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The Future is Still Ahead: Methodologies for Discovering New Antimicrobials within the World Biodiversity

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Abstract: The use of antimicrobials is essential to treat infectious diseases worldwide. However, the abusive and/or inappropriate use of these molecules have contributed to the development of resistant bacterial strains, restricting the current treatment options. This resistance phenomenon boosts the need for searching new antibacterial molecules to treat human and animal populations. Currently places and countries with high biodiversity such as Brazilian Amazon forest and Brazil, which is member of a group with 17 mega diverse countries, are pointed as promising environments for identifying new antibiotics and still have to be explored. However this biotechnological potential is far from getting to the population as these countries neither open their biodiversity to others nor efficiently explore it. In this work we aim to briefly review some of the simplest *in vitro* and *in silico* laboratory methodologies to identify new antimicrobial prototypes to stimulate these countries to explore their own biodiversity as well as to other countries that are interested on researching natural products and new molecules. This work intend to contribute to unveil this unexplored biodiversity world as well as to improve the development of new therapeutic options to fight against bacterial infections worldwide.

Keywords: Antimicrobial activity; Bacterial resistance; Screening methods; *In silico* toxicity, *In vitro* cytotoxicity.

1. Introduction

World Health Organization classifies bacterial infections among the top ten causes of death in the world [1-3]. Currently bacterial resistance is a serious public health problem and an enormous challenge as the commercial antibacterials have become inefficient against several multiresistant strains [4]. Bacterial resistance is a natural genetic event that allows the micro-organism to adapt to the environment being a real growing threat to therapeutics effectiveness [5, 6].

Currently a variety of antibacterials with different chemical groups and structures are used in human and veterinary medicine to treat several infectious diseases in order to reduce morbidity and mortality [1-3]. However the indiscriminate and abusive use favored the selection and spread of resistant bacteria. Therefore, bacterial infections that were previously treated at low cost are now driving the government concerns to the seriousness of the situation of health of their countries population [2].

According to the literature, advances in the development of synthetic antibacterials and recent discoveries of new drugs isolated from natural sources may represent huge contributions to fight against bacterial resistance [7, 8]. As the development of a new antibacterial requires years of research with high cost and risks of failure to the pharmaceutical industries, most common approaches in drug design is to perform structural modifications on the already existing molecules, which allows cross-resistance. In fact, creating new antibiotic classes or structures is a more rare situation, which turn molecules from natural sources even more interesting due to the novelty of their composition and/or conformation as well as mechanisms [9, 10].

The process of discovering a new antibiotic can be divided into two phases: i) discovery (known as preclinical or basic research) and ii) development (or clinical). In the initial stages, studies are generally focused on the

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identification and optimization of molecules with clinical potential, capable of representing new chemical entities using *in vitro* and *in vivo* (biological -experimental) or virtual (*in silico*) environments [11].

In the *in vitro* stage, the biological properties of an antibacterial molecule include antimicrobial and cytotoxic analysis including inhibition of bacterial biofilm, some initial pharmacokinetic characteristics and brief evaluation of the adverse effects [12].

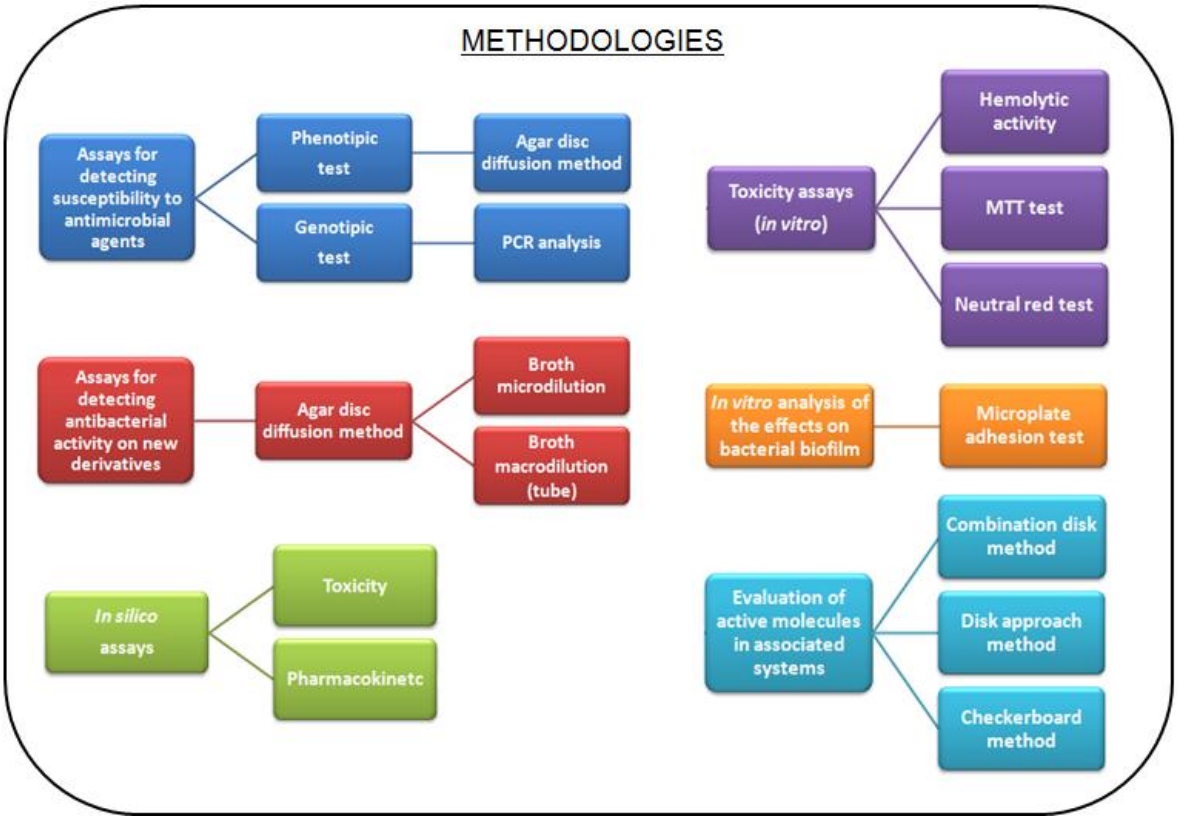
The discovery of antibiotics represents significant advances in modern medicine. However, after the introduction of an antibacterial into the market, the emergence of resistant bacterial is somehow expected and may occur [13]. Thus, greater investments in the development of new antibacterials are always required as well as a greater control over those on the market. The use and development of treatment alternatives (*eg*. combined therapy) to improve the spectrum of action is relevant when it comes to multiple bacterial etiology and resistance [14].

In order to be effective, new antibacterials should not be affected by pre-existing resistance against the current antibacterials classes [15]. Therefore, new tools have been introduced such as molecular modeling or *in silico* study to discovery and select new molecules using novelty and toxicological filters (Druglikeness and Drugscore), allowing a better planning [16].

Due to the emergence and spread of multiresistant bacterial strains and slow antibiotics discovering process, it is important to search new options including in the biodiversity of some countries such as Brazil (*eg*. Brazilian Amazon forest). Therefore, there is a need to support these countries to explore their own natural biotechnological sources as they restrict the access to themselves and to others with rigid laws and bureaucratic processes. On that purpose, they should find a way to pursue and overcome this challenge. This includes selecting methodologies to identify the antimicrobial and toxicity profiles choosing the one that best fit into their lab conditions and financial support .

The aim of this work is to briefly describe some *in vitro* and *in silico* techniques used for identifying new antibacterial molecules. They have been used as low cost standardized simple technologies and tools (Figure 1).

Figure-1. *In vitro* and *in silico* techniques for identifying of new antibacterial molecules.

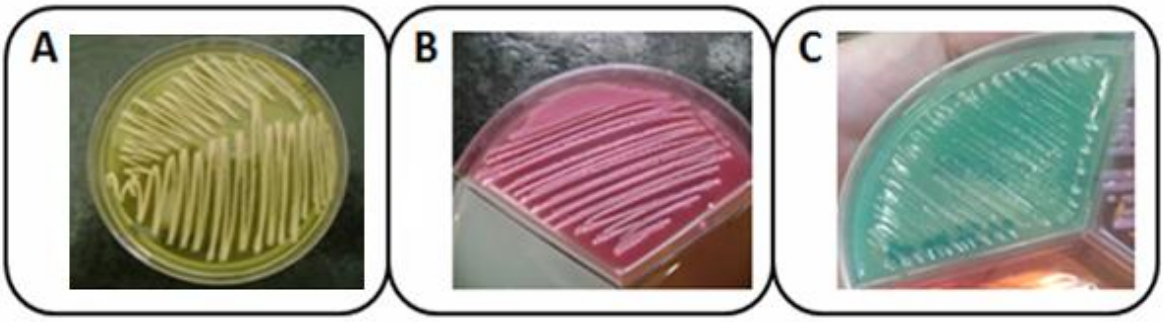


2. Bacterial and Reference Strains

2.1. Bacterial strains

The collection of the bacterial samples should meet the basic requirements AND , be transported in an appropriate way to the laboratory. For the identification of the microorganism, the growth characteristics of the colonies should be observed (eg., the use of selective and differential media), besides the specific biochemical characterization (Figure 02). After the identification of the culture, the bacterial sample must be stored in culture medium with adequate cryopreservation and temperature [17].

Figure-2. Examples of culture medium and strains: (A) Culture of *Staphylococcus aureus* on salted mannitol agar, (B) *Escherichia coli* on MacConkey agar, and (C) *Pseudomonas aeruginosa* on cetrimide agar.



2.2. Reference Strains

The identification, phenotypic characteristics and sensitivity profile of the reference strains must be determined and have a reliable origin, coming from a reference laboratory that performs phenotypic and molecular tests. The stock culture of the reference strains should be stored in the laboratory at -20°C, whereas the working culture, known as the weekly or monthly subculture of the stock culture, should be maintained between 4°C and 8°C. The maximum number of five consecutive cultures from the original strain must be observed to avoid compromising their phenotypic characteristics [18].

3. Assays for Detecting Susceptibility to Antimicrobial Agents

3.1. Phenotipic Test - Agar Disc Diffusion Method

Taking into account the intrinsic or natural resistance of certain bacterial species, antibacterial agents of different pharmacological classes should be selected to be used in the phenotypic characterization of resistance by the qualitative disk diffusion method of Bauer, *et al.* [19].

Colonies bacterial on TSA agar (Tryptic Soy Agar) should be transferred to a test tube containing 3 mL of 0.85% sterile saline solution. The bacterial inoculum suspension should be adjusted to a concentration equivalent to 0.5 (1.5 x 10^8 CFU / mL) of the McFarland standard scale by turbidity visual comparison. So, a sterile swab should be introduced into the saline solution with the bacterial suspension, and the inoculum should be spread throughout the surface of the Mueller Hinton agar plate, aiming to obtain homogeneous growth.

Then the discs with antibacterial agents will be laid on the plate making a slight pressure to ensure the contact of the same with the surface of the culture medium. After the incubation period at 35°C ± 2°C for 24 hours, the diameters of bacterial growth inhibition (halo) around each disc should be measured and interpreted in accordance with the Clinical and Laboratory Standards Institute [20]. For control, reference strains should be used under the same conditions. All tests shall be performed in triplicate.

3.2 .Genotipic Test - PCR Analysis

Polymerase Chain Reaction (PCR) technology allows the detection and amplification of several specific regions of the genome of an infectious agent using different primers in a single reaction. The protocol to be used depends on the microorganism involved and the type of characteristic being investigated [21].

Briefly, bacterial DNA extraction and the use of a mixture of deoxynucleoside triphosphate, Taq DNA polymerase, Tris-HCl (pH 8.4), KCl, and $MgCl_2$, in addition to specific primers are required. The PCR reactions are carried out in a programmable thermal controller, programming the time and temperature of the cycles of amplification of denaturation, annealing, DNA extension and final extension steps. A positive result is indicated by the presence of an amplified DNA fragment, revealed by run in a electrophoresis chamber on 2% agarose gel, at specific voltage and time. The reference strains should be used as negative and positive controls [22].

4. Assays for Detecting Antibacterial Activity on New Derivatives

4.1. Agar Disc Diffusion Method

A tradicional method for screening antibacterial activity of the new substances or extracts is the disk diffusion method of Bauer, *et al.* [19]. It is based on the inhibition of bacterial growth. A 5 mm diameter sterile filter paper is impregnated with 3 µL of the stock solution of the diluted molecules (5 mg/mL) in dimethyl sulfoxide solvent (DMSO) and placed on the surface of the Mueller Hinton agar plate [23].

Reference strains and known antibiotics should be used as positive control for both Gram positive and negative inhibition evaluation [20] and disks with DMSO, as negative control. As the molecules, substances or extracts are unknown, any halo of inhibition detected indicates some susceptibility, thus the quantitative tests should be performed.

4.2. Broth Microdilution/Macrodilution (Tube) Methods

4.2.1. Determination of Minimum Inhibitory Concentration (MIC)

MIC determination can be performed by the broth serial microdilution method using sterile 96-well polystyrene microplates (BSMIM) or broth serial macrodilution (tube) method (BSMAM).

In BSMIM, each well of the microplate receives 100 µL of the Mueller Hinton broth culture medium, except for the first row, in which 200 µL of the culture medium is added with the molecule to be tested (1 mg/mL in DMSO). Then serial dilutions of ratio 2 are performed at decreasing concentrations from 512 µg/mL to 0.5 µg/mL, with the final volume of 100 µL in each well.

The bacterial inoculum can be prepared by direct colony suspension in 0.85% sterile saline solution until is reached the turbidity of 0.5 of the McFarland scale standard solution. Then it is diluted in sterile saline solution (1:10), and is added 5 µL of the bacterial suspension in each well to final bacterial concentration of 5 x 10^5 CFU/mL. The plates should be capped and incubated in an incubator at 35°C ± 2°C for 16 to 20 hours [20]. After that, 15 µL of 0.01% resazurin (7-hydroxy-3H-phenoxazine-3-one-10-oxide) diluted in sterile distilled water is added and the place is reincubated at 35°C ± 2°C for 4 hours. The MIC values are visually determined after revelation with resazurin, a blue color indicator that is oxidized, in the presence of viable cells, to resofurin, a reddish-colored substance indicating bacterial growth. MIC is defined as the lowest concentration of an antibacterial agent capable of completely inhibiting visible bacterial growth *in vitro* [20].

In this experiment, the culture medium with the bacterial suspension but without the unknown molecule, can be used as positive control, and the pure culture medium and the DMSO without the bacterial inoculum, as negative controls, as well as reference strains. All tests should be done in triplicate.

4.2.2. Determination of Minimum Bactericidal Concentration (MBC)

For the determination of MBC using BSMIM or BSMAM samples, 10 µL of the wells/tubes showing no visible bacterial growth, as well as the controls of the experiment are seeded on different plates containing Mueller Hinton agar, followed by incubation at 35°C ± 2°C for 18 hours.

After counting the number of colonies, MBC is established as the lowest concentration of antibacterial agent capable of eliminating 99.9% of the bacterial inoculum [24].

4.2.3. Detecting of Tolerance

The phenomenon of antibacterial tolerance is defined as the possibility that the bacterium may be sensitive to the MIC of the antibiotic, with the capacity to survive in the presence of the antibiotic, needing a highter MBC [25]. Tolerance can not be detected by usual qualitative susceptibility tests, nor by the determination of MIC only [26].

For the determination of the tolerance of the bacteria, the calculation of the MBC / MIC ratio should be performed. The tolerant bacteria show a rate equal to or above 32, so MBC values are five or more dilutions higher than that of MIC [27].

5. Toxicity and Pharmacokinetc in Silico Assays

Advances in bioinformatics have made it possible to screen toxicity *in silico*. By exploring the chemical properties of molecules in the computer, due to availability of a large virtual database of structural arrangements, we are able to compare the chemical structure of a molecule under study, performing the so called "virtual screening" [28].

Thus, the available computational methods allow planning and studying new drugs, optimizing the development of bioactive molecules. These *in silico* methodologies are used to prioritize and/or guide the selection of the most promising molecules to be further investigated [29]. In this context, *in silico* analysis for the prediction of ADMET properties (absorption, distribution, metabolism, excretion and toxicity) has been highlighted in the preclinical studies, being used as a complement to the *in vitro* and *in vivo* studies, with the advantage of the low cost and the ability to reduce the use of animals in toxicity trials [30].

5.1. ACD/Labs

Advanced Chemistry Development Inc. (ACD/Labs) presents a predictive approach, providing theoretical values of probability and occurrence of a property with prediction of reliable indexes. The program is available at http://www.acdlabs.com/resources/ilab/ and uses computational tools for comparison with similar fragments of more than 100,000 molecules previously characterized from carcinogenicity, chronic and acute toxicity studies with report of adverse effects on various organs.

5.2. Admetsar

It is a software that aims to analyze and predict parameters such as absorption, distribution, metabolism, and excretion. Its database contains 95,629 chemical substances, including FDA-approved drugs, literature experimental molecules, pesticides, environmental agents and industrial chemicals. This program is free access, and can be found on http://lmmd.ecust.edu.cn:8000. The results are represented in text and the reference for consultation is available on it.

5.3. LAZAR

Lazy structure-activity relationships is available on http://lazar.in-silico.de/predict and uses two databases: Carcinogenic Potency Database (CPDB) used for analysis of carcinogenicity and distributed structure searchable toxicity (DSStox) for carcinogenicity and mutagenicity. The mode of presentation of predictions is based on the quantitative structure-activity relationship (QSAR) and biological similarities, providing a confidence index of each prediction that can vary from 0 to 1.

5.4. Osiris Property Explorer

This program allows the theoretical evaluation of parameters related to toxicity risks, such as mutagenicity, tumorogenicity, irritant and reproductive effects, as well as molecular properties, such as the calculation of lipophilicity (cLogP), solubility (logS) and molecular weight. It also calculates Druglikeness (DL) and Drug-score (DS) values and is available on http://www.organic-chemistry.org/prog/peo. The intensity of the theoretical toxic risks of certain fragments is represented by a color gradation system, where the red color indicates high theoretical risk of undesirable effects, the yellow intermediate theoretical risk and the green, low theoretical risk.

5.5. Toxread

The program is free of charge, available on http://www.toxgate.eu/download.php and works with four toxicity rules: Benigni/Bossa, Sarpy, Irfan and CRS4, predicting mutagenicity. The program allows the choice of at least 3 similar molecules for comparison with the target molecule, being the analysis carried out by the fragmentation of the molecule into clusters. Parameters as degree of similarity that can range from 0 to 1 and experimental data are provided, as well as the result is expressed in figures and colors that indicate which grouping is mutagenic or not.

6. Toxicity Assays (*in vitro*)

There are several methods for evaluating the *in vitro* cytotoxicity of chemical agents using mammalian cell lineages, which would be less expensive to analyze a higher number of antimicrobial agents. The *in vitro* quantitative assays use different parameters to identify cell proliferation and death, defining the toxicity of the compound in a particular cell culture, due to the intrinsic ability of the compound to cause damage to cellular functions [31].

6.1. Hemolytic Activity

The in vitro hemolysis test determines the degree of lysis of erythrocytes and the release of hemoglobin, caused by the action of a certain substance, indicating the hemocompatibility profile.

The fresh blood use from a healthy adult donor who has not used any substances that may interfere with the experiment for at least 15 days, it should be collected in 3.2% citrated tubes, washing three times with 1.0 mL of 1X PBS (Phosphate Buffer Solution) and centrifuging at 2500 RPM for 10 minutes at each wash. After dilution of the blood into 5 mL of PBS, 594 µL of the diluted blood was withdrawn in previously prepared microtubes with 6 µL of the different concentrations of the agents to be tested, in addition to the controls (final concentration 1%) and PBS negative control. The hemolysis tubes should be incubated in a water bath at 37°C for 3 hours, followed by centrifugation at 2500 RPM for 10 minutes. After removal of the supernatant, 100 µL should be pipetted into the 96-well microplate. The release of hemoglobin is quantified by spectrophotometry at 545 nm [32].

6.2. MTT Test

The MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) colorimetric assay is based on the detection of the damage induced by the unknown molecule in the cellular metabolism. Thus when it evaluates the activity of mitochondrial dehydrogenases, the cell viability can be quantified by the reduction of MTT (yellow color salt) to formazan crystals (blue color salt) by cleavage by mitochondrial enzymes.

Cells cultured in sterile flasks using Dulbecco's Modified Eagle's Medium (DMEM) culture medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL of streptomycin, are kept in an incubator at 37°C with 5% carbon dioxide (CO_2). The culture medium should be changed every two days and 0.2 g/L of EDTA (Ethylenediamine Tetraacetic Acid) in PBS should be used after cell monolayer formation.

Subsequently, in sterile microplate with 96 flat bottom wells, 1 x 10^4 cells/well are incubated with the unknown molecules in two-fold serial dilutions according to MICs values. After 24 hours at 37°C in a 5% CO_2 chamber, the wells should be washed with 100µL of PBS (1mM), and added 100 µL of MTT (1mg/mL). The plate is reincubated for 1 hour at 37°C in a 5% CO_2. Then the MTT solution is removed, and 100 µL of DMSO is placed in each well [33]. The DMEM culture medium and fetal bovine serum alone are used as growth controls. The tests should be performed in triplicate.

The absorbance (in optical density) of formazan crystals using a spectrophotometer at 490 nm is direct related to the number of viable cells. Thus the average percentage of cell viability is calculated, for each molecule concentration.

According to the cell viability percentage, the classification of the molecule can be estimated using the cytotoxicity scale [34]. Viable cells (%) are also used to calculate the cytotoxic concentration (CC50), defined as the molecule concentration able to cause 50% lysis or cell death, by linear regression from a dose-response analysis.

5.3. Neutral Red Test

The neutral red assay is based on the absorption and binding of the cationic dye (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) to the lysosomal matrix of viable cells. In damaged cells the dye is not retained [35].

After cell monolayer formation in the 96-well microplate, different concentrations of the unknown molecule are added and incubated for 48 hours at 37°C. Then, 200 µL of the 0.033% neutral red solution should be added followed by incubation at 37°C for 2 hours in a 5% CO_2 chamber. Thus after removal of the solution and washing the cells with DMEM medium, dye is incorporated into the cells and analyzed after the addition of 100 µL of a solution containing 50% ethanol, 1% acetic acid and distilled water. The plate should be kept at room temperature for 10 minutes and then the optical densities were read in a spectrophotometer at 540 nm. The viability (%) should be calculated with the formula AT / AN X 100, where AT and AN are the absorbances of the treated and non treated cells, respectively. Non-toxic concentrations are those that lead to cell viability greater than 90% when compared to the control cells [34].

7. *In vitro* Analysis of the Effects on Bacterial Biofilm

7.1 - Microplate Adhesion Test

Initially, bacterial colonies are inoculated in 2 mL TSB (Tryptone Soy Broth) with 1% glucose, shaking at 37°C for 20 hours. Then, the diluted inoculum (1:100) must be incubated for 24 hours at 37°C with the unknown molecules substance extracts, in different concentrations (1/2, 1/4, 1/8 and 1/16 of the MIC value).

The reading should be performed on a microplate reader at 570 nm, which correspond to growth stage (Reading 1). Then the wells should be washed carefully with distilled water to avoid removal of cells. This cell layer must be fixed by heating at 65°C to 70°C for 1:30 hour until the plate is completely dry, followed by a new reading under the same conditions (Reading 2 - Dry plate). After drying, 200 µL of 1% violet crystal must be added to each well of the plate and after 1 minute, the dye is discarded and the microplate is rinsed again with running water. Then the plate should be heated at 65°C to 70°C for another 1:30 hour. The violet crystal is then dissolved in 200 µL of 70% alcohol and the optical density read (Reading 3 - Dissolved in alcohol). For dilutions, two new 96-well microplates are used, diluted in water (1:10) in each well (180 µL of distilled water + 20 µL of well contents) (Reading 4 - Diluted in water 1:10), and with the another dilution in water (1:20) of each well (190 µL of distilled water + 10 µL of the well contents) with a new reading (Reading 5 - Diluted in water 1:20). As positive control, a reference strain known as a major biofilm produce should be used, whereas as negative control, water [36].

8. Evaluation of Active Molecules in Associated Systems

The combination of two or more antimicrobials may be necessary for the treatment of mixed infections in which: a) not all microorganisms are sensitive to the same antibiotic; b) the combined treatment may be more effective against a micro-organism than the use of a single antibacterial agent; c) there is a possibility of using non-toxic amounts of two antibiotics; or d) in preventing or retarding the development of bacterial resistance to a drug [26].

The pharmacodynamic interaction of antibiotics may cause modification in their biochemical action, being able to cause similar effects or opposites [37]. Synergism can be verified with drugs that have the same mechanisms of action (additive), which act in different ways (summation) or with those acting on different pharmacological receptors (potentiation). Therapeutic or toxic effects may arise from synergistic associations [38].

8.1. Combination Disk Method

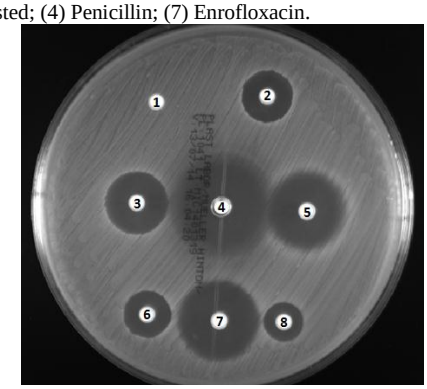
This method is used for the *in vitro* evaluation of the interaction of the active molecules associated with each other. It uses the same preparation of the discs, standard inoculum and Mueller-Hinton agar as the Kirby-Bauer susceptibility test [19, 23]. In the case of the combined disk, 1.5 µL aliquots of the stock solution of each of the molecules are added, totaling 3 µL.

This technique is based on comparing the halos of the bacterial growth inhibition produced by the molecules alone with that of both molecules. When the disc with the antibacterial combination exhibits halo greater than the sum of the halos of the isolated antibacterials, it demonstrates the additive or synergistic effect, whereas the smaller halo represents antagonism or competition [27].

8.2. Disk Approach/Double Diffusion Method

This method allows to detect of the interaction of the active molecules with the bacteria and the competition with the main commercially available antibacterials. The methodology followed the protocol already described in the previous item, but placing the disks in a distance in that must be equal to or slightly greater than the sum of the rays of the inhibition halos produced individually. In the non-interfering combination (Figure 03), the independent growth inhibition halos are formed but the antibacterial does not interfere with the action of the other molecule tested. In the synergic combination is observed increase or annealing of the zones of inhibition between the two molecules tested. The antagonistic effect of the combination shows the distortion of the halos at the interface of the zones of growth inhibition [27].

Figure-3. Growth inhibition halos of *S. aureus* ATCC 25923 obtained in the disc approach method: (1) DMSO negative control; (2) Vancomycin positive control; (3), (5), (7) and (8) Molecules tested; (4) Penicillin; (7) Enrofloxacin.



8.3. Checkerboard Method

This technique is based on the combination of two antimicrobial agents that can be carried out in test tubes or microtiter plates [20]. Double serial dilutions of each molecule tested are established including at least the 2X MIC value. Drug A is diluted in the microplate in serial concentrations vertically, while B is diluted horizontally. The protocol provides all possible combinations, from the highest concentration to the lowest of both molecules [27]. The following formulas were used to calculate the ΣFIC (the cumulative Fractional Inhibitory Concentration index): FIC of drug A = (MIC of drug A in combination)/(MIC of drug A alone), FIC of drug B = (MIC of drug B in combination)/(MIC of drug B alone) and ΣFIC = FIC of drug A + FIC of drug B. The results must be interpreted according to FIC indexes as follows: synergistic (ΣFIC: ≤0.5), additive (ΣFIC: >0.5 and ≤1), indifferent (ΣFIC: >1 and ≤4), and antagonistic (ΣFIC: >4) [39].

9. Conclusions

The selection of more promising compounds is of importance for the discovery of antibacterials. It is well known that thousands of molecules are abandoned throughout the search for new compounds due to inadequate toxicity and/or pharmacokinetics. However, chemical compounds with therapeutic potential can also be discarded out by errors in the execution of laboratory procedures or by the lack of knowledge of more accessible options to the availability of each laboratory. In this article we briefly described some techniques for searching prototypes promising in world biodiversity. Ultimately, it is expected that these strategies may contribute to the selection of the most appropriate *in vitro* and *in silico* techniques for each biological source. This select may allow the development of new investigations involving better therapeutic efficacy of the new drugs *in vivo*.

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