Antibacterial Activity and Mechanism of Nickel Nanoparticles against Multidrug Resistant *Pseudomonas aeruginosa*

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**ABSTRACT**

Burns patients are at high danger of nosocomial disease, and *Pseudomonas aeruginosa* is one of the mainly common causes of wound and systemic infection resulting in significant morbidity and mortality in burns patients. The appearance of multidrug resistant strains is up surging leading to problematical control. The aim of this study was to isolate and identify MDR-*P. aeruginosa* from the burn patients held in the burn unit and study the antibacterial activity and mechanism of Nickel Nanoparticles solution on bacterial isolated and evaluate by molecular and pathological techniques. This study was carried out on the Burn patients in Tikrit teaching hospital in Tikrit city / Iraq from January, 2017 to June, 2017. The scientific samples were collected using sterile cotton swabs from 60 patients with burn infection. *P. aeruginosa* that were recognized by cultural characteristics, Gram stain, and biochemical reactions. The results of the laboratory cultural of 60 cotton swabs used showed to isolated 35(58.3%) *P. aeruginosa* isolated and all bacterial isolates were resistant to Doxycycline hydrochloride, Penicillin, Cotrimoxazole, Ciprofloxacin, Cephalosporin and Penicillin. The study showed that the *Nickel Ferrite (NiFe₂O₄)* used to inhibit the growth of bacterial isolated by using different concentrations the MBC killer concentration was 256 μg / L and the lowest inhibitory concentration to *P. aeruginosa* was MIC 32 microgram / L). Molecular studies included the observation of the most important molecular changes at the level of DNA prior to and treatment with nanoparticles. Many variations were observed on the studied bacterial isolated. Variations include the appearance and disappearance of DNA and its different numbers when treated with nanoparticles. As for the results of the histopathological, it was found that the injury of mice with three antibiotic resistance isolates emerged after five days and the symptoms were heat, redness and swelling of the skin and the release of yellow and green purulent secretions from the place of injury. Which were treated with antimicrobial and nanoparticles together was faster than the time of the healing of nanoparticles treated only.

**Keywords:** Antibacterial, activity, Mechanism, Nickel Nanoparticles, Multidrug, Resistant *Pseudomonas aeruginosa*


**INTRODUCTION**

*Pseudomonas aeruginosa* is Gram negative bacteria belong to the proteobacteria phylum (1). *P. aeruginosa* is one of the most dangerous opportunistic infectious agent of man (2).Which involved in hospital infection and cause disease in immunocomprised patients One of the chief cause of serious infection in burn patients, resulting in mortality as high as 50% (3).

*P. aeruginosa* is chiefly a nosocomial pathogen. According to the centers for Disease Control and Prevention ( CDC), the overall rate of *P. aeruginosa* infection in United States hospital averages about 0.4 percent ( 4 per 1000 discharges ), and the bacterium is the fourth mainly commonly isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquire infection .It has been estimated that about ten percent of all hospital-acquired infections are caused by *P. aeruginosa*, and for immune-compromised patients the mortality rate range from 20 to 70 % (4).When pathogenic bacteria are toward the inside host tissue, unlike substances called virulence factors are free from the bacteria. Virulence factors causes injury to tissues through toxicity and invasiveness and allow for organization of bacteria. *P. aeruginosa* create a diversity of virulence factors, both extracellular and cell-associated yield. (5).Pyocyanin is the chief phenazine dye produced by *P. aeruginosa* and has been shown to contribute to its pathogenicity. The presence of pyocyanin is simple to
detect due to its blue color that turn stationary phase cultures of *P. aeruginosa* green, and is usually found to stain infected tissues, pus, or dressings (6).

Despite the variable degree of burns, disease remains the most common cause of mortality among burn patients (7). Burn wound infection can be caused by bacteria, fungi, or viruses. The emergence of multi-drug-resistant strain of bacteria and fungi have increased the occurrence of burn wound infections, sepsis, and connected death (8). As recognized by Infectious Disease Society of America, the usually recovered organisms from dirty patients in the burn ICU are members of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) group of pathogens (9).

When contagious bacteria are invading a host, toxic substances are produced by the microorganisms that cause injury to the host tissues. These substances are called virulence factors and let the bacteria to establish in the host. The host responds to the bacterial invasion with attack of inflammatory cells such as neutrophils which free cytotoxic enzymes, oxygen radicals and inflammatory mediators which cause further injury to host tissue. This host response machine is also contributing to the non-healing stage of the infected wound (10). Once recognized in a wound, the bacterium is almost impossible to remove with antibiotics due to biofilm formation. All wounds are dirty by bacteria, meaning that microorganisms are present but not replicating. Wounds become dirty from endogenous sources such as the gastrointestinal tract, the surrounding skin, the environment or from the healthcare provider (11). The classic symbols of infection in wounds include swelling, redness, pain, heat, purulence, and impair function. Chronic wounds can also show symptom such as low transcutaneous oxygen tension, expansion of necrotic tissue, foul odor and wound breakdown as well as deterioration and discoloration of granulation tissue and increased friability (12).

However wounds or burns disturb the wall and decline the immune system, allow opportunistic pathogens such as *P. aeruginosa* to take benefit. The hospital environment tends to grow the multi drug resistance *P. aeruginosa* strains, raise the rate of complication caused by MDR pathogens. *P. aeruginosa* is an opportunistic pathogen, often acquire in hospital environments and is often linked with respiratory and urinary infections, in burn injuries wounds, and in chronic wounds (1). Mechanism of resistance to antibiotics in *P. aeruginosa* are either base on non-mutational intrinsic resistance or mutational acquire resistance. Fluoroquinolones and Aminoglycosides are two main classes of antibiotics used in the treatment of *Pseudomonas* infection. *Pseudomonas* readily develops resistance to these agents, reducing the antibiotic efficiency (11).

Nickel has a strong antimicrobial potential, which has been use since the ancient times. But with the advent of antibiotics development, the medical applications of Nickel as antimicrobial were declined (13). Antimicrobial effect of Nickel can be improved by manipulating their size at nano level. Because of their alter in physiochemical properties, Nickel nanoparticles have emerged as antimicrobial agents due to their high surface-area-to-volume ratio and the unique chemical and physical properties (14). Nickel nanoparticles having size in the range of 10–100 nm show strong bactericidal potential against both Gram-positive and Gram-negative bacteria (15). The bactericidal action of Nickel nanoparticles against the pathogenic, MDR as well as multidrug-susceptible strains of bacteria was studied by many scientists, and it was prove that the Nickel nanoparticles are the potent weapons against the MDR bacteria such as *Pseudomonas aeruginosa*, ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Streptococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Staphylococcus aureus* (VRSA).

**Material and Methods**

**Nickel Ferrite (NiFe₂O₄) Nanoparticles (NFNPS).**

Obtained from the College of Science, Department of Chemistry and preparation manner Sol-Gel Auto Combustion Method (11).

**Animals Experiments**

In this study, 30 mice, *Mus musculus* male and female were obtained from the veterinary home of the college of Veterinary Medicine. All three mice hatches were placed in a plastic cage, the dimensions of the cage were 8x15x30 cm, and the cages were sprayed with wood and the temperature of the room was about 22±25. The animals were fed on the blackberry consisting of 35% yellow corn, 20% soy bean, 10% animal protein, 1% dried milk and 1% of both vitamins and mineral salts.

**Samples Collection**
Collected 60 swabs samples were taken from Surgical wound infection by sterile cotton swabs and transported to laboratory as soon as possible in sterile Brain heart infusion broth that incubated at 37 C for at least 24-28 hours to increasing chances of isolation (16).

Isolation and identifications of *Pseudomonas aeruginosa*

Swabs from specimens were plated on *Pseudomonas* MacConkey agar, a single transparent non-lactose fermenting colony was selected then subculture on appropriate medium. The organism was over again grown on MacConkey agar plate to generate colorless colonies non lactose fermenter, *P. aeruginosa* expresses the expigment pyocyanin, which was blue-green in color, and the colonies will appear flat, large and oval and on the nutrient agar the colonies appearance yellow green in color. It has also a characteristic fruity smell, when identified provisionally as *P.aeruginosa* it was sub cultured on nutrient agar slant after incubated at 37 C° for 24 hours, then stored at 4C° in refrigerator (17).

**Vitek2 Compact System**

The newly redesigned colorimetric Vitek2 compact system with modernized highly developed expert system (AES) (bioMerieux, Marcy Etoile, France) was evaluate for its correctness and quickness to recognize clinical isolates and to distinguish several antimicrobial resistance (18).

**Antimicrobial Susceptibility Testing**

Disc diffusion technique, also known as the Kirby- Bauer method was carried out according to the Clinical and Laboratory Standard Institute guidelines (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) (19).

**Preparation of the Concentration of the Nanoparticle Solution**

The minimum bactericidal concentration (MBC) is the lowest concentration of an nanoparticle substance required to kill a particular bacterium and reduces the viability of the initial bacterial inoculums by ≥99.9%It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by sub culturing to agar plates that do not contain the test agent(20).

**MOLECULAR CHARACTERIZATION OF Pseudomonas aeruginosa**

**DNA isolation:** The isolated colonies were then cultured in Brain heart infusion broth and incubated at 37°C for 24 hours. Incubation 2ml bacterial culture was centrifuged at 6000 rpm for about 10 minutes. To the pellet 1ml of lysis buffer (10mM Tris HCl, pH 8; 0.5M EDTA; 0.5% SDS; 1M NaCl) was added and vortexed properly and incubated at 45 °C in water bath for 10 minutes Following incubation, 1ml of phenol: chloroform mixture (1:1) was added to the mixture and centrifuge at 10,000 rpm for 10 minutes. The upper aqueous layer was transferred and equal volume of chloroform: isoamyl alcohol mixture (24:1) was added and then 1/10th volume of 3M sodium acetate was added. The mixture are mixed properly and centrifuged at 10,000 rpm for 10 minutes. To the upper aqueous layer double the volume of chilled ethanol was mixed and added to precipitate the DNA and later centrifuged at 12,000 rpm for 10 minutes. The DNA extracted was the stored in 20-50ml of TE buffer and stored at 4°C for further use. The extract DNA was then quantify using the Nano drop spectrophotometer (ND-1000) to check for the cleanliness. The pure DNA obtain thus obtained was run on 0.8% agarose gel to confirm for the DNA bands (21).

**RAPD-PCR**

PCR reaction were perform with three commercial 10- mer primers (Invitrogen) with the following 5'-3' sequences: (OPH-14) 5-ACCAGTTGG-3 (OPO-11) 5-GACAGGAGGT -3 and(OPP-4) 5-GTGTCTCAGG -3. The total reaction size was 25µl, which contained 2µl of template DNA and 47.5µM primers. PCR was performed using the following protocol: 94°C for 5min, followed by 30 cycles of 94°C for 45sec, 55°C for 1 min, and 72°C for 1min, followed by 72°C for 5min. PCR products were visualized on 1.5% agarose gel electrophoresis. (22).

**Experimental design**

42 white mice were used after confirm their security from the diseases and were divided into five groups and each group consists of 6 mice excepted groups (III and V) were distributed as follows

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1- Group I (Control group):- 6 Mice were treated with distilled water only.
2- Group II (Infection groups):- 6 Mice were infected with Pseudomonas aeruginosa by contamination superficial skin bun with (1) ml from suspension of the bacteria.
3- Group III: 6 Mice were infected with Pseudomonas aeruginosa by contamination the superficial skin bun with (1) ml from suspension of the bacteria and treated with (NiFNPS) carrying with Vaseline.
4- Group IIII: 12 Mice were infected with Pseudomonas aeruginosa and treated with antibiotics (Doxycycline hydrochloride and Ciprofloxacin).
5- Group V: 12 Mice were infected with Pseudomonas aeruginosa and treated with antibiotics (Doxycycline hydrochloride and Ciprofloxacin) and nanoparticles.

Results and discussion

Isolation and identification:
Out of 60 clinical samples collected from the burn patients, “P. aeruginosa” was isolated from 35(58.3%) samples. The specimens of burn were cultured on MacConkey agar and Nutrient agar incubated for 18-24 hrs at 37°C. Then chose bacterial isolate that non fermentative of lactose on MacConkey agar and used morphological and biochemical tests. Results showed the isolates were Gram stain negative, oxidase positive, citrate positive, urea hydrolysis positive, catalase positive, bluish green pigmentation positive, indole production negative, voges proskauer negative and methyl red negative. Then confirmed laboratory diagnosis of Pseudomonas aeruginosa by using VITEK-2 Compact system. In this study, the most prevalent bacterium isolated from burn patients was “P. aeruginosa”. Similarly, other studies such as (23,24) also showed a prevalence of “P. aeruginosa” infection among burn patients to be 55.5% and 60.1% respectively. However, studies of (25,26) showed a prevalence of P. aeruginosa were 17.85% and 59.6% respectively. These variations in prevalence average among numerous studies could be associated with the disparities in hygienic practices of population or belong to ability of this bacterium to produce different virulence factors. These virulence factors encoding by virulence genes located in the chromosome of Pseudomonas aeruginosa that enable it to play a great role in the infection and geographical location.

Antibiotic Susceptibility Test:-
Study sensitivity bacterial isolated toward 5 Antibiotics are Doxycycline hydrochloride,, CO-Trimoxazole, Penicillin, Cephalosporin and Ciprofloxacin. The result showed all bacterial isolated to P. aeruginosa were resistant to all antibiotics as shown in Table (1).

<table>
<thead>
<tr>
<th>Isolate bacteria</th>
<th>Doxycycline hydrochloride</th>
<th>CO-Trimoxazole</th>
<th>Ciprofloxacin</th>
<th>Cephalosporin</th>
<th>Penicillin,</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
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In this study, 100% of isolates were MDR, which is slightly more than the finding of (27) in who found 69% isolates to be MDR. And similarity, (28) showed 100% MDR isolates in burns patients. High rate resistance to various antimicrobial agents particularly among nosocomial organisms were revealed and become serious challenge in diseases treating (29,30). Report of WHO agrees with the truth of wide use of antimicrobial agents in outside and inside of medicine has a critical role in dissemination of bacterial resistant strains by involving various resistance mechanisms like production of Beta-lactamase enzymes that destruct these drugs [31]. The great difficulty of the bacterial resistance is the misuse and overuse of antimicrobial agents by patients in addition to the doctors (32,33), and it could be because of the erratically uses of antimicrobial agents without laboratory diagnosis and antimicrobial sensitivity test.

MIC and MBC of Nanoparticle Solution (FNPS) on P. aeruginosa
Results of the study showed the impact of nanoparticles (NFNPS) used has the potential to inhibit the growth of bacterial isolates using different concentrations. MIC (32 μg /ml) and MBC (256 μg /ml) to *P. aeruginosa* as shown in Table (2).

<table>
<thead>
<tr>
<th>bacterial isolate</th>
<th>nanoparticle concentrations (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>-</td>
</tr>
<tr>
<td>MBC</td>
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MIC=Nogrowth(-),turbidity(+)and(0)control(Muller-HintonBroth+.MBC=growth(+)and sterile(-).

DNA Isolation

DNA was isolated from resistant *P. aeruginosa* by using the method described (38) and was then transplanted onto agarose gel as shown in the figure (1).

The amount and purity of DNA was determined by reading UV absorption at a wavelength of 260 and 270 nanometers using the (Nanodrop) device. If the amount of DNA extracted was very sufficient to achieve reactions (RAPD-PCR), ranging from 25-50 ng per micro liter and with purity 1.69 to 1.6.

Results of RAPD-PCR interactions

Differences in DNA were record among samples studied through

1-The presence of DNA bunds and multiply their absence.
2-Differences in molecular weights among bands.
3-Difference in number of bands.
4-Differences in the concentration of bands.

These results were obtained after a number of experiments to arrive at optimal communication conditions, where the reaction components were controlled (dNTPs, Taq DNA polymerase, MgCl₂, DNA concentration as well as the concentration of the same primers, the appropriateness of the program on the thermo cycler and the pipette decree used). This led to get products double clear and repeatable, as the RAPD Markers are sensitive to any change in the mechanism of their reactions and the environment surrounding the reaction where it is one of the reactions that are characterized by the trouble of replay and get the same result. Therefore, RAPD relations for each user’s primers were repeated once.

There are many variations in the different primers; the nanoparticles have a clear effect on the bacteria. Some caused full mutagen at a site on the gene, some of which have the most recent mutations in the appearance and disappearance of certain bands and differences in bands locations, note in the second and third primers of the Pseudomonas aeruginosa complete appearance of the gene and the difference in the size of the bands. Nanoparticles also have the effect of increasing the efficiency of antimicrobial agents by completely hiding the gene or causing mutations this variation in initiator link sites with DNA and the distance between these sites may be created due to mutations that have been cases of deletion, substitution and addition leading to a change in the order of rules complementary to the initiator sequence.

The results of the present study showed the effect of nanoparticles and their ability to inhibit the growth and killing of antibiotic-resistant bacteria isolates. The results of our study agreed with previous study (39, 40) in which the inhibitory effect of nanoparticles of nickels were investigated on Pseudomonas Aeruginosa and Escherichia coli resistant to Ampicillin and the Streptococcus pyogenes resistant to Erythromycin. They approved of the bacteriostatic effects of nanoparticles of nickle on bacteria. Pseudomonas aeruginosa has numerous antibiotic resistant factors due to its plasmid and chromosome making its antibiotic treatment very hard. Thus, new antibiotics fail to stop the mortality and morbidity it causes. The low penetrability of external membrane of the bacteria and the presence of drug efflux pump are between the mechanisms causing its drug resistance [41]. Furthermore, in 2005, Morones et al., investigate the bactericidal effect of nanoparticles of Nickel on four gram-negative bacteria including Pseudomonas aeruginosa, Vibrio cholerae, Escherichia coli and Salmonella Spp. They discovered that nanoparticles of nickel stick to the cell membrane of the bacteria disintegrate the membrane and disturb the penetrability of membrane through releasing nickel ion (42).
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**Results of the Experiment mice treatment with nanoparticles and antibiotics**

The results of the experiment infection of the mice with *P. aeruginosa* showed symptoms of inflammation after approximately five days. The symptoms were high fever, redness, painful, swollen, and yellow or green ulcerative ulcers around the burn skin. After the onset of infection and treatment of mice with nanoparticles and antibiotics and according to the groups reported, the results showed that the recovery time of the group treated with antimicrobial and nanoparticles together was faster than the time of recovery of mice treated with nanoparticles only.

The results of these studies similarity (43) the antibacterial activity of Ni nanoparticles in combination with different antibiotics “tetracycline and ciprofloxacin erythromycin and chloramphenicol against Gram positive and Gram-negative bacteria “Streptococcus pneumoniae Staphylococcus aureus, Shigella flexneri and Pseudomonas aeruginosa” with inhibition of bacterial biofilm activity about 65%.

The nanoparticles can interact directly or indirectly with DNA inducing mutations. (44) reported that direct or indirect interaction of nanoparticles with DNA results in varying biological and biochemical effects, leading in mutations and genomic instability.

**Results of Histopathology**

Results show the Histopathology to control skin, the epidermis formed from a thin layer of epithelial cells consisting of 2-3 raw. They are dark and bold especially the surface because of the layer of globins and this layer overlapping at the bottom with the layer under the epidermis, which has a fibrous tissue link made of fiber and the presence of the capillaries in this region continued with larger blood vessels in the area below it. The dermis layer contains fibrous fiber bundles and longitudinal sections of the hair follicles that are directed towards the epidermis and outside, in addition to the presence of
dense, continuous fatty tissue with the hypodermis layer. The dermis also contains hair follicles and sweat glands as shown in the picture (5Aan

Results show the Histopathology to infected area Pseudomonas aurogenosa. The dermis was formed by stratified squamous epithelium which appeared irregular pattern and had many degenerated cells especially in the basal layer. The surface of epithelium was covering by a strands of keratin, which other coat of this keratin appeared desquamated from the surface of epithelium. The dermis was contain in bundles of collagen fibers of irregular pattern infiltrated with WBCs and macrophages and these WBCs were diffused in between fat cell of hypodermis (Fig 6-A).

The hair follicles which are passing through the dermis and epidermis are surrounded by cortical follicular cells and sebaceous glands (Fig 6-B).
The results of the Histopathology to the treated area with nanoparticles to the *Pseudomonas aeruginosa* The epidermis was easily detected formed by stratified squamous epithelium with thin strands of keratin covering its surface, this epithelium was seen continuous in certain places with hair follicles and sacs of sebaceous glands. The dermis in between glands and hair follicle was formed by dense C.T. associated with a number of fibroblasts and WBCs (Fig 7-A).

The extension of dermis and hypodermis was forming by adipose tissue. Loose C.T. and skeletal muscle fibers with presence of WBCs infiltration (Fig 7-B).

The results of the Histopathology to the treated area with nanoparticles and Doxycycline hydrochloride to *Pseudomonas aeruginosa*, the epidermis was formed by stratified squamous layer of 3-4 layers covered by strands of epithelium. The dermis was containing densely C.T. of collagen bundles infiltrated by WBCs and present of fibroblasts. The WBCs also surrounding the hair follicles in the dermis (Fig 9-A,B).
The results of the Histopathology to the treated area with nanoparticles and Ciprofloxacin to *P. aeruginosa* shows the epidermis was forming by stratified squamous epithelium of 4-5 layers of cells resting on basement membrane. The outer layer of these cells was covering by keratin. The basal layer of squamous cells were continuous with outer layer of follicular cells of the hair follicles in the dermis. The dermis was containing the sebaceous gland also with hair follicles, collagen bundles forming the bulk of dermis with presence of WBCs infiltrations (Fig 9-A).

The deepest layer of hypodermis and subcutaneous tissue were containing adipose C.T. and skeletal muscle fibers inversed by loose C.T. infiltration with lymphocytes and macrophage (Fig 9B).

Based on the analyzed articles, it can be stated that dressings containing nanocomposites are very promising and are an excellent therapeutic option in wound healing. Higher healing speed, wound contraction reduction, hemostatic effect, bactericidal action, low cytotoxicity, among others, were the results achieved and/or confirmed in the studies [45]. Although it has been demonstrated in this systematic review that nanoparticle-based products have relevant advantages in wound treatment, there has been a lack of research on human beings, highlighting the indication of new studies for application in clinical practice with safety.

**Conclusion**

In conclusion antibacterial properties of nickel nanoparticles were carried out very successfully on human bacterial pathogens. The zones of inhibition were formed in the antimicrobial screening test indicated that the Ni NPs synthesized in this process has an efficient antimicrobial activity against pathogenic bacteria. Thus synthesized Ni NPs can be used in medical field due to their efficient antimicrobial function.

**Conflicts of Interest:** None.

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