Malays. Appl. Biol. (2019) 48(3): 149–156

PRODUCTION AND TOXICITY EVALUATION OF RHAMNOLIPIDS PRODUCED BY *Pseudomonas* STRAINS ON L6 AND HepG2 CELLS

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Accepted 22 May 2019, Published online 30 June 2019

ABSTRACT

Biosurfactant rhamnolipid (RL) production using renewable resource is gaining attraction for commercial application. In this study, RL produced from three different strains of *Pseudomonas* using glycerol as a carbon source was used to evaluate toxicity towards rat skeletal muscle (L6) and liver cancer (HepG2) cells. In the present study, *Pseudomonas aeruginosa* PAO1 produced the highest concentration of RL (1.53 ± 0.28 g/L) and able to reduce the surface tension (ST) value of water the lowest (29.1 ± 0.5 mN/m). Toxicity evaluation using MTT assay indicated that RL produced does not have a cytotoxic effect towards both cell lines except where 50% inhibition concentration (IC_{50}) was detected for HepG2 only at high concentration (100 µg/mL) for RL produced by *P. aeruginosa* PAO1. The RL produced by strains in this study is nontoxic with good ST reducing ability that has potential applications in food, cosmetics and pharmaceutical sector.

Key words: *Pseudomonas,* biosurfactant, rhamnolipid, cytotoxicity, marine bacteria

INTRODUCTION

Surfactant is an amphiphilic molecule that possesses distinct hydrophobic and hydrophilic molecules (Nitschke & Costa, 2007; Gudina *et al*., 2013). Complications from chemical surfactant usage such as the sustainability of manufacturing and harm towards the environment enabled biosurfactant to be viewed as an alternative (Otzen *et al*., 2016). Rhamnolipid (RL) is predominantly reported to be produced by *Pseudomonas* strains but recently other bacterial strains are found to produce RL (Abdel-Mawgoud *et al*., 2010). RL is a glycolipid biosurfactant that is composed of β-hydroxy fatty acid connected to rhamnose sugar molecule through carboxyl end (Deziel *et al.,* 2000; Sekon & Rahman, 2014). Studies on RL application varies from enhanced oil recovery, cosmetics, pharmaceutical, food, agriculture and household items (Piljac & Piljac, 2007; Singh *et al.,* 2007; Long *et al.,* 2013; Parry *et al.,* 2013; Sekhon & Rahman, 2014). The utilization of RL has been approved by the United States Food and Drug Administration as food additive (rhamnose moiety) (Nitschke & Costa, 2007). Evaluation of toxicity level of a bioactive compound are crucial prior to development of cosmetic products, food and pharmaceutical application (Piljac & Piljac, 2007; Parasuraman, 2011). Various studies have been conducted to determine the toxicity of RL where the toxicity of RL was evaluated using HepG2, Caco-2, Hela, MCF-7, HK-2 cell lines (Loftabad *et al*., 2010; Jiang *et al*., 2014). Moreover, RL produced by *P. aeruginosa* strains has been demonstrated to elicit the same level of cytotoxicity towards normal (HK-2 cell, primary hepatocyte) and cancer cell (HepG2, Caco-2, Hela, MCF-7 cells) (Jiang *et al.,* 2014). The toxic effect is due to the ability of RL

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to reduce surface tension (ST) of medium rather than changing the specific molecular structure that prevents selection of non-cancer cells. However, studies by Jiang and partners (2014) is starkly in contrast to results of the previous study that have proved that RL has higher toxicity sensitivity on human cervical cancer cells (Lotfabad *et al*., 2010. This preliminary study was done to evaluate the toxic effect of RL produced by different strains of *Pseudomonas.*

MATERIALS AND METHODS

Bacterial strains

Pseudomonas strains used for RL production are *P. aeruginosa* PAO1, *P. aeruginosa* UMTKB-5 and *P. putida* MAD32. All strains were maintained by streaking them onto nutrient-rich (NR) agar weekly.

Bacterial growth curve

The bacterial growth curve was plotted to determine the suitable time for carbon source introduction for RL biosynthesis. The growth curve was plotted for 24 hours and optical density (OD_{-660}) reading was taken at every 2 hours interval using UV-spectrophotometer SHIMADZU UV-1601 (Shimadzu, Japan) (Widdel, 2007).

Biosynthesis of RL

Production of RL was done in 1 L conical flask containing 200 mL working volume. Mineral salt medium (MSM) which contains 2.80 g/L KH_2PO_4, 3.3 g/L Na_2HPO_4 and 0.25 g/L $CO(NH_2)_2$ was supplemented with 200 µL of trace element, 200 µL of 0.25 g/L $MgSO_4.7H_2O$. This was followed by the addition of 20 g/L of glycerol and 7% (v/v) of precultured bacterial cells. The culture was incubated for 72 hours with shaking at 200 rpm, 30°C using rotatory incubator shaker Ecotron CH-4103 (INFORS HT, Switzerland). After 72 hours, RL containing supernatant was harvested by centrifuging the culture at 8000 rpm (4°C, 5 min) using HIMAC CR 22N (Hitachi, Japan) (Yin *et al.,* 2009).

Quantification of RL

Quantification of RL concentration in the supernatant was done using orcinol assay (Abdel-Mawgoud *et al.,* 2011). 400 µL of supernatant was added with 750 µL of diethyl ether (AR Grade). The mixture is vortexed, and the RL containing upper ether fraction was isolated into a microcentrifuge tube. This process was repeated twice. After leaving the tube containing upper fraction to dry overnight, 400 µL of pH 8 phosphate buffer was added into the tube, 100 µL of this mixture was mixed with 900 µL of orcinol assay and placed into the water bath (80°C, 30 minutes). Then, the solution was left in a dark place to cool down before absorbance reading was taken at 421 nm (Ballot, 2009). The absorbance reading was multiplied by a factor of 2.25 to determine RL concentration to consider the lipid portion and relative proportion of congeners of RL (Déziel *et al*., 2000).

ST measurement

ST of RL containing supernatant was measured using a platinum ring probe method (Abdel-Mawgoud *et al.,* 2011). Triplicate reading of ST measurement is recorded using tension meter Sigma-701 (Attension, Finland).

Extraction of RL

Extraction of RL from the supernatant was conducted as described by Yin *et al.* (2009). The pH of the supernatant was measured using Accumet *Basic, AB 15* (Fisher Scientific, Switzerland) and reduced to pH 2 using 6 M HCl. Ethyl acetate (AR Grade) was added to the supernatant in separating funnel at 1:1 ratio. The upper fraction was taken after shaking the funnel vigorously. This step was repeated twice, and the upper fraction was added with approximately 10 spatulae of anhydrous sodium sulphate (AR Grade) to 1 L of extract to remove excess moisture. Crude RL was separated using Buchi R200 (Buchi, Japan) rotatory evaporator at 40°C under vacuum pressure. Viscous RL obtained was dissolved in 0.05 M sodium bicarbonate. The pH of the solution was adjusted to pH 2 using 6 M HCl. The solution was kept at 4°C for 24 hours before being centrifuged at 12500 rpm for 15 min. The precipitate obtained was frozen at -80°C overnight in MDF-U537 (Sanyo, Japan) Biomedical Freezer before being lyophilized using Freezone 4.5 Freezer Dry System (Labconco, USA).

Cell line and toxicity evaluation

Rat skeletal muscle (L6) and liver hepato-cellular carcinoma (Hep G2) cell were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Gibco, USA) and Minimal Essential Medium (MEM) (Gibco, USA) respectively and supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin streptomycin (Senthilraja & Kathiresan, 2015). Maintenance and subculture of the cell line were done until suitable cell confluency (80%) was achieved. Cells were seeded into a 96-well plate at a concentration of 8.0×10^4 cell/mL. RL was dissolved in distilled water and added at different concentration and incubated for 24 hours. The concentration of RL tested was within the range of that by the study of Jiang and colleagues (2014). The viability of cell was

determined using 3-[4,5-dimethylthioazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT) solution where 20 µL of the solution was added into each plate and incubated for 4 hours in 5% CO_2 incubator (ESCO, Germany) (Freshney, 2010). Absorbance reading was taken at 570 nm using a microplate reader *Multiskan Ascent* (Thermo Lab, Finland) and standard curve of cell viability (%) against sample concentration was plotted. Inhibition concentration at fifty percent (IC_{50}) value was determined (Jiang *et al.,* 2014; Senthilraja & Kathiresan, 2015).

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ treated\ cell}{Absorbance\ of\ untreated\ cell} \times 100\%$$

Statistical analysis

All the data in this experiment were analyzed and presented as means ± standard deviation. The data were evaluated statistically using One-Way ANOVA and Post Hoc (Tukey's Test) using SPSS software.

RESULTS

The RL produced by different strains of *Pseudomonas* exhibited different yield and ST values. Table 1 shows the summarized results of RL production. The highest concentration of RL (g/L) is produced by *P. aeruginosa* PAO1 (1.53 ± 0.28 g/L) and has reduced the ST (mN/m) the lowest (29.1 ± 0.5 mN/m) compared to *P. aeruginosa* UMTKB-5 and *P. putida* MAD32.

Cell viability (%) upon RL treatment

Figure 1 to 3 depicts the comparison of cell viability (%) between L6 and HepG2 cell line which was treated with the different concentration of RL produced by the same strains of *Pseudomonas* bacteria. Overall, it can be observed that the cell viability at test concentration is not significantly different within each respective group (Tukey's HSD test $p < 0.05$). However, it can be observed in Figure 2 that the cell viability is less than 50%

Table 1. Production of RL by *Pseudomonas* strains using glycerol as carbon source

Strain	RL[a] (g/L)	CDW[b] (g/L)	$Y_{RL/CDW}$[c] (g/g)	$Y_{RL/S}$[d] (g/g)	ST (mN/m)
P. aeruginosa UMTKB5	1.04 ± 0.14	0.42 ± 0.01	2.46	0.05	30.1 ± 0.2
P. aeruginosa PAO1	1.53 ± 0.28	0.48 ± 0.02	3.25	0.08	29.1 ± 0.5
P. putida MAD32	0.16 ± 0.02	0.33 ± 0.02	0.44	0.01	45.7 ± 2.3

[a] RL concentration quantified using orcinol assay, [b] CDW, Cell dry weight, [c] $Y_{RL/CDW}$, yield of per gram of RL per gram cell CDW, [d] $Y_{RL/S}$, Yield of per gram of RL per gram of substrate. (N=3)

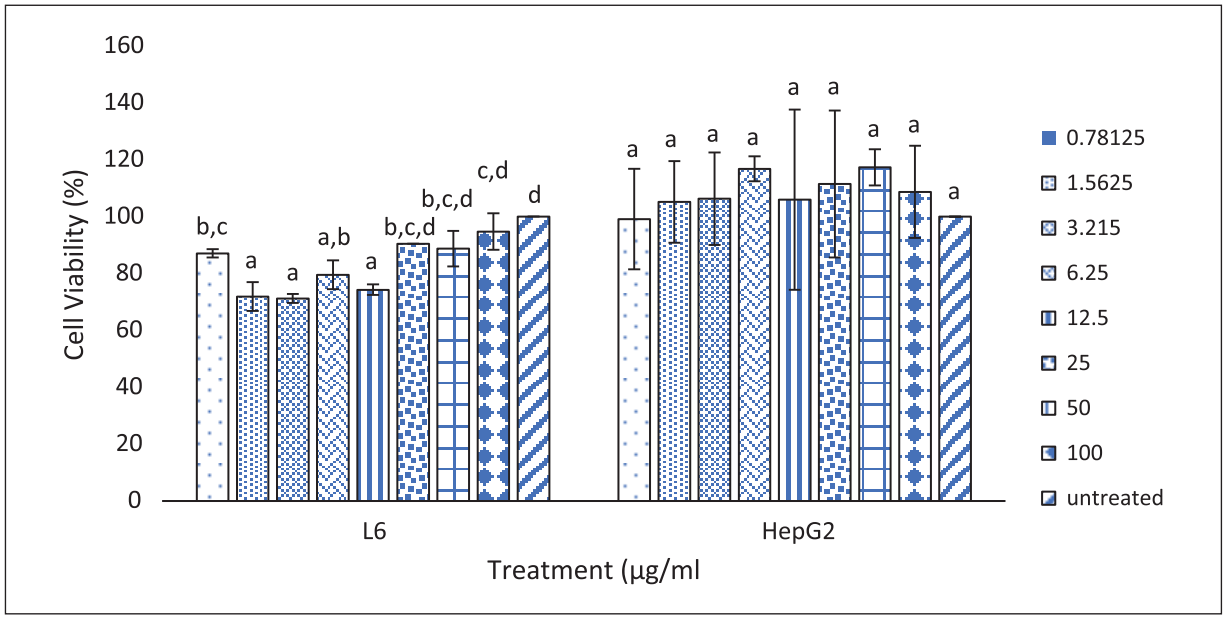

Fig. 1. The cell viability of L6 cells (%) and HepG2 (%) against RL (µg/mL) produced by *P. putida* MAD32. The value is a mean of three replicates. Mean data accompanied by different letters indicates significant difference within each representative group (Tukey's HSD test, $p < 0.05$).

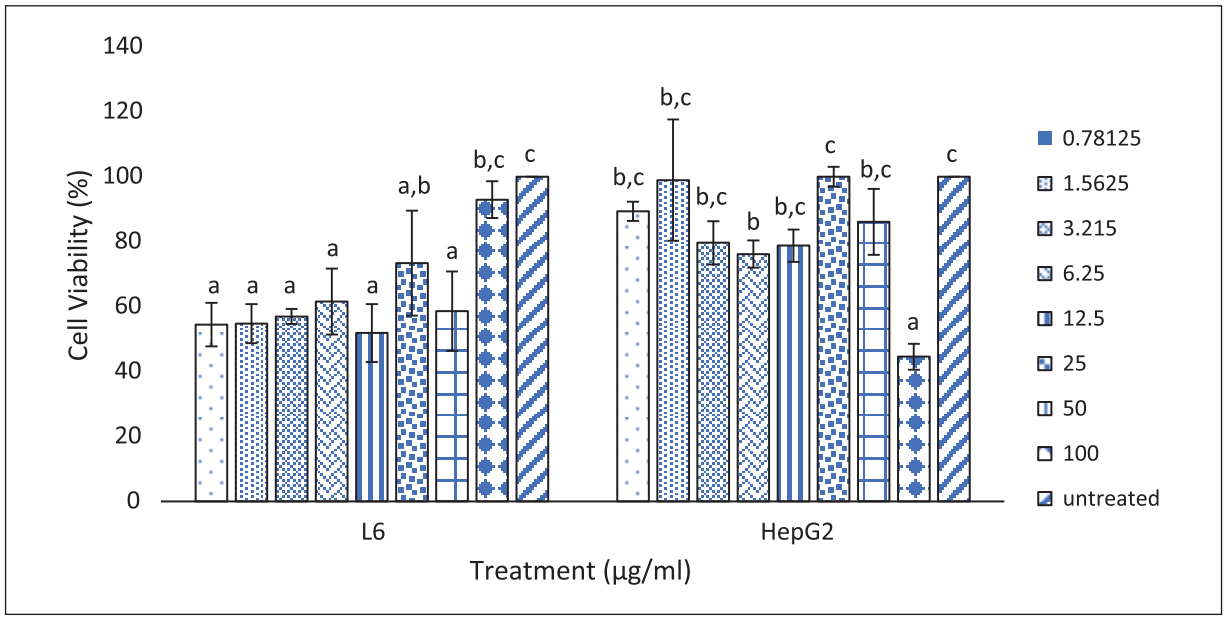

Fig. 2. The cell viability of L6 cells (%) and HepG2 (%) against RL (µg/mL) produced by *P. aeruginosa* PAO1. The value is a mean of three replicates. Mean data accompanied by different letters indicates significant difference within each representative group (Tukey's HSD test, $p < 0.05$).

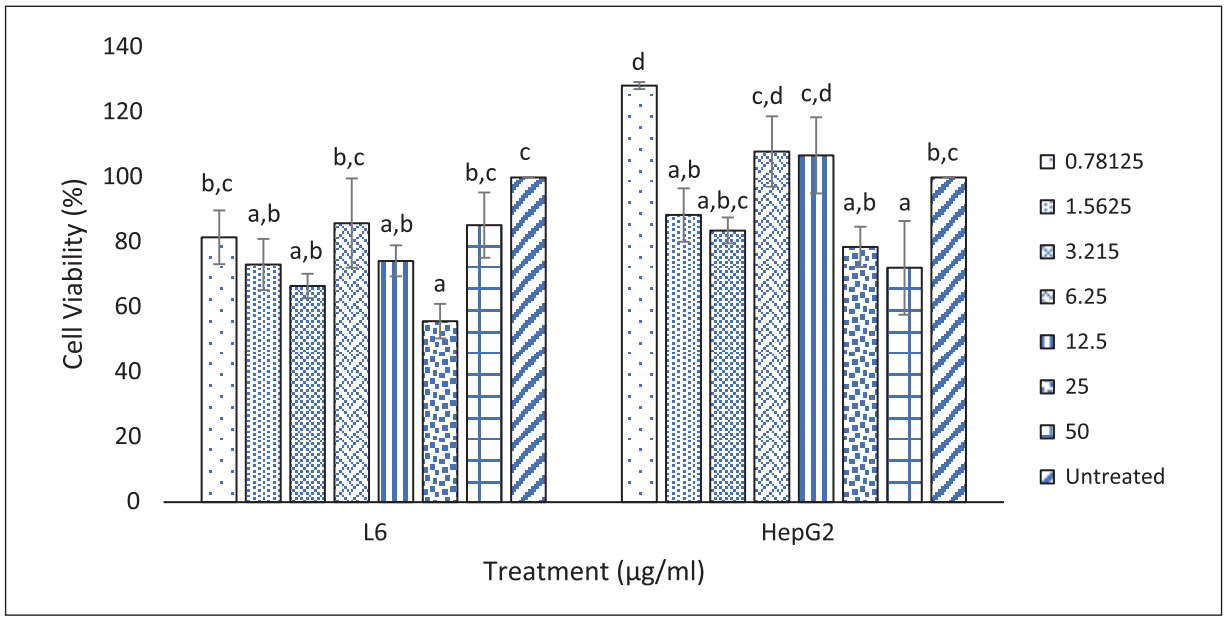

Fig. 3. The cell viability of L6 cells (%) and HepG2 (%) against RL (µg/mL) produced by *P. aeruginosa* UMTKB-5. The value is a mean of three replicates. Mean data accompanied by different letters indicates significant difference within each representative group (Tukey's HSD test, $p < 0.05$).

at a concentration of 100 µg/mL for HepG2 cells (Tukey's HSD test $p < 0.05$).

The percentage of inhibition (%) of cells upon RL treatment

Figure 4 to 6 depicts the comparison of the percentage of inhibition of L6 and HepG2 cell (%) against the log of RL concentration (µg/mL) based on a treatment by RL produced by same *Pseudomonas* strains. There is no graph available for HepG2 cell treated by *P. putida* MAD32 since there was no inhibition activity observed. Generally, the percentage of inhibition values (%) as less than 50%, therefore, IC_{50} concentration is not able to be determined. Furthermore, there is a relatively small difference in inhibition value as the concentration of RL increases and there is no significant difference between normal cell (L6) and cancer cell (HepG2).

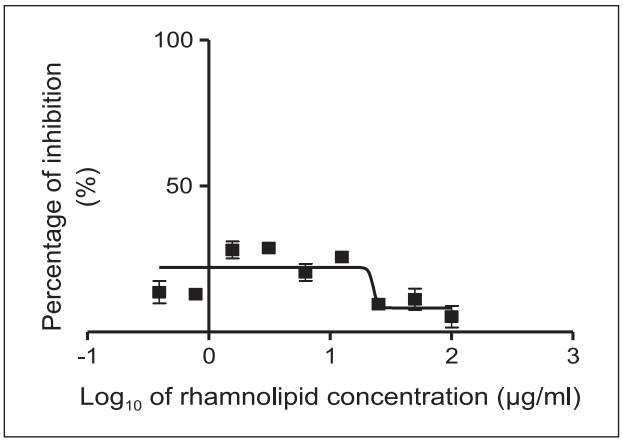

Fig. 4. The percentage of inhibition of L6 cells (%) against log of RL (µg/mL) by produced *P. putida* MAD32.

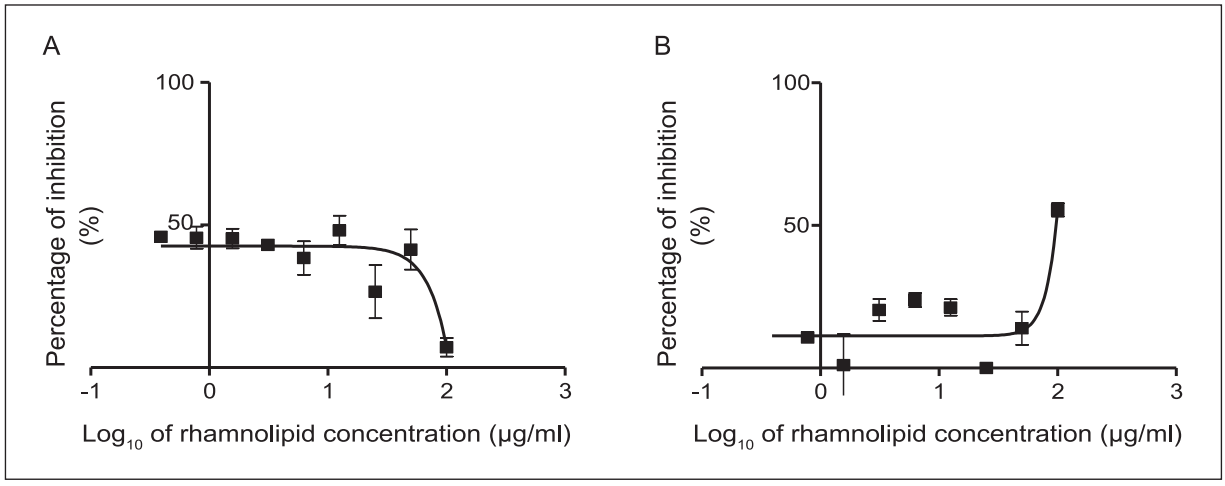

Fig. 5. The percentage of inhibition of (A) L6 cells (%) and (B) HepG2 cells (%) against log of RL (µg/mL) produced by *P. aeruginosa* PAO1.

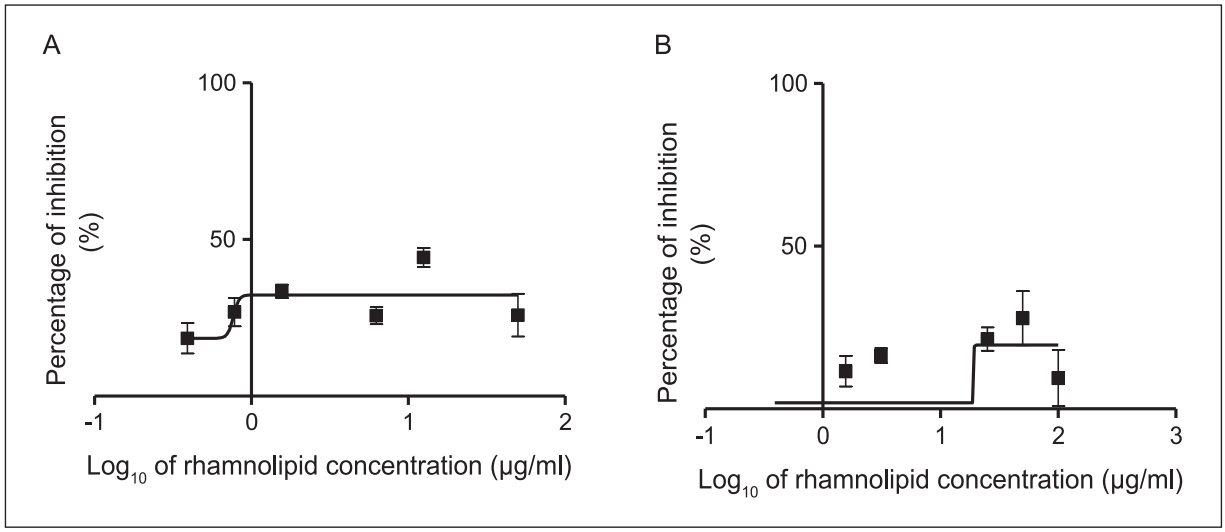

Fig. 6. The percentage of inhibition of (A) L6 cells (%) and (B) HepG2 cells (%) against log of RL (µg/mL) produced by *P. aeruginosa* UMTKB-5.

However, IC_{50} value can be observed for HepG2 cell treated with RL produced by *P. aeruginosa* PAO1 in Figure 5(B) where the value is almost at 100 µg/mL.

DISCUSSION

The production of RL is higher by *P. aeruginosa* PAO1 and UMTKB-5 compared to *P. putida* MAD32. A similar trend was observed in previous studies where RL production by *P. putida* strains are low (Wittgens *et al.,* 2011). The RL concentration produced by *P. aeruginosa* UMTKB-5 in this study was comparable to the previous study which uses the same strain (Rashid *et al.,* 2015). However, the cell dry weight (CDW) of this study is three times lower than the study by Rashid *et al*. (2015). The $Y_{RL/CDW}$ in this study is much higher indicating higher RL production per gram of cell. The difference in yield might be due to different nitrogen source used. Urea ($CO(NH_2)_2$) used in this study is known to support the growth of cell to higher biomass (Lee *et al.,* 2004; Azemi *et al.,* 2016).

ST activity occurs when the RL is able to reduce the intermolecular force between liquid molecules (Satpute *et al*., 2008; Azemi *et al*., 2016). According to Willimsen and Karlson (1996), good biosurfactant can reduce the ST of water by 20 mN/m. Later, Mulligen (2005), stated that a good surfactant can bring the ST of water from 72mN/m to 35 mN/m. Based on the ST readings *P. aeruginosa* PAO1 and UMTKB-5 can be considered good biosurfactants producing bacterial strain. Previous studies have shown that RL produced by *P. aeruginosa* strains can reduce the ST below 30mN/m (Muller *et al*., 2010). Furthermore, the ST values obtained for *P. aeruginosa* PAO1 in the previous study was 29.59 mN/m (Muller *et al*., 2010). These values fall within the range of reading obtained in this study. Based on the results it can be observed that *P. aeruginosa* UMTKB-5 showed as a more effective ST reducing ability than *P. aeruginosa* PAO1 by comparing the RL concentration (g/L) with the ST values (mN/m). This condition might have resulted due to the difference in congener structure of RL which can influence physicochemical properties exhibited (Abdel-Mawgoud *et al*., 2011).

Based on the toxicity study results it can be observed that RL produced by three different strains of *Pseudomonas* does not elicit potent toxic properties. Based on the cell viability results, the cell viability from the lowest to highest concentration did not differ much indicating that RL did not kill more cell as its concentration increases. All the cells treated with RL did not have a significant percentage of inhibition for the determination of IC_{50} values except for HepG2 cells treated with RL produced by *P. aeruginosa* PAO1.

The non-toxic properties of RLs produced by the *Pseudomonas* strains in this study might have been due to different congener of RL produced. The difference in congener structure and composition can affect how the RL affects the cells (Abdel-Mawgoud *et al*., 2011). In the previous study, mono-RL elicits cytotoxicity towards cell line (HepG2, Caco-2, MCF-7, HK-2) at around 100 mg/L while di-RL exhibited cytotoxicity at 150 mg/L towards the same cell type (Jiang *et al*., 2014). Surfactants such as RL have the ability to enhance membrane permeability of cell line and causes damage when the ST drops below a threshold (Xia & Onyuksel 2000; Koley & Bard, 2010). It was reported that ST of the culture medium of less than 41mN/m will elicit cytotoxicity towards cell line (Jiang *et al.,* 2014). The structure of RL will affect the ST properties which influence the cell viability. Jiang *et al*. (2014) concluded that the cytotoxicity of the RL towards the tested cell lines is due to the reduction of ST instead of specific molecular structure changes.

CONCLUSION

The RL obtained in this study still retains the ST reducing ability while being non-toxic. Thus, RL obtained in this study could potentially be utilized for commercial application such as emulsifiers in household products or cosmetics. However further studies are recommended on the characterization of RL congeners and cytotoxicity studies on different cell line to identify the potential industrial application.

ACKNOWLEDGEMENTS

This work was supported by the Exploratory Research Grant Scheme (ERGS) [ERGS/1/2013/STG07/UMT/03/02] awarded by the Ministry of Higher Education (MOHE), Malaysia and funding provided by IMB.

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