Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals

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Abstract

The resazurin assay utilising microtitre-plate, described by Drummond and Waigh in 2000, has been modified to achieve more accuracy in the determination of the minimum inhibitory concentration (MIC) values of natural products, including crude extracts, chromatographic fractions or purified compounds against various bacterial strains. This modified resazurin method is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products.

Keywords

Antibacterial assay; Resazurin; Plant extract; Serial dilution

1 Introduction

Antibiotics have revolutionised mankind’s health status, allowing treatment of life threatening infections. However with the increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotics. Most of the antibiotics available today come from natural origin, especially from various microbial or marine sources. Plants also produce compounds to protect themselves from microbial attacks. For screening natural products, e.g. crude extracts, chromatographic fractions or purified compounds for antibacterial activities, it is essential to employ an in vitro antibacterial assay that is simple, rapid, efficient, reliable, sensitive, safe and cost-effective. Moreover, most often the small quantities of natural products, especially purified compounds, that are available for antibacterial screening, can be a limiting factor in any viable screening programme. The conventional methods, e.g. disc diffusion method, may be time consuming and require significant quantities of the test materials, and there are also a few other problems associated with this method as discussed by Drummond and Waigh [3].
At the beginning of our research project, aimed at screening Scottish plants for antibacterial activities [1,2], the resazurin assay utilising a microtitre-plate as described by Drummond and Waigh in 2000 [3] seemed useful. This was because this method uses an indicator, resazurin, which allows the detection of microbial growth in extremely small volumes of solution in microtitre plates without the use of a spectrophotometer. However, it was soon realised that this published method incorporated changes in the concentration of both the test material as well as the bacterial suspension. Thus, although there is a serial dilution of the test materials, this method results in a decrease in the bacterial concentration serially, and as a consequence cannot be a ‘true’ indicator of the minimum inhibitory concentration (MIC) which is supposed to be assessed in this assay. Moreover, in a number of other antibacterial assays, the bacterial concentration is only approximate as they are compared to the Macfarland standard. This approach is subjective and results are often not reproducible in two different laboratories. To overcome these drawbacks, we have modified the resazurin assay, especially the dilution protocols, and utilised a standard concentration of bacterial suspension so that a ‘true’ MIC value can be obtained. By challenging the test materials with a standardised inoculum, reproducible and meaningful results can be obtained. We now present a detailed description of the modified resazurin method, which is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products.

2 Method

2.1 General

Incubator at 35 and 37 °C; pipettes of various sizes (Gilson); sterile tips, 100, 200, 500, and 1000 μL; 5 mL multichannel pipette (Fischer Supplies); centrifuge tubes (Fischer Supplies); vortex mixer (Fischer Supplies); centrifuge (Fisons); petridishes (Fischer Supplies); sterile universal bottles (Fischer Supplies); UV spectrophotometer (Shimadzu); sterile resazurin tablets (BDH Laboratory Supplies); sterile normal saline; sterile isosensitest broth (Oxoid); sterile isosensitest agar (Southern Group Laboratory, SGL); antibiotic solutions (Sigma–Aldrich); sterile solution of 10% (v/v) DMSO in water (Sigma–Aldrich).

2.2 Medium

Isosensitest medium was used throughout this assay, as it is pH buffered. Although NCCLS recommends the use of Mueller Hinton medium for susceptibility testing [4], the isosensitest medium had comparable results for most of the tested bacterial strains [5].

2.3 Use of standardised bacterial colony numbers

The method wherein turbidity is compared to Macfarland standards usually 0.5 is not able to give a standardised number of CFU for all strains, because this is operator driven and is thus subjective. It also makes it difficult to compare different bacterial species as they have differing optical densities. Thus to ensure that a uniform number of bacteria were always used, a set of graphs of killing/viability curves for each strain of bacterial species was prepared. A final concentration of $5 \times 10^5$ cfu/mL was adopted for this assay. Thus different strains and different bacterial species could be compared.

2.4 Preparation of bacterial culture

Using aseptic techniques a single colony was transferred into a 100 mL bottle of isosensitest broth, capped and placed in incubator overnight at 35 °C. After 12–18 h of incubation, using aseptic preparation and the aid of a centrifuge, a clean sample of bacteria was prepared. The broth was spun down using a centrifuge set at 4000 rpm for 5 min with appropriate aseptic precautions. The supernatant was discarded into an appropriately labelled contaminated waste beaker. The pellet was resuspended using 20 mL of sterile normal saline and centrifuged again.
at 4000 rpm for 5 min. This step was repeated until the supernatant was clear. The pellet was then suspended in 20 mL of sterile normal saline, and was labelled as Bs. The optical density of the Bs was recorded at 500 nm, and serial dilutions were carried out with appropriate aseptic techniques until the optical density was in the range of 0.5–1.0. The actual number of colony-forming units was calculated from the viability graph. The dilution factor needed was calculated and the dilution was carried out to obtain a concentration of $5 \times 10^6$ cfu/mL.

### 2.5 Preparation of resazurin solution

The resazurin solution was prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution.

### 2.6 Preparation of the plates

Plates were prepared under aseptic conditions. A sterile 96 well plate was labelled (Fig. 1). A volume of 100 μL of test material in 10% (v/v) DMSO or sterile water (usually a stock concentration of 1 mg/mL for purified compounds, and 10 mg/mL for crude extracts) was pipetted into the first row of the plate. To all other wells 50 μL of nutrient broth or normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use such that each well had 50 μL of the test material in serially descending concentrations. To each well 10 μL of resazurin indicator solution was added. Using a pipette 30 μL of 3.3× strength isosensitised broth was added to each well to ensure that the final volume was single strength of the nutrient broth. Finally, 10 μL of bacterial suspension ($5 \times 10^5$ cfu/mL) was added to each well to achieve a concentration of $5 \times 10^5$ cfu/mL. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each plate had a set of controls: a column with a broad-spectrum antibiotic as positive control (usually ciprofloxacin in serial dilution), a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 μL of nutrient broth instead (see Fig. 2). The plates were prepared in triplicate, and placed in an incubator set at 37 °C for 18–24 h. The colour change was then assessed visually. Any colour changes from purple to pink or colourless were recorded as positive (Fig. 1). The lowest concentration at which colour change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

### 3 Results

Resazurin is an oxidation–reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays [6]. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). A resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk [6,7].

The effectiveness of this modified resazurin assay has been demonstrated with methanol extracts of selected Scottish plants, purified compounds (Fig. 1), and the positive control ciprofloxacin (Table 1), and a direct comparison of the MIC determination of the antibiotics using the old and modified resazurin methods (Table 2) using the antibiotics norfloxacin, cefotaxime, and amoxicillin.

### 4 Concluding remarks

Choosing the correct assay to assess the antimicrobial potential of extracts and compounds is important for generating high-quality data with the greatest accuracy, speed and efficiency,
enabling the addition of potential new antimicrobial compounds and extracts to our armamentarium. This modified resazurin assay corrected the dilution inaccuracies, especially in relation to the MIC determinations, as described by Drummond and Waigh [3], and enabled results to be comparable for the test material for different bacterial strains. The method described here is easy to follow and accurate. The generation of an accurate MIC value, which can be compared to existing antibiotics, empowers the scientist with the knowledge to decide whether the extracts and compounds are worth pursuing further in terms of their antimicrobial potential.

References

Fig. 1.
Plates after 24 h in modified resazurin assay [pink colour indicates growth and blue means inhibition of growth; the test organism was *Staphylococcus aureus*; C₁, sterility control (test compound in serial dilution + broth + saline + indicator), no bacteria; C₂, control without drug (bacteria + broth + indicator); C₃, positive control (ciprofloxacin in serial dilution + broth + indicator + bacteria); A–D, Test compound/extract (in serial dilution in wells 1–12 + broth + indicator + bacteria)].
Fig. 2.
Structures of phytochemicals tested in the modified resazurin assay.
Table 1
MIC (mg/mL) determination using the modified resazurin assay

<table>
<thead>
<tr>
<th>Test materials</th>
<th>Bacterial strains</th>
<th>BC</th>
<th>CF</th>
<th>EC</th>
<th>ECP</th>
<th>KA</th>
<th>PM</th>
<th>PA</th>
<th>MRSA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achillea millefolium</td>
<td></td>
<td>—</td>
<td>—</td>
<td>2.5 × 10⁻¹</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5.0 × 10⁻¹</td>
<td>5.0 × 10⁻¹</td>
</tr>
<tr>
<td>Centaurea erythraea</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0 × 10⁻¹</td>
<td>1.0 × 10⁻¹</td>
</tr>
<tr>
<td>Chrysanthemum segetum</td>
<td></td>
<td>—</td>
<td>1.0 × 10⁻¹</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>5.0 × 10⁻¹</td>
</tr>
<tr>
<td>Euonymus europaeus</td>
<td></td>
<td>1.0 × 10⁻¹</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0 × 10⁻²</td>
<td>1.0 × 10⁻¹</td>
<td>1.0 × 10⁻¹</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Malva moschata</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0 × 10⁻²</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Genkawanin (4)</td>
<td></td>
<td>5.0 × 10⁻⁵</td>
<td>2.5 × 10⁻³</td>
<td>5.0 × 10⁻³</td>
<td>20.0 × 10⁻²</td>
<td>20.0 × 10⁻²</td>
<td>5.0 × 10⁻²</td>
<td>1.0 × 10⁻²</td>
<td>20.0 × 10⁻²</td>
<td>1.0 × 10⁻²</td>
</tr>
<tr>
<td>Imperatorin (4)</td>
<td></td>
<td>5.0 × 10⁻⁵</td>
<td>—</td>
<td>10.0 × 10⁻³</td>
<td>10.0 × 10⁻³</td>
<td>—</td>
<td>—</td>
<td>5.0 × 10⁻²</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isovitexin (2)</td>
<td></td>
<td>2.5 × 10⁻³</td>
<td>—</td>
<td>20.0 × 10⁻²</td>
<td>—</td>
<td>—</td>
<td>5.0 × 10⁻²</td>
<td>2.5 × 10⁻²</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Lappao A (3)</td>
<td></td>
<td>2.5 × 10⁻³</td>
<td>2.5 × 10⁻⁴</td>
<td>2.5 × 10⁻³</td>
<td>—</td>
<td>—</td>
<td>5.0 × 10⁻²</td>
<td>5.0 × 10⁻²</td>
<td>2.5 × 10⁻²</td>
<td>—</td>
</tr>
<tr>
<td>Oleuropein (5)</td>
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<td>—</td>
<td>—</td>
<td>5.0 × 10⁻²</td>
<td>—</td>
<td>2.5 × 10⁻²</td>
<td>1.0 × 10⁻³</td>
<td>20.0 × 10⁻²</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>2.5 × 10⁻⁸</td>
<td>2.5 × 10⁻⁷</td>
<td>2.5 × 10⁻⁷</td>
<td>2.5 × 10⁻⁶</td>
<td>2.5 × 10⁻⁶</td>
<td>2.5 × 10⁻⁷</td>
<td>2.5 × 10⁻¹⁰</td>
<td>2.5 × 10⁻⁵</td>
<td>2.5 × 10⁻⁰</td>
</tr>
</tbody>
</table>

BC, Bacillus cereus NCTC 9689; CF, Citrobacter freundii NCTC 9750; EC, Escherichia coli NCIMB 8110; ECP, Escherichia coli (penicillin resistant) NCIMB 4174; KA, Klebsiella aerogenes NCTC 9528; PM, Proteus mirabilis NCIMB 60; PA, Pseudomonas aeruginosa NCTC 6750; SA, Staphylococcus aureus NCTC 10788; MRSA, Staphylococcus aureus (MRSA) NCTC 11940.
### Table 2
Direct comparison of the MIC value determination of the antibiotics using the old (MIC-2) and modified resazurin methods (MIC-1)

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Gram stain</th>
<th>MIC values are in mg/mL</th>
<th>Cefotaxime</th>
<th>Norfloxacin</th>
<th>Amoxicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MIC-1</td>
<td>MIC-2</td>
<td>MIC-1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td></td>
<td>1.25 × 10⁻⁷</td>
<td>5.0 × 10⁻³</td>
<td>1.25 × 10⁻⁸</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td></td>
<td>5.0 × 10⁻⁷</td>
<td>2.0 × 10⁻⁵</td>
<td>5.0 × 10⁻⁸</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>+</td>
<td></td>
<td>5.0 × 10⁻⁷</td>
<td>2.5 × 10⁻⁵</td>
<td>5.0 × 10⁻⁸</td>
</tr>
<tr>
<td><em>Klebsiella aerogenes</em></td>
<td>_</td>
<td></td>
<td>5.0 × 10⁻⁶</td>
<td>5.0 × 10⁻⁵</td>
<td>5.0 × 10⁻⁸</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>_</td>
<td></td>
<td>5.0 × 10⁻⁷</td>
<td>2.5 × 10⁻⁶</td>
<td>5.0 × 10⁻⁷</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>_</td>
<td></td>
<td>1.25 × 10⁻⁷</td>
<td>1.25 × 10⁻⁵</td>
<td>5.0 × 10⁻⁸</td>
</tr>
</tbody>
</table>

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[^NCIMB]: NCIMB.
[^NCTC]: NCTC.