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Screening of Human Cytomegalovirus in Chemotherapeutic Patients

Saif Jabbar Yasir

Kufa University ,Faculty of science. Dept. of laboratory investigations.

Abstract: Blood samples were collected from 117 chemotherapeutic patients randomly. ELISA technique was used to find out the activity of humeral immunity among samples through measuring anti-HCMV IgM antibodies , where the result showed that IgM profile 25/117 (22 %) positive including 9/56 (16%) in male and 16/61 (26%) in female.

Regarding PCR technique, the study showed that among 117 patients gave 16 (14 %) PCR positive result including 7 (13%) in males and 9 (16%) in females; in contrast ; the results of control group were negative for anti-HCMV IgM antibodies in both ELISA and PCR technique.

The current study conclude that the higher prevalence of seropositivite for HCMV in this cases comparing with normal individuals and suggest that cytomegalovirus patients are at high risk for CMV infections. A significantly higher prevalence of CMV antibodies was observed in patients of all age-groups compared with controls. The PCR is a reliable and applicable tool for detection of HCMV study clinical samples. The recommendation of these results, it is suggested that detection of viral infection in this cases and medical control.

Key words: CMV, Chemotherapy, ELISA, PCR.

التحري عن الفايروس المضخم للخلايا البشري في مرضى السرطان الذين يخضعون للعلاج الكيميائي

د. سيف جبار ياسر

قسم الاحياء المجهرية / كلية الطب / جامعة الكوفة

الخلاصة: كان الهدف من هذه الدراسة هو دراسة العلاقة بين الفيروس ومرضى السرطان الدين يخضعون للعلاج الكيمياوي من خلال تشخيص الإصابة بالفايروس المضخم للخلايا البشرية في حالات العلاج الكيمياوي للسرطان في محافظة النجف من خلال التحري عن الأجسام المناعية المضادة نوع اي جي ام للفايروس المضخم للخلايا البشري في المصل وكشف الحمض النووي للفيروس المضخم للخلايا البشرية في عينات الدم، وذلك باستخدام تقنية تفاعل سلسلة البوليمر.

تم جمع عينات الدم من 115 مريضا بالسرطان تحت العلاج الكيماوي تضمنت 62 إناث و55 ذكور عشوائيا. تم استخدام تقنية الايزا لمعرفة فعالية المناعة الخلطية بين العينات من خلال تشخيص الأجسام المناعية نوع اي جي ام المضادة للفايروس المضخم للخلايا البشري، حيث ان النتيجة أظهرت أن الغلوبولين المناعي أعطى نتيجة موجبه لـ 25 (21%) اشتملت 9 (18 ٪) للذكور و 16 (17 %) للاناث.

وفيما يتعلق بتقنية البي سي ار، وأظهرت الدراسة أن من بين 115 مريضا 16 (12%) اعطت نتيجة موجبه لفحص البي سي ار تضمنت 8 (7 ٪) ا بالنسبة للذكور و 9 (9%) للاناث؛ مقابل ذلك مجموعة السيطرة اعطت نتائج سالبة لكل من فحص الأجسام المناعية المضادة للفايروس خلال فحص الاليزا وتقنية البي سي ار.

استنتجت الدراسة الحالية إلى انتشار كبير في إيجابية المصل للفايروس في المرضى خلال العلاج الكيمياوي مقارنة مع الأشخاص الطبيعيين وتشير إلى أن الحالات المذكورة هم عرضة للعدوى بالفايروس ولوحظ وجود ارتفاع معنوي كبير للاجسام المضادة للفايروس لدى المرضى بين جميع الفئات العمرية مقارنة مع مجموعة السيطرة. وان تقنية البي سي ار هي وسيلة يمكن الاعتماد عليها وقابلة للتطبيق لدراسة الكشف عن العينات السريرية. التوصية من هذه النتائج تقترح بإجراء الفحوصات للتحري عن وجود الاصابة الفايروسية بين هده الحالات والسيطرة عليها طبيا.

Introduction:

Cytomegalovirus (CMV) reactivation is an uncommon complication of chemotherapeutic treatments in patients with solid malignancies. *Human cytomegalovirus* is a member of *Herpesviridae* or herpesviruses. It is typically abbreviated as HCMV and is alternatively known as *Human herpesvirus 5* (HHV-5) [1]. Within *Herpesviridae*, HCMV belongs to the *Betaherpesvirinae* subfamily, which also includes cytomegaloviruses from other mammals [2].

HCMV infections are frequently associated with the salivary glands [2]. HCMV infection is typically unnoticed in healthy people, but can be life-threatening for the immunocompromised, such as HIV-infected persons, organ transplant recipients [3], or new born infants [2]. It can cause hydrops fetalis in infants. After infection, HCMV has an ability to remain latent within the body over long periods. CMV persists in the host because the viral genome encodes multiple proteins that interfere with MHC class I presentation of viral antigens. One viral protein blocks translocation of peptides into the lumen of the endoplasmic reticulum, while two other viral proteins cause degradation of MHC class I proteins before they reach the cell surface [4,5]. CMV infection may also "have a large impact on immune parameters in later life and may contribute to increased morbidity and eventual mortality [6].

The mode of HCMV transmission from person to person is entirely unknown but is presumed to occur through bodily fluids [7]. Infection requires close, intimate contact with a person secreting the virus in their saliva, urine, or other bodily fluids. CMV can be transmitted sexually and via breast milk, and also occurs through receiving transplanted organs or blood transfusions [5]. Although HCMV is not highly contagious, it has been shown to spread in households and among young children in day care centers [7].

The goal of this study was to study the relationship between the virus and chemotherapeutic through detection of Human cytomegalovirus (HCMV) infection in patients in AL-Najaf governorate by screening of anti-human cytomegalovirus IgM antibodies in the serum and detection of human cytomegalovirus DNA in blood samples, using PCR technique.

Materials and methods:

1. Study design:

The present study was conducted in Al-Sader teaching Hospitals in Al-Najaf governorate .The study period was from November 2014 to April 2015.

Serum samples were collected from 117 chemotherapeutic patients

aged (20 - 80) years whom admitted to the center of tumors diseases of the patients in AL-Sader teaching Hospital of Al-Najaf governorate. In addition to whole blood samples with EDTA were obtained from the same above mentioned persons for the purpose of detecting DNA of HCMV. Fifty healthy individuals (males and female) as a control group.

2. ELISA test:

The test was performed by using Cytomegalovirus (CMV) IgM ELISA test kit according to the manufacturing instructions (BioCheck, Inc.).

3. DNA-extraction and amplification kits:

DNA-extraction was performed using the (DNA-Sorb-B) kit according to the manufuncturing(Sacace Biotechnologies, Italy).

4. Detection of Serum Anti-CMV Antibody

HCMV virus-specific IgM antibodies were detected by indirect enzyme-linked immunosorbent assay . Sera obtained from our patients and control groups were collected and screened for the presence of anti-CMV IgM antibodies by means of a commercial enzyme immunoassay.

5.PCR Amplification and Thermocycling Conditions

PCR Amplification (CMV 500/800 IC). Target region Major Immediate-Early (MIE) gene.The procedure was applied according to the leaflet of the commercial kit (Sacace, Italy).

6. Agarose Gel Electrophoresis

The amplified PCR products were detected by agarose gel electrophoresis and visualized by staining with ethidium bromide.

7. Statistical Analysis

The Chi-square test was applied to determine the statistical significance of the data. P value of <0.05 was considered significant [8].

Results:

1.The results of ELISA test and PCR technique according to age groups :

Were revealed that IgM profile 25/117 (22%) positive including 9/56 (16%) in male and 16 (26%) in female.

Regarding PCR technique, the study showed that among 117 patients gave 16 (15 %) PCR positive result including 7 (13%) in males and 9 (15%) in females; in contrast ; the results of control group were negative for anti-HCMV IgM antibodies in both ELISA and PCR technique. Tables (1 and 2).

Table (1): showed that ELISA and PCR according to age group of male patients.

Age groups	Total No.	IgM	PCR
20 -35	10	2	2
36 - 50	16	3	2
51 - 65	16	2	2
66 - 80	14	2	1
Total	56	9 (16%)	7 (13%)

Table (2): showed that ELISA and PCR according to age group of femalepatients.

Age groups	Total No.	IgM	PCR
20 -35	12	4	1
36 - 50	21	5	4
51 - 65	17	4	3
66 - 80	11	3	1
Total	61	16 (26%)	9 (15%)

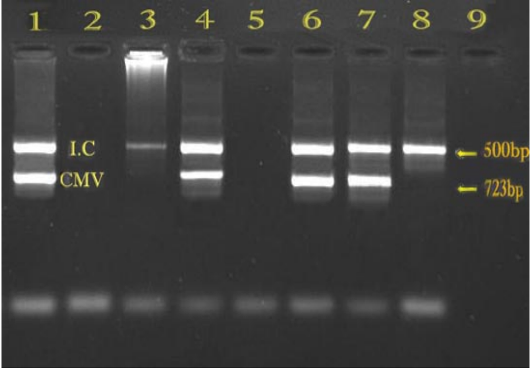

Figure 1: Ethidium bromide-stained agarose gel of PCR amplified products from extracted *HCMV* DNA amplified with primers MIE gene in blood samples .
Lane (1): Control positive of HCMV include 2 band (500bp refer to CMV and 723bp refer to internal control. Lane (2): Negative control. Lane (3,), samples show only 500bp band (CMV) refers to also positive results. Lane (4,6,7): sample show tow bands (I.C and CMV) refers to positive results. Lanes (5,9): show negative result. Lane (8): samples show only 500bp band (CMV) refers to also positive results.

Discussion:

This study revealed that out of the 117 samples 25 (22 %) were positive for anti-HCMV IgM antibodies and the seropositivity among the age group (51 - 65) was higher than other age groups. This result may explain the IgM positivity indicate that primary and

acute infection among patients and low activity of older age groups.

The current study has found that the infection rate at the general population was 16 (14 %) by using PCR technique. This study was the first at least in the study areas that use PCR tests for diagnosis of asymptomatic HCMV infections and there were no previous known research concerning such number of asymptomatic patients and risk groups in Al-Najaf governorate.

This study showed that no significant difference for the prevalence of CMV-IgM seropositivity between male and female patients. Tables (1,2). This results may indicate the presence of active viral infection according to detection of viral DNA in clinical samples.

Infection with CMV is common. In fact, it was recently reported that the seroprevalence of CMV in the United States is 50.4% [9]. Our results are similar to other study like [9]who found that the CMV is acquired through contact with infected body fluids, and the majority of infections are subclinical and asymptomatic

Several prospective studies have evaluated CMV reactivation in populations of patients with both solid and hematologic malignancies. [10] reported on a group of patients with acute myeloid leukemia receiving multi-agent chemotherapy. Of those patients with a positive CMV IgG and negative CMV IgM prior to starting chemotherapy, 35% developed CMV reactivation [11]. followed a group of ten patients with lymphoid malignancies treated with the monoclonal antibody alemtuzumab (anti-CD52), and noted that 100% experienced CMV reactivation at a median of 19 days after initiating therapy. Significant organ dysfunction occurred in two patients: one experienced hemorrhagic pneumonitis and another developed hepatitis.

Table (1,2) which correlated the rate of anti-HCMV IgM antibodies seropositivity, showed that patients with seropositivity in relation to the PCR results of viral DNA detecting in their blood. Detection of CMV antigen or DNA in blood is an indicator of CMV infection. Screening and treatment for allogeneic, our results are in agreement with [12-15] who revealed that the risk factors and outcome of CMV infection in patients having ALL or NHL receiving conventional chemotherapy. Our study shows that CMV infection was present in a large proportion of patients having prolonged ‘unexplained’ cytopenia after chemotherapy.

Though CMV reactivation has been less commonly reported in

patients with solid tumors, a case series by Kuo et al.2008 in patients with lung, rectal, and head and neck cancers noted that rates of CMV reactivation are actually similar to those in hematologic malignancies [16].The authors followed fifteen participants and performed serial testing for CMV DNA. Of the 15 patients, 14 experienced increases in their CMV viral loads. Symptomatology was directly correlated with CMV viral loads; those who were asymptomatic had a median viral load [17].

The prevalence among chemotheraputic patients was higher than among control group. It is concluded that patients with chemotherapy, are at high risk for CMV infections. The high prevalence of CMV infections might be responsible, at least in part, for the immunological disturbances and the susceptibility to other infections observed in this patients [18].

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Roles of Zinc, Magnesium, Selenium, and Carotenoids in Prevention of Prostate Cancer

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Summary: In the present study have been estimated of essential trace elements levels (zinc, magnesium, and selenium) and some carotenoids for evaluation of their roles in protecting prostate gland cells from various oxidative processes to avoid occur DNA mutation which results in the accidence of prostate cancer. By study 15 men suffering from prostate cancer and 13 healthy men aged 45 to 70 years. results of this study showed High significant decreased ($p< 0.001$) in all variables levels have been studied which include Zn, Mg, and Se minerals in addition of some carotenoids in patients with prostate cancer group compared to healthy men group.

Keywords: Prostate Cancer, Zinc, Magnesium, selenium, β-carotene, lycopene, carotenoids

دور الزنك والمغنيسيوم والسلينيوم والكاروتينيدات في الوقاية من سرطان البروستات

سعيد حسين عاصي و رياض شفاف حسين

وزارة العلوم والتكنولوجيا – دائرة بحوث المواد – بغداد – العراق

الخلاصة: أجريت هذه الدراسة لتقدير مستويات العناصر الضئيلة الاساسية (الزنك والمغنيسيوم والسلينيوم) وبعض الكاروتينيدات لاجل ان يتم تقييم دورها في حماية خلايا غدة البروستات من عمليات الاكسدة المختلفة تجنبا لحدوث طفرة وراثية في العامل الوراثي DNA تؤدي الى الإصابة بسرطان البروستات. من خلال دراسة 15 رجل مصاب بسرطان البروستات و13 رجل سليم تتراوح أعمارهم من 45 إلى 70 سنة. من خلال نتائج هذه الدراسة فلقد لوحظ انخفاضا معنويا كبيرا ($p<0.001$)في مستويات جميع المتغيرات التي شملها البحث والتي تضم معادن الزنك والمغنيسيوم والسلينيوم فضلا عن بعض الكاروتينيدات في مجموعة المرضى المصابين بسرطان البروستات بالمقارنة مع مجموعة الضبط.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer death in American men [1]. It is estimated that in 1997, there will be 209000 new prostate cancer cases and 41800 deaths from prostate cancer. Dietary factors which have been investigated include carotinoids such as beta – carotene, lycopene and the essential trace elements (Zn, Mg, and Se). The association of prostate cancer with the intake of certain dietary nutrients suggests that they may be useful preventive agents. The high concentration of zinc in the prostate suggests that zinc may play a role in prostate health. The concentration of zinc in the prostate is higher than that in any other soft tissue in the body [2]. Magnesium is the second most abundant intra cellular cation in the body, involved with over 300 biological activities [3]. Chronic inflammation may also play a key role in the progression from normal tissue to Prostatic Intraepithelial Neoplasia (PIN) and prostate cancer [4]. Several studies have shown an increased cancer rate in regions with low magnesium levels in soil and drinking water, and the same for selenium.

Selenium is an essential nutrient with a recommended dialy allowance of 70 µg in men. The variable distribution of Se in soil and foods produce geographical regions and populations with varying Se status. Dietary Se has been inversely associated with the risk of cancer since 1960s, Interest in selenium as a nutrient with potential preventive effects against prostate cancer was heightened in the mid-1990s, after reports from the Nutritional Prevention of Cancer Trial showed that men who received 200 µg selenium\d had a significantly reduced risk of this disease [5]. The chemopreventive effects of selenium may be due to its roles in cell cycle arrest, decreasing proliferation, inducing apoptosis, facilitating DNA repair by activation of p53, disruption of androgen receptor signaling, and being a key component of selenoenzymes [6-12], which incorporate selenium as selenocysteine, an infrequently occurring amino acid, into their active center[13].

Research emphasizing the biology of carotenoids was initiated after peto et al. suggested that β-carotene might be the primary anticancer agent in fruits and vegetables[14]. Numerous studies have shown that a high intake of carotenoid rich fruit and vegetables or high blood levels of β-carotene are associated with a reduced risk of cancer at a number of common

sites, such as prostate, lung, and stomach [15]. Lycopene is a carotenoid, an acyclic isomer of beta- carotene, and has no vitamin A activity [24]. It is a highly unsaturated, strait chain hydrocarbon containing 11 conjugated and 2 non- conjugated double bonds. Lycopene has antioxidant properties. Several studies have indicated that lycopene is an effective antioxidant and free radical scavenger.[25]

Materials and Methods

Chemical Reagents

β-Carotene, and lycopene (Sigma), Zinc, Mgnesium, and Selenium(Merck),95%n-hexane and ethyl acetate(fluka) for high performance liquid chromatography (HPLC), 99.8% ethyl alcohol (fluka) were used in the study.

1- Methods Determination of Serum Magnesium

Total magnesium in blood serum, was determined by utilizing atomic absorption spectrophotometer (flame method). Set of standard solution ranging (0.1 to 1 µg/ml) Mg was prepared. AA 7000 Shimadzu atomic absorption spectrophotometer was used

2- Determination of Serum Zinc

Total zinc in blood serum, was determined by utilizing atomic absorption spectrophotometer (flame method). Set of standard solution ranging (0.05 to 0.8 µg/ml) Zn was prepared. AA 7000 Shimadzu atomic absorption spectrophotometer was used

3- Determination of serum selenium

Total selenium in blood serum, was determined by utilizing atomic absorption spectrophotometer (flameless method). Set of standared solution ranging (10 to 110 ng/ml) was prepared. AA 7000 Shimadzu atomic absorption spectrophotometer was used4 -

4- Assessment of serum β-Carotene and lycopene

β- Carotene, and lycopene were measured simultaneously by high performance liquid chromatography (HPLC) in two groups of patients: 15 men with prostate cancer and 13 healthy men. Blood samples were collected from patients and healthy individuals for measure total serum β- Carotene and lycopene. Blood was collected via venipuncture, All tubes were protected from light ,allowed to clot, centrifuged at 3000 rpm for 10 min, and then stored at -30C^{o} until thawed for analyses by high performance liquid

chromatography HPLC. (454, 450 nm). UFLC Shimadzu HPLC was used.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). The statistical analysis of data was done by using Excel program and SPSS program (statistical package for social Science Version 11)., data between the groups were compared with Student's *t* test for continuous variables and chi-square t-test for continuous variables. Mann-Whitney's U-test was used for variables without normal distribution. A two-tailed P-value of <0.05 was considered significant

RESULTS

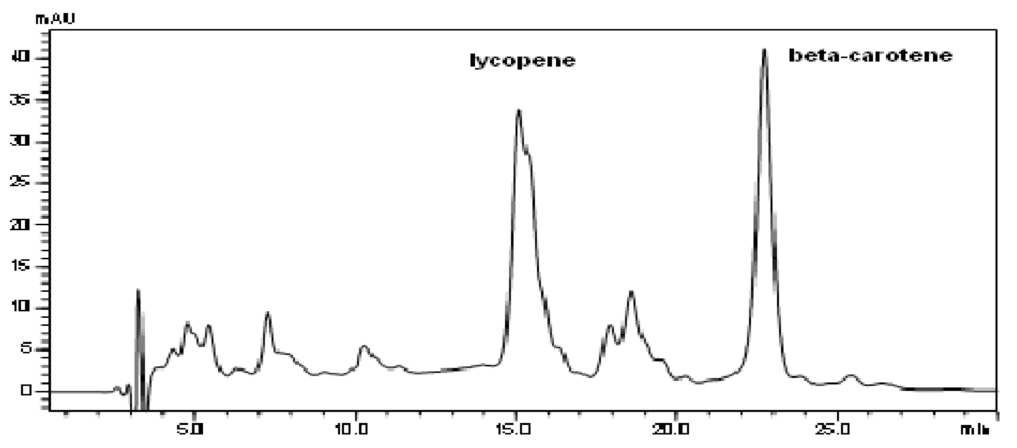

Figure1. Chromatogram lycopene and beta- carotene HPLC Technique

Table 1. Serum levels of Zinc, Magnesium, Selenium and beta-carotene in men with prostate cancer and healthy men groups

Parameters	Men with prostate cancer No.15	Healthy men (control group) No.=13	P value
Zinc µg/ml	0.65± 0.09	1.00 ± 0.08	p<0.001
Magnesium µg/ml	10.32± 1.22	24.00± 2.05	p<0.001
Selenium ng/ml	73.50 ± 8.20	115.00 ± 15.6	p<0.001
β- Carotene g/mlµ	0.12± 0.02	0.32 ± 0.07	p<0.001
Lycopene g/mlµ	0.09± 0.01	0.25 ± 0.05	p<0.001

Discussion

Table (1) shows the results of the present study where showed high significantly decreased ($P<0.001$) in levels of zinc, magnesium, selenium, beta-carotene, and lycopene in patients with cancer group compared to control group . figure (1) shows the chromatogram of lycopene and beta- carotene HPLC Device.

The epidemiology of prostate cancer is complex, with few established risk factors; those most established are family history, age, country, race, and testerone deficiency. The association of prostate cancer with intake of certain dietary nutrients suggest that they may be useful preventing agents.

In prostate cancer, decreased zinc levels are consistently observed in malignant tissue samples from different populations and at various stages of malignancy (Vartsky et al., 2003; Zaichick et al., 1997). Analysis of malignant prostate tissues showed a 60-70% reduction of zinc levels in comparison to those of the normal peripheral zone tissues (Zaichick et al., 1997). The plasma zinc level between patients with malignancy is also significantly lower than normal patients (Goel & Sankhwar, 2006).

The physiology and biochemistry of zinc and its importance in normal cellular and bodily function has been the subject of numerous reviews (Renty & Leslie 2007). The regulation and maintenance of a “normal” concentration and distribution of cellular zinc are essential to the function, metabolism, growth, proliferation and survival of cells. A significant clinical aspect of zinc is its role in the development and progression of malignancy. There is now compelling clinical and experimental evidence that zinc is an important factor in prostate cancer, and also in other types of cancer. In relation to essential tumor cell activities, the effects of zinc can be categorized as: intermediary metabolism and bioenergetics effects; motility and invasive effects; growth and proliferation effects. The actions of zinc on all three activities impose anti-tumor effects in malignant prostate cells and other tumor cells. This review will present the role of zinc in prostate malignancy about which most information exists; and which admittedly has been the focus of the research of the authors. Information has been accumulating that zinc also induces anti-tumor effects in other tumor cells.

The role of magnesium in cancer formation is complex. Numerous studies have focused on

the effect of magnesium deficiency on tumor incidence, where an unbalanced magnesium homeostasis is either magnesium deficiency or suplemmentation can affect the progression of existing tumors. It has been found that magnesium could impact carcinogenesis by two michnisms (Wolf et al. 2007), first, magnesium deficiency be association with inflammation, second, magnesium deficiency might be association with increased levels of free radicals. Both inflammatory mediators and free radicals might lead to oxidative DNA damage and thereby cancer formation (Castiglioni & Maier, 2011). Magnesium is also known to stabilize the structure of nucleic acids and is a vital co-factor of enzymes involved in DNA replication, repair and gene expression (Anastassopoulou & Theophanides, 2002). DNA repair mechanisms are responsible enomic stability and fidelity, thus any magnesium deficiency may contribute to defects in these systems and the appearance of DNA mutations. Accumulation of genomic alterations may thereby lead to tumor-genesis (Wolf et al. 2007).

As the epidemiology suggests that individuals with low Se levels are at higher risk of cancer, it appears that Se supplementation decreases the incidence of prostate cancer in men. Because of its effects on DNA repair , apoptosis and the endocrine and immune systems as well as other mechanisms, including its antioxidant properties. Selenium might play a role in the prevention of cancer. Epidemiological studies have suggested an inverse association between selenium status and the risk of prostate cancer (Dennert et al. 2011).

The strongest support for a chemoprevent effect of selenium in human prostate carcinogenesis comes from the national prevention of cancer trial , study to evaluate selenium

supplementation(200µg/d) which found 52% reduced risk factor in the incidence of prostate cancer (combs 2004).

The fact that LDL is a major transporter of β-carotene and lycopene in the circulation and that these carotenoids have the capacity to trap peroxyl radicals and quench singlet oxygen lends support to this hypothesis, β-carotene is a scavenger of peroxyl radicals, especially at low oxygen tension. This activity may be also exhibited by others carotenoids. The interaction of carotenoids with peroxyl radicals may proceed via an unstable β-carotene adduct radicals. Carotenoid adduct

radicals have been shown to be highly resonance stabilized and are predicted to be relatively un reactive. They may further undergo decay to generate non radical products and may terminate radical reactions by binding to the attacking free radicals. Carotenoids act as antioxidants by reacting more rapidly with peroxyl radicals than do unsaturated acyl chains. In this process, carotenoids are destroyed. Rao et al. found that serum levels of lycopene in prostate cancer patients were significantly lower than their age matched controls. It is hypothesized that prostate cancer patients perhaps lack the ability to isomerizes dietary lycopene and therefore do not absorb it efficiently. Our results are agreements with all studies above mentioned.

Conclusion

Present study indicates that Zn, Mg, and Se are minerals which play important role in prevention of prostate cancer in males in addition of some carotenoids such as beta-carotene wich play same role. It is well recommended that dietary intake of these nutrients as supplementation a day to take advantage of their antioxidant properties for the prevention of prostate cancer.

Acknowledgement

We thank all people to their kind help to accomplish this research.

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Polymer- nanoparticles composites for the reduction of the bacterial adherence to surfaces

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Abstract: The medical device is one of the sources of nosocomial infections; the adherence of the bacteria on the surface of this device is the first step in the medical device related infection. In this study nanocomposite of polymethyl methacrylate (PMMA)/titanium oxide nanoparticles (1, 5, 10, 20, 30 wt %) and copolymer acrylonitrile butadiene styrene (ABS)/titanium oxide nanoparticles (1, 5, 10, 20, 30 wt %) were used to investigate the ability of the nanocomposite for the reduction of the bacterial adherence to the surfaces. The microorganisms which is used in this study include (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumonia*). After an incubation of microorganisms with nanocomposites for 2 hours, it was found that the adhered bacterial cells were significantly reduced on all of the TiO_2-containing nanocomposites in the comparison with control polymer. The reduction is reached to 60.82% with10% TiO_2 in ABS for *P. aeruginosa.* The results of the *S. aureus* adherence on nanocomposite are revealed a significant reduction in the number of adhered bacteria and reached to 73.71% with 20% TiO_2 in PMMA. The effect of TiO_2 nanoparticales on the *K. pneumonia* adherence appeared very clear and the reduction uis reached to 90.04% with 10% TiO_2 in ABS.

Keywords: nanocomposite; TiO_2; bacterial adherence; nanoparticles.

مركب من البوليمر والجسيمات النانوية لتقليل التصاق البكتريا على السطوح

د. منى صبار الربيعي

استاذ مساعد، المعهد التقني\ بابل

الخلاصة: العدد الطبية هي احد مصادر الاصابات المرتبطة بالمستشفيات. والتصاق البكتريا على سطوح هذه العدد هو الخطوة الاولى في الاصابات المتعلقة بالعدد الطبية. في هذه الدراسة مادة مركبة مكونة من بولي مثيل ميثا كرليت PMMA مع جسيمات نانوية من اوكسيد التيتانيوم بتراكيز (1، 5، 10، 20، 30) كنسبة مئوية ومركب اخر مكون من كوبوليمر اكريلونايترل بيوتادين ستايرين ABS مع جسيمات نانوية من اوكسد التيتانيوم بتركيز (1، 5، 10، 20، 30) كنسبة مئوية استخدمت لاختبار قدرة المركب النانوي على تقليل التصاق البكتريا على السطوح. الاحياء المجهرية المستخدمة في هذه الدراسة شملت *P. aeruginosa ; S. aureus and K. pneumonia* وبعد حضن الاحياء المجهرية مع المركبات النانوية لمدة ساعتين وجد انه عدد الخلايا البكتيرية الملتصقة انخفض بشكل كبير على كل المعقدات النانوية مقارنة ببوليمرات السيطرة. الانخفاض في العدد وصل الى 60.82% مع بكتريا *P. aeruginosa* على سطح ABS بوجود 10% اوكسيد التيتانيوم. اما نتائج بكتريا *S. aureus* فقد اظهرت انخفاض في عدد الخلايا الملتصقة وصل الى 73.71% مع PMMA الذي يحوي 20 % اوكسيد التيتانيوم. وكان تاثير الاجسام النانوية على التصاق بكتريا *K. pneumonia* واضح جدا ووصل ال 90.04% مع مادة ABS الحاوية على 10% اوكسيد التيتانيوم.

Introduction

The first step in the initiation of biomaterials-related infection is microbial adhesion to the device and after that the proliferation of adhered bacteria leads to the produce of a biofilm, and infection. The surfaces of the medical devices are the places to the bacterial colonization and the adhesion. So that, the researcher tries to improve the properties of polymers in order to resist the bacterial adhesion for use in the medical devices.

Materials with nanoparticles may have properties which are effective to reduce the microbial adhesion, and biofilm growth. The studies indicated that the incorporating nanomaterial with the medical device can enhance the surface energy, in addition to that increase selected protein adsorption (1). In the nanomaterials, when the material particles become smaller the percentage of atoms at the surface area are increased. This may lead to the new properties of the nanoparticles. The ratio of the surface to the volume of the materials are increased and their electronic energy states become discrete, and this lead to the unique electronic, optical, magnetic, and mechanical properties of the nanomaterials (2).

Titanium Oxide (TiO_2) is an inert and cheap material, and well known as non-toxicity for the human. So that it is used in cosmetics and toothpaste (4). TiO_2 nanoparticle is insoluble (5). Titanium dioxide (TiO_2) is not classified as hazardous according to the United Nations' (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS) (6). TiO_2 is widely used as a self-cleaning and self-disinfecting surface (7).

The polymer is widely used in industries because it has the advantages such as low cost, low weight and good manufacturing flexibility (8). Polymethyl methacrylate (PMMA) is one of the thermoplastic material, and it has many applications in many technological and productive fields. Moreover, it has many advantages of good optical properties, chemical inertness, good mechanical properties,thermalstability, electrical properties, safety, and easy shaping (9). Polymers can be mixed together to produce combinations of properties. The different kinds of monomers are polymerized together and the result was named as copolymer. Acrylonitrile butadiene styrene (ABS) is an example of copolymer (10).

In this study, nanocomposite of polymethyl methacrylate/ titanium oxide nanoparticles and copolymer acrylonitrile butadiene styrene/ titanium oxide nanoparticles are used to investigate the ability of the

nanocomposite for the reduction of the bacterial adherence to surfaces.

Materials and Methods

Nanocomposites

The pieces of the two nanocomposites polymer, polymethyl methaacrylate (PMMA) and copolymer acrylonitrile butadiene styrene (ABS) with the different concentrations of the nanoparticale of TiO_2 (30-70 nm) are prepared kindly by Dr. Imad Disher college of material engineering, Babylon university in previous study (11).

In the present work, The composites of the polymethyl methacrylate /titanium oxide nanoparticles (1, 5, 10, 20, 30 wt %) are used. Also, the composites of the copolymer acrylonitrile butadiene styrene/titanium oxide nanoparticles (1, 5, 10, 20, 30 wt %) are used. Two sets of the polymer pieces are tested for each concentration. Control pieces which are polymer without TiO_2 also used.

Bacterial culture

The microorganisms which are used in this study include two clinical isolates of each species of (*Pseudomonas aeruginosa* and *Staphylococcus aureus* and *Klebsiella pneumonia*).

The microorganisms are streaked for the isolation on a nutrient agar plate (oxoid). After that, a single isolated colony is selected and used to inoculate 5 ml of nutrient broth. The bacterial culture is grown on a shaking incubator at 200 rpm for 18 hours at 37°C. Then, it is harvested by centrifugation at 3000 rpm for 10 min. After we removed the supernatant, the cells are washed with phosphate buffer solution (PBS) twice and resuspended with the PBS solution. The final concentrations of the bacterial cells are diluted approximately 10^6 CFU/mL for each isolated bacteria in 5 ml of PBS.

Adhesion experiments

Two pieces of the nanocomposites of each weight percentage are used. The nanocomposites pieces are sterilized with three times for 30 second rinses in 70% ethanol (12). The pieces are placed in tube which is contained 5 ml of bacterial suspension. Then the tubes are placed in the incubator at 37°C for 2 h. After that, the pieces of the nanocomposites are removed from the tubes with sterile forceps and washed three times with PBS to ensure the removal of the non-adherent bacteria. The pieces are placed in the tube which is contained 5 ml of PBS and vortex for 120 sec (13), in order to remove all the adherent bacteria from the nanocomposites pieces into the solution. Then, the solution is serially diluted in PBS, cultured on

nutrient agar and the numbers of CFU/ml are calculated.

Results and Discussion

The attachment of the bacteria to the surface is a first part in the process of development of the infection. The physicochemical properties of the surfaces play important role in this attachment.

The nanoparticles generally have different physical and chemical properties in comparesion to the fine particles. The smaller size of the nanoparticles means that a large portion of the atoms placed on the particle surface. So that, the properties of the surface, such as energy level, electronic structure is different from the fine particles (14).

The results of the present study are found that adhered bacterial cells are significantly reduced on all of the nanocomposites pieces which containing TiO_2 in comparison with control polymer.

Figures no. 1 and 2 demonstrate that the influence of the different concentrations of TiO_2 nanoparticales on the adherence of *P. aeruginosa* to PMMA and ABS respectively. The numbers of adhered bacteria are reduced significantly on all of the nanocomposites which containing TiO_2 and the reduction are reached to 60.82% with10% TiO_2 in ABS.

The results of the *S. aureus* adherence on the nanocomposite are shown in figures no 3 and 4. Also, the results are revealed the significant reduction in the numbers of the adhered bacteria which is reached to 73.71% with 20% TiO_2 in PMMA.

Figure no 5 and 6 are showed the effect of TiO_2 nanoparticales on the adherence of *K. pneumonia* on PMMA and ABS respectively. The reduction in the number of the adhered bacteria is very clear and it is reached to 90.04% with 10% TiO_2 in ABS.

Figure no 7 and 8 are showed the adherence of the different microorganisms on the polymers surface (PMMA and ABS respectively). The results are revealed that the *P. aeruginosa* is showed high adherence on the surface and the *K. pneumonia* is showed low adherence among tested microorganisms.

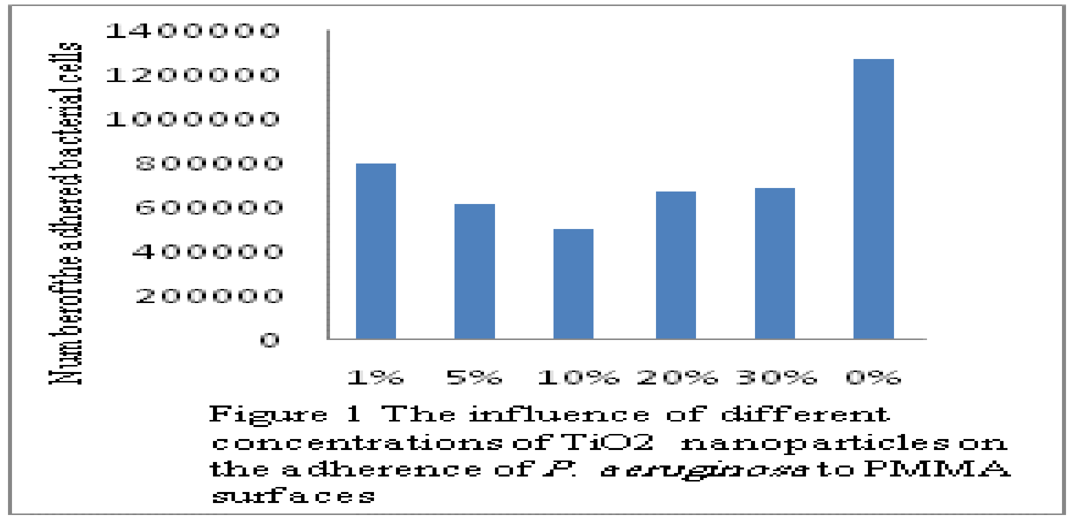

Figure 1 The influence of different concentrations of TiO_2 nanoparticles on the adherence of *P. aeruginosa* to PMMA surfaces

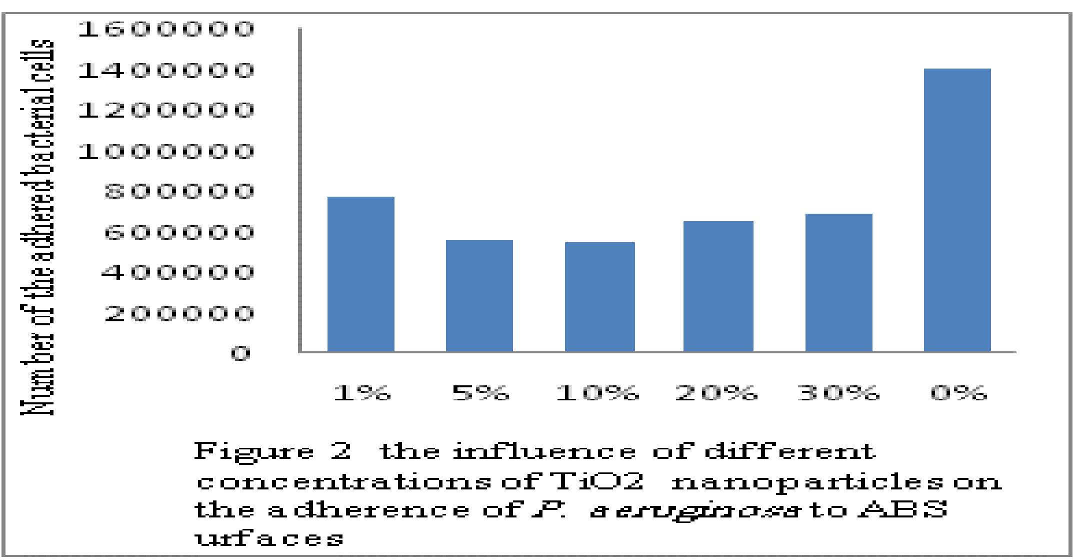

Figure 2 the influence of different concentrations of TiO_2 nanoparticles on the adherence of *P. aeruginosa* to ABS urfaces

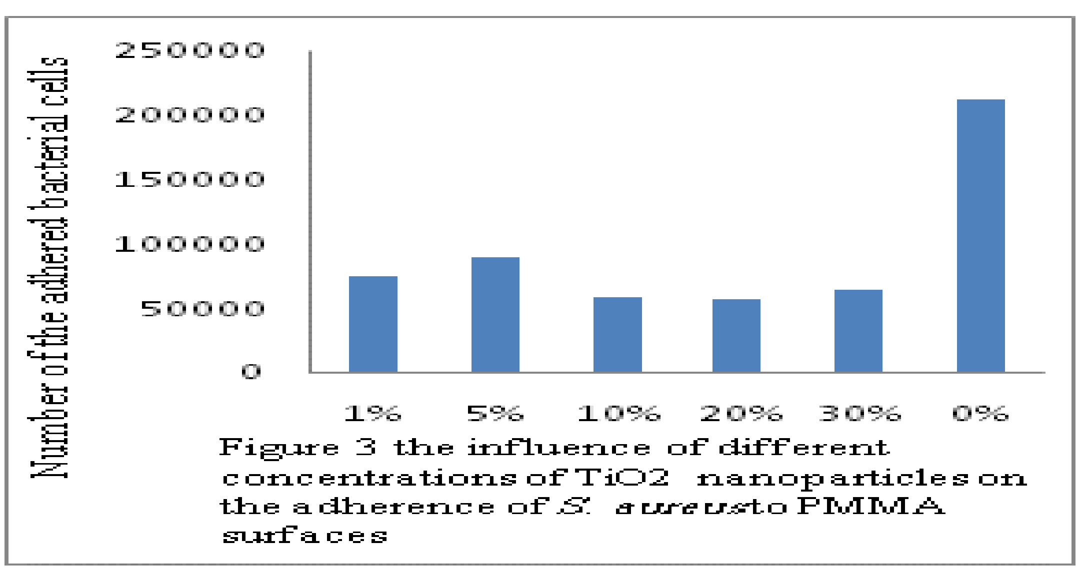

Figure 3 the influence of different concentrations of TiO_2 nanoparticles on the adherence of *S. aureus* to PMMA surfaces

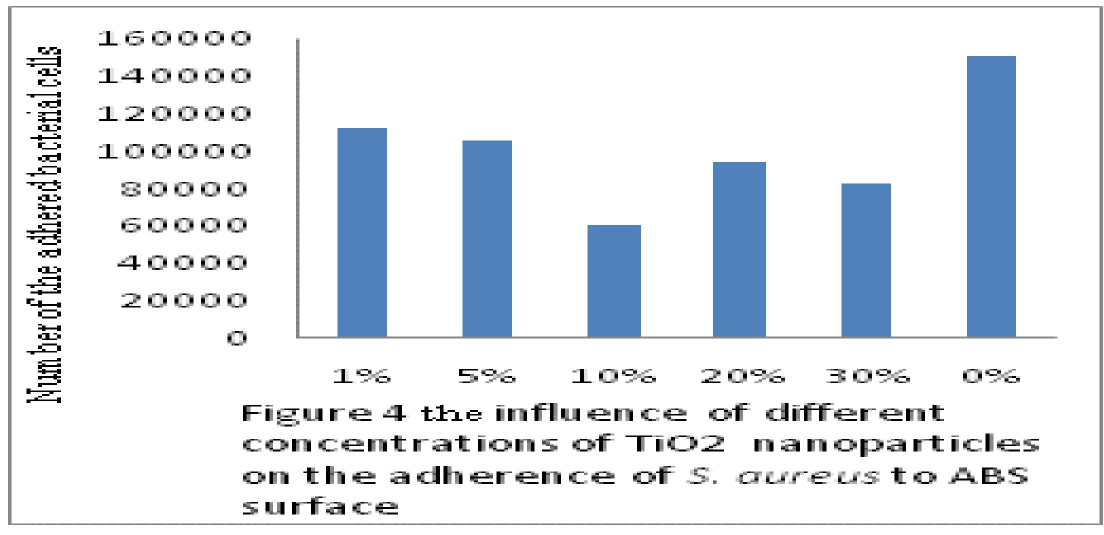

Figure 4 the influence of different concentrations of TiO2 nanoparticles on the adherence of *S. aureus* to ABS surface

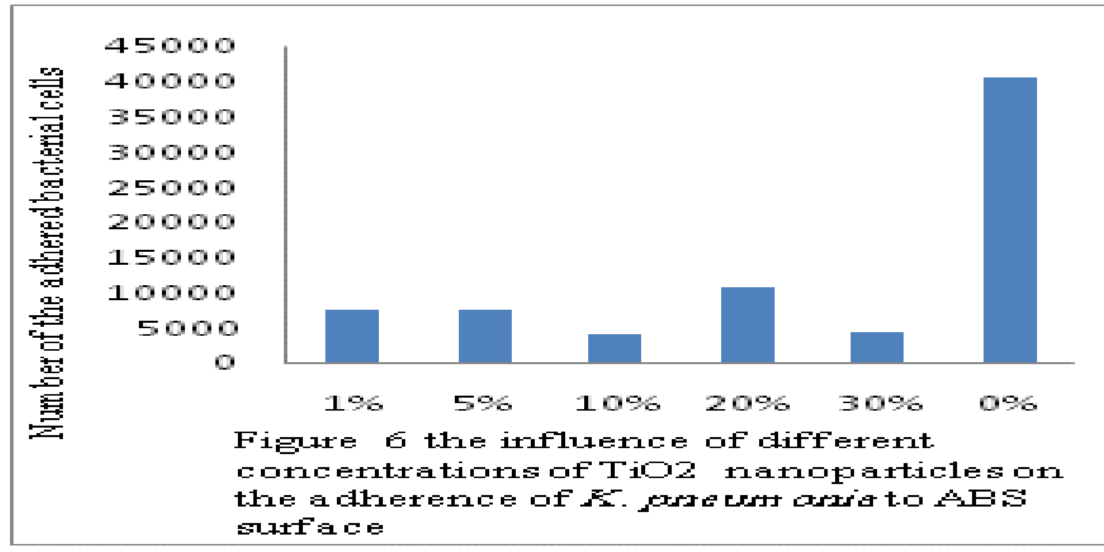

Figure 6 the influence of different concentrations of TiO2 nanoparticles on the adherence of *K. pneumoniae* to ABS surface

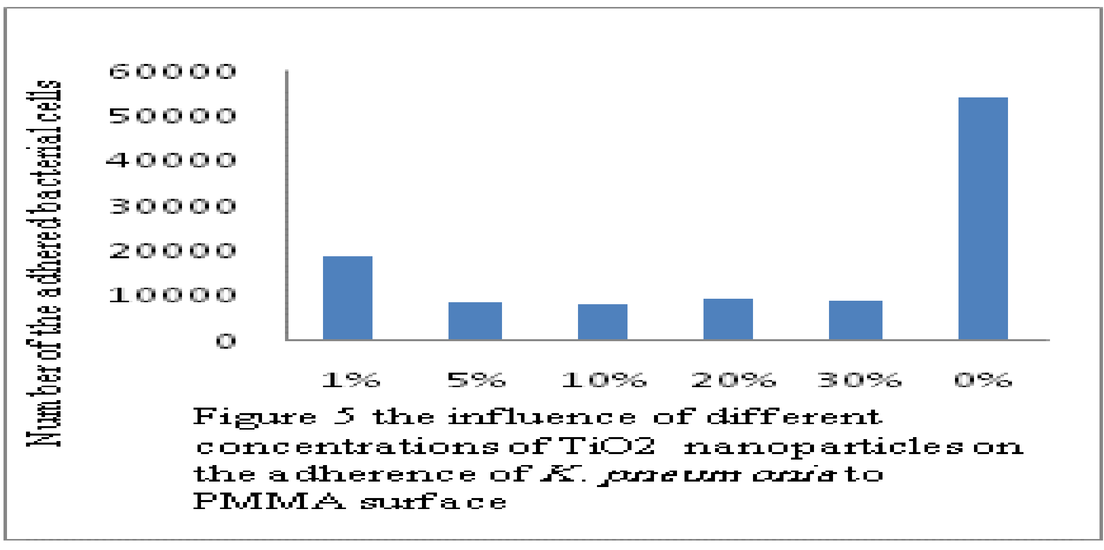

Figure 5 the influence of different concentrations of TiO2 nanoparticles on the adherence of *K. pneumoniae* to PMMA surface

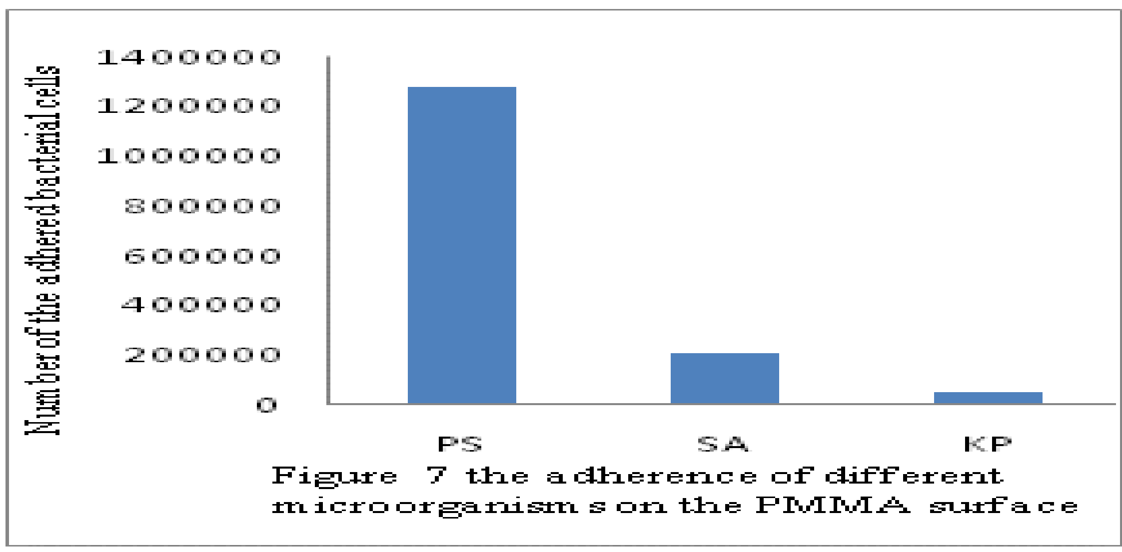

Figure 7 the adherence of different microorganisms on the PMMA surface

Many studies are showed that the materials with nanomaterial may have properties which are effective in the reduction of the microbial adhesion (15) (16). Also there are studies revealed that the addition of inorganic nanoparticles to polymers allows the change of the polymers physical properties (16).

Nabawia *et al*. (2014) are showed in their study that the mixing of TiO_2 nanoparticles with PMMA can improve the properties of the polymer which leads to the interesting technological uses (17). In adition to that, there is a study refered that the mixed nanoparticales with medical device surfaces can enhance surface energy, increase select protein adsorption, and promote protein bioactivity (1).

Holmes *et al*. (2009) are tested the modified surface with nanoparticle, the results showed that the coatings with silica nanoparticles significantly reduced adhesion of *Staphylococcus epidermidis* (90%), and *Pseudomonas aeruginosa* (15%) (15). There is no previous studies deal with the effect of TiO_2 nanoparticles on bacterial adhesion, but there is study on Gallium Oxide Ga_20_3 nanoparticles the researchers found that the Ga_20_3 nanoparticles reduced the bacterial adherence and the reduction reached to 46% in *S. aureus*, and 74% in *E coli* (18). Many researchers hypothesize that the rough of the material surface at the nanoscale may minimize flush contact between bacterial cell walls and the surface. This can inhibit electrostatic interactions which is necessary for adhesion to the surface (19).

We can conclude that the addition of nanomaterial to polymer can reduce the bacterial adhesion on polymer surface.

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Study the Impact of Some β- lactamase Encoding Genes on Swarming Phenomenon and Hemolysin Production by UPEC

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Abstract: Out of 74 urine specimens collected from patients suffering from urinary tract infections; 50 (67.56%) isolates were identify as *Escherichia coli*. Approximately, 78% of all isolates were identified as extended spectrum beta lactamases (ESBL) producer. Antibiotic susceptibility test was performed and ceftazidime was selected to complete this study by implying stress at sub-MIC on isolate harbor high number of resistance genes (N11) and compared with sensitive isolate (S). Only four β-lactamase coding genes were detected; bla_{TEM}, bla_{PER}, bla_{VIM} and $bla_{CTX-M-2}$ and N11have bla_{TEM}, bla_{PER}, bla_{VIM}. Besides, it was found that ceftazidime reduced the diameter of swarming seven fold when compared with control swarming plates (antibiotic free). Moreover, results of current work revealed that the presence of sub-MIC antibiotic stress was significantly affected the lytic activity of *E. coli* α-hemolysin by showing the absence of hemolysis of human RBCs when compared with control plates (antibiotic free blood agar).

Key words: Uropathogenic *E. coli*, β-lactamase, *E. coli* swarming**,** Hemolysin.

دراسة تأثير بعض الجينات المشفرة للبيتالاكتاميز على ظاهرة الحركة التموجية وانتاج انزيم الهيمولايسين في بكتريا القولون البولية

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الخلاصة: تم جمع 74 عينة ادرار من مرضى يعانون من خمج المجاري البولية ووجد ان 50 عزلة تعود لبكتريا *E. coli*.. شخصت 78% من عزلات E. coli على انها منتجة لأنزيمات البيتالاكتاميز واسعة الطيف. كما تم اجراء اختبار فحص الحساسية لسبعة مضادات حيوية وتم اختيار مضاد السيفتازيديم لاكمال هذه الدراسة لغرض تسليط اجهاد باستعمال التركيز تحت التركيز المثبط الأدنى على العزلة التي امتلكت اعلى عدد من جينات المقاومة (N11) وقورنت مع العزلة الحساسة (S). تم تشخيص اربعة جينات مشفرة لأنزيمات البيتالاكتاميز تضمنت bla_{TEM}, bla_{PER}, bla_{VIM}, $bla_{CTX-M-2}$ واظهرت العزلة المقاومة امتلاكها للجينات bla_{TEM}، bla_{PER}، bla_{VIM}. بالأضافة الى انه وجد ان مضاد السيفتازيديم ادى الى تقليص قطر الحركة التموجية بمقدار سبع مرات عند مقارنتها مع اطباق السيطرة الخالية من المضاد كما ان نتائج الدراسة الحالية بينت ان التركيز تحت المثبط الادنى أثر على الفعالية التحللية لهذه البكتريا من خلال ملاحظة اختفاء تحلل خلايا الدم الحمر للانسان عند مقارنتها مع اطباق السيطرة الخالية من المضاد.

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Introduction

Escherichia coli is one of the most important pathogenic bacteria that share the events of microbial contamination and cause about 90% of the urinary tract infection (UTI) and recurrent UTI, particularly in women. However, the importance of this pathogen comes from its ability to elaborate a wide spectrum of virulence factors. *E. coli* comprises a wide population of phenotypically and genetically highly variable organisms (1).

The discovery of antibiotics had a significant impact on lowering the incidence of UTI. On contrary, Extended spectrum beta lactamases (ESBL) produced by Enterobacteriaceae complicated the treatment of such infections (2).

ESBL covered a growing group of plasmid-mediated ß-lactamases which confer resistance to broad spectrum beta-lactam antibiotics. The species of Enterobacteriaceae producing this class of enzymes are increasing worldwide and this triggers an irritating alarm. Furthermore, high mortality rates are associated with infections caused by ESBL producing *E. coli*. Consequently, the emergence of ESBLs establishes a complicated yet real challenge for both clinical microbiology laboratories and clinicians (3).

Swarming motility is defined as a rapid multicellular bacterial surface movement powered by rotating flagella (4). Bacterial flagella can sense stimuli, such as chemicals and temperature, in the local environment, allowing bacteria to change swimming patterns to aid in survival (5).The bacterium moves through its medium by two states, the tumble and the run, that allows it to search for food and avoid noxious substances (6).

On the other hand, one of the most important secreted virulence factor of UPEC is α-haemolysin toxin. Production of toxins by colonizing *E. coli* may cause an inflammatory response, a possible pathway for UTIs symptoms (7). The aim of this work is to investigate the problematic ESBL producing *E. coli* in Iraq by identifying some of ESBLs coding genes and to investigate their effect on swarming and hemolysin production by UPEC.

Materials and Methods

Specimens collection

Sixty midstream urine specimens were collected randomly from patients presented with urinary tract infections attending Al-Yarmouk, Al-Numan, and Saint Raphael hospitals in Baghdad for the period from March 2014 to April 2014.

Isolation and Identification of *E. coli*

All specimens were streaked onto Blood agar (HiMedia, India)

and incubated at 37°C for 24 h. Thereafter suspected colonies were streaked onto MacConkey agar (Oxoid, England) and reincubated at 37°C for another 24 h. Pink colonies were selected and examined for Gram stainability, cultural, morphological characteristics, and conventional biochemical tests.

Antibiotic susceptibility test

Kirby-Bauer method was used as described by Morello *et al*. (8) to carry out the antibiotics susceptibility test for 7 different β lactam antibiotics including: ampicillin (10µg/disk), ampicillin / sulbactam (10/10µg/disk), amoxicillin / clavulanic acid (20/10µg/disk), cefalothin (30µg/disk), ceftazidime (30µg/disk) (all purchased form Bioanalyse, Turkey), imipenem (10µg/disk0, and meropenem (10µg/disk) (Both of them were provided by Mast, England). Each isolate was interpreted as susceptible, intermediate, or resistant to a particular antibiotic by comparison with standards inhibition zones (9).

Determination of minimal inhibitory concentration (MIC) (10).

Double serial dilutions (16-2048µg/ml) were prepared form a stock solution in addition to positive and negative controls were set up. 100 µl from10^8 CFU/ml bacterial suspension was added to all tubes except negative control tube and incubated at 37°C for 24hr. The lowest concentration that inhibit bacterial growth was considered as the MIC.

Detection of Extended- spectrum β- lactamase production (ESBL).

Disk replacement method was used according to that described by Al-Jasser (3) with some modifications. Two amoxicillin/ clavulanate disks (20/10µg/disk) were applied to a Mueller-Hinton plate inoculated with the test organism (*E. coli*). After one hour of inoculation at room temperature, these antibiotic disks are removed and replaced on the same spot by disks containing ceftazidime (30µg/disk) and aztreonam (30µg/disk). Control disks of these two antibiotics are simultaneously placed at least 30 mm from these locations. A positive test is indicated by an increase of zone of inhibition by□□5 mm for the disks which have replaced the amoxicillin/clavulanate disks compared to the control disks which are placed alone directly on inoculated Muller-Hinton plates. Inhibition zones were measured and recorded by a metric ruler (3).

Molecular detection of β-lactamases

ESBL-producing *E. coli* isolates were tested for the presence

of genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA\text{-}like}$, *bla*$_{CTX\text{-}M}$, *bla*$_{PER}$ and *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ by polymerase chain reaction (PCR).

DNA was extracted from all *E.coli* clinical isolates using Genomic DNA Mini Kit (Geneaid, Thailand). Purity and concentration of DNA were measured by Microspectrophotometer NAS99 (ACT Gene, USA).

Primer preparation

Forward and reverse primers (BioCorp, Canada) were chosen from previously published DNA sequences of *E. coli* described by Dallenne *et al.*(11).Table1 lists the sequences, names of the mentioned primer pairs as well as the molecular size of amplicons.

Table 1: Fragments of β-lactamases genes primers used in polymerase chain reaction (11).

id	Primer name	Primer sequence5'→3'	Gene targeted	Amplicon size(bp)
1	TEM_for	CATTTCCGTGTCGCCCTTATTC	*bla*$_{TEM}$	800
	TEM_rev	CGTTCATCCATAGTTGCCTGAC		
2	SHV_for	AGCCGCTTGCAAATTAAAC	*bla*$_{SHV}$	713
	SHV_rev	ATCCCGCAGATAAATCACCAC		
3	OXA_for	GGCACCAGATTCAACTTTCAAG	*bla*$_{OXA\text{-}1}$, *bla*$_{OXA\text{-}4}$, *bla*$_{OXA\text{-}30}$	564
	OXA_rev	GACCCCAAGTTTCCTGTAAGTG		
4	CTXM1_for	TTAGGAARTGTGCCGCTGYA*	*bla*$_{CTX\text{-}M}$ group-1	688
	CTXM1_rev	CGATATCGTTGGTGGTRCCAT*		
5	CTXM2_for	CGTTAACGGCACGATGAC	*bla*$_{CTX\text{-}M}$group-2	404
	CTXM2_rev	CGATATCGTTGGTGGTRCCAT*		
6	CTXM9_for	TCAAGCCTGCCGATCTGGT	*bla*$_{CTX\text{-}M}$group-9	561
	CTXM9_rev	TGATTCTCGCCGCTGAAG		
7	PER_for	GCTCCGATAATGAAAGCGT	*bla*$_{PER\text{-}1}$, *bla*$_{PER\text{-}3}$	520
	PER _rev	TTCGGCTTGACTCGGCTGA		
8	IMP_for	TTGACACTCCATTTACDG*	*bla*$_{IMP}$	139
	IMP_rev	GATYGAGAATTAAGCCACYCT*		
9	VIM_for	GATGGTGTTTGGTCGCATA	*bla*$_{IVIM}$	390
	VIM_rev	CGAATGCGCAGCACCAG		
10	KPC_for	CATTCAAGGGCTTTCTTGCTGC	*bla*$_{IKPC}$	538
	KPC_rev	ACGACGGCATAGTCATTTGC		

Primers utilized in this study were provided in lyophilized form, dissolved in sterile TE-Buffer (pH 7) to give a final concentration of 100 picomole/µl as recommended by the provider and stored in a deep freeze (-20°C) until use.

PCR

Reactants concentrations and conditions for multiplex PCR (ABI, USA) were summarized in Tables 2 – 4; while those for monoplex PCR were listed in Tables5-7.

Table 2: Reactants volumes and concentrationsused for the PCR amplification of bla_{TEM} and bla_{PER}

Reactant	Volume (µl)	Final concentration
Free nuclease water	14	-
Kapa Multiplex	25	-
DNA template	3	25 - 50 ng
TEM for	2	10 pmol
TEM rev	2	10 pmol
PER for	2	10 pmol
PER rev	2	10 pmol
Final concentration	50	-

Table 3: PCR program followed to amplify bla_{TEM} and bla_{PER}

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	3 Min	95
Denaturation	35	18 Sec	95
Primer annealing		38 Sec	59
Polymerization		38 Sec	72
Final extension	1	10 Sec	72

Reagents concentrations of bla_{KPC} and bla_{IMP} were similar to those described in Table2, except for the conditions of KPC primers were 0.2 pmole and 0.5 pmole for IMP primers. Amplification conditions are listed in Table4.

Table 4: PCR program followed to amplify *bla*$_{KPC}$ and *bla*$_{IMP}$ (11).

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	10 min	94
Denaturation	30	40 sec	94
Primer annealing		40 sec	55
Polymerization		1 min	72
Final extension	1	7 min	72

Reagents for *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$are similar to those listed inTable2, exceptprimers conditions for *bla*$_{CTX-M-1}$and *bla*$_{CTX-M-9}$were 0.4, 0.2, 0.4, and 0.4 pmol respectively. Amplification conditions are similar to Table 4,in exception to the annealing was at 60°C and the final extension for 10 min.

Table 5: Reactants volume and concentration employed for *bla*$_{VIM}$ amplification

Reactant	Volume (µl)	Final concentration
Free nuclease water	5.5	-
Kapa Multiplex	12.5	-
DNA template	3	25 - 50 ng
VIM for	2	10 pmol
VIM rev	2	10 pmol
final volume	25	-

Table 6: PCR amplification conditions for *bla*$_{VIM}$

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	3 Min	95
Denaturation	35	18 Sec	95
Primer annealing		32 Sec	60
Polymerization		32 Sec	72
Final extension	1	10 Sec	72

Reactants volumes and concentrations employed for *bla*$_{\mathbf{CTX-M-2}}$ amplification were the same as those described in Table 5.

PCR amplification conditions for*bla*$_{CTX-M-2}$were similar to those described in Table 6, except the

annealing was at 52°C for 38 sec and initial extension for 38 sec.
Reactants volumes and concentrations employed for *bla*$_{SHV}$ amplification were similar to those described in Table 6, except the primer concentrations were 0.4 pmol for both reverse and forward primers. Amplification conditions used for *bla*$_{SHV}$ were as listed in Table 7.

Table 7: PCR amplification conditions used to detect *bla*$_{SHV}$

Step	Number of Cycle	Time	Temperature (°C)
Initial denaturation	1	10 min	94
Denaturation	30	40 sec	94
Primer annealing		40 sec	60
Polymerization		1 min	72
Final extension	1	10 min	72

Reactants volumes and concentrations employed for *bla*$_{OXA}$ amplification were same conditions as those described for *bla*$_{SHV}$ amplification.

PCR amplification conditions of *bla*$_{OXA}$ were the same as those described in Table7.
Amplicons were visualized after running at 100 V for 1 hr on a 1.5% agarose gel containing ethidium bromide. A 100 and 800 bp DNA ladder were used as a size marker (11).

Effect of Ceftazidime stress on swarming of UPEC.

Swarming was determined as described by Li *et al*. (12). A single colony was isolated and grown overnight at 37°C in 1% Tryptone broth plus 0.5% NaCl. Ceftazidime antibiotic (1048µg/ml which is sub-MIC) was added to swarming media (1% Tryptone broth, 0.5% NaCl, and 0.25% agar, pH 7) after cooling to 50°C, poured into petri dishes. Control plates filled with swarming media without addition of antibiotic. Approximately, 20 µl of 1.5×10^{8} CFU/ ml was inoculated at the center of swarming plates. Thereafter, all plates were incubated for 24 h at 37°C. The diameter of the swarming zone was measured by a metric ruler and compared to control plates. This assay was done in triplicates.

Effect of Ceftazidime stress on Hemolysin production.

Hemolysin production was tested in accordance to the method described by Harley and Prescott (13). In brief; 20 µl of 1.5×10^{8}CFU/ml overnight *E. coli* N 11suspension stressed with sub MIC of Ceftazidime, same isolate was

grown in antibiotic free medium, and *E. coli* S was grown in antibiotic free medium, were spotted with 20µl of 1.5×10^8 CFU/ml of bacterial suspension onto Blood agar. Subsequently, all plates were incubated at 37ºC for 24 hours.

Statistical analysis

Data were presented as means ± standard deviation. ANOVA test, $LSD_{0.05}$, and T test were employed for means comparisons using Excel 2013 application.Differences were considered significant when $P \leq 0.05$.

Results and Discussion

Results showed that 50 specimens were identified to harbor *E. coli* which is tested by conventional morphological methods and biochemical analysis. A high percentage of resistance was identified against ampicillin 96%. However, 82% and 84% of isolates were resistant to β lactam/ β lactamase inhibitor antibiotic; ampicillin/sulbactam, amoxicillin/ clavulanic acid (Figure 1). These findings indictate the capacity of these isolates to produce β-lactamases.

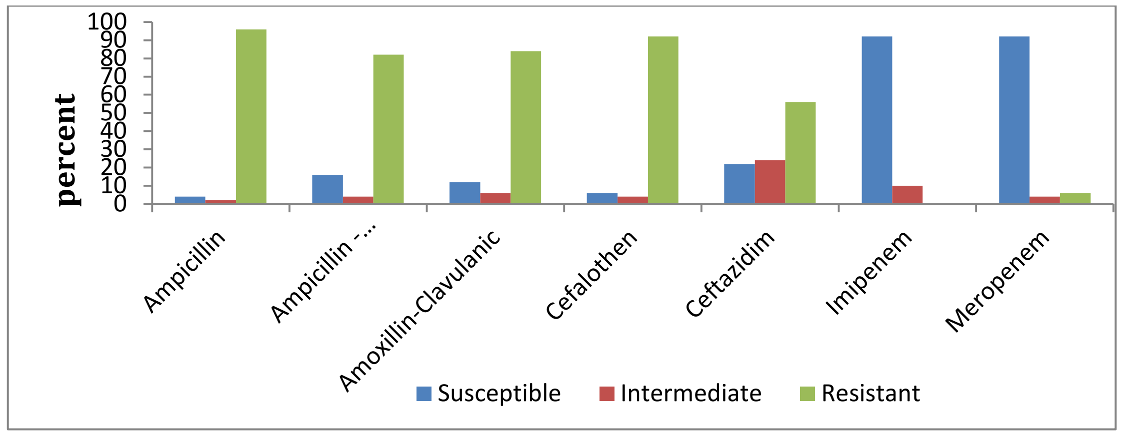

Figure 1: Antibiotic resistance of 50 UPEC

About 52% of all isolates were considered as ESBL due to resisting ceftazidime. Nevertheless, 82% of total isolates have the ability to produce β lactamase. While 92% of these isolates were sensitive to carbapenems (Figure 1).Results are presented in Figures 2, 3, and 4 demonstrated only four β-lactamase genes out of ten β-lactamase genes under investigation were detected; *bla*$_{PER}$ (520bp), *bla*$_{TEM}$ (800bp), *bla*$_{VIM}$ (390bp) and *bla*$_{CTX-M-2}$ (404bp) which comprised 4, 10, 12, and 18% of isolates, respectively. Nevertheless, *bla*$_{SHV}$, *bla*$_{OXA-1-like}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{IMP}$ and *bla*$_{KPC}$ were not detected.

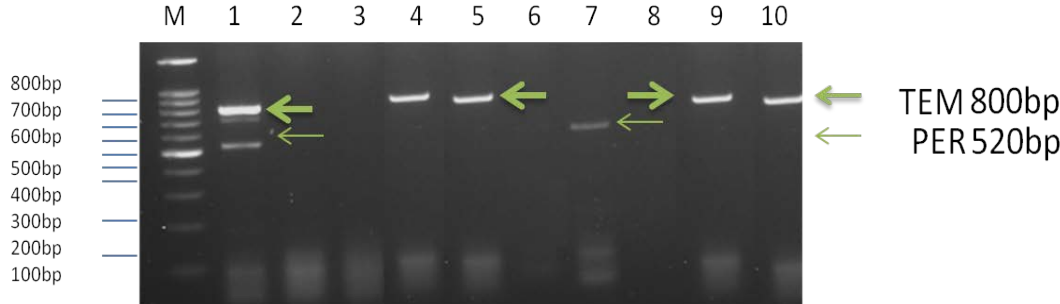

Figure 2: Analysis of the presence of bla_{TEM} and bla_{PER}among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-10 represent *E. coli* isolates 11, 12, 21, 22, 24, 25, 27, 28, 31, and 32, respectively.

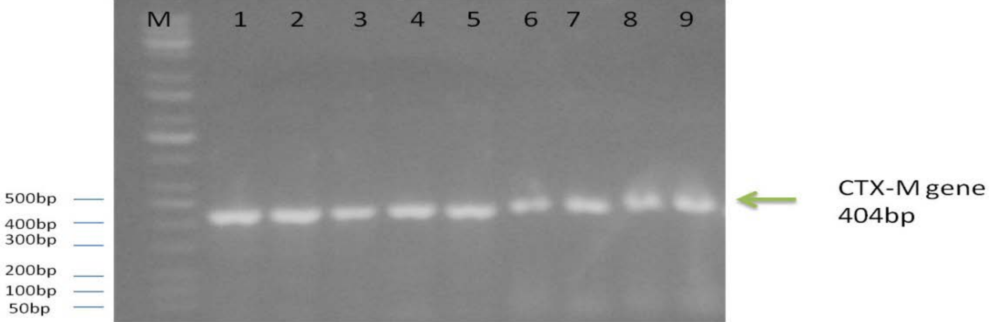

Figure 3: Analysis of the presence of $bla_{CTX\text{-}M\text{-}2}$among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-9 represent *E. coli* isolates 3, 26, 30, 31, 32, 35, 41, 48, and 49, respectively.

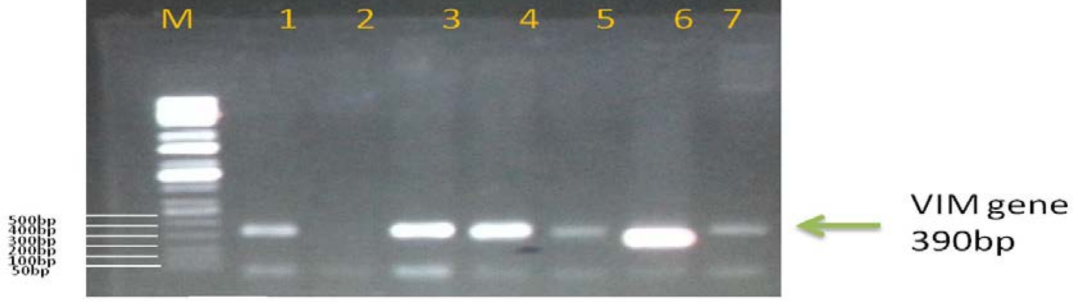

Figure 4: Analysis of the presence of bla_{VIM}among *E. coli* clinical isolates by PCR and run on agarose gel (1.5%) at 5 V/cm for 1 hour, stained with ethidium bromide and visualized on a UV transilluminator documentation system. Lane M: ladder, lanes 1-7 represent *E. coli* isolates 9, 10, 11, 23, 27, 38, and 46, respectively.

In a local study done by Aziz (14), the result revealed that the most common ESBL in *E. coli* were bla_{CTX-M} (CTX-M-14 and CTX-M-15) and bla_{TEM}; which represented 18 and 11% of total isolates tested, respectively. The current results can be strongly similar to the results revealed by Garrec *et al.* (15), who found that TEM was found at about 7.4% of *E. coli* isolates, and no isolate showed band of the presence of bla_{SHV} which, in turn, support our results since that local isolates appeared to lack this gene. Regarding bla_{CTX-M}, they found that 18 isolate of *E. coli* out of 107 (19.26%) *Enterobacteriaceae* isolates revealed the existence of $bla_{CTX-M.}$

In conclusion, ESBL *E. coli*local isolates are found to exist relatively at a high level among clinical isolates derived from UTI patients.

Effect of Ceftazidime stress on swarming of UPEC.

Current results showed that CAZ reduced the diameter of swarming seven fold when compared with control swarming plates (antibiotic free). The mean of swarming diameter for resistant isolate (N11)was about18.6± 2.3 mm in the presence of CAZ; nonetheless, it was 82± 2.6 mm when tested in CAZ free media. In regard to sensitive isolate (S), it developed a mean of swarming reached 84.3 ± 1.1 mm (Figure 3-7). Such finding is highly emphasized the impact of antibiotic on cells motility. In a conclusion, antibiotic stress immobilized bacterial cells and reduced their dissemination.

Such significant reduction ($P < 0.05$) (Figure 6) in swarming diameter could be assigned to the presence of resistance genes confer bacterial cells high capacity to distribute with high comfortable state, but when bacterial cells were exposed to stress of antibiotic, motility of cells was immobilized despite of the presence of these genes. The existence of antibiotic may affect transformation of swimming cells into swarming cells or reduction in secretion of wetting agents that are necessary for bacterial swarming (16).

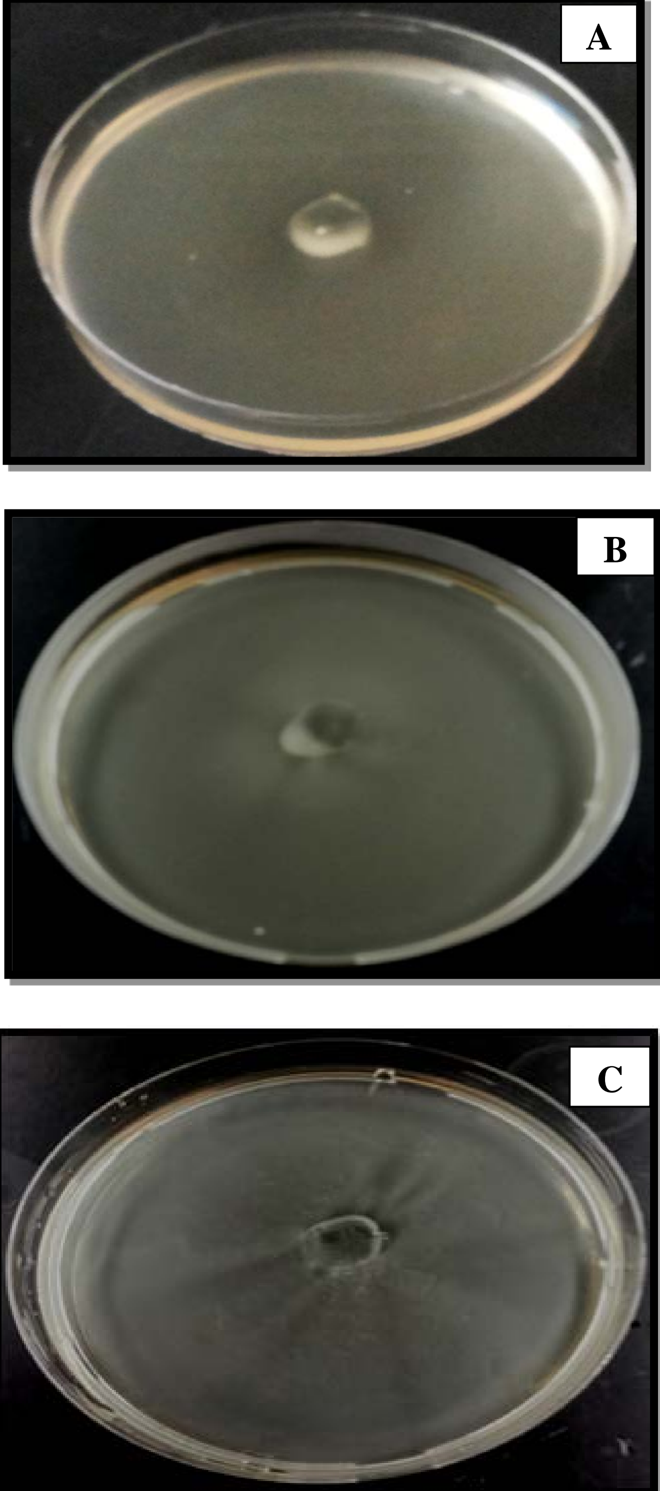

Figure 5: Effect of CAZ on swarming of *E. coli*. A) *E. coli* N 11 cultured on swarming medium supplemented with sub MIC of CAZ. B) same isolate cultured on CAZ free medium. C) *E. coli* S isolate spotted on antibiotic free medium.

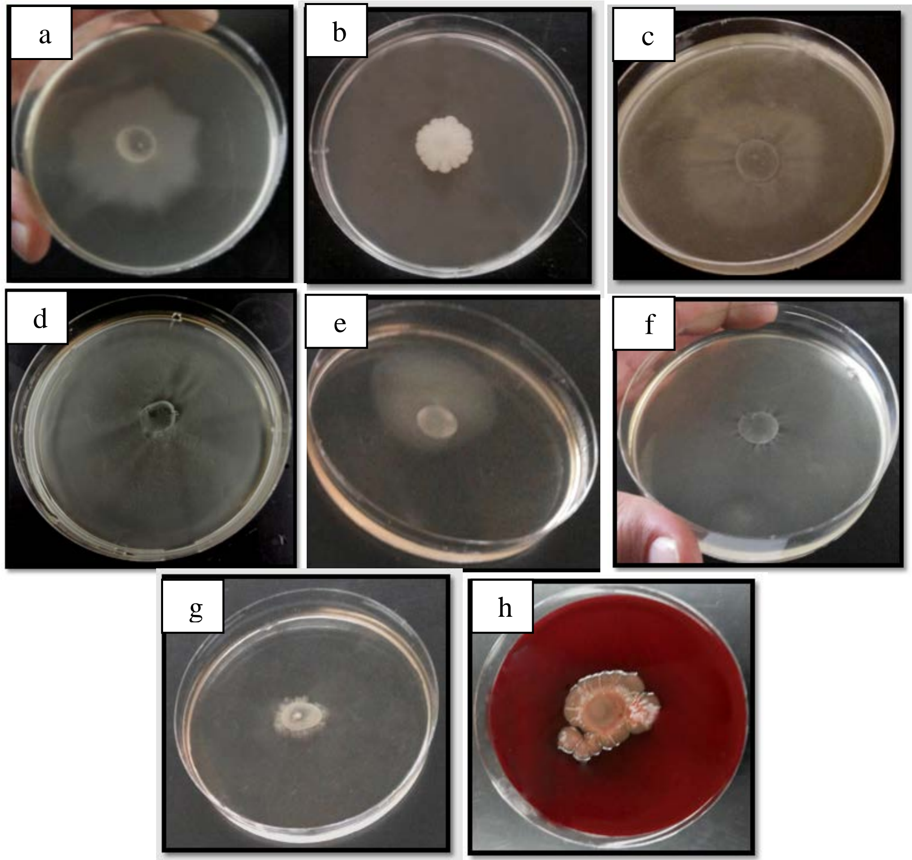

Figure 6: Ceftazidime stress caused reduction in swarming diameter of *E. coli*. Asterisk denotes to significant different (P< 0.05)

It is worthy to mention that *E. coli* isolates of the present work developed different patterns of swarming (Figure 7).

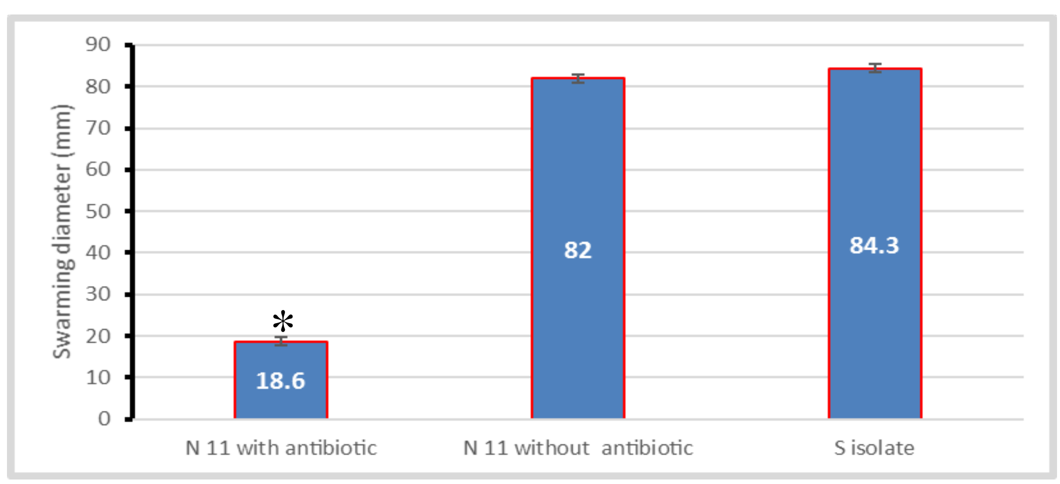

Figure 7: Different patterns of *E. coli* swarming developed on swarming media (a through g) and blood agar (h).

Effect of Ceftazidime stress on Hemolysin production.

Results of current work revealed that the presence of sub-MIC antibiotic stress was significantly affected the lytic activity of *E. coli* α-hemolysin by showing the absence of hemolysis of human RBCs when compared with control plates (antibiotic free blood agar). Effect of antibiotic stress on hemolysis is illustrated in Figures 8.

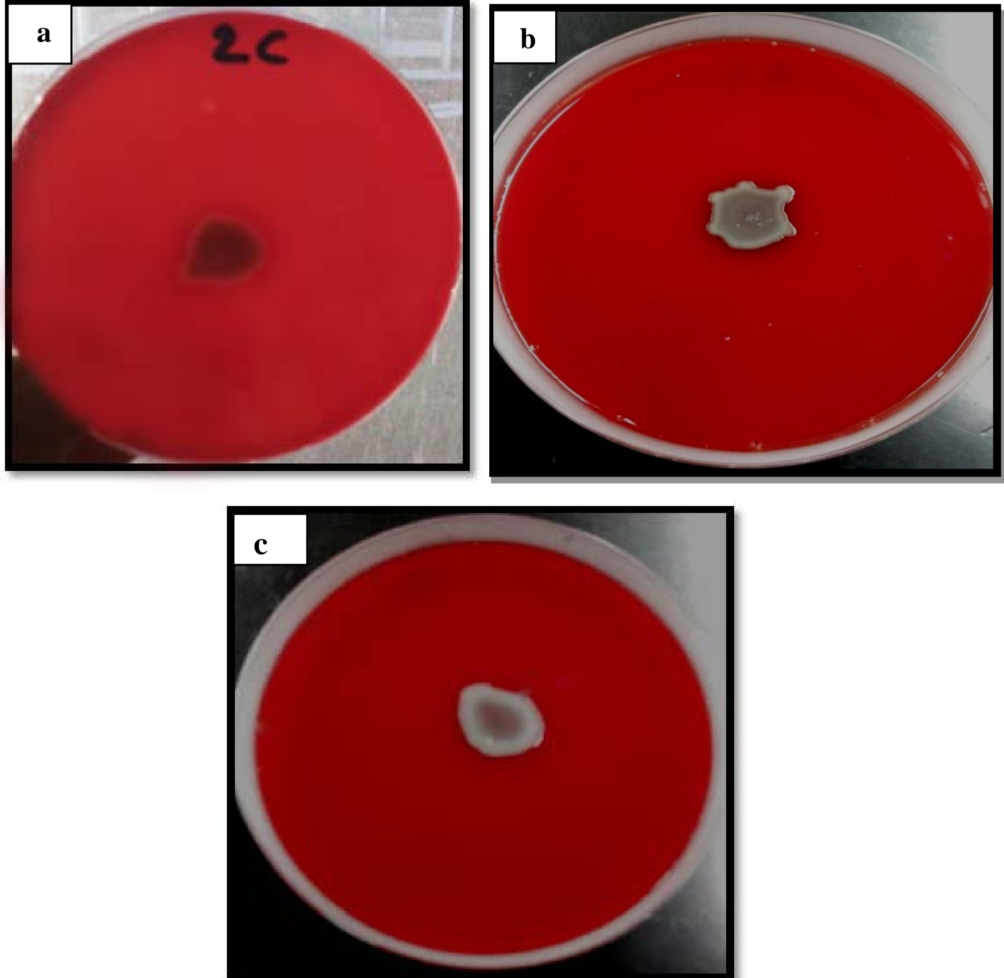

Figure 8: Effect of CAZ on hemolytic behavior of *E. coli*. a) N 11 isolate produces α-hemolysin in antibiotic free media. b) Inability of N 11 to produce α-hemolysin when exposed to sub MIC antibiotic stress. c) Absence of β-hemolysis on blood agar seeded with *E. coli* sensitive isolate S.

These current results suppose that the presence of resistance gene may have a correlation with the expression of α-hemolysin as that the sensitive isolate which lack resistance gene showed the inability to produce this toxin. When the resistant isolate showed loss of the

ability to produce hemolytic activity when exposed to antibiotic stress, this can lead to think that the hemolytic activity was suppressed and different reasons can be thought to play role in this suppression. One of them is that antibiotic reduced the resistant isolate ability to produce α-hemolysin or was produced in low concentration that was unable to permeabilize them and causing lysis of erythrocytes which responsible for causing cells death. On the other hand, the *hlyCBAD* operon encodes pro-alphahemolysin (hlyA), an acyltransferase that generates maturealpha-hemolysin, an ATPase (hlyB) and the inner membrane protein (hlyD), that along with the outer membrane protein TolC, and are responsible for toxin secretion, couldbe affected by antibiotic (17).

Another possible explanations are that antibiotic stress may affect the intracellularly activation by specific fatty acylation, or it may influence of a second activation step which takes place in the extracellular medium through binding of Ca^{2+}ions (18).

In addition, Ca^{2+}induces a change in HlyA conformation, that includes an increased surface hydrophobicity, in turn leading to the irreversible insertion of the toxin in the membrane (19) which may be reduced by the presence of antibiotic stress.

The present results may indicate that the presence of resistance gene play role in keep bacterial survival than expressing α-hemolysin as a fitness cost to overcome stresses of the surrounding environment.

In general, such differences in physiological processes between resistant and sensitive isolates could be attributed to that acquisition of resistant genes (in this case beta lactamases coding genes) applies additional metabolic activities represented by synthesizing new proteins and RNA; which, in turn, will affect the energetics of the cell and rendering them less fitted.

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Protective Role of Royal Jelly against Histological Effects of Aluminum Chloride on Testes of Male Rats

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Abstract: The aim of this study is to investigate the protective role of the royal jelly on the histological alterations of testes induced by aluminum chloride ($AlCl_3$). The first group of the rats was negative control group. The four treatment groups were received 20 mg $AlCl_3$ /kg body weight, one of them was considered the positive control group, The three other groups were received the same dose of $AlCl_3$ and subdivided according to the different concentration of the concentrations of royal jelly (50, 100 and 200 mg/kg). All treated doses were given orally by gastric intubation and the experiment was continued daily for 60 days. Due to experimental intoxication with $AlCl_3$, the microscopic examination for the testes in rats revealed numerous histological lesions in the seminiferous tubules and the interstitial tissue. In contrast, the histopathologic changes of testes were partially reversed by treatment with royal jelly and the testes appeared with nearly normal structure. It may be concluded that royal jelly revealed protective effect against the reproductive toxicity of $AlCl_3$.

Key words: royal jelly, aluminum, testes, seminiferous tubules, rats.

الدور الوقائي للغذاء الملكي ضد التغيرات النسجية لكلوريد الألمنيوم في خصى ذكور الجرذان

حسين بهاء دعيبل ، عبدالهادي عباس هادي

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الخلاصة: ان الهدف من هذه الدراسة هو تقصي الدور الوقائي للغذاء الملكي ضد التغيرات النسجية في الخصى المستحثة عن طريق كلوريد الألمنيوم. تم تخصيص المجموعة الأولى من الجرذان كمجموعة سيطرة (المجموعة السالبة)، أما المجاميع الاربع المعاملة فأعطيت 20ملغم كلوريد الألمنيوم/ كغم من وزن الجسم واحداها قد اعتبرت مجموعه السيطرة الموجبة اما الثلاث الباقية فقد تم إعطاءها الجرعة نفسها من كلوريد الالمنيوم وقسمت الى ثلاث مجاميع ثانويه استناداً إلى التراكيز المستعملة من الغذاء الملكي وهي 50 ،200,100 ملغم / كغم من وزن الجسم. أعطيت جميع الجرع فموياً عن طريق الانبوب المعدي واستمر الاعطاء يوميا لمدة ستون يوماً، بسبب السمية التجريبية من جراء المعاملة بكلوريد الالمنيوم، بينَّ الفحص المجهري للخصى حدوث أضرار نسجية عدة في النبيبات المنوية وفي النسيج البيني للخصية. بالمقابل، فإن التغيرات النسجية-المرضية للخصى قد تحسنت جزئيا من جراء المعالجة بالغذاء الملكي وظهرت الخصى قريبة من تركيبها الطبيعي. لذا يمكن الاستنتاج بأن الغذاء الملكي قد أظهر تأثيرا وقائيا ضد السمية التكاثرية لكلوريد الألمنيوم.

Introduction

Aluminum is the most abundant metal and the third most abundant element, after oxygen and silicon, in the earth's crust (1). Aluminum is one of the elements that enter widely in large quantities in daily life; so many sources contain aluminum within their contents as food additives, toothpastes and deodorants (2). Besides, aluminium compounds have several medical applications such as vaccine, antacids, antiperspirant, buffered aspirins, phosphate binders and allergen injections (3). It has been observed that the accumulation of this element to the extent of toxic concentrations causes a variety of environmental damages in terrestr ial and aquatic systems, so numerous studies have been dedicated to evaluate the toxic effects of aluminum in both human and animal, especially the potential influences of this element in the reproductive system. Moreover, the effect of aluminum on male and female fertility has become an area of growing concern (1).

Reproductive toxicity in human and animal can be initiated after being exposed to aluminum compounds. Testicular tissue damage has been associated with ingestion and accumulation of aluminum in target organs (4,5). Aluminum is able to interfere with several biological functions in regard to the reproductive toxicity including increased oxidative stress, alterations in membrane function, disruption in cell signaling, altering or inhibiting the enzyme activities and impairment of blood testis barrier (6).

On the other hand, the royal jelly (RJ) is one of the products of honeybee workers that secreted by the salivary glands and is devoted mainly as food for the queen throughout the larval period, while other nurse honeybee larvae are fed royal jelly for only three days (7). RJ can be exerting their positive effects on human body due to its natural pharmacological activities. It is usually used in folk and official therapy because it is consider as beneficial and effectual remedy. However, it is regarded as a controversial dietary supplement (8).

The pharmacological activities of RJ include the following: antioxidative activity, insulin-like action, hypotensive and blood regulatory actions, anti-inflammatory action (antibacterial, antiviral and fungicidal effects), wound healing effect, antihypercholesterolemic activity, hepatoprotective effect, immunomodulating effect, anti-allergic effect, antitumor action, tonic action, neurotrophic action and anti-aging effect. These important activities have been reviewed in some studies (7,8,9,10). Recent

studies have shown that the RJ has important functional effects, especially in the field of reproduction and fertility. So, because of its unique qualities and desired charact eristics has been used widely in medical treatments, health foods and in cosmetics (7).

The present study aims to investigate the histological toxic effects of aluminum chloride ($AlCl_3$) in the testis of albino male rats and protective role of the RJ towards the aluminum toxicity.

Materials and Methods

Royal jelly sample

The Royall jelly (RJ) sample was obtained from Hussein Al-zerjawi beekeeper in Najaf province (a region 160 km south Baghdad) during the year 2014. RJ sample were kept in cold condition and stored until its processing.

Experimental animals

Healthy adult albino Sprague-Dawley rats weighing between 215-288 gm were used in this experiment. They were housed in separated plastic cages at Faculty of Veterinary Medicine/ University of Kufa/ Iraq, and kept in controlled environment of 22-25 °C.

Commercial food (pellets) and tap water were provided to animals *ad libitum.* Rats were left to acclimatize for at least two weeks before the start of the experiment. None of the rats had any clinically evident infections.

Experimental design

Fifty sexually mature male rats were randomly distributed into five groups (10 rats each). All experimental rats, except normal control animals (negative group), were given orally by gavage daily with aluminum chloride ($AlCl_3$; $6H_2O$. Fluka Chemicals - Switzerland) at concentration 20 mg/kg body weight. This dose was used according to the previous study of Hala *et al*. (2010) (11).

The treated animals were subdivided into four groups. The positive control group did not treat with RJ, while the animals of the remaining three groups were received different concentrations of RJ (50, 100 and 200 mg/kg/day) respectively. The orally dosages of RJ were determined according to Galaly *et al*. (2014) (12). The concentrations of $AlCl_3$ and RJ that dissolved in distilled water depended on body weight. The period of exposure with $AlCl_3$ and treatment with RJ was continued for 60 days (13).

Histological technique

At the end of the experimental period, all rats anesthetized using a mixture of ketamine and xylazine i.m., and then they were sacrificed (14).Ordinary histological technique was followed to prepare slides from specimens of testes from all animal

groups to study the alterations that may be found in treated animal groups when compared to control groups. The processing and staining techniques were performed according to Bancroft and Stevens (1982) (15).

Histopathological examination

The stained sections of testes from each animal were observed under the compound microscope to evaluate the histological features and structural changes. Photomicrographs were taken by camera mounted microscope at different magnifications.

Results and Discussion

The microscopic examination of testicular sections of control rats showed normal histological structure. Photomicrographs of testes showed closely packed seminiferous tubules with little spaces between them in addition to obvious successive spermatogenic stages. Clumps of Leydig cells were obvious in the sections inside the interstitial spaces (Figures1&2).

In aluminum-treated group the histological appearance of testis after 60 days of exposure showed structural alterations as compared to the control group. The main histopathological changes were vacuolar degeneration of spermatogenic cells and Sertoli cells with clear necrotic debris in the seminiferous tubules which attained different shapes, appearance of multinucleated giant cells and/or pyknotic cells, irregularity of germ cell layers, abnormal distribution of spermatozoa in the lumens or no sperms in the lumen, thick and irregular basement membrane, interstitial edema, hyperplasia of Leydig cells, congestion of blood vessels, and intertubular space expansion (Figures 3&4).

On the contrary, the results of this study demonstrated that administration of RJ at graduated concentrations together with $AlCl_3$ result in progress enhancement in histologic testicular structure. Serious deleterious effects of $AlCl_3$ on testicular structure were reversed by RJ administration. The treatment of male rats with RJ at the dose 50 mg/kg showed continuous effectual damage induced by $AlCl_3$ on the testis tissue (Figures 5&6), while the using of RJ at 100 mg/kg showed mild improvement in histologic feature with occurrence of marked edematous fluid and spermatogenic arrest at spermatids stage (Figures 7&8).

Figure (1): Photomicrograph of testis section from control group showing closely packed seminiferous tubules with little interstitial connective tissue and normal distribution of cellular element. (H & E, 100×).

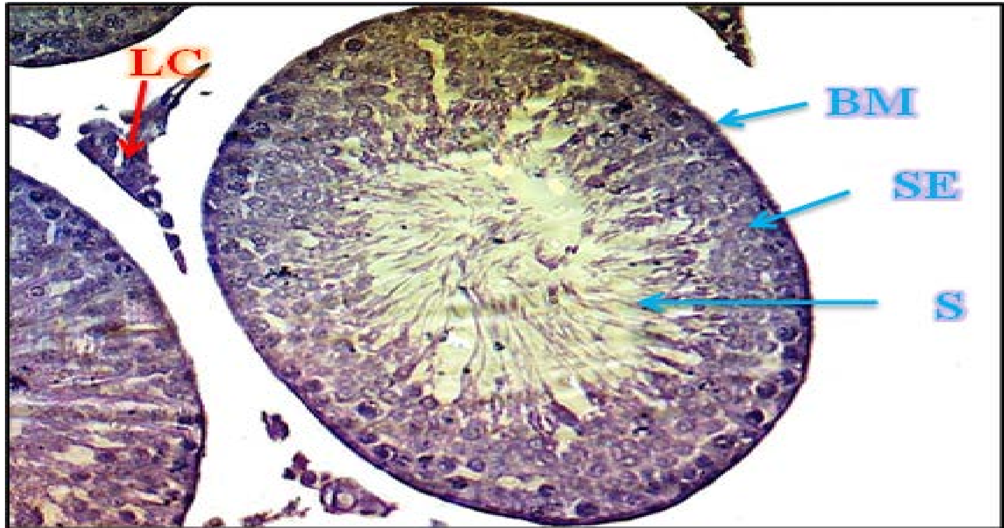

Figure (2): Photomicrograph of testis section from control group showing spermatogenic epithelium (SE), spermatozoa (S), Leydig cells (LC) and basement membrane (BM). (H & E, 200×).

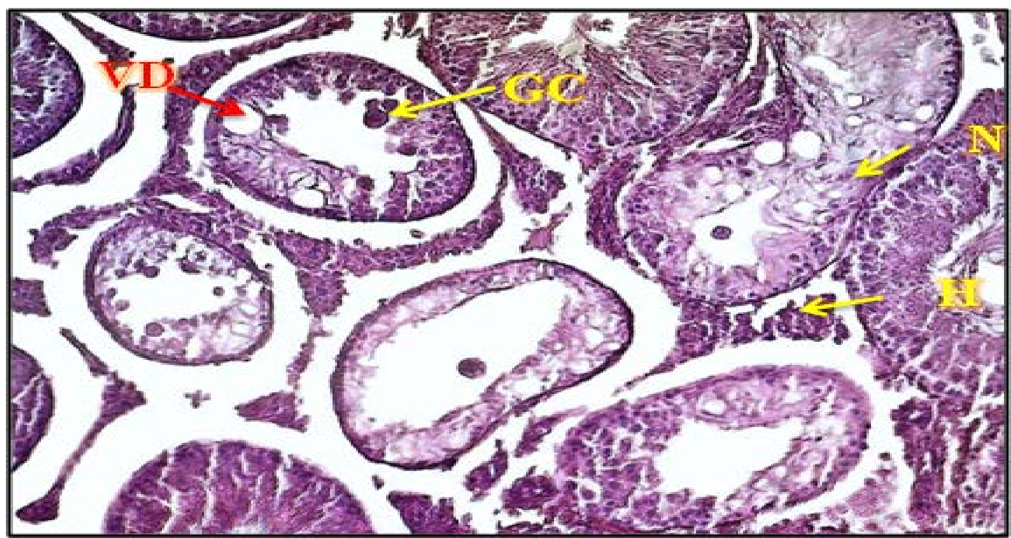

Figure (3): Photomicrograph of testis section from $AlCl_3$ -treated group (20 mg/kg) showing severe damage within the seminiferous tubules with inhibition of spermatogenesis. Note: vacuolar degeneration (VD) and necrosis (N) of germinal epithelium, formation of multinucleated giant cells (GC), and hyperplasia (H) of interstitial Leydig cells. (H & E, 200×).

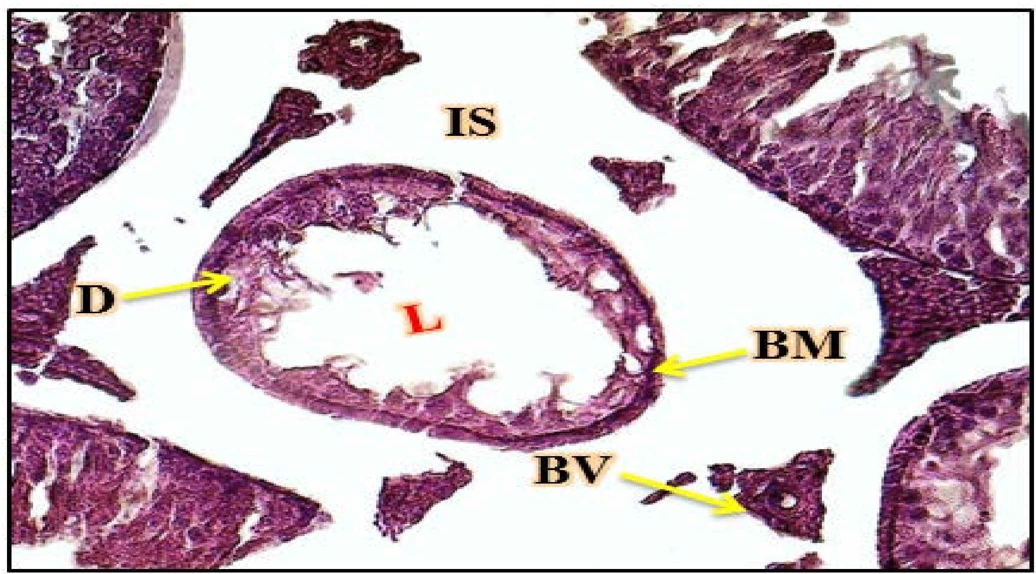

Figure (4): Photomicrograph of testis section from $AlCl_3$ -treated group (20 mg/kg) showing complete lesion in seminiferous tubule. Note: Degeneration (D) of spermatogenic cells and absence of spermatozoa, increase in the size of lumen (L), thick basement membrane (BM), increase in interstitial space (IS), and congested thick-walled blood vessels (BV). (H & E, 200×).

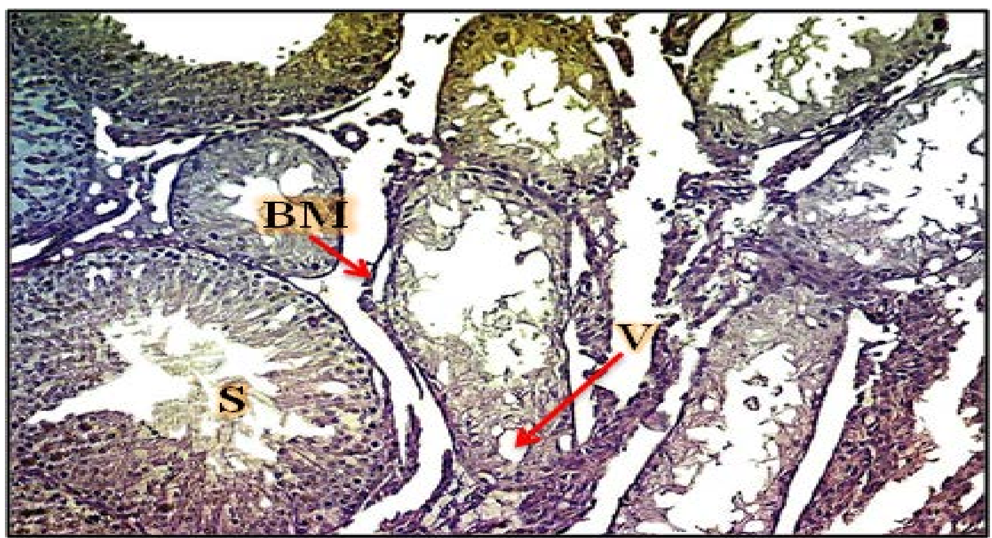

Figure (5): Photomicrograph of testis section from RJ-treated group (50 mg/kg) showing alterations of the general architecture in some seminiferous tubules. Note: disorganization of the germinal epithelium and occurrence of several large vacuoles (V), spermatogenesis arrest in some tubules and few fragmented spermatozoa (s) in the lumen, and irregular basement membrane (BM) . (H & E, 100×).

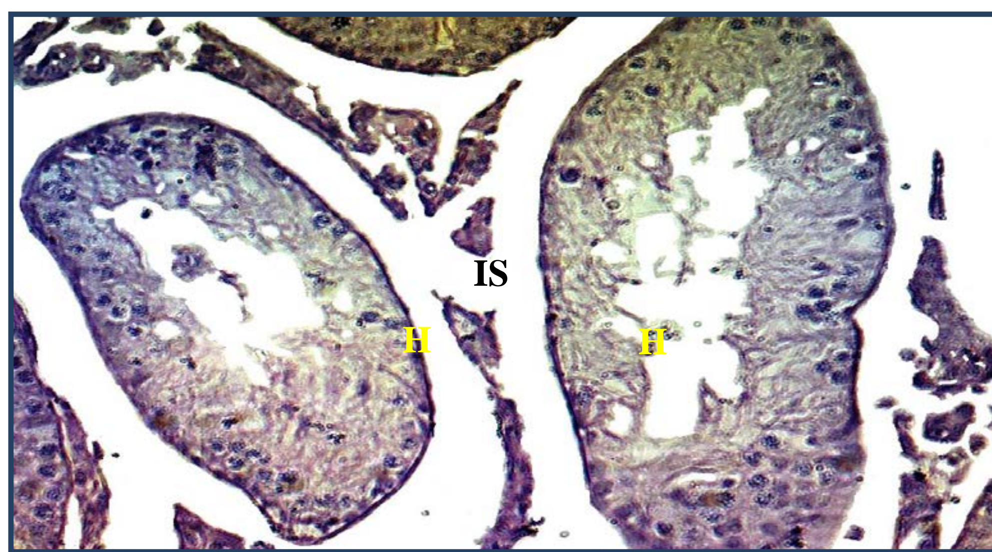

Figure (6): Photomicrograph of testis section from RJ-treated group (50 mg/kg) showing serious deleterious effects on testicular structure. Note: Hypoplasia (H) of the germinal epithelium with spermatogenic arrest at various stages of spermatogenesis, and marked increase in the intertubular spaces (IS). (H & E, 100×).

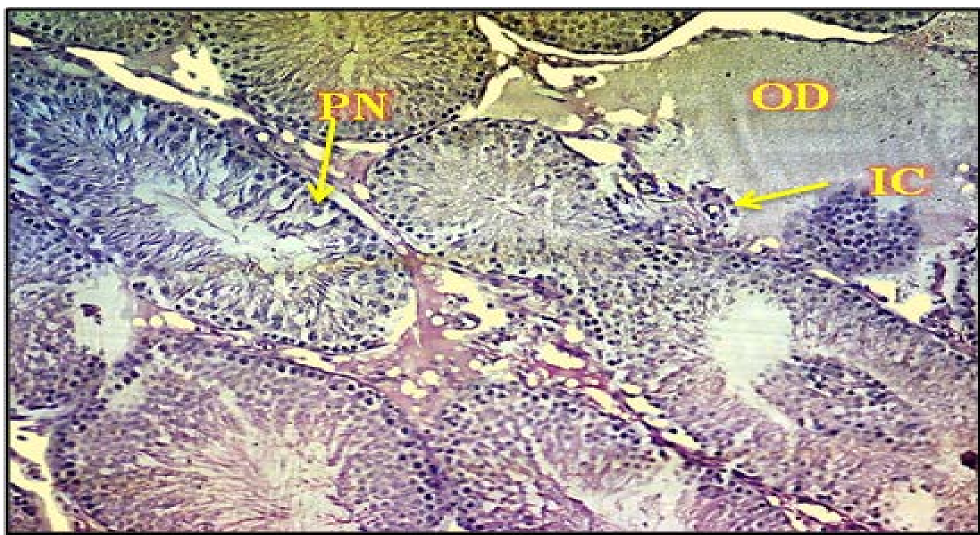

Figure (7): Photomicrograph of testis section from RJ-treated group (100 mg/kg) showing mild histological changes in the seminiferous tubules. Note: most of spermatogonia had pyknotic nuclei (PN), presence of homogenous acidophilic exudates or interstitial oedematous fluid (OD), and increase the inflammatory cells (IC) in the interstitium.(H & E, 100×).

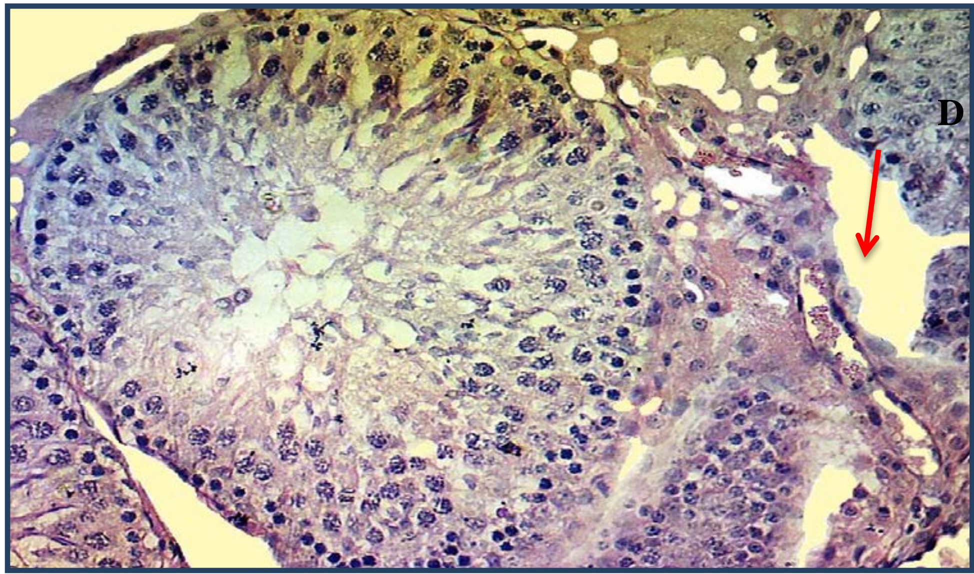

Figure (8): Photomicrograph of testis section from RJ-treated group (100 mg/kg) showing partial improvement in histologic feature. Note: arrest at the spermatid level in seminiferous tubules, arrest at the spermatid level in seminiferous tubule, interstitial edema and dilatation (D) of blood vessel. (H & E, 200×).

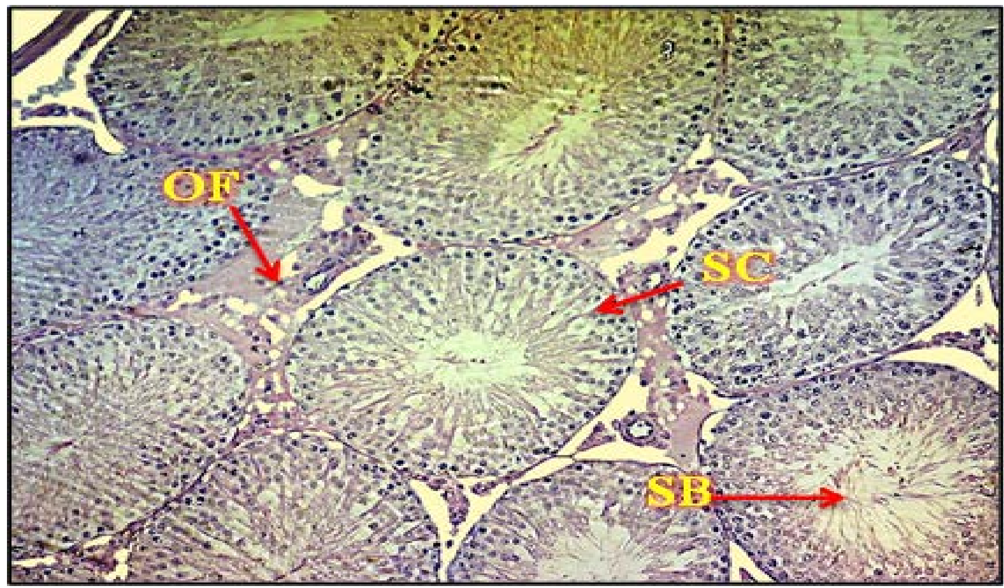

Figure (9): Photomicrograph of testis section from RJ-treated group (200 mg/kg) showing marked enhancement histological feature of seminiferous tubules. Note: different stages of normal spermatogenic cells (SC) with presence of sperm bundles (SB), and few areas of oedematous fluid (OF). (H & E, 200×).

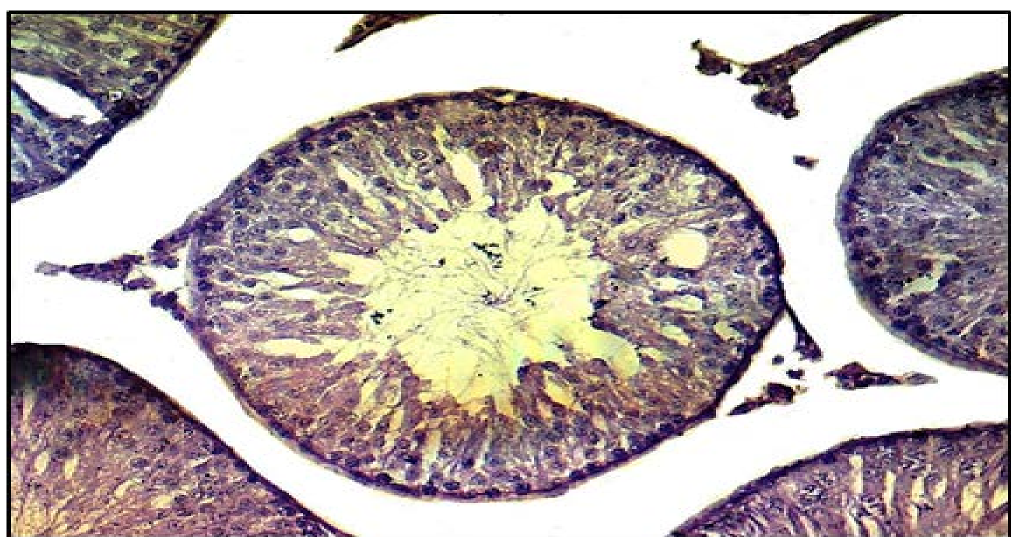

Figure (10): Photomicrograph of testis section from RJ-treated group (200 mg/kg) showing successive stages of normal spermatogenic cells of seminiferous tubule and normal distribution of epithelial lining. Damage reversed by RJ administration. (H & E, 200×).

The enhancement was evident at higher dose level of RJ (200 mg/kg) which include the restore arrangement of seminiferous tubules with apparently normal distribution of cellular elements. Also, sperm bundles retain to present in lumen of tubules (Figures 9 & 10).

Several studies performed on the human and experimental animals referred to the presence relationship between aluminum exposure and male reproductive toxicity. According to El-Demerdash *et al*. (2004) (16), the exposure to aluminum is responsible for increased oxidative stress and ROS which cause damage of cellular lipids, proteins and DNA. Consequently, these alterations might be representing the cause of degeneration and necrosis that revealed in the histology of testis.

Moreover, the cell membrane contains several highly unsaturated fatty acids which are easily attacked by free radicals leading to imitation lipid peroxidation which considered the rout for many pathological events (17). Furthermore, exposure to $AlCl_3$ may lead to a disorder in testosterone function resulting in deterioration in the spermatogenesis process in $AlCl_3$-treated group (18).

In the present work, the microscopic examination of aluminum-treated rats revealed the occurrence of multinucleated giant cells in the lumen of some tubules. Chinoy *et al*. (2005) (19) suggested that giant cells could be the result of faulty or failed chromosomal replication or cell division.

The hyperplasia of interstitial Leydig cells can be as a compensatory attempt in order to face the decline which occurred in testosterone level. This may result in alteration of GnRH levels which may affect LH secretion from the anterior pituitary lobe that stimulates Leydig cells to proliferate as a compensatory mechanism (20).

Presence of interstitial edema may result from the oxidative damage of capillaries endothelial cells through interference with their membranes, increasing their permeability (21). It was also reported that capillary filtration participates in interstitial fluid formation; thus, changes in capillary permeability and blood/lymph flow could modify the interstitial fluid (22). In addition, one study has reported that the congestion in blood vessel walls is attributed to hypertension in the surface blood vessels (23)

On the basis of our findings, histological observations in the present study proved the excellent recovery of testes after RJ treatment and this protection was dose dependent. RJ was used in this experiment in order to reverse or prevent toxic effect induced by aluminum based on some information which considered the RJ as a safe product when

administrated to male rats and mice and did not lead to any adverse effect (24).

The present study has established the ameliorating effect of RJ in the testicular structure and spermatogenesis process. The therapeutic efficacy of RJ may be attributed to its antioxidant activity against lipid peroxidation and free radical generation.

The beneficial biological properties of RJ components exhibit an antioxidant capacity and improved endogenous antioxidant defense system, thereby, histopathologic picture of testes were reversed by treatment with RJ to normal architecture. These results are in agreement with many reports (25, 26, 27).

Conclusion

This study showed that RJ has alleviating effect and protective role in testicular tissue, in the condition of aluminium -induced oxidative stress. Moreover, the RJ at concentration 200 mg/kg of body weight could be able to alleviate the disturbing effects of $AlCl_3$ and improved sexual efficiency of male rats.

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Detection of Coronavirus and Adenovirus Antibodies among Patients with Acute Respiratory Tract Infections

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Abstract:

Background: Acute Respiratory Tract Infections (ARTIs) are the major health problem worldwide, with high morbidity and mortality rates. Coronavirus and Adenovirus are common viral cause of respiratory tract infections.

Aim: The study aims to identify antibodies against Coronavirus and Adenovirus among patients with acute respiratory tract infections.

Patients and methods: The study carried out in Kirkuk governorate from the 10th November 2013 to 10th January 2014. Hundred and forty patients with signs and symptoms of acute respiratory tract infections, and 30 healthy individuals as control group .These patients admitted to Kirkuk General Hospital were examined for the presence of IgG antibodies against Coronavirus and each of IgG and IgM antibodies against Adenovirus in the serum by using Enzyme Linked Immunosorbent Assay (ELISA) technique.

Results: The results showed that were no case detected for Coronavirus IgG among total examined patients , while the rates of Adenovirus antibodies were 27.85% , 9.28% and 6.42% for Adenovirus-IgG , both IgG & IgM at same time respectively and Adenovirus-IgM. The highest rate of Adenovirus-IgG (61.53%) was among patients from rural area, while the highest rates of Adenovirus -IgM and for both IgG with IgM at same time (55.55% and 69.23% respectively) were found among patients from urban area. Also the rates of Adenovirus -IgM and for both IgG with IgM at same time were higher in women than that in men .

Conclusion: There was no case detected for Coronavirus IgG in this study , while the rate of Adenovirus was high. The rates of acute and recurrent Adenovirus infection were high. There were acute and recurrent infections.

Keywords: Acute Respiratory Tract Infections (ARTIs), Coronavirus and Adenovirus

التحري عن الأجسام المضادة للفيروس التاجي والفيروس الغدي ضمن المرضى ذوي الإخماج الحادة للجهاز التنفسي

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الخلاصة :

مقدمة: التهابات الجهاز التنفسي الحادة هي مشكلة صحية كبيرة على مستوى العالم والتي تسبب مستوى عال في معدل الاخماج والوفيات.يعتبر الفيروس التاجي والفيروس الغدي من الفيروسات الشائعة المسببة للأمراض التنفسية.

الهدف: هدفت الدراسة إلى تشخيص الأجسام المضادة لكل من الفيروس التاجي والفيروس الغدي في المرضى المخمجين بالتهابات المجاري التنفسية الحادة.

المرضى وطرق العمل: أجريت الدراسة في محافظة كركوك في الفترة الممتدة من العاشر من تشرين الثاني 2013 وحتى العاشر من كانون الثاني 2014. تم جمع نموذج الدم من 140 مريضا من المخمجين بالتهابات المجاري التنفسية الحادة وشملت الدراسة مجموعة اليسطرة 30 شخصا لا يعانون من الأعراض التنفسية من المراجعين لمستشفى كركوك العام للتحري عن الأجسام المضادّة نوع (جي) تجاه الفيروس التاجي و (أم وجي) تجاه الفيروس الغدي في مصل المرضى باستخدام طريقة الإنزيم المناعي المترابط الممدص.

النتائج: أظهرت النتائج انه لا وجود للأجسام المضادة نوع (جي)تجاه الفيروس التاجي في المرضي المفحوصين، بينما كان معدل الإصابة في الفيروس الغدي 27,85% ، 9,28% و6,42% و بالأجسام المضادّة نوع جي ، أم و جي على التوالي و أم . وكان أعلى معدل (61,53%) الأضداد الفيروس الغدي نوع جي بين المرضى القرويين وكـــــانت أعلى المعدلات (55,55% و 69و23%) الأضداد الفيروس الغدي نـــوع (ام)والاثنين (جي وأم) معا في نفس الوقت على التوالي في المرضى المدنيين. وكذلك معدل الأجسام المضادة تجاه الفيروس الغدي نوع (ام) و ام وجي في النساء اكثر مما في الرجال.

الاستنتاج: لا وجود الأضداد النوع جي تجاه الفيروس التاجي في مصل المفحوصين ضمن هذه الدراسة بينما كانت معدلات الإصابة بالفيروس الغدي عالية، هناك الاخماج حـــادة ومتكررة بالفيروس الغدي في المــــرضى المخمجين بالتهـــابات المجـــاري التنفسية.

مفاتيح البحث: اخمـــاج المجـــــــاري التنفسية الحـــــادة، الفيروس التـــــاجي، الفـــــيروس الغدي.

Introduction :

Acute respiratory tract infections (ARTIs) are the major health problem worldwide, with high morbidity and mortality rates. The most common viral respiratory tract infections are Coronavirus and Adenovirus.[1] Coronavirus genome are non segmented, single-stranded RNA of positive polarity, Coronaviruses are largely associated with respiratory and enteric infections in mammals and birds.[2] The large surface projections of the Coronaviruses gave them an appearance reminiscent of a crown, hence the name 'Coronaviruses'. The coronavirus was firstly isolated in human in the mid – 1960s [3]. It is responsible for approximately 30 % common cold, it is spread by the respiratory route and caused Severe Acute Respiratory Syndrome (SARS) form ingestion of aerosolized faeces via contamination of the hands and environment and usually occurs in

winter or spring and produce symptoms high fever (>38 ^{0}C)

,malaise, dry cough, shortness of breath, myalgia, bronchitis, headache and diarrhea. [4] Human adenoviruses belong to the *Adenoviridae* family and the *Mastadenovirus* genus. They are divided into six species, designated A to G with serotype 52.[5] Adenoviruses are medium-sized, non-enveloped icosahedral viruses a single linear, double-stranded DNA.[6] Adenovirus infections occur worldwide as epidemic, endemic and sporadic infections, the most common adenovirus serotypes causing respiratory infections are serotypes 1, 2 and 5 (species C) and serotypes 3 and 7 (species B). Adenovirus mortality varies from 6 to 70 % in pediatric and adult transplant patients ,a lower mortality rate (2%). The study aims to identify the seroprevalence of Coronavirus and Adenovirus patients with acute respiratory tract infection. [7]

Materials and Methods:

Pure serum were examined for the presence of IgG antibodies against Coronavirus and IgG and IgM against Adenovirus by using of Enzyme Linked Immunosorbant Assay (ELISA) technique(From komabiotech, USA) ,which was used according to manufacture instruction. The results were then interpreted on the basis of antibodies as seropositive or seronegative.

The present study was done from November 10th, 2013 to the 10th of January 2014 in Kirkuk province, five ml of venous blood were aspirated from 140 patients with acute respiratory infection and 30 healthy individuals as control group in Kirkuk General Hospital whom their ages (18-65 years old) from both male and female, the blood then let to be clotted and then centrifuged at 3000 round per minute (rpm) for 10 minutes twice.

Statistical Analysis

Computerized statistically analysis was performed using SPSS (Statistical Package for Science Services). Comparison carried out using Chi-square (X^2) and probability (P value). The P value $\leq$ 0.05 was considered statistically significant (S), less than 0.01 considered highly significant (HS) and greater than 0.05 was considered non significant.

Results :

Table (1) showed that there were no Coronavirus IgG for all the patients with acute respiratory tract infection tested by ELISA technique. Table(2) however showed the prevalence of Adenovirus –IgG, both IgG & IgM and Adenovirus –IgM were (27.85%) ,(9.28%) and (6.42%) for Adenovirus-IgG Adenovirus-IgM and for both Adeno IgG& IgM at the same time by using ELISA

respectively. Table (3) shows the relation of Adenovirus antibodies seropositive with residency of patients with acute respiratory tract infection . The highest rate of Adenovirus- IgG (61.53%) was found in patients from urban area ,while 38.47% was from rural area. The highest rate (55.55%) of Adenovirus-IgM was found in rural area. Also the highest rate (69.23 %) of both adenovirus - IgG and Adenovirus- IgM at the same time was from the rural area. Table (4) shows the relation of Adenovirus antibodies seropositive with sex of patients with acute respiratory tract infection .The highest rate of Adenovirus- IgG (53.84 %) was among male patients. The highest rate (66.66%) of Adenovirus-IgM and (61.53%) of both adenovirus - IgG and Adenovirus- IgM at the same time was among females .

Table 1: Seroprevalence of the *Coronavirus-IgG* Patients with Acute Respiratory Tract Infection By ELISA Technique.

Seroprevalence of *Coronavirus-IgG* antibodies	**No .**	**%**
Coronavirus- IgG(+)	0	0
Coronavirus- IgG(-)	140	100
Total	140	100

Table 2: Seroprevalence of the *Adenovirus-IgG* and *Adenovirus-IgM* Patients with Acute Respiratory Tract Infection By ELISA Technique.

Seroprevalence of *Adenovirus-IgG and IgM* antibodies	Patients with respiratory symptom		Control group	
	No.	%	No	%
IgM (-) and IgG (-)	79	56.44	25	83.33
IgM (-) and IgG (+)	39	27.86	2	6.66
IgM(+) and IgG (+)	13	9.28	1	3.33
IgM(+) and IgG (-)	9	6.42	2	6.66
Total	140	100	30	100

$X^2 = 12.37$ P = 0.021 P < 0.05 Significant

Table 3 : Relation of Adenovirus antibodies distribution with patient residency .

Residency	Seropositive of Adenovirus antibodies					
	Adenovirus- IgG		Adenovirus - IgM		Adenovirus(IgG & IgM)	
	No .	%	No.	%	No.	%
Urban	24	61.53	4	44.45	4	30.77
Rural	15	38.47	5	55.55	9	69.23
Total	39	100	9	100	13	100

X^2 = 6.3 P = 0.27 P < 0.05 Significant

Table 4 : Relation of Adenovirus antibodies distribution with sex .

Residency	Seropositive of Adenovirus antibodies					
	Adenovirus- IgG		Adenovirus - IgM		Adenovirus(IgG & IgM)	
	No .	%	No.	%	No.	%
Male	21	53.84	3	33.34	5	38.47
Female	18	46.16	6	66.66	8	61.53
Total	39	100	9	100	13	100

X^2 = 3.48 P = 0.92 P > 0.05 non Significant

Discussion:

Considering the importance of Coronavirus and Adenovirus infection transmission and its complications, it seems necessary to investigate the prevalence of these two virus infection among patients with acute respiratory tract infection, and varius serological techniques use for diagnosis this virus. Furthermore, no study of similarity has been published in Iraq. In this study, the Coronavirus – IgG was not detected in the total examined serum of patient with acute respiratory infection, this mean there is no past infection of Coronavirus for many years ago , this may due that these patient were not contacted with patient with Coronavirus infection or may due to these patient were follow the right roles for prevention of Coronavirus infection. While in other countries the detection of coronavirus-IgG antibodies were recorded results; in Cameron the rate was (3.23%),[8] in Singapore (3.50%)[9] and in Hong Kong (5.2%).[10] The results of Adenovirus infection among patients with acute respiratory tract infection patients by using ELISA

technique, were classified according to seropositive antibody type for Adeno-IgG represents past (chronic) infection ,Adeno - IgM represents the acute state of Adenovirus (primary) infection, and for both Adeno-IgG and Adeno-IgM at same time represent re-infection or reactivations of latent infection . In the presented study the rate of Adenovirus antibodies were (27.85%) , (6.42%) and (9.28%) for Adenovirus-IgG, Adenovirus-IgM and for both Adeno IgG& IgM at the same time respectively by using ELISA, respectively. (as shown in table 2). Similar results Adenovirus-IgG were recorded in Vienna (22%), in Egypt (25.3%), and in India (25.5%).[11,12,13] However, the low rate was recorded in Georgia (7.8%).[14]The current work revealed that adenovirus-IgM antibodies were similar rates found in Iran (6.3%),[15] in Palestine (6.8%),[16] in Turkey (7.7%) [17] and in Brazil (7.28%).[18]These different results from different studies may be due to differences in hygienic, socioeconomic status. In addition, the variations in results may be attributed to the fact that different ELISA kits used in the other studies from different companies may be with different reagents qualities and properties. The presented research revealed that adenovirus-IgM with IgG antibodies at the same time were found in 9.28% of patients with respiratory tract infections, the appearance of IgM with IgG at the same time represent re-infection or reactivation of latent infection. The higher value of adenovirus infection in rural area as compared to those of urban areas(as shown in table 3). On the other hand past infections rate was higher throughout urban areas. A hypothesis for the probable role of geographical influence upon Adenovirus seroprevalence might be the route of infection. In rural areas probably the main route through which the virus is transmitted from infants and young children due to poor sanitation , also low socioeconomic status has been found as a strong risk factor for acquisition Adenovirus in addition to hygiene practices . Regarding to the prevalence of Adenovirus antibodies in relation to the sex, the rate of Adenovirus- IgG (53.84 %) was among male patients. The highest rate (66.66 %) of Adenovirus-IgM and (61.53 %) of both adenovirus – IgG and Adenovirus- IgM at the same time was among females (as shown in table 4) . This may due to the females more contacts with infected patient specially children although the males more contacts with other patient outside the home mainly adults this may refer that the rate of adenovirus may in children higher than adults .

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Isolation of Anti-Candida Agent from *Penicillium spinulosum* strain C3-8

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Abstract: *Penicillium spinulosum* is xerophilic, psychrophilic and widely frequented fungus in the environment. This species was isolated as air contaminant fungus from Basrah province was grown at 27 °C on potato dextrose agar (PDA) and appeared as bluish green colonies with pink edges and their reversible was brown as wool or feather in shape with an aerial mycelial growth as well as exudates on the colony surfaces. Microscopically, the conidia with lactophenol stain were globose to oval shape and bluish-color, besides phialides and conidiophores were observed. The identification was confirmed by techniques of PCR and sequencing in addition to bioinformatics analysis of internal transcribed spacer (ITS) region as *Penicillium spinulosum* strain C3-8.This fungus was cultured on potato dextrose broth (PDB) at 28 °C for 7 days by using shaking incubator (200 rpm) and its crude mycelial extract produced bioactivity (inhibitory zones) values and 100µg/ml as MFCs against *C. albicans* and *C.glabrata*. The crude extract was analyzed by RP-HPLC revealed different purified fractions with various peaks and time values. All fractions were tested against *C. albicans* and gave that one fraction only produced inhibitory zone (25 mm) as bioactive appearance. The bioactive fraction was subjected into liquid chromatography mass spectrometry (LC-MS) and showed different molecular weights with various peaks, in addition the use of pure nystatin and ketoconazole for comparison in HPLC and MS analyses. The crude extract was stable in range of pH (2-9) and temperature degrees (4, 37 and 55 °C) and produced bioactivity against *C. albicans*, besides its concentrations (1mg/ml, 3mg/ml, 6mg/ml and 10 mg/ml) did not give any haemolysis zones against human RBCs.

Key words: Sequencing alignment of *Penicillium spinulosum* strain C3-8 and extraction of anti-Canidida agent.

عزل مضاد لخميرة الـ *Candida* من الفطر *Penicillium spinulosum* strain C3-8

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المستخلص: يعتبر الفطر *Penicillium spinulosum* من الفطريات التي تنمو في الضغوط الازموزية العالية والمحبة لدرجات الحرارة المنخفضة وهو واسع الانتشار في البيئة. تم الحصول على هذا النوع كتلوث من هواء محافظة البصرة وتم انمائه في درجة حرارة 27 °م على اكاردكستروز البطاطا واظهر مستعمرات خضراء مزرقة ذات حواف وردية وهي تشبه الصوف او زغب الطيور وغزلها الفطري ظاهر فوق سطح الاكارمحتوي على نضحات سائلة وان مقلوبها ظهر باللون الجوزي.مجهريا وباستخدام صبغة اللاكتوفينول ظهرت الكونيدات ذات شكل بيضوي ازرق اللون كما تم ملاحظة الـ Phialides والحوامل الكونيدية Conidiophores . تشخيص هذا النوع تم تأكيده باستخدام تقنيات تفاعل البلمرة والتتابع الجيني والـ Bioinformatics الخاصة بـ (ITS) internal transcribed spacerبانه يقع ضمن العترة *Penicillium spinulosum* strain C3-8. تمت زراعة هذا الفطر على مرق دكستروز البطاطا PDB في درجة حرارة 28 °م لمدة 7 أيام باستخدام حاضنة رجاجة (200 rpm) وان المستخلص الخام لغزله الفطري انتج فعالية حيوية (طوق تثبيط) و 100 مايكروغرام /مل كجرعة قاتلة ادنى MFC ضد نوعين من الـ *Candida* وهما *C. albicans*, *C.glabrata*. المستخلص الخام خضع لتقنية كروماتوكرافيا عالي الاداء (HPLC) وانتج اجزاء نقية مختلفة بقمم وفترات زمنية متباينة. تلك الاجزاء جربت ضد *C. albicans* حيث واحدة من تلك الاجزاء فقط هي التي انتجت فعالية حيوية . ان الجزء الذي انتج فعالية حيوية خضع لتقنية تحليل الطيف الكتلي واظهر بانه يحتوي على اوزان جزيئية مختلفة ذات قمم متباينة، أضف الى ذلك فقد استخدم النستاتين والكيتاكونوزول النقيين كمادتي سيطرة ومقارنة في تقنيات كروماتوكرافيا عالي الاداء والطيف الكتلي. لقد اظهر المستخلص الفطري الخام بانه ثابت الفعالية الحيوية في مديات (2-9) من الاس الهيدروجيني وفي درجات حرارة مختلفة (4، 37 و 55 °م) كما ان التراكيز (1 ملغم/مل، 3 ملغم/مل، 6 ملغم/مل و 10 ملغم/مل) العائدة له لم تظهر اي تحلل لكريات الدم الحمراء البشرية.

Introduction

Penicillium spinulosum is xerophilic, psychrophilic and widely frequented fungus in the environment producing finely roughed conidia as well as narrower phialides. This species was frequently isolated from soil and sometime from grain, as well as from paperboard orange juice cartons and ultra-pasteurized extended shelf life milk products as contaminant microorganism, and it does not produce any toxin in the milk products (1).

Many fungi, especially saprobic groups have ability to produce metabolic products including secondary metabolites which have been defined as smaller molecules that are produced when fungal nutritional sources are depleted. Production of these metabolites is in the stationary growth phase of fungi and often related with differentiation and sporulation. One function of secondary metabolites is as antifungal compounds for treatment of fungal pathogens (2).

The secondary metabolites are formed in fermentation process in which the organic compounds are chemically transformed by help of enzymes. Batch culture is one of fermentation type contains nutrients which are required for growth of organism and desired products (3).Extraction of external metabolite pattern is obtained by mixing of the

fungal supernatant with suitable solvent e.g, ethyl acetate, but the internal type is given by manipulation of mycelia with solvent such as acetone whereas mycelia are destroyed to take out the internal secondary metabolites outside in order to get these metabolites (4), (2).

The extracted metabolites are evaporated to get a solid crude extract, and identified by various chromatographically techniques examples HPLC, MS, NMR,GC-Mass, X-ray Crystallography, FTIR …etc as well as specific color testes and biological assays e.g, MICs, MFCs and cytotoxicity(5). Liquid chromatography mass spectrometry (LC-MS) technique is a qualitative and a quantitative analysis to identify unknown compound by determining molecular weights of the molecules as well as knowing structures of the compound through their fragments (6) and giving a highly degree identification of the compounds in the crude complex including fungal extract , so it is used to confirm fractions of HPLC technique whereas the latter technique can not give the confirmed identification (7).

Methodology

Identification of *Penicllium spinulosum*

Penicllium spinulosum was isolated from Basrah province as air contaminant by accident. *P.spinulosum* was grown on potato dextrose agar (PDA) at 27°C for five days and identified according to (8).

The molecular identification of *P. spinulosum* was made by using PCR, DNA sequencing and bioinformatics by LGC Genomics Sequencing Service (sequencing@lgcgenomics.com)in Germany to detect internal transcribed spacer (ITS) region by using the following primers and PCR conditions (Table,1).

Primers of *P. spinulosum* strain C3-8 :

ITS1: TCC GTA GGT GAA CCT GCG G

ITS4: TCC TCC GCT TAT TGA TAT GC

Table (1): PCR conditions of ITS

Steps	Temperature (°C)	Time (min.)
1	96	1
2	96	0:15
3	50	0:30
4	72	1:30

Steps: 2-4 40 cycles

This technique was used to identify the strains of the target fungal species. The fungal DNA sequence was subjected to BLAST of NCBI GenBank database based on various values of max score, total score, query cover percentage, E value and identity percentage. For selection of strain, less gaps and max score were used as evidences for such taxonomic confirmation.

Preliminary screening of anti-Candida

The preliminary screening of anti-Candida agent was performed by complete colony and disc of the colony from growing *P.spinulosum* strain C3-8 was formed on a plate of PDA, streaked with *C.albicans* and incubated at 37°C for 24 hr. The inhibition zone (IZ) was measured by calibrator (9).

Fermentation process

Fermentation assay method was made by The fungus was grown on PDA at 27 °C for five days. Two hundreds ml of PDB (pH: 5.1) were prepared in a flask (1000 ml) and sterilized by autoclave at 121°C for 15 min. Two discs (6mm diam.) from fungal colony were inoculated into flask. Incubation of the flasks was at 28°C in a shaker (200 rpm) incubator for 7 days (16).

Detection of anti-Candida agent in the fungal filtrate

Plates of SDA were prepared and inoculated with 100µl of 10^6 cell/ ml of *C. albicans*. Sterilized Whatman No.1 filter paper discs (6 mm in diameter) were prepared. The filter paper discs were soaked with the fungal filtrate and placed on the plate of *Candida*. The plates were made in duplicates. The plates were incubated at 37°C for 48 hr. and inhibitory zones (IZ) were measured by calibrator.

Extraction of anti-Candida agent (4)

Penicillium spinulosum strain C3-8 was grown on PDB for 7 days, the mycelia were separated from filtrate by three layers of filter papers. The mycelia were treated as the following:

*The mycelia were mixed with acetone and centrifuged (5000 rpm for 15 minute at 4°C) the filtrate was removed.

*The precipitate was washed by three solvents and as the following:

a) Absolute ethanol, the precipitate and ethanol were centrifuged (5000 rpm for 15 min. at 4°C to remove the supernatant and use of the precipitate.

b) Absolute methanol : the procedure was same as ethanol.

c) Absolute ethyl acetate: it was the same procedure as ethanol to get a precipitate substance. The precipitate was re-dissolved with acetone to form a solution, then it was carefully mixed with distilled water and final precipitate was made. The final precipitate was considered as crude anti-Candida agent from mycelia of *Penicillium spinulosum* strain C3-8.The crude anti-Candida agent was partially purified by its solubility in one solvent such as ethanol, methanol and dimethyl sulfoxide (DMSO). This test was repeatedly performed.

Anti-Candida bioactivity of fungal crude extracts

Plates of SDA were prepared and inoculated with 100µl of 10^6 cell/ ml of *Candida*. Wells (6 mm diam.) were made at the center of agar plates. 100µg/ml the fungal crude extract was placed in each well. The plates were incubated at 37°C for 48 hr. The appearance of inhibitory zone was examined by calibrator. Duplicate plates were made.

Characterization of fungal crude extract

1) Reverse-phase high performance liquid chromatography (RP-HPLC) (7).

The fungal extract and both pure nystatin and ketoconazole were analyzed by RP-HPLC as following steps:

1) The fungal crude extract was dissolved in dimethyl sulfoxide (DMSO) and evaporated at 55°C to obtain the crystalized extract and 10 mg were dissolved in 1 ml of RP-HPLC-methanol to obtain a solution (10mg/ml).

2) Solution of the fungal extract was carried out by HPLC (Dionex 5µm C_8 4.6 × 100mm) at flow rate 0.5 ml/min.

3) 50 µl sample solution (HPCL methanol) of 10mg/ml concentration of the extract solution was injected into column chromatography. The mobile phase was HPLC methanol : distilled water (85:15).

4) The purified extract was detected by UV-spectrophotometry (Dionex) at 254nm after 50 min. at 40°C.

5) Fractions of the purified fungal extract were tested against *C.albicans* to examine fungal bioactivity.

Bioactivity of purified compound against *C. albicans*

1) SDA plates were prepared and inoculated with 100µl of *C.albicans* (10^6 cell/ml).

2) Sterilized discs (6 mm diam.) of Whatman No.1 filter paper were soaked in purified extract of each fraction.

3) One disc was placed at the center of plate and incubated at 37°C for 48 hr.

4) Plates were examined for the appearance of growth inhibitory zones.

2)Liquid chromatography- mass spectrometry, LC-MS, (LC-MSD-Trap-SL)

The RP-HPLC-fractions of fungal extract and both pure nystatin and ketoconazole were analyzed by LC-MS as following steps:

1) 10µl of RP-HPLC-fraction of fungal extract which produced inhibitory zone, were added into 90µl of methanol (85% concentration) to obtain a mixture of methanol and RP-HPLC-fraction .

2) 5µl of the mixture were run on LCMS in mass spectrometry without fragmentation in addition to alternating positive and negative polarization and the peaks were observed.

3) Biological tests of fungal crude extract

a)Minimum fungicidal concentration (MFCs)

1) Five screw glass tubes were used, 9 ml of sterile SDB were placed in each tube and sterilized by autoclave at 121°C for 15 min.

2) 1000 µg/ml of fungal extract were added into first SDB tube , mixing by vortexer and 1 ml of the first tube was added into second SDB tube, thus a serial dilutions of different concentrations of the fungal crude extract were prepared (100 µg/ml , 10 µg/ml, 1 µg/ml , 0.1 µg/ml and 0.01 µg/ml).

3) 1 ml of fungal extract of each concentration was added into tubes containing SDB and inoculated with 100 µl of 10^6 cell/ml of *Candida*. Incubation was conducted at 37°C for 72 hr.

4) A full-loop of each tube was inoculated on SDA plate and incubated at 37°C for 48 hr to examine the fungal concentration which did not reveal growth of *Candida* species. MFC values were determined and compared with pure nystatin and ketoconazole.

b) Cytotoxicity assay of fungal crude extract against RBC

1) Preparation of blood agar medium plates with 7% of human blood.

2) Wells (6 mm diam.) were performed in each plate.

3) Four different concentrations of the fungal crude extract were prepared by dissolving in DMSO. These concentrations were 1 mg/ml, 3 mg/ ml, 6 mg/ ml and 10mg /ml and sterilized by Millipore filter paper (0. 2 µm).

4) 100 µl of each concentration was placed in each well.

5) The plate was incubated at 37°C for 24 hr to observe a haemolysis

zones around the wells. If haemolysis is found, it indicates the concentration is toxic for blood cells.

6) The test was performed in duplicates.

Optimum conditions of fungal crude extract

a) Effect of temperature on bioactivity of fungal crude extract

1)The fungal crude extract was left at 4°C, 37°C and 55°C for one hour and dissolved in DMSO to get 1000µg µg/ ml concentration.

2) Sterilized SDA plates were inoculated with 100 µl of 10^6 cell/ml of *C. albicans.*

3) Well was made in the plate and loaded with 100 µl of the fungal crude extract concentration and incubated at 37°C for 48 hr and inhibitory zone was measured.

b) Effect of pH on bioactivity of fungal crude extract

Two DMSO solutions which were 1000µg/ml concentrations of the fungal crude extract, one solution was at pH:2 and second one at pH :9. The solutions were sterilized by Millipore filter paper (0.2 µm). Adjustment of pH was made by the use of pH meter, and control of pH with NaOH and HCl.

2) The procedure was similarly made for steps of effect of temperature on bioactivity of the fungal crude extract protocol.

c) Effect of enzymes (pepsin and trypsin) on bioactivity of fungal crude extract

1)10000 µg/ml of both pepsin and trypsin were prepared. The solvent was DMSO.

2) 1000µg/ml of each enzyme were added into10000µg/ml of the fungal crude extract and thoroughly mixed to get a mixture of fungal extract with enzyme.

3) Sterilized SDA plates were inoculated with 100 µl of 10^6 cell/ml of *C. albicans.*

4) Wells were made in the plates and loaded with 100 µl of the mixture (step 2) and incubated at 37°C for 48 hr and inhibitory zone was measured

5) This test was performed in duplicates.

Results

Penicillium spinulosum grew on PDA and formed as shape of wool or feathers with an aerial mycelial growth. The colonies produced exudates on their surfaces. Microscopically, the conidia with lactophenol stain are globose to oval shape bluish-color as well as phialides and conidiophores were observed (Figures: 1, 2 ,3). Molecular identification (PCR, sequencing, bioinformatics of ITS and using NCBI revealed that this species within *P. spinulosum* strain C3-8 (Figure, 4).

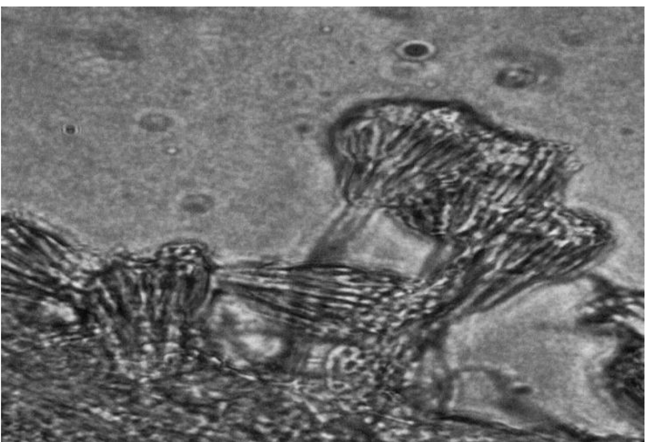

Fig.(1): Conidiophores and phialides of *P. spinulosum* strain C3-8 (magnification 100X)

Fig.(2): Colony of *P. spinulosum* strain C3-8 on PDA at 27 °C for 5 days.

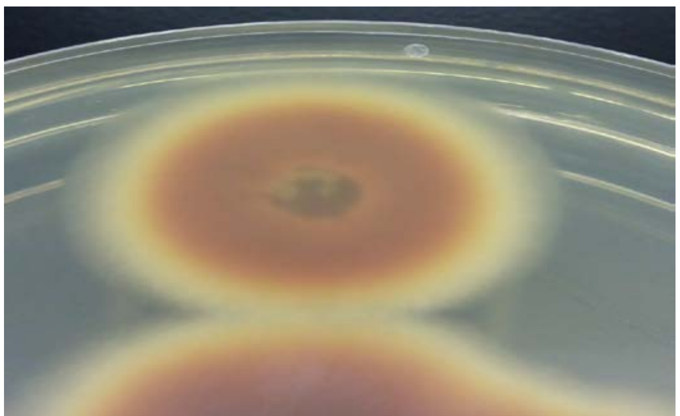

Fig.(3): Reversible colony of *P. spinulosum* strain C3-8 on PDA at 27 °C.

Penicillium spinulosum strain C3-8 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer ribosomal RNA gene, complete sequence

Sequence ID: gb|JQ717356.1| Length: 568 Number of Matches: 1

Range 1: 19 to 556 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
965 bits(522)	0.0	533/538(99%)	2/538(0%)	Plus/Plus

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Fig.(4).Sequencing alignment of *P.spinulosum* stran C3-8 by NCBI.

Preliminary screening of anti-Candida

Colony disks (6 mm in diameter) of *P. spinulosum* strain C3-8 revealed inhibitory zones (IZ) against *C. albicans* as 20 mm. (Figures: 5, 6).

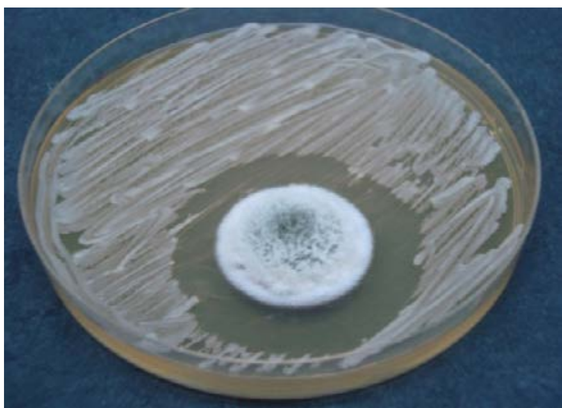


Fig.(5): Formation of inhibition zone by colony of *P. spinulosum* strain C3-8 against *C. albicans*.

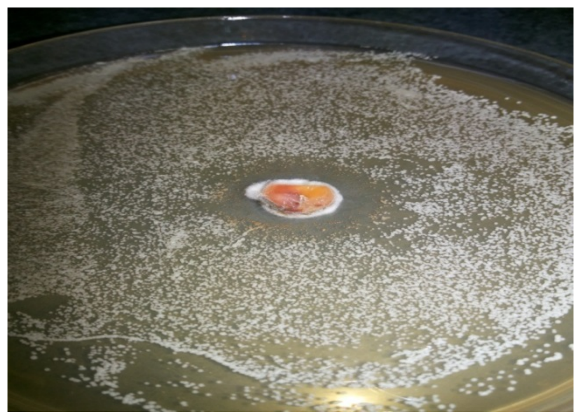


Fig.(6): Formation of inhibition zone by colony disc of *P. spinulosum* strain C3-8 against *C. albicans*.

Fermentation process

Penicillium spinulosum strain C3-8 was cultured in the fermentation medium (PDB) at $28C^0$ for 7 days in shaking incubator (200 rpm). The color PDB was changed from yellow into red by *P.spinulosum* strain C3-8 (Figure,7).

Fig.(7):Changing PDB from yellow into red color by growth of *P. spinulosum* at 28°C for 7 days using 200 rpm

Effect of *P. spinulosum* filtrate on growth of *Candida albicans*

Soaked filter paper discs in the filtrate of *Penicillum spinulosum* strain C3-8 did not show any inhibition zones against *Candida albicans*.

Extraction and solubility of anti-Candida

Extract of *Penicillium spinulosum* strain C3-8 was produced as a solid and red color (15 g/liter) in weight (Figure,8).It had complete solubility in DMSO and not soluble in Ethyl acetate (Table, 2).

Fig.(8): Extract of *P.spinulosum* strain C3-8.

Table (2): Solubility of *P.spinulosum* strain C3-8 extract in the different solvents.

Solvents	Solubility
DMSO	Complete solubility
Distilled water	Partial solubility
Ethanol	Partial solubility
Methanol	Partial solubility
Ethyl acetate	No solubility

Anti-Candida bioactivity of crude fungal extracts

The crude extract of *P. spinulosum* strain C3-8 revealed inhibitory zones against *C. albicans* and *C.glabrata* as 20 and 24mm. respectively (Table, 3). (Figures: 9, 10).

Table(3):Anti-Candida activity of crude extracts produced by *P. spinulosum* strain C3-8.

Inhibitory Zone(mm)	
C.albicans	*C.glabrata*
20	24

Fig.(9): Anti-Candida bioactivity of crude extracts against *C.glabrata*.

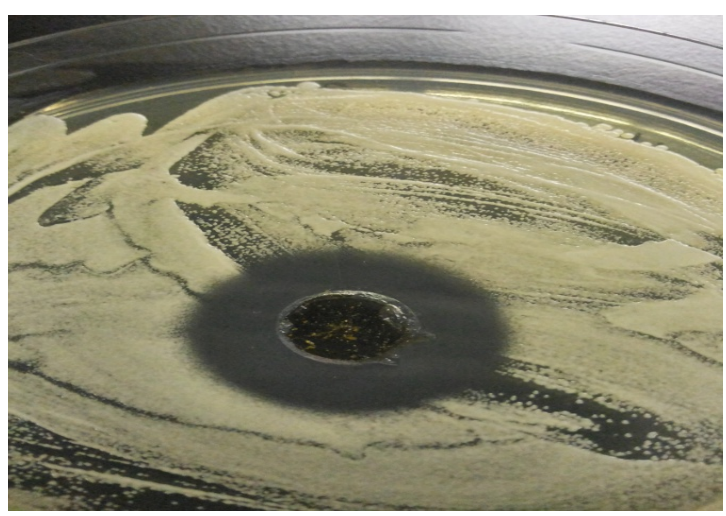


Fig.(10): Anti-Candida bioactivity of crude extracts against *C. albicans*.

Minimum fungicidal concentration (MFCs) of crude fungal extract

MFCs of fungal extract of *P.spinulosum* strain C3-8 revealed that 100 µg was recorded as MFC value against *C. albicans* and *C. glabrata* and compared with pure nystatin and ketoconazole (Table, 4).

Table (4): MFCs of *P.spinulosum* strain C3-8 against isolated *C. albicans* and *C. glabrata*.

MFCs (µg)		
Fungal extract	nystatin	ketoconazole
100	100	100

Assay of blood cytotoxicity of crude fungal extracts

Four concentrations (1mg/ml, 3mg/ml ,6mg/ml and 10mg/ml) of the crude extract did not show any haemolysis zones around wells of the blood agar plates (Table , 5).

Table (5): Blood cytotoxicity of crude fungal extract of *P. spinulosum* strain C3-8

Haemolysis Zones (mm)			
1mg/ml	3mg/ml	6mg/ml	10mg/ml
0	0	0	0
0	0	0	0

Some optimum conditions of crude fungal extract

The crude extract was stable in range of pH values (2-9) and three degrees (4 , 37 and 55°C) of temperature and it revealed bioactivity against *C. albicans*, while it was inactive in presence of pepsin and trypsin (Table, 6).

Table (6): Some optimum conditions of the crude fungal extract against *C.albicans* .

Inhibitory Zones (mm)		
Temperature (4, 37 and 55 °C)	pH (2 and 9)	Enzymes (pepsin & trypsin)
+	+	–

+ : presence of IZ, - : absence.

RP-HPLC analysis of fungal crude extract

Different peaks of RP-HPLC analysis at different times were appeared from crude extract of *P. spinulosum* strain C3-8, in comparison with pure nystatin and ketoconazole (Figures:11, 12,13).

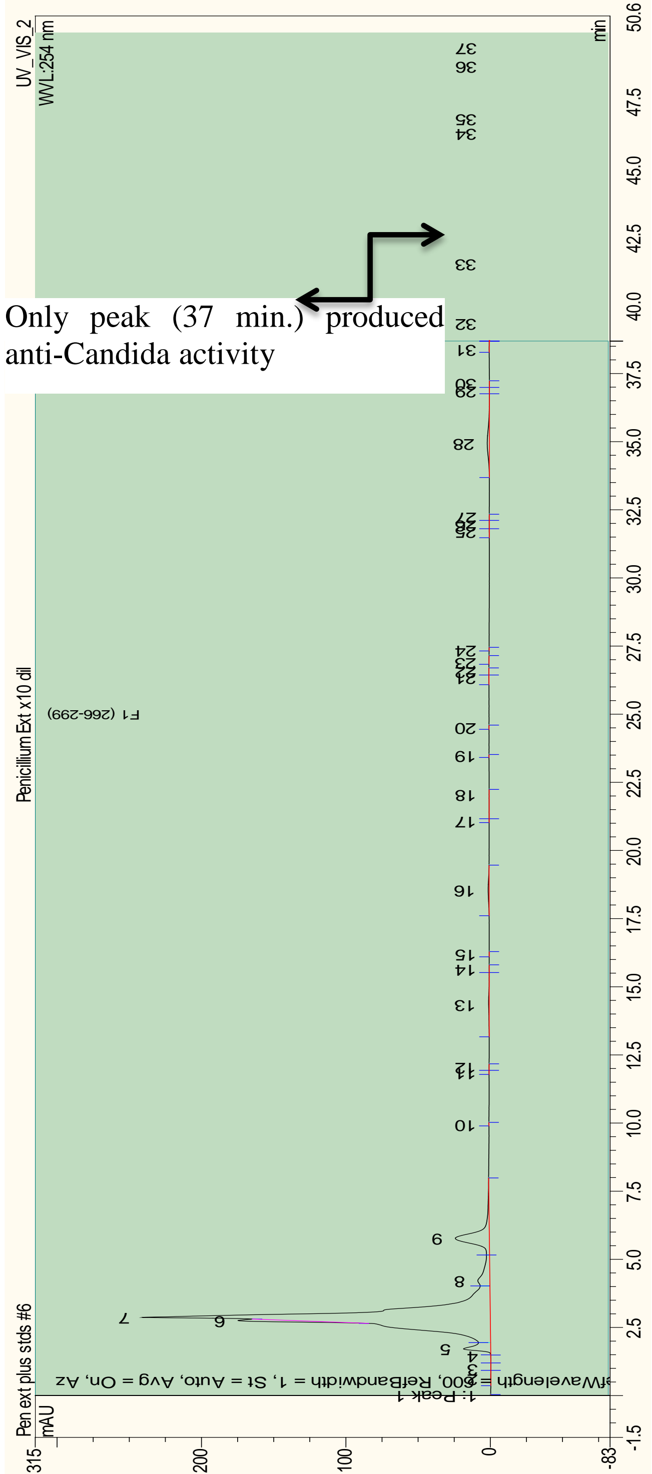


Fig.(11): RP-HPLC analysis of *P. spinulosum extract.*

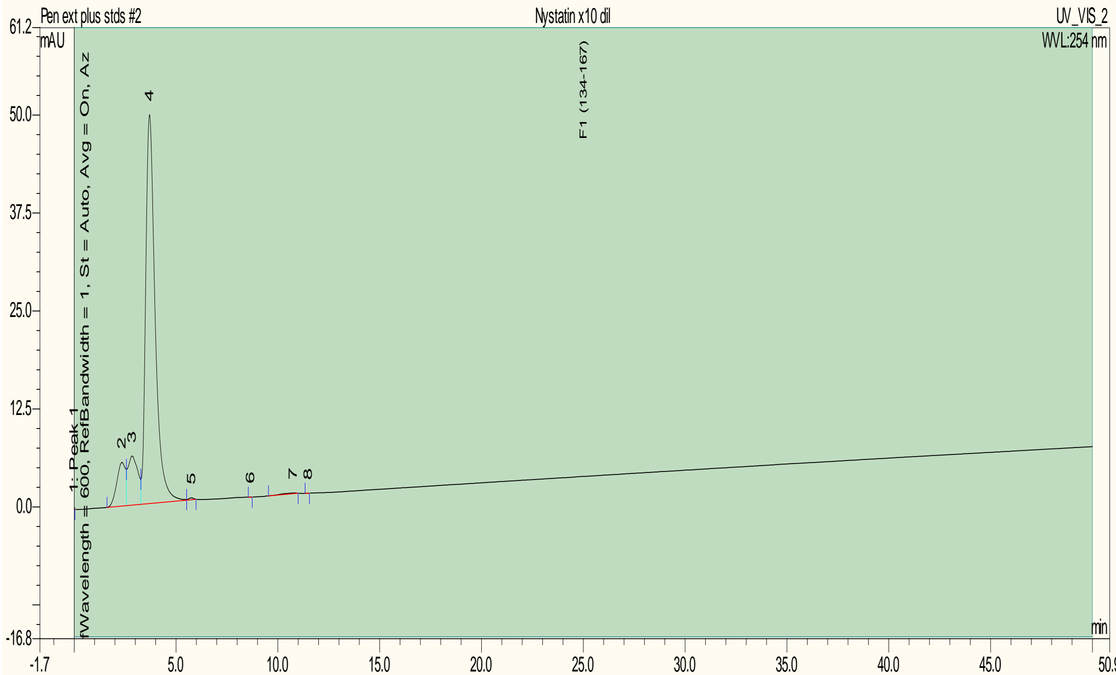


Fig.(12):RP-HPLC analysis of pure nystatin

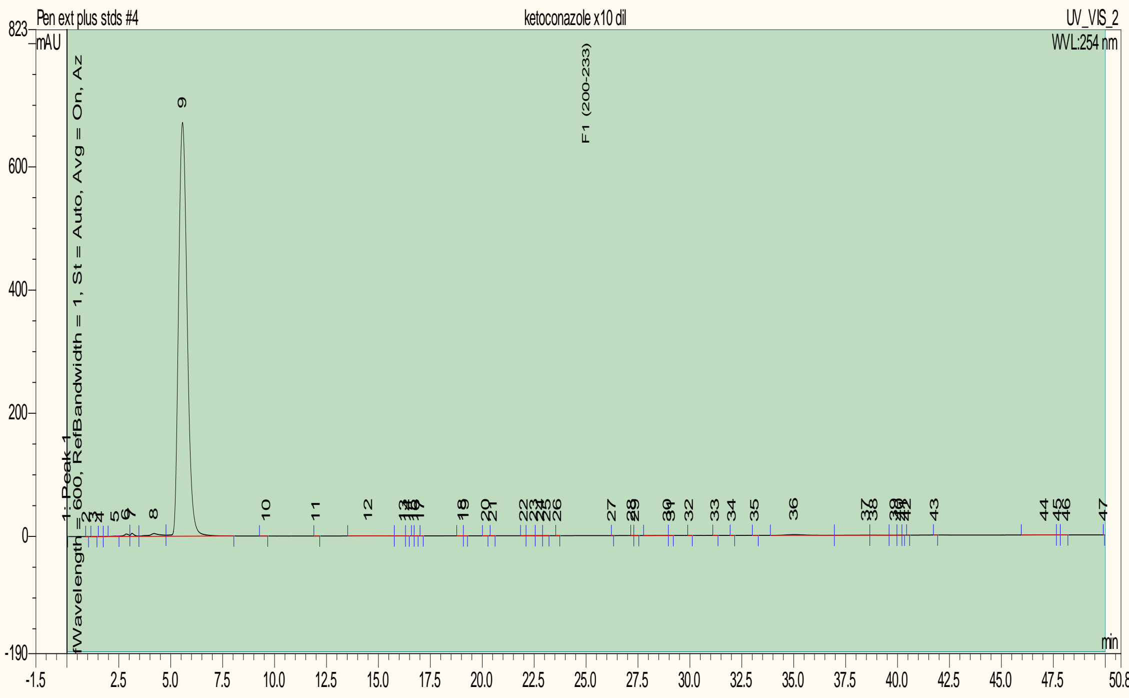


Fig.(13): RP-HPLC analysis of pure ketoconazole.

Bioactivity of RP-HPLC fraction against *C.albicans*.

RP-HPLC fractions of *P.spinulosum* strain C3-8 were tested against *C.albicans* and only one RP-HPLC fraction (37 minute.) produced IZ (25 mm) against *C. albicans* (Figure,14).

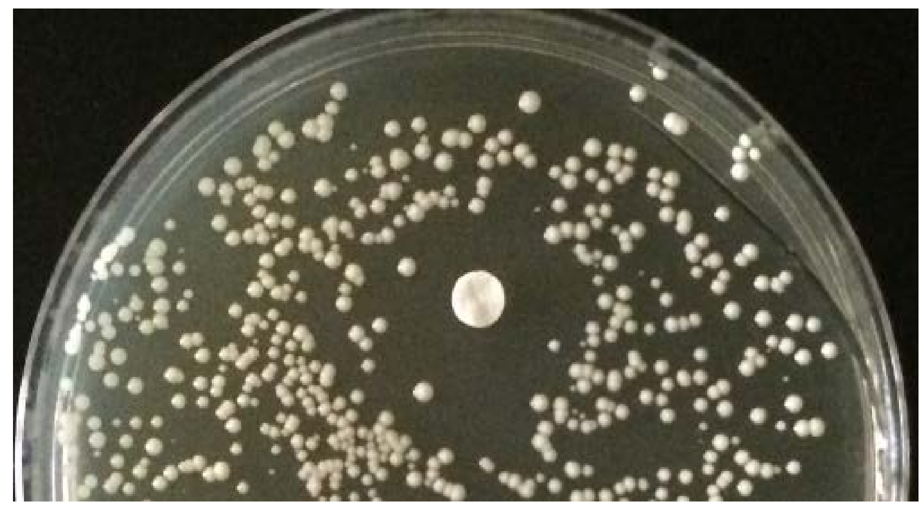
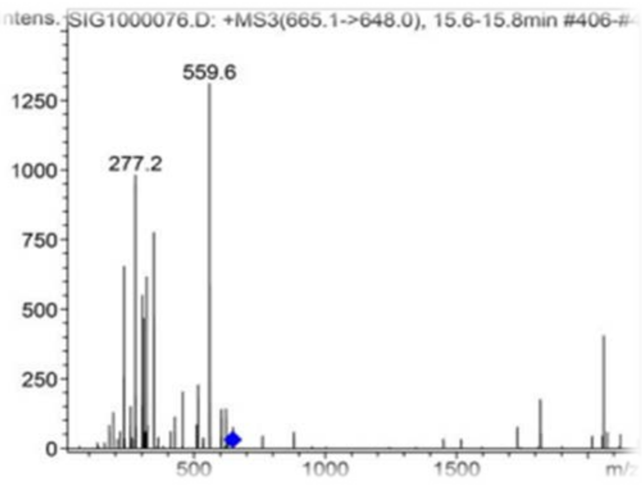


Fig.(14): Bioactive RP- HPLC-fraction against *C. albicans*

LC-MS spectrometry

The fraction which produced bioactivity against *C. albicans* was brought into LC-MS analysis to determine its molecular weight. In addition that, fractions of both pure nystatin ketoconazole were analyzed for comparison. The fractions revealed different peaks possessing 665.1 *m/z* (Figures: 15 , 16, 17).



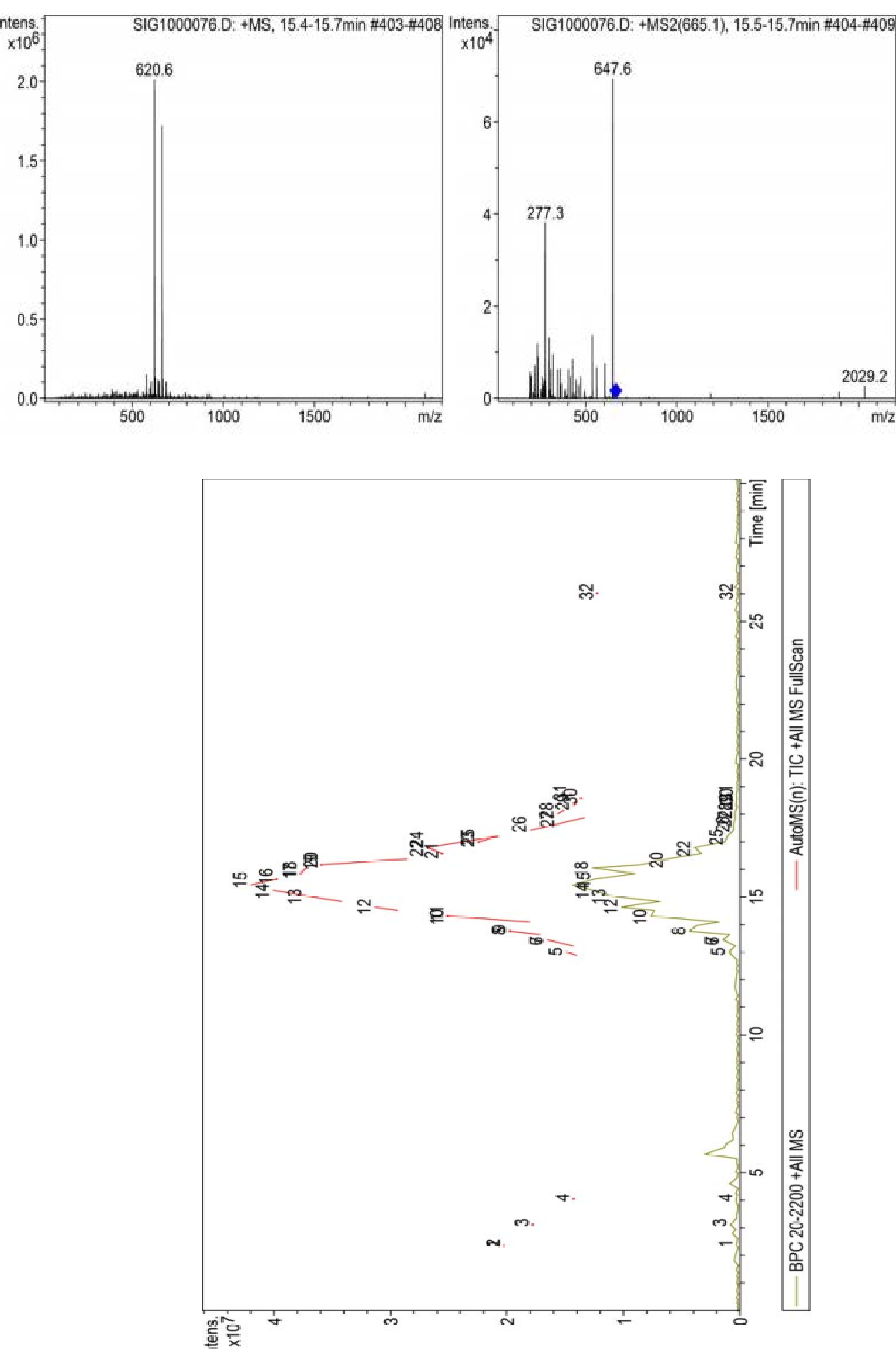
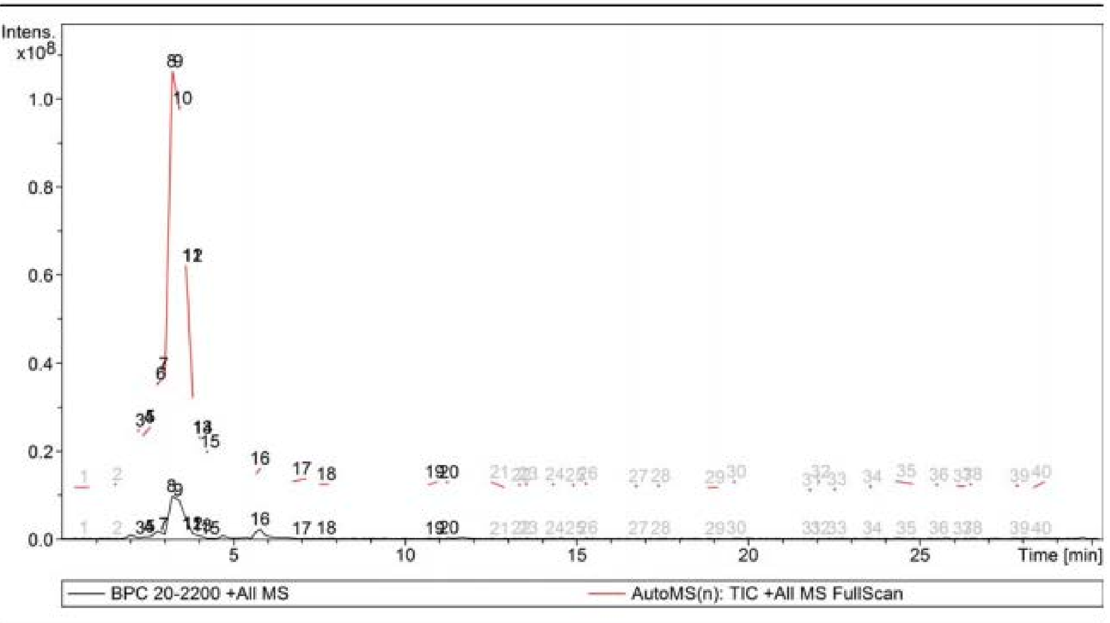


Fig.(15): LC-MS analysis of RP-HPLC bioactive fraction of *Penicillium spinulosum* strain C3-8.



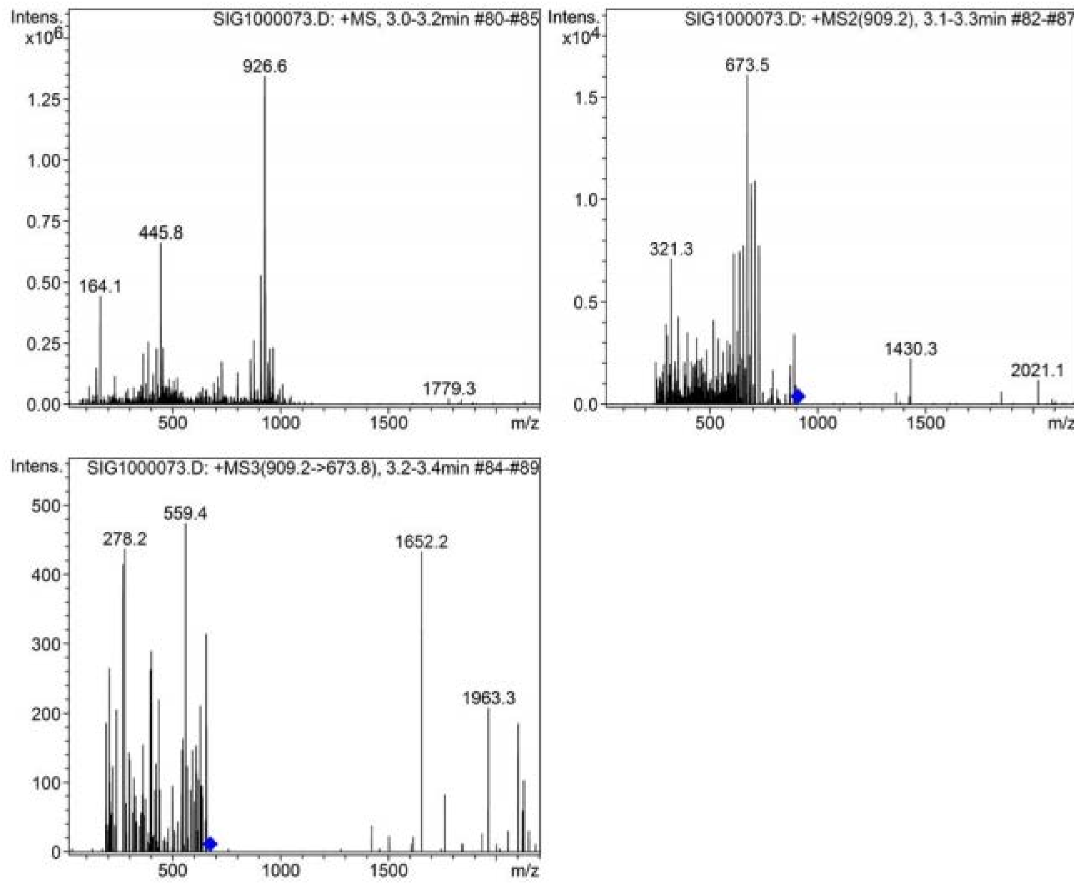


Fig.(16): LC- MS analysis of RP- HPLC pure nystatin fraction

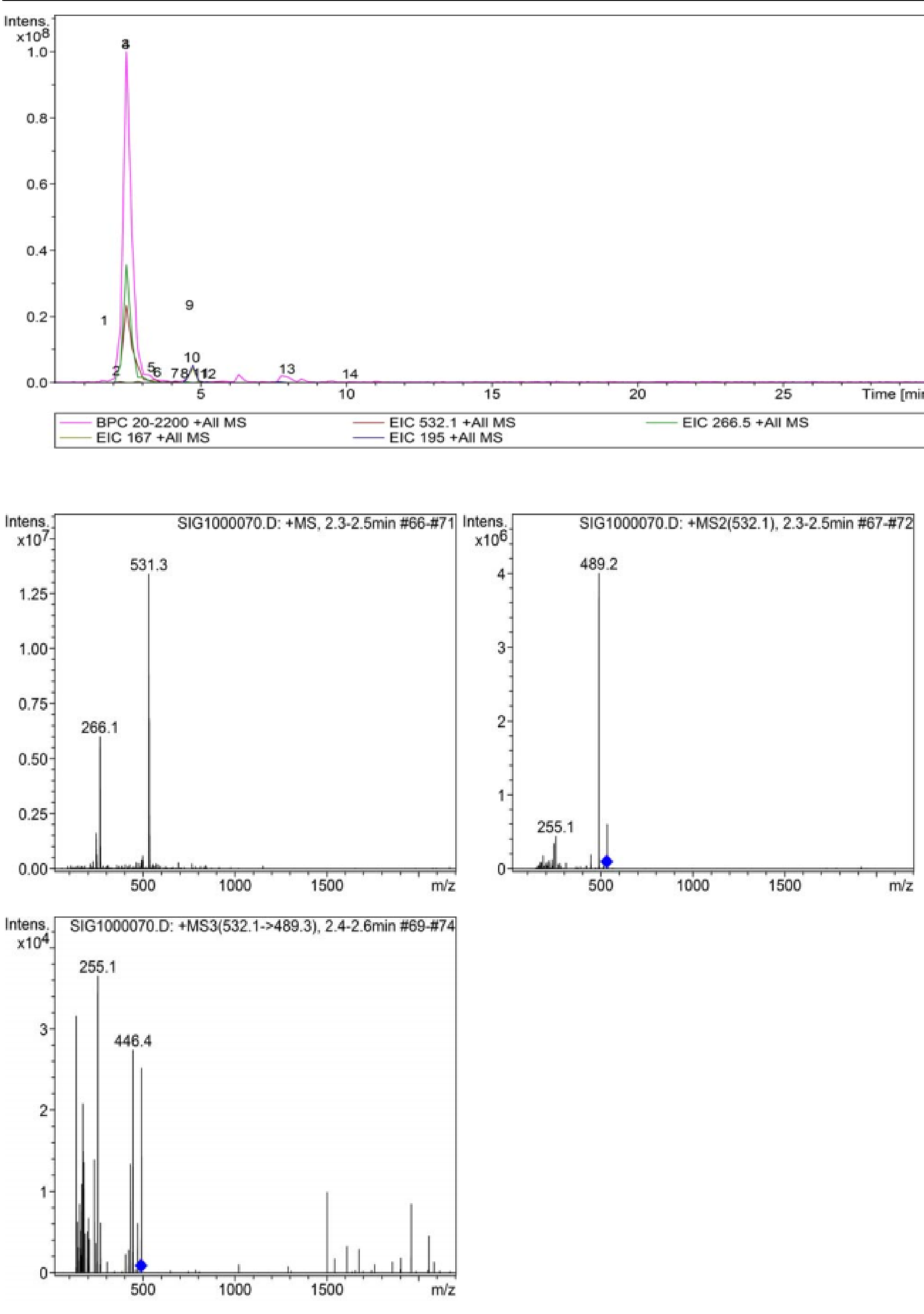


Fig.(17): LC-MS analysis of RP-HPLC pure ketoconazole fraction

Discusion

Screening and extraction of anti-Candida agent

Antifungal screening from fungi depended on the principles of antagonism in which one microorganism is inhibited or killed by other microorganism due to one is producer for bioactive secondary metabolites. The antagonism is performed in laboratory by culture of fungal colony disc in a plate containing suitable medium, inoculated with target microorganism and incubated at appropriate temperature degree for certain period, it will produce inhibitory zone around the target microorganism as preliminary screening of antifungal agent (9). The current study applied antagonism phenomenon by using colony disc from *Penicillium spinulosum* strain C3-8 against *C.albicans* and *C.glabrata* where produced inhibitory zone against them.

Fermentation process is defined as a growth of microorganisms including fungi in fermenting medium to get products including secondary metabolites such as anti-Candida agents. These metabolites comprise on two types: extracellular secondary metabolites are excreted outside of fungal cells (mycelia) while intracellular secondary metabolites are produced inside of the cells (2), (3). For this reason, this study showed that soaked filter paper discs in the filtrate of *P. spinulosum* strain C3-8 did not produce an inhibitory zone against *C. albicans* and indicated that the anti-Candida agent was excreted as intracellular metabolite.

Characterization of fungal extracts

High performance liquid chromatography (HPLC) is an analytical chemical technique used to separate a mixture into different components. Nevertheless, reverse phase high performance liquid chromatography (RP-HPLC) is one type of HPLC often used to analyze pharmaceutical products. This type contains a non-polar stationary phase (column) and moderately aqueous polar mobile phase. Sample mixture passes with polar mobile phase via column which is non polar, leads to retention time for molecules which have less polarity, while polar molecules are more readily elution for analyzed (10).Based on the principles of RP-HPLC, the present study showed several peaks of analyzed fungal crude extract with different retention times where HPLC-fractions were obtained (Figures: 11, 12, 13).

Another technique of chromatography was used to confirm identification of HPLC-fractions which is liquid chromatography- mass spectrometry (LC-MS) to measure mass-to-charge

ratio (m/z) of each molecules. LC-MS was done because highly degree identification of the compounds in the crude complex was given , in addition to HPLC technique can not give the confirmed identification (7).

LC-MS technique is a qualitative and a quantitative analysis to identify unknown compounds by determining molecular weights as well as knowing structure of the compound via their fragments (6). Based on the fraction approach from RP-HPLC which exhibited bioactivities against *C.albicans* , the bioactive fraction was analyzed by LC-MS and revealed several peaks with different masses for the fraction (Figures: 15 , 16, 17). Biologically, minimum fungicidal concentration (MFC) of the crude extract is defined as a dose in which there is no growth from tested microorganism on suitable medium. Serial macrodilution broth is a procedure to test the MFC of antibiotic including anti-Candida extract. Based on (11), the current study indicated that 100µg/ml as MFC of the fungal crude extract killed *C. albicans* and *C. glabrata* in SDB.

Cytotoxicity is a primary test to discriminate between toxin and antifungal because toxin is lethal in small dose while the antifungal is toxic in high doses. This assay was done in plates of blood agar with RBCs to produce haemolytic zone. Our fungal extract was tested against human blood red cells (RBCs).This study showed that all concentrations (1mg/ml,3mg/ml ,6mg/ml and 10mg/ml) of the fungal crude extract did not produce any haemolytic zone around RBCs. The absence of the zone may be attributed to the fungal extract was nontoxic agent. This result was agreed with other studies, *Penicillium spinulosum* did not produce mycotoxin in potato dextrose broth (12),(13).

Optimum conditions of fungal extracts:

The effects of pH, temperature and enzymes on antifungal activity are important factors in applied pharmacology including antifungal agents aspect to demonstrate the stability under these factors. For each antifungal, there is a range of pH for instance, ketoconazole that requires acidic pH (14).This study showed that the crude extract of *P. spinulosum* strain C3-8 was stable in range values (2-9) of pH as well as three degree (4-55°C) against *C. albicans* but it was inactive due to presence of pepsin and trypsin. The inactivity may be caused by using large amounts of those enzymes or due to fungal crude extract contain protein compounds in their structures were destroyed by pepsin and trypsin because they are two types of proteolytic enzymes can

destroy proteins lead to form peptides and free amino acids making the extract to be inactive in its mechanisms (15).

Conclusions:

There are fungi have ability to produce bioactive compounds are promising to be implemented as anti-Candida agents and can be used in pharmaceutical application after further investigations.

Acknowledgement

This paper is a part of PhD thesis was carried out in The Mycological Research Laboratory of Biology Department, College of Education for Pure Sciences, University of Basrah, and Laboratories of School of Biological Sciences, University of Reading (UK) in Laboratory of Professor Dr. Simon C. Andrews.

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Evaluation of Calprotectin level and Alkaline phosphatase among certain group of Hepatitis C patients treated with Interferon – Alpha in Babylon province

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Abstract: To evaluation of the interferon treatment among a group of patients with Hepatitis C virus and monitor the effect of such infection on calprotectin and alkaline phosphates in association with immunological therapy (Interferon), this idea might be refer to the state of gastrointestinal tract , neutrophil function as well as liver tissues of such patients, for this reasons 35 infected patients with hepatitis C infection enrolled in the present study as treated group with interferon as well as 17 apparently healthy person as a control group in Merjan medical city /Babylon Gastrointestinal tract diseases center(GIT) at the period between January– December 2014. For all patients and control samples hepatitis viral screen, Calprotectin were done by ELISA technique , viral load by Real Time - PCR technique as well as alkaline Phosphatase were done by using Cobas

C-111. The result of calprotectin (CP) concentration was showed that the young age group have lower level than old age group, while the level of alkaline phosphatase in patients <50 years higher than level in patients >50 years in reverse to calprotectin level at both age groups. Lower reduction of CP level at treatment period after 12 week of treatment while in post treatment was show that higher level of other patients groups . No significant difference (P>0.05) of both result in comparison between male and female results and the lowest level at viral load at 50 -100 million (iu/ml) while the upper level was noted in undetectable group as same as of RNA copy of hepatitis C virus.

Key word : -*Calprotectin alkaline phosphatase Hepatitis C virus ,interferone*

Introduction:

Calprotectin is a 36kDa calcium and zinc binding protein expressed by the gene S100 calcium-binding protein A8, S100A8. It accounts for 30 to 40% of neutrophils' cytosol. In vitro studies show it has bacteriostatic and fungistatic properties. Calprotectin is a very abundant neutrophil protein with many characteristics. It constitutes more than 60% of total proteins in the cytosol of neutrophil granulocytes, each neutrophil contains 25 picograms of calprotectin compared to about 30 picograms hemoglobin per erythrocyte (Tibble , 2000) . The main diseases that cause an increased excretion of faecal calprotectin are infectious colitis, Crohn's disease, Ulcerative colitis, and Neoplasms (cancer).

Increased concentrations will be found in response to infections and inflammation (Tibble , 2012). The Calprotectin syndrome presents with a clinical picture suggestive of zinc deficiency: children / young adults show growth retardation, bone marrow depression, immune deficiency, hepatosplenomegaly, arthritis and vasculitis; the concentration of calprotectin in their plasma is 2000 to 12000 times the normal. These huge amounts of calprotectin bind so much zinc that a functional zinc deficiency is created.Interferon-α has.immunomodulatory properties that may impact immune pathways that control and may even induce immune-mediated diseases. Patients with autoimmune diseases have been excluded from hepatitis c virus (HCV)clinical trials, and in clinical practice, these patients are not usually treated for HCV due to the high likelihood of inducing a flare of the immune-mediated condition (Jill *et al.,* 2010). Increasing evidence suggests the implication of CP in the diagnosis, follow up, assessment of relapses, and response to treatment in pediatric pathological conditions, such as enterocolitis, celiac disease, intestinal cystic fibrosis, glomerulonephritis, IgA nephropathy, malaria, viral infection, and cancer. Heritable disorder of CP metabolism was observed in an infant with hypercalprotectinemia /hyperzincemia and systemic inflammation. There is also a reported case of microcytic anemia and inflammation caused by an inborn error of zinc metabolism due to a dysregulation of CP metabolism (Henderson *et al.*, 2012).CP may play a role as an innate amplifier of inflammation in cancer development and tumor spreading. High levels of CP are expressed by bone marrow-infiltrating metastatic neuroblastoma cells (Joishy *et al.,* 2009). Specific blocking of pro-inflammatory mediators such as CP achieves

improvement and remission in infected children, an understanding of the biomarkers and pathological mechanisms during this early stage would possibly determine new therapeutic strategies and ensure optimal therapy for individual patients (George *et al.,* 2013).

The heterodimer calprotectin (S100A8/S100A9, mrp8/14) is a Toll-like receptor-4 ligand found in neutrophils and monocytes and is elevated in inflammatory conditions. Calprotectin levels in patients with limited systemic disease increased following treatment withdrawal and were significantly elevated in patients who relapsed compared with those who did not (Ruth *et al.,* 2013). Calprotectin (MRP8/14, S100A8/9), an example of a damage-associated molecular pattern and a marker of activation of innate immunity, has recently been demonstrated to have a role in autoimmunity and in induction of autoimmune T cells, exerting its action through TLR4 (Loser *et al.,* 2010).

Larger prospective trials of patients with generalized disease and long-term follow-up should enable us to further define its prognostic value in predicting disease relapse, with further investigations required to demonstrate whether calprotectin could be considered to have a role in vasculitis as a potential biomarker (Hrukova *et al.,* 2009).

Materials and Methods:

Thirty five infected patients with hepatitis C infection enrolled in this study as treated group with interferon therapy as well as 17 apparently healthy person as a control group. The viral load of hepatitis C - RNA copies was done Real time PCR(Bioneer) in GIT center of Merjan Medical City in period between January - December 2014. Viral detection and Calprotectin was done by ELISA technique according to manufactured instruction of Cuasabio compay . Alkaline phosphatse level was done by Cobac C 111 .

Results and Discussion:

The result of CP concentration was showed that the young age group has lower level than old age group and there is highly significant differences at LSD value 6.34 of all age group patients as well as after comparison with control group as mentioned in Figure (1). This result might be refer to the severity of viral infection in associated with interferon treatment give low neutrophil activity and low of proinflammatory (CP)cytokine synthesis . This result related to statement that said of calprotectin is antimicrobial and has induced

apoptosis in all cell types (human, animal, normal, malignant) tested(George *et al.,* 2013).. These effects can be reversed by addition of zinc; lack of calprotectin is not compatible with life (George *et al.,* 2013). This result agree with the conclusion ,that the prognostic importance of calprotectin in cirrhosis is confirmed and demonstrated as specific for certain liver disease. Plasma calprotectin concentrations were low in viral liver disease compared to patients with non-viral liver disease. Low calprotectin levels are indicated in viral liver disease, and an association between high ascites calprotectin levels and malignant ascites was observed (Homann *et al.,* 2003).

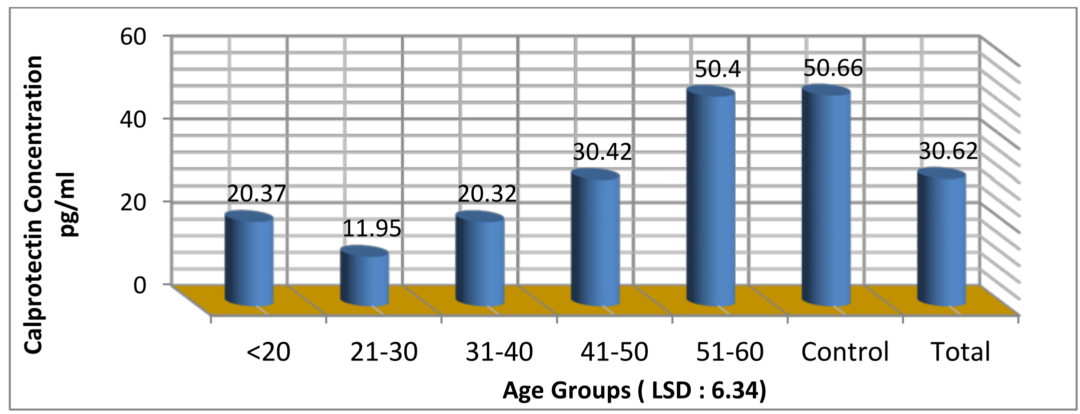

Figure (1) The calprotectin concentration (pg/ml) in different age groups in comparison with control.

In relation to calprotectin level the result of alkaline phosphatase (ALP) level showed that highly significant at all patients groups in compare with control group at LSD value 14.6 as in Figure (2) and show that the level in patients <50 years higher than level in patients >50 years in reverse to calprotectin level at both age groups . This result might be show that a directional relationship between alkaine phosphase and viral activtiy and indirect relation with calprotectin concentration at the same patients groups.

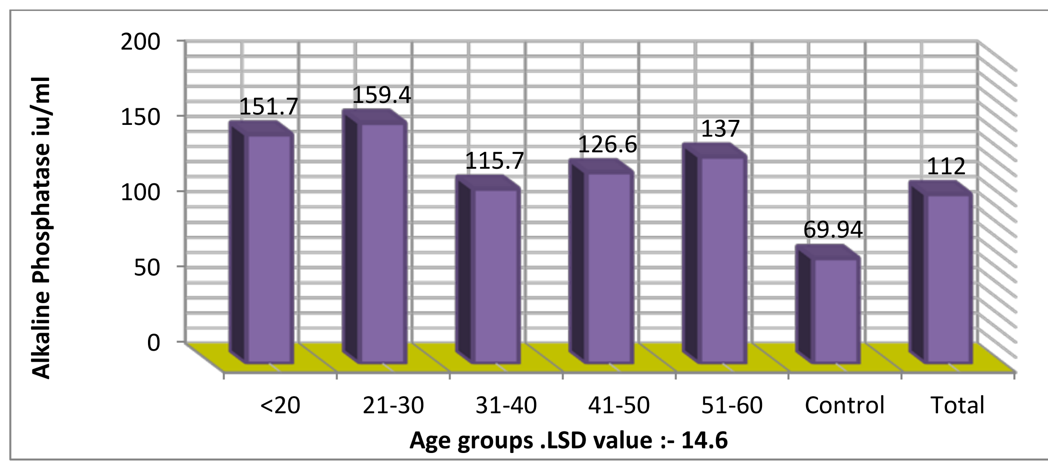

Figure (2): Alkaline phosphatase concentration in different age groups in comparison with control

The result of patients in association with state of treatment with interferon therapy as in Table (1) showed that highly significant decreased in all patients groups at LSD value 5.63 compare with control , lower reduction of level at treatment period after 12 week of treatment while in post treatment was show that higher level of-CP in patients groups , the eradication of virus activity at the end of treatment course give higher calprotectin concentration although it reduced in comparison with control group , this result might be show that reduced of disease activity and the immune response act normaly so the neutrophil activity is normal and the calprotectin level increased.

Table (1) Calprotectin concentration (pg/ml) according to state of patients of treatment in comparison with control.

Parameter	State of treatment	No of patients	Mean ± SD	LSD value
Calprotectin pg/ml	Pre Treatment	9	22.74 ± 2.92**	5.63
	After 12 Week	3	13.81 ± 1.37**	
	After 24 Week	9	21.35 ± 3.64**	
	After 48 Week	8	20.17 ± 3.92**	
	Post treatment	6	40.81 ± 6.96*	
	Control	17	50.66 ± 11.42*	
	Total	52	30.62 ± 3.67**	

(*) Significant Difference

( ) Highly significant Difference.**

The result of ALP concentration in state of treatment with interferon therapy shows that highly significant increased at all patients groups in comparison with control at LSD value 34.6 as in table (2) the lower level show after 12 week of treatment while higher level at pretreatment state of patients , this result might be show than slightly reduced in ALP level in association with treatment protocol when compareson of different result among patients groups.

Table (2) Alkaline phosphatase concentration according to state of patient treatment in comparison with control .

Parameters	State of patients	No.	Mean ± SD	LSD value
Alkaline Ph. Iu/l	Pre Treatment	9	145.0 ± 14.72	34.6
	After 12 Week	3	110.3 ± 5.95	
	After 24 Week	9	147.0 ± 22.17	
	After 48 Week	8	117.8 ± 5.12	
	Post treatment	6	122.3 ± 4.42	
	Control	17	69.94 ± 8.33	
	Total	52	112.0 ± 4.29	

This result relativily agreed with other studies such as Gerd *et al.,* (2010) who said that to investigate relapse predictors in chronic hepatitis C (CHC) patients with end-of-treatment response (ETR), after interferon-α (IFN-α) and ribavirin treatment. Higher levels of ALP prior to, during and after therapy seem to be associated with a higher risk of relapse in CHC patients . And the study of Lee *et al.,* (2007) mentioned thtat, since immune response and inflammation play key-roles in the *elimination of HCV, the higher pre*-treatment ALP levels in patients with Severe Viral response (SVR) may possibly reflect a higher degree of inflammation with a more sustained response to therapy. The distribution of studied parameters among male and female show that the result of both CP and ALP levels are highly significant difference ($P<0.01$) in compareson with controls in regarding to decreased of CP level while increased in ALP concentration. And no significat difference ($P>0.05$) of both result in compareson between male and female results. As shown in table (3).

Table (3) Calprotectin and alkaline phosphatase concentration according to sex groups in comparison with concentration.

Parameters	Sex	No.	Mean ± SD	P. Value of T - test
Calprotectin pg/l	Male	19	30.33 ± 1.59	0.009 (H. S.)
	Female	16	22.89 ± 2.14	0.004 (H.S)
	Control	17	50.66 ± 11.42*	Male and female 0.078 (N.S)
Alkaline Ph.	Male	19	139.05 ± 4.42	0.000 (H.S.)
	Female	16	124.62 ± 5.18	0.000 (H.S)
	Control	17	69.94 ± 8.33	Male and Female 0.067 (N.S.)

The result of both CP and ALP of patients shows highly significsnt (P<0.01) differences in compareson with control in regarding to decreased in CP and increased in ALP concentration as in result of Table (4) , this result might be refer to indirect proportional between ALP and CP among such patients (hepatitis) in association with interferon treatment protocol . This result disageement with the other study stated that Calprotectin is released from activated leukocytes leading to increased concentrations in plasma, serum, spinal fluid, synovial fluid, urine, saliva or stools is increased during bacterial infections or inflammation in relevant organs. (Tibble , 2012).

Table (4) Calprotectin and alkaline phosphatase concentration in comparison with control concentration.

Parameter	Variable	No.	Mean ± SD	P. Value of T - test
Calprotectin pg/ml	Test	35	30.62 ± 3.67	0.005 (H. S.)
	Control	17	50.66 ± 11.42*	
Alkaline Ph.	Test	35	132.45 ± 17.59	0.000 (H.S.)
	Control	17	69.94 ± 8.33	

The result in Table (5) show that the reverse correlation between CP and ALP among studied patients it mean that when increased of liver tissue damge and alkaline phosphatase lead to impaired in immune mediated mechanism of neutrophil and reduced the CP level on such patients without differentiate between treated and non treated patients with interferon.

Table (5) Correlation between calprotectin and alkaline phosphatase concentration among all patients group.

Parameter		Calprotectin	Alkaline Ph.
Calprotectin	Pearson Correlation	1	-0.542 **
	Sig. (2 – tailled)	.	0.001
	N.	35	35
Alkaline Ph.	Pearson Correlation	-0.542**	1
	Sig. (2 – tailled)	0.001	.
	N.	35	35

(**) Highly significant correlation (P<0.01).

(-) Reverse correlation.

This result disagree with the study of Felix *et al.*, (2011) who stated that the correlation analysis between elevated calprotectin concentrations and aetiology of cirrhosis (alcohol-related, HCV-related, HBV-related, autoimmune ehepatitis-related, cryptogenic cirrhosis) was not significant values.

The result of hepatitis C viral load (iu/ml) measured by using RT – PCR, the table (6) shows that the CP level according to viral load, the lowest level at viral load at 50 -100 million compare with control at LSD value 8.10 .

Table (6) Calprotectin concentration pg/ml according to hepatitis viral load in comparison with control

Viral load (iu/ml)	No.	CP concentration Mean ± SD	LSD value
>100 million	2	25.44 ± 0.28 *	8.10
50 -100 million	2	18.68 ± 0.11*	
1 – 50 million	7	21.91 ± 0.94*	
< 1 milion	2	32.80 ± 0.509*	
Undetactable	22	34.30 ± 1.72*	
Control	17	50.66 ± 11.42	
Total	52	30.62 ± 3.67	

The result stated in table (7) shows that highly significant increased in ALP of patients group in comparison with control , the higher levels are belong to viral load of >100 million and 1 -50 million while the lower level at 50 -100 million with LSD value is 20.14 .

Table (7) Alkaline phosphatase concentration according to hepatitis viral load (iu/ml) in comparison with control

Parameters	Viral load (iu/ml)	No.	Mean ± SD	LSD value
Alkaline Ph.	>100 million	2	143 .7 ± 9.85	20.14
	50 -100 million	2	125.4 ± 14.71	
	1 – 50 million	7	145.2 ± 16.34	
	< 1 million	2	135.6 ± 0.00	
	Undetactable	22	127.0 ± 4.57	
	Control	17	69.94 ± 8.33	
	Total	52	112.0 ± 4.29	

The result of RNA copy of virus is directly proportional with viral load with same result and percentage of CP and ALP as mentioned in Tables (8 and 9).

Table (8) Calprotectin concentration according to hepatitis RNA copy in comparison with control

Parameters	RNA Copies	No.	Mean ± SD	LSD value
Calprotectin pg/ml	>400 Million	2	25.44 ± 0.28 *	8.10
	100 -400	2	18.68 ± 0.11*	
	4 - 200	7	21.91 ± 0.94*	
	< 4 Million	2	32.80 ± 0.509	
	Less Than 200	22	34.30 ± 1.72	
	Control	17	50.66 ± 11.42*	
	Total	52	30.62 ± 3.67	

Table (9) Alkaline phosphatase concentration according to hepatitis RNA copy in comparison with control

Parameters	RNA Copies	No.	Mean ± SD	LSD value
Alkaline Ph.	>400 Million	2	143 .7 ± 9.85	20.14
	100 -400	2	125.4 ± 14.71	
	4 - 200	7	145.2 ± 16.34?	
	< 4 Million	2	135.6 ± 0.00	
	Less Than 200	22	127.0 ± 4.57	
	Control	17	69.94 ± 8.33	
	Total	52	112.0 ± 4.29	

The mean viral load and RNA copy of hepatitis C virus are 40.2 million (iu/ml) and 151.24 million (iu/ml) respectively and the higher percentage at undetactable group and less than 200 copy as Mentioned in Tables (10 and 11), this result might be refer to the accuarcy and senstivity of technique or good response of patient after treatment with interferon therapy.

Table (10) The percentage and mean viral load concentration among all patients group

Viral load (iu/ml)	Frequency	Percent	Mean viral load iu/ml
>100 million	2	5.7	40.2 million
50 -100 million	2	5.7	
1 – 50 million	7	20.0	
< 1 milion	2	5.7	
Undetactable	22	62.9	
Total	35	100	

Table (11) The percentage and mean of RNA copies among all patients group

RNA Copies	RNA Copies	Frequency	Percent	Mean RNA copies
Valid	>400 Million	2	5.7	151.24 million
	100 -400 million	2	5.7	
	4 - 200 million	7	20.0	
	< 4 Million	2	5.7	
	Less Than 200	22	62.9	
	Total	35	100	

In conclusion, r levels of CP and higher level of ALP as well as viral load of Hepatitis C virus prior to, during and after therapy with IFN-α underwent changesin their concentration . Further studies are required to clarify whether our observation could help to identify those patients who would benefit from extended therapy or replaced the treatment protocol for such patients as well as which patients have malignant risk or non .

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Epstein Barr Virus- Encoded Small Untranslated RNAs (EBERs)in Relation to Translational Expression of P27 Tumor Suppressor Gene in Patients with Bladder Tumors

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Abstract: This study was designed as retrospective research . A total number of (60) formalin-fixed, paraffin embedded bladder tissues were included. Among these cases fifty (50) tissue blocks with bladder neoplasia were furtherly divided into 2 groups:

1. Thirty (30) tissue blocks with invasive bladder cell carcinoma .
2. Twenty (20) tissue blocks from cases with benign bladder tumor.

In addition, ten (10) bladder tissue blocks without any significant pathological changes (apparently healthy) ,were included as a control group for this study. The age of these individuals (patients and control groups) were ranged between 18 and 72 years. Histopathological sections were made for these bladder biopsies and stained by hematoxylin and eosin for final definitive diagnosis. For detection of EBV,ultra sensitive version of In Situ Hybridization(ISH) method was used while the translational expression of P27 gene was immunohistochemically demonstrated. Positive EBV-EBERs -ISH reactions in malignant bladder tissues was detected in 13 out of 30 (43.3%) and in 20% (3 out of 20) of the benignbladder tissues. No positive – ISH reaction for EBV-EBERs was observed in the control bladder tissues.Statistically, the differences between the percentages of positive EBV –EBERs -ISH reactions in bladder cancer group with either benign bladder tumors or control groups are highly significant (P value = < 0.0001). Positive immunohistochemical reactions for P27 protein were observed in 16 cases (53.3 %) of bladder carcinoma and in7 cases (35%) benign bladder tumors while the control groups showed negative P27 immunostaining reactions.

Key word: EBERS-EBV, P27 Bladder Tumor, ISH, IHC

Introduction

Urinary bladder cancer is the fifth most common cancer in the western world and is responsible for about 3% of all cancer-related deaths. Approximately 55,000 new patients are diagnosed with bladder cancer annually in the United States, and 15,000 of them die of the disease each year (1).

Transitional cell (urothelial) carcinoma (TCC) is the most common urinary bladder neoplasm in the western world. Current pathogenetic concepts postulate that common urothelial neoplasms of the bladder arise via two distinct but somewhat overlapping pathways: papillary and nonpapillary (2).

Approximately 80% of urothelial tumors of the bladder are superficially growing exophytic papillary lesions that may recur but usually do not invade and metastasize. They originate from hyperplastic urothelial changes. The remaining 20% of urothelial tumors are highly aggressive, solid, nonpapillary carcinomas with a strong propensity to invade an metastasize (2,3).

Epstein Barr Virus is one of the eight known human herpesviruses. Its genome is a linear, double stranded DNA, about 170kb in length. Latently infected cells contain the genome as a circular plasmid in the nucleus. The terminal repeat (TR) sequences are present at both ends of the linear form of the genome and these repeats mediate the circularization in the infected cell, An unusually large tandemly repeated DNA sequence in the genome of EBV is known as the major internal repeat (IR1). The IR1 site divides the EBV genome into long and short unique sequences (UL and US). These sequences are filled with closely packed genes (4).

In addition, the EBV genome contains a viral cytokine, vIL-10, that was pirated from the host genome. This viral cytokine can prevent macrophages and monocytes from activating T-cells are required for EBV-dependent transformation of B-cell (5,6).

The small untranslated RNAs EBER-1 and -2 are accumulated at high levels during all forms of latency and regulate apoptosis through different mechanisms. EBER-1 interacts with the interferon-inducible protein kinase R (PKR), and inhibits its activation by double-stranded RNAs, protecting infected cells from IFN-induced apoptosis (7).

Epstein Barr virus encoded small RNA-2 has however a more prominent role in EBV-mediated growth transformation, as viruses lacking the coding sequence for this RNA were significantly less efficient in generating lymphoplastoid cell lines (LCLs) in vitro, and the cell lines generated

proliferated at much lower rates, due to reduced autochrine IL-6 production (8). These observations have been extended to epithelial cells lines, where EBERs induce the expression of growth factors that promote cell survival (9).In addition ,a recent study has found that EBV infection may have related to the initial occurrence or further development bladder carcinoma. In analysis of EBV and bladder cancer cases Grinstein et al.(10) observed that 37% (7/19) of bladder cancer cases evaluated displayed EBV by immunohistochemistry and PCR.

As for bladder cancer, (11), detected the EBV genome in 34% of whole bulk tissue samples with polymerase chain reaction (PCR), without specifying the infected cell population. Subsequently, (12) demonstrated EBV-encoded RNA within both carcinoma cells and infiltrating lymphocytes in 21%, only infiltrating lymphocytes in 7%, and only carcinoma cells in 3% of bladder cancers obtained from Taiwanese patients. To date, it remains unclear whether EBV has any significant role for pathogenesis in bladder cancers. In some bladder cancer cell lines, for example, the combination of cyclin E over expression and p53 loss induces centrosome amplification and chromosome instability (13). More generally, in tumor cells, cyclin E can be deregulated by a number of mechanisms, including gene amplification, down regulation of p27 or down regulation of the F-box protein Fbw7 (also called hCDC4), which tags phosphorylated cyclin E for proteosomal degradation (14).

Mutations in the gene encoding hCDC4 have been found in breast, ovarian, endometrial (15) and colorectal cancers (16) and are associated with elevated levels of cyclin E protein. More recently, the over expression of miR-27a in pediatric acute lymphoblastic leukemia (ALL) has been shown to suppress Fbw7 expression, leading to improper cell cycle progression and DNA replication stress, consistent with dysregulation of cyclin E expression (17) .They have found that deregulation of cyclin E can also occur through post-translational processing of the full-length cyclin E by an elastase-like protease to generate low molecular weight (LMW) isoforms.(18,19). Expression of these LMW isoforms in tumor cells leads to increased genomic instability(18) due to premature activation of CDC25C14 and shortening of the length of mitosis from nuclear envelope breakdown to prometaphase (20) .

Cyclin E levels in tumor tissue associated strongly with disease-specific and overall survival in patients with stage I, II and III disease but had no impact on outcome in patients with stage IV disease. cyclin E expression was found to be deregulated in ovarian cancer (21) as well as melanomas (22).

Invasive transitional-cell carcinomas (TCCs) have greater expression of cyclin E mRNA than do superficial TCCs or normal bladder cells (23). (24) showed that p27(Kip1) expression and cyclin E expression are down regulated as the stage of disease advances.

This study is aiming to analyze the rate of concordance of P27-gene translational expression and Epstein Barr Virus- Encoded Small Untranslated RNAs (EBERs) in bladder tissues from a group ofpatients with malignant and benign bladder tumors**.**

Materials and methods:

The study was designed as a retrospective one. It has recruited 60 selected formalin fixed, paraffin embedded bladder tissue blocks; among them, (25) tissue biopsies from bladder carcinoma with different grades as well as (20)tissues with benign bladder hyperplasia and apparently normal bladder tissue autopsies which were collected from the archives of Forensic Medicine Institute / Baghdad and used as bladder healthy control groups. The diagnosis of these tissue blocks were based on their accompanied records. A consultant pathologist reexamined all these cases to further confirm the diagnosis following trimming process of these tissue blocks.

In one hand, the detection of EBV by ISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed on 4μm paraffin embedded tissue sections usingdigoxigenin-labeled oligo-nucleotidesprobe which targets Epstein-Bar-Virus (EBV) EBER RNA. One section was mounted on ordinary glass slide and stained with hematoxyline and eosin, while another slide was mounted on charged slide to be used for ISH for detection of EBV.

For the In Situ Hybridization procedure,the slides were placed in 60c hot-air oven over night then the tissue sections were de-paraffinizedandthentreated by graded alcohols according to the standard methods and the details of processes for performing ISH reaction with this probe were applied according the instructions of the manufacturing company(Zyto Vision GmbH. Fischkai, Bremerhaven. Germany). The main steps for ISH procedure are:

Incubation of slides for 18 hrat 70°C on hot plate, then incubation of slides for 5 min in xylene. After that incubation for 5 min in 100% ethanol (alternatively, dewaxing protocols routinely used in immunohistochemistry procedures, e.g. 2-5 min xylene, 2-5 min 100% ethanol, 2-5 min 96% ethanol, 1-5 min 70% ethanol, can be used . Air drying of sections.then application (dropwise) Pepsin Solution(ES1) to the tissue/cell section and incubate

for 20-30 min at 37°C in a humidity chamber. After that we immersed slides in distilled water and drain off the water, air dried sections. Then add the probe to the center of a cover slip and place cover slip upside down on target area).

Denaturation of the slides at 75°C for 5 minon hot plate, thentransferred the slides to a humidity chamber and hybridize for 60 min at 37°C for DNA-targeting probes or at 55°C for RNA-targeting probes) and the post-hybridization and detection process that included removing the cover slip by submerging in 1x wash buffer TBS, then washed for 5 min in 1x wash B\buffer TBS (prepared by using WB5) at 55°C (should not perform this step on slides hybridized with Zytofast RNA (+) control probe(PF6) as this will reduce signal intensity). Then application ofAP-Streptavidin(AB9) drop wise (3-4 drops per slide) to the slides and incubate for 30 min at 37°C in a humidity chamber. Then washed in wash buffer TBS (prepared by usingWB5) and then twice times for 1 min in distilled water and application ofNBT/BCIP(SB4) drop wise (4 drops per slide) to the slides and incubated for 40 min at 37°C in humidity chamber.Then checking the color development in intervals of approx, 5-10 min using microscope. Lastly, washing three times for min in distilled water.After that covering the sections. Then the sections were embedded in an aqueous embedded medium, then final evaluation by light microscope.

Immunohistochemistry / Detection system (Abcam . England) was used to demonstrate the P27 tumor suppressor genes . This technique is based on the detection of the product of gene expression (protein) in malignant and normal cells using a specific monoclonal antibodies, i.e . Primary antibody for specific epitope (usually mouse antihuman monoclonal antibody) , which binds to nuclear targeted protein.

The bound primary antibody is then detected by secondary antibody (usually rabbit or goat anti mouse) , which contains specific label (in this context we used peroxidase labeled polymer conjugated to goat anti mouse immunoglobulin) . The substrate is DAB in chromogen solution , positive reaction will result in a browning color precipitate at the antigen site in tested tissues .

Rehydration process was done at room temperature which include : Slides were immersed in two changes of absolute ethanol for one minute each, then Immersion in ethanol (95%) for one minute each, after that immersed in ethanol (70%) for one minute each, finally immersion in distilled water for 5 minutes to remove residual alcohol. After that, slides were allowed to dry completely by incubating them

at 37°C for 5 minutes. Then we done digestion process by add proteinase K to the slides, and then the slides were incubated at 37°C for 15 minutes. Then the slides were dehydrated by immersing them sequentially in the following solution at room temperature for the indicated times, distilled water for 1 minute, 70% ethanol for 1 minute, 95% ethanol for 1 minute and 100% by incubating them at 37% for 5 minutes. Then we add the 20 µl of cDNA probe added to each section and slides were covered by cover slips be careful to avoid trapping any air bubbles. After that probe and target DNA were denaturized by placing the cover slipped-slides in pre-warmed oven at 95°C for 8-10 minutes, slides were transferred to a pre-warmed humid hybridization chamber and incubated at 37°C for overnight. Then the slides were allowed not dry out at any time during the hybridization and staining. All reagents used during hybridization and detection were warmed to room temperature. At the next day, slides were soaked in pre-warmed protein block at 37°C until the cover slips fell off and should be careful not to tear the tissue, and then the slides were allowed to remain in the buffer for 3 minutes, at 37°C after cover slips were removed. After that streptavidin-alkaline phosphatase conjugate reagent was added to tissue sections. Then slides were kept in a humid chamber at 37°C for 20 minutes. Then one to two drops of Slides were rinsed in detergent wash buffer for 5 minutes and then drained. After that One to two drops of 5-bromo3-chloro3-indoly/phosphate/nitro blue tertrazolium substrate- chromogen solution (BCIP/MBT) were placed on tissue section. Slides were incubated at 37°C for 30 minutes or until color development was developed completed. Color development was monitored by viewing the slides under the microscope. A dark blue colored precipitate forms at the complementary site of the probe in positive cells. Then the slides were rinsed in distilled water for 5 minutes, then counter staining process by immersion of the slides in Nuclear Fast Red stain for 30 seconds, then washing process was followed by immersion the slides for 1 minute in distilled water. After that Sections were dehydrated by ethyl alcohol, (95%, once for one minute then, 100% twice times for 2 minutes each); cleared by Xylene, then mounted with permanent mounting medium (DPX).

Chi –square test was used to detect the significance between variables of our study. All the statistical analyses was done by SPSS program(Version– 17)&P value was considered significant when p <0.05.

Results:

The studied groups :

The total number of Sixty studied bladder tissues that were included in this research work was distributed on two groups:-

1. Thirty tissues collected from cases with different grades of bladder carcinomas.
2. Twenty tissues collected from cases of bladder benign .
3. 3-Ten apparently healthy bladder tissues were used as control group . These were obtained and selected as an apparently healthy bladder tissues according to the histopathological examinations .

Techniques :

All these tissues were submitted for in situ hybridization (ISH) technique . Using standard histopathological criteria , these tumors were re-examined for final classification into benign tumor and malignant tumors .

Distribution of patients with bladder tumor and healthy control group according to their Age .

The archival specimens collected in this study were related to bladder tumor patients whom ages were ranged from fifteen years to seventy five years. The mean age of the patients with bladder carcinoma (41.7 $\pm$ 0.9 years) was higher than the mean age in the group of healthy control (35.8$\pm$ 0.74 years). There are significant statistical differences ($p< 0.05$) between different groups according to age (Table 1).

Table (1): Distribution of bladder tumor patients according to their age .

The Patients	N	Mean Age	S.D	S.E	Minimum	Maximum
Malignant Bladder Tumors	30	41.7	0.9	0.24	25.0	72
Benign Bladder Tumors	20	40.5	0.8	0.23	34	68
Healthy B. Tissues Control	10	35.6	0.74	0.13	18.0	63
Statistical Analysis	(P <0.05) = 0.009					

I.EBV-EBERs -Associated Bladder Tumors

The results of EBV- ISH among study groups

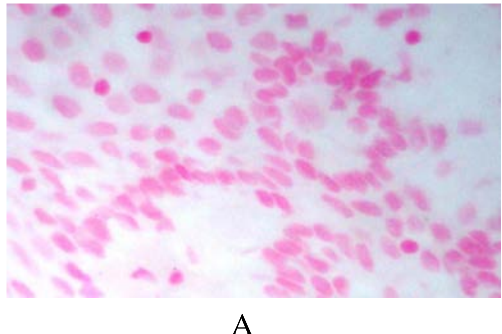
It was found after application and analysis of (ISH) results of EBV--EBERs in the tissues obtained from patients with bladder cancer as well as benign bladder tumors that (13) out of thirty patients with carcinoma of bladder showed positive in Situ

hybridization reaction where they constituted 43.3% of the total bladder cancer cases of this study (table 2 and figure 1). In the benign group, 15% has revealed positive signals, which represented 3 out of 20 cases in this group, whereas none of control group presented with positive signals for EBV-EBERs-ISH test. However , in comparison to the percentage of EBV -EBERs in healthy control group as well as in the group of benign bladder tumors, the differences between the percentages of EBV-EBERs in bladder cancers and each of these groups are statistically very highly significant (P value = < 0,001).

Table (2): Results of in situ hybridization for detecting EBV in tissues with bladder tumors.

Studied groups		EBV-EBERS -ISH		Total	Comparison of significance	
		Positive	**Negative**		**P-value**	Significance.
bladder Cancer	N	13	17	30	0.0001	Highly* Significant. (P<0.001)
	%	43.3	56.7	100		
Benign bladder	N	3	17	20		
	%	15	85	100		
The Control	N	0	10	10		
	%	0	100	100		

- The difference in signal scoring of positive reactions for EBV-EBERs between benign bladder tumors and bladder cancer groups (healthy controls are not part in this comparison, since all of them were negative) was statistically highly significant [HS] (P Kruskal-Wallis = 0.001).



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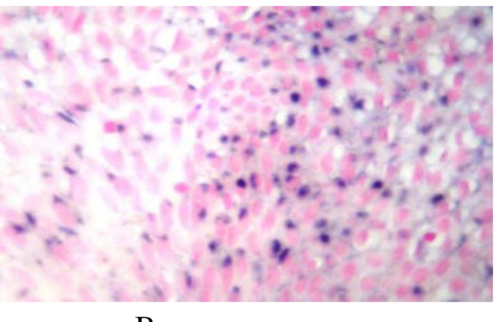

B

Fig.(1) :In Situ Hybridization (ISH) for EBV-EBERs -ISH Deduction Bladder Cancers Using Biotinylated -Labeled EBV-EBERs Probe ;Stained with NBT/ BCIP (Blue)and Counter Stained by Nuclear Fast Red(Red). A. Bladder Cancer with negative EBV-EBERs -ISH reactions (40X). B. Positive -EBV-EBERs -ISH reaction with strong score and high signal intensity(40X).

Co-existence of EBV-EBERs-ISH and P27 –IHC expression in tissues with bladder cancers.

The percentage of positive **P27**-tumor suppressor gene expression that associated with positive EBV-EBERs ISH reaction was constituted (53.3%:16 out of 30 cases) in bladder cancer group. Also, in benign bladder tumors the percentage of positive P27-Tumor suppressor gene expression was constituted (35%: 7 out of 20 cases) (table 3 and fig. 2). The statistical analysis showed significant association ($p<0.05$) on comparing the results (according to score) when group of bladder cancer was compared to benign and control group.

Table (3): Co-localization of EBERs along with P27 gene expression in tissues with bladder cancers.

Studied groups				EBV- EBERS-ISH		Total
				Positive	Negative	
Bladder Cancer	P27 IHC Reaction	Positive	N	7	6	13
			%	53.8	46.2	100
		Negative	N	9	8	17
			%	52.9	47.1	100
		Total	N	16	14	30
			%	53.3	46.7	100
Benign Bladder Tumors	Rb IHC Reaction	Positive	N	3	17	20
			%	15	85	100
		Negative	N	4	16	18
			%	28.6	71.4	100
		Total	N	7	13	20
			%	30	70	100
The Control	Rb IHC Reaction	Positive	N	0	0	0
			%	0	0	0
		Negative	N	0	12	12
			%	0	100	100
		Total	N	0	12	12
			%	0	100	100

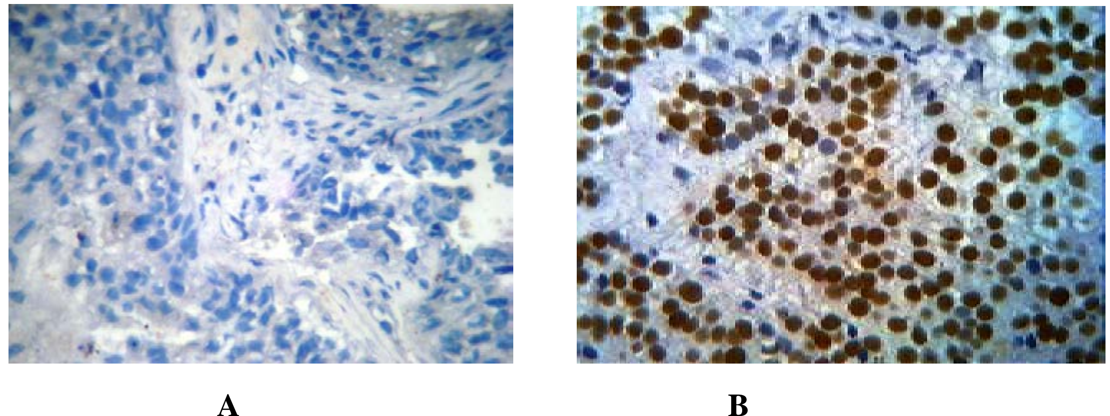

Fig.2 : Immunohistochemical results for P27 expression detection in bladder tumor; DAB chromogen stained (brown) and counter stained by Mayer's hematoxyline (blue); A. Bladder cancer with negative P27IHC reaction (20X).B. Benign Bladder with positive P27- IHC reaction (20X).

Discussion

In the current study, we found new evidence that there is an association between EBV-EBERs and bladder carcinoma and indicate the important role of EBV in bladder tumors. The positive EBV-EBERs -ISH reactions in malignant bladder tissues in the present study were documented in 43.3% while in the benign bladder tissues was detected in 20%. No positive – ISH reaction for EBV-EBERs was observed in the control bladder tissues. These result were in consistent with the finding of (11) who detected EBV genome in 34% of whole bulk tissue samples using PCR and (25) who demonstrated EBV-encoded RNA within both carcinoma cell and infiltrating lymphocytes in 21%, only infiltrating lymphocytes in 7%, and only carcinoma cells in 3% of bladder cancers obtained from Taiwanese population. (26) reported that EBER-expressing lymphocytes were detected in the bladder carcinomas in 26 out of 39 cases (66.7%) while all normal urinary bladder specimens showed negative results. The frequency of the infiltrating EBV-positive lymphocytes in the stromal region of bladder carcinoma was significantly higher in advanced T-stages than in earlier stages (26*)*.

These differences might be related to the geographic variation, the sensitivity of the probe used for ISH, or differences between the subjects studied, yet a definitive reason is not apparent.

The reason for EBV to exert its oncogenic influences in a particular patient is unknown but is probably associated with co-factors. Again it

is possible that HPV exerts its oncogenic influences in concert with co-factors including a possible collaboration with EBV (27). (28) reported that co-cultivation with viral producers resulted in an approximately 800-fold higher efficiency of infection than use of a cell-free system, indicating the importance of cell-to-cell contact in EBV infection.

Although the theory that an immunosuppressive environment in the tumor stroma allows virus persistence and replication is reasonable for our results, this is a highly selective look and further examinations are needed to support this theory.

In the present study the immunohistochemical positive results for P27 protein were observed in 16/30 cases (53.3 %) of bladder carcinoma and in7/20 cases (35%) benign bladder tumors while the control groups showed negative P27 immunostaining reactions.

Steeg and Abrams (29) reported that p27 and cyclin E play a central role in the transition from late G1 to S phase. Therefore, it is possible to assess the role of p27 and cyclin E in tumor recurrence and progression and therefore their value in predicting survival in patients with bladder cancer.

By analogy of the current results with other types of cancers, a significant inverse correlation of p27 protein levels with grade and stage was found. Low p27 protein levels are associated with poorer survival in breast (30) , gastric (31), prostate (32), upper uninary tract (16), and bladder cancers (24); (33). In addition, it has revealed that a low protein level of p27 was correlated with a negative prognosis for patients with lung (34), colorectal (35), and ovarian cancers (36). In the present study, low protein levels of p27 were associated with poorly differentiated grade bladder cancers and as such patients with these cancers could have unfavorable prognosis. It is likely that p27 affects differentiation pathways and acts as a tumor suppressor gene in different human tumors and therefore the evaluation of p27 protein levels may indicate the biological behavior of human tumors. Since p27 levels are mainly regulated by ubiquitin mediated proteasome degradation, which is targeted by cyclin ECdk2 phophorylation of p27 (37, 38), it is likely that loss of p27 expression and high expression of cyclin E are associated with cancer progression and unfavorable prognosis. In this respect, low p27 protein levels and increased protein levels of cyclin E are correlated, for example, with shortened survival in breast cancer (38, 39). There may be different regulatory mechanisms in the expression of these genes between

breast and bladder cancer. (24) suggested that cellular down-regulation of cyclin E may be an attempt to offset loss of p27 expression during tumor growth via a feedback inhibitory loop.

Low p27 was correlated with poorly differentiated grade, muscle invasion, lymph node involvement and poor survival in bladder cancer patients (24) and so could the low staining results of p27 in current study are possibly to be correlated with such sequels (38) .

Conclusions:

The highly significant translational expression of P27 gene as well as high rate ofoccurrence of EBV in bladder carcinomain our results could indicate for animportant role of these molecular and viral factors in the bladder carcinogenesisof subset of bladder malignant tumors.

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Isolation and Identification of Some Mycoplasma spp. from Septic Arthritis in Basra City

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Abstract: The aim of this study was to determine Mycoplasma as one of the causative agents of septic arthritis .Twenty– five of synovial fluids specimens from both sexes (14 male & 11 female) with different age groups were collected from patients attending to orthopedics and rheumatology clinics in Basra handled by physicians .

The results revealed 11 (44%) of synovial fluids samples were positive for mycoplasmas. *M. salivarium* was detected in 8(32%) synovial specimens, *Ureaplasm urealyticum* in 2(8%) of specimens *,and Mycoplasma fermentans was detected in 1(4%)* . Whereas 36% of synovial fluids were represent negative cultivation and *Staphylococcus aureus* and *Streptococcus pyogenes* were found in the synovial fluids of (16% , 4%) respectively on the other hand 24% of *M. salivarium* was isolated from 31-50 years and (4%) was isolate from 61-70 years which was exhibit the relationship between age and gender. Mycoplasma was frequently isolated from synovial specimens of knee (92%) followed by ankle specimens (8%). The present study leads to the isolation *M. salivarium*, *U. urealyticum* and *M. ferementans* as first time from synovial fluid in Iraq.

Key words : Mycoplasma spp. , Septic Arthritis , synovial fluids .

Introduction

Mycoplasmas are specific and unique species of bacteria , the smallest free-living organism known[1].The primary difference between mycoplasmas and other bacteria is that bacteria have a solid cell-wall structure and can grow in the simplest culture media, also bacteria are inhibited by penicillin[2]. *Mycoplasma*, can grow in tissue fluid blood, bone marrow of leukemic patients , urine and blood of HIV carries , saliva of healthy persons, joint fluids of patients with rheumatoid arthritis. So Mycoplasma can causes a typical pneumonia and responsible of trachiobronchitis , bronchitis ,and less sever upper respiratory tract infections in older children and young adults[3,4,5,6,7]. Mycoplasmas are present in the human Orapharynx including , *M. oral, M. salivarium* and *M.faucium* which produce ammonia and cause tissue damage.

Mycoplasma salivarium may be part of oral microbial flora and inhibits preferentially in gingival sulci and is suspected to play an etiological role in some cases of oral infections including periodontal diseases[8]. The mollicutes depend completely on their hosts for nutrients and refuge[9]. Septic arthritis ,may represent a direct invasion of joint space by various microganisms including bacteria *S. aureus,* S. pneumonia, *P. aeruginosa, H.influenza,* as well as large variety of viruses[10] .*M.hominis* and *M. fermentans* have been reported as a pathogenic microorganisms of chronic diseases such as RA[11,12] also known as infectious arthritis ,chronic arthritis is generally caused by pyogenic bacteria and termed as a septic arthritis. Ramirez *et al.*[13] found the hypothesis that *Mycoplasma* may be associated with the RA , however it cannot be demonstrated whether mycoplasmas are cofactor or whether they produced a secondary infection or occur more in those with abnormal immune systems who have RA.

Materials and Methods

The population studied was the patients attending to Basra general Hospital, Al-Sadr teaching hospital and private clinical in center of Basra city at different periods ,they were suffering from swelling and painful joints. A sample of twenty –five patients was selected (Collee,*et al* 1996) during the period from September 2007 – January 2009. The age of patients ranged from less ten to seventy years. A sample of fluid was taken from the swollen joint by physicians, sterile needle which inserted through the skin into joint. Direct examination by microscopic

slide with Gram stain and then a small amount of fluid (0.1 ml) was inoculated on the Monophasic-Diphasic Culture Setup(MDCS)[14].MDCS consist of pplo agar and broth, yeast extract, horse serum, …etc and Modified Mycoplasma Medium(MMM)[15] aerobically and other ordinary media (Blood agar base and MacConkey agar) to know if mycoplasmas or other bacteria were grown. After that, Giemsa and negative staining and biochemical tests were achieved.Clinical data were recorded in tenderized manner and processes by computer (spss, system)statistical analysis were performed by using chi – square with the revised significant difference.

Results

The identification of septic arthritis mycoplasmas was based on colonial morphologies growth properties, Giemsa stain and Negative staining. The colonies of a fried–egg appearance represented a prominent feature that started as a central dense growth in agar, as shown in figure (1, 2, 3).

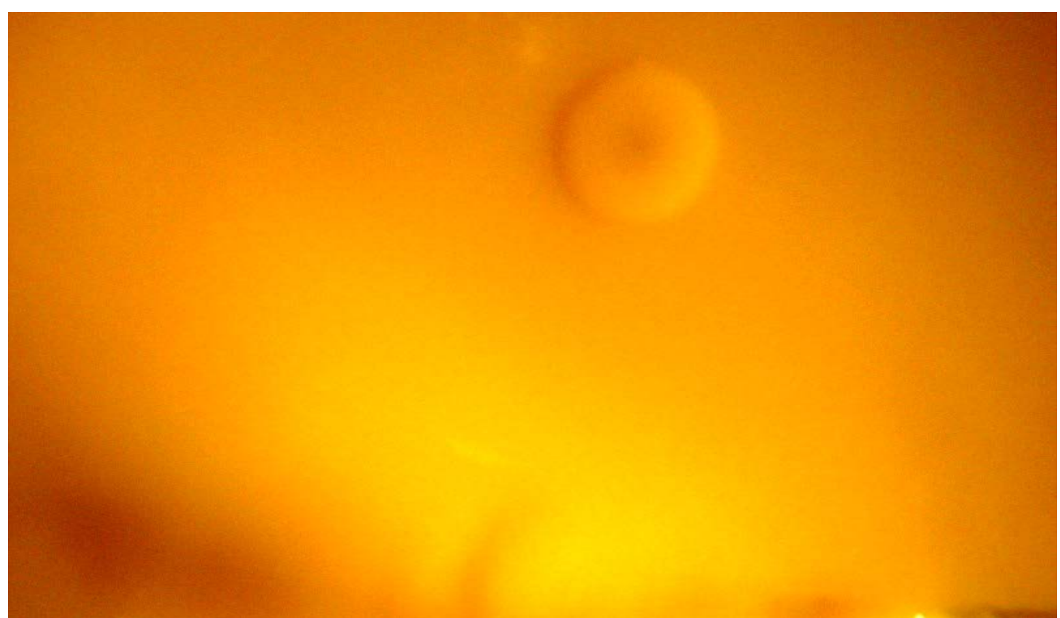

Fig 1. The fried egg shape like colony of *M. salivarium (X20).*

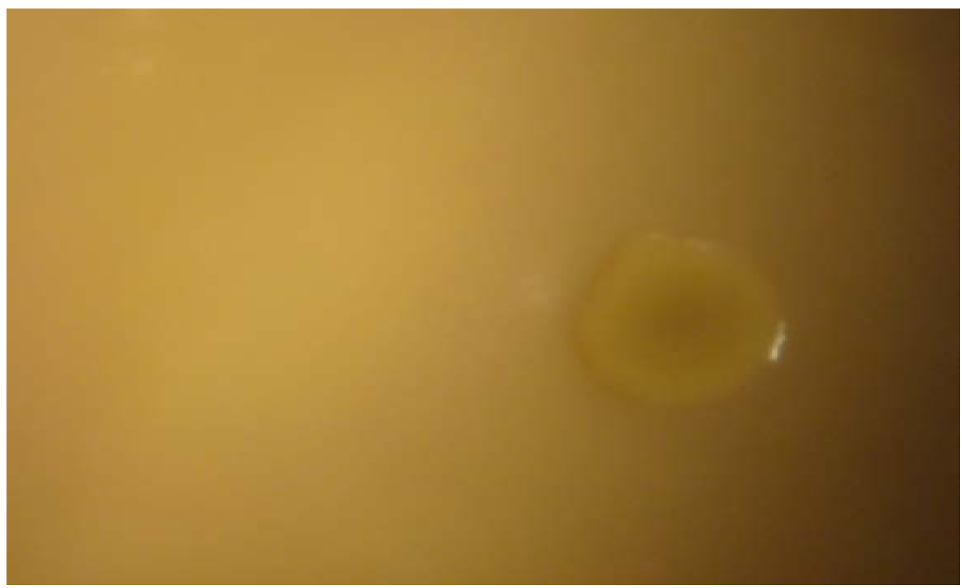

Fig 2. The fried egg shape like colony of *Ureaplasma urealyticum* (x20).

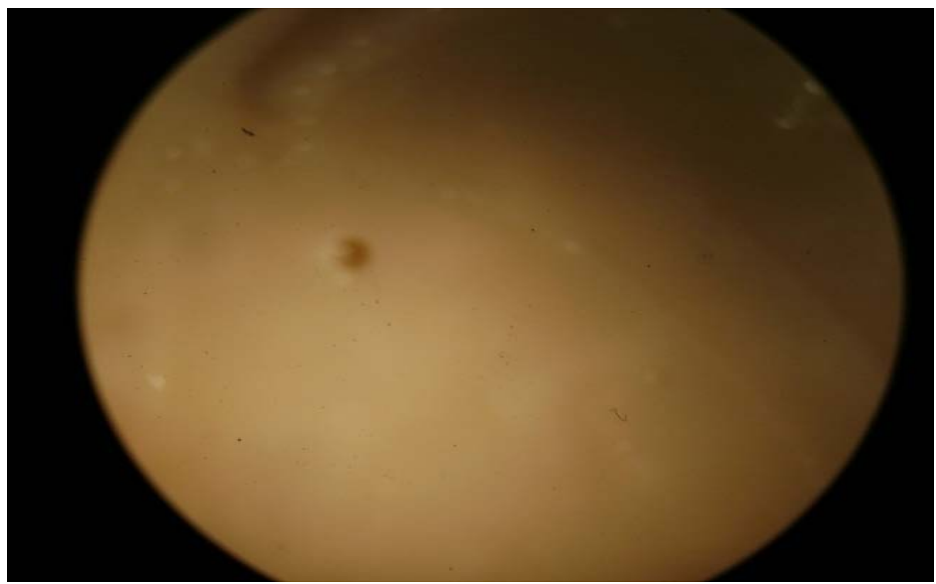

Fig 3. The fried egg shape like colony of *M. fermentans* (x 5).

Distribution of patients according to age groups:

Table (1) and Figure (4) demonstrated the distribution of pathogenic arthritis patients and age. The highest rates of samples 8 (32%) and 7 (28%) were associated with two age groups (31-40 and 41 -50 years) this represent a significant difference, namely (x^2 =53.440, $p<0.001$) whereas, the lowest rate was 1 (4%) with two groups(≤ 10, 11- 20 years).

Table (1). Distribution of septic arthritis cases among age groups

Age group	Patients	Percent %
10≤	1	4%
11-20	1	4%
21 -30	3	12%
31 -40	8	32%
41 -50	7	28%
51-60	3	12 %
61-70	2	8%
Total	25	100

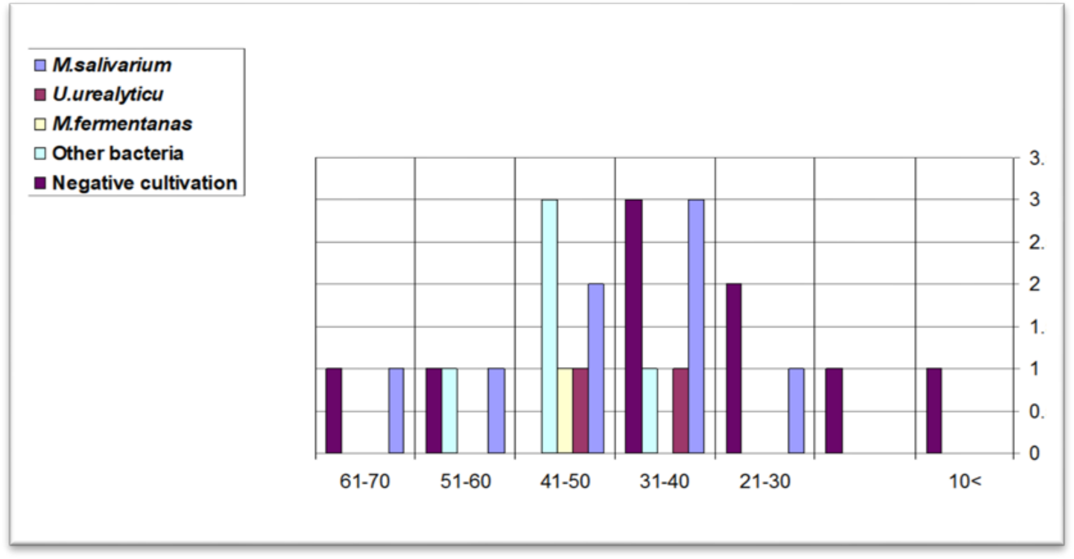

Fig 4. Distribution of the septic arthritis Mycoplasmas and other causes in patients with different age groups

Distribution of samples cases according to gender

The relationship between samples and sex are shown in table (2). The samples were made up of 14(56%) males and 11(44%) females. The comparison between the samples revealed non significant deference (X^2 =1.440 ; p<0.001).

Table (2). Distribution of study cases according to gender

Gender	Frequency	Percent
Females	11	44%
Males	14	56%
Total	25	100.0

x^2= 1.440 ; p< 0.001

Etiologic cause of septic arthritis

The results of the presents study revealed that there was a significant variation between mycoplasmas as a causative agents compared with other bacteria (x^2= 60.320 ; p< 0.001) as shown in table (3). A (32%) represents *M. salivarium* and 36 % negative cultivation of samples , whereas (16%) and (4%) represent septic arthritis with *Staphylococcus aureus* and *Streptococcus pyogenes* **.**

Table (3). Causative agent of septic arthritis

Cause of arthritis	Frequency (percent)
M. salivarium	8 (32%)
U. urealyticum	2 (8%)
M. fermentans	1 (4%)
Negative cultivatation	9 (36%)
Staphylococcus aureus	4 (16%)
Streptococcus pyogenes	1 (4%)

x^2= 60.320 p< 0.001

Joints affections

Table (4) exposes two clinical which features were recorded in septic arthritis patients. The common joints infected was knee joint which recorded (92%) and other joint affected was ankle joint with low percent, (8%). Besides that, the current study no diagnosed possible affection of hip , shoulder and elbow joints.

Table (4). Types of joint affection among study patients

Joints affection	Frequency (percent)
Knee joint	23 (92%)
Ankle joint	2 (8%)
Hip joint	-
Shoulder	-
Elbow joint	-

Discussion

Up to our knowledge, this study is the first in Iraq, in which Mycoplasmas were isolated as one of etiological agents associated to septic arthritis. This study was pronounced the associated of five microorganism to septic arthritis two bacterial species ; *S. aureus*, *Streptococcu pyogenes* ,and three Mycoplasma species : *M. salivarium, U. urealyticum* and *M. fermentans* which were defiant evidences for implicated of mycoplasmas with septic arthritis there were many samples which did not grow on ordinary medium or diagnosis by microscopic examination ,despite they were watched pus by naked eyes while growing on MMM or MDCS media .

Shibata *et al.*[8] referred *M. salivarium* pathogenic in the oral cavity and connective tissue ,but in the current study the *M. salivarium* was isolated from synovail fluid as in implicated factor in some patients such as septic arthritis. AL-Ghizawi[14] isolated *M. salivarium* from the oral cavity from healthy human while in this study *M. salivarium* was isolated as pathogenic agents for septic arthritis which exhibited that *M. salivarium* may act by entering into the individual cell of the body,when the conditions are suitable and deficiency immunity weakens as a result of the trauma accidents or overloud mechanisms *M. salivarium* target synovium membrane as pathogenic factor due to no *M. salivarium* isolated with SF as a normal flora.

Scott and Johnson *et al.*[16,17] found that M. salivarium occurred less often in both inflammatory and non inflammatory RA. Due to that it can be found in 10,20 and 30 years, without causing problems if a trauma occurs or upon genetic predisposition it triggered . That result comes in an agreement with the present study. Gilroy *et al.*[18] in their research isolated *M. fermentans* of some arthritis which was detected with 20% of French patients with chronic inflammatory arthritis. AL-Bahli ; Simihairi and

AL-Mossawi[19,20,21] isolated *U. urealyticum* with high frequency from urogenital tract while in the present study it is isolated from 2 patients with septic arthritis in favors of urorogenital tract as a suitable environment than synovail fluid . Grisold *et al.*[22] found that *M.salivareium* was detected in synovail fluid from 22 out of 33 patients with pain in anterior disc replacing the temprormandibular joints . Smith *et al.*[23] found that *S. aureus* was isolated from RA patients with (56%). The present study examined the relationship between gender and infection of joints with mycoplasmas, and men are more susceptible to the infection with mycoplasmas when compared with women . This was in agreement with previously proved study[24] as they studied patient with septic arthritis in 53 men and 38 women.

Holtom *et al.*[25] studied septic ankle arthritis and found that (73%) were male and (26.6%) female. According to the results obtained from the present study it had been found that the infection can be detected in the age groups above 20 years old for both genders and this agreed with Schaeverbeke *et al.,*[26] who isolated mycoplasma from 8 patients with age group above twenty years.

The present study showed that the most susceptible joints for infection of septic arthritis was knee joints (92%) and in much lower rate of infection was the ankle joints (2%) ts. Hoffman *et al.*[27] found that *M. salivarium, M. hominis* and *M. pneumoniae* have been identified as causing acute monoarthritis in humans , while *M. genitalium* and *M. pneumoniae* have been isolated from the joints of patients with polyarthritis . These results correspond to certain extent with the present study in that, *M. salivarium* is isolated from monoathritis in knee joints but, *M. hominis* and *M. pneumoniae* have not been detected here.

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A study of histological changes in the kidney of male albino mice administered with aqueous extract of chamomile Chamomilla recutita

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Abstract: The wide usage of Chamomilla recutita as anti-inflammatory and antioxidant plant, also its drinking is very effective to relief glomerulonephritis and sedative for uterus pain associated with menstruation cycle and after delivery.

This study designed to study histological changes associated with oral administration of aqueous extract of chamomile on kidney tissue.

The study included 40 male albino mice Mus musculus, their age ranged from (5-7) weeks , The mice were divided randomly to 5 groups and oral administered with 1 ml every day for 10 days :-

- Group G1: consider as control group and treated with normal saline.
- Group G2: was treated with aqueous extract of *chamomile with concentration of 3 gm /100 ml D.W.*
- Group G3: was treated with aqueous extract of chamomile with concentration of 5 gm /100 ml D.W.
- Group G4: was treated with aqueous extract of chamomile with concentration of 7 gm /100 ml D.W.
- Group G5: was treated with aqueous extract of chamomile with concentration of 10 gm /100 ml D.W.

The results showed the following histological alterations which correspond with concentration of chamomile extract, these changes include:

* The G2 sections had shown normal structure of kidney tissue

* The G3, G4 and G5 sections show :

1- Infiltration of extracellular fluids .
2- Several glomeruli demonstrate increase in cellularity of renal epithelial cells and deplesion of some of these cells in renal space of renal tubules.
3- Also necrosis of several renal tubules epithelium.
4- Hemorrhage (hematuria) had observed in several sections as a result of over dilation of renal blood capillary.
5- Atrophy in some renal tubules may be refer to loss of blood supply as a result of hematuria .
6- Infiltration and accumulation of inflammatory cells in the injurious sites.

Conclusion :the chamomile have negative effects on kidney tissue ,and the concentration of 3g /100ml D.W. the safest dose with less side effects.

Keywords: Histological changes, Chamomilla recotita, Kidney and albino mice.

دراسة التغيرات النسجية في كلى ذكور الفئران البيض المجرعة بالمستخلص المائي للبابونج

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الخلاصة : الاستعمال الواسع للبابونج كنبات مضاد للالتهابات ومضاد للاكسدة وكذللك اهمية شرب مستخلصه في علاج التهاب الكبيبات والنبيبات الكلوية وتقليل آلام الرحم في فترة الحيض وبعد الولادة. صممت هذه الدراسة لدراسة تغيرات النسجية في نسيج الكلية في الفئران لمجرعة بالمستخلص المائي للبابونج. شملت الدراسة استخدام ٤٠ فأر من ذكور الفئران البيض albino mice Mus musculus تراوحت اعمارها بين (٥-٧) أسابيع قسمت عشوائياً على خمس مجاميع وجرعت بجرعة مقدارها ١ مليلتر ولمدة ١٠ ايام :-

- المجموعة G1 : عدت مجموعة سيطرة وجرعت بالمحلول الفسيولوجي .
- المجموعة G٢ :جرعت بالمستخلص المائي للبابونج بتركيز ٣ غم لكل ١٠٠ مللتر ماء مقطر .
- المجموعة G3 : جرعت بالمستخلص المائي للبابونج بتركيز ٥ غم لكل ١٠٠ مللتر ماء مقطر.
- المجموعة G4 : جرعت بالمستخلص المائي للبابونج بتركيز ٧ غم لكل ١٠٠ مللتر ماء مقطر.
- المجموعة G5: جرعت بالمستخلص المائي للبابونج بتركيز ١٠ غم لكل ١٠٠ مللتر ماء مقطر .

اظهرت نتائج الدراسة الحالية :

*ان مقاطع النسجية G2 ظهرت طبيعية .
*اما G3,G4 ,G5 فقد اظهرت مقاطعها التغيرات التالية :

١- ارتشاح السوائل الالتهابية (الوذمة).
٢- زيادة الخلايا الظهارية المبطنة للنبيبات الكلويةوالكبيبات الكلوية وقد ظهرت متجمعه في بعض تجاويفها مما قد سبب انسداد بعض النبيبات الكلوية .
٣- حصول تنخر في بعض خلايا النبيبات الكلوية .
٤- حصول نزف دموي في بعض الاوعية الدموية الشعرية نتيجة للتوسع المفرط في اقطارها .
٥- ضمور بعض خلايا النبيبات الكلوية .
٦- ارتشاح وتجمع خلايا التهابية في المناطق المتضررة.

ومما تقدم نستنتج ان زيادة تركيز المستخلص المائي للبابونج له تأثيرات سلبية في نسيج الكلية وان التركيز ٣ غم لكل ١٠٠مل ماء مقطر هو التركيز الامثل بالنسبة لوزن الفئران.

Introduction

Chamomile Chamomilla recutita is one of most famous medicinal plants, it's belongs to compositae (Asteraceae) family [1] .It had antioxidant and anti-inflammatory effect [2] .Miller and colleges [3] had studied the effect of chamomile volatile oils on histamine secretion and they found that these oils stimulate mast cell degradation (histamine secretion) and thus it decrease allergy and asthma symptoms of bronchospasm. The chamomile extract had shown spasmolytic effect on guinea pigs intestinal smooth muscles and found that this effect correlates with concentration of extract [4]so that the

drinking of its extract is very effective for treatment of glomerulonephritis and spasm pain ,kidney pain , urine burning , urinary bladder inflammation and uterus pain during menstrual cycle and after delivery[5][6].
viola and others [7] had studied the effect of apigenin (flavones extracted from chamomile) on syntactical membrane of bovine cerebral cortex and they found there was high affinity of apigenin to bind to central benzodizepam receptor instead of flunitrozepam which act as ligand so that chamomile had hypotonic and relaxant effect.

Material and Methods

Extraction method: crude aqueous extract was used as extraction method to promote scientific and easy may method of extraction [8].Maceration method was used in extraction of active ingredients of chamomile, for concentration of aqueous extract of chamomile were prepared by weigh (3, 5, 7, 9) gm of chamomile powder then added to 100ml of D.W. then left for 30-60 minute in water bath with stirring then filtered by filter paper and used .

The prepared concentrations were (3gm/100ml), (5gm/100ml), (7gm/100ml), (10 gm/100ml).

Experimental animals: in this experiment 40 Swiss adult male albino mice were used with age ranged between (5-7) weeks and were put in plastic cages and provided by water and food ad libitum.

Experiment Design: the mice were grouped in 5 groups and oral administered by 1 ml every day for 10 days each group contain 8 mice:

1. control group (G1): was treated by normal saline.
2. group (G2): were treated with chamomile extract of 3gm /100ml D.W.
3. group (G3): were treated with chamomile extract of 5gm /100ml D.W.
4. group (G4): were treated with chamomile extract of 7gm /100ml D.W.
5. group (G5): were treated with chamomile extract of 10gm /100ml D.W.

Histological study: the mice were killed at eleventh day of experiment by cervical dislocation [9] and kidney were taken by common histological methods [10] then stained by harris hematoxylin and eosin and examined under light microscope.

Results and discussion :

1- The examination of H&E stained kidney sections of control group (G1) had shown renal corpuscles appear as dense rounded structures (glomeruli) surrounded by narrow spaces (bowman's space) ,the mass of cortical

tubules seen in section mainly consist of proximal convoluted tubules with lesser number of distal convoluted tubules Fig .1.

2- The result G2 sections had shown normal structure of kidney tissue Fig . 2.
3- While (G3) kidney sections showed in some region an atrophied renal corpuscles were observed with an increase in interstitial space the Atrophy of renal tubules may be refer to loss of blood supply as a result of hematuria [11] and the same effect had been appeared in renal biopsy of patients had took herbal medication for different purposes [12] ,also depletion of epithelial cells in renal space could be recognized Fig.3.
4- The kidney sections of G4 showed occludance in renal tubules in some regions as result of proliferation of tubular epithelial cells and the same had recognized in renal glomeruli (increase in cellularity) which narrowed the buman's space the proliferation of renal cells which alluded the pathogensis in various glomerulopahies[11] .also there was accumulation of edema and exudates fluid in interstitial space this may be to Infiltration of extracellular fluid ,this refer to antispasmodic effect of chamomile volatile oil and flavonoids which relax the blood vessels smooth muscle [13][14]which cause vasodilation then accumulation of edema fluids [13] and there was focal accumulation of inflammatory cells Fig.4.
5- The examination of G5 section revealed an increase in occludance in renal tubules in addition to depletion of tubular epithelial cells to the tubule lumen also we recognized increase in hemorrhage (renal hemturia) had observed in several section as a result of over dilation of renal blood capillary by antispasmodic effect of chamomile volatile oil and flavonoids which relax the blood vessels smooth muscle [13][14] that lead to destruction of renal blood vessels Fig.5,6 , or may be as Gopalkrishnan et al mentioned that massive hematuria due to renal papillary necrosis[15][16] ,Also several renal tubules epithelium appear necrotic, so that the normal simple cuboidal epithelial tissue was replaced by eosinophilic structurless debris in which cellular outline as well as the nucleus obscured Fig.5 the cellular degeneration were observed as a result of toxic phenolic compounds [17] these compounds bind to receptors on cell surface which lead to coagulation of cellular protein [15]then cellular necrosis . Lee et al had reviewed 58 cases of nephrotoxicity from Chinese

herbs and had documented that 7% of cases of nephropathy with acute tubular necrosis and acute renal failure as well as 12% of cases tubular dysfunction and 81% of cases sussered from renal interstitial fibrosis with sparse mononuclear cell infiltration[18] , and infiltration of inflammatory cells so as edema was very obvious to see in some region of sectionFig.6 , Chamomile toxic compounds like phenols compounds [17] [19] and tannins as protein coagulant [20] causes injuries in different renal tissue which is possibly related to the alterations in the metabolic state of the epithelial cells, these changes considered as a stimulus causing accumulation of inflammatory cells. The presence of inflammatory cells in the kidney after tubular obstruction is characteristic of this disorder [11] and the same Karkar had mentioned that the inflammation can be initiated by toxin substances or tissue necrosis. This achieved by intact regulatory immune system ,which includes pro– and anti–inflammatory cytokines chemokines, growth factors, complement cascade system, rennin-angiotensin system and different set of adhesion molecules expressed on leukocyte ,vascular endothelium ,in addition to neutrophiles ,monocyte ,macrophage and different subsets of T-Lymphocytes[21].From this study it has been concluded the concentration of 3gm/100ml D.w. is the most safe dose on kidney section and These achievements helped in researching into ways to modulate renal inflammation ,control severity of renal injury, promote regeneration and tissue repair and induce tolerance.

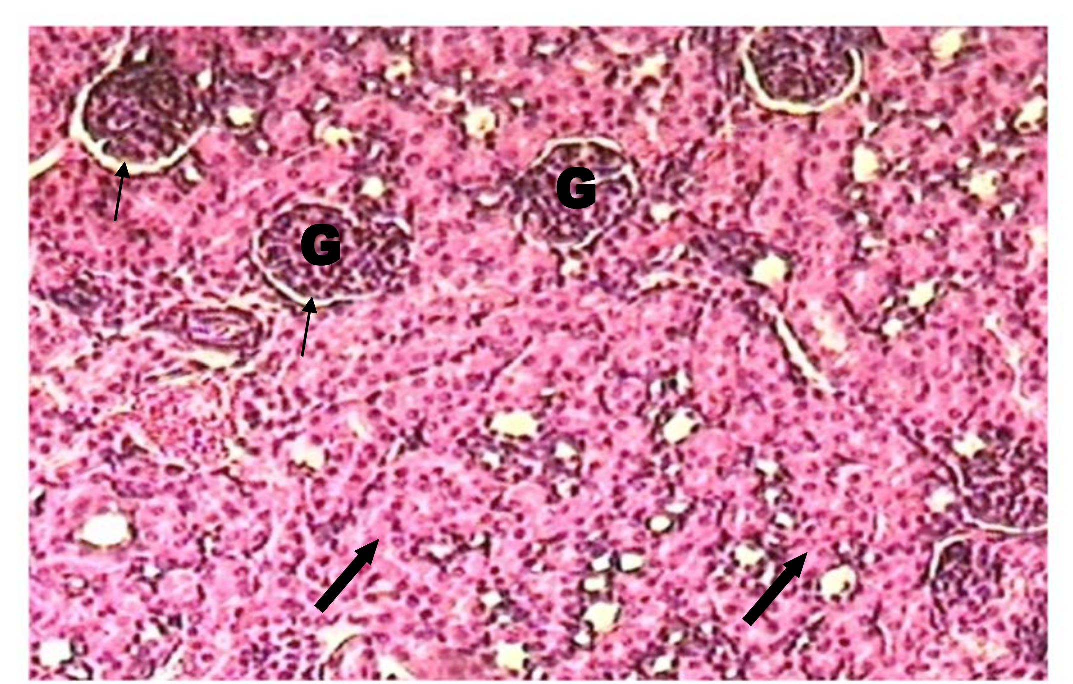

Fig.(1): section of kidney in control mice shows glomeruli(**G**) surrounded by bowman space (→)renal tubules(➡) H&E (300X).

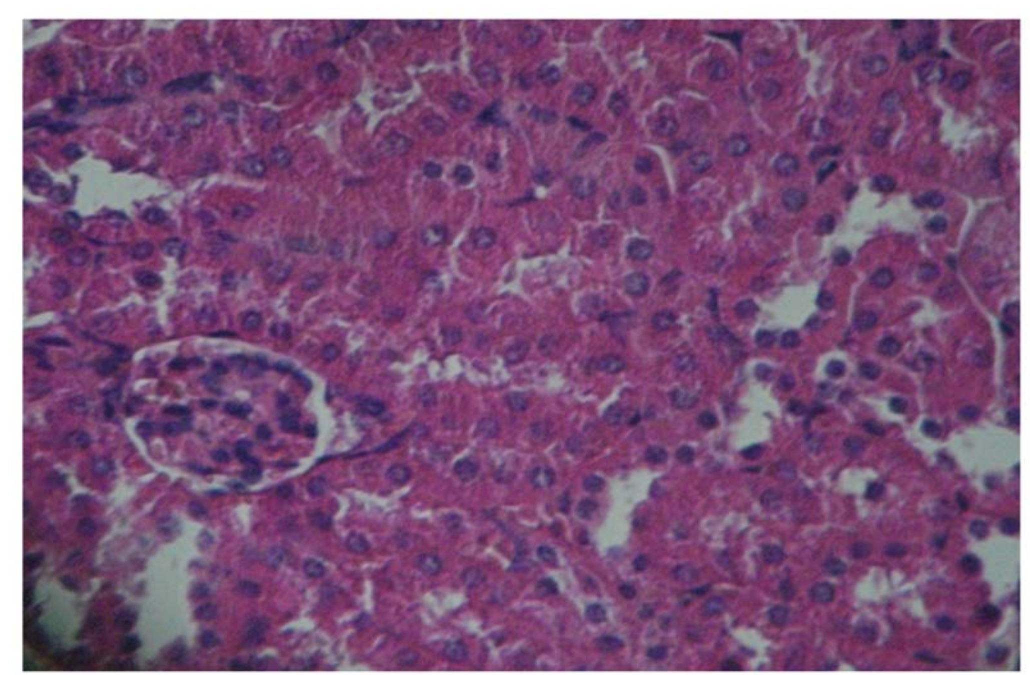

Fig.(2): section of kidney in mice treated with chamomile extract 3g/100ml D.W. shows normal tissue H&E(600X) .

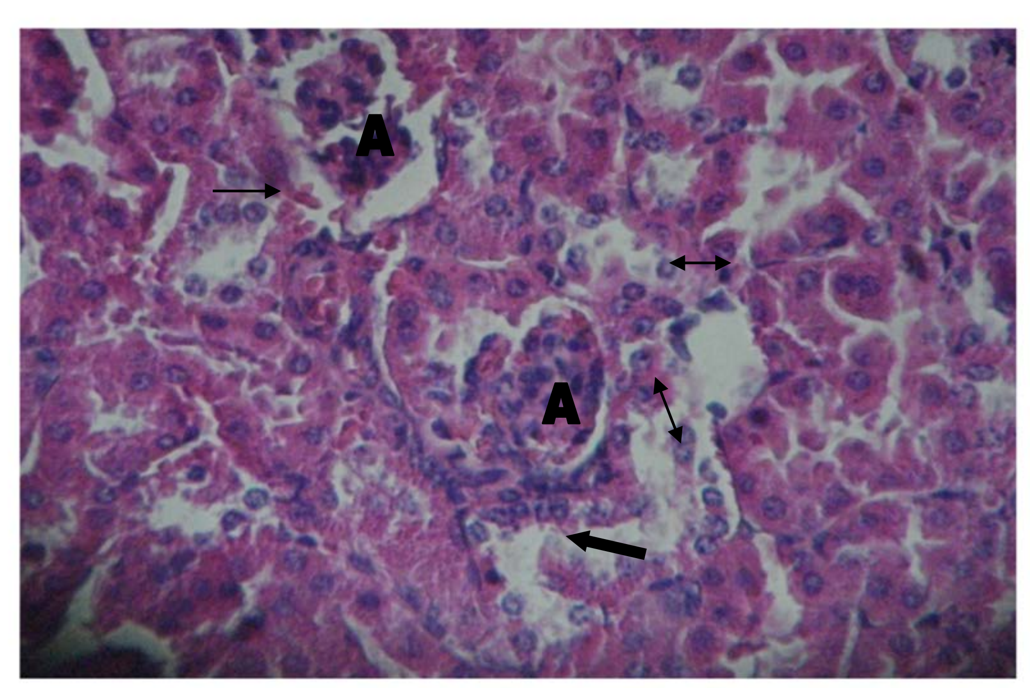

Fig.(3): section of kidney in mice treated with chamomile extract 5g/100ml D.W. shows atrophied glomeruli (**A**) ,depletion of renal epithelial cells (⟷) Hemorrhage (⟶) and accumulation of inflammatory cells(➡) H&E (600X).

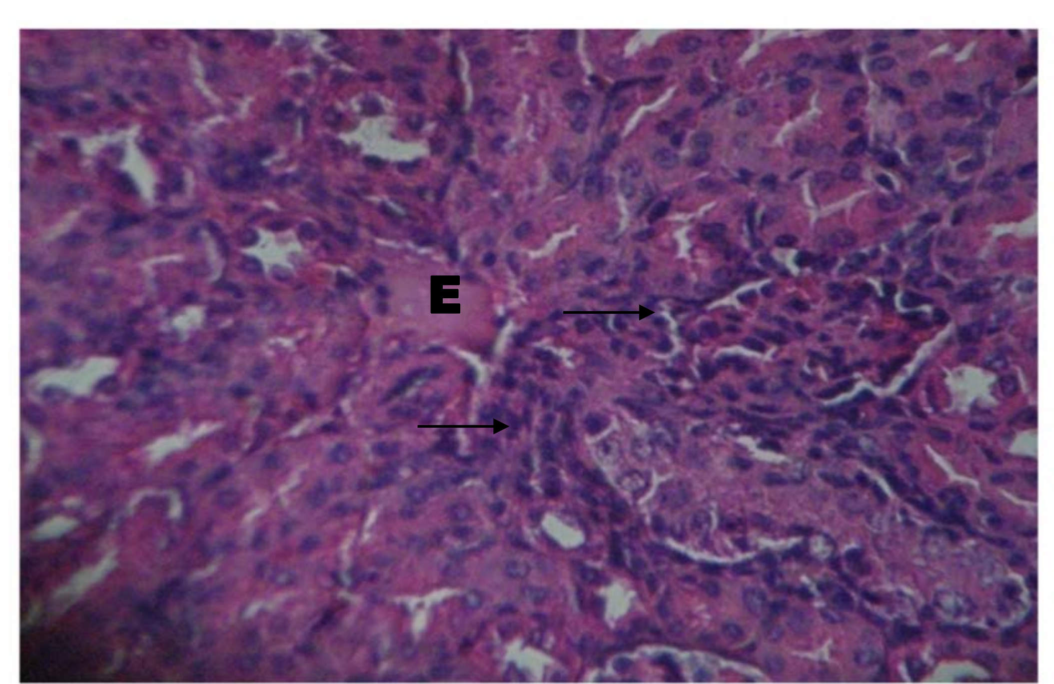

Fig.(4): section of kidney in mice treated with chamomile extract 7g/100ml D.W. shows increase in cellularity of both tubules and glomeruli , infiltration of edema (**E**) , accumulation of inflammatory cells(⟶)H&E (300X).

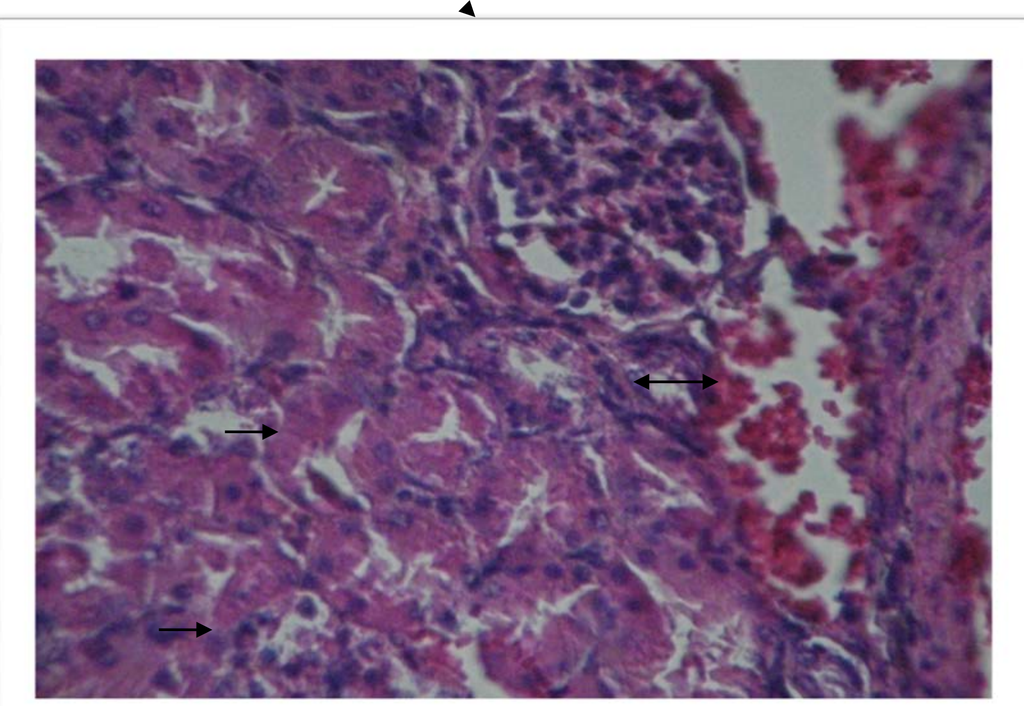

Fig.(5): section of kidney in mice treated with chamomile extract 10g/100ml D.W. shows necrotic renal epithelial cells (⟶) hemorrhage in blood vessels (⟷)

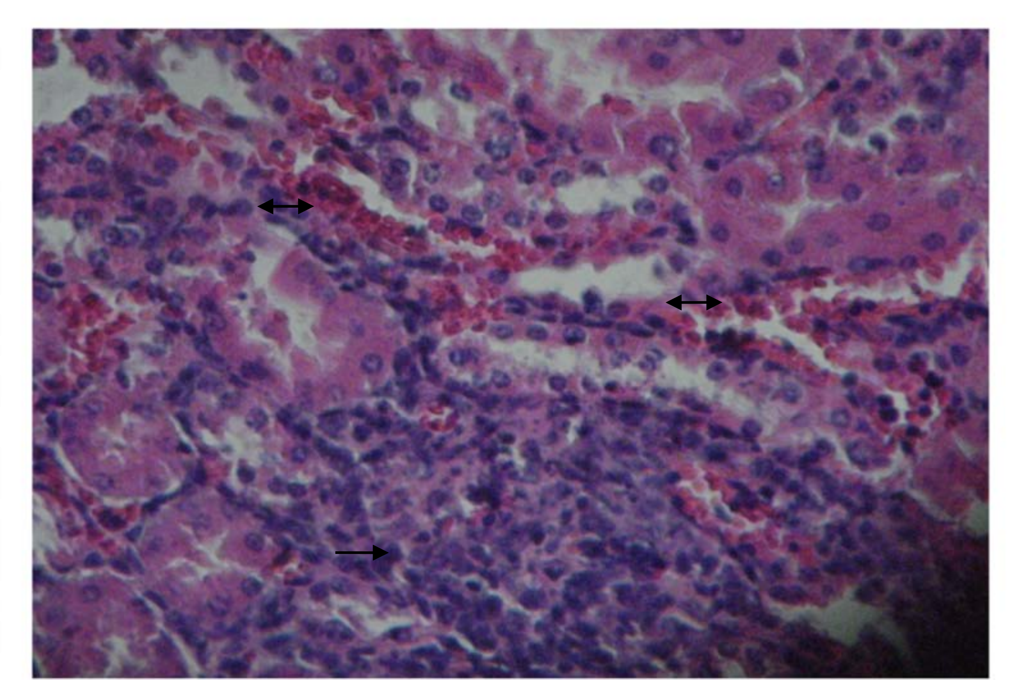

Fig.(6): section of kidney in mice treated with chamomile extract 10g/100ml D.W. shows Hemorrhage(⟷) , focal accumulation of inflammatory cells(⟶)H&E (600X).

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