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Extended Abstract

Antimicrobial, Anti Biofilm, and Anti Proliferative Activities of Lipopeptide Biosurfactant Produced by *Acinetobacter junii* B6

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Abstract

Lipopeptide biosurfactants (LPBs) are amphiphilic compounds produced by microorganisms exhibiting various biological activities. The main aim of the present study was to assess the in vitro antimicrobial, anti-biofilm, and cytotoxic effects of LPB produced by *Acinetobacter junii* (*Aj*L). We determined *Aj*L minimum inhibitory concentration (MIC) against both Gram-positive and Gram-negative bacteria as well as two fungal strains. Also, the anti-biofilm activity of *Aj*L against the biofilm produced by clinically isolated bacterial strains was investigated. The *Aj*L non-selectively showed activity against both Gram-positive and Gram-negative bacterial strains. The obtained results of the present study exhibited that the *Aj*L in concentrations nearly below critical micelle concentration (CMC) has an effective antibacterial activity. It was found that the MIC values of *Aj*L were lower than standard antifungal and it exhibited nearly 100 % inhibition against *Candida utilis.* The attained results of the biofilm formation revealed that *Aj*L disrupted the biofilm of *Proteus mirabilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* at 1250 µg/ml and 2500 µg/ml concentrations. The attained results of cytotoxic effect (determined by WST-1 assay) of the *Aj*L revealed IC_{50} of 7.8±0.4 mg/ml, 2.4±0.5 mg/ml, and 5.7±0.1 mg/ml, against U87, KB, and HUVEC cell lines, respectively. The results indicated that *Aj*L has a potential application in the relatively new field of biomedicine. 1. Introduction Biosurfactants are surface-active molecules with hydrophilic and hydrophobic moieties produced by different microorganisms such as bacteria, fungi, and yeasts that are mainly divided into two groups of glycolipid and lipopeptide. Lipopeptide biosurfactants (LPBs) are relevant molecules that exhibited antibacterial, antifungal, antiviral, and anti-adhesive activities . LPBs represent an extremely promising approach that can be replacing traditional antibiotics, which should be further explored. The antimicrobial properties of LPBs rely on different mechanisms to destroy target organisms as compared to conventional antibiotics. The strong antimicrobial activities of LPBs are ascribed to its ability to form ion-conducting channels in bacterial cell membranes by exploiting its detergent-like action on cell membranes, which called membrane active properties . Moreover, hydrophobic interactions provide the lipopeptide biosurfactants' affinity for membranes . Apart from their antimicrobial properties, LPBs showed the potential anti-biofilm

properties that form on the various surfaces particularly in surgical devices and implants . Biofilm formation is a natural phenomenon for most bacteria. The presence of extracellular polymeric matrix in biofilms exhibits reduced sensitivity to host defense systems, antibiotics, and other drugs, and thus, contribute to bacterial persistence in chronic infections. There are many reports about the effective biological activities of LPBs such as antimicrobial and anti-biofilm effects. Nowadays, intense researches are being focused on cancer as the most perilous areas in the biomedical field. Cytotoxicity of biosurfactants on different cell lines has gained much importance [10]. For example, the anti-proliferative and apoptotic properties of LPBs against human breast cancer (MCF-7 cells) were reported by Gudiña et al.. The inhibitory effect of Viscosin (a LPBs produced by Pseudomonas libanensis) on the prostate cancer cell line (PC-3M cells) was described by Saini et al.. In another study, the cytotoxic effects of LPBs on human colon cancer cell lines (HCT-15 and HT-29) were investigated .

The main aim of the present study was to evaluate the potential inhibitory activity of the AjL against biofilm producing bacterial pathogens isolated from clinical samples. Also, the antimicrobial activity of the AjL was investigated on three Grams positive and four Gram-negative bacteria. Furthermore, the cytotoxicity of the AjL was evaluated against U87 (glioblastoma cells line), KB (Mouth epithelial carcinoma cells) and HUVEC (human umbilical vein endothelial cells).

2. Materials and methods

Standard laboratory chemicals and a selection of reagents were obtained from the Sigma-Aldrich Company, (St. Louis, MO, USA). They included dulbecco's modified eagle's medium F12 (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (100 µg/ml), phosphate-buffered saline (PBS), trypan blue dye solution, trypsin- EDTA solution, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT), tryptic soy broth (TSB), dimethyl sulfoxide (DMSO), ciprofloxacin. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1 reagent) was prepared from Roche Holding AG (Basel, Switzerland). All eukaryote cell lines were obtained from Pasteur institute of Iran (Tehran, Iran). Bacterial and fungal sources were purchased from American type culture collection (Tehran, Iran).

The applied biosurfactant was partially purified from culture broth of A. junii B6 (GenBank accession KT946907) previously isolated from oil contaminated soil from the southwest part of Iran . Some physiochemical properties of the AjL such as CMC = 300 µg/ml and hydrophile-lipophile balance (HLB = 10) were determined.Bacterial cultures of three Gram-positive bacterial strains [Staphylococcus aureus (ATCC 29213), Bacillus subtilis (ATCC 6051), and Micrococcus luteus (ATCC 4698)] and four Gram-negative bacterial strains [Pseudomonas aeruginosa (ATCC 27853), Klebsiella neumonia (ATCC 13883), Escherichia coli (ATCC 25922), and Salmonella typhi (ATCC 6539)] were prepared in the Mueller-Hinton agar. The fungal strains used in this study included the Candida albicans (ATCC 10231) and C. utilis (ATCC 9950). The fungi were grown in Sabouraud dextrose agar medium. The antimicrobial activity was determined according to a modified microdilution assay protocol [16]. First of all, the microbial

inoculum was prepared (0.5 McFarland). Thereafter, the serial dilution (0.5–1200 µg/ml) of the AjL was prepared in a 96-well round-bottom microplate. Then, microbial strains were cultured into the desired well and incubated at 37 °C for 24 h in the case of bacterial strains and a longer incubation time of~48 h for fungal strains. In the next day, 40 µL INT solution (0.6 mg/ml) was added to each well, incubated for further 20 min at 37 °C, and the related absorbance was recorded at 490 nm. Un-inoculated media and inoculated media containing each microbial strain were applied as the negative and positive control, respectively. In addition, the antibacterial agent ciprofloxacin (concentration range of 0.5–8 µg/ml) and antifungal agent fluconazole (final concentration of 1–16 µg/ml) were used as standard control. The mortality rate (%) was calculated by the following equation :

(1)Mortality rate (%) = ((A-C) / (B–C)) × 100

Where A = test sample; B = positive control; C = negative control.

Anti-biofilm activity of AjL was examined against S. aureus, P. aeruginosa, and P. mirabilis as strong biofilm formers using the crystal violet-based microtiter plate method . After quantifying the bacterial MIC, TSB medium supplemented with glucose (1%) was inoculated with a loop full of bacterial colonies and then approximately 106 CFU/ml (100 µl) of the bacterial suspension were separately seeded into each well of 96-well microplates. Then, 100 µl from different concentrations of LPB (the final concentration range of 30–2500 µg/ml) were added to the desired wells. In parallel, 100 µL of the un-inoculated TSB medium was added to the desired well and considered as negative control. After 24 h incubation at 37 °C, the formed biofilm was fixed and stained by methanol and crystal violet, respectively. Then, the absorbance was recorded at 570 nm by a multimode microplate reader (BioTek Inc., Winooski, USA).U87, KB and HUVEC cell lines were grown in DMEM-F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and kept at 37 °C in a humidified atmosphere with 5% CO2. The medium was changed every other day.

An amount of 104 cells/well of each cell line were seeded in the 96 well microplate and incubated for 24 h. Next day, the medium was aspirated and 100 µl of each concentration of the prepared AjL were poured to each well. Then, microplates were incubated overnight at 37 °C and 5% CO2. On the next day, 10 µl of WST-1 stock solution was added to each well, and incubated for 4 h. Then, the absorbance was measured at 420 nm by a multimode microplate reader (BioTek Inc., Winooski, USA). Cell viability was expressed as 100% for untreated cells (control). All experiments were performed in triplicates and the viability (%) was calculated as the following equation (2):

(2)Viability (%) = (OD in treatment group/OD in control group) × 100

The inhibitory concentration required for 50% cytotoxicity (IC50) value was then determined after plotting of the viability percentage of inhibition versus the concentration.

The experimental data were analyzed using SPSS 21 for Windows (SPSS Inc. Chicago, U.S.A), employing basic statistical techniques. Other appropriate statistical software such as Microsoft Office Excel 2013 was also employed. One-way

analysis of variance (ANOVA) and t-test analysis were applied to determine the difference between the treatment groups and Tukey's multiple range test was used to find the significant difference among means at the probability level of ≤0.05.