

## Isolation and characterization of phenazine produced from mutant *Pseudomonas aeruginosa*

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### Abstract

Hundred and four isolates of *Pseudomonas aeruginosa* were screening and isolate SP9 identified as belong to *Pseudomonas aeruginosa*, and was selected according to it's high phenazine production. That isolate was subjected to mutation by different concentrations of N-methyl-N-nitro-N-nitrosoguanidine (NTG).

In 250 µg/ml of NTG, the mutant strain produced noticeable amount of phenazine more than isolate of strain SP9 which named SPm9. separation and characterization of phenazine was done by using TLC, HPLC and IR spectrum. Gel filtration was used for purification of phenazine with sephadex G25.

Antimicrobial activity of phenazine was done by using disk method for numbers of Gram-positive and Gram-negative isolates.

The results indicate that phenazine which produced from mutant strain SPm9 is a broad spectrum antibiotic follows like that produced from isolate strain Sp9. it is effective against *Bacillus subtilus*, *Staphylococcus aureus* and *Staphylococcus epidermidis* as well as *Escherichia coli* and *Salmonella typhi*. Phenazine was also found to be effective against *Candida albicans*.

### عزل وتشخيص الفينازين المنتج من عزلة طافرة لبكتريا *Pseudomonas aeruginosa*

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### الخلاصة

فحصت 104 عزلة من بكتريا *Pseudomonas aeruginosa* وانتخبت العزلة SP9 لقدرتها العالية على إنتاج الفينازين. طفرت العزلة SP9 باستخدام تراكيز مختلفة من المطفر الكيميائي N-methyl-N-nitro-N-nitrosoguanidine (NTG) في التركيز 250µg/ml من المطفر NTG أظهرت العزلة الطافرة إنتاجية ملحوظة من الفينازين بكميات أكثر من العزلة الأصلية غير الطافرة. تم عزل وتشخيص الفينازين باستخدام تقنيات TLC، HPLC و IR. استخدمت طريقة الترشيح الهلامي لتنقية الفينازين بواسطة Sephadex G25. وتم دراسة الفعالية الحيوية للفينازين باستخدام طريقة الأقراص مع عدد من الأحياء المجهرية السالبة والموجبة لصبغة غرام. أشارت النتائج إلى أن للفينازين، المنتج من العزلة الطافرة، مضاد حيوي ذو مدى واسع وله تأثير ضد *Bacillus subtilus*, *Staphylococcus aureus* و *Staphylococcus epidermidis* الموجبة لصبغة غرام وبالإضافة إلى *Escherichia coli* و *Salmonella typhi* السالبة لصبغة غرام. ووجد أيضا أن للفينازين تأثير ضد المبيضات البيضاء *Candida albicans*.

### Introduction

Phenazines constitute a large group of nitrogen containing heterocyclic compounds produced by a diverse rang of bacteria (1). Phenazine compounds produced by

fluorescent *Pseudomonas* competitiveness (2). The most widely studied phenazine producing *Pseudomonas* is *Pseudomonas aeruginosa*, a gram negative opportunistic bacteria of human, animals, insects, nematodes and plants (3,4,5). From 90-95% of *Pseudomonas aeruginosa* isolates produce pyocyanin (6) and which most of them characterized as phenazine compounds include phenazine-1-carboxylic acid (PCA), 1-hydroxy phenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (7, 6). More than 100 different phenazines structural derivatives have been identified in nature, and over 6000 compounds that contain phenazine as a central moiety have been synthesized (8), and these secondary metabolites have been studied intensively because of their broad spectrum antibiotic properties (9), against different bacterial and eukaryotic species. The side chain substituents on the phenazine backbone contribute to the biological activities of the compounds (10). Phenazine 1-carboxylate (PCA) secreted by *Pseudomonas fluorescens* contributes to biocontrol activity against fungal phytopathogens such as *Gaeumannomyces graminis* (11), and phenazine-1-carboxamide produced by *Pseudomonas chlororaphis* is able to inhibit the fungus *Fusarium oxysporum* (12). Previous studies showed that phenazine compounds also have antimicrobial activity against strains of *Bacillus subtilis*, *Candida albicans*, *Enterobacter aeruogenes*, and *Escherichia coli*, the phenazine also owing activity against *Mycobacterium tuberculosis* (13) and some plant pathogenic bacteria such as *Erwinia carotovora* and *Ralstonia solanacearum* (14). The objectives of the present work is to isolation and identification mutant *Pseudomonas aeruginosa* capable of phenazine production.

## Materials and Methods

- **Samples collection:** Hundred and four samples suspected to contain *Pseudomonas aeruginosa* were collected from different infections in human; Urine, Sputum, Ear and Wounds; from Al-Fallujah hospital and a clinical laboratory. *Pseudomonas aeruginosa* was enriched and then isolated by using cetrimid agar (Hi-Media, India) as selective medium which favor growth of *Pseudomonas aeruginosa* and inhibit other bacterial species. In addition to other media MacConkey's agar, King's A and B and Piocyanosel agar (Biomerix, France), which show a unique and easy characterize colonies. Cultures of *Pseudomonas aeruginosa* were grown on King's A medium at 37°C for 11 days, then 1ml of the suspension used to inoculate 100ml of Modified Pseudomonas P medium (PsP), consisted of the following: DL- alanin 2 g, Sodium citrate 10 g, Potassium sulfate 8.6 g, Potassium chloride 1.4 g, Magnesium sulfate 1.4 g, Dipotassium hydrophosphate 5 g. (15). All component were added to 1 liter of distilled water. pH was adjusted to 7.2-7.3 and sterilized by filtration through Millipore filters 0.45µm. The medium was suspended as 100ml in a 250ml Erlenmeyer flask and incubated at 37°C on rotary shaker at 110 rpm for 3 days.
- **Test microorganism:** *Candida albicans*, *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Bacillus subtilis* were used . These microorganisms were obtained from Baghdad university /College of science/ Biotechnology department and identified by using cultural properties and different biochemical tests as compared with schematic diagram of identification described by (16).
- **Standardization curve of Phenazine:** Standardization curve of Phenazine was done as follows:
- 1. **Wave length determination:** Maximum absorbance for phenazine was determined by scanning of wavelength using UV-visible spectrophotometer. Chloroform was used as reference sample, while 500 µg/ml of standard phenazine (sigma, U.S.A.) was used for scanning wavelength from (200-500 nm).

2. **Graf the standardization curve of phenazine antibiotic:** Standard phenazine were used. Different concentration of phenazine were used (50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml) for recording absorbance at 375 nm. (Fig. 1)

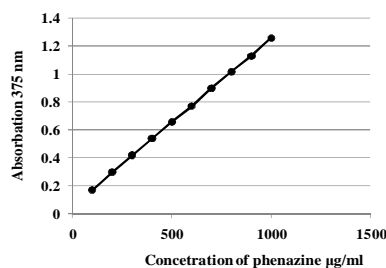


Fig (1): Standerization curve of phenazine

- **Measurement of pigment concentration:** Dark– blue chloroform extract was measured by UV-visible spectrophotometric analysis at 690 nm. Pure chloroform was used as blank. The following mathematical equation was used to caculate pyocyanin concentration: (17)

$$\text{Pigment concentration} = \frac{\text{Luminary density of pyocyanin suspension in wave length 690 nm}}{4.3 \times 10^6}$$

The result multiple with molecular weight of pyocyanin 210.2. The result measured with µg/ml.

Note:  $4.3 \times 10^6$  is extinction coefficient of crystalline sample of pure pyocyanin in wave length 690 nm (18).

- **Mutagenesis:** The methylating compound N-methyl-N-nitro-N-nitroso guanidine NTG (Pharmacia U.S.A.) was used as mutagenic agent following the method described by (19):

1. An amount (10 ml) nutrient broth was inoculated with *Pseudomonas aeruginosa* and incubated untile count achieved about  $5 \times 10^8$  cell/ml.
2. The culture centrifuged for 5 minutes at 5000 rpm to pellet the bacteria. Cells pellet of bacteria were then resuspended in equal volume of Tris-malic acid buffer prepared by Tris 6 g, Malic acid 5.8 g,  $(\text{NH}_4)_2\text{SO}_4$  1 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.25 mg. The volume was completed to 1 liter. pH was adjusted to 6 and autoclaved for 15 minute at  $121^\circ\text{C}$ . After medium has cooled to about  $50^\circ\text{C}$  aseptically  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g,  $\text{KNO}_3$  5mg was added.
3. Different concentrations of freshly prepared NTG (100, 150, 200, 250, 300 µg/ml) was added and cultures were incubated at  $37^\circ\text{C}$  without shaking for 30 minutes.

The treated culture was centrifuged then the pellets were resuspended in an equal volume of M56 minimal medium containing  $\text{Na}_2\text{HPO}_4$  8.2 g,  $\text{KH}_2\text{PO}_4$  2.7 g,  $(\text{NH}_4)_2\text{SO}_4$  1 g,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.25 mg. Per liter. pH was adjusted to 7.2 and autoclaved for 15 minutes at  $121^\circ\text{C}$ . After medium has cooled to about  $50^\circ\text{C}$   $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$  0.1 g,  $\text{KNO}_3$  5 mg was aseptically added and supplement with 10 ml of sterile 20% glucose solution.

4. The culture was recentrifuged and again pellets were resuspended in M56 minimal medium.
5. Serials dilutions were done of 10-1-10-10 which spreaded on nutrient agar and incubated in  $37^\circ\text{C}$  for 24 hours.
6. The treatment that gave Killing percentage 95%, was chose then count of residue bacteria was done using following equation:

$$\text{Killing percentage} = \frac{\text{Origin number of bacteria-residue number of bacteria}}{\text{Origin number of bacteria}} \times 100$$

7. Add 0.1 ml culture broth to PsP medium in dark condition at 37°C on rotary shaker at 110 rpm for 3 days.
- **Extraction of phenazine:** *Pseudomonas aeruginosa* mutant (SPm9) was grown at 37 °C in pigment production medium PsP, the cultures were incubated in rotary shaker at 110 rpm for 3 days. Phenazine antibiotics were extracted from the culture liquid depended on the method described by (20, 21) as follow:  
 Five milliliters of each culture was centrifuged (5000 rpm) for 30 minutes, and the supernatant was acidified (pH,2) with concentrated HCl. Then 5 ml of benzene was added. Samples were mixed for 1 hour and centrifuged at 5000 rpm for 30 minutes. Four milliliters of the benzene layer was decanted and dried under air. Samples were resuspended in 1 ml of 0.1 N NaOH, and the absorbance at 375 nm was determined.
- **Thin layer chromatographic method (TLC):** The technique of TLC was used as described by (22) with the some modification as follows:- The sheet (silica gel 60f-254, 0.2 mm, layer thickness and aluminium support, size 20×20 cm, Spain) was used for analyzing samples. Slotting line was marked 1 cm from bottom edge of the plate. A liquid of standard phenazine which dissolved with chloroform was spotted on the first position and the test samples spotted on other positions, the plate was left some minutes to dry in dark condition before development. The TLC plates were developed in chloroform-methanol (9:1 v/v) in an unlined and un-equilibrated glass tank, until the solvent front reached a mark distance from the spotting line. The plate was removed and left to dry. The plate examined under UV light and calculated Rf values.
- **High performance liquid chromatography (HPLC):** Analysis was performed with a Shimadzu LC-2010 AHT liquid chromatography (Japan) with Reodyne 7125, 20µl injector, a Shimadzu SPD-2010 A UV-visible detector set at 375 nm and the column of (250×4.6 mm) C18, 5 µm particle size was used. The mobile phase was 100% acetone nitrile. The flow rate was 0.5 ml.min<sup>-1</sup>. The column temperature was maintained at 30 °C.
- **Gel filtration chromatography:** An amount (3 ml) of crude phenazine was add to the column, which packaged by sephadex G25, accurately and eluted with Tris 0.01M with pH 7.5 contain 0.1% of Triton X100 and 0.25M sodium chloride buffer which prepared by dissolving 1.2114g of Tris with 14.61g of sodium chloride in some distilled water, pH 7.5, then 1ml of Triton X100 was added then the volume was completed to 1 liter of distilled water. The flow rate was 1 ml/ 3min, phenazine in eluted fractions were detected using UV-visible spectrophotometer at a wavelength of 375 nm. After the complete elution, the column was rewashed by Tris 0.01M; pH 7.5, contain 0.1% of Triton X100 and 0.25M sodium chloride buffer for 24 hours. Results were plotted as optical density and fraction number.
- **Dialysis:** Dialysis was done opposite to Tris 0.01M; pH 7.5, contain 0.1% of Triton X100 and 0.25M sodium chloride buffer for several days with repeated changes every days for discard from salts.
- **Biological activity of phenazine antibiotic:** Determination of phenazine activity was done by using paper disk method: Filter paper (watman) discs 5.5 mm in diameter are prepared beforehand and sterilized by autoclave at 121°C for 15 min, then sterile paper disk was taken by using forceps, an amount (50, 100 and 200 µg/ml) of phenazine antibiotic were precipitated on the sterile paper disk and placed a top the Muller-Hinten agar which spreaded with different test microorganism and incubated for 24 hour at 37°C. Then inhibition zone around the disk was measured.

## Results and Discussion

- **Identification of the isolates:** A number (104) of isolates suspected to be *Pseudomonas aeruginosa* were isolated from different infections in human included Urine, Sputum, Ear, Wounds and burns which collected from patients in Al-Fallujah hospital and clinical laboratories, the percentage of prevalence rates of *Pseudomonas aeruginosa* infection were 28.8% of burns, 19.2% of sputum, 21.1% of urine, 17.3% of ear and 13.4% of wounds. The isolates were identified as *Pseudomonas aeruginosa* using cultural and biochemical tests and grown on different media along with their ability to produce pyocyanin pigment. These tests were compared with schematic diagram proposed by (16).
- **Screening of the isolates:** Screening of the isolates was done according to their production of pyocyanin on Pseudomonas P medium as measured by (17). Isolate SP9 was selected as the highest producer which produce as much as 0.174 mg/ml. The SP9 strain then mutated by subjecting the wild type to (0, 100, 150, 200, 250, 300  $\mu\text{g/ml}$ ) of NTG. The results indicated that 250  $\mu\text{g/ml}$  was the concentration which produce 95% killing ratio. This concentration was used to develop the mutant which designated as SPm9. Results indicated that as much as 496  $\mu\text{g/ml}$  of phenazine was produced by mutant SPm9 as compared with wild type Sp9 281 $\mu\text{g/ml}$ .
- **Identification of phenazine:** Prior separation of phenazine from cultural medium was done by TLC with the solvent system chloroform: methanol (9:1 v/v). A band 2 with Rf 0.85 was characterized as related to phenazine as long as it gives biological activity when tested as shown in (Fig 2).

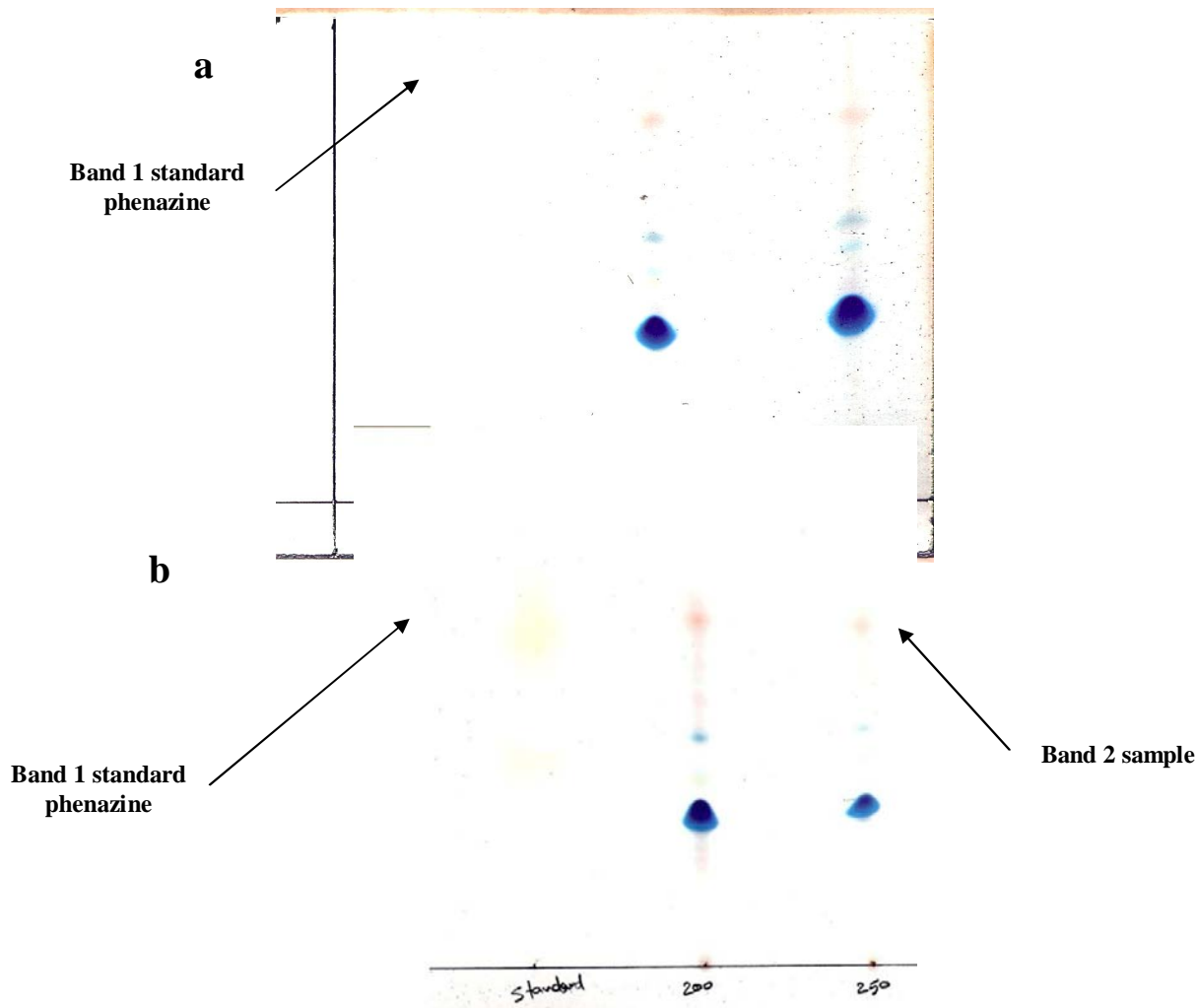
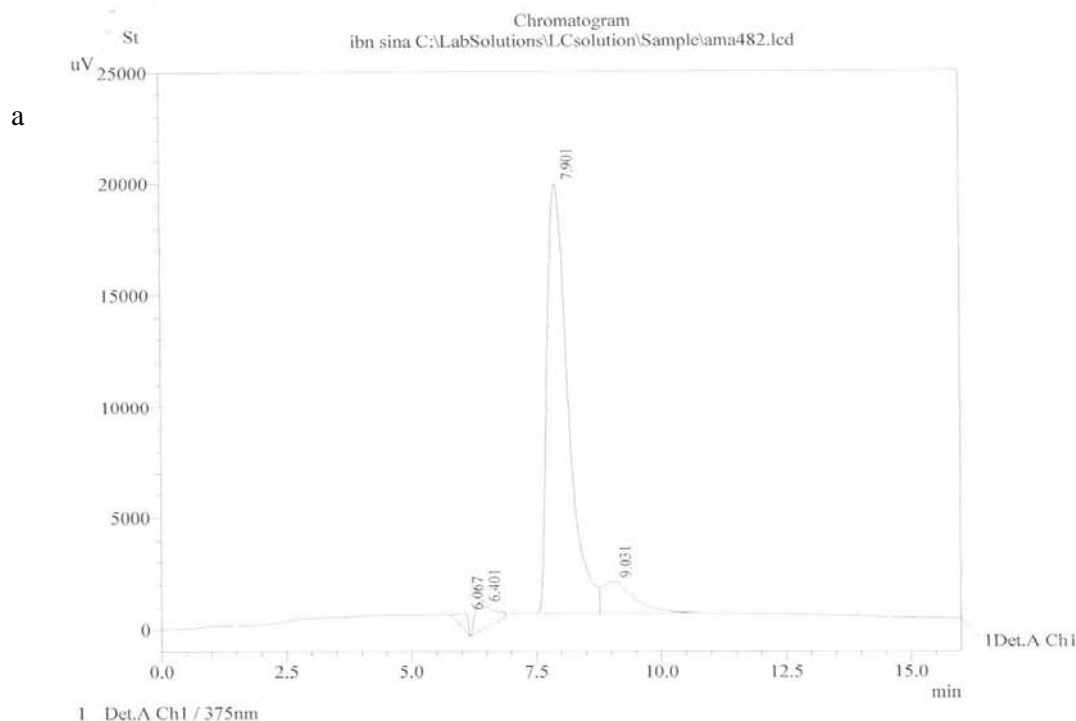
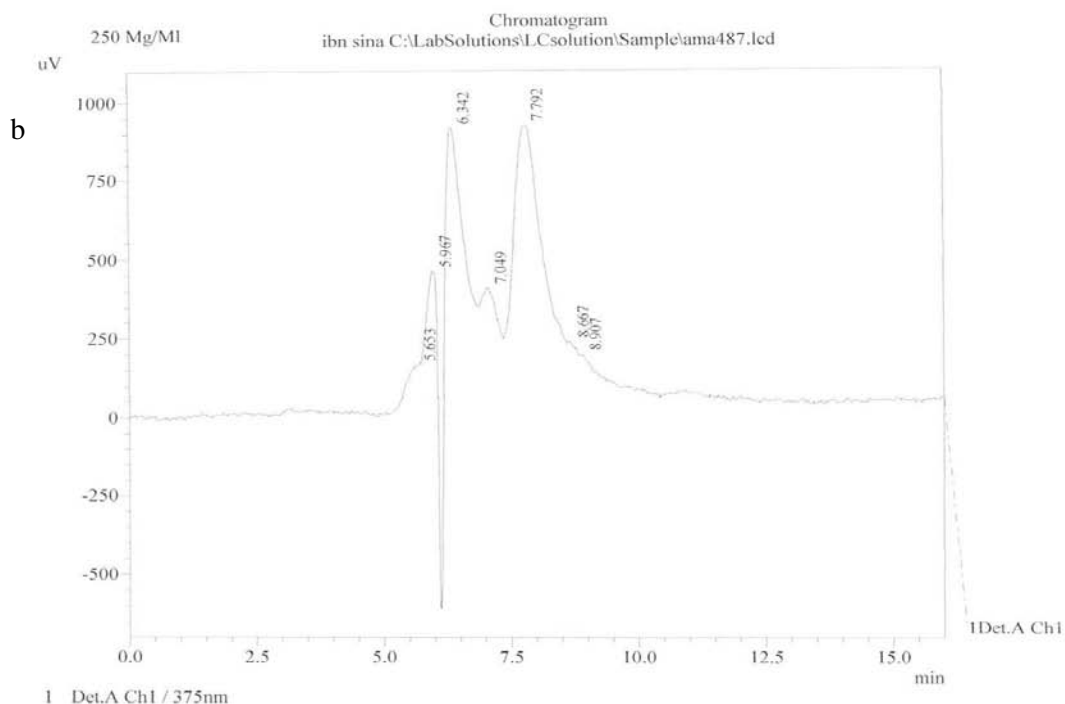


Fig. (2) Identification of phenazine by TLC a- concentration of NTG 100 and 150  $\mu\text{g/ml}$ . b- concentration of NTG 200 and 250  $\mu\text{g/ml}$ .

- **Identification of phenazine by HPLC:** A typical extraction of phenazine was done by using HPLC. Results shown in (Fig 3) which includes:
  - a. Standard phenazine represented by a peak with retention time of 7.9 min. HPLC analysis of phenazine from *Pseudomonas aeruginosa* isolate SP9 revealed the presence of typical peak of maxima at 375 nm. This result agree with that described by (23).
  - b. Presence of three peaks with a retention time 7.049 min, 7.792 min, 8.667 min compared with standard phenazine. These results agree with that of (24) who demonstrated the ability of *Pseudomonas aeruginosa* to produce many phenazine derivatives beside pyocyanin. Other studies reported that strains of *Pseudomonas aeruginosa* could produce a variety of redox-active phenazine compounds included pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxy phenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (7, 6).

The concentration of phenazine was calculated in the diagram as 496 µg/ml as compared with standard curve of phenazine antibiotic (Fig 3).

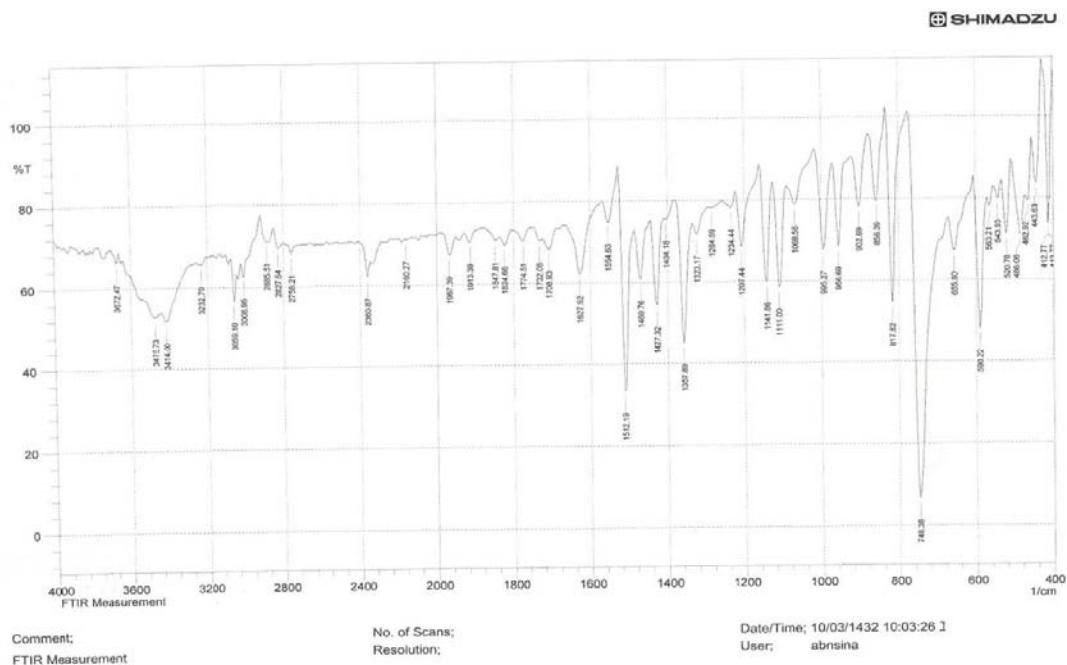




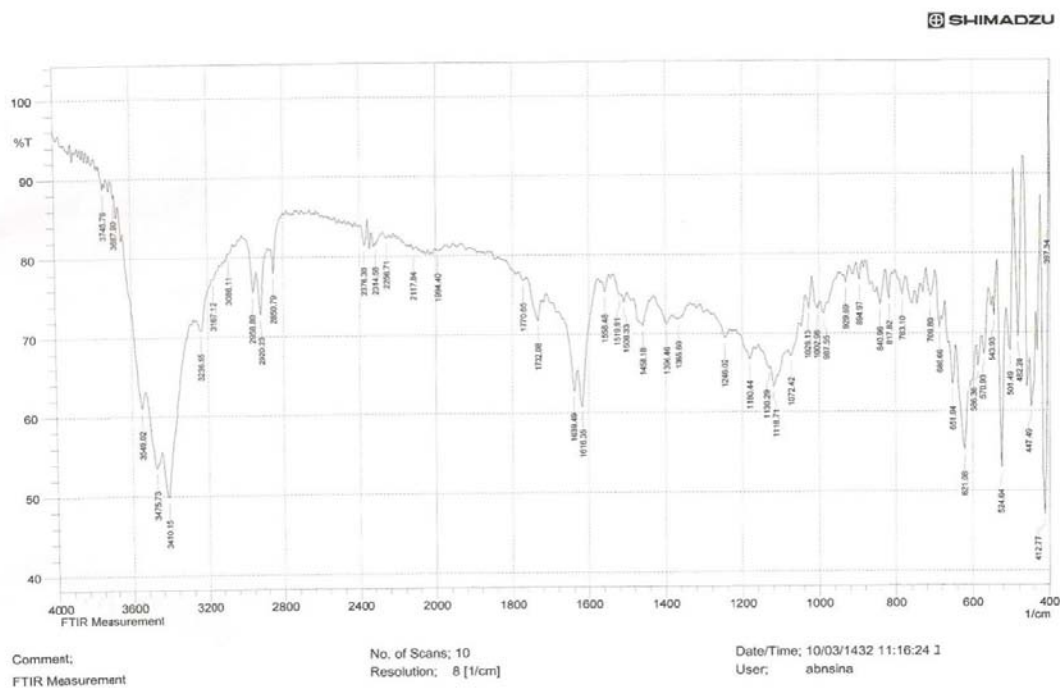
**Fig (3) Identification of phenazine by HPLC. a- standard phenazine. b- Sample of extract phenazine separation from SPm9 strain**

- **Identification of pheazine by IR:** Further identification of the metabolite separated from culture of SP9 was done by IR. The spectrum shown in (Fig 4) indicate the presence of phenazine as specified by side chains of the molecule as compared with standard which their results shown in (Table 1). These results agree with that of (13).

a



b

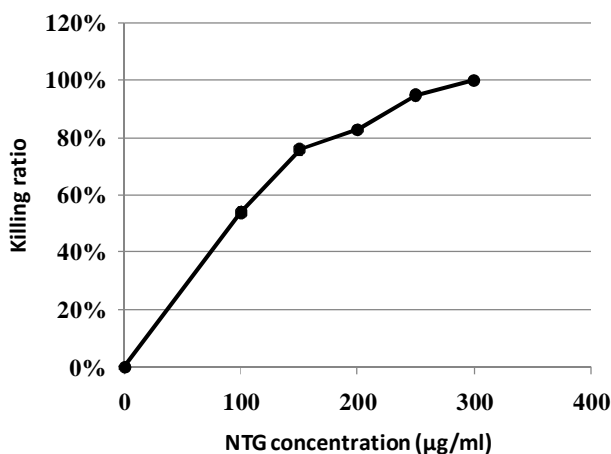


**Fig (4) FTIR measurements for: a- standard phenazine b- sample**  
**Table (1) Absorption bands of FTIR for cycle compounds of phenazine antibiotic**

compound	H-O	C-H aromatic	C=N	C=C aromatic	C=O	C-O	C-N	ip O-H	ip C-H aromatic	oop C-H aromatic
Standard phenazine	3475.73	3059.10	1627.90	1554.63	1469.76	1323.17	1284.59	1207.44	856.39	748.38
Sample with 250 µg/ml NTG	3475.73	3086.11	1639.49	1558.48	1458.18	1365.60	1246.02	1180.44	840.96	709.80



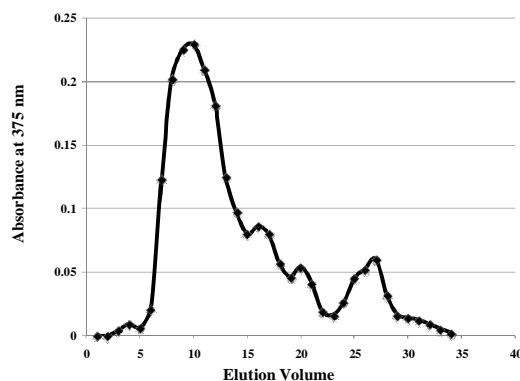
- **Mutagenesis:** Mutation experiment was conducted to obtain more productive strain. Survival curve for isolate SP9 was done to specify the most effective concentration of mutagen NTG . Results indicate that 250  $\mu\text{g/ml}$  of NTG reducing the number of cells to 95% as shown in (Fig 5). This concentration was used to develop the mutant strain.



**Fig (5) The effect of mutagen NTG on growth of *Pseudomonas aeruginosa* SP9**

A maximum amount of 496  $\mu\text{g/ml}$  of phenazine was obtained from mutated strain SPm9 as compared to that of wild type Sp9 (281  $\mu\text{g/ml}$ ) which indicated an increase in production of phenazine. The methylating compound NTG is one of the most potent mutagens yet discovered for bacteria. It induces primarily base transition mutation of the GC to AT type, although AT to GC transitions, transversions, and even frameshifts arise (19). So that mutagen used in many studies for increase the production of secondary metabolites of bacteria. A duplication in production was obtained when Al-Tememi 2000 (23) mutated *Pseudomonas aeruginosa* by using NTG. Byng and Turner, 1976 (25) reported the ability of mutants *Pseudomonas phenazinium* to synthesize nine other phenazines.

- **Purification of phenazine by Gel filtration:** Purification of phenazine was done by using gel filtration using sephadex G25 due to its low molecular weight (26). Results shown in (Fig 6) indicate the presence of fraction having activity at 375 nm. When these fraction pooled, the concentration measured spectrophotometrically, 200  $\mu\text{g/ml}$  an amount of was obtained as compared with standard phenazine.



**Fig(6) Gel filtration of Phenazine extraction from Spm9 strain by sephadex G25**

- **Biological activity of phenazine antibiotic:** Different concentration volumes of phenazine antibiotic (50, 100 and 200 µg/ml) were prepared for specify antimicrobial activity of phenazine against different microorganisms included: Gram-positive such as *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*; Gram-negative such as *Escherichia coli*, *Klebsiella spp.*, *Salmonella typhi* and *Pseudomonas aeruginosa*, and Yeast as *Candida albicans* . Results shown in (Fig 7) indicate that phenazine antibiotics shows high antimicrobial activity against all mentioned organisms except *Pseudomonas aeruginosa* and *Klebsiella spp.* MIC for phenazine was found to be 200µg/ml when purified phenazine was tested. This concentration was found to be effective against gram-positive bacteria specially *Staphylococcus aureus* and *Staphylococcus epidermidis* more than *Escherichia coli* and *Salmonella typhi* and have antimicrobial activity against *Candida albicans* more than *Escherichia coli* and *Salmonella typhi* but show less than that observed against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Results shown in (Fig 7) indicate that, all concentration of phenazine gave antimicrobial activity against all microorganisms tested except *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, and these results indicated that the purified phenazine have biological activity against gram negative as well as gram positive bacteria. We notice that the phenazine produced from mutant *Pseudomonas aeruginosa* (SPm9) have biological activities mimic that produced from isolate *Pseudomonas aeruginosa* (SP9). It was also have antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* more than *Escherichia coli* and *Salmonella typhi* and have antimicrobial activity against *Candida albicans* more than *Escherichia coli* and *Salmonella typhi* but less than that observed against *Staphylococcus aureus* and *Staphylococcus epidermidis*. These results are in agreement with that results of (13) who reported that phenazine antibiotic have antimicrobial activity against strains of *Bacillus subtilis*, *Candida albicans*, and *Escherichia coli*. However others demonstrated that activity of phenazine antibiotics are concentration dependent (27).

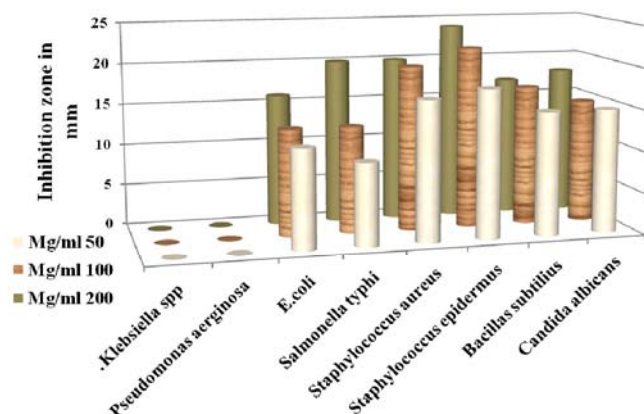


Fig (7): Antimicrobial activity of phenazine extraction from Spm9 against positive and negative bacteria and yeast

## Reference

1. Leland, S. & Elizabeth, A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnology process. Appl. Microbiol. Biotechnol., 86:1659-1670.
2. Mazzola, M.; Cook, R. ; Thomashow, L.; Weller, D. & Pierson, L. (1992). Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol., 58:2616-2624.
3. Jander, G.; Rahme, L. & Ausubel, F. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol., 182: 3843-3845.
4. Mahajan, S.; Tan, M.; Rahme, L. & Ausubel, F. (1999). Molecular mechanisms of bacterial virulence elucidated using *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. Cell, 96: 47-56.
5. Lauredo, I.; Sabater, A.; Ahmed, A.; Botvinnikova, Y. & Abraham, W. (1998). Mechanism of pyocyanin and 1-OH-PHZ induced lung neutrophilia in sheep airways. J. Appl. Physiol., 85: 2298-2304.
6. Smirnov, V. & Kiprianova, E. (1990). Bacteria of *Pseudomonas* genus, p. 100-111. Naukova Dumka, Kiev, USSR.
7. Budzikiewicz, H. (1993). Secondary metabolites from fluorescent Pseudomonads. FEMS Microbiol. Rev., 104: 209-228.
8. Mavrodi, D.; Blankenfeldt, W.; Thomashow, L. & Mental, M. (2006). Phenazine compounds in fluorescent *Pseudomonas* spp. Biosynthesis and regulation. Annual review of Phytopathol., 44:417-445.
9. Collee, J.; Fraser, A.; Marmion, B. & Simmons, A. (1996). *Pseudomonas*, *Stenotrophomonas*, *Burkholderia*, practical medical microbiology. 14<sup>th</sup> ed.
10. Jander, G.; Rahme, L. & Ausubel, F. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol., 182: 3843-3845.
11. Thomashow, L.; Weller, D.; Bonsall, R. & Pierson, L. (1990). Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. Appl. Environ. Microbiol., 56: 908-912.

12. Chin-A-Woeng, T. F. C.; Bloembergen, G. V.; Mulders, I.; Dekkers, L. & Lugtenberg, B. (2000). Root colonization by phenazine-1-carboxamide producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol. Plant-Microbe Interact.*, 13: 1340-1345.
13. Makarand, R.; Prashant, D. S.; Bhushan, L. & Sudhir, B. (2007). Detection, isolation and identification of phenazine -1-carboxylic acid produced by biocontrol strains of *Pseudomonas aeruginosa*. *J. Scientific and Industrial Res.*, 66:627-631.
14. Aunchalee, N.; Sukanya, A.; Chanokporn, P.; Paweena, P.; Saksit, C. & Chalerm, R. (2009). Synthetic, isolation of phenazine derivatives and their antimicrobial activities. *Walailak J. Sci. and Tech.*, 6(1):78-91.
15. Ralph, C. (1973). Studies on pigment production by *Pseudomonas aeruginosa*. M.S.C thesis, College of science, Taxes Tech university.
16. Holt, J. G.; Krieg, N. R.; Sneath, P. H; Staley, J. T. & William, S. T. (1994). *Bergey's Manual of Determinative Bacteriology*. 9<sup>th</sup> ed. William and Wilkins Co. Baltimore, London.
17. Frank, L. H. & DeMoss, R. D. (1959). On the biosynthesis of pyocyanin. *J. Bacteriol.*, 77:776-782.
18. Al-Mahmdy, L. R. (2003). Isolation and identification of *Pseudomonas aeruginosa* with the possibility of hypothetical map for its movement among the various sources. M.S.C thesis, College of science, Al-Mustansiriya University.
19. Carlton, B. & Brown, B. (1981). Gene mutation: Manual of methods for general bacteriology. p. 222-242. Gerhardt, P.; Murray, R.; Costihow, R.; Nester, E.; Wood, W.; Krieng, N. and Philips, G. (eds) American Society for Microbiology, Washington.
20. Chancey, S. T; Wood, D. W. & PiersonIII, L. S. (1999). Two component transcriptional regulation of N – Acyl – homoserine lactone production in *Pseudomonas aureofaciens*. *Appl. Environ. Microbiol.*, 65(6):2294– 2299.
21. Kumaresan, K.; Subramanian, M.; Vaitiyanathan, S.; Sevagaperumal, N.; Gopal, C. & Dilantha, F. (2005). Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. *Archives of Phytopathol. and Plant Prot.*, 38(1): 69 – 76.
22. Genevieve, G.; Sharief, B.; Sebastien, R.; Tjeerd, V.; Ben, J. & Guigo, V. (2006). Pip a novel activator of phenazine biosynthesis in *Pseudomonas chlororaphis*. *J. Bactriol.*, 188:8283-8293.
23. Al-Tmemei, M. B. (2000). Isolation and identification of phenazine antibiotic produced by *Pseudomonas aeruginosa*. M.S.C thesis, College of science, Al-Nahreen University.
24. Chin-A-Woeng, T.F. C.; Bloembergen, G. V. & Lugtenberg, B. J. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas aeruginosa*. *New Phytol.*, 157: 503-523.
25. Byng, G. & Turner, J. (1976). Isolation of Pigmentation Mutants of *Pseudomonas phenazinium*. *J. of General Microbiol.*, 97:57-62.
26. Leisinger, T. & Margraff, R. (1979). Secondary metabolites of the fluorescent pseudomonads. *Microbiol Rev.*, 43:422-442.
27. Stephen, S.; Baron, E. & John, J. (1981). Antibiotic action of pyocyanine. *Antimicrobial agent and Chemotherapy*. 20(5): 814-820.

**Bacterial Contamination of Imported Bulls Frozen Semen**

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**Abstract**

One hundred frozen semen straws (0.5 ml), have been taken from imported bulls, the frozen semen examination as well as evaluation in addition to bacteriological evaluation whether counting, isolation and antibiotic sensitivity, the results in current study showed all bacterial isolates were identified by using different biochemical tests and API-20 E system that used later to confirm identification, all isolated bacteria found in imported frozen semen were gram negative. 45 bacterial isolates in imported frozen semen distributed in 42 isolates *Stenotrophomonas maltophilia* and 3 isolates *Pseudomonas aeruginosa*, antibiotic susceptibility test for isolated bacteria by using 14 single antibiotic disks of commonly used drugs, most isolated *Steno. maltophilia* was susceptible to Ceftazidime, Ciprofloxacin, Cefotaxime, Gentamicin, Amikacin, Vancomycin, Erythromycin, Chloramphenicol and Tetracyclin. The results of current study showed individual movement  $61.3 \pm 2.00$ , viability of sperm  $76.2 \pm 2.06$ , percentage of dead sperms  $23.8 \pm 2.06$ , and abnormal sperms  $13.54 \pm 0.67$ .

**التلوث البكتيري في قصبات السائل المنوي المستورد للثيران**

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**الخلاصة**

أخذت 100 قصبة من السائل المنوي المجمد المستورد 0.5 مل، أجريت فحوصات تقييم السائل المنوي واختبارات العد والعزل الجرثومي على العينات واختبار فحص الحساسية للمضادات الحيوية المختلفة. كل الجراثيم التي عزلت شخصت، وأجريت الفحوصات الكيموحيوية التقليدية والتشخيص بنظام API-20E لتشخيص الأنواع الجرثومية المعزولة. كانت جميع الجراثيم المعزولة من عينات السائل المنوي المجمد المستورد سالبة لصبغة كرام، خمسة وأربعون عزلة جرثومية عزلت من السائل المنوي المستورد موزعه في 42 عزله من *Stenotrophomonas maltophilia* و 3 عزلات من *Pseudomonas aeruginosa*. اختبرت الحساسية للجراثيم المعزولة وتم استخدام 14 نوع من الأقراص المفردة للمضادات الحيوية الأكثر استخداما، أغلب عزلات *Steno. maltophilia* كانت حساسة للـ Ceftazidime, Ciprofloxacin, Cefotaxime, Gentamicin, Amikacin, Vancomycin, Erythromycin, Chloramphenicol و Tetracyclin, من ناحية أخرى كانت جميع الجراثيم المعزولة مقاومه بنسبة 100% لكل من الـ penicillin و Streptomycin و Amoxicillin. بينت نتائج الدراسة الحالية للعينات المستوردة ان الحركة الفردية  $61.3 \pm 2.00$  وحيوية الحيامن  $76.2 \pm 2.06$  والنسبة المئوية للحيامن الميتة  $23.8 \pm 2.06$  أما النسبة المئوية للحيامن المشوهة فكانت  $13.54 \pm 0.67$ .

**Introduction**

Artificial insemination has facilitated the choice of using the best possible bulls of proven fertility in improving the genetic makeup of the cattle population thus conveying

to the primary goal for breeding; to increase the productivity and the profitability of a particular commercial herd by increasing the number of offspring produced by selected genetically superior bulls. The use of AI made also possible to measure the performance of large numbers of progenies born after AI of many females using sperm doses from a single bull, thus allowing the accurate selection of bulls with desirable characters (1). Sperm cryopreservation contributes to the expansion of reproductive techniques, such as artificial insemination (AI) and in vitro fertilization, AI with frozen semen is essential in breeding and selection schedules contributing to increase production of domestic species. These schemes were well developed in dairy cattle (2). Presumed mechanisms of infection, causing infertility are the following: (a) Bacterial attachment to sperm; (b) an immobilizing factor produced by some bacteria; (c) immune system recruitment, and (d) alteration of glandular function (3). The pathogenic bacteria in the ejaculates can induce a defect in semen parameters, such as reduce sperm count, poor morphology and motility (4). It is already known that these parameters play a vital role in the fertility (5). The aim of study to know the types of bacteria present in frozen semen used for A.I.

### Materials and Methods

One hundred imported frozen semen samples was taken and make on these samples many examinations such as:

- **Frozen semen evaluation:** Taken out of frozen semen straws after storage and has been found important to thaw it by placing it (with effective separation from the water) in water bath at 37°C for 30 sec. After drying, both sides of frozen semen straw has been broken, in order to take a drop of semen after neglecting the first drop, and then make the following tests: Bacterial isolation, pH, Individual movement and The percentage of viability and morphologically abnormal sperm.
- 1. Bacterial isolation: Samples were taken from frozen semen and cultured on the nutrient broth, and another sample was cultured in tryptic soya broth, samples then were cultured in two ways:
  - A. Aerobically: Placing the samples were cultured in nutrient broth in incubator at temperature of 37°C degree and for a period of 24-48 hours, then were transported the growth from nutrient broth on three kinds of culture media (Blood agar, MacConky agar, nutrient agar) and cultured by streaking method, then placed in incubator at 37°C for a period of 24-48 hours, then was diagnosed types of bacteria that appeared on the culture media by using Gram stain and biochemical tests and by using Api-20E system.
  - B. Facultative anaerobic: Placing the samples were cultured in tryptic soya broth in candle jar for 7-14 days in order to note the presence of bacterial species that need for their live to 10% of carbon dioxide (CO<sub>2</sub>), such as *Brucella* and *Vibrio*, after the incubation were transported growth in the broth among Special media such tryptic soya agar and Brucella agar and placed in candle jar and put it in incubator under 37°C for 7-14 days until the colonies is a appear, diagnosis of the colonies by the way (6).
- **Bacterial Count:** Bacteria were counted by the way plate count method known as Miles and Misra (7). Make a series of tenfold dilution to semen sample mixing 0.9 ml of phosphate saline solution with 0.1 ml of semen sample to get dilution 1:10, and continuous to make series of dilution, then cultured the final three dilution on MacConky agar and then using a Pasteur pipette and incubation the petri dishes in incubator at 37°C for 24 hours and then calculate the number of colonies of bacteria.
- **Antibiotics sensitivity test:** According to 8 plates were prepared with Muller Hinton Agar, 3-4 similar colonies were selected to prepare pure culture, these colonies were transferred into about 5ml of trypticose soya broth incubated at 37 °C

for 2-8 hrs until light to moderate turbidity develops, turbidity adjusted by using MacFarland tube to  $1.5 \times 10^8$  cfu/ ml Dipasterile non toxic cotton swab on a wooden applicator into the standardized inoculums and rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid ,streak the surface of the plate with the swab three times, allow the inoculums to dry for 5-15 minutes with lid in place, antibiotics discs which include (Penicillin, Gentamycin, Streptomycin, Amoxicillin, Chloramphenicol, tetracyclin, Erythromycin, Ceftriaxone, Tobramycin, Vancomycin, Amikacin, Ceftazidime, Cefotaxime, and Ciprofloxacin) these disc were fixed by using aseptic technique and then incubated immediately at 37°C and examine after 24 °c, the complete inhibition zone was recorded.

2. **pH:** pH was evaluated for frozen semen by mean of special tape runway from 5.5 to 9.5 is intended for this purpose. Evaluated the pH of frozen semen revenue to (AI) center, local Vet. Clinics and imported.
3. **Individual motility (%):** Estimated percentage of the movement of individual sperm frozen semen taken from the (AI) center and local Vet. Clinics and imported in the following manner: A drop of thawed semen was placed over a warm slide and covered by cover slide. using light microscope 10 x 40 (400), several fields were examined to estimate the percentage of individual motility of sperm, which was assessed according to the method reported by (9). Considered the individual active progressive movement forwards the guide of the quality of screened semen.
4. **The percentage of viability and morphologically abnormal sperm:** Were measured as follows, a drop of thawed semen was mixed with drop of eosin stain and two drops of nigrosin stain, A thin smear of semen eosin- nigrosin mixture was done by using other slide and left to dry. Two hundred sperm were counted to calculate the percentage of viability sperm and morphologically abnormal sperm as in the following equation: The most important characteristic of the semen of bulls with high fertility is the low percentage of abnormal sperms, the percentage of abnormalities more than 30% indicate poor quality of semen and low fertility.

$$\text{Sperm Viability} = \frac{\text{No. of viable sperms}}{\text{Total No. of sperms}} \times 100$$

$$\text{Sperm Abnormality} = \frac{\text{No. of abnormal sperms}}{\text{Total No. of sperms}} \times 100$$

## Results and Discussion

- **Cultural results:** Colonies were obtained pure and separate through the culture, and growing on nutrient agar medium, blood agar medium, tryptic soya broth medium and then transferred on differential media such as MacConky agar, manitol salt agar characterization of colonies depends on the shape of colonies: The growing colonies on the MacConky agar lactose-non fermented were two types of bacteria, first Colonies was yellow or greenish yellow with Ammonia odor, on blood agar some strains may produce slight beta-haemolysis. The second one, colony was large, low, round, convex and smooth, sometimes surrounded by serrated growth, pale bluish green in color with a grape-like odor, some strains hemolyze blood.
- **Biochemical tests results:** Table (1) showed the results of some biochemical tests diagnostic and differential to isolated bacteria: The isolates can be biochemically distinguished from the known species and confirm the specific characteristics of each bacterial isolate. Furthermore, it has been possible to develop a practical laboratory test based on the characterized biochemical activities and can be devised a diagnostic strategy for the specified species of a bacteria (10). The biochemical

properties of the organism recorded in this study are the same as obtained by other workers (11,12,13). Our findings are partially agreed with the observations made by Collins and Patricia (14). The different pigments, which were produced on various media exhibited by *Pseudomonas aeruginosa* was considered due to high oxidative activity. This oxidative activity is the sole property, which is responsible to differentiate various species of the same genus (10).

**Table (1) Results of biochemical tests for isolated bacteria**

Type of bacteria		<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas aeruginosa</i>
Lactose fermentation MacConkey		NLF	NLF
Indol		-	-
Citrate		+	+
Urease		-	v
Phenylalanine		-	-
Gelatin liquefaction		+	+
Swarming		+	-
Motility on SIM		+	+
TSI	Reaction	ALK/ALK	ALK/ALK
	H <sub>2</sub> S	-	-
	Gas	-	-
Oxidase		-	+

NLF=no lactose fermentation

LF=lactose fermentation

V= variable reaction between species

ALK=alkaline reaction

+ =positive result

- = negative result

- **The results of biochemical tests by using API-20E system:** The API-20E system, is one of the most important diagnostic tests and more accurate diagnosis of most of the gram negative bacilli bacteria conformity with the results of conventional biochemical tests as shown in table (2).

**Table (2) Biochemical tests results for Api-20E system to diagnosis isolated bacteria**

APA-20E	<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas aeruginosa</i>
ONPC	-	-
ADH	-	+
LDC	+	-
ODC	-	-
CIT	+	+
H <sub>2</sub> S	-	-
URE	-	-
TDA	-	-
IND	-	-
VP	-	-
GEL	-	+
GLU	+	-
MAN	-	-
NO	-	-
SOR	-	-
RHA	-	-
SAC	-	-
MEL	-	-
AMY	-	-
ARA	-	-
OX	-	+

- **The results of bacterial isolation:** One hundred frozen semen samples have been tested, the bacteria isolated from imported frozen semen samples *Stenotrophomonas maltophilia* 42% and *Pseudomonas aeruginosa* 3%, while the samples have no



growth were 55%. Piasecka-Serafin (15) was first to demonstrate the possibility of translocation of bacteria from infected semen pellets to sterile ones in LN. From the results of bacterial isolation in different studies showed that presence of many types of bacteria in frozen semen according to many researchers (16,17,18,19 and 20). Different studies demonstrate that presence of bacteria in seminal fluid has been associated with infertility (21). Changes in sperm parameters that could account for this effect include reduced cell counts reduced motility or morphology alteration (22). *Stenotrophomonas maltophilia* was the most prevalent bacterial strain detected in cryopreserved samples (19). In current study, the percentage of *Stenotrophomonas maltophilia* more than other types of bacteria that may be return to it widely distributed including moist hospital environments as a contaminated bacteria (23) and also it found in liquid nitrogen (24). *Pseudomonas aeruginosa* is a frequently isolated contaminant of bull semen, (19) *Pseudomonas aeruginosa* can cause serious genital diseases in breeding bulls (25) and interfere with the sperm cell viability and probably lower the conception rate too (26, 27), Also if *Pseudomonas aeruginosa* is transmitted to the females genital tract at the time of breeding, it can result in infectious endometritis and associated subfertility (28).

- **Bacterial count:** The results of bacterial count in samples of imported frozen semen  $10.43 \pm 1.05$ ,  $12.49 \pm 0.03$  for bacteria *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* respectively.
- **The results of sensitivity test to antibiotics:** Susceptibility test Showed that most isolates of bacteria which was isolated from frozen semen resistance to most antibiotics used, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* isolated from samples of imported frozen semen resistant to Penicillin, Streptomycin and Amoxicillin in percentage 100%. Bacterial resistance to antibiotics as follows: *Stenotrophomonas maltophilia* resistant to Cefotaxime 33.3%, Gentamycin 40.5%, Tobramycin 95.2%, Amikacin 66.7%, Vancomycin 50%, Erythromycin 16.7%, Tetracycline 4.8% and Ceftriaxone 90.5%, while *Stenotrophomonas maltophilia* was sensitive 100% for Ceftazidime, Ciprofloxacin and Chloramphenicol. *Pseudomonas aeruginosa* resist 100% for each of Gentamycin, Tobramycin, Amikacin, Erythromycin, and Ceftriaxone. The resistance percentage for each of the Ciprofloxacin, Cefotaxime 33.3%, and 66.7% for Tetracycline, Vancomycin and Chloramphenicol as in table (3). Gentamycin, being broad spectrum, are more effective against gram-positive and negative bacteria (29, 30). Another explanation could be that some of the organisms, due to excessive use of the drug, had mutated and became resistant (30, 31). Alternatively, the sensitivity of SP (Streptomycin and Penicillin) against the microorganisms was reduced. Most bacterial isolates in current study were susceptible to Gentamycin, this results agreed with the results obtained by others (32, 33), this might be attributed to the action of Gentamycin that return to aminoglycosides group. The resistance *Pseudomonas* to Streptomycin and Penicillin were also reported by Palli *et al.* (34) and Aleem *et al.* (35). Lazarevic *et al.* (36) reported the wide uses to Penicillin and Amoxicillin in treatment may leads to high resistance to these antibiotics, also the random use of antibiotics causes a high resistance percentage (37).

**Table (3) The results of susceptibility test to antibiotics for isolated bacteria from imported frozen semen**

Type of bacteria		<i>Stenotrophomonas maltophilia</i>	<i>Pseudomonas aeruginosa</i>
Penicillin	No.	42	3
	%	100%	100%
Streptomycin	No.	42	3
	%	100%	100%
Ceftazidime	No.	0	0
	%	0%	0%
Ciprofloxacin	No.	0	1
	%	0%	33.3%
Cefotaxime	No.	14	1
	%	33.3%	33.3%
Gentamicin	No.	17	3
	%	40.5%	100%
Tobramycin	No.	40	3
	%	95.2%	100%
Amikacin	No.	28	3
	%	66.7%	100%
Amoxicillin	No.	42	3
	%	100%	100%
Vancomycin	No.	21	2
	%	50%	66.7%
Erythromycin	No.	7	3
	%	16.7	100%
Chloramphenicol	No.	0	2
	%	0%	66.7%
Tetracyclin	No.	2	2
	%	4.8%	66.7%
Ceftriaxone	No.	38	3
	%	90.5%	100%
Total	No.	42	3

- **Some characteristics of frozen semen.**

- pH:** The results of current study showed that the pH of the frozen semen tended to the neutral  $7.01 \pm 0.03$  table (4). Similar observations have been reported (38) as a results of current study, the pH ranged between 6.5-7.0, where researchers noted that a higher metabolic rate is expected when the pH of semen is maintained near neutrality 7.0. They suggested the importance of diluting semen in a buffered medium that resist changes in pH, so that maximum fertile life of the semen can be maintained (38).
- Percentage of sperm motility (%):** Sperm motility is the most parameter frequently used to measure sperm viability of the ejaculate and during the process of cryopreservation, and it has been related to fertility post artificial insemination (1). Sperm motility percentage of imported frozen semen  $61.3 \pm 2.00$  table (4). Decrease the percentage of sperm motility in the study of (1, 39)  $52.6 \pm 2.4\%$ , 50%, respectively compared with current study, that decrease might be due to the difference in breed, age, management-method of bulls. As well as, the percentage of sperm motility (forward movement) affected by freezing process because the spermatozoa exposures to different temperatures and PH with a long of neutral and freezing processes (40). Also the packaging, handling and freezing the semen in straws considered as an important causes of low quality semen (41).
- Percentage of sperm viability (Live and dead sperms %):** Semen with more than 30% initial dead spermatozoa may not be suitable for storage and freezing (42). Differential staining techniques have been used for determination of live and dead

spermatozoa (43). The results showed that the viability of imported frozen semen was  $76.2 \pm 2.06$ , (Table 4). The values of live spermatozoa in the current study are higher than the average values recorded by other worker (44, 42)  $61.6 \pm 2.59$  and  $43.5 \pm 1.46$ , respectively. This difference might be due to semen which has been frozen is difficult to assess with eosin as cryoprotectants, such as glycerol enhance penetration of the vital stain into the cell, thereby giving artificially high percentage of dead cells. Bearden and Fuquay (38) demonstrated the sperm viability which have a high correlations with fertility.

4. **Percentage of morphologically abnormal sperms:** Sperm morphology indicates normality or eventual deviations of the spermatogenesis and sperm maturation in the epididymis (1). The proportion of Morphologically abnormal spermatozoa in semen correlated negatively with fertility (45, 46). The result of abnormal sperms in present study was  $13.54 \pm 0.67$ , Table (4). The finding of present study was agreed with many investigators observed the incidence of spermatozoa with abnormal morphology in fertile bull to be 0-18% (47). The values of abnormalities recorded in the present study are lower than the average reported by Zalzal (44)  $34.1 \pm 2.24$ , the differences among the bulls of the studies which may be due to variation in general healthy body condition, their scrotal circumference, breed, age, genetic factors and the functions of accessory sex glands (48, 49). Moreover, collection frequency, precollection sexual stimulation, feeding regimen and climatic conditions can also influence the sperm abnormalities (50).

It was concluded from this study that the imported bull frozen semen might be contaminated by several types of bacteria and influence the semen pictures.

**Table (4) Some characteristics of frozen semen**

Characteristic	Imported straws (100)
pH	$7.01 \pm 0.03$
Individual motility (Motile sperm %)	$61.3 \pm 2.00^{ab}$
Live sperms %	$76.2 \pm 2.06^b$
Dead sperms %	$23.8 \pm 2.06^a$
Abnormal spermatozoa %	$13.54 \pm 0.67^a$

### References

1. Al-Makhzoomi, A. (2005). Sperm morphology in progeny-tested Swedish AI dairy bull sires. Swedish University of Agricultural Sciences (SLU), 54:1403-2201.
2. Medeiros, C. M.; Forell, F.; Oliveira, A. T. & Rodrigues, J. L. (2002). Current status of sperm cryopreservation: why isn't better. Theriogenology, 57:327-344.
3. Cottell, E.; McMorro, J.; Lennon, B. & Fawzy, M. (1996). Microbial Contamination in an IVF-embryo transfer system. Fertil Steril., 66: 776-780.
4. Khalili, M. A.; Pourshafie, M. R.; Saifi, M. & Khalili, M. B. (2000). Bacterial infection of the reproductive tract of infertile men in Iran. MEFSJ, 5:126-131.
5. Nunez-Calonge, R.; Caballero, P.; Rendondo, C. & Baquero, F. (1998). Urea plasma urealyticum reduces motility and induce membrane alteration in human spermatozoa. Hum. Rep., 13: 2750- 2761.
6. Cowan, S. T. (1977). Manual for the Identification of Medical Bacteria. 2<sup>nd</sup> Ed. Cambridge university press.
7. Miles, A. A.; Misra, S. S. & Irwin, J. V. (1938). The estimation of the bacteriocidal power of blood. J. Hyg., 38: 732-749.
8. Baurer, A. W.; Kirby, W. A.; Sherris, J. S. & Turk, M. (1966). Antibiotic susceptibility testing by a standardised single disc method. Am. J. Clin. Pathol., 45:493-496.
9. Dahmani, Y. (2009). Semen Evaluation Methods In Cattle. Magapor., 22:1-7.

10. Abro, S. H.; Wagan, R.; Tunio, M. T.; Kamboh, A. A. & Munir, M. (2009). Biochemical activities of bacterial species isolated from the frozen semen of cattle. *J. Agric. Soc. Sci.*, 5: 109-113.
11. Sharma, S. N. & Adhlakha, S. C. (1996). Text book of Veterinary Microbiology, 1<sup>st</sup> ed.
12. Nizamani, A. W. (1999). Studies on the bacterial flora of uteri of slaughtered sheep. M.Sc. Thesis, Faculty of A.H. and Veterinary Science Sindh Agriculture University, Tandojam, Pakistan.
13. Parkash, D. (2000). Bacteriological studies on mastitis in ewes and goats. M.Sc. Thesis, Faculty of A. H. and Veterinary Sciences Sindh Agriculture University, Tandojam, Pakistan.
14. Collins, C. H. & Patricia, M. L. (1999). Microbiological Methods, 8<sup>th</sup> ed., Company, New York.
15. Piasecka-Serafin, M. (1972). The effect of the sediment accumulated in containers under experimental conditions on the infection of semen stored directly in liquid nitrogen (2196 degree C). *Bull. Acad. Pol .Sci. Biol.*, 20:263-267.
16. Shalika, S.; Dugan, K.; Smith, R. D. & Padilla, S. L. (1996). The effect of positive semen bacterial and Ureaplasma cultures on in-vitro fertilization success. *Human Reprod.*, 11 (12): 2789-2792.
17. Mazzilli, F.; Delfino, M.; Imbrogno, N.; Elia, J. & Dondero, F. (2006). Survival of micro-organisms in cryostorage of human sperm. *Cell Tissue Bank*, 7:75-79.
18. Ibadin, O. K. & Ibeh, I. N. (2008). Bacteriospermia and sperm quality in infertile male patient at University of Benin Teaching Hospital. *Malaysian J. Microbiol.*, 4:65- 67.
19. Bielanski, A. & Vajta, G. (2009). Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. *Human Reprod.*, 24: 2457-2467.
20. Mozo-Martín, R.; Dahmani, Y.; Larraz, C. & Ubeda J. L. (2010). Main Bacterial species isolated from commercial seminal doses and antibiotic activity, *Magapor.*, 22:57-61.
21. Khalili, M. A.; Pourshafie, M. R.; Saifi, M. & Khalili, M. B. (2000). Bacterial infection of the reproductive tract of infertile men in Iran. *MEFSJ*, 5:126-131.
22. Toth, A.; Swenson, C. E. & O'Leary, W. M. (1978). Light microscopy as an aid in predicting urea plasma infection in human semen. *Fertil. Steril.*, 30:585-591.
23. Warren, J. R. (2007). Non-Fermentive Gram-Negative Bacteria. *Manual of Clin. Microbiol.*, 9<sup>th</sup> Ed., PP. 48-56.
24. Bielanski, A.; Bergeron, H.; Lau, P. C. & Devenish, J. (2003). Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiol.*, 46:146-152.
25. Borowicz, J. J.; Brishammar, S. & Gerhardson, B. (1995). A *Xanthomonas maltophilia* isolate tolerating up to 1% sodium azide in Tris/HCl buffer. *World J. Microbiol. Biotechnol.*, 11:236-237.
26. Eaglesome, M. D. & Garcia, M. M. (1995). Comparisons of antibiotic combinations to control pseudomonas aeruginosa in bovine semen. *Can. J. Vet. Res.*, 59:73-75.
27. Corona, A.; Cossu, I.; Bertulu, A. & Cherchi, R. (2006). Characterization of bacteria in fresh semen of stallions during the breeding season. *Anim. Reprod. Sci.*, 94:85-88.
28. Varner, D. D.; Scanlan, C. M.; Thompson, J. A.; Brumbaugh, G. W.; Blanchard, T. L.; Carlton, T. M. & Johnson, L. (1998). Bacteriology of preserved stallion semen and antibiotics in semen extender. *Theriogenology*, 50:559-573.
29. Ball, H. J.; Logan, E. F. & Orr, W. (1987). Isolation of mycoplasmas from bovine semen in Northern Ireland. *Vet. Rec.*, 121:322-324.
30. Shin, S. J.; Lein, D. H.; Patten, V. H. & Ruhnke, H. L. (1988). Anew antibiotics combination for frozen bovine semen. *Theriogenology*, 29:577-591.

31. Parusov, V. P. (1974). Disinfection for *Pseudomonas aeruginosa* contamination of bull semen. Vet. Bull., 44:2647-2654.
32. Ahmad, K. & Foote, R. H. (1985). Motility and fertility of frozen bull in tris-yolk and milk extenders containing amikacin sulfate. J. Dairy Sci., 68:2083-2086.
33. Cowan, S. T. (1977). Manual for the Identification of Medical Bacteria. 2<sup>nd</sup> Ed. Cambridge university press.
34. Naber, K. G.; Grimm, H.; Rosenthal, E. D. K.; Shah, P. M. & Wledemann, B. (1990). Resistance of Aminoglycosides the situation in the Federal republic of Germany. J. Inter. Med. Res., 18: 6- 26.
35. Palli, G. K.; Neporada, V. P.; Volyanskii, Y. U. L. & Korpan, A. I. (1975). Sensitivity of *Pseudomonas aeruginosa* to antimicrobial agents, Medicine Institute, Chernovitsy, Ukrainskaya, USSR, Moscow, 3:80-82.
36. Aleem, M.; Chaudhry, R. A.; Khan, N. U.; Rizvi, A. R. & Ahmed, R. (1990). Occurrence of pathogenic bacteria in buffalo semen. Buffalo J., 6:93-98.
37. Lazarevic, V.; Soldo, B.; Düsterhöft, A.; Hilbert, H.; Mauël, C. & Karamata, D. (1998). Introns and intein coding sequence in the ribonucleotide reductase genes of *Bacillus subtilis* temperate bacteriophage SPB. Proceedings of the National Academy of Sciences of the United States of America, 95: 1692-1697.
38. Jacoby, G. A. & Sutton, L. (1999). Pseudomonas susceptibility to sulbactam. Am. Soc. Microbiol., 33:583-584.
39. Bearden, H. J. & Fuquay, J. W. (2000). Applied Animal Reproduction. 5<sup>th</sup> ed. Upper Saddle, New Jersey: Prentice Hall Inc., PP. 138-147.
40. Barbas, J. P. & Mascarenhas, R. D. (2009). Cryopreservation of domestic animal sperm cells. Springer, 10:49-62.
41. Rajju, M. S. & Rao, A. R. (1982). Note on semen characteristics of crossbred and purebred bulls. Indian J. Anim. Sci., 52: 1230-1232.
42. Becker, W. C. (1977). Influence of glycerol levels, diluent and post-thaw temperature on motility and acrosomal maintenance in bovine semen frozen in plastic straws. J Anim. Sci., 44:1067-1071.
43. Kunbhar, H. K.; Raho, T. A. & Samo, M. U. (2011). In vitro fertility assessment of bull semen. Roavs., 2: 102-106.
44. Rochwerger, L. & Cuaniscu, P. S. (1992). Redistribution of a rat sperm epididymal glycoprotein after “*invitro*” and “*in-vivo*” capacitation. Mol. Reprod. and Develop., 31: 34-40.
45. Zalzal, S. J. (1989). Bacterial contamination in semen of Bulls in artificial insemination center. M.SC in Theriogenology. Surgery and Obstetrics department /College of Vet. Med./ Baghdad University.
46. Shamsuddin, M. & Larsson, B. (1993). In vitro development of bovine embryos after fertilization using semen from different donors. Reprod. Domest. Anim., 28:77-84.
47. Shamsuddin, M.; Rodriguez-Martinez, H. & Larsson, B. (1993). Fertilizing capacity of bovine spermatozoa selected after swim up in hyaluronic acid containing medium. Reprod. Fertil. Develop., 5: 307-315.
48. Sarder, M. J. U. (2004). Morphological sperm abnormalities of different breeds of bull and its impact on conception rate of cows in AI programme. Bangl. J. Vet. Med., 2: 129-135.
49. Leon, H.; Porras, A. A. & Galina, C. S. (1991). Effect of collection method on semen characteristics of zebu and European type cattle in the tropics. Theriogenology., 6: 349-355.
50. Sharma, M. L.; Mohan, G. & Sahni, K. L. (1991). Characteristics and cryopreservation of semen of Holstein-Friesian bulls under tropics. Indian J. Anim. Sci., 61: 977-979.

## Impact of Lactic acid bacteria (LAB) as probiotic against bacterial pathogen from *Cyprinus carpio* L.

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### Abstract

The aim of the present study is to investigate the potential of lactic acid bacteria as a probiotic (biological diseases control). Six strains of lactic acid bacteria J<sub>1</sub>, J<sub>3</sub>, J<sub>4</sub>, J<sub>6</sub>, J<sub>7</sub>, J<sub>8</sub> was Isolated from intestines of the common carp *Cyprinus carpio* were compared as to the antagonistic activity against fish pathogen bacteria *Aeromonas hydrophila*, *Pseudomonas luteola*, *Pseudomonas aeruginosa*, *Serratia rubidaea* and *staph sp.* Different method were used for measuring growth by optical density, agar well diffusion and cross- streak method also screened from bile salt and antibiotic tolerance. All strains of LAB showed different spectra of inhibition against pathogenic bacteria. The highest inhibition measured against *serratia rubidaea* and *A. hydrophila*, moderate inhibition occur against *P. luteola* and *P. aeruginosa* and lower inhibition to *staph sp.* Also culture supernatant free fluid of all LAB showed no antagonistic activity against pathogenic bacteria moreover all strain show resistance to all antibiotic sensitivity OA2A-P disc except J<sub>1</sub>, J<sub>3</sub>, J<sub>4</sub> were sensitive to erythromycin in concentration 60 mcg after incubation for 48 hr and only J<sub>1</sub>, J<sub>3</sub>, J<sub>4</sub>, J<sub>6</sub> were exhibited tolerance reaction to bile salt at concentration not more than 3000 ppm the present study recommend to use lactic acid bacteria J<sub>6</sub> as a good probiotic remedy to the fish culture in Iraq.

تأثير بكتريا حامض اللاكتيك LAB كمعززات حيوية ضد البكتريا المرضية في

*Cyprinus carpio* L.

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### الخلاصة

هدفت الدراسة الحالية الى اختيار قدرة بكتريا حامض اللاكتيك كمعززات حيوية في السيطرة البايولوجية ضد البكتريا المرضية. قورنت ستة عزلات من بكتريا حامض اللاكتيك J<sub>1</sub>, J<sub>3</sub>, J<sub>4</sub>, J<sub>6</sub>, J<sub>7</sub>, J<sub>8</sub> فيما بينها والمعزولة من أمعاء اسماك الكارب الشائع (*Cyprinus carpio*) في فعاليتها التضادية ضد البكتريا المرضية للأسماك *Aeromonas Serratia rubidaea*, *Pseudomonas aeruginosa*, *Pseudomonas luteola*, *hydrophila* و *staph sp.* استعملت طرق مختلفة وهي قياس معدل النمو باستعمال تقنية درجة العتامة (O.D) والتنشيط باستخدام الانتشار بحفر الاكار agar well diffusion وطريقة النمو التضادي التعاكسي cross- streak method فضلا عن مقاومة البكتيريا للبنية للمضادات الحيوية والاملاح الصفراء. أظهرت جميع عثر البكتريا للبنية مستويات تثبيط تضادي مختلفة ضد البكتريا المرضية فكان اعلى تثبيط هو ضد بكتريا *Serratia rubidaea* و *Aeromonas hydrophil*. أما التنشيط المتوسط فقد ظهر ضد بكتريا *Pseudomonas aeruginosa* و *Pseudomonas luteola* وادنى تثبيط ظهر ضد *staph sp* إلا ان السائل الطافي الخالي من

البكتريا (cff) لجميع عثر البكتريا اللبنية لم يظهر أي فعالية تضادية ضد البكتريا المرضية. أظهرت جميع العثر البكتريا اللبنية مقاومة ضد المضادات الحيوية المستعملة في قرص فحص الحساسية OA2A –P disc ماعدا J1, J4, J3 كانت حساسة للارثرومايسين بتركيز 60 mcg بعد حضانة 48 ساعة وعثر J1, J3, J4, J6 مقاومة لأملح الصفراء بتركيز ليس اكثر من 3000 ppm. بينت الدراسة ان استعمال العزلة J6 من بكتريا حامض اللاكتيك كمعزز حيوي جيد للاستزراع السمكي في العراق.

## Introduction

Various authors have shown that lactic acid bacteria are a part of the normal intestinal flora of fish (1). Most of the evidence comes from salmonid species like Arctic charr (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*), (2). (3) described the presence of lactic acid bacteria, including *Lactobacillus* in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacteria composition and load in some fish like carp (*Cyprinus carpio*) which showed the highest content of lactic acid bacteria in the intestines. Intensive aqua farming accompanies several disease problems often due to opportunistic pathogens as evident from general aquaculture. High stocking densities, high food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria (4). Due to this negative balance of the microbial community in rearing water as well as in fish gut, the aqua culturists often face mass mortality of their stocks. The use of antibiotics and chemotherapy remains the method of choice as disease control strategy. The abuse of chemotherapeutics, especially antibiotics has resulted in development of multiple antibiotic resistant bacteria (5). Increased concern about antibiotic resistant microorganisms has led to several alternatives including the use of non-pathogenic microorganisms as probiotic (6). Probiotic concept has been widely applied for health promoting in farm animals, pets and aquatic animals (7, 8). Probiotics are usually defined as live microbial feed supplements which beneficially affects on the host animal by improving its intestinal microbial balance (9). Based on this definition, probiotics may include microbial adjuncts that prevent pathogens from proliferating in the intestinal tract (10). Lactic acid bacteria (LAB) are among the most important probiotic microorganism typically associated with gastrointestinal tract whereas they exercise beneficial effects. (11) suggested that probiotic bacteria would be found to be useful not only as food but also as biological controllers of fish disease and activators of nutrient regeneration. In the biological control in aquaculture emerge and since then the research effort has continually increased. *Bacillus sp.* in often antagonistic against other fresh water fish pathogenic bacteria (12,13,14) was reported to the similar experiments have shown that the inoculation of some probiotic strains, mainly lactic acid bacteria, increase fish survival after being challenged with fish pathogens. (15) Showed inhibition of *Vibrio. vulnificus* by LAB and stimulation of the non-specific immune response resulting in resistance to disease in the prawn fed on LAB incorporated diets. Selection of probiotic strains is achieved by screening procedures for several characteristics *in vitro*, such as inhibitory activities against several fish pathogens and gastric and intestinal secretions (16). In the present study we compared the antimicrobial activity of probiotic bacteria (LAB) J1, J3, J4, J6, J7, J8 which isolated in previous study by (17) from gastrointestinal tract of common carp fish *Cyprinus carpio* against pathogenic bacteria using different methods such as growth monitored by measuring the optical density (O.D), cross streaking and well diffusion method.

## Materials and Methods

- **Chemicals and media:** Analytical grade chemicals and dyes were obtained from Al-Kindi company for production of veterinary vaccines and drugs, bacteriological media were obtained from oxoid UK which include set of biochemical media, blood agar base, Nutrient agar, Nutrient broth tryptic soy agar, gas generating Kit and sense test disk from lamb GT. Manchester; England. MRS media were obtained from Himedia, and bile salt were supplied by sigma.
- **Bacterial strains:** bacterial strains includes:
  1. *Pseudomonas aeruginosa* were obtained from central health laboratory/ Baghdad /ministry of health.
  2. *Aeromonas hydrophila*, *Staph sp.* *Serratia rubidaea*, *Pseudomonas luteola* were obtained from the laboratory of fish pathology veterinary college of Baghdad University. All bacterial strains were cultivated in 10 ml of Nutrient broth. Bacterial cultivation was performed at 30°C for 20 h. Approximately 1 ml of bacterial culture was transferred to 9 ml of liquid medium and incubated at 30°C for another 18 h, cell concentration was then adjusted to obtain final concentration of 10<sup>6</sup> CFU/ ml for determination of antibacterial activity.
  3. lactic acid bacteria J1, J3, J4, J6, J7, J8 isolated from gastrointestinal tract of common carp fish in previous study by (17). Before use LAB strains were activated in MRS broth (18).
- **Bile Tolerance:** The modified method of (19) was used to determine bile tolerance of selected LAB. Before testing for bile tolerance, LAB strains were grown at 30°C for 24 hour in MRS broth without bile. One ml of the culture broth was poured on to MRS agar with bile salt concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 30°C for 48 hour.
- **Bacteriocin assay:** The isolates of lactic acid bacteria were propagated in MRS broth and incubated at 30°C for 48 hours. Cells were separated by centrifugation at 5000 rpm for 10 minutes The clear supernatants obtained were treated as follows:
  1. Clear free fluid supernatants without any treatment (CFF).
  2. Neutralizing clear free fluid supernatants by adjusted to pH 6.5-7.0 with 2N NaOH (NCFF). Cell free supernatant was passed through 0.22µm membrane filter and evaluated for antimicrobial activity by agar well diffusion method (20).The antagonistic effects of the culture supernatants of bacteriocin producing *Lactobacillus* were tested on various indicator organisms on Nutrient agar. All cultures were grown aerobically at 30°C for 48 hours. Inhibition zones around the wells were measured.
- **Inhibitory effect by agar well diffusion method:** The inhibitory effects of *Lactobacillus* strains on indicator organisms were carried by agar well diffusion assay. Petri dishes with nutrient agar that were previously inoculated with 0.1 ml of 24 hours old nutrient broth culture of individual test bacteria were poured. Once solidified, Petri dishes were stored for 2 hours at 4°C. Four wells of 5 mm diameter were made and filled with 50µl of culture supernatant. The inoculated plates were kept at 4°C for 2 hours and then incubated at 30°C for 24 hours. Inhibition zones around the wells were measured (20).
- **Inhibitory effect by O.D. measuring growth method:** According to (21) Five ml replicates of nutrient broth were individually supplemented with 2 ml of each of the



individual LAB strain supernatants. The each tubes were then inoculated with freshly grown culture of each indicator pathogenic bacteria respectively and incubated at  $28 \pm 2^\circ\text{C}$  for 48 hr and growth was recorded by measuring the optical density at 540 nm. Control tubes comprised of nutrient broth inoculated with indicator pathogenic bacteria respectively

- **Inhibitory effect by cross – strack method:** All the three LAB strains were streaked on Tryptone Soya Agar (TSA) plates containing 1.0% sodium chloride and incubated at  $28 \pm 2^\circ\text{C}$  for 48 hr. Freshly grown culture of five pathogen bacteria was streaked perpendicular to this growth and after incubation at  $28 \pm 2^\circ\text{C}$  observed for antagonism according to (15).
- **Antibiogram of LAB isolates:** The isolates were inoculated into MRS broth individually and incubated for 24 hr about 25 ml of MRS agar was seeded with the cultures of LAB isolates  $10^6$  CFU/ml mixed well. Poured in to sterile petriplates and stored at  $4^\circ\text{C}$  for 1hr to solidify the media (OA21-P) antibiotics in a single ring were placed up side down pressed on the top of the agar plates and kept again at  $4^\circ\text{C}$  for 1hr the plate were incubated at  $30^\circ\text{C}$  for 24 hr and 48 hr resistance was defined as the absence of a growth inhibition zone around the discs.

## Results and Discussion

- **Bile salt tolerance:** Six strain of LAB were isolated from gastrointestinal tract of common carp fish (*cyprinus carpio*) were tested for their ability to grow at bile sult of 2000, 3000, 4000 ppm in order to bile tolerant strains. Only J1, J3, J4, J6 strain were able to grow in MRS agar supplemented with 2000 and 3000 ppm bile salt while all strains were sensitive to grow in MRS ager supplemented with 4000 ppm bile salt (Table 1).

This is similar to the result obtained by (22) with the strains of *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM 10) and *Lactobacillus sake* (L2) were the most resistant to 3000 ppm bile salt at pH 6 (23) reported that the bile salt tolerance of the *Lactobacillus* strains were able to grow in MRS agar supplemented with 3000 ppm bile salt. It has been reported that certain strains of *Lactobacillus* are able to reduce this detergent effect by their ability to hydrolyze bile salt by bile salt hydrolase enzyme (BSH) (22), which are then readily excreted from the GI-tract (24). This particular enzyme decreases bile solubility and thus weakening its detergent effect.

**Table (1) Bile salt tolerance of lactic acid bacteria isolated from common carp fish**

Bile Salt Concentration (ppm)	Lactic acid bactria strains					
	J1	J3	J4	J6	J7	J8
2000	+	+	+	+	-	+
3000	+	+	+	+	-	-
4000	-	-	-	-	-	-

(+) tolerance (-) sensitive

- **Antibacterial activity against fish pathogens:** The antagonism of the six LAB strain J1, J3, J4, J6, J7, J8 was ascertained by as test tube well as by well diffusion and cross–streaking on TSA plates. Inhibitory effect by O. D. test tube nutrient broth containing cell free fluid supernatants (cff) of the six strains of LAB respectively failed to record turbidity after inoculating with the pathogen (*Aeromonas hydrophila*, *Pseudomonas luteola*, *Pseudomonas leutela*, *Serratia rubidaeeae*, *Staph .sp.* ) and incubating for 30 h

implying inhibition (Fig. 1, 2, 3, 4, 5) Turbidity measured as optical density was obtained in all the control tubes while tubes containing the LAB supernatants recorded low optical densities of the value of zero time. the culture media (supernatant) were used which showed highest inhibition effects of *Bacillus sp.* may be due to production of antibiotics bacteriocins, Lysozymes, proteases and hydrogen peroxide and the alternative of pH values by the production of organic acid (25).

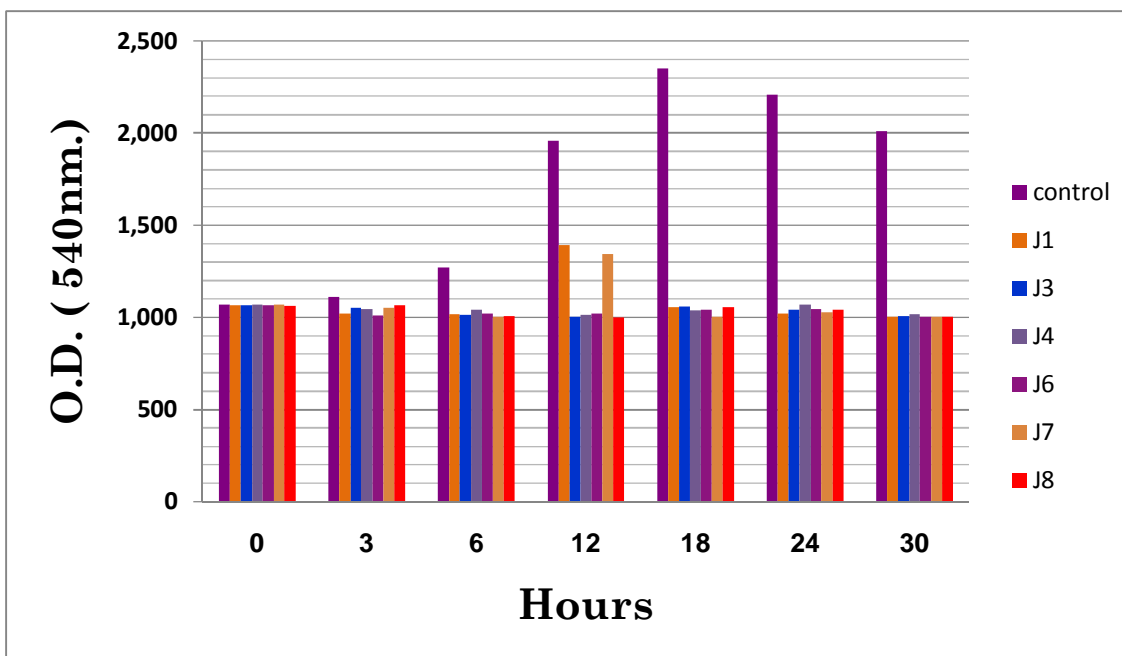


Fig. (1) Optical density of *Aeromonas hydrophila* (in test tube) *in vitro* containing six strains of LAB

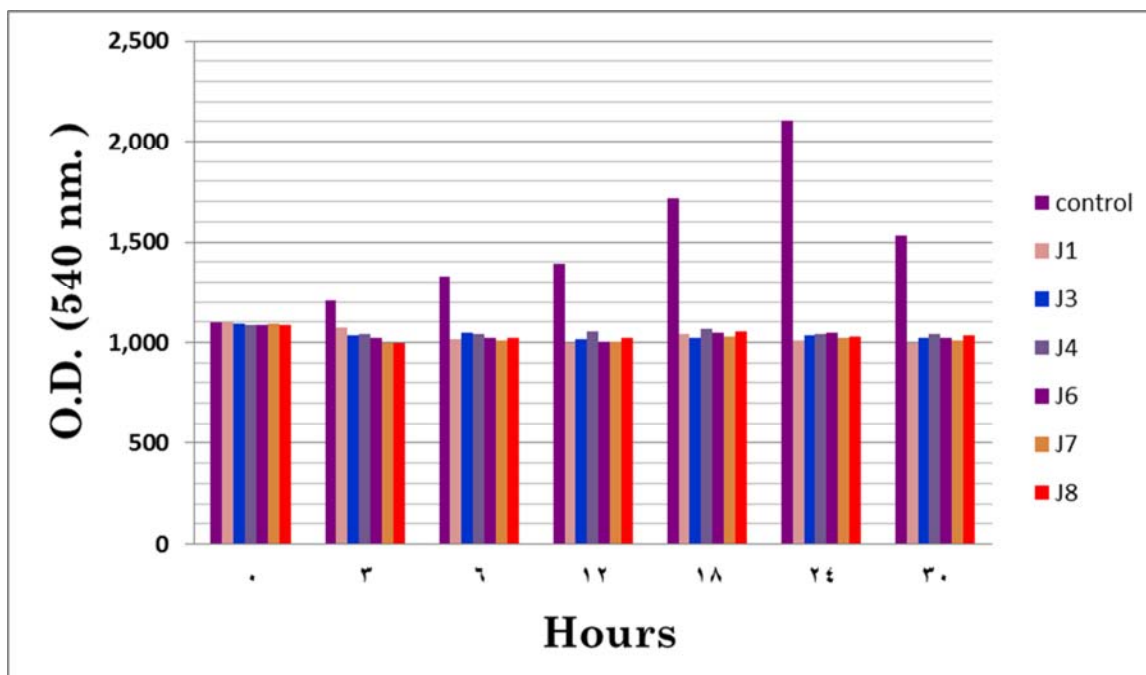


Fig. (2) Optical density of *Pseudomonas luteola* (in test tube) *in vitro* containing six strains of LAB

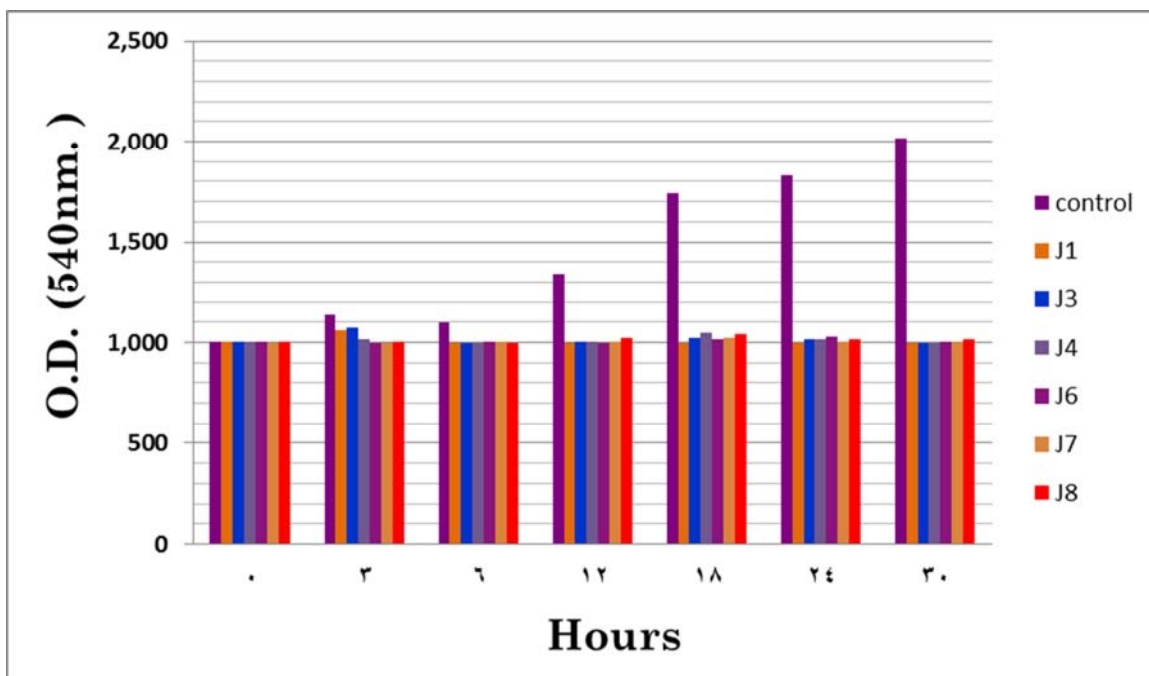


Fig. (3) Optical density of *Pseudomonas aeruginosa* (in test tube) *in vitro* containing six strains of LAB

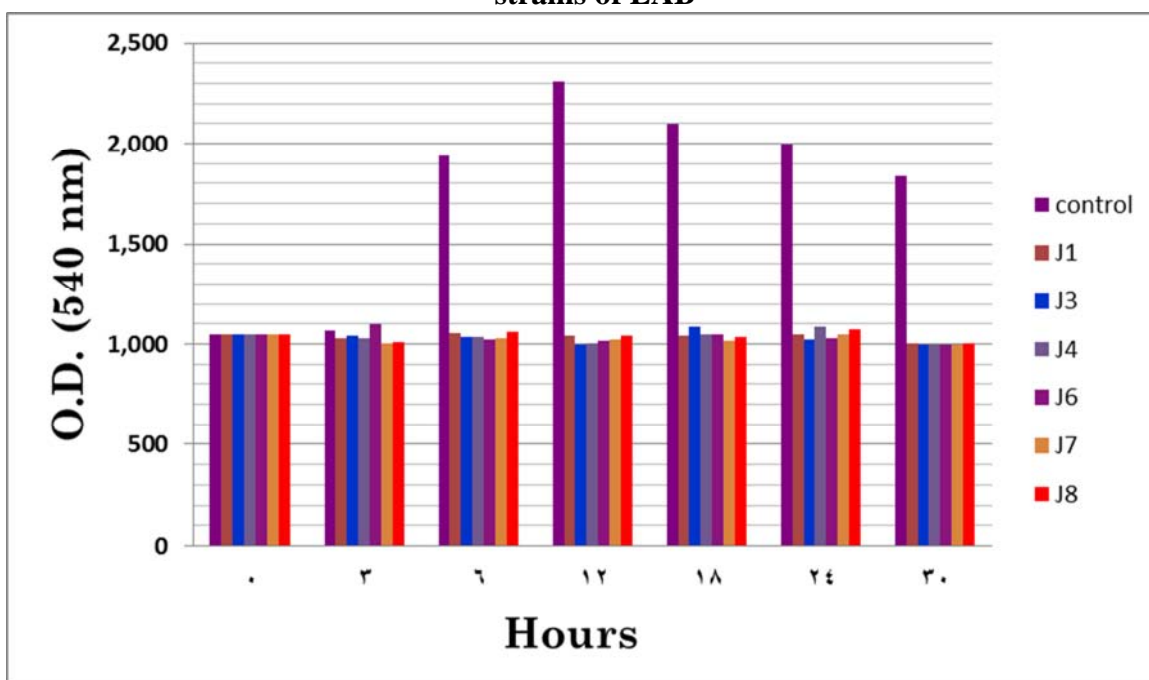
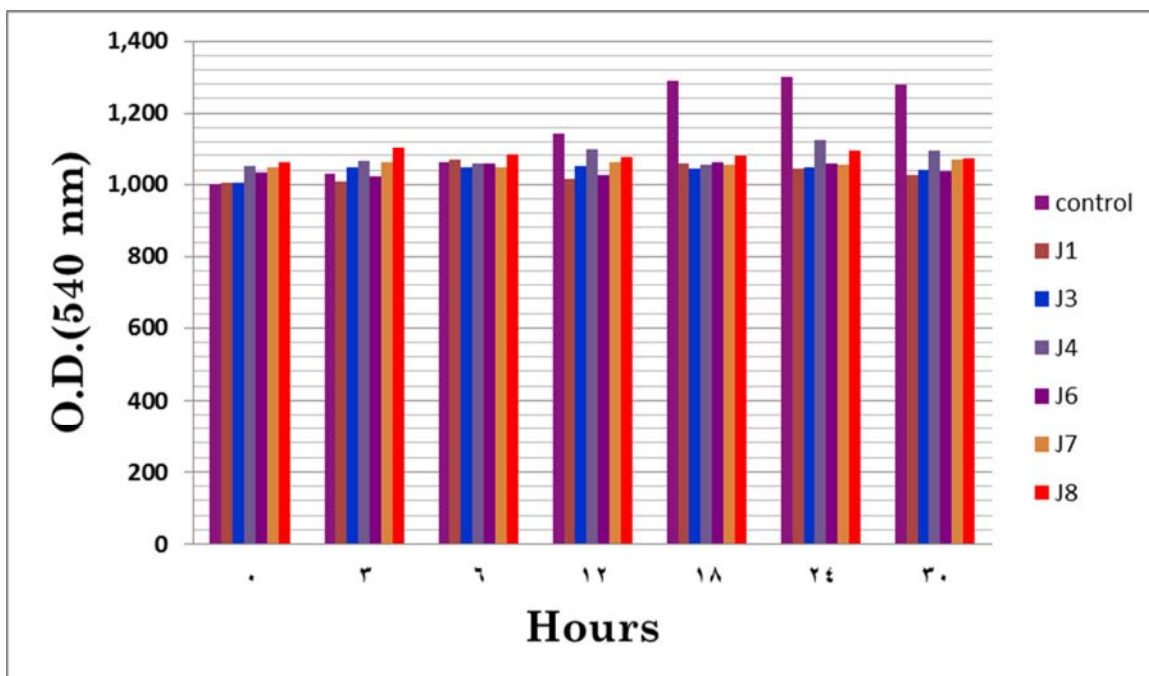


Fig. (4) Optical density of *Serratia rubidaea* (in test tube) *in vitro* containing six strains of LAB



**Fig. (5) Optical density of *Staph. sp.* (in test tube) *in vitro* containing six strains of LAB**  
**- Inhibitory effect by well diffusion and cross – streak method:** Six strains of LAB were assay for the ability to inhibit growth of (*Aeromonas hydrophila*, *Pseudomonas luteola*, *Pseudomonas aeruginosa*, *Serratia rubidaeeae*, *Staph. sp.*) by agar well diffusion method culture supernatants free fluid (cff) of LAB strains J1, J3, J4, J6, J7, J8 exhibited varying degree of inhibition activity against indicator (pathogen microorganism).

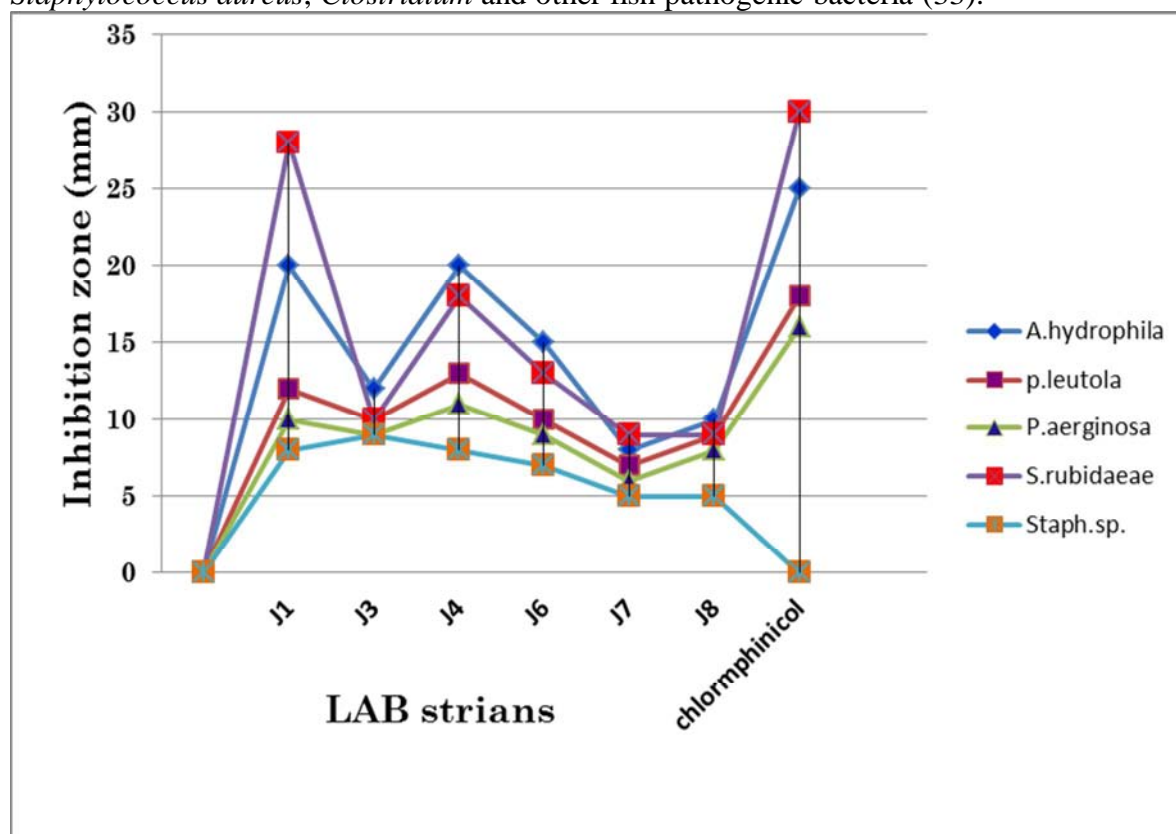
All LAB strain were exhibited highest antibacterial activity against *Serratia rubidaeeae* *Aeromonas hydrophila* respectively. Similar result were reported by (26) they showed the activity against *A. hydrophila* of 19 LAB strains including *Carnobacterium piscicola* and *Lactobacillus. plantarum*. The addition of freeze-dried *Carnobacterium. divergens* to compound feed did not improve the resistance of salmon fry challenged against pathogenic *A. hydrophila* (27). However, a similar dietary addition reduced the mortality rate of Atlantic cod fry when challenged against *Vibrio. anguillarum* (28). (29) found The inhibition zone 8 mm was observed on the plates inoculated with *Aeromonas hydrophila* by *Bacillus sp.* The moderate antimicrobial activity were against *Pseudomonas luteola*.

**Table (2) Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against pathogen microorganisms**

LAB strain	<i>Aeromonas hydrophila</i>		<i>Pseudomonas leutela</i>		<i>Pseudomonas aeruginosa</i>		<i>Serratia rubidaeeae</i>		<i>Staph. sp</i>	
	CFF	CFBH	CFF	CFBH	CFF	CFBH	CFF	CFBH	CFF	CFBH
<b>J1</b>	20 mm	nil	12 mm	nil	10 mm	nil	28 mm	nil	8 mm	nil
<b>J3</b>	12 mm	nil	10 mm	nil	9 mm	nil	10 mm	nil	9 mm	nil
<b>J4</b>	20 mm	nil	13 mm	nil	11 mm	nil	18 mm	nil	8 mm	nil
<b>J6</b>	15 mm	nil	10 mm	nil	9 mm	nil	13 mm	nil	7 mm	nil
<b>J7</b>	8 mm	nil	7 mm	nil	6 mm	nil	9 mm	nil	5 mm	nil
<b>J8</b>	10 mm	nil	9 mm	nil	8 mm	nil	9 mm	nil	5 mm	nil
<b>Chlormphenicol disc 30 mcg</b>	25 mm		18 mm		16 mm		30 mm		nil	

Note: CFF = culture supernatant, NCCF = culture supernatant adjusted to pH 6.5-7.0 with 1M NaOH,

Which, *Pseudomonas aeruginosa* spoil food at low temperatures as a result of its lipolytic and proteolytic activity (30). Control of *P. aeruginosa* by bacteriocin activity of (31) has been reported that *P. aeruginosa* controlled by bacteriocin activity of *L. casei* and *L. plantarum*. The low antimicrobial activity was against *Staph. sp.* There are many report about the antimicrobial activity of LAB most are against Gram positive bacteria (32) was reported to the isolated *Lactobacillus sp.*, both homofermenters and heterofermenters, were able to inhibit the human and fish pathogens by acid production when using a high glucose concentration. A few strains also inhibited both gram-positive and gram-negative fish and human pathogens with low (0.2%) concentration of glucose in the medium. Inhibitory activities of these strains have been usually detected against related species such as *Staphylococcus aureus*, *Clostridium* and other fish pathogenic bacteria (33).



**Fig. (6) Diameter of inhibition zone (mm) caused by antimicrobial activity of LAB strains against pathogen microorganisms**

Neutralized culture supernatants (CFBH) of all strains exhibited no inhibition (Table 2, Fig. 6). The inhibitory effect of LAB may be due to acid or the bacitracin-like substances or combination of both (34). (35) have revealed that no correlation was found between bacitracin activity, lactic acid and hydrogen peroxide production. They reported that *Lactobacillus* strains 228, 345 and 431 produced H<sub>2</sub>O<sub>2</sub> but did not demonstrate any inhibitory effect. Similar results were obtained (36) showed all strains of LAB may produce H<sub>2</sub>O<sub>2</sub> but did not show any inhibitory effect.

- **Antibiogram of LAB activity:** Behavior of all LAB strains J1, J3, J4, J6, J7 and J8 were resistance to all antibiotic sensitivity OA2A-p disc except the J1, J3, J4 were sensitive to erythromycin in concentration 60 mcg after incubation for 48 hr (Table 3).

**Table (3) Antibigram of LAB isolates determined by antibiotic sensitivity  
OA2A – PDisc**

Antibiotics	After 24hr							After 48hr					
	Concen Tration (mg)	J1	J3	J4	J6	J7	J8	J1	J3	J4	J6	J7	J8
<b>Erythromycin</b>	60 mcg	R	++	++	R	R	+	+++	++	+++	R	R	++
<b>Rifampicin</b>	15 mcg	R	R	R	R	R	++	R	R	R	R	R	++
<b>Colistinsulphat</b>	150mcg	R	R	R	R	R	R	R	R	R	R	R	R
<b>Penicillin</b>	2unit	R	R	R	R	R	R	R	R	R	R	R	R
<b>Kanamycin</b>	1000mcg	R	+	+	R	+	+	+	+	+	R	+	<u>R</u>
<b>Vancomycin</b>	5 mcg	R	<u>R</u>	<u>R</u>	R	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	<u>R</u>	R	+	+

R      resistance  
R     zone inhibition diameter less than 1 cm  
+      zone inhibition diameter > 1 cm  
++     zone inhibition diameter > 2cm  
+++    zone inhibition diameter > 3cm

Besides the production of antimicrobial substances, bile salt tolerance and antibiotic resistance the great variety of mechanisms have been proposed for the action of probiotics. Competition for adhesion receptors in the intestine, competition for nutrients and immune stimulation. Further investigations on these lines would throw more light into the actual mechanism of probiotic action in aquaculture. It was concluded that lactic acid bacteria do offer ample scope as probiotics showing antagonism towards pathogenic bacteria. As these probiotics were able to suppress pathogen growth in vitro and in vivo, it can be hypothesized that they have ability to colonize the gastrointestinal tract of prawn, which however, merits further confirmation. Consequently, they may prove to be suitable candidates for oral administration to farm fish, in commercial ventures to improve health and protect them against infection.

### References

1. Ringø, E. & Gatesoupe, F. J. (1998). Lactic acid bacteria in fish: a review. *Aquacult.* 160: 177-203.
2. Gonzalez, C. L.; Enicinas, J. P.; Garcia Lopez, M. L. & Otero, A. (2000). Characterization and identification of lactic acid bacteria from freshwater fishes. *Food Microbiol.*, 17: 383-391.
3. Kvasnikov, E. I.; Kovalenko, N. K. & Materinskaya, L.G. (1977). Lactic acid bacteria of freshwater fish. *Microbiol.*, 46: 619-619.
4. Austin, B. L. F.; Stuckey, P. A. W.; Robertson, I. E. & Griffith, D. R. W. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J. Fish Dis.*, 18: 93-96.
5. Sørum, H. (2006). Antimicrobial drug resistance in fish pathogens. In: Aarestrup, F.M.(Ed.), *Antimicrobial Resistance in Bacteria of Animal origin*. ASM Press, Washington DC, PP. 213-238.
6. Salminen, S.; Ouwehand, A.; Benno, Y. & Lee, Y. K. (1999). *Trends in Food Sci. Tech.* 10: 107.
7. Nousiainen, J.; Javaninen, P.; Setälä, J. & von Wright, A. (2004). Lactic acid bacteria as animal probiotics. P. 547-580. *In Lactic Acid Bacteria Microbiological and Functional Aspects* 3rd ed. Revised and Expanded.

8. Vaughan, E. E.; Heilig, H. G. H. J.; Ben-Amor, K. & de Vos, W. M. (2005). Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. *FEMS Microbiol.*
9. Fuller, R. (1989). Probiotics in man and animal. *J. Appl. Bacteriol.*, 66: 365-378.
10. Gatesoupe, F. J. (1994). Lactic acid bacteria increase the resistance of Turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio. *Aquatic living resources.* 7:277-282.
11. Yasuds, K. & Taga, N. A. (1980). Mass Culture Method For *Artemia salina* using bacteria as food. *Mer.*, 18:53.
12. Gatesoupe, F.J.(1999).The use of probiotics in aquaculture. *Aquaculture*, 180: 147-165.
13. Rengpipat, S.; Rukpratanporn, S.; Piyatiratitivorakul, S. & Menasaveta, P. (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiotic bacterium (bacillus S11). *Aquacult.*, 191:271.
14. Cai, Y.; Benno, Y.; Nakase, T. & Oh, T. K. (1998). Specific probiotic characterization of *Weissella hellenica* DS-12 isolated from flounder intestine. *J. Gent. Applied Microbiol.*, 44:311-316.
15. De Man, J.; Rogosa, M. & Sharpe, M. E. (1972). A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.*, 23: 130-135.
16. Byun, J. W.; Park, S. C.; Ben, Y. & Oh, T. K. (1997). Probiotic effect of *Lactobacillus* sp. DS-12 in flounder (*Paralichthys olivaceus*). *J. Gent. Appl. Microbiol.*, 43:305- 308.
17. Alfaragi, J. K. & Alsaphar, S. A. A. (2011). Isolation of lactic acid bacteria (LAB) species as probiotic from intestinal contents of common carp *Cyprinus carpio* L. publication acceptance. *J. Biotechnol Res. Center.*
18. Arihara, K.; Ota, H.; Itoh, M.; Kondo, Y.; Sameshima, T.; Yamanaka, H.; Akimoto, M.; Kanai, S. & Miki, T. (1998). *Lactobacillus acidophilus* group lactic acid bacteria applied to meat fermentation. *J. Food Sci.*, 63(3): 544-547.
19. Aslim. B.; Yuksekdog, Z. N.; Sarikaya, E. & Beyatli, Y. (2004). Determination of the bacteriocinlike substances produced by some lactic acid bacteria isolated from Turkish dairy products. *LWT*, 1: 2004.
20. Tagg, J. R. (1991). Bacterial BLIS. *ASM News Letters*, P.57.
21. Erkkila, S. & Petaja, E. (2000). Screening of commercial meat starter cultures at low pH in the presence of bile salts for potential probiotic use. *J. Meat Sci.*, 55: 297-300.
22. Pennacchia, C.; Ercolini, D.; Blaiotta, G.; Pepe, O.; Mauriello, F. & Villani, F. (2004). Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. *J. Meat Sci.*, 67: 309-317.
23. Maragkoudakis, P. A.; Zoumpopoulou, G.; Miaris, C.; Kalantzopoulos, G.; Pot, B. & Tsakalidou, E. (2006). Probiotic potential of *Lactobacillus* strains isolated from dairy products. *J. Int. Dairy*, 16: 189-199.
24. Verschuere, L.; Rombaut, G.; Sorgeloos, P. & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol. and Molecular Biol. Rev.*, 64: 655-671.
25. Lewus, C. B.; Kaiser, A. & Montville, T. J. (1991). Inhibition of food –borne bacterial pathogens by bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environmental Microbiol.*, 57:1683-1688.

26. Gildberg, A. A.; Johansen, J. & Bøggwald, J. (1995). Growth and survival of Atlantic salmon (*Salmo salar*) fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida*. *Aquaculture*, 138:23-34.
27. Gildberg, A.; Mikkelsen, H.; Sandaker, E. & Ringø, E. (1997). Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod *Gadus morhua*. *Hydrobiologia*, 352,279-285.
28. Vijayabaskar, P. & Somasundaram S. T. (2008). Isolation of bacteriocin Producing lactic acid bacteria from fish gut and probiotic activity against common fresh water fish pathogen *Aeromonas hydrophila*. *Biotechnol.*, 7: 124-128.
29. Unluturk, A. & Turantas, F. (1998). *Food Microbiol.* Mengi Tan Press, Cinarli, Izmir, P.605.
30. Kaya, M. (1992). Usage of different level of nitrite and different starter cultures in sucuk and their effects on the growth characteristic of *Listeria monocytogenes* and the some other some quality criteria of sucuk. Ataturk university, Inst. Doc. TH., P.127.
31. Adolfo, B. G. (2004). *Lactobacillus plantarum* 44A as a live feed supplement for freshwater fish. Ph. D Thesis, The Netherlands with Summaries in English, Dutch and Spanish, Wageningen Universiteit, Wageningen.
32. Shillinger, U. & Lucke, F. K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from.
33. Aslim, B.; Yuksekdog, Z. N.; Sarikaya, E. & Beyatli, Y. (2005). Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish Dairy Products., *LWT*. 38: 691-694.
34. Aroutcheva, A.; Gariti, D.; Simon, M.; Shott, S.; Faro, J.; Simoes, J. A.; Gurguis, A. & Faro, S. (2001). Defense factors of vaginal lactobacilli. *Am. J. Obstet. Gynecol.* 185: 375-379. Cited by Yuksekdog, Z. N., Beyatli, Y. & Aslim, B. (2004). Determination of some characteristics coccoid forms of lactic acid bacteria isolated from Turkish kefir with natural probiotics. *LWT*. 37: 663-667.
35. Nirunya, B.; Suphitchaya, C. & Tipparat, H. (2008). Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics *Songklanakarin Sci. Technol.*, 30:141-148.



## Role of Hydroxyapatite in Healing of Experimentally Induced Cutaneous Wound in Rabbits

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### Abstract

The study was carried out to throw some light on the effect of intra-incisional application of hydroxyapatite on healing of recovered skin documented by histopathological evaluation in rabbit. Thirty adult local breed rabbits were used, divided randomly and equally into two groups: the first group (Control group), subjected to wound of the skin from their back (about 5 cm in length) then sutured with silk (No. 4-0). Similar technique was used for treated group with the exception of local application of hydroxyapatite powder inside the incision prior to closure it. The operation was performed under the effect of general anesthesia represented by a mixture of xylazine-ketamine in a dose of 20 mg/kg and 35 mg/kg B.W., respectively, which were administered intramuscularly. During a clinical- follow of all animals, we trapped two cases of stitch abscess in the control group and one in treated group, which showed a response to medical treatment. Histopathological examination was performed on (3, 5, 7, 14 and 21) days post-wounding. Results revealed infiltration of large numbers of neutrophils with the formation of granulation tissue in the first periods in both groups, while fibrous connective tissue rich in dense collagen fibers were noticed in control group covered with necrotic tissue containing dead neutrophils and complete resolution of the skin with formation of fibrous connective tissue in the treatment group at the end of experiment. In conclusion, the Hydroxyapatite, promote wound healing because it attracts the macrophages and fibroblasts rapidly to the wound site.

### دور الهيدروكسي أبيتايت في شفاء الجروح الجلدية المحدثّة تجريبياً في الأرانب

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### الخلاصة

استهدفت الدراسة تسليط الضوء على دور الهيدروكسي ابيتايت في خياطة الجروح الجلدية المحدثّة تجريبياً في الأرانب معززة بدراسة مرضية- نسجية. اختير للبحث 30 أرنباً محلياً بالغاً، قسمت الحيوانات عشوائياً وبالتساوي إلى مجموعتين حيث عدت المجموعة الأولى (مجموعة سيطرة)، عمل لها جرح جلدي بطول 5 سم في جلد منطقة الظهر وتمت خياطته بخيط الحرير رقم (4.0)؛ أما المجموعة الثانية (مجموعة المعالجة) فعمل لها جرح جلدي بنفس الطول ثم وضع مسحوق الهيدروكسي ابيتايت داخل الجرح ثم تمت خياطته. أجري التداخل الجراحي لكل المجموعتين تحت تأثير المخدر العام المتمثل بمزيج الزايلازين - الكيتامين حيث حقن بالعضلة وبجرعة 20 ملغم/ كغم و 35 ملغم/ كغم على التوالي. ثم متابعة الحيوانات سريرياً حيث لوحظ وجود خمج لجرح الجلد في حيوانين عائدين لمجموعة السيطرة وحيوان واحد لمجموعة المعالجة حيث استجابوا للعلاج الطبي. أخذت مقاطع للفحص المرضي- النسجي من كافة الحيوانات خلال الفترات (3 و 5 و 7 و 14 و 21) يوماً من إحداث الجرح حيث أظهرت النتائج ارتشاح عدد كبير من خلايا العدلات مع تكون نسيج حبيبي في الفترات الأولى لكلتا المجموعتين، وفي نهاية التجربة تكون نسيج ليفي غني بالألياف الكولاجين مغطى بطبقة متخثرة تحتوي على العدلات الميتة في مجموعة السيطرة ولوحظ شفاء تام للجلد في مجموعة المعالجة. يمكن ان نستدل من النتائج التي حصلنا عليها بأن مسحوق الهيدروكسي ابيتايت له دور مهم في تسريع شفاء الجروح لأنه يجذب خلايا البلاعم وخلايا مولدة الكولاجين وبسرعة إلى الجرح.

## Introduction

Hydroxyapatite is a component of bone. It is a calcium phosphate mineral that also found in rocks and sea coral (1). Plastic surgeons use Hydroxyapatite implants made from sea coral since have been treated to that their structure and chemical make-up is almost identical to the Hydroxyapatite of bone. When implanted in the body the synthetic implant is accepted by the body and because of its porous nature allow normal tissue integration to take place. Also, the process to create Hydroxyapatite implant from sea coral involves intense heat that removes all proteins thus rendering the structure totally non-immunogenic (i.e. it does not provoke allergic reaction). In addition to solid Hydroxyapatite implants there is also injectable Hydroxyapatite. Also, there is past which is non-porous so bone and soft tissue growth does not occur (2,3). Many modern implants e.g. hip replacement and dental implants are coated with Hydroxyapatite. It has been suggested that this may promote osseointegration. Also, Hydroxyapatite is marked as a "bone-building" supplement with superior absorption in comparison to calcium. It is a second-generation calcium supplement derived from bovine bone (4,5). Facial augmentation with Hydroxyapatite has been used for correction of cheek, chin, jaw, nose, and brow bone. Solid Hydroxyapatite can be carved and trimmed to the requirements of the correction. Because bone and soft tissue grows into pores of the implant is securely held in place. One time the implant is partially resorbed and replaced by natural bone. Implants are usually well tolerated and causes some discomfort directly after the implantation. There may be swelling, aching, and numbness but these usually resolve after a week or so (6,7). The aim of the present study is concentrated on the role of Hydroxyapatite in healing of experimentally induced cutaneous wound in rabbits supported by clinico- histopathological evaluations.

## Materials and Methods

- **Experimental Animals:** A total of thirty adult local breed rabbits, of average age about (8-10) months and average weight (1.5-2) kg were used in this study. All rabbits were supplied from the same breeder, housed at the same conditions of temperature and humidity in especially designed wired cages in the animal housing of College of Veterinary Medicine, Baghdad University.
- **Pre-Operative Considerations:**
  1. Animals were fastened for 24 hrs for food and 12 hrs for water.
  2. The operative sites were prepared aseptically for surgical cutaneous incision.
  3. Anesthesia was accomplished by IM injection of Xylazine-Ketamine mixture in a dose of 20 mg/kg and 35 mg/kg B.W. respectively (8).
- **Experimental Design:** Animals were allocated into two main groups (15 rabbits for each group). The first group served as a control and the second group was the treatment group.
- **Technique of Skin Wound:** Animals were placed in ventral position, approximately 5 cm length incision was made on the skin of the back, bleeding was carefully arrested with fine hemostat. In the first group, skin was restitched with simple interrupted suture using non-absorbable thread (Silk No. 4-0). Then skin incision covered with sterile gauze to avoid contamination. In the second group, the same steps were applied with the exception of adding Hydroxyapatite powder (0.5 mg) inside the wound prior to suture it.

- **Post-Operative Cares and Follow-up:**
  1. Animals were received broad spectrum antibiotics represented by penicillin-streptomycin in a dose of 50.000 IU/kg and 30 mg/kg B.W. respectively injected IM for 5 days.
  2. The vital parameters which include, body temperature, pulse, and respiratory rate were monitored daily for the first week post-wounding.
  3. All animals were followed-up for one month post-surgically to record any abnormalities which may happen.
  4. The skin stitches were taken-off after 10 days when assuring complete healing or when secondary complications are not observed.
- **Histopathological Examination:** After 3, 5, 7, 14, and 21 days post-surgery, about 1 cm<sup>3</sup> from the skin and S/C tissue were retrieved for histopathological examination, 15 animals used for each group (3 animals\ period). Tissue samples were fixed in 10% buffered neutral formalin passed routinely and section into 5-6 micron, stained with Hematoxylin and Eosin (H & E) (9) and examined under light microscope.

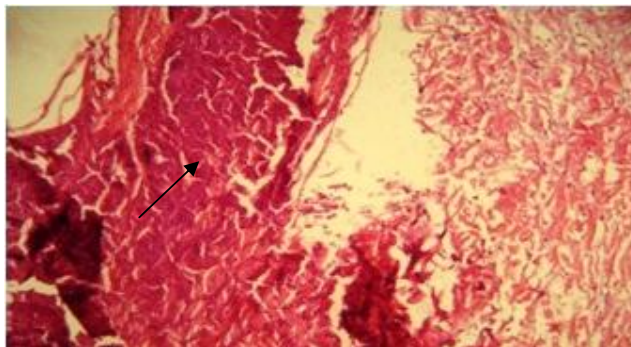
## Results

- **Clinical Follow-up:** The cardinal signs of inflammation (i.e. redness, swelling, heat and pain) were seen locally at the incision site. The signs gradually subsided, particularly from 48 hrs post-operation onward, and disappeared within (4-5) days. Decreased body activity and anorexia was found in all rabbits during the first 48 hrs post wounding. The skin wound in all rabbits healed by first intention with the exception of two rabbits related to the control group and one rabbit related to treated group which revealed partial sutures dehiscence due to sepsis (stitch abscess), which was drained by removing of one stitches in the dependent part and the cavity was cleaned, debrided then inserted with a dressing gauze soaked in 1% solution of tincture of iodine.

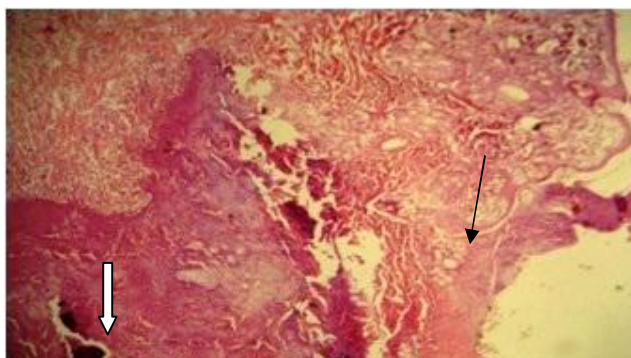
All the vital parameters (body temperature, pulse, and respiratory rate) were within the normal range except of three rabbits that showed wound infection in which there is fever which responded to treatment used as post-operative cares.

- **Histopathological Findings:**
- **At Three Days:** In control group, the microscopical picture showed ulceration of the skin surface with infiltration of large numbers of neutrophils within the dermal area (Fig.1), while in treatment group, deep ulceration of the skin surface with infiltration of large number of neutrophils deeply in the dermis and on the ulcerated surface with deposition of small amount of calcium salt (Fig.2).
- **At Five Days:** The control group show similar pathological lesions as mentioned in previous period. In treatment group, there were large area of calcifications within the dermis and subcutaneous tissue (Fig.3).
- **At Seven Days:** In control group, the ulcerated surface was infiltrated with large number of neutrophils with hydropic degeneration of remnant epithelial cells of the epidermis with large numbers of dead neutrophils on the surface (Fig.4). In treatment group, there was proliferation of granulation tissue containing large number of congested blood capillaries and young fibroblasts beneath the hyalinized and the ulcerated surface which infiltrated with large number of dead neutrophils (Fig.5).
- **At 14 Days:** Histopathological section in control group revealed proliferation of granulation tissue beneath the ulcerated surface which was covered by fibrin network (Fig.6). Also in treatment group, there was formation of granulation tissue containing large numbers of dilated blood capillaries with complete regeneration of the surface epithelium (Fig.7).

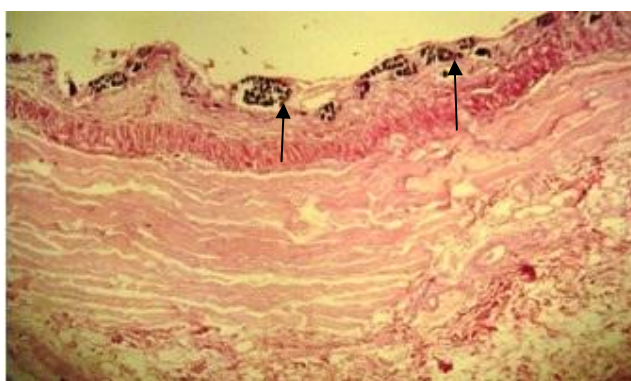
- **At 21 Days:** At this time, in control group, there was formation of fibrous connective tissue with dense collagen fibers infiltrated with dead neutrophils and covered with necrotic tissue (Fig.8). In treatment group, complete resolution of the skin with formation of fibrous connective tissue in between and surrounding the calcified areas (Fig.9).



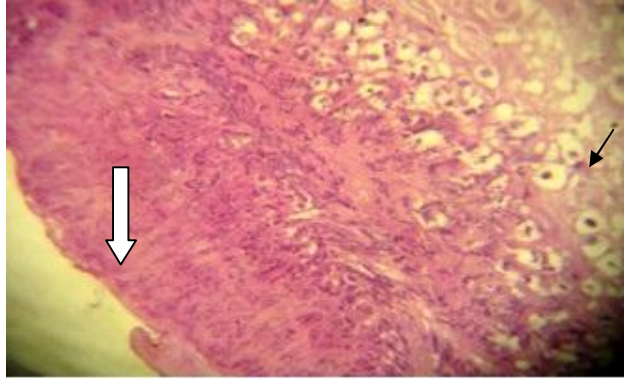
**Fig. (1) Microscopic picture of skin related to control group at 3 days showed ulceration of the surface with infiltration of large number neutrophils (arrow) (H & E×10)**



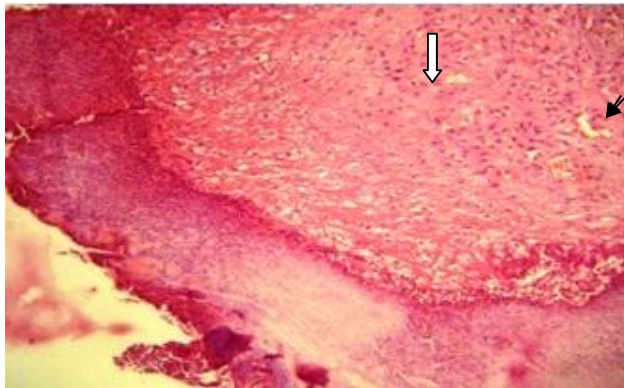
**Fig. (2) Microscopic picture of treated group at 3 days showed deep ulceration of the skin surface (black arrow) with infiltration of large number of neutrophils and small areas of calcification (white arrow) (H & E × 40)**



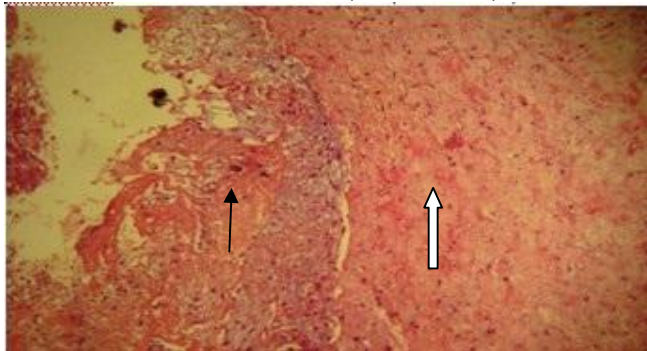
**Fig. (3) Microscopic picture of treated group at 5 days showed multiple areas of calcification (arrows) within the subcutaneous tissue (H & E×10)**



**Fig. (4) Microscopic picture of control group at 7 days showed infiltration of neutrophils with hydropic degeneration (black arrow) and large number of dead neutrophils on the surface (white arrow) (H & E  $\times 10$ )**

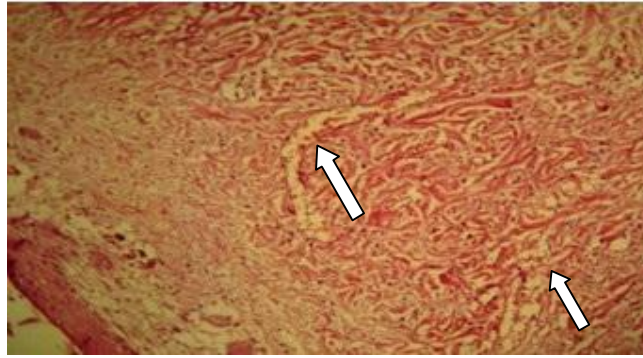


**Fig. (5) Microscopic picture of treated group at 7 days showed proliferation of granulation tissue containing large numbers of congested blood capillaries (black arrow) and young fibroblast cells (white arrow) beneath the hyalinized and ulcerated surface (H & E  $\times 40$ )**

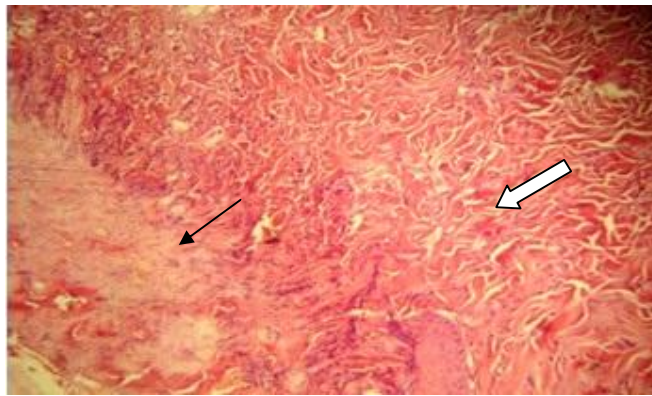


**Fig. (6) Microscopic picture of control group at 14 days showed proliferation of granulation tissue (white arrow) beneath the ulcerated surface which is covered with fibrin network (black arrow) (H & E  $\times 40$ )**

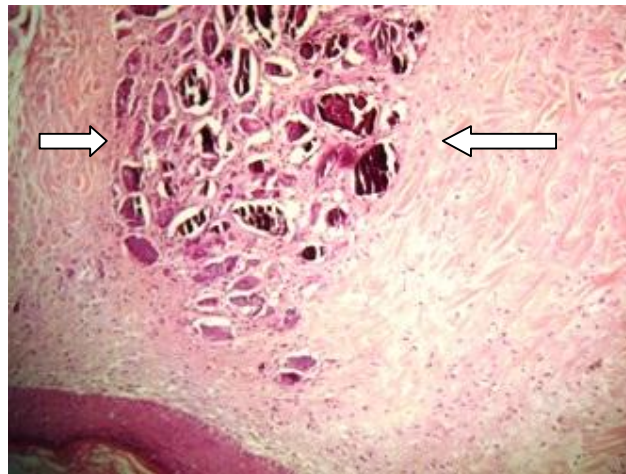




**Fig (7) Microscopic picture of treatment group at 14 days showing formation of granulation tissue containing dilated blood capillaries(white arrows)with complete regeneration of surface epithelium (H & E  $\times 40$ )**



**Fig (8) Microscopic picture of control group at 21 days showed formation of dense fibrous connective tissue(white arrow)covered with necrotic tissue containing dead neutrophils (black arrow) (H & E  $\times 40$ )**



**Fig. (9) Microscopic picture of treatment group at 21 days showed complete resolution of ulcerated surface with formation of fibrous connective tissue in between and surrounding the calcified areas (white arrows) (H & E  $\times 10$ )**

## Discussion

Based upon the results obtained, it gave a clarity of the success of Hydroxyapatite in promoting of wound healing. No serious health problems were recorded after the follow-up with the exception of three rabbits suffered from minor non-specific complications represented by wound infection which can be explained as a possible complication of surgery. Schessel *et al.*, (10) referred that bacteria can enter the body during surgery or after surgery through suture and drain site or by way of open wound. Some infections could occur later (perhaps weeks or months) after surgery and could be related to trauma or minor injuries, such as scratch, this might induce infection, initiated in the skin then spreading inside. Hydroxyapatite has been widely used to produce biomaterials. It had a higher promoting effects on wound healing, re-epithelialization and matrix formation (11). The present results showed inflammatory reaction and an intense foreign body reaction and infiltration of large numbers of neutrophils were observed by day three post-operation, where as tissue growth into the uncoated control implants was much slower and took place mainly on their surface. The reaction in apatite-coated groups decline after post-operative day 14 because Hydroxyapatite favored angiogenesis and attract macrophages and fibroblasts to the wounded area (12). Tissue damage leads to the release of basic fibroblast growth factor normally sequestered within intact cells and extracellular matrix (13). In addition, bleeding and hemostasis in a wound, up regulated cellular receptors for vascular endothelial growth factor and potentiates this growth factor's effects (14). Endothelial cells exposed to thrombin also release gelatinase A, which promotes the local dissolution of basement membrane which is an essential early step of angiogenesis (15). Wound angiogenesis is amplified by inflammation, macrophages and monocytes release myriad angiogenic factors as they migrate into the wound bed (16). It is well known that Hydroxyapatite attract macrophages to the wound area. Recent studies have identified macrophages as critical regulators of fibrosis like myofibroblasts which are derived from either resident tissue populations, or from bone marrow immigrants. Studies now suggest that pathogenesis of fibrosis is tightly regulated by distinct macrophages population that exerts unique functional activities throughout the initiation, maintenance, and resolution phases of fibrosis (17). The promotion of further calcification by Hydroxyapatite supports previous observations which referred that the presence of Hydroxyapatite nucleators enhanced further accumulation of calcium, due to low-activation energy pathways provided by the presence of Hydroxyapatite particles (18). In conclusion, the Hydroxyapatite materials promote wound healing and degraded *in vivo* and can be good candidate in area when fast proliferation of connective tissue is desirable.

## References

1. Straub, D. A. (2007). Calcium supplementation in clinical practice: A review of forms, doses and indications. *Nutri. Clin. Pract.*, 22(3): 286-296.
2. Rigo, E. C.; Boschi, M. O.; Yoshimoto, S. J.; Konig, M. J. & Carbonari, A. S. (2004). Evaluation *in vitro* and *in vivo* of biomimetic hydroxyapatite coated on titanium dental implants. *Mater. Sci. and Engineering*, 24: 647-651.
3. Kundu, B.; Soundrapandian, C.; Nandi, S. K.; Datta, B. K. & Mandal, T. K. (2010). Development of new localized drug delivery system based on ceftriaxone-sulbactam composite drug impregnated porous hydroxyapatite: A systemic approach for *in vitro* and *in vivo* animal trial. *Pharm. Res.*, 27(8):1659-1576.
4. Rungsiyakull, C. O.; Li, W.; Li, R. & Swian, M. (2008). Effect of fully porous-coated technique on osseointegration of dental implants. *Aust. J. Basic and Appl. Sci.*, 32: 189-193.

5. Woo, A. S.; Jang, J. L.; Liberman, R. F. & Weinzweig, J. (2010). Creation of a vascularized composite graft with cellular dermal matrix and Hydroxyapatite. *Plast. Reconstr. Surg.*, 125(6): 1661-1669.
6. Lobato, J. V.; Hussain, N. A. & Lopes, M. A. (2008). Clinical applications of titanium dental implant coated with Hydroxyapatite composite. *J. of Nanomanufacturing*, 2: 135-144.
7. Fouda, M. F.; Nemat, A.; Gawish, A. & Ayman, R. B. (2009). Does the coated of titanium implants by Hydroxyapatite affect the elaboration of free radicals? An experimental study. *Aust. J. Sci.*, 3(2): 1122-1129.
8. Gary, L. W. & Donald, D. H. (1996). A comparison of ketamine and the combination of ketamine-xylazine for effective surgical anesthesia in the rabbit. *Lab. Anim. Sci.*, 26(5): 804-806.
9. Prophet, E. B.; Mills, B. & Arrington, J. B. (1992). Laboratory methods in histotechnology. Washington: Armed Forced Institute of Pathology, P. 275.
10. Schessel, S.; Ralph, G. & Ran, K. (2002). The management of postoperative disrupted abdominal wall. *Am. J. Surg.*, 184(3): 263-268.
11. Okabayashi, R.; Nakamura, M.; Tanaka, Y.; Nagal, A. & Yamashita, K. (2009). Efficacy of polarized Hydroxyapatite and silk fibroin composite dressing gel on epidermal recovery from full-thickness skin wounds. *Biomed. Mater. Res.*, 90(2): 641-646.
12. Tommila, M.; Jokinen, J.; Wilson, T.; Forsback, A.; Saukko, P.; Penttinen, R. & Ekholm, E. (2008). Bioactive glass-derived Hydroxyapatite-coating promotes granulation tissue growth in subcutaneous cellulose implants in rats. *Acta Biomater.*, 4: 354-361.
13. Vlodavski, I.; Fuks, Z. & Ishai, R. (1991). Extracellular matrix resident basic fibroblast growth factor implication for the control of angiogenesis. *J. Cell. Biochem.*, 45: 167-176.
14. Tsopanoglou, N. E. & Maragoudakis, M. E. (1999). On the mechanism of thrombin induced angiogenesis. Proliferation of vascular endothelial growth factor activity on endothelial cells by up regulation of its receptors. *J. Biol. Chem.*, 274: 23969-23976.
15. Nguyed, M.; Arkell, J. & Jackson, C. J. (2001). Human endothelial gelatinases and angiogenesis. *Int. Biochem. Cell. Biol.*, 33: 960-970.
16. Growther, M.; Brown, N. J.; Bishop, E. T. & Lewis, C. F. (2001). Microenvironmental influence on macrophage regulation of angiogenesis in wound. *J. Leukoc. Biol.*, 70: 478-490.
17. Wynn, E.; Thomas, A. & Luke, Barron, F. (2010). Macrophages : Master regulators of inflammation and fibrosis. *Semin. Liver Dis.*, 30(3): 245-257.
18. Blumenthal, N. C. (1989). Mechanisms of inhibition of calcification. *Clin. Orthop. Relat. Res.*, 247: 279-289.



## Isolation and identification of *Salmonella* species from local cheeses in Sulaimani province

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### Abstract

A total of 120 random samples of local (Fresh, non- salted) cheese were collected from different markets of Sulaimani city during the period from October 2009 to June 2010. The results showed that three 2.5% out of the total 120 cheese samples were found contaminated with *Salmonella* species. *Salmonella enteritidis* was the only serotypes that have been found.

### عزل وتمييز انواع السالمونيلا من الجبن المحلي في مدينة السليمانية

إيمان ظاهر عارف

كلية الطب البيطري/ جامعة السليمانية

### الخلاصة

اجريت هذه الدراسة على مئة وعشرون عينة عشوائية من الجبن المحلي (الطري وغير المملح) والتي جمعت من اسواق مدينة السليمانية خلال الفترة من شهر تشرين الأول 2009 ولغاية شهر حزيران 2010 ولقد أظهرت النتائج ان 3 فقط من هذه العينات كانت ملوثة بالسالمونيلا بنسبة 2.5%. تم عزل نوع واحد فقط وهو *Salmonella enteritidis*.

### Introduction

During the last 25 years, there has been a continued increase in milk production and consumption of fermented dairy products (1). Fermented dairy products are cheese, yogurt and fermented milk. They are fermented by the addition of a starter culture such as lactic acid bacteria which are responsible for the production of lactic acid from lactose that gives the product its characteristic flavor. Cheese is one of the most consumed milk products (2). Cheese is an excellent source of protein, fat and minerals such as calcium, iron and phosphorus, vitamins and essential amino acids and therefore is an important food in the diet of both young and old people (3, 4). Family of *Enterobacteriaceae* contains many species which cause hazard to the consumer, other species are important from economic point of view as milk products. Salmonellosis may causes an outbreak of gastroenteritis (5, 6). Such infection not only has economic importance in agriculture and animal husbandry but also is believed to be major factor in the transmission of *Salmonella* to humans via food chain (7). Cheese like other types of food is exposed to contamination with microorganisms during the production process. Fresh cheese such as cottage and white cheese may be subjected to spoilage by coliforms, yeast and molds that enter as post-pasteurization contaminants (8). During cheese processing stages microbes can be introduced by cross-contamination from raw milk which is the raw material or from infected humans handling the food (9). The aim of this study was to investigate the contamination of locally-produced non fermented soft white cheeses with *Salmonella* species.

## Material and Methods

A total of 120 samples of local Fresh-non salted white cheese were collected randomly from local markets in Sulaimani city during the period from October 2009 to June 2010. The Samples were directly transferred by refrigerated containers to the laboratory and analyzed immediately without further storage. Briefly, Twenty five grams of cheese were suspended in 225 ml of buffered peptone water and incubated for 24 h at 37° c for pre-enrichment. One milliliter of pre- enrichment broth was transferred to 10 ml of tetrathionate broth (Selective Enrichment) and incubated for 24 h at 42 °c. One loop-full of tetrathionate broth was streaked onto *Salmonella Shigella* agar and Brilliant green agar (Selective media) and incubated at 37° c for 24 h incubation. Following that, the suspected *Salmonella* colonies were transferred simultaneously to Kligler and Urea agar base tubes by stabbing the butt and streaking the slope, and incubated for 24 h at 37° c (10). Slide agglutination test was performed for the tubes showing reactions typical of *Salmonella*, using somatic polyvalent *Salmonella* serum (11). The isolated *Salmonella* strains were undergone serotyping at the Institute of Public Health, Baghdad, Iraq.

## Result and Discussion

The results showed that three out of the total 120 cheese samples were found to be contaminated with *Salmonella* species. A total of 2.5% *Salmonella* serotypes were identified. *Salmonella enteritidis* was the only serotypes that have been found. The contamination of cheese may be due to the low Quality of raw milk used in cheese making or could be due to unsanitary conditions during processing and handling of cheese (12). The presence of the bacteria is inversely related to the presence of salt in cheese, the cheese used in this study was unsalted so it was not surprise to find *Salmonella* contaminants. The low percentage of contamination may be due to the season in which the study was performed, where the low temperatures of winter can affect the growth of *Salmonella* species that usually grow out 37° c (13). This study recommends the consumers to pasteurized and salted cheese rather than unsalted type which encourage the growth of *Salmonella* especially in bad stored cheese. Since dairy products manufacturing, handling and distribution in Iraq are carried out under primitive conditions therefore *Salmonella* contamination was expected and, so it's highly recommended that strict hygienic conditions should be adopted during manufacturing and handling of such products, besides that local markets and processing should be periodically inspected by specialists (10). In addition to that, pasteurized milk should be used for the manufacturing of local soft white cheese with its preservation inside the brine.

## References

1. Magda, M.; Madeha, N.; Safaa, Y. & Nagwa, M. (2010). Mineral content and microbiological examination of some white cheese in Jeddah, Saudi Arabia during summer 2008. Food and Chem. Toxicol., 48:3031–3034.
2. McGee, H. (2004). Cheese on Food and Cooking. Revised ed. Scribner. PP. 51–63.
3. Tripathi, R. M.; Raghunath, R.; Sastry, V. N. & Krishnamoorthy, T. M. (1999). Daily intake of heavy metals by infants through milk and milk products., 227 (2-3): 229-235.
4. O'Connor, C. B. (1993). Traditional cheese making manual. Audiotutorial module 3. ILCA (International Livestock Centre for Africa), Addis Ababa, Ethiopia.P.11.

5. El-Kouly, A. M; Hafez, R. S. & Mahmoud, M. D. (1995). Occurrence of some food poisoning bacteria in Egyptian soft cheese. Beni-suef. Vet. Med. Res., 7(1):342-355.
6. Ahmed, R.; Soule, G.; Walter, H.; Demezuk, W.; Clark, C.; Khakhria, R.; Ratnam, S; Marshall, S.; Woodward, D.; Johnson, W. & Rodgers, F. G. (2000). Epidemiology typing of *Salmonella* enteric serotype enteritidis in a Canada-wide outbreak of gastroenteritis due to contaminated cheese. J. Clin. Microbiol., 38(6): 2403-2406.
7. Morris, J. G. (1997). Current trends in human-diseases associated with food of animal origin. J.AVM.A., 209:2045-2047.
8. Sadek, Z. I.; Hosny, I. M.; El-Kholy, W. L. & El-Dairouty, R. K. (2009). Comparative Investigation for detection of food borne microorganisms in Egyptian hard Cheese, Ras using conventional and fast biochemical tests. Global Vet., 3 (3): 189–195.
9. Temelli, S.; Anar, S.; Sen, S. & Akyuva, P. (2006). Determination of microbiological contamination sources during Turkish white cheese production. Food Control., 17 (11): 856–861.
10. Ali, H. H. (2005). *Salmonella* serotypes isolated from local cheese in Mosul city. Iraqi J. Vet. Sci., (19) 1:27-30.
11. Forbes, B. A.; Sahm, F. D. & Weissfeld, S. A. (2007). Diagnostic Microbiology Twelfth edition. Mosby, Inc, an affiliate of Elsevier Inc.
12. Omer, I. A. & Osman, A. O. (2007). Microbiological properties and sensory characteristics of white cheese (*Gina bayda*) collected in Zalingei Area, West Darfur State. J. of Animal and Vet. Sci., 2: 61-65.
13. Sliq, S.; Horra, S. & Younes, A. (2010). Detection and Isolation of *Salmonella* in Some Syrian Fresh White Cheese. J. Agr. Sci., (26) 1:321-305.

**Cytotoxic effect of *Chelidonium majus* on cancer cell lines**

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**Abstract**

Cancer is a growing health problem coming next to the cardiovascular diseases regarding the morbidity and mortality with some international geographical variation according to its type. The purpose of the present study was to investigate potential *in vitro* cytotoxic effect of the aqueous extract of *Chelidonium majus* on two human cancer cell lines (pelvic: RD and cervical: HeLa). The extract exhibited a time- and concentration-dependent inhibitory effect on both cell lines. The highest significant inhibitory effect of extract recorded after 72 hrs of exposure at highest concentration (1000 µg/ ml), it was achieved 58.4% and 65.1% on RD and HeLa, respectively. The treatment with 500 and 1000 µg/ml of extract caused significant reduction in viability of RD and HeLa cells during all periods of exposure. These results revealed that *C. majus* possessed cytotoxic effect on cancerous cell lines, which makes it has possible role in medical oncology.

التأثير السمي للمستخلص المائي لنبات عروق الصباغين *Chelidonium majus* في بعض

الخطوط الخلوية السرطانية

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**الخلاصة**

يعتبر السرطان مشكلة صحية متنامية تأتي بعد أمراض القلب والشرابيين من حيث معدل الأضرار والوفيات مع الاختلاف في التوزيع الجغرافي العالمي اعتماداً على نوع السرطان. إن الغرض من هذه الدراسة هو فحص القابلية السمية في الزجاج للمستخلص المائي لنبات عروق الصباغين (*Chelidonium majus*) ضد الخطتين الخلويتين لسرطان العضلات المخططة البشري (RD) وسرطان عنق الرحم (HeLa). لقد أظهر المستخلص تأثيراً تثبيطياً معتمداً على التركيز المستخدم منه ومدة التعريض تجاه كلا الخطتين الخلويتين، فظهر التأثير التثبيطي المعنوي الأعلى عندما عوملت الخلايا بالتركيز 1000 مايكروغرام/ مل لمدة 72 ساعة، حيث وصل 58.4% و 65.1% لخلايا RD و HeLa على الترتيب. وأبدى المستخلص بالتركيزين 500 و 1000 مايكروغرام/ مل تأثيراً تثبيطياً معنوياً في كلا خطي الخلايا لجميع مدد التعريض المستخدمة. هذه النتائج تُظهر بأن المستخلص يمتلك تأثير سمي ضد الخطوط الخلوية السرطانية، والذي يجعل له دوراً محتملاً في علاج الأمراض السرطانية.

## Introduction

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasoning, beverages, cosmetics, dyes and medicine. Most of this therapy involves the use of plant extracts or their active components (1). At least 250.000 species of plants do exist, out of which more than one thousand plants have been found to possess significant anticancer properties (2). The National Cancer Institute (NCI) collected about 35.000 plant samples from 20 countries and has screened around 114.000 extracts for anticancer activity (3). *Chelidonium majus* Linn. (greater celandine, swallow-wort, and bai-qu-cai in Chinese) is the only species of the tribe Chelidoneieae of the Papaveraceae family. It is rich in various types of isoquinoline alkaloids. The phytochemical and pharmacological properties, including spasmolytic, antiulcer, anti-inflammatory, antibacterial, antiviral, antifungal and antioxidant activity of *C. majus* have been reviewed, which is of great interest for its use in Asian, European and Chinese herbal medicines (4, 5, 6, 7). *C. majus* has been traditionally used for treatment of gastric ulcer, astric cancer, oral infection, liver disease, and general pains. The extracts of *C. majus* are proven to be safe as components of veterinary and human phytopreparations, and of oral-hygiene agents (8). Previous chemical studies of *C. majus* have reported the isolation of isoquinoline alkaloids such as chelidonine, chelerythrine, sanguinarine, berberine, coptisine, and *dl*-stylophine (9). Among them, chelindonine and protopine exhibited anti-tumor activity, and protoberberine showed antibacterial and antiviral activity, while sanguinarine and chelerythrine had anti-inflammatory activity (9). Currently, one key component of a comprehensive preclinical screening and drug development program at the NCI is the cell-line screen (10). In Iraq, many efforts were begun for finding alternative treatment of cancer disease (11). Our study aimed at evaluate the cytotoxic effect of *C. majus* extract against malignant cell lines.

## Material and Methods

- **Preparation of the *C. majus* extract:** Distilled water was added to powdered plant leaves in a ratio of (1:5 W/V) with continuous stirring for 3 days at room temperature (18-25°C). The mixture was then filtered through a piece of soft cloth, filter paper and centrifugation to remove all the residual materials. The clear solution of the extract was dried at 45°C by using oven and kept at 4°C until use (12).
- **Cell lines:**
  - Rhabdomyosarcoma (RD) cell line was kindly provided by Iraqi Center for Cancer and Medical Genetics Research (ICCMGR). This human cell line was derived from a biopsy specimen obtained from a pelvic rhabdomyosarcoma of a 7-year-old Caucasian girl (13).
  - A HeLa cell line was kindly provided by ICCMGR. This human cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer on October 4, 1951. These cells are treated as cancer cells, as they are descended from a biopsy taken from a visible lesion on the cervix as part of Mrs. Lacks' diagnosis of cancer. A debate still continues on the classification of the cells (14).

Both cell lines were cultured in RPMI 1640 medium (Bio Whittaker) containing 20% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

- **Assay for cytotoxic activity:** Cell cultures in the micro-titration plate were exposed to a range of plant extract concentrations during the log phase of growth and the effect was determined after recovery time. The following protocol (15) was

performed on *C. majus* extract: After trypsinization, cell suspension seed in a micro-titration plates at 50000 cells/ml RPMI-1640 growth medium with serum 5% used for seeding. Plates then incubated for 24 hours. By using maintenance medium (free of serum), two-fold serial dilutions were prepared starting from 1000 µg/ml ending with 62.5 µg/ml. After incubation for 24hrs, and when the cells are in the exponential phase, remove the medium and add extract serially across the plates using replicates of four wells per concentration. Add fresh growth medium to the control wells. Only 20 µl of each concentration added for each well. 20µl of maintenance medium added to each well of control group. The time of exposure were 24, 48 and 72 hours. The plates sealed with self adhesive film then returned to the incubator at 36.8 °C. After the end of the exposure period, the medium decanted off and cells in the wells gently washed by adding and removing 0.1 ml sterile phosphate buffer saline two times. Finally, 50µl of 0.01% crystal violet dye added. After 20 min. the stain was washed gently with tap water for three times. The plate was left until become dry. The optical density of each well was read by using a micro-ELISA reader at 495 nm transmitting wavelength (15, 16). The percentage of inhibition was calculated according to the following equation (17):

$$\text{Inhibition \%} = 100 - [(\text{optical density of test wells} / \text{optical density of control wells})] \times 100$$

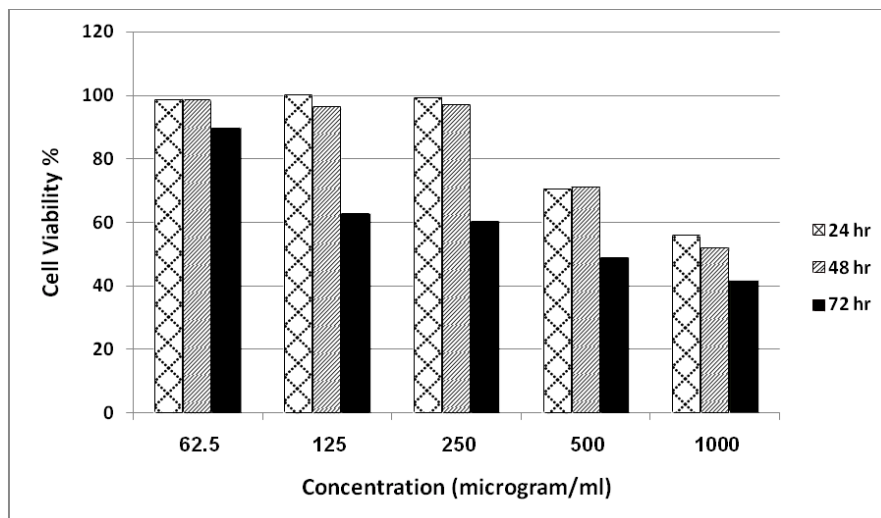
- **Statistical analysis:** Analysis of variance (ANOVA) and the least significant difference (LSD) were used for the statistical analysis. These calculations were carried out according to program SPSS version/10.

## Results

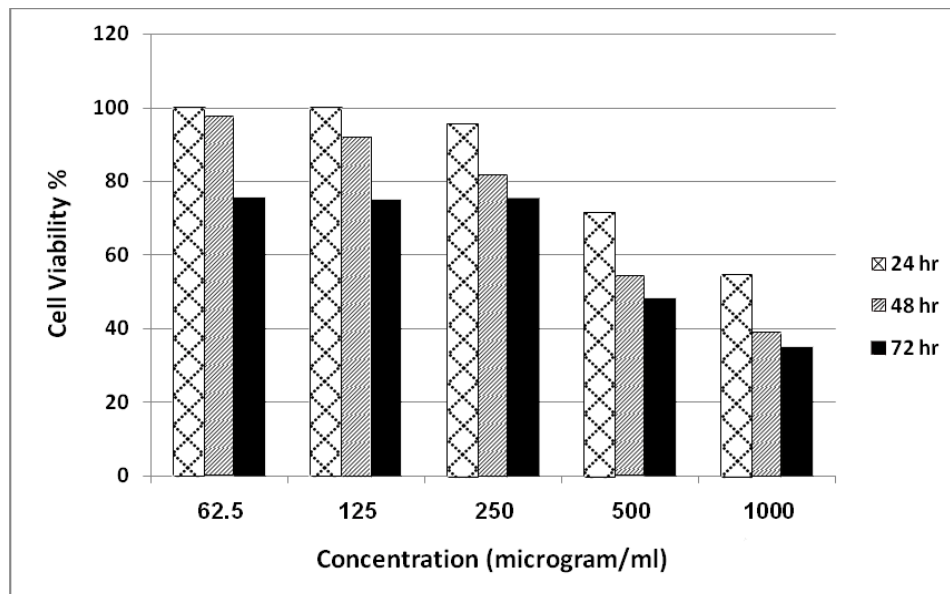
The cytotoxic effect of two-fold of *C. majus* extract during 24, 48 and 72 hrs of exposure on RD and HeLa cell lines are shown in Table (1) . The extract showed a time- and concentration-dependent effect on viability of both cell lines. Viability decreased with time reaching its lowest after 72 hrs of treating with all concentrations used. The treatment with 1000 µg/ml after 72 hrs caused highest reduction in viability of RD and HeLa cells thus reaching 41.6% and 34.9% (percentage of inhibition 58.4% and 65.1%), respectively (Fig. 1, 2). Interestingly, treatment with 500 and 1000 µg/ml of extract caused significant reduction in viability of RD and HeLa cells during all periods of exposure. At 72 hrs duration particularly, the significant reduction of RD viability observed with 125, 250, 500 and 1000 µg/ml concentrations (Table 1 ). Compared with inhibition of RD cells, HeLa inhibition by the extract was higher. This reflected the sensitivity of HeLa cells compared with RD cells.

**Table (1) A comparison of growth inhibition percentage of RD and HeLa cells, by aqueous extract of *Chelidonium majus* during three periods of exposure**

Concentration (µg/ml)	RD			HeLa		
	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
62.5	1.4	1.2	10.4	0.5	2.6	24.5
125	---	3.6	37.4*	0.4	8.1	25.1
250	0.8	3.2	39.7*	4.6	18.4	24.8
500	29.3*	28.7*	51.2*	28.7*	46.0*	51.8*
1000	44.2*	48.3*	58.4*	45.5*	61.1*	65.1*



**Fig. (1) Effect of aqueous extract of *Chelidonium majus* on viability of RD cells**



**Fig. (2) Effect of aqueous extract of *Chelidonium majus* on viability of HeLa cells**

### Discussion

Cancer chemotherapy with strategies using foods and medicinal herbs has been regarded as one of the most visible fields for cancer control (18). *Chelidonium majus* L. has a long history as being useful for the treatment of many diseases over the world. The plant contains, as major secondary metabolites, isoquinoline alkaloids, such as sanguinarine, chelidonine, chelerythrine, berberine and coptisine. Other compounds structurally unrelated to the alkaloids have been isolated from the aerial parts: several flavonoids and phenolic acids.(1). In this study, crude extract was used because of the biological activity, if proven to exist, might be lost during the process of purification from the crude extract (19). Furthermore, it was proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities, and that the benefit of a diet rich in fruit and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (20). Water soluble compounds may present in the aqueous extracts. Kamuhabwa

and his colleagues reported that polar compounds, responsible for anticancer activity are water soluble (21). The classical method for evaluating the effect of deleterious treatments on cell is based on proportion of inhibition (17), which indicate the rate of inhibition of cell growth (22) or percentage of toxicity (23). These parameters are used in present study for evaluation of cytotoxic effect of *C. majus* extract. In this study, *C. majus* aqueous extract showed a time- and concentration-dependent effect on viability of both cell lines. The same effect appeared when Jagetia and Rao studied the effect of *Tinospora cordifolia* extracts on HeLa cells (24). Also The aqueous extract of *Moringa oleifera* showed good cytotoxicity on HeLa cells which was concentration dependent (25). Yang and his colleagues reported that the saponin ginsenoside, isolated from *Panax notoginseng*, inhibits the cell growth of HeLa cells in a concentration- and time-dependent manner, with an IC(50) value of 150.5 $\pm$ 0.8 mcg/ml after 48 hr of incubation (26). Compared with inhibition of RD cell, HeLa inhibition by *C. majus* aqueous extract was higher. This reflected the resistance of RD cells compared with HeLa cells. Harput and his colleagues showed in their study on effects of The water extract of *Moltkia aurea* Boiss that RD cells were more resistant to the extract compared with Hep-2 (human larynx epidermoid carcinoma) and L-20B (transgenic murine L-cells) (27). Aqueous extract of *C. majus* at higher concentrations exert a direct cytotoxic effect on both malignant cell lines. This finding indicate that *C. majus* may be useful as cancer chemotherapeutic agents. The most accepted explanation for the cytotoxic effect of plant extract is the ability of plants to induce the programmed cell death in cancerous cells, as attempt to arrest their proliferation. A number of food items as well as herbal medicine have been reported to produce toxic effects by inducing programmed cell death (28).

### References

1. Craig, W. J. (1999). Health-promoting properties of common herbs. *Am. J. Clin. Nutr.*, 70: 491S-499S.
2. Mukherjee, A. K.; Basu, S.; Sarkar, N. & Ghosha, A. C. (2001). Advances in cancer therapy with plant based natural products. *Current Med. Chem.*, 8:1467-1486.
3. Shoeb, M. (2006). Anticancer agents from medicinal plants. *Bangladesh J. Pharmacol.*, 1: 35-41.
4. Colombo, M. L. & Bosisio, E. (1996). Pharmacological activities of *Chelidonium majus* L. (Papaveraceae). *Pharmacol. Res.*, 33(2):127-134.
5. Then, M.; Szentmihályi, K.; Sarkozi, A. & Varga, I. S. (2003). Examination on antioxidant activity in the greater celandine (*Chelidonium majus* L.) extracts by FRAP method. *Acta. Biologica Szegediensis.*, 47(1-4):115-117.
6. Yilmaz, B. S.; Ozbek, H.; Citoglu, G. S.; Ugras, S.; Bayram, I. & Erdogan, E. (2007). Analgesic and hepatoprotective effects of *Chelidonium majus* L. *J. Fac. Pharm.*, 36 (1):9 – 20.
7. Zuo, G. Y.; Meng, F. Y.; Hao, X. Y.; Zhang, Y. L.; Wang, G. C. & Xu, G. L. (2009). Antibacterial Alkaloids from *Chelidonium majus* Linn (Papaveraceae) against clinical isolates of methicillin-resistant *Staphylococcus aureus*. *J. Pharm. Pharmaceut. Sci.*, 11 (4): 90-94.
8. Kosina, P.; Walterova, D.; Ulrichova, J.; Lichnovský, V.; Stiborová, M.; Rýdlová, H.; Vičar, J.; Krečman, V.; Brabec, M. J. & Šimánek, V. (2004). Sanguinarine and chelerythrine: assessment of safety on pigs in ninety days feeding experiment. *Food Chem. Toxicol.*, 42:85-91.
9. Cho, K. M.; Yoo, I. D. & Kim, W. G. (2006). 8-Hydroxydihydrochelerythrine and 8-hydroxydihydroSanguinarine with a potent acetylcholinesterase inhibitory activity from *Chelidonium majus* L. *Biol. Pharm. Bull.*, 29:2317-2320.



10. Takimoto, C. H. (2003). Anticancer drug development at the Us National cancer institute. *Cancer Chemother. Pharmacol.*, 52(Suppl 1): S29-S33.
11. Yaseen, N. Y.; Hussein, S. M.; Saleh, F. S. & Mohammad, M. H. (2008). Plant and biological extracts in cancer therapy. Iraqi Centre for Cancer and Medical Genetics Research, Baghdad, Iraq.
12. Harborne, J. B. (1973). *Phytochemical methods*. Halsted press, John Wiely & Sons, New York. P. 278.
13. McAllister, R. M.; Melnyk, J.; Finklestein, J. Z.; Adams, E. C. & Gardner, M. B. (1969). Cultivation *in vitro* of cells derived from a human rhabdomyosarcoma. *Cancer.*, 24(3): 520-526.
14. Kaplan, G.; Levy, A. & Racaniello, V. R. (1989). Isolation and characterization of HeLa cell lines blocked at different steps in the poliovirus life cycle. *J. Virol.*, 63(1): 43-51.
15. Freshney, R. I. (1994). *Culture of animal cells: A manual for basic technique* (3<sup>th</sup> ed.). Wiley-liss, A John Wiley & Sons Inc. publication, New York.
16. Mahony, D. E.; Gilliat, T.; Dawson, S.; Stockdale, E. & Lee, S. H. (1989). Vero cell assay for rapid detection of *Clostridium perfringens* enterotoxins. *Appl. and Env. Microbiol.*, PP. 2141-2143.
17. Chiang, L. C.; Chiang, W.; Chang, M. Y. & Lin, C. C. (2003). *In Vitro* Cytotoxic, Antiviral and Immunomodulatory Effects of *Plantago major* and *Plantago asiatica*. *The Am. J. of Chinese Med.*, 31: 225-234.
18. Jo, E. H; Hong, H. D.; Ahn, N. C.; Jung, J. W.; Yang, S. R.; Park, J. S.; Kim, S. H. & Kang, K. S. (2004). Modulations of the Bcl-2/Bax family are involved in the chemopreventive effects of licorice root in MCF-7 human breast cancer cell line. *J. Agric. Food Chem.*, 52:1715-1719.
19. Harborne, J. B. (1984). *Phytochemical Methods*. (2<sup>nd</sup> ed.), Chapman & Hall, London, P. 5.
20. Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J. Nutr.*, 134:3479S-3485S.
21. Kamuhabwa, A.; Nshimo, C. & DeWitte, P. (2000). Cytotoxicity of some medicinal plant extract used in Tanzanian traditional medicine. *J. Ethnopharmacol.*, 70: 143- 149.
22. Gao, Y.; Den, J.; Xiang, Y.; Ruan, Z.; Wang, Z.; Hu, B.; Guo, M.; Teng, W.; Han, J. & Zhang, Y. (1999). Smoking, related cancers, and other diseases in shanghai: a 10-year prospective study. *Zhonghua-Yu-Fang-Yi-Xue-Za-Zhi.*, 33(1): 5-8.
23. Betancur-Galvis, L. A.; Morales, G. E.; Forero, J. E. & Roldan, J. (2002). Cytotoxic and antiviral activity of Colombian medicinal plant extract of the *Euphorbia* genus. *Mem. Inst. Oswaldo Cruz. Rio de Janeiro.*, 97: 541-546.
24. Jagetia, G. C. & Rao, Sh. K. (2006). Evaluation of cytotoxic effects of dichloromethane extract of Guduchi (*Tinospora cordifolia* Miers ex Hook F & THOMS) on cultured HeLa cells. *eCAM.*, 3(2):267-272.
25. Nair, Sh. & Varalakshmi, K. N. (2011). Anticancer, cytotoxic potential of *Moringa oleifera* extracts on HeLa cell line. *J. of Nat. Pharmaceuticals*, 2(3): 138-142.
26. Yang, Z. G.; Sun, H. X. & Ye, Y. P. (2006). Ginsenoside Rd from *Panax notoginseng* is cytotoxic towards HeLa cancer cells and induces apoptosis. *Chem. Biodivers.*, 3(2):187-97.
27. Harput, U. S.; Nagatsu, A. & Saracoglu, I. (2012). Antioxidant and cytotoxic effects of *Moltkia aurea* Boiss. *Rec. Nat. Prod.*, 6(1): 62-66.
28. Thatte, U.; Bagadey, S. & Dahanakar, S. (2000). Modulation of programmed cell death by medicinal plants. *Cell Mol. Biol.*, 46: 199-214.