**Evaluation of** **antimicrobial and antioxidant activity of Rhamnolipids biosurfactant Produced by *Pseudomonas aeruginosa***

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**ABSTRACT** Rhamnolipids are the glycolipid biosurfactant that are produced by different *Pseudomonas* species, it shows antioxidant and antimicrobial activity. Rhamnolipid biosurfactant was tested as antioxidant agent, the results showed 22.7 %, 47.4 %, 79.8 %, 85 % and 91.4 % of antioxidant activity at the concentrations 5, 10. 15, 20 and 25 mg/ml respectively. Cytotoxicity of the rhamnolipid biosurfactant was examined also at different concentrations against human erythrocytes. Hemolysis of the erythrocytes was observed at concentrations 100, 75, 50, 40 and 35 mg/ml, whereas the results exhibited no hemolysis at concentrations 25 and 15 mg/ ml. The findings of antimicrobial activity showed the rhamnolipid biosurfactant had antimicrobial effect against the microorganisms at different concentrations such as toward *Bacillus cereus* and *Klebsiella pneumoniae*, while lower inhibitory effect toward *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The rhamnolipid biosurfactant was shown lower inhibitory effect against fungal strains *Candida albicans* and *Aspergillus niger*. The lower minimum inhibitory concentration (MIC) values of rhamnolipid biosurfactant toward the investigated microorganisms was 2 mg/ml toward *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and3 mg/ml for *Staphylococcus aureus, Enterobacter cloacae, Bacillus cereus,* *Proteus mirabilis, Candida albicans* and *Aspergillus niger.* The study concluded that rhamnolipid biosurfactant exhibited effective antioxidant activity, no hemolysis at lower concentrations and has high antimicrobial effect.

**KEYWORDS** Rhamnolipid; Antimicrobial; Antioxidant; Cytotoxicity;*Pseudomonas aeruginosa*

**1. Introduction**

The antibiotics abuse has been led to the development of multi-drug resistant pathogens to commercially marketed antibiotics. The combatting of resistant infections emergence is required to search for development of novel antimicrobial drugs having broad spectrum antimicrobial activity (Terreni *et al.* 2021). The problem of the increasing resistance by pathogens to some antimicrobial compounds has paid attention to invest the natural compounds with various mechanisms of action as suitable alternatives to existing antibiotics (Álvarez-Martínez *et al.* 2020). A number of biosurfactants compounds have been exhibited antimicrobial activity against various human pathogenic bacteria, making them a suitable substitute for existing antimicrobial agents as potent therapeutic agents (De Giani *et al.* 2021).

Rhamnolipids are the glycolipid biosurfactant that are produced by different bacterial species such as *Pseudomonas aeruginosa*, *Pseudomonas* *plantarii,* *Pseudomonas chlororaphis*, *Pseudomonas putida, Pseudomonas fluorescens, Burkholderia pseudomallei*, *Burkholderia thailandensis*, *Burkholderia plantarii*, *Burkholderia glumae* and *Serratia rubidaea* SNAU02 (Costa *et al.* 2011; Nalini and Parthasarathi 2013; Irorere *et al.* 2017; Tan and Li 2018).

*Pseudomonas aeruginosa* is major producer of the rhamnolipid biosurfactant, which is widely distributed in the environment that can be available in variety of habitats, where it survives in these habitats due to its extraordinary physiological abilities (Moradali *et al.* 2017). Rhamnolipids are sustainable and show excellent physicochemical properties, which make them interesting for utilizing the rhamnolipids in cosmetic, food, pharmaceutical, and detergent manufacturing (Sekhon Randhawa and Rahman 2014). Rhamnolipids are good food additive as food preservatives and texturizing agents such as antimicrobials, antioxidants, emulsifiers and stabilizers that can be used in food processing (Nitsche and Silva 2018).

Lotfabad *et al. (*2013) reported remarkable inhibitory activity of rhamnolipids biosurfactants against Gram-positive bacteria and two fungi species *Chaetonium globosum* and *Penicillium funiculosum,* while no showed inhibition effects against Gram-negative bacteria and two fungi species *Aureobasidium pullulans* and *Penicillium chrysogeum.* While, Ndlovu *et al.* (2017) revealed the antimicrobial activity of rhamnolipid biosurfactants produced by *P. aeruginosa* ST5 against variety of opportunistic and pathogenic microorganisms, including antibiotic resistant *Staphylococcus aureus* and *Escherichia coli* and the fungal pathogens including *Candida albicans* and *Cryptococcus neoformans.*

The exact mechanism action of rhamnolipid biosurfactant against microbes is not completely understood and unknown but, it is supposed that the plasma membrane of the cell is the target, as the rhamnolipid has an amphipathic nature that allows it to interact with the phospholipids of the plasma membrane (Magalhães, and Nitschke 2013). The current study aimed to assessantimicrobial and antioxidant activity of rhamnolipids biosurfactant formed by *Pseudomonas aeruginosa*.

**2. Materials and Methods**

**2.1. The rhamnolipid biosurfactant Sample**

The rhamnolipid biosurfactant sample utilized in current study was extracted from *Pseudomonas aeruginosa* isolated in previous study from hydrocarbon contaminated soil (Alyousif *et al.* 2020a). The extracted rhamnolipid biosurfactant was purified and characterized in previous study (Alyousif *et al*. 2020b).

**2.2. Antioxidant Activity of rhamnolipid biosurfactant**

The method carried out according to Barros *et al.* (2007) with the modification in the present study. One ml of each sample at the concentrations 5, 10. 15, 20 and 25 mg/ml of DMSO was added to 1 ml of 0.2 mM methanolic DPPH of freshly prepared DPPH solution. The reduction of DPPH radicals was measured at 517 nm after incubation in the dark for 30 min. Ascorbic acid was used as the positive control with same concentration of the samples. The percentage of scavenged DPPH radical was calculated using the following formula:

DPPH radical scavenging % = [(A0 – A1)/A0] x 100

Where, A0 is the absorbance of the DPPH solution and A1 is the absorbance of the sample.

**2.3. Cytotoxicity Assay of rhamnolipid biosurfactant**

The toxicity of rhamnolipid was tested against human erythrocytes according to method of He *et al.* (1994). The blood suspension was prepared by adding 1 ml blood into 20 ml of physiological saline. The concentrations of rhamnolipid (15, 25, 35, 50, 75 and 100 mg/ml) were dissolved in DMSO. The test was performed by adding 100 µl of varying concentrations to 2 ml of blood suspension, and then the tubes were incubated at 37 °C and the turbidity of solution monitored after (3-24 h). The positive result was turned the blood solution into turbid, while the negative result was turned the blood solution into clear. Blood suspension with tap water was used as positive control and blood suspension with normal saline was used as the negative control in addition to the DMSO as a control. The concentrations which gave a turbid solution, due to lysing of erythrocytes as an indication of toxicity degree of rhamnolipid to the erythrocytes.

**2.4. Determination of antimicrobial activity of the rhamnolipid biosurfactant**

The antimicrobial effect of the rhamnolipid biosurfactant was carried out according to Nanda and Saravanan (2009). The antimicrobial effect was measured using agar well diffusion method. The bacterial isolates (*Bacillus cereus, E. coli, Enterobacter cloacae, Klebsiella pneumonia, Proteus mirabilis, P. aeruginosa* and *S. aureus*) and fungal strains includes *Candida albicans* and *Aspergillus niger* were cultured and activated individually. The rhamnolipid biosurfactant was dissolved in DMSO at concentrations (5, 10,15, 20 and 25 mg/ ml) using serial rhamnolipid dilution technique. The antimicrobial effect of the rhamnolipid biosurfactant was determined by measuring the inhibition zones diameter against each strain.

**2.5. Determination of Minimal Inhibition Concentration (MIC)**

The minimal inhibition concentration (MIC) valueof the rhamnolipid biosurfactant was carried out according to T.C *et al.* (2019). MIC was determined by a serial dilution technique using agar well diffusion method. A 10 mg/ml stock solution of the rhamnolipid biosurfactant in DMSO was diluted to concentrations 1, 2, 3 and 4 mg/ml. The minimal inhibition concentration (MIC) value was determined against a panel of pathogenic isolates.

**3. Results**

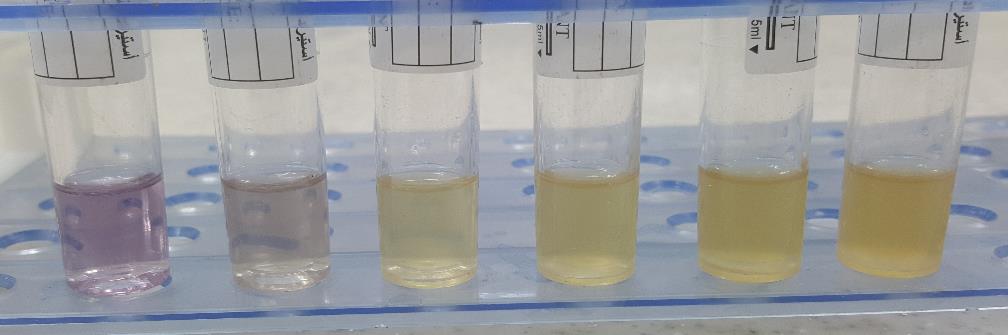
**3.1. Antioxidant activities of rhamnolipid biosurfactant**

The antioxidant activity results of rhamnolipid biosurfactant showed 22.7 %, 47.4 %, 79.8 %, 85 % and 91.4 % at the concentrations (5, 10. 15, 20 and 25 mg/ml) respectively as in figure (1). The rhamnolipid biosurfactant revealed effective antioxidant activity toward DPPH in a concentration dependent manner. In fact, at 25 mg/ ml, the rhamnolipid biosurfactant showed a potential scavenging effect of 91.4 %, which is four-times higher than that obtained at 5 mg/ ml (22.7 %).

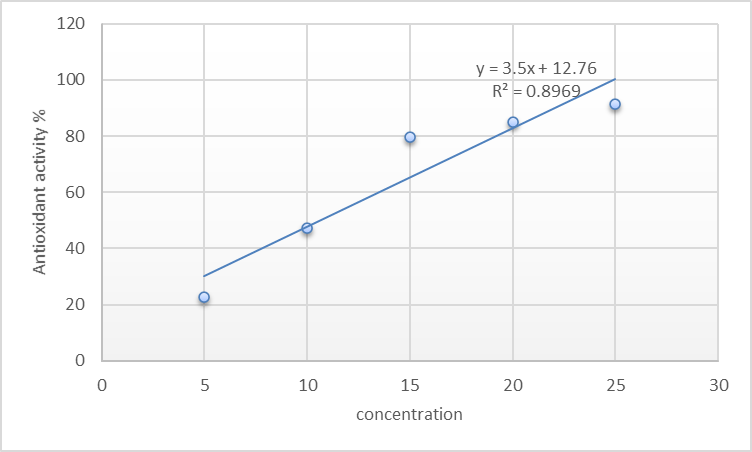
**Fig. 1:** Antioxidant activity of different concentrations of rhamnolipid biosurfactant companying to the standard antioxidant ascorbic acid.

The colour solution was changed from violet to yellow, it turns into a non-radical form after saturation of the electronic layer led to a loss of violet color, which is due to lack of absorption in terms of time at a wavelength of 517 nm as shown in figure (2).

**Fig. 2:** The antioxidant activity of different concentrations (mg/ml) of rhamnolipid biosurfactant using the DPPH method.



DPPH 5 mg 10 mg 15 mg 20 mg 25 mg

 The antioxidant activity of rhamnolipid biosurfactant was assessed by calculating the IC50 value to determine the concentration of rhamnolipid biosurfactant required to inhibit 50% of free radical DPPH present in the mixture. High-IC50 values refer to low antioxidant activity. The IC50 value of rhamnolipid biosurfactant showed 10.6 mg/ml as in figure (3) Thus, the low IC50 value refer to the high antioxidant activity.

**Fig. 3:** the curve of IC50 value for rhamnolipid biosurfactant.

**3.2. Cytotoxicity assay of rhamnolipid biosurfactant**

Cytotoxicity of the rhamnolipid biosurfactant was examined at different concentrations (15, 25, 35, 50, 75 and 100 mg/ml) against human erythrocytes. Hemolysis of the erythrocytes was observed at concentrations (100, 75, 50, 40 and 35 mg/ml) to a similar degree as in a positive control of tap water, whereas the results exhibited no hemolysis at concentrations (25 and 15 mg/ ml) to a similar degree as in a negative control of DMSO exhibited no hemolysis as in figure (4).



water 100 75 50 35 25 15 normal saline DMSO

**Fig. 4:** Cytotoxicity activity of rhamnolipid biosurfactant at different concentrations (15, 25, 35, 50, 75 and 100 mg/ml) against human erythrocytes.

**3.3. The antimicrobial activity of rhamnolipid**

The results of antimicrobial activity showed that the rhamnolipid biosurfactant had antimicrobial effect against all the tested pathogenic isolates at all concentrations (5, 10, 15, 20 and 25 mg/ml) in values were proportional to the rhamnolipid biosurfactant concentrations as shown in figure (5) and table (1). The rhamnolipid biosurfactant was shown higher inhibitory effect against *Bacillus cereus* (21/5, 23/10, 24/15, 25/20 and 25/25 mm/mg)and *Klebsiella pneumoniae* (19/5, 20/10, 22/15, 23/20 and 24/25 mm/mg), while lower inhibitory effect against *Staphylococcus aureus* (15/5, 19/10, 20/15, 20/20 and 20/25 mm/mg)and *Pseudomonas aeruginosa* (16/5, 19/10, 20/15, 20/20 and 20/25 mm/mg)*.* The rhamnolipid biosurfactant was shown lower inhibitory effect againstfungal strains *Candida albicans* (15/5,18/10, 19/15, 19/20 and 19/25 mm/mg) and *Aspergillus niger* (14/5, 15/10, 15/15, 15/20 and 16/25 mm/mg).

*Enterobacter* sp

25

15 20

kkkkk

*Bacillus cereus*

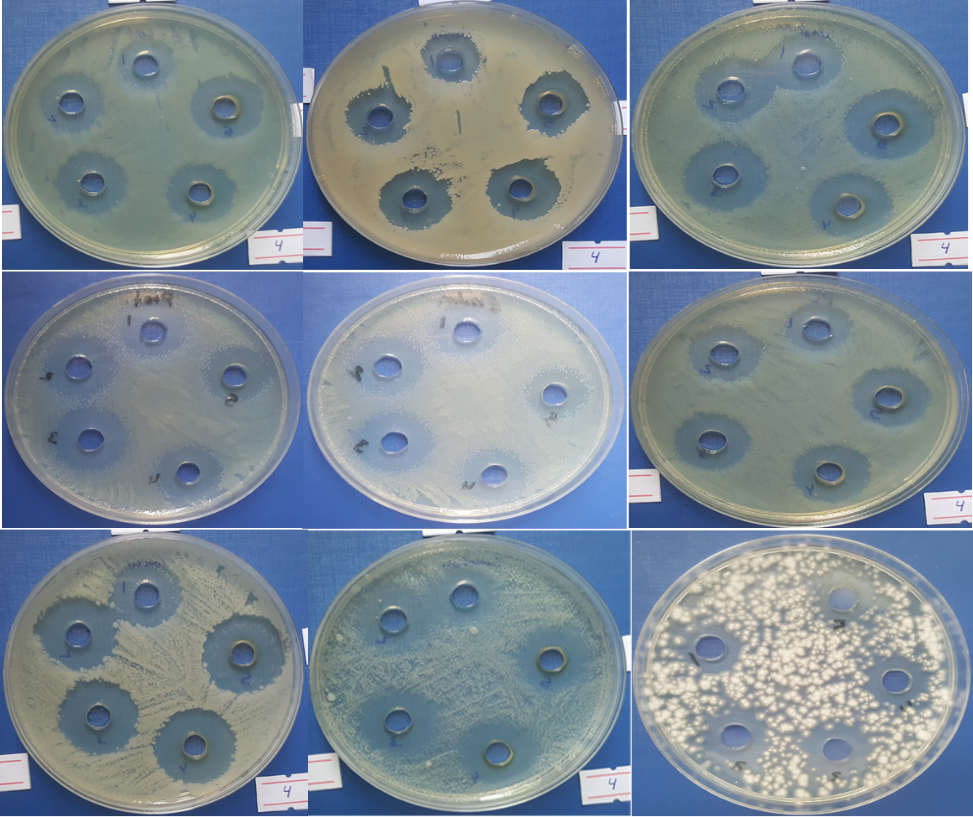
5

10

25

15

20



**Fig. 5:** The antimicrobial activity of rhamnolipid biosurfactant at different concentrations (5, 10,15, 20 and 25 mg/ ml) against nine pathogenic isolates including seven bacteria and two fungi

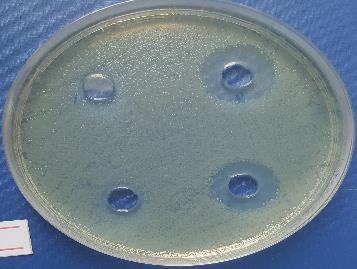
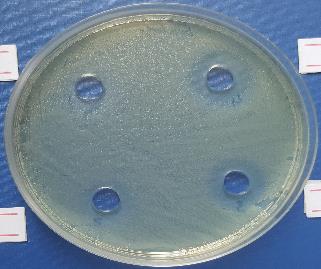
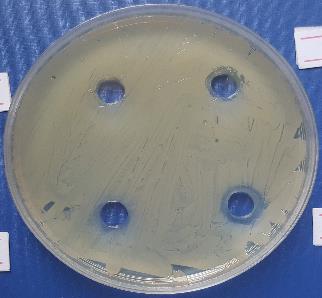
**Table 1:** The antimicrobial activity of rhamnolipid biosurfactant against nine pathogenic isolates including seven bacteria and two fungi

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Microorganisms | Zone Inhibition Diameters (mm).  Rhamnolipid concentration mg/ml. | | | | |
| 5mg | 10mg | 15mg | 20mg | 25mg |
| *E. coli* | 16 | 19 | 21 | 21 | 22 |
| *Staphylococcus aureus* | 15 | 19 | 20 | 20 | 20 |
| *Klebsiella pneumoniae* | 19 | 20 | 22 | 23 | 24 |
| *Proteus mirabilis* | 19 | 19 | 22 | 22 | 15 |
| *Enterobacter cloacae* | 16 | 17 | 20 | 20 | 24 |
| *Pseudomonas aeruginosa* | 16 | 19 | 20 | 20 | 20 |
| *Bacillus cereus* | 21 | 23 | 24 | 25 | 25 |
| *Candida albicans* | 15 | 18 | 19 | 19 | 19 |
| *Aspergillus niger* | 14 | 15 | 15 | 15 | 16 |

**3.4. Determination of Minimal Inhibition Concentration (MIC)**

The MIC values of rhamnolipid biosurfactant against the cultures of pathogenic isolates are shown in figure (6). The rhamnolipid biosurfactant showed lowest minimum inhibitory concentration (MIC) values against the pathogenic isolates, it was 2 mg/ml for *E. coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and3 mg/ml for *Staphylococcus aureus, Enterobacter cloacae, Bacillus cereus,* *Proteus mirabilis, Candida albicans* and *Aspergillus niger* as shown in in table (2).

**Fig. 6:** Minimal inhibitory concentration of rhamnolipid biosurfactant using concentrations 1, 2, 3 and 4 mg/ml against nine pathogenic isolates including seven bacteria and two fungi



**Table 2:** Minimal inhibitory concentration of rhamnolipid biosurfactant against nine pathogenic isolates including seven bacteria and two fungi

|  |  |
| --- | --- |
| Microorganisms | MIC values for rhamnolipids mg/ml |
| *E. coli* | 2 |
| *Staphylococcus aureus* | 3 |
| *Klebsiella pneumoniae* | 2 |
| *Proteus mirabilis* | 3 |
| *Enterobacter cloacae* | 3 |
| *Pseudomonas aeruginosa* | 2 |
| *Bacillus cereus* | 3 |
| *Candida albicans* | 3 |
| *Aspergillus niger* | 3 |

**4. Discussion**

**4.1. Antioxidant activities of rhamnolipid biosurfactant**

The antioxidant activity of the rhamnolipid biosurfactant was examined with DPPH scavenging test. This test has based on the ability of rhamnolipid to act as DPPH free radical scavengers which is a stable free radical having an unpaired valence electron at one nitrogen atom bridge (Abdollahia *et al.* 2020). As showed in figure **(**2) the rhamnolipid biosurfactant revealed effective activity of antioxidant against DPPH free radical in a concentration dependent manner. where, at 25 mg/ ml, the rhamnolipid biosurfactant showed a potential scavenging effect of 91.4 %, which is four-times higher than that obtained at 5 mg/ ml (22.7 %). When the DPPH free radical encounters a substance of hydrogen-donor, the free radical would be scavenged and the absorbance is reduced due to changing its color from purple to yellow, the rhamnolipid antioxidant activity was due to the free radical neutralization by transferring electrons (Jemil *et al.* 2017). The powerful DPPH scavenging activity of rhamnolipid biosurfactant could be attributed to the content of unsaturated fatty acids and reducing power of rhamnolipid biosurfactant could be increased with increasing of unsaturated fatty acids content (Abdollahia *et al.* 2020). Haque *et al.* (2020) reported the maximum antioxidant activity of rhamnolipid produced by *Marinobacter litoralis* was 72.6% at 5 mg/ml. Abdollahi *et al.* (2020) showed the results which indicated that both surfactin and rhamnolipids biosurfactant had antioxidant activity but surfactin revealed higher antioxidant activity than rhamnolipids. Whereas, the low antioxidant activity of rhamnolipid biosurfactant produced from *P. aeruginosa* MN1 may be attributed to lower content of unsaturated fatty acids. The antioxidants compounds are considered as essential additives that are used for preservation of different products in pharmaceutical, cosmetic and food industries by hindering oxidative rancidity of lipids and retarding their spoilage (Ohadi *et al.* 2017).

**4.2. Cytotoxicity assay of rhamnolipid biosurfactant**

Cytotoxicity of the rhamnolipid biosurfactant was examined against human erythrocytes. The results exhibited that rhamnolipid biosurfactant had no hemolysis at concentrations mg/ ml (25 and 15), where the erythrocytes were not precipitated as shown in figure (4). the lower sediment layer appeared in red color, which represents the human blood, while the upper layer represents the rhamnolipid as well as the physiological solution, if the compound was toxic, would result in the degradation of the red blood cells. However, in the present study, the rhamnolipid obtained from *P. aeruginosa* showed no toxic effect at concentrations 25 and 15 mg/ ml on the erythrocytes which considered it as non-cytotoxic biosurfactant that could be used as a possible biological material in various clinical aspects. Al-waely, (2013) revealed that all the concentrations he used of rhamnolipid did not show any hemolysis and then did not cause any cytotoxicity towards the erythrocytes. The rhamnolipid biosurfactant appears to be suitable and great alternatives to be employed as effective and safe therapeutic agent.

**4.3. The antimicrobial activity of rhamnolipid**

The findings of the current study show that the rhamnolipid biosurfactant had an antimicrobial effect against all the tested microorganisms (bacteria and fungi) at all concentrations that were used. The inhibition zones against the tested microorganisms were increased with increasing the concentrations of rhamnolipid biosurfactant as shown in table (1). The mechanism action of antimicrobial activity of rhamnolipid biosurfactant against microorganisms is not completely understood but, it is suggested that the cellular plasma membrane is the target, as the rhamnolipid have an amphipathic nature that allows it’s to interact with phospholipids of plasma membrane. Another suggestion about the mechanism action of the rhamnolipid was increasing the membrane permeability of microbial cells with consequent alteration of this membrane causing cell damage (Magalhães and Nitschke 2013). Lotfabad *et al. (*2013) reported results which exhibit remarkable inhibitory effect of two rhamnolipids biosurfactants against Gram-positive bacteria. While none of two rhamnolipids biosurfactants exhibited inhibition effects on Gram negative bacteria. The two rhamnolipids biosurfactants showed high inhibitory effect against *Chaetonium globosum* and *Penicillium funiculosum,* while none of them showed inhibition effects on *Aureobasidium pullulans* and *Penicillium chrysogeum* and two biosurfactants revealed different inhibitory behaviors against *Aspergillus niger*. De Freitas Ferreira et al. (2018) examined the antimicrobial activity of rhamnolipid with different pH values (from 5.0 to 9.0) against food pathogens. The antimicrobial rhamnolipid activity against the Gram-positive bacterial pathogens such as *Staphylococcus aureus, Listeria monocytogenes* and *Bacillus cereus* was pH dependent manner and favored at more acidic conditions while the Gram-negative bacterial pathogens such as *Salmonella enterica* and *Escherichia coli* (EHEC) revealed resistance at all pH levels which studied. The rhamnolipids are anionic biosurfactants when at pH condition of neutral or alkaline while, at acidic conditions, they behave as nonionic. The antimicrobial rhamnolipid activity can be increased in acid food, favoring the control of the Gram-positive bacteria present in acidic products.

**4.4. Determination of Minimal Inhibition Concentration** **(MIC)**

The MIC values of rhamnolipid biosurfactant against the cultures of tested microorganisms are shown in table (2). The minimal inhibition concentrations (MICs) are the lowest an antimicrobial compound concentration which inhibits the microorganisms growth after incubation period. MIC depends on the types of microorganisms and an antimicrobial compound itself. The effectiveness of an antimicrobial compounds by inhibiting the microbial growth will increase with the increasing the concentrations of antimicrobial compounds used in the experiment (Andrews 2001). The antimicrobial activity of rhamnolipid biosurfactant is attributed to their effect on plasma membrane permeability. Mendoza *et al.* (2020) examined the antimicrobial activity of the crude rhamnolipids biosurfactants produced by *Pseudomonas fluorescens*, *Pseudomonas poae* and *Pseudomonas libanensis* toward two species of Gram-negative bacteria including (*E. coli*, *Serratia marcescens*) and two species of Gram-positive bacteria including (*B. cereus*, *S. aureus*) by the conventional MIC. The rhamnolipid biosurfactant had a positive relation between increasing concentrations and the inhibition zone toward test microorganisms. The rhamnolipids from *Pseudomonas libanensis* had the lowest value of MIC among the other types of rhamnolipids, which indicate its ability against the tested bacteria.

**5. Conclusions**

The rhamnolipid biosurfactant exhibited antioxidant effect toward DPPH in a concentration dependent manner, no hemolysis at lower concentrations and has significant antimicrobial effect toward gram-negative bacteria, gram-positive bacteria and fungi. Based on these results, the isolated rhamnolipid biosurfactant from *Pseudomonas aeruginosa,* which could be utilized in various medical and pharmaceutical purposes.

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**Authors’ contributions**

WHA, YYYA designed the study, NAA performed the lab­oratory work, WHA, YYYA analyzed the data, NAA wrote the manuscript. All authors read and approved the final version of the manuscript.

**Conflicts of Interest**

All authors declare that there is no conflict to interest.

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