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Molecular – Bioassay Methods: Complementary Approaches for Development and Evaluation of Anti Infective Marine Product

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ABSTRACT

The current trends in drug development employ biotechnological approach to expedite effective drugs discovery program. Molecular biotechnological approach, in combination with bioassay, is practical in attaining effective drugs since the two platform methods complement each other by target identification, as well as compound screening, profiling and validation. The research on antimicrobial properties of marine products, targeting membrane function through membrane permeabilizing ability, has been carried out using molecular- and cellular-based approaches. The molecular approach for the screening of membrane permeabilizing peptide gene in local marine organism was found to successfully amplify a conserved gene sequence of the antibacterial peptide gene. Bacterial membrane permeabilizing ability of the methanolic extract was indicated through alteration of mRNA nucleotides, genes coding for membrane development in *Staphylococcus aureus* (MRSA) and the non-methicillin resistant strains. The alteration of nucleotides affected the transportation of lysine to the phospholipid bilayer of bacterial membranes, resulting in incomplete membrane structure, eventual lysis and cell death. Through cellular approaches, the methanolic extract of marine organisms affecting membranes of *S. aureus,* were confirmed. In specific, the extract showed a good inhibitory activity against *S. aureus* through plate and tube methods, and the cellular assay illustrated the penetration of fluorescence dye in treated bacterial pathogens, similar to the pathogens treated with positive antibiotic controls. The research constitutes a scientific advancement in the field of medical treatment of drug resistant bacteria and a forefront study of drugs discovery program focusing on drugs target genes.

Keywords: Membrane permeabilizing, drugs target, antibacterial peptide gene

INTRODUCTION

The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from various alternative sources. The efforts can be futile if the rate of alternative agent discovery is not fast enough since pathogens keep improving on developing resistance mechanisms. New drug discovery approaches have to be amplified by searching the best infective targets in resistant pathogens, coupled with complementary anti-infective agent discovery, from diverse sources including natural marine products. Methicillin–resistant *Staphylococcus aureus* (MRSA) is a virulent organism which causes substantial infection-related morbidity and mortality in hospitalized patients. MRSA is now one of the most common causes of bacterial nosocomial infections, accounting for 40–70% of *S. aureus* infections in intensive care units (Zetola *et al.,* 2005). The emergence of high levels of penicillin resistance, followed by the development and spread of strain resistant to the semi-synthetic penicillin (methicillin, nafcillin, and oxacilin), macrolides, tetracyclines, and aminoglycoside, has made therapy of staphylococcal diseases a

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global challenge. The rate of MRSA infection is increasing and its treatment is not only expensive, but available drug of choice is toxic to human host. The aim of the current study was to validate the inhibitory activity of the MRSA and non-MRSA strains by methanolic marine extract from local marine resource. The validation tests, based on two approaches namely bioassay and molecular assay, were attempted. The validation of an active extract for antibacterial agent screening purpose generally involved an experimental demonstration that inactivation of a gene product leaves the bacterial cell non-viable.

MATERIALS AND METHODS

Methanolic Extract Inhibitory Determination

The antibacterial activity of methanolic extract, on MRSA and non-MRSA strains, was determined according to the methods outlined by Kirby-Bauer (1966) for antibacterial disc diffusion assay and minimum inhibitory concentration (MIC) assay using tube dilution method, followed by colonial growth determination of treated bacterial cells on Mueller Hinton Agar. The treatment controls were Luria Bertani broth with no extract, broth and discs with positive and negative antibiotics, respectively. Following the MIC dosages determination, test bacterial strains were grown in broth containing extract and antibiotics diluted in two folds.

Molecular Assay

Studies on active compound from marine resource were conducted to perform prediction of genes affected by these compounds. Primers for RT-PCR were manually designed based on the interest region from gene coding for the target genes obtained from the public domain MRSA database. Genes targeted are genes related to membrane development of MRSA.

Partial expression profiles of bacterial target genes after the treatment with extract and controls were determined using the RT-PCR analysis. Methods and reagents used were according to the manufacturer's instructions stated on the commercial kit (Master Pure, Alleights) with slight modifications. Total RNA were extracted from the treated and untreated MRSA on MIC's plate, using the RNA Extraction kit. The total RNA was then converted into cDNA using the Monster Script (Alleights), which was also according to the manufacturer's instruction for the first and second strands, before projecting it to the Reverse Transcriptase PCR (RT-PCR) using prior designed primers.

The PCR was carried out in a total volume of 25 µl based on the methods introduced by Maniatis (1989) with some modifications. Standard PCR amplification steps were based on Sambrook *et al.* (1989) with some modifications for thirty cycles. The RT-PCR product was run on 1.2% agarose gels, stained in 0.2 µg/ml of ethidium bromide and viewed under UV light (Alpha Imager™ 2200, Alpha Innotech). The bands of interest were extracted with QIAquick gel extraction kit (Qiagen) and sent for commercial sequencing.

Profiling

The sequence analyses were carried out using the DNAsis and Biology Workbench alignment to align the sequencing product for the treated, untreated MRSA and membrane genes from the database. The alignments were done to determine the nucleotide changes after the treatment of marine extract.

Bioassay Validation

Bacterial cells previously treated in two folds, diluted extract and antibiotic controls were subjected to fluorescence dye assay to determine the cellular penetration of the extract and antibiotics. Bacterial cells (0.5 Mc Farland), antibiotics or marine organism extract and SYTOX green dye (5 µM) were combined and dispensed into black micro plate at 100 µl/well. The black micro plate was incubated at 37°C and the fluorescence intensities were measured with the fluorescence multi-well plate reader (SpectraMAX GeminiXS), at an excitation wavelength 485 nm and emission wavelength of 530 nm for every 30 minutes up to 6 hours of treatment.

RESULTS

Methanolic Extract Inhibitory Determination

The minimum inhibitory concentration (MIC) of the marine methanolic extract was indicated as 10.2 mg/ml for *S. aureus* ATCC 29247 and 12.8 mg/ml for *S. aureus* ATCC 700698 (*Fig. 1*).

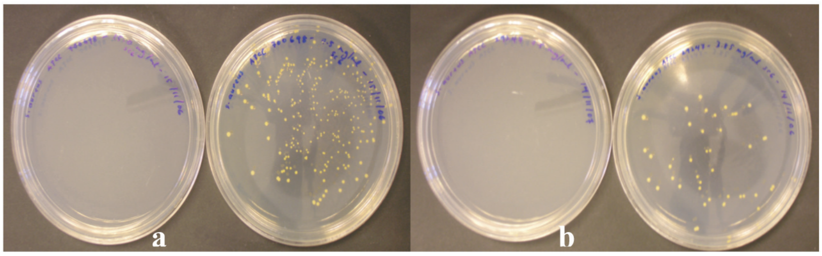

Fig. 1: Minimal inhibitory concentration of MRSA treated with marine organism extract (a) ATCC 700698; (b) ATCC 29247

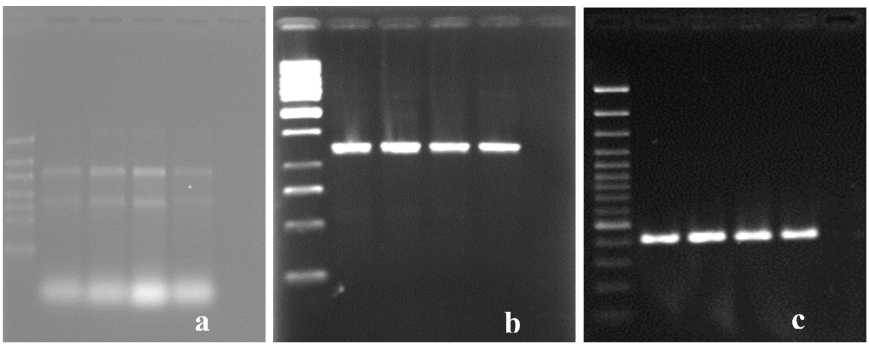

Fig. 2: a) RNA band of treated and untreated MRSA and non MRSA reference strain, ATCC 700698 and ATCC 29247; b) Amplification of a band from extracted RNAs at a size of 1241bp; c) Amplification of a band from extracted RNAs at a size of 406 bp

Molecular Assay

RT - PCR resulted in a single amplification band at 1241 bp and 406 bp respectively, for both strains of ATCC 29247 and 700698 which were treated with marine extract and controls (*Fig. 2*).

Target Identification

From the gene bank database, the predicted genes were encoded for proteins related to membrane development in *S. aureus.*

Profiling

The bands upon purification and commercial sequencing showed changes in the nucleotide sequences of the treated bacterial strains, but no changes in the untreated strains (*Fig. 3*).

Validation

Bacterial cell membrane permeabilizing assay is indicated by the changes in the fluorescence intensities of the treated bacterial strains, as shown in *Fig. 4.* The results showed obvious

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29247U_membF_complete          TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
membF_[921_-_2180]_AF145699    TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
29247T_mprF_complete           TGGCGACATTCTTCACTTACGCTTCATATATTTTAATAACATGGTTAGCT
                               **************************************************

29247U_membF_complete          ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGA-
membF_[921_-_2180]_AF145699    ATTATTTTTGTTCTGCTTATTGTAGCTTTCCGTAGAGCACGTAGGTTGAA
29247T_membF_complete          ATTATTTTGTGGCGACATTCTTCACTTACGCTTCATATATTTTAAT-AAC
                               ********    *  *  *  *  *  *   * *    *  *   *  *
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Fig. 3: Alignment of sequencing results from the treated, untreated S. aureus *and gene bank sequence of a putative membrane synthesis gene*

increase in the intensities of fluorescence in *S. aureus*, which was treated with marine extract and positive control, using membrane inhibitor antibiotic at MIC and 2x MIC for both strains ATCC 29247 and ATCC 700698 in the first 2 hours. After 2 hours of treatment, the intensities were found to slowly increase until 5 hours of post-treatment, and after which, there was no increase detected in the fluorescence intensity. As for *S. aureus* treated with negative control of membrane non-inhibitor antibiotic, the intensities were generally constant with no significant fluctuation throughout the experimental duration of 6 hours.

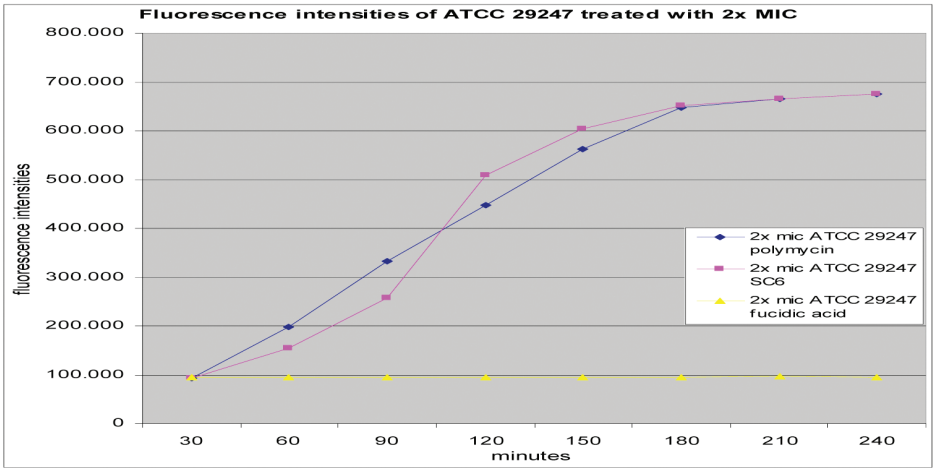

Fig. 4: Fluorescence intensities of ATCC 29247 treated with positive antibiotic control, marine methanolic extract, and negative antibiotic controls

DISCUSSION

Marine methanolic extract is a polar extract which comprises of compound with potential bacterial membrane permeabilizing ability. The extract at a minimum concentration of 10.2 mg/ml was found to inhibit the growth of non MRSA, strain ATCC 29247, and at a higher minimum inhibitory concentration of 12.8mg/ml for MRSA, strain ATCC 700698. When investigated at the molecular level, the inhibitory activity of the extract exhibited bands at two different positions for the MRSA and non-MRSA strains of the treated and untreated controls after the RT-PCR analysis, respectively. The amplification of these bands, visualized on gel after electrophoresis, suggested that the respective genes (with and without treatment) did not affect the partial expression, based on the size of the bands, respectively. However, upon purification, and the sequencing analysis by aligning the sequences of treated, untreated, and sequence of putative membrane development gene from the GenBank, showed there were nucleotide changes only in the treated samples. These findings indicate that the extract has the ability to alter the membrane of *S. aureus*.

In determining the effect of the extract on bacterial membrane integrity, the intensities of SYTOX green fluorescence dye in *S. aureus* cells (treated with marine methanolic extract at the MIC values) increases with time. SYTOX green is an organic compound which fluoresces upon interaction with nucleic acid. When the extract disrupts the membrane integrity of *S. aureus*, the dye enters the cell and easily colours the nucleic acid. In this study the increase in the intensities of fluorescence was also seen in the positive antibiotic control. The fluorescence intensities were found to increase drastically during the first 120 minutes of treatment, and this indicated that the membrane integrity was affected and the dye coloured the nucleic acid. Accordingly, the negative antibiotic control did not affect the bacterial cell membrane integrity and thus

showed a constant fluorescence intensity even after 6 hour of incubation in the antibiotic.

The present study validated the bacterial inhibiting activity of the methanolic marine extract of a local invertebrate species. The significance of the present study is that the two approaches in combination could be used to confirm the activity of the extract and elucidate the activity at the molecular level. The fluorescence dye bioassay approach clearly illustrated the mechanism of the methanolic extract and the positive antibiotic control in destructing the viability of the test bacterial strains via the integrity of bacterial membrane. As membrane plays an important role for cell integrity, the disruption of bacterial cell membrane thus provokes cell leakage and exposes the inner part to antibacterial compounds which serve as an effective alternative to cause the mortality of bacterial cells. An added advantage of the membrane, as an infective target, is the conservative nature of the structure, whereby the cell membrane is found and formed in all cells and the possibility of mutation is very low, making the membrane a readily available target.

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