LAB-SCALE PRODUCTION OF RHAMNOLIPID BY *PSEUDOMONAS AERUGINOSA* A3 AND STUDY ITS SYNERGISTIC EFFECT WITH CERTAIN ANTIBIOTICS AGAINST SOME PATHOGENIC BACTERIA

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ABSTRACT

This study was aimed to obtain the considerable amount of the biosurfactant (rhamnolipid) from local isolate of *Pseudomonas aeruginosa* on industrial-scale. A 7L laboratory bioreactor was appointed to accomplish this task and all conditions of production were set as previously by batch culture experiments. Bioreactor with 3.5 L working volume was conducted for 120 h to obtain 16 g/L at 30ºC and 6.5 pH. The supernatant of production medium was used to reduce the surface tension of water from 72 to 27 mN/m, and the emulsification index was reached to 67%. The product was extracted with a mixture of solvents, purified by silica gel using a column of glass chromatography (3.5 × 30cm), and characterized by TLC and FTIR where it turn out that the biosurfactant is composed of both mono- and di-rhamnolipid. Mono-RL was tested for its antibacterial activity against some clinical isolates of Gram-positive and Gram-negative bacteria to obtain the MIC, which was about 250µg/ml, also at same concentration, the toxicity against Rat embryonic fibroblast (REF) cell line was reached to 23%. The combination effect of Mono-RL with some antibiotics was studied against pathogenic clinical isolates revealed that there is a synergistic effect with ampicillin against *S. aureus* and *Serratia marcescens*, with cefotaxime against *E. coli* and *Kelbsiella pneumoniae*, same as with tetracycline.

Keywords: biosurfactant, bioreactor, emuslification, chromatography

انتاج الرامنولبد بوساطة *PSEUDOMONAS AERUGINOSA* A3 على المقاييس المختبرية ودراسة تأثيره التآزري مع مضادات حيوية معينة مضادة لبعض البكتريا الممرضة

أيوب مرتضى الشيخ فخري ناظم حسن حيدر عبد الكريم جاسم هاشم
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المستخلص

تهدف الدراسة إلى الحصول على كميات كافية من مستحلب الرامنولبد المنتج من عزلة محلية لبكتريا *Pseudomonas aeruginosa* على المستوى الصناعي، حيث استخدم المخمر الصناعي بحجم 7 لتر مع تثبيت كل الظروف المناسبة للانتاج والتي اجريت باستخدام مزارع الوجبة الواحدة. استمر الانتاج في المخمر بحجم نهائي للوسط 3.5 لتر لمدة 120 ساعة للحصول على كمية للمستحلب مساوية إلى 16 غم/لتر بدرجة حرارة 30 مئوية ودالة حموضة 6.5. استخدم راشح الوسط الانتاجي في خفض الشد السطحي للماء من 72 الى 27 ميلي نيوتن/متر وبمعامل استحلاب 67%. استخلص المنتج بالمذيبات ونقي بالسيلكا جيل باستخدام كروماتوغرافيا الزجاج وبعمود الفصل ذو الابعاد (3.5 × 30سم)، كذلك تم توصيف المنتوج المنقى بوساطة كروماتوغرافيا الطبقة الرقيقة وجهاز قياس طيف الاشعة تحت الحمراء حيث تبين ان المنتج مكون من احادي وثنائي الرامنولبد. استخدم احادي الرامنولبد كعامل مضاد للبكتريا ضد عدد من العزلات المحلية السريرية الموجبة والسالبة لصبغة غرام لغرض تحديد التركيز المثبط الادنى، كذلك كان التأثير السمي لنفس التركيز ضد الخلايا الليفية لجنين الجرذ هو 23%. درس التأثير التآزري الناتج من دمج احادي الرامنولبد مع مضاد الامبسلين وكان واضحا ضد كل من *S. aureus* و *Serratia marcescens*، كما اظهر تأثيرا تآزريا مع مضاد السيفوتاكزيم ضد كل من *E. coli* و *Kelbsiella pneumoniae*، كما هو الحال مع مضاد التتراسايلكلين.

الكلمات المفتاحية: مستحلب، مخمر،استحلاب، كروماتوغرافي

***Received:18/1/2019, Accepted:8/4/2019**

INTRODUCTION

There are multiple features which make biosurfactants (microbially-produced surfactants) a promising alternative for chemically-synthesized ones in industrial applications, owing these features to their higher effectiveness and biodegradability at extreme temperature, pH and salinity conditions, lower toxicity and environmental friendly. These molecules are biosynthesized by a number of microorganisms (21). *Pseudomonas aeruginosa* seems to produce rhamnolipid to some degree from substrates regardless of their hydrophobicity. This microorganism adapted for growing and biosynthesize surfactants in complex forms such as wastes and soil systems (22). The cellular metabolic pathway is directed toward rhamnolipids production at the end of exponential growth phase when nitrogen has been completely consumed (28). There's a big need for optimization of fermentation conditions in order to increase the product yield of biosurfactants as well as improving their specificity to overcome the barriers that limit industrial production and exploiting of microbial surfactants (26). The most abundant forms of rhamnolipids are rhamnosyl-B-hydroxydcanoyl-B-hydroxydecanoate (Rha-C_{10}-C_{10}), which is a mono-rhamnolipoid, and rhamnosyl-rhamnosyl-B-hydroxydcanoyl-B-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}), a di-rhamnolipid. Although, some rhamnolipids contain one or two rhamnose moiety linked to only one 3-hydroxyfatty acid (37). The activity of RL depends on the surface active compound quantity until the concentration of critical micelle (CMC) is obtained. Based on the theories of classic surfactant aggregation, the molecules of surfactant are integrate into the structure of water, which called monomers in aqueous phase, and aggregate at the interface of air-liquid or liquid-liquid at concentrations less than CMC (14). Once RL molecules reach its threshold, they associate to each other to form aggregates. These micelles when form in water, their tails, which are hydrophobic, form a core that encapsulates the hydrocarbons, and the hydrophilic polar heads maintain their contact with water by forming an outer shell. In the case of oil, the process is reversed (reversed micelle) where the hydrophobic part forms the outer shell and the polar head inside (29). Furthermore, various structures auto-assembled by RL when the concentration above the CMC, such as vesicles or lamella, depending on the concentration of RL and pH value (19). Three sequential reactions involved in the production of rhamnolipids. The first, which is responsible of synthesis of fatty acid dimer moiety, carried out by an enzyme, RhlA. The second reaction is catalyzed by RhlB, a membrane-bound rhamnosyltransferase, uses 3-(hydroxyalkanoyloxy) alkanoic acid (HAA) and deoxythymidine diphospho-l-rhamnose (dTDP-L-rhamnose) as precursors, producing mono-rhamnolipids. These are in turn the substrates, jointly with dTDP-L-rhamnose, producing di-rhamnolipids by the loosely bound to inner membrane, RhlC (27, 37). Glycolipids conquer a minor part of the molecular structure of the cell and commonly placed with lipids associated with external cell surfaces. Interestingly, glycolipids assist in molecular transfer across cell membrane as well as known to be involved in the biosynthesis of proteoglycans and glycoproteins (12). There are an increasing number of pathogens which are unsusceptible to treatment with common antibiotics; this challenges human health as well as other organisms. Therefore, the need for searching and development of novel pharmaceuticals characterized by a wide spectrum of activity, low toxicity as well as cost effective that can be applied for human, veterinary medicine and plant is of great importance (16). Biosurfactants can disturb the structure of biological membranes due to their interaction with membranous proteins as well as phospholipids (43). One of the major problems of hydrophobic antibiotics is that the difficulty in their uptake bu cells. So RL are tested for enhancing the activity of such hydrophobic antimicrobials (25). The aim of the present work is to produce rhamnolipid from *P. aeruginosa* A3 in bioreactor and testing its synergistic effect with antibiotics against resistant clinical isolates.

MATERIALS AND METHODS

Production of RL in Bioreactor

The bench-top LAMBDA MINIFOR laboratory bioreactor was used for RL production in optimized conditions. This

model is easy to handle and all important cultural conditions can be measured and controlled. The minimum working volume was 3.5 L of the 7 L capacity of the bioreactor vessel. The instrument is equipped with different sensitive probes for temperature, pH, air and agitation system The production medium was composed from the modified Proteose-peptone ammonium salts (PPAS), as follows (g/l): NH_4Cl, 1.0; KCl, 1.5; $MgSO_4$, 0.19; Tris HCl, 14.5; proteose-peptone 2.0; CTAB, 0.2; the media was autoclaved for 15 mis at 121ºC after the addition of 2.5% glycerol as carbon source, then 0.1% (v/v) trace elements solution composed of (g/l): $FeSO4.7H_2O$, 2.0; $MnSO_4.H2O$, 1.5; $(NH_4)6Mo7O24.4H_2O$, 0.6; $ZnSO4.7H_2O$, 0.29; and $CuSO4.5H_2O$, 0.25; sterilized by cellulose acetate Millipore filter (0.22µm), was added to the medium when warmed down after autoclaving (35). Seventy ml of the previous production medium (2%) that contain 1×10^6 cfu/ml was used to inoculate the bioreactor containing same production medium with the daily addition of 5 ml of the remaining spent medium after sterilization with cellulose acetate Millipore filter (0.22µm) as a good elicitor for RL production.

Biosurfactant extraction

The broth culture was extracted from biomass by centrifugation 10,000 rpm for 15 mins, then the pH of crude culture was adjusted by 1 N HCl to pH of 2, left overnight for precipitation, the latter was collected through centrifugation at 10,000 rpm for 20 mins, while the above supernatant extracted with an equivalent volume of chloroform: methanol (2:1 v/v). The median scum layer was separated and the superior aqueous phase re-extracted with similar volumes of solvents above. The merged extracts were concentrated by evaporation of solvents at 45° C to obtain viscous honey-colored extract. The yields were determined gravimetrically in terms of g/L (36, 41).

Biosurfactant purification

The RLs components were separated from the crude mixture as follows. Heat activated silica gel 60 slurry in chloroform was decanted onto a column of a glass chromatography (3.5 × 30cm). Two grams of crude extracted RL were dissolved in chloroform (4 ml) and added onto the column. Then the column washed with chloroform at a flow rate of 60 ml/hr to elute neutral lipids totally, followed by two steps of chloroform/methanol addition at volumes of 250 and 200 ml with 50:3 and 50:5 ratios, respectively, for separation of mono-RL. Final elution was done by same solvents above at 50:50 ratios with pure methanol, each volume of 100 ml, to elute di-RL. The fractions composition was checked by thin layer chromatography (34).

Analyzing of biosurfactant

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) technique was used to characterize the biosurfactants purification to determine their types. The process was conducted by TLC plates (10 × 10 cm) coated with silica gel. Separation and analysis of the produced biosurfactant proceed through a solvent system comprised chloroform, methanol and acetic acid in a ratio of 65:15:2, respectively. Then the plates were sprayed with resolving solution composing of 0.15g of orcinol dissolved in 8 ml of 60% H_2SO_4 and the final volume completed to 50 ml with deionized distilled water, left for 10 mins in oven at 110ºC. The appearance of brown spots was considered as positive test for the glycolipid biosurfactant. The spots were compared with standard RL and according to the R_f value which is rate of retention flow calculated by the formula:

R_f= Distance of sample spot/ Distance of solvent (42).

Fourier Transform Infrared Spectroscopy (FTIR) spectral analysis

The functional groups and chemical bonds were illustrated using FTIR spectrometry (Shimadzu 8400, Japan). The spectrum was limited at the range of 4000-650 cm^{-1} with resolution of 4 cm^{-1}

Surface tension measurement

The surface tension of an aqueous solution was measured by the Wilhelmy platinum plate with a QBZY-2 Tensiometer (China). Fifteen ml of supernatant was poured into 50 ml glass beaker and put onto the tensiometer platform. The measurement was carried out at 25±1 C after dipping the plate in the solution until monitoring the value of supernatant surface tension following the procedure of measurement written in the manual of the

instrument. Between each measurement, the Wilhelmy plate was rinsed with acetone and burned by alcohol burner. For more accurate value, the average of three records was used in the study (32).

Critical Micelle Concentration (CMC) and critical micelle dilution (CMD)

For CMC and CMD analysis, produced RL were subjected to serial twofold dilutions then measuring the surface tension. CMC is the minimum concentration that reduces the surface tension to the lowest level, while CMD is the maximum dilution that reduces the surface tension to the bottom level (7). The CMD was determined by ten-fold dilution, CMD^{-1} and CMD^{-2}. For measuring critical micelle concentration (CMC) of rhamnolipid after purification, concentrations range (5-500 mg/L) was prepared at room temperature (23). For a positive control, the CMC of sodium dodecyl sulfate (SDS) (100-10,000 mg/L) was prepared while the negative control is the distilled water. The CMC as well as the surface tension at the point of CMC were specified from the cut-point in the surface tension of these solutions and recorded (31).

Dry weight cell determination

After cultivation for 5 days, the fermentation broth was centrifuged at 8,500 g for 20 min to remove bacterial cells. 10 mL of the supernatant was taken to detect emulsification index (E_{24}) and surface tension. The collected bacterial cells allowed to dry in oven to obtain the dry weight, which is reported in terms of g/L (6).

Measurement of antibacterial activity of mono-rhamnolipid

Antimicrobial activity of purified mono-RL was determined according to values of minimum inhibitory concentration (MIC), which is the lowest concentration of biosurfactant needed to inhibit the visible growth of a given organism on Mueller Hinton agar after incubation time. A method of two-fold serial dilution of rhamnolipid was used to quantify antimicrobial activity. The process was assessed against *S. aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Streptococcus iniae*, *E. coli*, *Serratia marcescens* and *P. aeruginosa*. The susceptibilities were specified by micro-dilution technique using 96 well plates (U-shaped). A volume of 200ul of stock solution (µg/ml) for each rhamnolipid dissolved in Mueller-Hinton broth (MHB) was transferred into the first well of each row and two-fold serially diluted in subsequent wells by mixing with 100ul of MHB. A bacterial suspension of 100ul (1×10^6 cfu/mL) was added to these wells to extent final inoculum size of 5×10^5 cfu/well and incubated for 24 h at 37°C. The plates tested for visible growth and compared with rhamnolipid-free control wells. The MIC determined as the lowest concentration at which the tested microorganism does not pretend visible growth (33). All tests were conducted in triplicate.

Maintenance of cell cultures

Rat embryonic fibroblast (REF) Cell lines were maintained in RPMI-1640 medium supplemented with 10% Fetal bovine, penicillin 100 µg /mL, and streptomycin 100 µg/mL. Cells were passage using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37 °C to perform cytotoxicity assay for the produced rhamnolipid (40).

Cytotoxicity Assays

For determining the cytotoxic effect of mono-RL, the assay of MTT cell viability was done with the aid of 96-well plates. Cell lines were seeded at 1×10^4cells/well. After 24 h. or achieving confluent monolayer, cells were treated with RL. The viability of cells was measured after 72 h of treatment by discarding the medium, adding 28 µL of 2 mg/mL solution of MTT and then reincubating the cells for 2.5 h at 37 °C. After removing the MTT solution, the crystals remained in wells were solubilized through the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by incubation for at 37 °C for 15 min in oscillatory shaker (1). The absorbency at 492 nm (test wavelength) was determined on a microplate reader; the assay was conducted in thrice. The rate of cell growth inhibition (the percentage of cytotoxicity) was calculated as the following equation:-

Cytotoxicity = A-B/A *100

Where A and B are the optical density of control and test, respectively

Synergistic effect with antibiotics

The purified mono-RL was tested for synergistic effect with some antibiotics against selected pathogenic isolates in well-plate method after combination, such as ampicillin, cefotaxime, gentamycin, azithromycin, tetracycline, vancomycin. Ciprofloxacin was used as positive test for antibiotic activity against same isolates. Both MIC and CMC of RL were mixed with same concentration of antibiotic discs used in this experiment and placed in pre-cut wells of Muller Hinton agar cultured with pathogenic agents.

RESULTS AND DISCUSSION

Production of RL in bioreactor

After 120 h of incubation at 30ºC with steady favorite conditions, the surface tension of solution reached its minimum value (27.2 mN/m) with emulsification activity 67% and biomass 2.7 g/l. The RL concentration was 16 g/l (Figure 1).

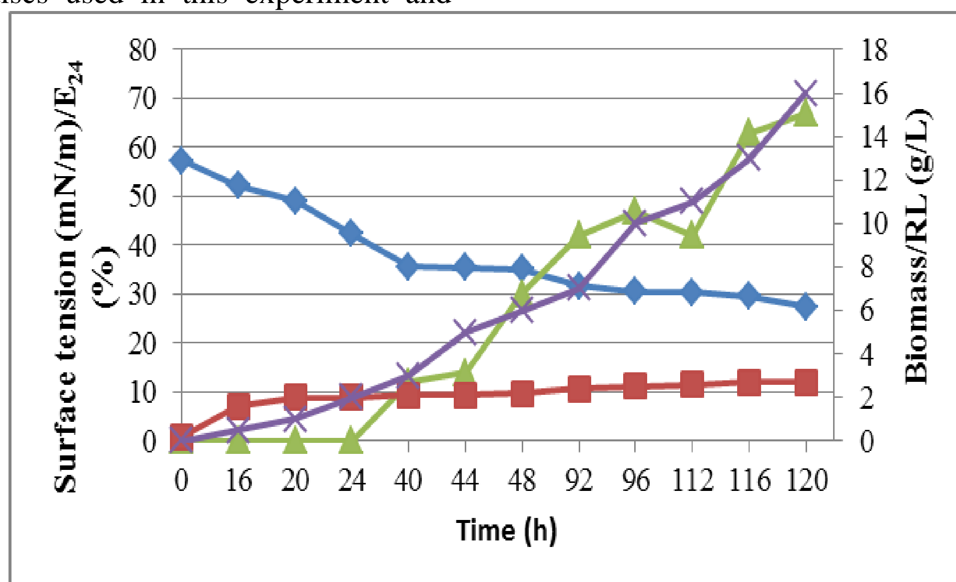

Figure 1. Production of RL by *P. aeruginosa* A3 in bioreactor; biomass(■); RL, (×); surface tension, (◆); E24%, (▲)

The dry cell weight was nearly constant after 24 hrs of production till the end of the experiment, while there was a continual increasing in RL production as well as emulsification index, whereas the surface tension decreased gradually. This experiment confirms that RL is a secondary metabolite. The maximum RL concentration produced by *P. aeruginosa* in a lab-scale fermenter was 22.5 g/L with biomass concentration was 5.3 g/L (3), while Noh *et al*., (24) obtained 23.6 g/L of RL from *P. aeruginosa* USM-AR2.

Analysis of biosurfactant by thin layer chromatography

After purification of crude RL by column chromatography, the separated fractions were analyzed using TLC. The spots appeared on the TLC plates were corresponding to rhamnsoe domains (R_f value 0.41 and 0.17) which represent mono- and di-rhamnolipids respectively (Figure 2). This indicates that this isolate (*P. aeruginosa* A3) produce a mixture of mono- and di-RL. The preliminary characterization of this type of biosurfactant was a glycolipid (13). Three distinct peaks (0.17, 0.34 and 0.53) were observed in the study conducted by Bharali *et al*., (5) to produce RL from *P. aeruginosa* grown on biodiesel, while only two spots (R_f of 0.9 and 0.6) observed by Das *et al*., (10) for mono and di-RL, respectively. These differences in R_f values is correlated to the congeners of the biosurfactant mixture depending on the production isolate and growth conditions.

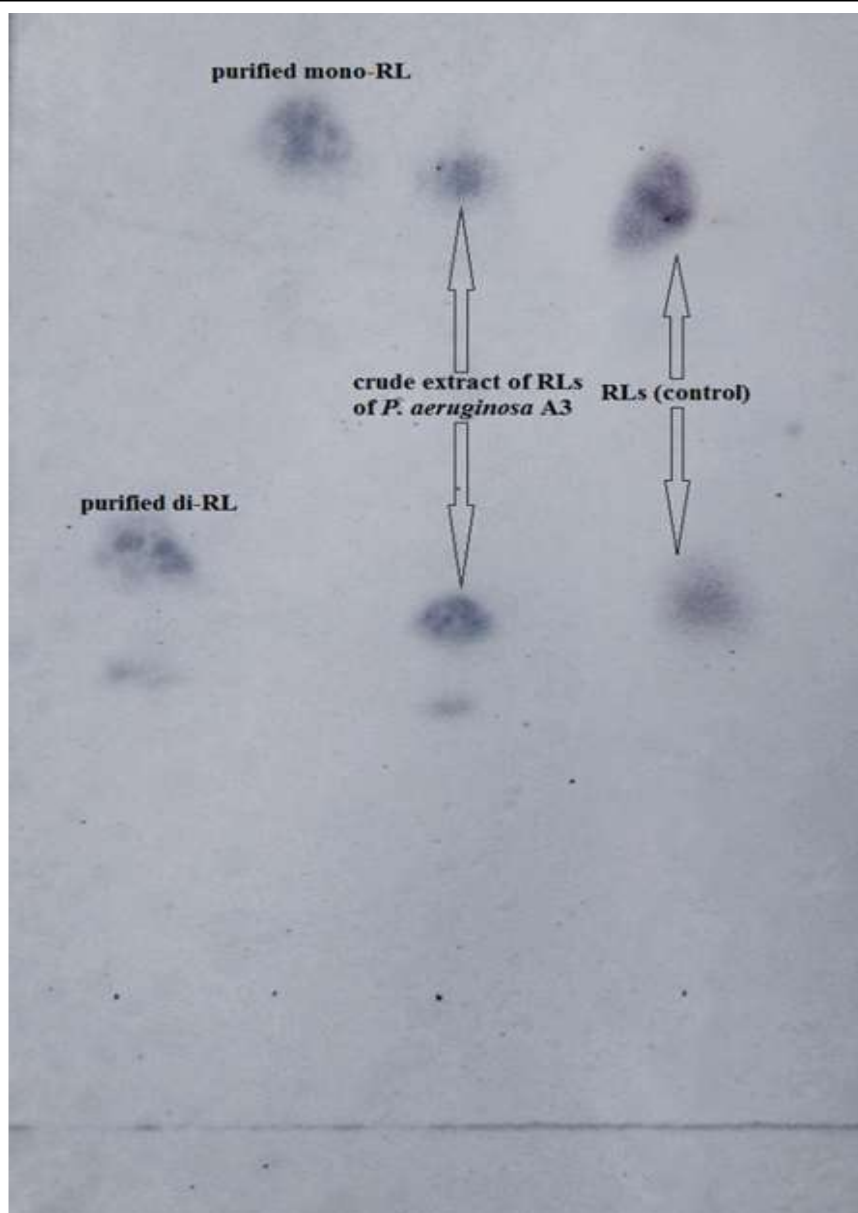

Figure 2. Analyzing of rhamnolipid by TLC

Fourier Transform Infrared Spectroscopy (FTIR) spectral analysis

Many major peaks are appeared in the spectrum of mono-RL, which are the (C-H) model between 2825 and 2954 cm-1, and the carboxylic acids extending between 1500 and 1740 cm-1. The broad peak at 1737 cm-1 comprises both the carbonyl (C=O) of the ester group, and the carboxylic acid part. At 1560 cm-1, the band corresponds to the COO of the rhamnolipid. The region below 1500 cm-1 contains overlapping peaks assigned to specific frequencies of RL group. Peaks at 1461 and 1379 cm-1 related to the (CH) modes, and the bands series between 1100 and 1300 cm-1 are associated with ester vibrations (C-O-C) (Figure 3). The total mono-RL concentration can be related to the absorbency at 1738 for the ester (C=O) band (17). The C-H and O-H groups refer to the rhamnose molecules of RL (18).

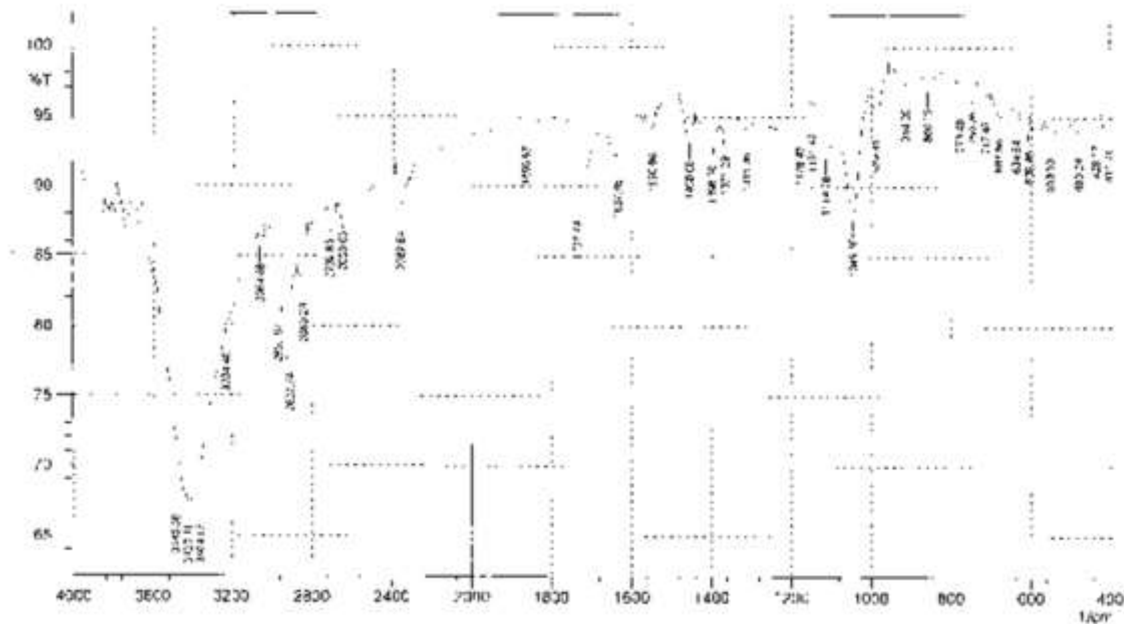

Figure 3. Characterization of RLs by FTIR, A: mono-RL, B: di-RL

Critical Micelle Concentration (CMC)

The mono-RL produced by *P. aeruginosa* A3 was able to reduce the surface tension of distilled water to 27 mN/m when the CMC value is 40 mg/L. This means that only 40mg/L of mono-RL is needed to reduce the surface tension to the minimum level. These values were different with Samadi *et al*., (33) who found that 20 mg of RL was able to reduce the surface tension to 24mN/m. However; the synthetic surfactants generally had a higher CMC values that RL. Sodium dodecyl sulfate show CMC of 2200 mg/L. The results have shown the effectiveness of mono-RL produced by *P. aeruginosa* A3 isolate.

MIC

It was obtained that 250 µg/mL was the MIC for most of bacterial used in the experiment; however, there's no MBC effect for the rhamnolipid even when the concentration was 1 g/L. This confirms that no bactericidal effect for the RL (Figure 4). These results are consistent with the cytotoxicity of RL on REF cell line, where the applicable toxic concentration was 23% corresponding to 250 µg/ml. The MIC of Mono-RL produced from *P. aeruginosa* MN1 against several Gram-positive bacteria was 25 mg/L, while it needed more than 1600 mg/L to affect the Gram-negative bacteria. However, RL-mixture and di-RL needed 50 mg/L to inhibit the Gram-positive bacteria (33).

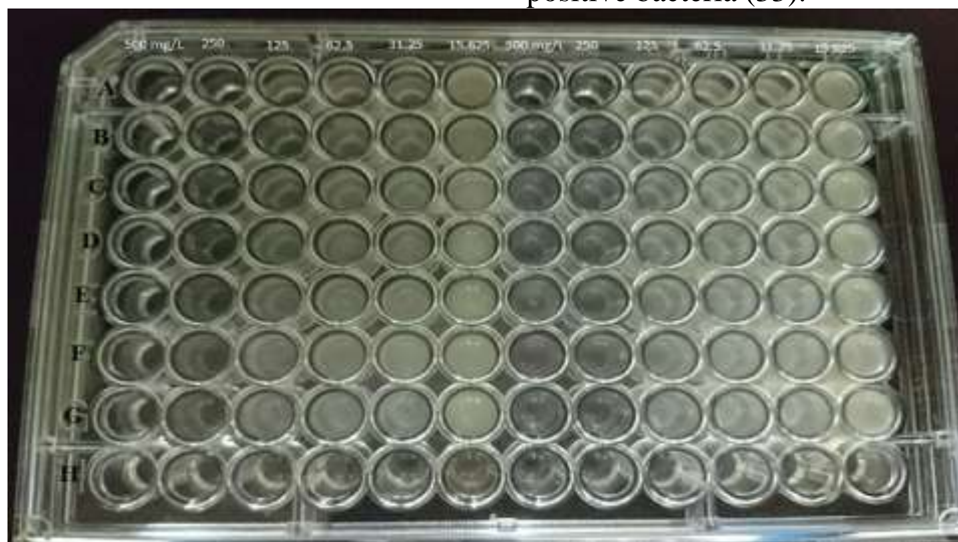

Figure 4. The effect of purified mono-RL on growth of bacterial cells, A, *S. aureus*; B, *E. coli*; C, *Serratia marcescens*; D, *Streptococcus iniae*; E, *Klebsiella pneumoniae*; F, *Proteus mirabilis*; G, *P. aeruginosa*, H, control.

Cytotoxicity effect

The cytotoxicity of mono-RL in concentration of 250 µg/mL (the same concentration used for MIC experiment) was 23% on REF cell line (Figure 5), this concentration is considered as acceptable for animal cell line because of low toxicity which gives a strong indication that mono-RL could be used as antimicrobial therapy. Also, the higher concentrations (500 and 1000 µg/mL) are less than 50% toxicity, which gives an indication about the high difference between the toxic dose and effective dose for antimicrobial therapy.

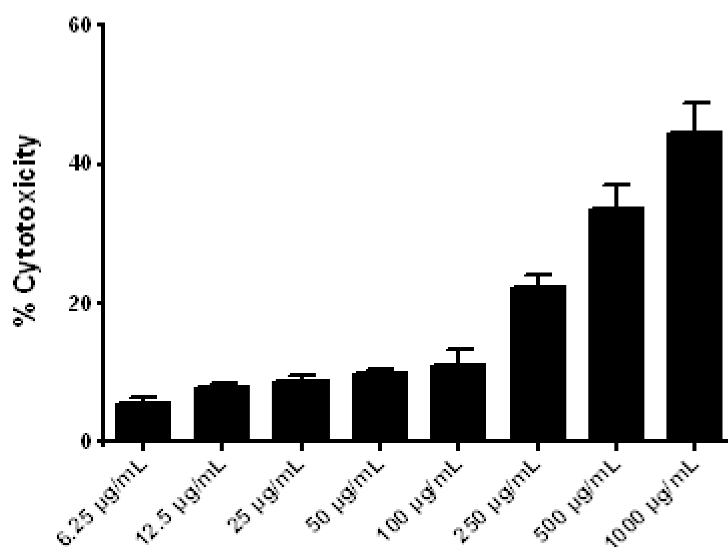

Figure 5. Cytotoxicity effect of mono-RL in REF cell line

Synergistic effect of mono-RL with antibiotics

The requirement for new antimicrobial agents to beat bacterial antibiotic-resistance leads to investigation for modern antimicrobial strategies. Thus, the combination of different antimicrobial compounds in order to increase their efficacy is considered as a magnificent solution for the problem. A synergistic association is when two or more agents act to enhance efficacy compared with single ones (11). Probably, the biosurfactant interfere with bacterial membranes and forming pores that permit the entrance of antibiotics into the cells, which increase the antimicrobial agent activity, owing to the amphiphilic nature of the surfactants, which destabilize lipid packing of biological membranes, altering integrity and penetrate these coats through hydrophobic interactions, that lead ultimately to an increase of the antibiotic impact (15). The best combination effect was found when clinical isolates were resistant to both mono-RL and ampicillin, but sensitive when these two antimicrobial agents were combined together in their MIC (Table 1 and Figure 6) (8, 9). A similar study conducted to demonstrate that concentrations of RL lower than CMC could increase the quantity of anionic phospholipids in membranes of *B. subtilis* 168 that lead to more sensitivity to sulfonates antimicrobials (39). The major differences in the composition of phospholipids in different bacterial species, and this will ultimately determine their response to antimicrobial compounds. A research conducted by Sotirova *et al.*, (38) was demonstrated that RL in concentration beneath the CMC could decrease the number of outer mambrane proteins (OMPs) of *P. aeruginosa* NBIMCC 1390 that lead to changes in pathogenisity and antibiotic sensitivity. These effects include the proteins composition and ultimately alter the membranous organization of pathogens under investigation. Any reduction in the OMPs could decrease the tightness of the outer membrane and, finally, increasing the passage of hydrophobic compounds, but still has no effect on cell viability, unless accompanied with another bactericidal agent.

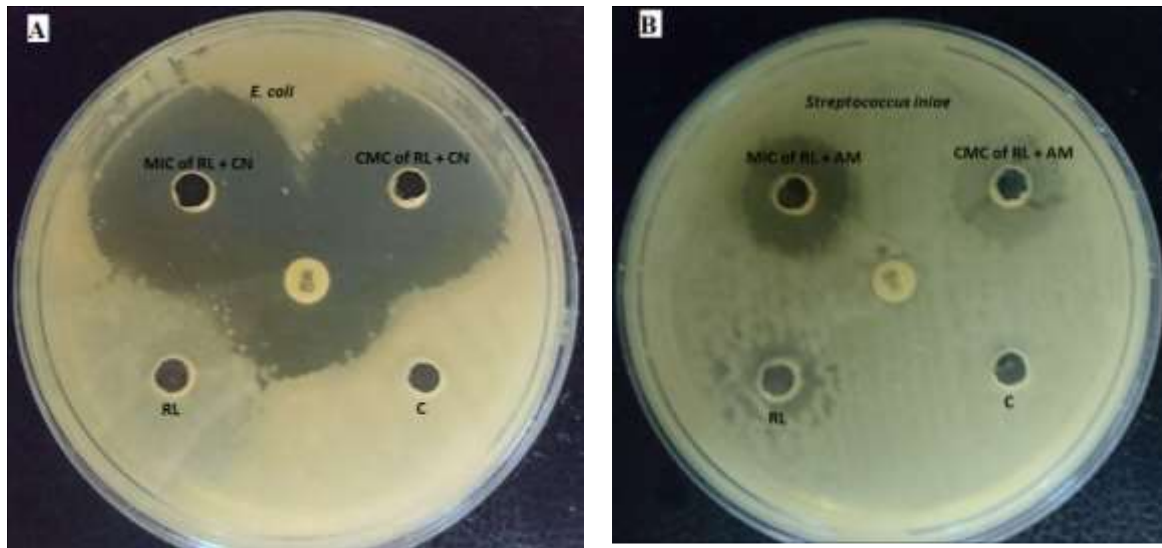

Figure 6. Combination effect of mono-RL with antibiotics against clinical isolates A: *E. coli*, B: *Streptococcus iniae*, CN: gentamycin, AM: ampicilin, C: control

Table1. Synergistic effect of mono-RL with different antibiotics

	Ampicillin disc (25µg/ml)	Mono-RL (250µg/ml)	MIC combine (250µg/ml)	CMC of RL combine (40µg/ml)
S. aureus	S	R	S	S
E. coli	R	R	S	S
Serratia marcescens	R	R	S	S
Streptococcus iniae	R	R	S	I
Klebsiella pneumoniae	S	R	S	S
Proteus mirabilis	S	S	S	S
P. aeruginosa	I	I	S	S
	Cefotaxime disc (30µg/ml)	**Mono-RL**	**MIC combine**	**CMC of RL combine**
S. aureus	I	R	S	S
E. coli	R	R	S	S
Serratia marcescens	R	R	S	R
Streptococcus iniae	S	R	S	S
Klebsiella pneumoniae	R	R	S	S
Proteus mirabilis	S	S	S	S
P. aeruginosa	R	I	S	S
	Gentamycin disc (30µg/ml)	**Mono-RL**	**MIC combine**	**CMC of RL combine**
S. aureus	R	R	S	S
E. coli	S	R	S	S
Serratia marcescens	S	R	S	S
Streptococcus iniae	I	R	S	I
Klebsiella pneumoniae	S	R	S	S
Proteus mirabilis	S	S	S	S
P. aeruginosa	S	I	S	S
	Azithromycin disc (15µg/ml)	**Mono-RL**	**MIC combine**	**CMC of RL combine**
S. aureus	S	R	S	S
E. coli	S	R	S	S
Serratia marcescens	S	R	S	S
Streptococcus iniae	I	R	S	I
Klebsiella pneumoniae	R	R	S	S
Proteus mirabilis	S	S	S	S
P. aeruginosa	S	I	S	S
	Teteracyclin disc (30µg/ml)	**Mono-RL**	**MIC combine**	**CMC of RL combine**
S. aureus	R	R	S	I
E. coli	R	R	S	S
Serratia marcescens	R	R	S	I
Streptococcus iniae	I	R	S	I
Klebsiella pneumoniae	I	R	S	I
Proteus mirabilis	S	S	S	S
P. aeruginosa	I	I	S	S
	Vancomycin disc (30µg/ml)	**Mono-RL**	**MIC combine**	**CMC of RL combine**
S. aureus	S	R	S	S
E. coli	R	R	S	S
Serratia marcescens	R	R	S	S
Streptococcus iniae	R	R	S	I
Klebsiella pneumoniae	R	R	S	I
Proteus mirabilis	R	S	S	S
P. aeruginosa	R	I	S	S

R: Resistance, S: Susceptible, I: intermediate

Lipopolysaccharide has an important role in the resistance of bacteria to antimicrobial agents (30). The type and amount of LPS is a crucial factor in the interactions between bacteria and their environment (20). Any loss in LPS due to exposure to RL, even at low concentrations, resulted in increasing of cell surface hydrophobicity (2). The liberation of LPS form cell surface happened after solubilization of the outer membrane due to binding and aggregating of biosurfactant on the membrane (38). Applying RL in concentration much below its CMC could affect the composition of bacterial membranes by increasing their permeability, and, resulting in bactericidal effects of antibiotics at lower concentrations than the accepted worldwide (39)**.** In this study, the optimum conditions for RL production by local *Pseudomonas aeruginosa* isolate was conducted in a bioreactor for industrial-scale production to obtain 16 g/L of biosurfactant. Extraction and purification carried out to conclude that this RL is consists of both mono- and di-RL. The antibacterial activity and cytotoxicity of mono-RL was performed to assign specific concentration for further experiments, which was 250 µg/ml; this concentration was combined with some antibiotics to overcome the resistance of some clinical isolates to such antibacterial agents.

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