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A biological study of chitinase produced by clinical isolates of *Pseudomonas aeruginosa* and detection of *ChiA* responsible gene

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ABSTRACT

All isolates in this study were diagnosed as *P. aeroginosa* according to the 16srRNA gene. Only two isolates were produced chitinase on chitin agar medium and were positive to the chiA gene. Most of the isolates exhibited high sensitivity (95%) and (90%) to Imipenem and Carbenicillin, respectively, and the resistance to Amoxicillin + Clavulanic acid was shown (80%), while revealed a variable degree in their response to others antibiotics. The crude extract activity and specific activity for extracted chitinase enzymes were 33 U/ml and 18.644U/mg, respectively. The enzyme was purified by different steps include: precipitating with saturation 70% of ammonium sulfate and then applied on ion-exchange chromatography using DEAE- cellulose column and then employ the Sephadex G-200 column for gel filtration chromatography. The purification fold and yield was 28.5%. The molecular weight of the purified enzyme was determined by SDS-PAGE method, and it appeared at 50 kDa. The results of the MTT assay showed that the chitinase has a cytotoxic effect on cancer liver cell lines at a concentration of 100 μg/ml and increased gradually at a concentration of 600 μg /ml, while it showed no or less cytotoxic effect on normal embryonic liver cell line (WRL-68). Chitinase enzyme appeared a higher antibacterial activity at concentration 600 μg/ml and a lower activity at concentration 400 towards clinical isolates of *S. aurous* and *E. coli*. The study of histopathological effects was exhibited little morphological changes on cells of liver tissues.

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INTRODUCTION

Pseudomonas aeruginosa is a pathogenic bacteria grows under both aerobic and anaerobic conditions, it gram-negative bacterium, motile,non-lactose fermenting, it implicated and caused several types of infections like UTIs, gastrointestinal system, eye, ear, bone, joints, bacteremia cystic fibrosis, burns and wounds infections and infection of the respiratory system (Schurek *et al.*, 2012). It is considered the most common reason for pneumonia, urinary tract infections and bacteremia (Fazeli and Momtaz, 2014). It has many virulence factors aimed at pathogenicity occur. These factors flagella, Exopolysaccharide, pili, and set of enzymes such as elastase, proteases, sialidases or called neuraminidase,

lipases, pigments, siderophores, as well as a several toxins such as exotoxin A and the Type III Secretion System (T3SS) toxins like ExoU, ExoY, ExoT, and ExoS (Harper *et al.*, 2014; Mahdavi *et al.*, 2017). Additionally, the bacterium tends to form biofilms (Mahdavi *et al.*, 2017). This bacterium is a virulent agent possesses a trend to expand the resistance to most types of available antibiotics. This natural (intrinsic) resistance to numerous antibiotics inflicts severe curative troubles for clinicians (Długaszewska *et al.*, 2016). There is several literatures that referred to the ability of bacteria in production of chitinases enzymes (Thompson *et al.*, 2001; Medeiros *et al.*, 2018; Khoushab and Yamabhai, 2010) which hydrolytic enzymes that hydrolase chitin to monomers and oligomers, Chitin is the most copious polysaccharide present in nature, in two figures as α-chitin, or as β-chitin (Khoushab and Yamabhai, 2010), Chitin is the chief constituent of arthropod exoskeletons and connective tissues, fungi cell wall, and is present in some aquatic organisms such as fish (Dahiya *et al.*, 2006). Generally, many genes responsible such as *ChiA, ChiB, ChiC,* and *ChiD* encoded for Chitinase were pointed out in these bacteria with different enzymatic activities (Ramaiah *et al.*, 2000). The medical applications for chitinase enzyme used as antibacterial and anticancer models (Krithika and Chellaram, 2016).

MATERIALS AND METHODS

Isolation and Identification

Forty isolates were obtained from postgraduate students in the biology department of the college science of Al-Mustansiriya and Baghdad University isolated from different clinical sources, all isolates were recognized and tested by, cultural, microscopic, biochemical and Vitek 2 system. These isolates were re-confirm by the molecular method. A specific primers f' 5' GCACTTTAAGTTGGGAGGAAC '3 for and r 5' CTTTACGCCCAGTGGCCGTAA '3 were used for amplification 144 bp of 16srRNA gene using PCR technique (Hillenbrand *et al.*, 2011). The reaction mixture of PCR in total volume was 25 μl included 12.5 of PCR green master mix, 1 μl of each primer and 2 μl of DNA template. The rest volume was completed with sterile de-ionized D.W, then vortexed. The PCR steps were, 95C° for 5 minutes, 30 cycles of 95 C° for 30 seconds, 60 C° for 30 second, 72 C° for 1 minute, and a final extension at 72 C° for 10 minutes.

Detection of *ChiA* gene

Specific primers were used for amplification 225 bp of *ChiA* gene encoded for chitinase 5′-TACGACTCACTATAGGGCGA-3′ and 5′-ACTCAAGCTATGCATCCAACGC-3′ (Ramaiah *et al.*, 2000). PCR reactions were the same as 16srRNA, as mentioned above, except the annealing temperature was 58 C°. Finally, PCR products were visualized by agarose gel electrophoresis.

Antibiotics susceptibility

The Kirby - Bauer disc diffusion method was performed (Morello *et al.*, 2006). All isolates were investigated for determination their susceptibility vs. 12 antibiotics were milligrams in weight, included Cefixime(5), ml/disc, Ciprofloxacin(5), Norfloxacin(5), Tetracycline(30), Carbenicillin(30),Cefotaxime(10), Imipenem(10), Gentamycin(10), Amikacin(30), Ceftazidime(30), Azetreonam(30) and Amoxicillin+Clavulanic acid(30).

Chitinase production

The medium contains Na_2HPO_4 6g, KH_2PO_4 3g, NH_4Cl 1g, NaCl 0.5g, yeast extract 0.05g, agar 15g, and colloidal chitin 1% w/v as a substrate in 1L deionized D.W. The isolates were investigated by inoculating them on the medium and incubated at 37ºC for 96 hr. The positive result was monitored based on the clear zone around the colonies. The enzymatic activity measured in nutrient broth media supplemented with colloidal chitin. The culture centrifuged in cooling condition at 10,000 rpm for 15 min. and the extract used for chitinase assay (Saima *et al.*, 2013).

Colloidal chitin Preparation

Chitin powder (40g) was added to 300 ml of HCl and maintained for 60 min. At 30°C with shake continuously. Chitin was settled as a colloidal suspension by adding 1 liter of distilled water gradually. The suspension was filtrated and washed by suspending in 2.5 L of distilled water, and washing was triplicate until the pH of the suspension reached a neutral range (Hsu and Lockwood, 1975).

Determination of optimal conditions for the production of Chitinases. The incubation period

One hundred ml of the production medium was inoculated with 10 ml of overnight bacterial suspension and incubated at 37ºC for four different periods (1, 2, 3,4,5 day). Then, Chitinase activity was measured (Saima *et al.*, 2013).

The temperature

Ten ml of fresh bacterial culture was transported to 100 ml of the prepared production medium previously, then incubated at different temperatures (20, 30, 37, 40, 50, 60°C) for 48 hrs and the chitinase activity was calculated, but pH was determination. Briefly, 10 ml of bacterial suspension was

transferred to 100 ml of prepared media with different pH values (4-10), and then incubated at 37°C for 48hrs; the chitinase activity was assayed (Saima *et al.*, 2013).

Measurement of chitinase activity

Microliter plate's 96-well were used for the Appreciation of chitinase activity. The crude enzyme (1 ml) was added to the reaction mixture consisting of 1 ml of 1% colloidal chitin and phosphate buffer (0.05, pH 7.0). After incubation at 30°C for 60 min. The reaction mixture centrifuge at 4°C for 15 min. the resulting supernatant (100 μL) was transferred in a new plate and add 100 μL of Schales' reagent. The plate covered with aluminum foil and was incubated for 15 min. at 100°C after cooling read the absorbance at 420 nm spectrophotometry (Ferrari *et al.*, 2014).

Determination of molecular weight of chitinase

The protein content was detection by the Bradford method (Bradford, 1976) and the molecular weight of chitinase was estimated by Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) according to a clarification of (Laemmli, 1970).

Purification of chitinase enzyme

Generally, The most enzymes purified by the same traditional purification methods, in this study chitinase crude extract collected was submitted to different steps of purification, including precipitation by ammonium sulfate, dialysis of crud enzyme, and passed through DEAE-cellulose ion-exchange chromatography column with dimensions (2.5×30 cm), and purification by gel filtration chromatography using Sephadex G-200 gel filtration column with dimensions (2.5×25cm), the first and second columns were equilibrated with Tris-HCl buffer pH 7. The Elution was the flow rate of 5ml/3min using the same buffer for equilibration, the absorbance of fractions have been measured at 280 nm, and the activity of the enzyme was calculated in all fractions.

Determination of anti-cancer

MTT assay has been achieved for the investigation effect of chitinase on cell lines; a microliter plate with 96 wells was used to perform this experiment. Two types of cell lines were examined, HepG2 (liver cancer cells) and WRL-68 (normal liver cell), were obtained from the Biotechnology Research Center, Al-Nahrain University. The cells were treated in duplicate with different concentrations of chitinase enzyme (100-600) μg/ml then, incubated for 24 hours to detect the cytotoxic effect of chitinase against a selected cell line, 10 microliters of MTT solution was dropped to each well, then the plates were incubated for 4 hours at 37°C. The absorbance was measured at 570 nm, to determine the cytotoxicity of chitinase vis both cell lines. The inhibitory rate of cell division was assayed by the equation, (Freshney, 2011).

$$Inhibition\ rate\ \% = \left(\frac{A-B}{A}\right) \times 100$$

A - where the optical density of control

B - where the optical density of the tested sample

Study the histological effect of chitinase

All the mice in this experiment were obtained from the National Center For Drug Control and Research (NCDCR) in Baghdad; it aged 4 weeks and weighed 20 grams. The mice divided into 5 groups, each containing 6 mice. These groups were injected through intraperitoneal with 0.5ml of stock purify chitinas and from each two-fold dilution prepared from the stock (400,500,600) μg/ml, using normal saline for dilution, on another hand two of mice were injected with 0.5ml normal saline as a control. For studying histopathological changes, the dissected liver was used and the histological sections were performed according to (Humason, 1972). Briefly, the organs were fixed by 10% formalin solution for 8 hrs (it was prepared by 10 ml formalin + 90 ml 0.9% NaCl), then washing with tap water for 2-5 min. then passing through a gradient concentrations of alcohol (50%, 70%, 80%, 90% and 100%) for 180 min. in each concentration. After that cleared by xylol for 60 min, and then the fixed organs saturated with paraffin at 60°C for 3 hr., by embedded in pure paraffin to form the blocks were sectioned not more than 4-5 μm in thickness by using microtome machine. These parts fixed on glass slides by using Myer's albumin and left for drying at 37°C at a suitable time. For staining, the sections were embedded in Haematoxylin stain for 10-15 min., followed by washing with tap water and acidic alcohol for1-2 min, and then washed by tap water. The second stain is Eosin stain; it was used for 15-30 sec. And then washed by D.W. For hydration, serial concentrations of alcohol were then used (70%, 90%, and 100%) for 2 min. in each concentration. In addition, the sections re-cleared by xylol for 10 min. Then Canada balsam dropped carefully on slides and covered by cover slide to examine under a light microscope.

Antibacterial activity assay

Antibacterial activity of chitinase was run by well diffusion method depending on (Medeiros *et al.*, 2018), against two types of pathogenic bacteria, *E. coli* and S. *aureus*. Bacterial suspension existed on Muller Hinton agar, and then the plates were punctured with a 6 mm wells, using the sterile tip. Different concentrations of chitinase enzyme were prepared (100, 200,300,400,500,600) μg/ml poured

into the wells, incubation at 37°C for 24 hrs., The inhibitions zones were measured. The experiment was done for three replicates.

RESULTS AND DISCUSSION

Molecular Study

Simplex polymerase chain reaction (PCR) is considered an important, powerful tool in the identification of many medical microorganisms and plays an important role in scientific researchers through the detection of bacterial genes interest (Thong *et al.*, 2011).

Detection of 16srRNA gene

Amplification of the 16srRNA gene of isolates was performed to confirm the identification of bacteria, specific primers for the conserved region of 16srRNA were used for amplification of bacterial gene by PCR technique and then PCR products were separated on agarose gel Figure 1. The results reported that all isolates of *P. aeroginosa (100%)* had 16srRNA gene band with (144bp), this results agreed with others studies were used 16srRNA gene to identify the same bacteria and demonstrated this gene sensitivity was (100%) (Farhud *et al.*, 2017; Shehab, Z. H. and AL-Rubaii, 2019); 16*SrRNA* gene is highly conserved in bacteria, and act as a housekeeping gene, therefore its give a reliable result in identification of bacteria (Janda and Abbott, 2007). There are many reasons for using molecular techniques to detect pathogenic bacteria, classical diagnosis methods (cultural, microscopic, biochemical tests) are less accurate and require more time (2-5) days and most have inherent limitations. Therefore, the genotypic detection depending on certain gene was used as confirmatory test provided a rapid diagnostic of bacteria collected from patients who suffered acute infection and cultures are negative (Altaai *et al.*, 2014).

Detection of Chitinase gene *ChiA*

Conventional PCR amplification was performed to detect the presence of *ChiA* gene coding for chitinase enzyme by using specific primers, as shown in Figure 2. The results were showed that only two isolates (5%) were positive for amplification fragments of the *ChiA* gene at 225bp, and 38 (95%) isolates were negative. The results of the current investigation agreed with (Dahiya *et al.*, 2006) who founded that (3.3%) of *P. aeroginosa* isolates possess the chitinase gene, other study revealed the presence of *ChiC* gene encoding chitinase enzyme in some *Pseudomonas spp* isolates, but our results differed from a previous study by (Zhong *et al.*, 2015) they founded that all isolates of *P. aeroginosa* were negative to

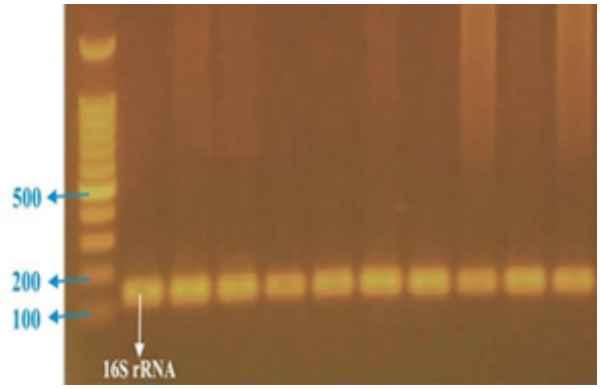

Figure 1: Agarose gel electrophoresis of (1%) for amplified 16SrRNA gene (144 bp) for *Pseudomonas aeruginosa* isolates compared with (1kbp) DNA ladder lane at 1.5 hr 5 volt/cm

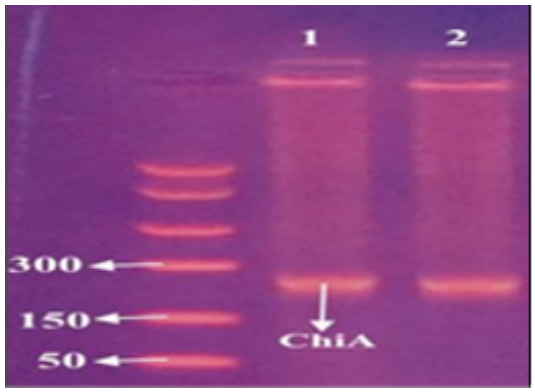

Figure 2: Agarose gel electrophoresis of (1%) for amplified of Chitinase gene ChiA (255 bp) for *Pseudomonas aeruginosa* isolates compared with (1kbp) DNA ladder lane at 1.5 hr 5 volt/cm

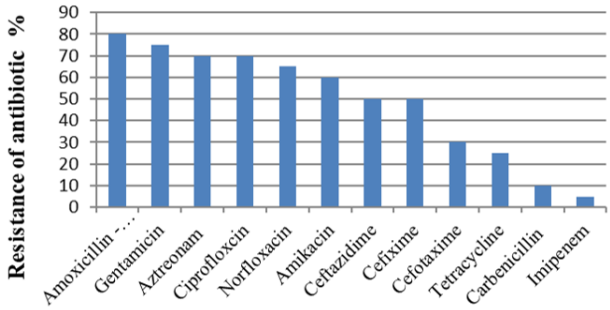

Figure 3: Antibiotics susceptibility test of *Pseudomonas aeruginosa* isolates

ChiA gene, but other isolates of gram-negative bacteria such as *Vibrio spp, E. coli, pseudomonas spp* and *Serratia spp* were positive for *chiA* gene.

Antibiotics susceptibility test

Antibiotic susceptibility profile was performed for *P. aeruginosa* isolates against 12 kinds of antibiotics related to eight different classes used in the hospitals routinely. All isolates had been examined to susceptibility test according to Clinical and Laboratory

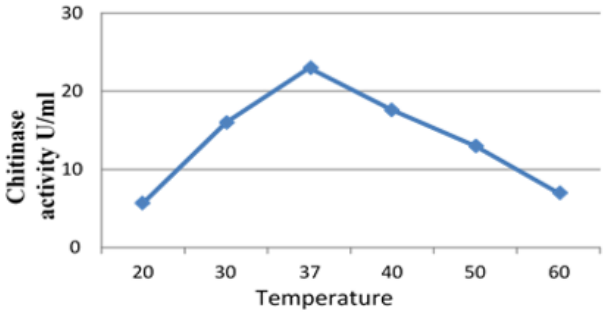

Figure 4: Effect of temperature degree on Chitinase activity produced from *Pseudomonas aeruginosa*

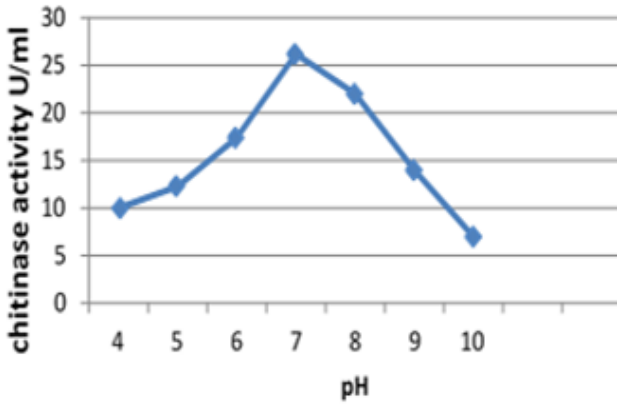

Figure 5: Effect of pH values on Chitinase activity production from *Pseudomonas aeruginosa*

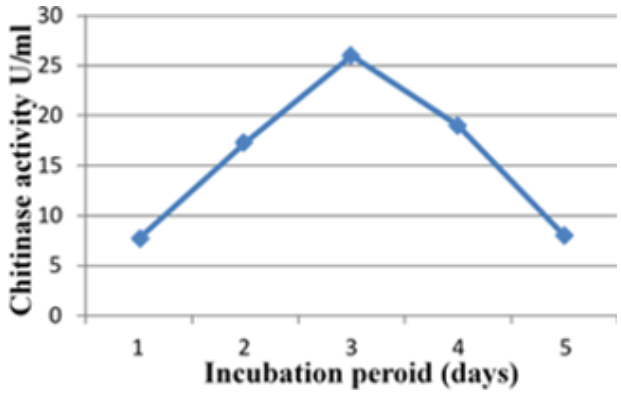

Figure 6: Effect of Incubation period on chitinase production from *pseudomonas aeruginosa*

Standards Institute (CLSI, 2016) as shown in Figure 3, and this assay was achieved by widespread Kirby-Bauer disk diffusion method.

The results of current research demonstrated a high resistance of *P. aeruginosa* isolates (80%)to Amoxicillin - Clavulanic acid, (75%)to Gentamicin, (70%) to Aztreonam and Ciprofloxacin (65%) to Norfloxacin, (60%) to Amikacin, (50%) to Ceftazidime and Cefixime, then isolates began gradu-

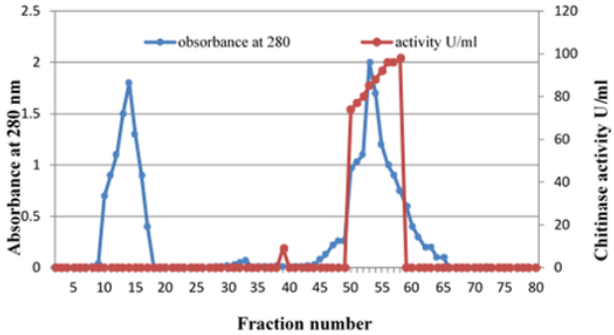

Figure 7: Ion exchange chromatography for chitinase purification from *pseudomonas aeruginosa* isolate by using DEAE-cellulose column (2.5×30 cm) equilibrated with Tris-HCl buffer pH 7.5), eluted with sodium chloride 0.5,1, and 1.5 M in flow rate 5ml/3min for each fraction

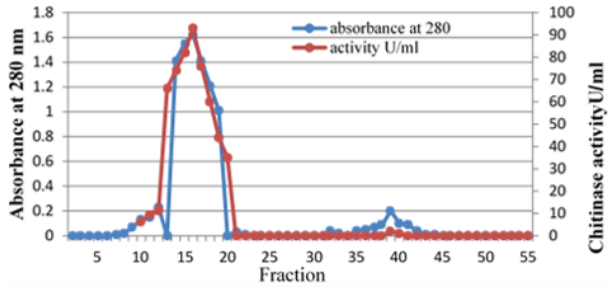

Figure 8: Gel filtration chromatography for chitinase purification from *pseudomonas aeruginosa* isolate by using sephadex G-200 column (2.5×25cm) at flow rate 5ml/3min

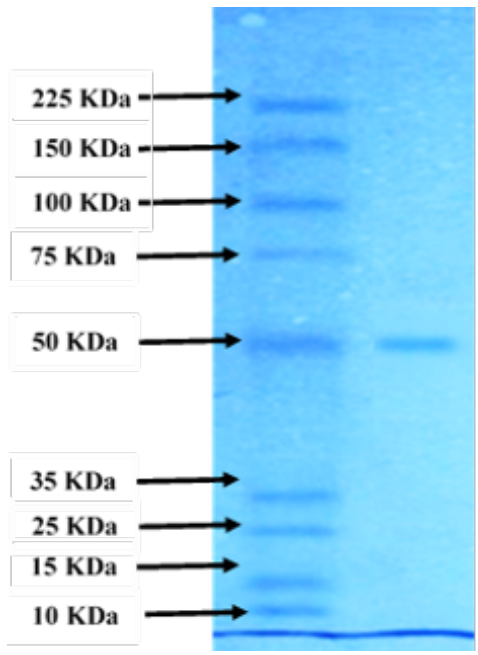

Figure 9: SDS-PAGE of purified chitinase produced by *pseudomonas aeruginosa*

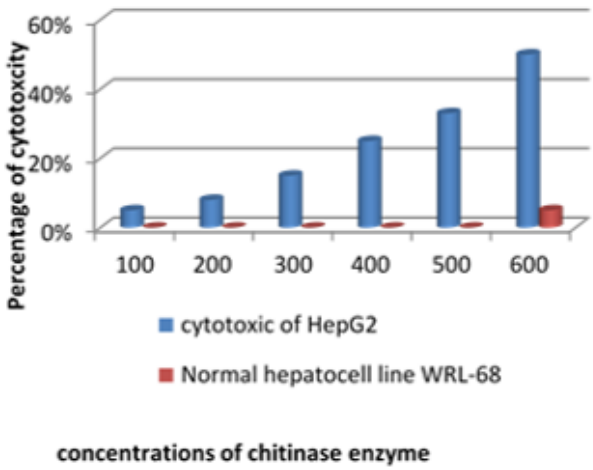

Figure 10: MTT of cytotoxic ability of purified chitinase against cell lines

ally to decline resistance with Cefotaxime (30%), Tetracycline (25%). While most of the isolates were sensitive to Imipenem (95%) and Carbenicillin (90%). The resistance of bacteria for B-Lactams antibiotics (Amoxicillin - Clavulanic acid, Aztreonam, Ceftazidime, Cefixime and Cefotaxime) can be explained to β-lactamase enzymes which hydrolyze the typical four-membered β-lactam ring by disrupting the amide bond, this the main machinery of resistance to β-lactam drugs to bacilli Gram-negative bacteria, so showing unsuccessful activity of this antibiotics (Blair *et al.*, 2015). These enzymes production is changeable through chromosome, plasmid and predominately correlated with encoding integron and transposons as mobile genetic elements that are easy to transfer among bacterial strains (Qing *et al.*, 2014). Gentamicin and Amikacin, these are two types of an important drug belong to the aminoglycosides groups. They act by inhibiting protein synthesis inside the bacterial cell by bounding with the 30S Ribosomal Subunit of the bacterial ribosome and damaging the safety of bacterial cell membranes. This inhibits the creation of amino acids and then proteins (Shakil *et al.*, 2008). The resistance of aminoglycosides is complicated; they are relying on some enzymes like acetyl transferases and phosphotransferases (EFSA, 2008). Ciprofloxacin and Norfloxacin are members of the fluoroquinolones group. They are synthetic broad-spectrum antibacterial drugs. Fluoroquinolones affect bacterial growth by inhibiting of Topoisomerase II (DNA gyrase) enzyme, which the most important goal in G -ve bacteria through intervention with DNA replication (Aldred *et al.*, 2014). Tetracycline is related to the Tetracyclines group, and they disrupt protein synthesis by bounding reversibly to the bacterial 30S ribosomal subunit and forbidding the aminoacyl tRNA from binding to the A position of the ribosome (Nelson and Levy, 2011). Tetracycline likewise links to the bacterial 50S ribosomal subunit and may alternative the cytoplasmic membrane ingredient and causing intracellular components leakage of bacterial cells. Recently, nanoparticles used as an alternative to antimicrobial agents such as Aluminum silver and titanium Oxide Nanoparticles (Jwad *et al.*, 2019; Saleh *et al.*, 2019; Wilson, 2014). In general, the resistance to different classes of antibiotics attributed to many reasons, changing the permeability of the cell membrane, modifications in the target sites structures and efflux pumps mechanism, which reduce the antibiotics concentrations (Shaikh *et al.*, 2015). Our results showed that Imipenem is the best choice, followed by Carbenicillin for therapy from different infections caused by *P. aeruginosa* bacteria. From our results, it is a clear sharp that clinical isolates of *P. aeruginosa* show a variety in their response for different antibiotics according to isolating source, the antibiotic concentration, the treatment period and its costs, its modus of taking, its side effects on the patients, and development of multidrug resistance remain determinants inducing orientation to synthesis new antibacterial agents for treatment.

Screening for chitinase production

All isolates were investigated to determine their ability to produce chitinase enzymes. The results were showed that two isolates only were able to produce this enzyme by formed a clear zone around colonies ranged from 5 to 15 mm. The isolate (P5) was appeared the most effective for Chitinase production with the highest diameter (15) mm. Nevertheless, the enzymatic activity and specific activities were 33 U/ml and 18.644 U/ml, respectively Table 1. On chitin media agar, it was detected as a Chitinase producer. Hence,this isolate was used to complete further experiments in this study, while, others isolates were not chitinase producers, the crud of enzyme extract was obtained by growing the selected isolate (P5) in 5 ml of Luria Beritani broth medium and incubated in shaking incubator, conditions of incubation were (with temperature 37^{o}C, PH 8and 72h, and centrifuged under refrigeration, then the supernatant acted crude enzyme extract.

Optimum temperature

The results were shown that the chitinase activity was recorded a highest at value 23 U/ml at 37°C, while decreased to 17.6 U/ml in 40°C, also in lowest temperature was the enzymatic activity is 5.7 U/ml at 20°C Figure 4. Temperature is one of the most important factors affecting the enzyme pro-

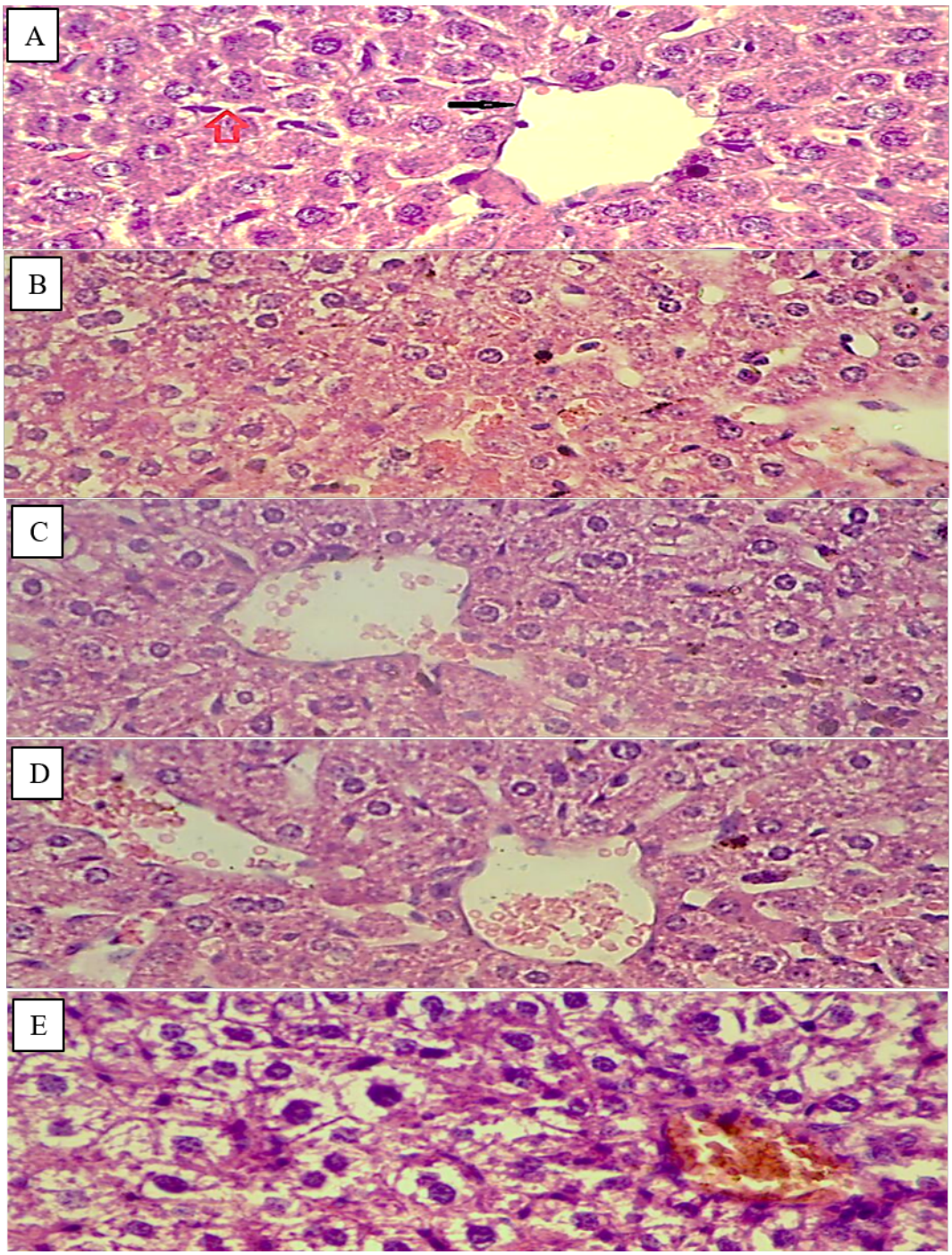

Figure 11: Histological Impact of Purified chitinase on mouse liver where, A: Control section of Normal tissue (non- treated). B: Section of the hepatocyte cells treated with chitinase (300μg/ml) showing very little effect. C: Section of the hepatocyte cells treated with chitinase (400μg/ml) showing few accumulations of cholesterol and with mild congestion. D: Section of the hepatocyte cells treated with chitinase (500μg/ml) showing mild accumulation of cholesterol and with mild congestion. E: Section of the hepatocyte cells treated with chitinase (600μg/ml) showing more accumulation of cholesterol material with congestion

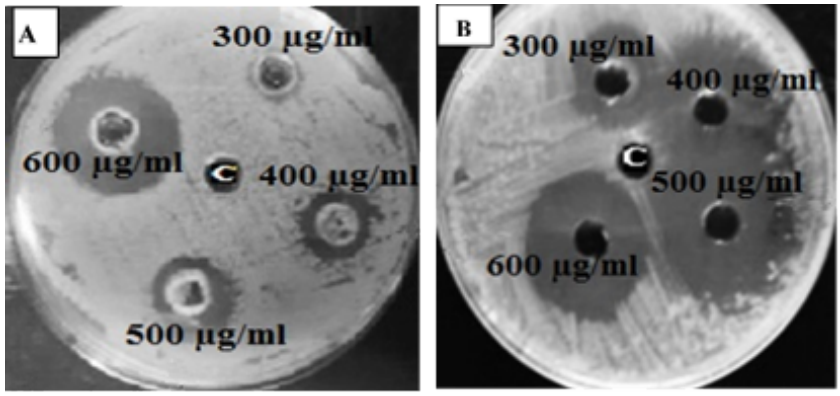

Figure 12: Antibacterial effect of purified chitinase on pathogenic bacteria, where, A: *Staphylococcus aurus* B: *E.coli*

Table 1: The purification steps of chitinase from pseudomonas aeruginosa isolate

Purification steps	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification fold	Yield %
Crude enzyme	100	33	1.77	18.644	3300	1	100
Precipitation with 65% saturation of $(NH_4)SO_4$	37	63.3	1.18	53.644	2342.1	2.88	70.9
Ion exchange chromatography (DEAE-Cellulose)	10	94.2	0.821	114.7	942	6.15	28.5
Gel filtration chromatography	10	78	0.44	177.3	780	9.51	23.6

duction for any enzymatic reaction; the temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing, its characteristics of the configuration of the enzyme such as destroy the non-covalent bonds or hydrogen bonds, which give protein stability (Uyar *et al.*, 2011). These results are compatible with (Hashim and Nema, 2018). The suitable temperature for the chitinase activity of *Aeromonas hydro*phlic was 37°C. The old report that mentions the enzyme produced from *P. fluorescens* had an optimal activity at a temperature range of 22–38°C (Alhasawi and Appanna, 2017), in another hand these results are inconsistent with (Wang *et al.*, 2008) who noted that the optimum temperature of chitinase activity was 50°C.

Optimum pH

The pH effects on the production of the enzyme because it has a role in the solubility and ionic situation of nutrients in the medium and for the microorganisms growing in this medium also, its effect on the production and stabilization of the enzyme and the growth of microorganisms (Asgher *et al.*, 2007). The results have shown that the highest of chitinase activity (26.2 U/ml) was increased in pH (7), while decreased after raised values of pH Figure 5. The result of the current research was compatible with the results recorded by (Lang *et al.*, 2010). They referred to enzymatic activity was increased at pH 7. In another study, the optimal activity exhibited by chitinase was observed al pH 8.

Incubation period

The highest chitinases activity was peaked to (26U/ml) after 3 days of incubation and then decreased with increasing the incubation period at 4 and 5 day with (19 and 8 U/ml respectively) Figure 6, this may be attributed to the change conditions along this period such as decreasing of nutrients, O_2 and increasing toxic metabolites which inhibit the growth, and digest of enzyme all these reasons result in decreased enzyme production (Nochure *et al.*, 1993). The results agreed with (Bhatt *et al.*, 2012; Kumar *et al.*, 2017), who

noted that Bacillus sp and Streptomyces sp produced the highest chitinase after 72 hr. of incubation. Another study founded that the optimum incubation period for chitinase activity from pseudomonas sp was 4 days (Lang *et al.*, 2010).

Chitinase purification

Precipitation by ammonium sulfate and dialysis

The Precipitation of enzyme by ammonium sulfate salts was considered one of the most important methods for concentration and it is atypical as the beginning step in purification. Ammonium sulfate is practicality and effective because of its cheapness, high solubility, lack of toxicity on the stability of enzymes (Bhatt *et al.*, 2012). The chitinase crude obtained from the extraction step was exposed to different saturation values; the results appeared that the best rate for proteins precipitated in saturation with 75% ammonium sulfate. The activity and specific activity was 63.3U/ ml and 53.64 U/mg, respectively Table 1, the sample was subjected dialysis against Tris- HCl pH 8. Another report has appeared that the precipitation at 75% ammonium sulphate saturation gave chitinase maximally with a specific activity of 11.81 U/mg and 5.67 fold purification (Subramaniam *et al.*, 2012).

Ionic Exchange Chromatography

This method is depending on the charge of the molecule surface, the protein and buffers conditions, the net charge of the protein, either a positive or negative (Irwin *et al.*, 1976). Ionic exchange chromatography was used after precipitation by ammonium sulfate powder and dialysis steps, the dialyzed chitinase was applied on DEAE-cellulose column, equated and rinse with an enough volume of Tris-HCl buffer (pH 8), the attached proteins that carry negatively charged were then eluted using ascending concentrations of NaCl 0.5, 1, and 1.5 M. The results appeared in Figure 7 showed that there was one peak of protein, which appeared after washing step and none of them has enzyme activity while two protein peak appeared after elution, one peak showed chitinase activity focused in fraction parts (50-60) eluted by 0.5M sodium chloride. All these peaks detected at 280 nm. The two protein peaks that collected after the elution step were assayed for chitinase activity. Fractions that have enzyme activity collected and concentrated. The specific activity of the enzyme reached 114.7U/mg with 6.15 purification fold and enzyme yield 28.5%. These results proved that chitinase has a negative charge due to bounded with the anionic ion exchange (DEAE-cellulose). The purification by (Senol *et al.*, 2014) shown the chitinase of *Bacillus subtilis* was up to 28.4 purification fold with a specific activity of 197.14U/mg after passage the enzyme through the DEAE cellulose exchanger column.

Gel filtration chromatography

The last step in the purification of chitinase in the gel filtration chromatography technique. The fractions were collected and have enzymatic (chitinase) activity applied on Sephadex G-200 formerly equilibrated with 0.1 M Tris-HCl buffer (pH7). Sephadex G-200 characterized by simple preparation, high recovery fast running, high stability and re-use many times in another separation (Stellwagen, 1990). Results showed that two peaks of chitinase activity appeared after elution with Tris- HCl buffer, and the Fractions representing chitinase activity were pooled and concentrated for further study Figure 8. In addition, Protein concentration, activity and specific activity of chitinase summarized in Table 1 that showed there is a decrease in the activity and protein concentration of the purified enzyme (78 U/ml) and (0.44mg/ml) respectively, but there was an increase in specific activity (177.3U/mg), Purification fold (9.51). In a similar study, the purification of chitinase with 75% ammonium sulfate saturation and followed by Sephadex G-100 gel permeation achieved to 13.75 fold purification with a specific activity of 1. 43 U/mg (Gangwar *et al.*, 2016).

Determination of molecular weight by SDS-PAGE

The molecular weight of chitinase was detected by SDS-PAGE and results showed only one bond for purified chitinase with molecular 50 KDa Figure 9. In general, the electrophoresis technique on polyacrylamide gel was considered as another step of purification. This result agrees with other studies that reported the molecular weight of chitinase from *Stenotrophomonas matophilia* was 50 kDa when determined by SDS PAGE technique (Shaikh *et al.*, 2018). In another study, the molecular weight of chitinase from *Bacillus sp*. was 41.68 kDa (Cheba *et al.*, 2016).

Anti-cancer activity of chitinase

In the present research, the cytotoxic ability of purified chitinase against cell lines was detected by MTT colorimetric assay. Two types of cell lines were selected to improve it, which were hepatocellular cancer cells (HepG2) and normal liver cell lines (WRL-68), which used as control. The cell lines were treated with different concentrations of chitinase enzyme 100,200, 300,400,500, and 600 μg/ml for 24 hours as were shown in Figure 10, the results revealed that the cytotoxicity for HepG2 was (5% at 100 μg/ml, then, the cytotoxicity effect was in increased gradually with higher concentrations until to reach 50% at 600 μg/ml, while WRL-68

cells appeared no or less cytotoxicity when treated with all concentrations of purified enzyme from the results that recorded in Figure 10, we can conclude that chitinase enzyme has a potential cytotoxic effects on cancer cells compared with normal cells. It is a local recently study by (Hashim and Nema, 2018) reported to the role of chitinase purified from *Aeromonas hydrophila* in killing 50%and 65% of breast cancer cells (MCF-7 and prostate cancer cells (PC3) respectively, and was no or less toxic for normal liver cells WRL-68. The reason behind killing or destroy cancer cells by chitinase may be attributed to receptors (polycarbohydrates) founded on the surfaces of many transformation cells (cancer cells), chitinase bind with carbohydrates and digested them. Thus, the enzyme enhances damage and killing any tumor cells appeared these carbohydrates (Pan *et al.*, 2005), While, the normal liver cells don't appear these receptors on their surfaces, so don't affect after treated with chitinase. Others studies confirmed the medical importance of bacterial chitinase as an anticancer (Pan *et al.*, 2005; Krithika and Chellaram, 2016) there are a few articles about prokaryotes s chitinase, but in eukaryotes, there were expanded literature such as Kavsan and his workers (Kavsan *et al.*, 2011) demonstrated that expression of CHI3L1 gene encoding for Chitinase 3-like 1 protein significantly increased in various tumor cells compared with normal tissues, CHI3L1 Protein decrease the doubling time of cancer cells (293 cells). Hence, it can be used as one of the critical targets for treating many cancer cells. Another study referred that M1 activated macrophage produced family 18 chitinases, were 8 worked together with some proteases, and damage different cancer cells in vivo and in vitro (Pan, 2012). Fine study deal with the role of chitinase enzyme in stimulation immune system, Here, it was pointed to some chitinases and Chitinase like proteins can be considered as an important and nonspecific marker for activation of macrophage and stimulation of T helpers cells that play an essential role in innate immunity against microorganisms containing chitin (lysis them), and destroying tumor tissues. Therefore, it can be used as an alternative drug for cancer therapy (Elmonem *et al.*, 2016).

Several bacterial enzymes were produced used as anti-tumor or have inhibition role in cancer cell division such as elastase (Al-Rubai, 2017) or Hyaluronidase (Salih *et al.*, 2019). In another hand Alattar and his coworker refer to, there is a link between extracellular production (crude hemolysin) of Shigella dysenteriae with inhibition in growing of RD and L20B cell lines through Cytotoxicity (Alattar *et al.*, 2018).

Study the histological effect of chitinase

The results of this experiment showed there is no effect for chitinase against hepatic tissue at low concentration (100,200,300) μg/ml. But, the concentration (600)μg/ml of purified chitinase was showed more accumulation of cholesterol material with more congestion, While, the concentrations 400,500 μg/ml of purified chitinase were show mild congestion figures compared with control Figure 11. These results were parallel with other study showed that CHIT involved in the modulation of the tissue remodeling processes in fibroblastic hepatic tissue in low concentration (Malaguarnera *et al.*, 2006).

Study of antibacterial activity (*in vitro*)

The antibacterial activity of chitinase enzyme was done against two types of pathogenic bacteria, one of them was G-ve bacteria E. *coli*, and another was G+ve bacteria *S. aureus* using by well diffusion method, the chitinase was used at different concentrations (100, 200,300,400,500,600) μg/ml. The results of this study appeared that higher effect against both bacterial isolates at 600 μg/ml concentration, and lower effect at 400 μg/ml concentration while appeared variable results in their effectiveness at 300 and 500 μg/ml concentration Figure 12. The enzyme revealed more activity against G+ve bacteria compared with G-ve bacteria, the difference in the response of both G-ve and +ve bacteria against the same purified chitinase may be due to the difference in the structure of cell wall between them bacteria. The gram-negative bacteria have an outer membrane contained lipopolysaccharide structure and a thin layer of peptidoglycan, which made the cell wall more resistant to the enzyme, while gram-positive bacteria have a thick outer layer of peptidoglycan make it more susceptible to it. It is interesting to find that our results were compatible with other global report referred that purified chitinase by *ps. aer*uginous has antibacterial activity against E. coli and *S. aureus* isolates. Certain articles have applied the chitinase as a natural bacteriocidal (Wohlkönig *et al.*, 2010; Yang and Yu, 2014; Farag *et al.*, 2016) toward various bacterial isolates includes *s.aureus* and *E. coli* (Lin *et al.*, 2009; Farag *et al.*, 2016).

CONCLUSION

Chitinases enzymes showed lysozyme activity, as measured by the ability of these proteins to hydrolyze and cleavage the chains of peptidoglycan caused lysis of bacterial cells.

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