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Research Article

Evaluation of bacterial biosurfactant activities as an anticancer and antibiofilm agent

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Abstract
Rhamnolipids are glycolipid biosurfactants produced by *Pseudomonas* sp. that can be applied in many fields, such as medicine, pharmaceuticals, cosmetics and food processing. The rhamnolipid utilized in the present study was produced from *Pseudomonas aeruginosa* which was isolated from hydrocarbon-contaminated soil. Different rhamnolipid concentrations were evaluated as anticancer agents against cancer cell lines, including the Hela cell line and the L20B cell line, and as antibiofilm agents against four pathogenic bacteria, including *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. Results showed that the rhamnolipid inhibited the proliferation of the cervical cancer cell line (Hela) during exposure. The inhibitory effect of rhamnolipid against the Hela cell line increased with the increasing concentration of rhamnolipid. The 750 µg/ml concentration recorded a higher inhibitory effect, while the 50 µg/ml concentration recorded a lower inhibitory effect against the Hela cell line. Similarly, the concentration of 750 µg/ml recorded a higher inhibitory effect, while the concentration of 62.5 µg/ml recorded a lower inhibitory effect against the L20B cell line. The results exhibited the best rhamnolipid activity as an antibiofilm agent against pathogenic bacteria at a concentration of 1 mg/ml for *E. coli*, *B. cereus*, and *K. pneumoniae*, while exhibiting the best antibiofilm activity against *Staphylococcus aureus* at a concentration of 2 mg/ml when incubated with different concentrations of rhamnolipid. The rhamnolipid showed high effectiveness as antibiofilm and anticancer agent, which constitutes a promising agent for use against pathogenic bacteria to prevent the formation of biofilm and an alternative therapeutic agent as an anticancer.

Keywords: Anticancer, Antibiofilm, Rhamnolipid, Biosurfactant, *Pseudomonas*

INTRODUCTION

Numerous biosurfactant compounds have demonstrated antibacterial activity against various human pathogenic bacteria, which qualifies them as a strong alternative therapeutic agent instead of present antimicrobial agents (De Giani *et al.,* 2021; Alyousif *et al.,* 2023). Rhamnolipids are glycolipid biosurfactants produced by Pseudomonas species. They comprise a hydrophilic group that includes one or two L-rhamnose molecules, which are glycosidically linked to a hydrophobic group of one or two fatty acids (Rahimi *et al.,* 2019). Rhamnolipids can be applied in many fields, such as medicine, pharmaceuticals, cosmetics, food processing, bioremediation of pollutants and agriculture (Almansoory *et al.,* 2019; Jiang *et al.,* 2020). The potential medical application of rhamnolipids has increased during the past decade as a biofilm control agent and anticancer agent (Chong and Li, 2017; Fracchia *et al.,* 2019).

Cancer represents an extremely complex disease affecting millions of people worldwide. The treatment of cancer by chemotherapy is highly toxic, non-specific and non-selective. However, there is a need for the development of new anticancer drugs (Kaur and Verma, 2015). The Continuous discovery of compounds from natural sources, such as bacteria, is anticipated to yield a variety of and unexpected chemicals with fascinating biological features, such as anticancer activity (Janakiram *et al.,* 2015). Several types of microbial surfactants, such as lipopeptides and glycolipids, preferentially limit the spread of cancer cells and disintegrate cell membranes triggered by apoptosis (Gudiña

et al., 2013). The rhamnolipids compounds interact with various animals' cell membranes and/or surroundings owing to their surface activity and can thus be considered possible cancer therapeutics or components of drug delivery systems (Paulino *et al.,* 2015). Rhamnolipid has been proven to have considerable antiproliferative activity against the human breast cancer cell line (MCF-7) against the insect cell line (Thanomsub *et al.,* 2006). The possibility of genetically altered bacteria for their greater production rates makes microbial manufacture of anticancer medications advantageous compared to their recovery from natural sources such as plants (Ari *et al.,* 2024).

Microbial biofilms exhibit a distinct bacterial physiology with a multicellular phenotype fundamentally different from planktonic bacteria. They are often linked to chronic and resistant healthcare-associated infections (Banat *et al.,* 2014). Anti-adhesive activity is a well-known property of biosurfactants, which is the ability to reduce the adhesion of microbial cells to solid surfaces and infection sites, thus inhibiting biofilm formation (Abdollahi *et al.,* 2020). Its most notable biosurfactants are those produced by bacteria with antimicrobial, anti-adhesive, or anti-biofilm activity against a wide range of pathogenic and opportunistic bacteria (Bolhassani, 2015). The crude biosurfactant produced by *Pediococcus acidilactici* and *Lactobacillus plantarum* inhibited adhesion and biofilm formation of *Staphylococcus aureus* and also can affect expression levels of biofilm-related genes and interfere with signaling molecules releasing (AI-2) in quorum sensing systems (Yan *et al.,* 2019).

Rhamnolipids have shown effective activity against the biofilm of pathogenic *Bordetella bronchiseptica*, they have been reported to disrupt preformed biofilms such as biofilm formed by *Bacillus pumilus* (Banat *et al.,* 2014). The present study aimed to investigate the biological activity of identified rhamnolipid produced by *Pseudomonas aeruginosa* as anticancer agent against cancer cell lines and an antibiofilm agent against pathogenic bacteria.

MATERIALS AND METHODS

Production of biosurfactant

The rhamnolipid biosurfactant used in the present study was produced by the *Pseudomonas aeruginosa* strain M4 (Accession no. MK607451.1), which was isolated from hydrocarbon-contaminated soil in a previous study (Alyousif *et al.,* 2020a). The rhamnolipid was isolated and purified in a separate study (Alyousif *et al.,* 2020b).

Rhamnolipid biosurfactant activity as an anticancer agent

Cell Lines and Culture Conditions

The Hela and L20B cell lines were obtained from the IRAQ Biotech Cell Bank Unit in Basrah and maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 50% confluence twice a week and incubated at 37 °C and 5% Co_2 (Al-Shammari *et al.,* 2019).

Combination cytotoxicity assays

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenylte trazolium Bromide assay (MTT) is cell viability assays were conducted on 96-well plates to determine the cytotoxic effect of rhamnolipid. Hela and L20B cell lines were seeded at 1 × 10^4 cells per well. After 24 hours, or once a confluent monolayer was achieved, the cells were treated with the tested rhamnolipid at six concentrations (50, 62.5, 100, 250, 500, 750, and 1000 µg/ml). Cell viability was assessed 72 hours post-treatment. First, the medium was removed, and 28 µl of a 2 mg/ml MTT solution was added. The cells were then incubated for 2 hours at 37 °C. After discarding the MTT solution, the remaining crystals in the wells were dissolved by adding 100 µl of DMSO (Dimethyl Sulphoxide) and incubating at 37 °C for 15 minutes with shaking (Al-Shammari *et al.,* 2019). Absorbance was determined using a microplate reader at 620 nm, and the assay was performed in triplicate. The inhibition rate of cell growth (percentage of cytotoxicity) was calculated using the following equation:

Proliferation rate as (PR)= B/A*100 Eq.1

Where A represents the mean optical density of untreated wells, B denotes the optical density of treated wells, and IR is calculated as 100 - PR.

Rhamnolipid activity as antibiofilm agent

Determination of biofilms forming by pathogenic bacteria

Four pathogenic bacteria, local isolates (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus*) were used in the present study provided by the Applied Microorganisms Laboratory, Biology Department, College of Science, University of Basrah. The biofilms formation by pathogenic bacteria was evaluated by the Congo red agar (CRA) method (Deka, 2014). Pathogenic bacteria were cultivated on CRA and the plates were incubated for 24 h at 37 °C. Positive results on CRA were read as forms of black rise colony with dry crystalline consistency.

Determination of antibiofilm by tissue culture plate (TCP) method

The tested bacteria were activated overnight at 37 ˚C in Tryptic Soy broth (TSB) containing 1% glucose for 24 h. Different concentrations of rhamnolipid (1, 2, 4, and 8 mg/ml) were prepared in TSB with 1% glucose. A microtiter plate combined 180 µl of each rhamnolipid concentration with 20 µl of the tested bacteria. A culture

without rhamnolipid was the positive control, while TSB alone was the negative control. The procedure was conducted and accomplished as described by (Mathur *et al.*,2006). The optical density of each well was read at 630 nm using a reader of the microtiter plate.

RESULTS

Rhamnolipid activity as an anticancer agent

Results showed that the rhamnolipid had an inhibitory effect on the proliferation of cervical cancer cell line (Hela) during the exposure period as shown in Fig. 1.

The inhibitory effect of rhamnolipid against Hela cell line was increased with the increasing concentration of rhamnolipid, as shown in Fig. 2. The viability of Hela cells line, which was treated with concentrations of 50, 62.5, 100, 250, 500, 750 and 1500 µg/ml of rhamnolipid, was 42.59%, 13.54%, 7.08%, 6.46%, 6.34%, 6.31% and 6.46% respectively with IC_{50}= 61.71 µg/ml. The concentration of 750 µg/ml was recorded a higher inhibitory effect and the concentration of 50 µg/ml was recorded a lower inhibitory effect against Hela cells line. The rhamnolipid biosurfactant had little effect on the viability of normal cell growth.

L20B cells line even when used at high concentrations as shown in Fig. 3; Figure 4. The viability of L20B cells line, which was treated with concentrations 50, 62.5, 100, 250, 500, 750 and 1500 µg/ml of rhamnolipid were 62.03%, 62.11%, 61.68%, 52.64%, 44.19%, 40.92%, 42.47% and 48.41% respectively.

Rhamnolipid activity as antibiofilm agent

Congo red agar method was used for the detection of biofilm formation by pathogenic bacteria. The results showed that all 4 pathogenic bacterial isolates formed strong biofilm by giving black rise colony with dry crystalline consistency as shown in Fig. 5.

The results of TCP method used for evaluation the effect of rhamnolipid of *P. aeruginosa* against biofilm-forming pathogenic bacteria are shown in Table 1.

The results showed rhamnolipid activity as antibiofilm agent by TCP method, which exhibited the best antibiofilm activity against pathogenic bacteria at a concentration of 1 mg/ml for *E. coli*, *B. cereus* and *K. pneumonia*

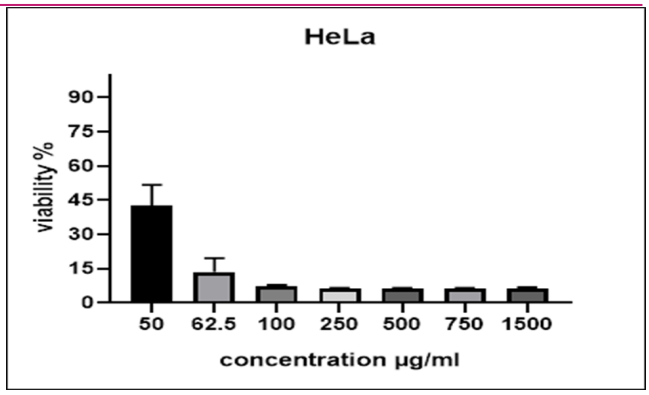

Fig. 1. *Inhibitory effect of different concentrations of rhamnolipid against Hela cell line*

while exhibiting the best antibiofilm activity against *S. aureus* at a concentration of 2 mg/ml when incubated with different concentrations of rhamnolipid and the effect decrease by increasing the concentration of rhamnolipid, compared with control positive.

DISCUSSION

Rhamnolipid activity as an anticancer agent

Cancer is a dreadful disease that shows many complications in treatment due to problems with drug efficacy and harmful side effects on healthy cells. The main challenge in discovering a drug potentially treating cancer to find an effective drug with minimal toxicity to normal cells (Talib *et al.,* 2021).

In the present study, the results showed that the rhamnolipid biosurfactant had an inhibitory effect on the proliferation of cervical cancer cell line (Hela) during the exposure period, as shown in Fig. 1. The inhibitory effect of rhamnolipid biosurfactant against Hela cell line was increased with the increasing concentration of rhamnolipid as shown in Fig. 2. The concentration of 750 µg/ml was recorded as a higher inhibitory effect. The results showed the rhamnolipid had little effect on the proliferation of L20B cell line during the period of exposure as shown in Fig. 3. The concentration of 62.5 µg/ml of the rhamnolipid was recorded as a higher viability of L20B cell line.

The induced cancer cell death process by rhamnolipid suggested several mechanisms leading to a series of

Table 1. Rhamnolipid activity as antibiofilm agent by TCP method determined by read O.D. at wave length 630 nm.

Bacteria	Concentration of rhamnolipid (mg/ml)				Control	
	C1 (1 mg/ml)	C2 (2mg/ml)	C3 (4mg/ml)	C4 (8mg/ml)	+	-
Escherichia coli	0.054	0.097	0.159	0. 687	0.648	0.027
Bacillus cereus	0.054	0.067	0.591	0.612	0.490	0.041
Staphylococcus aureus	0.036	0.026	0.091	0.163	0.609	0.029
Klebsiella pneumonia	0.018	0.577	0.073	0.352	0.383	0.083

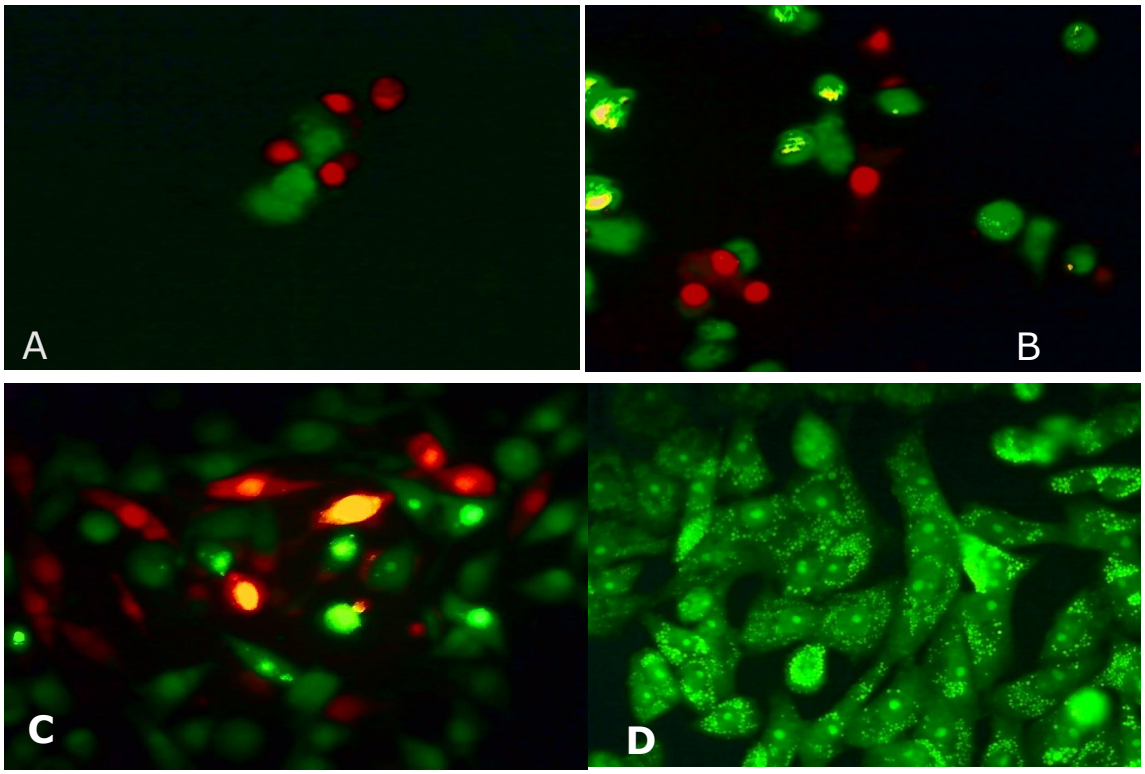

Fig. 2. *Viability of Hela cells line* ***A.*** *Cells treated with 62.5 µg/ml of rhamnolipid showing the viability with13.54%,* ***B.*** *Cells treated with 250 µg/ml of rhamnolipid showing the viability with 6.46%,* ***C.*** *Cells treated with 750 µg/ml of rhamnolipid showing the viability with 6.31%* ***D.*** *Untreated control cells showing Hela cells line as green live in control.*

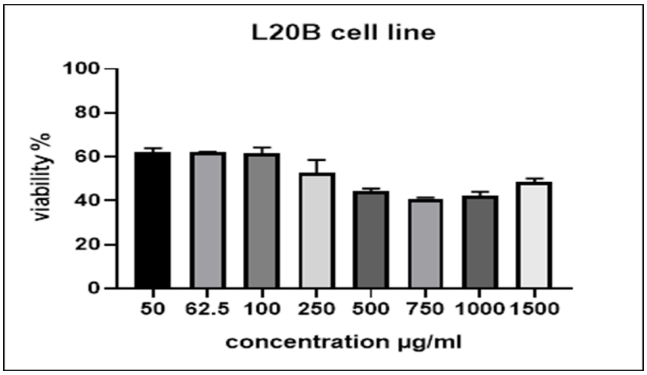

Fig. 3. *Inhibitory effect of different concentrations of rhamnolipid against L20B cell line. The concentration of 750 µg/ml was recorded a higher inhibitory effect and concentration 62.5 µg/ml was recorded a lower inhibitory effect against L20B cells line*

events involved in the destruction of cancer cells by apoptosis. The morphological alterations in treated cancer cells were DNA fragmentation, margination, chromatin condensation and plasma membrane blebbing. The apoptotic process is concentration-independent due to efflux pump, which activated after incubation with the higher dose of the tested substance (Chaudhry *et al.,* 2022).

Many researchers reported the activity of the rhamnolipid against cancer cells, which showed a variety of activity depending on the type of cancer cell line and the concentrations of rhamnolipid in addition to the type of rhamnolipid. Christova *et al.* (2013) investigated the cytotoxic effect of mono-rhamnolipid on cancer cell lines, including HL-60, BV-173, SKW-3 and JMSU-1. RL-1. The mono-rhamnolipid revealed 50 % inhibition of cellular viability at lower concentrations. Furthermore, mono-rhamnolipid demonstrated inhibition of the proliferation of BV-173 pre-B human leukemia cells by induction of apoptotic cell death. Rahimi *et al.* (2019) investigated the cytotoxicity of mono- and di-rhamnolipid against MCF-7 cells in a concentration-dependent manner, revealing apoptotic characteristics in treated MCF-7 cells. The overexpression of the p53 gene in the cell is a sign of apoptosis cell induction in treated MCF-7 cells with rhamnolipid. The promising results about the effect of rhamnolipid on cancer cells can encourage further research on the rhamnolipid towards developing natural, therapeutic anticancer agents.

Rhamnolipid activity as an antibiofilm agent

The bacteria resistance to various antimicrobial treatments has been increased by biofilm. Consequently, efforts are being made to identify potent antimicrobial compounds impervious to bacterial resistance mechanisms, including those present in biofilms (Penesyan *et al.,* 2015). The way that rhamnolipid disrupts or modifies the morphology of the cell wall or how it displays the effectively disconnected architecture of biofilms that constitute individual cells may all have an impact on the formation of biofilms (Skariyachan *et al.,* 2018).

Silva *et al.* (2017) reported the ability of rhamnolipid to

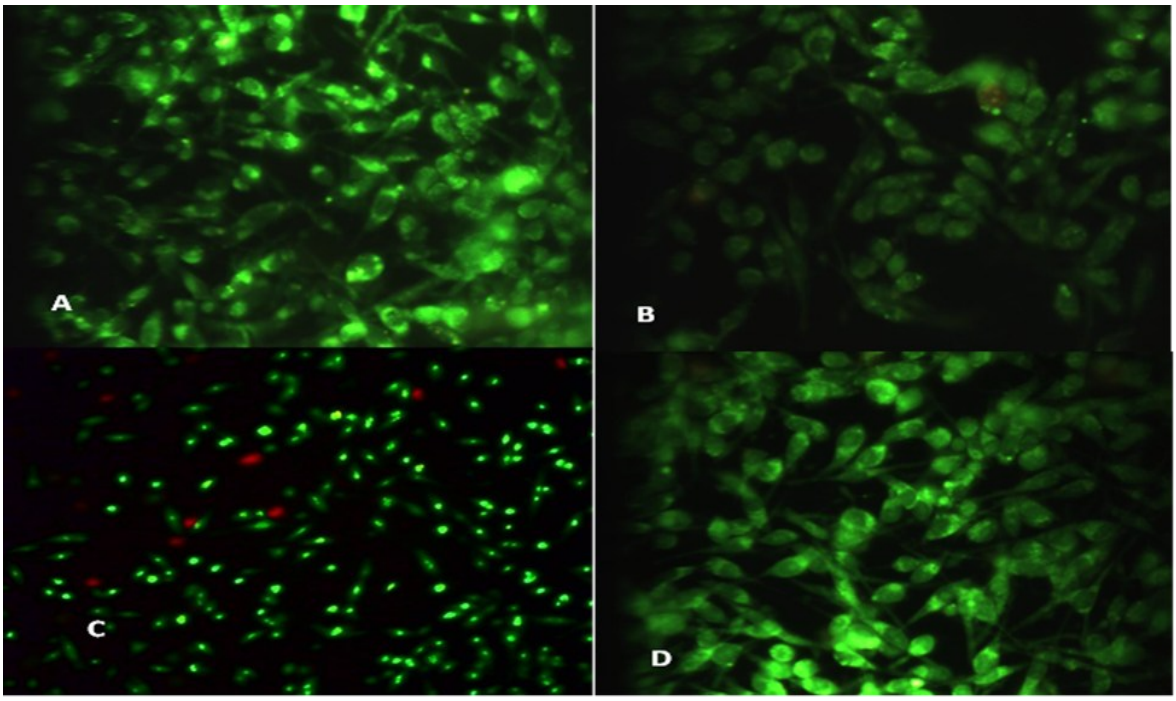

Fig. 4. *Viability of L20B cells line **A**. cells treated with 62.5 µg/ml of rhamnolipid showing the viability with 62.11% **B.** cells treated with 250 µg/ml of rhamnolipid showing the viability with 52.64%, **C.** cells treated with 750 µg/ml of rhamnolipid showing the viability with 40.92%, **D.** Untreated control cells showing L20B cells line as green live in control.*

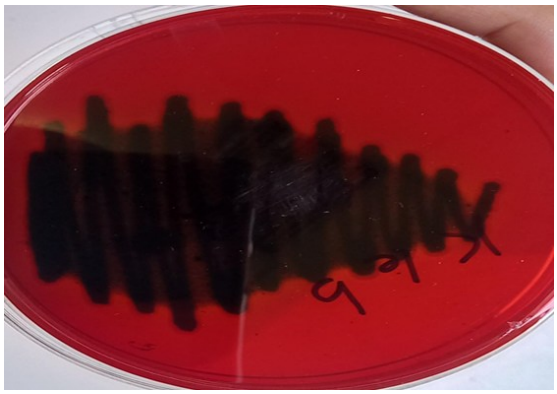

Fig. 5. *Biofilm formation by Escherichia coli using Congo red agar method*

remove *S. aureus* biofilms at 25˚C and 1% concentration when reducing around 35% of biofilm biomass while removing 86% when using skim milk. Yamasaki *et al.* (2020) observed that rhamnolipid inhibited all investigated oral bacteria's growth and biofilm formation. A study conducted by Al-Razn and Abdul-Hussein (2021) concluded that rhamnolipid prevents the adhesion and formation of biofilm by pathogenic bacteria isolated from a diabetic foot infection. Rhamnolipid extracted from *P. aeruginosa* showed anti-biofilm, anti-adhesive and preformed biofilm disruption activities against biofilm-forming pathogenic bacteria. In the present study, rhamnolipid showed high effectiveness as an antibiofilm and anticancer agent, which constitutes a promising substance for using in the medical field against pathogenic bacteria to prevent the formation of biofilm, in addition as a cancer treatment substance as an alternative to chemical treatment because of increasing resistance by pathogens to some antimicrobial substances that suggests it as a good alternative for synthetic compounds.

Conclusion

The present study showed that the rhamnolipid inhibited the proliferation of cervical cancer cell line (Hela) and L20B cells during exposure. The inhibitory effect of rhamnolipid against Hela cell line was increased with the increasing concentration of rhamnolipid. Also, rhamnolipid showed activity against biofilm formed by pathogenic bacteria with low concentration. Therefore, the rhamnolipid is a suitable alternative to be utilized as an effective and safe therapeutic agent to control pathogenic bacteria and cancer.

Conflict of interest

The authors declare that they had no conflict of interest.

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