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ASSESSMENT AND EVALUATION OF METHODS USED FOR ANTIMICROBIAL ACTIVITY ASSAY: AN OVERVIEW

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ABSTRACT

The emergence of new infectious diseases, recurrence of several infections and microbial resistance encouraged researchers towards confirmatory development of effective antimicrobials with wide margin of safety using various screening protocols. The Clinical and Laboratory Standards Institute (CLSI) methods for the assessment of antimicrobial activity are widely accepted by "regulatory authorities" worldwide. However, these methods are intended to test pure compounds such as antibiotics whereas optimization is required at extract level. The deficiency of optimized *in vitro* protocols for the assessment of antimicrobial activities has led to variations in results among researchers. This direct comparison of results led to false conclusions regarding the efficacy of antimicrobial agents. Several methods have been widely employed in the evaluation of antimicrobial activities of various target compounds. Many researchers have either incorporated two different methods or one single method. However, the conclusion cannot be drawn exclusively from the results obtained while employing only the preliminary antimicrobial test methods such as disc diffusion. Hence, the objective of this review article is to report various screening protocols for the determination of antimicrobial activity of both known and unknown antimicrobials.

KEYWORDS: Antimicrobials, antibiotics, drug resistance, clinical and laboratory standards institute (CLSI) guidelines, screening protocols

INTRODUCTION

Long before mankind with the discovery of microorganisms, the idea that certain compounds had curative potential, characterized as antimicrobial principles, was well accepted. Since

ancient times, man has used antimicrobials to treat infectious diseases and some of these antimicrobial drugs are included as routine medicines in the treatment of various ailments (Rios and Recio 2005).

Antimicrobials are the substances that kill or inhibit the growth of microorganisms such as bacteria, fungi or protozoans (Khan and Hanee 2011). The history of antimicrobials begins with the observations of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. The discovery of antimicrobials like penicillin and tetracycline paved the way for better health for millions around the world; hence, antimicrobial drugs are used in the treatment of various diseases.

This area of research faces many intrinsic problems because of methodologies used to perform the assays in order to confirm the efficacy of test antimicrobials. Especially, the intrinsic natures of plant secondary metabolites, the most extensively used sources for developing antimicrobials, affect the outcome of the antimicrobial assays employed by the soluble property of plant secondary metabolites and their extraction methodologies. The clinical and laboratory standards institute (CLSI) methods for the assessment of antimicrobial activity are widely accepted by "regulatory authorities" worldwide (Das *et al.,* 2010). However, these methods are intended to test pure compounds such as antibiotics and optimization is required for testing crude extract materials (Hammer *et al.,* 1999). The deficiency of optimized *in vitro* protocols for the assessment of antimicrobial activities of antimicrobials has led to variations in results among researchers. This direct comparison of results led to false conclusions regarding the efficacy of antimicrobial agents (Sarker *et al.,* 2007). Methods that have been widely employed in the evaluation of antimicrobial activities include disc diffusion, well diffusion and broth dilution. Antimicrobial activities reported in the literature have been evaluated with different sets of methodologies, level of sensitivity, amount of test compounds and microorganisms (Valgas *et al.,* 2007). Many research findings either incorporated two different methods or just one single method and the most commonly used have been the disc diffusion (Kiska 1998). However, the conclusions cannot be drawn exclusively from results obtained through the disc diffusion method since it is well established that this method is not suitable for the evaluation of non-polar samples (Rios *et al.,* 1998; Rios and Recio 2005). Hence, standardization of antimicrobial assays is quite difficult and there is no single assay that is compatible with all antimicrobial compounds. There are some advantages and disadvantages associated with each of the methods, therefore

researchers will have to decide and use of methods for best suited results. This emphasizes the fact that if identical assays are not used, it will not be feasible to compare results.

The manifestation of new infectious diseases, increase in pathogen resistance and renaissance of several infections encouraged researchers at both national and international levels towards the development of new protocols for the evaluation of antimicrobial activity. Therefore the current review covers various screening protocols for the determination of antimicrobial activity of various samples from different origins.

Screening protocols

Diffusion

This method is preferably used for the screening of hydrophilic substances because they can easily diffuse through the solid agar medium. The chemical nature and volatility of drug affect rate and quantity of drug diffusion that may leads to inconsistent results (Hewitt and Vincent 1989; Southwell *et al.,* 1993). This is particularly true in the screening of antifungal drugs where long incubation periods are required (Janssen *et al.,* 1987). This method produces least consistent and irreproducible results but the main advantage is that many samples can be tested using one plate.

Pour plate disc diffusion (PPDD)

Disc diffusion refers to the diffusion of an antimicrobial substance of a defined concentration from discs, tablets or strips, into the agar gel medium that has been inoculated with the pure microbial culture. It is based on the determination of an inhibition zone directly proportional to the microbial sensitivity to the antimicrobial agents present in the disc. The diffusion of the antimicrobial agent into the inoculated culture media results in a gradient of antimicrobial activity. When the concentration of the antimicrobial becomes so low then it can no longer inhibit the growth of the microorganism, resulting in inhibition zone. The diameter of inhibition zone is related to minimum inhibitory concentration (MIC) for the particular microorganism. The zone of inhibition becomes inversely proportional with the MIC of the test microorganism. Generally, the larger the inhibition zone, the lower the MIC. However, inhibition zone depends on the concentration and diffusibility of antibiotic in the disc (OIE 2008).

The microbial colonies are inoculated and incubated until their specific optical density (OD) at 600 nm reaches to give starting inoculum of approximately 10^8 cfu/mL. Inoculum is mixed

with specific quantity of molten soft agar and poured immediately onto the base layer of agar medium. The plates are allowed to solidify at room temperature. The discs are impregnated with the test sample and placed onto the top layer of agar plates. The diameter of inhibition zone is measured. This technique is cheap and easy in modifying test antimicrobial discs when required. This technique can test against large numbers of isolates and identify a subset of isolates for further testing by other methods, such as determination of MICs. As compared to well in agar method, reproducible results are obtained and can be used for the quantitative determination of antimicrobial substances (Kelmanson *et al.*, 2000; Jagessar *et al.*, 2008; Othman *et al.*, 2011). The role of bioactive substances in controlling pathogens has been determined by this technique (Bajpai *et al.*, 2010a; Bajpai *et al.*, 2011a).

Streak plate disc diffusion (SPDD)

This method is more laborious and time consuming as compared to PPDD. In this method the microorganisms are streaked on nutrient agar plates and incubated for a particular period. By this way single colonies can be obtained easily. The colonies are picked and cultured in broth until they reach their specific OD at 600 nm to give a starting inoculum of approximately 10^8 cfu/mL. A sterile cotton pad is used to inoculate agar plates by streaking over the surface with rotation to ensure even distribution of the inoculum. The discs are impregnated with the test sample and placed onto the top layer of agar plates. The diameter of inhibition zone is measured (Samy *et al.*, 2006; Othman *et al.*, 2011). The antimicrobial activity of several extracts and pure compounds against different microbes was determined (Jagessar *et al.*, 2008).

Well in agar (WA)

In this method, microbial suspension is spread uniformly over the agar plates using sterile glass rod spreaders, to get uniform distribution of microbes. Consequently, wells of between 510 mm are aseptically made on the agar media using a sterile cork borer. Desired amount of drug material is aseptically filled into the well. Later the plates are placed at room temperature for a time period to allow diffusion of drug into the agar. Then the plates are incubated for particular period and the diameter of inhibition zone is recorded (Samy *et al.*, 2006; Mathabe *et al.*, 2006; Othman *et al.*, 2011). This method is laborious, time consuming and difficult to get consistent and reproducible results because of uniform size of wells and uniform diffusion of drug material. However, this method has been used by some researchers

for the determination of antimicrobial activity of several compounds (Namdeo *et al.*, 2009; Bajpai *et al.*, 2010a; Othman *et al.*, 2011).

Radial diffusion assay (RDA)

The gel is prepared and mixed with diluted bacterial culture. Later the gel is poured into circular Petri dishes, incubated and stained in dilute solution of coomassie blue dye. The staining solution is decanted, replaced with dilute solution of acetic acid and dimethyl sulfoxide. The diameter of inhibition zone is measured (Lehrer *et al.*, 1991). Several researchers have determined microbicidal activity of several compounds or extracts (Takemura *et al.*, 1996; Du Toit and Rautenbach 2000). RDA gives a good indication of antimicrobial activity through the formation of inhibition zones. This method, however, is not as sensitive as the previously described methods. At very low concentrations, the test sample might not diffuse far enough into the gel to form a visible inhibition zone. The micro-gel well diffusion assay, in contrast, only depends upon the localized downward diffusion of the test sample. Because the reaction area is not as large as that of the RDA, diffusion of molecules of varying molecular sizes is also no longer a limiting factor.

Poison food technique

The antifungal activity of the drug material is determined by this method (Grover and Moore 1962; Mishra and Tiwari 1992; Nene and Thapilyal 2002). In this method the drug material is dissolved separately and pipetted aseptically to Petri dishes containing fungal growth media. Control plates (without drug) are inoculated following the same procedure. A fungal disc of appropriate size mycelial material, cut from the periphery of fungal culture with the help of a cork borer, is inoculated aseptically to the center of the prepared treatment and control Petri dishes. The plates are sealed and incubated at a temperature of 26 ± 2°C. The efficacy of the treatment is evaluated by measuring the average of perpendicular diameters of colony. Percentage inhibition of radial mycelial growth is evaluated by comparing the colony diameter of poisoned plate (with drug sample) and non-poisoned plate (with distilled water) and calculated using the formula given below.

$$\% \text{ Mycelial inhibition} = dc - dt/dc \times 100$$

Where dc and dt are the average diameters of mycelia colony in control and treated groups, respectively (Verma *et al.*, 2008).

Spore germination assay

This method is used to determining antifungal activity of samples of different origin (Nair *et al.*, 1991; Bajpai *et al.*, 2008; Johnson *et al.*, 2011). The fungal stocks are prepared by stamping agar plates containing growing mycelia with a sterile hollow cork borer, producing circular small pieces from the agar plate. They are suspended in sterile distilled water in an Eppendorff tube at a particular temperature until required. The cultures are grown on fungal medium Petri dishes in an incubator at a particular temperature. The dishes are incubated in a way to avoid evaporation and accumulation of moisture on the inside of the dishes. Spores are germinated and are harvested when the cultures are fully sporulated. The Petri dishes are flooded with sterile distilled water and then gently rubbing the culture to release the spores. The spore suspension is filtered and washed with sterile distilled water. The obtained spore suspension is transferred directly to sterile tubes after harvesting, and centrifuged. The supernatant is removed and the pellet is re-suspended in sterile distilled water. This process washes the spores free of any debris and remaining medium. Finally, the spores are re-suspended in sufficient quantity of sterile distilled water and shaken. This produces a high density spore suspension. The spore suspension is taken on a slide and the samples are added in the form of thin layer. The slides are placed under moistened conditions, incubated at room temperature and are fixed in lacto phenol blue. Percentage spore germination is calculated according to the following formula.

$$\% \text{ Spore germination} = \text{No. of germinated spores/ Total spores} \times 100$$

Micro-gel well diffusion assay

In this assay, the microtiter plates are blocked with diluted solution of casein in phosphate buffer saline PBS and sterilized under UV light. The first column of microtiter plate receives only gel served as control. The other wells receive microbial suspension and gel in same ratio. The drug sample is applied in the wells; the microtiter plate is covered and incubated at 37°C. The light dispersion is determined using a microtiter ELISA plate reader. The plates are preserved by adding hydroalcoholic solution of formaldehyde to each well. The gel is stained by adding diluted solution of Coomassie brilliant blue stain (Du Toit and Rautenbach 2000). The presence of bacteriocins in *Lactobacillus* and determination of its antimicrobial activity by micro-gel well diffusion assay has been reported (Toba *et al.*, 1991).

On the other hand, agar or agarose is advantageous in detecting contamination (Barry 1980). Results from the agarose based assays are also easily manipulated through the use of fixing

agents and staining agents. By using a fixing agent it is possible to halt the experiment at any specific time, as well as to preserve the experiment for an undetermined period of time and by using a staining agent it is possible to create a visual record of the result.

Broth based turbidometric assay (TB)

In this assay protocol, the broth culture is prepared and diluted to give a starting inoculum of approximately 10^9 cfu/mL. The required quantity of inoculums and drug sample is pipetted into 96 well plates. The optical density of each well is recorded using a microplate reader at 600 nm (Noga *et al.,* 1994). Survival index (SI) is calculated as per the following formula (Bexfield *et al.,* 2008).

SI = (OD_{600} of test sample at corresponding point/ OD_{600} at mid-log of control of bacterial growth of test sample) × 100

The effective concentration (EC_{50}) is also calculated from SI values (Alexander *et al.,* 1999). The antimicrobial activity of different compounds or extracts is determined by calculating minimum inhibitory concentration (MIC, usually expressed in mg/ml or µg/ml) (Namdeo *et al.,* 2009). The use of a liquid broth as a growth medium permits instant drug-microbes cell interaction, as opposed to the agar or agarose method, where it depends on the rate of diffusion. In this case more consistent and reproducible results are obtained.

Microtiter broth dilution method

The broth dilution method is simple to determine the lowest concentration of antimicrobials or even a single isolate that inhibits the growth of the microorganism being tested. However, the MIC does not always represent an absolute value. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the microorganisms and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution. It has the added advantage that the same tubes can be taken for minimum bactericidal concentration (MBC) tests also. The dilution methods appear to be more reproducible and quantitative than agar disc diffusion but less flexible in monitoring the programme. However, some samples are usually tested in doubling dilutions, which can produce inexact MIC data.

In this method drug sample is dissolved and diluted to get the required concentration. The first column of microtiter plate receives only broth served as a control while remaining wells

receive desired amount of bacterial suspension. Required amount of the drug sample is pipetted into all wells excluding controls. The microtiter plate is covered and incubated for a given period. Light dispersion is determined using an ELISA plate reader (Lehrer *et al.*, 1991; Amsterdam 1996; Steinberg and Lehrer 1997; Hancock 1997). MICs of antibiotics against fungal species from body fluids are determined by this method (Fujita 1996). The role of secondary metabolites in controlling pathogens has been evaluated by this method (Bajpai *et al.*, 2010a; Bajapai *et al.*, 2010b; Bajapai *et al.*, 2011b). The use of a microtiter plate is more economical as compared to the use of a culture dish. Furthermore, determining inhibition by the spectrophotometric method using a microtiter plate reader is more sensitive and accurate than measuring zones of inhibition. Because of less labour cost, microtiter broth dilution method is ideal for screening large numbers of samples to give consistent and reproducible results.

In agar dilution method different concentrations of antimicrobial agent incorporates into an agar medium, using two-fold serial dilutions. Later definite amount of bacterial inoculum is added to the agar plate. The results are considered as the most reliable for the determination of MIC. This method tests numerous bacteria using single plate, easily manipulate experimental conditions and semi-automation is also possible. Besides, this method is tedious, time consuming, expensive, difficult to maintain purity of inoculum, and complicated for the interpretation of results (NCCLS 2002; OIE 2008).

Measurement of extracellular ATP concentration

The possible effect of antimicrobial drugs on the permeability of the cytoplasmic membrane for small solutes, internal and external ATP pools of microorganisms is determined by this method. There is a rapid inhibition of the energy metabolism of microorganisms when the cells are exposed to antimicrobial substances. The mode of inhibition may involve glucose utilization or membrane interactions because extracellular ATP is known to serve as a mediator of cell-to-cell communication by triggering a variety of biological responses in various cells through ligation of membrane binding purinergic receptors (Gill and Holley 2004). The microbial cultures are prepared and incubated for a particular period. The culture is centrifuged, and pellets are collected and washed with a buffer. A cell suspension is prepared, and an emulsifying agent is added to it. Samples are added to the solution, centrifuged and then incubated in ice to prevent ATP loss. The extracellular ATP concentration is determined using a luminometer after the addition of glycine buffer and

luciferase-luciferin to supernatant. The intracellular ATP concentrations is determined by adding desired quantity of cell suspension to buffer containing surfactant and then sonicating to disrupt the microorganisms. Subsequently, luciferase-luciferin is added to the mixture and the ATP concentration is measured by luminometer. The antimicrobial synergistic effect of linolenic acid and monoglyceride against bacterial pathogens is determined (Lee *et al.*, 2002).

Viability test (Methylene blue dye exclusion assay)

Dye exclusion assay uses methylene blue, trypan blue, nile blue, eosin, and amethyst violet that enter microorganism in which selective permeability of plasma membrane is destroyed or compromised. This method is time consuming and gives reproducible results. This procedure describes the percentage of dead or injured cells in a population by measuring the optical density of cell suspension (Macfarlane 1936; Sehrek 1936; Scharff and Maupin 1960; Parker 1961; Novelli 1962).

The microbial cells are cultured, harvested and treated with mercuric chloride for 2 hrs, which causes irreversible disruption of cell membrane. The samples are prepared with percentages of damaged cells (0-100%). The mixed cells are centrifuged and the pellets are stained by methylene blue stain which is taken up by cell membrane. The optical density of the cells is measured by an UV/VIS spectrophotometer (Bonorat and Mares 1982). The antimicrobial investigation of several bioactive compounds against pathogenic bacteria and fungi has been reported (Mahboubi and Kazempour 2009).

Measurement of medium acidification

The proton pumping activity of microbes is determined by monitoring glucose induced acidification of the external medium by measuring the pH using electrodes. The addition of glucose to cell suspension results in the extrusion of acid due to the action of proton translocating H^+-ATPase, which is a plasma membrane located ATP driven proton pump belonging to the P-type ATPase superfamily (Eraso and Gancedo 1987). The change in external pH upon the addition of glucose disturbs the homeostasis.

In this method, microbial cells are cultured, harvested and suspended in cold sterile distilled water. Different concentration of drug material is added to it. The mixtures are incubated and then at different time intervals 2% glucose solution is added to it. The pH is monitored at regular intervals (Monk *et al.*, 1995; Hammer *et al.*, 2004). The proton translocating ATPase-

mediated activity of several antimicrobial compounds has been reported (Elias *et al.*, 1999; Ahmad *et al.*, 2010).

Assay of potassium ions (K^+) efflux

The cell membranes of microorganisms provide a barrier to the passage of small ions such as H^+, K^+, Na^+ and Ca^{2+} and allow cells and cellular organelles to control the transport of different compounds. This permeability barrier role of cell membranes is linked to many cellular functions, like maintenance of energy status of the cell, energy transducing processes, solute transport, regulation of metabolism and control of turgor pressure (Booth 1985; Poolman *et al.*, 1987; Trumpower and Gennis 1994). The anti-microbial action of drugs results in increased potassium ion efflux due to detrimental effect on cell membrane structure and function. K^+ is the major cytoplasmic cation of growing bacterial cells, involved in several key functions of bacterial cells. This ion plays a role in the activation of cytoplasmic enzymes, the maintenance of turgor pressure and possibly the regulation of the cytoplasmic pH (Bakker and Mangerich 1981)). Different studies showed that an efflux of potassium ions is a first indication of membrane damage in bacteria (Uribe *et al.*, 1985; Sikkema *et al.*, 1995; Heipieper *et al.*, 1996).

Extracellular K^+ concentration of cell suspension is determined using a combination of potassium selective/reference electrode (pH/mV meter). The standard curve of free K^+ ion at ppm concentrations is prepared. The net K^+ ion leakage of drug treated cell suspension is expressed as a percentage of total free K^+ ions in lysed cells of microbes (Cox *et al.*, 1998; Paul *et al.*, 2011).

Release of cellular material

This method indicates whether the microbial cells are alive or dead. The microbial suspension is prepared. Microbial cells are grown in culture broth overnight at 37ºC for bacteria whereas 48 hrs at 26ºC for fungi. The cells are centrifuged, harvested, washed with chelating agent and distilled water. The absorbance of the suspension is fixed. The drug material is added to cell suspension. The cells are centrifuged at different time interval and the absorbance of supernatant is determined using an UV/Vis spectrophotometer. The results are expressed as percentage of absorbing material with respect to time (Carson *et al.*, 2002). The intracellular solutes are released from growing or resting cells of microbes as a result of drug mediated stimulation of degradative enzymes. The released materials are small molecules which are apparently derived from preexisting RNA and protein, but not from DNA. Leakage of

cellular material might cause cell death which seems to be one of the mechanisms of antimicrobial action (Joswick *et al.*, 1971; Heipieper *et al.*, 1992).

Scanning electron microscopy (SEM)

This protocol examines minor changes in the cell morphology. The mechanism of antimicrobial activity is thought to be the degradation of cell wall, membrane proteins, binding of proteins, release of cellular material, coagulation of cytoplasm and depletion of the proton motive force (Burt 2004). The microbial culture is taken in a test tube and centrifuged, pellets are collected and fixed in glutaraldehyde solution containing sodium phosphate buffer with repeatedly washing. Then the pieces are fixed in osmium tetroxide (OsO_4) with repeatedly washing with buffer. The sample is dehydrated by a series of ethanol or tertiary butanol and then with propylene oxide. The samples are dried, coated with a thin layer of gold and are examined using SEM (Hayat 1981; Kockro *et al.*, 2000). This technique is used to determine the effect of various extracts or compounds on morphological changes of different microbes (Bajpai *et al.*, 2010a; Bajpai *et al.*, 2010b; Bajpai *et al.*, 2011a; Leela and Satirapipathkul 2011).

Transmission electron microscopy (TEM)

In TEM, high energy monochromatic and coherent electronic beam is used to probe the material under study. One of the most important advantages of TEM over other techniques is that information can be obtained from both real and reciprocal space. In real space, the structure and morphology of the microorganism can be studied in detail with high resolution and magnification (Fredrickx *et al.*, 2003). The microbial suspension is taken in a test tube and a paper disc impregnated with drug material is placed in it. The suspension is incubated at particular temperature for particular period. The mixture is centrifuged, and pellets are collected and fixed in glutaraldehyde solution. Pellets are washed with buffer solution and again fixed with osmic acid solution. The cells are embedded in molten agar and dehydrated by a series of ethanol. Then cells are polymerized using propylene oxide and Spurr resin. The microtome sectioning of polymerized samples are done and observed under TEM (Horiuchi *et al.*, 2001; Oonmetta-aree *et al.*, 2006). Transmission electron microscopy is used to study the biocidal action of drugs against the microorganisms. The TEM results confirmed the abnormalities in the cellular structure, indicating cell death (Sondi and Sondi 2004).

Effect of hydrostatic pressure

High hydrostatic pressure is a novel technology that can be applied in the food industry to inactivate food-borne pathogens (Hoover 1993; Knorr 1993; Palou 1997) with minimum loss of essential characteristics and nutritional properties of foods (Palou 1997). This process physically disrupts cell membrane, creating membrane leakage and can be explained by alteration of intermolecular structure and conformational changes at the active site of the enzymes (Suzuki and Kitamura 1963; Koo *et al.,* 2011). The inactivation of microbes by lysozyme, denatured lysozyme and lysozyme derived peptides occurs under high hydrostatic pressure (Masschalck *et al.,* 2001). The heat sealed microbial suspension is placed in a high hydrostatic pressure vessel which is filled with aqueous solution of mobil hydrosol to hydrostatically transmit pressure (maximum limit 689 Mpa). When the pump is started, the intensifier of the pump is driven to increase pressure to a desired point and the timer starts. When the set time ends, the control system sends a signal to open the valve and the pressure is released.

Oxygen consumption

The methylene blue dye can be taken up by dead or severely damaged cells, but not by living cells. Based on this fact, a method is developed which permits quantitative determination of injured cells in microbial populations. The microbial cells are stained using different concentrations of methylene blue for a given period. The cells are washed and resuspended in a phosphate buffer to measure O_2 consumption using the Clark-type oxygen electrode (Bonorat and Mares 1982).

This method was used to determine antimicrobial activity of some chalcone derivatives which inhibited electron transport system in the respiratory chain as well as oxygen consumption in microbial cells (Haraguchi *et al.,* 1998). The site of respiratory inhibition of chalcone is thought to be between Coenzyme Q and Cytochrome c in the bacterial respiratory electron transport chain (Haraguchi *et al.,* 1998).

Flow cytometric assay

The anionic dye bis-(1, 3-dibutyl barbituric acid) trimethine oxonol has low binding affinity for intact membranes and is limited to the outer regions of the cell membrane in living bacteria. Membrane depolarization leads to an uptake of dye inside the cell and binds to lipid rich compounds within the cell resulting in an increasing fluorescence signal, detected by a flow cytometer (Deere *et al.,* 1995). Hence, intact non-fluorescent and damaged fluorescent

bacteria can be well differentiated. The antibacterial activity of peptides (human β-defensin proteins) in tissue extract is evaluated by flow cytometric assay using a membrane sensitive dye, which detects depolarization in membrane potential (Jepras *et al.*, 1997). The defensins show broad spectrum dose-dependent activity against bacteria and fungi. The advantage of flow cytometric test is the possibility to quantify the antimicrobial effect of defensins at the target site, the cell membrane and the membrane potential. Moreover this method is very sensitive but expensive (Nuding *et al.*, 2006).

Bioluminescence assay

Bioluminescence is the production and emission of light by a living organism. It is a naturally occurring form of chemiluminescence where energy is released by a chemical reaction in the form of light emission. The luciferin reacts with oxygen to create light and luciferase enzyme catalyzes the reaction, which is mediated by cofactors such as Ca^{+2} or ATP. In bacteria, the expression of genes related to bioluminescence is controlled by an operon called the Lux operon (Hastings 1983; Lee and Camilli 2000; Lehtinen *et al.*, 2003).

When antimicrobial substances are membrane active, they disrupt the proton motive force (PMF). Disturbances of PMF deplete the amount of intracellular ATP, which can be measured using bioluminescence assay based on luciferase reaction (McElroy and DeLuca 1985; Tauriainen *et al.*, 1999; Ennahar *et al.*, 2000)

$$ATP + O_2 + \text{D-luciferin} = AMP + PPi + CO_2 + \text{oxyluciferin} + \text{light (~560 nm)}.$$

In this assay protocol, the microbial broth cultures are prepared and incubated. Absorbance is measured and MIC are determined (Manome *et al.*, 2003; Yamashoji *et al.*, 2004). Microbial culture is mixed with lysis reagent. The mixture is homogenized and incubated. After this luciferin-luciferase reagent is added to it and the emitted light is measured using luminometer which is expressed as relative light units (RLU), proportional to ATP concentration (Chappelle and Levin 1968). The $CIATP_{80}$ and $CIATP_{50}$ are evaluated as the concentration of antimicrobial agent inhibiting 80% and 50% of the bioluminescence. Quinone bioluminescence is determined by incubating microbial cultures with luminol-quinone reagent. The intensity of emitted light is measured. The $CIQNO_{80}$ and $CIQNO_{50}$ are evaluated as the concentration of antimicrobial agent inhibiting 80% and 50% of the bioluminescence. The effect of ellagic acid against oral pathogens in human, thereby reducing the incidence of dental caries with the use of ATP bioluminescence assay (Loo *et al.*, 2010).

Time kill study

The synergistic and antagonistic activity of different drugs alone or in combination against microbes is determined by the time kill assay. This method also gives idea about the bacteriostatic and bactericidal nature of the drug. In this method the microbial suspension is incubated with a test drug. The activity of the drug is verified by MIC determination against the reference strain of microbes. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergism is observed when the effect of the combined substances is greater than the sum of the individual effect. Several *in vitro* time kill studies of antimicrobial agents are determined using this assay system (Ogunmwonyi *et al.*, 2010).

XTT colorimetry

The XTT colorimetry assay is simple, inexpensive and widely used for quick, accurate determination of microbial strain susceptibility, assessment of batch to batch consistency during vaccine production and confirmation of microorganisms in purified samples (Kairo *et al.*, 1999; De Logu *et al.*, 2003; Tunney *et al.*, 2004; Cerca *et al.*, 2005; Moriarty *et al.*, 2005; Vande Sande *et al.*, 2005). This method is based on the reduction of a tetrazolium salt 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) that indirectly measures antimicrobial activity by evaluating electron transport system (Bensaid *et al.*, 2000). In this colorimetric assay, the broth culture of bacteria is added to a microtiter plate. The test samples are added to it and mixed properly. Desired quantity of mixture from each well is transferred onto a new plate supplemented with XTT/Menadione reagent. After incubation the plates are again gently shaken and the absorbance is measured using an ELISA plate reader. Percent reduction in formazan formation is calculated (Pettit *et al.*, 2005). The ≥ 50% reduction in XTT colorimetry assay is considered for antimicrobial activity whereas < 50% reduction is considered little or no antimicrobial activity. By this method, antimicrobial potential of several test compounds can be determined using following formula (Al-Bakri and Afifi 2007).

100% – [(Test absorbance at 490 nm − Blank absorbance at 490 nm)/ Negative control absorbance at 490 nm] × 100

Susceptibility assay using bacterial enumeration

The microbial cultures and the drug material are added to microtiter plate. Plates are gently shaken and sufficient quantity of planktonic culture is taken to determine the number of the

viable cells by the serial dilution method. All dilutions are placed on nutrient agar plates that are incubated. The antimicrobial activity of the test material and standard is compared. A mean (n = 3) reduction in viable count of ≥ 90% is considered for antimicrobial activity (Pettit *et al.*, 2005; Al-Bakri and Afifi 2007). Percent viable count reduction is calculated using the following formula.

100% – [(Experimental well viable count)/ (DMSO well viable count)] × 100

Bioautography

This technique can be employed in the bioactivity guided fractionation and isolation of therapeutically active markers using chromatographic fingerprinting with good localization of biological activity (Hostettmann 1999; Nostro *et al.*, 2000; Schmourlo *et al.*, 2004). An advantage of the bioautographic method is the possibility of using mobile phases containing low volatility solvents as n-butanol, in case of its complete removal before carrying out tests. However, highly acidic or alkali solvents remaining on a TLC plate after long drying time inhibit possible bacterial growth. In paper chromatography−bioautography, the developed chromatogram is placed onto the inoculated agar gel containing microorganisms, facilitating the diffusion of test material from chromatogram to agar gel (Goodall and Len 1946). Bioautography methods are usually divided into three categories, agar diffusion or contact bioautography, immersion or agar overlay bioautography and direct bioautography (Rios *et al.*, 1998). Antimicrobial screening of several test components against microorganisms has been reported by this method (El-Gendy *et al.*, 2008).

Agar diffusion or contact bioautography

In this assay, the samples are applied on TLC plate which is covered with thin layer of agar gel. The microbial suspension applied on agar plate either mixing with agar gel or swabbing with a cotton swab for a period of time to permit diffusion. Later the plates are removed and the agar layer is incubated. The zones of inhibition appear at the places, where the antimicrobial compounds are in contact with the agar layer (Beghe and Kline 1972; Hamburger and Cordel 1987). The antimicrobial activity of several test compounds against pathogenic microbes has been reported (Valgas *et al.*, 2007).

Immersion or agar overlay bioautography

In immersion bioautography, the plates are dipped or covered with agar medium, which after solidification are inoculated with the suspension of test microorganisms. The plates are incubated for a specified period of time. The inhibition or growth bands are visualized after

staining with dyes. Low temperature facilitates better diffusion of test material into the agar surface. This method is a combination of contact and direct bioautography, because the antimicrobial compounds are transferred from the chromatogram to the agar medium, as in a contact method, but the agar layer remains onto the chromatogram surface during the incubation and visualization, as in direct bioautography (Harborne 1973). Several antimicrobial compounds have been screened for their biological efficacy using this method (Suleimana *et al.*, 2010).

Direct bioautography

In this assay, the specific amount of drug material is applied to the TLC plates, which is developed using appropriate mobile phase. The microbial suspension is sprayed onto a TLC plate (Meyer and Dilika 1996; Schmourlo *et al.*, 2004). The plates are incubated and sprayed with visualizing agent (tetrazolium salts), which are converted by microbial dehydrogenase to intensely colored formazen (Meyer and Dilika 1996; Dilika *et al.*, 1996; Silva *et al.*, 2005; Runyoro *et al.*, 2006). Clear white zones against a purple background on the TLC plate indicate antimicrobial activity (Horvath *et al.*, 2002).

The description of above mentioned protocols for their practical antimicrobial applications has been given in Table 1.

Future prospects

The protocols discussed in the current review are used for the anti-microbial screening of different drugs at different levels. There is constant need for the development of newer protocols because the origin, chemical nature, purity, solubility and availability aspects of the drugs may affect the usefulness of the anti-microbial screening protocols. Moreover, antibiotic resistance issues necessitate the continued discovery and development of new antibacterial agents. Hence, with the development of new antimicrobial agents, there is the requirement of the development of new antimicrobial protocols which are not only useful for the screening of antimicrobial compounds but also helpful in the study of mechanism of action of antimicrobial drugs and can also give an idea regarding the development of new antimicrobial agents. Nonetheless, the impetus to develop highly sensitive method for a number of drugs is encouraging. Although the development of protocols may ultimately determine the utility and effectiveness of anti-microbial agents, alternative approaches continue to move forward.

Table 1: Applications of antimicrobial screening protocols

Drugs	Test microorganisms	Protocols used	References
Acalypha wilkesiana	*Escherichia coli, Staphylococcus aureus*	Well diffusion, Turbidometric assay	Othman *et al.*, 2011
Alpinia galanga	*S. aureus*	Release of cellular material, TEM[a]	Oonmetta-aree *et al.*, 2006
Ballota undulata	*E. coli*	Bacterial enumeration	Al-Bakri and Afifi 2007
Cleistocalyx operculatus	*S. aureus, Enterococci*	Scanning electron microscopy	Dung *et al.*, 2008
Duabanga grandiflora	*E. coli, S. aureus*	Pour plate, Streak plate disc diffusion	Othman *et al.*, 2011
Euphorbia fusiformis	*Bacillus subtilis, S. aureus*	Well in agar, Disc diffusion	Natarajan *et al.*, 2005
Gramicidin	*Micrococcus luteus*	Radial diffusion, Broth dilution	Du Toit and Rautenbach 2000
human β-defensin 3	*E. coli, S. aureus*	Flow cytometric assay	Nuding *et al.*, 2006

Table 1 continued

Hypericum triquetrifolium	***B. subtilis***	**XTT colorimetry**	**Al-Bakri and Afifi 2007**
Linolenic acid	*B. cereus, S. aureus*	ATP measurement	Lee *et al.*, 2002
Melaleuca alternifolia	*Candida albicans, C. glabrata*	pH measurement	Hammer *et al.*, 2004
Melaleuca alternifolia	*C. albicans, S. aureus*	Potassium ion efflux	Cox *et al.*, 1998
Oscimum gratissimum L.	*Slerotonia fructicola*	Spore germination	Grover and Moore 1962
Polyalthia longifolia	*Coilletotrichum*	Poison food technique	Mishra and Tiwari 1992
Saccharomyces cerevisiae	*E. coli*	Hydrostatic pressure	Bang and Chung 2010
Saccharomyces cerevisiae	*Rhodotorula glutinis, Euglena gracilis*	Viability test Potassium ion efflux Oxygen consumption	Bonorat and Mares 1982
Tetracycline	*Salmonella enterica serovar*	Bioluminescence assay	Vesterlund *et al.*, 2004
SXT[d]	*S. aureus*	Time kill assay	Zander *et al.*, 2010
β-1actams antibiotics	*Enterobacter* species	MIC, Disc diffusion	Kiska 1998
Eicosapentaenoic acid	*F. oxysporum*	MIC, Spore germination assay	Bajpai *et al.*, 2008

Table 1 continued

Metasequoia glyptostroboides	***S. typhimurium***	**MIC, SEM[b]**	**Bajpai *et al.*, 2010**
Brassica oleracea	*Candida* species	MIC, SEM, Cell viability	Bajpai *et al.*, 2011a
Metasequoia glyptostroboides	*Xanthomonas* species	MIC[c]	Bajpai *et al.*, 2011b
Nothapodytes foetida	*B. subtilis, S. aureus, E. coli*	MIC, Zone of inhibition	Namdeo *et al.*, 2009

[a] Transmission electron microscopy; [b] Scanning electron microscopy; [c] Minimum inhibitory concentration; [d] Sulfamethoxazole-trimethoprim.

CONCLUSION

The concept of test screening protocols may apply not only to food industry but also to cosmetic and pharmaceutical industries to screen several antimicrobial test compounds. However, it is quite difficult to regularize antimicrobial assays and there is no single assay that is well suited with all compounds. In the view of this fact this review focuses on different antimicrobial assays useful for the screening of various test material with the aim of obtaining optimal methods that provide consistent and reproducible data.*RST and PS contributed equally in this paper

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