CHARACTERIZATION OF ANTIBACTERIAL SUBSTANCE PRODUCED BY *Pseudomonas Fluorescens*

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Abstract

Pseudomonas fluorescens isolate was isolated from soil. The antibacterial activity of crude extract of this isolate was determined against pathogenic bacteria (Klebsiella pneumonia, Staphylococcus aureus) which are isolated from patients with skin infections. The extraction of this substance was done and study it characterization after purified by silica gel chromatography, It was stable within range of pH (5-9) at 37 ºC for 24 hr. and inactivated by serum at concentration > 50%. The substance was soluble in methylene chloride, chloroform, acetone, sodium hydroxide and hydrochloric acid and it was insoluble in water, methanol, ethyl acetate and hexane.

توصيف مادة مضادة للبكتيريا و المنتجة من قبل *Pseudomonas Fluorescens*

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الخلاصة

عزلت عزلة لبكتريا Pseudomonas fluorescens من التربة وشخص انتاجها للمادة المضادة للنمو البكتيري, حيث ثبطت نمو كل من البكتريا الممرضة Klebsiella pneumoniae Staphylococcus aureus والتي عزلت من المرضى المصابين بالتهابات جلدية. تم استخلاص هذه المادة ودرست بعض صفاتها بعد ان تم تنقيتها بأستخدام كروماتوغرافيا هلام السليكا, حيث لوحظ أنها تبقى مستقرة في مدى من الـ pH يتراوح ما بين (5–9) بدرجة 37 º م لمدة 24 ساعة, ولكن فقدت فعاليتها الحيوية عند المعاملة بالمصل بنسة 50% فما فوق. كما وجد ان لها القابلية للذوبان في كل من كلوريد المثيلين, الكوروفورم, الاسيتون, هيدروكسيد الصوديوم و حامض الهيدروكلوريك ولكنها غير ذائبة في الماء, الميثانول, أسيتات الاثيل و الهكسان.

Introduction

Pseudomonas fluorescens are Gram-negative rod shaped bacteria that inhabit soil, plants, and water surfaces (1). They are nonpathogenic and lack virulence factors of other plant pathogens. In Pseudomonas fluorescens Pf-5, enzymes that degrade plant cell walls and their components such as cellulase, pectinase, or pectinlyase are not present (2). Also it's demonstrated a hemolytic activity and as a result has been known to infect blood transfusions (3).

Strains of P. fluorescence showed known biological control activity against certain soil-borne phytopathogenic fungi and have the potential to produce known secondary

metabolites such as; Siderophore, hydrogen cyanide (HCN) and protease, which showed antagonistic activity against these fungi (4).

In this study we described the production, partial purification and some properties of one antibacterial substance produced by P. fluorescens.

Material & Methods

Isolation of P. fluorescens

P. fluorescens isolated from soil and was grow on nutrient agar (Difco) containing 5% yeast extract (Difco). Identified based on biochemical test and Morphological characteristics of the isolated bacterium (5).

Tested bacteria

The tested bacteria were isolated from patients with skin infections at Al- Yarmouk teaching hospital, the isolates were grow on nutrient agar and kept at 4 ºC until used.

Identification of Staphylococcus aureus and Klebsiella pneumoniae were based on their biochemical tests and Morphological characteristics (5)

Extraction & Purification of antibacterial substance

The antibacterial substance was extracted according to Sarangamat et al.(6), by adding 10ml of methylene chloride to 200ml of fresh broth culture of P. fluorescens and then shaking for 2hr. resultant emulsion was passed through layers of cotton to break the emulsion. After separation of the aqueous phase and organic solvent phases, the relatively inactive aqueous phase was discarded without further processing and the methylene chloride phase was evaporated under reduced pressure. The residue was then dissolved in small volume (1-5) ml of methylene chloride, filtered, and evenly absorbed onto dry silica gel (Fluka) (3g). After evaporation of the solvent, the charged silica gel was layered over the top of previously peaked, dry silica gel column (2.6 by 10cm). The column was washed exhaustively with methylene chloride (250 ml) to remove some orange – yellow inactive pigments. The main active band (yellow) was eluted from silica gel column with mixture of acetone and methylene chloride (1: 10 v/v) (300 ml). After solvent was evaporation, the resulting bright yellow residue was dissolve in (2-3) ml of methylene chloride, to which was added (0.5-1) ml of absolute methanol. The solution mixture was warmed on a water bath until a few needle – shaped crystals were formed in the container. At this stage the solution was kept overnight at 10ºC to crystallize the antibacterial substance.

Detection of antibacterial activity:

For detection of antibacterial activity, an agar spot test was used. Overnight culture of P. fluorescens was spotted onto the surface of agar plates (nutrient agar) and incubated for 24 hr. at 37 ºC to allow colony development. Approximately 5×10^5 cells of the K. pneumoniae and S. aureus strains were inoculated onto 7ml of an appropriate soft agar (0.7% w/v) and poured over the plate onto which the produce organism had been grown. Incubation at 37 ºC, after 24hr. the plates were checked for inhibitory zones. Inhibition was scored positive in the zone was wider than 2mm (7).

Stability of antibacterial substance activity

1. pH effects

Sample of antibacterial substance (solution of crystals in methylene chloride) was adjusted to pH value in the range (1-12) using 1N HCL and 1N NaOH. Antibacterial activity was then measured by the agar spot test. Plates were seeded with 0.1 ml of 24 hours inoculated broth of tested microorganism (approximately 5×10^5 CFU/ml). The agar plate was dried, and then 5µl of the antibacterial substance at different pH were spotted on to the surface of the plate. The inoculated plates were incubated at 37°C for 24 hours. The diameter of the inhibition zones was measured (8).

2. Serum effects

Sample of antibacterial substance was diluted in human serum of healthy volunteers in concentrations (25, 50, 75, and 100) % (v/v). Antibacterial activity determined by the agar diffusion (9). Petri dishes containing nutrient agar were inoculated with K. pneumoniae and S. aureus isolates by using sterile swabs, six evenly spaced holes (5mm) in diameter were made in the agar of each plate with sterile cork borer. To identify the intrinsic antibacterial activity of the diluents, control wells were filled with sterile 0.9% saline and undiluted serum. Test wells contained full strength (100, 75, 50, 25) % of antibacterial substance with serum. Three replicate plates were prepared. After the plates were inoculated equal volume 200 µl of each concentration was expressed into each well. Test

plates were then incubated at 37 ºC for 24hr. and zones of inhibition were measured with millimeters. A clear area around test wells indicated antibacterial substance had retained its antibacterial activity.

3. Solubility in organic solvents:

Sample of antibacterial substance (crystals) was treatment with organic solvents (methylene chloride, chloroform, acetone, sodium hydroxide (2N) and hydrochloric acid (2N), methanol, ethyl acetate, hexane and water in concentration 1:10 (w/v) (6).

Results & Discussion

Pseudomonas fluorescens produces a variety of secondary metabolites including siderophores and antibiotics such as phycocyanin, pyrolnitrin and pseudomonic acid, was investigated in in vitro and in vivo that showed antimicrobial activity against pathogenic bacteria and fungi (10, 11).

Our results showed that P. fluorescens isolate produced a yellow colored substance when grown in nutrient yeast extract broth. Antibacterial substance was determined from silica gel column. The antibacterial activity detected in the third peak (Fractions 25-30) (Fig.1).

The results of the agar spot method shown; the substance was stable, by measuring the inhibition zone size against the K. pneumoniae and S. aureus isolates within the range pH (5-9) at 37ºC for 24hr. and outside these limits there was loss of activity (Table 1). The highest activity was detected at pH (7) at 37ºC, whereas, mupriocin more active at pH 5.0 and pH 5.5 against bacteria (12); our results shown the antibacterial activity of substance produced by P. fluorescens was active at neutral pH, but exhibited reduced activity at acidic pH.

The result of the agar diffusion method showing no intrinsic antibacterial activity (measured by inhibition zone size) was found for (50, 75, and 100) % concentration of serum with antibacterial substance against the K. pneumoniae and S. aureus isolates (Table 2), also Mupirocin was highly bound (95%) to the protein of human serum, and as a consequence, the activity of the antibiotic was reduced in the presence of human serum (12).

Our results showed the antibacterial substance produced by P. fluorescens was soluble in methylene chloride, chloroform, acetone, (2N) NaOH and (2N) HCL, and was insoluble in methanol, ethyl acetate, hexane and water. This finding was agreed with Sarangamat et al. (6). Antimicrobial activity was not lost when treated with organic solvents (13).

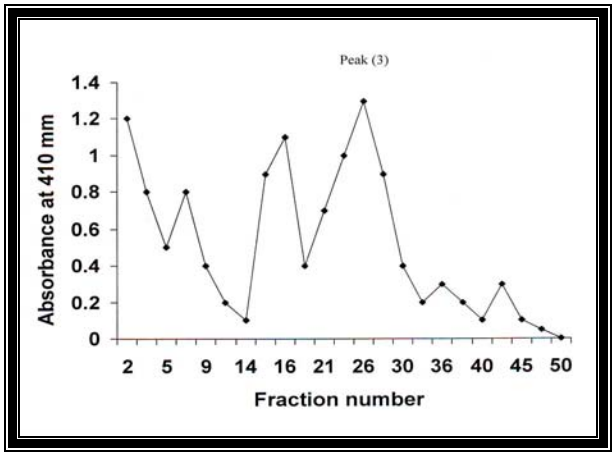

Fig (1): Purification of antibacterial substance produced by *Pseudomonas fluorescens*

Table (1): Activity of antibacterial substance against *S. aureus* and *K. pneumoniae* in the pH ranges (1-12).

pH	Inhibition zone diameter (mm.)	
	S. aureus	*K. pneumoniae*
1	-	-
2	-	-
3	-	-
4	1.7	1.2
5	1.8	1.2
6	4	2
7	6	3
8	5	2
9	5	3
10	2	1
11	-	-
12	-	-

(-) No inhibition zone

Table (2): Activity of full strength and diluted antibacterial substance against *Staph. aureus* and *K. pneumoniae*.

Bacteria	Inoculums and diluents antibacterial substance	Inhibition zone diameter (mm.)			
		100%	75%	50%	25%
Staph. aureus	No diluent	14			
	0.9% saline (control)	-			
	serum	-	-	3	8
K. pneumoniae	No diluent	10			
	0.9% saline (control)	-			
	serum	-	-	2	5

***S. aureus* , *K. pneumoniae*: 10^5 cfu/ml (colony – forming unit)**
(-)No inhibition zone

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