Original
ISSN 2413-0516

Synergistic Effect of Purified Protease Enzyme with Antibiotics on Pathogenic *S. Aureus In-Vitro* and *In-Vivo*

Haifa Sadoon Abdulwahhab[1*], Khawlah Jebur Khalaf[1], Raghad Akram Aziz[2]

[1]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.
[2]Department of Biology, College of Basic Education, Mustansiriyah University, Baghdad, Iraq.
*Correspondence to: Haifa Sadoon Abdulwahhab (E-mail: hayfasadoon.ph.d.mic.2020@uomustansiriyah.edu.iq)
(Submitted: 16 September 2024 – Revised version received: 27 September 2024 – Accepted: 10 October 2024 – Published online: 26 October 2024)

Abstract

Objective: The goal of this study is the detection of the *P. aeruginosa* isolate for production the protease, the optimal conditions for produce, extraction, purification and characterization of the enzyme and study the synergistic effect of protease with antibiotics on *S. aureus.*

Methods: Among (110) isolates from clinical sources, only (74) isolates identified as *P. aeruginosa* isolated from Baghdad hospitals. Detection of the optimal isolate for production the protease enzyme and the optimal conditions its produce, purification of enzyme by using ammonium sulfate, ion exchange chromatography and gel filtration, enzyme characterization of PH and temperature activity and stability, the study of synergistic effect of protease with antibiotics on *S. aureus*.

Results: The optimal conditions to produce protease is pH (8), (37)°C, (BHI) broth, and during (48 hrs.). The precipitation saturation ratio (80%). Ion-exchanges (DEAE and CM) Cellulose and Gel filtration have specific activity ((121.25), (161.6), (660.53)) units/mg)) respectively. The characterization done by pH and temperature activity purified protease was active in (138.51 units/ml) at pH (8), and stable in pH (8), the temperature for protease activity is (158.21 units/ml) at (37)°C. The synergistic effect of purified protease with antibiotics on *S. aureus in-vitro*,was detected using the agar diffusion method, the effectiveness of the prepared hydrogel types, the results showed *S. aureus* was more sensitive isolate to prepare (Gel & Protease & Ceftriaxone) the diameter of the inhibition zone reached (44 mm), the synergistic effect we noticed when using a (Gel & Ceftriaxone & Protease) healing was observed in time (five days) with wound healing without effect.

Conclusion: In this study, it was discovered that mixing (protease + hydrogel + Ceftriaxone) accelerates the wound healing without leaving traces.

Keywords: Peptide Hydrolases, anti-bacterial agents, staphylococcus aureus.

Introduction

P. aeruginosa belong for Gram-negative bacteria, opportunistic, causes various infections. It is one of the most prevalent pathogens in wound infections and delayed healing process. *P. aeruginosa* thrives at (37–42)°C and the fact that it can also grow at (42)°C distinguishes it from other fluorescent-type *Pseudomonas* species.[1]

P. aeruginosa can produce four types of pigments included: pyocyanin, pyoveridin, pyorubin and pyomelanim,as well as this genus might be split in to (2 groups) fluorescent and non-fluorescent based on their synthesis of pigments. Also *P. aeruginosa* is known for producing biofilm that are resistant to antibiotics.[2]

P. aeruginosa is known for producing biofilm that are resistant to antibiotics and also it producing virulence factors include flagella, pilli, exopolysaccharides, elastase, pigments, proteases, etc. Proteases are important enzymes secreted by *P. aeruginosa* and one of its types is alkaline protease, proteases used in many industrial and food fields as well as the health fields.

Also proteases have catalytic role in protein hydrolyzing and effects on bacteria and cancer cells through several mechanisms included modification of external membrane and inner membrane etc, these mechanisms can cause bacterial death[3] this study aims to produce protease from *P. aeruginosa* and study the synergistic efficiency of protease with antibiotics on *S. aureus*

Material and Methods

Identification of P. aeruginosa

Single colony was transferred, well-fixed, and stained with Gram stain on a microscope slide. After that, the bacterial colonies were moved to (MacConkey and Cetrimide Pseudo) agar in an aerobic environment for routine microbiological culture studies. The IMViC, oxidase and catalase test were performed as the final identification.

Screening of the Protease Producing from P. aeruginosa Isolates and Extraction Enzyme:

The method (Senior, 1999)[2] was followed to screen and estimate the protease enzyme qualitatively from *P. aeruginosa* isolates, to then estimate it quantitatively according to the method (Jain *etal.*, 2017)[4], then extract the enzyme according to the method (Barequet,2004)[5] to then estimate the activity of the enzyme based on the method of the scientist (Senior, 1999)[2].

Optimum Conditions for Protease Production

Optimum Culture Medium

The optimum production medium of *P. aeruginosa* (P15) was studied by using different types of media, included ((Casein, Luria, Van Gundy (VG) medium, salt casein and BHI) broth). Then enzyme activity and specific activity were estimated.

Optimum Incubation Period

Determining the ideal time for enzyme production through the incubation of production medium for (24, 48,72) hrs., the impact of the incubation period on the protease production through isolate of *P. aeruginosa* (P15) was investigated. Specific activity and enzyme activity were estimated.

Optimium Temperature

The production medium has been incubated at various temperatures (20, 25, 30, 35, 37, 40, and 42)°C, respectively, after that enzyme activity and specific activity were estimated.

Optimum pH

To establish the ideal (PH) for protease production, the production medium's PH was prepared with varying PH values from (6 to 12). After then the medium was incubated for (24 hrs) at (37)°C. The specific activity enzyme activity were estimated.

Effect of Ventilation on Enzyme Production

To know the effect of ventilation on enzyme production, I followed the method.[6]

Protease Purification

After the enzyme extraction step, the method (Hussein, 2016)[7] was followed to precipitate the (crude enzyme) using (ammonium sulfate) with a precipitation rate of (80%). After that, the (dialyzed enzyme) was added to the ion-exchange chromatography DEAE-Cellulose column as mentioned by (Hamdan *etal*., 2018)[8]. While gel filtration chromatography was used as a final step by following the method (Andrews,1964)[9] and the concentration and specific activity for protease were estimated by applying the method (Bradford, 1976) and (Dasilva *etal*., 2017)[10] respectively.

Enzyme Characterization

The protease was characterized by determining or knowing its molecular weight according to the method (Andrews, 1964)[9] and knowing the extent of the effect of pH and temperature on the activity and stability of the enzyme by applying the method (Peter and Galloway, 1990)[11]

Detection of Biofilm Formation Isolates

The microtiter plate method referred to by (Shanmugasundaram *etal*., 2012)[12] was used to identify pathogenic isolates biofilm formation.

Determination of MIC for Ceftriaxone Against Biofilm Formation Isolates

Using the micro dilution method according to[13] to determine the minimum inhibitory concentration (MIC) the antibacterial activity Ceftriaxone.

Effect of Ceftriaxone on Biofilm Formation

Using co-incubation studies, the antibiofilm activity of Ceftriaxone against (MDR) bacterial isolates from skin infections was measured in accordance with the methodology outlined by[14]

Determination of MIC for Doxycycline against Biofilm Formation Isolates

As the same determination of (MIC) for Ceftriaxone against Biofilm formation isolates

Effect Doxycycline on Biofilm Formation

In the same effect of Ceftriaxone on biofilm formation, the antibiofilm activity regarding Doxycycline against (MDR) bacterial isolates from skin infections was assessed.

Antibacterial Activity of Purified Protease on Biofilm Formation Isolates

With the use of microdilution method according to (Elshikh *etal*., 2016)[13] and depending on (MIC) values, the antibacterial activity protease against biofilm formation isolates was evaluated.

Effect of Protease on Biofilm Formation Isolates

By using co-incubation tests, the antibiofilm activity regarding protease enzyme against biofilm-forming bacterial isolate *S. aureus* from skin infections was measured in accordance with the methodology outlined by[15]

Determination of Combined Effect of Protease and Ceftriaxone by Microdilution Checkerboard Method

The synergistic effect of protease combined with ceftriaxone against *S. aureus* (, (MICs) of Ceftraxone and protease were separately determined by the standard microdilution method, according to[14]

Determination of Combined Effect of Protease and Doxycycline by Microdilution Checkerboard Method

The synergistic effect of protease combined with Doxycycline against *S. aureus*,evaluated as the same determination of combined effect of protease and Ceftriaxone by Microdilution checkerboard method.[14]

Preparation of the Hydrogel

We followed the method[16] with modifications to prepare of the hydrogel

Hydrogel Test

Measurement the swelling ratio, ability to spread and viscosity:-
The swelling ratio, ability to spread and viscosity for the hydrogel model measure according to mentioned in.[16]

Antibacterial Effect of Prepared Wound Hydrogel

The effect of prepared hydrogel with protease was studied against *S. aureus* isolated from wound using well diffusion methods according[16]

Treatment of Wounds Infection Using Prepared Wound Gel

The experiment was conducted as mentioned in[16] with some modification.Mice were divided into groups including:

Group 1: Mice without any infection without any pathogenic bacteria (control -), (5 mice).
Group 2: Mice were infected with *S. aureus* bacteria without treatment and were (control +), (10 mice).
Group 3: Induction wound infected by S. aureus treatment with gel (50%) only (8 mice).
Group 4: = = = = = = = = with gel (50%) + Ceftriaxone (8 mice).
Group 5: = = = = = = = = with (gel + protease) (8 mice).
Group 6: = = = = = = = = with (Ceftriaxone gel + protease) (8 mice).

Results

Isolation, Identification of P. aeruginosa and Culture Characteristic

In this study, (110) isolates were collected from different clinical sources. Only (74) isolates belong to *P. aeruginosa*. Were cultured on Pseudo-Cetrimide agar under aerobic conditions at (37)°C and incubated for (24–48) hrs.

On the pseudo-Cetrimide agar the colonies of *P. aeruginosa* isolates appear yellow-green to blue colored. The *P. aeruginosa* colonies on the MacConkey agar medium appeared round, small, convex, rough colony with irregular edges, whitish or creamy in color and has fruity odor.

Detection and Screening the Ability of P. aeruginosa Isolates to Produce Protease Enzyme

Qualitative Assay

Screenings about protease production by *P. aeruginosa* were used for detecting the ability of these isolates to grow on skim milk agar medium. A total of (74) isolates of *P. aeruginosa* were used in screening for protease production to period (24 h/48h) as shown in table 1 & 2

In our study, 56(75.67%), 61(82.43%) isolates positive for protease production at (24h, 48 h) respectively.

Quantitative Assay

Protease producing isolates were measured again quantitatively by the spectrophotometer had been used to detect the released amount of protease from the degradation of the casein at (A280) during (24 & 48) hrs. The results explained in figure 1 & 2

The results showed the isolate of *P. aeruginosa* (P15) showed the highest proteolytic activity both the qualitative and quantitative assay during (24,48) hrs.

Determination of Optimum Conditions for Protease Production

The optimal conditions including the best conditions for *P. aeruginosa* (P.15) to produce the protease enzyme. The result show the best medium is (BHI) broth with specific activity (91.21) units/mg, as shown in figure 3.

The results in figure 4 showed that the optimal temperature for producing the protease is (37)°C, with a specific activity of (88.9) units/mg of protein.

The optimal incubation period to produce the protease enzyme is (48) hrs at (37)°C and specific activity (80.59) units/mg. figure 5.

On the other hand, it was observed that the enzymatic activity increases when using a shaking incubator, figure 6. The results showed that the best production of the enzyme was at pH (8), as the specific activity reached (91.02) (unit/mg), as figure 7.

Table 1 **Screening protease producer *pseudomonas aeruginosa* isolates at (24h)**

Number of isolates	Protease production	Non-Protease production
74	56	18
Percentage	75.67	24.32

Table 2 **Screening protease producer *pseudomonas aeruginosa* isolates at (48h)**

Number of isolates	Protease production	Non-Protease production
74	61	13
Percentage	82.43	17.56

Extraction and Purification of Protease from P. aeruginosa (P.15) Isolate

Preparation of Protease Crude

The *P. aeruginosa* (P.15) was grown under optimum conditions, inoculated into (250 ml) of (BHI) broth as a productive medium, at (37° C) for (48) hrs. in shaking incubator (200) r.p.m then centrifuged by cooling centrifuge at (4°C, 6000 g, 20 min), supernatant which was filtered by Millipore filter (0.22 µm). The protease activity and specific activity were (40.25 unit/ml) and (65.983 unit/mg) respectively and the concentration of protein was (0.610 mg/ml).

Purification of Protease Enzyme

Ammonium Sulfate Precipitation

The optimal range for enzyme precipitation was at the saturation ratio of 80% when ammonium sulfate was utilized at different saturation rates (40, 50, 60, 70 and 80%), as shown in table 3.

The enzyme activity and specific activity (121.25) units/mg and (123.096) units/mg respectively and the protein concentration (0.985 mg/ml) with a purification of fold (1.865) and enzyme yield (72.298%).

Ion Exchange Chromatography (DEAE-cellulose)

Ion exchange chromatography patterns showed one protein peaks in washing steps and three peaks in gradient elution (0.1–1M) NaCl. Tubes (12–21) exhibited the highest protease activity and the enzyme activity appears in washing fractions, which means protease had a positive charge enable it to bind

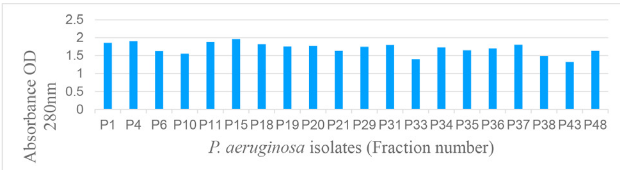

Fig. 1 **Spectrophotometer reading at (A280) after (24hours).**

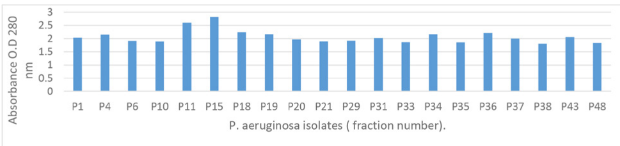

Fig. 2 **Spectrophotometer reading at (A280) after (48hours).**

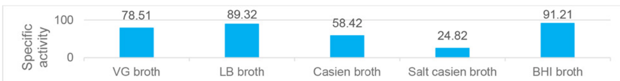

Fig. 3 **The best medium for protease production from *P. aeruginosa* (P.15).**

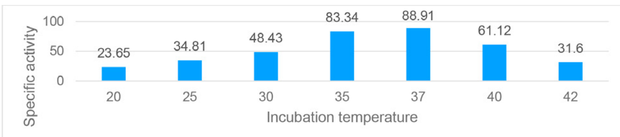

Fig. 4 **The best temperature for protease production from *P. aeruginosa* (P.15).**

Table 3 **Purification steps for Protease produced by *Pseudomonas aeruginosa* (P15)**

Purification Steps	Vol. (ml)	Enzymic Activity (U/ml)	Total Activity (U)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude extract	250	40.25	10062.5	0.610	65.983	1	100
Concentration by Ammonium Sulphate 80%	60	121.25	7275	0.985	123.096	1.865	72.298
Ion exchange chromatography DEAE-Cellulose	30	155.25	4657.5	0.350	443.571	6.722	46.285
Ion exchange chromatography CM-Cellulose	25	161.6	4040	0.302	535.09	8.109	40.149
Gel Filtration Sephadex G-100	20	172.4	3448	0.261	660.53	10.010	34.265

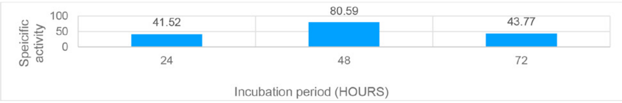

Fig. 5 **The best incubation period for protease production from *P. aeruginosa* (P.15). without the shaking incubator.**

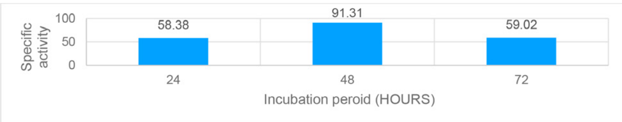

Fig. 6 **The best incubation period for enzyme production with presence of a shaking incubator**

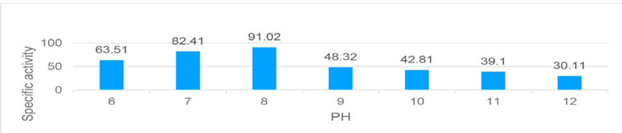

Fig. 7 **The best PH on protease production from *P. aeruginosa* (P.15).**

with the resin of ion exchange which has negative charge, figure 8.

The protease activity and specific activity were (155.25 units/ml), (443.57 units/mg) respectively, and a fold of purification (6.722) and enzymatic yield (46.285%) as explained in table 3.

(CM-Cellulose) Ion Exchange Chromatography

Tubes (43–52) exhibited the highest protease activity, (CM-Cellulose) patterns showed one peak in washing steps and one peak in gradient elution (0.1–1M) NaCl. The enzyme activity appears in elution fraction, which means protease had negative charge unable it to bind with the resin which has a positive charge. The fraction gathered and experienced for (protease and specific) activity were (161.6 units/ml), (535.09 units/mg) respectively, and a fold of purification (8.109) and enzymatic yield (40.149)% as explained in table 3, figure 9.

Gel Filtration Chromatography

Results in figure 10 showed the appearance of two peaks, the first peak (fraction tubes 15–30) included protease activity (172.4 units/ml) protein concentration (0.261 mg/ml) with specific activity (660.53 units/mg) and the purification fold

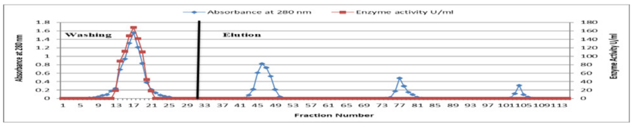

Fig. 8 **Ion exchange chromatography(DEAE-Cellulose).**

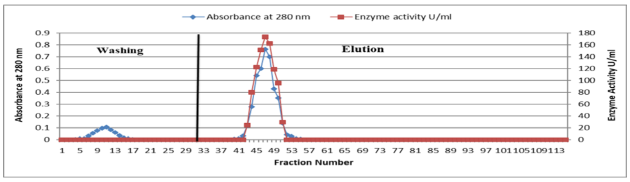

Fig. 9 **CM-Cellulose column for purification of protease produced by *P. aeruginosa*.**

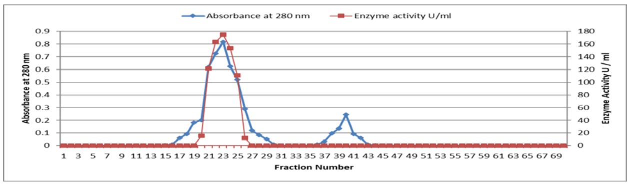

Fig. 10 **Gel filtration chromatography of protease produced by the *Pseudomonas aeruginosa*.**

was (10.01) with a yield of enzyme (34.265%) as mentioned in table 3. The protease obtained from *P. aeruginosa* was further purified and characterized using the Sephadex G-100 chromatography system.

Characterization of Purified Protease

The Characterization of purified protease includes study the effect of (molecular weight measurement, PH and temperature) on protease activity and stability.

The (M.W) of the protease was estimated by using gel filtration method using a standard curve,the molecular weight of this enzyme was estimated at about (19,952) Daltons, figure 11.

Effect of Ph on Protease Activity

The effect of pH on the activity of the enzyme purified from *P. aeruginosa* (P.15) was studied with pH ranges ranging from (5–11), the optimal pH for enzyme activity was (8) and the rate of enzyme activity (138.51) unit/ml. figure 12.

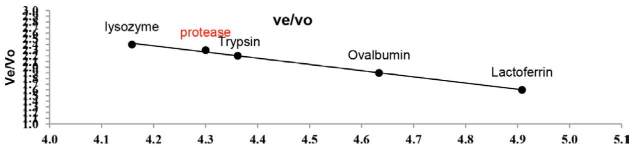

Fig. 11 **Standard curve to estimate molecular weight of protease using gel filtration by using standard proteins.**

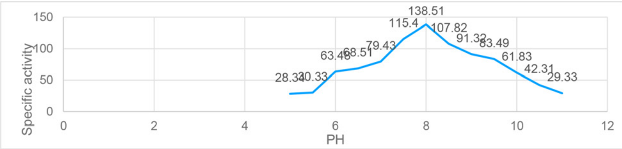

Fig. 12 **The optimum pH for protease activity.**

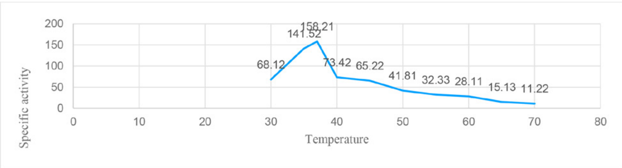

Fig. 13 **The optimum temperature for protease activity.**

Table 4 **Detection of Biofilm formation of clinical isolates**		
	Bacterial isolate	**Biofilm formation**
1	*P. aeruginosa*	Non
2	*P. aeruginosa*	Weak
3	*P. aeruginosa*	Strong
4	*P. aeruginosa*	Non
5	*P. aeruginosa*	Strong
6	*S. aureus*	Weak
7	*S. aureus*	Strong
8	*S. aureus*	Strong
9	*S. aureus*	Moderate
10	*S. aureus*	Strong
11	*S. aureus*	Moderate
12	*K. pneumoniae*	Non
13	*K. pneumoniae*	Non
14	*K. pneumoniae*	Non
15	*K. pneumoniae*	Non
16	*K. pneumoniae*	Weak
17	*Serratia marcescence*	Non
18	*Serratia marcescence*	Weak
19	*Serratia marcescence*	Weak
20	*Serratia marcescence*	Weak
21	*Serratia marcescence*	Weak
22	*E. coli*	Weak
23	*E. coli*	Non
24	*E. coli*	Strong
25	*E. coli*	Weak
26	*E. coli*	Strong

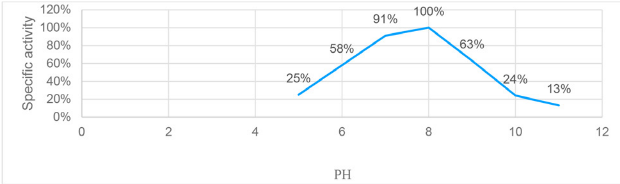

Fig. 14 **The optimum PH for protease stability.**

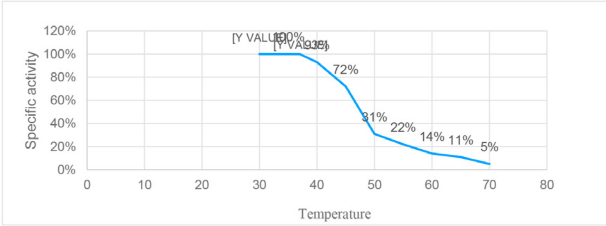

Fig. 15 **The optimum temperature for protease stability.**

Effect of Temperature on Protease Activity

The effect of different temperatures on the effectiveness of the enzyme purified from the bacterium *P. aeruginosa* (P15), which ranged between (30–70)°C,

The effectiveness of the enzyme was observed when the temperature between (30–37)°C, as it reached (158.21) unit/ml at (37)°C,while at (70)°C, where the lowest specific activity reached (11.22) unit/ml, figure 13.

Effect of Ph on Protease Stability

The optimal pH for enzyme stability appeared in a range between (7–8), as the remaining activity when incubating the enzyme with pH (7) was (91%), and its full effectiveness was maintained at pH (8) (100%), figure 14.

Effect of Temperature on Protease Stability

It was noted that the enzyme kept its full effectiveness at a temperature of (30–37)°C, After that, the effectiveness of the enzyme gradually decreased, reaching its lowest effectiveness at the temperature of (30–37)°C, fig (17). Temperature (70)°C, and the effectiveness was (5%) as showed the figure 15.

Detection of Biofilm Formation Isolates

The isolates obtained from this study, which amounted to (26) different isolates, included: *S. aureus* (6), *P. aeruginosa* (5), *S. marcescence* (5), *K. pneumonia* (5) and *E. coli* (5). The results of examining the biofilm formation of these isolates show in table 4.

In this study, noted that most bacterial isolates showed an ability ranging from weak, moderate, and strong to form biofilm was evaluated using the microtiter plate technique and determined by comparing O. D values of dyed adherent cells.

The *S. aureus* showed an average ability to produce Bio-film, one of the reasons that enabled *S. aureus* isolates to form a highly cohesive biofilm is their ability to secrete nuclease enzymes that work to decompose eDNA, which is one of the primary components in building the biofilm and making the cells able to bond with each other[16]

Antibiotic sensitivity test for Biofilm formation isolates

Some isolates that biofilm formation (11 isolates) from *P. aeruginosa* (3) *S. aureus* (6) and *E. coli* (2) isolated from burns and wounds were evaluated for sensitivity against (11) type of antibiotics (Doxycycline (DXT), Tobramycin (TOB), Amikacin (AK), Ceftazidime (CAZ), Imipenem (IMI), Ceftriaxone (CTX/RO), Tetracycline (TE), Cefepime (FEP), Gentamicin (CN), Aztreonam (ATM)) and Metheicillin (MET).

The obtained data had been recorded according to the appearance zone surrounding the antibiotic disc using the disc diffusion technique. The isolates were categorized into three groups: resistant, intermediate, and sensitive. Figure 16.

All isolates (11 isolates) showed varied levels of resistances to antibiotics. All isolates had been resistant to Cefepime (FEP) and Ceftriaxone (CTX/RO) with (100)%, Where the results revealed that all isolates showed resistance to the Amikacin (AK) except one isolate (E. coli) with (90.90)%. Also results revealed that the (4 isolates) of *S. aureus* from (6 isolates) had been resistant to Aztreonam (ATM), Ceftazidime (CAZ) and Tetracycline (TE) with (66.66)%, where the (3 isolates) of *S. aureus* from (6 isolates) had been resistant to Doxycycline (DO/DOX) and Methicillin (MET) with (50)%. Finally, the results show the (1 isolate) of *S. aureus* had been resistant to Gentamicin (CN) with (16.66)%. The results showed that all isolates were (100)% resistant to Doxycycline (DO) and Methicillin (MET).

Also results showed that the (5 isolates) of *S. aureus* from (6 isolates) were sensitive to Tobramycin (TOB) and Imipenem (IPE) with (83.33)%, will (3 isolates) of *S. aureus* from (6 isolates) were sensitive to methicillin (MET) and Doxycycline (DO/DOX) with rate 50%, also results revealed that the (2 isolates) of *S. aureus* from (6 isolates) had been sensitive to Gentamicin (CN) and Aztreonam with (33.33)%, finally Ceftazidime had a sensitive rate of (16. 66)%.

Anti-Bacterial Activity of Purified Protease and Effect on Biofilm Formation

Purified protease from *P. aeruginosa* (P.15) had been used to determine the MIC of purified protease at concentrations ranged from (20–0.03) mg/ml against *S. aureus* isolates. As compared to the control, purified protease (MIC) against *S. aureus* isolates evaluated and had been (10 mg/ml).

The formation of biofilms in *S. aureus* isolated from wounds were suppressed by purified protease, and had been reduced after treatment with purified protease at different incubation periods (24, 48, 72) hrs compared with control. Biofilm formation is inhibited which had been observed (53.06%) against *S. aureus* isolated after (24) hr, and (13.100) after (72) hrs. (Table 5).

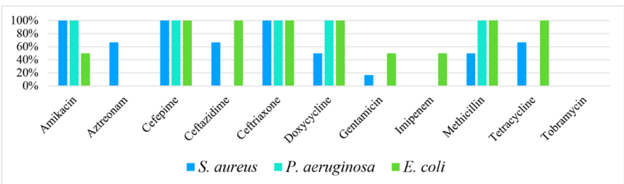

Fig. 16 **Antibiotic susceptibility test.**

Table 5 **Inhibition of biofilm formation by purified protease at different incubation periods.**

	Bacterial isolates	% Biofilm inhibition by purified protease		
		Incubation period		
		24	48	72
1	***Staph. aureus***	38.311%	21.035%	1.67%
2	***Staph. aureus***	53.06%	33.64%	13.100%

Combined Effect of Protease with Ceftriaxone and Doxycycline by Micro Dilution Checkerboard Method

Protease have been reported to show antimicrobial activity, was studied against gram-positive bacteria *S. aureus* using the standard microdilution method, and interaction between the protease and (Ceftriaxone) and (Doxycycline) separately were estimated by calculating the fractional inhibitory concentration (FIC index) of the combination. through checkerboard assay.

Results demonstrated that Protease purified by *P. aeruginosa* (P.15) revealed the maximum antibacterial effect, the improvement in antibacterial effect was seen when combined with Ceftriaxone and Doxycycline. Synergistic effect was shown Table 6 and 7.

This study affords a most important report of the synergistic activity of Protease with Ceftriaxone and Doxycycline against pathogenic isolates.

Combination therapy is applied with the purpose of receiving advanced the antimicrobial spectrum, lessening toxicity, avoiding the emergence of resistant mutants during therapy and finding synergistic antimicrobial activity. Synergism of a combination of antibiotics can be quantified as fractional inhibitory concentration indices (FIC) derived from a checkerboard microdilution method, this method provides a dynamic observation of the bactericidal effect of the antimicrobial agents[17]

Table 6 **Combined activity of protease with Ceftriaxone against *S. aureus***

	Bacterial isolate
	S. aureus
MIC of protease (mg/ml)	10
Concentration of protease in combination (mg/ml)	0.62
MIC of Ceftriaxone (µl/ml)	7.8
MIC of ceftriaxone in combination with protease ((µl/ml)	3.9
FIC	0.562
FIC INDEX	Synergistic

Table 7 **Combined activity of protease with Doxycycline against *S. aureus***

	Bacterial isolate
	S. aureus
MIC of protease (mg/ml)	10
Concentration of protease in combination (mg/ml)	0.62
MIC of **Doxycycline** (µl/ml)	15.6
MIC of **Doxycycline** in combination with protease ((µl/ml)	1.9
FIC	0.183
FIC INDEX	Synergistic

Hydrogel Test

Swelling Ratio

In a test to detect the ability of the hydrogel to swell, the preparations showed an ability to retain water, and from the results we note the ability of the Carbopol 934 hydrogel to retain large amounts of water when prepared at a concentration of 50% according to the application of the swelling equation[16]

Ability to Spread

The ability of the gel to spread was determined, as the diffusion value reached (0.5) cm within (5 min). A good gel has the ability to spread in the shortest possible time, as mentioned[16]

Viscosity

The viscosity value obtained using the Viscometer VR3000 was (772C.p) at a temperature of 37°C.

Antibacterial Effect of Prepared Wound Hydrogel

S. aureus that make up the biofilm was identified, which showed resistance to most antibiotics, in order to test the effectiveness of the (hydrogel alone), (protease + gel), (Ceftriaxone + gel), and mixed (gel + protease + Ceftriaxone) at other times, in inhibiting growth. *S. aureus* isolate was detected using the agar diffusion method, as the inhibition results were obtained from measuring the diameters of the inhibition zones for the isolates growing on the test medium, as shown in Table 8.

The results showed that the *S. aureus* bacteria was more sensitive to the prepare (Gel & Protease & Ceftriaxone),the diameter of the inhibition zone (44 mm).

Treatment of Wound Infections using Prepared Gel

The protease enzyme has activity against *S. aureus* that are resistant to most antibiotics. In thismore study the effect of the protease enzyme on wound infections and their treatment, it was necessary to use (gel) to be a carrier material and help spread the enzyme in animal tissues.

Experimental wounds were created in the skin of the mice according to selection of *S. aureus* bacteria to contaminate wounds the most affected group is the group treatment with mixture of (gel + Ceftriaxone + protease), where the period of healing of the wound was (5 days). In addition to a decrease in the, this group also possesses a high inhibitory activity against the S aureus bacteria and its toxic products contaminating the wound area.

Table 8 **Antibacterial activity of prepared wound hydrogel**

Bacterial isolate	Gel	Gel & protease	Gel & Ceftraxone	Gel & protease & Ceftraxone (mixed)
S. aureus	24mm	25mm	38mm	44mm

Discussion

Under a microscope, *P. aeruginosa* is (-Ve) bacteria. Gram stain showed very small rods, single or in pairs and non-spore forming bacteria

The ideal conditions for producing protease, ideal PH (8) with specific activity (91.02) units/mg, at (37°C) as the specific activity (88.9 units/mg), this result was in agreement with;[5] best medium is BHI broth with a specific activity (91.21 units/mg) and the results agree with the study of[7] The ideal hours are (48 h) with specific activity (80.59 units/mg). the best ratio for precipitating protease was (80%) saturation of ammonium sulfate.

Study done by[7] demonstrate the purified protease from *P. aeruginosa* was precipitation a round (80%) with total protease activity and specific activity 125.3 U/ml, 112.77 U/mg respectively

In DEAE-Cellulose the protease activity and specific activity were (155.25 units/ml), (443.57 units/mg) respectively and a fold of purification (6.722) and enzymatic yield (46.285%) while in study done by[6] results noticed that purification of protease has negative net charge, with protease specific activity was (10.8 mg/ml), purification fold (18.3) and yield (78%).

While, CM-Cellulose the protease activity and specific activity were (161.6 units/ml), (535.09 units/mg) respectively, and a fold of purification (8.109) and enzymatic yield (40.149%). The S aureus isolate had sensitive to (Gel + protease + Ceftriaxone) as the diameter of inhibition zone reached (44 mm), while the synergistic effect we noticed when using a (Gel & Ceftriaxone & Protease) healing was observed in time (5 days) with wound healing without effect.

Conclusion

In this study, it was discovered that mixing (protease + hydrogel + Ceftriaxone) accelerates the wound healing without leaving traces.

Conflict of Interest

None.

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