



Antimicrobial, anti-biofilm, and anti-proliferative activities of lipopeptide biosurfactant produced by *Acinetobacter junii* B6

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ARTICLE INFO

Keywords:

Antimicrobial
Anti-biofilm
Anti-proliferative
Lipopeptide
Biosurfactant
Acinetobacter junii

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* (AjL). We determined AjL minimum inhibitory concentration (MIC) against both Gram-positive and Gram-negative bacteria as well as two fungal strains. Also, the anti-biofilm activity of AjL against the biofilm produced by clinically isolated bacterial strains was investigated. The AjL non-selectively showed activity against both Gram-positive and Gram-negative bacterial strains. The obtained results of the present study exhibited that the AjL in concentrations nearly below critical micelle concentration (CMC) has an effective antibacterial activity. It was found that the MIC values of AjL were lower than standard antifungal and it exhibited nearly 100% inhibition against *Candida utilis*. The attained results of the biofilm formation revealed that AjL 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 AjL revealed IC₅₀ 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 AjL 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 [1]. Lipopeptide biosurfactants (LPBs) are relevant molecules that exhibited antibacterial, antifungal, antiviral, and anti-adhesive activities [2]. 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 [3]. Moreover, hydrophobic interactions

provide the lipopeptide biosurfactants' affinity for membranes [4]. Apart from their antimicrobial properties, LPBs showed the potential anti-biofilm properties that form on the various surfaces particularly in surgical devices and implants [5]. 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 [6,7] and anti-biofilm [8,9] 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 Gudíña et al. [11]. The inhibitory effect of Viscosin (a LPBs produced by *Pseudomonas*

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<https://doi.org/10.1016/j.micpath.2019.103806>

Received 20 February 2019; Received in revised form 15 October 2019; Accepted 15 October 2019

Available online 17 October 2019

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libanensis) on the prostate cancer cell line (PC-3M cells) was described by Saini et al. [12]. In another study, the cytotoxic effects of LPBs on human colon cancer cell lines (HCT-15 and HT-29) were investigated [13].

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

2.1. Materials

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-disulphophenyl)-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).

2.2. Production of lipopeptide biosurfactant

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 [14,15]. Some physiochemical properties of the AjL such as CMC = 300 µg/ml and hydrophile-lipophile balance (HLB = 10) were determined [15].

2.3. Antimicrobial activity assay

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 pneumonia* (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) [17]:

$$\text{Mortality rate (\%)} = ((A-C) / (B-C)) \times 100 \quad (1)$$

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

2.4. Anti-biofilm activity of the AjL

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 [19]. 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).

2.5. Cell culture

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.

2.6. Cytotoxic effects

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) [18]:

$$\text{Viability (\%)} = (\text{OD in treatment group} / \text{OD in control group}) \times 100 \quad (2)$$

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

2.7. Statistical analyses

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.

3. Results and discussion

3.1. Antimicrobial activity of AjL

The antimicrobial activity of the AjL was determined on the basis of minimum inhibitory concentration (MIC). The results of the antimicrobial activity of AjL, on both Gram-positive and Gram-negative bacteria as well as two fungal strains, were summarized in Table 1. The growth of all bacterial strains and two *Candida* pathogens (*C. albicans* and *C. utilis*) were inhibited by AjL at the lowest concentration (5.0 µg/mL) (Table 1). Interestingly, the MIC values of AjL were lower than standard antifungal (fluconazole) and it exhibited nearly 100%

Table 1

Antifungal and antibacterial activities of the AjL compared to the standard antimicrobial drugs.

Compound Microorganisms	AjL	Ciprofloxacin	Fluconazole
	MIC ($\mu\text{g/ml}$)		
<i>Staphylococcus aureus</i>	5.0	2.0	–
<i>Bacillus subtilis</i>	5.0	4.0	–
<i>Micrococcus luteus</i>	5.0	1.3	–
<i>Pseudomonas aeruginosa</i>	5.0	2.7	–
<i>Klebsiella pneumoniae</i>	5.0	2.0	–
<i>Escherichia coli</i>	5.0	2.0	–
<i>Salmonella typhi</i>	5.0	2.0	–
<i>Candida albicans</i>	5.0	–	8.0
<i>Candida utilis</i>	5.0	–	8.0

inhibition against *C. utilis* (Table 1 and Fig. 1). LPBs exhibited the antimicrobial activity [19,20], but to date a few studies on the activity of LPBs isolated from *A. junii* have been reported. The AjL non-selectively showed activity against both Gram-positive and Gram-negative bacterial strains. This is quite contrasting to earlier reports on antimicrobial actions of the biosurfactants where the LPBs have been reported to be active mostly against Gram-positive or Gram-negative bacterial strains [7]. This may be due to the production of different LPBs isoforms, which shows an antagonistic effect on both Gram-positive and Gram-negative bacteria. It has been reported earlier that different isoforms of the LPBs are being produced depending on the microorganism, substrate used and the culture conditions employed [21,22]. According to the obtained results of the present study, the mortality percentage were dose-dependent and with increasing of the concentration the mortality percentage were decreased (Fig. 2). This kind of the response might be because of the functional mechanisms of the AjL interactions with membrane structures [23,24]. The proposed hypothesis is that the antimicrobial activity of LPBs is relative to their amphiphilic characteristic that permits interaction with phospholipids modifying cytoplasmic membrane permeability due to pore formation with the consequent leakage of cell components and cell death [24–28]. Also, the different concentration of LPBs has affected on the discompose of the phospholipid bilayer [24]. In previous work, it was demonstrated that the CMC of the utilized AjL was 300 $\mu\text{g/ml}$ thus, above this concentration, AjL molecules self-aggregate to form structures as micelles,

vesicles or lamellas [15]. So, at concentrations nearly under CMC value, AjL penetrates readily into the cell membrane, where it is completely miscible with the phospholipids. On the other hand, at higher concentrations of AjL (above the CMC values), they form micelles that cannot participate in the microbial membrane destruction [15,22,24,29,30]. Similar results were reported by Carrillo et al. [24] where they proposed a molecular mechanism of membrane permeabilization by surfactin. They found that surfactin might induce pore formation underlying the antibiotic action of these LPB. This study also suggested that this compound also exhibits a strong membrane destabilizing action at concentrations below its CMC.

Modification of bacterial surface hydrophobicity is another suggested mechanism by which the biosurfactants affect on microbial pathogens. In the case of Gram-negative bacterial strains, such modification leads to release of lipopolysaccharides (LPS) which is important for their pathogenicity. For example, Al-Tahhan et al. [31] described that rhamnolipids from *Pseudomonas aeruginosa* ATCC 9027 even at concentrations less than the CMC could enhance the surface hydrophobicity of the bacterial cell and consequently, released LPS from the bacterial membrane and increased cell membrane instability.

3.2. Anti-biofilm activity

The results from anti-biofilm activity showed that the formed biofilm of *S. aureus*, *P. mirabilis*, and *P. aeruginosa* were disrupted up to 35%, 10%, and 32%, respectively, with the AjL at 1250 $\mu\text{g/ml}$ and reached to 52%, 31%, and 70% respectively, at the concentration of 2500 $\mu\text{g/ml}$, as presented in Fig. 3. Also, statistical analysis showed the significant difference at $p \leq 0.05$ between experimental groups. The biofilm production is a natural phenomenon that protect the microorganisms (biofilm producers) against external toxic factors [32] and make them resistance to the antibiotics. On the other hand, elimination of the biofilms is too difficult. According to the attained results, AjL showed the potential to decrease the biofilms formation of the applied pathogens (Fig. 3). Previous studies showed that biosurfactants were more effective than many traditional biofilm inhibitors [8,33]. The mechanism involved on anti-biofilm activity observed to LPBs remains unclear. Nevertheless, it is conscious among researchers that most biosurfactants can increase bacterial surface hydrophobicity and destabilize lipid packing due to their amphipathic nature. Finally, these changes increase the permeability of the membrane cells and

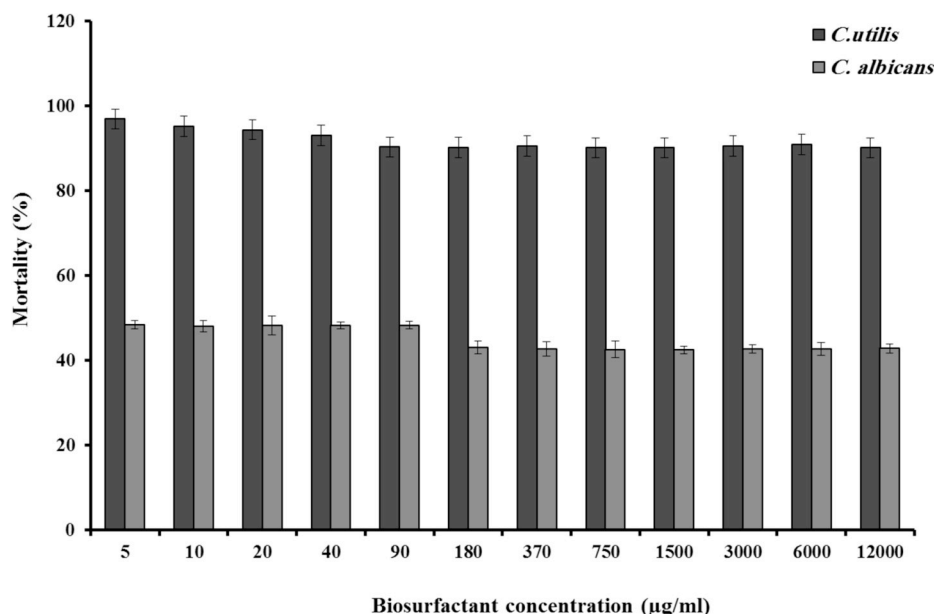


Fig. 1. Antifungal activity of the AjL at different concentration.

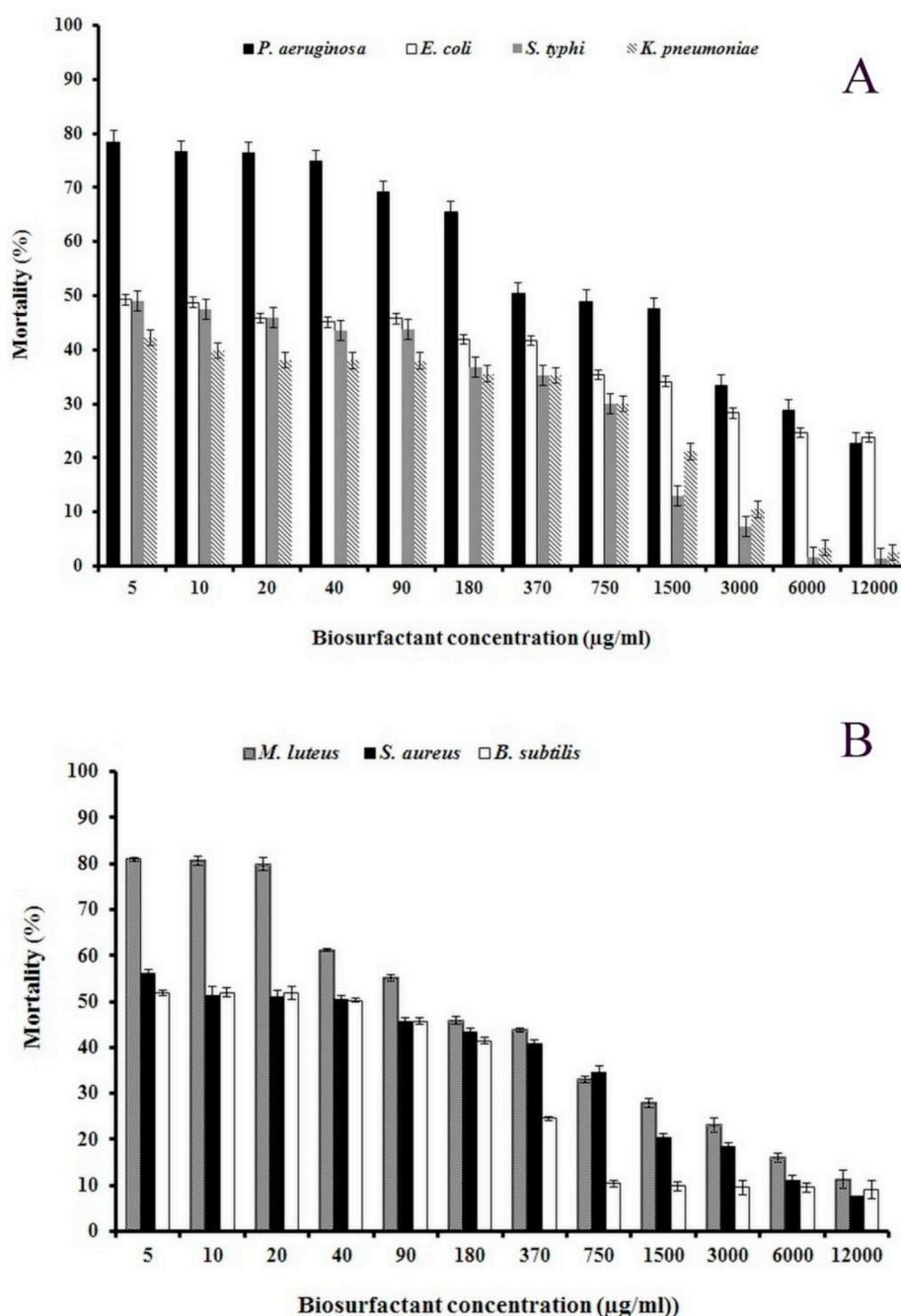


Fig. 2. Antibacterial activities of the AjL at different concentration on the Gram-negative bacteria (A) and Gram-positive bacteria (B).

consequently, reduced microbial adhesion to solid surfaces [25,33,34]. Also, Yan et al. [35] reported the mechanism of anti-biofilm activity by microbial surfactant isolated from lactic acid bacteria against *Staphylococcus aureus* CMCC 26003. Results showed that biosurfactants affect expressions of biofilm-related genes (*cida*, *icaA*, *dltB*, *agrA*, *sortaseA* and *sarA*) and interfere with the release of signaling molecules in quorum sensing systems.

3.3. Cytotoxic activity

The cytotoxic effect of the AjL on two cancer cell lines (U87 and KB) and normal cells (HUVEC) was determined by WST-1 assay (Fig. 4). Calculation of the IC₅₀ values for AjL against U87, KB, and HUVEC cells lines revealed IC₅₀ of 7.8 ± 0.4 mg/ml, 2.4 ± 0.5 mg/ml, and 5.7 ± 0.1 mg/ml, respectively. WST-1 assay confirmed that the

cytotoxic effect of the AjL on KB cells was higher than U87 cells. Also, the obtained results showed that the normal cell line (HUVEC) represented higher viability (74%) than cancer cells (64% and 65% for U87 and KB, respectively) at the lowest concentration (0.25 mg/ml) of AjL. The reported literature also showed the anti-proliferative effects of LPBs [36]. Abdelli et al. [6] reported that IC₅₀ of surfactin produced by *Bacillus safensis* F4 was 0.66 mg/ml and 1.17 mg/ml for T47D breast cancer cells and B16F10 mouse melanoma cells, respectively. In another study, the biosurfactants (BS-LBI and BS-LZ9) extracted from *Lactobacillus casei* showed toxicity effect on epithelial cell line (HEp-2) with IC₅₀ ranging from 109.1 ± 0.84 to 129.7 ± 0.52 mg/ml [37]. Pradhan et al. [38] investigated the anti-proliferative effect of two different biosurfactants S9BS (glycolipid biosurfactants) and CHBS (lipopeptide biosurfactants) extracted from *Lysinibacillus fusiformis* S9 and *Bacillus tequilensis* CH, respectively on human embryonic kidney

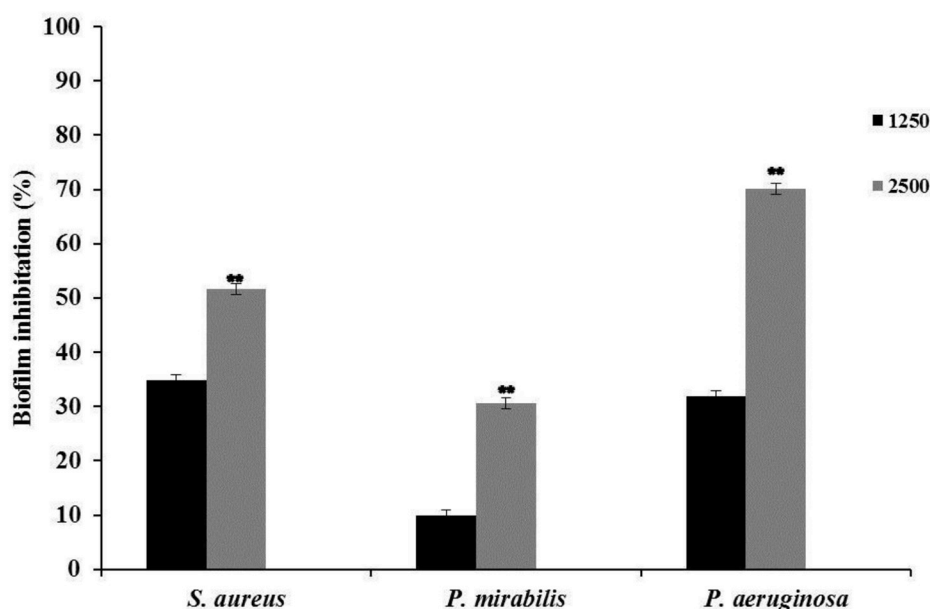


Fig. 3. Anti-biofilm activity of the AjL on the three biofilm producers; *S. aureus*, *P. mirabilis*, and *P. aeruginosa*.

cancerous cell (HEK-293). They concluded that the cytotoxicity effect of CHBS as LPBs ($IC_{50} = 100 \mu\text{g/ml}$) was less than glycolipid biosurfactant S9BS with IC_{50} of $75 \mu\text{g/ml}$. Many investigations have been conducted to find the mechanism of biosurfactant's induced anti-proliferative effect. Numerous studies have reported that biosurfactants could penetrate into cells by channel formation or diffusion of ions across the membrane barrier and membrane solubilization by a detergent-like mechanism [6,39,40]. Also, Pradhan et al. [38] reported that biosurfactants S9BS and CHBS induce apoptosis in HEK-293 cell lines by specific interaction/incorporation of its lipid moiety into the plasma membrane leading to the formation of pores and loss of cell integrity. In another study, Liu et al. [41] investigated the molecular mechanism of cell toxicity by LPBs and concluded that the fatty acid moiety of LPBs strongly interacts with the acyl chain of the phospholipids and the peptide moiety interacts with the polar head group of the lipids to penetrate in Bcap-37 cell lines.

4. Conclusion

To sum up, AjL was effective in inhibiting both Gram-positive and Gram-negative bacteria as well as fungal strains. The anti-biofilms assays revealed that AjL was able to decrease the biofilm formation of the applied pathogens. AjL represented lower toxicity on normal cells compared to those of cancer cell lines. However, more investigations should be performed to know about the related mechanism(s) involved in both cytotoxic and antibacterial effects.

Acknowledgements

This work was financially supported by a grant from Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran (95000232).

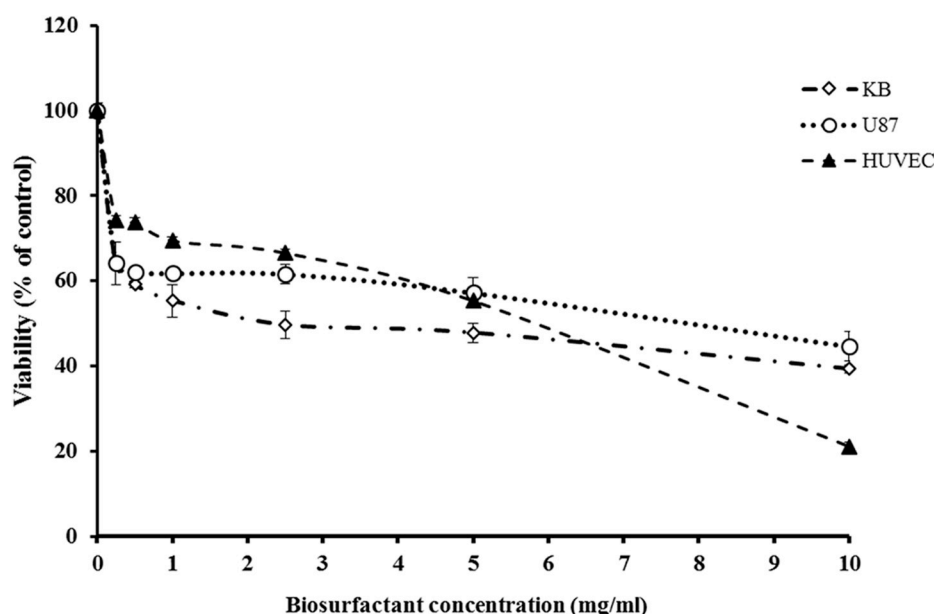


Fig. 4. Cytotoxic effect of the extracted AjL on U87, KB, and HUVEC cells lines.

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