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Using alamarBlue® Cell Viability Assay to Screen for Putative Antimicrobial Products

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Abstract

The number of pathogens resistant to antibiotics in hospitals and communities is on the rise. The costs associated with the treatment and work loss run into billions of dollars annually. Our research is focused on discovering new compounds with antimicrobial properties. With that goal in mind we have screened 30 crude plant extracts obtained from a phytochemical company. The plant extracts were chosen based on the reactions shown against pathogens from the formal research to follow up in this research. We screened these extracts on a mini panel of BSL 1 pathogens (*Candida albicans, Aspergillus niger, Staphylococcus aureus, Staphylococcus epidermidis,* and *Escherichia coli*) in 96 well plates. The alamarBlue® cell viability assay was used to screen against the pathogens. Viability was determined by reaction of alamarBlue®, which changes the color from blue to pink/red. Crude extracts with minimum inhibitory concentration (MIC) at or < 256 μg/ml were prioritized for further study. Four extracts showed activity against both *Candida albicans* and *Aspergillus niger* and three extracts demonstrated activity against *Aspergillus niger* only. At this moment, we are not ready to reveal the name of plant extracts screened from this research since the further research on the extracts are still ongoing. The alamarBlue® viability assay is a cost-effective, quick and easy method to test for antimicrobial activity. The method can be easily utilized by any researcher interested in drug discovery and new antimicrobial products.

Keywords: alamarBlue®, Drug Discovery, Microbial Viability Assay, Natural Products

1. Introduction

The number of antibiotic resistant to bacteria in hospitals and the community is increasing, thus this is one of the issues threatening public health. Therefore, this leads to the need for new antibiotics. The current trend among big pharmaceutical companies is to focus on enhancing the effectiveness of existing antibiotics. According to a survey, 67% of all approved antibacterial compounds are either natural products or their derivatives[1,2]. We screened these extracts on a mini panel of biosafety level (BSL) 1 pathogens (*Candida albicans, Aspergillus niger, Staphylococcus aureus, Staphylococcus epidermidis,* and *Escherichia coli*) in 96 well plates using alamarBlue® cell viability assay. Viability was determined by the oxidation-reduction indicator, resazurin, which is contained in alamarBlue®. The indicator fluoresces and changes the color from blue to pink/red when the oxidized form of resazurin is reduced to resorufin by the cell metabolism[3,4]. The main idea of the use of alamarBlue® was to screen 30 crude plant extracts obtained from a phytochemical company against pathogen. The brighter the color has shown, the higher its cellular proliferation of the pathogen is after reaction with extract; if the cell growth was inhibited by extract, the color reagent would stay blue. After the extracts and reagent were added and incubated, the viability of the cell metabolism was determined by measuring minimum inhibitory concentration (MIC). alamarBlue® was selected as an indicator in this

research because the method can be easily utilized by any researcher interested in drug discovery and new antimicrobial products.

2. Methodology

2.1. Preparation For Cultures

Five organisms were selected to test the viability of the alamarBlue® assay: three bacteria (*Staphylococcus aureus, Staphylococcus epidermidis and Escherichia coli) and* two fungi *(Candida albicans* and *Aspergillus niger)*. The pathogens were cultured using Luria-Bertani (LB) medium which is most widely used for the growth of microbes. Each pathogen was transferred from glycerol stocks to the LB agar plates and incubated overnight, and then from the plates to the conical tubes with LB broth again. The cultures were incubated overnight at 30°C (for fungi) or 37°C (for bacteria).

2.2. Alarmarblue® Viability Assay Protocol

The liquid culture of pathogen, *Staphylococcus aureus,* was incubated with LB broth overnight before screening. The absorbance of the prepared liquid culture was adjusted to 0.125 optical density (OD) at 550 nm using a spectrophotometer. 30 crude plant extracts were alternatively labeled #1 to #30. Starting with 1024 µg/ml, the 10 different concentrations (1024, 512, 256, 128, 64, 32, 16, 8, 4, and 2 µg/ml) of the antimicrobial extracts were made with LB Broth using two-fold serial dilution method. Aliquots were distributed as follows: 180 µl of LB broth or sterile DI water (negative controls); 180 µl of prepared dilutions of each extract and 10 µl of the liquid cultures were added to each well. The dilutions were placed in order of highest (1st column) to lowest (10nd column) concentrations. Two controls were made; LB broth, pathogen, and plus color reagent (11nd column), and the color reagent only (12nd column). Four replicates were made for each extracts. The plate was incubated for two hours at the proper incubation temperature either 30 or 37℃ for the fungi and bacteria, respectively. A 10 µl aliquot of the color reagent alamarBlue® was added after the two hour incubation to each well[5] (figure 1). The plate was incubated for an additional hour to react with color reagent. MIC was measured. The rest of pathogens, *Staphylococcus epidermidis, Escherichia coli, Candida albicans* and *Aspergillus niger,* were tested with the same protocol.

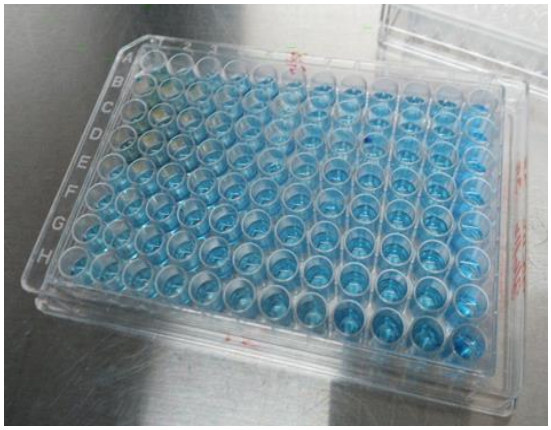

Figure 1. The original color of alamarBlue® with the dilutions of plant extracts before the reaction with pathogen.

3. Results and Data

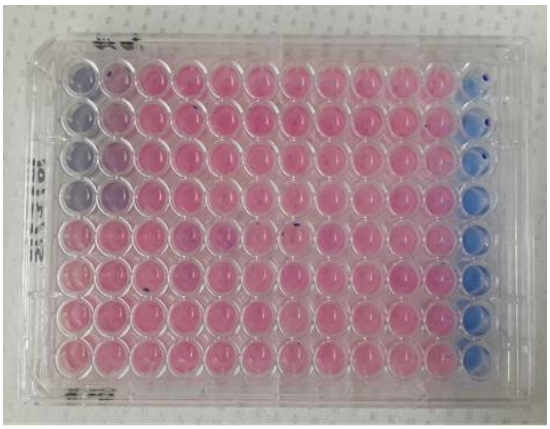

Figure 2. The 96 well plate of the extract #1 (top four rows) and #2 (bottom four rows) after the reaction with *S. aureus.*

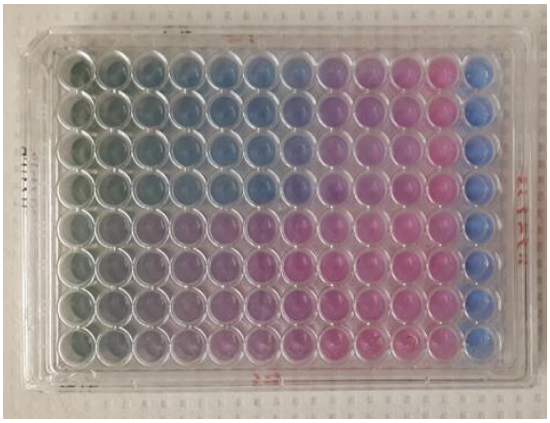

Figure 3. The 96 well plate of the extract #15 (top four rows) and #14 (bottom four rows) after the reaction with *A. niger*.

None of concentrations of the extracts, #1 and #2, did not work against *S. aureus* (figure 2). However, the plate of *A. niger* showed remarkable reactions with the extract #15 (top four rows) and #14 (bottom four rows). The MIC of the extract #15 was 16 μg/ml; the MIC of the extract #14 was 512 μg/ml (figure 3).

Table 1. MIC's of each extracts showed the reactions (at or < 512 μg/ml) against *A. niger* and *C. albicans.*

Pathogen	Extract	MIC (μg/ml)	Pathogen	Extract	MIC (μg/ml)
Aspergillus niger	#7	256	***Candida albicans***	#15	32
	#10	512		#16	256
	#14	512		#25	256
	#15	32		#26	512
	#16	256			
	#25	128(~64)			
	#26	256(~128)			
	#27	256(~128)			

Table 2. MIC's of each extracts showed the reactions (at or < 512 µg/ml) against *S. aureus* and *S. epidermidis*.

Pathogen	Extract	MIC (µg/ml)	Pathogen	Extract	MIC (µg/ml)
Staphylococcus aureus	#11	256	***Staphylococcus epidermidis***	#1	512
	#14	512		#4	256
	#15	32		#18	512
	#16	256		#29	512
	#25	512		#30	512

Table 3. MIC's of each extracts showed the reactions (at or < 512 µg/ml) against *Escherichia coli*; no activities of extracts observed.

pathogen	Extracts	MIC (µg/ml)
Escherichia coli	#1-30	No activities shown

The color changes were observed at the concentrations of a few extracts against the specific pathogens. Eight extracts worked against *A. niger;* four extracts worked against *C. albicans;* five extracts worked against *S. aureus*; five extracts worked against *S. aureus;* and no extracts showed reactions against *E. coli.*

4. Discussion

The color of the indicator was changed from blue (oxidized form) to pink (reduced form) if there were organisms metabolized under extract. This means the extract did not inhibit the growth of organism. If the color stayed blue, that means the organisms were inhibited to grow by the extract at the certain concentration because there was no organism left metabolizing. If the color appeared purple, which is the mixture of blue and pink colors, there are still organisms survived. Thus, the data of column with purple color was ignored for the more accurate value of MIC. Due to the original color of plant extracts, mostly green color, there may be different shades of initial blue colors when they were combined with alamarBlue®. In figure 2, the extracts did not work at any concentrations. The organisms were survived the extract with the reagent indicating the pink color. The highest concentration (first column) of the extract #1 also did not work because of its purple color of the indicator. In the figure 3, the concentrations of extract #15 (top four rows) showed that the MIC was 16 µg/ml because of the blue color at the column of the concentration at 16 µg/ml (6nd column from the left). At 8 µg/ml, it turned out to purple color which was ignored as possible MIC. The blue color is greenish at the first and second columns due to the original color of the plant extract. However, the MIC was measured at the very lower concentration, the two highest concentration can be disregarded from the possible MIC. The MIC of the extract #14 was measured with the same method, which was indicated at 512 µg/ml. Consequently, the extract, #15, showed the reactions at the lowest concentrations against *A. niger*, *C. albicans*, and *S. aureus*, and it will be the extract that we will follow-up as a priority. Interestingly, the data indicated that there are distinct reactions between gram-positive and gram-negative organisms; there was no reactions of any extracts against *E. coli* which is gram-negative organism.

5. Conclusion

The alamarBlue® viability assay is a cost-effective, quick and easy method to test antimicrobial activity. The method can be easily utilized by any researcher interested in drug discovery and new antimicrobial products. At this moment, we are not ready to reveal the name of plant extracts screened from this research since the further research on the extracts are still ongoing. Therefore, it is crucial to conduct further research such as additional fractionation, isolation and characterization to find out the compounds that each plant extracts contain. Also, biological activities will need

to be evaluated to identify the active compounds. We hope that this project will contribute to advancements in medical and pharmacological areas in most need of overcoming antimicrobial resistance.

6. Acknowledgements

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