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A Convenient Microdilution Method for Screening Natural Products Against Bacteria and Fungi

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Abstract

Candida albicans, *Cryptococcus neoformans*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (methicillin-susceptible and -resistant strains) are some of the most common pathogens of immunocompromised individuals. Since multi-drug resistance of these microorganisms is a major medical problem, we propose a convenient microdilution method for screening of natural products in a search for new antimicrobial agents that would be active against these organisms. To enable the screening process under standard laboratory conditions, *Cr. albidus* and *M. smegmatis* were used as model-organisms in place of their pathogenic counterparts. Antibiotics were used as positive controls, and their MIC values were in agreement with the MIC ranges recommended by the National Committee for Clinical Laboratory Standards. Organic plant extracts from *Lemna minor* and *Ilex cornuta* were used for developing the microdilution assay. The method can be used as a reliable tool for discovering antimicrobial agents with novel chemistry. It is adopted for organic extracts at a microgram scale and is currently being used for screens of more than one thousand extracts from the National Cancer Institute Open Repository.

Keywords: Antibacterial, antimycobacterial, antifungal activity, natural product screening, organic plant extracts, microdilution assay.

Introduction

Individuals with immune deficiency, caused by chemotherapy, HIV infection, or immune-suppression for organ transplantation, suffer from various bacterial and fungal diseases. In many cases, there are no effective treatments for these diseases. Before the AIDS pandemic, cryptococcosis was an unusual infection and now it may become incurable in the AIDS population (Davey et al., 1998; Jessup et al., 1998). Multi-drug resistant tuberculosis is another serious medical problem in immuno-compromised individuals (Drlica, 1996). In addition, the frequency of serious nosocomial bacterial and fungal infections is rising due to the use of newer and more powerful antimicrobial agents (Rodriguez-Tudela et al., 1996). As additional new antimicrobial agents are being found, microorganisms become more resistant to existing chemotherapies (Guiraud et al., 1999). Thus, there is a continuous need for novel antimicrobial compounds that would be effective against these and other pathogens.

There are many currently-available methods for antibacterial and antifungal testing and for determining minimal inhibitory concentrations (MICs) of various agents. Some of these methods include a disk-diffusion test, reference broth macrodilution methods [National Committee for Clinical Laboratory Standards (NCCLS) M7-A4 and M27-A], and their microdilution adaptations (NCCLS M7-A4, 1997; NCCLS 27-A, 1997). However, they are labor-intensive, time-consuming, require substantial laboratory space, generate large amounts of waste, and thus are not suited for routine screening of natural products for their antimicrobial activities. Recently, Eloff (1998) proposed a sensitive and quick microplate method to determine the MIC of plant extracts for bacteria. In this micro-dilution technique, tetrazolium salts were used to indicate growth of *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Several limitations of Eloff's method have been observed. We also investigated the use of this method for *Mycobacterium smegmatis*, *Candida albicans* and *Cryptococcus albidus*.

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In order to provide an optimized method for antimicrobial screening of natural products, we propose here a convenient micro-dilution method. For piloting assays, we used organic plant extracts from *Lemna minor* L. (Lemnaceae) and *Ilex cornuta* Lindl. (Aquifoliaceae) in our investigation. These species are virtually untested for the target organisms under study here. Previously, the medium in which *L. minor* was grown has shown a moderate activity against *Ca. albicans*, *Saccharomyces cerevisiae*, and some filamentous fungi (Porter, 1995). The panel of microorganisms included the most common bacterial and fungal pathogens of immunocompromised individuals. In addition to *Ca. albicans*, *P. aeruginosa*, *S. aureus* and methicillin-resistant *S. aureus* (MRSA), two model organisms, *Cr. albidus* and *M. smegmatis* were introduced. The pathogenic counterparts of these model organisms, *Cr. neoformans* and *M. tuberculosis*, respectively, were considered to be too hazardous for routine investigatory screening. There are no currently-available micro-dilution methods for natural product screening using these model organisms.

Materials and Methods

Microorganisms

The following organisms were used in this study: *Ca. albicans* ATCC 90028, *Cr. albidus* ATCC 34140, *M. smegmatis* ATCC 14468, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, and methicillin-resistant *S. aureus* ATCC 43300. The bacteria, except for *M. smegmatis*, were maintained and assayed in liquid Luria-Bertani medium (Atlas, 1997) and the fungi in yeast malt broth (Difco). Glycerol medium [4 g tryptic soy broth (Difco), 10 ml glycerol, 200 ml H_2O] was used for *M. smegmatis*.

Inoculum preparation

In general, 100 µl of a pure organism culture was added to 75 ml of an appropriate medium and a fresh organism culture was grown on a shaker under specific conditions (Table 1). The pure cultures were diluted with sterile water, to give the NCCLS recommended MIC values of the control antibiotics, as follows: *Ca. albicans* 1 : 10,000; *Cr. albidus* 1 : 500; *M. smegmatis* 1 : 10; *P. aeruginosa* 1 : 33, MRSA; *S. aureus* 1 : 50. The number of colony forming units (CFU) per single well on the 96-well plate was determined for each organism (Table 1). The cultures were then grown and diluted exactly under the same conditions each time an experiment was performed.

Antibacterial and antifungal drugs

Flucytosine and isoniazid were purchased from Aldrich, while amphotericin B, gentamycin, ketoconazole, oxacillin, and vancomycin were from Sigma. All drugs, except for amphotericin B and ketoconazole, were dissolved in water to obtain stock solutions of 200 µg/ml. Stock solutions of amphotericin B and ketoconazole were prepared in dimethyl sulfoxide (DMSO) at 0.8 mg/ml and 8 mg/ml concentrations, respectively. The solutions were filter sterilized using 0.22 µm nylon microfuge filters (Micron Separations Inc).

Extracts

Fresh *L. minor* plant material and *I. cornuta* bark, leaf, wood, and fruit materials were collected locally, dried, powdered, and extracted with $MeOH/CH_2Cl_2$ (1 : 1). The solvent was removed on a rotary evaporator. Standard extract solutions were prepared by resuspending the dried extracts in $MeOH/CH_2Cl_2$ (1 : 1) at 1 mg/ml concentration. Aliquots of 100 µg of a given extract were prepared on a 96-well polypropylene plate by placing 100 µl of the standard extract solution into each well and drying it in a centrifugal concentrator (Savant Speed-Vac). The plates were stored at –20 °C until use. Each 100 µg aliquot of dried organic plant extract was then reconstituted in 25 µl of DMSO.

Microdilution assays

The organic plant extracts were first screened at a concentration of 100 µg/ml. For every experiment, a sterility check (water, medium, and 2.5% DMSO), negative control (water, medium, 2.5% DMSO, and inoculum), and positive control (water, medium, inoculum, 2.5% DMSO and water-soluble

Table 1. Culture and bioassay conditions for the organisms in this study.

Organism	Final CFU, CFU/ml	Culture Growth Period, h	Culture Growth and Plate Incubation Temperature, °C	Plate Incubation Period, h
Ca. albicans	4×10^2	18	35	48
Cr. albidus	2.6×10^4	24	24	48
M. smegmatis	2.5×10^4	48	35	72
P. aeruginosa	1.3×10^7	18	35	18
MRSA	8.6×10^6	18	35	24
S. aureus	7.2×10^6	18	35	18

antibiotic, or DMSO-soluble antibiotic at 2.5% DMSO) were included. In general, the 96-well plates were prepared by dispensing into each well 95 µl of sterile H_2O, 5 µl of extract dissolved in DMSO, 80 µl of an appropriate medium, and 20 µl of the inoculum.

In order to determine the MIC values of the positive controls every time an experiment was performed, eight two-fold serial dilutions were performed. If the drug was initially dissolved in water, 100 µl of the liquid drug, 95 µl of H_2O, and 5 µl of pure DMSO were added and mixed in the first well. Then, 100 µl of the solution was used for downstream serial dilutions in seven consecutive wells, each already containing 100 µl of water. The last 100 µl were discarded. For DMSO-soluble drugs, 195 µl of water were added to the first well together with 5 µl of the drug, and the serial dilutions performed. The final volume in each well was 200 µl.

Contents of each well were thoroughly mixed with a multi-channel pipetter, and the microplates were incubated at temperatures and for periods of time appropriate to the organism under study (Table 1). Growth of the microorganisms was determined by absorbance at 750 nm on an automated microplate reader (SpectraMax Plus Spectrophotometer, Molecular Devices). Prior to taking spectrophotometer readings, contents of all wells were thoroughly mixed with a multichannel pipetter to resuspend clumped cells at the bottom of the wells in a solution. In the case of *P. aeruginosa* and *M. smegmatis*, highest reproducibility was achieved without the mixing step.

All of the extracts were first subjected to a single screen against each of the six organisms at 100 µg/ml. The activities of the extracts were confirmed in duplicate or triplicate and their MIC values determined as for the positive controls. The MIC values were considered as the lowest concentration of a drug or extract that does not completely inhibit the growth of the microorganism (the first well in which there is an observable growth of the organism).

The organic *L. minor* and *I. cornuta* extracts were also evaluated in a microdilution assay utilizing tetrazolium salts to indicate the bacterial growth (Eloff, 1998). The method was reproduced for *P. aeruginosa* and *S. aureus*, but not for MRSA. For the first time, we also attempted using *M. smegmatis* and the two yeasts in this method. The *p*-iodonitrotetrazolium violet was purchased from Sigma.

Results and Discussion

The MIC values of the positive controls for the non-model microorganisms were in agreement with those reported by NCCLS (NCCLS M7-A4, 1997; NCCLS 27-A, 1997) and other literature (Isenberg, 1992). The resistance of MRSA was confirmed by testing that strain with oxacillin. The MRSA was resistant to oxacillin (MIC 6.2–12.5 µg/ml) and gentamycin (MIC > 50 µg/ml). A strain of *S. aureus* is methicillin-resistant if the MIC value for oxacillin is greater than 4 µg/ml (NCCLS M7-A4, 1997) (Table 2).

The experimentally determined MIC values for the two model organisms, *C. albidus* and *M. smegmatis*, were compared with the MIC values listed for *C. neoformans* (Pfaller et al., 1995; NCCLS M27-A, 1997) and *M. tuberculosis* (McEvoy, 1999), respectively. There were substantial differences between the MIC values for the model organisms and their pathogenic counterparts. The isoniazid MIC value in tests against *M. smegmatis* was determined to be within

Table 2. Minimal Inhibitory Concentrations (µg/ml) of positive controls determined experimentally* and equivalent literature values for comparisons.

Positive Controls	*Ca. albicans*	*Cr. albidus*	*M. smegmatis*	*P. aeruginosa*	*S. aureus*	MRSA
Amphotericin B	0.2–2.5	50				
	(0.5–2)[a]	(0.25–1.0)[b]				
Flucytosine	0.8–1.6	>50				
	(0.5–2)	(1–4)[b]				
Ketoconazole	12.5	12.5				
	(0.03–16)	(0.03–16)[b]				
Isoniazid			0.8–1.6			
			(0.02–0.2)[c]			
Gentamycin				0.8	0.8–1.6	>50
				(1–4)	(0.12–1)	(≥16)
Oxacillin					0.1–0.2	6.2–12.5
					(0.12–0.5)	(≥4)
Vancomycin					0.8	0.8
					(0.5–2)	(0.5–2)

* values based on at least three experiments.

[a] values in parentheses are MIC values reported in the literature.

[b] literature value for *C. neoformans*.

[c] literature value for *M. tuberculosis*.

0.8–1.6 µg/ml range, whereas the reported MIC of isoniazid against *M. tuberculosis* was 0.02–0.2 µg/ml (McEvoy, 1999). The MIC values for amphotericin B and flucytosine against *Cr. albidus* were experimentally determined to be 50 and >50 µg/ml, respectively. In contrast, the MIC ranges of the same drugs listed for *C. neoformans* were 0.25–1.0 and 1–4 µg/ml, respectively (Pfaller et al., 1995; NCCLS M27-A, 1997). The only MIC value that was the same for both the pathogenic and model *Cryptococcus* strains was that of ketoconazole (12.5 µg/ml) (Table 2).

To determine the maximum volume of DMSO that could be used to dissolve solid extracts and that would not inhibit growth of the microorganisms, we investigated tolerance of all six organisms to DMSO. *Cr. albidus* was found to be the most sensitive organism and its growth was significantly inhibited by levels of DMSO higher than 2.5% of the well final volume (0.2 ml). Other organisms were able to tolerate up to 5% DMSO. Since the time and space efficiency of the bioassay could be greatly improved if all of the extracts were treated in the same manner when testing them against all of the six organisms, we decided to dissolve the organic extracts at concentrations that would provide a final bioassay concentration of 100 µg/ml, and at the same time would not exceed a final DMSO percentage of 2.5%.

Initially, we also encountered difficulty while trying to dissolve the organic extracts in 2.5% DMSO : H_2O mixture. Even at low concentrations (<500 µg/ml), the solid extracts were not completely soluble. When the extracts were first dissolved in pure DMSO, filter sterilized using 0.22 µm nylon microfuge filters, and then mixed with sterile water to make up 2.5% DMSO/water/extract solution, the extracts precipitated from the solution. To overcome this problem, 100 µg solid extract aliquots were prepared on a separate polypropylene 96-well plate. The extracts were then dissolved in pure DMSO at an appropriate concentration and added to 95 µl sterile water on a sterile bioassay 96-well plate. Occasionally, extracts appear mildly cloudy at the final 100 µg/ml bioassay concentration. As the extract concentration increased, the cloudiness became more pronounced. It is suggested that for crude screening purposes, besides reading the plates off on an automated microplate reader, the plates are also evaluated visually. It is also recommended that in order to avoid the solubility dilemma in determination of the final MIC values of extracts, the serial dilutions of extracts could be first performed in pure DMSO on a polypropylene plate and then appropriate extract/DMSO aliquots transferred onto a bioassay plate. In that way, one can avoid serial dilutions in water and thus reduce chances of precipitation of a solid extract. It has also been assumed, for crude screening purposes, that once the extracts are dissolved in pure DMSO, they are also sterilized, and thus a very costly and time-consuming step of filter sterilizing them was omitted.

It has been observed that the 96-well plate method is least reproducible for *M. smegmatis*. It is highly recommended that the plates are evaluated visually as well as on a microplate reader. Since *M. smegmatis* grows in large clumps of cells, even when its growth is inhibited, a false reading can be obtained on a microplate reader due to dead cells laying on the bottom of a well. A full growth of an organism can also be easily observed and monitored as a thick whitish layer of cells on the top of a liquid mixture in a 96-well plate well.

When we tried to test the positive controls and organic plant extracts using the tetrazolium salt microdilution method (Eloff, 1998), we encountered several problems. First of all, the method proposed by Eloff is suitable only for acetone soluble extracts, since the crude extractions were performed only with acetone. We extracted fresh plant materials with MeOH and CH_2Cl_2 in order to extract components with a wide spectrum of polarities. The majority of our organic (MeOH/CH_2Cl_2) extracts did not dissolve in acetone, and it was impossible to use our extraction solvent in this procedure, since methylene chloride was not compatible with sterile polystyrene plates. Secondly, we could only reproduce the tetrazolium salt method for *P. aeruginosa* and the MIC of gentamycin was determined to be 0.4 µg/ml. We could not reproduce the same method for *S. aureus* (ATCC 29213) as described by Eloff (1998). After *p*-iodonitrotetrazolium violet was added to the wells to observe bacterial growth, red color developed in all wells, and the MIC of gentamycin was >50 µg/ml. The method appeared not to be useful for *M. smegmatis* and the two yeast organisms. Since *M. smegmatis* is a very slow growing organism, it was impossible to determine if bacterial growth occurred after 10–30 min., as suggested by the author of the method. Even though we used two-day-old cultures for inoculation and incubated plates up to 48 hours after the tetrazolium dye was added, the color did not develop well. In the case of *Ca. albicans* and *Cr. albidus*, color did not develop after 10–30 min, nor after two days. In addition, determination of the exact MIC values of the positive controls and the extracts posed problems. After the color was developed, there was no clear end-point that would indicate growth or lack of growth of an organism. The color in wells appeared as less red, red, or more red, rather than as clear white (indicating lack of organism growth) and red (indicating organism growth). None of the investigated plant extracts exhibited significant activity using this method.

None of the *L. minor* and *I. cornuta* organic plant extracts exhibited significant activity against the six microorganisms. *I. cornuta* leaf extract was active against *M. smegmatis* at 100 µg/ml. The extracts were used mainly for method development purposes, and it was not expected that they would be active at these low concentrations. Currently, over 1000 organic plant extracts from the National Cancer Institute Open Repository are being screened for activities at ≤100 µg/ml concentration using our method. Several extracts have demonstrated significant activities (MIC < 10 µg/ml).

In conclusion, a convenient microdilution method for screening of natural products against bacteria and fungi was developed and optimized. Based upon our studies, we have

been able to apply the method, perform antimicrobial screening and MIC testing on large numbers of organic extracts, while working on a nanogram-to-microgram scale. Future work will involve high-throughput screening of various organic extracts from natural products. Extracts active against the model organisms will be further tested against their pathogenic counterparts. Chemistry of the most active and taxonomically unique extracts will be investigated through bioassay-guided fractionation in a search for structurally-novel compounds.

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