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Colorimetric microdilution assay: Validation of a standard method for determination of MIC, $IC_{50\%}$, and $IC_{90\%}$ of antimicrobial compounds

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ABSTRACT

The emergence of multiresistant bacteria directly impacts on the search for new compounds with antimicrobial activity, and it is important the improvement of new techniques are able to determine the minimum inhibitory concentration (MIC) of antimicrobial compounds. The microdilution technique is widely used for saving culture media, reagents and compounds to be tested. However, the literature does not describe a colorimetric method capable of correlating absorbance with concentration of viable microorganisms (CFU mL^{-1}). Therefore, the novelty of this work was the standardization and validation of a colorimetric and quantitative method capable of determining the MIC of several compounds with antimicrobial activity and the conversion of absorbance values to CFU mL^{-1}. The conditions carried out for the method were: the use of 0.125% (*w/v*) 2,3,5-triphenyltetrazolium chloride (TTC) solution added after 22 h of incubation at 35 °C, followed by 2 more hours of incubation and subsequent reading in a spectrophotometer. The tested microorganisms were: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). The method was validated and showed linearity ($R^2 > 0.95$), precision (RSD < 26%), accuracy (75% to 122%) and robustness ($p > 0.05$). The validated parameters ensured the harmonization of methodology to determine not only MIC as well as inhibitory concentrations of 50% ($IC_{50\%}$) and 90% ($IC_{90\%}$) of the antimicrobial compounds.

1. Introduction

Antibacterial drugs revolutionized medicine since the discovery of penicillin in 1928, and for > 60 years have been regarded as a cure for all diseases related to infections. However, the development of each new antibiotic was followed by the emergence of resistant microorganisms (WHO, 2014).

As a result, microbial resistance has become a global problem and a major threat to public health, especially in the case of multiresistant bacteria (Blaskovich, 2018; Salles et al., 2013; WHO, 2014). And it is noticed that since the 1980s, more precisely since 1987 there is no record of discovery of any new class of antimicrobial agents (Silver, 2011).

In view of the difficulty obtaining new compounds with antimicrobial activity, it is necessary to develop and optimize methods that assist in the identification of these substances from natural sources (Balouiri et al., 2016; Valgas et al., 2007).

Currently, several methods can be used to evaluate antimicrobial activity *in vitro*. Among them are the dilution methods, which cover macrodilution and microdilution, in which the substances to be tested are added to a liquid culture medium with a microorganism test. The determination of growth, after an appropriate incubation period, can be performed either directly by visual reading, or using spectrophotometry (Balouiri et al., 2016; CLSI, 2015).

The macrodilution and microdilution techniques are considered quantitative due to their ability to determine the minimum inhibitory concentration (MIC), which is the lowest concentration of antimicrobial agent able to visibly inhibit the microorganisms growth (Kuper et al., 2009).

There are several advantages in the use of microdilution, including sensitivity, reproducibility, convenience of having commercial plates prepared with antibiotics, economy of space and reagents, and the possibility to use automated reading systems to facilitate the generation of reports (Eloff, 1998; Jorgensen and Ferraro, 2009). Such advantages

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have lead to widespread use of this technique in the determination of the MIC of compounds with potential antimicrobial activity (Ayres et al., 2008; Bona et al., 2014; Eloff, 1998; Gabrielson et al., 2002; Morjan et al., 2015; Rahman et al., 2004; Silva et al., 2011).

Due to the existence of factors that may affect the sensitivity of the dilution method, it is necessary to know the experimental conditions and to make rigorous standardization in the execution of the tests (Ostrosky et al., 2008), because MIC can undergo variations according to the concentration of inoculum used, the culture medium and incubation conditions, in addition to the method of reading the results (Alves et al., 2008; Bidlas et al., 2008; Eloff, 1998; Kuper et al., 2009; Rios et al., 1988).

Among these factors, the most difficult one to standardize is reading the results, because the visual reading of turbidity can lead to an underestimation of bacterial growth. However, this issue has been solved with the use of growth indicators such as the tetrazolium salts, which are reduced in the presence of the microbial metabolism changing color and making reading easier. The 2,3,5-triphenyltetrazolium chloride (TTC) is a tetrazolium salt widely used in the MIC determination and is a colorless compound when solubilized with water, however in the presence of metabolically active bacteria is reduced to red-colored formazan which is directly proportional to the quantity of viable cells. Concomitantly, the readings in a spectrophotometer is also an auxiliary tool (Arthington-Skaggs et al., 2002; Gabrielson et al., 2002; Johnson et al., 1985; Moussa et al., 2013; Rios et al., 1988; Tengerdy et al., 1967).

In fact, a colorimetric method capable of correlating absorbance with viable microorganisms concentration (CFU mL^{-1}), is it not described in the literature. Such quantitative data can determine the inhibitory concentrations of 50% ($IC_{50\%}$) and 90% ($IC_{90\%}$) of the tested microorganisms and thus assist in determining the potency of antimicrobial compounds.

In this perspective, considering the need for identification of new compounds with antimicrobial activity and the necessary rigorous standardization of analytical methodologies for the evaluation of susceptibility tests, it is justified to validate a microdilution method that is effectively quantitative that is capable of providing CFU and allowing the determination of MIC and the potency of antimicrobial compounds against the following microorganisms: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231).

There is currently no colorimetric method described in the literature that proposes the conversion of absorbance values in CFU mL^{-1}. Therefore, the validation of this colorimetric method is novel and innovative adding a new contribution to the literature.

2. Material and methods

2.1. Microorganisms

Standard strains of the bacteria *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and the yeast *Candida albicans* (ATCC 10231) were used.

The inoculum preparation, with turbidity equivalent to 0.5 on the McFarland scale was performed from colonies with a period of growth between 20 and 22 h at 35 °C, in tryptone soya agar (TSA, OXOID®) for the bacteria, and Sabouraud dextrose agar (Sabouraud, BD®) for the yeast, which were transferred to tubes containing saline solution and the turbidity was adjusted using the densitometer DEN-1 Biosan®.

For quantification of microorganisms, serial dilutions were performed in saline (0.9%) and then aliquots of 1 mL of each dilution were plated in duplicate by pour plate method with TSA for the bacteria and Sabouraud agar for *C. albicans*. After incubation at 35 °C/24 h the results of the counting were expressed in CFU mL^{-1}.

2.2. Antimicrobial solutions

The solutions of chloramphenicol at concentrations of 3000 μg mL^{-1}, 1000 μg mL^{-1} and 100 μg mL^{-1} were prepared in 5% hydroalcoholic solution. The solutions of ketoconazole at concentrations of 100 μg mL^{-1} and 1000 μg mL^{-1} were prepared in 2% methanolic solutions.

2.3. Cellular viability solution indicator (2,3,5-triphenyltetrazolium chloride)

The 2,3,5-triphenyltetrazolium chloride (TTC) solutions at concentrations of 0.5%(*w/v*), 0.25% (w/v) and 0.125%(w/v) were prepared with sterile distilled water, filtered by sterilizing membrane (Polyethersulfone (PES) 0.20 μm Filtropur S - Sarstedt®) and stored in sterile flasks covered with aluminum paper under refrigeration (2 to 8 °C). The solutions were used for a maximum of 7 days after preparation.

2.4. Preliminary toxicity assay of the TTC before and after the incubation period

For each microorganism test, 2 sterile tubes were used in triplicate, containing each 1.5 mL of broth (Mueller Hinton for bacteria and Sabouraud for yeast) + 1.5 mL of saline + 300 μL of the microorganism suspension (0.5 MC Farland). In one of the tubes 600 μL of the TTC solution 5 mg mL^{-1} was added and both were homogenized and incubated 22 h for bacteria and 46 h for yeast. After incubation, the indicator solution was added to the tube that contained no TTC and both were incubated again for 2 h. After the total incubation time of 24 h for the bacteria and 48 h for the yeast, the visual reading of the tubes was performed.

Before and after a period of incubation quantification of microorganisms was performed by plate count.

2.5. Toxicity assay with different concentrations of TTC

For each microorganism, 5 sterile tubes were used in triplicate. To the first tube were added 4 mL of broth + 4 mL of saline solution + 800 μL of inoculum, and then an aliquot of 500 μL was withdrawn for counting of colonies. After 22 h of incubation at 35 °C, the tube was homogenized and, aliquots of 2 mL were transferred to the other four tubes. In three of these 400 μL of TTC solution was added in concentrations of 0.5% (*w/v*), 0.25% (w/v) and 0.125% (w/v). To the remaining content of the tube (control), 400 μL of sterile water was added.

The four tubes were incubated for 2 h and after this incubation time the dilution and counting of the CFU mL^{-1} present in each tube were performed.

2.6. Preliminary assays in tubes (macrodilution)

The tests for each microorganism were performed using 9 tubes labeled from “A” to “H”, to which were added 2 mL of Mueller Hinton broth for the growth of bacteria and Sabouraud broth for the growth of *C. albicans*. Then, 2 mL of the antimicrobial solution (100 μg mL^{-1}) was added (chloramphenicol for bacteria and ketoconazole for yeast) to tube A, and serial dilution was performed to obtain the following concentrations of antimicrobial agent in each of the tubes: 50 μg mL^{-1} (tube 1), 25 μg mL^{-1} (tube 2), 12.5 μg mL^{-1} (tube 3), 6.25 μg mL^{-1} (tube 4), 3.12 μg mL^{-1} (tube 5), 1.56 μg mL^{-1} (tube 6), 0.78 μg mL^{-1} (tube 7) and 0.39 μg mL^{-1} (tube 8).

After serial dilution, 2 mL was discarded from the contents of tube 8, and then 200 μL of standardized inoculum suspension was added to each tube. A blank tube was also prepared, containing 2 mL of broth + 2 mL of the solution of antimicrobial agent (100 μg mL^{-1}) + 400 μL

of saline. For the negative control tube 2 mL of broth +2 mL of the solution used to dissolve the antimicrobials was added (ethanol 5% or methanol 2%) + 400 µL of the suspension of microorganisms.

The tubes were incubated at 35 °C/22 h and 25 °C/46 h for bacteria and yeast respectively, after this period, 400 µL of indicator solution was added to test tubes and 800 µL to negative and blank control tubes. After further incubation for two hours, the readings were performed in a spectrophotometer at 540 nm followed by dilution and plating for quantification of microorganisms present in each test tube and in the control tube.

Tests were carried out with different indicator concentrations 0.5% (*w/v*), 0.25% (w/v) and 0.125% (w/v) to visually check the sensitivity of the TTC without the microbial growth, and a scan was performed in the visible range (400 to 800 nm).

Subsequently, due to the lack of inhibition of the proliferation of *P. aeruginosa* with the tested concentration (100 µg mL^{-1}) tests were carried out using the initial concentration of 3000 µg mL^{-1} of chloramphenicol. For the tests of *C. albicans*, due to the difficulty of growth of microorganisms and lack of development of red coloration, the incubation was performed at 35 °C/22 h and the initial concentration of ketoconazole was 1000 µg mL^{-1}.

2.7. Microdilution tests

The microdilution tests were performed in sterile round-bottomed 96-well microplates and different microplates were used for each microorganism.

100 µL of broth (Mueller Hinton for bacteria and Sabouraud broth for yeast) were added in all the wells of the plates. In the first well (line A) of each column (columns 1 to 11) was added 100 µL of antimicrobial solutions (100 µg mL^{-1} of chloramphenicol for *S. aureus* and *E. coli*; 1000 µg mL^{-1} of chloramphenicol for *P. aeruginosa*; and 1000 µg mL^{-1} of ketoconazole for *C. albicans*) and in column 12 was added 100 µL of hydroalcoholic or methanolic solution (vehicle in which the antimicrobial agent was diluted). Then serial dilutions were performed by passing 100 µL of wells 1 to 10 of the line A to the wells of line B and so forth, resulting in the following proportions: 1:2(A), 1:4(B), 1:8(C), 1:16(D), 1:32(E), 1:64(F), 1:128(G), 1:256(H). In each test hole (columns 1 to 10) 10 µL of the respective standardized inoculum was added (with turbidity equivalent to a 0.5 McFarland scale). In the blank wells (column 11) 10 µL of saline solution was added (0.9%). For the negative control (column 12) 10 µL of inoculum was added. The schematic representation of the methodology is presented in Fig. 1.

The plates were incubated at 35 °C for 22 h. After incubation, 20 µL of TTC solution 0.125% (*w/v*) was added into each well, and the plates were incubated again for 2 h. After the incubation, visual readings were performed by observing the development or not of red color originating from the reduction of TTC (colorless) to formazan (red), the lowest concentration was considered to be where there was no color development to determine the MIC. The readings were performed in microplate photometer (Multiskan™ FC) at 540 nm.

2.7.1. Validation of a standardized microdilution method

The validation of the method was performed according to the recommendations of the United States Pharmacopeia (USP, 2018) for the validation of alternative microbiological methods, being evaluated by the following parameters:

Linearity - After the microplates were read in a spectrophotometer, aliquots from 5 replicates of each concentration were diluted and plated in duplicate, and the results of the counts were expressed in CFU mL^{-1}.

For analysis of spectrophotometric readings results, the absorbances of all wells (test and negative controls) were first blanked by subtracting the absorbance value of the blank wells. Subsequently, the mean counts (CFU mL^{-1}) of 5 replicates of at least 5 different concentrations of antimicrobial were correlated in graphs with averages of absorbances obtained in a spectrophotometer in quintuplicate, in order to obtain the straight line equation of each microorganism, which allows the conversion of absorbances (y) in concentration of microorganisms (x), determination coefficient (R^2), and the linearity was evaluated through the analysis of this parameter.

Limit of detection and Quantification - Based on growth curves in order to evaluate the linearity the calculations of the limit of detection and the limit of quantification of the method were performed, in accordance with the ICH (2005), applying the following equations:

$$LD = \frac{3.3\,\sigma}{S} \quad LQ = \frac{10\,\sigma}{S.}$$

where:σ = standard deviation of linear coefficients of the curves of replicates$S$ = *linear coefficient of the final growth curve*

Precision - this was evaluated in two concentrations with 10 replicates, being the relative standard deviation (RSD) calculated by dividing the standard deviation by the average of the data and the result multiplied by 100. For the assessment of intermediate precision, the experiment was repeated on another day of work and the RSD was calculated by the standard deviation and average of 20 replicates (sum of two experiments).

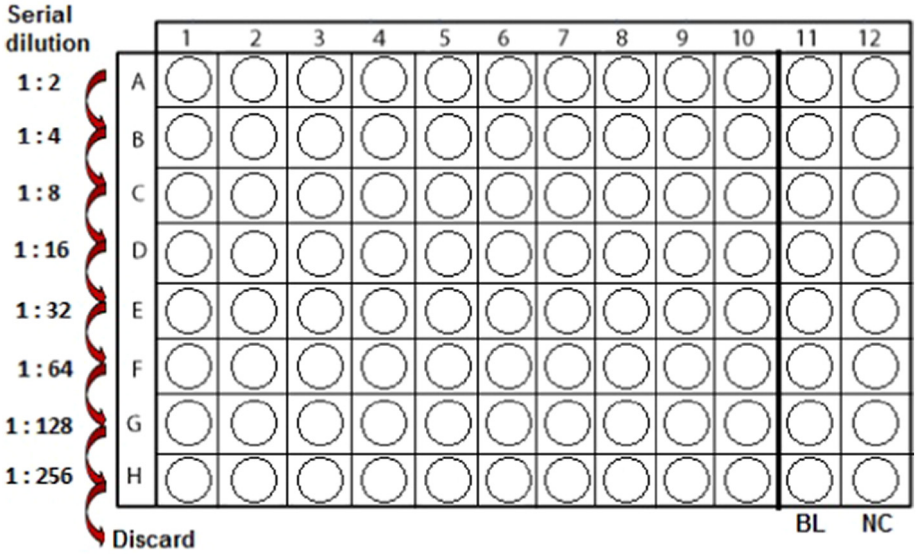

Fig. 1. Representation of serial dilution in 96-well plates. BL (Blanc); NC (Negative Control).

Accuracy - 5 concentrations were used within the working range for each microorganism with 5 replicates for each concentration. Calculations were performed using the equations of straight lines obtained for linearity of each microorganism and substituting the values of y by absorbance values of the readings in a spectrophotometer. The results of calculations were then compared with the practical values obtained by means of the CFU on plates and the percentage of recovery was verified.

Interval - The interval was selected considering the concentration of microorganisms that can be determined with precision, accuracy and linearity, observing the limit of quantification.

Specificity - it was assessed by development of color only in the presence of viable microorganisms from the reduction of TTC. The verification of the reduction or not of the TTC was performed with visual readings and in a spectrophotometer. Thus, the wave length used must be specific for the formazan color absorption originated from the TTC (540 nm).

Robustness - Controlled variations were performed in incubation temperature (30 °C and 33 °C), dilution of the inoculum used (1:2 and 1:10), and different incubation times before (18 h and 20 h) and after (4 h and 6 h) the addition of the TTC solution. Such variations were evaluated individually in 5 replicates to the standard method and for each pre-determined variation. After readings in a spectrophotometer, the values of absorbances of all the assays were blanked by subtracting the value of the absorbance of the blank wells, later converted into CFU mL^{-1}, applying the absorbance values in the equations of linearity of each microorganism. The results were analyzed by Analysis of Variance (ANOVA) in the software Statistica® following the model ONE-WAY ANOVA and Tukey test ($\alpha = 0.05$). Before performing statistical tests, the homogeneity of the replicates and the data normality were verified by Kolmogorov-Smirnov test.

2.7.2. Determination of the minimum inhibitory concentration (MIC) of antimicrobial compounds

The MIC determination was performed after application of the validated method, as described in the topic "2.7 Microdilution tests ", and two different methods of readings were then performed:

a) visual readings, being the latest concentration written down, in triplicate, in which there was no development of red coloration for each one of the microorganisms.
b) Spectrophotometric readings, being the values of absorbances blanked by subtracting the value of the absorbance of the blank wells, and then applied in the equations of straight (linearity) of each microorganism, determining the concentration of microorganisms (CFU mL^{-1}) present in each well. The collected data were presented in the form of bar charts with standard deviations and subsequently were statistically analyzed according to the model ONE-WAY ANOVA and Tukey test.

The MICs determined by visual readings were compared with the spectrophotometric readings by observation of graphics, as well as by means of the statistical analyses performed.

2.7.3. Determination of the inhibitory concentration of 50% ($IC_{50\%}$) and 90% ($IC_{90\%}$) of the microorganisms

At least 5 replicates of 5 concentrations of each microorganism was used. After applying the method and reading performance in a spectrophotometer, the absorbance values obtained were applied by straight lines equations, to check the linearity of the method, for each microorganism. The values of CFU mL^{-1} obtained were treated with logarithmic function and plotted in a scatter plot correlating the log of CFU mL^{-1} with concentrations of the tested antimicrobial agents.

To determine the $IC_{50\%}$ and $IC_{90\%}$, the average of the values of CFU mL^{-1} of negative control wells were multiplied by 0.5 and 0.1, in order to obtain the number of microorganisms corresponding to 50% and 10% of the total growth. The values were then transformed by logarithmic function and applied as a value of "x" in the straight line equations obtained from the correlation between the concentration of antimicrobials and the log CFU mL^{-1} of the microorganisms.

3. Results and discussion

3.1. Preliminary assays

TTC was chosen as cell viability indicator since it exhibited several advantages such as MTT and INT exhibited higher toxicity, and the resazurin and XTT undergo color alteration under reduction, when compared to four other colorimetric indicators of growth, 3-[4,5 dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium carboxanilide inner salt (XTT), 2-[4-iodophenyl-]3-[4-dinitrophenyl]-5-phenyltetrazolium chloride (INT) and resazurin (Alamar Blue), because the. The TTC, besides having lower cost than the XTT, being economically more viable, presents color only when reduced, which facilitates both visual reading and in spectrophotometer (Rahman et al., 2004).

Before these advantages, TTC was used widely as an aid in determining the antimicrobial activity of compounds, due to the fact that the development of coloring by microbial metabolism helps the visualization of the growth of microorganisms, facilitating the microdilution assay reading (Rahman et al., 2004; Tengerdy et al., 1967; Milenković et al., 2015; Morjan et al., 2015; Moussa et al., 2013).

However, as it can be seen in Table 1, some data compiled from the literature are presented demonstrating the lack of standardization regarding the concentration of TTC used, the incubation time before and after the addition of the indicator, as well as the type of reading used.

For the determination of the TTC concentration to be used in the tests, as well as the contact time of the TTC with microorganisms, preliminary tests of toxicity and sensitivity in tubes were carried out, in order to verify the influence of concentration on the growth of microorganisms as well as the development of coloration in the presence of viable organisms.

First an assay was performed to verify if the presence of TTC for a longer period would influence the growth of microorganisms. The

Table 1
Lack of standardization of the techniques of microdilution.

Author	Prior Incubation	TTC concentration (*w/v*)	TTC volume (% vol. total)	Subsequent Incubation	Reading
Silva et al., 2011	35 °C/ 24 h	0.50%	20 µL (20%)	1 h	Visual
Ayres et al., 2008	35 °C/ 18 h	0.50%	20 µL (20%)	3 h	Visual
Eloff, 1998	37 °C / overnight	0.20%	40 µL (40%)	10–30 min	Visual
RAHMAN et al., 2004	–	0.005%	–	37 °C/ 16 h	540 nm and Scanner
Gabrielson et al., 2002	–	0.01%	–	28 °C / overnight	540 nm and Scanner
Moussa et al., 2013	37 °C/ 3 h	0.50%	100 µL* (10%)	20 min	480 nm
Bona et al., 2014	35 °C/ 24 h	0.50%	20 µL (13%)	36 °C/ 3 h	Visual
Morjan et al., 2015	37 °C/ 24 h	0.50%	20 µL (20%)	–	Visual

Note: * Analysis performed with 1 mL and not in microplates as the others.

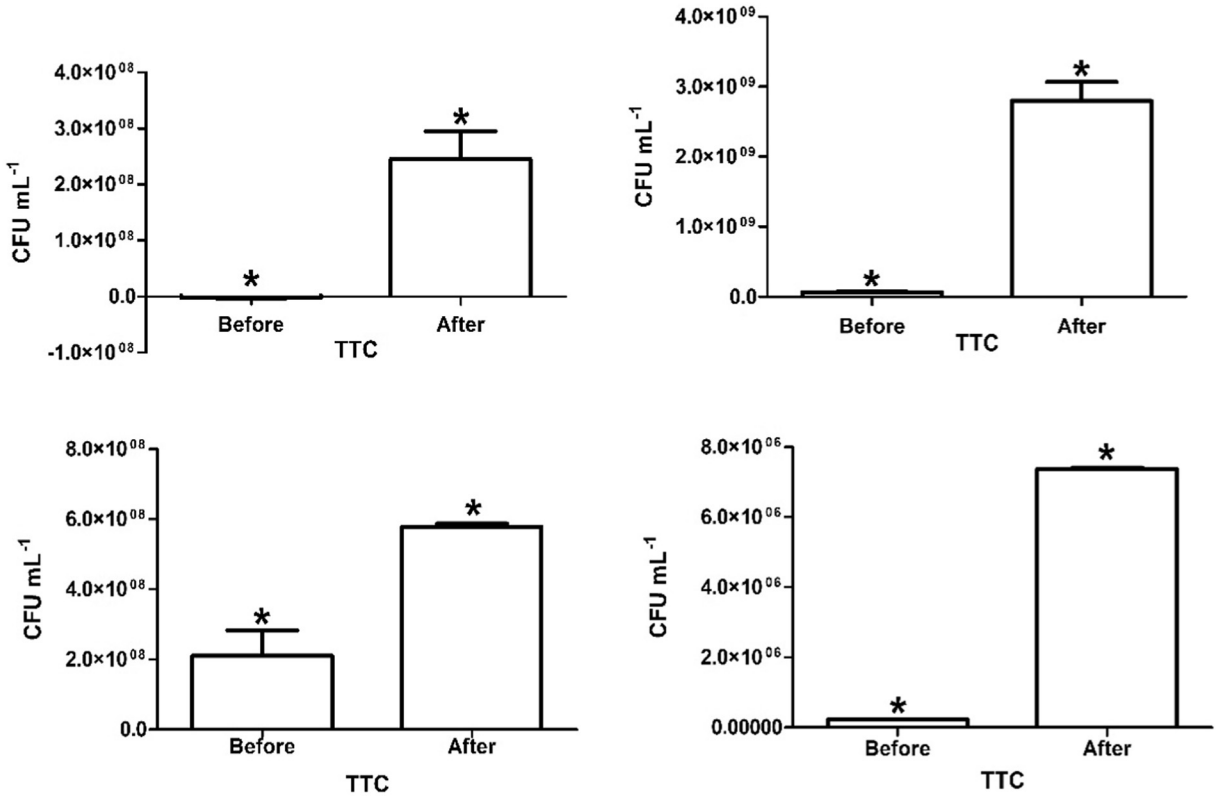

Fig. 2. - Growth of microorganisms in tubes with addition of TTC (0.5%) before previous incubation (24 h of contact with TTC) and after previous incubation of 22 h (plus 2 h of contact with TTC). A) *S. aureus;* B) *E. coli;* C) *P. aeruginosa;* D) *C. albicans*. *statistically significant between before and after.

concentration used for this test was 0.5% (5 mg mL^{-1}), which is the highest concentration used in accordance with a literature survey (Table 1). The results demonstrate that an incubation period of 24 h in the presence of TTC showed statistically significant ($p < 0.05$) toxicity when compared with a contact time of only 2 h with the previous incubation without TTC solution for 22 h. The results are presented in Fig. 2.

The results of cell growth or death presented in Fig. 2 were calculated as the difference between the number of CFU mL^{-1} present before and after the total period of incubation.

In this way, the addition of TTC should only be performed after a preliminary period of incubation for 22 h. The contact of the TTC with microorganisms for 2 h was enough for the development of red color visible to the naked eye. For this reason, different concentrations of TTC (0.5, 0.25, 0.125%) were tested to determine the lowest concentration capable of converting the TTC into colorful formazan without affecting the visual readings. According to Gabrielson et al. (2002), it is desirable to minimize the indicator concentration, because it may interfere with the bacterial growth, and interact with other components added for testing to the growth medium, as the compounds to be tested. However, an excess of growth indicator is necessary to ensure that this does not become a limiting factor in the development of color (Gabrielson et al., 2002).

The concentration of TTC chosen was the lowest concentration tested (0.125%), which showed no statistically significant toxicity ($p > 0.05$), as can be observed in Table 2, and it was able to develop visible color after 2 h of incubation in the presence of metabolically active organisms (Fig. 3). It gets close to the concentration used by Eloff (1998), who determined that the smallest effective concentration for the TTC was 2 mg mL^{-1} (0.2%). Rahman and colleagues (2004) defined that the concentration to be used in their assays should be 0.005%. However, the TTC solution was added to the plate previously and after the water evaporation, it was added to the culture medium that already contained the inoculum diluted, and the period of incubation in the presence of the TTC was much higher (16 h). It should also take into consideration that in this method only 20 μL of 0.125% TTC solution is added to a final volume of 130 μL (100 μL of broth with sample + 10 μL of inoculum + 20 μL of solution of TTC), thus in each well the final concentration of TTC is 0.019%, a concentration close to that used by Eloff (1998) but the contact time is much smaller.

Regarding the TTC sensitivity, images of tests performed in tubes are presented in Fig. 3, demonstrating that the use of TTC 0.125% does not harm the sensitivity of the method, and it is possible to determine the MIC of the antimicrobial compounds even at the lower concentration of TTC used. Because of this association of low toxicity and good sensitivity, this was the concentration of growth indicator chosen for the assays and validation of the method.

Taking into consideration that the TTC is reduced to formazan, from reddish to purple coloring, in the presence of viable microorganisms, scans were performed in a spectrophotometer in the visible range (400 to 800 nm) with the aim of determining the wavelength with adequate absorption to read of the tubes containing TTC in the presence of different concentrations of viable microorganisms. The wavelength of 540 nm was chosen on the basis of the results presented in Fig. 4 agreeing with the results presented in the literature of readings at this wavelength (Gabrielson et al., 2002; Rahman et al., 2004).

The coloring intensity could be perceived both visually and in the format of the spectrum obtained, because the tubes that have not developed red coloration due to the bacteriostatic activity of chloramphenicol showed an almost linear spectrum and near the base line, as can be seen in the three spectra shown in Fig. 4 (A, B and C). The tubes in which there was a growth of bacterial population and, therefore, reduction of the TTC, presented a spectrum with two bands of absorption characteristics which can also be observed in three spectra of Fig. 4 (A, B and C). For *C. albicans* it was not possible to visualize the characteristic bands of absorption shown in Fig. 4 (D). However, it was

Table 2
Evaluation of toxicity with different concentrations of TTC.

Microorganisms	TTC 0%	TTC 0.125%		TTC 0.25%		TTC 0.5%	
	CFU mL^{-1}	CFU mL^{-1}	*P* value	CFU mL^{-1}	*P* value	CFU mL^{-1}	P value
S. aureus	7.4×10^7	6.3×10^7	0.0570	5.7×10^7	0.0003*	4.8×10^7	0.0001*
E. coli	4.5×10^8	2.6×10^8	0.0850	1.8×10^8	**0.0350***	2.0×10^8	**0.0406***
P. aeruginosa	1.2×10^8	5.4×10^7	0.1080	1.9×10^7	**0.0044***	1.2×10^7	**0.0035***
C. albicans	7.0×10^6	7.0×10^6	0.9570	6.3×10^6	0.0960	5.4×10^6	**0.0150***

Note: * Values of $p < 0.05$ demonstrate a statistically significant compared to TTC 0% CFU mL^{-1}.

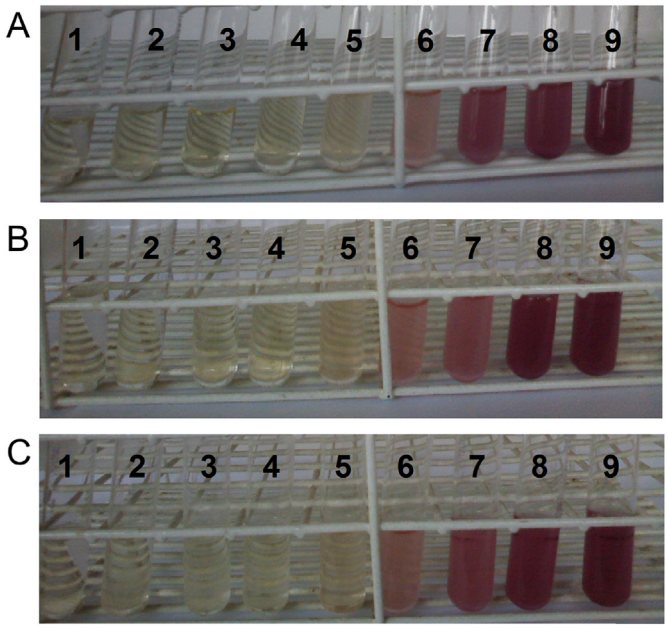

Fig. 3. Verification of sensitivity for different concentrations of TTC solution A) 0.5% (*w/v*); B) 0.25% (w/v); C) 0.125% (w/v), in different concentrations of antibiotics: Tubes 1) 50 μg mL^{-1}, 2) 25 μg mL^{-1}, 3) 12.5 μg mL^{-1}, 4) 6.25 μg mL^{-1}, 5) 3.12 μg mL^{-1}, 6) 1.56 μg mL^{-1}, 7) 0.78 μg mL^{-1} and 8) 0.39 μg mL^{-1}.

possible to show an increase in the coloring intensity due to the increased absorption of the spectra as the concentration of ketoconazole decreases.

According to Moussa et al. (2013), upon being reduced in the presence of bacteria, the TTC forms a compound of red color that is directly proportional to the quantity of active cells (Moussa et al., 2013). This fact was confirmed in view that the increase in absorption was proportional to the number of viable microorganisms and intensity in the red coloration.

3.2. Microdilution assays

The Microdilution assays were standardized and carried out so that the method could be validated.

The conditions set for the method were: the use of solutions of chloramphenicol at concentrations of 1000 μg mL^{-1} for the experiments with *P. aeruginosa* and 100 μg mL^{-1} for *E. coli* and *S. aureus*; solution of ketoconazole in the concentration of 1000 μg mL^{-1} for *C. albicans*; adding 10 μL of inoculum; incubation at 35 °C for 22 h, followed by the addition of 20 μL of TTC solution 0.125% and re-incubation for over 2 h, for both the bacteria and yeast, followed by readings. In Fig. 5 the experiments are presented which were also used for the validation of the method.

It is possible to observe in Fig. 5 (A, B and C) that the development of coloration is more intense for the bacteria when compared to the yeast *C. albicans* (Fig. 5 D). Even among the bacteria, it was possible to observe that the intensity of the reduction of the TTC was different. For this reason individual curves of correlation between the absorbance and the concentration (CFU mL^{-1}) were built for each tested microorganism.

After the visual readings, spectrophotometric readings (540 nm) were also performed, although Eloff (1998) argued that attempts to determine the turbidity of microcultures with microplate reader failed, bearing in mind that some microorganisms clustered at the bottom of the well and others remained in suspension (Eloff, 1998). More recent studies performed with readings in a spectrophotometer and scanner showed good results (Gabrielson et al., 2002; Rahman et al., 2004).

According to Gabrielson et al. (2002), the shape and the color of the pellet may vary according to the microorganism and the revelator used. For this reason, it is important to standardize the indicator to be used and to analyze separately each one of the microorganisms.

Moreover the precipitation of components present in the extracts of certain plants and even the green color of some extracts in high concentrations caused problems (Eloff, 1998). This is definitely a limitation of the method, considering that compounds that precipitate form emulsion, as for example oils that lead to the turbidity of the medium, may cause an erroneous interpretation of a false growth through an increase in absorbance. However, each case must be examined, because the use of control wells as “blank” can solve these problems in some situations.

In the cases described above, when even with the adjustment of the blank spectrophotometric reading is not reliable, it is still possible to determine the MIC of compounds through the visual reading, being affected only by the correlation between the absorbance and the number of CFU mL^{-1} present in each well. In the case of visual reading, it will not only be possible to effectively determine the MIC of compounds that have similar color to formazan resulting from the reduction of TTC.

3.2.1. Validation of the standardized method

3.2.1.1. Linearity. As observed in growth curves represented in Fig. 6, it is perceived that all microorganisms showed linearity, since the values of R^2 are > 0.95, as recommended by the United States Pharmacopeia (USP, 2018) for alternative microbiological methods. Where it is not possible to view 5 points separately, there is overlap of points on the first, because despite being different values the scale is too large and did not allow the differentiation between the first values that are much lower.

The equations of straight line depicted in Fig. 6, are of fundamental importance for the transformation of the values of absorbance in CFU mL^{-1} and therefore are used for the assessment of other validation parameters of the method, and to the determination of MIC and $IC_{50\%}$ and $IC_{90\%}$.

3.2.1.2. Specificity. The method is specific, with development of color occurring only in the presence of microorganisms that are reducers of TTC with active metabolic activity (Fig. 5). Because in the wells where there is growth inhibition of microorganisms resulting from the action of antimicrobial agents and in the wells of positive control there can be

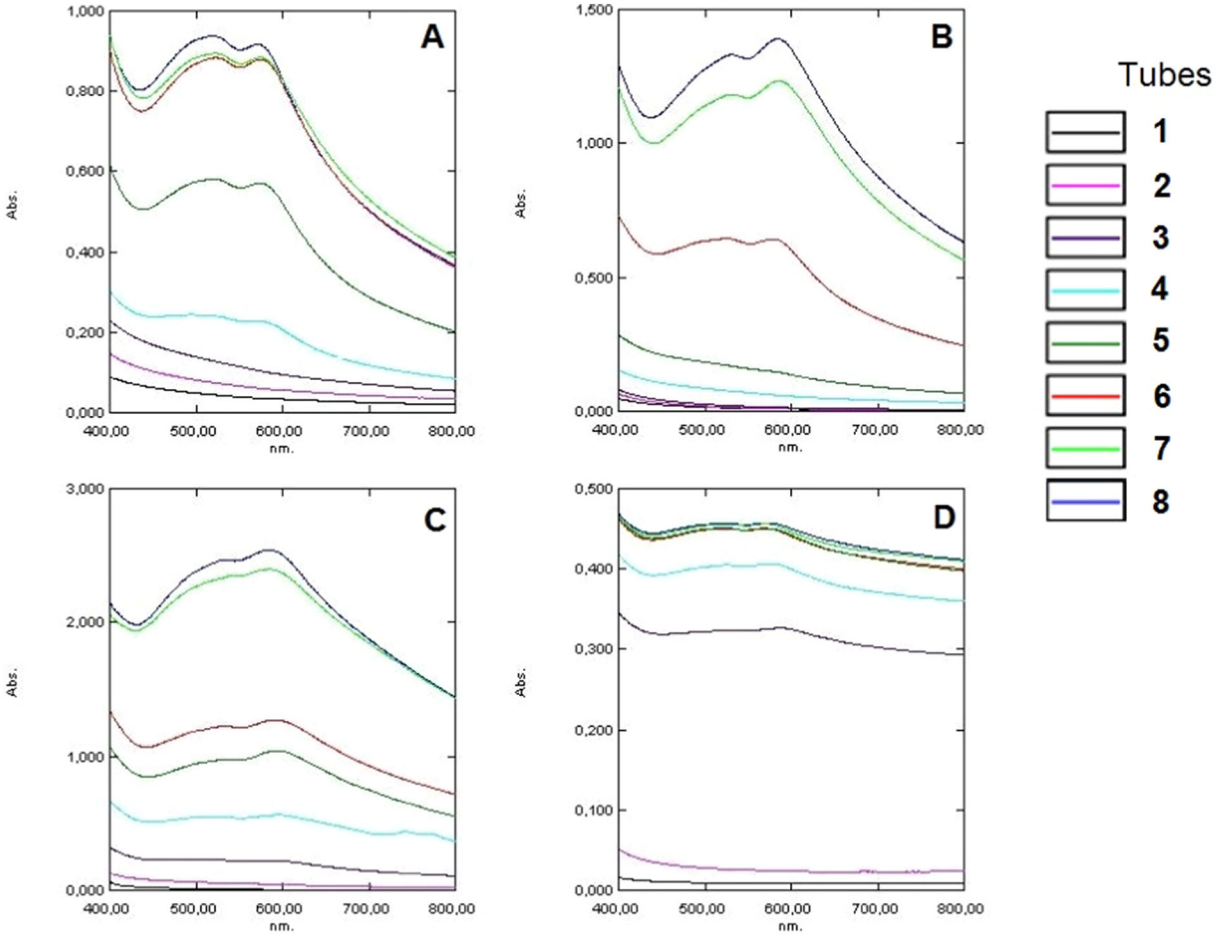

Fig. 4. Absorption spectra obtained by scanning (400-800 nm) of tests in tubes with TTC 0.125%: A) *S. aureus*; B) *E. coli*; C) *P. aeruginosa*; D) *C. albicans*.

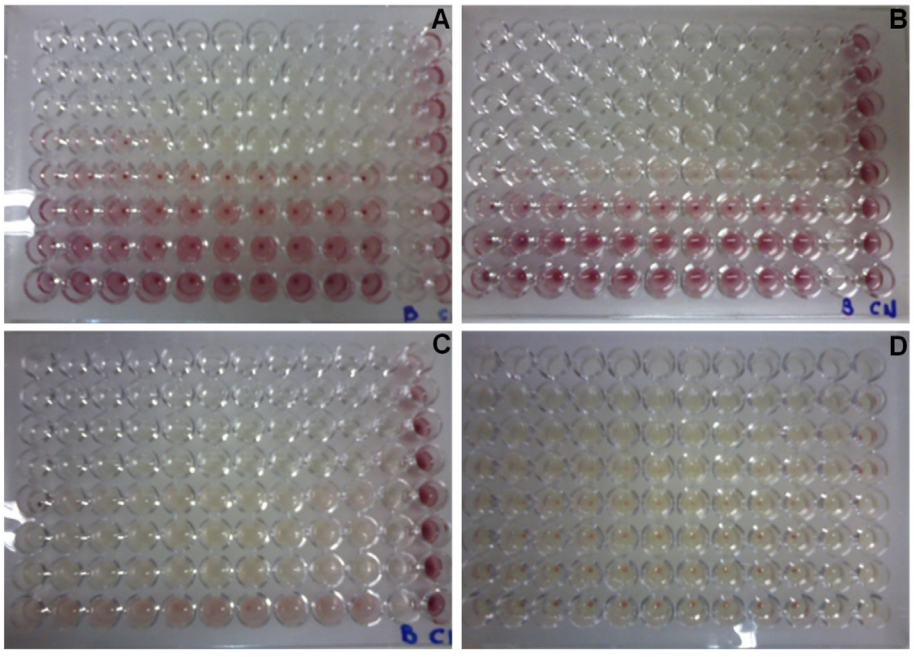

Fig. 5. Growth in microplate with gradient concentration of antimicrobials and with addition of TTC 0.125%: A) S. aureus; B) E. coli; C) P. aeruginosa; D) C. albicans.

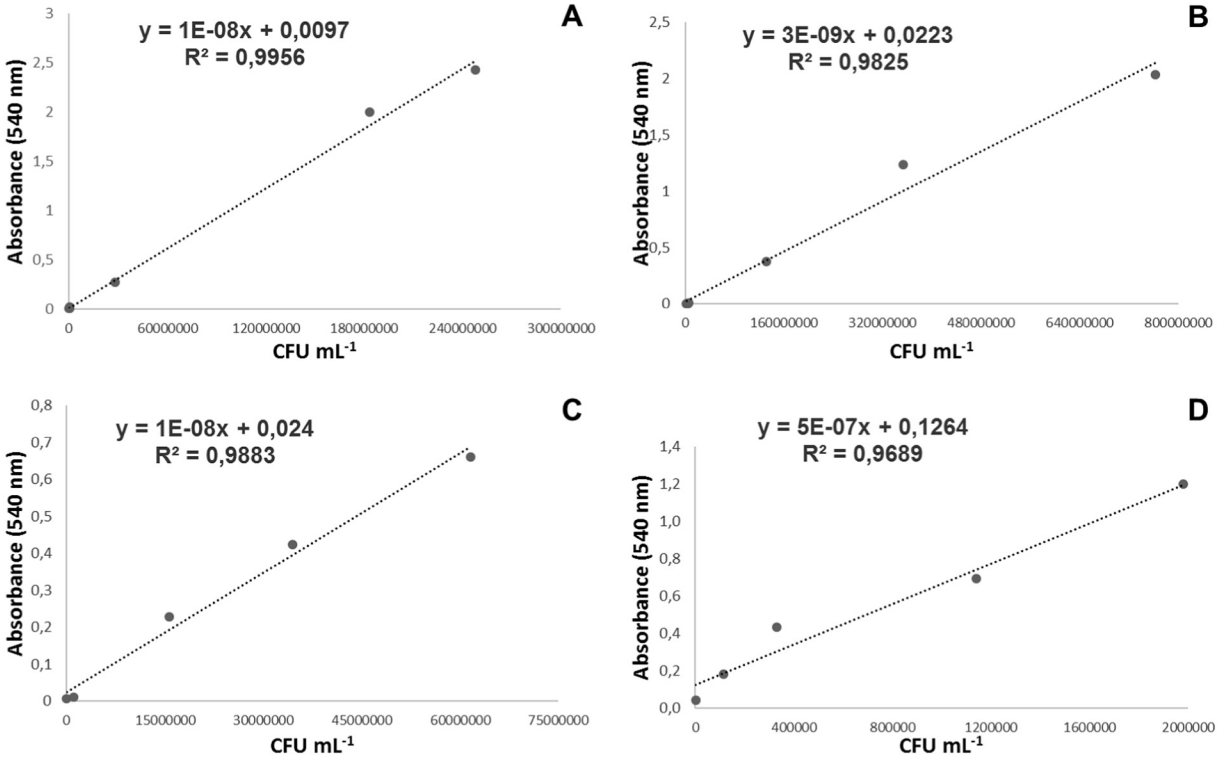

Fig. 6. Correlation among the readings in a spectrophotometer and the counts in plates: A) *S. aureus*; B) *E. coli*; C) *P. aeruginosa*; D) *C. albicans*.

Table 3
Results obtained for the precision assay.

Microorganisms	Antimicrobial* (μg mL^{-1})	Counts** (CFU mL^{-1})	RSD (%)
S. aureus	3.125	1.2×10^8	10.4
	0.78	1.9×10^8	5.9
E. coli	3.125	1.2×10^8	13.0
	0.78	6.7×10^8	10.1
P. aeruginosa	31.25	3.4×10^7	25.1
	7.8	3.3×10^7	25.3
C. albicans	31.25	1.2×10^6	10.8
	7.8	1.9×10^6	12.2

Note: * Refers to chloramphenicol for bacteria and ketoconazole for *C. albicans*. ** Mean of 10 replicates.

Table 4
Intermediate precision.

Microorganisms	Antimicrobial* (μg mL^{-1})	Counts** (CFU mL^{-1})	RSD (%)
S. aureus	3.125	1.0×10^8	20.3
	0.78	1.7×10^8	15.9
E. coli	3.125	1.5×10^8	22.5
	0.78	7.6×10^8	14.7
P. aeruginosa	31.25	3.4×10^7	23.1
	7.8	3.7×10^7	22.4
C. albicans	31.25	1.2×10^6	11.0
	7.8	1.9×10^6	12.1

Note: * Refers to chloramphenicol for bacteria and ketoconazole for *C. albicans*. ** Mean of 20 replicates.

no development of color, as well as, in the blank wells, where there are no microorganisms and, therefore, it is possible to state that there is no reduction in the TTC in consequence of other components present.

3.2.1.3. Precision. Precision indicates the degree of concordance among the results of individual tests to apply the procedure repeatedly. According to the American Pharmacopeia (USP, 2018), an interval between 15% and 35% in relative standard deviation (RSD) is considered acceptable. Thus, it is realized that the method presented precision for all the tested microorganisms, as shown in Table 3.

The highest RSD occurred for *P. aeruginosa* and this fact can be correlated to the tendency of this species to form biofilms. Therefore, the adhesion of these bacterial biofilm aggregates to the edges or the surface may cause interference in readings by a spectrophotometer and lead to a greater variability of results. However, despite a greater variation in experiments performed with *P. aeruginosa*, the method was precise, because the RSD was within the limits of 15–35% (USP, 2018).

The results of an evaluation of the intermediate precision of the method are presented in Table 4. The RSD obtained for the four microorganisms were lower than 30%, indicating that the method also has intermediate precision (USP, 2018).

3.2.1.4. Accuracy. Accuracy represents the closeness of the results obtained experimentally in relation to the results obtained by the traditional method and is usually expressed as a percentage of recovery of microorganisms and should be at 100 ± 30% (BRASIL, 2016; USP, 2018).

The percentages of recovery for *S. aureus* ranged from 75 to 113% and for *E. coli* from 85 to 114%, while for *P. aeruginosa* and *C. albicans*, the variation found was 90 to 122% and 100 to 121% respectively, as it can be seen in Table 5. Thus, the method is considered accurate for the four tested microorganisms.

3.2.1.5. Limit of detection (LD) and the limit of quantification (LQ). Table 6 shows the limits of detection and quantification defined respectively as the smallest number of microorganisms capable of being detected and determined with precision and accuracy under the established experimental conditions.

The limits of detection and quantification were determined based on growth curves using the equations recommended by ICH (2005), which were presented in Section 3 Materials and Methods.

The limits of detection and quantification are close among themselves for each microorganism, however, are high when compared to

Table 5
Evaluation of the Accuracy.

Microorganisms	Antimicrobial* (μg mL^{-1})	Counting plates (CFU mL^{-1})	Absorbance correlation* (CFU mL^{-1})	Recovery percentage (%)
S. aureus	12.5	6.1×10^5	6.9×10^5	113
	6.25	2.8×10^7	2.6×10^7	92
	3.12	1.7×10^8	1.3×10^8	75
	0.78	1.8×10^8	2.0×10^8	111
	0.39	2.5×10^8	2.4×10^8	97
E. coli	6.25	5.3×10^6	5.4×10^6	101
	3.12	1.3×10^8	1.2×10^8	95
	1.56	3.5×10^8	4.0×10^8	114
	0.78	7.6×10^8	7.0×10^8	92
	0.39	1.0×10^9	8.5×10^8	85
P. aeruginosa	62.5	1.0×10^6	9.2×10^5	91
	31.2	3.4×10^7	4.2×10^7	122
	15.6	2.6×10^7	2.3×10^7	90
	7.8	3.5×10^7	3.2×10^7	91
	3.9	6.2×10^7	6.6×10^7	107
C. albicans	125	1.1×10^5	1.1×10^5	101
	62.5	5.3×10^5	6.2×10^5	116
	31.2	8.5×10^5	1.0×10^6	121
	15.6	1.1×10^6	1.1×10^6	100
	3.9	2.0×10^6	2.1×10^6	108

Note: *Values obtained from correlation of the absorbance readings with the line equations of each organism.

Table 6
Limits of detection and quantification of the method.

Microorganisms	Limit of detection (CFU mL^{-1})	Limit of quantification (CFU mL^{-1})
S. aureus	1.7×10^7	5.3×10^7
E. coli	2.9×10^7	8.8×10^7
P. aeruginosa	2.0×10^6	6.1×10^6
C. albicans	2.4×10^5	7.2×10^5

the technique of counting in plates. However, this is not a failure of the method, but it occurs because in this method the initial concentration of microorganisms added is notably high, approximately 1.5×10^7 CFU mL^{-1} and, as a result of the use of chloramphenicol (bacteriostatic) and ketoconazole (fungistatic), the concentration of microorganisms detected in counting in plates must be similar to the number of microorganisms that were inoculated.

3.2.1.6. Interval. The intervals at which the method is applicable to the quantification of microorganisms tested are shown in Table 7. This parameter represents the range between the lowest and the highest concentration of microorganisms which have been determined with precision, accuracy and linearity.

3.2.1.7. Robustness. Robustness is expressed as the degree of reproducibility of results obtained with pre-established variations from normal conditions of the test. The results of the variations evaluated are shown in Table 8.

For all the microorganisms, the method did not endure great variations in dilution of the inoculum (1:10), as well as the addition anticipation of TTC in 4 h. However, for *P. aeruginosa,* there was no statistically significant difference for this last condition, and the *p* value was very close to 0.05 (0.073841), indicating a non-recommendation to add TTC in 4 h.

Table 7
Interval to which the method is applicable.

Microorganisms	Lower Limit (CFU mL^{-1})	Higher Limit (CFU mL^{-1})
S. aureus	5.3×10^7	2.5×10^8
E. coli	8.8×10^7	7.6×10^8
P. aeruginosa	6.1×10^6	6.2×10^7
C. albicans	7.2×10^5	2.0×10^6

For *S. aureus* in addition to the variations mentioned above, there was also a statistically significant difference with marked change of incubation temperature (30 °C). For *C. albicans* even the mildest dilution of the inoculum (1:2) led to divergent results of the standard method. Therefore, for *C. albicans,* the concentration of the inoculum is a critical factor.

Upon observing the method applied to four microorganisms it is possible to generalize that the method is robust and supports not major alterations in incubation temperature, which can vary from 30 to 35 °C (except for S. aureus, whose variation can occur between 33 and 35 °C). Small variations in the concentration of the inoculum were also well tolerated, except for *C. albicans*. Variations in advance of the time of addition of the TTC in 2 h were incurred for the four microorganisms, such as the delay in two hours in reading.

Regarding variations in the concentration of the inoculum, according to Eloff (1998), the inoculum size has a great effect on MIC values of compounds. Due to other reports in the literature about the influence of inoculum concentration on the determination of MIC (Alves et al., 2008; Bidlas et al., 2008), the importance of evaluating the variation of the concentration of the inoculum on the robustness of the method was defined. Thus, the results presented show that if the variation in the concentration of the inoculum is wide there are differences that can lead to modification of MIC.

3.3. Determination of MIC of antimicrobial compounds

The MICs were determined by visual readings, being considered the lowest concentrations where there was no development of red color. The results of visual readings were compared with the results obtained by spectrophotometric readings.

The results of spectrophotometer readings were interpreted after conversion of absorbances in CFU mL^{-1} applying the straight line equations shown in Fig. 6 and the determination of MICs was performed using statistical analysis. After checking the concentration that showed growth with significant statistical difference when compared to the holes where there was total inhibition of microbial growth, MIC was defined. The results are presented in Table 9 and Fig. 7 in the form of bar charts with standard deviations.

Observing the results presented, it was verified that the concordance of visual readings with the spectrophotometric readings statistically analyzed and also with the graphical presentation of the data (Fig. 7), demonstrating that, with the aid of TTC, the visual reading becomes reliable. However, it is important to emphasize that the visual reading only allows the determination of the MIC, being important to conduct data analysis, bearing in mind that with the naked eye, the red coloration may not be intense and may be difficult to visualize, although, by spectrophotometry it is easily detectable.

In addition, one should consider that the quantification of microorganisms is only possible from the spectrophotometric readings. Therefore, if the objective to use the method is to determine the number of microorganisms inhibited by the compound tested, or determine the $IC_{50\%}$ and $IC_{90\%}$, it is necessary to perform the spectrophotometric reading.

3.4. Determination of the inhibitory concentration of 50% ($IC_{50\%}$) and 90% ($IC_{90\%}$) of the microorganisms

To determine the $IC_{50\%}$ and $IC_{90\%}$ it was necessary to build curves of correlation between the concentration of microorganisms (CFU mL^{-1}) and the concentration of antimicrobial agent used. However, upon correlating directly the values of CFU mL^{-1}, the graph did not provided a straight line but a curve. After applying logarithmic function to the data, it was possible to obtain a straight line. The results and the

Table 8
Results of the evaluation of the robustness.

Variations		P value			
		S. aureus	*E. coli*	*P. aeruginosa*	*C. albicans*
1	T°C incubation period: 30 °C	**0.000142***	0.331557	0.996026	0.137579
2	T°C incubation period: 33 °C	0.820828	0.971823	0.856457	0.248811
3	Dilution of the inoculum: 1:2	0.449547	0.320429	0.353119	**0.001219***
4	Dilution of the inoculum: 1:10	**0.000657***	**0.000142***	**0.000207***	**0.000141***
5	Incubation period prior to adding the TTC: 18 h	**0.000241***	**0.038541***	0.073841	**0.036095***
6	Incubation period prior to adding the TTC: 20 h	0.981406	0.380264	0.999999	0.996181
7	Reading after 4 h of adding the TTC (5)	**0.000825***	0.571446	**0.022754***	0.409712
8	Reading after 4 h of adding the TTC (6)	1.000000	0.486054	0.967295	0.998954
9	Reading after 6 h of adding the TTC (5)	**0.038658***	0.941957	**0.005557***	0.821352

Note: * Values of $p < 0.05$ demonstrate a statistically significant difference.

Table 9
Determination of MIC for each antimicrobial.

Microorganism	MIC (μg mL^{-1}) visual reading	MIC ANOVA (*p* value*)
S. aureus	12.5	12.5 μg mL^{-1} (0.000204)
E. coli	6.25	6.25 μg mL^{-1} (0.000147)
P. aeruginosa	125	125 μg mL^{-1} (0.024426)
C. albicans	250	250 μg mL^{-1} (0.000149)

Note: MIC values correspond to chloramphenicol for bacteria and ketoconazole for *C. albicans*.

respective equations are shown in Fig. 8.

The values of CFU mL^{-1} obtained by conversion of absorbance values of negative control, applying the straight line equations for shown in Fig. 6, were multiplied by 0.5 and 0.1 to obtain the number of microorganisms corresponding to 50% and 10% of the total growth. Then these values were treated with the logarithmic function and applied to straight line equations, as shown in Fig. 8, to determined the $IC_{50\%}$ and $IC_{90\%}$, shown in Table 10.

4. Conclusion

The conditions established and validated were: the use of 100 μL of broth (Mueller Hinton for bacteria and Sabouraud broth for yeast) and 100 μL of antimicrobial solutions that were diluted serially. Then there was the addition of 10 μL of the respective standardized inoculum (with turbidity equivalent to a 0.5 McFarland scale) and after 22 h of incubation at 35 °C, 20 μL of the solution of 2,3,5-triphenyltetrazolium chloride (TTC) 0.125% (*w/v*) was added in all the wells, followed by 2 more hours of incubation and subsequent reading on a spectrophotometer (540 nm). Finally, the conversions of absorbances in CFU mL^{-1} were performed applying the equations of the line for each microorganism.

Concerning the use of TTC, this demonstrated efficacy upon improving the specificity of the method, and the concentration of 0.125% showed no significant toxicity to the tested microorganisms.

Considering that the validated method is linear, precise, accurate, robust and quantitative, it can be applied to the screening of antimicrobial compounds from plant extracts, synthetic extracts, bioactive molecules, and for the assessment of the potency of antimicrobial drugs

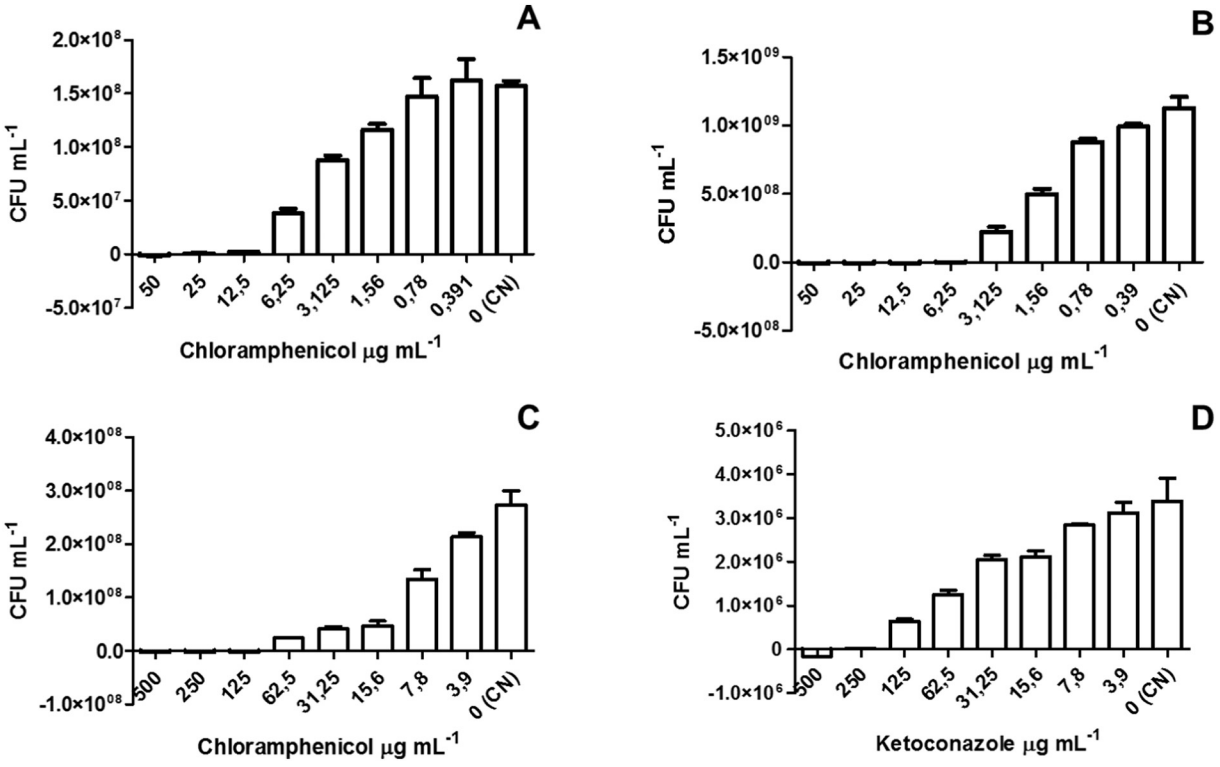

Fig. 7. MIC verification: A) *S. aureus;* B) *E. coli*; C) *P. aeruginosa*; D) *C. albicans*.

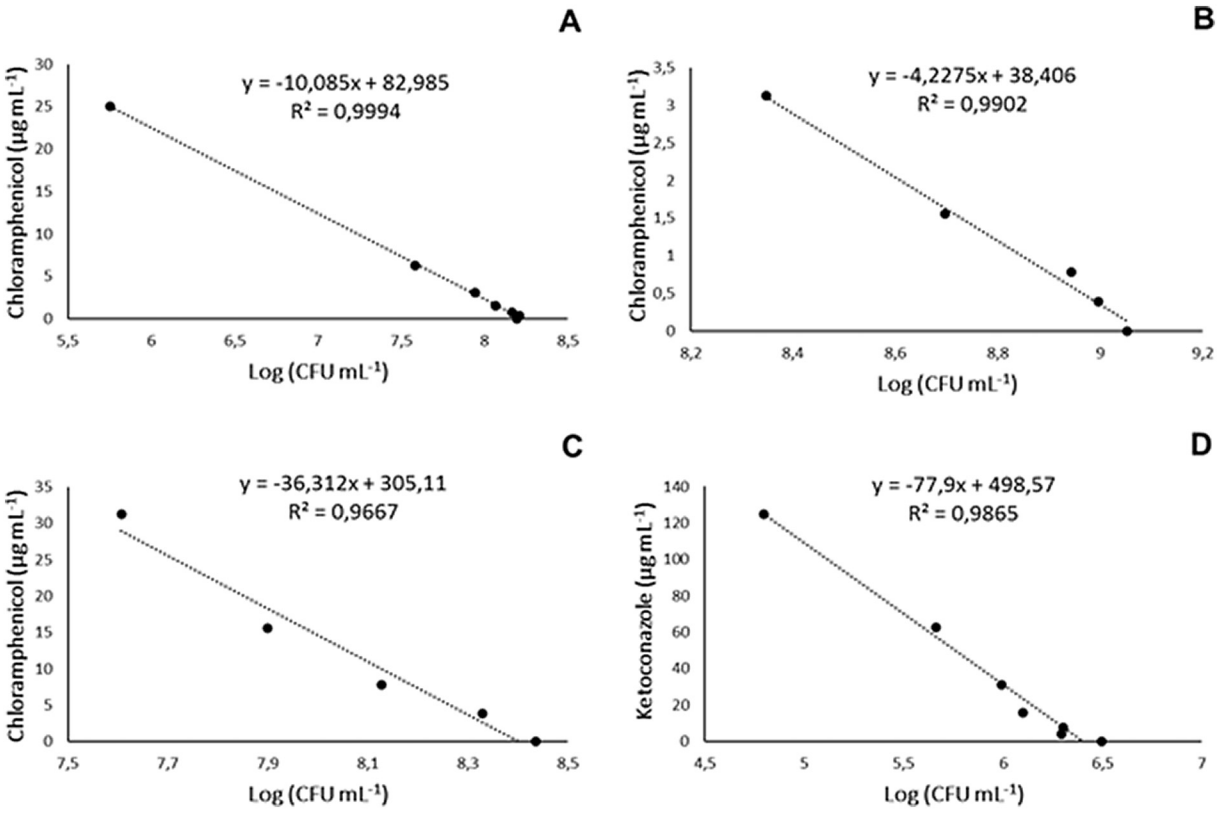

Fig. 8. Correlation between the concentration of tested antimicrobials and microorganisms: A) *S. aureus;* B) *E. coli*; C) *P. aeruginosa*; D) *C. albicans*.

Table 10
Determination of $IC_{50\%}$ and $IC_{90\%}$.

Microorganism	NC (CFU mL^{-1})	Log (NC x 0.5)	Log (NC x 0.1)	$IC_{50\%}$ µg mL^{-1}*	$IC_{90\%}$ µg mL^{-1}*
S. aureus	1.6×10^8	7.89	7.2	3.36	10.41
E. coli	1.1×10^9	8.75	8.05	1.41	4.36
P. aeruginosa	2.7×10^8	8.13	7.44	9.7	35.1
C. albicans	3.1×10^6	6.19	5.5	16	70.45

Note: *For the bacteria concentrations relate to chloramphenicol and to *C. albicans* refer to ketoconazole. NC = negative control.

in general.

It is a method of easy execution that provides a large amount of data at the same time due especially to the readings in a microplate photometer. It is economical due to the use of small quantities of culture medium and compound tested.

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