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COMPARATIVE REVIEW OF MAIN METHODS FOR ANTIMICROBIAL TESTS

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Review Article

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ABSTRACT

Bacteria present great importance in the clinic, where the mechanisms to combat these pathogens appear more and more to find a substance with low cost, good pharmacological properties and low toxicity. It can be mentioned that the main microbiological methodologies found in the literature for the detection of bacterial and fungal activity can be of three types: bio autographic, dilution and diffusion. Generally the techniques used are disk diffusion, cavity diffusion, agar paste, agar dilution and broth dilution. These assays are quantitative methods in which the effects can be classified. Experiments as an in vitro experimental model are the results of competition. The different types according to the method employed, such as diffusion assays in the medium, are capable of influencing the oxidation of compounds that favor the development of bacterial membrane. Objective: to gather the main methodologies applied in the antibacterial test and those that help to corroborate with the antimicrobial research, where comparisons will be made between methodologies applied by the authors, but with the intention of providing the execution protocol for each of the cited trials. The materials were collected in the main databases, using articles, books, technical manuals and international regulations, all in the most current contexts. Conclusion: the research gathered seven methodologies applicable directly to the antibacterial tests, followed by five tests that corroborate with the antibacterial results for the development of a possible pharmaceutical alternative, such as the experimental toxicity test with Drosophila megalogaster; Photodynamic Therapy (PDT) and cytoprotection against Microbial Heavy Metal Models.

INTRODUCTION

The search for efficient drugs to combat bacterial infections has revolutionized the focus of research. Wave with the discovery of antibiotics in the 1930's led to a drastic reduction in mortality caused by microbial diseases. It is known that bacterial resistance is a spontaneous phenomenon, which was later favored after the large-scale use of antibiotics in the fight against infectious diseases, often without previous knowledge of which microorganism was treated [1, 2].

Taking into account that bacterial resistance is a reality, it should be noted that it is an enormous genetic capacity to transmit genetic material of resistance to another bacterium, besides the fact that acquiring resistance to the effect of drugs used as therapeutic agents happens all the time [3]. The prior knowledge of which microorganism is being controlled is of great importance, and which substances most sensitive to these pathogens will greatly favor the decrease of these resistant bacterial [5, 33]. The main method used to find an antimicrobial susceptibility profile is known as antibiogram, a test commonly done in clinical microbiology laboratories [6].

For the determination of the sensitivity profile, several methodologies are available, but those considered as gold standard, such as agar dilution and broth microdilution, may present different reading forms as colorimetric method with reazasurin or by reading in absorbance in the ELISA. Although it can be done by routine laboratory trials, although they provide quantitative results minimum inhibitory concentration (MIC) has qualitative techniques, such as disk diffusion, although they are easy to perform are not able to predict MIC, possibly through the interaction of the medium with some oxidizable compounds [7, 8].

The main methodologies for detecting the antimicrobial activity found in the literature for fungi and bacteria can be of three types: dilution and diffusion. The agar diffusion methodologies are a quantitative method where the effects can be graded and

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can be performed through disk, well or model techniques. Taking into account that several methodologies that can be approached for antibacterial assays, and the understanding that each method is able to prove the presence of biological activity or not, this material was made with the main methods used for antibacterial assays.

METHODOLOGY

We used articles, technical manuals and standards present in the National Committee for Clinical Laboratory Standards (NCCLS) describing the different types of methodologies used for antibacterial assays found in the literature up to the year 2018. The work is not aimed at comparisons but rather as a guideline for the execution of each methodology applied by the authors, in order to provide knowledge from how to prepare each of the tests until the reading with the current form, and other complementary tests that corroborate for the cited tests [9].

Bacterial assays

Determination of Minimum Inhibitory Concentration–MIC

Macrodilution

Assays involving macrodilution are laborious, where small numbers of replicates are used [10,11]. Initially, bacterial samples should be reactivated in Brain Heart Infusion (BHI) medium and incubated in a bacteriological oven at 37°C for 24 hours [18]. After incubation, samples should be transferred to test tubes containing 5 mL of BHI broth, additional incubation at 37°C for 24 h. The inoculum should then be adjusted using a spectrophotometer in appropriate cuvettes to determine absorbance. The wavelength used generally ranges from 420 nm to 660 nm [9].

To begin the assay, 0.9 ml of the culture medium with different concentrations and 0.1 ml of bacterial inoculum adjusted to (1 to 3 X 108 CFU/ml) should be added to microtubes. The assays are given in duplicates having the microtubes incubated at 37°C for 24 hours. After the incubation, a visual reading is made to show the bacterial growth, translated by the turbidity [18]. An aliquot with a loop is then collected in each tube, so that the material is seeded in Mueller-Hinton agar for further incubation at 37°C for 24 hours and reading to corroborate with the other results [9].

Following reading after incubation, 20 µl of 0.01% sodium breath oxidation reduction indicator should be added, waiting one hour for the results to be read according to [10], at which time blue staining demonstrates bacterial inactivity and red, bacterial activity. Regarding the seeded plates, after the incubation period, they were inspected for observation of the surface growth of bacterial colonies [13].

Microdilution-determination of intrinsic activity through CIM

The assays to determine the minimum inhibitory concentration [14] after bacterial reactivation made in BHI at the concentration prescribed in the label, as lineages should be transferred to 3 mL of 0.9% NaCl solution and then shaken with the aid of vortex apparatus to form a suspension of 105 CFU / mL and adjusted to Mcfarland scale 0.5%. Then, 150 µL of the inoculum will be added to the medium containing 1,350 µL of BHI (10%) which at the end is 1,500 µL, it should be transferred to each labeled eppendorf or "micro centrifuge tubes [15].

The product in turn is weighed 10 mg which can be increased according to the concentrations to be tested. Then, 1 ml of Dimethylsulfoxide (DMSO-Merck, Marmstadt, Germany) is diluted to give an initial concentration of 10 mg/ml, which will then be diluted in sterile water to a concentration of 1024 µg/mL [14].

After mixing with vortexing the solution containing BHI + inoculum with total of 1,500 µL is mixed, 100 µL of this solution is transferred into each well of the microdilution plate, then it must be micro diluted with 100 µL of the product to be tested, starting in the first cavity until the penultimate, keeping the last cavity free of dilution of the test product, this in all the repetitions, being necessary to be maintained as control of microbial growth, then takes to stove adjusted 24 h at 37°C [16].

The CIM reading can be done by adding 20 µL of reazasurin to each well. Reazasurin is a dye that, when it comes into contact with some type of living microorganism, changes its chemical structure, turning its coloration from a bluish tone (absence of microorganism) to pink (confirmed) that there is presence of viable microorganism cells. If the researcher has the need to compare and/or confirm bactericidal or bacteriostatic function, the CBM minimum bactericidal concentration assay may obtain this data, noting that this should be done before reasurin is added, subcultured with 0.5 µL of each well to a petri dish with labeling of each well/concentration on the plate then incubated in an oven for 24 h 37°C. After a period of 24 hours/37°C, each concentration that has been subcultured is read, if there was bacterial growth or not [12,17].

Currently the ELISA equipment is another form of reading used in several laboratories, being able to provide a form of interpretation where the generated data can provide graphs that guarantee the fidelity of the tests besides informing the cellular viability, providing directly to what extent the product was able to act under the microorganism, such as the 50% inhibition of bacterial growth known as inhibition concentration in 50% of microorganism (IC50) [12].

For readings made at ELISA, there is a need for product dilution controls and sterility control. In the dilution control, 1,350

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µL of 10% BHI medium + 150 µL of saline will be obtained, the purpose of which is to compare the dilution of the product with the microorganism, with the dilution of the product with saline only, in order to compare those that precipitate, besides generating an average of the triplicate decreasing of the control of dilution, which would result in the precise growth of the microorganism. The sterility control will provide information of the exemption of contaminants that make unfeasible the reliability of the tests [12,16].

Microdilution - Antibiotic activity modifying effect direct contact

To verify the modifying effect of the antibiotics against the strains tested, the method proposed by [14]. Eppendorf tubes containing the sub-inhibitory concentration (MIC/8), 10% BHI content according to the sub-inhibitory concentration volume and 150 µL of the bacterial suspension (corresponding to 10% of the solution). For the control, eppendorf tubes were prepared with 1.5 mL of solution containing 1,350 µL of BHI (10%) and 150 µL of microorganisms' suspension. The plate was filled numerically by adding 100 µL of this solution into each well. Subsequently, serial dilution was performed with 100 µL of the antibiotic discarded in the penultimate well. The plates were incubated at 37°C for 24 h, and then 20 µL of resazurin was added for determination of viable cells by staining [12].

Determination of minimum inhibitory concentration (MIC) for fungi

The assays for determining the minimum inhibitory concentration for fungi can be carried out using the broth dilution technique using quadruplicate-labeled 96-well plates, with 100 µL of double-concentrated sabouraud dextrose broth (CSD) + fungal suspension being added thereafter performed serial dilution with 100µL of the natural product at the concentration of 4,096 µg/mL micro diluted in the first 2048 µg/mL well to the penultimate 2 µg/mL. The last well is not diluted, as it will serve to control fungal growth, in addition, sterility controls of the dilution medium of the natural product and of the antifungal should be performed, preferably. The plates should be incubated at 37°C for 24 hours and after that the minimum fungicidal concentration (CFM) test for subsequent reading in the ELISA (Termoplate®) device is initiated, generating a cell viability curve and IC50 [18]. For the antiseptic modification; tests described by [19] with modifications to fungal means.

In this test it is verified whether the action of the natural product is capable of causing on the yeasts some dimorphism by inhibiting the emission of hyphae. Firstly, they must install sterile and humid micro morphological chambers to promote the growth of yeasts. Within each test tube should contain 3mL in all, having 1 mL of detoxified Potato Dextrose Agar (PDA) depleted to cause yeast stress and thus emitting hyphae, + test substance in CSA/2 (8,192 µg/mL) , CSA/4 (4,092 µg/mL) and CSA/8 (2,048 µg/mL), then poured into lamina. The petri plates with lamina are inoculated by a calibrated loop (1 µg) where two parallel grooves are made on the already solidified medium, then covered by a sterile microscopical coverslip. After incubation and after 24 h (37°C) the culture was visualized by light microscopy. A control was made for yeast growth whose hyphal emission was stimulated by the impoverishment of the medium, as well as a control with the commercial antifungal fluconazole, for comparative purposes. The assays were performed according to [18] and in compliance with the experiments of [18] with some modifications.

Measuring the extension of filamentous structures

Possibly it is necessary a high resolution microscope the most appropriate are from the optical model (AXIO IMAGER M2-3525001980-ZEISS- Germany), coupled to a computer with Zen software it was possible to measure the extensions reached by the hyphae, and the values of reduction after fractions. Where photos of each slide will be taken across all fields for measurements of growth controls and compared to tests [18].

Disk diffusion

Firstly, the strains to be reactivated in the culture medium usually employed are: Brain Heart Infusion BHI and Agar Mueller Hinton. For the test tubes 4 to 5 mL of BHI is transferred with the aid of a pipette, after which bacterial sample is reactivated in incubation of 24h/37°C. Then with the aid of the sterile swab the microorganisms are replicated from the inoculum to the sterilized petri dish previously prepared with Mueller Hinton, where the smear should be repeated more than twice. After this step the disks used for control in one plate and the ones soaked by the test substances in another one should be used, if enough space can be made in the same plate then incubated for future reading [20,21].

The methodology is performed according to [2], adapted by [7,22] where first the solutions of the compounds are prepared in 10%, 5%, 0.6% and 0.3%. The diffusion assay should be prepared by adding initially weighing the test substance and bringing it to the concentration to be evaluated which according to [14], initially of 1024 µg/mL below significance for clinical use, 10 µL of the solution to be tested in its respective concentration on sterile filter paper discs of 6 mm diameter should be added at the concentrations to be evaluated. The application of this method is limited to fast growing microorganisms, being aerobic or aerobic facultative. The intent is that the test substance is able to inhibit the growth of the microorganism or prevent its proliferation. The evaluation is comparative against a reference biological standard (positive control) generally a broad spectrum antibiotic, the zone or halo of growth inhibition is measured starting from the circumference of the well or disk, to the margin where there is growth of microorganisms and compared to the size of the halo found in the test substance [10].

Diffusion for essential oil

First reactivation of the lines is done by inoculating previously in BHI 3.7%, and later they are seeded in plates with Heart

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Infusion Agar - HIA incubated by 24/37°C. Then for minimum inhibitory determination - DIM the oil should be weighed 50 µg dissolved in 50 µL of 1:1 DMSO and further prepared 1:2 serial dilutions (50-1,562 µg oil). After reactivation period, the strains should be transferred and adjusted McFarland scale (105 CFU/mL) with equivalent turbidity, later with the aid of sterile swab or loop should be made seed in the base of plates with more suitable means for its test, generally HIA.

A quantity of 100 µL of each of the dilutions will be applied to the top petri dish to be incubated at 37°C for 24 hours. DIM is defined as the minimum inhibitory dose per volume of air capable of suppressing the growth of the microorganism in a closed system [12]. The controls should be prepared with medium and only the solvent as a test, free of the presence of the oil [23].

Activity modifying the action of antibiotics in vitro by gaseous contact (essential oil)

The possible combination with antibiotics may be added disks of their antibiotics preferably, then the plates should be inverted and a volume of 50 µL of each concentration will be placed inside the lid. Three plaques are used per microorganisms, the antibiotic disks and the substance in the lid being placed on the first plate, the second placing the antibiotic and DMSO on the lid and the third only the antibiotic disks [24].

The plates were then incubated for 24 h at 37°C and the results read with the help of a pachymeter, the diameters of the inhibition halos of the controls being compared to the Inhibition Halo Diameters Interpretative Standards established by [9]. While the diameters of the inhibition halos in the three conditions are measured and evaluated to ascertain statistical significance [23].

Diffusion in cavity

Diffusion assays using cavities have become an alternative for antibacterial assays, according to the methodology described by [19] the substance studied should be solubilized in DMSO and then distilled water to obtain a solution in the desired concentration.

Having as culture media BHI and Agar Mueller Hinton prepared according to the manufacturer. The reactivation is done in BHI after the incubation period with the help of the swab embedded in the bacterial sample is replicated to petri dish with Mueller Hinton being repeated smear more than twice. Thus perforations of the 6 mm wells are performed using cannulae, sealing them later with Mueller Hinton Agar medium.

Each well is filled with 50 uL of tested substance and at the concentration determined by MIC, after procedure the plates are taken to the incubator for 24 hours/37°C. The results are measured with pachymeter considering as halos positive activity greater than 6 mm. The final result is given by the arithmetic mean of the diameters of the inhibition halos in triplicates being compared with halos of the controls, possible antibiotics used for comparative purposes [16,19].

Other techniques being used by researchers worldwide

Stainless steel cylinders

The methodology used to describe this in [10], using references [11,25]. The technique consists of the application of stainless steel cylinder in medium under the culture medium then addition of the sample on the cylinder, in study [4] a 200 µL of the extracts of medicinal plants, and did not have essay reports with essential oil using this methodology. As a positive control, bacteria were used chloramphenicol (40 µg/mL; 200 µL) and water as negative control. The assay consists of both bacteria and fungi.

Antibacterial assays using stainless steel cylinders were also proposed by [25] where they adapted the cylinders in the method of diffusion in agar. A bacterial suspension at 108 CFU/mL was used, challenging 200 µL of glycolic extracts from a plant species. At the end as a positive control, ampicillin was used.

Light-mediated antibacterial evaluation- Photodynamic Therapy (PDT)

The assay for the evaluation of light-mediated antibacterial activity is known as Photodynamic Therapy - PDT, which is capable of causing inactivation of microorganisms. Known for the first time more than 100 years ago, when Oscar Raab [27] reported the lethal effect of acridine hydrochloride on Paramecia caudatum.

At first the technique has been developed as a therapeutic option for cancer, based on a concept in which a non-toxic photosensitizing agent can be localized preferentially in certain tissues and subsequently activated by light generating singlet oxygen and free radicals which are cytotoxic to the cells of target tissue [27]. Currently the PDT has been used to treat infections in addition to the scope of the research trials to eliminate microorganisms in vitro, suggesting its usefulness as an adjuvant to current disinfection techniques [4,28].

For the assays the bacteria should be reactivated in more suitable medium, then 10 µL of the test solution is added to the blanks (100 µg/disc) in triplicate. The photosensitivity assessment should be performed simultaneously to avoid false positive results. While a plate is exposed to UVA light (5 W/m2, 315-400 nm, from a Osram Dulux S Blue UVA 9W/78-G23 lamp, with maximum emission at 350 nm) for 2 hous, the another must be kept in the dark for the same period. The emission flow rate shall be monitored with a digital ultraviolet light meter to avoid possible variations in emission values and consequently erroneous

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results. Soon the plates were incubated at 37°C for 24 hours, followed by reading the diameters of the growth inhibition zones with a digital caliper [26].

The positive control used for growth inhibition was ampicillin (10 µg/disc). Disks with ethanolic solution (100%) of 8-methoxypsoralein 8-MOP (10 µg/disc) is being used as a positive control of phototoxic activity. Then discs with DMSO (10 µL/disc) solvent were used to dilute product which is used as negative control [26].

Cytoprotection against toxic metals microbial models

The presence of metals in the body is able to generate free radicals, which in large concentrations cause serious damage to the DNA, inducing lipid peroxidation, depletion of thiols groupings. Phytoremediation is still under study to determine which plants can be used to remove, contain, transfer, stabilize or render toxic metals harmless. First test must be done to evaluate minimum inhibitory concentration (described methodology by microdilution). However, another M9 TRIS medium of mineral growth can be used, such as the BHI itself used in the top methodology [9,12].

After the results of the CIM, the eppendorf should contain the sub-inhibitory concentrations of the samples (extract, not being informed some test with oil), as the microorganisms must be standardized 105 CFU/mL in M9 TRIS medium with 2% glucose.

Then add to 100 µL of solution containing medium + microorganism + extract then microdilution with mercury chloride (in 10 mM concentration), after the microdilution it should be incubated for 48 h at 37°C. Subsequently expected the incubation period is carried out the Minimum Bactericidal Concentration, given by the lowest concentration capable of inhibiting the growth of the microorganisms. The subcutaneous is made in plate with HIA medium, withdrawing with a handle from each microdiluted well to the plate with HIA, then incubated for 24hours at 37°C. CBM is given by the lowest concentration capable of inhibiting bacterial growth, taking into account growth control [9,12].

Experimental toxicity test with Drosophila megalogaster

They are flies commonly found in fruits and contain approximately 1,500 species in the family Drosophilidae [12]. *Drosophila melanogaster*, is an emerging model system in toxicological research. The toxicity test is carried out by means of the evaluation of survival and negative geotaxia on exposure to the test substance through fumigation. Generally (Harwich strain) D. melanogaster are obtained from the National Species Stock Center, Bowling Green, OH. Adult flies should be transferred to 130 ml glass containers, containing filter paper. Next, 1 ml of 20% sucrose should be added to the filter paper remaining on the floor of the flask and the amounts of 1æL, 5æl and 10æl of the substance to be tested on the container lid, finally the flies are added in the amount of 20 per bottle. The test period should be controlled in 12-hour light/dark cycles with controlled temperature at 26°C and relative air humidity of ± 60%. The survival and geotaxia readings of the flies should be performed in periods of 3, 6, 12, 24, 36 and 48 hours. While analyzing the toxicity data a two-way ANOVA test is performed using GraphPad Prism 6.0 software and then performed a Tukey multiple comparison test. The conclusive reading can be seen through death, deficits of locomotion after the time intervals of exposure and those that did not present any visible change [29,30].

Interferences in the mentioned methodologies

The conditions that are performed interfere directly in the susceptibility of the diffusion and dilution methods, so the need to understand the condition that the experiments are made and the standardization of the same according to the relevant regulations, provide important considerations like [10]:

Culture mediums: The culture medium is one of the factors that can influence the bacterial growth and consequently the future results. The formation of the blisters may influence and further hamper the assessment of antimicrobial susceptibility, for example, those that diffuse poorly through agar, such as polymyxin [31].

Availability of oxygen: In study [10], reports that research by [32], observed a relation that the availability of oxygen can influence the multiplication of aerobic, anaerobic or facultative microorganisms.

Pipetting errors: The lack of calibration of the pipette may interfere with the volume and will be pipetted and consequently the exact concentration of the substances to be evaluated, which leads the authors to use the means of the triplicates or quadruplicates, which will reduce the margin of error.

Discs: The thickness and the uniformity of the agar are essential for the good resolution of the results, it must be controlled strictly its volume in order to avoid formation of superficial streaks and bubbles [32]. The discs used in the microdilution assays may influence the results, if they are not of a larger size or less than 6 mm; they must be sterilized [10].

Calibration of inoculum: Each strain used for each test separately presents unique characteristics, there is a standard to be followed for a perfect incubation, each strain has an incubation time greater than another, the nutrients that favor its growth vary, and consequently the amount of CFU used cannot escape of standards, such as the McFarland Scale 0.5%.

CONCLUSION

The "screening" under the main methodologies used in the antimicrobial activity were externalized, providing in depth

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knowledge to prepare and analyze the results. Highlighting the corroborating tests to elucidate the compounds that act, in addition to the prior knowledge of how to choose a good pharmaceutical formulation for the use of that compound found. The work provides sufficient material for assistance in the search for methodologies that can be applied under antimicrobial activity and previous toxic, with current context and following standards considered gold. Finally, it is important to evaluate the interfering factors, establishing parameters according to the need of the method used for the given objective.

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