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Detection of lipase, protease and biofilm of *Staphylococcus* spp isolated from imported minced meat

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Abstract: The aims of this research were isolation of *Staphylococcus* spp from imported minced raw red meat, evaluation of bacterial ability to produce lipases and proteases at different temperatures and screening of biofilm formation. Seventeen different samples of minced red meat imported from India(Al Kafeel and Anwar Karballa) and Australian were collected from different markets in Baghdad/ Iraq from July 2013 to March 2014. One hundred and forty staphylococcal isolates were picked from mannitol salt agar. Forty eight isolates (34%) were mannitol fermantor and a part of twenty five isolates (17.8%) were coagulase positive, they identified as *S. aureus*, whereas, ninety two isolates (65.75%) were non mannitol fermentor, which they identified as *S. epidermidis*. Monitoring of antibiotic susceptibility showed that all staphylococcal isolates were highly sensitive to penicillin, erythromycin, and chloramphenicol. While, 100 and 20 isolates(71.4%, 14.2%) were resistant to nalidexic acid and tetracycline respectively. Screening of protease production reveled that all staphylococcal isolates were proteases producers. The bacterial isolates that incubated at 37℃ produced proteases after 24 h, however, proteases activity could be detected after 72 h at 20℃ incubation. Very low growth of staphylococcal isolates were recognized at 4℃ incubation and proteases enzyme could not detected even after one week. Only 30 and 20 of mannitol fermented and non mannitol fermented *Staphyloccocus* spp gave fluorescent orange color on rhodamine B plates which indicated for the production of the true lipases when they were incubated at both 37℃ and 20℃. Both lipases and esterases could be detected from five isolates of mannitol fermented *Staphyloccocus* spp using tween 80 plates, two other isolates produced lipases. Five isolates from Non mannitol fermented *Staphyloccocu*s spp produced lipases without esterase. Biofilm was formed by both mannitol fermented and non mannitol fermented staphylococcal isolates depending on the temperatures in which 100% of isolates formed biofilm at 20℃ and 71.4% at 37℃.

Key words: meat contamination, *Staphylococcus*, mannitol fermentation, coagulase, antibiotic susceptibility.

التحري عن اللايبيز والبروتيز والاغشية الحيوية لبكتريا *Staphylococcus* spp المعزولة من اللحوم الحمراء المفرومة المستوردة

خلود عبد الاله محمد[1] و حيدر شنون كريم[2] وعمار محمد جواد وصفاء عبد الرحيم محمود وانعام محمد موسى جعفر

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الخلاصة: هدف البحث الحالي الى عزل انواع بكتريا *Staphyloccocus* من اللحوم الحمراء المفرومة المستوردة، تقييم قابيلة العزلات على انتاج انزيمات البروتييز واللايبيز وبدرجات حرارة مختلفة كما تم تحديد قابلية البكتريا على تكوين الاغشية الحيوية. تم جمع سبعة عشر نموذجا من اللحم الاحمر المستورد المفروم من الاسواق المحلية لمحافظة بغداد/ العراق من مصدر هندي(انوار كربلاء والكفيل) ومن مصدر استرالي وخلال الفترة من ايلول 2013 ولغاية اذار 2014، وعزلت مئة واربعون عزلة من على سطح وسط اكار المانتول ملح. وجد ان ثمان واربعين عزلة كانت مخمرة لسكر المانتول ممثلة 34% وقد كانت خمسة وعشرون عزلة تنتج انزيم الكواكيوليز بنسبة 17.8% وشخصت بانها *S. aureus*. اثنان وتسعون عزلة غير مخمرة للمانتول وبنسبة 65.75% شخصت بانها *S. epidermidis*. بين فحص الحساسية ان العزلات جميعها حساسة للبنسلين والاريثرومايسين والكلورامفينكول وان 100 و20 عزلة بنسبة 71.4% و 14.2% هي مقاومة للناليديكسك والتترايسكلين على التوالي. تنتج العزلات جميعها انزيم البروتييز بعد 24 ساعة عند حضن البكتريا بدرجة 37 ْم وبعد 72 ساعة عند الحضن بدرجة 20 ْم بينما كان النمو ضعيفا جدا عند حضن العزلات بدرجة 4 ْم ولم يتم انتاج الانزيم بعد اسبوع من الحضن. تبين ان 30 و20 عزلة بكتيرية مخمرة وغير مخمرة تعطي لون برتقالي متألق على اطباق صبغة الرودامين ب وبكلا درجتي حرارة 20 ْم و37 ْم ودل ذلك على انتاج انزيمات اللايبيز الحقيقية. اعطت خمس عزلات بكتيرية مخمرة للمانتول انزيمات اللايبيز والاستريز وعزلتين انزيمات اللايبيز وحدها على اطباق التوين 80 وانتجت خمس عزلات بكتيرية غير مخمرة للمانتول انزيمات اللايبيز فقط. كونت العزلات البكتيرية المخمرة وغير المخمرة للمانتول الاغشية الحيوية اعتمادا على درجة الحرارة وبنسبة 100% بدرجة 20 ْم و71.4% بدرجة 37 ْم.

الكلمات المفتاحية: تلوث اللحوم،*Staphylococcus* spp ، تخمير المانتول، كواكيوليز، حساسية المضادات الحيوية.

Introduction:

Meat is considered as an important food for human being. Worldwide, there is a big concern about food born diseases. Contaminated meat were already came from processing, catering and domestic environment (1). Bacteria were possess many common ways to live and organized on raw and process meat mediated by their ability to produce hydrolysis enzymes such as proteases and lipases at different temperatures. In addition, the adherence onto the surfaces and forming an organized communities referred to as biofilm (2); stainless steel, glass, rubber, polyproline surfaces can be contaminated by either spoilage or pathogenic bacteria, which under certain conditions adhere to these surfaces , initiating the cell growth and leading to the biofilm formation (3). *S. aureus* has been frequently found on surfaces of different origin food processing materials being responsible for the outbreak related to the consumption of fresh and processed foods (4). Food poisoning

caused by *S. aureus* depends on the ability of the strain to survive in substrates, multiply under a variety of conditions and produce several extracellular substrate (5). Proteases and lipases are the most important hydrolysis enzymes which may cause illness, enterotoxin may also play an important role in food toxification (6). However, illness might be occurred with no viable bacteria could be isolated from the suspected food stuff. Regarding these aspects, this study was aimed to isolate of Staphylococcal spp from imported minced raw red meat, evaluation of bacterial ability to produce lipases and proteases at different temperatures 4, 20 and 37℃, and screening of biofilm formation.

Materials and methods:

Samples collection and bacterial isolation:

Seventeen out imported minced red meat were collected from different markets in Baghdad, from July 2013 to March 2014.

Bacterial isolation was carried out by culturing 25 gm of each sample into 250 ml of brain heart infusion broth(BHI) and incubation for 18 h at 37℃. Cultured were poured into mannitol salt agar, golden or pale isolated colonies were picked and propagated on nutrient agar for further steps (7).

Coagulase activity was determined by the agglutination method, briefly several colonies were mixed with antiserum specified for coagulase. The clot formation was recorded as positive result.

Antibiotic Susceptibility Test:

Antibiogram was conducted for each isolate by standard Bauer Kirby method (8). Muller Hinton agar plates were used for bacterial culturing, disc of penicillin (10U), erythromycin (30mcg), tetracycline (30mcg), nalidexic acid (30mcg) and chloramphenicol (30mcg) were subjected over the bacterial growth, plates were incubated for 18h at 37℃, diameter of cleared zones were recorded for results.

Screening of Proteases and Lipases Enzymes

Screening of proteases and lipases enzymes production from staphylococcal isolates was carried out at three different temperatures including 4, 20 and 37℃.

Proteases production was assayed on nutrient agar plates containing 1% casein. The hallow zone that appeared around the growth indicates to the activity of proteases enzyme (9).

Two methods were adopted to evaluate lipases production, the tween 80 method and rhodamine B method. Tween 80 was incorporated at 1% into nutrient agar medium, the appearance of hallow zone is indicated for the esterase activity while the appearance of turbid zone

is indicated for the lipases activity (10, 11).

Fluorescent dye, the rhodamine B, was used for true lipases detection according to (12). Briefly, 1mg/ml rhodamine B dye was dissolved in water and sterilized by filtration. 1% of rhodmine B solution and 2.5% of separately presterilized olive oil were added to the growth medium which composed of sterilized nutrient agar. Test medium was mixed thoroughly and poured into plates petridishes. Lipases activity was monitored by irradiated plates under UV light using UV transilluminator, and lipases positive isolates gave orange fluorescent color.

Detection of Biofilm and Slime Formation

Two different methods were used in detection of biofilm formation; tube method and congo red method which described by Christensen *et al*. (13) Bose *et al.* (14), Gundogan *et al.* (15). BHI medium containing 1% sucrose was divided into 5 ml in glass vials sterilized, cooled and inoculated by test bacteria, incubated statically at 4, 20 and 37℃ for 72 h. The growths were withdrawn, and the glass vials were air dried. Biofilm formation was recorded as +++ (heavily positive), ++ (moderate positive), + (weak positive) and – for negative biofilm formation. Congo red stain 0.8% was incorporated into BHI agar containing 1% sucrose. Bacterial isolates were streaked over plated and incubated at different temperature for 72 h, the black colonies with a dry crystalline consistency indicated to the biofilm formation; while, bright red to black colonies were established as a slime formation.

Results and Discussion:

One hundred and forty different isolates were picked out from mannitol salt agar for screening of *Staphyloccocus* spp from seventeen different imported minced raw meat samples. Forty eight isolates (34%) were had mannitol fermentation ability , and they appeared as small slimy moisture, pale colonies surrounded by yellow colored which are indicated for acid production from mannitol fermentation (Fig1); only 25 isolates were appeared as golden colonies, they coagulase positive and they identified as *S. aureus*, they represent 17.8% of total staphylococcal isolates . Whereas, ninety two isolates (65.7%) were non mannitol fermentation, small white colonies with dry moisture and coagulase negative reaction, they identified as *S. epidermids*.

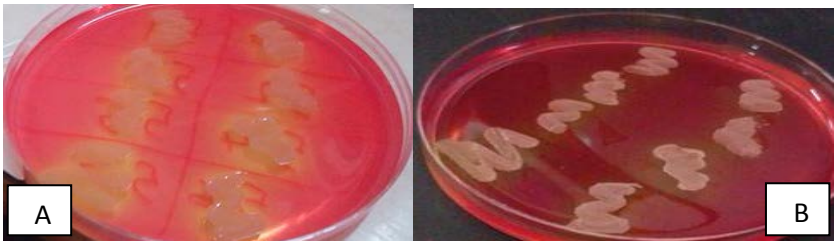

Figure 1: Cultures of staphylococcal isolates on mannitol salt agar
A= mannitol ferment isolates;B= non mannitol ferment isolates

Recent study of screening of contaminated imported raw red meat by *staphylococcus* spp showed no contamination could be observed in which the incidence of *Staphylococcus* spp frequencies was too low for all samples that taken over the particular period of time and from different places in Baghdad they ranged from 60 CFU/ ml to 120 CFU/ ml, sample location and the time period were summarized in table(1). APHA (16) revealed that the red meat is considered as a contaminated with *Staphylococcus* spp only when sample contains more than 1×10^2 colonies. *Staphylococcus* contamination of raw red meat may be introduced through worker handling, skin lesion, sneezing and coughing or from soil and water contamination.

Table (1): isolation of *Staphyloccocus* spp from imported minced red meat from different markets in Baghdad

location	Isolation period	Samples no.	Bacterial no/plate	Mannitol ferment	Non-mannitol ferment	Meat label
Zuafarania	July	1	8	3	5	**Al-Kaffel**
Bab-al-muaadam	August	1	6	4	2	**Al-Kaffel**
Zuafarania	August	1	6	3	3	**Al-Kaffel**
Bab-al-Muaadam	September	1	8	-	8	**Anwar Karbala**
Zuafarania	September	2	20	6	14	**Anwar Karbala**
Hai al- Amel	October	3	30	15	15	**Al-Kaffel**
Hai al- Ameel	December	2	8	4	4	**Al-Kaffel**
Ghazalia	February	2	15	5	10	**Australian**
Zuafarania	March	1	8	3	5	**Al-Kaffel**
Ghazalia	March	2	25	10	15	**Australian**
Total no.		17	150	48	92	

- **Non- expired red meat imported from India and Australia**

The study results indicated that coagulase negative *Staphylococcus* were more prevalence (82.2%) from coagulase positive *Staphylococcus* (17.8%) in raw minced imported red meat, this assumption was in accordance with previous study of Udo et al (17) , Citak and Duman (18) and Guandan and Ataol (15) who showed that CNS was more prevalent than *S. aureus* in various food. Isolation of coagulase positive *Staphylococcus* from imported raw red meat have an important value for public health because such these isolates may be a causative agent for food poisoning. Generally, it has been considered that the number of *S. aureus* need to be 7×10^5 CFU/gm of food to produce the toxin that cause illness, however, neither the absence of *S. aureus* nor the presence of a small number of organism could provide a complete assurance that red imported meat are safe. This is because that production of toxins could be adequate to cause food poisoning as mentioned by (19, 20).

In this study monitoring of antibiotic susceptibility showed that all staphylococcal isolates were highly sensitive to penicillin, erythromycin, and chloramphenicol in which the radius of inhibition zones ranged from 5 to 13mm for penicillin, 6-14mm for erythromycin and 5-10mm for chloramphenicol (Figure 2), while, 100, 20 isolate with a percentage of 71.4%, 14.2% showed to be resistant to nalidexic acid and tetracycline respectively. Susceptible isolates for nalidexic acid and tetracycline gave inhibition zones ranged from 5-3 mm and 13-17mm. It has been suggested that the meat industry is contributing to the antibiotic resistance. Our results were not in agreement with Gundogan and Aotaol (15) who found many isolates were resistant to antibiotics including ampicillin, tetracycline, methecillin and gentamycin of different *staphylococcus* spp isolated from different food samples. High antibiotic sensitivity could be attributed to that the source of contamination of the raw red meat like the environment not from pathogenic specimens, for example it can be obtained from human during handling of food, by which humans, are considered as a good reservoir for *S. aureus* in their nose or in their throat. In addition Staphyloccocal isolates may come from soil contamination or water. Monitoring of antibiotic susceptibility patterns of environmental isolates of *Staphyloccocus* is very important because *staphylococcus* has emerged as a reason of hospital and community acquired infections, and associated with a verity of clinical infections.

Figure(2): antibiotic susceptibility test for *Staphyloccocus* spp isolated from minced imported red meat

Recent screening for protease production found that all staphylococcal isolates belong to coagulase positive and coagulase negative isolates were proteases producer, and the clear zone appeared around bacterial growth was indicated for protease activity. Bacterial isolates incubated at 37 ℃ produced proteases after only 24 h while proteases activity could be detected after 72 h at 20 ℃ incubation. Very low growth of staphylococcal isolates was observed at 4℃ incubation and proteases enzyme could not be detected even after one week incubation. This may be due to that of the 37 ℃ is the optimum temperature for *Staphyloccocus* growth, retardation of bacterial growth could be happened at non-optimal temperatures such as refrigerator or at saved time.

Other researchers have found that the proteolysis activity of staphylococcal strains reached to 23.5%, while Parkash *et al.* (21) reported that among *S. aureus* strains that isolated from milk, did not show any proteolytic activity. Proteolytic activity of bacteria facilitate their surviving in a protein rich environments, where the proteases enzyme degrade proteins into long or short peptide or even to amino acids and thus may cause meat spoilage. On the other hands, protease enzymes have also been reported in staphylococcal associated infections and lesions, in which they considered as a major virulence factor in *Staphyloccocus*. Takeuchi *et al*., (22) reported that proteases positive isolates of *S. aureus* might be responsible for dermatitis in young chickens. Other workers found that 65.9 % of CNS and 26.6% of CPS isolated from acne lesions had proteases activity (9).

Differences in the results were obtained from lipases screening assays, where only 30 and 20 of mannitol fermented and non mannitol fermented *Staphyloccocus* spp gave florescence orange color on rhodamine B plates incubated at

both 37°C and 20°C indicating for the production of true lipases, while, both lipases and esterase could be detected from 5 isolates of mannitol fermented *Staphyloccocu*s spp using tween 80 plates, and two other isolates produced lipases. Moreover, only 5 isolates from Non mannitol fermented *Staphyloccocus* spp produced lipases without esterase as presented in figure (3) and table (2).

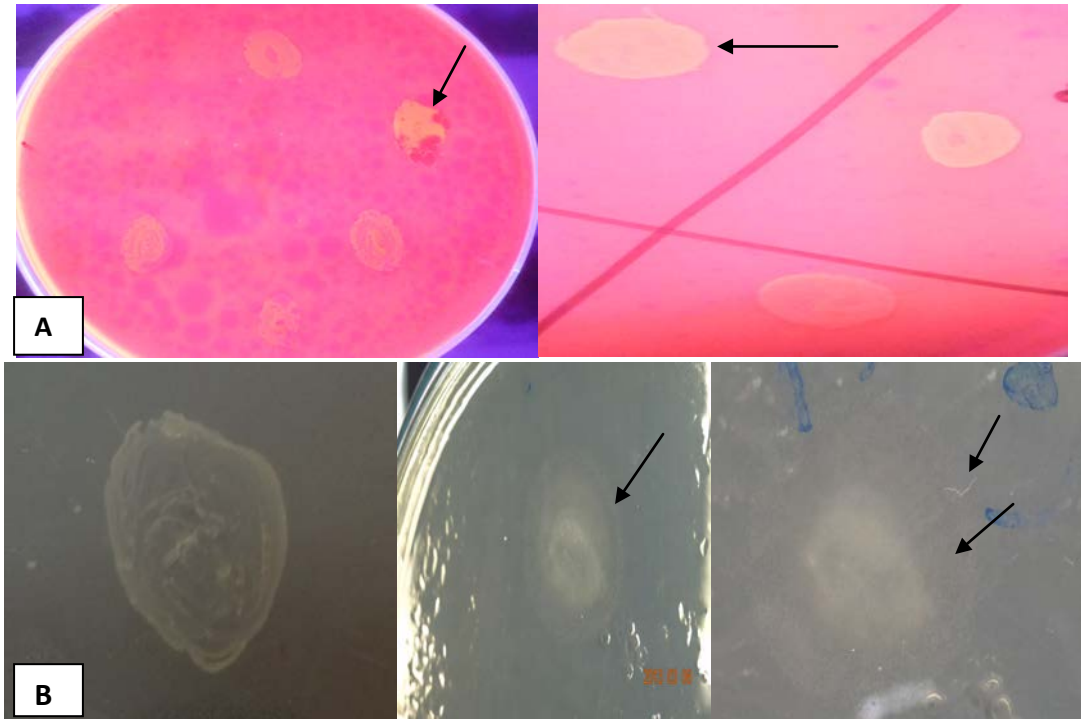

Figure (3): Screening of lipases enzymes for *Staphyloccocus* spp by different methods.

A= rhodamine B plates assay (positive result).

B= tween 80 plates assay (left= -ve, middle= lipase only, rhight= lipase and esterase).

Table (2): Screening of lipases enzymes for *Staphyloccocus* spp isolated from minced imported red meat at different temperatures

Lipase assay		Temperature °C	Mannitol fermented	% percentage	Non mannitol fermented	% pecentage
Rhodamine B		37	30	62.5	20	21.7
		20	30	62.5	20	21.7
		4	-		-	0
Tween 80	**lipase**	37	2	4.16	5	5.4
		20	2	4.16	5	5.4
		4	-	0	-	0
	esterase	37	-	0	-	0
		20	-	0	-	0
		4	-	0	-	0
	Lipase+ esterase	37	5	10.4	-	0
		20	5	10.4	-	0
		4	-		-	0

The production of bacterial lipases depends on many factors might influence the biosynthesis of lipases from microorganisms on cultural medium. These factors include medium composition especially (fatty acids or oils), pH and temperature. This assumption was in accordance with Pogaku *et al*. (23) who found that medium composition effects on the production of lipases from *Staphyloccocus* strain isolated from oil contaminated soil.

This research found that Biofilm was formed by both mannitol fermented and non mannitol fermented staphylococcal isolates, and depending on the temperatures. The adherence of bacterial growth to the surface of the glass vials indicating for biofilm formation while the black color that appeared over bacterial growth on congo red plates indicating for biofilm formation. In addition, the presence of red color bacterial growth with moisture texture indicated for slime production as shown in figure (4).

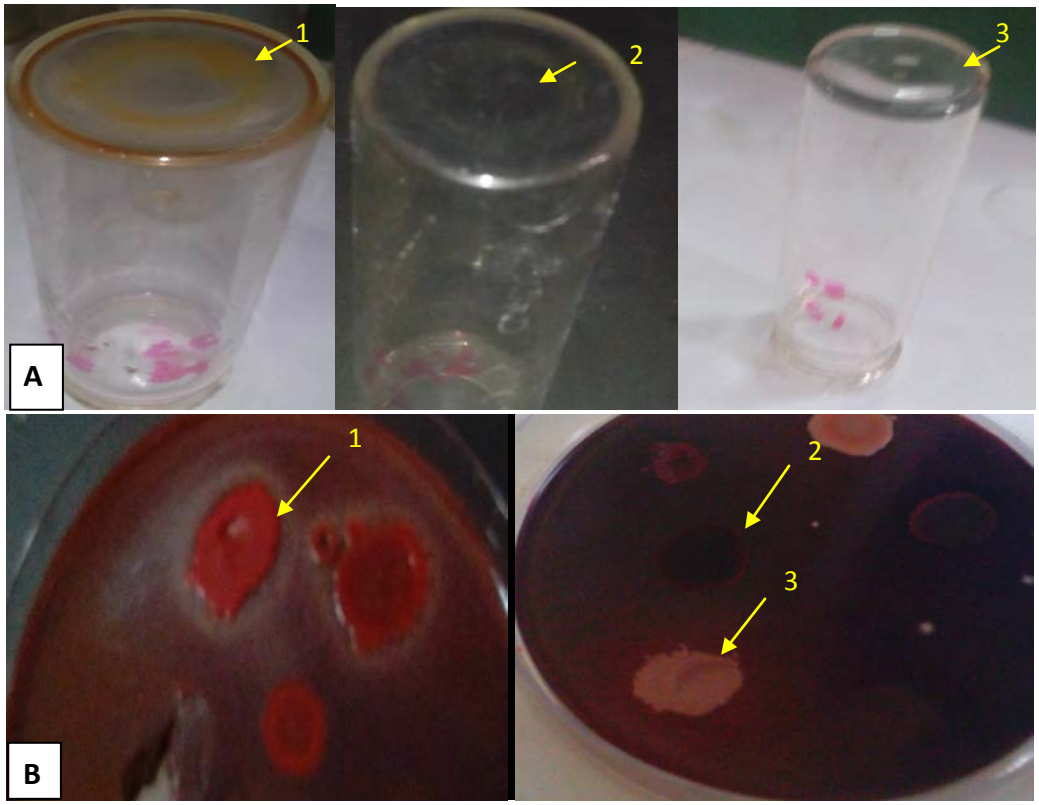

Figure (4): Screening of biofilm formation by *Staphyloccocus* spp isolated from minced imported red meat
A= tube method (1=+++, 2=++, 3=+)
B= congo red method(1= red color for slime formation, 2= black color for biofilm, 3= -ve).

All 140 staphyloccocal isolates representing 100% were formed biofilm at 20℃, among them only of 28 staphyloccocal isolates appeared as red color indicating for slime production while only 100 isolates representing 71.4% were formed biofilm at 37℃ and divided into 40 and 60 isolates from mannitol fermented and non mannitol fermented isolates respectively. Furthermore, no slime production could be detected at 37℃ from all isolates; no biofilm formation was achieved at 4 ℃ even after two weeks incubation. Using the glucose incorporated brain heart infusion medium to screen biofilm formation at three different temperatures gave a good insight on the behavior of bacterial isolates in their natural niche of raw red meat. The biofilm is a serious risk in the food industry because removal of irreversibly adhered cells is difficult and required application of strong mechanical force or chemical interruption of the microbial adhesion using surfactant, sanitizers or heat. There is a high probability

that the irreversibly adhered cell will remain even after pasteurization as presented by Silva Meira *et al.* (24).

Conclusions:

The recent study has concludes that Staphyloococus number that found per gram of meat was acceptable, which contains different type of staphyloccocal isolates including mannitol fermintor (coagulase positive and coagulase negative) and non- mannitol fermentor. The existing of coagulase positive *Staphyloccocus aureus* from minced imported meat may contribute public health risk. Different *Staphyloccocus* spp were susceptible to penicillin, erythromycin, tetracycline and chloramphenicol. Resistant to nalidixic acid was detected for 100 bacterial isolates. Staphyloccocal isolates produced many enzymes at different temperatures. lipase, estraces and protease were detected from isolates, which they also formed biofilm and slime layer which might cause a serious problem in meat industry, causing disease for human beings and animals.

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Purification and characterization of inulinase production from *Bacillus cereus* (Be9)

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Abstract: Fifty isolates of *Bacillus*spp were obtained from rhizosphere soil of compositae plant roots.The ability of inulinase production by these isolates was screened. *Bacillus* Be9 which isolated from soil of lettuce root was the highest inulinase producer; it was identified as *Bacillus cereus*.Inulinase was purified by two steps included ion exchange chromatography using DEAE- cellulose column and gel filteration by Sephacryl S-100 column. The ion exchange step showed the presence of three isoforms of the enzyme. The three isoforms were subjected to additional purification by gel filtration. The final specific activity of inulinase was 166.6 U/mg with 3.41 fold of purification and 45.4% yield. The characteristics of purified inulinase were studied. The optimum pH of enzyme activity and stability was 7.0, and the maximum enzyme activity was observed at 45^{o}C. The thermal stability for the enzyme was at (40-60)^{o}C and the enzyme retained more than 75% of its activity after incubation at 80% for 30 min. 10mM of Na^{+2}, Mn^{+2}, Ca^{+2}, Mg^{2}, enhanced enzyme activity 116.9%,115%, 113.8% and 112% respectively. Different levels of inhibition ranged from (0-92%) revealed when enzyme was treated with Cu^{+2}, K^{+}, Zn^{+2}, Ni^{+2}, Fe^{+2}, Hg^{+2} at 10Mm. Results showed that inulinase is a metalloenzyme since the enzyme lossed its activity when treated with EDTA and 65.9% when treated PMSF.

Keywords: Purification, characterization, inulinase, Bacillus cereus.

تنقية وتوصيف انزيم الانيولينيز المنتج من بكتريا*Bacillus cereus* Be9

فاطمة عبد الحسين غضبان و سناء برهان الدين

قسم علوم الحياة / جامعة بغداد

الخلاصة: تم الحصول على 50 عزلة بكتيرية تنتمي الى جنس *Bacillus* من التربة المحيطة بجذور نباتات العائلة المركبة واختبرت قدرة هذه العزلات على انتاج انزيم الانيولينيز، وبينت النتائج ان العزلة *Bacillus* Be9 المعزولة من تربة الخس هي اكفأ عزلة منتجة للانزيم وقد شخصت على انها احدى سلالات *Bacillus cereus*.

نقي الانزيم Inulinase المنتجمن العزلة Be9 بخطوتين تضمنت كروماتوغرافيا التبادل الايوني باستخدام عمود DEAE-Cellulose والترشيح الهلامي في عمود sephacryl S-100 ، اسفرت عملية التبادل الايوني عن ظهور ثلاث صور انزيمية ، خضعت الصور النزيمية الى خطوه تنقيه اضافية تمثلت بخطوة الترشيح الهلامي ، حيث بلغت الفعالية النوعية 166.6وحدة/ ملغم بروتين بعدد مرات تنقية 3.41 وبحصيلة انزيمية 45.4%.

درست بعض صفات الانزيم المنقى حيث بلغت قيمة الرقم الهيدروجيني الامثل لفعالية وثبات الانزيم 7 واعلى فعالية عند درجة حرارة 45م ^{o} وكان الثبات الحراري الامثل للانزيم بين 40 – 60 م^{o} لمدة نصف ساعة .وحافظ الانزيم على اكثر من 75%من فعاليته عند حضنه بدرجة 80م^{o} لمدة 30 دقيقة .

درس تاثير بعض الايونات الفلزية في فعالية انزيم الانيولينيز ولوحظ زيادة في فعالية الانزيم عند حضنه مع ايونات الصوديوم، المنغنيز ،الكالسيوم والمغنيسيوم و بتركيز 10ملي مولر وبلغت الفعالية الانزيمية للانيولينيز 116.9% ، 115% ، 113.8% و 112 % على التوالي كما لوحظ نسب مختلفة من التثبيط تراوحت بين 0-92% عند معاملته بايونات النحاس ،البوتاسيوم ،الخارصين ،النيكل ،الحديد و الزئبق . انزيم الانيولينيز هو من الانزيمات المعدنية حيث انخفضت فعاليته عند معاملته بالعامل المخلبي EDTA إلى الصفر والى 65.9% عند معاملته بPMSF.

Introduction

Microbial inulinases belong to an important class of industrial enzymes that have gained increasing attention in the recent years because of its wide spectrum of applications including: ultra-high fructose syrup obtaining from inulin, bioethanol production, inulo-oligosaccharide production, single-cell oil and single-cell protein production,citric acid, butanediol, alcohols and lactic acid production (1). Inulin is a well-known fructan particularly abundant in some plants belonging to families Asteraceae, Campanulaceae, Poaceae, Liliaceae and Amaryllidaceae (2). It is made of linear chains of d-fructofuranose molecules linked by *β*- 2,1-glycosidic bonds and has a d-glucose moiety at the reducing end. Inulin and its partially hydrolyzed products (fructooligosaccharides) have gained significant importance in food and pharmaceutical industries.

Fructooligosaccharidesare popular functional food components due to their beneficial health properties, such as bifidogenic nature, low calorie diet and rich source of dietary fibre (3). Inulinase, as a kind of hydrolases, can be divided into endoinulinase and exoinulinase. The endoinulinases, without invertase activity, can only cut the internal linkages in inulin to yield inulooligosaccharides, while the exoinulinases remove the terminal fructose residues from the non-reducing end of the inulin to yield fructose or glucose (4). The purpose of this study was to purifyand characterizeinulinaseenzymefrom*B. cereus*Be9.

Materials and Methods

Collection of Samples:

Sixty samples from rhizosphere soil of compositae plant roots included (Lettuce, Cabbages, Cauliflower, Leek and Barley) were collected in sterile containers and transported to the laboratory until usage.

Isolation of *Bacillus* spp.

One gm of each soil sample was added to 9 ml of sterile water and shaked to homogenize, then heated to 80˚C for 15 min in water bath. Serial dilutions were made for each sample by using sterile water. From each dilution(0.1ml) was spreaded on a nutrient agar plates, and incubated aerobically at 37^{o}C for 24 hrs.The growing colonies were purified by sub culturing on nutrient agar for many times until pure culture was obtained.Bacterial isolated were obtained and identified as Bacillus spp. according to the morphological and microscopic examination.

Screening forinulinase production from *Bacillus* spp.

Semi-quantitative method

The bacterial isolates were cultured on inulin agar medium consisted of (0.5g Inulin, 0.4 g Pepton, 10mM MgSo4.7H_2O and 2g Agar-Agar dissolved in 100 ml D.W).The pH was adjusted to 7.0 and sterilized at 121 ^oC for 10 min. The plates were incubated at 40˚C for 48hrs. Colonies that displayed growth, were selected for furthur experiments and transferred to fresh plates.

Quantitative method

Ten ml of inulin broth medium consisted of (0.5g Inulin, 0.4g Pepton and 10mM Mgso4.7H_2O dissolved in 100 ml D.W) were inoculatedwith 0.1ml of selected isolates(O.D=0.4at 600nm) and incubated at 40ºC for 48 hrs. The cells were precipitated by cooling centrifuge at 6000 rpm. Supernatant were assayed for inulinase and invertase activity by measuring reducing sugars released from inulin and sucrose, respectively.

Assay of Inulinase Activity (5)

The inulinase activity was assayed by determining the reducing sugars formed during incubation of soluble enzyme. Enzyme extract 0.2ml was added to 0.8ml of reaction solution 1% inulin(1g of inulin powder in 100ml of 0.1M of Potasium phosphate buffer pH7.0) and the mixture was incubated at 40ºC for 30 minutes. The enzyme reaction was stopped by adding 1ml of DNSA to each tube. Then incubate d in boiling water bath for 5 minutes and cooled in ice bath. Five milliliter of D.W.was added to each tube and mixed well and the optical density of the solution were measured at 540nm. The enzymatic activity was calculated based on the standard curve of fructose. One unit(U)of inulinase activity was defined as the amount of enzyme that produces 1µmol of fructose per minute under the specified conditions.

Assay of InvertaseActivity (6)

Enzymatic activity of invertase was estimated by the same previous steps as in inulinase assay except of 1% sucrose was used as a substrate instead of 1% inulin and the enzymatic activity was calculated based on the standard curve of mixture of glucose and fructose.One unit (U) of invertase activity was defined as the amount of enzyme that hydrolyzes 1µmol of sucrose per minute under the specified conditions. Protein concentration in the supernatant was determine by method described by Lowry *et al*.,(7)..

Enzyme activity (U/ml)=O.D (540 nm)/(Slope x volume of enzyme xincubation period) Protein concentration (mg/ml)=O.D(600 nm)/ (Slope x 1000)

Calculation of specific activity

Specific activity(U/mg protein) = Enzyme activity(U/ml)/Protein concentration(mg/ml)

Extraction of enzyme

Fifty ml of inulin broth pH 7.0 was inoculated with 0.5ml of activated bacterial suspension (O.D=0.4 at 600nm) and incubated at 40˚C for 48hrs.The enzyme was extracted by cooling centrifuge at 6000 rpm for 30 min. Activity of enzyme and protein concentration were assayed for supernatant and specific activity was calculated.

Enzyme purification

Inulinase produced by the selected *Bacillus* isolate was purified by several steps as mentioned bellow:

Precipitation of inulinase with ammonium sulphate

The crude inulinase solution (supernatant) was precipitated with different concentration of ammonium sulphate (30- 90%) saturation under cooling condition, the precipitates were separated by cooling centrifuged at 10000 rpm for 30 minutes and dissolved in small amount of potassium phosphate buffer pH7.0. The final volume of solution, the activity of enzyme and protein concentration were measured and specific activity was assayed .

Concentration of enzyme

The crude inulinase solution (supernatant) was concentrated bysucrose. The activity of enzyme, protein concentration and specific activity were determined.

Ion exchange chromatography

Five ml of concentrated enzyme by sucrose solution was loaded on DEAE – cellulose column (24×2 cm). The column was washed with 0.1M potassium phosphate buffer pH7.0 at flow rate of 30 ml/h, and eluted with gradient (0.1 - 0.5 M) NaCl solutions. Fractions of 5ml/tube were collected. The enzyme activity and optical density at 280 nm were measured . The fractions with high inulanase activity were collected, volume, enzyme activity and protein concentration were estimated, then this sample (active fractions) was concentrated with sucrose.

Gel filtration chromatography

The concentrated enzyme solution (5ml) was loaded on Sephacryl S-100 (70 x1.6 cm), and equilibrated with 0.1M potassium phosphate buffer pH7.0. The enzyme was eluted with same buffer. The fractions were collected with 5ml volume at flow rate 20 ml/h. The enzyme activity and optical density at 280 nm were measured.The active fractions were collected, enzyme activity and protein concentration were measured.

Characterization of inulinase

Determination of the optimum temperature for inulinase activity

Amount of 0.8ml. of reaction solution(1% inulin) was incubated at different temperature (25,30, 37, 40,45,

50, 60 and 70) °C. 0.2ml. of purified enzyme was added to reaction solution at each temperature and incubated for 30min, then the enzyme activity was assayed.

Determination of inulinase stability at different temperatures

One ml of purified inulinase was incubated in water bath at different temperature(25, 37, 40, 45, 50, 60, 70, 80and 90) °C for 30 min and immediately transferred into an ice bath. The enzymatic activity was measured and the remaining activity was calculated (%) and plotted against the temperature. The remaining activity was estimated according to the following equation:

$$\text{Remaining activity (\%)} = \frac{\text{Activity after treatment}}{\text{Activity before treatment}} \times 100$$

Determination of optimum pH of inulinase activity

Amount of 0.2ml of purified enzyme was addedto 0.8ml. of reaction solutionat different pH values (4.0-10.0) and incubated at 45°C for 30 min. Then enzyme activity was assayed.

Determination of inulinase stability at different pHs

Equal volumes of purified enzyme and buffer solutions with pH range (4 to 10) were incubated at 45C for 30 min. and cooled in ice bath. The enzymatic activity for each treatment was measured and the remaining activity (%) for inulinase was calculated.

Determination of metal ions and inhibitor effects on inulinase activity

One ml. of purified enzyme was mixed with 1ml. of 10mM. metal ions solution of ($ZnCl_2$, $NiCl_2$, NaCl, $HgCl_2FeCl_3$, $CaCl_2$, $MnCl_2KCl$,$CuSO_4$, $MgSO_4$). EDTA and PMSF solution and incubated at 45C for 30 min. then enzyme activity was assayed and remaining activity was calculated**.**

Results and discussion

Isolation of *Bacillus* spp.

Sixty samples were collected from rhizosphere soil of compositae plant roots. Fifty bacterial isolates were obtained (Table 1) and identified as *Bacillus* spp. according to morphological and microscopic examination .

The result showed that the highest number of isolates (20) was obtained from rhizosphere soil of Lettuce plant roots. (Table 1). Rhizosphere soil samples and compositae plant material are common sources of inulinase – producing microorganisms. This might be attributed to the adaptation of some strains to inulin-containing plants, which is generally found in compositae (8). Inulin can be found in many plant species from mono- and dicotyledonous families, such are Liliaceae, Amaryllidaceae, Gramineae and Compositae (9).

Table 1: *Bacillus* isolates obtained from different sources

Sources of isolates	Number of *Bacillus* isolates	Code number of isolates
Lettuce	20	Be1,Be2, Be3, Be4, Be5, Be6, Be7, Be8, Be9, Be10, Be11, Be12, Be13 Be14, Be15, Be16, Be17, Be18,Be19,Be20
Cabbages	13	Bc1,Bc2,Bc3,Bc4,Bc5,Bc6,Bc7, Bc8,Bc9,Bc10,Bc11,Bc12,Bc13
Cauliflower	8	Bu1, Bu2, Bu3, Bu4, Bu5, Bu6, Bu7, Bu8
Leek	6	Bl1, Bl2, Bl3, Bl4, Bl5,Bl6
Barley	3	Bb1, Bb2, Bb3
Total	50	

Screening for inulinase producing *Bacillus*

Semi-quantitative screening

Inulin agar medium was used for screening the inulinase production. The results showed that 25 isolates from 50 isolates were able to produce inulinase by utilization inulin as sole sources of carbon in medium at 40˚C for 48 hrs incubation. (10)isolated 50 bacterial strains on the basis of their growth on agar plates containing inulin as sole carbon source. The rapid growth and healthy colonies of these isolates on inulin based media indicated positive inulinase activity.

Quantitative screening

The twenty five Bacillus spp. isolates were selected according to their growth on inulin agar plate for quantitative screening for inulinase production using DNSA method (16). Results in table (2) indicated that all bacterial isolates were inulinase producer. Among them *Bacillus* (Be9) was the most efficient one, the specific activity was (59.5 U/mg) protein, while the specific activity for the other isolates were ranged between (21.5-58.3) U/mg protein.

Table (2): Production of inulinase by *Bacillus* isolates grown in inulin broth medium pH 7.0, for 48 h. incubation at 40°C.

Isolate symbol	Specific activity (U/mg) towards:		I/S ratio
	Inulinase (I)	Invertase (S)	
Be9	59.5	0.0	-
Be7	58.3	0.0	-
Bl4	58.0	0.0	-
Bc3	56.6	3.5	16.17
Bc1	56.2	0.0	-
Be2	53.7	2.2	24.41
Bc6	52.9	3.1	17.06
Bb3	50.2	2	25.1
Be3	47.9	0	-
Be5	47.3	2	23.65
Bc10	46.0	4	11.5
Bu6	44.7	5	8.94
Bl2	43.0	0.0	-
Bb2	41.8	2	20.9
Bc5	35.7	0.0	-
Be13	33.0	0.0	-
Bu7	30.8	0.0	-
Bl5	25.7	3.4	7.56
Be15	25.2	0.0	-
Be19	25.0	0.0	-
Bc12	23.7	0.0	-
Be20	22.9	0.0	-
Be11	21.9	9	2.43
Be17	21.8	5.9	3.69
Bu3	21.5	0.0	-

The I/S ratio varied between (0.0 - 25.1).The ability of the bacterial isolate to produce high level of extracellular inulinase comparing with the production of invertase has been confirmed.The differences in the inulinase production among these isolates might be due to the activity of genes encoded inulinase synthesis and source of isolation (11).The ratio invertase by inulinaseactivitys (S/I) is an important parameter because it shows if the enzyme is a true

inulinase (S/I<100) or an invertase (S/I>100) (12).

The isolate (Be9) was subjected to further biochemical tests according to Bergys Manual of Systematic Bacteriology (13)

Purification of inulinase

Crude extract of *B.cereus* Be9 after production under the optimum conditions was purified by several steps including:

Precipitation with ammonium sulphate

Cell free extract of *B. cereus* was subjected to ammonium sulphate precipitation with different ratio of saturation (30-40-50-60-70 80 and 90%). The results indicate that 50% saturation gave specific activity 23.5 U/mg protein figure (1). The result indicates that there was decrease in the specific activity (23.5 U/mg) compared with the crude extract (50 U/mg) that's mean loss the most of enzyme activity in this step , therefore ammonium sulphate step was left out. During the initial purification of the inulinase from*A.niger*with ammonium sulphate fractionation was adopted. However, all activity of the enzyme was lost and therefore this step in the purification was left out (14)**.**

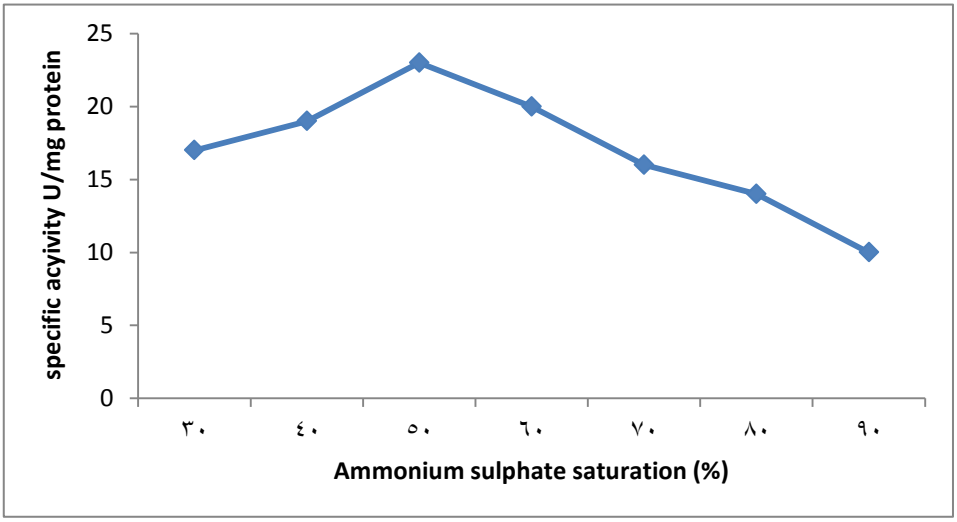

Figure (1): Specific activity of *B. cereus* Be9 inulinase after precipitation with different ammonium sulphatesaturation(%)

Concentration with sucrose

The crude extract of *B. cereus* Be9 after production under optimum conditions was concentrated with sucrose and the results showed increase in activity (18.5 U/ml) with specific activity (66.07 U/mg) and 84.09 % yield.

Ion exchange chromatography

The result showed three protein peaks in the eluted fractions, the three

peak represented inulinase, located at fractions (50-60), (69-93) and (99-114) respectively, which was eluted with (0.2 M) , (0.3M) and (0.4M) of NaCl solution, respectively. The presence of three activity peaks, means that the enzyme have three isoenzyme (Figure 2). These result indicated that inulinase has a negative net charge since it bounds with anionic ion-exchange. The specific activity increased in this step to 80.66 U/mg proteins. The fold of purification was 1.66 with 55% recovery (Table 4).

Multiple isoenzyme forms of this enzyme are common in the purified enzyme from microorganisms. (15) found four different inulinase isoforms, in the extracellular extracts of *Fusraiumoxysporum*.

Gel filtration chromatography:

Results in figure (3) showed that the eluted fraction contained one protein peak and one peak represent enzyme activity between the fractions (14-29).The results in table (4) showed increases in the specific activity of the enzyme 166.6 U/mg protein, with 3.41 fold of purification with 45.4 % yield.Gel filtration chromatography is a bead-formed gel prepared from agarose. It can be used under most of the conditions encountered in gel filtration. It has broad fractionation ranges which makes them suitable for characterizing or cleaning up samples containing components of diverse molecular weight (16).(17) observed that*B. smithii* T7 was purified by gel filtration on superdex 75, the specific activity 1105.5 U/mg , with 31.4 fold and 27.3% yield

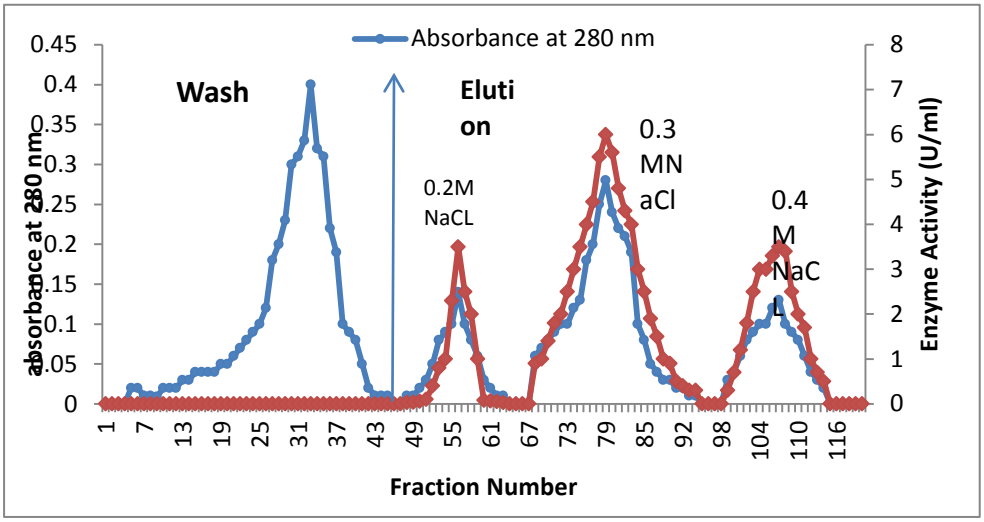

Figure (2): Purification of inulinase produced by *B. cereus* Be9 using ion exchange chrmatography DEAE-Cellulose column (1.5x30 cm) and equilibrated with 0.1M potassium phosphate buffer pH7, the fraction were collected with 5ml/tube at flow rate 30 ml/h, and eluted with gradient (0.1-0.5M) NaCl solutions.

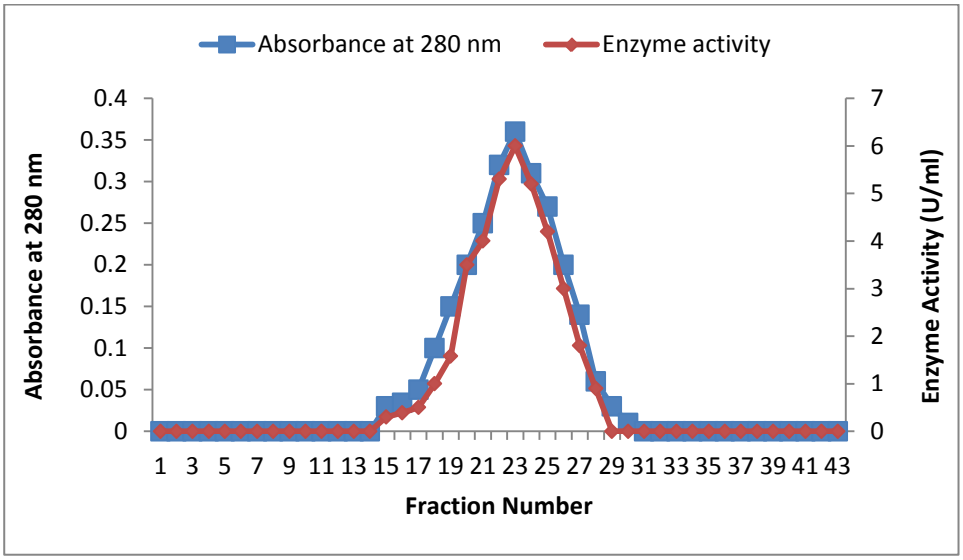

Figure (3): Purification of inulinase produced by *B. cereus* Be9 using gel filtration chromatography Sephacryl column S-100 (1.5x70 cm) equilibrated with 0.1M potassium phosphate buffer pH7 and eluted with the same buffer fraction volume 5ml/tube at flow rate 20 ml/h.

Table (4): Purification steps of inulinase from *B. cereus* Be9

Steps of purification	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Fold of puri.	Yield (%)
Crude extract	50	4.4	0.09	48.8	220	1	100
Concentration with sucrose	10	18.5	0.28	66.07	184	1.36	84.09
Ion exchange chromatogrpahy by DEAE-Cellulose	10	12.1	0.15	80.66	121	1.65	55
Gel filtration chromatography Sephacryl S-100	10	10	0.06	166.6	100	3.41	45.4

Characterization of inulinase

Effect of temperature on inulinase activity

The results showed that enzyme activity increases with temperature increasing within the range of 25°C. to 45°C. it reached its maximum value (5.5 U/ml.) at 45°C., a reduction in enzyme activity was observed above 45°C. figure(4).

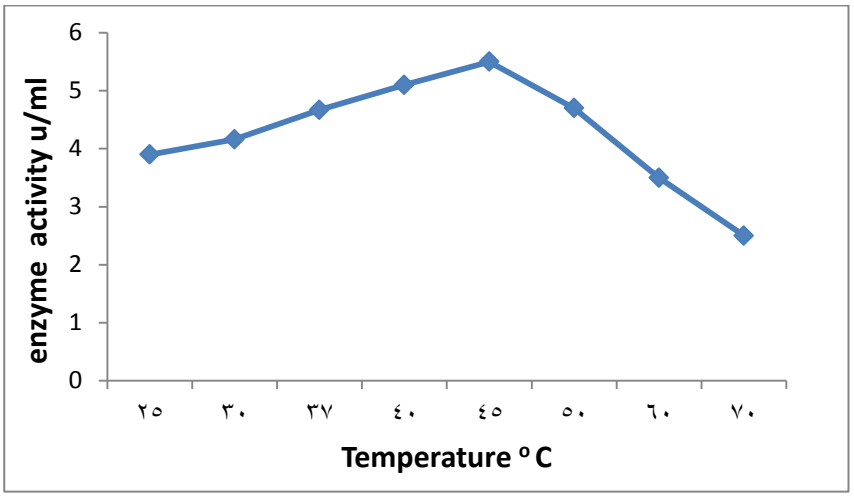

Figure (4): Effect of temperature on the activity of purified inulinase produced by *B. cereus* Be9

The decrease in enzyme activity at high temperatures may be due to the destruction of enzyme or changes in its tertiary structure (18). Similar reports were obtained for *Bacillus* thermophilic strain (19).

Optimum temperature for enzyme stability

The results indicated that the inulinase enzyme that produced from *B. cereus* Be9 isolate was stable until temperature 60 °C the remaining activity was 93 % then decreased to 82 % , 75.7 % and 69.4 % at 70 , 80 and 90C^{o} respectively (Figure 5). This indicating that the inulinase which produced from *B. cereus* Be9 was stable at high temperature and hence it remains active at temperature above the normal or physiological temperature, this property is suitable for used of enzyme in application which needs high temperature.Higher thermostability of industrially important enzyme brings down production costs because lower amount of enzyme is required to produce the desired product inulinase from mould, yeast and bacteria have been studied but only a few of these enzymes have an optimum temperature at 60°C or higher, as required for industrial applications (20).

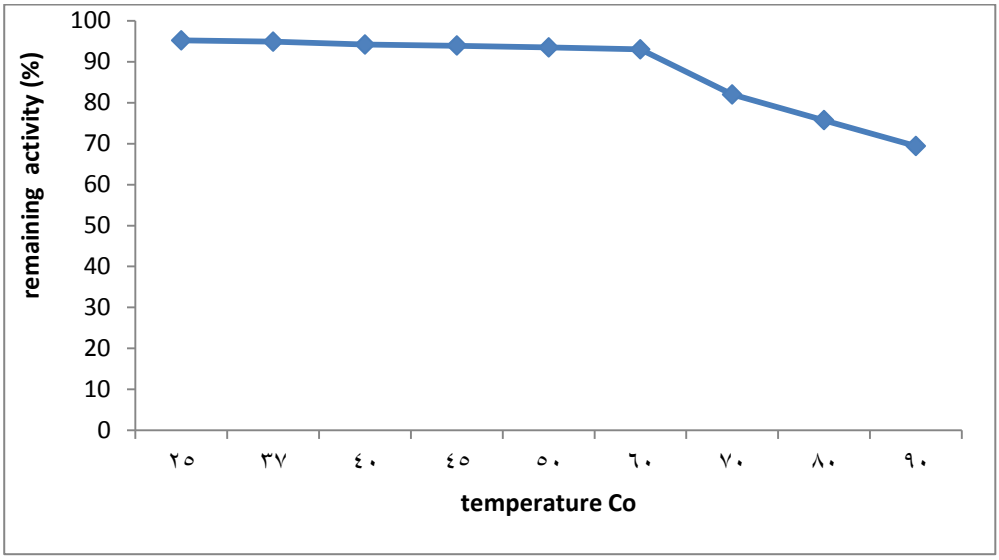

Figure (5): Effect of temperature on purified inulinase stability produced by *B .cereus* Be9.

Inulinase from *B. stearothermophilus*KP 1289 was active between 30 and 75°C, with an optimum at 60°C. The enzyme was classified as a thermostableexo-inulinase (21).

Optimum pH for inulinase activity

The results showed that inulinase was active in a wide range of pH (4.0-10.0) but it was more active at pH 7.0 than other value, the activity was (5.4U/ml). Figure(6).

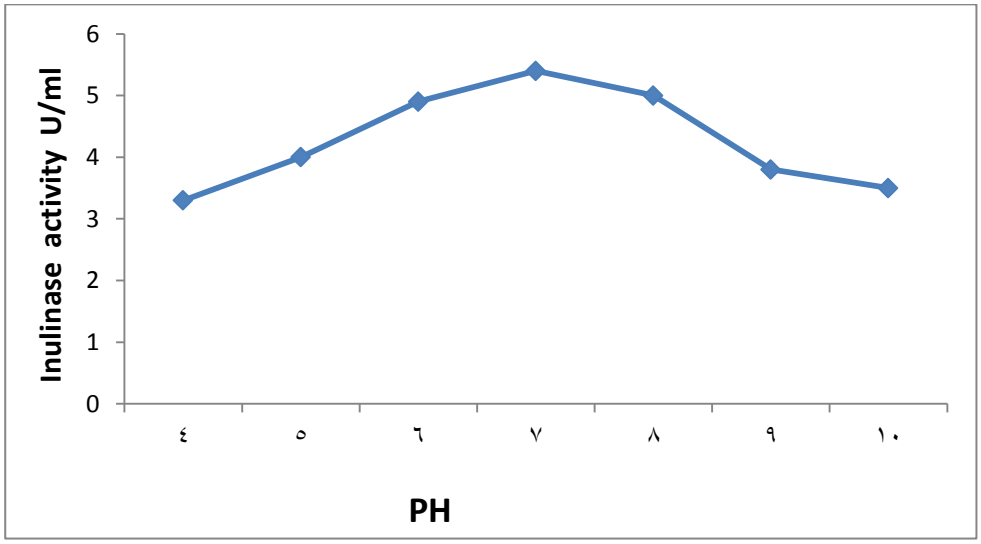

Figure (6): Effect of pH value on purified inulinase activity of *B. cereus* Be9

The pH can exert its effect on enzyme activity in different ways; on the ionization of groups in the enzyme's active site, on the ionization of groups of substrate, or by affecting the conformation of either the enzyme or the substrate which could explain the decrease in the activity at value of acidity pH 4.0 and alkalinity pH 10.0 (22). Generally, inulinase from fungal strains shows pH optima between 4.5 and 7.0, from yeast strains between 4.4 and 6.5, and from bacterial strains between 4.8 and 7.0 (23).

Optimum pH for inulinase stability

The results showed that inulinase has good stability in pHs 6 - 8 with highest remaining activity at pH 7 (89%) (Figure 7). The activity decreased slightly at pHs 9 and 10 the remaining activity were 79% and 68% respectively

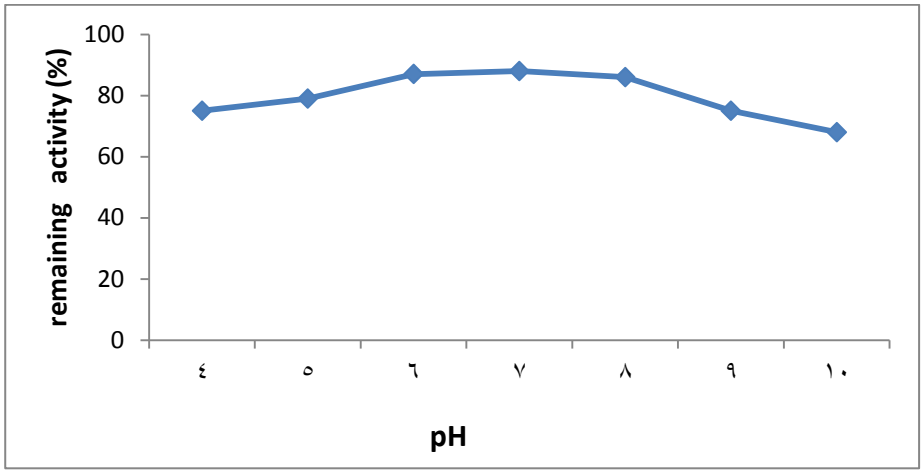

Figure (7) : Effect of pH on purified inulinase stability produced by *B. cereus* Be9.

The effect of pH on the enzyme stability could be explained in the formation of improper ionic form of enzyme or the active sites and irreversible inactivation. Stability of the enzyme depends on many factors such as temperature, ionic strength, chemical nature of buffer, concentration of various preservatives, concentration of metal ions, substrate and enzyme conecentration (24).

The effect of pH on stability of inulinase from*Bacillus spp.* showed that the enzyme had optimum pH for stability at 6.0-8.0 pH (25).

Metal ions and inhibitor effects on inulinase activity

Results mentioned in table (5) showed that the inulinase activity increased when incubated with 10 mM Na^{+2} ,Mn^{2+}, $Ca^{2+}$and Mg^{2+} as this concentration is effective, the remaining activity were 116. 9% ,11.5%, 11.3.8% and 11.2% respectively while, Hg^{+2}caused complete inhibition. whereas K^{+} , Ni^{+2} , Zn^{+2} ,Fe^{+2} and Cu^{+2} had a slight inhibitory effect on activity at the same concentration.Suggesting that some

metal ions had a capability to protect enzyme against denaturation. Where, (26) mentioned that these metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature Enzyme activity is sensitive to the presence of specific substances that bind to the enzyme and cause conformational change in the enzyme. Conformational change is the change in the shape of the molecule, in this case the active site of the enzyme (27).

Table (5): The effect of metal ions on inulinase activity produced from *B. cereus* Be9

Metal ion	Concentration (mM)	Remaining activity(%)
Control	-	100
NaCl	10	116.9
$MnCl_2$	10	115
$CaCl_2$	10	113.8
$MgSO_4$	10	112
KCl	10	92
$ZnCl_2$	10	85
$NiCl_2$	10	84.3
$FeCl_3$	10	74.9
$CuSO_4$	10	60.4
$HgCl_2$	10	0.0

Inhibition of the enzyme with Hg^{+2} indicate the presence of SH groups in the proteins (28) and suggesting the importance of thiol-containing amino acids residues in the enzymes function. The present results can be compared with the study of (29) who also similarly reported that the activity of inulinase from *A. niger*decreased in the presence of Ag^{+2}, Hg^{+2}, Cu^{+2} and Mn^{+2}. The effect of metal ion may due to the formation of complex with ionized inulinase resulting in changing solubility and behavior at the substrate interfaces, transition metal ions may change the conformation of protein to a less stable form by interaction with enzyme surface charge which could markedly affect the ionization of some amino acid residues (30).Most of monovalent cations such Na^{+} increase the activity of many enzymes due to their role in protection of enzyme structure and hence creating a suitable reaction state (31). Study on inulinase from *B.* cereus MU-31 indicated that the presence of 1mM pb^{2+} and Hg^{+2} inhibited the inulinase activity (32)

The effect of inhibitor agents on the enzyme activity

The result in table (6) showed that serine proteinase inhibitor (PMSF) decreased inulinase activity

produced from *B. cereus* Be9 to 65.9% at 10 mM which indicate that this inulinase may be belonge to serine inulinase.Inulinase inhibited strongly by EDTA the remaining activity was 0.0 at 10 mM this means that this enzyme is metalloenzyme which requires metal ion for activity, and removal of metal ion from enzyme structure leads to entire loss in enzyme activity.

Table (3-6): Effect of the inhibitor agents on inulinase purified from *B.cereus* Be9 activity

Material	Concentration (mM)	Remaining activity(%)
Control	-	100
PMSF	10	65.9
EDTA	10	0.0

According to the report of (33), EDTA have inhibitory effect on some inulinase activity demonstrating that the characterized enzymes were metalloenzymes. (28) found only 6% of the activity of inulinase from *A. fumigates* was obtained in presence of 1 mM of EDTA.Inulinasepurified from a Marine Yeast *C. aureus* G7a was strongly inhibited by phenylmethanesulphonyl fluoride (PMSF) and EDTA (33).

Conclusion

The local isolate of *Bacillus cereus* Be9 is an efficient inulinase producer. Purification indicated that produced inulinase had negative net charge and can be purify by two steps of chromatoghraphy. The enzyme of *B. cereus* Be9 stable at different pH values (6-8) and temperature (40-60) °C. *B.cereus*Be9 inulinase is considered to be metalloenzyme .

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Survival rates of *Bulinus truncatus* as a way to determine the molluscicidal activity of *Ricinus Communis* extracts

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Abstract: The effects of *Ricinus Communis* leaves and bark extracts to the snail *Bulinus truncatus* were assessed by determination the survival rates. The study was showed different effects of the extracts of *R. communis* on the snail *B. truncatus*. These effects are represented by death, escaping from exposure media and imbalance of snail behavior. LC50 values 96hrs. of leave extracts with stock solutions 50, 100 and 200g/L were (32, 11 and 6.4%) respectively while the LC50 values 96hrs. of bark extracts with stock solutions 50, 100 and 200 were (191, 54 and 43%) respectively. Leave extracts of *R. communis* were more toxic to *B. truncatus* than bark extracts. The aim of this study is to detect the effects of leave and bark extracts of *R. communis* on the snail vector of *Schistosomiasis* in order to control the bilharzia disease.

Key words: survival rates, *Ricinus communis, Bulinus truncates*

نسب بقاء القوقع *Bulinus truncatus* كطريقة لقياس فعالية مستخلصات نبات الخروع في ابادة القواقع

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الخلاصة: تم تقييم تاثيرات مستخلصات اوراق وقلف نبات الخروع *Ricinus communis* في القوقع *Bulinus truncatus* عن طريق تحديد نسب البقاء. الدراسة لاحظت تاثيرات مختلفة لمستخلصات نبات الخروع*R. communis* في القوقع اعلاه. تمثلت تلك التاثيرات بالموت والهروب من وسط التعرض الاختلال في سلوك القوقع. متوسط التراكيز نصف المميتة خلال 96 ساعة تعرض لمستخلص الاوراق من المحلول الخزين 50 و 100 و200 غرام/لتر كانت 32 و11 و6.4 % على التوالي بينما التراكيز نصف المميتة لمستخلص القلف من المحلول المخزن 50 و100و200 غرام/لتر كانت 191و54و43 % على التوالي. مستخلصات اوراق الخروع *R. communis* كانت اكثر سمية لقوقع *Bulinus truncatus* من مستخلصات القلف. الهدف من الدراسة هو قياس تاثيرات اوراق وقلف نبات الخروع للقوقع الناقل الحيوي لمرض البول الدموي للسيطرة على مرض البلهارزيا.

كلمات مفتاحية: نسب البقاء ، ***Ricinus communis*، *Bulinus truncatus***

Introduction

Molluscicides have a history of success and failure in the control of Schistosomiasis. The high cost of imported synthetic compounds, along with increasing concern over the possibility of snail resistance to these compounds and their toxicity in non-target organisms, have given a new impetus to the study of plant molluscicides [1].

Bioactive products of plant origin have become the focus because they are less expensive and less hazardous to the environment than their synthetic counterparts [2].

The castor oil plant, *Ricinus communis* (Euphorbiaceae) is a species of flowering plant. It belongs to a monotypic genus, *Ricinus*, and subtribe, Ricininae. The common name "castor oil" comes from its use as a replacement for castoreum, a perfume base made from the dried perineal glands of the beaver (*castor* in Latin). It has other common names as palm of Christ and *Palma Christi* [3].

R. communis is widespread in the southeastern Mediterranean Basin, Eastern Africa, India, and tropical regions. Castor beans seeds (which despite its name) are the source of oil, and wide uses. The seeds are containing (40 – 60) % oil and rich with triglycerides, as ricinolein. Also they are containing a toxin matter as a ricin, that present in lower concentrations in the plant. The stems are varying in pigmentation. The fruit is a spiny, greenish capsule containing large, oval, shiny, bean-like, highly poisonous seeds with variable brownish mottling.

The flowers are borne in terminal panicle-
like inflorescences of green or, red monoecious flowers without petals [4, 5].

R. communis oil has many uses in medicine and other applications. Alcoholic extract of the leaf was hepatoprotective in rats. Methanolic extracts of the leaves of *R. communis* were nontoxic when used in antimicrobial testing of rats [6].

The mode of action of *R. communis* on central nervous system in mice at low and high doses was determined. A water extract of the root bark showed analgesic activity in rats. Antihistamine and anti-inflammatory properties were found in ethanolic extract of *Ricinus communis* root bark. *Extracts of R. communis* exhibited a caricidal and insecticidal activities against *Haemaphysalis bispinosa* Neumann (Acarina: Ixodidae) and hematophagous fly *Hippoboscam aculate* leach
(Diptera: Hippoboscidae)[7].

Molluscicides of plant origin have gained greater importance since it is believed that natural products are ecologically sound and culturally than synthetic ones. A large number of plant families which possess natural molluscicidal activity have been identified. The plant phytochemicals derived from plant resources can be used as an alternative to the synthetic molluscicides . *R. communis* is the host plant of the common castor butterfly (*Ariadne merione*), the Eri silkmoth (*Samia cynthia ricini*), and the castor semi-looper Moth (*Achaea janata*). It is also used as a food plant by the larvae of some other species of Lepidoptera, including *Hypercompe hambletoni* and the nutmeg (*Discestra trifolii*) [8].

The toxicity of castor beans is due to the ricin . If ingested, symptoms may be delayed by up to 36 hours but commonly begin within 2–4 hours. These include a burning sensation in mouth and throat, abdominal pain, purging and bloody diarrhea. Within several days there is severe dehydration, a drop in blood pressure and a decrease in urine. Unless treated, death can be expected to occur within 3–5 days; however in most cases a full recovery can be made[9].

Poisoning occurs when animals or humans, ingest broken seeds, intact seeds may pass through the digestive tract without releasing the toxin. Toxicity varies among animal species: four seeds will kill a rabbit, five a sheep, and six an ox or horse, seven a pig, and eleven a dog. Ducks have shown incredible resistance to the seeds: it takes an average of 80 to kill them. The toxin provides the castor oil plant with some degree of natural protection from insect pests such as aphids.

Three terpenoids and a tocopherol-related compound have been found in the aerial parts of *Ricinus communis* [10].

The aim of the present study was to evaluate molluscicidal activity of *Ricinus communis* against *B. truncatus* to explore the full potential use of these extracts as molluscicides in future.

Methods and materials

Collection of snails

Bulinus truncatus samples snails were collected from June to August weekly. Samples were collected from Al-Rasheed district (30km)south of Baghdad. Study area is including station near street number 37 (connect between Al-Rasheed districts and Tigris river) abscissae of site (33 8 32.83 North, 44 25 37.20 East). The snails were collected from small canal beside main canal called Muhyii River. Zooplankton net was used to collect the snails and plastic bags in the

channel were collect to obtain the snails attached on their surfaces weekly. Snails were placed in 5 L plastic containers with amount of water from the river. Snails were identified according to [11].

The snails were fed with the extracts of leaves of alfa alfa plant 10ml per 50L daily. The collected snails isolate , identified according to standard keys of snails then they are acclimatized to laboratory conditions (T 25± 3) before testing for two days. Snails were cultivated in laboratory with get method of McClelland as described by [12].

Preparation of Extracts

The aquatic extract of the leaves and bark of *R. comminus* were prepared, concentrated and dried. The leaves and bark were dried in the shade and shredded in a hand mill (Estrella®, model 41B) and an electric mill (Moulinex®) and sifted through a number 30 mesh to obtain a fine powder and left in a cool dry place. A weighted amount of the extract was made up to desired concentrations in water for analysis. Leaf and bark powder of *R. comminus* (50, 100 and 200g.) were macerated in 1 L of distilled water for 24 h and placed in glass flasks. The macerate was filtered through cotton gauzes in a plastic funnel to get crude extracts.

Bioassays and evaluation of molluscicidal activity

Bioassays were conducted in the laboratory by determined the survival rates. The W. H. O. method (II) for testing for molluscicides was followed, exposure for 96 hours and recovery periods for 24 hours were made in all the tests. Survival rates were counted and Lc50s were determined. To monitor the susceptibility of snails and to compare its potency with the extracts while the lethal concentrations and their 95% confidence limits were determined by probit analysis[13].

Treatments

Three doses (50, 100 and 200g /L) of leaves and bark were used to produced stock solutions . From these solutions, serial dilutions were prepared :

For leaves at 1, 2, 3, 4 and 5 %.

For bark 2, 4, 6, 8 and 10 %.

Nine individuals of snail were test in each concentration with three replicates and the average of these experiments was calculated and compered with control.

Statistical analysis

The effect of aquatic extracts was applied by different methods and evaluated by LC50. This

parameter was determined for each exposure period (24, 48, 72 and 96 hrs.) in all concentrations. LC50 was calculated mathematically according to the correlation equations (equation 1) . The concentration which give a result of 13.5 of survival axes at equations was considered as LC50 at equation 2 (median lethal concentration= median survival concentration) [14].

Y=ax+ b……..……..(1)

Where

Y : y axes (survival rates)

a: intercept

X : x axes (concentrations)

b : the slob of average line

LC50=13.5-b/x ……(2)

LC50: median lethal concentration(median survival concentration)

13.5 : median of total number of tested snails (27 individuals)

Regression analysis of relationships between concentrations and survival rates in different periods of exposure were recorded and plotted by Excel [15].

Results and Discussion

1. The snail *B. truncatus* exposed to *R. communis leaves* extracts

1.a. Stock solution (50 g/L)

Survival rates and LC50 values

High percent of snail survival rates were noticed as follow: 99.3% (after 24 and 48 hrs. of exposure), 98.7 % (after 72hrs. of exposure) and 98.1 % (after 96hrs. of exposure) (Table 1).

Table(1) Numbers of *B. truncatus* survival exposed to *R. communis* leafs extracts (50, 100 and 200g/l) for 96hr.

Stock solution of leaves 50, 100 & 200g/L		Numbers of survival No. of snails in each con.= 27			
DW	**Stock sol. /ml**	**24hr exposure**	**48hr exposure**	**72hr exposure**	**96hr exposure**
(100)Con.	0	27	27	27	27
99	1	27	27	27	27
98	2	27	27	27	27
97	3	27	27	27	27
96	4	27	27	27	27
95	5	26	26	25	24
50g/L	Total	161	161	160	159
	%	99.3	99.3	98.7	98.1
100g/L	%	98.7	83.3	83.3	83.3
200g/Ll	%	87	81.4	81.4	81.4
Increasing of doses caused decreasing of percent of survival rates					

Table (2) Acute toxicity of *R. communis* extracts to snail *B. truncatus* according to correlation equations.

Time of exposure	***R. communis extracts***					
	Leaves - LC50			**Bark - LC50**		
	50g/L	100g/L	200g/L	50g/L	100g/L	200g/L
24 hr.	95	48	8	-	-	191
48 hr.	95	11	6.9	191	120	74
72 hr.	48	11	6.9	191	75	54
96 hr.	32	11	6.4	191	54	43

Table(3) Correlation equations of Dose-Effect of *R. cummunis* leaves extracts to snail of *B. truncatus* for 96hr. of exposure.

Doses ***R. cummunis*** **leaves extracts**	**Correlation equations**		**R^2**
50g /L	24hr.	Y=-0.1429x+27.19	0.4286
	48hr.	Y=-0.1429x+27.19	0.4286
	72hr.	Y=-0.285x+27.381	0.4286
	96hr.	Y=-0.4286x+27.571	0.4286
100g /L	24hr.	Y=-0.2857x+27.381	0.4286
	48hr.	Y=-1.314x+28.619	0.5889
	72hr.	Y=-1.314x+28.619	0.5889
	96hr.	Y=-1.314x+28.619	0.5889
200g /L	24hr.	Y=-1.8x+28	0.9529
	48hr.	Y=-2.0286x+27.571	0.9538
	72hr.	Y=-2.0286x+27.571	0.9538
	96hr.	Y=-2.0571x+26.81	0.9105
Doses ***R. cummunis*** **bark extracts**	**Correlation equations**		**R^2**
50g/L	24hr.	----------	---------
	48hr.	Y=-0.0714x+27.19	0.4289
	72hr.	Y=-0.0714x+27.19	0.4289
	96hr.	Y=-0.0714x+27.19	0.4289
100g/L	24hr.	----------	--------
	48hr.	Y=-0.1143x+27.238	0.6857
	72hr.	Y=-0.1857x+27.429	0.6898
	96hr.	Y=-0.2571x+27.619	0.6312
200g/L	24hr.	Y=-0.0714x+27.19	0.4286
	48hr.	Y=-0.1875x+27.429	0.6898
	72hr.	Y=-0.2571x+27.619	0.6312
	96hr.	Y=-0.3286x+27.81	0.5889

Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be in 32% concentration after 96 hr. of exposure. At the first three days of exposure (24= 48 and 72 hr.) LC50s were calculated to be in the concentrations (95 and 48%) respectively (Table 2).

Dose-response correlations

Similar dose-response relationship between the tested extracts and tested

snails was appearance at 24hr. and 48hr. of exposure with correlation factor 0.4286 . Also clear relationship was appeared at 72 and 96 hr. of exposure with same correlation factor 0.4286 (Table 3) (Figures 1,2 and 3).

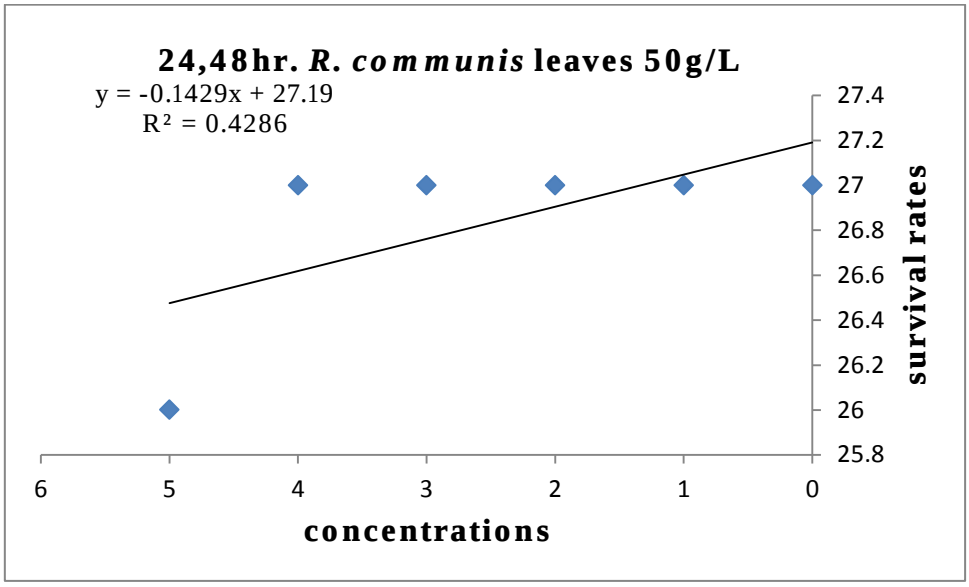

Figure 1: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 24 and 48 hr. of exposure.

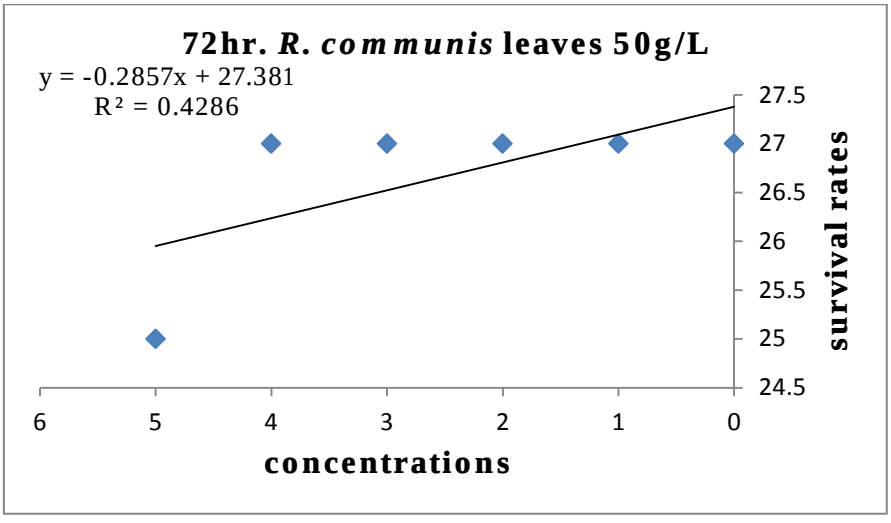

Figure 2: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 72 of exposure.

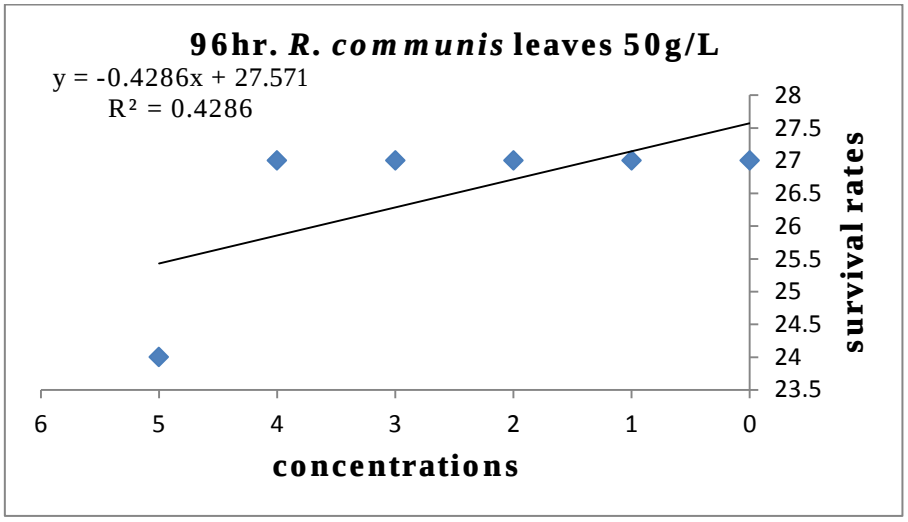

Figure 3: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 96 hr. of exposure.

1.b. Stock solution (100 g/L)

Survival rates and LC50 values

High percent of snail survival rates were noticed as 98.7% (after 24 hrs. of exposure) and 81.4 % (after 48, 72 and 96hrs. of exposure) (Table 2). Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be in 48% concentration after 24 hr. of exposure. At 48, 72 and 96 hrs. of exposure, LC50s were calculated to be in the concentration of 11% (Table 3).

Dose-response correlations

Clear dose-response relationship between the tested extracts and tested snails was appearance at 24hrs. of exposure with correlation factor 0.4286 . Also Similar relationship was appeared at 48, 72 and 96 hr. of exposure with same correlation factor 0.5889 (Table 3)(Figures 4 and 5). **1.c. Stock solution (200g/L)**

Survival rates and LC50 values

High percent of snail survival rates were noticed as 98.7% (after 24 hrs. of exposure) and 81.4 % (after 48, 72 and 96hrs. of exposure) (Table 1). Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be really in 48% concentration after 24 hr. of exposure. At 48, 72 and 96 hrs. of

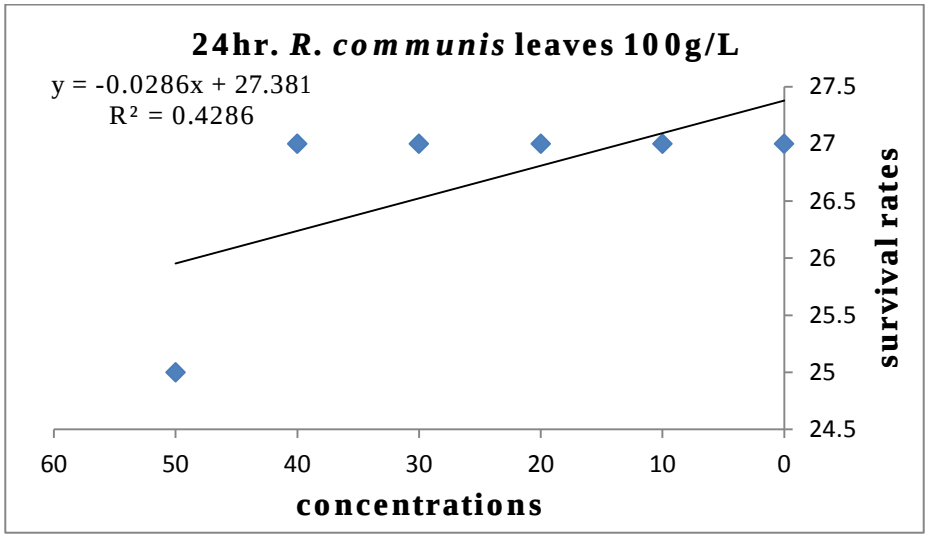

Figure 4: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 24 hr. of exposure.

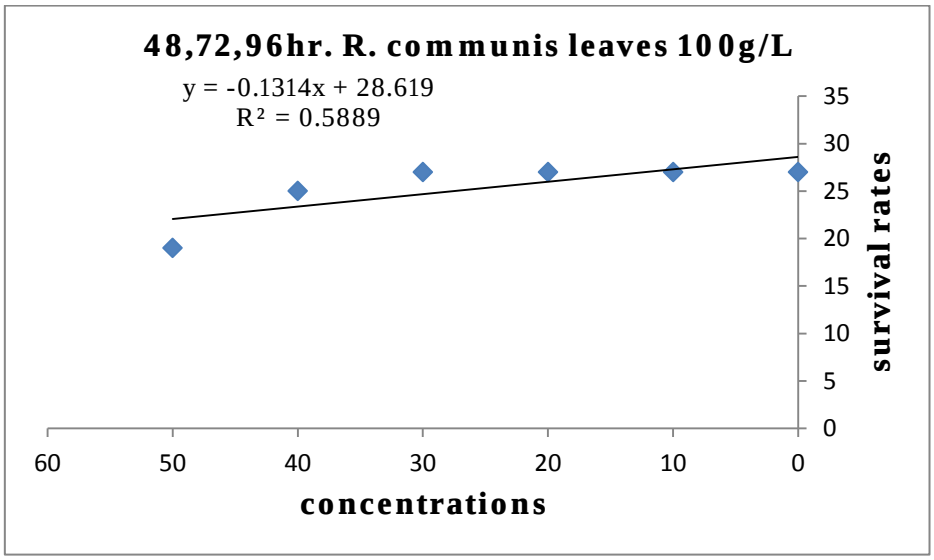

Figure 5: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 48, 72 and 96 hr. of exposure.

exposure, LC50s were calculated to be really in the concentration of 11% (Table 2).

It's clear that there was a significant decrease in the survival rates of snails exposed to tested extracts compared to the control group. Also we were noticed decreasing of survival rates follow up to increasing of exposure time (Table 4).

Table (4) Survival rates of *B. truncatus* exposed to *R. communis* leafs extracts with time.

Period of exposure	Control (zero time)	24hr	48hr	72hr	96hr
%Survival rates	100	99.3	99.3	98.7	98.1
Increasing of exposure time were caused of decreasing of survival rates.					

It's clear that there was a significant decrease in the survival rates of snails exposed to tested extracts follow up to increasing of doses of extracts (Table 5).

Table (5) Survival rates of *B. truncatus* exposed to *R. communis* leafs extracts with doses.

Doses of exposure	0g/L	50g/L	100g/L	200g/L
%Survival rates	100	98.1	83.3	81.4
Increasing of doses were caused of decreasing of survival rates.				
Increasing of doses were more effective to decreasing of survival rates than increasing of exposure time.				

This finding agrees with finding which showed marked reduction in the survival rate of snails treated with concentrations of different plant extracts compared to control [16, 17].

Dose-response correlations

Clear dose-response relationship between the tested extracts and tested snails was appearance at 24hrs. of exposure with correlation factor 0.9529 . Also, similar relationship was appeared at 48 and 72hrs. of exposure with same correlation factor 0.9538. The result was showed dose-response relationship between the tested extracts and tested snails at 96hrs. of exposure with correlation factor 0.9105 (Table 3) (Figures 6,7 and 8).

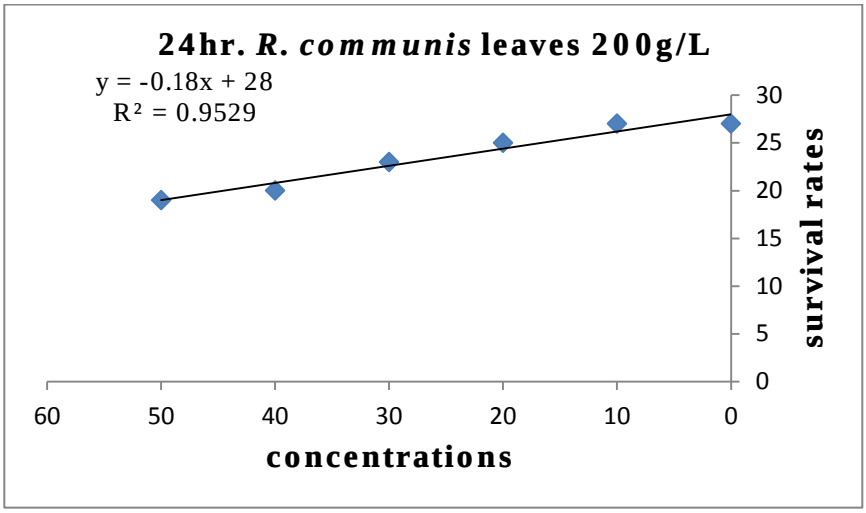

Figure 6: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 24 and 48 hr. of exposure.

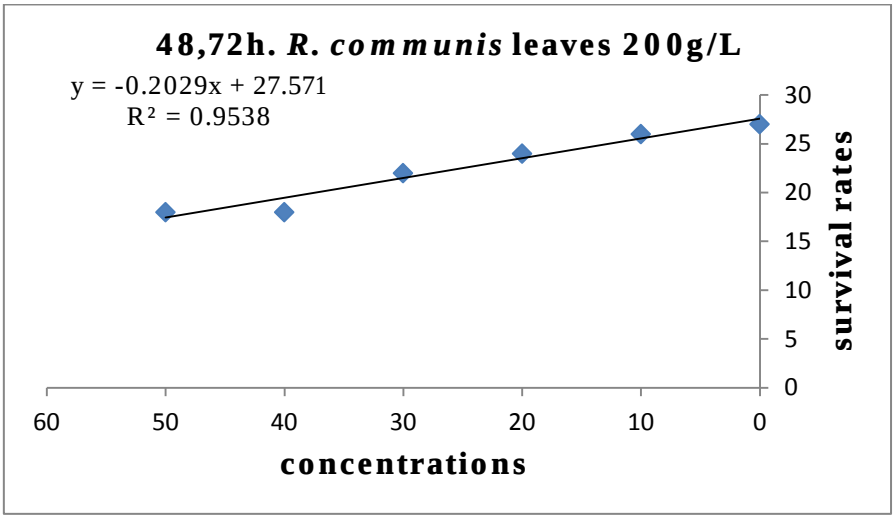

Figure 7: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 48 and 72 hr. of exposure.

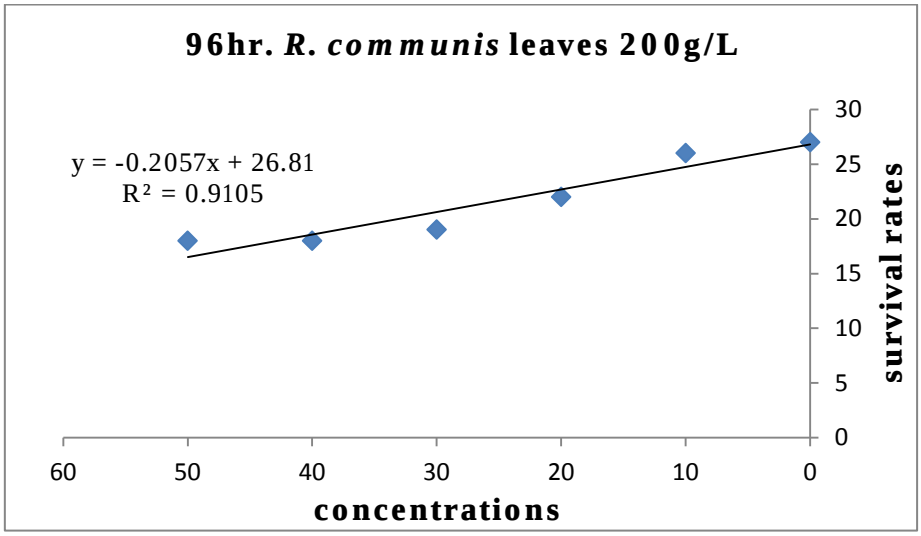

Figure 8: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* leaves extracts for 96hr. of exposure.

2. *B. truncatus* exposed to *R. communis* bark

2.a. Stock solution (50 g/L)
Survival rates and LC50 values

High percent of snail survival rates were noticed as 87% (after 24 hrs. of exposure) and similar survival rates as 83.3 % (after 48, 72 and 96hrs. of exposure) (Table 6).

Table(6) Numbers of *B. truncatus* survival exposed to *R. Communis* bark extracts (50, 100 and 200g/l) for 96hr.

Stock solution of bark 50g/L		Numbers of survival No. of snails in each con.= 27			
DW	**Stock sol. /ml**	**24hr exposure**	**48hr exposure**	**72hr exposure**	**96hr exposure**
(100)Con.	0	27	27	27	27
98	2	27	27	27	27
96	4	27	27	27	27
94	6	27	27	27	27
92	8	27	27	27	27
90	10	27	26	26	26
5g/L	Total	162	161	161	161
	%	100	99.3	99.3	99.3
100g/L	%	100	98.7	98.1	97.5
200g/L	%	99.3	98.1	97.5	96.9

Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be really in 191% concentration after 48, 72 and 96 hrs. of exposure (Table 2).

Dose-response correlations

No dose-response relationship between the tested extracts and tested snails was appearance at 24hrs. of exposure . But similar relationship was appeared at 48, 72 and 96hrs. of exposure with same correlation factor 0.4286 (Table 3) (Figure 9).

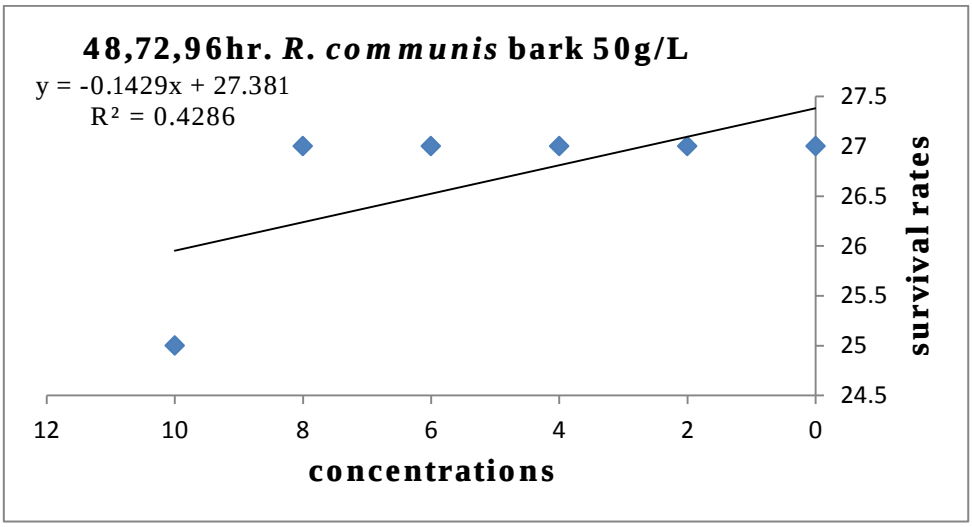

Figure 9: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 72 and 96hr. of exposure.

2.b. Stock solution (100 g/L) Survival rates and LC50 values

High percent of snail survival rates were noticed as 100% (after 24 hrs. of exposure) and similar survival rates as 99.3 % (after 48, 72 and 96hrs. of exposure) (Table 6). Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be really in 120, 75 and 54% concentration after 48, 72 and 96 hrs. of exposure (Table 2).

Dose-response correlations

No dose-response relationship between the tested extracts and tested snails was appearance at 24hrs. of exposure. But there were relationship at 48, 72 and 96hrs. of exposure with correlation factors 0.6857, 0.6898, and 0. 6312 respectively (Table 3) (Figures 10, 11 and 12).

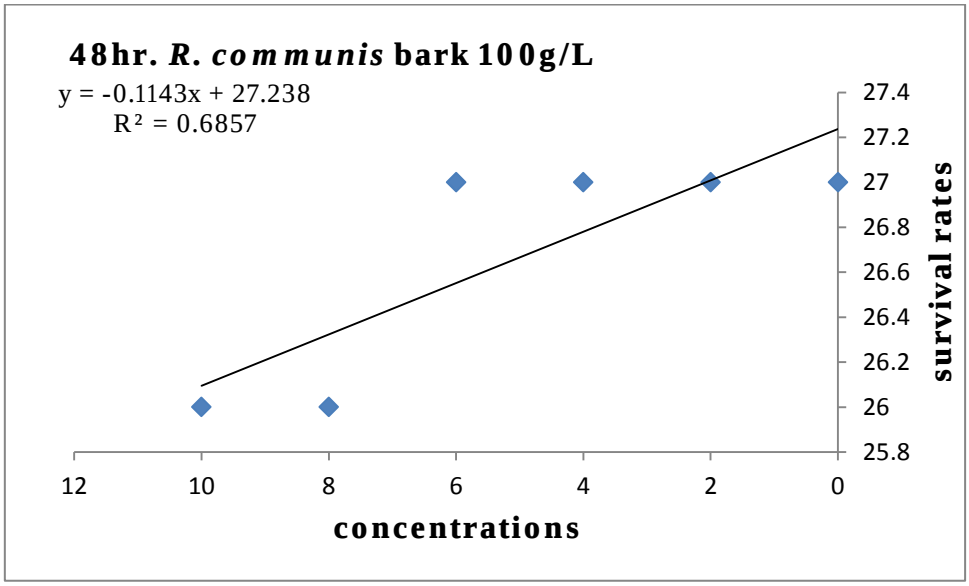

Figure 10: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 48hr. of exposure.

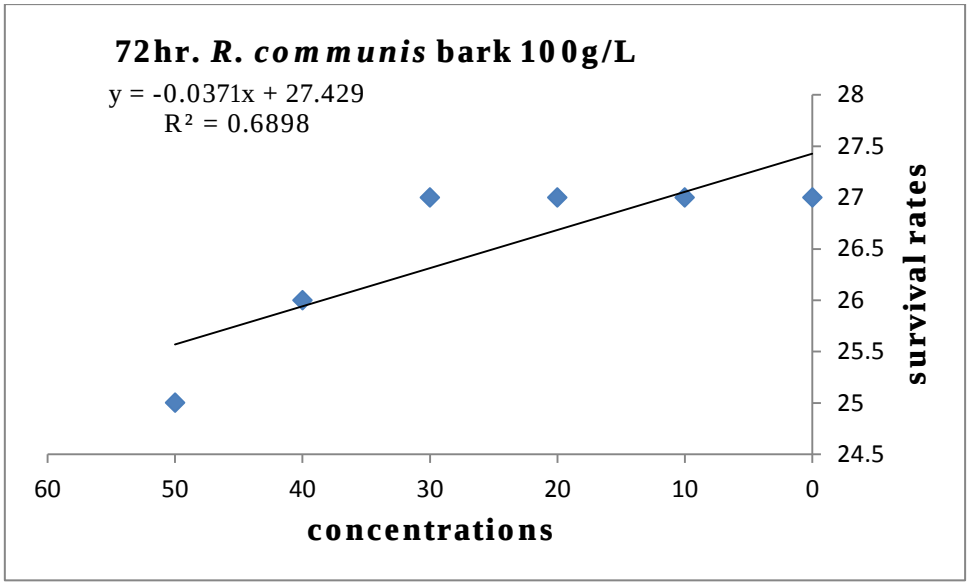

Figure 11: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 72hr. of exposure.

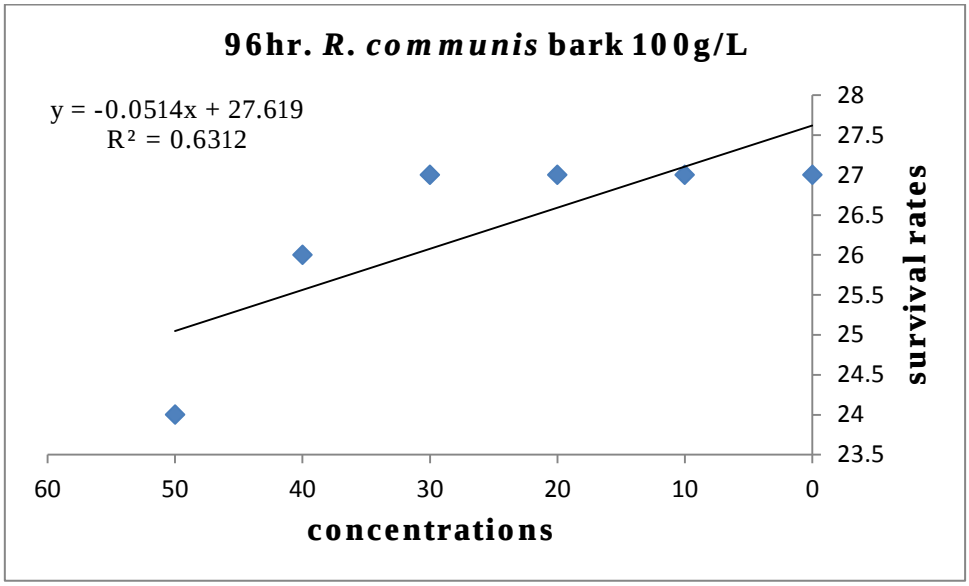

Figure 12: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 96hr. of exposure.

2.c. Stock solution (200 g/L)

Survival rates and LC50 values

High percent of snail survival rates were noticed as 99.3% (after 24 hrs. of exposure), 98.1 % (after 48hrs. of exposure), 97.5 %(after 72hrs. of exposure) and 96.9 % (after 96hrs. of exposure) (Table 6). Median lethal concentration of mortality of *B. truncatus* exposed to the aquatic extracts was calculated to be really in 191, 74, 54 and 43% concentration after 24, 48, 72 and 96 hrs. of exposure respectively(Table 2).

Dose-response correlations

The study was found that there were dose-response relationship between the tested extracts and tested snails at 24, 48, 72 and 96hrs. of exposure with correlation factors 0.4286, 0.6898, 0.6312 and 0.5889 respectively (Table 3)(Figures 13, 14, 15 and 16).

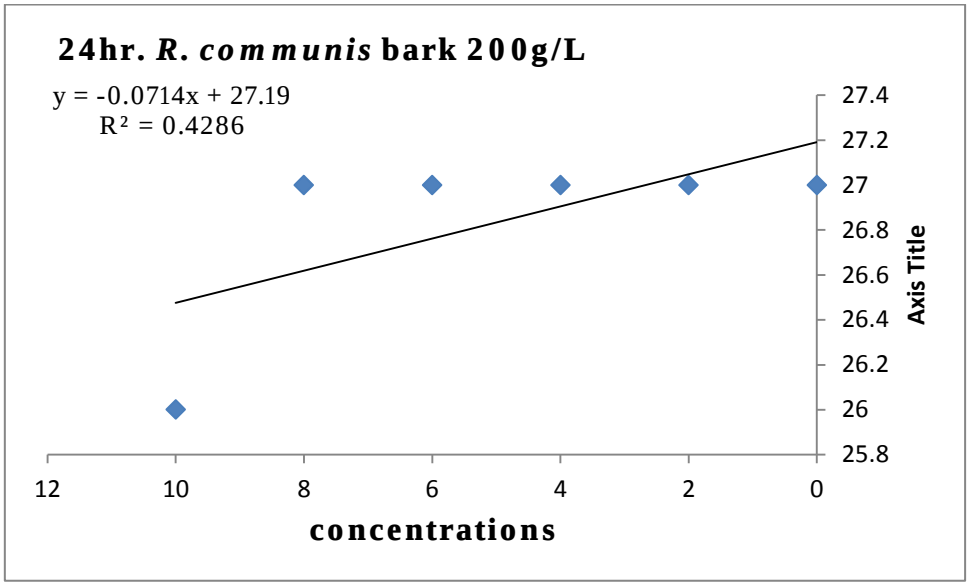

Figure 13: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 24hr. of exposure.

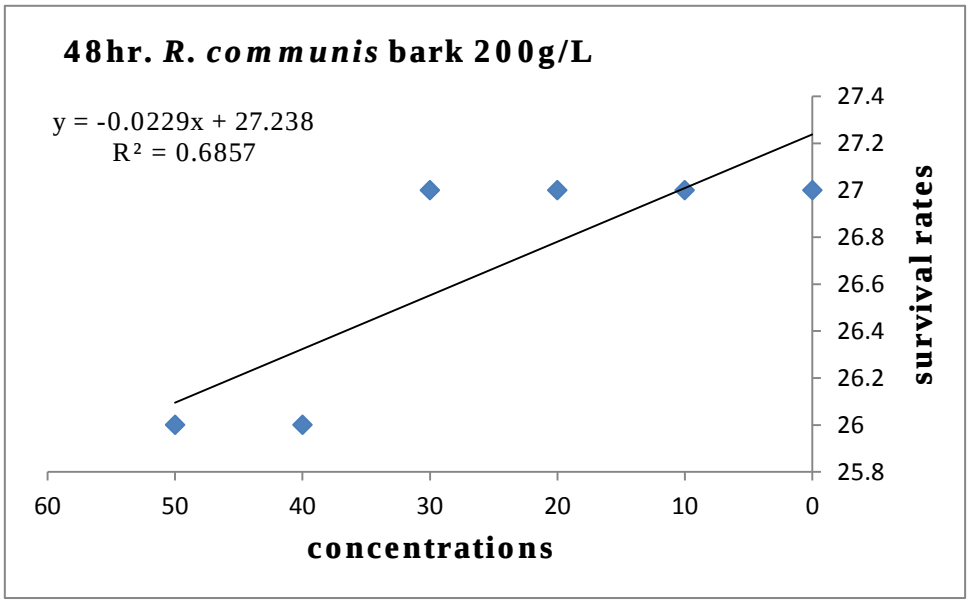

Figure 14: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 48hr. of exposure.

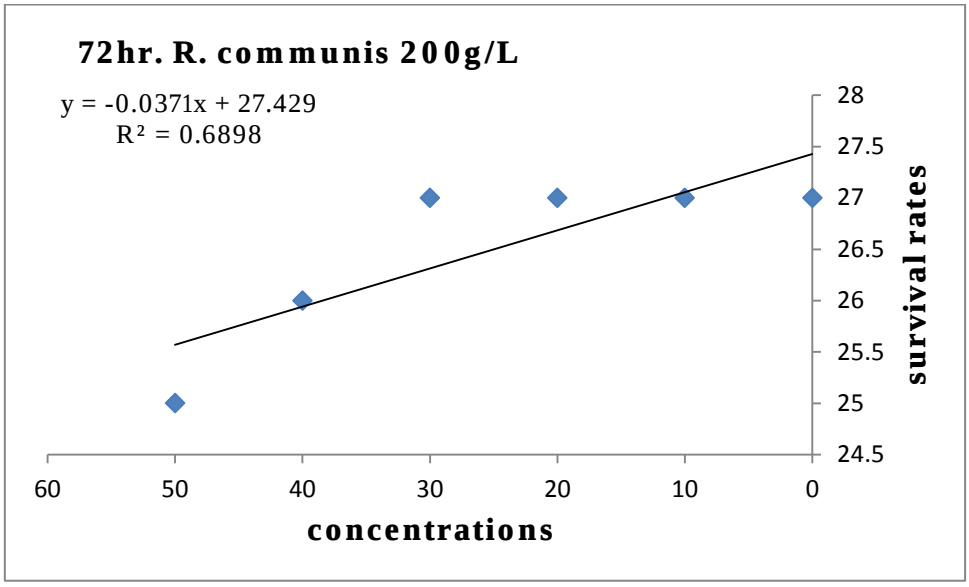

Figure 15: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 72hr. of exposure.

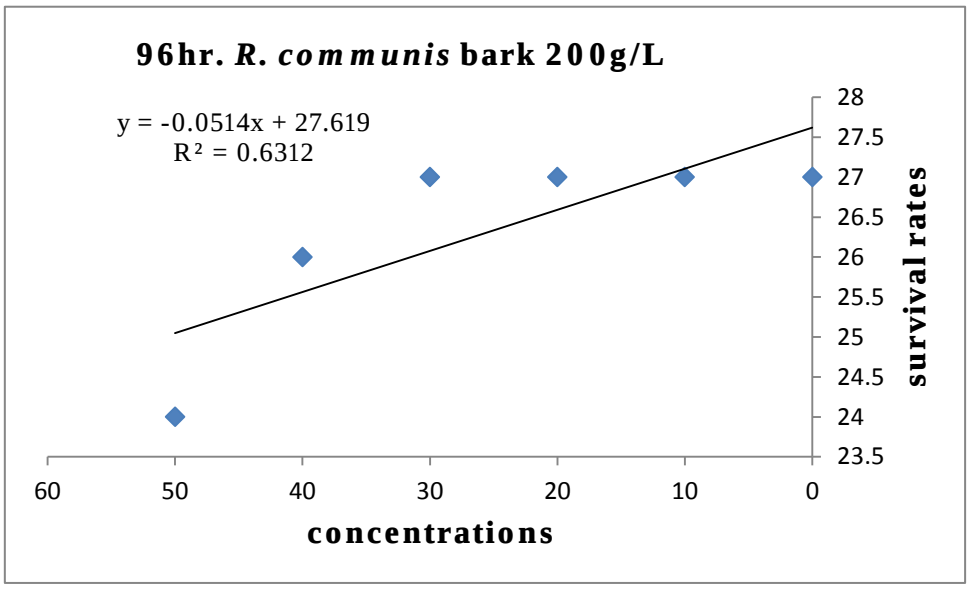

Figure 16: Relationship between survival rates of *B. truncatus* snails and concentrations of *R. communis* bark extracts for 96hr. of exposure.

It's clear that there was a significant decrease in the survival rates of snails exposed to tested extracts compared to the control group. Also we were noticed decreasing of survival rates follow up to increasing of exposure time (Table 7).

Table(7) Survival rates of *B. truncatus* exposed to *R. communis* bark extracts with time.

Period of exposure	24hr	48hr	72hr	96hr
%Survival rates	100	99.3	99.3	99.3
Increasing of exposure time was caused of decreasing of survival rates				

It's clear that there was a significant decrease in the survival rates of snails exposed to tested extracts follow up to increasing of doses of extracts (Table 8).

Table(8) Survival rates of *B. truncatus* exposed to *R. communis* bark extracts with doses.

Doses of exposure	0g/l	50g/l	100g/l	200g/l
%Survival rates	100	99.3	97.5	96.9
Increasing of doses were caused of decreasing of survival rates.				
Increasing of doses were more effective to decreasing of survival rates than increasing of exposure time.				

It was found that these extracts were cause effect and death to snail of *B. truncatus* and dose and time dependent. These results were agreed with applied study of water extract of *T. tetraptera* which used a concentration of 15, 20 and 25mg/L in Nigeria. Also these results were agreed with a histopathological study of *T. tetraptera* extract on *Bulinus* (Phyopsis) *globosus, Biomphalaria glabrata* and *Physa waterlotti* . The effect of the extract on various snail tissues was found to be time and concentration dependent [16].

The mechanism of these extracts activity was demonstrated by produced significant reductions on the glycogen and protein content and molluscicidal action on the carbohydrate metabolism of the snail [15].

The mechanism of activity of extracts on the snails was included registration in many organs as kidney, hepatopancreas and gastro-intestinal tract.Further effects of *T. tetraptera* extracts to *B. glabrata* and *Lymnaea columella* snail as growth and egg production were recorded in some studies[14].

The molluscicidal effect of nicotinanilide was evaluated and compared with niclosamide against different stages of the fresh water snail *Lymnaea luteola* eggs, immature, young mature, and adults and the calculated values of lethal concentration (LC50 and LC90)[15].

The mechanism by which these leaf extracts killed snail is not exactly known and will require further studies for elucidation.

Conclusion

Plants leaves and bark are contains some bioactive substances for use as molluscicides . These substances can use locally with simple technology. If these substances are proved as a sufficiently toxic and ecological sound, it should be possible to develop culturally acceptable and inexpensive molluscicides. Synthetic molluscicides are expensive and in addition, may lead to problems of toxicity to non-target organisms and deleterious long-term effects in the environment. The possible development of resistance in Schistosomiasis transmitting snail is another important factor. The use of plants with molluscicidal properties is a simple, inexpensive and appropriate technology for control of the snail intermediate host. We conclude that *Ricinus communis* extracts may be used as a potent molluscicide.

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Molecular detection of class1 Integron and pattern of antibiotic resistance in *Pseudomonas aeruginosa* isolated from burn patients in Karbala province\ Iraq

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Abstract: *Pseudomonas aeruginosa* is a problematic opportunistic pathogen in immunocompromised patients like burn patients. It is naturally resistant and able to acquire resistance by transposable genetic elements like integrons. This study investigated the presence and spread of class1 integrons amongst 60 clinical isolates of *P. aeruginosa* obtained from burn patients and evaluated their antibiotic susceptibility for the determination of multidrug-resistance (MDR). Cutaneous pus samples were collected from 158 hospitalized burn patients during the period from December 2012 to June 2013 in Karbala province\ Iraq. Bacterial isolates were identified by using conventional biochemical tests and then identification was confirmed by using VITEK-2 compact system. All isolates were analyzed for antibiotic susceptibility by the disk diffusion method using the antipseudomonal agents: Imipenem, Meropenem, Piperacillin, Ceftazidim, Aztreonam, Ciprofloxacin and Gentamicin. The detection of class1 integron was performed by the PCR method. Resistance results were as the following: Imipenem 58.33%, Meropenem 66.67%, Piperacillin 86.67%, Ceftazidim 51.67%, Aztreonam 43.33%, Ciprofloxacin 46.67% and Gentamicin 91.67%. MDR was found in 55 (91.67%) out of 60 *P. aeruginosa* isolates. Class1 integron was found in 59 (98.33%) of the isolates tested. The extensive dissemination of class1 integron gene in *P. aeruginosa* isolated from burn unit is considered dangerous and the increasing resistance to carbapenems in addition to widespread MDR among the isolates reveals the association of this type of resistance with the high dissemination of class1 integron detected in this pathogen predicting epidemiology.

Keywords: *P.aeruginosa*, class1 integron, carbapenem, MDR, burn.

الكشف الجزيئي للإنتكرون- صنف1 ونمط المقاومة للمضادات الحيوية في بكتريا *Pseudomonas aeruginosa* المعزولة من مرضى الحروق في محافظة كربلاء/ العراق

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الخلاصة: تعد الزوائف الزنجارية *P. aeruginosa* مسببات مرضية انتهازية خطرة للمرضى هابطي المناعة كمرضى الحروق. فهي مقاومة طبيعياً وبإمكانها اكتساب المقاومة بواسطة عناصر جينية متنقلة مثل الإنتكرون. تم التحري في هذه الدراسة عن وجود وانتشار الانتكرون- صنف1 في 60 عزلة سريرية لبكتريا *P. aeruginos* المستحصلة من مرضى الحروق وقيمت حساسيتها للمضادات الحيوية لغرض تحديد المقاومة للادوية المتعددة (MDR).. جمعت نماذج تقيح جلدي من 158 شخص من مرضى الحروق الراقدين في المستشفيات خلال الفترة بين كانون الأول 2012 ولغاية حزيران 2013 في محافظة كربلاء/ العراق. شخصت العزلات البكتيرية باستخدام الاختبارات الكيميوحيوية التقليدية ثم تم تأكيد التشخيص من خلال استخدام جهاز VITEK-2 compact. أجري اختبار الحساسية للمضادات الحيوية لجميع العزلات بواسطة طريقة انتشار الأقراص باستخدام المضادات الآتية: إميبينيم، ميروبنيم ، ببراسلين، سيفتازيديم، أزترونام، سبروفلوكسين و جنتمايسين وكشف عن الإنتكرون- صنف1 بواسطة طريقة تفاعل إنزيم البلمرة المتسلسل (PCR). نتائج المقاومة كانت كالآتي: 58.33% للإميبينيم ، ,66.67% ميروبينيم ، 86.67% ببراسلين ، 51.67% سيفتازيديم ، 43.33% أزترونام، 46.67% سبروفلوكسين و 91.67% للجنتمايسين. ظهر ت المقاومة للأدوية المتعددة في (91.67%) 55 من أصل 60 عزلة *P. aeruginosa* و وجد الإنتكرون- صنف1 في (98.33%) 59 من العينات المدروسة. إن الانتشار الواسع لجين الإنتكرون- صنف1 في بكتريا *P. aeruginosa* المعزولة من وحدة الحروق يعتبر خطراً وإن المقاومة المتزايدة للكاربينيم، بالإضافة لوجود المقاومة للأدوية المتعددة الواسع الانتشار بين العزلات يظهر ارتباط هذا النوع من المقاومة مع الانتشار الواسع للإنتكرون- صنف1 في هذا المسبب المرضي بما ينبئ بالوبائية.

Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen for humans, animals, and plants [1]. It is an aerobic Gram-negative rod, possessing a strictly respiratory metabolism. The organisms are usually (1.5-5) µm in length and (0.5-1.0) µm in width, and are motile due to the presence of flagella [2]. Existence of this bacterium is in soil and aquatic environments [3]. It is an important pathogen in immunocompromised patients, such as patients suffering from AIDS, cancer, burn wounds and cystic fibrosis (CF) [4]. It requires minimal nutrition and can tolerate a wide range of temperatures. Therefore, infections caused by it are often difficult to eradicate. Also, it is resistant to many antibiotics, disinfectants and has the ability to acquire resistance [5] besides that it exhibits intrinsic resistance to several antimicrobial agents [6]. Burn injury is a major problem in many areas of the world and it has been estimated that 75% of all deaths following burns are related to infection [7]. *P. aeruginosa* developes antimicrobial

resistance rapidly, which complicates medical treatment of infections. It is frequently isolated from patients and hospital environments and has been implicated as the cause of nosocomial infections in burn patients [8]. Carbapenem compounds such as (Imipenem and Meropenem) are highly potent broad-spectrum antimicrobial agents. They play an important role in the treatment of infections caused by *P. aeruginosa* [9]. Many of the bacterial pathogens associated with epidemics of human disease have evolved into multidrug-resistant (MDR) forms (resistance to three or more first line classes (beta-lactams, aminoglycoside, and fluroquinolone)) subsequent to antibiotic use [10, 11]. Most treatment failures are related to inappropriate initial antibiotic therapy with insufficient coverage of MDR pathogens, the rationale for using combinations of antibiotics to cover MDR gram negative bacteria [12]. Carbapenems still as the main antimicrobials for treating infections due to MDR *P. aeruginosa*, but the development of carbapenem resistance may significantly compromise their efficacy [13]. These antibiotics, therefore, remain as the last therapeutic option for treatment of serious infections caused by *P. aeruginosa*. As a result, the recent appearance of carbapenem resistant *P. aeruginosa* isolates is considered a major healthcare problem [5]. Resistance mechanisms in these bacteria are explainable by both the mutation of genes and the change of action mechanisms in chromosomal and transferable gene elements like transposons or integrons [14, 15]. Integrons are transportable genetic particles which can carry the antibiotic resistance genes [16, 15]. They are located in many different parts of plasmids and chromosomes [17, 15]. They are potentially major agents in the dissemination of MDR among gram-negative bacteria, especially in Pseudomonas [18]. The presence of class 1 integrons is a matter of great concern, as this is one of the most efficient genetic elements responsible for the spread of antibiotic resistance in the hospital environment [19, 20]. This study aimed to investigate the presence and spread of class1 integrons in *P. aeruginosa* isolated from burn patients and to evaluate antibiotic susceptibility of those bacterial isolates for the determination of multidrug-resistance (MDR).

Materials and methods

Bacterial Isolates

Sixty *P. aeruginosa* isolates were recovered from 158 cutaneous pus samples collected by using sterile cotton swabs from burn patients between December 2012 and June 2013 in Karbala province\ Iraq. The

bacterial identification as *P. aeruginosa* was achieved by the standard microbiological tests such as Gram stain, oxidase test, catalase test, growth on MacConkey agar, growth on cetrimide agar, blood haemolysis, motility, liquefaction of gelatin, growth at 42°C and at 4°C, pigment production, Kligler's Iron test and IMViC tests (indole, methyl red, Voges-Proskauer and citrate) [21]. Then identification was confirmed by using VITEK-2 compact system according to the manufacturer company, bioMérieux (France). The isolates were maintained in nutrient broth medium containing 40% glycerol at -20ºC [22, 23].

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test was performed by the disk diffusion test (DDT) according to Kirby Bauer's method depending on the protocol of the Clinical and Laboratory Standards Institute (CLSI) 2012 [24]. The following antimicrobial discs were used: Imipenem (10 µg), Meropenem (10 µg), Piperacillin (100 µg) (Bioanalyse, Turkey), Ceftazidime (30 µg), Aztreonam (30 µg), Ciprofloxacin (5 µg) and Gentamicin (10 µg) (Himedia, India).

DNA Extraction

Bacterial genomic DNA was extracted from all isolates by the employment of ExiPrep™ plus Bacteria Genomic DNA Kit (Bioneer, Korea) and by using the ExiPrep™ 16 plus instrument (Bioneer, Korea). After bringing out the DNA, all samples were kept in -20 °C until the PCR stage.

Class 1 Integron PCR

Class 1 Integron was determined for all the isolates by using the targeting gene, *int*I1; its primers sequences were, F5'-TCT CGG GTA ACA TCA AGG-3' and R5'-ACATGCGTGTAAATCATCGTC' [25]. Amplification of DNA was carried out by the PCR reaction which was performed in a final volume of 25µl which contained 2 µl extractions of DNA, 1 µl (10 pmol) from forward primer and 1 µl (10 pmol) from reverse primer, 12.5 µl from Go Taq® Green Master Mix (Promega, USA) and 8.5 µl of nuclease free water provided by Go Taq® Green Master Mix. The reaction was performed in a Veriti thermal cycler (USA) after achievement of the optimization for the PCR conditions. The amplification reactions for *int*I1 gene included one cycle of initial denaturation at 94 ºC for twelve minutes followed by 35 cycles of denaturation at 94 ºC for one minute, annealing at 57 ºC for one minute and extension at 72 ºC for 2

minutes. Moreover, one cycle for the final extension at 72 ºC for ten minutes was performed [25]. PCR reaction was conducted in the presence of negative control (containing nuclease free water instead of DNA template). After performing PCR reaction, electrophoresis of PCR products was conducted in 1.5% agarose gel for 90 minutes at 70 volt [26]. Then, the results were evaluated under UV light by using UV transilluminator for the observation of DNA bands at 302 nm. After PCR, the specific band with the size of 500 base pairs (bp) was considered as a fragment of intIl gene.

Statistical analysis

The Statistical Analysis System (SAS) [27] was used to analyze the antibiotic susceptibility results. Chi-square test was used to significant compare between percentages in this study. *P- values* equal to or less than 0.01 were considered statistically highly significant.

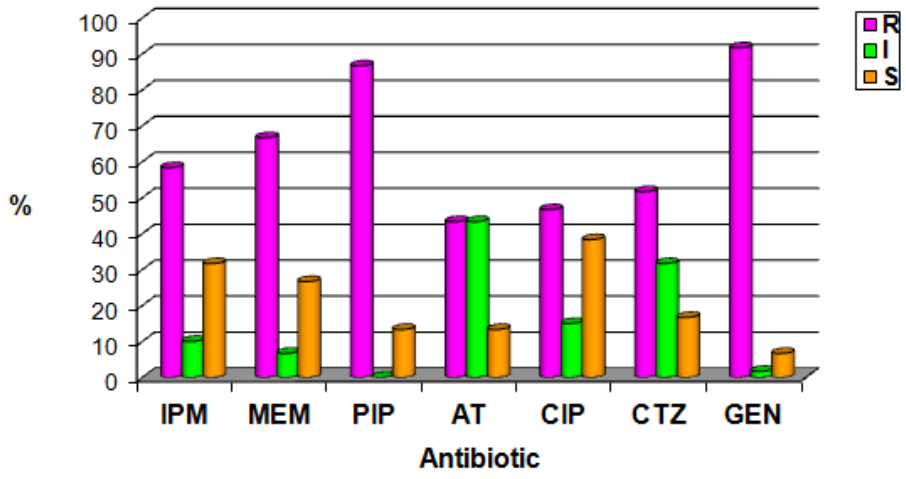
Results and discussion

Cultural and biochemical identification revealed that sixty three (63) *P. aeruginosa* isolates were recovered from 158 samples. Confirmed identification by using VITEK-2 compact system resulted in sixty (60) *P. aeruginosa* isolates (37.97%) were recovered from the 158 samples and three (3) out of sixty three (63) previously identified by the morphological and biochemical tests were considered as *Pseudomonas putida*. It was confirmed by Lyczak, *et al*. in 2000 [28] and Ulku, *et al.* in 2004 [29] that *P. aeruginosa* resistance to many antibiotics and antiseptics and it's so commonly occurrence in the environment make it extremely likely that an individual suffering severe burns or wounds will be challenged with this opportunistic microorganism before the wounds can heal. Most of the sixty *P. aeruginosa* isolates in this study were highly resistant to all antibiotics used particularly the β-lactams and the aminoglycoside (Gentamicin) and resistance levels were largely variable for each antibiotic and revealed highly significant values (p <0.01) for all antibiotics as shown in the table (1) and figure (1) depending on DDT.

Table-1: Antibiotic susceptibility distribution in total samples according to DDT

Antibiotic	R % (No.)	I % (No.)	S % (No.)	*P value* [a]
IPM	58.33 (35)	10.00 (6)	31.67 (19)	0.0023
MEM	66.67 (40)	6.67 (4)	26.67 (16)	0.0019
PIP	86.67 (52)	–	13.33 (8)	0.0001
AT	43.33 (26)	43.33 (26)	13.33 (8)	0.0028
CTZ	51.67 (31)	31.67 (19)	16.67 (10)	0.0013
CIP	46.67 (28)	15.00 (9)	38.33 (23)	0.0025
GEN	91.67 (55)	1.67 (1)	6.67 (4)	0.0001

Abbreviations: R: resistant; I: intermediate; S: sensitive; IPM: Imipenem; MEM: Meropenem; PIP: Piperacillin; AT: Aztreonam; CIP: Ciprofloxacin; CTZ: Ceftazidime; GEN: Gentamicin. **Total No. of samples = 60**. **a:** ***P-value*** was calculated using the Chi-square test in terms of the R, I & S group.



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In this study, the highest frequency resistance revealed was against the Gentamicin while the less resistance was against Aztreonam followed by Ciprofloxacin. Our study revealed that Piperacillin resistanc rate is 86.67 which is similar to Mohammed [30] findings in 2012 that reported 93.3% of *P.*

aeruginosa isolates were resistant to Piperacillin and close to Haran (2012) [31] who found that resistance to Piperacillin is 88%. *P. aeruginosa* is naturally resistant against penicillins such as Piperacillin as reported by Ibezim in 2005 [32] making this study result reasonable. In contrast, it crossed with Al-Doory (2012) finding, 35.8% resistance showing high considered difference [33]. Low resistance was shown against Ciprofloxacin 46.67% but still higher than 20.6% belonging to Al-Doory results in 2012 [33]. The result of our study was similar to the result of Mohammed (2012) which was 54.6% [30]. In 2002, Lambert mentioned that Ciprofloxacin, belong to fluoroquinolone, inhibits bacterial growth by binding to A subunit of DNA gyrase [34]. In this study, *P. aeruginosa* sensitivity to Gentamicin belonging to aminoglycosides group has shown notably high resistance 91.67%. This result agree with AL-Khazali [35] who has found in 2009 that resistence of *P. aeruginosa* isolated from burns and wounds to Gentamicin was 89.% and disagree with Mohammed in 2012 [30] and Al-Doory (2012) [33] results that were 60% and 35.9 respectively. The aminoglycosides inhibit protein synthesis in bacterial cell by binding to 30S subunit of the ribosome and *Pseudomonas* sp. were resistant to aminoglycoside primarily due to changes in the target enzymes and inactivation of the antibiotics as mentioned by Lambert in 2002[34] and Matsuo *et al.* in 2004 [36]. In this study, *P. aeruginosa* isolates revealed high resistance (51.67%) for the fourth generation of cephalosporin Ceftazidime. This result coincides with the finding of Al-Doory (2012) [33] that reported 61.6% Ceftazidime resistance rate and disagrees with resistance rates, 89.8% and 82.6% of Al-Muhannak (2010) [37] and Mohammed (2012) [30], respectively. But it's in contrast with Gailiene *et al.* (2007) who have found that resistance of *P. aeruginosa* to Ceftazidime is 12.8%, the differences seem to be significant [38]. The increased prevalence of Ceftazidime resistant *P. aeruginosa* may be related to the increased use of beta lactam antibiotics such as amoxicillin and Ceftazidime. Selective pressure resulted from the use of antimicrobial agents is a major determinant for the emergence of resistant strains. The elevated resistanc of *P. aeruginosa* isolates in burn unit to carbapenems, Imipenem 58.33% and Meropenem 66.67% in this study is so close to Al-Doory findings in 2012 which showed that resistance results for Imipenem and Meropenem were 53.3% and 53.2%, respectively [33]. However, the difference in result is apparent compared with Al-Shwaikh (2006) [39] who found that all *P.*

aeruginosa isolated from burn and wound infections were sensitive to Imipenem 100%, also this result disagrees with Gailiene *et al*. (2007) findings which showed that resistance results of *P. aeruginosa* against Imipenem and Meropenem were 23.9% and 11.3% respectively [38]. Disagreement is continued to the results obtained in Baghdad in 2012 by Mohammed [30] who showed extremely low resistance against Imipenem and Meropenem, 8% both. Pseudomonads may develop resistance to carbapenems through combined mechanisms such as target inaccessibility, stable derepression of AmpC β-lactamase, overexpression of efflux systems and production of Metallo-β-lactamases (MBLs) as reported in 2002 by Livermore [40]. This study revealed a moderate resistance level against the monobactam (Aztreonam) which reached 43.33% conflicting with the elevated resistance rate obtained by Al-Muhannak in 2010 [37] who found that resistance level was 59.3% and differs than the high results in 2012, 84% and 81.3% of Haran and Mohammed, respectively [31, 30]. However, Moazami-Goudarzi and Eftekhar reported in 2013 that the increase in antibiotic resistance is mostly due to extensive use of antibiotics such as ciprofloxacin, β-lactams and aminoglycosides in the burn centers as well as non-availability and high costs of other effective drugs [41]. In this study, out of total (60) *P. aeruginosa* isolates, carbapenem resistant isolates were 41 (68.34%), while the intermediate-sensitivity isolates were 2 (3.33%) and the sensitive isolates were 17 (28.33%) as shown in figure (2).

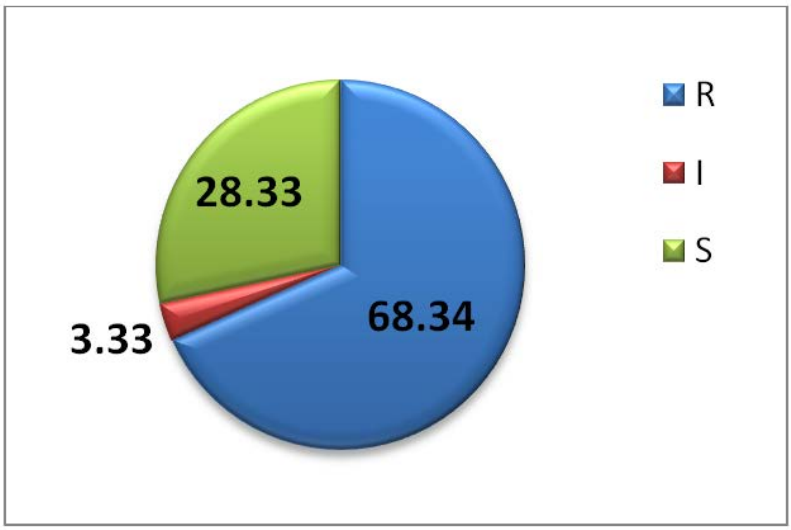

Figure-2: Carbapenem susceptibility rates by DDT. R: resist; I: intermediate; S: sensitive.

These results are elevated in comparison with the result obtained by Mohammed [30] who found that carbapenem resistace in 75 *P. aeruginosa* isolated from different source cases in Baghdad province in 2012 were 16 (21.3%) and the sensitive ones were 59 (78.7%). In this study, the prevalence of carbapenem resistance in burn unit is high which agrees with Yousefi *et al*. (2010) in Iran who found that out of 160 *P. aeruginosa* isolate, 93 (58.1%) isolates were sensitive to Imipenem, 61 (38.1%) were resistant and 6 (3.8%) of isolates showed intermediate resistance and have observed that hospitalization in burn units and ICU wards had significant association with Imipenem non-susceptible isolates [5]. Thus, they concluded that the high prevalence of antimicrobial resistance observed among *P. aeruginosa* isolates underlines the strict consideration in antibiotics use at clinical settings. In this study, out of 60 *P. aeruginosa* isolates, 55 (91.67%) were MDR. Depending on bacterial isolate consideration as non-susceptible to an antimicrobial agent when it tested resistant, intermediate or non-susceptible by using clinical breakpoints as interpretive criteria by CLSI (2009) [42], theses MDR isolates were distributed in 43 (78.18%) carbapenem non-susceptible and 12 (21.82%) carbapenem susceptible as shown in table (2).

Table-2: Total MDR in *P. aeruginosa* isolates and its distribution according to Carbapenem susceptibility

Resistance Feature	All (%)	Carbapenem	
		Non-susceptible (%)	susceptible (%)
MDR	55 (91.67)	43 (78.18)	12 (21.82)
Total	60 (100)	44 (73.33)	16 (26.67)

The results of the present study is higher than the findings of Zarei-Yazdeli *et al*. [43] who found in 2014 that 75.3% of *P. aeruginosa* isolates were MDR and close to the Iraqi study of Al-Shammary in 2013 [44] who found that MDR was 100% in *Pseudomonas* spp. The prevalence of MDR isolates has been increasing worldwide and poses a serious problem in hospital settings. Emerging carbapenem resistance in *P. aeruginosa* isolates have limited therapeutic options for treatment of MDR P. aeruginosa which are considered as the last

line of drugs for treatment of infections caused by these organisms as was shown by Fraenkel *et al*. in 2006 [45] and Aloush *et al*. (2006) [46]. Chitkara and Feierabend mentioned in 1981 that burn hospitals often harbor MDR *P. aeruginosa* that can serve as the source of infection [47]. As confirmed by Pagani *et al*. in 2004, the spread of these organisms is often difficult to control as *P. aeruginosa* exhibits intrinsic resistance to several antimicrobial agents [6]. Furthermore, some of resistance genes have been found as gene cassettes in integrons, which could play an important role in the transfer of these resistance genes to other bacteria as shown in 2006 by Yan *et al*. [48] and by Phongpaichit *et al*. in 2007 [49]. This is confirmed by the fact that several studies have reported, the presence of different resistance genes including aminoglycosides in class 1 integron as reported in 2005 by Fonseca *et al*. [50]. In 2009, Chen *et al*. concluded from their study that integrons were prevalent and played an important role in MDR *P. aeruginosa*, which may provide some important surveillance information reflecting the antibiotic selective pressure in this specific region [51]. Poonsuk *et al*. found in 2012 that clinical use of antibiotics may increase selective pressure for MDR strains and for horizontal gene transfer [52]. This could pose a serious threat to the efficacy of antibiotics used for treating infections caused by clinically significant pathogens including *P. aeruginosa*.

Class 1 Integron gene was detected after amplification with PCR technique using the primer *int*I1. The amplicon, (500 bp) size was detected on agarose gel (Figure-3).

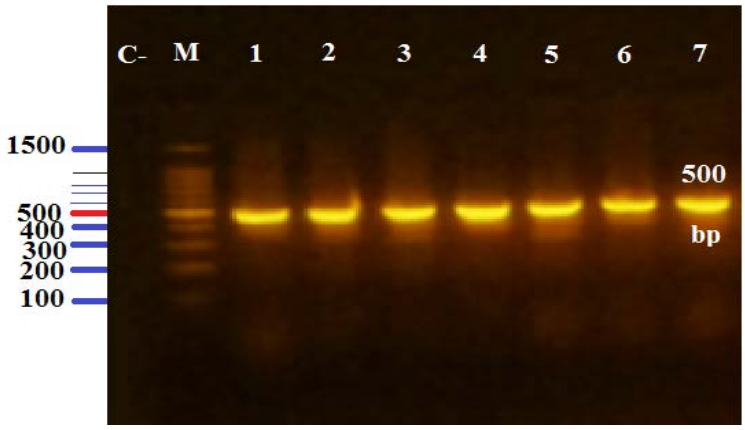

Figure-3: Ethidium bromide stained agarose gel (1.5%) showing PCR amplification products (500 bp) with *int*I1 primers for *P. aeruginosa* extracted DNA indicating class 1 Integron gene. Lane C-: negative control; lane M: 100 - 1500 bp ladder; lane 1 - 7: positive *P. aeruginosa* class 1 Integron genes. Electric current was allowed at 70 volt for 90 min.

Depending on the detection of Integrase gene for the investigation of class 1 integron [25], PCR assay revealed that 59 (98.33%) out of the 60 *P. aeruginosa* isolates were integrase positive (Figure-4), which confirm the extremely high dissemination of class 1 integron at the burn unit in Karbala province in Iraq.

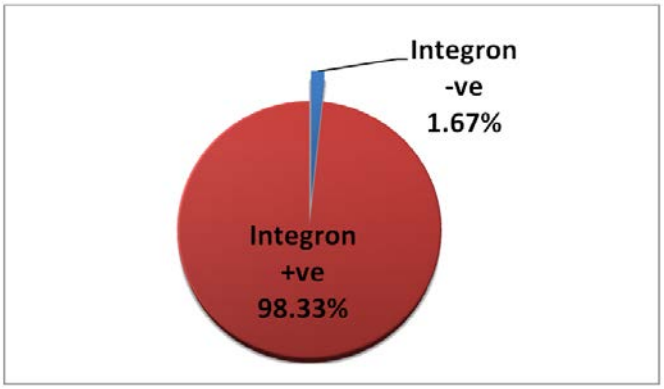

Figure-4: Dissemination of antibiotic resistance gene, class 1 Integron among *P. aeruginosa* isolated from burn patients

This result agrees with Zarei-Yazdeli *et al*. (2014) in Iran [43] whose PCR results showed that 82.6% of *P. aeruginosa* carried class 1 integron but it disagrees with the Malysian study presented in 2009 by Lim *et al*. [20] that revealed 19% integron positive *P. aeruginosa* isolates and differs than study of Yousefi *et al*. (2010) [5] who found that 56.3% of *P. aeruginosa* isolates carried this gene In Iran. The latter is close to (57%) class 1 integron positive result of Odumosu *et al*. in Nigeria in 2013 [53]. This mobile genetic element plays an important role in the dissemination of resistance genes among bacteria. It captures and integrates gene cassettes by site specific recombination and converts them to functional genes as shown by Recchia and Hall in 1995 [17] and in 1997 [54]. This study achieved in Karbala province in Iraq disagrees with the Iraqi study achieved in Thi-Qar province in 2013 by Al-Shammary [44] who used CS primers for amplification of the variable region of class 1 integron responsible for carbapenem resistance in *Pseudomonas* spp., showed that only 4 out of 25 (16%) *Pseudomonas* isolates carried the mentioned gene. Another study in Iraq achieved in Najaf province in 2014 by Hindi *et al*. [55] found that the prevalence of class 1 integron was 20% in Salmonella enterica detected by intI gene. Out of 60 *P. aeruginosa* isolates tested in this study, only one (1.67%) isolate was Integron negative. This investigation revealed that class1 Integron was

carried by both Carbapenem resistant and Carbapenem sensitive isolates. These resulted data are in accordance with Fonseca *et al*. (2005) findings that reported 56.6% of imipenem nonsusceptible and 32.5% of imipenem susceptible isolates were positive for class 1 integron [50]. This result is also agreeing with kouchaksaraei *et al*. (2013) [56]. Class 1 integrons contribute to the emerging problem of antibiotic resistance in human medicine by acquisition, exchange, and expression of resistance genes embedded within gene cassettes as mentioned in 2014 by Jechalke *et al*. [57]. Class 1 integrons frequently located in plasmids and transposons. These have the ability to undergo horizontal transfer and contribute to rapid dissemination of antibiotic resistance genes among bacterial isolates according to Fluit and Schmitz (1999) [58]. The findings of Hsiao *et al*. in 2014 suggest that antibiotic-resistance genes captured by class 1 integrons in *P. aeruginosa* isolates under constant antibiotic-selective pressure are frequently found in hospital environments [59]. Class 1 integrons are commonly found in *P. aeruginosa* isolated from the clinical samples as demonstrated by Zarei-Yazdeli *et al*. in 2014 [43]. Therefore, the transfer of antibiotic resistance genes is often related to these integrons. Strateva and Yordanov (2009) found that MDR *P. aeruginosa* isolates have an extraordinary ability to acquire integron associated antibiotic resistance genes to adapt to their adverse environmental growth conditions in the hospital setting [60]. Othman *et al*. reported in 2014 that it is of vital importance to regularly monitor nosocomial infections in burn units and undertake culture and sensitivity tests to select the most effective antibiotics for treatment in order to control the MDR threat [61].

Conclusions

Our results showed a high prevalence of class 1 integron gene in most of *P. aeruginosa* isolates recovered from burn patients. The increasing resistance to carbapenem is due to its increasing usage especially Meropenem. The widespread MDR among *P. aeruginosa* isolates is associated with frequency dissemination of class 1 Integron. This extensive dissemination of the gene is considered dangerous due to its responsibility in resistance dissemination among this bacterial population or other genera by horizontal transmission predicting epidemiology. Therefore, it is important to emphasize the control of contamination and investigate resistant strains especially at burn unit to prevent their dissemination and it is recommended to limit the use of carbapenems to reduce their threatening fate of resistance.

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Detection of *GliP, GliZ, GliA and GliJ genes* of gliotoxin in *Aspergillus fumigatus* by using PCR

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Abstract: Gliotoxin is an important virulence factor of *Aspergillus fumigatus*.The biosynthesis of this mycotoxin is regulated and expressed by the present of *GliP* and *GliZ* genes. This study was aimed to identified *Aspergillus fumigatus* isolates, as well as to detection of the important gliotoxin genes by using conventional PCR. To achieve this, DNA was isolated from twenty *A. fumigatus* isolates using commercial kit. The yield of the DNA extracted was in range of (65-210) ng/µl with purity of (1.5-1.9). Specific species identification of *A. fumigatus* isolates were achieved by using specific primer. The results showed that all isolates have positive results to primer (*Afumi*). The molecular detection of gliotoxin genes using PCR showed that *GliA* and *GliJ* were detected in all isolates; *GliZ* was detected in 17 isolate; while *GliP* was detected in 15 isolates.

Key word: *Aspergillus fumigatus*, gliotoxin

التحري عن جينات الغليوتوكسين *GliP* و *GliZ* و *GliA* و GliJ في فطر *Aspergillus fumigatus* باستخدام تقنية PCR

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الخلاصة: يعد الغليوتوكسين هو اهم عامل الضراوة لفطر *A.fumigatus*. عملية التصنيع الحيوي لهذا السم تتم بواسطة تنظيم وتعبير الجينين *GliP* و*GliZ*. ان هذه الدراسة تهدف الى تشخيص فطر*A.fumigatus* و تحري عن الجينات المهمة المنتجة للغليوتوكسين باستخدا تقنية PCR التقليدية. ولتحقيق ذلك، تم عزل الحمض النووي من عشرين عزلة لفطر *A.fumigatus* . كان العائد من الحمض النووي المعزول يتراوح بين (65-210) نانوغرام / ميكرولتر مع نقاوة تتراوح بين (1،5-1،9). وقد تم تشخيص عزلات فطر *A.fumigatus* باستخدام بادئات محددة. وأظهرت النتائج أن جميع العزلات لها نتائج إيجابية للبادئ (*Afumi*).. بينما أظهر التحري الجزيئي للغليوتوكسين باستخدام PCR ان جينات *GliA* و*GliJ* وجدت في جميع العزلات.وان جين *GliZ*,وجد في 17 عزلة. في حين ان جين *GliP* وجد في 15 عزلة.

1. Introduction

The filamentous fungus *A. fumigatus* is the most pathogenic, and is responsible for approximately 90 % of all invasive aspergillosis infections (Dagenais and Keller, 2009). The fungus *A. fumigatus* is a pulmonary pathogen capable of inducing disease in those with pre-existing pulmonary malfunction (e.g. asthma, cystic fibrosis ,tuberculosis, lung cancer) or undergoing immunosuppressive therapy prior to organ transplantation (Denning, 1996a; Fraser, 1993; Daly & Kavanagh, 2001).Three forms of aspergillosis are recognized clinically: saprophytic, allergic and invasive. Invasive aspergillosis (IA) is the most serious form of disease as it involves the invasion of viable tissue and may produce a mortality rate of 40–90% in immunosuppressed patients (Denning, 1996b, 1998). Non-*Aspergillus fumigatus* species may also cause invasive disease (Walsh & Groll, 2001). *A. fumigatus* produces several secondary metabolites, some of which are believed to play important roles during the infection process (Kamei & Watanabe, 2005; Ben-Ami *et al*., 2009). Gliotoxin, an important secondary metabolite of *A. fumigatus* (Kupfahl *et al*., 2008), belongs to the class of epipolythiodioxopiperazines (ETPs) and has pleiotropic immunosuppressive properties that include induction of apoptosis in macrophages and lung epithelial cells, inhibition of nuclear factor *κ*-B activation, and inhibition of phagocytosis (Sugui *et al.*, 2007; Kupfahl *et al.*, 2006). Several groups have recently reported that gliotoxin plays an important role in invasive aspergillosis (Orciuolo *et al.*,2007; Kwon-Chung & Sugui,2009); however, the mechanism remains unclear. which contains a transannular disulfide bridge of unknown origin (Fox and Howlett, 2008).

The *Gli* biosynthetic cluster, which directs gliotoxin production in Aspergillus fumigatus, was identified in 2004 and contain 13 genes (Gardiner *et al.*, 2004; Schrettl *et al.*, 2010. *GliZ* works as a transcriptional regulator of gliotoxin biosynthesis (Bok *et al.*,2006), while *GliP* encodes a nonribosomal peptide synthase that catalyzes the first biosynthetic step in gliotoxin synthesis (Cramer *et al*.,2006). However, the extracellular export mechanism of gliotoxin, which is deemed critical for the virulence and self-protection of the fungus from the toxin, remains unknown. *GliA*, play a significant role in the tolerance to gliotoxin and protection from extracellular gliotoxin in *A. fumigatus* by exporting the toxin. This also allows the fungus to evade the harmful effect of its own gliotoxin production. Moreover,

GliA contributes to the virulence of *A. fumigatus* through gliotoxin secretion. (Wang *et al*., 2014).While, *GliJ* gene encoded metal-dependent dipeptidase that it's one of four-enzyme cascade that Convert Glutathione Conjugates into Transannular Disulfide Bridges in gliotoxin biosynthesis pathway (Scharf *et al*., 2013). The aims of the work presented here were to specific identification of *A. fumigatus* and detection of some gliotoxin genes using specific PCR.

2. Materials and methods

2.1. *A. fumigatus* culture conditions

Twenty *A. fumigatus* isolates (obtained from university of Baghdad / department of biotechnology) were used in this study; 18 isolate from patients with invasive aspergillosis and two from environment. *Aspergillus* cultures were grown in potato dextrose agar (PDA) (Himedia-India) at 37 ^{0}C for up (7-10) days. Stocks were maintained on Sabouraud dextrose agar (SDA) (Oxoid-UK).

2.2. Genomic DNA extraction

The DNA was extracted from 20 *A. fumigatus* isolates by using Reagent Genomic DNA Kit (Geneaid-Taiwan) to produce a rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by nanodrop. Genomic DNA integrity was detected by running on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light (Sambrook *et al*., 1989).

2.3. Primer selection and PCR assay

Five decamers of oligonucleotides primers sequence were used in a lyophilized form and were dissolved in sterile deionizer distilled water to give a final concentration of (10pmol/µl). One primer set was used for identification of *A. fumigatus* and the anther four primers sets were used for detection of gliotoxin genes. The primers and their sequences were showed in table (1)

Amplification reactions were performed in a volume 25 µl (PCR PreMix(Promega),(final reaction volume = 25 µl). Amplification was carried out using a thermocycler (Eppendorf-Germany), using the following program:- cycle of 5 min at 95°C for initial strand separation, followed by 40 cycles of 1min at 95˚C for denaturation and 45sce at 58˚C for anneling and 1min 72˚C for primer extension. Finally, 1 cycle of 10 min at 72˚C was used for the final extension, and this program resulted by optimization.

Approximately, 7µl of PCR amplified products were separated by electrophoresis in 1.2% agarose gels (1.5 hrs, 5V/cm, 1X Tris-borate

buffer). Gels were stained with ethidium bromide, PCR products were visualized by U.V transilluminator and then were imaged by gel documentation system. The amplified products usually consist of one discrete band**,** the size of PCR products estimated by comparing with the marker DNA ladder (100-2000) bp (Bioneer)

Table (1): The names & sequences of the specific primers used in the study.

No.	Primers	Sequence (5'-3')	fragment size (bp)	Purpose
1	*AfumiF1* *AfumiR1*	GCCCGCCGTTTCGAC CCGTTGTTGAAAGTTTTAACTGATTAC	136	Identification of *A. fumigatus* (Walsh; 2011)
2	*GliAf* *GliAr*	TTTGCGATCAACGAACTCTG CCCTTGACGGACTGGAAGTA	161	Gliotoxin detection (Gardiner; 2005)
3	*GliJf* *GliJr*	CTCTGATCGACGGCCATAAT TCGAGCTGTTGGAGTGTCTG	288	
4	*GliPf* *GliPr*	AAACCCCTGTGAATGCAGAC CCCCTTGAGATGAAAGGTGA	173	
5	*GliZf* *GliZr*	TCCAGAAAAGGGAGTCGTTG ACGACGATGAGGAATCGAAC	177	

3. Results and discussion

3.1 Genomic DNA Extraction

The DNA was extracted efficiently by using Reagent Genomic DNA Kit. The Purity and concentration were measured by using the standard method (Sambrook *et al*., 1989).The yield of The yield of the extracted DNA was in range of (65-210) ng/µl with purity of (1.5-1.9).

3.2. PCR Analysis

3.2.1. Identification of *A. fumigatus*

In this study, PCR technique was used to examined for specificity identification of *A. fumigatus* isolates. The results of this study was demonstrated that *Afumi* primer based PCR method had high sensitivity and specificity in detecting of *A.fumigatus*. An amplicon corresponding to 136 bp in size was seen after agarose gel electrophoresis PCR was tested with twanty different *A.fumigatus*.

Figure(1) shows that the genomic DNA of all isolates was recognized and complementary to *Afumi* gene sequence and represented by presence of single band in molecular weight 136bp.

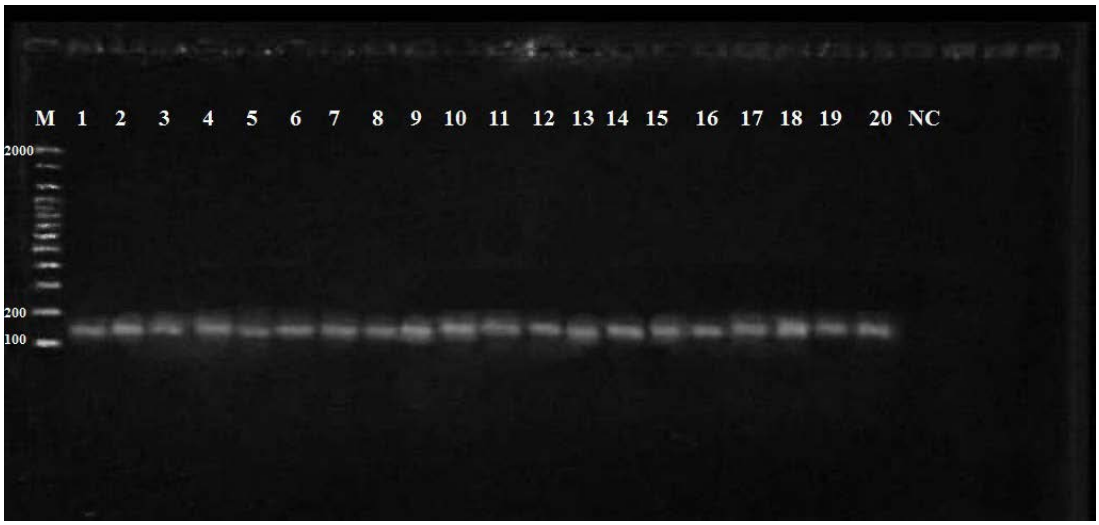


Figure (1): PCR product for *Afumi* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.

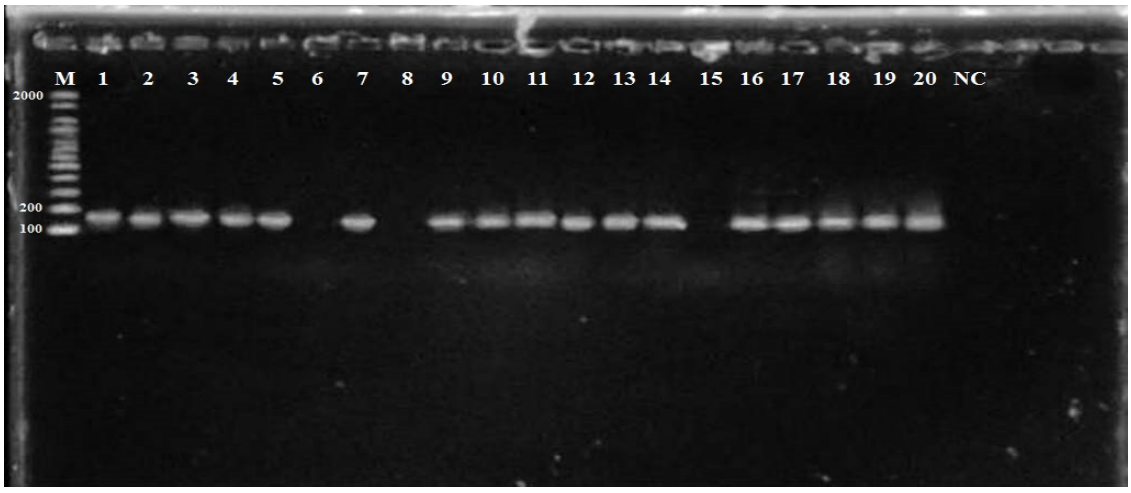
3.2.2. Gliotoxin detection

1.*GliP* primer

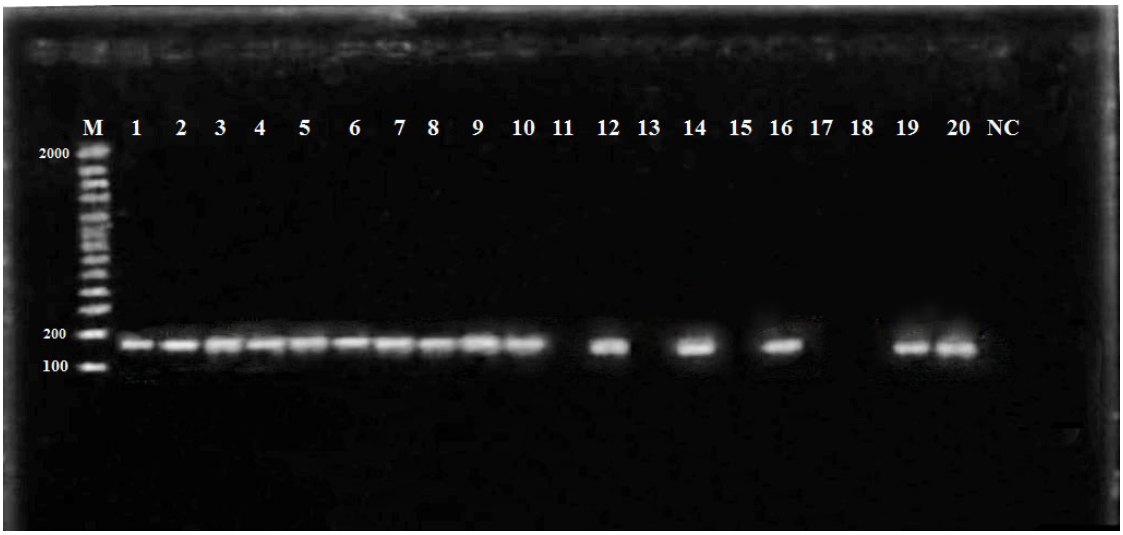
PCR results of amplified genomic DNA extracted from *A. fumigatus* isolates, showed in figure 2, the isolates number (1,2,3,4,5,7,9,10,11,12,13,14,16,17, 18,19 and 20) have positive results and represented by presence of single band in molecular weight 173bp but isolates number (6,8 and 15) have negative results and represent by absence of band in molecular weight 173bp (Figure 2).

2 *GliZ* primer

PCR results of amplified genomic DNA extracted from *A. fumigatus* isolates, showed in figure 3, the isolates number (1,2,3,4,5,6,7,8,9,10,12,14,16,19 and 20) have positive results and represented by presence of single band in molecular weight 177bp but isolates number (11,13,15,17 and 18) have negative results and represent by absence of band in molecular weight 177bp (Figure 3).



Figure(2) :PCR product for *GliP* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.



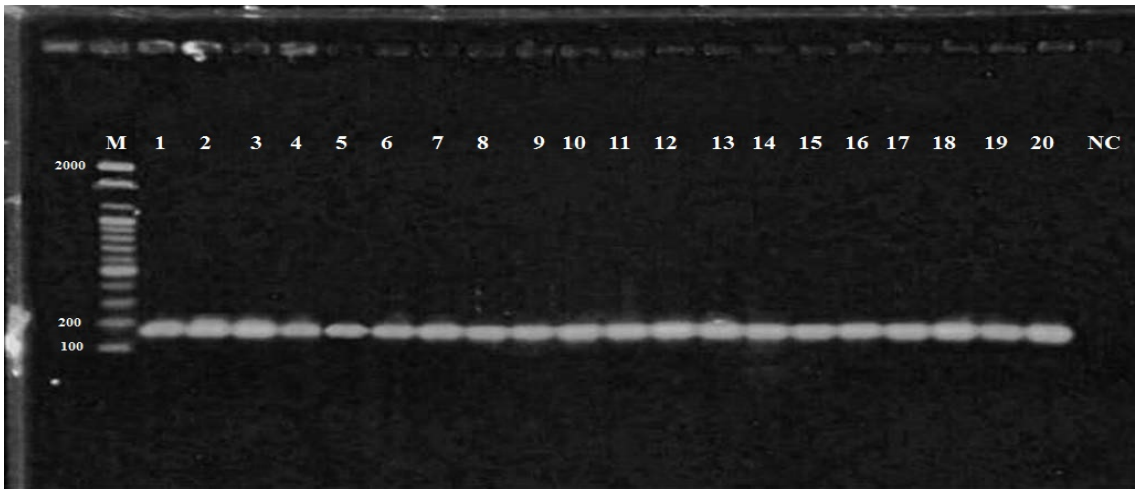
Figure(3) :PCR product for *GliZ* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.

3 *GliA* primer

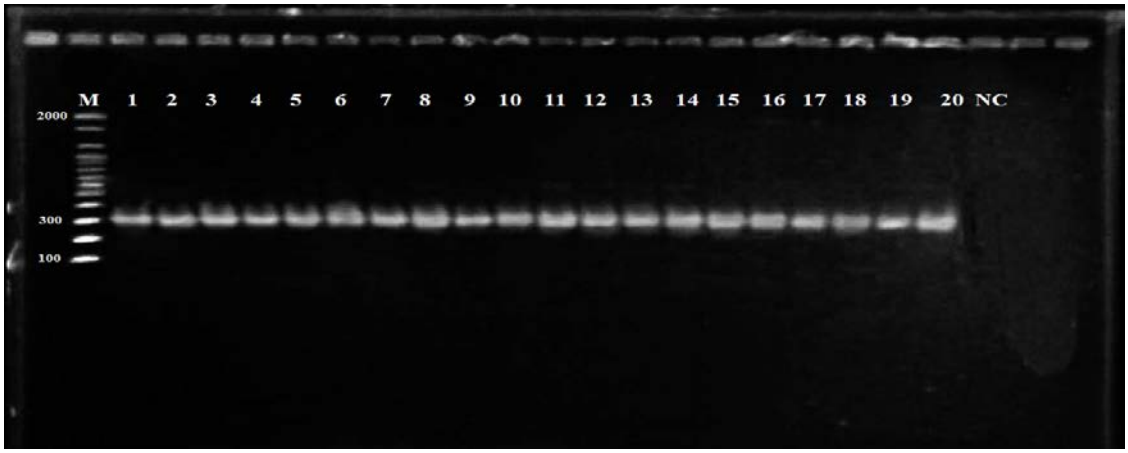
PCR results of amplified genomic DNA extracted from *A. fumigatus* isolates, showed in figure 4, all isolates have positive results and represented by presence of single band in molecular weight 161bp (Figure 4).

4 *GliJ* primer

PCR results of amplified genomic DNA extracted from *A. fumigatus* isolates, showed in figure 5, all isolates have positive results and represented by presence of single band in molecular weight 288 bp (Figure 5).



Figure(4): PCR product for *GliA* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.



Figure(5) :PCR product for *GliJ* primer for DNA samples of *A. fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: negative control.

There are two transcription gene redulators for the biosynthesis of gliotoxin that have been described that is the transcriptional regulater *LaeA* and transcriptional regulater *GliZ* (Bok, *et al*., 2006). These 2 transcriptional gene regulaters are very important and they work hand in hand with the enzyme that catalyzes the first step of gliotoxin biosynthesis. This enzyme is encoded by the gene *GliP* and the enzyme is called the nonribosomal peptide synthetase (Sugui, *et al*.,2007) since gliotoxin is known as the nonribosomal peptide toxin (Cramer, *et al*., 2006). so this explain presence of *GliP* and

GliZ genes and un ability of some isolates to produce gliotoxin that due to unexpression of this genes or surrounding condition not stimulate to produce gliotoxin. All PCR result of gliotoxin genes are summarized in table (2) so this explain presence of *GliP* and *GliZ* genes and un ability of some isolates to produce gliotoxin that due to unexpression of this genes or surrounding condition not stimulate to produce gliotoxin.

Sugui, (2007) deletion of the *GliP* gene in *A. fumigatus* resulted in abrogation of gliotoxin synthesis, failure to induce apoptosis in mammalian cells, and a reduced ability to

inhibit the oxidative burst in human neutrophils in vitro. Most importantly, the *GliP*Δ mutant showed reduced virulence in two different mouse strains, indicating that, contrary to previous reports (Kupfahl,2006), gliotoxin is a virulence determinant of *A. fumigatus*. Disruption of the *GliP* gene resulted in elimination of gliotoxin production, confirming the role of *GliP* in the biosynthesis of gliotoxin(Cramer, 2006).

Bok, (2006) demonstrated that LaeA, a nuclear protein, regulated several putative fungal virulence factors, including synthesis of gliotoxin and other secondary metabolites as well as conidial morphology and have role for gliotoxin in *A. fumigatus* pathogenesis and development of IA (Bok, 2005;2006). To genetically elucidate the contribution of loss of gliotoxin to the *laeA* phenotype,bok, (2006) deleted *GliZ*, a putative transcription factor located in the gliotoxin gene cluster; deletion of *GliZ* resulted in loss of gliotoxin production in vivo and in vitro.This loss was associated with absence of transcription of a biosynthetic gene in the gliotoxin gene cluster.

Wang, (2014) suggest that, through its capacity to export gliotoxin extracellularly, *GliA* functions to protect the fungus from the harmful effects of extracellular gliotoxin, which strongly suggests that *GliA* also contributes to protection from its own produced gliotoxin by constantly exporting the toxin. *GliA* disruption caused the fungus to be highly susceptible to extracellular gliotoxin. In addition to *GliA*, was that the amount of gliotoxin was reduced not only in extracellular but also in intracellular spaces, which suggests that gliotoxin production was greatly reduced by the *GliA* disruption.

While Dhingra, (2012) was suggested that, expression levels of *GliZ* were greater in the *veA* overexpression strain, in spite of the observed low levels of *GliP* expression and reduced gliotoxin accumulation. When *GliZ* was overexpressed in a *veA* wild-type background, high levels of both *GliP* and concomitant gliotoxin accumulation were observed. It is

possible that *veA* in *A. fumigatus* could influence the expression of structural genes in the gliotoxin clusters by other unknown mechanisms in addition to the effect on the expression of *GliZ*.

While *GliJ* gene encoded metal-dependent dipeptidase that it's one of four-enzyme cascade that Convert Glutathione Conjugates into Transannular Disulfide Bridges in gliotoxin Biosynthesis pathway (Scharf *et al*., 2013).

Table (2): PCR analysis for gliotoxin genes of *A. fumigatus*

Primers	Samples																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	NC
AFU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GliA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
GliZ	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	-	+	+	-
GliP	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-
GliJ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

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Real-time PCR assay for rapid detection of *Haemophilus influenzae* in children with meningitis

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Abstract: *Haemophilus influenzae* still causing bacterial meningitis among infants and young children in developing countries and the traditional methods for identification this bacterium needs a lot of time. Thus, the aim of this study was to detect both capsulated and nontypable infections of *H. influenzae* using *hpd* gene based real-time PCR technique. Seventy five cerebrospinal fluid (CSF) specimens were collected from children less than five years old diagnosed with suspected bacterial meningitis. All specimens were tested by culture and real-time PCR assay with a specific primer pair and probe from *hpd* region for detection of *H. influenzae*. Eleven (14.7%) CSF specimens had positive culture results while 26 (34.6%) CSF specimens were identified as *H. influenzae* by real-time PCR technique. Fifteen cases (57.7%) were occurring in children less than one year and the ratio of infection in male:female was 1.4:1. In conclusion, real-time PCR assay based on *hpd* was an accurate and rapid method for detection of *H. influenzae* in the CSF of children with meningitis.

Key words: *H. influenzae;* CSF; *hpd* gene, Real-time PCR; Meningitis.

اختبار تفاعل سلسلة البلمرة-اللحظي للكشف السريع عن بكتريا *Heamophilus influenzae* في الاطفال المصابين بمرض التهاب السحايا الجرثومي

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الخلاصة: لاتزال بكتريا *Haemophilus influenzae* تسبب التهاب السحايا الجرثومي بين الرضع والأطفال الصغار في البلدان النامية، والطرق التقليدية لتشخيص هذه البكتيريا تحتاج إلى الكثير من الوقت. لذا هدفت هذه الدراسة الكشف عن الاصابات الناتجة من بكتريا *H. influenzae* المحاطة بكبسولة وغير المحاطة بكبسولة باستخدام تقنية تفاعل سلسلة البلمرة- اللحظي بالاعتماد على جين *hpd*. جمعت خمسة وسبعون عينة من السائل النخاعي من اطفال تقل اعمارهم عن خمس سنوات من العمر يتوقع اصابتهم بمرض التهاب السحايا الجرثومي. تم اختبار كل العينات باستخدام الاوساط الزرعية واختبار تفاعل سلسلة البلمرة- الاحظي باستخدام زوج من البوادئ ومجس المتخصصة لتضخيم منطقة جين *hpd* للكشف عن بكتريا *H. influenzae*. اظهرت النتائج أحد عشر (14.7٪) عينة من السائل النخاعي نتائج موجبة في الوسط الزرعي للبكتريا في حين تم تشخيص 26 (34.6٪) عينة من السائل النخاعي بتقنية تفاعل سلسلة البلمرة- اللحظي حاوية على الجين المدروس. كما بينت الدراسة وجود خمسة عشر اصابة (57.7٪) في اطفال تقل اعمارهم عن سنة واحدة وان نسبة الإصابة في الذكور: الإناث كانت 1,4: 1. نستنتج من ذلك ان اختبار تفاعل سلسلة البلمرة- اللحظي المعتمد على جين *hpd* هو اختبار دقيق وسريع للكشف عن *H. influenzae* في السائل النخاعي للاطفال المصابين بألتهاب السحايا.

Introduction

Haemophilus influenzae is an opportunistic bacterial pathogen divided into two major categories: capsulated from a-f serotypes and noncapsulated or nontypable (NT) strains (1).

Meningitis is the major cause of morbidity and mortality among infants and children below the age of 5 years. The role of *H. influenzae* as the major pathogen in meningitis was evidenced in many studies in developing countries especially serotype b (2,3).

Real-time PCR is one of the molecular techniques that used for detection of *H. influenzae* characterized by its short time condition (less than 2 hours) and sensitivity (4). Different target genes were amplified for diagnosis of *H. influenzae* such as *16SrDNA, ompP6* (5) and *hpd* (6,7).

hpd is the gene encoding protein D that is highly conserved surface lipoprotein found in all *H. influenzae* strains and the organization of this gene is homologous in different serotypes and biotypes (8). Also, the *hpd* in the NT and *H. influenzae* serotype b was antigenically and genetically conserved despite it is localized on the surface of bacteria (9,10).

From our previous reports (11,12), *H. influenzae* serotype b proved to be responsible for meningitis in Iraqi children less than 5 years old. However, NT case was detected in CSF of one child. Therefore detection of capsulated and NT *H. influenzae* was needed by choosing a conserved gene found in all *H. influenzae*. Thus, the aim of this study was to detect both capsulated and NT infections of *H. influenzae* using *hpd* based real-time PCR.

Material and Methods

Collection of CSF specimens

During 2010, seventy five CSF specimens were collected from children less than five years old from different Iraqi hospitals included 11 cases of confirmed *H. influenzae* meningitis by culture of CSF and biochemical test; 30 cases of probable meningitis who having symptoms with turbid CSF, elevated protein > 100mg/dl, decreased glucose<40mg/dl or WBC> 100mg/dl with more than 80% neutrophil; 26 cases of suspected meningitis who having symptoms of meningitis as well as 8 cases of other meningitis as control group. One milliliter of CSF was transported to the Central Health Laboratory with ice bags and stored at -20 $^{\circ}$C until used. Data included age, gender, sign and symptoms, WBC and RBC count, glucose, and protein levels for in each case were registered.

Extraction of Genomic DNA

An overnight culture of bacteria was used for extraction of DNA by

wizard genomic DNA purification kit (Promega, USA) while 100 µl of CSF specimens was used for extraction of DNA Mini Kit (Geneaid, Thailand) according to manufactures instructions. The DNA concentration and purity were determined by using a spectrophotometer.

TaqMan Real-Time PCR

hpd #3 primers and probe (6) were selected to amplify the *hpd* of *H. influenzae*. The forward primer (5´-GGTTAAATATGCCGATGGTGT TG-3´) and the reverse primer (5´-TGCATCTTTACGCCACGGTGT A-3´) were used to amplify a 151 bp fragment. The amplified product was detected with a TaqMan oligonucleotide probe (Fam-5´-TTGTTACACTCCGT "T" GGTAAAAGAACTTGCAC -3´-SpC6 and the " T " was BHQ1 fluorescent dye).

PCR mixture was carried out in a total volume of 25µl included a TaqMan universal master mix (Applied Biosystem, USA), 100 nM of each primer, 300 nM of the probe, and 100 ng/µl template DNA. Quantitative real-time PCR was carried out in ABI prism 7300 real-time PCR in 96 well PCR plates. The reaction was initiated by activation of Taq polymerase at 95˚C for 10min, followed by 40 cycles consisting of 15sec denaturation at 95˚C and 1min annealing at 60˚C. The amplicon was detected by measuring fluorescences and analyzed by applied biosystem software. Negative control was prepared in parallel with each run. The sample was considered positive for *H. influenzae* DNA when the exponential shape increased in fluorescence during the first 35 cycles of amplification, whereas the negative result was assigned as no amplification when the Ct value was greater than 40 (6).

Statistical analysis

All data were analyzed using the SPSS IBM version 20. Chi-Square was used for find the relationship between age and infection with *H. influenzae* as well as between months of year and infection. The P value $\leq$ 0.05 was considered statistically significant.

Results and Discussion

Although the culturing method is the represented gold standard for detection of *H. influenzae,* it had lower sensitivity than PCR assay. The results of study demonstrated that the identification rate of *H. influenzae* from 75 specimens by *hpd* based real-time PCR was 34.7% (26 specimens included 11 cases of confirmed *H. influenzae* meningitis, 9 cases of probable meningitis and 6 cases of suspected meningitis) whereas the culture confirmed cases rate was 14.7% (11 specimens). Therefore, the sensitivity of culture

was 42% in comparison to real-time PCR for detection of *H. influenzae* in CSF specimens. It was recorded that identification of *H. influenzae* by real-time PCR is more efficient than the culture method (13). From these data, it may suggest that the negative results of culture may return to earlier administration of antibiotic prior to specimen collection. WHO (14) confirmed that 50% of CSF cultures were negative for *H. influenzae* in children treated with antibiotics prior CSF specimen collection.

In addition, the results above proved the accuracy of the molecular techniques especially in negative culture specimens and the real-time PCR based *hpd* was successful to detect the both capsulated and NT *H. influenzae* that previously reported on our work (11,12). Other studies recorded that real-time PCR targeting the *hpd* was more sensitive and specific for detection of *H. influenzae* (capsulated and NT) when used directly on clinical specimens than other traditional methods (6,15,16).

Our finding showed significantly relationship between age of children and infection with *H. influenzae* (P value ≤ 0.05), 15(57.7%) cases of *H. influenzae* were occurred in children less than one year and 11 (42.3%) cases were distributed in children aged 1 to 4 years (Figure 1).

In the estimation by WHO (14), it was found nearly 60% of *H. influenzae* type b occurred in children less than one year. The fact that children less than one year were more susceptible to *H. influenzae*, especially type b is related to lack of anticapsular antibody in this age. Older children have anticapsular antibody and reached to adult level in 4 years (17).

The distribution of *H. influenzae* between males and females was studied. It was found that 57.7% (15 cases) of male had infected with *H. influenzae* while the percentage of infected females was 42% (11 cases) and the ratio of infection in male:female was 1.4:1.

This result was agreed with Johnson *et al.* (18) which male to female ratio was1.5:1 and significantly higher male preponderance was seen in the children under five years old. The possible explanation for this differences outcome between males and females are males may be genetically and physiologically more susceptible to infection which human males are more susceptible to dysentery, gonorrhea, meningitis, pneumonia, rabies, syphilis, tetanus and certain types of cancers than females (19).

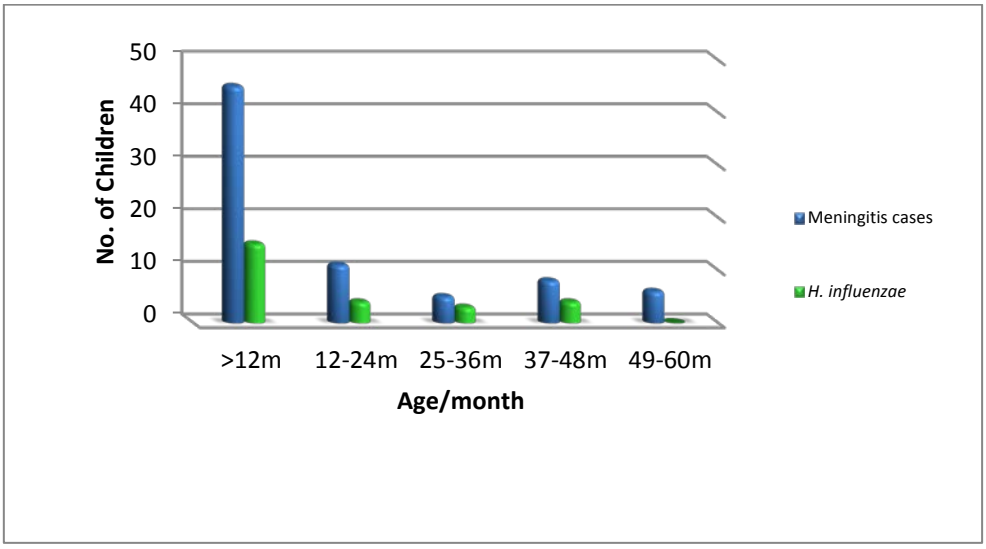

Figure 1: Effect of age on the children infected with *H. influenzae* meningitis.

However, the results observed that infection with *H. influenzae* not affected by months of year (P value > 0.05), they showed a bimodal seasonal pattern that one peak of *H. influenzae* infection occurred in autumn to winter seasons and another peak occurred during summer season (Figure 2).

Some epidemiological studies showed a bimodal seasonal pattern with one peak during September to December and another peak during March to May (20) whereas other reports not showed seasonal variation in *H. influenzae* meningitis (3).

Other investigators showed that *H. influenzae* was more frequently isolated during rainy season particularly the antimicrobial resistant isolates may be strongly associated with increasing number of antibiotic prescriptions during the winter season which promotes selection of resistance among pathogens colonizing the nasopharynx and oropharynx (21).

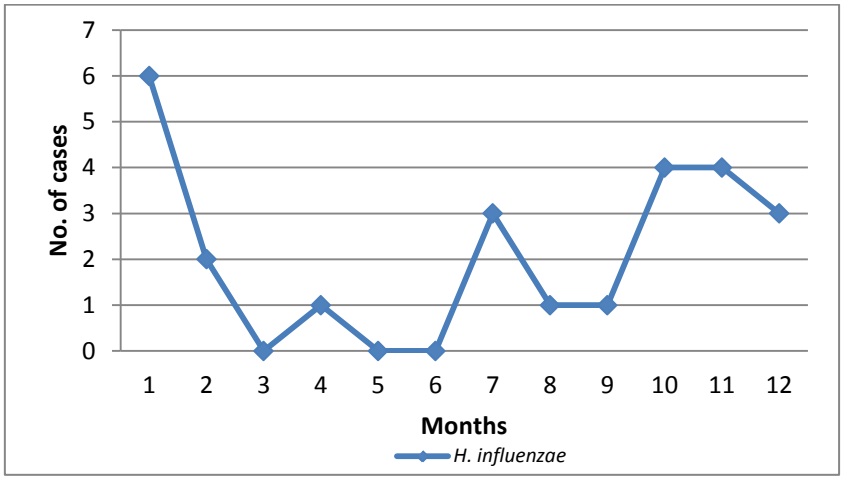

Figure2. Seasonal distribution of *H. influenzae* in children infected with meningitis.

Increased antimicrobial consumption was common in Iraq particularly in influenza season may indicate the possibility increase of *H. influenzae* infection as the secondary infection followed the influenza which increased in autumn and winter seasons.

In conclusion, real-time PCR assay based on *hpd* was an accurate and rapid method for detection of *H. influenzae* in the CSF of children with meningitis.

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Level and Distribution of Selected Trace Elements in fish tissues (*Cyprinus carpio* and *Barbus luteus*) and sediments from Main outfall drain river near the center of Al-Nassiriya city

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Abstract: The present study was conducted to investigate the concentration and distribution of four trace metals cadmium, cobalt, copper and lead, in exchangeable and residual phases of the sediment in main outfall drain and tissues (gills, liver and muscle) of two important commercial species of fish (*Cyprinus carpio* and *Barbus luteus)* during summer season 2014. Also the present study include some physical and chemical properties of this ecosystem. The results showed clear coordination between air and water temperature in all study locations. Air and water temperatures ranged between (34.5-39.31) ºC and (26.22-32.01) ºC respectively. pH, salinity, dissolved oxygen and biological oxygen demand ranged between (7.3-8.6), (4.3-9.01) ‰, (6.0-7.70) mg/L and (2.26-3.25) mg/L respectively. Localized variations were noticed in percentages composition of sediment contents of clay, silt and sand. Total organic carbon ranged between (0.28-1.85) in study stations. The mean concentrations of Cd, Co, Cu and Pb in exchangeable and residual phases as follow Cd (4.22,0.14),Co(12.40,19.33), Cu(4.76,12.71) and Pb(29.21,1.85) µg/g dry weight respectively.The present study showed a differences in concentrations of studied metal in different tissues of fish, these concentrations varies from one species to another and the tissues of same species also showed differences in concentration of studied metals. This due to the nature and the function of the tissue and ability of fish on regulating the level of the metals in their bodies during the uptake and elimination processes.

Keywords: Trace elements, Main outfall drain, *Cyprinus carpio*, *Barbus luteus* and Sediments.

مستوى وتوزيع العناصر النزره في انسجة الاسماك (الكارب الاعتيادي والحمري) ورواسب المصب العام قرب مركز مدينة الناصرية

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الخلاصة: أجريت هذه الدراسة لقياس تراكيز وتوزيع أربع من العناصر النزره (الكادميوم ،الكوبلت ،النحاس والرصاص) في نهر المصب العام للرواسب بجزيئيها المتبادل والمتبقي وأنسجة (الغلاصم ،الكبد والعضلات) لنوعين من الأسماك التجارية المهمة جمعت من المصب العام خلال موسم الصيف 2014. وكذلك شملت الدراسه الحالية دراسة بعض الخصائص الفيزيائيه والكيميائية لذالك النظام البيئي.أظهرت نتائج الدراسة توافق واضح بين درجة حرارة الهواء ودرجة حرارة الماء في جميع المواقع اذ تراوحت بين (34.5-39.31)°م و(26.22-32.01) °م ،وكانت قيم الاس الهيدروجيني ذات مدى ضيق في جميع المواقع وتراوحت بين (7.3-8.6). سجلت قيم الملوحة والأوكسجين المذاب والمتطلب الحيوي للأوكسجين بين(4.3-9.01)جزء بالألف،(6.00-7.70)ملغم/لتر و(2.26-3.25) ملغم/لتر.سجلت اختلافات موقعيه في النسب المئوية لمكونات الرواسب من الطين والغرين والرمل تراوحت معدلات محتوى الكاربون العضوي الكلي في الرواسب بين (0.28-1.85)%.بلغت معدلات تراكيز العناصر النزرة في الرواسب بجزيئيه المتبادل والمتبقي كالأتي الكادميوم (4.22,0.14)، الكوبلت (12.40,19.33)، النحاس(4.76,12.71) والرصاص(29.21,1.85) (مايكروغرام/غرام) وزن جاف على التوالي. بينت الدراسة وجود تراكيز متباينة من العناصر النزرة في أنسجة كلا النوعين من الأسماك. إن تراكيز العناصر المدروسة تختلف في تراكمها من نوع لأخر، وتختلف في أنسجة النوع الواحد. وهذا يعود إلى طبيعة ووظيفة الأنسجة وقابلية الأسماك على تنظيم مستويات هذه العناصر في أجسامها خلال عمليات الأخذ والأزاله.

الكلمات ألدالة: العناصر النزرة، نهر المصب العام، الكارب الاعتيادي، الحمري والرواسب

Introduction:

The expansion in the production of huge amounts of chemical materials, and the increase in these materials annually are due to the global industrial development, especially of the chemical industries like petrochemicals ,paper industry, paints ,plastics, and electric instrument industries, but these lead to environmental crease identifying by environmental pollution [1] .

Trace metals are generally released in aquatic environments in different ways and accumulation of these metals is dependent on the its concentrations, the type of aquatic organisms and the exposure period [2].

Many studies in different regions from the world have represented using the sediment of rivers and estuaries and fish as an indicators for pollution by trace metals [3,4,5,6,7]

Fishes are known for the ability to concentrate heavy metals in their muscle and since the play important role in human nutrition, they need to be carefully screened to ensure that

unnecessary high level of some toxic trace metals are not being transferred to man through fish consumption [8]. This study is geared towards determining the distribution of trace metals in the fish part and sediment Main outfall drain river in Al-Nassiriya city, with the view to establishing abase line data on the current pollution status of the river. The results obtained from this study would also provide information for background levels of metals in the sediments and fish species of the river contributing to the effective monitoring of both environment quality and health of the organisms inhabiting the river.

Materials and Methods :

Study area :

Main outfall drain is a river use to discharge the effluents of agriculture activites from its both side. It is extended from Al-Shaklawiya near Baghdad north until Al-Basrah at the south with length about 565 km[9]. It is dividing into three sectors (North, Mid and South), the south sector (which the present study area is a part of its) extended from the end of the mid sector until Shatt Al-Basarah in the south, with length about 165 km. The discharge of water is 220m^3/sec in this sector[10]. New branch was opened in this sector with length 7 km, use to transform the water to the marshes south Al-Nassiriya city.

Three stations were selected in the south sector of this river to implemented, the present study, these are station 1(St.1) was near Al-Holandee bridge and the general carriage in the center of Al-Nassiriya city, St.2 was 20 km far from the first station, while St.3 was in the beginning of the new branch (Fig.1).

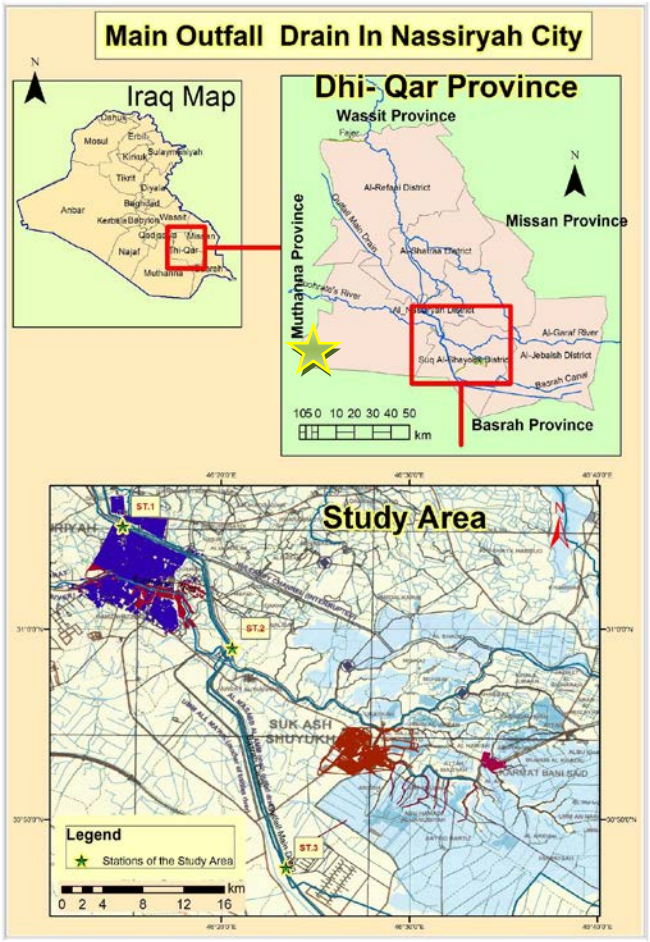

Figure 1: Map of the study area showed the study stations.

Materials and Methods :

Fish samples and sediments were collected from Main outfall drain river from locations as shown in Fig.(1) during summer/2014.

Fish samples were captured from the study area by using gill nets 25*25 mm mesh size. The captured fish were then placed in polyethylene bags and frozen immediately and transferred to laboratory. In the laboratory, the fish washed with deionized water, standard length and weight were measured to the nearest mm. and mg. respectively, then the abdominal cavity of each specimen was opened and the organs gills and liver were separated, whereas muscle was taken from the left posterior side of each fish, tissues were then dried under 105° for 24 hr. The determination of metals in fish sample was done according to the following procedure described by [3].

Sediments were obtained by van ven grab sampler from representation sites, the surface sediment about 5 cm was used for the present study. Trace metals analysis were preformed on the <63µm fractions of the sediment, which had been separated by sieving after oven drying and grinding. The determination of the trace metals in the exchangeable fraction of the sediment were done following the procedure described by [11] , whereas those in the residual fractions of the sediment was determined following their procedure described by [3].Trace metals were extracted in triplicate from sediments and fish samples. Cd, Co, Cu and Pb were determined in air/acetylene flam atomic absorption spectrophotometer AAS-model SP 9 pye-unicam. Sediment texture was analyzed and the percentage of three size fractions (sand, silt and clay) were calculated according to [12,13]. Total organic carbon (TOC %) in the sediment were determine according to [14].

Results and Discussion:

The mean water quality data for the studied stations are presented in Table (1). The air temperature during the study period for each location in Main outfall drain ranged between (34.5-39.31)°c, the lowest value was (34.5)°c at station 1 in May , whereas the highest value was (39.31)°c at station 3 in August. The water temperature for the study area ranged between (26.22-32.01)°c, the lowest at station 1 was (26.22)°c in May, while the highest in station 3 was (32.01)°c in August. Temperature is an important factor, which regulates the biogeochemical activities in the aquatic environment [15].

All metabolic and physiological activities and life processes are greatly influenced by water temperature [16].Water temperature is related with solar radiation and air temperature in the present study, water temperature follows changes in air temperature because of increase the surface area in comparison with volume. There are differences in the temperature among the stations over the day and that come from the different time of samples taking. These are agreed with the [17,18]. The water salinity values for all stations varied between (4.3-9.01)‰, the lowest value (4.3) ‰ was recorded in June at station 3, the highest value (9.01) ‰ was recorded in August at station 1. The higher values of salinity was observed in the study for the Main outfall drain because this river used as a drainage water supply and this due to the high level of dissolved salts. As, the highest salinity values were registered during the summer months , that was caused by decreasing of the water levels and increasing of the

evaporation ratio [19]. Furthermore, the dissolved ions are concentrated by evaporation and diluted by freshwater input , these results were in agreement with the previous studies of [20,21]. Water pH values during the study period for all stations in Main outfall drain ranged between (7.3-8.6). The pH was in alkaline level, it has being known that Iraq water mainly tend to be alkaline, this agree with results which obtained by [22,21]. The daily differences in pH values were because of removing carbon dioxide from bicarbonate due to photosynthesis activity during day hours [23] or in water, with high algal concentration. pH varied, reaching values as high during the day when algae are using carbon dioxide in photosynthesis and pH drops during the night when the algae respire and produce carbon dioxide [24].The maximum value of dissolved oxygen was (7.70)mg/l in May at St3., while the minimum value was(6.00) mg/l in August at St.1. The lower value of DO at St 1. may be due to higher the degradation of organic substances, the latter process reduce the DO content in water, [25]showed the amount of DO in water is decreasing with the increasing of organic substances, which use the energy of organic substances by chains of microorganisms which use DO as oxidize factor in degradation process. Maximum biological oxygen demand has been recorded at station 1 in August, while the minimum value has been at station 3 in May. The results of the present study showed that the BOD_5 in station 1 were higher than other stations and that caused by adding different amounts of domestic and sewage waste, agriculture runoff, urban runoff and near the station from the center city. Hot months were recorded high (BOD_5) value because of microorganisms activity [26], these results agreement with [27].

Concentrations of trace metals in sediment (exchangeable and residual phases) at different stations under study are presented in Table(2), Sediments acts as archive for many pollutants one of them is trace metals. Acknowledge of the concentration and distribution of trace metals in the sediment can therefore play a key role in defecting sources of pollution in aquatic ecosystem [28].

In the present study, the concentrations of all studied trace metals recorded higher concentrations at station 1 more than the other stations, this may be due to the high organic carbon content and the high percentage of fine grain size (silt and clay) of sediment texture. [29] has indicated that the concentration of trace metals in the sediment are affected by many factors such as grain size of the sediment texture, Total Organic Carbon percentage (TOC%)

content and carbonate content of the sediment as well as physical and chemical parameters. Lower concentrations of trace metals were recorded at station 3 because this station represent new branch and had less exposure to this type of pollutants. TOC content and amount of fine grain size(silt and clay) in the mentioned station was recorded lower value comparing with other stations (Fig. 2) respectively, while station 2 recorded relatively higher value of trace metals more than those in st.3 because of , in this station high traffic density of fishing boats. Trace metal pollution in sediment can affected the water quality and bioaccumulation of metals in aquatic organisms, resulting in potential long-term implication on human health and ecosystem [30]. The present study showed that the different tissues of *C.carpio* and *B. luteus* fish were varied from one to another in their accumulation of trace metals (Table 3). The results of the present study showed that, liver accumulate and concentrate highest concentrations of Cu in comparison with other metals in both species. [31] reported that the liver was the major site for Cu accumulation because liver is the responsible organ in controlling the toxicity of heavy metals. [32] found the same results in Tiliapia fish collected from Nassar lakes as well as obtained by [33, 34 and 35]. Co accumulated in liver of *C. carpio*, whereas it accumulated in the gills of *B. luteus*. Generally, the different distributions of trace metals in the body tissues may be due to the physiological nature of the tissues in the species, and this indicates that the different species have different patterns of accumulation. [36] have indicated that the accumulation and distribution of metals in the fish tissues depend on the duration of exposure, physiological condition of the metal and the environmental factors around the fish.

The high concentration of Co in these tissues in both species may be related to its concentrations in sediment. It can be pointed from the concentrations of Pb and Cd that it has been accumulated with lower levels in muscles of the fish in comparison with its concentrations in either the gills and liver. Lead and cadmium have been found in the kidney of *C.carpio* and in the liver of *B. luteus* except Cd could be found in gills of the same species as compared to other tissues. This indicates that the difference in the accumulation patterns of Pb and Cd in fish tissues depend on uptake and elimination rates of metals [37]. The results of the present study agree with those found by other researchers [38].

Low concentrations of trace metals were recorded in the muscles

of both species. The present results agree with those reported by [7]. The concentrations of most metals in most tissues of *C. carpio* were higher than in *B. luteus* which promote the mentioned phenomenon. [39] have indicated that differences in metal accumulation in fish bodies may be due to the differences in their diet during the growth of the species.

Table(1): Mean values of selected environmental factors in the study area during the study period.

Station	phases	Cd	Co	Cu	Pb
1	**Exch.**	6.03±0.04	14.56±0.51	7.61±0.50	32.32±2.80
	Resid.	0.08±0.02	21.7±1.40	12.77±1.05	2.46±0.48
2	**Exch.**	4.02±0.09	12.25±0.98	4.44±1.11	29.32±12.04
	Resid.	0.06±0.02	20.1±0.06	9.79±1.21	1.78±0.13
3	**Exch.**	2.26±0.42	10.4±0.80	2.25±1.24	26.00±11.76
	Resid.	0.04±0.02	16.1±0.06	5.8±2.33	1.31±0.30
Mean exch.		4.22±0.27	12.40±0.64	4.76±0.95	29.21±8.86
Mean resid.		0.14±0.02	19.33±0.51	12.71±1.53	1.85±0.30

Table(2): Mean concentration ±SD of trace metals in sediment(exchangeable and residual phases) µg/g dry weight in the study area during the study period.

Months	Stations	Air temp.°c	Water temp.°c	Salinity (‰)	pH	Do (mg/l)	BOD (mg/l)
May	1	34.5	26.22	6.01	8.4	7.60	2.3
	2	35.2	27.10	5.16	7.6	7.66	2.28
	3	36.4	27.80	5.06	7.4	7.70	2.26
June	1	34.9	26.66	7.56	8.3	6.7	3.1
	2	35.5	27.54	5.19	7.5	6.75	3.09
	3	37.2	28.20	4.30	7.3	6.80	3.07
July	1	37.12	30.00	7.65	8.45	6.2	3.15
	2	38.22	31.12	5.35	7.79	6.3	3.12
	3	39.0	31.03	4.90	7.6	6.4	3.10
August	1	37.86	30.24	9.01	8.6	6.00	3.25
	2	38.13	31.96	8.26	7.87	6.1	3.14
	3	39.31	32.01	8.00	7.75	6.1	3.13

Exch.= Exchangeable phase Resid.= Residual phase

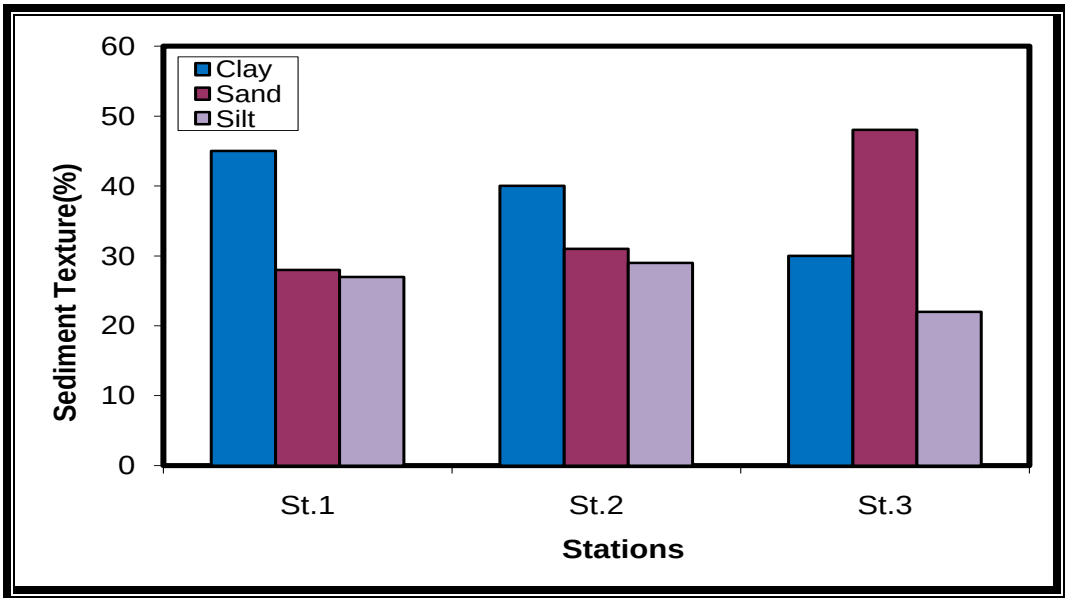

Figure (2): Percentage of clay, silt and sand in sediments for all locations of the study.

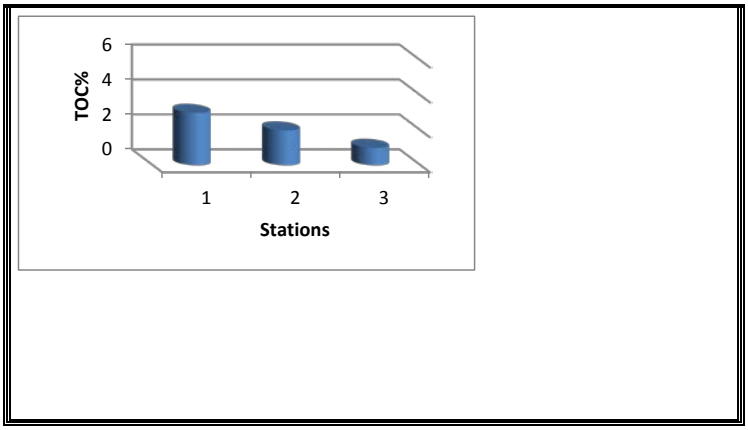

Figure (3): Mean values of total organic carbon (TOC %) variations for all locations of the study.

Table(3): Concentration of trace metals (Mean±SD) µg/g dry weight in different organs from fish

A-*Cyprinus carpio.*

Organs / Metals	N.	Gill	Liver	Muscle
Cd	30	0.11±0.005	0.08±0.01	0.05±0.005
Co	30	10.13±0.56	23.76±1.45	10.45±0.98
Cu	30	13.45±1.57	20.77±0.31	14.14±0.64
Pb	30	14.62±0.0.18	13.46±0	6.42±0.69

B- *B. luteus*

Organs / Metals	N.	Gill	Liver	Muscle
Cd	30	$0.11\pm2.314E^{-18}$	0.04±0.01	0.02±0.005
Co	30	22.18±0.19	2.26±0.04	1.80±0.005
Cu	30	3.94±0.53	92.82±5.83	12.84±0.18
Pb	30	2.08±0.02	8.16±1.23	2.39±0.23

N: Number of samples

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Study the Association of Interleukins 6 and 7 Levels with Insulin Resistance in Iraqi Females with Diabetes Mellitus Type 2

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Abstract: The present study was planned to determine the serum levels of interleukins (IL)-6, IL-7 in type 2 diabetes mellitus (T2DM), as well as, insulin were quantitatively determined in patients and control subjects by means of sandwich ELISA test using commercially available kits. Also (body mass index; BMI) and homeostatic model assessment (HOMA-IR and HOMA-β٪) were calculated. Fifty five Iraqi T2DM female patients and 30 control subjects matched for age and ethnic background were also included. The mean serum level of IL-6 and 7 (6.31 *vs*. 2.77 and 25.18 *vs*. 8.98 pg/ml) respectively, while the mean serum level of insulin (18.75 *vs*. 6.28 µIU/ml) and HOMA-IR (3 *vs*. 0.8) was significantly higher in T2DM patients as compared to controls. HOMA-β٪ showed a significant decreased mean in T2DM patients as compared to controls (48.9 *vs*. 72.3٪). The receiver operator curve (ROC) analysis of the forthcoming variations revealed the descending order of FPG and HbA1C (0.999), HOMA-IR (0.985), serum IL-7 (0.975), FSI (0.972), serum IL-6 (0.922) & (HOMA-β٪) (0.772) that showed significant variation. In conclusion, high level of IL-6, IL7 were associated with an increased risk of T2DM especially in obese diabetic females.

Key wards: Interleukin-6, Interleukin-7, Insulin Resistance, Diabetes Mellitus Type 2.

دراسة علاقة مستويات الانترلوكينات 6 و7 مع مقاومة الأنسولين عند النساء العراقيات المصابات بداء السكري النوع الثاني

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الملخص: أجريت الدراسة الحالية بهدف تقييم المستوى المصلي للانترلوكينات-6 و7 فضلا عن هرمون الأنسولين لدى مرضى داء السكري النوع الثاني، التقدير الكمي تم باستخدام تقنية الالايزا من خلال عدد القياس التجارية المتوفرة. كما تم حساب معامل كتلة الجسم (BMI)، مقاومة الأنسولين (HOMA2). أجريت الدراسة على 55 امرأة عراقية مصابة بداء السكري النوع الثاني ولغرض المقارنة اعتمدت 30 امرأة من الأصحاء. اظهر المستوى المصلي للانترلوكينات- 6 و7 زيادة معنوية (6.31 مقابل2.77 و25.18 مقابل 8.98 بيكوغرام/مل) ، كما اظهر المستوى المصلي لهرمون الأنسولين زيـــــادة معنوية وهـــــي (18.75 مقابل 6.28 مايكرو وحدة عالمية/مل) و مقاومــة الأنســـولين (HOMA-IR) (3 مقابل 0.8) ، في حين اظهرت النسبة المئوية لوظيفة خلايا بيتا في البنكرياس (HOMA-β%) انخفاض معنوي وهو (48.9 مقابل 72.32٪) في المرضى مقارنة بالاصحاء.
للتمييز بين مرضى داء السكري و مجموعة السيطرة استخدم تحليل Receiver Operator Curve (ROC) والذي أظهر الترتيب التنازلي بحسب الأهمية للمؤشرات التي أظهرت فروقا معنوية وكان الترتيب سكر بلازما الدم الصائم و HbA1c (0.999) و - HOMA IR (0.985) والمستوى المصلي للانترلوكين-7 (0.975) والمستوى المصلي لهرمون الأنسولين (0.972) والمستوى المصلي للانترلوكين-6 (0.922) هذا يعني ان جميع المتغيرات تمكنت من إشغال منطقة معنوية من ROC.
نستنتج من ذلك ان المستويات العالية للانترلوكينات- 6 و7 ترتبط مع زيادة خطورة داء السكري من النوع الثاني خصوصا في النساء السمينات.

الكلمات المفتاحية: انترلوكين- 6 ، انترلوكين-7، مقاومة الانسولين ، مرض السكري النوع الثاني

Introduction

Type 2 diabetes mellitus is characterized by three pathophysiological abnormalities; impaired insulin secretion, peripheral insulin resistance, and excessive hepatic glucose production [1].

The term "insulin resistance" indicates the presence of an impaired biological response to either exogenously administered or endogenously secreted insulin, this is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscles and by impaired suppression of hepatic glucose output [2].

Hyperglycemia is also consistently associated with increased oxidative stress. Oxidative stress is a component of cellular damage and has an important role in the pathogenesis of a number of human diseases including atherosclerosis. Mechanisms that contribute to increased oxidative stress in diabetes may include not

only increased non-enzymatic glycosylation and auto-oxidative glycosylation but also to decreasing antioxidant defence potential [3].

One of the markers of inflammation is interleukin 6 (IL-6) [4]. It is a pleiotropic cytokine, produced mostly by adipocytes, fibroblast, endothelial cells, and activated leukocytes and monocytes [5]. IL-6 mainly is known as a chief regulator of acute-phase inflammatory response [6]. However, a critical role of IL-6 in the transformation from acute to chronic inflammation was established as well [7].

Many proinflammatory cytokines play a central role in inflammatory reaction and were shown to increase the risk of T2DM [8]. These pro-Inflammatory cytokines can enhance insulin resistance directly in adipocytes, muscle and hepatic cells, leading to systemic disruption of insulin sensitivity and impaired glucose homeostasis. Increased levels of these pro-inflammatory cytokines lead to hepatic production and secretion of acute-phase proteins [9].

High serum levels of IL-6 also seem to be associated with insulin resistance and type 2 diabetes [10].

IL-7 has recently been identified as a new adipokine whose expression and secretion are increased in the obese human adipose tissue [11]. Whether this endogenous production of IL-7 has any physiological function in adipose tissue and metabolism is still unknown. IL-7 is a constitutively secreted cytokine primarily produced in bone marrow and peripheral lymphoid organs [12]. IL-7 is critically required for lymphocyte development and homeostasis [13]. The key role of IL-7 in lymphocyte homeostasis was shown to rely on its control of basal lymphocyte glucose metabolism [14], maintaining high glucose uptake and expression of GLUT1 therefore allowing adequate glycolytic flux [15]. Beside its primary function in the regulation of the activation, growth and survival of lymphoid cells, it has been suggested that IL-7 can also act on non-lymphoid cells. Indeed, IL-7 has been shown to induce the production of proinflammatory IL-1, IL-6, IL-8 and TNFα by monocytes [16]. IL-7 is produced primarily by epithelial cells and is found predominantly in the thymus, bone marrow, and intestine [17].

T2DM has been seen that insulin resistance is often an ancillary metabolic derangement in type 2 diabetes characterized by postprandial hyperglycemia and compensatory hyperinsullinemia.

Overt diabetes results when beta cells of pancreas no longer can afford excess insulin secretion in compensation of insulin resistance [18]. But exact underlying mechanism that may lead to insulin

resistance is still unclear. Obesity is the leading cause by some school of thoughts [18, 19].

Insulin resistance (IR) is a key feature of type 2 diabetes and obesity [20]; as yet the molecular mechanism responsible for IR is not fully understood. Recently, the chronic inflammation has been proposed to be involved in the pathogenesis of IR [21, 22]. Compared to the healthy subjects, plasma levels of the proinflammatory markers are increased (e.g., TNF-α, IL-6, and resistin), while the anti-inflammatory markers are decreased (e.g., adiponectin, leptin) in the obese subjects with type 2 diabetes [21, 22].

In this study, we investigated the relationship between IR with IL-6 and 7 in women with type 2 DM. We investigated also the association between IL-6 and 7 with the age, BMI, W-H R, HbA1c and F.P.G.

Material and Methods

The present study was carried out in the National Diabetes Center for Treatment and Research at Al-Mustansiriya University between July 2012- March 2013. A total of 55 patients of type2 diabetes mellitus (females of age group37-66 years.), the diagnosis of T2DM was made on the basis of the recommended criteria by WHO [23]. Thirty age and sex matched (females) healthy individuals served as controls who attended for routine health check up at the center. None of the healthy control was taking any medicine or dietary supplement; they were selected after detailed physical examination and laboratory tests.

Samples collection: After 12 hrs fasting 5 ml. venous blood collected in plain tubes, the samples were allowed to clot for half an hour following which a samples were centrifuged for 15 minutes at 4000 rpm. Then serum was stored immediately at -20C until use.

Serum glucose was determined by a glucose oxidase method (Randox Company, U.K.) [24].

Serum concentrations of IL-6, IL-7 were determined by ELISA using a commercial kit manufactured by Ray biotech. Company, USA and insulin determined by ELISA using commercial kits (DRG diagnostics Company, Germany and USA). Micro ELISA system (washer & reader) (Thermo, Germany) and Incubator (Gallenkamp, U.K.) were used in ELISA determination. Glycated hemoglobin was measured by using the Variant Hemoglobin A1C program developed by BIO-Rad.

Weight was measured using standardized beam weight scales without footwear and with only light clothes. standing with the feet together. BMI is defined as weight in kilogram per height in meters

squared and is independent of gender and age. HOMA-IR was Height was measured with the subjects barefoot and determined using the equation of Wallace *et al* **HOMA-IR = (FPI × FPG)/405** and **HOMA- β% = (360 × Insulin/ Glucose – 63)%**, Where FPI is fasting plasma insulin concentration (μIU/ml) and FPG is fasting plasma glucose (mg/dl). **Statistical Analysis**

All Data are presented as mean ± standard deviation (S.D.), and differences between means were assessed by the Student t test. Cohen's d was used to compare the effect size for variables. Differences were considered significant at $P < 0.05$ [25]. All statistical analysis was performed using SPSS statistical software (version 19). Receiver operator curve (ROC) analysis was also employed**.** The statistical significance, direction and strength of linear correlation between two quantitative variables, one of which being a non-normally distributed variable, was measured by Spearman's.rank linear correlation coefficient, and a probability (P) value less than the 0.05 was considered statistically significant**.**

Results

Table no.1shows the average ages of the control and diabetic subjects were (49.00±8.90) and (51.00±6.90) years, respectively, ($P =0.260_{[NS]}$). The mean BMI values of both groups were in the range of obesity and the mean value in diabetic group was higher (32.7 ± 5.30) than in healthy group (30.8 ± 4.50kg/$m^{2)}$ although the difference was statistically non-significant (P=0.110). Also there was a significant (P<0.001) elevated of FPG level in type 2 diabetic patients as compared to control group.

Serum IL-6 and 7 were significantly (P<0.001) higher in type 2 diabetes mellitus females when compared to control. The level of serum insulin was significantly (P<0.001) higher in type 2 diabetic patients compared to control. The normal levels of IL-6and 7 were (2.77 ± 1.19pg/ml), (8.98 ± 2.4pg/ml) elevated in the diabetic patient who recorded (6.31 ± 2.67pg/ml) and (25.15 ± 11.45 pg/ml) respectively.

As shown in table 1, the mean insulin resistance estimated using HOMA-IR formula was significantly higher among cases with T2DM (3 ± 1.3) compared to healthy controls (0.8 ± 0.4). The positive effect of T2DM on increasing insulin resistance evaluated as a very strong effect (Cohen's d = 2.1). The other index of insulin sensitivity estimated by HOMA-β٪ formula was the percentage of beta cell function. The mean for this estimate was significantly lower in cases with T2DM (48.9 ± 22.1 %) compared to healthy controls (72.3 ± 25.2%).

The negative effect of T2DM on reducing beta cell activity also evaluated as a strong effect (Cohen's d =1), it was however less affected in comparison to insulin resistance.

As shown in table 2 and figures 1-2, the tested parameters can be ranked according to its magnitude affection by T2DM as a chronic disease process according to ROC area. This ranking can also be used to compare the validity of these parameters in the context of case-control.

As shown in table 3 an abnormally high fasting plasma glucose positive for T2DM would be defined as ≥112.5 mg/dl. At this cut-off value the test would be 98% sensitive, 100% specific and 98.8% accurate. An abnormally high fasting serum insulin.

Table 1: Comparison of various parameters between controls and T2DM cases

Parameters			Mean ± S.D	Cohen's d	P-value
AGE	Years	cases	51.00±6.90	0.244	0.260
		controls	49.00±8.90		
BMI	Kg/m^2	cases	32.7 ± 5.30	0.653	0.110
		controls	30.8 ± 4.50		
W-H R		cases	0.91 ± 0.05	0.653	0.990
		controls	0.91± 0.04		
F.P.G	mg/dl	cases	196.3 ± 56.1	2.244	<0.001
		controls	96.3 ± 8.6		
IL-6	pg/ml	cases	6.31 ± 2.67	1.59	<0.001
		controls	2.77 ± 1.19		
IL-7	pg/ml	cases	25.15 ± 11.45	1.77	<0.001
		controls	8.98 ± 2.4		
Insulin	pg/ml	cases	18.03±5.53	2.623	<0.001
		controls	6.09±1.63		
HOMA-IR		cases	3 ± 1.3	2.1	<0.001
		controls	0.8 ± 0.4		
HOMA-β٪		cases	48.9 ± 22.1	1.0	<0.001
		controls	72.3 ± 25.2		

*__BMI__ (body mass index) *__W-H R__ (waist to hip ratio) *__F.P.G__ (fasting plasma glucose) *__IL__ (interleukin) * __HOMA-IR__ (insulin resistance) * __HOMA-β٪__ (the percentage of beta cell function)

Table 2: ROC area for selected parameters when used as test to predict a diagnosis of T2DM differentiating it from healthy controls

	ROC area	**P**
Fasting plasma glucose (mg/dl)	0.999	<0.001
HbA1c %	0.999	<0.001
Insulin resistance (HOMA2)	0.985	<0.001
Serum IL7 (pg/ml)	0.975	<0.001
Fasting serum insulin (µiu/ml)	0.972	<0.001
Serum IL6 (pg/ml)	0.922	<0.001
Percent beta cell function (HOMA-β٪)	0.772	<0.001

positive for T2DM would be defined as ≥ 11.85 µiu/ml. At this cut-off value the test would be 92.2% sensitive, 96.8% specific and 93.9% accurate. An abnormally high HbA1c% positive for T2DM would be defined as ≥5.9%. At this cut-off value the test would be 98% sensitive, 100% specific and 98.8% accurate.

The optimum cut-off value for cytokines was a serum level of IL-6 ≥2.95 pg/ml associated with 100% sensitivity, 66.7% specificity and 87.3% accuracy. A serum IL-6 concentration below this for T2DM would be defined as ≥ 11.85
cut-off value is not compatible with a diagnosis of T2DM. On the other extreme a serum IL-6 level ≥ 6.02 pg/ml would be highly specific (100%) and
none of the healthy controls would have a serum level as high as this level.

The abnormally high serum IL-7 was defined as a serum concentration ≥13.35 pg/ml, which was associated with 93.9% sensitivity, 96.7% specificity and 94.9% accuracy. The cut-off value associated with highest sensitivity (100%) was ≥7.6. A serum IL-7 concentration below this cut-off value is not compatible with the diagnosis of T2DM. On the other extreme a serum IL-7 level ≥15.19 pg/ml would be highly specific (100%). None of the healthy controls would have a serum level as high as this level.

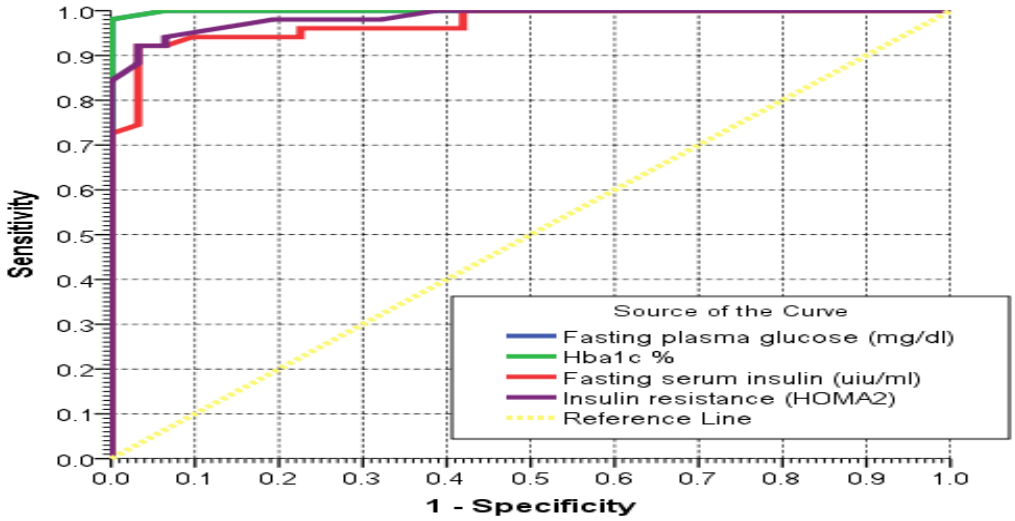

Figure 1: The ROC curve showing the trade-off between sensitivity (rate of true positive) and 1-specificity (rate of false positive) for FPG, FSI, HOMA-IR and HbA1c when used as test to predict a diagnosis of T2DM differentiating it from healthy controls

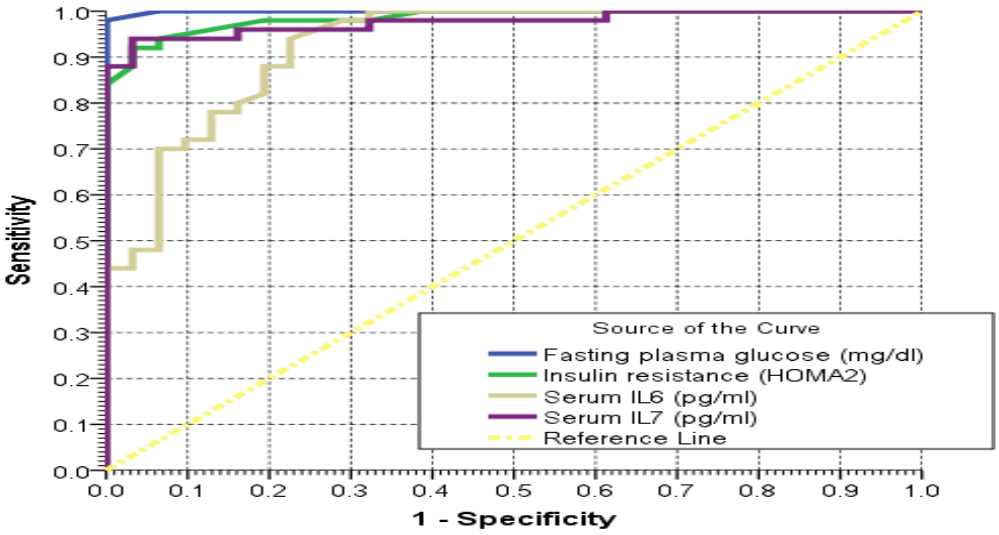

Figure 2: The ROC curve showing the trade-off between sensitivity (rate of true positive) and 1-specificity (rate of false positive) for FPG, HOMA-IR, IL-6 and IL-7 when used as test to predict a diagnosis of T2DM differentiating it from healthy controls

Table 3: Validity parameters for selected indices when used as test to predict a diagnosis of T2DM differentiating it from healthy controls.

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy
Fasting plasma glucose (mg/dl)			
109.5 (Highest sensitivity)	100.0	93.5	97.6
112.5 (Highest specificity) (Optimum cut-off)	98.0	100.0	98.8
HbA1c %			
5.8 (Highest sensitivity)	100.0	93.5	97.6
5.9 (Highest specificity) (Optimum cut-off)	98.0	100.0	98.8
Serum IL6 (pg/ml)			
2.95 (Highest sensitivity) (Optimum cut-off)	100.0	66.7	87.3
6.02 (Highest specificity)	42.9	100.0	64.6
Serum IL7 (pg/ml)			
7.60 (Highest sensitivity)	100.0	36.7	75.9
13.35 (Optimum cut-off)	93.9	96.7	94.9
15.19 (Highest specificity)	87.8	100.0	92.4
Fasting serum insulin (uiu/ml)			
6.14 (Highest sensitivity)	100.0	58.1	84.1
11.85 (Optimum cut-off)	92.2	96.8	93.9
14.87 (Highest specificity)	72.5	100.0	82.9
Insulin resistance (HOMA-IR)			
0.95 (Highest sensitivity)	100.0	61.3	85.4
1.45 (Optimum cut-off)	92.2	93.5	92.7
1.95 (Highest specificity)	84.3	100.0	90.2
Percent beta cell function (HOMA β٪)			
36.6 (Highest specificity)	33.3	96.7	57.3
61.0 (Optimum cut-off)	80.4	63.3	73.9
102.3 (Highest sensitivity)	100.0	16.7	68.5

Among glucose metabolism related parameters, it was found that the abnormally high insulin resistance (IR) measured by HOMA-IR was defined as ≥1.45, which was associated with 92.2% sensitivity, 93.5% specificity and 92.7% accuracy. The cut-off value associated with highest sensitivity (100%) was ≥ 0.95. A calculated insulin resistance below this cut-off value is not compatible with a

diagnosis of T2DM and can therefore exclude a diagnosis of T2DM with 100% confidence. On the other extreme a calculated insulin resistance ≥ 1.95 would be highly specific (100%). None of the healthy controls would have such a high IR. A subject positive at this highly specific cut-off value would be diabetic with 100% confidence.

As shown in table 4, although subjects with highest insulin resistance had a slightly higher mean age, the differences observed were not statistically significant.

The mean BMI was lowest among subjects with lowest insulin resistance As shown in table 4the positive trend between BMI and insulin resistance was of a weak magnitude and failed to reach the level of statistical significance. A similar, but weaker and statistically insignificant positive trend was observed for waist hip ratio with insulin resistance.

The mean F.P.G was highest among diabetics with highest insulin resistance compared to the other two insulin resistance categories. It showed a statistically significant strong positive (direct) linear correlation with insulin resistance (figure 3).

The serum HbA1c showed a statistically significant moderately strong positive (direct) linear correlation with insulin resistance (figure 4).

The serum IL-6 showed a statistically significant weak positive (direct) linear correlation with insulin resistance (figure 5).Serum IL-7 on the other hand, healthy controls would have a serum level as high as this level with 93.9% sensitivity, 96.7% specificity.

Table 4. Pearson correlation coefficients of IL-6, IL-7 with HOMA-IR and other parameters

Parameters	IL-7 pg/m	IL-6 pg/m	HOMA-IR
Age years	r=0.41; P=0.003	r=0.601; P<0.001	r=0.05; P=0.73 [NS]
BMI kg/m^2	r=-0.008 ; P=0.96 [NS]	r=0.034; P=0.82 [NS]	r=0.196; P=0.17 [NS]
W-H R	r=0.155 ; P=0.28 [NS]	r=0.144; P=0.32 [NS]	r=0.079; P=0.58 [NS]
HbA1c	r=0.118; P=0.41 [NS]	r=0.264; P=0.06 [NS]	r=0.618; P<0.001
F.P.G mg/ml	r=0.089; P=0.54 [NS]	r=0.118; P=0.42 [NS]	r=0.555; P<0.001
IL-6 pg/ml	r=0.37; P=0.008	1	r=0.304; P=0.032
IL-7 pg/ml	1	xxxxxx	r=0.122; P=0.39 [NS]

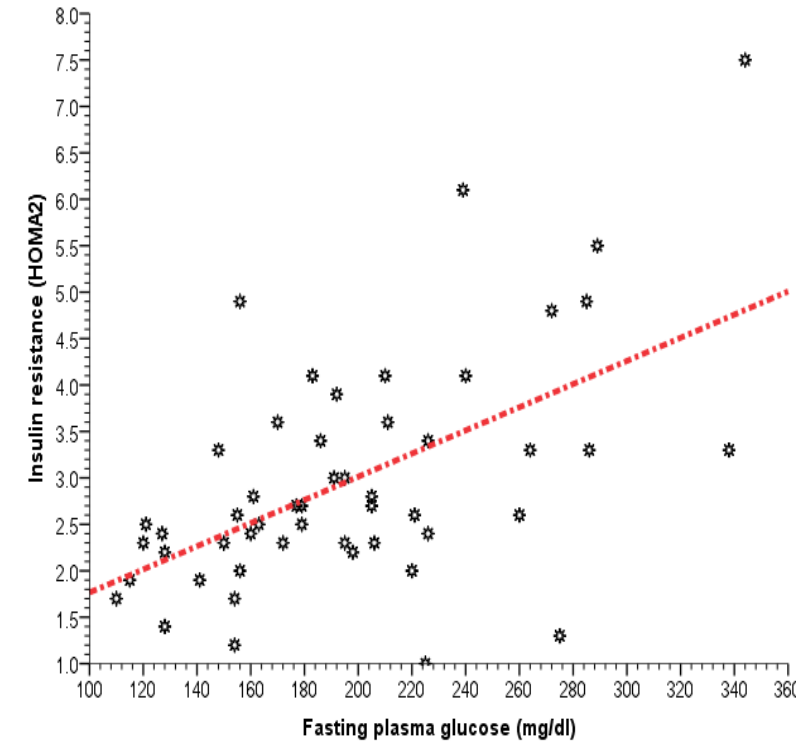

Figure 3: The association between insulin resistance and F.P.G (mg/dl)

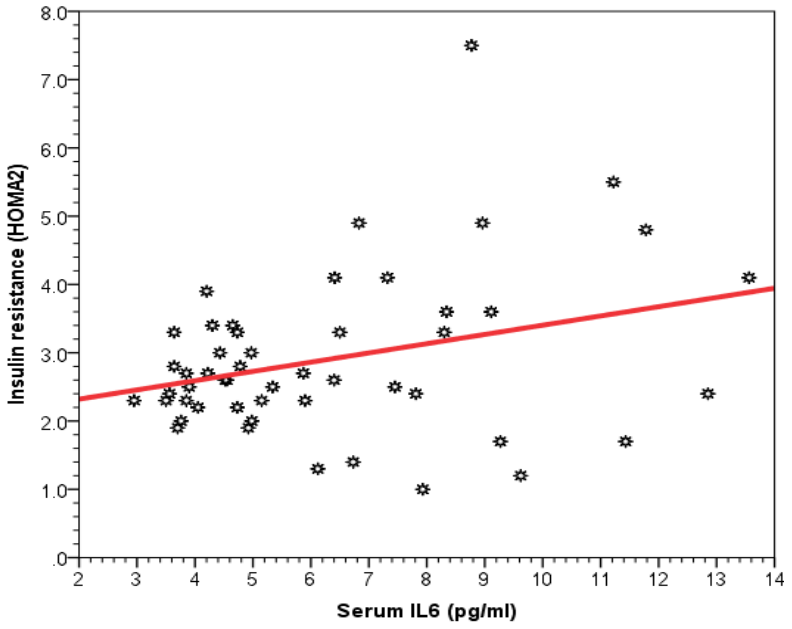

Figure 5: The association between insulin resistance and serum IL-6 (pg/m)

showed no important or statistically significant linear trend with insulin resistance.

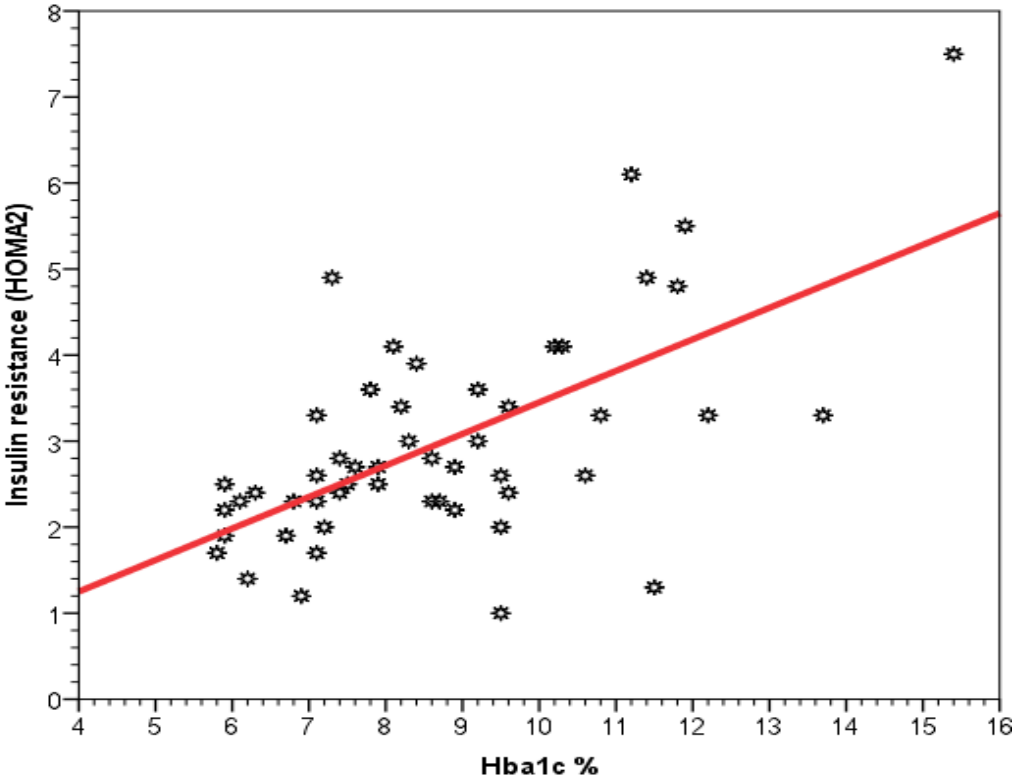

Figure 4: The association between insulin resistance and HbA1c %

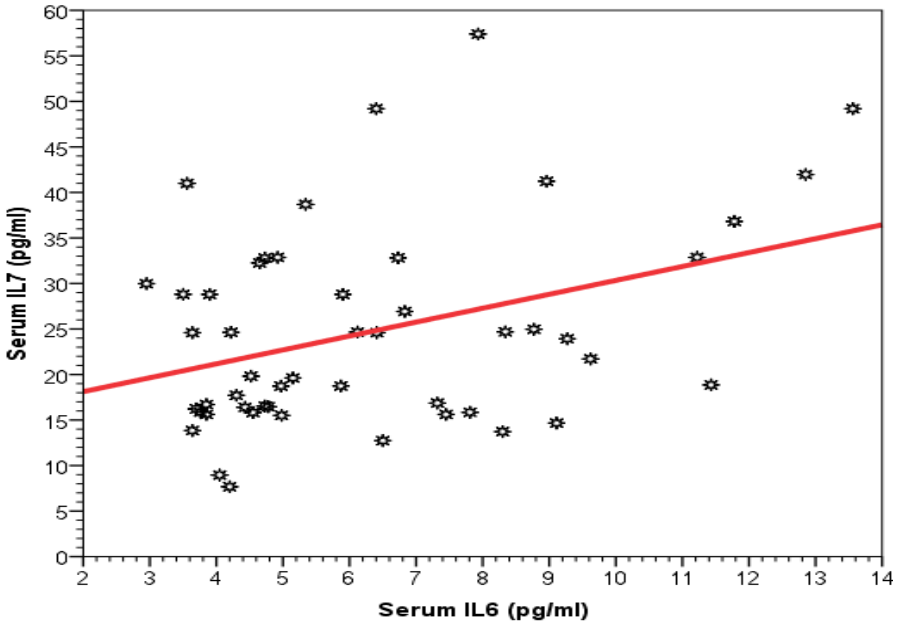

Figure 6: The association between serum IL-7 (pg/ml) and serum IL-6

The mean serum IL-6 was significantly highest among diabetics with highest serum IL-7. The positive linear trend between the two inflammatory markers was moderately strong and statistically significant (table 4, Figure 6).

The mean HbA1c% was obviously higher among diabetics belonging to the highest quartile of serum IL-6 compared to the mean observed in the other IL6 categories. The differences observed in mean however failed to reach the level of statistical significance. The positive linear trend of serum IL-6 observed with HbA1c was important, but failed short of statistical significance.

Discussion

In this study diabetic patients showed significantly higher levels of IL-6 than did the controls**.** Excessive weight gain leads to increased macrophage infiltration of white adipose tissue, and increased central adiposity is associated with a chronic inflammatory state, induced by proinflammatory mediators, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and C-reactive protein (CRP) [26].

Inflammatory mediators may also be increased in T2DM and insulin resistance, and inhibit insulin signaling through activation of Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB [27,28].

Adipose tissue secretes a number of bioactive molecules, such as TNF-α, and IL-6 collectively called adipokines, which affect peripheral insulin sensitivity and may be important players in the development of T2DM and atherosclerosis, on the other hand, these molecules expressed by the human macrophages can modulate chronic inflammation and have an impact in cardio metabolic complications such as atherosclerosis [29]. Increased production of these adipokines occurs with expanding obesity, particularly visceral obesity, by both the adipocytes and the nonfat cells, mostly macrophages that infiltrate the adipose tissue [30].

Indeed, pro inflammatory cytokines such as IL-6 have been demonstrated to inhibit glucose transport in adipocytes [31]. The inhibition of insulin metabolism is likely due to competing mechanisms in the post receptor signaling pathways of the insulin receptor [32].

The inflammatory regulator IL-6 has also emerged as a factor that is implicated in hepatic insulin resistance. However, the role of IL-6 in the etiology of insulin resistance is not fully understood. Impaired insulin receptor signaling and insulin-dependent glycogen synthesis were found in HepG2 cells and primary mouse hepatocytes acutely pretreated with IL-6 [33].

IL-6 caused reduced insulin signal transduction in the liver of mice [34]. These results seemed to be consistent with the hypothesis that IL-6 may have a negative effect on insulin resistance. But in healthy humans under the optimal condition, treatment by recombinant human IL-6 (rhIL-6) at a physiological concentration neither impaired the whole body glucose disposal, nor increased endogenous glucose production [35].

In this study we found that diabetic patients showed significantly higher levels of IL-7 than did the controls**.** In human, several lines of evidence suggest a significant role for IL-7 in inflammatory diseases, including T2DM. Firstly, under normal conditions,

local IL-7 production acts on resident T. cells through IL-7 receptor (IL-7R; a heterodimer composed of IL-7Rα and IL-2Rγ chains) to promote their differentiation, survival and homeostasis [36]. IL-7 is produced by stromal cells and intestinal epithelial cells, suggesting a role for IL-7 in modulating immune responses in the intestinal microenvironment. Secondly, elevated serum concentrations of IL-7 have been detected in patients with T2DM, although IL-7 is mostly known as a key regulator of lymphocyte homeostasis, we recently demonstrated that it also contributes to body weight regulation through a hypothalamic control. Previous studies have shown that IL-7 is produced by the human obese white adipose tissue (WAT) yet its potential role on WAT development and function in obesity remains unknown [37].

Indeed, constitutive IL-7 over-expression leads to glucose intolerance and insulin resistance, traits that are commonly associated with lipo dystrophy in both animals and humans [38].

Insulin resistance (IR) is a state in which a given concentration of insulin produces a less-than-expected biological effect. It is closely related to glucose metabolism, lipid metabolism, hypertension, and obesity.

Insulin resistance not only accelerates the development of liver fibrosis and liver cancer but also interferes with anti-viral treatment. IR significantly improves following antiviral therapy in CHC patients, indicating that early diagnosis and treatment of IR not only reduces viral replication, but also prevents disease progression [39].

Hyperinsulinemia is considered as indicative of insulin resistance. Biological actions of insulin are essential for regulation and maintenance of glucose homeostasis. Insulin resistance is defined as decreased sensitivity or responsiveness to the metabolic actions of insulin. It plays an

important role in the pathogenesis of diabetes, and it has shown to be a feature predictor of many health related adverse outcomes including coronary artery disease and stroke. Moreover, IR is a feature of a number of syndromes related to abnormal reproductive endocrinology, such as polycystic ovarian syndrome (PCOS) and premature adrenarche. Therefore, it is of great interest to quantify insulin sensitivity and resistance in humans to investigate the pathogenesis and epidemiology of major public health problems and to follow the clinical course of patients on various therapeutic regimens [40].

Pancreatic islet β-cells perform a critical role in the regulation of whole animal fuel homeostasis by secreting insulin in response to nutritional and hormonal signals. Deterioration of β-cell mass and function are key events in the development of T2DM [41].

In T2DM, the reduction in beta-cell function is associated with the loss of glucose-stimulated insulin secretion (GSIS) and the reduction of beta-cell mass [42]. Insulin secretion is a complex mechanism with multiple points of regulation [43]. Briefly, glucose is transported through cell membrane by the effect of insulin secreted by beta-cells and high-capacity of glucose transporter (GLUT) and metabolized by glucokinase that generates glucose-6-phosphate.

All the tested parameters had some validity in predicting T2DM, since the ROC area was significantly higher than the ROC area of an equivocal test of 0.5. It is not a discovery to find Fasting plasma glucose, HbA1c, Insulin resistance (HOMA-IR) and Fasting serum insulin among the parameters associated with highest validity, providing an almost perfect test (ROC area > 0.97) for prediction of T2DM.

Observation of a positive significant correlation between serum HbA1c and insulin resistance Observation of studied patients. Elevated HbA1c level was related to decreased insulin secretion, β-cell function loss, poor glycemic control and higher BMI.

The results of this study agreed with other studies [44, 45] that demonstrated positive (direct) linear correlation of IL-6 with insulin resistance in T2DM subjects.

Inflammatory processes, particularly increased pro inflammatory cytokine production and activation of immune system, are associated with obesity, and have been implicated in the pathogenesis of atherosclerosis, metabolic syndrome, insulin resistance, and diabetes mellitus [46]

The adipose tissue plays a com-mon role in the relationship between

cytokines and insulin resistance. Inflammatory cytokines might induce insulin resistance by direct actions on insulin- signaling post receptor molecules or by inducing central obesity through activation of the hypothalamic- pituitary-adrenal axis [47].

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Evaluating Interferon-γ (IFN-γ) as Biomarker for Juvenile Idiopathic Arthritis and Adult Onset Rheumatoid Arthritis in Samples of Iraqi Patients

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Abstract: Etiopathogenic mechanisms that lead to arthritis diseases in particular juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) are complex, but cytokines have been suggested to mediate important effects in disease severity and activity, type of administrated therapy and in clinical subtypes of JIA, and one of these cytokines is interferon-γ (IFN-γ), which was assessed in sera of 49 JIA and 43 RA Iraqi patients, as well as 20 JIA controls (CI), and 17 RA controls (CII). Levels of IFN-γ showed a significant increase ($P \leq 0.05$) in both groups of patients compared to their controls (52.1 ± 2.1 *vs*. 28.1 ± 1.8 and 66.0 ± 2.7 *vs*. 35.2 ± 2.8 pg/ml, respectively). These variations were also subjected the effect of disease severity and activity, type of administrated therapy and clinical subtypes of JIA. It was concluded that IFN-γ plays an important role in the pathogenesis of JIA and RA, and may represent a good biomarker for each of the two diseases.

Keywords: Interferon-γ, Juvenile Idiopathic Arthritis, Rheumatoid Arthritis

تقييم الانترفيرون ـ كاما كمؤشر حيوي لالتهاب المفاصل مجهول السبب في اليافعين والتهاب المفاصل الرثوي للبالغين في عينات من المرضى العراقيين

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الخلاصة: تعد آليات النشوء والامراضية لمرض التهاب المفاصل معقدة وبالخصوص التهاب المفاصل مجهول السبب في اليافعين والتهاب المفاصل الرثوي للبالغين، ولكن يعتقد بان للحركيات الخلوية دورا هاما في احداث المرض وتحديد شدته ونشاطه ، فضلا عن تأثيرها على نوع العلاج المستخدم في كلا النوعين.كما انها تؤدي دورا مهما في تحديد الانواع الفرعية لمرض التهاب المفاصل مجهول السبب في اليافعين. تم تقييم المستوى المصلي للانترفيرون-كاما في 49 عينة لمرضى عراقيين يافعين مصابين بالتهاب المفاصل مجهول السبب ،43 عينة لمرضى عراقيين بالغين مصابين بالتهاب المفاصل الرثوي و 20 عينة سيطرة لمرض التهاب المفاصل مجهول السبب في اليافعين (سيطرة 1) و17 عينة سيطرة لمرض التهاب المفاصل الرثوي للبالغين (سيطرة 2). أظهرت النتائج مستويات الانترفيرون–كاما زيادة معنوية ($P > 0.05$) في مصل مرضى التهاب المفاصل مجهول السبب في اليافعين والتهاب المفاصل الرثوي للبالغين مقارنة مع مجاميع السيطرة (52.1 ± 2.1 *vs.* 28.1 ± 1.8 and 66.0 ± 2.7 *vs.* 35.2 ± 2.8 pg/ml) على التوالي. وقد خضعت هذه التغايرات لتأثيرت شدة المرض ونشاطه ونوع العلاج والانواع الفرعية لمرض التهاب المفاصل مجهول السبب لليافعين .ونستنتج ان الانترفيرون–كاما يؤدي دورا هاما في امراضية كل من التهاب المفاصل مجهول السبب لليافعين والتهاب المفاصل الرثوي للبالغين ويمثل مؤشرا حيويا جيدا لكلا المرضين.

Introduction

Arthritis is the most common cause of joint pain and physical disability worldwide; including Iraq (1,2), but juvenile idiopathic arthritis (JIA) and rheumatoid arthritis (RA) are the two major groups of arthritis characterized by chronic joint inflammation (3). The criteria of International League of Associations for Rheumatology (ILAR) defined JIA as arthritis in one or more joints that begins before the age of 16 years, persists for at least six weeks, and excludes all other known conditions that cause similar symptoms (4-7). Based on such criteria, the JIA patients are distributed into six main subgroups; oligoarthritis (OLI), polyarthritis (POL), systemic arthritis (SA), enthesitis-related arthritis (ERA), psoriasis arthritis and undifferentiated arthritis. Oligoarthritis patients are further classified into persistent (POLI) and extended (EOLI), while polyarthritis is classified into RF positive (RF+ve POL) and RF negative (RF-ve POL) (8,9).

Rheumatoid arthritis is a further type of arthritis, which is characterized by an inflammation of the synovium and a destruction of cartilage and bone (10). However, details of RA pathogenesis are also

not well-characterized, although there is strong evidence that immune components; especially T and B lymphocytes or their products (i.e. cytokines and antibodies) are involved in disease progression and pathogenesis (11,12). Accordingly, RA is suggested to be a cytokine-mediated disease, and studies demonstrated that assessment of IL-2, IL-6, IFN-γ and TNF-α serum levels may confirm a disease activity (13).

Cytokines play a prominent role in the etiopathogenic mechanism of JIA and RA, and one of them is interferon-γ (IFN-γ) or type II interferon, which is a critical cytokine for innate and acquired immunity against different infections (viral, some bacterial and protozoal infection) (14). It is an important activator of phagocytes and inducer of MHC expressions and its aberrant expression has been associated with a number of autoimmune diseases (15). The importance of IFN-γ in the immune system originates in part from its ability to inhibit viral replication, but most importantly from its immune-stimulatory and immune-modulatory effects (16,17). It is produced predominantly by natural killer (NK) cells as part of the innate immune response, while during antigen-specific immunity, Th1 lymphocytes are the major producer of IFN-γ (18). It is also responsible of shifting the immune response toward a Th1 type and suppression of Th2 response via IL-12 induction and IL-4 suppression, in addition, IFN-γ enhances TNF-α synthesis (19). Among JIA patients, although the data that support the mediator effects of IFN-γ in active disease are not overwhelmed, Gattorno *et al*. (20) reported elevated of IFN-γ serum levels in systemic JIA patients, regardless of the disease activity. However, it also demonstrated that the overnight stimulation of the systemic JIA patients peripheral blood mononuclear cells (PBMCs) showed no difference between active JIA patients and healthy controls in the absolute number of IFN-γ producers cells (21). In addition, further independent gene expression evaluations were not able to detect an IFN-induced genetic signature within the PBMCs of patients with active systemic JIA; therefore the role of IFN-γ in systemic JIA still requires further investigations (22).

A similar argument has also been held in RA, and the role of IFN-γ in pathogenesis of disease is still a matter of controversy, but it is well-known fact that the disease is characterized by an accumulation of effector Th1 lymphocytes that target joints and result in cartilage and bone damage (23,17). In addition, IFN-γ has been demonstrated to be a disease limiting factor in animal models of RA (collagen-induced arthritis; CIA), in which IFN-γ

administration was associated with amelioration of disease severity, while progression of CIA was worsen in IFN-γ genetically deficient mice (24). Furthermore, IFN-γ has also been found to inhibit the production of IL-1β-induced cartilage-degrading matrix metalloproteinase when cultured with synovial tissue specimens from RA patients (25), and furthermore, a neutralization of IFN-γ was found to inhibit arthritis (26).

The present study came to inspect deeply IFN-γ role in both diseases with special reference to JIA, because and for the best knowledge of investigator, such cytokine have not been determined in Iraqi JIA patients.

Materials and Methods

Subjects

A total of 129 Iraqi Arab subjects (patients and control) were enrolled in the study. They were distributed as 49 juvenile idiopathic arthritis (25 females and 24 males; age range: 2.5 – 16 year), 20 JIA controls (control I; 12 females and 8 males; age range: 3 – 16 years), 43 rheumatoid arthritis (23 females and 20 males; age range: 23 – 60 years) and 17 RA controls (control II; 9 females and 8 males; age range: 18 – 50 years). The patients were referred to the Rheumatology Units at Baghdad Teaching Hospital and Imamein Kadhimein Medical City in Baghdad for diagnosis and treatment during the period July 2013 – April 2014. The diagnosis was made by the consultant medical staff at the two hospitals, and it was based on a clinical examination, X-ray findings and laboratory tests. For JIA and its subtypes, inclusion and exclusion criteria are those defined by the International League of Associations for Rheumatology (ILAR) for active JIA (8). Rheumatoid arthritis patients were diagnosed according to the revised diagnostic criteria established by the American College of Rheumatology (ACR), 2010. The 2010 criteria included tender and swollen joint counts, C-reactive protein (CRP), anti-cyclic citrullinated peptide (anti-CCP) antibodies or rheumatoid factor (RF), and symptom duration (27).

Both JIA and RA patients were under therapy, but different protocols were followed. The patients were either treated with methotrexate (MTX: single oral weekly dose of 5–15 mg, Enbrel (etanercept: single weekly subcutaneous dose of 25 mg), corticosteroids (oral methylprednisolone: single daily dose of 50 mg) or disease-modifying anti-rheumatic drugs (DMARDs), which included leflunomide (daily oral dose of 5 mg), sulfasalazine (daily oral dose of 500 – 1000 mg), imuran (azathioprine; daily oral dose of 50 mg), or hydroxyl-chloroquine (daily oral dose of 200

– 500 mg). In a further group of patients, the therapy included a combination of the four protocols (combined group). Normally, blood samples were taken from patients seven days-post last dose. It is also worth to mention that there was a group of patients in which the therapy was discontinued for a period of at least the last six month. Based on the forthcoming data, the patients were distributed into groups on the basis of some principles. In the first, the ACR functional classification (ACRFC) of disease severity was adopted (28). The second principle is Disease Activity Score (DAS). In JIA patients, DAS-27 was estimated. It yields a score ranging from 0 to 57, or can be simplified as low (0 – 3), moderate (4 – 10) and high (11 – 57), while for RA; DAS-28 was estimated (29). A DAS28 value > 5.1 corresponds to a high disease activity, 3.2 – 5.1 corresponds to a moderate disease activity and < 3.2 corresponds to a low disease activity (30). The calculations of DAS-28 were carried out online using the DAS-28 calculator, which is available online (http://www.das-score.nl). The patients were also distributed into groups according to the type of administrated therapy, and for the necessity of analysis, the patients were distributed into four main groups, which were group I that received all types of therapies with the exception of Enbrel and corticosteroids; groups II and III were treated with Enbrel and corticosteroids, respectively and group IV, in which the therapy was discontinued or has not received any therapy. Finally JIA patients were classified into four clinical subtypes; oligoarthritis (OLI), polyarthritis (POL), systemic arthritis (SA) and enthesitis-related arthritis (ERA). Oligoarthritis patients were further classified into persistent (POLI) and extended (EOLI), and polyarthritis into RF positive (RF+ve POL) and RF negative (RF-ve POL), as suggested by ILAR (9).

In addition to patients and control subjects were included. For JIA, they were ascertained from the Pediatric Healthcare Hospital (Al-Kadhimyah, Baghdad), and they were healthy according to the point view of consultants, which was based on a clinical examination and laboratory tests as those of JIA patients. RA controls were blood donors who were apparently healthy.

Blood Collection

From each participating subject, 5 ml of venous blood was collected using disposable syringes. Two ml were dispensed in EDTA tube, and used in the assessment of total leukocyte count and ESR. The remaining blood was transferred to two a plain tube, which was left in the refrigerator (4°C) until clotting for approximately 30 min., and by

then they were centrifuged (3000 rpm) for 15 min. in a temperature cooled centrifuge (4°C). After centrifugation, the serum was separated and distributed into aliquots (0.25 ml) in 0.5ml Eppendorf tubes, which were frozen immediately at -20°C until assessment of other laboratory parameters (a maximum of 12 weeks).

Diagnostic Tests

Each participating subject was tested for the following parameters : total leukocyte count, ESR, CRP, rheumatoid factors (RFs), liver function tests and renal function tests. These tests were used to assess the disease severity and activity.

Serum Level of IFN-γ

Sera of patients (JIA and RA) and both controls (I and II) were assessed for the level of IFN-γ by means of an ELISA method that was based on similar principles, in which ELISA kit was employed in such assessments (PeproTech Company; UK), ELISA system(Human Reader HS,USA).

Statistical Analyses

The results of present study were tabulated in a DATA sheet of SPSS (statistical package for social sciences) version 13.0, which was used to achieve the statistical analyses. The data were presented as mean ± standard error (S.E.), and significant differences between means were assessed by ANOVA (analysis of variance), followed by either LSD (least significant difference) or Duncan test.

Results

Total JIA and RA Patients

Serum level of IFN-γ was significantly increased in JIA patients compared to their controls (52.1 ± 2.1 *vs*. 28.1 ± 1.8 pg/ml). The same observation was also made in RA patients, in which a significant increased level of IFN-γ was observed in the patients compared to their control (66.0 ± 2.7 *vs*. 35.2 ± 2.8 pg/ml). However, the level of IFN-γ was significantly decreased in JIA patients compared to RA patients. Also, JIA controls showed a significant decreased level of INF-γ compared to RA controls (Table 1).

Table 1: Distribution of IFN-γ Serum level among juvenile idiopathic arthritis and rheumatoid arthritis patients with their controls.

Groups		No.	IFN-γ Level Mean ± S.E. (pg/ml)	Total %
Juvenile Idiopathic	**Patients**	49	52.1 ± 2.1^{B}	65.0
	Control I	20	28.1 ± 1.8^{D}	35.0
Rheumatoid Arthritis	**Patients**	43	66.0 ± 2.7^{A}	65.2
	Control II	17	35.2 ± 2.8^{C}	34.8

Different superscript letters: Significant difference ($P \leq 0.05$) between means.

Patients (JIA and RA) Distributed by Disease Severity Scale

Serum level of IFN-γ was similarly presented in the four groups of JIA patients distributed by disease severity scale (I: 53.6 ± 3.9; II: 53.5 ± 3.5; III: 47.6 ± 4.1; IV: 51.5 ± 6.0 pg/ml), but these means were significantly higher than mean of controls (28.1 ± 1.8 pg/ml). A similar observation was also made in RA patients, in which the IFN-γ mean was significantly increased compared to their controls (69.5 ± 4.6, 69.8 ± 4.7, 66.9 ± 11.1 and 69.0 ± 3.0 *vs*. 35.2 ± 2.8 pg/ml) respectively. (Figure 1).

JIA and RA Patients Distributed by Disease Activity Score

A similar manner of distribution for IFN-γ level was observed in JIA and RA patients with respect to DAS, in which patients at the three scores (Low, Medium and High) recorded a significant increased serum level of IFN-γ compared to their controls (JIA: 52.0 ± 7.7, 41.0 ± 3.8 and 54.0 ± 2.3 *vs*. 28.1 ± 1.8 pg/ml, respectively; RA: 78.7 ± 6.2, 62.9 ± 3.7 and 72.5 ± 10.5 *vs*. 35.2 ± 2.8 pg/ml, respectively). (Figure 2).

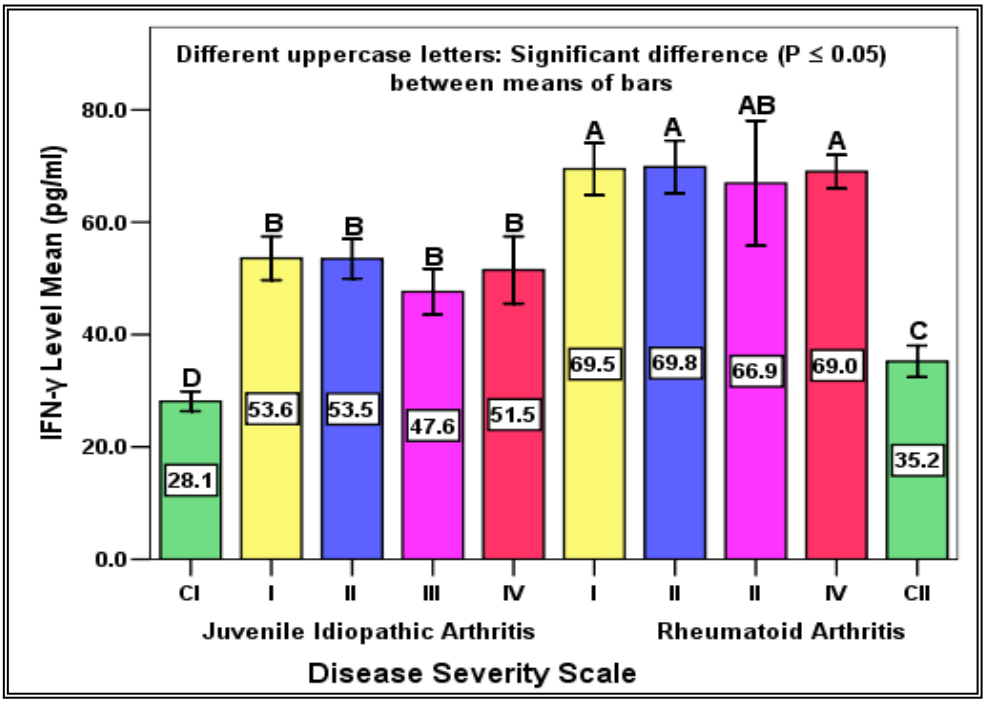

Figure 1: Distribution of IFN-γ Serum level among juvenile idiopathic arthritis and rheumatoid arthritis patients by disease severity scale (C: controls).

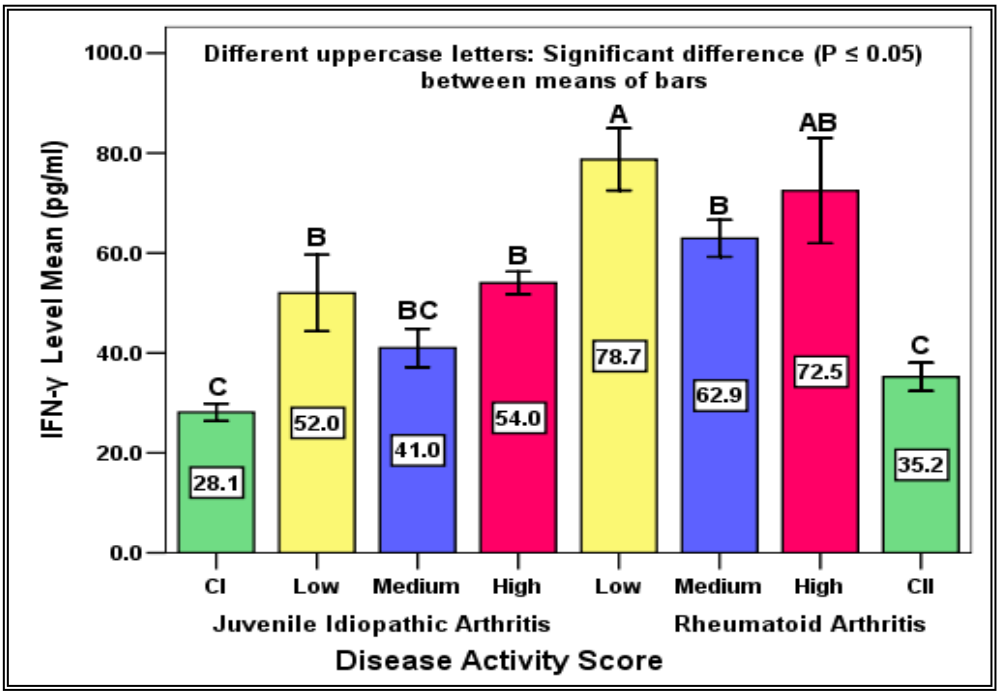

Figure 2: Distribution of IFN-γ Serum level among juvenile idiopathic arthritis and rheumatoid arthritis patients by disease activity score (C: controls).

Patients (JIA and RA) Distributed by Type of Therapy

Serum level of IFN-γ showed a difference between the four means of RA patients, but they were significantly higher the mean of their controls (57.5 ± 5.1, 60.5 ± 5.8, 65.7 ± 4.6 and 75.3 ± 5.6 *vs.* 35.2 ± 2.8 pg/ml, respectively). In JIA patients, the four groups of therapy were observed with approximated level means (54.8 ± 2.8, 50.2 ± 7.7, 49.5 ± 4.7 and 48.7 ± 5.3 pg/ml, respectively), but they were significantly higher than the mean of their control (28.1 ± 1.8 pg/ml). (Figure 3).

JIA Patients Distributed by Clinical Subtypes

Serum level of IFN-γ maintained a mean of 56.7 ± 4.2, 50.7 ± 4.4, 56.5 ± 4.1, 45.5 ± 6.4, 52.6 ± 7.0 and 49.4 ± 16.4 pg/ml, in clinical subtypes of JIA patients (pOJIA, eOJIA, RF+ve POJIA, RF-ve POJIA, SJIA and EJIA, respectively); however, these means were significantly higher than mean of their controls (28.1 ± 1.8 pg/ml) (Table 2).

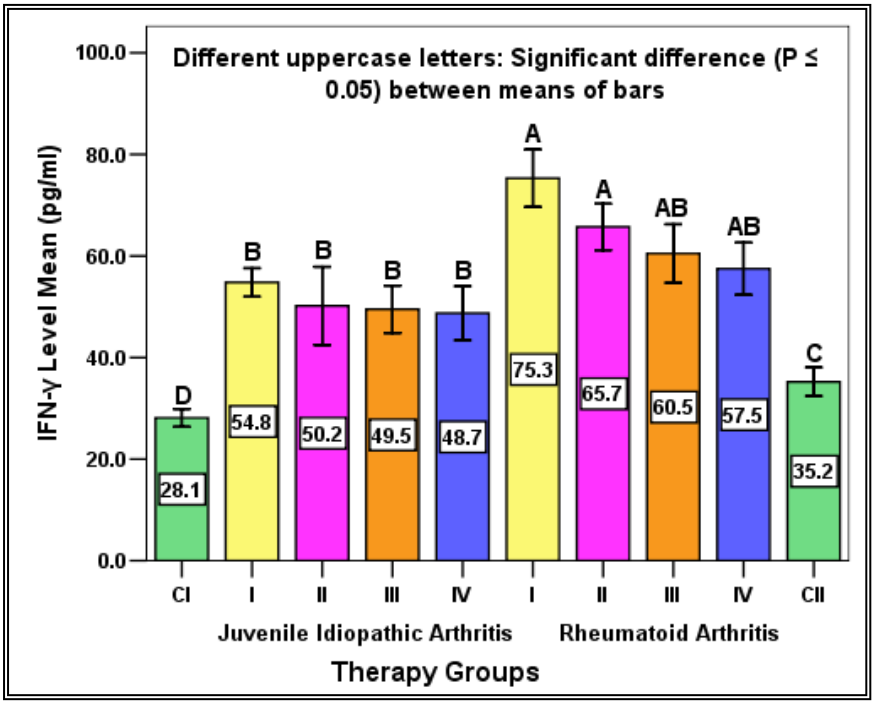

Figure 3: Distribution of IFN-γ Serum level among juvenile idiopathic arthritis and rheumatoid arthritis patients by type of therapy (C: controls).

Table 2: Distribution of IFN-γ Serum level among juvenile idiopathic arthritis patients by clinical subtypes and controls.

JIA Clinical Subtypes		No.	IFN-γ Level Mean ± S.E. (pg/ml)	Total %
Oligoarthritis	**Persistent**	16	56.7 ± 4.2^{A}	16.7
	Extended	12	50.7 ± 4.4^{A}	14.9
Polyarthritis	**RF Positive**	5	56.5 ± 4.1^{A}	16.6
	RF Negative	11	45.5 ± 6.4^{A}	13.4
Systemic Arthritis		3	52.6 ± 7.0^{A}	15.4
Enthesitis-Related Arthritis		2	49.4 ± 16.4^{A}	14.6
Controls		20	28.1 ± 1.8^{B}	8.3

Different superscript letters: Significant difference ($P \leq 0.05$) between means

Discussion

The significant increased serum level of IFN-γ in JIA patients is well-correlated with the results of Mahendra *et al*. (31), van der Ham *et al*. (32) and Breda *et al*. (33), and these observations suggest a pathogenic effect for IFN-γ in etiology of JIA. However, two further investigations may contradict such profile and were unable to report a significant variation in IFN-γ level between JIA patients and controls (34,35). Similarly, the results in RA patients are consistent with those of Meyer *et al*. (36), Tukaj *et al*. (19) and Pavlovic *et al*. (37) investigations, in which a significant increased serum level of INF-γ was reported in RA, especially in early diagnosed patients. The present results also declared that the level of IFN-γ was significantly decreased in JIA patients compared to RA patients; an observation that also shared by van der Ham *et al*. (32).

Serum level of IFN-γ was distributed similarly in the four groups of JIA, but these means were significantly higher than means of their controls, and these findings were also suggested by van der Ham *et al*. (32) as well as Sikora *et al*. (38). In addition, a similar manner of distribution for IFN-γ level was observed in JIA and RA patients with respect to their response to DAS and therapy; observations that were also recorded by Jarvis *et al*. (39), who demonstrated a significant increased serum level of IFN-γ in patients compared to their controls, while among each group of patients (JIA or RA), there were no significant DAS- or therapy-associated variations in the serum level of IFN-γ were observed, and in RA patients, Milman *et al*. (40) also demonstrated a similar finding.

Taking these results in a collective manner may confirm the immunopathogenic role of INF-γ in both types of arthritis, and accordingly it has been suggested that its serum level might be a good marker of the RA activity (19). However, a negative correlation between INF-γ serum level and disease activity in RA patients has also been found, and Serbian RA patients treated with INF-γ showed a significant improvement of the clinical parameters (37). Such confliction in findings may be related to the general role of cytokines as key mediators of inflammation in the course of autoimmune arthritis and other immune-mediated diseases, and uncontrolled production of pro-inflammatory cytokines such as IFN-γ can promote autoimmune pathology (41). It might be therefore concluded that IFN-γ plays an important role in the pathogenesis of JIA and RA, and represented a good biomarker for each of the two diseases.

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Improvement and Partial Purification for Pectinase production from *Pesudomonas aeruginosa*

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Abstract: Soil samples were collected with the aim to isolate pectinase producing from bacterial strains. Bacterial isolates obtained from these samples were grown on a selective medium containing pectin as a sole source for carbon and energy. Results showed that one isolate out of twenty were able to produce pectinase with the most efficient production. The specific activity of pectinase in culture filtrate of the most efficient isolate was 1.1 U/mg protein. This isolate was identified according to its morphological and biochemical characteristics as *Pesudomonas aeruginosa*. Pectinase produced by *Pesudomonas aeruginosa* was purified throughout partially purification including ammonium sulfate precipitation. Purification results showed that the specific activity of the partially purified enzyme was 80 U/mg protein and 86 U/mg protein. The production of pectinase was improved by using UV-rays to achieve random mutagenesis , the highest production for the mutants was 29 U/mg protein as specific activity.

Key words: Pectinase, *Pesudsomonas aeruginosa*, Purification, UV-rays.

تحسين وتنقية جزئية للبكتنيز المنتج من بكتريا *Pesudomomnas aeruginosa*

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الخلاصة: جمعت عينات تربة بهدف عزل سلالات بكتيرية منتجة للبكتنيز، نميت العزلات البكتيرية المزولة من هذه العينات على وسط انتخابي متضمن البكتين كمصدر وحيد للكاربون والطاقة ، بينت النتائج ان عزلة واحدة فقط من عشرين عزلة قادره على انتاج البكتنيز بكفاءه اعلى. وكانت الفعالية النوعية للبكتنيز في الراشح البكتيري للعزلة هي 1,1 وحدة/ملغم بروتين. شخصت العزلة طبقا للصفات المظهرية و الكيمياء حياتية على أنها تعود الى النوع *Pesudomonas aeruginosa*. نقى البكتنيز المنتج بواسطة *Pesudomonas aeruginosa* جزئيا بالترسيب بكبريتات الامونيوم . نتائج التنقية تبين ان الفعالية النوعية للآنزيم المنقى كانت 80 وحدة/ملغم بروتين و 86 وحدة/ملغم بروتين. لتحسين انتاج البكتنيز بواسطة الاشعة فوق البنفسجية كمطفر عشوائي، اعلى انتاج كان للطافر الذي اعطى فعالية نوعية 29 وحدة/ملغم بروتين.

Introduction

Many of the pathogenic bacteria and fungi for the plant are produce pectinases enzymes for invading the host tissues. These enzymes are essential in the lysis of dead plant material by nonpathogenic microorganisms, but they are assist in the recycling of carbon compounds in the environment (1, 2). Many of the studies have been studied the production of pectinases from various microorganisms. But there are difficulties in the supply of appropriate substrate might be the most problem to develop such studies. A substrate should be provide all necessary nutrients to the microorganism, if not, it must be necessary to supplement them externally (3). Investigation of pectinases is a important issue in the enzymology due to their applications in pharmaceuticals, food, agricultural products, bioremediation process and the treatment for degumming of textile fiber (4, 5). Pectinases are lysis one of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary call walls of young plant cells (6, 7).

Materials and Methods

Microorganism and identification

In this study the biochemical and morphological characteristics were applied to identification of the selected isolate, API 20E system was used to confirm the results of identification for *Pesudomonas aeruginosa*. The bacterium was maintained on nutrient agar at 4 °C and sub cultured weekly.

Isolation and screening of the pectinase producing bacteria

Pectin degrading bacteria screening was obtained using pectin agar plates aiming to investigate the presence of the enzyme that has the effectiveness of pectin degraded. Pectin agar medium contained 5 g/ l citrus pectin, 1 g/l yeast extract, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.1 g/l, $MgSO_4$. 7H2 O, 0.11g/l $CaCl_2$, and 20 g/l agar, (pH 7.0). 100 µl of all dilution of each bacterial isolates were spread on pectin plates and then incubated at 30 ºC for 48 hrs. Plates were flooded with potassium iodide solution, the clear zones were appeared around the colonies indicating pectin hydrolysis (8).

Pectinase production

Pesudomonas aeruginosa was grown in a broth medium that contained the following categories (g/L) pectin (1.0)gm, ammonium dihydrogen sulphate (0.14) gm, potassium dihydrogen phosphate (0.2) gm, potassium hydrogen phosphate (0.6) gm, and magnesium sulphate (0.02) gm. The pH was adjusted to 7.0. 50 ml of the broth medium was autoclaved in a flask and cooled to the incubation temperature (30°C) and inoculated with 0.5 ml of a

pure culture that had grown for 90 hrs at 30 °C and shacked with 140 rpm. After every 6 hrs of incubation the bacterial cells was filtrate and recovered by centrifugation (6000 g 10 min. 4 °C) and used for further analyses (9).

Enzyme assay

Pectinase activity was estimated with colorimetric method of miller (1959) and absorbance of the colour developed was measured at 540 nm. One unit of pectinase was defined as amount of pectinase which catalyses the formation of 1 µmol of galacturonic acid per min (10).

Improvement of the Pectinase production

*Pesudomonas aeuroginos*a was subjected to UV as mutagenic agent to achieve random mutagenesis, in order to obtain isolates mutant with higher productivity of the enzyme than the wild-type isolates. Different durations time was used (1, 2, 3, 4, 5, 10 and 15 seconds). Culture of *Pesudomonas aeruginosa* inoculated in nutrient broth at 18 hrs was pelleted of 10 ml, then washed and resuspended in phosphate buffer (pH 7.0) (11). Petri dish contained 4 ml of bacterial suspension was subjected to UV-light with a distance of 30 cm and was irradiated. The bacterial suspension was mixed during irradiation. 100 µl of bacterial suspension was diluted and spread on nutrient agar, in order to calculate the lethality rate. The growing mutants colonies were cultivated on the selected medium to obtain their ability for pectinase production (12).

Partial pectinase purification

The culture flask after the incubation was centrifuged under refrigerated (4 ºC) condition at 10000 rpm for 10 min and the supernatant was filtered by Millipore filter (0.45 mm) to remove the cells. The filtrate was precipitated with different concentration of ammonium sulphate solution up to the saturation level from 60 to 80% with magnetic stirrer at 4 ºC for overnight, the precipitate was collected by cooling centrifuge (8000 rpm for 10 min), then the pellet was resuspended in phosphate buffer (pH 8.0) and dialyzed against 2 changes of buffer under magnetic stirrer at 4 ºC for 24 hr in order to obtain proper purification. An amount of partially purified enzyme was quantified (13).

Results and Discussion

Isolation and screening of pectinolytic bacteria

Isolation of the most efficient pectinolytic bacteria was done by the inoculums of soil samples inoculated on pectin plates. The Results indicated that one isolate from the collected isolates was possess the ability of pectinase production,

which forming 11 mm in diameter clear zone (Table 1). The specific activity was determined for the selected isolate by using filtrate production broth medium given 1.1 U/mg protein (14).

Table (1): Ability of the isolates for pectinase production after incubation for 48 hrs at 30 ºC.

Isolate No.	Diameter of clear zone mm	Isolate No.	Diameter of clear zone mm
1	9	11	0
2	10	12	**11**
3	0	13	0
4	0	14	0
5	0	15	7
6	0	16	8
7	9	17	9
8	7	18	10
9	0	19	0
10	0	20	0

Identification of the bacterial isolate

The results indicated that the most efficient isolate in pectinase production belong to species of *Pesudomonas aeruginosa*. The results were in agreement with (15).

Purification of pectinase

The specific acrivity was calculated before and after the precipitation to determine the increasing in the specific activity, the results indicated that an extracellular pectinase from the culture media was purified about 4.0-fold, specific activity of 80 U/mg and a final yield of 46% by applying single step of purification of precipitation using ammonium sulfate (Table 2). There are increasing in the specific activity, and the result was similar to study of Bhardwj and Garg (2012) and Rastegari *et al* (2014) ,those reported that there are increasing in the specific activity through the steps of purification and through using ammonium sulfate.

Table (2): Purification steps for pectinase produced by *Pesudomonas aueroginosa*.

Purification step	Vol. (ml)	Enzy. Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude Enzyme	65	0.80	0.04	20	52	1.0	100
$(NH4)_2SO_4$	15	1.60	0.02	80	24	4.0	46
Dialysis	13	1.97	0.023	86	26	4.3	50

Improvement of pectinase activity

The plates were incubated in dark place at 37 ºC for 24 hrs to determine survivals and detection of the killing percentage that more than 90%.The results indicated that only six out of twenty mutants were given an increase in pectinase production than the wild type, and the mutant *Pesudomonas aueroginosa* M3 was the most efficient in the production of pectinase with 29 U/mg protein as specific activity and 26 fold of increase compare with the wild type (Table 3), on the other hand, 14 isolate showed lower pectinase production. The plates were incubated in dark place at 37 ºC for 24 hrs to determine survivals and detection of the killing percentage that more than 90%. LD90 was occurred at 3 seconds and most of the selected isolate was lost after 5 seconds of irradiation (Figure 1).The result is accordance to those of Al-gelawi and Al-makadci (2007) who reported that the *Pesudomonas aeuroginos*a was UV sensitive and the lethality increased with the increase of UV dose.

Table (3): The specific activity for mutants compare with wild type.

Mutant No.	Specific activity (U/mg)	Fold of increase
***Pesudomonas aueroginosa* M1**	1.1	1
***Pesudomonas aueroginosa* M2**	15	14
***Pesudomonas aueroginosa* M3**	**29**	**26**
***Pesudomonas aueroginosa* M4**	24	22
***Pesudomonas aueroginosa* M5**	21	19
***Pesudomonas aueroginosa* M6**	14	13

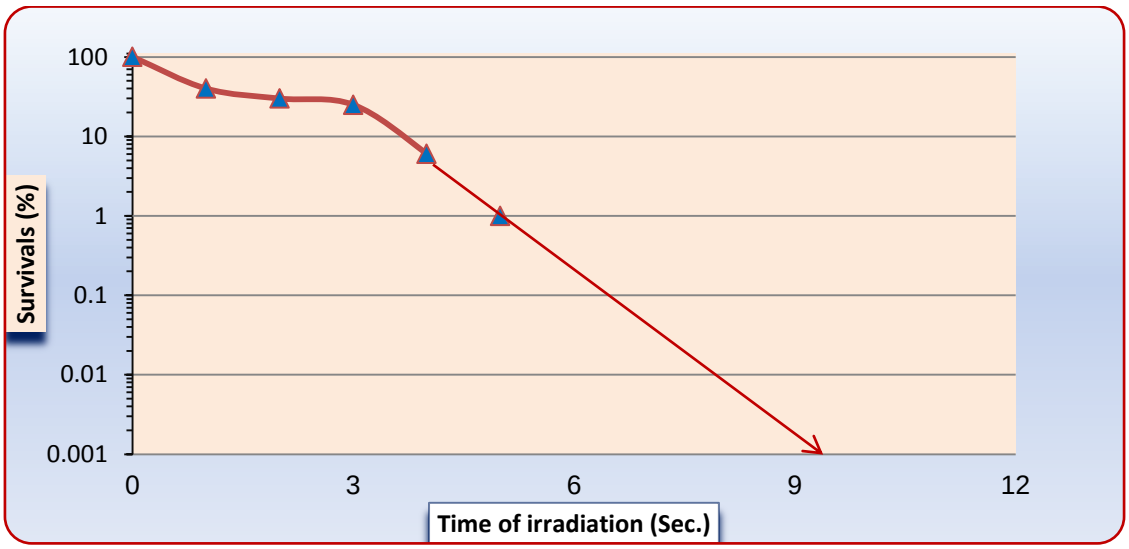

Figure (1): Survivals of *Pesudomonas aeuroginos* after the irradiation by UV-light for various periods.

The conclusions indicate that the production of pectinase was improved by using UV-rays to achieve random mutagenesis, and the highest production for the mutants was 29 U/mg protein as specific activity.

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Environmental study on the use of the electric device mosquito killer (model: MD-20WA) and histological study of its effects on the livers and on weight

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Abstract: During this study evaluated the efficiency of mosquito killer and insects device (model: MD-20WA) by electrocution since been subjected male laboratory rats M.musculus of the device to different time periods and see the impact on the weight and liver tissue and enzymes, where the divided groups to the control group G1 The group exposed to 1 hour G2 and 12 hours G3 and 24 hours G4and 36 hours G5. The results showed significant decrease ($p < 0.05$) in the rate of body weight in the G5, compared with the rest of the totals. The histopathological changes it has been observed that the group G2 and G3 the liver tissue appeared normal approach to the tissues of animals control milestones either G4 Group tissues of animals showed few pathological changes intensity The group G5 which got pathological changes more severe than its peers and compared with the G1. The study also indicated for rising significantly ($p < 0.05$) In the concentration of enzyme AST, ALT in the serum of the two sets of G4, G5, compared with the control group and the other groups. Conclusion The free Radicals have Roles of the Toxicity of Environmental.

Keywords: the electric device mosquito killer, histological study, liver, wieght male mice, AST,ALT, Free radicals.

دراسة بيئية حول استخدام الجهاز الكهربائي قاتل البعوض (model: MD-20WA) ودراسة تأثيراته على الوزن و على نسيج الكبد

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الخلاصة: تم خلال هذه الدراسة تقيم كفاءة جهاز قاتل البعوض والحشرات (model: MD-20WA) بالصعقة الكهربائية إذ تم تعرض ذكور الفئران المختبرية *M.musculus* للجهاز الى فترات زمنية مختلفة ومعرفة تأثير ذلك على الوزن و نسيج الكبد وانزيماته، حيث قسمت الحيوانات الى 5 مجموعات، السيطرة G1 والمجموعة المعرضة الى 1ساعة G2 و12 ساعة G3 و24 ساعة G4 و 36 ساعة G5 . بينت النتائج حصول انخفاض معنوي ($p < 0.05$) في معدل وزن الجسم في G5، مقارنة مع باقي المجاميع. اما التغيرات النسيجية فقد لوحظ ان المجموعة G2 وG3 ظهر فيها نسيج الكبد بمعالم طبيعية مقاربة لأنسجة حيوانات السيطرة اما المجموعة G4 اظهرت انسجة حيواناتها تغيرات مرضية قليلة الشدة أما المجموعة G5 حصل فيها تغيرات مرضية أكثر شدة من نظيراتها ومقارنة مع G1.كذلك اشارت الدراسة الى حصول ارتفاع معنوي($p < 0.05$) في تركيز انزيمي AST,ALT في المصل لمجموعتي G4,G5 مقارنة مع مجموعة السيطرة وباقي المجاميع. وتستنتج الدراسة ان للجذور الحرة ادوار في سمية البيئة .

Introduction

We have the latest human change in the ecological balance in many of exploiting areas and invest the territory in order to assess a new balance and resist the animals and harmful plants focused to the use of certain chemical products which are increasing the quantity and seriousness of the day [1] and the right big health damage to non-target organisms such as pet birds and the bees and humans [2]. There are multiple types of pesticides vary according to the nature of their work, each type of pesticides including a group or groups of chemical compounds similar or per differ them from the other. The synthesis of pesticides could be organically , and may share some plant sources in preparation [3] [4].Recently, there was special phenomenon of the use of electrically stunned plane insect devices .This devices have different types and sizes .Also they attract insects by neon publisher UV at a wavelength of 365 Nanometer .The clamp was dumbfounded by insects and throw it designated for that purpose by the stairs [5]. Interestingly, there many warnings and precautions set by the manufacturers of this type of device like placed inside the places , rooms and closed the windows to prevent the attraction of insects abroad .This wringing to indicated that the equipment generates UV and generates ozone gas [6] Ozone is blue gas and this color is composed of three atoms of oxygen chemical formula O3. This gas is one of the toxic gases and not exceed per slim atmosphere [7]. On the contrary, the upper layers of the atmosphere, contain the ozone in the atmosphere. Ozone in lower cause major damage, Inhalation a fraction of the ozone with the air causes the headache and affects the lungs[22],[23],and weaken their resistance to the bacteria may cause Crash cells [8]. The study aims was to investigate the efficiency of this device in the fight against insect mosquitoes and the study of its effect on the tissue and cellular composition of the liver of laboratory mice.

Materials and methods

Insect killer one as electrical device

Got the device from the local markets, carries the brand name kill pest (model: MD-20WA) factory by Kim Thuan Phat / China Company dimensions (40 × 25) cm contains Two contains neon bulb length of one 30 cm card consumption of 35 watts hedged its made internal network iron is connected to an electric current insects device ,external network is made of aluminum.

Laboratory animals

Used in this study, male adult mice type of *Mus musculus* L. strain BALB / C weight (25-30) g, which have been bred in the animal house under controlled conditions of temperature (20-25) ° C and fed diet B integrated animal.

Experiment tests:

Electric shock device commented on the rise 3 meters above ground level in a closed room doors and windows. Then samples of adult mosquitoes C.pipiens brought by 20 insect for each test period with operating control device for a period of 1 hour then calculated the percentage of the loss of adult mosquitoes. Repeated the same experiment in terms of the number of insect except for increasing the duration of exposure to electric shock device and by 12,24,36 hours and then calculated the percentage of loss in each experiment.

In order to study the side effect of the device electric shock to humans ,We use the laboratory mice as model .Animals at all stages of the experiment put under living situation similar in terms of lighting, temperature, and provided the stuff and water. we divided rats into five groups each group of five mice. The first group G1 considered a control, the second group G2 and placed in front of electric shock device for 1 hour and the third group G3 for 12 hours and the group G4 for 24 hours and the group G5 for 36 hours, then autopsy on animals of each group after the end of the time of exposure of the device, the liver eradication and preserved in formalin concentration of 10%, then the samples sent to the laboratory histological preparations then samples were examined with a microscope and photographed.

Statistical analysis

The result were expressed as mean ± SD and analyzed statistically by Spss system ver.20. The association between the body weight pre- and after exposure to device were analyzed by using student t-test and ANOVA at levels of ($p<0.05$).

Results & discussion:

Body weight (g)

Results in Figure (3-1) described the weight changes after exposure for device mosquito killer. The results showed no significant differences ($0.05 \leq p$) in the weights of the bodies of the male rate rats sets of G2 and G3 & G4 , compared with the control group(G1) , while animals of G5suffered from the loss of weight was elevated to the level of significance ($p <0.05$).

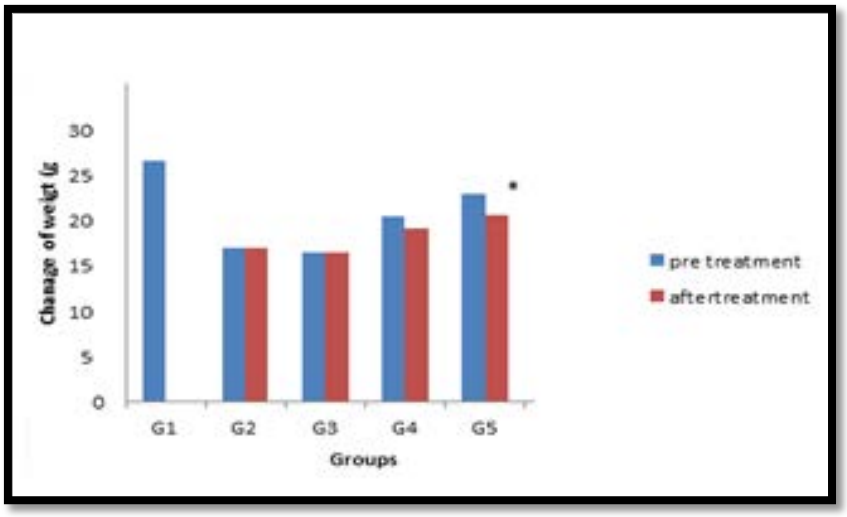


Fig. (3-1): Effect of exposure of device mosquito killer on weight changes for mature male mouse of study groups.
***Means a significant change when (p <0.05).**

The Liver

The table (1.1) show impact of the use device in the effectiveness of the liver enzyme ALT, AST enzymes to mature male albino rats, the results showed a significant increase ($p \leq 0.05$) in the concentration of enzymes in serum of male rats for G4 &G 5, compared with G 2 &G 3 and control group G.

Table (1.1) : Effect of electric device (model: MD-20WA) on ALT, AST enzymes concentrations in white male rats.

Group Parameter	G1(control)	G2	G3	G4	G5
ALT Conc.% U/L	8.9±0.407	8.050±0.238	9.10±0.201	11.880±0.420*	14.917±0.128*
AST Conc.% U/L	10.193±0.332	9.854±0.555	12.050±0.550	14.417±0.561*	17.025±0.583*

*** means significant difference at (p <0.05) between groups**

The enzymes carrying Amin ALT, AST enzymes that reflect Apprenticeship liver that concentrations by high ones ubiquitous in liver cells, as it exists enzyme AST high concentrations in the liver and kidneys [9] and it is their focus in the blood gives a picture of how their activities in those members, especially the liver. When looking at the results of the present study, we find that effect of explosion of device may have caused an increase of the effectiveness of the enzymes ALT, AST serum indicates that these enzymes have leaked quantities of high tissue of the liver to body fluids, particularly blood serum that high dropout reflects the get damage in the tissues of the body, especially the liver, which means that in the body fluids approached or perhaps exceeded class toxicity. as used effectiveness enzymes carrying Amin baseband phosphatase in serum as a barometer sensitive about the extent of changes pathological or physiological you get in the liver result damaged cellular winning result of injuries pathological cases or physiological toxicity requiring additional activity in the metabolic processes by the liver, if What is learned that increase the concentration of the enzyme GPT in serum is an indication of damage to the liver cells and to a lesser extent in other tissue cells,[10] &[11]The increase suggests changes in the concentration of enzyme GOT to corruption occurs in most cells of vital organs such as the liver and other [12] The study showed that pathological changes in the liver tissue resulting from the use of device. The study showed that pathological changes in the liver tissue resulting from the use of device

Return to the kind of reactions are called photochemical reactions return to the viability of oxygen (Protein content in the tri-state in nature) to interact with the electronic irritants cases of reactive molecular or interact with free radicals formed during the reaction. In addition, the oxygen absorbs light wavelength of less than 200 nanometers disintegrated into two atoms of oxygen, as in the equation:

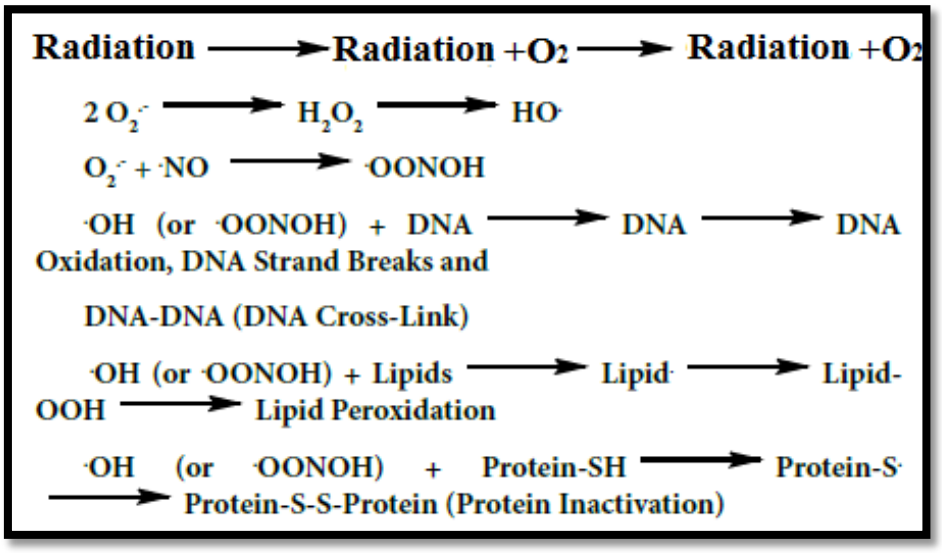
$O_2 + h\gamma\ (\ \lambda < 200\ nm\ ) \longrightarrow 20^{o}$

The oxygen atoms react with oxygen molecular to form O_3 molecular

$O + O_2 \longrightarrow O_3$

Ozone is formed weak absorption in the visible region (near 600 nm) and strongly in the UV region (below 320 nanometers).[13].

Ozone is a toxic gas that is not naturally present at lower atmospheric levels. However, it is one of the primary pollutants present in photochemical smog. Ozone is a strong oxidizing agent and has been linked to liver inflammation, and sinusoid epithelial damage. It has been suggested that these damages are initiated by free radicals formed from the decomposition of secondary ozonides, a reaction product of ozone with unsaturated lipids [14].As (Fig.2) Ozone also has been reported to induce damage to cellular DNA via free radical mediated reactions [15].



Figure(2):Impact of Ozone for induce damage to cellular DNA via free radical mediated reactions.

The current study showed pathological changes in the textile in the study, which included Members of the liver. It may cause exposure to radiation for a long time ranging from one hour to 36 hours, to the emergence of pathological changes in most tissues studied varied severity of the fabric to another and from one area to another in the same fabric. While it is resulting in radiation exposure for 12 hours to the appearance of normal tissue milestones approach to the tissues of animals control. The group suffered a 24-hour radiation ,the tissues of animals showed low-intensity pathological changes in comparison with the group exposed for 36 hours radiation ,which demonstrates the

arrival of the intensity of radiation exposure to toxic class which has produced all damaged monuments textile liver .

The picture shows (-1-) passage histologically for liver a rat control, which appears as a component of almost circular shape lobules, and shows the central vein average per lobule, as it extends it hepatocytes radially in the form of ropes called chords liver and permeates the liver cells make way or hepatic Sinusoids. Not noticed any change in the textile and textile clear sections of the livers of rats G2. The tissues sections rats G4,G5, has seen congestion of blood vessels and bleeding bloody, image (3a, 3b, 3c), as well as the emergence and bleeding bloody between sinusoids liver and the emergence intensify Chromatin some nuclei liver and called Pyknosis a form of necrosis Of hepatocytes with the presence of hemorrhage in the vein pyloric Portal veins (4). The severity of the bloody eased congestion in histological sections of the livers of animals in the third set .

The study structural changes in the lymph Members of the reticular endothelial system , a result of exposure to chemical and radiological an important indicator to assess the toxic effects - immune resulting from these materials [16], and the attic was this side take an important place in the present study was to assess the size of the deviations mice immune to males as a result of the explosion for ozone reached a degree of toxicity. histological sections of the liver showed the existence of the state of tension and blood vessels where he appeared in male rats, which is due to the relaxation of Arteries.

He noted [17] that exposure periods UV that come with the sun or artificial light therapy lamps are the two things very worry for human health ,the effect radiation on the skin, including dermatitis and weaken the immune system. According to [18] that the ultraviolet radiation term UVA and medium UVB and short UVC can destroy the protein collagen and thus accelerates aging skin[21].the interactions electric discharge converts some oxygen into ozone O_3, which strongly dissociates of something organic compounds or for some metals or their oxides [19], [20] reviled that the ozone.

Once the organism enters the body decomposes into oxygen, O_2 and free electron unstable attacks the membranes of living cells reasoned case of lipid peroxidation performer to rupture of membranes and flow the hemoglobin through them and this proved the picture of 5 for the case of hemorrhage and necrosis of the cells.

Acknowledgement

The researcher wish to grant this work for my friends that supported and contributed to completion this work. Special thanks to S.S Inam Ali Tsear Environment Department \ College of Science University of Kufa for the great supporting during the study.

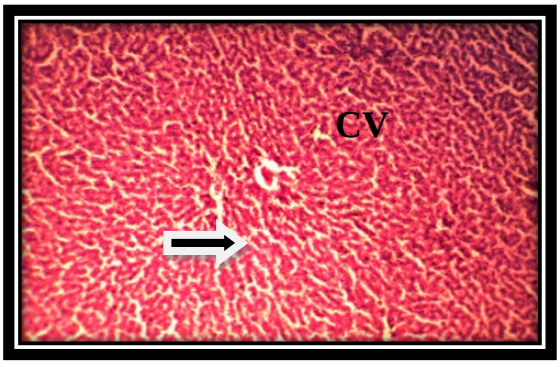


Image 1: A section in the liver of mouse in thecontrol group G1, represented by the existence of a natural senesoides ( ) &central vein (CV)(200x hematoxylin & Aiiosin)

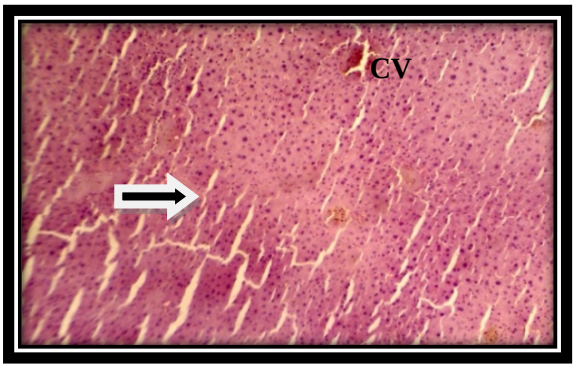


Image 2: A section in the liver of mouse in the G2 , Natural landmarks tried to control. Are ropes around the central vein (CV) and interspersed senesoides ( ) (200x hematoxylin &Aiiosin)

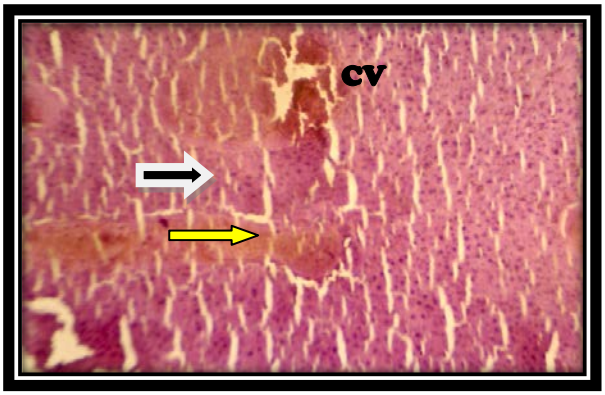
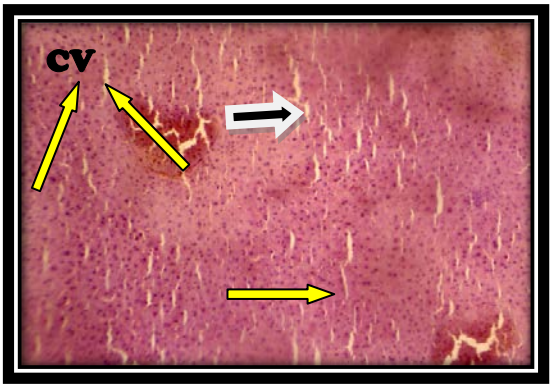


Image (3): section in the liver of mouse (G4) in which senosids ( ) and arranged in a sling Around the central vein (CV) dilated and contain hemorrhage ( ) (Allosan – hematoxylin 200 x)



Imag(3b) section the liver of a rat (G3) in which liver cells swollen show ( ) and arranged in a sling around( ) the central vein (CV) dilated and contain hemorrhage ( ) (Allosan – hematoxylin200 x)

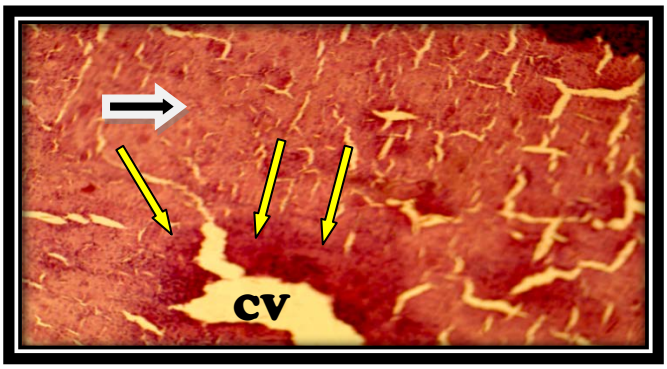


Image (3d) section in the liver of a rat G3 in which liver cells (senosoid) swollen & nicrosis show (➡) and arranged in a sling around the central vein(CV) very dilated and contain hemorrhage (➡) (Allosan – hematoxylin 200 x)

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Histological and biochemical effects of silver nanoparticles on kidneys of female quail (*Coturnix coturnix*)

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Abstract: This study was designed to identify the effect of silver nanoparticles on histology and biochemistry(during measurement of urea and creatinine levels) of the kidney.(45) quail (females) were collected from agricultural research center in Abu-Ghraib, divided into (4) groups including: T1 (12 quails were dosed with 4ppm), T2 (12 quails were dosed with 8ppm) and T3 (12 quails were dosed with 12 ppm) of silver nanoparticles solution for (60) days.As well as T4 (included 9 females as control group).After the dosing period , toxic responses were assessed by histopathologic and biochemical parameters. Biochemical studies showed significant decrease in the level of urea and creatinine, dosed groups with silver nanoparticles showed histopathological alterations in kidney tissue , included congestion of blood vessels, infiltration of inflammatory cells, exudate, hyperplasia in each of blood vessel walls and capsule, atrophy of tubules,hydropic degeneration, hemorrhage in each of cortex, medulla and glomerulus , hyaline degeneration of tubules, amyloid precipitation in each of glomerulus and blood vessels, sloughing of the epithelial cells of renal tubules from basement membrane and hypertrophy in the tubules. Severity of these effects varied depending on individual differences between samples and concentration of silver nanoparticles, which appeare more in samples were dosed with highest concentration.

Keywords: Silver nanoparticles, quail, kidney, urea, creatinine

التأثيرات النسجية و الكيموحيوية لجسيمات الفضة النانوية على كلى اناث طائر السمان (Coturnix coturnix)

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الخلاصة: صُممت هذه الدراسة للتعرف على تأثير جسيمات الفضة النانوية على نسجية و بايوكيميائية (من خلال قياس مستوى اليوريا و الكرياتنين) الكلى لاناث طائر السمان.اذ تم جمع (45) انثى لطائر السمان من مركز البحوث الزراعية في ابو غريب و قُسمت الى (4) مجاميع تضمنت:م1 (12 طائر جُرعت ب 4ppm)،م2 (12طائر جُرعت ب8ppm)،م3(12 طائر جرعت ب 12ppm) من محلول جسيمات الفضة النانوية لمدة (60) يوما. فضلاً عن مجموعة السيطرة م4 (تضمنت 9 طائر). بعد انتهاء فترة التجريع تم تقييم الاستجابات السامة من خلال قياس التأثيرات النسجية المرضية و المؤشرات الكيموحيوية.أظهرت الدراسة الكيموحيوية حصول نقصان معنوي في مستوى اليوريا و الكرياتنين، بينما تضمنت التغايرات النسجية المرضية لنسيج الكلى للمجاميع المجرعة بجسيمات الفضة حصول احتقان الاوعية الدموية،ارتشاح الخلايا الالتهابية،وذمة،فرط التنسج لكل من جدران الاوعية الدموية و المحفظة،ضمور النبيبات الكلوية،تنكس مائي، نزف دموي في كل من القشرة و اللب و النبيبات الكلوية، تنكس زجاجي لخلايا النبيبات الكلوية، ترسب الامايلويد في كل من الكبيبات الكلوية و الاوعية الدموية، انفصال الخلايا الظهارية للنبيبات الكلوية عن الاغشية القاعدية فضلا عن تضخم النبيبات الكلوية، و تباينت شدة التأثيرات تبعا للفروق الفردية بين العينات و تركيز جسيمات الفضة النانوية اذ تزداد شدة التأثير في العينات المجرعة بالتراكيز الأعلى.

Introduction

Nanotechnology deals with nanoparticles which have size between (1-100)nm and have a high ratio of area to size(1).Nanosilver is one of the most commonly used in several fields because of it's antibacterial properties and antifungal properties (2), antiviral properties (3)and anti-inflammatory properties(4). Silver nanoparticles have stronger germicidal capability which can kill up to (650)different type of bacteria(2) . These effects of ionic nanosilver is due to many mechanisms such as attachment to bacterial cell membranes and then penetrate the cell, causing variations in the cell membrane causing cell death(5) , silver nanoparticles have the ability to release silver ions which interferes with the thiol groups in vital enzymes, leading to inhibition(6) , silver ions also cause inhibition of respiratory enzymes and generate reactive oxygen species(ROS)which attack the cell itself causing death(7).These properties may be play asadverse effects on human health and the environment and cause of high toxicity of nanosilver (8). The aim of present study to identify the effect of silver nanoparticles on histology and biochemistry of quail kidneys.

Materials and methods

(45) female quail (2-3 months age) were collected from agricultural research center in Abu-Ghraib and divided into (4) treatments which included : T1 (12 quails were dosed with 4ppm), T2(12 quails were dosed with 8ppm) and T3(12quails were dosed with 12ppm) of silver nanoparticles solution by oral consumption and one group T4(9 quails)was used as control group (given distilled water). Silver nanoparticles solution prepared by Nanoparspanada Iranian company (4000ppm and average size of these particles 50-150). Treatment groups have been given silver nanoparticles solution, T1(4ppm: 1 ml in the morning, 1ml in the evening), T2(8ppm 1ml in the morning and 1ml in the evening), T3 (12ppm 1ml in the morning and 1ml in the evening) and T4 group have been given distelled water, for (60)days as an dosing period . After the end of dosing period , birds were sacrified by discapitation and then blood samples were collected for serum separation which required for biochemical study. birds were dissected and kidney samples were isolated and fixated with Bouin's fluid, histological slides were prepared and stained with hematoxyline-eosin stain according to Bancroft and Stevens (9).Slides were examined by using light

microscope (Meiji) with camera (Canon) used for picturing.

Results

Histological examination

Microscopic examination was revealed that the kidney in control group consist of two regions which include the cortex (has glomerulus, distal and proximal tubules) and medulla (has hinle loops and collecting duct) (Figure:1). The low concentration of silver nanoparticles (4ppm) induced congestion of blood vessels ,slight hemorrhage in cortex, precipitation of proteins in some of glomeruli and cells of renal tubules which named (amyloid and hyaline degeneration) (Figure:2), serous exudate in interstitial substances, severe hemorrhage and dissociation of interstitial in medulla (Figure:3).While histological alterations in the kidney of birds were dosed with 8ppm manifested by the presence of infiltration of inflammatory cells , hemorrhage, sloughing of epithelial cells in most of renal tubules , hydropic degeneration for some of renal tubules cells (which increase in samples were dosed with 12ppm (figures 4,7) and amyloid deposition increased in glomeruli compared with 4ppm, serous exudate , atrophy in most of the renal glomeruli (Figure:5).While the high concentration of silver nanoparticles induced a sever infiltration ,increase dissociation of interstitial substances of medulla, sever amyloid deposition in both glomeruli (Figure:6) and blood vessels(figure:7), pycnosis and karyolysis (Figure:8), more sloughing of renal tubule cells than the other concentrations, atrophy of glomeruli (Figure:9) and renal tubules (Figure:10,11), hyperplasia for each of blood vessels (Figure:10), capsules , and renal tubule cells and hypertrophy of renal tubules were observed (Figure:11).

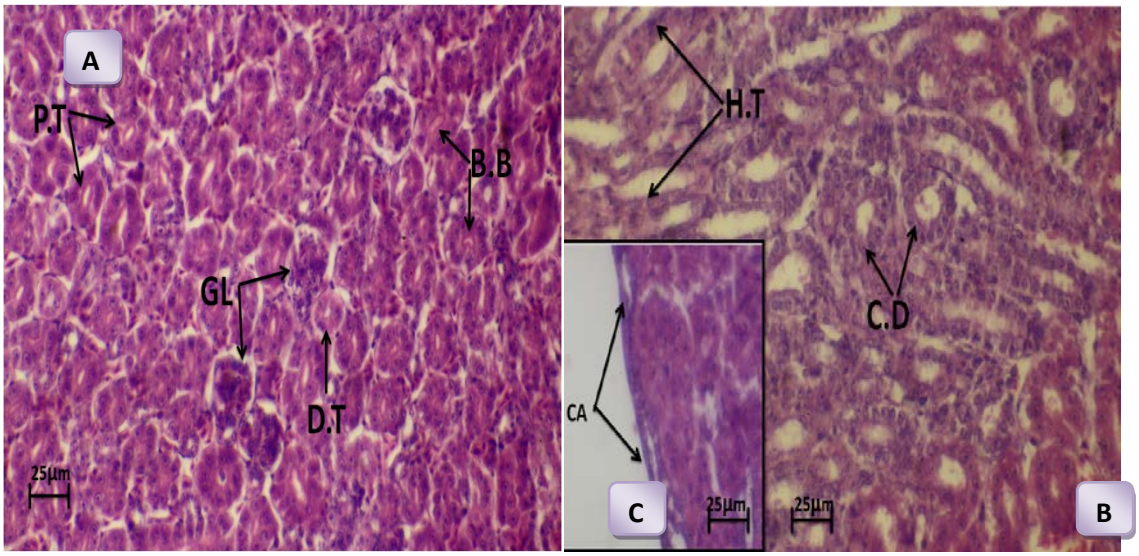


Figure (1): Section in the kidney of control group shows (A): Cortex - GL: Glomerulus, P.T:Proximal Tubules, D.T:DistalTubues, B.B:Brush Border. (B&C): Medulla- C. D: Collecting Duct, H.T: Hinle Tubules, CA:Capsule(H&E) .

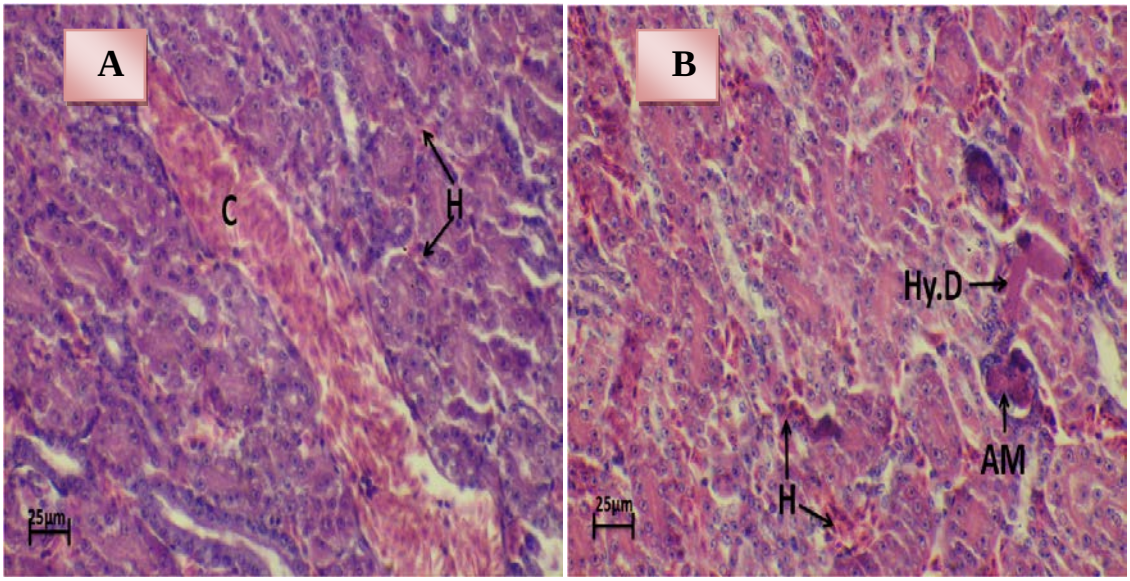


Figure (2): Section in the kidney of quail dosed with 4ppm shows: (A) -C:Congestion.(B) -Hy. D: Hyaline Degeneration, AM: Amyloid, and H: Hemorrhage. (H&E).

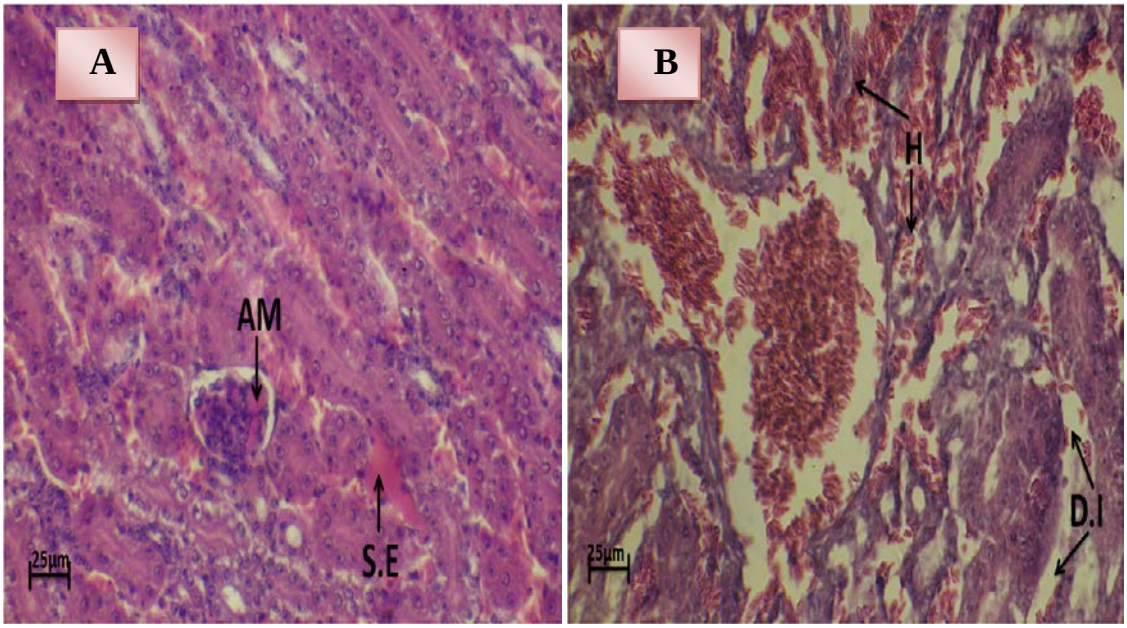


Figure (3): Section in the kidney of quail dosed with 4ppm of silver nanoparticles shows:(A) - S.E: Serous Exudate, AM: Amyloid. (B) - H: Hemorrhage , and D.I: Dissociation of Interstisial substances. (H&E).

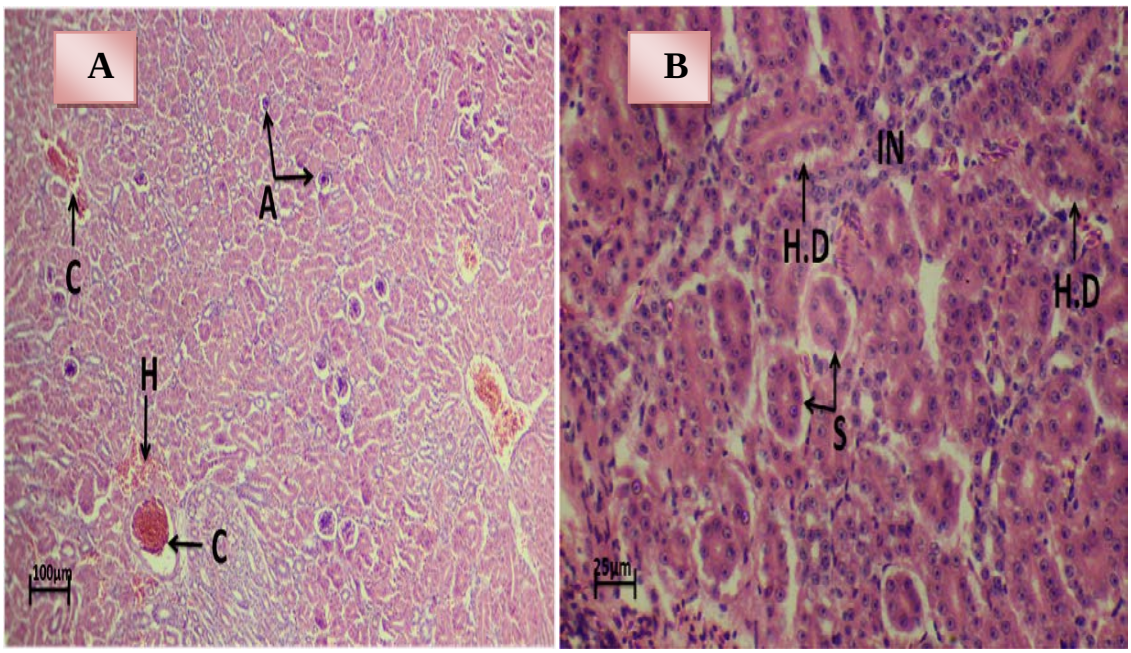


Figure (4): Section in the kidney of quail dosed with 8ppm of silver nanoparticles shows: (A) -C:Congestion, A:Atrophy of glomerulus, H: Hemorrhage , (B) - I:Infiltration, H.D: Hydropic Degeneration, and S:Sloughing . (H&E) .

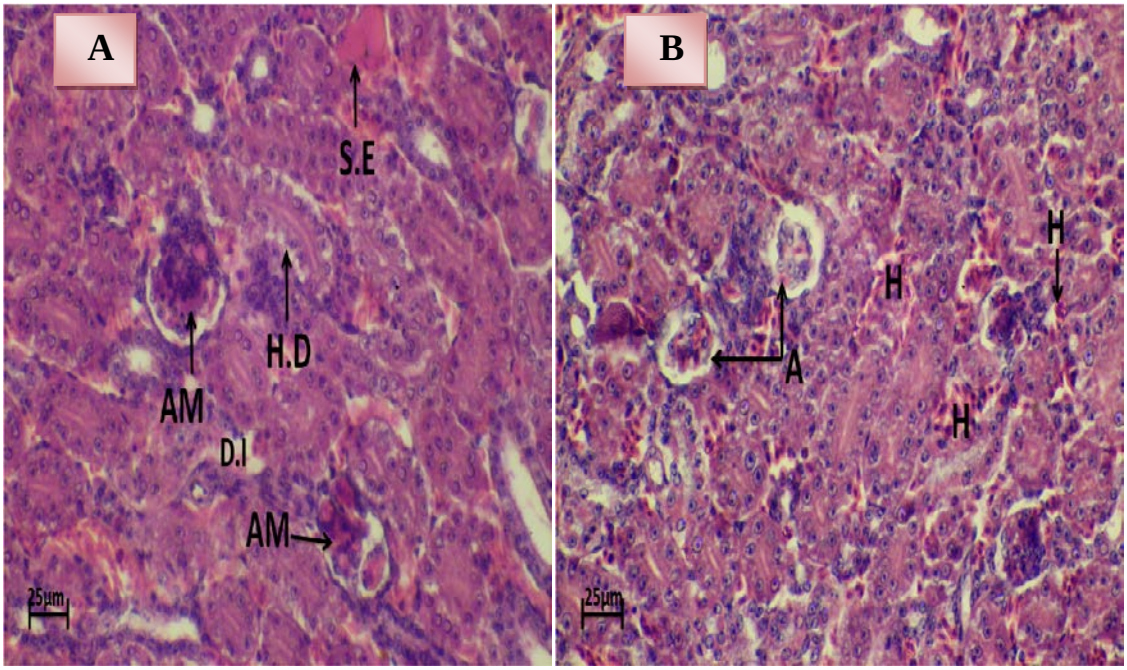


Figure (5): Section in the kidney of quail dosed with 8ppm of silver nanoparticles shows:(A) -S.E:Serous Exudate, AM:Amyloid, D.I:Dissociation of Interstisial substances, H.D: Hydropic Degeneration , (B) - A:Atrophy of glomerulus, and H: Hemorrhage. (H&E) .

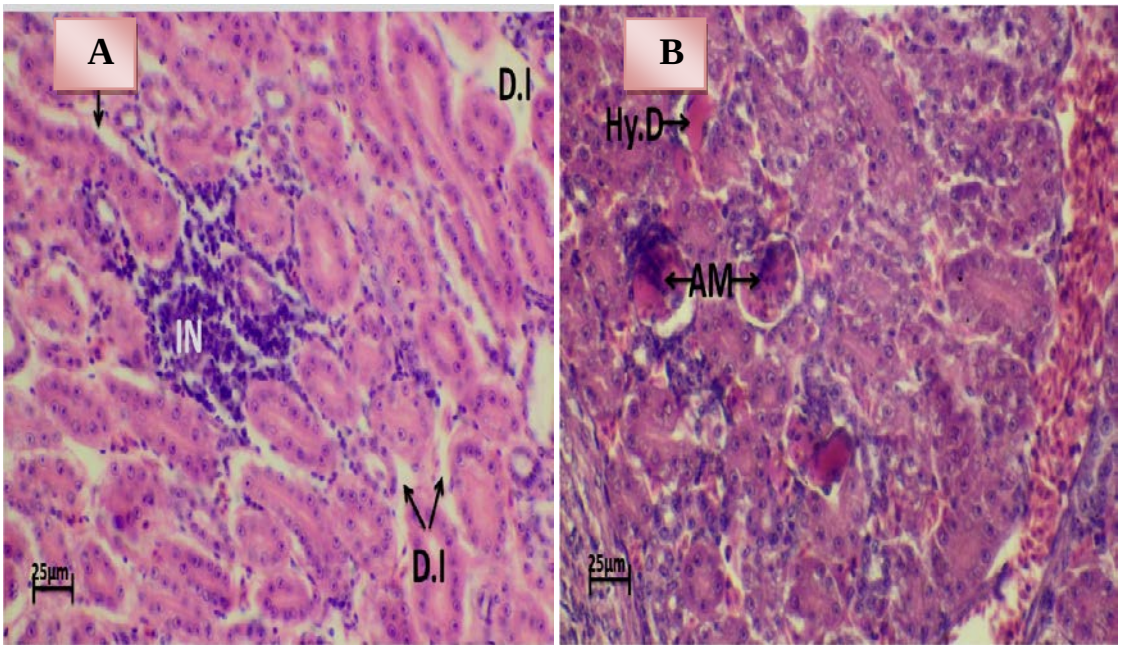


Figure (6): Section in the kidney of quail dosed with 12ppm of silver nanoparticles shows:(A)- D.I:Dissociation of Interstisialsubstances,IN: Infiltration, S:Sloughing, (B) - AM:Amyloid and Hy.D:Hyaline Degeneration . (H&E) .

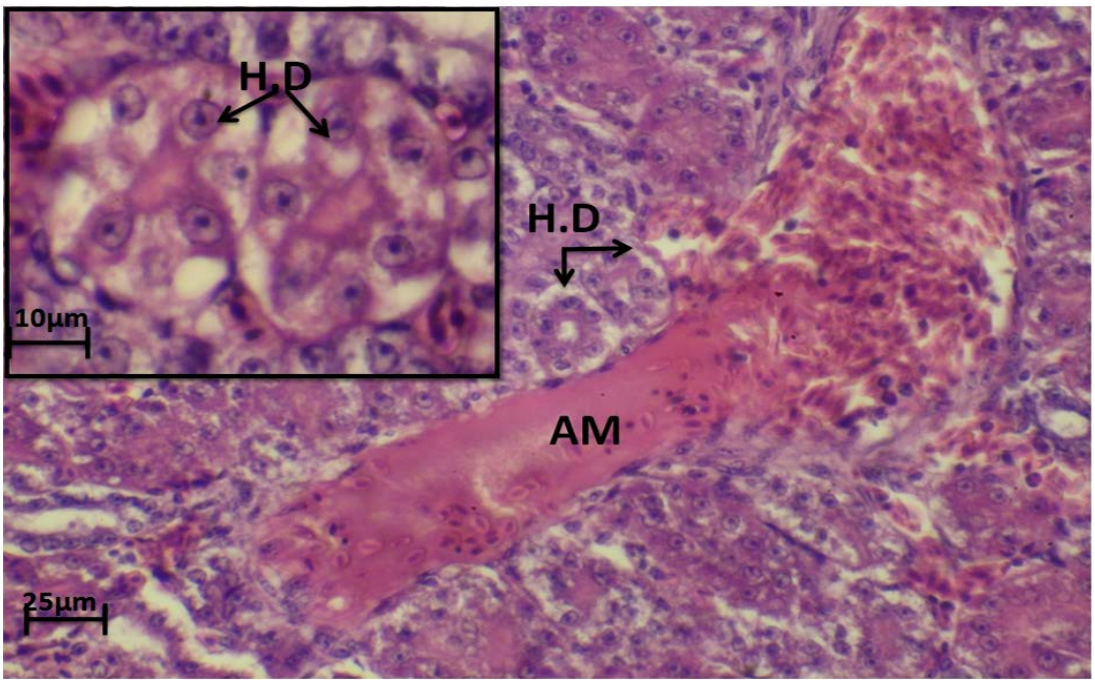


Figure (7): Section in the kidney of quail dosed with 12ppm of silver nanoparticles shows: (A) - H.D: Hydropic Degeneration , (B)-AM:Amyloid and H.D: Hydropic Degeneration . (H&E) .

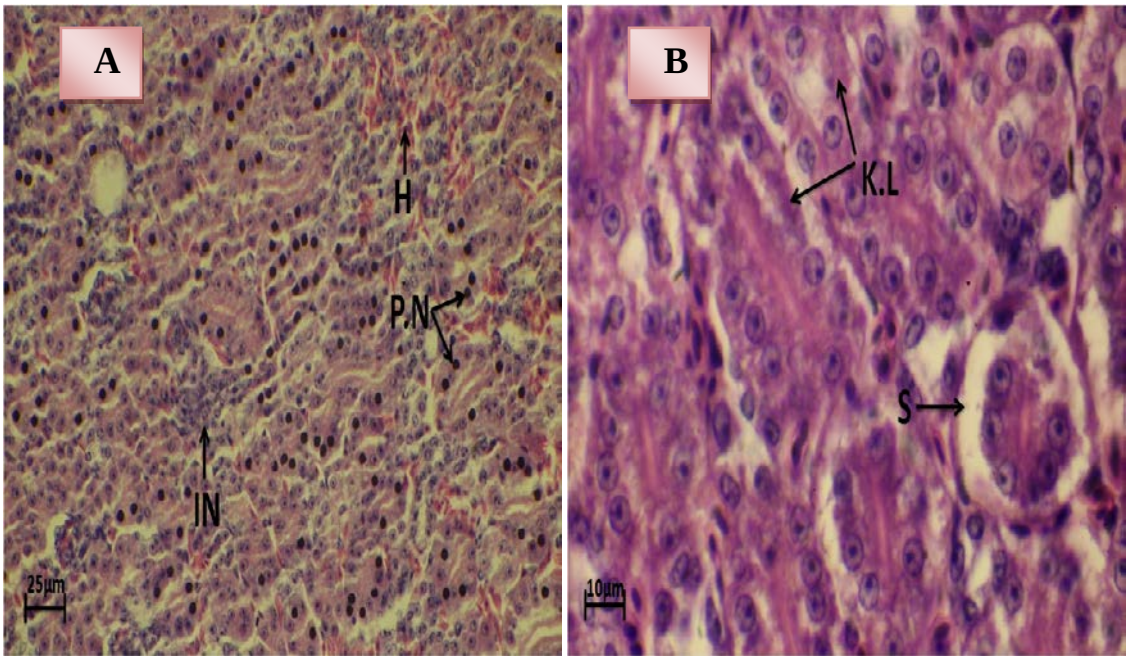


Figure (8): Section in the kidney of quail dosed with 12ppm of silver nanoparticles shows: (A) – P.N: Pycnotic Nuclei, IN:Infiltration, H:Hemorrhage, (B) – K.L: Karyolysis and S: Sloughing . (H&E) .

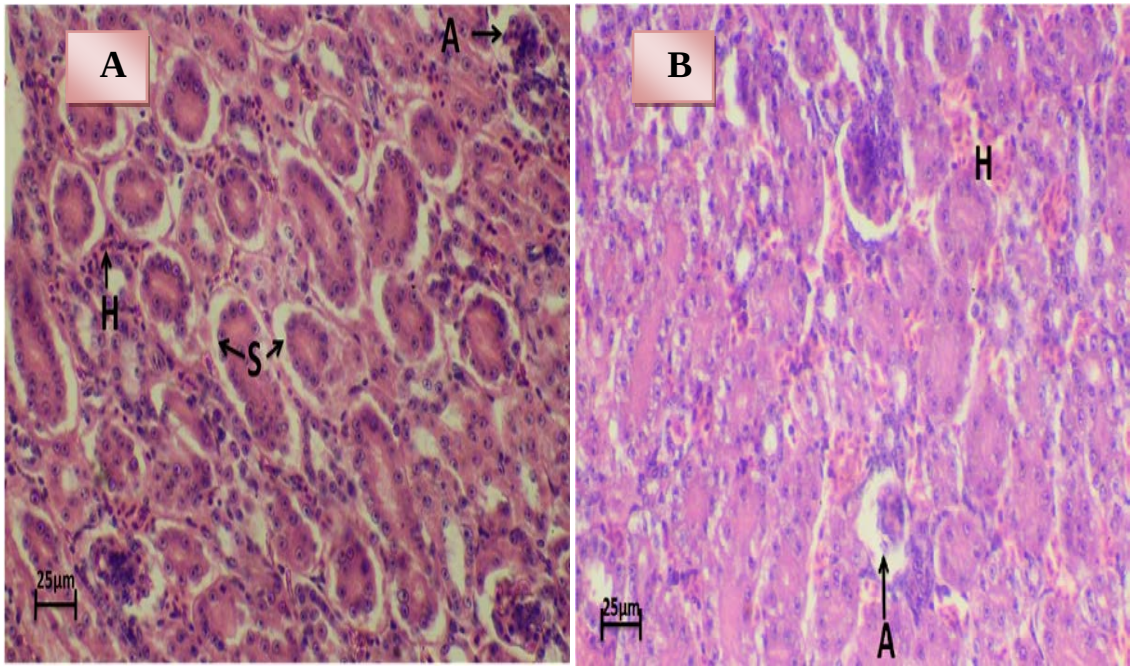


Figure (9): Section in the kidney of quail dosed with 12ppm of silver nanoparticles shows: (A) –S: Sloughing ,H:Hemorrhage, (B) – A:Atrophy . (H&E) .

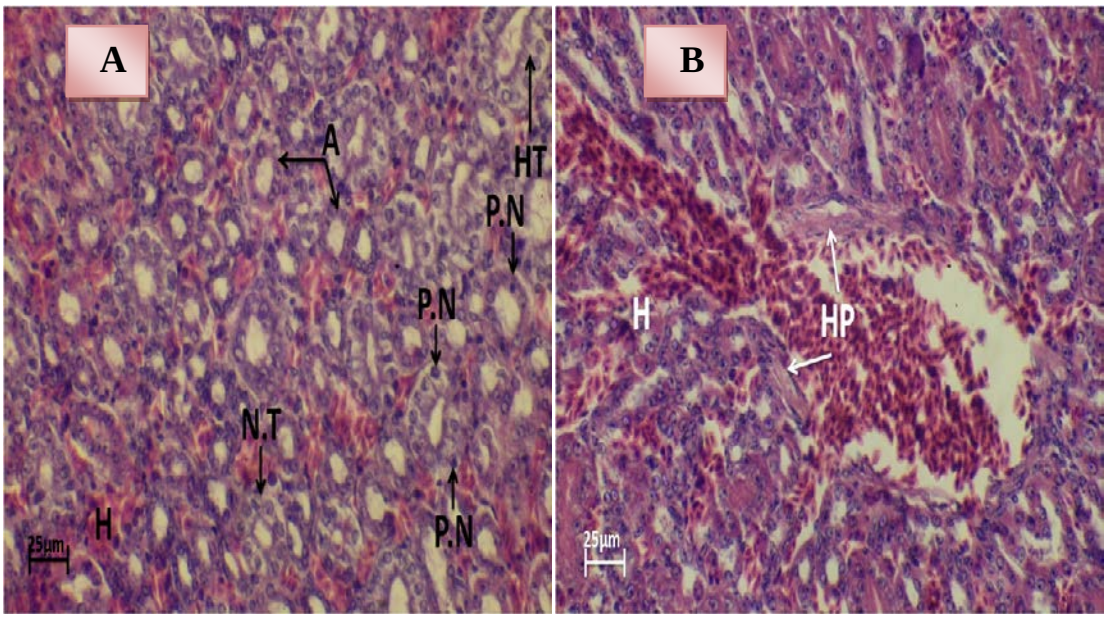


Figure (10): Section in the kidney of quail dosed with 12ppm of silver anaoparticlesshows:(A) -P.N:Pyknotic nuclei, HT:Hypertrophy, A:Atrophy, N.T:Normal Tubules. (B) – HP:Hyperplasia, H:Hemorrhage . (H&E) .

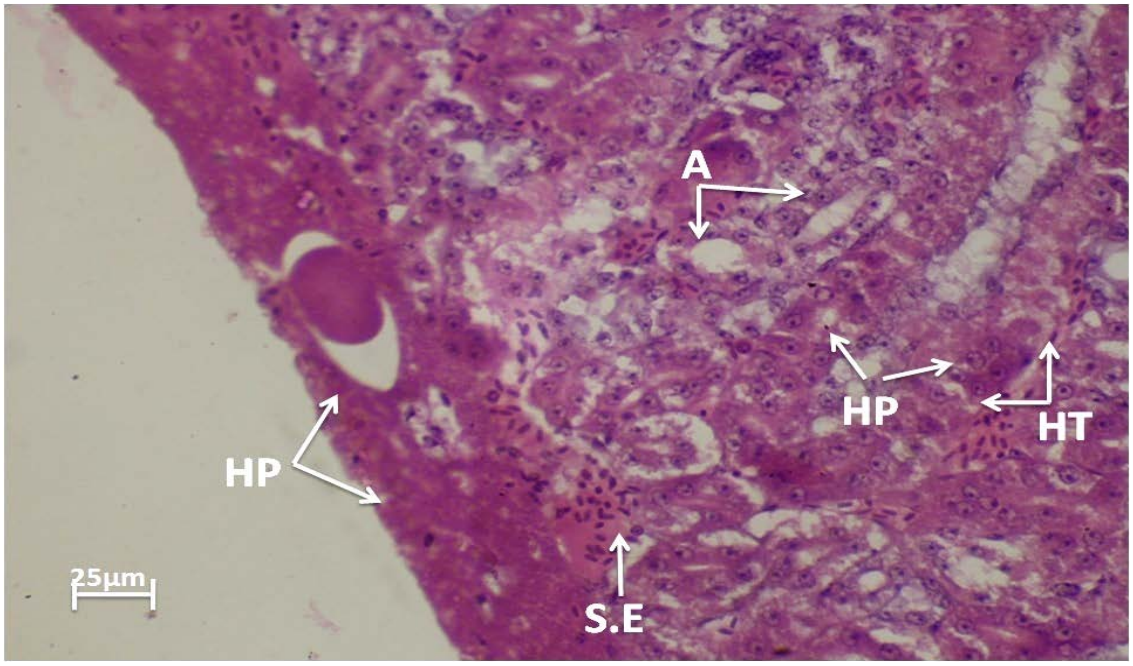
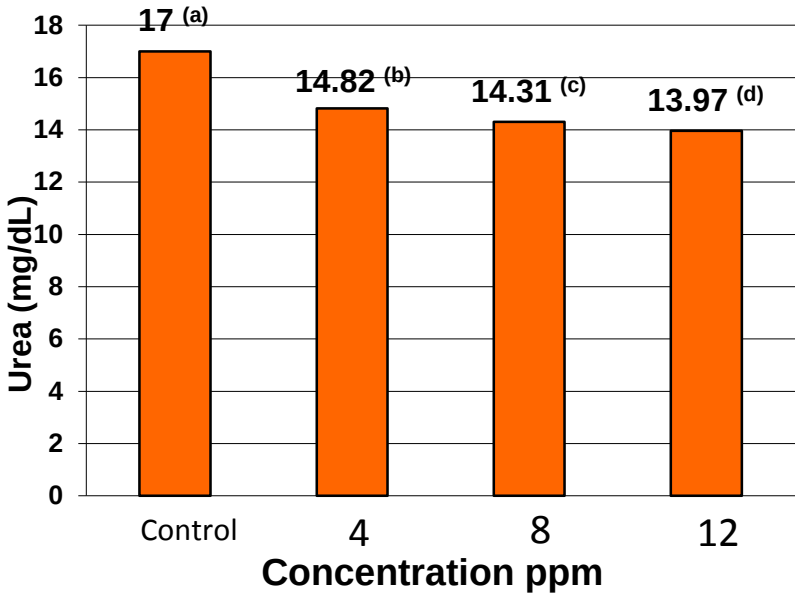


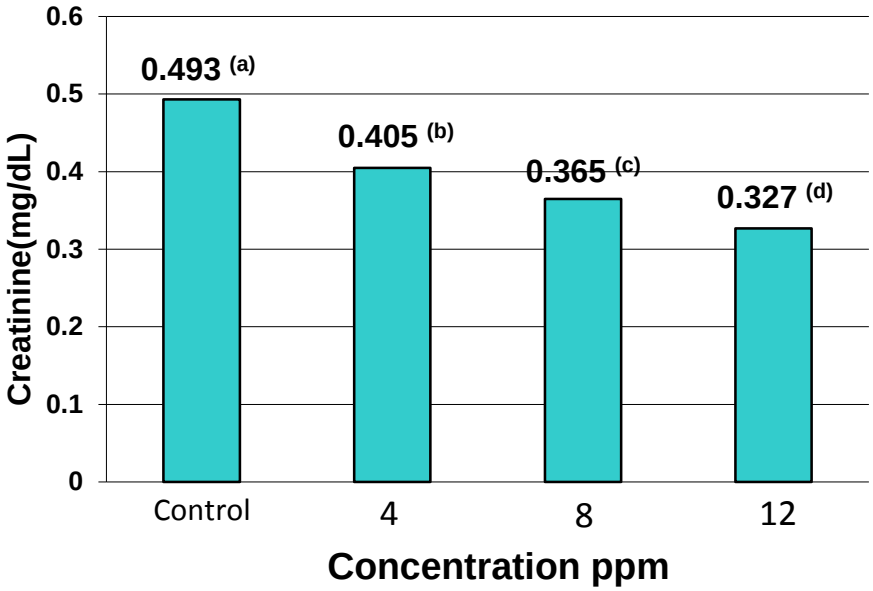
Figure (11): Section in the kidney of quail dosed with 12ppm of silver nanoparticles shows: HT: Hypertrophy, HP:Hyperplasia, A:Atrophy and S.E:Serous Exudate . (H&E) .

Serum biochemistry

The results of measuring the amount of blood urea and creatinine showed that, there is a statistically significant decrease($P \leq 0.05$) in the level of urea and creatinine were observed in all groups were dosed with silver nanoparticles when compared with the control group (Figure 12,13).



Figure(12): Effect of silver nanoparticles concentrations(4,8,12) in urea level of quail. Different letters mean significant (P≤ 0.05)



Figure(13): Effect of silver nanoparticles concentrations(4,8,12) in creatinine level of quail. Different letters mean significant (P≤ 0.05)

Discussion

1.Histological study

The skin , respiratory system and digestive system represent the common ports for silver nanoparticles penetrating in the body because of it's small size, and digestive system represents the most one port that allows silver nanoparticles to enter the body in form colloidal suspension, the absorbent particles associates via digestive tract with plasma proteins will transport and distribute throughout all various organs such as liver, kidney and heart(10). The toxicity of silver nanoparticles primarly occurs through activation of oxidative stress which in turn caused lipid peroxidation via liberation of free radicals in the body which attacks cellular membranes and liberates reactive oxygen species. Thus, the increment accumulation of these species stimulate inflammatory responses (11).

The first effects resulting from inflammatory responses, cause congestion of blood vessels in renal tissues (cortex and medulla) in samples dosed with 4ppm, and the congestion intensity increase in samples dosed with higher concentration. Moreover, the explanation of this changes in tissues may be due to the effect of silver nanoparticles on blood vessels walls lead to dilation of the blood vessel and at the end the vessels full with blood (12). In response to changes occurs in blood vessels will lead to infiltration of inflammatory cells, which included tissues of kidneys' cortex and medulla which was observed in samples dosed with 8ppm. This pathological case increase with samples dosed with 12 ppm , the infiltration is happening due to leukocytes penetration of blood vessels that cause response for some chemical mediators, because of endothelial cells of blood vessels contraction which causing increment in space size among endothelial cells that lining blood vessel or due to absence of desmosomes that lies among endothelial cells of blood vessels by effects of silver nanoparticles as well as red blood cells abilities to adhesion with each other forming roloux which in turn causing rush of white blood cells from the middle of the vessel into the vessel's circumference according to marginal theory and adhesive to blood vessel's walls, as well as amoebic movement of white blood cells which in turn plays a role in penetration of vessel's walls to reach the affected tissue and do its phgocytotic function (13).

Silver nanoparticles caused extensive histological changes in blood vessels' walls. It has been observed serous exudate in interstitial spaces of cortex and

medulla which appeared with low proportion in samples dosed with 4ppm and 8 ppm while appeared with higher amounts in samples dosed with 12ppm , as studies mentioned that the histological changes caused serous exudate resulting from increment of interstitial spaces size among endothelial cells of blood vessel by effect of inflammation that causing malfunction in hydrostatic pressure which is greater than osmotic pressure and causing rush of macromolecules represented by small size proteins-containing fluids (caused serous exudate) outside the blood vessels and thus accumulation in interstitial spaces of the damaged tissue(13,14). Also, silver nanoparticles caused acute effect on blood vessels' walls by causing rupture in these vessels and leak of red blood cells which pass throughout interstitial spaces of the damage tissue and this condition called hemorrhage(13),the present study showed low hemorrhage in cortex and medulla of kidneys in samples dosed with 4ppm which is gradually increasing in higher concentrations, as well as effect of silver nanoparticles on walls glomerulus capillaries caused destruction and hemorrhage of glomerulus which appeared in samples dosed with 8ppm and 12ppm.

Silver nanoparticles caused deposition of different types of substances in intracellular and lead to swelling. Thus, water deposits in epithelial cells of renal tubules which demonstrated by results of the present study in most kidneys samples and for all concentrations,the main reason for the water degeneration is inhibition of glycolysis by nanosilver ions via inhibition of oxidative phosphorylation process causing decrease in ATP production and lead to inhibition of enzyme Na+, K+-ATPase of cellular membrane (15) therefore, causing sodium influx inside the cells and pottasuim exit of causing swelling of mitochondria also water accumulation inside the cells lead to swelling (14) . As well as proteins deposition represented by hyaline degeneration inside renal tubules cells for each of cortex and medulla in samples dosed with 4 ppm which gradually increase with concentrations increment and this result agrees with many studies (16,17,18) on rats, guinea pigs and fishes. The proteins deposition occurs due to accumulates of protein droplets in the cells' cytoplasm as final result of atrophy process or damage of the cells which represented by accumulation of the non natural proteins resulting from amino acid decomposition especially immuno-globulin and carbohydrates as a result of some

cells death because of inflammation caused by silver nanoparticles(12). As well as amyloid deposition which is represented by cellular homogenous substances deposit in situations outside the cells especially in situations adjacent to the blood vessels due to malfunction occurs to protein synthesis by effect of silver ions which released from silver nanoparticles and may immuno-globulins phagocytosis occurs and light chains of globulin taken by the cells and after partial decomposition the protein fibers excreted as amyloid(12,14). Amyloid deposition has been observed around basal membranes of glomerulus capillaries and blood vessels in most samples.

Most of renal glomerulus showed higher ability to resist silver nanoparticles damage and adaptation with changes that caused by silver nanoparticles via atrophy incidence which represented by decreasing in the size of epithelial cells in glomerulus tissue due to lack of food and oxygen supply (19) Moreover, the atrophy not limited to epithelial cells of glomerulus tissue but included connective tissue especially interstitial substance of kidney (cortex and medulla) causing disintegration of interstitial spaces and blood vessels walls thickness of kidney's tissue in samples dosed with 8ppm and 12ppm, while thickness of renal tissue capsule was observed in samples dosed with 12 ppm, this was confirmed by the study done by (17) which included effect of silver nanoparticles on Guinea pig tissues, so increment of capsule and blood vessels walls thickness indicates on hyperplasia in connective tissue and this indicates on fibers formation from fibroblasts stimulated by nanosilver, which represent a behavior or adaptation of tissue to resist nanosilver effect(19).

Additionally, silver nanoparticles damage was observed on basement membrane which renal tubules cells based on it, through impact on hemidesmosomes and causing epithelial cells sloughing from basal membrane toward tubule's center leading to decreasing cavity size of renal tubule(20). In addition, the histological alterations included epithelial cells nuclei of renal tubules causing pyknosis when chromatin condensing as dark mass, and one of necrosis cases resulting from negative effect of silver nanoparticles on chromatin substance, also karyolysis occurs when chromatin substrate decomposition (19).

As well as, the greatest effects on renal tubules of cortex was observed in group dosed with 12 ppm of silver nanoparticles which represented by hypertrophy of renal tubule's size and hyperplasia of epithelial cells lining the tubules. As mentioned by (14) that

synchronization of hypertrophy and hyperplasia considered abnormal indication to form abnormal tissue which considered the initiate step of neoplasia. Therefore, the present study showed nanosilver capability to induce unprogrammed division of the cells and increased size of renal tubules.

2.**Biochemical study**

The kidneys are important organ in the body which plays a main role in pH regulation of body fluids through re-absorption and maintenance balance of sodium, potassium, hydrogen and remove metabolism wastes (19). Urea and creatinine tests are the most important indicators to measures renal efficiency to remove wastes resulting from metabolic process in the blood, so abnormal increasing or decreasing in urea and creatinine levels indicates for renal failure (21). The results of present study showed significant decrease in urea level and this agree with results of a study done by(22) on mice. Probably, urea leveldecreased due to silver nanoparticles capability to create free radicals which in turn attack hepatocytes causing alterations for hepatocytes which has been supported by histological sections of this study, thus negatively affects on urea cycles in liver tissues, and probably ammonia resulting from proteins catabolism in the body not able to transform into urea leading to decrease in urea level (22). Also, nanosilver particles may interfere with urea cycle enzymes that produced by hepatocytes causing inhibition of these enzymes and then malfunction occurs to urea cycle in treatment groups as compared to control groups.

Creatinine is the most sensitive indicator than urea to measures renal function, its level associated with skeletal muscles mass(21). The present study was observed significant decrease in creatinine level when compared with control group due to impairment of food intake by birds and dependent on food storage in skeletal muscles or may due to silver nanoparticles effect on amino acid (1) especially glycine and arginine, which play important role in creatine synthesis, causing damage in these amino acids and then decrease in creatine and creatinine level.

Conclusion

Histopathological findings from the kidneys showed in all dosed group by silver nanoparticles have induced low and sever injuries such as congestion, infiltration, hyaline and hydropic degeneration, amyloid, atrophy, hypertrophy and hyperplasia of renal tubules.As well as silver nanoparticles have produced alterations on urea and creatinine levels.Therefore, the

concentrations used in this study can not be use as anti-microbial agents.

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Detection of *Enterobacter* spp. in Iraqi infants patients with meningitis and nectroiziting enterocolitis

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Abstract: This study was design to detected *Enterobacter* spp. from infants patient suffered from meningitis and nectroiziting enterocolitis. Three hundred (350) clinical samples were collected include (blood, cerebrospinal fluid, stool and urine) were collected from infants patient suffered from meningitis and nectroiziting enterocolitis. This study is the first to report the isolation of *Enterobacter spp.* among the preventive isolates using Vitek-GN2 system gave 33/350 (9.4%) isolates of *Enterobacter spp.* isolates include (*E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *E.hormaechei*, *E.kobei*, *E.ludwigii*) that isolates show differences between percentages of each isolate presence were non-significant (P<0.05). The results of antibiotic susceptibility was determined using Vitek-2GN system; *Enterobacter spp.* isolates showed 100% resistance to cefazolin, ceftazidime , Nitrofurantion, Trimethoprim \sulfamethoxazole, and cefoxitin.

Keywords: *Enterobacter spp.*, NEC. *Enterobacter spp.*

التحري عن *Enterobacter spp.* في الاطفال العراقيين الرضع المصابين بالتهاب السحايا والتهاب الامعاء والقولون التنخري

ياسمين سليم حسن الجبوري و منى الموسوي

قسم علوم الحياة-كلية العلوم للبنات-جامعة بغداد

الخلاصة: اجريت هذه الدراسة للتحري عن *Enterobacter spp.* لدى الاطفال الرضع.تم جمع 350 عينة سريرية تشمل (الدم، الخروج،الادرار ،وسائل النخاع الشوكي) من الرضع المصابين بالتهاب السحايا والتهاب الامعاء والقولون التنخري .سجلت هذه الدراسة العزل الاول لجرثومة*Enterobacter spp.* بين العزلات باستخدام نظام الفايتك GN2 اعطى 350/33 (9.4%) عزلة من *Enterobacter spp.* وتشمل (*E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *E.hormaechei*, and *E.ludwigii*) .الاختلافات بين النسب المئوية لكل عزلة كانت غير معنوية (P<0.05) . اظهرت نتائج المقاومة للمضادات الحيوية باستخدام نظام الفايتك GN2 عزلات *Enterobacter spp.* مقاومة 100% لكل من cefazolin, ceftazidime Nitrofurantion, Trimethoprim \sulfamethoxazole,, وcefoxitin .

Introduction

Enterobacter is a genus of common Gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria of the family Enterobacteriaceae. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts and in those who are on mechanical ventilation(1).

The urinary and respiratory tracts are the most common sites of infection.

The genus *Enterobacter* is a member of the coliform group of bacteria. It does not belong to the fecal coliforms group of bacteria, unlike *Escherichia coli*, because it is incapable of growth at 44.5 °C in the presence of bile salts. Some of them showed quorum sensing properties as reported before. (2)

Two clinically important species from this genus are *E. aerogenes* and *E. cloacae*.

The genus *Enterobacter* ferments lactose with gas production during a 48-hour incubation at (35-37) °C in the presence of bile salts and detergents. It is oxidase-negative, indole-negative, and urease-variable. (2, 3).

Treatment is dependent on local trends of antibiotic resistance. (3) Cefepime,afourth-generation cephalosporin from the β-Lactam antibiotic class.

Imipenem (carbapenems) is often the antibiotic of choice.

Aminoglycosides such as amikacin have been found to be very effective, as well.

Quinolones can be an effective alternative.

A recent study has shown that the presence of *Enterobacter cloacae* B29 in the gut of a morbidly obese individual may have contributed to the patient's obesity. Reduction of the bacterial load within the patient's gut, from 35% *E. cloacae* B29 to non-detectable levels, was associated with a parallel reduction in endotoxin load in the patient and a concomitant, significant reduction in weight, Furthermore, the same bacterial strain, isolated from the patient, induced obesity and insulin resistance in germfree mice that were being fed a high-fat diet. The study concludes that *E. cloacae* B29 may contribute to obesity in its human hosts through an endotoxin-induced, inflammation-mediated mechanism.(4)

Materials and Methods:

A total of 350 clinical samples {100 samples from each blood, stool and urine from patients with necrotizing enterocolitis NEC , while 50 samples from cerebrospinal fluid (CSF) from patients with meningitis were especially collected from 100 patients under 2 years of age , the information and clinical

signs was recorded according to patient's symptom .Method was done as described by Kim *et al*., (2008) for isolation ofpresumptive *Enterobacter spp.* from clinical sample as follow .(5)

From each clinical sample 0.5 ml of blood, 0.5 ml urine (mid steam), 0.5 ml of CSF (through a spinal tap) and fecal swab from stool} was aseptically introduced into 10 ml of buffered peptone solution (PBS) and incubated at 37°C for (18-24) hrs. 1mL of preenriched buffered solution was added to 10 ml of Enterbacter enrichment broth (EEB) and incubated at 37°C for (18-24) hrs. The enriched culture was streaked onto selective media Hicrome *Enterobacter* Agar (HEA) .Up to 33 presumptive *Enterobacter spp.* colonies that exhibited during culture on HEA were selected for culture on Trypton Soy Agar (TSA) at 25°C for (48-72) hrs.

Antimicrobial susceptibility test

All the confirmed isolates were tested to antibiotic resistance using Vitek-2 GN system according to production company in AL.Mahmudia Hospital .The tested antibiotics (n=17) includes (Ampicillin, Ampicillin\sulbactam, Pipercillin \Tazobactam, Cefazolin, Cefoxitin, Imipenem, Meropenem,Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Ceftazidime, Ceftriaxone, Cefepime, Levofloxacin , Nitrofurantion, and Trimethoprim \sulfamethoxazole).

Results and Discussion

Table (1) shows 33(9.4%) isolates which appear blue-green colonies on HEA, raised colonies (1-2) mm diameter with or without halos after (18-24) hr. at 37 ºC and microscopic examination appears as single or double chains and motile were considered presumptive *Enterobacter spp*.

Table (1): Percentagesofpresumptive *Enterobacter* spp. isolates and its characteristics on culture mediaaccording to thetype of sample

Samples (No.)		Characteristics on HESA (No. of presumptive *Enterobacter* spp. Isolates)	% of total samples	Characteristics on TSA (No. of presumptive *Enterobacter* spp. Isolates)	% of total isolates
Clinical samples (350)	Blood (100)	Blue –green (6)	1.42	Wight pigment (5)	15.20
	Stool (100)	Blue –green(19)	4.85	Wight pigment (17)	51.51
	Urine (100)	Blue –green(12)	3.14	Wight pigment (11)	33.33
	CSF (50)	0	0.0	0	0.0
Total	350	37	9.41	33	100

Biochemical tests

All the tested isolates (n=37) that gave yellow pigmented on TAS give the same result to the biochemical tests. The urease, oxidase, coagulase, indol and H2S production gives negative result, while catalase, citrate and motality test gives positive result table (2).These results showed conformity with the *Enterobacter spp*.(6)

Table (2): Biochemical tests of the presumptive *Enterobacter spp.* Isolates

Test	Results
Urease , Oxidase , Coagulase , Indol , **Sodium thiosulphate** production	Negative
Catalase , Citrate , Motality	Positive

API 20 E test

The tested isolates (n=33) that gave positive results in the biochemical tests appeared same results in API 20E test. 33 isolates showed conformity with the *Enterobacter cloacae*. The results of API 20E for *Enterobacter spp.* revealed that

Arginine dihydrolase (ADH), Omithine decarboxylase(ODC) and Sodium citrate (CIT) were positive, Ortho-nitro-phenyl-galactopyranoside (ONPG), Sodium thiosulphate (H_2S), Lysine decarboxylase (LDC), Urea (URE), Tryptophane (TDA) and Indole production(IND) were negative. Voges–Proskauer (VP) and gelatin (GEL) were also positive and all sugars Glucose (GLU), Mannitol (MAN), Rhamnose (RHA), Sucrose (SAC), Sorbitol (SOR), Melibiose (MEL), Amygdalin (AMY) and Arabinose (ARA) were positive except indole production which was negative.

The API 20E biochemical kit has been reported not to be a reliable tool for the confirmation of the identity (7). Reported that the misidentification of strains by the API 20E biochemical kit was due to its limited biochemical gallery. The latter kit consisted of a wide variety of fermentable carbon sources and was able to correctly identify strain *E. cloacae* rather than as *C. sakazakii*.(8).

Vitek -2 system

Thirty three isolateshowed excellent identification confidence with the *Enterobacter* spp. that included *E.cloacae* spp. *dissolvens*, *E.cloacae* spp. *cloacae*, *E.hormaechei*, *E.kobei* and *E.ludwigii* with identification values (94 –99)%, Four presumptive isolates (on HESA)were tested by Vitek GN2 system. The isolates *C.sakazakii* that showed excellent identification confidence with the *C.sakazakii* organism in Vitek-GN2 with the identification values or probability (98 - 99)% table(3). Differences between percentages were non-significant (1.378).

Table (3) :The identification values of *C.sakazakii* and *Enterobacter spp.* by Vitek-2 system.

No. of isolates	Type of organisms	Identification values (%)
16	*E.cloacae* spp. *dissolvens*	99
8	*E.hormaechei*	98
2	*E.cloacae* spp. *cloacae*	96
1	*E.ludwigii*	96
1	*E.kobei*	94
Chi-square value	----	1.378 NS

NS: Non-significant. (P≤ 0.05)

Oonaka *et al*. (9) identified 52 strains of *Enterobacteriaceae* isolated from PIF using VitekGN2 compact system (Biomerieux) with the identification values of (80 – 99)%.

Table4 shown distribution of *Enterobacter* spp. among the presumptive isolates according to the type of samples using Vitek-GN2 system gave 33 isolates of*Enterobacter* spp. (100%) that included *E.cloacae* ssp. *cloacae* (63.64%) , *E.cloacae* ssp. *dissolvens* (6.1%) , *E.hormaechei* (24.24%) , *E.kobei* and *E.ludwigii* (3.03%) of each one.*E.cloacae* ssp. show increase in the significant between percentages (P<0.01).

Blood samples showed increase in the significant between percentages (P<0.01).Urine sample shows decrease in the significans between percentages (4.054), while stool sample shows no significant between percentages (P<0.05).

From these results which indicated that Vitek-2 system have ability to distinguish between *Enterobacter* spp. (*E.cloacae* ssp. *cloacae and E.cloacae* ssp. *dissolvens*, *E.hormaechei*, *E.kobei*, *E.ludwigii*) rather than as *E. cloacae* only, While API-20E system does not have ability to distinguishbetween *E.cloacae* and other *Enterobacter* spp.

As shown in table (4) it is indicated that *E.cloacae* ssp.*cloacae and E.cloacae* ssp. *dissolvens*that's the common isolate (73%) from *Enterobacter spp.* (*E.hormaechei*, *E.kobei, E.ludwigii*). These results agree with(10) whom shown to be of clinical significance by the report of several outbreaks of sepsis in neonatal intensive care units in Brazil and the USA that causes by *E. cloacae*. Differences between percentages of *Enterobacter spp.* were significant (P<0.01).while Differences between percentages of each isolate were non- significant.

Table (4) : Distribution of *Enterobacter spp* among the isolates according to type of samples using Vitek-2 system.

Type of samples (No. of isolates)		*E.cloacae* ssp. *Cloacae*	*E.cloacae* ssp. *dissolvens*	*E. hormaechei*	*E.kobei*	*E. ludwigii*	Chi-square value
Clinical samples	Blood (5)	2(1.43)	1 (0.86)	1(0.57)	1(0.3)	0(0.00)	0.064 NS
	Stool (17)	10(0.86)	0(0.00)	6(1.7)	0(0.00)	1(0.57)	0.072 NS
	Urine (11)	10(2.86)	1(0.86)	0(0.00)	0(0.00)	0(0.00)	0.0669 NS
Total (37)		22(59.46)	2 (5.41)	7(18.92)	1(2.7)	1(2.7)	11.032 **

NS: Non-significant.

Species of the *Enterobacter cloacae* complex are widely encountered in nature, but they can act as pathogens. The biochemical and molecular studies on *E. cloacae* have shown genomic heterogeneity, comprising six species: *Enterobacter cloacae, Enterobacter asburiae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigii* and *Enterobacter nimipressuralis, E. cloacae* and *E. hormaechei* are the most frequently isolated in human clinical specimens. The *Enterobacter* spp. has taken on clinical significance and has emerged as nosocomial pathogens from intensive care patients. The National Nosocomial Infections Surveillance System reported data on nosocomial bacteremia from 1976 to 1989 in the USA, and the National Healthcare Safety Network (2008) reported that *Enterobacter* spp. account for approximately 5% of nosocomial bacteremia cases; these data have not varied. Another multicenter study focused on 24,179 cases of nosocomial bloodstream infections between 1995 and 2002 showing *Enterobacter* spp. to be among the most ten commonly isolated nosocomial pathogens with a greater incidence in intensive care unit wards. *Enterobacter* spp. account for approximately 88.1% from isolates, *E. cloacae* is an important nosocomial pathogen responsible for bacteremia and lower respiratory tract, urinary tract and intra-abdominal infections, as well as endocarditis, septic arthritis,

osteomyelitis and skin and soft tissue infections. The skin and the GI tract are the most common sites through which *E. cloacae* can be contracted. *E. cloacae*have emerged as one of the most commonly found nosocomial pathogen in neonatal units, with several outbreaks of infection being reported. *E.hormaechei* is commonly isolated as a nosocomial pathogen of clinical significance; it has been reported in several outbreaks of sepsis in neonatal intensive care units in the USA and in Brazil, where the outbreak zoriginated from contaminated parenteral nutrition. *E. ludwigii* and *E. kobei* have taken on clinical significance and have emerged as nosocomial pathogens from intensive care patients (11).

Antimicrobial susceptibility test

The results of antibiotic susceptibility has been that done using Vitek-2 system *Enterobacter spp.* isolate was 100% resistant to cefazolin, ceftazidime , Nitrofurantion, Trimethoprim \sulfamethoxazole,and cefoxitin. *Enterobacter spp.* isolates are high sensitive to many antibiotics includes (Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Ampicillin, Ampicillin\sulbactam, Pipercillin \Tazobactam, Ceftazidime, Ceftriaxone, Cefepime Azetreonam and augmentin). The results in the present study agreement with previous studies. Jacoby (12) that has found *Enterobacter spp.*isolates locally shown 100% resistance to cefazolin, ceftazidime ,and cefoxitin. And with Pintado, *et al.*, (13) that found *Enterobacter spp.*were susceptible to Trimethoprim \sulfamethoxazole. The results of this study not agreement with our study. Gupta *et al*.,(14) that found *Enterobacter spp.*were resistant to Quinolones this result is not agreement with our study. Lowman *et al*.,(15) that they found *Enterobacter spp.*resistance to ampicillin these results of this study not agreement with our study. In 2006, Paterson published a good review of resistance among various Enterobacteriaceae.(16). Ritchie *et al*., (17) published a good discussion regarding antibiotic choices for infection encountered in the ICU.

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Effect of Diabetes mellitus on certain sperm function parameters before and after *in vitro* activation the *Glycyrrhiza glabra* as motility stimulant

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Abstract: Diabetes mellitus is one of the most common endocrine diseases that present in many infertile patients. This study was conducted to overwhelm the negative response of diabetic semen to *in vitro* activation techniques using a medium containing herbal motility stimulants (*Glycyrrhiza glabra*).The medium used for *in vitro* activation was Hams F12 with 0.1% *Glycyrrhiza glabra (G.glabra)*and two techniques was performed namely wash and spin technique and density gradient technique. The patients were divided into six groups according to male infertility factors .The results showed that the mean of sperm concentration in the six groups by using two activation techniques with free-*G. glabra* medium and with 0.1% *G. glabra* medium, was significantly (P<0.001) lower than that of before activation. Whereas ,the percentage of sperm motility and morphologically normal sperm(MNS) after activation by using density gradient technique with medium containing 0.1% *G. glabra* was significantly (P<0.001) higher in all the groups than that of before activation and after activation by using wash and spin technique. According to the data of present study, the best result of certain sperm characters were noticed when using a medium containing 0.1% *G. glabra* with density gradient technique for diabetic men. This data can be utilized to enhance the output of diabetic patient through assisted reproductive technologies programs.

Keywords: Diabetes mellitus, endocrine, infertile**,** *Glycyrrhiza glabra*

تأثير داء السكري على معايير النطف الرئيسيه قبل وبعد التنشيط في الزجاج باستخدام عرق السوس كمحفز للحركة

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الخلاصة: داء السكر هو أحد أمراض الغدد الصماء الاكثر شيوعا التي توجد في العديد من المرضى العقيمين . تهدف الدراسه ألى التخلص من التاثيرات السلبية لداء السكر على المني باستخدام التنشيط في الزجاج باستخدام محفز حركة مسحوق عرق السوس..استخدم في هذه الدراسه وسط زرعي Hams F12 كما تم استخدام اثنين من التقنيات تسمى الاولى النبذ المركزي وسباحه النطف , والثانية الانحدار التكاثفي. تم تقسم المرضى الى سته مجاميع تبعا الى عوامل العقم عند الرجال:المجموعه الاولى المرضى المصابين بوهن النطف- المجموعه الثانية المرضى المصابين بوهن النطف مع قله في عددها- المجموعه الثالثة رجال يعانون من حالة وهن النطف مع زيادة في نسبة الشكلياء غيرالطبيعيه- المجموعه الرابعة رجال يعانون من وهن النطف مع وجود عدد خلايا دائريه غير طبيعي .المجموعه الخامسة رجال غير عقيمين و مصابين بالسكر.المجموعه السادسة الرجال غير عقيمين وغير مصابين بالسكر(مجموعة سيطرة). اظهرت النتائج بان تركيز النطف باستخدام التقنيتان للتنشيط مع وسط زرعي خالي من عرق السوس أومع وسط زرعي يحتوي على 0.1٪ من عرق السوس ذات فروقات معنويه $P < 0.001$ عند المقارنة مع التركيز قبل التنشيط.علاوة على ذلك فان نسبه النطف ذات فعالية عالية ونسبة شكلياء طبيعية بعد التنشيط باستخدام تقنيه الانحدار التكاثفي مع وسط زرعي يحتوي 0.1٪ عرق السوس معنويا ($P < 0.001$) أعلى من قبل التنشيط وبعد التنشيط باستخدام تقنيه النبذ المركزي وسباحه النطف . ولوحظ بان الخلايا الدائرية قد اختفت بعد التنشيط باستخدام تقنيه الانحدار التكاثفي مع أوبدون 0.1٪ عرق السوس. نستنتج من الدراسة الحالية ، بأن أفضل النتائج الخاصة بمعايير النطف الرئيسية قد وجدت عند استخدام تقنيه الانحدار التكاثفي مع وسط زرعي يحتوي على 0.1٪ من عرق السوس للرجال المصابين بمرض السكري. ويمكن استخدام هذه البيانات لتعزيز نتائج مرضى السكري من العقيمين عند التوصية لاجراء برامج التقنيات المساعدة على الانجاب.

Introduction

Fertility is defined as the capacity to reproduce or the state of being fertile. Whereas, infertility as defined by the American Society for Reproductive medicine (ASRM) is a disease of the reproductive system that impairs the body ability to perform the basic function of reproduction. Thus, typically it means the inability to achieve pregnancy after one year of unprotected intercourse [(1).] In approximately 40% of infertile the male partner is either the sole or a contributing cause of infertility. Male infertility refers to the inability of a male to achieve a pregnancy in a fertile female, and it is commonly due to deficiencies in semen and semen quality that is used as a surrogate measure of male infertility[(2).] Now a day's assisted reproductive technologies (ART) have become more accessible to the general population, enabling infertility couples to hope that they can materialize their aspirations for healthy offspring later in life. It is well established that and good reproductive outcome has been female's age [(3).] Infertility in either male or female or both factors .One of male factor infertility is

asthenozoospermia which is defined as a reduction in sperm motility of less than 50%. Extreme temperatures and delayed analysis after sperm collections leading to the negative false sperm values. Numerous factors can affect sperm motility such as structural defect of sperm, increased semen viscosity, different infections, immunological factors, diabetes mellitus and others[4]. Diabetes mellitus is a disease characterized by elevated blood glucose levels than normal values. It is the result of defective insulin secretion and /or action. The resulting of chronic hyperglycemia is associated with damage to and subsequent dysfunction of various organs, especially the blood vessels, nerves, heart, eyes، and kidneys .There are two major types of diabetes. Type1 is insulin dependent diabetes mellitus. Type 2 is insulin independent Diabetes mellitus [5]. On the other hand it had been renewed interest in using extracts of medicinal plants in the treatment of various diseases, since they are natural products, easy to get and are also cheap .There is a wide interest in medicinal plants in Iraqi now and because male infertility is an important problem ,it was thought necessary to investigate the effect of one of these plants, namely Licorice (*Glycyrrhiza glabra*) on stimulation of human sperm motility. Extracts of this plant has been reported to cause a reduction in prostatic enlargement in prostatic cancer in human when it is mixed with eight different herbs [6], and consumption of the roots of the plants improve semen parameters in rams .At the same time *in vitro* addition of licorice to the culture medium used for gametes preparation and embryonic development was first used in Iraq since 2003[7]. However the semen of diabetic men was facing a fluctuation failure in preparation in vitro for assisted reproduction purposes. Therefore, the objective of the present study was to overwhelm the negative response of diabetic semen to *in vitro* activation techniques. The study will investigate the comparison of certain sperm function parameters of diabetic men semen between two sperm preparation techniques namely; Density gradient centrifugation technique and swim up technique (wash and spin) using Hams F12 medium with /without addition of 0.1% *Glycyrrhiza glabra* extrat.

Materials and Methods

1.Patients

Three hundred twenty patients were involved in the investigation. Their ages ranged between 18- 60 years old. The patient divided into two main groups. Eighty men were fertile that were both diabetic and non diabetic (control) .The other 240 patient were infertile suffering from Diabetes mellitus for more than 5 years, namely: Diabetic with

asthenozoospermia, Oligoasthenozoospermia ,asthenoteratozoo spermia and asthenozoospermia with leukocytosis.

2. Preparation of *Glycyrrhiza glabra* Extract:

The major method for preparation of *Glycyrrhiza glabra* extract is aqueous method 1000 gm. of licorice in granular powder moistened with boiling water and percolated until the licorice was decay. Then ammonia solution was added to the percolate, filtrates and evaporates until back pilular mass having a characteristic of sweet taste powder was prepared. The *Glycyrrhiza glabra* extract was obtained from the (Al-Ahliya Flavours & Fragrances Co. Ltd. IRAQ). The *Glycyrrhiza glabra* has been stored in well-closed container protected from light and moisture [(8).]

3. Preparation of *Glycyrrhiza glabra* Extract for *in vitro* Sperm Activation:

The concentration of *Glycyrrhiza glabra* working solution was prepared by adding 10 mg from *Glycyrrhiza glabra* extract to 10 ml PBS (0.1%) in plastic test tubes contained broad spectrum antibiotic (Ampicillin 0.004g) to prevent bacterial growth[(9).] The media which used for activation was prepared by adding 0.3 ml of *Glycyrrhiza glabra* suspension(30%) to 0.7 of Ham's F-12 Media. The solution was filtered using Millipore filter (0.45 µM) & (0.25µM), and then pH was adjusted to reach (7.2-7.4) [(10).]

- Seminal Fluid Analysis

The sample of seminal fluid was collected after 3-5 days of sexual abstinence directly into a clean container, the specimen was examined by macroscopic and microscopic examinations according to the standard form of WHO (1999).

- In *vitro* Sperm Activation Techniques

Two methods of In vitro sperm activation have been used in this study done according to Makkar, et al.[(10)] .

1- Wash and spin technique (Swim up) with the use of Hams –F12 alone and Hams F12 with *Glycyrrhiza glabra* medium 0.1%.
2- Density gradient technique with the use of Hams F12 alone and Hams F12 with *Glycyrrhiza glabra* medium 0.1%.

- Statistical Analysis

Data of present study were expressed as mean ±SEM and analyzed using Analysis of variance (ANOVA) .When F value reach the significant level, least significant test (LSD) was used to compare between the results [(11)].

Results

Table (1)shows that the mean of sperm concentration before activation of non diabetic fertile men was significantly(P<0.001) higher than that of diabetic fertile and infertile men in the four groups. After activation the sperm concentration was significantly (P<0.001) higher by using density gradient technique compared with using the wash and spin technique in fertile non diabetes and diabetic fertile and infertile groups except oligoasthenozoospermic men. The sperm concentration in diabetic fertile was significantly (P<0.001) higher than that of other diabetic infertile groups by using both techniques and media. However, the sperm concentration in oligoasthenozoospermic men following the activation by density gradient technique was improve compared to using wash and spin technique.

Table 1: Mean of sperm concentration following *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Sperm Concentration(m/ml)						
	Non D.M fertile	D.M fertile	D.M. Astheno spermia	D.M Oligo Astheno spermia	D.M. Astheno terato spermia	D.M. Astheno Spermia With leukocytosis	LSD
Before activation	73±1.9 A-a	67.2±2.70 A-b	62.1±2.54 A-c	11.4±0.7 A-d	61±2.2 A-c	61.6±2.1 A-c	1.95
Wash and spin technique alone	31.7±1.2 B-a	23.2±1.66 B-b	21.9±1.40 B-c	5.5±0.5 B-d	20±0.14 B-e	20.1±0.3 B-e	
Wash and spin technique with 0.1%G.g	33.2±1.4 C-a	24.8±1.48 B-b	23.3±1.44 B-b	6.0±0.5 B-c	21.6±056 B-d	21.5±0.4 B-d	
Density Gradient technique alone	35.7±0.8 D-a	32.9±1.4 C-b	30.4±1.2 C-c	6.1±0.3 B-d	30.6±1.3 C-c	29.1±1.2 C-c	
Density Gradient technique with 0.1% G.g	38.9±0.7 E-a	36.8±1.3 D-b	33.4±1.0 D-c	6.9±0.3 B-d	33.8±1.2 D-c	32.1±0.9 D-c	
LSD	2.2						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05
Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

Table (2) revealed that the active sperm motility (Grade A) pre-activation in fertile men (43.3±1.4) was significantly (P<0.001) higher than that of infertile diabetic men in four groups .The percentage of sperm motility (Grade A) increases after activation in all semen samples of the six groups .There was a significant (P<0.001) elevation by using density gradient technique of fertile non-diabetic and diabetic samples compared with the four semen samples of diabetic infertile men. At the same time the percentage of sperm motility grade A in diabetic men complaining from asthenoteratozoospermia and asthenospermia with leukocytospermia was significantly(P<0.001) increase compared to diabetic men complaining from asthenozoospermia alone and men with oligoasthenozoospermia by using wash and spin technique or density gradient technique with both media.

Table (2): Mean of sperm motility (Grade A) following *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Sperm motility Grade A						
	Non D.M fertile	D.M fertile	D.M Astheno spermia	D.M. Astheno Oligospermia	D.M. Astheno teratospermia	D.M. Astheno spermia with leukocytospermia	LSD
Before activation	43.3±1.4 A-a	41±2.8 A-b	4±1.4 A-c	1.5±0.7 A-d	6.3±1.4 A-e	6.5±1.6 A-e	1.75
Wash and spin technique alone	50.8±1.6 B-a	45±2.6 A-b	8.4±1.3 B-c	5.8±0.7 B-d	14±1.6 B-e	12.5±1.3 B-f	
Wash and spin technique with 0.1%G.g	56±1.6 C-a	48±2.4 A-b	11±1.0 B-c	7.5±0.7 B-d	18±1.6 B-e	16.5±1.3 B-f	
Density Gradient technique alone	61±1.4 D-a	52.3±1.9 B-b	15.3±1.0 C-c	9.2±0.87 B-d	22.5±1.7 C-e	21±1.2 C-f	
Density Gradient technique with 0.1% G.g	66.5±1.5 E-a	58.5±1.8 C-b	19.5±1.1 D-c	12.3±0.7 B-d	28±1.6 D-e	26±1.0 D-f	
LSD	4.201						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05

Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

The activity of sperm motility Grade B before activation in diabetic infertile men (4groups) was significantly (P<0.001) lower than that of fertile diabetic and non-diabetic men. Following *in vitro* activation by density gradient technique with and without adding 0.1% *Glycyrrhiza glabra*, there was a significant (P<0.05) increase in active sperm motility grade B in diabetic men complaining from asthenoteratospermia and asthenospermic with leukocytospermia compared to diabetic men complaining from asthenospermia only and oligoasthenozoospermia (Table 3) .Although, not significant increase in active sperm motility grade B was occurred in fertile and diabetic fertile men following the activation by both techniques, there was a significant (P<0.001) elevation compared to other groups.

Table (3): Mean of sperm motility (Grade B) following the *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Sperm motility Grade B						
	Non D.M fertile	D.M fertile	D.M. Astheno spermia	D.M. Oligo astheno spermia	D.M. Astheno terato spermia	D.M. Astheno spermia with leukocytosis	LSD
Before activation	33.5±1.8 A-a	29.5±2.2 A-b	9.0±1.7 A-c	7.0±1.6 A-d	8.2±1.1 A-cd	10.5±1.5 A-ce	1.8
Wash and spin technique alone	34.5±1.7 A-a	31.1±2.3 A-b	13.3±1.6 A-c	11.8±1.6 B-c	14.5±1.1 B-d	16±1.7 B-d	
Wash and spin technique with 0.1%G.g	35.2±1.5 A A-a	32±2.4 A-b	17±1.8 A-c	14.9±1.3 B-d	17.5±1.3 B-e	18±1.3 B-e	
Density Gradient technique alone	35.4±1.5 A-a	32.3±2.5 A-b	19.8±1.5 A-c	17.3±1.3 B-d	23±1.1 C-e	22±1.1 B-e	
Density Gradient technique with 0.1% G.g	35.5±1.5 A-a	33.3±2.6 A-b	22.5±1.3 A-c	20.4±1.5 B-d	25±1.0 C-e	27.5±1.1 C-f	
LSD	4.419						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05

Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

In this table (4), sperm motility Grade C was significantly (P<0.001) higher before the activation in diabetes infertile men (oligoasthenospermia) (asthenoteratospermia) compared with non diabetic and diabetic fertile men and no significant (P>0.05) differences with the rest of diabetic infertile groups. After activation, there was a significant (P<0.001) elevation in sperm motility grade C all infertile groups compared to fertile groups by using both techniques and media as shown in table (4). The sperm motility grade C in non diabetic fertile men after the activation was not founded leading to a highly significant (P<0.001) difference compared with fertile and infertile diabetic groups.

Table (4). Mean of sperm motility (Grade C) following *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Sperm motility Grade C						
	Non D.M fertile	D.M Fertile	D.M.Asthe no spermia	D.M.Oligo astheno spermia	D.M. Astheno terato spermia	D.M. Astheno spermia with leukocytosis	LSD
Before activation	18±0.73 A-a	19±2.8 A-a	20.5±1.1 A-a	23±1.3 A-b	23±2.6 A-b	21.7±1.4 A-c	1.903
Wash and spin technique alone	10.9±1.0 B-a	19.5±2.6 A-b	25.3±1.0 B-c	29±1.2 B-d	28±2.2 B-d	29.5±1.7 B-d	
Wash and spin technique with 0.1%G.g	7.3±1.7 B-a	15.8±2.3 A-b	28.4±1.0 B-c	32.5±1.2 B-d	32.5±2.0 B-d	32±1.6 B-d	
Density Gradient technique alone	3.1±1.5 B-a	11.8±2.1 A-b	31.5±1.0 B-c	34.9±0.8 B-d	30.5±1.3 B-e	34±1.4 B-f	
Density Gradient technique with 0.1% G.g	0±0.0 B-a	9.6±1.9 A-b	33.5±0.7 B-c	38.4±0.8 B-d	27.6±1.7 B-e	32±1.6 B-f	
LSD	4.661						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05

Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

The mean of morphologically normal sperm before activation appears significantly (P<0.001) lower in diabetic asthenoteratozoospermic men compared with other groups. Although ,the density gradient technique with and without adding 0.1% *Glycyrrhiza glabra* revealed a significant improvement compared to before activation of its corresponding group, there was a significant (P<0.001)reduction compared to other groups. The same observation was significantly(P<0.001)revealed regarding men complaining from asthenospermia and leukocytospermia compared with other groups as shown in table (5).

Table (5): Mean of Morphologically normal sperm following the *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Morphologically normal sperm (%)						
	Non D.M fertile	D.M fertile	D.M. Astheno spermia	D.M. Oligo astheno spermia	D.M. Astheno terato spermia	D.M. Astheno spermia with leukocytosis	LSD
Before activation	73.6±1.6 A-a	65.4±2.9 A-b	63.6±1.7 A-b	54.9±1.6 A-c	21.5±2.5 A-d	36.7±2.0 A-e	2.28
Wash and spin technique alone	74±1.7 A-a	66.1±2.9 A-b	63.9±1.7 A-c	56±1.7 A-d	22±2.5 A-e	37.5±2.2 A-f	
Wash and spin technique with 0.1%G.g	75.9±1.8 A-a	67.6±3.0 A-b	66±1.4 A-b	61.5±1.8 A-c	24.2±2.8 A-d	39.2±2.5 A-e	
Density Gradient technique alone	89.6±1.1 B-a	77.5±1.8 B-b	74±1.4 B-c	68±1.9 B-d	46.8±1.7 B-e	59.6±1.8 B-f	
Density Gradient technique with 0.1% G.g	90.6±1.2 B-a	79±1.6 B-b	77.6±1.6 B-b	71.6±1.1 B-c	49.1±1.5 B-d	60.5±1.9 B-e	
LSD	5.593						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05

Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

The mean of round cells in all semen samples involved in this study is shown in table (6).The number of round cells before activation was significantly (P<0.001) higher in diabetic men with asthenospermia and leukocytospermia compared with other semen samples. However the round cells in asthenoteratozoospermic men were significantly (P<0.001) higher than non-diabetic and diabetic fertile men .After activation ,there was a significant (P<0.05)reduction in the number of round cells in the six groups by using density gradient technique of both media compared with wash and spin technique with Hams F-12 alone . At the same time a significant (P<0.05) decrease in the mean of round cells was observed between density gradient technique of both media ,and wash and spin technique with 0.1% *Glycyrrhiza glabra* medium of diabetic men complaining from asthenoteratozoospermia and men complaining from asthenozoospermia with leukocytospermia as shown in table (6).

Table (6): Mean of round cells following the *in vitro* activation by two techniques and media used for the semen of fertile, diabetic fertile and infertile men.

Method of activation	Round cells (cell/HPF)						
	Non D.M fertile	D.M fertile	D.M. Astheno spermia	D.M. Oligo astheno spermia	D.M. Astheno terato spermia	D.M. Astheno spermia with leukocytosis	LSD
Before activation	0.9±0.31 A-a	0.1±0.1 A-b	1.8±0.4 A-c	3.1±0.5 A-d	11.1±1.2 A-e	21.3±2.2 A-f	0.75
Wash and spin technique alone	0.40.16 A-a	0.1±0.1 A-a	1.3±0.3 A-b	2.6±0.4 B-c	7±0.6 B-d	14.5±2.1 B-e	
Wash and spin technique with 0.1%G.g	0.1±0.10 A-a	0±0 A-a	0.5±0.2 B-a	1.3±0.3 C-b	4.6±0.7 C-c	8.3±0.7 C-d	
Density Gradient technique alone	0±0.0 A-a	0±0 A-a	0±0 B-a	0±0 D-a	0±0 D-a	0±0 D-a	
Density Gradient technique with 0.1%G.g	0±0.0 A-a	0±0 A-a	0±0 B-a	0±0 D-a	0±0 D-a	0±0 D-a	
LSD	1.857						

Different small letters mean the significant differences between the diabetic groups(the raw) at P<0.05

Different capital letters mean the significant differences between the activation techniques (the columns)at P<0.05

Discussion

The results of this study refer a significant improvement in certain sperm parameters following *in vitro* activation by the two techniques and media in normozoospermic non diabetic and diabetic fertile men. These results were suspected because no abnormalities were founded in the semen of those men. However there was a significant reduction in sperm concentration in both techniques and media used in non diabetic and diabetic men following *in vitro* activation. This is due to the failure of the dead and poor motility sperms to swim up and travel from pellet to the upper layer of culture medium. These results were clarified the beneficial effect of preparation techniques by the removal of dead, immotile spermatozoa and semen debris in such way only high quality motile spermatozoa were harvested and poor quality spermatozoa were absent in the post activation medium [12]. These results were in agreement with other studies [13]. Even that the sperm concentration by using density gradient technique alone or with medium containing 0.1% *Glycyrrhiza glabra* was given better results than that of wash and spin technique especially in the case of(Oligoasthenospermia). This observation may resulted from the superior output production of density gradient technique when collected all the sperms with sufficient active motility and morphology for insemination by any type of ART world wild centers [14].

However**,** the sperm concentration in the semen of diabetic men was more reduced than normal in non diabetic subjects before and following *in vitro* activation. It has been reported that DM alters conventional sperm parameters. In addition, DM causes histological damage of the epididymis, with a negative impact on sperm transit. Various mechanisms may explain the sperm damage observed in patients with DM. These include endocrine disorders, neuropathy, and increased oxidative stress. Moreover, DM decreases serum testosterone levels. This is associated with a steroidogenetic defect in Leydig cells .Consequently all these abnormalities leading in the decrease of sperm concentration. [15]. On the other hand, sperm motility and semen volume were found to be about 30 and 60% less. Poor semen quality has also been reported in diabetic men, including decreased sperm motility and concentration, abnormal morphology and increased seminal plasma abnormalities. [15]. However all these abnormalities that founded in the diabetic men with different male infertility factors especially with asthenozoospermia were overwhelmed by *in vitro*

activation techniques and the best results were obtained following the addition of 0.1% *Glycyrrhiza glabra* to Hams F-12 medium and using density gradient technique. Thus, the culture medium enhances different sperm function parameters following *in vitro* activation technique namely; sperm concentration, total sperm motility percentage and grade activity of forward progressive movement. Moreover, culturing of sperms with licorice extract – Hams-F12 medium result in a significant increase in the percentages of sperm motility and grade activity of forward movement (grade A and grade B) of all the groups of asthenozoospermic samples. These results were similar to the results reported by other studies [17]. The *Glycyrrhiza glabra* has estrogenic activity [18]. Estrogens are known to improve sperm characteristics including sperm motility and grade activity in addition to induction of hyperactive motility [19]. Furthermore, it has been noticed that the *Glycyrrhiza glabra* contains Ca^{+2}, potassium, glucose, fructose, vitamin E, vitamin C and many other substances e.g.: Zn^{+2}, sucrose, amino acid [20]. All these substances can stimulate sperm motility and the grade activity of forward movement. The sugars are considered to be a source of energy for sperm motility. Fructose is one of the principle energy substrate for spermatozoa and an activator factor of mammal spermatozoa[21] , the protein and amino acids, which sustain and maintain sperm osmolality and in turn integrity of sperm cell membrane[22]. Moreover, vitamins E and C are major chain – breaking antioxidants in sperm membranes and appears to have a dose dependent protective effect [23]. The current study recorded enhancement in the percentage of morphologically normal sperms(MNS) with the significant decrease in the number of round cells in diabetic men who complaining from asthenospermia ,oligoasthenospermia , asthenoteratospermia and leukocytospermia following *in vitro* activation .The most frequently and markedly affected in the semen of diabetic men being the morphology and/or volume of ejaculate, [24].Therefore, the activation of semen of diabetic men have any of male infertility factors resulted in a significant improvement in the percentage of MNS by using density gradient technique with and without *G. glabra* .Whereas the activation by wash and spin technique with and without using *Glycyrrhiza glabra* in the medium did not increase the percentage of MNS in different male factor infertility semen. It was well known that wash and spin technique should only be used for normozoospermic semen and not recommended for other

abnormal semen quality factors [25]. Thus density gradient is the preferred technique for sperm processing for ART. Accordingly, the MAS and the number of round cells that founded in teratoasthenospermic and leukocytospermic patients were drown down through the density gradient activation technique. But the comparison between the results of those men and other male infertility factors showed the lowest positive responses in sperm motility, MAS and round cells. It has been reported that high levels of ROS were leading to cause male factor infertility, contributing to poor semen parameters, reduced sperm vitality, impaired sperm function and fertilization, and DNA damage [26]. Thus the increase in round cells (If specifically the leukocytes) can produce up to 1,000 times more ROS compared with spermatozoa under physiologic conditions [27]. The sperm membrane remodeling process during spermatogenesis may be the common origin for both abnormal spermatozoa and ROS. Failures in the process, such as head-tail attachment abnormalities, incomplete acrosome development, or sperm cytoskeleton alterations, can lead to the creation of ROS and abnormal sperm morphology [28].

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