non-toxicity have made it a standard tool across biological, pharmaceutical, and environmental research[63].

The Alamar blue assay protocol involves diluting to desired concentrations in suitable growth medium, filter sterilizing, and preparing stock solutions of test compounds or extracts (e.g., 20 mg/mL). Inoculate microplate wells (96-well microtiter plate) with microbial suspension (bacteria or fungi) in growth medium, adding test compounds at desired concentrations. Include positive (antimicrobial agent) and negative (no treatment) controls. Add Alamar Blue reagent (typically 10% of well volume, e.g., 10–20 µL per 100–200 µL well). Incubate for 1–4 hours at 37°C (time depends on the organism and metabolic rate)[62,64]. After this incubation period, the wells are examined for any alterations in color. A transition from blue to pink or purple signifies the existence of live cells, indicating metabolic activity and cellular viability (Fig. 5). On the other hand, limited alteration or no color change shows that the microorganisms have been inhibited or killed. To quantify the results, measure absorbance (570 nm and 600 nm) or fluorescence (excitation 560 nm, emission 590 nm). Calculate percent reduction relative to controls; a decrease in color change or fluorescence indicates antimicrobial activity[65].

iii. Flow Cytometry

Flow cytometry is a powerful analytical technique for analyzing the physical and chemical properties of cells or suspended particles as they flow in a fluid stream past a laser.[66]. This technique has emerged as a pivotal technique for assessing antimicrobial activity, offering rapid and accurate results compared to traditional methods. The effects of antimicrobial agents on microbial cells, such as modifications in cell viability, membrane integrity, and metabolic activity, can be evaluated using flow cytometry[67].

To perform the assay, prepare a bacterial suspension and treat it with the antimicrobial agent at relevant concentrations. Incubate for a specified time, then stain the cells with viability dyes (e.g., SYTO9 for live cells, propidium iodide for membrane-compromised/dead cells[68,69]. The stained cell suspension is subsequently passed through a flow cytometer instrument. Analyzed 10,000 cells per sample, collecting data on fluorescence and light scatter. The emitted fluorescence signals are captured by detectors and analyzed to determine the microbe cell viability, integrity of the membrane, metabolic activity, and other characteristics of the cell. The obtained data can be further examined to assess the antimicrobial efficacy of the evaluated medicines[69].

Researchers can evaluate the effects of antimicrobial drugs on microbial populations by comparing treated and untreated samples. Flow cytometry-based assays can be adapted for both research and clinical use, enabling early detection of antimicrobial resistance and supporting targeted therapy decisions. It uses fluorescent dyes to distinguish live, damaged, or dead bacteria after antimicrobial exposure[70].

iv. Microplate Assay

Microplate assay was primarily developed to analyze the in vitro screening of botanical extracts and provide the quantitative evaluations based on the inhibitory potential of the antimicrobial substance. The principle of the assay is based on the detection of antimicrobial substances through a 96-well microtiter plate[71,72,73](Fig. 5). The microplate correlates the antimicrobial compounds of the plant extracts with the pathogen growth. The interpretations are based on the determination of MIC ranging from MIC0–100[73]. The assay holds major potential for determination of antagonistic as well as synergistic effects of antimicrobial agents.

Conclusions

Microbial infections in the past few decades have become a global concern and threat. Significant etiological factors such as resistance, mutations, and other abiotic-biotic factors are known to cause significant mortality and morbidity to live life. Therefore, research on novel methodologies related to antimicrobial agents, antimicrobial susceptibility, and other drug investigations. These research areas need to be updated, developed, and improvised due to extensive resistance and new strains worldwide. The techniques related to antimicrobial susceptibility are standardized timely through agencies like the CLSI which gather, develop, and update information regarding new techniques, novel drugs, and other information regarding antimicrobial activities. With the higher resistance rate, natural plant-based antimicrobial agents need to be studied and hence could expand to new chemical free, cost-efficient antimicrobial substances in the future. New methodologies regarding the botanical assays need to be evaluated through different microbial techniques. Therefore, the new age agriculture sector needs standardized extensive research for the future generations of living life including humans, plants, and animals.

This review combines multiple antimicrobial bioassays and provide a platform for protocols related to susceptibility assay with principles, procedures with illustrations, advantages and disadvantages. For future investigations, this information will allow precise and optimized data for microbial infections and diseases and ultimately global healthy life to living beings.

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