ANTIBACTERIAL SCREENING METHODS FOR EVALUATION OF NATURAL PRODUCTS

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Introduction

The use of natural products as therapeutic agents has become increasingly popular; however, research into the antimicrobial activity of these products has been hampered by the lack of standardized and reliable *in vitro* screening methods. The lack of standardized methods also makes direct comparison of results between studies impossible. The various methods used are disc diffusion, well diffusion, agar dilution and broth dilution.

Both the diffusion and dilution methods have been widely used for many years to accurately measure antibacterial activity and are routinely used in antibacterial susceptibility testing. It is important to note, however, that the substances normally tested by these methods are generally hydrophilic in nature and so the tests have been optimized to this condition (Janssen *et.al.*, 1987).

Among vast numbers of methodologies have been used relating to antimicrobial activity of natural products, the most popular methods employed are disc/well diffusion or the agar/broth dilution method, however, while many laboratories use the disc diffusion method, for example, most use different incubation times, different agar recipes and different volumes of test substance. Antimicrobial activity based on the broth dilution technique show enormous variations in technique, methodology and the choice of surfactants and solvents such as Tween 20, dimethylsulphoxide (DMSO) and ethanol (Flamini *et al*.,1999, Hammer *et al*., 1999).

Essential oils are volatile, insoluble in water, viscous and complex substances and the simple tests described above are inadequate for the antimicrobial testing of oil. There is, therefore, a need for a standard and reproducible method for assessing the antibacterial activity of the oils. Broth dilution method, using 0.02% Tween 80 to emulsify the oils, has been shown to be the most accurate method for testing the antimicrobial activity of the hydrophobic and viscous essential oils (Hood *et al*., 2003). When evaluated against a range of oils, this method provided the most reliable and correct results and allowed direct comparison of the antibacterial activity of the test oils, irrespective of viscosity and hydrophobicity.

The major difficulties associated with screening of natural products for antibacterial activity:

(1) The viscosity or hydrophobicity associated with essential oil creates difficulty in obtaining a stable dispersion of the oils in aqueous media.
(2) The problems associated with the diffusion of lipophilic oil components through agar
(3) The determination of the number of viable bacteria remaining after incubating with the oil.

Methods

Agar Absorption Assay

1. Allow 20 ml nutrient agar plates to dry in a 37°C incubator for 30 min.

2. Add 500 µl of the natural product (selected oil) was pipetted onto the surface of the agar and spread the oil over the surface of the agar plate using a sterile glass spreader.
3. Let the plates to dry leaving them upright at room temperature for 30 min. There should not be any visible oil on the surface.
4. Spread 500 µl of the selected bacteria over the essential oil treated nutrient agar plate.
5. Incubate the plates overnight at 37°C and count the bacterial colonies.

The agar absorption assay produce the least consistent and irreproducible results.

Agar Dilution Assay

1. Overlay 5 ml agar containing 2% essential oil, with and without 0.02% Tween 80, on 15 ml of molten nutrient agar plates.
2. Two control plates should be used, one with agar alone and another with agar plus 0.02% Tween 80 (final concentration).
3. Streak single colony of each bacterium onto the surface of the agar and incubate at 37°C overnight.
4. Bacterial growth can be measured on a scale of zero (no growth) to four (growth of control).

Incorporation of the oil into the agar, in the presence or absence of Tween 80, result in inconsistent growth of bacteria and therefore irreproducible results (Hood *et al*., 2003).

Disc Diffusion Assay

1. Take 20 ml dried (at 37°C for 30 min) nutrient agar plates.
2. Spread an overnight culture of bacteria (0.5 ml) over the surface of the agar plate using a sterile glass spreader and incubated at 37°C for a 30 min.
3. Add 10 µl of each oil on sterile 6 mm blank antimicrobial susceptibility discs.
4. Keep the oil impregnated discs onto the inoculated surface of the agar plate (maximum of five discs per plate).
5. The agar plates incubated overnight at 37°C and the zones of bacterial inhibition recorded.

This protocol can be modified to improve oil solubility as follows.

(a) 10 ml Tween 80 in sterile molten agar (final concentration Tween 80 0.02%, 0.1% and 1%) poured on to a 10 mL prepared nutrient agar plate. Susceptibility discs were then placed on the Tween 80/agar layer.
(b) Tween 80 (final concentration 0.5%, 1%, 2.4% or 5%) added to the essential oil prior to application to the susceptibility disc.

The concentration of Tween 80 that resulted in the largest zone of inhibition varied for each essential oil and there was significant variation in size of zone of inhibition with different concentrations of Tween 80 (Hood *et al*., 2003).

Well Diffusion Assay

1. Inoculate 19 ml of molten nutrient agar with 0.5 ml of overnight culture.
2. The inoculated agar then poured into petri plates and allowed to set.
3. Create 4 wells using a 6 mm cork borer.
4. Add 100 µl of either undiluted oil or nutrient broth into these wells, incubate the plates at 37°C overnight and record the zones of inhibition.

This method gives very inconsistent results.

Broth dilution assay:

1. The oil can be emulsified by adding 10 µl of 10% aqueous solution of Tween 80 to 90 µl of essential oil (1/10 volume) followed by vortexing.
2. Then add nutrient broth in 10-20 µl aliquots with brief vortexing to make the final volume 1.5ml
3. An overnight bacterial culture (500 µl) then added to each tube.
4. Three control tubes, a) 0.02% Tween 80 in nutrient broth, b) 100 µl of essential oil, 0.02% Tween 80 and nutrient broth and one containing only nutrient broth were also prepared.
5. Incubate the test tubes with shaking at 37°C for 12 h.
6. Then dilute each suspension serially (10-fold) with sterile nutrient broth to a final concentration of 10^{-7}.
7. Plate 500 µl of the 10^{-5}, 10^{-6} and 10^{-7} dilutions onto nutrient agar plates using an alcohol flamed glass spreader. The plates incubated overnight at 37°C and the bacterial colonies counted.

Tween 80 detergent has been reported to have various effects on bacteria at concentrations as low as 0.05%, 0.5% and 1%. The reported effects observed include a bacteriostatic action, inhibition of nucleic acid synthesis and alteration of fatty acid composition, respectively. Therefore, it was deemed essential that the concentration of Tween 80 in essential oil testing should be kept below 0.05% to minimize any adverse effects.

Among all the methods described the broth dilution method is becoming the most widely used method.

References:

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