ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

# Response of Diabetic Feet's Pathogenic Bacteria to Heavy Metal Salts.

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**ABSTRACT**

This investigation aimed at the study of the effect of heavy metals salts solutions on the pathogenic bacteria isolated from diabetic Feet's patients. Isolation and identification of isolated bacteria based on biochemical, morphological and molecular biology techniques (PCR using 16S rRNA primer). In this experiment, 27bacteria was isolated and identified; it included 8 *Escherichia coli*, 7 *Staphylococcus spp*., 5 *Pseudomonas spp*., 4 *klebsiella spp*. and 3 *Proteus spp*. It was confirmed by 16S rRNA gene sequence analysis. Also these bacteria tested for susceptibility to commonly used antibiotics and found that mainly meropenem, and cephoperazone +sulbactam were the most effective antibiotics against bacteria. On studying the physiology and growth response of these bacteria to different concentration of some selected heavy metal salt (e.g. copper & zinc) solutions with different concentrations (10 µM– $10^7$ µM), Revealed that the most effective concentration of heavy metals salt solutions CuSo4 & ZnSo4 are ($10^5$ µM). These solutions were tested on the tissue of patients to make sure that these concentrations were nontoxic. After detection of the nontoxic and most inhibitory concentration to different pathogenic bacteria. These obtained results can be applied for treatment of diabetic feet's pathogenic bacteria as a lotion.

**Keywords:** Heavy metals, Diabetic feet, Pathogenic bacteria, Antibiotics.

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ISSN: 0975-8585

## INTRODUCTION

Foot infections are one of the main complications of diabetes mellitus and are a significant risk factor for lower extremity amputation. They are usually caused by bacteria. An important component in treating these infections is providing effective antimicrobial therapy [1].

Due to the important roles of heavy metals (zinc, arsenic, cadmium, mercury and nickel) in islet function and diabetes [2], we can use it as treatment of diabetic foot infection.

Heavy metals also have an inhibitory effect on Pathogenic bacteria, where the study by Maillard in (2001) described the bactericidal mechanisms of action of heavy metals as silver and the differences in effectiveness against bacterial groups. While its role in the control of bacteria such as *Pseudomonas aeruginosa* is well recognized [3].

Another study in 2007 Found that, Zinc is an essential trace element in the human body and its importance in health and disease is appreciated; Zinc confers resistance to epithelial apoptosis through cytoprotection against reactive oxygen species and bacterial toxins. Bacterial cultures are exposed to different concentrations of various heavy metals solutions and the growth response is determined. On the other hand, those heavy metal ions inhibited the bacterial growth and metabolism [4].

## MATERIAL AND METHOD

**Chemicals:**

Most chemicals were purchased from Sigma chemicals co. ADWIC Egypt. Chemicals of gel electrophoresis were from Promega co. U.K. The used water was distilled using water distillation apparatus (D4000).

**Study design**:

This is a prospective study in which the pathogenic bacteria from infected wounds (of 22 consecutive diabetic patients found in the National Institute of Diabetes & Endocrinology) were cultured on using aerobic and anaerobic microbiological techniques. Isolates were identified by morphological, staining methods, biochemical tests and molecular techniques

(16S sequencing rDNA). Isolates were also tested for susceptibility to commonly used antibiotics, then the growth and metabolism of the isolates were tested in response to different concentrations of some heavy metals salt solution (e.g. Zinc and Copper) (10 µM–$10^7$ µM). Finally we detect the most bacterial inhibitory, human non-cytotoxic concentrations of metal salt solution to different pathogenic bacteria.

**Collection and Culture of Isolates**:

Bacteria were isolated from the abscess of infected diabetic feet of patients using sterilized cotton swabs that were submerged into saline solution (Sodium chloride 0.9%). Subsequently, the swab was carefully withdrawn and covered to prevent contamination.

The collected bacterial isolates was cultured on MacConkey, blood and chocolate agar media, then were incubated at 37° C for 24 h, Pure colonies were obtained. Pure bacterial colonies were re cultured them on Muller Hinton agar media and incubated at 37° C for 24 h, now we have fresh pure colonies that can reserved for long time at -70°C to be use in next steps.

**Identification of bacteria by biochemical tests:**

Bacterial identification in our laboratory in brief, isolates were first evaluated based on plate morphologies (after overnight growth). Pure bacterial colonies were identified by morphological and biochemical characteristics. The tests performed included Gram staining, Motility, Catalase, Oxidase, Indole,

ISSN: 0975-8585

Methyl Red, Voges Proskauer, Citrate Utilization production, Urease production, Triple Sugar Iron, Mannitol and MacConkey agar[5].

**Identification of bacteria isolates by Polymerization chain reaction (PCR): Identification by 16S rRNA gene sequence analysis:**

Bacterial DNA Extraction:

The DNA extracted is used as the template for PCR to amplify a segment of about 500 or 1.500bp of the 16S rRNA gene sequence. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique [6].

PCR primer:

16S forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3'
16S reverse primer: 5'-CGYTAMCTTWTTACGRCT-3'

**16S rRNA sequencing Reaction mixes (100 µl) were set up as follows:**

10 mMTris/HCl, pH 8•3; 50 mMKCl; 2•5 mM MgCl2; 200 µM (each) dATP, dCTP, dGTP and dTTP; 1•25U Taq DNA polymerase (Genei Bangalore, India); 0•1 µM (each) primer; and 4 µl DNA template. Reaction mixtures, following a 'HOT start', were subjected to the following empirically optimized thermal cycling parameters: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, followed by a final extension at 72°C for 5 min[7],[8].

The generated DNA sequences were assembled by aligning the forward and reverse sequences. This consensus sequence is then compared with a NCBI database library (http://blast.ncbi.nlm.nih.gov) [6]. The Nucleotide sequences of bacteria are shown in table (1).

**Table 1: Identification of bacteria by PCR**

| Isolate No | Patients Age Range (year) | Disease Duration Range | Types of bacteria | Nucleotide Sequences of one bacteria of each type |
|---|---|---|---|---|
| **(1-8)** | (50-75) | (1month-6years) | ***E.coli(Gr-ve)*** | Ggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtatgaagaaggccttcgg gttgtaaagtactttcagcggggaggaagggagtaaagttaatacctttgctcattgacgttacccgca gaagaagcaccgcctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttaatcg gaattactgggcgtaaagctcacgcaggcggtttgttaagtcagatgtgaaatccccgggctcaa cctgggaactgcatctgatactcgcaagcttgagtctcgtagaggggggtagaattccaggtgta gcggtgaaatgcgtagagatctggaggaataccggtggcgaaggcggccccctggacgaagac tgacgctcaggtgcgaaagcgtggggagcaaacaggattagataccctggtagtccacgccgta aacgatgtcgacttggaggttgtgcccttgaggcgtggcttccggagctaacgcgttaagtcgacc gcctggggagtacggccgcaaggttaaaactcaaatgaattgacgggggcccgcacaagg |
| **(9-13)** | (50-59) | (13days-4years) | ***Pseudomonas Spp.*(Gr-ve)** | Ctcggtcaaggcctgtcgcgcggcgagcggatggactgggccattcaaaaagccaccgagctgg gcgtcagcgaaattacgccaattgtcagcgaacgctgcgaggtgcgcctcaaggacgaacgtgcc gagaagcgtcaggcacactggcagcagatcgcgatcagcgcctgtgagcaatgtggtcgctccgt ggtgccggtgattcatgctccgatgccgctggccgagtggatcaagcacaccgaagccgacctgaa actggtcgtgcacccggtggccgaacccctgaccagccatgacaagcccgccagtctggcccttctg attggtccggaaggcggtctgaacgatgcggaagtcactcaggcgcaagacgcaggcttccacg ccgcgcgccg |

ISSN: 0975-8585

| | | | | |
|---|---|---|---|---|
| **(14-17)** | (35-67) | (1month-1year) | ***Klebsiella Spp.*** **(Gr-ve*)*** | Gacaccgtccgttttagcttagtggctgaggccgctgacgtcgccgaatgcgacaccctgatctactac<br>tggccaaagaacaaaccagaggcgcagttccagctgataaacctgctctctctgctgccggtgggaag<br>cgacattttggtcgtcggtgtaaaccgcagcggcgtccgcagcgcggagcagatgctggctgaatacg<br>cgccattaaacaaagtggatagcgcccgccgctgtggcctctttcatggccgtctggaaaagcagc<br>cgaccttcgacgccgacgcattctggggcgaatacacactggataatctgacgattaaaaccctgcc<br>gggcgtcttcagccgcgacggtctggacgtcggcagccagctgctgctctccaccctcgagccgcata<br>ccaaagggaaagtgctcgacgtcggctgcggcgcgggcgtgctggccgccgcgctggccagccat<br>tcaccgaaagtgcgcctcaccctgtgcgacgtcagcgcccggcggtcgaagccagccgcgcga<br>cgcttgccgccaacggcctggctggcgatgtgtttgccagcaacgtcttctccgaggtcaatggtcg<br>cttcgacatgatcata |
| **(18-20)** | )35-80) | (2months-2years | ***Proteus Spp*****. (Gr- ve*)*** | Ggggaatattgcacaatgggcgcaagcctgatgcagccatgccgcgtgtatgaagaaggccttaggg<br>ttgtaaagtactttcagcggggaggaaggtgataaggttaataccttgtcaattgacgttacccgcag<br>aagaagcataggctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttaatcggaat<br>tactgggcgtaaagcgcacgcaggcggtcaattaagtcagatgtgatagccccgagcttaacttggg<br>aattgcttctgaaactggttggctagagtcttgtagaggggggtagaattccatgtgtagcggtgaaat<br>gcgtagagatgtggaggaataccggtggcgaaggcggccccctggacaaagactgacgctcaggt<br>gcgaaagcgtggggagcaaacaggattagataccctggtagtccacgctgtaaacgatgtcgattta<br>gaggttgtggtcttgaaccgtggcttctggagctaacgcgttaaatcgaccgcctggggagtacggc<br>cgcaaggttaaaactcaaatgaattgacgggggcccgcacaagcggtggagcatgtggtttaattcg<br>atgcaacgcgaagaaccttaccta |
| **(21-27)** | (35-72) | (2months-6years) | ***Staphylococcus Spp.*****(Gr-ve*)*** | Ttaacttttaatgatcaaacagtttatttatgtgaaattgctgacataacaagtgataaaattgaa<br>gttgatttataagaaaaacaaaatattaatacagaattgccagttgatgttacgatttgcagtggacta<br>atcaaagctgacaaatatgagtggatgctacaaaaagctactgaattgggtgcttcatcatttataa<br>ctgtgagcatggaacgttcaattgttaaattaaacgaggcaaaagttgccaaaaagatagagcgttgg<br>caaaaaataattaaagaagctgctgaacaaagctatcgtttagtgattccatctattcaattcgagt<br>cgaatttaaaattaatttgtgatactattgataattatgactatattcttattgcatatgaagaggaa<br>gcaaaagatggtgagttaagcaatttcaagcaaactttacaacaattcaatgctcaggataaagt<br>gttgatgatttttggtcctgagggtggtttgtcagaaaatgaaaatg |

## RESULT AND DISCSSION

Table2. Identification of bacteria by biochemical tests.

| Isolate Number | Patients Age Range (year) | Disease Duration Range | Bacteria and Stainability | Biochemical Tests Used To Identify Gram –ve Bacilli | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Glucose Fermentation | Lactose Fermentation | Urease | Oxidase | Indol (SIM) | Citrate | Methyl Red / Voges-Proskauer) |
| (1-8) | (50- 75) | (1month-6years) | *E.coli* (Gr-ve) | + | + | - | - | + | - | MR+ and VP- |
| (9-13) | (50-59) | (13days-4years) | *Pseudomonas Spp.* (Gr-ve) | - | - | - | + | - | - | MR- and VP- |
| (14-17) | (35-67) | (1month-1year) | *Klebsiella Spp.* (Gr-ve) | - | + | - | - | - | + | MR- and VP+ |
| (18-20) | (35-80) | (2months-2years | *Proteus Spp.* (Gr-ve) | + | - | + | - | - | - | MR- and VP- |
| | | | | Biochemical Tests Used To Identify Gram +ve Cocci | | | | | | |
| | | | | Blood Agar Plate (BAP) | Mannitol Salt Agar (MSA) | Catalase | Oxidase | | | |
| (21-27) | (35-72) | (2months-6years) | *Staphylococcus Spp.* (Gr-ve) | β-hemolytic | + | + | - | | | |

The results of identification have showed that the total number of bacteria was 27 which included 5 types of bacteria (*E.coli, Staphylococcus, Pseudomonas, Klebsiella* and *Proteus)* as shown in table(2).

*Escherichia coli* was the most common isolate, being recovered from (29.63%) of total number of bacteria in this connection. In another study, it was found that *Staphylococcus aureus* is both a common colonizer of human skin and the most frequently isolated pathogen in diabetes foot infections. The spread of diabetes foot infections to soft tissue and bony structures is a major causal factor for lower-limb amputation. It is therefore of great importance to differentiate colonizing from infecting strains of *Staphylococcus aureus*[9].

Also another research in 2013 Conclude that Forty percent of diabetic foot infections was poly microbial. *Staphylococcus* and *Pseudomonas* were the most common Gram-positive and Gram-negative organisms [1]. Other organisms in our experiment were *Staphylococcus spp*. (29.6%), *Pseudomonas spp*. (18.52%), *Klebsiella spp*. (14.81%)and *Proteus spp*.(11.11%),

**Table3: Percent of types of bacteria in diabeticfeet.**

| Totalnumber / Type of Bacteria | Present (+ve) | Absent (-ve) | Percent (%) |
|---|---|---|---|
| ***ESCHERICHIACOLI*** | 8 | 19 | 29.63 |
| ***StaphylococcusSpp.*** | 7 | 20 | 25.93 |
| ***PseudomonasSpp.*** | 5 | 22 | 18.52 |
| ***KlebsiellaSpp.*** | 4 | 23 | 14.81 |
| ***ProteusSpp.*** | 3 | 24 | 11.11 |

In this regard a study which by previous South Indian authors found that the distribution of gram-negative bacteria (57.6%) is more common than that of gram-positive ones (42.3%) and it is contrary to the viewpoint that diabetic foot infections are frequently mono microbial [10].

Antibiotics are necessary for treatment of Diabetic foot infection but not sufficient to overcome inadequate vascular supply, poor glycemic control or improper wound care[11].

In this respect, the management of wound infection has long tested man's ingenuity. The advent of antibiotics in the 1950s revolutionized the control of bacterial infections, but with the recent escalating prevalence of bacterial resistance there has been renewed interest in the use of topical antimicrobials, particularly silver, iodine, honey and larval therapy [12]. In our experiment mainly meropenem, and cephoperazone +sulbactam were the most effective antibiotic against bacteria as shown in table (4).

ISSN: 0975-8585

Table4. Sensitivity of bacteria to antibiotics. Sensitive: (+) Resistant: (-) Intermediate: (-+)

| No. | Antibiotics / Bacteria | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *E.coli* | - | + | + | - | - | - | + | + | + | -+ | - | - | - | - | - | + | + | - | + | -+ | - | - |
| 2 | *E.coli* | + | + | + | -+ | -+ | - | + | + | + | + | - | - | - | - | - | + | + | - | + | -+ | - | - |
| 3 | *E.coli* | - | + | + | - | - | - | + | + | + | + | - | - | - | - | - | + | + | - | + | - | - | - |
| 4 | *E.coli* | + | - | + | - | - | - | - | - | + | + | - | - | - | - | - | + | + | - | - | - | - | - |
| 5 | *E.coli* | - | - | + | - | - | - | - | - | + | - | - | - | - | - | - | + | - | - | - | - | - | - |
| 6 | *E.coli* | + | - | + | - | - | - | - | - | + | + | - | - | - | - | - | + | + | - | - | - | - | - |
| 7 | *E.coli* | - | - | + | - | - | - | - | - | + | + | - | - | - | - | - | + | - | - | - | - | - | - |
| 8 | *E.coli* | - | -+ | + | - | - | - | + | - | + | + | - | - | - | - | - | + | -+ | - | - | - | - | - |
| 9 | *Pseudomonas Spp.* | -+ | -+ | - | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | *Pseudomonas Spp.* | - | -+ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 11 | *Pseudomonas Spp.* | - | - | - | - | - | - | + | - | - | - | - | - | --- | - | - | - | - | - | - | - | - | - |
| 12 | *Pseudomonas Spp.* | - | + | + | - | - | - | + | - | + | - | - | - | - | - | - | + | - | - | + | - | - | - |
| 13 | *Pseudomonas Spp.* | -+ | + | + | - | -+ | - | + | - | + | -+ | - | - | - | - | - | + | - | - | + | - | - | - |
| 14 | *Klebsiella Spp.* | - | - | - | - | - | - | - | - | + | + | - | - | - | - | - | + | - | - | - | - | - | - |
| 15 | *Klebsiella Spp.* | + | - | + | - | - | - | - | - | + | + | - | - | - | - | - | + | + | - | - | - | - | - |
| 16 | *Klebsiella Spp.* | - | -+ | - | - | - | - | - | + | + | + | - | - | - | - | - | + | - | - | + | - | - | - |
| 17 | *Klebsiella Spp.* | + | - | + | - | - | - | -+ | - | - | + | - | - | - | - | - | + | - | - | - | - | - | - |
| 18 | *Proteus Spp.* | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | + | - | - | - | - |
| 19 | *Proteus Spp.* | + | - | + | - | - | - | - | - | + | + | - | - | - | - | - | - | + | - | - | - | - | - |
| 20 | *Proteus Spp.* | - | + | + | - | - | - | + | + | + | + | - | - | - | - | - | + | + | - | + | - | - | - |
| 21 | *Staphylococcus Spp.* | + | - | - | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - | - | - | - | + |
| 22 | *Staphylococcus Spp.* | + | - | - | - | - | - | - | + | - | - | + | + | - | - | + | + | + | - | - | - | - | - |
| 23 | *Staphylococcus Spp.* | - | - | - | - | - | - | - | + | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| 24 | *Staphylococcus Spp.* | - | - | - | - | - | - | - | + | - | - | + | + | - | - | + | + | + | - | - | - | - | - |
| 25 | *Staphylococcus Spp.* | + | - | - | - | - | - | + | + | - | - | + | + | - | - | - | + | + | - | - | - | + | - |
| 26 | *Staphylococcus Spp.* | - | - | - | - | - | - | - | - | - | - | + | + | - | + | - | + | - | - | - | - | - | - |
| 27 | *Staphylococcus Spp.* | - | - | - | - | - | - | - | + | - | - | - | - | - | + | - | + | - | - | - | - | - | - |

**Symbols:** 1-Cefoxitin 2- Cefepime 3- Cephoperazone+ Sulbactam 4- Ceftazidime 5-Amoxicillin+Clavulinic 6- Tobramycin 7- Piperacillin-tazobactam 8- Levofloxacin 9- Meropenem 10- Tigecycline 11- Vancomycin 12-Linezolid 13- Doxycyclin 14- Erythromycin 15- Azithromycin 16- Imipenem 17- Ertapenem 18- Ceftriaxone 19- Ciprofloxacin 20- Ambicillin+ Salbactam 21-Oxacillin 22-Cefaclor.

ISSN: 0975-8585

In our study for sensitivity of bacteria to heavy metal solutions (e.g. Zinc& Copper) we found the most effective and nontoxic concentration Cuso4 &Znso4 solutions are ($10^5$ µM) as shown in table (5).

**Table 5: Sensitivity of bacteria to heavy metals (zinc& copper) salt solutions**

| | | Sensitivity to Cuso4(µM) | | | | | Sensitivity to Znso4.7H2O(µM) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Isolate No | Heavy metal Bacteria | 10 | $10^2$ | $10^3$ | $10^5$ | $10^7$ | 10 | $10^2$ | $10^3$ | $10^5$ | $10^7$ |
| 1 | *E.coli* | - | - | -+ | + | -+ | - | - | -+ | + | -+ |
| 2 | *E.coli* | - | - | - | + | + | - | - | + | + | + |
| 3 | *E.coli* | - | - | + | + | -+ | - | - | - | + | + |
| 4 | *E.coli* | - | - | + | + | + | - | - | -+ | + | + |
| 5 | *E.coli* | - | - | -+ | + | + | - | - | -+ | + | + |
| 6 | *E.coli* | - | - | + | + | - | - | - | + | + | + |
| 7 | *E.coli* | - | - | -+ | -+ | + | -+ | -+ | - | + | + |
| 8 | *E.coli* | - | - | - | -+ | + | - | - | -+ | + | + |
| 9 | *Pseudomonas Spp.* | - | - | + | + | + | - | - | -+ | + | + |
| 10 | *Pseudomonas Spp.* | -+ | - | + | + | -+ | - | - | -+ | + | + |
| 11 | *Pseudomonas Spp.* | - | - | -+ | -+ | + | - | - | + | + | + |
| 12 | *Pseudomonas Spp.* | - | - | -+ | -+ | + | - | - | + | + | - |
| 13 | *Pseudomonas Spp.* | - | - | -+ | -+ | + | - | - | -+ | + | + |
| 14 | *Klebsiella Spp.* | - | - | -+ | + | + | - | - | + | + | + |
| 15 | *Klebsiella Spp.* | - | - | -+ | + | + | - | - | - | + | + |
| 16 | *Klebsiella Spp.* | - | - | + | + | - | - | - | - | + | + |
| 17 | *Klebsiella Spp.* | - | -+ | - | + | -+ | - | - | -+ | + | + |
| 18 | *Proteus Spp.* | - | - | + | -+ | -+ | - | - | -+ | + | + |
| 19 | *Proteus Spp.* | - | - | + | + | -+ | - | -+ | -+ | + | + |
| 20 | *Proteus Spp.* | - | - | + | + | + | - | -+ | + | + | - |
| 21 | *Staphylococcus Spp.* | - | - | -+ | + | + | - | - | - | + | -+ |
| 22 | *Staphylococcus Spp.* | - | - | -+ | + | + | - | - | - | + | + |
| 23 | *Staphylococcus Spp.* | - | - | - | + | + | - | - | -+ | + | + |
| 24 | *Staphylococcus Spp.* | - | - | + | + | + | - | - | -+ | + | + |
| 25 | *Staphylococcus Spp.* | -+ | -+ | + | - | + | - | - | -+ | + | - |
| 26 | *Staphylococcus Spp.* | - | - | + | -+ | + | - | - | + | + | -+ |
| 27 | *Staphylococcus Spp.* | - | -+ | -+ | -+ | + | - | - | + | + | + |

**Sensitive: (+) Resistant: (-) Intermediate: (-+)**

In this regard, Ionic silver (at a concentration of $10^{-9}$ to$10^{-6}$ mole/L) is bactericidal, fungicidal, virucidal and protozoicidal. Although silver has been used for many centuries and in wound management for a long time, its bactericidal mechanisms of action are still not fully understood. Silver has now assumed a prominent position in wound care[3].

ISSN: 0975-8585

## CONCLUSION

*Escherichia coli* and *Staphylococcus spp*. were the most common causes of diabetic foot infections. Anaerobic organisms are still a common cause for infection, although the prevalence is less. These wounds may require use of combined antimicrobial therapy for initial management [13].

We try to treat diabetic feet's pathogenic bacteria using the most effective and nontoxic concentration ($10^5$ µM) of heavy metals salts (e.g. Copper &Zinc) as a lotion.

## ACKNOWLEDGEMENT

The authors would like to express their sincere gratitude to Dr. Mohamed Gamal Farahat , Lecturer Botany and microbiology Department- Faculty of science-Cairo University.

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