



Improving of Antibacterial Activity for Antibiotics by Purified and Characterized Lectin from *Acinetobacter baumannii*

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Abstract: Lectin has a wide range of applications in many fields as anti-tumor, anti-insect, antiviral and antifungal drug. Here we reported lectin production from *Acinetobacter* as there is no report on lectin production from *Acinetobacter*. In the present study a novel strain, *Acinetobacter baumannii* S₃ isolated from sputum samples gave the highest production level of lectin by microscopic glass slide and microtiter plate methods and the hemagglutination activity was not blood-type specific. Lectin was purified to homogeneity with a recovery yield of 72.72% and 40.76 fold of purification by using ammonium sulfate at 35% saturation followed by QAE-Sephadex ion exchange chromatography and sephadex G-200 gel filtration chromatography. The optimum activity of purified lectin was found at 30°C and pH 7. The best cofactors for lectin activity were Mn⁺⁺ and Mg⁺⁺ with relative activities of 132 and 122%, respectively and severely inhibited in presence of Fe³⁺, Cu²⁺ and detergents. Lectin led to increase antimicrobial activity of antibiotics and had synergism effect, thus lectin may be useful adjuvant agent for the treatment of many bacterial infections in combination with antibiotics.

Key words: lectin, *Acinetobacter baumannii*.

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Introduction

Acinetobacter baumannii is aerobic small gram-negative cocco-bacilli (1). *A. baumannii* is ubiquitous in clinical and natural environments. The skin was the first location for colonization followed by colonization of oropharynx secretions, respiratory secretions, urine, irrigating and intravenous solutions (2). It is an important opportunistic pathogen that causes a variety of nosocomial infections especially pneumonia, urinary tract, blood stream, endocarditis, meningitis and surgical wound infections

(3). *A. baumannii* is successful in colonizing and persisting in the hospital environment, in addition to survive on artificial surfaces because their ability to resist desiccation and to form the biofilm (4, 5).

The colonization on human mucosal surface tissues is a crucial step for an infection. The successful colonization need to bind to the epithelial cells of these tissues for resistance the host's defense mechanisms. The attachment of bacteria to the host tissues need adhesion factors and the bacteria fail to survive if they lost these adhesions. The adhesion factors

called lectins and can bind to the glycoconjugates on the surface of other cells thus they are considered from an important virulence factor (6).

Lectins are defined as a divalent or multivalent carbohydrate-binding proteins of non-immune origin that can agglutinate cells or precipitate polysaccharides and glycoconjugates. They may also be glycoproteins, that have at least one non-catalytic domain which showed reversible binding to specific monosaccharides or oligosaccharides, that can agglutinate human and/or animal erythrocytes by binding to the carbohydrate moieties on the surface of erythrocytes and agglutinate the erythrocytes, without altering the properties of the carbohydrates (7). Lectins are originally called phytohemagglutinins since they were first found in plants and later termed as agglutinins or hemagglutinins due to its ability to agglutinate of human and animal erythrocyte. Lectins are ubiquitous in nature and can be found in almost all living organisms such as plants, animals (vertebrates and invertebrates), algae, fungi, microorganisms and viruses(8). Most lectins play a crucial role in different biological processes, particularly in host defense mechanisms, inflammation, and metastasis. The lectin isolated from ground bean seeds reduced the viability of hepatoma and leukemia cells and revealed an inhibitory activity toward human immunodeficiency virus-1 reverse transcriptase (9). In addition, several lectins isolated from fungi possess immunomodulatory, mitogenic, and antitumor activities (10). The wide applications and variety of uses of lectins reveal the need to isolate lectins from new local sources since lectins are very expensive. Therefore; there is a

continuing need to search another sources to find strain has an ability to produce new and high yield of lectin. Because of no report about lectin production and purification by *A. baumannii*, thus the aim of this study to demonstrate lectin production by *A. baumannii*, purification, characterization of lectin and examination of antibacterial activity for combination of lectin with antibiotics.

Materials and Methods

Sample Collection

The study included a total of 80 samples of which 40 were from sputum and 40 were from blood collected from patients at different hospitals in Baghdad city.

Bacteriological Analysis

The collected microbial sources were transported to the laboratory. Samples were plated primarily onto blood agar and MacConkey agar which was incubated at 37°C for 24 hour. The bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of systemic bacteriology. Suspicious isolates were presumptively identified by using colony morphology, negative oxidase test, positive catalase test and inability to motile coccobacilli (11). Further, the *A. baumannii* isolates were confirmed by using API 20E biochemical kit and the Vitek 2 system by using Vitek GNI card (bio Mérieux, France) according to the manufacturer's instructions.

Screening for Lectin Production

1-Semi-Quantitative Analysis on Microscopic Glass Slide

Twenty five μl of bacterial suspension at dilution 10^{-9} was mixed with 25 μl of 0.02M Phosphate Buffer Saline (PBS) pH 7.2 on glass slide, then 25 μl of blood suspension for several animal erythrocytes such as chicken, horse, goat, sheep, rabbit, and human erythrocytes A⁺, B⁺, AB⁺ and O⁺ at a concentration of 3 %, blending the mixture well by wooden chopsticks and added over the bacterial suspension then the glass slide examined for agglutination. An agglutination within 5 minutes refer to positive result (12). The control was PBS with blood instead of bacterial suspension.

2- Quantitative Analysis in Microtiter Plate (Hemagglutination Assay)

A serial two-fold dilutions of bacterial suspension or lectin solution (50 μl) in microtiter U-plate with 0.02M Phosphate Buffer Saline (PBS), pH 7.2 was mixed with the same volume of a 3% suspension of animal and human erythrocytes in the same buffer and incubated at 37°C for 2 hours. The activity was expressed as hemagglutination units (H.U.). One H. U. was defined as the inverse of the highest dilution still capable of causing agglutination (13).

Estimation of Protein Content

The protein content of lectin was determined by using Bradford dye method with BSA as a standard (14). The absorbance at 280 nm was used to estimate the protein concentration in column eluates.

Extraction and Purification of Lectin

The selected bacterial isolate was grown on Colonization Factors Antigens (CFA) medium described by Kohno *et al.* (15) that composed of the following (g/L): gasamino acid, 10;Yeast extract, 1.5; Mg SO₄,0.05; MnCl₂,0.005 at 37°C for 24 hour, then the cells was harvested by centrifugation at 8000rpm for 30 min, washed twice and re-suspended in 0.02M Phosphate Buffer Saline (PBS) pH 7.2. Cells were disrupted by glass beads for 50 min at 4°C using the vortex. Residual whole cells and cell membrane fragments were removed by centrifugation 8000rpm for 20 min. The resulting supernatant was used as the starting point to establish the hemagglutination activity of lectin in crude cell extracts.

The supernatant was fractionated with ammonium sulfate at concentrations 20-80% saturation and the obtained precipitate after centrifugation at 8000 rpm for 30 min was suspended in 0.02M Phosphate Buffer Saline (PBS) pH 7.2 and the hemagglutination activity and protein concentration were measured. The dialyzed protein was applied to a QAE - Sephadex column (3×25cm) previously equilibrated with the same buffer. The column was washed with the same buffer and the elution was made with a salt gradient containing 0.1–0.5M NaCl. The hemagglutination activity for each fraction was assayed as described above. The fractions that revealed significant peak of activity were mixed together and applied to a Sephadex G-200 column (2×90cm) previously equilibrated with the same buffer. Elution was performed with the same buffer, the fractions that revealed the protein and hemagglutination activity in the same peak were mixed and

transferred to a new sterile tube for further study. The active peak represented the purified lectin.

Characterization of Purified Lectin

1-Effect of Temperature on Lectin Activity

The optimum temperature of lectin were determined by measuring the hemagglutination activity at various temperatures (30, 35, 40, 45, 50, 55, 60, 65 and 70°C).

2-Effect of pH on Lectin Activity

The optimum pH for lectin activity was determined by carrying out the reaction at different pH values using different buffers (0.1M phosphate, pH 4.0-7.0, 0.2M Tris-HCl, pH 7.5-9.0).

3-Effect of Additives on Lectin Activity

One ml of different salts such as NaCl, KCl, CaCl₂, CuSO₄, CoCl₂, MgCl₂, MnCl₂, ZnSO₄ and FeCl₃ and different detergents like Tween-20, Tween-40, Tween-60, Tween-80, Triton X-100 and SDS were mixed with 1 ml of purified lectin and incubated at 37°C for 2 hour, then the hemagglutination activity was determined as described earlier.

Effect of Lectin on Antibiotic Activity

The ceftazidime and samacycline antibiotics were screened for antimicrobial activity using the macro

dilution method(16) against *S. aureus* and *E. coli*. To determine the minimum inhibitory concentrations (MICs), Each antibiotic was dissolved in distilled water to give a stock concentration of 10000 µg/ml. The stock concentration of each antibiotic was filterilized using 0.22 µm millipore filter. Twofold serial dilutions of each antibiotic was made with nutrient broth to give concentrations ranging from 1 to 5000 µg/ml. one hundred microliter of 10⁴ cfu/ml bacterial suspension (*S. aureus* and *E. coli*, separately) was added to the sterile capped test tubes. 50µl from each dilution was placed in wells (7 mm in diameter) on Mueller-Hinton agar medium. All tubes and plates were then incubated at 37°C for 18 to 24hour. The lowest concentration of antibiotic showing no visible growth in case of tubes using and inhibition zone in case of plates using was recorded as the minimum inhibitory concentration (MIC). Combination of purified lectin with ceftazidime and samacycline separately were tested by using the same methods as above against *S. aureus* and *E. coli*, since one hundred microliter of each bacterial suspension and lectin were added separately to two fold serial dilutions of the antibiotic. 50µl from each dilution was placed in wells (7 mm in diameter) on Mueller-Hinton agar medium. All tubes and plates were then incubated at 37°C for 18 to 24 hour. The lowest concentration of antibiotic showing no visible growth in case of tubes using and inhibition zone in case of plates using was recorded as the minimum inhibitory concentration (MIC).

Results

Isolation of *Acinetobacter baumannii*

The results revealed that 6 (7.5%) isolates of *A. baumannii* were obtained out of 80 samples. Among these six isolates,

4(67%) were isolated from sputum and 2(33%) were from blood (figure 1).

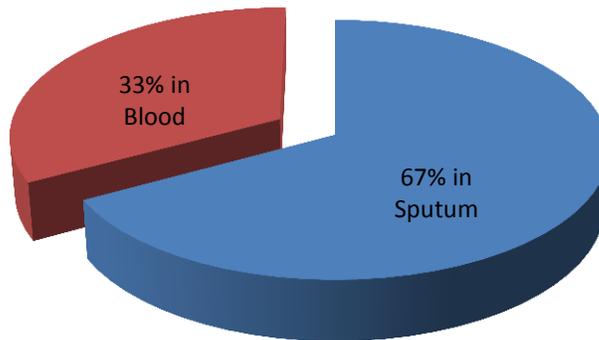


Figure 1: Isolation rates of *A. baumannii* from different clinical specimens

Detection of Lectin Producers

1-Semi-Quantitative Analysis on Microscopic Glass Slide

The results of hemagglutinating on the glass slide revealed that all bacterial isolates gave high hemagglutination activities for human erythrocytes (A⁺, B⁺, AB⁺ and O⁺) while the others animal erythrocytes such as rabbit and chicken erythrocytes gave low hemagglutination, in contrast, sheep, goat and horse

erythrocytes which give no hemagglutination activity (table 1). *A. baumannii* S₃ showed higher hemagglutination value (+++++) with blood group O⁺, followed by the isolates (S₄ and S₁) that emerged the values of agglutination (++++), while the isolate *A. baumannii* B₂ gave the lowest level (++) of lectin production. Among the tested blood groups, blood group O⁺ and blood group B⁺ were with good activity than blood groups A⁺ and AB⁺.

Table 1: Differences of hemagglutination activities of *Acinetobacter baumannii* isolates to various erythrocytes by microscopic glass slide method

Isolate No.	Human erythrocytes				Animal erythrocytes				
	A ⁺	B ⁺	AB ⁺	O ⁺	Rabbit	Sheep	Goat	Horse	Chicken
<i>AbS</i> ₁	*+++	++++	++	++++	++	-	-	-	+
<i>AbS</i> ₂	++	+++	+	+++	+	-	-	-	++
<i>AbS</i> ₃	+++	++++	++	+++++	++	-	-	-	++
<i>AbS</i> ₄	++	+++	+	++++	++	-	-	-	+
<i>AbB</i> ₁	++	++	++	++	+	-	-	-	+
<i>AbB</i> ₂	+	++	+	++	+	-	-	-	+

+: Weak hemagglutination ++: Moderate hemagglutination +++: Good hemagglutination
 ++++: Very good hemagglutination +++++: Excellent hemagglutination -: No hemagglutination

2- Quantitative Analysis in Microtiter Plate (Hemagglutination Assay)

All bacterial isolates gave hemagglutination activities in microtiter plate and the result demonstrated that *A. baumannii* S₃ had higher titer (128U/ml)

of hemagglutination in O⁺ blood group and B⁺ blood group (64U/ml). In contrast, lower titers revealed in blood group A⁺ followed by AB⁺ in addition to low titer detected in other animal erythrocytes (table 2).

Table 2: Differences of hemagglutination activities of *Acinetobacter baumannii* isolates to various erythrocytes by microtiter plate method

Isolate No.	Human erythrocytes				Animal erythrocytes					With group O ⁺	
	A ⁺	B ⁺	AB ⁺	O ⁺	Rabbit	Sheep	Goat	Horse	Chicken	Protein Conc. (mg/ml)	Specific activity (U/ml)
<i>AbS</i> ₁	32	64	16	64	16	-	-	-	8	5.98	10.70
<i>AbS</i> ₂	8	32	4	32	8	-	-	-	2	3.65	8.76
<i>AbS</i> ₃	32	64	16	128	16	-	-	-	8	9.33	13.71
<i>AbS</i> ₄	8	32	4	64	16	-	-	-	8	7.32	8.74
<i>AbB</i> ₁	8	16	8	16	4	-	-	-	4	2.21	7.23
<i>AbB</i> ₂	8	8	4	16	4	-	-	-	2	2.14	7.47

Extraction and Purification of Lectin

Lectin was extracted by using the glass beads, the hemagglutination activity increased to 256U/ml with specific activity was 119.62U/mg. The lectin was purified using standard techniques including ammonium sulfate fractionation

with (20-80%), QAE-Sephadex ion exchange chromatography and sephadex G-200 gel filtration chromatography. The crude extract was subjected to fractionation with ammonium sulfate and the results revealed that the saturation ratio 35% achieved specific activity 363.12 U/mg, with 3.03 purification folds

and 84.8% yield. Proteins were separated from other substances co-extracted by PBS in the crude extract by precipitating it with ammonium sulfate. This process of protein precipitation is called salting-out where a salt solution decreases the solubility and precipitates the proteins. Salt solutions such as ammonium sulfate and magnesium sulfate absorb the water of hydration surrounding and protecting the protein in the extract, causing the latter to coagulate (17). Ammonium sulfate precipitation followed by dialysis step to remove this salt from the sample and lead to concentrate the product. It is important to remove an ammonium sulfate from the precipitated solution since their presence lead to give false positive agglutination. After dialysis, the sample applied to QAE -Sephadex ion

exchanger. The elution with 0.1 to 0.5M NaCl gradient showed three peaks of proteins and the first peak of protein contained the hemagglutination activity as shown in figure (2). The specific activity was increased to 1077.89 U/mg with 9.01 fold of purification and 78.78% the yield of lectin. Gel filtration chromatography with sephadex G-200 was the last step of lectin purification. The eluted fractions of this step contained two peaks of protein. The protein of the second peak showed hemagglutination activity (figure 3). In this final step the specific activity reached to 4876.19 U/mg with 40.76 fold of purification and a yield of 72.72%. The results of lectin purification were summarized in (table3).

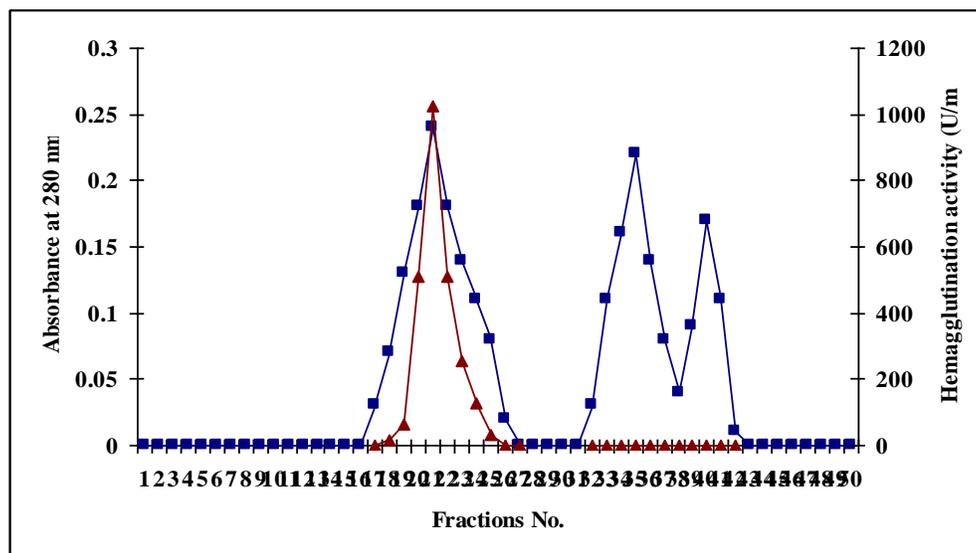


Figure 2: Purification of lectin from *Acinetobacter baumannii* using ion exchange on QAE -Sephadex column (3x25 cm) at flow rate 0.3 ml/min and fractions of 3 ml/tube were collected. (▲) refer to hemagglutination activity, (■) refer to protein concentration

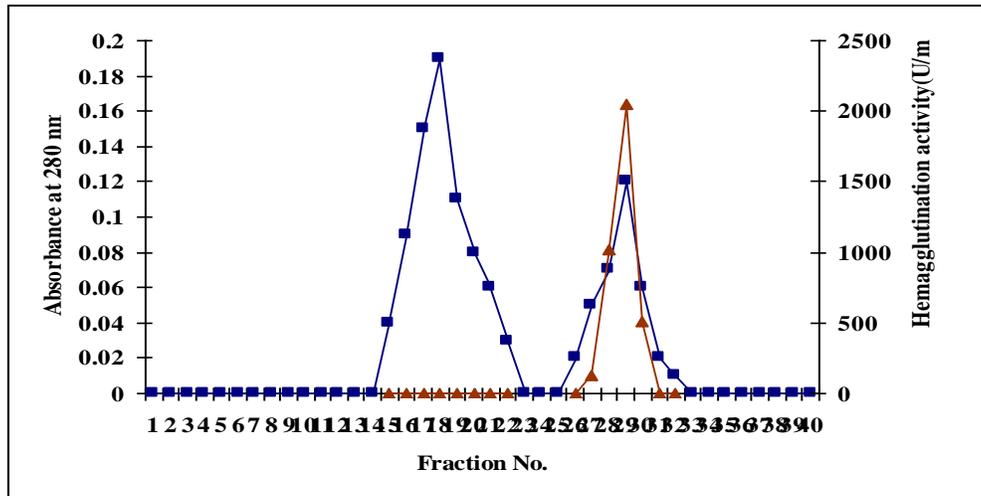


Figure 3: Purification of lectin from *Acinetobacter baumannii* using gel filtration on sephadex G-200 column (2x90 cm) at flow rate 0.5 ml/min and fractions of 5 ml/tube were collected. (▲) refer to hemagglutination activity, (■) refer to protein concentration.

Table 3 : Summary of treatment for purification of lectin from *Acinetobacter baumannii*

Purification step	Size (ml)	Hemagglutination activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	66	256	2.14	119.62	16896	1	100
(NH ₄) ₂ SO ₄ saturation	28	512	1.41	363.12	14336	3.03	84.8
QAE -Sephadex	13	1024	0.95	1077.89	13312	9.01	78.78
Sephadex G-200	6	2048	0.42	4876.19	12288	40.76	72.72

Characterization of Lectin

1-Effect of Temperature on Lectin Activity

The optimum temperature of purified lectin activity was 30°C (Figure 4), and

the hemagglutination activity decreased with increasing the temperature and found that the lectin becoming completely inactive at 55-70°C.

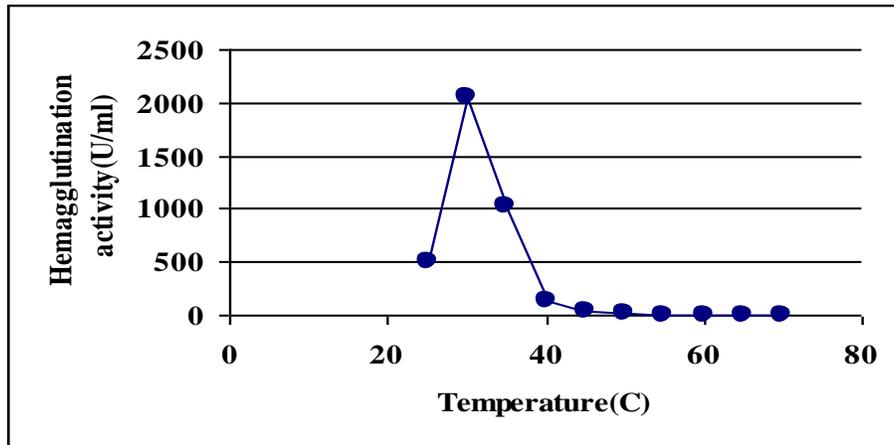


Figure 4: Effect of temperature on activity of lectin from *Acinetobacter baumannii*

2-Effect of pH on Lectin Activity

The optimum pH of purified lectin was 7 (Figure 5) and the hemagglutination

activity decreased with in pH values that higher or lower than this value.

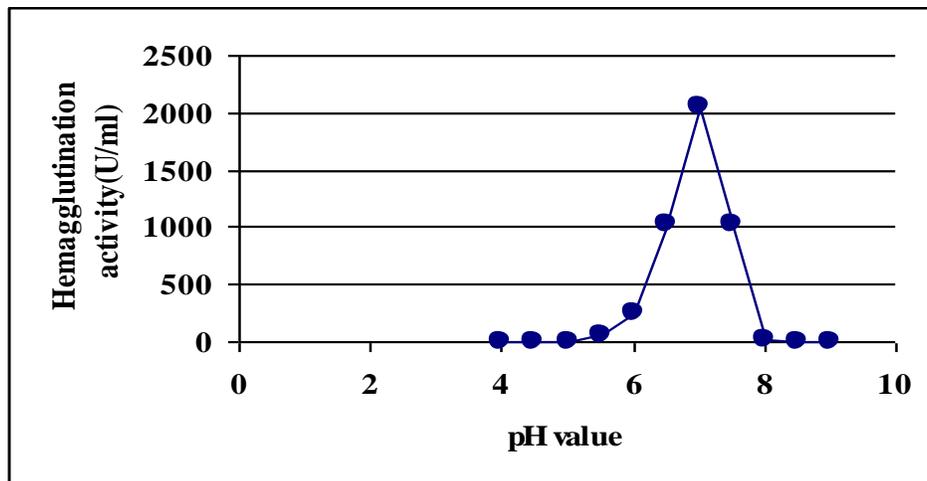


Figure 5: Effect of pH on activity of lectin from *Acinetobacter baumannii*

3-Effect of Additives on Lectin Activity

The effect of additives on lectin activity was studied by mixing each of the

additives with purified lectin. The metal ions Mn^{2+} and Mg^{2+} led to increase lectin activity with relative activities of 132 and 122%, respectively. The other metal ions

such as K^+ , Na^+ and Ca^{2+} had no effect on lectin activity, In contrast, the remaining additives such as Zn^{2+} , Cu^{2+} , Co^{2+} and Fe^{3+} and all studied detergents inhibited

the hemagglutination activity of lectin (table 4).

Table 4: Effect of salts and detergents on lectin activity

salt	Remaining activity(%)	Detergent	Remaining activity(%)
NaCl	100	Tween-20	18
KCl	100	Tween-40	21
CaCl ₂	100	Tween-60	6
CuSO ₄	13	Tween-80	13
CoCl ₂	63	Triton X-100	8
MgCl ₂	122	SDS	14
MnCl ₂	132		
ZnSO ₄	55		
FeCl ₃	34		

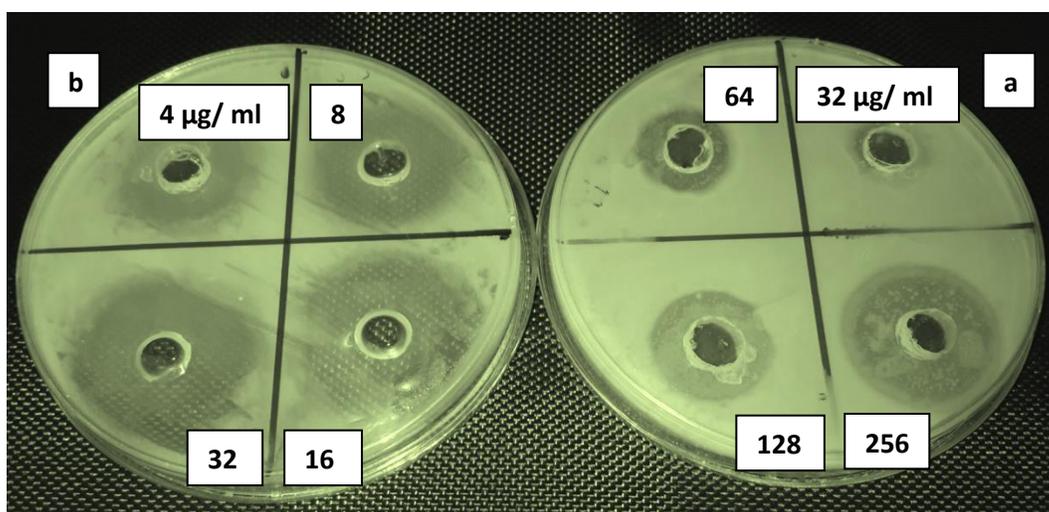
Effect of Lectin on Antibiotic Activity

The preliminary study showed that the extracted lectin alone had no activity against bacteria. The ceftazidime showed weak antibacterial activity against the reference strains *S. aureus* and *E. coli*, since their MICs were 256 and 1024 $\mu\text{g/ml}$, respectively. In contrast, the combination of lectin with ceftazidime has strong antibacterial activity against *S. aureus* and *E. coli*, respectively. The MICs of ceftazidime decreased to 32 and 128 $\mu\text{g/ml}$, respectively, also the same results were obtained in the case of using

agar diffusion method where the diameters of inhibition zones reached to 28 mm at 32 $\mu\text{g/ml}$ for *S. aureus* and 26 mm at 128 $\mu\text{g/ml}$ for *E. coli* as shown in table(5) and figure(6). The samacycline also revealed very weak antibacterial activity against these reference strains, since their MICs were 5000 and 2500 $\mu\text{g/ml}$, respectively, but in combination with lectin its activity was enhanced. The MICs of samacycline decreased to 1024 and 512 $\mu\text{g/ml}$ with diameters of inhibition zones of 23 and 17 mm for *E. coli* and *S. aureus*, respectively.

Table 5: MICs of antibiotics for *S. aureus* and *E. coli* isolates in tubes and plates with and without lectin

Antibiotic	Bacteria	MIC for antibiotic alone ($\mu\text{g/ml}$)	Diameter of inhibition zone (mm)	MIC for antibiotic + Lectin ($\mu\text{g/ml}$)	Diameter of inhibition zone (mm)
Ceftazidime	<i>E. coli</i>	1024	19	128	26
	<i>S. aureus</i>	256	22	32	28
Samacycline	<i>E. coli</i>	2500	20	512	23
	<i>S. aureus</i>	5000	13	1024	17



**Figure 6: a) Zones of inhibition of ceftazidime concentrations against *S. aureus*
b) Zones of inhibition of ceftazidime concentrations and lectin combinations against *S. aureus***

Discussion

A. baumannii isolates were isolated from lower respiratory tract with higher rate (18). Also Mammina *et al.* (19) reported that out of 36 *A. baumannii* isolates, 26(72.2%) isolates were from respiratory tract secretions. *Acinetobacter* spp. isolates were isolated from blood in low rate and this indicating their ability to cause many infections (2). Reason for the difference in the proportion of isolation of these bacteria return to the difference in samples collection, the health conditions

of patients as well as the variation in the number of samples taken for the study, and the random using of antibiotics. In a study done by Koljalg *et al.* (20) revealed that the isolation source of bacteria has important role in their adhesion and aggregation, since the isolated *A. baumannii* from wounds and blood samples showed a less gathering compared with isolates that isolated from the lower respiratory tract and environmental isolates. Russo *et al.*, (21) reported that more than 60% of the acquired pneumonia infections in

hospitals caused by gram negative bacteria including *A. baumannii*. The highest titer found with blood type O⁺ since the activity reached to 2048U/ml for *A. baumannii* (22). *A. baumannii* has type1 Pilli that have the ability to hemagglutinate the erythrocytes that contain mannose and this type of pilli considered from the virulence vectors that facilitate the adhesion of bacteria to epithelial cells (23). In a study reported by Mesa *et al.* (24) found that the purified lectin from *Artocarpus camansi* led to agglutinate O⁺ and A⁺ blood types in higher levels and B⁺ blood type in lower level, in contrast, it did not agglutinate AB⁺ blood types. while the purified *Prunella vulgaris* lectin showed an ability to agglutinate chicken RBCs and inability to agglutinate of human blood groups (25). The lectin produced by *Mycobacterium smegmatis* agglutinated erythrocytes for different animals such as rabbit, cow and goat and also nonspecifically agglutinated human A⁺, B⁺ and O⁺ erythrocytes (26). Glass beads that works for easily disrupted cells, is inexpensive, multiple samples can be used at the same time and leads to obtain a high concentration of protein. vortex beading that lead to extract the periplasmic proteins, is similar to sonication in that the sample and beads are mixed and placed on a vortex to induce mechanical beating and It is important to cool the sample (27). The sequence in purification steps led to increasing in the specific activity for lectin and the time for hemagglutination activity got shorter may refer to the efficiency of the purification process and the decrease of the contaminating materials. The lectin from *Prunella vulgaris* was purified by ammonium

sulphate, Sephadex G-100 and DEAE-cellulose chromatography and showed a fold purification of 7.66 and a yield of 51.69% (11). The soluble hemagglutinin (HA) (cholera lectin) produced by *Vibrio cholerae* strain CA401 was purified to apparent homogeneity by a sequence of ammonium sulfate fractionation, gel filtration and preparative isoelectric focusing with specific activity of 110 U/mg and a yield of 0.02% (28). The purified lectin from *Sophora alopecuroides* exhibiting optimum temperature at 30°C (29). The activity of lectin purified from *Schizophyllum commune* started to decrease at 55°C and is completely lost at 65°C after 30 min (10). The activity of lectin produced by *Sophora alopecuroides* was enhanced at very high acidic pH and inhibited at high basic pH(29). The activity of lectin purified from *Schizophyllum commune* was not markedly affected by pH and the maximum activity being retained at pH 5.0 to 9.0(10). The hemagglutination activity of purified lectin from *Sophora alopecuroides* was stimulated by Mn²⁺(29). In addition, the lectin activity from the Seeds of *Cissus poplunea* was enhanced by D-galactose and MgCl₂ (30). According to the results of the effect of lectin on antibiotic activity, lectin led to enhance ceftazidime activity with about ten times and samacycline with five times in comparison with controls(antibiotic alone). These finding indicate that lectin has increased antimicrobial activity of antibiotics and had synergism effect, thus lectin may be useful adjuvant agent for the treatment of many infections in combination with antibiotics.

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