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Production , Extraction and Purification of *Bacillus licheniformis* FH4-IRQ Alkaline Phosphatase

Hassan Majeed Rasheed Alhilli and Shatha Salman Hassan

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Abstract: The optimum conditions for ***Bacillus licheniformis*** FH4-IRQ alkaline phosphatase production on PB ALP medium were determined as :, arabinose 3% , maltose 2% and 3% , cellobiose 1% and 2% , gave high alkaline phosphatase productivity , pH 9 , incubation temperature 45-50 C. Alkaline phosphatase was produced after 18 hr of inoculation and it increased during the beginning of stationary phase .Alkaline phosphatase was precipitated by ammonium sulphate (60%-95% saturation) and purified using DEAE Sepharose CL6B ion exchange and Sephacryl S 300 gel filtration , 34.85% of total enzyme was recovered with specific activity 7.066 U/mg and 71.59 folds of purification . The result refer that the ***Bacillus licheniformis*** **FH4-IRQ** prefer high temperature with alkali conditions to produce alkaline phosphatase without depending on specific carbon source.

Key words: ***Bacillus licheniformis*** **, alkaline phosphatase , purification , extraction**

انتاج واستخلاص وتنقية انزيم الفوسفاتيز القاعدي من بكتريا *Bacillus licheniformis* FH4-IRQ

حسن مجيد رشيد الحلي و شذى سلمان حسن

قسم علوم الحياة- كلية العلوم-جامعة بغداد

حددت الظروف المثلى لانتاج الفوسفاتيز القاعدي من بكتريا *Bacillus licheniformis* FH4-IRQ ب 3% ارابينوز 2%او 3% مالتوز 1%او 2% سيلوبايوز والتي اعطت اعلى انتاجية للانزيم مع رقم هيدروجيني 9 وحضانة بمدى حراري بين 45-50 . انتج الانزيم بعد 18 ساعة من التلقيح وازداد تركيزه خلال طور الثبات . استعملت كبريتات الامونيوم برجة اشباع 60%-95% لترسيب الانزيم ونقي باستعمال المبادل الايوني DEAE Sepharose CL6B وهلام الغربلة Sephacryl S300 . تم استرداد 34.85% من الانزيم الكلي مع فعالية نوعية 7.066 وحدة/ملغرام مع 71.59 عدد مرات التنقية . النتائج تشير ان *Bacillus licheniformis* FH4-IRQ تفضل ظروف حرارة عالية وقاعدية لانتاج الانزيم دون الاعتماد على مصدر كاربوني محدد .

Introduction

Alkaline phosphatase (E.C.3.1.3.1) is belongs to the class of hydrolases and acts on phosphate groups. This enzyme catalyzes the hydrolysis of almost every phosphomonoester to give inorganic phosphate and the corresponding alcohol, phenol or sugar, and also catalyzes transphosphorylation reactions in presence of large concentration of a phosphate acceptor. It is a homodimeric enzyme and each active site region contains three metal ions, two zinc and one magnesium ion, all necessary for enzymatic activity **.** In nature alkaline phosphatases are found in many organisms, both prokaryotes and eukaryotes . The phosphatases are produced by bacteria , fungi, fish and mammals are relatively abundant .They are absent from higher plants**(1).** Alkaline phosphatase have been found in variety of micro-organism including and ***Bacillus*** species **(2).**

B. lechiniformis alkaline phosphatase represents extracellular enzyme that led to get pure enzyme with less steps than with intracellular or membrane bound form **(3)** .Several methods were used for alkaline phosphatase extraction and purification such as : precipitation by ammonium sulphate , ion exchange and gel filtration **.Yamane and Maruo (1978)** extracted alkaline phosphoesterase (APases) with alkaline phosphodiesterase (APDase) from the membrane fraction of ***B. subtilis*** 6160-BC6 and from the culture fluid of ***B. subtilis*** RAN 1. The purification done in several steps : precipitation by ammonium sulphate, DEAE A-25 , Celite 545 and Sephadex G-200 with 315 folds of purification **(4)**.

This stady aimed to characterize alkaline phosphatase from local isolated strain of ***Bacillus*** and to know the best growth conditions for alkaline phosphatase production.

Materials and Methods

Alkaline phosphatase production medium (5)

This medium was referred as **PB ALP production medium** (PB indicated Pandy and Banik) , it composed from glucose(1 gm),peptone (0.5 gm) ,yeast extract (0.1 gm) , $MgSO_4.7H_2O$ (0.02 gm) , KH_2PO_4 (0.002 gm) , NaCl (0.5 gm) and $CoCl_2$ (0024 gm . All components were dissolved in 90 ml D.W. , pH was adjusted to 8.5 and then volume was completed to 100 ml by D.W. and sterilized by autoclaving at 121˚ C for 15 min .

ALP production with different growth conditions(6)

Different incubation temperature

PB ALP medium (100 Ml) was inoculated with 4 Ml of bacterial culture (Bacillus licheniformis FH4.IRQ. that isolated in Iraq from potato and identified by 16 sRNA in GTCA company in Germany)(24 hr age) and incubated for 96 hr at different temperature . temperature used were (25 , 30 , 37 , 40 , 46 , 50 , 55 and 60) ˚C.

Incubation periods

PB ALP medium was inoculated with inoculum 24 hr and incubated in shaker water bath 46˚C , 175 rpm , for 96 hr. Samples (1 ml of culture) were taken every 1 hr to determine the biomass and ALP concentration.

Carbon sources

One hundred ml of PB ALP medium prepared with different carbon sources by replace the glucose by:- D-fructose , D-xylose , maltose , L-arabinose , D-mannose , glycerol phosphate di sodium salt , glycerol , sucrose , dextran , cellobiose , lactose , raffinose , ribose , sorbose , trehalose and mannitol at three different concentrations ,1% , 2% and 3%.

The medium was prepared , autoclaved and inoculated with 4 ml (24 hr age) bacterial culture and incubated in shaker water bath 175 rpm at 46 ˚C , for 96 hr .

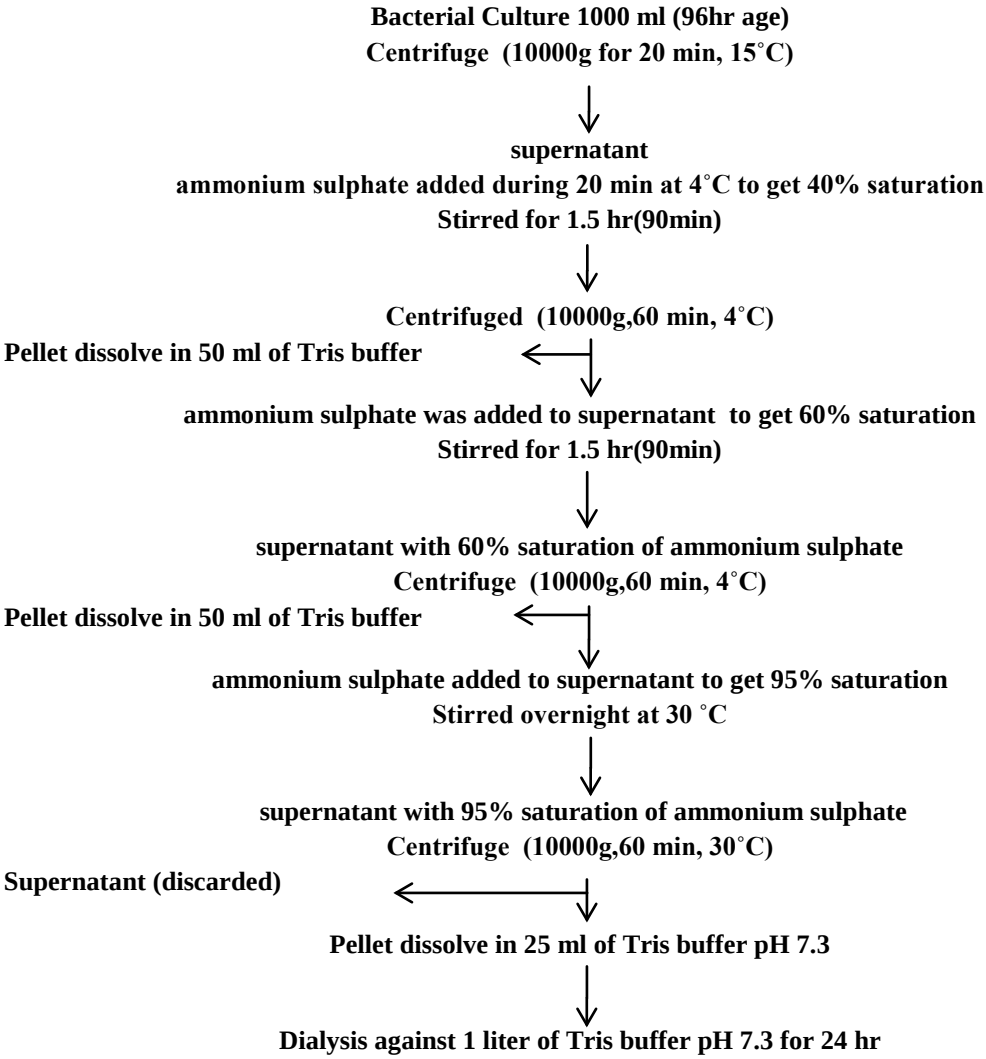
Different pH values

One hundred ml of PB ALP medium were prepared with different pH value . pH were adjusted by using three kinds of buffers : MES buffer, for acidic pH (5,6,7), Tris – acetate buffer for neutral and alkaline pH (7,8,9), CAPS buffer for extremely alkaline pH (9,10,11). the pH value were adjusted and inoculated with 4 ml (24 hr age) bacterial culture and incubated in shaker water bath 175 rpm at 46 ˚C , for 96 hr .

ALP purification

One litre of PB ALP medium (pH 8.5)was inoculated with 40 ml of bacterial culture and incubated for 96 hr at 50ºC .

Different saturation percentages of solid ammonium sulphate (35% , 40% ,50%, 60% , 70% , 80% , 90% , 95% and 100%) were used for batch wise precipitation of alkaline phosphatase. The following scheme explains the steps of ALP precipitation by ammonium sulphate.



Scheme showing Alkaline phosphatase precipitation by ammonium sulphate

Purification

Ion exchange chromatography by DEAE Sepharose CL6B

Tris base with pH was adjusted by acetic acid to 7.3 (for wash) .The same buffer was prepared with addition of sodium chloride in different amount to get the following concentrations :- (0.1 , 0.2 , 0.3 , 0.4 , 0.5 , 0.6 , 0.7 , 0.8 , 0.9 , 1.0 , 5.0) M (for elution).

After the dialysis of precipitated enzyme , it mixed with DEAE-Sepharose CL6B for 15 nin with gentle shaking . washing buffer was

added and stirred for 10 min then it separated , elution buffers were added as in washing buffer starting from the lower NaCl concentration to the highest one.

Purification by gel filtration

Sephacryl S-300 column (1x30)cm (Sigma Aldrich) was used for alkaline phosphatase purification.

Results and Discussion

Bacillus licheniformis was cultured on ALP production media at different conditions , the best growth temperature for alkaline phosphatase production ranged from 45 to 50º C ,sharp decrease in ALP production was noticed at 60ºC , in 25 to 30ºC the production was very low and it increase gradually with increasing of temperature to 50ºC(fig 1) , this may be as a result to the growth phase that alkaline phosphatase produce in , so , in low temperature this mean longer time to reach to end of log phase or beginning of stationary phase while the higher temperature decrease the time to reach to stationary phase .Hulett and Campbell used 55ºC as growth temperature for ALP production from ***Bacillus licheniformis*** **(5)** and many researches depended on this temperature for ALP production from ***Bacillus licheniformis*** **(7)** , some change on media were described by Spencer and co workers but without changing in incubation temperature**(8)**,the temperature 55ºC still used **(9 ,10, 11),** while another researchers used 37ºC as incubation temperature for alkaline phosphatase production from ***Bacillus subtilis*** and ***Bacillus*** in general **(12) .**

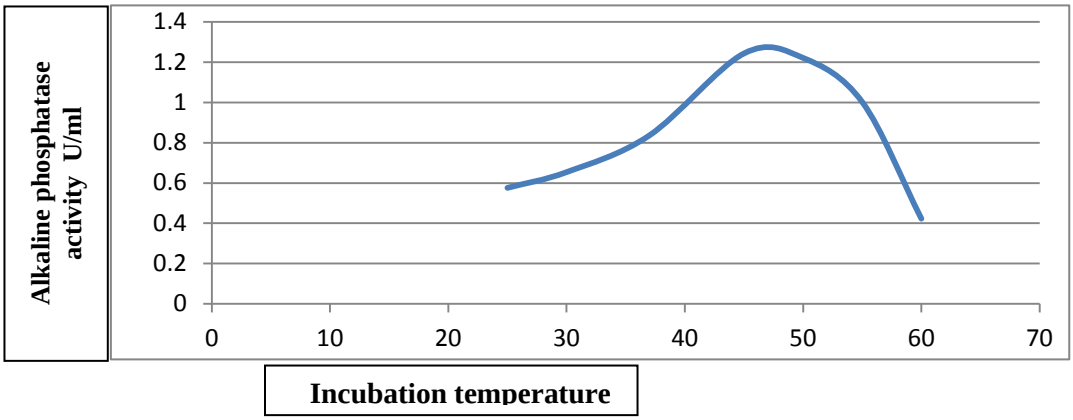


Figure 1: Effect of incubation temperature on alkaline phosphatase production from *Bacillus licheniformis* FH4-IRQ for 72 hr incubation.

Alkaline phosphatase production at different pH values

Wide range of pH values of media were used to determine the best one for alkaline phosphatase production.
Highest productivity seemed between pH 8 to 9 (1.09 -1.13 U/ml) , While pH 7 shows decrease in ALP production . Sharp decrease (0.211 and 0.43) U/ml happened in acidic pH (5 and 6) and extreme alkaline pH (11) 0.364 U/ml, (fig 2) . as in temperature , most researches depend on modified DM medium with pH value equal to 7.2**(9)** While Pandey and Banik (2010) (16)use their medium with pH value equal to 8 , Mahesh *et al*., (2010) used their medium with pH value equal to 8.8 **(12) .** pH is important factor in three dimensional conformation for any enzyme , in other bacteria , the alkaline phosphatase found inside cells or in periplasmic space that protect enzyme durin its synthesis from pH variation in culture media , while in Bacillus , alkaline phosphatase is external enzyme that be intact with the pH of media so it's important to adjust pH at appropriate value for enzyme conformation.

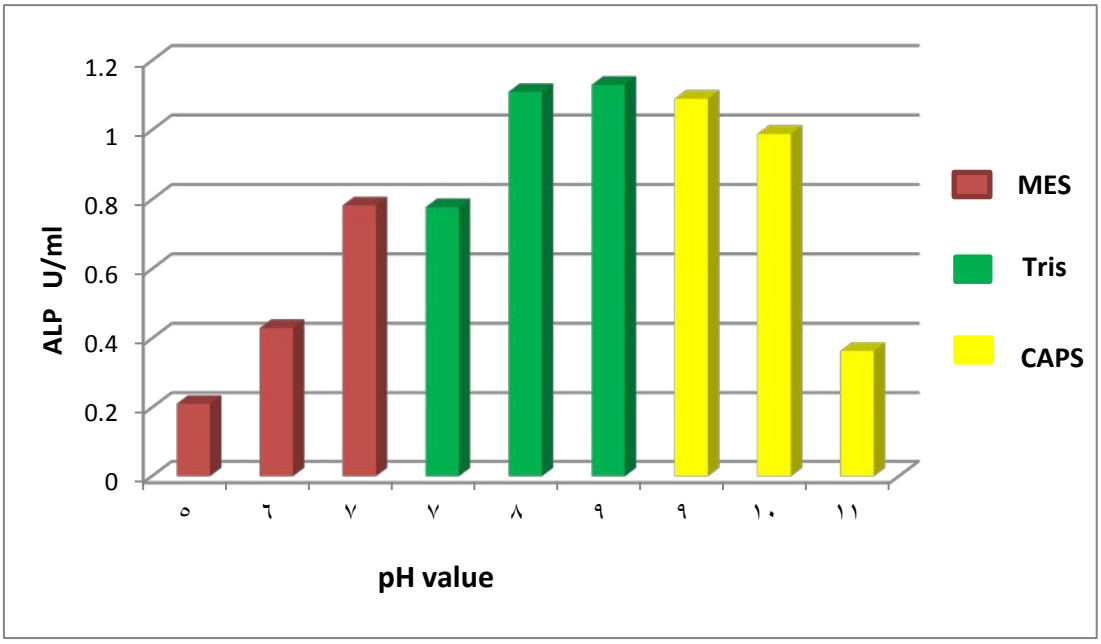


Figure 2: Effect of pH on alkaline phosphatase production from *Bacillus licheniformis* FH4-IRQ.

Effect of carbon sources on alkaline phosphatase production

Sixteen different carbon sources at three concentrations were used to determine the best one for alkaline phosphatase production from ***Bacillus licheniformis*** **FH4-IRQ.** isolate . The best sugars for alkaline phosphatase production were cellobios 1% (1.96U/ml),2%(1.96U/ml) and 3% (1.578U/ml), arabinose 3%(1.822U/ml) , maltose 2% (1.878U/ml) and 3%(1.9U/ml) , these carbohydrates gave productivity more than others (fig 3) .

Alkaline phosphatase was produced from ***Bacillus licheniformis*** by using DM medium containing fructose as a carbone source and Spencer did the same but with cobalt chloride addition **(5, 19)** . DM medium modified by Spencer and co workers is still used for the same purpose. Pandey and Banik prepared medium for alkaline phosphatase production from ***Bacillus*** that depend on glucose as carbon source **(6).**

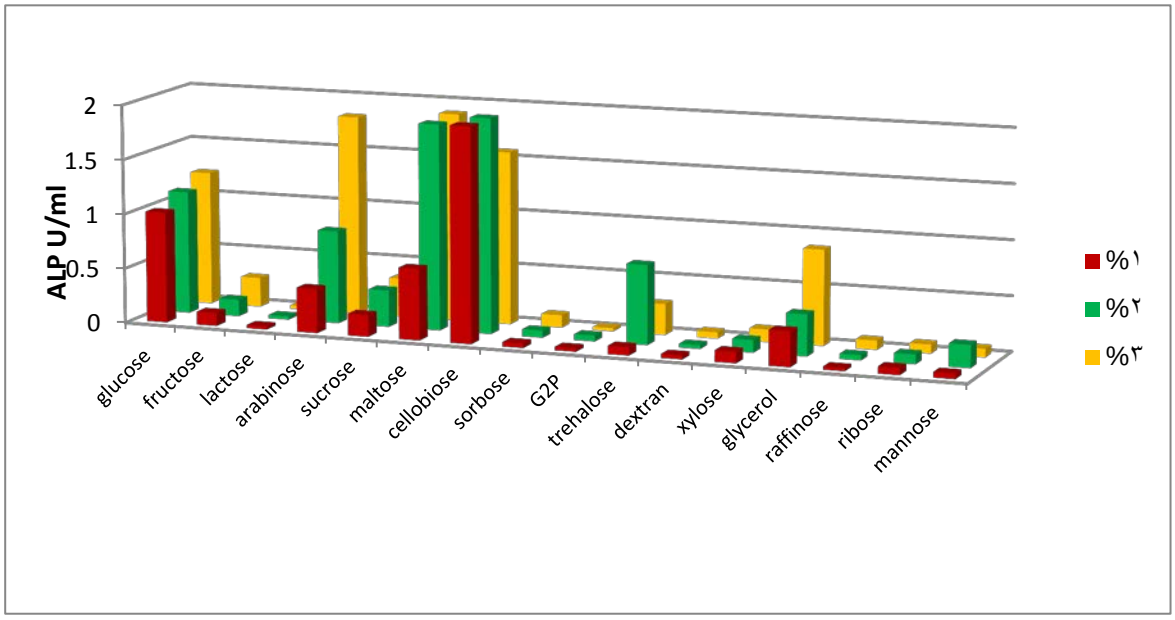


Figure 3: Effect of carbon source type and concentration on alkaline phosphatase production from *Bacillus licheniformis* FH4-IRQ. Isolate.

Alkaline phosphatase purification

Precipitation of alkaline phosphatase by ammonium sulphate

The result showed that (1) 81.4 % of enzyme was precipitated in the saturation range between 60-95% (table 1) . Many proteins were precipitated in saturation percentage below 60% , . Unwanted proteins can be removed from a protein solution mixture by salting out as long as the solubility of the protein in various concentrations of salt solution is known **(13).** The activity increased after dialysis(enzyme were dialyzed against 1 liter of Tris buffer) from 2.035 U/ml to 2.643 U/ml with specific activity 0.775 U/mg and 7.85 fold of purification .

Qader and co workers used 40% of ammonium sulphate saturation for alkaline phosphatase precipitation of ***Bacillus subtilis*** **(14) .** Mahesh and co workers used ammonium sulphate for ALP precipitation from ***Bacillus*** **spp**. ,but they didn't mention the saturation percentage that they used **(12).**

Ion exchange chromatography

The enzyme activity appear in elution fractions (0.5 – 0.7 M of NaCl), (fig 4) **,** that is mean ALP had negative charge enable it to bind with the resin of ion exchange . Most of alkaline phosphatase activity was eluted in 0.6 M of NaCl

Table 1: alkaline phosphatase precipitation at different ammonium sulphate saturation percentage.

Saturation % of $NH_4(SO_4)_2$	35%	40%	50%	60%	70%	80%	90%	95%	100%
ALP activity U/ml	0.003	0.013	0.147	0.198	0.343	0.692	1.017	1.148	1.167
ALP activity %	0.2	1.11	12.6	17	29.4	59.3	87.1	98.4	100

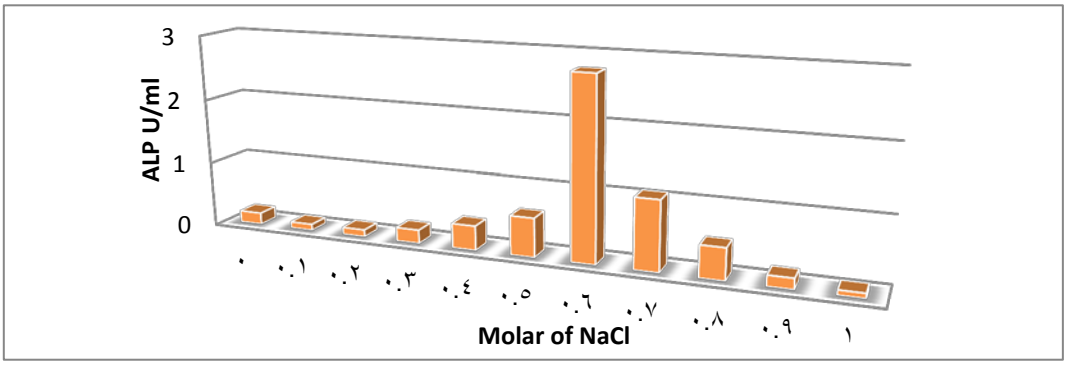


Figure 4: Alkaline phosphatase elution by NaCl from DEAE Sepharose CL6B ion exchange, fraction volume 15 ml .

The partially purified alkaline phosphatase had specific activity 5.198 U/mg with 41.9% recovery and 52.66 fold of purification . the sample was dialyzed against 1 liter of Tris acetate buffer pH 7.3 and concentrated by amicon tube to 1 ml to prepare it for gel filtration step.

Gel filtration by Sephacryl S-300

Three peaks of proteins were obtained ,in the elution of gel filtration, alkaline phosphatase activity was determined in the second one in the fractions 17-23 (fig 5), the enzyme recovery was 34.85% with specific activity 7.066 U/mg and 71.59 fold of purification (table 2) .Hulett and co workers recovered 10.28% of ALP of ***Bacillus licheniformis*** **MC14** (**(10) .**Mori and co workers recovered 12% of the total ***Bacillus staenothermophilus*** ALP **(15)** Sugahara and co workers recovered 30% from the total enzyme with 33 fold of purification from ***Bacillus subtilis*** **168 (16).** Mahesh and co workers recovered 36% of alkaline phosphatase from ***Bacillus*** **spp.(12)** Kostadinova and Marhova (2010) recovered 11.2% of ALP from ***Bacillus cerus*** with 282 fold of purification**(17).**

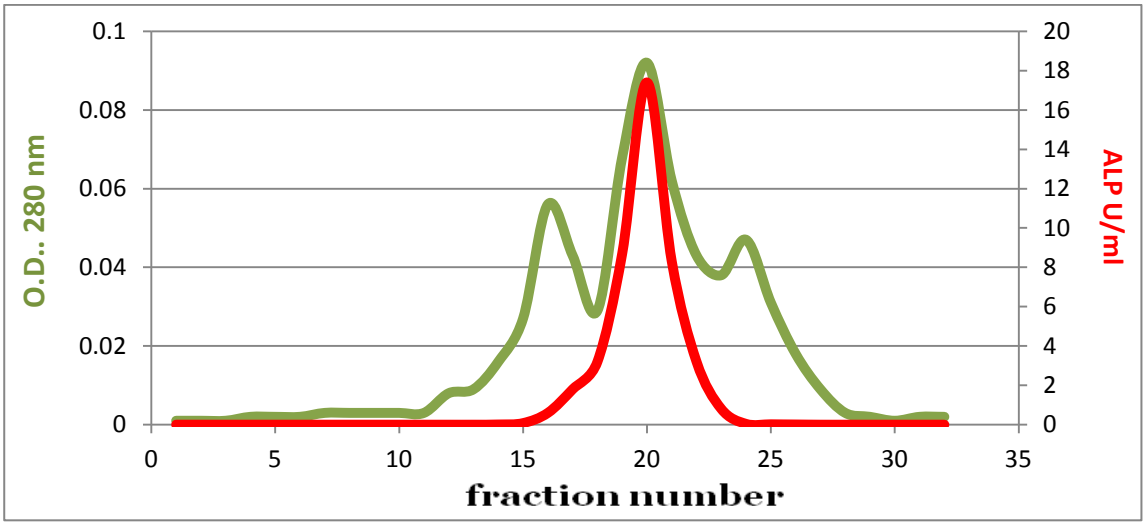


Figure 5: gel filtration chromatography with Sephacryl S-300 (1x30 cm) , elution buffer : 0.01M Tris acetate buffer pH 8 , fraction volume 1 ml , flow rate 0.4 ml/ min.

Table 2 : *Bacillus licheniformis* FH4-IRQ alkaline phosphatase purification steps.

Step	Volume (ml)	Activity (U/ml)	Total activity (U)	Proteins (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Fold of purification	Recovery (%)
Crude	100	0.987	98.7	10.198	1018.9	0.0987	1	100
Ammonium sulphate precipitation	30	2.643	79.29	3.4	102	0.775	7.85	80.3
DEAE-Sepharose CL6B	15	2.76	41.4	0.531	7.965	5.198	52.66	41.9
Sephacryl S300	3	11.47	34.4	1.613	4.84	7.066	71.59	34.85

Detection of Alkaline phosphatase purity by electrophoresis

The purity of ***Bacillus licheniformis* FH4-IRQ** alkaline phosphatase was checked by native Polyacrylamid gel electrophoresis, the enzyme seems as one band (fig 6) indicating the purity of ALP. The band appeared in the area that equivalent to the standard protein with molecular weight 120000 Dalton, that the native alkaline phosphatase (with two subunits) have this molecular weight .(standard proteins bands not shown here)

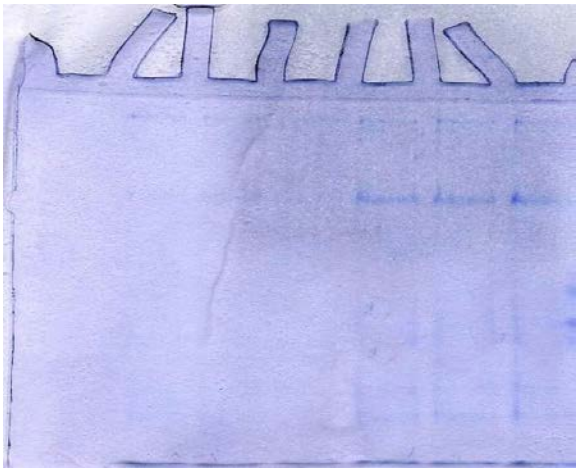


Figure 6: Conventional polyacrylamide gel electrophoresis of alkaline phosphatase purified from *Bacillus licheniformis* FH4-IRQ. Isolate.

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Detection of Mitochondrial Cytochrome C Oxidase subunit I (COI) Gene as a Molecular Marker for identification local *Rhopalosiphum* Aphid (Homoptera: Aphididae)

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University of Baghdad / College of Science / Department of Biology

Abstract: Aphid species are usually identified by microscopic examination of morphological features. This process requires significant expertise and is difficult in immature aphid stages that lack many diagnostic morphological features in addition to the characterization of polymorphism to this group so resolution is required the molecular techniques for aphid diagnosis. Since there were no molecular data or specific primers used to identify the local species. Thus, the current study aimed to detect of mitochondrial cytochrome C oxidase subunit I gene as a molecular marker. Lep primer was applied to local *Rhopalosiphum* aphids as the most common primer for aphid samples identification which were previously characterized by morphological features. Only 5% of samples was not be detected by this primer. We conclude the importance for sequencing and analysis of DNA sequences to design a specific primer for local *Rhopalosiphum* aphids that could improve the misidentification problems that are intrinsic with morphological features.

Keywords: *Rhopalosiphum*, Molecular Identification and Iraqi aphid.

الكشف عن جين Mitochondrial Cytochrome C Oxidase subunit I (COI) كعلامة جزيئية لتشخيص العزلات المحلية للجنس *Rhopalosiphum* (Homoptera: Aphididae)

رؤيا صفوان كمال، حيدر بدري علي

جامعة بغداد / كلية العلوم / قسم علوم الحياة

الخلاصة :عادة ما تشخص انواع المن من خلال استخدام طريقة الفحص المجهرى للصفات المظهرية. هذه العملية تتطلب خبرة كبيرة ومن الصعب تطبيقها على الاطوار غير الناضجة التي تغيب فيها الكثيرة من الصفات المظهرية التشخيصية بالإضافة إلى وصفها بتعدد المظهرية لذا فأن الحل هو تطبيق التقنية الجزيئية للحامض النووي في تشخيص إلمن. ولعدم وجود أي بيانات جزيئية متاحة للعزلات المحلية من الجنس *Rhopalosiphum* ، بالتالي عدم وجود بادئ متخصص لهذه العزلات المحلية. وهكذا، فإن الدراسة الحالية هدفت الى التحري عن **Mitochondrial Cytochrome C Oxidase subunit I (COI)** كعلامة جزيئية للمن. تم استخدام البادئ LEP مع هذه العزلات المحلية من الجنس *Rhopalosiphum* والذي يعد الأكثر شيوعا للمن والتي تم تشخيصها مسبقا من خلال الصفات المظهرية. فشل هذا البادئ في التحري عن 5% من النماذج فقط. نستنتج من هذا أهمية دراسة وتحليل تتابع الحمض النووي لتصميم بادئ متخصص للعزلات المحلية للجنس *Rhopalosiphum* ، حيث يمكن ذلك أن يحل مشاكل التشخيص الخاطئ للصفات المظهرية.

الكلمات المفتاحية: ***Rhopalosiphum*** ،التشخيص الجزيئي، الفونا العراقية للمن.

Introduction

Aphids species are a little group in compared with other insects species but their diversity is very high because of polymorphism reality (1). Aphids are important pests, with over 250 species found on agricultural crops worldwide (2). Classification of aphids is according to their morphological traits like other insects. It means that main differences or main similarities of samples are being compared and they are being located in their own location (3). Aphid classification is being discussed very much today especially in one main category: their family numbers (4) .There have been many morphological anatomical studies conducted on aphids which have prepared background for systematic studies about them (5). Aphids have high ability for adaption and changing and their morphology is being affected by environmental factors. Many ecological physiological factors affect morphological form of aphids (1). Using of Molecular technique for species-level identification may, at first aspect, seem to represent an appropriate use of new technology to solve an old problem—identifying and classifying of this group. Animal mitochrondrial DNA (mtDNA) is a small circular molecule ranging in size from 15 to 18 kilo base pairs (bp) (6). Insect mtDNA consists of thirty-seven genes including two ribosomal RNA (rRNA) genes, twenty two transfer RNA (tRNA) genes and thirteen protein coding genes (7; 8; 9). The region of mtDNA containing cytochrome oxidase I and II (COI-II) has been used in systematic and population genetic studies of insects, including aphids (10; 11; 12). Molecular identification techniques provide not only objective and reliable data in the form of DNA sequences to identify Aphid species, thereby serving to corroborate morphological identifications, but also can provide good evidence for constructing phylogenetic relationships (13). The aphid genus *Rhopalosiphum* Koch, 1854 comprises 20 species worldwide (14; 15; 16). Eight species of this genus are registered in Europe (17), three of them ***Rh. maidis*** (Fitch, 1856), Rh. padi (L., 1758) and ***Rh. rufiabdominale*** (Sasaki, 1899) were recorded from Iraq (18). We applied here Lep primer to local *Rhopalosiphum* aphids as a common primer for aphid samples identification which was previously characterized by morphological features.

Materials and Methods

Taxonomic Sampling

We examined about 82 individual Aphid specimens obtained from different locations in Baghdad, Diala and Babylon

provinces. on different grasses plant such as wheat, barley, corn and others. Specimens were identified to species level by the second author. Taxonomy and nomenclature were as described by Remaudie`re and Remaudie`re (14), Nieto Nafria et al. (19), Eastop and Blackman (20) and Favret (21). All the aphid specimens used for molecular analyses were collected and preserved in 95 or 99% ethanol, and the samples for maceration and slide preparation were collected in 70-80% ethanol. Morphological terminology, measurement range, and location of each character follow Heie (22) and Blackman and Eastop (23). All the examined specimens were deposited in the Iraqi Natural History Museum, University of Baghdad.

DNA Extraction

DNA was extracted from 2-3 individuals of each sample with the Geneaid DNA Mini extraction kit for (Tissue), according to the standard protocol recommended by the manufacturer, with some modification. DNA of the filtrate was eluted according to kit instructions with 40µl DNA elution buffer and stored at -20 C° until use.

Amplification for COI Gene

The cytochrome c oxidase I gene was amplified with LepF (5′-ATTCAACCAATCATAAAGATATTGG-3′) (forward) and LepR (5′-TAAACTTCTGGATGTCCAAAAAATCA-5′) (reverse) (24). The DNA fragments to be analyzed were amplified using AccuPower_ PCR PreMix (BIONEER, Corp., Daejeon, Korea) in 50µl reaction mixtures containing 0.4 µM each primer and 100-200 ng of genomic DNA template. The PCR amplification protocol was performed according to the following procedure: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 60 s; annealing temperature 46.7 °C for 30 s; and extension at 72 °C for 60 s and final extension at 72°C for 5 min.

Results and Discussion

Morphological analysis:

The characters used for identification and differences between the collected species were morphometric parameters of the followings: length of Ant.I, Ant.II, Ant.III, Ant.IV, Ant.V, Ant.VI, Ant.VIb for antennal segments I, II, III, IV, V, VI, and the base of Ant.VI, respectively; PT (processus terminalis) ; ratio of Ant VI BASE to PT (‘ANT PT/BASE’) is a frequently used discriminant; length of last two segments and the number of accessory hair of rostrum and usually form a combined structure (R IV+V), the length of which is often compared

with that of the 2nd segment of the hind tarsus (HT II); and length of SIPH (siphunculus) which is often compared with length of cauda. According these morphological traits from about 120 aphid samples only two aphid species were identified from some middle Iraq provinces on different grasses plant. These species were ***Rhopalosiphum maidis*** and ***Rh. Padi*.** Because of one of important specifics of aphids is similarity of their morphological traits which make their identification difficult (25) many samples identified to ***Rhopalosiphum*** genus taxa only

Molecular analysis:

DNA amplification: The result of the PCR amplification from initial denaturation at 95 C° for 5 min, followed by 35 cycles of 95 C° for 60 s; annealing temperature 46.7 C° for 30 s; and extension at 72 C° for 60 s and final extension at 72 C° for 5 min. Amplification gave approximately 710-bp fragments from all amplified DNA templates shown in figures 1.

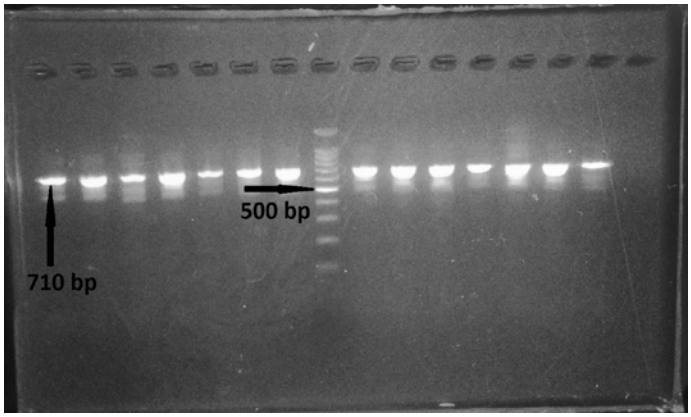

Figure 1. 1.0 % agarose gel of PCR amplicons partial of Mitochondrial Cytochrome C Oxidase subunit I (COI) Gene of aphid specimen derived from primer pairs LepF and LepR. 1000-100bp DNA Ladder

Since Amplification of Mitochondrial Cytochrome C Oxidase subunit I (COI) Gene to separate Iraq local *Rhopalosiphum* species using a common primer pairs as LepF and LepR primers, failed to give a good difference size bands between *Rh. padi* , *R. maidis* and other *Rhopalosiphum* species, also In this respect, not only the COI analysis did not recover the differences between *Rhopalosiphum* spp. but also doesn't improve the differences between several aphid genera (26). So this study confirmed the importance to designing specific primers that show a size difference

between *R. padi* and the other *Rhopalosiphum* species. Jean-Christophe *et al*. (27) designed a specific primer sequences based on the complete sequence of the leucine plasmid of the bacterial endosymbiont of *Rh. Padi* to diagnosis of breeding system of this aphid species. While Bulman, *et al.* (28) used other specific primers located in ribosomal small (SSU) and large subunit (LSU) RNA genes to study to separate New Zealand *Rhopalosiphum* species. This study emphasized the need to design specific primers to identify local *Rhopalosiphum* species. And studying the sequences of all amplified DNA fragments templates products obtained in this study is extraordinary for overcoming of the misidentification problems that are inherent with morphological features.

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Role Cytolethal distending toxin B (CdtB) in *Salmonella enterica* serovar Schwarzengrund

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Abstract: Cytolethal distending toxin (CDT) expressed in various Gram-negative pathogenic bacteria is a virulence factor that induces distention or apoptosis in the host cells through DNA damage. *Salmonella enterica* serovar Schwarzengrund isolates from clinical samples were screened for *cdtB* by PCR. To understand the role of CdtB in *S.* Schwarzengrund, a *cdtB* deletion mutant strain (Δ*cdtB*) was constructed. *In vitro* cultured rat epithelial cell were infected with a wild type strain and its isogenic Δ*cdtB* to determine whether the strains of *S.* Schwarzengrund are responsible for typical signs of cytolethal distending intoxication, including cytoplasmic distension, and nuclear enlargement of host target cells. The results showed that rat intestinal epithelial cells infected with *S.* Schwarzengrund became distended and the nuclei were larger than those infected with Δ *cdtB.* In addition, the wild type strain induced an increased cytotoxic LDH (Lactate Dehydrogenase) release from rat epithelial cells up to 72 h after infection as compared with its isogenic *cdtB* mutant. In conclusion, the CdtB from *S.* Schwarzengrund damages DNA, resulting in cytoplasmic distension and nuclear enlargement of rat epithelial cells after 72 h of infection, similar to that from *S.* Typhi. Therefore, CdtB produced from non-typhi *S.* Schwarzengrund strains may play an important role in pathogenesis in host cells.

Key words: Cytolethal distending toxin B, *Salmonella* Schwarzengrund

دور جين CdtB في بكتريا السالمونيلا انتريكا نوع Schwarzengrund

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الخلاصة : يفرز سم CdtB في انواع مختلفة من بكتريا المرضية السالبة لصبغة كرام والذي يعتبر عامل ضراوة مسببا انتفاخ للسايتوبلازم والنواة او موت الخلايا المبرمج للمضيف بسبب الاضرار التي يسببها الى DNA . سالمونيلا نوع Schwarzengrund عزلت من عينات مرضية وتم استخدام تقنية PCR لمعرفة وجود جين *cdtB* في تلك العزلات. لفهم دور CdtB في بكتريا السالمونيلا نوع Schwarzengrund تم احداث طفرة وراثية (Δ*cdtB*) بأزالة هذا الجين من بكتريا السالمونيلا. مختبريا تم استخدام الخلايا الظهارية لامعاء الفئران اصيبت ببكتريا السالمونيلا نوع Schwarzengrund الحاملة لجين *cdtB* وبكتريا سالمونيلا نوع Schwarzengrund تم أزالة جين *cdtB* منها لتحديد فيما ان بكتريا سالمونيلا نوع Schwarzengrund المسؤولة عن احداث انتفاخ في السايتوبلازم ونواة خلية المضيف. أظهرت النتائج أن الخلايا الظهارية في أمعاء الفئران المصابة ببكتريا السالمونيلا نوع Schwarzengrund الحاملة لجين *cdtB* أصبحت منتفخة وكانت نواة أكبرمقارنة مع خلايا الظهارية لامعاء للفئران المصابة ببكتريا السالمونيلا نوع Schwarzengrund تم أزالة جين *cdtB* منها. بالاضافة الى ذلك أظهرت النتائج ايضا أن الخلايا الظهارية للفئران المصابة ببكتريا السالمونيلا Schwarzengrund الحاملة للجين *cdtB* بعد 72 ساعة من الاصابة تفرز كمية كبيرة من انزيم LDH(Lactate Dehydrogenase) مقارنة ببكتريا سالمونيلا Schwarzengrund تم أزالة جين *cdtB* منها . ومن خلال النتائج تبين ان cdtB في بكتريا سالمونيلا نوع Schwarzengrund له دور مهم في تلف DNA مسببة بذاك انتفاخ سايتوبلازم ونواة خلايا الظهارية لامعاء للفئران بعد 72 ساعة من الاصابة وهذه العلامات مشابة لبكتريا سالمونيلا التيفوئيد .لذلك CdtB المنتج من بكتريا سالمونيلا نوع Schwarzengrund يلعب دورا مهما في امراضية خلايا المضيف.

1. Introduction

Salmonella infection is one of the major food-borne illnesses in the United States. So far, more than 2,610 serovars of *Salmonella enterica* have been recognized from all over the world, and almost all are able to cause some illness in humans and animals. One of the most important *Salmonella* serovars are *Salmonella* Schwarzengrund which is still an important cause of human illness (CDC, 2010). *S.* Schwarzengrund is spreading internationally from chicken to humans in Thailand and from imported food products to humans in Denmark and the USA (Aarestrup *et al*., 2007). There are various strategies for invasion during interaction with the hosts to create a better environment for bacterial replication (Mattoo *et al*., 2007). DNA damage is a one of these strategies by bacterial toxins that accomplish these strategies is cytolethal distending toxin B (CdtB) (Mattoo *et al*., 2007). CdtB was discovered in *Campylobacter* spp. and other pathogens such as enterohaemorrhagic *E. coli*, *Shigella dysenteriae*, and *Helicobacter hepaticus* which is causing distention of both cytoplasm and nucleus (Argon *et al*., 1997). In other hands CdtB also was characterized in *Salmonella* Typhi as one of the "Typhoid Toxins" which possibly has important roles in unusually lengthy persistence and spread of *S.* Typhi, causing fatal systemic diseases (Spano, *et al*., 2008 &Haghjoo &Galan, 2004) . Previous studies have indicated that comparative genome sequence analysis of *Salmonella* enterica subspecies *enterica* revealed two subpopulations, called clade A and clade B. CdtB was present in clade B isolates, including *S.* Schwarzengrund, and only a small subset of clade A, including *S.* Typhi .The CdtB shares between clade B (*S.* Schwarzengrund) and clade A (*S.* Typhi) suggesting that this gene may has been horizontally transferred between them (Bakker *et al*., 2011). Furthermore, the *cdtB* gene could develop severe symptoms similar to typhoid fever in patients with non-typhoidal *Salmonella,* including *S.* Schwarzengrund. Therefore, an understanding of role of CdtB in non-typhoidal *Salmonella* (*S.* Schwarzengrund) is necessary for elucidating invasive salmonellosis caused by *S.* Schwarzengrund.

2. Materials and methods

2.1. Bacterial strains

Thirty isolates of *S.* Schwarzengrund of clinical origin were obtained from the Arkansas of Department Health (AHD).(Arkansas State - USA).

2.2. PCR detection of cdtB.

S. Schwarzengrund isolates were screened for the *cdtB* gene by the simplex PCR method using single set primers (Skyberg *et al*., 2006), using the following primers: forward primer 5'- GCCA TGGAAAAACCTGTTTTTTTCCTT CTG -3' and reverse primer 5'- GCTCGAGACAGCTT CGTG CCAAAAAGGCTA -3.The template DNA from the isolates was extracted from overnight cultures by using the DNeasy ® Blood and Tissue kit (Qiagen, Valencia, CA, USA). The PCR reaction mixture with a final volume of 10 µl, 1 µl of template DNA , 200 µM of each dNTP, 0.25 µM of forward and reverse primers, 2.5 units of *Taq* DNA polymerase (Qiagen) and 1 × PCR buffer . Cycling conditions included 95 °C; 30 cycles of 40 s at 94 °C, 60 s at 66.5 °C, and 90 s at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were analysed by electrophoresis in 1× Tris-acetate-EDTA buffer at 50 V for 85 min on 1.2% agarose gels.

2.3. *Construction of cdtB mutant*

Deletion of *cdtB* in *S.* Schwarzengrund strain was created by using lambda Red mutagenesis (Datsenko and Wanner, 2000). Briefly, primers were designed to amplify a *kan* cassette encoded on plasmid pKD4, which is flanked by FLP recombinase target (FRT) site (Datsenko and Wanner, 2000). PCR products were generated by using pairs of primers (kanF-cdtB ACTACAAAGTTATGACCTGGAA TCTTCAGGGCTCTTCAGTGTAG GCTG GAG CT GCTTC and kanR-cdtB AATAAGGTGCTCGATCGACAAT GACCCCATAATCTACATAT GAATATCCTCCTTAG. The PCR products were purified by using PCR purification kit, treated with *DpnI* (New England BioLabs, Ipswich, MA) to eliminate methylated template DNA and introduced into *S.* Schwarzengrund carrying the plasmid pKD46 (which encodes the lambda red recombinase system under the control of an L-arabinose-inducible promoter) by using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA) with a 0.2 cm cuvette (Bio-Rad Laboratories) to generate the Δ*cdtB::kan* and selected by incubating on an LB plate containing 50 mg/L kanamycin. To resolve the antibiotic cassettes, the temperature sensitive plasmid pCP20 was introduced to the strain Δ*cdtB::kan* to encode the FLP recombinase.

2.4. *Cell and bacterial culture conditions*

The rat intestinal epithelial cell (REC) line (CRL 1589) was used. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and gentamicin (50 µg/ml, Sigma) at 37°C in a humidified atmosphere of 5% CO_2. The cell line was seeded at a density of 5 x 10^4 cells ml^{-1} into 24-well plates (1 ml per well) for biological assays. *S.* Schwarzengrund was grown and maintained in LB broth (BD Biosciences, Boston, MA).

2.5. *Cytolethal distending toxin assay*

RECs were seeded into 6-well tissue culture plates at a density of $1x10^5$ cells per well in 1.5 ml medium. The plates were incubated at 37ºC in an incubator with 5% CO_2 for 22-23 h before bacterial inoculation. The media were replaced with fresh DMEM containing 10% FBS 30 min before inoculation with the *Salmonella* (wild type and mutant *cdtB* strain). The cell lines were infected at an MOI of 200 and incubated for 1 h at 37ºC in 5% CO_2 incubator. Cells were then washed 3 times with pre-warmed PBS. DMEM with gentamicin (100 µg/ml) was added to each well and the 6-well plates were incubated for 1 h. Cells were washed 3 times with pre-warmed PBS, again. The cells were then incubated for a wide range of times (24 to 72 h) at 37ºC and 5% CO_2. By this time, the wells were washed 3 times with PBS, fixed with 3% paraformaldehyde, and observed microscopically by light microscope for demonstration of morphological changes. This experiment was duplicated once. The nuclear morphology of control and rat intestinal epithelial cells inoculated with *Salmonella* (wild type and *cdtB* mutant strains) was examined by using a fluorescent microscope (Evos All In One digital inverted microscope). After aspirating growth medium and washing with PBS, the cells were fixed with a 3% paraformaldehyde solution for 10 min at room temperature. At the end of the incubation, each culture was washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 2 min, and stained with propidium iodide (Sigma). The cells were examined under a fluorescent microscope (Aragon *et al*., 1997).

2.6. *Detection of Lactate Dehydrogenase Activity*

RECs were harvested at various time intervals (1, 24, 48 and 72 h) after infection. The medium was then removed to determine the extracellular lactate dehydrogenase

(LDH) content. The intracellular LDH contents and the LDH released in the medium were analyzed by the cytotoxicity detection kit (LDH; Promega) according to instructions provided by the manufacturer. The absorbance was read at 490 nm using an immunoassay system (Dynatech, Cambridge, MA). The LDH efflux was expressed as a percentage of the total LDH release.

2.7. *Statistics*

The statistical differences between wild type *cdtB* and its isogenic *cdtB* mutant strains was calculated by one-way ANOVA test followed by Duncan's test using Sigma-plot (SigmaPlot 11 Systat Software Chicago Ill USA).

3. Results

3.1. Detection of cdtB gene in S. Schwarzengrund *isolates by PCR*

All isolates of *S.* Schwarzengrund used in this study were positive for *cdtB* by PCR (Fig. 1).

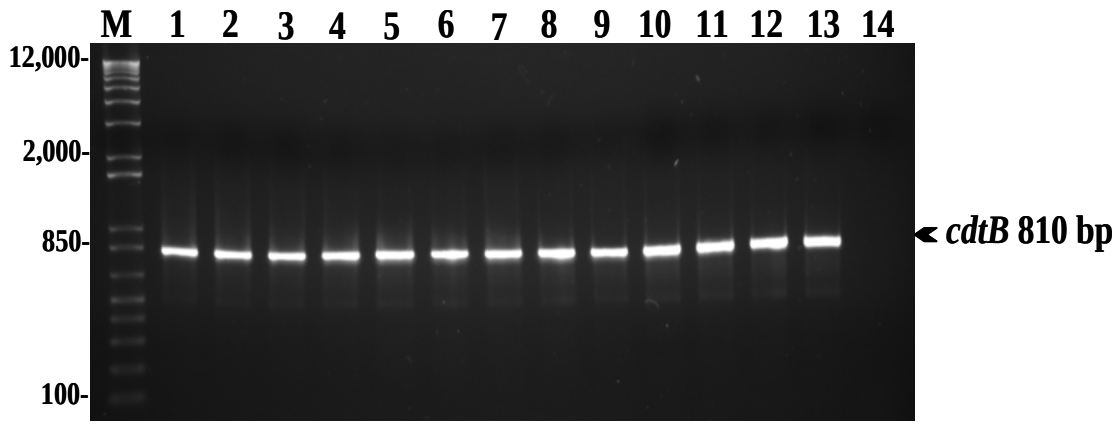

Fig. 1. Agarose gel electrophoresis of *cdtB* PCR products amplified from *S.* Schwarzengrund. Lane 1: M kb ladder; 2 to 13: *S.* Schwarzengrund isolates; Lane 14: negative control.

3.2. Cytolethal distending toxin assay

The cytolethal distending toxin assay was performed on the RCE cell lines for 24 to 72 h. The results showed nuclei in the RECs were distended and larger than the control and the mutant *cdtB* during various periods of time (Fig. 2).

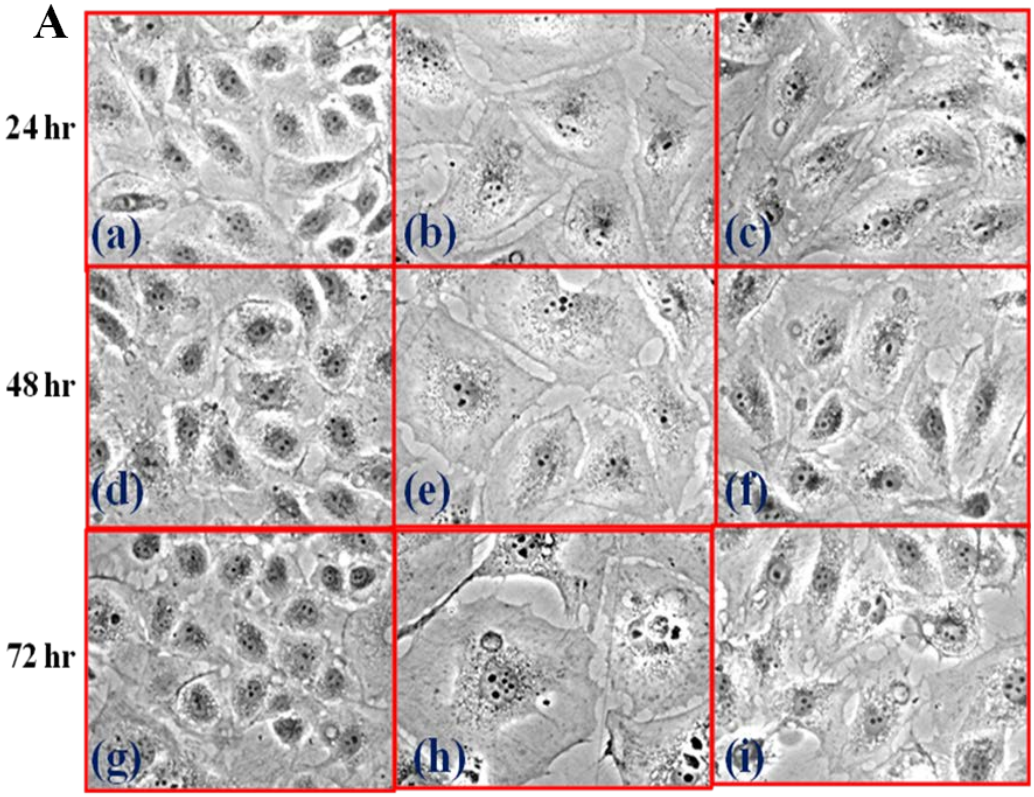

Fig. 2.. Effect of wild type and its isogenic ΔcdtB on rat intestinal epithelial cell morphology. (a), (d) and (g) control for various periods of time (24 h to 72 h). (b) and (c) Rat intestinal epithelial cells infected with wild type and mutant cdtB, respectively, for 24 h. (e) and (f) cells infected with wild type and mutant cdtB, respectively, for 48 h. (h) and (i) cells infected with wild type and ΔcdtB, respectively, for 72 h.

During 48 to 72 h of infection. In addition, the RECs appeared greatly enlarged, with diameters approximately three times those of the control cells, and in some cases more than five times. After 24 to 48 h of infection, cells infected with the *S.* Schwarzengrund mutant of *cdtB* did not show signs of toxicity, such as distension of both cytoplasm and nucleus (Fig. 2). After the RECs were incubated with the wild type strain and its isogenic Δ*cdtB*, they were stained with propidium iodide and examined under a fluorescent microscope. The fluorescent microscope images show that the RECs with *S.* Schwarzengrund mutant of *cdtB* had small nuclei (Fig.3).

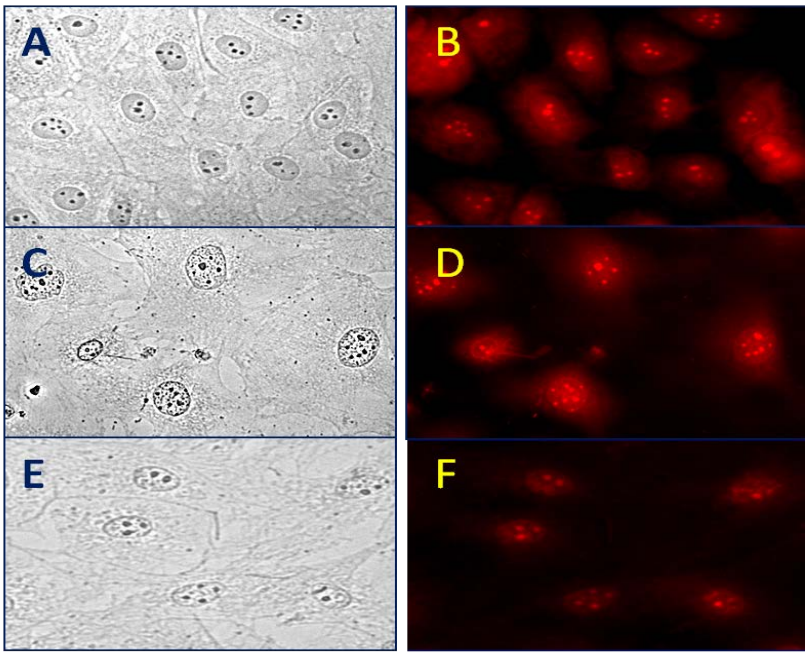

Fig. 3. Fluorescent photomicrographs (A and B) control for 72 h. (C and D) Rat intestinal epithelial cells incubated with wild type *cdtB* for 72 h. (E and F) Rat intestinal epithelial cells have been infected with Δ *cdtB*. All images shown were stained with propidium iodide at the same magnification (40 X) of the fluorescent microscope.

In contrast, the RECs with the *cdtB* wild type strain had a greater nuclear size after 72 h of infection. These results combined indicate that CdtB displays the same signs of toxicity in the host cells as other bacterial pathogens, encoding *cdtB*.

3.3. Detection of Lactate Dehydrogenase (LDH) Activity

The LDH activity of the infected RECs was measured to confirm cell death, to determine whether the *S.* Schwarzengrund strains made the host cells extracellularly release cytosolic LDH to the culture medium upon disruption of the plasma membrane integrity, resulting in apoptosis (or necrosis) and cellular toxicity. In RECs infected with the *cdtB* wild type, there was a significant difference in cytosolic LDH release ($p < 0.05$) from RECs within 72 h after infection (Fig. 4)

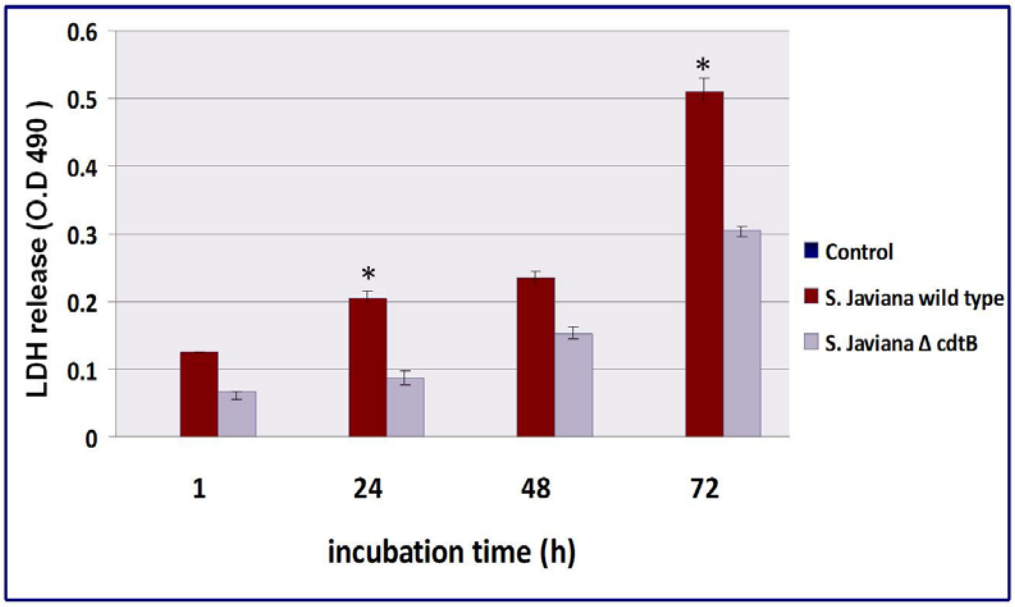

Fig. 4. LDH release from rat epithelial cells after various times (1 to 72 h) of infections with *S*. Schwarzengrund wild type or its isogenicΔ *cdtB*. The histogram shows the relative amounts of LDH released(OD 590) from the indicated cells by *S*. Schwarzengrund wild type of *cdtB* and its isogenic Δ*cdtB*.compared to the control and RECs infected with Δ*cdtB*. In addition, cytotoxicity did not differ significantly between the control and the *cdtB* mutant- infected monolayers of the rat's intestinal epithelial cells. Within 24 to 48 h of infection, there was no observed significant difference between cytotoxic LDH release in the wild type *cdtB* strain and its isogenic Δ*cdtB* (Fig. 4). These results suggest that cytotoxicity was CdtB-dependent in the rat intestinal epithelial cells.

4. Discussion

Cytolethal distending toxin B (CdtB) has been found in several Gram-negative bacterial pathogens, which induce severe host cell distension of infected target cells (Johnson, and Lior,1987; Escalas *et al*., 2000; Shenker *et al*., 1999). The *cdtB* as an essential virulence gene has been detected among *Salmonella* serovars especially *S.* Typhi, which has been correlated with prolonged persistence and systemic disease (Spano *et al*., 2008). In this study we demonstrate that *S.* Schwarzengrund CdtB a non-typhoidal serovar, causes distension of the cytoplasm and nuclear in RECs.

This study showed that all *S.* Schwarzengrund isolates from clinical

samples contained *cdtB*, which encoded CdtB toxin. The results of this study suggest that *cdtB* might have been horizontally transferred between *S.* Typhi and non-typhoidal serovars, into the bacterial DNA chromosome (Bakker *et al*., 2011). To understand the role of CdtB in *S.* Schwarzengrund , a *cdtB* deletion mutant was constructed .The construction of the *cdtB* deletion mutant determined the responsibility of *cdtB* for typical signs of cytolethal distending intoxication (cytoplasmic distension and nuclear enlargement) and LDH. A cell line model was established to investigate how CdtB induced these typical signs. RECs were infected with wild type *cdtB* strain and its isogenic Δ*cdtB* to satisfy the cytolethal distending criteria. This study shows that the RECs became distended and the nuclei were more enlarged than in the controls and the Δ*cdtB* strain 72 h after infection. The distension of the cytoplasm and nuclei in RECs cultures infected with the *cdtB* wild type strain of *Campylobacter* sp.was originally reported by Johnson & Lior (Johnson and Lior, 1988). Other studies, by Haghjoo and Galan (2004) and Spano *et al.* (2008), showed cellular distension in HeLa cells after infection with CdtB of *S.* Typhi. These results indicate that CdtB induces the same characteristic cellular distention in the host cells, as do other bacterial pathogens that encode the CdtB subunit. In addition, these results show that RECs infected with the wild type *cdtB* strain and examined by a fluorescent microscope showed expanded nucleoli compared with its isogenic *cdtB* mutant, suggesting that the *cdtB* gene plays an essential role in nuclear enlargement (Dassanayake *et al*., 2005). These data are contrary to those of Elwell *et al* (2000) and Spano *et al.* (2008), whose studies suggest that the CdtB from *S.* Typhi is sufficient to induce clear signs of intoxication, such as cellular distension.

Furthermore, we characterized the cytotoxicity of *cdtB* wild type and its isogenic mutant by using LDH activity as an indicator of cell membrane integrity, which leads to either apoptosis or necrosis. RECs infected with *cdtB* wild type showed a significantly increased level of cytotoxicity as compared to isogenic Δ*cdtB* that appeared as a low level of cytotoxicity after 72 h of infection. This observation was consistent with previous studies, which show that some bacterial pathogens that encode *cdtB* wild type releas high levels of LDH compared with isogenic Δ*cdtB* (Rabin *et al*., 2009; Purdy *et al*., 2000).

In conclusion, *S.* Schwarzengrund strains caused cytoplasmic and nuclear enlargement together with LDH towards RECs that are characteristic of CDT activity of other pathogen encode *cdtB* . Further studies on purification and expression of CDTB toxin in non-typhoidal *Salmonella* strains (*S.* Schwarzengrund) will reveal the role of this toxin in pathogencity of this important serovar.

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The Hypoglycemic and Hypolipidemic Activity of Aqueous Green Tea Extract in Normal and Alloxan-Induced Diabetic Male Albino Rats

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Abstract: This study was designed to investigate the hypoglycemic and hypolipidemic activity of aqueous green tea (GT) extract in normal and alloxan-induced diabetic rats. Forty adult male albino rats weighing 180-200 g were divided into four groups of 10 rats each: The first group was served as control; the second group was treated with GT (250 mg/kg b.w); the third group was affected by inducing experimental diabetes by intraperitonial injection of alloxan (150 mg/kg b.w); and the fourth group was affected by inducing diabetes and treated with GT (250 mg/kg b.w). Level of blood glucose and lipid profile [total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C)] were evaluated at the ending of the experimental period which was designed for 4 weeks.

The results revealed that blood glucose level was significantly ($P < 0.05$) less in the GT-treated group than in the control. Also, diabetic rats treated with GT showed a significant ($P<0.05$) reduction in blood glucose level when compared with diabetic group. Levels of TC, TG, LDL-C and VLDL-C were significantly ($P < 0.05$) lower while the level of HDL-C was significantly ($P < 0.05$) higher in the GT-treated group than that in the control. There was a significant ($P<0.05$) increase in levels of TC, TG, LDL-C and VLDL-C and a significant ($P<0.05$) decrease in level of HDL-C in diabetic rats when compared to the control group. However, administration of GT extract restored the levels of serum lipids to normal values.

Keywords: Green Tea, Alloxan, Rats.

فعالية خفض السكر وتقليل الدهون للمستخلص المائي للشاي الاخضر في ذكور الجرذان البيض الطبيعية والمصابة بالسكري المستحدث بالالوكسان

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الخلاصة: صممت هذه الدراسة للتحري عن قابلية المستخلص المائي للشاي الاخضر على تقليل سكر ودهون الدم في الجرذان الطبيعية والمصابة بالسكري المستحدث بالالوكسان. تم تقسيم 40 من الجرذان الذكور، تراوحت اوزانهم بين 180-200 غرام، الى اربعة مجاميع متساوية: اعتبرت المجموعة الاولى مجموعة سيطرة، عوملت المجموعة الثانية بالشاي الاخضر (250 mg/kg b.w) ، تم استحداث السكري بالالوكسان (150 mg/kg b.w) في المجموعة الثالثة، اما المجموعة الرابعة فعوملت بالشاي الاخضر بعد استحداث السكري. بعد مرور اربعة اسابيع، المدة المحددة للتجربة، تم تقدير مستوى الكلوكوز في الدم وكذلك صورة الدهون [الكوليستيرول الكلي، الشحوم الثلاثية، البروتين الدهني العالي الكثافة، البروتين الدهني الواطئ الكثافة، و البروتين الدهني الواطئ الكثافة جداً.
اظهرت النتائج بأن مستوى الكلوكوز في الدم كان أقل معنوياً في المجموعة المعاملة بالشاي الاخضر مقارنة مع مجموعة السيطرة. كذلك، اظهرت المجموعة المعاملة بالشاي الاخضر بعد استحداث السكري انخفاض مستوى الكلوكوز في الدم مقارنة مع المجموعة المصابة بالسكري. مستويات الكوليستيرول الكلي، الشحوم الثلاثية، البروتين الدهني الواطئ الكثافة، والبروتين الدهني الواطئ الكثافة جداً كانت منخفضة معنوياً بينما مستوى البروتين الدهني العالي الكثافة كان مرتفعاً معنوياً في المجموعة المعاملة بالشاي الاخضر مقارنة مع مجموعة السيطرة. كانت هناك زيادة معنوية في مستويات الكوليستيرول الكلي، الشحوم الثلاثية، البروتين الدهني الواطئ الكثافة، والبروتين الدهني الواطئ الكثافة جداً وانخفاضاً معنوياً في مستوى البروتين الدهني العالي الكثافة في المجموعة المصابة بالسكري مقارنة مع مجموعة السيطرة. وعليه فأن اعطاء مستخلص الشاي الاخضر يعيد مستويات دهون المصل الى القيم الطبيعية.

الكلمات المفتاحية: الشاي الاخضر، الالوكسان، الجرذان.

Introduction

Green tea (GT) is a widely consumed popular beverage produced from freshly harvested leaves of the tea plant, *Camellia sinensis*. The main constituents of GT are polyphenols and the major polyphenols are catechins. The major catechins present in GT are epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) (1). Evidence suggests that GT and its catechins possess antioxidant, antiatherogenic, antiinflammatory, and anticarcinogenic properties (2,3).

Obesity has increased at an alarming rate in recent years and is now a worldwide health problem. The investigation of the antiobesity properties of food components is a popular field of research, because it may lead to the discovery of new naturally occurring agents to prevent or treat obesity. There are studies on GT and GT components, especially catechins and caffeine, demonstrating their effects on energy expenditure, fat oxidation, and blood lipids (4); in addition to their beneficial effects for reduction of body weight, body mass index and body fat (5).

Diabetes mellitus (DM) is a metabolic disorder of the endocrine system affecting carbohydrate, lipid and protein metabolism. The worldwide survey reported that the DM is affecting nearly 10% of the population **(**6). Diabetes mellitus results in hyperglycemia and is characterized as type 1 in absolute insulin deficiency or type 2 in insulin resistance due to receptor insensitivity to endogenous insulin (7). Beside hyperglycemia, several other factors including dislipidemia or hyperlipidemia are involved in the development of micro and macro vascular complications of DM which are due to qualitative and quantitative abnormalities in lipoproteins (8).

The use of herbal products for medicinal benefits has played an important role in nearly every culture on earth for many years; the search for anti-diabetic products will continue to focus on plants and other natural resources (9). Antihyperglycemic effects of these plants are attributed to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes (10). In animal models studies (11,12), it has been suggested that EGCG and other catechins help prevent hyperglycemia by enhancing insulin activity and possibly by preventing damage to β-cells. On the other hand, several clinical investigations reported that GT treatment reduced fasting blood glucose and improved glucose tolerance in healthy and diabetic subjects (13,14).

Among the potential health benefits of GT, its lipid-lowering effect has been well documented in animal models of hyperlipidemia (15). Evidence suggests that GT inhibits the intestinal absorption of dietary lipids, including TC, TG, and other lipophilic compounds (16). Evidence also suggests that GT and its principal catechin, EGCG, interfere with the emulsification, digestion, and micellar solubilization of lipids (17).

Materials and Methods

Experimental Animals

Forty adult male albino rats (*Rattus norvegicus*) weighing 180-200 g were used in this study; they were provided by the animal's house of the College of Science, University of Baghdad. The experimental rats were housed in plastic cages in a room under standard environmental conditions (26 ± 2°C; 12/12 h light/dark cycle); feed and water were provided *ad libitum*.

Experimental Design

Following acclimatization for one week, the rats were divided into

four groups, each containing 10 rats as followed:

Group I (Control group): Rats were not receiving any drug during the period of the study.

Group II (GT group): Rats were given aqueous GT extract at a dose of 250 mg/kg b.w. orally.

Group III (Diabetic group): Rats were injected with alloxan at a dose of 150 mg/kg b.w. intraperitoneally.

Group IV (Diabetic-GT group): Rats first were injected with alloxan at a dose of 150 mg/kg b.w. intraperitoneally, and then they were given aqueous GT extract at a dose of 250 mg/kg b.w. orally.

The experimental period was designed for 4 weeks. Body weights were monitored for all the rats at the beginning and at the ending of the experimental period.

Preparation of Aqueous Green Tea Extract

The aqueous extract of GT was made by soaking 250 mg of GT powder in 1 liter of boiling distilled water with continuous mixing for 10 minutes. Then the solution was mixed for 15 minutes faraway from heat. The mixture then was filtered through filter paper to remove all the residual materials. The clear solution of the extract was dried at 45°C by using oven for 24 hours and kept at 4°C (18). Solution of GT extract was freshly prepared on daily bases and provided to the rats orally in a dose of 250 mg/kg b.w by clean drinking bottles.

Induction of Diabetes

Rats were made to fast for 12 hours prior to alloxan administration. Diabetes was induced by a single intraperitoneal injection of 150 mg/kg b.w. monohydrated alloxan (Sigma, St. Louis, MO, USA) dissolved in sterile normal saline. After 72 hours, blood samples were collected from the tail vein of the animals for evaluation of blood glucose levels. Animals presenting glucose levels above 250 mg/ dl were considered diabetic and used for the experiment (19).

Blood Sample Collection

At the end of the experimental period which was 4 weeks and after overnight food deprivation, rats were deeply anesthetized by exposure to ether. Blood samples were collected by heart puncture and transferred into plastic tubes and then centrifuged at 3000 rpm for 10 minutes. After centrifugation, sera were separated for biochemical assays.

Determination of Blood Glucose Levels

Blood glucose level was determined by the glucose-oxidase principle using the one touch electronic glucometer Accu-Chek

Advantage (Boehringer, Germany) instrument (20).

Estimation of Lipid Profile

Serum lipids concentrations were estimated spectrophotometric ally using commercial kits (Biolabo SA, France). Total cholesterol (TC) and triglycerides (TG) were measured enzymatically (21,22), whereas the high-density lipoprotein cholesterol (HDL-C) was estimated by precipitation technique (23). According to Friedewald equation (24), very low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C) were calculated as:

Results of the changes in the body weight of the four experimental rat groups (Table 1) revealed that body weight did not differ between the groups at the beginning of the experiment. After 4 weeks, there was a significant ($P<0.05$) decrease in the body weight of the GT group when compared with the control group.

VLDL-C = TG/5 and LDL-C = TC - (VLDL-C + HDL-C).

Statistical Analysis

Data were reported as the mean ± SD. The results were analyzed statistically using analysis of variance (ANOVA) applicable to a completely randomized design. The significance among means was tested depending on Duncan multiple range tests using SPSS program. A possibility of *P* value ($p<0.05$) was considered as significant differences between means (25).

Results and Discussion

Body weight of the diabetic group was significantly ($P<0.05$) lower than that of the control group, GT group, and diabetic-GT group. Likewise, the body weight of the diabetic-GT group was significantly ($P<0.05$) lower than that of the control group, but it was significantly ($P<0.05$) higher than that of the diabetic group.

Table 1: Effect of GT extract on body weight in normal and diabetic rats

Groups → / Body weights ↓	Control group	GT group	Diabetic group	Diabetic-GT group
Initial body weight (g)	188.7±7.3[a]	192.2±4.2[a]	190.4±6.5[a]	194±5.2[a]
Final body weight (g)	214.8±3.8[a]	202.6±5.3[b]	184.7±6.4[c]	200.5±4.6[b]

- Values are presented as mean ± SD of 10 rats in each group.
- Similar letters indicate no significant differences.
- Different letters indicate significant differences at $P< 0.05$.

The biological properties of GT have led to a developing body of scientific research related to its association with multiple chronic diseases, particularly obesity (26). The current study revealed that GT extract decreased body weight gain; this finding is in agreement with Sayama *et al.* (27) who reported that the addition of GT powder to the diet suppressed fat accumulation and body weight increase in mice. Several mechanisms have been postulated to account for the beneficial effects of GT on weight control and prevention of obesity; the mechanistic studies have suggested that GT decreases lipid and carbohydrate absorption, increases lipid metabolism, inhibits de novo lipogenesis, and increases carbohydrate utilization (28). Catechins may have a major role to play in these weight losing effects of GT, as they are potent inhibitors of enzymes such as glucose-6-phosphate dehydrogenase that may have a major role to play in the development of obesity (29). Also, the mechanism by which catechins reduce body fat may be related to the increase of energy expenditure (30). On the other hand, it was reported that EGCG had an inhibitory effect on acetyl-CoA carboxylase which is essential for *in vitro* fatty acid biosynthesis (31). Moreover, the weight-loss effect of EGCG may have been due to a reduction in food intake (26).

Weight loss, one of the clinical features of diabetes, seen in alloxan-induced diabetic rats may be due to the degeneration of the adipocytes and muscle tissues to compensate for the energy lost from the body due to frequent urination and over-conversion of glycogen to glucose. While the significant increase in body weight in diabetic-GT group compared with diabetic group, suggests that GT extract may have a positive anabolic effect by decreasing the degeneration of the adipocytes and muscle tissues through improving glucose metabolism (32).

The blood glucose levels of the rats at the end of the experimental period are given in table 2. Blood glucose levels of the GT-treated rats were significantly ($P<0.05$) lower than those in the control rats. Diabetic rats showed a significant ($P<0.05$) increase in the levels of blood glucose as compared to the normal rats. Diabetic rats treated with GT showed a significant ($P<0.05$) reduction in blood glucose levels when compared with diabetic group, but they still significantly higher than that of the normal rats.

Table 2: Effect of GT extract on blood glucose levels in normal and diabetic rats

Groups	Blood glucose levels (mg/dl)
Control group	125.6 ± 6.3^{a}
GT group	111.3 ± 4.2^{b}
Diabetic group	272.5 ± 5.4^{c}
Diabetic-GT group	188.4 ± 3.7^{d}

- Values are presented as mean ± SD of 10 rats in each group.
- Different letters indicate significant differences at $P< 0.05$.

As mentioned in the above table, treatment of the rats with alloxan resulted in a significant increase in the levels of blood glucose. Similar results were reported in previous study which demonstrated that alloxan produces oxygen radicals, which destroy pancreatic *beta*-cells and cause severe hypoinsulinaemia (type I diabetes) that is responsible for the hyperglycemia seen in alloxan-treated animals (33).

On the other hand, the results indicated that GT extract reduced blood glucose levels in both normal and alloxan-induced diabetic rats. However, it is still significantly higher in the diabetic rats than normal rats. Findings from both epidemiological observations and laboratory studies suggest several mechanisms by which GT and its components may influence glucose metabolism and diabetes. Several studies reported that the hypoglycemic effect of GT was attributed to the presence of polyphenols, catechins and the water-soluble polysaccharides fraction (34). It has been found that tea catechins inhibit the carbohydrate digestive enzymes such as α-amylase, intestinal sucrase, and α-glucosidase in the intestines of the rats, which suggests that glucose production may be decreased in the gut, thus lowering glucose concentrations (34,35). Several studies have also shown that GT has an effect on glucose tolerance and insulin sensitivity. Anderson and Polansky (11) reported that GT increases insulin activity, and that the predominant active compound is EGCG. While Wu *et al.* (36) demonstrated that EGCG does not only regulate the glucose level in blood, but also may rehabilitate damaged *beta*-cells, which are responsible for producing insulin. Moreover, GT has also been shown to enhance insulin sensitivity by increasing insulin-stimulated glucose uptake in adipocytes (11). It was also found that GT consumption significantly decreased blood glucose levels by increasing hepatic glycogen level in alloxan-diabetic rats possibly through

reactivation of the glycogen synthase system (as a result of increased insulin secretion) and decreasing liver glucose-6-phosphatase activity, which is mainly responsible for releasing glucose molecules to the blood by converting glucose-6-phosphate to glucose (37,11). All these mechanisms may be responsible for the anti-hyperglycemic effect of GT seen in the normal and diabetic rats in this study.

Results of lipid profile (Table 3) showed that the levels of TC, TG, LDL-C and VLDL-C were significantly ($P<0.05$) lower while the level of HDL-C was significantly ($P<0.05$) higher in the GT-treated rats than that in the control rats. There was a significant ($P<0.05$) increase in levels of TC, TG, LDL-C and VLDL-C and a significant ($P<0.05$) decrease in level of HDL-C in diabetic rats when compared to the normal rats. However, administration of GT extract to these rats restored the levels of serum lipids to normal. The results revealed a significant ($P<0.05$) decrease in levels of TC, TG, LDL-C and VLDL-C and a significant ($P<0.05$) increase in level of HDL-C in the diabetic GT group compared to diabetic group In the current study, we found that aqueous GT extract imposed significant beneficial changes in the lipid profile of the normal and alloxan-induced diabetic rats. The levels of TC, TG, LDL-C and VLDL-C were significantly lower while the level of HDL-C was significantly higher in the GT-treated rats than that in the control rats. While alloxan caused abnormality in all lipid concentrations of the rats represented by an increase in levels of TC, TG, LDL-C and VLDL-C and a decrease in level of HDL-C, administration of GT extract to the alloxan-induced diabetic rats restored the levels of serum lipids to normal. These finding are in agreement with those observed by Kuhn *et al.* (38) who reported that GT drinking has been associated with lowered serum levels of TC, TG, and LDL-C, and high serum levels of HDL-C.

Table 3: Effect of GT extract on lipid profile in normal and diabetic rats

Groups → / Lipid profile ↓	Control group	GT group	Diabetic group	Diabetic-GT group
Total cholesterol (mg/dl)	90.23±1.36[a]	82.56±1.09[b]	106.45±1.61[c]	92.81±1.47[a]
Triglycerides (mg/dl)	87.55±1.55[a]	74.43±1.42[b]	103.84±1.71[c]	89.63±1.23[a]
HDL- cholesterol (mg/dl)	34.57±0.16[a]	40.24±0.42[b]	28.58±0.71[c]	33.47±0.95[a]
LDL- cholesterol (mg/dl)	38.51±0.28[a]	27.43±0.37[b]	56.90±0.04[c]	41.41±0.21[a]
VLDL-cholesterol (mg/dl)	17.51±0.75[a]	14.89±0.24[b]	20.97±0.36[c]	17.93±0.52[a]

- Values are presented as mean ± SD of 10 rats in each group.
- Similar letters indicate no significant differences.
- Different letters indicate significant differences at $P< 0.05$.

The hypolipidemic effect of GT, which was presented in this study, could be attributed to a reduction in cholesterol absorption and to an increased excretion of and cholesterol; another proposed action is the inhibition of cholesterol synthesis in the liver (39). Previous studies suggested that GT extract reducing activity of cholesterol and lipid oxidation in rats by significant hypolipidemic action of polyphenols and catechins that will be responsible for the observed effects and reducing the TC, TG, LDL-C and VLDL-C, and increase HDL-C (40,41). The lipid-altering effects of GT catechins occur because of their influence at various points in cholesterol metabolism (17). For instance, GT extracts inhibit the absorption of lipids as well as cause inhibition of cholesterol synthesis; besides this, GT extracts also cause upregulation of the LDL receptor (42).

The significant decrease in lipid profile may be due to presence of flavinoids in GT extract that will inhibit the oxidation of LDL and cholesterol *in vitro* (43). Additionally, it has been reported that a GT extract inhibited gastric and pancreatic lipases in vitro; tea catechins are thought to be responsible for the lipase inhibitor in GT extract (44).

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Morphometric study of the cerebellum cortex of the albino rats treated with Pregabalin drug during the postnatal period

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Abstract: The cerebellum is the largest part of the mammalian hindbrain; it was the dynamic portion of the vertebrate central nervous system that has highly complex but orderly organization. The cerebellum cortex has a striking morphology consisting of folia and fissures and variety of cells morphologically and functionally, so it considered an ideal system to study the development of the central nervous model in the mammals in both prenatal and postnatal periods. The ontogenesis of the cerebellum in the mammals takes place along period of the development from the embryogenesis to the postnatal time after delivery to reach to the morphologically and physiologically maturity form, therefore these stages are very critical and sensitive to any internal or external effects that may be occur during the growth more than the mature cerebellum, like drugs, pesticides, and other environmental agents.

The antiepileptic drugs which are used in the control and treatment many neurological disorders or as analgesic agents in the infants, children and pregnant women like epilepsy are one of the most drugs that have harmful effects on the growth and development of the nervous system, Pregabalin (PGB) (Lyrica®) is the last generation of the antiepileptic medications. The study was aimed to deal with the quantitative assessment of the toxicity effect of the Pregabalin drug on the postnatal development of the cerebellum of the albino rats. The results of the morphometric assessment of the postnatal development in this study showed the weight of the developing cerebellum increased normally in the control neonates, while in the treated neonates with PGB, the results showed significant ($P<0.05$) decreased in the weight especially after postnatal 15 with unstable levels of the weight of the cerebellum. The other morphometric results of the area, perimeter of the cerebellum in the treated group with PGB were showed significant ($P<0.05$) decreased when compared with the control group. While the measurements of the anterior-posterior diameter (APD)and transverse diameter (TD) values indicated the toxic effect of the PGB on the development of the cerebellum cortex during Postnatal stages, The previous results indicate that the low antiepileptic dose of the Pregabalin (150 mg/kg B.wt/ day) may be safer to use by the pregnant women with certain neurological diseases as epilepsy than the other higher doses (300 and 600 mg/kg B.wt/ day) on the embryonic development of the cerebellum.

Key words: Pregabalin, postnatal, cerebellum

دراسة قياسية للتاثير السام لعقار الببريكابالين في التكوين النشوئي لمخيخ الجرذان البيض خلال مرحلة ما بعد الولادة

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الخلاصة: يُعد المخيخ الجزء الاكبر من الدماغ الخلفي للبائن ويعتبر الجزء الحركي في الجهاز العصبي المركزي في الفقريات ويتميز بصفات مظهرية غايه في التعقيد والتنظيم الدقيق.تمتلك قشرة المخيخ مظهراً مميزاً مؤلف من اخاديد وشقوق وانواع مختلفه من الخلايا متباينه مظهرياً ووظيفياً لذلك يعتبر المخيخ هو النموذج المثالي لدراسة التكوين النشوئي للجهاز العصبي في اللبائن في كل من المرحلة الجنينية ومابعد مرحلة الولادة.تحدث عملية التكوين النشوئي للمخيخ في اللبائن على مدى واسع من التكوين ابتداءً من المرحلة الجنينية ولغايه مابعد الولادة للوصول الى النضج المظهري والوظيفي لذ لم تعتبر هذه المراحل حساسة وحرجة جدا تجاه التأثيرات الداخليه او الخارجية التي من الممكن ان تحدث خلال النمواكثر من المخيخ الناضج ومنها العقاقير والمبيدات الحشرية وبعض العوامل البيئية.تعتبر الادوية المضادة لمرض الصرع والتي عادة ماتستعمل كمسكنات للالام في علاج العديد من الاضطرابات العصبية في الاطفال الرضع والنساء الحوامل واحدة من اكثر الادوية ضرراً على نمو ونشوء الجهاز العصبي ، دواء البريكابالين هو الجيل الاخير من ضمن هذه الادوية.تهدف هذه الدراسة الى تقدير كمي للتأثير السام لدواء البريكابالين في التكوين النشوئي للمخيخ في مرحلة مابعد الولادة في الجرذان البيض.

Introduction

Early in postnatal life, the brain continues to undergo extensive development. The cerebellum is an ideal model system for studying the development of the central nervous system, because it has a morphologically unique structure, and a special dynamic development, During the postnatal development of the mammalian cerebellum there a distinct histological feature during a process was called the foliation that gives the special morphology of the cerebellum; the folia increase the surface of the cerebellum, and thereby accommodate an increase in cell number, which in turn facilitated the acquisition of more complex functional circuited (1). Brain growth varies among mammals, but comparisons of brain development between species are possible, the developmental ages of human and rats' embryos or fetuses are comparable when anatomical features and histological landmarks are similar in appearance between the two species, even though their exact chronological ages are different (2).

The timing of the brain growth spurt for each species can be used as a marker of developmental age that is a means to identify comparable chronological ages. The brain growth of rats and mice occurs postnatally, with peak growth velocity on postnatal day 7 and postnatal day 10 (P7-P10) and ends in the third week (3). While in humans, the brain growth period starts during the third trimester of pregnancy, with peak growth velocity around the time of birth (4)

The cerebellum is late-developing neuronal structures, completing formation after 34 weeks of gestation in humans and during the first 2 weeks of postnatal life in the rat. Within this period both the structure of the cerebellum and cerebellar-dependent behaviors matures rapidly (5)

In the rodents the cortex of the cerebellum undergoes complex ontogenesis during the first 3 postnatal weeks (6) and very active cell proliferation occurs.

The Pregabalin (PGB) have a reproductive toxicity in animal models, therefore the Food and Drug Agency (FDA) in 2009 classify the Pregabalin within category c, that include drugs that have an adverse effect on the fetus (7), and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks. Also no adequate data about its execrated in the breast milk of human however, it is present in the milk of rats. (8) Therefore we try in this study to investigate the toxicity effect of the Pregabalin drug on the postnatal development of the cerebellum during the breast feeding in albino rats.

Materials and Methods

Animals

After isolation of the sexually mature females which at the estrous stage by examine the vaginal smears under light microscope, the isolated females put in breeding cages each 2 females with one mature male and left overnight.

Early in the next morning, copulation was confirmed by examining the females, and notices the presence of the vaginal plug or the sperms in slides microscopically in the vaginal swabs.

In this work the gestational day zero was defined as the day when spermatozoa were observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ*, then females were transferred to separate cages without males and stay until the appropriate days to isolated the embryos (9)

One high toxic dose of PGB; 200mg/kg/B.wt was used, depending on the toxicological profile of the material safety data sheet of the Pfizer report (2010) this dose was administrated to the pregnant rats from the first dpc, until the 21 day postnatal day (PND21) after normal delivery. the concentration was diluted with appropriate volume of D.W to obtain the equivalent concentration in rats with according to the body weight.

The drug solution was given with a volume of 1-2 ml. orally using polyethylene orogastric tubes connected to a hypodermic syringe in appropriate size. The dosage was in milligram per kilogram body

weight (mg/Kg) given twice a day (twelve hourly). D.W was used as the vehicle. The control group was administrated with the same volume of distilled water.

Litters

Full – term pregnant female rats were allowed for normal vaginal delivery. The litters were left with their dams. Postnatal day zero (P0) was defined as the day of parturition as many researches were mentioned (3), so the after birthday was labeled as the first post-natal day (PND1).The administration of the drug to the dams was continued until PND21.

In this study we selected the PND 12, 13,15,20,21, at each selected postnatal day, 4 pups were anesthetized lightly by diethyl ether solution and scarified by decapitation with scissors, the weight of neonates, whole brains ,and cerebellum were used **,**the cerebellum were fixed in Bouin's solution, and then transformed to 70% ethanol.

Results and Discussion Weight (g)

Figure (1) was revealed that the weight of the developing cerebellum was significantly increased gradually in the control group along the selected postnatal ages. While in the treatment group the weight of the developing cerebellum was increased irregularly, at PND12, 13 (0.205±0.06 g) when compared with the control group (0.08±0.01 g), then decreased rapidly during PND15 (0.147±0.04 g) and also at PND21 (0.27±0.06 g) near the value of the control group (0.140±0.04 g) and (0.29±0.09 g) respectively. And the weight was increased significantly ($P<0.05$) again at PND20 and reached the highest value (0.31±0.08 g), when compared with the control group (0.15±0.06 g). We tried in this study to demonstrate the effect of the Pregabalin drug on the ratio between the weight of the developing cerebellum and the whole brain of the neonate's rats at the selected ages.

In figure (2) the results revealed that the ratio between the brain and the developing cerebellum was high in the control group and the ratio was increased with the proceed of the ages and the growth of the cerebellum in the neonates. While in the treated group the ratio was raised during PND 12, 13, 15 only, but statistically these changes in the treatment group were not significant when compared with the control group.

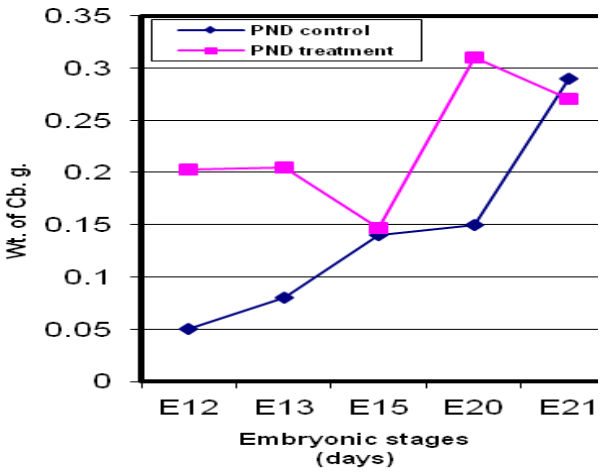


Figure (1) Effect of pregabalin(200 mg/kg body weight/day) on weight of the Cerebellum(g) during postnatal days in both control and treatment group

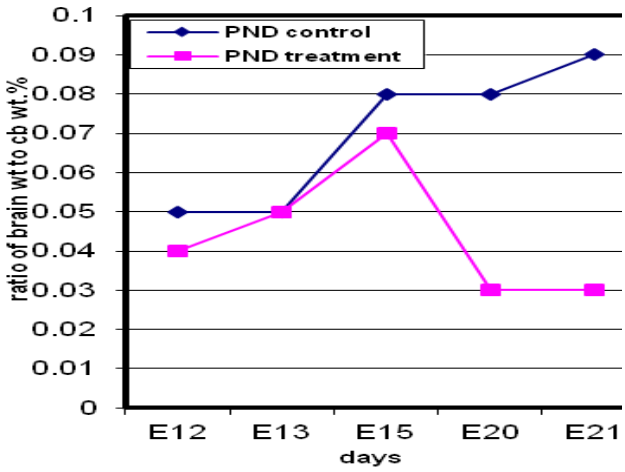


Figure (2) Effect of the Pregabalin (200 mg/kg body weight/day) on the ratio of brain weight /weight of Cerebellum % during postnatal days in both control and treatment group

Many studies referred that brain weight is relatively unaffected by the changes in the total body weight during the postnatal period, unlike the other organs which their weight affected by the changes in the weight of the whole body during the development of the animal, delayed brain development and smaller brains are seen in juvenile animals when caloric or nutrient restriction during early postnatal development, also the brain during the development became more effected and sensitive to any metabolic or physiologic changes with different degree from one area of the brain to another, for example the area that

mature after birth, i.e. cerebellum; Therefore in this study the weight of the cerebellum was recorded specifically with the ratio of the whole brain weight to the cerebellum weight (10).

In the rats the normal development of the cerebellum during postnatal period in rats the weight increased normally with the progress the old of the neonates from PN12 to PN21 which represent the final stage of the development of the cerebellum. But when noticed the cerebellum weight in the treatment group with the Pregabalin drug the results showed abnormal unstable values between the increased and the decrease levels, this may be due to the response to the effect of the drug which lead to many degenerative and apoptosis effects on the cerebellum cells in the cortical layer during the late stages of the postnatal development of the cerebellum, this was supported by the earlier research with many other of antiepileptic drugs which showed that these drugs were effected on the weight of the many regions of the brain in the rats (11) .

The main biochemical factors in the development of the brain are the neurotrophins and many growth factors that provide trophic support to developing neurons, so may be the chemical action of the Pregabalin was play a critical role in changing the chemical nature of these chemicals and interfered with their chemical pathways. Our perhaps the Antiepileptic drug cause sensitive neurons to undergo apoptotic death in the developing rat forebrain, these findings apply to compounds that block voltage-gated sodium channels and other ions that important in the development (12)

The more important indicator about the dangerous role of the antiepileptic drugs in the development of the brain that the neurotoxicity of these drugs is associated with impairment of the neurotrophins and survival-promoting signals in the brain and depressed an endogenous neuroprotective system in the brain that crucial for neuronal survival during development (13,14)

Cerebellum Area (Sqmm.)

The measurement of the area of the developing cerebellum at postnatal period in the rats by using the morphometric method showed in Figure(3) revealed that the area in the control group was increased and stay constant after PND15, although the sudden decrease occur at PND13. While in the treatment group the area of the developing cerebellum was smaller significantly ($P<0.05$) than the control group along the ages in this study, but in the treated group itself the area level was not changed significantly ($P<0.05$) with the proceed of the development compared with control.

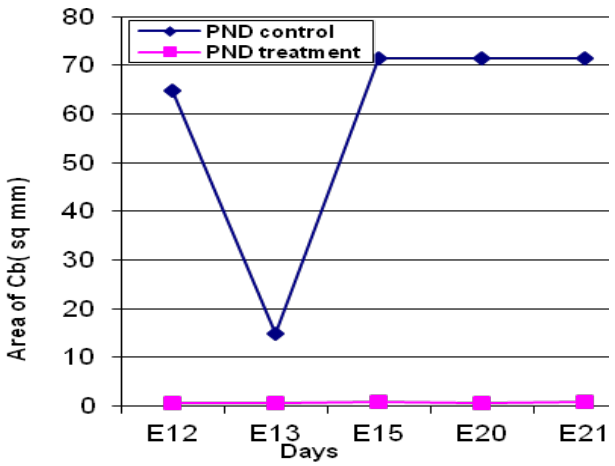


Figure (3) Effect of the Pregabalin (200 mg/kg body weight/day) on Area Sq mm. of postnatal cerebellum in both control and treatment group

Cerebellum Perimeter (mm)

The results in (Figure 4), revealed that perimeter of the developing cerebellum in the control group increased significantly ($p<0.05$) at PND12 (37.22±1.36 mm) and decreased at PND13, with rapid elevation at PND 15, 20 and decreased significantly ($p<0.05$) again at PND21. But the treatment with Pregabalin caused significant ($P<0.05$) decrease in the perimeter values when compared with the control group. Whereas there were no effect of the treatment on the perimeter level between the postnatal days.

Measurement of the Anterior-Posterior (APD) and Transverse Diameter (TD) (mm)

The statistical analysis for the results, (Figure 5) showed that the APD value was at the higher value at PND12 in the control group (7.93±0.34 mm) then the values were decreased gradually with the continuous development of the cerebellum and reached its lower values at PND21 (4.32±0.09 mm). While in the treated group, the APD values of the developing cerebellum were significantly ($P<0.05$) under there in the control group, the APD value was decreased 4.73 mm compared with the control group.

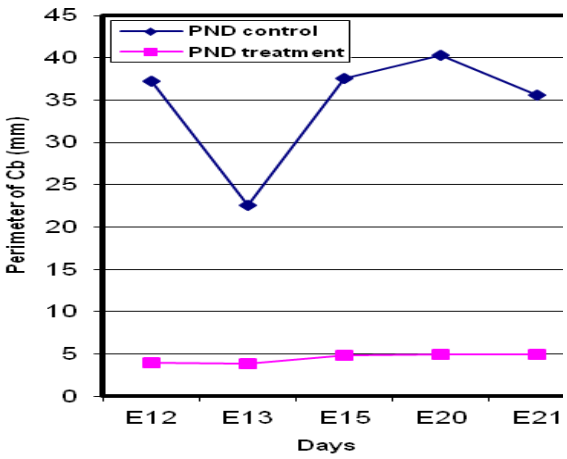


Figure (4) Effect of Pregabalin (200 mg/kg body weight /day) on Perimeter of postnatal cerebellum in both control and treatment group

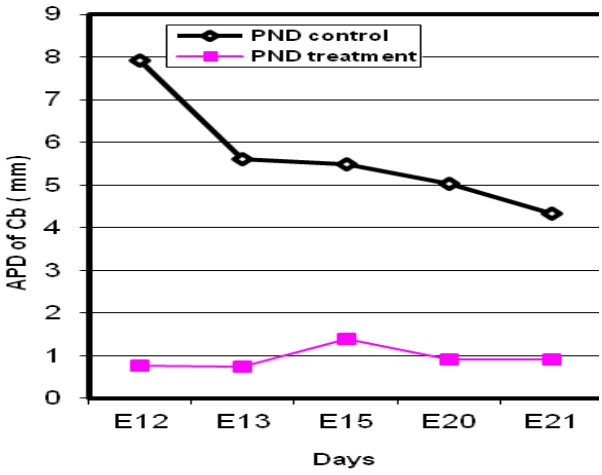
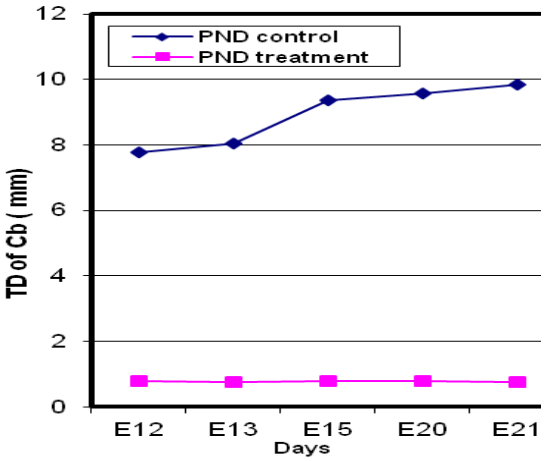


Figure (5) Effect of Pregabalin (200 mg/kg body weight/day) on ADP mm. of postnatal cerebellum in both control and treatment group

The results of the TD morphometric diameter of the neonates cerebellum in the control group (Figure 6) indicated that the TD value at PND12 (7.77±0.23 mm) increased gradually with the progressed postnatal period, while the treatment with Pregabalin caused a significant decrease in TD value (0.787±0.04 mm) at the E12 when compared with the control group, and stay stable with the postnatal days progress, generally the TD value of the all treated cerebellum was decreased 8.14 mm compared with the control group.



Figure(6) Effect of the Pregabalin (200) mg/kg body weight/day) on TD mm. of postnatal cerebellum in both control and treatment group

The linear morphometric use as a screening test for development toxicity testing is questionable, the brain and all tissues are composed of cells and their products, and the important question in developmental neurotoxicity study is to determine the potential effects of a compound on the developing nervous system during the differences in the morphometry of the organ. (15)

Neurotoxic chemicals that affect brain development by interfering with DNA synthesis or inhibiting or delaying migration of neurons have the potential to reduce the numbers of the cells present in variety of neuroanatomical regions and therefore the dimensions of these regions.

These varieties may be the primary toxicant–related and for this reason some form of the morphometric analysis usually is conducted in developmental neurotoxicity studies (16)

The US Environmental Protection Agency (EPA) has been refining the requirements of neurotoxicology studies for the registration of new chemicals with the aim of assessing and evaluating the potential functional and morphologic hazards to the developing nervous system, both in utero and during suckling.

The important question in developmental neurotoxicity study is determine the potential effects of a compound on the developing nervous system and how these produced changes effect on the morphology and function. (15).Therefore this study was to attempt to report the changes in the morphology of the developing cerebellum of the rats from embryonic stages to the postnatal period when the histological development of the cerebellum complete.

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Evaluation of INF- γ and IL-4 in early and late cutaneous leishmaniasis patients with single and multiple sores

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Abstract: Ninety-five patients infected with cutaneous leishmaniasis (CL) at AL-Karama Hospital in Baghdad were included in this study; 60 individuals were infected with single sore, and 35 with multiple sores. The study also included thirty healthy individuals were treated as a control group. Serum samples from all groups mentioned above were tested for cellular immune response at different stages of the disease activity (early and late) by estimation of gamma interferon (IFN-γ) and interleukin-4 (IL-4) using Enzyme-Linked Immunosorbent Assay (ELISA) kits. The results of this study revealed highly significant increase ($P<0.01$) in IFN-γ levels which were detected in CL patients with single and multiple sores (mean 67.30 ± 59.41pg/ml) in comparison to control groups (mean 3.81±1.79 pg/ml) however, a significant differences were ($P>0.01$) noticed among patients with single sores (mean 47.2 ± 27.80pg/ml) and multiple sores (mean 87.4 ± 30.52 pg/ml) in the mean concentration of this cytokine. There was no significant differences ($P>0.01$) among patients with single sore (mean 51.04 ± 15.0pg/ml) and multiple sores (mean 63.70± 20.32 pg/ml) in the mean concentration of IL-4.Finally this study revealed that: Th-2 predominates during the early stage of the disease then shifts to Th-1 that proceed in the late stage, but both cytokines increased in CL patients in comparison to control group. This finding suggested that the immune response of CL infection is possibly regulated by both Th-1 and Th-2, and multiple sores´ patients showed an increase of IFN- γ (mean 87.4 ±30.52 pg/ml) and IL-4 (mean 63.70±20.32pg/ml) levels than single sore patients with mean value (47.2 ± 27.80pg/ml) and (51.04±15.0 pg/ml) respectively. These results indicated that multiple sores patient's immune response is higher than the others with single sores.

Keywords: *Leishmania tropica*, IFN- γ, Single and multiple sores.

تقييم الاستجابة المناعية الخلوية في المراحل المبكرة والمتاخرة لمرضى اللشمانيا الجلدية ذوي القرحة الواحدة وذوي القرح المتعددة

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الخلاصة: شملت هذه الدراسة 95 شخصاً مصاب بداء اللشمانيا الجلدية من مستشفى الكرامة في بغداد، 60 شخص منهم مصاب بقرحة واحدة 35 شخص مصاب بقرح متعددة تراوحت من 2 إلى 10 قرح، شملت الدراسة 30 شخص أصحاء للمقارنه.تم قياس الاستجابة المناعية الخلوية للعينات المذكورة أعلاه في مراحل مختلفة من المرض (في بداية ونهاية المرض) عن طريق تقييم(γ -IFN) و (IL-4) باستخدام عدد الاليزا ELISA kits.أظهرت نتائج الدراسة الحالية مايأتي :بينت الدراسة ان هناك زيادة عالية في مستوى (IFN-γ) في مرضى اللشمانيا ذوي القرحة الواحدة وعديدي القرح pg/ml(67.30±59.41) بالمقارنة مع الأشخاص الأصحاءpg/ml (3.81±1.79) وأظهرت النتائج ان هنالك فرق معنوي في مستواه بين المرضى ذوي القرحة الواحدة pg/ml(47.2±27.80) وعديدي القرح pg/ml(87.4±30.52). وأظهرت النتائج انه لا يوجد فرق معنوي في مستوى (IL-4)بين المرضى ذوي القرحة الواحدة pg/ml(51.04±15.0) وعديدي القرح pg/ml(63.70±20.32).اثبتت هذه الدراسة مايلي :إن الاستجابة المناعية (Th-2) تسود خلال المرحلة المبكرة من المرض في مرضى اللشمانيا الجلدية ثم تتحول إلى الاستجابة (Th-1) والتي تستمر في المرحلة النهائية من المرض لكن الزيادة الحاصلة في مستويات المحركات الخلوية تقترح إن المناعة في مرضى اللشمانيا الجلدية يتم تنظيمها من قبل كلا الاستجابتين (Th-1) و (Th-2) وقد وجد إن المرضى عديدي القرح اظهروا زيادة في مستويات كل من (IFN-γ) pg/ml (87.30.52±30.52) و(IL-4) pg/ml(63.70±20.32) عند مقارنتهم مع المرضى ذوي القرحة الواحدة وpg/ml(47.2±27.80) و pg/ml(51.04±15.0) بالتعاقب .

Introduction

Leishmaniasis is one of the most diverse and complex of all vectors borne diseases. It is caused by an obligatory intracellular protozoan parasite belonging to the genus *Leishmania* (1). *Leishmania* infection is transmitted to susceptible mammalian hosts by the bite of a female sand fly (subfamily Phlebotominae). The immune response to *Leishmania* infection is cell mediated. The organism lies exclusively intracellular, mainly inside macrophages as replicating amastigote. The outcome of infection will depend on whether the host mounts primarily a T-helper Th-1 or Th-2 response (2). Studies in animals suggest that the same parasite epitope can induce a Th-1 response in animals with resolving infection or a Th-2 response in others with disease progression (3).

IFN-γ is a highly secreted protein, secreted mainly by activated T-lymphocytes and natural killer cells. It is involved in a wide range of physiological processes, including antiviral, immunoregulatory and anti-tumour properties, cell proliferation and apoptosis as well as stimulation and repression of a variety of genes (4, 5). IFN-γ induces the production of nitric oxide (NO) in phagocytic cells that harbor *L. major* which leads to destruction of the parasite (6). Interleukin-4 (IL-

4) is a cytokine produced primarily by activated T lymphocytes, mast cells and basophiles. It's an important regulator of isotype switching, inducing IgE production in B lymphocytes. In addition, IL-4 has also been shown to have anti-tumor activity both in vivo and in vitro. (7, 8 and 9). IL-4 stimulates the differentiation of naive CD4 T cells into Th-2 cells capable of producing Th-2 associated cytokines IL-4, IL-5, IL-10 and IL-13, and promotes antibody production and IgE class switching by B cells. IL-4 also functions as a powerful inhibitor of IFN- γ producing CD4 T cells and suppressor of protective Th-1 immune responses (10). IL-4, along with related cytokine IL-13, triggers macrophages to undergo alternative activation and is associated with parasite survival and persistence of infection (11, 12 and 13). The Th-1\Th-2 was associated with resistance and susceptibility to leishmanial infection respectively (14).

Materials and Methods

Subject selection

The individuals studied were classified into two groups:

1. Group one leishmaniasis patients

This group included ninety-five patients infected with cataneous leishmaniasis (Baghdad boil) (35 males and 60 females), age range (6-60) years. They were diagnosed depending on the clinical picture and laboratory diagnosis (smears and cultures). This group classified into 60 patients with single sore (One lesion) and 35 patients with multiple sores (two-10 lesions)

2. Group two control

This group included (30) healthy individuals, age range (6-60) years, (18 males and 12 females).

Blood samples collection

Five ml of venous blood was collected from each individual used. Serum samples were collected from AL-Karama Teaching Hospital. The collected samples were centrifuged and the serum was collected and dispensed in plastic eppendorf tubes then stored at -20ºC until used for serological tests.

Determination of IFN-γ

Principle of the assay

The assay max human IFN-γ ELISA kit is designed for detection of human IFN-γ in plasma, serum and cell culture samples. This assay employs a quantitative sandwich enzyme immunoassay technique that measures human IFN-γ in less than four hours. IFN-γ in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for IFN-γ, which is recognized by a streptavidin –

peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured. The absorbance of color was recorded by a microplate reader at a wavelength of 450 nm immediately.

Determination of IL-4
Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and any IL-4 present is bound by the immobilization antibody. After washing away any unbound substances were removed. An enzyme-linked polyclonal antibody specific for IL-4 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of IL-4 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Statistical analysis

Experimental data were analyzed by using (ANOVA) test. P value < 0.01 was considered statistically significant.

Results and Discussion

This study showed that IFN-γ levels were significantly higher (P< 0.01) in leishmaniasis patients (mean 67.30 ± 59.41pg/ml) in comparison to control group (mean 3.81±1.79 pg/ml). The results of this study also showed that the levels of this cytokine in early CL patients (28.00± 11.31 pg/ml) were significantly (P<0.01) lower than levels in late CL patients (106.68± 32.33pg/ml).

The results in general showed that IFN- γ level was significantly (P<0.01) higher in multiple sores patients (mean 87.4 ±30.52 pg/ml) than its level of single sore patients (mean 47.2± 27.80 pg/ml) (Table 1).

Table (1): Mean concentration ± SD of IFN- γ in the sera of 95 subjects in pg/ml.

Patients	NO. of patients	Mean conc. ± SD of IFN- γ (pg/ml)
CL	95 (100%)	67.30 ± 59.41
Single	60 (63.61%)	47.2±27.80
Multiple	35 (36.84%)	87.4±30.52

There was a significant increase of IL-4 levels in the sera of CL patients (mean 57.37± 68.62 pg/ml) in comparison to the control group (mean 33.42± 9.36 pg/ml).
The current results showed that this cytokine level in multiple sores patients (mean 63.70 ± 20.32pg/ml) is higher than the levels in single sore patients (mean 51.04 ± 15. pg/ml) but this difference was not significant (P>0.01) (Table 2) and early CL patients have a significantly (P<0.01) higher level of IL-4 (mean78.70±62.40 pg/ml) in comparison to late CL patients (mean 36.05 ± 5.94pg/ml).

Table (2): Mean concentration ± SD of IL-4 in the sera of 95 subjects in pg/ml.

Patients	NO. of patients	Mean conc. ± SD of IL-4 (pg/ml)
CL	95 (100%)	57.37±68.62
Single	60 (63.61%)	51.04±15.0
Multiple	35 (36.84%)	63.70±20.32

Outcome of the infection is critically dependent on the activation of one of the two subsets of CD4 T cells, Th-1 and Th-2.
From the results obtained, it can be considered that the immune system tend towards cell mediated immunity due to the increased levels of IFN- γ in CL patients in general in comparison to the control group. This elevation is due to the activation of host macrophages to eliminate *Leishmania* parasites, because macrophages, the terminal effector cells, are the exclusive hosts for the survival and multiplication of *Leishmania* parasites (15, 16), any attack of the immune system to kill the parasite has to involve this cell. The decisive question therefore is how to activate the macrophage to use its lethal machinery to kill *Leishmania*. It is now clear that NO produced by the inducible NO synthase (iNOS) is the most important mechanism of the macrophage to kill *Leishmania in vitro* and *in vivo*. IFN-γ is by far the most important cytokine leading to induction of iNOS (17).

The importance of IFN-γ in NO induction was demonstrated by the finding that IFN-γ, alone among a number of cytokines, was capable of independently enhancing iNOS transcription and NO release from stimulated mouse peritoneal Macrophages (18).

IFN-γ production is to a large extent induced by IL-12 and down-regulated by IL-4 (19, 20). The resistant hosts mount a Th-1 response (high IFN-γ low IL-4) whereas in susceptible hosts a Th-2

response (low IFN-γ high IL-4) predominates (21).

IL-4 is the signature cytokine associated with Th2-type immune responses and is associated with non-healing forms of cutaneous disease in mice (22).

The higher levels of IL-4 that we noticed in these results especially with those of multiple sores agreed with the fact that IL-4 has been shown to be associated with disease progression and susceptibility was also reported by (23).

IL-4 stimulates the differentiation of naive CD4 T cells into Th-2 cells capable of producing Th-2 associated cytokines IL-4, IL-5, IL-10 and IL-13, and promotes antibody production and IgE class switching by B cells. IL-4 also functions as a powerful inhibitor of IFN-*γ*-producing CD4 T cells and suppressor of protective Th-1 immune responses (24), this fact corresponded with our results of low levels of IFN-*γ* and the high levels of IL-4 in early infection with cutaneous leishmaniasis. IL-4, along with related cytokine IL-13, trigger macrophages to undergo alternative activation and is associated with parasite survival and persistence of infection (25, 26 and 27).

Cutaneous leishmaniasis is characterized by a mixed Th-1/Th-2 immune response during the early phase then Th-2 is detected and converts to an evident Th-1 type of immune response documented by lymphocyte proliferation and IFN-γ secretion (28).

29 and 30 also supported that the susceptibility is strongly correlated with a predominant Th-2 cytokine response whereas resistance is correlated with a Th-1 response.

Many studies had shown that during the early infection with *leishmania major* hosts exhibited mixed Th-1/Th-2 responses of CD4 cell population, then induces a transient Th-2 type response and then shifts to a Th-1 response associated with healing (31) induction of this Th-1 type of response partly depends on the activation of IFN-γ producing CD4 T cells and in the absence of CD8 T cells, the Th-2 response is sustained.

In the current study, the results showed this type of cytokine shift, characterized by lower levels of IFN-γ in the early stage of the disease (mean 28.00±11.31pg/ml) and then the levels increase later (106.68 ±32.33 pg/ml) these findings agreed with (32) who suggested that in patients with CL a Th-2 response transiently predominated during the early phase of infection and was followed by the development of Th-1 response during the late

course of lesion development, this suggestion is completely corresponded with our results of high levels of Th-1 cytokine (IFN-γ) and low levels of Th-2 cytokine (IL-4).

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Investigation of bacterial contamination and Concentrations of some heavy metals in Several commercial Marks of Fruit Juices and Soft Drinks in Baghdad city\ Iraq

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Abstract: Fifty samples of soft drinks and juices were collected randomly from different markets in Baghdad city, to determine the concentrations of some heavy metals (Copper, lead and cadmium) and the bacterial contamination. The results showed that the presence of bacteria in samples of soft drinks and juices varying degrees that percentage 6 from 20 samples (soft drinks) 26%, and percentage 8 from 30 samples (fruit juices) 36%. The higher concentration of Copper (1.29 ppm) was record in the sample 38 (Al-Rawabi pomegranate), while the samples 36(Mizo pineapple) , and sample 49 (original fruits) were no detection of it in them. The sample 15 (Miranda orange)contains the higher concentration of Cadmium(20.01 ppm) , but the sample 30 (Rani pomegranate) contains the lowest concentration (0.002 ppm). When the concentrations of lead were measure , the higher value (12.3 ppm) was record in the sample 16 (Shani fruit syrup), but the lowest value (0.002 ppm) was found in the sample 27 (Rani orange).

Key words: contamination of soft drinks, heavy metals , bacterial quality.

فحص التلوث البكتيري وتراكيز بعض العناصر الثقيلة في عدة ماركات تجارية من عصائر الفواكه والمشروبات الغازية في مدينة بغداد \ العراق

ايثار كامل المياليي

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الخلاصة: تم جمع 50 نموذج من المشروبات الغازية والعصائر عشوائيا من مناطق مختلفة في مدينة بغداد، وذلك لتحديد تراكيز بعض العناصر الثقيلة (النحاس والرصاص والكادميوم) والتلوث البكتيري . اظهرت النتائج تواجد البكتريا في بعض نماذج المشروبات الغازية والعصائر بنسبة 6 نماذج من اصل 20 نموذج (المشروبات الغازية)اي حوالي 26% ، وبنسبة 8 نماذج من 30 نموذج (للعصائر) اي حوالي 36%. سجل اعلى تركيز للنحاس(1.29 جزء بالمليون) في النموذج 38 (Al-Rawabi pomegranate) ، بينما لم يسجل اي تركيز للعنصر في النموذجين 36 (Mizo pinapple) و 49 (Original fruit). احتوى النموذج 15 (Miranda orange) على التركيز الاعلى من الكادميوم (20.01 جزء بالمليون)، لكن التركيز الاقل (0.002 جزء بالمليون) سجل في النموذج 30 (Rani pomegranate). وعند قياس تراكيز الرصاص، سجلت اعلى قيمة (12.3 جزء بالمليون) في النموذج 16 (Shani fruit syrup)، ووجدت اقل قيمه (0.002 جزء بالمليون) في النموذج 27 (Rani orange) .

الكلمات الافتتاحية : تلوث المشروبات الغازية ، النوعية البكتيرية

Introduction:

Fruit juices are becoming an important part of the modern diet in many communities . They are nutritious beverages and can play a significant part in a healthy diet because they offer good taste and a variety of nutrients found naturally in fruits. Juices are available in their natural concentrations or in processed forms (1).

There is an increase concern about the health effects in humans due to continuous consumption of food contaminated with heavy metals. The extent of this contamination depends on several complex factors .One of them being specific metabolic and homeostatic mechanism operating in the type of food and tissue considered (2).

Toxicological and environment studies have prompted interest in the determination of toxic elements in drink. The ingestion of drink is an obvious means of exposure to metals, not only because of environmental contamination and contamination during processing (3).

Heavy metals composition of drinks is of interest because of their essential or toxic natural. For example iron, zinc, copper, and manganese are essential, while lead, cadmium, nickel, and mercury are toxic at certain levels (4).

Facility modernization and quality manufacturing are required to prevent heavy metal contamination in foods and thus the possible health hazards to the consumer (5). For each element, there is a range of safe and adequate exposure, within which homeostatis is able to maintain optimal tissue concentration and functions. Every trace element is potentially toxic when the range of safe and adequate exposure is exceeded. A living organism has powerful mechanisms that maintain the plateau (6)

Some chemical and bacterial changes were occur in the stored juice cans with different range of temperature, these changes were differ according to storage period, temperature and the kind of microorganism which contain in them. The extent of microorganism which led to alcoholic fermentation as result of fermentative yeasts for the saccharidesin juices , so at last produce alcohol and carbon dioxide (7),Also Some bacterial species ,when it found in juices which led to lactic acid fermentation(8).

Materials and Methods

Fifty samples of soft drinks and juices which collected from different markets in different areas of Baghdad were collected .

Many of traditional markets of soft drinks and juices were chose to

study the concentrations of copper, cadmium and lead in them and assess the microbial quality(table.1).

Table 1: The samples of study and production companies (Soft Drinks & Juices Samples)

Soft drinks and juices samples	Production companies
Seven Up(Box)	Baghdad Soft Drinks Company distinction from Seven Up World
Pepsi(Box)	Baghdad Company for soft drinks under license from PepsiCo you
Miranda orange (Box)	Baghdad Company for soft drinks under license from PepsiCo you
Miranda orange (Box)	Baghdad Company for soft drinks under license from PepsiCo you(Contains source Ffinelalanin)
Miranda apple (Box)	Baghdad Company for soft drinks under license from PepsiCo you
Seven Up free on sugar free on color and caffeine(Box)	Baghdad Soft Drinks Company distinction from Seven Up World
ULUDAG gazoz(Strawberry) (1930)(bottle)	Soft drink Strawherry flavored Company Ooloda_ Turkey
Gazoz (orange)(bottle)	Ooloda production company Made in Turkey
Coca cola(bottle)	Made in Iraq
Coca cola (Box)	Production under license from Coca-Cola Company by an oasis of soft drinks, juices, mineral water and plastic sheeting company. Factory Karbala _ Iraq
Sinalco Lemon (Box)	Production license of soft and firm distinction from Sinalco World . Yousefah_ Iraq

ULUDAG Gazoz (Lemon)(bottle)	Production Ooloda Pak _ Turkey
Miranda (Lemon)(bottle)	Baghdad_ Iraq Sinalco license from you
Miranda (orange)(bottle)	Baghdad_ Iraq Sinalco license from you
Shani fruit syrup(Box)	Baghdad_ Iraq Sinalco license from you
Fanta (orange) (Box)	Babylon _ Iraq
Sprite (Lemon)(Box)	Babylon _ Iraq
Fanta Citrus (Box)	Babylon _ Iraq
Cola Delta Wafi	Babylon _ Iraq
Duo mount (bottle)	Soft drink production Baghdad _ Iraq Under license from PepsiCo soft drinks
(Dalia Top grapes)	Production Zain Middle East Company Baghdad _ Iraq
Rani granules (orange) (Box)	Made in Dubai United Arab Emirates in with industrial Morgan Company
Rani peach granules	Made in Dubai United Arab Emirates in with industrial Morgan Company
Mizo Kamaruddin (bottle)	Gallant factory for the manufacture and packaging juices limited Saudi Arabia
Spring orange juice (Box)	Company Spring (Saudi Arabia)
TROPIX Drink Kamaruddin (Box)	Gallant factory for the manufacture and packaging juices limited Saudi Arabia
Rani (Orange) (bottle)	Made in Dammam, Saudi Arabia Aujan factory
Rani Cocktail Fruits (bottle)	Made in Dammam, Saudi Arabia Aujan factory
TROPIX Cocktail Fruits	Importer in Iraq Companies House Radwan for General Trading Baghdad

Rani Pomegranate (bottle)	Made in Dammam in with Aujan factory Saudi Arabia
Rani (Carrots and orange) (bottle)	Made in Dammam in with Aujan factory Saudi Arabia
Rani Kamaruddin (bottle)	Made in Dammam in with Aujan factory Saudi Arabia
Ugarit (Lemon)	Importer in Iraq Ugarit commercial production company Syria
Ugarit (orange) (Box)	Importer in Iraq Ugarit commercial production company Syria
Ugarit (Pears) (Box)	Importer in Iraq Ugarit commercial production company Syria
Mizo Pineapple (bottle)	Importer in Iraq Company sky Radi General Trading importer in Syria . Saudi origin
Ugarit apricot(Box)	Syria
AI_Rawabi Pomegranate (bottle)	Lebanon's approval of the Black Forest Britain_ London
Ugarit grapes	Syria
Mizo (Lemon) (bottle)	Production company stock factory for the manufacture and packaging of origin Saudi Arabia
Rauch mango juice (Box)	Made by the company Rauch Austria
Dandanah Natural grape juice (Box)	Said factory production Jeddah Saudi Arabia
Dandanah Natural Peaches juice (Box)	Said factory production Jeddah Saudi Arabia
AI_Rawabi Kamaruddin Apricot (Bottle)	Made in Lebanon permission of the Black Forest Britain _ London
J`us juice Cut pineapple (bottle)	Made in United Arab Emirates Dubai

AI_Rawabi Raisin red grapes (bottle)	Made in Lebanon permission of the Black Forest Britain _ London
Dalia (Orange) (Box)	Made in Kuwait
Dalia Red grapes (Box)	Made in Kuwait
Original Fruits (Box)	Made in Saudi Arabia
Dalia Peaches (Box)	Made in Kuwait

Total plate count method was depends to test the bacterial count in studied samples, nutrient agar was used for this purpose , while Macconkey agar was depends to detect the numbers of fecal coliform , especially *Eschericia coli.*(9).

Atomic absorption spectrum was depends to measure the concentrations of copper, cadmium and lead according to (10).

Results and Discussion:

There is a potential range of soft drink & juices products to become contaminated with microorganisms. The range of microorganisms associated with way to maintain the samples and preservatives .

The results of Bacteria in Soft drinks & juices were indicated that all examined samples had shown negative growth except in some samples that given in tables 2., which explains that the percentage of contamination in soft drinks samples was 26% , while was 36% for juice samples Fecal coliform was recorded with number higher (18,6,4,,2,and 3 cell\1ml) than permissible value(zero\1 ml) in the samples 5,7,8,9 and 12 cell\1ml), while the exceeded values (40,20,25,17,70,121,70,300,350,200 ,250,300,260,16 and 14 cell\1ml) were recorded in samples 4,5,6,16,21,24,25,41,42,43,44,45,46 ,47and 49),respectively (table.3). When there is appropriate conditions for the contamination that leads to the living microorganisms and fungal growth and yeast

pH values do not exceed the common range in soft drinks and juices(table 3),usually with acidity side.

Table 2:Bacteria growth in Soft drinks & juices in percentages

Samples	Months	The percentage of contaminated
Soft drink	Jan.2015_Mar.2015	**26%**
Juices	Mar.2015_Apr.2015	**36%**

Table 3: Fecal coliform , Total Plate Count and pH in the studied samples.

No.	Samples	Size ml	Fecal Coliform (cell\1 ml)	Total plate count (Cell\1ml)	PH	Note
1	Seven Up(Box)	330	Zero	Zero	3.20	Permitted
2	Pepsi(Box)	330	Zero	Zero	2.53	Permitted
3	Miranda orange (Box)	330	Zero	Zero	3.8	Permitted
4	Pepsi diet (Box)	330	Zero	40	2.66	Permitted
5	Miranda apple (Box)	330	18	20	2.8	Not Permitted
6	Seven Up free on sugar free on color and caffeine(Box)	330	Zero	25	3.67	Permitted
7	ULUDAG (Strawberry) gazoz (bottle)	250	zero	6	3.5	Not Permitted
8	Duo Mount (bottle)	250	zero	4	2.42	Not Permitted
9	Gazoz orange (bottle)	250	zero	2	2.76	Not Permitted
10	Coca cola (bottle)	250	Zero	Zero	2.4	Permitted
11	Coca cola (Box)	330	Zero	Zero	2.6	Permitted
12	Sinalco Lemon (Box)	330	zero	3	3.1	Not permitted
13	ULUDAG Gazoz Lemon (bottle)	250	Zero	Zero	3.41	Permitted
14	Miranda Lemon (bottle)	250	Zero	Zero	2.92	Permitted
15	Miranda orange (bottle)	250	Zero	Zero	2.7	Permitted

16	Shani fruit syrup(Box)	250	Zero	17cell/ml	3.23	Permitted
17	Fanta (orange (Box)	250	Zero	Zero	2.99	Permitted
18	Sprite (Lemon)(Box)	250	Zero	4cell/ml	2.98	Permitted
19	Fanta Citrus (Box)	250	Zero	1cell/ml	2.87	Permitted
20	Cola Delta Wafi	250	Zero	Zero	2.98	Permitted
21	Dalia Top grapes	330	Zero	70cell/ml	2.7	Permitted
22	Rani granules orange (Box)	240	Zero	Zero	2.32	Permitted
23	Rani peach granules	240	Zero	Zero	3.2	Permitted
24	Mizo Kamaruddin (bottle)	296	Zero	121 cell/ml	4.1	Not Permitted
25	TROPIX Drink Kamaruddin (Box)	330	Zero	70 cell/ml	4.32	Permitted
26	Spring orange juice (Box)	250	Zero	Zero	3.19	Permitted
27	Rani (Orange) (bottle)	300	Zero	Zero	3.96	Permitted
28	Rani Cocktail Fruits (bottle)	300	Zero	Zero	4.11	Permitted
29	TROPIX Cocktail Fruits	300	Zero	Zero	4.21	Permitted
30	Rani Pomegranate (bottle)	300	Zero	Zero	4.1	Permitted
31	Rani (Carrots and orange) (bottle)	300	Zero	Zero	5.21	permitted
32	Rani Kamaruddin (bottle)	300	Zero	Zero	5.30	Permitted
33	Ugarit (Lemon)(Box)	330	Zero	Zero	3.87	Permitted
34	Ugarit (orange) (Box)	330	Zero	Zero	4.54	Permitted
35	Ugarit (Pears) (Box)	330	Zero	4 cell/ml	4.7	Permitted
36	Mizo Pineapple (bottle)	296	Zero	Zero	3.19	Permitted
37	Ugarit apricot(Box)	330	Zero	Zero	4.55	Permitted
38	AI_RawaBi Pomegranate (bottle)	275	Zero	Zero	3.65	Permitted
39	Ugarit red grapes (Box)	330	Zero	Zero	3.86	Permitted
40	Mizo (Lemon) (bottle)	296	Zero	Zero	4.22	Permitted
41	Rauch mango juice (Box)	355 ml	Zero	300	3.88	Not permitted

42	Dandanah Natural grape juice (Box)	355 ml	Zero	350	3.2	Not permitted
43	Dandanah Natural Peaches juice (Box)	355	Zero	200	5.23	Not permitted
44	AI_RawaBi Kamaruddin Apricot (Bottle)	275	Zero	250	4.34	Not permitted
45	J`us juice Cut pineapple (bottle)	330	Zero	300	3.45	Not permitted
46	AI_RawaBi Raisin red grapes (bottle)	275	Zero	260	6.54	Not permitted
47	Dalia (Orange) (Box)	250	Zero	16	3.98	Permitted
48	Dalia red grapes (Box)	250	Zero	Zero	3.44	Permitted
49	Original Fruits (Box)	250	Zero	14	4.98	Permitted
50	Dalia Peaches (Box)	250	Zero	4	6.23	Permitted

Most fruit juices contain sufficient nutrients that could support microbial growth. Several factors encourage, prevent or limit the growth of microorganisms in juices , the most important are PH, hygienic juices, the most important are PH, hygienic practice and storage temperature and concentration of the preservative(11).

storage of products at refrigerator temperature or below is not always best for the maintenance of desirable quality of some fruits. Water used for juices preparation can be a major source of microbial contaminants such as total coliforms, fecal coliforms, fecal streptococci, Spoilage yeasts such as *Saccharomyces cerevisiae*, can tolerate acidic environments .It should also be noted that change in PH could transform a food in to one which can support the growth of pathogens (11).

Metals are present in foods (including drinks) either naturally or as a results of human activities such as agricultural practices, industrial emissions, car exhausts ,or contamination during manufacture(12) Food and beverage contamination may also occur due to raw materials and water used (13).

Three heavy metals concentrations (Cu, Cd and pb) were measured in the studied samples.

In nature ,Cu occurs in rocks, water , air and it is essential for normal growth and metabolism of all living organism (14).

The results were revealed that the higher concentration of Cu (1.29 ppm) was recorded in the sample 38 (Al-Rawabi),but do not detectable in both 36 (Mizo pineapple) and 49 (Original fruits) samples. Also most of the studied samples were exceeded the permissible values (0.01ppm)by WHO , and Iraqi standard (0.05 ppm).

20 ppm concentration of Cd was recorded as a higher concentration in the sample 15(Miranda orange) ,but the lowest value 0.002 ppm was recorded in sample 30 (Rani Pomegranate).these results explains the recorded values exceed the permissible value (0.005and 0.05 ppm) which proposed by WHO and Iraqi standard, respectively. These results were identical to these were recorded by (15 and 16).

Lead known to induce reduced cognitive development and intellectual performance in children and increase blood pressure and cardiovascular disease in adults (17).

It was noticed that the higher concentration of pb (12.3 ppm)was found in the samples 16(Shani fruit syrup) while the lowest was recorded in sample 27 (Rani orange) (Table.4) .

As a conclusion , most of the studied samples do not suitable or healthy to use by human but so harm to health according to their microbial assessment and the concentrations of Cu, Cd and Pb.

Table 4: Range and the Mean of Heavy metals in Soft drinks and Fruit Juices (ppm).

No.	Cu	Cd	Pb
1	0.1534	1.077	2.106
2	0.1966	0.527	1.536
3	0.014	6.718	5.677
4	0.037	2.579	2.03
5	0.2487	3.914	0.386
6	0.018	2.761	0.85
7	0.001	0.418	1.93
8	0.476	5.447	1.32
9	0.124	1.54	0.543
10	0.236	0.425	0.17
11	0.037	0.763	0.39

12	0.004	1.536	1.67
13	0.169	15.23	0.002
14	0.159	1.231	0.006
15	0.156	20.01	10.02
16	0.217	1.236	12.3
17	0.020	0.828	0.754
18	0.587	0.52	0.731
19	0.476	0.74	2.40
20	0.272	0.01	3.01
21	0.396	0.70	1.92
22	0.471	1.05	1.12
23	0.134	0.67	1.93
24	0.027	0.96	0.85
25	0.087	0.02	0.64
26	0.393	0.53	1.25
27	0.403	0.03	0.002
28	0.236	1.46	0.17
29	0.315	0.64	0.84
30	0.174	0.002	0.006
31	0.474	0.04	0.64
32	0.058	0.70	0.39
33	0.537	1.32	0.50
34	0.524	1.03	0.66
35	0.655	1.76	0.68
36	ND	1.90	0.10
37	0.840	0.64	1.32
38	1.296	0.007	0.09
39	0.0254	0.006	0.01
40	0.0294	0.75	0.04
41	0.033	1.45	0.12
42	0.0238	0.38	0.08
43	0.270	1.321	0.09
44	0.0328	2.31	0.01
45	0.0651	0.71	0.03
46	0.0087	0.88	0.011
47	0.0118	2.27	0.07
48	0.0044	3.45	0.25
49	ND	2.65	0.07
50	0.1966	2.08	2.01
Range	ND-1.296	0.002-20.01	0.002-12.3
Mean	0.226	1.984	1.274

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Diagnostic and Epidemiological Study of *Toxoplasma gondii* for Students of Thi-Qar University by ELISA and Real-Time PCR Techniques

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Abstract: Toxoplasmosis is caused by an obligate intracellular tissue protozoan parasite *Toxoplasma gondii*, which is able to infect humans as well as other warm blooded domestic and wild animals. The infection has a world-wide distribution with approximately one-third of the world population estimated to be exposed to this parasite The present study was performed for the first time in Thi-Qar province to estimate the prevalence of toxoplasmosis among the university students. Blood samples were collected from 319 (111 males and 208 females) apparently healthy students, they have ages between (18-42) years attended from different colleges of Thi-Qar university (students of morning and evening study), during the period from October 2013 to April 2014. Enzyme linked Immunosorbant Assay (ELISA), was used to evaluate the presence of anti-*Toxoplasma* IgM and IgG antibodies and detection of *B1* gene of *T.gondii* DNA by Real-Time Polymerase Chain Reaction (RT-PCR). The results indicated that 21.94% of students were anti-*Toxoplasma* antibodies using ELISA test. The study demonstrated that no significant difference in infection percentages between colleges where students studied, although the highest seropositivity students was 47.83% in Education Sports College whereas it did not score any infection with toxoplasmosis in College of Education for Pure Science . The current study also showed that the highest positive percentage with *T.gondii* of male students was 28.57% in College of Engineering while the highest percentage with *T.gondii* of female students was 30.43% in College of sports education. The present study revealed that the acute infection of toxoplasmosis which presented IgM and both IgM and IgG was high at small age young students (18-22) years while the chronic infection which presented IgG was high at old students (38-42) years. The present study demonstrated that seroprevalence of toxoplasmosis was significantly associated with close contact with cats. The present study revealed an association between blood group system and *Toxoplasma* infection with highest prevalence among samples of blood group A^+ 30.65% and lowest prevalence in samples of blood group B^+ 17.44%.

Besides the serological diagnosis of *T.gondii*, Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection *B1* gene of *T.gondii* DNA in the blood of students. Out of 319 students only 6.26% showed positive for toxoplasmosis among those 2.50% were males and 3.76% were females. The positive result in RT-PCR analysis were distributed on the patterns of the anti-*Toxoplasma* antibodies by ELISA test , Any positive blood samples with IgM, IgG and both IgM and IgG respectively have not been found whereas 20 positive cases of no anti –*Toxoplasma* antibodies.

Real-Time PCR test in student's blood has advantages in detection of recent or active toxoplasmosis.

Keywords: *Toxoplasma gondii*, ELISA, risk factors, Real-Time PCR, B1 Gene, university students.

دراسة تشخيصية ووبائية لطفيلي المقوَّسة الكوندية *Toxoplasma gondii* لطلبة جامعة ذي قـــار باستعمال تقنيتي الاليزا و تفاعل سلسلة البلمرة ذو الوقت الحقيقي

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[1]مديرية التربية في ذي قار/ اعدادية النصر للبنات
[2و3]جامعة ذي قار /كلية العلوم /قسم علوم الحياة

الخلاصة: المقوسة الكوندية *Toxoplasma gondii* هو طفيلي يعيش داخل الخلايا حيث انه يصيب عدد كبير من سكان العالم لكن ليس من الشائع ان يسبب امراض خطيرة. انجزت الدراسه الحاليه لاول مرة في محافظة ذي قار لمعرفة انتشار داء المقوسات في طلاب الجامعة. جمعت عينات الدم من319 (111 ذكور و208 اناث)طالب تتراوح اعمارهم 18-42 سنة من مختلف الكليات لجامعة ذي قار (الدراسة الصباحية والمسائية) خلال المدة من شهر تشرين الاول013 2 الى نهاية شهر نيسان2014 ،قد استخدم فحص الادمصاص المناعي المرتبط بالانزيم(ELISA) لتقييم وجود الاجسام المضاده للمقوس نوع IgM وIgG واستخدمت تقنية تفاعل سلسلة البلمرة ذو الوقت الحقيقي للكشف عن الجين *B1* في الحامض النووي DNA لطفيلي المقوسة الكوندية. اظهرت الدراسة (21,94%) من الطلبه يحملون الاجسام المضادة للمقوسة الكوندية كما اظهرت الدراسة عدم وجود فروق معنوية لنسبة الاصابة بين الكليات و رغم ذلك فأن اعلى نسبة اصابة كانت (**47,83%**) في كلية التربية الرياضية بينما لم تسجل اية اصابة في كلية التربية للعلوم الصرفة كذللك سجلت الدراسة ان اعلى نسبة اصابة للذكور**28,57%** في كلية الهندسة بينما كانت أعلى نسبة اصابة للاناث 30,43% في كلية التربية الرياضية. بينت الدراسة ان الإصابة الحاده لداء المقوسات المتمثلة IgM وكلا من IgG و IgM كانت في الفئة العمرية الاصغر18-22 سنة بينما الاصابة المزمنة المتمثلة IgG كانت في الفئة العمرية الاكبر38-42 سنة.

بينت الدراسة أن التماس مع القطط له علاقة معنوية مع الانتشار المصلي لداء المقوسات كذللك . أظهرت ان هنالك علاقة بين الاصابة بداء المقوسات ومجاميع الدم حيث سجلت اعلى نسبة اصابة 30,65% بين الطلبة اللذين يحملون مجموعة الدم +A واقل نسبة اصابة 17,44% بين الطلبة اللذين يحملون مجموعة الدم +B . استخدمت الدراسة بالاضافة الى التشخيص السيرولوجي للمقوسة الكوندية تقنية تفاعل سلسلة البلمرة ذو الوقت الحقيقي للكشف عن الجين *B1* في الحامض النووي DNA لطفيلي المقوسة الكوندية في دم الطلبة حيث سجلت الدراسة 6,26% حالة موجبة لداء المقوسات من اصل 319 طالب بينهم2,50% ذكور 3,76% اناث.النتائج الموجبه التي شخصت بواسطة تقنية Real-Time PCR قد وزعت حسب انواع الاجسام المضادة فلم تسجل الدراسة اي حالة موجبة مع IgM و IgG و كلا من IgG وIgM بينما سجلت 20 حاله موجبة في العينات السالبة للاضداد. من مميزات تقنية Real-Time PCR انها سجلت الاصابة الحديثة لداء المقوسات في دم الطلبة.

Introduction

Toxoplasma gondii is a protozoan parasite that causes the disease toxoplasmosis. It is a very common parasitic infection in humans and other warm-blooded animals,with approximately a third of the world's human population estimated to have been exposed to the parasite. Toxoplasmosis can be asymptomatic or can have more severe consequences such as congenital birth defects, eye disease, or potentially fatal toxoplasmic encephalitis in immune-compromised individuals (1).

Sexual replication of the parasite occurs only in domestic cats and wild felidae (definite hosts), while asexual replication occurs in both intermediate and final hosts (2, 3). Oocysts are passed in the feces of cats and become infectious within 21 days of being shed.Tachyzoites survive and multiply only in an intra -cellular location while tissue cysts containing few or many bradyzoites occur in the tissues of infected

animals within a week of infection(4) .

Ingestion of tissue cysts in infected meat and oocysts from soil, food, or water contaminated with cat feces are the two major routes of transmission(5). Rarely, transmission of *T.gondii* occur through blood transfusions and organ transplantations (6*).* Real-time PCR has been used to amplify and quantify DNA from the *T. gondii B1* gene (Costa *et al*., 2000).

Material and Methods

Serological test

The sera of all cases were tested for the presence of specific IgM and IgG anti-*Toxoplasma* antibodies via ELISA kits (BioChik Diagnostics Company, USA) according to the manufacture's instructions

Isolation of genomic DNA from whole blood

DNA was extracted from the whole blood samples of the study groups using acommercial purification system (Reagent Genomic DNA extraction kit (Invitrogen. USA) following the manufacture's instruction for DNA purification from blood. Purified DNA molecules were stored at -40°C, after estimation of DNA concentration and purity, The extracted genomic DNA from whole blood samples was checked by using Nanodrop spectrophotometer (THERMO.USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

Real-Time PCR

Real-Time PCR basedTaqMan probe was performed for rapid detection of *T. gondii* according to method described by Meihuilin *et al*. (7). Real-Time PCR TaqMan probe and primers were used for amplification of conserved region B1 gene in *T. gondii* .These primers were provided by (Bioneer Company. Korea) as showed in following table (1). The Real-Time PCR amplification reaction was done by using (AccuPower® DualStarTM qPCR PreMixBioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as following table (2):

Table (1): Real-Time PCR TaqMan probe and primers

Primer	Sequence		Product size
B1 primer	F	TCCCCTCTGCTGGCGAAAAGT	94bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
B1 probe	5-6FAM-TCTGTGCAACTTTGGTGTATTCGCAG -TAMRA-3		

Table (2): The qPCR master mix

RT-PCR master mix	Volume
DNA template	5µL
Forward B1 gene primer (20pmol)	2.5µL
Reverse B1 gene primer (20pmol)	2,5µL
TaqMan B1 gene probe (20pmol)	2.5µL
DEPC water	37.5 µL
Total	50µL

These qPCR master mix reaction components that mentioned in table above were added into AccuPower® DualStarTM qPCR PreMixtubes which containing Taq DNA polymerases, dNTPs, 10X buffer for TaqMan probe amplification. Then tubes placed Exispin vortex centrifuge at 3000rpm for 3 minutes, after that transferred into MiniOpticon Real-Time PCR system and applied the following thermocycler conditions as the following table (3):

Table (3): Thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	45
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

Statistical analysis

Data were analyzed with chi-square and P value < 0.05 was considered statistically significant**.**

Results

1.Incidence of Infected Students Accor- ding to Test Type

The present study carried out on 319 apparently healthy students from Thi-Qar university that included in this study, 111 male students and 208 female students, to elucidate *Toxoplasma gondii* infection by using Enzyme Linked Immunosorbent Assay (ELISA), as well as Real Time Polymerase Chain Reaction (Real time-PCR). Results presented in this study showed that overall the prevalence of toxoplasmosis were 70(21.94%) among those 24(7.52.%) were males and 46(14.42%) were females by using ELISA test while the overall prevalence of toxoplasmosis were 20(6.26%) among those 8(2.50%)were males and 12(3.76%) were females by using Real time quantitative PCR, Figure (1).

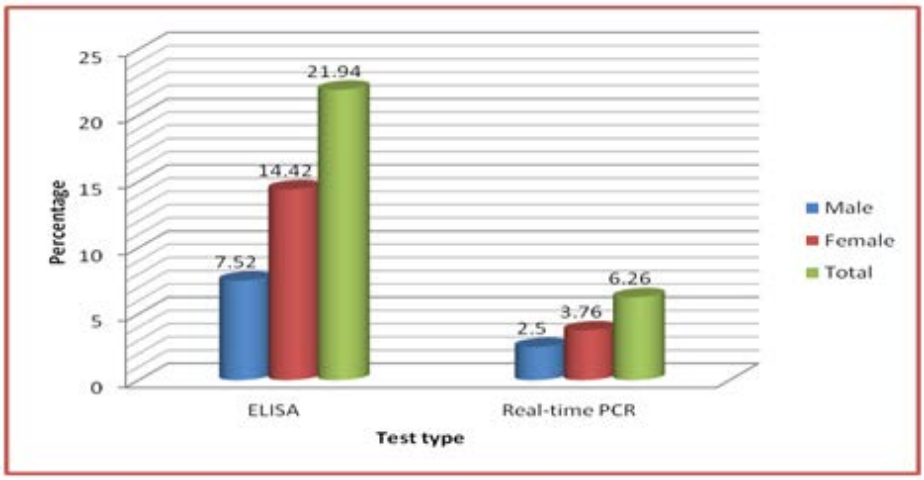

Figure 1: Distribution of students infected with toxoplasmosis according to test type.

2. Distribution of Infected Students According to College and the Sex by ELISA test

The present study showed that no significant difference in infection percentage between faculties of students (p>0.05), although the highest seropositivity students was 11(47.83%) in College of Sport Education whereas it did not score any infection with toxoplasmosis in College of Education for Pure Science. Also, the current study showed that the highest positive percentage with *T.gondii* of male students was 6 (28.57%) in College of Engineering while the highest percentage with *T.gondii* of female students was 7 (30.43%) in College of sport education. table (4).

Table 4: Distribution of students infected with toxoplasmosis from Thi-Qar University according to sex and college

S	Colleges	No.	Male / Female	No.	%	Total +ve No.	Total +ve %	-ve No.	-ve %
1	Sciences	34	10	2	5.88	5	14.70	29	85.29
			24	3	8.82				
2	Compute& Science mathematics	25	5	1	4.00	3	12.00	22	88.00
			20	2	8.00				
3	Agriculture & Marshes	24	9	0	0.00	2	8.33	22	91.67
			15	2	8.33				
4	Education for human science	28	11	2	7.14	5	17.85	23	78.57
			17	3	10.71				
5	Nursing	30	5	5	16.66	6	20.00	24	80.00
			25	5	16.66				
6	Pharmacy	20	4	1	5.00	7	20.00	16	80.00
			16	3	15.00				
7	Education sports	23	11	4	17.32	11	47.83	12	52.17
			12	7	30.43				
8	Literature	22	8	1	4.54	4	18.18	18	81.82
			14	3	13.63				
9	The media	14	2	0	0.00	2	14.29	12	85.71
			12	2	14.28				
10	Engineering	21	15	6	28.57	8	38.10	13	61.90
			6	2	9.52				
11	Law	24	4	0	0.00	6	25.00	18	75.00
			20	6	25.00				
12	Medicine	14	6	4	28.57	6	42.86	8	57.14
			8	2	14.28				
13	Education for pure science	14	7	0	0.00	0	0.00	14	100
			7	0	0.00				
14	Administration & Economy	26	14	2	7.69	8	30.77	18	69.23
			12	6	23.07				
Total		319	111	24	7.52	70	21.94	249	78.06
			208	46	14.42				
Statistical analysis		Cal. X^2=14.68;Tab. X^2 (df=13 ; α=0.05) =22.36							

3. Incidence of Infected Students from Thi- Qar University According to Age by ELISA test

The present result showed that the highest infection rate of toxoplasmosis occurred in age group 23-27 years of students .Whereas, in relation to age group students who had been positive for IgM and students who had been positive for both IgM and IgG against *Toxoplasma* showed same results characterized by the presence of high positive percentage 14(5.81%) at age group 18-22 years and the study did not score any infection in those students at age groups(28-32), (33-37) and (38-42) years respectively . Students had been positive for anti- *Toxoplasma* IgG antibodies showed variable results characterized by the presence of high percentage 2 (28.57%) at the

age group of 38-42 whereas the lowest one was 20 (8.30%) as noticed at the age group 18-22 years. There was a significant difference between age group and toxoplasmosis of students ($p<0.05$) as shown in table (5).

Table 5: Percentage distribution of students from Thi-Qar University infected with toxoplasmosis according to age.

Age groups (year)	IgM^+ve		IgG^+ve		IgM^+ve & IgG^+ve		Total anti-*Toxoplasma* Abs		No anti-*Txoplasma* Abs		Total	
	N0	%	N0	%	No	%	No	%	No	%	No	%
18-22	14	5.81	20	8.30	14	5.81	48	19.92	193	80.08	241	100
23-27	3	5.45	13	23.64	1	1.81	17	30.90	38	69.10	55	100
28-32	0	0.00	2	20.00	0	0.00	2	20.00	8	80.00	10	100
33-37	0	0.00	1	16.67	0	0.00	1	16.67	5	83.33	6	100
38-42	0	0.00	2	28.57	0	0.00	2	28.57	5	71.43	7	100
Statistical Analysis	$X^2=43.3^{**}$		$X^2=38.05^{**}$		$X^2=50.66^{**}$		$X^2=115.85^{**}$					
	$TabX^2(df=6,\alpha=0.05)=9.98$						$TabX^2(df=2,\alpha=0.05)=5.66$					

4. Incidence of Infected Students with Toxoplasmosis According to Contact with Cats

The presentstudy demonstrated that seroprevalence of toxoplasmosis was significantly associated with close contact with cats of students infected with toxoplasmosis ($p<0.05$) they recorded 9 (31.03%) and 61 (21.03%) respectively, as shown in figure (2).

5. Incidence of infected students with toxoplasmosis according to blood groups

Blood group is of interest that the present study revealed an association between blood group system and *Toxoplasma* infection with highest prevalence among samples of blood group A+ 19 (30.65%) and lowest prevalence in samples of blood group B^+ 15 (17.44%). There was a significant difference between blood groups ($p<0.05$) as shown in table (6).

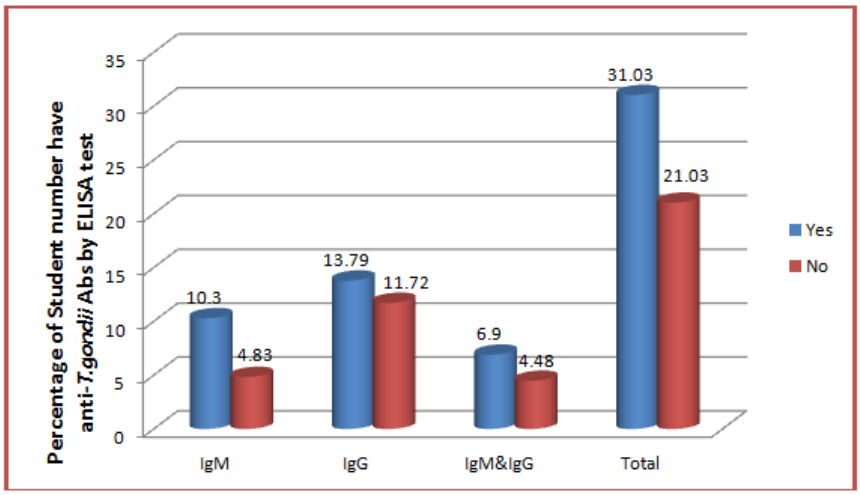

Figure 2: Distribution of students infected with toxoplasmosis according to contact with cats.

Table 6: Distribution of students infected with toxoplasmosis according to blood groups

Blood groups	Anti-*Toxoplasma* Abs		No Anti-*Toxoplasma* Abs		Total	
	No.	%	No.	%	No.	%
A^+	19	30.65	43	69.35	62	100
B^+	15	17.44	71	82.56	86	100
AB^+	5	25.00	15	75.00	20	100
O^+	26	20.31	102	79.69	128	100
Statistical analysis	Cal. X^2 = 30.37 **					
	Tab. X^2 (df=7 α=0.05)= 9.48					

6. Detection of *T.gondii B1* gene by Real-Time quantitative PCR

Besides the serological diagnosis of *T.gondii* Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection of *T.gondii* DNA in the blood of students .*T. gondii* DNA was successfully extracted and analyzed by RT-PCR technique, the study revealed that out of 319 studnts only 20 (6.26%) showed positive toxoplasmosis as shown in figure (3).

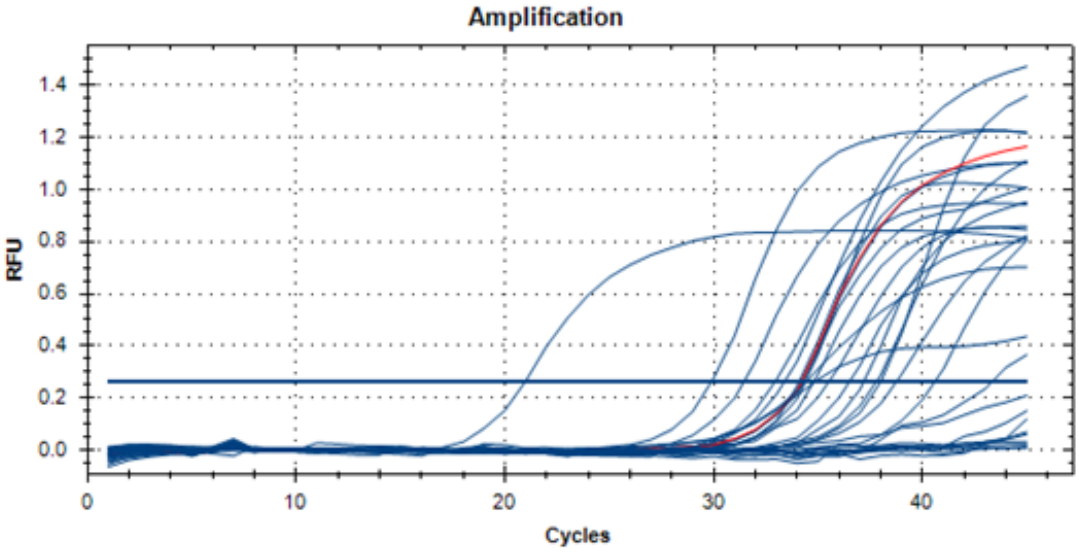

Figure 3 : Real-Time amplification plot of B1 gene in *T.gondii* from blood samples of students .Where, Blue plots: 20 positive samples, Red plot: Positive control (DNA *Toxoplasma gondii*).

7. Relation between Real-time PCR Analysis and ELISA Results

Students 20 (6.26%) who recorded positive results in RT-PCR analysis were distributed on the patterns of the anti-*Toxoplasma* antibodies by ELISA test , any positive cases with IgM, IgG and both IgM and IgG respectively have not been found whereas there are 20 positive cases of no anti – *Toxoplasma* antibodies as shown in table (7).

Table 7: Real Time PCR analysis distribution to anti-Toxoplasma antibodies in students.

Real-time PCR / ELISA-test	Real-time PCR +ve		Real-time PCR -ve		Total	
	No.	%	No.	%	No.	%
ELISA +ve	0	0	70	21.94	70	21.94
ELISA-ve	20	6.27	229	71.79	249	78.06
Total	20	6.27	299	93.73	319	100

Discussion

1. Seroprevalence of Toxoplasmosis by ELISA test

The prevalence rate of our study was (21.94%) by ELISA test, this result was agreed with the regional and universal trend for toxoplasmosis infection ratio, where a quarter to one third of various populations showed immunity (8; 9). Our result was in agreement with other studies in some Arab countries in Saudi Arabia by (10) who found that the incidence rate was 21.8% and in Sudan by (11) who revealed that the infection rate was 20.7%. Moreover the result of our study was in agreement with several studies in world, in Lowa seroprevalence by IFA test was 20.4% in Veterinary students by (12), in Mexico seroprevalence by using ELISA in high school students aged (14-25) years old was 22.4% by (13), in Iran seroprevalence by using ELISA IgG in students 22.6% by (9). While the result of our study lower than seroprevalence reported by (14) in Brazil who recorded seropositive toxoplasmosis of undergraduate students attending from Nursing and Science Colleges was 39%. The variation and similarities in results may be related to several factors, including cultural patterns and climatic, nutrition habits, sample size, age, target population, sampling method, types of laboratory tests and tools (15, 1)

2. Distribution of Infected Students According to College and the Sex by ELISA test

The current study showed that there is no significant difference in infection percentage between faculties of students ($p>0.05$) 05), although the highest seropositivity students was 11(47.83%) in College of Sport Education whereas it not scored any infection with toxoplasmosis in College of Education for pure science. Also, the current study showed that the highest positive percentage with *T.gondii* of male students was 6 (28.57%) in College of Engineering while the highest percentage with *T.gondii* of female students was 7 (30.43%) in College of Sports Education.

This variation in result may be attributed to differences in cultural level, nutritional habits, age or rural urban area (15).

3. Incidence of Infected Students According to Age by ELISA test

The age group students who had positive for IgM and students had positive for both IgM and IgG against *Toxoplasma* showed same results characterized by the presence of high positive percentage 14 (5.81%) at age group 18-22 years. Students had positive for anti-*Toxoplasma* IgG antibodies for showed variable results characterized by the presence of high percentage 2(28.57%) at the

age group of 38-42 years. This gives a clear idea that the acute infection of toxoplasmosis which presented IgM and both IgM and IgG was high at young adult human while the chronic infection which presented IgG was high at old adult human, this results agreed with other studies in same province by (16, 17, 18).

High prevalence values of infection with *T.gondii* were found in young adult human, this probably happened due to more frequent *Toxoplasma* contact in childhood and adolescence ,through cats contact, soil exposure (19) .Additionally ,in acute stage of *T.gondii* infection, IgM antibodies estimation appears early in the course of infection while IgG antibodies to *T.gondii* usually appear within 1 to 2 weeks later of appearance IgM and peak within 1 to 2 month, then fall at variable rates and usually persist for life (20). However, when IgG antibodies against *T.gondii* were present in blood they indicate that *T.gondii* cyst was already present in the tissue (21).

4. Incidence of Infected Students with Toxoplasmosis According to Contact with Cats

The current study demonstrated that seroprevalence of toxoplasmosis was significantly associated with close contact with cats. This result is in accordance with several studies (22, 23,24 25, 26).While the result disagreed with several studies (13, 18,27).

Cats rearing and stray cats are widely spread in Thi-Qar governorate, this may increase chance of contact with cats litter or contaminated food or water by parasite oocysts and this may explain the strong association between seropositivity and exposure to cats..Felids are the only definitive hosts responsible for shedding oocysts that contaminate the environment and become infective for long time in water or soil (1).

5. Incidence of infected students with toxoplasmosis according to blood groups

Blood group is of interest that the present study revealed an association between blood group system and *Toxoplasma* infection with highest prevalence among samples of blood group A+ and lowest prevalence in samples of blood group B^+ There was a significant difference between blood groups ($p<0.05$)**.** This result agrees with (28, 29) who recorded that the highest prevalence among samples of blood group A+.

The present result of this study is a possibility that the parasite utilized glycoconjugates, which characterize the blood phenotypes of the ABO blood group system, as a potential receptors (30, 31).

6. Detection of *T.gondii B1* gene by Real-Time quantitative PCR

Besides the serological diagnosis of *T.gondii* Real-Time PCR (RT-PCR) technique was used to confirm the infection with *T.gondii* by detection *B1* gene of *T.gondii* DNA in the blood of students. *Toxoplasma gondii* DNA was successfully extracted and analyzed by RT-PCR. The diagnostic value of PCR for the detection of *T.gondii* in blood samples has been evaluated from both immune competent and immune compromised patients (32, 33, 34).

The study revealed that out of 319 students only 20 (6.26%) showed positive toxoplasmosis among those 8 (2.50%) were male and 12 (3.76%) were female. Statistically, there was no significant difference between them ($p>0.05$). The positive result was higher in this study than those recorded by (34) in Turkey, who demonstrated that rate was (1.3%) and with (36) in Taiwan, who recorded no active parasitemia was detected by Real-time PCR assay, while the rate was lower in this study than those recorded by (37), who recorded that the rate infection of toxoplasmosis was(69%,**)** and other studies in Iraq (18, 38, 39) who showed that the rate was (38.0%, 17.7% and 16%) respectively of aborted women .

The explanation of these differences stated by other researcher may be resulted from the use of only healthy students for both sexes in this study which gave findings that may therefore differ from findings in other population.

The result of negative blood group phenotypes (A-, B-, AB- and O-) were not considering in this study, because of their small size number.

7. Relation between Real-time PCR Analysis and ELISA Results

Students 20 (6.26%) who recorded positive results in RT-PCR analysis were distributed on the patterns of the anti-*Toxoplasma* antibodies , it did not find any positive case with IgM, IgG and both IgM and IgG respectively whereas 20 positive cases of no anti –*Toxoplasma* antibodies .This result was in- line with the result obtained by (36) who showed that out of 1783 blood from healthy blood donors were tested for the presence of *T.gondii* antibodies and DNA using ELISA and RT-PCR respectively, 5(0.28%) 166(9.3%), tested positive for anti-*Toxoplasma* IgM and IgG respectively but no active parasitemia of positive ELISA result was detected by Real-time PCR assay .Another study by (40) who revealed that no active parasitemia of anti- *Toxoplasma* IgG was detected by Real-time PCR assay. Also, these results agreed with a number studies which have already shown that a positive PCR

result is not always accompanied by positive serology indicating local synthesis of antibodies (41, 39).

Current diagnosis of toxoplasmosis dependent on serological detection may fail to detect specific anti-*Toxoplasma* IgG or IgM during the active phase of *T. gondii* infection, because these antibodies may not be produced until after several weeks of parasitemia. Therefore, in this study highly specific molecular has been used as Real-time PCR based TaqMan probe and primers to amplify the *T. gondii B1* gene for detection of *Toxoplasma gondii* (7).

Conclusion

Real-Time PCR technique is more sensitive and specific than serological tests. The present result showed that 77.89% percent of females in Thi-Qar province are susceptible to acute *Toxoplasma* infection during the child -bearing years, and therefore their infant are susceptible to congenital toxoplasmosis

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Comparing the activity of some local and commercial antibiotics

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Abstract: A comparison has been performed between 5 local antibiotics and 5 commercially imported antibiotics of the same kind: Ampicillin, Amoxicillin, Cefotaxime, Tetracycline, and Clarithromycin, in an aim to compare the efficiency of local and commercial antibiotics. Bacterial sensitivity against these antibiotics was detected. Disk diffusion method and Minimal Inhibitory Concentration (MIC) was used to detect antimicrobial sensitivity. Results showed an increase in the inhibition zone of local antibiotics in general compared to commercial antibiotics , especially for Cefotaxime which showed significant increase in the inhibition zone of the local product compared to the commercial product against *Staphylococcus aureus* (23mm vs. 17mm) and *Pseudomonas aerugenosa* (17mm vs.7mm), both at ($P<0.05$). Similarly, Tetracycline against *Klebsiella spp* (32mm vs. 26mm) and Clarithromycin against *Proteus mirabilis* (11mm vs. 7mm).The MIC test showed parallel results with the disk diffusion method. In conclusion, local antibiotics have good activity and must be encouraged and supported rather than commercial antibiotics.

Keyword: Antibiotics, Bacterial sensitivity, Disk diffusion method, and Minimal inhibitory concentration.

مقارنة فعالية بعض المضادات الحيوية المحلية والمستوردة

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الخلاصة : تمت مقارنة 5 مضادات محلية و 5 مضادات مستوردة من نفس النوع وهي : اموكسيلين والاميبسيلين و سيفوتاكسايم وتتراسايكلين والكلاريثرومايسين ،و الهدف مقارنة فعالية المنتوج المحلي والمنتوج المستورد التجاري و تم التحري عن المقاومة البكتيرية لهذه المضادات . تم استخدام طريقة الانتشار بالاقراص وطريقة التركيز المثبط الادنى للتحري عن الحساسية المضادة للبكتيريا. اظهرت النتائج زيادة في منطقة التثبيط للمضادات المحلية مقارنة بالمضادات المستوردة بشكل عام، ولكن بشكل خاص بالنسبة الى مضاد السيفوتاكسايم اظهر زيادة معنوية اعلى في مناطق التثبيط للمنتوج المحلي منه للمنتوج المستورد وبالذات ضد بكتيريا *Staphylococcus aureus* (23 ملم مقابل 17 ملم) وكذلك بكتيريا *Pseudomonas aerugenosas* (17 ملم مقابل 7 ملم) ($P<0.05$). وبشكل مشابه مضاد التتراسايكلين ضد بكتيريا *Klebsiella spp* (32 ملم مقابل 26 ملم) واخيرا مضاد الكلاريثرومايسين ضد بكتيريا *Proteus mirabilis* (11 ملم مقابل 7 ملم). اما نتائج التركيز المثبط الادنى فقد كانت موازية لنتائج الانتشار بالاقراص ، لذلك نستنتج ان المضادات المحلية ذات فعالية جيدة ولابد من تشجيعها ودعم صناعتها.

الكلمات المفتاحية : المضادات ، الحساسية البكتيرية ، طريقة الانتشار بالاقراص ، التركيز المثبط الادنى.

Introduction

Antibiotics must have selective toxicity for the Microbe, several hundreds of compounds with antibiotic activity have been isolated from microorganisms over the years, but only a few of them are clinically-useful. The reason for this is that only compounds with selective toxicity can be used clinically (1). The selective toxicity of antibiotics means that they must be highly effective against the microbe but have minimal or no toxicity to humans. It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms (2).

Antibiotics are very important in our daily lives as they represent the main and important way to treat different kinds of bacterial infections especially the dangerous infections of bacterial causes such as meningitis, pneumonia, osteomyelitis, ect (3, 4, 5). Locally in our country antibiotics are produced by factories which has a long experience in this field, but with the dramatic open exportation most of the antibiotics has been exported to our country and many are from untrusted sources and with absence of quality testing . These commercial antibiotics began to compete the local antibiotics, and the consumer started to favor the commercial upon the local product. The aim of the present study was to test the quality of some local and commercial antibiotics in a goal to encourage the local product.

Materials and Methods:

A- Disk diffusion method: Since the introduction of antibiotics the disc technique has been used by various workers for their assay and also for the detection of bacterial sensitivities. Kirby-Baure method was used according to (6) to carry out antimicrobial susceptibility for 5 local and 5 commercial antibiotics.

1-preparation of antibiotic discs:

Using whattman filter paper. Discs were cut out with a size of 6.5 mm by using a cork borer, then the discs were separated out in a large glass petri dish and sterilized in a hot air oven 50-60 C^0 overnight.

Stock solution of antibiotic was prepared to take an appropriate volume which give the reference concentration for each one:

1-**Ampicillin** (Local) 500 mg was obtained from SDI-IRAQ Company.

- **Ampicillin** (Commercial) 500 mg was obtained from LYKA Company.

2-**Amoxycillin** (Local) 500 mg Obtained from SDI-IRAQ Company.

-**Amoxycillin** (commercial) 500 mg Obtained from KOPRAN Company.

3-Tetracycline (Local) 250 mg obtained from SDI-IRAQ Company.

-**Tetracycline** (commercial) 250 mg Acquired as APCYCLINE-250 from Ajanta pharma limited company.

4-**Cefotaxime** (Local) 250 mg Obtained from SDI-IRAQ Company.

-**Cefotaxime** (commercial) 250 mg obtained from PHARMA INTERNATIONAL Company.

5-**Clarithromycin** (Local) 250 mg Obtained from SDI-IRAQ Company.

-Clarithromycin (commercial) 250 mg obtained from CLARANTA Company.

Each antibiotic was prepared as a stock solution not less than 1000 µg/ml. From stock solutions the appropriate volume was taken to accommodate its concentration in the discs. For example Ampicillin was prepared in a conc. Of 1.25 mg/L (1250 µg/ml), since the volume which is added to disc is 0.02 ml (20µl) then 1.25*20= 25µg (which is the final antibiotic concentration in the disk).

Table (1): Antibiotic concentrations in micrograms for each disc.

Antibiotic	Disc conc. *µ*g/disc
Ampicillin	25
Amoxicillin	10
Cefotaxime	30
Tetracycline	10
Clarithromycin	5

After preparation of known conc. of each antibiotics, a volume of a drop (0.02ml) from each was transferred by micropipette to each disc (to reach the reference concentration in the disc for each antibiotic) Table 1. Then a sterile syringe needle pierced in the center of a blank filter paper disc, the syringe then was held upright with the disc above and the required conc. is discharged gently by micropipette on the disc till the disc was thoroughly soaked. The moist discs were placed on stainless steel wire cloth and dried in moving air oven at 60C^{o} for 5 minutes separately. Discs were then placed in sterile dry vials until they were used (7).

2- Organism, Culture medium, and Inoculms:

Purified isolates of *Staphylococcus. aureus*, *Staphylococcus. Epidermidis*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia. coli and Klebsiella sp*. (which were previously diagnosed in the laboratories of biology department /College of science/Baghdad University) used as test organisms.

Mueller –Hinton agar medium was used (to prepare the media for the inoculation of bacterial swabs) according to manufacture direction.

The inoculums for bacterial strains were prepared by taking four to five pure colonies from an overnight growth using a sterile loop and emulsified in sterile normal saline. Gently dilution was performed till the turbidity was comparable visually to [0.5 Mcfarland] turbidity standard with inoculums density approximately 10^8 CFU/ml (6). For each bacterial strain a sterile swab was dipped into its standardized cell suspension and squeezed gently by rotating the swab to remove the excess fluid. The entire surface of each agar plate was inoculated by streaking the swab in 3 different direction to ensure a uniform growth, the plates were allowed to dry for 5-10 min.

3- Applying antibiotic discs and reading inhibition zones:

Antibiotic discs were placed on agar and pressed firmly to insure they contact with the agar by using a sterile forceps and approximately the same distance from edge of plate and from each other because of reflection wave effect from the edge which give inaccurate zone. The plates then were incubated for 18-20 hr. at 35-37C°.

Inhibition zone developed around the antibacterial discs for each antibiotic and was measured by using a metric ruler in millimeter (mm) measuring the edge of clear zone (no growth area). There are many factors that affect diameter of inhibition zone such as thickness of medium (not more 4-6 mm as it reduce size of the zone) and too thin medium may be not standarized and exaggerated or ill-defined zone appearance , also humidity and the age of medium may affect the zone size (8).

Each isolate was interpreted as susceptible, intermediate or resistant to a particular antimicrobial agent by comparison with standard inhibition zones mentioned in table 2.

Table 2: Inhibition zone of the antibiotic disc according to (9).

No.	Antibiotic disc	Code	Disc potency Mg/disc	Diameter of Inhibition zone (mm)		
				susceptible	intermediate	resistance
1	Ampicillin	AM	25	≥ 18	15-17	≤ 14
2	Amoxicillin	AX	10	-	-	-
3	Cefotaxime	CFM	30	≥ 23	15-22	≤ 14
4	Tetracycline	TE	10	≥ 15	12-14	≤ 11
5	Clarithromycin	CLR	5	-	-	-

A- Minimal Inhibitory Concentration (MIC) preparations:

Methods of dilution susceptibility testing are used to determine the minimal concentration of an antimicrobial agent to inhibit or kill a microorganism. This can be done by dilution of the antimicrobial agent in either agar or broth media. Antimicrobials are tested in serial dilutions (two fold). (10)

Stock solutions for each antibiotic were prepared as previously mentioned and suitable ranges of antibiotic concentrations for the organisms to be tested were chosen, Table 3, Then a double folded serial dilutions (each according to its own solvent and diluent) (11) for the range of each antibiotic was prepared from the stock solution according to (12).

Stock solutions were freezed and thawed only one time and then they were discarded. Table 4 shows the solvent, diluents, and storage conditions for antibiotics.

Table (3): antibiotic concentration ranges for organisms under test.

Antibiotic	Concentration range Mg/ml				
	E.coli	***Pseudomonas aeruginosa***	***Proteus mirabilis***	***Klebsiella spp***	***Staphylococcus spp***
Ampicillin	0.25-128	-	-	0.15-64	0.5-512
Amoxicillin	0.25-128	-	-	0.15-64	0.25-128
Cefotaxime	0.15-256	0.5-128	-	0.06-128	0.5-128
Tetracycline	0.06-256	-	2-1024	0.25-128	0.5-512
Clarithromycin	-	-	-	2-1024	0.5-256

Table (4): Preparation and storage of antibiotic solutions (11).

Antibiotic	Solvent	Diluent	4ºC	-20ºC
Ampicillin	[a]	water	7 days	NR
Amoxicillin	[a]	water	7 days	NR
Cefotaxime	water	water	10 days	6 months
Tetracycline	Ethanol	water	-	NR
Clarithromycin	DMSO	water	-	-

[a] Saturated $NaHCO_3$ solution ; NR = not recommended ; DMSO = dimethylsulphoxide

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Statistical Analysis:

Statistical analysis was done using the statistical analysis system-SAS (2010) program to study the effect of factors in study traits.

The statistical significance of difference in means of continuous dependent (normally distributed function of random variable) between two groups was assessed by independent samples t-test.

Results and Discussion:

Five local and 5 commercial antibiotics were tested for their sensitivity against six bacterial strains of Gram positive and Gram negative bacteria. The sensitivity was compared between local and commercial produced antibiotics.

Inhibition zones were compared to standard inhibition zones to determine susceptibility of bacterial strains against antibiotics enrolled in the study, Table (2).

Inhibition zones of antibiotics against bacterial strains in general showed diameters of local antibiotics were slightly higher than those of the commercial antibiotics, and the resistance of some bacterial strains like *Proteus mirabilis* and *Pseudomonas aeruginosa* was the same for both local and commercial produced antibiotics, Table (5).

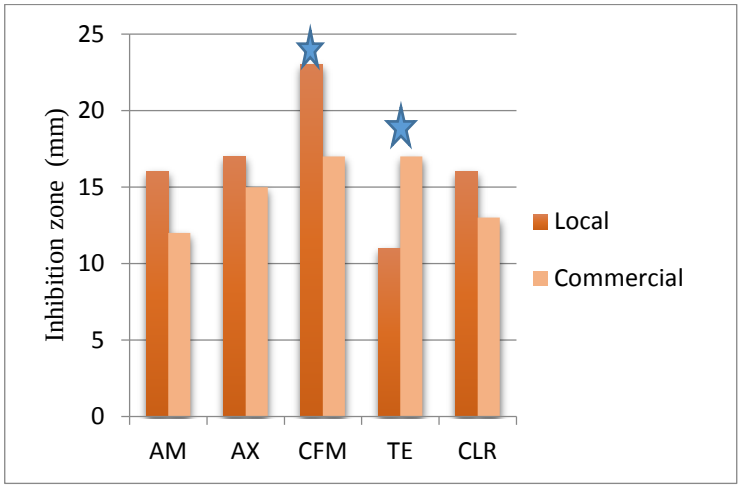
Table 5: Inhibition zone size (mm) of the antibiotics against different bacterial strains.

Antibiotic / Bacteria	Ampicillin		Amoxicillin		Cefotaxime		Tetracycline		Clarithromycin	
	L	C	L	C	L	C	L	C	L	C
Staphylococcus aureus	16	12	17	15	23	17	11	17	16	13
Staphylococcus epidermidis	17	13	19	17	25	22	11	18	35	36
Escherichia coli	R	R	R	R	R	R	28	26	16	13
Klebsiella spp	23	21	23	22	23	20	32	26	19	15
Proteus mirabilis	R	R	R	R	R	R	R	R	11	R
Pseudomonas aeruginosa	R	R	R	R	16	R	R	R	R	R

L= Local; **C**= Commercial; **R**= Resistant.

The effectivity of antibiotics were relatively equal between local and commercial antibiotics against *S.aureus* , except for Cefotaxime as the local product showed a significant increase compared to the commercial product (23mm versus 17mm) at ($p < 0.05$) ,whereas for Tetracycline the commercial product showed the significant increase compared to the local one (17mm versus 11mm) at ($p < 0.05$), fig (1,2) . The effectivity of

antibiotics were also relatively equal between local and commercial antibiotics against *S.epidermidis* , except for Tetracycline the commercial product showed the significant increase compared to the local one (18mm versus 11mm) at ($p < 0.05$), fig (3,4). Staphylococci are inherently susceptible to most antibiotics, except those with a purely Gram negative spectrum. However, b-lactamase production
evolved rapidly in Staphylococci (13, 14). The resistance to such antibiotics can be related to multiple antibiotic resistance carried on plasmids (15).

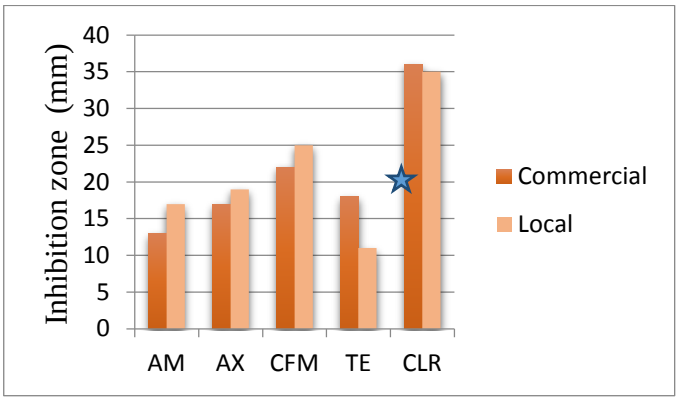


AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

Figure 1: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *S.aureus* to some commercial and local antibiotics.

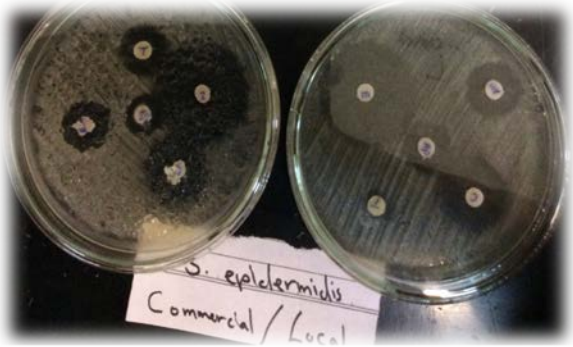
AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

Figure 2: Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *S.aureus* to some commercial and local antibiotic.



AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

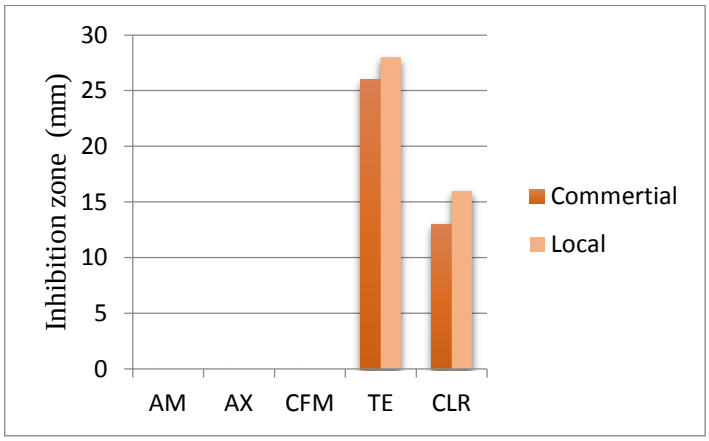
Figure 3: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *S.epidermidis* to some commercial and local antibiotics.



AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

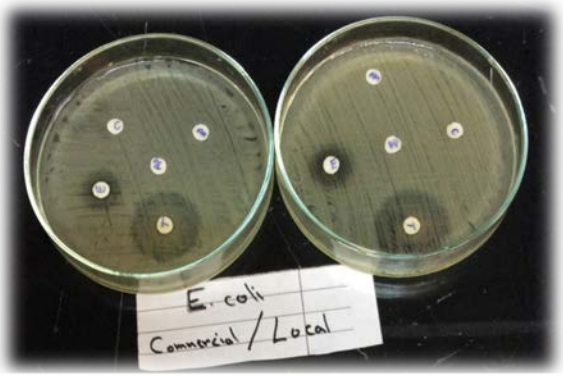
Figure 4 : Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *S.epidermidis* to some commercial and local antibiotic.

The local antibiotic product of Tetracycline and Clarithromycin gave inhibition zone (more effective) larger than the commercial one against ***E.coli*** , while resistant against Ampicillin, Amoxillin, and Cefotaxime, fig (5,6), the resistance may be related to extended spectrum β-lactamase (ESBLs) that mediate resistance in such Gram negative bacteria (16). All local antibiotics are better than the commercial one in effectiveness against *Klebsiella* especially Tetracycline which showed a significant increase in the inhibition zone for the local product compared to the commercial product (32mm versus 26mm) at ($p < 0.05$), fig (7,8).



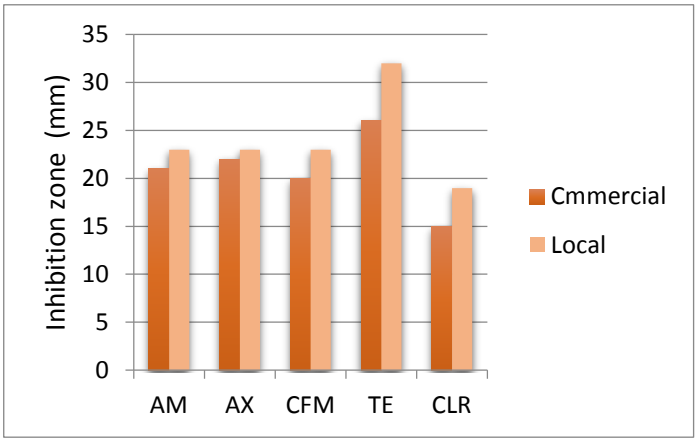
AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

Figure 5: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *E.coli* to some commercial and local antibiotics.



AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

Figure 6: Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *E.coli* to some commercial and local antibiotic.



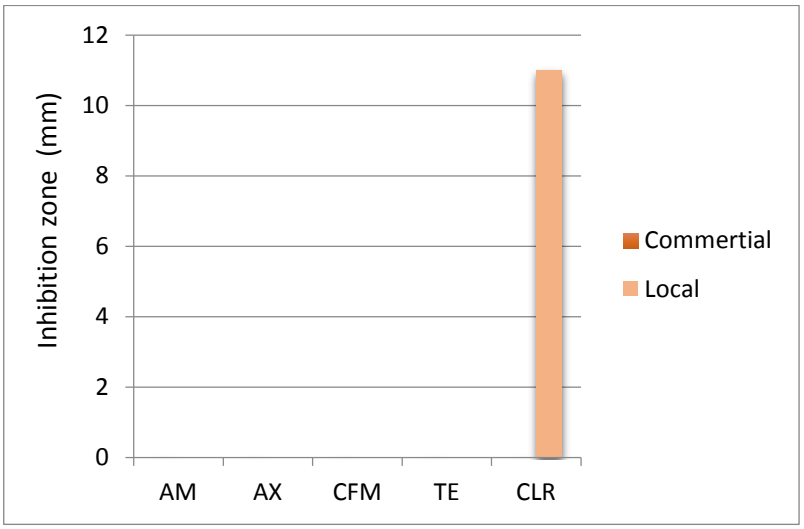
AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

Figure 7: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *Klebsiella sp* to some commercial and local antibiotics.

AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

Figure 8: Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *Klebsiella sp.*to some commercial and local antibiotic.

Local Clarithromycin was effective in sensitivity against *Proteus mirabilis* which was resistant to the commercial product, fig (9, 10). Local Cefotaxime showed an intermediate effect against *Pseudomonas aeruginosa* which was resistant to the commercial product, fig (11, 12). *Proteus mirabilis* and *Pseudomonas aeruginosa* were both resistant to all the other antibiotics (local and commercial) in spite of their action which is mostly on Gram negative bacteria (17). This resistance to these antimicrobial agents has been increased with prescription of years, because of the random use of these antimicrobial agents. On the other hand, these bacteria had the ability to produce β-lactamases, especially (ESBLs), as well as their ability to transfer genetic elements carrying the genes of these enzymes which show a number of mutations leading to resistance (18), in addition to other mechanisms like alteration of the target site or alteration in the access to the target site by modification of penicillin binding proteins (PBPs) (19). Minimal inhibitory concentrations of local antibiotics in general showed a lower concentration that inhibited the growth of the bacterium than the commercial antibiotic which needed a higher concentration to inhibit the growth of the bacterium. Commercial product of Tetracycline showed better effect against Gram positive bacterium (*S. aureus* and *S. epidermidis*) with the inhibition in the growth of bacterium in a lower concentration than the local product,

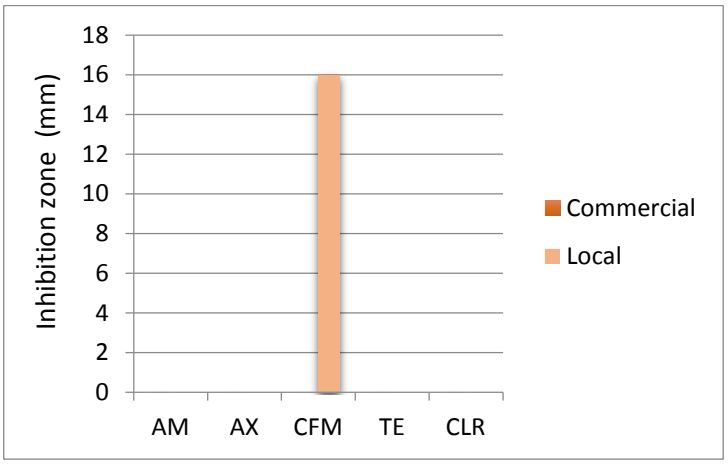


AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

Figure 9: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *Proteus mirabilis* to some commercial and local antibiotic.

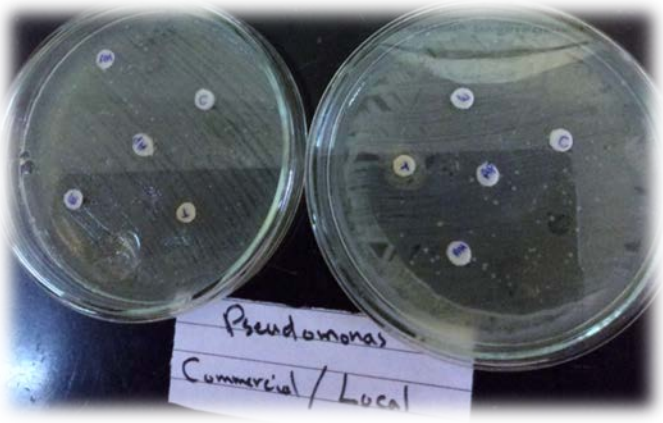
AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

Figure 10 : Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *Proteus mirabilis* to some commercial and local antibiotic.



AM=Ampicillin, **AX**=Amoxillin, **CFM**=Cefotaxime, **TE**=Tetracycline, **CLR**=Clarithromycin.

Figure 11: Diagram explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *to Pseudomonas aeruginosa* some commercial and local antibiotic.



AM=Ampicillin, **AX**=Amoxillin, **C**=Cefotaxime, **T**=Tetracycline, **E**=Clarithromycin

Figure 12: Picture explain antibiotic sensitivity by disk diffusion method determined by inhibition zone (mm) of *Pseudomonas aeruginosa* to some commercial and local antibiotic.

Local product of Cefotaxime against *S.aureus* , Tetracycline against *Klebsiella* and Clarithromycin against *Proteus mirabilis* showed the better effect in the inhibition of the bacteria , Table (6). These results also parallel the results of the disc diffusion method with minor differences which were detected by the interpretation of both tests.

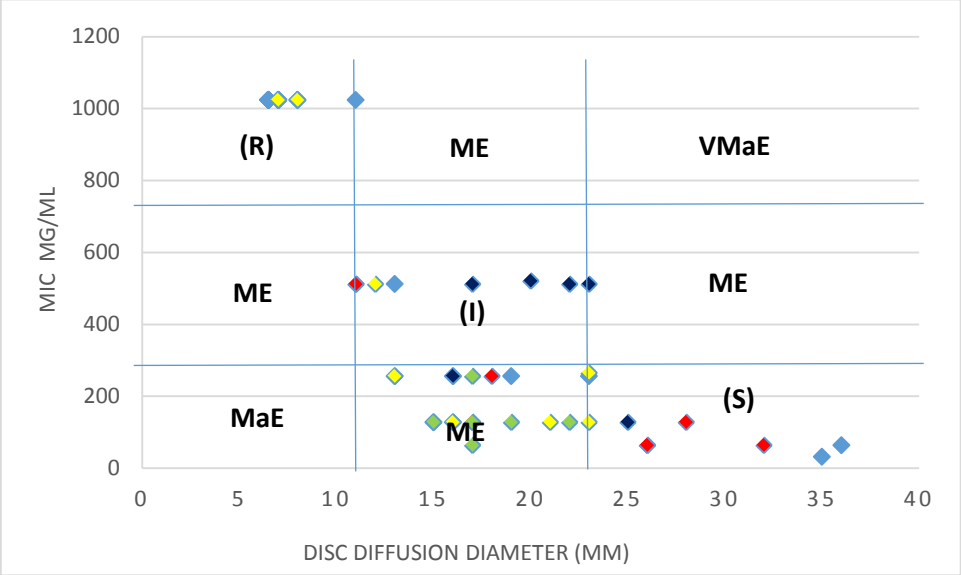
Table 6: MIC of antibiotics against bacterial strains under study.

Antibiotic / Bacteria	Ampicillin µg/ml		Amoxicillin µg/ml		Cefotaxime µg/ml		Tetracycline µg/ml		Clarithromycin µg/ml	
	L	C	L	C	L	C	L	C	L	C
Staphylococcus aureus	128	512	64	128	64	512	512	128	32	256
Staphylococcus epidermidis	32	256	64	128	32	64	512	128	32	128
Escherichia coli	**R**	**R**	**R**	**R**	512	1024	32	64	256	512
Klebsiella	32	64	32	64	64	128	16	64	32	64
Proteus mirabilis	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	512	**R**
Pseudomonas aeruginosa	**R**	**R**	**R**	**R**	512	**R**	**R**	**R**	**R**	**R**

L= Local; **C**= Commercial; **R**= Resistant for the highest concentration of the antibiotic.

Errors in Interpretation and reporting results:

In the interpretation of test results there are possibilities for errors to occur. Based on impact of errors in treatment of patient they are classified as minor errors, major errors and very major errors. This can be achieved by comparing disk diffusion, which is widely used to report with the MIC, which is reference method. The following flow chart of our results, Fig (13) shows resistance, intermediate, sensitivity and a small number of minor errors interpretation according to (20), However, the accuracy of disk susceptibility methods can be evaluated in comparison with E-test interpretive criteria (21) available from the Clinical Laboratory Standards Institute (CLSI) (22).



R= resistant; **I=** intermediate; **S**= sensitive; **ME**= minor error; **MaE**=major error; **VMaE**= very major error.

Figure (13): Flow chart shows the errors interpration.

In conclusion the local antibiotics product are as much effective as the commercial antibiotics and in some cases even better in the susceptibility against pathogenic bacterial strains, thus it must be supported and encouraged as a national less expensive effective drugs.

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Immunoglobulins IgG, IgA, IgM, complement C3 and C4 levels in sera of patients with polycystic ovary syndrome and the risk of cardiovascular diseases

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Abstract: Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of reproductive age. This study was designed to explore the clinical utility of humoral components of the immune system as a predictive of cardiovascular risk in PCOS patients. Women with PCOS (n =52) were recruited from Kamal AL-Samaraee Hospital.17 healthy, age-matched female volunteers were recruited as control subjects. We measured serum immunoglobulin (IgG,IgA,IgM), complement(C3,C4) concentrations in both groups. Results: Compared with controls, women with PCOS had significant increase ($p<0.05$) in C3(147.630±42.557vs. 54.611±14.177mg/dL),C4 (30.382± 12.549vs.15.741±4.917mg/dL),IgG (1011.811±310.347vs.746.788±210.311mg/dL),IgA(250.100±104.467vs. 140.311±6.783mg/dL), and IgM(194.700±81.976 vs. 126.023±32.103mg/dL) levels. C3 of patients group showed positive significant correlation with C4($r=0.311$,$p=0.025$) and IgM($r=0.276$,$p=0.047$). IgG positively correlated with IgA($r=0.358$,$p=0.009$) and with testosterone($r=0.275$, $p=0.047$), also IgM showed positive correlation with C3/C4. Conclusion: increase circulating Immunoglobulin and complement component might serve as a signal for the presence of an immune response that may increase cardiovascular risk in PCOS patients.

Key Words: Polycystic ovary syndrome, Immunoglobulins, complement C3, C4.

مستويات البروتينات المناعية IgG, IgA, IgM والمتممات C3 , C4 في مصول المريضات بمتلازمة تكيس المبيض كدليل للتنبأ بخطر الاصابة بامراض القلب الوعائية

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الخلاصة: يعتبر مرض متلازمة تكيس المبيض من امراض غدد الصماء الشائعة عند النساء في سن الانجاب. صممت هذة الدراسة لغرض استكشاف امكانية استخدام بعض مكونات النظام المناعي للتنبؤ باحتمالية خطر الاصابة بامراض القلب الوعائية عند مريضات متلازمة تكيس المبيض.تم استحصال 52 عينة من مريضات في مستشفى كمال السامرائي و17 عينة مطابقة بالعمر لنساء اصحاء كمجموعة سيطرة.تم قياس مستويات Ig M, Ig A, IgG وC4,C3 في مجموعة المرضى والسيطرة.النتائج: بالمقارنة مع مجموعة السيطرة ، فقد وجدت زيادة معنويه في مستويات

C3(147.630±42.557vs.54.611±14.177mg/dL),C4(30.382±12.549vs.15.741±4.917mg/dL), IgG(1011.811±310.347vs.746.788±210.311mg/dL),IgA(250.100±104.467vs. 140.311±6.783mg/dL), and IgM(194.700±81.976 vs. 126.023±32.103mg/dL)

اظهر C3ارتباطا معنويا موجبا مع C4 و IgM . وارتباط معنوي موجب بين IgG و IgA.ايضا تم ايجاد ارتباط معنوي موجب بين كل من IgG وبين التوستوستيرون ،و IgM ونسبة C3/C4.

الاستنتاج: زيادة تركيز هذة البروتينات قد يكون اشارة الى وجود استجابة مناعية عند المريضات بمتلازمة تكيس المبيض والتي ممكن ان تستخدم للتنبؤ الى زيادة خطر الاصابة بأمراض القلب الوعائية.

Introduction:

Polycystic ovary syndrome (PCOS) is a common endocrine disorder, characterized by Hyperandrogenism, chronic anovulation, polycystic ovaries proved by ultrasonography, and frequently morbid obesity, which is associated with infertility, frequent menstrual, hirsutism and frequent miscarriages (1, 2).

There are various reports about the prevalence of PCOS according to racial and genetic differences, it ranges from 2- 20% (3).

Many of these women show a cluster of cardiovascular risk factors, such as obesity, insulin resistance, abnormal glucose tolerance, lipid abnormalities, and hypertension (4, 5). Serum complement components are increased in many disorders including obesity, dyslipidemia, insulin resistance, type-2 diabetes, and cardiovascular diseases (6, 7). Interestingly, the effects of complement seem to be context and organ-dependent (8). Increasing evidence points to multiple functions of the complement system beyond pathogen killing. It is known that the complement system and immunoglobulins are the main components of humoral immunity. Their activation is known to be involved in many cardiovascular diseases (9, 10). Also elevated some

immunoglobulins were established with myocardial infarction (11), atherosclerosis (12, 13). Activated macrophages produce the cytokine C3, which is the third complement component (14), these cells mainly concerned with the development of atherosclerotic plaques (15).

We aimed to investigate whether inflammatory proteins, including complement C3, C4 and immunoglobulin are altered in polycystic ovary syndrome and related to cardiovascular diseases risk.

Materials and Methods:

Study populations

A case-control study including fifty two women diagnosed with PCOS by Kamal AL-Samaraee Hospital from October 2014 to April 2015, according to Rotterdam criteria (16), and seventeen healthy controls, matched for age, was enrolled.

Fasting blood samples were performed in the early follicular phase (between days 2 and 5 after the last menstrual period). Exclusion criteria was any woman who had any medical history or women with a triglyceride level more than 200 mg/dl and total cholesterol level more than 240 mg/dl were also excluded. Hormonal analyses were performed in both groups: follicle-stimulating hormone (FSH), luteinizing hormone, free testosterone, prolactin (MiniVidas Analyzer). Serum immunoglobulins and complement C3and C4 were determined by radial immune diffusion plate kit (LTA, Italy) (17).

Statistical analysis was done with SPSS statistical software. Data are presented as the means ± standard deviation (SD). Comparisons of two independent groups were made using Student's t-test (analysis of variance ANOVA to analyze the difference between group means). The Pearson test was used for correlation. P values < 0.05 were considered to be significant.

Results and Discussion:

The mean age(± SD) of patients group did not differ from that of control group (27.87± 5.629 *vs.* 27.41±5.767 years), while body mass index (kg/m^2) of patients group showed significant increase ($P< 0.05$) in comparison with control group (31.43±5.53 *vs.* 22.64± 1.67). Significantly higher serum concentrations were observed in complement C3, C4 and all Immunoglobulins in patients group as compared with controls, as shown in table1.

Table 1: Immunoglobulins and complement (C3and C4) levels in PCOS and control groups.

Group statistics- Independent samples test-t-test for equality of means					
Parameters	**Groups**	**N**	**Mean**	**Std. Deviation**	**P value**
C3(mg/dL)	Control	17	54.611	14.177	.000
	Patients	52	147.630	42.557	
C4(mg/dL)	Control	17	15.741	4.917	.000
	Patients	52	30.382	12.549	
C3 / C4	Control	17	3.812	1.853	.009
	Patients	52	5.342	2.072	
IgA(mg/dL)	Control	17	140.311	6.783	.001
	Patients	52	250.100	104.467	
IgG(mg/dL)	Control	17	746.788	210.311	.007
	Patients	52	1011.811	310.347	
IgM(mg/dL)	Control	17	126.023	32.103	.001
	Patients	52	194.700	81.976	

Note:Results are expressed as mean ±SD (standard deviation)
P-Value considered significant if it was less than 0.05

Figure 1 shows positive correlations between serum IgG and IgM (r = 0.358, P = .009), C3 and C4(r = 0.311, P = 0.025), and C3 and IgM (r = 0.276, P = 0.047). IgG correlated significantly with C3/C4 ratio (r = 0.278, P = 0.039) and with free testosterone (r = -0.275, P = 0.048). Other hormones did not correlate with immunoglobulins and both complements C3 and C4.

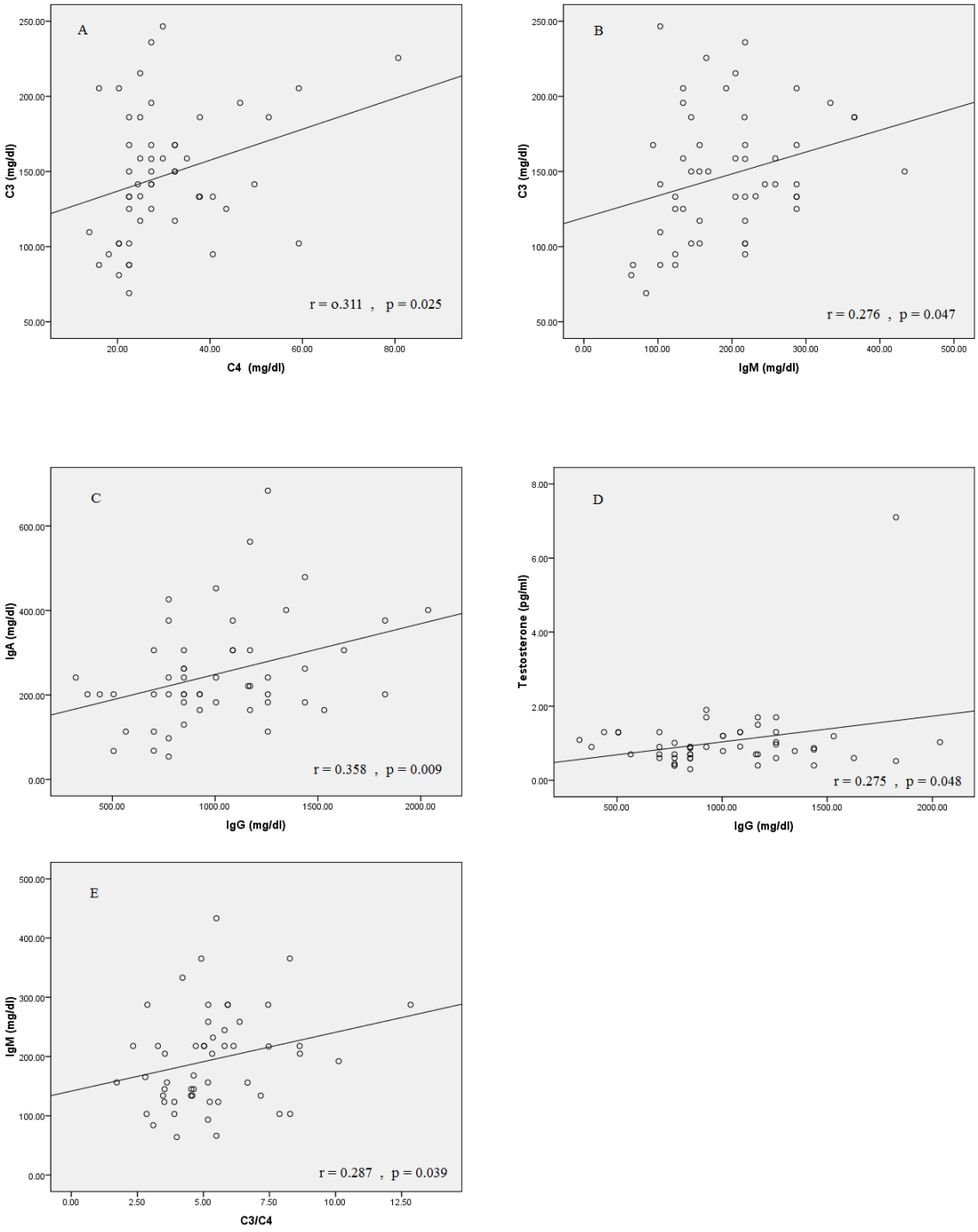

Figure 1: Pearson correlation between immunoglobulins and complements C3 and C4. A: C3 and C4; B: C3 and IgM; C: IgG and IgG; D: IgG and testosterone; E: IgM and C3/ C4.

Our results showed that, in comparison with age-matched controls, women with PCOS have increased levels of immunoglobulins and complements C3 and C4.

There is increasing evidence that patients with PCOS have increased cardiovascular risk compared with controls. It has been estimated that myocardial infarction, carotid atherosclerosis, extensive coronary artery disease are more likely in patients with PCOS than women with normal ovaries (18,19,20,21).

Many studies confirmed that women with PCOS have elevated acute phase proteins levels; CRP and ferritin (22, 23), endothelial dysfunction (24), and increased leukocytes (25), indicating that they may have an increased cardiovascular risk.

Liver is the source of complement proteins that may deposit in different tissues however several organs synthesize these proteins including heart and endothelium (26, 27).

The association of total immunoglobulins with cardiovascular disease remains the subject of debate. It has been proposed that serum IgA, IgG and IgE in men with dyslipidemia have been speculated that may be associated with cardiovascular disease (10), but others (12, 28) have suggested that the increase in IgM and IgG levels might be a consequence of the primary involvement of C3, as there was no independent association of IgM and IgG with atherosclerosis and myocardial infarction.

It was found that low levels of complement C3 and C4 together were indicated with the high level of C9 in follicular fluid compared to the plasma. This was explained that blood-follicle barrier increases its permeability during follicle maturation which allows diffusion of plasma proteins (29).

It was demonstrated that vascular endothelial growth factor (VEGF), which is required for oocyte maturation, is inhibited by complement activation, and could be one of the causes for infertility in women with PCOS (30).

Although C3 was found to be independently less predictive to metabolic syndrome, it was suggested that C3 in women with this syndrome have cardiovascular risk more than normal one (7), these findings have clinical and public health implications.

Our data was consistent with that of other researches (31, 32) in increased C3 level of PCOS group while disagree with other study (33).

Our result showed significant increase in serum C3/C4 ratio of PCOS group compared with controls. It was concluded that increasing this ratio is a novel marker for recurrent cardiovascular

events in acute coronary syndrome (34).

Many researchers found an association between elevated free testosterone, blood pressure, abnormal lipids metabolism with cardiovascular diseases in PCOS, suggesting that hyperandrogenism increases the cardiovascular risk in PCOS (35, 36, 37). However, the association between hyperandrogenism and cardiovascular risk is not universally accepted (38). Therefore many studies may be useful to find out the interaction between immune signals and androgens that may provide biomarkers for a pre-disease existence in women with PCOS at risk to develop cardiovascular diseases.

Determination of these clusters of proteins in larger controlled studies, may serve this goal in women with PCOS at risk to develop cardiovascular diseases in order to demonstrate their potential roles in poly cystic ovary syndrome and its complications as well as planning of therapeutic strategies.

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Localization study for lysosome enzyme (Beta-hexosaminidase) in the social amoeba *Dictyostelium discoideum*

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Abstract: *Dictyostelium discoideum* possesses many kinds of lysosomal enzyme family member. Those lysosomal enzymes appear to be very similar in homology to those found in higher eukaryotes. There is growing evidence that lysosomes are not only degradative organelles but are also involved in other critical cellular processes. Beta-hexosaminidase (NagA) is one of the most important lysosome enzymes. We used the model organism *D. discoideum* to study this protein. One separate mutant cell line was first analyzed, cell line was overexpressed (Green fluorescent protein) GFP-tagged NagA (GFP-NagA). Microscopic analysis of cells expressing GFP-NagA revealed that NagA was associated with lysosomes. Subjecting these cells to selected lysosome prob provided additional support for these findings. To test if the protein is found in the Golgi complex and the endosome, we used wheat germ and RITC-Dextran. Compared with wild-type cells, the GFP-NagA over-expressing cells were susceptible to osmotic stress.

Key words: *Dictyostelium*, lysosomal enzymes, Beta-hexosaminidase
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دراسة لموقع البروتين المسوؤل عن انزيم الحالة البياهكسيامنيديز في اميبا الدكتيستليم

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الخلاصة: قد اثبت ان اميبا *Dictyostelium discoideum* تمتلك انواع مختلفة من الانزيمات الحالة . وتتعتبر هذه الانزيمات الحالة مشابهة للانزيمات الموجودة في الكائنات حقيقية النواة. هنالك عديد من الادلة العلمية التي تبث ان الوظيفة الاساسية للانزيمات الحالة ليت فقط تحليل العضيات وانما يمكن ان تدخل في كثير من العمليات الخلوية المهمة . انزيم البيتاهكسامنديز احد اهم الانزيمات الحالة . استخدمت اميبا الدكتيستليم لدراسة بروتين انزيم البيتاهكسامنديز. تم استخدام خلايا مطفرة تفرط في انتاج البيتاهكسامنديز. بينت دراسة التحليل المكرسكوبي ان بروتين البيتاهكسامنديز مرتبط بالجسم الحال او المحلل . وبتعريض خلايا الدكتيستليم الى انواع مختلفة من الصبغات تم اضافة ادلة اضافية على موقع البروتين. ولغرض اختبار اذا كان البروتين موجود في اجسام كولجي والاندوسوم تم استخدام wheat germ and RITC-Dextran . وتم تعريض الخلايا المحتوية على بروتين البيتاهكسامنديز بلاصافة الى خلايا السيطرة الى عدد حالات الضغط الازموزي.

Introduction

Lysosomes are specialized organelles that exist in all eukaryotic cells. A lysosome contains lumen which contains hydrolytic enzymes (1). The function of the lysosome is degrading macromolecules. Lysosomes not only have a role as terminal degradative organelles, but there are also other cellular processes which lysosomes are involved such as cell surface receptor regulation and antigen presentation (2). The importance of the lysosomal system is in that there are over 50 lysosomal storage diseases that have been described in humans. Beta-hexosaminidase is one of the most important lysosome enzymes that can catalyze the hydrolysis of terminal non-reducing N-acetyl-D-hexosamine residues in N-acetyl-β-D hexosaminides (3).

Dictyostellium is a free living soil amoeba with a haploid genome containing six chromosomes. It is used for studying the signaling pathways regulating endocytosis and phagocytosis (4). *Dictyostellium* is a suitable organism to study lysosome enzyme processing and targeting (5). *D. discoideum* offers a suitable model system for the study of lysosomal enzymes. *Dictyostelium* lysosomes seem to be fundamentally similar to those in higher eukaryotes in terms of their physical properties and enzyme content. It has much larger amounts of material are available due to the phagotrophic nature and ease of growth of the organism (6). *D. discoideum* lysosomal enzymes are synthesized on membrane- bound polysomes and co- translationally inserted into the RER (7). These proteins are then core glycosylated (5). After glycosylation,
oligosaccharides are modified as a precursor form of the lysosomal enzymes is transported through the secretary pathway to lysosome (5). A helpful fact is that is the sorting of lysosomal enzymes in these cells may not require receptors at all (8). Therefore, we can use it to study the pathways for these enzymes. To assess the localization of Beta-hexosaminidase in *Dictyostelium*, full length GFP-NagA overexpression was tested and analyzed.

Materials and Methods

We used two cell lines AX4 and the GFP-NagA (9). The cell lines were grown axenically meaning at 21^{o}C in shaking culture at 150 rpm in HL5 medium. HL5 medium is composed of 1% glucose, 1% oxoidproteose peptone, 0.5% yeast extract (Fisher Biotech, Fair lawn, New Jersey), 2.4 mM Na2HPO4, with 8.8 mM KH2PO4 at pH 6.5. 300 mg/ml of streptomycin sulfate and 100 mg/ml of ampicillin was added to the media to prevent bacterial contamination (Sigma). Additionally, for the transformed cells, the HL5 medium supplemented with 10 mg/ml of G418, an antibiotic used for mutant cells selection (Invitrogen). In order to reduce background fluorescence from the HL5 medium, all cells were incubated in "Loflo" medium for 24 hours before any

experiments utilizing fluorescence microscopy (www.dictybase.org).

Creation of *D. discoideum* cell line with NagA

Dictyostelium NagA cDNA was subjected to PCR using primers that SalI and XhoI recognition sites on it. The PCR product was ligated into the TA vector (Invitrogen) and sequenced. In order to ligate, the pDneo2a-GFP vector previously cut with SalI and XhoI, the NagA PCR TA products was digested with SalI and XhoI. The pDneo2a-GFP-NagA vector was then transformed into *Dictyostelium* cells by electro-transfection.

GFP Visualization

First, cells were cultured to a density of approximately 1-4 × 10^6 cells/ mL, harvested, and allowed to settle on a glass cover slip. To visualize the cell fluorescence, the cells were viewed and photographed using a Nikon Eclipse 90i microscope equipped with 12V-100W halogen lamp, external transformer, with a built-in fly-eye lens, and NCB11, ND8, ND32 filters. The Bright fields filter and BrightLine® GFP Filter Set up with 1000X magnification were also used.

LysoTracker Staining

In order to visualize the V-H+ATPase vesicles, such as those found in the lysosomes cells were cultured to a density of 1-4 × 10^6 cells/ mL and then allowed to adhere to a Falcon® 35×10 mm Petri dish containing Loflomedium. The HL5 medium removed and replaced with fresh medium containing 100 nM LysoTracker® vital dye (Molecular Probes, Eugene, OR), and the cells were incubated for 30 minutes at room temperature. LysoTracker is used to stain lysosomes because it fluoresces in acidic compartments (5.2 pKa). The live cells were imagined using a BrightLine® DAPI Filter Set and BrightLine®GFP Filter Set on a Nikon Eclipse 90i microscope with 1000 times magnification.

Endosome Visualization: RITC Dextran Loading

By using rhodamineisothiocynate-dextran (RITC- dextran, Sigma Aldrich) a fluid internalized by the endosomes, but not degraded, the co-localization of the enzymes with the endosomes can be tested (10). In *Dictyostelium*, the red fluorescence of RITC is typically used to stain the endosomes. The cells were harvested and permitted to settle on a 35×10 mm Petri dish containing 2 ml of HL5 medium. Then, 40 µL of 100 mg/ml RITC- dextran was added to the cells, and they were incubated for 60 minutes. After that, cells were

collected, washed, and allowed to settle on a glass cover slip. The cell lines were photographed using the BrightLine® TXRED filter set to visualize the endosomes while the BrightLine® GFP filter set was used to visualize the GFP fluorescence.

Golgi Visualization: Wheat Germ Agglutinin Staining

Wheat Germ Agglutinin (WGA) (Sigma Aldrich) is used as an indicator for the Golgi apparatus; it is a Golgi-specific stain (11). First, cells were collected and allowed to settle on a glass cover slip. Then, 50 µl of Texas Red-labeled WGA (1.0 ug/ml) was added to the cells. The cells were incubated at 37^{o}C for 10 minutes, and then they were washed twice with Loflo medium. Cell fluorescence was visualized using the BrightLine® TXRED filter set. The Golgi appeared red, while the BrightLine® GFP filter was used for comparison with GFP localization.

Results

GFP-NagA is located within the lysosome:

Dictyostelium NagA Co-localize Lysosome and Endosome Systems. Immunofluorescence microscopy was used to find out the intracellular location of the GFP tagged NagA. The over-expressing cell lines GFP-NagA was subjected to fluorescence microscopy to infer a possible location. As a control, AX4 cells were transformed with an empty vector, and it was found that the GFP was evenly distributed throughout the cell (not shown.) All cell lines showed areas of GFP fluorescence in membrane vesicle areas (see arrows) thought to be lysosomes, post-lysosomes and endosomes Figure 1(A). To confirm the localization of the GFP-tagged enzymes to the lysosomes shown in Figure1 (B), all cell lines were subjected to fluorescence microscopy following staining with the known *Dictyostelium* LysoTracker. LysoTracker stains acidic organelles, including lysosomes (12). NagA and GluA over-expressing cells showed GFP co-localization with the LysoTracker.

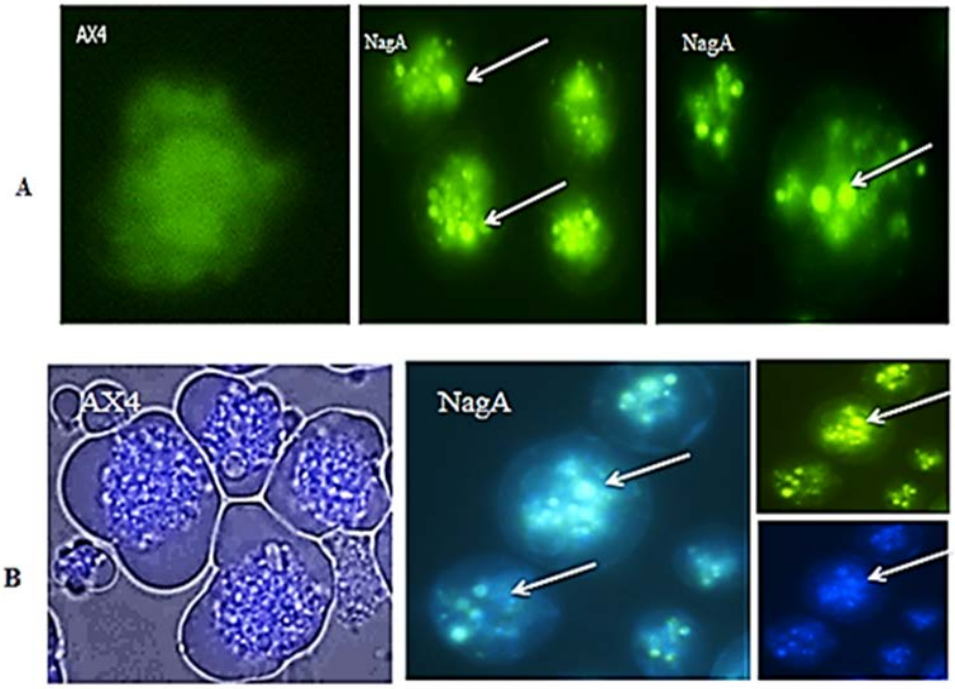

Figure 1: The expressed GFP-NagA protein associates with lysosome organelle marker protein. (A) GFP images of WT-AX4 control cells with no GFP expression and GFP-NagA over-expressing cells showing strong GFP fluorescence in a spot thought to in lysosome (indicated by arrows). (B) LysoTracker visualizes the location of the GFP-NagA. Distribution of LysoTracker® dye in the AX4 cell line. Overlaid images of LysoTracker® blue (DAPI) with GFP visualization in the GFP-NagA showed co-localization of NagA with lysosomes (indicated by arrows).

GFP-NagA are Associated with Early and Late Endosomes

The presence of GFP-Nag A proteins in other organelles was also investigated. The presence of these enzymes in endosomes has been investigated by comparing the overlap between GFP –NagA with endosomes that have been marked with RITC-dextran. Analysis of the immunofluorence data revealed a discernible partial overlap after 60 minutes of RITC-dextran loading indicating early endosomes. The AX4 cell line was used as a control Figure 2.

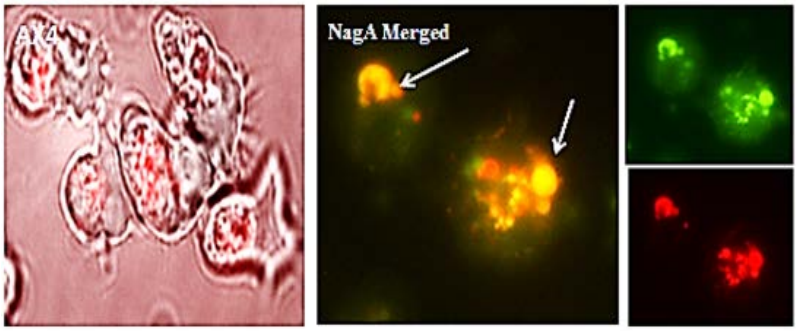

Figure 2: GFP-NagA associates with endosomes. The vesicles of the endocytic system from endosome to lysosome are shown as red stained membranes due to the uptake of RITC-dextran after 60 minutes of treatment. WT-AX4 cells with the endocytic system shown in red. GFP-NagA cells show, mostly, association with the endosomal system (indicated by arrows).

***Dictyostelium* NagA showed little association with the Golgi**

In order to confirm the localization of GFP-tagged NagA proteins with the Golgi membranes, cell lines were subjected to fluorescence microscopy following staining with Wheat Germ Agglutinin (WGA); WGA is a known *Dictyostelium* Golgi marker. GFP-NagA over-expressing cells showed little co-localization with the Golgi marker WGA Figure 3.

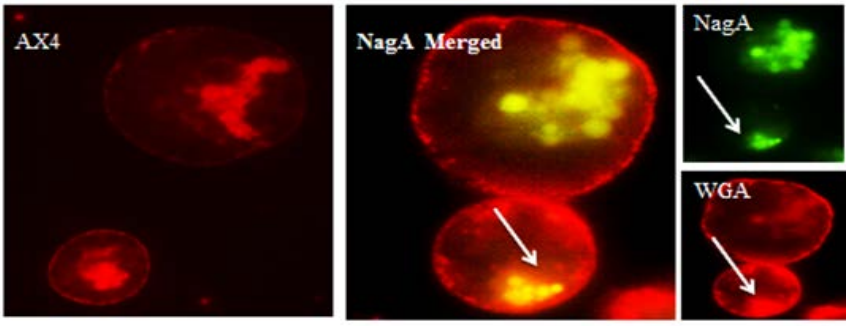

Figure 3: Immunofluorescence visualization of both GFP and WGA in NagA over-expressing cells shows little Golgi association. Images of GFP-NagA over-expressing cells, and mutant cell lines stained with known Golgi marker WGA (shown in red) and GFP fluorescence (shown in green), and of merged images (shown in yellow). WT-AX4 cells showing the WGA stained Golgi. GFP-NagA over-expressing cells showing the WGA- stained Golgi in red (indicated by arrows), and the GFP-NagA in green (indicated by arrows). The merged image shows little co-localization of GFP with the WGA- stained Golgi (yellow).

Effects of osmotic stress in NagA over-expressing cell line:

To investigate the function of the NagA protein in the CV system, cell lines were subjected to osmotic stress. Figure 4 presents GFP-NagA mutant cell lines compared to wild-type cells and pDneo2a-GFP (controls) under isotonic, hypotonic, and hypertonic environments. Under isotonic conditions, the wild type AX4 and pDneo2a-GFP cell lines maintained an amoeboid shape. However, the GFP-NagA cell line overexpressing cells were altered in appearance; their morphology was more rounded, apparently due to the effects of the over-expression of the NagA protein. To test the cellular appearance under stress condition, all cell lines were incubated in hypotonic stress. First, the shape of GFP-NagA expressing cells seen were more rounded in appearance compared to the control cells. While there was considerable swelling compared to both the control cultures of AX4 and GFP expressing cells under hypotonic condition. Under hypertonic stress, GFP-NagA appeared similar to the control cells with slightly enlarged vacuoles.

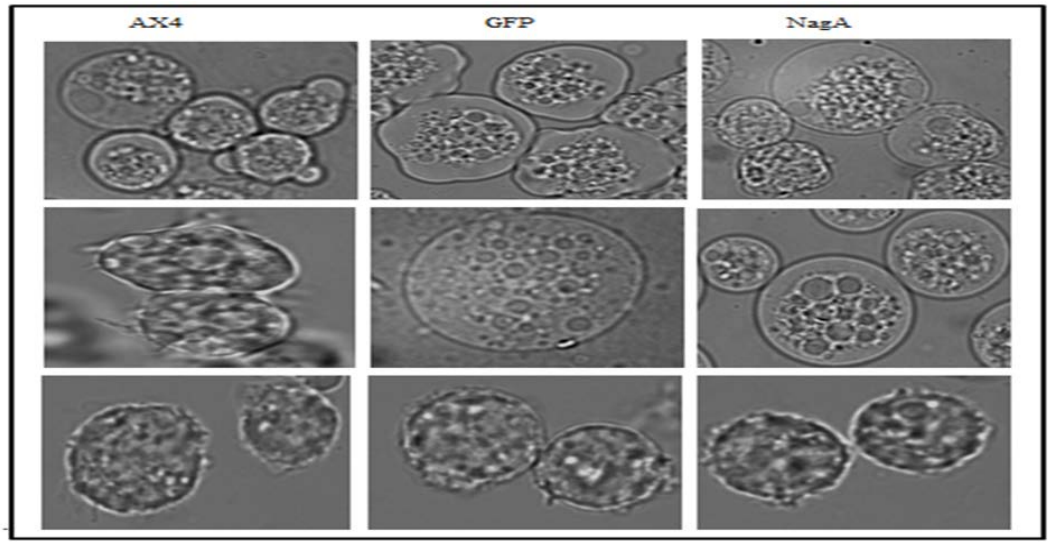

Figure 4: GFP-NagA cells are defective in osmoregulation. (A) Cellular appearance in isotonic environment (HL5 medium). The GFP- NagA cells appear different with enlarged contractile vacuoles compared to other cell lines. (B) Appearance in a hypotonic environment: The GFP-NagA expressing cells were extremely rounded with more and larger contractile vacuoles compared to AX4 and pDneo2a-GFP cells. (C) Appearance in a hypertonic environment: Cells expressing GFP- NagA had slightly enlarged vacuoles.

Discussion

GFP-NagA expression gene construct was transformed into *Dictyostelium discoideum* via electroporation. The transformed cell

lines were over-expressing GFP-NagA fusion proteins. We were able to identify GFP-NagA within the cell by using fluorescence microscopy.

Interestingly, the biosynthesis of lysosomal enzymes in *Dictyostelium* is analogous to that in mammalian cells (13). Frist, synthesized lysosomal hydrolases are synthesized as membrane-bound, N-glycosylated precursor proteins in the ER and afterward transported to the Golgi. Conversely, mammalian cells where lysosomal enzymes are targeted to lysosomes through the recognition of mannose 6-phosphate (M6P) sugars by MPRs. The sorting machinery recognizing M6P sugars is not characterized in *Dictyostelium* (12).

It has also been previously demonstrated by Martial in 1971 that the lysosomal enzyme extracts from yeast cells is localized in the vacuoles. Furthermore, many of proteins that localized either within the vacuole or outside of the plasma membrane. In yeast cells, the vacuole is an important organelle and it contains a many types of hydrolytic enzymes.

Analysis of the results demonstrated that DdNagA localized with lysosomes as we predicted. LysoTracker staining of the lysosomes in GFP-NagA cell lines were shown to co-localize with the GFP-NagA protein. Interestingly, the DdNagA appears to be more enriched in the lysosome. It is possible that NagA synthesis and transported from the Rough endoplasmic reticulum to the Golgi complex and ultimately lysosomes at a distinctly faster rate.

When cells were stained with WGA, a Golgi-specific stain, (10) in this study, we found there was little association between the lysosome and the Golgi apparatus. This may be because the Golgi apparatus is known to be the main site of sorting of newly synthesized proteins destined to the lysosomal compartment. Actually, the mature shape of lysosome moved to their destination (lysosome). Basically, this process happened in the lumen of the Golgi apparatus and occasionally on the surface of the plasma membrane. In addition, loading the mutant cells with RITC dextran for 60 and 180 minutes showed that the fourth mutants partially associate with early endosomes. This may indicate that acidic endosomal/lysosomal compartments may be essential for complete the proteolytic processing of lysosomal enzymes in *Dictyostelium* (12).

The CV network is a protective mechanism that prevents the cell from bursting by expelling the excess water under hypotonic stress. Defects of the CV complex can cause cells to swell and lysis (14).

Our data show that the DdNagA protein is not co-localized with the CV in *Dictyostelium* but that they may have a role in its function. NagA overexpressing cell phenotypic appearance may be due to the defects of proteins that are essential for homeostasis of the CV regulation. The CV system is a post-Golgi compartment, as indicated by the presence of O-glycosylated proteins in its membranes (15,13) and overexpression of lysosomal enzymes may alter the membrane flow or protein trafficking to the CV. In previous studies, RabD was localized to the CV and endo-lysosomes suggested that an uncharacterized membrane transport system connecting the endo-lysosomal and CV system exists in D. discoideum (16). There is a link between vascular function and osmoregulation came from studies of mutants defective in vacuolar protein sorting.

In this study, we present evidence that the lysosomal enzymes NagA associate with the lysosomes and endosomes and partially with the Golgi membranes. Their differing localization concentrations may contribute their functioning in the other cellular processes. We also showed that these proteins function in osmoregulation of the cell. Further experiments will be needed to show the exact role of the lysosomal sorting.

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Chronic toxicity effects of 2,4-D herbicide on common carp (*Cyprinus carpio* Linnaeus, 1758)

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Abstrac: The chronic toxic effects of (2,4-D) herbicide on the common carp was determined by three concentrations(20, 40 and 60)mg/l of eight *Cyprinus carpio* fish for six weeks. The behavioral, histological and hematological changes were used as a standard to observe the chronic effects through the study period; they included the clinical symptoms by erratic swimming, hyperactivity, loss of equilibrium with respiratory effects such as rapid gill movement and swim near the water surface. Blood parameters were changed in Red Blood Cell R.B.C., White Blood Cell W.B.C., Packed Cell Volume P.C.V.,HaemoglobinHb and liver enzyme (GPT & GOT).Values elevated significantly according to the pesticide concentration ($P\leq0.001$) and exposure periods ($P\leq0.05$) in comparison with control. The histological examination of the gills, liver and kidney of the common carp fish after chronic exposure to 2,4-d herbicide showed pathological changes and alterations such as fusion of the secondary lamella,epithelial lifting ,blood congestion,epithelial hypertrophy of the lamellar epithelium and epithelial necrosis. In the liver, pathological changes were seen, including hepatocytes hypertrophy, cytoplasmic vacuolation, blood congestion and cellular necrosis.Degenerative changes of the renal epithelium, necrosis in renal tubules and occlusion of the tubular lumen where the pathological alterations in the kidney.

التأثيرات السمية المزمنة لمبيد الاعشاب 2,4-د على اسماك الكارب الاعتيادي (*Cyprinus carpio* Linnaeus,1758)

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الخلاصة: حددت التاثيرات السمية المزمنة لمبيد (2، 4 – د) لاسماك الكارب الاعتيادي التي عرضت مجموعة من ثمانية اسماك,لثلاث تراكيز(20 و 40 و 60)ملغم/لترمن المبيد لستة اسابيع . استخدمت التغيرات السلوكية و النسيجية والدموية كثوابت لدراسة التاثيرات المزمنة خلال فترة الدراسة،التي تضمنت الاعراض السريرية والمتمثلة بالسباحة بشكل غير منتظم وفقدان التوازن مع ظهور علامات تنفسية مثل زيادة حركة الغطاء الغلصمي والسباحة قرب سطح الماء. اما في دراسة التأثيرات المزمنة فقد اظهرت فحوصات الدم انخفاضا معنويا حسب تركيز المبيد عند مستوى معنوية ($P\leq0.001$) ومدة التعرض عند مستوى معنوية ($P\leq0.05$) مقارنة بمجموعة السيطرة في كل من كريات الدم الحمراء والبيضاء وحجم الخلايا المرصوصة وتركيز الهيموكلوبين وانزيمات الكبد (GOT & GPT) للاسماك المعاملة عند مقارنتها بمثيلاتها المقاسة قبل المعاملة. اظهر الفحص النسيجي في كل من (الغلاصم ، الكبد والكلى) لأسماك الكارب الاعتيادي بعد التعرض المزمن لمبيد 2, 4-d وجود تشوهات نسيجية في الغلاصم ادت الى انشطار الصفائح الغلصمية الثانوية وارتفاع الخلايا الطلائية، احتقان الاوعية الدموية الموجودة في الغلاصم ، فرط التنسج (زيادة عدد الخلايا الطلائية) وتنخر الخلايا الطلائية . وكذلك حدوث تغيرات نسيجية في الكبد ادت الى تضخم الخلايا الكبدية ، حدوث فجوات في السايتوبلازم ،تنخر الخلايا الكبدية واحتقان دموي. اما الكلى فقد ادت التغيرات النسيجية الى حصول تغيرات انحلالية في الظهارة الكلوية، تنخر في النبيبات الكلوية وانسداد في التجويف الانبوبي.

Introduction:

Fresh water are highly vulnerable to pollution since they act as immediate sinks for the consequences of human activity always associated with the danger of accidental discharges or criminal negligence (1).Some of these pollutants are directly discharged by industrial plants and municipal sewage treatment plants, others come from polluted runoff in urban and agricultural areas (2).Most of human activities lead to production of too many contaminants that the major part of them enter to the aquatic systems. The majority of organic contaminants is degraded by biologic system, but other compounds, such pesticides and heavy metals, are resistant to the degradation and remains in the aquatic environments for a long time (3).One of the important factors that contaminate the natural habitat is agricultural pesticides. These substances used against pest, undesirable herbs and agricultural diseases were found to have adverse effects on the environment (4). Pesticides are often observed in water due to direct application in water, storm runoff and spray drift or vapor transport (5,6). Herbicides, also commonly known as weed killers, are pesticides used to kill unwanted plants. Selective herbicides kill specific targets, while leaving the desired crop relatively unharmed (7). These herbicides may enter from agricultural run-off, industrial terrestrial ecosystems effluent and other sources into aquatic media and produce undesirable side effects on biological and functional properties by changing the species composition of an algal community (8).Toxicity test is a biological test usually with an invertebrates, fish or small mammal to determine the adverse effects of a compound or effluents (9).

Chronic toxicity tests commonly refer to the exposure occurring at low concentration over a long period of time, several weeks or months (9).

Materials and methods

Experimental fish:

Live specimens of the common (*Cyprinuscarpio*L., 1758) (30±5 g) were purchased from a hatchery in Al-Musayyib City, Iraq. Fish were transferred to aquarium for acclimated to the laboratory conditions for one week. Fish were fed commercial food during the acclimation period under the continuous aeration condition and were fasted for 24 hrs before the start of the experiments. There was a simultaneous control group together with the actual experiments. The control group was kept in experimental water without adding the 2,4-D pesticide keeping all other conditions constant.

Water:

Water (dechlorinated tap water) was used in the experiment in a glass aquarium. The temperature, dissolved oxygen (DO), and the pH of the water in the aquaria were checked before the experiment and kept constant through the experiment and its value shown in table 1.

Table 1: Physical and chemical properties of the experiment water .

Physical and chemical properties	Range
Temperature (c°)	21-25
Dissolved oxygen (D.O) (mg/L)	5-7
Hydrogen ion concentration(pH)	7.3- 7.5
Electrical conductivity (µs/cm)	900- 1150
Salinity (ppt)	0.576-0.736

Toxicant:

The toxic compound used was 2,4-d (2,4-dichlorophenoxyacetic acid), the esteric toxic compound**.**

Chronic Toxicity Experiment:

Three groups were used and each group consists of eight fish in each aquarium that exposure for (20, 40 and 60) mg /L. In addition to control group, for six weeks. Water was replaced every 48 hr. To remove the wastes. Fish were feed regularly for once per day (10).

Behavioral changes in chronic test:

The behavior of the fish was studied through compare the movement of the control in aquaria as well as movement gills and the overall balance of the body with the exposure fish and recorded observations from the first day of exposure (11).

Hematological tests:

Blood samples were taken by way of puncturing the caudal vessel by cutting dorsoventrally through the caudal peduncle, collect blood by anticoagulants micro -hematocrit tubes (EDTA tube) (12). RBC&WBC count, PCV%,Hb (hemoglobin estimation)and Red blood cells Constants were tested.

Histological changes:

The tissue samples have been taken from the following organs: gills, liver, and kidney.

Serological tests:

The liver enzymeSerum Glutamic Oxaloacetic Transaminase

(SGOT) and Serum Glutamic Pyruvic Transaminase(SGPT) were tested.

Results And Discussion:

Fish are relatively sensitive to changes in their environment and have a relatively long lifespan compared to other aquatic organisms(12). Behavioral changes are the most sensitive indication of potential toxic effects(12). There were swimming problems, sudden jumping, loss of balance and swimming disorders, the fish were observed to have breathing difficulties and tried to breathe air from the surface. They jumped suddenly towards the surface before they motionlessly sank to the bottom. Fish blood is a pathophysiological indicator of the whole body function and therefore blood parameters are important in diagnosing the structural and functional status of fish exposed to a toxicant (13). Their changes depend on the fish species, age, the cycle of sexual maturity and health condition (14).water quality is one of the major factors, responsible for individual variations in hematology of fish as they are sensitive to slight fluctuation (15).Mean values of haematological analysis under control and treated groups with 2,4-d are given in Table 2.

RBC, WBC, PCV and Hb, SGOT and SGPT in control test results and treatments 20, 40 and 60 mg/l shows the highest average rate as compared to control. The increased pesticide doses and the period of exposure had significantly affected the number of RBC, WBC, PCV and Hb, SGOT and SGPT in examined common carp fish .RBC, WBC, PCV and Hb levels were reduced after exposure to the pesticide 2,4-d. Haematological parameters of fish are highly variable between and within species and seasons (16), with the values of individual indicators differing relative to temperature, season, sex, food, and the type of culture (17).Studies have shown that when the water quality is affected by toxicants, any physiological changes will be reflected in the values of one or more of the hematological parameters (18). Blood cell responses are important indicators of changes in the internal and external environment of fishes. In fish, exposure to chemical pollutants can induce either an increase or decrease in hematological levels depending on fish species, age and cycle of sexual maturity (19)**.** Red blood cells are the most numerous formed elements in blood. Any alteration in the number (quantitative) or morphology (qualitative) of RBCs from normal values can cause various pathological disorders in fish under stressful conditions (20).The result of this test has found**.**

Table (2): Mean ± SD of the values of RBC, Hb, PCV, MCV, MCH, MHCH, WBC, SGOT and SGPT of carp fish subjected to 3 doses of pesticide and 3 exposure periods.

Variable	Exposure Period	Mean ± SD			
		Common Carp			
		Dose mg/L			
		Control	20	40	60
R.B.C. $\frac{X10^6}{mm^3}$	2 weeks	2.57±1.7	2.5±0.3	1.5±0.6	1.1±0.2
	4 weeks		2.46±0.2	2.3±0.3	0.93±0.05
	6 weeks		2.2±0.4	1.3±0.5	0.87±0.06
Hb. gm/100ml	2 weeks	12.3±0.5	11.3±0.9	10.0±1.4	10.2±0.4
	4 weeks		9.3±0.6	10.3±0.6	9.5±0.4
	6 weeks		10.3±0.8	10.0±0.5	9.2±0.2
PCV %	2 weeks	35.3±1.6	35.0±1.4	32.3±1.7	30.3±0.6
	4 weeks		33.0±2.2	30.5±0.7	30.0±0.0
	6 weeks		32.7±1.4	33.0±1.5	29.0±1.7
M.C.V. f.I.	2 weeks	137.9±4.2	140.4±6.9	235.0±75.6	277.0±17.3
	4 weeks		136.8±1.9	135.6±11.4	321.8±15.4
	6 weeks		149.0±8.1	258.6±59.4	340.7±42.9
M.C.H. p.g	2 weeks	48.1±1.8	45.4±1.6	60.0±9.9	93.7±8.9
	4 weeks		39.5±1.4	46.2±6.4	102.6±3.3
	6 weeks		47.3±5.6	79.2±22.6	108.5±16.4
M.C.H.C. %	2 weeks	34.8±0.45	32.3±0.9	30.9±1.4	33.7±1.2
	4 weeks		28.9±1.2	33.8±2.0	31.9±0.54
	6 weeks		31.7±2.6	30.2±1.7	31.7±0.78
W.B.C. $\frac{X10^3}{mm^3}$	2 weeks	45.3±1.6	30.4±0.5	24.0±1.2	21.0±0.9
	4 weeks		22.0±1.3	23.1±1.4	18.7±0.4
	6 weeks		19.5±0.4	17.0±0.8	13.0±0.9
SGOT U/l	2 weeks	15.0±1.7	17.3±1.6	23.0±0.9	30.0±1.7
	4 weeks		16.0±1.2	26.0±1.4	31.0±1.4
	6 weeks		20.0±0.9	27.0±1.5	33.0±1.2
SGPT U/l	2 weeks	16.0±1.5	19.0±1.4	26.0±1.3	32.3±1.5
	4 weeks		20.3±1.5	26.7±1.8	35.0±1.1
	6 weeks		23.0±1.3	31.0±1.4	40.7±0.8

RBC, WBC, PCV and Hb, SGOT and SGPT in control test results and treatments 20, 40 and 60 mg/l shows the highest average rate as compared to control. The increased pesticide doses and the

period of exposure had significantly affected the number of RBC, WBC, PCV and Hb, SGOT and SGPT in examined common carp fish .RBC, WBC, PCV and Hb levels were reduced after exposure to the pesticide 2,4-d. Haematological parameters of fish are highly variable between and within species and seasons (16), with the values of individual indicators differing relative to temperature, season, sex, food, and the type of culture (17).Studies have shown that when the water quality is affected by toxicants, any physiological changes will be reflected in the values of one or more of the hematological parameters (18). Blood cell responses are important indicators of changes in the internal and external environment of animals. In fish, exposure to chemical pollutants can induce either an increase or decrease in hematological levels depending on fish species, age and cycle of sexual maturity (19)**.** Red blood cells are the most numerous formed elements in blood. Any alteration in the number (quantitative) or morphology (qualitative) of RBCs from normal values can cause various pathological disorders in fish under stressful conditions (20).The result of this test has found that the highest mean R.B.C. value (2.5±0.3) was recorded in a sample of 20 mg/L after two weeks exposure and almost similar to that of control sample (2.57±1.7) and the lowest mean value (0.87±0.06) was found in the sample of 60 mg/L after six weeks exposure (Fig 1). Analysis of variance shows clear significant differences of various pesticide doses ($P \leq 0.001$) and the period of exposure ($P \leq 0.05$) upon fish red blood cells (Appendix1). Also, the LSD value (0.437) confirms such significant differences. Hemoglobin serves to transport oxygen from gills to different tissues of the fish in the form of oxyhaemoglobin and carbon dioxide from tissue to the gills in the form of carboxyhaemoglobin and its concentrations reflect the supply of an organism with oxygen and the organism itself tries to maintain them as much stable as possible (21).In case of Hb, highest mean value (11.3±0.9) was recorded in the sample of 20 mg/L pesticide dose after two weeks exposure while the lowest mean value (9.2±0.2) was found in fish samples treated with 60mg/L pesticide and six weeks exposure (Fig 2). Analysis of variance of Hb data shows significant impacts of both pesticide doses ($P \leq 0.001$) and exposure period ($P \leq 0.05$) on fish blood Hb (appendix2). Also, the least significant difference value was found to be 1.1 that reveals significant differences between certain examined data, particularly between those of 20 mg/L and 60mg/L.Haematocrit (Hct) or packed cell volume (PCV) expresses

the volume of RBCs in 100 ml of whole blood. Any deviation from its normal values can lead to various pathological conditions. For packed cell volume (PCV), mean values were varied from 29.0±1.7 in blood sample of a fish treated with 60mg/L after six weeks exposure to 35.0±1.4 of that treated with 20mg/L and four weeks of exposure (Fig 3). However, the lowest mean value shows no significant difference with that of control sample (35.3±1.6).Analysis of variance of these data reveals only significant differences (P≤0.001) of different pesticide concentrations but the exposure periods had no such effects (P>0.05) on these values (Appendix3). Furthermore, LSD test (LSD= 2.185) failed to give any significant differences between data related to exposure periods. MCV gives an indication of the status or size of RBCs (15).Regarding M.C.V. test, the highest mean value (340.7±42.9) was recorded in a sample of common carp subjected to 60mg/L pesticide after six weeks and the lowest mean value 135.6±11.4 in the sample of that treated with 40mg/L pesticide and four weeks exposure which was almost similar to that of control sample (Fig 4).Analysis of variance test shows the only significant differences of different pesticide dose (P≤0.001) on these values while exposure periods had no such impacts (P>0.05) and least significant value 122.714 shows differences only between some of these data (Appendix4).In case of M.C.H. test, the current study has found that the highest mean value 108.5 ± 16.4 of carp sample treated with 60mg/L pesticide dose after six weeks of exposure periods while the lowest mean 39.5 ±1.4 was recorded in that of 40mg/L with four weeks of exposure while the control sample had a mean of 48.1±1.8 (Fig 5).The analysis of variance test shows significant influence of pesticide various does (P≤0.001) on these data while no significant influence was found for the exposure periods (P>0.05) on the values of M.C.H. test (Appendix5). Also least significant test (LSD=28.685) shows significant differences between some of these data.The MCHC is a good indicator of red blood cell swelling or shrinkage (21).In M.C.H.C. test, the obtained results were found to vary from 28.9±1.2 in common carp sample treated with 20 mg/L pesticide dose after four weeks exposure to 33.8±2.0 of that subjected for 40mg/L pesticide dose (Fig 6). However, all these data were lower than that of control (34.8±0.45) sample.No significant differences of both pesticide different doses and various exposure periods (P>0.05) upon these data due to analysis of variance. Furthermore, the least significant difference value (LSD=5.588) failed

to detect any significant differences between these values (Appendix6).The changes in blood parameters may cause by distraction or haemolysis of red blood cell by the effect of the herbicide that cause anemia and decrease in hemoglobin, the decrease in hemoglobin may cause by the effect of the herbicide on the metabolic enzyme of haeme (22), also haemodilution is another cause for decrease hemoglobin that occur to maintenance the respiration and gas exchange, the secretion of catecholamines under stress condition increase flow of blood to gills with increase of permeability of gills tissue, also increase taking water by the fresh water fish (haemodilution)(23). The decreases in white blood cell (leucopenia) cause by stress factor by the toxic effect of the pesticide by increase the activity of Pituitary gland (24).this study has found that higher mean value 30.4±0.5 was recorded for the sample treated with 20mg/L pesticide and two weeks of exposure while the lowest mean value 13.0±0.9 was in the sample subjected to 60mg/L pesticide dose and six weeks of exposure (Fig 7). However, all the data of examined common carp had values much lower than that of control sample (45.3±1.6).Analysis of variance of these results shows high significant impacts ($P \leq 0.001$) of both pesticide and exposure periods on the percentage of W.B.C. and these differences were confirmed by the value of LSD (1.539) these data (Appendix7). Since liver is the main metabolic centre, where most of the xenobiotics are metabolized and detoxified. It is therefore, one of the forefront organs of the body, which faces the major onslaught of an unwanted chemical invasion. The liver function is therefore, likely to be disturbed under such circumstances (25). The main hepatic cellular component to be affected by the ambient toxicants seems to be the cell membrane. Aquatic toxicants either have increased the membrane permeability causing enhanced leaching out of the enzymes, or reduced the permeability forcing the enzymes to accumulate in the cells. Cellular damage is another reason for decreased synthesis of enzymes in living organisms. The transaminases, GOT and GPT are two key enzymes considered as a sensitive measure to evaluate hepatocellular damage and some hepatic diseases (26). The increased serum aminotransferases might reflect myocardial and hepatic toxicity. Leading to extensive liberation of the enzymes into the blood (27). The results indicate that the pesticide affect the liver cells and GOT and GPT activities.In case of SGOT test, the current results show that highest mean value (33.0±1.2) was recorded in a sample of common carp treated with 60

mg/L and six weeks exposure while the lowest mean value 17.3±1.6 was found in the sample of that subjected to 20 mg/L and two weeks exposure (Fig 8).Analysis of variance test shows high significant difference of both pesticides does and exposure periods (P≤0.001) on these data. LSD values (1.843) give clear differences between these values (Appendix8).SGPT test has given mean values varied from 19.0±1.4 in common carp sample treated with 20 mg/L pesticide with two weeks to 40.7±0.8 in that subjected to 60mg/L pesticide with 6 weeks of exposure (Fig 9). All these data were significantly (P≤0.05) higher than that of control sample (16.0±1.5).Analysis of variance shows high significant influences of both pesticide and exposure periods (P≤0.001) on these data and the least significant value (2.169) show clear differences between them (Appendix9).

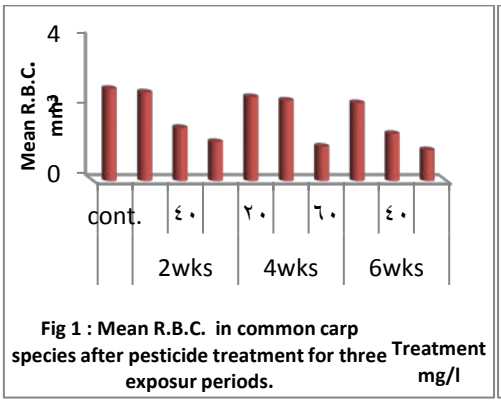

Fig 1 : Mean R.B.C. in common carp species after pesticide treatment for three exposur periods.

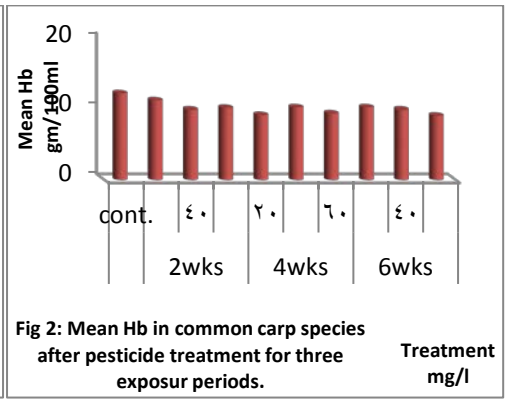

Fig 2: Mean Hb in common carp species after pesticide treatment for three exposur periods.

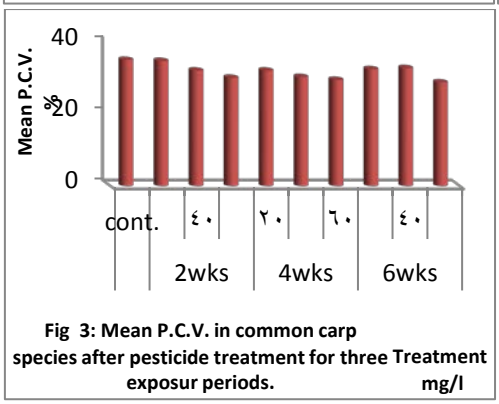

Fig 3: Mean P.C.V. in common carp species after pesticide treatment for three exposur periods.

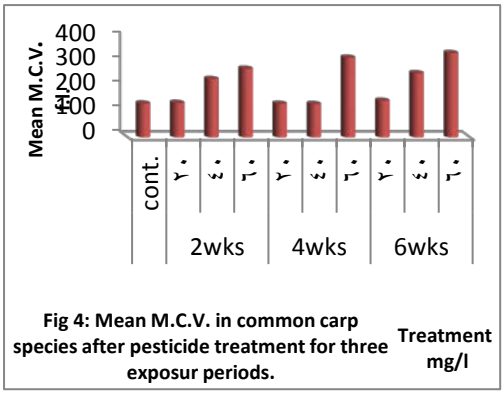

Fig 4: Mean M.C.V. in common carp species after pesticide treatment for three exposur periods.

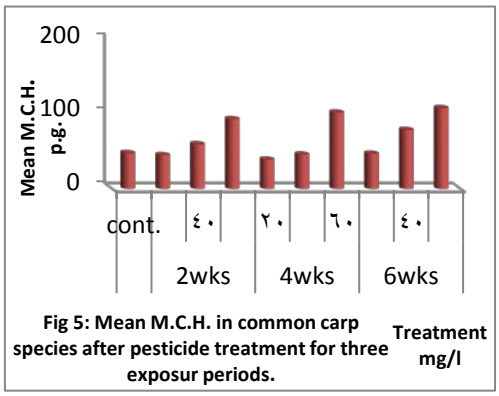

Fig 5: Mean M.C.H. in common carp species after pesticide treatment for three exposur periods.

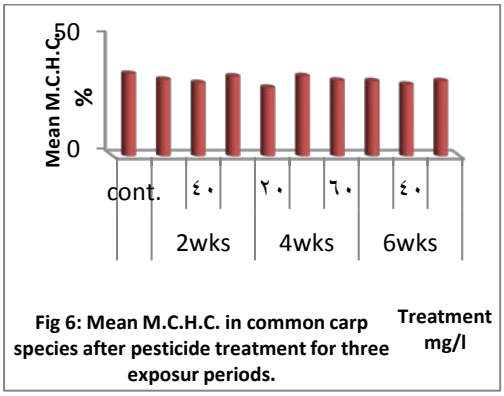

Fig 6: Mean M.C.H.C. in common carp species after pesticide treatment for three exposur periods.

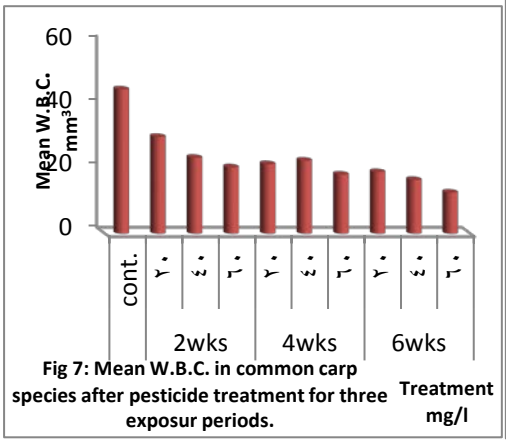

Fig 7: Mean W.B.C. in common carp species after pesticide treatment for three exposur periods.

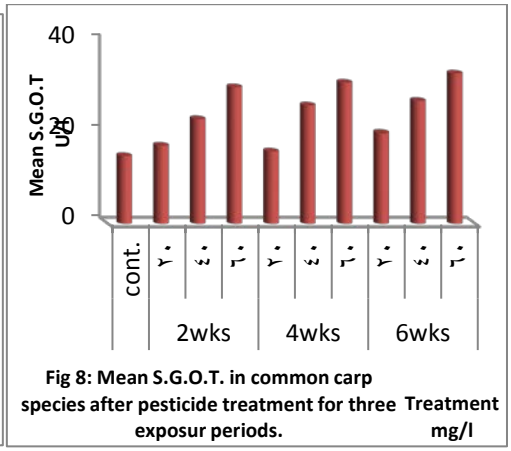

Fig 8: Mean S.G.O.T. in common carp species after pesticide treatment for three exposur periods.

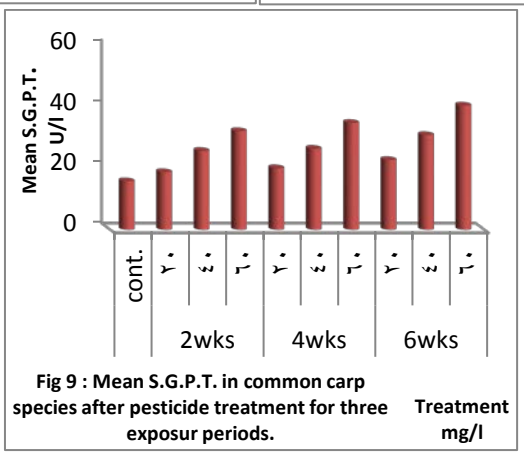

Fig 9 : Mean S.G.P.T. in common carp species after pesticide treatment for three exposur periods.

Histopathological changes in *C.carpio*:

Histopathological changes have been used as important biomarkers in environmental monitoring that allows examining specific target organs. The histological results observed in all tissues of *C. carpio* in the present study indicates that sub lethal concentrations caused moderate to severe alteration in gill ,liver and kidney, which are an important organs performing a vital function like detoxification, respiration, osmoregulation, acid base balance, etc. (28).Gill is one of

the most important organs directly in contact with pollutants and any kind damage of the gill tissue leads to disorder in the gas exchange process and also the decrease of ion regulation efficiency via this organ (29) Histopathology of gill is the appropriate bio-indicator to pollution monitoring (30). Various lesions in gill were recorded in the present study such as epithelial hyperplasia and hypertrophy, fusion of some secondary lamellae, Telangiectasis and mononuclear cells infiltration and lifting of epithelial cells,dilatation of central venous sinus,epithelial necrosis, and vasodilatation of the centralvenous sinus, blood congestion that shown infigures (10, 11, and 12).

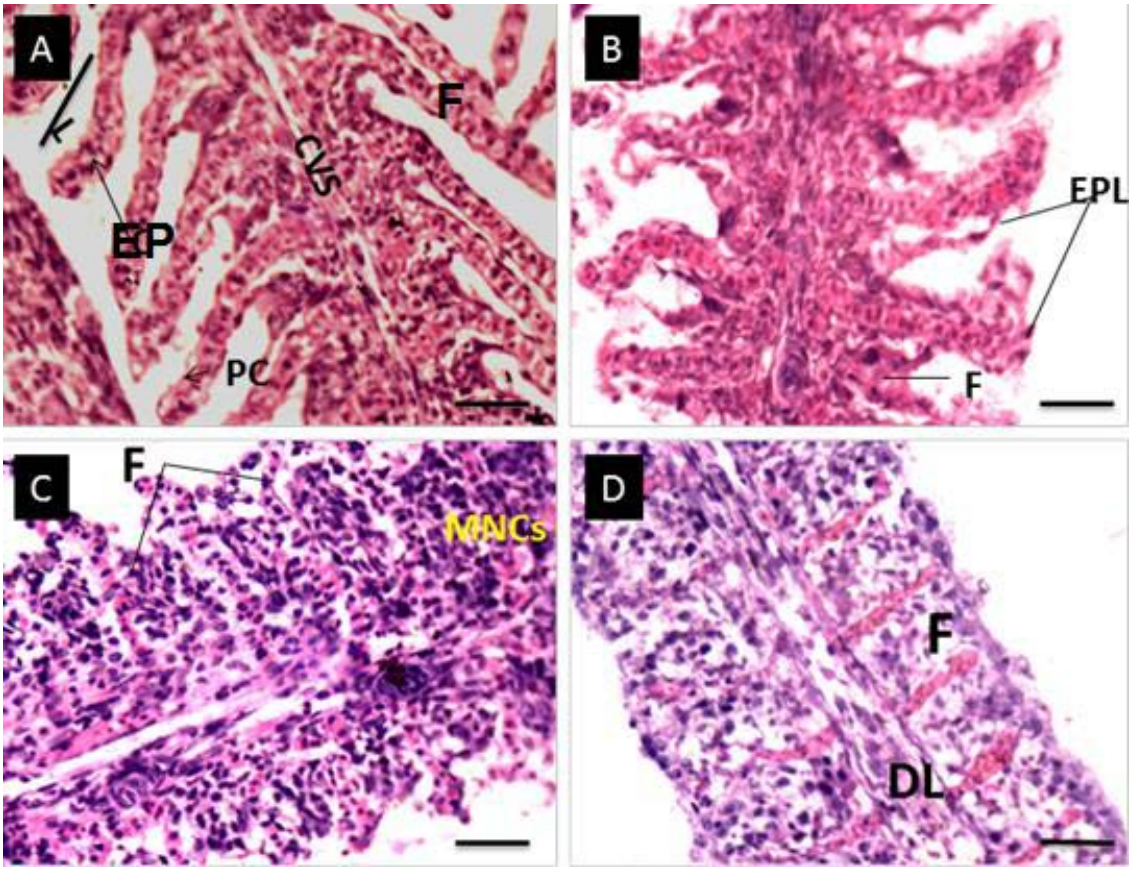

Figure 10: Photomicrographs of gill filament of *C. carpio* [A] normal aspect of the gill, showing primary filament (F), pillar cell (PC) epithelial cell (EP) and central venous sinus (CVS); [B] gill section treated with 20mg/L for 2 weeks showing epithelial lifting (EPL) with partial fusion of the secondary lamella (F); [C] gill section treated with 40mg/L for 2 weeks showing moderate fusion of the secondary lamella (F) with mononuclear cells (MNCs) infiltration; [D] gill section treated with 60mg/L for 2 weeks showing complete fusion of the secondary lamella and dilatation of CVS. H&E; 40x.

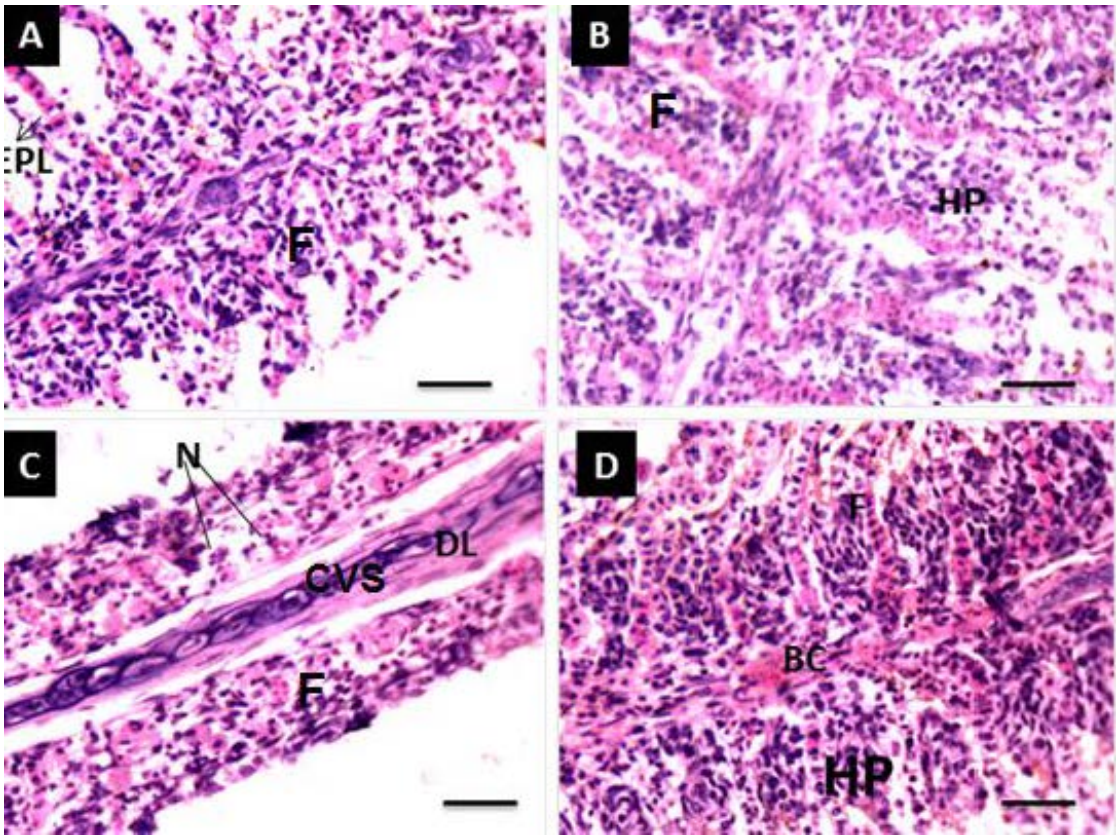

Figure 11: Photomicrographs of the gill filament of *C. carpio* [A] gill section treated with 20mg/L for 4 weeks showing epithelial lifting (EPL) with partial fusion of the secondary lamella (F); [B] gill section treated with 40mg/L for 4 weeks showing moderate fusion of the secondary lamella (F) with hyperplasia of the epithelial cells (HP); [C&D] gill section treated with 60mg/L for 4 weeks showing epithelial necrosis (N), vasodilatation of the central venous sinus (CVS), hyperplasia of the epithelial cells, blood congestion (BC) and complete fusion of the secondary lamellae (F). H&E; 40x.

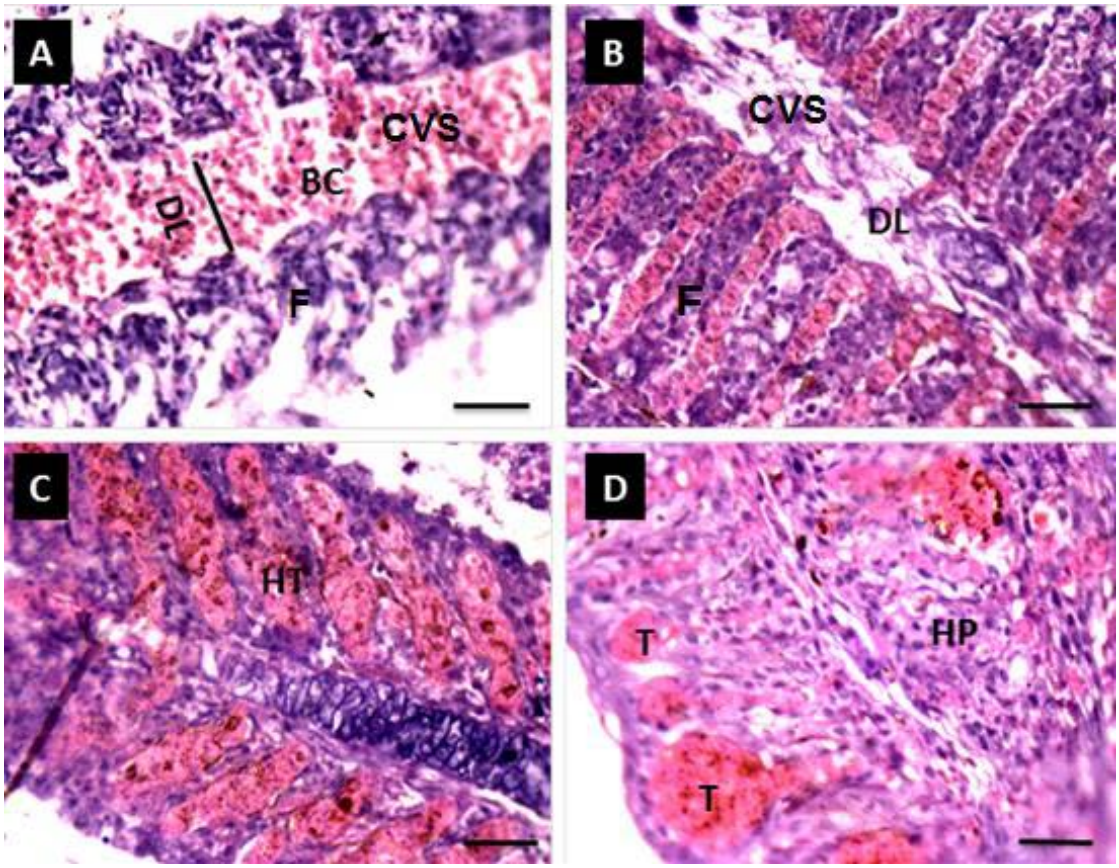

Figure 12: Photomicrographs of the gill filament of *C. carpio* [A] gill section treated with 20mg/L for 6 weeks showing partial fusion of some secondary lamellae (F), blood congestion (BC) with vasodilatation of the central venous sinus (CVS); [B] gill section treated with 40mg/L for 6 weeks showing fusion of the secondary lamella (F) with vasodilatation of CVS [C&D] gill section treated with 60mg/L for 6 weeks showing epithelial hypertrophy of the lamellar epithelium (HT), complete fusion of the secondary lamella, epithelial hyperplasia (HP) and telangiectasis at the tips of secondary lamellae (T). H&E; 40x.

The histological alteration of the gills, liver and kidney were observed and this was more pronounced in higher concentrations and exposure time.The toxicity rate of each organism increased with an increase in the concentration. The lifting of lamellar epithelium could serve as a defense mechanism, because separation of the epithelium of the lamellae increases the distance across which pesticide must diffuse to reach the blood stream (31).The lifting of lamellar epithelium is probably due to severe edema (32). Cell proliferation which results in hyperplasia is one of the major histological changes observed in fish exposed to pesticide (33) and which leads to lamellar fusion

observed in the present study. Hyperplasia of gill epithelium would not only decrease the surface area available for oxygen diffusion (34) but would increase the oxygen distance between water and blood, which in turn could cause tissue hypoxia (35). Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae are examples of defense mechanisms, since; in general, these result in the increase of the distance between the external environment and the blood thus, serve as a barrier to the entrance of contaminants(36).Hadi and Alwan (2012) suggested that the gill hyperplasia may increase the epithelial thickness so as to retard into the blood stream(37). Cell proliferation with thickening of the gill filament epithelium may lead to the lamellar fusion (38). The lifting of lamellar epithelium is other histological change observed, probably induced by the incidence of severe edema (32). Edema with lifting of lamellar epithelium could be served as a mechanism of defence, because separation the epithelial of the lamellae increases the distance across which waterborne pollutants must diffuse to reach the bloodstream (39).Lamellar telangictasis resulted from rupture of pillar cells and capillaries under the effect of pesticide pollution and leads to an accumulation of erythrocytes in the distal portion of the secondary lamellae (37). These histopathological changes of the gills likely resulted in hypoxia, respiratory failure problems with ionic and acid-base balance (39).The organ most associated with the detoxification and accumulation process is liver and due to its function, position and blood supply, it is also one of the organs most affected by contaminants in the water (40). The histological changes in the liver were the same in (2, 4 and 6 weeks) after exposure to different concentration (20, 40 and 60 mg/L) that shown in figure (13).

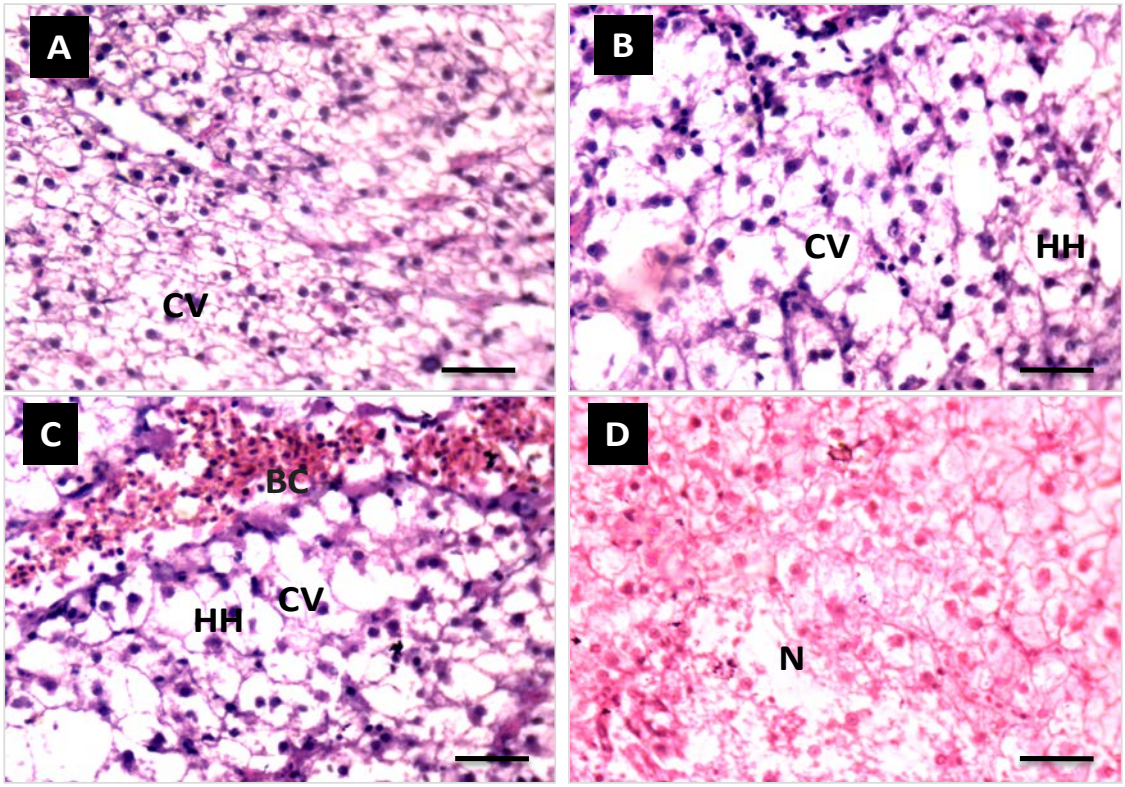

Figure 13: Photomicrographs of the liver of *Cyprinus carpio* exposed to different concentration of 2,4-d herbicide for 6 weeks [A] Hepatic tissue exposed to 20mg/L shows cytoplasmic vacuolation (CV) [B] hepatic tissue exposed to 40mg/L shows hepatocytes hypertrophy (HH) and cytoplasmic vacuolation (CV); [C&D] hepatic tissue exposed to 60mg/L showing cytoplasmic vacuolation (CV), hepatocytes hypertrophy (HH), blood congestion (BC) and cellular necrosis (N). H&E. 40x.

Various changes observed in the liver in the present study likeCytoplasmic vacuolation, Hepatocytes hypertrophy and cytoplasmic vacuolation and blood congestion and cellular necrosis.It also plays a prominent role in fish physiology, both in anabolism (protein, lipid, carbohydrate) and catabolism (glycogenolysis, detoxification) and it acts as a storage centre for many substances, mainly glycogen. Hepatocytes may thus be expected to be the primary targets of toxic substances, providing an excellent biomarker of aquatic pollution (41). The monitorization of histological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in the field and experimental studies (38). Vacuolation of the hepatocytes probably due to metabolic damage related to exposure with 2,4-d contaminated water (42).

Hypertrophy is generally characterized by an increase in cellular size. Exposure to compounds that induce proliferation of the endoplasmic reticulum membranes can be regarded as an example of hypertrophy (43). Vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver (40). The vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances
in the parenchymal cells and the rate of their release into the systemic circulation (37). Vacuole formation was considered by Mollendroff (1973) as a cellular defence mechanism against substances injurious to hepatocytes and this mechanism responsible for collecting the injurious elements and preventing them from interfering with the biological activities of these cells(44).The alterations in liver due to toxicity impact are often associated with a degenerative necrotic condition (45).As in higher vertebrates, the kidneys of fish perform an important function relate to electrolyte and water balance and the maintenance of a stable internal environment. Following the exposure of fish to toxic agents, histological alterations have been found at the level of the tubular epithelium and glomeruli (46). Ortiz *et al*. (2003) found kidneys of fish receive the largestproportion of post-branchial blood, andtherefore renal lesions may be good indicators of environmental pollution (47). Various changes happen in kidney tissue in various exposure periods like cellular swelling of the renal epithelium, melanomacrophage aggregation, degenerative changes of the renal epithelium. Necrosis in renal tubules with depletion in hemopiotic tissue,degenerative changes in the form of cytoplasmic vacuolation, infiltration of protein substances in tubules, occlusion of the tubular lumen, necrosis in the tubular epithelium that shown in Figures (14,15).

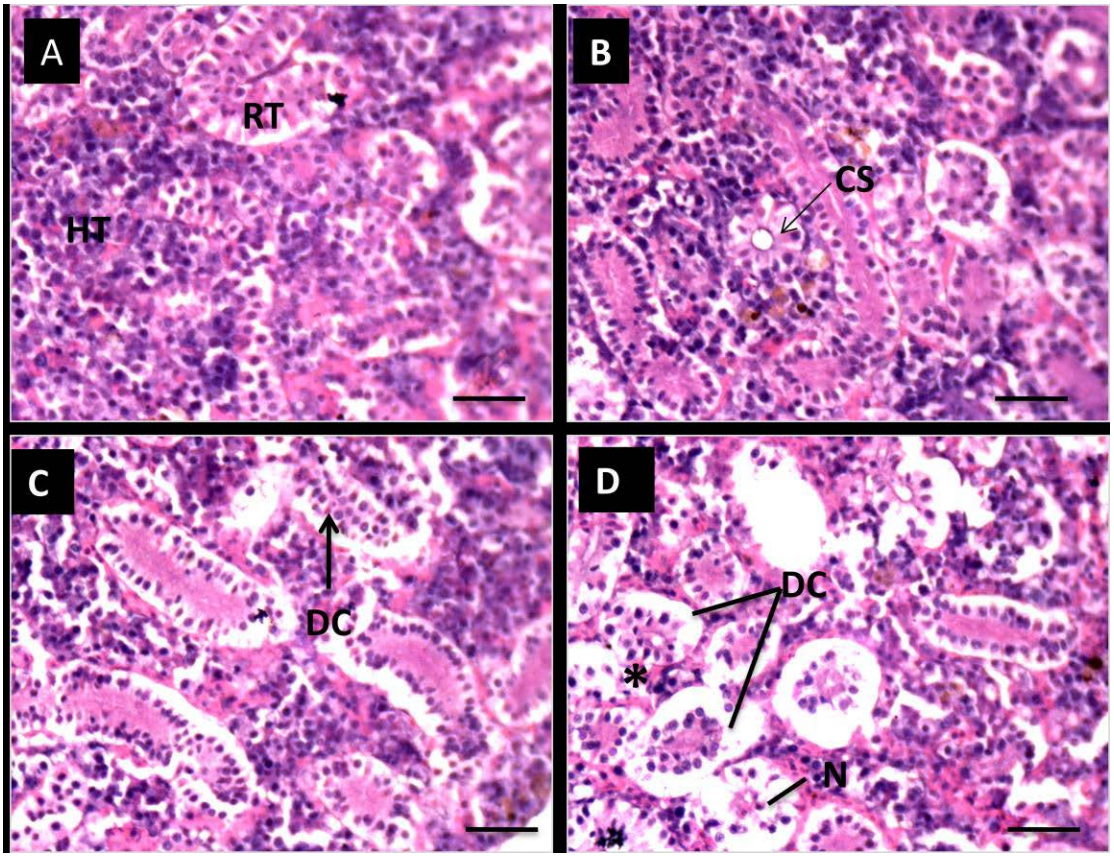

Figure 14: Photomicrographs of the kidney of *Cyprinus carpio* [A] Normal renal tubules showing the well defined haemopiotic tissue (HT) and renal tubules (RT); [B] kidney section exposed to 20mg/L for 2 weeks showing cellular swelling of the renal epithelium (CS); [C] kidney section exposed to 40mg/L for 2 weeks showing degenerative changes (DC) in the form of cytoplasmic vacuolation; [D] kidney section exposed to 60mg/L for 2 weeks degenerative changes (DC) in the form of cytoplasmic vacuolation and acute cellular degeneration of the tubular epithelium, necrosis in the tubular epithelium (N) and slight reduction hemopiotic tissue (*). H&E. 40x.

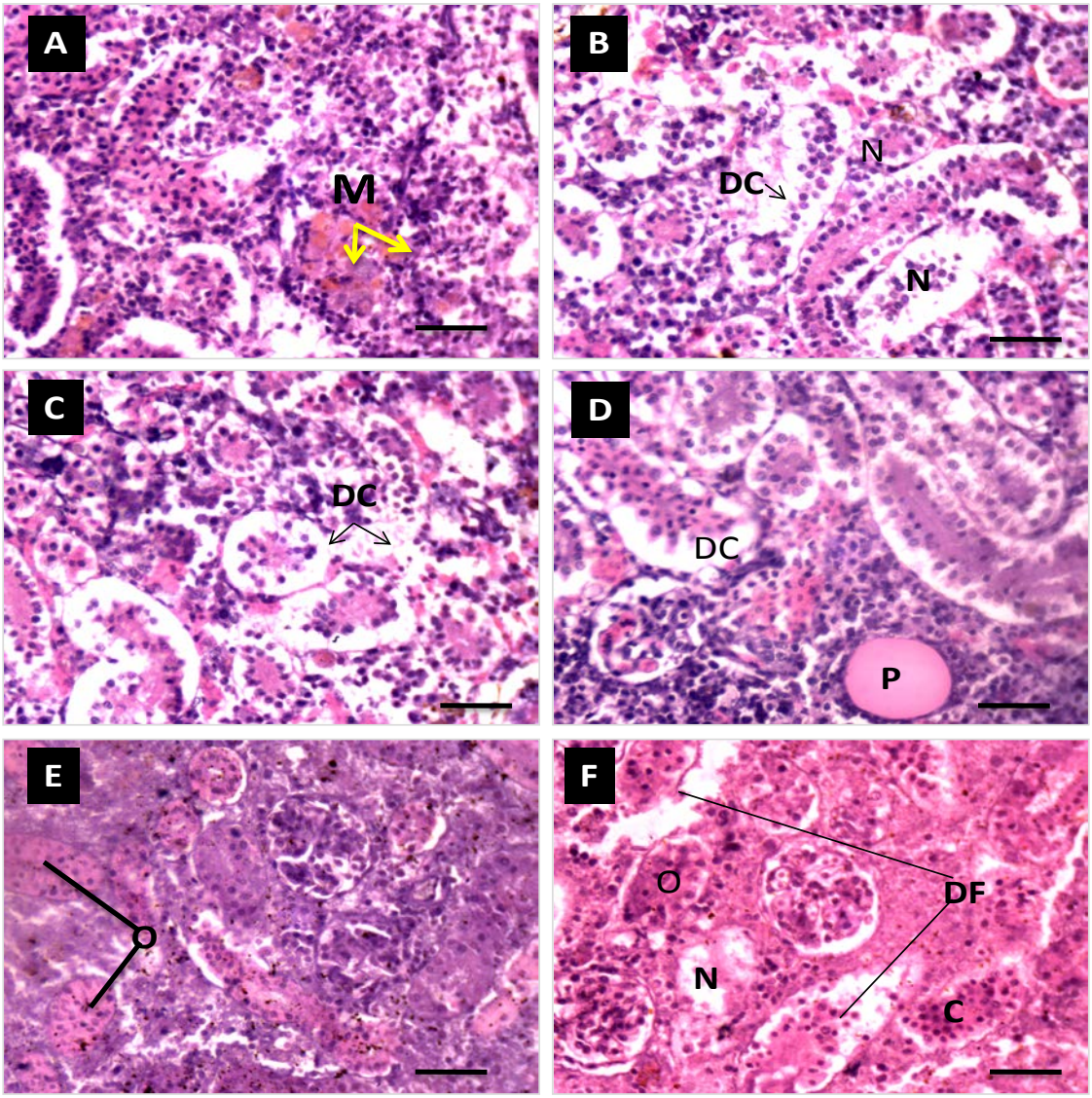

Figure 15: Photomicrographs of the kidney of *Cyprinus carpio* [A] Kidney section exposed to 20mg/L for 4 weeks showing melanomacrophage aggregation (M); [B] kidney section exposed to 20mg/L for 6 weeks showing degenerative changes of the renal epithelium (DC), necrosis in renal tubules (N) with depletion in hemopiotic tissue; [C] kidney section exposed to 40mg/L for 4 weeks showing acute degenerative changes (DC) in the form of cytoplasmic vacuolation); [D] kidney section exposed to 40mg/L for 6 weeks showing (DC) and infiltration of protein substances in tubules (P); [E] kidney section exposed to 60mg/L for 4 weeks showing occlusion of the tubular lumen (O); [F] kidney section exposed to 60mg/L for 6 weeks showing occlusion of the tubular lumen (O), necrosis in the tubular epithelium (N), deformation in renal tubules architecture (DF) and reduction in hemopiotic tissue. H&E. 40x.

The kidney is a major site for toxic effects due to a wide variety of environmental pollutants (48). Because of water reabsorption taking place in the distal tubules, relatively high concentrations of toxins may have an effect on renal cells. The renal contents may become acidified in some renal segments, which may provide an interaction with toxic substances.

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Potential anti - biofilm activity of human enteric *Lactobacillus acidophilus* on urophaogenic *E.coli* (UPEC) biofilm formation ability

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Abstract: It has been suggest that biofilm formation by uropathogenic *E.coli* (UPEC) is associated with recurrence and persistence of urinary tract infections (UTIs). The conventional strategies of antibiotic therapy not only proved to be sub – optimal efficacy but also led to the development of multi – drug resistant strains of UPEC. One promising alternative therapy is the use of certain probiotics particularly *Lactobacillus spp*. to prevent and treat recurrent complicated and uncomplicated UTIs. The present study evaluated the antagonistic and anti – biofilm activities of eighteen human derived enteric and vaginal *Lactobacillus* isolates on multi – drug biofilm producer UPEC, isolated from recurrent UTI.. Eight isolates of assessed *Lactobacillus* isolates were showed clear inhibitory actions against UPEC planktonic cells, with average of 8 – 18mm diameter of inhibition zones, the enteric isolate *L acidophilus* Lf1 showed the largest inhibition zone in agar – well diffusion technique. Significant ($P<0.05$) reduction of biofilm formation ability was proved in UPEC when treated by *L. acidophilus* (Lf1) crude cell free supernatant (CFS), with about seven- folds reduction in biofilms densities . The neutralized and H_2O_2 excluded CFSs of Lf1 also exhibited well anti – biofilm activity, the H_2O_2 excluded CFS was displayed superior reduction in biofilm formation abilities in that the percentage reduction values average was of about 75- 83 %, While neutralized CFS pronounced slightly less reduction percentage against biofilms, with an average values of about 55 -64 % . Bacteriocins was the most potent anti – biofilm agent , the 80% saturation fraction of Lf1 bacteriocins was pronounced the higher inhibitory potential on biofilms abilities of UPEC, as biofilm reduction values average was of about 80.5-83.5%.

Key words: Probiotics, *L. acidophilus*, UPEC, Biofilm

الفعالية التثبيطية المحتملة لبكتريا *Lactobacillus acidophilus* المعوية على قدرة تكوين الاغشية الحيوية في بكتريا القولون البولية Uropathogenic *E.coli* (UPEC)

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الخلاصة: تشير الدلائل الى ان خمج المسالك البولية المتكرر و المتسبب من بكتريا القولون البولية UPEC له علاقة بتكوين الغشاء الحيوي من قبل هذه البكتريا، و ستراتيجيات العلاج التقليدية المتبعة و المتمثلة في استخدام مضادات الحياة اثبتت انها دون المستوى المطلوب للشفاء التام كما ان استخدامها ادى الى تطوير سلالات ذي مقاومة متعددة للعلاجات المتاحة . و اعتمدت البروبايوتك Probiotics بالأخص بكتريا اللاكتوباسيلس *Lactobacillus* spp. كبديل واعد لمنع وعلاج خمج المسالك البولية المتكررة المعقد و غير المعقد . في الدراسة الحالية تم تقييم تضاد وتثبيط الاغشية الحيوية لبكتريا القولون البولية من قبل ثمانية عشر عزلة لبكتريا اللاكتوباسيلس المعوية والمهبلية المشتقة من الانسان. أظهرت ثمانية من عزلات بكتريا اللاكتوباسيلس المختبرة تثبيط واضح ضد بكتريا القولون البولية بتقنية الانتشار في حفر الهلام و بمتوسط اقطار مناطق تثبيط 8 – 18 ملم و كانت العزلة المعوية *L. acidophilus* Lf1 أكفأ العزلات المختبرة . انخفضت معنويا ($p<0.05$) قابلية تكوين الاغشية الحيوية في عزلات بكتريا القولون البولية وبحوالي سبعة مرات عند معاملتها مع الطافي الخام لبكتريا Lf1 ،كما ان الطافي المتعادل والمزال منه البيروكسيد كان مثبطا لتكوين الاغشية الخلوية ولكن تفوق الطافي المزال منه البيروكسيد في قدرته الاختزالية في تكوين الاغشية الخلوية على الطافي المتعادل وبمعدل متوسط اختزال 75 – 80% ، في حين كان معدل متوسط اختزال الطافي المتعادل اقل بقليل وبمعدل 55 – 64% . وثبطت بكتريوسينات Lf1 الاغشية الخلوية لبكتريا القولون البولية بكفاءة عالية حيث ان البكتريوسين المجزأ بنسبة تشبع 80% خفض قدرة تكوين الاغشية الخلوية بنسبة 80.5-83.5 % .

Introduction:

Urinary tract infections (UTIs) are a major public health concern and also represent one of the most common hospital - acquired infections. Most of uncomplicated UTIs are caused by *E.coli*, accounting for up to 80 % of community – acquired and approximately 50% of nosocomial UTIs(1) The origin of these strains is frequently the patient's own intestinal flora. In comparison to commensals strains, UPEC present several virulence factors , which enable the bacteria to colonize the urinary tract and persist in face of highly effective host defense. (2) . A common problem in UTIs is recurrence, even in patients without anatomic abnormalities, it estimated that 20 to 30 % of UTI patients of UPEC will have a recurrence infection within 3 to 4 months of acute infection. The high incidence of recurrent UTI (rUTI) suggests that many individuals do not develop protective immunity to uropathogens, and it's a consequence of UPEC aggregation into the biofilm - like communities within bladder , depending upon type 1 pili expression (3). Recurrent UTI caused by UPEC represent classical biofilm problem and in that biofilm formation may be considered as another pathogenic determinant for UPEC which allows the strains to persist a long time in the urinary tract and interfere with clearing out of effect of hydrodynamic forces and killing activity of host defense mechanism. Such infection may be difficult to treat as they exhibit multi – drug resistance(4). For the treatment and prevention of UPEC-related UTIs including recurring infections, the use of low dose once daily or post-coital antimicrobials have been a cornerstone (5). However, even with urine concentrations of antibiotics far exceeding minimal inhibitory concentrations, UPEC reservoirs in tissues were not eradicated effectively(6). Therefore, some alternative non-antimicrobial based therapeutic approaches such as probiotics, which has been demonstrated to be health – promoting, and specific probiotics have been shown to be effective in treatment or prevention of many gastrointestinal and urogenital infections. The potential application of probiotics is continuously widening, with new evidence accumulating to support their therapeutic and prophylactic of many diseases, including the urinary tract infections (UTIs).The use of probiotics against UTI has previously been assessed(7) .However, while there is evidence that *Lactobacillus*

spp. have an effect on UTI, their mechanism of action has thus far not been elucidated, with most studies relying on circumstantial evidence. In all, biosurfactants, bacteriocins, lactic acid and hydrogen peroxide. seem to be inhibitory for UPEC growth and biofilm formation, while adversely affecting fimbrial structure and adhesion and upregulating immunogenic membrane proteins(8). The goal of this study was to assess the in vitro antimicrobial – antibiofilm bacteriostatic activity of selected human derived *Lactobacillus* strains, against UPEC .

Material and method:

Organisms

Isolation and identification of *Lactobacillus*

Two different sources were used for isolation of *Lactobacillus*, ten fecal samples from 10-12 week old healthy fully breast-fed infants and eight vaginal samples from a healthy reproductive age women. Samples cultured in de Man, Rogosa and Sharp (MRS) broth supplemented with 5 µg /ml erythromycin, the tubes were incubated anaerobically (anaerobic jar and gas pack) at 37°C for 48 hr. The grown cultures plated on MRS plates, smooth small convex whitish to creamy glistening colonies were isolated and plated several time on MRS agar plates. *Lactobacillus* isolates were identified to species level depending on sugar fermentation profile (9).

Uropathogenic E. coli (UPEC) isolates

Fifty UPEC isolates were used in study, these isolates were isolated from patients suffering from rUTI in previous study(10) .

antibiotic susceptibility

The antibiotic susceptibility testing for UPEC isolates was done by using Kirby – baure disk diffusion method. The antibiotic disks which were used; cephalothin ,nitrofurantion ,gentamicin ,cefixime, ampicillin, tetracycline, amikacin ciprofloxacin , meropenem and imipenem. The zone size around each antibiotic disk was measured by millimeter.

Biofilm assay

Method described by (11) was followed for production and assay of biofilm in UPEC isolates. In brief, Overnight cultures of individual bacterial isolates was grown in brain heart infusion (BHI) broth supplemented with 1 % glucose, cultures were diluted in BHI broth and adjusted in comparison to MacFarland tube 0.5, aliquots 200 µ

l from the each culture were deposited to the wells of a 96 - well polystyrene microtiter plate and incubated under constant conditions at 37℃ for 24hr . wells were washed after incubation twice with PBS pH7 to remove loosely adherent cells, attached cells were stained with 0.1 % methylene blue solution. Plates were gently shake to help the colorant to get the bottom of the wells and incubated at room temperature for 15min.Wells were washed twice and the quantitative analysis of biofilm production was performed by extraction with ethanol alcohol , and absorbance of the methylene blue present in the distaining solution (ethanol) was measured at 630 nm by ELISA reader (Beckman coulter, Austria). Control was set up with methylene blue binding to the wells exposed only to the culture medium without bacteria. Assay was performed triplicate and the mean biofilm absorbance value was determined for each tested bacterial strain and was calculated as follow:

Biofilm degree = Mean OD 630 of tested bacteria - Mean OD 630 of control

Biofilm antibiotic susceptibility testing

The highest biofilm producer UPEC isolates (E1 , E 17 , E 24 and E 47) were selected to be testing, following the procedure of (12).Procedure mentioned previously for biofilm production was followed, before the staining step, the antibiotics meropenem(MEM) and ciprofloxacin(CIP) containing media with MIC concentration added separately to the each biofilm containing well, plates were incubated for another 24 hr. at 37 ℃ . then the wells were washed and biofilm was stained with methylene blue .

***Lactobacillus* antagonistic activity assay**:

The antagonistic activity of the isolated Lactobacilli cell – free supernatants (CFS), were analyzed against planktonic cells of UPEC strong biofilm producers isolates (E1, E17, E 24 and E 47), using agar-well diffusion assay (13).

Anti – biofilm activity of *L. acidophilus* CFS

potential anti – biofilm activity of L. acidophilus (Lf1) was investigated to ensure probiotic bioactivity on biofilms of UPEC isolates (E1, E17, E 24 and E 47).

Biofilm producing procedure previously described was followed, before staining step 100 µl of crude, neutralized, and H2O2 excluded CFSs were individually added to wells, plates incubated at 37°C for 24hr. and subsequently biofilm densities was detected by staining with methylene blue.

Anti – biofilm activity of Lf1 bacteriocins

Neutralized and catalase treated CFSs of Lf1 was precipitated with different concentration of ammonium sulfate (50 – 80 %) saturation under cooling condition, the precipitates at different saturation were collected by centrifugation (8000 rpm, 20min, 4°) and dissolved in small volume of 0.05 phosphate buffer pH 7. The suspensions was dialyzed against same buffer at 4°C overnight. Anti – biofilm activity was estimated to each obtained fraction as previously described .

Statistical analysis

The study data were compared using Statistical Analysis System - SAS (2010) and the least significant difference (LSD) . Difference were considered significant when $P< 0.05$.

Results and discussion

UPEC biofilm formation : All the UPEC isolates showed biofilm formation ability with different potential capacity under the same conditions of experimentation (fig. 1). The isolates were confined between two groups, strong biofilm producers 21 isolates (42%) and 29 isolates (58%) weak producers. The UPEC isolates ; E 1, E 17 , E 24 and E 47 produced a thickest biofilm with O.D 0.862 , 0.425 , 0.523 , and 0.436 respectively (these isolates were selected to carry out the rest of experiments through this study). Obviously, the isolate E 1 was achieved the maximum biofilm thickness, while minimum biofilm producer was the isolate E49.

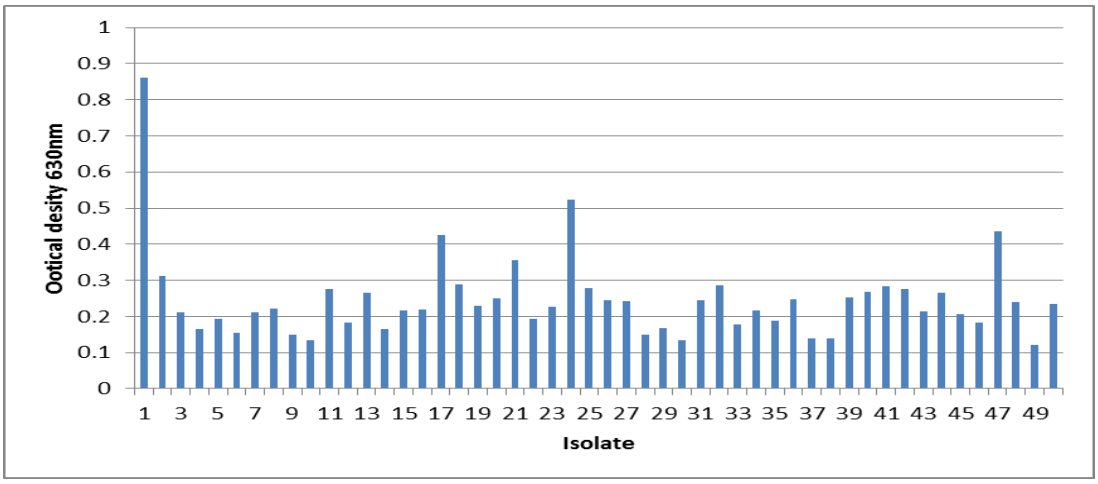

Figure: 1 Biofilm Production Capacity (O.D630) Of UPEC isolates

UPEC isolates showed a remarkable high capability to form biofilm in this study, as 100% of tested isolates were showed biofilm phenotype positive . The prevalence of biofilm production was thus higher than reported in previous studies (14). Biofilm formation is more in repeated UTI's. This shows that biofilm formation associated with repeated UTI which correlates with other studies. Previous studies also reported a higher incidence (74%) of biofilm in repeated UTI. This may explain why *E. coli* are more prevalence in UTIs than other associated microorganisms, and this shows that the biofilm formation is mostly companied with repeated UTIs. The difference in biofilm thickness resulted from different reasons such as; differences in isolates capacity to form biofilm(15), and potential biofilm capacity in *E.coli* are depends on several surface determinants, which are profoundly induce biofilm formation on the population level such as, expression of curli , type l fimbriae , flagella and motility, autotransporter proteins, F conjugative pilus, and exopolysaccharids production (16). The primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from each from each isolate play an essential as well as important role (17).

Antibiotic Susceptibility

UPEC isolates exhibited multi – drug resistance (MDR), the isolates showed high degree of resistance to cephalothin ,nitrofurantion ,gentamicin ,cefixime, ampicillin, tetracycline, and amikacin, with a percentage resistant pattern of 100, 96, 94, 84, 80, 78, 24, % respectively, Among the tested antibiotics, ciprofloxacin and meropenem were the most effective with 100% sensitivity, followed by imipenem 90% sensitivity (fig.2).

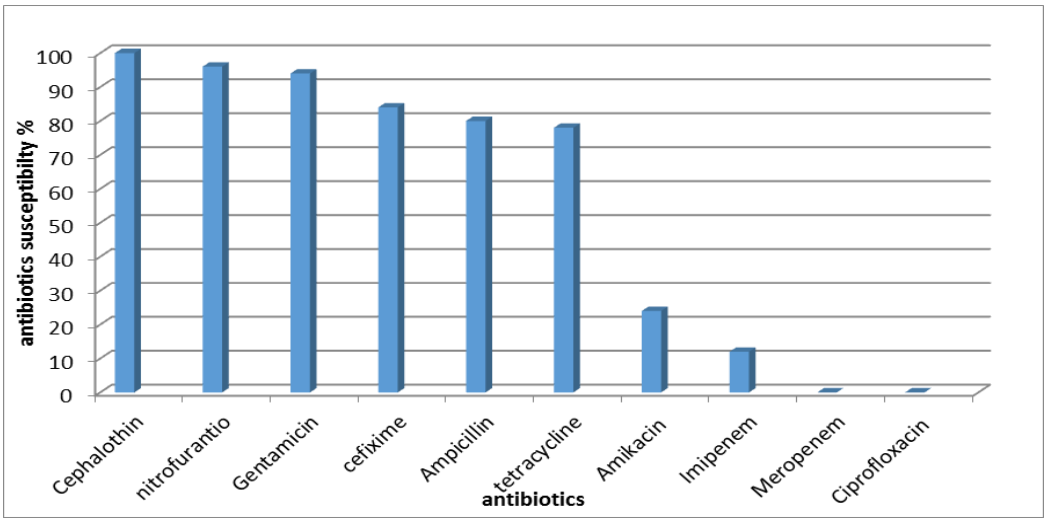

Figure 2 : UPEC antibiotics susceptibility pattern

From the results, the UPEC isolates showed high degrees of resistance to the most of tested antibiotics, the increasing frequency of MDR *E. coli* strains is worrisome, since this agent is being frequently prescribed for uncomplicated UTIs and has been considered as the first-line empirical treatment for more than 30 years (18). The obtained results agree with some degree to the findings of (19), which were demonstrate that UPEC was resistant to Cephalexin (100%) . The high magnitude resistant rate to nitrofurantion antibiotic in this study was an interesting finding and it seems to be rare case and is non congruent with the previous findings which is much higher than reported in contemporary literature. The highest global resistant rate was recorded for nitrofurantion against *E. coli* isolates from UTI patients was 17.8%(20).

The high degree of sensitivity to fluoroquinolone and meropenem have important clinical implication in the context of empirical use of these two antimicrobial therapies , it has been seen that quinolones and carpenems are valid option as an empirical therapy of uncompilicated UTIs but a careful use is recommended, to avoid the selection and the spread of the resistant strains (21). It is interesting to note that these antibiotics are only available for intravenous administration and provided on prescription only. Hence, the route of administration of these antibiotics may have reduced its misuse which had led to the reduction in the emergence of resistant bacterial strains.

Determination of MIC and MBC values

MEM and CIP were the most effective drug against UPEC planktonic cells, with minimum inhibitory concentration (MIC) values about of 2- 8 µg /ml and 32 – 125 µg /ml respectively. The minimum bactericidal concentration (MBC) values for both antibiotics were two-fold higher than MIC values (table. 1).

Table 1 The MICs and MBCs of MEM and CIP for UPEC isolates

	MEM		CIP	
UPEC isolates	**MIC µg/ ml**	**MBC µg/ml**	**MIC µg/ml**	**MBC µg/ml**
E1	8	16	125	250
E17	2	4	62	125
E24	4	8	32	64
E47	2	4	32	64

Biofilms antibiotic susceptibility :Significant reduction ($p< 0.05$) was observed in biofilms formation ability of UPEC isolates when treated with antibiotics. MEM was the most effective anti – biofilm drug . It was achieved maximum reduction rate about seven – folds against E1 biofilms as the biofilm O.D630 reduced from 0.765 (pre – treated) to 0.11(post - treatment), while CIP was showed much lower rate of

reduction in that the maximum reduction rate was about four – folds against E1 , where the O.D fall from0.765 to 0.178 (tab.2).

Table 2 : Absorbency of UPEC isolates biofilms treated with MEM and CIP

UPEC isolates	Pre-treatment ($O.D_{630}$)	Post – treatment ($O.D_{630}$)	
		MEM	CIP
E1	0.765±0.245	0.011±0.031	0.178±0.134
E17	0.677±0.311	0.188±0.127	0.15±0.094
E24	0.489±0.279	0.020±0.115	0.177±0.077
E47	0.522±0.143	0.170±0.043	0.167±0.121

Based on the obtained data the MEM was more effective as anti – biofilm agent than CIP, as the biofilm reduction rate are more significant in treatment with MEM than CIP. Despite the obtained results, all the antibiotic treated biofilms showed decline in absorbance but no antibiotic strictly inhibited the biofilm formation capacity of treated isolates. This finding strongly suggest the most efficient antibiotic which succeeded in the killing the planktonic cells of UPEC failed to effectively kill all bacterial cells within the biofilm which will be able to establish a new biofilm. The biofilm infections are 10 to 1000 more resistant to effective antibiotic. The biofilm-associated resistance to antimicrobial agents begins at the attachment phase and increases as the biofilm ages. The mechanism of biofilm-associated antimicrobial resistance seems to be multifactorial and may vary from organism to organism. The mechanism for enhance biofilms antibiotic resistance is believed to involves alteration in gene expression leading to a phenotype different between the planktonic and sessile (22). The more radical hypothesis for biofilm resistance to antibiotics is that the majority of sub lethally damaged cells in the population commit suicide (apoptosis), thereby providing some protection to the survivors (persisters). The presence of persister cells and small-colony

variants has been associated with enhanced antibiotic resistance of many organisms in biofilms (23) .

Isolation and Identification of *Lactobacillus*:

All the screened feces and vaginal swabs samples exhibited the presence of Lactobacillus , so eighteen lactobacilli isolates were identified depending on sugar fermentation profile. the isolates were fall into two species *L. acidiphilus* and *L. fermentium*. The distribution of these two species was as the following ; in feces samples, 6 (60 %) *L.acidophilus*, and 4 (40 %) *L. fermentium* , and within vaginal swab samples, 6 (75%) *L.fermentium*, and 2(25%) *L.acidophlius*.

Lactobacillus antagonistic activity:

Eight (44.4%) of tested lactobacilli isolates were demonstrated significantly higher antagonistic activity against the target bacteria UPEC , with an average inhibition zones 8 – 18 mm in diameter. The enteric isolate *L. acidophilus* Lf1 was the most potent than the other isolates, it exhibited larger inhibitory zone (15-18) mm , and the most sensitive UPEC isolates to antagonism of lactobacilli was the isolate E47, other the seven rest lactobacilli isolates showed less and variable inhibitory activities (table- 3). It was clear from the obtained data the enteric *L. acidophilus* Lf1 was the potent antimicrobial producer isolate, so it was selected as potentially probiotic isolate to investigate the anti – biofilm activity in subsequent experiments. Although all tested *Lactobacillus* life along in same environment, gut or vagina, and these two sites in healthy human body induce the production of different microbial inhibitory substances by *Lactobacillus*, as was reported previously, 99% of *Lactobacillus* strains make at least one of antimicrobial substances (24). The failure of other tested strains to inhibit target bacterium might due to bacterial metabolic activity changes in response to surrounding environment (in vitro) compared with their native habitat, and a lack of cell

Table 3: Antagonistic activity of *L.actobacillus spp*. against UPEC planktonic cells

	Diameter of Inhibition zones (mm)			
Lactobacillus isolates	**E1**	**E17**	**E24**	**E47**
Lf1	**18**	**16**	**15**	**15**
Lf2	**15**	**17**	**14**	**12**
Lf3	**0**	**0**	**0**	**0**
Lf4	**0**	**0**	**0**	**0**
Lf5	**0**	**0**	**0**	**0**
Lf6	**0**	**0**	**0**	**0**
Lf7	**0**	**0**	**0**	**0**
Lf8	**0**	**0**	**0**	**0**
Lf9	**13**	**16**	**12**	**15**
Lf10	**14**	**12**	**14**	**13**
Lv1	**0**	**0**	**0**	**0**
Lv2	**0**	**0**	**0**	**0**
Lv3	**9**	**11**	**10**	**8**
Lv4	**0**	**0**	**0**	**0**
Lv5	**10**	**11**	**9**	**8**
Lv6	**0**	**0**	**0**	**0**
Lv7	**15**	**13**	**14**	**15**
Lv8	**0**	**0**	**0**	**0**

– cell contact between the indicator bacterium (UPEC) and the producer bacterium by the used detection assay procedure, agar - well diffusion technique (25).

The inhibitory action of *Lactobacillus* (antagonism) in vitro consider to be multifactorial, mainly due to accumulation of main primary metabolites, such as, ethanol, H_2O_2, CO_2, and organic acids. In a particular lactic and acetic acids from hexoses sugar fermentation, are responsible for the decrease of pHs in microbial residing. The accumulation of lipophilic organic acids and the concomitant reduction in pH of the milieu results in broad - spectrum inhibition activity against Gram-positive and Gram-negative bacteria (26). Lipophilic acids antagonistic effects against of many potential

pathogenic bacteria attributed to the penetration of microbial cellular membranes and intracellular dissociate to produce hydrogen ions, which interfere with essential metabolic functions. The acidic pH inhibition on the microbial growth act on three levels: the enzymatic activity, the membrane permeability and bioavailability of some nutrients which depends on ionic balance (27*).* *Lactobacillus* also are capable of producing antimicrobial compounds such as, bacteriocins and bacteriocins-like substances. These compounds are also responsible for the inhibitory effects. Bacteriocins are biologically active protein moieties with bacteriocidal mode of action . Two main features distinguish the majority of bacteriocins from classical antibiotics: bacteriocins are ribosomally synthesized and have relatively narrow killing spectrum (28), bacteriocins gain entry into the target cells by recognizing specific cell surface receptor then kill the cell by forming ion – permeable channel in the cytoplasmic membrane, by nonspecific degradation of cellular DNA, inhibiting the protein biosynthesis through the specific cleavage of 16s rRNA, or by cell lysis(29). Most of reported *Lactobacillus* bacteriocins fall into class l bacteriocins (lantobiotics), The antibacterial activity of lantobiotics based on interaction with the bacterial membrane, they binds specifically to phosphoethanolamine which results in inhibition of phospholipase A2 and various other cellular functions. Most of bacteriocins l dissipate the proton motive force (PMF) of target cells, via pore formation(30).

Anti-biofilm activity of *L.acidophus* CFS :The anti – biofilm activity of *L. acidophilus* (Lf1) crude CFS was investigated against UPEC isolates biofilms. Significant difference ($P<0.05$) was found in O.D values pre and post treated biofilms, the isolate E1 biofilm was the most affected one, hence the absorbency decreases about seven- folds from 0.733(control) to 0.101. The isolate E47 biofilm was the less affected by inhibitory activity of Lf1. It represented about three – folds reduction in biofilm ability, whereas, the O.D reduced from 0.499 to 0.156 (table.4).

Table 4 : Anti- biofilm activity of *L. acidophilus* Lf1 CSF on UPEC biofilms

UPEC isolates	Pre – treatment $O.D_{630}$	Post –treatment $O.D_{630}$
E1	0.733±0.101	0.101±0.011
E17	0.588±0.122	0.100±0.088
E24	0.501±0.11	0.188±0.022
E47	0.499 ±0.103	0.156±0.144

The inhibitory effect of *Lactobacillus* CFS on biofilm formation was shown in previous study (31). Similarly , in another related study, the inhibition effect of *L. rhamnosus* GG were observed on *E.coli* growth and biofilm formation (32). The attachments and subsequent invasion of epithelial cells by *E.coli* require asset of genes (33). Previous studies have reported that probiotic components down regulate these genes expression in *E.coli* genome thereby preventing its attachment process (34), and the attachment step involves many surface presented proteins in *E.coli* biofilm construction, the reduced expression of these proteins may be the important mechanism which contributes to the anti- biofilm effect of probiotic bacteria on pathogens(35). Moreover, lactobacilli co-aggregate with uropathogens to block their adhesion and/or displace previously adherent uropathogens from the urinary tract. This co-aggregation can create a microenvironment in which the inhibitory substances produced by the lactobacilli can concentrate on the pathogens that inhibit and block the adhesion stage of uropathgens, which in turn interfere with attachment phase of biofilms formation (36). Besides the crude CFS, also termed acid CFS, L. acidophilus belonged to obligate homofermentative group , in which ferment hexoses and produced strictly lactic acids and no more other organic acids, the reduction in pH of surrounding milieu may suppress AI-2 mediated QS , which regulate many functions of *E. coli* including motility and establishment of biofilm (37).

The exclusion of organic acid and H_2O_2 from Lf1 CFSs. clearly affected the anti – biofilm activity of Lf1. H_2O_2 excluded CFS was most effective anti- biofilm agent in that it exhibited superior inhibition against UPEC biofilm with a percentage reduction values average of about 83 – 75 %, the highest reduction value recorded for E17 biofilm. While neutralized CFS pronounced slightly less reduction percentage against biofilms, with an average values of about 64 – 55 % (fig.3) .

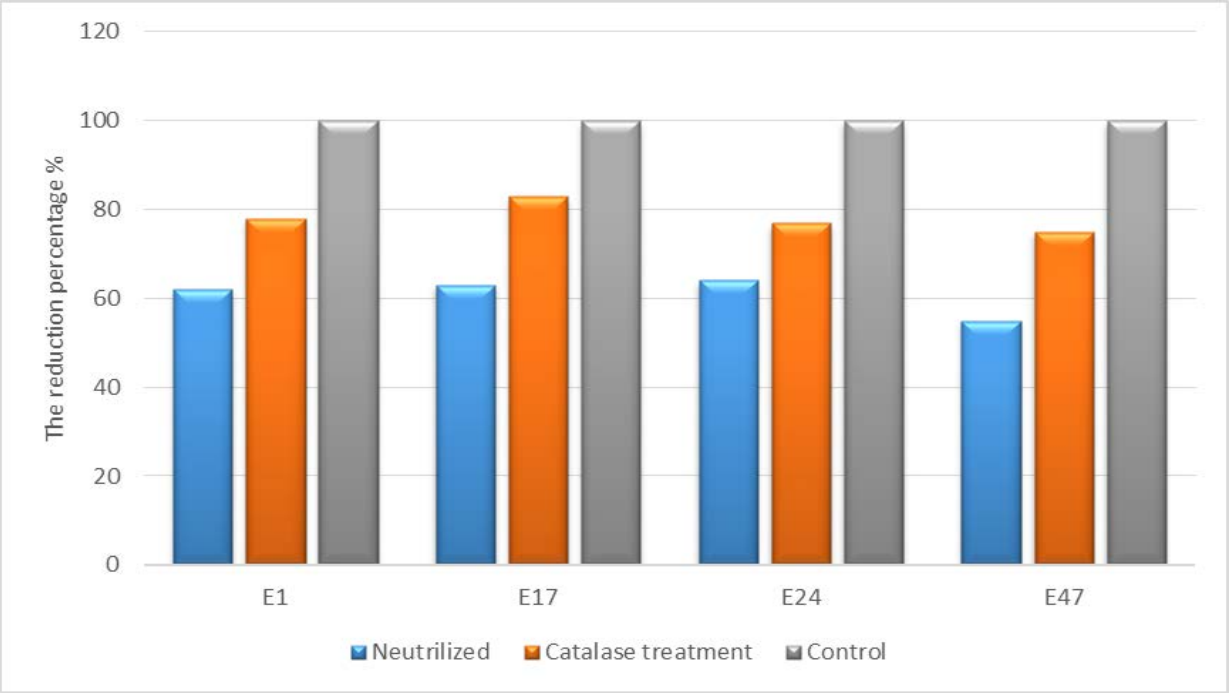

Figure 3 : The Anti – biofilm activity of neutral and H2O2 excluded CFS of Lf1 on UPEC isolates (E1, E17, E24,E 47), expressed as percentage of biofilm reduction%

The neutral and H_2O_2 excluded CFSs displayed anti – biofilm behavior, although the results were variable, and the obtained results absence the role of peroxide in the antagonistic effect, but in same time show a leading role of other metabolites including organic acids in that the neutralized CFS was showed less anti – biofilm behavior , providing and confirming that the antagonistic effect of *Lactobacillus* is multifactorial. Some in vitro studies results suggested the biofilm inhibition seems to be pH and H_2O_2 dependent (38), and there are some

evidence supporting the notion that the cooperation of lactic acid and H_2O_2 are the key factors in resisting urogenital infections, other studies demonstrated that H_2O_2 is the most toxic lactobacilli product on Gram – negative pathogens (39), but (40) have proved that *Gardnerella vaginosis* biofilms allow to survive in presence of lactobacilli derived H_2O_2, (41) attributed biofilms formation inhibition to the change of oxidation – reduction potential as consequence of lactobacilli metabolites accumulation rather than acids and peroxide. it seems anti –biofilm features of lactobacilli is strains dependent and each strain independently exert its antagonistic feature against bacterial pathogens.

Anti – biofilm activity of bacteriocins:

Ammonium sulfate fractionated bacteriocins of *L. acidophilus* Lf1 displayed a considerable anti-biofilm activity against four biofilm producer isolates of UPEC ,and the highest anti-UPEC biofilm was recovered at 80% saturation, with a percentage reduction of biofilm formation of about 80.5-83.4%, and the less potential percentage reduction was recorded at 50% saturation , with average values of about 50.7 – 68.9% (fig.4).

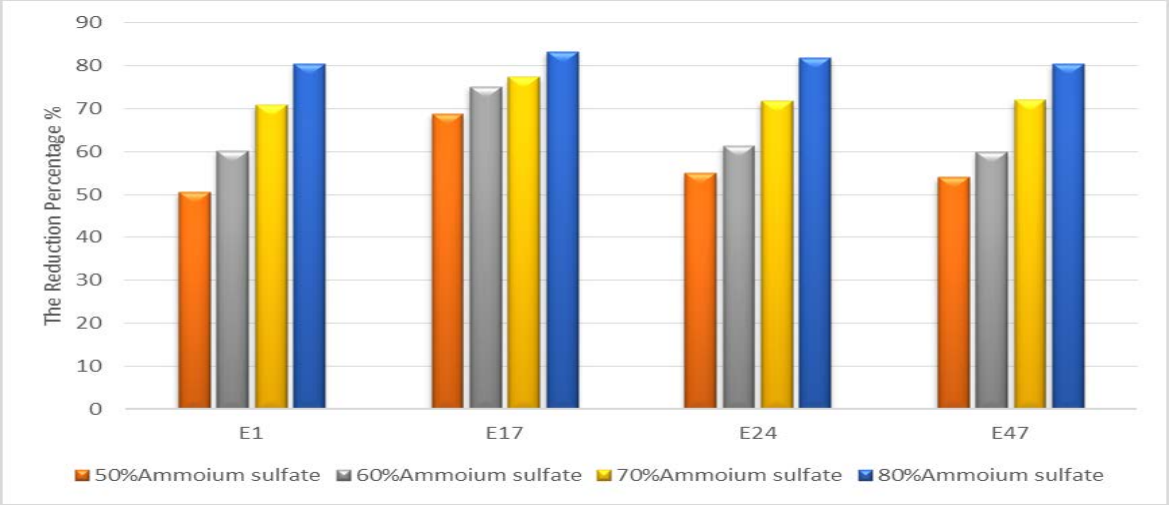

Figure 4: The Inhibition of UPEC biofilm formation by *L. acidophilus* Lf1 bacteriocins in various saturation degrees, expressed as percentage% of biofilm reduction.

Obtained results in related to bacteriocins look encouraging, since to our knowledge is one of bacteriocins which showed such high anti – biofilm activity, other bacteriocins produced by *L. acidophilus* and other lactobacillus spp. show lower activities against E.coli . However, successful inhibition of E.coli biofilms formation has been observed (41) . Although the sensitivity of Gram – negative bacteria biofilms to probiotic bacteriocins is not common, but *L. acidophilus* has already been reported to produce bacteriocins effective on some human urogenital pathogens (42). The mechanisms lies in bacteriocins anti – biofilm effect can be explained by affection on cellular membrane instability and permeability via formation of ionic channels, lead to the disruption and loss ability of propelling force, in that cells are disturbed in some sense(43), besides bacteriocins diminish the quorum signals (acyl – homoserinlactone) and these signals are necessary for biofilms formation, hence such diminishing of signals may cause interruption in biofilm production (44).

Conclusion:

The result of this study suggest that the anti- biofilm potential of enteric *L. acidophilus* is likely bacteriocin – based feature, and it may be beneficial to use bacteriocins fraction as a partial replacement or adjunct to antibiotic therapy to help in treating recurrent UTIs caused by UPEC.

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Detection of some virulence factors of *Aeromonas hydrophila* isolated from local freshwater fish

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Abstract: From sixty two bacterial samples obtained from fish of Baghdad local market. According to the cultural phenotype and biochemical tests, 23 (37.09%) samples were identified as *Aeromonas hydrophila* and depending final identification confirmed by API 20. All bacterial strains of *Aeromonas. hydrophila* showed a different capacity in hemolytic activity, 12 (52%) strains showed β-hemolytic activity, 9 (39%) isolates were showed α-hemolytic activity and 2 (8.69%) isolates were λ-hemolytic activity. All isolates produce proteases, lipase DNase with a variable capacity. Examination susceptibility to commonly used antibiotics referred to the high resistance rate among *A.hydrophila* strains isolated from local freshwater fish. We found that all strains enrolled for this study were resistant to ampicillin,Amoxicillin, Cefotaxime while it was fully sensitive to amakacin and azithromycin. Free cell supernatant of different strains of*Aeromonas hydrophyla* showed cytotoxic effect on HepG2 cells with different severity.

Key word: *A.hydrophila*, cytotoxicity, virulence factors, antibiotic susceptibility.

التحري عن بعض عوامل الفياعة لبكتريا *Aeromonas hydrophila* المعزولة من اسماك المياة العذبة المحلية

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الخلاصة: من مجموع اثنان وستون عينة جمعت من اسماك المياه العذبه تم الحصول عليهامن الاسواق المحلية في بغداد تم تشخيص اعتمادا على النمط المظهري والتفاعلات البايو كيميائية *Aeromonas hydrophila* على انها (23)37% والتاكيد باستخدام نظام تشخيص PIA 20.

قابلية مختلفة على تحلل الدم حيث ان 12عزلة اي بنسبة (52%) كانت اظهرت جميع السلالات المعزوله لهذه البكتريا ذات تحلل نوع بيتا و9 عزلات اي بنسبة (39%) اظهرت تحلل من نوع الفا و عزلتان ليس لها القابلية على تحلل الدم اي النوع كاماز جميع العولات كانت تمتلك القابلية على تحلل البروتين و تحلل الدهون والاحماض النوويه.

اشارت اختبارات الحساسية للمضادات الحيويه شائعة الاستعمال الى ارتفاع نسبة المقاومة للمضادات الحيوية لجميع السلالات المعزوله لهذه البكتريا من اسماك المياه العذبه وبشكل عام كانت مقاومة للامبسلين والاموكسيسلين والسيفاتاكسايم بينما كانت جميع العزلات حساسه الى الاميكاسين والازثرومايسين . اظهرت النتائج ان جميع سلالات بكتريا*Aeromonas hydrophila* المعزولة لها تأثير سمي على خلايا السرطانيه للكبد *وبشكل متغاير باختلاف السلالة.*

Introduction

Aeromonas.hydrophila inhabited a wide diversity of habitats such as fresh and salt water fish , seafood and soil (1). It is an opportunistic pathogen could cause disease in cold and warm blooded animal and could causes infection in human (2). *Aeromonas hydrophila* have the ability to produce a numerous of virulence factors which play important role in the pathogenicity of this bacteria and cause different disease in fish and human. Previous studies showed the effect of many diseases caused by these bacteria in the fish although it was found as a part of the normal flora normally found in the fish (3). *A. hydrophila* could cause many diseases in human such as wound infection, sepsis and food born gastroenteritis (4). Direct contact with the water or soil and during food preparation contaminated with these bacteria could cause disease in human (5). *A. hydrophila* possess a wide range of virulence factors such as Aerolysin, Enterolysin, Cytotoxin and Lipopolysaccharide endotoxin in addition to enzymes such as Protease ,Elastase, Gelatinase and DNase(1), these factors increase bacterial pathogenicity .Increasing bacterial resistance to antibiotics among this bacteria make it to be an emerging pathogen that threatens human (6).Previous studies indicate to the immunological and cytotoxic effect which was reported by these bacteria (7). Our study investigated the distribution of *A.hydrophila* among local fish purchase from Baghdad markets and studying some virulence factors and cytotoxic activity.

Material and methods

Bacterial isolation and identification

Bacterial samples were collected from fresh water fish at morning time within 6 hours after fishing. Samples were collected aseptically from the outside surface, gills, and intestine of the fish by using sterile swabs, grown on brain heart infusion then on Aeromonas isolation agar medium and MacConky agar (HImedia, India) ,and incubated for 24 hr at 37Ċ as described by (1). A purified isolated colony from each sample was cultured on Brain Hart Infusion (BHA) medium and enrolled for further studies. A phenotypic characters on culture media and microscopic features were recorded. Biochemical tests were done according to (9). All strains were confirmed using EPI 20 E (Biomerix Co., France) test .

Virulence factors of *A. hydrophila*

Hemolytic activity

This test was done by streaking purified isolates on blood agar

containing 5% human blood (2), Incubated for 24hr at 37Ċ. The areas around colonies were recognized to determine the hemolytic activity.

Detection of slim layer production by Congo red agar

This test was done according to the method of (7). By using sterile loop, dispread loop full of 18 hr bacterial growth of each strain separately on brain heart agar containing (0.08% w/v Congo red, incubated for 48 hr at 37Ċ . Detection black ground colonies under obliquely reflected light. colonies that took up the dye and appeared red to black had the high ability to produce the slime layer while colonies that looked pale red were considered to have moderate to low slim production.

Detection of protease and lipase formation:

Detection of bacterial ability to produce protease was done according to (13). Overnight culture was speared on 2% w/v agar agar (Himedia , India) containing 10% skimmed milk (Himedia , India) incubated at 37Ċ for 24 hr. Determination of protease activity for each strain in broth culture filtrate it was done according to (14).

Bacterial ability to produce lipase was detected as the method (15), by streaking loop full overnight growth on Tween 20 agar and recognizing lysis around bacterial colonies. Antibiotic susceptibility test was done by Disc diffusion method according to (Atlas et al., 1995)

Cytotoxic effect of bacterial supernatant

For the detection of the cytotoxic effect of bacterial supernatant on eukaryotic cell it was used HepG2 cell line was used (Al- Nahrain research center). Cell culture was prepared as described by (16). Hep G2 cell line was grown in minimal essential medium (MEM) solution (Invitrogen, Lofer, Austria) supplemented with 10% fetal bovine serum, 100µg /ml penicillin and 100µg/ml streptomycin. Culture was incubated with 5%Co2 and 95%humidity at 37Ċ. Centrifugation and preparing a suitable dilution after counting cell viability by trypan blue. 1×10^4 cells were cultured in 100µl of medium in each well of the 96 flat bottom tissue plates. Plates were incubated for 48hr at 37°C in an incubator supplemented with (5%) CO2 until formation confluentmonolayer on the internal surface of microtiter plate.culture media were removed and monolayer of Hep G2 exposed to cell free filtrate of bacterial supernatent and its dilutions, PBS was used as substituted in control. Cytotoxic changes were

recorded and cell viability was measured by MTT assy spectrophotometrically. Cytotoxic change of HepG2 treated with cell free filtrate were recorded at timely intervals of incubation. Each experiment was measured in triplicate and compared with control.

Statistical analysis Statistical analysis was performed by one-way ANOVA at significance level $p<0.05$, when significant differences were detected, Values are expressed as means ± Standard Error (SE).

Results and discussion

From sixty two samples isolated from different body site of fresh water fish from local markets in Baghdad 23(37%) isolates were identified as *Aeromonas.hydrophila* grown on aeromonas isolation agar (Himedia, India). Colonies appered round small to medium in size, convex, transparent to Pale on MaConky agar because it was non lactose fermenter. Microscopical examination showed gram negative bacilli motile, non-spore former. Biochemical reactions results showed in a Table1.

Table 1 Biochemical reactions of *A.hydrophila* identification.

Gram reaction	-
Motility	+
Catalase, oxidase	+
Lactose utilization	-
TSI	19(82.6%)K/Ag 4(17.6) K/A
Nitrate reduction	+
citrate utilization	+
Indole production	+
Urease test	-
Lipase	+
protease test	+
DNase	+

(K/Ag alkaline bottom and alkaline slant, K/A acid bottom and alkaline slant).

Hemolytic activity of *A.hydrophila* was examined on blood agar. 12(52%) were showed to be B-hemolytic activity, 9(39%) showed

partial hemolytic and 2(8.69%) were λ hemolysis. There were slight differences when comparing this result with previous studies (12) which referred that all isolates which was isolated from diseased fish was beta hemolytic. Our result was in a compatibility with (1) which showed variation in hemolytic capacity in different strains.

The ability of bacteria to uptake Congo red and appeared with dark color when it was cultured on Congo red agar used as indicator for slim layer production as a virulence factor.

All isolates of *A.hydtophila* obtained from this study showed variation in slim layer production around dark color colonies this in turn referred to different ability in disease effectivity. 10 (43.4%) isolates were high slim layer producer (+++), 10(43.4%) moderate (++) and3 (13.1%) isolates were weak (+). this mean there was variation in ability of pathogenesis as shown in previous studies (11). The slim layer production highly attributed to the pathogenicity, it appears to inhibit PMN, chemotaxis, phagocytosis and antimicrobial drugs (18). Another study showed that all isolates obtained from clinical cases were having the ability to produce slim viscous material around colonies when it was grown on Congo red agar. (1).

Detection of protease activity was done qualitatively on agar containing casein and determining specific activity by using cell free filtrate as a crude enzyme.

Five isolates from each type of α &β hemolytic and two of λ-hemolytic *A.hydrophila* were enrolled in this test. Protease activity measurement was done according to the method of (3) showed in a table-2 .

Table (2) Hemolytic and protease activity of different strains of *A.hydrophyla* isolated from fish

B-hemolytic *A.hydrophyla*		**α-hemolytic *A.hydrophyla***		**λ-hemolytic *A.hydrophyla***	
strain	Protease activity µ/ml	strain	Protease activity µ/ml	strain	Protease activity µ/ml
A6	100.2	A5	106.3	A1	90.2
A4	122.4	A9	124.1	A7	92.3
A11	145.8	A15	130.4		
A13	131	A17	125		
A22	140.3	A20	98.3		

Protease is considered as a virulence factor and it's related to *A.hydrophila* pathogenicity. Our study showed all isolates produce protease and there was variation between strains in protease production clarified on casein agar represented in clear zone around *A.hydrophila* colonies of different strains. The highest value of protease activity was beyond *A.hydrophila* A11 (145.8) while the lowest was beyond A1 (90.2), so the most effective one was A11 in protease production. Previous studies (1) showed there were differences in a number, type and quantities of proteases depending on *A. hydrophyla* strain variation, and protease activity affected by incubation temperature & culture media.

Antibiotic susceptibility was performed for twelve isolates of *A.hydrophila* which were enrolled for protease activity measurement to the most common used antibiotics in Baghdad Table (3).

Table (3) Antibiotics susceptibility of different strains of *A.hydrophila*

Antibiotic	Bacterial strains of *A.hydrophila*												%R
	A1	A7	A5	A9	A15	A17	A20	A6	A4	A11	A13	A22	
Ampicillin (10µg)	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	100
Amoxicillin 30µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	100
Cefotaxime 30µg	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	100
Imipenem u	S	S	S	S	S	S	S	S	S	**R**	S	S	8.3
Azithromycin 30µ	S	S	S	S	S	S	S	S	S	S	S	S	0
Chloramphenicol 25µ	S	S	**R**	**R**	S	S	S	S	S	S	S	R	25
Trimethoprim 5µ	S	**R**	S	S	S	S	**R**	**R**	S	**R**	S	S	33.3
Ciprofloxacin 15µ	S	S	S	**R**	S	**R**	**R**	S	S	**R**	S	S	33.3
Gentamicin10µ	S	S	S	S	**R**	S	S	**R**	**R**	**R**	S	S	33.3
Amikacin 30µg	S	S	S	S	S	S	S	S	S	S	S	S	0

Our study reported that all isolates enrolled for antibiotic susceptibility test were resistant to Ampicillin, Amoxicillin, Cefotaxime. All *hydrophila* strains were sensitive with a ratio of (100%) to Azithromycin and Amikacin while it ranged between sensitive and resistant to other antibiotics under study. *A.hydrophila* . Antibiotic resistance was increased around the world as indicated (11), and clinical isolates were more resistance than strains which were isolated from a water environment (8).Antibiotic resistance genes located chromosomally or on plasmid and this resistance could be transferred from other bacteria.

Cytotoxic effect was measured for *A.hydrophila* strains (A6, A4, A11, A13, A22, A5, A9, A15,A17,A20,A1and A7). Cytotoxic effect was detected by all strains under study with the variation depending on strain. Cell shrinkage, detachment and rounding were observed in HepG2 as a cytotoxicity change. Vacuolation effect on Hep G2 cell line with 6hr after supernatant addition, complete cell death was observed with in 72hr except strain which have inhibition rate 98±4.22.

Table (4) Inhibition rate of bacterial cell free filtrate on Hep G2 cell line. Values represent average ±SE (%)

Bacterial strain	Viability inhibition rate at6h	Viability inhibition rate% after 72h	Bacterial strain	Viability inhibition rate after 6h	Viability inhibition rate after 72h
A6	72± 4.11	98±4.22	A9	98±2.55	100
A4	85±0.11	100	A15	95±4.35	100
A11	97±5.12	100	A17	96±2.22	100
A13	97±2.22	100	A20	95±5.42	100
A22	95±3.21	100	A1	92±3.24	100
A5	92±3.35	100	A7	94±5.33	100

Free cell filtrate of *A.hydrophyila* contains many extracellular products which have a pathogenic effect on eukaryotic cells, a variety of virulence factors, including cytotoxic and cytopathic enterotoxins, aerolysins, , proteases, haemagglutinins and lipases (1), and are invasive to cultured cell lines (20). Previous studies refer to this cytotoxicity due to aerolysin which is a pore-forming toxin secreted by human pathogen *A. hydrophila* that causes vacuolation in the cytoplasm of baby hamster kidney cells (19)and capable of producing a vacuolation effect in HeLa cells. Our study agree with (7) which reported that all isolates of *A. hydrophila* have cytotoxic effect started with 5 hr after treatment with cell free supernatant and with (12) which indicated cytotoxic effect of *A.hydrophila* on vero cells line.

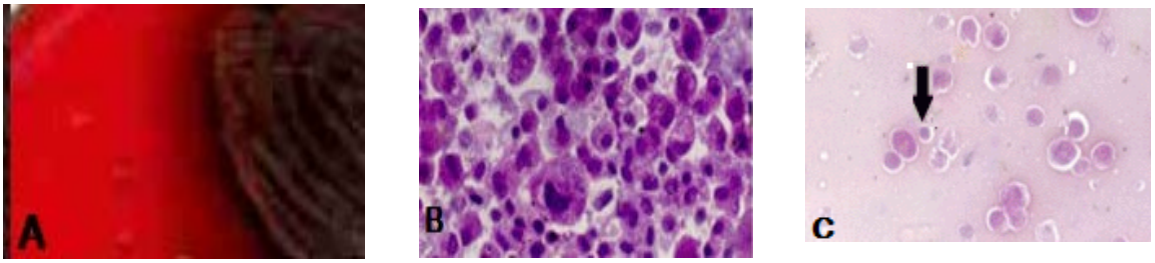

Figure 1 :A- *A.hydrophila* on congo red agar B- Hep G2 cell line control C- cytotoxic effect of free cell supernatant on Hep G2 (100x)

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Histopathological and Functional Changes in Kidney of male Rabbits infected with *Pseudomonas aeruginosa* DNA

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Abstract: This study was designed to clarify the histopathological and functional changes in kidney caused by bacterial DNA released upon bacterial autolysis or killed by antibiotic.12 males of rabbits were divided into two groups, each group included 6 rabbits. First group[G1]was considered as control injected with phosphate buffered saline[PBS],second group[G2]was injected into the urinary bladder via inoculums[50µl] were injected by the aid of catheter[0.6mm in diameter] both groups feed *ad Libitum* with conventional diet. The main histopathological changes in kidneys were infiltrated inflammatory cells, macrophages and neutrophils adjacent blood vessels, damage of glomerular and massive necrosis in the tubules. That resulted showed changes in kidney function such us significant increase ($P<0.05$) in plasma levels of creatinine and urea.

Key words: Histopathology, kidney, *pseudomonas aeruginosa* DNA, Inflammation.

التغايرات الامراضية النسجية والوظيفية في كلية ذكور الارانب المصابة بدنا بكتريا *Pseudomonas aeruginosa*

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الخلاصة: صممت هذه الدراسة لتفسير التغييراتالامراضية النسجيه والوظيفية في كلية ذكور الارانب بسبب الاصابة بالدنا البكتيري المنطلق من تحلل البكتريا الذاتي او المقتولة بواسطة المضادات الحيوية ، حيث استخدمت 12 من ذكور الارانب قسمت الى مجموعتين كل مجموعة ضمت 6 ارانب. المجموعة الاولى (G1) مجموعة السيطرة حقنت بمحلول فسيولوجي (دارئ الفوسفات) والمجموعة الثانية (G2) حقنت داخل المثانة البولية عن طرق الاحليل الذكري ب (50µl) من دنا بكتريا *Pseudomonas aeruginosa* بوساطة انبوب حقن بقطر(0.6mm) وكلا المجموعتين اعطيت الغذاء بشكل حر ومستمر . تضمنت التغايرات المرضية لنسيج الكلية ارتشاح الخلايا الالتهابية وخلايا البلعم الكبيرة والخلايا العدلة قرب الاوعية الدموية وحصول تلف في الكبيبات وتنخر كبير في الانابيب البولبة وكذلك لوحظ تغيير في وظائف الكلية بزيادة معنوية ($P<0.05$) في مستوى الكرياتنين واليوريا في بلازما الدم.

Introduction

Humans have the majority two of kidneys one on either side of the abdomen, kidneys clear toxins from blood. Urea is the most important part of the waste products that are taken out by the kidney ,the kidney also regulate acid concentrations ,as well as maintaining water balance in the body by excreting urine-water is mixed with urea to produce urine[1]

Bacteria or viral infections often induce various histopathogical changes, immune and autoimmune syndromes, infections of kidney can trigger the onset and exacerbation of immune complex glomerulonephritis [2-3].

Bacterial DNA causes potent immune responses stimulation the first report on the immune stimulatory properties of bacterial DNA date back to Tokunaga and colleagues in 1984 due to the presence of un methylated CPG di nucleotides in a particular base sequence context termed' CPG motif' importantly, the immunostimulatory activity is found in bacteria, yest , viruses, insect and nematode DNA[4].

Microbial DNA liberated at the site of infection converts immature antigen-presenting cells [APCs] to mature.

Professional APCs, it triggered B cells proliferation, differentiation, resistance to apoptosis, and release of cytokines .They induce natural killer cell activity and proinflammatory cytokines release from mononuclear cells [5] and effector molecules such as nitric oxide [6]. Furthermore, bacterial DNA leads to septic shock and death in sensitive animals [7]. DNA of periodontal pathogens, prophyromonas gingivalis and tannerella forsythia, stimulates cytokine production in human monocytic cells through Toll like receptor 9[TRL-9] and nuclear factor kappa B signaling [8].

The present study aimed to elucidate the histopathological and functional changes in kidneys of male Rabbits caused by bacterial DNA released upon bacterial autolysis or killed by antibiotic, hence, many inflammatogenic reactions will be established leading to serious tissue damage.

Materials and Methods

Isolation and identification

Pseudomonas aeruginosa was isolated from sputum of 3 years old child suffering from cystic fibrosis, streaked on MacConkey agar plates and citramide agar [all media were

purchased from Himedia India] incubated at 37ºC for 24h. Thereafter, the grown colonies were identified according to Holt *et al*[9] and Bernere and Farmer [10].Biochemical tests were carried out according to Forbes et al.[11].API-Staph system was employed to confirm the identification.

Bacterial DNA extraction and purification

DNA was extracted from *P .aeruginosa* following the procedure applied by Harely and Prescott[12].

An overnight tryptic soy broth culture of *P .aeruginosa* was obtained .One ml of this culture was transferred into a microcentrifuge tube which was spin for 10 seconds.The supernatant was removed carefully and 600μ l of cells lysate solution [tris-EDTA-SDS]were added , gently pipet up and down to resuspend the bacterial pellet and incubated at 80ºC for 5minutes. Thereafter the sample was slowly cooled at room temperature and 3ml of RNase solution were added , mixed 25 times by inverting the microcentrifuge tube at 37ºC for 30 minutes, and cooled to room temperature. 200ml of protein precipitation solution [ammonium acetate] were added, vortexed very gently for 20seconds. The sample was microcentrifuged for 3minutes at 14000 rpm pellet the protein, supernatant was poured into a clean tube. Then, 600 µl of 100 % isopropanol was added, the tube was capped, mixes very gently by inverting the tube at least 50 times, centrifuged at 14000 rpm for 1 minute to pellet the DNA. The supernatant was poured off and the liquid was drain onto an absorbent towel .600 µl of 70 % ethanol was added and the tube was inverted several times, subsequently, the 70 % ethanol was decanted and 600µl of absolute ethanol was added and the tube was inverted several times, centrifuged at 14000 rpm for 1 minute then the supernatant was poured off very slowly. The DNA pellet was air dried for at least 15 minutes. Then 100µl of the hydration solution [tris-EDTA] were added in a water bath at 65ºC for 1 hour.

Animals

Sexually mature local rabbits (n=12) of an average body weight 1650+150 g and 14-15 months old. Animals were kept in the department of Biology, college of Science, University of Baghdad under the laboratory conditions [12 h light; 12 h dark] photoperiod with controlled room temperature 25-28ºC good ventilation and were feeding and tap water *ad l*ibitum.

Experimental design

The rabbits were randomly divided into two groups each group were kept into four steel box cages [three rabbits per cage].The bladder was emptied from urine by pressing on abdominal area .Urethra and surrounding area were sterilized with 70 % ethanol then apoly ethylene [13] .The control group [G1] was given normal physiological saline [0.9/ NaCl] injected intraurethrally.

The second group [G2] treated with 20ml of 10µg /ml bacterial DNA was injected directly intraurethraly by aid of 0.6mm in diameter catheter to events injury pathological changes.

Histological preparation

Histological examination was done by fixing 1cm +1cm of the rabbit kidney in 10% formaline saline and embedded in paraffin wax .Tissues block were sectioned 5 μ m thick and stained with haemotoxylin , eosin and PAS reaction (periodic acid-Schiff) [14].

Estimation of kidney Function

At the end of the experiment, blood samples were taken by cardiac puncture and blood was collected in clean EDTA tubes .Then plasma was collected by centrifugation (3000rpm) for 15 minutes and stored at -20ºC. kits of creatinine and urea were purchased from Jab kit company J.T Barcelona[spain] kits Creatinine was determined by kinetic method described by [15], determination of urea was done according to the enzymatic method [16].

Statistical analysis

Data wear presented as a mean ± standard deviation (SD) [17]. T test was employed for means comparison $P \leq 0.05$ is considered significant [18].

Results and discussion

Concentration and Purity of DNA

A spectrophotometer was used to determine the absorbance of DNA solutions with the use of distilled water as blank.

The absorbance of *P. aeruginosa* DNA at 260nm and 280nm were 0.334 and 0.181, respectively. Accordingly, the concentration of DNA was 167 µg/ml and the purity ($OD_{260/280}$ ratio) was 1.84. As a consequence, the extracted DNA was considered pure given that it was within the expected range of 1.7 to 2 [25].

Histopathological of kidney

Histological study showed a typical structure organization of the kidney in the untreated rabbits

(Figure-1).While the kidneys of rabbits treated with *P.aeruginosa.*

DNA showed several types of kidneys damage, such as infiltrated of flammatory cells ,macrophages and neutrophils around blood vessels and massive necrosis in the tubules (Figure-2). Also showed especially neutrophils in areolar connective tissue around swelling blood vessels and necrotic change in the adjacent tubules [Figure-3] In agreement with previous study [19] reported that when the microbe degraded or autolysed for any reason such as killing by antibiotic or processing by the antigen presenting cell DNA will released, hence, it will evoke the inflammatogenic reaction causing serious problems. The infiltrated of the macrophage in the kidneys treated with DNA due to the worsening of glomerular damage was associated with marked macrophages infiltration [20-21]. Macrophages activity and B cell function contribute to pathogenesis of renal disease [22-23].

CPG-DNA can aggravated immune complex GN via multiple pathways including the increased chemokine and chemokine receptors expression by macrophages infiltration [24]. Observed infiltration of neutrophils around blood vessels and massive necrosis in the tubules and vacuolation due to the bacterial DNA invade the superficial umbrella cells and replicate to high levels, forming intercellular bio- films a process that induce TLR4 mediated cytokine response that recruits neutrophils to site of infection [25-26].observed the accumulation of mononuclear [flammatory cells] in glomerular capillaries and adjacent tubules [Figure-4].

If glomerular injury is severe enough to damage the glomerular accumulation of, monocytes and macrophages, may form as a result of an inflammatory reaction to immune complex formed to non-glomerular antigen-antibody reaction to intrinsic glomerular antigens, as in anti-glomerular basement membrane disease [27-28].

An inflammation effects of DNA may result from the production of cytokines and chemokines [29] .Recognition of microbial DNA by TLR9 in mammalian phagocytic cells triggers an immune stimulatory cascade that culminates in the maturation ,differentiation and /or proliferation of multiple cell types ,together these cells secrete cytokines and chemokines that create pro inflammatory immune stimuli and other mediators of inflammation in addition to reactive oxygen species generation [30,31].thus ,this cells population soluble products are

crucial to the observed inflammation response to DNA[32].

Kidney function

Investigation of the blood collected from the male rabbits injected *P. aeruginosa* DNA results in elevation of plasma creatinine and urea levels compared with the control group[G1] .The results revealed a significant (P<0.05)increase in serum creatinine and urea levels of group exposure to of group [G2] compared with control group[Table-1]. Increased serum creatinine and urea levels refers to an inflammation of the glomerulus , which is the unit involved infiltration in the kidney this inflammation typically results in one or both of the nephritic or nephritic syndromes also known glomerulonephritis [33,34] Bacteria or viral infections often induce flares of various histopathological changes ,immune and autoimmune syndromes in kidney infection can trigger the onset and exacerbation of immune complex glomerulonephritis (GN) [33-34].

Renal bacterial infections also trigger activity that may induce progressive loss of renal function up to end –stage renal disease [33-35]

Table -1-: Effect of *Pseudomonas aeruginosa* DNA on kidney functions

Group	Urea mg/dl	Creatinine mg/dl
G1	16 ± 2.72	0.6 ± 0.23
G2	*31 ±3.24	*2.2 ± 0.65

Significant deference P<0.05 *

Acknowledgment

Author would like to offer special thanks to Prof. Dr. Harith Jabbar Fahad Al-Mathkhury Department of Biology, college of Sciences, University of Baghdad for preparation of *Pseudomonas aeruginosa* DNA.

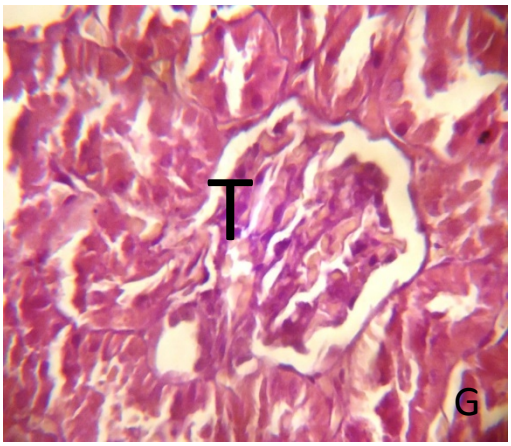

Figure 1: A corss section in kidney of control rabbit showing normal glomerulus (G) and uriniferous tubules (T). H&E. 40X.

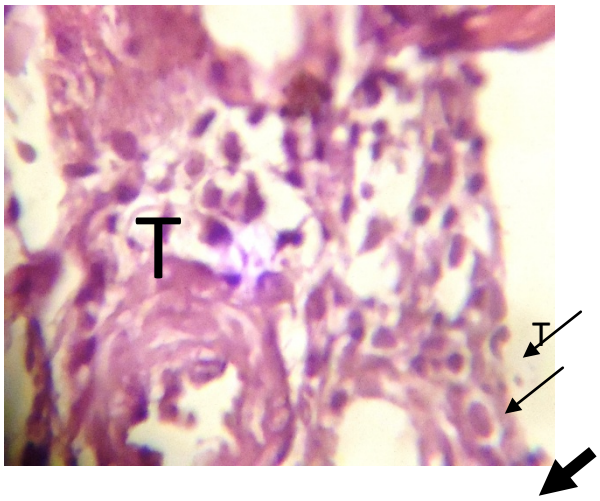

Figure 2: A corss section in kidney of rabbit treated with *P. aeruginosa* DNA showing infiltration of inflammatory cells; neutrophils (thick arrow), macrophages (thin arrow), adjacent blood vessel (BV) and massive necrosis in the tubules (T). H&E.40X.

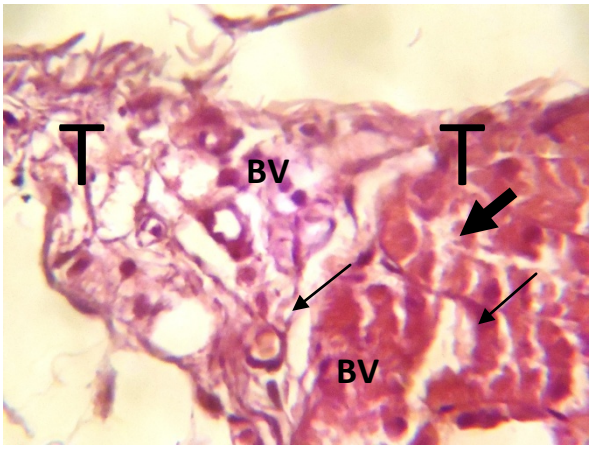

Figure 3: A corss section in kidney of rabbit treated with *P. aeruginosa* DNA showing light infiltration of inflammatory cells especially neutrophils (thick arrow) in areolar connective tissue around swelling blood vessel (BV) and necrotic changes (thin arrow) in the adjacent tubules (T). H&E.40X.

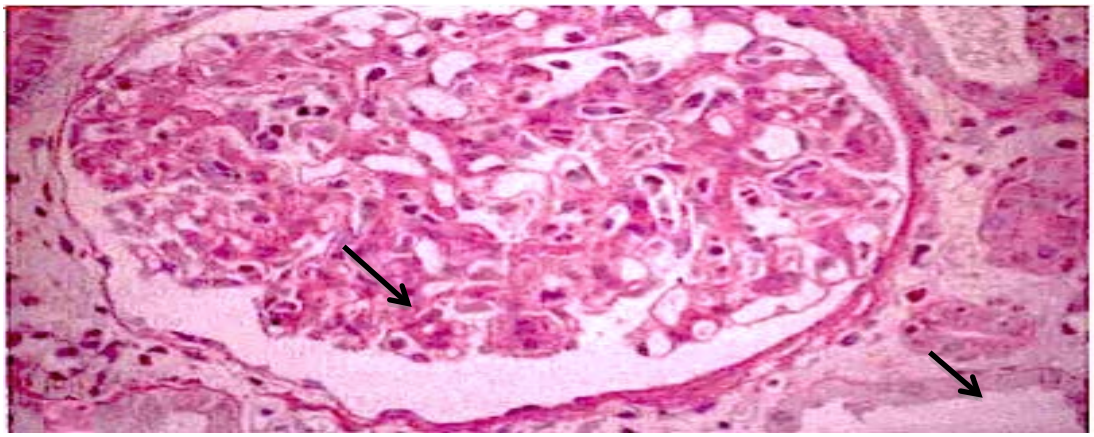

Figure 4: A cross section in kidney of rabbit treated with P.aeruginosa DNA showing accumulation of mononuctear in flammatory cells in glomerular capillaries (glomerulitis) and adjacent---- tubulesPAS 80X.

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Inhibitory activity of colicin crude exract against different isolates of Enterobacteriacea in both planktonic and biofilm state

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Abstract: Colicins are antibacterial proteins produced by bacteria, which can kill bacterial strains closely related to a produced species, in order to reduce environmental competitors for acquiring nutrients and living space. In vitro experiment was carried out on the antibacterial activity of colicin crude extract against different isolates *(E.coli,Klebsiella pneumoniea,Proteus mirabilis and Salmonella typhi)* using well assay method for planktonoc cells. Biofilm killing assay was also used against biofilm embedded cells by using microtiter plate. The results showed that colicin extract was active against these isolates when found as planktonic and biofilm state.The higher effect of colicin crude extract was observed against *E.coli* and *S.typhi* biofilm in compare with other isolates and there was significant effect of colicin extract against all isolates.

Key words: *E.coli*,Colicin, Biofilm

التأثير المثبط لمستخلص الكولسين الخام ضد عزلات مختلفه من العائله المعويه في حالة وجودها بشكل عالق وبشكل غشاء حيوي

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الخلاصة: الكولسينات هي بروتينات مضادة للجراثيم تنتج بواسطة البكتيريا، والتي يمكن أن تقتل سلالات بكتيرية مرتبطة ارتباطا وثيقا بالانواع المنتجة، من أجل الحد من التنافس البيئي للحصول على المغذيات ومكان العيش. التجربة اجريت على النشاط المضاد للبكتيريا لمستخلص الكولسين الخام ضد العزلات المختلفة *Escherichia coli,Klebsiella pneumoniea,Proteus mirabilis and Salmonella typhi)*, باستخدام طريقة الحفر بالنسبه للعالق البكتيري .طريقة القتل للغشاء الحيوي ايضا اجريت باستخدام الشريحه العياريه ذات الحفر. النتيجة التي تم الحصول عليها أظهرت أن مستخلص الكوليسين الخام كان فعالا ضد هذه العزلات عندما وجدت بشكل عالق وبشكل غشاء حيوي .ان اعلى تاثير للكولسين الخام تم ملاحظته ضد الغشاء الحيوي لعزلات *(E.coli and S.typhi)* مقارنة بالعزلات الاخرى وان هناك تاثير معنوي لمستخلص الكولسين الخام تجاه جميع العزلات.

Introduction

The colicins are protein compounds produced by and active against, *Escherichia coli* and others members of Enterobacteriaceae family. At least 34 different colicins have been described and found to share an interesting number of features (1). Colicins, a class of antimicrobial compounds produced by bacteria, are thought to be important mediators of intra- and interspecific interactions, and are a significant factor in maintaining microbial diversity (2). Colicins are plasmid-encoded 3-domain antibacterial proteins that are produced during times of stress and active against *Escherichia coli* and closely related bacteria (3). Colicins kill sensitive bacteria in three main and distinct ways .The most frequent mechanism is the formation of ion channels in the plasma membrane (pore formation), resulting in membrane depolarization (4,5). The opening of the pore also induces a phosphate and sometimes K+ efflux, which leads to depletion of cytoplasmic ATP (6).

Less frequent is the nuclease activity of colicins, which can be directed against the chromosomal DNA (acting as a nonspecific DNA endonuclease) or a specific endonuclease against 16S-rRNA. The least frequent is degradation, catalyzing the hydrolisis of the b-1, 4 bond between N-acetyl glucosamine and N-acetylmuramic acid in the glycan backbone of the bacterial cell wall or inhibition of synthesis of wall peptidoglycan or murein inducing the formation of spheroplasts and consequently, cell lysis (7,5).

Biofilm is a microbially derived sessile community characterized by cells that are attached to an abiotic or living surface and embedded in a matrix of extracellular polymeric substances that they have produced. This polymicrobic community has an altered phenotype and it is physiologically different from planktonic microorganisms. Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming (planktonic) state (8, 9, 10). Centres for Disease Control and Prevention estimate that 65% of human bacterial infections involve biofilms. It has been demonstrated that the antimicrobial agents required to kill bacteria in its biofilm embedded state is 10- 1000 times more concentration than the amount necessary to kill the same amount of free-swimming bacteria cells (11, 12,13). The aim of this project was to test the antibacterial activity of colicin crude extract against different *spp*.from Enterobacteriaceae that grow as planktonic and biofilm state and to compare the efficiency in which these colicins kill cells that grow on

solid media, and solid surfaces (biofilms).

Material and Methods

Specimen's collections

One hundred and five specimens included: urine (50), stool (25), Blood (10) ,ear swab (10) and sewage (10), were collected in sterilized containers from four hospitals in Baghdad including Al-Imam Ali hospital, Ibn Al-baladi Hospital, Fatema Al-Zahraa Hospital and Al-sader Hospital.

Isolation and identification

In the laboratory within aseptic conditions, the collected specimens were streaked directly on MacConkey agar and EMB agar (Himedia/India) and incubated for 24h at 37ºC. Pink colonies were picked and recultured on another MacConkey and EMB agar. Further identification tests included the morphological characteristics and biochemical tests were carried out depending on (14).Finally API E20 system was done.

Detection of colicinogenic isolates by Cup assay method:

Method reported by (15) was followed for screening colinogenic isolates. Loopful from an overnight Luria bertani (LB) broth culture of each producer isolates is heavily streaked on Brain heart infusion agar and incubated at 37C for 18 hr. Wells were made by using cork borer 8 mm diameter. Suspension of indicator isolate were spread on the surface of Muller hinton agar then left to dry at 37 C for 10 min,and for each isolate plates were used (Diplicate). Discs removed from agar medium were sticked gently on the surface of the medium spread by the sensitive isolate ,then incubated at 37 C for overnight. Sensitivity was detected by measuring the zone of inhibition; the sensitive strain was taken from Al-Mustansiriya university-College of science, while other indicator isolates such as *Klebsiella pneumoniea* and *Proteus mibabilis* was isolates from urine samples, *Salmonella typhi* was isolated from stool samples from patients in different Baghdad hospitals. All these isolates were identified by using different biochemical test.

Extraction of crude non-bound colicin

Method described by (16)was followed to achieve colicin crude extract.The overnight culture of bacterial isolates in volume 2.5 ml of LB broth was used to inoculate100 ml of sterile LB broth supplemented with 5 % glycerol and in shaker incubater.At cell density of about $3x10^8$ (14hr incubation of late log phase), Mitomycin C was added at concentration of 2 µg / ml,incubation continued with shaking for another 3 hr.The culture

was centrifuged at 5000Xg for 30 min in cooling centrifuge.The supernatant was taken for assay of colicin activity and protein determination. For estimation of protein concentration in colicin crude extract, Bradford method (17) was used.

Detection of colicin activity by well assay

The quantitative determination of colicin in bacterial culture was performed by using the Wells Assay that described by (18)**.**Two fold dilution of crude extract was prepared (1/2, 1/4, 1/8). The indicator strain was cultured on Muller Hinton agar after comparsion with Macferland tube ($1x10^8$), wells were made in the plate by cork borer, different dilution from the crude colicin was added in these wells..The clear zone was seen after overnight incubation**.**

Invitro inhibitory activity of colicin extract on planktonic

Two fold dilutions for crude extract of colicin was used. All supernatants were cultured on Brain heart infusion agar in order to confirm the absence of E.coli cells. Thereafter, they were stored at 4°C until the assay. Well diffusion method described by (18) was followed to detect colicin extract inhibitory effect*.The MIC of colicin crude extract was detected by this assay.

Biofilm assay

Method described by(19) was followed to achieve biofilm formation:

To study the ability of adherence, Five non-producing *E.coli* isolates, 7 isolates of *Klebsiella pneumonia, 7* isolates of *Proteus mirabilis and 2 isolates of salmonella typhi* were grown in trypticase soy broth (TSB) containing 1% glucose in 96-well polystyrene tissue culture plates and incubated at 37◦C for 24 h under aerobic conditions. After incubation, the planktonic cells were washed ten times with deionized water, and the adhering bacterial cells in each well were fixed with 200 µl of absolute methanol for 20 mints. The plates were emptied and left to dry overnight. The adhering cells were stained with 200 µl of 0.1% crystal violet for 15min, and excess stain was rinsed off. The plates were washed with distilled water and air-dried overnight. The crystal violate dye bound to the adherent cells was dissolved with 1ml of 95% ethanol per well, and the plates were read at 490nm using a spectrophotometer. The experiment was performed in triplicates, and the absorbance of wells containing sterile TSB was used as the negative control the result calculate as in table (1).

Table(1): Classification of bacterial adherence by tissue culture plate method (19)

OD values	Adherence	Biofilm formation
< OD c	Non	Non
OD < OD ≤ 2*ODc	Weakly	Weak
2*ODc < ODt≤ 2*ODc	Moderately	Moderate
4ODc<OD t	Strong	High

E. coli. K .pneumoniea, P. mirabilis and S. typhi

After incubation, all wells were washed with D.W for the elimination of unattached cells. Afterward, 200µl of 0.1% crystal violet was added to each well, shaking the plates three times to help the colorant to get the bottom of the well. After 15 minutes at room temperature, each well was washed with 200 µl D.W. This process was repeated three times. The crystal violet bound to the biofilm was extracted later with 200µl of ethyl alcohol, and then absorbance was determined at 490 nm in an ELISA reader (Beckman coulter, Austria). Controls were performed with crystal violet binding to the wells exposed only to the culture medium with bacteria.

Inhibitory effect of crude extract of colicin on biofilm as treatment.(3).

To detect the inhibitory effect of crude colicin on biofilm, method described by (3) was followed, the isolates of *K. pneumoniae,E.coli,P.mirabilis and Salmonella typhi* were selected to be assayed according to inhibition activity of colicin against planktonic cells of it on plate agar. Same protocol described earlier was followed to produce a biofilm. Then, before the staining step, the previously prepared crude colicin containing media were added to the biofilm containing wells: Subsequently, the tray was incubated for another 24 hours at 37ºC, after incubation period, all wells were washed and stained as the same procedure described above.

Results and Discussion

Isolation and Identification of *E.coli*

From one hundred and 5 specimens, the bacteria Escherichia *coli* formed (55 isolates) were collected from different sources. Isolates were identified by standard microbiological procedures (Gram staining, colonial morphology, catalase test, cytochrome oxidase reaction, motility, biochemical

tests). Results of the present study revealed that the highest percentage of *E.coli* isolates was in urine samples (63.63%) compare to other sources, stool (18%), blood (5.45%), sewage (9.09) and ear swab (3.63%) because *E.coli* is considered the main causative pathogen involved in recurrent UTI in women, which is responsible for approximately 80% of all episodes of infection (20) .Uropathogenic *E. coli* cause 90% of the urinary tract infection. The bacteria colonize from the faeces or perineal region and ascend the urinary tract to the bladder (21). UPEC strains are characterized with specific virulence factors closely related with colonization and persistence of bacteria in the urinary tract. These factors include adhesins or fimbriae, siderophore systems, and toxins (22, 23).

Screening the colicinogenic isolates

Locally isolates *E.coli* were screened in order to select the efficient isolates in colicin production .The ability of these isolates in colicin production was assayed after culturing at 37 C in Brain heart infusion agar, then wells were made on this agar and put on Muller hinton agar that contained the sensitive strain.so the antagonistic effect against the sensitive strain was detected by measuring inhibition zone according to cup assay method (figure 1).

Figure (1):Cup assay method for detection coliciongenic *E .coli*

Results indicated in table (2) showed that 30 isolates (54.54%) from 55 isolates were colicin producers according to inhibition zone against the sensitive strain. Diameters of inhibition zone ranged from 12 to 45 (mm).

Local study by (24) reported that 30.55% isolates were colicin producers while others not, This

study was not agreed with the present study that show(54.54%), while Tishvarian,(25) in another local study showed that 70.83% from *E. coli* isolates were colicinogenic, and this result is higher than the present study .

Table (2):Frequency of colcinogenic isolates depending on diameter of inhibition zone against sensitive strain

Id	*E. coli* isolates	Diameter of inhibition zone	Id	*E. coli* isolates	Diameter of inhibition zone
1	E 1	25 mm	**16**	E 67	45 mm
2	E 2	17 mm	**17**	E 71	15 mm
3	E 12	20 mm	**18**	E 72	15 mm
4	E 13	15 mm	**19**	E 75	42 mm
5	E 20	17 mm	**20**	E 80	15 mm
6	E 27	20 mm	**21**	E 83	12 mm
7	E 51	12 mm	**22**	E 85	15 mm
8	E 52	12 mm	**23**	E 87	33 mm
9	E 54	13 mm	**24**	E 88	35 mm
10	E 55	15 mm	**25**	E 89	20 mm
11	E 57	12 mm	**26**	E 93	15 mm
12	E 58	15 mm	**27**	E 96	15 mm
13	E 62	15 mm	**28**	E 97	15 mm
14	E 64	40 mm	**29**	E 99	17 mm
15	E 66	12 mm	**30**	E103	35 mm

Feldgarden and Rily, (26) demonstrated that most *E. coli* are resistant to most colicins. On average, 93% of the isolates were resistant to any one colicin, and 33% were multiply resistant to all the colicins tested.

Cell free extract of colicin

The free extract was achieved according to (16) by using shaker incubator for 14 hr ,cooling centrifuge and mitomycin C. One of the most efficient ways of producing large amount of colicin is by inducing the bacteriocinogenic strain with a suitable metabolic inhibitor, one of the most effective is mitomycin C (27,28). Numerous studies carried out for induction of different colicins, have demonstrated that increased synthesis of colicin occurs in response to a wide range of DNA damaging physical agents such as ultraviolet (UV), radiation (29); antibacterial agents, such as Mitomycin C (MMC) (16); The phenotypic assay for mitomycin C-inducible bacteriocin production detects virtually all strains bearing a colicin gene (30).In previous study

by (31), addition of 0.25 µg /ml of mitomycin found to increase colicin production. glycerol Glycerol was used as an enhancing agent where (32) and (33) observed that the production of these antagonists was best by using minimal media. The protein concentration was detected by Bradford assay. The result showed that protein concentration was between 250 - 1111 µg /ml.

Invitro inhibitory activity of colicin extract on planktonic cells

To confirm the antibacterial activity of colicin extracted from 6 colicinogenic isolates contain, their MIC were determined against 4 species of Enterobacteriaceae (*E. coli, K. pneumoniae, P. mirabilis* and *Salmonella typhi*).MIC was detected by 2 fold dilution preparation ,Results were showed that different concentration effected on planktonic cell in Petri dish by well assay method (table 3). According to these results , the MIC value against these isolates was selected to study the effect of colicin extract on biofilm formed by different isolates of enterobacteriaceae. The MIC on plate agar was determined and measured depending on the activities of the antimicrobial proteins against planktonic bacteria (34).

Table (3): The minimum inhibitory concentration MIC of colicin extract (µg/ml) against different isolates of Enterobacteriaceae

colicinogenic isolate	MIC (µg/ml)			
	E.coli 95	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Salmonella typhi***
E54	40.58	40.58	No effect	40.58
E57	57.42	57.42	57.42	No effect
E58	No effect	127.11	127.11	127.11
E71	163.43	81.71	81.71	81.71
E75	245.51	245.51	122.57	122.57
E103	62.64	No effect	125.299	31.32

From the table above, it can be noticed that colicin extract from (E54), (E57) and (E58) colicinogenic isolates affected on all other isolates in the same concentration but there was no effect on *P.mirabilis* in case (E54) ,*S.typhi* in case (E57) and *E.coli* in case of (E58). Colicin exract from isolate (E71) affected in *E.coli* isolates in concentration higher than that affected on the other isolates in this study ,isolate (E75) affected on *E.coli* and *K.pneumoniae* in the

same concentration while affected on *P.mirabilis* and *S.typhi* in other concentration .colicin extract from isolate (E103) affected in all test isolates in different concentrations, high concentration affected on *P.mirabilis* while low concentration on *S.typhi* but there was no effect on *K.pneumoniae*. It was found that colcinogenic isolates (E71) and (E75) affected on all isolates. The results revealed that highly effective isolate was (E54) because gave inhibition zone in lower concentration in compared with other colicinogenic isolates.

The crude extract of colicin isolated from producer local isolate showed a wide activity spectrum on other gram negative bacteria in different concentrations (table 3), this was an agreement with (35) and (36) who mentioned that colicin produced by some isolates of *E.coli* are effective against *Vibrio metschni, Pseudomonas ,Nisseria kovii,K. pneumoniea* and some Gram positive bacteria.

The results of present study also showed that colicin extract was effective against *S. typhi* and *P. mirabilis* .Lyon *et al.*,(37) reported that the colicin had the ability of inhibiting the growth of one or more species and/or strains of pathogenic Enterobacteriaceae, as for example, *Shigella* spp. such as *Shigella dysenteriae, Shigella fexneri, Shigella boydii, Shigella sonnei*, and like; *Salmonella* spp. such as *S. typhi, S. typhimurium, S.paratyphi* A, *S. choleraesuis*, and like; *Escherichia* spp. such as *E.coli* strain 0157:H7, *E. afreundii*, and like; and *Enterococcus* spp. This report was in an agreement with present study. colicins H and G were shown to exhibit inhibitory activity against *Salmonella* strains isolated from clinical cases (38).

Colicin type E1 was also shown to exhibit inhibitory activity against *Listeria monocytogenes,* the causative agent of human listeriosis in broth culture and in ready-to-eat (RTE) products (39) while colicins H and G were shown to exhibit inhibitory activity against *Salmonella* strains isolated from clinical cases ,Due to an increasing prevalence of antibiotic resistant *Salmonella* strains, alternative strategies to fight these foodborne pathogens are needed. *E. coli* L1000 appears to be a promising candidate in view of developing biotechnological alternatives to antibiotics against *Salmonella* infections (38). Jordi *et al.*,(41) found that 20 kinds of *E. coli* could express colicin, which inhibited five kinds of Shigatoxin-producing *E. coli* (O26, O111,O128,O145,andO157:H7).
These *E. coli* can cause diarrhea and hemolytic uremic syndrome in humans.

Biofilm formation

Biofilm cultivation in polystyrene microtitre plates was carried out essentially as described by (42) and (19). Briefly, overnight cultures of *E.coli, K.pneumoniae,P.mirabilis* and *S.typhi* strains grown in TSB (0.1 % glucose) medium and with dilution 1:100. The result of the study of the *K.pneumoniae* showed high ability to biofilm formation then followed by *E.coli, P.mirabilis* and *S.typhi* . According to calculation of Atshan *et al* .,(19), this indicated the *K.pneumoniae* and *E.coli* isolates produce strong biofilm while *P.mirabilis* and *S.typhi* produced moderate biofilm .

Table (4): Types of isolates that used for biofilm formation.

	Isolates				
Biofilm formation	***E. coli***	***K. pneumoniea***	***P. mirabilis***	***S. typhi***	**Control**
O.D	0.266	0.3	0.226	0.2	0.06
Classification	Strong	Strong	Moderate	Moderate	

In natural environments, bacteria often form biofilms, microbial communities in which bacteria adhere to an abiotic or biotic surface via surface charges as well as production of pili, fimbriae and exopolysaccharides. Microbial cells in biofilms show distinct properties, particularly resistance to antibiotics, disinfectants, shear stress and the immune system (43).

Inhibitory effect of crude colicin against biofilm as treatment

It was found that crude colicin extract from 6 isolates was affected against all isolates biofilm as treatment, as showed in figure (2). From this figure it can be noticed that biofilm of differrnt isolates was affected by colicin crude extract from colicinogenic isolates,it was found that higher effect for *E.coli* isolate was achieved by colicinogenic isolate (E71) and (54) while colicinogenic isolate (E58) was not affected against planktonic cells in petri dish, so was not used in the experiment against biofilm.

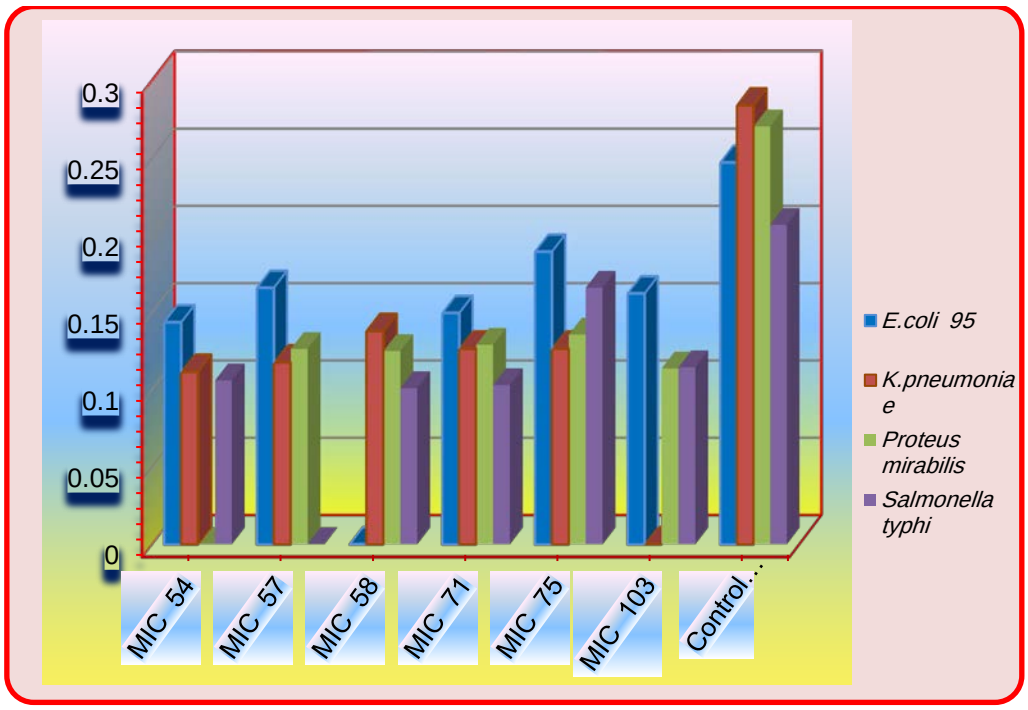

Figure (2): The effect of colicin crude extract on biofilm formation

In addition ,*K.pneumoniae* was affected by crude extract of all colcinogenic isolate in the same proportion and isolate (E103) had no effect in plnktonic state so was not used against biofilm. The result indicated that *P.mirabilis* was affected by colicin extract of all isolates and the isolate (E54) had no effect on planktonic cells so it was not used against *Proteus* biofilm. The result also revealed that *S.typhi* biofilm was affected higher than any of other isolates, and the higher effect was achieved by colcinogenic isolates (E103),(E71) and (E58). Harry and Walker,(3**)** showed that 2 days culture of BW25113 strain grown as biofilms at ambient temperature were resistant to the cytotoxic action of colicin E3 and E9 but sensitive to colE1 with the minimal biofilm eradication concentration However, when this same strain was cultured as biofilms for 3 days at ambient temperature.

Kamenšek *et al.*,(44) reported that regrowth of cultures treated with the subinhibitory concentrations of colicin M indicate an adaptive response to the stress through the activation of the envelope and other stress responses. While neither an increase in colanic acid or biofilm production was detected, in the natural environment, activation of the various above-described stress responses might allow rapid adaptation, including mature biofilm formation, in the event of a sudden decrease/absence in antimicrobial concentrations.

Klebsiella pneumoniae is an important biofilm forming organism responsible for a wide range of infections placing it among the eight most important nosocomial pathogens (45).So using colicin extract as effective antibacterial agent gave acandidate for using it as treatment for infections that caused by this pathogen. There was no information about antibacterial activity of colicin extract against biofilm that formed by different spp of enterobacteriaceae.

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Impact of air pollution on some morphological and physiological characteristics in some evergreen plants in Baghdad city ,Iraq

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Abstract: The present experiment was done to determine the impact of ambient air pollution on some biological factor in *Eucalyptus camaldulensis*, *Albizia lebbeck*, *Conocarpus erectus* plants using one site in Baghdad city. Various morphological and biochemical characteristics of the plants were studied including(number and density of stomata, number of dust particle,total chlorophyll , leaf area and leaf area index , dry weight, fresh weight, number of filaments, percentage of Pb and Cd). Suggesting the activation of protective mechanism in these plant under air pollution stress, and also the plant make physiological adjustments to compensate for that environmental stress. According to our result Albizia tree are more useful to reduce air pollution than the ether plants under study.However, more research is necessary to evaluate the contribution of individual and in combination of air pollution on crop production and its losses.

key words : *Air pollution , Heavy material, LAI ,Ever green trees, total Chlorophyll.*

تأثير تلوث الهواء على بعض الخصائص المظهرية والفسيولوجية في بعض النباتات دائمة الخضرة في مدينة بغداد ،العراق

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المستخلص: أجريت دراسة لمعرفة بعض التأثيرات البيولوجية لتلوث الهواء على *Eucalyptus camaldulensis* و *Albizia lebbeck* و*Conocarpus erectus* في أحدى موقع داخل مدينة بغداد . شملت الدراسة العديد من الصفات المظهرية والبايوكيميائية ومها (عدد وكثافة الثغور ،عدد دقائق الغبار العالقة ، الكلوروفيل الكلي ، المساحة الورقية ودليل المساحة الورقية ،الوزن الرطب والجاف ، عدد الزوائد والشعيرات الورقية فضلا عن نسبة الرصاص والكادميوم في الأوراق). بينت الصفات ان هذا النباتات كانت تحت تأثير التلوث الهوائي الأمر الذي أدى إلى حدوث بعض التغيرات فيها لتتحمل هذا الإجهاد البيئي . استنادا إلى هذه النتائج اتضح ان أشجار Albizi*a* كانت الأفضل في تقليل تلوث الهواء مقارنة بباقي النباتات قيد الدراسة .

أوصت الدراسة على ضرورة إجراء دراسات أخرى لمعرفة تأثيرات تلوث الهواء على إنتاجية المحاصيل الزراعية .

Introduction

Air pollution is one of the severe problems world facing today. It deteriorates ecological condition and can be defined as the fluctuation in any atmospheric constituent from the value that would have existed without human activity (Tripathi and Gautam, 2007). In recent past, air pollutants, responsible for vegetation injury and crop yield losses, and causing increased concern (Joshi and Swami, 2007). It is a major problem arising mainly from industrialization. Pollutants could be classified as either primary or secondary. Pollutants that are pumped into the atmosphere and directly pollute the air are called primary pollutants while those that are formed inthe air when primary pollutants react or interact are known as secondary pollutants (Agbaire, 2009). All combustion release gases and particles into the air. These can include sulphur and nitrogen oxides, carbon monoxide and soot particles, as well as smaller quantities or toxic metals, organic molecules and radioactive isotope (Agbaire and Esiefarienrhe, 2009). Concentrations of most air pollutants in Baghdad City have shown a downward trend in recent years, but they are generally in many instances worse than natural ambient air; thus, all pollutants (except CO) fluctuated between high and below limits certified by Iraqi and international standards. It has been observed that the concentrations of the most pollutants are high in summer in comparison to the other seasons. The peak concentrations of pollutants are linked to traffic density, private generators, and chemical processes in the atmosphere(Rabee,2014). *Eucalyptus camaldulensis* is a tree of the genus *Eucalyptus,*family Myrtaceae. Itis one of around 800 in the genus. It is a plantation species in many parts of the world but is native to Australia and plays an important role in stabilizing river banks, holding the soil and reducing flooding .*E.camaldulensis*is the most widely planted eucalypt worldwide. Great variation is shown within this species and provenance is very important as the tolerances and characteristics vary widely. It can grow as high as 230 feet. Its 4 - 12 inch leaves are dark green and shiny. Its blue-gray bark peels to reveal a cream-colored inner bark (Seyyednejad and Koochak,2011). *Conocarpus erectus*, family: Combretaceae**,** is a low branching evergreen shrub or tree. The species is usually a shrub 1.5 to 4 m in height but can become a tree up to 20 m or more in height. The root system consists mainly of laterals and fine roots that are dark brown, weak and brittle, and have a corky bark. Leaves have slightly winged petioles, 0.5 to 1 cm long, with 2

dot-like glands. Leaf blade edges are un toothed and usually have several dot-like glands near vein angles on the lower surface. The fragrant greenish flowers are tiny, less than 2 mm across, and crowded in ballsless than 6 mm in diameter in terminal and lateral clusters. Flowers are mostly bisexual, but some trees bear heads of male flowers. *Albizia lebbeck* L.(Benth), family, Fabaceace, is a tropical and subtropical tree native to deciduous and sub- deciduous forest of Burma, bangladesh, India and Sri Lanka (Parrotta, 2005). The tree can grow up to 20-30 m in humid tropics. The bark is flaky, pale grey (or yellowish to greenish-violable).The leaves are pinnate with 2-4 pairs of pinnulae having 4-10 pairs of bright green leaflets rounded at both ends. *Albizia lebbeck* tree produces greenish-yellow to white flowers (2.5-7.5 cm in diameter) which are fragrant. The tree produces numerous light grey pods (10-30 cm long;2-5cm wide)ratting in wind. The tree grows poorly on heavy clays, but grows well on fertile, well-drained, loamy soils, in areas that receive from 600-2500 mm of rain per year. However, this species is also capable of tolerating years with as little as300 mm of rainfall. This tree is nitrogen-fixing, and tolerates acidity, alkalinity, heavy and eroded soils, waterlogged soils, and drought. Older trees can survive grass fires and intense night frost, and although these events will kill off above ground growth of young trees, new growth normally follows (DFSC,2000).

The aim of this study is reduces the air pollutions by using Three species of evergreen plants. In order to determine the effects and alterations due to air pollutants in plants, the present study examines the impact of air pollution on *Eucalyptus camaldulensis* , *Conocarpus erectus* and *Albizia lebbeck*

Material and method:

Study area

This study was conducted between Oct.2014 to June. 2015. The area of study is situated in west of Baghdad city (33°17′51″N44°17′26″E) in the main streets of AL-amiriya town. This is designated as polluted site(table1&2).A replicates of the plant sample leaves in regions were taken immediately to the laboratory for analysis. The plants under study were 3-5 years old.

Table 1: Values of temperature and relative humidity and measured during study periods in Baghdad (Humadi ,2014).

Variables	Baghdad							
	Nov.	Dec.	Jan.	Feb.	March	April	May	June
Temp. (c°)	25.0	24.0	22.0	19.0	25.0	33.0	37.0	40.0
RH. %	57.0	57.0	53.0	65.0	51.0	47.0	40.0	20.0

Table 2: Concentration valuesof Pb and Cd measured during study periods in Baghdad (Humadi ,2014).

Variables	Baghdad							
	Nov.	Dec.	Jan.	Feb.	March	April	May	June
Pb_{ppm}	3.0	5.7	0. 2	4.5	3.0	0.4	3.8	6.5
Cd_{ppm}	0.1	ND	ND	0.0	0.3	0.2	0.2	0.0

ND= none detected

Morphological measurements:

Leaf Area Indexes the amount of one-sided leaf area per unit area of ground. (Chen and Black, 1992).

Fresh weight (g):

10 fresh leaves (from each plant) were weighted.

Dry weight (g):

20g of fresh leaves (from each replicates) were dried over night in an oven at 70°C and re weight to obtain the dry weight.

Number of stomata:

Stomata number in each species was determined by using microscope under the objective lenses of 40X and ocular lenses of 10X, terminologies in respect of stomata complex types follow (Abulrahman and Oladele,2003).

Density of stomata:

Stomata density was determined by counting the number of stomata per square millimeter based on the entire leaf surface .stomata index was determined as number of stomata per square millimeter divided by the number of stomata plus number of epidermal cells per square millimeter multiplied by 100 (Abulrahman and Oladele,2003). Sample of 5 replicate were used for each of the parameters.

Number of filaments:

Number of filament in each species were determined by using microscope under the objective lenses of 40 X and ocular lenses of 10 X.

Number of dust particle:

Number of dust particle and it's diameter(0.05-0.19mm) in each species was determined by using microscope under the objective lenses of 40 X and ocular lenses of 10 X.

Biochemical measurements

Chlorophyll percentage

Total chlorophyll in the leaves of selected plant were determined by use of acetone 80% according to the method described by Lichtenthaler (1987).

Leaf is an important plant organ, and is associated with photosynthesis and evapotranspiration; therefore, leaf area measurements are required in most physiological and agronomic studies involving plant growth.

Lead and cadmium:

To determined the concentrations of Pb and Cd, all leaves were oven – dried in the laboratory at 105^{o}C for 24 h. Then these samples were digested with a 3: 2 : 2 mixtures of HNO_3- H_2SO_4 – HCl. The digested solutions were analyzed by atomic absorption spectrophotometer (Perkin-Elmer odel 5000).

Statistical analysis: The experiment was conducted in randomized complete block design. The data were analyzed by using the statistical software mstatc in order to determine the significant percentage under P $\leq$0.05.

Results and Discussion

Leaf area (cm^2)and Leaf area index:

Figure (1) illustrate the result of study, in which higher significant value of leaf area and LAI were (115.5 cm^2 , 45.045) for Albizia leaves, while lower value of leaf area and LAI were (16 cm^2, 4.800) for Conocarpus leaves ,respectively.

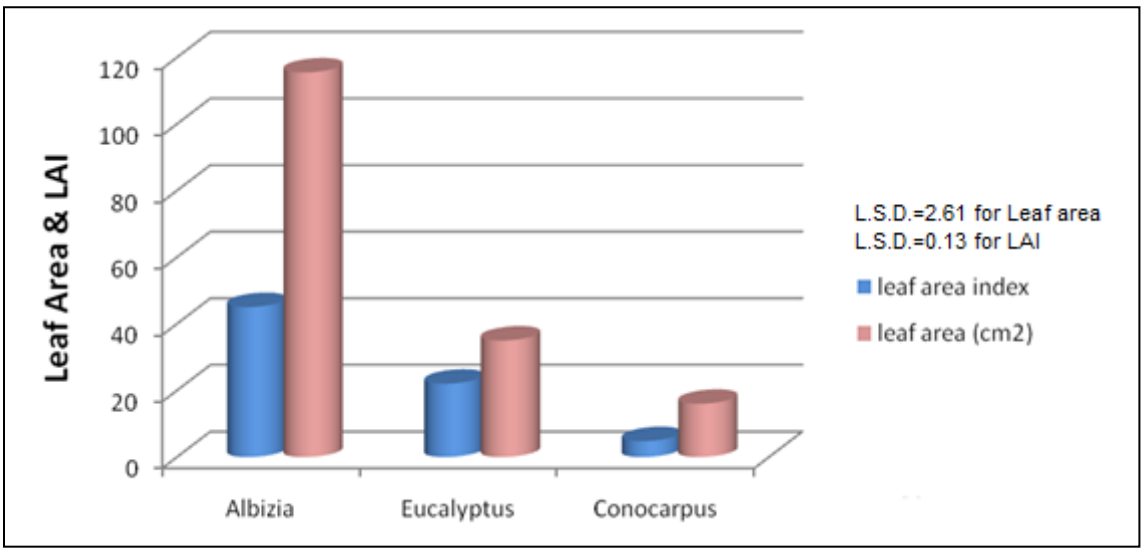


Fig.1:Leaf area and leaf area index values of plant's leaves

Leaf area is one of the most important bio-meteorological variables to be characterized. It is an inventory of the population of leaves that are absorbing light and momentum and are exchange heat, moisture, CO_2 and trace gases with the atmosphere. From a micrometeorological perspective an increase in leaf area index increases light interception and the source/sink strength for heat, water and CO_2 exchange. It can also start a negative feedback loop by increasing drag on wind, decreasing wind velocity that acts to reduce mass and energy exchange (Albertson *et al.*, 2001). Lower wind velocity in the canopy will

also act to accentuate profiles of temperature, humidity and CO_2 in the vegetation, which in turn will have feedbacks with physiological resistances linked to the stomata (Baldocchi,2012).Plants provide an enormous leaf area for impingement, absorption and accumulation of air pollutants to reduce the pollutant level in the air environment (Warren, 1973; Shannigrahi *et al.*, 2004, Chauhan and Joshi , 2010).

2. Leaf Dry and Fresh Weights(g):

The leaves of the trees have an important role in retention of the particulate matters; they are mostly affected when the fresh and dry weight increase. Figure (2) shows the air pollution significant effects on dry and fresh weight of the three plants leaves .The highest dry and fresh weight were (1.66 , 4.19 g) for the Albizia leaves ,while the lowest were (1.44, 3.39 g) for the Eucalyptus leaves , respectively.

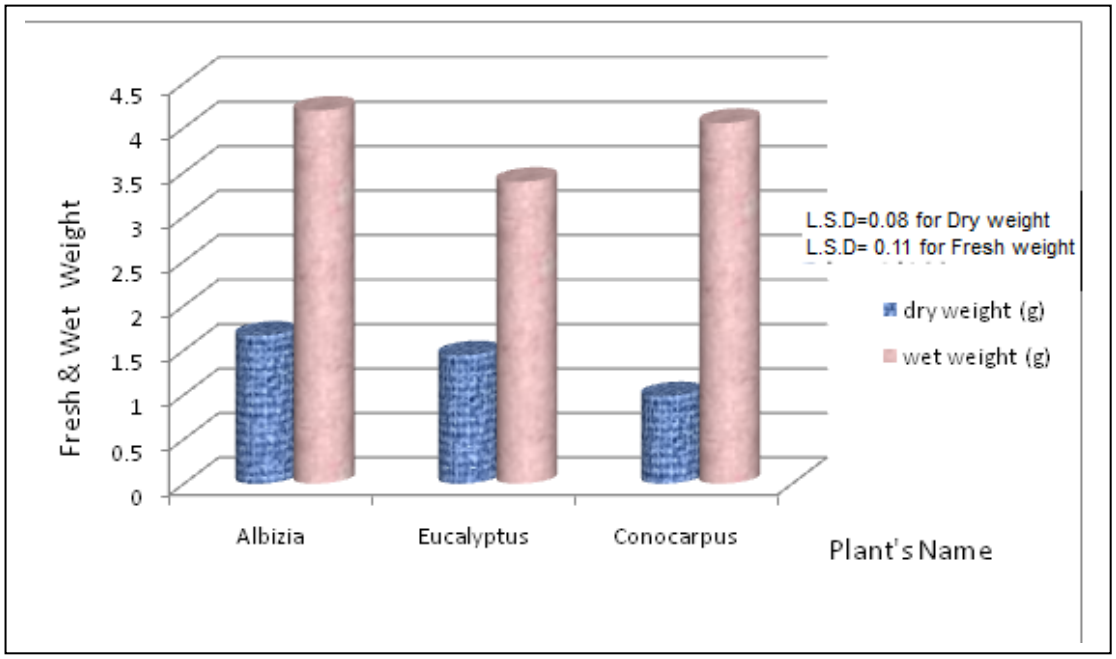


Fig.2: dry & fresh Weight of plant's leaves

Air pollution has become a serious environmental stress to the plants due to increasing industrialization and urbanization during last few decades (Rajput and Agrawal, 2004). The particulates and gaseous pollutants, alone and in combination, can cause serious setbacks to the overall physiology of plants (Ashenden and Williams, 1980; Mejstrik, 1980). Of all dry and fresh plant parts weight , the leaf is the most sensitive part to the air pollutants and several other such external factors (Lalman and Singh, 1990). Reduction in leaves dry and fresh weight may be attributed to the inhibition of chlorophyll formation and damage of leaf tissue which in turn the not photosynthetic grains. Lalman and Singh (1990) concluded that the air pollution fumigated plants undergo several reversible and irreversible physiological and biochemical changes as reflected by foliar injury reduced leaf area and biomass. Decreased photosynthetic activity and poor plant growth apparently reduced the dry matter and yield (Khan and Khan, 1993). Air pollution decreases the total chlorophyll and dry matter production (Mandloi and Dubey, 1988: Katiyar and Dubey, 2000, Chauhan and Joshi 2010).

3. Number and Density of stomata(mm^2):

The leaves of the species studied are hypoamphistomatic *i.e* having more stomata on lower epidermis than on the upper

epidermis figure.(3-a,b,c). All three species possessed large density with stomata number. The data presented table(3)support that the Albizia species was the highest stomatal density and stomatal number (8.5,990) in mm^2,while Conocarpus was the lowest stomatal density and stomatal number (4.8,165) inmm^2 between the three species, respectively.

Table (3-a): Stomata Density and number of stomata in three plants leaves(mm^2) of the Upper surface .

Plants Names	***A. lebbeck***	***E. camaldulensis***	***C. erectus***
Stomata Density	0	5.6	5.0
Number of Stomata	0	198	80
L.S.D for Density=0.12		L.S.D for Number=0.91	

Table (3-b): Stomata Density and number of stomata in three plants leaves(mm^2) of the Lower surface .

Plants Names	***A. lebbeck***	***E. camaldulensis***	***C. erectus***
Stomata Density	8.5	6.2	4.8
Number of Stomata	990	220	165
L.S.D for Density=0.56		L.S.D for Number=13.09	

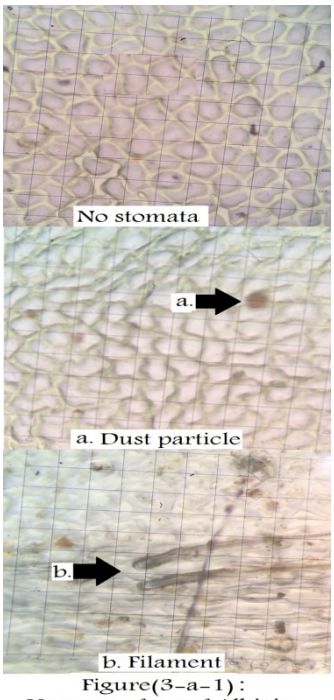
4. Number of Dust Particle(mm^2)

A number of characteristics of dust are important in considering its impacts. Dust can have both a physical and a chemical impact. Dust falling onto plants may physically smother the leaves. Thus the absolute level of deposition is important. This is affected by dust emission rates, meteorology and conditions on the leaf surface. Dust can also physically block stomata. Figure (5) shows that the dust particles were accumulated more on leaves of Albizia (209) in mm^2 , which has more filament on leaves surface(33) in mm^2,while it was less accumulated on leaves of Eucalyptus(176)in mm^2 which do not have filament on leaves surface. Although the leaves of Conocarpus was the lowest in dust particle accumulation (132), also it contain filament on leaves surface as shown in figure (3c),and maybe because of the smallest LAI of the three plants

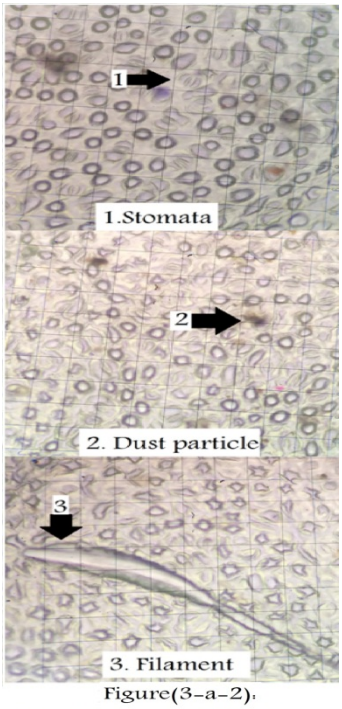
under study figure(1) . The dust forms a hard crystalline crust on the leaf surface, which dissolves releasing solutions of calcium hydroxide into the intercellular spaces. This causes cell plasmolysis and death. Finally, heavy cement/lime dust deposition can lead to growth reduction for many tree species(Farmer, 1993). Particulate matter such as cement dust, magnesium-lime dust and carbon soot deposited on vegetation can inhibit the normal respiration and photosynthesis mechanisms within the leaf. Cement dust may cause chlorosis and death of leaf tissue by the combination of a thick crust and alkaline toxicity produced in wet weather. Dust on leaves blocks stomata and lowers their conductance to CO_2, simultaneously interfering with photosystem II. cement dust forms crusts on the surface of leaves, twigs and flowers. This inhibits gaseous exchange from the surfaces of plant parts. Such crust on the leaves also inhibits light penetration and consequently reduces photosynthesis.(Gheorghe1 and Barbu,2011). dust on leaves blocks stomata and lowers their conductance to CO_2, simultaneously interfering with photosystem II. cement dust forms crusts on the surface of leaves, twigs and flowers. This inhibits gaseous exchange from the surfaces of plant parts. Such crust on the leaves also inhibits light penetration and consequently reduces photosynthesis.(Gheorghe1 and Barbu,2011).

5.*Leaf total Chlorophyll* (mg/g leaf)

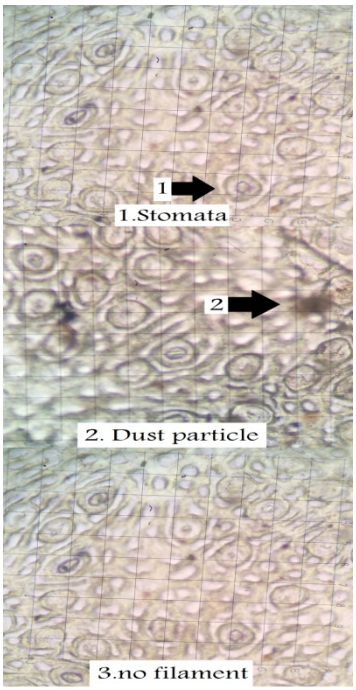
Total chlorophyll content decreased significantly in response to air pollutants. Figure(6)showed significant reduction in total chlorophyll,the total chlorophyll percentage is higher in Conocarpus leaves (0.100mg/g leaf) than other two plant's leaves , while it is the lowest in Eucalyptus leaves (0.044 mg/g leaf) . The chlorophyll is the essential components for photosynthesis, and occurs in chloroplasts as green pigments in all photosynthetic plant tissues. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone and ether.



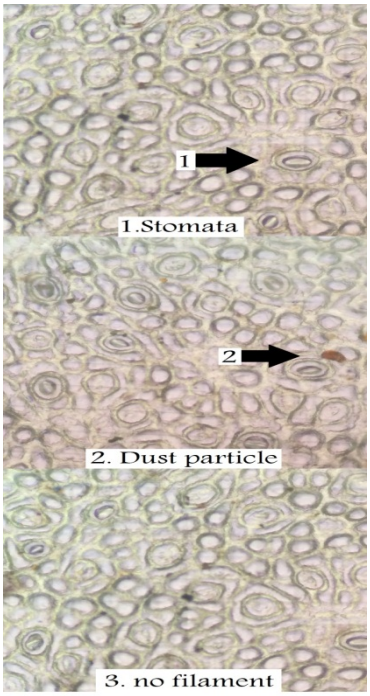
Figure(3-a-1) :
Upper surface of Albizia



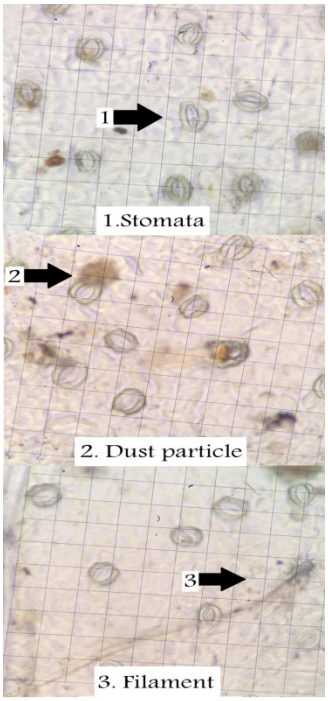
Figure(3-a-2):
Lower surface of Albizia



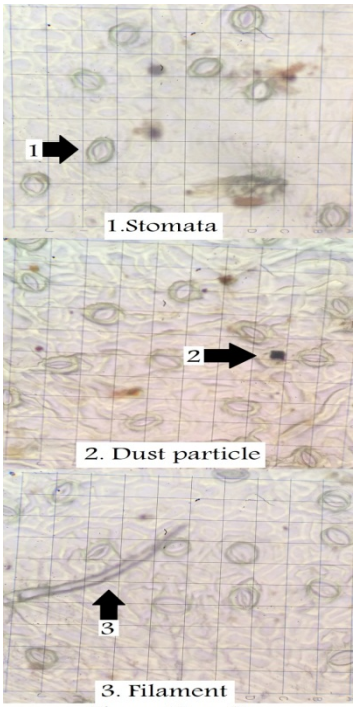
Figure(3-b-1):
Upper surface of Eucalyptus



Figure(3-b-2):
Lower surface of Eucalyptus



Figure(3-c-1):
Upper surface of Conocarpus



Figure(3-c-2):
Lower surface of Conocarpus

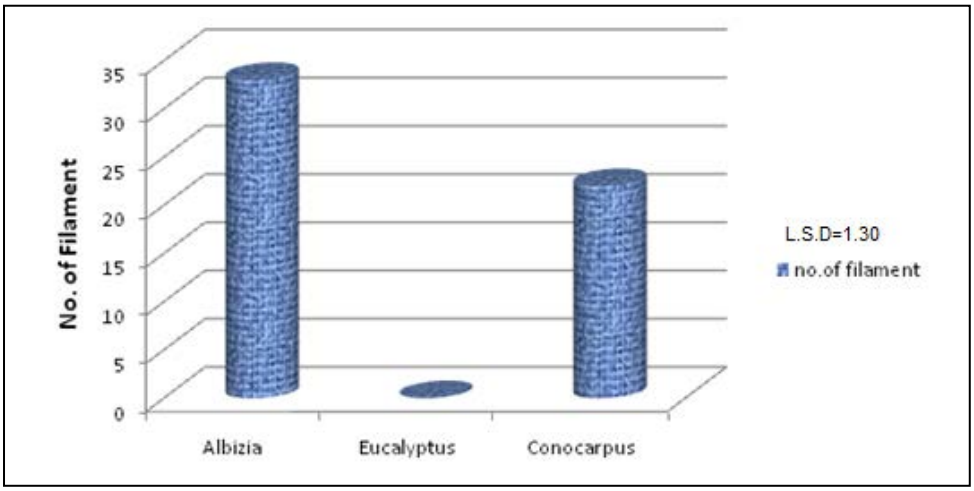


Fig. 4 :No. of Filament in Plant's Leaves

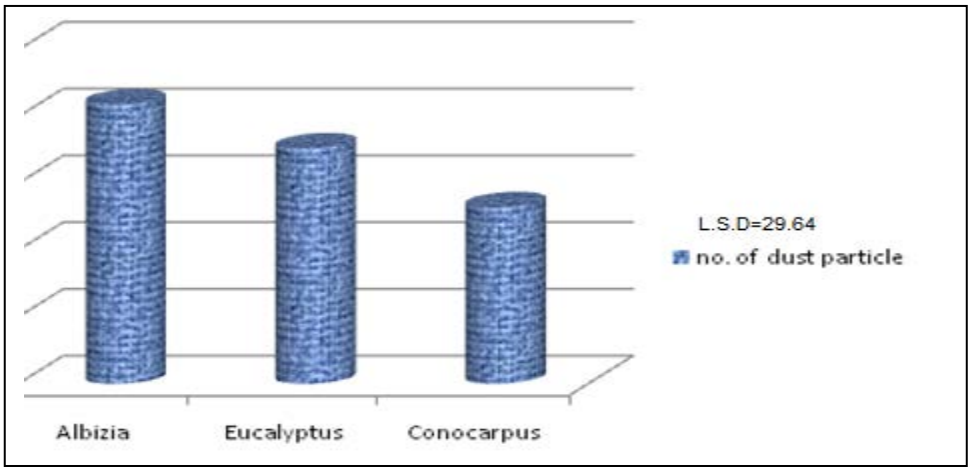


Fig. 5 :No. of Dust Particle in Plant's Leaves

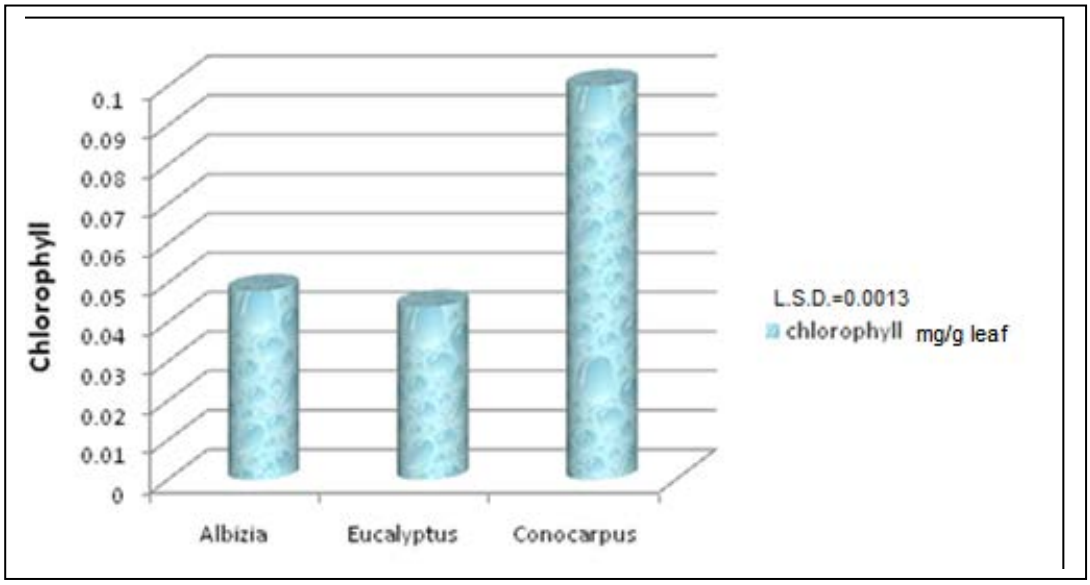


Fig.6:Chlorophyll (mg/g) of Plant's Leaves

6.*Leaf Cadmium and Lead content (%).*

Result shows in figure (7). That Pb and Cd concentration respectively (82, 4.5%) is higher in leaves of Albizia among three plants, while it is lowest in leaves of Conocarpus (57, 3.0%) , respectively. Cadmium is a relatively element non essential to plant, animal and humans. Its presence in living organisms is unwanted and harmful. An increased level of Cadmium in the air, water and soil increases it's uptake by living organism. It is taken up by plant and animal through them also by humans. This lead to cadmium cycle :soil-plant-animal-man. Lead is a microelement naturally present in trace amounts in all biological materials,i.e. in soil ,water, plants and animals. (Gheorghe1 and Ion, 2011**).** It has no physiological function in the organism (Seyyednejad and Koochak,2011). The main sources of lead contamination are smelting works, application of waste water treatment sludges to soil, transportation , rain , snow , hail and others. Air quality in urban areas is often degraded due to emissions from various sources (e.g., cars, factories, power plants) associated with urban development and high concentrations of people. In addition, the trees in urban areas have the ability to air quality and reduce greenhouse gas concentrations by both reducing emissions and directly removing

pollutants from the atmosphere. Trees can reduce pollutant emissions by reducing building energy via tree shade, blocking winter winds and reduced air temperatures. Tree leaves (shade) and reduced air temperatures also affect the levels of ultraviolet radiation at ground level and human comfort.

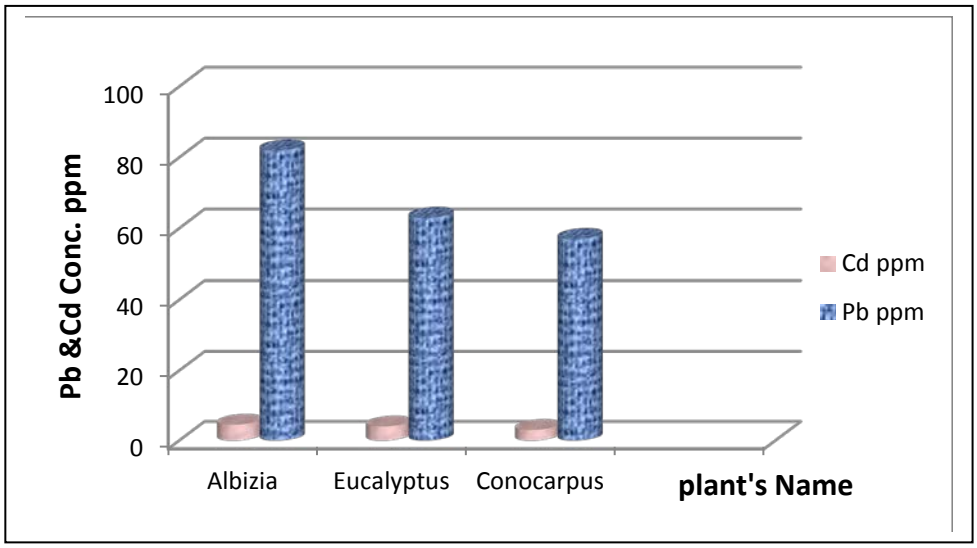


Fig. 7 : Pb &Cd Conc. in Plant's Leaves

Conclusion

The study clearly show that air pollutants have detrimental effects on morphological characteristics, (leaf area and leaf area index , dry weight, fresh weight, number and density of stomata , number of stomata , number of dust particle , number of filaments, total chlorophyll on leaves plants). Changes in morphological characteristics maybe due to percentage of Pb and Cd on leaves plants which mustard plants directly corresponded to the levels of air pollution at Baghdad city . The study elucidates that air pollution emitted from urban and industries adversely affecting the ambient air and agricultural production. It is very clear that urban and industrial air pollution has become a serious threat to agricultural production grown adjacent to urban and industrial areas. According to our result Albizia tree are more useful to be planted in Baghdad city to reducing air pollution .

However, More research is necessary to evaluate the contribution of individual and in combination of air pollutant on crop production and its losses.

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