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Immunomodulator Activity of Biosurfactant Extract from Serratia Marcescens

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Abstract: This study was conducted in order to examine the immunomodulator effect of this biosurfactant material and test for its protective effect against infective dose (LD50) of *Salmonella typhi* as an indicator of the effectiveness of cellular immunity and *Staphylococcus aureus* as an indicator of the effectiveness of humeral immune response in normal and immunosuppressive mice. The results showed that animals treated with extract before 24 h with an LD50 dose of *S. typhi* and *S. aureus* bacteria respectively were protected and the survival rate of injected mice increased with increasing concentration of extract in compare with control group. The survival rate reached to 100% and 80% in normal mice respectively but in immunosuppressive mice reached to 60% and 40% only, when injected with 80 mg of biosurfactant. In addition, this extract was examined for its mitogenic effects in the lymphocyte transformation test compared with phytohemagglutinin (PHA). The statistical analysis of results showed that there was no significant difference between the percentage recorded when using PHA(80.6%) and both 80 mg/ml (85.1%) and 40mg/ml (71.9) of biosurfactant in compare to negative control and low concentration of biosurfactant. The effect of extract on migration of polymorphonucler cell was examined also and the result showed significant decrease in diameter of migration area especially at 80mg/ml concentration (11.5mm) in compare with negative control. It can be concluded that biosurfactant can act to stimulate immune system and may working to increase the ratio of lymphocyte transformation and migration of PMN.

Key words: Immunomodulator · *Serratia marcescens* · Biosurfactant

INTRODUCTION

The immunomodulator are compounds with abroad immunostimulater activity, some of which have specific activity such as vaccines and target particular proteins, while others are nonspecific, which are working to increase the immune response. In general, the critical roles of immunostimulant are to assist the function of phagocytic cells and stimulate each of complement system, lysozyme activities and natural killer cells. Moreover, these components provoke to improve the antibody response, which enhance the protection against infectious disease [1]. Primary and secondary metabolites are produce by different types of bacteria, which can be used as biological active compounds in the medical field through its various potential activities; for instance, they work as anticancer, antimicrobial, anti-adhesiveness and possess therapeutic activity and immunomodulator activity [2].

The microbial surfactant (surface-active agent) are metabolites, generally secondary compounds produced especially by bacterial cell surface or may be secreted extracellular. These compounds are classified by their chemical composition (glycolipids, lipopeptides, polysaccharides, phospholipids, fatty acids and natural lipids) and microbial origin (bacteria, filamentous fungi and yeasts) [3].

Among the different categories of the surfactant, lipopeptides produced by *Serratia marcescens* possess the surface activities and antibiotic properties, with high usability of work as immunomodulator agents or enzyme inhibitors [2, 3].

The lipopeptide surfactant is considered as a valuable applicant in the field of industrial processes due to its exceptional properties. The physical and chemical features including, the stability at extreme temperature or pH values, low toxicity, high biodegradable additionally it can be produced from inexpensive waste product [4].

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The lipopeptide biosurfactant can be used as an anti-inflammatory agent and a novel potential therapeutic. The biosurfactant can decrease sepsis related mortality when injected before 36 hours of infection with pathogenic bacteria in a rat model. Additionally its material can act as potent nontoxic and non-pyrogenic immunological adjuvant when mixed with conventional antigen, which enhances the specific immune response [3].

The action of antibacterial activity of biosurfactant is dependent on the lysis of bacterial cell through its ability to interact with the phospholipids of the cellular membrane. This leads to enhance nonspecific permeability of the cytoplasmic membrane [5].

This study was considered as an initial attempt to justify the potential effect of biosurfactant on immune cell and pathogenicity of bacteria during infection to get the possibility to use these compounds as a alternatives to many antibiotics in the future to treatment for diseases or as a stimulator of immune system.

MATERIALS AND METHODS

Bacterial Isolates: *Serratia marcescens, Salmonella typhi* and *Staphylococcus aureus* were supplied by microbiology lab in the department of biology, college of science, Karbala University. The identification of these bacteria was carried out according to biochemical tests and API system [6, 7].

Laboratory Animals: All experiments were done on 150 male albino mice aged 8 – 10 weeks. Their weight was 23 + 3 grams at the start of experiments. Before carrying out the experiment, they were divided into several groups, each group was kept in separate plastic cage at 20-25°C and they had free access to food and water.

- Twenty mice were used to determine the toxicity test
- Fifty mice were separated into two groups each one consisted of 25 mice were used to determine the lethal dose 50(LD50) for *S. typhi* and *S. aureus*.
- Forty mice were separated into two group each one consisted of 20 mice were used to determine the effect of biosurfactant on survival rate of normal mice infected with *S. typhi* and *S. aureus*
- Forty mice were injected with 5mg/ kg of dexamethasone subcutaneous 5 days prior to the treatment to develop immunosuppressed cause in mice and these mice were separated into two group each one consisted of 20 mice were used to determine the effect of biosurfactant on survival rate of immunosuppressed mice infected with *S. typhi* and *S. aureus* [8].

Biosurfactant Production

Detection Biosurfactant Production by S. Marcescens: The screening of biosurfactant strain *(S. marcescens*) was assayed qualitatively using blood hemolysis test methods and drop collapse method [9, 10].

Extraction of Biosurfactant: Depending on the way described by Ahmed and Hassan [5] and Alves *et al.* [11] the biosurfactant was extracted from *S. marcescens* culture, followed by drying the suspension and conversion to powder in an oven at 37 °C and it was preserved in refrigerator until used.

Toxicity Test: This test was carried out according to Anyanwa and okolo [12] with some modifications. Ten male mice were injected with 100 mg/ml of biosurfactant. The toxic symptoms during 10 days after injection was observed. The ratio of weight of the liver and spleen to the weight of the animal body was determined as well.

Determination of LD_{50} for S. typhi and S. aureus: The LD_{50} for *S. typhi* and *S.* into two group each one consisted of 20 mice were used to determine the effect of biosurfactant on survival rate of normal mice infected with *S. typhi* and *S. aureus* bacteria was determined as describe by Cruickshank [7], depending on the equation mentioned in the way to Reed and Muench [13].

Immunological Parameters

Prophylactic Effect of Biosurfactant Extract Against S. Aureus and S. typhi: Three normal mice groups (5 mice in each group) were injected with three concentrations of biosurfactant (80 – 40 – 20 mg\ml) intraperitoneal and after 24h these mice were injected with LD50 of *S. aureus*. These steps were repeated with three immunosuppressive mice groups as well as with five normal and five immunosuppressive mice that injected with normal saline instead of extract. Then the number of dead mice was determined every day for a weak period [8, 14].

The experiment was done one more time, but mice injected with *S. typhi* instead of *S. aureus* bacteria.

Effect of Extract on Lymphocyte Transformation: The percentage of transformed cells had been calculated from the ratio between the numbers of transformed cells to the total number of cells that counted in prepared slide oil emersion [15]

$$Tcell\% = \frac{No.of\ transformed\ cell\ (lymphoblast)}{Total\ No.of\ lymphocyte\ cell} \times 100$$

Effect of Extract on Migration of Polymorph Nuclear (PMNs) Cells: The suspension of PMNs in RPMI 1640 medium was prepared as described by Chech and Lahrer [16] to give a final concentration of 1 X 10^6 cells \ml. Then the migration of these cells was conducted by adopting the method described by Nonoyama *et al* [17] and calculated the movement distance of PMN cells that is used to determine the migration inhibition factor (MIF) as mentioned by Federlin *et al.* [18] by following:

$$MIF = \frac{Area\ of\ migration\ with\ antigen}{Area\ of\ migration\ without\ antigen}$$

If MIF index was ≤ 0.8 mm, regard as positive value.

Statistical Analysis: The statistical analysis has been run out by analysis of variance (ANOVA) test. The results have been shown as mean±SD. The least significant difference was used to determine the difference between mean.

RESULTS AND DISCUSSION

Screening of Biosurfactant Produced Bacteria: The blood agar hemolysis method was used to determine the ability of bacteria to produce biosurfactant depending on the fact that biosurfactant has the ability to hemolysis red blood cells, as a result, among different species of *S. marcescens* one isolate had chosen as an efficient bacteria to extract biosurfactant material. The relationship between blood hemolytic activity and ability of isolated bacteria to produce biosurfactant was determined by many studies, so has become important steps to prove this relationship and determine the biosurfactant activity by isolated bacteria [19]. The data of several studies indicate that the purified biosurfactant is sufficient to lysis red blood cells *in vitro* in addition genetic and biochemical analysis confirmed the work of biosurfactant as hemolysis and this hemolytic activity may contribute to destroy and irritation of blood cell [20].

Extract and Determine of Biosurfactant: Depended on modified oil collapse method, the surface effects of biosurfactant secreted in cell free supernatant or extract from the bacterial chosen strains were determine.

The results of current study were illustrated that the drop taken from prepared sample was collapsed and showed spreading movement in the oil-coated wells, meaning found suitable amount of biosurfactant in tested sample. The diameter of sample drop was increase with biosurfactant concentration increase. The sample drops diameter was at least 0.5 mm larger than the water drop diameter, which was used as negative control because not collapsed and observed as bead.

Many of the previous studies concluded that the drop of biosurfactant suspension collapsed and spread on oil coated surface, where indicated it depends mainly on reducing the force or interfacial tension between oil and biosurfactant drop, therefore the reduction of inter facial tension become as selection criterion for biosurfactant capacity of microorganism in liquid medium, in contrast to water, that not spread because the hydrophobicity of oil that keep the water drop groped [21].

Toxicity Assay for Extract: Two injected mice with extract were sacrificed every two days until the tenth day to observe the toxic change that was caused by extract. The result of this study revealed that none of these mice showed any toxicity syndrome in addition there was no significant difference (P ≤0.05) in the body and liver weight of the injected mice in contrast to control mice injected with PBS as shown in Table (1).

This result approaches with many studies; Anyanaw [12] who noticed the non – toxic effect of lipopeptide biosurfactant of *S. marcescens* to mice, as well as Desai and Banat [22] who illustrated the low toxicity of microbial biosurfactant that added a veritable advantage for this material compared to biosurfactant manufactured chemically.

Prophylactic Activity of Biosurfactant Against *S. Typhi* and *S. Aureus* Bacteria

Ld50 of Tested Bacteria: The result of this experiment found that dose, which cause the death of half No. of injected mice during 5 days was 2.37x10^6 cell / ml for *S. aureus* and 1.44 x 10^5cell / ml for *S. typhi* as illustrated in Figures 1 and 2 respectively.

Table 1: Effect of biosurfactant on growth of mice and organ indexes

		10 mice injected with 100 mg \ ml of biosurfactant			10 mice injected with PBS (control)		
No. of sacrificed mice every two day	Several of day	Body weight (g) M+SD	Kidney weight (g) M+SD	Liver weight (g) M+SD	Body weight (g) M+SD	Kidney weight (g) M+SD	Liver weight (g) M+SD
2	2	24.36+2.80	0.005+0.001	0.045+0.007	25.10+2.84	0.005+0.006	0.044+0.006
2	4	25.27+1.43	0.006+0.003	0.056+0.006	25.90+0	0.005+0.003	0.056+0.008
2	6	26.10+0.85	0.006+0.004	0.058+0.003	27.66+10.04	0.006+0.003	0.055+0.007
2	8	27.22+4.21	0.007+0.006	0.055+0.007	28.19+11.31	0.007+0.006	0.057+0.001
2	10	27.80+2.68	0.008+0.001	0.059+0.001	28.88+8.59	0.007+0.003	0.39+0.003
		P≥0.05	P≥0.05	P≥0.05	P≥0.05	P≥0.05	P≥0.05

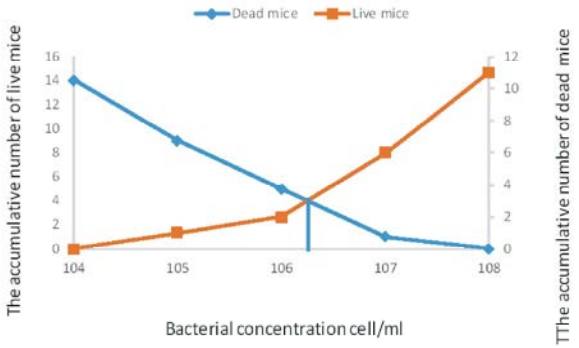

Fig. 1: LD50 in mice injected with *S. aureus*

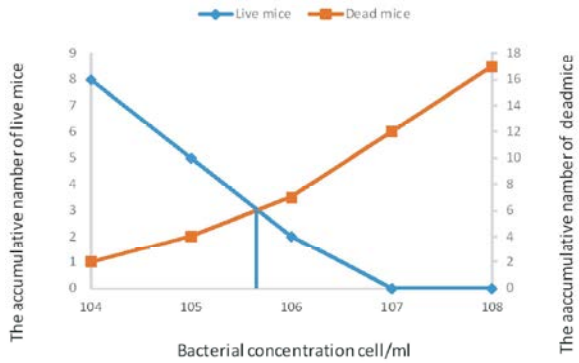

Fig. 2: LD 50 in mice injected with *S. typhi*

The results of LD 50 obtained in the current study differs from most previous studies result, where these differences are attributable to the pathogenesis of bacterial strain used in the current experiment as well as the force of immune system of animal used [23]. Also believed that the reason for this variation may be due to the source of bacterial isolates used in the experiments.

Prophylactic Activity of Biosurfactant: The result of this study observed increase in the number of live mice (normal and immune suppressive) infected with LD50 of *S. aureus* and *S. typhi* bacteria with increasing concentration of the biosurfactant in compare with control group. The survival rate reached to 100% in normal mice injected with 80 mg of biosurfactant and LD50 of *S. aureus* but in immunosuppressive mice it reached to 60% only in compare to control group 20% and 0% in normal and immunosuppressive mice respectively as shown in Table (2), whereas the survival rate in normal mice injected with 80 mg of biosurfactant and LD50 of *S. typhi* bacteria reached to 80% and 40% in immunosuppressive mice in compare to control group (0% in normal and immunosuppressive mice respectively) as shown in Table (3).

When studying the prophylactic activity of biosurfactant it is believed that the reason for effects described in results may be due to the ability of the injected biosurfactant to stimulate cellular and humeral body defense either increase the number of PMN cells or stimulate the enzymes involved in the complement pathway and may stimulate directly macrophage cell that plays a key role in the elimination of bacterial invader. Many researchers proved this idea and explained the antibacterial activity of biosurfactant through its work as an inhibitor of fibrin clotting and cyclic AMP phosphodiesterase or work as immunostimulator [24]. These compounds often lead to the induction of apro – inflammatory cytokine response that promotes elimination of the pathogenic bacteria, through its ability to promote neutrophil recruitment to the site of infection by acting as chemo attractants [25].

Effect of Extract on Lymphocyte Transformation: The statistical analysis of results showed that there was no significant difference ($P \leq 0.05$) between the percentage of lymphocyte transformation recorded when using PHA (80.6%) and percentage recorded when using 80 mg / ml (85.1%) and 40 mg / ml (71.9%) of biosurfactant, in compare to negative control and low concentration 20 and 10 mg / ml of biosurfactant which showed the highly significant difference when compared to both PHA and high concentration of the same material as shown in Table (4).

Table 2: Effect of biosurfactant on survival rate of normal and immunosuppressive mice infected with *S. aureus* bacteria

Type of mice	Biosurfactant concentration	Number of mice	Number of survival mice after 5 day	Survival rat
Normal mice	0	5	1	20%
	20	5	2	40%
	40	5	3	60%
	80	5	5	100%
Immunosuppressive mice	0	5	0	0%
	20	5	1	20%
	40	5	1	20%
	80	5	3	60%

Table 3: Effect of biosurfactant on survival rate of normal and immunosuppressive mice infected with *S. typhi*

Type of mice	Biosurfactant concentration	Number of mice	Number of survival mice after 5 day	Survival rat
Normal mice	0	5	0	0%
	20	5	1	20%
	40	5	2	40%
	80	5	4	80%
Immunosuppressive mice	0	5	0	0%
	20	5	0	0%
	40	5	2	40%
	80	5	2	40%

Table 4: Effect of biosurfactant on lymphocyte transformation

Concentration of biosurfactant	The percentage of transformed cells
0	33.8±2.151
10	50.6±10.20
20	54.8±1.82
40	71.9±1.00
80	85.1±4026
PHA	80.6±2.75

LSD (0.05) =8.557

Table 5: Effect of biosurfactant on migration of PMN cells

Concentration of biosurfactant mg / ml	Diameter of migration area mm	Migration inhibition factor
0	16.60±1.79	1
10	15.5±3.40	0.013
20	15.8±1.06	0.95
40	13.5±2.93	0.81
80	11.5±1.64	0.69
PHA	8.24±2.04	0.46

LSD (0.05) = 4.07

From the results of effect of extract on possible lymphocyte transformation reference to the viability of this material to stimulate cellular mediated immune response, by being able to work to stimulate the transformation of lymphocyte cell [26, 27].

The high response of lymphocyte cell to high concentration of biosurfactant may be attributed to the ability of this material to induce cell differentiation instead of cell proliferation and this glycolipids material usually induce the stage of apoptosis of malignant cells and their cytotoxic effect was noticed on cancer cell viability [28].

Effect of Extract on Migration of Polymorph Nuclear Cells: The result listed in Table (5) shows a decrease in diameter of PMN migration area when using different concentrations of biosurfactant where the decline was not significant at 10, 20 and 40 mg / ml concentration of these materials to reach 15.5, 15.8 and 13.5 mm respectively but was significant at 80 mg / ml concentration, 11.5 mm in compare with negative control. In addition, the 80 mg / ml concentration gave value approach to positive control PHA that reach to 8.24 mm.

In other words, all the concentrations of the biosurfactant that used in this study and negative control were within normal value for migration (because its value of migration inhibition factor (MIF) was 1, 0.013, 0.95 and 0.81 except 80 mg / ml and positive control PHA, which were inhibitor for migration (the MIF value was 0.69 and 0.46 respectively).

The inhibition of migration of PMN cell away from inflammation site is one of immunological means to prevent development of injury, where that process is done with help of T cell that able to secrete migration inhibition factors, such as proinflammatory cytokines [29,30].

Therefore, the ability of high concentration of biosurfactant to act as migration inhibition factor is one of the supporters of the possibility to use of that material as immune modulator to stimulate lymphocyte cells [31].

CONCLUSIONS

Serratia marcescens bacteria produce a very active compound called biosurfactant agent, this agent is particularly interesting because of its high surface activities and therapeutic potential.

This study is regarded as an attempt to detect the ability of biosurfactant to stimulate immune system where it was noted that the high concentration of biosurfactant assist laboratory animals to resist bacterial infection and may working to increase the ratio of lymphocyte transformation and migration of PMN.

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